Research Article

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Targeted photoimmunotherapy for cancer

Abstract: Photodynamic therapy (PDT) is a clinically approved procedure that can exert a curative action against malignant cells. The treatment implies the administration of a photoactive molecular species that, upon absorption of visible or near infrared light, sensitizes the formation of reactive oxygen species. These species are cytotoxic and lead to tumor cell death, damage vasculature, and induce inflammation. Clinical investigations demonstrated that PDT is curative and does not compromise other treatment options. One of the major limitations of the original method was the low selectivity of the photoactive compounds for malignant over healthy tissues. The development of conjugates with antibodies has endowed photosensitizing molecules with targeting capability, so that the compounds are delivered with unprecedented precision to the site of action. Given their fluorescence emission capability, these supramolecular species are intrinsically theranostic agents.

Keywords: Targeted photodynamic therapy, cancer, antibody, photosensitizer, reactive oxygen species, fluorescence

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Introduction

Light and health. These two words have been related for 3,000 years since the Greeks introduced heliotherapy, namely, the use of the sun as a therapeutic agent (Figure 1) [1]. Although the potential of light has been long recognized, it is only at the end of the 19th century that its therapeutic potential was fully proved when Finsen demonstrated the efficacy of phototherapy in the treatment of *Lupus vulgaris* [2], a tubercular condition of the skin. For this, he was awarded the Nobel Prize in Physiology or Medicine in 1903.

At the beginning of the 20th century, Raab and von Tappeiner were investigating the effects of acridine dyes on protozoa when, during a thunderstorm, the protozoa died. After this episode, they hypothesized that the light-mediated cytotoxicity of these dyes was caused by the transfer of energy from light to the chemical (called photosensitizer [PS]) [3].

A few years later, von Tappeiner and the dermatologist Jesionek demonstrated the first therapeutic medical application of this phototoxicity using eosin and white light in the treatment of skin tumors [4]. Shortly after, von Tappeiner and Jodlbauer demonstrated that oxygen was required to induce this photosensitization reaction [5], and in 1907, they introduced the term "photodynamic action" [6] to describe this phenomenon at the foundation of the photodynamic treatment.

In the battle against microbes, the initial promising results of the photodynamic effect have not been considered for further developments due to the introduction of antibiotics in the 1940s. It is worth to note that in the last few years, the increasing severity of the antimicrobial drug resistance problem has drawn the attention of governments and several international organizations. The urgency to find alternative ways to tackle bacterial infections has led to a progressive reintroduction of the photodynamic effect as a potential and efficient antimicrobial treatment since one of its advantages is that it does not induce drug resistance [7–15].

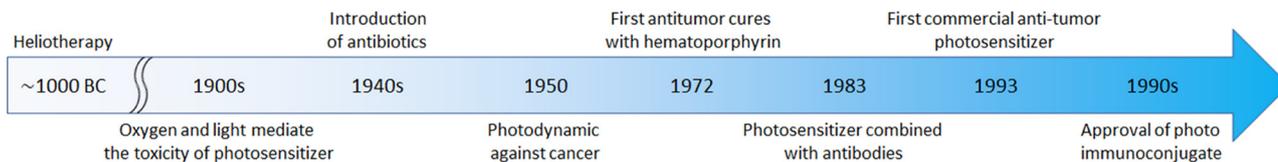However, despite this recently renewed interest in the photodynamic treatment against microbial infections,

Heliotherapy		Introduction of antibiotics		First antitumor cures with hematoporphyrin		First commercial anti-tumor photosensitizer	
~1000 BC	1900s	1940s	1950	1972	1983	1993	1990s
	Oxygen and light mediate the toxicity of photosensitizer		Photodynamic against cancer		Photosensitizer combined with antibodies		Approval of photo immunoconjugate

Figure 1: Historical development of photodynamic therapy.

most advancements in PDT occurred in relation to the treatment of tumors and noninfectious diseases (such as vitiligo and others) [16,17]. Since tumor cells exhibited significantly greater uptake and affinity for porphyrins and porphyrin derivatives compared to normal tissues [18], these photosensitizing molecules were introduced to detect otherwise invisible malignancies by exciting their red fluorescence emission.

It was only in 1972 that the first antitumor treatment with photo-activated PS was demonstrated by Diamond et al. Specifically, it was shown that injection of hematoporphyrin (Hp) into mice with subcutaneous brain tumors, followed by 24 h light exposure, induced necrosis of the malignant cells [19]. The demonstration of its potential applications for cancer treatment quickly led to a series of therapeutic medical trials, in which clinical PDT was introduced. Kelly and Snell were the first in 1976 to report the use of Hp derivative (HpD) in humans for the treatment of bladder cancer via photo-activation of the PS [20], paving the way for the development of PDT as an effective anticancer modality.

After further successes in its clinical application, the development of photodynamic therapy culminated in 1993, when the first commercial PS agent, an Hp derivative, was developed and put on the market with the brand name Photofrin [2]. Being the first PS to gain approval for clinical PDT, Photofrin carried some disadvantages (e.g., prolonged patient photosensitivity), which prompted the development of second- and third-generation photosensitizers [2].

The PS is one of the three components needed to induce the photodynamic effect, together with visible light and molecular oxygen (Figure 2). Once the photosensitizing molecule is administered to the target structure, a source of nonionizing light with a suitable wavelength excites the PS, whose various interactions with oxygen naturally occurring in the biological environment lead to the production of reactive oxygen species (ROS) such as free radicals and singlet oxygen [14]. These reactive species oxidize different types of biomolecules (nucleic acids, lipids, and proteins) inside the target cells, causing partial or complete destruction of sensitive structures.
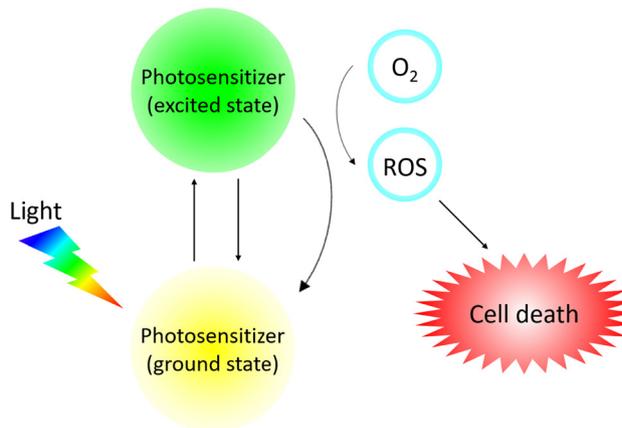

Figure 2: Diagram of photophysical and photochemical reactions during PDT. PS absorbs photons from a light source, and in its triplet excited state reacts with molecular oxygen, producing reacting species which then cause cell death.

Damages to these structures are crucial to optimize the cytotoxic efficiency of PDT [21].

Compared to other existing cancer treatments, PDT presents some remarkable advantages (Figure 3) and can be used in both outpatient and surgical procedures, with the first one being the most often performed.

Thanks to dedicated laser or LED sources, light can be directed very precisely on the malignant tissue. Furthermore, PDT requires short treatment time, and usually few or no scars remain. Unlike radiotherapy, PDT can be repeated at the same location many times, and it is generally less expensive than other cancer treatments [16]. In treating certain kinds of cancers and precancers, PDT has proved to be a very powerful practice, which can work as well as surgery or radiation therapy [22].

Admittedly, PDT also has some adverse effects. The generation of ROS causes oxidative stress and local hypoxia (resulting in a decrease in pH) [23], and the induced inflammatory reactions release cytokines [24]. During and after PDT, these lead to the sensation of discomfort and pain (such as burning, prickling, and stinging) [24]. When treating large areas, pain can be reduced by using daylight PDT instead of conventional PDT or by increasing progressively the irradiance [23,24].

PDT for cancer

Advantages	Disadvantages
• No long-term side effects • Few or no scars remain • Very precise • Little invasiveness • Outpatient and surgical procedures • Takes a short time • Can be repeated at the same location many times • Cheaper than other cancer treatments	• Can only treat areas that can be reached by light • Cannot treat disseminated cancer metastasis • Accurate light delivery is crucial • Tissue oxygenation is crucial • Photosensitivity after treatment • Pain • Inflammation • Allergic reactions • Flu-like symptoms • Nerve palsy, transient amnesia

Figure 3: Advantages and disadvantages of antitumor PDT.

After PDT, common inflammation (such as erythema) or uncommon (such as crusting, infection, scarring, and urticarial reaction) adverse effects may appear. Other uncommon adverse effects of PDT are changes in hair growth (both increase and loss), dyspigmentation, and allergic reactions [24].

Some rare adverse effects of PDT comprise nerve palsy, transient amnesia, hypertension (observed in patients with known hypertension), and systemic flu-like symptoms (observed in immunosuppressed patients) [24].

PDT can even lead to skin cancer, but its role is not fully understood because in reported cases patients often have a predisposition to skin cancer [23]. The concept of an immunocompromised district can explain the development of skin cancer: the area exposed to PDT light undergoes immune dysregulation that can lead to less resistance than the surrounding areas [23].

Even if PDT will never replace them due to its limitations (Figure 3) [16], it will probably play an increasingly adjuvant action. In some countries, PDT has become the leading modality for the treatment of nonmelanoma skin cancer [25].

Most PSs are fluorescent, and thus, they combine therapeutic and diagnostic properties, which make them intrinsically theranostic agents [21,26,27].

In PDT, most PSs are administered systemically through direct injection in the body: even if these molecules preferentially accumulate in the malignant cells, they show a lack of specificity to target cancer cells. In these cases, an accurate application of light is necessary to act only on the interested tissues.

To enhance the selectivity against cancer cells and preserve the integrity of surrounding normal tissue, PS with improved molecular-targeting properties has been developed by exploiting the conjugation of PS with targeting moieties.

In particular, monoclonal antibodies (mAbs) proved to be effective targeting carriers for cancer therapy because of their capability to address tumor cells overexpressing antigens on the plasma membrane [28].

In 1983, Levy developed an anticancer treatment that combined the phototoxic effects of PSs and the target-seeking ability of antibodies and then named photo-immunotherapy (PIT) [29]. This technique, derived from PDT, uses PSs associated with mAbs to allow higher accuracy in the treatment: the photo-immuno-conjugate (PIC) improves the selectivity of the PS to act on targeted cells with minimal effects on the surrounding healthy cells, as demonstrated in several preclinical and clinical studies [16]. Nowadays, several mAbs, among the ones approved for therapeutic use by FDA, are used in PIT to target tumor-associated proteins such as CD20 (rituximab, ibritumomab tiuxetan, and tositumomab), CD33 (gemtuzumab), CD52 (alemtuzumab), HER2/neu (trastuzumab), and EGFR (cetuximab, panitumumab) [18].

The aim of this short review is to summarize the basic principles and provide a concise selection of recent advances in PIT, considering in particular, the development of constructs based on PS nongenetically fused to mAbs. Excellent reviews have recently provided a detailed description of the synthetic strategies that have been proposed, and the reader is referred to them for these aspects [30–33]. The biological and clinical aspects of PDT have been discussed in recent reviews as well [21,34–36].

Basic principles of photodynamic effect and the induced cellular damages

As mentioned earlier, the photodynamic effect requires three main components: the PS, visible light, and molecular oxygen (O_2). Each of these elements is nontoxic by itself: only when combined together, they initiate a photochemical reaction that culminates in the production of cytotoxic compounds that cause irreversible cellular damage and, consequently, tumor reduction or elimination.

PDT can be described as a two-step process. First, the PS is administered to the patient, leading to significant accumulation in malignant cells after some time. Subsequently, the area of interest is irradiated by visible radiation at a wavelength that matches the absorption properties of the PS [37]. Once the PS is excited, it interacts with the surroundings, made up of different types of biomolecules, and with molecular oxygen [34]. These interactions can follow two diverse pathways, named Type I and Type II reactions [38].

In Type I processes, electron transfer from the excited PS to molecular oxygen or biomolecules such as nucleic acid, lipids, and proteins leads to the formation of reactive oxygen species (ROS). The local generation of these species can cause cell damage and death [34]. The reaction through which the PS in its excited triplet state transfers its energy to molecular oxygen ($O_2(X^3\Sigma_g)$, or simply $^3O_2$) forming singlet oxygen ($O_2(a^1\Delta_g)$, or simply $^1O_2$) is commonly known as Type II process [34] and is the most common pathway in PDT [34].

Once formed in the cell environment, ROS and singlet oxygen induce extensive damage to proteins, lipids, and other biomolecules in the irradiated area. This causes direct death of tumor cells through different types of pathways, depending on the intracellular location of the PS [39].

The Type I and Type II processes are commonly represented with an extended Jablonski diagram as shown in Figure 4. A photon of proper wavelength is absorbed by the ground state PS, which is thus promoted to a short-lived excited state (S_n). From S_n, the PS undergoes rapid energy loss through vibrational relaxation (VR) and internal conversion (IC) to the first excited state singlet state (S_1). S_1 is an unstable energy level (with lifetime in the range 10^{-9}–10^{-7} s), which quickly relaxes to the ground state via the generation of heat or emission of light (fluorescence).

Alternatively, the lower-energy, long-lived triplet state (T_1) can be populated from S_1 via a process named intersystem crossing [40]. Since T_1 is long lived ($\sim 10^{-6}$ for air equilibrated solutions), it can interact with surrounding molecules in the cell environment, including cell membrane components and molecular oxygen [38].

In Type I reactions, PS in the excited singlet state S_1 or in the triplet state T_1 interacts with substrates that can be oxidized or reduced.

In Type II processes, the T_1 state of the PS directly interacts with molecular oxygen, naturally occurring in the cell environment in its ground triplet state $^3O_2$. The resulting Dexter-type energy transfer generates singlet oxygen ($O_2(b^1\Sigma_g)$ or $O_2(a^1\Delta_g)$) and at the same time depopulates the PS excited triplet state [2].

The $O_2(b^1\Sigma_g)$ (157 kJ/mol) state is extremely short lived, and it quickly relaxes to the lower energy state $O_2(a^1\Delta_g)$ (or simply $^1O_2$) (94 kJ/mol, with a lifetime of 10^{-6}–10^{-3} s, depending on the environment). The oxidizing species $^1O_2$ is considered to be the principal cytotoxic agent in PDT [2].

Although $^1O_2$ is highly reactive, its action is limited to a distance of ~ 100 nm from the site of sensitization, given its short lifetime ($\approx 3.5\,\mu s$) [38]. Indeed, the distance traveled in water by free-diffusing singlet oxygen during its lifetime is quite short in comparison to the typical scale of the cell environment. Therefore, an effective way to enhance $^1O_2$ cytotoxic activity is to deliver the photosensitizer as close as possible to sensitive molecular species in the targeted tumor cells.

In the cell environment, singlet oxygen can react with different structures and organelles such as mitochondria, tubulin, lysosomes [41], vesicles, and membranes, where the molecular components most involved in the photo-induced damages are proteins, DNA, and lipids [42]. In the case of proteins at physiological pH, $^1O_2$ reacts at the significant rate constants ($10^7\,M^{-1}\,s^{-1}$) with high electron density amino acids such as cysteine, histidine, methionine, tyrosine, and tryptophan [43]. These reactions compromise the functionality of proteins, leading to impairment of normal cell reactions. Due to its reduction potential, the oxidative activity of singlet oxygen on nucleic acids occurs preferentially on guanine, implying consequent damage of the double bonds in C4 and C8 of the purine ring. Finally, singlet oxygen is electrophilic and therefore reacts with unsaturated lipids to form highly reactive compounds (lipid hydroperoxides), which initiate chain reactions of free radicals, leading to extensive cytotoxic damage.
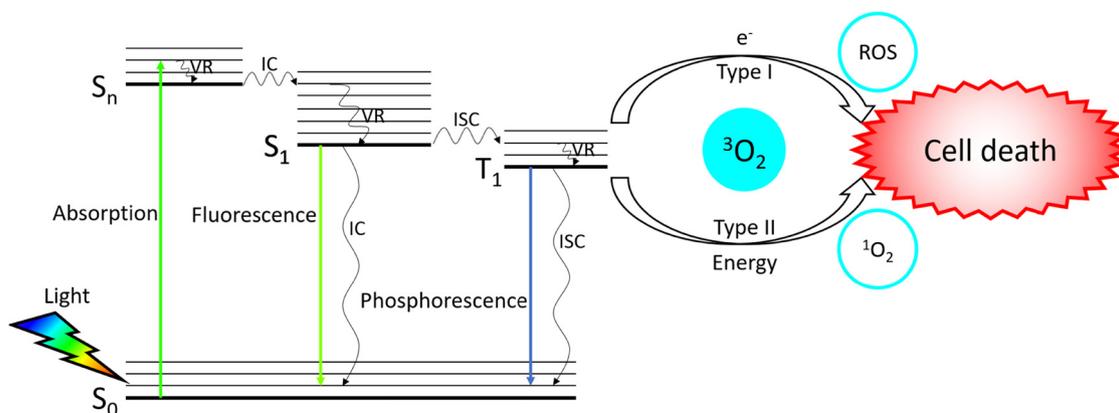
Figure 4: Extended Jablonski diagram showing the photophysical and photochemical processes relevant to PDT pathways. The PS in the ground state absorbs a photon and is promoted to an excited singlet state, S_n, from which it undergoes nonradiative relaxations (vibrational relaxations [VR] and internal conversion [IC]) to the lowest vibrational substrate of the lowest electronically excites singlet state. The molecule may undergo intersystem crossing (ISC) to the triplet state. The triplet state of PS can interact with oxygen by two mechanisms, named Type I and Type II.

The cytotoxic action derived from the extensive damages to molecular species by photoinduced ROS may lead to different cell death pathways. These include apoptosis, necrosis, or autophagy. Some studies suggest that the main route of cell death caused by PDT is apoptosis, which can occur through two different pathways: an extrinsic process (through death receptor [DR]) and an intrinsic process (through mitochondrial pathways) [39,44]. It was proposed that different doses of PDT can result in different types of cell death, and high dose (high light fluence and/or high PS concentration) leads to necrosis, whereas low dose leads to apoptosis [45]. Besides damages to tumor cells, the cytotoxic action of PDT results in vascular damages and activation of an immune response (Figure 5), which address the antigens and the numerous molecules that are released after the cell damage and destruction [44].

It is important to stress the fact that unless the photodynamic action is confined to the tumor cells, similar but unwanted damages may be observed also in surrounding healthy tissues.
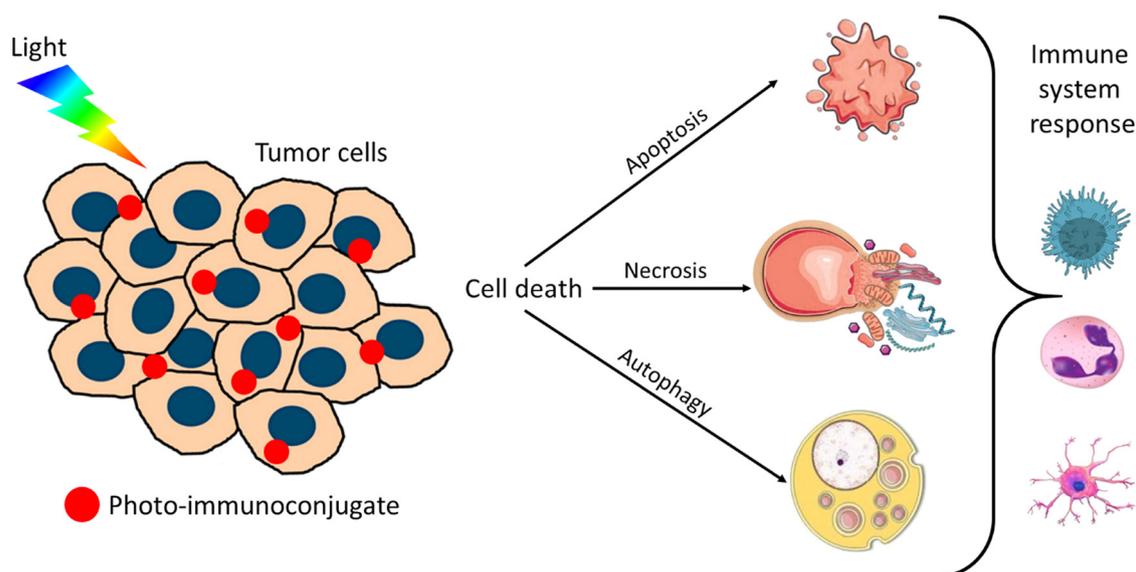

Figure 5: Effect of PDT on tumor cells. After irradiation, cells undergo one of the three main pathways of cell death; then, the released molecules activate the immune response.
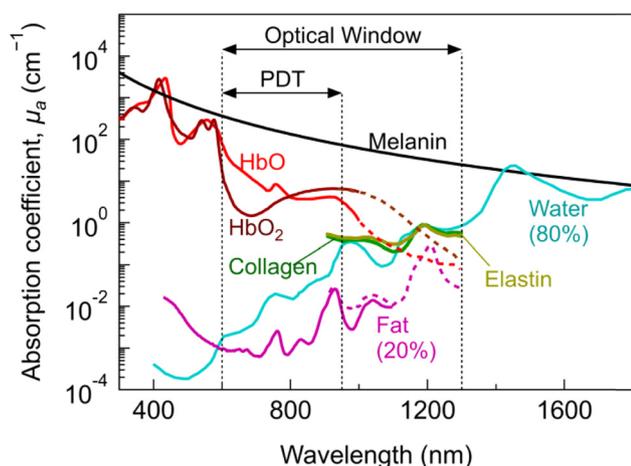
Figure 6: Absorption coefficient as a function of wavelength for several tissue constituents. Reproduced from Algorri et al. [47].

Photosensitizers

In principle, an ideal PS should be endowed with the following properties [46]:

- Strong absorption (with a high absorption coefficient) in the red or near-infrared (650–800 nm), where penetration in tissues is deeper (PDT window in Figure 6).
- High quantum yield of triplet state formation (Φ_T), the energy of the triplet state above ~94 kJ/mol, long lifetime of the triplet state (τ_T in the long µs range), and high quantum yield of singlet oxygen formation (Φ_Δ).
- Low toxicity in the dark, i.e., in the absence of light, the PS must not be harmful.
- High accumulation in the target area.
- Excellent biocompatibility securing a rapid disposal by the body.
- High chemical stability and low photobleaching (to allow sustained photoinduced singlet oxygen production).

The first generation of PSs consisted of hematoporphyrin derivatives (HpDs). As previously recalled, in 1993, a HpD called Photofrin® (porfimer sodium) was approved for clinical use, and it was used for several types of cancer despite its disadvantages: (i) low chemical purity; (ii) intense accumulation in the skin causing a prolonged photosensitivity (even 2–3 months after administration); and (iii) the wavelengths range of its absorption, which does not allow a good penetration into tissues [48].

Motivated by these drawbacks, an intense research activity was undertaken to develop new photosensitizing agents with defined chemical identity, improved photophysical characteristics, and enhanced tumor selectivity.

Second-generation PSs have higher chemical purity, the higher quantum yield of singlet oxygen formation, and have higher tissue penetration (thanks to their absorption peaks falling in the red portion of the visible spectrum or in the near infrared). Second-generation PSs were developed following different synthetic strategies, focusing on the modification of the macrocycle or the substituents, or considering different molecular architectures [49,50]. This has led to the development of several molecular families, including chlorins (Foscan®), metalloporphyrins (Llutrin®, Lutex®), verteporphin (Visudyne®), pheophorbides (Tookad®), phtalocyanines, porphycenes, protoporhyrin IX precursor (Hexvix®, Metvix®, Levulan®), cyanines, dipyrromethenes, hypericin, phenothiazines (methylene blue, toluidine blue), purpurins (Purlytin®), and xanthenes (Rose Bengal) [51]. Other compounds comprise also new structures based on BODIPY, squaraine, and fullerene cages.

In spite of the numerous types of photosensitive compounds that have been discovered and developed, only a small number of photosensitizers are clinically approved, mostly based on the tetrapyrrole structure (Table 1). Some

Table 1: Clinically approved photosensitizers [53]

Name	Approved country	Cancer type
Photofrin	Worldwide	Lung, gastric, bladder, cervical, and esophageal cancers
Ameluz/Levulan	EMEA, USA, Austria, China	Actinic keratosis, Human Papilloma Virus
Metvix	EMEA, USA, Canada	Actinic keratosis, basal cell carcinoma
Cysview	EMEA, USA	Bladder cancer
Foscan-Temoporfin	EMEA, Norway, Iceland	Neck cancer, head cancer
Visudyne -Verteporfin	Norway, China	Age-related macular degeneration, basal cell carcinoma, lung
Laserphyrin-Talaporfin	Japan	Lung cancer, glioma
Photochlor	USA	Basal cell carcinoma, lung, head and neck cancers
Photosens	Russia	Age-related macular degeneration, breast, lung, stomach, skin cancers
Hemoporfin	China	Prader-Willi syndrome

of them were proposed for palliative treatment, such as Foscan® that was approved in 2001 for the treatment of advanced head and neck cancer [52].

Spectral properties (absorption and fluorescence emission) for selected PS molecules are reported in Figure 7.

Porphyrins play important biological roles (e.g., they are involved in oxygen transport), and they find applications in many fields, from medicine to imaging. They contain four 5-membered heterocyclic (pyrrole) rings, linked in a highly conjugated cyclic array with 22 π electrons, resulting in a characteristic absorption in the visible spectrum (from the Greek *porphyra*, purple) [54]. Their typical absorption spectrum consists of an intense and narrow absorption band at approximately 400 nm (Soret or B band) and less intense Q bands at about 500–650 nm. Their typical fluorescence quantum yield ranges between 0.1 and 0.3, and they possess high singlet oxygen yields [54].

Chlorophyll derivatives exhibit the basic structural skeleton of chlorin [55]. Compared to porphyrins, chlorins display 18 π electrons delocalized system [54] and two hydrogen atoms in the pyrrole ring, causing a lower solubility in an aqueous environment [56] and a red shift of the absorption bands. Moreover, chlorins show a higher molar extinction coefficient than porphyrins in the red, and their Q bands are at longer wavelengths (500–700 nm) [33].

Phthalocyanines have a structure deriving from the cyclic condensation of four isoindole units linked together by nitrogen atoms. Some of the advantages are simplicity of synthesis, availability of pure compounds, and high absorption in the red region [57]. They usually have 18 π electrons available [54]. An example is IRDye 700DX, a water-soluble phthalocyanine derivative, often chosen for its strong photocytotoxicity when brought in close vicinity of the cellular membrane.

Despite the therapeutic achievements accomplished in the treatment of certain types of cancer with the second-generation PSs, their lack of specificity resulted in poor targeting capabilities toward malignant cells, with negative consequences such as a long time for the drug to reach the tumor area, unspecific accumulation in healthy surrounding cells, and the need to use a high dose of PS to obtain a significant therapeutic effect [58,59]. To increase selectivity, second-generation PSs were combined with specific target molecules such as surface markers or antibodies directed to specific antigens [28,39].

Targeting tumor cells with antibodies

Photo-immunotherapy (PIT) is based on the use of photo-immunoconjugates (PICs), delivery systems where conjugation of a PS to specific antibodies enables the recognition of and binding to cancer-associated antigens [30], combining the advantages of the photodynamic effect with the high specificity of antibody molecules. PDT together with immunotherapy not only allows a higher selective killing of targeted cancer cells but also increases the immune-stimulatory response triggering innate and adaptive immune reactions related to specific cell death pathways [60].
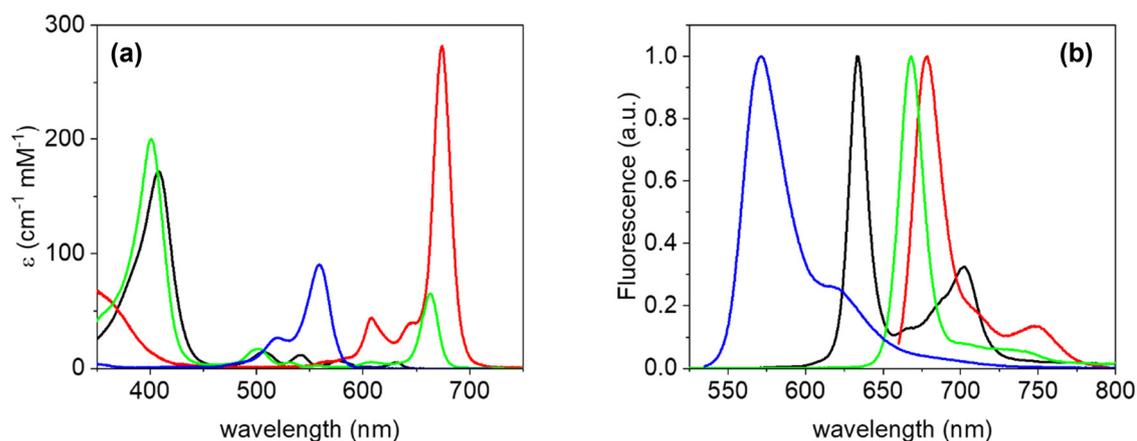

Figure 7: Representative absorption and fluorescence emission spectra by selected photosensitizing molecules. Molar absorption coefficients (a) and normalized fluorescence emission (b) spectra for protoporphyrin IX dimethyl ester in chloroform (black), zinc phthalocyanine in pyridine (red), Chlorin e6 in ethanol (green), and rose bengal in basic ethanol (blue).

The overexpression of certain receptors on the cell surface is correlated with carcinogenic transformation and proliferation of malignant cells. The inhibition of cell growth and tumor spreading can be obtained by targeting those receptors via tailored PICs specifically designed for the malignancy under treatment [28]. In PIT, the array of PS delivery structures spans from monoclonal antibodies (mAbs) to nanobodies (functional active sites of antibodies) [28], each of them able to recognize a specific tumor-associated cell surface antigen.

In clinical treatments, antibodies are used for their high affinity, i.e., low dissociation constants (K_d in the order of nM), and for a wide variety of highly, or exclusively, expressed antigens in many tumor types, which have a relevant role in promoting metastasis, angiogenesis, proliferation, and drug resistance [28].

Nevertheless, the overall success of PIT depends not only on the ability of the antibody to target its antigen but also on the cytotoxic action of the PS once conjugated to the carrier. Examples of fully functional constructs that have arrived at the clinical or preclinical phase comprise cetuximab, panitumumab, and trastuzumab conjugated with IRDye700DX [28].

Interestingly, in spite of the large number of available PSs, just a few of them have been used in PIT. In particular, as shown in the pie chart in Figure 8, IRDye 700DX and chlorin e6 were chosen in around 63% of the photoactive mAb-PS complexes reported in the literature examined in this review. Representative compounds are also reported in Figure 8.

Various strategies have been developed over the years to produce antibody conjugates [31]. Some methods of direct conjugation of the PS to an antibody are as follows:
- Via lysines: The lysine residues present on the antibody can bind the activated PSs with an isothiocyanate group (NCS) or succinimidyl ester (NHS) [30,33].
- Via cysteines: The thiol groups of cysteine residues on the antibody can be used to bind PSs modified with a maleimide or succinimide group. Cys offer more predictable and suitable conjugation sites than lysines due to their lower abundance [43].
- Via carbodiimide coupling: Carbodiimide reagents (as EDC and DCC) are used to activate the carboxyl groups present on the PS that subsequently are coupled with the amino of the antibody [28,33,59].
- Via a "click" path (or CuAAC, copper-azide-catalyzed alkyne cycloaddition reaction) [30,31,33,59,61].

A possible problem arising from these approaches is that conjugation may occur at the antibody–antigen recognition site, and in this case, the targeting activity (immunoreactivity) could be inhibited or even lost. One strategy to overcome this problem is to genetically modify the antibody and mutate potentially reactive groups (Lys, Cys,...) located near the binding site, although this requires to check whether the removal of these amino acids from the antigen-binding site affects the ability of the antibody to bind the epitope [30].

Another critical feature is the possible influence of a high degrees of labeling on mAb immunoreactivity [30]. In particular, the number of PSs per antibody (degree of labeling, DOL) is difficult to control and therefore often mixtures of PICs with heterogeneous compositions (both in terms of DOL and conjugation position) are obtained, also different from batch to batch. However, well-defined protocols, with strictly controlled reagent concentrations, solvent, and reaction times, allow to achieve a certain degree of reproducibility [59].

To improve the intracellular accumulation of PICs in malignant cells, a charge excess on the construct can be exploited. Cationic PICs were found to have a 17 times higher uptake efficiency than free PS and 12 times higher than anionic ones. This larger accumulation of cationic PICs is due to their interaction with the overall negative charge of the cell membrane of cancer cells (malignant cells have a more negative charge than healthy cells) [30]. However, it is worth noting that several factors determine the effectiveness of the construct. On the one hand, binding to a specific site of the cell membrane seems to be the crucial feature to induce the cellular death, more important than the specific intracellular location of the PIC, as shown by studies using trastuzumab-conjugated to a phthalocyanine [45]. On the other hand, *in vitro* studies showed that PSs conjugated with some internalizing mAbs seem to be able to produce higher photocytotoxic effects than PSs conjugated with noninternalizing mAbs [30].

Cancer cell lines and molecular targets

A wide array of different cell lines has been examined to assess the photosensitizing efficiency and targeting capabilities of PICs. Figure 9a sorts these cancer lines by their occurrence in the examined literature. The most investigated cancer type in PIT studies is breast cancer (12.4%), followed by malignant pleural mesothelioma (MPM; 8.8%) and colon and gastric cancer (8 and 6.6% respectively).
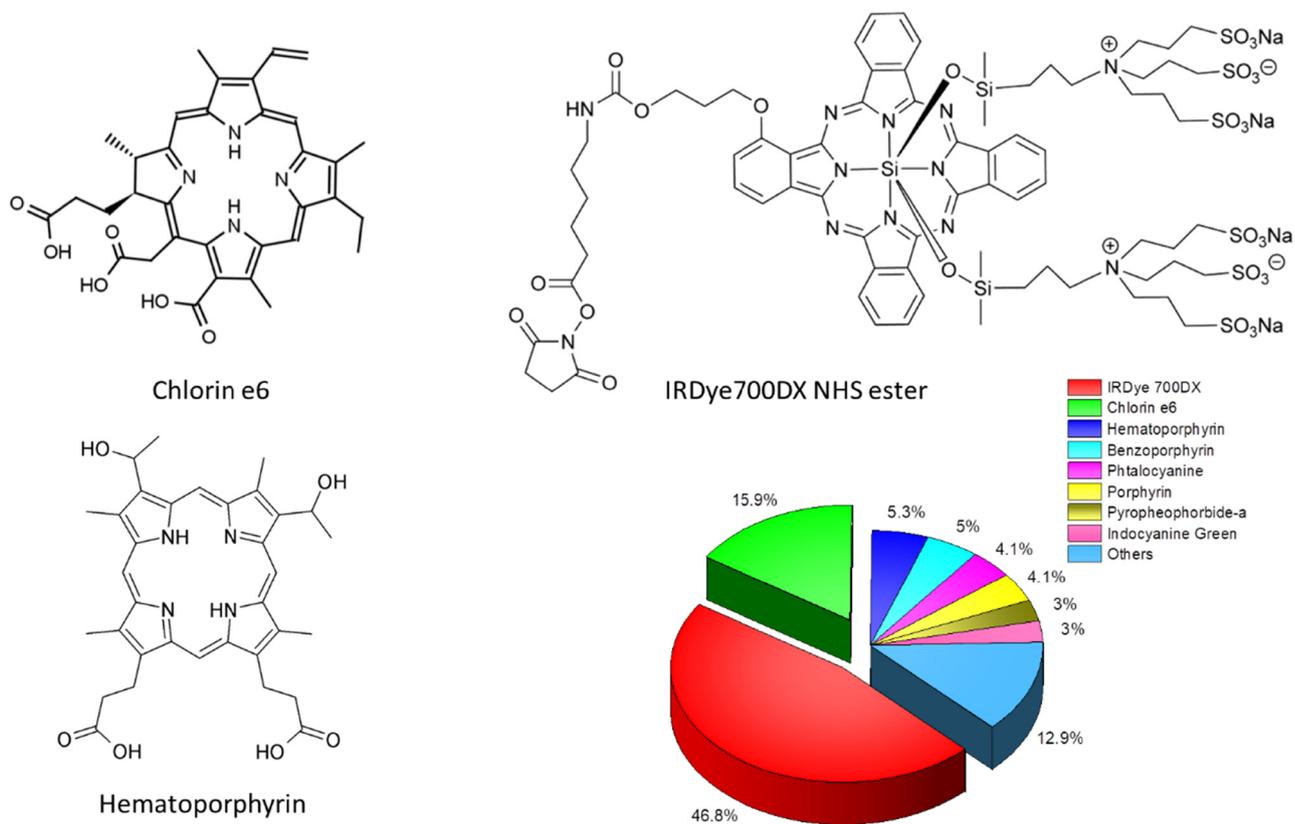
Figure 8: Representative PS molecules used in PICs and visual representation of the distribution of PS in the examined PIT literature.

The molecular targets of PICs are cancer-associated antigens resulting from an aberrant overexpression of nonmutated proteins [62]; these molecular targets are recognized by the PIT agents, whose photo-activation can inhibit or halt cancer progression by triggering cell death mechanisms. Moreover, direct damage on cancer
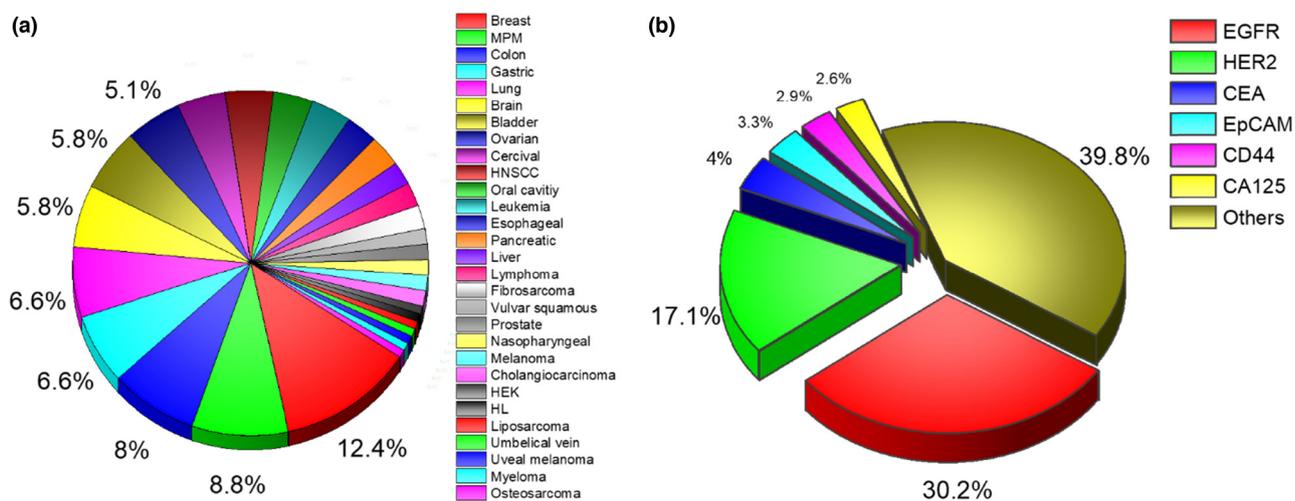

Figure 9: (a) Pie chart visualization for the occurrence of cancer cell lines used in different PIT studies. Only cell types with more than 5% of occurrence are listed with the corresponding label. (b) Visual representation of the antigens most frequently targeted by antibodies in PIT. The pie chart shows that the most recurrent antigen in the examined PIT studies is EGFR, immediately followed by HER2, CEA, EpCAM, CD44, and CA125. Receptors with less than 2% occurrence account for almost 40% of the analyzed papers, indicating that a variety of molecular targets has been considered.

cells induced by reactive oxygen species and produced during PIT can provoke an inflammatory reaction at the targeted tumor site [63]. Consequent stimulation of the immune response of the organism increases its beneficial effects.

Tumor-associated antigens targeted by PICs are usually presented to the extracellular space on the plasma membrane: in general, once a mAb-based conjugate is bound to the target, it is often retained on the plasma membrane due to its molecular weight (110–140 kDa) [30]. On the other hand, PICs based on antibody fragments and nanobodies are likely to be internalized thanks to the presence of receptor-mediated endocytosis [30]. In many cases, antibodies are used to decorate the external surface of liposomes and nanoparticles, providing them with the aforementioned targeting capabilities.

Since the range of antigens that are exclusively or highly expressed in tumors is very broad, the variegate arsenal of PICs for targeting different cancer cell types has experienced a remarkable development in the past few years. The analysis of the literature evidenced that the most common membrane receptors exploited as targets are EGFR, HER2, CEA, EpCAM, CD44, and CA125 (see Figure 9b). In the following, we briefly summarize some recent studies on PICs targeting these receptors.

Cancer antigen 125 (CA125), also called mucin 16 (MUC16), is a transmembrane glycoprotein of the mucin family. Its extracellular domain includes many highly conserved tandem repeats, and two of these regions (OC125 and M11) are recognized by anti-CA125 antibodies [64]. CA125 is overexpressed in ovarian cancer and also expressed on the epithelial surface of various organs, including the respiratory tract [65]. It is a commonly used marker for ovarian cancer diagnosis, response to treatment, and prediction of recurrence [64].

Binding of CA125 receptor was achieved by the antibody OC125 [66,67] and its fragment $F(ab')_2$ [68–72].

In PICs against CA125, the most used PS is chlorin e6 [66,67,69–72], but hematoporphyrin [68] was also used. A study combining PIT with a chemotherapeutic agent (cisplatin) demonstrated increased cytotoxicity [70].

By comparing cationic and anionic PICs, it was found that cationic ones have higher tumor selectivity, lead to a greater cellular uptake of PSs with larger tumor reduction, and can stimulate endocytosis and lysosomal degradation of the PIC [69,71,72].

A second target that has been studied in detail is a cluster of differentiation 44 (CD44). CD44 is a transmembrane glycoprotein involved in cell interaction, cell adhesion, and migration. It exists in various forms generated by alternative splicing. CD44 acts as a receptor for a variety of molecules such as hyaluronic acid, osteopontin, and collagen. It is expressed in many mammalian cells and overexpressed in many tumor-regulating cell metastasis, including colon and stomach cancer [73].

The most used antibody in the recognition of the CD44 receptor is IM7 [74–79]. In addition, generic antibodies [80–82] and the BIWA 4 antibody [83] were also used.

The isoform CD44v8-10 has been determined as a promising biomarker. Therefore, an anti-CD44v antibody was linked to methylene blue, and its effectiveness in PIT against gastric cancer was demonstrated [84].

On the PS side, IRDye 700DX was adopted in several PIT studies against CD44-expressing cells [74–81]. In addition to other phthalocyanines [83], the use of PSs of the chlorophyll family, chlorine [83], and chlorine e6 [82] was also reported.

In one case, the PS was not directly bound to the antibody, but the PIC was obtained by conjugating the PS to a biotinylated antibody using neutravidin [80].

Some studies have shown that it is possible to increase the effectiveness of PIT by combining the action of PICs with other molecules. Examples include the short-term interleukin-15 (a cytokine that activates natural killer and B- and T-cells) [74]; the PD-1 blockade (it enhanced the preexisting tumor antigen-specific T-cell response) [76,78]; a systemic CTLA4 immune checkpoint inhibitor [75]; and a second PIC (with an anti-CD25 antibody) [77].

Finally, by running blood through a thin extracorporeal tube, PIT can be used to eliminate circulating cancer cells from the bloodstream [82].

A third molecular target is the carcinoembryonic antigen (CEA), a glycoprotein involved in cell adhesion and immunologically characterized as CD66 [85]. It can be found at aberrant levels in patients with primary colorectal cancer or other malignancies; it seems to play a crucial role in important cellular functions such as cell proliferation and protection against apoptosis [28].

The use of several antibodies to target CEA in PIT has been reported: 35A7 [86], C2-45 [87], F11-39 [88], M5A [89], single-chain Fvs MFE-23 [37], as well as other generic antibodies [90–94].

A variety of PSs have been introduced in these PICs. For the porphyrin family, benzoporphyrin [94], hematoporphyrin [93], photofrin II [94], porphyrin [86,88], and verteporfin [37] was reported. Other PSs include pyropheophorbide-a [37] for the chlorophyll family and IRDye 700DX [87,89–92] for the phthalocyanine family.

Phototoxicity and predominant localization on the plasma membrane of PICs were found to be equivalent after 1 and 6 h of incubation with CEA-positive tumor cells. In the same study, PICs unexpectedly maintained

significant phototoxicity even under hypoxic conditions [87]. Intravenous administration of PICs leads them to accumulate mainly in the liver and then in the tumor area, while the intratumoral administration of PIC results in longer retention in the tumor without accumulation in normal tissues [93].

Repeated PIT treatment arrested the cancer growth for a longer period of time than a one-time PIT treatment [89].

Perhaps, the most studied molecular target is the epidermal growth factor receptor (EGFR, also called ErbB1 or HER1). EGFR is a transmembrane protein that binds specific ligands, including the epidermal growth factor (EGF). Binding leads to a series of conformational changes that activate specific EGFR signaling pathways, going from cell proliferation to the blocking of apoptosis [95]. It is the most widely utilized membrane receptor and the first molecular target against which mAbs (such as cetuximab and panitumumab) were developed for anticancer

therapy. Playing a crucial role in both normal and cancerous cells, it has been used in clinical practice for the treatment of metastatic colorectal and head and neck cancer. It is overexpressed in many cancers, including head and neck, breast, lung, colorectal, prostate, kidney, pancreas, ovary, and bladder [95].

Approved antibodies (cetuximab [96–114], panitumumab [102,111,115–147], and trastuzumab [148]) have been used to develop PICs to target cancer cells that overexpress the EGFR receptor.

A comparative study on two monoclonal antibodies targeting EGFR and showing internalization (Figure 10) demonstrated lower antitumor efficacy *in vivo* (lower accumulation into the tumors and more rapid hepatic catabolism) of PICs based on cetuximab compared to those using panitumumab [102].

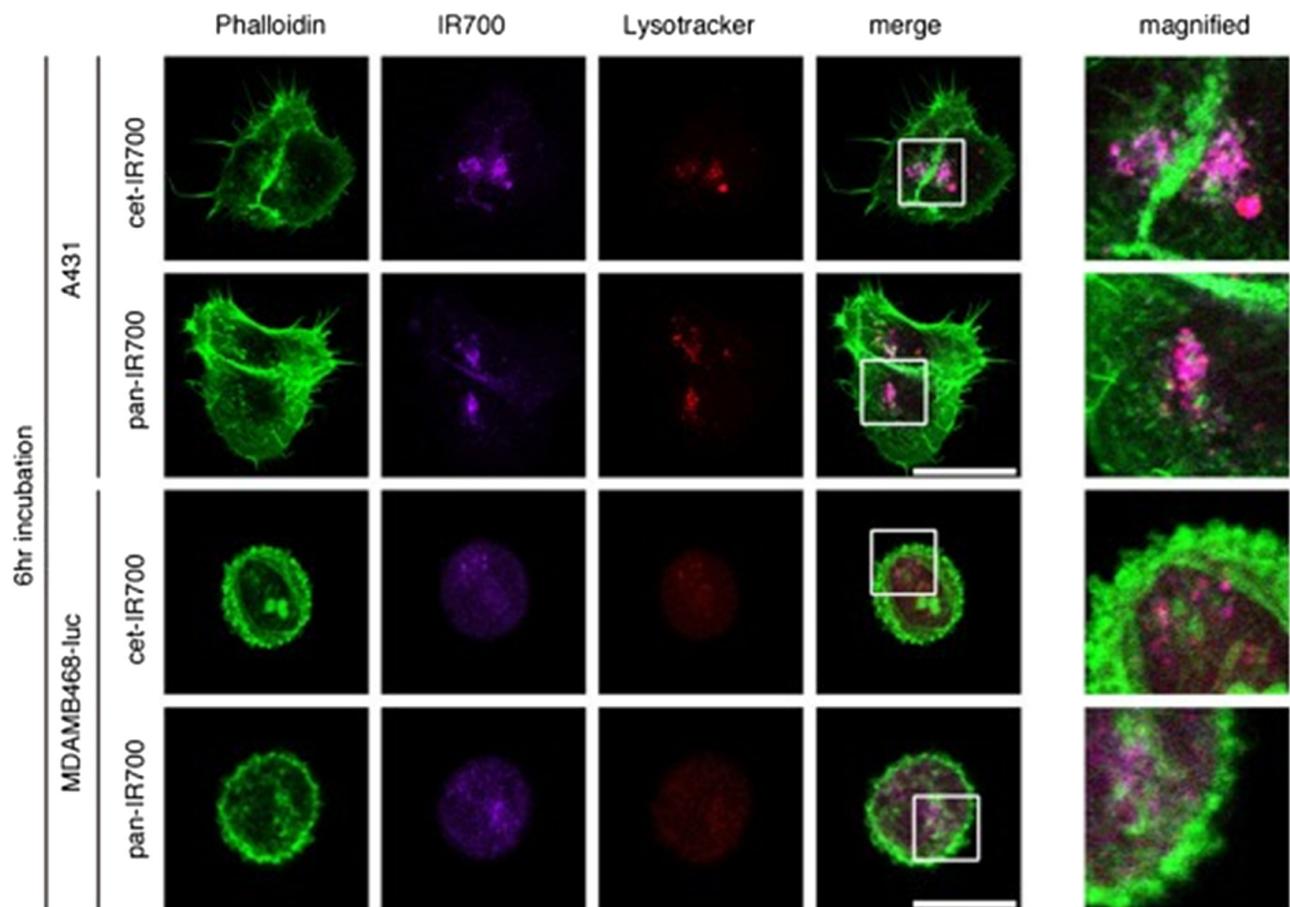Additional antibodies that have been evaluated are 425 [83,149,150], 7D12 [109,151,152], A225 [153], C225

Figure 10: Cellular internalization of the PIC. The EGFR-positive A431 and MDAMB468-luc cells were incubated with cetuximab-IR700 (cet-IR700) or panitumumab-IR700 (pan-IR700). Immunostaining was performed with phalloidin (actin membrane detection, green) and LysoTracker (lysosome detection, red). After 6 h of incubation, PICs were internalized into the lysosome (colocalization with lysotracker). Scale bar = 25 µm. Reproduced from Sato et al. [102].

[154–156], can225 [157], and other generic antibodies [94,158,159]. Some studies have exploited the greater permeability of antibody fragments [150,151,160] or affibodies [161].

The most commonly used PS in PIT against EGFR receptor is IRDye 700DX [96–100,102,109,111,115–118, 120–124,126–129,134–149,151,152,157,160–164]. Porphyrins used in PICs are benzoporphyrin [94,101,105,106,108,110, 112,153–155] and photofrin II [94]. For the chlorophyll family, the following PSs were used: chlorin [83], chlorin e6 [103,114,150,156,158,159], and pyropheophorbide-a [104]. Additional PSs include Cy5.5 [156], indocyanine green [125], and IRDye 800CW [100,123,133]. Finally, the use of antibody-labeled nanoparticles [108,112,159], micelles [114,151], or liposomes [106] has been also explored. A study in which a PS was encapsulated in a viral envelope and tagged with an antibody showed that the complex was internalized [158].

A greater antitumor efficacy was obtained by combining the action of PICs that target different receptors [131,143,149] or by combining the action of a PIC with a chemotherapeutic agent such as cisplatin and paclitaxel [103], doxorubicin [104], or irinotecan [108].

Another strategy used to destroy tumor cells is fractional administration of PIC followed by repeated and systematic near IR light irradiation [137] or PIC dose splitting and repeated use of light exposures [100] because PICs penetrate deeper into the tissue, thus increasing the cytotoxic efficacy after the second session of PIT [99].

Due to the localization of the antigen–PIC complex, light activation may induce physical stress within the cell membrane leading to an increase in the transmembrane water flow, which then causes cell necrosis [111].

As exemplified in Figure 11, the massive entry of solvent molecules is responsible for membrane swelling and rupture with the release of intracytoplasmic contents into the extracellular space. Time-lapse fluorescence imaging of cells expressing GFP, allowed to visualize fast dispersion of the fluorescent protein upon rupture of the cell membrane [98].

The effectiveness of PIT depends not only on the density of the receptors but also on the intrinsic biological properties of the tumor cell lines [113]. PIT has distinct effects on cells with different shapes (spindle shaped and spherical shaped), where the adhesive cells demonstrated region-specific cell membrane rupture occurring
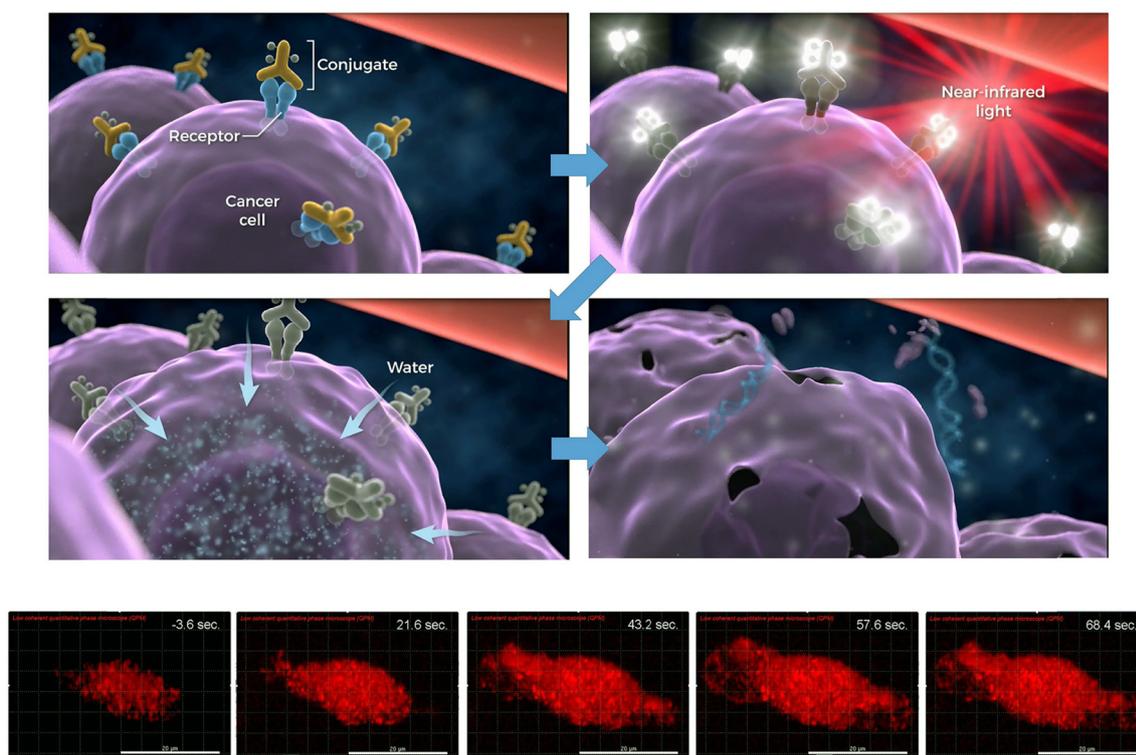

Figure 11: Cartoon visualization of the photoinduced damages on the cell membrane and the subsequent entry of solvent molecules upon permeabilization. The bottom line shows time-lapse images for cellular cytotoxicity induced by NIR-PIT. Reproduced from ref. [165]. https://pubs.acs.org/doi/10.1021/acs.accounts.9b00273. Further permission related to the material excerpted should be directed to the ACS.

first on the distal free edge of the cell near the site of adhesion [117]. Due to the effect of PIT, rapid cell expansion may be followed by rupture of the membrane (inducing a rapid decrease in the blood flow) [116] or the cell membrane can become highly permeable to larger molecules as bubbles and ruptures form on it [98].

Hours after PIT, the tumor vessels become supraphysiologically permeable, and circulating PICs can readily leak into the already-treated tumor space where it can bind with viable cancer cells, and this is the so-called super enhanced permeability and retention (SUPR) effect in which PIT induces the death of cancer cells leading to an immediate and dramatic increase in vascular permeability with consequent accumulation of nanomaterials inside the tumor treated with PIT. Due to the increase in vascular permeability immediately after PIT, PICs can access more tumor areas after treatment with PIT [145]. Repeated exposures of near IR light starting 3 h after the initial PIT produced superior results than single-light exposure regimes and equal to or greater than longer NIR light exposures [139]. After PIT, there is a limited time-window during which nanosized particles (as liposome-containing daunorubicin) could be administered to augment the effects of PIT [133]. The SUPR effect allowed for a fivefold increase in the accumulation of a liposomal chemotherapy (DaunoXome), leading to more effective therapy than PIT alone or daunorubicin [130]. In addition, PIT greatly reduces the speed of the blood, while the blood vessel is not damaged or thrombosed. The low blood flow speed implies a long time of circulation of the drug, and the dilated central vessels can lead to a slowdown of the peripheral flow and an increase in the volume of the drug pool in tumor vessels [127].

The use of imaging markers to follow, in real-time or in the early stages after PIT, enabled the visualization of the efficacy of the treatment and the cell death. The fluorescent localization showed that, in tumor tissue, PIC concentration increased during the 60-minute time window after injection [136] and that the amount of PIC peaked at 24–48 h after injection [97]. Studies on luciferase–luciferin indicate therapeutic effects in the early phase as well as in the late phase after PIT, while GFP fluorescence imaging (Figure 12) is able to report therapeutic effects on longer time scales [119].

EpCAM

The epithelial cell adhesion molecule (EpCAM) is expressed in a wide range of human carcinomas and therefore is considered a potential target for cancer treatments. EpCAM, also called CD326, is a transmembrane glycoprotein involved in cell signaling, proliferation, and differentiation. It was originally identified as a tumor-associated antigen due to overexpression in rapidly
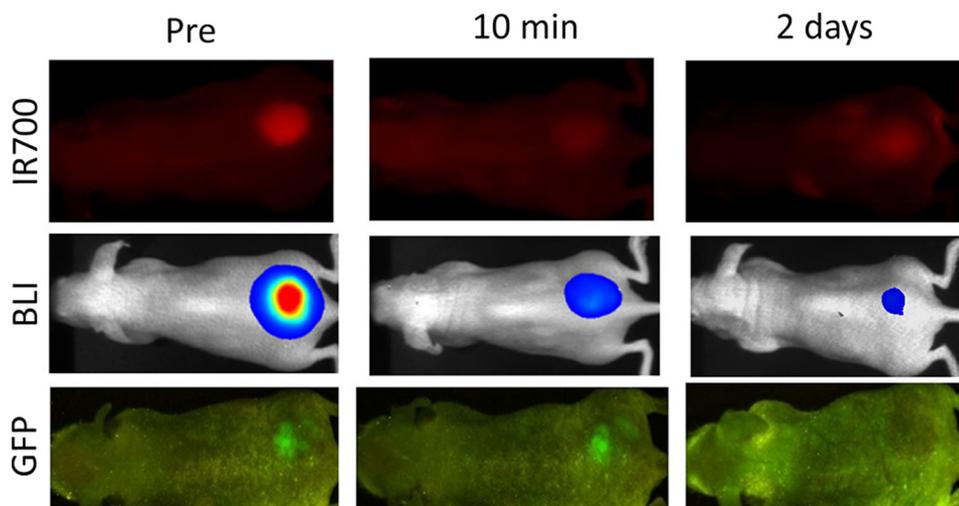

Figure 12: *In vivo* monitoring of photoimmunotherapy effects. Real-time images of A431-luc-GFP tumor-bearing mice. Before NIR-PIT panitumumab-IR700 (red) accumulates in the tumor. Immediately after irradiation, the fluorescence decreases, while after 48 h, it shows an increase (probably due to the injection of PICs into the tumor region). Luciferase–luciferin photon counting (color gradient) gradually decreased, indicating progressively cell death. Immediately after irradiation, GFP fluorescence (green) is almost constant, but after 48 h, it shows a strong decrease (suggesting cell death). Reprinted with permission from Maruoka et al. [119]. Copyright 2017 American Chemical Society.

growing epithelial tumors, being highly expressed in carcinomas [166].

A human EpCAM-targeting monoclonal antibody (3–17 l) has been used in combination with the photosensitizer TPCS2a for a photochemical internalization approach [167]. *In vitro* experiments showed that 3–17 l is a good candidate for the diagnosis of EpCAM-positive tumors, potentially relevant for antibody–drug conjugations. Other PICs able to recognize EpCAM have been engineered with the 17.1 A antibody [86,168–170], some generic whole antibodies [80,171,172], or the scFv antibody [149].

Different PSs were used for PIT against cells expressing the EpCAM antigen including porphyrin [86,173], chlorin e6 [168–170], the phthalocyanine IRDye 700DX [80,149,171], and mitoxantrone [172].

In addition to being directly conjugated to antibodies, PSs have been bound via a linker molecule (neutravidin) [80] or incorporated into nanoparticle-based micelles [172].

HER2

HER2, also called Erb2, is a transmembrane glycoprotein expressed in many tissues, and it facilitates cell growth. HER2 plays a role in the development of carcinogenic diseases, and it is overexpressed in approximately 15–30% of breast cancers. Furthermore, it was found that such an overexpression occurs also in other cancers, including ovary, bladder, lung, colon, head, and neck [174].

The most common strategy for targeting the HER2 antigen on cancer cells is to use the clinically approved antibody trastuzumab [98,99,111,117,123,124,141,143,144, 148,154,163,175–195]. Other antibodies used against HER2 are pertuzumab [186] and C6.5 [37,196,197]. Affibodies were employed to increase the concentration of the drug at the tumor site, exploiting the higher permeability to small ligands [198–201].

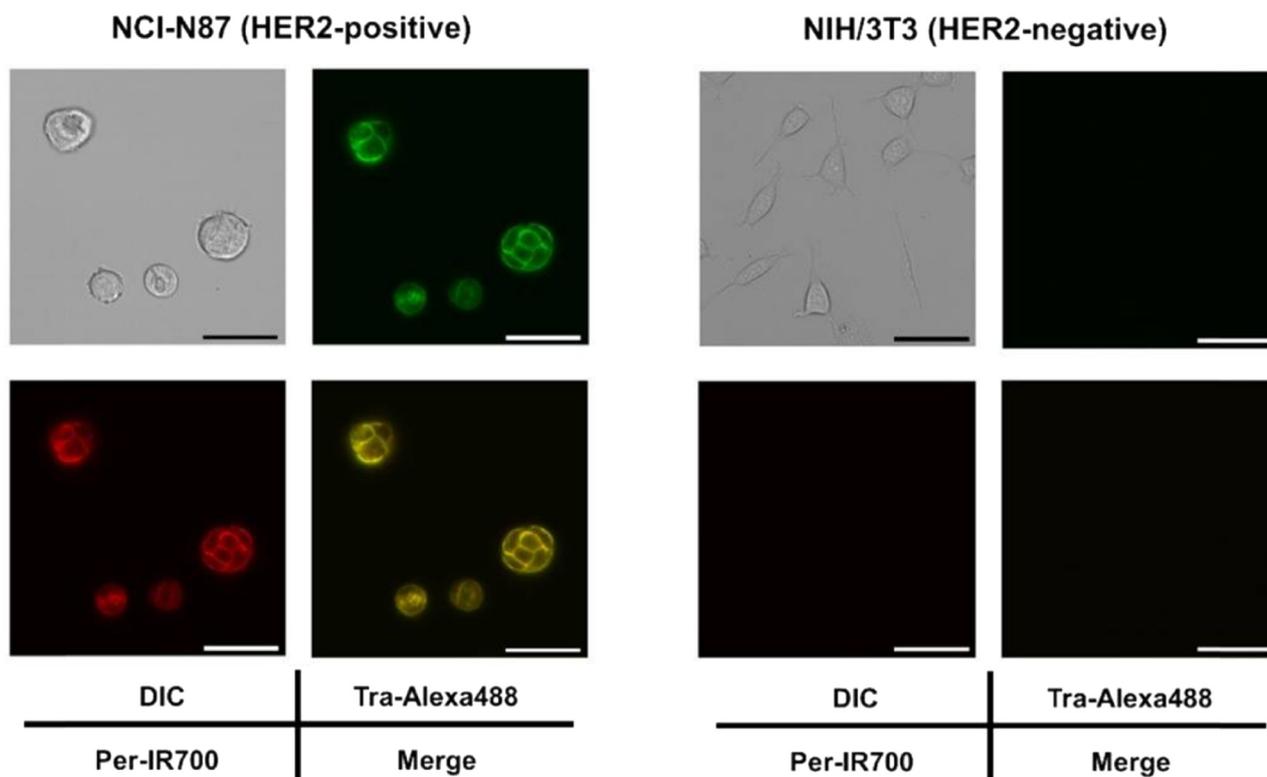The more commonly employed PS in constructs against HER2 is IRDye 700DX [98,99,111,119,123,124,141,

Figure 13: PICs targeting the cell receptor with different antibodies. Both trastuzumab-Alexa488 (green) and pertuzumab-IR700 (red) target HER2. After 3 h of incubation, their fluorescence is localized on the cell surface of the HER2-positive NCI-N87 cells (left) but not in the HER2-negative NIH/3T3 (right). DIC: differential interference contrast. Scale bar = 50 μm. Figure reproduced under a Creative Commons Attribution 3.0 License from ref. [186].

143,144,148,175,177–179,181,185–193,198,201], although a variety of other compounds were also used.

The combined action of two PICs targeting different receptors [143] or the same receptor but with different antibodies [186,202] demonstrated improved efficacy. As an example, Figure 13 shows that PICs based on monoclonal antibodies recognizing different epitopes of HER2 can only target cells expressing the specific antigen recognized by the antibodies.

Also for this case, the cytotoxic effect of PIT was also combined with the chemotherapeutic agent (5-fluorouracil [188], doxorubicin [203], and rapamycin [184]) or the cytotoxic agent (maytansinoid [185]).

Targeting of HER2-negative cancer cells was also obtained with an adenoviral vector that transduced HER2-extracellular domain into HER2-negative human cancer cells [191].

Interestingly, improved cell killing was reported after a second PIT session because the first PIT session kills primarily cells on the surface of the tumor but, due to increased vascular permeability, PICs administered immediately after PIT penetrated deeper into the tissue [99].

Light activation of HER2-bound PIC causes conformational changes in the shape of the antigen–antibody complex that induces physical stress within the cell membrane, leading to an increase in the transmembrane water flow, which eventually causes the cell bursting [111]. Although plasma membrane perforations caused by PIT may be minimal, this damage is irreversible and worsens over time causing the cell death [179]. After PIT, the cell membrane forms bubbles and ruptures, becoming highly permeable to larger molecules and the release of molecular patterns associated with the damage induces maturation of dendritic cells [98]. After PIT, in adhesive cells with different shapes (spindle shaped, spherical shaped), peripheral portions of the cell membrane near the site of adhesion are particularly vulnerable to the effects of PIT (likely because these sites exhibit higher baseline surface tension) [117].

Conclusion

The development of photoimmunoconjugates, delivery supramolecular systems endowed with targeting capability and photosensitizing properties, has allowed an important step forward in PDT, providing unprecedented tissue and cellular selectivity, and improved phototoxic action.

Thanks to the interaction between the antibody and the target antigen molecule, PICs are brought in close vicinity of sensitive cellular components that are damaged when the PS is excited by the visible light, thus leading to enhanced phototoxicity relative to the photosensitizer devoid of any targeting functionality.

The concomitant stimulation of the immune-stimulatory response triggering innate and adaptive immune reactions related to specific cell death pathways potentiates the direct photoinduced damages and open perspectives for the development of personalized, cancer-specific therapy.

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