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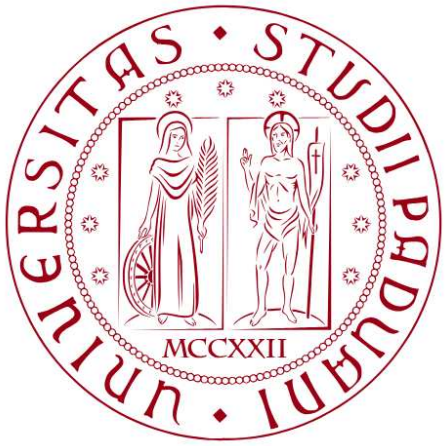
Corso di Laurea in Fisica



ELABORATO DI LAUREA

La proteina KillerRed: proprietà biofisiche e utilizzo nella terapia sperimentale del cancro

The KillerRed protein: biophysical properties and use in experimental therapy for cancer

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Contents

Abstract

Photodynamic therapy (PDT) is a non-invasive treatment used in the therapy for both cancerous and non-neoplastic diseases, such as dermatoses and infections. Its key elements are light, photosensitizers (PSs) and molecular oxygen. Most chemical PSs used nowadays in PDT produce reactive oxygen species (ROS) through type II photoreaction, and tend to have side effects, such as long term sensitivity to light.

In this thesis, we focus on the KillerRed protein (KR), a genetically encoded red fluorescent photosensitizer, characterized by a dimeric structure, derived from the green fluorescent protein (GFP). We delve into the structure and properties of KR, its targeting capabilities and its delivery strategies via different means (from viral vectors to nanoparticles). We also examine its application in cancer treatment, and the potential advantages of its use in PDT, alone as well as in combination with other therapies.

Introduzione

La terapia fotodinamica (PDT) è un approccio non invasivo per il trattamento di diverse malattie, tra cui infezioni e tumori. Gli elementi fondamentali della terapia fotodinamica sono i fotosensibilizzatori (PSs), la luce e l'ossigeno. Queste tre componenti interagiscono tra loro tramite due diverse reazioni che portano alla produzione di specie reattive dell'ossigeno (ROS), le quali danneggiano la cellula ospite in cui vengono prodotti, portandola alla morte. I PSs chimici comunemente utilizzati producono ROS soprattutto tramite la reazione fotodinamica di tipo II, che dipende fortemente dalla concentrazione locale di ossigeno. Ci sono svantaggi intrinseci all'uso dei PSs chimici, come ad esempio la possibilità di accumularsi anche in tessuti sani e la fotosensibilità a lungo termine. Per trovare soluzione a questi problemi, si è ricorso all'ingegneria genetica per produrre fotosensibilizzatori geneticamente codificati, che possono venire espressi in maniera selettiva in determinati compartimenti cellulari ed essere trasportati in maniera specifica a siti di lesione tumorale. In questo lavoro di tesi analizzeremo i processi della PDT e focalizzeremo l'attenzione sulla proteina fluorescente rossa KillerRed (KR), un fotosensibilizzatore geneticamente codificato, di struttura dimerica, derivato dalla proteina fluorescente verde (GFP). KR è un PS che, attivato dalla luce di eccitazione, produce ROS principalmente tramite reazioni di tipo I, caratteristica che, non richiedendo la stessa concentrazione di ossigeno necessaria per la fotoreazione di tipo II, sembra rendere possibile l'uso della PDT anche nell'ambiente tumorale, tipicamente ipossico. Descriveremo la struttura e le proprietà di KR, le sue applicazioni, le strategie di targeting genetico a livello sub-cellulare e le strategie di trasporto a livello sistemico, evidenziando i vantaggi delle nanotecnologie in questo campo applicativo. Discuteremo altresì i vantaggi e gli svantaggi dell'applicazione di KR sia da sola che in combinazione con altre terapie, evidenziando potenziali emergenti e sfide future.

1 Introduction

Photodynamic therapy (PDT) is a non-invasive approach for the treatment of various diseases, including infections and tumors. The fundamental elements of photodynamic therapy are photosensitizers (PSs), light and oxygen. These three components interact with each other through two different reactions that lead to the production of reactive oxygen species (ROS), which damage the host cell in which they are produced, leading to its death. Commonly used chemical PSs produce ROS primarily through type II photodynamic reactions, which are strongly dependent on the local concentration of oxygen. There are intrinsic disadvantages to the use of chemical PSs, such as the possibility of accumulating in healthy tissues and long-term photosensitivity. To address these issues, genetic engineering has been used to produce genetically encoded photosensitizers that can be selectively expressed in specific cellular compartments and specifically transported to tumor lesion sites. In this thesis work, we will analyze the processes of PDT and focus on the red fluorescent protein KillerRed (KR), a genetically encoded photosensitizer with a dimeric structure, derived from the green fluorescent protein (GFP). KR is a PS that, when activated by excitation light, primarily produces ROS through type I reactions, a characteristic that, not requiring the same concentration of oxygen necessary for type II photoreactions, appears to make PDT possible even in the typically hypoxic tumor environment. We will describe the structure and properties of KR, its applications, genetic targeting strategies at the sub-cellular level, and systemic transport strategies, highlighting the advantages of nanotechnologies in this field. We will also discuss the advantages and disadvantages of applying KR both alone and in combination with other therapies, highlighting emerging potentials and future challenges.

2 Basic principles of PDT

The PSs used in PDT consist of a chromophore domain and an effector domain [29]. The chromophore domain is the site where the PS absorbs light. The effector domain is responsible for the specific localization of the PS within the target cells, and for the generation of ROS in response to light activation. This is the beginning for a series of different processes that lead to cell death in the treated area. Cells may die in different ways, through inflammatory response, apoptosis, autophagy, necrosis and degeneration [13].

2.1 PDT reactions

The photoactivation process

Photon absorption promotes the PS from its fundamental singlet energy state

S_0 to a singlet excited state S_1. The active state of the photosensitizer is a triplet excited state, T_1[1], which is obtained from the singlet S_1 by spin conversion through intersystem crossing (ISC, which is a non-radiative relaxation process) with a specific quantum yield[2], Φ_{ISC}, [8], [27].
This modification to the excited state is the main reason of the longer lifetime of the triplet state compared to the singlet state (it is a forbidden transition since it causes the change in spin multiplicity, therefore it is less likely to occur, and this explains why its lifetime is longer, up to a factor of 10^4). This longer lifetime increases the interaction probability of the excited compound with other molecules in the environment [27].

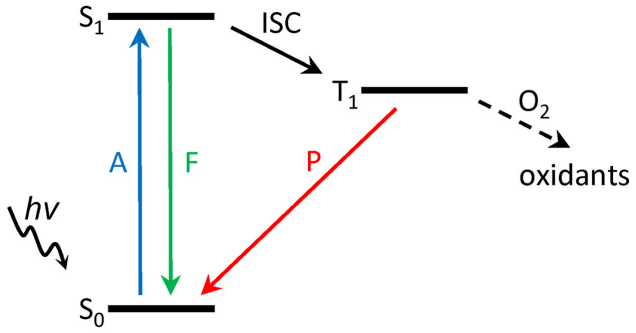

Figure 2.1: Jablonski Diagram [27] showing the different processes that can occur when a PS molecule in its ground state absorbs a photon. The excited PS can relax in a radiative way by emitting light (fluorescence, emission in $\simeq 10^{-9}$ s). Alternatively, it can relax in a non radiative way, for example via intersystem crossing (ISC), transiting in the excited triplet state (it could also relax by emitting heat). From the triplet state, the molecule can emit light via phosphorescence (emission in $\simeq 10^{-3}$ s) or, if the environment is rich in molecular oxygen, it can transfer its energy to the surrounding oxygen molecules generating oxidants. In both radiative processes the emitted photons have longer wavelength than the absorbed photons due to intervening energy loss.

[1]By *singlet state* we mean a state in which the electrons have opposite spins, while by *excited triplet state* we mean an excited state in which two electrons (one with higher energy than the other) have the same spin.

[2]The quantum yield is an index defined for radiation-induced processes as the ratio between the number of events of interest that occur and the number of photons absorbed. For example, we can define the Φ_{ISC} "the number of events that lead to intersystem crossing per photons absorbed"; Φ_f, the fluorescent quantum yield, as the number of times the excited singlet state S_1 relaxes to its original ground state by emitting a quantum of fluorescence: $\Phi_f = \frac{k_f}{k_f + \Sigma k_{nr}}$, where k_{nr} is the rate constant for all non-radiative relaxation processes, as internal conversion and intersystem crossing, and k_f is the rate constant for radiative relaxation (fluorescence).

Type I and II reactions

The formation of the T_1 state is the first step towards the generation of ROS. We now take a closer look at the processes, named Type I and Type II photoreactions, that take place when the PS resides in this long-lived excited state within an environment rich in oxygen.

Type I reactions occur when the triplet state photosensitizer receives an electron from the environment (or when a hydrogen is transferred) and becomes a radical species. If in presence of molecular oxygen, the reduced photosensitizer reacts with it and generates a superoxide anion radical. This $O_2^{\cdot -}$ superoxide can become hydrogen peroxide H_2O_2 by reacting with hydrogen from its environment, and is next converted into the hydroxyl radical ($HO^{\cdot}$). This chain of reactions leads to the oxidative stress that can kill the cell [8]. This mechanism can only take place if the triplet state of the photosensitizer and the target are close to one another [27].

Type II reactions occur when the triplet excited state transfers all its excess energy directly to molecular oxygen (whose ground state is a triplet state), forming singlet oxygen ($^1O_2$) which has "extremely strong oxidizing properties" [8].

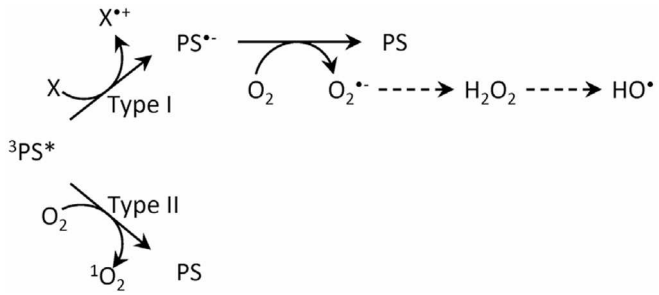

Figure 2.2: Type I and type II reactions. In type I reactions, the photosensitizer interacts with the substrate X, gaining a negative charge from it. The radical form of the photosensitizer interacts with oxygen, transferring its negative charge, and returns to its ground energy state. The negatively charged oxygen then undergoes a series of reactions that terminate with the generation of an hydroxyl radical. In type II reactions, energy is directly transferred from the photosensitizer to molecular oxygen, generating singlet oxygen. Image from [27].

The reason why the PSs and oxygen molecules interact directly with one another in type II photoreactions depends on spin factors. Most organic compounds are characterized by a singlet ground state that is excited into a triplet state. In contrast, oxygen molecules have a triplet ground state, and are excited into a singlet state. This is the main reason why PSs molecules do not damage the cell and its structures directly, but rather interact with oxygen species [8].

There are two possibilities for the excited PS to transfer energy directly to the oxygen molecule (type II reactions): by Förster resonance energy transfer (FRET) or by Dexter electron transfer [18]. In FRET, the excited state of the donor (PS^*)

generates an oscillating dipole that induces another oscillating dipole in the acceptor (O_2 in ground state) by resonance. The oscillatory movement is transmitted by a virtual photon, and the efficacy of this energy transfer is proportional to d^{-6}, where d is the distance between molecules [20]. The second mechanism, known as Dexter electron transfer, requires the two molecules to be in physical contact with one another (closer than about 10 Å; this guarantees that there is an appreciable overlap between the wave functions of the two electrons that will be exchanged). The donor (which is the PS^*) than transfers its excited electron to the acceptor (O_2), and gains from it an electron in the ground state. The energy exchange thus takes place through physical exchange of electrons [20]. For both mechanisms, an overlap between the emission energetic spectrum of the excited PS and the absorption energetic spectrum of the oxygen molecule is required [18], [20].

For most PSs, excitation triggers both Type I and Type II reactions. However, there could be a preference towards one of the two, depending on the intrinsic properties of the photosensitizer, surrounding environment, oxygen concentration and pH. It is assumed that the most influential process in the efficacy of PDT is type II reaction, but if there is scarcity of oxygen, type I reaction may prevail. It is still not clear how each oxidant species contributes to cell death [27]. It is thought that different pathways that lead to cell death are dependent on the location of the PS inside the cell. Thus, damage to mitochondria can lead to apoptosis, while the loss of integrity of the cell and its membrane can lead to necrosis. Damage to endoplasmic reticulum or lysosomes may promote autophagy [13].

2.2 Light administration in PDT

Illumination and light characteristics heavily influence the efficacy of PDT, depending on the properties of each PS. Dosimetry of light is particularly complicated, since light has to pass through different media, with different optical properties. Reflection, refraction, absorption and scattering are all effects to be taken into account. Absorption, in particular, is what limits most the application of PDT to solid tumors, since light can penetrate only for a small distance inside tumors (less than a centimeter in most cases) [17]. Light administration techniques are constantly researched (use of LED lights and lasers, use of fiber optical sources, even bioluminescence, and so on [29]). What seems crucial in experimenting with light (both *in vitro* and *in vivo*) is the light administration protocol. Exposure to high intensity laser in a brief interval of time will decrease PDT efficacy, since the procedure will consume all the oxygen available, without carrying out the desired cancer-killing effects. For this reason, pulsed light delivery is considered the strategy of choice and may limit thermal injuries to healthy

tissues [7]. Pulse frequency, light intensity and repetition rate are all important parameters for PDT [17]. Other approaches have been also tried, such as, for example, "metronomic PDT" [4], (see next paragraph).
Other aspects must be duly considered too. Thus, in any experiment, it is necessary to exclude that light itself promotes cell death (in the absence of PS).
Also, light may modify the PS itself, changing its properties, in two processes called "photomodification" and "photodegradation". Photobleaching is a particular form of photodegradation. ROS produced from the activation of the PS damage the PS itself, specifically its chromophore, hampering its fluorescence emission.

2.2.1 Metronomic PDT and fractionated light

Metronomic PDT (mPDT) is a therapeutical approach in which cancerous tissues are treated for a long period of time (e.g. days) with a low intensity light (typically power densities lower than 10 mW/cm^2). In a seminal study, Davies and Wilson treated CNS-1 luciferase transfected (Luc$^+$) astrocytoma in rats (for its histopathological similarities with human gliomas) with 5-Aminoluvelinic acid (5-ALA) and light delivery via tetherless, light-weight, LED-based fiber coupled optical sources [4]. They monitored the development of small tumors using bioluminescence imaging (BLI)[3] followed by histology [4] to determine whether mPDT could be utilized in post-surgical therapies to remove the remaining cancer cells, prolonging life expectancy for glioma patients.
The first part of the study involved rats 7-days post tumor implantation. BLI showed an average tumor intensity of $(1.0 \pm 0.1)\times 10^5$ counts, corresponding to a tumor diameter in the range [0.5-0.72] mm . PDT with the same light dose of an acute treatment (up to 230 J), but prolonged over a 24 hour period eliminated small tumors, without affecting healthy brain tissue. Histological staining for apoptosis (TUNEL) revealed few apoptotic cells inside the tumor and none in healthy tissues.
In the second part of the study, tumor bearing rats were treated 10 days after tumor implantation. The tumors showed a mean BLI signal intensity of $(6 \pm 1)\times 10^5$ counts, with a diameter of [0.82-1.2] mm (determined by post-mortem histology). This time, the treatment consisted of 24-48h of illumination with powers of 2, 1 and 0.5 mW respectively. The results showed tumor relapse in the treatment groups was delayed over a 2-fold longer time interval compared to the control groups, with a tumor mean diameter at day 26 of 0.6 ± 0.2 mm for the 24h treatment group and 0.46 ± 0.09 mm for the 48h treatment group. A 4 days (96h)-mPDT treatment was the most successful procedure, as no BLI sign

[3]The images were collected and analyzed with the commercial software *Living Image: Xenogen*

of tumor above that of the background was detected up to 26 days after tumor implantation, with a reduction of terminal BLI intensity of $97 \pm 8\%$ compared with the control group [4].
Despite these positive outcomes, the mPDT protocol is scarcely researched due to its intrinsic technical difficulties, first of all the delivery of light in a continuous manner in living patients. Its results are very promising though; further research on survival rates and tumor re-growth is needed.

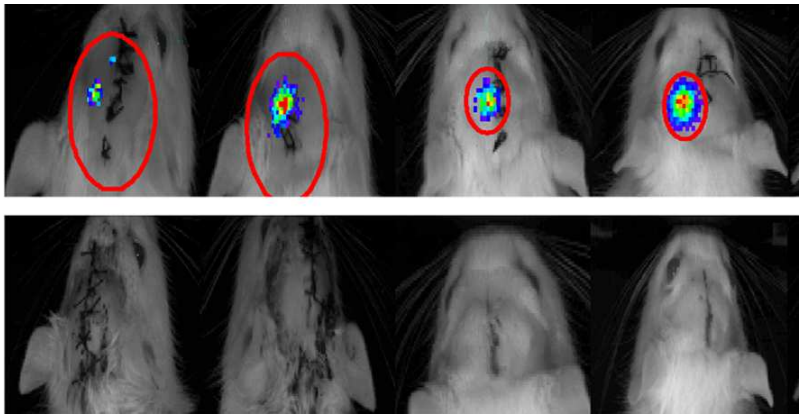

Figure 2.3: Bioluminescence imaging data from animals treated with 24h mPDT. Top panel: animals bearing tumor 7 days after implantation. Bottom panel: animals bearing tumor at day 8 after implantation, after 24h of mPDT (fluence rate 2 mW, total dose of 178 J). BLI images were taken with an exposure of 5 minutes 10 minutes after the administration of luciferin. Image modified from [4].

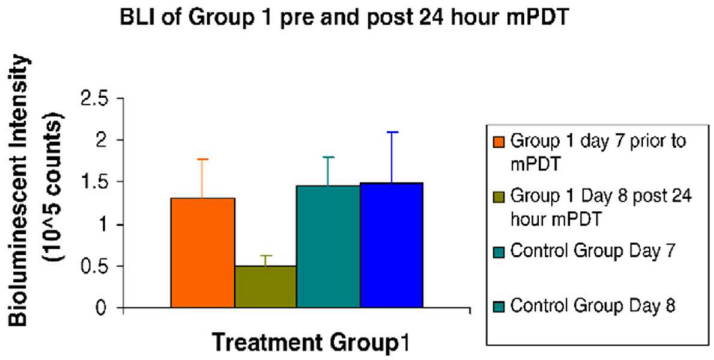

Figure 2.4: Data of bioluminescent intensity of group 1 before and after 24h mPDT. The treatment caused a reduction in BLI intensity of $60 \pm 20\%$. Image from [4].

Another preclinical study by Leroy and Vermandel (2017) [9] tested the efficacy of 5-ALA interstitial PDT (iPDT) with fractionated light compared to previous studies on continuous light in acute treatments (non mPDT), using three groups of rats presenting U87 human glioblastoma multiforme: one is the sham-control group, the second was treated with a 2-fraction administration of light and the latter with a 5-fraction administration of light. They delivered 25 J of light with 30 mW/cm^2, wavelength of 635 nm, in both treated groups. They have found through immunohistology that the two fractionated light administrations stimulated intratumoral necrosis, peritumoral edema and an infiltration of macrophages. In addition to this, they saw that the group treated with 5-fraction showed better apoptotic response, but also a marked angiogenesis due to hypoxia. To solve these problems, fractionated light administration could be enhanced by the use of anti-angiogenic factors and by using different fractionated illumination patterns to decrease hypoxia. Light fraction proved overall efficient in this preclinical study, and more feasible than mPDT (due to its necessity of continuous administration of light and PS) [9].

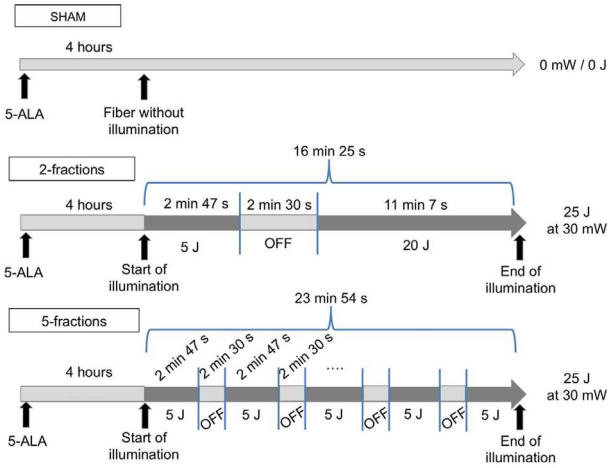

Figure 2.5: Treatment protocols in fractionated PDT in mice presenting U87 glioblastoma. Image from [9].

Summarizing, light administration is a very important feature to consider in PDT, particularly for cancer therapy. The focus of PDT should not be exclusively the photosensitizer, but it should be a combination of PS, light administration and dose, in relation to the type of cancer to be treated [17].

2.3 PDT selectivity and systemic anti-cancer response

Photosensitizers can be broadly classified in two groups: (i) chemical and (ii) **PDT selectivity**

genetically encoded. In both cases, it is important that phototoxic reactions occur in the designed, pathological area. Selectivity of PS activation is particularly important in cancer treatment. The affinity of a PS for low density lipoproteins (LDLs) is an important feature to consider in cancer treatment [8]. LDLs are constituents of the cell membrane and tend to accumulate in rapidly dividing cells, such as cancer cells, which are characterized by enhanced expression of LDL receptors on the cell membrane. Most chemical PSs are hydrophobic and can combine with LDLs.
Genetically encoded photosensitizers may be targeted by various means to a given cell sub-population, offering the advantage of localized production of ROS.

PDT and systemic anti-cancer response

PDT also leads to a systemic response from the organism, affecting the vascular system that supplies the tumor and stimulating the immune system. The destruction of the cancer tissue is correlated to vascular occlusion due to coagulation processes and local inflammation. Vascular occlusion is caused by the damage caused by ROS directly to tumor blood vessels, activating clotting processes and generating thrombi which deprive the tumor of much needed nutrients, contributing to cancer cell death. Furthermore, the destruction of the pathological tissue allows direct interaction between immune cells and cancer cells, stimulating an immune and inflammatory response [8]. PDT has demonstrated the ability to induce immunogenic cell death (ICD) and release tumor-associated antigens during cancer cell destruction, resulting in the activation and proliferation of CD8+T lymphocytes. Nevertheless, its therapeutic impact on metastasis and recurrence remains limited [14].

3 Photosensitizers

The focus of this thesis is the genetically encoded KR protein, but before delving into its properties, it is important to highlight the characteristics of a good PS, and the evolution of PDT from the beginning of the XX century until today.

3.1 Characteristics of the ideal PS

PSs are defined as "substances capable of absorbing light with a specific wavelength, triggering photochemical or photophysical reactions" [8]. There are some characteristics that have to be taken in consideration when evaluating the potentials of a photosensitizer [8]:

- Neglectable presence of chemical impurities and stability of the compound at room temperature;

- Specificity of excitation wavelength. The absorption maximum should be in a range of wavelength between $[600 - 800]$ nm, as longer wavelength do not provide enough energy for the photoactivation process; the minimum of absorption should be in a wavelength range between $[400 - 600]$ nm, to avoid excessive stimulation by direct exposition to sunlight;
- The PS absorption band should not overlap with any absorption band of other substances in the human body (as for example hemoglobin or melanin);
- The PS should not have a toxic effect in the dark. It should also be easily soluble in the tissue of interest in the human body;
- High selectivity for pathogenic tissues: an *exogenous* photosensitizer should be easily removed from healthy tissues, while it should remain long enough in neoplastic cells, at least several hours, to minimize undesired phototoxic effect;
- Photosensitizers should be easily produced, with low cost and high availability.

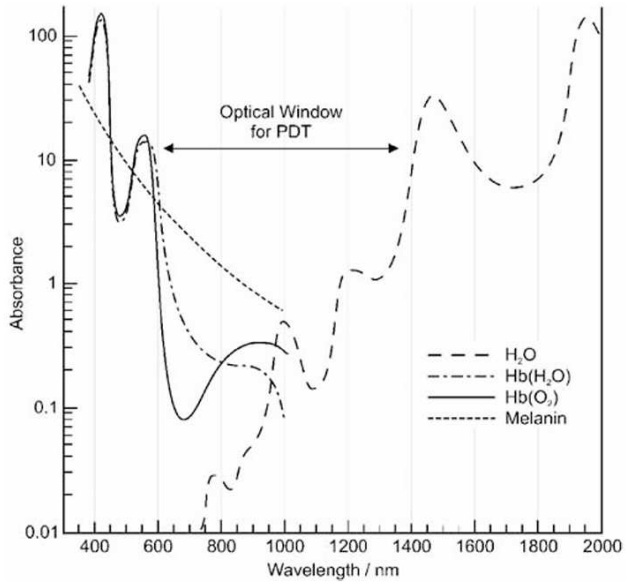

Figure 3.1: Visual description of the *optical window for PDT*. The effective range is even more restrictive than the one presented in the picture, since a wavelength $\lambda > 850$ nm has insufficient energy to trigger PDT. Image from [17].

3.2 Brief history of PDT

First generation photosensitizers

The first observation of the phototoxic effect of a PS is attributed to a medical student in Munich, Oscar Raab. While experimenting with protozoa treated with dyes, Raab noted fluorescence emission from those that had been irradiated, correlated with oxygen consumption and toxic effects that culminated with the death of the organisms. Raab's mentor, Professor von Tappeiner, explained this phenomenon in 1904 and named it "photodynamic effect". The first medical application of the photodynamic effect dates 1905, when they tried to treat skin cancer with an eosin solution[4]. These initial attempts, however, did not reach a wide audience and the therapeutic potential remained unexploited for decades.
It was only in the 1970s that PSs regained scientists' interest. Dr. Thomas Dougherty, who at the time was working in Roswell Park's Department of Experimental Biology, state of New York, and his colleagues worked with a hydrophilic porphyrin mixture, named "hemato-porphyrin derivative" (HpD), obtained by chemical modification of hematoporphyrin (Hp), the first porphyrin used for PDT. HpD performed better than Hp in many respects, showing improved selectivity for tumor tissue and a reduced photosensitizing effect on normal skin.
Porfimer sodium (Photofrin) is a PS derived from HpD, which is widely used in PDT for the treatment of tumors, including esophageal and endobronchial cancers. However, there were also many limitations to its clinical use, as for example the low chemical purity (as it is a mixture of 60 molecules), a maximum of absorption at 630 nm that leads to poor tissue penetration and a long half-life, which leads to skin hypersensitivity to light for weeks (the compound tends to accumulate in the skin) [8].

Second generation photosensitizers

These shortcomings of Photofrin prompted the search for other molecules that could better serve the purpose. Hematoporphyrin derivatives and synthetic PSs are so called "second generation PSs", characterized by higher chemical purity, improved tissue selectivity, deeper penetration of the excitation light due to a peak absorption in the range [650-800] nm. In addition, hematoporphyrin derivatives have a higher yield of singlet oxygen, resulting in an enhanced treatment potential, reduced side effects and faster clearance from the body. The main issue is their limited water-solubility, a problem that calls for other drug administrations solutions [8].

[4]Eosin refers to several fluorescent acidic compounds that bind to and form salts with basic, or eosinophilic, substances such as proteins containing amino acids residues like arginine and lysine, staining them dark red or pink due to the effects of bromine on eosin. Besides staining proteins in the cytoplasm, eosin can also be used to stain collagen and muscle fibers for microscopic examination. Structures that stain easily with eosin are called eosinophilic. In histology, Eosin Y is the most commonly used form of eosin for staining purposes

Third generation PSs comprise molecules that show further improvements in targeting neoplastic formations and reduced unwanted effects on the surrounding healthy tissues. The main issue is the delivery of the drug in loco, which is an open field of research. There are many ways to increase the selectivity of a PS for pathologic tissue, including the combination with molecules that target specific receptors, the combination with LDLs (for the reasons discussed in paragraph 2.3), the conjugation with antibodies or receptors specific for tumor tissues, and so on.

Third generation photosensitizers

Other delivery pathways such as nanomolecules, electroporation and genetic encoding are showing promising results [8]. In the next chapter, we will delve in the use of the KR protein in PDT.

4 The KR protein

The KR protein is the first fluorescent protein designed specifically to be phototoxic by Bulina's team in 2006 [27], [13]. The research for a fully genetically encoded PS stemmed from the main disadvantage that all known PSs (including the genetically encoded ones) required the injection of an external compound in order to function properly. The research begun with the study of the fully genetically encoded Green Fluorescent Protein (GFP), that is an inefficient PS (this is likely due to the shell-structure that surrounds the chromophore, preventing ROS generation) [2]. Bulina's team focused on the phototoxic effect on *Escherichia coli* cells of proteins that had a GFP-like structure: they found that the KR protein could kill effectively cells when stimulated by radiation, without displaying toxicity in the dark [2].

4.1 KR structure and properties

The KR protein was derived via mutagenesis from anmCP2 (a non-fluorescent chromoprotein found in a species of bioluminescent hydrozoan jellyfish) by 20 amino acids substitutions [2]. Crystallographic studies show that KR is characterized by a particular structure: 11-stranded β-barrel structure - that resembles the Green Fluorescent Protein's (GFP) own structure - with a tendency to dimerize. The amino acids substitutions obtained via engineering determine a pore in the β-barrel structure, forming a water channel that leads to the chromophore; it is thought that Glu68 (glutamic acid) and Ser119 (serine), which are adjacent to the chromophore, are key reactive residues for KR [7]. This water channel is thought to be a key factor for the superior efficacy of KR in PDT, since the chromophore isn't shielded from the surrounding environment [27]. The chromophore,

named QYG, is characterized by three main amino acids that determine its properties: Gln65-Tyr66-Gly67 [13].

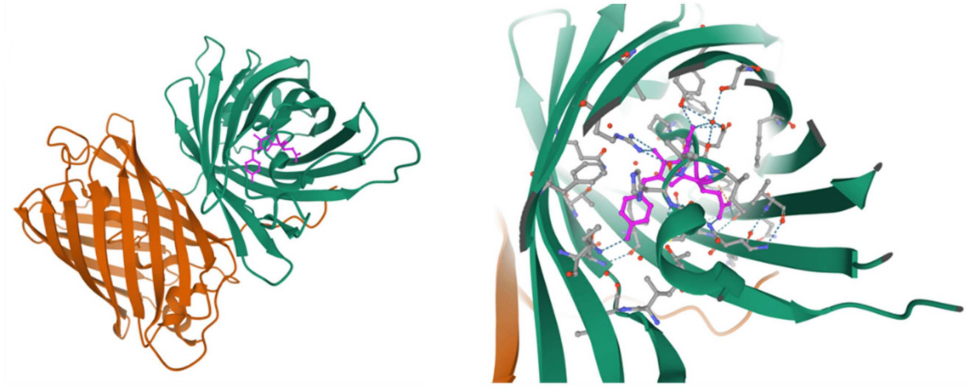

Figure 4.1: KR tridimensional structure. The chromophore domain is shown in pink, at the center the $\beta-barrel$ structure. Image from [13].

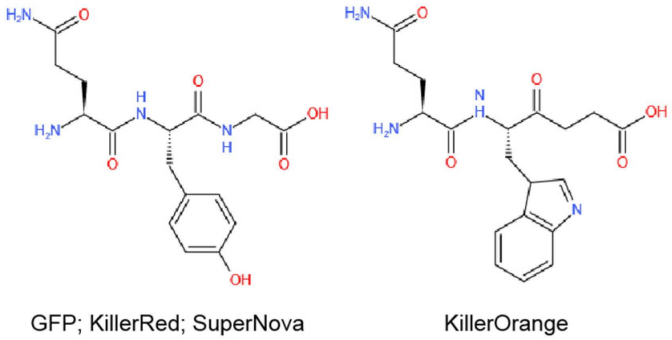

Figure 4.2: KR chromophore domain compared with other chromophores (GFP, SuperNova, KillerOrange). Image modified from [13].

The KR protein can absorb wavelengths in the green-yellow region [540-580] nm, and emits in the red region of the visible spectrum. The absorption maximum is at 585 nm, while the emission maximum is at 610 nm [2]. In the initial experiments performed by Bulina's team, KR was stimulated with white light (1 W/cm^2) and killed 96% of *E. coli* cells in a 10 minute illumination interval; after 20 minutes of illumination, almost no viable cells were detected. They also investigated the wavelength dependence of the cell-killing capacity using a uniform intensity of 35 mW/cm^2, and found that KR was most efficient with light in the [540-580] nm region, while it was almost ineffective in the blue region, [460-490] nm, in agreement with absorption spectrum of KR, suggesting that the chromophore mediated the phototoxic effect [2].

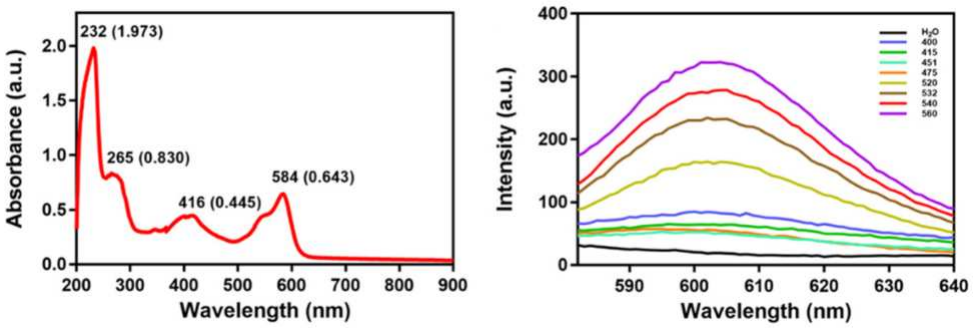

Figure 4.3: Left: KR absorption spectrum. Right: KR fluorescence emission spectra excited with different wavelengths of visible light. Image from [13].

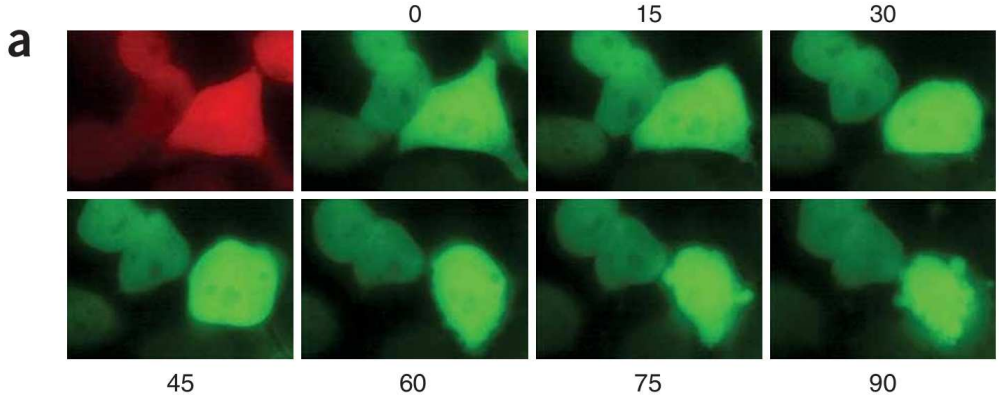

Figure 4.4: Eukaryotic cells expressing both KR and AcGFP1 (a green fluorescent protein). The cells were irradiated with green light for 10 minutes (TRITC filter set, 100x objective). The first image shows the fluorescence of KR before irradiation. The other images, identified by the corresponding number, represent the cells after irradiation at intervals of 15 minutes, the first image being taken immediately after irradiation. The fluorescent protein makes it possible to observe conformational changes in the irradiated cells, showing the phototoxicity of KR. Note that KR is fully photobleached after illumination. Image from [2].

Properties of fluorescent proteins							
Protein	No. AA	Chromophore	λ_{ex} (nm)	λ_{em} (nm)	Φ_f	$\Phi_{^1O_2}$	$O_2^{\cdot -}$ formation
KillerRed	239	QYG	585	610	0.25	0.000	Yes
SuperNova	271	QYG	579	610	0.30	-	Yes
GFP	238	TYG	395, 475	508	0.77	-	-

Table 1: Main characteristics of some fluorescent proteins, data from [13]. λ_{ex} stands for *excitation wavelengths* while λ_{em} stands for *emission wavelengths*; Φ_f is the fluorescence quantum yield, $\Phi_{^1O_2}$ is the $^1O_2$ quantum yield.

As shown in Table 1, the KR protein possesses a low fluorescence quantum yield

(note that fluorescence competes with intersystem crossing, which is desired for PDT) and a neglectable $^1O_2$ quantum yield[5]. The KR protein was shown to promote the photosensitized formation of $O_2^{\cdot -}$, meaning that the compound mainly works through type I reactions. For this reason, KR is thought to be effective even under low concentration of oxygen, which is a major issue for type II PDT, hampering its use in the naturally hypoxic tumor microenvironment [13].

As mentioned in paragraph 2.2, light administration is a major factor in PDT. There are controversies about the most suitable laser stimulation strategy for the KR protein, particularly regarding the use of continuous vs. pulsed laser stimulation. A study by Kuznetsova et al. (2015) used tumor spheroids of HeLa Kyoto cells expressing KR in the nucleus, in fusion with histone 2B, (concentration 13 ± 1 μM, approximately 10^6 molecules per cell) with both a continuous laser (CW) or a pulsed laser, with an irradiance of 160 mW/cm^2, with different exposure time, to achieve fluences[6] of 140, 170 or 200 J/cm^2, values comparable with those used *in vivo*. The pulsed laser was characterized by a wavelength $\lambda = 584$ nm, pulse duration of 15 ns and pulse repetition rate of 10 Hz, while the CW laser had a $\lambda' = 593$ nm. The percentage of necrotic and apoptotic cells was assessed with specific fluorescent dyes (necrotic cells were Propidium iodide-positive, apoptotic cells were Annexin-positive). The results showed that, in spite of identical photobleaching, spheroid destruction was different between CW and pulsed irradiation, and it was generally increasing with light dose. Irradiation with the pulsed laser resulted in lower residual cell viability at each light dosage [7]. Spheroids treated with CW were mostly necrotic, while spheroids treated with pulsed irradiation were mostly apoptotic [7].

[5] *The quantum yield of a particular oxidant is defined as the fraction of optical excitations that result in the formation of that oxidant* [27].

[6] PDT fluence refers to the amount of light energy delivered per unit area, typically measured in Joules per square centimeter (J/cm^2).

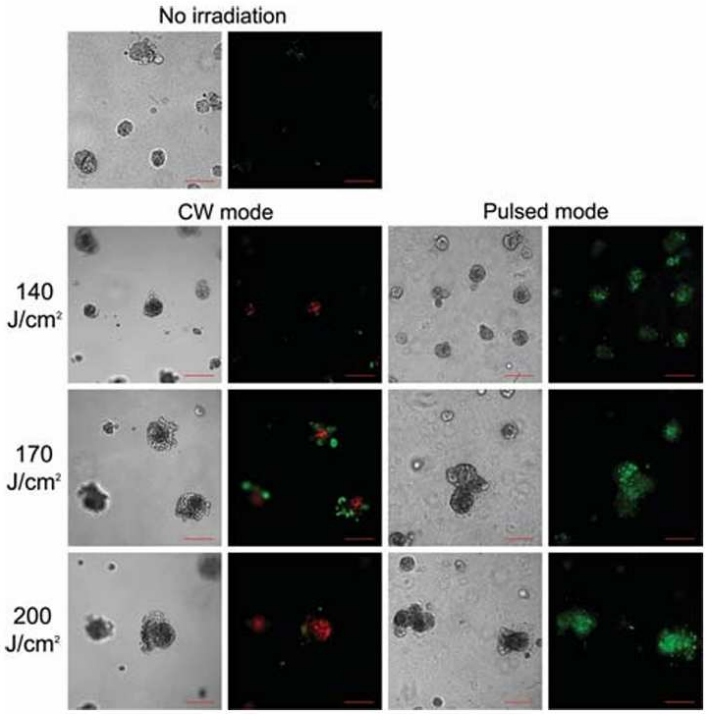

Figure 4.5: Cell death analysis in KR-expressing tumor spheroids at different light fluences. Images were acquired in transmitted light or fluorescence emission. Staining for necrosis: PI, red; staining for apoptosis: Annexin V-FITC, green. Bar length: 200 µm. Image from [7].

4.2 KR photobleaching

Kuznetsova et al. (2015) also investigated KR photobleaching properties. KR was particularly susceptible to photobleaching, likely due to the structure of the protein itself, particularly the water-channel. In the study, the Authors tried to determine if a photobleaching-based dosimetry was possible as the fading of fluorescence results from the direct attack of ROS or singlet oxygen on the chromophore. Therefore, it should be possible to assess the efficiency of photochemical reactions and predict the outcome of the therapy by measuring the rate of photobleaching. Kuznetsova et al. (2015) used the same irradiation regimens (CW and pulse mode) described above. Photobleaching was assessed with irradiances in the range of 20-160 mW/cm^2, with a total fluence of 200 J/cm^2. Fluorescence was measured every 5 minutes during irradiation, either with a laser scanning microscope or on a fluorescence inverted microscope. The results showed that KR photobleaching was linearly dependent on the fluence in the range [20-150] J/cm^2. The maximum photobleaching was limited to approximately 80%, irrespective of power density. The Authors were unable to determine whether the residual fluorescence was due to the shape of the spheroids themselves or to the

presence of highly photostable KR molecules in the population [7].

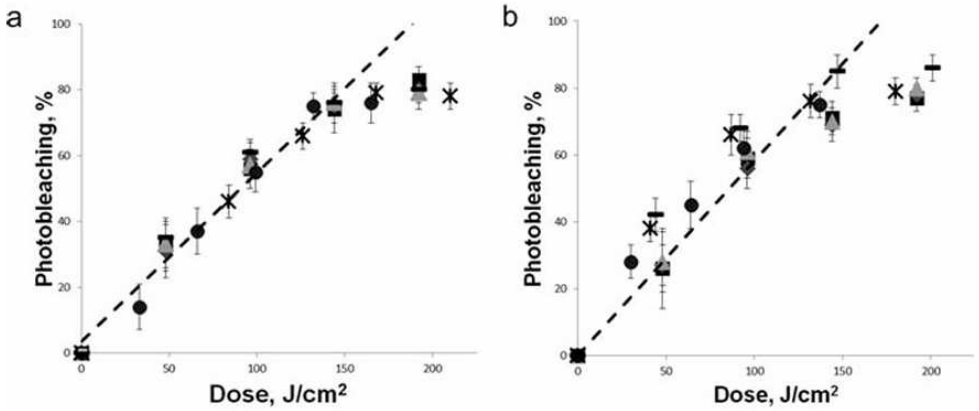

Figure 4.6: Photobleaching analysis of KR in tumor spheroids at different irradiances; a) CW laser irradiation, b) pulsed laser irradiation. ■: 20 mW/cm^2, ◇: 50mW/cm^2, ▲: 70 mW/cm^2, •: 110 mW/cm^2, ∗: 140 mW/cm^2, -: 160 mW/cm^2. The results are expressed as mean ± SD, with a population of $n = 15$ spheroids. The dashed lines represent a linear approximation, with $R^2 = 0.98$ and 0.91 for a) and b) respectively. Image from [7].

4.3 KR applications

The location of the PS is fundamental, both at a cellular and subcellular level. Since ROS have a short lifespan, they can only interact with the substrate within a 1 µm radius [7], often in the range of few nm [13], [29].
As a genetically encoded photosensitizer, KR can be targeted to different subcellular compartments using suitable signal peptides [16].

4.4 KR as endogenous PS

4.4.1 Membrane-Targeted KR

The integrity of the cell plasma membrane is vital to the survival of the cell. Membrane-targeted KR (mem-KR) addresses this exact necessity of the cell. ROS produced by PDT damage the plasma membrane by changing the conformation of its lipidic components via lipid peroxidation, compromising its stability and leading to necrosis [13]. It is to be noted that a precise localization of ROS generation is required, as widespread ROS diffusion is detrimental to adjacent healthy cells. Signal peptides act as targeting signals, allowing the cellular transport machinery to direct proteins to specific intracellular or extracellular destinations [24]. Once

[7]Note that a cell has typically a diameter in the span [20-40] µm.

a protein has reached its intended location, the signal peptide is usually cleaved by a signal peptidase, meaning most mature proteins do not retain their signal peptides. While signal peptides are generally located at the N-terminus, the targeting sequence for proteins destined for peroxisomes is found on the C-terminal extension [1]. For example, the N-terminus of KR can be modified to add the first 20 amino acids of the GAP43 protein (MLCCMRRTKQVEKNDEDQKI), which contain a palmitoylation site and act as a membrane localization signal (MLS). This modification causes the lipidated protein to attach to the membrane (the protein is associated with the membrane on the cytoplasmic side). Another commonly used sequence for plasma membrane targeting is that of the LCK protein: LCK(1-26) palmitoylation (MGCGCSSNPEDDWMENIDVCENCHYP) is added at the N-terminus of KR. The addition of palmitic acid occurs on cysteine 3. The final result is the same. This targeted localization can significantly improve the therapeutic efficacy of PDT by increasing the efficiency of ROS production and damage to the targeted cells [3].

4.4.2 Mitochondria-Targeted KR

Utilizing KR targeted to mitochondria enables cell killing via the apoptotic pathway. KR can be targeted to mitochondria by using a *mitochondria localization signal* (MTS) such as the mitochondrial presequence of human cytochrome *c* oxidase subunit VIII (SVLTPLLLRGLTGSARRLPVPRAK) [19]. Mitochondria are especially vulnerable to ROS since they are deeply involved in cell metabolism. Leakage of mitochondrial contents in the cytosol is likely to cause major damage to the cell itself [13], [29].
When KR is expressed in neurons photoactivated mem-KR results in the death of the cell, whereas photoactivated mt-KR results in a non-lethal organelle fragmentation. There are three potential reasons to explain this phenomenon. In the first place, mitochondria can efficiently control oxidative stress, slowing the diffusion of ROS and neutralizing them. Second, damaged mitochondria seem to avoid interaction with other organelles in neurons, so as to limit oxidative damage. Lastly, proteasomes degrade the activated caspase-3, to limit its detrimental action in the cell and avoid cell death [13], [5].
Apart from neurons, PDT with mt-KR shows promising results *in vivo*, leading to cell death through different pathways: caspase-dependent/ independent apoptosis and lastly autophagy [13], [23]. The caspase dependent apoptosis is caused by an increased permeability of the mitochondrial membrane due to ROS produced by the photoactivation of mt-KR allowing the release of cytochrome C, which initiates the caspase pathway. The caspase-independent pathway begins when the mitochondrial membrane is depolarized, or ROS cause mitochondrial rupture and dysfunction. Phototoxicity of mt-KR can also induce PARK2/PARKIN-

dependent mitochondrial autophagy [13], [30].
There is also a major downside to consider: mt-KR has shown to compromise muscle growth and development in worms (*Caenorhabditis elegans*), even without irradiation [23]. Further studies are required to ensure safety of mt-KR for PDT [13].

4.4.3 Nucleus-Targeted KR

Nucleus-Targeted KillerRed (nuc-KR) can be generated by fusing it in frame with various nuclear localization sequences such as Nucleoplasmin (KRPAATKK-AGQAKKKK, [6]), **c-Myc** (PAAKRVKLD, [15]) or p53 (PPKKKP, [11]). Light-stimulated nuc-KR generates ROS that can damage DNA with precise spatio-temporal control (chemical PSs lack such precision). The exact mechanism of DNA damage is incompletely characterized.
Nuc-KR has been explored for cancer therapy. KR fused with histone subunits, as for example 2A subunit (H2A), or fused to the nuclear lamina protein B1, can trigger oxidative stress that increases DNA double strand damage and can interrupt the cell cycle, limiting proliferation and leading to cell death ultimately [13].

4.5 Delivery strategies of KR Gene *in Vivo*

DNA strands need to be carried inside the cell, since DNA alone cannot pass through the cell membrane due to its properties: a negative charge, a steric factor and hydrophilicity. To achieve this, various possibilities are currently investigated. We can broadly differentiate between viral and non-viral delivery strategies [13].

4.5.1 Viral delivery strategies

Viral vectors offer the possibility to express permanently or temporarily exogenous DNA inside the host cell. Viral vectors can be distinguished into integrating and non-integrating vectors. Integrating vectors integrate their DNA into the host genome, providing stable, long-term gene expression but with a higher risk of insertional mutagenesis. Non-integrating vectors do not integrate their DNA into the host genome, reducing the risk of insertional mutagenesis and providing transient gene expression, suitable for short-term therapeutic applications. Transduction of the KR gene with non-integrating vectors proved successful in pre-clinical trials [13].
Takehara and colleagues designed TelomeKiller, a specific recombinant replicating adenovirus vector that expresses KR when activated by the human telomerase

reverse transcriptase (hTERT)[8] promoter [13], [25]. TelomeKiller administration proved successful when injected inside the tumor itself, where it produced KR efficiently in the host cell and the concentration of the PS inside the tumor did not decrease over time [25]. In recent studies, the Authors showed TelomeKiller inhibited the growth of a lung cancer and promoted the deletion of metastases under illumination with orange light (590 nm, 180 mW/cm^2, 60 min). TelomeKiller also proved successful in treating a malignant melanoma (stimulation with 589 nm, 300 mW/cm^2, 45 min) [13], [26].

4.5.2 Non-viral delivery strategies

There are also downsides to the use of viral vectors in general, as they may cause aberrations in DNA, potential immunogenicity due to the capsid, and the viral capsid might have too little capacity to carry the complete exogenous gene into its desired location. To overcome these difficulties, non-viral delivery methods have been investigated. Non-viral vectors can be generally defined as *an assembly of cations that complex DNA into small-sized particles* [13]. Non-viral vectors offer many advantages: low costs of production, simple preparation, less immunogenicity, no recombination potential and no limits in genome delivery.

Cationic polymers

Cationic polymers, as for example chitosan (CS) and polyethylene, are suitable carriers for the KR gene, as they can interact with negatively charged nucleic acids [13]. Liao et al. (2014) used membrane-targeted KR in a photosensitizing ternary complex consisting of CS/pKR-mem/γPGA[9]: CS shielded the KR gene from nuclease degradation, while γPGA aided in dissolving CS/DNA complexes inside the cells via electrostatic repulsion, enhancing the expression of the gene [13]. Cells expressing mem-KR showed loss of integrity of the cell plasma membrane and reduced viability after illumination (540-560 nm, 55 mW/cm^2, 30 min) [12]. Also, the phototoxic effect of KR decreases with time, suggesting safety of this PS [13].

Polyethylene

Polyethylene (PEI) has also been employed for KR transfection. In this study, Tseng et al. (2015) employed a pH-responsive complex that could enhance the absorption of p53[10] and pKR-mem sequences inside the acidic microenvironment of the tumor. Such complex is composed of branched PEI and a complex of

[8] Telomerases are ribonucleoprotein enzymes that can elongate telomeres. These enzymes are composed by two parts: hTERT is the catalytic site of human telomerase, which elongates the telomeres by adding nucleotides; TERC is the RNA component that codes for the nucleotides to add. Telomerases are important in cancer therapy as they are expressed in 90% of cancers, while they are absent in most somatic healthy cells.

[9] Poly γ-glutamic acid.

[10] p53 is a transcription factor involved in cancer suppression.

PEG[11], histidine and glutamic acid. The study showed that a single administration, followed by light irradiation (593 nm, 100 mW/cm^2, 20 min), could be quite effective, reducing the development of the tumor while improving life expectancy in the experimental model (athymic BALB/c nude mouse) [28].

Cationic polymers, however, show different shortcomings. Their usage is limited by charge-mediated toxicity, incompatibility and nonspecific interactions with blood. To overcome these downsides, cationic derivatives of natural polymers have been used, displaying lower toxicity and immunogenicity, as for example Pullulan, a linear homopolysaccharide, and the polysaccharide hydroxyethyl starch (HES) [13]. Self-assembly nanosystems have been studied to carry p53 and pKR inside the tumor environment, proving to be more efficient than monotherapy in some tumors [13].
Despite all progress made, further research is still needed to assess the safety of these non-viral carriers.

4.6 KR as exogenous PS

Genetically encoded KR has the advantage of high selectivity even at the level of subcellular compartments, as we discussed above. This approach, though, is not devoid of risks. Delivery of the KR gene is a major problem to face. Stability of the compound during administration and the degradation by enzymes are two issues that need to be properly addressed. In addition, potential gene toxicity due to the casual recombination of the sequence might be very dangerous to the patient. To overcome such risks, KR can also be used in protein form as an exogenous photosensitizer.
Carriers of KR protein can be broadly divided into inorganic nanoparticles and lipo/membrane nanocarriers.

4.6.1 KR protein delivery based on inorganic nanoparticles

Nanoparticles are suitable carriers for the delivery of KR protein, as they have high loading capacity, good stability and can be modified with additional functional groups to display different characteristics [13]. They have high chemical stability and corrosion resistance under physiological conditions [13].

Mesoporus silica nanoparticles

Mesoporus silica nanoparticles (MSNs) have some properties that make them suitable as drug carriers: biocompatibility, biodegradation and a porous, flexible structure. Proteins such as KR can be carried by MSNs thanks to the porous structure and electrostatic interaction. MSNs can be prepared with amine or

[11]Polyethylene Glycol; this biocompatible compound enhances solubility of other molecules in water and is used in pharmaceutical therapies.

carboxyl modifications on the surface, to display a positive or negative charge. In a study from Shi et al. (2019), charged MSN were used in combination with an outer layer of lipids to prevent the MSN-KR complex from bursting during transportation. Their study confirmed that KR proteins maintain correct folding after delivery, showing fluorescence and ROS production [22]. This delivery strategy is very efficient *in vitro*, but it does not overcome the problem of poor tissue penetration of light when applied *in vivo* [13].

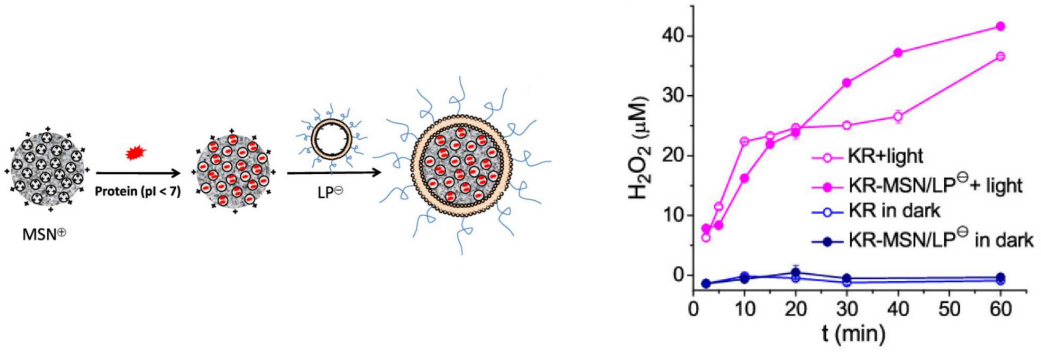

Figure 4.7: Left: scheme of uploading the delivery system. pI is an index for the charge of the protein: KR is negatively charged, since pI=5.1. Right: H_2O_2 (ROS) production of irradiated and non irradiated KR when uploaded on MSN/LP^- complexes. Concentration of KR: 180 μM; irradiation with LED light (10 mW/cm^2), for 2.5, 5, 10, 15, 20, 30, 40, 60 min. Image elaborated from [22].

Upconversion nanoparticles

Upconversion nanoparticles (UCNPs) are particles that can convert low-energy light into high-energy light [13]. They can be excited at longer wavelengths than KR (absorption maximum at 585 nm) that can penetrate the tissue further than 2-3 mm of green light. For this characteristics, UCNPs are thought to be effective in performing KR-PDT deeper in tissues, as they can be excited even by near infrared radiation (NIR). There have been studies in which covalently bound KR/green-emitting UCNPs (emission at 545 nm) complexes show efficacy in 1 cm depth compared to KR alone, under NIR light stimulation (980 nm) [10].
There is still room for advancement of this technology, as for example, increasing the loading efficiency, increasing light-conversion efficiency and reducing illumination times [13].

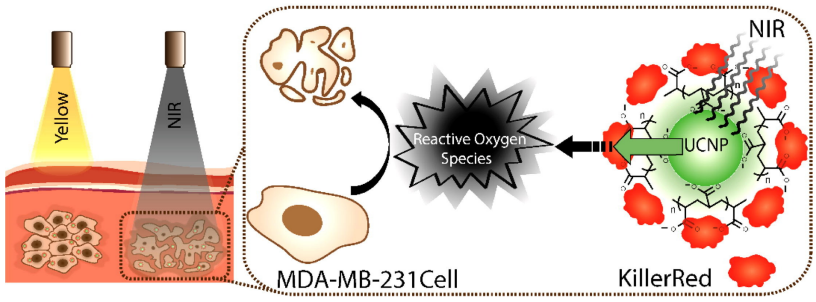

Figure 4.8: Scheme of PDT delivery via UCNPs. Image from [10].

4.6.2 KR protein delivery based on lipo/membrane nanocarriers

Liposomes consist in encapsulating membranes composed by one or more bilayers of hydrophobic phospholipid around the core [13]. They are known to be effective drug carriers, as they have biocompatibility and low immunogenicity, they can carry and protect from physiological conditions both hydrophilic and hydrophobic molecules and they can self-assemble. The major downside to this delivery strategy is the risk of unexpected drug leakage, which can induce toxicity in healthy tissues. Also, this approach does not resolve the difficulty to excite KR deeper in tissues [13].

5 KR in combination with other therapies

Cancer therapy is often a combination of different approaches to enhance the chances of survival and the complete remission from the disease, limiting side effects of each therapy (chemotherapy, radiotherapy, surgical resection and so on). There are ongoing studies to test photodynamic combinational therapies.

5.1 KR-mediated PDT combined with virotherapy

Virotherapy is a clinical approach that makes use of viruses as therapeutic agents. Viral vectors have the advantage of high infectivity and stable gene expression, compared to plasmid-based gene delivery [13]. The main challenge is to expand the use virotherapy from intratumoral injection to systemic delivery, to treat deep or metastatic tumors.
Recombinant adeno-associated virus serotype 2 (AAV2) seems to be a promising vector. Functionalized AAV2 nanoparticles can decrease the unit dose, the risks of AAV-directed immune response, ectopic expression, and oncogene activation [13]. A new approach uses magnetic-field-enforced delivery to target magnetic

nanoparticles (MNPs) to specific locations, improving vector accumulation and virion infectivity in those designed places. Adeno-associated viruses expressing the KR gene (AAV-KR) are delivered in the desired location thanks to iron oxide nanoparticles and a magnetic field. PDT with magnetic guidance has shown induced tumoral cell death via apoptosis, reducing tumor growth (laser, 1.5 mW, 20 min), and eliminating even chemotherapy-resistant cancer cells [13].
Another delivery system based on magnetized AAV2-KR used a hypoxia-responsive carrier based on lactate production. This carrier self-assembles, and its main components are hyaluronic acid (HA), lactate oxidase (LOX) and magnetized AAV2-KR [13]. LOX and magnetic fields provide specific release inside the lactate-rich and hypoxic microenvironment of the tumor. LOX can produce H_2O_2 from lactate oxydation, which then induces bioreduction of HA, allowing the electrostatical dissociation of the AAV2-KR from the carrier. The results are promising, since the experiment carried out *in vivo* show limitation in tumor growth and significant reduction in tumor weight 2 weeks after illumination (laser, 1.5 mW/mm^2, 20 min) [13].
Another approach to virotherapy from Takehara's group targets the tumor with the already cited TelomeKiller, with the promoter of the human telomerase reverse transcriptase (hTERT) [25]. This study showed that TelomeKiller was a very efficient strategy for virotherapy, as it combined the oncolytic effect of the replicating adenovirus with the phototoxicity of KR during PDT, reducing cancer volume and depleting the metastases in proximal lymphonodes.
A major problem of this approach remains the low penetration depth of the excitation light (about 2 mm) and the administration of the drug. An intratumoral injection was required, as intravenous administration was neutralized by adenovirus-specific antibodies [25].

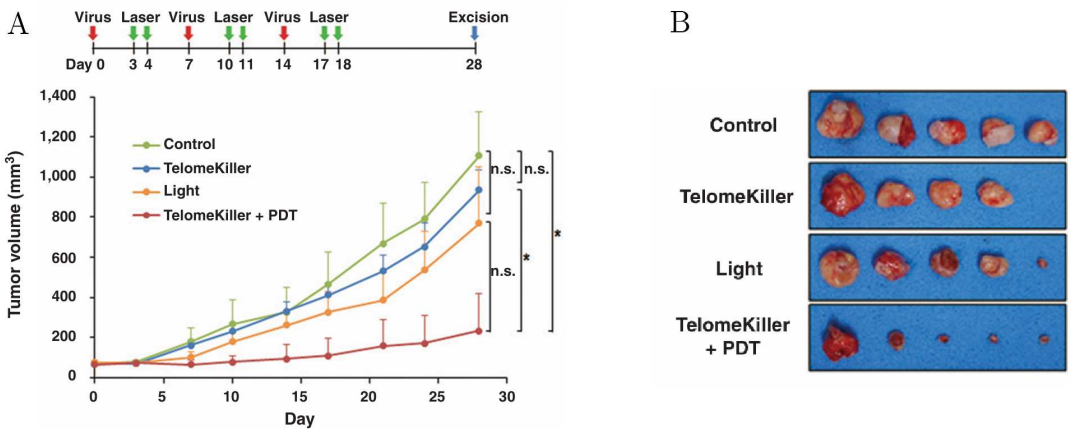

Figure 5.1: Treatment of subcutaneous HCT116 in a xenograft tumor model. A) Light and drug administration protocol and data of tumor volume vs. time. Data are expressed as mean ± standard deviation. Significant differences between treatments were assessed using Student t test, with a p-value < 0.05. Comparison marked with $*$ are statically significant, while n.s. stands for non significant. B) Images of isolated tumors from each different group. Image modified from [25].

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5.2 KR-mediated PDT combined with immunotherapy

Immunotherapy is a therapeutical strategy that seeks the involvement of the immune system, which is manipulated to recognize and activate immune response towards tumors that were not recognized in the first place. Combining PDT and immunotherapy is a feasible way to overcome the shortcomings of immunotherapy on its own (low response rates, autoimmune response, nonspecific inflammation). PDT is suitable for combined immunotherapy as it promotes the release of antigen factors and DAMPs[12] from dying tumor cells, fostering dendritic cells (DCs[13]) maturation and activating an immune response [13].
One study from Serebrovskaya et al. (2009) developed 4D5scFv-KR, a combination of the antibody fragment 4D5scFv (specific as anti-p185$^{HER-2-ECD}$) and KR which maintained the characteristics of both component separately: antigen affinity and photodynamic effects. The results demonstrated that the 4D5scFv-KR complex was effective in targeting cancer expressing p185$^{HER-2-ECD}$ (ovarian carcinoma SKOV-3 cell line) and killing it when exposed to light (white light, 1 W/cm^2, 10 min), while free KR or the 4D5scFv singularly did not affect cell viability. In addition, the 4D5scFv-KR complex did not affect non-expressing p185$^{HER-2-ECD}$ cell lines (CHO cells), proving the high specificity of the combination complex [21]. In the same study, the Authors tested the combination of

[12]Damage-Associated Molecular Patterns.
[13]Dendritic cells are antigen presenting cells in the mammalian immune system.

this new complex with the antitumoral agent cisplatin[14] on living cells and found a lower cell viability in this treatment combination than with either treatment singularly [21]. The results of this study are promising, but it is important to test these new approaches in xenograft tumors[15] *in vivo*.

There are also other problems to face, as for example the insufficient penetration depths of the KR stimulating wavelength and the long-term photosensitivity.

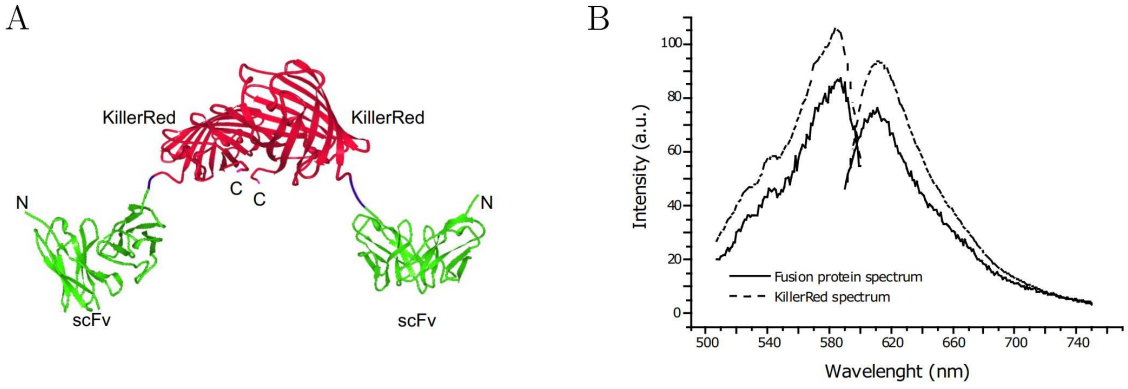

Figure 5.2: A) Scheme of the 4D5scFv-KR complex, elaborated from the Protein Database PDB (1fve and 1g7k). The antigen fragments are linked to the N-termini of dimerized KR. B) Excitation and emission spectra of KR and of the immunophotosensitizer complex. Fluorescent properties remain unaltered. Image modified from [21].

[14]Cisplatin is an important antineoplastic chemotherapic agent. It interferes with the cellular cycle by binding to the DNA.

[15]Xenograft tumors are tumor models derived from patients' cancer and transplanted in experimental organisms, as for example rats.

6 Conclusion

PDT is an interesting and promising approach for cancer therapy, as it is non invasive or minimally invasive and very selective. There are still many challenges to overcome to achieve its clinical application, as for example the complete control of light dosage and the research for efficient PSs that don't cause long term photosensitivity in the patient. Light administration remains a main challenge in every treatment. It is very important to focus not only on the properties of the PS itself, but also on the characteristics of the disease to treat. A cancer-centered perspective is essential when dealing with PDT. Light features (continuous versus pulsed light, repetition rates, frequency, penetration depth and so on) and administration techniques (fractionated light administration or mPDT) depend heavily on the disease to treat.

The KR protein is a promising photosensitizer that works mainly through type I photoreaction, with good results even in hypoxic microenvironments. It has many desirable features, such as high phototoxicity and ROS production, high biocompatibility and the possibility of genetic encoding inside host cells. The KR protein offers advantages both as a genetically encoded photosensitizer (as for example an optimal spatio-temporal control in ROS production inside the cell itself) and as an "exogenous" photosensitizer (with no risk of potentially dangerous genetic modifications). Every form of KR requires specific delivery strategies that are still under investigation, as for example viral delivery with the use of adenoviruses, or non-viral delivery with the aid of cationic polymers and polyethylene. Nanoparticles, as magnetic nanoparticles (MNPs) and upconversion nanoparticles (UCNPs), seem to be very promising in aiding the delivery of this PS, improving the efficacy of PDT. In particular, UCNPs seem to be a promising solution to overcome limited light penetration depths, as they can be excited with NIR radiation.

There are also other future prospects to consider. The KR protein prompted the study for other genetically encoded photosensitizers, that might improve the already satisfying characteristics of KR itself. SuperNova is a new red fluorescent protein derived from KillerRed, but unlike KR, it doesn't tend to dimerize, reducing the steric encumbrance that might interfere with cellular functions.

Lastly, combination therapy often proves more efficient in cancer therapy than monotherapies. Many attempts to combine PDT with virotherapy or immunotherapy are being attempted with promising results, even for what concerns metastatic cancers.

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