ANTICANCER RESEARCH *33*: 2823-2832 (2013)

Review

Tumor-targeted Photodynamic Therapy

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Abstract. *Photodynamic therapy (PDT) is a well-established clinical treatment modality for various diseases, including cancer. It involves the topical or systemic administration of a photosensitizer, followed by selective irradiation of the target lesion with a specific wavelength of non-ionizing light, which triggers oxidative photodamage and subsequent death of the targeted cells. Due to this two-step therapeutic process, PDT is a safe and minimally-invasive therapy. Nevertheless, classical non-targeted photosensitizers lack sufficient tumor selectivity and are taken up in the neighboring normal tissues, resulting in undesirable adverse effects. To overcome this obstacle, diverse tumor-targeting approaches have been developed. In this article, we discuss the current strategies and rationale regarding tumor-targeted PDT.*

Conventional cancer treatments, including chemotherapy, radiation therapy and surgical intervention, are effective but substantially invasive for patients. Photodynamic therapy (PDT) is a minimally-invasive therapeutic modality that requires systemic administration of a photosensitizer (PS) followed by irradiation of the disease site with a light of a specific wavelength to activate the PS. When irradiated, the PS absorbs a photon and first transforms from its ground singlet state to an excited singlet state, and then undergoes intersystem crossing to a long-lived triplet state, or it loses the absorbed energy by heat/fluorescence emission or other photophysical phenomena (1). The triplet state PS stimulates two competing photochemical pathways, generally called type I and type II reactions. The type I reaction involves electron/proton transfer directly from the PS to cellular organic substrates, yielding free radicals or radical ions. These radicals react with molecular oxygen to produce reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion and hydroxyl radical. The type II reaction involves direct energy transfer from the triplet state PS to molecular oxygen, forming another ROS, singlet oxygen. Both types of reactions cause oxidation of various cellular molecules and can induce cell death *via* multiple pathways of apoptosis, necrosis and/or autophagy. In particular, singlet oxygen is thought to be the primary photochemical product and the pivotal mediator of the cell death induced by PDT. Singlet oxygen has a very short lifespan (<40 ns in biological systems) and a limited radius of action (<20 nm) (2). Therefore, the primary site of photodynamic damage is highly proximal to the area of its production and is dependent upon the subcellular localization of the PS. The molecular mechanisms of PDT-induced cell death are reviewed in detail elsewhere (3, 4).

Since the PS is ideally harmless without excitation by light, PDT can be a safe and promising modality for cancer treatment if selective accumulation of PS in tumor cells is achieved, and provided that strictly tumor-focused light irradiation is possible. However, because the tumor selectivity of typical PSs is insufficient, and it is practically difficult to irradiate tumor cells alone (because normal cells in or proximal to the lesion are also irradiated), tumor-specific PS delivery becomes crucial to avoid unwanted damage to healthy tissues (5, 6). In order to achieve enhanced PS accumulation in the target tumor site, two strategies, namely passive and active targeting, have been developed. In this review, we discuss these strategies for tumor-targeted PDT. Although another system using 'activatable' PDT agents, which are activated by the tumor-specific microenvironment or tumor-associated enzymes and become photodynamically active only in tumor cells, but not in normal cells, deserves attention as an alternative means of achieving tumor-targeted PDT, the details are beyond the scope of this review [for information, see reviews by Verhille *et al*. (7) and Lovell *et al*. (8)].

Passive Targeting

Passive targeting takes advantage of physiological and morphological differences between normal and tumor tissues to achieve tumor-selective delivery and accumulation of the PS. Because of the uncontrolled proliferation of tumor cells,

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Key Words: Drug delivery, photodynamic therapy, photosensitizer, tumor targeting, review.

solid tumors have an abnormal tissue architecture and composition that limits the uptake and distribution of drugs (9). Tumor blood vessels often have a defective cellular lining composed of disorganized, loosely-connected, branched, overlapping or sprouting endothelial cells (10). This makes the tumor endothelium very leaky. Furthermore, solid tumors have poorly developed lymphatic drainage, allowing an accumulation of extravasated macromolecules in the extravascular space around the tumor neovasculature. Thus, systemically administered nano-sized drugs or delivery vehicles tend to preferentially accumulate in tumors compared to normal tissues. Such an effect is referred to as the enhanced permeability and retention (EPR) effect (11). Extracellular matrix (ECM) components in the tumor stroma, including collagen, elastin and hyaluronan, are thought to be the major barrier to drug penetration within solid tumors. From the view of drug retention, however, the ECM can act rather advantageously; for example, photoactive anticancer porphyrin derivatives were reported to preferentially interact with collagen and localize in tumor tissues (12).

Another physiological abnormality of tumor tissues is their acidic microenvironment. Hyperproliferative tumor cells have a high metabolic rate and grow beyond the supply of oxygen and nutrients (13). To obtain extra energy, tumor cells employ an enhanced level of anaerobic glycolysis, which results in the production of a large quantity of lactic acid, resulting in intratumoral hypoxia and a low pH state (14, 15). The hydrophobic characteristics of PSs with carboxylic groups are increased under such conditions, and the PSs are distributed preferentially in the tumor cells by diffusion through the plasma membrane. Because hydrophilic molecules generally remain in the systemic blood circulation, while hydrophobic ones tend to extravasate and be retained in tumor tissue, the accumulation of a PS in tumors is enhanced as its degree of hydrophobicity increases. Most of the classical PSs are hydrophobic, but such PSs can easily aggregate in the aqueous environment and are currently not suitable for intravenous use for this reason. Therefore, many of the current PSs being used are often encapsulated in nanostructures, such as liposomes and polymeric particles, or are conjugated with hydrophilic polymers. These nanostructures not can only overcome the solubility problems of the PSs, but can also selectively accumulate in tumor tissues due to the EPR effect (16). Because of their large molecular size, the nanostructures do not easily diffuse back into the blood vessels, resulting in a prolonged retention in the tumor.

Because the efficacy of PDT is thought to be mainly attributable to the production of singlet oxygen, two classes of nanostructures have been developed with different molecular characteristics, based on the respective strategies regarding the mode of singlet oxygen production, namely biodegradable and non-biodegradable nanostructures, and both have been used in PDT. Basically, the PSs involved in biodegradable nanostructures have to be released to produce singlet oxygen, while the PSs in non-biodegradable nanostructures may not need to be released, but oxygen species must be able to freely diffuse in and out of the structures (Figure 1).

Targeting tumors using biodegradable nanostructures. Biodegradable nanostructures have received much attention as a possible means of delivering hydrophobic PSs. Their main advantages are their high drug loading capacity, the large variety of materials and manufacturing processes available, and in particular, the potential to ensure the controlled release of the PS. Biodegradable nanostructures are made of natural or synthetic compounds that are degraded in a biological environment by hydrolytic processes and enzyme-catalyzed degradation. The chemical composition and architecture of these materials can be tailored to accommodate the PS with various degrees of lipophilicity, charges and molecular weights.

Liposomes, which are lipid vesicles with a uni- or multilayered membranous structure, are the most intensively studied pharmaceutical carrier, and have been shown to have great potential for clinical use. Several studies demonstrated an effective accumulation of liposomal PS in tumor cells and an enhanced PDT response (17). Molinari *et al*. investigated the efficiency of the transfer and phototoxicity of a photosensitizing agent, *meta*-tetra (hydroxyphenyl) chlorin (m-THPC), loaded in several formulations of mixed liposomes formed by dimyristoyl-*sn*-glycero-phosphatidylcholine (DMPC) with the cationic 'gemini' surfactant, which contains two polar heads and two aliphatic chains, against human glioblastoma cells (18). The addition of the gemini surfactant to natural phospholipid liposomes enhances the transfection activity and strengthens the liposome bilayer, preventing leakage of encapsulated materials. They showed that the cationic DMPC liposomes can transfer m-THPC to glioblastoma cells more efficiently than the same chlorin in the pharmaceutical form (Foscan®), and can significantly increase the phototoxic effect. Using m-THPC-loaded DMPC/gemini liposomes, Bombelli *et al*. reported similar results against human colon cancer COLO206 cells (19).

In the case of PDT for skin cancer, Pierre *et al*. encapsulated 5-aminolevulinic acid (ALA), a naturally-occurring precursor of the photosensitizing species protoporphyrin IX (PpIX) in heme biosynthesis, into liposomes with a lipid composition similar to that of the stratum corneum, containing ceramide, palmitic acid, cholesterol and cholesterol sulfate (no phospholipids), in order to enable the ALA to penetrate the epidermal barrier (20). This liposome formulation successfully delivered ALA to the target skin layer, illustrating the importance of using a molecular composition suitable for the target tissue. Venosa *et al*. tested

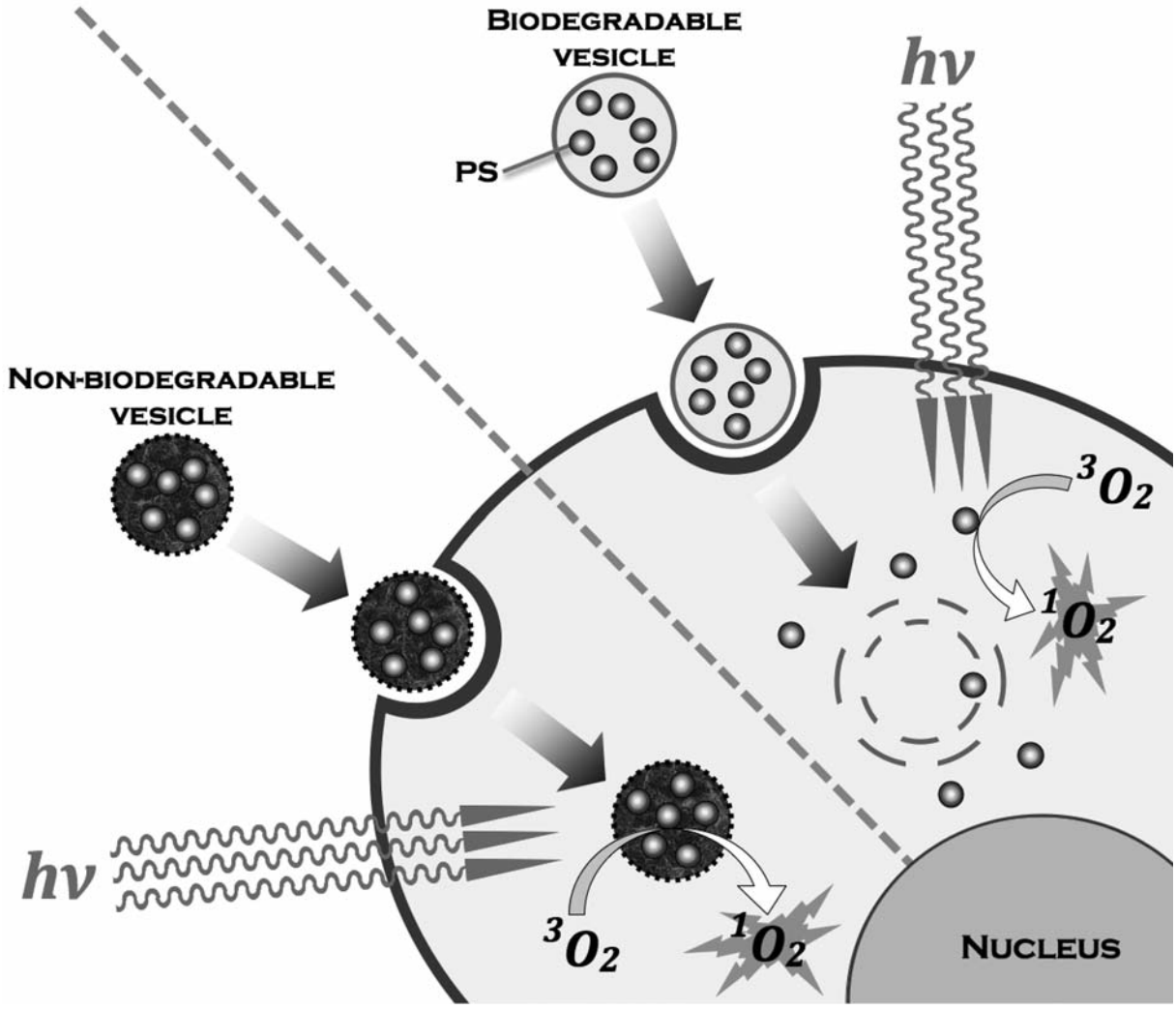

Figure 1. *A schematic representation of the mode of singlet oxygen production by photodynamic therapy (PDT) using photosensitizer (PS)-loaded biodegradable or non-biodegradable nanovesicles. Both nanovesicles translocate into the cytosol of the target cells via adsorptive or receptor-mediated endocytosis. PSs in the biodegradable vesicle are released and deployed to certain cellular organelles after vehicle degradation, and then produce cytotoxic singlet oxygen ($^1O_2$) from ground state molecular oxygen ($^3O_2$) upon irradiation with the excitation light (hν). On the other hand, PSs loaded in the non-biodegradable vesicle remain encapsulated, and the $^3O_2$ that has diffused inside the structure can be excited at the site by light-irradiated PSs, generating $^1O_2$, which then diffuses to exert phototoxic effects.*

mixed liposomes, consisting of phosphatidylcholine and phosphatidylglycerol or phosphatidylcholine and phosphatidic acid, for the delivery of the undecanoyl ester of ALA, which can be loaded in liposomes with higher efficiency than ALA (21). Interestingly, the incorporation of undecanoyl-ALA into liposomes with varying compositions did not improve the rate of porphyrin synthesis from the ALA ester, due to poor cytoplasmic release of the contents, suggesting that the appropriate molecular architecture of liposome-favoring endocytosis and preventing the interaction of highly lipophilic PS the with cellular membrane is needed to ensure the effective intracellular release of the molecule and the subsequent phototoxicity. Thus, entrapment ability and delivery efficacy are not necessarily positively correlated.

The major drawback of conventional liposomes is their short plasma half-life due to their rapid clearance by the cells of the reticuloendothelial system, primarily in the liver. A number of methods have been suggested to enable long-term circulation of liposomes *in vivo*, including coating the liposomal surface with biocompatible polymers, such as poly (ethylene glycol) (PEG) and poly (ethylene oxide), in order to confer a 'stealth effect' on the particles (22); that is, these polymers form a protective layer over the liposome surface and shield the liposome from interactions with opsonizing proteins.

Synthetic polymer-based micelles are also attractive drug carriers for passive targeting. Among the various molecules, poly (DL-lactide) (PLA) and poly (DL-lactide-co-glycolide) (PLGA) are the most popular biodegradable polymers because of their long history of clinical use, favorable degradation characteristics and possibilities for sustained drug delivery (23). To modulate the balance between hydrophilicity and hydrophobicity, co-polymers that consist of a mixture of lactic acid polymers and glycolic acid polymers have been developed. Increasing the molar ratio of glycolide in the copolymer has been shown to increase the hydrophilicity and the biodegradation rate. In a series of studies of *meso*-tetra (*p*-hydroxyphenyl) porphyrin (p-THPP)-containing micelles, Konan *et al*. prepared three types of biodegradable nanoparticles (50:50 PLA:PLGA, 25:75 PLA:PLGA and PLA) (24, 25). They revealed that the polymer compositions did not significantly affect many of the physical characteristics of the nanostructure, such as the polymer molecular weight, particle size or drug-loading capacity, but that they clearly exhibited different phototoxicities against EMT-6 murine mammary tumor cells in the following order: 50:50>25:75>PLA. This suggests that the phototoxicity of the PS is highly affected by the nature of the co-polymer, which influences the rate of uptake and the intracellular concentration of the PS. Ding *et al*. incorporated 5,10,15,20-tetrakis(*meso*-hydroxyphenyl)porphyrin (m-THPP), into PEG-PLA co-polymer-based micelles and systematically investigated the relationship between the loading extent of m-THPP and the phototoxic effect on HN5 head and neck cancer cells and H2009 lung cancer cells (26). They demonstrated that 2% m-THPP-loaded PEG-PLA micelles generated a greater amount of singlet oxygen than 10% loaded micelles did, and that 2% and 10% loaded micelles had comparable phototoxicity against HN5 and H2009 cells, but that the latter also exhibited a dark toxicity (*i.e*. a non-photo-specific toxicity). This indicates that there is an optimal level of PS entrapment to exert an effective phototoxic effect.

Natural biodegradable materials have also been extensively investigated in terms of their potential use for constructing a PS-delivering vehicle due to their abundance, biocompatibility and unique characteristics. Chitosan, a natural product-based polymer obtained by the alkaline deacetylation of chitin, is one such representative biodegradable material, and has been used as a support material for gene delivery, cell culture and tissue engineering (27). Lee *et al*. developed PpIX-conjugated glycol chitosan nanoparticles as tumor-homing drug carriers with a cellular on/off system (28). The amphiphilic PpIX-glycol conjugates formed a stable nanostructure under aqueous conditions, wherein conjugated PpIX molecules formed hydrophobic inner cores and were covered by the hydrophilic glycol polymer shell. Based on the nanoparticle structure, the particles exhibited a self-quenching effect, that is, they were in an 'off' state with no phototoxicity upon light exposure. However, upon cellular uptake, the compact nanoparticle structure was gradually degraded to generate strong singlet oxygen generation when the region was irradiated. Furthermore, compared to free PpIX, PpIX-glycol nanoparticles exhibited prolonged blood circulation, enhanced tumor targeting and improved therapeutic efficiency in HT-29 (human colon cancer) tumor-bearing mice.

Recently, an iodinated form of glycol-chitosan polymer which is chemically-embedded with the photosensitizing agent chlorin e6 (Ce6) was designed and prepared by Lim *et al*. (29). This novel nanostructure has an enhanced capacity for generating singlet oxygen by the intraparticle heavy-atom effect (30), along with high tumor targetability in SCC7 (squamous cell carcinoma) tumor-bearing mice, thanks to the biocompatible glycol chitosan-coated exterior, with a positive charge and tumor-homing characteristics. Other PS-containing nanostructures formulated with natural polymers, such as cyclodextrin, human serum albumin, hyaluronic acid and alginate, have also been reported to be useful in *in vivo* and *in vitro* PDT studies.

Targeting with non-biodegradable nanostructures. Non-biodegradable nanostructures have several advantages over biodegradable systems. Firstly, they are highly stable to fluctuations in pH and temperature, so they can maintain their integrity over much longer periods of time, and work as catalysts to produce ROS from molecular oxygen. Secondly, the molecular properties of the nanostructures, including their size, shape and porosity, can be readily controlled during their preparation. The pore size can be adjusted to <1 nm in diameter, which prevents the loaded PS from leaking out, but is large enough to permit the free diffusion of molecular and singlet oxygen (31).

Polyacrylamide polymers have been used for the synthesis of non-degradable nanostructures for drug delivery. Tang *et al*. prepared polyacrylamide-based nanostructures encapsulating methylene blue (MB), a widely used PS, the clinical application of which is problematic owing to its propensity for rapid enzymatic inactivation (32). They demonstrated that the nanoparticles provide a good protection for the embedded MB against reduction by diaphorase enzymes, and that they significantly improve the photodynamic efficacy of MB against C6 glioma cells. A polyacrylamide-based nanostructure was also used to encapsulate a near infrared (NIR) photosensitizing agent, 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphyrin tetra(*p*-toluenesulfonate) (TMPyP), as reported by Gao *et al*. (33). Light in the NIR range can traverse biological tissues very efficiently due to the low scattering and energy absorption by water and hemoglobin, thus leading to minimal tissue invasion and deeper (up to 10 cm) tissue penetration (34). The authors of that study revealed that the potential toxicity of TMPyP is circumvented by its permanent encapsulation in non-degradable particles, but at the same time, the TMPyP can still

kill C6 glioma cells efficiently by producing singlet oxygen upon exposure to NIR light.

Other major non-biodegradable nanostructures are silica-based or metallic particles. Silica-based nanostructures are chemically-inert and stable *in vivo*, and various methods for their synthesis allowing for precise control of the particle size, shape and porosity have been extensively reported. Prasad's group has developed a highly stable and versatile formulation of ultrafine organically modified silica (ORMOSIL) nanostructures entrapping water-insoluble PS 2-devinyl-2-(1-hexyloxyethyl) pyropheophorbide (HPPH) (35). Compared with free HPPH, ORMOSIL-HPPH exhibited much higher uptake by UCI-107 (human epithelial ovarian carcinoma) and HeLa cells and had significant phototoxicity. ORMOSIL was also utilized to encapsulate a highly lipophilic PS, phthalocyanine 4 (Pc4), by Zhao *et al*., which improved the solubility, stability and PDT efficacy of the molecule against A375 and B16-F10 melanoma cells, compared to free Pc4 (36). However, Simon *et al*. showed that a silica-based nanostructure containing PpIX was accumulated in tumors of xenografted mice better than free PpIX, but that it also accumulated in healthy tissues, especially the liver (37), so special care has to be taken when examining the *in vivo* use of these compounds, and further improvements, such as the use of surface modification of the particles with a targeting molecule, may be necessary.

Gold nanoparticles are also widely used as drug nanocarriers. They have a remarkable capacity to absorb and scatter light due to localized surface plasmon resonance (SPR) effects, and also have a unique property in that they can efficiently convert the absorbed light to heat *via* a photothermal effect (38). These photophysical characteristics have been utilized for imaging techniques and hyperthermia therapy, providing a platform for the system of theranostics. In contrast to silica-based nanoparticles, gold nanostructures can be fabricated to have an extremely small size of only a few nanometers. Furthermore, unlike the silica nanoparticles, where the PS is confined to the core, it is possible to attach many PS molecules to the surface of gold nanoparticles because they have a large surface area (39). Consequently, the produced singlet oxygen is able to diffuse into the tumor cells much more easily. Cheng *et al*. prepared PEGylated gold nanoparticles loaded with Pc4, and showed highly effective tumor targeting with a greatly shortened delivery time required for the PDT response in a tumor-bearing mouse model (40). Gold nanorods, which are another type of gold nanostructure, have also received significant attention. Because of their anisotropic shapes, gold nanorods exhibit two distinct bands of SPR, a weak transverse SPR band at ~520 nm, similar to that of gold nanospheres, and an intense longitudinal SPR band, which can be tuned from the visible to NIR (650-900 nm) regions by increasing their aspect ratios (41). A notable example of targeted PDT using gold nanorods was reported by Jang *et al*. (42). They prepared gold nanorods conjugated with the PS pyropheophorbide-a (PPa) *via* a cleavable peptide linker that is specifically recognized by a tumor-associated protease, matrix metalloprotease-2 (MMP2). In their study, the intrinsic fluorescence and phototoxicity of the conjugated PPa was suppressed in its native state by energy quenching due to the surface energy-transfer properties of the gold nanorods, becoming activated only after cleavage by MMP2. The authors demonstrated that the conjugated PPa exhibited a significant production of singlet oxygen and phototoxicity against an MMP2-overexpressing cell line, HT1080 (human fibrosarcoma), whereas no apparent damage was shown in MMP2-negative BT20 cells. Thus, this novel enzyme-activatable PDT agent may be useful for NIR fluorescence imaging as well as for PDT. In addition to gold nanostructures, successful bioimaging and targeted PDT using other nanostructures made of metals, such as iron oxide, barium manganese oxide or NaGdF4 doped with Yb and Er, were also reported.

Active Targeting

Although the EPR effect-based passive targeting forms the basis of targeted therapy, it suffers several limitations. For example, because certain tumors, especially those of early stage, do not exhibit the EPR effect, and because the permeability of blood vessels may not be the same throughout a single tumor, delivery systems that depend only on passive targeting mechanisms inevitably face intrinsic limitations to their specificity and efficacy (43). To overcome these limitations, it is desirable for PS-delivering carriers to have the ability to actively bind to the specific cells after extravasation. Of course, such an active targeting process cannot be separated from a passive process because it occurs only after the molecule has passively accumulated in tumors, but active targeting is expected to lead to more effective intratumoral accumulation, with lower systemic toxicity, and in the case of targeting with internalizing ligands, to higher intracellular concentrations of the PS. During active targeting, the targeting ligands are attached to PS molecules or on the surface of the PS-loaded nanostructures to lead to specific binding to appropriate antigens or receptor molecules overexpressed by the tumor cells or tumor vasculature, but not by normal healthy cells.

Antibody-based targeting. Active targeting with a monoclonal antibody (MAb) has been the gold standard for drug delivery systems over the past two decades due to the tremendous antigen specificity. In the field of tumor-targeted PDT, the specific term 'photoimmunotherapy' (PIT) has been used to describe the approach in which a PS conjugated with antibody or an antibody fragment against a tumor- or tumor vasculature-associated antigen is used. Unlike conventional antibody-based

immunotherapies, the MAb used in PIT does not necessarily need to have an intrinsic effector function. This is thought to be advantageous for MAbs with potential systemic toxicity. PS-MAb conjugates require a high PS to MAb ratio in order to exert significant phototoxicity, but overloading of the PS may lead to a loss of binding activity of the MAb. An intact MAb contains more lysine residues available for conjugation than do smaller antibody fragments or the single-chain variable fragments (scFvs), so PS loading is less likely to occur at the region required for antigen recognition when an intact MAb is used, sustaining immunoreactivity. However, different antibody formats significantly affect the pharmacokinetic profiles *in vivo*. The use of an intact MAb slows the blood clearance, leading to an increased chance of the agent accumulating in vital organs; moreover, these agents have a poor ability to diffuse throughout the tumor mass due to their large molecular size. In contrast, the scFv format exhibits a rapid blood clearance and easily diffuses within the tumors (44).

The proteins in the epidermal growth factor receptor (EGFR) family are often overexpressed in many types of human tumors, and are known to be internalized by endocytosis, making them one of the molecular targets for intracellular drug delivery. Savellano *et al*. conjugated a clinically approved benzoporphyrin derivative (verteporfin) to the anti-EGFR MAb cetuximab, and showed that the conjugate effectively targeted and photodynamically killed EGFR-overexpressing A431 (epidermal carcinoma) and Ovcar5 (ovarian cancer) human cancer cells, whereas free verteporfin exhibited no specificity (45). Using two different PEGylated anti-EGFR2 (HER2) MAbs conjugated with PPa, similar positive results were reported by Savellano *et al*. (46). The authors demonstrated that multiepitope-targeted PIT with a mixture of two PPa-MAb conjugates significantly enhanced the PIT efficacy against SKOV3 (human ovarian cancer) and SKBR3 (human breast cancer) cells with a high degree of specificity, compared to free PPa and each PPa-MAb alone. Kuimova *et al*. also reported the successful intracellular imaging and effective PIT using anti-HER2 scFv conjugates with PPa or verteporfin (47). One of the most striking studies on PIT against EGFR was reported by Mitsunaga *et al*. (48). In their study, a novel NIR phthalocyanine, IRDye700DX, was conjugated to an anti-HER1 MAb (trastuzumab) and anti-HER2 MAb (panitumumab). Target-specific photodynamic effects were observed in both *in vitro* and *in vivo* experiments. Importantly, unlike conventional PIT agents, which usually require translocation into the cytosol in order for them to exert their phototoxicity, the IRDye700DX-MAb conjugates were substantially effective when bound to the target cell membrane, with no need for internalization. These characteristics of IRDye700DX were further supported by a recent report of our group, in which the dye was conjugated to a human anti-carcinoembryonic antigen MAb (49). Thus, IRDye700DX-based PIT seems to be promising and may be applicable for cancer treatment, especially for drug-resistant tumor cells (*e.g*. cancer stem cells) with a high drug efflux capacity. Another notable study of anti-EGFR-mediated active targeting was reported by Kuo *et al*. (50). The authors prepared anti-EGFR-conjugated gold nanorods which were coated with a hydrophilic and anionic photosensitizer, indocyanine green, which simultaneously served as a photodynamic and photothermal therapeutic agent to kill cancer cells. They demonstrated that the combined PDT and hyperthermia more efficiently killed A549 human lung cancer cells than PDT or hyperthermia alone, and the system also served as an effective imaging probe.

Targeting via ligand–receptor interactions. Cognate ligands of the tumor-associated membrane receptors have also been utilized for tumor-targeted PDT. As described above, the EGFR is often overexpressed in various types of human cancers making EGF an attractive molecule for tumor targeting. Lutsenko *et al*. synthesized conjugates of EGF with aluminum or cobalt disulfonated phthalocyanine, and found that these EGF-PS conjugates had higher photodynamic activity against human MCF-7 breast cancer cells than did unconjugated phthalocyanines (51). They also showed *in vivo* efficiency of the conjugates against B16 melanoma in tumor-bearing mice.

Another attractive ligand that can be used with a PS carrier is low-density lipoprotein (LDL), which solubilizes and transports hydrophobic molecules, such as cholesterol. Because cholesterol is a vital component of biological membranes and is essential for the growth and viability of all cells, hyperproliferative tumor cells and tumor vascular endothelial cells necessarily demand a high concentration of LDL; therefore they often express the LDL receptor at much higher level than normal cells (52). Furthermore, LDLs have a very large payload for hydrophobic drugs, and most hydrophobic PSs are believed to be extensively incorporated into the lipoproteins (53). Collectively, these properties would make LDLs suitable PS carriers for active tumor targeting. Song *et al*. designed and synthesized tetra-t-butyl silicon naphthalocyanine with two long alkyl chains of oleate for efficient incorporation of naphthalocyanine (NIR-PS) into LDL (54). Reconstituted LDL exhibited preferential uptake by human hepatoblastoma (HepG2) tumors compared to normal tissues after intravenous injection in tumor-bearing mice, and enabled for non-invasive NIR imaging, demonstrating the feasibility of theranostics using this PS-LDL complex. Schmidt-Erfurth *et al*. prepared Ce6-conjugated LDL by carbodiimide coupling (55). Covalent binding to LDL significantly increased the cellular uptake of Ce6, which was dependent mainly on a LDL receptor-mediated mechanism, for Y79 human retinoblastoma cells compared to free Ce6. The Ce6-LDL conjugate also exhibited effective phototoxicity against Y79 cells.

Of note, covalent PS conjugation to LDL can be disadvantageous. Urizzi *et al*. examined how two different methods of PS loading onto LDL affected the photodynamic efficiency (56). When aluminum tetrasulfonated phthalocyanine (AlPcS4) was non-covalently inserted into the phospholipid monolayer of LDL, the dye exhibited a significant increase in phototoxicity against A549 cells. In contrast, LDL covalently loaded with the AlPcS4 exhibited little phototoxicity, even at a 10-fold-higher drug dose. It seems reasonable to postulate that the covalent labeling of the apolipoprotein of LDL with the PS greatly reduced the LDL receptor recognition, rendering the derivative photo-inactive. Alternatively, the intracellular destination of the PS-LDL conjugate may be altered so that the site becomes unsuitable for the compound to exert efficient phototoxicity. Such an explanation may be supported by the study reported by Obochi *et al*. (57). In their report, the effect of human serum components on the photodynamic activity of zinc phthalocyanine (ZnPc) was studied. They revealed that LDL inhibited the cellular uptake of ZnPc by Chinese hamster fibroblasts (V-79) compared to incubation of ZnPc with the same cells in serum-free medium, whereas it increased the photodynamic efficiency of the ZnPc, suggesting that LDL facilitates the localization of the ZnPc at cellular targets susceptible to photodynamic damage. On the other hand, high-density lipoprotein increased the ZnPc uptake by 23%, but the photodynamic efficiency was basically unaffected. Thus, the cellular distribution of the PS is vitally important for its photodynamic activity. In addition to LDL, various proteins, such as insulin, bovine serum albumin, transferrin and factor VII, have been utilized as tumor- or tumor vasculature-targeted PS carriers that are internalized *via* the corresponding receptor protein, which is up-regulated in certain types of cancer.

Some non-protein ligands have also been successfully applied for tumor targeted-PDT. Folates are vitamins involved in one-carbon metabolism and *de novo* nucleotide synthesis, and have a high affinity for the folate receptor (FR). The FR is one of the receptors that are overexpressed in numerous tumors, but not in most normal tissues. Upon receptor binding, folates are endocytosed *via* what is believed to be a non-destructive pathway, thus allowing folate conjugation to represent a potential strategy for tumor-targeted PS delivery. Using folate-PEG-cholesterols and solid lipid nanoparticles (SLN), which are biodegradable nanostructures made of solid lipid, Stevens *et al*. developed FR-targeted hematoporphyrin-stearylamine (HpSa) (58). HpSa-SLN greatly increased the cellular uptake and phototoxicity of the PS in FR-positive KB cells, a human oral epidermal carcinoma cell line. An *in vivo* study on a folate-based PS targeting was recently reported by Syu *et al*. (59). In their study, a folate-conjugated polymeric m-THPC delivery system was developed to improve the tumor targeting of the PS. Their results demonstrated that folate-conjugated m-THPC-loaded micelles are specifically taken up and accumulate in xenograft KB tumors, preventing photodamage to the healthy tissues.

Another vitamin, retinoic acid, was also used as a tumor-targeted PS carrier, with the expectation that there would be synergic or additive antitumor effects due to the differentiation-inducing capability of retinoid *via* the its nuclear receptor (RAR). Sibrian-Vazquez *et al*. synthesized a series of porphyrin-retinamides containing retinoic acid covalently linked to the para-phenyl position of *meso*-tetraphenylporphyrin (60). The porphyrin-retinamides were well taken up by RAR-positive neuroblastoma SK-N-DZ cells, and one of them showed clear, but moderate, photo- and dark-toxicities against SK-N-DZ, but not against HEp2 cells. Thus, the ligands of nuclear receptors such as retinoic acid, steroids and other hormones, could be useful molecular candidates for PS-targeting due to their high affinity to the nuclear receptor and their considerable contribution to tumor cell function. In fact, the use of estrogen receptor ligands has been extensively studied, especially in estrogen receptor-overexpressing breast cancer cells (61, 62). However, to date, the preparation of PS-conjugated nuclear receptor ligands that have adequate photodynamic activity and binding affinity after PS conjugation has yet to be fully achieved. Therefore, the methodology needs further improvement.

Conclusion

Although various strategies have been developed for tumor-targeted PDT, it seems unlikely that a universal approach will be established. This is because of the innate properties of tumors; that is, their heterogeneity and dynamic transition of the molecular basis of the cancer cell itself in terms of the tumor vasculature and tumor stroma. This transition involves changes in the expression profiles of tumor-associated antigens, the acquisition of drug resistance and/or the development of intra- or peri-tumoral physiological barrier functions. In order for substantially effective PDT against tumors to be developed, a tailored PDT, in which an appropriate targeting strategy, molecular targets and targeting molecules are carefully selected corresponding to the tumor type and the stage of disease, would be necessary. From the perspective of the prevention of tumor metastasis and recurrence, PDT-induced antitumor immune responses also need to be taken into account.

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Received April 4, 2013
Revised May 30, 2013
Accepted June 3, 2013