UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

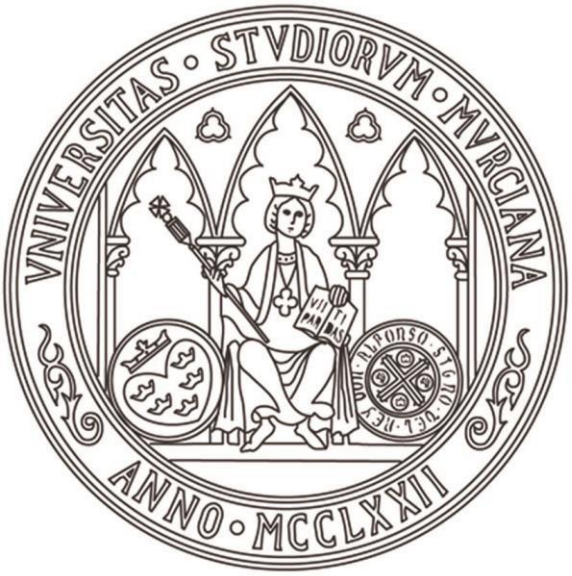
TESIS DOCTORAL

Development of novel coumarin- and ruthenium-based photodynamic therapy anticancer agents

Desarrollo de nuevos compuestos basados en cumarina y rutenio para la terapia fotodinámica del cáncer

D. Enrique Ortega-Forte

2023



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Autor: D. Enrique Ortega-Forte

Director/es: D. José Ruiz López

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D./Dña. Enrique Ortega Forte

doctorando del Programa de Doctorado en

Programa de Doctorado en Química Básica y Aplicada

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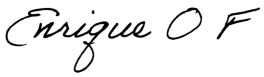

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La presentación de la Tesis Doctoral titulada "Development of novel coumarin- and ruthenium-based photodynamic therapy anticancer agents", realizada por D. ENRIQUE ORTEGA FORTE, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 20 de junio de 2023

Este trabajo cierra un capítulo importante de mi vida. Comencé esta etapa como estudiante universitario y me voy como investigador científico, habiendo aprendido a trabajar en el laboratorio, habiendo desarrollado mis propias hipótesis e ideas y habiendo puesto mi granito de arena en la investigación de esta enfermedad. He disfrutado cada día, cada paso y cada proceso de este camino. Gracias a todos los que de una manera u otra han contribuido a este trabajo.

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ABSTRACT

Photodynamic therapy (PDT) is a clinically-approved modality for the treatment of cancer. In this therapy, light is used to activate a pharmacological substance called photosensitizer (PS) and convert molecular oxygen into cytotoxic reactive oxygen species (ROS), which induce cancer cell death. The prospect of using this light-mediated anticancer strategy is attractive since it allows selective cancer targeting and low invasiveness owing to the spatial and temporal control over drug activation. However, the inherent oxygen dependency of PDT and the poor penetration of light into biological tissues hampers its therapeutic efficacy. This Thesis explores the development of different families of chemical compounds as novel photodynamic anticancer agents aimed to address some of the major limitations of current PDT agents. The Introduction section includes an intensive revision of the state-of-the-art in oncological PDT and in the current development of organic fluorophores, transition metal complexes and metal-organic conjugates as PSs. The Results and Discussion sections is divided into 3 chapters. The Chapter I presents a library of organic fluorophores based on COUPY coumarin derivatives and its applicability as PDT agents upon visible light irradiation. Through a systematic study, a structure–activity relationship rationale was established, which allowed the identification of three lead compounds with potent phototherapeutic anticancer activities under normal and low-oxygen conditions (hypoxia) and minimal toxicity toward normal cells. Acting as mitochondria-targeting compounds, their photobiological mechanisms of action were further elucidated. The Chapter II comprises the development of cyclometalated Ru(II) polypyridyl complexes of the formula $[Ru(C^N)(N^N)_2]^+$ as biologically-compatible green light photosensitizers with high phototherapeutic efficacy under hypoxia in cancer cells under short times of irradiation. Finally, the Chapter III of this manuscript addresses the development of a novel hypoxia-active PS based on the conjugation of a cyclometalated Ru(II) polypyridyl complex to a near-infrared (NIR) NIR-emitting COUPY coumarin. Spectroscopic and photobiological studies revealed that this metal-organic conjugate exhibits high photoactivity toward cancer cells after highly penetrating NIR light irradiation under hypoxia, which could circumvent tissue penetration issues and alleviate the hypoxia limitation of PDT. Overall, this research work sets the stage for the development of novel coumarin and ruthenium-based photodynamic anticancer agents and paves the way to the obtention of highly potent, NIR- and hypoxia-active PSs with advantageous chemical and biological properties. In conclusion, this Thesis contributes to the development of novel classes of organic and inorganic compounds as anticancer tools in PDT.

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INTRODUCTION

1. Photodynamic Therapy for cancer

Nature uses light to control essential chemical reactions in living systems. The photosynthesis, which converts light and carbon dioxide into sugars and oxygen, or the synthesis of vitamin D, which requires sunlight for its production, are great examples of this. In the same way, light has been used in medicine for more than three thousand years. Ancient civilizations in Egypt, India and China applied light for the treatment of skin diseases such as psoriasis or vitiligo.[1] At the end of the 19th century, Niels Finsen used concentrated light radiation to treat lupus vulgaris, which earned him the 1903 Nobel Prize in Medicine and cemented the use of light-driven approaches as a feasible tool to treat diseases.[2] At the turn of the 20th century, a medical student named Oscar Raab observed that paramecia incubated with acridine orange died when exposed to sunlight.[3] His supervisor, Herman Von Tappeiner, and dermatologist Albert Jesionek also reported that white light could destroy skin tumours in the presence of eosin, describing the phenomenon as “photodynamic action”.[4] But research on the therapeutic application of light to patients consolidated in the 1970s, when Thomas Dougherty and co-workers eradicated over a hundred different tumours using hematoporphyrin derivative in combination with red light.[5] This ground-breaking light-based therapy, which is now referred to as Photodynamic Therapy or PDT, has since then advanced into the clinical setting as a novel treatment modality for cancer.

Cancer is a leading cause of death in the world. Accounting for 10 million deaths worldwide every year, it is a major contributor to the global disease burden.[6] Projections forecast that cancer burden will dramatically increase within the next decades.[6] Currently, it is estimated that one out of three people will suffer cancer during their lifetime (data corresponding to the International Agency for Research on Cancer, 2022).[7] In females, breast cancer is the most frequently diagnosed and a dominant cause of death, accounting for 25 % of the total cancer cases and 16 % of cancer deaths.[7] In males, lung cancer ranks first of incidence in the vast majority of countries and represents 14 % of all cancer cases and 22 % of the total cancer deaths.[7] Currently, the most common causes of cancer death are lung cancer, colorectal cancer and liver cancer.[7] The mortality trend is increasing in economically developed countries due to aging and associated risk factors such smoking habits, physical inactivity, unhealthy diets or exposure to carcinogens.[7] However, against a widely held belief, more than two-thirds of all cancer deaths occur in low- and middle-income countries.[8]

Cancer is a collective term used to describe a group of more than one hundred different diseases. Traditionally, cancers are classified according to the tissue from which they arise.[9] For instance, carcinomas arise from epithelial cells and account for more than 80% of cases. Sarcomas, in contrast, derive from connective or muscle tissue, while lymphomas originate from lymphoid cells. Each category contains many subdivisions based on the location, specific cell type and other histological features.[9] But cancer diseases share common cellular properties and are driven by the disruption of certain molecular networks. At the cellular level, cancer is expressed by unregulated cell growth and division. Two heritable properties define cancer cells: 1) they proliferate in defiance of normal controls on cell division and form a tumour (or neoplasm, meaning literally “a new growth”), and 2) they are able to invade and colonize surrounding or distant tissues (malignancy).[10] If cells within a neoplasm have not yet become invasive, the tumour is considered as benign. However, when tumour cells acquire the ability of invasiveness, they cross the basement membrane, enter bloodstream vessels and spread to other sites of the body, stablishing secondary tumours through a process known as metastasis.[11] Metastatic dissemination is considered the cause of most cancer deaths.[12] However, even when a cancer has metastasized, its origins can be traced to a single primary tumour derived from a single aberrant cell with an initial genetic mutation.[10] Notwithstanding, the progeny of this cell undergoes many further heritable changes, including both genetic and epigenetic alterations, before they become cancer cells.[13] Many lines of evidence indicate that carcinogenesis requires a considerable number of independent somatic mutations occurring at repeated rounds of proliferation (**Figure 1**).[14] For example, proto-oncogenes such as *ras*, *myc* or *bcl-2* code for proteins involved in important cellular processes that regulate normal cell division, cell differentiation and cell death.[15] Overproduction of these proteins leads to the loss of control over such processes of the cell. Proto-oncogenes may become activated by mutation events that increase the expression level to become oncogenes, which trigger carcinogenesis.[15] Cells also possess tumour-suppressor genes. Inactivation of a tumour suppressor gene such as *p53*, *Rb* or *PTEN* by random mutations or epigenetic events removes the restrictions that regulate controlled cell proliferation.[15] Overall, alterations in key cell regulatory genes can confer cancer cells advantages with respect to non-cancer cells, *i.e.,* high proliferation rate or continuous proliferation in defiance of control signals.[10] As genetic damage accumulates, an increasingly heterogeneous population of cells develops.[16] This is known as clonal expansion, whereby cancers evolve in a reiterative process of mutations and selection.[16,17] Just as in evolution of species, the competing subclones within a tumour are subject to natural selection according to a Darwinian-like process of evolution.[17] The offspring of the best-adapted cancer cells will continue to divide and will become the dominant clones within the tumour.[16] The genetic makeup of cancer cells can be dramatically different between clones, providing an intratumoral heterogeneity that has tremendous clinical implications.[18]

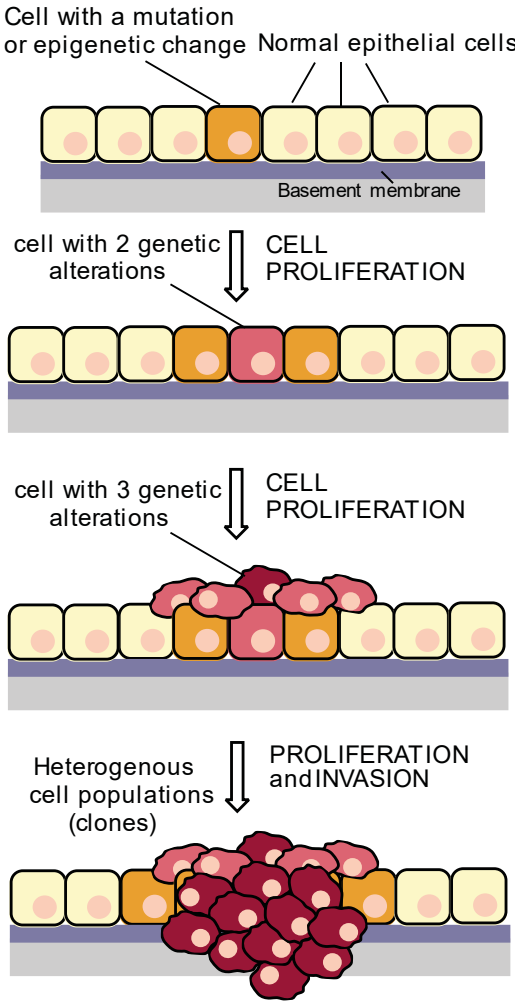

Figure 1. Clonal evolution in cancer. Schematic diagram of cancer progression.

As part of an evolutionary process, cancer cells acquire additional genetic alterations during tumour progression. These abnormalities not only include defects in the controls of cell cycle progression and proliferation. Stress and cell survival pathways are also upregulated in cancer cells, thereby disabling cell death induction signals and molecular pathways involved in the response to stress or DNA damage.[15] Moreover, due to the tumour growth demands, cancer cells require elevated rates of protein synthesis and often exhibit dysregulation of the protein translation machinery.[19] Altogether, these elements cause cancer cells to be selected to survive, grow and divide.

But a tumour is not simply a group of cancer cells. Fibroblast, adaptive and innate immune cells, secreted factors, extracellular matrix and blood vessels in the surrounding conform the so-called tumour microenvironment, which strongly influence the behaviour of cancer cells.[20] The dynamic and reciprocal interplay between cancer cells and components of the tumour microenvironment supports cancer cell survival, invasion and metastasis.[20] For instance, tumours are typically

hypoxic and have an acidic extracellular pH due to their metabolism.[21] Yet the tumour microenvironment can take over existing blood vessels and promote the formation of new ones (angiogenesis) to restore oxygen levels, remove metabolic waste and ensure nutrient supply.[20] Eventually, the best-adapted cell subclones of a given tumour will metastasize, founding new colonies in distant environments. All these drivers of cancer progression have been defined as the "hallmarks of cancer".[22,23] The main hallmarks include growth-suppressor evasion, growth signalling self-sufficiency, invasiveness, replicative immortality, induction of angiogenesis and resistance to cell death (**Figure 2**).

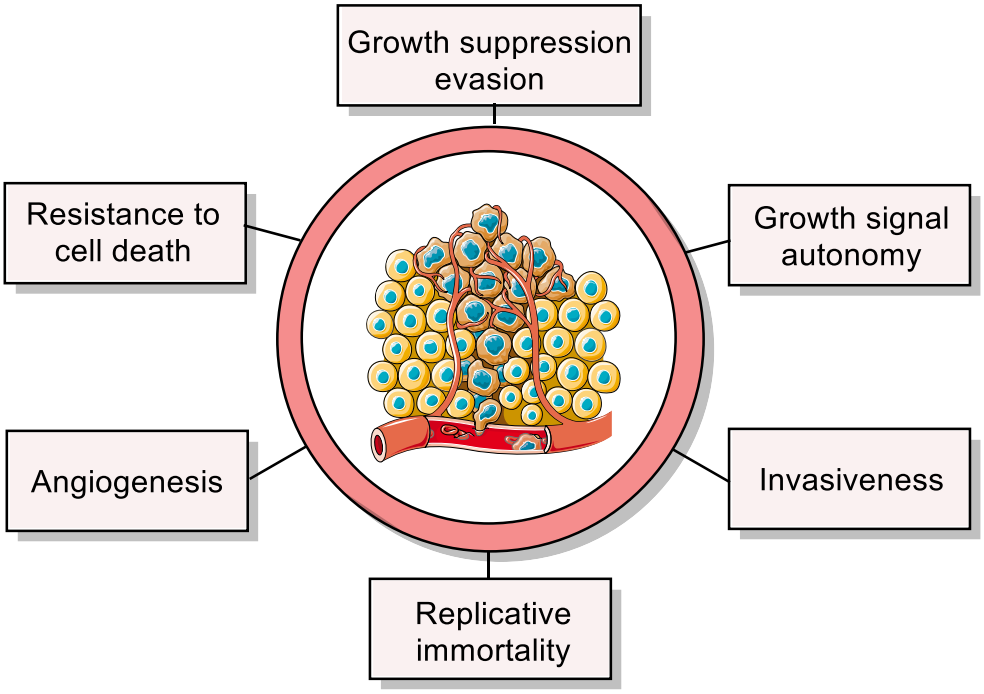

Figure 2. The main hallmarks of cancer. Adapted and modified from reference [22].

Conventional treatment options for cancer include surgery, radiation therapy or chemotherapy. However, some cancers are not amenable to surgery, and high recurrence rates have been associated with surgical resection of tumours.[24] Radiation therapy utilizes local ionizing radiation to cause extensive DNA damage, which results in chromosomal aberrations that lead to cancer cell death. Due to high-energy radiation, this therapy also carries risks of inducing secondary neoplasms and is limited by the cumulative radiation dose.[25] Therefore, chemotherapy constitutes the frontline choice for most malignancies.[26] In fact, most cancer patients receive chemotherapy at some point throughout the course of the disease. Chemotherapy relies on the use of chemical drugs that cause lethal cytotoxicity to cancer cells. The attack is usually directed towards DNA to impair cell replication, against essential metabolic pathways or against oncogenic proteins that are

overexpressed in malignant cells. For example, cisplatin (*cis*-diamminedichloridoplatinum(II), **Figure 3a**), a clinical drug used in over half of all chemotherapy regimens, induce DNA cross-linking adducts that stop cell division and ultimately produce cell death.[27] 5-Fluorouracil is widely employed in clinic as an antimetabolite that blocks nucleic acid synthesis (**Figure 3b**),[28] whereas the mode of action of imatinib, used to treat chronic myeloid leukaemia, is the inhibition of an activated tyrosine-kinase protein (**Figure 3c**).[29] Many other anticancer molecules have been developed in the past decades with a variety of different mechanisms of action.[26] Ideally, chemotherapeutic drugs would only interfere with cellular processes that are unique to cancer cells. However, most currently available drugs in the market do not specifically target cancer cells, but rather affect all proliferating cells in the human body, both normal and neoplastic. Therefore, chemotherapy is often associated with systemic adverse effects. Moreover, drug resistance usually develops, either acquired upon prolonged chemotherapeutic treatment with suboptimal doses or as intrinsic phenomena.[30] Recent evidence suggests that intra-tumour heterogeneity is a major obstacle to chemotherapy because, although a given drug can efficiently eliminate malignant cells harbouring specific genetic lesions, other cells driven by alternative oncogenic pathways (or defective tumour-suppressor pathways) survive the treatment.[31] More recently, targeted therapy and immunotherapy in combination with other modalities have entered as therapeutic tools in the armamentarium against cancer.[32,33]

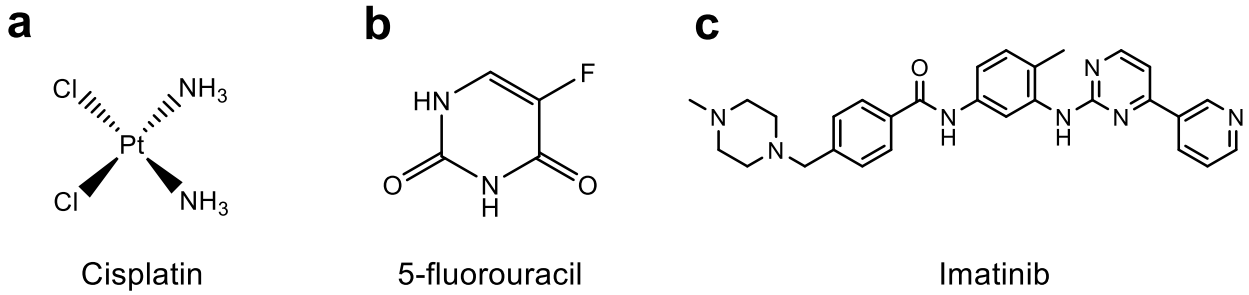

Figure 3. **Representative anticancer chemotherapy drugs used in clinic.** These molecules also possess three representative mechanisms of action in chemotherapy, being **a)** cell replication blockage, **b)** impairment of metabolic function and **c)** enzymatic inhibition.

In contrast to the conventional treatments, PDT represents an alternative antitumor oncologic intervention. Since its inception in the early 20th century and its first clinical application in the 1970s, PDT has emerged as an attractive medical technique for the treatment of multiple cancer types. In this therapy, light is used to activate an otherwise non-active pharmacological substance (*i.e.,* a photosensitive drug). Light provides spatial and temporal control over drug activation, which increases the selectivity with respect to conventional chemotherapeutic drugs.[34] Furthermore, light

can be safely administered in a non-invasive manner, either by irradiating superficial body areas such as the skin, or minimally invasive with the aid of endoscopes or optical fibres. Owing to its unique mechanism (**section 2**), PDT could circumvent many of the challenges posed by intra-tumour heterogeneity and cause effective antitumour activity in a controlled fashion towards a wide variety of cancers. Overall, although its full potential has yet to be shown, the range of applications of PDT, the negligible systemic effects associated and the recent technological improvements to deliver light into human body make this strategy a valuable therapeutic option of cancer treatment.

In addition to PDT, another technique that involves the combination of light with chemotherapeutic compound, called photoactivated chemotherapy (PACT), is currently gaining attention.[35] In PACT, photocaged compounds are used.[35,36] The chemical structure of a PACT drug changes by irreversible light-induced reactions such as ligand ejection.[36] Therefore, the main cytotoxic outcome of PACT is the release of the cytotoxic species after light irradiation. In contrast to PDT, the mechanism of PACT is non-catalytic and does not require oxygen, which is of particular interest to treat oxygen-depleted system like hypoxic tumours (**section 3.1.**).

2. Principles of Photodynamic Therapy

PDT involves three individually non-toxic components, which combine to induce cellular or tissue damage (**Figure 4**). The first component of PDT is the photosensitizer (PS) —a photosensitive molecule that can absorb light. During a PDT treatment, a non-toxic dose of the PS is injected either locally or systemically to the patient. Although the PS will initially distribute over the human body in a non-specific manner, ideally it would accumulate into the target tissue (*i.e.,* the tumour area) after an incubation period that usually varies from 5 min to 24 h.[37] The second component involves the application of light of a specific wavelength to the target tissue, which activates the PS. The light-activated PS interacts with the third element of PDT —molecular oxygen. Different from the first two components, molecular oxygen is endogenously present at the cellular level. The photoreactions between the PS and molecular oxygen generate reactive oxygen species (ROS), which mediate cellular toxicity. Noteworthy, these reactions occur in the vicinity of the light-activated PS. Therefore, the biological response of this photodynamic process takes place only in the immediate local areas of tissue that have been exposed to light. The photogenerated ROS induce oxidative stress during PDT, interrupting normal cell functions that finally drive tumour cell death. A great advantage of the PDT strategy is that sensitization is catalytic in principle, with a single PS molecule producing many equivalents of cytotoxic ROS. Overall, the combination of these three components, which ideally do not have toxic effects on their own, allows for selective and precise

treatment, unlike conventional chemotherapeutics that induce systemic toxicity or ionizing radiation therapy that damages adjacent normal tissue. Furthermore, PDT treatment can be combined either before or after surgery, chemo- or radiotherapy without compromising these therapeutic modalities.[38]

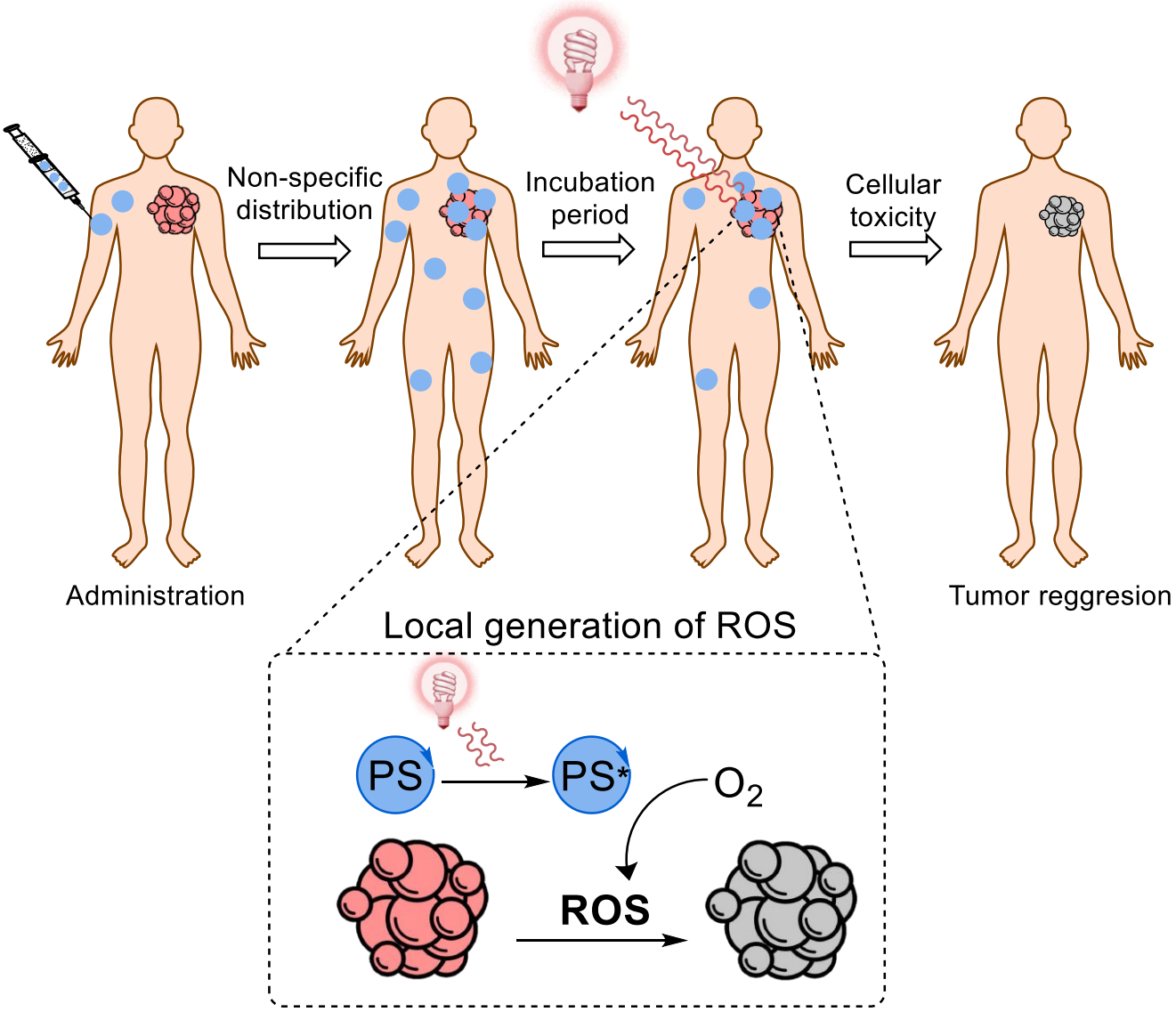

Figure 4. **Schematic representation of a PDT treatment.** Pale red = tumour, blue = PS; PS* = activated photosensitizer, dark red = light irradiation, grey = destroyed tumour.

Light irradiation is a critical parameter for the PDT procedure and correlates directly with the penetration depth of light into biological tissues. The light tissue penetration relies on the absorption and scattering properties of biological components, which limit the actual reachable depth of PDT.[39,40] Although optical penetration depth vary from tissue to tissue, it is strongly dependent on

the wavelength of light.[39,40] Shorter wavelengths in the ultraviolet (UV) or blue region of the electromagnetic spectrum penetrate least efficiently through tissue than longer wavelengths within the visible (VIS) region such green or yellow light. Red and near-infrared (NIR) radiations, which lie in the so-called “tissue transparency window” or “optical window of biological tissues” (600–900 nm), will penetrate much deeper. Whereas blue or green light penetrate a few hundreds of micrometres, red and NIR light can reach several millimetres, typically 3–10 mm depending on the tissue.[41]

Since the photodynamic action should be confined to the cancer tissue and avoid damage to healthy tissues, the choice of the wavelength of light during a clinical procedure is based on the tumour depth, size, shape and accessibility.[38] Light at shorter wavelengths will be appropriate for superficial or endoscopically accessible cancers, whereas red or NIR wavelengths would be suited for interstitial delivery of PDT, especially for the treatment of large and/or deep-seated neoplasms.[38,42] Thus, the limited depth of UV or VIS light penetration may hinder the efficacy of PDT towards solid tumours. However, despite longer wavelengths are preferred for their deeper tissue-penetrating ability, the chosen wavelengths for a PDT treatment need to be adjusted according to tumour phenotype and depth. For example, clinical experiences on the use of Photofrin with red light at 630 nm for bladder cancer treatment showed that healthy, underlying bladder tissue was also damaged.[43] Due to these adverse effects, Photofrin-PDT fell into disuse as an option for bladder cancer treatment.[44] Nowadays, a metal-based PS (TLD-1433) is currently undergoing clinical trials for this indication using green light activation at 520 nm, which matches tumour bladder depth and therefore avoids damage to healthy urotheial tissue layers.[45]

Regarding to light sources, both lasers and incandescent light sources have been employed in PDT with similar efficacy.[40] The first lasers used in PDT were pumped dye lasers.[46] However, these lasers are large, costly and require maintenance. The advent of diode lasers, which are lighter, portable and cost-effective have replaced dye lasers in the clinical setting. Besides, light-emitting diodes (LEDs) emerged as an alternative light source devices for PDT.[47] Light in any colour can be generated by LEDs with bandwidths of approximately 5% of the central wavelength and high fluence rates.[40] Moreover, LEDs have the advantage of being low-cost, portable and require no access to advanced medical equipment. In any case, dosimetry and calibration of the light device is mandatory, as well as adjustments on total dose, light exposure time and fractionation schemes.[40] During oncological PDT, light irradiation of target tissue can be achieved by various methods, including the use of LED arrays or diffuser fibres for topical irradiation, cylindrical diffusing fibres for interstitial PDT and balloon catheters for light delivery into oesophageal tissues.[48] Noteworthy, artificial light is not imperatively required. Recently, daylight PDT has been developed for the treatment of superficial skin cancer.[49] In this treatment modality, PSs are topically applied, and sunlight exposure is allowed in a dosimetry-controlled fashion. The procedure has shown to be cost-effective and pain-reducing compared to other conventional PDT protocols.[49]

In the clinical setting, accurate dosimetry is crucial to achieve maximal therapeutic effects for PDT delivery. To ensure that the overall delivered optical power is accurate, light source calibration is necessary, with fluence and irradiance being fundamental parameters. Energy is generally measured in joules (J). The amount of energy delivered per unit area is known as fluence, sometimes called the dose exposure, and is given in joules per square centimetre (J/cm^2). The rate of energy delivery is known as power and is measured in watts (W). One watt equals to 1 J per second (W = J/s). Therefore, power delivered per unit area is known as the irradiance or power density, given in watts per square centimetre (W/cm^2). As such, the exposure area and exposure time are used to calculate total light dose:

$$\text{fluence} = \frac{(\text{power} \times \text{time})}{\text{area}}$$

2.1. Photodynamic reactions

As previously stated, during PDT a light-responsive PS generates ROS, which induce cell death. The photoreaction mechanisms are complex but can be divided into two main pathways called Type I and Type II (**Figure 5a**). Whereas Type I mechanism involves electron transfer reactions, Type II pathway relies on energy transfer processes.

The photosensitization processes begin with the absorption of light (**Figure 5b**). When the PS is irradiated with a specific wavelength of light, the absorption of a photon brings the PS from its ground electronic state into an excited singlet state ($^1PS^*$). This is a short-lived species (nanoseconds) and decays quickly to the ground state upon emission of fluorescence. The next productive step is an intersystem crossing (ISC) process, whereby the spin of $^1PS^*$ inverts to form an excited triplet state ($^3PS^*$). Since the transition $^1PS^* \rightarrow {}^3PS^*$ is spin-forbidden, this triplet state has a relatively long lifetime (microseconds), which allows the excited PS to interact with nearby molecules. The long-lived excited triplet state can also decay back to the ground state by emission of light (phosphorescence). However, the slower the decay of this triplet state, the more time the PS has to interact with its environment. At this point, the excited triplet $^3PS^*$ may undergo two kinds of reactions aforementioned: Type I (electron transfer) or Type II (energy transfer). In a Type I reaction, electron or proton transfers between the $^3PS^*$ and biomolecules in the surrounding environment result in the generation of radical anions or cations, respectively. These generated radicals then react with oxygen or water to produce different types of ROS such as superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^\bullet$) or hydroperoxyl radical ($HO_2^\bullet$).[50,51] Alternatively, in a type II mechanism, the $^3PS^*$ directly transfers its energy to molecular oxygen (itself a triplet in the ground state, $^3O_2$) to generate a reactive singlet oxygen species ($^1O_2$). Hence, in systems with high oxygen

content, Type II reactions predominate, whereas in oxygen-depleted systems, a Type I mechanism will be favoured.[51,52] Nevertheless, Type I and Type II reactions can occur simultaneously, and the ratio between them depends on the photochemistry and photophysics of the PS, the availability of molecular substrates and the concentration of cellular oxygen. Most current PSs operate primarily *via* type II mechanism, with the production of $^1O_2$ being the main sensitization pathway.[53]

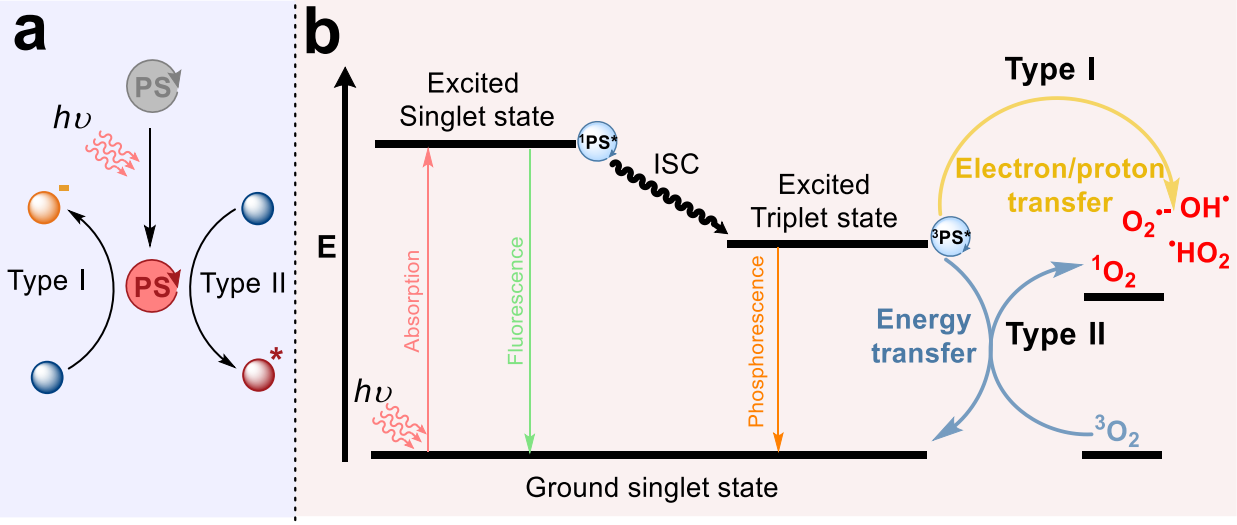

Figure 5. Photodynamic reactions. a) Schematic representation of Type I and Type II-PDT reactions. **b)** Jablonski energy level diagram for PDT.

Both Type I and Type II PDT processes ultimately lead to the build-up of ROS, which are highly reactive with biological molecules, including proteins, lipids and DNA. Amino acids residues are among the primary targets of an oxidative attack on proteins and enzymes, which may modify their bioactivity.[50] ROS can also oxidize lipids to form hydroperoxides, thereby disturbing lipid function and eliciting cell toxicity.[54] DNA may also be oxidatively damaged by different ROS either at nucleic bases or at the sugar residues.[55] Oxidative damage on DNA can lead to strand breaks, as well as cross-linking with proteins.[50] Although cells can mobilize a set of adaptive mechanism to repair DNA and cope with oxidative stress, excessive damage may cause mutations, induce cell cycle arrest or lead to cell death.[56] Due to their short lifetime (nanoseconds), ROS and $^1O_2$ will react with biomolecules that are proximal to the intracellular location of the PS and typically affect substrates within a radius of ~100 nm.[57]

It is worth to note that ROS are essential for biological functions. They modulate signal transduction pathways involved in stress response and cell survival by reacting and modifying the structure of proteins, transcription factors and genes to alter their functions.[56] Mounting evidence suggest that cancer cells exhibit adaptive mechanisms to tolerate ROS stress; with such

adaptations contributing to malignant transformation, metastasis and drug resistance.[56] However, a moderate increase in the level of ROS can lead to transient cellular alterations, whereas a severe increase may cause irreversible oxidative damage leading to cell death.[56] In this sense, PDT reactions are aimed to shift the redox balance of cancer cells, thus inducing an overall increase in ROS levels that will cause cell death when a certain tolerability threshold is surpassed.

2.2. Anticancer mechanism of action of photodynamic therapy

The photoinduced anticancer activity of PDT depends on several factors: the type of PS, its concentration, metabolism, location and biodistribution, the time of light irradiation, the interval time between the PS administration and irradiation and the availability of molecular oxygen. All these factors determine the efficiency of ROS production in the tumour tissue. Since central to PDT is the generation of ROS, it is evident that these oxygen species will be responsible for the mechanism of action of PDT. The downstream targets of PDT-photogenerated ROS include 1) tumour cells, 2) tumour vasculature and 3) the immune system.[58] In essence, excessive ROS levels trigger an irreversible oxidative damage to cancer cells, destroy the tumour microvasculature, which weakens blood vessels and halts tumour growth, and induce an inflammatory response that can stimulate an immunoprotective response (**Figure 6**).

Photodynamic reactions may elicit different cell death mechanisms that lead to tumour destruction. PDT typically induce cancer cell death by apoptosis, necrosis or autophagy regardless of the cell cycle phase. Among them, apoptosis seems to be the preferred mode of cell death following PDT because ROS such as $O_2^{\bullet-}$, $OH^{\bullet}$ and H_2O_2 usually cause cytochrome c release from mitochondria, which activates this cell death pathway.[59] Apoptosis is an adenosine triphosphate (ATP)-dependent process characterized by cell shrinkage, DNA fragmentation and the formation of the so-called apoptotic bodies.[60] In necrosis, in contrast, cells undergo swelling and loss of membrane integrity, which leads to depletion of ATP stores and leakage of intracellular components.[60] While apoptosis is a programmed cell death tightly controlled by a number of different proteins, necrosis has been described as a passive endpoint state of cell lysis and death. Autophagy is a highly regulated self-digestion process by which cells degrade damaged organelles. Since photodynamic damage occurs in close proximity to the PS location, the mode of cell death induced in PDT is largely dependent on the subcellular accumulation of the PS. For example, mitochondria- or endoplasmic reticulum-targeting PSs usually trigger apoptotic cell death upon

irradiation.[61,62] However, if the PS accumulates in the plasma membrane or into lysosomes, ROS build-up could rather provoke necrosis or autophagy-dependent cell death.[62] Light fluence and PS concentration are also factors contributing to the type of cell death, with high fluences and/or PS concentrations producing necrosis and low doses mainly inducing apoptosis.[62] Besides conventional types of cell death (*i.e.,* apoptosis, necrosis and autophagy), the emergence of new methodological approaches has greatly expanded our knowledge of the molecular mechanisms derived from PDT action, with other non-conventional modes of cell death being reported (*i.e.,* ferroptosis, pyroptosis or mitotic catastrophe).[63]

Furthermore, tumours cannot grow without blood supply. To secure this, the tumour microenvironment promotes angiogenesis by the secretion of angiogenic growth factors.[64] In general, solid tumours tend to have leaky vascularity, with tortuous and irregular vessels, impaired lymphatic drainage and elevated production of permeability factors.[64] This may serve as for PS accumulation into tumours by exploiting the enhanced permeability and retention effect.[65] Photoactivation of a PS in the vicinity of the tumour microenvironment, either in endothelial cells or in blood vessels, can cause damage to the endothelial-cell layer. This photodamage is usually characterized by the loss of tight junctions between cells, leading to basement membrane exposure, vascular leakage and subsequent platelet aggregation.[66] Altogether, the vascular damage derived from PDT has demonstrated to play an important role in the long-term tumour response.[66]

Although tumour ablation could be achieved by direct cancer cell killing and vascular damage, the curative outcome of PDT also derives from boosting the host immune system. Early studies in the late 1980s reported infiltration of immune cells such as lymphocytes and macrophages into PDT-treated tumours, suggesting the activation of the immune response.[67] PDT-mediated oxidative stress triggers signal transduction pathways that increase the expression of heat shock proteins and inflammatory cytokines, which in turn induce the arrival of macrophages and neutrophils.[68] Macrophages are committed to remove damaged cancer cells, while neutrophils express major histocompatibility complex (MHC) molecules to orchestrate the adaptive immunity response.[68] Macrophages and neutrophils then provide antigens to dendritic cells, which enable cross-presentation to $CD8^+$ cytotoxic T lymphocytes.[68] These immunostimulatory effects of PDT can produce long-lasting tumour-specific immunity *via* activation of memory T-cells.[68]

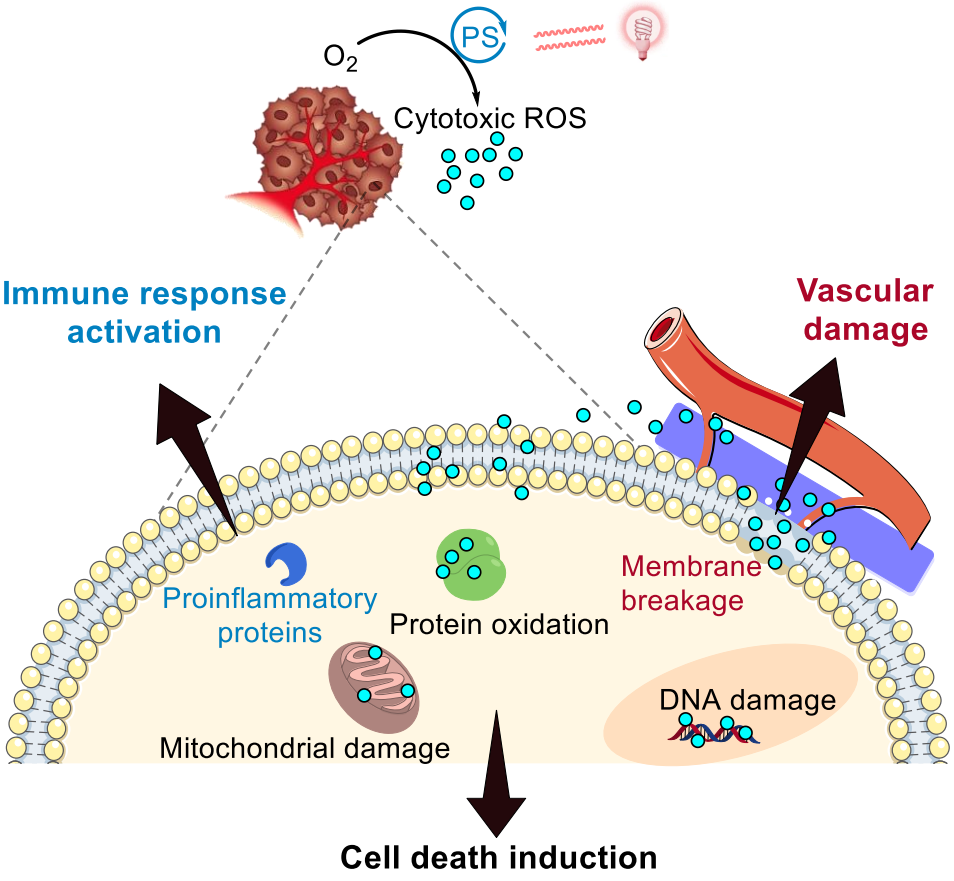

Figure 6. Anticancer mechanisms of action of PDT

3. Limitations of photodynamic therapy

PDT holds the potential to become a standard pillar of cancer treatment. However, currently it is only being used in the treatment of superficial cancers, towards neoplasms that are readily accessible to endoscopes or optical fibres, or as an adjuvant in the surgical procedures of primary lesions.[69] One of the reasons why PDT has not yet become a mainstream modality comes down to its limitations towards solid, bulky or deep-seated tumours. These types of tumours contain large subregions where oxygen concentration is low, ranging between 1% – 2% O_2 (hypoxia limitation).

Likewise, PDT cannot be applied to advanced metastatic or disseminated cancers since irradiation of the whole human body results a fairly complicated task with the current available technologies. As a localized treatment modality, an appropriate PDT dose (the product of light fluence and PS concentration) must reach every part of the neoplasm to be effective. In essence, poor tissue penetration by both light and PS limits the effectiveness of PDT. Inadequate drug penetration throughout the tumour volume or, as already stated, insufficient light penetration into

tissues prevent the delivery of PDT at enough doses, hindering the efficacy of a given PDT treatment (tissue penetration depth limitation).

However, accurate dosimetry during PDT is complex due to the dynamic interactions between light, PS and oxygen, rendering difficulties to be accurately determined.[70] This means that often clinical PDT treatments under-illuminate certain anatomical regions while over-illuminate others.[70] Under-illumination would lead to poor control of residual tumour regrowth, whereas over-illumination might induce excessive toxicity to surrounding normal tissues.[70] In general, clinical trials are designed for maximal tumour ablation rather than normal tissue preservation, and normal tissue injuries have been commonly reported as a result (collateral damage limitation).[70]

3.1. Hypoxia

Solid human tumours contain regions with very low levels of molecular oxygen, a condition known as hypoxia. The presence of these intratumoral regions at low oxygen concentrations was postulated by Thomlinson and Gray in 1955.[71] At that time, it was known that cells under hypoxia showed resistance to radiotherapy.[72] This led to the discovery of nitroimidazoles, which are small molecules that mimic the effects of oxygen and thereby sensitize hypoxic cells to radiation therapy. However, clinical trials with nitroimidazoles in combination with radiotherapy did not result in significant improvements except for certain cancer types such as head and neck cancers.[73] This raised the question of whether hypoxia was a hallmark of solid cancers. With the introduction of the oxygen electrode in the 1990s, which enabled accurate measurements of oxygen tension in tumours, investigators found that oxygen concentrations varied across tumoral regions but were much lower compared to normal tissues.[74] This posed several challenges for cancer therapy.[75] Regarding to radiotherapy, tumour hypoxia has been associated with poor prognosis because of the resistance of hypoxic cells to radiation treatment.[76] On the other hand, hypoxic cells are generally resistant to chemotherapy for several reasons. First, cells under hypoxia are distant from blood vessels and therefore cannot be easily accessed by some anticancer drug molecules.[77] Second, hypoxia selects for cells that have low proliferation rates and low apoptosis-mediated cell death rate, which might lessen anticancer drug activity.[77] And last, hypoxia upregulate drug resistance genes including P-glycoprotein encoding genes that detoxify drug molecules more effectively.[76] When it comes to PDT, hypoxia represents a major limitation owing to a lack of oxygen available for photosensitization. Since the mechanism of action of PDT relies on the generation of ROS, its therapeutic efficacy is intrinsically hampered by reduced oxygen levels.

Hypoxia is one of the greatest challenges of PDT treatment and has thus been referred to as the Achilles' Heel of this therapeutic modality. The dependence on O_2 to trigger singlet oxygen

photosensitization considerably precludes PDT activity in hypoxic tumours. Furthermore, PDT is itself an oxygen-consuming process that can aggravate hypoxia within cancer cells.[58] Tumour hypoxia can occur directly, either as a result of the existence of hypoxic cells that live despite poor blood supply or induced by PDT through fast depletion of oxygen being consumed in photochemical reactions, and indirectly through vasculature damage, which would also lead to exhaustion of blood supply.[66] Altogether, oxygen consumption during PDT exacerbate hypoxia, leading to treatment failure and resistance. The resultant hypoxic cells adapt their molecular signalling pathways to cope with the anaerobic state through the upregulation of the hypoxia-inducible factor 1 (HIF-1).[78] HIF-1 is a heterodimeric protein consisting of two subunits, HIF-1α and HIF-1β. In normoxia, HIF-1α is constitutively degraded in the cytoplasm, but hypoxia induces its translocation to the nucleus, where it binds to HIF-1β, enabling the active form of HIF-1.[78] The overexpression of HIF-1 is strongly correlated with chemoresistance of hypoxic tumour cells and negative prognoses.[78]

In an effort to generate photocytotoxicity despite hypoxia, various strategies have been developed. Hyperbaric oxygenation of the tumoral tissue, oxygen delivery *via* nanoparticles or perfluorocarbon or water splitting process are some of the methods adopted to deal with depletion of tissular oxygen.[37] Fractionated illumination with controlled, short intervals of light and periods has also shown to improve tumour response.[79] Several other strategies to overcome hypoxia in PDT have been explored.[80] The research focus is now on the development of novel PSs that can circumvent the hypoxia limitation.

3.2. Tissue penetration depth

PDT efficacy is also hindered by the penetration capability of light into the tumoral tissue. Different interactions of light with tissue, including reflection, refraction, scattering and absorption, limit how far light can diffuse into tissue.[69] As previously mentioned, this results in poor penetration light depth, which severely hinders phototherapy and limits PDT potential to topical and superficial tumours. Optical penetration depth at which the light intensity drops to $1/e$ (~37%) of the initial intensity lies between 50 and 100 μm for UV and blue light (λ = 400–450 nm).[69] The penetration depth of green light (500–550 nm) is a few hundred micrometres, due to restriction by highly absorbing molecules in tissue such as melanin, haemoglobin and cytochromes.[46,69] Red and NIR light (600–900 nm) may reach the largest penetration depth, typically between 3–10 mm.[41,69] As such, even using NIR light, only tumours located ≤1 cm deep can be reached by PDT.

To extend the therapeutic depth, different strategies are under development. For example, upconversion nanoparticles that convert incident NIR light into shorter wavelength emissions (*e.g.* visible or UV) to indirectly activate conjugated PSs have shown promise against deep-seated

tumour in mice.[81] Another strategy involves the delivery of targeted, self-luminescent nanoparticles into tumours that are inaccessible to external irradiation. These self-luminescent nanoparticles can then activate PSs via resonance energy transfer to treat deeper lesions.[82] To tackle the penetration depth issue, multi-photon activation has been advocated as advantageous since it may push activation wavelengths towards the NIR region. Two-photon absorption PDT is the most frequently used.[83]

Proposed by the 1963 Physics Nobel Prize winner Göeppert-Mayer, two-photon absorption allows to red-shift excitation wavelengths to the tissue transparency window.[84] Whereas in conventional PDT the PS absorb one photon at a given wavelength, in two-photon PDT two photons are absorbed simultaneously with each photon contributing half of the required energy. This two-photon excitation (TPE) populate the same excited state as with one-photon with twice the energy.[83] For example, a PS that absorb light at 420 nm can be activated by two-photon 840 nm irradiation. As a result, the incident radiation would be in the NIR region, allowing larger penetration into the tissue and increasing the depth of PDT. However, not all PSs are valid for TPE. A large two-photon absorption cross-section along with high photostability is mandatory.[83] Some organic molecules with extended conjugation systems fulfil these requirements and are suitable for two-photon PDT.[83] Transition metal complexes also possess high two-photon absorption cross-section in the NIR region.[85] Nonetheless, to achieve absorption of multiple photons simultaneously, high power femtosecond solid-states lasers are needed, which increases the expenses of this technique as they are difficult to operate and require specialized personnel.[86] All these issues outweigh the advantages of TPE and hamper its clinical use.[86]

3.3. Collateral damage

As the PS accumulates into tumoral tissue and neovasculature, successful PDT treatment can ablate the tumour. However, if certain amounts of PS infuse into normal tissue that is exposed to light, severe tissue morbidity can be expected. Moreover, some PS will concentrate to a lesser degree in off-target organs including the skin for periods of several week post-infusion.[70] This might derive into skin photosensitivity problems derived from unintentional sunlight exposure, which constitute one the most common adverse-effect symptoms of clinical PDT. In a clinical study with 2031 patients, evaluation of the side effects of PDT determined that pain and edemas, in conjunction with itching was experienced by the majority of subjects.[87] Other less common side effects included crusting, pustules or erosions in the affected area, probably as a result of necrotic cell death induction in adjacent tissues, along with sunlight sensitivity problems.[88] On the other hand, the residues of PS may also cause serious side effects, with toxic metabolite accumulation in

different organs. Nevertheless, all these unwanted effects are easily manageable from a clinical perspective, which may be leveraged in combinatorial therapies of PDT and chemotherapy.

Many of these limitations (hypoxia, penetration depth and collateral damage) are partly due to the incomplete knowledge about the required light dose to be delivered to the tumour without damaging the adjacent normal tissue, and partly due to the flaws in the properties of the PSs currently available. As it will be discussed in **section 4**, development of effective PSs that overcome the major limitations of PDT has become a daunting task in the research field.

4. Photosensitizers

An ideal PS is expected to have several properties for a successful clinical development.[89] In terms of pharmacokinetics, ideal PSs should present an appropriate retention time in the body to preferentially accumulate in tumour tissue and show rapid clearance from normal tissue.[89] This would maximize treatment selectivity and minimize phototoxic side effects. Amphilicity is a highly desirable structural characteristic to achieve these targeting properties.[89] When systemically introduced, an amphiphilic PS can travel through the blood stream without aggregation or degradation owing to its hydrophilicity until it arrives to the target tissue, where some degree of lipophilicity will allow entry to tumour cells.[89] Moreover, the ideal PS should present aqueous solubility for formulation, good biocompatibility and high photostability upon light irradiation. These are critical points since PSs that are rapidly metabolized or degraded only provide a small window for light activation, whereas metabolically robust and stable molecules offer longer term control and less side effects.[34] As for the photophysical properties, high absorption (molar extinction coefficient, ε) at long wavelengths is desired.[90] Negligible dark cytotoxicity is another must-have property for a clinically successful PS.[90] Along with this presumably low toxicity in the dark, high light-induced amplification cytotoxicity (photoactivation) is needed. The parameter to optimize in anticancer PDT is thus the difference in toxicity between dark and light conditions. The most common determination of phototoxicity is called phototherapeutic index (PI) and is usually calculated from *in vitro* cellular assays. The PI is defined as the ratio between the concentration of the PS needed to cause 50% of cell inhibition in the dark (dark IC_{50}) and that measured after light irradiation (light IC_{50}). In essence, this dark to light IC_{50} ratio measures how much cytotoxic a PS is after light irradiation compared to dark conditions. Evidently, high PIs are correlated with better performance of the PS toward cancer cells, although PI values are specific for the conditions of the assay and might change upon minimal

alterations in the experimental variables (incubation time, cell line, light dose, light intensity and irradiation time). Other key requirements for a PS to efficiently generate ROS are a high quantum yield of the triplet state formation and an adequate triplet lifetime to interact with molecular oxygen or other biomolecules. Since a longer lifetime of the excited-state PS (PS*) presumably warrants a more efficient conversion of molecular oxygen into ROS, a relationship using the Stern–Volmer equation can be established:

$$\tau_0/\tau = 1 + \kappa\,\tau_0[O_2]$$

where τ_0 and τ are the lifetimes of the excited PS* in the absence and presence of oxygen, respectively, and κ is the rate constant for the diffusion-limited reaction. Then, PSs with longer lifetimes would be most likely to generate more ROS. Nevertheless, with so many disparate criteria to be tackled, it is difficult for a given PS to fulfil all the requirements. Current PSs approved for clinical use do not satisfy many of these criteria, although extensive research work is in progress to overcome the salient challenges of PDT.[91]

4.1. Current clinical photosensitizers

Development of PSs can be distinguished into three generations. First-generation sensitizers are based on the naturally-occurring porphyrin structure and its derivatives. Porphyrins are 18 π-electron aromatic macrocycles with an absorption spectrum characterized by a strong $\pi - \pi^*$ transition (Soret peak) and four Q bands in the visible region.[53] Their heterocyclic tetrapyrrolic structure resembles that of haems and chlorophylls, which are functional pigments in biology either in the electron transport chain or in photosynthesis, respectively. Porphyrins possess unique advantages in PDT such as visible-light activation and high $^1O_2$ generation efficiency.[53] The first porphyrin PS was the hematoporphyrin derivative (HpD), a complex mixture of porphyrinic compounds prepared from crude hematoporphyrin treated with acetic and sulphuric acids.[58] HpD was used during the 1970s and 1980s to treat skin, lung and oesophageal cancers with successful rates.[58] However, the poor purity and off-target accumulation in normal cells of HpD led to the discovery of Photofrin® (sodium porfimer), an oligomeric mixture of purified and hydrolyzed HpD. Photofrin® became the first approved PDT agent for bladder cancer in 1993.[92] Since then, it has been widely used to treat various cancers (**Table 1**). To date, Photofrin® is the only PS which has received approval worldwide for cancer therapy, although the European Medicines Agency has recently withdrawn this approval.

Despite Photofrin® successfully eliminates tumours upon 630 nm laser light, the low molar extinction coefficient requires the use of higher PS concentrations, leading to slow body clearance,

non-specific accumulation and skin photosensitivity complications.[93] To address these limitations, second-generation PSs with higher purity and less post-treatment photosensitivity were developed. Some of these PSs include the biosynthetic precursors of Protoporphyrin IX (PpIX) such as Levulan® (5-aminolevulinic acid) and Metvix® (methyl aminolevulinate), which are clinically approved for the treatment of basal cell carcinoma (**Table 1**). Modifications of the porphyrin ligand also led to new structurally different PSs, including chlorins (Foscan®, Radachlorin® and Laserphyrin®), bacteriochlorins (Tookad® soluble), phthalocyanines (Photosens® and Purlytin®) or metalloporphyrins (Lutex®).[52] These new classes of PS efficiently absorb light at longer wavelengths in their Q bands and have been employed in clinical settings against a variety of cancers (**Table 1**).[93] As shown in **Table 1**, most of the current PSs on clinical use are based on the tetrapyrrolic structure (*i.e.,* porphyrin, chlorin, phthalocyanine), which strongly dictates their photophysical and bioactive properties (**Figure 7**). Due to this shared structural scaffold, similar drawbacks have been associated to all of them, including 1) poor aqueous solubility, 2) poor photostability, 3) tedious synthesis and purification, 4) lack of selectivity and 5) long clearance times and skin photosensitivity problems.[94] Therefore, there is an urgent need on the improvement of these PSs and the discovery of new scaffolds for PDT. Therefore, there is an urgent need on the improvement of these PSs and the discovery of new chemical scaffolds for PDT. Much of the current research in the PDT field is focused on the development of the so-called third-generation of PSs that circumvent such limitations, being the modification of the parent structure of well-stablished sensitizers a common strategy.[95] The tetrapyrrolic ring system possess 12 positions that might be substituted with different groups (e.g., carboxylic acid, hydroxyl, sulfonate, ammonium, carbonyl or pyridinium), providing an enormous variety of possible derivatives.[95] Besides, porphyrin, chlorin and phthalocyanine structures can also be oxidized or extended.[95] Another strategy consists of the incorporation of a metal ion in the tetrapyrrolic scaffold.[37] Metalation allows for stabilization of the compound towards metabolic liabilities while improving photostability.[37] Furthermore, the presence of a central heavy atom may increase the ISC efficiency, thereby impacting on the ability to trigger PDT reactions.[37] However, despite research efforts in the development of metal-containing tetrapyrrolic PSs, only a few compounds have advanced into clinical settings.[37] Photosens®, for example, is an aluminium-containing phthalocyanine compound that has received clinical approval in Russia for different cancer types (**Table 1**). The 762 nm light-activated PS Tookad® contains a palladium-coordinated bacteriochlorin, which is a chromophore used by certain bacteria for photochemical processes, and is commonly applied in vascular-targeted PDT.[96] On the other hand, Purlytin®, a tin-coordinated phthalocyanine PS, and Lutex®, a texaphyrin-based PS with a lutetium metal center and axially-bound acetate ions, entered clinical trials in the United States of America for cancer treatment.[52]

Table 1. Overview of clinically approved PSs or under clinical trials.[a]

Agent	Chemical name	Scaffold	Stage	Cancer type
Photofrin®	sodium porfimer	porphyrin	approved worldwide	oesophageal, endobronchial, bladder and lung cancer
Metvix®	methyl 5-aminolevulinate	porphyrin precursor	approved worldwide	basal cell carcinoma
Levulan®	5-aminolevulinic acid	porphyrin precursor	approved worldwide	basal cell carcinoma and squamous cell carcinoma
Foscan®	temoporfin	chlorin	approved in European Union	head and neck squamous cell carcinoma
Radachlorin®	chlorin mixture (e6, p6 and purpurin)	chlorin	approved in Russia	basal cell carcinoma
Laserphyrin®	talaporfin	chlorin	approved in Japan	brain and lung cancer
Photosens®	sulfonated aluminium phthalocyanine	phthalocyanine	approved in Russia	lung, liver, breast, skin and gastrointestinal cancer
Tookad® soluble	padeliporfin, WST11	bacteriochlorin	approved in European Union, Mexico and Israel	prostate cancer
Purlytin®	rostaporfin	phthalocyanine	in clinical trials	basal cell carcinoma
Lutex®	Motexafin lutetium	porphyrin	in clinical trials	brain, breast, cervical, and prostate cancers

[a]Data from https://www.clinicaltrials.gov/

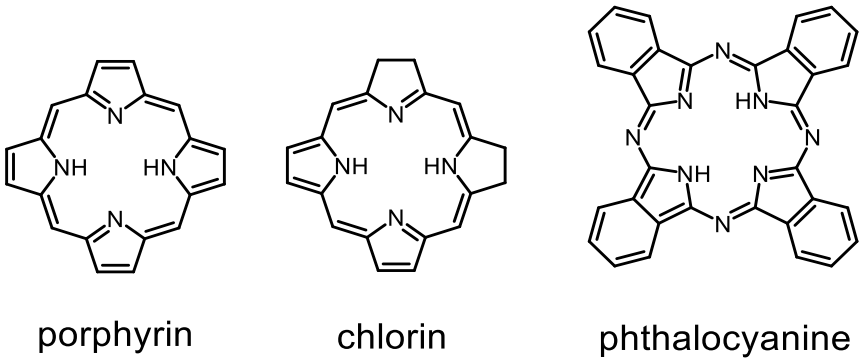

Figure 7. Structure of the main chemical scaffolds found in conventional organic photosensitizers.

Some other strategies are aimed at improving tumour-tissue specificity. For instance, the covalent attachment of antibodies or targeting ligands based on biomolecules or small molecules (peptides, carbohydrates, folic acid and others) that preferentially bind to surface receptors expressed on cancer cells has been reported to enhance tissue selectivity.[97,98] PSs have been also conjugated to known chemotherapeutic drugs, such as 5-fluorouracil and cisplatin, for dual cyto- and phototoxic action.[99] Recent endeavours in nanotechnology *via* PS encapsulation have been remarkably fruitful, leading to early-stage applications for targeted anticancer phototherapy.[100,101] Nonetheless, many research efforts have been devoted towards the development of new scaffolds for effective PDT. As it will be discussed in **sections 4.2** and **4.3**, recent progress in the development of organic fluorophores and transition metal complexes as PSs, from molecular design to applications, has led to innovative approaches for enhanced PDT and the discovery of new PSs. Metal-based conjugates have also received attention recently as an emerging strategy to develop new PDT agents (**section 4.4**).

4.2. Organic fluorophores as photosensitizers

Fluorescence imaging has greatly advanced our understanding of cell and molecular biology. Fluorophores based on small organic molecules are indispensable tools in bioimaging, either for visualization of molecular structures and dynamics or for the detection and quantification of chemical species within biological systems.[102] In particular, fluorescence sensors that can be specifically targeted to certain organelles have been remarkably attractive in cancer research because the majority of chemical and biologically-relevant processes in cancer take place inside subcellular organelles.[103] Since organic fluorophores are usually designed to absorb light and to possess large molar extinction coefficients for real-time imaging, they offer a good opportunity to

develop novel PSs for efficient anticancer PDT. In fact, most of the current PSs, either clinically approved or in clinical trials, are based on organic fluorophores.

Besides porphyrin, chlorin and phthalocyanine analogues, novel PSs PSs based on organic fluorphores have been developed in recent years, *e.g.* cyanine, boron dipyrromethene, xanthene and coumarin (**Figure 8**).[95] Among them, cyanine dyes have been widely used in both fluorescence diagnosis and phototherapy. Exemplary, indocyanine green (ICG), a Food and Drug Administration (FDA)-approved fluorophore for use in medical diagnosis, has also been used in PDT (**Figure 8**).[104] Although ICG exhibits low toxicity and relatively high absorption extinction coefficient, its poor $^1O_2$ quantum yields provide low PI values.[105] Moreover, the electron-rich heptamethine chain of ICG can suffer from self-sensitized photo-oxidation during light irradiation, which leads to decomposition or photobleaching.[106] Hence, development of novel, efficient and photostable cyanine-based PSs has been attempted. For example, Shi *et al.* found that the heptamethine cyanine IR-780 (compound **1**, **Figure 8**) showed better $^1O_2$ photogeneration capacity and higher stability than ICG.[107] The derivatization of **1** with pyridinium moieties (**2**) improved the PDT effect.[108] Incorporation of heavy atoms such as iodine atoms into the indole ring of an heptamethine cyanine (**3**) has shown to enhance the $^1O_2$ production and reduce the tumour burden in mouse models (**Figure 8**).[109] Moreover, the addition of 2,2,6,6-tetramethylpiperidinyloxy (TEMPO) to the cyanine scaffold demonstrated to enrich the ISC process and the long-lived triplet state of the PS (**4**), thus improving its anticancer photoactivity against HeLa cells.[110] Another group of cyanine-based PSs are hemicyanine chemosensors, which benefit from intramolecular charge transfer mechanisms that can be modulated depending on the electron-donating ability of the donor groups at the *meso*-position.[111] By linking this hemicyanine structure to the drug 5'-DFUR, the resulting PS **5** was able to kill tumour cells upon NIR irradiation through a dual mechanism involving PDT and chemotherapy via the release of 5'-DFUR (highlighted in blue in **Figure 8**).[112]

Phenothiazine derivatives are another class of organic cationic dyes with phototherapeutic potential. With a wide range of activities, including antimalarial and antidepressant, the phenothiazinium dye methylene blue (MB) has been also used in the clinic for PDT applications (**Figure 8**).[113] However, despite its water-solubility and the high $^1O_2$ quantum yield, MB presented unspecific targeting which results in poor tumour accumulation.[113] Benzo-phenoxazine compounds such as Nile blue, commonly used for histological staining, have also been of interest as PSs (**Figure 8**).[114] Although the original Nile blue is a poor PS candidate, substituting the oxygen atom of the oxazine scaffold with sulfur improved its PDT efficiency. Peng and co-workers found that conjugation of this sulfur-containing Nile blue analog to a biotin targeting moiety (**6**) results in a potent Type I $O_2^{\bullet-}$ generator even in low-oxygen environments upon NIR-light irradiation.[115]

Among organic fluorophores in PDT, boron dipyrromethene (BODIPY) is one of the most extensively studied class due to its chemical and photo-physical properties such as easily

modifiable structure and intense absorption in the visible region (**Figure 8**). Introduction of iodine or bromine atoms to the π-conjugated skeleton of BODIPY can enhance the ISC *via* the heavy atom effect.[116] In general, the presence of a high atomic number atom favours the spin-forbidden process and increase the ISC capacity of the excited PS.[116] For example, hexaiodinated BODIPY has been described as an aggregation-enhanced luminescent molecule (compound **7**) where iodine atoms favour the ISC process and the generation of $^1O_2$.[117] In another approach, introduction of a π-electron rich system such as thiophene into the BODIPY dye together with the incorporation of bromide atoms (**8a** – **8d**) increased the ISC yield and improved $^1O_2$ production.[118] Bromo-substituted BODIPYs bearing thienopyrrole moieties have also been reported as potent NIR-active PSs (**9a** and **9b**, **Figure 8**).[119] Nevertheless, other heavy-atom free approaches have been investigated; for example, by introducing sulfur atoms into the BODIPY scaffold and modifying the *meso*-position to tune the photophysical properties.[120] This strategy has proven to render potent mitochondria-targeted BODIPY derivatives (**10a** – **10d**) with light IC_{50} values in the nanomolar range against HeLa cancer cells.[120] Many other strategies, either through heavy atom effect or *via* structural modifications, have been proposed to develop BODIPY-based PSs.[121]

Xanthenes such as rhodamine and fluorescein derivatives have also attracted attention in PDT. In particular, the discovery of the mitochondria-specific dye Rhodamine123 as an anticancer agent[122] fuelled the used of these xanthene derivatives in cancer phototherapy because of their large molar extinction coefficients, strong absorption in the visible region and aqueous solubility and photostability, which are favourable features for PDT (**Figure 8**).[123] The xanthene dye Rose Bengal has also been used as a photosensitizing agent in ophthalmology.[124] Leveraging the heavy atom effect, rhodamine dyes have been successfully substituted with bromine, resulting in PSs with decent anticancer photoactivity in several cell lines (**11**, **Figure 8**).[125]

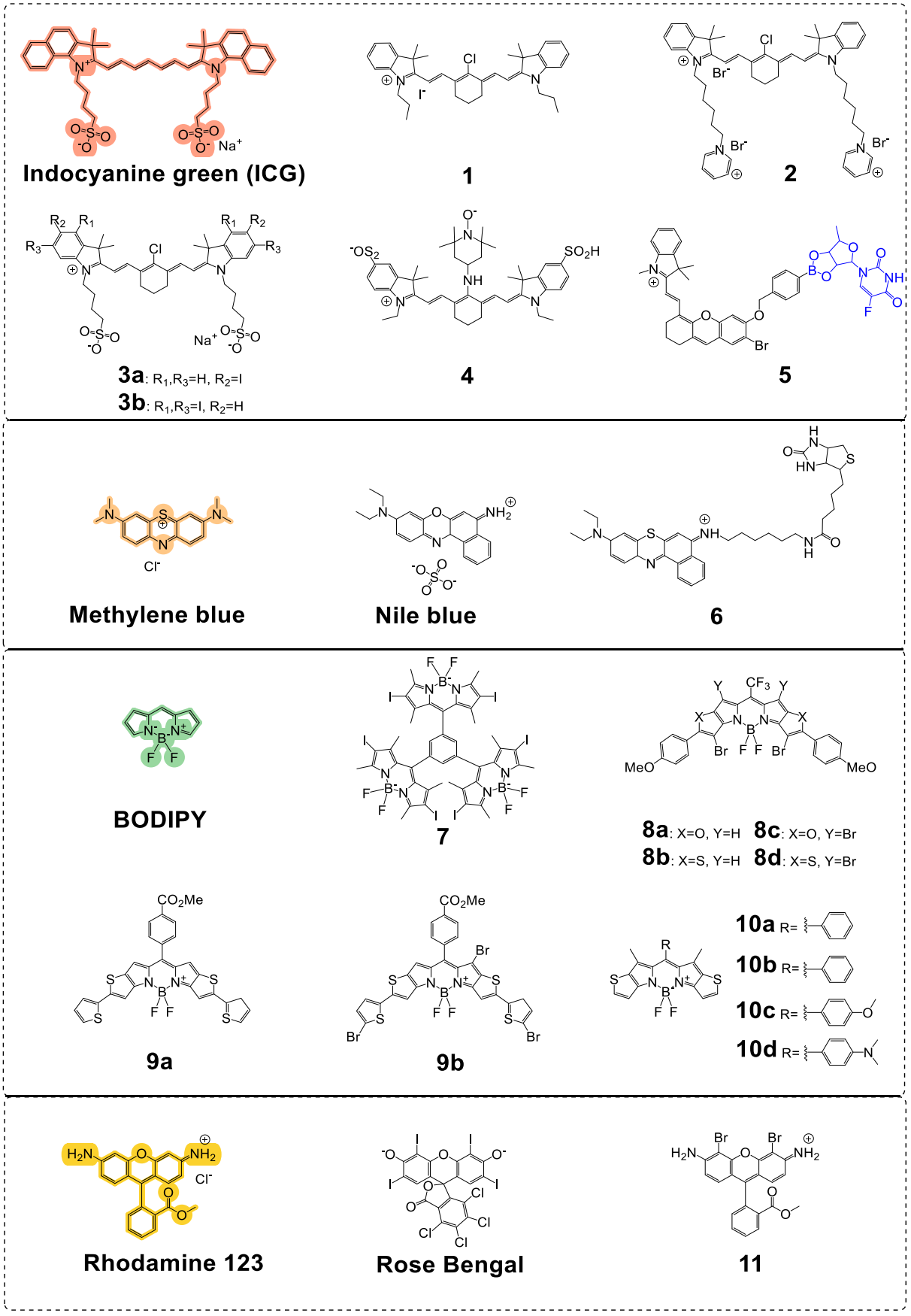

Figure 8. A representative set of common classes of organic small-molecule fluorophores studied as photosensitizers for cancer therapy, sorted by structure and emission colour.

Coumarins are also privileged scaffolds for the development of novel organic-based PSs. Both synthetic and naturally-occurring coumarins are well-known for their broad pharmacological properties including antibiotic, antiviral and anticancer activities, and are also employed in food and cosmetic industries.[126] Some coumarins such as psoralens (**Figure 9**) have been successfully used in medicine for centuries to treat cutaneous disorders like psoriasis and vitiligo in combination with light.[127] Psoralens are furocoumarins, consisting of a furan ring fused to the coumarin scaffold, that naturally occur in plants. However, psoralens require the use of UV light, with the added risk of developing malignant melanomas.[128] Besides, this furocoumarin class generally suffers from poor aqueous solubility and tumour selectivity, which increments the risk of adverse effects such as skin photosensitivity.[129] Yet the coumarin skeleton can be modified to red-shift the absorption and emission maxima, which would avoid the toxicity associated to short wavelengths of light, and to increase water solubility.[126] Coumarin derivatives with an extended π-system at the 3-position and bearing triethylene glycol chains have been reported as water-soluble, potent anticancer PDT agents upon one- and two-photon excitation (**12a** – **12e**, **Figure 9**).[130] In a different approach, Zhao and co-workers developed some cationic coumarins with different alkyl chains (**13a** and **13b**) operating in the red and NIR region of the electromagnetic spectrum.[131] These coumarins also showed excellent biocompatibility, efficiently generated ROS and inhibited the growth of both cancer cells and Gram-positive bacteria through photodynamic reactions.[131] Incorporation of onium salts as an electron acceptor group to 7-diethylaminocoumarin, which serves as electron donor group, enables the construction of a donor-π-acceptor system (**14a** – **14c**) that efficiently generates Type I-ROS *via* far-red and NIR light irradiation.[132] Such coumarins preferentially accumulated in mitochondria, and induced mitophagy and apoptosis *in vitro* and tumour ablation *in vivo* upon 808 nm irradiation.[132] A similar approach was explored by Teng and Yoon and collaborators, who introduced a rigid electron-donating group at the 7 position of the coumarin scaffold and conjugated to a rhodamine containing an oxonium ion on the 3-position (**15**).[133] Under 690 nm laser irradiation, **15** exhibited potent PDT activity against *Staphylococcus aureus* bacteria in mouse models.[133] Marchán *et al.* have developed a new class of coumarin-based derivatives in which the carbonyl group of the lactone ring in the classical coumarin scaffold has been replaced by cyano(4-pyridine/pyrimidine)methylene moieties with the aim of creating a strong push-pull effect. Such coumarin-pyridine fluorophores were named COUPYs (**16a** – **16k**, **Figure 9**).[134–136] Small modifications on the structure of COUPY dyes, which do not alter the overall molecular size of the molecule, allows the modulation and fine-tuning of their spectroscopic properties and subcellular localization.[134–136] Moreover, besides exhibiting interesting photophysical properties for bioimaging applications, such as far-red and NIR emission, large Stokes' shifts and brightness, COUPY fluorophores possess attractive characteristics for PDT including aqueous solubility, excellent cell

membrane permeability, mitochondria selectivity and high photostability.[134] Within the framework of this doctoral thesis, we have demonstrated that COUPY dyes exhibit potent anticancer photoactivity upon visible light irradiation either alone (See **Chapter I** in the Results and Discussion section) or conjugated to metal complexes (See **Chapter III** in the Results and Discussion section). Furthermore, the coumarin scaffold has been exploited in many other ways to develop both fluorogenic probes and phototherapeutic agents.[137]

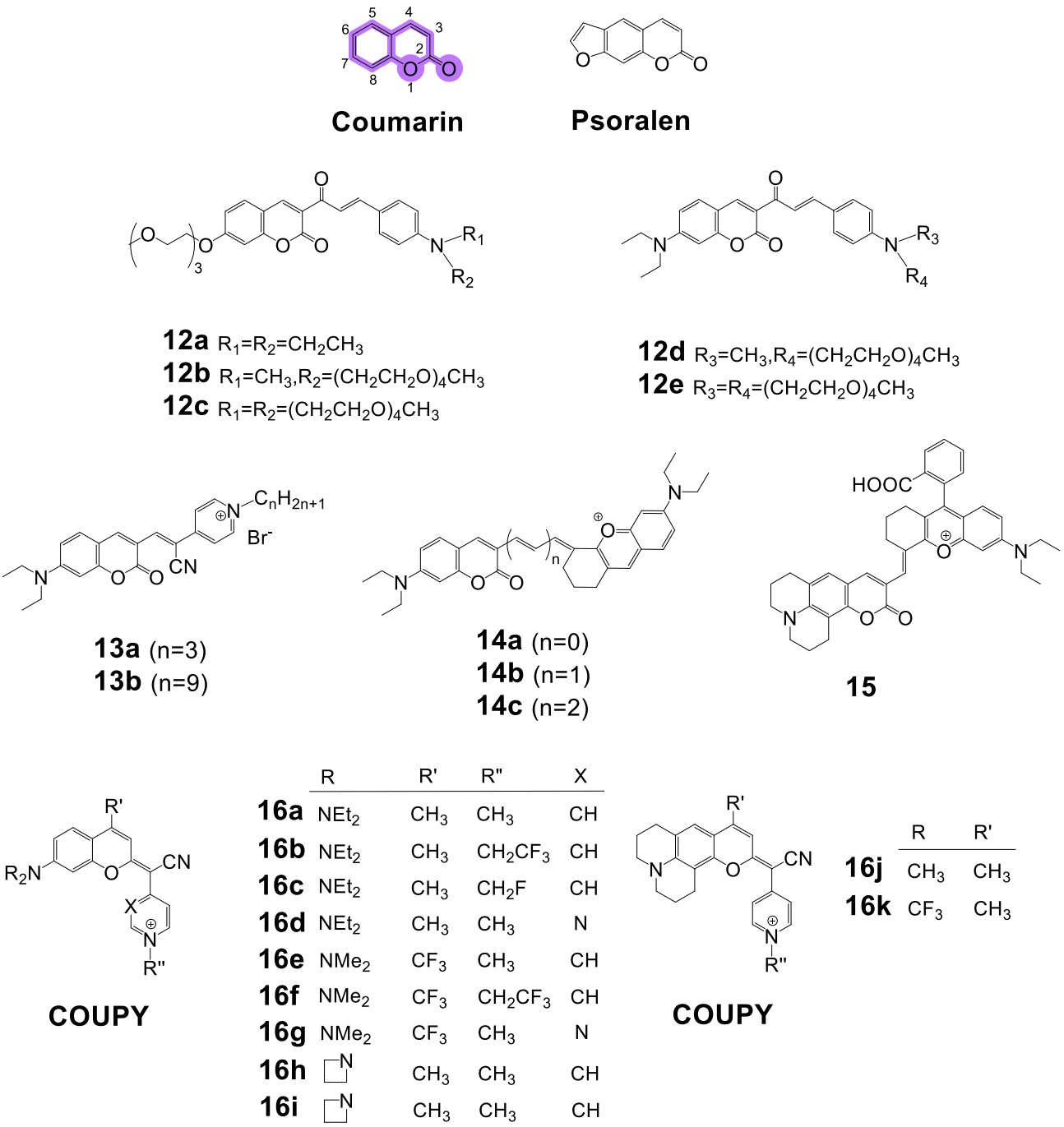

Figure 9. Coumarin scaffold and some coumarin fluorophores studied as photosensitizers for cancer therapy.

4.3. Transition metal complexes as photosensitizers

Although organic-based PSs have dominated the phototherapeutic market for decades, transition metal complexes are of increasing interest as alternative photodynamic agents in the recent years. The serendipitous discovery of cisplatin in 1969 by Rosenberg and collaborators revolutionized anticancer chemotherapy and spurred the development of new metal-based drugs with alternative modes of action.[138] Since then, metallodrugs have entered in clinical trials or have been approved for clinical use due to their diversity of three-dimensional structural scaffolds, wide range of applications in medicine and innovative mechanisms of action.[139] In the same way, the diversity of coordination and oxidation states, together with the steric and electronic variety that can be accomplished by combining different metals and coordinated ligands have also been exploited to develop metal-based PDT sensitizers.[140]

Metal complexes offer some advantages over classical organic PSs, including increased water solubility, improved stability and the possibility of quantification in biological samples by inductively-coupled plasma mass spectrometry (ICP-MS) techniques.[140] In general, due to the full occupation of their *d* orbitals, transition metal complexes are kinetically stable and thermodynamically inert, which allows for electron transfer reactions without interference of ligand exchange.[141] Transition metal complexes also provide robustness to avoid photobleaching.[141] Besides, metal-based sensitizers leverage luminescent properties that benefit from large Stokes shift, minimal self-quenching and long emission lifetimes to track their localization in biological systems.[141] In fact, the ability of metal complexes to tune the electronic states can also be exploited to target different subcellular organelles, including mitochondria, endoplasmic reticulum or lysosomes.[142]

However, much of the attention directed to metal-based PSs is due to their spectroscopic and electrochemical properties. In contrast to the π–π^* electronic transitions that trigger PDT reactions in organic PSs, transition metal complexes possess more excited electronic configurations that can be exploited in PDT (**Figure 10a**).[141] These electronic transitions can occur on the metal (metal-centred, MC), within the ligand (intraligand, IL), or between the metal and the ligands *via* charge-transfers: metal-to-ligand charge transfer (MLCT) or ligand-to-metal charge transfer (LMCT). In addition, charge-transfer states can also take place between two metals (metal-to-metal charge transfer, MMCT) in the case of bi- or multimetallic complexes or between two ligands within the complex (ligand-to-ligand charge transfer, ILCT).

As previously discussed, transition metal complexes can access triplet excited states more easily than organic fluorophores due to spin-orbital couplings derived by the heavy atom effect, which increases the rate of ISC.[143] Depending on the metal oxidation state, the atomic number and the

field strength of the ligand, *d* orbitals in transition metal complexes arrange in different energy levels, namely t_{2g} and e_g.[143] Upon excitation, electrons of the metal *d* π-orbitals are excited to higher energy levels and ISC leads them to populate long-lived triplet states, which allow the PS to interact with molecular oxygen or other biomolecule substrates.[141] Indeed, the different reactivities of the excited-state electronic configurations and the large quantum yields have prompted researchers to rationally design metal complexes with photobiological mechanisms that are unachievable with organic PSs.[45] Among transition metal-based PSs, ruthenium(II), iridium(III) and platinum(II) complexes are the most studied (**Figure 10b**). Yet the electronic arrangements of these metallic compounds are quite different. Ru(II) complexes are $4d^6$ centres with hexa-coordinated octahedral architectures and Ir(III) complexes exhibit $5d^6$ octahedral coordination, whereas Pt(II) complexes are $5d^8$ centres with planar quadrilateral geometries. Other transition metals including Rh(II), Os(III),[144] as well as first-row metal complexes have also been explored for phototherapeutic applications although in a lesser extent.[145]

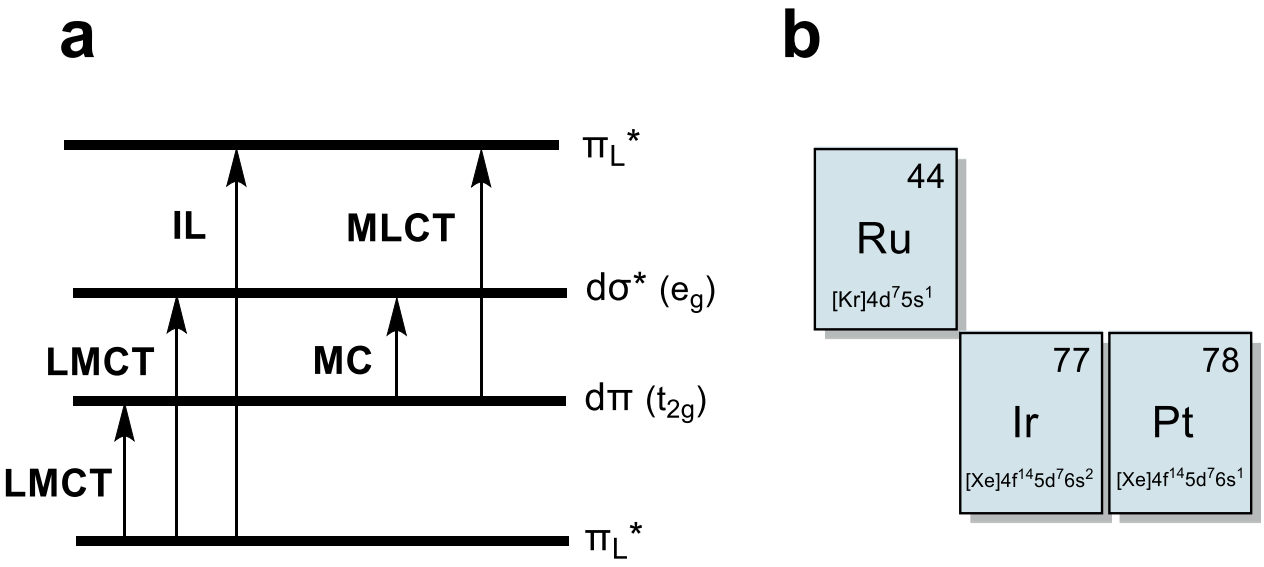

Figure 10. **a**) Main electronic transitions available to transition metal complexes with octahedral symmetry. LMCT: ligand-to-metal charge transfer, IL: intraligand, MC: metal-centered, and MLCT: metal-to-ligand charge transfer. **b**) Atomic number and outer electron arrangement of main metals used in anticancer PDT.

Ru(II) complexes are the most in-depth investigated metal-based PSs in cancer phototherapy, mostly because of their attractive photophysical and pharmacological properties. Added to these properties are the relative ease by which these compounds can be synthesized. Notwithstanding, the first studies on Ru metallodrugs involved ammine- and chlorido-containing Ru(III) complexes due to their similarity to cisplatin.[146] In the late 1980s, Keppler and co-workers communicated a novel class of Ru(III) anticancer agents of the type trans-[$RuCl_4(HIm)_2$] where HIm is imidazole that culminated in the discovery of KP1019 ((IndH)[*trans*-$RuCl_4$(indazole)$_2$]) and its sodium salt derivative KP1339 (sodium *trans*-[tetrachloridobis(1*H*-indazole)ruthenate(III)]),[147] which entered phase I clinical trials for the treatment of platinum-resistant colorectal cancers (**Figure 11a**).[148] The exciting results with such complexes prompted the development in the 1990s of another class of structurally related compounds bearing dimethyl sulfoxide (DMSO) as ligand.[149] After several optimization studies on Ru(III)-DMSO compounds, Alessio and co-workers developed sodium *trans*-[RuCl4(DMSO)(HIm)] or NAMI-A, which entered in clinical trials as an antimetastatic drug (**Figure 11a**).[150] Although clinical evaluations with NAMI-A were discontinued due to insufficient efficacy, its success along with that of KP1019/KP1339 boosted the development of new Ru-based anticancer compounds.[151] Since then, Ru-based compounds have also become an active focus of research in anticancer drug development.[152–154] In particular, “half-sandwich” Ru(II) compounds bearing the η^6-arene moiety, such as RAPTA derivatives introduced by Dyson *et al.*[155] or RM175 and its analogues by Sadler *et al.*,[156] have experienced a strong impetus as chemotherapeutics. Other Ru(II) arene complexes have been developed with novel, alternative modes of action including histone deregulation[157] or protein translation inhibition (**Figure 11b**).[19,152] It is worth noting that Ru(II) complexes have generally shown short retention times in animals,[158] which is a highly desirable trait in the development of chemo- and phototherapeutic drugs. Nevertheless, octahedral Ru(II) polypyridyl complexes have attracted much attention in PDT research since selected ligands can be combined in a combinatorial fashion, enriching the availability of fine-tuned PS molecules.[159] Albeit some Ru(II) arene complexes have been reported for cancer phototherapy,[160] the main research lines are currently devoted to Ru(II) polypyridyl complexes, which are gaining a momentum with the Ru(II) complex TLD-1433 having just entered in phase 2 clinical trials as PDT agent for the treatment of bladder cancer (**Figure 11a**).[45]

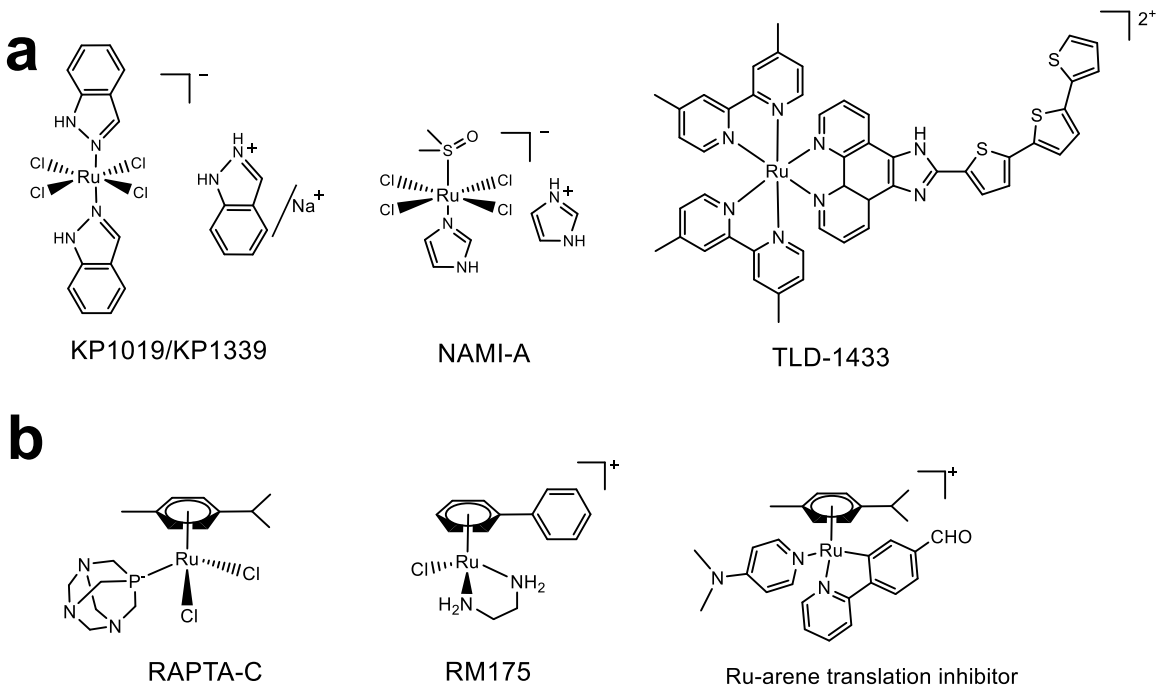

Figure 11. **Ruthenium complexes used in cancer therapy**. a) Ru-based metallodrugs that have entered into clinical trials. b) Examples of Ru(II)-arene complexes investigated as anticancer chemotherapeutic agents.

The archetypal Ru(II) polypyridil complex $[Ru(bpy)_3]^{2+}$ (**Ru1**, **Figure 12**), where bpy is bipyridine, was synthesized in 1936 by Burstall,[161] although the biological activity of such compounds was reported in 1952.[162] Two decades later, in 1972, Adamson and Demas reported the electron transfer mechanisms of **Ru1**, initiating the development of Ru(II) polypyridyl complexes as PSs.[163] By modifying the bipyridine ligands of **Ru1**, the photophysical photochemical and physicochemical properties, including solubility, luminescence, photostability, absorption and cellular localization, can be judiciously modulated. For instance, extension of the π system or the presence of electron-donating or electron-withdrawing functional groups directly impact on the spectroscopic properties of Ru(II) polypyridyl complexes.[164] According to density functional theory (DFT) calculations, introduction of a vinyl dimethylamino electron-donating substituent in **Ru2** reduced the HOMO-LUMO energy gap (**Ru3**), whereas changing the phenanthrene ligand to bathophenanthrene (**Ru4**) red-shifted the absorption tail to allow photoactivation at 595 nm.[164]

In general, octahedral Ru(II) polypyridyl complexes are +2 positively charged. However, changing the electronic states of these molecules greatly influence the bioactivity. For example, introduction of extra charges in $[Ru(bpy)_3]^{2+}$ ligands by the attachment of tertiary ammonium groups render a highly positively charged Ru(II) complex, **Ru5**, with improved water-solubility and cellular internalization, and thus higher photocytotoxicity.[165] In the same way, introduction of negative charges may significantly impact on lipophilicity and thus alter their cellular localization and the

biological activity.[166] Compound **Ru6**, with a net charge of +2 accumulated in mitochondria and induced necrosis after irradiation, whereas sulfonate-substituted **Ru7**, which is a -4 negatively-charged molecule, localized outside mitochondria and triggered apoptosis.[166]

The dipyridophenazine (ddpz) ligand is one of most used ligands in Ru(II) polypyridyl complexes.[167] Complex **Ru8** ($[Ru(bpy)_2dppz]^{2+}$ (**Figure 12**) was first reported in 1990 by the Barton group as a luminescent DNA intercalative probe.[168] This complex is not luminescent in aqueous solution but displays intense luminescence in the presence of double-helix DNA, with $>10^4$ enhancement factor.[168] The same "light-switch" mechanism was also observed for $[Ru(phen)_2dppz]^{2+}$ complex **Ru9**, where *phen* is phenanthroline.[169] The phenomenon attracted much attention in phototherapy since ROS generation proximate to DNA can be used for DNA photocleavage. Both **Ru8** and **Ru9** bearing dppz ligands display strong visible absorption arising mainly from a ^{3}MLCT excited state, which can be quenched by molecular oxygen.[170] When irradiated with light at 420 nm, the initially populated ^{1}MLCT state of such complexes relaxes to the lowest-energy ^{3}MLCT with almost unity efficiency. Changing the dppz ligand by benzodipyridophenazine (ddpn) results in the loss of ^{3}MLCT emission, yet prolonged excited-state lifetimes have been reported for $[Ru(bpy)_2(dppn)]^{2+}$ **Ru10**.[171] This long-lived $\pi - \pi^*$ triplet state corresponds to ^{3}IL excited states and is centred on the dppn ligand.[171] Therefore, the use of a π-expansive ddpn provides Ru(II) systems such as **Ru10**, which exhibit higher $^1O_2$ photogeneration than its ddpz-containing parent compound (**Ru8**) by different wavelength treatments.[172] Importantly, the change of functional groups on the ligand can also interfere with charge distribution, hydrophilicity and overall bioactivity of Ru(II) polypyridyl PSs. Gasser and colleagues designed a series of substituted $[Ru(bpy)_2dppz]^{2+}$ complexes bearing different functional groups on the ddpz ligand (**Ru11 – 16, Figure 12**).[173] These complexes efficiently intercalate in DNA and promote light-induced DNA cleavage, exhibiting PI values up to 150 in HeLa cells upon 420 nm irradiation.[173] Additionally, Gasser and Chao and collaborators also reported two $[Ru(bpy)_2dppz]^{2+}$ complexes with OH (**Ru17**) and OMe (**Ru18**) functional groups which targeted cell membrane and cell cytoplasm, respectively.[174] Moreover, **Ru17** and **Ru18** exhibited potent photocytotoxicity towards HeLa cancer cells upon one-photon and two-photon excitation, respectively.[174]

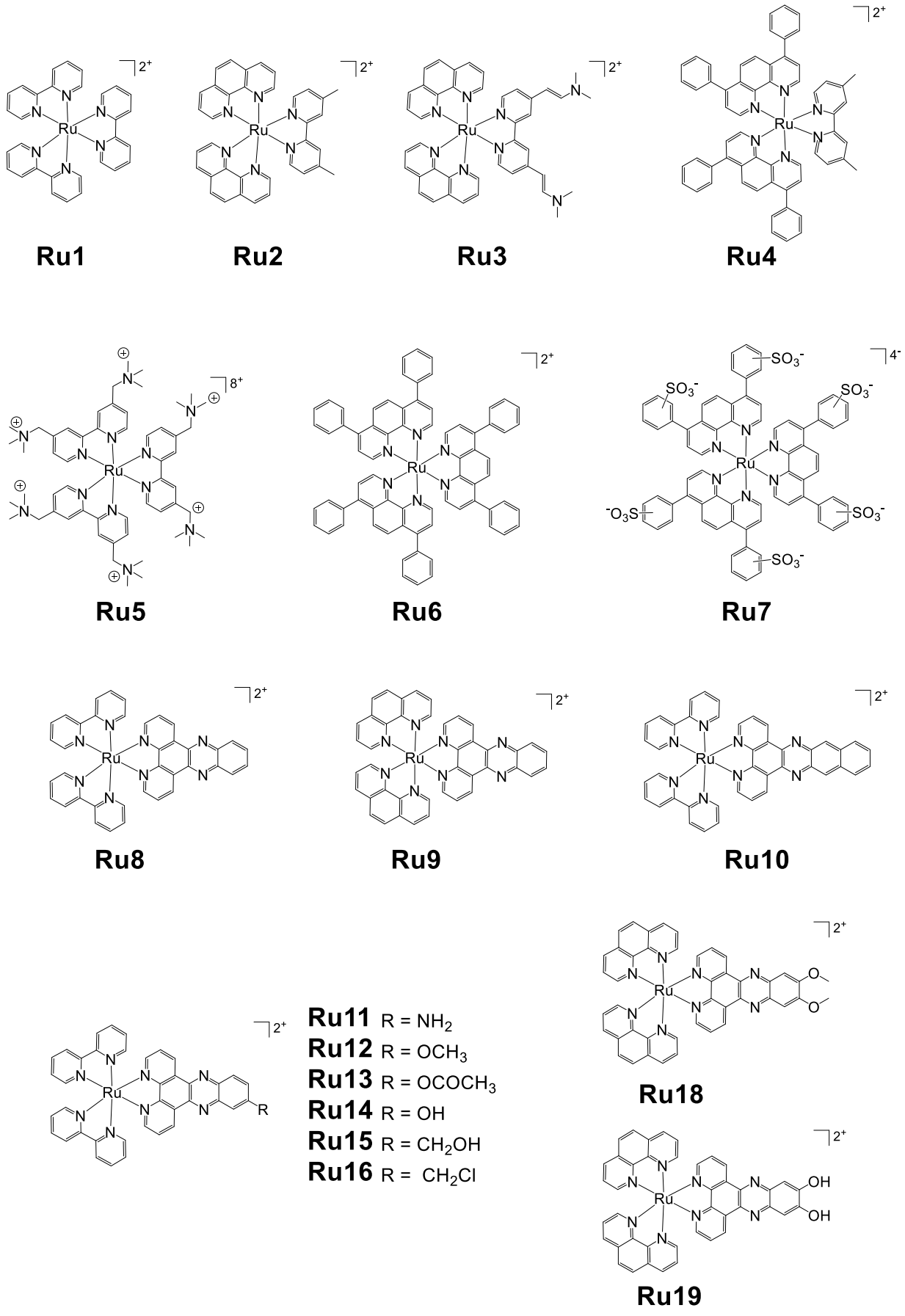

Figure 12. Ru(II) complexes studied as photosensitizers for cancer therapy.

In order to extend the absorption window of Ru(II) PSs into the red and NIR spectral region, different strategies have been explored. One of them was reported by Zhao *et al.*, who prepared a series of Ru(II) polypyridyl complexes (**Ru19** – **25**, **Figure 13**) where one bpy moiety was replaced by Schiff base (iminopyridine or iminoquinoline derivatives) followed by the incorporation of a dppn ligand.[175] These modifications red-sifthed the ^{1}MLCT absorption, improved population of long-lived ^{3}IL states and increased photocytotoxicity against A549 and HepG2 cells with PI values up to 763 upon 650 nm light irradiation.[175] The use of chromophoric ligands such as bis(1,8-naphthyridine)-based ligands has proven to be a useful strategy to shift absorption towards the NIR region.[176] Very recently, the McFarland group reported a series of complexes of the type [Ru(NNN)(NN)(L)], with 2,2'-(4-(*tert*-butyl)pyridine-2,6-diyl)bis(1,8-naphthyridine) as the ligand NNN, phen or dppn as NN ligands and a monodentate 4-picoline ligand. Exemplary, complexes **Ru26** and **Ru27** showed NIR absorbing properties and immunoprotective PDT activity against aggressive melanoma.[176]

Two-photon absorption PDT has been also employed to achieve NIR excitation, and several Ru(II) polypyridyl complexes have been designed.[177,178] There are excellent reviews that have summarized Ru(II)-based PSs for two-photon PDT.[159,179] Cyclometallation represents another strategy to red-shift the excitation of metal-based PSs, with $[Ru(NN)_2(C^N)]^+$ being the archetypal scaffold. McFarland *et al.* studied the effect of π–expansive ligands of cyclometalated Ru(II) complexes (**Ru28** – **Ru31, Figure 13**) and compared their photobiological properties with their N^N counterparts.[180] The use of cyclometalating ligands dramatically increased the dark cytotoxicity of **Ru28** – **Ru30**, whilst **Ru31** with dppn ligand was inactive in the dark and exhibited PI >1400 in SK-MEL-28 cells after visible light irradiation.[180]

In 2011, MacDonnell and Wolf *et al.* showed that Ru(II) complexes containing bithienyl groups appended to phen ligands fuelled long-lived excited states, revealing a potential for energy-harvesting applications such as photovoltaics and solar energy conversion.[181] Inspired by these compounds, McFarland and co-workers parallelly developed Ru(II) complexes containing α-oligothienyl groups for use in PDT.[182,183] Optimization studies with such complexes led to the discovery and development of TLD-1433 (**Figure 11a**), a Ru(II) polypyridyl complex with an imidazo[4,5-*f*][1,10]phenanthroline ligand appended to an α-terthienyl group and two 4,4′-dimethyl-2,2′-bpy coligands.[45] The π-expansive ligand has a ^{3}ILCT state energy that is lower than that of the ^{3}MLCT, which allows efficient $^1O_2$ production with long wavelengths despite very low molar extinction coefficients.[45] As previously mentioned, TLD-1433 became the first Ru(II)-based PS to advance to human clinical trials for bladder carcinoma, and its mode of action is related to dual Type I and Type II PDT action.[184] In the clinic, TLD-1433 is directly injected into the bladder wall and irradiated at 520 nm using an optical fibre with a diffuser.[45] TLD-1433 completed phase 1b study in 2018 and is currently in phase 2 (Clinicaltrials.gov identifiers NCT0353635, NCT03945162).

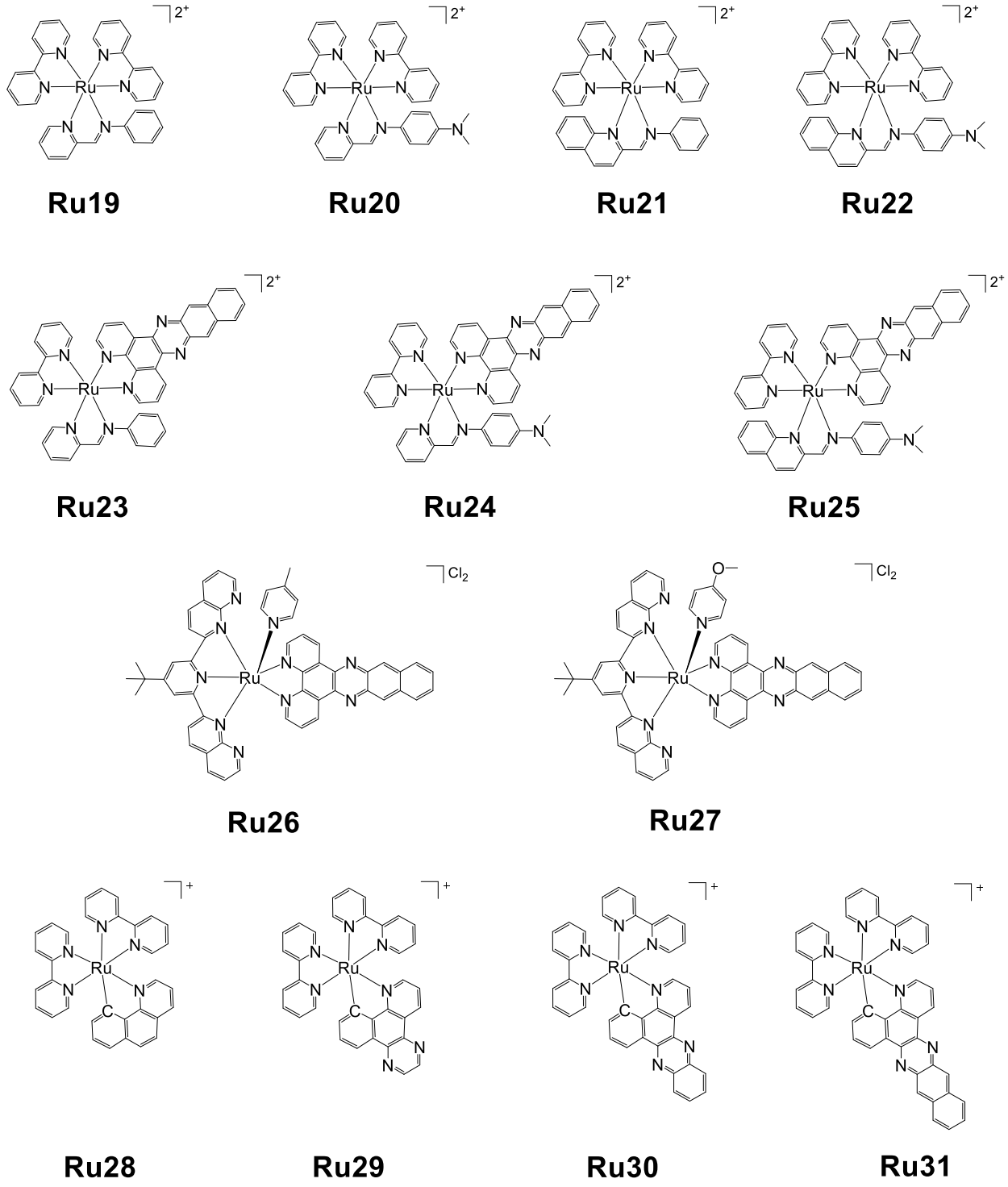

Figure 13. Ru(II) complexes with π-expansive ligands studied as photosensitizers for cancer therapy.

Another relevant aspect of Ru(II)-based PS design is the steric hindrance. Introduction of steric strains in the coordination sphere of Ru(II) systems *via* bulky ligands may lead to their dissociation upon light irradiation. The Glazer group showed that the unstrained complex **Ru32** acts as a

classical PDT agent whereas strained complexes **Ru33** and **Ru34** undergo ligand photoejection, thereby acting as PACT agents as well (**Figure 14**).[185] This instilling oxygen-independent strategy was later explored by McFarland *et al.*, which introduced two strain-inducing ligands into the TLD-1433 analogue.[186] The resultant complexes **Ru35** and **Ru36** behave as extremely potent PACT-PDT dual cytotoxins with outstanding PI values of >5800 under hypoxia in SK-MEL-28 cells (**Figure 14**).[186]

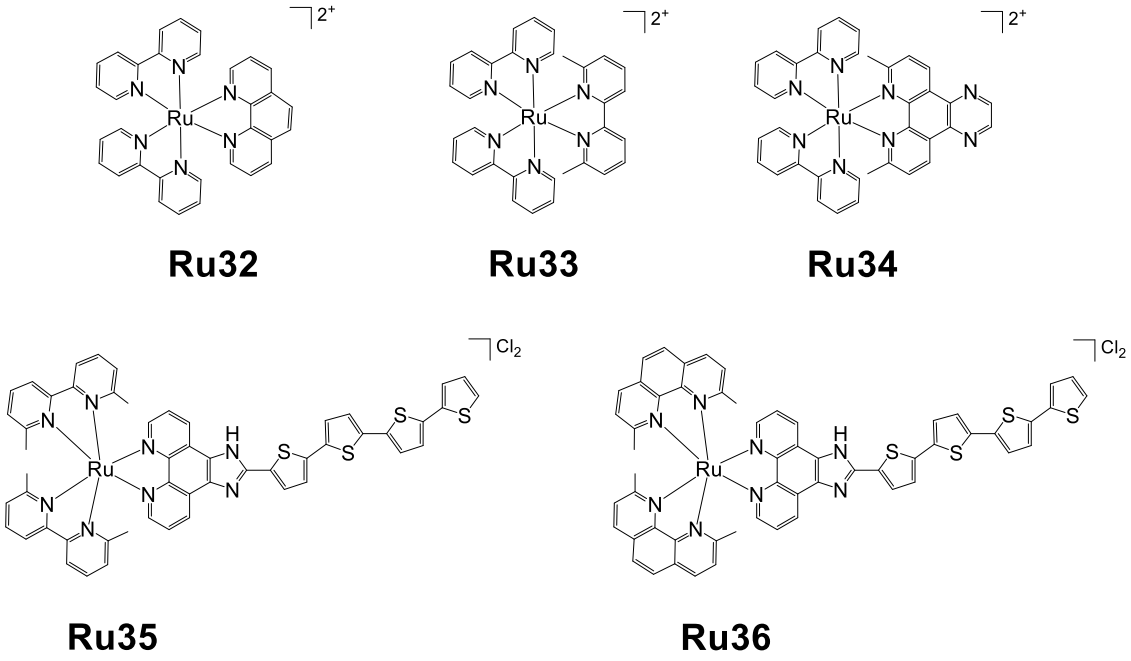

Figure 14. Strained Ru(II) complexes reported as dual PACT-PDT agents.

Beyond Ru(II) PSs, Ir(III) complexes have gained increasing interest for anticancer PDT. Similar to the development of Ru-based phototherapeutic drugs, octahedral cyclometalated Ir(III) complexes possess attractive particularities as PSs (**Figure 15c**) REF.[142] Particularly, octahedral Ir(III) complexes of the formula $[Ir(C^N)_2(N^N)]^+$ can harbour different cyclometalated and/or ancillary ligands in their structure. This enables wider modifications of photochemical and photophysical properties as well as efficient decoupling of ^{3}MLCT excited states, which are sensitive to oxygen, and can either be emissive or interconvert to other excited states to trigger PDT reactions depending on the coordinated ligands.[187] In summary, their long emission lifetimes, together with the ability to colour-tune the emissions upon ligand modification, and their high ROS generation efficiency have turned these Ir(III) complexes into promising, theragnostic PSs.[187]

Studies with Ir(III) complexes bearing monodentate heterocyclic ligands (**Ir1** – **Ir3**, **Figure 15**) have shown that 425 nm light irradiation triggers ligand dissociation, generating $^1O_2$ in the process.[188] Complexes with bidentate 2,2'-bisimidazole ligands, in contrast, provide more

photostability to Ir(III) complexes **(Ir4** – **Ir8**).[189] The alkyl substituents (methyl, ethyl, propyl, butyl) increase both lipophilicity and $^1O_2$ generation in that order (**Ir5<Ir6<Ir7<Ir8**), with PI values up to 150 towards HeLa cells upon 450 nm light irradiation.[189] One strategy to achieve ROS generation using Ir(III) PSs is to control their singlet and triplet energy levels by the choice of cyclometalated ligands. Kwon and co-workers designed four Ir(III) complexes with different energy levels and distinct $^1O_2$ generation efficiency by incorporating different phenylpyridine and phenylquinoline ligands to the metal centre (**Ir9-Ir12**).[190] Light irradiation of these complexes induced cancer cell death mediated by oxidative protein cross-linking and aggregation.[190]

Despite the absorption tail of Ir(III) complexes fall within the visible range, their main absorption bands lie in the UV region. This usually limits PDT performance and might produce undesired photodamage. One common strategy to extend the absorption of the complexes towards the red region of the spectrum includes the introduction of π-conjugated systems on C^N and/or N^N ligands. In this sense, Sun and McFarland prepared five heteroleptic Ir(III) complexes with π-expansive cyclometalating 2,3-diphenylbenzo[*g*]quinoxaline (dpbq) ligands and different diimine ligands with varying degrees of π-conjugation (N^N = bpy **Ir13**, phen **Ir14**, pqu **Ir15**, bqu **Ir16** and quqo **Ir17**).[191] This systematic variation of the ligands proved to dramatically influence the PDT activity of the complexes, with **Ir17** being the most phototoxic compound with red-light irradiation (PI = 273 against SK-MEL-28 melanoma cells).[191] Varying the degree of π-conjugation in Ir(III) complexes have also been explored by the groups of Ruiz and Brabec.[192] The authors found that the dark cytotoxicity of phosphorescent biscyclometalated Ir(III) complexes (**Ir18** – **22**, **Figure 15**) was reduced when increasing the largeness and degree of π-conjugation of the N^N ligand, while the activity was potentiated by visible light, suggesting a synergy of chemo- and phototherapy towards cancer cells.[192]

Another attractive feature of Ir(III) complexes is their ability to act *via* Type I pathway, particularly relevant to kill cancer cells under challenging hypoxia conditions. The groups of Ruiz and Brabec reported a series dipyridophenazine iridium(III) complexes (**Ir23** – **Ir31**) as $O_2^{\bullet-}$ generators following visible light irradiation.[193] Worthy of note, the location of the 4-(trifluoromethyl)benzyl derivatives **Ir29** and **Ir31**, which showed the highest PIs toward cancer cells, varied between mitochondria and endoplasmic reticulum.[193] This adds emphasis on how Ir(III) PSs can be accordingly modified to accumulate in specific cellular organelles to induce effective PDT activity.[193]

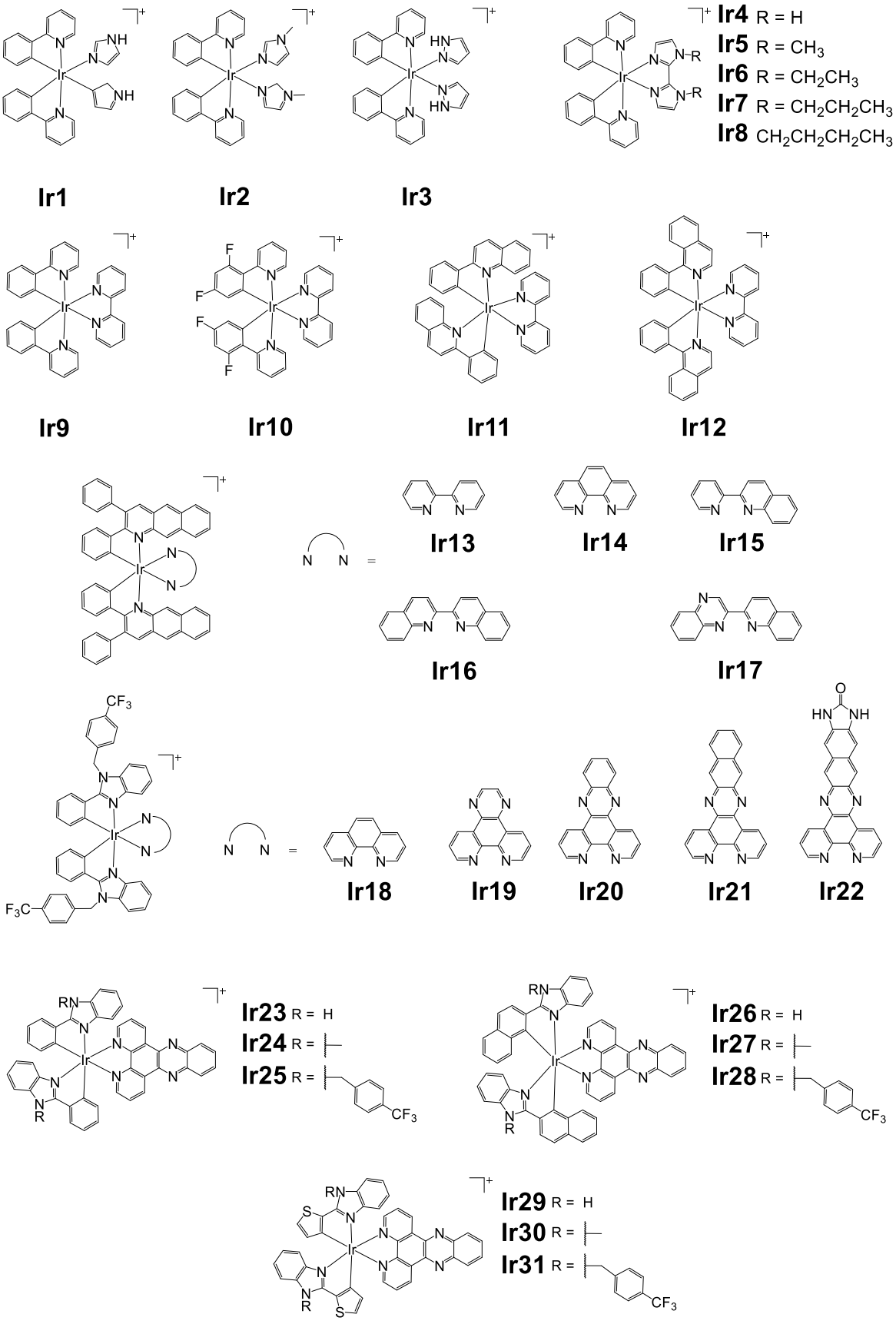

Figure 15. Ir(III) complexes studied as photosensitizers for cancer therapy.

Due to the clinical success of platinum drugs, phototherapeutic applications of cyclometalated Pt(II) complexes have become an intense subject of research. Pt(II) complexes possess the ability to photogenerate $^1O_2$ owing to the heavy atom effect.[194] For instance, the platinum compound **Pt1** (**Figure 16**) was reported as a nuclear-targeted luminescent Pt(II) PS with extremely high $^1O_2$ yields (0.95, 355 nm).[195] Neutral Pt(II) 2,6-dipyrido-4-methyl-benzenechloride compound (**Pt2**) has shown to effectively kill cisplatin-resistant cells upon 405 nm light irradiation following PDT reactions.[196] Brabec and co-workers developed a cisplatin analogue where an ammine group was substituted by a 1-methyl-7-azaindole moiety (**Pt3**) which can bind to DNA and generate $^1O_2$ upon 365 nm irradiation.[197] Attachment of catecholate or diketonate ligands bearing anthracene and pyrene moieties to Pt(II) centers (**Pt4** and **Pt5**) have also been considered for PDT, as they exert high PI values in cancer cells under broadband visible light irradiation.[198,199] NIR-active Pt(II) complexes have also been designed. In 2017, Pinho e Melo and collaborators prepared a platinum(II) 4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine-fused chlorin (**Pt6**), which emit simultaneous fluorescence and phosphorescence in the NIR region owing to the tetrapyrrolic structure of the chlorin.[200] Phototoxicity studies determined that **Pt6** achieved submicromolar IC_{50} values in melanoma A375 cells after irradiation.[200] In another approach, Mao *et al.* combined three Pt(II) centres with triphenylamine bridges, which resulted in a highly photoactive Pt(II) complex, namely **Pt7**, upon 450 nm light irradiation.[201] Pt(II)-based PDT has also been explored to combat both cancer and bacterial infections using cycloplatinated(II) 2-benzoazole-phenolato N^O complexes (**Pt8** – **Pt10, Figure 16**).[202] In this work, Ortega-Forte *et al.* found that low doses of blue light (450 nm) activated these Pt(II) complexes to effectively eliminate *in vitro* both multi-resistant bacteria and lung cancer cells *via* ROS production.[202] Many other PDT strategies using Pt(II) complexes have been explored,[179,194] with particular attention being also paid to photoactivatable Pt(IV) complexes for PACT.[203]

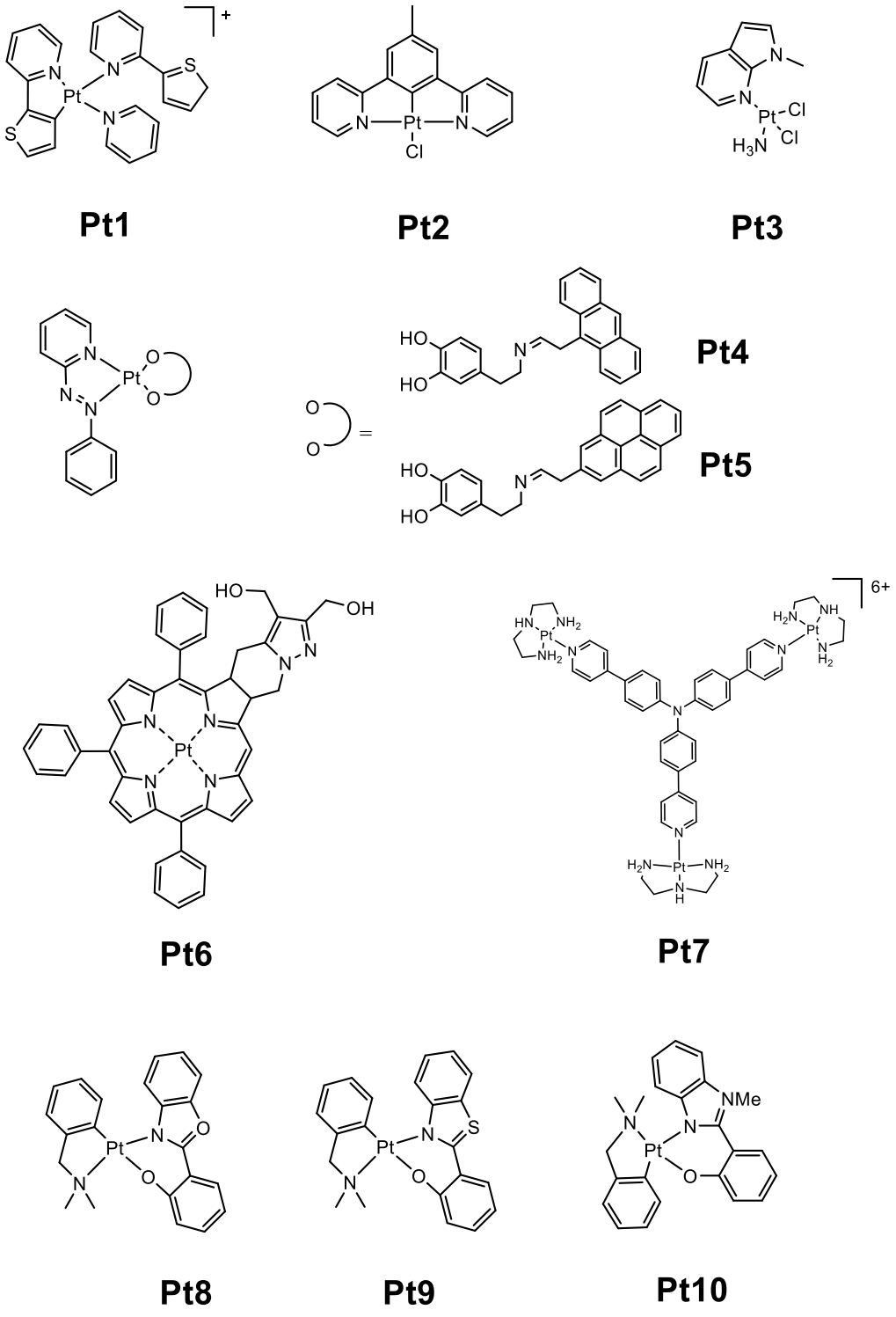

Figure 16. Photoactive Pt(II) complexes studied as anticancer PDT agents.

4.4. Metal-organic conjugates as photosensitizers

Incorporation of organic fluorophores into metal-based scaffolds to form conjugates or dyads represents a powerful strategy for bringing out the best properties of both organic and inorganic PSs. The merge of organic and inorganic molecules into new photo-responsive systems represents a frontier area of research in numerous applications involving photochemistry and PDT.[204] In particular, the strategic fusion of transition metal complexes with one or more organic fluorophores generates new molecular species with unique excited state energy or electron transfer processes between the subunits.[204] On the one hand, transition metal atoms improve stability, solubility and accessibility to various electronic states as it has been discussed in the previous section. On the other hand, organic fluorophores can shift absorption and emission spectra of PSs, improve specific targeting and provide an additional low-energy excited state which permits a second photoreactivity mechanism that can lead to a dual switching PDT behaviour.[184] Importantly, conjugation of metal-based scaffolds to organic-based moieties also serves to extend the excited state lifetime of the dyads. The lifetime extension of a metal complex when conjugated to an organic ligand was first described in the 1970s by Wrighton *et al.*, who demonstrated that tethering pyridine chromophores to a Re(I) complex resulted in long-lived MLCT states.[205] Later, Ford and Rodgers lengthened by ten-fold the ^{3}MLCT lifetime of some Ru(II) complexes by covalently attaching pyrene residues with a flexible link, opening an avenue to enhance the light-harvesting properties of conjugated PS.[206]

Pyrenes represent one of the most common organic moieties used in metal-based PS conjugates. Pyrenyl-appended Ru(II) complexes benefit from an excited state equilibration process between the pyrene ^{3}IL state and the Ru(II) ^{3}MLCT state.[207] Such Ru(II)-pyrene dyads (**C1** and **C2**) act as Type I/Type II PDT agents with nanomolar cytotoxicity in metastatic melanoma cells upon light irradiation both under normoxia and hypoxia (**Figure 17**).[208] A recent work on an Ir(III) complex bearing a pyrene moiety revealed a bidirectional energy transfer from the pyrene part to the metallic center and then back to the triplet excited state of the pyrene ligand (**C3** and **C4**).[209] This “ping-pong” type energy transfer dramatically increased the triplet excited-state lifetime of **C4** (microseconds) compared to parent complex without pyrene **C3** (nanoseconds), allowing a strong ROS production.[209] However, the installation of organic chromophores to a metal complex or the extension of π-conjugated systems such as pyrene may undermine the lipophilicity of the compound. Adding extra-charges to the metal conjugates could circumvent that issue.[210] In this sense, the Sun and McFarland groups prepared a series of pyrene-containing Ir(III) polypyridyl complexes with +3 charge (**C5** – **C7**).[210] Photobiological studies showed that pyrene-modified Ir(III) conjugates **C6** and **C7** reached PI values of 248 and >435 toward SK-MEL-28 cancer cells.[210]

A straightforward approach to prepare a stable, long wavelength-absorbing PS is to attach a cyanine-based fluorophore to the coordination centre of a transition metal complex. Yang *et al.* synthesized the Ir(III)-cyanine conjugate **C8**, which displays intense absorption in the NIR region (**Figure 17**).[211] **C8** was further encapsulated into nanoparticles, and the resultant nanoPDT agent showed effective generation of $^1O_2$ and tumour ablation *in vivo* after irradiation with 808 nm laser. Pt(II)-cyanine conjugates have been also studied as PSs. In 2018, the group of Hartman prepared **C9** by coordinating an heptamethine cyanine dye to a Pt diamino fragment.[212] Photobiological evaluation of **C9** determined that after accumulation in mitochondria, NIR light triggered both $^1O_2$ production and photorelease of the Pt(II) ligand owing to the photodetachable O^O bidentate donor, thus acting as a dual PDT-PACT agent.[212]

Rhodamine and BODIPY scaffolds have also been shown to red-shift the absorption when directly chelated to transition metal complexes or conjugated to the ligand. In 2019, Wong and co-workers designed a chelating ligand consisting of a rhodamine-tethered bpy, which could readily coordinate to transition metal centres such as Re(I), Ir(III), Rh(III) and Pt(II) (**C10** – **C13**, **Figure 17**).[213] The combination of these metal centres to the rhodamine unit improved rhodamine triplet excited state and $^1O_2$ formation. Conjugates **C10** – **C13** exerted potent mitochondria-targeted PDT effects *in vitro* and *in vivo* upon visible light irradiation.[213] BODIPY represents widely used scaffold to construct BODIPY-metal conjugates with advantages for cell imaging and PDT. For example, Xie *et al.* prepared a 4-methoxystyrylbenzene substituted BODIPY ligand to extend absorption to the NIR and then conjugated it to a Pt centre (**Figure 17**).[214] This Pt(II)-BODIPY conjugate, **C14**, allowed *in vivo* NIR imaging and efficient tumour ablation with 620 nm light irradiation.[214] Similarly, the He group developed a Pt(II)-BODIPY using a BODIPY-modified fluorophore containing bis(pyridin-2-ylmethyl)amine (DPA) as chelating ligand (**C15**).[215] Conjugate **C15** was sequestered in lysosomes and light irradiation triggered lysosomal escape and promoted entry into the nuclei, contributing to photoinduced DNA damage.[215] In the same way, Ru(II) and Ir(III) complexes have been functionalized with BODIPY to leverage both the light-harvesting properties from BODIPY and the efficient ISC promoted from transition metals.[216] In this regard, Zhao *et al.* have designed BODIPY-functionalized biscyclometalated Ir(III) PSs (**C16** and **C17**) *via* acetylide bonds with high molar absorption at longer wavelengths and long-lived ^{3}IL triplet states.[217] In another work, the same group prepared Ru(II)-BODIPY analogues and found that the triplet excited state lifetimes were much larger for **C18** and **C19** and provided higher $^1O_2$ quantum yields than their iridium counterparts.[218] However, tethering a fluorophore such as BODIPY on the metal complex without any π-conjugation or linker between the organic part and the metal coordination centre might be less efficient in terms of improving visible light absorption of the conjugate.[218]

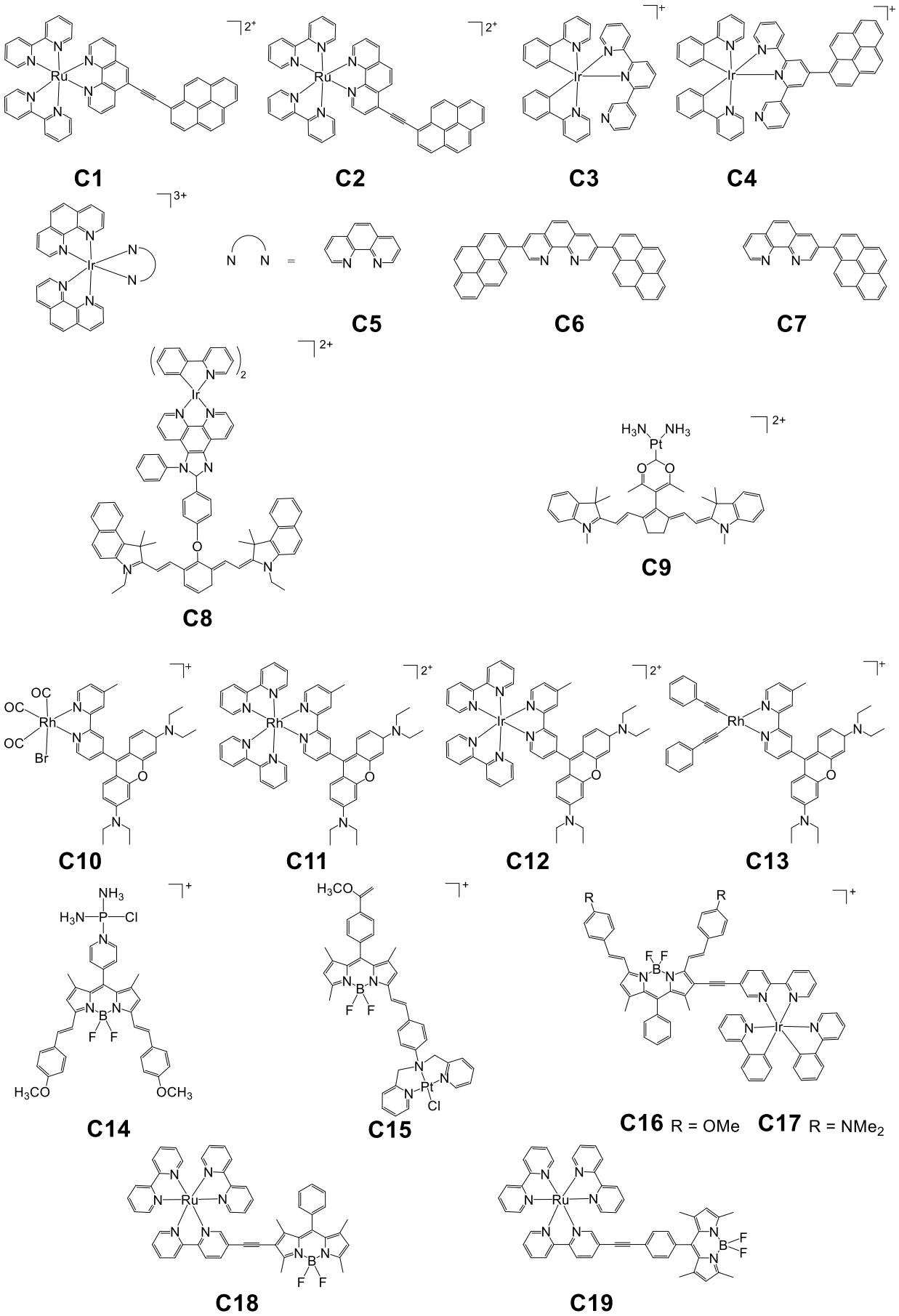

Figure 17. Metal-organic conjugates studied as photosensitizers for cancer therapy.

Researchers have also witnessed inroads in coumarin-based conjugates to improve their photobiological potential.[219] As mentioned in **section 4.2**., coumarin fluorophores are amenable to structural modifications which facilitate the introduction of substituents to the coumarin skeleton that enable bathochromic shifts in the absorption and emission spectra, variations of the push-pull effect or increases in intramolecular charge transfers.[137] All these modification strategies can harness novel PDT agents when connected to metal complexes. For instance, Zhao *et al.* found that tethering coumarin molecules to Ir(III) and Ru(II) complexes (**C20** – **C23**) *via* an imidazole-phenanthrene ligand resulted in visible-light harvesting systems with strong absorption and long-lived triplet excited states that show potential utility in PDT (**Figure 18**).[220,221] The extra phenyl linker between the coumarin unit and the imidazole (**C21** and **C23**) shifted the absorption of the complexes towards blue region of the electromagnetic spectrum.[220,221]

Besides, coumarins are known for their drug-likeness properties and for their organelle-targeting abilities, which can be used to drive metal-based PSs to specific subcellular locations. As such, Huang *et al.* developed a family of Ir(III)-coumarin conjugates (**C24** – **C26**) that preferential accumulate into mitochondria (**Figure 18**).[222] Furthermore, these conjugates behaved as potent photocatalysts, with the ability to photoinduce NAD(P)H oxidation and generate ROS, thereby causing important redox imbalances that lead to cell death after blue and green light irradiation.[222] The complex **C25** also showed photochemotherapeutic activity against orthotopically subcutaneous transplanted tumors in mice models.[222]

Metal-coumarin conjugates have also drawn attention for their ability to produce Type I ROS, an interesting point to alleviate the hypoxia limitation of PDT. A coumarin-modified cyclometalated Ru(II) complex (**C27**) with potent PDT activity has been smartly developed for such indication (**Figure 18**).[223] The light-harvesting ability of the coumarin unit, together with their electron-donating ability, modulated the energy level of the attached cyclometalated Ru(II) complex. Compared to the parent Ru(II) complex, the conjugated version showed lower oxidation potential, and thus the feasibility of PDT reactions *via* Type I pathway. Hydroxyl radicals are believed to be the main species generated after irradiation, which would explain the potent photocytotoxicity under low oxygen tension (5% O_2).[223] The therapeutic efficacy of **C27** was also demonstrated *in vivo* in a xenografted tumour model showing minimal side effects.[223] Marchán and Ruiz *et al.* also developed a Type I-PDT agent based on the conjugation of an Ir(III) complex to a far-red emitting COUPY coumarin (**C28, Figure 18**).[224] The complex displayed high cellular accumulation into the cytoplasm of HeLa cells and showed high photocytotoxicity upon green and blue light even under hypoxic conditions (2% O_2). In this case, superoxide anions $O_2^{\bullet-}$ are produced after visible light irradiation, which were responsible for cell death since **C28** showed no photocytotoxicity towards HeLa cells pre-treated with the $O_2^{\bullet-}$ scavenger tiron. Consistent with this was the oxygen quantum yields of the

complex, which were below 0.01 in phosphate buffered-saline.[224] In addition, the mechanism of action of **C28** was also studied in-depth in prostatic cancer stem cells, finding that its photoactivation caused elevation of calcium flux, induced apoptosis and stimulated autophagy.[225] In a posterior work, the groups of Marchán and Ruiz explored structural modifications of the Ir(III)-COUPY conjugate **C28** either through variations of the spacer between the coumarin moiety and the transition metal complex using flexible or rigid linkers (**C29** and **C30,** respectively) or through modifications of the COUPY scaffold (**C31** and **C32**).[226] All these Ir(III)-coumarin conjugates exhibited potent photocytotoxicity towards cancer cells, especially against A2780cis cisplatin-resistant ovarian cancer cells both in 2D and 3D cell culture models, and efficiently generated ROS upon 520 nm light irradiation. Structure-activity relationship studies revealed that incorporation of flexible or rigid spacers improved the PI values of the conjugates **C29** and **C30** under hypoxia compared to the parent conjugate **C28**.[226]

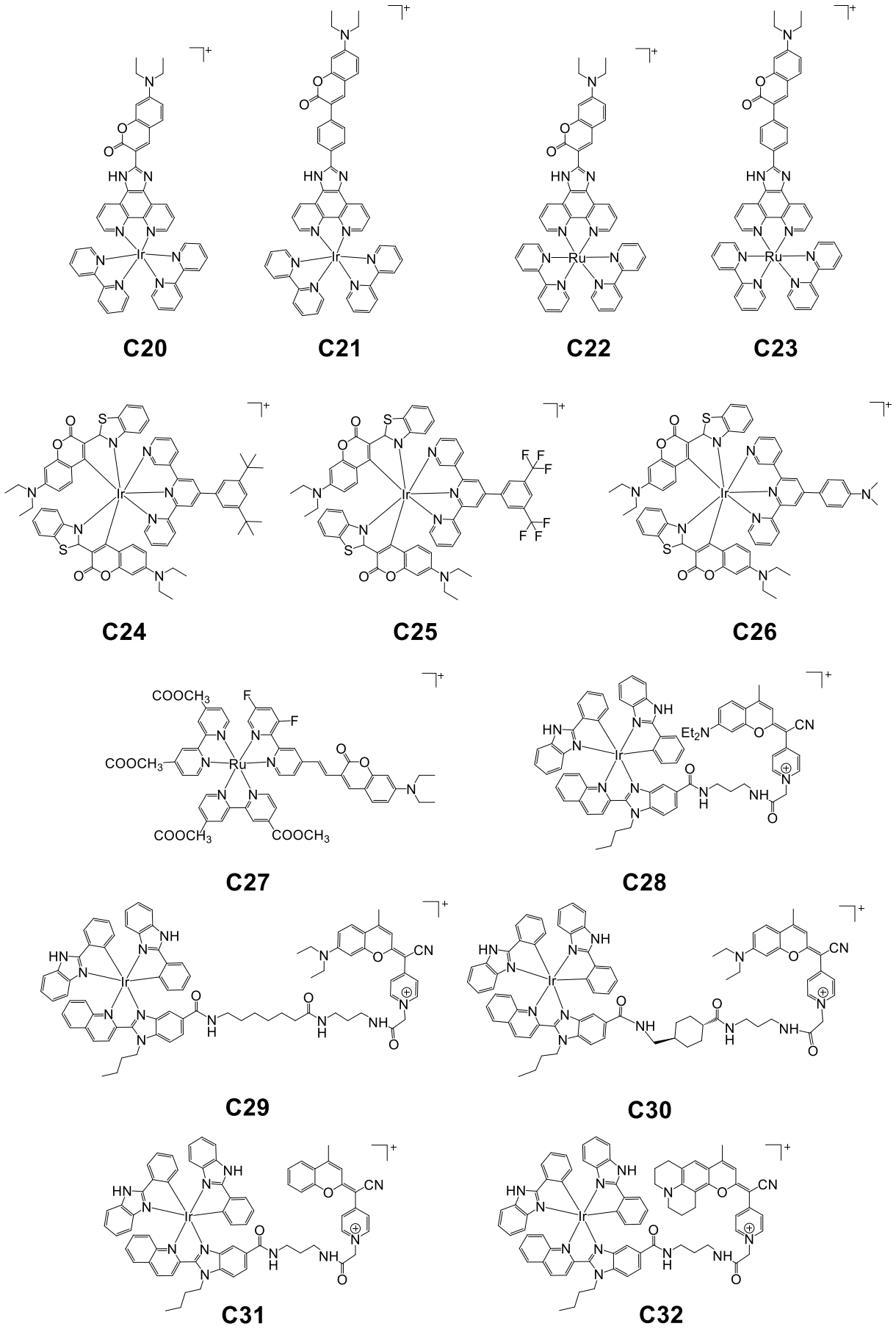

Figure 18. Metal-coumarin conjugates studied as photosensitizers for cancer therapy.

5. Aims

This thesis aims to explore the development of novel photodynamic anticancer agents from chemical synthesis to biological evaluation. The general aims of the present thesis thus embrace the rational design and synthesis of the compounds, the determination of their photophysical and photochemical properties, the biological characterization against cancer cells and the identification of the best hit candidates for anticancer PDT through structure-activity relationship (SAR) studies. The work is structured in 3 chapters. **Chapter I** is focused on assessing the potential of coumarin-based COUPY fluorophores as novel PSs in anticancer PDT. **Chapter II** comprises the development of a new family of cyclometalated Ru(II) polypyridyl-based PSs. In **Chapter III**, the development of a NIR-activatable PS is described. This PS was designed through the conjugation of the best performing cyclometalated Ru(II) complex from **Chapter II** to a near-infrared emitting COUPY coumarin (**Chapter III**).

The specific objectives for each chapter are:

Chapter I:

- Determination of the cellular uptake and localization of different COUPY fluorophores *via* confocal microscopy.
- Determination of cyto- and photocytotoxicity of a novel library of COUPY fluorophores against cancer cells *in vitro* under normoxia and hypoxia.
- Establishment of structure-activity relationships and identification of hit candidates for PDT.
- Evaluation of ROS photogeneration *in vitro* using specific scavengers.
- Elucidation of the mode of action of hit candidates in the dark and under irradiation using flow cytometry and fluorescence microscopy-based assays.

Chapter II:

- Synthesis and characterization of octahedral cyclometalated Ru(II) complexes with the formula $[Ru(N^N)_2(C^N)]^+$ with 2-aryl-benzimidazole ligands where aryl group is phenyl, biphenyl or naphthyl.
- Synthesis of modified N^N ligands with π-expanded conjugated system.
- Photophysical and photochemical characterization of parameters relevant to the PDT activity of the complexes.

- Determination of lipophilicity, generation of ROS in cell-free systems and photocatalytic oxidation of NADH.
- Determination of cyto- and photocytotoxicity against cancer cells *in vitro* under normoxia and hypoxia.
- Establishment of structure-activity relationships and exploration of the π-expansive N^N ligand effects on the complexes to identify the best hit candidates for PDT.
- Evaluation of ROS photogeneration *in vitro* using specific scavengers.
- Elucidation of the mode of action in the dark and under irradiation using flow cytometry and fluorescence microscopy-based assays.

Chapter III:

- Synthesis and characterization of a new PDT agent based on the conjugation between a NIR-emitting COUPY fluorophore and a highly potent cyclometalated Ru(II) complex *via* an amide-bond linker.
- Photophysical and photochemical characterization of the novel Ru(II)-COUPY conjugate relevant to its PDT activity.
- Determination of lipophilicity, cellular uptake and localization of the compounds in cancer cells.
- Determination of cyto- and photocytotoxicity of the novel Ru(II)-coumarin conjugate against a panel of cancer cells *in vitro* as well as of the Ru(II) complex and coumarin precursors.
- Photocytotoxicity screening using different light treatments regimes (from red light to NIR light treatment).
- Elucidation of the mode of action in the dark and under light irradiation.
- Evaluation of ROS photogeneration *in vitro* upon NIR light irradiation.

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RESULTS AND DISCUSSION

CHAPTER I. Development of COUPY coumarins as novel photodynamic therapy anticancer agents

PUBLICATION:

Enrique Ortega-Forte[1], Anna Rovira[2], Albert Gandioso[2], Joaquín Bonelli[2], Manel Bosch[3], José Ruiz[1,*], Vicente Marchán[2,*]. COUPY Coumarins as Novel Mitochondria-Targeted Photodynamic Therapy Anticancer Agents. *Journal of Medicinal Chemistry*. **64**, 17209–17220 (2021). https://doi.org/10.1021/acs.jmedchem.1c01254

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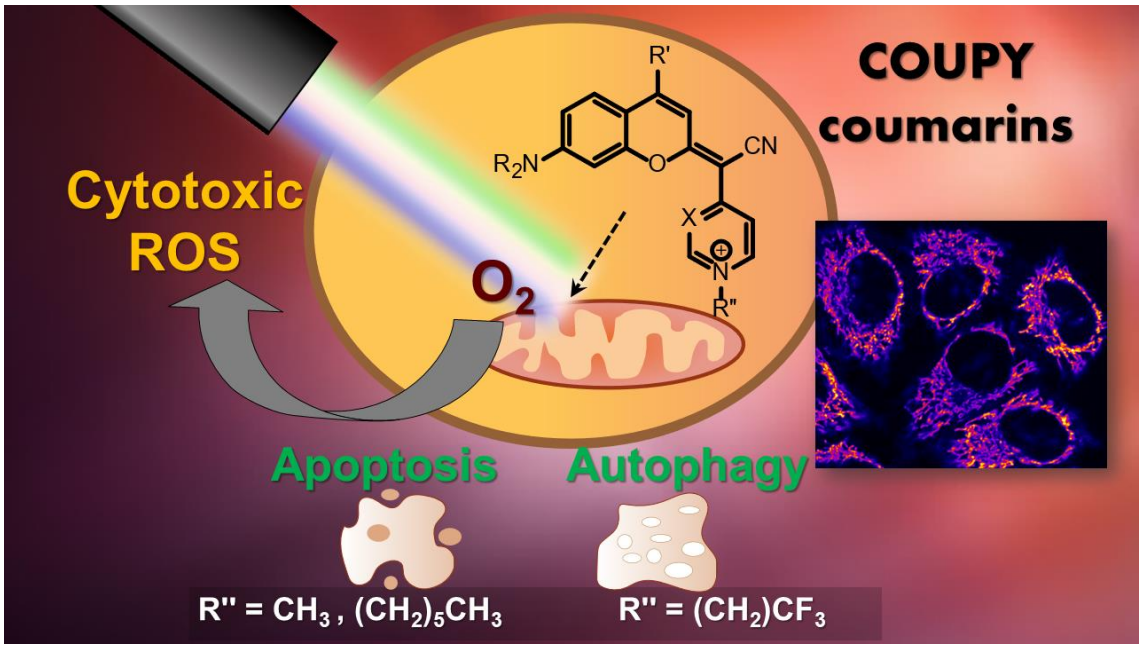
Abstract

Photodynamic therapy (PDT) for cancer treatment has drawn increased attention over the last decades. Herein, we introduce a novel family of low-molecular-weight coumarins as potential PDT anticancer tools. Through a systematic study with a library of 15 compounds, we have established a detailed structure–activity relationship rationale, which allowed the selection of three lead compounds exhibiting effective in vitro anticancer activities upon visible-light irradiation in both normoxia and hypoxia (phototherapeutic indexes up to 71) and minimal toxicity toward normal cells. Acting as excellent theragnostic agents targeting mitochondria, the mechanism of action of the photosensitizers has been investigated in detail in HeLa cells. The generation of cytotoxic reactive oxygen species, which has been found to be a major contributor of the coumarins' phototoxicity, and the induction of apoptosis and/or autophagy have been identified as the cell death modes triggered after irradiation with low doses of visible light.



CHAPTER II. Development of novel cyclometalated Ru(II) polypyridyl-based photodynamic therapy anticancer agents

PUBLICATION:

Francisco J. Ballester,[a] Enrique Ortega,[a] Delia Bautista,[b] M. Dolores Santana[*a] and José Ruiz[*a] Ru(II) photosensitizers competent for hypoxic cancers via green light activation. *Chemical Communications*. **56**, 10301–10304 (2020). https://doi.org/10.1039/D3SC01844J

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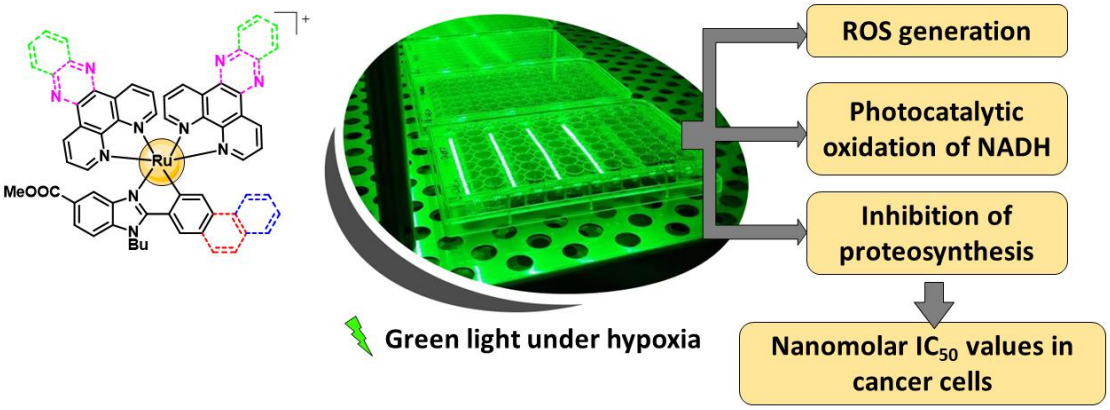
Abstract

A family of five heteroleptic complexes [Ru(C^N)(N^N)$_2$][PF_6] (HC^N = methyl 1-butyl-2-arylbenzimidazolecarboxylate; N^N = polypyridine) has been synthesized to act as biologically-compatible green light photosensitizers with phototherapeutic indexes up to higher than 700 under hypoxia (2% O_2) in HeLa cancer cells under short time of irradiation.



CHAPTER III. Development of a Ru(II)-coumarin conjugate as a novel near-infrared light-activatable photodynamic therapy anticancer agent

PUBLICATION:

Enrique Ortega-Forte[1], Anna Rovira[2], Marta López-Corrales,[2] Alba Hernández-García,[1] Francisco José Ballester,[1] Eduardo Izquierdo-García,[3] Mireia Jordà-Redondo,[4] Manel Bosch,[5] Santi Nonell,[4] María Dolores Santana[1], José Ruiz,[1,*] Vicente Marchán[2,*]and Gilles Gasser[3,*]. A near-infrared light-activatable Ru(II)-coumarin photosensitizer active under hypoxic conditions. *Chemical Science*. (2023). https://doi.org/10.1039/d3sc01844j

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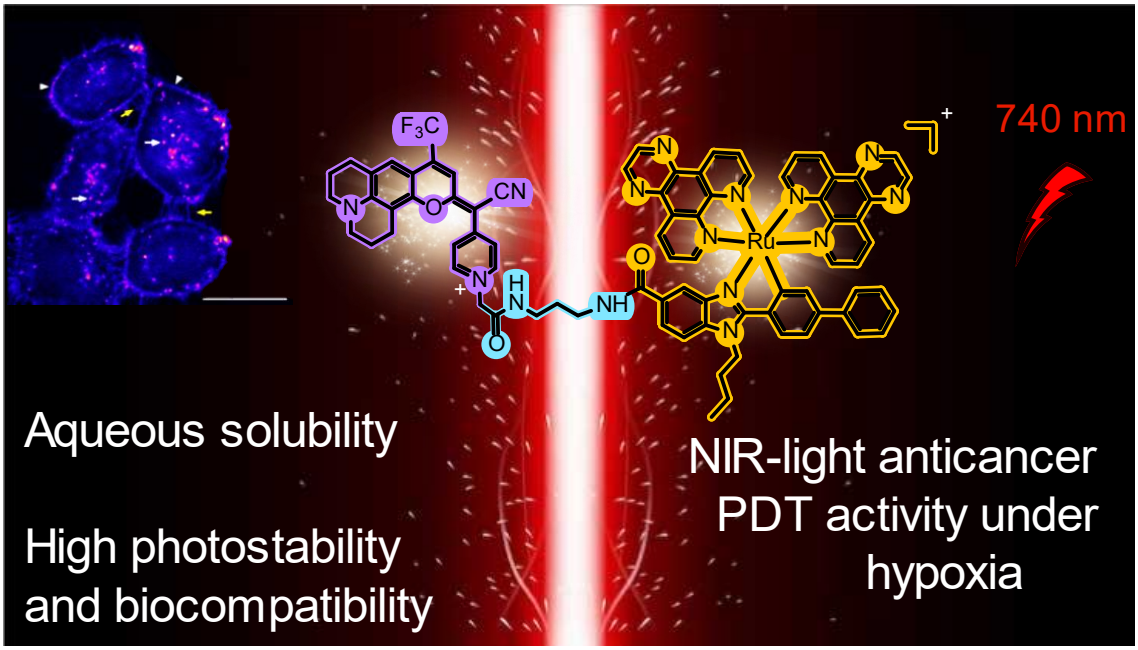
Abstract

Photodynamic therapy (PDT) represents a promising approach for cancer treatment. However, the oxygen dependency of PDT to generate reactive oxygen species (ROS) hampers its therapeutic efficacy, especially against hypoxic solid tumors. In addition, some photosensitizers (PSs) have dark toxicity and are only activatable with short wavelengths such as blue or UV light, which suffer from poor tissue penetration. Herein, we developed a novel hypoxia-active PS with operability in the near infrared (NIR) region based on the conjugation of a cyclometalated Ru(II) polypyridyl complex of the type [Ru(C^N)(N^N)$_2$] to a NIR emitting COUPY dye. The novel Ru(II) coumarin conjugate exhibits water solubility, dark stability in biological media and high photostability along with advantageous luminescent properties that facilitate both bioimaging and phototherapy. Spectroscopic and photobiological studies revealed that this conjugate efficiently generates singlet oxygen and superoxide radical anions, thereby achieving high photoactivity toward cancer cells upon highly penetrating 740 nm light irradiation even under hypoxic environments (2% O2). The induction of ROS-mediated cancer cell death upon low energy wavelength irradiation along with the low dark toxicity exerted by this Ru(II) coumarin conjugate could circumvent tissue penetration issues while alleviating the hypoxia limitation of PDT. As such, this strategy could pave the way to the development of novel NIR and hypoxia active Ru(II)-based theragnostic PSs fuelled by the conjugation of tunable, low molecular weight COUPY fluorophores.



CONCLUSIONS

Three novel types of photodynamic anticancer agents corresponding to the main existing classes of photosensitizers (*i.e.*, organic fluorophores, transition metal complexes and metal-organic conjugates) have been developed.

On the one hand, a library of fifteen coumarin derivatives was synthesized and characterized and the applicability of such compounds as PSs was investigated in detail (**Chapter I**). Confocal microscopy studies confirmed excellent cell membrane permeability and cellular uptake in HeLa cancer cells. Determination of *in vitro* cytotoxicity and photocytotoxicity in cancer cells both under normoxia and hypoxia revealed important SARs and thus enabled the identification of three hit candidates with phototherapeutic indexes higher than 71 under broadband visible light (400 – 700 nm) irradiation. ROS photogeneration studies indicated that specific species (*i.e.*, peroxyl radicals in normoxia and singlet oxygen in hypoxia) were predominantly raised in cancer cells after irradiation. Further biological evaluations in cancer cells revealed that, although the three hit candidates accumulated in mitochondria and photoinduced apoptosis, the N-alkylated hexyl group-containing COUPY coumarin significantly depleted the mitochondria membrane potential and caused autophagy-dependent cell death.

On the other hand, a library of five cyclometalated Ru(II) polypyridyl complexes of the formula $[Ru(C^N)(N^N)_2]^+$ was prepared and in-depth studies on their photochemistry and photophysics were performed (**Chapter II**). ICP-MS studies confirmed high intracellular accumulation after exposure of the compounds to HeLa cancer cells. Comprehensive photobiological evaluations were carried out to assess the ability of these compounds to act as PDT agents after green light (520 nm) irradiation. *In vitro* cytotoxic and photocytotoxic activities in cancer cells confirmed the suitability of the complexes as PSs since phototherapeutic indexes up to 750 were obtained under hypoxia. SAR studies revealed that the Ru(II) polypyridyl complex containing a methyl 1-butyl-2-arylbenzimidazolecarboxylate ligand and dpq or phen as N^N ligands showed the best cyto- and photocytotoxic profiles. Singlet oxygen and hydrogen peroxide were the main ROS species found in cancer cells upon irradiation under normoxia, whereas superoxide anions were raised under hypoxic conditions. In addition, the mode of action of the hit candidates involved inhibition of protein translation.

By taking advantage of the photodynamic anticancer properties of the Ru(II) polypyridyl complexes developed in **Chapter II**, and of the rich, easy-modifiable physicochemical properties of the COUPY fluorophores, their conjugation was exploited for developing a novel Ru(II)-coumarin conjugate

(**Chapter III**). Based on the antecedents, the Ru(II) complex containing dpq N^N ligands and the methyl 1-butyl-2-arylbenzimidazolecarboxylate ligand was selected for its prominent activity under hypoxia, and was then conjugated to a julolidine-fused trifluoromethyl-containing COUPY coumarin derivative with NIR-emitting properties. The novel Ru(II)-COUPY conjugate exhibited water-solubility, dark stability, photostability and luminescent properties. Confocal microscopy and ICP-MS studies confirmed that the conjugate accumulate in the cytoskeleton and in the cell membrane to a lesser extent. A phototoxic chromatic screening using red (620 nm), deepred (645 nm), far-red (670 nm) and near-infrared (740 nm and 770 nm) light conditions against HT-29 cells confirmed the photoactivity retention properties of the conjugated upon highly-penetrating near-infrared (740 nm) light, at which the clinical drug Protoporphyrin IX was inactive. Spectroscopic and photochemical characterizations revealed that type I superoxide anions and type II singlet oxygen were formed upon irradiation even under hypoxia, which was further confirmed by *in vitro* cellular studies. Overall, the ROS-mediated cancer cell death upon low-energy doses of light irradiation at near-infrared wavelengths could improve tissue penetration and alleviate the hypoxia limitation of current PDT agents.

RESUMEN EN CASTELLANO

La terapia fotodinámica (PDT, por sus siglas en inglés) es una modalidad terapéutica no invasiva para el tratamiento del cáncer. En esta terapia, una sustancia farmacológica denominada fotosensibilizador se activa por la acción de la luz y convierte el oxígeno molecular en especies reactivas de oxígeno (ROS) citotóxicas. La acumulación de estas ROS en el interior celular induce la muerte de las células cancerígenas. La principal ventaja de la PDT reside en que el uso de la luz permite controlar la acción farmacológica en el tiempo y en el espacio. Esto permite dirigir selectivamente el tratamiento hacia el tejido tumoral, pues únicamente donde se aplica la luz se producirán las reacciones fotoquímicas que median la toxicidad celular. De esta manera se evita el daño a las células sanas y al tejido circundante. Después de la exposición de luz a una específica longitud de onda, el fotosensibilizador pasa de su estado singlete fundamental a su estado singlete excitado y a continuación pasa al estado triplete excitado mediante un cruce entre sistemas, desde donde tiene lugar la reacción fotoquímica. Dependiendo del mecanismo, las reacciones fotodinámicas pueden clasificarse en dos, Tipo I y Tipo II. Mientras que el Tipo II es un mecanismo de transferencia de energía directa al oxígeno molecular para producir oxígeno singlete ($^1O_2$) como especie reactiva citotóxica, las reacciones Tipo I consisten en procesos de transferencia electrónica entre el fotosensibilizador y biomoléculas adyacentes en el medio biológico, generando radiacles como el anión superóxido ($O_2^{\bullet -}$) el radical hidroxilo ($OH^{\bullet}$) o el hidroperóxido ($HO_2^{\bullet}$). Estos ROS interaccionan con distintas biomoléculas de la célula tumoral como el ADN, las proteínas o los lípidos, alterando sus funciones normales y provocando su muerte celular. Además, las reacciones fotodinámicas que se desencadenan durante la PDT tienen un efecto fototóxico sobre la vasculatura del tejido tumoral, causando daños en el epitelio y destruyendo el microambiente tumoral. Asimismo, el daño oxidativo mediado por la PDT activa vías de señalización moleculares que incrementan la expresión de proteínas y factores proinflamatorios, las cuales inducen la activación de la respuesta inmunitaria contra las células tumorales. Sin embargo, la eficacia clínica de la PDT está limitada por tres factores principalmente. El primero es la dependencia del oxígeno de esta terapia. Como la concentración de oxígeno presente en los tumores es normalmente más baja comparada con el tejido sano, las reacciones fotodinámicas no pueden ocurrir y, en consecuencia, el tratamiento falla. El segundo factor es la escasa penetración de la luz en los tejidos biológicos, de manera que el tratamiento de tumores profundos y/o poco accesibles para la aplicación de luz limita considerablemente la eficacia terapéutica de esta técnica. Mientras que la irradiación mediante longitudes de onda corta como aquellas en la región azul espectro electromagnético pueden penetrar cientos de micrómetros en el tejido biológico, la penetración óptica de la luz con longitudes de onda en la región del rojo o cercana al infrarrojo (NIR) es capaz de penetrar varios centímetros. Por tanto, el empleo de una fuente de luz con longitudes de onda

altas presenta mayores ventajas para conseguir garantizar la eficacia de la PDT contra tumores sólidos o profundos. El último factor comprende los efectos adversos que pueden derivar de esta terapia. Aunque la mayoría de estos efectos no deseados pueden mantenerse bajo control a nivel clínico, como el dolor o la fotosensibilidad cutánea, la causa principal de su aparición se debe normalmente a la toxicidad de los fotosensibilizadores en la oscuridad, a su acumulación inespecífica en tejidos no tumorales o a una aplicación del tratamiento PDT a dosis inadecuadas. Por lo tanto, es necesaria la investigación en el descubrimiento y el desarrollo de nuevas clases de fotosensibilizadores que superen las limitaciones de aquellos agentes para PDT que se encuentran en uso clínico.

Esta tesis pretende explorar el desarrollo de diferentes familias de compuestos químicos como nuevos agentes fotodinámicos anticancerígenos con el fin de abordar y superar las principales limitaciones de los fotosensibilizadores actuales. La Introducción consiste en una revisión sobre la PDT como modalidad de tratamiento del cáncer. Además, se realiza una revisión intensiva sobre los distintos tipos de fotosensibilizadores que se están investigando hasta la fecha. En primer lugar, se presentan los fotosensibilizadores de uso clínico en PDT anticancerígena. Un fotosensibilizador debe cumplir una serie de requisitos para poder tener un fin clínico, aunque principalmente debe presentar dos: 1) una elevadad fototoxicidad y 2) una baja toxicidad en oscuridad. El índice fototóxico, definido como la relación entre la toxicidad en la oscuridad y la toxicidad al ser irradiado con luz, nos permite conocer la eficacia de un fotosensibilizador dado hacia las células cancerígenas. Hasta ahora los fotosenbilizadores aprobados para uso clínico para el tratamiento con PDT son fundamentalmente porfirinas, ftalocianinas y clorinas. Pero su eficacia clínica está limitada por la corta vida de su estado excitado y el bajo rendimiento cuántico para producir ROS. Estas limitaciones, unidas a sus efectos secundarios, han promovido un importante campos e investigación dedicado a la búsqueda de nuevos fotosensibilizadores. En este contexto, los compuestos orgánicos con propiedades fluorescentes (fluoróforos orgánicos) están siendo investigados como fotosensibilizadores. En la Introducción se hace especial énfasis en los derivados de cumarina, que presentan propiedades fotoquímicas y fotofísicas modulables idóneas para PDT. Por otro lado, los compuestos de metales de transición, que contienen en su esfera de coordinación un metal de transición como el rutenio, el iridio y el platino, entre otros, ofrecen características particulares para el desarrollo de agentes para PDT. Propiedades fotofísicas como la longitud de onda de absorción o los rendimientos cuánticos y propiedades fotoquímicas como la elevada estabilidad de complejos metálicos pueden ajustarse de acuerdo con los requisitos de esta terapia. Además, los compuestos basados en metales de transición permiten seleccionar y modificar los ligandos ciclometalados y/o los ligandos auxiliares para optimizar la eficacia de los fotosensibilizadores resultantes. Por último, los conjugados metálicos que combinan compuestos basados en metales de transición con fluoróforos orgánicos representan una clase emergente de

fotosensibilizadores. La introducción incluye una revisión de los avances en el desarrollo de conjugados metálicos como fotosensibilizadores, cuya principal ventaja es la potenciación de las propiedades de ambos tipos de compuestos, orgánicas e inorgánicas, en una sola molécula.

La sección de Resultados y Discusión se divide en 3 capítulos de acuerdo con cada tipo de fotosensibilizador. En el Capítulo I se describe una familia de fluoróforos orgánicos basados en derivados de cumarina. El diseño de estos compuestos consiste en la incorporación de derivados de piridina o pirimidina al esqueleto de la cumarina clásica, creando una familia de compuestos de cumarina-piridina/pirimidina denominados COUPY, por sus siglas en inglés. En total se analizó la aplicabilidad de 15 compuestos COUPY como agentes PDT bajo irradiación de luz visible en modelos celulares de cáncer *in vitro* que incluían células tumorales de ovario, células tumorales de cuello de útero y células sanas de riñón. Se llevó a cabo un estudio sistemático de los compuestos para establecer relaciones estructura-actividad que permitieron la ulterior identificación de tres candidatos con alta actividad fototerapéutica en células cancerígenas en condiciones de hipoxia, con índices de fotoactivación de 71. Además, se evaluó la bioactividad de los compuestos en líneas celulares no tumorales en condiciones de oscuridad, obteniendo una baja toxicidad. Los ensayos por microscopía confocal determinaron que estos compuestos acumulan principalmente en la mitocondria y el nucleolo de las células cancerígenas. Posteriormente se dilucidaron los mecanismos fotobiológicos de acción de estos compuestos tras una irradiación con una fuente de luz LED de banda ancha en el espectro electromagnético visible mediante ensayos celulares guiados por fluorescencia y por citometría de flujo. Estos ensayos confirmaron que los compuestos inducen muerte celular programada por apoptosis tras la irradiación. Curiosamente, la presencia del grupo funcional N-alquilo de la piridina es un modulador clave para inducir autofagia como mecanismo de acción secundario.

El Capítulo II comprende el desarrollo de complejos ciclometalados de rutenio(II) de tipo polipiridilo con fórmula $[Ru(C^N)(N^N)_2]^+$. Los compuestos sintetizados fueron caracterizados químicamente y se determinaron sus propiedades químicas y fotoquímicas. El estudio explora el efecto del sistema π-conjugado en los ligandos N^N y el efecto de la modificación el ligando ciclometalado. Asimismo, se determinaron la estabilidad y fotoestabilidad de los complejos en medio biológico, confirmando su estabilidad en dichas condiciones. En cuanto a la fotoquímica de los compuestos, se determinando los valores de rendimiento cuántico y su capacidad para foto-oxidar catalíticamente la coenzima NADH. A continuación, se ensayó actividad antiproliferativa *in vitro* de los compuestos contra células tumorales de ovario con y sin resistencia a cisplatino y en células tumorales de cuello de útero tanto en condiciones de oscuridad como bajo irradiación con luz verde con lámparas LED centradas a una longitud de onda de 530 nanómetros. En condiciones de oscuridad, los compuestos inducen la inhibición de la síntesis de proteínas y provocan muerte celular programada mediada por apoptosis. Cuando se someten a irradiación con luz verde, la

fotoactivación de los compuestos genera ROS y produce efectos citotóxicos en las líneas celulares de cáncer utilizadas. Las relaciones estructura-actividad permitieron identificar dos compuestos cuyos índices de fotoactivación en condiciones de oxígeno normales son del orden de 76. A su vez, se determinó la actividad fototerapéutica en condiciones de hipoxia, con índices de fotoactivación mayores de 769. Las principales especies reactivas involucradas en el mecanismo de acción son el oxígeno singlete (mecanismo Tipo I) y peróxido de hidrógeno (mecanismo Tipo II) en condiciones de oxígeno normales, mientras que en condiciones de hipoxia se encontraron elevados niveles de anión superóxido. La habilidad de producir ambos tipos de mecanismos PDT, sumado a las diferencias de especies ROS involucradas dependiendo de la cantidad de oxígeno en el ambiente, podrían explicar la elevada fotoactividad de este tipo de compuestos de rutenio en hipoxia.

Por último, en el Capítulo III de este manuscrito se aborda el desarrollo de un nuevo fotosensibilizador basado en la conjugación de un complejo ciclometalado de Ru(II) polipiridilo con una cumarina COUPY con emisión en la región del espectro del infrarrojo cercano (NIR). Los posteriores estudios espectroscópicos revelaron que este conjugado metal-cumarina es capaz de producir simultáneamente reacciones PDT de Tipo I y Tipo II. Se realizó un estudio fotobiológico en diferentes líneas cancerígenas, así como un screening de la fotoactividad del conjugado y de los precursores con diferentes regímenes de irradiación que abarcaban desde rojo (620 nanómetros) hasta el infrarrojo cercano (770 nanómetros) pasando por tratamientos de luz con radiaciones en el rojo profundo y el rojo lejano. Los resultados revelaron una elevada fotoactividad del conjugado frente a las células cancerígenas irradiadas con luz NIR (740 nanómetros) en condiciones de hipoxia. En cambio, el fotosensibilizador de uso clínico, la protoporfirina IX, demostró no ser activa en las mismas condiciones de ensayo. Dado que la radiación de luz a longitudes de onda en la zona del NIR es más penetrante que la luz visible con longitudes de onda menores, este fotosensibilizador podría servir para sortear tanto la limitación tanto de la penetración óptica en tejidos como la escasa actividad terapéutica de la PDT en condiciones de hipoxia.

Este trabajo de investigación sienta las bases para el desarrollo de nuevos compuestos basados en cumarina y rutenio con elevada actividad fototerapéutica, y allana así el camino para la obtención de nuevos fotosensibilizadores activos en hipoxia y que operan en el NIR. En conclusión, esta Tesis ha contribuido a la investigación y desarrollo de nuevas clases de compuestos orgánicos e inorgánicos como herramientas anticancerígenas para la PDT.