Approaches to improve photodynamic therapy of cancer

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1. ABSTRACT

Photodynamic therapy (PDT) is a clinically approved method of tumor treatment. Its unique mechanism of action results from minimal invasiveness and high selectivity towards transformed cells. However, visible light used to excite most photosensitizers has rather limited ability to penetrate tissues resulting in insufficient destruction of deeply seated malignant cells. Therefore, novel strategies for further potentiation of the anticancer effectiveness of PDT have been developed. These include combined treatments with surgery, chemo- and radiotherapy, strategies targeting cytoprotective mechanisms induced in PDT-treated cells, as well as attempts aimed at enhancement of PDT-mediated antitumor immune response. Moreover, new photosensitizers and novel light sources are being developed. Impressive progress in nanotechnology and understanding of tumor cell biology rise hopes for further improvements in this elegant and promising method of cancer treatment.

2. INTRODUCTION

The beginnings of photodynamic therapy (PDT) date back to the end of the XIXth century when Oscar Raab observed killing of light exposed microorganisms incubated with acridyne dyes (1). Few years later PDT was used for the first time in the treatment of human cancer by von Tappeiner and Jesionek who cured a skin tumor using a combination of eosine and visible light (2). Hematoporphyrin derivative (HPD) isolated from porcine blood was the first photosensitizer (PS) approved for human use (3). Its further derivatization led to the development of Photofrin - a potent PS successfully tested in 1970s in the treatment of human cancers by Dr. Thomas Dougherty who is considered one of the pioneers of modern PDT. However, it is a photosensitizing protoporphyrin IX precursor - ALA (delta-aminolaevulinic acid) that is nowadays the most widely used for clinical PDT.

PDT is a two-step procedure that consists of three components: a photosensitizer, light and ground state

Table 1. Clinically approved and/or tested photosensitizers

Chemical group	Photosensitizer	Wavelength (nm)	References
Approved			
Porphyrins or porphyrin precursors	Photofrin	630	(140)
	ALA[1]	635	(141)
	ALA esters	635	(142)
Chlorins	Foscan	652	(143)
	Verteporfin	690	(144)
In clinical trials			
Chlorins	HPPH[2]	665	(145)
	Purlytin	660	(146)
	Talaporfin	660	(147)
	Fotolon	660	(148)
Phthalocyanines	Silicon phthalocyanine	675	(149)
Bacteriochlorins	TOOKAD	762	(150)
Texaphyrin	Motexafin lutetium	732	(151)

Abbreviations: [1]ALA - delta-aminolaevulinic acid, [2]HPPH - 2-[1-Hexyloxyethyl]-2 Devinyl Pyropheophorbide-a

oxygen. First, a photosensitizing agent is administered. After the time needed for the PS to accumulate in patient's tissues, the tumor is irradiated with light of the wavelength corresponding to PS absorbance band. At the presence of molecular oxygen this cold photochemical reaction leads to robust generation of singlet oxygen and, subsequently, reactive oxygen species (ROS) such as superoxide ion, hydroxyl radical or hydrogen peroxide are formed. These highly reactive molecules immediately react with cellular macromolecules leading to their oxidative damage and, eventually, to the cell death in the mode of necrosis, apoptosis or autophagy. Antitumor activity of PDT is dependent not only on its direct cytotoxicity but also on disruption of tumor vasculature and induction of acute inflammatory response that can further lead to the development of systemic immunity (4-5).

In 1993 in Canada PDT with Photofrin was first clinically approved for the treatment of the superficial bladder cancer. Now PDT is widely used in the treatment of early stages of esophageal and bronchial cancers and certain precancerous lesions such as Barrett's esophagus as well as in the palliative treatment of a number of advanced tumors. The list of the indications for the use of PDT is still being expanded - the procedure is also registered in some countries for the treatment of skin, stomach, cervical and head and neck tumors. Ongoing clinical trials might lead to the approval of the use of PDT in such challenging malignancies as pleural mesothelioma or brain tumors in the nearest future.

3. MAJOR ADVANTAGES AND DISADVANTAGES OF PDT - WHAT NEEDS IMPROVEMENT OR MIGHT BE IMPROVED?

The dual specificity of PDT is ensured by (i) enhanced PS accumulation in tumors and (ii) selective illumination of the diseased area. PDT can be safely used with standard antitumor therapies such as surgery, chemo- and radiotherapy without diminishing their clinical efficacy. Moreover, PDT is also effective in the treatment of chemo- and radio-resistant tumors. PDT has not been reported to be mutagenic since none of the clinically approved PS accumulates to the cell nucleus. PDT of skin tumors results in excellent cosmetic outcomes. The procedure is not harmful to the connective tissue and does not induce scaring. Use of PDT in restoring of the bronchial or esophageal lumens in advanced tumors outweighs standard procedures such as thermoablation and

enables retention of intact tissue anatomy and function. Furthermore, PDT lacks long term and generalized side effects common for chemo- or radiotherapy. It is also worth mentioning that PDT can be performed in an out-patient setting that reduces costs of patient care. The construction of a wearable low irradiance organic light-emitting diodes (LEDs) improved the ambulatory treatment of non-melanoma skin cancers.

Availability of different PS and various light sources makes photodynamic therapy a complex and to some extent complicated procedure. In majority of cases it needs to be optimized for every patient, which might be a non-desirable feature for a standard therapy. As none of the clinically approved PS is tumor-specific, off-target photosensitivity is a clinical problem. For Photofrin, skin photosensitivity lasts up to 4-6 weeks and is associated with an increased risk of sunburns. The visible light used for PS excitation does not penetrate tissues deep enough to eliminate tumor cells in more deeply located parts of the tumor. This limits therapeutic efficacy and might be the cause of the tumor relapse. For many years PDT has been considered to be an expensive therapy due to high prices of PS and light sources. Currently, the latter is not a significant problem since light sources such as LEDs are available at reasonable prices.

There is a myriad of research directions that might result in improved clinical efficacy of PDT. This review focuses only on few of them, such as search for new photosensitizers, development of new light sources, targeting cytoprotective mechanisms induced in PDT-treated tumor cells, establishing more effective combined treatments utilizing PDT, enhancement of the PDT-induced immune response and, finally, new nanotechnology-based techniques.

4. NEW PHOTOSENSITIZERS

Today, over a dozen of photosensitizers are approved for human use or tested in clinical trials. PS belong to several structural groups such as porphyrins (Photofrin) or their precursors (ALA, ALA esters), chlorins (Foscan, Verteporfin, Purlytin, Fotolon or Talaporfin), bacteriochlorins (TOOKAD), and texaphyrins (LuTex) (Table 1). All these PS are excited with light of wavelength between 630 and 760 nm, but so far none is considered an ideal compound to be used in photodynamic therapy.

The most important features of an ideal photosensitizer determined by optimal photophysical and photochemical properties are: (i) high potency, (ii) long absorption wavelength, (iii) good pharmacokinetics, (iv) high degree of tumor tissue selectivity, and (v) minimal systemic toxicity. The molar absorption coefficient, quantum yield of triplet formation and triplet state life-time are the parameters that account for the PS efficiency in singlet oxygen and ROS generation, so the higher they are, the lower PS and light doses are required to achieve a robust response (6). The optical window for light corresponds to the range between 600-850 nm. The low energy limit is dictated by the minimal energy of the triplet state sufficient to produce singlet oxygen, while the high energy limit is restricted by the tissue penetration depth, since human tissues are more transparent for longer wavelengths. The fate of a compound in the organism strongly depends on its hydrophobicity. It determines not only tissue penetration by a PS but also its half-life and clearance and thus influences the undesirable systemic phototoxicity. Finally, the obligatory feature of an ideal PS is its selectivity towards cancer cells in comparison with normal tissues. It restricts the photo-damage to malignant cells, enables the use of lower PS concentration and limits off-target side effects (7). Ironically, the optimization of one feature usually causes the worsening of the other. For instance, the increase of a PS hydrophobicity can result in its longer half-life in plasma, slower clearance, and prolonged post-treatment photosensitivity, but it usually increases PS selectivity towards tumor cells.

The clinical success of PDT is strongly dependent on PS characteristics. Therefore there are extensive studies aimed to improve them. Modifications involve chemical alterations of the existing moieties, attachment of polymers and biomolecules, development of new formulations and carrier systems and search for new chemicals with photosensitizing properties (8). Some of these improvements are discussed below.

The attachment of small molecule functional groups to existing scaffolds is the oldest and most widely exploited approach of PS improvement. In an approved PS Foscan, the modification of chlorin scaffold with amphyphylic hydroxyphenyl groups, results in high singlet oxygen yield, relatively high hydrophobicity, but prolonged skin sensitivity (9). More hydrophylic metalloporphyrin modifications, like in TOOKAD (10), or anionic lutetium texaphyrin derivative Lutrin (11) are characterized by deeper tissue penetration, higher water solubility, reduced plasma half-life and lead to decreased retention in tissues and skin light hypersensitivity. Phthalocyanine derivatives containing alkyl chains (12), bulky substituents (13) or polyamines (14) are good examples of successful modification in order to balance their hydrophilic/hydrophobic characteristics. The increase of the amphyphylic nature of the zinc-phthalocyanines with bulky 1-napthol-5-sulphonic acid prevents their aggregation and contributes to improved photodynamic properties.

The attachment of polymers, ligands and biomolecules is a more complex PS modification. Coupling chlorines with polyethylene glycol (15), beta-cyclodextrins (16) or with albumin (17) outstandingly improves tissue penetration, solubility and other properties of the PS. In order to increase tumor tissue selectivity, there are attempts to conjugate PS with ligands for receptors that are preferentially distributed on the surface of tumor cells, such as folate (18) or LDL (19), which direct them to cancer cells and reduce non-specific normal tissue retention and toxicity. Finally, the conjugates of PS with monoclonal antibodies targeting tumor-associated antigens (TAA) are being evaluated in preclinical experiments with some promising results (20). However, these large complexes have limited ability to reach solid tumors, therefore conjugates with antibody fragments are possibly a better option (21).

Another way of PS delivery which improves tumor tissue selectivity is the use of molecular carriers, such as liposomes, ethosomes and nanoparticles (22-23). New cationic liposome-based formulations containing chlorine-based Foscan demonstrate high degree of selectivity towards malignant gliomas (24). It is worth mentioning that these special carriers ameliorate not only selectivity but also general tissue penetration, especially as far as hydrophilic sensitizers such as ALA are concerned (22).

Recently there are attempts to apply semiconductor quantum dots (QD) as potential new PS. Theoretically they are able to generate singlet oxygen directly via TET (Triplet Energy Transfer) or indirectly via FRET (Förster Resonance Energy Transfer) by activating PS molecule conjugated with them (25-26). Noteworthy, the absorption wavelength of QD can be adjusted by simple change of their size, shape and composition, making them versatile tools for PDT. However, important issues of toxicity (most QD contain heavy metals) currently preclude their clinical use. However, recently developed heavy metal-free quantum dots showing bright emissions in the visible and near infra-red region of the spectrum can eliminate these problems. Fullerens are another type of nanoparticles considered as potential photosensitizers. Due to absolute water insolubility they have to be modified with hydrophilic groups. Their potency and the mechanisms of cancer cell killing are currently under investigation (27).

5. NEW LIGHT SOURCES

Another consideration relevant to development of PDT is associated with the use of new light sources and better modes of light delivery. Pump dye lasers, diode lasers, lamps with appropriate optical filters as well as light-emitting diodes are currently being used in PDT (28). Due to the development of fiber optics light can be successfully delivered to virtually any organ. Significant progress in LED technology enabled design of small sources suitable for ambulatory PDT. Moreover, due to the construction of optical fibers equipped with diffusing tips light can be easily delivered to the lumen of the digestive tract or to the urinary bladder.

Recently, novel and intriguing solutions have been proposed to improve PDT including chemiluminescence PDT (CL-PDT) already tested *in vitro* (29). Chemiluminescence defines light generation in a

chemical reaction that might be catalyzed by various enzymes such as firefly luciferase - an enzyme oxidizing D-luciferin to oxyluciferin. With this technique light can be generated strictly within the tumor cells. In luciferase gene-transfected NIH3T3 murine fibroblasts Rose Bengal excitation induced cytotoxic effects which were restricted only to the transgene-expressing, but not bystander cells. This technique might serve as a challenging alternative superior to current PDT in terms of its selectivity (29). Similar approach led to the development of PhoTO-Gal, a thiazole orange (TO)-based photosensitizer, which is activated by beta-galactosidase (Gal). PhoTO-Gal was demonstrated to kill beta-Gal-expressing HEK293 cells but not the cells lacking the enzyme. PDT with such novel photosensitizers can result not only in attenuated and prolonged light sensitivity but might also serve as a handy tool for reporter enzyme expression-specific cell killing (30).

A novel light sources that have been recently developed can split light beam into two, four or even eight separate beams of equal power with only minor total power loss (31). Such devices allow several beams of light to be independently distributed and can be used for PDT in patients with either numerous small adjacent tumors or a single tumor of a large size.

Wilson *et al.* reported on the development of two tetherless, fiber-coupled optical light sources based on diode lasers or LEDs for *in vivo* delivery of interstitial metronomic PDT (mPDT – a novel PDT-based technology discussed below) (32). The latter light-emitting device is ultralight and weighs only 16,5 g. The prototypes have been well tolerated in preliminary trials in tumor-bearing rats and have been shown to provide stable levels of continuous performance for up to 5 days. Being tetherless (all components such as light source, battery, circuitry, fiber and fiber coupling are contained within a self-cooling package) they can be easily and continuously worn by animals for several days.

PDT seems to be an ideal treatment modality to be administered and performed in an out-patient settings. A portable LED devices for PDT of skin tumors meet these needs (33). A prototype diode array, weighing approximately 21g is comprised of 37 diodes cast in an epoxy core, a diffuser, a timer and a battery pack serving as a power source. This device can be easily attached to the surface above the tumor and the patient may safely return home. The illumination will be initiated after the time needed for the PS accumulation in tumor. This solution spares time of the healthcare staff and significantly lowers costs of the therapy. A recent open pilot study showed that PDT utilizing a similar device composed of organic LEDs was associated with lower pain comparing with traditional PDT (34).

Two-photon PDT (briefly discussed below) promoted the development of femtosecond lasers delivering pulses of light of 800 nm wavelength at 1 kHz frequency (35). Such pulses provide the necessary high peak power (kW-MW) while still maintain low average power that prevents photothermal damage of healthy tissues.

Luminescent semiconductor nanocrystals also known as quantum dots (QDs) demonstrate some unique and fascinating optical properties such as sharp and symmetrical emission spectra, high quantum yields, broad absorption spectra, good chemical- and photo-stability and the ability of the emitted wavelength tuning. Moreover, the photoluminescence of QDs is exceptionally bright and stable (36-37). Their challenging characteristics might be desirable for the use of QDs as alternate light sources in PDT.

Recently, a novel combination of radiation- and photodynamic therapy in the mode of Self Lightning Photodynamic Therapy (SLPDT) has been developed (38). In this technique, nanoparticles (NP) emitting scintillation or persistent luminescence attached to phtotosensitizers can be used as *in vivo* PDT agents. Upon exposure to ionizing radiation NPs emit scintillation luminescence, which further activates PS. As a consequence, singlet oxygen is produced enhancing ionizing radiation-mediated cell killing. Since luminescence emitted by NPs is persistent, short-time exposure to X-rays can be followed by prolonged PS excitation. In this setting no external light source is required to trigger photodynamic reaction (38-39).

6.   TARGETING   CYTOPROTECTIVE MECHANISMS IN PDT-TREATED CELLS

Although PDT is generally considered to be a potent, selective, and safe anticancer therapy, it is usually not efficient in long-lasting tumor control. At least to some extent this limitation results from induction of cytoprotective mechanisms, which help tumor cells to survive PDT-triggered oxidative stress. Identification and targeting of these rescue reactions turned out to be an attractive strategy for augmentation of antitumor effects of PDT.

6.1. ROS-scavenging enzymes

Singlet oxygen and reactive oxygen species contribute directly to the cytotoxic activity of PDT. Thus, PDT-mediated induction of ROS-scavenging enzymes serves as a potent cytoprotective mechanism limiting PDT effectiveness and promoting tumor cells survival. In mammalian cells, superoxide dismutase (SOD) and catalase are the primary antioxidants that do not require glutathione for their function, while secondary antioxidants (glutathione peroxidases, glutathione-S-transferases, thioredoxin/thioredoxin reductase system, peroxiredoxins) rely on glutathione availability (40).

Superoxide dismutase catalyzes the reaction which turns superoxide anion into less toxic products: hydrogen peroxide and oxygen (41). SOD activity was shown to be induced by PDT and to protect tumor cells from PDT-induced cytotoxicity. Inhibition of this enzyme seems to be a reasonable approach for potentiating of antitumor PDT efficacy (42). Sodium diethyldithiocarbamate, a SOD inhibitor, was shown to augment cutaneous photosensitization (43). Moreover,

treatment of cancer cells with 2-metoxyestradiol, an endogenous estrogen metabolite and the SOD inhibitor, significantly augmented antitumor activity of PDT both *in vitro* and *in vivo* (42). Catalase, the main hydrogen peroxide-removing enzyme converting it into water and oxygen, also plays a cytoprotective role in PDT-induced oxidative stress. However, in the majority of published studies only exogenous enzyme was used thus the role of endogenous catalase in PDT resistance has not been completely determined (44).

The glutathione activity-associated antioxidant systems regulate cellular redox balance due to the ease of thiol groups oxidation and the rapidity of their regeneration. A number of studies have shown a protective role of thiol groups in PDT-treated cells (45). The repair of lipid peroxides (LOOHs) generated in cellular lipid bilayers upon oxidative injury is probably the most important role of the glutathione-dependent systems in protection from PDT-induced damage (46). Depletion of glutathione with buthionine sulfoximine significantly potentiates the antitumor effects of PDT both *in vitro* and *in vivo* (45). Low-dose ALA-PDT was shown to induce thioredoxin (TRX) expression that was associated with tumor cell death prevention (47). Moreover, methylene blue-mediated PDT altered the expression of peroxiredoxins (PRXs) (48), however the exact role of this enzyme family in PDT effectiveness remains to be elucidated.

6.2. Handling of damaged proteins
Proteins encompass up to 70% of cellular dry mass and, as a consequence, are the major targets for PDT-generated reactive oxygen species (40). ROS-mediated protein damage leads to disruption of the proteins structure and loss of their biological activity. To survive, cells activate protective mechanisms that repair oxidatively damaged proteins and trigger dispose mechanisms for their elimination. Oxidatively-damaged proteins become "clients" for heat shock proteins (HSPs) – molecular chaperones that help in protein folding and prevent their aggregation. PDT has been shown to induce expression of various HSPs (49-51). Moreover, impairment of HSP90 function with geldanamycin analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) was reported to potentiate cytotoxic activity of PDT both *in vitro* and *in vivo* (52).

The majority of currently used photosensitizers localize to the membranes of the endoplasmic reticulum (ER). Thus PS excitation and robust local generation of ROS result in enhanced ER protein damage. Excessive accumulation of misfolded proteins in the ER leads to the development of a complex reaction commonly referred to as ER stress. PDT has been recently shown to induce ER stress due to the robust accumulation of oxidatively-damaged ER-located protein aggregates that are detrimental to the cell (53). To survive, the cell must degrade them, mainly via the ubiquitin-proteasome system (UPS) or, as it has been recently shown, via autophagy. PDT induces proteasome activity and increases cellular accumulation of polyubiquitinylated proteins (53). Preincubation of cancer cells with proteasome inhibitors significantly improves

cytostatic/cytotoxic activity of PDT (53). Moreover, the treatment combining Photofrin-PDT and proteasome inhibitors led to 60-100% of total cures in tumor-bearing mice (53). Since both PDT and proteasome inhibitors are registered for human treatment, the latter combination is of significant clinical importance and awaits further studies.

PDT-damaged proteins can be subsequently degraded in lysosome-like structures called autophagosomes in a process referred to as autophagy. Autophagy plays an important role in both cytoprotection and induction of cell death (54). It has been recently shown that PDT utilizing various photosensitizers that localize to the ER induces autophagy (55-56). It seems that autophagy offers protection from the phototoxic effects in low-dose PDT but with increased oxidative stress it serves as an alternate mechanism of cell death (57).

6.3. Mechanisms not directly associated with ROS scavenging
Some intracellular enzymes such as heme-oxygenase-1 (HO-1) are not directly engaged in ROS scavenging, yet have been shown to play an important role in protection against PDT-induced cytotoxicity. HO-1 catalyses breakdown of heme to biliverdin, carbon monoxide and iron ion (Fe^{2+}). PDT has been shown to increase HO-1 expression at both mRNA and protein levels (58-59). Moreover, inhibition of HO-1 enzymatic activity with Zn (II) protoporphyrin IX sensitizes cancer cells to Photofrin-PDT-mediated damage and seems a good rationale for further potentiating of antitumor effects of PDT (58).

The tumor cells can protect themselves against PDT-induced cytotoxicity by decreasing intracellular PS concentration. This effect can be achieved by: (i) decreased photosensitizer uptake via diminished expression of alpha-2macroglobulin/LDL receptor-related proteins (60), (ii) increased removal of photosensitizer via multidrug–resistance (MDR) protein (61) and (iii) enhanced photosensitizer metabolism, for instance, conversion of ALA-induced protoporphyrin IX to heme by ferrochelatase (62-63).

7. COMBINATIONS OF PDT WITH OTHER TREATMENT MODALITIES

In the majority of cases a single treatment modality is not able to cure cancer. PDT induces tumor cells death through activation of a variety of intracellular signaling pathways, eventually leading to apoptosis, necrosis or autophagy-associated cell death, induction of antitumor immune response and disruption of tumor vasculature. Therefore, due to the diverse antitumor effects, PDT is often used in combination with other established treatment modalities such as surgery, radiotherapy or chemotherapy.

Surgery is one of the standard treatments of solid tumors. However, even extremely precise surgery may leave some minute islands of tumor cells that promote tumor regrowth. Administration of a photosensitizer before

surgery and subsequent illumination of the site of the disease enables better identification of tumor cells. The procedure, commonly referred to as photodiagnosis (PD), has been extensively studied. For example, Zhong W. *et al.* evaluated the use of benzoporphyin-derivative monoacid ring A (Verteporfin) and microendoscope-derived light for successful fluorescence imaging of ovarian cancer cells (64). Moreover, PDT of the tumor bed can be performed at the time of the surgery. It has been shown that the combination of PDT with surgery results in significant reduction of metastases, promotes development of the antitumor immune response and decreases the rate of tumor relapses (65-67).

PDT has also been successfully combined with radiotherapy. Administration of a photosensitizer before radiation was shown to potentiate antitumor effect of PDT (68). The interplay between PDT and radiotherapy might be two-sided. PDT has been shown to sensitize cancer cells to radiotherapy (69) and, conversely, radiotherapy has improved anticancer efficacy of PDT (70-71). However, it has to be emphasized that the final result of this combination strongly depends on the PDT dose, fluence rate and the time between administration of the photosensitizer and tumor irradiation.

The antitumor effectiveness of combinations of PDT with standard chemotherapy has been evaluated since 1983 when Creekmore et al. demonstrated synergistic interaction between PDT and actinomycin D in in vitro treatment of mouse lymphocytic leukemia cells (72). However, subsequent studies utilizing PDT in combination with various chemotherapeutic agents, such as anthracyclines, platinum compounds, antimetabolites, microtubule inhibitors and others showed that interactions observed can be synergistic, neutral or antagonistic, depending on the drug, time interval between PDT and chemotherapeutic application, as well as on the tumor type (73).

Another well-studied approach is combination of PDT with bioreductive drugs. PDT strongly potentiates anti-tumor effectiveness of mitomycin C and nitromidazole by favoring the drug activation through the PDT-induced tumor hypoxia (74-75). The results of a clinical study evaluating the use of mitomycin C together with ALA-PDT in the group of patients with recurrent superficial bladder cancer are promising (76).

PDT is known to be a strong inducer of proangiogenic factors, such as COX-2 and VEGF, which impair the PDT outcome. These observations resulted in development of successful combinations of PDT with antiangiogenic agents. A number of studies showed synergistic antitumor interactions between PDT and COX-2 inhibitors (77-79). Moreover, the combination of PDT with monoclonal antibodies targeting VEGF reduced tumor volume and increased animal survival (80).

Several studies have confirmed the value of maintaining good tissue oxygenation for antitumor effects of PDT (81-83). Combination of PDT treatment with hyperbaric oxygen (84-86), perfluorochemical emulsions that increase tissue oxygenation (87) or administration of erythropoietin - a cytokine promoting erythrocyte renewal (88) have been proven more effective than PDT alone.

It is worth mentioning that majority of presented studies have been performed *in vitro* or in animal models. Therefore, further clinical trials evaluating the clinical potential of the combinations of PDT with other treatment modalities are urgently needed.

8. ENHANCEMENT OF PDT-MEDIATED IMMUNE RESPONSE

It has been documented that besides direct cytotoxic effects on tumor cells PDT can induce host antitumor immune response. The concept of the important role of immune response in the PDT outcome dates back to 1996, when Korbelik *et al.* demonstrated substantially lower therapeutic effects of PDT in immunodeficient SCID mice relative to normal, immunocompetent mice (89). Subsequent follow-up studies confirmed these findings and revealed that intact immune system is indispensable for the PDT success (90-91). The particular significance of this phenomenon reflects the possible influence of antitumor immune responses not only against a primary tumor, but also against tumor cells disseminated in the organism or localized outside the treatment site. Moreover, increasing number of promising results from clinical trials confirm the important role of PDT-mediated immune response in the therapy outcome (92-93).

Although there are reports that direct PDT can impair immune cells activity and thus exert immunosuppressive effects (94), the vast majority of studies suggest significant immunostimulatory potential of this therapy. PDT-treated tumor cells undergo apoptotic and necrotic cell death, thereby they can serve as a massive source of tumor antigens, like damaged, misfolded or mislocalized tumor-derived proteins, lipids, fragments of damaged extracellular matrix, which can play a role of DAMPs (damage-associated molecular patterns), and contribute to immune system stimulation (95).

The development of antitumor host immune response by PDT is a consequence of the induction of many different innate and adaptive immunity mechanisms and a complex interplay between them. It has been demonstrated that almost immediately after PDT an acute local inflammatory response is launched, accompanied by cytokine release, immune cell infiltration, activation, and subsequently the initiation of specific, antitumor adaptive immunity (90-91, 96). However, in most observed cases it is not strong enough to completely eradicate tumor cells. Therefore, many different studies are aimed at further enhancement of the PDT-mediated immune response. The strategies of augmenting the anti-tumor immune response induced by PDT can be grouped into five main categories (Table 2).

8.1. Immunoadjuvants

Immunostimulators that strengthen the efficacy of PDT can be assigned to two different groups: toll like receptor (TLR) ligands and activators of the alternative

Table 2. Selected combinations of PDT[1] and immunotherpay of cancer

Strategy	Treatment modality	Results of combination treatment	Ref
PDT + immunoadjuvants	killed *Corynebacterium pravum* delivered after [2]HPD-PDT	improvement of PDT	(98)
	Schizophyllan administered before Photofrin-PDT	increased cure rate	(99)
	Mycobacterium cell wall extract + various photosensitizers	increased cure rate increased tumor infiltration with leukocytes	(97)
	killed *Streptococcus pyogenes* OK432 before HPD-PDT	improvement of PDT	(152)
	Bacillus Callmette-Guerin (BCG) + various photosensitizers	increased cure rate increase in memory T cells	(153)
	zymosan	activation of complement cascade increased cure rate reduced number of recurrent tumor	(102)
	glycated chitosan from shrimp shells with Photofrin-PDT	doubled long-term animal survival compared with PDT alone	(100)
	CpG oligodeoxynucleotide (ODN) with Radachlorin-PDT	Hsp70[3] release IFN-gamma[4] production by CTLs[5] suppression of tumor growth	(154)
	intratumoral injection of gamma-inulin after PDT with various photosensitizers	reduction of tumor re-growth rate massive CTLs infiltration	(101)
PDT + cytokines	recombinant human TNF-alpha[6], Photofrin -PDT	additive effects in tumor growth retardation	(155)
	GM-CSF[7] gene introduced into tumor cells, Photofrin-PDT and Verteporfin-PDT	increased cure rate higher cytotoxic activity of tumor associated macrophages	(107)
	recombinant G-CSF[8], Photofrin-PDT	attenuated tumor growth and prolonged animal survival	(106)
	low dose PDT in combination with recombinant TRAIL[9] and FasL[10]	increased apoptosis of tumor cells	(109)
	intratumoral injection of adenoviral particles containing murine IL-12[11] gene	increase in the number of CTLs suppression of tumor growth complete regression of 9-mm sized tumor in all animals	(108)
PDT + adoptive immunotherapy	peritumoral or iv injection of NK[12] cells expressing IL-2 gene, immediately after mTHPC[13]-PDT	tumor growth retardation increase in tumor-free mice	(113)
	intra- and peritumoral macrophage infusion	stimulated cell-mediated antitumoral activity increased survival rate	(114)
	intratumoral injection of immature dendritic cells, Photofrin-PDT	stimulation of CTLs and NK increased cure rate	(111)
	intratumoral injection of DC[14], chlorin PS-PDT	better cure rate regression of untreated tumors induction of IFN-gamma - producing CTLs	(112)
PDT + introduction of foreign antigens	tumor cells transduced with EGFP[15], Verteporfin-PDT	generation of anti-EGFP antibodies complete tumor eradication resistance to rechallenge	(115)
	tumor cells stably expressing beta-galactosidase, Verteporfin-PDT	generation of specific CTLs complete tumor eradication regression of untreated contralateral tumor	(156)
PDT + anticancer therapeutics	DMXAA[16], Photofrin-PDT	induction of TNF production in tumor tissue reduction in tumor volume retardation of tumor regrowth	(117)
	Imiquimod, ALA[17]-PDT	increased number of responses	(116)
	low-dose cyclophosphamide, Verteporfin-PDT	depletion of Tregs[18] decreased TGFbeta[19] secretion increased cure number resistance to tumor rechallenge	(118)

Abbreviations: [1]PDT – photodynamic therapy, [2]HPD-hematoporphyrin derivative, [3]Hsp-heat shock protein, [4]IFN-gamma-interferon gamma, [5]CTL – cytotoxic T cell, [6]TNF-tumor necrosis factor, [7]GM-CSF-granulocyte macrophage colony stimulating factor, [8]G-CSF-granulocyte colony stimulating factor, [9]TRAIL-TNF-related apoptosis-inducing ligand , [10]FasL – Fas ligand, [11]IL-interleukin, [12]NK-natural killer cell, [13]mTHPC-m-tetrahydroxyphenylchlorin, [14]DC-dendritic cell, [15]EGFP-enhanced green fluorescent protein, [16]DMXAA-5,6-dimethylxanthenone-4-acetic acid, [17]ALA-delta-aminolaevulinic acid, [18]Treg-regulatory T cell, [19]TGF-beta-transforming growth factor beta

pathway of the complement cascade. The former activate antigen presenting cells like macrophages and dendritic cells by binding their pattern recognition receptors, mostly TLRs. Many different known sources of TLR ligands have been combined with PDT: killed bacterial cell walls (97-98), schizophyllan (99), shrimp chitosan (100) and others (Table 2). The complement system activators shown to promote tumor specific cytotoxic T-cells proliferation in response to PDT are zymosan and gamma-inulin (101-102). In most cases the intratumoral administration of immunoadjuvants prior to or immediately after PDT augments inflammatory reaction and triggers the immunostimulatory program which results in the specific antitumor memory T-cell generation, better overall PDT cure rate and prolonged animal survival.

8.2. Cytokines

It is well documented that PDT induces local and systemic cytokine secretion (103-105). However, there are experimental examples indicating further stimulation of the immune system by the administration of various cytokines in combination with PDT. Recombinant cytokines, cytokine genes introduced into tumor cells, or cytokine genes encapsulated in adenoviral particles were delivered intratumorally or intravenously prior to or immediately post PDT. The mechanisms of immune response stimulation

reflect the activity of the cytokine and involves stimulation of granulopoesis (106-107), immune cell mobilization and activation (108), as well as increased apoptosis of tumor cells (109). The combination treatment results in attenuated tumor growth, prolonged animal survival and in some cases complete tumor regression (Table 2).

8.3. Adoptive immunotherapy

Adoptive transfer of normal and genetically-modified immune cells has been shown to be effective in some cases of cancer immunotherapy. Apart from the obvious effectiveness of lymphocytes isolated from PDT-cured rats against the same tumor cells growing in syngeneic animals (110), there are also examples of efficient improvement of PDT outcome with intratumoral injection of immature dendritic cells (111-112), modified human natural killer (NK) cells expressing IL-2 (113), or activated macrophages (114). Interestingly, immune cells delivered to PDT-treated tumor site have been shown to retain their viability and functionality, and improved PDT cure rate in several models utilizing different photosensitizers.

8.4. Introduction of foreign antigens

Tumors stably transfected with foreign genes like beta-galactosidase or green fluorescent protein (GFP), derived from distantly related organisms, are very easily cured with PDT. Moreover, cured animals are extremely resistant to rechallenge with the same, foreign gene-modified tumor cells, suggesting the development of strong antitumor immune response. These results unequivocally reveal that introduction of remote antigens to tumor cells combined with PDT elicits specific cytotoxic T-cell mediated immune response and generates memory T-cells (115).

8.5. Anticancer therapeutics

Most of the aforementioned combination treatment modalities of PDT remain challenging regarding their clinical application perspective. The ideal cancer treatment should effectively destroy primary tumor, induce specific anti-tumor immune response to eradicate metastases and prevent tumor recurrence, and also should be feasible to be applied in patients. Regarding the latter condition, the use of anticancer drugs that potentiate antitumor immunity together with PDT appears promising and worth exploring in future studies. So far there are single reports on efficient synergistic effects of PDT utilizing various photosensitizers combined with antitumor drugs influencing immune system, including immunostimulatory agent imiquimod (116), TNF-alpha-inducing drug DMXAA (117), and regulatory T cell-depleting cyclophosphamide (118). It is noteworthy that antitumor agent dose, treatment schedule and overall combined therapy scheme are the critical parameters determining the treatment success rate and therefore should be optimised.

An important issue relevant to development of PDT-mediated anti-tumor immunity is associated with the PDT regimen. The recent data from both animal studies and clinical trials published by Gollnick *et al.* suggest that PDT regimen that is the most effective for direct tumor cell killing differs from PDT regimen that induces the most potent immune response (92, 119). Moreover, some promising results have been obtained from the studies on the use of tumor cells treated *in vitro* with PDT as anti-tumor vaccines (120-121), however the detailed discussion of these points is beyond the scope of this review.

9. OTHER NEW DIRECTIONS

9.1. Photochemical internalization

Specific drug delivery and its selectivity are among the most important issues for the safety and efficacy of virtually any anticancer therapy. Many macromolecular therapeutic agents are unable to directly penetrate cell membrane and thus mainly enter the cell through endocytosis. Berg and co-workers proposed a technology designed to deliver the active therapeutic content of endocytic vesicles into the cytosol by induction of a photodynamic damage in a process named photochemical internalization (PCI) (122). In this process, light-activated PS generates highly reactive singlet oxygen that damages lipid bilayer, what results in rapture of the vesicle membranes and leads to the release of its contents into the cytoplasm. Since singlet oxygen formation is restricted to the PS localization and the molecule has a very short half-life, therapeutic molecules localized in the vesicle matrix are not harmed by this procedure (123). PCI might not only enhance anticancer efficacy of therapeutic macromolecules but also decrease the development of drug-related side effects to the illuminated tissues. Moreover, the PCI strategy might serve as a mechanism of delivery of plasmids or adenoviruses for the purpose of gene therapy (124-125). The most desirable photosensitizers to be used in the PCI are amphyphylic disulphonated compounds that localize to the vesicle membranes or tetrasulphonated ones that localize to the matrix of the endocytic vesicles (126). Successful delivery of divert therapeutic agents such as bleomycin or immunotoxins (EGF–saporin and cetuximab–saporin) by means of PCI has already been reported (127-130).

9.2. Metronomic PDT

As far as chemotherapy is concerned, the term 'metronomic' describes continuous treatment with low doses of anticancer drugs. By analogy, metronomic PDT (mPDT) is an approach to constantly deliver both PS and light at low doses (131). The idea of mPDT arose from the observations that conventional PDT of brain tumors applied as a single, high-dose treatment induces surrounding tissue necrosis and life-threatening brain edema (132). In experimental tumor models ALA-mPDT has been shown to induce tumor cell apoptosis rather than necrosis (131). In patients, successful delivery of PS and light for the purpose of mPDT seems to be a significant technical challenge. In animals, prolonged PS delivery can be achieved by adding of ALA to the drinking water. This strategy has been proved effective in the treatment of astrocytomas in rats and papilomas in rabbits (131). Light sources used in mPDT have to be designed in a way guaranteeing appropriate light delivery in a minimally invasive manner for an extended period of time without compromising

Table 3. Examples of nanoparticles (NPs) use as photosensitizer carriers in PDT

Nanoparticles	NP size [nm]	Photosensitizer	Bio-degradability	Ref
PLGA[1]	n.a.[2]	ZnPc[3]	+	(157)
Gold NPs	2-4	Phthalocyanine	-	(158)
PLGA	660	BChl-a[4]	+	(159)
ORMOSIL[5]	30	HPPH[6]	-	(160)
Polyacrylamide NPs	30	MB[7]	-	(161)
SMNPs[8]	20-30	PHPP[9]	-	(162)
SNPs[10]	110	Hypocrellin A	-	(163)

Abbreviations: [1]PLGA-poly(lactic-co-glycolic acid, [2]n.a. – non assessed, [3]ZnPc-zinc(II) phthalocyanine, [4]BChl-a, bacteriochlorophyll-a; [5]ORMOSIL- organically modified silica, [6]HPPH-2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-a; [7]MB- methylene blue, [8]SMNPs- silica-based magnetic nanoparticles, [9]PHPP- 2,7,12,18-Tetramethyl-3,8-di-(1-propoxyethyl)-13,17-bis-(3-hydroxypropyl)porphyrin, [10]SNPs- silica nanoparticles

patient's movement. To date, two such tools have been proposed. The first one is based on a diode laser and has been tested in tumor-bearing rabbits while the second one is based on high luminescence LEDs and has been used in the treatment of rat tumors (133).

9.3. Nanoparticle-based PDT

Nanoparticles (NP) are multifunctional molecules with size varying between 1 and 1000 nm. They have already been widely used in various research areas and, as expected, might bring challenging new solutions to PDT. NPs can be characterized by divert features valuable for their biological applications: (i) NPs can be used to deliver hydrophobic drugs into the bloodstream (134); (ii) the molecules are easily biodegradable; (iii) their small size allows deeper tissue penetration and, finally, (iv) their functional groups can easily be modified. Numerous materials (such as inorganic oxides, metal, and organic compounds) has been used to obtain NPs with most desired properties (135). For the purpose of PDT, there are three main directions of the NPs use: (i) as singlet oxygen generating agents (136), (ii) as luminescent particles (38), and (iii) as PS carriers (137) (Table 3).

9.4. Two photon PDT

Visible light currently used for PDT varies in wavelength from 600 to 760 nm. For this spectrum, light penetration into the tissues is limited only to a few millimeters and puts in question the efficacy of the treatment of large tumors. Light of longer wavelength has better tissue-penetrating properties but its energy is not sufficient for photosensitizer excitation. Two-photon absorption (TPA) is a phenomenon of simultaneous absorption by a molecule of two photons that altogether provide appropriate energy for its excitation. For TPA near-infrared light can be used, bringing the potential of deeper light penetration into the tissues reaching even 2 cm in a xenograft tumor model in mice (138). Moreover, if the laser beam is highly focused, the site of reaction is better specified and surrounding tissue damage is diminished (139). Hence, development of proper light sources as well as photosensitizers with well-described and optimized TPA properties is urgently needed.

10. CONCLUSIONS

PDT is considered to be a selective and potent anticancer therapy. However, its therapeutic potential might still be enhanced by the development of novel combined treatments and by further optimization of the PDT setting. It is worth mentioning that PDT can be safely used together with other treatment modalities such as surgery, chemo- or radiotherapy without compromising their efficacy. The PDT-mediated singlet oxygen - a highly reactive molecule that, to our knowledge, is not naturally eliminated in mammalian cells, gives an attractive and challenging opportunity for further potentiation of anticancer treatment efficacy. Finally, the majority of currently used antitumor treatment methods result in immunosuppression that might worsen their outcome. PDT serves as a therapeutic regimen that not only induces acute inflammatory reaction but also has been shown to promote systemic immunity that is crucial for fighting metastases and establishing long-term tumor regrowth control.

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