Photodynamic Therapy

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Abstract

Photodynamic therapy (PDT) is a clinically established treatment modality for a range of cancers. It utilizes the combined action of photosensitizer, light, and molecular oxygen to generate reactive oxygen species (ROS), particularly singlet oxygen, to eradicate malignant cells and tissues. The therapeutic outcome depends largely on the performance of the photosensitizer. For cancer treatment, only a few PDT drugs, including porfimer sodium, temoporfin, and aminolevulinic acid, have been clinically approved. Unfortunately, they still suffer from a number of drawbacks. As a result, development of new generations of photosensitizers that are more efficient and tumor selective, have a wider scope of action, and produce less side effect is under intensive investigation. In addition, various approaches have been actively explored to enhance the tumor-targeting property of photosensitizers. It is commonly believed that PDT exerts its antitumor effects through three different biological mechanisms. Firstly, the ROS generated through the photosensitization process can trigger apoptotic or necrotic response, leading to direct tumor cell death. Secondly, the photodynamic action can target the blood vessels so as to block the nutrient and oxygen supplies to the rapidly proliferating tumor cells. Finally, PDT can also enhance antitumor immunity which is important not only in killing the tumor cells but also in preventing recurrence. The treatment efficacy of PDT can further be improved in combination therapy where it is used together with drugs that are cytotoxic, anti-angiogenic, or immunogenic. This chapter aims to give an overview of the principle and development of this innovative approach for cancer treatment.

Keywords

Photodynamic therapy • Reactive oxygen species • Photosensitizer • Tumor targeting • Nanoparticle • Cell death • Anti-angiogenesis • Antitumor immunity • Antitumor vaccine • Combination therapy

Introduction

Light has long been used for medicinal purposes. Photodynamic therapy (PDT) has emerged as a promising treatment modality for a variety of premalignant and malignant diseases. It involves the combined use of three individually nontoxic components, viz., photosensitizer, light, and molecular oxygen, to produce a toxic effect. In the presence of light, the photosensitizer is activated and converts endogenous molecular oxygen into cytotoxic reactive oxygen species (ROS). The ROS generated react rapidly with biological substrates, leading to apoptotic or necrotic cell death. An ideal photosensitizer is nontoxic without illumination. Thus, with a specific delivery of light and preferably also of the photosensitizer, the toxic effect can be confined to a localized region. Such specificity makes PDT a promising approach in treating various diseases, including cancer [1–5].

Cancer is the greatest threat to human health in modern society, despite of many significant scientific and technological breakthroughs. Classical cancer therapies like surgical removal, radiotherapy, and chemotherapy are still widely used, but their invasiveness and low specificity have been deterring. Only in the late 1990s were targeted therapies clinically available. Compared with the classical therapies, PDT is relatively noninvasive and has fewer side effects, higher tolerance of repeated doses, and higher specificity that can be achieved through precise delivery of light.

To achieve a desirable therapeutic outcome, the efficacy of the photosensitizer, for example, the efficiency in generating ROS and the selectivity for tumor cells, is the most important. Although PDT appears to be a promising approach, only a few photosensitizers, for example, porfimer sodium, temoporfin, and aminolevulinic acid, have been clinically approved for different oncological conditions. Unfortunately, these drugs still have some deficiencies such as weak absorption of tissue-penetrating red light, sustained skin photosensitivity, and low initial selectivity, among others. Thus, throughout the years, optimization of their photophysical and biological characteristics, as well as the development of novel photosensitizers with improved properties, particularly those which are tumor targeting, has been the major focus in PDT research.

History of Photodynamic Therapy

Light has been used for thousands of year to treat diseases. In ancient China, Egypt, and India, it was used for different skin problems. The importance of phototherapy was fully recognized in 1903 when the Nobel Prize in Physiology or Medicine was awarded to Finsen in "recognition of his contribution to the treatment of diseases, especially lupus vulgaris, with concentrated light radiation, whereby he has opened a new avenue for medical science."

Although there was also some use of light together with special chemicals in treating skin conditions in the past, formal recognition of photodynamic activity was absent until about a hundred years ago [2, 6]. Raab was the first to exploit the interaction between light and the fluorescence compound acridine to exert cytotoxic effect on a *Paramecium*. Later, von Tappeiner successfully used topical eosin and white light to treat skin tumor and coined the term "photodynamic action." Since then, there have been extensive researches on the photosensitizers. Most of the studies were focused on the use of hematoporphyrin and its derivatives, first on tumor detection and subsequently on tumor treatment. In the 1970s, Diamond showed that hematoporphyrin can be used as a photosensitizing agent to kill rat glioma both in vitro and in vivo. Later, Dougherty reported the first successful, large-scale clinical application of PDT using hematoporphyrin and red light to treat skin cancer in human patients.

More and more promising results were obtained. Finally in 1993, Photofrin (porfimer sodium), a derivative of hematoporphyrin, was approved in Canada as the first drug for PDT in the treatment of bladder cancer. Two years later, it was also

approved in the United States of America for use in esophageal cancer. Over the next two decades, a number of other photosensitizers have received approval from regulatory authorities in various countries for various malignant conditions. The list includes Levulan (5-aminolevulinic acid, ALA), Metvix (methyl aminolevulinate), Foscan (meta-tetra(hydroxyphenyl)chlorin, m-THPC), and Verteporfin (benzoporphyrin derivative monoacid ring A). A number of other photosensitizers are currently under scientific research or in clinical trials. It is envisioned that more drugs will become available in the near future. In addition to the wide applications in multiple types of cancer, including skin, esophageal, lung, colon, head and neck, digestive system, prostate, bladder, and lung cancers, PDT can also be used in skin conditions like acnes and psoriasis, age-related macular degeneration, and antibacterial therapy in infectious diseases.

Photochemistry of Photodynamic Therapy

Photodynamic Reaction

The three components of PDT are all nontoxic individually. However, illumination of the photosensitizer will lead to the production of toxic ROS (Fig. 1). Upon absorption of light with appropriate wavelength, the photosensitizer will be excited from the stable, ground state to a transient, excited singlet state. The singlet state photosensitizer can go back to the ground state by emitting fluorescence; such property makes the photosensitizer a good diagnostic tool for superficial cancers. Alternatively, the singlet state photosensitizer can also be converted by intersystem crossing to the relatively more stable triplet state.

Two photodynamic reactions can occur before the triplet state photosensitizer returns to the ground state. Type I reaction involves the removal of proton(s) from or transfer of electron(s) to nearby molecules, for example, protein, fatty acid, or water. This process generates different free radicals which react with molecular oxygen to

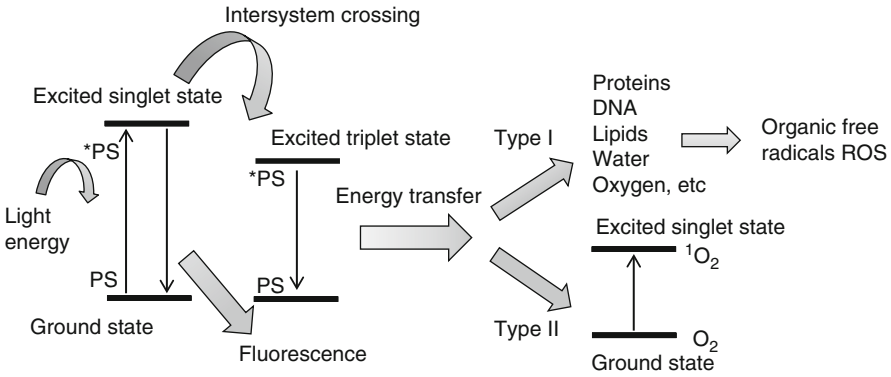

Fig. 1 A modified Jablonski diagram showing the photosensitization process

produce different ROS, including superoxides and hydroperoxyl and hydroxyl radicals, as well as hydrogen peroxide. In contrast, type II reaction involves the direct transfer of energy from the excited triplet photosensitizer to the ground state triplet oxygen. As a result, reactive singlet oxygen is generated. While the two photodynamic reactions can occur simultaneously, for most photosensitizers, type II reaction predominates, making singlet oxygen as the major type of ROS generated during PDT [1, 5].

The ROS generated are extremely unstable. For example, singlet oxygen has a short life-span of about 0.04 μs. Thus, it has only a very short effective reaction range (about 0.02 μm) from its site of formation. The subcellular localization of the photosensitizer will determine the organelles primarily damaged by the treatment and, subsequently, the biochemical pathway and process involved [4].

Light Source

A suitable light source is critical in making the photosensitizer toxic in the right place and at the right time. Several types of PDT light source are available, including broadband lamps, light-emitting diode lamps, and lasers. Among them, lasers are most frequently used. Laser light has the characteristics of monochromaticity, coherence, and collimation. These properties allow a narrow beam of light with high intensity, which can transmit into target tissue with great precision. The fact that laser can be focused onto a tiny spot contributes to the specificity of PDT. Only the photosensitizer present in the illuminated tumor site will be activated whereas those nearby in the non-illuminated normal tissue will not result in any adverse side effects. Due to their accessibility to light, dermatological malignancies are most conveniently treated by PDT. For internal cancers, light delivery to the target area is more challenging. Nevertheless, with the development of optimal fiber-optic delivery devices, for example, fiber-optic cable inside endoscope, it is now possible for laser to be directed to cavity or areas inside the body, and hence PDT is useful also in treating esophagus, lung, stomach, and bladder cancers [5, 7].

The wavelength of the applied light should match with the absorption peak of the photosensitizer so as to have adequate activation. For clinical usage in PDT, laser with wavelength between 650 and 850 nm is most appropriate. At a longer wavelength ($>$850 nm), the excited photosensitizer does not have sufficient energy to excite oxygen and produce ROS. In contrast, at a shorter wavelength ($<$650 nm), light cannot effectively penetrate into tissue. Thus, it is preferable for photosensitizer to have an absorption peak within this phototherapeutic window (650–850 nm) so that it can be activated by tissue-penetrating light to initiate the photodynamic reactions [5].

The timing of illumination is also important. The drug-light interval refers to the time lag between photosensitizer administration and light exposure. Upon systemic administration, the photosensitizer will remain in the vascular system for some time before entering into the tumor cells. When light is applied within this short period, the PDT effect will mainly be on the vascular system, causing thrombus formation which indirectly kills the tumor cells. However, if the drug-light interval is long

enough for the photosensitizer to get into the tumor cells, the ROS generated inside the cell upon illumination will kill the cell directly [8]. It is also possible to have multiple illuminations so as to trigger both anti-vascular and direct cytotoxic effects. Different photosensitizers and different formulations of the photosensitizers have different pharmacokinetics in the body. The exact timing and dosimetry of the illumination needed for individual photosensitizer need to be optimized.

Photosensitizer

First Generation of Photosensitizers

Photofrin is the first photosensitizer approved for clinical application in 1993. It is a hematoporphyrin derivative. Studies on the photodynamic action of hematoporphyrin started more than a hundred years ago. Hematoporphyrin was first isolated from dried blood. Subsequent acetylation and reduction, together with partial purification, yielded a hematoporphyrin derivative which was twice as potent as hematoporphyrin in phototoxicity. Further purification yielded Photofrin which represents the first generation of photosensitizers [6].

Although it is still the most common photoactive drug used clinically and in research, Photofrin suffers from a number of deficiencies [1, 9]. It is a mixture of monomeric (with different forms) and oligomeric (dimers to hexamers) porphyrins, including some components which are not photoactive. The complex composition is a major concern on the reproducibility of the photosensitizer action. As a porphyrin, Photofrin shows a maximum absorption peak around 400 nm (the B or Soret band). Unfortunately, light at this region cannot pass through tissue. Photofrin has another much lower absorption peak (Q band) at 630 nm that could only allow a tissue penetration of ~5–10 mm in therapeutic PDT. The short wavelength of the Q band is an obvious limitation of Photofrin. In a clinical setting, the drug-light interval for Photofrin-PDT is long (about 2–3 days), during which the patient must be protected from light. This also renders the effective dose of Photofrin and the therapeutic outcome more unpredictable. Moreover, Photofrin lacks tumor selectivity and has prolonged retention in the body. Patients treated with Photofrin have to avoid direct sunlight and even bright indoor light while wearing protective clothes and sunglasses for weeks afterward in order to minimize skin photosensitivity. Such inconvenience lowers the quality of life of the patients.

Second Generation of Photosensitizers

Because of the drawbacks of using Photofrin, research has been ongoing to search for a second generation of photosensitizers with improved physicochemical and biological activities. A perfect photosensitizer should be a pure compound which is easy to synthesize. It should have a long wavelength for the Q band to allow better absorption of tissue-penetrating red light. It should be soluble in the body fluid so that it can be carried via the bloodstream to the tumor site efficiently. It should have a rapid clearance from the body. Preferentially, it should also possess some selectivity to the tumor cells [5, 10].

Most of the second-generation photosensitizers still employ the tetrapyrrole ring of porphyrins as the basic structure. To modify, one approach is to expand the macrocycle, for example, in the production of phthalocyanines, while the second approach is to reduce one or more of the porphyrins' pyrrole rings to give chlorins. Both of these modifications result in a preferred redshift of the Q-band absorption to 650–700 nm.

In phthalocyanines, the hydrophobic nature of the macrocyclic skeleton favors the formation of aggregates, resulting in poor solubility in aqueous environment and inefficient singlet oxygen generation. These characteristics will limit their application in PDT. In the last decade, a substantial number of phthalocyanine derivatives have been prepared by rational modifications of the metal center (silicon, zinc, or aluminum) or peripheral atoms of the polycyclic rings. These derivatives possess different charges and polarity and for some of them also improved PDT properties [9]. Similarly, different chlorin derivatives, for example, meta-tetrahydroxyphenyl chlorin, monoaspartyl chlorin-e6, and methyl pheophorbide a, have also been developed for use as second-generation photosensitizers [5] (Fig. 2).

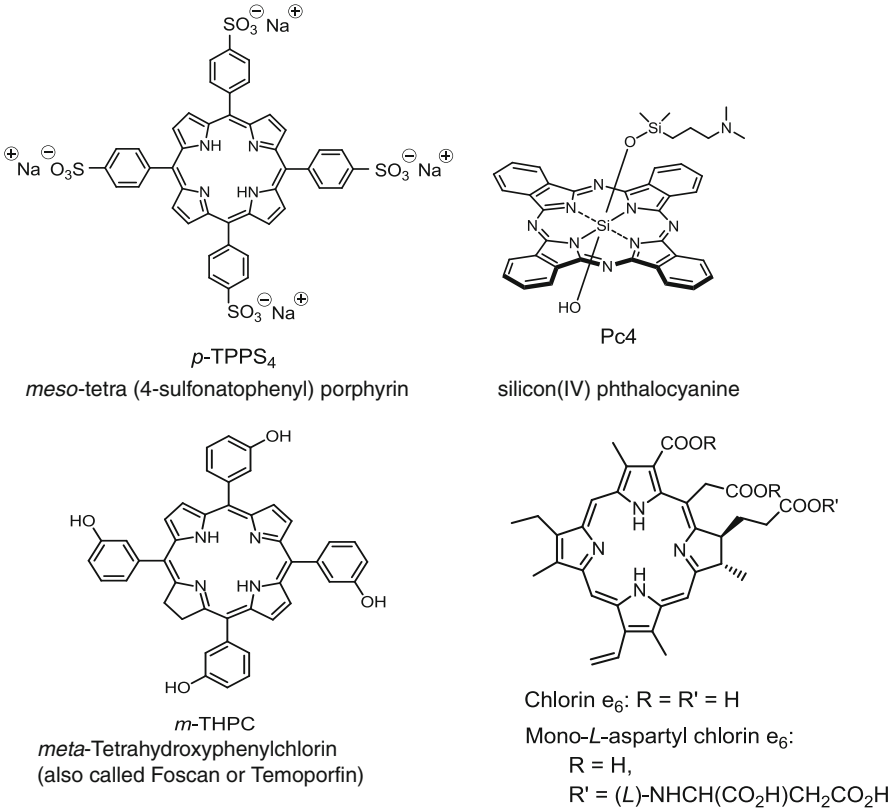

Fig. 2 Examples of second-generation photosensitizers

Apart from the classical tetrapyrrole derivatives, boron dipyrromethene (BODIPY) dyes are emerging as another class of promising photosensitizers [10]. As a versatile class of functional dyes, BODIPY derivatives possess many desirable chemical and photophysical properties, for example, ease of chemical modification of the skeleton, high extinction coefficient, environment insensitivity, resistance to photobleaching, and relatively high stability and solubility in aqueous media. BODIPY dyes have been used for a long time as fluorescence imaging probes. More recently, different modifications have been made to depress the fluorescence yield and hence enhance the singlet to triplet intersystem crossing for ROS generation. Among the BODIPYs, aza-BODIPYs are particularly promising as the modification results in a redshift of the Q band to the near-infrared region (ca. 680 nm).

Some photosensitizers are applied as prodrugs and do not have the aromatic central ring structure by themselves, for example, 5-aminolevulinic acid (ALA) and its derivative methyl aminolevulinate. ALA is a natural precursor in the biosynthesis of heme, an iron-containing porphyrin, which is an important component in proteins, for example, hemoglobin. With a downregulation of ferrochelatase, the final enzyme in heme biosynthesis, topical application of ALA, as a pro-photosensitizer, on skin lesions will allow its bioconversion to proceed until the production of protoporphyrin IX (PPIX). The accumulation of photoactive PPIX is ready for illumination in PDT. Although the absorption maximum of PPIX is just around 630 nm, the wavelength and the penetration of light is not a major concern as ALA is generally used in treating superficial malignant and nonmalignant dermatological conditions [11].

Third Generation of Photosensitizers

While the second-generation photosensitizers possess improved physicochemical properties and potent cytotoxicity, most of them lack selectivity for tumor cells. In those cases, the specificity of PDT can only rely on direct illumination of the tumor site as the drugs are distributed throughout the whole body. Therefore, there has been a need for the third-generation photosensitizers which can be specifically localized in the tumor cells [3]. This will minimize any potential side effects. At the same time, the specificity also allows the use of a lower overall dose to achieve the therapeutic outcome.

Based on the physiological differences between tumor cells and normal cells, different strategies have been explored for directing the photosensitizer to the tumor cells [3, 12] (Fig. 3). Similar strategies have been used for the targeting of other chemotherapeutic agents as well. The first approach is based on the overexpression of certain antigens or receptors in certain types of tumor cells. By conjugating with antibodies that recognize such antigens or ligands that bind to such receptors, the photosensitizer is expected to have stronger interaction with the tumor cells. Attaching targeting component to the photosensitizer may also help to improve the solubility of the photosensitizer in the biological environment. The second approach exploits the specific microenvironment of the tumor tissue for the activation of the photosensitizer which is applied as a prodrug [13]. In this case, while the inactive

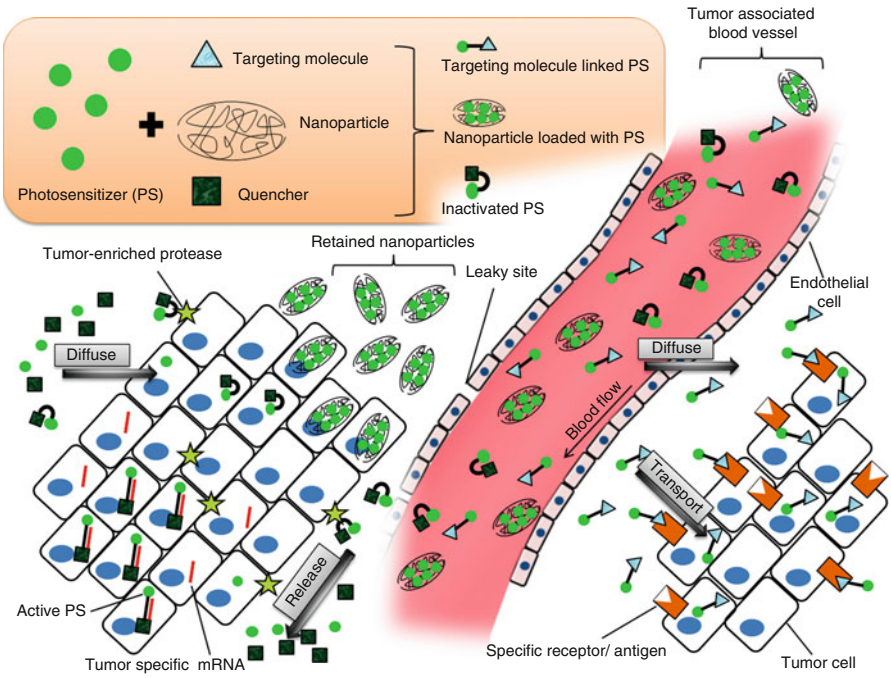

Fig. 3 Strategies used to direct the photosensitizer to the tumor site

prodrug is distributed all over the body, it will only be activated and exert its effect in the tumor cells. Finally, the third approach involves the encapsulation of the photosensitizers in colloidal carriers, such as liposomes, polymeric micelles, and different types of nanoparticles [7, 14]. These nanomedicines usually have a longer circulation time and significant accumulation in tumors through the enhanced permeability and retention (EPR) effect because of the larger molecular size [15]. The external surface of the nanocarriers can be further functionalized to achieve additional targeting effects.

Modified Photosensitizers for Targeted Therapy

Photosensitizers Linked to Targeting Molecules

Photosensitizers Conjugated with Small Ligands

Tumor cells differ from normal cells in the uncontrolled cell growth and proliferation. To allow the more active metabolism, some tumor cells have an overexpression of receptors or transporters to allow more efficient uptake of essential nutrients. Consequently, one way to target the drug to the tumor cells is by conjugating with these small molecules.

Folate is a pterin-based vitamin required by cells in the biosynthesis of nucleotide, the basic component of DNA. A number of folate-conjugated photosensitizers, including porphyrins, pheophorbides, chlorins, and bacteriochlorophyll, have been synthesized to target the folate receptor which is overexpressed in many types of tumor cells. For example, using the xenograft nude mouse animal model, Gravier et al. [16] showed that folate-conjugated m-THPC had an enhanced accumulation in the human head and neck carcinoma KB cells when compared with the human colon carcinoma HT-29 cells, consistent with the much higher expression of folate receptor in the former type of cells.

Another approach is to conjugate the photosensitizer with polyamines. Polyamines are naturally occurring compounds that play multifunctional roles in a number of cell processes including cell proliferation and differentiation. Rapidly dividing cells such as tumor cells require a large amount of polyamines to sustain the rapid cell division. Part of these materials can be biosynthesized internally, while the majority is imported from exogenous sources through active and specific polyamine transporters. These features have led to the use of polyamines as potent vectors for the selective delivery of drugs into tumor cells. For example, Jiang et al. [17] have synthesized a series of polyamine-appended phthalocyanines. The conjugated photosensitizers showed an increase cellular uptake and enhanced photodynamic activities, although the actual uptake pathway remained elusive. Moreover, the polyamine moieties, which are protonated under physiological conditions, can enhance the hydrophilicity and reduce the aggregation tendency of the photosensitizer.

Photosensitizers Conjugated with Peptides

Synthetic peptide with appropriate sequences can specifically bind to different surface markers on tumor cells. For example, photosensitizers conjugated with peptide specifically targeting the overexpressed epidermal growth factor receptor in tumor cells have been synthesized [18]. Conjugation of the photosensitizer with the more general peptide sequence, for example, cell-penetrating peptide or nuclear localization signal, is another important strategy in drug delivery [19]. These peptides, usually rich in basic amino acids, enhance the cellular uptake of the photosensitizer by the improved electrostatic interaction with cell surface molecules, for example, glycosaminoglycans which are highly expressed on the membrane of tumor cells, and the negatively charged lipids. These peptide-linked photosensitizers also increase the drug concentrations within tumor tissue with the EPR effect believed to play a major role. The use of the peptide signaling sequence might even have the potential to direct the drug into specific organelles or divert the photosensitizer away from the efflux pathway.

Photosensitizers Conjugated with Antibodies

The surface marker on tumor cells can be recognized by an antibody. Thus, another approach in photosensitizer targeting is to conjugate the drug with an antibody that can recognize these tumor-specific antigens. As an example, Jankun [20] conjugated hematoporphyrin with CYT-351, an antibody that recognizes the prostate-specific membrane antigen, and demonstrated that it had an improved targeting delivery and

accumulation in the LNCaP human prostate cancer cells. While this approach is useful, the storage of antibody is inconvenient and the possibility of cross-reactivity may lead to other problems in a clinical setting. The large size of the antibodies may hinder tissue penetration and lower cellular uptake. As a result, smaller antibody fragments, such as single-chain Fv fragments, have received considerable attention as alternative targeting carriers [21].

Photosensitizers Activatable at Tumor Site

In this approach, the photosensitizer is administered in an inactive, prodrug form to be activated only at the tumor site [13]. The photosensitizer is usually connected with a quencher through a cleavable linker. The close proximity of the two units inhibits the ROS generation by the photosensitizer. Upon interaction with an appropriate stimulant in the tumor site, the linker is cleaved. The separation of the photosensitizer and the quencher restores the photoactivity of the former. The most common method to cleave the linker is through the action of protease which is overexpressed in the tumor site, for example, cathepsin and matrix metalloprotease.

A similar approach was employed in the development of a dimeric photosensitizer targeting the drug-resistant bacteria [22]. Two photosensitizing units are linked via a β-lactam ring. Because of the close proximity, self-quenching occurs and the dimeric species lacks phototoxicity. In the methicillin-resistant *Staphylococcus aureus*, β-lactamase is present as an evolved mechanism for inactivating the β-lactam ring-containing penicillin type of antibiotics. This enzyme will cleave the β-lactam ring in the inactive, dimeric photosensitizer. Thus, the photosensitizer exerts its effect only in the drug-resistant bacteria but not in other cells.

The two photosensitizing units can also be linked via an oligonucleotide. Gao et al. [23] coupled two chlorin-e6 molecules to the opposite ends of an oligonucleotide with sequence complementary to the tumor marker survivin mRNA. In the presence of the survivin mRNA in breast cancer cells, the oligonucleotide loop of the molecular beacon hybridizes to its target. The extension of the beacon disrupts the dimerization of the photosensitizer and restores its photodynamic properties.

Photosensitizer Encapsulated into Nanoparticles

The photosensitizer can be encapsulated in nanoparticles for carrying it to the tumor site [7, 14]. The framework serves as a protective layer, preventing hydrolysis or enzymatic degradation of the photosensitizer in the blood and interstitial fluid. By itself, the nanoparticles can facilitate passive targeting of the photosensitizer by the EPR effect as the cellular retention ability of a substance is directly related to its molecular size. To minimize the interaction with serum proteins, nanoparticle can be covered with a layer of polyethylene glycol. This could prevent its recognition as a foreign substance by the body defense mechanism; otherwise, it would be removed rapidly from the blood circulation through the reticuloendothelial system.

Nanoparticle coated with polyethylene glycol also provides an amphiphilic environment for lipophilic photosensitizer. Upon interaction with the hydrophobic plasma membrane of the cell, the photosensitizer can be released from the nanoparticles and delivered into the cell cytoplasm.

Besides making use of the EPR effect, additional targeting can also be achieved by having specific functional and targeting groups attached onto the vehicles, either by covalent modification or simply through adsorption. One example is the use of humanized anti-DR5 antibody-targeted chitosan/alginate nanoparticle for the delivery of the photosensitizer meso-tetra(*N*-methyl-4-pyridyl) porphine tetra tosylate formulation [24]. DR5 is a member of the cell surface tumor necrosis factor receptor superfamily and is always upregulated in various types of tumor cells. The anti-DR5 antibody on the nanoparticles facilitates the photosensitizer uptake in the human colorectal carcinoma HCT116 cells via receptor-mediated endocytosis. In addition, the binding of anti-DR5 antibody to DR5 also triggers off an apoptotic signal in the tumor cells through the recruitment and activation of caspase-8. In this case, antibody conjugation not only allows active targeting to tumor cells but also exerts additional cytotoxic effect through the activation of the receptor.

Different nanoparticles have been used for the encapsulation of photosensitizer; among them mesoporous silica nanoparticles (MSN) and gold nanoparticles are of particular interest. MSN are mesoporous (with pore diameter between 2 and 50 nm), nano-sized materials built up by the assembly of silica units. The silica framework in MSN is rigid, chemically stable, and resistant to mechanical stress, heat, and pH. These physical characteristics, together with its high biocompatibility (low or zero intrinsic cytotoxicity), make MSN an ideal photosensitizer delivery system [25, 26]. Another desirable property of MSN as a photosensitizer carrier is its amphiphilic nature (with internal pore surface hydrophobic and external particle surface hydrophilic) which enhances the cell entering efficiency of most hydrophobic photosensitizers. The particle size and pore size of MSN are tunable and hence adjusting photosensitizer loading is possible. In addition to the photosensitizer, other drugs can also be loaded into the MSN at the same time to allow combination therapy.

Gold and gold-containing compounds have long been used in medical practice. In nanocrystalline forms, gold exhibits intriguing physicochemical and optical properties which can be modulated by changing the nanostructures (such as nanospheres, nanorods, nanocubes, nanocages, and nanoshells) and their dimensions. The unique plasmonic and photothermal properties, together with the good biocompatibility, high chemical stability, and ease of fabrication and surface functionalization, of gold nanoparticles enable them to be used as multifunctional nanoplatforms for multimodal imaging, photothermal therapy, and targeted delivery of various therapeutics, including photosensitizer [27, 28]. Gold nanoparticles serve as an ultra-efficient energy quencher of excited photosensitizer through their surface-energy-transfer properties. Drugs on the gold nanoparticles remain inactive in their native state until they are released. Photosensitizer conjugated onto the surface of gold nanorods via a protease-cleavable peptide linker has been prepared by Jang and Choi [29]. The photodynamic efficiency was found to be higher in the HT1080 (matrix metalloprotease-2 positive) than the BT20 (matrix metalloprotease-2 negative) cells.

Biological Effects of Photodynamic Therapy

Direct Cytotoxic Effect on Tumor Cells

Effect on Subcellular Organelles

After the photosensitizer is taken into a cell, it may enter into a specific subcellular compartment according to its physicochemical properties, for example, amphiphilicity, hydrophobicity, and charge. The functional group(s) present on the photosensitizer may also govern its subcellular distribution. Owing to its unstable nature, the ROS generated can only exert its destructive effect in the local vicinity. Therefore, the effect of PDT on cells is related to the subcellular localization of the photosensitizer. Among the various organelles, mitochondria, endoplasmic reticulum, and lysosome are the most crucial targets in PDT, as they are all potential starting points of cell death signaling pathways in the presence of oxidative stress [30] (Fig. 4). However, it has to be noted that most of the photosensitizers have diverse subcellular distribution. Moreover, the subcellular localization can also be cell type and photosensitizer concentration dependent.

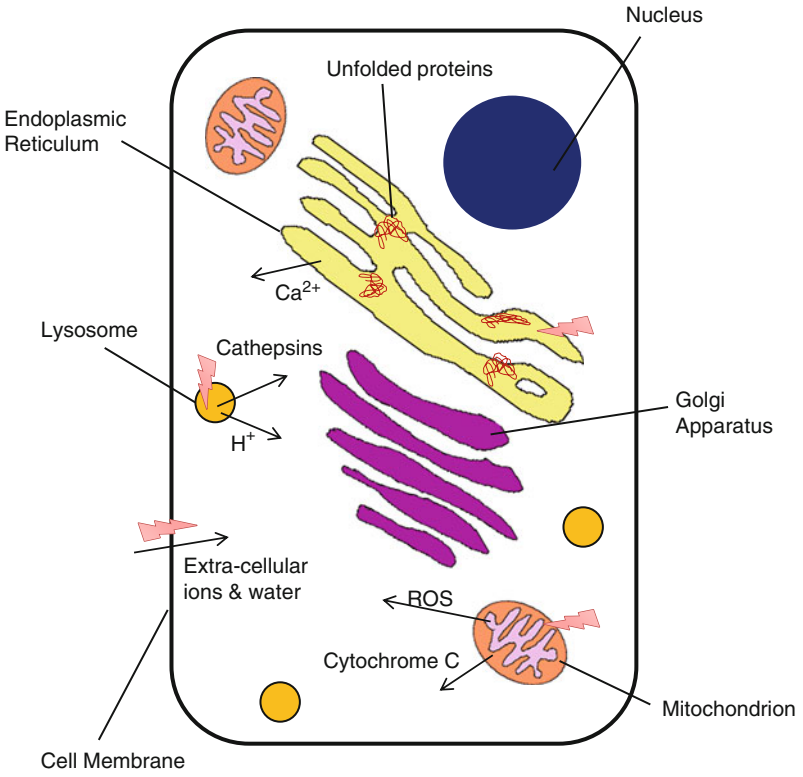

Fig. 4 Effect of photodynamic action on subcellular targets

Mitochondrion is the most popular subcellular target in photosensitizer research. This organelle is responsible for generating metabolic energy (adenosine triphosphate, ATP) to maintain normal cell functions. For those photosensitizers residing in mitochondria, in addition to the primary ROS generated directly from the photosensitizer, there is also secondary ROS generation from the damaged mitochondria [31]. The collapse of mitochondrial membrane disrupts the coupling efficiency of the electron transport chain. Consequently, there is extra ROS generation, causing further mitochondrial membrane damage. Such a vicious cycle accelerates the cell death process. The membrane damage also results in the release of an effective cell death triggering protein, cytochrome c. These characteristics make mitochondrial damage considered as the most efficient way to kill the tumor cells. The reducing environment in mitochondria has also been exploited to achieve localized activation of the photosensitizer. For example, Park et al. [32] have synthesized a prodrug in which chlorin-e6 is linked to a quencher via a disulfide bond which can be reduced in the mitochondria, thus releasing the active photosensitizer for photodynamic action. PDT with this photosensitizer is effective in inhibiting the growth of the human cervical carcinoma KB cells in a xenograft nude mouse model.

Endoplasmic reticulum (ER) is another important target in PDT [30]. It is the site of protein folding and posttranslational modifications. In the presence of ER stress, for example, PDT-induced oxidative stress, unfolded or misfolded proteins will accumulate. Unfolded protein response (UPR) will be triggered to rescue the cell. However, when the condition becomes irreversible and beyond its repairing capacity, UPR will initiate the cell death pathway. ER is also a key organelle for intracellular calcium level regulation. Disturbed intracellular calcium homeostasis has also been proven to be related to the activation of apoptosis. Besides cytotoxicity, photosensitizer acting on ER is particularly interesting because of its potential in activating beneficial inflammatory response. Photodamage by ER-targeting hypericin induced the expression of heat shock protein (HSP)-70 and calreticulin on the tumor cell surface in the human urinary bladder cell line T24 [33]. The expression of these proteins on the dying tumor cell surface is critical in preventing posttreatment cancer recurrence [34].

Lysosome is a highly acidic ($pH < 5$) organelle where aged and malfunctioned proteins are digested and recycled. For this reason, lysosome is rich in cathepsins, a group of acidic hydrolytic enzymes. Activation of photosensitizers in this organelle leads to lysosomal membrane permeabilization (LMP), resulting in the release of cathepsins into the cytosol. These enzymes can induce apoptosis and/or necrosis, depending on the severity of lysosomal damage. When LMP is less severe, apoptosis could be initiated by cathepsins B and D through the activation of the pro-apoptotic protein BH3 interacting-domain death agonist, which subsequently directs the activation of caspases, resulting in apoptosis. However, when massive LMP occurs, the cytosol is rapidly acidified and proteins will be digested by the cytosolic pH-active lysosomal cathepsins. Such uncontrolled destruction of proteins inside the cell eventually forces the cell to undergo necrosis [30].

Besides mitochondria, ER, and lysosome, there are also photosensitizers targeting Golgi apparatus, nucleus, and cell membrane, although the cell death pathways

involved are less well elucidated. Photosensitizers targeting the cell membrane usually cause necrosis due to the influx of extracellular ions. The biological effect of PDT-induced nuclear damage is relatively unexplored. Nevertheless, based on the action of other DNA-targeting drugs, photodynamic action in the nucleus is expected to kill the cell effectively.

Cell Death Mechanisms

Regardless of subcellular localization, the generation of ROS would eventually subject the cell to the cell death pathway. In the simplest sense, cell death could be classified as apoptosis, necrosis, and autophagy. Multiple signaling cascades are concomitantly activated in tumor cells exposed to photodynamic stress [30]. The exact cell death pathway that the cell would undergo depends on multiple factors [4], including the subcellular localization of the photosensitizer, the dose of the photosensitizer, the drug-light interval, the fluency rate of the light source, as well as the total light dose applied in the process.

In general, the use of a low PDT dose, i.e., low drug dose, short drug-light interval, low fluency rate, and low total light dose, would result in apoptosis. When compared with necrosis, apoptosis is a better controlled cell death process. Although the apoptotic pathway could be initiated in different subcellular organelles and hence has divergent upstream signaling pathways, most of them would finally activate the executioner protease, caspase-3, which serves as the converging commitment point of apoptosis. Caspase-3 would further activate downstream deoxyribonucleases and proteases for controlled self-destruction. In the process, the cell would gradually shrink and be fragmented into small membrane-bounded apoptotic bodies. These apoptotic bodies, because of the presence of phosphatidylserine on the external layer of the membrane, would be recognized and engulfed by phagocytes of the immune system. As the cellular membrane remains intact in apoptosis, there would not be any leakage of cellular content into the surroundings.

In contrast, necrosis is a less controlled cell death process. The use of higher PDT dose is more likely to trigger necrosis of tumor tissue as the excessive amount of ROS thrown into the system would nonspecifically destroy all the cellular machineries that regulate the cell death process. In most cases, this massive damage would disrupt the cell membrane. The tumor cells will burst and release the cellular content into the surroundings. As these leakages contain different proinflammatory factors, local inflammation will be induced.

Traditionally, apoptosis is regarded as a safer and preferred form of cell death in cancer therapy so as to avoid any complication of autoimmunity. Nonetheless, recent reports suggested that the induction of appropriate inflammatory response through necrosis may assist the development of antitumor immunity [35]. In reality, regardless of the PDT dose, the resultant cells will not be entirely apoptotic or necrotic as the tumor stroma is consisted of a heterogeneous population of cells that absorb different amount of photosensitizer from the environment. An optimal proportion of apoptotic and necrotic cells in the tumor tissue may lead to improved therapeutic outcome in PDT.

The third mechanism of cell death is autophagy which may also be considered as a cell-protecting mechanism. In the process, a double membrane structure, the autophagosome, is formed in the cytosol to sequester the damaged proteins and organelles. The autophagosome then fuses with lysosome where the worn-out proteins and organelles are digested and recycled. This process can be triggered when the photodamage occurs in the mitochondria and/or ER. If the damage is not severe, this action could maintain the cell function by removing the damaged parts, and hence is cell protective. However, when the damage is too severe, the over self-digestion would eventually lead to autophagic cell death with morphologic and biochemical features distinct from both apoptosis and necrosis. In this regard, photosensitizer targeting the lysosome should provide more efficient photo-killing, as it could promote autophagic stress and suppress the autophagic pro-survival function [36].

Destruction of Tumor-Associated Blood Vessel

Vascular Photodynamic Therapy

To support its rapid growth, tumor cells will secret certain factors to induce the establishment of novel blood vessels for adequate nutrient supply. Hence, the tumor-innervating blood vessel is another target in antitumor therapy, including PDT. Two PDT approaches can be used to ablate the blood vessel. First, the photosensitizer can be conjugated with antibodies or ligands that recognize protein markers on the tumor-associated endothelial cells in the blood vessel. One example is the conjugation of a chlorophyll a based photosensitizer to the cyclic Arg-Gly-Asp short peptide which targets the upregulated $\alpha_v\beta_3$ integrins on the cell surface of tumor neovasculature [37]. The second approach is by vascular photodynamic therapy (VPDT) in which the photosensitizer is illuminated and activated while it is still in the bloodstream after systemic administration.

VPDT differs from classical PDT by having a much shorter drug-light interval. The interval is long enough to allow the photosensitizer to get into the tumor-innervating blood vessel but is not sufficient for it to get into the tumor cells. Illumination at such an appropriate time will damage specifically the endothelial cells in the blood vessel. Substances released from the damaged endothelial cells can trigger thrombosis, blocking the blood supply, and hence the nutrient and oxygen supplies, to the tumor. Using an animal model, Byrne et al. [8] showed that illumination immediately following the administration of the photosensitizer BF-2 tetraaryl-azadipyrromethene can trigger the regression of the implanted MDA-MB-231 mammary tumor in mice through a vascular-targeting mechanism.

Induction of Hypoxia

Following VPDT, the oxygen supply to the local tumor would be cut off. This hypoxic environment would induce additional effects apart from directly causing cell death. Under normal condition, ATP is degraded stepwise to hypoxanthine, xanthine, and then uric acid. The final two steps are catalyzed by the enzyme

xanthine oxidase and require molecular oxygen as substrate. A hypoxic environment after VPDT is not favorable for these reactions and would lead to an accumulation of (hypo)xanthine in the cells. Once the oxygen supply resumes, the accumulated (hypo)xanthine will be converted to uric acid within a short period. As this reaction also generates ROS, a further round of damage would be imposed onto the tumor tissue to assist tumor eradication [38]. Besides such ischemia reperfusion injury, the high concentration of uric acid turns the tumor microenvironment into a pro-inflammatory state. This can also facilitate the development of antitumor immunity.

The principle of VPDT is to cut off oxygen and nutrient supplies to the tumor. Ironically, there are also reports suggesting that PDT could induce angiogenesis. During hypoxia, the hypoxia-inducible factor-1α (HIF-1α) of the tumor cells will be upregulated [38]. HIF-1α is known to regulate a number of downstream pro-survival signals that rescue the stressed cells and promote the generation of new blood vessels. There are also reports suggesting that PDT itself could trigger an oxygen-independent activation of HIF-1α that further enhances the angiogenic potential of the treated tumor tissue [39]. As a consequence, if PDT failed to totally eradicate the tumor, the remaining tumor tissue could become more aggressive as a result of the increase in pro-survival signals and angiogenic ability.

Induction of Antitumor Immunity

Immuno-editing Hypothesis

Over the past few decades, scientists working on chemotherapy have focused on compounds with selective cytotoxic effect on tumor cells but not normal cells. In most cases, the selective toxicity of these compounds is a result of the higher proliferation rate of the tumor cells. The antitumor potential of the immune system is largely ignored. Most in vivo studies simply use nude mice, an immunodeficient strain lacking T cells, as the animal model. In some cases, the immune system is even abolished together with the tumor cells as immune cells are also rapidly proliferating cells. It is only about 10 years ago when scientists started to recognize the importance of immune system in the battle against cancer. The immunosurveillance hypothesis was formulated. Later on, it was refined to form the immuno-editing hypothesis in which the three Es, i.e., elimination, equilibrium, and escape, in the phase of cancer growth are being emphasized [40].

The immuno-editing hypothesis describes the three phases that neoplastic cells must withstand before they could turn into a malignant disease. The “elimination” phase is the initial phase of tumor development where the immune system detects and kills any cells that possess slight antigenic differences due to spontaneous or induced genetic changes. At this stage, the amount of mutated cells is far less than the capacity that the immune system can handle. Thus, there would not be any tumor growth in the body. Gradually, this process would select those mutated cells that are less susceptible to attack from the immune system. When these cells accumulate and reach the upper limit that the immune system could handle, it enters into the “equilibrium” phase. Although the immune system is still capable to control the

number of mutated cells in the body, any oncogenic trigger could break the equilibrium and push the system into the "escape" phase where the amount of mutated cells is beyond the capacity of the immune system. The mutated cells survived at this stage are less susceptible to immune attack. Taking together, the immune system is not only host protective, but can also be tumor promoting through chronic inflammation and immunoselection of poorly immunogenic variants. Accordingly, a successful cancer therapy should include reduction of tumor burden and an enhanced ability for the immune system to recognize malignant cells.

Activation of Immune System

The ability of PDT to induce antitumor immunity was first demonstrated in detail by Korbelik et al. [41]. In this study, tumor (EMT6 mammary sarcoma) was implanted into both normal BALB/c and severe immunodeficient (scid) mice. The tumors disappeared shortly after Photofrin-PDT in both populations. However, the tumor relapsed quickly in the scid mice, whereas the normal BALB/c mice remained tumor-free at the end of the 3-month experimental period. Adoptive transfer of the immune cell T lymphocytes into the scid mice was successful in delaying the recurrence of the treated tumor. In another experiment, the mice were inoculated with tumor cells at two distinct sites. After the administration of the photosensitizer, only one of the tumors was illuminated while the other was kept in dark. The illuminated tumor regressed as a result of direct cytotoxicity of PDT. The non-illuminated one also regressed, indicating the presence of systemic antitumor immune response. Finally, the tumor-bearing animals cured by PDT could resist rechallenge from the same kind of tumor cells. All these observations suggest the presence of a strong PDT-induced antitumor immunity.

More and more efforts are being spent to optimize the PDT protocols and understand the mechanisms involved in the activation of the immune response. Shortly after PDT, the damaged tumor cells would release factors into the bloodstream which cause an extensive infiltration of neutrophils into the tumor site. The neutrophil secretes proinflammatory factors to recruit phagocytes, such as macrophage and dendritic cell, in an attempt to clear the damaged tumor cells and debris. These will also lead to a potentiation of local inflammatory response via the secretion of cytokines such as interleukin-1β, interleukin-6, and tumor necrosis factor-α [1, 42]. Afterward, the phagocytes would migrate back to the lymphatic system to prime T cells and/or B cells to develop systemic antitumor immunity against the tumor cells. Recently, natural killer cell is also shown to be involved in the development of immunologic memory against tumor via its interaction with cytotoxic T lymphocytes [42], although its exact role in PDT-induced immunity remains to be established. While it is now obvious that PDT can induce antitumor immunity, how it can break loose the tolerance response in the "escape" phase of tumor development remains to be clarified.

Immunogenic Cell Death

Immunogenic cell death is a relatively new concept arose from a study trying to evaluate the antitumor effect of the anthracycline type of chemotherapeutics.

Contrary to the general belief at the time, it was found that apoptosis resulted from the treatment could induce inflammatory response. Comparison of the cell surface proteome between immunogenic and non-immunogenic apoptotic cells revealed that the key difference was the presence of calreticulin on the immunogenic apoptotic cells [43]. This finding leads to an extensive search of molecular determinants that possess similar proinflammatory function, which are collectively named as damage-associated molecular patterns (DAMPs).

DAMP is a set of proteins or metabolic products hidden inside the cells under normal situation, but become exposed on the cell membrane or released into the surroundings when the cells are under stress [34]. PDT could lead to the cell membrane exposure or release of HSP from tumor cells. HSP is a family of molecular chaperones involved in structural folding of both newly synthesized and stress-modified proteins. Intracellular HSP are powerful anti-apoptotic proteins. However, when exposed or released, HSP could assist antigen uptake and the antigen presentation process of dendritic cells [44]. Recently, it has also been shown that PDT with hypericin could lead to the exposure of calreticulin on tumor cells and the release of ATP from the stressed cells [45], which could assist the activation and functional maturation of dendritic cells. The ultimate result of the successful induction of DAMP is that the adaptive arm of the immune system, involving T cells and B cells, could be primed more efficiently and effectively. For instance, a stronger secretion of proinflammatory factors, such as interleukin-1β and interleukin-12, by dendritic cells, an enhanced killing activity of T cells toward tumor cells, and the rejection of tumor rechallenge after ablation of the first one by PDT have all been shown to be correlated with the induction of DAMP following PDT. On an applied front, evidence of the DAMP hypothesis initiates the development of PDT vaccine to treat cancer.

Photodynamic-Therapy-Derived Antitumor Vaccines

Apart from direct PDT, patients could benefit from PDT-induced antitumor response via a vaccination protocol [46]. There are two types of PDT-derived vaccines, viz., whole tumor cell lysate generated by PDT and dendritic cells which have previously been exposed to PDT-treated tumor cell lysate.

For whole tumor cell lysate, the tumor cell line used to induce tumor in the host is subjected to PDT in vitro. Then, the PDT-treated tumor cells are irradiated with gamma or X-ray at a lethal dose to ensure that no living tumor cells remain. Finally, these attenuated tumor cells are injected back to the host as a vaccine. For the dendritic cell vaccine, the whole tumor cell lysate, i.e., the first type of vaccine, is co-incubated with dendritic cells obtained from the host. Afterward, the antigen-loaded dendritic cells are injected back to the host as the vaccine. The only difference between the two types of vaccine is on whether the activation of dendritic cells occurs in vivo or in vitro.

The use of dendritic cell vaccines is particularly promising. For example, PDT tumor lysate-pulsed dendritic cells could not only inhibit tumor establishment from injected viable tumor cells (mammary EMT6 tumor) but also slow down the rate of growth in fully established (i.e., late-stage) solid tumors [44]. The effect is tumor specific. The vaccine is useful only for the specific tumor from which the vaccine is

prepared, but has no effect on other kinds of tumor cells. The use of PDT-derived vaccines is free of adjuvants, so the risk of developing hypersensitivity after vaccination is minimized. Being least invasive and able to destroy both local and distant tumors via specific antitumor immune response, PDT-derived vaccine has the potential of being developed as an immunotherapy.

Combination Therapy

To improve therapeutic outcomes, PDT can be used together with other anticancer treatment modalities [1]. The three antitumor mechanisms of PDT are exerting direct cytotoxicity on the tumor cells, inducing vascular damage and strengthening antitumor immunity. Each of these three actions can be further enhanced with the use of appropriate drugs in combined therapy. The combination of modalities with different mechanisms has several advantages, for example, enhanced therapeutic efficacy, reduced side effects, and retarded drug-resistance problem. In most cases, additive or synergistic effects have been observed which can also allow a reduction in the doses of the anticancer drugs given to patient.

PDT Combined with Cytotoxic Agents

Cytotoxic drug is routinely used to kill tumor cells. For combined PDT and chemotherapy, the simplest approach is to have sequential administration of a cytotoxic drug, followed by a photosensitizer, or vice versa. Photofrin-induced PDT and genistein, a soy ingredient, are both well-established anticancer cytotoxic agents. In combination treatment, the efficacy of inducing apoptosis in the human thyroid cancer SNU 80 cells is much higher than that of individual treatment with PDT or genistein [47].

Another approach is to conjugate the photosensitizer with the drug, for example, platinum complexes which disrupt DNA in the nucleus. A zinc(II) phthalocyanine conjugated with an oxaliplatin derivative has been reported for dual chemo- and photodynamic therapy [48]. The introduction of the oxaliplatin derivative enhances the cellular uptake and intracellular ROS generation efficiency of the phthalocyanine unit, resulting in a higher cytotoxicity. The IC_{50} value, i.e., the dose required for 50% inhibition of cell proliferation, of the conjugate against the human colon adenocarcinoma HT-29 cells is 0.11 μM, fivefold lower than that of the photosensitizer reference compound without the platinum complex and eightfold lower than that of oxaliplatin, demonstrating that the two anticancer components work in a cooperative fashion.

PDT Combined with Anti-angiogenic Agents

Angiogenesis is essential for tumor growth. Although PDT can exert an anti-vascular effect under the right condition, there is also the possibility that it could stimulate

angiogenesis via the induction of HIF-1α. To ensure damage of the blood vessels, anti-angiogenic treatment can be used together with PDT to enhance the therapeutic effect [39].

Tumor angiogenesis is a complicated physiological process regulated by a variety of endogenous pro- and anti-angiogenic factors. Proangiogenic factors include different growth factors, for example, vascular endothelial growth factor, epidermal growth factor, etc. The binding of these factors onto their respective receptors will trigger the downstream signaling pathway involving the enzyme tyrosine kinase. These are all potential target points for inhibiting angiogenesis. Combined therapy of PDT with drugs blocking the interaction between the growth factor and the receptor or inhibiting tyrosine kinase has been reported with enhanced antitumor effect [39].

Another approach is to use agents to disrupt the already mature and established tumor-associated blood vessel. Seshadri and Bellnier [49] demonstrated the use of the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) together with Photochlor (a pyropheophorbide-a derivative) in combination therapy. DMXAA exhibits only moderate antitumor activity when used alone, but it is commonly used together with other treatments like chemotherapy or radiation. When used with Photochlor, the antitumor activity was significantly enhanced in the BALB/c mice bearing murine colon adenocarcinoma CT-26 tumor model. DMXAA could significantly reduce the dose of photosensitizer required to obtain the same therapeutic effect. For the same dose, ~60% of the mice receiving combined treatment remained tumor-free, compared with ~10% for DMXAA only, and 0% for photosensitizer monotherapy, at the end of the 60-day experimental period.

PDT Combined with Immunoactive Agents

Although PDT can activate antitumor immunity by itself, a combined PDT-immune therapy tends to boost the strength of PDT-mediated antitumor immunity and block the immunosuppressive side of the immune system for better outcome.

To enhance PDT-mediated immunity, components of the immune system could be artificially administrated or activated in the host [46]. For example, treatment with granulocyte colony-stimulating factor can potentiate the antitumor effect of Photofrin-PDT in reducing tumor growth and prolonging the mouse survival time. Neutrophil, an immune cell responsible for maintaining optimal T cell response, was stimulated in the process. To facilitate antigen acquisition and presentation for optimal activation of adaptive immune response, autologous dendritic cells can be injected into the tumor shortly after the PDT procedure. The results indicate that the dual therapy is more effective than either procedure alone.

While combination therapy is useful in enhancing antitumor immunity, it can also be used to turn down the tumor-derived immunosuppression. Regulatory T cells (Treg) are responsible for dampening the overwhelming immune system back to normal state in inflammation. By doing so, Treg could prevent the over-activated immune system from killing the host. Treg is found inside the tumor tissue and thus

can downregulate the tumoricidal effect of the immune system. To deplete the Treg population, a low dose of cyclophosphamide was used together with verteporfin in combined treatment. The 120-day post-PDT survival was raised from 0% in the monotherapy groups to 70% in the combined treatment group of mice bearing the aggressive reticulum cell sarcoma J774 derived tumor [50].

Summary

PDT is a promising approach in cancer treatment. It is relatively noninvasive and specific toward tumor cells. The specific delivery of light and the use of tumor-targeting photosensitizers could restrict the PDT-induced toxicity within the tumor region and hence minimize the side effects. The central philosophy of PDT in cancer treatment is to generate ROS inside the tumor stroma to cause direct destructive effect toward both tumor cells and the tumor-innervating blood vessels. The released cellular content would then potentiate the development of antitumor immunity that helps to clean up the remaining tumor cells. Although direct cytotoxicity and the anti-vascular effect can kill the localized tumor cells, they fail to tackle the problem of metastasis where a minute amount of tumor cells has already escaped from the primary site. The third antitumor mechanism in PDT is on the activation of the host immune system which might be more important in this respect, in view of the fact that most cancer patients die, not because of the localized tumor, but because of cancer metastasis. More and more research is being conducted on the development of more powerful and tumor-targeting photosensitizers, as well as devices for more efficient delivery of the activating light. Clinical trials are being carried out to investigate the efficacy of PDT for different cancers. Although the present review focuses on the use of PDT in oncology, it has to be emphasized that the ROS generated is detrimental not only to tumor cells but also to other living organisms including bacteria, fungus, and virus. Thus, with further improvements, PDT is expected to find wide applications in various clinical fields.

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