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Photosensitizer-antibody conjugates for detection and therapy of cancer

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Abstract

Photodynamic therapy (PDT) is a promising approach for the treatment of superficially localized tumors. A limitation, however, is the lack of selectivity of the photosensitizers, which can result in severe toxicity. In this overview, the possibilities for using monoclonal antibodies (MAbs) for selective delivery of photosensitizers to tumors, are discussed. This approach is called photoimmunotherapy (PIT). For PIT to be successful, sufficient amounts of sensitizer should be coupled to the MAb without altering its biological properties. A challenging aspect herein is the hydrophobicity of therapeutic photosensitizers. Options for direct and indirect coupling of photosensitizers to MAbs are evaluated, while pros and cons are indicated. Special attention is paid to the quality testing of photoimmunoconjugates, as this information is important for further optimization of PIT. Results obtained thus far with PIT in in vitro and in vivo model systems are discussed. Despite the encouraging progress made, showing the high selectivity of photoimmunoconjugates, PIT still awaits initial clinical evaluation.

Keywords: Photoimmunotherapy; Photodynamic therapy; Photoimmunoconjugates; Monoclonal antibody; Tumor targeting

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1. Introduction

Photodynamic therapy (PDT) is a therapeutic modality especially applied for the treatment of superficially localized tumors. In this approach, a photosensitive dye (photosensitizer) is injected intravenously (i.v.) or intraperitoneally (i.p.), where after it accumulates more or less selectively in the tumor. Due to the higher sensitizer concentration in the tumor compared to surrounding normal tissue, the tumor can be visualized by sensitizer fluorescence. For therapeutic application, the tumor becomes exposed to laser light, whereby the excitation of the photosensitizer results in the production of reactive species such as singlet oxygen, which are toxic for the tumor [1]. Light in the red or near-infrared region is used with a wavelength, which is maximally absorbed by the sensitizer. This maximum absorption wavelength of the sensitizer is an important parameter in PDT, since light of a longer wavelength penetrates deeper in the tissue, thereby making treatment of larger tumors possible. Another approach facilitating the treatment of larger tumors is the use of interstitial illumination techniques. PDT has been applied clinically for the treatment of a variety of tumor types [2]. The most promising results thus far have been obtained in head and neck and esophagus cancer, locoregional breast cancer recurrences and basal cell carcinoma. PDT has also attracted attention in relation to several other clinical applications, but these will not be subjects of the present overview.

Despite its promising results, current PDT leaves much to be desired. A limitation is the lack of selectivity of the photosensitizers, which can result in severe normal tissue damage after PDT of large surface areas, like in the treatment of disseminated i.p. tumors or mesothelioma. Furthermore, PDT can result in skin phototoxicity, with the consequence that patients must stay out of bright sunlight for several weeks following the administration of the photosensitizer. An option to overcome these problems is to couple the photosensitizer to monoclonal antibodies (MAbs) directed to tumor-associated antigens. This so-called photoimmunotherapy (PIT) aims at the selective delivery of photosensitizers to the tumor. With this approach the problem of cutaneous phototoxicity might be reduced, as the skin is poorly permeable for macromolecules like immunoglobulins (vide infra).

2. Critical targets for photodynamic effects

The potentially critical cellular and subcellular targets for PDT have been intensively studied with unconjugated photosensitizers. Besides direct cell killing as a result of phototoxic cell damage, indirect effects also seem to play an important role in the destruction of tumor tissue. This information might be important, when aiming to hit the hypersensitive cellular and subcellular sites with PIT. Since singlet oxygen has a short lifetime (<0.04 μs) and a radius of action (<0.02 μm), which is short in comparison with the diameter of a tumor cell (±10 μm), targeting of the photosensitizer to the most critical cellular structures has to be performed with some precision [3].

2.1. Direct effects with unconjugated photosensitizers

Direct cytotoxicity results from incorporation of the sensitizers into cellular membranes. Membrane damage leads to swelling, bleb formation, shedding of vesicles containing cytosolic enzymes, and inhibition of membrane enzymes.

Several studies point at the mitochondrial membrane, as being one of the critical cellular structures in PDT with free photosensitizers. For porphyrins it has been demonstrated that after initial localization in the plasma membrane, they accumulate in the nuclear membrane and other organelles, especially mitochondria and lysosomes [4]. The specific pattern of localization, however, possibly depends on the cell type, the sensitizer and/or the time of sensitizer exposure. Wilson et al. [5] compared the subcellular localization of Photofrin® in PDT-sensitive RIF-1 cells and PDT-resistant RIF-8A cells, using confocal fluorescence microscopy. Whereas Photofrin® strongly localized in the inner mitochondrial membrane of RIF-1 cells, this was not the case for RIF-8A cells, suggesting the importance of mitochondrial damage for phototoxicity.

Care should be taken, however, when interpreting Photofrin® localization data because this sensitizer consists of numerous porphyrin species with different fluorescence quantum yields. Therefore, fluorescence measurements do not necessarily reflect the most important photodynamically active porphyrin species at any given binding site. To circumvent this problem, Woodburn et al. [6] synthesized a range of pure, monomeric porphyrin derivatives, varying in hydrophobicity and charge. The subcellular localization was studied in C_6 glioma and V79 Chinese hamster lung fibroblast cells using confocal laser scanning microscopy. In general, cationic porphyrin derivatives localized in mitochondria, whereas those with a more anionic character appeared to localize in lysosomes. In a subsequent study to assess the phototoxicity of these compounds in C_6 cells, a significant correlation was found between subcellular localization and degree of phototoxicity [7]. The most phototoxic compounds (those with cationic side chains) accumulated in mitochondria.

Additional information on the involvement of mitochondria in PDT-induced damage came from studies of Chiu et al. [8]. They showed that photodynamic treatment of mouse L5178Y-R cells with silicon phthalocyanine tetrasulfonate ($SiPcS_4$) caused release of cytochrome *c* into the cytosol, which is a critical step in the mitochondrial pathway of apoptosis. This release was not the result of immediate damage to the mitochondrial membrane, as it occurred 15 min after illumination.

Fabris et al. [9] determined the intracellular localization and phototoxicity of zinc phthalocyanine (ZnPc) as a function of the incubation time in the rat embryo fibroblast cell line 4R. After 2 h incubation, fluorescence microscopy showed that ZnPc was present in the Golgi apparatus, and to a lesser extent in the plasma membrane. After 24 h, ZnPc was still present in the Golgi apparatus, but mitochondrial localization could be clearly observed as well. Necrosis, due to loss of plasma membrane integrity and depletion of intracellular ATP, was the prevailing mode of cell death after 2 h incubation. In contrast, illumination performed after 24 h incubation caused only partial inhibition of plasma membrane activities, and cell death occurred largely by apoptosis.

The main subcellular target of *m*THPC-mediated PDT has not yet been defined. A diffuse cytoplasmic distribution of *m*THPC was observed in V79 Chinese hamster lung fibroblasts [10], murine myeloid leukemia M1 cells [11] and HT29 human colon adenocarcinoma cells [12] using fluorescence microscopy. The endoplasmatic reticulum, mitochondria, Golgi apparatus and nuclear membrane were stained by *m*THPC, whereas no *m*THPC fluorescence was observed in the nucleus. Yow et al. [13] showed in two nasopharyngeal carcinoma cell lines (HK1 and CNE2) that *m*THPC-mediated PDT ruptured the mitochondria, indicating that mitochondria are an important subcellular target.

A number of studies have addressed the possible involvement of DNA damage in PDT phototoxicity, but have come to contradictory conclusions. Ramakrishnan et al. [14] observed, in L5178 mouse lymphoma cells treated with chloroaluminium phthalocyanine, phototoxicity to be correlated with the formation of DNA–protein cross-links, and also with the number of DNA strand breaks observed. In contrast, Dougherty et al. [15] concluded that PDT has generally a low potential of causing DNA damage, since most sensitizers do not accumulate in cell nuclei to a large extent. This latter finding does not

mean, however, that DNA would not be sensitive for photodynamic effects, as was illustrated by Sobolev et al., who are aiming for targeting photosensitizers to the cell nucleus [16].

2.2. Indirect effects with unconjugated photosensitizers

The vasculature seems to be the most critical target for indirect photodamage. As a result of PDT-induced damage to the vascular endothelium, hypoxia, anoxia and deprivation of nutrients in the tumor might arise. The underlying mechanism of action, however, might depend on the sensitizer used: PDT with Photofrin® or mono-L-aspartyl chlorin e_6 leads to vessel constriction and thrombus formation [17,18], while the use of phthalocyanines causes vascular leakage [19].

Another indirect effect is the induction of a strong inflammatory reaction [20]. After photodynamic treatment, destroyed tumor cells are phagocytosed by macrophages. These antigen-presenting cells can process tumor-specific antigens and present them on their membrane surface, thereby inducing T lymphocyte mediated cellular immunity.

3. Tumor targeting with monoclonal antibodies (MAbs)

Several factors are known to affect the efficiency of tumor targeting by MAbs in vivo. These factors are related to the antigenic target, the MAb, as well as the tumor. Before results on the production of photosensitizer-MAb conjugates and the evaluation of these conjugates in in vitro and in vivo models are summarized, the influence of each of these factors on the efficiency of targeting will be discussed.

3.1. The target antigen

The hybridoma technology introduced in 1975 by Köhler and Milstein [21] enabled the development of MAbs specifically directed against each particular cellular antigen. In this procedure mice are mostly immunized with tumor cells or with a purified tumor antigen. After fusion of spleen cells from the immunized mouse with myeloma cells, a hybridoma cell clone can be selected that produces a MAb with the desired antigen specificity. Nowadays, also in vitro routes to high affinity MAbs can be explored by using phage display libraries [22].

An ideal antigen for tumor targeting is highly expressed by all tumors in the patient population, at the outer cell surface of all tumor cells, and not by normal tissues. Unfortunately, tumor-specific antigens have only been found in experimentally induced tumors and not in so-called spontaneous tumors. Most identified antigens in human tumors represent tumor-associated antigens, which are present on tumor tissue but are also detectable on normal tissues. Expression of target antigen in normal tissues can be acceptable for PIT, if the tissue is poorly accessible for MAbs, or if the tissue does not become exposed to normal daylight or laser light during treatment.

Another limitation is that antigen expression on tumor cells is neither uniform nor static. In most of the tumors, a percentage of cells will lack the target antigen in question. To deal with the problem of heterogeneous antigen expression, several research groups started the exploitation of antigens on tumor stroma [23] or neo-vasculature [24–26] for tumor targeting with MAbs. This latter might be especially attractive because markers of angiogenesis are expressed by a diversity of tumor types and they are well accessible for MAbs. What is more, the vasculature is considered to be a critical target for photodamage (see Section 2.2).

Shedding of an antigen by the tumor into the blood is considered to be a disadvantage, since circulating antigen can trap the injected photoimmunoconjugate before it reaches the tumor, and this makes tumor targeting less efficient.

After binding of the conjugate to a surface antigen, the formed complex can be internalized by the tumor cell. Because of the intracellular localization of at least a part of the critical structures for phototoxicity, internalization might make a conjugate more effective in PIT [16]. Although data on the internalization of photoimmunoconjugates are scarce, it can be anticipated that the three parameters antigen, antibody and photosensitizer all may have their specific effects on the rate of internalization of the conjugate, on the intracellular trafficking and catab-

olism of the conjugate, and on the level and site of photosensitizer retention. With respect to the latter, accumulation at the hypersensitive cellular and subcellular sites will make the conjugate more effective in PIT (see Section 2).

3.2. The monoclonal antibody

The uptake of a MAb in the tumor depends on the antigen recognized by the MAb, as well as on its molecular size. An intact MAb is a large immunoglobulin molecule with a weight of 150 kDa, which is much larger than the size of a photosensitizer (Mw < 1 kDa). Such large molecules have a limited capability for penetrating a tumor. Moreover, the residence time of an intact MAb in blood is long, resulting in low tumor to normal tissue ratios. For obtaining more favorable ratios, the use of smaller MAb fragments such as $F(ab')_2$ (Mw = 100 kDa), Fab (50 kDa) and Fv (25 kDa) can be an advantage, because smaller fragments penetrate more easily than whole immunoglobulins. Therefore, they have the potential for targeting a larger proportion of the tumor cells, with higher tumor to nontumor ratios at a earlier time after administration. However, the fraction of the administered MAb dose accumulating in the tumor (expressed as percentage of the injected dose per gram of tissue=%ID g^{-1}) is higher for intact MAbs than for fragments [27], which is important for delivery of sufficient amounts of sensitizer to the tumor.

$F(ab')_2$ and Fab fragments can be obtained by proteolytic cleavage of the intact MAb. Recombinant DNA technology can be used to produce the smaller derivatives, such as Fv fragments [28,29]. At the current stage of development, no convincing data are available supporting the choice of either intact MAbs or fragments for PIT. Whether a MAb should have a low or high affinity, and should be administered at low or high dose, strongly depends on the specific MAb used for targeting. These variables are not only influencing MAb uptake and retention in the tumor, but also the homogeneity of MAb distribution throughout the tumor.

Administration of a murine MAb to a patient usually results in a human anti-mouse antibody (HAMA) response. Owing to the presence of HAMAs, a subsequent administration of the MAb can lead to rapid clearance of the injected MAb from the blood, thus preventing efficient tumor targeting. Moreover, an anaphylactic reaction can occur. Arming the MAb with a non-self agent, like for example a photosensitizer, might further increase immunogenicity. To avoid HAMA responses the MAb molecule can be reshaped to human-mouse chimeric (cMAbs) or even humanized (hMAbs) versions by using recombinant DNA techniques. Alternatively, fully human MAbs can be developed, by using phage display libraries or transgenic mice [30].

3.3. The tumor

After i.v. administration to the patient, the large MAb molecules (or conjugates) have to pass several physiological barriers before binding to the antigen [31–33]. When the tumor is reached, the MAb distributes throughout the vascular compartments of the tumor. The vascularization pattern depends strongly on the site of the tumor. Within a tumor the vascularization pattern can be heterogeneous, with extensive vascularization in the vital regions and limited vascularization in the necrotic areas. The average perfusion in tumor tissue is lower than in normal tissue. To reach the tumor cells, MAbs have to pass the blood vessel walls. In normal tissues the barrier offered by endothelial cells varies greatly. In liver, spleen and bone there is virtually no barrier, because the endothelium is fenestrated and the basement membrane is lacking. In contrast, particularly endothelium of lung and skin is poorly permeable for macromolecules. This latter is important, as coupling of photosensitizers to MAbs might reduce cutaneous phototoxicity in this way. In tumor, the endothelium is usually fenestrated, even in tumors arising from tissues that normally have no fenestrated capillaries. In addition, the basement membrane of the tumor endothelium is frequently defective and this likely gives rise to increased permeability. Once the MAb molecules have crossed the blood vessel wall, they have to move through the extracellular space of the tumor stroma by diffusion and convection before they reach the tumor cells. Another factor influencing antibody movement throughout the tumor is the intratumoral pressure. This pressure increases upon tumor growth, and may lead to a reduction of blood flow and reduced extravasation of the MAb. As a consequence, small tumors

show higher MAb uptake levels than large tumors [34,35]. For this and other reasons, PIT seems especially attractive for the eradication of small superficially localized tumors. When the MAb finally has arrived in the peripheral cell layer of a tumor nest it has to pass intercellular junctions before reaching inner cell layers.

The trafficking of MAbs after i.p. administration, strongly depends on the particular MAb or MAb conjugate, and the site of the target antigen. If the target antigen is accessible from the peritoneal cavity, the MAb can bind directly. Alternatively, the MAb can enter the blood circulation and follow the routing as described above for i.v. administration. As a consequence, i.v. and i.p. administrations may lead to different uptake levels in tumors and normal tissues, and to different MAb distribution patterns throughout the tumor deposits [36].

3.4. MAbs for tumor targeting

Nowadays, MAbs with proven in vivo capacity for selective tumor targeting are available for virtually each tumor type. Therefore, PIT does not meet limitations from that point of view. To date, the American Food and Drug Administration (FDA) has approved 11 MAbs/MAb conjugates for therapy (and several others for diagnosis), the majority in the past 4 years for the treatment of cancer such as Rituxan, Herceptin, Mylotarg, Campath, and Zevalin [37–39]. At least 400 other MAbs/MAb conjugates are in clinical trials worldwide, among which are a proportion in phase III trials for treating cancer [39].

All the distinguished anti-cancer MAbs recognize tumor-associated antigens, which implies that they also target some normal tissues. Targets exploited thus far comprise signal transduction pathways (e.g. the HER2-receptor, the Epidermal Growth Factor Receptor, and the Vascular Endothelial Growth Factor), cluster designation (CD) molecules (e.g. CD20, CD22, CD33, CD44, CD52), and oncofetal antigens (e.g. CEA). Besides that, antigens are exploited that are not expressed on tumor cells themselves but on endothelial or stromal cells of the tumor [23–26].

Several strategies have been described to enhance the potency of tumor-selective MAbs. Construction of human antibodies of the IgG1 or IgG3 isotype might provide the MAbs with the capacity for mediating antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). These kinds of toxicity can be effective for the eradication of single tumor cells or small cell clusters in patients, but will be insufficiently effective in combating bulky tumors, especially when the patient is immunocompromized. As an alternative, the MAb can be directly armed with a variety of cell killing agents such as toxins, conventional chemotherapeutics, super-toxic drugs, or radionuclides. Alternative ways for MAb-mediated tumor targeting comprise the use of immunoliposomes or pretargeting strategies, e.g. by using bispecific MAbs.

The success of MAbs thus far has been restricted by the fact that normal cells are also targeted, leading to dose limiting toxicity. With respect to this, the use of photoimmunoconjugates in PIT is an interesting alternative approach as the targeting step is followed by a physical activation step as a second possibility to increase specificity.

4. Photosensitizers for targeting

A large number of photosensitizers have been evaluated in PDT experiments. In this section, we limit ourselves by summarizing the characteristics of the sensitizers, which have frequently been used in PDT as well as in PIT, and still are attracting attention nowadays.

4.1. Haematoporphyrin derivative (HpD)

HpD is prepared by treatment of haematoporphyrin (Fig. 1A) in acetic acid with 5% sulfuric acid as a catalyst (“HpD Stage I”), followed by treatment with an alkaline solution and neutralization. From the resulting solution so-called HpD Stage II is purified. The latter consists of a mixture of mono-, di- and oligomers, all containing the porphyrin moiety.

In 1961, the applicability of HpD Stage II as a diagnostic agent was demonstrated [40]. In the 1970s, its therapeutic potency became clear [41,42]. Because the oligomeric fraction of HpD Stage II appeared to be

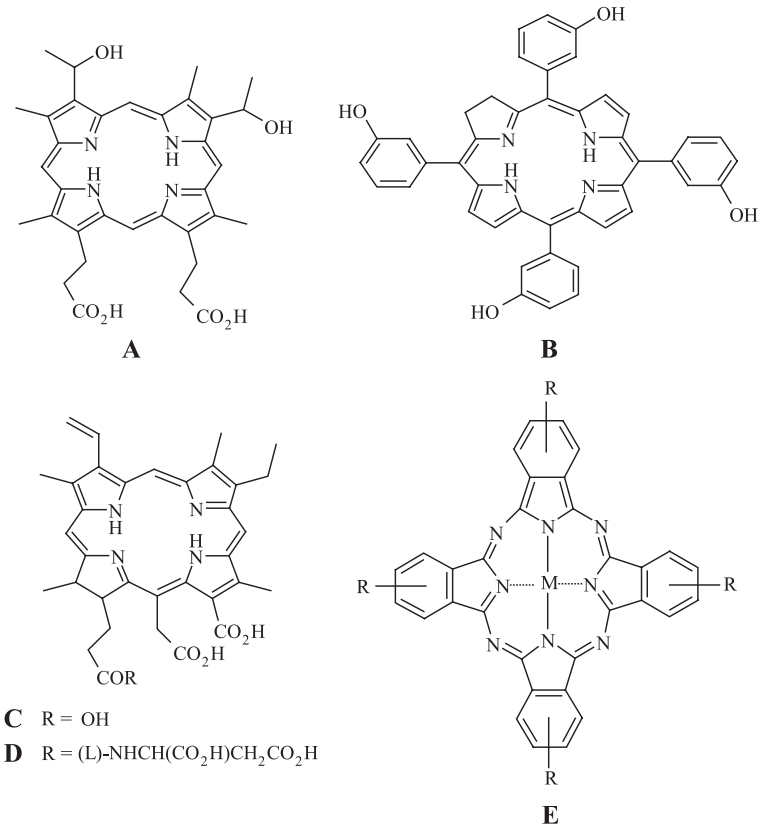

Fig. 1. Chemical structures of (A) haematoporphyrin, (B) *m*THPC, (C) chlorin e_6, (D) mono-L-aspartylchlorin e_6, and (E) phthalocyanine.

largely responsible for phototoxicity, purification methods were developed to provide enrichment of the oligomers [43]. This resulted in the commercial product Photofrin®, which has been registered for the palliative treatment of totally and partially obstructing cancers of the esophagus, as well as for lung cancer [15].

Nowadays, Photofrin® is still the only world-wide registered photosensitizer for treatment of cancer, despite the following limitations: first, even after purification it consists of about 60 compounds, and therefore, it is difficult to reproduce its composition. Such complexity also hampers reproducible coupling of Photofrin® to MAbs. Second, the compound mixture has a suitable absorption maximum at 630 nm, however, its molar absorption coefficient at this wavelength is very low (1170 M^{-1} cm^{-1}). Therefore, high sensitizer and light doses have to be delivered to the tumor. Third, the uptake in tumor tissue is not very selective. Finally, cutaneous photosensitivity, caused by uptake of the sensitizer in the skin is rather long lasting with Photofrin®-based PDT. For that reason, patients treated with Photofrin® have to avoid sunlight for about 4–6 weeks, which appears difficult and inconvenient in practice. An additional difficulty is that the alertness of the patient is not triggered during the light exposure itself, as superficial burns and accompanying pain become manifest just several days later.

Because the characteristics of Photofrin® were far from optimal, several new sensitizers have been developed. Most effort was put into the development of porphyrin derivatives such as benzoporphyrin derivative monoacid ring A (BPD, absorption maximum at 690 nm, molar extinction coefficient of 3.6×10^4 M^{-1} cm^{-1}) and other chlorins, because

the porphyrin moiety is an efficient generator of singlet oxygen.

4.2. Chlorins

The chlorin structure consists of a porphyrin skeleton with one carbon–carbon double bond converted to a carbon–carbon single bond, resulting in absorbance at a wavelength in the far-red region of the spectrum. *Meta*-tetrahydroxyphenylchlorin and mono-L-aspartylchlorin e_6 are the two most frequently applied chlorin-type sensitizers.

4.2.1. Meta-tetrahydroxyphenylchlorin (mTHPC)

*m*THPC (or temoporfin, commercially produced as Foscan®; Fig. 1B) was first described by Bonnett et al. [44], in their study on a series of *meso*-tetrahydroxyphenyl porphyrin derivatives. Its photochemical properties are favorable: it has a strong absorption (molar extinction coefficient 2.2×10^4 M^{-1} cm^{-1}) at an absorption maximum of 652 nm. In an extensive comparative in vivo study, *m*THPC appeared to be the most phototoxic compound of this series, with lowest normal tissue toxicity [45].

Nevertheless, also *m*THPC shows lack of selectivity [46]. In relation to this, Hettiaratchy et al. [47] reported on the incidence of phototoxicity in a group of 14 healthy volunteers after a single dose of 0.100–0.129 mg kg^{-1}. Six men developed severe superficial burns on the left forearm and more superficial burns on other body areas. Wound healing was much slower than with conventional thermal injury. In a reaction to these observations, Scotia (manufacturer of *m*THPC at those times) reported that in a group of 957 healthy volunteers and patients, only 22 (2.3%) showed phototoxicity [48]. The company attributed the high incidence of the adverse reactions described by Hettiaratchy et al. [47] to problems during the administration of the drug, resulting in leaking out of the drug. This can lead to delayed and prolonged photosensitivity reactions in the affected tissues. While the FDA rejected *m*THPC in 2000, the European Medicines Evaluation Agency (EMEA) approved the sensitizer in 2001 for the palliative treatment of patients with advanced head and neck squamous cell carcinoma failing prior therapies and unsuitable for radiotherapy, surgery or systemic chemotherapy.

Exploitation of *m*THPC in PIT is challenging from the chemical point of view, as this photosensitizer is poorly water-soluble and lacks a functional moiety for direct coupling to MAbs (Fig. 1B).

4.2.2. Mono-L-aspartylchlorin e_6

Because the natural compound chlorophyll *a*, which has a chlorin-type structure, is sensitive to auto-oxidation and, therefore, unsuitable for PDT, several derivatives of this compound have been developed. Under vigorous alkaline conditions chlorin e_6 is formed (Fig. 1C). This compound has only moderate in vivo activity, as showed by Kostenich et al. [49] in xenograft-bearing mice. Therefore, chlorin e_6 was further modified, resulting in a large family of derivatives. The most prominent member of this group is mono-L-aspartylchlorin e_6 (NPe6, Fig. 1D), which has an absorbance peak at 654 nm and a molar extinction coefficient of 4.0×10^4 M^{-1} cm^{-1}.

Chlorin e_6 has extensively been evaluated in PIT approaches. It's carboxyl moieties not only improve the water-solubility of this photosensitizer, but also provide opportunities for coupling to MAbs (Fig. 1C and D).

4.3. Phthalocyanines (Pc)

Since the 1930s, the group of phthalocyanines, especially the Cu-complexes, has been used as commercial pigments, e.g. in ball-point inks. For PDT, they are very interesting because of their high extinction coefficients (up to 2×10^5 M^{-1} cm^{-1}) in the far-red region (650–700 nm). Furthermore, the synthesis of phthalocyanines (Fig. 1E) is rather straightforward. Phthalocyanines form chelates with a variety of metal ions. Most phototoxic appeared to be the $zinc^{II}$- and $aluminium^{III}$-complexes [50,51], because both ions lengthen the triplet state lifetime of the sensitizer.

Because the phthalocyanine moiety is very hydrophobic, sulfonated derivatives were developed in an attempt to increase hydrophilicity. Unfortunately, upon sulfonation mixtures of compounds were obtained, which could not be fully separated. Paquette et al. [52] studied in Chinese hamster lung fibroblast cell line V-79 the cellular uptake and phototoxicity of sulfonated phthalocyanines. Uptake and toxicity appeared to be related to the degree of sulfonation, as both increased

in the series $AlPcS_4 \rightarrow AlPcS_3 \rightarrow AlPcS_2$. Due to its hydrophilicity, $AlPcS_4$ is taken up by the cells less efficient than the amphiphilic *cis*-$AlPcS_2$. In BALB/c mice bearing EMT-6 mammary tumors, $AlPcS_2$ was 10 times more phototoxic than $AlPcS_4$ [53].

Because $AlPcS_4$ is the most hydrophilic member of the sulfonated phthalocyanines, it has been recognized as the sensitizer of choice for evaluation in PIT approaches.

5. Development of photoimmunoconjugates

MAbs have been recognized as attractive carrier molecules for selective delivery of photosensitizers to tumors. However, several obstacles have to be faced in the development of high quality photoimmunoconjugates, i.e. conjugates in which the MAb is loaded with photosensitizer without loss or alteration of its biological properties. The photosensitizer of choice should contain a functional moiety for direct or indirect covalent linking to the lysine [54–71], thiol [72–78] or carbohydrate groups [80–87] of a MAb, otherwise such a moiety has to be created. When multiple functional moieties are present, careful attention should be paid to the occurrence of intra- and inter-molecular cross-links during conjugation. A serious problem met in the coupling of therapeutic photosensitizers is the poor water-solubility of these agents. In fact, most therapeutic photosensitizers tend to aggregate in aqueous media due to their hydrophobic skeleton [88,89]. The hydrophobicity of the coupled photosensitizer, as well as the type, number, and arrangement of charged groups, can strongly influence the physicochemical properties of the MAb, resulting in alteration of pharmacokinetics, biodistribution [56,64,68,69,73,89], specific and non-specific binding [77,88] and internalization [72,90]. The use of spacers/linkers can be considered to create a distance between the hydrophobic sensitizer and the MAb [65,67,88,91]. The use of poly-linkers such as dextran [79,81–83,92], polyglutamic acid [80,84,85], poly-L-lysine [72,74–78], polyvinyl alcohol [54,55,93,94], and *N*-(2-hydroxypropyl)methacrylamide [95] might be attractive as they can increase the water-solubility and the sensitizer:MAb ratio of the conjugate.

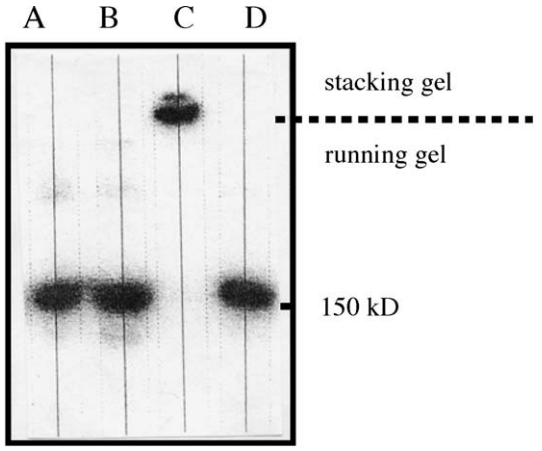

Fig. 2. Illustration of the phototoxicity of *m*THPC to the integrity of ^{125}I-cMAb U36. Fifty microgram of ^{125}I-cMAb U36 were incubated in 500 μl of MeCN/0.9% NaCl (1:4, v/v) at pH 9.5: with 25 μg *m*THPC in the dark (lane A), with 25 μg *m*THPC in the light under N_2 atmosphere (lane B), with 25 μg *m*THPC in the light (lane C), and without *m*THPC in the light as a control (lane D). After 1 h of incubation, SDS-PAGE and Phosphor Imager analysis was performed.

Of major importance for the reproducible production of photoimmunoconjugates is that all reactions, including the modification of the photosensitizer, conjugation and subsequent purification are performed in subdued light, and that solvents are saturated with nitrogen to eliminate the presence of oxygen. In the presence of light and oxygen the sensitizer can produce singlet oxygen, which will affect the integrity of the MAb. Fig. 2 illustrates this effect for the coupling of *m*THPC, when these precautions are not taken. In this particular example the MAb was impaired in such a way that it could not penetrate a 7.5% SDS-PAGE gel anymore.

6. Testing the quality of photoimmunoconjugates

The success of PIT will, among others, depend on the quality of the photoimmunoconjugates with respect to tumor selectivity and degree of accumulation. Before reviewing the results obtained with PIT thus far, we first will discuss some of the quality tests exploited to evaluate (i) the reproducibility of the conjugate production, (ii) the integrity and antigen binding capacity of the MAb upon coupling of the

photosensitizer, (iii) the tumor targeting capacity of the conjugate, (iv) the efficacy of the conjugate in PIT in in vitro and in vivo tumor models. Only with this information, further optimization of conjugates becomes possible.

Analytical procedures can be used during the production of the photoimmunoconjugates, as well as for the characterization of the final product. As the MAb is functioning as a targeting vehicle, and unstable conjugates will result in inefficient delivery of sensitizer to the tumor, it is important to check the stability of the photoimmunoconjugate, for example by incubating the conjugate at 37 °C in human serum [56,58,64,65,68,69,96]. Assessment of the sensitizer:MAb molar ratio (substitution ratio) can be performed by spectroscopy using the distinct absorbance of sensitizer (400–700 nm) and MAb (280 nm). Alternatively, protein-mass-spectrometry can be used [97], while some photosensitizers allow radiolabeling as a facile approach to calculate the exact number of dye molecules per MAb molecule [64,93]. Knowledge of this ratio is relevant for assessment of dose–response relationships. Besides that, several research groups have observed a ratio-dependent blood clearance of conjugated MAbs [56,58,97–99], including MAbs conjugated with photosensitizers, indicating that tumor targeting becomes less efficient when the MAb is overloaded. Photosensitizer-MAb conjugates with molar ratios varying between 0.5:1 and 70:1 have been described in literature, the higher ratios being mostly obtained in combination with polylinkers.

The integrity of a photoimmunoconjugate can be analyzed by high-performance liquid chromatography (HPLC) and electrophoresis (SDS-PAGE), using the native unconjugated MAb for comparison. Quantification of protein bands/peaks can be facilitated when a radiolabeled MAb is used for conjugation [56,58,61,64,65,67–69,72,95,96].

The next step is to evaluate whether both components in the photoimmunoconjugate have retained their essential functional properties upon coupling, being antigen binding for the MAb and singlet oxygen production for the photosensitizer. To assess the immunoreactivity of the conjugated MAb in comparison to the native unconjugated MAb, binding experiments can be performed. These can be enzyme-linked immunosorbent assay (ELISA) types of assays with relevant purified antigen or target antigen expressing cells [54,55,73,76–78,93,100,101], or fluorescence imaging assays [62,66,71,102]. The easiest way for quantitation, however, is to use radiolabeled conjugate in combination with antigen expressing target cells [58,64,68,69,82,83] or antigen-coated Sepharose beads [56,57,63,65,67,79,80,96]. Specificity of binding can be proven by blockage of conjugate binding with excess of native unconjugated MAb, or by the use of cells lacking the target antigen.

Also the properties of the sensitizer can change upon coupling. Spectroscopy might reveal changes in the absorption spectrum [77]. For the coupling of $AlPcS_4$ to BSA via a 6-aminohexanoic acid spacer in aqueous media, spectroscopy showed a decrease in absorption of the characteristic peak of the monomer at 677 nm besides an absorption increase at 644 nm, indicating aggregation of the photosensitizer [88]. Aggregate formation might also impair singlet oxygen production, expressed as the singlet oxygen quantum yield (i.e. the ratio of moles of singlet oxygen generated by the photosensitizer relative to the moles of photons that are absorbed by the photosensitizer in the ground state) [88,102–104]. Several tests to measure singlet oxygen production or overall oxidative activity have been described using, for example, *N*,*N*-dimethyl-4-nitrosoaniline [79,80], L-tryptophan [88,91] or 2′,7′-dichlorofluorescin diacetate [94,105,106] as substrate for photooxydation. The latter substrate is of particular interest as its oxidation results in the fluorescent derivative 2′,7′-dichlorofluorescein, which can be used to visualize the intracellular sites where reactive oxygen species are produced [105,106].

The efficacy of photoimmunoconjugates in PIT can be measured in vitro by exposing antigen expressing target cells for a certain time to a range of conjugate concentrations, followed by washing and illumination of the cultures. During illumination, cell cultures are incubated in phenol red-free medium to prevent light absorption by the medium. Several types of assays are exploited to evaluate PIT efficacy. The trypan blue dye exclusion test [71,72,77,102,105] and the colony formation assay [72,77,79,82,83,105–107] both analyze the viability of individual target cells. In contrast, colorimetric assays, such as those using 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT assay)[54,55,65,67,74,91,95], sulforhodamine

B (SRB assay)[64,68–70], or naphthol blue–black [92], assess the overall growth effect on the total cell population. While the MTT assay measures mitochondrial dehydrogenase activity, the other two assays analyze cellular protein content. Valuable controls for proper interpretation of the in vitro efficacy data, and the contribution of the MAb carrier molecule herein, are: addition of equivalent amounts of free sensitizer or non-specific photoimmunoconjugate with illumination, addition of conjugate, unconjugated MAb or free sensitizer without illumination, and illumination only. An IC_{50} value can be estimated based on cell/colony counts or absorption values, and is defined as the concentration that corresponds to a reduction in survival or growth of 50% compared with values of control cells.

Furthermore, several procedures can be applied to evaluate whether photoimmunoconjugates rely on target cell internalization for optimal efficacy in PIT. This can be done by fluorescence microscopy after incubation of cells at 37 and at 4 °C as a non-internalization control [16,67,94,95]. Alternatively, cells can be solubilized after incubation and washing, and fluorescence can be measured in a fluorometer [72,77,79,92]. Finally, MAb and/or photosensitizer can be radiolabeled to facilitate quantitation [70,72,88,95,105].

In vivo evaluation of photoimmunoconjugates comprises measurement of their biodistribution as well as evaluation of their efficacy in PIT. Biodistribution can be assessed by extraction of the photosensitizer from tissue samples and quantitation of the photosensitizer by fluorescence spectroscopy [74,76,79,84,108], by fluorescence imaging [56–58,60,61,63,66,90] or by use of radiolabeled MAbs in photoimmunoconjugates [64,65,67,68,93,96]. Although the latter method is easy, one has to realize that the tissue levels of radioactivity and photosensitizer might be different due to their different excretion routes after catabolism of the conjugate [64]. In in vivo PIT procedures the photoimmunoconjugate is usually administered systemically to tumor-bearing nude mice, by intravenous [64,66–68,76,78,93,108] or intraperitoneal injection [73,75,79,84,85]. The tumor becomes illuminated with light at an appropriate wavelength and energy dose at an appropriate time after the injection, which corresponds to a maximum photosensitizer accumulation in the target tissue. Volumes of treated and control tumors are calculated by measuring the dimensions of the tumors using calipers. Alternatively, survival is chosen as endpoint.

7. Photoimmunoconjugates for tumor detection

The use of MAbs for selective delivery of photosensitizer to tumors has been studied for about 20 years. The most convincing proof that MAbs are well qualified for this purpose, has been obtained in studies on photoimmunodetection. In 1991, Pèlegrin et al. [56] described the direct coupling of fluorescein to an anti-CEA MAb, and the evaluation of the conjugates in mice bearing established human colon carcinoma xenografts. Fluorescein was covalently coupled to the ^{125}I-labeled MAb at substitution ratios ranging from 4 to 19. Conjugates with a molar ratio up to 10, showed an optimal immunoreactivity. However, for conjugates with a molar ratio of 10, the biological half-life in xenograft-bearing nude mice was reduced by about 40% compared to unconjugated MAb, or conjugates with a substitution ratio of 4. As a consequence, tumor uptake was also reduced by 25%. Moreover, the fluorescence intensity per fluorescein molecule diminished with the increase of the fluorescein-MAb molar ratio. Despite their impaired quality, more extensive biodistribution and tumor detection studies were performed with conjugates containing 8–10 fluorescein molecules. This compromise was required to obtain a sufficiently high fluorescence signal for tumor detection. Conjugates were shown to be more effective in tumor detection than Photofrin®: despite an injected dose of MAb-conjugated fluorescein which was 136 times lower than the Photofrin® dose, the observed fluorescence intensity of the tumor was 8-fold higher with the conjugate. Folli et al. [57] confirmed the feasibility of these conjugates for tumor detection in six patients with primary colorectal cancer. Besides this, they also observed a more rapid blood clearance of the conjugate, after a simultaneous injection of ^{125}I-labeled MAb containing 10–14 fluorescein molecules and ^{131}I-labeled unconjugated MAb.

Despite their promising initial clinical results, Folli et al. [58] concluded that fluorescein was not the optimal candidate photosensitizer to continue their efforts in photoimmunodetection. Firstly, the fluorescein excitation and emission wavelength of 488 and

515 nm, respectively, are too low for satisfactory tissue penetration, and secondly, the exciting laser light induced non-specific yellowish autofluorescence in several normal tissues, which can interfere with optimal tumor detection. For this reason they switched to indocyanine, a sensitizer with longer excitation and emission wavelengths of 640 and 667 nm, respectively. In studies similar to those as previously described for fluorescein [56], Folli et al. demonstrated that indocyanine:MAb E48 conjugates with a molar ratio up to 2, are optimal with respect to immunoreactivity and in vivo targeting of A431 xenografts (Fig. 3). Moreover, a conjugate with an indocyanine-MAb ratio of 2 was more efficient in tumor detection than a fluorescein-MAb conjugate with a ratio of 6.

Other research groups have confirmed the potential of cyanine-MAb conjugates for tumor detection in animal models [60,61,63,66,96]. Gutowski et al. [96] used indocyanine-MAb 35A7 conjugates with a substitution ratio of 2–3 in nude mice bearing LS174T peritoneal carcinomatosis to assess the feasibility of intraoperative photoimmunodetection. The fluorescence status of 333 biopsies was compared with their histological analysis. Detection of very small nodules with a mass <1 mg or a diameter <1 mm was possible. The overall sensitivity was 90.7% (100% for nodules >10 mg vs. 78% for nodules ≤ 1 mm), while the overall specificity was 97.2%. On the basis of these encouraging results, clinical evaluation of intraoperative photoimmunodetection was advocated.

An interesting application of photoimmunodetection is its combination with PIT. In this approach, photoimmunodetection can be used to delineate the tumor to be treated with PIT, and to monitor tumor response upon PIT. Soukos et al. [66] demonstrated the potential of this approach in the hamster cheek pouch carcinogenesis model. They used MAb C225 directed against EGFR, as this receptor is overexpressed in precancerous oral lesions. Cyanine-MAb C225 conjugates (substitution ratio of 2.3) appeared to be a promising diagnostic tool for delineation of the premalignant lesions, as well as for monitoring the efficacy of PIT with chlorin e_6-C225 conjugates.

A similar strategy was explored in another field of application: eradication of neovasculature, which causes loss of vision in some ocular disorders. In a study of Birchler et al. [63], the scFvL19 antibody fragment was used, which has a high affinity for the ED-B domain of fibronectin, a marker of angiogenesis. Selective uptake of indocyanine-MAb conjugates (substitution ratio of 0.7) in newly formed blood

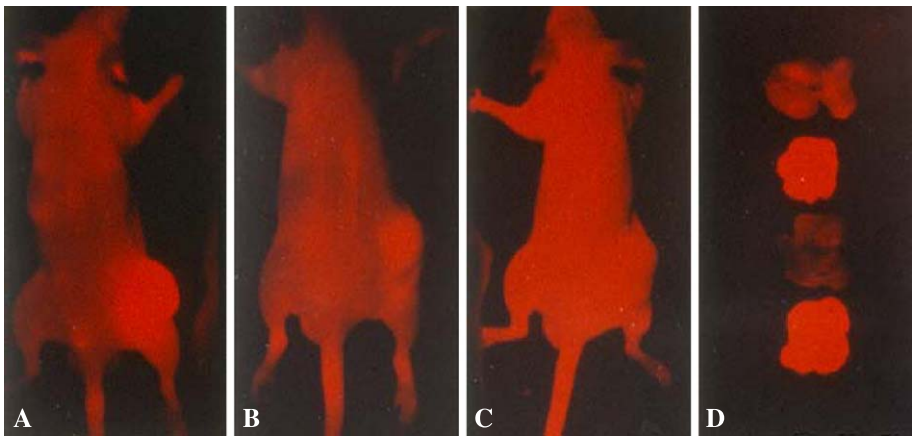

Fig. 3. Photoimmunodetection of A431 xenografts in nude mice; (A) 24 h after i.v. injection of 100 μg ^{125}I-MAb E48-(indocyanine)$_2$ conjugate, representing only 1 μg of indocyanine coupled to MAb. The red fluorescence from indocyanine-MAb conjugate can be detected across the mouse skin; (B) control mouse given injection i.v. of 100 μg of irrelevant IgG coupled to 1 μg of indocyanine; (C) control mouse given injection i.v. of 15 μg of free indocyanine; (D) photoimmunodetection of tumors resected from nude mice 24 h after injection of control irrelevant IgG-(indocyanine)$_2$ (upper tumor), or MAb E48-(indocyanine)$_2$ conjugate (second upper tumor), or no antibody and no dye (third tumor, barely visible), or only with 15 μg of free dye (lower tumor). Laser irradiation was performed at 640 nm, using a Kodak Wratten filter 70 for detection (figure reproduced with permission from Ref. [58], American Association for Cancer Research).

vessels was demonstrated in a rabbit model of ocular angiogenesis. Moreover, PIT with chlorin e_6-scFvL19 caused complete and selective occlusion of ocular neovasculature and promoted apoptosis of the corresponding endothelial cells. Occlusion of neovasculature was confirmed by the use of the diagnostic indocyanine-MAb conjugate. A similar strategy can be applied for the eradication of neovasculature in tumors.

Aforementioned data demonstrate that photosensitizers can be coupled to MAbs, and exploited for diagnostic purposes, with full maintenance of the tumor targeting characteristics of the MAb. Unfortunately, neither fluorescein nor indocyanine can be used therapeutically due to their low yield of singlet oxygen.

8. Photoimmunoconjugates for cancer therapy

8.1. Photoimmunotherapy with HpD-MAb conjugates

The first in vitro and in vivo studies, showing that photoimmunoconjugates had superior selective antitumor effects in PIT over drug or MAb alone, were described about 20 years ago [109,110]. For this purpose, the group of Levy in Vancouver, Canada, coupled HpD directly to an anti-myosarcoma MAb by carbodiimide catalyzed peptide bond formation. Their reported substitution ratio of about 60, is much higher than would have been expected based on the number of amino groups present in a MAb molecule. This phenomenon might be caused by the absorption of HpD and HpD aggregates onto hydrophobic sites of the MAb [78]. Whatever the case, the reproducibility of this coupling procedure appeared to be a problem [54]. Data from antigen binding studies were not provided in detail, while conjugate integrity tests or biodistribution studies were not performed. Therefore, several options are left open to explain the minimal efficacy of those conjugates in mice, even when bearing very small tumor load (5×10^4 tumor cells).

Also Pogrebniak et al. [107] coupled HpD directly to a MAb (designated 45-2D9), resulting in conjugates with a substitution ratio between 4.2 and 39.5. MAb 45-2D9 recognizes a cell surface protein, which is expressed on NIH 3T3 cells transformed with the *ras* oncogene (clone 45-342), but not on the parental cell line and another transfectant (clone 45-342A). In vitro PIT studies with the HpD-MAb conjugate revealed just a 4-fold difference in cell survival comparing the antigen-positive 45-342 cell line with the antigen-negative 3T3 cell line. The problem with this conjugate was best illustrated in in vitro binding assays. Although the conjugate showed binding to antigen-positive target cells, this binding appeared to be non-specific, as unconjugated MAb was just partly able to compete. The authors used an attractive in vivo model, comprising of nude mice bearing an antigen-positive tumor in the left flank and an antigen-negative tumor in the right flank, for proving the antigen selectivity of their MAb. Injection of ^{125}I-MAb 45-2D9 revealed selective binding, being 11.5 times higher for the antigen-positive tumor. However, the biodistribution of the HpD-MAb conjugate was not fully evaluated, and therefore, profound data on the targeting capacity of this photoimmunoconjugate are lacking. Although HpD-MAb conjugates resulted in significantly more long-term cures in tumor-bearing mice than free HpD at the same concentration, it remains far from clear whether this photoimmunoconjugate was optimal for in vivo PIT application.

Berki et al. [100,102] used a HpD-MAb conjugate, prepared essentially as described by Mew et al. [109], for the in vitro elimination of T cells in a mixed cell population. The photoimmunoconjugate induced T cell death in PIT at a 10-fold lower concentration compared to unbound HpD, and had the advantage of being selective. This method can be useful for selective destruction of one cell population in a heterogeneous cell mixture, e.g. in bone marrow transplants for tumor cell elimination (purging) or for T cell depletion to avoid graft versus host reactions.

After initial studies with HpD-MAb conjugates, the group of Levy switched to antibody conjugates with the benzoporphyrin derivative (BPD-MAb conjugates, see Section 4.1) and banished methods for direct conjugation of photosensitizers to MAbs. As indicated above for HpD, reproducibility is one problem in direct conjugation techniques. Another problem is the random coupling of high numbers of hydrophobic photosensitizers throughout the MAb molecule, which might alter the physico-chemical properties of the MAb. To avoid such problems, BPD was first linked to a modified PVA backbone (Mw $\pm$ 10 kDa) at a ratio of about 25:1, followed by

conjugation of 1–3 of these carrier molecules to MAb lysine residues using heterobifunctional linking technology [54]. Jiang et al. [55] used this technique to couple BPD to MAb 5E8, a MAb directed against a glycoprotein expressed on the surface of human squamous cell carcinoma of the lung. While the conjugate showed 80% binding using an ELISA with antigen expressing A549 target cells, also some binding was observed with a control MAb T48-conjugate. This tendency for stickiness was also observed when the specific and control conjugates were tested for efficacy in PIT in vitro: LD_{50} values for BPD, BPD-MAb T48 and BPD-MAb 5E8 were 150, 100 and 10 ng ml^{-1} when tested on antigen-positive A549 cells, and 85, 58 and 45 ng ml^{-1} when tested on antigen-negative M-1 cells. In a later study, the biodistribution of the agents was evaluated after i.v. administration to A549-bearing nude mice [93]. For this purpose ^{14}C-BPD was used. Although the highest tumor uptake of ^{14}C-BPD was obtained upon administration of the ^{14}C-BPD-MAb 5E8 conjugate, dramatically high uptake levels were also observed in liver, lung, spleen and kidney, indicating entrapment of these conjugates in tissues with a highly developed reticuloendothelial system. No in vivo PIT studies were performed with these particular BPD-MAb conjugates.

In vivo PIT data on the potential of BPD-MAb conjugates were obtained by Hemming et al. [108], using an anti-EGFR MAb and the hamster cheek model of squamous cell carcinoma. BPD was coupled to the specific anti-EGFR MAb and an anti-CEA control MAb, as described before [54]. Although the in vitro binding characteristics were not described, a strong aspect of this study is the in vivo biodistribution evaluation of the conjugates. To this end, tumor-bearing hamsters received either 2.5 mg kg^{-1} BPD, 1 mg kg^{-1} BPD-anti-EGFR, 1 mg kg^{-1} BPD-anti-CEA (both conjugates containing 120 μg BPD per mg MAb), or PBS. Uptake of BPD in tumor and normal mucosa was assessed at 6 h after injection, by homogenization of the tissues and extraction of the sensitizer. The administration of the free sensitizer resulted in almost equal BPD levels in tumor and normal mucosa (7.8 ± 0.7 and 5.0 ± 0.8 μg g^{-1}, respectively). With the specific conjugate a 26-fold better selectivity was observed (6.8 ± 0.6 μg g^{-1} for tumor and 0.26 ± 0.09 μg g^{-1} for normal mucosa), whereas the control conjugate resulted in BPD tissue levels below the detection limit. These data also indicated that the anti-EGFR MAb was highly capable for efficient delivery of BPD to the tumor, as a 20-fold higher amount of BPD had to be administered to obtain similar tumor uptake levels. Similar tumor uptake values resulted in a similar efficacy in PIT: the 1 month survival was 67% for the BPD group and 80% for the BPD-anti-EGFR group.

8.2. Photoimmunotherapy with chlorin e_6-MAb conjugates

The group of Hasan studied MAb conjugates with the photosensitizer chlorin e_6 monoethylenediamine monoamide. They coupled the photosensitizer through its carboxyl groups to dextran, polyglutamic acid (PGA) or poly-L-lysine polylinkers, and these carriers were subsequently attached to the Fc carbohydrate moiety of the MAb [82]. This method has the theoretical advantage that binding of the carrier occurs at a point distal from the antigen binding sites of the MAb, thus minimizing the possibility for impairment of immunoreactivity. With dextran or PGA as linker, 30–36 sensitizer molecules were coupled to anti-Leu-1, a MAb directed against T cells [79,80]. The binding of the conjugates was slightly decreased, to about 70–85% of that of the unconjugated MAbs. Nevertheless, in vitro PIT studies demonstrated that the conjugates were effective as selective phototoxic agents. No data on the in vivo efficacy of the dextran-linked conjugate have been published.

Biodistribution studies were performed with i.p. injected PGA-linked chlorin e_6-MAb OC125 conjugate in a murine ovarian cancer model of i.p. growing NIH-OVCAR3 cells [84]. For comparison, also the biodistribution of equivalent amounts of free sensitizer and PGA-chlorin e_6 was evaluated. Animals were sacrificed at various time points between 3 and 168 h after injection, and the amount of sensitizer was quantified by extraction followed by fluorescence spectroscopy. For both the photoimmunoconjugate and the free sensitizer, peak tumor concentrations were reached at 24 h. However, the absolute tumor concentrations were only 2–3-fold higher for the conjugate than for the free sensitizer. These results were disappointing, especially because the photoimmunoconjugate showed lower tumor to nontumor

ratios than the free sensitizer. In vivo PIT studies were performed with the same conjugate [85]. Seven days after i.p. injection of 30×10^6 NIH-OVCAR-3 cells, mice received 0.5 mg kg^{-1} sensitizer equivalents, and 24 h later illumination was performed at 5 J m^{-2} using a cylindrical diffusing tip fiber. Treatment was repeated every 48 h, for a total of three or four treatments. The median survival for the three and four times treated mice was 47 and 58 days, respectively, and 38 days for control mice. A reference group receiving the unconjugated sensitizer was not included in this study.

Photoimmunoconjugates with the positively charged poly-L-lysine linker have been most extensively studied. To determine the effect of charge of the conjugate on cellular uptake, biodistribution and efficacy in PIT, negatively charged conjugates were also produced. To this end, after coupling of the sensitizer to the carrier, the complex was polysuccinilated. A first study, in which the biodistribution of cationic and anionic chlorin e_6-OC125F(ab′ $)_2$ and unconjugated F(ab′ $)_2$ were directly compared in nude mice bearing i.p. ovarian cancer, was described by Duska et al. [73]. To facilitate analyses, OC125F(ab′ $)_2$ was radiolabeled with ^{125}I. Biodistribution analysis at 3 and 24 h after i.p. injection revealed that the amount of ^{125}I retained in tumor as well as normal organs was highest for unmodified OC125F(ab′ $)_2$, lower for cationic chlorin e_6-OC125F(ab′ $)_2$, and lowest for anionic chlorin e_6-OC125F(ab′ $)_2$. A recognized advantage of the cationic conjugate over the anionic one, was its much higher cellular uptake after binding to the target antigen, which resulted in higher phototoxicity [72]. In contrast, after i.v. administration the anionic conjugates appeared to be superior. Under these conditions cationic conjugates became rapidly cleared from the blood [76,78]. Therefore, the group of Hasan hypothesized that polyanionic conjugates perform better after i.v. delivery, while polycationic conjugates performs better after intracavitary delivery.

The efficacy of aforementioned cationic and anionic conjugates was determined in nude mice for several tumor types, and appeared consistent with the biodistribution data. In an i.p. ovarian cancer model (15×10^6 NIH-OVCAR-5 cells, 1 mg kg^{-1} sensitizer equivalent, 25 J cm^{-2} 24 h p.i.; three treatments repeated every 72 h), the cationic chlorin e_6-OC125F(ab′ $)_2$-conjugate (substitution ratio 15) showed after i.p. administration a better efficacy than the anionic conjugate and the free sensitizer [75]. The median survival of the cationic group was 41 days, compared with 35 days for the anionic group and 37 days for the free sensitizer and control groups.

Anionic conjugates, in this case using i.v. injected MAb 17-1A for tumor targeting, were evaluated in a hepatic metastasis model of HT29 colorectal cancer cells in nude mice [78]. PIT (0.25 mg kg^{-1} sensitizer equivalent, 80 J cm^{-2} 3 h p.i.) with the i.v. injected anionic chlorin e_6-17-1A conjugate (substitution ratio 8.5) led to an increased median survival of 102 days in comparison to 77 days for the mice receiving free sensitizer and 63 days for control mice. The efficacy of the cationic conjugate was not determined.

The use of carrier units as described in aforementioned studies has the advantage of increasing the loading of MAbs with sensitizer, but this happens at the expense of MAb specificity especially when charged groups are introduced. Several researchers, therefore, coupled chlorin e_6 directly to the MAb, at a low substitution ratio [66,86,101]. The group of Hasan directly coupled chlorin e_6 to the anti-EGFR MAb C225 at a substitution ratio of 4.8 [66]. Pilot studies in the previously described hamster cheek pouch model for premalignant oral lesions revealed the potential of PIT (65 μg chlorin e_6 per animal, 80 J cm^{-2} 48 h p.i.) with i.v. injected conjugate to reduce overexpression of EGFR in dysplastic areas. Additional studies have to be performed to evaluate whether photodestruction of EGFR will result in inhibition of cellular proliferation and malignant progression.

Another chlorin photosensitizer attracting much attention in clinical PDT studies is *m*THPC. Thus far, only Vrouenraets et al. [64] have described the coupling of this sensitizer to MAbs. Before conjugation, the sensitizer was radiolabeled with ^{131}I and tetracarboxymethylated. This latter modification was of paramount importance to make this apolar agent better water-soluble and to create a functional moiety suitable for esterification and conjugation to lysine residues of a MAb. In this study ^{131}I-*m*THPC was coupled to ^{125}I-labeled MAbs directed against squamous cell carcinoma of the head and neck (HNSCC). Biodistribution studies in nude mice bearing the HNSCC xenograft line HNX-OE, showed that the tumor selectivity of MAb U36-coupled *m*THPC was strongly improved in compar-

ison with free *m*THPC. The accelerated clearance of the photoimmunoconjugates, however, resulted in decreased tumor uptake levels: about 8 and 14%ID g^{-1} tumor for conjugates containing 1.8 and 0.9 *m*THPC molecules per MAb, respectively, as compared with 19.5%ID g^{-1} for the unconjugated ^{125}I-MAb. Thus far, these conjugates have only been evaluated in PIT in vitro. Pilot studies showed that PIT (25 J cm^{-2} at 652 nm) with *m*THPC was highly toxic when coupled to the internalizing anti-EGFR MAb 425 (A431 cells, IC_{50} 7.3 nM). However, this was not the case when coupled to the non-internalizing MAb U36. The *m*THPC-MAb U36 conjugate exhibited very low phototoxicity to UM-SCC-22A cells, despite the fact that the MAb U36 defined antigen is abundantly expressed on these cells. Based on these findings and data of others [16,72,105], the authors hypothesized that the critical target for PIT might be localized intracellularly, and they therefore, recommended the use of internalizing MAbs for PIT. In line with this, they hypothesized that more hydrophilic sensitizers (than *m*THPC), which in free form do not readily pass the cell membrane and are, therefore, ineffective in PDT, would be effective when coupled to internalizing MAbs. In a subsequent study, they confirmed this hypothesis with the compound 5-[4-[5-(carboxyl)-1-butoxy]phenyl]-10,15,20-tris-(4-methylpyridiniumyl)-porphyrin iodide (TrisMPyP-ΦCO$_2$H) [68]. This hydrophilic porphyrin derivative just served as a model compound, as its weak absorption ($\varepsilon = 7.0 \times 10^3$ M^{-1} cm^{-1}) at an absorption maximum of 595 nm makes it of limited value for PIT. In vitro PIT data showed that this photosensitizer was indeed effective when coupled to an internalizing MAb, but not as unconjugated compound or conjugated to a non-internalizing MAb.

8.3. Photoimmunotherapy with Pc-MAb conjugates

Vrouenraets et al. [79] further investigated the concept of using internalizing MAbs for PIT by using the therapeutically better suited hydrophilic sensitizer aluminum phthalocyanine tetrasulphonate ($AlPcS_4$). Conjugation of $AlPcS_4$ was performed via its tetra-glycine derivative. Preliminary in vitro data showed that particularly the internalizing $AlPcS_4$-MAb 425 conjugate was highly toxic to A431 cells (25 J cm^{-2} at 675 nm) (Fig. 4). This conjugate was 7500 times more toxic than the free compound (IC_{50}s: 0.12 vs. 900 nM), and about 60 times more toxic than *m*THPC-MAb 425 in the same model. In

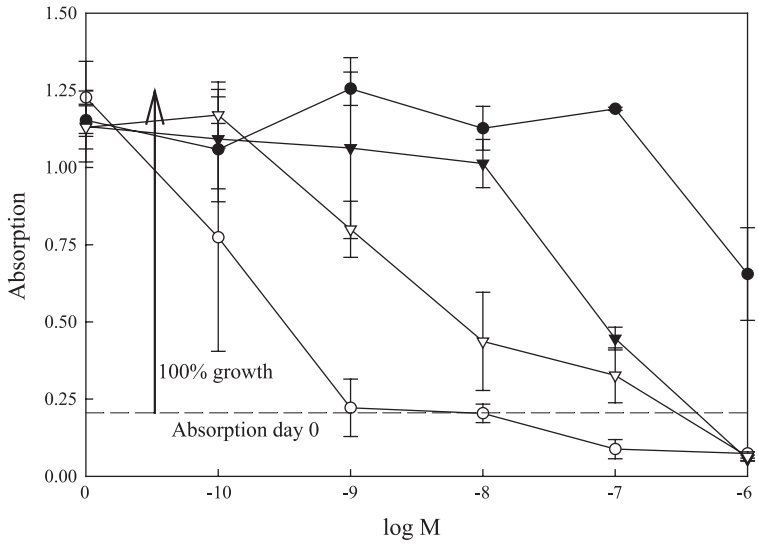

Fig. 4. The antiproliferative effect of $AlPcS_4$ and $AlPcS_4$-MAb conjugates with sensitizer:MAb ratio 2 on A431 cells upon illumination with 25 J cm^{-2} (SRB assay). Free $AlPcS_4$ (●), $AlPcS_4$-mMAb 425 (○), $AlPcS_4$-cMAb U36 (▽), and $AlPcS_4$-mMAb E48 (▼). Results of triplicate experiments are indicated (mean ± S.D.). The molarity (M; *x*-axis) of the free or conjugated $AlPcS_4$ is indicated logarithmically.

an extended in vitro evaluation, *m*THPC- and $AlPcS_4$-MAb conjugates were directly compared using 5 HNSCC cell lines as target and three MAbs (BIWA 4, E48 and 425) for targeting [70]. In general, *m*THPC-MAb conjugates were hardly effective, while $AlPcS_4$-MAb conjugates were highly toxic to all five cell lines. Especially $AlPcS_4$-BIWA 4 conjugates, directed against the CD marker CD44v6, were effective with IC values ranging from 0.6 to 5.4 nM. A strong correlation was observed between phototoxicity of $AlPcS_4$-conjugates and the total binding capacity (internalized and surface bound) of these conjugates. In contrast to their previous findings, these more extensive studies revealed that efficacy was not correlated with internalization capacity only.

Despite these encouraging in vitro results, the in vivo behavior of the $AlPcS_4$-MAb conjugates was not optimal. Although $AlPcS_4$-MAb U36 was capable for selective tumor targeting in HNX-OE-bearing nude mice, uptake was less than for the unconjugated MAb. Conjugates with the higher substitution ratio (2.4 vs. 1.2) were cleared more rapidly than the unconjugated MAb, a phenomenon also previously observed with the two other directly coupled sensitizers *m*THPC and TrisMPyP-ΦCO_2H. With respect to this, encouraging results were obtained by Carcenac et al. [65]. They reported the coupling of $AlPcS_4$ to the anti-CEA MAb 35A7 via a mono five-carbon spacer chain, at various substitution ratios. Conjugates with 5, 12 or 16 $AlPcS_4$ molecules per MAb 35A7 molecule were evaluated in nude mice bearing the human colon carcinoma xenograft line T380, and displayed a fully congruent biodistribution, comparable to the unconjugated MAb 35A7. These results are remarkable, as this is the first report demonstrating the coupling of a therapeutic photosensitizer to a MAb without affecting its biodistribution characteristics. Inert coupling of photosensitizers to MAbs was thus far only achieved with diagnostic photosensitizers (see Section 7 and Fig. 3). A likely explanation for the more optimal in vivo behavior of these conjugates in comparison to the conjugates developed by Vrouenraets et al. is the presence of a spacer chain, which creates a distance between the hydrophobic backbone of the sensitizer and the MAb.

Notwithstanding the favorable biodistribution characteristics, the initial in vitro PIT results with $AlPcS_4$-MAb 35A7 conjugates were disappointing. Treatment of the colon carcinoma cell line LoVo revealed an IC_{50} value of 0.35 μM (light dose 50 J cm^{-2} at 676 nm). More recently, they tested in their systems the hypothesis that the use of internalizing MAbs might improve therapeutic outcome [67]. For this purpose the transfected cell line SKOv-CEA-1B9 was developed, expressing comparable levels of ErB2 and CEA antigen. The non-internalizing anti-CEA $AlPcS_4$-MAb 35A7 conjugate induced 68% growth inhibition at 2.50 μg ml^{-1} (0.35 μM) $AlPcS_4$ equivalents. In line with the results obtained by Vrouenraets et al., the internalizing anti-ErB2 $AlPcS_4$-MAb FSP77 conjugate was much more effective. Under the same experimental conditions 51% growth inhibition was induced at a sensitizer concentration of 0.04 μg ml^{-1}.

9. Conclusions

During the last decade conjugated and unconjugated MAbs became part of the armature used for diagnosis and treatment of cancer. MAbs are also capable for selective delivery of photosensitizers to tumors, as was best illustrated in photoimmunodetection studies. In the initial studies with fluorescein, conjugates with a high photosensitizer:MAb ratio were used to obtain high amounts of dye molecules in the tumor, sufficient for visualization. However, the in vivo tumor targeting potential of these conjugates was not optimal, due to overloading of the MAb. Introduction of indocyanine, which has better photochemical characteristics for photodetection than fluorescein, allowed the use of conjugates with lower substitution ratios. These conjugates had retained the full tumor targeting capacity of the unconjugated MAb.

In PIT, not only tumor selectivity and degree of accumulation of the photoimmunoconjugates appear to be important for efficacy, but also the intracellular uptake [16,67–69,72]. Two approaches have been explored to increase internalization: (1) modification of the physico-chemical properties of the conjugate, (2) targeting to an antigen, which internalizes upon binding of the conjugate. The group of Hasan best documented the first approach by using poly-L-lysine linkers to prepare cationic chlorin e_6-MAb conjugates. These conjugates are efficient transporters of photo-

sensitizers, due to their high substitution ratio. However, cationization of the MAb impaired the antigen specificity of the MAb, as became clear most prominently after i.v. injection of the conjugates. The second approach makes use of MAbs directed against internalizing antigens and was explored by Vrouenraets et al. [68,69] and Carcenac et al. [67]. Both groups aim at the direct coupling of photosensitizers to MAbs, without alteration of the biological characteristics of the MAb. Very encouraging are the results of Carcenac et al. [65], who showed the loading of a MAb with up to 16 $AlPcS_4$ groups using a spacer chain, without altering its biodistribution characteristics. This is an important achievement in the development of PIT. Since $AlPcS_4$ was shown to be a very potent photosensitizer once entered into the target cells, these results hold promise for further PIT development.

The data summarized herein show the possibilities to design optimal photosensitizer immunoconjugates, and to make the step to clinical PIT. Key issues to be considered are: specificity of the MAb, photochemical properties of the photosensitizers, and optimal control over the preparation, tumor targeting and intracellular delivery of the photoimmunoconjugate.

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