Milestones in the development of photodynamic therapy and fluorescence diagnosis†

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Many reviews on PDT have been published. This field is now so large, and embraces so many subspecialities, from laser technology and optical penetration through diffusing media to a number of medical fields including dermatology, gastroenterology, ophthalmology, blood sterilization and treatment of microbial-viral diseases, that it is impossible to cover all aspects in a single review. Here, we will concentrate on a few basic aspects, all important for the route of development leading PDT to its present state: early work on hematoporphyrin and hematoporphyrin derivative, second and third generation photosensitizers, 5-aminolevulinic acid and its derivatives, oxygen and singlet oxygen, PDT effects on cell organelles, mutagenic potential, the basis for tumour selectivity, cell cooperativity, photochemical internalization, light penetration into tissue and the significance of oxygen depletion, photobleaching of photosensitizers, optimal light sources, effects on the immune system, and, finally, future trends.

Introduction

The history of photodynamic therapy (PDT) began in 1900 with O. Raab's observation that a combination of light and acridine was toxic to paramecium.[1] H. von Tappeiner, C. Ledoux-Lebards, A. Jodlbauer, A. Jesionek, W. Hausmann, F. Meyer-Betz, A. Policard, H. Fischer, H. Auler, G. Banzer, F. H. J. Figge, S. K. Schwartz, R. L. Lipson, E. J. Baldes, K. R. Weishaupt, T. J. Dougherty, amongst others, were the early pioneers in the development of PDT.[2–4] From the 1970s an increasing number of scientists and medical doctors worldwide joined the PDT field. Experimental PDT has become an established treatment for some diseases (Table 1). More than 8000 original articles and 600 reviews on PDT have been published. This field is now so large, that it is impossible to cover all aspects in a short review. Here we will concentrate on a few aspects, which we regard as basic in the development of PDT.

Hp, HpD and Photofrin

Already in 1912 Meyer-Betz demonstrated that hematoporphyrin (Hp) was an extremely powerful photosensitizer.[5] The interest for PDT was reignited in the early 1950s, after the experiments of Figge *et al.* which demonstrated that Hp had tumour localizing properties.[6] The need for purification of Hp was realized, since chromatography showed that it contained many components.[7] Treatment with sulfuric and acetic acids was introduced, and led to hematoporphyrin derivative (HpD).[7] It turned out that HpD contained even more components than commercial Hp. However, HpD had better tumour localizing properties than crude Hp.[8] Chromatographic analysis of tumour extracts showed that it was mainly the "impurities" that were retained in the tumours. Many groups embarked on attempts to identify these impurities,[9–11] and it was concluded that they were composed of dimers, oligomers and aggregates of porphyrins. Kessel *et al.* showed that diethers and diesters were central.[12] Aggregates showed tumour localizing abilities, but had low fluorescence yields and photosensitizing efficiencies.[13,14] Kessel proposed that aggregates were monomerized in tumour cells and became trapped, rendering the cells photosensitive (Moan's personal communications). HpD was further chromatographically purified by Dougherty's group to Photofrin, which is still the most widely used clinical PDT photosensitizer.[11,15]

Second and third generation photosensitizers

The first generation photosensitizers (HpD, Photofrin) have several drawbacks, such as contamination with impurities, relatively low absorbance at 630 nm, where tissue penetration of light is not optimal, and prolonged skin photosensitivity lasting up to 6–8 weeks.[15–17] The second generation photosensitizers (phthalocyanines, naphthalocyanins, benzoporphyrins, chlorins, purpurins, texaphyrins, porphycenes, pheophorbides, bacteriochlorins, *etc.*) were introduced to overcome these problems.[16,18–22] They may be obtained as chemically pure substances, effective generators of singlet oxygen, have high absorbance in the region of 650–850 nm, and give photosensitivity lasting for only a short time. The second generation photosensitizers *meta*-tetra hydroxyphenyl chlorin (m-THPC; Foscan, Biolitec AG) and benzoporphyrin derivative monoacid A (BPD-MA; Visudyne, QLT Inc. and Novartis Opthalmics) are approved for clinical use (Table 1).

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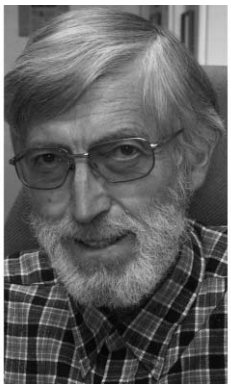

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Several second generation photosensitizers are currently under clinical evaluation. These include BOPP (boronated porphyrin; Pacific Pharmaceuticals Inc.), Npe6 (mono-*N*-aspartyl chlorin e6, talaporfin; Meiji Seika Kaisha, Ltd), hypericin (Pharmaceuticals Inc.), $AlPcS_n$ (sulfonated aluminium phthalocyanine, Photosense; State Research Center), chlorin e6 derivative (Photodithazine; State Research Center), ATMPn (porphycene; Glaxo-Wellcome Inc.), HPPH (2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a, Photochlor; Roswell Park Cancer Institute), motexafin lutetium (MLu, lutetium(III) texaphyrin, Lu-Tex, Antrin; Pharmacyclics Inc.), SnET2 (tin ethyl etiopurpurin, Sn etiopurpurin, rostaporfin, Photrex; Miravant Medical Technologies), *etc.*[3,23,24]

Second generation photosensitizers bound to carriers for selective accumulation in the tumour, are often called "third generation photosensitizers".[16,17] Photosensitizers conjugated with biomolecules, such as monoclonal antibodies, liposomes, *etc.*, have been developed.[16,17,25]

5-Aminolevulinic acid and its derivatives

Earlier research on the biochemistry of porphyria diseases showed that porphyrins can be endogenously produced.[26–30] Each of the porphyrias has a specific enzyme defect in the pathway of heme biosynthesis. There are 8 enzymes involved in the synthesis of heme, and, with the exception of the first one, defects of these enzymes lead to tissue accumulation and excessive excretion of porphyrins and/or their precursors, such as 5-aminolevulinic acid (ALA) and porphobilinogen. Heme biosynthesis is normally so tightly regulated that the concentrations of intermediate products are below the threshold of photosensitization. In 1951

Table 1 Approved drugs for use in PDT and fluorescence diagnosis

Chemical name	Tradename	Indication (countries, year)	Company, website
Hematoporphyrin derivative (HpD), porfimer sodium	Photofrin	Superficial bladder, gastric, cervical, esophageal, lung and endobronchial cancers (more than 120 countries, from 1993)	Axcan Pharma http://www.axcan.com/http://www.photofrin.com/
Benzoporphyrin-derivative monoacid ring A (BPD-MA), verteporfirin	Visudyne	Age-related macular degeneration (more than 70 countries, from 2001)	QLT Inc. And Novartis Opthalmics http://www.qltinc.com/Qltinc/main/mainhome.cfmhttp://www.visudyne.com/
Meta-tetra hydroxyphenyl chlorin (m-THPC), temoporfin	Foscan	Head and neck cancer (EU, Norway, Iceland, 2001)	Biolitec AG http://www.biolitec.com/
5-Aminolevulinic acid (ALA)	Levulan	Actinic keratoses of face or scalp (USA, 1999)	DUSA Pharmaceuticals, Inc. http://www.dusapharma.com/http://www.levulanpdt.com/
Methyl aminolevulinate (MAL)	Metvix	Actinic keratosis, basal cell carcinoma (EU and Australia, from 2001)	PhotoCure ASA http://www.photocure.com
Hexyl aminolevulinate (HAL)	Hexvix	Fluorescence diagnosis of bladder cancer (Sweden, 2004, EU, 2005)	PhotoCure ASA http://www.photocure.com

Berlin *et al.* demonstrated that excess administration of exogenous ALA bypasses the cellular feedback control mechanism in normal organisms and leads to abnormally large quantities of protoporphyrin IX (PpIX) in humans.[31,32] In 1975, Batlle *et al.* found that addition of ALA to the culture medium of soybean callus, a vegetable tumour, led to accumulation of porphyrins associated with fluorescence during UV exposure, inhibition of growth, and, finally destruction of tissue.[33] Clinical research on erythropoeitic protoporphyria revealed that PpIX was an efficient photosensitizer, causing skin photodamage *via* mitochondrial destruction.[31,33] In 1987, two groups proposed to use ALA as a porphyrin precursor in PDT.[34,35] Malik and Lugaci[34] demonstrated that exogenous ALA-induced PpIX together with light led to inactivation of Friend erythroleukemic cells. Peng *et al.*[35] reported that after administration of ALA to tumour-bearing mice, porphyrin fluorescence was induced in tumour, skin, kidney, liver, but not in muscle and heart tissues. In 1990 Kennedy *et al.*[36] reported the first clinical trials using ALA-PDT for the treatment of malignant and precancerous skin abnormalities. Introduction of ALA as a PpIX precursor was nothing less than a milestone in the development of PDT. This has several reasons: ALA is the only PDT agent that is a biochemical precursor of the photosensitizer, the one that is naturally produced in the body, and shows low cytotoxicity alone. Endogenously produced PpIX is rapidly cleared from the body (24–48 h) because natural clearance mechanisms exist. ALA can be administered systemically (intravenously, orally) or topically. Topical delivery of ALA avoids systemic photosensitivity, because the drug can be selectively applied on the areas to be treated. A short time interval (1–8 h, depending on the mode of administration) is needed between the administration of ALA and the maximal accumulation of PpIX in target tissues.[37–39]

Besides its usefulness in therapy, ALA can also be applied for diagnostic purposes:[40–43] after topical or systemic ALA application PpIX is induced in epithelial tumours, with a high tumour-to-surrounding tissue ratio, and the tumours can be visualized under exposure to blue light. Fluorescence images can be utilized either for a directed biopsy sampling or as an aid during surgery.[44]

In 1999 ALA (Levulan, DUSA Pharmaceuticals) was approved for the treatment of actinic keratoses (Table 1). However, the hydrophilic nature of the ALA molecule was thought to limit its penetration through biological membranes. Several methods, such as use of different formulations (creams, lotions, gels, *etc.* alone, with penetration enhancers and/or iron chelators), physical methods (curettage, ultrasound, iontophoresis, electroporation and electrophoresis) and chemical derivatization of ALA, were proposed to improve ALA delivery and porphyrin production. A large number of derivatives have been synthesised in the search for compounds that penetrate the plasma membrane of targeted cells and diffuse through epidermal layers more easily than ALA itself does. So far, most of the results indicate that many of the esters are more efficient in inducing porphyrin accumulation in cells in *vitro* than ALA itself. ALA methyl ester (Metvix, PhotoCure ASA) is widely used for the treatment of skin premalignancies and malignancies and ALA hexyl ester (Hexvix, PhotoCure ASA) is being developed for the diagnosis of bladder cancer (Table 1). PDT with topically applied ALA and one of its derivatives is an effective treatment for lesions less than 2 mm in depth.[37] New ALA derivatives are continuously being designed.[45,46] ALA-PDT is now the most widely practised form of PDT. Therapy and diagnosis with ALA are being used in dermatology,[4,37,47,48] gynaecology,[49–54] urology,[55–58] gastroenterology,[59–62] neurosurgery,[44] *etc.*[63] In the future low doses of ALA-PDT will probably turn out to be useful for photorejuvenation[64–66] and photochemoprevention of skin tumours.[67–70]

Oxygen and singlet oxygen

In 1902 Ledoux-Lebards observed that eosin killed paramecia with high efficiencies in flasks enriched with oxygen, and postulated that the presence of oxygen is essential for photoinactivation.[71] Later von Tappeiner and Jesionek,[72] and Straub[73] demonstrated the requirement of oxygen in photosensitization reactions. In 1931 Kautsky and de Bruijn proposed that singlet oxygen might be a reactive intermediate in dye-sensitized photooxygenation.[74] Singlet oxygen was recognized through the works of Schenck and Foote.[75–77] They concluded that photosensitization can proceed in two ways, defined as Type I and II mechanisms.[75–77] In a Type I reaction, the excited photosensitizer reacts directly with a substrate, leading to transfer of a proton or an electron, thereby

forming radicals which may react with oxygen and produce reactive oxygen species. Alternatively, in a Type II reaction, the triplet-state photosensitizer transfers its energy directly to molecular oxygen, forming an excited state known as singlet oxygen. This highly reactive form of oxygen reacts with and cause damage to many biological molecules, including lipids, proteins, and nucleic acids.[78–82] Singlet oxygen was first proposed by Weishaupt *et al.* as the cytotoxic agent responsible for photoinactivation of tumour cells.[83] It was shown that PDT is dependent on oxygen both *in vitro*[84–86] and *in vivo*.[87] When the oxygen concentration is reduced from 5% (the concentration in normal tissue) to 1%, the PDT effect is halved.[86] The PDT effect is mainly mediated through generation of singlet oxygen.[88,89] The short lifetime of singlet oxygen[90,91] explains why PDT damage occurs close to cell and tissue regions of high photosensitizer concentrations and why PDT has a low genotoxic potential.[92–99]

The basis for tumour selectivity

Several theories have been proposed for the mechanisms explaining why photosensitizing drugs are selectively taken up and/or retained in tumour tissues. These theories are based on special properties of tumours cells, or on physiological differences between tumours and normal tissues. The topic has been reviewed earlier, and little new information has accumulated after the reviews appeared.[100–105] In summary, the following factors are involved: a low tumour pH (related to poor vascularity of tumours leading to enhanced glycolytic activity followed by an increase in lactate levels) causes preferential accumulation of drugs that protonate and become more lipophilic as they enter acid tumours *via* the blood supply.[106] Tumours contain many macrophages that can ingest and monomerize aggregated photosensitizers as well as lipoprotein bound drugs.[107] More low-density lipoprotein (LDL) receptors are found on the surface of tumour cells than on the surface of normal cells.[108] Lipophilic photosensitizers preferentially bind to lipoproteins.[108] Tumours have a poor lymphatic drainage and a leaky vasculature.[107,109] Differences in water content and in other physiological parameters between tumours and normal tissue play roles for tumour localization of drugs.[110] A large interstitial space is often found in tumours.[110] A higher content of collagen seems to be present in several tumours than in normal tissues.[110]

Tumours are already, prior to PDT, starved of oxygen,[111] and additional PDT-induced vascular damage may inactivate them selectively.[87,112–116]

Alterations of metabolic steps in heme synthesis may be the main reasons for increased ALA-induced accumulation of PpIX in neoplastic cells and tissues. Thus, in some malignant cells and tissues the porphobilinogen deaminase activity is increased,[27,117–120] while the ferrochelatase activity is reduced.[27,117,119,121] Since ferrochelatase catalyzes the insertion of ferrous iron into the PpIX ring, the size of the labile iron pool influences PpIX accumulation.[122] The importance of available iron on PpIX production was demonstrated by using iron chelators.[123–126]

As mentioned above, tumours and normal tissues differ with respect to physiological structure. This may affect PpIX production and accumulation, and lead to tumour selectivity. Due to inflammation many tumours may have a slightly elevated temperature.[127,128] The rate of biosynthesis of PpIX increases steeply with increasing temperature.[129–131]

Stratum corneum is the main barrier for penetration of topically applied drugs from the skin surface into tumours and other tissues.[132] When ALA, or its derivatives, are applied topically on cutaneous tumours, some tumour selectivity is caused by a compromised stratum corneum.[38,103]

Differences between tumour and normal cells with respect to proliferation, differentiation, mitochondrial content, pH *etc.* may lead to selective PpIX accumulation and retention.[100,133–139] Thus, the reasons for selective PpIX accumulation in neoplastic and altered tissues may be of enzymatic, morphological or environmental character. Intricate interactions may exist between these factors, dependent on the nature of disease, its localization and stage, and on the PpIX precursor used, as well as on its application mode and time.[100]

Light sources

Since non-invasive PDT depends on aimed light delivery, it can be applied only to tumours and other lesions that can be reached by light, either directly or through optical fibres. The light source and the light delivery systems are two of the fundamental importance in PDT.[140,141] There is a need for designing optimal combinations of photosensitizers, light sources and treatment parameters for all PDT applications.

Light entering tissue is absorbed by the dominant chromophores (hemoglobin, melanin and water). This determines how deeply the light will penetrate. Each chromophore absorbs light at different wavelengths differently. As determined by the absorption spectra of the chromophores, the penetration depth will change with the wavelength. The so-called "optical window" of living tissue is between 600 nm (above the absorption of heme) and 1300 nm (below the absorption of water). Thus, to get optimal depths of action, one has to use photosensitizers absorbing in this region, preferably at the largest possible wavelengths. In principle, photons up to 1240 nm (corresponding to the energy gap between ground state oxygen and that of singlet oxygen) might be used. However, one has to allow for the singlet–triplet energy gap of the photosensitizer, and photons above about 850 nm will hardly generate triplet states with high enough energy to produce singlet oxygen.

The choice of light source should be made in consideration of the depth of the lesion, and, the chosen wavelength has to be within the absorption band of the photosensitizer. For treatment of deep lesions it is desirable to apply a photosensitizer with a high absorbance as far as possible into the red region. Many of the second generation photosensitizers absorb at longer wavelengths than the traditionally used 630 nm for HpD.

Aggregated and monomeric photosensitizers have different absorption spectra and different fluorescence quantum yields.[142] The optimal wavelength should give maximal quantum yield of singlet oxygen at maximal depth. The action spectrum of the photosensitizer with respect to cell photoinactivation needs to be determined.[143–147] The action spectrum describes the relative effectiveness of different wavelengths in producing the desired biological response. These spectra have the same shape as the fluorescence excitation spectrum of the photosensitizer, indicating that primarily non-aggregated molecules generate singlet oxygen.[143–147]

The oxygen concentration changes during PDT because of vessel damage and direct consumption of oxygen in the

photochemical process itself. This will lead to a change of the penetration spectrum (haemoglobin and oxy-haemoglobin have different absorption spectra) and has to be paid attention to when optical wavelength is sought.[148]

Light absorption leads to heat generation. Generally, fluence rates above about 150 mW cm^{-2} will give hyperthermia.[149] Recent studies have shown that a low fluence rate is preferable since depletion of oxygen occurs at high fluence rates.[115,150–154] At the same time, the exposure time needs to be considered. Thus, the fluence rate influences the direct photochemical oxygen consumption and, therefore, plays a critical role in clinical PDT.[153,154]

A number of different light sources have been used in PDT, lasers and non-coherent sources.[140,141] Lasers produce high intensity, coherent, monochromatic light. This light can be focused into and led through optical fibres and, thus, delivered directly to the target. Argon dye, potassium–titanium–phosphate (KTP) dye, metal vapour, copper and gold and, most recently, diode lasers have been used for clinical PDT around the world.[140,141,155] Lasers emit continuous wave or pulsed light, with pulse lengths down to a few fs. It has been hypothesized that high-intensity pulsed light could penetrate deeper into tissues than continuous wave light by causing a transient decrease in the absorption of chromophores in tissue by the first part of the pulse.[156] This process allows the rest of the pulse to pass through tissue with less attenuation. Whether a pulsed laser is better than a continuous wave light source in PDT is still unclear and contradictory data can be found in the literature. Most clinical studies have shown no significant differences in PDT efficacy of pulsed and continuous wave light.[156–160]

An advantage of using lasers is that they can be easily coupled into fibre optic delivery systems to reach otherwise inaccessible locations such as urinary bladder, lung and digestive tract.[140,141,161] For dermatology, however, non-laser sources are superior to laser systems because of their large illumination field, low cost, small size, and simple construction.[140,161–163] Non-coherent halogen, xenon arc and metal halide lamps, fluorescent tubes, light emitting diodes (LED) and intense pulsed light sources (IPL) are the most frequently applied light sources for PDT in dermatology. The broad emission spectra of these lamps give some disadvantages, such as thermal effect or difficulty in light dosimetry. Even if a combination of PDT and hyperthermia (due to IR radiation) seems to be advantageous, it should be avoided in skin since hyperthermia may be associated with more pain. Light exposure using a laser at a defined wavelength allows accurate light dosimetry at the surface of the lesion. For broad-band sources the depth of light penetration, the extinction coefficient of the photosensitizer, and the spectral intensity can all vary across the bandwidth of light used. Therefore, the light doses reported with the use of a laser, filtered light and white light are not directly comparable.

Lasers and non-coherent light sources have been used for PDT and usually show similar efficacies.[140,164,165] Since coherence is lost within a few tenths of a millimetre of penetration into human tissue, this property is not of any importance for PDT.

No single light source is ideal for every possible indication for PDT, even with the same photosensitizer. Choice of light sources should be based on: photosensitizer absorption (fluorescence excitation and action spectra), disease (location, size of lesions, accessibility), its reliability, simplicity of maintenance, cost and size. Even if the optimal light source is chosen, the clinical efficacy of PDT is dependent on the pattern light delivery: Total light dose, light exposure time, fluence rate, fractionation mode.

Photodegradation of photosensitizers

Almost all photosensitizers are degraded by light through singlet oxygen mediated processes: (1) the macrocycles of the photosensitizer molecules are fragmented, resulting in loss of absorbance and fluorescence, and (2) the photosensitizer molecules are altered, forming fluorescent photoproducts, which are often photosensitizers themselves and usually more water-soluble than the parent compounds.[166–171] The main photoproducts of porphyrin photodegradation are probably non-fluorescent. It was proposed that one pathway of photodegradation of porphyrins, such as of Hp, HpD, PpIX and mesoporphyrin, might be that the porphyrin is epoxidised at the double-bond between the ring and the methine bridge.[172] Formation of bilirubin and biliverdin might be the result.[172] These pigments are quite photolabile. Most photosensitizers are photodegraded and phototransformed in first order processes, *i.e.* with the degradation rates independent on the initial dye concentration. However, in biological samples photodegradation does not follow exponential kinetics due to heterogeneous binding in biological samples and oxygen depletion during light exposure.[167,173–176]

There are large differences in the photostability of the different photosensitizers. Generally, water-soluble dyes tend to be more stable than lipophilic ones, at least when present in cells and tissues.[172,177] The reason for this might be related to the intracellular localisation of the photosensitizer. Factors other than solubility are also important for the photostability of a photosensitizer. Binding of a photosensitizer to a protein generally decreases its photostability. Aggregates of a photosensitizer are more photostable than monomers.[142]

Photobleaching of a photosensitizer may limit its efficacy in PDT and has to be taken into account when choosing optimal light fluences and photosensitizer concentrations. On the other hand, photobleaching can prevent photodamage to normal tissue adjacent to the tumour area.[167,174] Photobleaching requires singlet oxygen, just as tumour destruction does. Thus, photodegradation rates may be used for clinical dosimetry.[175–179]

Damage to organelles

Subcellular targets and cellular responses associated with PDT can be different for different photosensitizers.[180] Damage to cell membranes after PDT has been early shown by a number of methods: ESR,[181] electron microscopy,[182,183] microscopic demonstration of blebbing and cell expansion.[34] Membrane effects are also causing increased attachment of cells to a substratum (supposedly also to the intracellular matrix) after PDT, and decreased attachment of suspended cells.[184–188] These membrane effects may be of great importance for reducing the metastatic potential of surviving cells in a tumour.

Damage to microtubules caused by PDT, notably with water soluble photosensitizers, leads to accumulation of cells in mitosis and subsequently to deaths.[189,190]

Since anionic photosensitizers localize outside the nucleus, little DNA damage is caused by PDT.[191] Only a small fraction of DNA, localized close to the nuclear membrane is damaged by PDT.[95,191]

The optimal way to kill cells in tumours has been debated for decades. PDT can kill cells by apoptosis and/or necrosis directly, or indirectly by closing the tumour microvasculature. Furthermore, stimulation or suppression of the host immune system may occur.[114,192–196] Recently, Kessel *et al.* proposed that also autophagy may be triggered by PDT and play a role.[197,198] The mode of cell death is dependent on photosensitizer type, on PDT dose and on cell genotype.[199] The subcellular localization of the photosensitizer is a key factor for the outcome of PDT.[200] Mitochondrially localized photosensitizers are able to induce apoptosis.[201] Lysosomally localized photosensitizers can elicit either a necrotic or an apoptotic response. In the plasma membrane (where several photosensitizers accumulate) rescue responses, apoptosis and necrosis can be initiated. Several protein phosphorylation cascades may be involved in regulation of the response to PDT. Lysosomal effects are the basis for photochemical internalization (PCI), a novel cancer therapeutic method related to PDT.[202,203] By PCI lysosomally localized toxins, immunemodulating molecules and DNA can be released into the cytoplasm of cells. We showed that a significant fraction of the lysosomes in a cell can be ruptured by PDT without inactivating it.[204] Release of lysosomally localized fluorophores leads to an increase of the fluorescence intensity caused by dilution and deaggregation and was early demonstrated both *in vitro* and *in vivo*.[96,97,205]

Immune effects

Canti and co-workers showed that less metastasis occurs after PDT than after surgery of tumours in mice.[206] This is certainly related to PDT effects on the immune system, although increased substratum binding and decreased ability of suspended cells to attach may play roles. Immune effects of PDT have been studied over decades.[194,206–210] Crosslinks of proteins on the cell surface, modulation of antigen presentation and direct effects on cells of the immune system have been demonstrated. In contrast to surgery, radiotherapy and chemotherapy, which are immunosuppressive in nature, PDT causes acute inflammation, expression of heat shock proteins, invasion and infiltration of the tumour by leukocytes, and may increase the presentation of tumour-derived antigens to T cells.[207]

PDT resistance, heat-shock or stress protein expression, and gene activation induced by photosensitizer-mediated oxidative stress are not fully elucidated yet.[207,211–215] Understanding of the molecular mechanisms of cellular responses after PDT will contribute to improvements of PDT.

Cell cooperativity

It was early shown that cells subjected to PDT communicate.[216] Thus, when cell colonies are treated, a non-random (non-Poisson distributed) inactivation occurs: there is an overweight of colonies with all cells undamaged and of colonies with all cells inactivated.[216,217] In PDT-treated monolayers, patches of damaged cells are frequently seen. This is not due to clonal effects, but rather to "rescue" or "killing" factors transmitted between the cells. Such cooperativity, which has been demonstrated for a number of tumour cell lines and for several photosensitizers,[218–222] almost certainly play a role for the efficiency of PDT and may explain the surprisingly deep necrosis sometimes observed after PDT.

Future trends

Many new second generation photosensitizers have been developed and a few of them are already in clinical trials. Future photosensitizers, third generation photosensitizers, are still in the initial stages of research. Improved photosensitizer delivery may allow more non-oncological diseases to be treated with PDT. The design of new photosensitizers which may attach to foreign DNA, RNA and protein may allow use of PDT against bacteria, viruses, fungi, *etc.* Nanobiotechnology may be very useful in PDT, allowing for effective and targeted photosensitizer delivery.[223–225]

Quantum dots have a potential as photosensitizers for PDT applications.[226] They may be used in the future to photosensitize other PDT agents or molecular oxygen through an energy transfer process, but further studies are needed.[227]

Another new approach in PDT is based on the strong two-photon absorption in certain newly developed organic molecules.[228,229] The therapeutic volume of two-photon PDT is very small and localized only within a small spot of a focused laser beam, where high light intensities are obtained. Thus, the photodynamic effect is highly localized, which is of advantage when treating sensitive tissue. Another benefit of two-photon PDT could be a higher penetration depth in tissue compared to conventional PDT, since longer wavelength can be used to generate singlet oxygen.[228,229]

Since PDT and other treatment modalities (radiation therapy, chemotherapy, hyperthermia) damage different targets, PDT combined with these modalities can be useful since dose reduction may be possible (drug, light, treatments times) with similar, or even better results than obtainable by single treatments. Potential side effects, as skin photosensitivity and systemic toxicity can thus be lowered. The effective treatment depth can be increased by combining PDT with other therapies. Combinations with immunoconjugates,[230] chemotherapy,[231–235] ultrasound,[236,237] radiation therapy,[236–241] electric current,[242] hyperthermia,[243–246] surgery,[247–251] application of bioreductive drugs,[252,253] microtubule inhibitors,[254] glucose injection,[255–258] or anti-angiogenic drugs[259] give good results *in vitro* and *in vivo*, but further investigations are needed before combination therapies can be introduced in clinical practice.

The major limitation of chemotherapy of certain tumours is the development of multidrug-resistant tumour cells, making complete response difficult to obtain. A number of researches have studied the effects of PDT on multidrug-resistant cells.[260–263] Their results, although still inconclusive, have opened the possibility of treating multidrug-resistant tumours with PDT.[264]

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