Combination of Light and Ru(II) Polypyridyl Complexes: Recent Advances in The Development of New Anticancer Drugs

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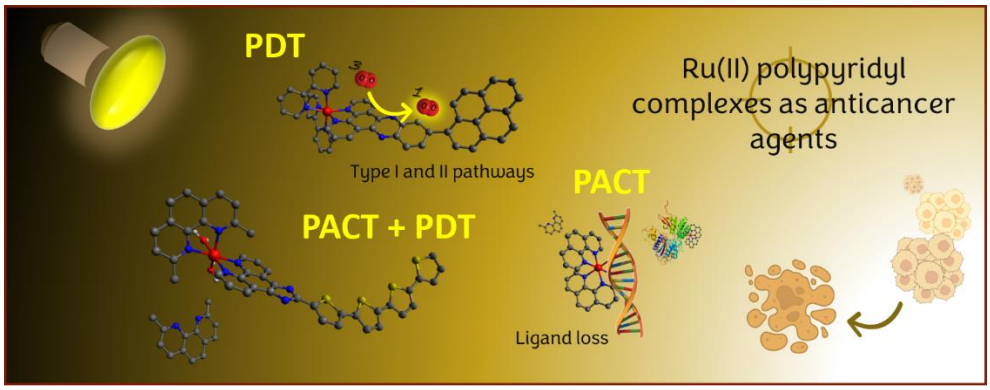
Graphycal Abstract



Ru(II) polypyridyl complexes are a versatile class of compounds to be exploited in the design of photoresponsive anticancer agents.

Abstract

The increasing impact of cancer on worldwide mortality makes the research of novel chemotherapeutic agents a current and challenging issue. In the recent years, transition metal complexes have attracted much interest in this field, with cisplatin and its analogues being largely employed in the treatment of a variety of cancers. However, several issues, such as scarce selectivity and severe side effects, makes it urgent to develop alternative solutions. In this scenario, Ru(II) polypyridyl complexes (RPCs) have emerged as promising systems to be used in photodynamic therapy (PDT) and, more recently, in photochemotherapy (PACT), taking advantage of the spatio-temporal control over the drug activation ensured by light. Their versatile chemical-physical repertoire may be exploited to design both substitutionally inert photosensitizer agents for PDT and photolabile complexes for PACT, the latter featuring oxygen-independent mechanisms of action, that are of relevance considering the generally hypoxic environment of tumors. Herein, we reviewed the recent opportunities brought by the use of RPCs in PDT and PACT. Our aim was to provide a general and comprehensive overview of the numerous mechanisms of action that are made accessible by these different, but often complementary, approaches, in a work that could be of use to both the experienced scientist as well as the young researcher who approaches this topic for the first time. A particular emphasis is put on those RPCs that were subjected to an *in vitro* biological evaluation and whose mechanisms of action were, at least partially, disclosed.

Keywords Ru(II) Polypyridyl Complexes, Photodynamic Therapy, Photoactivated Chemotherapy, Multiple-active Agents, Photoresponsive Compounds.

Contents

1. Introduction

Worldwide, cancer still is a major cause of death, since current therapies are not so efficient to provide the best outcome for patients. Much progress is being made against the disease over the last century, new therapeutic strategies are being developing but it still represents a hard challenge and there is a compelling need for more selective treatments, preferentially harming tumor tissues and not healthy tissues.

In this regard, transition metal complexes have long been employed as chemotherapics for various cancer types, in that they possess the ability to interfere with biological processes, especially those involving DNA [1,2].

Platinum-based drugs, of whom cisplatin embodies the lead compound, held court in the last decades [3,4]. Nonetheless several issues (poor solubility, high systemic toxicity, severe side effects, induction of intrinsic or acquired drug resistance, low selectivity for DNA and inefficient bioavailability) [4–10] necessitate the developing of alternative metal complexes.

Some ruthenium(II) complexes possess a long known biological activity [11–13] and have been exploited for interaction with DNA, cellular imaging and as therapeutics [14–16]. Properties such as tolerable biological profiles and different modes of action make them appealing candidates for cancer therapy [17,18]. Ruthenium(II) complexes are indeed emerging as adjuvants to platinum-based chemotherapics [19], even outclassing them from certain points of view. For example, they show lower systemic toxicity, hence fewer side effects, as a consequence of their higher selectivity for cancer *vs* healthy cells [14,15,20,21], which can be in turn a result of a higher uptake by the former than the latter [22]. Notably, ruthenium complexes have shown to overcome several resistance mechanisms of platinum anticancer drugs, achieving an adequate accumulation and retention in platinum-resistant cancer cells [23–29] and making their way to a possible therapeutic alternative to cisplatin.

To date, the resistance mechanisms associated with ruthenium complexes themselves have been barely studied. Gaiddon *et al.* [30] reported the first comprehensive investigation on this topic, and found that a high expression of the ABCB1 gene is correlated with a reduced cytotoxicity of cyclometalated complexes, concluding that a cotreatment with inhibitors of ABC transporters (*e.g.* verapamil) may improve the efficacy of the ruthenium compound.

A possible general strategy to enhance the selectivity towards cancer cells is the use of prodrugs, that display their biological activity only once arrived at the tumor site, following specific activation. This can be the result of either internal (endogenous) stimuli, such as hypoxic or reducing environment, pH variation, enzyme concentration, hormone level, glucose and biomolecules, all related to the pathological characteristics, or external (exogenous) stimuli, such as temperature, light, magnetic field, ultrasound, electric pulse or high energy radiation, that are located in the outside of the physiological environment. Only in the latter case, however, there is complete control on the fate of the compound, in other words spatial and temporal management over the generation of toxic species, avoiding those side effects typically encountered with more conventional approaches. Moreover, the activation could be obtained by multiple triggers. In this respect, Wang *et al.* recently reported an interesting review on the topic of stimuli-responsive therapeutic metallodrugs [31].

Ruthenium complexes can be considered as prodrugs, both exploiting the chance of ruthenium to be present in its complexes in two oxidation states (III: inactive form; II: active form) with a consequent *in situ* "activation by reduction" (target-specific delivery) [32,33], as well as exploiting a light-triggered activation, that represents the most employed approach so far [34–36]. When looking at anticancer agents, light activation can be categorized in Photodynamic Therapy (PDT) and Photoactivated Chemotherapy (PACT). The former entails the generation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), whereas the latter involves an irreversible photochemical reaction that leads to cell death upon light irradiation *via* the generation of cytotoxic photoproducts (photoreleased ligands and/or Ru(II)-based frameworks). This review presents the most recent examples of ruthenium complexes, classified depending on their use in PDT, PACT or their combination, when possible.

Literature on PDT, PACT and RPCs is very rich [37,38,47–51,39–46]. However, the present reviews usually focus only on some specific points of view rather than providing a comprehensive and systematic discussion on the use of RPCs in both PDT and PACT. In general, the mechanisms of action of photoresponsive compounds are not always clearly described and in those cases where PDT and PACT are presented together in the same survey, the discussion of compounds belonging to each of these categories appears to be often unbalanced, with a common tendency to dedicate less space to PACT-based compounds [25,52]. Importantly, hybrid compounds combining multiple modalities of actions and multiple targeting abilities are commonly underestimated and/or suffer from the lack of a clear and detailed classification.

Our aim was therefore to fill these gaps, through the realization of a work that is entirely dedicated to photoresponsive RPCs, with the effort to provide the Reader a comprehensive and systematic overview of those complexes that were recently developed as PDT, PACT and hybrid multi-target and multi-active compounds.

A particular emphasis was placed on the structural modifications that could be exploited to optimize the characteristics of RPCs as photoresponsive agents, as well as on the most recent solutions that were undertaken to overcome some key features that still obstacle the translation into the clinical use of this challenging class of compounds.

All the references discussed herein are treated from a more chemical point of view, only mentioning some clinical aspects. Finally, nanosystems are intentionally excluded from the discussion, not to excessively weigh down the reading and following our expertise.

2. Use of Ruthenium complexes in Photodynamic Therapy (PDT)

PDT is a treatment to destroy cancerous and cells after light activation through production of ROS species, mainly singlet oxygen ($^1O_2$). It takes advantage of the synergic action of three components, namely a photosensitizer (PS), molecular oxygen and light (usually visible), that are harmless individually but form a combination useful to obtain a therapeutic action in a two-step procedure, consisting first in the administration of the PS followed by irradiation of the target tissue. The PS could be administered locally or systematically; light irradiation at a suitable wavelength promotes the excitation of the PS from the ground state to the singlet excited state $^1PS^*$. A nonradiative intersystem crossing process (ISC) provides then the triplet excited state $^3PS^*$, that subsequently interacts directly or indirectly with molecular oxygen, going back to the ground state. PDT can then proceed by two photo-processes, namely Type I and Type II (Fig. 1):

- in Type I mechanism, the excited triplet state of PS and nearby biomolecules exchange an electron or a proton, producing radicals with very short lifetime (about 1 ns) that can instantaneously react with water or molecular oxygen to give ROS (*e.g.* superoxide anion $O_2^{\bullet-}$, hydroxyl radical $^{\bullet}OH$ or hydrogen peroxide H_2O_2);
- in Type II mechanism, the excited triplet state of PS transfers energy to molecular oxygen in its ground triplet state ($^3O_2$) with formation of cytotoxic singlet oxygen ($^1O_2$). The latter possesses a half-life of 40 ns in the biological environment [53] and is among the most potent cytotoxic species known, able to damage the cell by promptly reacting with the surrounding biomolecules.

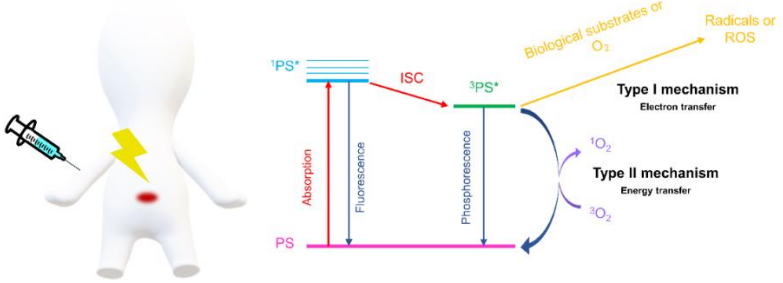


Fig 1. Mechanism of action of PDT.

In other words, the PS is a prodrug: the species responsible for the real therapeutic action of PDT are the reactive species generated upon light irradiation, that, following both mechanisms, can cause damage to several biological targets through multiple pathways, finally bringing the cell to death by apoptosis or necrosis. Mainly, PDT proceeds through Type II mechanism [54,55], due to the highly reactive nature of $^1O_2$ ($^1\Delta_g$ state 95 kJ mol^{-1} higher in energy than $^3O_2$) [56] that can target unsaturated lipids and certain amino acids side chains as well as the nitrogenous bases of nucleic acids [25,57].

It is to say that it is not always easy to distinguish between the two photo-processes, moreover they often occur together [58]: the properties of both PS and substrate, the environment and the PS concentration usually make the difference between the two. Some drawbacks need to be taken into consideration when pointing at one mechanism at the expense of the other, for instance tests to discriminate between intermediate species (*e.g.* $^1O_2$ and $O_2^{\bullet-}$) are not always reliable and conclusive, also given that they are carried out in an environment different from the biological one, and the detection of a small amount of a species does not necessarily refer to a prevailing mechanism.

PDT has been evolving as a promising therapeutic approach for certain types of localized cancers [59] (including skin, esophagus, lung and bladder cancers [60–63]), and was clinically approved for the treatment of some of them [64,65].

It is gaining momentum by virtue of properties such as a minor cross-resistance with other pharmaceuticals, due to different mechanisms of action [36], and a major selectivity, that limits the drug activity, thus the generation of toxic species, only at the site of light administration. This guarantees minimal side effects and extreme spatial and temporal control, also by virtue of the very fast reaction of singlet oxygen, that affects only the surrounding biomolecules. Moreover, PDT efficacy is not affected by radio- or chemo-resistance [59].

Nevertheless, the risk of occurring of resistance and cancer recurrence during PDT treatment is real (*vide infra*), yet it could be tackled by adopting several approaches. The combination of PDT with other therapeutic modalities is a major strategy [28], along with dual action compounds, possessing cytotoxicity both in dark and light [27], multi-targeting and dual-therapy ability [66]. An enhanced cancer-targeting could also be of help in overcoming resistance [26], as well as other specific mechanisms, such as the inhibition of enzymes involved in the degradation of anticancer drugs [67], the exploitation of the high concentration of a tumor intracellular component [68] or the conjugation with anti-cancer molecules [29,69].

An ideal PS for PDT should possess properties such as strong light absorption, photostability, amphipathic nature (so to cross cell membranes but still having good water solubility [70,71]), noticeable ISC efficiency for ROS production [72], photosensitizing efficiency, targeting tumor ability and in-depth penetration [73]. The PS should also have low to no toxicity in the dark, in order to fully control the toxicity of the system.

Most of the PSs currently applied in clinics belong to the tetrapyrrole family (porphyrins, phthalocyanines and chlorins), since they match some of the photophysical and biological requirements of a good PDT agent and also show fluorescent properties, providing theranostic abilities [74,75]. An advantage of conjugating a substrate with porphyrins is their ability to exploit the enhanced permeation and retention (EPR) effect, that allows for passive accumulation and retention in the tumor site; unfortunately, even if documented in murine models and humans, this topic is still controversial [76–79].

Among the photoactive organic tetrapyrrolic derivatives that have been clinically approved as PDT drugs are included Photofrin, Chlorin e6, Visudyne and Foscan (Fig. 2) [80]. Photofrin is the first approved PS for clinical PDT by FDA (1993) for the treatment of esophageal and non-small cell lung cancers, while Foscan has been approved in the UK for the treatment of several cancer types [53,81].

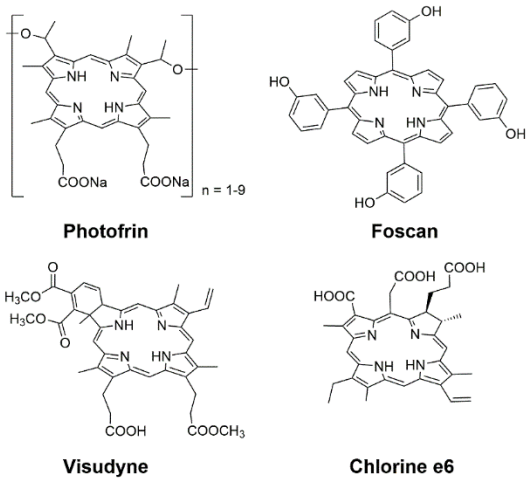


Fig. 2. Chemical structures of clinically approved or under-clinical developing photosensitizing agents: a) Porfimer Sodium (Photofrin), b) Temoporfin (Foscan), c) Verteporfin (Visudyne), d) Chlorine e6.

Nevertheless, porphyrins suffer by drawbacks that restricts their biological and medical applications [82] such as inadequate solubility, stability, pharmacokinetics and penetration depth in addition to low selectivity for cancer tissue, tendency to aggregate in water, slow clearance and, as a consequence, light sensitivity [83,84].

A promising alternative is represented by metal-complexes, four metal-based PSs being indeed clinically approved: WST11 (Pd(II)-based, approved for vascular-targeted of prostate cancer), Lutex (Lu(III)-based, lutetium texaphyrin, for cervical intraepithelial neoplasia), Purlytin (Sn(IV)-based, for age-related macular degeneration) and TLD1433 (first ruthenium complex approved as PS for cancer treatment with PDT and actually in phase II of clinical trials against non-muscle invasive bladder cancer, ClinicalTrials.gov Identifier: NCT03053635: TLD1433) [85–91] (Fig. 3). Besides TLD1433, other ruthenium complexes entered clinical trials, but containing Ru(III) instead of Ru(II) species: imidazolium(imidazole)-(dimethylsulfoxide)tetrachlororuthenate(III) (NAMI-A) [92,93], indazolium trans-tetrachlorobis(1H-indazole)ruthenate-(III) (KP1019) [94,95] and its sodium salt KP1339 [24,93,96]. While further clinical developments of NAMI-A and KP1019 were prevented by, respectively, low efficacy and low solubility, KP1339 and TLD1433 are currently undergoing clinical trials. Moreover, some new Ru(II) scaffolds are being investigated in preclinical studies for their anticancer activity, such as the ruthenium complex RAPTA-C in combination with erlotinib [97].

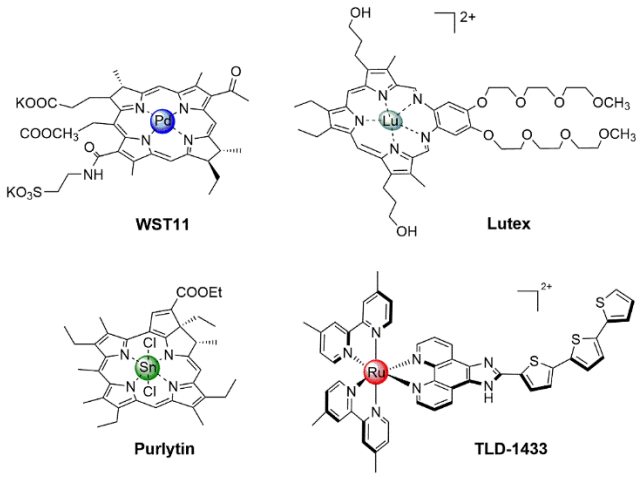


Fig. 3. Chemical structures of clinically approved or under-clinical developing metal-based photosensitizing agents.

Ru(II) complexes can be both obtained by inserting the metal center into conventional porphyrin-based PSs as well as by designing new ruthenium polypyridyl complexes (RPCs) [98].
RPCs represent the most promising class of PSs, showing many of the already cited favorable properties (good water solubility, easy clearance from the body [99], rich visible light absorption, high efficiency of ROS generation, stability and biocompatibility [100]). They show long-lived triplet metal-to-ligand charge transfer (^{3}MLCT) excited states upon excitation with visible light by virtue of the heavy atom effect [101,102]. Moreover, the choice of suitable ancillary ligands may favorably tune the photophysical and biophysical properties of the complexes [103–112]. The cytotoxicity may derive from different mechanisms, such as the production of singlet oxygen or other ROS, but also through the release of biologically active ligands or the generation of reactive intermediates capable of covalently bond to biological targets [113].
Many of the examples reported in the following are derivatives of the $[Ru(bpy)_3]^{2+}$ complex (Fig. 4), that often is taken as a comparison due to favorable properties such as a very high singlet oxygen quantum yield (Φ_Δ) (> 0.5 in CH_3CN, 0.2 in D_2O) [114].

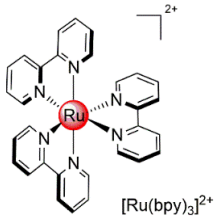


Fig. 4. Chemical structure of the $[Ru(bpy)_3]^{2+}$ complex.

Despite its clinical promise, the use of RPCs in PDT has not reached clinical applications yet, apart from the iconic McFarland's' compound TLD1433. This can be ascribed to the empirical nature of the clinical PDT protocols [115], but also to a series of limitations that may affect the three basic components of PDT, namely the PS (RPCs), light and oxygen. Among the most common drawbacks of PSs are the low solubility in water and biocompatibility, poor efficiency in the generation of cytotoxic ROS species, weak cellular uptake and targeting capabilities. Moreover, the white-light absorption by the residual PS may cause undesired side effects, such as skin sensitization, that force to maintain patients in the dark for long time periods following treatments. On the other side, a major issue that concerns the use of light may stem from its scarce ability to penetrate into tissues, especially when using PSs with non-optimal absorption profiles, whereas, regarding the dependence of PDT by molecular oxygen, this still represents a crucial obstacle to be overcome in order to allow for the application of PDT in hypoxic tumors.
Therefore, an intensive research work is currently devoted to overcoming the above-mentioned issues. As an example, Yoon and coworkers recently published a mini review in which they got through some innovative strategies which can make PDT effective even in hypoxic environments [116] (more details in chapter 2.3) and, in another survey, the same authors highlighted the potentiality of adopting novel supramolecular approaches to achieve enhanced photodynamic activities and lower adverse side effects [117].
The following section of this review is dedicated to the description of some of the most recent progresses that were achieved in the development of RPCs-based PSs for application in PDT. In light of the drawbacks described above, the RPCs are discussed on the basis of the three components of PDT, focusing on compounds designed to maximize the PS performances (paragraph 2.1), or to allow for the use of more penetrating radiations (paragraph 2.2) or finally to go beyond the PDT reliance on oxygen (paragraph 2.3).
Lastly, we would like to bring to the Reader's attention many causes of reflection coming from a McFarland's recent review, that in our opinion should be taken into account when planning a research study on PSs for PDT [25]. The review introduces transition metal complexes used in PDT from a tumor-centered approach, trying to highlight what is missing and what is possible to do to bring more clinical efficacy to this technique and what structural features control the nature of the triplet excited state of Ru(II) complexes. Basing on a classification of PSs starting from Photofrin to the next generations of PSs, McFarland highlighted the drawbacks that each successive step tried to overcome (mainly scarce tissue selectivity and low absorption of red light) and concluded that the desired improvements cannot be usually obtained simultaneously. At the same time, some clues were derived that may help the development of more efficient systems, such as the need for single agents instead of mixtures of compounds, the importance of increasing the

selectivity for cancer cells and the hydro-solubility of compounds, also by the use of formulations, and the necessity for longer absorption wavelengths, without decreasing but preferentially enhancing the absorption intensity. Many published works listed the "ideal" properties a PS should possess, but McFarland reported that no current PS presents all of them at the same time and even if such a candidate did exist, it would not been suitable for all cancers and phenotypes. Moreover, a more realistic interpretation of the listed criteria is proposed in the review. For example, the ideal requests for i) an efficient generation of $^1O_2$ and ii) a strong absorption in the PDT window (700-900 nm) have been re-stated as the requests for i) $^1O_2$ quantum yields remaining high even in hypoxic environment and for ii) molar absorption cross sections being large in a clinically useful wavelength range. This implies, for example, that PSs able to work with either an oxygen-dependent or -independent mechanism are strongly desired. Moreover, they rationalized that some π-extended Ru(II) complexes, in the past considered to be non-suitable for PDT, being their absorption outside the therapeutic window, could be instead appropriate candidates *via* low-lying spin-forbidden ^{3}IL states, that could be populated with red light to give phototoxic effects. Anyway, a very desirable property of a PS is to be panchromatic, in other words activable at any wavelength (vis-NIR), allowing a therapy to be tailored. Finally, many papers are devoted to studies on organelle specific PSs, but McFarland pointed out that such an ability, even if interesting, is useless if the compound is not able to reach the target tissue or the oxygen level is not sufficient or light does not penetrate where it is needed. In the following some recent examples will be reported, but we agree with McFarland observations, so we suggest to always bear them in mind when considering a particular case.

2.1 Performance of the PS in the PDT approach

2.1.1 Increasing the cellular uptake

The cellular uptake may occur through different mechanisms, both energy-dependent and independent [111,118], and depends on a subtle interplay between properties such as hydrophilicity/lipophilicity, overall charge and steric hindrance of the complexes, so the modulation of the uptake is crucial to the PDT efficacy, and can also drive the intracellular localization.

Among the strategies that have been adopted in the recent years to increase the uptake is included the encapsulation of RPCs into proteins [119], polymers or carriers (*e.g.* nanoparticles) [120,121], but even if this is a common approach, it goes beyond the scope of the present review. In this section we focused our attention on some recent RPCs that were optimally designed to evaluate how the presence of specific ancillary ligands and their chemical modifications can influence the cellular uptake, in an effort to improve the outcomes of these systems as PDT agents. The structures of the complexes discussed in this section are reported in Fig. 5.

Different types of fragments can be attached to the coordinated ligands to tune the hydrophilicity/lipophilicity and the bio-compatibility of the complexes: i) *PEG moieties* have been inserted in a series of ruthenium(II) phthalocyanines (RuPcs) (**1a-c**) [122], where, notably, the least hydrophilic compound **1c** proved the best cytotoxicity toward bladder cancer cells upon irradiation with red light, notwithstanding its lowest cellular uptake within the series. The Authors suggested that the highest intracellular solubility of **1c** could account for a more efficient generation of ROS; ii) *amino acids* such as tyrosine and tryptophan have been conjugated to $[Ru(bpy)_2(dmbpy)]^{2+}$ complexes (**2a** and **2b**, respectively) [123], with the more lipophilic **2a** performing better as a PDT agent than **2b** in 2D and 3D cellular models *via* the formation of $^1O_2$, cleavage of DNA and apoptosis; iii) *long alkyl chains* (**3a,b**) [124] and iv) *halogen groups* (**4a-e**) [125] have been introduced into the structure of RPCs revealing that if the bulky substituents prevent the interaction with the target the PDT activity does not increase, notwithstanding the enhanced cellular uptake [125].

Zhu and coworkers recently reported the first example of ion-pairing between a RPC ($[Ru(TMP)_3]^{2+}$, **5**) and lipophilic counterions (a series of 19 chlorophenols) as a successful strategy to enhance the cellular uptake, providing a new mechanism that could be exploited for cell-impermeable RPCs [126].

Another strategy used to enhance the lipophilicity of the complexes is cyclometalation, which could induce dramatical changes in the cellular uptake and localization of the complexes: some examples are reported in paragraph 2.2.1.2.

A series of RPCs containing sulfonate groups (**6a-d**) [127] with similar photophysical and photochemical properties was chosen to study the effect of the different overall charges on cytotoxicity, showing a decrease of the dark toxicity moving

from the cationic to the neutral to the anionic complexes while light irradiation produced a similar and enhanced cytotoxicity for all complexes, indicating a similar and fast cellular uptake and $^1O_2$ production. The overall charge also affected the cellular localization of the complexes [127]. The study of a series of ruthenium-based polyamidoamine polymers with different overall charges revealed instead an increased PDT efficiency for the cationic compound Ru-PhenAN (**7a**) compared to the anionic Ru-PhenISA (**7b**). This is probably due to the unusual ability of a macromolecule such as **7a** to accumulate in the nucleus [128].

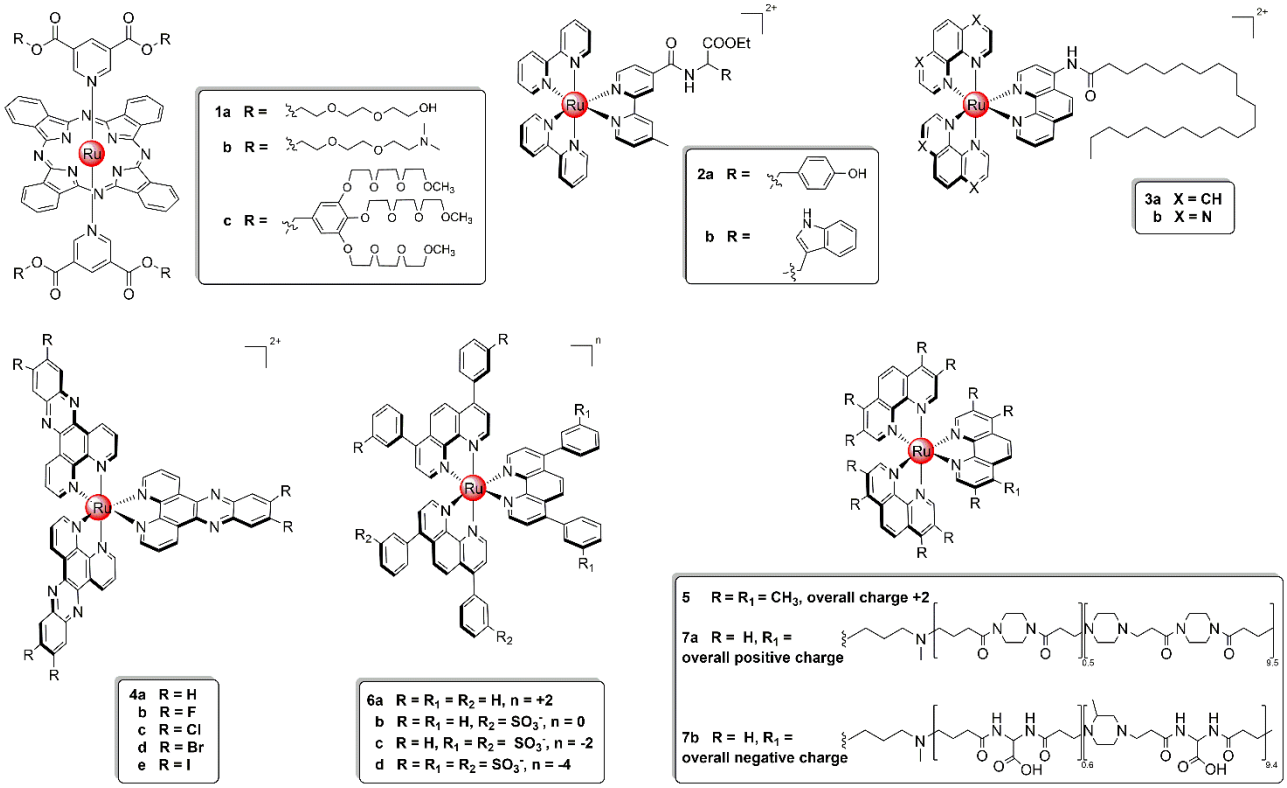


Fig. 5. Chemical structures of the complexes discussed in paragraph 2.1.1.

2.1.2 Changing functional groups and steric hindrance

The photophysical, photochemical and photobiological properties of the ruthenium(II) complexes can be optimized by changing the ligands coordinated to the metal center or the functional groups attached to them. Besides the cellular uptake, from ancillary ligands depend also key properties such as the charge distribution and the mode-of-action of light-triggered toxicity. The variation of ligands at the axial positions of RPCs may indeed switch from a complex with potential application as a PS (**8a**) to a complex with no photosensitizing potential (**8b**) [129]. The structures of the complexes discussed in the following are reported in Fig. 6.

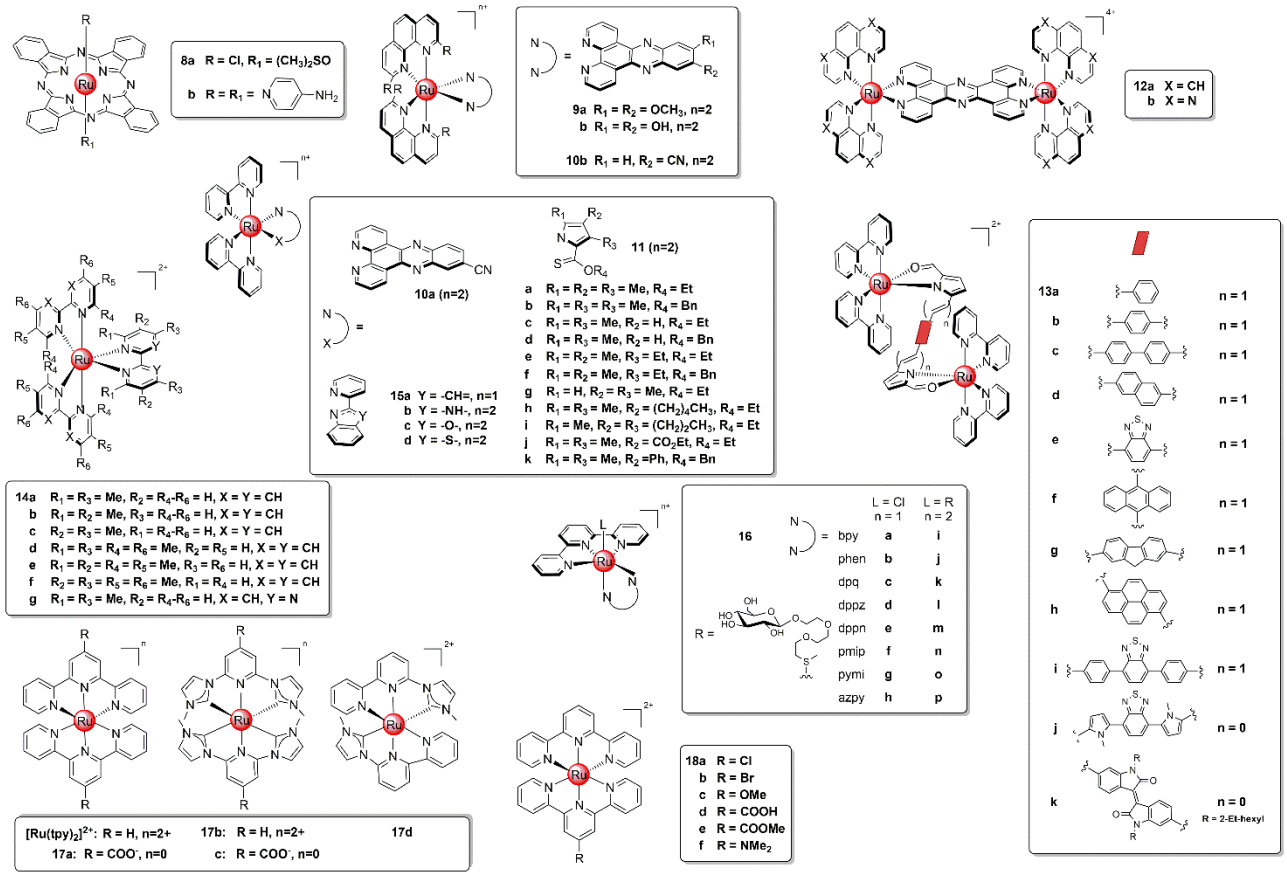


Fig. 6. Chemical structures of complexes discussed in paragraph 2.1.2.

Even a small structural variation can deeply affect the biological properties of RPCs. The 7,8-dimethoxy (**9a**) or -dihydroxy (**9b**) derivatives of $[Ru(phen)_2(dppz)]^{2+}$ show different cellular uptake and localization, dark toxicity and ability to penetrate HeLa multicellular tumor spheroids (MCTSs), with **9a** performing better in all respects than **9b** [130]**.** Quite the opposite, the 7-cyano derivatives of $[Ru(bpy)_2(dppz)]^{2+}$ (**10a**) and $[Ru(phen)_2(dppz)]^{2+}$ (**10b**) from the same group did not show any photocytotoxic effect, due to low $^1O_2$ production [131]. McFarland *et al.* investigated the variations brought on a series of heteroleptic ruthenium(II) complexes (**11a-k**) bearing substituted pyrroles decorated with ethyl and benzyl thionoesters [91]. All compounds embody promising alternatives to traditional chemotherapeutics, being more cytotoxic than cisplatin in both cancer cell lines studied (HL-60 and SKMEL28 cells); cytotoxicity within the series depends both on the substitution pattern about the pyrrole ring and on the type of thionoester, with groups in R2 and R4 mutually conditioning, as well as on the cell line employed, with **11b**, **11j** and **11h** emerging as the best *in vitro* PDT agents [91].

The substitution of phen co-ligands in $[\{Ru(phen_2)\}_2(tpphz)]^{4+}$ (**12a**) with TAP to give the $[\{Ru(TAP_2)\}_2(tpphz)]^{4+}$ complex (**12b**) has been reported by the Thomas group [132], revealing a different photochemical behavior. The new complex is indeed emissive in both aqueous and CH_3CN solution, with a TAP-based ^{3}MLCT emissive state, contrarily to the parent compound (**12a**), which is quenched in aqueous solution and shows a tpphz-based ^{3}MLCT emissive state. **12b** is able to strongly bind to both duplex and quadruplex DNA, leading to the quenching of the photoexcited state, along with the generation of guanine radical cation sites as photo-oxidization products [132]. Moreover, due to its photodamaging mechanism independent of ROS and $^1O_2$ generation, its localization in PDT specific sites (nuclei and mitochondria) and the high two-photon cross-section (TPACS, σ_2 = 90 GM), **12b** is a good candidate as PS for PDT at tissue depths, hence in hypoxia conditions. The complex can be also considered a theranostic agent, since it possesses its own luminescence through which the tissue penetration can be directly monitored. In a tumor spheroid of the human skin cancer C8161 melanoma (complex 100 µM), the irradiation at 60 mW with 900 nm laser light produced the eradication of the proliferative region after only 15 min of light exposure, with luminescence observed throughout the thickness of the investigated sample up to ~240 ± 20 µm, even if with a gradient, and phototoxicity also obtained in hypoxic environment [133].

The McFarland group investigated a series of bis[pyrrolyl Ru(II)] complexes (**13a-k**) focusing on the role the central organic chromophore plays on the spectroscopic and biological properties of the compounds [134]. Depending on the chromophore, the compounds span a broad range of activity, moving from *in vitro* phototoxic agents to traditional cytotoxic agents. Systems with conjugated aromatic hydrocarbon linkers (pyrene **13h**, fluorene **13g**, naphthalene **13d**) possess the largest PIs, with **13h** proving as the most potent PS for *in vitro* PDT, due to a phototoxicity index (PI: IC_{50} in the dark/ IC_{50} upon light irradiation) of 27000 *vs* >6000 and >3500 for **13d** and **13g**, respectively (broadband visible light dose, 100 J cm^{-2}, 28 mW cm^{-2}) and a high generation of singlet oxygen. **13h** has one of the larger PI reported to date and is also active towards HL-60 tumor spheroid model and bacteria (*S. mutans* and *S. aureus*) [134].

The insertion of ligands able to introduce strain within the complex may lead to their dissociation upon irradiation. Such a strategy could be exploited to turn the photoactivity toward PACT. Only few examples are discussed here, many others will be presented starting from chapter 3. Glazer *et al.* studied a series of seven Ru(II) complexes (**14a-g**) [113] containing isomeric tetramethylsubstituted bipyridyl and bypyrimidin-type ligands, five of which (**14a,b,d,e,g**) featuring strain-inducing methyl groups that make them susceptible to ligand loss upon irradiation through the activation of a photodissociative pathway from a ^{3}MC excited state, becoming prone to form covalent bonds to biomolecules. Compounds **14c** and **14f** are instead unstrained and possess long-lived ^{3}MLCT excited states, which make them emissive and able to produce singlet oxygen. A structure−activity relationships study revealed that: i) the bis-heteroleptic complexes are always more cytotoxic than tris-homoleptic ones, whatever the mechanism; ii) the ligand ejection is faster for heteroleptic compounds and the dark toxicity is lower; iii) the bipyrimidine compound **14a** is less potent compared to the corresponding bipyridine one **14g**; iv) the more methyl groups number the more potency, but also dark activity; v) surprisingly, even if the compounds do not show absorption in the red region of the spectrum, some of them can be activated with red light, showing cytotoxicity (**14a, 14b**) and production of singlet oxygen (**14f**) [113].

Bioisosteric substitutions have been investigated in a series of Ru(II) complexes with 2-(2-pyridyl)benzazole groups containing either two dmphen or two bpy ancillary ligands (**15a-d** and **90a-d**, see paragraph 3.2 and figure 34) [135] that confer the compounds, respectively, the strained (**90a-d**) or unstrained (**15a-d**) attribute. Whilst the former are active both under dark and light conditions, the latter are toxic against leukemic cells only upon light irradiation. Compounds **90a** and **15a** showed the lowest IC_{50} values upon light irradiation (0.034 and 0.18 μM, Loctite Indigo LED array (≈ 450 nm), light dose of 29.1 J cm^{-2}), undergoing ligand ejection, while **15d** and **15c** displayed the highest PIs (88 and 224) and are able to generate singlet oxygen, even if this mechanism is not thought to be the only one. The compounds are thus to be considered both for PDT and for PACT [135].

The Bonnet group investigated the potential for either PDT or PACT of eight terpyridine chloride complexes (**16a-h**) and their thioether-glucose conjugates (**16i-p**) of general formula $[Ru(tpy)(N\text{-}N)(L)]^{+/2+}$ (N-N=bpy, phen, dpq, dppz, dppn, pmip, pymi or azpy, $L=Cl^-$ or 2-(2-(2-(methylthio)ethoxy)ethoxy)ethyl-β-d-glucopyranoside), where the choice of the ancillary ligand makes the complex more suitable for photocaging (see also paragraph 3.2), PACT or PDT [136]. Most chloride complexes resulted non-toxic in the dark, so were most of the glycoconjugated complexes, for which the uptake is hampered by the highly hydrophilic nature of the conjugates. The only exception is [**16m**]$(PF_6)_2$ that can be considered an excellent PDT agent, even if a PACT activity could not be ruled out. The authors tried to overcome this issue by changing the protocol and allowing for the photoactivation outside the cell, followed by cellular uptake. This strategy resulted in an increased PI for [**16l**]$(PF_6)_2$, that can be suggested as a PACT agent. The present study suggested the five complexes [**16a**]Cl–[**16c**]Cl, [**16f**]Cl and [**16g**]Cl for the photocaging of thioether-based biologically active compounds, while [**16d**]Cl and [**16e**]Cl, featuring the known intercalating agents dppn and dppz, as potential PDT or PACT agents. Complex [**16f**]Cl shows an exceptional cellular uptake as well as production of singlet oxygen, both superior to [**16d**]Cl and [**16e**]Cl, but not accompanied by cytotoxicity, and [**16h**]Cl does not result suitable either as PACT carrier or for phototherapy, due to prevention of ^{3}MC excited state population [136].

Most Ru(II) complexes for PDT feature three bidentate ligands having long-lived excited states; on the contrary, systems with two tridentate ligands are less common since they mostly exhibit low quantum yields and very short excited-state lifetimes, not being able to efficiently produce singlet oxygen. Nonetheless, they offer the advantage of being achiral, avoiding the possible formation of isomers with different interactions with chiral biological targets. Glazer *et al.* investigated bis-tridentate Ru(II) complexes involving N-heterocyclic carbene (NHC) ligands (**17b-d**) in comparison with terpyridine analogues ($[Ru(tpy)_2]^{2+}$, **17a**) [137], the former showing more favorable excited-state lifetimes, $^1O_2$ production and photocytotoxicity than the latter upon irradiation at 405 and/or 450 nm, according to their absorption

profile (**17b**: 405 nm; **17c**: both wavelengths; **17d**: 450 nm) (Fig. 7). **17b-d** represent the most potent light-activated bis-tridentate complexes reported so far (IC_{50}/PI in HL-60 cells for **17b-d**, respectively: 12.5 μM/24 (405 nm); 3.5 μM/86 (405 nm) and 5.6 μM/54 (450 nm); 9.1 μM/37 (450 nm)) [137]. Gasser and co-workers recently reported a systematic investigation of Ru(II) tpy-based complexes (**18a-f**) [138], that generally show favorable excited state lifetimes and luminescence only at low temperature (77 K), but still are able to bind to DNA *via* different interaction types (electrostatic interactions, intercalation and groove binding) and cleave it upon light irradiation. The study demonstrated cytotoxicity in the micromolar range only for complex **18f**, but without selectivity between cancer (HeLa) and healthy (PE-1) cells, probably due to the very short excited state lifetimes of this complex [138].

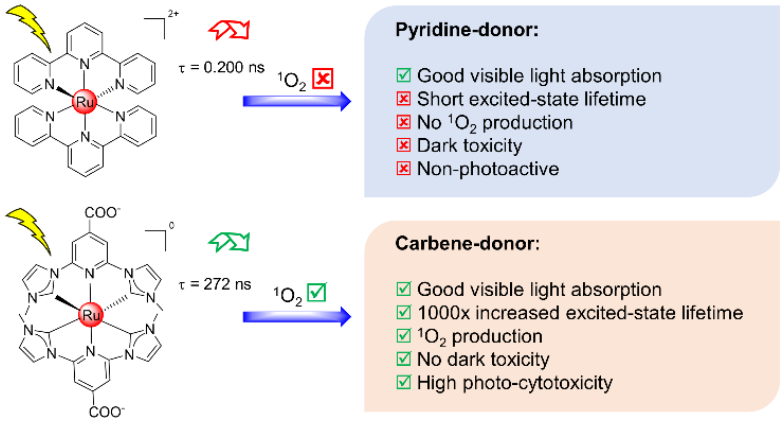


Fig. 7. Comparison between characteristics of N-heterocyclic carbene ligands and terpyridine analogues (complexes $[Ru(tpy)_2]^{2+}$ and **17c**).

2.1.3 Conferring targeting ability

2.1.3.1 Cancer-targeted selectivity

An ideal PS should accumulate primarily in the tumor cell and show a high phototoxicity index, in other words it should be non-toxic in dark but able to acquire toxicity in the presence of suitable light. However, a common drawback of PSs used in PDT is that they often suffer from poor selectivity toward cancer cells and inefficient activation of cell death during cancer treatment. This would lead to the increase of the dosage requested by the PS to be effective and, consequently, to the occurrence of off-target toxicity, thus limiting the *in vivo* application [139]. In this paragraph are described some of recent examples of RPCs developed in an effort to improve this specific limitation: the relative structures are reported in Fig. 8-10.

The employment of PSs with enhanced tumor-specific uptake or photosensitization is therefore highly desirable. To reach the goal, PS structures can be engineered as to take advantage of the peculiar physiological properties of tumors, exploiting the over- or specific expression of particular receptors on the cancer cell [26,140–143], the key role [144] or the higher concentration of some biological agents in the tumor cell [68] and the affinity for DNA [145].

Molecules such as tamoxifen [140], biotin [26,146,147] and the Arg-Gly-Asp peptide c(RGDfK) [141] have been conjugated to RPCs to target compounds **19-21** toward cancer cells overexpressing, respectively, receptors for estrogen (ER; MCF-7 ER+ breast cancer cells), biotin (BR; A549R cisplatin resistant cancer cells) and $\alpha_v\beta_3$-integrin (Fig. 8). The latter are highly expressed on activated endothelial cells and many tumor cells but are neither expressed in resting endothelial cells nor in most normal cells. The tamoxifen conjugate **19** shows selective ER-mediated uptake: its dark toxicity is low toward MCF-7 cells, so is the phototoxicity toward ER- breast cancer cells and non-cancerous cells, whereas significant phototoxicity is observed toward MCF-7 cells, causing cell death by late apoptosis or necrosis and disruption of lysosomes [140]. The biotin conjugate **20** shows similar photophysical properties and phototoxicity to the

biotin-free analogue, but is much more selective for BR-overexpressing A549R cancer cells compared to normal HLF-a cells, both in 2D and 3D models [26]. Biotin-conjugation proved successful as tumor-targeting strategy also in many other cases (see complexes **34c,e**; **37d**, *vide infra* [146,147]). The peptide conjugate **21** accumulates in the mitochondria of $\alpha_v\beta_3$-integrin-overexpressing U87MG cancer cells and induces cell death by apoptosis upon irradiation, being a promising PDT therapeutic both in 3D MCTSs and *in vivo* models [141]. All three complexes are efficient two-photon PSs and generators of singlet oxygen.

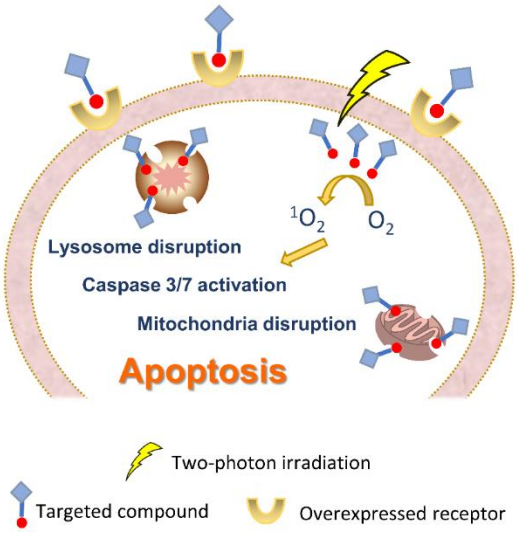


Fig. 8. Schematic illustration of the mechanism of targeted compounds **19**, **20** and **21** in estrogen receptors-, biotin receptors- and $\alpha_v\beta_3$-integrin-overexpressing cancer cells, respectively.

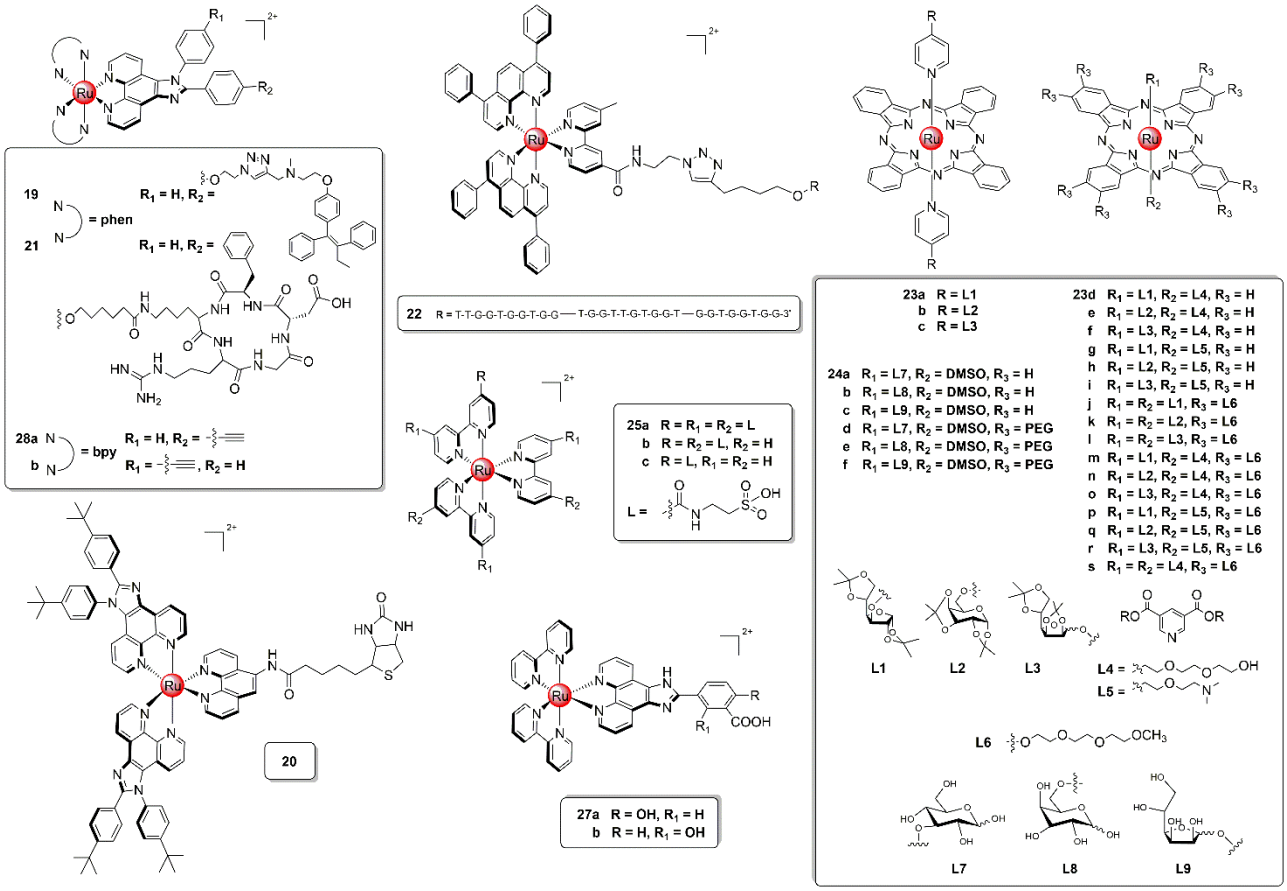


Fig. 9. Chemical structures of some complexes discussed in paragraph 2.1.3.1.

The Gasser group very recently reported the first example of a RPC directly conjugated to an aptamer (AS1411), **22**, able to selectively target MCF-7 cancer cells *via* the nucleolin protein, that is expressed in the nucleoli of cells and thus overexpressed in a wide number of cancer cell lines [148]. Complex **22** was synthesized capitalizing the photosensitization properties of a previously reported complex from the same group (*vide infra*, complex **46f** [149]) by covalently linking the 5'-end of the AS1411 aptamer to the Ru(II) complex via the insertion of an azide linker [148].

The conjugation of carbohydrates is also a possible strategy to gain entry into tumor cells, due to the overexpression of carbohydrate-binding proteins, such as galectins and glucose transporters GLUT1 and GLUT3. Ketal-protected (**23a-s**) [142] and deprotected (**24a-f**) [143] carbohydrate moieties (glucose, galactose and mannose) have been introduced at the axial positions of RuPcs, some of them also featuring PEG units either on peripheral sites of Pcs or/and at axial positions to increase the solubility in aqueous medium. Unfortunately, in both cases the carbohydrate functionalization did not return a better tumor cellular uptake, but positively impacted the phototoxicity, with **23d-i** (PS9–14) best performing within the protected series and **24a-c** within the deprotected one [142,143].

Conjugation with taurine can be exploited to selectively target brain cancerous cells [144]. Among the three taurine-functionalized $[Ru(bpy)_3]^{2+}$ complexes **25a-c** those displaying multiple and symmetrical substitutions **25a-b** show the best selectivity and lifetime; **25a** in particular exhibits phototoxicity upon excitation at 458 nm through production of ROS being active against the F98 brain cancer line [144].

Chao *et al.* exploited the higher intracellular GSH concentration in cancer cells than in normal cells to build the first example of a GSH-activable dinuclear ruthenium(II) complex (**26**) [68] as PS for two photon PDT (TP-PDT) and increase the tumor targeting ability and selectivity of the PS. Such a strategy favors both the selective intracellular drug activation, due to the GSH-based reducing microenvironment in tumor cells, as well as the overcoming of drug resistance, due to an elevated level of intracellular GSH. The reduced form of the Ru(II) complex $\mathbf{26_{red}}$ is generated by intracellular GSH in HeLa cells upon both one-photon excitation (450 nm) and two-photon excitation (810 nm). It shows low and similar cytotoxicity in the dark both in 2D and 3D cell models, but it becomes much more toxic to MCTSs under two-photon irradiation (PI = 19) *via* production of ROS, being able to inhibit cancer growth and kill cancer cells in a 3D model. The oxidized form $\mathbf{26_{ox}}$, on the contrary, is not able to produce singlet oxygen [68] (Fig. 10).

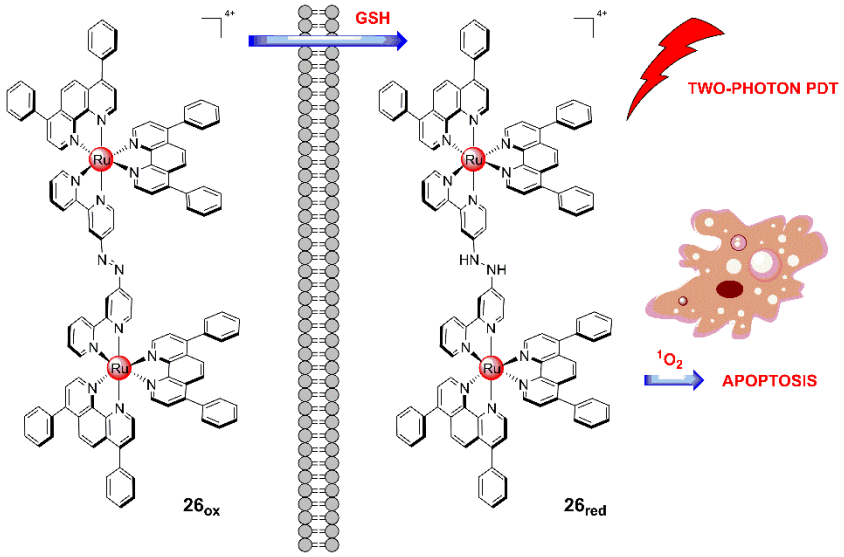


Fig. 10. Schematic illustration of GSH activation and phototoxicity of compound **26**.

The insertion of phenanthroimidazole moieties produced the two RPCs $[Ru(bpy)_2(phcpip)](ClO_4)_2$ (**27a**) and $[Ru(bpy)_2(ohcpip)](ClO_4)_2$ (**27b**) with selective phototoxicity toward cancerous cell lines due to their strong molecular binding to DNA [145].

Interestingly, Chao and coworkers recently reported two new RPCs (**28a,b**) containing an alkyne group at different positions as the first examples of Ru(II) complex-based bioorthogonal two-photon PSs [150]. They are active against triple-negative breast cancer (TNBC), which is one of the most malignant breast cancer types where three of the most

common therapeutic targets are lacking (estrogen receptor, ER, progesterone receptor, PR, and human epidermal growth factor receptor-2, HER2). For this reason, the Authors used a bioorthogonal labelling strategy to selectively label biomolecules *via* the copper-catalysed azide–alkyne cycloaddition (CuAAC) reaction. Featuring a faster reaction rate, **28b** resulted the more promising candidate and proved able to bind the artificially labelled plasma membrane of TNBC cells, provoking a strong damage of the membrane upon irradiation. **28b** is an efficient singlet oxygen generator and two-photon PS both in monolayer and 3D MCTSs, showing high selectivity for TNBC compared to normal cells.

2.1.3.2 Targeting DNA and organelles

Due to the higher proliferation rate of cancer cells over healthy cells, that is regulated by DNA, increasing the affinity for DNA could preferentially drive the complex to tumor cells. The metal center could act as an electrophile binding site for the nucleophilic functional groups of DNA, while the insertion in the complex of aromatic units could favor the interaction with DNA: covalent binding as well as electrostatic, intercalating and groove binding are the main modes of interaction between metal complexes and DNA.

Different approaches to target DNA revealed promising. A supramolecular strategy allowed to obtain a complex able to target and photocleave DNA by self-assembly of hexa-β-cyclodextrin appended ruthenium polypyridyl (6CD-Ru) and adamantane-modified anthracene (ADA-AN) in water (**29**, Fig. 11) [151]. The appended CD moieties provide the complex with good water solubility, while the six anthracene groups increase the affinity for DNA *via* intercalation, consequently enhancing the photocleavage ability upon irradiation with visible light [151]. The tetra-positively charged complexes **30a,b** exploit instead the electrostatic interactions to bind to DNA *via* cationic peripheral Ru(II)-complexes and anionic phosphate groups, favoring a partial insertion of Ru(II) portions into the DNA backbone [152] (Fig. 12). In **30b** strong interactions form between the Zn(II)-macrocycle core and the DNA minor groove. Both complexes can photocleave the DNA molecule in the presence of O_2, indicating a catalytic mechanism dependent on ROS [152]. Many other examples of Ru(II) compounds able to bind to DNA and to damage it are reported throughout this review [67,123,158–164,132,138,145,153–157].

Addressing the PDT agent to a specific organelle could also be a good strategy to increase the selectivity for cancer cells. Altering the lipophilicity and the overall charge by varying ancillary ligands and functional groups or by cyclometalation could dramatically change the localization of the complex. Nevertheless, an increased lipophilicity could result in a higher affinity of the complex for serum proteins: this aspect should be taken into account, since it could be either an unwanted or a desired feature, depending on the intended application of the complex.

Mitochondria and nucleus are popular targets: considering the lipophilicity and negative potential of the mitochondrial outer membrane, lipophilic cations such as Ru(II) complexes are able to accumulate into mitochondria. The addition of chloroalkyl units, such as the alkylating agent Chlorambucil (**132**, *vide infra* [69]) or chloromethyl groups (**66a-c**, *vide infra* [165]), can favor the selective uptake by mitochondria, as well as the conjugation with a peptide (**21**, *vide supra* [141]) or the insertion of thiophenyl groups (**50a**, *vide infra* [162]), that revealed as good strategies to specifically target mitochondria. Many other examples of complexes that selectively accumulate in mitochondria and nucleus have been reported throughout this review [28,113,121,160]; nevertheless, it should be taken into account that localization in the former could translate into dark toxicity, while in the latter could cause unfavorable DNA mutation [109,168]166. Targeting different organelles is therefore highly desirable.

To this purpose, lysosomes are to be considered alternative and interesting targets: due to their detoxification function towards metallic chemotherapeutics, they are able to reduce off-target toxicity. Moreover, since lysosomes represent endocytosis final destination, hydrophilic cationic metal complexes, that are not able to diffuse into cells but can enter cells through endocytosis, can be exploited to target these organelles (**19**, **135**, *vide supra* and *infra* [29,140]).

The insertion of a lysosome targeting group such as morpholine drives two new water soluble organo-metallic Ru(II)–arene complexes ([Ru(η^6-p-cymene)(L)Cl_2], **31a**, and [Ru(η^6-p-cymene)(L)(PTA)Cl], **31b**) to both the nucleus and lysosomes. The two complexes proved as innovative PDT agents able to generate singlet oxygen, damaging cancer cells to death by apoptosis [169] 167 (Fig. 12). In the complexes, the ligand L is made up of a naphthalimide-based fluorescent probe linked to the morpholine unit, while the role of PTA is to increase the amphiphilic character and reduce the hydrolysis rate, besides having DNA damaging properties. The complexes resulted photocytotoxic under low power blue LED light (448 nm) and could be considered as theranostic agents, due to their cellular imaging potential. Also the

insertion of BODIPY into Ru(II)-complexes has frequently been reported to drive the localization to lysosomes (**35**, *vide infra* [170] 168)

While several examples of Ru(II) complexes targeting lysosomes are reported in this review, only a few reports on Golgi-apparatus-targeting metal complexes are present in the literature so far [171–174] 169-172 and none of these contain Ru(II). Monitoring the Golgi apparatus by using luminescent metal-complexes is considered to be a helpful and innovative strategy to understand the cellular response to DNA damage, that leads indeed to dispersal of the apparatus throughout the cytoplasm. Wu and coworkers presented a new complex using a Ru(II) pyridine succinimidyl ester complex and sphingosine lipid (**32**, Fig. 13) [115]; the complex is taken up by HeLa, A549 and MCF-7 cells by endocytosis and specifically accumulates in the Golgi-apparatus. Upon irradiation at 488 nm its cytotoxicity is activated through production of singlet oxygen and the Golgi apparatus gets damaged, finally resulting in its dispersal, with evident cellular morphological changes [115].

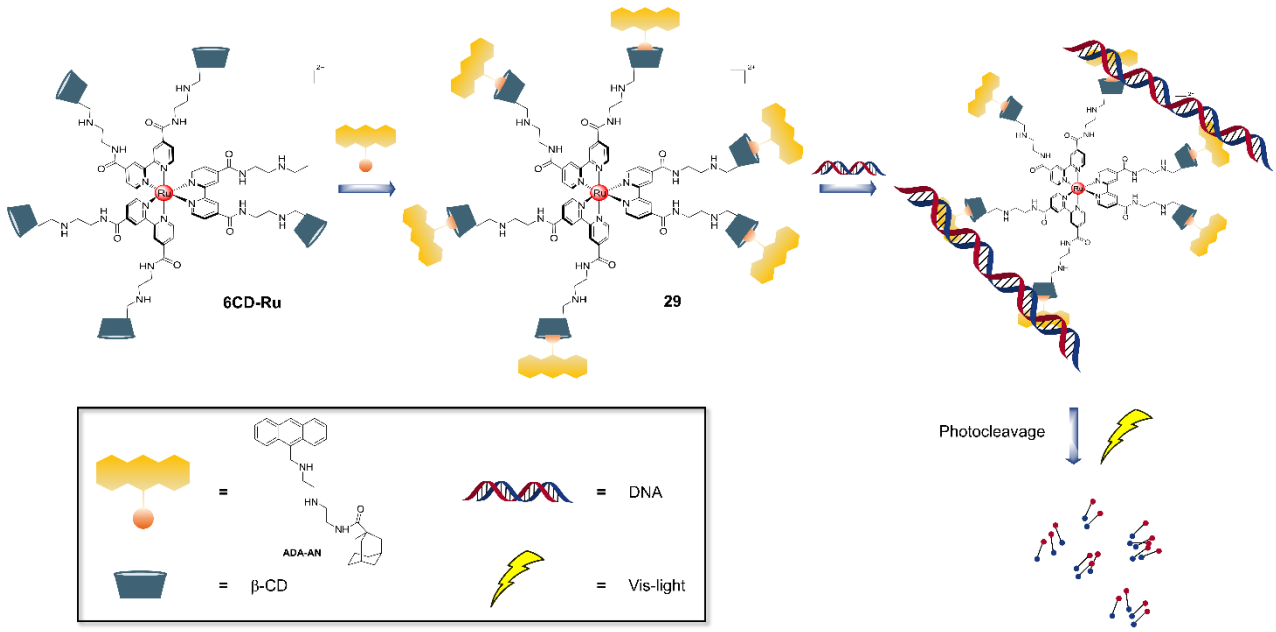


Fig. 11. Schematic representation of intercalation and photocleavage of DNA using compound **29**.

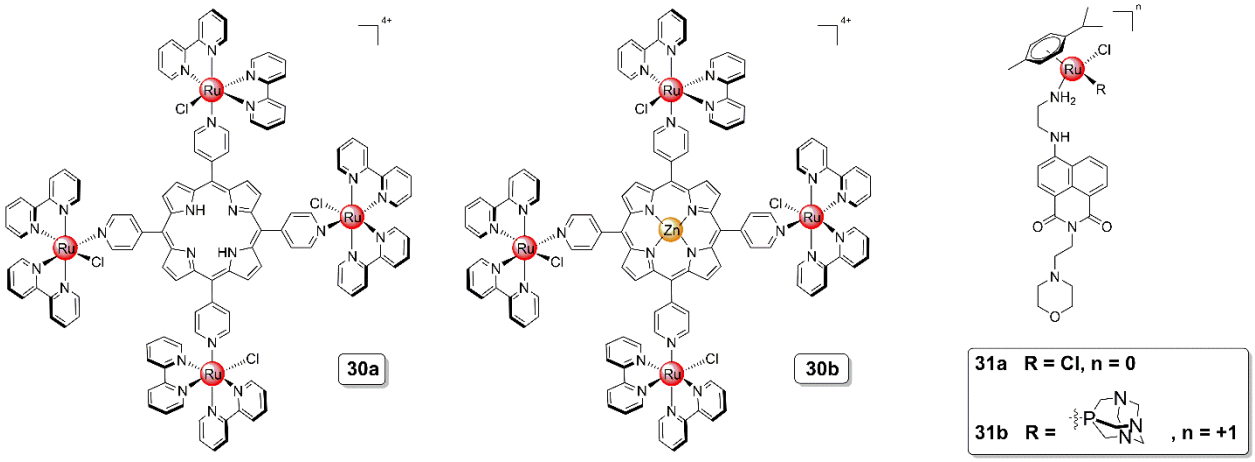


Fig. 12. Chemical structures of complexes **30a,b** and **31a,b**.

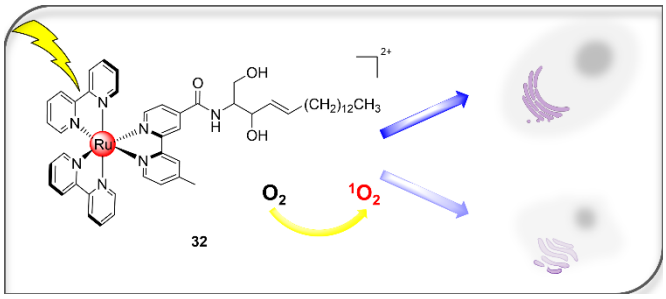


Fig. 13. a) luminescent emission of compound **32** and b) its Golgi apparatus localization along with PDT treatment effects.

2.2 The role of light in the PDT approach

The key role of a PS for PDT is the light absorption, aimed at producing singlet oxygen or other typologies of ROS. Light should be administrated in the red or NIR region of the spectrum (phototherapeutic window, 600–900 nm), so to guarantee sufficient PDT efficacy in deep tissues and concurrently avoid damage from high energy light irradiation [170,175–178] 168, 173-176. A common drawback of PDT approaches arises indeed from the fact that the requested light to activate the PSs does not fit such a requisite and its intensity necessarily drops when crossing thick tissues to reach deep tumor sites [53]. Most photoresponsive materials are in fact activable only employing UV or short-wavelength visible light, limiting biomedical applications due to the scarce tissue penetration [177] 175 and, as for UV light, to their damage to biological systems.
On the other hand, the PDT efficacy depends on the ROS yield, that in turn is a result of the triplet excited state lifetime of the PS. Therefore, a shift toward the therapeutic window, along with an enhanced absorption in the same region [175,179] 173,177 and the extension of the triplet excited state lifetimes, are desirable properties for suitable PDT-based RPCs to be possibly used to treat deeply seated tumors.

2.2.1 Boosting visible light harvesting properties

2.2.1.1 Appending chromophores

Appending a strong absorbing molecule, such as a chromophore, to the ligand could be a successful strategy to obtain a PS activable in the NIR region of the spectrum.
In this sense, BODIPY is one of the most investigated chromophores by virtue of peculiar qualities, like easy functionalization, strong absorption in the visible and NIR regions, narrow emission, good photostability and negligible photobleaching [180–182]178-180. The structures of the BODIPY-based complexes discussed in the following are reported in Fig. 14-15.

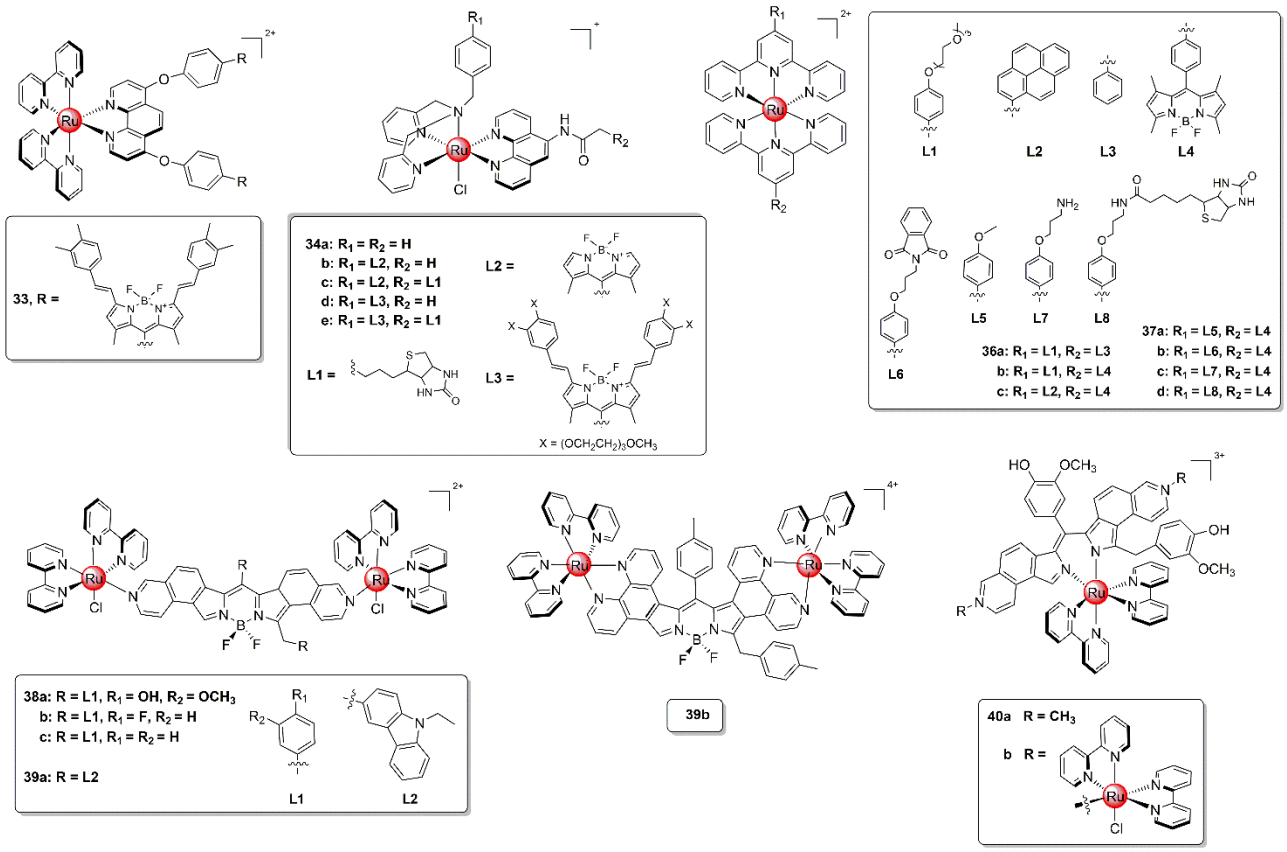


Fig. 14. Chemical structures of BODIPY-based complexes discussed in paragraph 2.2.1.1.

Several Ru(II) complexes decorated with two distyryl BODIPY units absorbing in the red region of the spectrum and being phototoxic have been reported by different groups (**33-35**). Amomg them, **33** performs better as for singlet oxygen generation than both the BODIPY dye alone and the Ir(III) analogue, notwithstanding the distance between the coordination center and BODIPY unit, that, on the contrary, hampers the performance of the Ir(III) analogue [183] 181. **34e** features, besides PEGylated-BODIPY, a tumor-targeting biotin moiety and exhibits both high PI (>5000 at 600−720 nm in A549 cancer cells) as well as large singlet oxygen quantum yield (Φ_Δ = 0.65 in DMSO) [146]. Notably, the lysosome-targeting and NIR absorbing Ru(II)–BODIPY conjugate **35** was found to delay and inhibit tumor growth in *in vivo* experiments (BALB/c mice bearing A375 tumor xenografts) while revealing a nontoxic essence (Fig. 15) [170] 168.

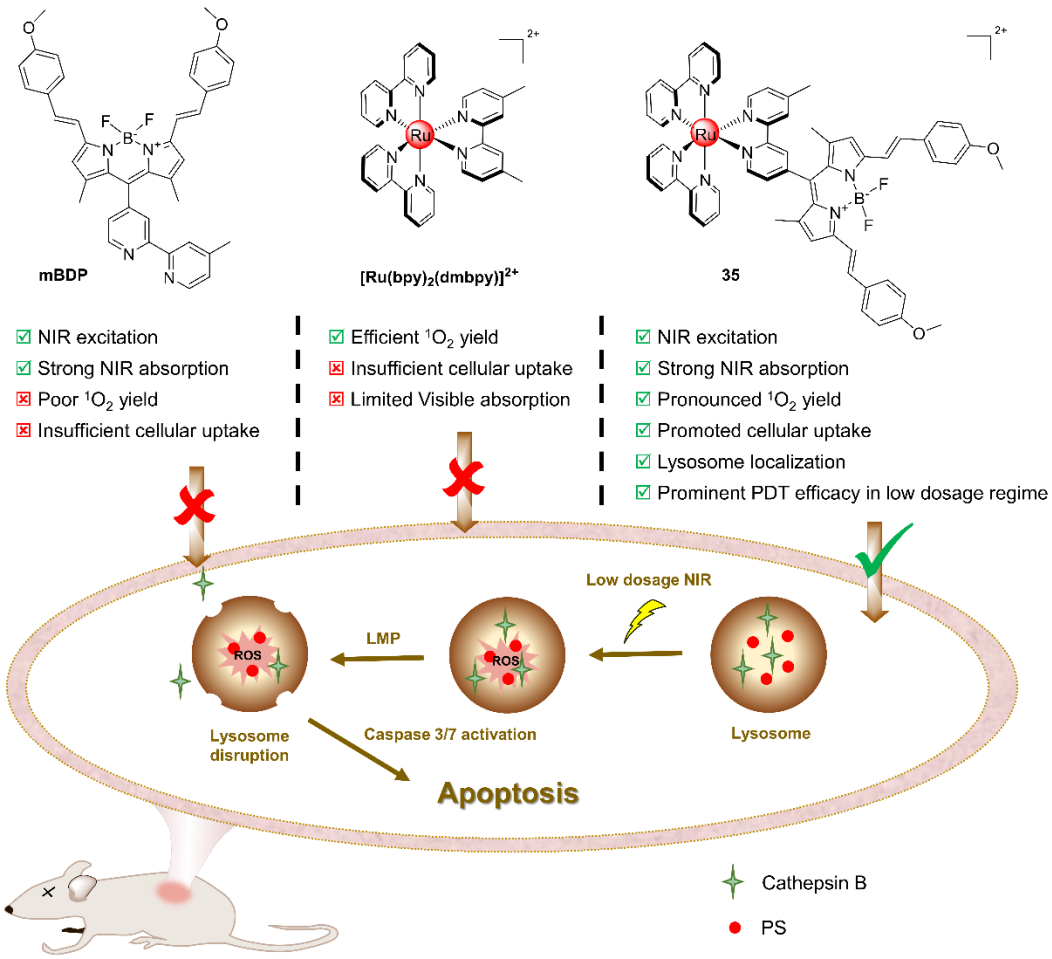


Fig. 15. Structures of compound **35** and its precursors, schematic illustration of the design strategy and supposed molecular behavior in PDT.

36a-c are among the few examples of Ru(II) bis-terpyridine complexes [184] 182; such compounds are generally characterized by strong ^{1}MLCT absorption in the visible range but possess weak ligand field strength due to the distortion of the coordination angles, which favors the thermal decay associated with the low-lying ^{3}MC excited state and leads to a short-lived triplet excited state. The conjugation with BODIPY and/or pyrene chromophores in **36b,c** allows for the achievement of strong visible absorption (green regions, 450-600 nm) along with low-lying $^3\pi,\pi^*$ states that prolong the triplet excited state lifetime to tens of microseconds. As a direct consequence, the complexes exhibit large singlet oxygen generation, that provides the complexes with phototoxicity towards A549 lung cancer cells upon excitation at 500 nm [184] 182.

Another series of BODIPY-decorated bis-terpyridine Ru(II) complexes containing different pendant groups (methoxy in **37a**, substituted phthalimide in **37b**, amine in **37c** and biotin in **37d**) was recently reported [147], with all complexes showing good properties as PDT agents (strong absorbance in the visible region, high Φ_Δ values and good stability) and PDT activity much higher than that of the structurally similar **36a-c**. This is noteworthy, considering that generally Ru(II)-bis-terpyridine systems do not possess the photophysical requirements for a PDT agent, and that unsubstituted BODIPY also usually has a minor photodynamic effect. This suggests that a suitable combination between a BODIPY unit and a Ru(II) bis-terpyridine core can produce a system having a photodynamic effect. **37d** features a biotin moiety that confers cancer-cell-targeting properties through uptake by overexpressed vitamin receptors, and works both through a type-II process (singlet oxygen production) and a type-I process (superoxide anion radical) upon photoexcitation. It shows the highest photocytotoxicity within the series with a remarkable PI value of >1400 in HeLa cancer cells (with a low light dose activation, 400−700 nm, 2.2 J cm^{-2}), along with a reduced activity in noncancerous HPL1D cells. Moreover, **37d** shows emission properties that could be exploited for cellular imaging (theranostic agent) [147].

Swavey *et al.* presented a series of new BODIPY-based Ru(II)-complexes, incorporating π-extended pyrroles (**38a-c** [159] and **39a** [163] or phenanthro-fused pyrroles **39b** [163], showing interesting spectroscopic properties within the PDT window (high molar absorptivities, singlet oxygen production). The red-shift is more likely due to the destabilization of BODIPY π-orbitals following the direct coordination *via* the nitrogen donor atoms present on the π-extended pyrroles of the BODIPY core. Complex **39b** showed a more efficient photocleavage activity towards plasmid DNA than complex **39a**, probably due to its higher positive charge that shortens the distance travelled by singlet oxygen [163]. The same group reported two ruthenium(II) complexes involving a dipyrromethene ligand and one or three Ru(II) centers (**40a,b**): the metal ion features the same structural place holder role as BF_2 in BODIPY dyes, aligning the π-system of the dipyrrin. Both complexes reached MLCT transitions into the PDT window (600 nm to 850 nm) and were able to photocleave plasmid DNA through efficient generation of singlet oxygen [158].
Other chromophores have been investigated for the same purpose, revealing successful in red-shifting the absorption of the complexes into the therapeutic window. The structures of the complexes discussed in the following are reported in Fig. 16-18.

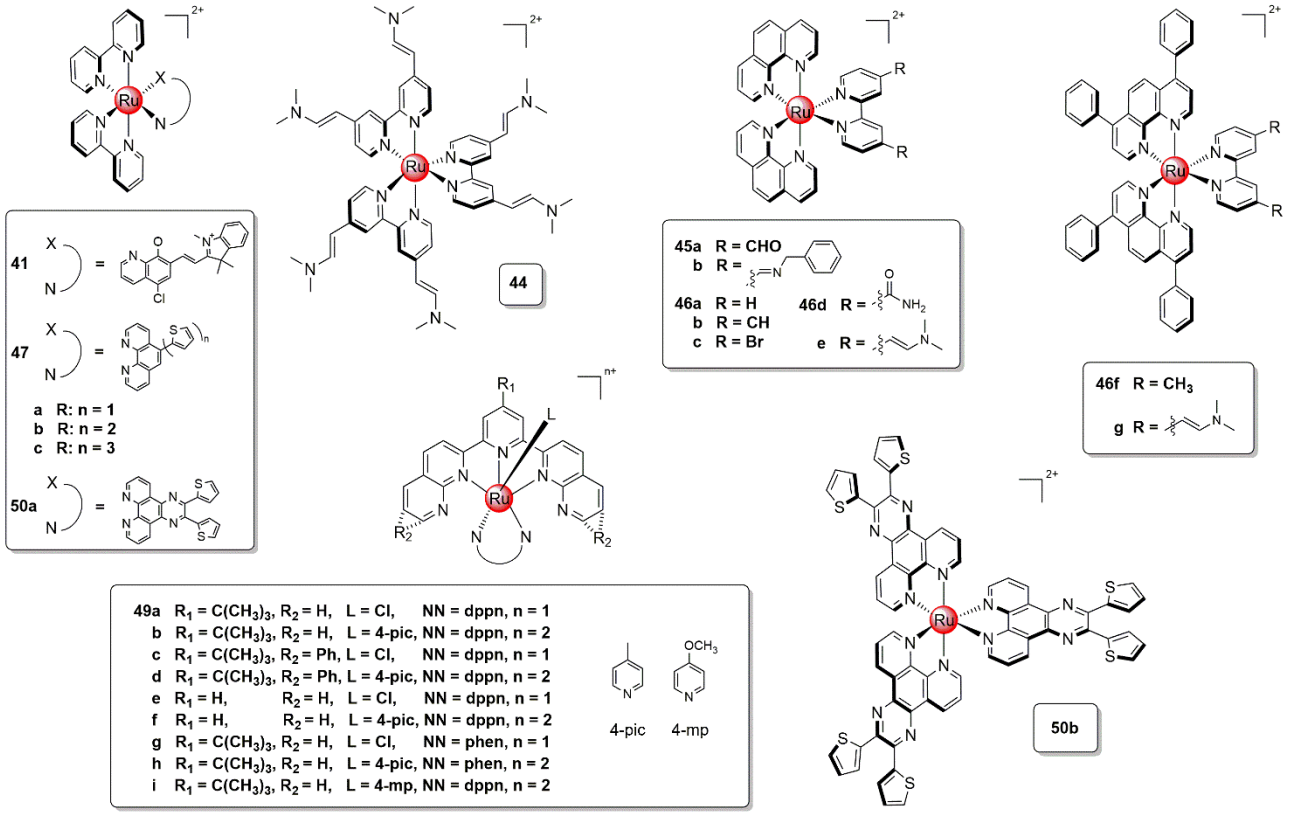


Fig. 16. Chemical structures of some complexes based on chromophores other than BODIPY discussed in section 2.2.1.1.

Wang and coworkers reported a new Ru(II) complex featuring the 5-chloro-8-oxyquinolate ligand incorporated into a merocyanine scaffold $[Ru(bpy)_2(Cl\text{-}7\text{-}IVQ)]^{2+}$ **41** that represents the first RPC able to photocleave DNA *via* •OH pathway, with an MLCT absorption maximum above 600 nm (649 nm, $\varepsilon_{649nm} = 1.73 \times 10^4\ M^{-1}\,cm^{-1}$) [157].
Zhao *et al.* reported a series of RPCs (**42a-g**) where the bpy moieties were first replaced by a Schiff base and then by a further dppn ligand to shift the ^{1}MLCT absorption and improve the photobiological activity *via* the population of long-lived intraligand (^{3}IL) excited states (Fig. 17) [185] 183. In particular, **42g** shows an excellent phototoxicity against A549 and HepG2 cells under 650 nm irradiation with PI values of 763 and 613, respectively [185] 183.

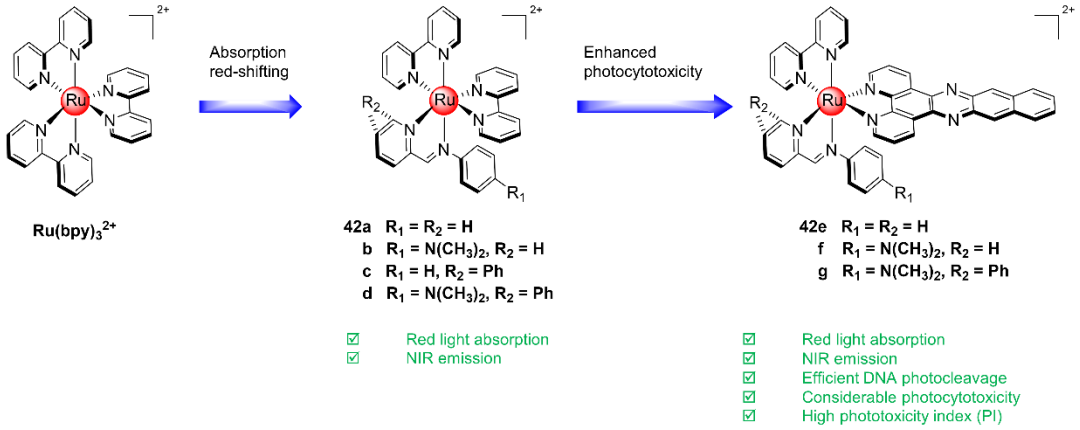


Fig. 17. Structures of compounds **42a-g** and schematic illustration of the design strategy.

Sun *et al.* reported the first example of a red-light responsive Ru(II)-based PS ([Ru(tpy)(Cl-7-IVQ)I]$^{+}$ (**43**) able to selectively locate into lysosomes, that proved to be a PDT agent *via* singlet oxygen generation both *in vitro* and *in vivo* [186] 184. The PS activation is achieved by exciting at 660 nm, by virtue of the lowest energy absorption band attributed to a MLLCT transition from the hybrid Ru(dπ)-Cl-7-IVQ(π) orbital to the π*(Cl-7-IVQ) orbital. The cellular uptake and location could be monitored by fluorescence emission from the ligand Cl-7-IVQ by exciting at 450 nm.

The Gasser group contributed with many examples of RPCs with red-shifted absorption. The introduction of vinyl dimethylamino groups [Ru(bpy)$_3$]$^{2+}$ (**44**) [114] or aldehyde substituents on [Ru(phen)$_2$(bpy)]$^{2+}$ (**45a**) [84] provoked a strong red-shift of the MLCT transition, with an absorption tail towards the 600-900 nm window. **44** resulted photocytotoxic notwithstanding the low production of singlet oxygen due to its high uptake into HeLa cells [114]. The conjugation of **45a** with benzylamine gave a potential PS for PDT (**45b**), with toxicity in the low micromolar range against cancerous HeLa cells but only activable from 450 nm up to 540 nm [84]. The introduction of a vinyl dimethylamino group produced the desired bathochromic effect also on [Ru(phen)$_2$(bpy)]$^{2+}$ and [Ru(bphen)$_2$(bpy)]$^{2+}$ scaffolds (**46e,g**), but again poor photophysical properties, while the methylation of [Ru(bphen)$_2$(bpy)]$^{2+}$ to give **46f** produced both a red-shifted absorption and favorable properties such stability in human plasma, cytoplasmatic localization in HeLa cells and phototoxicity in the nanomolar range upon excitation at 595 nm both in 2D and 3D cellular models [149].

The McFarland group also proposed several examples within this topic, mainly being Ru(II)-oligothienyl π-extended systems. They found that by increasing the number of thienyl groups (n) in **47a-c** the nature of the emissive state did not change, in other words the π* acceptor orbital of the ^{3}MLCT state is the same in the three complexes [187] 185. On the contrary, n modified the nonradiative ^{3}MLCT relaxation pathways: complexes **47b** and **47c** showed different excited state absorption (ESA) profiles, with long-lived ^{3}IL states involving ^{3}ππ* transitions localized on the thienyl groups that can be populated only when n ≥ 2. Despite the low absorption in the red-NIR region ($\varepsilon_{625\ nm}$ << 100 M^{-1} cm^{-1}), the complexes are red-phototoxic against SKMEL28 and HL60 cancer cells through the generation of singlet oxygen [187] 185. The same group studied several NIR absorbing RPCs of [Ru(NNN)(NN)(L)] type, made up by a tridentate polypyridyl ligand NNN, chosen to shift the absorption toward the NIR region, a bidentate π-expanded ligand NN, providing access to ligand-localized triplet states sensible to O_2, and a monodentate ligand L, capable of modulating the chemical and photochemical stabilities of the complexes and the biological toxicity. Complexes **48a-e** revealed phototoxic to cancer cells, including highly pigmented B16F10 melanoma cells [188] 186. The tpbn (NNN) moiety lowers the ^{1}MLCT/^{3}MLCT energies, while the IP-appended thiophenes (NN) fragment allows for the population of the ^{3}ILCT excited state, but only when n = 4 (**48e**). The intensity of the ^{3}ILCT band in the UV-visible region is weak and its energy is slightly higher than that of ^{3}MLCT, nonetheless, in **48e** the ^{3}ILCT state is still accessible and primarily contributes to the photobiological activity, conferring **48e** the highest PI values in all tested cell lines, regardless the low and similar production of singlet oxygen across all complexes (PI_{vis} for all compounds in A375 cells: 2-90; in B16F10 cells: 2-29; in SKMEL28 cells: 4-88. PI_{733nm} for all compounds in A375 cells: 1-8; in B16F10 cells: 1-7; in SKMEL28 cells: 1-12) (Fig. 18). This study brought to light that the achievement of MLCT absorption at longer wavelengths occurs to the detriment of the population of the long-lived ^{3}ILCT state, negatively affecting the production of singlet oxygen and the related biological activity [188] 186.

Lifshits and coworkers recently conducted a structure-activity relationships (SAR) study on [Ru(NNN)(NN)(L)] complexes **49a-i** [189] 187. This study allowed to establish that compounds featuring the chromophoric tpbn ligand and the pyridyl axial ligand (**49b,f,i**) possessed absorption at shorter wavelengths (λ_{max} = 720 nm *vs* 790-800 for **49a,d,e,g** and 900 nm for **49c**) but better stability and water solubility, together with the largest singlet oxygen quantum yields and PIs, by virtue of the fact that such compounds are the only ones with accessible ^{3}IL states. The most promising compound **49b**, under red light irradiation, is phototoxic against melanoma cells, that usually are more resistant to PDT, and, in addition, proved to stimulate anti-tumor immune responses both *in vitro* (B16F10 mouse melanoma cell line) and *in vivo* (immuno-competent, syngeneic B16F10 tumor model). Indeed, beyond the local anticancer activity, PDT is reported to perform immunomodulatory functions that can prevent cancer recurrence [190–199]188-197 and can be advantageously coupled with surgery [189,200] 187-198.

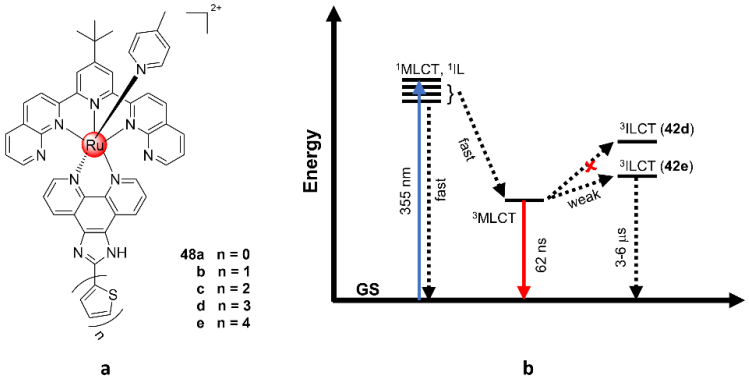


Fig. 18. a) Structures of compounds **48a-e**; b) Jablonski diagram depicting the proposed excited state pathways in the series.

Also Wang *et al.* exploited the insertion of thienyl groups into RPCs, producing two new complexes (**50a,b** [162]) that show different optical properties, in that **50b** displays a rare and very strong solvatochromism, with a shift of 112 nm between CH_2Cl_2 (588 nm) and H_2O (700 nm), whereas **50a** possesses a visible emission maxima at 600-625 nm in all of the solvents tested. Both complexes interact with Calf Thymus-DNA by intercalation mechanism and are able to generate singlet oxygen. **50a** proved to be a promising PDT agent, also able to selectively target and image mitochondria thanks to its emission properties [162].

2.2.1.2 Cyclometalation

Cyclometalation represents another strategy to shift the excitation wavelength of a PS towards the red, exploiting the π donor properties of the phenylene ligand. The structures of the complexes discussed in the following are reported in Fig. 19.

An unwanted effect of the cyclometalated compounds reported so far is their inherently dark cytotoxicity [201,202] 199,200; however, recently the McFarland group presented four cyclometalated analogues of TLD1433 (**51a-d**) [203] 201 among which **51d** proved as a PS for PDT, being cytotoxic against SKMEL28 melanoma cells only upon light irradiation at 532 nm (fluence of 100 J cm^{-2} delivered at a rate of 35 mW cm^{-2}) with a PI as high as >1100. **51c**, by contrast, emerged as a potent chemotherapic agent, being selectively cytotoxic to cancer cells over normal cells in the dark [203] 201.

The ability of compounds to be cytotoxic both in the dark and even more upon light exposure could be exploited to overcome cancer cell resistance mechanisms. The new π-conjugated cyclometalated ruthenium compounds **52a-g** are active towards two cisplatin resistant gastric cancer cell lines (AGS and KATO III) and show enhanced phototoxicity due to singlet oxygen production (3 watts LED lamp, λ_{ex}=590 nm, light dose=20.3 J cm^{-2}) [27]. Among a new family of five

heteroleptic cyclometalated Ru(II) complexes of the type $[Ru(CN)(NN)_2][PF_6]$ (NN = phen, dpq and dppz; CN = deprotonated methyl 1-butyl-2-arylbenzimidazolecarboxylate, **53a-e**), only compounds **53b,d** showed a good PS activity under hypoxia upon irradiation with green light, featuring PI values greater than 769 and 588, respectively, towards HeLa cells [204] 202. PDT effects originate from ROS production upon photoirradiation, that is H_2O_2, $^{\bullet}OH$ and $^1O_2$ under normoxia, and H_2O_2 and $^{\bullet}O_2^-$ under hypoxia [204] 202.

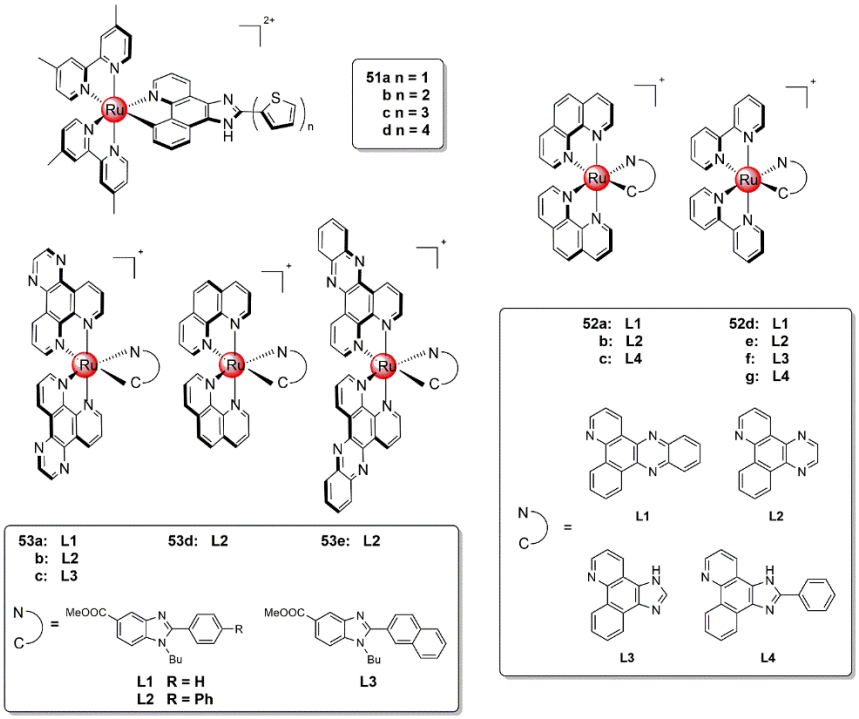


Fig. 19. Chemical structures of cyclometalated complexes discussed in paragraph 2.2.1.2.

2.2.1.3 Two-photon absorption (TPA)

The request to activate photoresponsive materials with NIR light can be reached not only through the use of a "canonical" red-shifted one-photon absorption (OPA) but also thanks to the simultaneous absorption of two photons, through the challenging two photon absorption technique (TPA) [90,205–208]90,203-206 (Fig. 20).

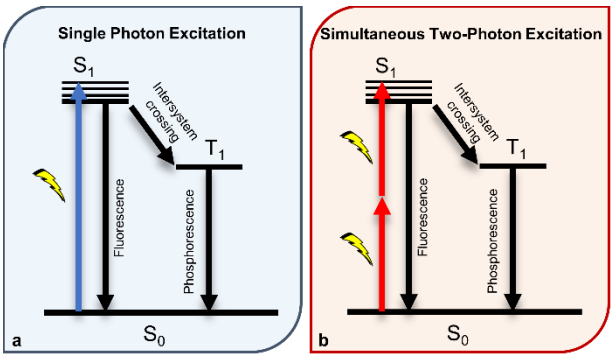


Fig. 20. Comparison between one-photon (a) and simultaneous two-photon excitation (b).

According to this method, the use of a low energy NIR irradiation would permit to minimize the possible light-induced photodamage of the PS, but it also guarantees an enhanced light penetration depth, that, at lower wavelengths is generally limited by absorption and light scattering effects of biological tissue (Fig. 21).
In addition, TPA allows for a high spatial resolution, due to its dependence to the square of the light source intensity, contrary to the linear dependence on the intensity of the one-photon (OP) process. For this reason, TPA only occurs at the focal point of the laser and requires intense laser beams or focused laser pulses with a high photon density [90,209–213]. 90, 207-211

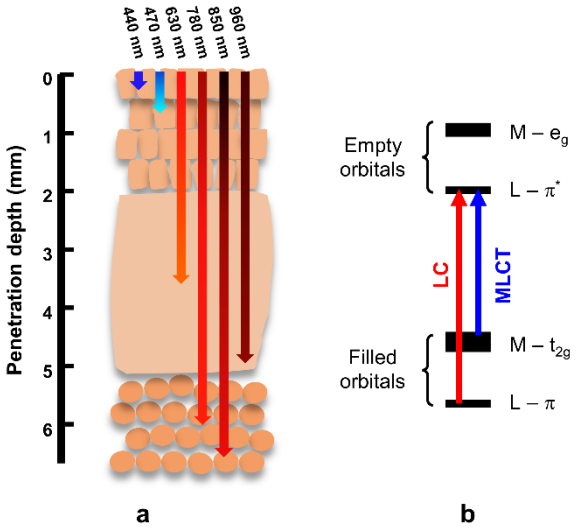


Fig. 21. a) Light penetration depth into skin. Hemoglobin, melanin, water and fat mainly limit the optical biological window between 600 and 900 nm, respectively absorbing the former two at the lower end and the latter two at the upper end. b) Simplified MO scheme of the frontier orbitals for octahedral d6 complexes like $[Ru(bpy)_3]^{2+}$.

To be used in two-photon PDT (TP-PDT), a PS must be able to absorb two photons simultaneously, in other words a large TPACS is required (σ_2 is expressed in Goeppert-Mayer (GM) with 1 GM = 10^{-50} cm^4 s·$molecule^{-1}$). Unfortunately, σ_2 is too low for most commercial PSs, hence a higher TP laser power is needed, at the cost of possibly causing irreversible biological damage to the normal organism [214]212.
Finally, this technique progressed in the past few decades to become a practical tool for bio-imaging and cancer phototherapy, nevertheless more technological innovations are required to treat a relatively large *in vivo* target such as a solid tumor [212]210. The structures of the complexes discussed in the following are reported in Fig. 22.
The already cited complexes **9a** [130] and **12b** [132]**,** and complex **72** [215]**213** (*vide infra*) show TPACS (245 GM, 90 GM, 1371 GM, respectively) much higher than clinical approved PSs such as H_2TPP (<20 GM at 800 nm) [216]214 and tetraphenylporphyrin (σ_2 = 2.2 GM) [217]215, and other two-photon bioactive RPCs and organometallic compounds recently reported [23,90,105,205,214,218–221]23,90,105,203,212,216-219. These complexes represent large conjugated, planar and symmetrical structures, all properties that make a PS prone to achieve efficient TPA. As for **72**, the extension of the molecular orbital of Ru(II) moiety onto the Pt metal center and the stiffening of the structure upon self-assembly are responsible for the increased TPA activity [215]213. Many other TP-light responsive compounds are cited throughout this review [28,65, 66,134,135,138,144,214–216,217,218]28,65,66,134,135,138,144, 212-214,215,216.
The insertion of a rigid π-conjugated electron-donating group such as trans-stilbene on a RPC produced both a red-shifted OPA and exceptionally high TPACS to complexes **54a-g**, with σ_2 values ranging from 150 to 2175 GM. Different groups have been inserted in the trans-stilbene moieties, the complexes being able to generate $^1O_2$ with a phototoxicity

mediated by apoptosis and paraptosis pathways both in 2D and 3D cellular models. The most promising complex **54g** was evaluated inside a mouse model, confirming its ability to act as an efficient PS for PDT [226] 220.
Thanks to these encouraging results, a SAR study was then performed on **54g** [227]221. This study highlighted how even subtle structural modifications on a given ligand could markedly affect the physical-chemical properties, and so the therapeutic outcomes, of the final complexes. To this aim, compounds **54h-o** were synthesized and studied, with only **54j,k**, bearing hydrophilic groups, being worthy of further development due to a phototoxicity that is similar to **54g** on cancer cells but lower on normal cells, and a favorable increased hydrophilicity [227] 221. The lead compounds **54f,g** were recently modified by changing the ancillary ligands to give compounds **55a-d** where two bpy were substituted by two phen or bphen moieties [228] 222. The new compounds show a red-shifted OPA (595 nm) and a very strong TPA (800 nm, 1600 GM) that enables them to be used for the treatment of deep-seated or large tumors. Notably, all complexes were tested on exceptionally large MCTSs (800 μm diameter) and were found to fully penetrate them and produce singlet oxygen even at the hypoxic center, being able to completely eradicate the MCTSs. In particular, **55b** was phototoxic in the low μM range upon OP or TP irradiation and was also active on mice bearing an adenocarcinomic human alveolar basal epithelial tumor [228] 222.

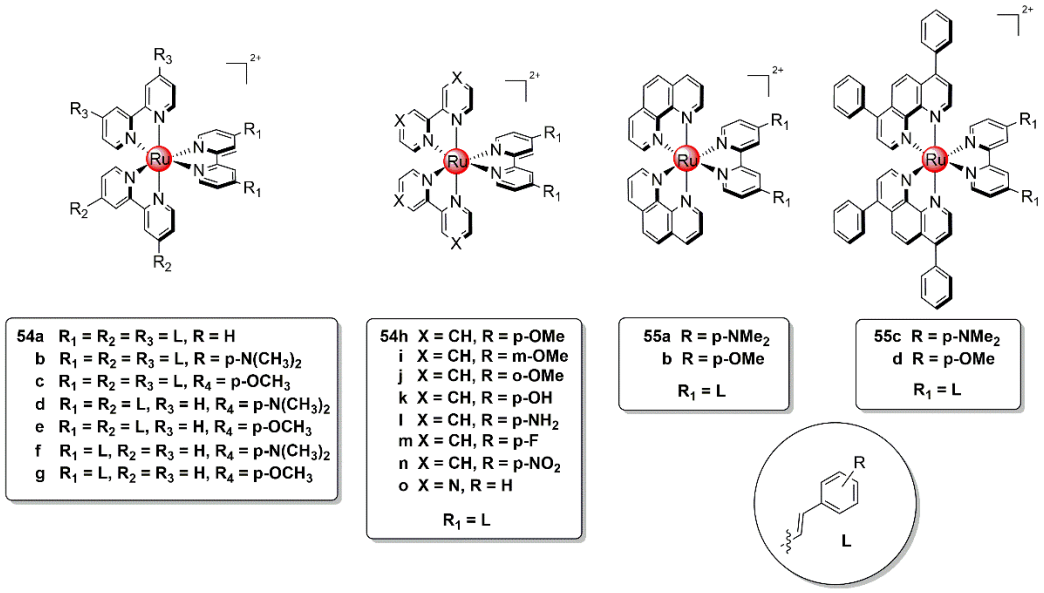


Fig. 22. Chemical structures of TPA complexes discussed in paragraph 2.2.1.3.

2.2.2 Prolongation of the Ru(II) triplet excited state lifetime

Ru(II) complexes showing prolonged excited state lifetimes could be useful PDT agents endowed with high phototoxicity, in that the triplet state lifetime is crucial to the production of singlet oxygen, even in hypoxic condition, which is a typical tumor issue [229]223.
The access to low-lying, long-lived intraligand ^{3}IL states could help extending the triplet state lifetime [25]. The insertion of organic chromophores into the structure of RPCs, besides boosting light absorption, is a way to populate the desired ^{3}IL excited states [230]224: they generally lie in a lower energy level than their ^{3}MLCT states, so equilibriums towards ^{3}MLCT states are suppressed and extension of the triplet states lifetime is achieved. The structures of the complexes discussed in the following are reported in Fig. 23.

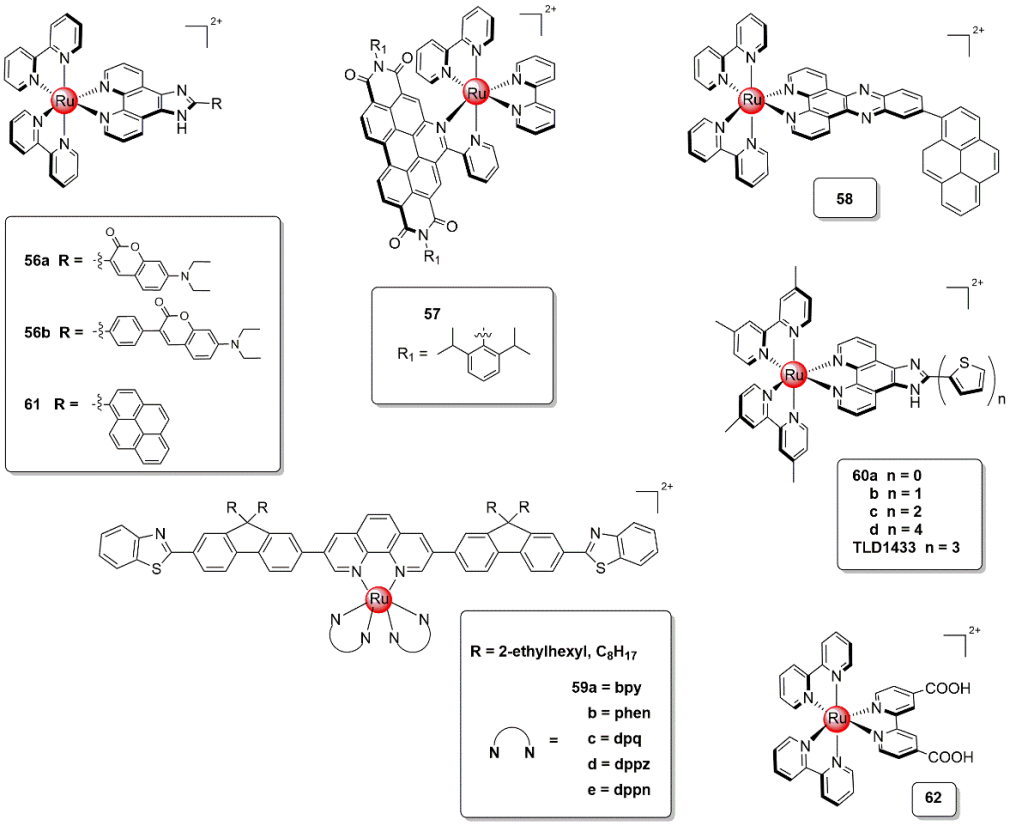


Fig. 23. Chemical structures of complexes discussed in paragraph 2.2.2.

The conjugation of RPCs with coumarin, ab-PBI and pyrenyl groups succeeded in providing complexes with extended triplet excited state lifetimes (**56a,b**: 12–15 μs [160], **57**: 4.2 μs in degassed dichloromethane [166]225, **58**: 7 μs in deaerated CH_3CN [231]226). Complexes **56a,b** and **58** showed a good production of singlet oxygen, the latter being phototoxic against A549 and MCF-7 cells, with exceptionally large PIs (1030 and 3004, respectively) [231] 226.
McFarland and coworkers reported on heteroleptic trisdiimine Ru(II) complexes to be used both as PSs for PDT and as Reverse Saturable Absorbers (RSAs), applications that share the request for long intrinsic lifetimes and high triplet yields [232]227. Benzothiazolylfluorenyl (BTF)-substituted phenanthroline ligand was combined with bpy, phen, dpq, dppz or dppn (**59a-e**), ancillary ligands known to affect the photophysics of the complexes by enhancing the π-conjugation. Attempts to build structure-activity relationships across this series in two cell lines failed, still the most π-extended complex **59e** showed to be the most promising as PDT agent. It is indeed the only complex showing the lowest-energy triplet state localized on dppn and not associated with the BTF-substituted phenanthroline ligand, with the longest triplet lifetime within the series (41.2 μs). **59e** also proved phototoxic and selective toward melanoma cells and possibly as theranostic agent, due to intracellular luminescence both in the dark and even more after light irradiation [232] 227.
The McFarland group also reported very recently a study of the photophysical properties that form the basis of the observed phototoxicity of TLD1433 [25,89,233]228 and analogues (**60a-d**) [230,234]224,229. The long-lived excited states and consequently the biological activity depend on the energy of the non-emissive ^{3}IL state, that, in turn, is a result of the number of conjugated thiophene moieties. When the ^{3}IL state is too high in energy, it is not accessible compared to ^{3}MLCT and does not contribute to the phototoxicity: this is the case of none to one thiophene groups (**60a,b**). The presence of two thiophene groups (**60c**) makes the ^{3}IL and ^{3}MLCT states close in energy, and the result is a delayed ^{3}MLCT emission due to population of this state from ^{3}IL. It is only in the case of three and four thiophene rings (TLD1433, **60d**) that the ^{3}IL state is the lowest-lying triplet and plays a predominant role in the excited state relaxation, being responsible for the efficient oxygen sensitization and, consequently, an increased phototoxicity (Fig. 24) [230,234] 224,229.

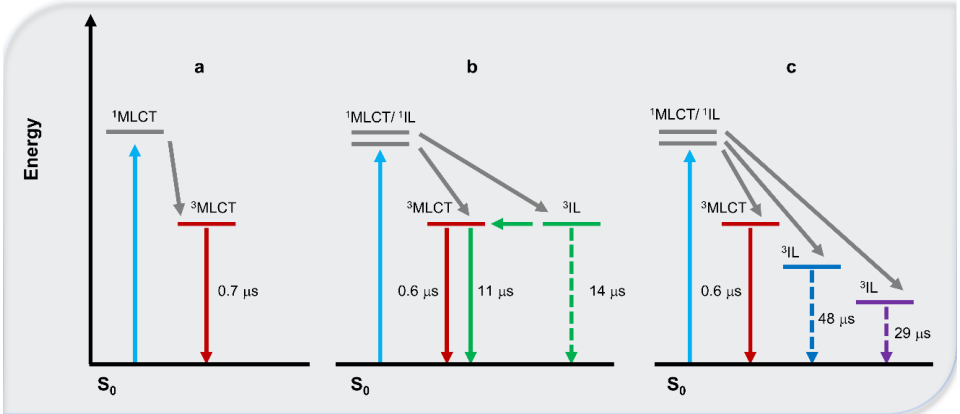


Fig. 24. Jablonski diagrams of the photophysical models that describe the ns-ms excited state dynamics of TLD1433 and complexes **60a-d** upon excitation at 410 nm in deaerated water; a) **60a,b**; b) **60c**; c) TLD1433, **60d**. Grey arrows represent processes occurring faster than the investigated timescale.

Very interestingly, the McFarland group investigated the photophysics of $[Ru(bpy)_2(ippy)]^{2+}$ (**61**) in simulated biological fluid, with the very final aim of understanding the mechanistic aspects of PDT under more biologically relevant conditions [156]. This is the first example of a systematic study on the effects of ionic strength and DNA intercalation on the photophysical properties of a PS, resulting in a great influence of these factors. In particular, an additional excited state can be detected at high ionic strength besides the excited state ^{3}MLCT-^{3}IL equilibration, prolonging the lifetime and possibly coming from interactions between ions and the PS; moreover, due to the restricted rotation of the DNA-intercalated pyrenyl unit, the DNA binding accelerated the nonthermalized photodynamics of **61** and removed some deactivation pathways [156].
Another way to increase the production of ROS is to exploit the oxygen vacancies (OVs), in that they can offer more carriers for surface reactions that lead to the generation of reactive oxygen species (ROS), such as $^1O_2$, H_2O_2, $\cdot O_2$, $\cdot$OH. Zhou *et al.* developed a BiOBr semiconductor engineered with OVs and functionalized with a ruthenium PS (**62**) that shows very interesting therapeutic properties for PDT in cancer cells, both *in vitro* and *in vivo*, coming from the OVs-based increment of ROS generation upon light irradiation [235]230.

2.3 The role of oxygen in the PDT approach

The reliance on molecular oxygen is a crucial feature of PDT, that may discourage the further translation of such approach into clinic for the treatment of different types of cancer. Indeed, the rapid growth of tumor cells and the imperfect vasculatures of tumor environments usually cause insufficient oxygen supply, particularly in those regions that are close to the core of solid tumors [236–240]231-235. In addition, there is a continuous consumption of oxygen during the PDT process [215,241]213,236 and the hypoxic environment could favor the onset of cancer resistance to PDT and failure of the radio- and chemotherapy [242,243]237,238.
Therefore, notwithstanding these aspects represent major complications with PDT, which can potentially threaten its effectiveness in hypoxic regions [239,244]234,239 and enhance the risk of relapse [245]240, an increasing attention is currently devoted to the development of suitable solutions to overcome such critical dependence [246–249]241-244. Indeed, besides the employment of oxygen-independent light-triggered modalities of action that are made accessible by PACT mechanisms [250,251]245-246, to which the second part of the present review is entirely dedicated to (chapter 3), several innovative options might be adopted to the same aim.
In an interesting minireview, Yoon and coworkers recently reported about the use of some pioneering strategies used to address hypoxia in PDT, classifying the different approaches in three categories, namely i) O_2-replenishing methods that raise the oxygen level prior to or during the PDT treatment, ii) new PDT paradigms that lower the need for oxygen, and iii) combinations between PDT and other hypoxia-activated or O_2-independent therapeutic modalities [116].

A recently proposed organometallic drug complex combined with a supramolecular interaction strategy (**63**) belongs to the first category [252]247. Compound **63** is an efficient *in situ* O_2 self-supplier, synthesized *via* host-guest interactions followed by self-assembly into Ru(II)-containing supramolecular metallodrug micelles in aqueous solution, in which the Ru center is able to catalyze the decomposition of H_2O_2 to produce O_2 (catalase-like activity). Singlet oxygen was then produced upon irradiation under IR (660 nm, $\Phi_\Delta = 0.27$), damaging lysosomes and mitochondria, thus enhancing cell cytotoxicity. The anticancer activity was evaluated on A549 cells, revealing that sufficient O_2 is provided *in situ* for chemotherapy and PDT synergetic oncotherapy, while tests on O_2 sensitive HCT116 cells (pre-exposed to normoxic or hypoxic conditions) revealed that **63** is able to overcome the chemotherapeutic drug-resistance caused by the hypoxic environment. On the other hand, curcumin and camptothecin contained in the complex can, respectively, control cell proliferation and apoptosis and inhibit the DNA synthesis. **63** thus represents a remarkable example of a dual action drug, acting by chemotherapy and PDT (Fig. 25, see also paragraph 4.1.2) [252] 247.

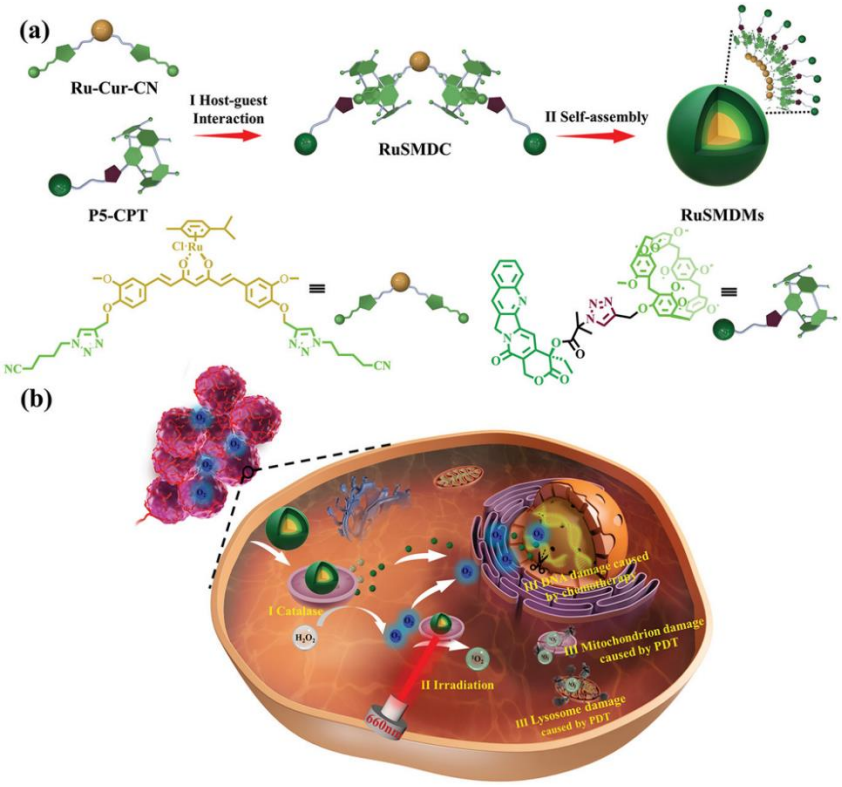


Fig. 25. a) Formation and self-assembly of **63** in water. b) In situ oxygen self-supply of **63**. (I) **63** acts a s a catalase and converts H_2O_2 to O_2; (II) generation of singlet oxygen upon red light irradiation; (III) synergistic anticancer effect between PDT and chemotherapy. Reprint with permission from Ref. [252]247, © 2021 Wiley-VCH GmbH.

Type I PDT is part of the second category, in that it seems to perform well even under low O_2 conditions [238,253,254]233,248,249. This process is also more appealing if thinking that ROS generated from the type I mechanism is even more toxic than $^1O_2$ from the type II mechanism [255]250. Indeed, cytotoxic superoxide anions and hydroxyl radicals are produced by type I process *via* surrounding substrates reducing the direct dependence on oxygen, moreover through disproportionation reactions the superoxide anion generates oxygen and hydrogen peroxide: the H_2O_2 accumulation within the cell can further lead to the Haber–Weiss or Fenton reaction to give toxic hydroxyl radicals, whereas the production of oxygen reduces the hypoxic condition [256]251.

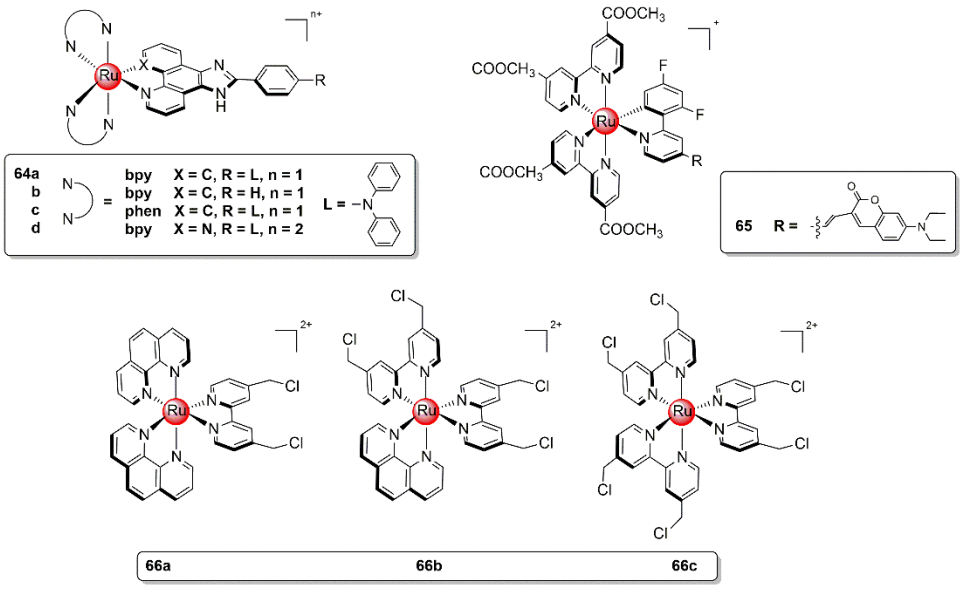


Fig. 26. Chemical structures of complexes discussed in paragraph 2.3.

Despite the offered advantages, *type I* PDT is still a less investigated field. To be a good candidate for *type I* process, a PS should possess good electron donating ability, in other words a low oxidation potential [254,257]249,252. Good PS candidates for the *type I* process are represented by both Ru(II) complexes, featuring both excellent redox properties and light-harvesting ability, such as the already cited complex **40b** [158] and complexes **64a-d** (production of $OH^{\bullet}$ and $O_2^{-\bullet}$ upon irradiation under hypoxic conditions) [256]251, as well as cyclometalated Ru(II) complexes, featuring strong δ-donating ability with a remarkable cathodic shift of the oxidation potential, such as **65** (formation of hydroxyl radicals under hypoxia and in *in vivo* efficacy on a model of hypoxic solid tumor) [257]252.

A supplemental to *type I* PDT comes from the ability of some Ru(II) complexes to both harvest light and initiate photoredox reactions in the cell, that may lead to the formation of non-oxygen radicals that can be employed to exert cytotoxicity through the *type I* mechanism. The three chloromethyl Ru(II) complexes **66a-c** reported by Wang *et al.* exhibited phototoxicity under hypoxic conditions through photoreduction dehalogenation reactions. Indeed, the resulting carbon radicals, produced in the presence of NADH, replaced ROS in damaging DNA and led to cancer cells death by apoptosis [165].

The third category of the Yoon review relates to combinational techniques and includes both the co-delivery of a PS and hypoxia-responsive prodrugs or anti-angiogenesis inhibitors as well as the combination of PDT with other O_2-independent techniques, such as photothermal therapy (PTT: employment of photothermal agents to convert light energy to heat) [258,259]253,254 and immunotherapy [260,261]255,256. More in general, the combination between PDT and other techniques holds a great promise in the development of novel anticancer alternatives. With this regard, chapter 4 of the present paper was entirely dedicated to dual-action tools combining PDT with other PACT-based techniques, as well as to multiple-active approaches involving different modes of action.

Therrien and coworkers proposed instead a new strategy that in principle allows for the transportation of singlet oxygen to cells *via* anthracene ruthenium metalla-assemblies **67a-c** (Fig. 27). Unfortunately, different spectroscopic techniques showed that even if anthracene moieties can form endoperoxide derivatives by reacting with oxygen, they lose such an ability upon formation of the metalla-assembly, maybe due to electronic or steric constraints [262]257.

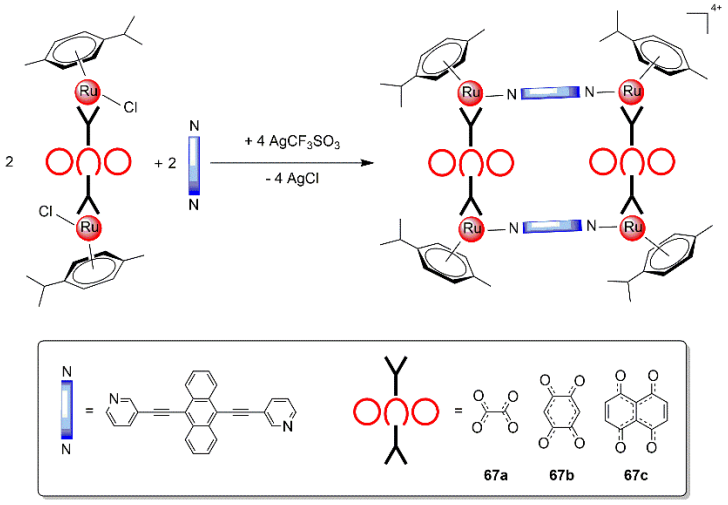


Fig. 27. Synthesis of metalla-rectangles **67a-c**.

2.4 Poly-metallic complexes

Ru(II) containing homo- and hetero-polynuclear complexes have been studied as PDT drugs, both often performing better than the related monometallic precursors.

The Thomas group reported for the first time Ru(II) dinuclear dppn-homoleptic **68a** and dppz/dppn-heteroleptic **68b** complexes [153] (Fig. 28), all of them being able to produce singlet oxygen and to be taken up by both cisplatin sensitive and resistant human ovarian carcinoma lines, displaying phototoxicity with high PI values (A2780/A2780cis cells, broad-spectrum irradiation with 14 J cm^{-2}: **68a**: ≥200/≥500; **68b**: 3/≥14). Moreover, the mixed dppz/dppn complex **68b** is a DNA light-switch, indicating the DNA intercalation of the Ru(II)-dppz moiety: thus, notwithstanding the lower PI, its luminescence makes it a lead for the development of theranostics [153]. Other examples of Ru(II) homopolynuclear complexes to be used in PDT are reported throughout this review [68,132,158,159,163].

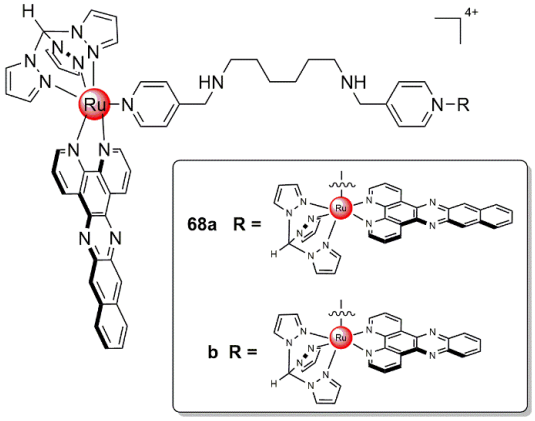


Fig. 28. Chemical structures of homo-polynuclear complexes **68a,b**.

Heterometallic Pt(II)/Ru(II) systems are being largely investigated, showing synergistic effects between the metal centers and often enhanced cytotoxicity and tumor selectivity compared to cisplatin (Fig. 29). Several heterodinuclear supramolecular ruthenium based-complexes contain a cis-$PtCl_2$ (cisplatin-like) moiety as bioactive unit, that guarantees an enhanced interaction with DNA compared to the parent compound and/or cisplatin.

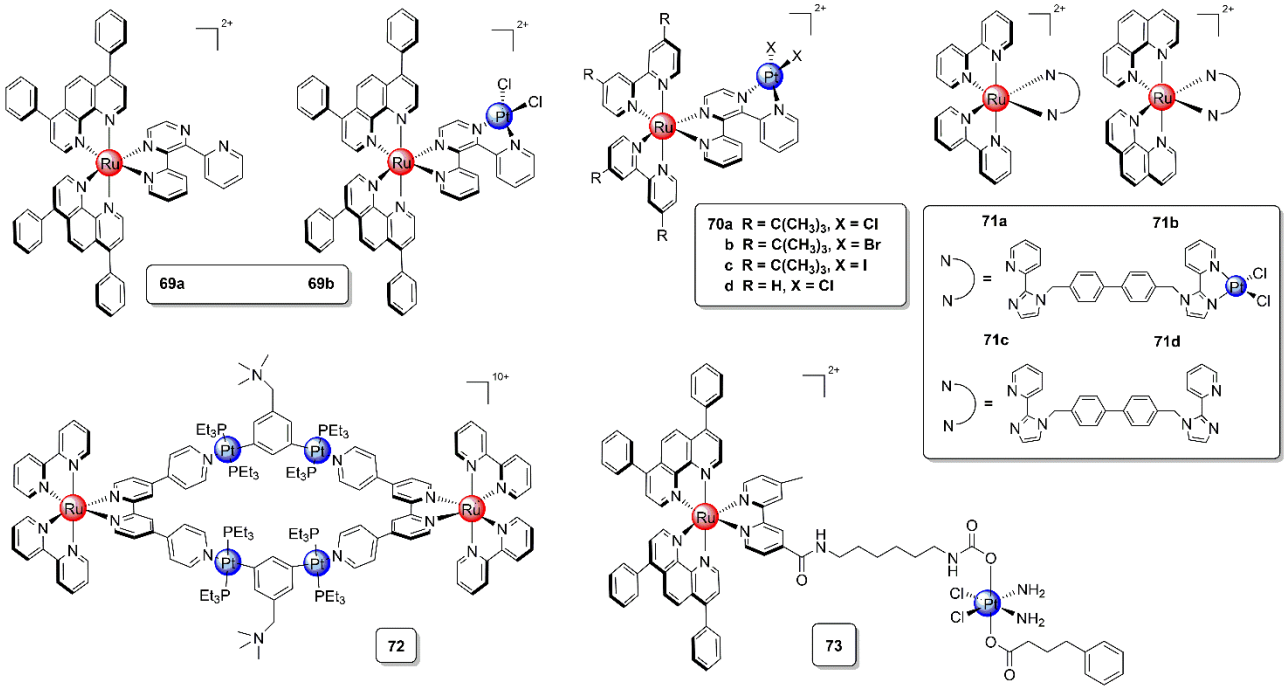


Fig. 29. Chemical structures of Pt/Ru hetero-polynuclear complexes discussed in paragraph 2.4.

The evolution of the Ru-polyazine monometallic complex **69a** [263]258 by insertion of a cisplatin-like unit on the dpp moiety produced a Ru-Pt compound **69b** [155] where the binding to DNA is thermally activated through the cisplatin-like moiety while the DNA photocleavage is photochemically activated through the Ru-center**.** Interestingly, **69b** seems to work even in the absence of oxygen upon blue light irradiation, contrarily to the precursor **69a** [155].

Complexes $[Ru(^tBu_2bpy)_2(\mu\text{–}dpp)PtX_2]^{2+}$ (X = Cl, Br, I) **70a-c** and in particular $[Ru(bpy)_2(\mu\text{–}dpp)PtCl_2]^{2+}$ **70d** [154] are able to photocleave the DNA through $^{\bullet}OH$ and $O_2^{\bullet-}$ pathways. Interestingly, **70a-c** did not show the same emission of the mononuclear complex at 716 nm, deriving from the lowest lying ^{3}MLCT, probably due to an electron-transfer process from the photo-excited Ru(II) unit to the Pt(II) one which quenches the emission [154].

Complexes $[Ru(bpy)_2(BPIMBp)PtCl_2]^{2+}$ (**71a**) and $[Ru(phen)_2(BPIMBp)PtCl_2]^{2+}$ (**71b**) can covalently interact with DNA, contrarily to their parent complexes $[Ru(bpy)_2BPIMBp]^{2+}$ (**71c**) and $[Ru(phen)_2BPIMBp]^{2+}$ (**71d**) [264,265]259,260. All complexes are photocytotoxic towards MCF-7 cancer cells upon irradiation at 450 nm, with Pt(II) containing complexes also inducing autophagy, and, by virtue of that, being potential PDT agents possibly able to overcome cisplatin resistance.

Stang and coworkers exploited coordination-driven self-assembly to obtain the heterometallic Ru–Pt metallacycle **72**, where the Pt(II) metal center provides the complex with properties suitable for PDT (NIR emission, strong two-photon absorption, high singlet oxygen generation), while the charge of the metallacycle favors the cell internalization and localization in both mitochondria and nuclei. The complex is phototoxic and damages mitochondria and nuclei; notably, it is effective in *in vivo* studies at low light doses with minimal system toxicity [215]213.

Complex **73** is an example of heterometallic Ru(II)–Pt(IV) able to behave as a multitargeting and dual action agent (*vide infra*) [66]. For more examples of mixed ruthenium-platinum complexes with medicinal applications, Jain recently reported a comprehensive review including Ru(III), Ru(II), Pt(IV) and Pt(II) [266]261.

The combination of Ru(II) with transition metal ions is not limited to Pt(II), as shown by the polymetallic complexes reported in Fig. 30 and 31.

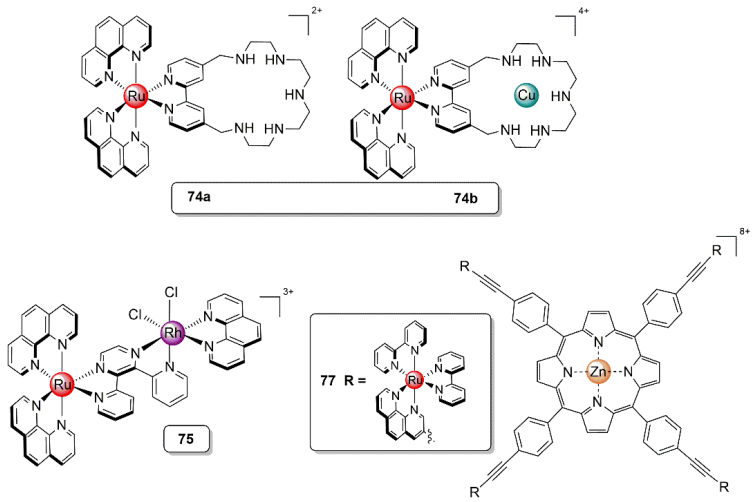


Fig. 30. Chemical structures of hetero-polynuclear complexes containing transition metal ions different from platinum.

Our group reported two new polypyridyl complexes containing Ru(II) **74a** and Ru(II)/Cu(II) **74b** metal ions [267,268]262,263. The peculiar macrocyclic moiety gives the ruthenium complexes both water solubility, thanks to the protonable amine sites, as well as a favored interaction with DNA, due to electrostatic and hydrogen bonding interactions between ammonium groups on the complex and phosphate groups on DNA. Both complexes are able to cleave a plasmid DNA upon light irradiation, producing single (**74a**) or double strand breaks (**74b**); moreover, both complexes display increased cytotoxicity upon irradiation at 411 nm, with **74b** being more active, highlighting the key role of the coordinated copper ion in the biological activity of such compounds [268]263. The Brewer group reported the Ru(II)/Rh(II) bimetallic complex $[(phen)_2Ru(dpp)Rh(phen)Cl_2]^{3+}$ **75** showing a strong electrostatic interaction with DNA, probably involving groove binding, that seems to be crucial for the covalent photobinding and photocleavage of DNA. Upon light irradiation **75** gives rise to a highly reactive ^{3}MMCT state able to react with DNA in the absence of oxygen, providing selective tumor-toxicity by using a less energetic excitation source than other rhodium-based bioactive molecules [161]. Recently, Chao and coworkers reported the first example of a mitochondria-targeting hetero-binuclear Ir(III)–Ru(II) complex **76** that acts both as a PACT and a PDT agent (Fig. 31) [28]. It was developed to overcome cancer resistance to cisplatin, thanks to the combination between the spatial and temporal controlled irradiation in tumor cells offered by PDT and the higher susceptibility of mitochondria to the oxidative damage caused by PSs. The complex proven stable in the dark, but it undergoes photodissociation upon irradiation at 450 nm, the typical MCLT peak for the ruthenium complex (PACT agent). Moreover, the complex is able to produce singlet oxygen only under stepwise irradiation at 450 nm followed by 405 nm, the latter being attributed to the iridium moiety (PDT agent and mitochondria targeting). Thanks to this synergistic therapeutic effect between PACT (photodissociation upon irradiation at 450 nm and mDNA damaging) and PDT (irradiation at 405 nm and production of $^1O_2$), the complex is up to 250 fold more active than cisplatin towards two cisplatin resistant cancer cell lines, A549R and SGC-7901/DDP, accumulating in the mitochondria where it causes mitochondrial dysfunction, inducing death by apoptosis [28].

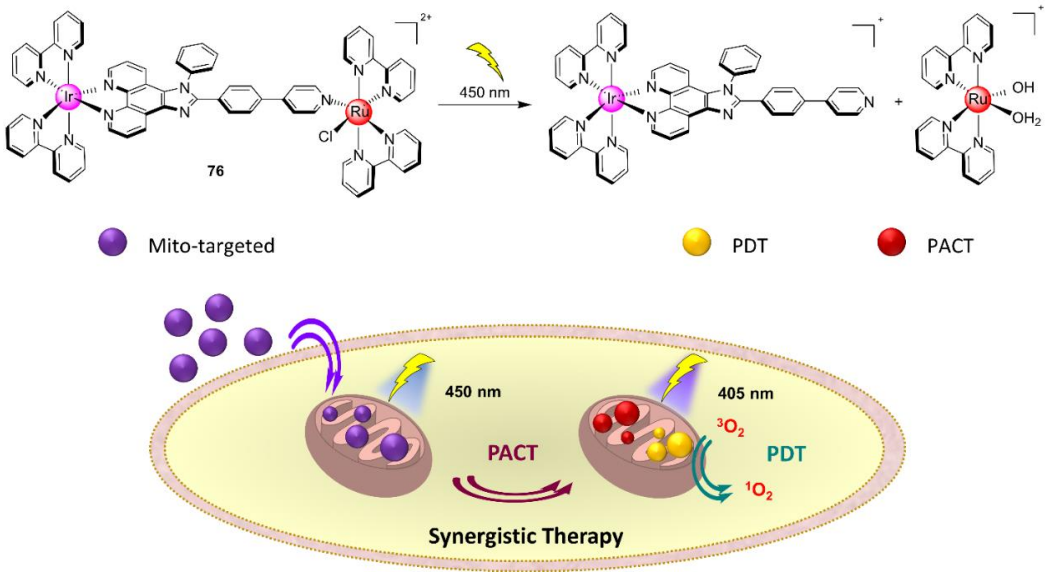


Fig. 31. Structure of complex **76** and a schematic mechanism of synergistic therapy.

A first example of a Zn-porphyrin decorated with four Ru(II)-phen complex units has been recently reported **77**, showing phototoxicity through singlet oxygen production (Φ_Δ = 0.45 %) when irradiated with red light (620–630 nm). The complex produces more $^1O_2$ than the analogous complex bearing only one Ru(II)-phen center, accounting for the cumulative effect of multinuclear decoration and a better PDT efficiency [269]264.

3. Ruthenium(II) complexes as photoactivated chemotherapy (PACT) agents

The reliance on molecular oxygen of PDT is one among the most crucial issues that seriously threaten its development in clinical trials [248,270–272]243,265-267 (see also paragraph 2.3). This scenario has therefore guided researchers to investigate systems able to exert cytotoxic effects through O_2-independent pathways, looking for suitable alternatives to be applied not only in normoxic conditions but even in the typically hypoxic environment of tumors.

In this context, a promising opportunity is represented by photoactivated chemotherapy (PACT). This approach, which was firstly named by the group of Sadler in 2009 [273]268, makes use of biologically active compounds that are converted into ideally non-toxic prodrugs by the use of light-cleavable protecting groups. Then, light irradiation promotes the deprotection of these bioactive ligands, permitting to release strong biological activity.

The versatile chemistry of RPCs makes this class of compounds extremely interesting for its potential use in the design of PACT compounds [98]. Their biological activity typically arises from the light-mediated dissociation of labile ligands (L) from metal-based prodrug forms (Ru-L) (Fig. 32). It is generally accepted that, irradiation of Ru-L promotes the generation of a triplet excited state of metal-to-ligand character (^{3}MLCT), through ultrafast ISC from ^{1}MLCT state. Then, differently from pathways involving the interaction with other molecules, such as O_2 in PDT, this state interconverts into a low-lying ^{3}MC state, which possesses a strong dissociative character (M-L (σ^*)) and enables the substitution of a ligand by a solvent molecule, typically water (Fig. 32a) [251,274]246,269. Consequently, light-activation results in Ru(II) aqua species (Ru in Fig. 32b) capable of interacting with lipids and proteins, or to covalently bind to DNA in a cisplatin-like fashion. In addition, the photoreleased ligands (L in Fig. 32b) may possess their own antitumoral activity.

It can also be noted that during this process neither the metal nor the ligand are oxidized or reduced, as instead occurs to other classes of inorganic PACT compounds, commonly based on Pt(IV) [275–277]270-272 or Co(III) [278]273, whose biological activity stems from the generation of cytotoxic Pt(II) or Co(II) species upon photoreduction in the cellular environment.

Therefore, the application of photosubstitutionally labile RPCs in PACT may bring to new photoresponsive inorganic compounds working through O_2-independent mechanisms of action, while preserving the high selectivity ensured by

the spatio-temporal control of irradiation. This opened the way to the development of a series of promising PACT agents, that exhibited remarkable anticancer activities both under normoxic (21% O_2) and hypoxic conditions (1% O_2) (*vide infra*) [250,279]245,274.

It should be also highlighted that the boundary between the different mechanisms of action, as well as among the possible biological targets, of this class of compounds is extremely thin; in addition, the question about which is the cytotoxic species among the ligand-deficient metal product or the liberated ligand, is often a complex item to solve and it has been the subject of some controversy [280]275. Some interesting perspectives on this topic were recently reported by the group of Bonnet [281,282]276,277. Moreover, the biological activity of these compounds is not always simply associated to a single modality of action but, in several cases, it depends on the simultaneous occurrence of more than one of them (see chapter 4). Although this variable scenario complicates the classification of this class of compounds into sharp-cut boundaries, in the following part of this review we attempted to provide a description of RPCs-based PACT agents on the basis of the most relevant modalities of action that affect their biological activities.

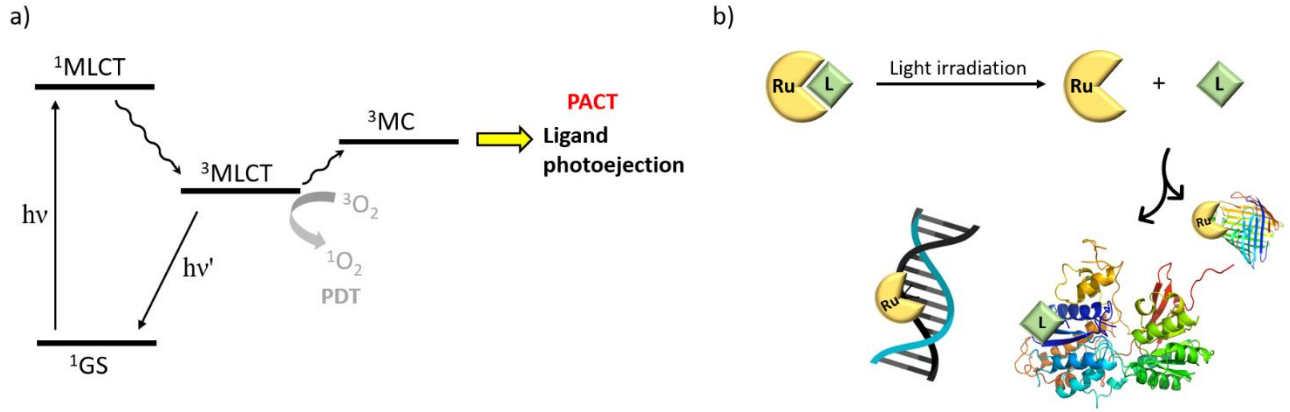


Fig. 32. a) Jablonski diagram of photosubstitutionally labile Ru(II) complexes for application in PACT and b) general mechanism of action of Ru(II)-based PACT agents with possible different biological targets.

3.1 Ru(II)-based PACT complexes: comparison with their use in PDT

PACT is often compared to PDT, especially in the research of photoresponsive inorganic compounds for application in the oncology field.

Besides the reliance on molecular oxygen, which certainly represents the most important disadvantage of Ru(II)-based PDT complexes when compared to PACT-based ones, other relevant differences among these two approaches can be evidenced. A crucial point is that, in PDT, the production of cytotoxic species by a photostable photosensitizer is a catalytic process, whereas in PACT the photochemical activation can only occur once. Indeed, Ru(II)-based PACT complexes typically require high toxicity of the photoproduced compounds to result in high efficiency.

Another point concerns the different modes of action of metal complexes in these two methods. PDT mostly works *via* ROS production whereas PACT involves the generation of variable cytotoxic agents that can vary from DNA-binding metal products to protein inhibitors. On one side, ROS production may trigger a strong immune response, helping against tumor recurrence [199]197, but on the other side, it also may provoke tissue necrosis, with loss of membrane integrity and cell lysis [283]278, that can ultimately contribute to pain during PDT treatments [284]279. From this point of view, milder forms of light-activated cell death pathways, such as apoptosis, are accessible with Ru(II)-based PACT compounds [285–288]280-283. Last but not least, contrary to PDT, that has been already approved in clinics, none among the RPCs investigated as PACT agents have advanced to clinical trials yet. This allows the PACT research to possibly take advantage of the knowledge acquired by the clinical advancement of PDT, for example in the application of fiber-optic technology for delivering light to regions of the body that are hardly accessible to light, namely tumors of internal organs, such as pancreas, head, neck and prostate [289–291]284-286.

Therefore, in light of these considerations, PACT should be considered not competitive but complementary to PDT, potentially offering valid alternatives in those cases where Ru(II)-based PDT compounds may lack of efficacy.

3.2 Light-induced generation of active Ru(II) photoproducts: Ru(II) photocisplatin agents

The hypoxic nature of the most aggressive and drug-resistant tumors has remarked the importance to adopt modalities of treatments that can elicit a therapeutic effect through oxygen-independent mechanisms of action. More than two decades ago, the efforts in this field brought to the discovery that thermally inert octahedral metal complexes could be used in combination with light to mimic the thermal chemistry between cisplatin and DNA [292]287. This peculiar activity relied on the photochemical release of labile ligands, upon population of dissociative ^{3}MCexcited states, which led to the generation of ligand-deficient metal complexes (*vide supra*). Such photoproducts could temporarily coordinate solvent molecules or directly react with Lewis bases such as DNA nucleobases, and thus could be exploited to disrupt the topological integrity of the biopolymer, the key mechanism by which cisplatin pursues its typical activity. The possibility to use light to control the cytotoxicity of such systems, which were then referred to "photocisplatin" reagents [292,293]287,288, soon appeared a precious opportunity to eliminate the widely debated side effects associated with cisplatin and, at the same time, to comply with the important request to develop alternative approaches that do not rely on the presence of molecular oxygen.

Turro and coworkers were among the first to exploit the rich chemical-physical repertoire of RPCs in this strategy, in 2004 [294]289. They showed that photolysis of *cis*-$[Ru(bpy)_2(NH_3)_2]^{2+}$ (**78** in Fig. 33) resulted in the sequential loss of NH_3 ligands, with quantum yields of ligand photoejection processes (Φ_{LD}) of 0.024 and 0.018 under 350 and 400 nm irradiation, respectively. This led to the formation of the bis-aqua complex *cis*-$[Ru(bpy)_2(H_2O)_2]^{2+}$, which exhibited covalent binding properties toward single and double stranded DNA (Fig. 33).

Since that time, the interest in Ru(II)-based photocisplatin agents has greatly increased and several PACT compounds based on RPCs was reported in literature.

In this paragraph we provide an overview of the most representative examples of Ru(II)-based PACT agents developed to date, whose biological activities are mainly associated to the generation of Ru(II) photoproducts, rather than to the photodissociating ligands, ideally barely active in this modality of action.

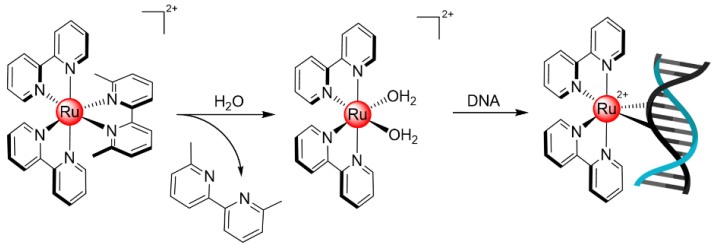


Fig. 33. Cisplatin-like mode of action of a typical Ru(II)-based DNA photobinding agent.

As it can be easily envisaged, the biological activity of this class of compounds is closely related to the photoinduced ligand dissociation efficiencies. These, in turn, are markedly affected by electronic and steric features of metal compounds (stereoelectronic effects) [295,296]290,291.

As an example, contrary to *cis*-$[Ru(bpy)_2(NH_3)_2]^{2+}$(**78** in Fig. 33), which easily undergoes photodissociation of the two monodentate ligands NH_3, the release of the bidentate ethylenediamine (en) and diphenyl-ethanediamine (ph-en) ligands from $[Ru(bpy)_2(en)]^{2+}$ and $[Ru(bpy)_2(ph\text{-}en)]^{2+}$ (**79** and **80** in Fig. 34) is more difficult due to the chelation effect (Φ_{LD} = 0.002(1) and 0.003(1), respectively, for ligand exchange with Cl^-) [297]292. However, ligand photoejection can be facilitated by lowering the ligand coordination ability towards the Ru(II) center, as it occurs for **81** and **82**, where the weaker coordinative bis-thioether ligands result in significantly improved photoejection quantum yields (Φ_{LD} = 0.019 and 0.027 for **81** and **82**, respectively).

Steric effects also play a crucial role in the photochemistry of such compounds. In particular, the insertion of bulky substituents in the ruthenium architectures can be used to distort the geometry of the resulting pseudo-octahedral complexes and lower the energy of the ^{3}MC states, allowing their thermal population upon excitation to the ^{3}MLCT

states [298–300]293-295. This facilitates the ligand loss and the consequent generation of active Ru(II)-aquo species. Thereby, the use of strain-inducing substituents has been widely employed in the design of a variety of light-responsive ruthenium compounds.

A series of DNA photobinding agents was achieved through the introduction of two bulky methyl groups on the bpy, the DNA intercalating dpq or in the DNA "light-switch" dppz ligands, in the corresponding complexes **84**, **85** and **86** (Fig. 34) [107,301]107,296. Irradiation of these compounds afforded the selective photoejection of methylated ligands, while no photodissociation was displayed by the analogue, unstrained complex **83**. This resulted in a ligand-deficient Ru(II)-aqua species that, contrary to intact forms of metal complexes, featured DNA photobinding properties and mediated increased photoinduced anticancer activity.

In this approach, sterically hindered ligands are generally expected to dissociate from complexes upon irradiation. However, irradiation can even promote the ejection of a non-hindered moiety, as it was shown for $[Ru(bpy)_2(dpphen)]^{2+}$ (**87**), whose irradiation (λ = 460 nm, 100 mW/cm^2) determined the quantitative dissociation of the less hindered bpy ligand [302]297.

This "unusual" ligand substitution was explained considering the key role played by two twisted bpy moieties and/or the extended Ru–N(bpy) distances in the asymmetrical distortion of the complex: the ejection of bpy appeared to be facilitated by the free rotation of the pyridine ring about the C2-C2' bond, which became possible after the first Ru-N was broken. On the contrary, the plane of the hindered dpphen was more resistant to distortion, rendering such ligand less prone to photoejection.

Obviously, increasing the number of strain-inducing substituents in metal complexes will further enhance the distortion in the geometry of metal complexes, providing a suitable way to improve their photoreactivity and, also, biological behaviors.

As an example, the incorporation of two bulky dmphen ligands in **89** (Fig. 34) returned a *ca.* 10-fold faster photodissociation of the methylated ligand after irradiation (λ > 400 nm, 200 W), if compared to the less hindered compound **88**, with $t_{1/2}$ values of 4 min and 42 min, respectively [303]298. Of worth noting, the high photoreactivity of **89** was paralleled by a strong photo-induced anticancer activity against leukemia cells HL60, with IC_{50} values in the sub-micromolar range (19-fold more potent than cisplatin) and PI > 1880 (λ > 400 nm, 410 W, 3 min).

The $[Ru(dmphen)_2]^{2+}$ scaffold was also employed by Havrylyuk *et al*. in a series of heteroleptic Ru(II) complexes where different 2-(2-pyridyl)benzazole moieties were introduced to improve the photoreactivities of compounds by virtue of the increased steric clash (**90a**-**d**, Fig. 34) [135]. However, though these compounds were effective in killing HeLa 60 cells upon irradiation (PI = 10 for the lead compound **90a**, light exposure: LED, λ > 450 nm, 29.1 J/cm^2), the high dark toxicities compromised their further development as PACT agents. Later, the same group continued this work by performing a SAR study on a series of $[Ru(dmphen)_2]^{2+}$-containing complexes featuring regular 3-(pyridyl-2-yl)-pyrazol(in)e (**91a**-**d**, **91g**-**i**) and inverse 1-(pyrid-2-yl)-pyrazoline (**91e**, **91f**) as photolabile, non-toxic ligands [304]299. Structural modifications of the pyrazol(in)e ligands influenced the photoreactivity of these compounds but also strongly affected their anticancer profiles. In particular, they showed that the coordination of regular pyrazol(in)e ligands to the Ru(II) bis-dimethylphenanthroline scaffold resulted in photoresponsive compounds with higher photochemical properties and biological potential if compared to the analogous complexes with inverse pyrazolines, whereas the insertion of a phenyl ring to the 1N-pyrazoline cycle was fundamental to improve the photoinduced ligand release in aqueous media. Interestingly, the replacement of the chlorine atom in the pyridyl-pyrazole ligand **91h** by a carboxylic group in the monocharged **91i**, allowed to achieve a 15-fold lower dark cytotoxicity without altering the sub-micromolar phototoxicity, resulting in the largest PI observed across the series (PI = 146, HeLa 60 cells, light exposure 29.1 J/cm^2).

It can be noted that the employment of these heterocycles-based co-ligands combined intrinsic steric clash with extended π-conjugation. This is a powerful combination that can be exploited to activate Ru(II) complexes using lower energy, more penetrating radiations, in close analogy to the pioneering work on the first, quinoline-containing, DNA photobinding agents (**92** and **93**), that displayed anticancer activity upon red and NIR irradiation (PI up to 9.2, λ > 650 nm, 7 J/cm^2, HL60 cells) [305]300.

The importance of extended π-conjugated ligands in allowing the photoactivation of Ru(II)-based PACT compounds within the therapeutic window was also observed by Sun *et al.*, who studied the behavior of a series of complexes containing bpy, tpy and biq ancillary ligands and photolabile acetonitrile molecules, after irradiation through tissues with different thickness [306]301. Among these compounds, only the π-extended biq-containing ones, such as

$[Ru(tpy)(biq)(CH_3CN)]^{2+}$ or $[Ru(biq)_2(CH_3CN)_2]^{2+}$ (**94** in Fig. 34), were effectively activated with low-energy red light, thanks to absorption tails that extended up to 750-780 nm. In particular, though light was consistently attenuated by the thickness of tissue, red-light was still able to activate **94** in pork tissue up to 16 mm and led to a significant cell death in HeLa cells after passing through 8-mm thickness (laser light, λ = 671 nm, 110 mW cm^{-2}, 30 min).

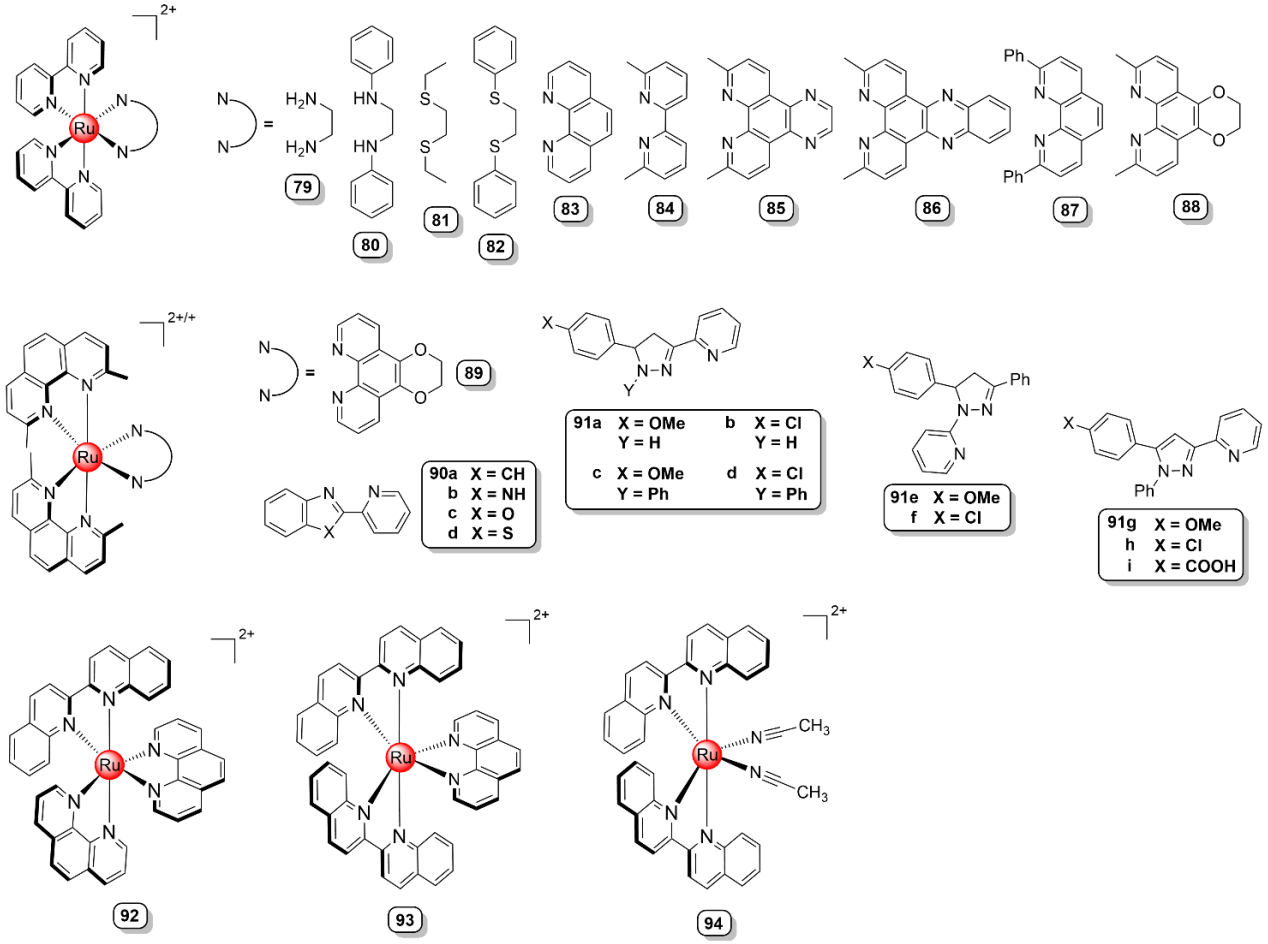


Fig. 34. Ru(II)-based PACT agents promoting the photoinduced generation of unsaturated Ru(II) centers with DNA binding properties.

Considering the central role of DNA as cellular target of Ru(II)-based PACT agents, poor cellular uptake and nuclear localization would pose a serious obstacle to the utility of this class of compounds. Therefore, modulating the nature of ancillary ligands to increase the hydrophobicity of metal complexes and impart higher cellular and/or nuclear uptake, represents a clear opportunity to improve cytotoxicity.

The group of Bonnet widely explored this topic [281,307]276,302. Following their work on the use of biologically harmless thioether ligands, such as *N*-acetylmethionine or biotin, as photocleavable protecting groups for the active $[Ru(tpy)(bpy)]^{2+}$ scaffold [308]303, they demonstrated that modifying the bidentate ligand (NN) in $[Ru(tpy)(NN)(hmte)]^{+}$ complexes featuring the photolabile hmte ligand (**95**-**97**, Fig. 35), markedly affected their biological activities [309]304. Indeed, the substitution of the bpy ligand in the hydrophilic complex **95** (LogP = -3.28 ± 0.31) with 3,3'-biisoquinoline and di(isoquinolin-3-yl) amine in the more lipophilic **96** (LogP 2.10 ± 0.27) and **97** (LogP 0.45 ± 0.10) compounds, permitted to switch from a biologically inactive compound (**95**), due to its poor penetration in cancer cells, to two promising PACT agents (**96** and **97**), that displayed higher cellular uptake and increased cytotoxicity upon green light irradiation (PI from 3.8 to 4.5 against A549 and A431 cancer cells, λ = 520 nm, 38 J/cm^2).

Bphen is another ligand which was widely employed to achieve improved lipophilicity, in keeping with the pioneering work of Puckett and Barton showing that the cellular transport of dppz complexes of Ru(II) can be significantly enhanced by the insertion of this lipophilic unit [103,310]103,305.

In this regard, Fayad and coworkers described a correlation between hydrophobicity, cellular-uptake and cytotoxicity in a series of compounds of general formula $[Ru(NN)_2(6,6'\text{-dmpby})]^{2+}$, with NN = bpy (**84**, Fig. 34), phen, bphen (**98**) and

sulfonate-bphen (**99**), and containing 6,6'-dmbpy as photolabile ligand (Fig. 35) [311]306. Cell viability assays on A549 and triple negative breast cancer (MDA-MB-231) cells indicated that the highest activity (PI from 4.6 to 6.6, λ = 460 nm, 100 mW/cm^2) was possessed by the most lipophilic compound, **98** (LogP = 0.42), which was also internalized by cancer cells up to ten times more than the bpy-containing analogue **84** (LogP = -2.95).

The bphen ligand was also modified to impart steric clash to ruthenium complexes. In compound **100** (Fig. 35), the introduction of two methyl groups in the 2,9 positions of a bphen unit promoted the photoejection of both bpy and the methylated bphen ligand, resulting in anticancer activities strongly dependent on the cell line tested [312]307. In particular, light-activation (460 nm, 100 mW/cm^2) of **100** promoted a potent effect in B16-F10 melanoma cells, with a remarkably high phototherapeutic index ($IC_{50\ light}$ = 0.11 µM, $IC_{50\ dark}$ > 100 µM, PI > 900). Compared to the unstrained and more hydrophilic control $[Ru(bpy)_2(phen)]^{2+}$ (LogP = -2.82), which resulted almost non-toxic either in dark or light ($IC_{50dark/light}$ > 100 µM), **100** (LogP = -1.57) exhibited an up to 35-times higher cellular internalization. A more recent work extended the study of **100** to human malignant melanoma cells (A375) cells, reporting a c.ca 70-fold more potent light-induced effect than cisplatin ($IC_{50\ dark}$ = 43.10 µM, $IC_{50\ light}$ = 0.13 µM, PI = 340) [286]281. The modes of cellular uptake of this compound were also investigated; among several possible routes, including simple passive diffusion and endocytosis, active transport was found to be the main intracellular transport.

Cytotoxicity and lipophilicity can be clearly strongly related to each other. Naturally, exceptions to this general concept can also be found, as showed, for example, by Zhout *et al*. for series of DNA photobinders containing a photolabile py molecule and dppz (**101a**) or variously fluorinated dppz units (**101b**-**d**), as non-photolabile ligands [288]283. Among these compounds, the highest potency ($IC_{50light}$ ~ 8 µM, IC_{50dark} > 200 µM, in HeLa and SKOV-3 cell lines, λ_{irr} = 470 nm, 27 J/cm^2) was found for the difluorinated **101c**, which displayed lower cellular uptake and nuclear accumulation if compared to **101b** and **101d**. The better activity of **101c** was instead attributed to the greater affinity of this compound towards DNA, thus suggesting a key role of fluorine atoms in strengthening the interaction with the biopolymer, likely through enhanced electronic and hydrogen bonding interaction.

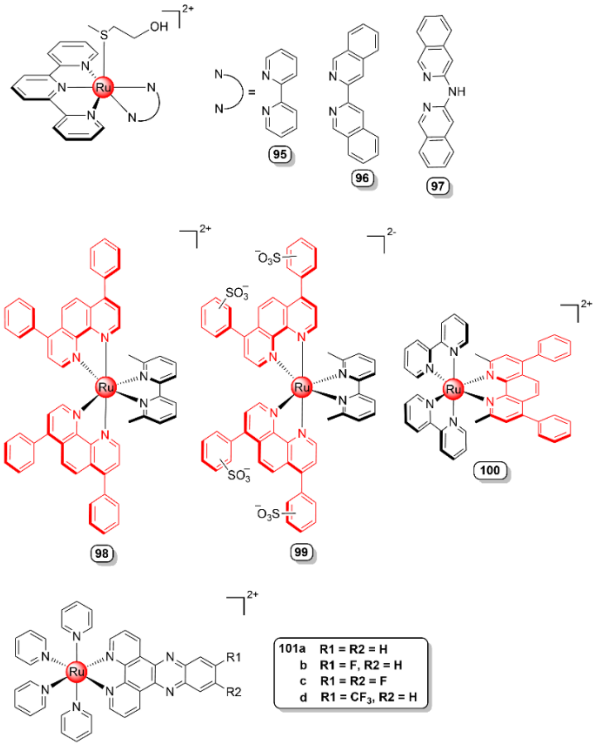


Fig. 35. Bonnet's complexes **95**-**97**, strained RPCs containing the lipophilic bphen ligand (in red) to improve cellular internalization (**98**-**100**) and Zhout's dppz-containing complexes **101a**-**d**.

In the rational design of strained RPCs as PACT agents, a point that should be highlighted is that high photoreactivities emerging by "cell-free" experiments are not always paralleled by the effective generation of phototoxins *in vitro*.

Indeed, in the cellular environment the efficiency of photodissociation processes can be hampered by a series of decay pathways. In addition, sequestration of both the starting ruthenium compound and/or the Ru(II)-photoproduct by "non-essential" proteins or other biomolecules could undermine their access to more crucial cellular targets, nullifying their biological effects. The occurrence of these processes depends on several factors, such as the structural features of metal complexes, in a way that is not always easily predictable.

In this regard, J. Roque *et al.*, recently showed that slight differences in the point of attachment of a naphthalene residue to the IP ligand in $[Ru(6,6'\text{-dmbpy})_2(1\text{-NIP})](PF_6)_2$ and $[Ru(6,6'\text{-dmbpy})_2(2\text{-NIP})](PF_6)_2$ (respectively compounds **102a** and **102b** in Fig. 36), containing the photolabile 6,6'-dmbpy ligand, had a surprising effect on their biological activity [313]308. In fact, though **102a** and **102b** possess nearly identical Log $P_{o/w}$ and photoejection rates, only the former compound displayed a notable phototoxicity under hypoxic conditions (1% O_2) in SK-MEL-28 malignant melanoma cancer cells ($EC_{50\ light}$ of 6.94 ± 0.12 µM, PI = 15, blue light exposure, 20 mW/cm^2). The lack of phototoxicity of **102b** (both in normoxia and hypoxia conditions) was assumed to be due to the structural differences among the two compounds.

Interestingly, the same authors further showed that functionalizing with methyl or methoxy groups the inactive isomer **102b** made it as phototoxic as the active isomer **102a**, under normoxia conditions. On the other side, methoxy variants of **102a** were inactive [314]309. Therefore, apparently innocuous structural modifications can exert larger effects on the photocytotoxicity of ruthenium compounds than their lipophilicity and photoreactivities would suggest.

Lastly, besides DNA, which certainly represents the most common target of Ru(II)-based PACT compounds, other biological substrates can play the role of final targets of Ru(II)-photoproducts, opening the way for the development of new metal-based drugs with different mechanisms of action and considerably widening their applications.

Bataglioli *et al.* recently demonstrated that the photoreactivity of compounds **102c** and **102d** (Fig. 36), can be successfully exploited to modulate the amyloid beta (Aβ) peptide aggregation [313,315]308,310, a crucial issue in the progression of the Alzheimer's disease (AD) [316]311. The photoinduced loss of a 6,6'-dmbpy unit from these complexes afforded the formation of cis-exchangeable coordination sites, able in turn to covalently bind the Aβ peptide through its histidine residues. This resulted in large amorphous aggregates, bypassing the generation of toxic low MW oligomeric species of the peptide and thus providing a critical insight into the pathophysiology of AD. Docking studies also indicated that the more hydrophobic phen ligand contained in these compounds was critical to favor the interaction between metal complexes and Aβ fibrils, as the bpy-containing analogue **102e** displayed weaker binding affinity.

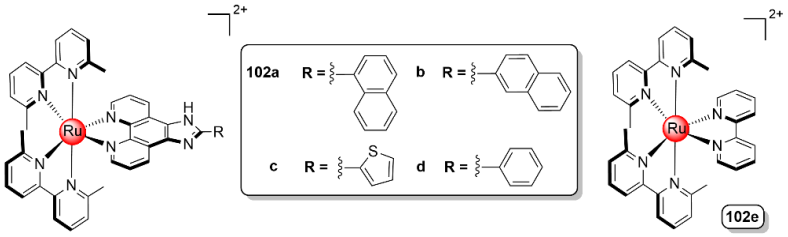


Fig. 36. Ru(II)-based PACT agents **102a**-**e**.

Besides RPCs, Ru(II) arene complexes, such as those featuring the general formula $[\eta^6\text{-arene})Ru(NN)(X)]^{n+}$ (NN: bidentate ligand; X: leaving group), represent another large family of Ru(II) compounds with promising prospects as photocisplatin agents. These systems display a typical "piano stool" structure and their antitumoral activity is generally attributed to the generation of Ru(II)-hydrates, able to covalently bind to DNA following the photoinduced de-arenation or the dissociation of the monodentate ligand. However, this topic has been recently reviewed elsewhere and the Reader is directed to other works for a more detailed discussion [49,317]49,312.

3.3 Ruthenium(II) complexes as photocages for bioactive compounds

"Photocaging" represents an intriguing alternative to DNA photobinders to elicit cytotoxic effects through the spatiotemporal control over the release of molecules with antitumoral activities by using light.

In this strategy, a biologically active compound (caged compound) is bound to photolabile protecting groups (caging scaffolds) resulting in composite systems (prodrugs) termed “photocages”. Until the photocage remains intact, the toxicity of the caged compound is “masked” whereas, upon light-activation, the bond between the caged molecule and the caging scaffold is irreversibly broken, leading to the release of the bioactive unit and unleashing its biological effect. Therefore, alongside with the increase of selectivity which stems from the light-driven control over the drug activation, this method potentially permits to lower a series of side effects arising from non-selective toxicity and low bioavailability issues, commonly associated to the use of active ligands in their “free” forms. Indeed, the camouflage of bioactive compounds may change several of their chemical-physical properties, including water solubility and/or tendency to form aggregates, just to name a few. For decades, since their first introduction by Engels and Schlaeger for the protection of adenosine [318]313, researchers have employed organic-based photoremovable protecting compounds (PPGs) to control the release of a wide variety of bioactive molecules, including hormones, neurotransmitters, anticancer agents and kinase inhibitors [319–323]314-318. However, aside rare exceptions [324,325]319,320, their removal typically requires high-energy UV light, which possesses poor tissue penetration and can lead to tissue damages. Moreover, important biological warheads, such as nitrogen-containing heterocycles, thioethers and nitriles, cannot be protected with PPGs, limiting their applicability in biological systems.

In this respect, RPCs offer an orthogonal approach to their organic counterparts. In fact, the coordinated ligands, including functional groups that cannot be caged with PPGs, can be easily liberated upon light irradiation [326,327]321,322. In addition, ancillary ligands can be optimally modified to allow for ligand deprotection by using less toxic and more penetrating radiations. It is appropriate to specify that, unlike Ru(II)-based photocisplatin compounds (paragraph 3.2), whose biological activity is linked to Ru(II)-based photoproducts, in the photocaging strategy the focus is on the biological effect provoked by the photoreleased ligand, whereas the empty cage should be ideally non-toxic to prevent interferences with the activity of the liberated drug. Therefore, being aware of the subtle differentiation between these modalities of action, in this paragraph we focused on systems whose light-mediated biological activity is mainly associated, or is supposed to be associated, to the liberated caged compounds rather than to the remaining Ru(II) photoproducts. However, additional (minor) contributions from the latter, such as the capacity to bind to DNA of certain Ru(II) fragments, such as $[Ru(bpy)_2]^{2+}$ and $[Ru(phen)_2]^{2+}$, cannot be completely ruled out. A more in-depth discussion of photoresponsive Ru(II) complexes purposely designed to involve multiple modalities of action will be handled in chapter 4.

The “photocaging approach”, has been largely used to control the release of a wide variety of bioactive molecules, optimally derivatized with nitrile, pyridine, imidazole, amine, diazine and thioether functionalities to allow for the metal coordination to ruthenium photocages. In this area, an astonishing work was performed by Etchenique and coworkers, which gave birth to a new class of Ru(II)-photocages for the light-controlled release of nitrogen-containing neurotransmitters. The first member of this class was the compound $[Ru(bpy)_2(4AP)_2]^{2+}$ [328]323, whose irradiation promoted the liberation of 4-aminopyridine (4AP), a widely known potassium channel blocker, resulting in modulation of the neuronal activity of isolated ganglia of *Hirudo medicinalis* leech. Later, the same group continued to exploit the good caging and photoreactive properties of the $[Ru(bpy)_2]^{2+}$ fragment to release multiple biologically active agents from a single precursor [329]324 and to cage g-aminobutyric acid (GABA) [330–332]325-327, nicotine [333]328, serotonin [330,334]325,329 dopamine [208]206, glutamate [335]330 and, more recently, L-arginine [336]331.

Since this pioneering work, the typology of caged molecules inserted into Ru photocages was promptly expanded. Turro and Kodanko employed the $[Ru(bpy)_2]^{2+}$ core to cage a series of nitrile-containing inhibitors of various cysteine cathepsins, a class of proteases associated with tumor progression, angiogenesis and metastasis in several cancers, especially in metastatic breast and prostate cancer [337,338]332,333. In the majority of these cases, coordination of enzyme inhibitors to metal centers hampered their interaction with the active sites of enzymes and blocked their activity. This was restored upon liberation of the active molecules from metal compounds following irradiation, achieving spatiotemporal control over their proteolytic activity. As an example, Cbz-Leu-Ser(OBn)-CN was released upon light-activation of compound **103** (Fig. 37), leading to an up to 88-fold decrease of the activity of cathepsin K compared to dark conditions and resulting in an increased photocytotoxicity in a 3D model of prostate carcinoma cells [339]334.

The $[Ru(bpy)_2]^{2+}$ fragment was also preserved in a recent photoresponsive compound against the influenza virus. Irradiation with visible light (λ = 450 nm, light dose 0.84 J/cm^2, 4 min) of the computationally designed compound **104** promoted the complete ejection of a gallic acid derivative, a potent inhibitor of the RNA-dependent RNA polymerase of

the influenza virus (PA_N), and led to a marked inhibition of the enzymatic activity (IC_{50} = 7.4 ± 2.2 μM) compared to dark conditions (IC_{50} > 100 μM) [340]335. Similarly, Vásquez and coworkers controlled the release of the histidine-containing tripeptide Arg-Gly-His (RGH) from compound **105** modulating the *in vitro* Ni(II)-dependent nuclease activity by using LED light (455 nm) [341]336.

In keeping with the use of $[Ru(NN)_2]^{2+}$ scaffolds as protecting groups, Renfrew *et al*. replaced bpy with ancillary phen ligands to control the liberation of the imidazole-containing anticancer agents econazole and 6-mercaptopurine (6-MP), respectively from compounds **106** [342]337 and **107** [343]338 (Fig. 37). In case of **107,** irradiation with blue light increased the cytotoxicity against MCF-7 breast cancer cells (PI = 4, LED, λ = 465 nm, 8.5 J/cm^2) whereas, in the dark, the complex was over 20-fold less toxic than the free drug, thus offering a suitable way to deliver 6-MP in a safer prodrug form.

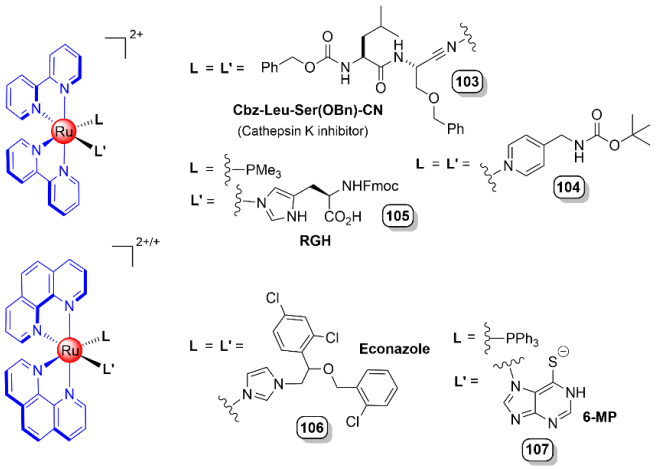


Fig. 37. Examples of ruthenium based photocages employing the $[Ru(NN)_2]^{2+}$ fragment (NN = bpy or phen) as protecting group for **L/L'** bioactive compounds.

Besides the $[Ru(NN)_2]^{2+}$ core, caged complexes of the general formula $[Ru(tpy)(NN)\mathbf{L}]^{2+}$, containing **L** as a monodentate bioactive compound, represent another common and valid alternative.

The $[Ru(tpy)(NN)]^{2+}$ scaffold possesses only one coordination site available for caging monodentate ligands and efficiently allows the photoinduced liberation of pyridine-, nitrile- and thioether-functionalized bioactive compounds (*vide infra*) [344–346]339-341. Analogously to the previous paragraph, sterically hindered NN ligands were commonly used to facilitate photoejection processes. It has been demonstrated, for example, that the insertion of bulky diimine ligands, such as 6,6'-dmbpy or biq, determines a major distortion of the **L**-metal bond, weakening the σ- and π-back-bonding to the metal and thus favoring the consequent photoejection of **L** [298,344]293,339. Importantly, high photoejection efficiencies should not be reached at the expense of significant loss of thermal stabilities, a feature that generally accompanies the increased distortion of the pseudo-octahedral geometries of this class of compounds.

The group of Turro employed the $[Ru(tpy)(NN)]^{2+}$ core to develop a series of Ru(II)-photocaging compounds containing various inhibitors of important enzymes. In keeping with their work on cysteine cathepsin, they showed that an optimal placement of the Ru-caging groups in the epoxysuccinyl inhibitors of compounds **108a** and **108b** (Fig. 38) provided an up to 10-fold control (for **108b**) over the permanent inactivation of cathepsin L with blue light (λ ≥ 395 nm, 10 min) [347]342.

Cytochrome P450 enzymes (CYPs) are an important class of heme-containing proteins that catalyze key biological transformations [348]343 and whose inhibitors can be conveniently caged into Ru(II) complexes [349,350]344,345. In this respect, irradiation of the Turro's compounds **109a** and **109b** afforded the release of abiraterone (ABI) to selectively inactivate the multifunctional enzyme cytochrome CYP17A1, involved in steroidogenesis and upregulated in prostate cancer [351]346. In particular, **109a** displayed a higher photoreactivity compared to **109b**, due to the greater steric hindrance imparted by 6,6'-dmbpy compared to biq (Φ_{500} = 0.018 (1) vs 0.0043(2) in water), and its photoactivation (λ ≥ 395 nm, 250 W) determined a tight dose-dependent response in ABI-sensitive DU145 human prostate carcinoma cells. This effect was attributed to the liberation of ABI rather than to $[Ru(tpy)(6,6'\text{-dmbpy})(OH)]^{2+}$, that is scarcely able to generate ROS. On the contrary, **109b** suffered by greater dark toxicity, thus making the $[Ru(tpy)(6,6'\text{-dmbpy})]^{2+}$ core as

the preferred one to cage CYP inhibitors. An analogous strategy was then followed by the same group to control the photorelease of imatinib, a tyrosine kinase inhibitor used in the treatment of nonresectable gastrointestinal stomach tumors, from compounds **110a**-**b** (Fig. 38)[352]347.

Renfrew and coworkers applied the $[Ru(tpy)(NN)]^{2+}$ (NN = bpy, biq) structures to improve the therapeutic profile of the pyridine-containing CHS-828 (compounds **111a** and **111b**) [353]348, a potent anticancer agent based on the inhibition of the nicotinamide phosphoribosyltransferase (NAMPT), an enzyme that is abnormally upregulated in a wide variety of cancers [354,355]349,350. The caging of CHS-828 in **111a**-**b** resulted in water-soluble and stable complexes, with lower dark toxicity compared to the free ligand and capable to induce increased toxicity against A549 and MCF-7 human carcinoma cells when irradiated (PI values from 4.4 to 10.2, λ = 465 nm, 8.5 J/cm^2), due to the photorelease of CHS-828. Another potent inhibitor of NAMPT, STF-31, was inserted by the group of Bonnet in compounds **112a** and **112b** [250]245. Irradiation of these complexes with red light (20.6 J/cm^2) promoted the release of the cytotoxic inhibitor (Φ_{625} = 0.058 ± 0.080 vs 0.013 ± 0.019 for **112a** and **112b** respectively, in water), resulting in an up to 18-fold reduction of the NAMPT activity with respect to dark. In the case of **112b**, light-activation induced a 3- to 4-fold increase in cytotoxicity whether the cells were cultured with 1% or 21% O_2, respectively. This provided one among the first examples of the effectiveness of a photocaging agent under hypoxic conditions. Interestingly, in contrast to Kodanko *et al*., who applied the $[Ru(tpy)(6,6'\text{-dmbpy})]^{2+}$ core to cage ABI, the dmp-analogue **112a** suffered by high thermal instability, hampering its application in PACT.

A further demonstration that PACT agents can be both activated under hypoxia and normoxia was recently reported by the same group, through the caging of an analogue (R) of marine alkaloid rigidins, an emerging class of microtubule-targeting agents with antitumoral activity at nanomolar concentrations, in the photosensitive compound **113** (Fig. 38) [356]351. Its green light irradiation (522 nm, 38 J/cm^2) promoted the inhibition of tubulin polymerization directly in A549 cancer cells and led to significant PIs in both normoxic and hypoxic conditions. As an example, almost identical PI values were reported in lung A549 cells under normoxic and hypoxic conditions (4 and 4.1 respectively), whereas high phototoxicities were preserved in hypoxic skin A375 and A431 cells (PI values >16 and >12, respectively). *In vivo* studies also probed the effectiveness of such compound in xenograft A549 model in nude mice upon intraperitoneal injection and green light activation. Moreover, according to Renfrew [353]348, the authors remarked the promising characteristics of $[Ru(tpy)(bpy)]^{2+}$ as protecting group, as the activity of R was strongly reduced through its insertion in **113** whereas it was completely restored upon photocleavage.

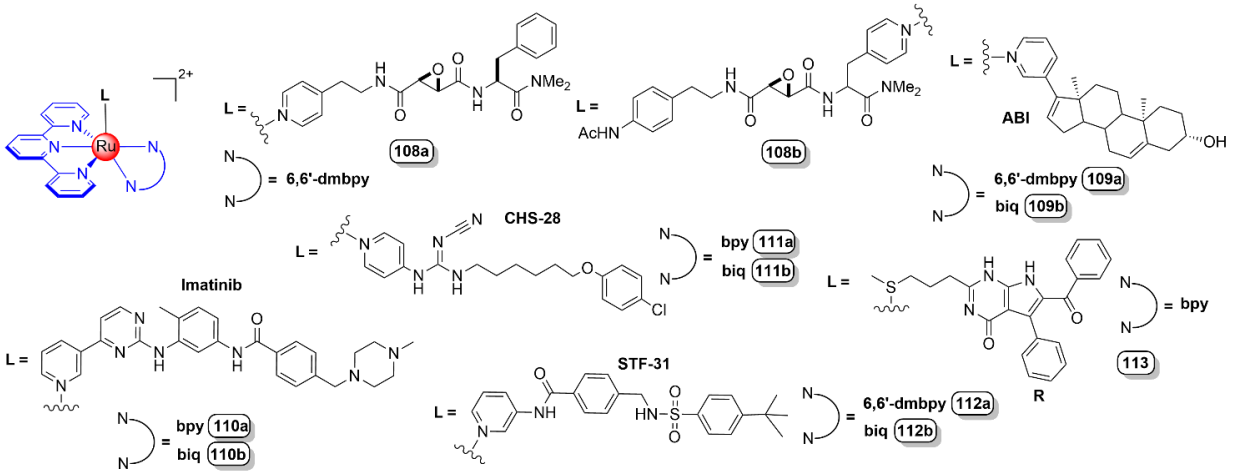


Fig. 38. Ruthenium based photocages employing the $[Ru(tpy)(NN)]^{2+}$ scaffold as protecting group for **L** bioactive ligands.

In addition to bidentate and tridentate ancillary ligands, frameworks with higher denticity can be also employed to further extend the photophysical and photochemical properties of Ru(II) photocages. Indeed, their use may increase the structural rigidity of metal complexes, lowering the occurrence of non-radiative relaxation processes following absorption of light, while maintaining absorption profiles in the visible region and the capacity to effectively photorelease nitrile- and nitrogen-containing active molecules.

Several systems belonging to this new item were reported by Turro and Kodanko, who developed a solid-phase method [357]352 to rapidly synthesize a family of ruthenium caging groups based on the use of TPA_m (tris(2-pyridylmethyl)amine) or TPA_m derivatives [358]353. The photoreactivities of the resulting complexes were described in terms of steric and electronic effects [358,359]353,354, but also on the basis of an unusual role played by orbital mixing of excited states. This was the case of the 1-isocyTPQA$_m$-containig compound **115** (Fig. 39), whose higher photoreactivity relative to the analogue TPA_m-containing **114a** (Φ_{400} (**115**) = 0.033(3) and Φ_{350} (**114a**) = 0.012(1), for the first CH_3CN exchange process in water) was explained considering that ligand dissociation in **115** was mediated by a highly mixed ^{3}MLCT/$^3\pi\pi$ excited state, instead of the typical ^{3}MC state [326,360]321,355. However, notwithstanding the potential of these protecting groups and the successful employment of TPA_m to control the photorelease of the cathepsin K inhibitor Cbz-Leu-NHCH$_2$CN from **114b** (Fig. 39) [361]356, this class of compounds is relatively "new" and examples of their *in vitro* application are still rare. To the best of our knowledge, the only exceptions are represented by compounds **114c-e**, that liberated harmless N-monodentate ligands upon light exposure and showed low cytotoxicity against human prostate carcinoma PC-3 cells under dark conditions, thus indicating the potential of TPA_m agents as photocaging groups [362]357.

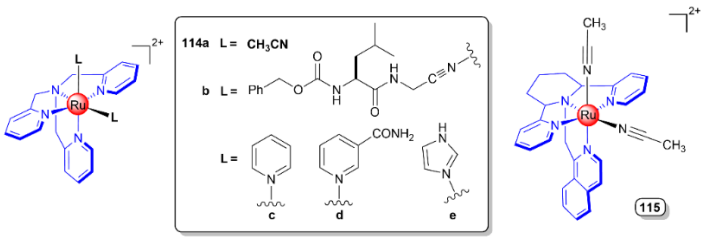


Fig. 39. Examples of the use of the tetradentate TPA_m ligand in Ru(II)-based photocages.

Lastly, it should be remarked that the evaluation of the "suitability" of a given Ru(II) scaffold for photocaging purposes is of outmost importance to keep ruthenium photocages from suffering by several drawbacks, that may seriously threaten their biological application. As an example, a high photoreactivity may be undermined by considerable dark toxicity and/or thermal instability. In addition, the lack of a systematic comparisons in the literature between the proposed Ru(II)-scaffolds further complicates the establishment of most effective/promising protecting groups for PACT applications.

An interesting view on this topic was recently presented by the group of Glazer, which studied a series of compounds with the general formula [Ru(tpy)(NN)**L**], featuring harmless monodentate and photolabile ligands (**L**), from which they derived the most relevant properties that an "ideal" photocage should possess [363]358.

The authors individuated five key properties: (i) peak of lowest energy absorption, (ii) photoreactivity (Φ_{ps}), (iii) stability under biological conditions, (iv) dark cytotoxicity and (v) light-induced cytotoxicity, illustrated in radar charts of Fig. 40. In this view, an "ideal" photocage should possess high values for points (i-iii) and low ones for points (iv and v). This scheme could be also applied to compounds featuring multiple modalities of action (see chapter 4), with the only difference that, in the second case, high light-induced cytotoxicity (v) is preferable considering the additional activity of Ru(II) photoproducts. According to that, the model compound **116a**, featuring bca as bidentate ligand and pyrazine as non-toxic photolabile ligand, turned out to fulfill several of the above mentioned requirements for pure photocaging applications, including low toxicity both in dark and upon light-activation (IC_{50} > 100 µM in a HL-60 cell line). On contrary, the higher phototoxicity of **116b** (PI value > 3.6) would made the [Ru(tpy)(btz-qui)] scaffold more indicated in the design of dual action agents.

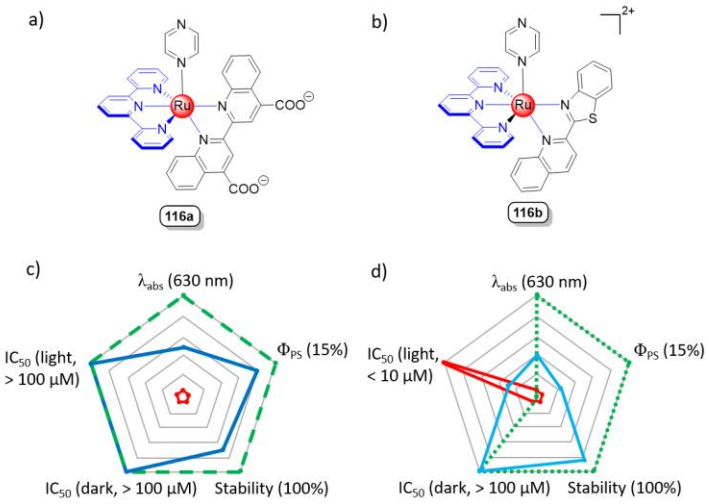


Fig. 40. Ru(II) photocaging compounds **116a** (a) and **116b** (b) together with the radar charts of the key properties for an ideal photocaging compound (c) and a dual action agent (d). In c and d are respectively reported the radar charts for compound d **116a** (solid blue), compared to an ideal pure photocaging agent (dashed green) and for compound **116b** (solid aqua), compared to a "dual action" agent (dotted green). In solid red are shown hypothetical negative controls. The axes scale from center to perimeter as follows: absorbance, 420 - 630 nm; Φ_{PS}, 0 - 15%; stability (%); and IC_{50}, 0 - 100 µM; optimal parameters are specified in parentheses.

4. Multiple-modality mechanism of action

Combining different mechanisms of action into unique molecular systems represents a challenging opportunity in the design of new potential therapeutic drugs. Many recent examples are reported in the literature regarding compounds acting through multiple modalities, that may be different techniques, targets, activation stimuli or therapeutic activities.

4.1 Dual action

"Dual-" or "multiple action-" based Ru(II) complexes may provide a way to achieve improved potency compared to separate approaches, by virtue of the simultaneous occurrence of different light-induced anticancer mechanisms while providing a precious opportunity to overcome some among their most critical drawbacks.

4.1.1 PDT/PACT

The combination of PDT and PACT modalities into dual action PDT/PACT agents permits to handle, on one side, the reliance on molecular oxygen of PDT and on the other side, the non-catalytic action of PACT-based compounds.
From a photophysical point of view, multiple photoreactive compounds usually rely on the photo-triggered population of competitive excited states, with relaxation pathways that ultimately lead to different PACT and PDT mechanisms. As depicted in Fig. 41a, light-induced excitation brings to the population of triplet excited states, following rapid intersystem crossing from singlet manifolds. This energy can be dissipated in part through the lowest-lying ^{3}IL/^{3}ILCT states, able to sensitize ROS through Type I or Type II pathways, and in part *via* thermal population of dissociative ^{3}MC states, giving rise to photochemical substitution reactions.
The latter pathway may result in the photorelease of bioactive ligands and/or active Ru(II)-photoproducts, that in turn may have different biological targets (chapter 3). In addition, a few compounds with intermediate modalities of action to these options are also possible. Therefore, several scenarios are feasible in the design of multiple action-based compounds. In this respect, a schematic representation of some among the common combinations between different mechanisms of action associated to a multiple active Ru complex bearing a "classical" dppn ligand, for ROS sensitization, and a bioactive ligand (**B**), is depicted in Fig. 41b.

As it can be envisaged, the number of variables in this panorama makes it difficult to classify this class of compounds into distinct, well defined, categories. However, in the following paragraphs we grouped some among the most representative examples of dual/multiple active compounds on the basis of the different modalities of action to which they rely, namely i) ROS sensitization and DNA interaction, ii) ROS production and photochemotherapy and iii) photochemotherapy and DNA interaction. Moreover, examples of compounds with dual targeting and dual stimuli modalities are also reported.

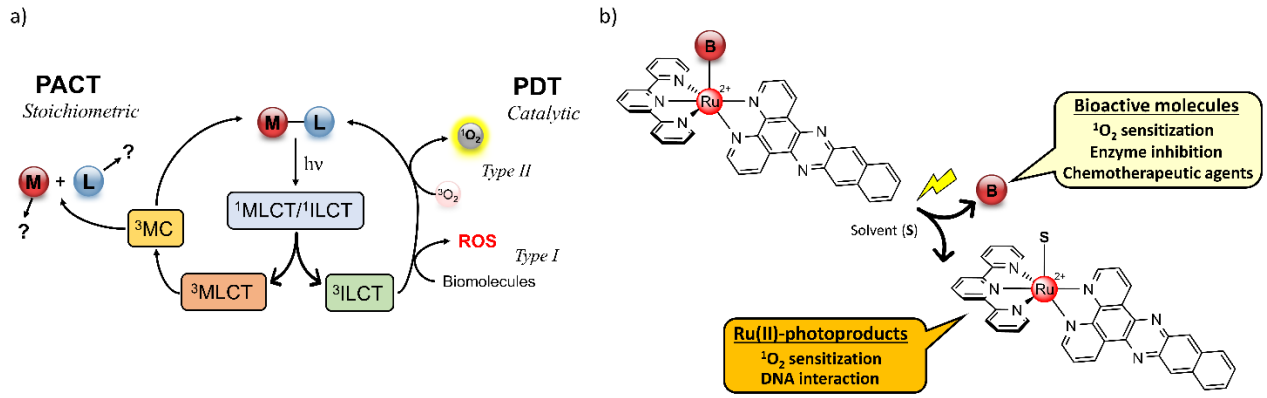


Fig. 41. a) Competing excited state relaxation pathways for dual/multiple active photoresponsive Ru(II) complexes and b) some among the possible combinations between the modes of action of a multiple-active compound with the general formula $[Ru(tpy)(dppn)(\mathbf{B})]^{2+}$ (**B** = bioactive ligand).

4.1.1.1 PDT/PACT agents combining ROS sensitization and DNA interaction

In this section are described those systems featuring simultaneous production of ROS, mainly $^1O_2$, and Ru(II) photoproducts able to interact with DNA, *i.e.* through covalent binding, thus exerting an additional photocisplatin-like mode of action. Complexes involving the release of bioactive ligands from metal centers will be treated instead in paragraphs 4.1.1.2 and 4.1.1.3.

The group of Turro was among the first to report that irradiation of optimally designed RPCs may result in both production of $^1O_2$ and dissociation of photolabile ligands capable of covalent binding to DNA [298,364,365]293,359,360. For example, the efficient $^1O_2$ sensitization by $[Ru(bpy)_2(dppn)]^{2+}$, imparted by the presence of low-lying π-π* excited states located on the dppn ligand, was combined with the capacity of $[Ru(bpy)_2(CH_3CN)_2]^{2+}$ to easily undergo photoinduced ligand exchange in the dual-active compound $[Ru(bpy)(dppn)(CH_3CN)_2]^{2+}$ (**117**) [366]361 (Fig. 42), which exhibited a great phototherapeutic margin (PI = 711) in HeLa cells, being almost 650- and 2.5-fold more potent than the precursors.

Bonnet and coworkers preserved the use of the π-expansive dppn ligand by caging the D- and L-glucose thioether conjugate (2-(2-(2-(methylthio)-ethoxy)ethoxy)ethyl-β-glucopyranoside) in the dual active enantiomeric complexes **16m-a** and **16m-b**, that showed submicromolar phototoxicities against A549 human lung carcinoma and MCF-7 human breast adenocarcinoma cancer cells (EC_{50} < 1 μM, PI values between 9.6-30, λ_{irr} = 454 nm, 3.1 J/cm^{-2}) [367]362. Such results were rationalized on the basis of two distinct factors: the release of the aquo complex $[Ru(tpy)(dppn)(H_2O)]^{2+}$, which interacts with DNA at an exceptionally high 400:1 base pair/Ru ratio and the good singlet oxygen sensitization by the same achiral photoproduct (Φ_Δ = 0.52(1) in methanol, λ_{irr} = 460 nm) [368]363, for which, mitochondrial DNA seemed to be the most likely target. The photophysical properties and the biological behavior of **16m-a** were further compared with those of a series of complexes where other bidentate spectator chelating ligands, including dppz and pmip, replaced the dppn group (see chapter 4.1.1.2 for more details) [136]. However, **16m-a** showed the highest potential as dual-action PACT/PDT agent, thus highlighting the key role played by the presence of the dppn ligand to afford both adequate $^1O_2$ production and interaction with biological targets.

From these studies it also emerged the fundamental role played by the steric and electronic features of the monodentate ligand L in the photoreactivity of $[Ru(tpy)(dppn)(L)]^{2+}$ -based compounds.

Indeed, while **16m-a** and **16m-b** effectively underwent photosubstitution of glucose conjugates, the analogous py-containing complex $[Ru(tpy)(dppn)(py)]^{2+}$ (**16m-c**) does not display ligand exchange upon excitation ($\Phi_{500} < 0.0001$ in CH_3CN), but only produces $^1O_2$, with near-unit efficiency ($\Phi_\Delta = 0.98(6)$ in methanol, $\lambda_{irr} = 460$ nm) [298,364]293,359.
However, the replacement of the dppn moiety by the sterically hindered dmdppn to give $[Ru(tpy)(dmdppn)(py)]^{2+}$ (**118a**) allows to recover dual reactivity, by making the dissociative ^{3}MC excited state more accessible while maintaining the dmdppn-centered $^3\pi\pi^*$ as the lowest-energy excited state. As a result, complex **118a** undergoes both py dissociation ($\Phi_{500} = 0.053(1)$ in CH_3CN) and produces $^1O_2$ upon irradiation ($\Phi_\Delta = 0.69(9)$ in methanol, $\lambda_{irr} = 460$ nm) [364]359.
Turro, Kodanko *et al.* recently reported on the potential of this compound in a comparative study with other three complexes where the dmdppn ligand was adorned with flanking phenyl (**118b**), 2,4-dimethylphenyl (**118c**) and 2,4-dimethoxyphenyl (**118d**) groups at positions 10 and 15, to prevent DNA intercalation without affecting the photophysical properties of the Ru(II) complexes [164] (Fig. 42). Due to more favored deactivation processes through competitive dissociative states in the aryl-substituted complexes, **118b**, **118c** and **118d** displayed a more efficient py-photosubstitution relative to the non-substituted derivative **118a**, together with good $^1O_2$ sensitizing properties. Despite the flank aryl groups blocked the ability of intercalate into DNA, these systems were able to interact with the biopolymer in an electrostatic manner and their photoactivation induced an increased toxicity in cancer cells, suggesting that intercalation may be not necessary for bioactivity. However, **118a** resulted the most active compound across the series, exhibiting a ca. 7-fold enhancement of toxicity upon irradiation in MDA-MB-231 triple negative breast cancer cells ($EC_{50\ light} = 4.6 \pm 0.5$ μM $EC_{50\ dark} = 34 \pm 3$ μM, PI = 7.4, $\lambda_{irr} = 460$-470 nm, 170 J/cm^2, 15 min) [164].
It can be noted that, in these systems, the singlet oxygen generation is raised by a Ru(II)-coordinated ligand but may be also imparted by a photosensitizer unit which is released upon excitation.
An example of the latter approach was recently described by Kodanko and coworkers, who showed that the pyridine functionalized BODIPY ligand in compound **119** engaged a double action: i) it effectively sensitized $^1O_2$ even once photoreleased from the ruthenium complex ($\Phi_\Delta = 0.23(4)$, $\lambda_{irr} = 460$ nm in MeOH) and ii) it acted as "antenna group", affording to elicit a considerably cytotoxic effect (PI > 140 against MDA-MB-231 triple negative breast cancer cells) by using low-energy light ($\lambda_{irr} = 520$-530 nm, 15 min, 50 J/cm^2) [369]364. A similar approach was also followed by Jin and coworkers in complex **120** (Fig. 42), where a photolabile pyrene-modified tpy group (tpy-pyr) enabled the compound to sensitize $^1O_2$ ($\Phi_\Delta = 0.18$, $\lambda_{irr} = 450$ nm in MeOH) and acted as antenna group for two-photon excitation in the NIR region ($\sigma_2 = 300$ GM at 800 nm) [224]365. Commonly, the tpy is not a photolabile ligand due to its tridentate character but, in this compound, the presence of an uncoordinated pyridine weakened the ligand field due to steric effect, making tpy-pyr dissociable upon irradiation. Upon activation ($\lambda_{irr} = 740$ nm, 1 W/cm^2), **120** potently inactivated a series of cancer cells, including hypoxic and cisplatin resistant A549 cells, with $IC_{50\ light}$ value of 0.21 μM and PI of 285 in the latter case (IC_{50}(cisplatin) > 100 μM). Moreover, both the BODIPY- and the pyrene-based ligands displayed fluorescence turn-ON once released from **119** and **120** respectively, thus making possible to visualize their intracellular ligand dissociation and cellular localization.

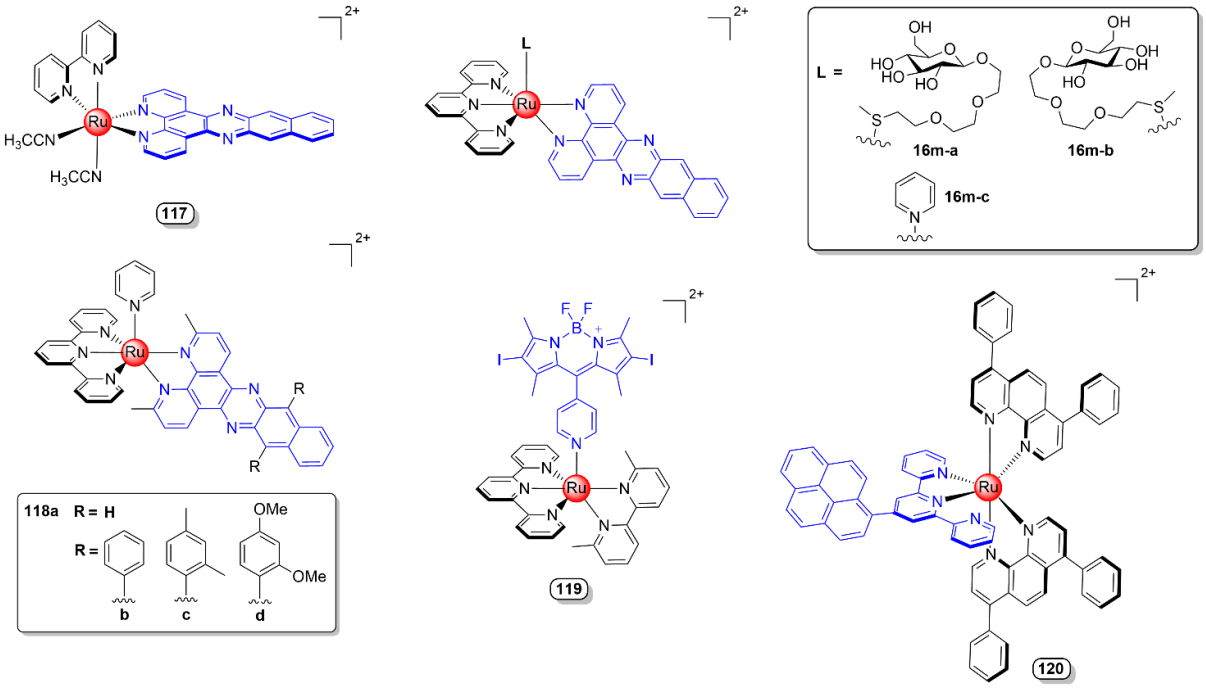


Fig. 42. Ru(II)-complexes with dual reactivity: $^1O_2$ sensitization and photoproduction of Ru(II)-based frameworks able to interact with DNA. In blue are evidenced the moieties responsible for ROS production.

The nature of co-ligands is a clear tool to modulate the photoreactivity of RPCs, and thus, also their potential as dual action agents.

In this respect, Mc Farland and coworkers demonstrated that increasing the number of thiophene units (nT) appended to the IP ligand in $[Ru(6,6'\text{-dmbpy})_2(IP\text{-nT})]^{2+}$ (n = 1-3), incorporated for ROS generation, permitted to switch from a roughly pure photocisplatin agent, namely compound **102c** (n = 1, see also paragraph 3.2), to the dual PACT/PDT compound **102c2** (n = 3) [315]310 (Fig. 43), which displayed an excellent phototherapeutic margin ($EC_{50\ light}$ = 0.2 ± 0.1 µM, $EC_{50\ dark}$ = 150 ± 8 µM, PI = 750) in HL-60 cells upon photo-activation (λ_{irr} = 400-700 nm, 100 J/cm^2). However, authors envisaged that, besides $^1O_2$ sensitization and DNA covalent binding by the Ru(II)-photoproduct, the redox and conductive properties typically possessed by α-oligothienyl systems of n > 2 may also play a role (see paragraph 2.2.2 for more details).

Most recently, the potential of analogous complexes bearing a further thiophen ring (IP-4T) on the IP ligand and 6,6'-dmbpy (**102c3**) or dmphen (**102c4**) as strain-inducing ligands, were also investigated (Fig. 43)[279]274. Besides their capacity to both sensitize $^1O_2$ (Φ_Δ = 43% and 65% for **102c3** and **102c4**, respectively) and undergo photoselective ligand loss of 6,6'-dmbpy or dmphen (Φ_{PS} = 0.47% and 0.28% for **102c3** and **102c4**, respectively), these systems were found to elicit unprecedented phototoxicities toward cancer cells both under normoxia (18.5% O_2) and hypoxic (1% O_2) conditions. In particular, **102c4** was active at concentrations as low as 170 pM in normoxia and near 10 nM in hypoxia and exhibited phototherapeutic indexes as large as > 500,000 in normoxia and > 5,800 in hypoxia under blue light treatments (453 nm, 100 J/cm^2), against human melanoma SKMEL28 cells. These are the largest values reported to date for any compound class. Interestingly, a lower activity was possessed by **102c3**, suggesting that, besides the pivotal function played by the IP-4T ligand on the impressive activity of these compounds, also the identity of co-ligands played a role, although their liberated forms do not appear as the source of cytotoxicity.

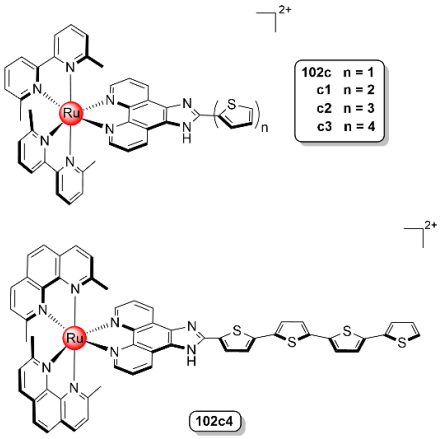


Fig. 43. Ru(II)-bis-heteroleptic complexes containing IP ligands appended with n thiophene units (nT = 1-4), reported by McFarland and co-workers, including compound **102c4**, which features the highest phototherapeutic indices reported to date for any class of compounds.

In addition to the sensitization of $^1O_2$, photocisplatin binding agents can be also combined with the production of free radical species.

Wang and coworkers developed a series of photoresponsive RPCs (complexes **121** and **122**, Fig. 44) that underwent dissociation of the pyridine sulfonic acid (py-SO_3) group under visible light irradiation (λ_{irr} > 470 nm), *via* Ru-O homolysis [370,371]366,367. This led to the formation of both metal-based DNA photobinders and radical species, namely py-$SO_3^{\cdot}$, able to photocleave the biopolymer in hypoxic conditions. However, the poor phototoxicity displayed by these systems toward lung adenocarcinoma A549 cells prompted the same group to synthesize compound **123**, where two more lipophilic bphen moieties replaced the bpy-based ancillary ligands [372]368. Such modification greatly improved the cellular uptake of this compound (see also paragraphs 2.1.1 and 3.2), which exhibited a 6-fold higher cell uptake in cisplatin-resistant human ovarian adenocarcinoma SKOV-3 compared to the more hydrophilic and scarcely active analogue **121** (LogP -2.19), with a considerable fraction of **123** (69%) being localized in the nuclear region. The enhanced cell uptake and nuclear accumulation levels were retained the main reasons for the high phototoxicities displayed by this compound against a series of cell lines, including SKOV-3 and A549 cells ($IC_{50\ light}$ values in the 200-400 nM region, PI = 42-55, λ_{irr} = 470 nm, 22.5 mW cm^{-2}, 30 min).

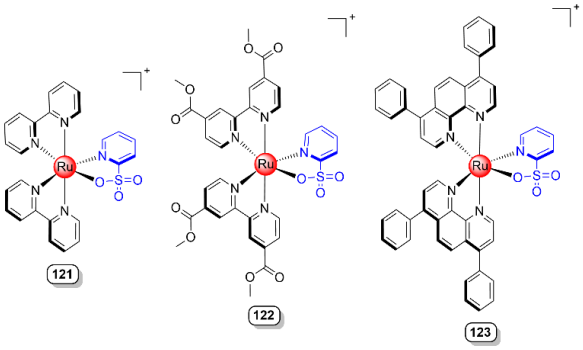


Fig. 44. Photoresponsive RPCs containing a photolabile pyridine sulfonic group developed by Wang and coworkers.

Lastly, besides photoinduced DNA damaging and ROS production, peculiar ancillary moieties can be exploited to endow the resulting Ru(II) complexes with additional light-induced anticancer mechanisms.

In this respect, Wang, Zhou *et al*. recently showed that light exposure of the nitro-anthraquinone-containing compound **124b** (Fig. 45) determined an intramolecular electron transfer from the excited Ru center to the anthraquinone ligand, resulting in intracellular NAD(P)H depletion (photoredox catalysis activity), generation of $O_2^{\cdot-}$ (PDT activity) and photorelease of the DNA damaging agent $[(biq)_2Ru]^{2+}$ (PACT action), thus integrating three functions in the same photoreactive dyad [373]369.

Compared to the anthraquinone-free complex **124a**, **124b** showed enhanced phototoxicity against a series of cancer cells, including cisplatin-resistant A549 cells, in which, upon photoactivation (λ_{irr} = 600 nm, 22.5 mW/cm^2, 40.5 J/cm^2), it led to a considerably higher toxicity ($IC_{50\ light}$ of 0.96 ± 0.02 µM, PI = 21) than cisplatin (IC_{50} = 61.2 ± 3.2 µM). Once again, the PACT modality of this compound permitted to preserve a considerably activity even under hypoxic conditions (3% O_2) ($IC_{50\ light}$ = 6.5 ± 0.5 µM, $IC_{50\ dark}$ = 33.9 ± 2.3 µM, PI = 5, A549 cells).

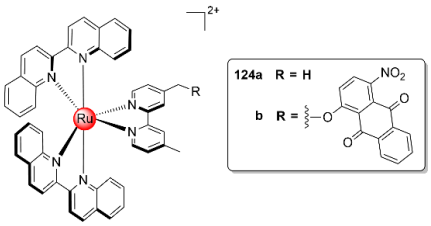


Fig. 45. Multiple active compound **124b** together with its analogue **124a**.

4.1.1.2 PDT/PACT agents combining ROS sensitization and photochemotherapy

In the design of dual/multiple action PACT/PDT agents, ROS sensitization can be also combined with the photorelease of bioactive molecules, such as inhibitors of essential enzymes for cancer cells that play crucial roles in their biosynthesis and metabolisms (see also paragraph 3.3).

In this field much work has been undertaken by the group of Turro, who showed that the insertion of the cathepsin K inhibitor Cbz-Leu-$NHCH_2$-CN and the tyrosine kinase inhibitor imatinib, respectively in **125** [368]**363** and **126** [352]347 (Fig. 46), resulted in dual photoactive compounds able to both deliver the bioactive ligands with spatiotemporal control (Φ_{450} (**125**) = 0.00014(3), in water, Φ_{500} (**126**) = 0.073(1), in acetonitrile) but also to produce $^1O_2$ with good yields (Φ_Δ (**125**) = 0.64(3) and Φ_Δ (**126**) = 0.57(7) in methanol, λ_{irr} = 460 nm). Analogously to some cases of the previous paragraph, the latter property was imparted by the presence of dppn/dppn-based ancillary ligands, which also conferred singlet oxygen sensitizing features to Ru(II)-photoproducts, albeit with lower efficiencies compared to their intact forms (Φ_Δ = 0.52(1) and 0.22(2) for $[Ru(tpy)(dppn)(H_2O)]^{2+}$ and $[Ru(tpy)(dmdppn)(H_2O)]^{2+}$, respectively, in methanol, λ_{irr} = 460 nm) [352,368]347,363. On the other side, in **125** the replacement of the dppn moiety with a bpy ligand returned a complex only able to ligand dissociation [368]363.

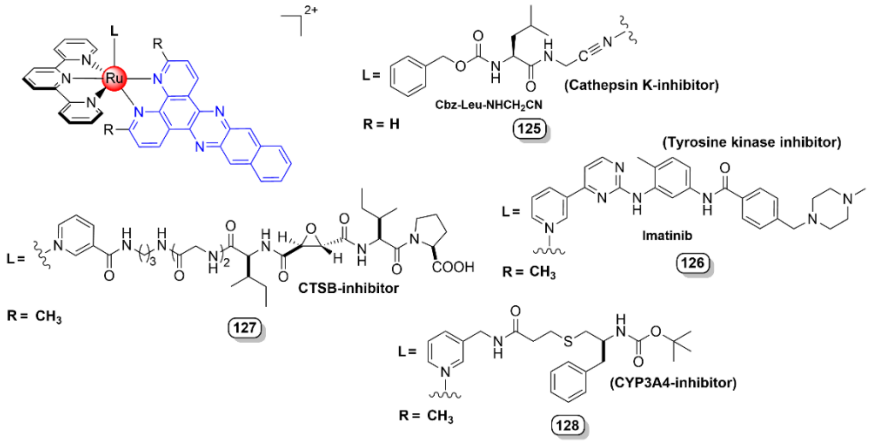


Fig. 46. Ru(II)-based PACT agents with dual reactivity: $^1O_2$ sensitization and photorelease of chemotherapeutic agents.

Enzyme-targeting may also offer the advantage to deliver phototoxic RPCs specifically to tumor sites, exploiting the overexpression of selected enzymes in a variety of cancers [374,375]370,371.

This principle, sketched in Fig. 47, was adopted by Arora and coworkers in the design of compound **127** (Fig. 46), where a highly potent and selective inhibitor of cathepsin B (CTSB), that is a cysteine protease with invasive and metastatic behaviour [376]372, was used as "delivery vehicle" for the ruthenium compound [377]373. The targeting ability of this compound were combined with the effective $^1O_2$ sensitization (Φ_Δ = 0.58(3), λ_{irr} = 460 nm, in methanol) and the irreversible inhibition of CTSB upon the photoejection of the inhibitor (Φ_{500} = 0.0070(6) in 2% CH_3OH/H_2O). As a result,

127 exhibited a selective activity toward cancer *vs* non-cancer cells, with a reduction of cell viability from 90% to roughly 60% upon irradiation in MDA-MB-231 triple negative human breast cancer (TNBC) (λ_{irr} > 395 nm, irradiation time 35 min). Again, the substitution of dmdppn in **127** with dmbpy or dppn returned complexes only able to photodissociate or to produce $^1O_2$, respectively [377]373.

Another important aspect in the adoption of a targeted enzyme-based approach is represented by the possibility to inactivate specific enzymes that are responsible for the fast metabolism of administered drugs and can thus compromise their chemotherapeutic effect. Therefore, photorelease of enzyme inhibitors can be used in synergy with drugs to provide a way to enhance their therapeutic efficacy.

An example of this strategy was recently reported by Toupin *et al.* [378]374, who caged an analogue of ritonavir, an inhibitor of CYP3A4 (the most abundant liver and intestinal P450 isoform that oxidizes several drugs and other xenobiotics relevant to human health) [379]375, in compound **128**. Besides the $^1O_2$ production (Φ_Δ = 0.59(6), λ_{irr} = 460 nm, in methanol), irradiation of **128** liberated the ritonavir analogue (Φ_{500} = 0.024(4) in CH_3CN), which in turn blocked the intracellular CYP3A4-dependent metabolism of vinblastine, the anticancer drug primarily metabolized by CYP3A4 *in vivo*. As a result, a potent synergism between **128** and vinblastine was observed in prostate adenocarcinoma DU-145 cells (combination index CI values < 1).

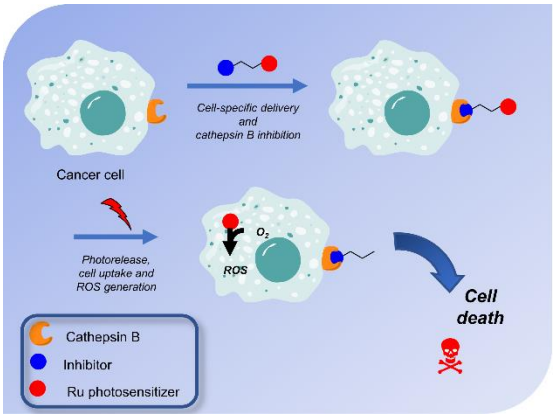


Fig. 47. Dual therapeutic for tumor-specific delivery, cysteine cathepsin inhibition and cell death through generation of ROS.

4.1.1.3 PACT/PACT agents combining photochemotherapy and DNA interaction

Two PACT modalities can be combined in the same molecular tool to give the simultaneous photorelease of a molecule with antitumoral properties and the generation of active metal-based photoproducts, capable of interacting with different biological substrates, such as DNA or proteins [296]291, thus providing a way to develop multi-targeted compounds.

The group of Turro was among the first to apply this concept, demonstrating that 5-cyanouranil (5-CNU), an active derivative of the chemotherapy agent 5-fluorouracil (5-FU), largely used in the treatment of colorectal and breast cancers [380]376, could be successfully photoreleased from $[Ru(bpy)_2(5\text{-}CNU)_2]^{2+}$ [381]377 and $[Ru(tpy)(5\text{-}CNU)_3]^{2+}$ [382]378, leading to the production of Ru(II)-aquo complexes with DNA binding properties. In keeping with this approach, crucial enzymes for cancer cells have been caged into dual-active Ru(II) complexes capable of covalently binding to DNA upon light-activation. Three inhibitors of cytochrome P450s were liberated from compounds **129a**, **129b** and **129c** (Fig. 48) by Glazer and coworkers, enhancing the protein inhibition activity upon light-exposure (up to 136-fold for **129c**, LED array, 28 J/cm^2) but also generating the active $[Ru(bpy)_2(H_2O)_2]^{2+}$ framework [67]. This potentially offered an additional mechanism of action beyond enzyme inactivation, even though, in this specific case, a study by the group of Bonnet highlighted that the high hydrophilicity of this Ru(II)-photoproduct may compromise its ability to cross cell membranes and thus, to effectively reach the nuclear targets [281]276. Kumar *et al*. reported on the insertion of saccharin, an inhibitor of carbonic anhydrase IX (CA IX, a prognostic marker for highly aggressive tumor in hypoxic stress), in the dpq- and dppz-containing complexes **130a**-**b** [383]379. Irradiation (λ_{irr} = 365 nm) of compounds determined saccharin dissociation and promoted DNA photocleavage through covalent binding of the activated Ru(II)-

photoproducts, under anaerobic conditions. Similarly, the group of Zhang exploited the nitrile function contained in bicalutamide (BC), an androgen receptor (AR) antagonist involved in the progression of prostate cancer [384]380, to cage this molecule into complexes **131a-c** [385]381. Coordination to Ru(II) blocked the binding of BC to the receptor, owing to the large steric structures of the complexes that impeded the BC interaction with the binding site of AR. Blue light irradiation (λ_{irr} = 465 nm, 10 mW/cm^2) determined the release of BC and the generation of active Ru(II) fragments. This led to a more potent anticancer effect if compared to the one of free BC ($IC_{50\ light}$ = 8.9 ± 0.6 µM and PI = 9.6 in case of **131a**, *vs* IC_{50} ~ 45 µM for BC in androgen-sensitive human prostate adenocarcinoma LNCaP cells), and thus remarked that the synergistic action between enzyme-targeting and generation of DNA damaging agents can be effectively exploited to achieve superior efficacy compared to the use of the sole enzyme inhibitor.

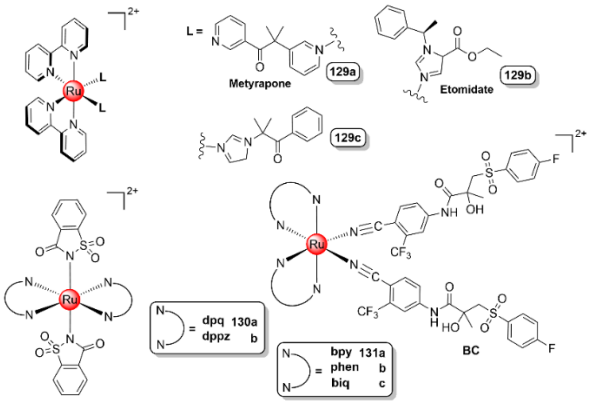


Fig. 48. Examples of Ru(II) compounds combining the release of a molecule with antitumoral properties and the generation of DNA metal binding agents upon photolysis.

4.1.2 Combinations of other techniques

Combining RPCs with chemotherapeutic agents could offer the advantage of a synergistic effect between PDT/PACT and chemotherapy [252]247, but also other benefits, such as the *in situ* optical monitoring of the therapeutic outcome. Su *et al.* investigated indeed a new heteroleptic PDT-based Ru(II) complex functionalized with the alkylating agent Chlorambucil (CHL-RuL, **132**, Fig. 49) as an image-guided chemo-photodynamic combined theranostic agent, thanks to the coupling with a coumarin group [69]. The insertion of CHL in the Ru-complex served to overcome some issues shown by the drug, such as stability, selectivity and resistance, and to favor the selective uptake by mitochondria. The alkylating activity of conjugated CHL was unfortunately lowered by the bulky structure of the whole complex. Anyway, the mitochondrial accumulation returned higher cytotoxicity both in the dark and upon light exposure under one-photon or two-photon irradiation [69]. A new porphyrin-RAPTA conjugate **133** (Fig. 49) revealed a dual clinical potential brought about by the porphyrin moiety, as for the PDT ability, and by the RAPTA component, as for the anticancer properties in the dark. The latter seemed to originate from the enhanced cellular internalization of the cationic porphyrin [386]382. The new RPC **134** (Fig. 49), featuring an expanded conjugate structure, showed both an enhanced singlet oxygen generating ability, that determined a photocytotoxicity with a PI > 11.5, and a further antitumor activity due to an increased cellular uptake mediated by light-triggered ROS production, that determined the cell membrane damaging [387]383. A recently reported Ru(II) complex decorated with the Chinese medicine herb rhein (**135**), widely used in multiple pharmacological applications (antibacterial, anti-inflammation, anticancer), showed lysosome-targeted properties, high singlet oxygen quantum yield and favorable phototoxicity against cancer cells *via* an autophagy pathway (irradiation at 450 nm, 3.5 mW/cm^2), together with activity towards cisplatin-resistant A2780R cells [29] (Fig. 50).
The use of Photobiomodulation (PBM) in combination with other techniques has not been frequently reported, indeed PBM, which uses light sources in the red or NIR spectral region (700-1100 nm), is employed to stimulate, heal or regenerate damaged or dying tissues. Nonetheless, PBM can work in synergy with other techniques, performing better than the single ones by virtue of the PMB-induced higher ATP levels in the cell, that allow for increased cellular uptake and apoptosis. Da Silva and coworkers reported two RuPcs, the tetrahedral [Ru(Pc)] **136a** and the octahedral *trans*-[Ru(NO)(NO_2)(Pc)] **136b** to be used in a dual PDT/PBM strategy (Fig. 49). While PDT generated ROS, PMB activated NO

dissociation from **136b** with reactive nitrogen species (RONS) production upon light irradiation. As a consequence of the synergistic effect between singlet oxygen and nitric oxide, **136b** exhibited a higher phototoxicity than **136a** against human melanoma (A375) cells. The PBM-induced increased cell metabolism, that translated into ATP production and seemed to enhance the uptake of the complex and the apoptosis [388]384.

Photoredox catalysis (PC) has been singularly employed in combination with PDT or PACT in anticancer treatments and helped reaching significant progress. An example of a PACT/PC synergism was recently showed by Shuang *et al*., who reported that the insertion of strong electron-withdrawing -NO_2 groups into the PIP ligands of the two mitochondria-localized complexes **137c**-**d**, made possible to photo-catalyze the oxidation of NADH, a key target due to its role in intracellular redox balance and mitochondrial electron transport chain. On the other side, the photodissociation of a py ligand generated Ru(II)-frameworks able to covalently bind DNA [225]385. Thanks to such a dual function (NADH depletion and binding of mitochondrial DNA), two-photon irradiation (λ_{irr} = 800 nm, 1 W/cm^2, 30 min) of **137c**-**d** increased the potency towards cisplatin-resistant human lung adenocarcinoma cell line A549/DDP under both normoxic and hypoxic conditions, compared to the NO_2-free analogues **137a**-**b**, that only displayed a PACT mechanism.

Other examples combining PDT and PC have already been described throughout this review [165,373]369 and a recent highlight on this topic was published by Raza and coworkers [389]386.

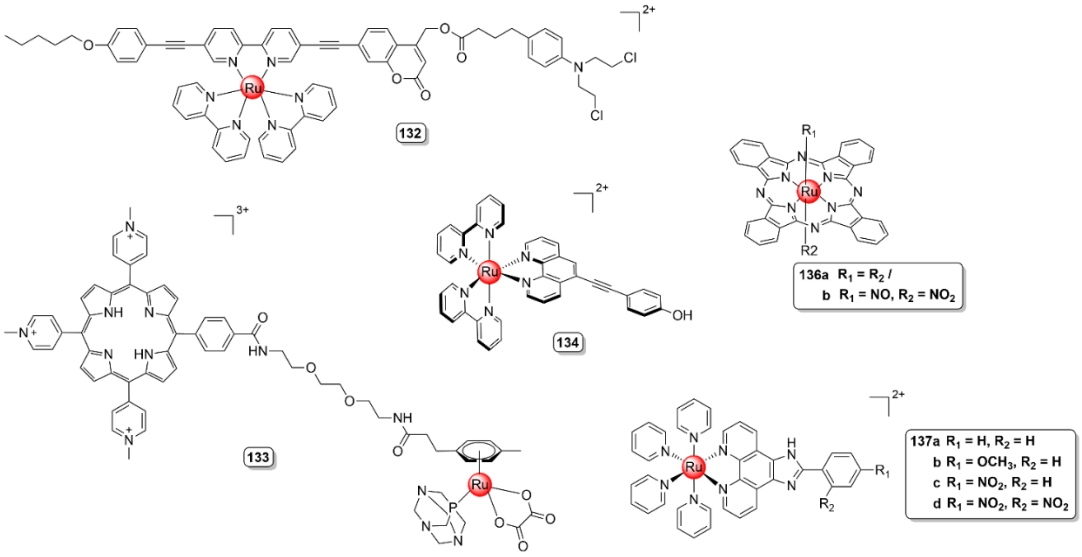


Fig. 49. Chemical structures of some complexes discussed in chapter 4.1.2.

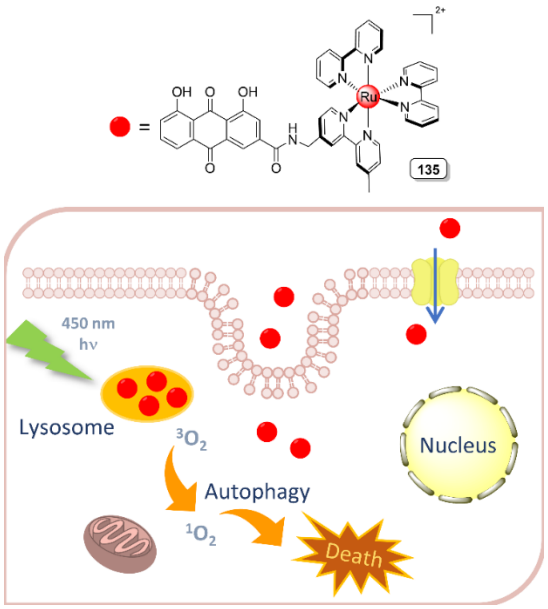


Fig. 50. Chemical structure of complex **135** and illustration of its PDT in cancer treatment through autophagy pathway.

4.1.3 Theranostics

Theranostic agents allow for combining therapeutics and diagnostics, providing a way to visualize the cellular localization of a compound but also to monitor the proceeding of phototherapy.

An example of this approach was reported by Mao *et al.*, who inserted the TEMPO moiety in the two PDT compounds **138a-b** (Fig. 51) to gain enhanced cellular uptake and production of intracellular ROS. Besides the conferred increase in photo-toxicity, the TEMPO moiety also acted as an oxidative stress indicator, thanks to its conversion to diamagnetic species upon exposure to ROS. This renewed its fluorescence emission, allowing for the monitoring of the PDT response directly in the cellular environment [390]387. Similarly, Yuan *et al.* designed a NBD-containing polypyridyl Ru(II) complex (**139**), which, on one hand, acted as a PDT agent *via* singlet oxygen generation, and on the other hand, as a luminescent probe, being able to respond to the production of H_2S, that increased during PDT. This in turn permitted to monitor the phototherapy process by detecting the changes in the probe's luminescence signal [391]388.

An analogues strategy was also followed by Geri *et al*., who designed the theranostic, BODIPY (boron-dipyrromethene)-containing, PACT agent **140** (Fig. 51). This compound belongs to light-activated carbon-monoxide-releasing compounds (photoCORMs), that represents a more general class of prodrugs whose biological effects are mediated by the photorelease of CO molecules and in which RPCs were largely exploited [392–395]389-392. Irradiation of **140** (λ = 350 nm, 6 mW/cm^2, 10 min) determined the liberation of two CO molecules, leading to antiproliferative effects that were markedly cell line dependent. At the same time, the fluorescent BODIPY functionality allowed to easily monitor the cellular uptake in epidermoid carcinoma A431 and human embryonic kidney HEK293 cells through laser scanning confocal microscopy, evidencing an unspecific accumulation in the endoplasmic reticulum and mitochondria, being the latter a key target of photoCORMs.

Many other Ru(II)-based photoCORMs, along with RPCs-caging compounds of other small bioregulators such as NO, have been recently reviewed elsewhere [396,397]393,394. Other examples of RPCs-based theranostic candidates were described throughout this review [69,132,133,147,153,162,186,232,235].69,132,133,147,153,162,184,227,230

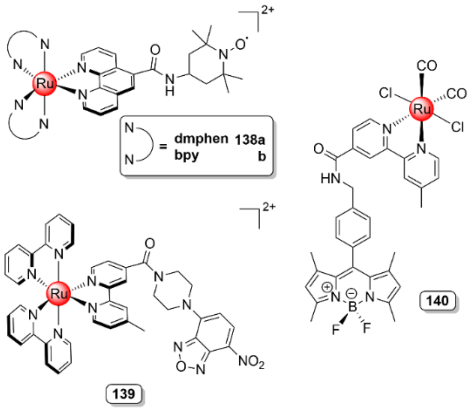


Fig. 51. Chemical structures of the complexes discussed in chapter 4.1.3.

4.2 Dual targeting

The combination of multiple compounds addressing different cancer sites could be a good strategy to boost the biological potency, still the risk to increase the dark toxicity due to the employment of multiple agents is to be taken into account. A single photoresponsive compound able to act on two different sites is therefore desirable.

Chao *et al.* reported five new fluorinated Ru(II) complexes (**141a-e**) which showed excellent TP-PDT properties and good singlet oxygen quantum yields (Fig. 52) [222]395. The complexes localized both on cytomembranes and mitochondria, which, following two-photon irradiation, were simultaneously ablated, leading to HeLa cells destruction. **141d** showed the largest PI in HeLa MCTSs upon both one-photon (37.1) and two-photon light irradiation (101.6) and was employed for *in vivo* study, showing the ability to inhibit the growth of HeLa tumors [222] 395. The same authors also exploited a combined targeting ability toward tumor cells and mitochondria to overcome some PDT limitations, such as insufficient doses of PS, oxygen and light at the target site. The insertion of a glucose unit into Ru(II) complexes (**142a-d**, Fig. 52) conferred them the capacity to accumulate in cancer cells and mitochondria using TP-PDT [63]. The most promising **142b** complex was tested *in vivo* and proved useful for regressing tumors in mice [63].

Recently, the Gasser group combined a Pt(IV) and a Ru(II) center to obtain the multi-targeting and multi-action anticancer agent **73** (Fig. 29), able both to target different organelles and to act *via* multiple cytotoxic mechanisms, offering the advantage of overcoming drug resistance [66]. Following an energy-dependent endocytosis uptake, Pt(IV) was reduced to Pt(II) and the axial ligands (Ru(II) complex and phenylbutyrate) were released. Each moiety possessed a different function: cisplatin migrated to the nucleus and acted as the chemotherapeutic drug, helped by the phenylbutyrate that can de-condense the chromatin, while the Ru(II) complex moved to the Golgi apparatus and acted as a PS for PDT, generating singlet oxygen upon light irradiation (from 480 to 595 nm) [66]. These studies therefore provided evidence that Ru(II) complexes can interact with more than their aimed targets and highlighted the importance of keeping an open mind when evaluating the therapeutic activity of this class of compounds.

More dual targeting PSs have been described throughout the present review (nucleus + mitochondria [133,215]133,213; mitochondria + lysosomes [223]396), whereas several examples of multitargeting PACT-based RPCs (enzymes + DNA) were described in paragraph 4.1.1.3.

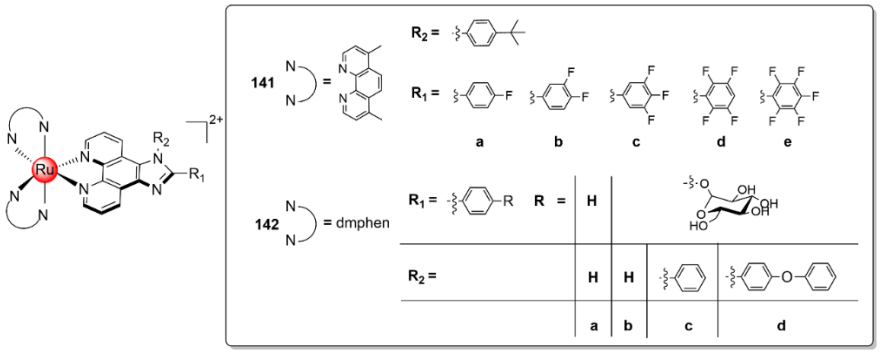


Fig. 52. Chemical structures of the complexes discussed in paragraph 4.2.

4.3 Dual stimuli

Finally, other modalities of drug-activation are possible besides light. In this last section a few cases of pH-triggered photoresponsive systems are reported. A series of morpholine containing RPCs (**143a-d** in Fig. 53) displayed pH-dependent luminescence and ability to produce singlet oxygen *via* photoinduced electron transfer (PET) mechanism [223]396. A stepwise mechanism was proposed, that first entails the photodamage of lysosomes, from which the PS is released and attracted to the mitochondria due to the cationic nature, and then the damage of mitochondria. Following the damage of lysosome and the increase in the lysosomal pH, the $^1O_2$ production raised accordingly. **143d** exhibited the highest $^1O_2$ quantum yield at every pH value (67.2% at pH 5; 86.6% at pH 8) and can be considered as an eligible candidate for *in vivo* TP-PDT [223]396. Similarly, Papish *et al.* investigated a series of pH-activated metallo-prodrugs (pHAMPs) (**144a-d** in Fig. 53) containing the protic ligand 6,6'-dihydroxy-2,2'-bipyridine (6,6'-dhbp) [398]397, whose protonation degree depends on the pH, to study how the charge can influence their passive diffusion. To this aim, LogP was measured as a function of pH to estimate the passive diffusion into cancer cells and hypoxic tumors, that are known to acidify their extracellular surroundings. All complexes were readily deprotonated in cell media at pH 7.4, complex **144c** showing the highest lipophilicity at each pH value studied and the best cellular uptake. This study suggested that a good uptake is required but is not sufficient for light driven toxicity, since cancer cell lines not showing the highest uptake are more phototoxic than others with a better uptake [399]398.

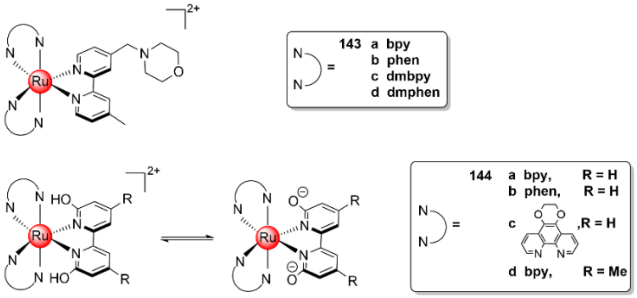


Fig. 53. Chemical structures of the complexes discussed in paragraph 4.3.

5. Outlook and future directions

RPCs represent an intriguing class of metallodrugs whose rich and versatile chemical physical repertoire makes them suitable tools for the design of novel light-responsive compounds with antitumoral properties. However, notwithstanding photodynamic therapy has been clinically approved for the treatment of a wide variety of cancers (see also chapter 2) and considering the enormous potential of RPCs not only as PDT but also as PACT agents and multi-active light-responsive compounds, it is disappointing that, to date, only the Mc Farland's compound TLD1433 has entered clinical trials as anticancer drug. Indeed, crucial challenges still need to be addressed in order to fill the gap between the molecular design of RPCs and their translation into clinic. Some critical aspects have already been discussed throughout this survey, which are briefly recalled in this paragraph, whereas new perspectives are herein highlighted to provide an idea on where the efforts in this field of research are currently going.

A first challenge may stem from the lack of absorption of RPCs in the PDT window, and thus from the poor tissue penetration of the required activating light. However, this drawback can be overcome by tuning the absorption profiles of Ru(II) compounds *via* ligand modifications (see also chapter 2.2.1): a straightforward way usually involves the extension of the π-systems of the coordinated ligands but also the use of cyclometalated ligands, or polypyridyl ligands further modified with organic-based "antenna" groups, may help in shifting the absorption profiles towards the red. Alternatively, the use of the two-photon absorption (TPA) strategy to overcome this issue is undoubtedly exciting.

The oxygen-dependence of RPCs developed as pure PDT agents represents another major challenge. As discussed in chapter 2.3, several innovative strategies can be employed to overcome this crucial weakness, such as O_2-replenishing methods, that raise the oxygen level prior to or during the PDT treatment, or Type I- rather than Type II-based cytotoxic mechanisms, or finally the combinations of PDT with other O_2-independent therapeutic modalities, that are treated more in detail in chapter 4.

In this last regard, besides the suitability in hypoxic environments made accessible by PACT-based pathways, the combination of multi-therapeutic and/or multi-targeting abilities into unique molecular tools may help to overcome crucial issues in phototherapeutic treatment, such as the risk of cancer recurrence. Therefore, notwithstanding the design of these novel, multiple active compounds is an emerging field of research and further efforts are needed to improve their therapeutic outcomes, we believe that such hybrid systems will attract an ever-growing attention.

Another major challenge might arise from the possible incapacity of the photoresponsive compounds to effectively reach the tumoral target. The fast and unregulated tumor growth typically leads to sub-optimal tumor vascularization, which, in turn, results in hypoxic regions that are far from functional blood vessels. Therefore, the chemical-physical features of the single molecules, including charge and lipophilicity, should be finely tuned to gain optimized tumor penetration and selectivity abilities. Alternatively, novel drug delivery strategies can be developed with the same scope, overcoming issues such as high *in dark* toxicity and unspecific drug distribution, and thus making possible to considerably limit the related side effects.

Noteworthy, notwithstanding many encouraging *in vitro* results, more detailed insights into cell death mechanisms are needed for a better understanding of the modalities of action of photoresponsive RPCs. Moreover, their biological activities are often determined by using different experimental set up (*i.e*., different biological protocols and/or irradiation conditions), thus making it difficult to establish which is the most promising compound, among many reported in the literature, to be used in the treatment of a certain type of cancer. Indeed, the demand for the generation of a SAR database of RPCs with PDT/PACT activities is becoming increasingly urgent, as also pointed out elsewhere by other authors [25].

Importantly, there is still a crucial lack of assays performed under hypoxia as well as *in vivo* studies, which precludes the full assessment of the clinical potential of this class of compounds.

These critical gaps require to be urgently addressed over the next years.

Last but not least, we agree with a recent perspective from Bonnet [282]277, that the distrust of the medical community towards the idea of anticancer phototherapy together with the disinclination of pharmaceutical companies to invest in these new therapeutical alternatives should not be ignored. This general diffidence may arise from some crucial disadvantages associated with the currently commercialized PDT drugs (pain, photosensitivity, tissue clearance) and that are commonly generalized to all phototherapeutic agents, including PACT and multifunctional compounds.

In light of these considerations, we envisage that only the development of photoresponsive compounds with excellent performances (high phototherapeutic index, activity in hypoxic environments, low dark toxicity, capacity to be triggered by highly penetrating radiations, just to name a few) in combination with an efficient light penetration could convince both clinicians and big pharma companies to take RPCs into serious consideration. Furthermore, novel pioneering and multidisciplinary approaches aimed to improve crucial therapeutic outcomes, such as targeting ability and minimization of undesired photosensitization effects, are expected to translate this versatile and challenging class of compounds into clinic in the near future.

6. Conclusions

Ruthenium polypyridyl complexes are emerging as potential chemotherapics alternative to platinum-based drugs. They can be used as light-triggered prodrugs with a spatio-temporal control by using a PDT or PACT approach. Even if there are no ideal complexes for both techniques, several *ad-hoc* structural modifications are being exploited to play with factors such as cellular uptake, steric hindrance, oxygen dependence and light harvesting properties, trying, often successfully, to overcome the main limitations of the two techniques. Conferring targeting properties to the compounds is also a good strategy to enhance the performance of the complexes. Moreover, the combination between PDT and

PACT proved significantly superior to the individual use, exploiting different mechanisms of action and, above all, overcoming both oxygen requirements of PDT agents and the stoichiometric action of PACT-based compounds.
However, despite all the progress made in the field in the recent years, ruthenium complexes basically did not enter clinic, thus more studies are required. Possibly, a multidisciplinary approach spanning chemistry, biology and medical science could help reach the goal of entering routine clinics, even if this is not always possible to achieve in an ordinary research laboratory and could be, indeed, one of the main reasons for exclusion from clinics of these extremely challenging classes of compounds.

Acknowledgements

Financial support from *Fondo Sociale Europeo REACT EU* (PON Ricerca e Innovazione 2014-2020) and from University of Urbino (Department of Pure and Applied Sciences – Grants DISPEA FUSI PROG2019 e DISPEA MACEDI PROG2021), are gratefully acknowledged.

Abbreviations

ab-PBI	azabenz-annulated perylene bisimide
azpy	2-(phenylazo)pyridine
bca	2,2’-biquinoline-4,4’-dicarboxylic acid
biq	2,2’-biquinoline
BODIPY	boron dipyrromethene (4-bora-3a,4a-diaza-s-indacene)
bphen	4,7-diphenyl-1,10-phenanthroline
BPIMBp	1,4’-bis-{(2-pyridin-2-yl)-*1H*-imidazol-1-yl)methyl}- 1,10-biphenyl)
bpy	2,2’-bipyridine
BTF	benzothiazolylfluorenyl
btz-qui	2-benzothiazol-2-yl-quinone
Cl-7-IVQ	5-chloro-7-(2-(1,3,3-trimethyl-*3H*-indol-1-ium-2-yl)-vinyl)quinolin-8-olate
6,6’-dmbpy	6,6’-dimethyl-2,2’-bipyridine
dmp	4,7-dimethyl-1,10-phenanthroline
4,4’-dmbpy	4,4’-dimethyl-2,2’-bipyridine
dmdppn	3,6-dimethylbenzo[i]dipyrido[3,2-a:2’,3’-c]phenazine
dmphen	2,9-dimethyl-1,10-phenanthroline
dpp	2,3-bis(2-pyridyl)pyrazine
dpphen	2,9-diphenyl-1,10-phenanthroline
dppn	benzo[*i*]dipyrido[3,2-*a*:2’,3’-*c*]phenazine
dppz	dipyrido[3,2-*a*:2’,3’-*c*]phenazine
dpq	dipyrido[3,2-*d*:2’,3’-*f*]quinoxaline
en	ethylenediamine
GSH	glutathione
hmte	2-(methylthio)ethanol
ip	1*H*-imidazo[4,5-*f*][1,10]phenanthroline
ippy	2-(1-pyrenyl-*1H*-imidazo[4,5-*f*]-[1,10]phenanthroline
ISC	intersystem crossing
1-isocyTPQA	1-(((*2R*,*6S*)-2,6-bis(pyridin-2-yl)piperidin-1-yl)-methyl)isoquinoline)
MCTS	multicellular tumor spheroid
NADH	*β*-nicotinamide adenine dinucleotide
1-NIP	2(naphthalene-1-yl)-*1H*-imidazo[4,5-*f*][1,10]phenanthroline
2-NIP	2(naphthalene-2-yl)-*1H*-imidazo[4,5-*f*][1,10]phenanthroline
ohcpip	2-(2-hydroxyl-3-carboxyphenyl)imidazo[4,5-*f*][1,10]phenanthroline
OPA	one-photon absorption
Pc	phthalocyanine
PEG	polyethylene glycol
phcpip	2-(3-carboxyl-4-hydroxyphenyl)imidazo[4,5-*f*][1,10]phenanthroline

phen	1,10-phenanthroline
ph-en	diphenyl-ethanediamine
PI	phototoxicity index
PIP	2-phenylimidazo[4,5-*f*[1,10]phenanthroline
pmip	2-(4-methylphenyl)-*1H*-imidazo[4,5-*f*][1,10]phenanthroline
PS	photosensitizer
PTA	1,3,5-triaza-7-phosphatricyclo-[3.3.1.1]decane
pymi	(E)-N-phenyl-1-(pyridin-2-yl)methanimine
ROS	reactive oxygen species
RPC	ruthenium polypyridyl complex
RuPc	ruthenium phthalocyanine
tap	1,4,5,8-tetraazaphenanthrene
$^{t}Bu_2bpy$	4,4'-di(*tert*-butyl)-2,2'-bipyridine
TEMPO	2,2,6,6-tetramethyl-piperidine-1-oxyl free radical
tmp	tetramethylphenanthroline
TNBC	triple-negative breast cancer
TPA	two-photon absorption
TPA_m	tris(2-pyridylmethyl)amine
TPACS	two-photon absorption cross section
tpbn	2,2'-(4-(*tert*-butyl)pyridine-2,6-diyl)bis(1,8-naphthyridine)
tpphz	tetrapyridophenazine
tpy	2,2':6',2''-terpyridine

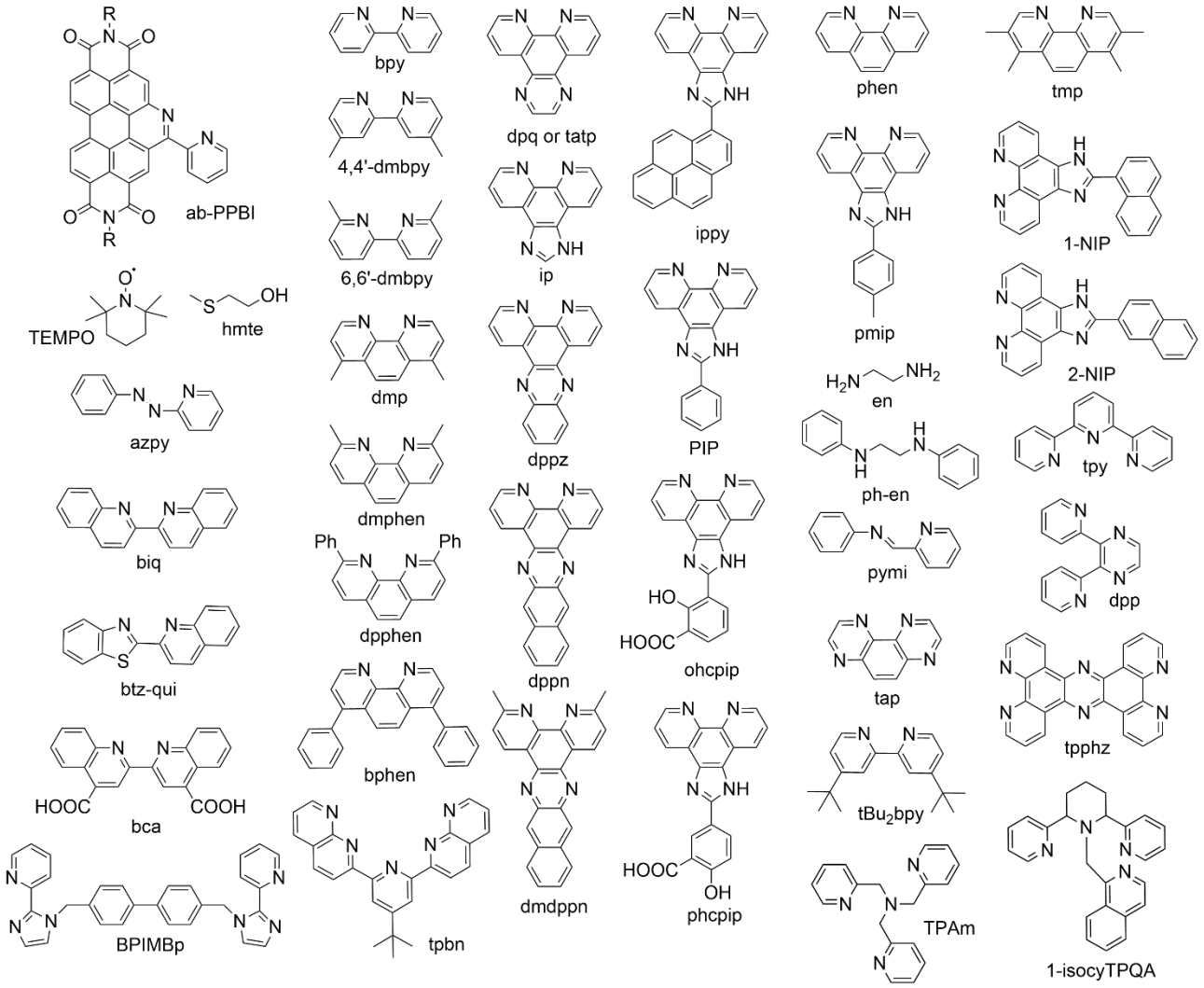


Fig. 54. Schematic representation of some ligands cited throughout this review.

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