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Review

Strategies for Enhanced Photodynamic Therapy Effects†

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ABSTRACT

Photodynamic therapy (PDT) is a treatment modality for the selective destruction of cancerous and nonneoplastic pathologies that involves the simultaneous presence of light, oxygen and a light-activatable chemical called a photosensitizer (PS) to achieve a cytotoxic effect. The photophysics and mechanisms of cell killing by PDT have been extensively studied in recent years, and PDT has received regulatory approval for the treatment of a number of diseases worldwide. As the application of this treatment modality expands with regard to both anatomical sites and disease stages, it will be important to develop strategies for enhancing PDT outcomes. This article focuses on two broad approaches for PDT enhancement: (1) mechanism-based combination treatments in which PDT and a second modality can be designed to either increase the susceptibility of tumor cells to PDT or nullify the treatment outcome-mitigating molecular responses triggered by PDT of tumors, and (2) the more recent approaches of PS targeting, either by specific cellular function-sensitive linkages or *via* conjugation to macromolecules.

INTRODUCTION

Photodynamic therapy (PDT) is defined as the site-directed generation of cytotoxic effects upon targeted light administration to activate photosensitive chemical compounds. Light activation of chemicals or dyes for photo-killing was first introduced in the beginning of the 20th century (1) and is gaining recognition as an effective modality against cancerous (2,3) as well as noncancerous diseases (4). To produce cytotoxic effects, PDT requires three components to be present simultaneously: light (5,6), oxygen (7,8) and a photosensitizer (PS) (9). Various PS have been approved for clinical use (10,11), including porfimer sodium (Photofrin)[a] for the treatment of lung and digestive tract cancers and Barrett's esophagus; aminolevulinic acid (ALA) for the treatment of actinic keratoses; and temoporfin (Foscan), which is approved in the European Union and Japan for the treatment of head and neck cancers. In 1993, Photofrin was initially clinically approved for the treatment of bladder cancer, and is currently the most commonly used PS. However, Photofrin has poor selectivity between tumor and normal tissues, and due to its long clearance time, produces skin photosensitivity that can last for several weeks following treatment (14). Second-generation PS such as verteporfin (Visudyne), which is approved for the treatment of age-related macular degeneration (AMD) (15), have improved tumor selectivity and a quicker clearance, resulting in less prolonged skin phototoxicity. In addition to a somewhat preferential accumulation of the PS in the tumor *vs* in normal tissue, selectivity in PDT is achieved by precisely directing light irradiation to the tumor (3). While the current regimens used for PDT may be adequate for palliation, improved treatment responses (and perhaps even a targeting modality) may be necessary for more complex anatomical sites with localized but intricate three-dimensional structures, such as abdominal tumors (ovarian cancer) or tumors in the thoracic cavity (lung cancer).

In this article, we will focus on approaches to improving treatment outcomes of PDT of cancer by using mechanism-based combination therapies, by enhancing PS targeting, by the utilization of cellular function-sensitive linkages and by the more traditional methods using various carrier-mediated targeting systems.

COMBINATION THERAPY

Photodynamic therapy is gaining recognition as a treatment option for many cancerous as well as nonneoplastic pathologies, and the clinical results are promising. However, for PDT to evolve as a first-line curative modality, both our understanding of PDT mechanisms and the designing of improved treatments based on this understanding promise to provide an enhanced therapeutic response (16). Combination regimens comprising PDT and a secondary treatment can be designed to increase the effectiveness of PDT by either: (a) reducing potentially detrimental molecular responses triggered by surviving tumor cells following PDT, or (b) increasing the susceptibility of tumor cells to PDT. In Fig. 1, "Approach (a)" is based on prosurvival molecular responses such as angiogenesis and inflammation, which are elicited by PDT. Another important factor governing the cytotoxic effects of PDT is hypoxia; PDT can cause hypoxia by oxygen consumption (17,18) and by vasculature damage (19,20). Hypoxia is a major stimulus for angiogenesis, which is mediated by the

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[a]Photofrin has been systemically used in thousands of patients for more than 20 years and some of the early work includes Dougherty (12) and Tsukagoshi (13).

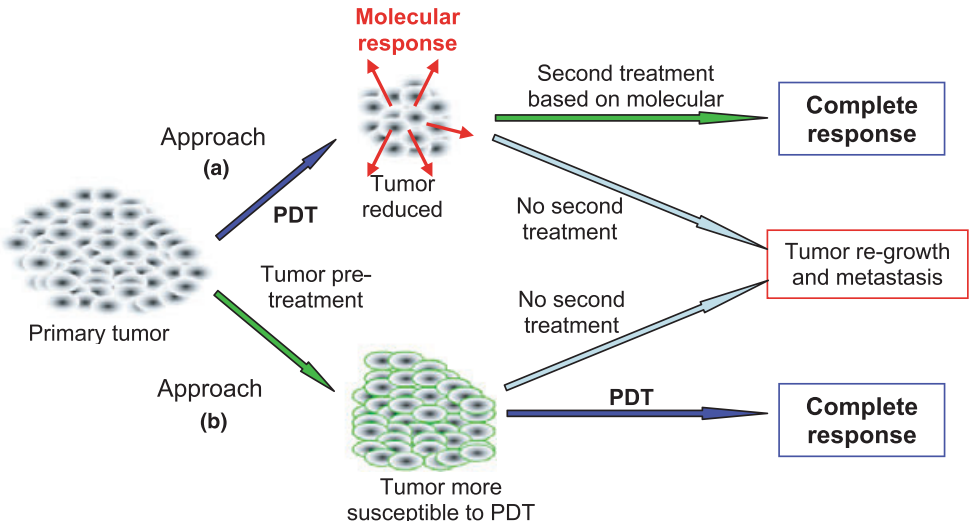

Figure 1. Mechanism-based photodynamic therapy (PDT) combination regimens. There are two basic strategies for combining PDT with a second modality: (a) a secondary treatment is given following PDT, depending on the molecular response of surviving cells to PDT, and (b) the tumor is treated prior to PDT, increasing susceptibility to PDT.

vascular endothelial growth factor (VEGF), a potent angiogenic molecule (21).

Several groups, including ours, have reported an increase in the synthesis and secretion of VEGF following subcurative PDT (22–24), and are investigating the molecular responses of PDT-treated tumors to design novel mechanism-based combination treatments to improve PDT efficacy (and long-term patient health). Ferrario *et al*. have demonstrated an improved treatment response by combining photophrin-mediated PDT with antiangiogenic drugs (23), with cyclooxygenase-2 (24) and with the matrix metalloproteinases (MMPs) inhibitor prinomastat (25). Kosharskyy *et al*. recently reported that subcurative PDT in an orthotopic model of prostate cancer increases VEGF secretion by 2.1-fold and raises the percentage of animals with lymph node metastases (25, Table 1). PDT followed by the administration of an angiogenic agent, TNP-470, abolished the VEGF increase and reduced local tumor growth more effectively when compared with the administration of TNP-470 before PDT. In addition, animals treated with PDT + TNP-470 showed negligible weight loss when compared with that for animals under other treatments at the time of killing (> 3 g). This suggests that combination treatments reduce disease-related toxicities that generally cause extreme weight loss (26) as seen in Table 1, which summarizes the results obtained from combined therapy. Such a mechanism-based approach that directly inhibits VEGF secretion could enhance the therapeutic potential of both PDT and antiangiogenic treatments, and merits further investigation (26). Therefore, the combination of PDT and antiangiogenic treatments may improve therapeutic outcomes in cancer patients and reduce treatment-related toxicities from a given monotherapy.

"Approach (b)" in Fig. 1 involves the development of combination regimens for ALA-based PDT, wherein the effectiveness is enhanced by increasing the susceptibility of tumor cells to PDT. ALA-PDT is clinically approved for the treatment of actinic keratosis, but is being increasingly applied to the treatment of skin and other cancers. This therapy relies upon the conversion of endogenously administered ALA into an active PS, protoporphyrin IX (PpIX). One drawback of ALA-PDT is that the depth of light penetration is limited, which means that PDT of deeper tissues may be subcurative, resulting in unwanted molecular responses that can be detrimental to treatment. Several years ago a connection was discovered between cell differentiation therapy and increased generation of PpIX from ALA, resulting in increased PDT effectiveness (27). The strong tendency of tumor cells toward cell proliferation rather than differentiation prevents cells from reaching maturation (28). Differentiation-promoting agents such as vitamin D or methotrexate, which are approved for cancer treatments, can induce tumor cell differentiation, thereby bypassing defective growth regulation mechanisms and rendering tumor cells more susceptible to antitumor therapies. Further study of the link between differentiation therapy and improved ALA-PDT revealed that key enzymes

Table 1. Treatment response in mechanism-based combination therapy.

Groups	Weight loss* (mean ± SE, g)	Prostate volume† (mean ± SE, mm^3)	Prostate weight† (mean ± SE, mg)	Lymph node metastasis (%)
A. Control	6.1 ± 0.5	761 ± 110	1200 ± 200	40
B. PDT	5.4 ± 0.5	407 ± 134	700 ± 100	72
C. TNP-470	3.1 ± 0.9	484 ± 94	820 ± 120	20
D. TNP-470 + PDT	4 ± 0.4	696 ± 170	800 ± 100	25
E. PDT + TNP-470	−0.3 ± 0.9	277 ± 116	460 ± 160	20

Nearly five to eight animals are used in each group. *Weight loss is calculated by subtracting weight of the animal at the time of killing to weight before injection. †Determined postkilling.

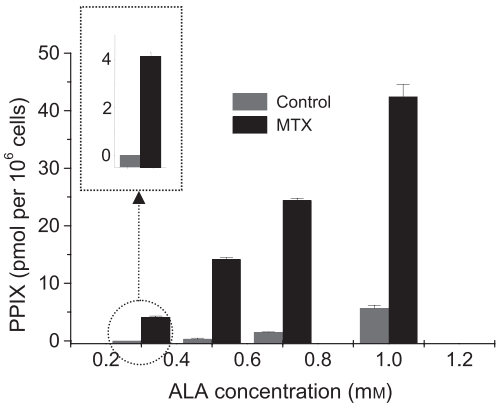

Figure 2. Methotrexate (MTX) enhances protoporphyrin IX (PpIX) accumulation in LNCaP cells over a range of aminolevulinic acid (ALA) concentrations. Cells were pretreated with MTX or medium alone for 72 h, then incubated for 4 h in different ALA concentrations before harvest. (Inset) Enlargement of the graph at 0.3 mM ALA; note that MTX enhancement of PpIX occurred even at the lowest ALA dose.

involved in PpIX synthesis are upregulated by differentiation therapy, which results in the generation of higher cellular levels of PpIX and increased ALA-PDT efficacy (29, Fig. 2). We have demonstrated in an orthotopic prostate cancer model (unpublished) that methotrexate produces a response in solid tumors that enhances the therapeutic effect of ALA-PDT. Methotrexate pretreatment not only enhanced tumor PS levels resulting in increased tumor control, but also nullified the increased metastases caused by PDT. As seen in these promising results, the development of novel PDT combination regimens is a significant step toward improving the outcome of PDT both in terms of local tumor control and metastasis reduction.

TARGETING SPECIFIC CELLULAR FUNCTION-SENSITIVE LINKAGES

Specificity can be improved by not only targeting a PS to a specific site of action, but also by creating an active form of a PS from an inactive (quenched) form by using specific cellular functions (such as enzymes) at the site of action. In this case, an inactive PS is administered such that it is activated (and produces cytotoxic effects) only at the site of the lesion (Fig. 3). This method has been utilized for both cancer and antimicrobial PDT. In cancers, proteases such as MMPs and cathepsins are thought to be crucial for invasion, metastasis and angiogenesis. Funovics *et al*. described the use of various imaging probes designed specifically for locating proteases to be used as targets in the detection of tumors and other diseases (30). Considering the elevated levels of MMPs in tumors, Pham *et al*. designed a near-infrared fluorescent donor linked *via* a protease substrate peptide linker to a fluorescent or nonfluorescent acceptor for sensing tumor-associated MMP-7 activity (31). The underlying principle is that in a fluorescence resonance energy transfer (FRET) between the donor and the acceptor, the absence of proteases results in the quenched fluorescence for the donor; however, in the presence of proteases, the substrate protein is cleaved, releasing the FRET interaction between the donor–acceptor fluorophore, which results in a four-fold increase in the fluorescence signal for an initially quenched molecular dye. A large number of fluorescent probes have been described in detail for proteolysis as a tool in drug discovery (32,33).

Although it is difficult to specifically demarcate between cancerous cells and noncancerous cell markers, functional-sensitive linkages have been significantly beneficial for picking specific markers for infectious diseases such as microbial infections, where enzymatic expressions are clearly differentiated from the host environment. Xing *et al*. have used quenched near-infrared fluorogenic substrate for imaging β-lactamase activity using a β-lactam substrate flanked by a FRET-mediated quenched fluorophore with a nonfluorescent acceptor QSY21, which are released in the presence of enzymes and serve as a biomarker for imaging gene expression in living animals (34). Similarly, these β-lactamase cleavable linkers have been previously used in cancer chemotherapy as antibody-directed enzyme prodrug therapy (35,36).The enzymatic compositions of bacterial and mammalian cells are significantly different, offering an opportunity for capitalizing the difference for achieving high selectivity against bacterial cells. Therefore, our group is focusing on exploiting the strategy of a cleavable lactam-ring containing constructs for anti-microbial PDT of β-lactamase producing phenotypes of bacteria. Figure 4 shows our results with the pictorial representation of a β-lactamase activatable cephalosporin-PS prodrug, in

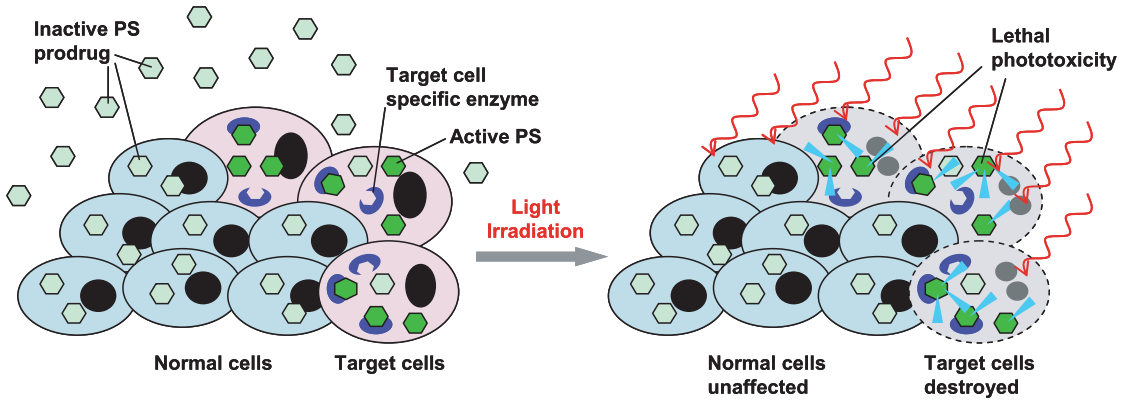

Figure 3. Targeting specific cellular function-sensitive linkages. An inactive photosensitizer (PS) prodrug is taken up in all cells but only activated by certain enzymes expressed in target cells. The target cells containing the active PS are killed upon light irradiation.

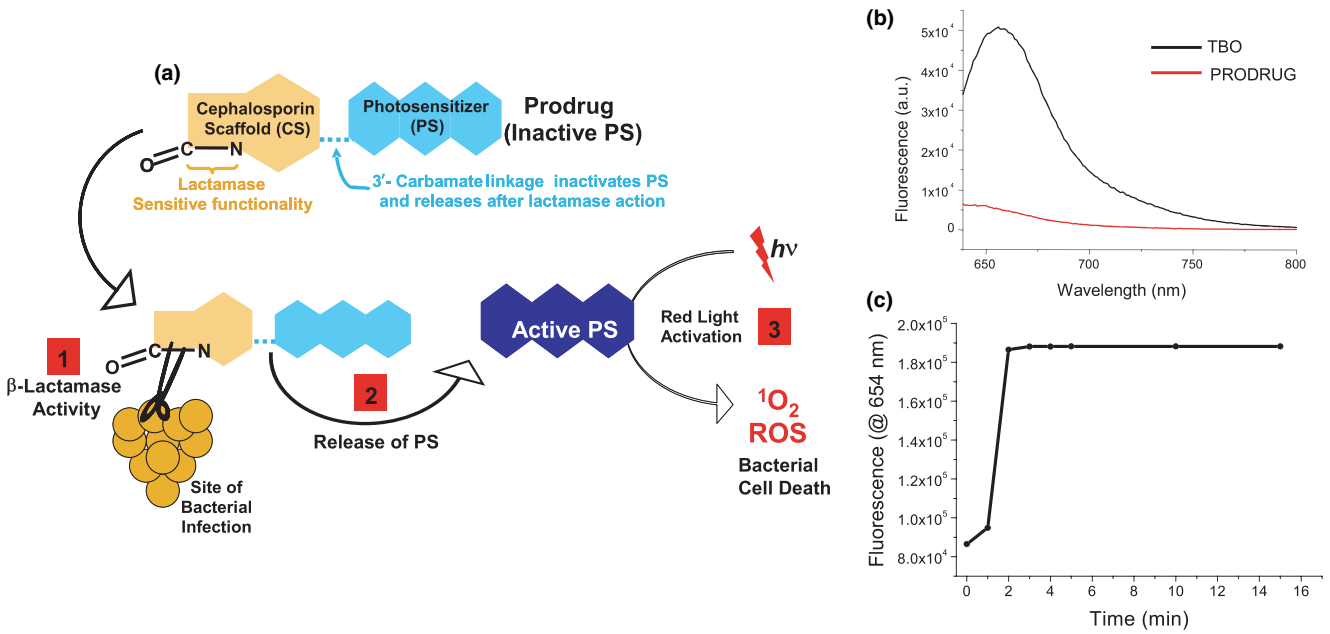

Figure 4. (a) Schematic rationale for proposed prodrugs. The prodrug is comprised of toluidine blue O (TBO) as a photosensitizer (PS) conjugated to a cephalosporin scaffold (CS). The hydrolytic opening of the β-lactam ring will occur only in or adjacent to bacterial cells where β-lactamase is abundant. This results in the release of photoactivatable PS, which with red light illumination will cause bacterial cell death. (b) The fluorescence emission spectra show a nearly eight-fold reduction in emission (ex. 635 nm), signifying quantitative quenching of the PS. (c) The time-dependent fluorescence emission for the PS release from the prodrug in the presence of the β-lactamase enzyme (from *Enterobacter cloacae*) shows an effective PS release within the first 2 min of incubation.

which toluidine blue O is used as the inactive PS, and unlike conventional antibiotics in which hydrolysis of the β-lactam ring by β-lactamases inactivates the antibiotic, the lactam ring opening of the prodrug will release the PS and make it light activatable for photo-killing.

The strategy of nonspecific enzyme susceptibility has been applied to PDT of a neuroblastoma solid tumor by Krinick *et al.* (37). The authors prepared a polymer-bound PS, mesochlorin e6 monoethylene diamine disodium salt (Mce6), *via* an enzymatically degradable oligopeptide (glycylphenylalanylleucylglycine; G-F-L-G) side chain, which is susceptible to lysosomal degredation by cathepsin B. Compared with a nondegradable polymer in which the bound PS is not released, the PS-bound polymer containing the oligopeptide sequence was more effective *in vivo*. However, the target enzyme is not specific to cancer cells and a modest selectivity is achieved. So far, this strategy has been proved to be of considerable potential in the targeting of dyes and decreasing the side effects; further studies are needed to apply this strategy to tumor-related PDT.

ENHANCEMENT OF PDT BY THE PS CONJUGATION TO CARRIER MOLECULES

Current methods to increase the specific accumulation of PS at the target site involve the use of various formulations, including polymer-PS conjugations or the encapsulation of drugs in colloidal carriers such as oil dispersions, liposomes and polymeric particles (38). These available methods show improved selectivity but are not effective enough to allow exclusive accumulation of the PS in target cells, and warrant further improvement. A recent review by Solban *et al.* (39) discusses in detail PDT-targeting methodologies, including passive PS-targeting and active PS-targeting (active targeting is achieved by attachment to carrier moieties that are specifically overexpressed on cancer cells). As PS diffusion *in vivo* is a dynamic process, dependent on a variety of factors such as cell size, shape and the condition of the vasculature (which in tumors tend to be more leaky), there is some discrimination observed for PS accumulation between cancerous and normal tissues (40,41). To achieve the optimal PDT response, PS are typically delivered well in advance to allow for the maximal PS-accretion in the target tissue prior to light delivery. The physiological properties of the PS also play an important role in PS diffusion. For example, hydrophilic molecules remain longer in circulation, while hydrophobic molecules diffuse comparatively faster into cellular compartments (39). Thus, depending on the postadministration time, the PS will have accumulated predominantly in either the cellular or the vascular tumor compartments, which provides the opportunity to target either site using the same PS. Fluorescence microscopic studies indicated that tumor localization of verteporfin, a hydrophobic PS, moved from being predominantly within the tumor vasculature at 15 min after injection, to being predominantly within the tumor parenchyma 3 h after injection (42). In general, PDT targeting of the cellular compartment causes direct tumor cytotoxicity (42–44), while the PS confined within the tumor vasculature upon irradiation will result in extensive vascular damage and shutdown (20,45–48). Thus, cells are killed by tissue ischemia (49,50). Such a vascular mechanism is understood to be involved in PDT with verteporfin in AMD (51) as well as with TOOKAD in human prostate cancer (52) and rat C6 glioma xenographs (53). The effect of combining both short-interval and long-interval PDT

on inhibiting tumor growth was more effective than each form of PDT individually (54). Foscan works under the same principle as verteporfin, although the PS-light interval required to obtain an optimal tumor-to-normal tissue PS ratio is about 4 days (48,55,56), with only a modest tumor selectivity. Consequently, there is a need for specific carriers to direct PS to target tissues (38,57). Various carrier molecules, such as low-density lipoproteins (LDLs) (58,59), oil dispersions, antibodies (60), peptides (61), polymeric nanoparticles (NP) and polymers are used to facilitate PS delivery. The nature of the interaction between the carrier moiety and the PS depends on their physical characteristics and could be mediated by hydrogen bonding, van der Waals forces, π-bond stacking, hydrophobic interactions, physical entrapment and ionic pairing.

As discussed above, the dynamic nature of the PS diffusion *in vivo* means that selectivity between the tumor and normal tissue is not enough to prevent significant damage to adjacent tissues during PDT. The most commonly used strategy to improve PS delivery to a specific target is to attach a carrier moiety with PS. Some of the commonly used targeting approaches are described below:

Serum proteins

Upon systemic administration, most drugs bind to various serum proteins, including high- and LDLs and albumins. PS are also associated with plasma proteins for their transport and distribution. *In situ* generation of these carrier systems can lead to improved PDT action as they may enhance intracellular accumulation of the dye *via* receptor-mediated endocytosis with improved targeting (62). Serum albumin has been widely studied as a carrier for various PS using both covalent and noncovalent methods of conjugation (62). PS–albumin conjugates target scavenger receptors (63,64) that are expressed in high numbers on macrophages. These macrophages represent a significant portion of the tumor mass in certain cancers. Another significant carrier molecule in the blood is LDL; it has been shown that many PS bind to LDL in circulation (65,66). Neovascular endothelial cells and tumor cells generally have a high expression of LDL receptors due to increased cell proliferation (67). Thus, hydrophilic PS bound to LDL can be preferentially accumulated into proliferating endothelial cells by the LDL receptor-mediated endocytosis pathway. The role of LDL receptors as carrier molecules to improve phototoxicity has been investigated using various PS, including amphiphilic hematoporphyrin (58), hydrophobic zinc phthalocyanine (58), benzoporphyrin derivative (66,68) and chlorin e6 (69,70).

Antibodies

Employing monoclonal antibodies (MAbs) as antigen/receptor-specific carriers for selective delivery of PS to the tumor is also known as photoimmunotherapy (PIT) (71). The basic idea evolves around the specific recognition of MAbs for molecular markers present on the tumor cell surface. PIT involves the use of PS-immunoconjugates (PICs), which are made by the covalent attachment of one or more PS molecules to an antibody that targets a specific cell-surface antigen. Whole MAbs with a molecular weight of about 150 kDa (71,72) and smaller MAb fractions (73,74) ranging from 25 to 100 kDa have been studied in conjugation to various PS in PIT. A PS can be directed to the tumor target by utilizing internalizing MAbs (60). Following receptor-mediated endocytosis, PICs undergo sorting to endosomes and lysosomes where the antibody is partially degraded and the PS is released (72). An example of this is the development of a PIC by conjugating verteporfin with C225, a MAb that targets the epidermal growth factor receptor (EGFR), which is overexpressed on the surface of many cancer cells. PIT with C225 PIC showed a nearly 90% reduction in ovarian cancer metastasis with cells overexpressing EGFR while no significant affect on EGFR-negative cells was observed (75). The development and testing of PICs for detection as well as the treatment of tumor cells is reviewed in detail by van Dongen *et al.* (76). Though MAbs provide a specifically directed approach toward particular antigens, most identified human tumor markers are expressed in all tissues (normal and tumor tissue). This limits the application of PIT to tumors with overexpressed target antigens. Furthermore, most PICs are localized in endosomes or lysosomes following receptor-mediated endocytosis. Lysosomally located PS are often less efficient than those that are mitochondrial or membrane bound in terms of inducing cell death (77).

Synthetic peptides

A recent review on enhancing the selectivity of PDT describes in detail the applications of synthetic peptides and polymeric compositions (*e.g.* polylysine, polyarginine) as PS carriers for applications in tumor targeting and antimicrobial therapy (78). Peptides have advantages over other carrier molecules due to their small size, relative ease of synthesis and high affinity for binding to receptors. A range of peptide sequences have been used successfully to direct PS to target tissues which express molecules such as gonadotropin-releasing hormone (79), angiogenic factors (80), VEGF receptor-2 (81,82) and neuropilin-1 (NRP-1) recombinant chimeric protein using VEGF receptor-specific heptapeptide (83). Synthetic peptides are also used as a nuclear localization signal (NLS) (84) as the nucleus is the most sensitive site in a cell, and the proximity of the PS to the nucleus results in enhanced cytotoxicity (85). NLS peptides have also been coupled to other carrier systems, such as LDLs, to provide a potentially selective entry pathway into malignant cells that overexpress the LDL receptor (86), or polymers (87) that provide improved targeting of tumor sites. These peptides need to be chemically modified for stability and efficiency, and common strategies include using pseudo amino acids and cyclic peptides (78). Synthetic peptides are also gaining recognition as specific tumor markers for diagnosis and have been attached to fluorescent dyes or NP for the selective imaging of cancer cells. For example, a recent study showed a disease-specific library-derived fluorescent probe for the early detection of cancer (88). The authors used a phage clone (G1) displaying the peptide sequence IAGLATPGWSHWLAL, fluorescently labeled with the near-infrared fluorophore AlexaFluor 680, and evaluated its ability to bind and target PC-3 prostate carcinomas. The fluorescently labeled phage clone (G1) had a tumor-to-muscle ratio of approximately 30 to 1 and prostate tumors (PC-3) were readily detectable by optical imaging methods (88).

Polymers

Biodegradable polymers are extensively used for drug delivery, and when conjugated to a PS, are known to increase PDT specificity and/or uptake in tumors or other pathological lesions. These polymers as carriers alter the pharmacokinetics, the biodistribution (with less skin photosensitivity) and decrease the phototoxicity to normal tissue (89). Considering the vast number of publications in this area, several recent articles have been selected to discuss currently employed techniques. Polymers can be used as micelles or encapsulating agents to solubilize poorly soluble PDT agents such as meso-tetratphenylporphine (TPP). Encapsulation of TPP into polyethylene glycol/phosphatidylethanolamine conjugate (PEG-PE)-based micelles and immunomicelles (bearing an anticancer monoclonal 2C5 antibody) resulted in significantly improved anticancer effects of the drug in murine (LLC, B16) and human (MCF-7, BT20) cancer cells (90). The most abundantly used biodegradable polymer to enhance the physical properties of PS is PEG. Compared with the nonpegylated conjugate, the attachment of PEG (pegylation) to a poly-L-lysine-chlorin e6 conjugate increased the relative phototoxicity in an ovarian cancer cell line (OVCAR-5), while reducing it in a macrophage cell line (J774). This suggests that the pegylation of a polymer–PS conjugate improves tumor targeting (91). In another *in vitro* study, a PEG-modified photofrin liposome (PF-PEG-Lip) was prepared and demonstrated a slower release of photofrin with a significantly higher phototoxicity when compared with an unpegylated PF-Lip conjugate (92). Considering these advantages, polymers are extensively utilized in dual modes of targeting, as discussed in the next section.

Composite targeting

With the goal of obtaining the full potential of PDT, strategies have been explored in which more than one carrier moiety was conjugated with a PS for the selective targeting of cells and tissues. Considering the complex nature and architecture of tumor tissues, it is essential to design a carrier system that will maximize the cytotoxic effect on tumor cells with a minimum effect on healthy cells. Tijerina *et al.* (93) compared toxicity mechanisms in human ovarian carcinoma A2780 cells treated with a polymer-bound PS either containing or lacking the NLS and/or an oligopeptide spacer. Variations in apoptosis and necrosis were observed after photoactivation, indicating various factors that play an important role in the cytotoxicity of conjugates, such as the charge, the hydrophobicity, and the nature and composition of the conjugates. These studies provide a platform for the design of optimal PDT delivery systems; NP as an example of such multimodal or composite carriers that can tag a PS with more than one targeting moiety are discussed in the following section.

Nanoparticles are at the leading edge of the rapidly developing field of material science in nanotechnology, which influences both diagnostics and therapeutics. NP range in size from 1 to 1000 nm, and have beneficial properties such as biodegradability, large surface areas, high PS loading and ease of functional modification (94). The variety of NP constructs is increasing rapidly and it would be impossible to cover all of them in this review. Therefore, we have focused on the emerging use of NP on PS encapsulation for PDT. Figure 5 shows a pictorial representation of a typical NP application for PDT. The PS is encapsulated within the hydrophobic core of the NP. The outer layer, made up of hydrophilic PEG, is derivatized with a targeting moiety that selectively binds to tumor cell-surface motifs. Upon irradiation, the tumor cells are destroyed without affecting the surrounding normal tissue.

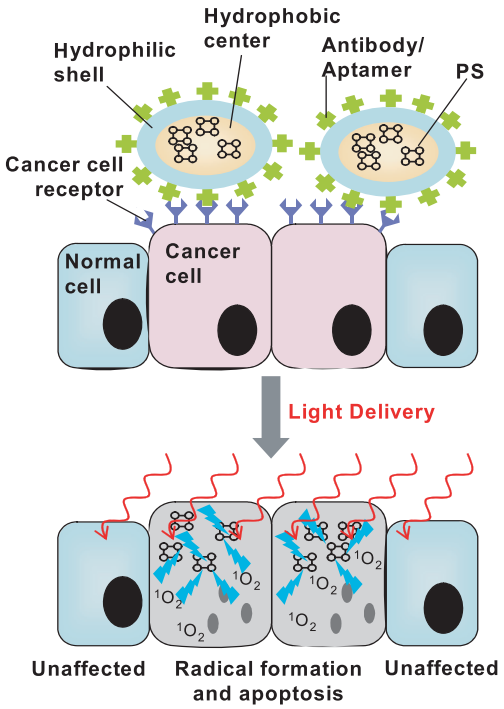

Figure 5. Targeted nanoparticle (NP)-mediated delivery of photosensitizer (PS) for selective photodynamic therapy of tumor cells. The NP targeting moiety can be an antibody or aptamer. Upon light irradiation, the PS selectively kills tumor cells, while not affecting normal cells.

Various polymers have been employed in the generation of NP to achieve biocompatibility, coupled with ease of PS conjugation or encapsulation, and modifiable surface groups. The most common NP for embedding PS are the polymers of polylactic acid (PLA) or polylactic-co-glycolic acid (PLGA) (95–99). For example, NP of PLA or PLGA, loaded with the PS hypericin, showed higher photoactivity than a free PS in a NuTu-19 cell ovarian cancer model derived from Fischer 344 rats (100).

Recently, we investigated the uptake and phototoxicity of the biodegradable PLA-PEG NP encapsulating PS, verteporfin, in two human ovarian cancer cell lines, OVCAR3 (ErbB3-expressing) and PA-1 (ErbB3 lacking). The free PS and the NP-encapsulated PS were taken up by the tumor cells in equal amounts. However, the aptamer-linked fluorophore that targets ErbB3 selectively labeled the ErbB3-expressing OVCAR3 cells, while the PA-1 cells, which lack ErbB3, were unlabeled (Fig. 6). The inability of the untargeted NP to distinguish between ErbB3-expressing and ErbB3-lacking cells demonstrates the need for targeting moieties attached to the NP.

Various biomolecules, such as antibodies, aptamers or peptides, can be used as targeting moieties in conjugation with NP as carriers. Although antibodies are excellent and the most abundantly used targeting moieties for certain applications

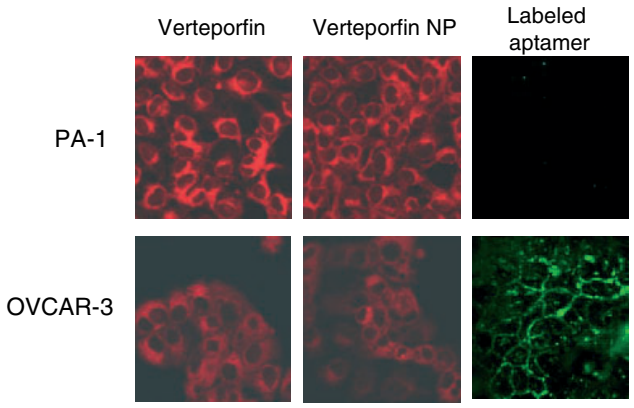

Figure 6. Confocal microscopy of ErbB3-expressing OVCAR-3 and ErbB3-lacking PA-1 cells following incubation with verteporfin (left column), NP-encapsulated veterporfin (middle column) and a fluorescein-labeled A30 ErbB3 aptamer (right column).

(101), aptamers have the advantage of smaller size, ease of isolation and lack of immunogenicity. Aptamers or nucleic acid ligands are short oligonucleotides that fold into well-defined three-dimensional architectures, enabling specific binding to molecular targets (102,103). Pegaptanib sodium (Macugen) is an aptamer clinically approved for the treatment of AMD (104). A large number of aptamers have been generated for cancer diagnosis and therapy, providing a foundation for their potential as targeting agents for PDT (105–108). Despite their advantages, in comparison with antibodies, fewer functional aptamers have been identified (109). Thus, many oligonucletide aptamers that are important for cancer research are not yet available. The susceptibility of aptamers to nuclease degradation is their major pitfall. Although the incorporation of chemically modified nucleotides at specific points along the nucleotide chain has increased resistance to nucleases (110), this has also made the chemical synthesis of functional aptamers difficult and costly.

Reddy *et al.* (111) have recently reported the first application of targeted NP for PDT. In this study, a multifunctional polyacrylamide NP for targeting brain tumors was used for encapsulating Photofrin (the PS), with iron oxide as the imaging agent. A tumor-homing peptide, F3, which selectively targets tumor cells and angiogenic vasculature, was attached to the NP surface *via* a PEG spacer. The F3-targeting moiety significantly enhanced the tumor NP localization; considerable magnetic resonance imaging contrast enhancement was achieved in i.c. rat 9L gliomas following intravenous NP administration. An improved treatment efficacy was observed in the animals, which exhibited a significantly enhanced overall survival compared with animals treated with nontargeted Photofrin-encapsulated particles or with Photofrin alone.

SUMMARY

We have presented two broad strategies for the enhancement of PDT treatment response. These involve: (1) mechanism-based combination treatments in which PDT and a second modality can be designed to either increase the susceptibility of tumor cells to PDT or nullify the treatment-outcome-mitigating molecular responses triggered by PDT of tumors, and (2) some of the more recent approaches of PS targeting, either by specific cellular function-sensitive linkages or *via* conjugation to macromolecules. Specifically, we show that PDT outcomes can be improved by the development of new combination regimens, by the chemical synthesis of conjugates that target cellular functions exclusively and by specific carrier systems that are designed to target tumor cells in more than one way. It is not our intent to suggest that a given strategy is superior *a priori*; in fact, each comes with significant challenges. For instance, rationally designed combination treatments may require extensive mechanistic studies and may be patient-specific. The best results in this case may be obtained with individualized regimens, which would be a complicated undertaking. In the case of targeted PDT, potential challenges include the complexity of new chemistry, the purification of conjugates and the low PS-loading onto carrier molecules. The development of specific cellular function-sensitive linkages for cancer targeting also faces a difficulty in that it may be easier to develop functional targeting approaches for antimicrobial PDT, as the molecular expression of enzymes is more clearly distinguished from the surrounding host environment when compared with the functional differences between tumor cells and normal cells. Further exploration of strategies that target the PS to tumor cells and mechanism-based combination therapies that enhance the treatment outcomes of PDT may lead to new and improved treatments for a variety of cancers and other pathologies, including for those diseases that are resistant to other therapies.

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