Immuno-photodynamic therapy of cancer

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II

Current challenges and opportunities of photodynamic therapy against cancer

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Current challenges of photodynamic therapy against cancer

The most notable of challenges for PDT (Fig. 1), include: I) undesirable distribution of PS after intravenous administration, II) attenuation of light through tissues that can result in incomplete light delivery to the tumor area, III) hypoxia in the tumor environment that depletes the oxygen available for PDT, IV) incomplete or transient tumor vasculature disruption after PDT and subsequent angiogenesis or vessel repair, V) partial tumor destruction after PDT followed by tumor relapse, or VI) insufficient PDT-mediated induction of antitumor immune responses. These efficacy-reducing factors and recent efforts for improvement, either by enhancing PDT or by combination with other modalities, will be addressed.

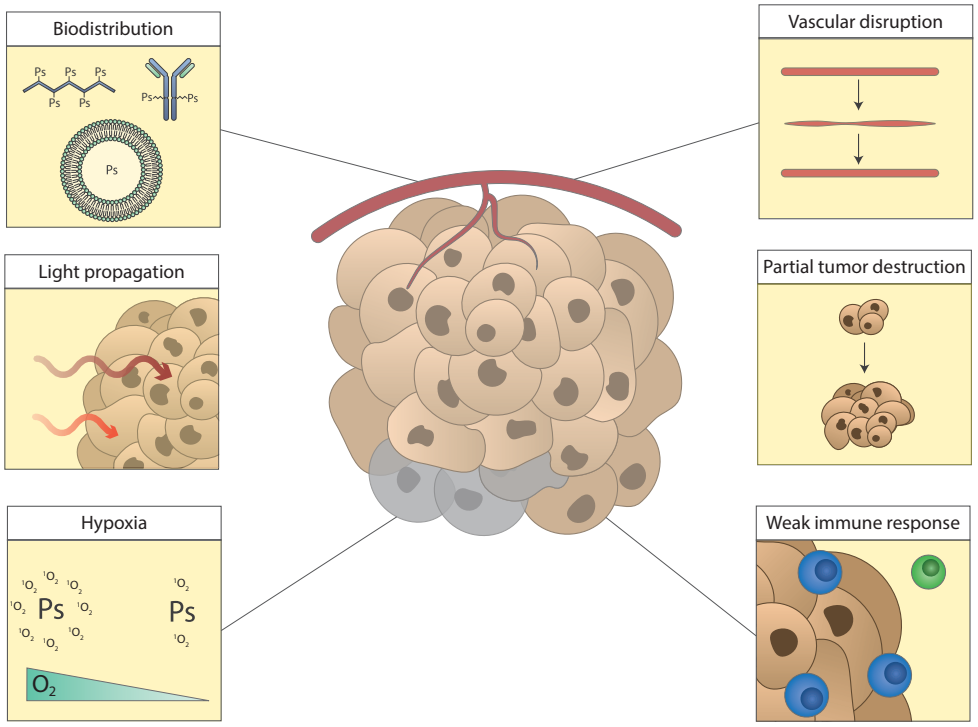

Figure 1 Current challenges of photodynamic therapy

The current challenges of PDT: I) unfavorable biodistribution of PS, II) limited light propagation through tissues, III) reduced number of ROS through hypoxia in the tumor, IV) temporary vascular disruption followed by repair, V) partial tumor destruction followed by tumor relapse, VI) insufficient induction of antitumor immune responses.

Biodistribution of Photosensitizers

A longstanding challenge of PDT is related to the poor tumor distribution that many PS tend to present after administration, reducing accumulation of PS in the tumor and potentially hampering complete diffusion of PS throughout the tumor. This may result in a reduced number of active molecules in the tumor area as well as throughout the various tumor layers, and induce an incomplete destruction of the tumor area. Furthermore, an unfavorable biodistribution may facilitate prolonged periods of unwanted retention in non-target tissues. This is known as photosensitivity and often affects patients when substantial amounts of PS remain in the skin. For these reasons, research has focused on improving the distribution of PS to enhance antitumor efficacy and reduce off-target accumulation. This is often achieved by employing carrier systems, including antibodies, liposomes and nanoparticles (NPs), that may facilitate a more favorable biodistribution. Encapsulation in or association with these carrier systems introduces a degree of control in the distribution of the PS-carrier complex. Several carriers that have been employed to improve the biodistribution of PS will be discussed.

Nanoparticles

Many nanoparticle (NP)-based carriers have been created and characterized in vitro.[1–8] Two studies reported the use of 9-fluorenylmethoxycarbonyl -L-lysine that encapsulated chlorin e6 (Ce6)[9] and a gold NPs carrying a derivative of the poorly soluble Zn(ii)-phthalocyanine and polyethylene glycol (PEG), [10] respectively. Both particles accumulated in tumors over time, and induced a tumor growth inhibition after PDT in murine models. In another paper, a carrier consisting of polyhedral oligomeric silsesquioxane was PEGylated and crosslinked with Ce6.[11] Distribution to xenografted U14 murine cervical tumors was higher compared to free Ce6. Furthermore, the tumor accumulation of the particles was higher in the tumors compared to the heart, liver, lung, spleen and kidneys. PDT with the particles induced a tumor growth inhibition that was significantly better than PDT with free Ce6, showing the potential of carriers for PS. The PS PpIX was conjugated to PEGylated glycol chitosan to form NPs that display increased tumor accumulation after i.v. administration compared to its non-PEGylated equivalent and free PpIX with fluorescence intensity in the tumor being increased compared to the heart, liver, spleen, lung and kidneys.[12] In addition, PDT with the NPs induced significantly enhanced tumor growth inhibition versus its non-PEGylated version as well as PpIX alone.

Several studies investigated the use of NPs that require activation before treatment, functioning as pre-drug carriers for PS that introduce a measure of controlled release. For example, Ce6 was incorporated into PEGylated NPs, either alone[13] or with chemotherapy[14], that disintegrate at acidic environments encountered inside cells and in which Ce6 was quenched before disintegration. These particles displayed enhanced accumulation in tumors compared to free Ce6 and PDT with the particles

showed an increased tumor growth inhibition compared to free Ce6. In another study, Ce6 was encapsulated in NPs that consist of imidazole that disintegrate in response to $^1O_2$, to allow control of Ce6 release.[15] These NPs accumulated in tumors after i.v. administration at increased amounts compared to free Ce6 significantly enhanced tumor growth inhibition for the $^1O_2$ responsive micelles compared to $^1O_2$ unresponsive micelles, to free Ce6 and untreated. In another approach, Ce6 was incorporated into NPs together with a near-infrared (NIR) dye in which Ce6 was quenched until the NPs were activated by photobleaching the NIR dye, allowing photo-activation of the NPs.[16] In mice, the NPs distributed to tumors over time and treatment with the NPs led to significantly enhanced tumor growth inhibition compared to the particle without photobleaching, and compared to NPs without the NIR dye in which Ce6 was not quenched. The authors also report that edema was reduced at locations where no photobleaching was performed compared to a non-quenched version of the NP, indicating the ability of the activatable NPs to reduce off-target effects. Taken together, these results show the potential of (activatable) NPs for enhancing the tumor distribution of PS, both for increased therapeutic effect and reduced retention in off-target organs.

Antibodies for tumor targeting

Antibodies are a frequently studied carrier for PS with a high degree of selectivity as a result of antibody-determined specificity. Several antibody-PS conjugates were created and evaluated in vitro.[17–19] In the preclinical setting, Benzoporphyrin Derivative (BPD) was conjugated to the EGFR-blocking antibody Cetuximab (Cet-BPD).[20–22] The BPD was linked in a ratio that allows quenching of fluorescence and partial deactivation of phototoxicity. The results indicate that Cet-BPD is internalized, like Cetuximab, into the lysosome as part of the EGFR trafficking pathway where it dissociates, thereby restoring BPD's fluorescence and phototoxicity. In a mouse model, BPD-Cet displayed increased tumor selectivity compared to free BPD (Verteporfin),[23] and was found to increase the maximum tolerated photodynamic dose up to ~17 times compared to free BPD. Administration of Cet-BPD without illumination decreased metastatic burden compared to control in nude mice with orthotopic EOC tumors, characterized by disseminated metastases, and PDT treatment further reduced metastatic burden, which was significantly enhanced in conjunction with paclitaxel. Together, these data show that Cet-BPD can be used as an efficient antitumor modality. The PS IRDye700DX was conjugated to human anti-human C2-45 antibody that is specific for carcinoembryonic antigen (CEA).[24] The conjugate, termed 45IR, displayed CEA-specific binding to cells and induced strong CEA-specific cell death after illumination with NIR light in various CEA-positive cells. PDT with 45IR was found to effectively inhibit tumor growth in murine models, showing its potential in a preclinical setting. The PS IRDye700DX dye was also conjugated to basiliximab and panitumumab, that was administered as a cocktail for PDT, inducing a growth inhibition in tumor-bearing mice.[25] In another study, Ce6 was conjugated to trastuzumab, an antibody specific for the HER2 antigen.[26] This conjugate displayed a 6-fold increase in the accumulation in

tumors when compared to free Ce6, showing the enhanced distribution. Finally, the anti-EGFR antibody panitumumab was coupled to IRDye700DX, and investigated in a postsurgical setting to remove microscopic tumor remnants.[27] PDT with this conjugate showed significantly enhanced tumor control after removal of 50% and 90% of the tumors compared to resection only and administration of the conjugate without illumination. Together, these results show the benefit of antibodies as carriers for PS to enhance tumor accumulation. Moreover, it shows that antibody-PS conjugates can display a dual mode of action, whereby the therapeutic potential of the antibodies is combined with PDT in a single agent.

Peptides

Peptides have also been used as carriers for PS, including Tat (a cell-penetrating peptide)[28], albumin[29] and biotin[30] that have been evaluated in vitro. For in vivo studies, folic acid was conjugated to the PS pyropheophorbide A, displaying enhanced tumor accumulation tumors that express folate receptor versus free pyropheophorbide a, but much les in tumors that do not express the folate receptor.[31] In mice, PDT with the conjugate induced complete regressions of folate receptor-expressing tumors, providing a proof-of-concept for the approach. A different study employed the αvβ6 integrin-targeting peptide, designated as HK, that was functionalized with graphene coated with the PS HPPH.[32] The accumulation of the construct in tumors was shown to be increased compared to free HPPH or PEGylated constructs without the HK peptide. PDT with the construct resulted in significant tumor growth inhibition as well as increased expression of CD70 and CD40 in $CD11c^+$ DCs in tumor-draining lymph nodes and enhanced IFN-γ levels in serum, suggesting that the treatment induces an antitumor immune response. Recently, virus-like particles (VLPs) derived from human papillomavirus (HPV) were shown to preferentially bind to several cancer over non-cancer cell lines,[33] by binding to Heparan sulfate proteoglycans.[34–36] Based on this, these HPV-based VLPs were conjugated to the PS IRDye700DX that was intended for the treatment of primary uveal melanoma. The resulting PS, AU-011, was shown to induce necrosis in uveal melanoma xenografts in rabbits.[37] Expanding on this, AU-011 was combined with checkpoint blockade antibodies anti-CTLA-4 and anti-PL-L1, inducing complete or near-complete responses, respectively, in TC-1 tumor-bearing mice.[38] These results indicate that peptides hold great potential as carriers for PS by enhancing their tumor accumulation and improving antitumor efficacy after treatment.

Extracellular vesicles

Recently, extracellular vesicles (EVs) were investigated as drug carriers for PS. EVs are nanosized cell-derived vesicles and are involved in communication between cells, as well as in immunological processes such as antigen presentation[39]. They consist of a lipid-bilayer with several membrane proteins and can encapsulate various types of lipids, proteins and genetic information including DNA, mRNA, lncRNA and

microRNA.[40–44] In cancer treatment, they were reported to be promising carriers for microRNAs,[45,46] doxorubicin[47] and paclitaxel.[48] Moreover, several studies investigated EVs as carriers for the PS meta-tetra(hydroxyphenyl)chlorine (mTHPC).[49,50] They show enhanced tumor accumulation and improved tumor growth inhibition of the mTHPC-EVs compared to liposomal formulations of mTHPC (Foslip) or free mTHPC. In a follow-up study, mTHPCs-EVs were shown to induce infiltration of immune cells into murine tumors, but this did not result in complete rejection of the infiltrated tumors.[51] In a different study, M1-like macrophage-derived EVs were used as carriers for Ce6 and a pH-responsive prodrug version of doxorubicin.[52] These EVs were able to polarize M2-like macrophages to an M1-like phenotype and induce strong antitumor effects in tumor-bearing mice, significantly enhancing survival compared to all relevant controls. Another approach used EVs as a carrier for Zinc Phthalocyanine (ZnPc), a potent but poorly soluble PS.[53] The ZnPc-EVs displayed a preferential uptake in cancer cells over immune cells in vitro and were shown to accumulate in tumors after administration in mice. PDT with the ZnPc-EVs induced a strong tumor growth inhibition, without inducing notable off-target toxicities. Together, these data show the potential of EVs as carriers for PS in PDT treatment.

Sensitizer-loaded immune cells

In a more experimental study, gold nanoparticles were crosslinked to Ce6 molecules, conjugated to NHS-PEG-aCD3 antibodies and loaded into cytokine-induced killer (CIK) cells as a delivery system.[54] CIK cells are purified human PBMCs that are cultured in presence of IFN-γ, anti-CD3 and IL-2 before further use. In mice, the Ce6-NHS-PEG-aCD3-CIK cells showed enhanced accumulated in tumors compared to Ce6-NHS-PEG-aCD3 not loaded in CIKs. In mice, unloaded CIK alone induced a tumor growth inhibition, however, this was significantly enhanced after PDT with Ce6-NHS-PEG-aCD3-CIK. This study show that immune cells can also be used as carriers for PDT.

Light propagation through tissues

Attenuation of light in tissues limits the maximum tissue-penetration of light. This may result in incomplete illumination of the tumor area if the depth of the tumor exceeds the maximum light penetration used for PDT (reviewed in [55,56]). This depth is usually several millimeters, up to approximately a centimeter into the tissue measuring from the surface of illumination, but is highly dependent on the wavelength used and the properties of the tissue. For PDT in patients, this can result in partial destruction of the tumor and its surrounding vasculature, leading to tumor regrowth. Reducing the attenuation of light through tissues to optimize PDT involves a tradeoff, whereby wavelengths in the deep red or NIR spectrum generally penetrate further into tissues, but contains reduced energy compared to smaller wavelengths for the generation of ROS. This can, fortunately, be compensated by increasing the fluence but must be performed in a manner that prevents tissue

heating as a consequence of the high energy of the light.

NIR-absorbing sensitizers

Several PS that allow absorption in the NIR region have been under recent investigation. In line with this, novel PS consisting of black titania[57] or conjugated polymer nanoparticles (CP-NPs)[58] were shown to produce heat for photothermal therapy (PTT) and ROS for PDT after illumination with NIR light. Both PS distributed to the tumor area, and induced a tumor growth inhibition in tumor-bearing mice. A different NIR-light absorbing molecule consists of $NaYbF_4$ NPs,[59] that were shown to significantly inhibit tumor growth after intratumoral administration followed by PDT. Luciferase-expressing cells were engrafted inside the tibias of nude mice as a model for bone metastasis and treated with PDT after injection of the NPs into the bone matrix. Tumor growth inhibition was also observed in this setting compared to control, indicating that more deep-seated tissues can be treated with PDT using these NPs. A new kind of green titania with absorption in the NIR region was conjugated to triphenylphosphonium (TPP) to target mitochondria.[60] PDT treatment with NIR light on a single tumor in mice bearing two tumors on opposite flanks significantly inhibited tumor growth in the treated tumors compared to the internal control tumor, or versus animals treated with NIR light alone, the PS alone or untreated animals. Of note, the clinically approved indocyanine green (ICG) is a fluorescent dye that was investigated extensively in the preclinical setting due to its diagnostic and therapeutic properties,[61–66] but its instability in the body has hampered its use in the clinic. To this end, an encapsulated form of ICG was investigated in patients, showing an improved clinical response against basal cell carcinoma.[67] Another sensitizer, a bacteriochlorin named redaporfin[68], was recently characterized and investigated for its potential in vascular-targeted PDT, showing strong antineoplastic activity in murine models through deprivation of the blood supply.[69] Redaporfin-PDT was shown to induce immunogenic cell death by selective destruction of the endoplasmic reticulum and the Golgi apparatus.[70] Moreover, vascular-PDT with redaporfin was shown to induce infiltration of neutrophils, a systemic increase of IL-6, increased levels of IFN-γ-producing or $CD69^+$ $CD4^+$ and $CD8^+$ T cells and an increased $CD4^+/CD8^+$ T cell ratio.[71] The same study reports that the therapeutic effect of redaporfin-PDT was dependent on neutrophils and $CD8^+$ T cells, but not $CD4^+$ T cells. In the clinic, redaporfin has shown high efficacy in combination with checkpoint blockade antibody treatment in a case report against head and neck cancer[72] and has been in a phase I/II trial for the same condition (NCT02070432). Together, these approaches attempting to enable the use of higher wavelengths for optimal tissue penetration are promising.

Upconversion nanoparticles

Another approach involves utilization of upconversion nanoparticles (UCNPs), that absorb multiple photons at a certain wavelength and converts these into a photon

through an anti-Stokes shift with shorter wavelengths that therefore contains more energy and can be used to excite a PS for PDT.[73,74] As a proof of concept, many UCNPs were created and characterized.[75–82] For in vivo application, Gao et al. designed UCNPs that were loaded with ZnPc as a PS and conjugated to c(RGDyK) to target the vasculature surrounding the tumor.[83] The UCNPs displayed enhanced accumulation after administration in the tumor area compared to UCNPs lacking c(RGDyK). PDT with this UCNP induced enhanced vascular permeability in the tumor area which subsequently increased the tumor accumulation of injected particles. To test the efficacy of the UCNPs, 1 cm pork tissue was placed between the tumor and the light source, after which PDT was combined with chemotherapeutic agent Doxil. This treatment regimen resulted in a significantly enhanced tumor inhibition rate of the modified UCNP versus Doxil alone, showing that the UCNPs enabled PDT in deep-seated tissues. In other studies, Ce6-loaded[84,85] UCNPs and $AgBiS_2$-loaded[86] UCNPs were created, inducing significant tumor growth inhibition after PDT at high wavelengths for upconversion. Similarly, UCNPs were created that also contained doxorubicin for chemotherapy.[87] PDT with the particles administered intratumorally induced a tumor growth inhibition, but did not provide a clear benefit over UCNPs that do not include doxorubicin. Taken together, these data indicate the potential of approaches that attempt to overcome the limitations of light propagation through tissues.

Hypoxia in the tumor area

The hypoxic environment that is commonly observed in solid tumors can be a challenging factor for the efficacy of PDT. Hypoxia is defined by a limited availability of O_2, the main acceptor for the energy transfer of light, resulting in a small available amount at the site of action. This can reduce photodynamic damage through singlet oxygen generation and, therefore, diminish therapeutic effect. Recent efforts to address hypoxia in the context of PDT involve the use of PS that do not require large amounts of oxygen for their function. For example, NIR-absorbing gold nanorods that induced plasmonic heating upon illumination, were functionalized with endoperoxides that can induce the release of singlet oxygen as a result of plasmonic heating. In this way, the PS functions as its own source of oxygen thereby circumventing hypoxia.[88] Other sensitizers were developed to remain cytotoxic under hypoxic conditions in vitro, and await further testing in vivo.[89,90]

Sensitizers that function as their own source of oxygen

One study used nanocomposites that facilitate light-dependent splitting of water molecules to generate oxygen and contain the PS PpIX to consume the oxygen molecules for PDT.[91] This nanocomposite was shown to increase singlet oxygen generation in vitro under hypoxic conditions compared to free PpIX as well as induce significant 4T1 cell viability reduction in both hypoxic and normoxic conditions. In contrast, PpIX-PDT with the same light regimen only resulted in pronounced cell

death in normoxic, but not hypoxic, conditions. Moreover, the construct was shown to distribute to murine tumors and PDT in tumor-bearing mice induced an enhanced growth inhibition compared to PpIX alone, showing the potential of the approach. In another study, Ce6-loaded manganese ferrite nanoparticles were created that can catalyze the H_2O_2 present in the tumors to generate O_2 for PDT.[92] These particles also strongly reduced cancer cell viability under normoxic as well as hypoxic conditions. In tumor-bearing animals, the particle reduced the level of hypoxia in the tumor, and induced an improved tumor growth inhibition after PDT compared to all relevant controls. One study loaded nanoparticles that contain perfluorocarbon (PFC) as an O_2 carrier and ICG as a PS into red blood cell membranes.[93] The resulting particles enhanced accumulation in tumors compared to the NPs not loaded into red blood cell membranes. Tumor-bearing mice treated with PDT showed an improved tumor growth inhibition for the particle compared to all relevant controls without inducing cures. Similarly, the PS IR780 was loaded into nanodroplets containing PFC with a O_2-loading capacity to alleviate tumor hypoxia.[94] The construct induced cancer cell death under hypoxic conditions, and PDT in tumor-bearing mice induced significant tumor growth inhibition versus all relevant controls. These data show the feasibility of sensitizers that are their own source of oxygen.

Hypoxia-responsive prodrugs

Another study employed the use of hypoxia-responsive prodrug TPZ, that was co-encapsulated with ICG in PEGylated PLGA-based NPs and conjugated with iRGD.[95] These particles induced a strong reduction in cancer cell viability under normoxic and hypoxic conditions in vitro, whereby TPZ specifically improved the cell death of the NPs under hypoxic conditions. The particles accumulated in murine tumors to a much larger degree than the particles lacking iRGD or free ICG, as well as compared to other organs. In tumor-bearing mice, PDT with the particles induced a statistically significant tumor growth inhibition and reduction of lung metastasis after treatment was compared to all relevant controls. A different approach involved the use of UCNPs functionalized with the PS Rose Bengal (RB) for RB-PDT and a hypoxia probe, installed on the surface of red blood cells with folic acid inserted into the membrane for a measure of tumor-selectivity.[96] In hypoxic environments, the hypoxia probe can release O_2 release from oxygenated hemoglobin upon illumination, strongly reducing cancer cell viability in vitro. The folic acid addition enhanced the tumor accumulation after administration, and PDT with the UCNPs enhanced tumor growth inhibition compared to the relevant controls. These studies show the potential of using hypoxia-responsive drugs for PDT.

Diffusion of oxygen in the tumor

In another study, hyaluronidase was used to disrupt the extracellular matrix in the tumor to enhance the diffusion of oxygen and, potentially, the efficacy of nanoparticle-mediated PDT.[97] Biodistribution data showed a 2-fold increase in tumor

accumulation when treated with intratumoral hyaluronidase before administration of the NPs. Treatment with hyaluronidase also increased the oxygen content throughout the tumor and enhanced the tumor growth inhibition induced by PDT versus the same treatment without hyaluronidase, indicating the potential of extracellular matrix modeling prior to PDT.

Together, these data show the potential of PDT-enhancing modalities that alleviate or circumvent the hypoxic environment often observed in tumors.

Vascular disruption

Photodynamic therapy can be performed with the intention of disrupting the vasculature by damaging the endothelial layer of the tumor vasculature, often by employing types of PS (e.g., verteporfin, padoporfin and padeliporfin) that mostly retain in the vasculature after administration. This type of PDT is called vascular-targeted PDT (VTP) and has been shown in a preclinical setting to be an effective antitumor approach with excellent safety profiles.[71,98] However, incomplete or temporary vessel shutdown in addition to angiogenesis that restores the tumor vasculature are main factors in relapse after treatment.[99–101] To address this, several strategies have been under recent investigation.

Tumor vasculature disrupting agents

To improve treatment outcome, VTP can be combined with compounds that further disrupt or 'normalize' tumor vessels to effectively treat the tumor. One example of this approach utilized inhibitors of the phosphatidylinositol 3-kinase (PI3K) pathway in combination with verteporfin-PDT,[102] as the PI3K pathway was shown to promote endothelial cell survival and proliferation after PDT treatment. The study identified an inhibitor of the anti-apoptotic Bcl-2 family protein Mcl-1 that induced enhanced apoptotic capability combined with Verteporfin-PDT compared to treatment with either modality alone. In mice, this combination induced a stronger tumor growth inhibition than either modality alone, showing the feasibility of the approach.

Specific targeting of the vasculature

Another study used EGFP-EGF1 conjugated nanoparticles that encapsulated the PS hematoporphyrin mono-methylether (HMME), to target the vasculature for PDT.[103] The EGFP-EGF1 ensured preferential uptake by tissue factor-overexpressing cells which is aberrantly expressed on angiogenic vessels[104], enabling accumulation in the tumor vasculature versus non-conjugated particles in vivo as well as ex vivo. This tissue factor-targeting particle was tested in a follow-up study for the treatment of lymphoma in murine models,[105] showing a tumor growth inhibition in tumor bearing mice. Another approach employed VTP with the PS IR700 dye

by conjugation to a platelet-derived growth factor receptor b (PDGFR-b)- specific affibody.[106] PDGFR-b is abundantly expressed by the pericytes surrounding the tumor vasculature and is therefore a potential for vasculature targeting. The affibody-IR700 conjugate displayed a high affinity for PDGFRb and was shown to induce cell death in $PDGFRb^{+}$ pericytes, but not in PDGFRb- tumor cells. In mice, the conjugate was shown to distribute efficiently to tumors and to a slightly lesser extent to the liver and kidneys after intravenous injection. Ex vivo analysis showed co-localization of the conjugate with PDGFRb, confirming its targeting capacity. PDT using the PDGFRb-IR700 conjugate facilitated a tumor growth reduction in tumor bearing mice, showing the feasibility of the approach. In a different study, the hypoxia-responsive prodrug TPZ, was loaded into micelle aggregates consisting of the PS TPC (5-(4-carboxyphenyl)-10, 15, 20-tris (3-Hydroxyphenyl) chlorin) and a novel angiogenic-vessel targeting (AVT) cyclopeptide.[107] The rationale to this approach is to target PDT-induced angiogenesis and convert TPZ into its active form in the hypoxic tumor environment. In mice, the construct was shown to accumulate in tumors to a higher degree than particles lacking the AVT cyclopeptide. PDT with the construct induced a significant tumor growth inhibition compared to all control groups in mice bearing two tumors on opposite flanks.

Using VTP to enhance combination treatments

In another study, VTP using the PS WST-11 was found to enhance the tumor retention of radioisotope ^{90}Y- conjugated to DOTA-AR, a bombesin-antagonist peptide.[108] Bombesin is known to bind the gastrin-releasing peptide receptor (GRPr), that is overexpressed in multiple human cancers including prostate cancer. Radiolabeled DOTA-AR was previously found to bind to GRPr and was specifically incorporated into PC-3 human prostate xenografts.[109] However, approximately two thirds of injected radiolabeled DOTA-AR were washed out within the first 24 h. To improve this, VTP with WST-11 was applied before administration, improving the retention of ^{90}Y-DOTA-AR in PC-3 tumors. Ex vivo analysis showed increased TUNEL and reduced CD31 staining, indicative of successful VTP. The combination VTP and ^{90}Y-DOTA-AR, showed enhanced therapeutic efficacy in PC-3 tumor-bearing mice versus either treatment alone, indicating a synergistic effect. In another study, VTP using WST-11 has been combined with fractionated radiotherapy to improve treatment outcome in prostate cancer models.[110] They show that VTP induces a tumor growth inhibition with improved survival in tumor-bearing mice, and that the combination of PDT with fractionated radiotherapy further enhances the survival, providing a proof-of-concept for future studies. Recently, PDT with Radachlorin, a Ce6-based photosensitizer, was shown to enhance the accumulation of circulating NPs in murine models.[111] In mice, Radachlorin-PDT was shown to completely disrupt the vasculature and strongly inhibit tumor growth, resulting in the accumulation and retention of PEGylated poly-lactic-co-glycolic acid based NPs in the tumor. Analysis of the tumor area revealed that the NPs distributed homogenously throughout the tumor area, and that the majority of NPs are associated with immune cells of myeloid origin, among which are phagocytic cells. The results show the potential of

combining VTP with NP-based therapeutics that benefit from targeting tumor-associated myeloid cells.

Together, these data show the potential of approaches that focus on increasing or exploiting the vasculature disruption induced by PDT. In addition, they underline the need for additional research to investigate more opportunities for improvements of VTP and combinations that further exploit the effects induced by the treatment.

Partial destruction of the tumor

An arduous challenge of PDT is a partial, incomplete, destruction of the tumors that results in survival tumor cells after treatment, followed by a rapid regrowth and tumor progression. One method to overcome this is by addressing the other challenges and opportunities of PDT that are described in this review, or by improving the photosensitizer and protocol used for treatment. Another strategy to improve the destruction of the tumor is by combining PDT with other cytotoxic modalities such as chemotherapy, to further reduce the amount of viable cancer cells after treatment. As with the other efforts, there are many in vitro indications of the feasibility of this approach that precede testing in a preclinical setting.[112–120]

Combinations with chemotherapeutic agents

In one study, the PS vinyl-substituted tetraphenylethylene (TPEPY) was integrated with chemotherapeutic agent Mitomycin C (MMC), thereby quenching its activity as a PS and simultaneously inhibiting the cytotoxicity of MMC.[121] This prodrug construct was converted into its active form by glutathione, which is present inside cells, enabling its cytotoxic potential. In vitro, the conjugate caused a slight reduction in cancer cell viability in absence of light and a strong reduction in viability in presence of light, that could be prevented by pretreatment of the cells with glutathione (GSH)-inhibitor BSO or ROS-scavenger vitamin C. After this, the conjugate was PEGylated to obtain NPs that were injected intratumorally in tumor-bearing mice. Following this, an increasing intensity of TPEPY fluorescence was observed, showing the activation of the conjugate in vivo. PDT with the conjugate induced a statistically significant tumor growth inhibition compared to all relevant control, indicating the feasibility of this approach. Recently, the PS IR-780 dye and mitochondrial-acting anti-cancer drug lonidamine (LON) were co-encapsulated into cationic liposomes that localize to mitochondria after cellular uptake.[122] Illumination of these liposomes was shown to efficiently generate singlet oxygen and to raise local temperature, causing LON to be released. The liposomes were shown to accumulate in murine tumors to a higher degree than in the liver, lung and kidneys. PDT in tumor bearing animals with the liposomes that contained IR780, with or without lonidamine, induced complete and lasting tumor regressions. These data show the antitumor potential of liposome-encapsulated IR780 dye, but could not display the efficacy of LON in this setting, showing the need for additional research.Several approaches

utilized nanosized carriers that contained a PS and a chemotherapeutic agent e.g., pyropheophorbide and SN38, the active metabolite of topoisomerase inhibitor Irinotecan,[123] paclitaxel and sinoporphyrin sodium,[124] RGD functionalized UCNPs with pyropheophorbide a methyl ester and doxorubicin,[125] that achieved similar results in murine models. They all show accumulation in tumors and tumor growth inhibition following PDT, further strengthening the potential of combining PDT with chemotherapy.

Another study investigated a highly complex particle consisting of magnetic mesoporous silica nanoparticles (M-MSNs) with the PS Ce6 and doxorubicin adsorbed onto its surface.[126] Moreover, alginate/ chitosan polyelectrolyte multilayers were assembled around the surface to create pH-responsive particles and shRNA for P-glycoprotein was additionally adsorbed onto this to alleviate multidrug resistance. The particles released their content more readily at decreasing pH and induced cytotoxicity to cancer cells in vitro. In tumor-bearing mice, PDT induced a significant tumor growth inhibition compared to controls. However, the benefit of the particles versus the particles without the pH-responsive layer and shRNA's was minor, and the direct role of the shRNA for P-glycoprotein as well as the pH-responsive layer were not assessed properly, complicating an evaluation of their role in this setting. Although the strategy is interesting, additional research is required to determine whether this particle has a clear benefit over more simple approaches that are similarly effective. Of note, one study combined cisplatin chemotherapy with 5-ethylamino-9-diethyl-aminobenzo[a] phenothiazinium chloride (EtNBS)-mediated PDT.[127] Tumor bearing mice with very large tumors (~800 mm^3) were treated with EtNBS-PDT and induced a very strong tumor regression versus either modality alone, indicating the potential of this combination large tumors, that are frequently resistant to therapy.

Combination with other antineoplastic agents

In a different study, the antitumor agent and heat-shock protein (HSP)90 inhibitor tanespimycin in addition to the PS IR-820 were encapsulated into temperature-sensitive liposomes.[128] Illumination of the liposomes resulted in increased temperatures by IR-820-mediated PTT that triggered the release of tanespimycin. The liposomes induced light-dependent cytotoxicity in cancer cells and increased HSP90 expression. Intravenous administration of the liposome in tumor-bearing mice resulted in accumulation in the tumor in addition to the liver, lung and kidneys. In mice, PDT treated after intratumoral administration of the liposomes induced a significant tumor growth inhibition versus all relevant controls. Unfortunately, the biological activity and antitumor effects of tanespimycin were not further investigated, complicating an accurate evaluation of its function in this setting.

Taken together, these studies show the feasibility and potential of combining PDT with other cytotoxic modalities to enhance the destruction of the tumor area. Many of the studies measured the antitumor efficacy only up to early timepoints after treatment, complicating an evaluation of the effects on survival and showing the

need for additional research.

Insufficient PDT-induced antitumor immune responses followed by tumor progression

Several successful efforts to enhance the therapeutic outcome after PDT utilize the antitumor immune responses initiated by the treatment. This approach benefits from all aspects of PDT, which therein functions as a tumor-debulking and/or vasculature-disrupting modality that can induce an acute inflammation in the tumor microenvironment that has been shown to result in tumor-specific responses.[38,129–131] Immunotherapy, in turn, is aided by the tumor destructive capacities of PDT which inhibits the tumor growth, disrupts the dense mass often observed in solid tumors and can convert the immunosuppressive environment into a more inflammatory state.[130–137] PDT-induced antitumor immune responses have been shown to be essential for complete tumor clearance and progression-free survival in murine models, mostly by $CD8^+$ T cell depletion studies that resulted in abrogation of the antitumor effect.[129,131,138–140] However, many tumors remain resistant to PDT resulting in tumor outgrowth in spite of immune responses after treatment. Although it has been shown that some cancers display low endogenous levels of the DAMP calreticulin (CRT), thereby reducing phagocytic clearance and failing to induce immune responses,[141] the exact mechanisms underlying the evasive mechanisms of tumors in the context of PDT are mostly unknown and may vary between tumor models. Several efforts have been undertaken to improve the efficacy of PDT-induced antitumor immune responses. These efforts can be divided into strategies that utilize PDT to generate or enhance tumor vaccines, strategies that combine PDT with various different forms of immunotherapy, and strategies that combine PDT with immune checkpoint inhibition.

PDT-generated or enhanced tumor vaccines

Several studies utilize PDT to induce ICD in cancer cells and enable access to previously inaccessible (neo)epitopes, that initiates the maturation of dendritic cells (DCs). Such DCs, often called PDT-DCs, function as antitumor vaccines and are either generated in vitro by co-incubation of PDT-treated tumor cells with DCs or in-situ after treatment of the tumor in vivo.[142–149] In one study, PDT-DCs were generated and used to treat glioma-bearing mice.[150] In vitro, PDT treatment of glioma cells was shown to induce surface-exposure of the DAMPs CRT, HSP70, HSP90 in addition to an increase in extracellular DAMPs adenosine triphosphate (ATP) and high mobility group box 1 (HMGB1). PDT-DCs were then generated by treating glioma cells with Hypericin (Hyp)-PDT after which the cells were co-incubated with BMDCs. In a prophylactic setting, high survival rates were observed versus no surviving animals for control or mice treated with freeze/thaw (F/T, as a model for necrosis) incubated DCs. Neutralization of either HGMB1, CRT, extracellular ATP or

treatment with antioxidants all reduced the efficacy of the PDT-DC vaccine, showing the importance of DAMPs as well as PDT in this setting. Moreover, administration of PDT-treated tumor cells in absence of DCs significantly reduced mouse survival rates, indicating enhanced efficacy for PDT-DCs over injection of PDT-treated tumor cells. Furthermore, $Rag1^{-/-}$ mice did not respond to treatment with PDT-DCs, underlining the importance of the adaptive immune system. Brain-infiltrating immune cells after PDT-DC treatment showed increases in total T cells, $CD4^+$ T cells, $CD8^+$ T cells and Th17 cells as well as reduced amounts of regulatory T cells (Tregs) compared to control mice. In a therapeutic setting, PDT-DC treatment was combined with temozolomide (TMZ) chemotherapy and induced a strong increase in survival versus either modality alone. As expected, the TMZ treatment was found to reduce the absolute numbers of intra-brain mononuclear cells and CD8+ T cells. However, the PDT-DC vaccine treatment reversed this effect for mononuclear cells, but not for $CD8^+$ T cells. Furthermore, the PDT-DC treatment reduced the amount of brain Tregs compared to control and treatment with TMZ alone. These results show the potential of PDT-generated DC vaccines in the treatment of glioma-bearing mice and underline the importance of DAMP generation and the presence of a functional adaptive immune system for the efficacy of such treatments.

In another study, Hyp-PDT was shown to enhance surface exposure of CRT, HSP70 and HSP90, and reduce the levels of "don't eat me" signal CD47.[151] Moreover, Hyp PDT induced phagocytosis of cancer cells by DCs, and induce upregulation of maturation markers CD80, CD86 and CD40 to a larger extent than DCs co-cultured with F/T treated cancer cells. In animal models, the PDT-DCs were found to be potent inducers of IFN-γ-secreting $CD8^+$ T cells from autologous T cells and initiated a reduction in the total amount of $CD4^+$ $CD25^+$ Foxp3 cells. Furthermore, the PDT-DCs were shown to inhibit tumor growth in a prophylactic setting, strongly enhancing survival versus F/T treated LLCs and other relevant controls. CTLs obtained from the immunized mice were shown to efficiently induce cancer cell death ex vivo for PDT-DC mice, significantly enhanced compared to mice vaccinated with PDT-treated cancer cells lacking DCs, indicating the existence of tumor-specific T cells after treatment with PDT-DC. In another study, the effect of light fluence on the functional maturation of DCs was investigated.[146] To this end, cancer cells were treated with 5-ALA-PDT at different fluences ranging from 0.125-2 J/cm^2. A fluence of 0.5 J/cm^2 was shown to induce the largest proportion of early apoptotic cells of all fluences tested, that subsequently induced the highest IFN-γ production in BMDCs after co-incubation. Furthermore, this early apoptosis-inducing PDT regimen was shown to induce morphological hallmarks of DC maturation and displayed strong upregulation of maturation markers MHC-II, CD80 and CD86. In addition, mice treated with the PDT-DCs generated by the early-apoptosis PDT regimen were protection from tumor challenge, whereas animals vaccinated with F/T-DCs were not. These results indicate that 5-ALA PDT at a regimen that induces a high proportion of apoptotic cells can induce strong DC maturation that can prevent tumor outgrowth in a prophylactic setting. Together, these results strongly indicate that PDT-induced oxidative stress can exert potent immune stimulation and that vaccination with PDT-treated tumor cells, administered directly or after co-incubation with DCs, can

reduce tumor growth and enhance survival.

Taking a different approach, Kleinovink et al. performed a study that combined PDT with tumor-specific vaccination against TC-1 and RMA tumors.[129] Radachlorin-PDT induced a tumor growth delay in TC-1 tumors without inducing complete responses. Serum analysis showed a significant increase in HMGB-1 serum levels in PDT treated mice compared to control. The tumor-draining lymph nodes displayed an increase in total numbers of $CD8^+$ T cells, tumor (TC-1)-specific $CD8^+$ T cells and $CD11c^+$ cells versus non-tumor draining lymph nodes in PDT-treated and versus dLNs and ndLNs in control mice. In a therapeutic setting, combination of PDT with vaccination significantly improved survival of mice compared to either treatment alone, showing the potential the approach. Moreover, all cured animals rejected a secondary tumor challenge, indicating the existence of immunological memory. In a distant tumor model, the combination also enhanced survival of tumor-bearing mice compared to either treatment alone.

Together, the data show the efficacy of combining PDT with cancer-vaccinations for treating primary as well as metastatic tumors.

Combination with immunostimulatory agents

Several studies have effectively combined PDT with immunotherapeutic agents that improve the antitumor efficacy. In one study, exogenously administered CRT was used to boost PDT-generated immune responses.[152] The antitumor efficacy of Ce6-treated cancer cells injected into tumor-bearing mice was enhanced by pre-incubation of the cancer cells with recombinant CRT or cell surface CRT-inducing agent mitoxantrone prior to injection. In addition, the tumor response to mTHPC-PDT was shown to be significantly enhanced by administering CRT as an adjuvant. The same effect was not observed in NOD-SCID mice, underlining the role for the immune system in this process. Another approach attempted to target Tregs for death with PDT by using anti-CD25 antibodies conjugated to Ce6.[153] The antibody was shown to bind to $CD25^+$ $CD4^+$ T cells after intravenous administration in murine models. PDT effectively depleted $CD25^+$ $CD4^+$ T cells and led to an increase in infiltrating $CD8^+$ T cells, but not $CD4^+$ T cells, compared to isotype-Ce6, anti-CD25 alone and untreated. Furthermore, the amount of intratumoral IFN-γ producing $CD8^+$ T cells and IFN-$\gamma^+CD107a^+CD8^+$ cytotoxic T cells were increased by the regimen. PDT also induced a significant tumor growth inhibition in mice compared to isotype-Ce6, anti-CD25 alone and untreated. Another approach consisted of mitochondria-directing particles that contained the PS IR-820 and the toll-like receptor (TLR)-ligand CpG for PDT and immunotherapy.[154] This particle displayed mitochondria enrichment and was able to induce strong cancer cell death, while the CpG in the particles was shown to retain biological activity. In mice, the particle accumulated in the tumor and PDT induced significant growth inhibition compared to the particle without CpG, showing the importance of CpG in this setting. Recently, the efficacy of Radachlorin-PDT combined with NPs containing two TLR-ligands and a leukocyte-attracting agent was investigated.[131] The combination induced strong

antitumor responses in several murine tumors, significantly enhancing survival and induced an abscopal effect in distant tumors. The observed effects were shown to depend on the presence of $CD8^+$ T cells, as depletion completely abrogated the antitumor efficacy. Moreover, the combination was reported to convert the immunosuppressive tumor microenvironment from cold (immunosuppressed) to hot (pro-inflammatory). Finally, the treatment was shown to function as an in-situ vaccination modality that induced tumor-specific, oncoviral- or neoepitope-directed, CD8+ T cells against the respective tumors.

In another study, a core consisting of the IDO inhibitor 1-methyltryptophan (1MT) was coupled to the PS PpIX through a peptide sequence that is cleaved by caspase-3 for (PDT-) inducible release of 1MT.[155] PDT with the construct induced an enhanced tumor growth inhibition compared to treatment with either PpIX-PDT or 1MT treatment alone. Furthermore, the regimen reduced the amount of metastatic tumor nodules in the lung treatment, suggesting an abscopal effect. Analysis of immune cell populations in blood and spleen revealed reduced percentages of $CD4^+$ T cells and increased CD8/CD4 ratio, indicating 1MT activity in vivo. Finally, TNF-a, IFN-y and IL-17 were increased while IL-10 was reduced after treatment in both the primary and metastatic lung tumors. Similarly, a chlorin-based particle was created that also contained an IDO inhibitor, displaying strong cytotoxicity in several cancer cell lines.[156] In mice, PDT with the particles induced a significant tumor growth inhibition in treated and distant tumors whereas the PDT with the particles without the IDO inhibitor induced a tumor growth inhibition on treated, but not untreated tumors. Percentages of $CD45^+$ cells and $CD4^+$ T cells were increased in both treated and untreated tumors after PDT, $CD8^+$ T cells were only increased in the untreated tumors, while B cells as well as neutrophils were only increased in the treated tumors. These results show the potential of combining PDT with IDO inhibitors and suggest a strong involvement of the immune system in the therapeutic efficacy.

These papers show that PDT combines well with different forms of immunotherapy, underlining the ability of immunotherapy to complement and enhance the antitumor efficacy of PDT.

Combination with immune checkpoint blockade antibodies

Several studies have combined PDT with immune checkpoint blockade antibodies to enhance the antitumor efficacy of PDT. To this end, UCNPs containing PS Ce6 and TLR-7 agonist imiquimod (R837) were employed and combined with anti-CTLA-4 antibodies.[157] In vitro, PDT with the particles induced cancer cell death, and incubation of PDT-treated cancer cells with BMDC induced upregulation of maturation markers CD80 and CD86. In mice, PDT with the particles on tumors induced DC maturation in the tumor-draining lymph nodes and elevated blood levels of IL-12p40, IFN-y and TNF-a at 3 days after treatment. Moreover, PDT combined with CTLA-4 treated on mice bearing two tumors on opposite flanks, induced complete and lasting responses in both treated and distant (untreated) tumors, in contrast to all relevant controls. The addition of anti-CTLA-4 antibody treatment to PDT treatment with

the particle induced increased amounts $CD8^+$ T cells and reduced the numbers of Tregs in tumor infiltrates. In addition, PDT combined with anti-CTLA-4 antibody treatment led to elevated IFN-y levels in serum versus PDT treatment alone. As a control for immunological memory, cured mice were rechallenged with C26 tumors after which the majority of mice were protected from tumor challenge. Another approach consisted of VTP with WST-11 combined with anti-PD-1/PD-L1 antibodies.[158] In tumor-bearing mice, only the full combination provided a significant tumor growth delay in addition to an increased progression-free survival. Furthermore, the combination led to an increase in the $CD8^+$:Treg and conventional T cells (Tconv):Treg ratios and was shown to reduce the number of metastatic lesions in the lung compared to either modality alone. In distant tumors, infiltrating lymphocyte populations were analyzed, but no significant differences were found. Also, CD8:Treg and Tconv:Treg ratios appeared to be lower in the distant tumors for the combination compared to VTP alone, and no differences in proliferating T cells were shown. Lastly, human xenografts were shown to upregulate expression of PD-L1 after VTP with WST-11, suggesting a rationale for initiating human trials investigating the combination of VTP and anti-PD-L1 antibody treatment.

Several other studies successfully combined PDT with anti-PD-L1 antibodies.[159,160] One such study combined the PS Fe-5,10,15,20-tetra(p-benzoato)porphyrin (TBP) with anti-PD-L1 antibody treatment.[161] Fe-TBP produced singlet oxygen under both normoxic and hypoxic conditions due to its ability to convert H_2O_2 to O_2, which can subsequently be used to yield singlet oxygen. In mice, PDT with Fe-TBP induced complete regressions in tumor-bearing animals. These regressions were shown to be $CD4^+$ T cell, $CD8^+$ T cell and B cell dependent, as depletion of these cells significantly diminished the antitumor effects. Furthermore, Fe-TBP PDT on primary tumors combined with anti-PD-L1 antibodies also strongly inhibited the growth of distant tumors versus both treatments alone. Cured mice were protected from tumor rechallenge, indicating the existence of immunological memory. Lastly, PDT and anti-PD-L1 antibody treatment induced increased amounts of total $CD45^+$ cells in primary tumors as well as increased amounts of $CD4^+$ and $CD8^+$ T cells in primary and distant tumors versus untreated animals, further indicating involvement of the immune system in the treatment response. Similarly, a different study investigated pH-responsive PEGylated NPs with a mitochondria-directing agent that encapsulate catalase enzymes to alleviate hypoxia by conversion of H_2O_2 to O_2 were loaded with Ce6 for PDT.[162] These particles displayed preferential localization to mitochondria and induced efficient light-dependent toxicity to cancer cells in hypoxic areas compared to particles lacking catalase. Accumulation of the particle was observed mostly in the liver, followed by the tumor, but PDT still induced a significant tumor growth inhibition compared to the relevant controls. Combination of PDT and anti-PD-L1 antibody treatment induced significant tumor growth inhibition on both treated and distant tumors, whereas PDT with the particle in absence of anti-PD-L1 antibody treatment only induced significant tumor growth inhibition on primary (treated) tumors. Furthermore, this combined regimen increased the percentages of $CD8^+$ T cells in the tumor and IFN-y levels in sera after treatment, indicating involvement of the immune system. A different study investigated core-shell NPs encapsulating

chemotherapeutic agent oxaliplatin and the PS pyrolipid combined with anti-PD-L1 antibodies.[163] In vitro, PDT with the NPs induced ICD through increased exposure of CRT in cancer cells. In tumor-bearing mice, PDT induced a tumor growth inhibition in two different models, and increased the serum levels of IFN-γ, IL-6 and TNF-a after treatment. Furthermore, PDT performed on primary tumors combined with anti-PD-L1 antibodies induced strong tumor growth inhibition in both primary and distant tumors compared in two different models. In another study, zinc porphyrin silica NPs loaded with R837 and combined with anti-PD-L1 antibodies.[164] The R837 was released at low pH and promoted dendritic cell maturation, inducing conversion of the tumor microenvironment into an inflammatory state. PDT with the NPs combined with anti-PD-L1 induced strong antitumor effects and an abscopal effect and was shown to increase the $CD8^+/CD4^+$ ratio as well as the percentage of CTL in both primary and distant tumors.

Together, these studies show that PDT combined with checkpoint blockade antibodies is a highly effective treatment option with strong antitumor efficacy on both primary and models of metastatic tumors.

Recent advances of clinical photodynamic therapy

The use of PDT as a standalone treatment or in combination, in trials and in the clinic has been summarized previously.[55,165–167] Recent clinical trials include combining interstitial PDT using porfimer sodium with standard of care chemotherapy or immunotherapy in patients with locally advanced or recurrent head and neck cancer (NCT03727061). This trial is currently recruiting, and could provide valuable insights in the efficacy of interstitial PDT in patients when combined with chemotherapy or immunotherapy, possibly allowing a comparison of these combinations. One other trial is investigating the efficacy of 5-ALA PDT combined with anti-PD1 antibody Nivolumab in patients with malignant pleural mesothelioma (NCT04400539). This pilot trial is not yet recruiting, but could be pivotal in determining the efficacy of PDT combined with immune checkpoint inhibition in humans that has shown great promise in preclinical models. A different trial is investigating PDT using porfimer in an intraoperative setting sodium and immune checkpoint inhibition in patients with non-small cell lung cancer that display pleural disease (NCT04836429). This trial is currently recruiting and could improve our understanding of PDT-induced immune stimulation in an intraoperative setting. Finally, a trial dedicated to understanding the immune response following 5-ALA PDT in patients with basal cell carcinoma has been initiated (NCT05020912). This trial is currently recruiting and determines several immunological response parameters after treatment, possibly providing novel insights related to the immune response induced by PDT in humans.

Concluding remarks

The field of PDT has been developing at a steady pace, enhancing the treatment efficacy by addressing one or several of the limitations of PDT. Many improvements have been made related to the biodistribution of PS that enhance their accumulation in the tumor. This increases the number of PS in the tumor and therefore theoretically also the ability of these PS to induce damage to the tumor area. Improvements related to the penetration depth of light used for treatment have also been made, increasing the area where photodynamic effect can occur in larger tumors. Moreover, interesting PS have been developed that partially alleviate or circumvent the hypoxic state present in the tumors, that may increase the damage to cells in the tumor area. These efforts all enhance the direct tumor-killing capacity of PDT, reducing the number of viable cancer cells after treatment compared to previously applied PDT modalities. In addition, combination of PDT with other antineoplastic agents can further enhance the tumor growth inhibition and improve survival after treatment. Many of these combinations were shown to be effective against primary tumors, but were not shown to induce an abscopal effect. In this regard, combinations of PDT and immunotherapy were highly effective, inhibiting the growth of both primary and distant, metastatic tumors in a preclinical setting. Although some trials that investigate PDT with immunotherapy have been initiated, the combination has not been thoroughly investigated in humans. Future trials will have to determine whether the efficacy observed in a preclinical setting reflects the treatment outcome in the clinic.

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