Resistance to Targeted Anti-Cancer Therapeutics 5
Series Editor: *Benjamin Bonavida*

Valentina Rapozzi
Giulio Jori *Editors*

Resistance to Photodynamic Therapy in Cancer

Springer

Resistance to Targeted Anti-Cancer Therapeutics

Volume 5

Series Editor
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For several decades, treatment of cancer consisted of chemotherapeutic drugs, radiation, and hormonal therapies. Those were not tumor specific and exhibited severe toxicities in many cases. But during the last several years, targeted cancer therapies have been developed. Targeted cancer therapies—sometimes called "molecularly targeted drugs—are drugs or other agents (e.g., anti-bodies) that block the growth and spread of cancer by interfering with specific gene products that regulate tumor cell growth and progression".

We have witnessed in the last decade a significant explosion in the development of targeted cancer therapies developed against various specific cancers. These include drugs/antibodies that interfere with cell growth signaling or tumor blood vessel development, promote the cell death of cancer cells, stimulate the immune system to destroy specific cancer cells, and deliver toxic drugs to cancer cells. One of the major problems that arises following treatment with both conventional therapies and targeted cancer therapies is the development of resistance, preexisting in a subset of cancer cells or cancer stem cells and/or induced by the treatments. Tumor cell resistance to therapies remains a major problem and several strategies are being considered to reverse the resistance to various manipulations. *Resistance to Targeted Anti-Cancer Therapeutics* focuses on the basic and translational research behind the molecular mechanisms of resistance found in many kinds of anti-cancer therapeutics.

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Resistance to Photodynamic Therapy in Cancer

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In Memoriam of Dr. Giulio Jori –

The scientific community is sad to learn of your early departure. You have been a leader in the PDT field, and you have contributed significantly to the benefit of mankind. We also remember you as a great friend, an excellent teacher, and a compassionate collaborator. You will always be remembered, and we are forever indebted to you. This book is dedicated to you and your family.

Valentina Rapozzi, PhD., Co-Editor

Benjamin Bonavida, PhD., Series Editor

Preface

Photodynamic therapy (PDT) represents an already well consolidated but still gradually expanding approach to the treatment of solid tumors, which is based on the combined action of three elements—a tumor-localizing photosensitizing agent, oxygen and specific intervals of visible light wavelengths, which leads to the generation of highly cytotoxic reactive oxygen species (ROS) (that is, *in primis* singlet oxygen). This technique has so far obtained the approval in several countries for the palliative or curative treatment of tumors localized in different organs, and in particular, the non-melanoma skin cancer, head and neck, gastroenteric apparatus, lungs, prostate and brain. Moreover, the application of PDT is also gaining attention for the treatment of diseases outside the oncological field, such as blood sterilization, age-related macular degeneration, and a variety of infectious diseases including those caused by antibiotic-resistant bacteria, recalcitrant rheumatoid arthritis and a number of cutaneous pathologies.

For many years, one favorable aspect of PDT has been assumed to be represented by the low probability of selecting photoresistant strains of malignant cells, a consequence of the multi-target mode of action of photosensitized processes; in most cases, an irreversible damage is induced to different proteins, unsaturated lipids and nucleotides. This should minimize the possibility for cells to develop protective strategies, including the activation of anti-oxidant processes. However, the development of thorough in-depth studies on the factors controlling the response of cells to repeated PDT treatment has provided different examples pointing out the not-too-rare gradual generation of cell clones poorly susceptible to the damaging effects of photosensitizing agents. Such observations raised the question of the consequences of cell resistance to PDT in a clinical scenario, especially when the PDT treatment needs to be repeated in the case of recurrences or insufficient response of the neoplastic lesion to the primary PDT treatment.

This volume addresses this above issue by assembling selected contributions from investigators who are authoritatively involved in front-running studies focused on the basic mechanisms of PDT and the translation of the results obtained in such studies to the clinical utilization of this technique. The volume starts with a chapter providing an overview of the main features of PDT at molecular, cellular and tissue levels. In addition, the response of various types of tumors to PDT in

human patients is described and the advantages or limitations of the utilization of PDT in the individual fields are discussed. In order to provide an exhaustive description of the current "state of art" of our knowledge on the modalities by which photosensitized processes can promote the selection of cells clones exhibiting poor susceptibility to the attack by ROS and the practical importance of such processes, the volume contains a total of ten additional chapters which deal with three aspects of tumor resistance to PDT.

Part 2 is centered on the mechanisms which have been shown to be most frequently responsible for the induction of cell resistance to PDT. In particular, a correlation is attempted between the probability and rate of the formation of resistant cells and the sequence of the main physical, chemical and biological events which lead to the eventual cell killing after the initial electronic excitation of the photosensitizer: according to the currently available information, cell death after photodynamic inactivation can occur via three concurrent pathways—random necrosis, apoptosis, and autophagy. Moreover, the possible involvement of tissue vasculature in the development of resistance to PDT is taken into attentive consideration, since the impairment of neo-formed blood vessels is well known to often play a major role in the PDT-induced damage to neoplastic lesions. Finally, methodologies are described for the isolation of PDT-resistant cells in order to facilitate a detailed examination of the most prominent characteristics of such cells at both functional and morphological levels. Such observations are essential to devise optimal ways for preventing the generation of PDT-resistant cells or obtaining their specific inactivation.

Part 3 of the volume reviews different strategies currently used to sensitize tumor cells to PDT with an aim to pilot the photosensitized process for counteracting or at least minimizing the probability to stimulate the selection of resistant malignant cells. The information provided by the investigations carried out at molecular and subcellular levels are being exploited in order to identify modes of regulation of PDT resistance that depend on the targets of the photosensitized processes, most of all the nature of the subcellular compartments which represent the binding sites of the added photosensitizer, since they are involved in the early stages of the photoprocess. Specific examples are given based on the involvement of specific factors, such as GRP78-targeting subtilase cytotoxin and survivin gene knockdown.

Part 4 of the volume outlines emerging and apparently very promising approaches to adequately control the tendency to induce resistance of tumor cells to PDT. One approach is based on the manipulation of the mechanisms regulating the intracellular formation of the tumor sensitizer protoporphyrin IX from the exogenously administered pro-drug 5-aminolevulinic acid (ALA); this approach is usually known as ALA-PDT and is increasingly utilized for the treatment of skin tumors. An alternative approach exploits the novel possibilities opened in the field of PDT by the introduction of multi-functional nanoparticles as carriers of photosensitizing agents to tumor tissues. In particular, the possibilities to utilize such nano-vehicles to target specific receptors preferentially expressed by malignant cells, thus, enhancing the selectivity of the PDT action and, thus, the scope of this approach

is critically outlined. Finally, the practical applications of such approaches for the PDT treatment of melanoma are exemplified.

We wish to mention that one potentially very important contribution to this volume, dealing with the relationship between the chemical structure of the photosensitizing agents and the development of tumor cell resistance to PDT by Janet Morgan, a well-known expert on this topic, is unfortunately missing since the author regretfully passed away while she was engaged in the preparation of her chapter.

Valentina Rapozzi
Giulio Jori

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Part I
Introduction

Chapter 1
Basic and Clinical Aspects of Photodynamic Therapy

Valentina Rapozzi and Giulio Jori

Abstract Photodynamic therapy (PDT) was originally developed for the treatment of solid tumors by the concerted action of a tumor-localizing agent, named as the photosensitizer, molecular oxygen and visible light source. The photoinduced cytotxic species are mainly represented by singlet oxygen ($^1O_2$), even though the formation of NO has been observed in selected situations. Optimal results are generally obtained by using porphyrins and their tetrapyrrolic analogues as the photodynamic agents, since these compounds display an intense absorbance of the red spectra wavelengths, which are endowed with a particularly deep penetration into most biological tissues. While several porphyrins exhibit an intrinsic preferential affinity for malignant tissues, the selectivity of the tumor localization can be significantly enhanced by pre-binding the photosensitizer with a targeting agent, such as an antibody or a vehicle (e.g., glycoproteins, peptides, oligonucleotide aptamers, growth factors, lipoproteins) directed against antigens or receptors which are specifically present at the surface of tumor cells. Recently, novel perspectives have been opened by the association of the photosensitizer with nanoparticles, which can be made to be multifunctional, thereby allowing the simultaneous delivery of photoactivatable moieties acting by different mechanisms, as well as of phototherapeutic and photodiagnostic agents. The chemical structure of the photosensitizer and the nature of the possible carrier have important consequences with regards to the distribution of the photosensitizer among different compartments of the tumor tissue, such as neoplastic cells, blood vessels or the non-vascular stroma, as well as its localization in different subcellular sites; these would obviously affect the mechanism of photoinduced tumor damage, modulating the competition between necrosis, apoptosis and autophagia, the importance of photoinduced hypoxia, and the balance between enhancement of the immune response and immunosuppression. So far, about 250 randomized clinical trials have been officially reported for

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PDT of tumors, and essentially all types of solid tumors with the exception of melanotic melanoma have been found to be positively responsive to the photodynamic treatment. Thus, PDT is currently considered as a reasonable option for the treatment of a variety of malignant lesions for curative or palliative purposes by using either external or endoscopic irradiation approaches via non-coherent or laser light sources.

Keywords Photodynamic therapy · Photophysics and photochemistry · Subcellular localization · Mechanisms of cytotoxicity · Delivery systems · Clinical implications

Abbreviations

ALA	5-aminolevulinuic acid
AIF	apoptosis-inducing factor
APAF-1	apoptosis-activating factor 1
COX-2	cyclooxigenase-2
DAMP	damage-associated molecular pattern
DC	dendritic cells
DFF	DNA fragmentation factor
DLI	drug light interval
EPR	enhanced permeability and retection
GRP	glucose-regulated protein
HIF	hypoxia inducible factor
HO-1	heme oxygenase-1
HP	hematoporphyrin
HPD	hematoporphyrin derivative
HS1	heat shock protein 1
LED	light emitting diode
MBs	molecular beacons
MMP	mitochondrial membrane potential
MMP-9	matrtix metallopeptidase 9
$^1O_2$	singlet oxygen
PARP	poly(ADP/ribose) polymerase
PCI	photochemical internalization
PDT	photodynamic therapy
PEG,	polyethylene glycol;
PLA2	phospholipase A2
PLC	phospholipase C
PpIX	protoporphyrin IX
PS	photosensitizing agent
RES	reticulo endothelial system
ROS	reactive oxygen species

RIP1	receptor interacting protein 1
SOD	superoxide dismutase
TfR	transferring receptors
VEGF	vascular endothelial growth factor

Introduction

Basic Aspects of Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) is a clinically approved, minimally invasive therapeutic procedure that has been so far utilized largely in the oncological field since it has proven to act efficaciously and preferentially against malignant tissues [1]. The procedure involves the systemic or topical administration of a photosensitizing agent (PS), which is intrinsically non toxic at photodynamically active concentrations; following the accumulation of significant amounts of PS by the neoplastic lesion, the electronic excitation of PS by irradiation with visible light wavelengths corresponding to one of its absorption bands, and in the presence of oxygen, initiates a series of physical, chemical and biological events which eventually lead to tumor necrosis. This process is of multi-factorial nature, since it generally involves direct killing of neoplastic cells, damage of the tumor vasculature, triggering of an anti-tumor immune response and induction of local inflammatory reactions [2].

Thus, PDT appears to act through the combined action of three elements: light, photosensitizer and oxygen. The cytotoxic species are represented by reactive oxygen species (ROS), generated via electron or energy transfer from the initially photoexcited PS, the main reactivity being associated with singlet oxygen ($^1O_2$), which can most efficiently attack a variety of subcellular components, such as aromatic and sulphur-containing amino acid side chains, guanosine nucleotides, unsaturated lipids and steroids [3]. Therefore, one critical factor controlling the successful outcome of the PDT of tumors is the requirement of a sufficiently large concentration of oxygen in the tumor tissue, which is obviously correlated with the presence of an extensive degree of vascularisation. Since the initially present oxygen concentration is progressively reduced during irradiation and the consequently induced photooxidative processes, attention must be paid to any important depletion of oxygen in order to avoid a partial response of the tumor to the photodynamic treatment [4].

As regards the choice of the PS, one essential property for an optimal response of the tumor consists in the quantum yield of $^1O_2$ formation. A simplified scheme of the photophysical pathway leading to conversion of molecular oxygen to its excited singlet derivative is shown in Fig. 1.1 for a typical photosensitizing agent, namely protoporphyrin IX. Thus, absorption of one visible light photon by the PS promotes one electron to the first excited singlet state (^1P), which can either decay to the original ground state by radiative (fluorescence emission) or non-radiative (largely thermal) pathways, or undergo the so called intersystem crossing to the

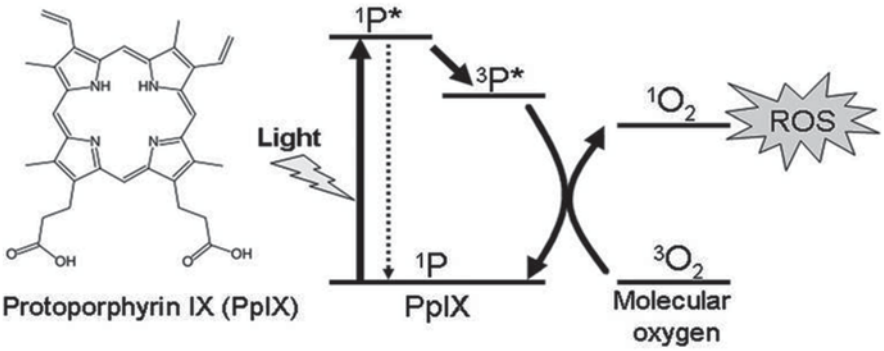

Fig. 1.1 Schematic illustration of photosensitization processes illustrated by a modified Jablonski diagram. The PS (PpIX) initially absorbs a photon that excites it to the first excited singlet state and this can relax to the more long lived triplet state. This triplet PS can interact with molecular oxygen leading to the formation of reactive oxygen species (ROS) and singlet oxygen

first excited triplet state (^{3}P). This species is usually endowed with a relatively long lifetime in the sub-millisecond range in liquid media. Hence, it can play a major role in diffusion-controlled processes, and in particular the transfer of at least a fraction of the absorbed energy to oxygen, which is a diradical, hence a triplet in its ground state, and is promoted to the hyper-reactive singlet state.

A number of *in vitro* studies pointed out that the $^1O_2$ quantum yield value, which can ensure a satisfactory response of the malignant tissue, should be greater than 0.5 [5]. In principle, the production of $^1O_2$ requires an energy of 22.5 kcal/mole, hence, any PS absorbing light in the 400–800 nm wavelength interval should have sufficiently high level electronically excited states, especially the lowest triplet state, to warrant the production of $^1O_2$ [6]. However, for the PDT of tumor, the most active PSs are represented by porphyrins and their tetrapyrrolic analogues (e.g., phthalocyanines, naphthalocyanines, chlorines, bacteriochlorines and porphycenes) which are characterized by relatively intense absorption bands in the 600–800 nm spectral interval, namely, light wavelengths which exhibit the maximum penetration power into most biological tissues [7]. This will guarantee the uniform illumination of larger volumes of the tumor tissues, as well as a selective photochemical effect owing to the lack of competitive absorption of the incident light by endogenous chromophores. On the other hand, photons with a wavelength longer than 800 nm do not provide enough energy to excite oxygen to its singlet state, and do not have the capacity for forming a substantial yield of reactive oxygen species upon irradiation.

Other pertinent desirable properties of photosensitizing agents are (a) the degree of purity, namely the PS should be a single pure compound to facilitate a more precisely defined pharmacokinetic behaviour, as well as quality control analysis with low manufacturing costs and prolonged shelf life and (b) a high stability to irradiation with visible light, minimizing the often occurring process of photobleaching [7, 8], which implies a decrease in the amount of the photochemically active principle in the course of light delivery. Most importantly, the PS should be endowed with

good pharmacokinetic properties that achieve a large degree of selectivity in its accumulation by the tumor in comparison with normal tissues, *in primis* the peritumoral districts. The ratio between the PS concentration in the tumor and the surrounding tissue is depending on a variety of factors and it is sometimes difficult to predict the time course of PS distribution *in vivo* [9]. In this connection, the kinetics of PS uptake and release by different tissues and the serum are often complex and can be influenced by (i) the chemical structure of the PS (ii) the PS distribution among serum proteins immediately after its intravenous injection (iii) the biochemical and morphological characteristics of the tumor, (iv) the possible association of the PS with a carrier or a targeting agent and (v) the modality of PS clearance from the body. In general, porphyrin derivatives are eliminated by the liver-bile pathway, due to the hydrophobic properties induced by their flat aromatic tetrapyrrolic skeleton; only highly hydrophilic porphyrins, such as the octacarboxylic uroporphyrin, undergo a preferential clearance via kidneys [9]. Traditionally, the interval between PS administration and the beginning of the irradiation procedure is rather long (>24 h), in order to allow for the PS to diffuse away from normal tissues, while lymphatic drainage of drugs from tumors is usually less efficient. However, recent reports [10] suggest that the tumor response may be more pronounced when light is delivered at a shorter drug-light interval since the PS is still present in the blood vessels, thus producing marked vascular damage. In any case, none of the photodynamically active porphyrin-type PS have been found to accumulate in the cell nuclei, an important aspect since it implies the lack of DNA damage, at least in the early stages of the photodynamic process, which could be carcinogenic or provoke the development of PDT-resistant clones.

One special case of an anti-tumor photosensitizing agent is represented by the so-called pro-drugs, such as 5-aminolevulinc acid (ALA) and its esters, that take advantage of a natural biosynthetic pathway to generate the photoreactive species. These compounds are metabolically converted to protoporphyrin IX, a porphyrin derivative with good photosensitizing properties [11]. Most frequently, ALA and ALA esters are applied topically or supplied orally and they are very frequently used to treat localized tumors, especially non-melanoma skin cancer [12].

Finally, the success of the PDT treatment is dependent on the selection of appropriate light sources and the irradiation conditions. As mentioned above, the 600–800 nm spectral region is most convenient for the *in vivo* treatment of tumors by PDT; this wavelength interval is also called the “optical window” owing to the greater penetration power into tissues [13]. The extent of penetration of 750 nm light into poorly melanotic tissues can reach 2 cm. Longer wavelengths are insufficiently energetic, while the tissue penetration of infrared light starts to decrease around 900 nm due to the absorption of water. As a consequence, both lasers and non-coherent light sources have been used for PDT and most often show similar efficacies [13]. Originally, argon-pumped dye lasers have been used for PDT at a clinical level, however, these sources have been abandoned since they resulted to be cumbersome, expensive and inefficient. On the contrary, diode lasers are small and cost-effective, are simple to install, have automated dosimetry and calibration features and a longer operational life. Light emitting diodes (LEDs) represent alter-

native light sources with relatively narrow spectral bandwidths and allow a precise light dosimetry in spite of non-coherence [14]. Lasers can be coupled into fibers with diffusing tips to treat tumors in the internal organs, such as the urinary bladder, brain, and the digestive tract. Inflatable balloons, covered on the inside with a strongly scattering material, shaped to fit an organ, are also commercially available. It is quite feasible to pilot a light beam in solid organs deep in the body under image guidance.

The critical parameters for planning an irradiation protocol are represented by the fluence rate (mW/cm^2) and the total light fluence (J/cm^2). As a matter of fact, the light dosimetry during PDT is a most difficult issue. In general, the fluence rate should be carefully controlled to avoid or minimize the risk of the advent of thermal effects, which would decrease the selectivity of the photodynamic process for tumors. Values lower than 150 mW/cm^2 are recommended, especially in the case of poorly vascularised tumors. The overall delivered amount of light (fluence) is dependent on the size of the diseased area, location and accessibility of the lesion, and characteristics of the tumor; hence, it is not possible to provide reliable directions *a priori*. Integrated systems that measure the light distribution and fluence rate either interstitially or on the surface of the tissues being treated are currently available [15]. Moreover, novel modalities of light delivery, such as fractionated or metronomic irradiation, are under investigation, but it is currently too early to draw definite conclusions about their efficacy.

Uptake and Subcellular Localization of Photosensitizers

The subcellular localization of PS is of special importance, since it determines the localization of the primary damage (Fig. 1.2). Indeed, ROS and in particular $^1O_2$ have a short half-life and react with oxidable substrates close to their site of generation (i.e. the average endocellular pathway of $^1O_2$ is about 20 nm during its lifetime) [17]. Several factors can affect the localization pattern of a PS, such as its chemical properties, the mode of delivery, the time interval after its systemic administration and the biochemical/morphological characteristics of a given tumor [17]. The understanding of the principles controlling PS localization is important for choosing the most effective PS for each application. The structural features that determine the subcellular localization pattern are: (a) the net ionic charge which can range from −4 (anionic) to +4 (cationic) (b) the degree of hydrophobicity expressed as the logarithm of the n-octanol/water partition coefficient (c) the degree of asymmetry present in the molecule. Hydrophobic PSs, with two or less negative charges, can diffuse across the plasma membrane, and then relocate to other intracellular membranes. These PSs present a good uptake by cells *in vitro*, above all at low concentrations (<1 μM) in the medium. Less hydrophobic PSs, with more than 2 negative charges, are taken up by endocytosis because they are too hydrophilic to diffuse across the plasma membrane [18]. PSs which are positively charged and are also hydrophobic can localize in the mitochondria [19]; this is probably due to the

Fig. 1.2 Cellular signaling pathways leading to apoptosis in cells after PDT. Initial targets of PDT-generated ROS depend on PS localization and include the mitochondria, lysosomes, endoplasmic reticulum, plasma membrane and PS binding to Bcl-2. (Castano AP et al.) [16]

influence of the mitochondrial membrane potential as well as the lipid bilayer of the membrane [20]. The PSs (such as silicon phtalocyanine Pc-4 and benzoporphyrin derivative monoacid ring A) that localize in the mitochondrial membranes exert their primary action in this site and are generally very rapid inducers of apoptosis, contrary to PSs localized in lysosomes or the plasma membrane [21–23]. A very early step occurring upon illumination is the release of cytochrome c from the mitochondria into the cytosol of treated cells [24–26] due to a rapid loss of the mitochondrial membrane potential (MMP) [26]. The release of cytochrome c can have various consequences: inhibition of the respiratory chain [25] that could lead to necrosis through deficient ATP production, but also to apoptosis by activation of the caspase pathway. The formation of the complex of cytochrome c, dATP, apoptosis-activating factor 1 (APAF-1) and procaspase-9 promotes the generation of active caspase 9 and can in turn cleave pro-caspase 3 [27–29]. The resulting active caspase 3 is responsible for the cleavage of a large number of proteins [30] including the DNA fragmentation factor (DFF) and the poly(ADP-ribose) polymerase (PARP) that are involved in the final steps of the apoptotic process.

In addition to this intrinsic caspase pathway, there can be a delayed activation of caspase 8, the enzyme involved in the extrinsic apoptosis pathway, mediated by the CD95/FAS receptor and its ligand [31]. Probably, this mechanism is initiated by some PSs (such as Rose Bengal) which are localized in the plasma membrane [32]. The activation of caspase 8 consequently leads to cytochrome c loss, caspase 3 activation and apoptosis. The mechanism underlying the release of cytocrome c and activation of caspases can be modulated by proteins of the Bcl-2 family. The PS localized in the plasma membrane can induce a number of signaling pathways stimulating both phospholipase A2 (PLA2) and phospholipase C (PLC), which are membrane-associated enzymes [33].

Several PSs localize in lysosomes and upon illumination they can cause cell death via two different routes: the release of lysosomal enzymes in the cytosol, or relocalization of the PS after illumination to other targets [34]: these can be located in the cytoplasm or more specifically in the nucleus [35, 36]. The photo-oxidation of lysosomes causes lysosomal membrane rupture and leakage of cathepsins [37, 38] that induce Bid cleavage and MMP [38] leading to apoptosis. This process is very slow compared with that induced by mitochondrial based PSs [39, 40]. Probably this is due to the tendency of PSs with greater degrees of aggregation to accumulate in lysosomes.

Mechanisms of PDT-Mediated Cytotoxicity

The mechanisms involved in the killing of tumors by PDT seem to be a complex interplay between direct and indirect (via vascular damage) effects on the neoplastic cells and based on the intratumoral localization pattern of the applied PS (Fig. 1.3).

Direct Effects

PDT can give rise to all three cell death pathways: apoptotic, necrotic and autophagy-associated cell death. Apoptosis is a generally major cell death modality involved in cell response to PDT. The mitochondrial outer membrane permeabilization after photodynamic injury is controlled by Bcl-2 family members and thought to be largely p53-independent [29]. After MMP there is the release of caspase activators, such as cytochrome c and Smac/DIABLO, or AIF (apoptosis-inducing factor) [29]. Phototoxicy is not propagated only through caspase-signaling but involves other proteases, such as calpains, as well as non-apoptotic pathways [29]. The inhibition or genetic deficiency of caspases only delay phototoxicity or shifts the cell death modality towards necrotic cell death [42]. The necrotic process can trigger the activation of RIP1 (receptor interacting protein 1) kinase, an excessive mitochondrial ROS production and an intracellular Ca^{2+}-overload [29, 43]. The third death pathway induced by PDT is autophagy [44, 45]. This is a lysosomal pathway for the

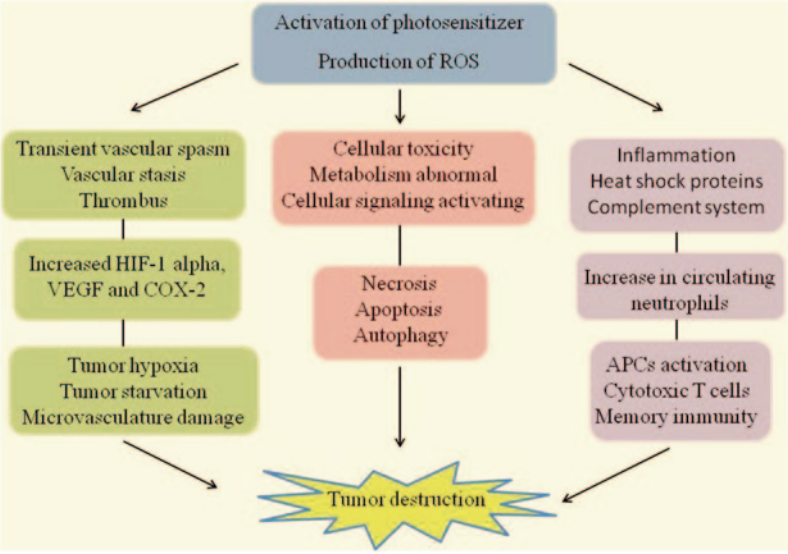

Fig. 1.3 Pathways of PDT-induced tumor cell death or destruction. The antitumor effects of PDT include three main mechanisms: direct tumor cell killing, vascular destruction, and immune system activation. Li at al. [41]

degradation and recycling of intracellular proteins and organelles. Autophagy can be stimulated by various stress signals including oxidative stress [46]. This process can have both a cytoprotective and a pro-death role following cancer chemotherapy, including those involving ROS as primary damaging agents [46]. Recently, several studies describe autophagy as a mechanism to maintain cell viability after photodynamic injury [45].

Indirect Effects

With respect to the indirect effects of PDT, it is important to consider the variation of tissue microcirculation [47]. A study by Star et al [48] reported what happened directly *in vivo* in rats comparing the microcirculation in a mammary tumor and in an adjacent normal tissue before, during and after PDT with a hematoporphyrin derivative (HPD). At the beginning, it was evident for blanching and vasoconstriction of the tumor vessels, then there were different heterogeneous responses such as the eventual complete blood flow stasis, hemorrhage, and in some larger vessels, the formation of platelet aggregates. Many reports directly implicated the endothelium as a primary target for PDT *in vivo* [49]. This is probably due to an increased sensitivity and a greater PS accumulation in exponentially growing endothelial cells compared to similarly proliferating tumor cells. Vascular injury may also be influenced by the drug light interval (DLI) [50]. It appears that the PS that have short

DLI achieve clinical success mainly by vascular ablation (vascular targeted PDT) in contrast to those with long DLI whose actions appear to be mainly cellular (cellular target PDT).

Cytoprotective Mechanisms:

"With photodynamic therapy (PDT), no matter what you do, if you are lucky, there is a pro-death response, however, simultaneously, there is a pro-survival molecular response, which mitigates the desired outcome with PDT**"**. This statement was reported by T. Hasan [51] that clearly expresses what happens in PDT. Literature data have described different cytoprotective mechanisms that cancer cells use to avoid the cytotoxic effect of PDT [29]: (i) the level of antioxidant molecules [52, 53] (ii) enzymes that can detoxify ROS such as superoxide dismutase (SOD) [54] and (iii) proteins whose encoding genes are themselves induced by PDT. NF-κB, a transcription factor which can be activated by reactive oxygen species including those generated by PDT, is involved in both apoptotic and anti-apoptotic activities, dependent on the model system studied [55, 56]. Some studies demonstrated that the inhibition of NF-κB by over-expression of the IκBα super-repressor or by the use of pharmacological inhibitors strongly sensitizes cancer cells to apoptosis induced by PDT [57, 58]. Other stress-related transcription factors induced by PDT include AP-1 (a protein complex composed by Jun, Fos, Maf and ATF-family proteins) [59], hypoxia inducible factor (HIF) [60] or Nrf2 [61]. PDT was shown to up-regulate heme oxygenase-1 (HO-1) expression and the mechanism is dependent on Nrf2 nuclear accumulation and on p38 MAPK and PI-3K activities. Because of the antioxidant activity of HO-1, it can be envisioned that Nfr2-dependent signal transduction can control cellular protection against the PDT-mediated cytotoxic effect [61].

A number of proteins involved in cellular stress responses has been shown to be regulated by PDT. Heat shock protein 1 (HS1), which is proposed to be involved in a cellular rescue response, was found to be phosphorylated by phthalocyanine 4/PDT [62]. HSP70 was induced most efficiently by PS which preferentially localizes in lysosomes [63]. There are different results about the role of PDT in stress proteins that might be due to the different cell lines used, or the different subcellular localization of the PSs. The heat shock proteins, which are known chaperones for damaged proteins, might be involved in the rescue response of cells after PDT damage. Another group of stress-induced proteins are the glucose-regulated proteins (GRP), which have a chaperon function in the endoplasmic reticulum. In addition, they can serve as intracellular calcium stores and are involved in the resistance against chemotherapeutic drugs [64].

One well-characterized signalling cascade is represented by the pathway that mediates mitogenesis upon stimulation by growth factors. A downstream event in the mitogenic pathway is the activation of ERK, a member of the MAPK family of kinases. Different PS modulate in different ways the ERK-activation [65]. PDT was able to activate the SAPK/JNK pathway, as well as the P38/HOG1 protein [66–69].

Both SAPK/JNK and p38/HOG1 are important in the stress response of cells to external stimuli, including $^1O_2$ [70].

Importance of the Tumor Microenvironment in PDT

The tumor microenvironment consists of malignant cancer cells, connective tissue and different host cells including endothelial cells, pericytes, and inflammatory leukocytes (macrophages and neutrophils) [71, 72]. Leukocytes, recruited into tumors, stimulate the endothelium, and indirectly activate tumor vascularization and also neutrophil recruitment in the tumors can be followed by vascular endothelial growth factor (VEGF) and matrix metallopeptidase 9 (MMP-9) release with associated angiogenesis and invasion [72, 73]. Tumor-associated macrophages exhibit a phenotype that favors tissue growth, angiogenesis and tissue remodeling. Cellular factors associated with PDT, such as necrosis, apoptosis, and hypoxia, can function as stimuli within the tumor microenvironment. Emerging data suggest that PDT-mediated changes to the tumor microenvironment can modulate treatment responsiveness. Preclinical investigations indicate that combining PDT with targeted therapies directed at attenuating the pro-survival actions of the tumor microenvironment can enhance the therapeutic potential of PDT [74]. Several laboratories have also shown that PDT can induce the expression and/or activation of additional proangiogenic molecules including HIF-1, VEGF, COX-2 and prostaglandins, TNF-α, matrix metalloproteinases (MMPs), integrins, IL-6, and IL-8 within the tumor microenvironment [74]. Procedures suppressing angiogenesis and inflammation should improve tumor responsiveness.

Role of Nitric Oxide in PDT

Another important crucial aspect that involves the efficacy of PDT is the role of nitric oxide (NO). This radical, produced by many cells in the human body, controlling important functions in tumor progression, may have a major influence on the outcome of cancer photodynamic therapy [75]. Tumor models characterized by low production of NO are more sensitive to PDT treatment [75]. It is important to consider that in the tumors with high NO levels, vascular events including vasoconstriction, ischemia, hypoxia and inflammatory reactions induced during PDT may be reduced. However, elevated NO levels may maintain vessel dilation during PDT treatment, resulting in increased tumor oxygenation, that enhances the oxygen-dependent generation of oxidative stress [76]. In addition to the NO level present in the tumor, it is important to consider the role of NO induced by PDT. Gupta et al in 1998 [77] reported for the first time that Pc4-PDT induces the release of NO by the NO synthases. Subsequently, different laboratories reported the induction of NO from different PS with apparently contrasting results. While some

studies demonstrated a protective effect induced by NO [78, 79], others reported an increase of apoptosis in tumor cells induced by high levels of NO [80].

There are different molecular pathways modulated by NO that can stimulate or arrest the tumor growth and these particular pathways can also modulate the cell response to PDT. In particular, NO can exert a cytoprotective role in tumor cells treated with PDT, activating protein kinase G (PKG) through a cGMP–dependent mechanism, leading to the suppression of caspase activity [78, 79, 81]. Proteins that can be increased by NO and are involved in reducing the cell PDT response are the antioxidant heme oxygenase-1 (HO-1) [82], the heat shock proteins (HSP) that are inversely correlated with sensitivity to PDT [83, 84], and the anti-apoptotic Bcl-2 [78, 85].

Other molecular pathways modulated by NO that able to influence the PDT tumor response are (i) the WAF1/CIP1/p21 pathway [86, 87] that, together with an overexpression of p53, can induce the blockade of the cell cycle and activate the apoptotic pathways [77, 88]; (ii) the HIF-1 alpha protein, involved in angiogenesis, inflammation and cell proliferation [89, 90]; and (iii) the NF-κB/Snail/YY1/RKIP circuitry [58, 80, 91].

PDT and the Immune System

PDT has a significant effect on the immune system [92–94] which can be either immunostimulatory or immunosuppressive. PDT frequently provokes a strong acute inflammatory reaction observed as localized edema at the targeted site [95]. The inflammation elicited by PDT is a tumor antigen non-specific process orchestrated by the innate immune system [96]. PDT appears particularly effective in producing an abundance of alarm/danger signals, also called damage-associated molecular patterns (DAMPs) or cell death-associated molecular patterns (CDAMPs) [96]. Among cytokines involved in the regulation of the inflammatory process, the most critical role in the tumor PDT response is mediated by IL-1β and IL-6 [97, 98]. Blocking the function of various adhesion molecules was proven to be also detrimental to the PDT response [97, 98]. On the other hand, blocking anti-inflammatory cytokines, such as IL-10 and TGF-β, can markedly improve the cure rates after PDT [96].

Numerous pre-clinical and clinical studies have demonstrated that PDT can influence the adaptative immune response resulting in the potentiation of adaptive immunity, or immunosuppression. The precise mechanisms leading to potentiation vs. suppression are unclear, however, it appears they are dependent upon the treatment regimens, the areas treated and the PS type utilized [99, 100].

PDT can activate both humoral and cell-mediated anti-tumor immunities, although the importance of the humoral response is unclear. As PDT efficacy is reduced in the absence of CD8+ T cell activation and/or tumor infiltration [101–103], most mechanistic studies have focused on the means by which PDT potentiates CD8+ T cell activation. It is clear that the induction of anti-tumor immunity following PDT is dependent upon the induction of inflammation [104]. PDT–induced

acute local and systemic inflammation is postulated to culminate in the maturation of dendritic cells (DCs) [98]. Mature DCs are critical for the activation of tumor-specific CD8+ T cells and the induction of anti-tumor immunity [98, 105, 106].

Delivery and Selectivity of PS

A key limitation in PDT is the poor water solubility of many PSs and their tendency to aggregate under physiological conditions. In addition, the accumulation and selective recognition of target tissue are still not optimal for many clinical applications.

To improve PS delivery to the target tissue, nanomedicine offers nano-agent strategies [107]. Nanoparticles can increase the solubility of hydrophobic drugs and offer the benefits of hydrophobicity and proper size to accumulate in the tumor tissue via the enhanced permeability and retention (EPR) effect [108]. Selective accumulation may be improved by the modification of the surface area using other ligands, which offers an attractive strategy to increase drug delivery to cancer cells and, thereby, keeping them away from healthy tissues that are sensitive to the toxic effect [109, 110]. The ideal delivery system should be biodegradable, have a small size and a high loading capacity, minimum immunogenicity and be non-toxic; moreover, it should not cause side effects, and demonstrate prolonged circulation in the body after administration, minimal self-aggregation tendency and selectively accumulate in therapeutically effective concentrations in the required area with little or even no uptake by non-target cells [109, 111].

A major drawback of using nanoparticles is that such moieties are rapidly removed from the circulation after administration by macrophages of the reticuloendothelial system (RES) [109, 110]. The circulatory half-life of nanoparticles can be significantly augmented using functionalized lipids in their construction. Such nanoparticles show longer circulation in the blood, demonstrate less reactivity towards serum proteins and are susceptible to RES uptake.

The most widely used polymeric steric stabilizer is polyethylene glycol (PEG). This is a water-soluble polymer that presents low toxicity, non-immunogenicity, protein resistance, and can be synthetically prepared in large quantities with high purity [109–112]. There are many ways to modify PSs to improve the effect of PDT. PS can be modified by encapsulation in delivery agents such as liposomes [113], micelles [114–117], ceramic based nanoparticles [118], gold nanoparticles [119, 120], and polymer nanoparticles [121]. Liposomes are able to encapsulate hydrophobic as well as hydrophilic drugs. Liposomal formulations show the ability to decrease the tendency of PS to aggregate and improve the tumor-selective accumulation [113].

Carbon nanotubes are another distinct possibility to deliver PS to required tissues [122]. These structures are synthesized by rolling sheets of carbon into hollow tubes that are single-walled, double-walled or multi-walled. They can be modified to carry active agents or targeting groups which can be bound covalently [123].

Carbon nanotubes absorb light in the near-infrared region and can cause cell death inside living cells due to excessive local heating [124].

To increase the selectivity and specific localization of the PS in the tumor, it is possible to use active targeting which relies on conjugates with a receptor-targeting moiety and a PS. One example includes monoclonal antibodies such as herceptin, folate-modified nanocarriers [110], antibodies against transferrin (Tf) receptors (TfR), which are over-expressed on the surface of many solid tumors. Different combinations can be achieved using other specific ligands such as vitamins, glycoproteins, peptides, oligonucleotide aptamers, growth factors, lipoproteins and other useful tools to target nanoparticles to cancer cells [110, 113, 125]. A technique termed photochemical internalization (PCI) was developed by Berg [126, 127]. This procedure relied on co-incubating cells with a macromolecule that is needed to be delivered into the cell cytoplasm and a PS such as aluminium phthalocyanine disulfonate. Both of these molecules were incorporated into lysosomes with the PS localizing in the lysosomal membrane. On delivery of the correct amount of light the lysosome was ruptured by photochemical damage to its membrane, thus releasing the intact macromolecule into the cytoplasm. This technology has been used to increase intracellular delivery of genes [128], viruses [129], peptide nucleic acids [130] and ribosome inactivating proteins [131]. PCI has been shown to potentiate the biological activity of a large variety of macromolecules and other molecules that do not readily penetrate the plasma membrane.

Recently, the concept of PDT molecular beacons (MBs) was developed. This derives from the use of MBs as fluorescent probes with high target specificity [132, 133]. The PS is linked to a quenching molecule, so that it is inactive until the linker is cleaved by a target-specific enzyme. The linker may be also an antisense oligonucleotides (hairpin) loop, which is opened by hybridization to complementary mRNA. The most important characteristics of MB is that tumor selectivity no longer depends solely the PS delivery, but also on the tumor specificity of the unquenching interaction and selectivity of the beacon to this interaction. Recently, asymmetric hairpin beacons were described to balance high quenching efficiency with 2-step activation (cleavage and dissociation) to enhance tumor cell uptake [134].

Clinical Indications for PDT

Photodynamic therapy has been so far largely developed as a tool for the treatment of solid tumors. The first clinical application of PDT goes back as far as 1903, when von Tappeiner and Jesionek from the University of Munich reported the successful treatment of malignant skin lesions by the combined action of eosin and sunlight [135]. von Tappeiner coined the term photodynamic to describe this phenomenon [136], in order to distinguish the photoinduced cell killing from the sensitization of photographic plates that was also extensively studied at that time. The introduction of porphyrins, in particular hematoporphyrin (Hp) and its derivative HpD, as tumor photosensitizers was performed by Schwartz et al. [137], but the definite impulse

to the use of PDT in oncology was provided by Dougherty in 1973. He founded a research group specifically devoted to PDT initially using a product, known with the commercial name of Photofrin, i.e. a heterogeneous porphyrin sample enriched in Hp dimers and oligomers [138]. As of now, a few thousand patients were treated by PDT worldwide, using a number of porphyrins or porphyrin analogues as tumor localizers and photosensitizers: of the large variety of tumors examined, none was found to be unresponsive, with the exception of melanotic melanoma. Details of the results obtained in the about 250 clinical trials officially reported for PDT can be found in recent reviews [139]. Thus, the conclusion can be safely accepted that PDT represents a reasonable option, sometimes even as a primary treatment, in the oncological field, even though caution still exists owing to the limited number of adequately randomized trials carried out by the various centers [140].

As one would expect, the largest number of PDT applications was focused on the treatment of pre-malignant and malignant cutaneous lesions. This is largely due to the development of ALA and its derivatives as PDT agents [11, 13], which can be most often topically applied using gel-type formulations. ALA-PDT is currently approved for the treatment of actinic keratosis, mucous dysplasia, and basal cell carcinoma. This therapeutic modality is also effective against squamous cell carcinoma, the response rate being at least as favourable as that typical of cryotherapy and 5-fluorouracil. Complications seen in these series were limited to cutaneous photosensitivity, and local pain following therapy was usually controlled by oral analgesics. Recent observations appear to indicate that the complete response rate of such cancer lesions can increase to above 95 % using novel formulations of ALA, such as nanoemulsions or patch-based applications. These favourable features of PDT are further increased by the excellent cosmetic results obtained, for example in comparison with surgery. Importantly, Kaposi's sarcoma was shown to respond very favourably to systemic PDT.

Similar promising effects were observed upon application of PDT in the field of head and neck tumors [141, 142]. Thus, PDT treatment of early carcinomas of the oral cavity, pharynx and larynx in the presence of Photofrin or Foscan, a tetra-meta-hydroxyphenyl-chlorin, gave quite efficient responses with no detectable permanent damage to the normal tissue: this treatment resulted in the preservation of vital functions, including swallowing and speech. In some cases, such as patients affected by squamous cell carcinoma of the oral cavity, a cure rate of 100 % was reported after a 5 years follow up. In general, a small number of recurrences was noticed for the treatment of these tumors, which were efficiently taken care of by repeated PDT or local surgery. Very interestingly, Hopper et al. [143] treated early oral cancer, characterized by lesions up to 2.5 cm in diameter, with Foscan-PDT and they observed a disease-free survival of 75 % after two years. ALA-PDT was also tried for head and neck tumors, but the extent of tumor response was clearly lower than that measured for Foscan-PDT.

In the case of the gastrointestinal (GI) tract, the most intensively studied applications of PDT include Barrett's oesophagus and various levels of dysplasia, as well as early oesophageal cancer [144]. Such diseases involve superficial and frequently large mucosal areas, which are readily accessible to light piloted to the treatment

site by means of optical fibres. PDT clearly acts in a markedly less invasive mode in comparison with other approaches, such as oesophagectomy, which are usually associated with significant morbidity and an about 5 % mortality rate. The conclusions reached by a general analysis of the various clinical trials performed for this application clearly point out that PDT is an effective, safe and minimally invasive first-line treatment for patients affected by mucosal adenocarcinoma and Barrett's dysplasia. It is worth underlining that patients, who do not exhibit a complete response to PDT , show appreciable improvements upon subsequent exposure to radiotherapy [145]. It also seems that the combined treatment of patients affected by high grade dysplasia in Barrett's oesophagus with PDT plus omoprazole led to a definitely greater cure rate than those obtained when either therapy was applied alone. Thus, these findings support the possibility to use PDT in association with other therapeutic modalities if necessary.

PDT was also occasionally applied for the treatment of GI cancers occurring beyond the oesophagus, including early duodenal and ampullary tumors, unresectable pancreatic carcinomas, and bulky colon and rectal cancer; in most cases, the aim of PDT application was essentially palliative. At present, the use of PDT for these diseases must be considered as experimental; thus, phase III studies have not yet been completed in spite of promising results achieved in the initial applications.

Typically, PDT can also be used as a palliative treatment for intraperitoneal malignancies [146], in particular if applied intraoperatively after surgical debulking the main tumor. In this case, the limited penetration of red light into the malignant tissue is of help to minimize the risk of damage to the underlying normal tissue layers. Similarly, space selectivity typical of visible light-promoted PDT was proven to safely treat prostate cancers sparing the surrounding normal tissue districts. In this connection, Foscan-PDT appears to be especially efficacious against prostate cancer. On the other hand, particularly promising results in this field have been recently proposed through the use of vascular-targeted PDT to be performed in the presence of Pd-bacteriopheophorbide (Tookad), which acts upon irradiation at short post-injection times [147]. Analogously, superficial bladder cancer is an attractive target for PDT: the geometry of the bladder allows for a homogeneous illumination of the area to be treated, by the pre-injection of light-scattering lipid material.

Lastly, brain tumors [148] and early lung cancer [149] are readily amenable to treatment by PDT with good selectivity in the destruction of the malignant tissue. For brain tumors, advantage is taken of the lack of porphyrin penetration across the blood-brain barrier. In several cases, the PDT treatment is applied intraoperatively, after fluorescence-guided resection, thanks to the red light emission typical of porphyrins upon visible light-excitation [150].

Overall, PDT can be definitely considered at the threshold of entering mainstream clinical practice in many oncological fields. Moreover, a careful engineering of the chemical structure of the porphyrin molecule is presently opening new pathways of medical applications of PDT, including microbial infections [151], waterborne diseases [152], non-cancerous skin lesions [153], and age-related macular degeneration [154].

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References

1. Agostinis P, Berg K, Cengel KA, Foster TH, Gollnick SO, Hahn SM, Hamblin MR, Juzeniene A, Kessel D, Korbelik M, Moan J, Mroz P, Nowis D, Piette J, Wilson BC, Gola J. Photodynamic therapy of cancer: an overview. CA Cancer J Clin. 2011;61:250–81.
2. Dolmans DE, Fukumura D, Jain RK. Photodynamic therapy for cancer. Natl Rev Cancer. 2003;3:380–7.
3. Jori G. Tumor photosensitisers: approaches to enhance the selectivity and efficiency of photodynamic therapy. J Photochem Photobiol B Biol. 1996;36:87–93.
4. Jori G, Coppellotti O. Inactivation of pathogenic microorganisms by photodynamic techniques: mechanistic aspects and perspective applications. Anti-Infect Agents Med Chem. 2007;6:119–31.
5. De Rosa M, Crutchley R. Photosensitised singlet oxygen and its applications. Coord Chem Rev. 2002;233:351–71.
6. Jori G, Reddi E. Second generation photosensitisers for the photodynamic therapy of tumors. In: Douglas R, Moan J, Ronto G, Editor. Light in biology and medicine. London: Plenum Press; 1999. pp. 253–66.
7. Ochsner M. Photophysical and photobiological processes in the photodynamic therapy of tumors. J Photochem Photobiol B Biol. 1997;39:1–8.
8. Reddi E, Ceccon M, Valduga G, Jori G, Bommer JC. Photophysical properties and antibacterial activity of meso-substituted cationic porphyrins. Photochem Photobiol. 2002;75:462–70.
9. Mroz P, Tegos GP, Gali H, Wharton T, Sarna T, Hamblion MR. Photodynamic therapy with fullerenes. Photochem Photobiol Sci. 2007;6:1139–49.
10. Allison RR. Photodynamic therapy: oncologic horizons. Future Oncol. 2014;10:123–4.
11. Pottier R, Krammer B, Stepp H, Baumgartner R. Photodynamic therapy with ALA: a clinical handbook. Cambridge: Royal Society of Chemistry; 2005.
12. Bourré L, Giuntini F, Eggleston IM, Mac Robert AJ. 5-Aminolaevulinic acid peptide pro-drugs enhance photosensitisation for photodynamic therapy. Mol Cancer Ther. 2008;7:1720–29.
13. Babilas P, Landthaler M, Szeimies RM. Photodynamic therapy in dermatology. Eur J Dermatol. 2006;16:340–48.
14. Paula LF, Santos RO, Menezes HD, Britto JR, Vieiram JB, Gomtijo PP, Oliveira AC. A comparative study of irradiation systems for phototherapeutic applications. J Braz Chem Soc. 2009;21:1–7.
15. Van den Bergh H. On the evolution of some endoscopic light delivery systems for photodynamic therapy. Endoscopy. 2008;40:392–407.
16. Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part two-cellular signaling, cell metabolism and modes of cell death. Photodiagnosis Photodyn Ther. 2005;2:1–23.
17. Peng Q, Moan J, Nesland JM. Correlation of subcellular and intratumoral photosensitizer localization with ultrastructural features after photodynamic therapy. Ultrastruct Pathol. 1996;20:109–29.
18. Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part one-photosensitizers, photochemistry and cellular localization. Photodiagnosis Photodyn Ther. 2004;1:279–93.
19. Dummin H, Cernay T, Zimmermann HW. Selective photosensitization of mitochondria in Hela cells by cationic Zn(II) phthalocyanines with lipophilic side-chains. J Photochem Photobiol B. 1997;37:219–29.
20. Rashid F, Horobin RW. Interaction of molecular probes with living cells and tissues. Part 2. A structure-activity analysis of mitochondrial staining by cationic probes, and a discussion of the synergistic nature of image-based and biochemical approach. Histochemistry. 1990;94:303–8.

21. Kessel D, Luo J, Deng Y, Chang CK. The role of subcellular localization in initiation of apoptosis by photodynamic therapy. Photochem Photobiol. 1997;65:422–6.
22. Kessel D, Luo Y. Mitochondrial photodamage and PDT-induced apoptosis. J Photochem Photobiol B. 1998;42:89–95.
23. Dahle J, Steen HB, Moan J. The mode of cell death induced by photodynamic treatment depends on cell density. Photochem Photobiol. 1999;70:363–7.
24. Granville DJ, Carthy CM, Jiang H, Shore GC, McManus BM, Hunt DW. Rapid cytochrome c release, activation of caspase, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. FEBS Lett. 1998;437:5–10.
25. Varnes ME, Chiu SM, Xue LY, Oleinick NL. Hotodynamic therapy-induced apoptosis in lymphoma cells: translocation of cytochrome c causes inhibition of respiration as well as caspase activation. Biochem Biophys Res Commun. 1999;255:673–9.
26. Kessel D, Luo J. Photodynamic therapy: a mitochondrial inducer of apoptosis. Cell Death Differ. 1999;6:28–35.
27. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science. 1998;281:1312–6.
28. Nunez G, Benedict MA, Hu Y, Inohara N. Caspases: the proteases of the apoptotic pathway. Oncogene. 1998;17:3237–45.
29. Buytaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochem Biophys Acta. 2007;1776:86–107.
30. Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 1999;6:99–104.
31. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science. 1998;281:1305–08.
32. Zhuang S, Lynch MC, Kochevar LE. Caspase-8 mediates caspase-3 activation and cytochrome c release during singlet oxygen–induced apoptosis of HL-60 cells. Exp Cell Res. 1999;250:203–12.
33. Agarwal ML, Larkin HE, Zaidi SI, Mukthar H, Oleinick NL. Phospholipase activation triggers apoptosis in photosensitized mouse lymphoma cells. Cancer Res. 1993;53:5897–902.
34. Berg K, Moan J. Lysosomes and microtubules as targets for photochemotherapy of cancer. Photochem Photobiol. 1997;65:403–9.
35. Berg K, Madslien K, Bommeer JC, Oftebro R, Winkelman JW, Moan J. Light induced relocalization of sulfonated meso-tetraphenylporphines in NHIK 3025 cells and effects of dose fractionation. Photochem Photobiol. 1991;53:203–10.
36. Peng Q, Farrants GW, Madslien K, Bommer JC, Moan J, Danielsen HE, Nesland JM. Subcellular localization, redistribution and photobleaching of sulfonated aluminum phthalocyanines in a human melanoma cell line. Int J Cancer. 1991;49:290–5.
37. Berg K, Moan J. Lysosomes as photochemical targets. Int J Cancer. 1994;59:814–22.
38. Reiners JJ Jr, Caruso JA, Matthieu P, Chelladurai B, Yin XM, Kessel D. Release of cytocheome C and activation of pro-caspase-9 following lysosomal photodamage involves bid cleavage. Cell Death Differ. 2012;9:934–44.
39. Noodt BB, Berg K, Stokke T, Peng Q, Nesland JM. Different apoptotic pathways are induced from various intracellular sites by tetraphenylporphyrins and light. Br J Cancer. 1999;79:72–81.
40. MacDonald LJ, Morgan J, Bellnier DA, Paszkiewicz GM, Whitaker JE, Litchfield DJ, Dougherty TJ. Subcellular localization patterns and their relationship to photodynamic activity of pyropheophorbide-a derivatives. Photochem Photobiol. 1999;70:789–97.
41. Li W, Ma Q, Wu E. Perspectives on the role of photodynamic therapy in the treatment of pancreatic cancer. Int J Photoenergy. 2012;12:1–9.
42. Kessel D. Relocalization of cationic porphyrins during photodynamic therapy. Photochem Photobiol Sci. 2002;1:837–40.
43. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature. 2005;434:652–8.
44. Buytaert E, Callewaert G, Vandenheede JR, Agostinis P. Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. Autophagy. 2006;2:238–40.

45. Reiners JJ Jr, Agostinis P, Berg K, Oleinick NL, Kessel D. Assessing autophagy in the context of photodynamic therapy. Autophagy. 2010;6:7–18.
46. Dewaele M, Maes H, Agostinis P. ROS-mediated mechanisms of autophagy stimulation and their relevance in cancer therapy. Autophagy. 2010;6:838–54.
47. Castellani A, Pace GP, Concioli M. Photodynamic effect of haematoporphyrin on blood microcirculation. J Pathol Bacteriol. 1963;86:99–102.
48. Star WM, Marijnissen HP, van den Berg-Blok AE, Versteeg JA, Franken KA, Reinhold HS. Destruction of rat mammary tumor and normal tissue microcirculation by hematoporphyrin derivative photoradiation observed in vivo in sandwich observation chambers. Cancer Res. 1986;46:2532–40.
49. Debefve E, Mithieux F, Perentes JY, Wang Y, Cheng C, Schaefer SC, Ruffieux C, Ballini JP, Gonzales M, van den Bergh H, Ris HB, Lehr HA, Krueger T. Leukocyte-endothelial cell interaction is necessary for photodynamic therapy induced vascular permeabilization. Lasers Surg Med. 2011;43:696–704.
50. Betz CS, Rauschning W, Stranadko EP, Riabov MV, Volgin VN, Albrecht V, Nifantiev NE, Hopper C. Long-term outcomes following Foscan [registered]-PDT of basal cell carcinomas. Lasers Surg Med. 2012;44:533–40.
51. Hasan T. Using cellular mechanisms to develop effective combinations of photodynamic therapy and targeted therapies. J Natl Compr Canc Netw. 2012;10(Suppl 2):S23–6.
52. Sattler UG, Mueller-Klieser W. The anti-oxidant capacity of tumor glycolysis. Int J Radiat Biol. 2009;85:963–71.
53. Frank J, Flaccs A, Schwarz C, Lambert C, Biealski HK. Ascorbic acid suppresses cell death in rat DS-sarcoma cancer cells induced by 5-aminolevulinic acid-based photodynamic therapy. Free Radic Biol Med. 2006;40:827–36.
54. Golab J, Nowis D, Skrzycki M, Czeczot H, Baranczyk-Kuzma A, Wilczynski GM, Makowski M, Mroz P, Kozar K, Kaminski R, Jalili A, Kopec M, Grzela T, Jakobisiak M. Antitumor effects of photodynamic are potentiated by 2-methoxyestradiol. A superoxide dismutase inhibitor. J Biol Chem. 2003;278:407–14.
55. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-kappa B control. Proc Natl Acad Sci U S A. 1997;94:10057–62.
56. Kasibhatla S, Genestier L, Green DR. Regulation of fas-ligand expression during activation-induced cell death in T lymphocytes via nuclear factor kappaB. J Biol Chem. 1999;274:987–92.
57. Matroule JY, Bonizzi G, Morliere P, Paillous N, Santus R, Bours V, Piette J. Pyropheophorbide-a methyl ester-mediated photosensitization activates transcription factor NF-kappaB through the interleukin-1 receptor-dependent signaling pathway. J Biol Chem. 1999;274:2988–3000.
58. Rapozzi V, Umezawa K, Xodo LE. Role of NF-κB/Snail/RKIP loop in the response of tumor cells to photodynamic therapy. Lasers Surg Med. 2011;43:575–85.
59. Almeida RD, Manadas BJ, Carvalho AP, Duarte CB. Intracellular signaling mechanisms in photodynamic therapy. Biochim Biophys Acta. 2004;1704:59–86.
60. Ferrario A, von Tiehl KF, Rucker N, Schwarz MA, Gill PS, Gomer CJ. Anti-angiogenic treatment enhances photodynamic therapy responsiveness in a mouse mammary carcinoma. Cancer Res. 2000;60:4066–9.
61. Kocanova S, Buytaert E, Matroule JY, Piette J, Golab J, de Witte P, Agostinis P. Induction of heme-oxygenase 1 requires the p38MAPK and PI3K pathways and suppresses apoptotic cell death following hypericin-mediated photodynamic therapy. Apoptosis. 2007;12:731–41.
62. Xue LY, He J, Oleinik NL. Rapid tyrosine phosphorylation of HIS in the response of mouse lymphoma L5178Y-R cells to photodynamic treatment sensitized by the phthalocyanine Pc 4. Photochem Photobiol. 1997;66:105–13.
63. Gomer CJ, RyterSW, Ferrario A, Rucker N, Wong S, Fisher AM. Photodynamic therapy-mediated oxidative stress can induce expression of heat shock proteins. Cancer Res. 1996;56:2355–60.
64. Liu H, Bowes RC 3rd, van de Water B, Sillence C, Nagelkerke JF, Stevens JL. Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca^{2+} disturbances, and cell death in renal epithelial cells. J Biol Chem. 1997;272:21751–9.

65. Assefa Z, Vantieghem A, Declercq W, Vandenabeele P, Vandenheede JR, Merlevede W, de Witte P, Agostinis P. The activation of the c-Jun N-terminal kinase and p38 mitofen-activated protein kinase signaling pathways protects HeLa cells from apoptosis following photodynamic therapy with hypericin. J Biol Chem. 1999;274:8788–96.
66. Tao J, Sanghera JS, Pelech SL, Wong G, Levy JG. Stimulation of stress-activated protein kinase and p38 HOG1 kinase in murine keratinocytes following photodynamic therapy with benzoporphyrin derivative. J Biol Chem. 1996;271:27107–15.
67. Klotz LO, Fritsch C, Briviba K, Tsacmmacidis N, Schliess F, Sies H. Activation of JNK and p38 but not ERK MAP kinases in human skin cells by 5-aminolevulinate-photodynamic therapy. Cancer Res. 1998;58:4297–300.
68. Klotz LO, Pellieux C, Briviba K, Pierlot C, Aubry JM, Sies H. Mitogen-activated protein kinase (p38-, JNK-, ERK-) activation pattern induced by extracellular and intracellular singlet oxygen and UVA. Eur J Biochem. 1999;260:917–22.
69. Xue L, He J, Oleinick NL. Promotion of photodynamic therapy-induced apoptosis by stress kinases. Cell Death Differ. 1999;6:855–64.
70. Briviba K, Klotz LO, Sies H. Toxic and signaling effects of photochemically or chemically generated singlet oxygen in biological systems. Biol Chem. 1997;378:1259–65.
71. Albini A, Tosetti F, Benelli R, Noonan DM. Tumor inflammatory angiogenesis and its chemoprevention. Cancer Res. 2005;65:10637–41.
72. Karin M. Cancer research in flames; tracking inflammation's role in promoting malignancy could lead to better treatments. Scientist. 2005;19:24–5.
73. Benelli R, Morini M, Carrozzino F, Ferrari N, Minghelli S, Santi L, Cassatella M, Noonan DM, Albini A. Neuthrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. FASEB J. 2002;16:267–9.
74. Gomer CJ, Ferrario A, Luna M, Rucker N, Wong S. Photodynamic therapy: combined modality approaches targeting the tumor microenvironment. Lasers Surg Med. 2006;38:516–21.
75. Korbelik M, Parkins CS, Shibuya H, Cecic I, Stratford MR, Chaplin DJ. Nitric oxide production by tumor tissue: impact on the response to photodynamic therapy. Br J Cancer. 2000;82:1835–43.
76. Reeves KJ, Reed MW, Brown NJ. Is nitric oxide important in photodynamic therapy? J Photochem Photobiol B. 2009;95:141–7.
77. Gupta S, Ahmad N, Mukhtar H. Involvment of nitric oxide during phthalocyanine (Pc4) photodynamic therapy-mediated apoptosis. Cancer Res. 1998;58:1785–8.
78. Gomes ER, Almeida RD, Carvalho AP, Duarte CB. Nitric oxide modulates tumor cell death induced by photodynamic therapy through a cGMP-dependent mechanism. Photochem Photobiol. 2002;76:423–30.
79. Bhowmick R, Girotti AW. Cytoprotective signaling associated with nitric oxide upregulation in tumor cells subjected to photodynamic therapy-like oxidative stress. Free Radic Biol Med. 2013;57:39–48.
80. Rapozzi V, Della Pietra E, Zorzet S, Zacchigna M, Bonavida B, Xodo LE. Nitric oxide-mediated activity in anti-cancer photodynamic therapy. Nitric Oxide. 2013;30:26–35.
81. Bhowmick R, Girotti AW. Pro-survival and pro-growth effects of stress-induced nitric oxide in a prostate cancer photodynamic therapy model. Cancer Lett. 2014;343:115–22.
82. Bhowmick R, Girotti AW. Signaling events in apoptotic photokilling of 5-aminolevulinic acid-treated tumor cells: inhibitory effects of nitric oxide. Free Radic Biol Med. 2009;47:731–40.
83. Hanlon JG, Adams K, Rainbow AJ, Gupta RS, Singh G. Induction of Hsp60 by photofrin-mediated photodynamic therapy. J Photochem Photobiol B. 2001;64:55–61.
84. Nonaka M, Ikeda H, Inokuchi T. Inibitory effect of heat shock protein 70 on apoptosis induced by photodynamic therapy in vitro. Photochem Photobiol. 2004;79:94–8.
85. Suschek CV, Krischel V, Bruch-Gerharz D, Berendji D, Krutmann J, Kröncke KD, Kolb-Bachofen V. Nitric oxide fully protects against UVA-induced apoptosis in tight correlation with Bcl-2 up-regulation. J Biol Chem. 1999;274:6130–7.

86. Ahmad N, Feyes DK, Agarwal R, Mukhtar H. Photodynamic therapy results in induction of WAF1/CIP1/P21 leading to cell cycle arrest and apoptosis. Proc Natl Acad Sci U S A. 1998:95:6977–82.
87. Ishida A, Sasaguri T, Kosaka C, Nojima H, Ogata J. Induction of the cyclin-dependent kinase inhibitor p21(Sdi/Cip1/Waf1) by nitric oxide-generating vasodilatator in vascular smooth muscle cells. J Biol Chem. 1997;272:10050–7.
88. Ho YS, Wang YJ, Lin JK. Induction of p53 and p21/WAF1/CIP1 expression by nitric oxide and their association with apoptosis in human cancer cells. Mol Carcinog. 1996;16:20–31.
89. Zhou J, Brüne B. NO and transcriptional regulation: from signaling to death. Toxicology. 2005;208:223–33.
90. Milla Sanabria L Rodriguez ME Cogno IS Rumie Vittar NB Pansa MF Lamberti MJ Rivarola VA. Direct and indirect photodynamic therapy effects on the cellular and molecular components of the tumor microenvironment. Biochim Biophys Acta. 2013;1835:36–45.
91. Bonavida B, Baritaki S. Dual role of NO donors in the reversal of tumor cell resi stance and EMT: downregulation of the NF-kB/Snail/YY1/RKIP circuitry. Nitric Oxide. 2011;24:1–7.
92. Korbelik M. Induction of tumor immunity by photodynamic therapy. J Clin Laser Med Surg. 1996;14:329–34.
93. Van Duijnhoven FH Aalbers RI Rovers JP Terpstra OT Kuppen PJ. The immunological consequences of photodynamic treatment of cancer, a literature review. Immunobiology. 2003;207:105–13.
94. Canti G., De Simone A, Korbelik M. Photodynamic therapy and immune system in experimental oncology. Photochem Photobiol Sci. 2002;1:79–80.
95. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M, Moan J, Peng Q. Photodynamic therapy. J Natl Cancer Inst. 1998;90:889–905.
96. Korbelik M. PDT-associated host response and its role in the therapy outcome. Lasers Surg Med. 2006;38:500–8.
97. Sun J, Cecic I, Parkins CS, Korbelik M. Neutrophils as inflammatory and immune effectors in photodynamic therapy-treated mouse SCCVII tumors. Photochem Photobiol Sci. 2002;1:690–5.
98. Gollnick SO, Evans SS, Baumann H, Owczarczak B, Maier P, Vaughan L, Wang WC, Unger E, Henderson BW. Role of cytokines in photodynamic therapy-induced local and systemic inflammation. Br J Cancer. 2003;88:1772–9.
99. Kousis PC, Henderson BW, Maier PG, Gollnick SO. Photodynamic therapy enhancement of antitumor immunity is regulated by neutrophils. Cancer Res. 2007;67:10501–10.
100. Hunt DW, Levy JG. Immunomodulatory aspects of photodynamic therapy. Expert Opin Investig Drugs. 1998;7:57–64.
101. Korbelik M, Cecic I. Contribution of myeloid and lymphoid host cells to the curative outcome of mouse sarcoma treatment by photodynamic therapy. Cancer Lett. 1999;137:91–8.
102. Abdel-Hady ES, Martin-Hirtsch P, Duggan-Keen M, Duggan-Keen M, Stern PL, Moore JV, Corbitt G, Kitchener HC, Hampson IN. Immunological and viral factors associated with the response of vulval intraepithelial neoplasia to photodynamic therapy. Cancer Res. 2011;61:192–6.
103. Henderson BW, Gollnick SO. Mechanistic principles of photodynamic therapy. In: Vo-Dinh T, Editor. Biomedical photonics handbook. Boca Raton: CRC Press; 2003. pp. 31–27.
104. Henderson BW, Gollnick SO, Snyder JW, Busch TM, Kousis PC, Cheney RT, Morgan J. Choice of oxygen-conserving treatment regimen determines the inflammatory response and outcome of photodynamic therapy of tumors. Cancer Res. 2004;64:2120–6.
105. Reis E, Sousa C. Activation of dendritic cells: translating innate into adaptive immunity. Curr Opin Immunol. 2004;16:21–5.
106. Sur BW, Nguyen P, Sun CH, Tromberg BJ, Nelson EL. Immunotherapy using PDT combined with rapid intratumoral dendritic cell injection. Photochem Photobiol. 2008;84:1257–64.
107. Paszko E, Ehrhardt C, Senge MO, Keller DT, Reynolds JV. Nanodrug applications in photodynamic therapy. Photodiagnosis Photodyn Ther. 2011;8:14–29.

108. Maeda H, Matsumura Y. EPR effect based drug design and clinical outlook for enhanced cancer chemotherapy. Adv Drug Deliv Rev. 2011;63:129–30.
109. Chatterjee DK, Fong LS, Zhang Y. Nanoparticles in photodynamic therapy: an emerging paradigm. Adv Drug Deliv Rev. 2008;60:1627–37.
110. Kozlowska D, Foran P, MacMahon P, Shelly MJ, Eustace S, O'Kennedy R. Molecular and magnetic resonance imaging: the value of immunoliposomes. Adv Drug Deliv Rev. 2009;61:1402–11.
111. Torchilin VP. Targeted pharmaceutical nanocarriers for cancer therapy and imaging. AAPS J. 2007;9:E128–47.
112. Chen B, Pogue WB, Hasan T. Liposomal delivery of photosensitizing agents. Expert Opin Drug Deliver. 2005;2:477–87.
113. Derycke AS, de Witte PA. Liposomes for photodynamic therapy. Adv Drug Deliv Rev. 2004;56:17–30.
114. Li B, Moriyama EH, Li F, Jarvi MT, Allen C, Wilson BC. Diblock copolymer micelles deliver hydrophobic protoporphyrin IX for photodynamic therapy. Photochem Photobiol. 2007;83:1505–12.
115. Van Nostrum CF. Polymeric micelles to deliver photosensitizers for photodynamic therapy. Adv Drug Deliv Rev. 2004;56:9–16.
116. Zhang GD, Harada A, Niushiyama N, Jiang DL, Koyama H, Aida T, Kataoka K. Polyion complex micelles entrapping cationic dendrimer porphyrin: effective photosensitizer for photodynamic therapy of cancer. J Control Release. 2003;93:141–50.
117. Nishiyama N, Morimoto Y, Jang W-D, Kataoka K. Design and development of dendrimer photosensitizer-incorporated polymeric micelles for enhanced photodynamic therapy. Adv Drug Deliv Rev. 2009;61:327–38.
118. Roy I, Chulchanskyy TY, Pudavar HE, Bergey EJ, Oseroff AR, Morgan J, Dougherty TJ, Prasad PN. Ceramic-based nanoparticles entrapping water-insoluble photosensitizing anticancer drugs: a novel drug-carrier system for photodynamic therapy. J Am Chem Soc. 2003;125:7860–5.
119. Hone DC, Walker PI, Evans-Gowing R, FitzGerald S, Beeby A, Chambrier I, Cook MJ, Russell DA. Generation of cytotoxic singlet oxygen via phthalocyanine-stabilized gold nanoparticles: a potential delivery vehicles for photodynamic therapy. Langmuir. 2002;18:2985–7.
120. Wieder ME, Hone DC, Cock MJ, Handsley MM, Gavrilovic J, Russel DA. Intracellular photodynamic therapy with photosensitizer-nanoparticle conjugates: cancer therapy using a Troian horse. Photochem Photobiol Sci. 2006;5:727–34.
121. Ricci-Junior E, Marchetti JM. Preparation, characterization, phototoxicity assay of PLGA nanoparticles containing zinc(II) phthalocyanine for photodynamic therapy use. J Microencapsul. 2006;23:523–38.
122. Zhu Z, Tang Z, Phillips JA, Yang R, Wang H, Tan H. Regulation of singlet oxygen generation using single-walled carbon nanotubes. J Am Chem Soc. 2008;130:10857–8.
123. Erbas S, Gorgulu A, Kocakusakogullari M, Akkaya EU. Non-covalent functionalized SWNTs as delivery agents for novel Bodipy-based potential PDT sensitizers. Chem Commun. 2009;4956–8.
124. Zhang XQ, Chen M, Lam R, Xu X, Osawa E, Ho D. Polymer-functionalized nanodiamond platforms as vehicles for gene delivery. ACS Nano. 2009;3:2609–16.
125. Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC. Nanoparticles in medicine: therapeutic applications and developments. Clin Pharmacol Ther. 2008;83:761–9.
126. Berg K, Prasmicakite L, Selbo PK, Hellum M, Bonsted, Hogset A. Photochemical Internalization (PCI)—novel technology for release of macromolecules from endocytic vesicles. Oftalmologia. 2003;56:67–71.
127. Berg K, Selbo PK, Prasmicakite L, Hogset A. Photochemial drug and gene delivery. Curr Opin Mol Ther. 2004;6:279–87.

128. Prasmickaite L, Hogset A, Berg K. Evaluation of different photosensitizers for use in photochemical gene transfection. Photochem Photobiol. 2001;73:388–95.
129. Hogset A, Ovstebo Engesaeter B, Prasmicakite L, Berg K, Fodstad O, Maelandsmo GM. Light-induced adenovirus gene transfer, an efficient and specific gene delivery technology for cancer gene therapy. Cancer Gene Ther. 2002;9:365–71.
130. Folini M, Berg K, Millo E, Villa R, Prasmickaite L, Daidone MG, Benatti U, Zaffaroni N. Photochemical internalization of a peptide nucleic acid targeting the catalytic subunit of human telomerase. Cancer Res. 2003;63:3490–4.
131. Selbo PK, Sandvig K, Kirveliene V, Berg K. Release of gelonin from endosomes and lysosomes to cytosol by photochemical internalization. Biochim Biophys Acta. 2000;1475:307–13.
132. Rai P, Mallidi S, Zheng X, Rahmanzadeh R, Mir Y, Elrington S, Khurshid A, Hasan T. Development and applications of photo-triggered theranostic agents. Adv Drug Deliv Rev. 2010;62:1094–124.
133. Menon JU, Jadeja P, Tambe P, Vu K, Yuan B, Nguyen KT. Nanomaterials for photo-based diagnostic and therapeutic applications. Theranostics. 2013;3:152–66.
134. Chen J, Liu TWB, Lo P-C, Wilson BC, Zheng G. "Zipper" molecular beacons: a generalized strategy to optimize the performance of activatable protease probes. Bioconj Chem. 2009;20:1836–42.
135. von Tappeiner H Jesionek A. Therapeutische versuche mit fluoreszierenden stoffen. Muench Med Wochenschr. 1904;47:2042–4.
136. von Tappeiner H. Zur kenntnis der lichtwirkenden stoffe. Dtsch Med Wocheschr. 1904;16:579–80.
137. Schwartz S, Absolon K, Vermund H. Some relationships of porphyrins, x-rays and tumors. Med Bull. 1955;27:7–13.
138. Lipson RL, Baldes EJ. The photodynamic properties of a particular hematoporphyrin derivative. Arch Dermatol. 1960;82:508–16.
139. Patrice T. Photodynamic therapy. In: Haeder D, Jori G, Editor. Cambridge: Royal Society of Chemistry; 2003.
140. Qiang YG, Yow CM, Huang Z. Combination of photodynamic therapy and immunomodulation: current status and future trends. Med Res Rev. 2008;28:632–44.
141. Bredell MG, Besic E, Maake C, Walt H. The application and challenges of clinical PD-PDT in the head and neck region: a short review. J Photochem Photobiol B. 2010;101:185–90.
142. Biel M. Advances in photodynamic therapy for the treatment of head and neck cancers. Lasers Surg Med. 2006;38:349–55.
143. Hopper C, Kubler A, Lewis H, Tan IB, Putnam G. mTHPC-mediated photodynamic therapy for early oral squamous cell carcinoma. Int J Cancer. 2004;111:138–46.
144. Sieron A, Namyslowski G, Misiolek M, Adamek M, Kawczyk-Krupka A. Photodynamic therapy of premalignant lesions and local recurrence of laryngeal and hypopharyngeal cancers. Eur Arch Otorhinolaryngol. 2001;258:349–52.
145. Cengel KA, Glatstein E, Hahn SM. Intraperitoneal photodynamic therapy. Cancer Treat Res. 2007;134:493–514.
146. Pinthus JH, Bogaards A, Weersink R, Wilson BC, Trachtenberg J. Photodynamic therapy for urological malignancies: past to current approaches. J Urol. 2006;175:1201–7.
147. Koudinova NV, Pinthus JH, Brandis A, Brenner O, Bendel P, Ramon J, Eshhar Z, Scherz A, Salomon Y. Photodynamic therapy with Pd-Bacteriopheophorbide (TOOKAD):successful in vivo treatment of human prostatic small cell carcinoma xenografts. Int J Cancer. 2003;104:782–9.
148. Kostron H. Photodynamic diagnosis and therapy and the brain. Methods Mol Biol. 2010;635:261–80.
149. Loewen GM, Pandey R, Bellnier D, Henderson B, Dougherty T. Endobronchial photodynamic therapy for lung cancer. Lasers Surg Med. 2006;38:364–70.

150. Stummer W, Novotny A, Stepp H, Goetz C, Bise K, Reulen HJ. Fluorescence-guided resection of glioblastoma multiforme by using 5-aminolevulinic acid-induced porphyrins: a prospective study in 52 consecutive patients. J Neurosurg. 2000;93:1003–13.
151. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease. Photochem Photobiol Sci. 2004;3:436–50.
152. Coppellotti O, Fabris C, Soncin M, Magaraggia M, Camerin M, Guidolin L, Jori G. Porphyrin-photosensitised processes in the prevention and treatment of water- and vector-borne diseases. Curr Med Chem. 2012;19:808–19.
153. Ibbotson SH. Topical 5-aminolevulinic acid photodynamic therapy for the treatment of skin conditions other than non-melanoma skin cancer. Br J Dermatol. 2002;146:178–88.
154. Bressler NM. Photodynamic therapy of subfoveal choroidal neovascularisation in age-related macular degeneration with verteporfin: two year results of two randomized clinical trials-tap report. Arch Ophthalmol. 2001;119(2):198–207.

Part II
Mechanistic Aspects Involved in Resistance to PDT

Chapter 2
Mechanisms of Resistance to Photodynamic Therapy: An Update

Adriana Casas, Christian Perotti, Gabriela Di Venosa and Alcira Batlle

Abstract Photodynamic Therapy (PDT) involves the combination of light and photosensitizer (PS). Therefore, it is possible for cells to develop resistance based on the doses of PS used or the light dose. The data compiled by several authors make it clear that the degree of cell resistance to PDT is highly dependent on the PS used; however, no cellular characteristics have yet been identified as predictors of PDT resistance. The mechanisms by which the treated tissue becomes resistant to the PS share some similarities to those found in general drug resistance and radio-resistance, and they are mainly related to both the bioavailability of the PS and to the mechanisms of detoxification of the generated reactive oxygen species. Among the features related to PDT resistance are: the expression of p-glycoprotein and ABCG2 transporters, the abrogation of apoptosis and autophagy, the induction of antioxidant defences, the induction of HSPs changes in cytoskeleton and adhesion, the induction of cyclooxygenases, the production of nitric oxide and hypoxia; these are some but not all of the factors involved in the development of resistance. As a general rule, all the authors that reported resistance to PDT have attributed this phenomenon to several factors acting in concert. In this chapter, we will review some of the most important aspects related to PDT resistance.

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Several approaches to reversing of PDT resistance have been developed. Among them, the use of PS linked macromolecules, which are internalized into cells via endocytosis and accumulated in the endosome/lysosome compartments, whereby they could efficiently be disrupted after irradiation by the mechanisms of photochemical internalization.

Keywords Apoptosis · Chemoresistance · Cross-resistance · Mechanisms · MDR · PDT · Photodynamic therapy · Photosensitizer · Porphyrins · Resistance

Abbreviations

ALA	5-aminolevulinic acid
BCRP	Breast cancer resistant protein
BPD-MA	Benzoporphyrin derivative monoacid ring A
CAM-DR	Cell adhesion-mediated drug resistance
CPO	[9-capronyloxy-tetrakis(methoxyethyl) porphycene]
DXR	Doxorubicin
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinases
EVs	Extracellular vesicles
FADD	Fas-associated via death domain
HIF1	Hypoxia-inducible factor 1
HO-1	Heme oxygenase 1
HSP	Heat shock proteins
HPPH	2-(1-hexyloxethyl)-2-devinyl pyropheophorbide-a
iNOS	inducible NO synthase
L-NAME	NG-Nitro-L-arginine methyl ester
LOX	Lipooxigenase
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MRP1	Multidrug resistant associated protein 1
m-THPC	5,10,15,20-tetra(*meta*-hyroxyphenyl) chlorin
m-THPP	meso-tetra(3-hydroxyphenyl)porphyrin
NO	Nitric oxide
NPe6	Mono-L-aspartyl chlorin e6
Pa-PDT	Pheophorbide a-PDT
PCI	Photochemical internalisation
P-gp	P-glycoprotein
PDT	Photodynamic therapy
PpIX	Protoporphyrin IX
PS	Photosensitizer

PII	Photofrin II
SnET2	Tin ethyl etiopurpurin I
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SPNO	Spermine NONOate
TOOKAD	Palladium-bacteriopheophorbide WST09
TPPS2a	Disulfonated meso-tetraphenylporphine
VEGF	Vascular endothelial growth factor

Introduction

The technique known as Photodynamic Therapy (PDT) uses a photosensitizer (PS) that can be applied either systemically or topically in a confined area, which upon excitation with visible light [1, 2] will react in the presence of oxygen to form cytotoxic oxygen species [3]. PDT involves the combination of light and photosensitizer, therefore, it is possible for cells to develop different levels of resistance based on the doses of PS used and/or the light dosage during treatment [4–7]

The mechanisms by which the treated tissue becomes resistant to the PS share some similarities to those found in general drug resistance, e.g. (i) differential uptake rate or efflux (ii) altered intracellular trafficking of the drug (iii) decreased drug activation, and (iv) increased inactivation of drug.

After PDT has been initiated, reactive oxygen species are produced [3], and consequently, antioxidant defense mechanisms are triggered to counteract the damage [8, 9]. As a result, Heat Shock Proteins (HSP) as well as other HSP-assisting proteins are activated [10]. In a later stage, increased repair of proteins, membranes and DNA, together with the induction of stress response genes and consequent activation of several survival pathways are also triggered [11].

Over the years, cell lines with different degrees of resistance to PDT have been isolated. Understanding the underlying mechanisms of the PDT resistance will help to improve the efficacy of PDT and can generate new combination protocols, such as chemo- or radiotherapy and PDT, for a better and a more efficient patient treatment.

In 1991, Luna and Gomer [6], using a fibrosarcoma cell line (RIF-1) and Photofrin II (PII), generated PDT-resistant cells using two protocols: short exposure (damage to the plasma membrane) and long exposure (damage to the organelles and enzymes). The levels of resistance obtained with both protocols were stable and increased cell survival by 2.5–3.0 logs and 1.2–1.5 logs, respectively, at the highest light dose used.

Using the same parental RIF-1 cell line, Singh et al. [5] were able to isolate and characterize two resistant cell lines based on the long exposure protocol (8 cycles of PII-PDT), obtaining what they called RIF-8A. These cells showed a similar degree of resistance (2.0 logs in survival). When they implanted the cells into mice, they

found PDT resistance *in vivo*. Moreover, the cells isolated from those tumors after PDT treatment also showed some degree of resistance *in vitro*; however, the resistance index was lower, suggesting a role of additional factors derived from both the host and the microenvironment.

Based on the afore mentioned studies and those from Mayhew et al. [4], it is clear that the degree of cellular PDT resistance was highly dependent on the PS used, but no cellular characteristics had been identified as predictors of PDT resistance in the generated clones. It is believed, however, that the chemical structure of the PS used is a key factor in the development of resistance, as the structure determines the intracellular accumulation of the PS [12–15].

In recent years, 5-aminolevulinic acid (ALA)-mediated PDT has become one of the most promising leads in PDT. ALA is the pro-drug of the PS Protoporphyrin IX (PpIX). Using ALA-PDT, we generated two resistant clones from a murine mammary adenocarcinoma cell line. The clones exhibited 6.7- and 4.2-folds increase in resistance, respectively, compared to the parental cell line. On the contrary, no evidence of PDT resistance was found in the response of human glioma spheroids to repetitive ALA-PDT [16] treatment, suggesting that resistance to PDT comprises a broad number of aspects, and not all cell types and cell models develop resistance to the same PS.

We have previously reviewed the main features of PDT resistant cells, and we compared them with features commonly found in chemoresistant cells. We found that many mechanisms of resistance to PDT are shared with chemotherapy and radiotherapy [17]. Contributing to PDT resistance are (1) the expression of p-glycoprotein and ABCG2 transporters (2) induction of early response genes and signal transduction pathways (3) abrogation of apoptosis and autophagy (4) induction of antioxidant defenses (5) HSPs induction (6) changes in cytoskeleton and adhesion (7) induction of cyclooxygenases (8) production of nitric oxide (9) survivin expression (10) and hypoxia. These are some, but not all, of the factors involved in the development of resistance. As a general rule, all the authors that have reported resistance to PDT have attributed this phenomenon to several factors acting in concert. In this chapter we will review some of the most important aspects related to PDT resistance.

Photosensitizer Uptake and Efflux in Resistant Cells

Results of recent studies suggest that PDT efficacy and/or resistance may depend on the uptake of PS [18] and alterations in the expression and function of key molecules involved in PS transport; these may be related to the emergence of resistance. PDT is also dependent on the cellular localization of the PII during treatment, and a key role has been given to the mitochondria. RIF-1 derivative resistant cells accumulated either slightly higher amounts of PS per cell compared to the parental cells with a lower amount of PS on a per mg protein basis [6] or similar amounts of PS but weaker co-localization with markers of the inner mitochondrial membrane

in the resistant variants. These findings suggesting that the inner mitochondrial membrane is a significant PII binding site and may be related to the mechanism of resistance [5, 19].

Our group also found that the amount of porphyrins accumulated per cell in ALA-PDT-resistant cells was similar to the parental cell line [7], but that the same amount was half of that found in the parental cell line protein content when expressed in a per mg basis. This means there is less available porphyrin to target the same amount of proteins [20]. If the amount of porphyrins and not the cell target molecules is the limiting factor in PDT damage, this feature can also induce resistance.

We have also reported alterations in the heme pathway that leads to a higher amount of hydrophilic porphyrins and lower amounts of PpIX in these ALA-PDT resistant cells. Since hydrophilic porphyrins such as coproporphyrin and uroporphyrin are known to have low efficacy as PSs, these factors could partly account for the development of resistance [21–23].

We have reported an observation of particular importance in ALA-PDT-based treatment—our resistance clones exhibit an increased number of mitochondria per cell. Since these organelles are responsible for the last step of ALA conversion into PpIX, it leaves no doubt about the key role of the mitochondria in PDT treatment [7]. In addition, PDT induces several forms of damage in the mitochondria, such as the inactivation of enzymes, uncoupling oxidative phosphorylation and the generation of toxic species, rapidly leading to apoptosis [24–29].

Sharkely et al. [30] also described the changes in the mitochondria found in their resistant clones. The organelles were smaller and more electron dense, with higher cristae density compared to the parental RIF-1 cell line. The total mitochondria area per cell in the resistant line was double that of the parental cell line, and the ATP content and succinate dehydrogenase activity were higher. However, the oxygen consumption rates were similar, suggesting an altered energy metabolism. On the other hand, the RIF-8A resistant cell line had a decreased mitochondrial potential.

In 1997, it was suggested that in the early stages of damage, the mitochondria protect themselves from oxidative stress by downregulating several mRNA and rRNA-encoding gene products [31]. Shen et al. found a reduction of the mitochondrial 16S rRNA and NADH dehydrogenase subunit 4 in the PDT-resistant variants of HT29 human colon adenocarcinoma [32]. While Singh et al. [33] found significant resistance to PDT in cell lines lacking mitochondrial DNA, resistance to alkylating agents or γ-irradiation, however, was not found; even though the mitochondrial function has been closely related to cell death by apoptosis [34], the resistance in the isolated lines was not due to changes in this mechanism.

In our ALA-PDT-resistant lines we found higher protein content, an increased number of mitochondria and a higher oxygen consumption rate [7]. However, when we normalized per ng protein content, the number of mitochondria was similar in both the resistant and the parental cell lines. Surprisingly, PpIX synthesis, which takes places in the mitochondria, was not increased in the resistant cells, suggesting some altered functions in these organelles.

The Role of P-glycoprotein in the Efflux of PS

It is not yet completely understood what causes the multidrug resistance (MDR) phenotype, and the lack of understanding remains a major problem in oncological treatments. The p-glycoprotein (P-gp or ABCB1) is encoded by the *MDR1* gene, and it is one of the ATP-binding cassette (ABC) drug transporters of the drug efflux pump class for a broad number of antineoplastics. It is also one of the main molecules involved in the development of MDR.

With respect to PDT, no overexpression of P-gp in RIF cells was found after multiple PDT treatments [5, 6]. In addition, similar doxorubicin (DXR) uptake was found when comparing RIF-8A to its parental cell line, and no cross-resistance to DXR was observed [5, 35]. However, cross-resistance to cisplatin treatment was observed [5, 35]. Additionally, our group found that the ALA-PDT resistant clones are not resistant to cisplatin, DXR, 5-fluorouracil, mitomycin C or methotrexate treatments.

PDT resistance in chemoresistant cell lines is also variable. The Chinese ovary hamster CHO-MDR line exhibited cross-resistance to PII-PDT, and this behaviour was correlated with a lower PII accumulation [5]. Similarly, cross-resistance to PDT was found in P388/ADR murine leukemia cells resistant to DXR, utilizing a cationic chlorin PS, and the impaired PS accumulation suggested a correlation between the resistance and the increased P-gp efflux pump activity [36]. However, other authors reported that the same murine leukemia P388/ADR cells were not cross-resistant to mesoporphyrin-PDT, suggesting the impairment is more related to the PS structure and its affinity for the P-gp [37].

The relationship between the MDR cell phenotype and the ALA-PDT response is not clear. Tsai et al. [38] found that MCF-7/DXR cells accumulated lower levels of PpIX from ALA, as compared to the parental MCF-7. However, the effect of ALA-PDT in MCF-7/DXR cells was less effective than in MCF-7 cells even when they showed similar amounts of PpIX. These results suggest that the resistant cells might possess intrinsic mechanisms that render them less sensitive to ALA-PDT, and their resistance is not related to the MDR efflux of PpIX.

Intracellular levels of ALA did not increase substantially upon incubation of the MCF-7 TX200 cells transfected with a P-gp inhibitor [39]. Similarly, Li et al. [40] showed that the P-gp inhibitor verapamil did not induce changes in PpIX levels in MDR-resistant leukemia cells exposed to ALA. More recently, Chu et al. [41] employing ALA derivatives, showed that the human uterine sarcoma cells MES-SA-Dx5 overexpressing P-gp exhibited reduced intracellular levels of PpIX derived from hexyl-ALA to a limited degree, and this mechanism could be reversed by using verapamil. Hexyl-ALA-PDT induced a decrease in MDR1 mRNA levels in MES-SA-Dx5 cells (resistant to DXR) together with a concomitant decreased expression of P-gp [41]. Similarly, pheophorbide-PDT of the multidrug-resistant HepG2 cells induced c-Jun N-terminal Kinase activation, leading to a down-regulation of P-gp [42].

It was also suggested that the intracellular localization of the PS has some influence on the MDR phenotype. Selbo et al. [43] found the MES-SA-Dx5 cells to be more resistant to PDT with disulfonated meso-tetraphenylporphine (TPPS2a) via a process not mediated by the P-gp mechanism. Their findings suggest the influence of different endocytic vesicle localization of the PS and that lysosomal targeting by PDT induces a stronger cytotoxic effect than PDT targeting the endosomes. According to Chu et al. [41] and Tang et al. [42], this finding may be related to an indirect down-regulation of MDR or a mechanism different from drug efflux.

Neither chlorin e6 accumulation nor efflux was modified in MCF-7/DXR overexpressing P-gp as compared to the parental line, but its subcellular distribution was different. In addition, a P-gp inhibitor restored the distribution of the PS and restored the response to chlorin e6-PDT [44].

In conclusion, it appears that MDR confers a degree of PDT resistance in some cases, and PDT resistance is strongly dependent on the structure of the PS and its affinity for the P-gp, as well as its intracellular distribution. However, no rules have yet been found to determine cross resistance.

Reversal of MDR by PDT

Sometimes, the MDR phenotype can be reversed by employing classic PDT. MCF-7 mammary carcinoma cells overexpressing P-gp or multidrug resistant-associated protein exhibited similar accumulation of chlorins, porphyrin-based PS and pheophorbides compared to the parental line [39], and the MCF-7/DXR, DXR resistant cells, were even more sensitive to meta-tetra(hydroxyphenyl)chlorin (m-THPC) [45].

Cheung et al. [46] showed that pheophorphide a-PDT was able to circumvent MDR in the P-gp overexpressing human uterine sarcoma MES-SA/Dx5 cells. Both intracellular accumulation of pheophorbide a and pheophorbide a-PDT-induced cell death were not abrogated by the MDR phenotype. Both activity and expression of MDR1 and P-gp were reduced by Pa-PDT treatment, and such reductions were attenuated by the ROS scavenger α-tocopherol. On the other hand, a higher light dose was needed to induce apoptosis in hexyl-ALA-PDT-treated MES-SA/Dx5 cells [47]. ALA–PDT also reversed the resistant phenotype of the cell lines LBR-D160 and LBR-V160 lines isolated from a murine T-cell lymphoid leukemia after increasing vincristine or DXR exposure [48].

In addition, alternatives to PDT have been developed to overcome chemoresistance. Palladium-bacteriopheophorbide WST09 (TOOKAD) is a new generation of hydrosoluble PS, which binds primarily to albumin and has a local effect in the vasculature, since it does not extravasate and remains constrained in the circulation. The effect of TOOKAD-PDT in HT29/MDR cells as well as derived xenografts shows that this therapy overcomes drug resistance [49].

Photochemical internalization (PCI) has been shown to help overcome chemoresistance in several MDR cell lines employing several PS and anticancer drugs [43,

50, 51]. PCI is a novel technology for the release of endocytosed macromolecules into the cytosol. Both PS and cytotoxic agents are localized in the same endocytic vesicle and internalized together into the target cell, where the PS is specifically localized to the vesicular membrane, and upon activation of PS by light, induces a release of the endocyosed macromolecules from their compartmentalization [52].

One mechanism for MDR is increased acidification of endocytic vesicles and increased cytosolic pH, so weak base chemotherapeutic agents, including DXR, are trapped in endocytic vesicles and exhibit a drug resistant phenotype. In MCF-7/ADR [50] cells that were preloaded with DXR, after PCI treatment the drug was released into the cytosol and entered cell nuclei, to the same extent than non-treated MCF-7 cells, thus, reversing the MDR phenotype by endo-lysosomal release of the drug.

Selbo et al. [43] also evaluated the reversal of resistance induced by PCI of macromolecules that were not targets of ABC drug pumps. Both MES-SA and MES-SA-Dx5 cell lines were equally sensitive to PCI of gelonin (ribosome-inactivating protein) using a low light dosage, even though the endocytosis rates were lower in the MDR cells. When higher light doses were employed MES-SA/Dx5 cells were more sensitive to PCI of gelonin than the parental cells. After adenoviral infection, PCI enhanced the fraction of transduced cells, in both cell lines, suggesting the potential use of PCI of macromolecular therapeutic agents that are not targets of P-gp as a strategy to eradicate MDR cancer cells. This PCI-mediated reversal of resistance circumvents ROS-induced photodamage in an apoptosis-independent manner [53].

In addition, some other approaches to reverse chemoresistance employing PDT utilize nanomedicinal tools to deliver the PS more efficiently and reduce the chances of being pumped out from the cytoplasm. In this regard, nanoparticle-based therapy integrative systems represent an emerging approach to overcome resistance. Ling et al. [54] recently reported the generation of tumor pH-sensitive magnetic nanogrenades (termed PMNs) composed of self-assembled iron oxide nanoparticles and pH-responsive ligands. These PMNs can readily target tumors via surface-charge switching triggered by the acidic tumor microenvironment, and are further disassembled into a highly active state in acidic subcellular compartments that “turns on” photodynamic therapeutic activity. These PMNs localized with high selectivity in mice tumors enabling pH-dependent PDT, and induced an increased therapeutic efficacy in drug-resistant tumors, showing a great potential for clinical applications.

ABCG2-mediated Efflux of PS and Its Role in Cross Resistance

Another ABC transporter found to be capable of inducing resistance, in addition to P-gp, is a novel member of the G subfamily and has been described in the breast cancer cell line MCF7/AdrVp. This transporter was named the breast cancer resistance protein (BCRP), and is currently referred to as ABCG2 [55, 56]. Like all

members of the ABCG subfamily, ABCG2 is a half transporter, and it is believed to function as a mechanism of defense against toxins, regulating the traffic of toxic metabolites from and into the organism [57]. Among the drugs that can be effluxed by ABCG2 are mitoxantrone, camptothecin-derived and indolocarbazole topoisomerase I inhibitors, methotrexate, flavopiridol, and quinazoline ErbB1 inhibitors [58].

Several photosensitizers have been reported to be substrates of ABCG2 [59], including ALA-induced PpIX [13, 14], Pheophorbide a [13, 15], chlorin e6 [60], pyropheophorbide-a methyl ester [60], 2-(1-hexyloxethyl)-2-devinyl pyropheophorbide-a (HPPH) [12, 16], benzoporphyrin derivative monoacid ring A (BPD-MA) [12] and Hypericin [61]. On the contrary, meso-tetra(3-hydroxyphenyl)porphyrin (m-THPP), m-THPC [15, 60], hematoporphyrin IX [60] and HPPH-galactose [12, 16] TPPS(2a), di-sulfonated meso-tetraphenylchlorin (TPCS(2a)) and di-sulfonated aluminium phtalocyanine (AlPcS(2a)) are not substrates of ABCG2.

Using an ABCG2 knockout mouse model, Jonker et al. demonstrated that this transporter is able to efflux PpIX and protect cells from photodamage [62]. A correlation was also demonstrated between the expression of ABCG2 and resistance to PII-PDT [63]. Robey et al. [64] suggested that this transporter may be involved in the development of PDT resistance. In their studies, they compared the ability of ABCG2 to transport pheophorbide a and other PS with a similar structure. Bronchoalveolar carcinoma cells (NCI-H1650 MX50) overexpressing the transporter were found to have reduced intracellular accumulation of pyropheophorbide a methyl ester, chlorin e6 and PpIX, while the intracellular accumulation of hematoporphyrin IX, m-THPP, and m-THPC was not altered [39]. Afterwards, in line with the above mentioned findings, when human embryonic kidney HEK-293 cells were transfected with ABCG2, they exhibited resistance to PDT employing Chlorin e6, pheophorbide a, pyropheophorbide a methyl ester and ALA but not to m-THPC.

Multidrug resistance is suggested to be an important mechanism for the survival of cancer stem cells during therapy [65]. ABCG2 is a putative cancer stem cells marker and the molecular determinant of the Hoechst 33342 side population phenotype, which is detected as a distinct cell population with low blue and red fluorescence in flow cytometry dot blots [66]. Selbo et al. [59] demonstrated that strongly amphiphilic PSs used for PCI-based drug delivery are not substrates of ABCG2. They employed the breast carcinoma cell line MA11, with a Hoechst 33342 side population of $>50\%$ as an ABCG2 high expression model. Pheophorbide a and Hoechst 33342 were used as positive control substrates of ABCG2. ABCG2-inhibition by fumitremorgin C did neither induce an increased accumulation of three PCI-photosensitizers: TPPS(2a), TPCS(2a) and AlPcS(2a) nor enhanced the response of the cells to PDT. The same results were also obtained with TPPS2a in the malignant glioma cell line U87 having a SP of $\sim 0.1\%$. In contrast, both uptake and PDT-induced cytotoxicity was strongly enhanced for Pheophorbide a when combined with fumitremorgin C. On the other hand, EGFR + /ABCG2 + MA11 cells exposed to PCI employing the targeting toxin EGF-saporin were responsive to the treatment [59].

It has been suggested that the expression of ABCG2 transporter is decreased in a variety of cancers. Analyzing a set of normal and cancer paired tissues, Gupta et al.

[67] found that human samples from colorectal and cervical cancers had a down-regulation of ABCG2 mRNA levels.

Using a variety of human and mouse cell lines with a range in the expression of ABCG2 transporter, Liu et al. [68] studied the effect of tyrosine kinase inhibitors, which are able to block the ABCG2 function. The set of cell lines employed included: BCC-1 cells (basal cell carcinoma) and RIF-1 fibrosarcoma cells with high ABCG2 expression, Colo 26 (colon carcinoma) with moderate expression and FaDU (head and neck squamous cell carcinoma) with no expression of the transporter. In ABG2-expressing cells, an efficient efflux of HPPH, BPD-MA and PpIX was observed, while PII and HPPH-galactose were minimally transported. Treatment with a tyrosine kinase inhibitor increased the accumulation of the PS BPD-MA, PpIX, and HPPH in ABCG2 expressing cells, but not in the non-expressing cells. It also enhanced PDT efficacy both *in vitro* and *in vivo* in a RIF-1 tumor model. These results demonstrate on one hand that the use of ABCG2 inhibitors may increase the efficacy of clinical PDT, and on the other hand they show the importance of the PS structure and the affinity for the transporter in the development of resistance.

Although Robey et al. [39] suggested that Photofrin was not a ABCG2 transporter, Usuda et al. [69] using A431 cells overexpressing ABCG2 found they were resistant to PII-PDT but not to Mono-L-aspartyl chlorin e6 (NPe6)-PDT, which is similar in structure to m-THPC, and resistance to PII-PDT was reversed using a non-tyrosine kinase inhibitor of ABCG2 (Fumitremorgin C). In addition, in 81 centrally located early lung cancer lesions, > or = 10 mm in diameter, the expression of ABCG2 was inversely correlated with the outcome of PII-PDT while the transporter expression did not seem to affect the antitumor effect of NPe6-PDT.

Curiously, the modulation of ABC transporters by a PS has also been reported. Using HT-29 colon cancer cells treated with hypericin but no light, an increase in the activity of ABCG2 and the multidrug-resistant associated protein 1 (MRP1) transporters was reported [70].

Hypoxia regions are very commonly present in tumors and this oxygen depletion has been found to up-regulate the expression of ABCG2. It can also promote cell survival by decreasing the intracellular accumulation of porphyrins and heme [71]. Since it is well known that PDT requires the presence of oxygen to achieve its effect, hypoxia may inhibit PDT-depleting oxygen but also increase the ABCG2 transporter, which diminishes the intracellular levels of PS.

In summary, the resistance conferred by ABCG2 and P-gp transporters is directly related to the cell line and the PS employed, and in some cases, the resistant phenotype can be reversed by using specific transporter inhibitors.

Recently, Goler-Baron and Assaraf [72] identified a novel mechanism of MDR in which ABCG2-rich extracellular vesicles (EVs) form in between attached neighbor breast cancer cells and highly concentrate various chemotherapeutics in an ABCG2-dependent manner, thereby sequestering them away from their intracellular targets. These authors also showed that illumination of EVs that accumulated photosensitive cytotoxic drugs such as imidazoacridinones and topotecan resulted in intravesicular formation of ROS and severe damage to the membranes in the

vicinity of EVs leading to cell death and overcoming of resistance. Furthermore, consistent with the weak base nature of imidazoacridinones, MDR cells that are devoid of EVs but contained an increased number of lysosomes were efficiently killed via photodynamic lysosomal rupture. Combining targeted lysis of imidazoacridinone-loaded EVs and lysosomes elicited a synergistic cytotoxic effect resulting in the reversal of MDR. In contrast, topotecan, a substrate of ABCG2, accumulated exclusively in EVs of MDR cells but not in non-MDR breast cancer cells. This exclusive accumulation in EVs enhanced the selectivity of the cytotoxic effect exerted by photodynamic therapy to MDR cells without harming normal cells.

Taking advantage of the increased number of lysosomes in MDR cells, the same group [73] developed an imidazoacridinones-based PDT approach to eliminate MDR cancer cells. MDR cells overexpressing the MDR efflux transporters ABCG2, ABCB1 or ABCC1 and their normal counterparts were used: non-small-cell lung cancer A549 cells and their ABCG2-overexpressing A549/K1.5 subline, ovarian carcinoma 2008 cells and their stable MRP1 transfectant 2008/MRP1, and non-small lung cancer cell line SW1573 and its ABCB1-overexpressing subline SW1573/2R160. PDT resulted in 10- to 52-fold lower IC-50 values of various imidazoacridinones, thereby, restoring parental cell sensitivity. Finally, *in vivo* application of PDT-imidazoacridinone to human ovarian tumor xenografts in the chorioallantoic membrane model revealed selective destruction of tumors and their associated vasculature after two consecutive treatments.

Apoptosis and Resistance to PDT and its Relationship with PS Structure

As a result of apoptosis induction by different stimuli, a series of signaling pathways are activated. As it has been previously addressed, PDT is known to up-regulate several signaling pathways, some of which on one hand may act as mediators or promoters of apoptosis as a result of the PDT treatment. On the other hand, some pathways are related to promote damage repair [74] and, therefore, could be linked to the development of resistance. In this regard, there are controversial evidences as some studies correlated the presence of apoptosis with the appearance of resistance, whereas in a few cases, altered apoptosis pathways have been found in cells resistant to PDT.

There have been different studies that linked the type of cell death and the PS subcellular localization [29]. Therapy can induce photodamage in the different organelles or in the plasma membrane. It has been demonstrated that anti-apoptotic proteins of the Bcl-2 family were targeted by PDT if the PS was localized in the endoplasmic reticulum (ER) or the mitochondria. On the other hand, when the plasma membrane was targeted by PDT, inhibition or delayed apoptosis and rescue responses were triggered [28, 75].

The subcellular localization of the PS varies according to its chemical nature and the molecular structure. Due to the hydrophobic nature of most PS they tend

to bind to membranes, e.g., ER, mitochondria, Golgi, lysosomes and plasma membrane [76]. Kessel et al. [77] evaluated the PDT responses to two structurally-related photosensitizing agents, using P388 murine leukemia cells. The PS tin etiopurpurin (SnET2) induced photodamage in lysosomes and mitochondria yielding a rapid apoptotic response. Using the analog tin octaethylpurpurin amidine (SnOPA), which targeted lysosomes, mitochondria and cell membranes yielded apoptotic evidence only after 24 h post PDT, suggesting photodamage to the membranes can delay or prevent an apoptotic response to PDT, thus resulting in resistance to PDT.

Along the same lines, Dellinger et al. [78] reported that PDT of cells exposed for a short period to high concentrations of PII exhibited an aborted form of apoptosis and attributed this to the leakage of cytoplasmic content through photodamaged membranes.

The Kessel group found that SnOPA and a monocationic porphyrin relocalized to the cytosol during irradiation, and this feature was correlated to delay or inhibition of apoptosis [75]. Employing m-THPC, SnET2 and 9-capronyloxy-tetrakis (methyoxyethyl) porphycene (CPO) in fluorescence localization studies, this group was also able to show that the PSs that theoretically targeted mitochondria, were also able to bind to a variety of intracellular membranes. These PSs generated damage through apoptosis, affected Bcl-2 levels, but did not affect the pro-apoptotic protein Bax. According to the accumulation site, the apoptotic response was induced soon after treatment with m-THPC and CPO, while the response was delayed when treated with SnET2 (associated with lysosomal photodamage) [79].

Utilizing positively charged porphyrins, Kessel et al. [80] showed that the position of the charge influenced the intracellular localization sites. The PS (5,10-di[4-(N-trimethylaminophenyl)-15,20-diphenylporphyrin) can penetrate the plasma membrane easily and target mitochondria, resulting in a rapid loss of membrane potential and triggering apoptosis. On the other hand, 5,15-di[4-(N-trimethylaminophenyl)-10,20-diphenylporphyrin with a different charge distribution targets lysosomes instead, resulting in extensive photodamage but with less effectiveness than the former pathway.

The same group using NPe6, which is an amphiphillic PS that targets the endosomal/lysosomal membranes, demonstrated that it is possible to induce indirect activation of apoptosis. Upon damage to these vesicle membranes, there is a leakage of enzymes that mediate the activation of the apoptotic lysosomal pathway involving the release of cathepsin B and cleavage of Bid which, in turn, can interact with the mitochondria leading to the release of cytochrome c and the activation of caspases -3 and -9 [81].

In addition to apoptosis, autophagy can also occur after PDT, especially when low light doses are applied [82]. This process acts as a pro-survival response due to recycling of damaged organelles, offering protection from photodamage [83, 84]. The role of authopagy in PDT resistance will be addressed in another chapter of this book.

Activation of Caspases and Expression of Apoptotic and Anti-apoptotic Proteins in PDT Resistant Cells

Almeida et al. [85] analyzed the intracellular signaling mechanisms in PDT. They found that there are two major apoptotic pathways, both of which involved the activation of caspase 8 or 9 (initiators) leading to the activation of caspases 3, 6 and 7 (effector caspases). In addition, the lysosomal pathway is a prelude for the mitochondria-mediated apoptosis after lysosomal photodamage [81].

As mentioned before, monocationic PS such as monocationic porphyrin localize to the plasma membrane, and during the first minutes of irradiation, relocalized to the cytosol [75]. With longer periods of irradiation, procaspases -3 and -9 are affected by photodamage, preventing an apoptotic response. Although this is not a general rule for all the PS that initially bind to the plasma membrane, these results indicate that the absence of apoptosis can result from photodamage to keypoints of the apoptotic program.

There were no changes in caspase 8 RNA expression levels according to studies carried by Wild et al. [86] where they analyzed 3 cells lines: a normal cell line (UROtsa, urothelial) and two tumor cell lines (RT4, urothelial; HT29, colonic) following ALA-PDT. Although there were no changes at the RNA level, they found a delay in the activation of caspase 8 only in the normal cells, suggesting that this caspase pathway activation might be a secondary way to ensure photodamage. According to caspase -8, the active caspase 3 fragment was found only in the normal urothelial cell line 1 h after photodynamic therapy. Combined data analyses suggest that photodynamic therapy *in vitro* leads to apoptosis in UROtsa and to necrosis in the tumor cell lines, respectively. The activation of caspases 8, 3, 6 and 7 was found in HeLa cells treated with BPD-MA-PDT [87].

Ruhdorfer et al. [11] carried out gene expression analysis in squamous cell carcinoma A-431 after ALA-PDT and found that the product of the '*Fas-associated via death domain*' (FADD) gene was strongly induced. FADD is an adaptor molecule, which via its death effector domain (DED) engages the DEDs of procaspase 8, which will become caspase 8 and triggering the apoptotic pathway.

It has recently been published that the anti-apoptotic protein Mcl-1 plays an important role in protecting cells from PDT-induced apoptosis. In contrast to the reduction in the anti-apoptotic proteins Bcl-2 and Bcl-xl, sub-lethal PDT induces an increase in Mcl-1 expression. Silencing Mcl-1 sensitizes tumor cells to PDT-induced apoptosis, and ectopic expression of Mcl-1 significantly delays Bax translocation to the mitochondria and inhibits caspase3 activity following PDT [88]. Mcl-1 expression is associated closely with the activated AKT signaling following PDT. Treatment with Celecoxib, a non-steroidal anti-inflammatory drug, downregulated Mcl-1 expression, and enhanced PDT-induced apoptosis both *in vitro* and *in vivo*. This down-regulation is closely related to the inhibition effect of Celecoxib on the AKT/GSK-3β pathway, and was blocked upon addition of the GSK-3β inhibitor LiCl or the proteasome inhibitor MG132. In addition, a loss in Mcl-1 by inhibiting AKT promoted PDT-induced apoptosis through the mitochondrial pathway [88].

It was reported that it is possible to circumvent PDT resistance in an apoptosis-independent manner. Since it was found that PCI of gelonin was more effective in the PDT-resistant MES-SA/Dx5 cells as compared to the normal lines, death-inducing signaling was studied. A low activation of caspase3 and a strong PARP I cleavage after PCI occurred in both cell lines. The PARP I activation was, however, stronger after PCI than after PDT in the MES-SA cells, but not in the MES-SA/Dx5 cells and, therefore, cannot explain the strong PCI effect in the MES-SA/Dx5 cells [53].

The Bcl-2 family is a large family of proteins that play a key role in the apoptotic pathway. It is composed of anti-apoptotic members such as Bcl-2, CED-9 and Bcl-XL, and pro-apoptotic members such as Bak, Bax, BNIP3, as well as the BH3-only subfamily (Bik, Blk, Hrk, BimL, Bad, Bid) [89, 90].

Since Bcl-2 proteins are frequently activated after PDT-treatment [91, 92], these proteins may be involved in the development of cell resistance. Upon PDT, when photodamage targets the mitochondria and/or the endoplasmic reticulum, the anti-apoptotic protein Bcl-2 is affected, while lysosomal photodamage results in the activation of pro-apoptotic protein Bid, also leading to apoptosis [29].

Shen et al. [32] employed PDT-resistant HT29 cells to perform gene expression analysis of apoptosis- related genes. This study showed an increased expression of Bcl-2 and heat shock protein 27 as well as a decreased expression of Bax in the PDT resistant cells. However, mRNA and protein expression levels for the propaptotic BNIP3 were increased. The same group reported that these resistant cells showed a significant increase in cisplatin sensitivity that correlated with the increased BNIP3 and decreased mutant p53 protein levels [93].

HL60 cells transfected with Bcl-2 were able to suppress apoptosis after BPD-MA-PDT treatment [91]. They also found that overexpression of Bcl-2 or Bcl-XL did not avoid apoptosis by preventing the release of cytochrome C but instead by blocking the activation of caspases [87]. Also, CHO cells overexpressing Bcl-2 showed a 2-fold increase of PDT-resistance compared to the parental cells [94, 95]. In addition, human gastric adenocarcinoma MGC803 and A 431epidermoid carcinoma cells transfected with the Bcl-2 antisense reversed the resistance to PDT [92, 96].

There is, however, some controversy regarding the use of Bcl-2 expression as a predictor for PDT response. Different groups have used PDT-treated tumor biopsies to try to correlate Bcl-2 expression and the outcome of the treatment. Kawaguchi et al. [97] found no correlation between Bcl-2 or p53 expression and local recurrence after PDT in squamous cell carcinomas of the bronchus. McGarrity et al. [98] reported that variable changes in Bcl-2 and p53 immunoreactivity were noted in normal and carcinoma tissues biopsies of esophageal tumors treated with PII-PDT, whereas Koukourakis et al. suggested that Bcl-2 expression is associated with a favorable outcome of PDT, which could be due to the selective degradation of Bcl-2 induced by PDT, leading to apoptosis by decreasing the Bcl-2/Bax ratio [99]. In this context, several groups have shown in different cell lines, PDT-induced damage to Bcl-2.

Human breast cancer MCF-7c3 cells expressing procaspase-3, known to respond to PDT via apoptosis, were transfected with wild-type Bcl-2 or Bcl-2 deletion mutants lacking the anchorage region to the membrane, resulting in a relative resistance to Pc 4-PDT. These results suggested that the deleted regions, which include a caspase-3 cleavage site, are not necessary for the inhibition of PDT-induced apoptosis. On the other hand, a C-terminal truncated Bcl-2 mutant provided no protection, indicating that the degree of Bcl-2 photodamage determines the level of sensitivity of the cancer cells to apoptosis in response to PDT. In addition, overexpression of Bcl-2 in MCF-7c3 cells inhibited the activation-associated conformational change of the pro-apoptotic protein Bax, consequently leading to a requirement of higher light doses to activate Bax [100].

Employing the same concept, Chiu et al. [101] reported studies of the role of Bax in apoptosis and cell killing caused by PDT. MCF-7c3 cells treated with antisense Bax yielded a 50% inhibition of PDT-induced apoptosis. Similarly, in a human prostate cancer cell line DU-145, which does not express Bax, apoptosis was completely blocked after Pc 4-PDT treatment, but despite the lacking of Bax the cells were equally sensitive to PDT. Since cells deficient in Bax remain as photosensitive as Bax-proficient cells, the authors concluded that the commitment to cell death is likely determined before the step of Bax activation and cytochrome c release or is independent of them.

As mentioned before, Usuda et al. [102] showed that PII-PDT can induce damage to Bcl-2 and, therefore, apoptosis. Employing different PS targeting different organelles showed that targeting the mitochondria, as PII-PDT, is more effective in inducing apoptosis and Bcl-2 photodamage, compared to a lysosomal target agent such as NPe6-PDT. The latest PS showed no damage to Bcl-2 and a delayed apoptosis. Also, Bcl-2 overexpressing cells were considerably more resistant to NPe6-PDT than the parental MCF-7c3 cells.

Using ATX-s10, a PS which localizes to mitochondria and lysosomes, it was reported that overexpression of wild-type Bcl-2 conferred relative resistance to MCF-7 cells to PDT. Inhibition of lysosomal cathepsins B and D protected MCF-7c3 cells from apoptosis induced by ATX-s10-PDT. This demonstrated that lysosomal photodamage can initiate an apoptotic response and this apoptotic pathway can be regulated by photodamage to Bcl-2 via the mitochondria [103] Using the Tao variant of 1c1c.7 murine hepatoma cells having lysosomal fragility, apoptosis was delayed and diminished upon exposure to NPe6-PDT, thus exhibiting resistance to the treatment [104].

The tumor suppressor P53 is the most frequently mutated gene in human tumors. Increased P53 expression affects its target genes expression and leads to cell cycle arrest or apoptotic cell death depending on the cell type and context [105, 106]. P53-deficient cells are often less responsive to chemotherapeutics, and this implies that it could also be responsible for PDT resistance. However, no conclusive reports are available to show that the down-regulation of this protein may induce resistance to PDT. On the other hand, cells lacking functional p53 fail to undergo apoptosis,resulting in continued proliferation.

Using hypocrellin as PS it was found that wild-type p53 transfected-HT29 human colorectal carcinoma cells were 2-fold sensitive to PDT than the parentals [96]. In the same trend, human promyelocytic leukemia HL60 cells which express the wild type p53 were also more sensitive to PDT, with both PII and tin ethyl etiopurpurin I (SnET2), compared to cells presenting deletions or mutations in P53. The human colon carcinoma cell line LS513 which expresses wild-type p53 was also more sensitive to PII-PDT compared to the mutated P53 counterpart [107]. In every case, the cell lines underwent rapid apoptosis in response to PDT [108]. Immortalized fibroblasts derived from Li-Fraumeni syndrome patients, that only have one p53 allele and is mutated, were less sensitive to PII-PDT compared to normal fibroblasts [109]. On the other hand, expression of the oncoprotein E6 which suppresses p53 function did not alter the response to PDT in LS513 and MCF-7 cells [109]. However, in spite of all the evidence involving p53 on photodynamic sensitivity, there is yet no conclusive association of p53 with the PDT response [97, 99].

An interesting and unexpected finding was that PpIX interacts with wild-type p53 *in vitro* and induces cell death of colon cancer cells in a p53-dependent and independent manners. PpIX might directly target p53 and stabilize it in human colon cancer cells HCT116 probably by disrupting the p53/MDM2 complex. This might lead to p53-dependent cell death via p53-regulated apoptosis in the dark, prior to irradiation and also upon irradiation [110].

Cellular Antioxidant Defense Mechanisms

It has been shown that the antioxidant defense mechanisms of the cell such as superoxide dismutases (SOD), the glutathione system and catalase or lipoamide dehydrogenase, antagonized the effects of PDT [111–114].

MCF-7 cells could be protected from PDT damage by transfecting them with the glutathione peroxidase gene, which helps with the removal of lipid hydroperoxides in living cells after $^1O_2$ exposure. In addition, it was found that human kidney 293 cells over-expressing glutathione S-transferase P1-1 had a reduced phototoxicity induced by Hypericin-PDT, by reversion of downregulation of the nutrient-sensing protein kinase mTOR and blocking apoptosis [115].

The glutathione system has been involved in chemoresistance [116]. In addition, a few authors reported that detoxification by glutathione conjugation was correlated with PDT resistance. Luna & Gomer [6] found an increase in the levels of reduced glutathione in their resistant cells RIF, but no alterations in the levels of glutathione peroxidase or superoxide. However, Singh et al. found no differences in glutathione levels in the RIF-8A resistant variant [35]. Our clones resistant to ALA-PDT [7] showed that the reduced glutathione content expressed on the basis of cell number increased two-fold, however, no differences were observed when expressed in per μg of protein, thus being difficult to evaluate the role of GSH detoxification in this system due to the different protein content in the resistant lines. However, the GSH:

porphyrins are higher in the resistant clones, suggesting an increased detoxifying activity per molecule of PS.

Mikesova et al. [117] compared the sensitivity of six colon-derived cancer cell lines to Hypericin-PDT, revealing a whole spectrum of responses from insignificant to high cytotoxicity. It was found that the cell line sensitivity was partially but not directly related to the intracellular Hypericin content, glutathione level or redox status, demonstrating partial but not direct correlation with resistance to PDT when considered separately but combination of these parameters are responsible for photocytotoxicity, thus reinforcing our theory of multifactorial features in the development of PDT resistance.

MES-SA/Dx5 cells resistant to DXR are cross-resistant to PDT. A DXR-induced increased expression of the ROS-scavenging proteins glutathione peroxidase GPx1 and GPx4 in MES-SA/Dx5 cells was indicated as the mechanism of resistance to PDT, in line with the reduction in PDT-generated ROS observed in this cell line. The MES-SA/Dx5 cells were also cross-resistant to ionizing radiation in agreement with the increased GPx1 and GPx4 expression [53].

In cancer cells resistant to DXR, overexpression of Glutathione transferase pi (GSTP1) is also suggested to influence the cellular redox status through the suppression of DXR conversion to semiquinone free radical and the subsequent production of ROS. Thus, the overexpression of GSTP1 is related to the development of drug resistance in cancer cells not only by increased detoxification of anticancer agents, but also by suppression of cellular ROS which induce cell death [118].

In the colon adenocarcinoma cell line LoVoDX resistant to DXR, oxidative changes induced by Photofrin-PDT were delayed in comparison to its DXR-sensitive counterpart LoVo. The expressions of GSTP1, a marker protein for photochemical toxicity, and secretory phospholipase A(2), a prognostic and diagnostic marker for colon cancers, were increased in both cell types after PDT. Increased SOD1 activity and TBARS levels in both cell lines, together with a decrease of protein-associated -SH groups were also evidenced. The increasing level of ROS following the oxidation of sulfhydryl cell groups and lipid peroxidation influence the activity of many transporters and enzymes [119].

Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, and their action is relevant to photodamage [120, 121]. The expression of the isoform SOD2 was found to be differentially regulated by ALA-PDT. Up-regulation was found in the urothelial RT4 tumor cells, not affected in the colonic HT29 tumor cell line and slightly down-regulated in the normal urothelial cells UROtsa [86] after ALA-PDT treatment.

Heat Shock Proteins in the Response to Photodamage

It is known that heat, chemical exposure, oxidation and PDT affect the expression of heat shock proteins (HSPs), which in turn are associated with modulation of cellular damage [9, 122]. HSP27, HSP34, HSP60, HSP70, HSP90, HSP110 [123, 124],

glucose regulated proteins GRPs (GRP74, GRP78, GRP94 and GRP100) [125] and heme oxygenase (HO-1) [126] have also been involved in protecting the cells from photodamage.

Luna & Gomer showed that their RIF PII-PDT-resistant cells increased the expression of HSP70 and H0-1 mRNA upon PDT treatment [6], without changing the expression of these proteins. The same group has previously reported that hyperthermia-resistant cells which overexpress HSP70 were not cross resistant to PDT [127].

Verwanger et al. [128] ran a cDNA array in human squamous cell carcinoma cells A-431 after treatment with ALA-PDT and found increased expression of HSP70. They also found increased expression of HO-1 following dark incubation with ALA, which was not further augmented after irradiation, which was ascribed to the need for heme degradation after PpIX cell loading.

HSP60, a chaperone found mainly in mitochondria, was shown to be overexpressed in colon cancer cells HT29 and in the PDT-resistant fibrosarcoma RIF-8A cells [129]. Using microarray analysis, the same group found that the mRNA of HSP27 was increased in these cells, which is known to be involved in the signaling pathway leading to apoptosis [130]. Moreover, cells stably transfected with HSP27 cDNA showed an increased survival after PII-PDT treatment, suggesting that this protein may play a key role in PDT resistance. Similarly, an increased expression of HSP27 mRNA was found in the HT29 human colon adenocarcinoma PDT-resistant cells [32].

HSP1 was also found to be phosphorylated and consequently activated after Pc 4-PDT of the mouse lymphoma L5178Y cells [131]. In the same way, several studies have shown that many stress response proteins are induced after PDT treatment [28]. The increased expression of HO-1 either by induction with hemin or by stable transfection of the gene, increased the resistance of colon adenocarcinoma C-26 tumor cells to PDT-mediated cytotoxicity. On the other hand, treatment of the cells with an HO-1 inhibitor augmented the rate of phototoxicity [132].

Treatment of cells with a calcium ionophore increased the expression of GRPs and also developed PDT resistance after long exposure to PII protocol (16 h) [125]. This study also indicated elevated levels of mRNA encoding GRP-78 and GRP-94 and an increase in GRP protein synthesis in RIF-1 cells even before irradiation, showing that the PS itself is capable of inducing an oxidative stress response. While a short exposure protocol (1 h) prior to illumination, resulted in minimal increase in GRP mRNA levels or GRP protein synthesis. These results suggest that subcellular localization of the PS, which is the factor mainly affected by time exposure, is correlated with GRP induction.

Since GRP78 mRNA and protein levels are elevated in response to PDT in various cancer cell lines, stable overexpression of GRP78 and its role in resistance to PDT was investigated. GRP78-targeting subtilase cytotoxin catalytic subunit fused with epidermal growth factor (EGF-SubA) sensitizes various cancer cells to Photofrin-mediated PDT. The combination treatment is cytotoxic to apoptosis-competent SW-900 lung cancer cells, as well as to Bax-deficient and apoptosis-resistant DU-145 prostate cancer cells. In these cells, PDT and EGF-SubA cytotoxin induce the

expression of an ER stress-associated apoptosis-promoting transcription factor. Although some apoptotic events such as disruption of the mitochondrial membrane and caspase activation are detected after PDT, there is no phosphatidylserine plasma membrane externalization or DNA fragmentation, suggesting that in DU-145 cells the late apoptotic events are missing. Moreover, in SW-900 cells, EGF-SubA cytotoxin potentiates PDT-mediated cell death but attenuates PDT-induced apoptosis. In addition, the cell death cannot be reversed by caspase inhibitor z-VAD, confirming that apoptosis is not a major cell death mode triggered by the combination therapy. Moreover, no typical features of necrotic or autophagic cell death are recognized. Instead, an extensive cellular vacuolation of ER origin is observed. Altogether, these findings indicate that PDT and GRP78-targeting cytotoxin treatment can efficiently kill cancer cells independent on their apoptotic competence and triggers an atypical, non-apoptotic cell death [133].

Changes in Cytoskeleton, Cell to Cell Adhesion and Adhesion to Substrate in PDT Resistant Cells

The extracellular matrix (ECM) is the non-cellular component present within all tissues and organs, and provides not only essential physical scaffolding for the cellular constituents but also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis. Cell adhesion to the ECM is mediated by ECM receptors, such as integrins, discoidin domain receptors and syndecans. Adhesion mediates cytoskeletal coupling to the ECM and is involved in cell migration through the ECM. Migration, cell adhesion, cell-to-cell communication differentiation and survival are common functions of the ECM [134, 135].

Integrins are the major class of surface receptors that attach to the extracellular matrix (ECM) and are responsible for a cell's interaction with its environment; these receptors process external signals into intracellular ones and induce a number of regulatory cascades. Ultimately, this can lead to a variety of cellular responses. Integrins are non-covalently attached heterodimer transmembrane receptors that consist of α- and β-subunits, forming a functional receptor. Signals that come from intracellular receptors can regulate adhesion, migration, growth, differentiation, and death of cells [136, 137].

The chemotherapy resistance phenotype has been often associated with altered expression patterns of cytoskeletal components and adhesion [138, 139]. In recent years, it has been established that the cell-cell and cell-matrix interactions involve the reorganization of the cytoskeleton and also involved the activation of multiple signaling pathways that can modify cell growth, survival and differentiation. Experimental data provided evidence that the anti-apoptotic pathways mediated by cell adhesion induce tumor resistance to different injuries. Cell adhesion mediates drug resistance (CAM-DR) in multiple myeloma, malignant lymphoma, acute and chronic leukaemias, as well as in pancreatic cancer, neuroblastoma, small cell and

non-small cell lung cancer, mesothelioma, colorectal carcinoma, and breast cancer, and is based on the observation that cells that adhere to ECM ligands are protected from undergoing apoptosis. Cell adhesion protects from death by radiation, and genotoxic chemotherapy [140, 141]. Cell adhesion to ECM mediated by integrins impact favorably to both normal and tumor cells, and it has been implicated in the development of radioresistance. This phenomenon has been called cell adhesion-mediated radioresistance, and it can be reversed by overexpression of integrin-linked kinases, with concomitant reduction of cell size and adhesion to ECM proteins [142, 143].

Many changes related to cell adhesion have been reported as a consequence of photodamage, such as inhibition of cell adhesion by PDT-BPD-MA [144]. In addition, Verteporfin-PDT induced a transient decrease in adhesion of human ovarian cancer cells OVCAR 3 to collagen IV, laminin, fibronectin, and vitronectin. Interestingly, after photosensitization, the β1- integrin on the cell surface could still react with anti- β1 antibody suggesting that the subunit was still structurally intact, although β1-integrin-containing focal adhesion plaques (a functional attribute) became diffuse. The loss in integrin function arises largely through intracellular damage rather than through direct damage to the integrin proteins on the cell surface [145].

Tumor cells are in general less adhesive than normal cells, which contributes to tumor cell detachment and metastasis. In this sense, fibronectin and its integrin receptors play a key role in tumor development. Rudhorfer et al. [11] using a squamous carncinoma cell line A-431 observed that ALA-PDT markedly downregulated the fibronectin gene. As a result of this downregulation, cells rounding up and detachment begin to occur, and as a consequence, migration and metastasis are increased.

PDT employing PII and BPD-MA applied to colon carcinoma cells induced a transient decrease in adhesiveness and in adhesion molecules expression [146]. The authors suggested that the decrease in adhesiveness could account for the decreased metastatic potential of PDT-treated cancer cells. No matter what the effect is, there is an impact on the metastatic ability of the PDT-surviving cells. It could either increase [147] or decrease [148], and these differences may be due to the PS employed, the light dosage, cell model and even tumor location.

Although not yet well understood the effects of PDT on the ECM, it is clear that PDT induces changes in ECM. It has been described that PDT can either decrease or increase adhesion to plastic, ECM and to endothelial cells [145, 149, 150]. BPD-MA PDT inhibited cell adhesion of normal human fibroblasts, causing neither change in ECM nor integrin expression [144].

Overhaus et al. tested the hypothesis that PDT alters the vascular wall matrix thereby inhibiting invasive cell migration, and as such, provides an important barrier mechanism to favorably alter the vascular injury response. In an experimental intimal hyperplasia model composed of untreated smooth muscle cells and fibroblasts seeded on control and PDT-treated 3D collagen matrix gels, they demonstrated that PDT reduces the invasiveness degree of smooth muscle and fibroblast migration rate, generating a matrix barrier to invasive vascular cell migration [151].

The main components of the cytoskeleton are tubulin, actin and the intermediate filaments, and are one of the targets for PDT [152]. Changes in the cytoskeleton system have been related to tumor progression and metastasis [150], and it also favors changes in the cell shape during apoptosis by promoting the apoptotic bodies formation [153].

Signaling from the extracellular space such as changes in cell shape, or cell detachment, are sensed and transmitted into the intracellular space via p53 mitogen-activated protein kinase (MAPK), extracellular signal regulated kinases (ERK) 1/2 or JNK signaling pathways among others. It was found that promotion of p38MAPK, ERK, JNK and Ras signaling pathways supported survival and/or apoptosis after Hypericin-PDT treatment. This group also found that after PDT there was an up-regulation of NEDD9 (also called HEF1). This protein, part of the CAS protein family, localizes at focal adhesion sites. NEDD9 early up-regulation could participate in apoptosis induction and execution, activation of JNK kinases and in the transition of 'flat' attached cells to rounded mitotic cells [154].

Integrins not only regulate cell adhesion, but also participate in the crosstalk with different growth factor receptors. These receptors can be phosphorylated upon binding of their ligand or by the binding of integrins in the absence of ligand. Sanovic's group [154] also showed a downregulation of genes enconding for integrin 3, integrin β1, and integrin 6 after Hypericin-PDT, thus reducing the transduction of signals from the ECM and cell adhesion. They also found thrombospondin-1, which is a ligand for integrin β1 was downregulated in A-431 cells after treatment.

Buytaert et al. treated bladder cancer cells with Hypericin-PDT and they found a downregulation of integrin 2 and β3 precursors [155]. Studies conducted by Galaz et al. [156] reinforced the hypothesis that integrins downregulation induces cell detachment and apoptosis. They have demonstrated that altered levels of β1-integrins favored cell detachment and apoptosis via E-cadherin loss after ZnPc-PDT.

However, integrins are not the only proteins responsible for cell detachment and the control of rearrangements of the actin cytoskeleton, since Hypericin-PDT induce up-regulation of the Rho family GTPase 3 causing similar effects by inhibiting integrin-based focal adhesions and formation of actin stress fibers leading to cell rounding [154]. Sanovic's group has also reported another detachment mechanism affected by PDT which also contributes to significant changes in cell morphology and decreases cell adhesion, which is the overexpression of Pleckstrin homology-like domain, family A, member 1 (PHLDA1) [154].

Our group found that the ALA-PDT adenocarcinoma resistant cells *in vitro* were less invasive and tend to migrate less, while *in vivo* their ability to metastasize were decreased compared to the parental cell line. The lower tumor uptake, latency time and growth rate suggested that anchorage-dependent adhesion was also impaired *in vivo* in the resistant clones. However, *in vitro* binding to the ECM protein collagen I was higher for both of the clones, but no overexpression of β1 integrin was found, which is the main molecule involved in collagen I binding [157]. In addition to a loss of actin stress fibers, the resistant clones also exhibited disorganized actin cortical rim, as well as E-cadherin, β-catenin (cell–cell adhesion proteins) and vinculin (cytoskeleton-associated protein) distribution [157]. These alterations of

the cytoskeletal and adhesion proteins can be probably correlated with the lower metastatic phenotype of the cells *in vivo*.

Another major cytoskeletal protein is vimentin, which is degraded in response to various inducers of apoptosis [158, 159]. When Jurkat cells were transfected with a caspase-resistant vimentin variant, Phthalocyanine-PDT-induced apoptosis was partly suppressed and delayed, suggesting that this cytoskeleton component has a major role in the development of resistance by impairing caspase-3 translocation [160].

Cell attachment as well as cell to cell interactions may influence the plating efficiency of cells. Some of the PII-PDT resistant variants from Luna & Gomer showed a reduced plating efficiency of up to 36–43 % [6]. In addition, a much higher number of these resistant cells was required to generate a tumor when injected into syngeneic mice compared to non-resistant ones. In our hands, ALA-PDT variants resistant cells had shown an impaired plating efficiency that correlated with a lower tumor take when injected into mice. This characteristic may be associated to the ECM changes in the PDT resistant cells [149]. In addition, Perry et al. found a correlation between PII-PDT sensitivity and plating efficiency when analyzing an array of lung cell lines with different histologies [161].

Since the cellular shape is an important factor that regulates the cell sensitivity to mitogens, this suggests that the proliferative rate is anchorage dependent [162]. The ECM components on which the cells grow *in vivo* or the substract used to culture cells *in vitro*, commands the shape of the cells, as well as the production of specific proteins coming from external signals [163]. Since ALA-PDT resistant cells tend to spread more than the parental cell line, we hypotetized that these cells grown in suspension would loose the resistance [7]. However, in our study, the resistance indexes of cells growing in suspension did not change in the resistant clones as compared to the parental cells. Also, no significant effect was found when ALA-PDT was performed in cells growing onto fibronectin coatings.

Similarly to some studies that suggested the cell size could be related to resistance to chemotherapy [164], there are some evidences that cell size could also be related to PDT resistance. In the study carried out by Luna & Gomer the PII-PDT resistant variants obtained from RIF fibrosarcoma cells were larger and the protein content was increased [6]; similar results were obtained in the variants isolated by Sharkley et al. [30]. An increased cell spreading together with an increased number of cells per colony were also observed. Supporting this trend of thoughts, Richter et al. [165] treated several human leukemia cell lines with BPD-MA-PDT, in comparison to normal lymphocytes, they found that the resistance was related to the cell sizes, with the smallest cells being the most vulnerable. Our group also found that ALA-PDT resistant cells had twice the volume and protein content increase compared to the parental line [7]. Since the plasma membrane is the main target for PDT damage [206], a larger cell exposes a greater surface area, suggesting the effectiveness of the treatment could be inferior in the resistant clones.

Nitric Oxide

As a general rule, depending on the cell type and the pathology, the gaseous radical nitric oxide (NO), could play either a protective or a toxic role in the cells. However, several studies have shown NO can induce many pathways to mediate chemoresistance. Inhibition of the proto-oncogene MYCN (v-myc myelocytomatosis viral related *oncogene*, neuroblastoma derived (avian)) and expression of a large set of ATP binding cassette transporters by NO, influence the chemoresistance outcome in neuroblastoma cells [166]. In malignant astrocytes, NO has been found to modulate radioresistance and chemoresistance against nitrosourea derivatives [167]. Using a blocking agent against all NO synthases reversed the resistant phenotype through induction of apoptosis in cisplatin-resistant ovarian cancer cells [168].

NO is not an effective oxidant *per se*, but under biological conditions could be converted to strong damaging oxidants. At low concentrations NO may act as an antioxidant in lipid membranes by scavenging chain propagating oxyl and peroxyl radicals [169], contributing to the overall cellular resistance to peroxidative stress. When this occurs during PDT, the outcome of the treatment may be impaired, even under nontoxic levels of exogenous NO as it was shown by Niziolek et al. [170] on PpIX-sensitized liposomes and breast tumor COH-BR1 cells treated with ALA-PDT.

iNOS (inducible nitric oxide synthase) modulates survival to confer chemoresistance in head and neck cancer [171]. The cytoprotective effects of NO in response to oxidative stress could be long-term in an indirect way. Cytokines can induce nitric oxide synthase to produce endogenous NO, conferring long-term hype in resistance to H_2O_2 or high-level NO cytotoxicity in hepatocytes [172, 173].

Resistance to ALA-PDT could be evidenced after rather long periods after exposure to NO; between 8 and 20 h after treatment of COH-BR1 tumor cells with the NO donor spermine NONOate (SPNO). A concomitant increase in HO-1 and ferritin levels was also observed. These cells exhibited an immediate radical-quenching effect of NO, but it also evoked a delayed cytoprotective response, suggesting that the protective mechanism involves the mobilization of "signaling" iron [174]. The same group reported in 2010 [175] that NO has the capacity to support apoptosis, and that the NO donor SPNO was able to inhibit necrosis but support apoptosis when cells were exposed to it before PDT. These observations were along with an increased activation of caspases-3 and -7. The effect of SPNO-supported apoptosis was more evident when comparing cells growing in glucose-deprived conditions to glucose-containing medium. They suggested that PDT resistant cells based their resistance on the membrane protection by NO and the maintenance of sufficient glycolytic ATP to sustain apoptosis.

The same group demonstrated that incubating lymphocytic leukemia L1210 cells with SPNO either immediately before or after light exposure PDT employing merocyanin 540 (a lipophilic dye that localizes primarily in the plasma membrane), photodamage was inhibited. They postulated that chain peroxidation triggered by iron-catalyzed turnover of nascent hydroperoxides generated by singlet oxygen attack on membrane lipids contributes significantly to phototoxicity, and that NO,

thus, acts cytoprotectively. Propagating radicals such as 5alpha-OOH are impaired by action of NO on photodamaged cells [176].

In our laboratory [177], we generated NO-resistant cells of murine the LM3 mouse breast adenocarcinoma called LM3-SNP, by successive exposures to the NO donor sodium nitroprusside (SNP). We have found this variant had no cross-resistance to ALA-PDT treatment. In parallel, we have also induced ALA-PDT resistance in these NO-resistant LM3-SNP cells, suggesting that resistance to NO did not interfere in the development of PDT resistance. Moreover, we found that several cell lines with different NO production levels responded in a similar way to ALA-PDT treatment [178], and that the modulation of NO levels did not modify the intrinsic response of various cells lines to PDT treatment.

Bhowmick et al. [179] reported that iNOS up-regulation after PDT treatment, induced an increase of tumor cell resistance. The inducible form iNOS was found to be up-regulated in breast tumor COH-BR1 cells after ALA-PDT treatment, while the other NOS isoforms nNOS and eNOS were unaffected. Also incubating the cells with L-NAME (NG-Nitro-L-arginine methyl ester), an NOS inhibitor, during PDT enhanced the activation of caspases-3 and -7 and apoptotic killing, suggesting that iNOS was acting as photoprotective. In line with these findings, exposure to the NO scavenger cPTIO enhanced ALA-PDT-induced caspases-3 and -7 activation and apoptotic death in the mentioned breast tumor cells.

Similar results were found by the same authors in prostate cancer PC-3 cells treated with PDT, which resulted in upregulation of iNOS, as well as caspases-3 and -7 activation and apoptosis stimulation after treatment with iNOS inhibitors and an NO scavenger. Cells surviving PDT had an increased cell cycle, but iNOS inhibition prevented this and the increase in cell cycle S-phase occupancy observed after irradiation, showing that upregulation of NOS/NO elicited both a pro-survival and pro-growth response. This was the first report of NO-dependent growth stimulation in cancer cells exposed to a PDT oxidative stress. The pro-survival response described could be a general phenomenon in NOS-expressing tumors subjected to PDT, and one that might seriously compromise treatment effectiveness unless counteracted. It has been proposed the use of the iNOS inhibitor GW274150 to overcome iNOS-mediated resistance to PDT [180].

Hypoxia

It is well known that most tumors undergo hypoxia, which is a therapeutic challenge since it can reduce the effectiveness of radiotherapy and PDT [181]. Tumor hypoxia can also induce proteomics and genomic changes in the cancer cells, therefore negatively affecting the therapeutic outcome. Tumor hypoxia influences the selection of malignant cells which have been able to overcome the microenvironment of nutrient deprivation, this making the tumor more aggressive and promoting the development of a more treatment-resistant disease.

PDT induces vascular damage and oxygen consumption resulting in tissue hypoxia which in turn may limit the efficacy of this treatment. Tumor cells may protect themselves against PDT-mediated damage by stabilizing the hypoxia-inducible factor 1 (HIF1)-alpha [99, 182]. It has been reported that PDT induces hypoxia and the expression of vascular endothelial growth factor (VEGF) via the HIF1-alpha pathway, which in turn promotes angiogenesis, thus enhancing tumor proliferation and survival. VEGF mRNA expression was induced in the lung cancer cell line SBC-3 after ATX-s10-PDT [183]. When the human esophageal cell line Het-1A was induced to overexpress HIF-1alpha, it displayed resistance to ALA-PDT. Moreover, knocking down of the HIF-1alpha restored the photosensitivity of the cells [184].

In order to overcome PDT resistance induced by hypoxia, some strategies have been developed. Through a concept called "arterial flow focalization", by controlled temporary endo or peri-vascular occlusion of the collateral arterial branch(es) upstream of the tumor, it is possible to redirect blood flow through the principal artery of the downstream tumor (organ), thereby increasing tumor arterial flow, and hence oxygen supply, thus increasing tumor PtO2 at the desired intensity and timing, in synchrony with radiotherapy, and greatly improving radiosensitivity. Chemotherapy and photodynamic therapy efficacy could be also be increased, by increasing the PtO2 and by improvement of tumor blood perfusion and hence drug delivery to the tumor [185]. Additionally, the more hypoxic cells can be preferentially targeted by bio-reductive drugs and hypoxia-directed gene therapy [186, 187].

Using a synthetic self-assembling peptide as a cellular scaffold, Alemany-Ribes et al. [188] recreated the *in vivo* limitation of oxygen and drug diffusion and its biological effect, which is the development of cellular resistance to therapy. Cells grown in the 3D cultures upregulated the expression of the hypoxia-responsive genes EGF and insulin-like growth factor binding protein 3 (IGFBP3), which act as oxygen markers. 3D cultures were found to be more resistant to PDT than traditional 2D monolayers, exhibiting a radial survival pattern in which the core of the construct maintained a larger percentage of living cells. After circulating oxygen stream during irradiation, complete cell death was observed under conditions in which cell viability had been 80% in the absence of oxygen flow. These data indicate that the high cellular survival observed under static conditions was due to the low oxygen concentration in the core of the construct, which created a protective microenvironment for cells, thus reinforcing the importance of cell hypoxia in the outcome of PDT

Conclusions and Future Directions

Recently, in addition to the development of PDT resistant cells as models to further study the impact of PDT on cellular targets, there have been an increasing interest in developing techniques related to the use of PS and light to overcome drug resistance to chemotherapeutic drugs. One of the mechanisms of the reversion of MDR takes advantage of the acidic microenvironment within drug vesicles.

Photochemical-mediated release of chemotherapeutic agents that are either targets or not targets of P-gp pumps, which are trapped in endocytic vesicles, has been shown to reverse the MDR phenotype. This approach can be employed for the treatment of any chemoresistant cell, but particularly cancer stem cells. In addition, illumination of ABCG2-rich extracellular vesicles that accumulate photosensitive cytotoxic drugs has been demonstrated to overcome resistance. Another approach uses nanomedicinal tools to deliver the PS more efficiently and to reduce the chances of being pumped out from the cytoplasm, e.g., pH-sensitive nanoparticles that enable pH-dependent PDT.

Combinations of PDT and targeted therapies, as well as the use of PS delivered in nanoparticles such as poly ethylene glycol-based, carbon nanotubes, dendrimers, carbon-based nanoparticles and polymeric micelles, are the most recent strategies to circumvent photoresistance and will be discussed in the next chapters.

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References

1. Gomer CJ. Photodynamic therapy. Oxford: Pergamon Press; 1987.
2. Dougherty TJ. Photodynamic therapy-new approaches. Semin Surg Oncol. 1989;5:6–16.
3. Weishaupt K, Gomer C, Dougherty T. Identification of singlet oxygen as the cytotoxic agent in photoactivation of a murine tumor. Cancer Res. 1976;36:2326–9.
4. Mayhew S, Vernon D, Schofield J, Griffiths J, Brown S. Investigation of cross-resistance to a range of photosensitizers, hyperthermia and UV light in two radiation-induced fibrosarcoma cell strains resistant to photodynamic therapy in vitro. Photochem Photobiol. 2001;73:39–46.
5. Singh G, Wilson B, Sharkey S, Browman G, Deschamps P. Resistance to photodynamic therapy in radiation induced fibrosarcoma-1 and chinese hamster ovary-multi-drug resistant cells in vitro. Photochem Photobiol. 1991;54:307–12.
6. Luna M, Gomer C. Isolation and initial characterization of mouse tumor cells resistant to porphyrin-mediated photodynamic therapy. Cancer Res. 1991;51:4243–9.
7. Casas A, Perotti C, Ortel B, Di Venosa G, Saccoliti M, Batlle A, Hasan T. Tumor cell lines resistant to ALA-mediated photodynamic therapy and possible tools to target surviving cells. Int J Oncol. 2006;29:397–405.
8. Henderson B, Miller A. Effects of scavengers of reactive oxygen and radical species on cell survival following photodynamic treatment in vitro: comparison to ionizing radiation. Radiat Res. 1986;108:196–205.
9. Gomer C, Ryter S, Ferrario A, Rucker N, Wong S, Fisher A. Photodynamic therapy-mediated oxidative stress can induce the expression of heat shock proteins. Cancer Res. 1996;56:2355–60.
10. Shackley DC, Haylett A, Whitehurst C, Betts CD, O'Flynn K, Clarke NW, Moore JV. Comparison of the cellular molecular stress responses after treatments used in bladder cancer. BJU Int. 2002;90:924–32.
11. Ruhdorfer S, Sanovic R, Sander V, Krammer B, Verwanger T. Gene expression profiling of the human carcinoma cell line A-431 after 5-aminolevulinic acid-based photodynamic treatment. Int J Oncol. 2007;30:1253–62.
12. Morgan J, Potter WR, Oseroff AR. Comparison of photodynamic targets in a carcinoma cell line and its mitochondrial DNA-deficient derivative. Photochem Photobiol. 2000;71:747–57.
13. Peng Q, Moan J, Farrants GW, Danielsen HE, Rimington C. Location of P-II and AlPCS4 in human tumor LOX in vitro and in vivo by means of computer-enhanced video fluorescence microscopy. Cancer Lett. 1991;58:37–47.

14. Lin CW, Shulok JR, Kirley SD, Cincotta L, Foley JW. Lysosomal localization and mechanism of uptake of Nile blue photosensitizers in tumor cells. Cancer Res. 1991;51:2710–9.
15. Van Hillesberg R, van der Berg J, Kort W, Terpstra O, Wilson J. Selective accumulation of endogenously produced porphyrins in a liver metastasis model in rats. Gastroenterology. 1992;103:647–51.
16. Madsen SJ, Sun CH, Tromberg BJ, Hirschberg H. Repetitive 5-aminolevulinic acid-mediated photodynamic therapy on human glioma spheroids. J Neurooncol. 2003; 62:243–50.
17. Casas A, Di Venosa G, Hasan T, Al Batlle. Mechanisms of resistance to photodynamic therapy. Curr Med Chem. 2011;18:2486–515.
18. Luna M, Ferrario A, Rucker N, Gomer C. Decreased expression and function of alpha-2 macroglobulin receptor/low density lipoprotein receptor-related protein in photodynamic therapy-resistant mouse tumor cells. Cancer Res. 1995;55:1820–3.
19. Wilson B, Olivo M, Singh G. Subcellular localization of photofrin and aminolevulinic acid and photodynamic cross-resistance in vitro in radiation-induced fibrosarcoma cells sensitive or resistant to photofrin-mediated photodynamic therapy. Photochem Photobiol. 1997;65:166–76.
20. Hilf R, Smail D, Murant R, Leakey P, Gibson S. Hematoporphyrin derivative induced photosensitivity of mitochondrial succinate dehydrogenase and selected cytosolic enzymes of R3230AC mammary adenocarcinomas of rats. Cancer Res. 1984;44:1483–88.
21. Nelson JS, Sun CH, Berns MW. Study of the in vivo and in vitro photosensitizing capabilities of uroporphyrin I compared to photofrin II. Lasers Surg Med. 1986;6:131–6.
22. Okunaka T, Eckhauser ML, Kato H, Bomaminio A, Yamamoto H. Aizawa K, Sarasua MM, Koehler KA. Correlation between photodynamic efficacy of differing porphyrins and membrane partitioning behavior. Lasers Surg Med. 1992;12:98–103.
23. Strauss WS, Sailer R, Schneckenburger H, Akgun N, Gottfried V, Chetwer L, Kimel S. Photodynamic efficacy of naturally occurring porphyrins in endothelial cells in vitro and microvasculature in vivo. J Photochem Photobiol. 1997;39:176–84.
24. Musser DA, Oseroff AR. The use of tetrazolium salts to determine sites of damage to the mitochondrial electron transport chain in intact cells following in vitro photodynamic therapy with Photofrin II. Photochem Photobiol. 1994;59:621–6.
25. Hilf R. Mitochondria are targets of photodynamic therapy. J Bioenerg Biomembr. 2007;39:85–9.
26. Boegheim JP, Lagerberg JW, Dubbelman TM, Tijssen K, Tanke HJ, Van Der Meulen J, Van Steveninck J. Photodynamic effects of hematoporphyrin derivative on the uptake of rhodamine 123 by mitochondria of intact murine L929 fibroblasts and Chinese ovary K1 cells. Photochem Photobiol. 1988;48:613–20.
27. Salet C, Moreno G. Photosensitization of mitochondria. Molecular and cellular aspects. J Photochem Photobiol B. 1990;5:133–50.
28. Moor AC. Signaling pathways in cell death and survival after photodynamic therapy. J Photochem Photobiol B. 2000;57:1–13.
29. Kessel D. Death pathways associated with photodynamic therapy. Med Laser Appl. 2006;21:219–24.
30. Sharkey S, Wilson B, Moorehead R, Singh G. Mitochondrial alterations in photodynamic therapy-resistant cells. Cancer Res. 1993;53:4994–9.
31. Crawford DR, Wang Y, Schools GP, Kochheiser J, Davies KJ. Down-regulation of mammalian mitochondrial RNAs during oxidative stress. Free Radic Biol Med. 1997;22:551–9.
32. Shen XY, Zacal N, Singh G, Rainbow AJ. Alterations in mitochondrial and apoptosis-regulating gene expression in photodynamic therapy-resistant variants of HT29 colon carcinoma cells. Photochem Photobiol. 2005;81:306–13.
33. Singh K, Russell J, Sigala B, Zhang Y, Williams J, Keshav K. Mitochondrial DNA determines the cellular response to cancer therapeutic agents. Oncogene. 1999;18:6641–6.
34. Wang X. The expanding role of mitochondria in apoptosis. Genes Dev. 2001;15:2922–33.
35. Moorehead R, Armstrong S, Wilson B, Singh G. Cross-resistance to cisplatin in cells resistant to photofrin-mediated photodynamic therapy. Cancer Res. 1994;54:2556–9.

36. Kessel D, Woodburn K, Skalkos D. Impaired accumulation of a cationic photosensitizing agent by a cell line exhibiting multidrug resistance. Photochem Photobiol. 1994;60:61–3.
37. Kessel D, Erickson C. Porphyrin photosensitization of multi-drug resistant cell types. Photochem Photobiol. 1992;55:397–9.
38. Tsai T, Hong RL, Tsai JC, Lou PJ, Ling IF, Chen, CT. Effect of 5-aminolevulinic acid-mediated photodynamic therapy on MCF-7 and MCF-7/ADR cells. Lasers Surg Med. 2004;34:62–72.
39. Robey RW, Steadman K, Polgar O, Bates SE. ABCG2-mediated transport of photosensitizers: potential impact on photodynamic therapy. Cancer Biol Ther. 2005;4:187–94.
40. Li W, Zhang W, Ohnishi K, Yamada I, Ohno R, Hashimoto K. 5-aminolevulinic acid-mediated photodynamic therapy in multidrug resistant leukemia cells. J Photochem Photobiol. 2001;60:79–86.
41. Chu ES, Yow CM, Shi M, Ho RJ. Effects of photoactivated 5-aminolevulinic acid hexyl ester on MDR1 over-expressing human uterine sarcoma cells. Toxicol Lett. 2008;181:7–12.
42. Tang PM, Zhang DM, Xuan NH, Tsui SK, Waye MM, Kong SK, Fong WP, Fung KP. Photodynamic therapy inhibits P-glycoprotein mediated multidrug resistance via JNK activation in human hepatocellular carcinoma using the photosensitizer pheophorbide a. Mol Cancer. 2009;8:56–68.
43. Selbo PK, Weyergang A, Bonsted A, Bown SG, Berg K. Photochemical internalization of therapeutic macromolecular agents: a novel strategy to kill multidrug-resistant cancer cells. J Pharmacol Exp Ther. 2006;319:604–12.
44. Merlin JL, Gautier H, Barberi-Heyob M, Teiten MH, Guillemin F. The multidrug resistance modulator SDZ-PSC 833 potentiates the photodynamic activity of chlorin e6 independently of P-glycoprotein in multidrug resistant human breast adenocarcinoma cells. Int J Oncol. 2003;22:733–9.
45. Teiten M, Bezdetnaya L, Merlin J, Bour-Dill C, Pauly M, Dicato M, Guillemin F. Effect of meta-tetra(hydrophenyl)chlorin (mTHPC)-mediated photodynamic therapy on sensitive and multidrug-resistant human breast cancer cells. J Photochem Photobiol B. 2001;62:146–52.
46. Cheung KK, Chan JY, Fung KP. Antiproliferative effect of pheophorbide a-mediated photodynamic therapy and its synergistic effect with doxorubicin on multiple drug-resistant uterine sarcoma cell MES-SA/Dx5. Drug Chem Toxicol. 2013;36:474–83.
47. Chu ES, Yow CM. Modulation of telomerase and signal transduction proteins by hexyl-ALA-photodynamic therapy (PDT) in human doxorubicin resistant cancer cell models. Photodiagnosis Photodyn Ther. 2012;9:243–55.
48. Diez B, Ernst G, Teijo MJ, Batlle A, Hajos S, Fukuda H. Combined chemotherapy and ALA-based photodynamic therapy in leukemic murine cells. Leuk Res. 2012;36:1179–84.
49. Preise D, Mazor O, Koudinova N, Liscovitch M, Scherz A, Salomon Y. Bypass of tumor drug resistance by antivascular therapy. Neoplasia. 2003;5:475–80.
50. Lou PJ, Lai PS, Shieh MJ, MacRobert AJ, Berg K, Bown SG. Reversal of doxorubicin resistance in breast cancer cells by photochemical internalization. Int J Cancer. 2006;119:2692–8.
51. Adigbli DK, Wilson DG, Farooqui N, Sousi E, Risley P, Taylor I, Macrobert AJ, Loizidou M. Photochemical internalisation of chemotherapy potentiates killing of multidrug-resistant breast and bladder cancer cells. Br J Cancer. 2007;97:502–12.
52. Berg K, Selbo PK, Prasmickaite L, Tjelle TE, Sandvig K, Moan J, Gaudernack G, Fodstad O, Kjølsrud S, Anholt H, Rodal GH, Rodal SK, Høgset A. Photochemical internalization: a novel technology for delivery of macromolecules into cytosol. Cancer Res. 1999;59:1180–3.
53. Olsen CE, Berg K, Selbo PK, Weyergang A. Circumvention of resistance to photodynamic therapy in doxorubicin-resistant sarcoma by photochemical internalization of gelonin. Free Radic Biol Med. 2013;65:1300–9.
54. Ling D, Park W, Park SJ, Lu Y, Kim KS, Hackett MJ, Kim BH, Yim H, Jeon YS, Na K, Hyeon T. Multifunctional tumor pH-Sensitive self-assembled nanoparticles for bimodal imaging and treatment of resistant heterogeneous tumors. J Am Chem Soc. 2014 [Epub ahead of print]
55. Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, Ross DD. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA. 1998;95:15665–70.

56. Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res. 1998;58:5337–9.
57. Haimeur A, Conseil G, Deeley RG, Cole SP. The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. Curr Drug Metab. 2004;5:21–53.
58. Doyle LA, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene. 2003;22:7340–58.
59. Selbo PK, Weyergang A, Eng MS, Bostad M, Mælandsmo GM, Høgset A, Berg K. Strongly amphiphilic photosensitizers are not substrates of the cancer stem cell marker ABCG2 and provides specific and efficient light-triggered drug delivery of an EGFR-targeted cytotoxic drug. J Control Release. 2012;159:197–203.
60. Navone N, Polo C, Frisardi A, Batlle A. Mouse mammary carcinoma PBGase and hydroxymethylbilane synthetase. Comp Biochem Physiol B. 1991;98:67–71.
61. Calzavara-Pinton P. Repetitive photodynamic therapy with topical delta-aminolaevulinic acid as an appropriate approach to the routine treatment of superficial non-melanoma skin tumors. J Photochem Photobiol B. 1995;29:53–7.
62. Jonker JW, Buitelaar M, Wagenaar E, Van Der Valk MA, Scheffer GL, Scheper RJ, Plosch T, Kuipers F, Elferink RP, Rosing H, Beijnen JH, Schinkel AH. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci USA. 2002;99:15649–54.
63. Tsunoda Y, Usuda J, Imai K, Kubota M, Maehara S, Ohtani K. The expression of BCRP/ABCG2 causes resistance to Photofrin-PDT. Jpn J Laser Surg Med. 2008;28:355–61.
64. Robey RW, Steadman K, Polgar O, Morisaki K, Blayney M, Mistry P, Bates SE. Pheophorbide a is a specific probe for ABCG2 function and inhibition. Cancer Res. 2004;64:1242–6.
65. Moitra K, Lou H, Dean M. Multidrug efflux pumps and cancer stem cells: insights into multidrug resistance and therapeutic development. Clin Pharmacol Ther. 2011;89:491–502.
66. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001;7:1028–34.
67. Gupta N, Martin PM, Miyauchi S, Ananth S, Herdman AV, Martindale RG, Podolsky R, Ganapathy V. Down-regulation of BCRP/ABCG2 in colorectal and cervical cancer. Biochem Biophys Res Commun. 2006;343:571–7.
68. Liu W, Baer MR, Bowman MJ, Pera P, Zheng X, Morgan J, Pandey RA, Oseroff AR. The tyrosine kinase inhibitor imatinib mesylate enhances the efficacy of photodynamic therapy by inhibiting ABCG2. Clin Cancer Res. 2007;13:2463–70.
69. Usuda J, Tsunoda Y, Ichinose S, Ishizumi T, Ohtani K, Maehara S, Ono S, Tsutsui H, Ohira T, Okunaka T, Furukawa K, Sugimoto Y, Kato H, Ikeda N. Breast cancer resistant protein (BCRP) is a molecular determinant of the outcome of photodynamic therapy (PDT) for centrally located early lung cancer. Lung Cancer. 2009;67:198–204.
70. Jendzelovský R, Mikes J, Koval' J, Soucek K, Procházková J, Kello M, Sacková V, Hofmanová J, Kozubík A, Fedorocko P. Drug efflux transporters, MRP1 and BCRP, affect the outcome of hypericin-mediated photodynamic therapy in HT-29 adenocarcinoma cells. Photochem Photobiol Sci. 2009;8:1716–23.
71. Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE, Sarkadi B, Sorrentino BP, Schuetz JD. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem. 2004;279:24218–25.
72. Goler-Baron V, Assaraf YG. Structure and function of ABCG2-rich extracellular vesicles mediating multidrug resistance. PLoS One. 2011;6(1):e16007.
73. Adar Y, Stark M, Bram EE, Nowak-Sliwinska P, van den Bergh H, Szewczyk G, Sarna T, Skladanowski A, Griffioen AW, Assaraf YG. Imidazoacridinone-dependent lysosomal photodestruction: a pharmacological Trojan horse approach to eradicate multidrug-resistant cancers. Cell Death Dis. 2012;3:e293.

74. Oleinick NL, Morris RL, Belichenko I. The role of apoptosis in response to photodynamic therapy: what, where, why, and how. Photochem Photobiol Sci. 2002;1:1–21.
75. Kessel D. Relocalization of cationic porphyrins during photodynamic therapy. Photochem Photobiol Sci. 2002;1:837–40.
76. Reiners JJ Jr., Agostinis P, Berg K, Oleinick NL, Kessel D. Assessing autophagy in the context of photodynamic therapy. Autophagy. 2010;6:7–18.
77. Kessel D, Luo Y, Deng Y, Chang CK. The role of subcellular localization in initiation of apoptosis by photodynamic therapy. Photochem Photobiol. 1997;65:422–6.
78. Dellinger M. Apoptosis or necrosis following Photofrin photosensitization: influence of the incubation protocol. Photochem Phorobiol. 1996;64:182–7.
79. Kessel D, Castelli M. Evidence that Bcl-2 is the target of three photosensitizers that induce a rapid apoptotic response. Photochem Photobiol. 2001;74:318–22.
80. Kessel D, Luguya R, Vicente MG. Localization and photodynamic efficacy of two cationic porphyrins varying in charge distribution. Photochem Photobiol. 2003;78:431–5.
81. Kessel D, Luo Y, Mathieu P, Reiners JJ Jr. Determinants of the apoptotic response to lysosomal photodamage. Photochem Photobiol. 2000;71:196–200.
82. Kessel D, Oleinick NL. Initiation of autophagy by photodynamic therapy. Methods Enzymol. 2009;453:1–16.
83. Kessel D, Arroyo AS. Apoptotic and autophagic responses to Bcl-2 inhibition and photodamage. Photochem Photobiol Sci. 2007;6:1290–5.
84. Kessel D, Oleinick NL. Photodynamic therapy and cell death pathways. Methods Mol Biol. 2010;635:35–46.
85. Almeida RD, Manadas BJ, Carvalho AP, Duarte CB. Intracellular signaling mechanisms in photodynamic therapy. Biochim Biophys Acta. 2004;1704:59–86.
86. Wild PJ, Krieg RC, Seidl J, Stoehr R, Reher K, Hofmann C, Louhelainen J, Rosenthal A, Hartmann A, Pilarsky C, Bosserhoff AK, Knuechel R. RNA expression profiling of normal and tumor cells following photodynamic therapy with 5-aminolevulinic acid-induced protoporphyrin IX in vitro. Mol Cancer Ther. 2005;4:516–28.
87. Granville DJ, Carthy CM, Jiang H, Shore GC, McManus BM, Hunt DW. Rapid cytochrome c release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. FEBS Lett. 1998;437: 5–10.
88. Song J, Chen Q, Xing D. Enhanced apoptotic effects by downregulating Mcl-1:evidence for the improvement of photodynamic therapy with Celecoxib. Exp Cell Res. 2013;319:1491–504.
89. Reed JC. Bcl-2 family proteins. Oncogene. 1998;17:3225–36.
90. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes. 1999;13:1899–911.
91. Granville DJ, Jiang H, An MT, Levy JG, McManus BM, Hunt DW. Bcl-2 overexpression blocks caspase activation and downstream apoptotic events instigated by photodynamic therapy. Br J Cancer. 1999;79:95–100.
92. Srivastava M, Ahmad H, Gupta S, Mukhtar H. Involvement of Bcl-2 and Bax in photodynamic therapy mediated apoptosis. J Biol Chem. 2001;276:15481–8.
93. Zacal N, Espiritu M, Singh G, Rainbow AJ. Increased BNip3 and decreased mutant p53 in cisplatin-sensitive PDT-resistant HT29 cells. Biochem Biophys Res Commun. 2005;331:648–57.
94. He J, Agarwal M, Larkin H, Friedman L, Xue L, Oleinick N. The induction of partial resistance to photodynamic therapy by the protooncogene BCL-2. Photochem Photobiol. 1996;64:845–52.
95. Kim H, Luo Y, Li G, Kessel D. Enhanced apoptotic response to photodynamic therapy after bcl-2 transfection. Cancer Res. 1999;59:3429–32.
96. Zhang WG, Ma LP, Wang SW, Zhang ZY, Cao GD. Antisense bcl-2 retrovirus vector increases the sensitivity of a human gastric adenocarcinoma cell line to photodynamic therapy, Photochem Photobiol. 1999;69:582–6.

97. Kawaguchi T, Yamamoto S, Naka N, Okishio K, Atagi S, Ogawara M, Hosoe S, Kawahara M, Furuse K. Immunohistochemical analysis of Bcl-2 protein in early squamous cell carcinoma of the bronchus treated with photodynamic therapy. Br J Cancer. 2000;82:418–23.
98. McGarrity TJ, Peiffer LP, Granville DJ, Carthy CM, Levy JG, Khandelwal M, Hunt DW. Apoptosis associated with esophageal adenocarcinoma: influence of photodynamic therapy. Cancer Lett. 2001;163:33–41.
99. Koukourakis M, Corti L, Skarlatos J, Giatromanolaki A, Krammer B, Blandamura S, Piazza M, Verwanger T, Schnitzhofer G, Kostandelos J, Beroukas K. Clinical and experimental evidence of Bcl-2 involvement in the response to photodynamic therapy. Antinacer Res. 2001;21:663–8.
100. Usuda J, Azizuddin K, Chiu SM, Oleinick NL. Association between the photodynamic loss of Bcl-2 and the sensitivity to apoptosis caused by phthalocyanine photodynamic therapy. Photochem Photobiol. 2003;78:1–8.
101. Chiu SM, Xue LY, Usuda J, Azizuddin K, Oleinick NL. Bax is essential for mitochondrion-mediated apoptosis but not for cell death caused by photodynamic therapy. Br J Cancer. 2003;89:1590–7.
102. Usuda J, Hirata T, Ichinose S, Ishizumi T, Inoue T, Ohtani K, Maehara S, Yamada M, Tsutsui H, Okunaka T, Kato H, Ikeda N. Tailor-made approach to photodynamic therapy in the treatment of cancer based on Bcl-2 photodamage. Int J Oncol. 2008;33:689–96.
103. Ichinose S, Usuda J, Hirata T, Inoue T, Ohtani K, Maehara S, Kubota M, Imai K, Tsunoda Y, Kuroiwa Y, Yamada K, Tsutsui H, Furukawa K, Okunaka T, Oleinick NL, Kato H. Lysosomal cathepsin initiates apoptosis, which is regulated by photodamage to Bcl-2 at mitochondria in photodynamic therapy using a novel photosensitizer, ATX-s10 (Na). Int J Oncol. 2006;29:349–55.
104. Caruso JA, Mathieu PA, Joiakim A, Leeson B, Kessel D, Sloane BF, Reiners JJ Jr. Differential susceptibilities of murine hepatoma 1c1c7 and Tao cells to the lysosomal photosensitizer NPe6: influence of aryl hydrocarbon receptor on lysosomal fragility and protease contents. Mol Pharmacol. 2004;4:1016–28.
105. Shaw P, Bovey R, Tardy S, Sahli R, Sordat B, Costa J. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc Natl Acad Sci USA. 1992;89:4495–9.
106. Siognov RV, Haupt Y. The cellular response to p53: the decision between life and death. Oncogene. 1999;18:6145–57.
107. Fisher AM, Rucker N, Wong S, Gomer CJ. Differential photosensitivity in wild-type and mutant p53 human colon carcinoma cell lines. J Photochem Photobiol. 1998;42:104–7.
108. Fisher AMR, Danenberg K, Banerjee D, Bertino JR, Danenberg P, Gomer CJ. Increased photosensitivity in HL60 cells expressing wild-type p53. Photochem Photobiol. 1997;66:265–70.
109. Fisher AMR, Ferrario A, Rucker, N, Zhang S, Gomer CJ. Photodynamic therapy sensitivity is not altered in human tumor cells after abrogation of p53 function. Cancer Res. 1999;59:331–5.
110. Zawacka-Pankau J, Krachulec J, Grulkowski I, Bielawski KP, Selivanova G. The p53-mediated cytotoxicity of photodynamic therapy of cancer: recent advances. Toxicol Appl Pharmacol. 2008;232:487–97.
111. Kliukiene R, Maroziene A, Nivinskas H, Cenas N, Kirveliene V, Juodka B. The protective effects of dihydrolipoamide and glutathione against photodynamic damage by Al-phtalocyanine tetrasulfonate. Biochem Mol Biol Int. 1997;41:707–13.
112. Golab J, Nowis D, Skrzycki M, Czeczot H, Baranczyk-Kuzma A, Wilczynski GM, Makowski M, Mroz P, Kozar K, Kaminski R, Jalili A, Kopec' M, Grzela T, Jakobisiak M. Antitumor effects of photodynamic therapy are potentiated by 2-methoxyestradiol. A superoxide dismutase inhibitor. J Biol Chem. 2003;278:407–14.
113. Dolgachev V, Oberley LW, Huang TT, Kraniak JM, Tainsky MA, Hanada K, Separovic D. A role for manganese superoxide dismutase in apoptosis after photosensitization. Biochem Biophys Res Commun. 2005;332:411–7.

114. Oberdanner CB, Plaetzer K, Kiesslich T, Krammer B. Photodynamic treatment with fractionated light decreases production of reactive oxygen species and cytotoxicity in vitro via regeneration of glutathione. Photochem Photobiol. 2005;81:609–13.
115. Wang H, Qian S, Schafer F, Domann F, Oberley L, Buettner G. Phospholipid hydroperoxide glutathione peroxidase protects against singlet oxygen-induced cell damage of photodynamic therapy. Free Radic Biol Med. 2001;30:825–35.
116. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst. 2000;92:1295–1302.
117. Mikešová L, Mikeš J, Kovaľ J, Gyurászová K, Culka L, Vargová J, Valeková B, Fedoročko P. Conjunction of glutathione level, NAD(P)H/FAD redox status and hypericin content as a potential factor affecting colon cancer cell resistance to photodynamic therapy with hypericin. Photodiagnosis Photodyn Ther. 2013;10:470–83.
118. Aliya S, Reddanna P, Thyagaraju K. Does glutathione S-transferase Pi (GST-Pi) a marker protein for cancer? Mol Cell Biochem. 2003;253:319–27.
119. Kulbacka J, Chwilkowska A, Bar J, Pola A, Banas T, Gamian A, Saczko J. Oxidative alterations induced in vitro by the photodynamic reaction in doxorubicin-sensitive (LoVo) and -resistant (LoVoDX) colon adenocarcinoma cells. Exp Biol Med (Maywood). 2010;235:98–110.
120. Wright KE, MacRobert AJ, Phillips JB. Inhibition of specific cellular antioxidant pathways increases the sensitivity of neurons to meta-tetrahydroxyphenyl chlorin-mediated photodynamic therapy in a 3D co-culture model. Photochem Photobiol. 2012;88:1539–45.
121. Kimani SG, Phillips JB, Bruce JI, MacRobert AJ, Golding JP. Antioxidant inhibitors potentiate the cytotoxicity of photodynamic therapy. Photochem Photobiol. 2012;88:175–87.
122. Schlesinger M. Heat shock proteins. J Biol Chem 1990;265:12111–4.
123. Gomer CJ, Luna M, Ferrario A, Rucker N. Increased transcription and translation of heme oxygenase in Chinese hamster fibroblasts following photodynamic stress or Photofrin II incubation. Photochem Photobiol. 1991;53:275–9.
124. Curry PM, Levy JG. Stress protein expression in murine tumor cells following photodynamic therapy with benzoporphyrin derivative. Photochem Photobiol. 1993;58:374–9.
125. Gomer CJ, Ferrario A, Rucker N, Wong S, Lee AS. Glucose regulated protein induction and cellular resistance to oxidative stress mediated by porphyrin photosensitization. Cancer Res. 1991;51:6574–9.
126. Jozkowicz A, Was H, Dulak J. Heme oxygenase-1 in tumors: is it a false friend? Antioxid Redox Signal. 2007;9:2099–117.
127. Gomer C, Rucker N, Wong S. Porphyrin photosensitivity in cell lines expressing a heat-resistant phenotype. Cancer Res. 1990;50:5365–8.
128. Verwanger T, Sanovic R, Aberger F, Frischauf AM, Krammer B. Gene expression pattern following photodynamic treatment of the carcinoma cell line A-431 analysed by cDNA arrays. Int J Oncol. 2002;21:1353–9.
129. Hanlon J, Adams K, Rainbow A, Gupta R, Singh G. Induction of HSP60 by Photofrin-mediated photodynamic therapy. J Photochem Photobiol. 2001;64:55–61.
130. Wang H, Hanlon J, Rainbow A, Espiritu M, Singh G. Up-regulation of HSP27 plays a role in the resistance of human colon carcinoma HT29 cells to photooxidative stress. Photochem Photobiol. 2002;76:98–104.
131. Xue LY, He J, Oleinick NL. Rapid tyrosine phosphorylation of HS1 in the response of mouse lymphoma L5178Y-R cells to photodynamic treatment sensitized by the phthalocyanine Pc 4. Photochem Photobiol. 1997;66:105–13.
132. Nowis D, Legat M, Grzela T, Niderla J, Wilczek E, Wilczynski GM, Głodkowska E, Mrówka P, Issat T, Dulak J, Józkowicz A, Waś H, Adamek M, Wrzosek A, Nazarewski S, Makowski M, Stokłosa T, Jakóbisiak M, Gołab J. Heme oxygenase-1 protects tumor cells against photodynamic therapy-mediated cytotoxicity. Oncogene. 2006;25:3365–74.
133. Firczuk M, Gabrysiak M, Barankiewicz J, Domagala A, Nowis D, Kujawa M, Jankowska-Steifer E, Wachowska M, Glodkowska-Mrowka E, Korsak B, Winiarska M, Golab J. GRP78-targeting subtilase cytotoxin sensitizes cancer cells to photodynamic therapy. Cell Death Dis. 2013;4:e741.

134. Järveläinen H, Sainio A, Koulu M, Wight TN, Penttinen R. Extracellular matrix molecules: potential targets in pharmacotherapy. Pharmacol Rev. 2009;61:198–223.
135. Frantz C, Stewart K, Weaver V. The extracellular matrix at a glance. J Cell Sci. 2010;123:4195–200.
136. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110:673–87.
137. Rippa AL, Vorotelyak EA, Vasiliev AV, Terskikh VV. The role of integrins in the development and homeostasis of the epidermis and skin appendages. Acta Naturae. 2013;5:22–33.
138. Cianfriglia M, Yassen A, Tombesi M, Samoggia P, Barca S, Caserta M. Expression of lymphocyte homing receptor gene is lost in multi-drug-resistant variant of human lymphoblastoid CCRF-CEM cells. Int J Cancer. 1991;49:394–7.
139. Thompson E, Paik S, Brunner N, Sommers C, Zugmaier G, Clarke R, Shima T, Torri J, Donhaue S, Lippman M, Martin G, Dickson R. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol. 1992;150:534–44.
140. Hazlehurst LA, Dalton WS. Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies. Cancer Metastasis Rev. 2001;20:43–50.
141. Schmidmaier R, Baumann P. ANTI-ADHESION evolves to a promising therapeutic concept in oncology. Curr Med Chem. 2008;15:978–90.
142. Cordes N, Meineke V. Cell adhesion-mediated radioresistance (CAM-RR): extracellular matrix-dependent improvement of cell survival in human tumor and normal cells in vitro. Strahlenther Onkol. 2003;179:337–44.
143. Cordes N. Overexpression of hyperactive integrin-linked kinase leads to increased cellular radiosensitivity. Cancer Res. 2004;64:5683–92.
144. Margaron P, Sorrenti R, Levy JG. Photodynamic therapy inhibits cell adhesion without altering integrin expression. Biochim Biophys Acta. 1997;1359:200–10.
145. Runnels JM, Chen N, Ortel B, Kato D, Hasan T. BPD-MA mediated photosensitization in vitro and in vivo: cellular adhesion and beta1 integrin expression in ovarian cancer cells. Br J Cancer. 1999;80:946–53.
146. Rousset N, Vonarx V, Eleouet S, Carre J, Kerninon E, Lajat Y, Patrice T. Effects of photodynamic therapy on adhesion molecules and metastasis. J Photochem Photobiol B. 1999;52:65–73.
147. Momma T, Hamblin MR, Wu HC, Hasan T. Photodynamic therapy of orthotopic prostate cancer with benzoporphyrin derivative: local control and distant metastasis. Cancer Res. 1998;58:5425–31.
148. Casas A, Di Venosa G, Vanzulli S, Perotti C, Mamome L, Rodriguez L, Simian M, Juarranz A, Pontiggia O, Hasan T, Batlle A. Decreased metastatic phenotype in cells resistant to aminolevulinic acid-photodynamic therapy. Cancer Lett. 2008;271:342–51.
149. Foultier M, Vonarx-Coinsmann V, Cordel S, Combre A, Patrice T. Modulation of colonic cancer cell adhesiveness by haematoporphyrin derivative photodynamic therapy. J Photochem Photobiol. 1994;23:9–17.
150. Vonarx V, Foultier M, de Brito X, Anasagasti L, Morlet L, Patrice T. Photodynamic therapy decreases cancer colonic cell adhesiveness and metastatic potential. Res Exp Med. 1995;195:101–16.
151. Overhaus M, Heckenkamp J, Kossodo S, Leszcynski D, La Muraglia G. Photodynamic therapy generates a matrix barrier to invasive vascular cell migration. Circ Res. 2000;86:334–40.
152. Lee C, Wu S, Chen L. Photosensitization by 3,3'-dihexyloxacarbocyanine iodide: specific disruption of microtubules and inactivation of organelle motility. Cancer Res. 1995;55:2063–9.
153. Ruoslahti E. Fibronectin and its integrin receptors in cancer (Review). Adv Cancer Res. 1999;76:1–20.
154. Sanovic R, Krammer B, Grumboeck S, Verwanger T. Time-resolved gene expression profiling of human squamous cell carcinoma cells during the apoptosis process induced by photodynamic treatment with hypericin. Int J Oncol. 2009;35:921–39.

155. Buytaert E, Matroule JY, Durinck S, Close P, Kocanova S, Vandenheede JR, de Witte PA, Piette J, Agostinis P. Molecular effectors and modulators of hypericin-mediated cell death in bladder cancer cells. Oncogene. 2008;27:1916–29.
156. Galaz S, Espada J, Stockert JC, Pacheco M, Sanz-Rodriguez F, Arranz R, Rello S, Canete M, Villanueva A, Esteller M, Juarranz A. Loss of E-cadherin mediated cell-cell adhesion as an early trigger of apoptosis induced by photodynamic treatment. J Cell Physiol. 2005;205:86–96.
157. Casas A, Sanz-Rodriguez F, Di Venosa G, Rodriguez L, Mamone L, Blázquez A, Jaén P, Batlle A, Stockert JC, Juarranz A. Disorganisation of cytoskeleton in cells resistant to photodynamic treatment with decreased metastatic phenotype. Cancer Lett. 2008;270:56–65.
158. Giese G, Schmidt J, Gilbert M, Albrecht R, Traub P. TPA induces apoptosis in MPC-11 mouse plasmacytoma cells grown in serum-free medium. Biol Cell. 1997;89:99–111.
159. Hashimoto M, Inoue S, Ogawa S, Conrad C, Muramatsu M, Shackelford D, Maliah E. Rapid fragmentation of vimentin in human skin fibroblasts exposed to tamoxifen: a possible involvement of caspase-3. Biochem Biophys Res Commun. 1998;247:401–6.
160. Belichenko I, Morishima N, Separovic D. Caspase-resistant vimentin suppressed apoptosis after photodynamic treatment with a silicon phthalocyanine in Jurkat cells. Arch Biochem Biophys. 2001;390:57–63.
161. Perry R, Matthews W, Mitchell J, Russo A, Evans S, Pass H. Sensitivity of different human lung cancer histologies to photodynamic therapy. Cancer Res. 1990;50:4272–6.
162. Dodson JW, Hay ED. Secretion of collagen by corneal epithelium. II. Effect of the underlying substratum on secretion and polymerisational epithelial cell products. J Exptl Zool. 1974;789:51–72.
163. Gospodarowicz D, Greenburg G, Birdwell CR. Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. Cancer Res. 1978;38:4155–71.
164. Brambilla E, Moro D, Gazzeri S, Brichon P, Nagy-Mignotte H, Morel F, Jacrot M, Brambilla C. Cytotoxic chemotherapy induces cell differentiation in small-cell lung carcinoma. J Clin Oncol. 1991;9:50–61.
165. Richter A, Kelly B, Chow J, Liu D, Towers G, Dolphin D, Levy J. Preliminary studies on a more effective phototoxic agent than hematoporphyrin. J Natl Cancer Inst. 1987;79:1327–2.
166. Porro A, Chrochemore C, Cambuli F, Iraci N, Contestabile A, Perini G. Nitric oxide control of MYCN expression and multi drug resistance genes in tumors of neural origin. Curr Pharm Des. 2010;16:431–9.
167. Esteban FJ, Horcajadas A, El-Rubaidi O, Luque-Barona R, Ibáñez G, García-Carriazo A, Segovia M, del Moral-Leal ML. Nitric oxide in malignant astrocytes. Rev Neurol. 2005;40:437–40.
168. Leung EL, Fraser M, Fiscus RR, Tsang BK. Cisplatin alters nitric oxide synthase levels in human ovarian cancer cells: involvement in p53 regulation and cisplatin resistance. Br J Cancer. 2008;98:1803–09.
169. Rubbo H, Radi R, Trujillo M, Kalyanaraman B, Barnes S, Freeman BA. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. J Biol Chem. 1994;269:26066–75.
170. Niziolek M, Korytowski W, Girotti AW. Chain-breaking antioxidant and cytoprotective action of nitric oxide on photodynamically stressed tumor cells. Photochem Photobiol. 2003;78:262–70.
171. Fetz V, Bier C, Habtemichael N, Schuon R, Schweitzer A, Kunkel M, Engels K, Kovács AF, Schneider S, Mann W, Stauber RH, Knauer SK. Inducible NO synthase confers chemoresistance in head and neck cancer by modulating survivin. Int J Cancer. 2009;124:2033–41.
172. Kim YM, Bergonia H, Lancaster JR. Nitric oxide-induced autoprotection of isolated rat hepatocytes. FEBS Lett. 1995;374;228–32.
173. Joshi MS, Ponthier JL, Lancaster JR. Cellular antioxidant and prooxidant actions of nitric oxide. Free Radic Biol Med. 1999;27:1357–66.
174. Niziolek M, Korytowski W, Girotti AW. Nitric oxide-induced resistance to lethal photooxidative damage in a breast tumor cell line. Free Radic Biol Med. 2006;40:1323–31.

175. Niziolek-Kierecka M, Pilat A, Korytowski W, Girotti AW. Apoptosis-accommodating effect of nitric oxide in photodynamically stressed tumor cells. Photochem Photobiol. 2010;86:681–6.
176. Zareba M, Niziolek M, Korytowski W, Girotti AW. Merocyanine 540-sensitized photokilling of leukemia cells: role of post-irradiation chain peroxidation of plasma membrane lipids as revealed by nitric oxide protection. Biochim Biophys Acta. 2005;1722:51–9.
177. Di Venosa G Casas A Fukuda H Perotti C Batlle A. No cross-resistance between ALA-mediated photodynamic therapy and nitric oxide. Nitric Oxide. 2005;13:155–62.
178. Di Venosa G Perotti C Fukuda H Batlle A Casas A. Sensitivity to ALA-PDT of cell lines with different nitric oxide production and resistance to NO cytotoxicity. J Photochem Photobiol B. 2005;80:195–202.
179. Bhowmick R, Girotti AW. Cytoprotective Induction of nitric oxide synthase in a cellular model of 5-aminolevulinic acid-based photodynamic therapy. Free Radic Biol Med. 2010;48:1296–301.
180. Bhowmick R, Girotti AW. Cytoprotective signaling associated with nitric oxide upregulation in tumor cells subjected to photodynamic therapy-like oxidative stress. Free Radic Biol Med. 2013;57:39–48.
181. Vaupel P, Harrison L. Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. Oncologist. 2004;9:4–9.
182. Ferrario A, von Tiehl KF, Rucker N, Schwarz MA, Gill PS, Gomer CJ. Antiangiogenic treatment enhances photodynamic therapy responsiveness in a mouse mammary carcinoma. Cancer Res. 2000;60:4066–9.
183. Okunaka T, Usuda J, Ichinose S, Hirata H, Ohtani K, Maehara S, Inoue T, Imai K, Kubota M, Tsunoda Y, Kuroiwa Y, Tsutsui H, Furukawa K, Nishio K, Kato H. A possible relationship between the anti-cancer potency of photodynamic therapy using the novel photosensitizer ATX-s10-Na(II) and expression of the vascular endothelial growth factor in vivo. Oncol Rep. 2007;18:679–83.
184. Askari JA, Buckley PA, Mould AP, Humphries MJ. Linking integrin conformation to function. J Cell Sci. 2009;122:165–70.
185. Reyal J. Arterial flow focalization could increase tissue oxygen partial pressure, or trigger endothelial shear stress—a new concept to overcome cancer hypoxia-induced radiotherapy resistance, or stimulate liver regeneration during fulminant hepatitis. Med Hypotheses. 2009;74:301–8.
186. Boyer M. Bioreductive agents: a clinical update. Oncol Res. 1997;9:391–5.
187. Patterson A, Williams K, Cowen R, Jaffar M, Telfer B, Saunders M, Airley R, Honess D, van der Kogel A, Wolf C, Stratford U. Oxygen-sensitive enzyme-prodrug gene therapy for the eradication of radiation-resistant solid tumors. Gene Ther. 2002;9:946–54.
188. Aemany-Ribes M, García-Díaz M, Busom M, Nonell S, Semino CE. Toward a 3D cellular model for studying in vitro the outcome of photodynamic treatments: accounting for the effects of tissue complexity. Tissue Eng Part A. 2013;19:1665–74.

Chapter 3
Tumor Microenvironment as a Determinant of Photodynamic Therapy Resistance

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Abstract The tumor microenvironment is a complex force to be reckoned with in terms of cancer treatment. Structure and composition of the tumor stroma, oxygenation status within the tumor, and expression and/or activation of proteins that mediate tumor progression can contribute to the efficacy of, or resistance to, various therapeutic modalities. Photodynamic therapy (PDT) is no exception—the oxygenation status and molecular makeup of the tumor and its stroma is critically important to the success of PDT. Moreover, the application of light therapy to a tumor can counteract the therapeutic benefit by altering the microenvironment. For example, PDT is capable of inducing hypoxia which can limit the extent of PDT damage (by consuming oxygen too rapidly), initiating angiogenesis which allows for reestablishment of the tumor vasculature, and activating survival signaling pathways and increasing expression of proteins which promote tumor progression. This chapter highlights key players in the tumor microenvironment that contribute to treatment failure as well as how resistance can be circumvented by overcoming these road blocks. Further, this chapter will discuss various technologies developed to monitor the tumor microenvironment in an effort to improve PDT dosimetry, allowing for personalized treatment that increases therapeutic efficacy.

Keywords Photodynamic therapy · Tumor microenvironment · Hypoxia · Stroma · Extracellular matrix · Epidermal growth factor receptor · Vascular endothelial growth factor · Fluence rate · Diffuse reflectance spectroscopy · Diffuse correlation spectroscopy

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Abbreviations

μW	microwatts
17-AAG	17-allylamino-17-demethoxygeldanamycin
3D	three dimensional
ABCG2	ATP binding cassette transporter 2
AKT	protein kinase B
ALA	aminolevulinic acid
AlPcS	Aluminum phthalo-cyanine-tetrasulfate
ATP	adenosine triphosphate
BFR	blood flow rate
BPD	benzoporphyrin derivative monoacid ring a
Ce6	Chlorin e6
cm	centimeter
COX-2	cyclooxygenase 2
DCS	diffuse correlation spectroscopy
DOT	diffuse optical tomography
DRS	diffuse reflectance spectroscopy
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMMPRIN	extracellular matrix metalloproteinase inducer
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
GRP-78	78 kDa glucose regulated protein
h	hour(s)
H_2O	water
Hb	deoxyhemoglobin
HbO_2	oxyhemoglobin
HIF-1α	hypoxia inducible factor 1 alpha
HPPH	2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide a
Hsp	heat shock protein(s)
MAPK	mitogen activated protein kinase
MLu	motexafin lutetium
MMP	matrix metalloproteinase
MPPa	pyropheophorbide a methyl ester
mTHPC	meso-tetrahydroxyphenyl chlorin
mW	milliwatts
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nm	nanometer
PDT	photodynamic therapy
PI3K	phosphoinositide-3 kinase
PpIX	protoporphyrin IX
RIF	radiation-induced fibrosarcoma
SCC	squamous cell carcinoma

Sec	seconds
SFDI	spatial frequency domain imaging
STAT3	signal transducer and activator of transcription 3
StO_2	hemoglobin oxygen saturation
tHB	total hemoglobin
TIMP-1	tissue inhibitor of metalloproteinases
VEGF	vascular endothelial growth factor

Introduction

As is the case with other forms of cancer therapy, photodynamic therapy (PDT) must face a comprehensive set of obstacles that are adeptly employed by tumors to mitigate the cytotoxic effects of treatment. The local tumor microenvironment is one such obstacle. Many tumors are characterized by poorly formed vascular and lymphatic systems that hinder attempts to treat the disease from many fronts. The presence of tortuous blood vessels of irregular composition and their lack of organization into a spatially well-distributed hierarchical network can compromise the delivery of drugs to a tumor, whether they be chemotherapeutic agents, molecular targeting drugs, or photosensitizers [1]. Further exacerbating this problem, drug extravasation into the tumor parenchyma can be impeded by high interstitial fluid pressures and/or the aberrant deposition of stroma [2]. The presence of tumor hypoxia in regions of chronic or intermittent poor perfusion contributes to resistance to oxygen-dependent treatments, such as radiation or PDT [3,4]. Moreover, a host of molecular alterations that facilitate the survival of tumor cells in their unfavorable environment also promote resistance or enable recurrence to treatment; for example, overexpression of multidrug resistance genes is associated with pump-mediated efflux of therapeutic drugs from tumor cells [5], aberrant activation of survival pathways can protect tumor cells from the insult of therapy [6], and upregulation of angiogenic factors can initiate vascularization needed for tumor regrowth after a sublethal insult [7]. The tumor microenvironment is undoubtedly a factor to be contended with in the treatment of tumors with PDT. Yet, just as with other therapies, there are means to overcome, avoid, or even take advantage of the unique characteristics of the tumor microenvironment to provide effective treatment using PDT.

The Hypoxia Story

A well-developed body of literature indisputably establishes the limitations that hypoxia can impose on tumor response to PDT. Notably however, the context of these, and the great majority of other investigations on the effect of hypoxia on PDT, is not based on the specific effects of pre-existing tumor hypoxia, but rather on the consequences of a rapid and severe hypoxia that can be created by PDT itself (Fig. 3.1;

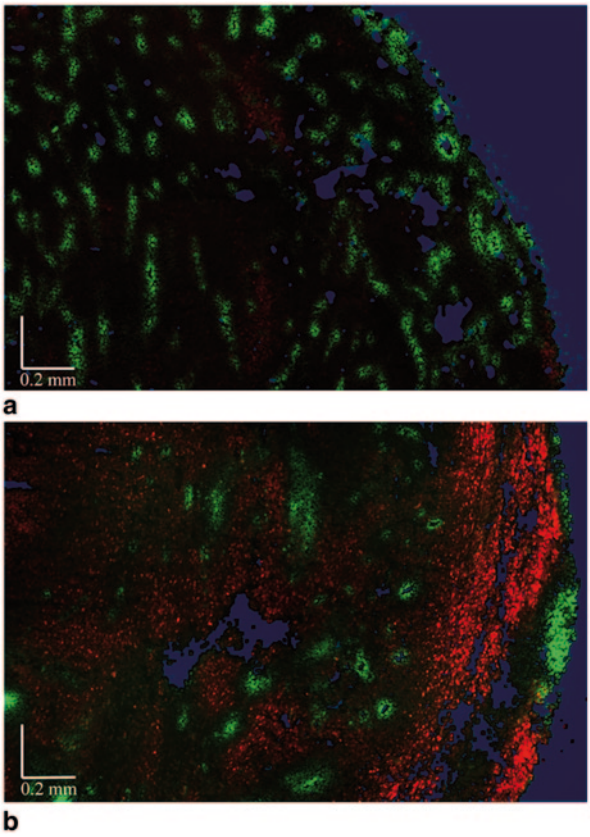

Fig. 3.1 PDT produces tumor hypoxia during the illumination period. Images of hypoxia in control (**a**) and PDT-treated (**b**) murine tumors labeled with the hypoxia marker EF3 ([2-(2-nitroimidazol-1[H]-yl)-N-(3,3,3-trifluoropropyl) acetamide]). Hypoxia is depicted in *red* and perfusion in *green*. Hypoxia labeling was performed over the course of 30 min of PDT (Photofrin, 5 mg/kg, 75 mW/cm^2, 135 J/cm^2) and perfusion was labeled just before tumor excision (at the conclusion of PDT) by i.v. injection of Hoechst 33342. PDT-induced increases in tumor hypoxia are visible in the intensity and extent of EF3 staining (*in red*). Reprinted with permission from Busch TM, et al. [11]

[8–11]). This hypoxia can result from oxygen consumption by the photochemical reaction, as well as from the consequences of ischemia that developed during the illumination period [4].

Fluence Rate Effects

Toward the goal of reducing PDT-triggered hypoxia during illumination, investigators have studied how its occurrence is related to the exposure parameters used for illumination. In particular, the fluence rate of illumination was identified for its role in altering tumor physiology during PDT whereby the lowering of fluence rate was found to help maintain the oxygenation of tumors during their illumination [12]. Data from murine tumor models reveal that lower fluence rate helps to preserve tumor oxygenation during illumination in cells most removed from perfused vasculature, which is the location where cells would be most expected to be at risk from the effects of photochemical oxygen consumption [11]. Furthermore, the lower fluence rate benefited the oxygenation of even tumor regions adjacent to the vasculature, which importantly demonstrates that PDT-created oxygen depletion

can encompass even the best perfused regions of a tumor [11]. Use of a low fluence rate is advantageous for many photosensitizers, with this benefit attributable to multiple mechanisms. For example, the hypoxia-combatting effects of slowing photochemical oxygen consumption could increase the cytotoxic effects of PDT to tumor cells and vascular endothelial cells, thus contributing to both the direct damage of tumor cells and the anti-vascular effects of PDT. Indeed, performing Photofrin-PDT at a low fluence rate produced more tumor cell kill while also augmenting the vascular effects of treatment [13]. Fluence rate effects on tumor hemodynamics during illumination are also detectable for PDT with 5-aminolevulinic acid (ALA) [14], albeit this result does not extend to all studies of ALA-mediated PDT [15]. In another mechanism of low fluence rate effect, the longer treatment times that are associated with delivery of equivalent fluence at a lower rate result in greater vascular damage due to the increased time that tumor blood vessels are subject to PDT [16, 17].

Therapeutically, the benefits of lower fluence rate are detectable through increases in treatment-created apoptosis [18, 19] and necrosis [20], as well as better long term tumor responses [17, 21]. Due to its apoptotic effect, use of low fluence rate may have the advantage of reducing normal tissue toxicity [18]. Light delivery at lower fluence rate can inhibit PDT-induced inflammation, under the condition that the full dose of an effective treatment regimen is delivered at the low rate [9]. Interestingly, low fluence rate can also abrogate PDT-induced immunosuppression [22]. In a rodent peritoneal model of ovarian cancer, Estevez et al [23] showed that even a small decrease in fluence rate from 30 to 20 mW/cm^2 for hexaminolaevulinate-mediated PDT of equal treatment length led to increases in the frequency of full-thickness necrosis of tumor nodules. In agreement with this observation, it is known that lower fluence rate leads to a more homogeneous PDT response throughout the tumor volume that is characterized by a more uniform oxygenation, vascular, and cytotoxic response to PDT [13].

Fractionated Illumination

Another light delivery approach to decrease the extent of PDT-created hypoxia during illumination incorporates the addition of interruptions in light delivery. The resulting fractionation of illumination serves to interrupt the progression of hypoxia development, thereby facilitating a recovery in the level of tumor oxygenation during the break(s) in light delivery. In a hyperfractionated approach, the interruptions in illumination are on the order of seconds to a minute and are repeatedly performed throughout the course of light delivery. The resulting cyclic patterns of decreasing (during illumination) and then increasing (during fractionation) oxygen concentrations can readily be measured [24]. The ideal lengths of the fractionation intervals have been modeled by several investigators [25] and are thought to be strongly dependent on the tumor microenvironment and in particular the capillary density of the tumor [26]. This dependency stems from the impact of capillary density on tissue reoxygenation when the oxygen-consumptive stress, in this case PDT, is removed. Toward this point, Xiao et al [27] studied the survival benefit of fractionated PDT in rat models of well-differentiated prostate tumors that were characterized by

well-distributed, perfused networks of blood vessels and anaplastic prostate tumors that exhibited inhomogeneous perfusion and poor blood flow in the tumor core. Fractionation intervals of 100 s of light on and light off significantly improved the therapeutic response of both models to interstitial PDT with a benzoporphyrin derivative (QLT0074). In rats bearing the anaplastic tumor model, fractionated PDT increased the duration of median survival from 26 days with continuous illumination to 51 days with fractionated illumination. In the well-differentiated tumor, the improvement with fractionated illumination amounted to an increase from 65 to 82 days median survival after PDT with continuous versus fractionated illumination, respectively. However, when the fractionation interval was substantially shortened to 1–5 s, a survival benefit was maintained only in the well-differentiated tumors (survival increasing further to 95 days). The authors credited the better perfusion of the well-differentiated tumor model as a contributing factor to the better response of this model to very short fractionation intervals.

In contrast to the hyperfractionated regimens described above, in some cases fractionated PDT is described as incorporating only a single interruption in illumination that can range from minutes to hours long. This is effectively a two-part illumination scheme that has been studied mostly in association with PDT using ALA or other photosensitizer precursors. An investigation of two-part illumination incorporating a 2 h break in light delivery for ALA-PDT of mouse skin revealed it to cause greater damage than the corresponding protocol with continuous illumination [28]. These studies were performed in the context of low fluence rate (in their case, 20 mW/cm^2), since use of low fluence rate with ALA-PDT can reduce pain that is associated with the excitation of ALA-created protoporphyrin IX (PpIX). Thus, it was also a goal of the authors to evaluate if two-part illumination at low fluence rate could be as effective as two-part PDT at high fluence rate (50 mW/cm^2). Results to the affirmative support the possibility of testing two-part light delivery at low fluence rate in clinical trials. The findings of a clinical trial of two-part ALA-PDT for superficial basal cell carcinomas at 50 mW/cm^2 do suggest that the long-term response (5 year follow-up) of patients to two-part illumination is superior to continuous illumination [29]. The mechanisms behind the improvement in PDT response with two-part illumination remain to be fully elucidated and likely will vary by specific treatment protocol. Evidence of a benefit to tissue oxygenation during the interruption in light delivery has been documented in some cases, as have increases in tissue levels of the photosensitizer PpIX, which is synthesized as a result of ALA administration, but the contribution of these effects to PDT cytotoxicity is not always clear [30–32]. Interestingly, the break in light delivery may also disproportionally benefit PDT cytotoxicity to cells that contain low levels of PpIX [29].

New Light Sources

Emerging technologies and new techniques of light delivery stand to further alleviate the development of PDT-induced hypoxia during illumination. The introduction of devices that illuminate at low fluence rates for extended periods of time,

Fig. 3.2 Example of a textile based light delivery device that facilitates illumination of curved surfaces. (Reprinted with permission from Cochrane C, et al. [35])

also known as metronomic PDT, have proven feasible in both animal models and clinical studies. In PDT of rabbits with intracranial carcinomas, Bogaards et al [33] demonstrated the capability to deliver daily doses of low fluence rate illumination at approximately 6 mW/cm^2 from light-emitting diodes. In the clinic, a lightweight and potentially disposable light-emitting diode device has demonstrated efficacy in the treatment of Bowen's disease and superficial basal cell carcinoma by providing for the extended delivery (3 h) of red light (peak 620 nm) at 5 mW/cm^2 to ALA-sensitized lesions [34]. The development of a flexible light delivery device that consists of textile-incorporated optical fibers emitting approximately 18 mW/cm^2 at 635 nm will further facilitate the delivery of low fluence rate PDT to irregular anatomical surfaces (Fig. 3.2; [35]). Moreover, treatment with even lower fluence rates may also be effective. Recent studies establish the cytotoxic effects of ALA-PDT at ultralow (17 mW/cm^2) fluence rate when spheroids are exposed to either single or repetitive illumination of 24 h in duration [36]. Finally, the use of sunlight as the excitation source for methyl aminolevulinate-PDT is being studied in dermatology for treatment of actinic keratosis and basal cell carcinoma. Resulting data suggest this approach to provide for less painful treatment that is equivalently effective to PDT with artificial light sources for the appropriately selected patient groups [37–39].

A Role for Tumor Stroma

Aside from the role that the vasculature of a tumor plays in determining its oxygenation status, there are numerous other ways through which the stroma of a tumor can alter PDT response. The stroma of a tumor consists of connective tissues that support the function of its parenchymal cells. Blood vessels and extracellular matrix (ECM) are major components of the tumor stroma, intermixed with cells of connective tissues, such as fibroblasts, and inflammatory cells such as lymphocytes, macrophages, and granulocytes [40]. Ample evidence exists for the effects of stroma on the responsiveness of a tumor to therapies such as chemotherapy and radiation [41],

and it can similarly influence outcomes to PDT. Furthermore, the interaction of PDT with stroma is governed by factors that are specific to the application of PDT, thus leading to a set of unique considerations that present as both opportunities and challenges.

Vascular Basement Membrane

Early observations in the field noted the tendency for porphyrins, used as photosensitizers for PDT, to localize to collagenous areas within tumors [40]. Collagens are most abundant among the proteins of ECM, a secreted scaffold that also includes other adhesive proteins, such as fibronectin and lamin, and functions to support the assembly of cells and regulate their communication [42]. An affinity of photosensitizer for collagen is suggestive of the possibility that extracellular matrix composition may affect the PDT response. Indeed, it has been demonstrated that tumors with a greater composition of collagen IV, a major component of basement membrane, are more sensitive to PDT in a manner that is dependent on photosensitizer localization to the stroma [43]. This sensitivity resulted from increases in PDT-mediated vascular damage. Specifically, tumors of murine radiation-induced fibrosarcomas (RIF) in which the collagen composition was approximately doubled to account for 9.3% of the tumor area responded to PDT with a decrease in blood flow to 25% of the pre-PDT level, compared to the maintenance of flow at 66% in tumors that contained less collagen. In parallel, the blood vessels from tumors that contained more collagen were more highly congested with fibrin accumulation after PDT than those from tumors that contained less collagen. These differences in vascular response were associated with an improvement in long-term tumor response; 25% of the more highly collagenated tumors did not recur after treatment with PDT whereas all of the tumors regrew in the group that contained less collagen ([43]; Fig. 3.3).

Several mechanisms exist by which PDT of stroma could augment tumor response. In vitro studies show that PDT promotes the adhesion of circulating cells to some proteins of the ECM, a process that could critically contribute to vascular congestion and ultimately thrombosis. Fungaloi et al [44] studied how PDT of human placenta collagen I and collagen II, and of ECM-derived from human umbilical vein endothelial cells, affected the subsequent adhesion of platelets to their surfaces. Significantly fewer platelets adhered to the treated surface of ECM, however, large increases were detected in platelet adhesion to the treated surfaces of collagen I and III. This increase in platelet binding to collagen could not be accounted for by increases in the capacity of the collagen to bind von Willebrand Factor, a circulating glycoprotein that facilitates platelet adhesion to exposed collagen. Consequently, the authors concluded that PDT altered the direct interaction of platelets with collagen, perhaps through treatment-induced change in collagen structure. Indeed, there is both in vitro and in vivo evidence that PDT produces crosslinking of collagen [45, 46].

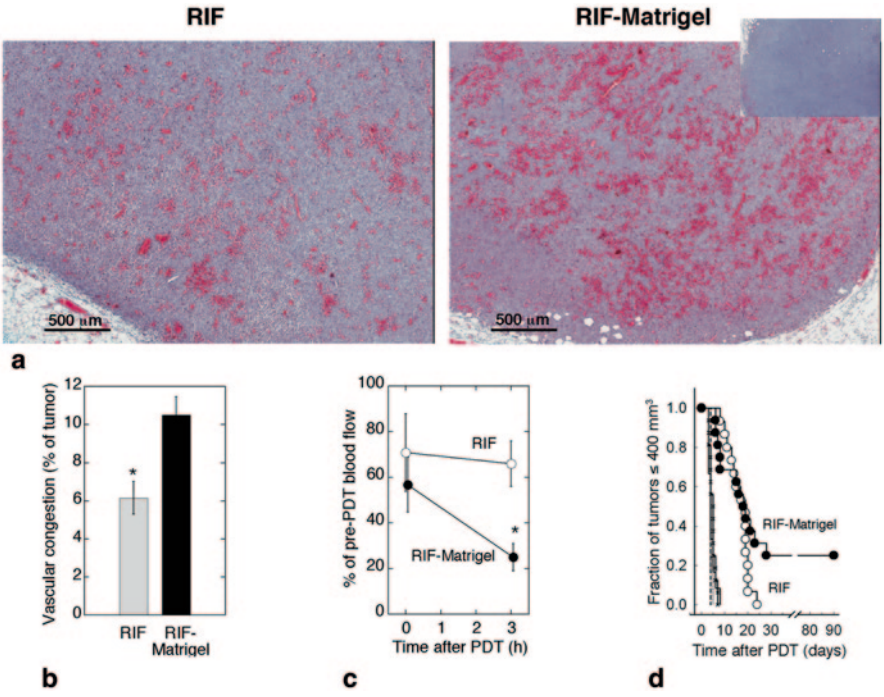

Fig. 3.3 PDT produces more vascular damage and has a better therapeutic effect in tumors that contain more collagen IV. Murine tumors supplemented with Matrigel Basement Membrane Matrix (RIF-Matrigel) at the time of tumor inoculation were characterized by a greater deposition of collagen IV in a vascular-associated pattern (9.3 % of the tumor area vs. 4.9 % in RIF-Matrigel vs. RIF tumor respectively; data not shown). PDT produced more vascular congestion (**a** and **b**) in the RIF-Matrigel model (inset in panel A shows untreated control). Correspondingly, RIF-Matrigel tumors experienced a greater reduction in tumor blood flow by 3 h after the completion of PDT (**c**), and long-term response to PDT was better in the RIF-Matrigel model (**d**). In the Kaplan-Meir plots (**d**), controls include untreated RIF (*dashed line*) untreated RIF-Matrigel (*dotted line*), light-only (*solid line*) and Photofrin-only (*crosshatch*). PDT performed with Photofrin (5 mg/kg) and 632 nm illumination at 75 mW/cm^2 to a dose of 135 J/cm^2. * indicates $p < 0.05$ for differences between RIF and RIF-Matrigel tumors. (Reprinted with permission from Maas AL, et al. [43])

In contrast to the above, others have shown that PDT promotes cell binding to ECM even in the absence of a treatment effect on ECM itself. In one investigation, PDT of an endothelial cell monolayer led to cell retraction and exposure of an underlying fibronectin matrix, which subsequently promoted the binding of PDT-naïve neutrophils [47]. PDT of fibronectin did not increase neutrophil binding. However, stimulation of endothelial cell retraction through means other than PDT did similarly promote neutrophil binding to the exposed matrix. These results suggest that PDT-induced damage of endothelial cells to expose basement membrane may contribute to vascular congestion and potentially thrombosis irrespective of an effect of PDT on the matrix itself. Moreover, the PDT-induced accumulation of neutrophils at the site of treatment has important implications in other aspects of

treatment response; for example, Kousis et al [48] demonstrated that neutrophil accumulation is involved in the development of inflammation and the induction of an antitumor immune response after PDT.

Taken together, the above indicates that in blood vessels that are highly associated with extracellular matrix, both the increased deposition of ECM as well as the PDT effect on ECM could potentially contribute to a stronger vascular response. Such an effect of ECM on vascular responses could further explain known discrepancies in the PDT responses of microvasculature versus that of larger vessels. Whereas PDT of microcapillaries frequently leads to thrombus formation and stasis of blood flow, treatment of larger diameter blood vessels can lead to endothelial cell denudation in the absence of thrombus formation [44]. This observation correlates with vessel-dependent variability in the collagen content of the vascular basement membrane whereby smaller arteries and veins can contain substantial amounts of collagen in their walls in contrast to, for example, that present in the basement membrane of a larger vessel such as the aorta [44]. The thoughts behind a collagen effect on size-dependent differences in vessel response to PDT must be reconciled against findings of PDT-created venule thrombosis in the presence of an intact endothelial cell layer [49]; nonetheless, however, the PDT response of larger vessels makes the therapy applicable to cardiovascular applications, where the ability of PDT to damage atherosclerotic plaques without stimulating restenosis is of interest to the field [46, 50].

Interstitial Stroma

In addition to the vessel-supporting ECM, the composition of the interstitial stroma of a tumor is also of consequence to disease progression and therapeutic resistance. Whereas tumors frequently exhibit degradation of the collagen IV that contributes to the basement membrane of endothelial and epithelial cells, they simultaneously contain increases in the deposition of fibrillar collagens, such as collagen I and III, which are major components of the interstitial ECM [51]. These fibrotic changes in ECM, together with an excess accumulation of cells such as fibroblasts, constitute a desmoplastic reaction that can be characteristic of tumors, including some breast and pancreatic malignancies [52, 53]. At a structural level, desmoplasia can contribute to treatment resistance by creating a barrier to drug delivery through promoting high interstitial fluid pressure, creating a physical obstruction to the passage of large molecular weight compounds, or directly binding and trapping therapeutic drugs [51]. The abundance of activated fibroblasts in the desmoplastic reaction also contributes to tumor progression, both by secreting ECM proteins such as collagen, as well as through signaling in positive feedback loops with malignant cells so as to promote tumor growth [53]. Furthermore, a loss of basement membrane and increases in interstitial collagen in tumors put the malignant cells in greater direct contact with extracellular matrix. This leads to the activation of molecular pathways that contribute to cell proliferation (e.g. through changes in the expression of cell

cycle proteins) or increase cell motility [e.g. through induction of integrin signaling and extracellular signal-regulated kinase 1/2 (ERK 1/2) activation], among other signaling-mediated changes associated with the malignant phenotype] [54].

Importantly, in terms of its interaction with interstitial stroma, PDT may not be subject to the same limitations faced by standard chemotherapeutic drugs. In studies of pancreatic cell lines, Celli et al [55] measured the cytotoxic effect of the chemotherapy drug gemcitabine, compared to PDT, when cells were grown on either standard tissue culture plastic or plates coated with growth factor-free Matrigel, a commercially available basement membrane preparation extracted from a murine sarcoma. Results with gemcitabine reproduced the known inhibitory effect of basement membrane on its cytotoxicity. However, the interaction of cells with Matrigel was of limited consequence to the killing potential of PDT, especially at high doses of light. Subsequently, Celli [56] published on the PDT response of 3D nodules of pancreatic cancer cells grown on a fibroblast-embedded "bed" of ECM. Results showed that nodules grown on the ECM with fibroblasts were equally sensitive to PDT as those grown in the absence of fibroblasts (Fig. 3.4). Again, this contradicts the treatment inhibitory effects known to result from the exposure of cancer cells to fibroblast-released factors in the study of therapeutic modalities other than PDT [57]. The effectiveness of PDT in the fibroblast-containing system could be a consequence of PDT-mediated cytotoxicity to the fibroblasts themselves, an observation that was noted as worthy of future investigations [56].

Cell-Matrix and Cell-Cell Associations

Broadly speaking, the impact of PDT on cell interaction with ECM could partly result from the effect of treatment on integrins. Integrins are transmembrane receptors that exist as heterodimers of R and T units and modulate cell adhesion to ECM, thereby, facilitating signaling between the extracellular and intracellular environment [58]. Integrin binding is involved in the association of cells to ECM and has been shown to play a role in the adhesion of PDT-treated cancer cells and normal cells (e.g., fibroblasts and neutrophils) to matrix proteins [47, 59–61]. However, numerous authors have reported that impaired cell-ECM attachment following PDT does not result from altered integrin expression [59, 60]. For example, a PDT-created loss in adhesion of human fibroblasts to collagen type I, human vitronectin, or human fibronectin was not associated with a loss in the expression of the integrins R_2, R_4, R_VT_5, T_1 or T_3 (where R_2, T_1, and T_3 mediated cell attachment to collagen; R_4, R_VT_5, and T_1 mediated attachment to fibronectin; and R_VT_5, T_1 and T_3 mediated attachment to vitronectin) [59]. Instead, PDT suppressed the phosphorylation of focal adhesion kinase (FAK), a protein tyrosine kinase that associates with the focal adhesions of integrin-mediated cell clustering on ECM and initiates the activation of signaling pathways that result from integrin binding [59]. In agreement with the hypothesis that PDT interferes with focal adhesion, others have shown PDT to alter patterns of focal adhesion formation [60, 61] and have documented that intracellular patterns of FAK expression are different between PDT-resistant versus parental

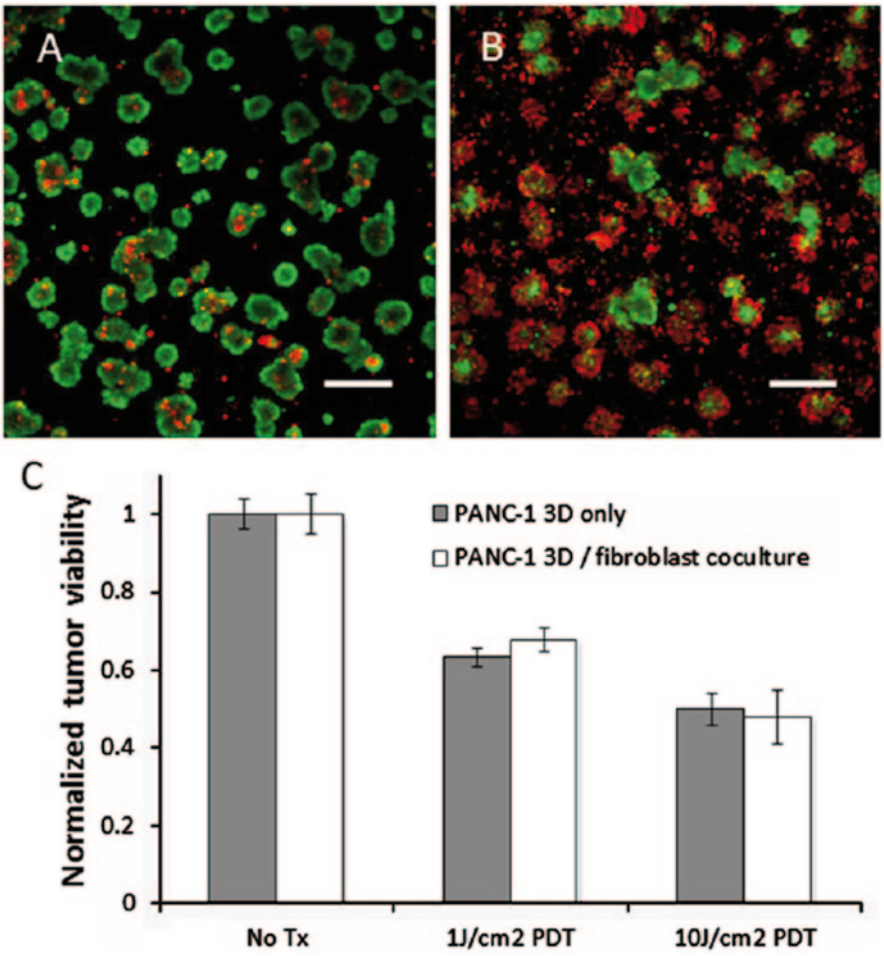

Fig. 3.4 PDT is equivalently cytotoxic to 3D nodules of pancreatic tumor cells (PANC-1) grown in the presence vs. absence of fibroblasts. Compared to control nodules (**a**), BPD-PDT (**b**) leads to cell death in pancreatic tumor nodules grown on a stromal bed that contains fibroblasts (*green* indicates viable cells via calcein staining; *red* indicates dead cells via ethidium bromide staining). The PDT-induced reduction in tumor viability was equivalent regardless of the presence (*white bars*) vs. absence (*gray bars*) of fibroblasts in the underlying stroma (**c**). (Reprinted with permission from Celli JP [56])

cell lines [62]. In addition to interfering with cell-matrix association, PDT can also disrupt the adhesion complexes formed by E-cadherin, a transmembrane protein that mediates cell-cell adhesion. These changes are temporally associated with the subsequent manifestation of PDT-induced apoptosis [63, 64].

The importance of the stroma in mediating the effects of PDT on solid tumors continues to emerge. It is clear that both structural (e.g. collagen) and cellular (e.g.

fibroblasts) components of the stroma have a part in determining the PDT sensitivity versus resistance of a tumor. Moreover, the precise nature of the interaction of PDT with stroma is intricately intertwined with the mechanisms through which a particular PDT regimen mediates its damage. In the case of vascular damage, improved consistency in the deposition of the subendothelial basement membrane and/or increases in its collagen content could increase the prevalence of outcome-benefitting microvascular congestion and thrombosis. In the case of tumor cell damage, the effect of PDT on cell-cell and cell-matrix interactions could potentially enable it to circumvent some of the limitations faced by other therapeutic modalities, making the presence of a dense interstitial matrix of less consequence to PDT than for other treatment options. Continuing research will further reveal the pathways by which stroma exerts an influence on PDT response thereby facilitating the development of approaches to bypass the limitations that it imposes and optimize the unique attributes of the PDT reaction with its components.

Mitigating the Molecular Microenvironment

In addition to the oxidative stress that is directly produced by PDT itself, therapy-resultant microenvironmental changes, such as hypoxia, inflammation, and vascular disruption can lead to alterations in protein expression and/or cellular signaling during and after treatment (Fig. 3.5)[65–67]. These signaling changes may counteract the therapeutic benefit of treatment, and even make a tumor more aggressive, thus, it is necessary to consider the impact of PDT on the molecular microenvironment. Several reports have highlighted "resistance" to PDT as a result of treatment-induced changes to tumor and endothelial cell biology [68, 69]. The most common molecules known to impair PDT response due to alteration in their expression or signaling following light treatment include the epidermal growth factor receptor (EGFR) [70–72], ERKs [73, 74], signal transducer and activator of transcription 3 (STAT3) [70], cyclooxygenase 2 (COX-2) [75, 76], heat shock proteins (Hsp) [67, 68, 77], matrix metalloproteinases (MMPs), and vascular endothelial growth factor (VEGF) [78–80]. These proteins contribute to tumor progression through processes that include the induction of survival signaling, cell growth and proliferation, promotion of cell motility and metastasis, inflammation, angiogenesis, and prevention of apoptosis.

Choice of treatment parameters influences the stimulation of molecular responses as they relate to microenvironmental effects. For example, fluence rate can either stimulate or impair the immune response to light treatment. Clinical reports demonstrate that low fluence PDT protocols appear to induce the immune response in patients with angiosarcoma, ultimately leading to a better long term therapeutic outcome than in patients treated with a high fluence rate [81]. Fluence rate can also elicit microenvironmental changes following PDT as a result of hypoxia-created stress since high fluence rates lead to greater depletion of oxygen compared to low fluence rate protocols [82]. This increase in hypoxia results in stabilization of the

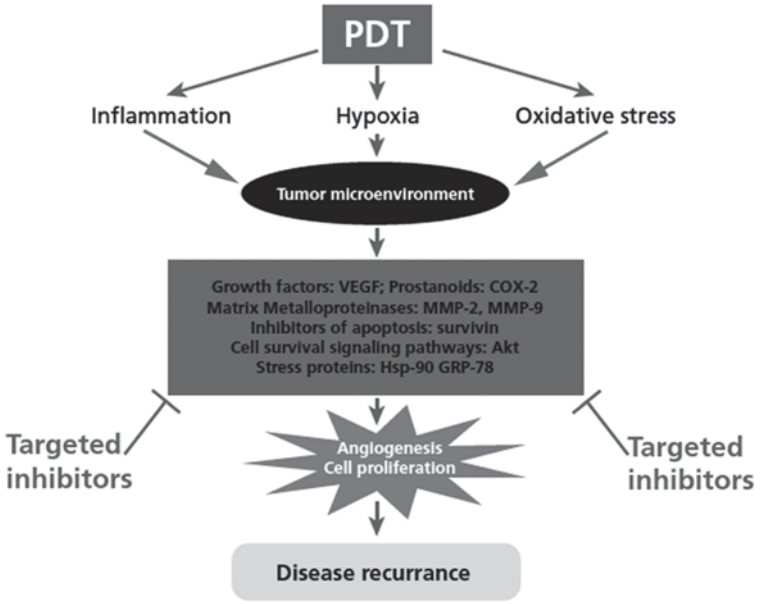

Fig. 3.5 Schematic highlighting the impact of PDT on the tumor microenvironment and how treatment resistance can develop. Included in this diagram are also proteins that are modulated by PDT and may be good candidates for molecular targeting agents. (Adapted from Gomer CJ [67], and reprinted with permission)

hypoxia inducible factor 1 α (HIF-1α), a transcription factor responsible for the induction of hundreds of genes that regulate processes such as survival and angiogenesis [77, 83]. Indeed, it is reported that use of high fluence rate hypericin-PDT results in increased HIF-1α expression and increased secretion of VEGF compared to low fluence rate PDT in human bladder carcinoma xenografts [82]. Inadequate deposition of light or nonhomogeneous distribution of photosensitizer further contributes to suboptimal treatment as a consequence of the induction of angiogenesis and expression of survival factors that lead to tumor progression [82]. Overcoming resistance to PDT involves understanding how microenvironment-initiated signaling of these pathways can compromise PDT response, as well as development of methods to counteract their altered activity toward a better treatment response.

Cell Signaling

The impact of PDT on cell signaling is well established, however, the mechanisms that underlie this effect remain under investigation. Numerous, and sometimes conflicting, reports exist demonstrating either increased or decreased expression of signaling molecules like EGFR, ERK, and STAT3, which can potentially contribute

to tumor recurrence or treatment resistance. EGFR is a cell surface receptor tyrosine kinase that is responsible for activation of several signaling pathways related to cell survival/protection from apoptosis (PI3K/AKT; STAT3) and cell growth/proliferation (MAPK; PI3K/AKT) [70, 72, 84, 85]. Aberrant expression or activation of EGFR is characteristic of numerous cancers, including non-small cell lung carcinoma, head and neck squamous cell carcinoma, breast carcinoma, and ovarian cancer [70, 84, 86, 87], and its activation can potentially be exacerbated by the use of PDT. For example, benzoporphyrin derivative monoacid ring a (BPD, Verteporfin)-mediated PDT is reported to increase phosphorylation of EGFR, as well as that of one of its downstream targets, STAT3. PDT also induces the nuclear translocation of these proteins in ovarian cancer and non-small cell lung carcinoma cell lines, which is suggested to initiate anti-apoptotic signaling, and thus contribute to treatment resistance [70].

Inhibition of EGFR in combination with PDT is reported to enhance treatment response in a number of different models, both in vitro and in vivo. Addition of the EGFR-targeting monoclonal antibody, cetuximab, to PDT (either in combination with or via conjugation of the antibody to a photosensitizing agent) inhibits tumor growth and increases cytotoxicity in bladder tumor xenografts [88], head and neck squamous cell carcinoma xenografts [89], and ovarian cancer tumor xenografts and cells [86, 90, 91]. EGFR inhibition can also be accomplished with the use of small molecule inhibitors, such as gefitinib and erlotinib; indeed, it has been reported that addition of PDT to gefitinib treatment in non-small cell lung carcinoma cell lines (which are frequently considered poor responders gefitinib) can induce greater cytotoxicity than PDT or gefitinib treatment alone [92]. Addition of erlotinib to PDT can attenuate EGFR activation and lead to increased cytotoxicity in ovarian cancer and non-small cell lung carcinoma cells [70]. Likewise, combining PDT with gefitinib is capable of inhibiting NF-κB activity, AKT-mediated signaling, and proteasome function [92]. PDT can briefly inhibit activation of one of the main effectors of MAPK signaling, ERK1/2, in laryngeal squamous cell carcinoma cells, effectively impeding cell motility (which is an indicator of metastatic potential) [74]. Others show that sustained inhibition of ERK1 activity following combination of PDT with tyrphostin (a tyrosine kinase inhibitor specific for EGFR) resulted in greater, synergistic cytotoxicity in colorectal adenocarcinoma cells [73].

Stress Response

The induction of proteins involved in cellular response to stress (e.g. COX-2, GRP78, Heat shock proteins, HIF-1α, survivin) is another common observation following PDT. Cyclooxygenase-2 (COX-2) is one of two isoforms of cyclooxygenase that is responsible for the conversion of arachidonic acid to prostaglandins, which are involved in inflammation and mitogenesis, among other functions [75]. COX-2 expression is associated with the development and progression of various malignancies—progression that can be further exacerbated by PDT as light treat-

ment is reported to induce the expression of biologically active COX-2 [75, 76]. Inhibition of COX-2 can be achieved with the use of either NS-398 (a commercially available COX-2 selective inhibitor) or Celecoxib (Celebrex®; a non-steroidal anti-inflammatory drug that selectively targets COX-2). Celecoxib is an FDA-approved agent indicated for osteoarthritis and rheumatoid arthritis, as well as management of acute pain in adults; but this drug is also under active investigation in the treatment of several types of cancer, often in combination with other treatment modalities. Preclinical investigation has demonstrated increased apoptosis of mouse mammary carcinoma cells when combining celecoxib with Photofrin-PDT [75]. When Photofrin-PDT was combined with NS-398, similar enhancements in therapeutic benefit were observed in RIF tumor xenografts [76]. Likewise, treatment of human cholangiocarcinoma xenografts with ALA-PDT and celecoxib resulted in a dramatic improvement in overall tumor response, compared to either therapy alone [93].

PDT can also induce the unfolded protein response, characterized by induction of several members of the heat shock protein family. Heat shock proteins (Hsp) are a class of chaperone proteins induced upon cellular stress whose primary function is to assist in proper protein folding, prevent protein aggregation, and stabilize partially folded proteins [68]. PDT modulates the expression and/or function of several members of the Hsp family, including GRP-78, Hsp27, and Hsp90, which are thought to contribute to treatment resistance [68, 77]. As with the proteins already discussed, resistance attributed to increased Hsp expression and activity can be overcome with the addition of drugs to counteract the induction of the stress response. For example, addition of 17-allylamino-17-demethoxygeldanamycin (17-AAG) to PDT was capable of increasing the curative response of a mouse mammary carcinoma tumor model. The improved treatment outcome following this combination therapy was attributed to inhibition of several molecular targets that are either associated with or induced by Hsp90, including HIF-1α and survivin [77].

Angiogenic Factors

VEGF is one of the prominent molecular factors induced by PDT that can lead to treatment failure. This cytokine is responsible for increasing blood vessel permeability and the growth, proliferation, migration, and differentiation of endothelial cells that are required for angiogenesis [94]. PDT-induced secretion of VEGF can stem from several effects of PDT on the physiologic and molecular microenvironment of tumors. First, vascular damage initiated by PDT can promote secretion of VEGF as the tumor attempts to re-establish its vasculature. Second, PDT is known to activate signaling cascades that lead to the secretion of VEGF, potentially providing an additional source of this cytokine following light therapy. Notably, many of the aforementioned molecules, such as HIF-1α and EGFR, can induce pathways which ultimately culminate in the secretion of VEGF, which is important for long term tumor progression by promoting angiogenesis and cell survival/motility [66, 79, 83–85].

Several groups have reported on PDT-induced increases in secretion of VEGF. In H460 human tumor xenografts, it was observed that treatment with BPD-PDT led to a nearly two-fold increase in human VEGF levels compared to untreated tumors [79]. Moderate increases in human VEGF have also been observed in the serum of mice bearing human bladder carcinoma cell xenografts following Hypericin-mediated PDT [82]. Even more dramatic increases in human VEGF levels were observed in human tumor xenografts of Kaposi's sarcoma treated with Photofrin-PDT [95]. As with EGFR signaling, the use of molecular targeting agents to combat this elevated VEGF secretion following PDT can be highly effective in improving long term therapeutic responses. Several preclinical studies have focused on the combination of PDT with inhibitors of VEGF, such as the anti-VEGF antibody, bevacizumab (Avastin®) [82, 94, 95].

Matrix metalloproteinases (MMPs) are endopeptidases that, upon activation, mediate the breakdown of the ECM to facilitate angiogenesis and cell motility. MMPs (specifically MMP-9) are also reported to recruit VEGF to the tumor microenvironment to assist in the formation of new vessels [65, 96]. It is important to note that MMPs are not typically expressed by tumor cells, but rather macrophages and the stromal cells of the matrix [65]. PDT is known to impact the expression of the proenzyme forms of several MMPs, including MMP-1, MMP-2, MMP-3, and MMP-9, as well as proteins which stimulate or impair their activity [65, 67, 97]. For example, Photofrin-PDT is reported to induce the expression of MMP-9 and the extracellular matrix metalloproteinase inducer (EMMPRIN), which activates MMP-9. Additionally, Photofrin-PDT decreases expression of the tissue inhibitor of metalloproteinases (TIMP-1), which is responsible for blocking MMP activity [98]. This study by Ferrario et al [98] further demonstrated that addition of the broad spectrum MMP inhibitor, Prinomastat, to PDT improved tumor responses in a mouse mammary carcinoma model.

Transport Proteins

Notwithstanding the induction of resistance-associated signaling pathways by PDT itself, the pre-existing expression of certain proteins in tumors can also impact the potential for effective treatment. Of particular note is the adenosine triphosphate (ATP) binding cassette transporter 2 (ABCG2). ABCG2 is an ATP-dependent drug-efflux pump that is expressed at the cell surface, mitochondrial membrane, and intracellular compartments of both normal and tumor tissues [99]. This transporter has been implicated in mediating drug resistance to chemotherapeutic agents as well as small molecule inhibitors used to treat cancer by pumping these drugs from the cell before they are able to accumulate and induce cytotoxicity. Certain photosensitizing agents of the porphyrin and chlorin families are also substrates for this transporter, and the efflux of these drugs via ABCG2 is reported to contribute to PDT resistance by decreasing the amount of photosensitizer available for the photochemical reaction in the intracellular compartment [100]. Specifically,

pyropheophorbide a methyl ester (MPPa), chlorin e6 (Ce6), 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide a (HPPH), BPD, hypericin, and PpIX generated from ALA are known substrates of ABCG2 [100, 101]. However, as with the signaling proteins just described, therapeutic response can be improved by combining PDT with inhibitors of ABCG2 [99].

The Importance of Combination Therapy to Overcoming Treatment Resistance

PDT resistance often arises from alteration of multiple molecular targets. As such, it is important to note that treatment resistance to PDT is unlikely to be attributed to a single molecular alteration. Rather, it is more logical to expect that PDT induces a host of molecular alterations within the treatment site, which function together to impair treatment response. For example, squamous cell carcinoma (SCC) associated with non-melanoma skin cancer can become resistant to methyl aminolevulinate-based PDT following repeated exposure. This resistance is related to several molecular alterations in resistant tumor cells, specifically in expression of the *EGFR, Cyclin D1,* and *ERK1/2* genes. These genes were observed to increase following PDT, thus rendering the SCC tumor cells more tumorigenic and attenuating the PDT response [69].

As discussed above, the addition of molecular targeting agents have been successful in improving tumor responses when combined with PDT. However, while these drugs are specific in their molecular targets, it should be noted that they can impact more than just their protein of interest. For example, EGFR inhibition can impact cellular signaling, which also includes activation of angiogenic factors like VEGF; consequently, EGFR inhibitors can block multiple factors that promote tumor progression. Nevertheless, the use of combinations of molecular targeting drugs provides more assurance of success when the inhibition of multiple pathways is desired. In an effort to provide a greater therapeutic impact, there has been preclinical investigation into the efficacy of combining multiple molecular targeting agents with PDT. Bhuvaneswari et al. [94] studied the combination of cetuximab and bevacizumab with hypericin-PDT in human tumor xenografts of bladder cancer. This treatment paradigm led to a dramatic inhibition of tumor growth, compared to treatment with PDT alone. Likewise, treatment of PDT with cetuximab and bevacizumab was more effective at rapidly inhibiting tumor growth than PDT combined with either drug alone.

In the translation of preclinical treatment paradigms to clinical protocols, it is important that the specificity of the inhibitor relative to species-dependent differences in the molecular targets (i.e. for human tumor xenografts inoculated in animal models) are considered when interpreting results. For example, in H460 human tumor xenografts of non-small cell lung cancer, PDT led to large increases in the secretion of VEGF from tumor-localized host (i.e. murine) cells. The human-specific antibody bevacizumab reduced levels of tumor (i.e. human) secreted VEGF after

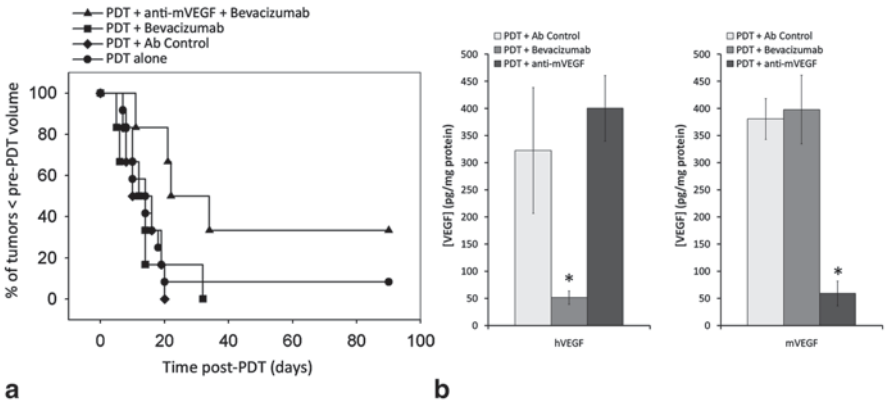

Fig. 3.6 The importance of considering tumor and host-derived molecular factors in evaluating treatment response. A tumor response assay shows that treatment with PDT in combination with anti-human (Bevacizumab) and anti-mouse VEGF (anti-mVEGF) antibodies improves therapeutic benefit compared to PDT alone or PDT in combination with anti-human VEGF antibody (**a**). This improved response highlights the importance of the host environment in mediating the treatment effect. Enzyme-linked immunosorbent assay (ELISA) reveals that the anti-human and anti-mouse VEGF antibodies are specific in decreasing secretion of their targets (**b**). Data are expressed as mean ± SE. $*p<0.05$ for comparison to PDT with antibody control. (Adapted from Gallagher-Colombo SM, et al. [79])

PDT, but it was not successful in abrogating the release of host VEGF, nor did it provide any long-term therapeutic benefit. An antibody against murine VEGF highly suppressed the release of host VEGF and its addition to the treatment regimen resulted in a more durable treatment response (Fig. 3.6) [79]. These data emphasize the importance of the host cells that compose the tumor microenvironment in dictating the therapy-determining activation of molecular pathways.

Microenvironmental Monitoring

Tumor microenvironment indisputably impacts response to PDT at the molecular, cellular and tissue levels. Thus, there is great potential to use information on the status of tumor physiology and/or hemodynamics during or surrounding treatment as a predictive or even dosimetric tool. This is best accomplished using noninvasive or minimally invasive techniques that can easily be applied in a clinical setting and provide the possibility of real time feedback. In this regard, optical techniques for characterization of the microenvironment are particularly well-suited to PDT. By definition, any treatment site appropriate for PDT is also accessible through optical measurement.

Techniques for Measuring Oxygenation and Blood Flow

Much work in the optical interrogation of tissue microenvironment has focused on contrast-free imaging, which relies on the inherent contrast of tissue rather than an exogenous agent. The contrast mechanisms available in tissue are changes in light scattering and changes in light absorption, with the primary scatters being water-lipid interfaces (e.g., cell and mitochondrial membranes). Absorption contrast, on the other hand, results primarily from a small number of intrinsic tissue chromophores. For most tissue, the primary absorbers are hemoglobin in its oxygenated and deoxygenated forms, and water, as shown in Fig. 3.7a. Differences in the concentrations of these absorbers lead to a wide variety of optical properties in living tissue [102]. The absorption of hemoglobin is complicated by the fact that it is confined to erythrocytes, which are in turn confined to vasculature. As a result, the absorption spectrum of hemoglobin exhibits a pigment packaging effect [103]. This essentially renders the hemoglobin centralized within large vessels to be invisible to optical measurements. Therefore, optical measurements of hemodynamics are inherently weighted toward the microvasculature. This is advantageous, as the microvasculature is responsible for supplying the majority of tissue with nutrients and oxygen, and is also the target of PDT damage with many treatment protocols.

The essential hemodynamics of tissue can be characterized by three related parameters: total hemoglobin content ([tHB]), hemoglobin oxygen saturation (StO_2), and blood flow rate (BFR). The relationship between these parameters is illustrated in Fig. 3.7b. [tHb] is proportional to the fraction of tissue occupied by blood. [tHb] can therefore be influenced by morphological variability, such as the density and tortuosity of vessels, and by dynamic changes such as vasodilation. Oxygen is supplied by the vasculature and diffuses radially into tissue. As oxygen leaves the vasculature, fewer hemoglobin molecules are bound to oxygen, so StO_2 is reduced along the length of the vessel. In normal tissue with regularly spaced capillaries, the tissue is well-oxygenated, oxygen diffusion is slow, and the reduction in StO_2 is moderate. In hypoxic tissue, the diffusion of oxygen is rapid, leading to significant reduction in StO_2. BFR is essentially a measure of how many erythrocytes pass

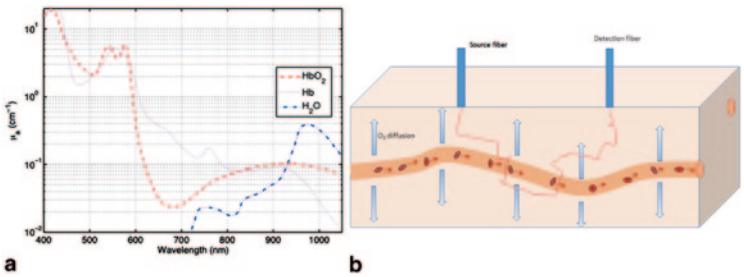

Fig. 3.7 Absorption spectra of oxy- and deoxy-hemoglobin and water, at concentrations typical of tissue (**a**). Plots are created using data from Finlay JC, et al. [103]. Schematic of diffuse optical detection in tissue, illustrating blood flow, oxygen diffusion, and light transport (**b**)

through a given volume of tissue per unit time. It is therefore influenced by both the velocity of blood flow in the vasculature and the capacity (or cross-sectional area) of the vessel. Increasing the BFR will effectively increase the oxygen supply and reduce the decrease in StO_2. BFR rate does not necessarily affect [tHB]; as long as the incoming and outgoing blood flow in a given tissue match, the [tHb] will remain constant. To this point, we have shown in mouse models that [tHb] can remain unchanged at the conclusion of PDT with an ischemia-creating protocol. With time, however, the progression of vascular shutdown at the center of the tumor in the presence of active peripheral blood flow led to *increases* in [tHb]. These increases could be attributed to increases in the deoxygenated form of hemoglobin, presumably as a result of blood accumulation in the tumor in the absence of effective egress [17]. Thus, the proper interpretation of hemodynamic parameters is aided by comprehensive knowledge of its components.

The absorption spectrum of tissue gives information about both [tHb] and StO_2 because, as stated above, the absorption of visible light by tissue is primarily due to hemoglobin. However, the absorption spectrum can further be broken down into contributions from deoxyhemoglobin ([Hb]), oxyhemoglobin ($[HbO_2]$), and water ($[H_20]$). [tHB] is the sum of [Hb] and $[HbO_2]$, and StO_2 is the ratio of $[HbO_2]$ to [tHb]. All these hemodynamic parameters can be monitored optically. In a typical measurement setup, light is launched into the tissue by an optical fiber, and collected by another fiber that is a defined distance away. The signal measured is composed mostly of multiply scattered light, which has no single pathlength. This signal is collectively referred to as diffuse reflectance, and the measurement of it as diffuse reflectance spectroscopy (DRS). As shown in Fig. 3.7b, the light collected by the detection fibers samples a large volume of tissue as it propagates. Optical measurements are therefore by nature volume-averaged measures of the tissue optical properties. To rigorously separate the effects of absorption from those of scattering requires measurements at multiple source-detector separations [104], time points [105], or over a wide spectral range where spectral features can be used to separate them [106].

The basic DRS measurement scheme has been adapted to a number of different geometries and modalities. In recent years, effort in this area has sought to expand DRS measurements from point measurements to images. For instance, diffuse measurements have been made interstitially during PDT of the prostate [107]. Combining multiple interstitial measurements produces a low-resolution image of the tissue. The logical extension of this strategy is to surround the tissue being interrogated with multiple light sources and detectors, and using multiple source-detector pairs to reconstruct a 3D map of the optical properties of the tissue, a technique known as diffuse optical tomography (DOT) [108]. DOT imaging has been used diagnostically for characterization of the lipid, water, and blood content of human breast tissue [109], and as part of treatment planning for prostate PDT [110]. Moreover, when cylindrical diffusing fibers are used as the light source for DOT, it is theoretically possible to also treat for PDT using the same setup. Such instrumentation would enable online dosimetry using DOT-measured hemodynamic parameters and move the use of microenvironmental measures for individualized treatment into the clinical setting [111].

It is not always practical to surround a target tissue with sources and detectors. When only the tissue surface is optically accessible, absorption and scattering images of the tissue can be reconstructed using spatial frequency domain imaging (SFDI). This technique projects a spatially-modulated light field on the tissue surface, and images the reflected light. Multiple images with different modulation of the incident light are then combined to reconstruct the scattering and absorption of the tissue [112]. The result is an image in which each pixel has essentially acted as a detector with multiple light sources. Quantitative measurement of tissue oxygenation using SFDI has been demonstrated in human subjects [113].

The above described techniques in DRS provide a snapshot of local hemoglobin content and saturation, but they do not provide a measure of a third essential component of tissue hemodynamics, that is blood flow. Assessment of blood flow requires a dynamic measurement, which can be accomplished optically using diffuse correlation spectroscopy (DCS). The basic setup for DCS is similar to that for DRS, but DCS looks at the changes in signal resulting from the motion of erythrocytes in the vasculature. In a static sample, the signal varies little with time, so the signal measured at one time will be highly correlated with the signal measured a short time later. Conversely, if a significant fraction of the cells in the sample are moving, the signal will vary quickly, and the temporal correlation of the signal will break down quickly. The time it takes for the correlation to break down can be directly related to BFR. As with DOT, DCS has been extended to 3D imaging, providing blood flow maps in addition to oxygenation and [tHb] distributions [114].

All diffuse optical measurements, from point measurements to 3D DOT images, have a fundamental resolution limit imposed by the optics of light transport in tissue. Because the light is scattered many times before being detected, the resolution of the measurement is limited by the range in possible paths that light can take from the source to the detector. This leads to several interesting characteristics of diffuse optical measurements. Increasing the separation between the source and detector has two effects: it increases the mean pathlength of the detected light, making the measurement more sensitive to scattering; it also samples a volume at greater depth in the tissue and averages over a larger volume. This characteristic has been exploited to separate blood flow measurements in different layers of tissue during PDT with findings that establish a microenvironmental advantage to low fluence rate [13]. In DOT, the depth-dependence is inherently taken into account in the reconstruction of the optical properties map, such that greater source-detector separations preferentially inform the reconstruction of optical properties at more distant points. Another effect of the volume-averaging inherent in diffuse optical measurements is a limitation on the scale of features that can be detected. In general, the pathlength over which the measurements average (mm to cm) is much greater than the size of absorption features such as capillaries (less than 1 mm) or scattering features such as membranes and organelles (microns). Despite this, scattering changes can provide information about the microscopic structure of tissue. This is because the wavelength dependence of the scattering spectrum depends critically on the size distribution of the scatterers. Scattering measurements can therefore provide information about cell morphology, such as the size of cell nuclei [115] and the density and shape of mitochondria [116] and lysosomes [117].

Importantly, both DCS and DRS show promise as microenvironmental-based predictive tools in PDT. Both types of measurements can detect the hemodynamic differences that high versus low fluence rates introduce to the local microenvironment during PDT. In murine tumors treated with ALA-PDT, Becker et al [14] found lower fluence rate (10 mW/cm^2) to produce smaller initial increases in blood flow followed by a recovery to higher levels of perfusion than that in tumors treated at higher fluence rates (35 and 75 mW/cm^2). This is consistent with expectations that low fluence rate produces less PDT-initiated ischemia during the illumination period [11]. Together, the presence of less treatment-induced ischemia and the oxygen-conserving photochemistry of low fluence rate can conserve tissue oxygenation during low rate illuminations. Indeed, Woodhams, et al [118] reported low fluence rate to lead to a slower rate of decline in the oxygen saturation of liver tissue during the course of light delivery for PDT mediated by aluminum disulphonated phthalocyanine. The power for hemodynamic monitoring to predict PDT-created cytotoxicity or treatment outcome has been demonstrated in rodent studies by several groups [119–122], including investigations that employed instruments based in DRS or DCS [119, 121, 122]. More recently, DCS has also been used to identify differences in the consistency of blood flow cycles in murine tumors that may be indicative of the sensitivity of the blood vessels to subsequent stresses such as PDT (Fig. 3.8; [123]).

Techniques for Measuring Photosensitizer Levels and Photobleaching

The distribution and levels of photosensitizer accumulation within the microenvironment can also play a major role in treatment outcomes. Quantification of the distribution of photosensitizers is important both for localizing areas of preferential accumulation of sensitizer and for assessing the optimal light dose based on sensitizer uptake. Fortunately, the majority of sensitizers in use today are fluorescent, allowing their detection via fluorescence spectroscopy. The basis of the geometry of the fluorescent measurement is similar to the DRS measurement, but now the source fiber launches excitation light at a wavelength strongly absorbed by the sensitizer, and the collection fiber collects the longer-wavelength emission from the sensitizer. Many of the challenges discussed in the last section regarding DRS also apply to in vivo tissue fluorescence measurement. In addition, the fluorescence signal depends on the effects of absorption and scattering at both the excitation and emission wavelengths. To quantitatively evaluate fluorescence signal, it is important to correct for these effects. This can be accomplished using a corresponding DRS measurement, if the fluorescence and DRS measurements are designed to sample similar volumes of tissue. As with DRS, fluorescence measurements can be extended to imaging modalities. In the context of PDT, a fluorescence version of SFDI imaging has been shown to generate quantitative maps of the distribution of the PpIX in preclinical models [124].

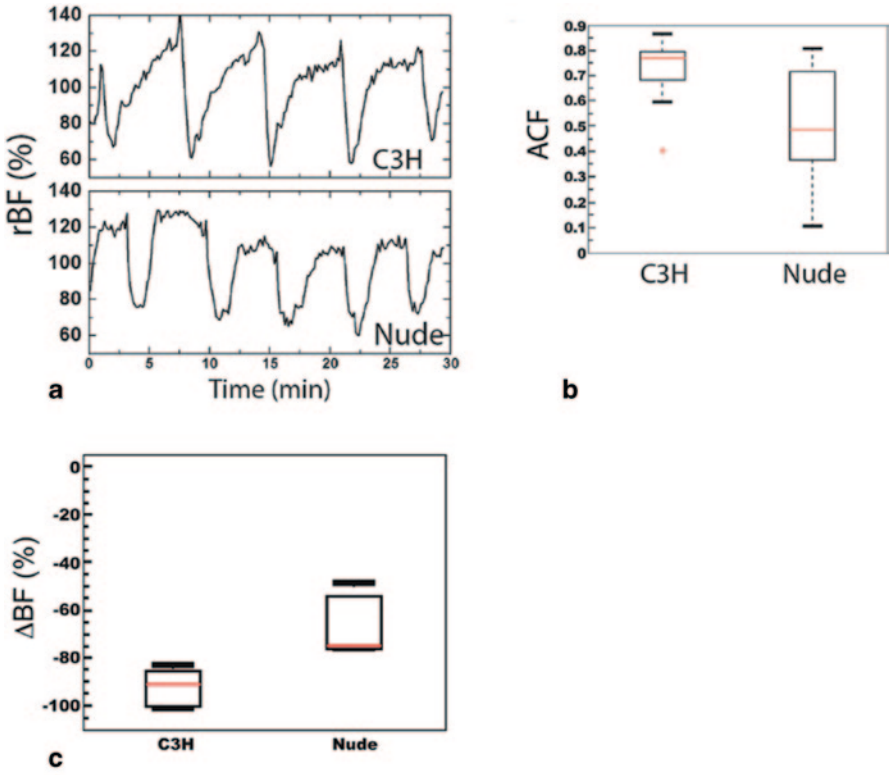

Fig. 3.8 Mouse strain leads to differences in cyclic patterning of tumor blood flow and vascular response to PDT. DCS was used to monitor tumor blood flow patterns in RIF tumors propagated in C3H vs. nude mice. Cycling of tumor blood flow was apparent in both mouse strains (**a**), however these cycles were significantly more regular in the C3H mice compared to the nude animals, as demonstrated by a higher autocorrelation function (ACF) in the C3H (**b**). The PDT-induced reduction in tumor blood flow was significantly greater in RIF tumors grown in C3H animals than in nudes (**c**). These data indicate an effect of host on the hemodynamic function and stress response of tumor blood vessels. (Printed with permission from Mesquita et al. [123])

In principle, it is also possible to determine sensitizer concentration based on DRS measurements. For sensitizers such as Photofrin and PpIX, the absorption maxima overlap with the much larger absorption peaks of hemoglobin. This makes it difficult to detect their absorption against the background of variable and heterogeneous tissue absorption. Other photosensitizers have absorption peaks at longer wavelengths, for example Motexafin Lutetium (MLu) at 732 nm [125] or aluminum phthalo-cyanine-tetrasulfonate (AlPcS,) at 680 nm [126], where the absorption background is lower and flatter, allowing their detection by DRS. In general, the optimal method of detection depends on the fluorescence yield and concentration of the sensitizer and the background optical properties [127]. As more long-wavelength sensitizers with greater tumor specificity are developed, absorption spectroscopy may play a larger role in sensitizer concentration assessment.

Just as the local change in tumor hemodynamic properties during illumination can inform on treatment outcomes, the PDT induced-change in photosensitizer levels can be similarly informative. The loss of sensitizer fluorescence resulting from photochemical reactions is defined as photobleaching. In general, photobleaching reduces the concentration of sensitizer available for PDT, and it may effectively limit the dose of reactive oxygen species that can be delivered. It does, however, have a practical use for dosimetry. Under the condition that the tissue damage and the photobleaching are mediated by the same mechanism, the destruction of the sensitizer can serve as a surrogate for tissue damage. This strategy is known as 'implicit dosimetry' to distinguish it from the 'explicit' strategy of separately quantifying the drug, light and oxygen components to predict tissue response [128]. When the damage is due primarily to singlet oxygen reactions, and the photobleaching is due to singlet oxygen reactions with the photosensitizer in its ground state, the local dose is related quantitatively to the local bleaching [129]. Fluorescence photobleaching of PpIX has been shown to correlate with skin reactions in preclinical models [130, 131], and has been used clinically to limit pain in PDT [132].

The relationship between photobleaching and tissue damage is reproducible if both are singlet-oxygen-mediated. It is important to keep in mind, however, that other bleaching mechanisms are possible. For instance, some photosensitizers can bleach by alternative reactions with cell substrates (e.g., interactions between the sensitizer triplet excited state and cell components). With bleaching mechanisms that are not singlet-oxygen dependent, photobleaching can actually be more efficient in hypoxic regions where the PDT effectiveness is lowest. The bleaching of intratumoral injected silicon phthalocyanine 4, for instance, is more efficient at higher incident fluence rates on tissue, which are presumably more oxygen-depleting, and shows no correlation with tumor growth delay [133]. In the case of Photofrin, multiple mechanisms of bleaching are possible, and the extent to which photobleaching reports on dose depends on the initial concentration of Photofrin [134]. Also, meso-tetrahydroxyphenyl chlorin (mTHPC) exhibits photobleaching that is oxygen-dependent, but does not strictly depend on dose [134, 135]. Given this variety in the relationship between photobleaching and response, it is essential that the correlation between photobleaching and dose be independently verified for a given sensitizer before it is used as a dose metric, for instance by comparison with direct singlet oxygen detection [135].

Conclusions

For the most effective treatment with PDT, it is important to consider the structure and function of the tumor microenvironment, at scales ranging from the molecular through the tissue level. Prior to even initiating light delivery, the pre-existing expression of transport proteins can impact the extent of photosensitizer accumulation within a tumor. The distribution and function of tumor blood vessels will determine levels of photosensitizer and oxygen in tumors. Furthermore, the composition of

vascular-associated basement membrane can alter the sensitivity of blood vessels to PDT-created damage. During illumination, treatment-created hypoxia can impede the cytotoxic and anti-vascular effects of therapy. In turn, the nature of these responses will dictate treatment-induced increases in the expression or activation of many molecules involved in angiogenesis and the cell stress response. Collectively, these microenvironmental factors contribute to the sensitivity versus resistance of solid malignancies to PDT, yet as our knowledge of each of these factors has grown, so too has the development of approaches to limit their negative consequences. Molecular targeting drugs have met with success in mitigating the results of undesired activation of signaling pathways by PDT. Alternative methods of light delivery and new developments in light delivery technology help to control hemodynamic effects during illumination. Up-and-coming research strives to more effectively harness the strengths of PDT in disrupting cell-cell and cell-matrix interactions toward applications in highly stromal disease. Finally, the development and application of treatment systems that incorporate online monitoring of PDT-induced changes in the tumor microenvironment could provide a means for the prediction of outcome. With this capability comes the possibility for early identification of a need for intervention with additional treatment, and ultimately the personalization of treatment through real time adjustment of light delivery based on microenvironmental response.

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References

1. Fukumura D, Duda DG, Munn LL, Jain RK. Tumor microvasculature and microenvironment: novel insights through intravital imaging in pre-clinical models. Microcirculation. 2010;17:206–25.
2. Provenzano PP, Hingorani SR. Hyaluronan, fluid pressure, and stromal resistance in pancreas cancer. Br J Cancer. 2013;108:1–8.
3. Fokas E, McKenna WG, Muschel RJ. The impact of tumor microenvironment on cancer treatment and its modulation by direct and indirect antivascular strategies. Cancer Metastasis Rev. 2012;31:823–42.
4. Busch TM. Local physiological changes during photodynamic therapy. Lasers Surg Med. 2006;38:494–99.
5. Ishikawa T, Kajimoto Y, Sun W, Nakagawa H, Inoue Y, Ikegami Y, Miyatake S, Kuroiwa T. Role of Nrf2 in cancer photodynamic therapy: regulation of human ABC transporter ABCG2. J Pharm Sci. 2013;102:3058–69.
6. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
7. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. Nat Med. 2011;17:1359–70.
8. Tomioka Y, Kushibiki T, Awazu K. Evaluation of oxygen consumption of culture medium and in vitro photodynamic effect of talaporfin sodium in lung tumor cells. Photomed Laser Surg. 2010;28:385–90.

9. Henderson BW, Gollnick SO, Snyder JW, Busch TM, Kousis PC, Cheney RT, Morgan J. Choice of oxygen-conserving treatment regimen determines the inflammatory response and outcome of photodynamic therapy of tumors. Cancer Res. 2004;64:2120–26.
10. Huang Z, Chen Q, Shakil A, Chen H, Beckers J, Shapiro H, Hetzel FW. Hyperoxygenation enhances the tumor cell killing of photofrin-mediated photodynamic therapy. Photochem Photobiol. 2003;78:496–502.
11. Busch TM, Wileyto EP, Emanuele MJ, Del Piero F, Marconato L, Glatstein E, Koch CJ. Photodynamic therapy creates fluence rate-dependent gradients in the intratumoral spatial distribution of oxygen. Cancer Res. 2002;62:7273–9.
12. Henderson BW, Busch TM, Snyder JW. Fluence rate as a modulator of PDT mechanisms. Lasers Surg Med. 2006;38:489–93.
13. Busch TM, Xing X, Yu G, Yodh A, Wileyto EP, Wang HW, Durduran T, Zhu TC, Wang KK. Fluence rate-dependent intratumor heterogeneity in physiologic and cytotoxic responses to photofrin photodynamic therapy. Photochem Photobiol Sci. 2009;8:1683–93.
14. Becker TL, Paquette AD, Keymel KR, Henderson BW, Sunar U. Monitoring blood flow responses during topical ALA-PDT. Biomed Opt Express. 2010;2:123–30.
15. van Leeuwen-van Zaane F, de Bruijn HS, van der Ploeg-van den Heuvel A, Sterenborg HJ, Robinson DJ. The effect of fluence rate on the acute response of vessel diameter and red blood cell velocity during topical 5-aminolevulinic acid photodynamic therapy. Photodiagn Photodyn Ther. 2014;11:71–81.
16. Seshadri M, Bellnier DA, Vaughan LA, Spernyak JA, Mazurchuk R, Foster TH, Henderson BW. Light delivery over extended time periods enhances the effectiveness of photodynamic therapy. Clin Cancer Res. 2008;14:2796–805.
17. Busch TM, Wang HW, Wileyto EP, Yu G, Bunte RM. Increasing damage to tumor blood vessels during motexafin lutetium-PDT through use of low fluence rate. Radiat Res. 2010;174:331–40.
18. Francois A, Salvadori A, Bressenot A, Bezdetnaya L, Guillemin F, D'Hallewin MA. How to avoid local side effects of bladder photodynamic therapy: impact of the fluence rate. J Urol. 2013;190:731–6.
19. Yamamoto J, Yamamoto S, Hirano T, Li S, Koide M, Kohno E, Okada M, Inenaga C, Tokuyama T, Yokota N, Terakawa S, Namba H. Monitoring of singlet oxygen is useful for predicting the photodynamic effects in the treatment for experimental glioma. Clin Cancer Res. 2006;12:7132–9.
20. Angell-Petersen E, Spetalen S, Madsen SJ, Sun CH, Peng Q, Carper SW, Sioud M, Hirschberg H. Influence of light fluence rate on the effects of photodynamic therapy in an orthotopic rat glioma model. J Neurosurg. 2006;104:109–17.
21. Sitnik TM, Hampton JA, Henderson BW. Reduction of tumor oxygenation during and after photodynamic therapy in vivo: effects of fluence rate. Br J Cancer. 1998;77:1386–94.
22. Frost GA, Halliday GM, Damian DL. Photodynamic therapy-induced immunosuppression in humans is prevented by reducing the rate of light delivery. J Invest Dermatol. 2011;131:962–8.
23. Estevez JP, Ascencio M, Colin P, Farine MO, Collinet P, Mordon S. Continuous or fractionated photodynamic therapy? Comparison of three PDT schemes for ovarian peritoneal micrometastasis treatment in a rat model. Photodiagn Photodyn Ther. 2010;7:251–7.
24. Zilberstein J, Bromberg A, Frantz A, Rosenbach-Belkin V, Kritzmann A, Pfefermann R, Salomon Y, Scherz A. Light-dependent oxygen consumption in bacteriochlorophyll-serine-treated melanoma tumors: on-line determination using a tissue-inserted oxygen microsensor. Photochem Photobiol. 1997;65:1012–9.
25. Naghavi N, Miranbaygi MH, Sazgarnia A. Simulation of fractionated and continuous irradiation in photodynamic therapy: study the differences between photobleaching and singlet oxygen dose deposition. Australas Phys Eng Sci Med. 2011;34:203–11.
26. Pogue BW, Hasan T. A theoretical study of light fractionation and dose-rate effects in photodynamic therapy. Radiat Res. 1997;147:551–9.
27. Xiao Z, Halls S, Dickey D, Tulip J, Moore RB. Fractionated versus standard continuous light delivery in interstitial photodynamic therapy of dunning prostate carcinomas. Clin Cancer Res. 2007;13:7496–505.

28. Middelburg TA, Van Zaane F, De Bruijn HS, Van Der Ploeg-van den Heuvel A, Sterenborg HJ, Neumann HA, De Haas ER, Robinson DJ. Fractionated illumination at low fluence rate photodynamic therapy in mice. Photochem Photobiol. 2010;86:1140–6.
29. de Vijlder HC, Sterenborg HJ, Neumann HA, Robinson DJ, de Haas ER. Light fractionation significantly improves the response of superficial basal cell carcinoma to aminolaevulinic acid photodynamic therapy: five-year follow-up of a randomized, prospective trial. Acta Derm Venereol. 2012;92:641–7.
30. Curnow A, Haller JC, Bown SG. Oxygen monitoring during 5-aminolaevulinic acid induced photodynamic therapy in normal rat colon. Comparison of continuous and fractionated light regimes. J Photochem Photobiol B. 2000;58:149–55.
31. van den Boogert J, van Staveren HJ, de Bruin RW, Siersema PD, van Hillegersberg R. Fractionated illumination for oesophageal ALA-PDT: effect on blood flow and PpIX formation. Lasers Med Sci. 2001;16:16–25.
32. Robinson DJ, de Bruijn HS, Star WM, Sterenborg HJ. Dose and timing of the first light fraction in two-fold illumination schemes for topical ALA-mediated photodynamic therapy of hairless mouse skin. Photochem Photobiol. 2003;77:319–23.
33. Bogaards A, Varma A, Zhang K, Zach D, Bisland SK, Moriyama EH, Lilge L, Muller PJ, Wilson BC. Fluorescence image-guided brain tumor resection with adjuvant metronomic photodynamic therapy: pre-clinical model and technology development. Photochem Photobiol Sci. 2005;4:438–42.
34. Attili SK, Lesar A, McNeill A, Camacho-Lopez M, Moseley H, Ibbotson S, Samuel ID, Ferguson J. An open pilot study of ambulatory photodynamic therapy using a wearable low-irradiance organic light-emitting diode light source in the treatment of nonmelanoma skin cancer. Br J Dermatol. 2009;161:170–3.
35. Cochrane C, Mordon SR, Lesage JC, Koncar V. New design of textile light diffusers for photodynamic therapy. Mater Sci Eng C Mater Biol Appl. 2013;33:1170–5.
36. Mathews MS, Angell-Petersen E, Sanchez R, Sun CH, Vo V, Hirschberg H, Madsen SJ. The effects of ultra low fluence rate single and repetitive photodynamic therapy on glioma spheroids. Lasers Surg Med. 2009;41:578–84.
37. Wiegell SR, Skodt V, Wulf HC. Daylight-mediated photodynamic therapy of basal cell carcinomas-an explorative study. J Eur Acad Dermatol Venereol. 2014;28:169–75.
38. Wiegell SR, Fabricius S, Gniadecka M, Stender IM, Berne B, Kroon S, Andersen BL, Mork C, Sandberg C, Ibler KS, Jemec GB, Brocks KM, Philipsen PA, Heydenreich J, Haedersdal M, Wulf HC. Daylight-mediated photodynamic therapy of moderate to thick actinic keratoses of the face and scalp: a randomized multicentre study. Br J Dermatol. 2012;166:1327–32.
39. Wiegell SR, Wulf HC, Szeimies RM, Basset-Seguin N, Bissonnette R, Gerritsen MJ, Gilaberte Y, Calzavara-Pinton P, Morton CA, Sidoroff A, Braathen LR. Daylight photodynamic therapy for actinic keratosis: an international consensus: International Society for Photodynamic Therapy in Dermatology. J Eur Acad Dermatol Venereol. 2012;26:673–9.
40. Peng Q, Nesland JM. Effects of photodynamic therapy on tumor stroma. Ultrastruct Pathol. 2004;28:333–40.
41. Lunardi S, Muschel RJ, Brunner TB. The stromal compartments in pancreatic cancer: are there any therapeutic targets? Cancer Lett. 2014;343:147–55.
42. Pazos M, Nader HB. Effect of photodynamic therapy on the extracellular matrix and associated components. Braz J Med Biol Res. 2007;40:1025–35.
43. Maas AL, Carter SL, Wileyto EP, Miller J, Yuan M, Yu G, Durham AC, Busch TM. Tumor vascular microenvironment determines responsiveness to photodynamic therapy. Cancer Res. 2012;72:2079–88.
44. Fungaloi P, Statius van Eps R, Wu YP, Blankensteijn J, de Groot P, van Urk H, van Hillegersberg R, LaMuraglia G. Platelet adhesion to photodynamic therapy-treated extracellular matrix proteins. Photochem Photobiol. 2002;75:412–7.
45. Overhaus M, Heckenkamp J, Kossodo S, Leszczynski D, LaMuraglia GM. Photodynamic therapy generates a matrix barrier to invasive vascular cell migration. Circ Res. 2000;86:334–40.

46. Waterman PR, Overhaus M, Heckenkamp J, Nigri GR, Fungaloi PF, Landis ME, Kossodo SC, LaMuraglia GM. Mechanisms of reduced human vascular cell migration after photodynamic therapy. Photochem Photobiol. 2002;75:46–50.
47. de Vree WJ, Fontijne-Dorsman AN, Koster JF, Sluiter W. Photodynamic treatment of human endothelial cells promotes the adherence of neutrophils in vitro. Br J Cancer. 1996;73:1335–40.
48. Kousis PC, Henderson BW, Maier PG, Gollnick SO. Photodynamic therapy enhancement of antitumor immunity is regulated by neutrophils. Cancer Res. 2007;67:10501–10.
49. Fungaloi P, Waterman P, Nigri G, Statius-van Eps R, Sluiter W, van Urk H, LaMuraglia G. Photochemically modulated endothelial cell thrombogenicity via the thrombomodulin-tissue factor pathways. Photochem Photobiol. 2003;785:475–80.
50. Drakopoulou M, Toutouzas K, Michelongona A, Tousoulis D, Stefanadis C. Vulnerable plaque and inflammation: potential clinical strategies. Curr Pharm Des. 2011;17:4190–209.
51. Egeblad M, Rasch MG, Weaver VM. Dynamic interplay between the collagen scaffold and tumor evolution. Curr Opin Cell Biol. 2010;22:697–706.
52. Arendt LM, Rudnick JA, Keller PJ, Kuperwasser C. Stroma in breast development and disease. Semin Cell Dev Biol. 2010;21:11–8.
53. Rucki AA, Zheng L. Pancreatic cancer stroma: understanding biology leads to new therapeutic strategies. World J Gastroenterol. 2014;20:2237–46.
54. Shields MA, Dangi-Garimella S, Redig AJ, Munshi HG. Biochemical role of the collagen-rich tumor microenvironment in pancreatic cancer progression. Biochem J. 2012;441:541–52.
55. Celli JP, Solban N, Liang A, Pereira SP, Hasan T. Verteporfin-based photodynamic therapy overcomes gemcitabine insensitivity in a panel of pancreatic cancer cell lines. Lasers Surg Med. 2011;43:565–74.
56. Celli JP. Stromal interactions as regulators of tumor growth and therapeutic response: a potential target for photodynamic therapy? Isr J Chem. 2012;52:757–66.
57. Hwang RF, Moore T, Arumugam T, Ramachandran V, Amos KD, Rivera A, Ji B, Evans DB, Logsdon CD. Cancer-associated stromal fibroblasts promote pancreatic tumor progression. Cancer Res. 2008;68:918–26.
58. Garmy-Susini B, Varner JA. Roles of integrins in tumor angiogenesis and lymphangiogenesis. Lymphat Res Biol. 2008;6:155–63.
59. Margaron P, Sorrenti R, Levy JG. Photodynamic therapy inhibits cell adhesion without altering integrin expression. Biochim Biophys Acta. 1997;1359:200–10.
60. Runnels JM, Chen N, Ortel B, Kato D, Hasan T. BPD-MA-mediated photosensitization in vitro and in vivo: cellular adhesion and beta1 integrin expression in ovarian cancer cells. Br J Cancer. 1999;80:946–53.
61. Uzdensky AB, Juzeniene A, Kolpakova E, Hjortland GO, Juzenas P, Moan J. Photosensitization with protoporphyrin IX inhibits attachment of cancer cells to a substratum. Biochem Biophys Res Commun. 2004;322:452–7.
62. Milla LN, Cogno IS, Rodriguez ME, Sanz-Rodriguez F, Zamarron A, Gilaberte Y, Carrasco E, Rivarola VA, Juarranz A. Isolation and characterization of squamous carcinoma cells resistant to photodynamic therapy. J Cell Biochem. 2011;112:2266–78.
63. Espada J, Galaz S, Sanz-Rodriguez F, Blazquez-Castro A, Stockert JC, Bagazgoitia L, Jaen P, Gonzalez S, Cano A, Juarranz A. Oncogenic H-Ras and PI3K signaling can inhibit E-cadherin-dependent apoptosis and promote cell survival after photodynamic therapy in mouse keratinocytes. J Cell Physiol. 2009;219:84–93.
64. Galaz S, Espada J, Stockert JC, Pacheco M, Sanz-Rodriguez F, Arranz R, Rello S, Canete M, Villanueva A, Esteller M, Juarranz A. Loss of E-cadherin mediated cell-cell adhesion as an early trigger of apoptosis induced by photodynamic treatment. J Cell Physiol. 2005;205:86–96.
65. Gomer CJ, Ferrario A, Luna M, Rucker N, Wong S. Photodynamic therapy: combined modality approaches targeting the tumor microenvironment. Lasers Surg Med. 2006;38:516–21.
66. Ortel B, Shea CR, Calzavara-Pinton P. Molecular mechanisms of photodynamic therapy. Front Biosci(Landmark Ed). 2009;14:4157–72.
67. Gomer CJ. Induction of prosurvival molecules during treatment: rethinking therapy options for photodynamic therapy. J Natl Compr Cancer Netw. 2012;10:S35–9.

68. Casas A, Di Venosa G, Hasan T, Al B. Mechanisms of resistance to photodynamic therapy. Curr Med Chem. 2011;18:2486–515.
69. Gilaberte Y, Milla L, Salazar N, Vera-Alvarez J, Kourani O, Damian A, Rivarola V, Jose Roca M, Espada J, Gonzalez S, Juarranz A. Cellular intrinsic factors involved in the resistance of squamous cell carcinoma to photodynamic therapy. J Invest Dermatol. 2014;134:2428–37. doi:10.1038/jid.2014.178 jid2014178 [pii].
70. Edmonds C, Hagan S, Gallagher-Colombo SM, Busch TM, Cengel KA. Photodynamic therapy activated signaling from epidermal growth factor receptor and STAT3: targeting survival pathways to increase PDT efficacy in ovarian and lung cancer. Cancer Biol Ther. 2012;13:1463–70.
71. Yang PW, Hung MC, Hsieh CY, Tung EC, Wang YH, Tsai JC, Lee JM. The effects of photofrin-mediated photodynamic therapy on the modulation of EGFR in esophageal squamous cell carcinoma cells. Lasers Med Sci. 2013;28:605–14.
72. Martinez-Carpio PA, Trelles MA. The role of epidermal growth factor receptor in photodynamic therapy: a review of the literature and proposal for future investigation. Lasers Med Sci. 2010;25:767–71.
73. Weyergang A, Selbo PK, Berg K. Sustained ERK [corrected] inhibition by EGFR targeting therapies is a predictive factor for synergistic cytotoxicity with PDT as neoadjuvant therapy. Biochim Biophys Acta. 2013;1830:2659–70.
74. Zhang H, Shen B, Swinarska JT, Li W, Xiao K, He P. 9-Hydroxypheophorbide alpha-mediated photodynamic therapy induces matrix metalloproteinase-2 (MMP-2) and MMP-9 downregulation in Hep-2 cells via ROS-mediated suppression of the ERK pathway. Photodiagn Photodyn Ther. 2014;11:55–62.
75. Ferrario A, Fisher AM, Rucker N, Gomer CJ. Celecoxib and NS-398 enhance photodynamic therapy by increasing in vitro apoptosis and decreasing in vivo inflammatory and angiogenic factors. Cancer Res. 2005;65:9473–8.
76. Ferrario A, Von Tiehl K, Wong S, Luna M, Gomer CJ. Cyclooxygenase-2 inhibitor treatment enhances photodynamic therapy-mediated tumor response. Cancer Res. 2002;62:3956–61.
77. Ferrario A, Gomer CJ. Targeting the 90 kDa heat shock protein improves photodynamic therapy. Cancer Lett. 2010;289:188–94.
78. Bhuvaneswari R, Gan YY, Yee KK, Soo KC, Olivo M. Effect of hypericin-mediated photodynamic therapy on the expression of vascular endothelial growth factor in human nasopharyngeal carcinoma. Int J Mol Med. 2007;20:421–8.
79. Gallagher-Colombo SM, Maas AL, Yuan M, Busch TM. Photodynamic therapy-induced angiogenic signaling: consequences and solutions to improve therapeutic response. Isr J Chem. 2012;52:681–90.
80. Solban N, Selbo PK, Sinha AK, Chang SK, Hasan T. Mechanistic investigation and implications of photodynamic therapy induction of vascular endothelial growth factor in prostate cancer. Cancer Res. 2006;66:5633–540.
81. Thong PS, Olivo M, Kho KW, Bhuvaneswari R, Chin WW, Ong KW, Soo KC. Immune response against angiosarcoma following lower fluence rate clinical photodynamic therapy. J Environ Pathol Toxicol Oncol. 2008;27:35–42.
82. Bhuvaneswari R, Yuen GY, Chee SK, Olivo. Hypericin-mediated photodynamic therapy in combination with Avastin (bevacizumab) improves tumor response by downregulating angiogenic proteins. Photochem Photobiol Sci. 2007;6:1275–83.
83. Ferrario A, von Tiehl KF, Rucker N, Schwarz MA, Gill PS, Gomer CJ. Antiangiogenic treatment enhances photodynamic therapy responsiveness in a mouse mammary carcinoma. Cancer Res. 2000;60:4066–9.
84. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F, Salomon DS. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006;366:2–16.
85. Nyati MK, Morgan MA, Feng FY, Lawrence TS. Integration of EGFR inhibitors with radiochemotherapy. Nat Rev Cancer. 2006;6:876–85.
86. Abu-Yousif AO, Moor AC, Zheng X, Savellano MD, Yu W, Selbo PK, Hasan T. Epidermal growth factor receptor-targeted photosensitizer selectively inhibits EGFR signaling and induces targeted phototoxicity in ovarian cancer cells. Cancer Lett. 2012;321:120–7.

87. Soukos NS, Hamblin MR, Keel S, Fabian RL, Deutsch TF, Hasan T. Epidermal growth factor receptor-targeted immunophotodiagnosis and photoimmunotherapy of oral precancer in vivo. Cancer Res. 2001;61:4490–6.
88. Bhuvaneswari R, Gan YY, Soo KC, Olivo M. Targeting EGFR with photodynamic therapy in combination with Erbitux enhances in vivo bladder tumor response. Mol Cancer. 2009;8:94.
89. Kim SG, Hong JW, Boo SH, Kim MG, Lee KD, Ahn JC, Hwang HJ, Shin JI, Lee SJ, Oh JK, Chung PS. Combination treatment of Cetuximab and photodynamic therapy in SNU-1041 squamous cancer cell line. Oncol Rep. 2009;22:701–8.
90. Mir Y, Elrington SA, Hasan T. A new nanoconstruct for epidermal growth factor receptor-targeted photo-immunotherapy of ovarian cancer. Nanomedicine. 2013;9:1114–22.
91. Del Carmen MG, Rizvi I, Chang Y, Moor AC, Oliva E, Sherwood M, Pogue B, Hasan T. Synergism of epidermal growth factor receptor-targeted immunotherapy with photodynamic treatment of ovarian cancer in vivo. J Natl Cancer Inst. 2005;97:1516–24.
92. Postiglione I, Chiaviello A, Aloj SM, Palumbo G. 5-aminolaevulinic acid/photo-dynamic therapy and gefitinib in non-small cell lung cancer cell lines: a potential strategy to improve gefitinib therapeutic efficacy. Cell Prolif. 2013;46:382–95.
93. Kim CH, Chung CW, Lee HM, Kim do H, Kwak TW, Jeong YI, Kang DH. Synergistic effects of 5-aminolevulinic acid based photodynamic therapy and celecoxib via oxidative stress in human cholangiocarcinoma cells. Int J Nanomedicine. 2013;8:2173–86.
94. Bhuvaneswari R, Yuen GY, Chee SK, Olivo M. Antiangiogenesis agents avastin and erbitux enhance the efficacy of photodynamic therapy in a murine bladder tumor model. Lasers Surg Med. 2011;43:651–62.
95. Ferrario A, Gomer CJ. Avastin enhances photodynamic therapy treatment of Kaposi's sarcoma in a mouse tumor model. J Environ Pathol Toxicol Oncol. 2006;25:251–9.
96. Jodele S, Chantrain CF, Blavier L, Lutzko C, Crooks GM, Shimada H, Coussens LM, Declerck YA. The contribution of bone marrow-derived cells to the tumor vasculature in neuroblastoma is matrix metalloproteinase-9 dependent. Cancer Res. 2005;65:3200–8.
97. Firczuk M, Nowis D, Golab J. PDT-induced inflammatory and host responses. Photochem Photobiol Sci. 2011;10:653–63.
98. Ferrario A, Chantrain CF, von Tiehl K, Buckley S, Rucker N, Shalinsky DR, Shimada H, DeClerck YA, Gomer CJ. The matrix metalloproteinase inhibitor prinomastat enhances photodynamic therapy responsiveness in a mouse tumor model. Cancer Res. 2004;64:2328–32.
99. Barron GA, Moseley H, Woods JA. Differential sensitivity in cell lines to photodynamic therapy in combination with ABCG2 inhibition. J Photochem Photobiol B. 2013;126:87–96.
100. Morgan J, Jackson JD, Zheng X, Pandey SK, Pandey RK. Substrate affinity of photosensitizers derived from chlorophyll-a: the ABCG2 transporter affects the phototoxic response of side population stem cell-like cancer cells to photodynamic therapy. Mol Pharm. 2010;7:1789–804.
101. Robey RW, Steadman K, Polgar O, Bates SE. ABCG2-mediated transport of photosensitizers: potential impact on photodynamic therapy. Cancer Biol Ther. 2005;4:187–94.
102. Sandell J, Zhu TC. A review of in-vio human optical properties and its impact on PDT. J Biophotonics. 2011;4:773–87.
103. Finlay JC, Foster TH. Effect of pigment packaging on diffuse reflectance spectroscopy of samples containing red blood cells. Opt Lett. 2004;29:965–7.
104. Farrell T, Patterson M, Wilson B. A diffusion theory model of spatially resolved, steady-state diffuse reflectance for the noninvasive determination of tissue optical properties in vivo. Med Phys. 1992;19:879–88.
105. Jacques S, Wang LH, Hielscher A. Time-resolved photon propagation in tissues. In: Optical_thermal response of laser-irradiated tissue. New York: Plenum Press; 1995.
106. Finlay JC, Foster TH. Hemoglobin oxygen saturations in phantoms and in vivo from measurements of steady-state diffuse reflectance at a single, short source-detector separation. Med Phys. 2004;31:1949–59.

107. Dimofte A, Finlay JC, Zhu TC. A method determination of the absorption and scattering properties interstitially in turbid media. Phys Med Biol. 2005;50:2291–311.
108. Boas DA, Brooks DH, Miller EL, DiMarzio CA, Kilmer M, Gaudette RJ, Zhang Q. Imaging the body with diffuse optical tomography. Signal Process Mag, IEEE. 2001;18:57–75.
109. Durduran T, Choe R, Culver JP, Zubkov L, Holboke M, Gaimmarco J, Chance B, Yodh A. Bulk optical properties of healthy female breast tissue. Phys Med Biol. 2002;47:2847–61.
110. Wang KK, Zhu TC. Reconstruction of in-vivo optical properties for human prostate using interstitial diffuse optical tomography. Opt Express. 2009;17:11665–72.
111. Liang X, Wang KK-H, Zhu TC. Feasibility of interstitial diffuse optical tomography using cylindrical diffusing fibers for prostate PDT. Phys Med Biol. 2013;58:3461–80.
112. Cuccia DJ, Bevilacqua F, Durkin AJ, Tromberg BJ. Modulated imaging: quantitative analysis and tomography of turbid media in the spatial-frequency domain. Opt Lett. 2005;30:1354–6.
113. Gioux S, Mazhar A, Lee BT, Lin SJ, Tobias AM, Cuccia DJ, Stockdale A, Oketokoun R, Ashitate Y, Kelly E, Weinmann M, Durr NJ, Moffitt La, Durkin AJ, Tromberg BJ, Frangioni JV. First-in-human pilot study of a spatial frequency domain oxygenation imaging system. J Biomed Opt. 2011;16:086015.
114. Zhou C, Yu G, Furuya D, Greenberg J, Yodh A, Durduran T. Diffuse optical correlation tomography of cerebral blood flow during cortical spreading depression in rat brain. Opt Express. 2006;14:1125–44.
115. Perelman LT, Backman V, Wallace M, Zonios G, Manoharan R, Nusrat A, Shields S, Seiler M, Lima C, Hamano T, Itzkan I, Van Dam J, Crawford JM, Feld MS. Observation of periodic fine structure in reflectance from biological tissue: a new technique for measuring nuclear size distribution. Phys Rev Lett. 1998;80:627–30.
116. Wilson JD, Bigelow CE, Calkins DJ, Foster TH. Light scattering from intact cells reports oxidative-stress-induced mitochondrial swelling. Biophys J. 2005;88:2929–38.
117. Wilson JD, Giesselman BR, Mitra S, Foster TH. Lysosome-damage-induced scattering changes coincide with release of cytochrome c. Opt Lett. 2007;32:2517–9.
118. Woodhams JH, Kunz L, Bown SG, MacRobert AJ. Correlation of real-time haemoglobin oxygen saturation monitoring during photodynamic therapy with microvascular effects and tissue necrosis in normal rat liver. Br J Cancer. 2004;91:788–94.
119. Pham TH, Hornung R, Berns MW, Tadir Y, Tromberg BJ. Monitoring tumor response during photodynamic therapy using near-infrared photon-migration spectroscopy. Photochem Photobiol. 2001;73:669–77.
120. Standish BA, Lee KK, Jin X, Mariampillai A, Munce NR, Wood MF, Wilson BC, Vitkin IA, Yang VX. Interstitial Doppler optical coherence tomography as a local tumor necrosis predictor in photodynamic therapy of prostatic carcinoma: an in vivo study. Cancer Res. 2008;68:9987–95.
121. Yu G, Durduran T, Zhou C, Wang HW, Putt ME, Saunders HM, Sehgal CM, Glatstein E, Yodh AG, Busch TM. Noninvasive monitoring of murine tumor blood flow during and after photodynamic therapy provides early assessment of therapeutic efficacy. Clin Cancer Res. 2005;11:3543–52.
122. Wang HW, Putt ME, Emanuele MJ, Shin DB, Glatstein E, Yodh AG, Busch TM. Treatment-induced changes in tumor oxygenation predict photodynamic therapy outcome. Cancer Res. 2004;64:7553–61.
123. Mesquita RC, Han SW, Miller J, Schenkel SS, Pole A, Esipova TV, Vinogradov SA, Putt ME, Yodh AG, Busch TM. Tumor blood flow differs between mouse strains: consequences for vasoresponse to photodynamic therapy. PLoS One. 2012;7:e37322.
124. Konecky SD, Owen CM, Rice T, Valdés Pa, Kolste K, Wilson BC, Leblond F, Roberts DW, Paulsen KD, Tromberg BJ. Spatial frequency domain tomography of protoporphyrin IX fluorescence in preclinical glioma models. J Biomed Opt. 2012;17:056008.
125. Finlay JC, Zhu TC, Dimofte A, Stripp D, Malkowicz SB, Busch TM, Hahn SM. Interstitial fluorescence spectroscopy in the human prostate during motexafin lutetium-mediated photodynamic therapy. Photochem Photobiol. 2006;82:1270–80.

126. Weersink RA, Hayward JE, Diamond KR, Patterson MS. Accuracy of noninvasive in vivo measurements of photosensitizer uptake based on a diffusion model of reflectance spectroscopy. Photochem Photobiol. 1997;66:326–35.
127. Davis SC, Pogue BW, Dehghani H, Paulsen KD. Tissue drug concentration determines whether fluorescence or absorption measurements are more sensitive in diffuse optical tomography of exogenous contrast agents. Appl Opt. 2009;48:D262–72.
128. Wilson B, Patterson M, Lilge L. Implicit and explicit dosimetry in photodynamic therapy: a new paradigm. Lasers Med Sci. 1997;12:182–99.
129. Georgakoudi I, Foster TH. Singlet oxygen- versus nonsinglet oxygen-mediated mechanisms of sensitizer photobleaching and their effects on photodynamic dosimetry. Photochem Photobiol. 1998;67:612–25.
130. Robinson DJ, de Bruijn HS, van der Veen N, Stringer MR, Brown SB, Star WM. Fluorescence photobleaching of ALA-induced protoporphyrin IX during photodynamic therapy of normal hairless mouse skin: the effect of light dose and irradiance and the resulting biological effect. Photochem Photobiol. 1998;67:140–9.
131. Boere IA, Robinson DJ, de Bruijn HS, Kluin J, Tilanus HW, Sterenborg HJ, de Bruin RW. Protoporphyrin IX fluorescence photobleaching and the response of rat Barrett's esophagus following 5-aminolevulinic acid photodynamic therapy. Photochem Photobiol. 2006;82:1638–44.
132. Cottrell WJ, Paquette AD, Keymel KR, Foster TH, Oseroff AR. Irradiance-dependent photobleaching and pain in delta-aminolevulinic acid-photodynamic therapy of superficial basal cell carcinomas. Clin Cancer Res. 2008;14:4475–83.
133. Baran TM, Foster TH. Fluence rate-dependent photobleaching of intratumorally administered Pc 4 does not predict tumor growth delay. Photochem Photobiol. 2012;88:1273–9.
134. Finlay JC, Mitra S, Foster TH. In vivo mTHPC photobleaching in normal rat skin exhibits unique irradiance-dependent features. Photochem Photobiol. 2002;75:282–8.
135. Jarvi MT, Patterson MJ, Wilson BC. Insights into photodynamic therapy dosimetry: simultaneous singlet oxygen luminescence and photosensitizer photobleaching measurements. Biophys J. 2012;102:661–71.

Chapter 4
Autophagy Induced by Photodynamic Therapy (PDT): Shaping Resistance Against Cell Death and Anti-Tumor Immunity

Abhishek D. Garg and Patrizia Agostinis

Abstract Autophagy is one of the main catabolic pathways in a eukaryotic cell, utilized for cellular self-degradation in order to support recycling and replenishing of biomolecular resources crucial for cell growth and survival. Autophagy is inherently a cytoprotective and prosurvival process yet in general, it has become clear through a number of studies that autophagy has a highly contextual role in cancer biology; depending on which stage, site or type of tumorigenesis or therapy intervention one is looking at the role of autophagy may end up varying from pro- to anti-tumorigenic. Several studies have shown that, depending on the photosensitizer under consideration, autophagy activated by PDT either contributes to therapy resistance (by suppressing cell death) or susceptibility (by facilitating autophagic cell death). Beyond cell death modulation, cancer cell-associated autophagy also assists in resistance against PDT by suppressing anticancer immune effector mechanisms. In this chapter, we have summarized the current state-of-the-art and the existing gap-in-knowledge concerning PDT-induced autophagy in cancer therapy susceptibility and resistance.

Keywords Anticancer immunity · Apoptosis · Cancer · Cell death · Chaperone-mediated autophagy (CMA) · Danger signals · Immunogenic cell death · Macroautophagy (MAP)

Abbreviations

CD	Cluster of differentiation
CMA	Chaperone-mediated autophagy
CRT	Calreticulin
DAMP	Damage-associated molecular pattern

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Ecto	Surface exposed or surface tethered
ER	Endoplasmic reticulum
HSP	Heat-shock protein
Hyp	Hypericin
ICD	Immunogenic cell death
IFN	Interferon
IL	Interleukin
MAP	Macroautophagy
PDT	Photodynamic therapy
Phox	Photo-oxidative
ROS	Reactive oxygen species

Introduction

Autophagy is one of the main catabolic pathways in a eukaryotic cell, utilized for cellular self-degradation in order to support recycling and replenishing of biomolecular resources crucial for cell growth and survival [1]. Autophagy is constitutively present in a cell at basal thresholds yet, not surprisingly, it is mainly induced under stressful conditions of nutrient deprivation or organelle-associated/subcellularly-localized stress [2]. Here, autophagy helps in replenishing nutrients through degradation of existing cellular components and eliminating (and recycling) the damaged organelle/subcellular entities, thereby, lowering cellular stress [1, 3]. For instance, serum starvation *in vitro* stimulates bulk autophagy which, by degrading cellular biomolecules or organelles, provides new nutrients resulting in maintenance of energy and metabolic homeostasis [4]. Similarly, if physiochemical stress is applied on a cell that results in damage to particular cellular organelles or subcellular locales then the signalling pathways and damage-related by-products produced by such a stress can compromise cellular integrity [4] e.g. stress on the ER can induce ER stress signalling pathways and oxidative stress which may curtail cellular survival [5]. In such cases, autophagy can help eliminate the damaged entity, thereby, limiting the stress signalling and prolonging cellular survival e.g. the autophagic removal of dysfunctional proteins and mitochondria can thwart the build-up of aggregation-prone proteins and the unwarranted creation of toxic reactive oxygen species (ROS) by mitochondria [6, 7]. Here, organelle targeted or organelle localized stress may stimulate more specific forms of autophagy e.g. reticulophagy (targeted towards damaged endoplasmic reticulum or ER), mitophagy (targeting the damaged mitochondria) or pexophagy (targeting the damaged peroxisomes) [2]. Overall, as evident, autophagy is inherently a cytoprotective and a prosurvival phenomenon.

Highest diversity of autophagic pathways has been demonstrated to exist in yeast [2]; mainly consisting of macroautophagy (MAP), piecemeal microautophagy of the nucleus, chaperone-mediated autophagy (CMA), cytoplasm to vacuole targeting and microautophagy [1]. In the mammalian system though, the existence of mainly MAP, CMA and microautophagy has been demonstrated [1, 3] while the existence

of other autophagic pathways remains as-yet-uncharacterized. Here, MAP (hereafter referred to as simply autophagy unless otherwise mentioned) is an evolutionarily-conserved catabolic pathway that has been studied most comprehensively in a mammalian perspective [4]. CMA and microautophagy on the other hand have been relatively much less studied in mammalian systems [1–3]. CMA and microautophagy tend to be much more target specific degradative processes, even if the damage is broad and not just confined to their targets [2]. Possibly in molecular terms, MAP demands a much more complex and diverse set of signalling modules than CMA or microautophagy—a conjecture that needs exhaustive analysis. Through MAP, cytoplasmic biomolecular components as well as whole organelles are sequestered as cargo into double-membraned vesicles termed as autophagosomes [1]. The membranes for the formation of such autophagosomes have been postulated to be derived from multiple sources including (but not limited to) the plasma membrane, the ER and the Golgi complex [2]. These autophagosomes are consequently fused with the lysosomes, where the autophagosome-cargo is degraded (by various lysosomal enzymes) and recycled [2]. Thus, during MAP, the autophagic cargo is indirectly fused with the lysosomes through the agency of autophagosomes [2].

Microautophagy entails (spatially or dimensionally confined) lysosome-based direct engulfment of cytoplasmic entities *via* lysosome-membrane based invagination, protrusion/septation and vesicle scission into the lumen [8]. On the other hand, CMA is a selective type of autophagy where the cytosolic chaperone heat shock cognate 70 (Hsc70) binds specific damaged proteins containing a motif identical or similar to the pentapeptide KFERQ and helps in conveying these particular proteins across the lysosomal membranes and into the lysosomal lumen for degradation [9]. This procedure of protein translocation across lysosomal membrane is assisted by the CMA-essential receptor, the lysosome-associated membrane protein 2A (LAMP2A) [9]. Of note, MAP and CMA (to a relatively limited extent) have been found to play important roles in cancer progression, cancer therapy response and cancer metabolic "microevolution".

Many excellent reviews have recently summarized, as well as described the molecular pathways mediating and regulating mammalian autophagy [4, 10–13]. Hence, we are avoiding a redundant discussion concerning the same in this book chapter and would like to refer the readers to these comprehensive reviews for further such specific reading. In this book chapter, we will be concentrating on the functional significance of autophagy induced by photodynamic therapy (PDT) in cancer therapy responsiveness.

The ability of PDT to induce cell death depends on an orderly course of action. This procedure starts with the build-up of a photosensitive drug or photosensitizer in cells followed by its activation (in presence of oxygen) *via* light irradiation (of suitable wavelength which corresponds to the excitation spectra of that exact photosensitizer); all of which collectively brings about the creation of ROS within the cell [14, 15]. These ROS or photo-oxidative (phox) stress has the capacity to cause cell death [16].

On many levels, PDT as a therapy is very different from usual systemic chemotherapeutics, loco-regionally applied chemotherapeutics and physical or

physicochemical anticancer modalities (e.g. radiotherapy) [17]. For instance, the unique dual-component therapeutic approach of PDT (i.e. photosensitive drug activated following excitation through light of relevant wavelength) such that each of these components is harmless alone but lethal when combined provides a therapeutic paradigm that is mechanistically interesting and multifactorial [18]. This situation is made more interesting by the fact that PDT can be used to generate subcellular organelle-specific stress since every photosensitive drug has a unique subcellular localization profile either confined to a particular organelle or overlapping across several [19] e.g. Hypericin can localize mainly to the ER [20] while 5-aminolevulenic acid (5-ALA) can localize mainly to the mitochondria (see Table 4.1). This is especially interesting when it comes to autophagy studies because of its tendency to "chase" damaged organelles or subcellular sites for degradation and recycling.

Thus, the unique nature of PDT allows higher flexibility and refined insights both in terms of clinical application and preclinical/biochemical analysis. These properties are very interesting when studying the role of autophagy in anticancer therapy response. In this book chapter, we will summarize the current state-of-the-art and the existing gap-in-knowledge concerning PDT-induced autophagy in cancer therapy susceptibility and resistance.

Autophagy Pathways in Cancer Progression and Therapy Response: State-of-the-Art and Emerging Trends

Prior to discussing the specific role of PDT-induced autophagy in cancer therapy response, it is very important to understand the general, broad role of autophagy in cancer progression. Such a context is crucial when extrapolating post-PDT functional results of autophagic activity to a wider "cancer therapy response canvas". In the following paragraphs, a bird's-eye-view of the role of autophagic pathways in cancer progression and general anticancer therapy response has been presented.

In cancer, there are three temporally distinct levels where autophagy (primarily MAP) plays crucial (contextually overlapping or contradictory) roles i.e. early steps of carcinogenesis before the formation of a clinically-relevant tumor, tumorigenesis before anticancer therapy and carcinogenesis during/after anticancer therapy [4]. The role of autophagy during early steps of carcinogenesis and in tumors before anticancer therapy is primarily shaped by the mutational, oncogenic and microevolutionary landscape of the cancer cells, the cancer cell-stromal cell-immune cell interactions and the metabolic stress associated with tumor microenvironment [4]. On the other hand, the role of autophagy during and after cancer therapy is shaped both by the pre-existing role of autophagy in an established tumor and the effects of the type of therapeutic intervention (on cancer cells, stromal cells and immune cells). Across all these levels, evidence suggests that autophagy is engaged by cancer cells as a vastly plastic and dynamic machinery to either contain the early steps in carcinogenesis or sustain the continued existence and development of established tumors after therapy [4].

Table 4.1 The effect of autophagy on cancer cell's therapeutic responsiveness to photodynamic therapy (PDT)

PDT-relevant photosensitizer	Sub-cellular localization	Experimental context	Role of autophagy in cancer therapy-response	Experimental intervention used for confirming autophagy's role	Ref.
Macroautophagy (MAP) and cancer's response to PDT					
5-ALA	Mitochondria	*In vitro* cell cultures	Pro-death	3-MA	[59]
5-ALA	Mitochondria	*In vitro* cell cultures	Pro-survival	ATG7 siRNA	[42]
9-capronyloxytetrakis(methoxyethyl)porphycene	ER Mitochondria	In vitro cell cultures	Pro-survival	ATG7 siRNA	[60]
Chlorin NPe6 and palladium bacteriopheophorbide WST11	Lysosome	In vitro cell cultures	Pro-death	ATG5 KO-cells ATG7 KO-cells	[32]
Chlorophyllin e4	Lysosome Mitochondria	In vitro cell cultures	Pro-survival	3-MA Bafilomycin A1	[33]
Chlorophyllin f	Lysosome Mitochondria	In vitro cell cultures	Pro-survival	3-MA	[34]
Graphene quantum dots	Intracytoplasmic vesicles	In vitro cell cultures	Pro-death	LC3B KO-cells	[61]
Hypericin	ER	In vitro cell cultures and ex vivo immune cell co-cultures	Pro-survival and Suppresses 'eat me' signal ecto-CRT and anti-cancer immune effector mechanisms	3-MA Bafilomycin A1 ATG5/p62 siRNA ATG5 KO-cells p62 KO-cells	[30, 35, 37, 62, 63]
Mesochlorin	Mitochondria	In vitro cell cultures	Pro-survival	ATG7 siRNA	[60]
mTHPC	ER	In vitro cell cultures	Pro-death	Wortmannin	[64]
Pc 4	Mitochondria ER	In vitro cell cultures	Pro-death	ATG7/LC3 siRNA	[65]
Pc 4	Mitochondria ER	In vitro cell cultures	Pro-death	3-MA Wortmannin	[66]

Table 4.1 (continued)

PDT-relevant photosensitizer	Sub-cellular localization	Experimental context	Role of autophagy in cancer therapy-response	Experimental intervention used for confirming autophagy's role	Ref.
Pc 4	Mitochondria ER	In vitro cell cultures	Pro-survival	ATG7 siRNA	[67]
Photofrin	Mitochondria Cellular Membranes	In vitro cell cultures	No effect	3-MA Bafilomycin A1	[46]
Platonin	?	In vitro cell cultures	Pro-death	3-MA	[68]
Rose bengal acetate	Cytoskeleton Mitochondria Golgi apparatus ER	In vitro cell cultures	Pro-death	3-MA	[69]
Verteporfin	Mitochondria ER	In vitro cell cultures	Pro-survival	CQ ATG7 siRNA	[70, 71]
Chaperone-mediated autophagy (CMA) and cancer's response to PDT					
Hypericin	ER	In vitro cell cultures	Pro-survival and Suppresses 'eat me' signal ecto-CRT	LAMP2A KO-cells	[5, 35]

3-MA—3-methyladenine; *5-ALA*—5-aminolevulinic acid; *CRT*—calreticulin; *Ecto*—surface exposed; *ER*—endoplasmic reticulum; *KO*—knock-out; *mTHPC*—m-tetrahydroxyphenylchlorin; *Pc 4*—(Silicon) Phthalocyanine

In case of early steps responsible for carcinogenesis, several studies have linked defective autophagy (mainly MAP but not specifically CMA) to increased carcinogenesis [21–23]. Here, mechanistically, the tumor suppressor function of autophagy has been accredited to the essential cell-autonomous effects of autophagy in mitigating damage (inflicted as a result of neoplastic transformation) and maintaining cellular integrity under circumstances of metabolic strain (brought about due to carcinogenic insults) [13]. Moreover, it has been observed that autophagy may also stop the progress of tumorigenesis by inducing oncogene-induced senescence, a process thought to prevent further tumor progression [24]. Of note, the ability of tumor suppressor genes and oncogenes to employ autophagic pathways may eventually govern the cargo selection by the autophagosome machinery, thereby influencing the 'functional plasticity' of autophagy during cancer progression [4].

Several studies have found that autophagy (both MAP as well as CMA) plays rather a pro-tumorigenic role, within the established tumor [4, 25]. Autophagy is commonly increased in advanced tumors, and the maximum levels are frequently found in inadequately oxygenated regions where the requirement for nutrients is augmented along with the necessity to endure quite a few forms of metabolic insults. Thus, clinically-relevant, advanced tumors exhibit an 'autophagy addiction' that is obligatory to preserve their energy equilibrium, through the recycling of intracellular components [13, 26]. Moreover, it has been reported that, cancer cell associated autophagy could also facilitate the metastasis of tumor cells by suppressing pro-death mechanisms encountered during the process of metastatic dissemination e.g. autophagy has been shown to stifle extracellular matrix (ECM) detachment-induced cell death, (i.e. anoikis), thereby raising the possibility that it could promote cancer cell survival in the blood stream following extravasation/ loss of interaction with the ECM [27]. In this scenario, autophagy can also be critical to maintain tumor cell "dormancy" upon extravasation and colonization of a distant site, until a robust cancer cell–ECM contact is re-established at a remote "seeding" site.

The above discussion outlines the cancer cell-autonomous functions of autophagy in carcinogenesis resulting in dynamic adaptation to stressful circumstances and preservation of proteome integrity as well as energy metabolism. However, recent research has clearly indicated that autophagy also regulates a range of cell-non-autonomous processes [4]. Such cell-non-autonomous (mostly paracrine) functions of autophagy have widespread impact on the tumor microenvironment and they seem to be regulated by the site, nature of the concerned soluble or cellular mediators, and the complexity of the tumor cell–stromal cell contacts [4]. For instance, autophagy may play a role in supporting the tumor-associated vasculature by supporting starvation and hypoxia-evoked angiogenesis on the level of the endothelial cells [28]. Similarly, accumulating observations suggest that autophagy may play an important role in immunosurvelliance of senescent or normal cancer cells. Here, both cancer cell-associated and immune cell-associated autophagy has been found to be play contextual roles (i.e. either immunoevasive or pro-immunity roles depending on the setting) in shaping the cancer cell-immune cell interactions [4]. Last but not least, recent research has also suggested a role for autophagy in cancer-associated fibroblasts in supporting energy metabolism and the expansion of adjacent epithelial cancer cells [4].

It is clear that autophagy plays a contextually dynamic role in cancer initiation as well as progression. Interestingly, this trend is mirrored for the autophagy's role in anticancer therapy response in general with therapy responses varying from unaltered or augmented, to decreased cancer cell killing upon autophagy blockade [4]. However, the majority of cancer therapy studies have revealed that cancer cell-associated autophagy plays a pro-survival role thereby reducing the cytotoxic effects of anticancer therapeutics. In principle, considering the well-established role of autophagy in stress mitigation, this is quite conceivable. Moreover, it has also emerged recently that therapy-induced autophagy in cancer cells has the capability to influence the interface between dying cancer cells and the immune system by modulating the emission of immunostimulatory danger signals [29] or damage-associated molecular patterns (DAMPs) [5, 30]. In general, depending on the anticancer therapy under consideration, the type of cellular stress they induce, and the autophagic cargo that is selected, cancer cell-associated autophagy can either augment the cancer immunogenicity or help in immunoevasion and suppression of anticancer immunity [3, 4].

It should be noted, however, that, under certain conditions (involving treatment with specific anticancer therapeutics), autophagy has been shown to encourage cell death; either by enabling the pro-apoptotic processes or by mediating "autophagic cell death", a type of cell death mediated (rather than simply accompanied) by autophagy [4]. It is important to note that so far, the pro-death or 'autophagic cell death' outcomes have been mainly attributed to MAP while CMA has been primarily shown to be a pro-survival pathway. The mechanisms of 'autophagic cell death' in the context of cancer are still elusive and require further extensive analysis.

Thus, in general, it is clear that autophagy has a highly contextual role; depending on which stage, site or type of tumorigenesis or therapy intervention one is looking at, the role of autophagy may end up varying from pro- to anti-tumorigenic.

At this point, an important question is, does this general trend concerning the contextual role of autophagy in anticancer therapy also applies to PDT or is it more resolved? Also, a mechanistically crucial question that general non-PDT research has been largely unable to answer is: is the pro- or anti-tumorigenic role of autophagy after anticancer therapy regulated by the subcellular location of the therapeutic stress? The latter question has high relevance and in our view only PDT is capable of answering such a question within a therapeutic context. In the following sections, the autophagy's role in PDT-based cancer therapy response has been analysed and discussed in details.

Autophagy Pathways and PDT-Induced Cancer Cell Death: The Ying and the Yang

A number of *in vitro* studies using various photosensitizers have been published over the last 6–7 years since the first analysis on the role of autophagy in PDT appeared [31]. Thus, in order to gain a wider view of the relevance of autophagy for

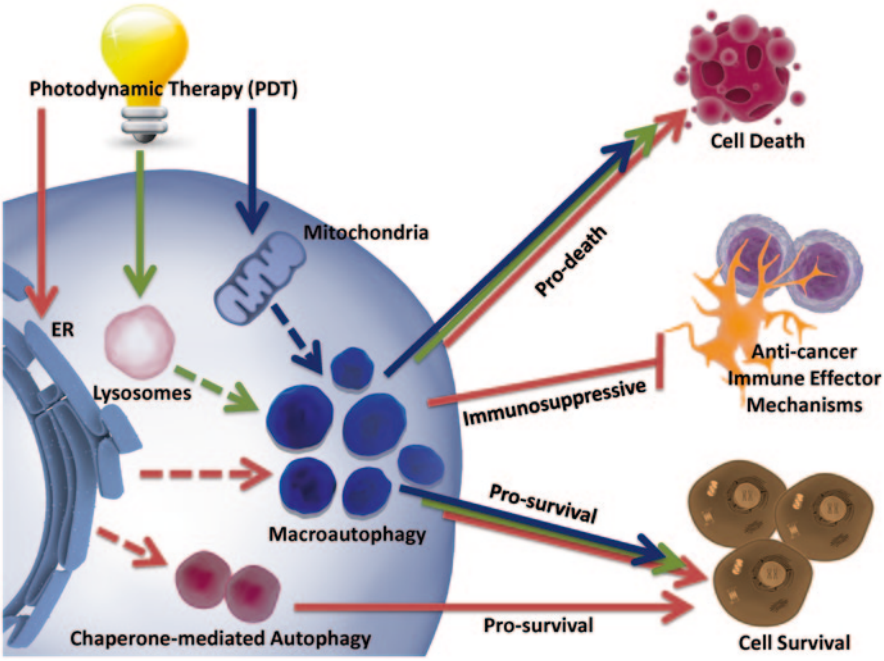

Fig. 4.1 The functional role of autophagy in cancer responsiveness to photodynamic therapy (*PDT*). Depending on the photosensitizer under consideration and the context (but independent of the photosensitizer's target subcellular organelle like the ER, mitochondria or lysosomes, autophagy activated by PDT either contributes to therapy resistance (pro-survival role demonstrated for macroautophagy and chaperone-mediated autophagy) or susceptibility (pro-death role *via* autophagic cell death, mainly through macroautophagy). Moreover, cancer cell-associated macroautophagy also assists in resistance against PDT by suppressing anticancer immune effector mechanisms

PDT-based therapy response, we surveyed all the studies that have used autophagy blockade strategies to ascertain the role of this molecular pathway in PDT responsiveness (see Table 4.1).

Interestingly, our survey showed that, there was nearly the same number of instances of autophagy (mainly MAP) playing a pro-survival role as there were instances where it played a pro-death role for different PDT paradigms (a paradigm here denoting the fact that each PDT paradigm utilizes a unique photosensitizer) (Table 4.1 and Fig. 4.1). Like for certain other therapies, for PDT as well, CMA was found to be mainly playing a pro-survival role although this has so far been tested only for one paradigm i.e. Hypericin-based PDT (Hyp-PDT). Thus, depending on the photosensitizer under consideration, autophagy activated by PDT either contributes to therapy resistance or susceptibility (Table 4.1 and Fig. 4.1).

What is the mechanistic basis for this balanced contextual role of autophagy in response to PDT? Specific subcellular localization of a photosensitizer doesn't seem to completely explain this contextual functional role of autophagy [31]. Localization of the photosensitizer in mitochondria, mitochondria/ER and lysosomes

(Table 4.1) all exhibited the ability to induce both pro-survival and pro-death autophagy depending on the photosensitizer or PDT dosage under consideration. Although it had been postulated previously on the basis of studies with chlorine NPe6-based PDT and palladium bacteriopheophorbide WST11-based PDT (both mainly targeting the lysosomes) that targeting lysosomes might cause mainly pro-death autophagy induction (or autophagic cell death) [32] yet more recent studies with different lysosome targeting photosensitizers (based on chlorophyllin e4 and f) showed the presence of pro-survival autophagy [33, 34]. However, in case of the latter study and various other studies mentioned in Table 4.1, only chemical inhibitors of autophagy (e.g. 3-MA, Bafilomycin A1, Wortmannin and CQ) have been utilized for autophagy blockade. It is important to mention here that chemical inhibitors of autophagy are not very specific and they exhibit the ability to inhibit various non-autophagy related processes that may also influence terminal read-outs of survival or cell death [2]. In this perspective, these results should be validated by genetic approaches, such as RNAi-based knock-down of autophagy relevant molecules (like ATG5, ATG7, p62 and LC3) since this strategy has a relatively higher chance of specifically ablating autophagy with minimal off-target effects [2]. Of note, wherever possible, results obtained in cells derived from animals with genetic knock-out of autophagy genes (e.g. ATG5 knock-out murine embryonic fibroblasts) need to be compared with RNAi-based knock-down of that particular molecule (e.g. ATG5 siRNA) since observations in these two systems might sometime differ due to pre-existing compensation mechanisms prevalent in the knock-out cells [30, 35, 36].

It is noteworthy that the precise molecular details behind PDT-induced autophagic cell death are currently unknown. On the other hand, molecular details behind autophagy-based resistance to PDT therapy on the level of cell death have started to emerge. Mainly using Hyp-PDT system, we have demonstrated that PDT-induced oxidative-stress causes significant accumulation of oxidatively damaged proteins [35, 37] and possibly other biomolecules (e.g. peroxidised lipids) at the subcellular site where the photosensitizer originally localized before PDT (since after PDT, several photosensitizers exhibit the tendency to re-localize)[3]. Following this, MAP is predominantly activated and strives to degrade and recycle the damaged organelle and subcellular locales affected by PDT in a spatiotemporally defined fashion [35, 37]. This cytoprotective activity of MAP ultimately leads to lowered PDT-associated cell death [35, 37]. For example, Hypericin primarily localizes in the ER and Hyp-PDT thereby causes oxidative ER stress, ER-to-mitochondria transfer of ROS through mitochondria-associated ER membranes and thereafter mitochondrial apoptosis [3, 20]. Subsequently, MAP is activated and initially strives to recycle the damaged ER (through reticulophagy) and later tries to recycle damaged mitochondria (through mitophagy) [35, 37]. This spatiotemporally defined MAP activity subsequently lowers ER stress and mitochondrial apoptosis [35, 37]. In this paradigm, we also found that CMA may co-exist with MAP such that CMA may help in recycling of specific damaged cytosolic proteins and thereby augment cancer cell survival [35, 37]. This cytoprotective role of CMA was found to be more pronounced when MAP was genetically inactivated (e.g. in ATG5 knockout cells)

[35, 37]. Thus, PDT-induced autophagy exerts resistance to cell death by recycling the damaged organelles and subcellular sites targeted by the PDT-induced oxidative stress.

It is important to note, though, that the "black-and-white" scenario described above is not applicable to all the PDT paradigms. A particularly interesting scenario that may in the future help in solving the problem about the "switch" that decides between pro-death and pro-survival roles of autophagy is the PDT settings based on Pc4 and 5-ALA. In both these cases, contradictory studies exist, that support pro-death as well as pro-survival role of autophagy for the same photosensitizer (Table 4.1). It is important to note that this scenario is not unheard of in general, as similar simultaneous contradictory instances have been reported for some other widely applied anticancer chemotherapeutics like bortezomib [38], temozolamide [39] and imatinib [40]. There are several processes or phenomena that can explain these contradictory observations. For example, technically speaking, the nature of cancer cell type used (different "cell culture clones" of cancer cell lines "evolving" spontaneously *in vitro*) and the type of autophagy blockade strategy applied can make a difference (considering that pharmacological inhibition of autophagy is prone to off-target effects) [2]. On the level of PDT treatment itself, differences can exist in terms of sub-cellular localization of the photosensitizers—a parameter that has been found to be highly prone to variations. Last but not least, on the mechanistic level, contextually different outcomes may exist due to the variations in the cross-talk between cell death-associated signalling and autophagy e.g. pro-apoptotic proteins like caspases or calpains can trigger the cleavage of autophagy-related proteins (like Beclin 1 or ATG5) that results often in a gain-of function, and similarly autophagy can target pro-apoptotic proteins (like active caspase 8) for degradation [41]; thus, deregulation of one pathway may lead to increased activation of the other pathway.

The latter point may well be applicable in the case of 5-ALA PDT [42], where the pro-death function of autophagy might have been observed due to the presence of a cancer system with predisposition towards autophagic cell death [42]. Several lines of evidences suggest that the glioma system is more susceptible to autophagy-inducing therapies (like temozolamide and imatinib) due to its tendency to undergo autophagic cell death, at least *in vitro* [39, 40, 43]. More specifically it has been observed that glioma cells are more likely to respond to therapy through excessive autophagy rather than apoptosis, possibly due to deregulated caspase signalling in glioma cells [43, 44]. However, more studies are required to establish this as a primary reason for such variations or bias.

Nevertheless, one thing that becomes clear from these observations is that it is the combination of the PDT setting and various technical or mechanistic parameters that may decide the ultimate functional role of autophagy in therapy response. Last but not least, the exact association between organelle-specific stress and functional autophagic outcome in terms of therapy response is still an enigmatic point that needs further attention perhaps through the agency of PDT combined with synthetic biology paradigms. One such promising synthetic biology paradigm is genetically-encoded photosensitizers (GEP) [45]. Chemical photosensitizers can exhibit varia-

tions in cellular localization especially with respect to certain cancer cell types or the photosensitizer concentration. Moreover, even if the chemical photosensitizers localize predominantly at a particular subcellular site, there is no way of excluding the possibility that a certain amount may have a slightly different localization. Such variations confound research into the link between organelle-specific stress and the functional autophagic therapy outcome. We envisage that GEPs [45] targeted to particular subcellular organelles may help in solving these issues and help characterize the organelle stress-autophagy link in cancer therapy response.

Direct Autophagy Inhibition via Photosensitizers: An Untapped Potential?

The contextual role of autophagy in shaping cancer responsiveness to different PDT paradigms is interesting. On the basis of such knowledge, autophagic cell death-inducing PDT paradigms can be applied to cancer types susceptible to pro-death autophagy. Similarly, PDT paradigms associated with pro-survival autophagy can become good candidates for combinatorial therapy with pharmacological autophagy inhibitors. However, it is noteworthy that the currently available pharmacological autophagy inhibitors have significant off target effects which depending on the context may or may not be desirable [2]. In such a scenario, a need has emerged to characterize new and more specific autophagy inhibitory strategies that are devoid of undesirable off target effects. Interestingly, two photosensitizers often utilized for PDT have been shown recently to have the capability to inhibit autophagy directly in the absence of light activation. Photofrin alone was shown to inhibit autophagosome formation under conditions of rapamycin treatment and starvation [46]. Preliminary data indicated that photofrin might be interfering with the process of autophagosome initiation [46], however, it remains unexplored. On the other hand, verteporfin was selected from more than 3500 drugs through an automated microscopy screening assay as a specific inhibitor of autophagosome accumulation [47]. Like photofrin, verteporfin was also found to inhibit autophagy under conditions of rapamycin treatment or starvation [47]. In fact, it also inhibited the accumulation of chloroquine-induced autophagosomes [47]. The authors confirmed that verteporfin inhibits autophagosome formation, sequestration and degradation [47].

As apparent from the above data, there is clear untapped potential associated with these photosensitizers' utilization as autophagy inhibitors. However, it is noteworthy that to inhibit autophagy with these photosensitizers it was necessary to reach high concentrations (up to 10 μM for verteporfin, for example); something that might be a roadblock for *in vivo* treatment. However, for *in vitro* mechanistic studies, these photosensitizers could be immediately useful. Nevertheless, since the above studies are primarily *in vitro* in nature it would be crucial to confirm whether photofrin and verteporfin also inhibit autophagy *in vivo* in tumors, at concentrations that are accompanied by minimal side-effects. Such inhibitors, if proven physiologically effective, can have significant advantages over current pharmacological

autophagy inhibitors both in terms of diagnostic tracing of autophagy inhibition as well as possibly on target therapeutic intervention. Further dedicated studies are required to tap into the potential of these photosensitizers as specific autophagy inhibitors.

Autophagy Pathways In Vivo and PDT Therapy Response: Uncharted Territories

In vitro cell cultures do not recapitulate the *in vivo* scenario where tumor cells interact with stromal cells or immune cells. As discussed previously, tumor stromal cells and immune cells can also have a significant impact on autophagy-based anticancer therapy outcome and responsiveness. Thus, in scenarios where the cancer cell-associated autophagy cross-talk with stromal or immune cells has significant impact on anticancer therapy, the *in vitro* results (as detailed in Table 4.1 for various PDT paradigms) may no longer be translatable *in vivo*. For instance, it was recently shown that while autophagy inhibition *in vitro* sensitized cancer cells to radiotherapy yet the same intervention reduced radioresponses *in vivo* due to lack of immunogenic signalling [48]. It is noteworthy that none of the *in vitro* results described for various PDT paradigms in Table 4.1 have as yet been tested *in vivo*. Co-culture analysis of cancer cell-associated autophagy crosstalk with immune cells was performed recently by us for Hyp-PDT (as discussed in the next section); however a more *in vivo* analysis on broader terms is required to completely characterize the tumor-level therapeutic relevance of autophagy in deciding cancer resistance or susceptibility to PDT.

Autophagy-Based Suppression of Anti-Cancer Immunity: An Emerging Paradigm in PDT Therapy Response

In recent times, it has emerged that the surface proteome and/or the secreted proteome of a dying cancer cell can be composed of particular danger signals or DAMPs that could facilitate the formation of a productive interface between the cancer cells and the immune cells that could reduce therapy resistance and instigate anti-tumorigenic immune reactions[49, 50]. To this end, a cancer cell death sub-routine capable of exhibiting a surface or secreted proteome “rich” in crucial DAMPs is immunogenic cell death (ICD) [16, 51–53]. We have recently shown that Hyp-PDT induces efficient *bona fide* ICD in several cancer model systems *in vitro*, *ex vivo* and *in vivo* [51, 54, 55]. Hyp-PDT induced ICD is immunostimulatory in character such that cancer cells undergoing this sub-routine can mediate potent anti-tumor immunity [18, 19, 52, 56, 57]. DAMPs found to be crucial for ICD and anti-tumor immunity (as also induced by Hyp-PDT) include—pre-apoptotically surface exposed calreticulin (ecto-CRT)—an ‘eat me’ signal, pre/early-apoptotically secreted

ATP—a 'find me' and inflammasome activating signal and mid/late-apoptotically released chaperones like HSP70/90– acting as TLR (toll-like receptor) agonists and 'find me' signals [51, 54, 55].

We have observed that following Hyp-PDT, cancer cell-associated autophagy (specifically MAP) has the potential to influence the interface between the dying cancer cells and the immune system by modulating the emission of DAMPs [30, 58]. More specifically, we found that following Hyp-PDT, autophagy suppressed the emission of ecto-CRT (without affecting ATP secretion) such that autophagy ablation caused approximately two-fold increase in ecto-CRT [30, 58]. Moreover, autophagy ablation in Hyp-PDT-treated cancer cells had functional effects on immunological determinants of anticancer immunity. Autophagy ablation in cancer cells treated with Hyp-PDT caused augmented phenotypic maturation of DCs, improved DC-derived IL-6 production and an increase of DC-mediated clonal expansion of (IFN-γ producing) $CD4^+$/$CD8^+$T cells [30, 58]. Thus, Hyp-PDT-induced autophagy (or MAP) in cancer cells was found to play an unprecedented role in therapy resistance by encouraging immunoevasion, suppressing the cancer cell immunogenicity and deteriorating elicitation of anticancer immunity (Fig. 4.1).

Interestingly, there were some indications that as far as emission of DAMPs is concerned, MAP and CMA may play conflicting roles following Hyp-PDT [5, 30, 58]. More specifically, we observed that fibroblasts lacking the CMA-essential gene *LAMP2A* are unable to exhibit ecto-CRT after Hyp-PDT [5]. Whether this effect of CMA on ecto-CRT finally translates into immunologically relevant consequences needs further investigation. However, as of now, on the basis of available data we can confidently say that MAP assists in resistance against Hyp-PDT by suppressing anticancer immune effector mechanisms.

Conclusion

Autophagy is one of the main catabolic pathways in a eukaryotic cell, utilized for cellular self-degradation in order to support recycling and replenishing of biomolecular resources crucial for cell growth and survival. Although, as such, autophagy is inherently a cytoprotective and prosurvival phenomenon, yet in general, it has become clear through a number of studies that autophagy has a highly contextual role in cancer biology; depending on which stage, site or type of tumorigenesis or therapy intervention one is looking at, the role of autophagy may end up varying from pro- to anti-tumorigenic. To this end, the unique nature of PDT allows higher flexibility and refined insights with respect to testing and estimation of autophagy-based cancer therapy responsiveness. Several studies have shown that, depending on the photosensitizer under consideration, autophagy activated by PDT either contributes to therapy resistance or susceptibility. Therapy susceptibility can be outlined by the ability of PDT to induce autophagic cell death—a molecularly scarcely explored phenomenon that needs further attention in the future. On the other hand, PDT-induced autophagy exerts resistance to cell death by recycling the damaged

organelles and subcellular sites targeted by the PDT-induced oxidative stress. Moreover, cancer cell-associated autophagy also assists in resistance against PDT by suppressing anticancer immune effector mechanisms. From all these observations it is clear that it is the combination of the photosensitizer's nature, cancer cell type, various autophagy-related technical or mechanistic parameters and cancer cell-immune cell cross-talk that may decide the ultimate functional role of autophagy in therapy response. However, this conclusion is based on largely *in vitro* and partly *ex vivo* results and, thus, there is an immediate need to carry out more *in vivo* analysis on broader terms to completely characterize the tumor-level therapeutic relevance of autophagy in deciding cancer resistance or susceptibility to PDT.

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References

1. Yen WL, Klionsky DJ. How to live long and prosper: autophagy, mitochondria, and aging. Physiology (Bethesda). 2008;23:248–62.
2. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2012;8:445–544.
3. Maes H, Van Eygen S, Krysko DV, Vandenabeele P, Nys K, Rillaerts K, Garg A, Verfaillie T, Agostinis P. BNIP3 supports melanoma cell migration and vasculogenic mimicry by orchestrating the actin cytoskeleton. Cell Death Dis. 2014;5:e1127.
4. Maes H, Rubio N, Garg AD, Agostinis P. Autophagy: shaping the tumor microenvironment and therapeutic response. Trends Mol Med. 2013;19:428–46.
5. Verfaillie T, Garg AD, Agostinis P. Targeting ER stress induced apoptosis and inflammation in cancer. Cancer Lett. 2013;332:249–64.
6. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell. 2011;147:728–41.
7. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008;132:27–42.
8. Li WW, Li J, Bao JK. Microautophagy: lesser-known self-eating. Cell Mol Life Sci CMLS. 2012;69:1125–36.
9. Massey AC, Kaushik S, Sovak G, Kiffin R, Cuervo AM. Consequences of the selective blockage of chaperone-mediated autophagy. Proc Natl Acad Sci U S A. 2006;103:5805–10.
10. Chen N, Karantza V. Autophagy as a therapeutic target in cancer. Cancer Biol Ther. 2011;11:157–68.
11. Liu EY, Ryan KM. Autophagy and cancer-issues we need to digest. J Cell Sci. 2012;125(Pt 10):2349–8.
12. Amaravadi RK, Lippincott-Schwartz J, Yin XM, Weiss WA, Takebe N, Timmer W, DiPaola RS, Lotze MT, White E. Principles and current strategies for targeting autophagy for cancer treatment. Clin Cancer Res. 2011;17:654–66.
13. White E. Deconvoluting the context-dependent role for autophagy in cancer. Nat Rev Cancer. 2012;12:401–10.

14. Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, et al. Photodynamic therapy of cancer: an update. CA Cancer J Clin. 2011;61:250–81.
15. Verfaillie T, van Vliet A, Garg AD, Dewaele M, Rubio N, Gupta S, de Witte P, Samali A, Agostinis P. Pro-apoptotic signaling induced by photo-oxidative ER stress is amplified by Noxa, not Bim. Biochem Biophys Res Commun. 2013;438:500–6.
16. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. The emergence of phox-ER stress induced immunogenic apoptosis. Oncoimmunology. 2012;1:786–8.
17. Garg AD, Nowis D, Golab J, Agostinis P. Photodynamic therapy: illuminating the road from cell death towards anti-tumor immunity. Apoptosis. 2010;15:1050–71.
18. Garg AD, Nowis D, Golab J, Vandenabeele P, Krysko DV, Agostinis P. Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. Biochim Biophys Acta. 2010;1805:53–71.
19. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. DAMPs and PDT-mediated photo-oxidative stress: exploring the unknown. Photochem Photobiol Sci. 2011;10:670–80.
20. Verfaillie T, Rubio N, Garg AD, Bultynck G, Rizzuto R, Decuypere JP, Piette J, Linehan C, Gupta S, Samali A, Agostinis P. PERK is required at the ER-mitochondrial contact sites to convey apoptosis after ROS-based ER stress. Cell Death Differ. 2012;19:1880–91.
21. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest. 2003;112:1809–20.
22. Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH, Jung JU. Autophagic and tumor suppressor activity of a novel Beclin1-binding protein UVRAG. Nat Cell Biol. 2006;8:688–99.
23. Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, Liang C, Jung JU, Cheng JQ, Mulè JJ, Pledger WJ, Wang HG. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat Cell Biol. 2007;9:1142–51.
24. Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, Tavarè S, Arakawa S, Shimizu S, Watt FM, Narita M. Autophagy mediates the mitotic senescence transition. Genes Dev. 2009;23:798–803.
25. Kon M, Kiffin R, Koga H, Chapochnick J, Macian F, Varticovski L, Cuervo AM. Chaperone-mediated autophagy is required for tumor growth. Sci Transl Med. 2011;3:109ra17.
26. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, Thompson CB. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell. 2005;120:237–48.
27. Fung C, Lock R, Gao S, Salas E, Debnath J. Induction of autophagy during extracellular matrix detachment promotes cell survival. Mol Biol Cell. 2008;19:797–806.
28. Du J, Teng RJ, Guan T, Eis A, Kaul S, Konduri GG, Shi Y. Role of autophagy in angiogenesis in aortic endothelial cells. Am J Physiol Cell Physiol. 2012;302:C383–91.
29. Garg AD, Martin S, Golab J, Agostinis P. Danger signalling during cancer cell death: origins, plasticity and regulation. Cell Death Differ. 2014;21:26–38.
30. Garg AD, Dudek AM, Ferreira GB, Verfaillie T, Vandenabeele P, Krysko DV, Mathieu C, Agostinis P. ROS-induced autophagy in cancer cells assists in evasion from determinants of immunogenic cell death. Autophagy. 2013;9:1292–1307.
31. Reiners JJ Jr., Agostinis P, Berg K, Oleinick NL, Kessel D. Assessing autophagy in the context of photodynamic therapy. Autophagy. 2010;6:7–18.
32. Kessel DH, Price M, Reiners JJ Jr. ATG7 deficiency suppresses apoptosis and cell death induced by lysosomal photodamage. Autophagy. 2012;8:1333–41.
33. Du L, Jiang N, Wang G, Chu Y, Lin W, Qian J, Zhang Y, Zheng J, Chen G. Autophagy inhibition sensitizes bladder cancer cells to the photodynamic effects of the novel photosensitizer chlorophyllin e4. J Photochem Photobiol B. 2014;133C:1–10.
34. Lihuan D, Jingcun Z, Ning J, Guozeng W, Yiwei C, Wei L, Jing Q, Yuanfang Z, Gang C. Photodynamic therapy with the novel photosensitizer chlorophyllin f induces apoptosis and autophagy in human bladder cancer cells. Lasers Surg Med. 2014;46:319–34.
35. Dewaele M, Martinet W, Rubio N, Verfaillie T, de Witte PA, Piette J, Agostinis P. Autophagy pathways activated in response to PDT contribute to cell resistance against ROS damage. J Cell Mol Med. 2010;15:1402–14.

36. Garg AD, Dudek AM, Agostinis P. Calreticulin surface exposure is abrogated in cells lacking, chaperone-mediated autophagy-essential gene, LAMP2A. Cell Death Dis. 2013;4:e826.
37. Rubio N, Coupienne I, Di Valentin E, Heirman I, Grooten J, Piette J, Agostinis P. Spatiotemporal autophagic degradation of oxidatively damaged organelles after photodynamic stress is amplified by mitochondrial reactive oxygen species. Autophagy. 2012;8:1312–24.
38. Kawaguchi T, Miyazawa K, Moriya S, Ohtomo T, Che XF, Naito M, Itoh M, Tomoda A. Combined treatment with bortezomib plus bafilomycin A1 enhances the cytocidal effect and induces endoplasmic reticulum stress in U266 myeloma cells: crosstalk among proteasome, autophagy-lysosome and ER stress. Int J Oncol. 2011;38:643–54.
39. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ. 2004;11:448–57.
40. Shingu T, Fujiwara K, Bogler O, Akiyama Y, Moritake K, Shinojima N, Tamada Y, Yokoyama T, Kondo S. Inhibition of autophagy at a late stage enhances imatinib-induced cytotoxicity in human malignant glioma cells. Int J Cancer. 2009;124:1060–71.
41. Gordy C, He YW. The crosstalk between autophagy and apoptosis: where does this lead? Protein Cell. 2012;3:17–27.
42. Coupienne I, Bontems S, Dewaele M, Rubio N, Habraken Y, Fulda S, Agostinis P, Piette J. NF-kappaB inhibition improves the sensitivity of human glioblastoma cells to 5-aminolevulinic acid-based photodynamic therapy. Biochem Pharmacol. 2010;81:606–16.
43. Jiang H, White EJ, Conrad C, Gomez-Manzano C, Fueyo J. Autophagy pathways in glioblastoma. Methods Enzymol. 2009;453:273–86.
44. Yuan J, Kroemer G. Alternative cell death mechanisms in development and beyond. Genes Dev. 2010;24:2592–602.
45. Bulina ME, Chudakov DM, Britanova OV, Yanushevich YG, Staroverov DB, Chepurnykh TV, Merzlyak EM, Shkrob MA, Lukyanov S, Lukyanov KA. A genetically encoded photosensitizer. Nat Biotechnol. 2006;24(1):95–9.
46. Luo S, Xing D, Wei Y, Chen Q. Inhibitive effects of photofrin on cellular autophagy. J Cell Physiol. 2010;224:414–22.
47. Donohue E, Tovey A, Vogl AW, Arns S, Sternberg E, Young RN, Roberge M. Inhibition of autophagosome formation by the benzoporphyrin derivative verteporfin. J Biol Chem. 2011;286:7290–300.
48. Ko A, Kanehisa A, Martins I, Senovilla L, Chargari C, Dugue D, Marino G, Kepp O, Michaud M, Perfettini JL, Kroemer G, Deutsch E. Autophagy inhibition radiosensitizes in vitro, yet reduces radioresponses in vivo due to deficient immunogenic signalling. Cell Death Differ. 2014;21:92–9.
49. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer. 2012;12:860–75.
50. Dudek AM, Martin S, Garg AD, Agostinis P. Immature, semi-mature, and fully mature dendritic cells: toward a DC-cancer cells interface that augments anticancer immunity. Front Immunol. 2013;4:438.
51. Garg AD, Krysko DV, Verfaillie T, Kaczmarek A, Ferreira GB, Marysael T, Rubio N, Firczuk M, Mathieu C, Roebroek AJ, Annaert W, Golab J, de Witte P, Vandenabeele P, Agostinis P. A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death. EMBO J. 2012;31:1062–79.
52. Kepp O, Tesniere A, Zitvogel L, Kroemer G. The immunogenicity of tumor cell death. Curr Opin Oncol. 2009;21:71–76.
53. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer. 2012. doi:10.1038/nrc3380.
54. Dudek AM, Garg AD, Krysko DV, De Ruysscher D, Agostinis P. Inducers of immunogenic cancer cell death. Cytokine Growth Factor Rev. 2013;24:319–33.
55. Garg AD, Krysko DV, Vandenabeele P, Agostinis P. Hypericin-based photodynamic therapy induces surface exposure of damage-associated molecular patterns like HSP70 and calreticulin. Cancer Immunol Immunother. 2012;61:215–21.

56. Galluzzi L, Kepp O, Kroemer G. Enlightening the impact of immunogenic cell death in photodynamic cancer therapy. EMBO J. 2012;31:1055–1057.
57. Zitvogel L, Kepp O, Kroemer G. Decoding cell death signals in inflammation and immunity. Cell. 2010;140:798–804.
58. Garg AD, Dudek AM, Agostinis P. Autophagy-dependent suppression of cancer immunogenicity and effector mechanisms of innate and adaptive immunity. Oncoimmunology. 2013;2(10):e26260.
59. Ji HT, Chien LT, Lin YH, Chien HF, Chen CT. 5-ALA mediated photodynamic therapy induces autophagic cell death via AMP-activated protein kinase. Mol Cancer. 2010;9:91.
60. Kessel D, Reiners JJ Jr. Apoptosis and autophagy after mitochondrial or endoplasmic reticulum photodamage. Photochem Photobiol. 2007;83:1024–8.
61. Markovic ZM, Ristic BZ, Arsikin KM, Klisic DG, Harhaji-Trajkovic LM, Todorovic-Markovic BM, Kepic DP, Kravic-Stevovic TK, Jovanovic SP, Milenkovic MM, Milivojevic DD, Bumbasirevic VZ, Dramicanin MD, Trajkovic VS. Graphene quantum dots as autophagy-inducing photodynamic agents. Biomaterials. 2012;33:7084–92.
62. Davids LM, Kleemann B, Cooper S, Kidson SH. Melanomas display increased cytoprotection to hypericin-mediated cytotoxicity through the induction of autophagy. Cell Biol Int. 2009;33:1065–72.
63. Rubio N, Verrax J, Dewaele M, Verfaillie T, Johansen T, Piette J, Agostinis P. p38(MAPK)-regulated induction of p62 and NBR1 after photodynamic therapy promotes autophagic clearance of ubiquitin aggregates and reduces reactive oxygen species levels by supporting Nrf2-antioxidant signaling. Free Radic Biol Med. 2013;67:292–303.
64. Francois A, Marchal S, Guillemin F, Bezdetnaya L. mTHPC-based photodynamic therapy induction of autophagy and apoptosis in cultured cells in relation to mitochondria and endoplasmic reticulum stress. Int J Oncol. 2011;39:1537–43.
65. Xue LY, Chiu SM, Oleinick NL. Atg7 deficiency increases resistance of MCF-7 human breast cancer cells to photodynamic therapy. Autophagy. 2010;6:248–55.
66. Xue LY, Chiu SM, Azizuddin K, Joseph S, Oleinick NL. The death of human cancer cells following photodynamic therapy: apoptosis competence is necessary for Bcl-2 protection but not for induction of autophagy. Photochem Photobiol. 2007;83:1016–23.
67. Separovic D, Kelekar A, Nayak AK, Tarca AL, Hanada K, Pierce JS, Bielawski J. Increased ceramide accumulation correlates with downregulation of the autophagy protein ATG-7 in MCF-7 cells sensitized to photodamage. Arch Biochem Biophys. 2009;494:101–5.
68. Chen YJ, Huang WP, Yang YC, Lin CP, Chen SH, Hsu ML, Tseng YJ, Shieh HR, Chen YY, Lee JJ. Platonin induces autophagy-associated cell death in human leukemia cells. Autophagy. 2009;5:173–83.
69. Panzarini E, Inguscio V, Dini L. Timing the multiple cell death pathways initiated by Rose Bengal acetate photodynamic therapy. Cell Death Dis. 2011;2:e169.
70. Fateye B, Li W, Wang C, Chen B. Combination of phosphatidylinositol 3-kinases pathway inhibitor and photodynamic therapy in endothelial and tumor cells. Photochem Photobiol. 2012;88:1265–72.
71. Andrzejak M, Price M, Kessel DH. Apoptotic and autophagic responses to photodynamic therapy in 1c1c7 murine hepatoma cells. Autophagy. 2011;7:979–84.

Chapter 5
Isolation and Initial Characterization of Resistant Cells to Photodynamic Therapy

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Abstract After photodynamic therapy (PDT), the apparition of resistant tumor cells can occur. Laboratory models are being developed in order to understand the potential mechanisms implicated in such resistance. In this sense, we describe the methods published for the isolation and characterization of tumor cells resistant to PDT. We also propose other unpublished procedures that could be of interest for the study of cells resistant to PDT. Factors such as the parental cell line, the photosensitizer (PS) (or prodrug), the photodynamic treatment conditions, the treatment interval, and the clonal or total population selection have to be taken into consideration. Treatment doses are generally high and repeated over time. The development of resistant cells to PDT could take several months. The characterization of resistant cell populations *vs* parental cells can be performed by using different cellular and molecular techniques, including: cell morphology analysis, intracellular PS content measurement, PS localization, migration and invasion capacity, expression and distribution of adhesion proteins, death proteins and evaluation of specific genes implicated in cell proliferation and survival. Transplantation mouse models also contribute to determine the biological activity of the PDT-resistant cells *in vivo*, allowing the evaluation of their tumorigenicity and aggressiveness. Laboratory cell models will help us to understand how resistance to anticancer PDT affects the biological and functional aspects of tumorigenicity *in vitro* and *in vivo*, which are necessary to improve the clinical results.

Keywords Isolation · PDT · Photodynamic therapy · Photosensitizer · Resistance · Tumor cells

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Abbreviations

ALA	δ-aminolevulinic acid
ALDH1	aldehyde dehydrogenase 1
BCC	basocellular carcinoma
BCRP	breast cancer resistant protein
BPD-MA	benzoporphyrin derivative monoacid ring A
CAM	cell adhesion molecule
EGFR	epidermal growth factor receptor
ERK	extracellular signal regulated kinases
HPPH	2-(1-hexyloxethyl)-2-devinyl pyropheophorbide-a
IAP	inhibitor of apoptosis protein
MAL	methyl δ-aminolevulinic acid
MAPK	mitogen-activated protein kinase
MDR	multidrug resistance
MRP	multidrug resistant associated protein
NMSC	non melanoma skin cancer
PHP	polyhematoporphyrin
P-gp	P-glycoprotein
PDT	photodynamic therapy
PpIX	protoporphyrin IX
PS	photosensitizer
PII	photofrin II
PPC	Zn(II) pyridinium-substituted phthalocyanine
SOD	superoxide dismutase

Introduction

Resistance to anti-cancer therapies is the main cause of their failure, leading to tumor progression and poor clinical prognoses. Thus, a deeper understanding of how resistance affects the biological and functional aspects of tumorigenicity is necessary to enhance the efficacy of cancer treatments. Resistance to chemotherapy as well as radiotherapy has been broadly studied; however, the process is far from being well understood. The effectiveness of the treatment for specific cancers is limited by drug resistance and, in the same way, recurrence after radiotherapy continues to pose a major obstacle [1–5]. Although it is not well documented, Photodynamic Therapy (PDT) of cancer can also induce tumor cell resistance in patients [6–9]. Therefore, the development of cellular and/or animal models, based on the selection of resistant cells, that allow a better understanding of this process, is an important goal in the research of the types of cancer in which this therapeutic modality is being applied, including cancers of the head, neck, lung, esophagus, urinary bladder, gynecological cancers and particularly, non-melanoma skin cancer (NMSC)

[10–15]. The complexity of genetic and epigenetic alterations of tumors invariably highlights complex situations, but the development of this kind of models will be very useful to perform systematic, molecular and functional studies, to analyze the mechanisms underlying PDT-resistance.

In cancer therapy, the first treatment usually kills most tumor cells; however, some tumors do not react properly to the therapy and resistant cancer cells can become even more aggressive after several cycles of treatment. In general, resistance can be classified into two types: (i) intrinsic, in which resistance-mediating factors pre-exist in the tumor cells before receiving treatment, and (ii) acquired, which is developed as a consequence of the treatment in tumors initially sensitive. Intrinsic resistance arises from a complex range of biochemical and molecular characteristics of the tumor which result in the cells death escape. Acquired resistance can be caused by different factors, including the limited amount of drug or radiation reaching the tumor, those affecting the tumor micro-environment, as well as mutations in tumor cells arising during treatment [16–19]. Other adaptive responses, such as increased expression of the therapeutic target and activation of alternative compensatory signaling pathways, have to be also considered. Furthermore, it is recognized that tumors can contain a high degree of molecular heterogeneity with genotypic or phenotypic variations [20–22]. This intratumoral heterogeneity implies that different parts of a tumor may have different properties, apart from the existence of different degrees of sensitivity to different treatments. Furthermore, the heterogeneity can lead to variations in the specific mechanisms of response induced by the therapy. In addition, in the acquired resistance, the tumors not only become resistant to a particular therapy originally used to treat them, moreover they may develop cross-resistance to other therapies with different mechanisms. This is particularly evident in chemotherapy, where tumor cells can become resistant to multiple drugs. Therefore, resistance can arise through therapy-induced selection of a cell population that developed resistant characteristics and/or from a resistant minor subpopulation of cells present in the original tumor with determined characteristics.

PDT can leave a significant number of surviving tumor cells which have been exposed to reactive oxygen species arising when the photosensitizer is excited by light, but insufficient to destroy them. Potential changes in the rates of cell division, death, mutation or migration would have direct effects on the tumor growth and also in the response to a new photodynamic treatment with biological consequences. In this context, we describe different methods to isolate tumor cells resistant to PDT as well as some of the resistant characteristics, which facilitate a better understanding of the mechanism of action of PDT to enhance its efficacy.

Isolation of PDT-Resistant Cells

A better understanding of the *in vitro*/*in vivo* characteristics of PDT-resistant cells allow us to study the long-term molecular, biochemical and cellular changes induced by the treatment. This can be exploited to selectively treat the surviving cells

with modified PDT protocols or with other therapies. Some drug-resistant cell lines for chemotherapeutic agents as methotrexate, vinblastine or terephthalanilide were first developed around 1960 by using *in vivo* mouse models [23]. The *in vitro* development of resistant cancer cell lines was early described in chemotherapy in 1970 [24]. The research isolated resistant cell lines from chinese hamster cells using an increased treatment dose with actinomycin D. The cells showed a 2500-fold greater resistance to the drug than parental cells, and these resistant cell lines were also cross-resistant to other chemotherapy drugs, such as vinblastine and doxorubicin. Many examples of drug-resistant cells isolation have been reported since then in the literature. In addition, isolation of resistant cells to other cancer therapies has also been described. Hahn and van Kersen [25], for instance, obtained heat-resistant cell strains from mouse radiation induced fibrosarcoma cells (RIF-1) by repeated heating (11 heating and regrowth cycles) of cells derived from survivors of previous heating cycles (60 min; 45 °C). They selected several thermally resistant strains derived from single cells that had survived. The resistant cells showed a growth rate and plating efficiency similar to that of RIF-1 cells and no obvious morphological abnormalities were described. In the case of PDT, resistant cells have been obtained by using exogenous PSs, such as Photofrin and phthalocyanines among others, and with the endogenous PS protoporphyrin IX (PpIX), formed from δ-aminolevulinic acid (ALA) or methyl δ-aminolevulinic acid (MAL) through the heme biosynthetic pathway [26–28].

Resistance against anticancer therapies has hardly been studied in animal models until very recently. The *in vivo* models provide the native microenvironment in which tumors reside, being, therefore, more "real" than the *in vitro* ones. However, although there are a wide number of published papers in chemotherapy [18–19], no much work has been performed to evaluate resistance to PDT [8, 26–28]. The most frequently used *in vivo* models are the mouse tumor allografts (or syngenic) and the human tumor xenografts, obtained by inoculating both immortalized mouse or human cancer cells, respectively. Small fragments from tumors showing intrinsic resistance to anticancer agents can also be injected. In addition, and to avoid the rejection of the implanted cancer cells, mice used for allografts or xenografts had impaired immune systems. Tumors induced by chemical or physical carcinogens (ultraviolet or ionizing radiation) can also be used to obtain cell lines, with innate or acquired resistance, due to a determined therapy.

Defining the Level of Resistance

In chemotherapy, drug-resistant cell models are generated in the laboratory, principally by repetitive exposures of culture cancer cells to increased concentrations of drugs. The surviving resistant cells are then compared to the parental sensitive ones using different assays (e.g. viability/proliferation assays, such as the MTT, or the clonogenic assay) [23, 29, 30]. Similar strategies have been used to generate PDT-resistant cells using repeated photodynamic treatments as it will be described later.

Cellular sensitivity is determined by exposing them to previously defined treatment conditions followed by assessing cell viability to achieve the Fold Resistance Index. In chemotherapy, the drug concentration that causes 50 % growth inhibition (IC50) is the main index used to determine the increase in resistance (Fold Resistance IC50 = Resistant Cell Line/IC50 of Parental Cell Line). In PDT, there is not a well-defined way to describe it. The Fold Resistance Index would refer to selected treatment conditions (PS concentration and dose light irradiation) to induce a lethality of 50 % (LD50) or 90 % (LD90) in the parental cell line (Fold resistance = Resistant Cell Line/Parental Cell Line).

To establish the level of drug resistance that occurs in the clinical treatment of cancer, the ideal situation would be to compare cell cultures established directly from cancer patients before and after chemotherapy. Data from chemotherapy have been recently summarized by McDermott et al. [23], indicating that the majority of cell lines developed after chemotherapy from patients with lung, neuroblastoma or ovarian cancers, showed from 2–5-fold increase in resistance to the agents from the IC50 value of the parent cell line. The fold increase in PDT-resistant *vs* parental cells, in general, is not so high, but it is considered as resistant variant a 1.5-fold increase over the parental cells.

Different models for PDT can be developed but, in general, once it has been selected the treatment conditions of drug concentration and light radiation dose to induce a LD50 or LD90, tumor cells are subjected to repeated PDTs and total resistant populations or specific resistant clones are selected from the mixed population.

The main objectives are to develop an *in vitro* model where repeated therapy is extensively used to achieve large fold resistance vs parental cells, and to obtain a stable phenotype in the resistant cells. Some factors have to be taken into account to create the model including the parental cell line and the treatment conditions (drug concentration and light radiation dose) used, that must be optimized depending on the parental cell line selected for use in developing the resistant model. The recovery rate after the treatment is also important since there can be differences between PSs, even at equivalently cytotoxic treatment conditions administered to cells. After the treatment with the selected dose, cells must be able to return to logarithmic growth, ensuring the selection of resistant cell subpopulations.

Selecting the Cellular Model

In cancer research, the study of the cellular and molecular bases of intrinsic and acquired resistance to cancer therapies, including PDT, could be performed by using mainly two types of *in vitro* models: (i) primary cell cultures, directly obtained from human or mouse tumors, and whose sensitivity or resistance to the anticancer therapy has to be later evaluated, and (ii) immortalized cancer cell lines, showing or not primary resistance.

In the first option, the ideal situation would be to select a chemotherapy and radiation untreated cell line since previous treatment may have already caused changes

in the resistance pathways and increased the expression of drug resistance markers that may not be relevant to the therapy being studied. However, cell lines derived from untreated human tumors are relatively rare and most of the cells used in these studies are been derived from treated tumors. In relation to immortalized cancer cells, many cell lines are available for each cancer type, carrying different genetic alterations to choose the most suitable *in vitro* models to investigate mechanisms of resistance to PDT. In the case of chemotherapy, for instance, there are two important sources to be checked: the Genomics of Drug Sensitivity in Cancer (GDSC, www.cancerRxgene.org), which is the largest public resource for information on drug sensitivity in cancer cells and molecular markers of drug response; and the Cancer Cell Line Encyclopedia (CCLE, www.broadinstitute.org/ccle) which includes data related with gene expression or chromosomal copy number [31, 32]. Therefore, there are numerous cell lines available and the selection will depend on the kind of study to carry out. In the case of PDT, not much research related to PDT-resistant cell selection has been performed, and those which have been carried out have used immortalized cell lines, including the mouse radiation-induced fibrosarcoma (RIF-1) cells [26, 33]; murine mammary adenocarcinoma (LM3) cells [28]; human colon adenocarcinoma (HT29) cells [34]; human lung adenocarcinoma (CL-5) cells; human melanoma (A435) cells and human breast carcinoma (MDA-MB-231) cells [35]; and human squamous cell carcinoma SCC-13 cells [8, 36].

Strategies for Selection of Resistant Cells

A validated protocol to reproduce resistance under the therapy-induced pressure in preclinical studies is based on an *in vivo/in vitro* selection of cancer cells with intrinsic or acquired resistance after chronic treatment. In any case, the scenarios to select resistant cells to PDT are multiple, and here we describe some of many different possibilities.

In Vitro Selection

In the *in vitro* system, apart from the cells selected (primary or established cell line) for the isolation of the resistant cells, it should be taken into account that tumors are heterogeneous and, then, their different subpopulations have different properties. Consequently, the cancer cell lines derived from them would be also heterogeneous [20–23]. In any case, and once the cell line has been chosen, there are three basic selection strategies for isolating anticancer therapy resistant cells: (1) selecting a small resistant population from the original culture; (2) selecting resistant clones and (3) selecting cells with determined molecular markers (Fig. 5.1a).

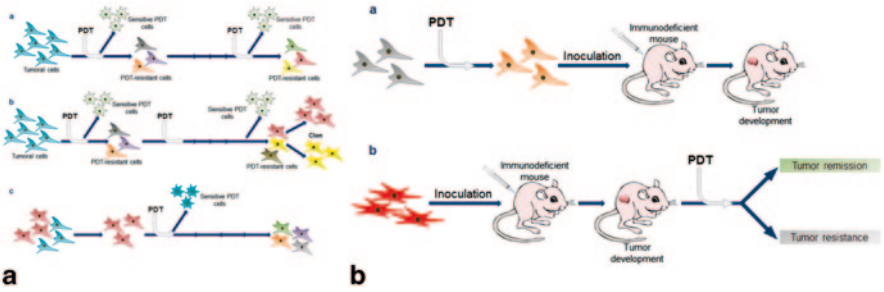

Fig. 5.1 Different ways to obtain *in vitro* (**a**) and *in vivo* (**b**) PDT-resistant cell populations. **a** The whole population is subjected to repeated treatments and a small percentage of potential heterogeneous cells can resist due to the development of mutations (*a*); cell clones with mutations and with new mutations developed after repeated PDT-treatments are selected. PDT would positively select first those cells that possess intrinsic resistant mechanisms (*b*); selecting cells expressing determined molecular markers and, then, expose them to PDT-treatments (*c*), **b** heterogeneous cell population is injected in the mice and then subjected to repeated PDT-treatments (*a*); cells are first subjected to repeated treatments and the resistant cell populations are inoculated in the mice (*b*)

1. *Selecting a Resistant Population from the Original Culture*

Basically, when a punctual treatment is given to a cell population, a small percentage of cells can resist, being responsible of the repopulation of the culture. This resistant cell population is again exposed to a new treatment and most probably the selected cells would be heterogeneous and differ from the original parental cells, due to the apparition of genetic or epigenetic alterations that promote their survival [23, 37, 38]. In this sense, heterogeneity has been seen in taxane-resistant models developed from human lung cancer cell lines [29, 37, 38] or in human breast cancer cells [30]. There is also another possibility, that the selection results in the isolation of a cell population that already had a resistant signature in the original culture. Indeed, this has been demonstrated for many drug-resistant models, which are often enriched with markers of cancer stem cells (CSCs). CSCs are thought to be responsible for tumor regeneration after chemotherapy and radiotherapy and they would also have a role in resistance to PDT.

Therefore, it is tempting to consider that from a heterogeneous group of initial cells, PDT would positively select those cells that suffer determined genetic alterations or possess intrinsic resistant mechanisms, while cells that do not have such acquired alterations or intrinsic mechanisms die after treatment. In addition, the therapy would progressively stimulate a higher expression of molecules that induce resistance, including additional mutations. The resulting resistant variant cells could be also finally selected, for instance, by cell sorting using specific molecular cell markers.

2. *Selecting Resistant Clones*

The second method is selecting resistant clones by limited dilution. Clonal selection has the advantage that isolated cells would be more resistant to the treatment than others within the same cell line [23, 39, 40]. However, it must be taking into

account that such clones are not necessarily the responsible for the tumor relapse and, eventually, for metastasis. For this method of isolation, two possible strategies can be followed. The first option consists on selecting resistant clones after treatment, subjecting such clones to a second therapy/drug and again selecting the most resistant clones that can be again subjected to new treatments. This was the protocol used, for instance, in the isolation of colchicine resistant cells from the carcinoma cell line KB3–1, treated with three stepwise increases drug treatment [39]. Clones were collected from each round of the selection strategy. The other option consists on selecting resistant clones after several rounds of treatments. This also would allow investigating heterogeneity within the developed drug-resistant model. In this sense, two cisplatin-resistant clones, obtained from a human colon cancer cell line (LoVo), were selected [40]. The clones showed morphologically distinct characteristics; one of them overexpressed the ABC efflux transporter P-glycoprotein, whereas the other clone did not. Similar heterogeneity has been also described in cisplatin-resistant models developed from a human pancreatic cancer cell line with a mutation in DNA repair protein BRCA2 [41].

3. *Selecting Cells with Determined Molecular Markers*

The third method for isolation resistant cells to cancer therapies is based on the expression of differential molecular markers in the tumor cells. Both intrinsic and acquired resistance to anticancer therapies result from numerous genetic and epigenetic changes, therefore, for an effective cell selection a combination of different markers, based on specific individual genotypic and phenotypic variations in the resistant that cells can be used. Taking into account that in chemotherapy cancer cell resistance occur at different levels, including activation of oncogenes and inhibition of tumoral suppressors, variations in drug influx/efflux or evasion from apoptosis, different markers could be employed to identify them [17, 42, 43]. In addition, stem cell characteristics are also important factors in the resistance process [1, 44]. In the case of PDT, all of these factors would be implicated in promoting resistance. Alterations in the expression of many different genes have been observed and, therefore, multiple signaling pathways are contributing to PDT resistance [12, 45–47].

A very important factor in drug resistance is mediated by proteins which belong to the ATP-binding cassette (ABC) transporter family, which increase drug efflux and, thus, reduce the intracellular drug concentration. Among these proteins, P-glycoprotein (MDR, Pgp or ABCB1), multidrug resistance protein 1 (MRP1 or ABCC1) and ABCG2 are the most frequently associated with multidrug resistance. These proteins are expressed at variable levels in cancerous cells [48]. Accordingly, ABCG2 is being used as an important marker for selecting cancer cells by flow cytometry and magnetic-associated cell sorting (MACS). ABCG2 can bind and efflux a wide range of structurally different classes of PS used preclinical and clinically, such as porphyrins and chlorins. It is expressed at different levels on cell lines used in many *in vitro* and *in vivo* tumor models for PDT which may affect phototoxic efficacy [49, 50]. Among the PSs that are substrates for ABCG2 they included Photochlor, Benzoporphyrin derivative monoacid ring A (BPD-MA, Verteporfin), Hypericin and Protoporphyrin IX (PpIX), after exogenous administration of ALA.

ABCG2 may reduce the intracellular levels of the substrate PS below the threshold for cell death in tumors treated with PDT, leaving resistant cells to repopulate the tumor [51–53].

Another important factor in resistance to drugs is the epidermal growth factor (EGFR). Alterations in the protein lead to sustained activation of the MAPK/ERK signal pathway in many human malignancies including skin, colorectal, ovarian, breast, and prostate cancers, and often correlates with the enhanced cellular proliferation and development of cancer metastasis [54, 55]. Therefore, it is an important potential factor in the resistance to PDT. In this sense, in general, in cells with a good response to PDT, down-regulation of EGFR has been noted in PDT-treated cells *in vitro* and *in vivo*, and it has been suggested that the decreased cell migration and the invasiveness in RIF-1-PDT-derived variants are related to the down-regulation of EGFR. Compared to parental CL1-5, A375 and MDA-MB-231 cells, ALA-PDT caused a reduction in the level of EGFR in PDT-derived variants, which correlated with the reduced migration and invasion in the PDT-derived variants [35]. However, it has been described that sustained ERK activation protected cells from PDT [56]. A recent study using A-431 squamous cell carcinoma of the skin and WiDr colorectal adenocarcinoma cells linked EGFR and ERK activation as potential predictive factors of response to PDT [57]. It has been also demonstrated in patients with a bad response to PDT as well as in the resistant PDT-SCC-13 cells the up-regulation of EGFR [8].

Finally, several evidences suggest that tumors contain a small subpopulation of cells, the cancer stem cells (CSC), which exhibit self-renewal capacity, proliferate infrequently, express several pluripotency genes and are responsible for tumor maintenance and metastasis [44, 58, 59]. These slow cycling cells are not impacted/affected by anti-cancer agents that kill rapidly growing tumor cells, although these need to be killed upon treatment to eradicate the tumor. If some, even a few, are left intact, they will be responsible for tumor drugs resistant and relapse. In fact, in recent years, CSCs have been identified in several cancers and have been proposed to explain the metastatic capacity, recurrence, and resistance to radio therapy and chemotherapy [44, 60, 61].

Some markers have been associated to CSC. For instance, in breast cancer, the stem cell population is CD44+/CD24 and CD133 marks cancer stem cells in brain tumors, colorectal carcinoma and pancreatic carcinoma. In head and neck squamous cell carcinoma a CD44+ population of cells possesses the properties of CSC, and ABCG2 and aldehyde dehydrogenase 1 (ALDH1) activity have also been reported to identify cancer stem cells in a host of cancer types [62–64] and also for skin cancer [65, 66].

CSCs have been identified and isolated using different approaches including flow cytometry and magnetic-associated cell sorting. Therefore, recently, Adhikary et al. [67] selected a cell population from the squamous cell carcinoma SCC-13 and A-431 cell lines by using aldehyde dehydrogenase 1 as marker. Such isolated cells formed spheroids and induced larger tumors with faster growing in immunocompromised mice as compared to non-selected cells. Spheroid-selected cultures were highly enriched on the expression of epidermal stem cell and embryonic stem

cell markers, basically of aldehyde dehydrogenase 1 (ALDH1), keratin 15, CD200, oct4 and trimethylated histone H3, among others. These studies indicate that the subpopulation of cells that possess stem cell-like properties enhance tumor forming potential and can be selected by cell sorting using the human epidermal stem cell markers. These results are very interesting and aim to (i) evaluate the expression of stem cells in PDT resistant cells and (ii) to select firstly cells with CSC markers and test the sensitivity to PDT.

In vivo Selection

Likewise, as in the case of the resistant cell selection by using *in vitro* systems, cancer cells, with intrinsic and/or acquired resistance previously subjected to repeated treatments, may be injected in immunodeficient mice. These strategies are based in the studies performed on resistance to drugs in chemotherapy [18]. In this case, there would be two basic selection strategies for isolating PDT-resistance cells in mice by using: (1) a determined original cancer cell population or (2) a resistant population obtained from an original culture subjected to repeated PDT treatments or cells showing the determined molecular markers (Fig. 5.1b). Cancer cells will be injected in the mice subcutaneously (s.c.) into the dorsal flank, or orthotopically by implanting tumor cells into the organ of origin. After the injection, tumor cells become palpable and can receive repeated treatments with the selected compound/PSs to induce tumor destruction. However, after a variable period of continuous treatment, if resistance occurs, the "remnant" tumor cells proliferate again and the tumor cells can now can be explanted and cultured for cellular and molecular resistance studies. In both cases, the tumoral environment in the host will contribute to select cells with resistant characteristics.

Not many *in vivo* studies have been performed for selecting PDT-resistant cells. Adams et al. [68] evaluated the response to *in vivo* PDT with Photofrin in tumors derived from RIF-1 mouse fibrosarcoma cells and in tumors derived from RIF-8A cells, which showed *in vitro* resistance to PDT. The authors found a significant reduction in the tumor volume similar for both RIF-1 and RIF-8A tumors, whereas the re-growth was significantly delayed for RIF-1 compared to RIF-8A tumors following PDT. They also evaluated the clonogenic survival of the cells obtained from explanted *in vitro* immediately following *in vivo* PDT treatment. Apart from this article, most of the studies performed in mice with different cell lines have been focused for determining the efficacy of PDT with different PSs. In this respect, many reports with different cell lines and PSs have been published. Some recent examples are: the murine mammary tumor 4T1 cells with HPPH as PS [50], the human colorectal carcinoma HCT116 cells with the chlorin-based photosensitizer DH-II-24 [69], the mammary MCF-7 cells and pheophorbide a [70], the non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) with chlorin e6– polyvinylpyrrolidone [71], among many others. In addition, these models have been used to evaluate the role of determined molecular markers in the PDT response. Hence, Tang et al. [72] studied the therapeutic potential of PDT in the multidrugresistance (MDR) human hepatoma cell line R-HepG2 with the photosensitizer pheophorbide a.

Examples of PDT Selection

In PDT, the generation of resistant cell variants will enable investigators to understand the molecular mechanisms of sensitivity to several photosensitizers, based on inherent and induced resistance in different cell lines. PDT-resistant cell lines have been obtained using various photosensitizers such as Photofrin, phthalocyanines or Nile Blue, as well as after exogenous incubation with precursors of PSs such ALA and MAL.

The first studies for the isolation of PDT-resistant cells were performed by Luna and Gomer [26]. They isolated PDT-resistant variants from the mouse radiation-induced fibrosarcoma (RIF-1) cell line, following a protocol of repeated porphyrin (Photofrin II, PII) incubation and light treatments. They used two incubation procedures, either an extended (16 h) or a short (1 h) incubation period to obtain resistant cells exposed to conditions with different intracellular photosensitizer localization. By cloning, they selected two individual colonies from each PDT porphyrin incubation time used. However, the morphological characteristics as well as the behavior of the different clones were different. All resistant variants had increased protein content and were larger than the parental RIF-1 cells. *In vitro* growth rates were similar. Flow cytometric analysis using propidium iodide showed the characteristic mixture of diploid and tetraploid subpopulations for the parental and one of the clones selected, whereas a complete tetraploid phenotype was present in the three other PDT-resistant variants.

Likewise, Singh et al. [27] induced resistant populations to PDT also from the RIF-1 tumor cells by repeated photodynamic treatment with PII (4 or 18 h of drug incubation) to the 0.1–1 % survival level, followed by regrowth from single surviving colonies. The resistance is shown as increased cell survival in the strain designated RIF-8A, compared to the wild-type RIF-1 cells, when exposed to increasing PII concentrations, 18 h of drug incubation and fixed light exposure. Resistance to PDT was also observed in Chinese hamster ovary-multidrug resistant (CHO-MDR) cells, compared to the CHO wild type cells by the same authors. These findings suggest that different mechanisms are responsible for PDT-induced resistance and multi-drug resistance. Lately, the same group, by using three different photosensitizers (aluminum phthalocyanine tetrasulfonate, AlPcS4; Nile Blue A and Photofrin), selected by their different localization properties, induced different resistant populations in three human cell lines: neuroblastoma (SK-N-MC), human colon adenocarcinoma (HT29) and human bladder carcinoma (HT1376) [34]. Cells were incubated for 1 h (Nile Blue) or 18 h (AlPcS4 and Photofrin) using two different drug concentrations and two different light doses. They evaluated the cell survival by the colony forming assay and the authors indicate that multiple cultures were performed from single surviving colonies. Cells were regrowth and treated again receiving between 8 and 14 cycles. Each treatment cycle was aimed at achieving survival levels in the 1–10 % range, and they considered as PDT-resistant variants those cells with over 1.5-fold increase in PDT resistance. Resistant cells were isolated by the colony forming assay. Under such conditions, they obtained several resistant cell lines from HT29 using the three PSs and from HT1376 using the PS Nile Blue. However, the isolated clones obtained from HT1376 with AlPcS4 or

Photofrin and those from SK-N-MC with any of the three PSs did not show resistance to PDT. All the cell lines showed different levels of intrinsic resistance. As the authors indicate, the variability in sensitivity to a single photosensitizer for different cell lines is not surprising. However, the different relative rankings with respect to resistance are very interesting and highlight the importance of the appropriate photosensitizer selection. Moreover, this could correlate with the understanding that the mechanisms and pathways of cellular death are sensitizer-specific. The authors suggest that a specific variation within the population or a selectively advantageous mutation during the repeated treatments facilitates the development of the resistant variants.

Using similar protocols, Casas et al. [28] isolated resistant clones of murine adenocarcinoma cells, LM3, after repeated ALA-PDT treatments. The authors used a fixed concentration, 0.6 mM, of the ALA pro-photosensitizer and varied the light doses (0.36–5.4 J/cm^2) to achieve survival levels in the 5–10 % range. The surviving cells were allowed to grow and were again subjected to a new cycle of ALA-PDT. The final population received a total of 13 cycles (LM3L13) and, afterwards, 8 clones were isolated by the limiting dilution method. The LD50 was defined as the light dose to kill 50 % of the cells at saturating concentrations of ALA. The resistance index to ALA-PDT was defined as LD50 resistant clone/LD50 LM3. In both cases, the resistant clones isolated showed a stable level of resistance.

On the other hand, Mayhew et al. [33], using polyhematoporphyrin (PHP) and Zn(II) pyridinium-substituted phthalocyanine (PPC) as PSs, isolated two RIF-1 resistant cell populations, and demonstrated a 5.7 and 7.1-fold increase in resistance, respectively. Both resistant strains were isolated following 15 cycles of photosensitization treatment with increasing sensitizer concentrations and fixed light doses. After the photosensitization cycles, the isolated strains were RIF-25R, from PHP treatment, and P10 strain, obtained after PPC treatment.

Milla et al. [36], using a cell line obtained from squamous cell carcinoma of skin (SCC-13 cells), developed resistance to PDT cells. The procedure followed was simple and based also as that previously described [26, 28]. Cells were incubated with a fixed concentration of MAL (1 mM) and, thereafter, exposed to different red light doses to cause survival rates of 5–10 %. The surviving cells were harvested 24 h after PDT and replated, allowing them to grow and then subjecting them to a new PDT treatment. The final population received 10 PDT cycles and two populations were selected: one subjected to 5 PDT cycles and the other exposed to 10 PDT cycles (SCC-5G and SC-13–10G, respectively). The resistance for each population was checked by the MTT assay, indicating that the PDT conditions required to obtain the last SCC resistant generations were more intense, from 7.31 J/cm^2 to obtain the 1st to 25 J/cm^2 for the 10th generation. In addition, these resistant cells have a higher viability after PDT, compared to the parental cells. In fact, the parental and the 1st generation cells exposed to PDT (MAL 1 mM and 7.31 J/cm^2 red light dose) had a viability of 10 %, while the 5th generation and the 10th generation had 85 and 95 % of viability, respectively.

Likewise, by using three different cell types, lung adenocarcinoma (CL1-5), breast carcinoma (MDA-MB-231) and melanoma (A375) cells, PDT-derived vari-

ants were established after five consecutive ALA-PDT treatments [35]. However, in this case, the authors indicated that the obtained populations did not show resistant properties and that the response to new PDT-treatments was similar to that of the parental cells.

On the other hand, there are no reports employing cell sorting methodologies for studying resistance to PDT, but some studies have been performed employing cells that highly express defined resistant markers. This is the case of the ATP-dependent transporter ABCG2, which is expressed at different levels in many cell lines used in *in vitro* and *in vivo* tumor models for PDT, which may affect their phototoxic efficacy [49, 50]. In addition, recently, Yu and Yu [73] treated primary cultures from a head and neck cancer (HNC) tumor with ALA-PDT and they studied the photosensitizing effect on CSCs markers, particularly ALDH1. They observed that ALA-PDT treatment significantly down-regulated the ALDH1 activity and reduced the CD44 positivity and stem cell signatures expression (Oct4 and Nanog) in sphere-forming cells. The authors concluded that ALA-PDT effectively reduced CSC-like properties, including ALDH1 activity, CD44 positivity, self-renewal and invasion. These findings can be considered the first study in which different CSC markers have been evaluated and related with the response to PDT.

Finally, it should be noted that the level of PDT resistance observed is, in general, less than that reported for most drug-resistant cell lines. Although the knowledge of drug resistance mechanism is far from being understood, drugs are quite specific and there are usually a single or a few subcellular targets (DNA, enzyme, receptors), as a direct effect of the treatment on the amplification of a membrane-bound glycoprotein transport system, decreased repair of a specific target or altered pathways. However, there are numerous sites and types of injury associated with PDT, and overlapping mechanisms are therefore involved in PDT-cytotoxicity and resistance. Therefore, modifying the sensitivity of cellular PDT targets or repair systems would not be expected to produce the same degree of resistance as observed with chemotherapeutic drugs, which are associated with a limited number of targets or mechanisms of action. Hence, the levels of resistance over a 1.5-fold increase in survival at the LD90 or LD50 are considered suitable in the generation of PDT-resistant cells. In addition, different reports indicated that the relative resistance to PDT for the tumor cell lines is photosensitizer-specific.

Initial Characterization of PDT-Resistant Cells

The determination of structural, biochemical, molecular and/or functional differences between the parental and the PDT-tumor resistant cells is a main goal in order to provide mechanisms underlying altered susceptibility to PDT. This chapter included in this volume, as well as the review already published by Casas et al. [28] point out the different cellular and molecular characteristics of the PDT-resistant cells. Overexpression/mutations of growth factors and growth factor receptors, as well as of signal transduction proteins, lead to sustained proliferative and survival

signaling and to an aberrant proliferation, which contribute to PDT resistance. Some examples that could be tested as molecular markers are (i) "gain-of-function" gene alterations, such as the PI3K/Akt/mTOR and MAPK/ERK pathways [74–77] (ii) inactivating mutations in tumor suppressor genes, such as the *retinoblastoma (RB), PTEN (phosphatase and tensin homologue deleted from chromosome 10)* and P53 [78–81] (iii) alterations in the machinery of apoptosis or autophagy, including overexpression of anti-apoptotic proteins like Bcl-2, IAPs survivin and inactivation of pro-apoptotic genes such as genes encoding caspases or proapoptotic Bcl-2 members [82–86] (iv) oxidative and stress genes and proteins, such as hemeoxigenase (HO), heat shock proteins (HSP), superoxide dismutase (SOD), glutathione peroxidase [87–90] and (v) proteins related with ATP-binding cassette transporter [49–53]. Therefore, here we only emphasized some aspects to help in an initial characterization of these resistant cells compared to the parental population from which they have been isolated.

Cell Morphology and Population Characteristics

In general, the results obtained in different laboratories related with the changes in the morphology of isolated resistant cells, compared with the appearance of the parental cells, are homogeneous. However, the results reported on cell dynamic characteristics are contradictory. It has been described that PDT-resistant cells change their morphology in relation to that shown by the parental cells. The resistant cells isolated after treatment with Photofrin II from RIF-1 showed an increase in cell size compared to that of the parental cells [26, 34]. Nuclear size was also increased. Total cellular protein content was, as well, significantly higher than that of the parental cells. Plating efficiency of the 1 h PDT-resistant variants was similar to that of the parental RIF-1 cells, whereas in the case of 16 h the PDT-resistant variants plating efficiency was reduced to 36–43 %. However, the cell doubling time for resistant and parental cell types was similar.

Similar results have been described for the resistant-PDT Clon 4 and Clon 8 (both isolated from LM3) in terms of protein content, being higher in the resistant clones (2-fold increase) [28]. However, the plating efficiency was significantly impaired (25–30 %) in both Clon 4 and Clon 8 compared to LM3, as well as the growth rate, which was also significantly decreased in the resistant clones compared with the parental LM3 (3.5-fold lower in Clon 8 than the control). An increase in the latency time has been reported for Clon 8 cells compared to the parental LM3 cells [91].

Similarly, there were no substantial differences on cell size, plating efficiency and distribution of the cells in the cell cycle between the SCC-13 cells and the PDT-resistant variants (unpublished results from our laboratory). It has also been described that SCC-13 cells present a diverse morphology [36]. SCC-13 parental cells showed a polyhedral to fibroblastic with long prolongations morphology, which was also observed in the resistant isolated generations; however, these cells had a higher proportion of fibroblastic forms and the cell colonies formed were more

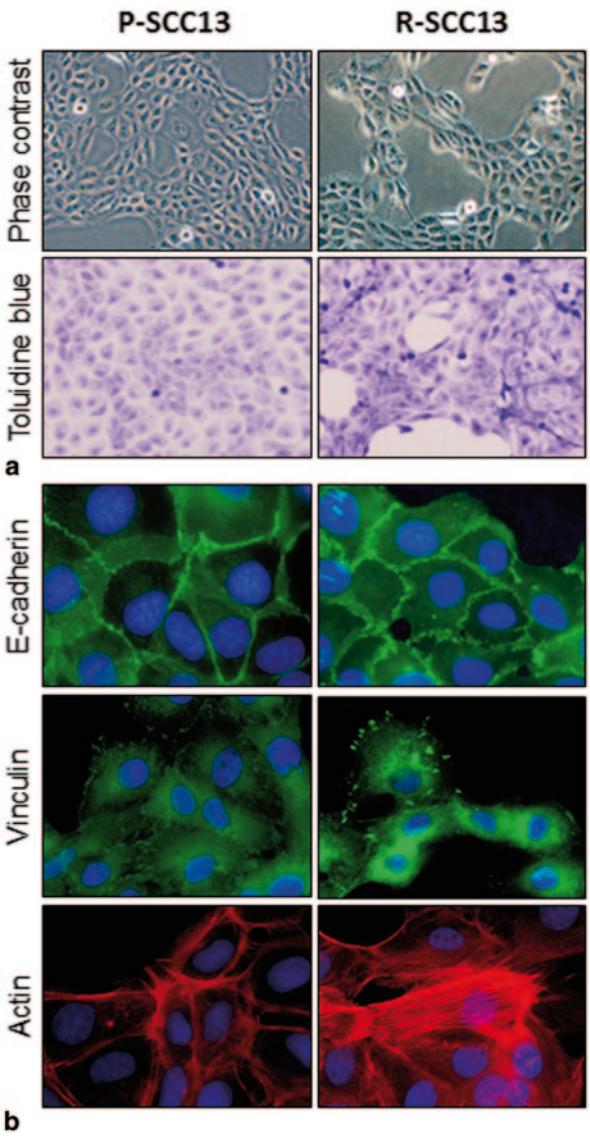

Fig. 5.2 Cell morphology of PDT-resistant cells compared to parental SCC-13 cells. **a** Resistant PDT-cells (R-SCC-13) show a more pronounced fibroblastic morphology compared to parental cells (P-SCC-13) when they are observed under phase contrast as well as after Toluidine blue staining. **b** E-cadherin expression is similar in both cell types, whereas higher expression of vinculin as well as higher amounts of thick stress fibers can be seen in the resistant SCC-13 population

expansive with respect to the parental populations (Fig. 5.2). This was also noted previously in Clon 4 and Clon 8 resistant cells. They exhibited a more fibroblastic, dendritic pattern, and a higher cell spreading than the LM3 parental line. At the subcellular level, electron microscopy showed that there were no noticeable differences on lysosomes and membranes among the lines, although the mitochondrial number per cell and per area was higher in both the resistant clones [28]. These results are in concordance with those previously published with RIF-1 cells [27, 92]. Thereby, the mitochondria in the resistant RIF-8A cells were smaller, while their

number per cell was higher than in the parental RIF-1 cells. In addition, the RIF-8A cells produced more ATP and demonstrated higher succinate dehydrogenase activity than the RIF-1 cells. The authors indicated differences in the efficacy and/or the mode(s) of energy production in the RIF-1 and RIF-8A and pointed out that, since the mitochondria are sensitive targets for porphyrin-mediated PDT [12, 93], the observed changes in structure and/or function (or both) of the mitochondrion may be involved in the PDT resistance seen in RIF-8A cells. In contrast, alterations related with the integrity and functionality of the mitochondria have been described in the established PDT-derived variants CL1-5/6A5, A375/3A5, and MDA-MB-231/1A5 isolated from CL1-5, A375, and MDA-MB-231, respectively. In this case, the mitochondrial membrane potential was significantly reduced. The authors indicated that, in these cells, the consecutive ALA-PDT treatments caused permanent mitochondrial damage in the established PDT-derived variants. In any case, these isolated variants are not considered as PDT-resistant cells [35].

In the human colon adenocarcinoma HT29 cells, PDT-resistant variants, selected from sequential PDT treatments by using different photosensitizers, showed downregulation in the mitochondrial genes coding for the 16S ribosomal RNA (rRNA) and nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 4. The authors also found, in the PDT-resistant variants, an increased expression of the gene encoding the Bcl-2 protein and downregulation of the gene encoding the Bax protein, suggesting that both the altered expression in the mitochondrion and apoptosis-regulating genes contribute to PDT resistance [94]. Similarly, it has been recently reported in three human head and neck squamous carcinoma cell lines, (UMSCC1, UMSCC14A, and UMSCC22A), treated with silicon phthalocyanine (Pc4) as a mitochondria-targeted photosensitizer, that they responded differently [95]. UMSCC1 and UMSCC14A cells were more resistant than UMSCC22A cells to Pc 4-PDT-induced cell death. The authors indicated that this differential response was due to the expression of the mitochondrial protein mitoferrin-2 (Mfrn2), an iron transporter of the mitochondrial inner membrane. PDT-sensitive cells expressed higher Mfrn2 mRNA and protein levels compared with the PDT-resistant cells.

Nuclear Analysis

A higher degree of nuclear heterogeneity is generally present in cultured PDT-resistant cells. Also, long nuclear connections can be found between nuclei of cells in division. Giant nuclei (polyploidy) are also observed in higher proportion in resistant cells related to parental cells [26, 36, 92]. In addition, it has been described an increase in the number of cells with micronuclei in the resistant SCC-13 cells (12 % ± 2.8) as compared to the parental SCC-13 cells (3 ± 1.3 %) [36]. All these results would be in agreement with the high rate of abnormal divisions observed, particularly at anaphase or telophase, with chromosomal material present in the middle of the two cells [36]. Micronuclei presence has also been described in many different resistant cells, such as the hepatocellular carcinoma HepG2 cell line (subjected to etoposide treatement) [96]and the human endometrial adenocarcinoma HEC-1 cells (subjected to paclitaxel treatment) [97].

Analysis of the karyotype revealed that most of the parental RIF-1 cells were diploid or tetraploid (40 and 80 chromosomes) and contained some abnormal chromosomes. The resistant RIF-8A variant cell karyotype was inconsistent, being the most frequently observed presence of the polyploidies of 120 chromosomes [27]. In addition, using a comparative genomic hybridization array (aCGH), Gilaberte et al. [8] reported that both resistant and parental SCC-13 cells present amplicons in the *3p12.1 CADM2, 7p11.2 EFGR* and *11q13.3 CCND1* genes, but the resistant cells showed a distinctive amplicon in 5q11.2 MAP3K1 not present in the parental cells. These changes detected by aCGH on CCND1, EFGR and MAP3K1 were confirmed by western blot, suggesting that genomic imbalances related to CCND1, EFGR and particularly MAP3K1 could be involved in the development of resistance of SCC to PDT. Previous studies indicated that PDT can produce single and double strand breaks, sister chromatids exchanges, chromosome aberrations and mutagenic alterations [98–101], supporting the results described in the resistant populations and indicating that such alterations could be related with the resistance process, as it has been also described in different resistant tumors treated with diverse chemotherapeutic agents [102–106].

PS Accumulation and Subcellular Localization

It has been proposed that the differential response to PDT could be due to different PS accumulation in parental and resistant cells. Hence, Luna and Gomer [26] have found that, generally, the amount of PII per cell was slightly increased in the resistant variant CL-8 cells as compared to the parental RIF-1 cells, although CL-1 cells retained approximately one-half of the amounts of PII. In addition, CL-8 cells have a 1.5-fold increase PDT resistance and CL-1 cells exhibited even higher (4.5-fold) in survival cells following PII incubation. Therefore, the authors indicated that PDT resistance exhibited by the CL-1 and CL-8 cells was not due solely to decreases in PII accumulation.

Likewise, since the amount of ALA/MAL-converted PpIX might affect phototoxicity, the examination of whether the differential cytotoxicity was due to the different PpIX contents in resistant *vs* nonresistant cells has also to be taken into account. Therefore, ALA-PDT did not cause significant differences in phototoxicity between the parental cells and the PDT-derived variants from CL1–5, A375, and MDA-MB-231 cancer cells [35]. PpIX accumulation was very low in CL1–5 cells and did not change significantly as the ALA concentration was increased; meanwhile, MDA-MB-231 cells produced relatively high PpIX content. It appears that the differential ALA-converted PpIX content would explain the differential phototoxicity among the parental and the derived CL-5 and MDA-MB-231 variants.

In the case of LM3 cells, Casas et al. [28] found that the amount of porphyrins synthesized by LM3 cells normalized by cell number was not significantly different from the resistant sublines (Clon 4 and Clon 8), but when expressed on a per μg protein basis, the porphyrin synthesis was increased 2-fold in the parental line.

In addition, Milla et al. [36] did not find differences in the production of PpIX after incubation with MAL between the parental SCC-13 and the resistant isolated populations.

With respect to the subcellular localization of the PSs, Casas et al. [28] described the distribution of endogenously synthesized PpIX after incubation with ALA, in the LM-3 parental cells as well as in the resistant clones. The parental and resistant populations exhibited a similar cytoplasmic PpIX localization, including mitochondria, lysosomes, the cell membrane and the Golgi apparatus. Similarly, localization of Photofrin and ALA-induced PpIX in the parental RIF-1 tumor cells and in the RIF-8 resistant to Photofrin was similar [107]. In both cell types, PSs are located mainly in the mitochondria. They also evaluated the uptake kinetics of Photofrin alone and after coincubation with mitochondria-specific probes (10N-Nonyl acridine orange, NAO or rhodamine-123, Rh-123) showing a stronger colocalization of Photofrin, NAO and Rh-123 in RIF-1 than in RIF-8 cells. The authors indicated that the differences in this binding may account for the PDT resistance in RIF-8A cells. However, it should be emphasized that in both cell types the subcellular localization was mitochondrial. In addition, Mayhew et al. [33] described that the two resistant strains also isolated from RIF-1 cells treated with PPC o PHP did not show differences in the localization of the PSs comparing with the parental cells and neither in drug uptake. The authors concluded that in both PDT-resistant strains, the increased resistance could not be attributed to the intracellular sensitizer localization.

In agreement with the results described above, no major differences have been found in PpIX localization among the parental and the resistant SCC-13 cells. PpIX was localized in the plasmatic membrane in all analyzed populations, but very low fluorescence intensity was also detected into lysosomes and mitochondria and in cytoplasm. PpIX was also observed in vacuoles at longer incubation periods using also organelle markers, such as Mitotracker or Lisotracker. There is a problem when the concentrations and the incubation times are low since PpIX fluorescence is very difficult to detect by optical resources due the immediate photobleaching of the PS under the microscopy exciting light of 460–490 nm [36].

Therefore, taking altogether the published data, it is not clear that differences between parental and resistant cells in the subcellular localization of the PS even in its intracellular accumulation would be the cause of the differential response to PDT after identical treatment conditions. Nevertheless, several reports indicated the importance of the ATP-binding cassette transporter protein (ABCG2) in the regulation of PSs transport in different cell lines and its role in the response to PDT [51–53, 108, 109]. Thus, many studies have to be performed to better determine the importance of intracellular accumulation of the PS in the response to PDT in resistant cells.

Cell Adhesion and Migration Abilities

Cell adhesion proteins play a crucial role in migration and invasion abilities of cancer cells. Therefore, the expression and distribution of the proteins implicated in

these processes would be also important to determine the resistance to PDT abilities of cancer cells [46, 47, 110–112]. Casas et al., [91, 113] showed that in mammary adenocarcinoma cells (LM3), E-cadherin is located at the plasma membrane connecting neighbor cells, but it is disorganized in Clone 4 and Clone 8 LM3-resistant cells. E-cadherin distribution was completely aberrant in the resistant clones, being situated in the numerous interdigitations which are present along cell to cell contacts. Similarly, β-catenin showed the same distribution pattern for E-cadherin in LM3 cells, being also disorganized in the interdigitations and showing a diffuse cytoplasmic distribution. In addition, the authors did not find significant differences in the expression of cell-substrate adhesion proteins β1-integrin, vinculin, FAK and phospho-FAK in the resistant clones, compared to LM3 cells. However, the vinculin distribution was different; whereas in LM3-parental cell, vinculin was confined to the focal adhesion points, a diffuse cytoplasmic pattern was observed in the resistant clones. FAK distribution was both cytoplasmic and nuclear, whereas phospho-FAK was confined to the focal adhesion points, and no differences were found among the distribution in the cell lines. Related with cell adhesion and migration, actin microfilaments constitute a basic cytoskeletal element [114–116]. Thus, whereas long stress fibers situated at the basal plane were present in LM3, this organization became perturbed in Clone 4 and Clone 8 cells. In Clone 4 cells stress fibers were shorter and only a few of them were found in Clone 8 cells. A fine, quite regular and continuous cortical F-actin layer was present in LM3 cells, whereas it was more irregular in Clone 4 and a waved pattern of cortical actin was observed in Clone 8 cells. No significant differences in the adhesion of the three cell lines to the ECM proteins fibronectin and laminin were found, whereas Clon 4 and Clon 8 adhesion ability to Collagen I were 1.3 and 2-fold as compared to LM3, respectively.

Milla et al. [36] did not find strong differences in the expression patterns and levels of E-cadherin and β-catenin between resistant and parental SCC-13 cells (Fig. 5.2). They also evaluated the expression levels of cell-substrate adhesion proteins β1-integrin, vinculin, FAK and phospho-FAK. In resistant cells vinculin and phospho-FAK showed a distribution in the center and in the cellular periphery, while in parental cells they were mainly in the center. Vinculin was localized at the end of the stress fiber in the three studied populations (Fig. 5.2). By western blot analysis, they observed that resistant cells had higher expression of β1-integrin, vinculin and phospho-FAK with respect to the parental cells. The pattern of the actin stress fibers showed that, in the resistant SCC-13 cells, F-actin was highly expressed in cortical regions and many cells showed conspicuous stress fibers as compared to parental cells. Unpublished results obtained in our laboratory revealed higher adhesion ability to Collagen I of the SCC-13 resistant cells compared to that of the parental cells (1.5-fold).

Motility is a key factor in the regulation of cancer cell invasion [42, 116, 117]. Therefore, to test this property, characterizing PDT-resistant cells is also important. There are several studies with different cell lines and PSs indicating that motility and invasion abilities are reduced after PDT. These studies include, for instance, the head and neck cancer cell lines KJ-1 and Ca9-22, treated with ALA [118], glioma spheroids obtained from human U373 and A172 cell lines treated with ALA [119],

nasopharyngeal carcinoma KJ-1 cell line treated with tetrahydroxyphenyl chlorine [120] or human ovarian cancer HO-8910 treated with hypocrellin B [121]. In relation with the PDT-resistant cells, Tsai et al. [35] indicated that the migration ability was permanently affected in the established PDT-derived variants CL1–5, A375 and MDA-MB-231 cancer cells. In fact, by using the scratch wound assay, the authors found a significant reduction in migration in those survived from ALA-PDT, suggesting that the photodamage induced by ALA-PDT caused the suppression of cell migration ability in these cells. In addition, invasion is also affected in ALA-PDT-derived CL1–5, A375 and MDA-MB-231 variants [35]. By using the Matrigel assay, Casas et al. [91] did not find significant differences between the LM3 and resistant clones (4 and 8). The authors also tested the chemotaxis or directional migration using control inserts, and saw that 100 % of LM3 cells migrated through the porous membrane, whereas only the 38 ± 8 % and 73 ± 0 % of Clones 4 and 8, respectively, were able to migrate, concluding that the resistant clones presented lower invasion abilities than the parental LM3 cells. The authors related the decreased abilities of the resistant cells with the alterations in the expression of adhesion proteins and microfilaments indicated above.

On the contrary, the ability of migration and closing wounds evaluated by the scratch wound assay in parental SCC-13 and in the PDT-derived resistant variants indicated that, whereas at early time (4 and 8 h) after starting the assay there were no differences in the migration capacity, however, the resistant cells showed higher capacity of closing wounds at longer times (12 and 24 h) compared to the parental cells [36].

All these differences in the results obtained the migration and invasion abilities of the resistant variants, these could be due to different factors, including (i) the cell line, (ii) the PS or prodrug used and (iii) the way of selecting resistant cells.

Tumor Induction and Metastatic Abilities in Mice

There are many animal models to study drug resistance with advantages and disadvantages as reviewed by Rottenberg and Jonkers [122] and Politi and Pao [123]. In general, cancer cell lines are injected into immunodeficient mice for testing tumorigenicity and metastatic abilities of cultured cancer cells. This is the strategy used by the investigators to test the characteristics of resistant cells to PDT. Luna and Gomer [26] evaluated the tumorigenic ability of the two PDT-resistant variants of the RIF-1 mouse tumor cell line obtained by repeated treatment with PII after two incubation times (16 h and 1 h). The authors injected variable number of cells (10 up to 10^6) into the flanks of immunocompetent C3H mice and found that the number of cells required to produce palpable tumors in 50 % of inoculated mice (latency time) was 10–20 for parental RIF-1 cells but it was higher (between 5×10^4 and 5×10^5) for the PDT-resistant variants. Similar results were obtained when athymic “nude” mice were used as host animals. Tumor-doubling time in C3H mice was similar for the parental RIF-1 line and for the 16-h P-II PDT-resistant variants (2–2.8 days).

However, the 1-h PDT resistant strains had increased doubling times, ranging from 3.9–4.6 days.

Similar results related to the ability to form tumors by resistant cells were found by Casas et al. [91]. They evaluated the ability of the parental LM3 cells and the PDT-resistant clones (Clon 4 and Clon 8) to grow subcutaneously in mice to form primary tumors and spontaneously to metastasize to the lung. An amount of 10^5 or 10^6 cells were injected in immunodeficient BALB/c mice. The authors found that tumor take (percentage of mice that developed palpable tumors at latency time) was decreased in the resistant clones compared with the LM3 line, most markedly in Clon 8. When 10^5 cells were injected, 30 % of mice developed tumors, whereas no tumors were developed by the resistant clones. Increasing the amount injected to 5×10^5 cells, tumor take was 100 % for LM3 cells, 60 % for Clon 4 and 30 % for Clon 8. Further increasing the amount injected to 10^6 cells, 100 % of mice injected with LM3 and Clon 4 developed tumors, and only 60 % of mice injected with Clon 8 did. The growth rate was also significantly decreased in Clon 4 compared with LM3, and Clon 8 growth delay was even more marked, 3.5-fold lower than the control. Latency time was similar for LM3 and Clon 4 whereas it was markedly longer for Clon 8 cells ($p < 0.001$). They also evaluated the spontaneous lung metastasis induced by LM3 and resistant clones and whereas LM3 cells metastasized to the lung in a tumor-size dependant way, Clones 8 and 4 almost did not induce nearly any metastasis at all. Only one small lung metastasis was found in one Clon 4 in the 7–19 mm tumor diameter range, whereas Clon 8 cells did not induce any metastasis at all. The authors related these results with the impaired changes in cell adhesion found in the resistant clones compared with parental LM3 cells. The conclusion of both studies indicated that the ability of the PDT-resistant cells to induce tumors is lower than that of the parental ones.

However, in the case of the article recently published by our laboratory [8], the results obtained after subcutaneous inoculation in immunodeficient mice of the squamous cell carcinoma SCC-13 cells and the obtained PDT-resistant populations were different (Fig. 5.3). Both the parental and the resistant cells formed progressively growing tumors, but the tumors induced by the PDT-resistant cells were bigger than those induced by the parental cells. Also the number of tumors was significantly higher in mice injected with resistant cells compared to those induced by parental cells. The differences between the mean number of tumors developed per mouse injected with the parental and resistant cells were statistically significant on days 15 and 30. We also evaluated the histological characteristics and whereas the tumors induced by parental SCC-13 cells were mostly well or moderately differentiated squamous cell carcinomas, those induced by PDT-resistant cells were mostly moderately or poorly-differentiated SCC, formed by atypical keratinocytes with nuclear pleomorphism even infiltrating skeletal muscle fibers (Fig. 5.3). The metastatic abilities of the resistant SCC-13 cells were not evaluated. Although not performed with PDT-resistant cells, previous results published by Momma et al., [124] using an orthotopic prostate cancer obtained by inoculation of the MatLyLu variant of the Dunning 3327 rat prostate cancer cell line, treated with benzoporphyrin derivative, found that PDT produced a significant increase in the mean number

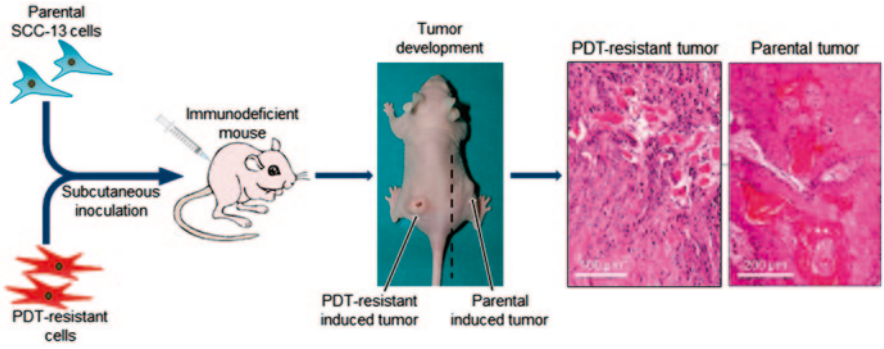

Fig. 5.3 *In vivo* tumor development after inoculation of parental squamous carcinoma cells SCC-13 cells and PDT-resistant cells in immunosuppressed mice. Parental and PDT-resistant SCC-13 cells are injected in the wright and in the left flanks of the mice, respectively. The tumor induced by the resistant variant is bigger compared to that of parental. Then, histopathological analysis revealed that the tumor induced by the resistant variant presents characteristics of poorly differentiated squamous cell carcinoma with cellular atypia and aberrant mitotic cells. In addition, squamous cells infiltrating the skeletal muscle can be observed. The tumor induced by the parental cells showed characteristics of well differentiated squamous cell carcinoma with dyskeratotic cells and keratin accumulations

of lung metastases. The authors indicated that different factors may need to be evaluated when considering PDT for primary prostate cancer.

It should be noted that the differences in the ability to induce tumors between PDT-resistant cells obtained in the different studies could be due to a variable number of factors. In the studies carried out by Luna and Gomer [26] and by Casas et al., [28] resistant clones were isolated, whereas in our case a resistant population was selected. As it has been indicated before, it is possible that, by using cloning methodology, the optimal resistant clones to study tumorigenicity in mice were not selected, whereas in the resistant cell population cells with different tumorigenic abilities are present. Obviously other factors can also contribute to the differences obtained, included the cell line, the PS and the experimental conditions.

Conclusions

Resistance constitutes a relevant unsolved problem in cancer therapy. Cancer cell culture assays, as well as mice models, are excellent tools available to diagnose intrinsic and acquired resistance, which may develop rapidly as the results of repeated treatments. Thus, a current challenge in PDT is modelling, both in cellular and animal systems, the characteristics associated to tumor resistance, that may reveal useful information from the molecular basis of intrinsic and acquired resistance to PDT.

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References

1. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer. 2013;13:714–26.
2. Longley DB, Johnston PG. Molecular mechanisms of drug resistance. J Pathol. 2005;205:275–92.
3. Gillet JP, Gottesman M. Mechanisms of multidrug resistance in cancer. Methods Mol. Biol. 2010;596:47–76.
4. Hombach-Klonisch S, Natarajan S, Thanasupawat T, Medapati M, Pathak A, Ghavami S, Klonisch T. Mechanisms of therapeutic resistance in cancer (stem) cells with emphasis on thyroid cancer cells. Front Endocrinol (Lausanne). 2014;5:37.
5. Al-Dimassi S, Abou-Antoun T, El-Sibai M. Cancer cell resistance mechanisms: a mini review. Clin Transl Oncol. 2014;16:511–6.
6. Maydan E, Nooothet PK, Goldman MP. Case reports: development of a keratoacanthoma after topical photodynamic therapy with 5-aminolevolunic acid. J Drugs Dermat. 2006;5:804–6.
7. Fiechter S, Skaria A, Nievergelt H, Anex R, Borradori L, Parmentier L. Facial basal cell carcinomas recurring after photodynamic therapy: a retrospective analysis of histological subtypes. Dermatology. 2012;224:346–51.
8. Gilaberte Y, Milla L, Salazar N, Vera-Alvarez J, Kourani O, Damian A, Rivarola V, Roca MJ, Espada J, González S, Juarranz A. Cellular intrinsic factors involved in the resistance of squamous cell carcinoma to photodynamic therapy. J Invest- Dermatol. 2014;134(9):2428–37.
9. Bardazzi F, Loi C, Magnano M, Burtica EC, Giordano F, Patrizi A. Methyl-aminolevulinic acid photodynamic therapy for actinic keratoses: an useful treatment or a risk factor? A retrospective study. J Dermatol Treat. 2014. (In press).
10. Juarranz A, Jaén P, Sanz-Rodríguez F, Cuevas J, González S. Photodynamic therapy of cancer. Basic principles and applications. Clin Transl Oncol. 2008;10:148–54.
11. Bredell MG, Besic E, Maake C, Walt H. The application and challenges of clinical PD-PDT in the head and neck region: a short review. J Photochem Photobiol B. 2010;101:185–90.
12. Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, Hahn SM, Hamblin MR, Juzeniene A, Kessel D, Korbelik M, Moan J, Mroz P, Nowis D, Piette J, Wilson BC, Golab J. Photodynamic therapy of cancer: an update. CA Cancer J Clin. 2011;61:250–81.
13. Neville JA, Welch E, Leffell DJ. Management of nonmelanoma skin cancer in 2007. Nat Clin Pract Oncol. 2007;4:462–9.
14. Morton CA, McKenna KE, Rhodes LE. British Association of Dermatologists Therapy G, Audit S, the British Photodermatology G Guidelines for topical photodynamic therapy: update. Br J Dermatol. 2008;159:1245–66.
15. Ortiz-Policarpio B, Lui H. Methyl aminolevulinate-PDT for actinic keratoses and superficial nonmelanoma skin cancers. Skin Therapy Lett. 2009;14:1–3.
16. Lackner MR, Wilson TR, Settleman J. Mechanisms of acquired resistance to targeted cancer therapies. Future Oncol. 2012;8:999–1014.
17. Rebucci M, Michiels C. Molecular aspects of cancer cell resistance to chemotherapy. Biochem Pharmacol. 2013;85:1219–26.
18. Rosa R, Monteleone F, Zambrano N, Bianco R1. In vitro and in vivo models for analysis of resistance to anticancer molecular therapies. Curr Med Chem. 2014;21:1595–606.
19. Wu Q, Yang Z, Nie Y, Shi Y, Fan D. Multi-drug resistance in cancer chemotherapeutics: mechanisms and lab approaches. Cancer Lett. 2014;347:159–66.

20. Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. Biochim Biophys Acta. 2010;1805:105–17.
21. Gerlinger M, Swanton C. How Darwinian models inform therapeutic failure initiated by clonal heterogeneity in cancer medicine. Br J Cancer. 2010;103:1139–43.
22. Saunders NA, Simpson F, Thompson EW, Hill MM, Endo-Munoz L, Leggatt G, Minchin RF, Guminski A. Role of intratumoral heterogeneity in cancer drug resistance: molecular and clinical perspectives. EMBO Mol Med. 2012;4:675–84.
23. McDermott M, Eustace A, Busschots S, Breen L, Crown J, Clynes M, O'Donovan N, Stordal B. In vitro development of chemotherapy and targeted therapy drug-resistant cancer cell lines: a practical guide with case studies. Front Oncol. 2014;4:40.
24. Biedler JL, Riehm H. Cellular resistance to actinomycin D in Chinese hamster Cells In vitro: cross-resistance, radioautographic, and cytogenetic studies. Cancer Res. 1970;30:1174–84.
25. Hahn GM, van Kersen I. Isolation and initial characterization of thermoresistant RIF tumor cell strains. Cancer Res. 1988;48:1803–7.
26. Luna MC, Gomer CJ. Isolation and characterization of mouse tumor cells resistant to porphyrin-mediated photodynamic therapy. Cancer Res. 1991;51:4243–9.
27. Singh G, Wilson BC, Sharkey SM, Browman GP, Deschamps P. Resistance to photodynamic therapy in radiation induced fibrosarcoma-1 and Chinese hamster ovary-multi- drug resistant cells in vitro. Photochem Photobiol. 1991;54:307–12.
28. Casas A, Perotti C, Ortel B, Di Venosa G, Saccoliti M, Battle A, Hasan T. Tumor cell lines resistant to ALA-mediated photodynamic therapy and possible tools to target surviving cells. Int J Oncol. 2006;29:397–405.
29. Ikeda R, Vermeulen LC, Lau E, Jiang Z, Kavanaugh SM, Yamada K, Kolesar JM. Isolation and characterization of erlotinib-resistant human non-small cell lung cancer A549 cells. Oncol Lett. 2011;2:91–4.
30. Zuo KQ1, Zhang XP, Zou J, Li D, Lv ZW. Establishment of a paclitaxel resistant human breast cancer cell strain (MCF-7/Taxol) and intracellular paclitaxel binding protein analysis. J Int Med Res. 2010;38:1428–35.
31. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehar J, Kryukov GV, Sonkin D, Reddy A, Liu M, Murray L, Berger MF, Monahan JE, Morais P, Meltzer J, Korejwa A, Jane-Valbuena J, Mapa FA, Thibault J, Bric-Furlong E, Raman P, Shipway A, Engels IH, Cheng J, Yu GK, Yu J, Aspesi P Jr, de Silva M, Jagtap K, Jones MD, Wang L, Hatton C, Palescandolo E, Gupta S, Mahan S, Sougnez C, Onofrio RC, Liefeld T, MacConaill L, Winckler W, Reich M, Li N, Mesirov JP, Gabriel SB, Getz G, Ardlie K, Chan V, Myer VE, Weber BL, Porter J, Warmuth M, Finan P, Harris JL, Meyerson M, Golub TR, Morrissey MP, Sellers WR, Schlegel R, Garraway LA. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature. 2012;483:603–7.
32. Gillet JP, Varma S, Gottesman MM. The clinical relevance of cancer cell lines. J Natl Cancer Inst. 2013;105:452–8.
33. Mayhew S, Vernon D, Schofield J, Griffiths J, Brown S. Investigation of cross-resistance to a range of photosensitizers, hyperthermia and UV light in two radiation-induced fibrosarcoma cell strains resistant to photodynamic therapy in vitro. Photochem Photobiol. 2001;73:39–46.
34. Singh G, Espiritu M, Yun Shen X, Hanlon JG, Rainbow AJ. In vitro induction of PDT resistance in HT29, HT1376 and SK-N-MC cells by various photosensitizers. Photochem Photobiol. 2001;73:651–6.
35. Tsai T, Tai Ji H, Chiang P, Chou R, Chang WW, Chen C. ALA-PDT results in phenotypic changes and decreased cellular invasion in surviving cancer cells. Lasers Surg Med. 2009;41:305–15.
36. Milla LN, Cogno IS, Rodríguez ME, Sanz-Rodríguez F, Zamarrón A, Gilaberte Y, Carrasco E, Rivarola VA, Juarranz A. Isolation and characterization of squamous carcinoma cells resistant to photodynamic therapy. J Cell Biochem. 2011;112:2266–78.
37. Breen L, Murphy L, Keenan J, Clynes M. Development of taxane resistance in a panel of human lung cancer cell lines. Toxicol In vitro. 2008;22:1234–41.

38. Breen L, Keenan J, Clynes M. Generation of lung cancer cell line variants by drug selection or cloning. Methods Mol Biol. 2011;731:125–33.
39. Akiyama S, Fojo A, Hanover JA, Pastan I, Gottesman MM. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. Somat Cell Mol Genet. 1985;11:117–26.
40. Yang LY, Trujillo JM, Siciliano MJ, Kido Y, Siddik ZH, Su YZ. Distinct P-glycoprotein expression in two subclones simultaneously selected from a human colon carcinoma cell line by cis-diamine dichloroplatinum (II). Int J Cancer. 1993;53:478–85.
41. Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, Villegas E, Jacquemont C, Farrugia DJ, Couch FJ, Urban N, Taniguchi T. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. Nature. 2008;451:1116–20.
42. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
43. Solyanik GI. Multifactorial nature of tumor drug resistance. Exp Oncol 2010;32:181–5.
44. Mertins SD. Cancer stem cells: a systems biology view of their role in prognosis and therapy. Anticancer Drugs. 2014;25:353–67.
45. Buytaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochem Biophys Acta. 2007;1776:86–107.
46. Casas A, Di Venosa G, Hasan T, Batlle A. Mechanisms of resistance to photodynamic therapy. Curr Med Chem. 2011;18:2486–515.
47. Milla Sanabria L, Rodríguez ME, Cogno IS, Rumie Vittar NB, Pansa MF, Lamberti MJ, Rivarola VA. Direct and indirect photodynamic therapy effects on the cellular and molecular components of the tumor microenvironment. Biochim Biophys Acta. 2013;1835:36–45.
48. Noguchi K, Katayama K, Sugimoto Y. Human ABC transporter ABCG2/BCRP expression in chemoresistance: basic and clinical perspectives for molecular cancer therapeutics. Pharmgenomics Pers Med. 2014;7:53–64.
49. Ishikawa T, Nakagawa H, Hagiya Y, Nonoguchi N, Miyatake S, Kuroiwa T. Key role of human ABC transporter ABCG2 in photodynamic therapy and photodynamic diagnosis. Adv Pharmacol Sci. 2010;2010:587306.
50. Morgan J, Jackson JD, Zheng X, Pandey SK, Pandey RK. Substrate affinity of photosensitizers derived from chlorophyll-a: the ABCG2 transporter affects the phototoxic response of side population stem cell-like cancer cells to photodynamic therapy. Mol Pharm. 2010;7:1789–804.
51. Robey RW, Steadman K, Polgar O, Bates SE. ABCG2-mediated transport of photosensitizers: potential impact on photodynamic therapy. Cancer Biol Ther. 2005;4:187–94.
52. Li W, Zhang WJ, Ohnishi K, Yamada I, Ohno R, Hashimoto K. 5-Aminolaevulinic acid-mediated photodynamic therapy in multidrug resistant leukemia cells. J Photochem Photobiol B. 2001;60:79–86.
53. Liu W, Baer MR, Bowman MJ, Pera P, Zheng X, Morgan J, Pandey RA, Oseroff AR. The tyrosine kinase inhibitor imatinibmesylate enhances the efficacy of photodynamic therapy by inhibiting ABCG2. Clin Cancer Res. 2007;13:2463–70.
54. Bianco R, Troiani T, Tortora G, Ciardiello F. Intrinsic and acquired resistance to EGFR inhibitors in human cancer therapy. Endocr Relat Cancer. 2005;12:159–71.
55. Roskoski R Jr. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol Res. 2012;66:105–43.
56. Tong Z, Singh G, Rainbow AJ. Sustained activation of the extracellular signal-regulated kinase pathway protects cells from photofrin-mediated photodynamic therapy. Cancer Res. 2002;62:5528–35.
57. Weyergang A, Selbo PK, Berg K. Sustained ERK [corrected] inhibition by EGFR targeting therapies is a predictive factor for synergistic cytotoxicity with PDT as neoadjuvant therapy. Biochim Biophys Acta. 2013;1830:2659–70.
58. Pincelli C, Marconi A. Keratinocyte stem cells: friends and foes. J Cell Physiol. 2010;225:310–5.
59. La Porta CA. Thoughts about cancer stem cells in solid tumors. World J Stem Cells. 2012;4:17–20.

60. Ishii H, Iwatsuki M, Ieta K, Ohta D, Haraguchi N, Mimori K, MoriI M. Cancer stem cells and chemoradiation resistance. Cancer Sci. 2008;99:1871–7.
61. Chiang AC, Massagué J. Molecular basis of metastasis. N Engl J Med. 2008;359:2814–23.
62. Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields JZ, Wicha MS, Boman BM. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC). Cancer Res. 2009;69:3382–9.
63. Chen YC, Chen YW, Hsu HS, Tseng LM, Huang PI, Lu KH, Chen DT, Tai LK, Yung MC, Chang SC, Ku HH, Chiou SH, Lo WL. Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head and neck squamous cancer. Biochem Biophys Res Commun. 2009;385:307–13.
64. Jiang F, Qiu Q, Khanna A, Todd NW, Deepak J, Xing L, Wang H, Liu Z, Su Y, Stass SA, Katz RL. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. Mol Cancer Res. 2009;7:330–8.
65. Ortells MC, Keyes WM. New insights into skin stem cell aging and cancer. Biochem Soc Trans. 2014;42:663–9.
66. Lang D, Mascarenhas JB, Shea CR. Melanocytes, melanocyte stem cells, and melanoma stem cells. Clin Dermatol. 2013;31:166–78.
67. Adhikary G, Grun D, Kerr C, Balasubramanian S, Rorke EA, Vemuri M, Boucher S, Bickenbach JR, Hornyak T, Xu W, Fisher ML, Eckert RL. Identification of a population of epidermal squamous cell carcinoma cells with enhanced potential for tumor formation. PLoS One. 2013;8:e84324.
68. Adams K, Rainbow AJ, Wilson BC, Singh G. In vivo resistance to photofrin-mediated photodynamic therapy in radiation-induced fibrosarcoma cells resistant to in vitro Photofrin-mediated photodynamic therapy. J Photochem Photobiol B. 1999;49:136–41.
69. Lim YC, Yoo JO, Park D, Kang G, Hwang BM, Kim YM, Ha KS. Antitumor effect of photodynamic therapy with chlorin-based photosensitizer DH-II-24 in colorectal carcinoma. Cancer Sci. 2009;100:2431–6.
70. Hoi SW, Wong HM, Chan JY, Yue GG, Tse GM, Law BK, Fong WP, Fung KP. Photodynamic therapy of Pheophorbidea inhibits the proliferation of human breast tumor via both caspase-dependent and—independent apoptotic pathways in in vitro and in vivo models. Phytother Res. 2012;26:734–42.
71. Ren Y, Wang R, Liu Y, Guo H, Zhou X, Yuan X, Liu C, Tian J, Yin H, Wang Y, Zhang N. A hematoporphyrin-based delivery system for drug resistance reversal and tumor ablation. Biomaterials. 2014;35:2462–70.
72. Tang PM, Chan JY, Zhang DM, Au SW, Fong WP, Kong SK, Tsui SK, Waye MM, Mak TC, Fung KP. Pheophorbide a, an active component in Scutellaria barbata, reverses P-glycoprotein-mediated multidrug resistance on a human hepatoma cell line R-HepG2. Cancer Biol Ther. 2007;6:504–9.
73. Yu CH, Yu CC. Photodynamic therapy with 5-aminolevulinic acid (ALA) impairs tumor initiating and chemo-resistance property in head and neck cancer-derived cancer stem cells. PLoS One. 2014;9:e87129.
74. Perona R, Sánchez-Pérez I. Control of oncogenesis and cancer therapy resistance. Br J Cancer. 2004;90:573–7.
75. Martinez-Carpio PA, Trelles MA. The role of epidermal growth factor receptor in photodynamic therapy: a review of the literature and proposal for future investigation. Lasers Med Sci. 2010;25:767–71.
76. Edmonds C, Hagan S, Gallagher-Colombo SM, Busch TM, Cengel KA. Photodynamic therapy activated signaling from epidermal growth factor receptor and STAT3: Targeting survival pathways to increase PDT efficacy in ovarian and lung cancer. Cancer Biol Ther. 2012;13:1463–70.
77. Rosenzweig SA. Acquired resistance to drugs targeting receptor tyrosine kinases. Biochem Pharmacol. 2012;83:1041–8.
78. Tong Z, Singh G, Rainbow AJ. The role of the p53 tumor suppressor in the response of human cells to photofrin-mediated photodynamic therapy. Photochem Photobiol. 2000;71:201–10.

79. Zawacka-Pankau J, Krachulec J, Grulkowski I, Bielawski KP, Selivanova G. The p53-mediated cytotoxicity of photodynamic therapy of cancer: recent advances. Toxicol Appl Pharmacol. 2008;232:487–97.
80. Lee SY, Luk SK, Chuang CP, Yip SP, To SS, Yung YM. TP53 regulates human AlkB homologue 2 expression in glioma resistance to Photofrin-mediated photodynamic therapy. Br J Cancer. 2010;103:362–9.
81. Nakanishi A, Kitagishi Y, Ogura Y, Matsuda S. The tumor suppressor PTEN interacts with p53 in hereditary cancer. Int J Oncol. 2014;44:1813–9.
82. Grossman D, McNiff JM, LI F, Altieri DC. Expression of the apoptosis inhibitor, survivin, in nonmelanoma skin cancer and gene targeting in a keratinocyte cell line. Lab Invest. 1999;79:1121–6.
83. Zaffaroni N, Pennati M, Colella G. Expression of the anti-apoptotic gene survivin correlates with taxol resistance in human ovarian cancer. Cell Mol Life Sci. 2002;59:1406–12.
84. Rodel F, Hoffman J, Distel L. Survivin as a radioresistance factor and prognostic and therapeutic target for radiotherapy in rectal cancer. Cancer Res. 2005;65:4881–7.
85. Ferrario Á Rucker N Wong S Luna M Gomer CJ. Survivin, a member of the inhibitor of apoptosis family is induced by photodynamic therapy and is a target for improving treatment response. Cancer Res. 2007;67:4989–95.
86. Yamamoto H, Yee Ngan C, Monden M. Cancer cells survive with survivin. Cancer Sci. 2008;99:1709–14.
87. Gomer CJ, Ryter SW, Ferrario A, Rucker N, Wong S, Fisher AM. Photodynamic therapy-mediated oxidative stress can induce expression of heat shock proteins. Cancer Res. 1996;56:2355–60.
88. Luna MC, Ferrario A, Wong S, Fisher AM, Gomer CJ. Photodynamic therapy-mediated oxidative stress as a molecular switch for the temporal expression of genes ligated to the human heat shock promoter. Cancer Res. 2000;60:1637–44.
89. Ferrario A, Gomer CJ. Targeting the 90 kDa heat shock protein improves photodynamic therapy. Cancer Lett. 2010;289:188–94.
90. Etminan N, Peters C, Lakbir D, Bünemann E, Börger V, Sabel MC, Hänggi D, Steiger HJ, Stummer W, Sorg RV. Heat-shock protein 70-dependent dendritic cell activation by 5-aminolevulinic acid-mediated photodynamic treatment of human glioblastoma spheroids in vitro. Br J Cancer. 2011;105:961–9.
91. Casas A, Di Venosa G, Vanzulli S, Perotti C, Mamome L, Rodriguez L, Simian M, Juarranz A, Pontiggia O, Hasan T, Batlle A. Decreased metastatic phenotype in cells resistant to aminolevulinic acid-photodynamic therapy. Cancer Lett. 2008;271:342–51.
92. Sharkey SM, Wilson BC, Moorehead R, Singh G. Mitochondrial alterations in photodynamic therapy-resistant cells. Cancer Res. 1993;53:4994–9.
93. Morgan J, Oseroff AR. Mitochondria-based photodynamic anti-cancer therapy. Adv Drug Deliv Rev. 2001;49:71–86.
94. Shen XY, Zacal N, Singh G, Rainbow AJ. Alterations in mitochondrial and apoptosis-regulating gene expression in photodynamic therapy-resistant variants of HT29 colon carcinoma cells. Photochem Photobiol. 2005;81:306–13.
95. Hung HI, Schwartz JM, Maldonado EN, Lemasters JJ, Nieminen AL. Mitoferrin-2-dependent mitochondrial iron uptake sensitizes human head and neck squamous carcinoma cells to photodynamic therapy. J Biol Chem. 2013;288:677–86.
96. Mukherjee A, Misra S, Howlett NG, Karmakar P. Multinucleation regulated by the Akt/PTEN signaling pathway is a survival strategy for HepG2 cells. Mutat Res. 2013;755:135–40.
97. Tanaka T, Toujima S, Tanaka J. Differential sensitivity to paclitaxel-induced apoptosis and growth suppression in paclitaxel-resistant cell lines established from HEC-1 human endometrial adenocarcinoma cells. Int J Oncol. 2012;41:1837–44.
98. Fiedler DM, Eckl PM, Krammer B. Does delta-aminolaevulinic acid induce genotoxic effects? J Photochem Photobiol B. 1996;33:39–44.

99. Evans HH, Horng MF, Ricanati M, Deahl JT, Oleinick NL. Mutagenicity of photodynamic therapy as compared to UVC and ionizing radiation in human and murine lymphoblast cell lines. Photochem Photobiol. 1997;66:690–6.
100. Rousset N, Keminon E, Eléouet S, Le Néel T, Auget JL, Vonarx V, Carré J, Lajat Y, Patrice T. Use of alkaline Comet assay to assess DNA repair after m-THPC-PDT. J Photochem Photobiol B. 2000;56:118–31.
101. Woods JA, Traynor NJ, Brancaleon L, Moseley H. The effect of photofrin on DNA strand breaks and base oxidation in HaCaT keratinocytes: a comet assay study. Photochem Photobiol. 2004;79:105–13.
102. Struski S, Doco-Fenzy M, Koehler M, Chudoba I, Levy F, Masson L, Michel N, Ulrich E, Gruson N, Bénard J, Potron G, Cornillet-Lefebvre P. Cytogenetic evolution of human ovarian cell lines associated with chemoresistance and loss of tumorigenicity. Anal Cell Pathol. 2003;25:115–22.
103. Qutob SS, Multani AS, Pathak S, Feng Y, Kendal WS, Ng CE. Comparison of the X-radiation, drug and ultraviolet-radiation responses of clones isolated from a human colorectal tumor cell line. Radiat Res. 2004;161:326–34.
104. Oudin C, Bonnetain F, Boidot R, Végran F, Soubeyrand MS, Arnould L, Riedinger JM, Lizard-Nacol S. Patterns of loss of heterozygosity in breast carcinoma during neoadjuvant chemotherapy. Int J Oncol. 2007;30:1145–51.
105. Roschke AV, Kirsch IR. Targeting karyotypic complexity and chromosomal instability of cancer cells. Curr Drug Targets. 2010;11:1341–50.
106. Cooke SL, Ng CK, Melnyk N, Garcia MJ, Hardcastle T, Temple J, Langdon S, Huntsman D, Brenton JD. Genomic analysis of genetic heterogeneity and evolution in high-grade serous ovarian carcinoma. Oncogene. 2010;29:4905–13.
107. Wilson BC, Olivo M, Singh G. Subcellular localization of photofrin and aminolevulinic acid and photodynamic cross-resistance in vitro in radiation-induced fibrosarcoma cells sensitive or resistant to photofrin-mediated photodynamic therapy. Photochem Photobiol. 1997;65:166–76.
108. Ogino T, Kobuchi H, Munetomo K, Fujita H, Yamamoto M, Utsumi T, Inoue K, Shuin T, Sasaki J, Inoue M, Utsumi K. Serum-dependent export of protoporphyrin IX by ATP-binding cassette transporter G2 in T24 cells. Mol Cell Biochem. 2011;358:297–307.
109. Barron GA, Moseley H, Woods JA. Differential sensitivity in cell lines to photodynamic therapy in combination with ABCG2 inhibition. J Photochem Photobiol B. 2013;126:87–96.
110. Hehlgans S, Haase M, Cordes N. Signalling via integrins: implications for cell survival and anticancer strategies. Biochem Biophys Acta. 2007;1775:163–80.
111. Hazlehurst LA, Dalton WS. Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies. Cancer Metast Rev. 2001;20:43–50.
112. Mierke CT, Kollmannsberger P, Zitterbart DP, Diez G, Koch TM, Marg S, Ziegler WH, Goldmann WH, Fabry B. Vinculin facilitates cell invasion into three-dimensional collagen matrices. J Cell Biol Chem. 2010;285:13121–30.
113. Casas A, Sanz-Rodriguez F, Di Venosa G, Rodriguez L, Mamone L, Blázquez A, Jaén P, Batlle A, Stockert JC, Juarranz A. Disorganisation of cytoskeleton in cells resistant to photodynamic treatment with decreased metastatic phenotype. Cancer Lett. 2008;270:56–65.
114. Blanchoin L, Boujemaa-Paterski R, Sykes C, Plastino J. Actin dynamics, architecture, and mechanics in cell motility. Physiol Rev. 2014;94:235–63.
115. Fogh BS, Multhaupt HA, Couchman JR. Protein kinase C, focal adhesions and the regulation of cell migration. J Histochem Cytochem. 2014;62:172–84.
116. Le Bras GF Taubenslag KJ Andl CD. The regulation of cell-cell adhesion during epithelial-mesenchymal transition, motility and tumor progression. Cell Adh Migr. 2012;6:365–73.
117. Robertson DS. The development of tumor cell characteristics. J Cell Physiol. 2014;229:705–10.
118. Yang TH, Chen CT, Wang CP, Lou PJ. Photodynamic therapy suppresses the migration and invasion of head and neck cancer cells in vitro. Oral Oncol. 2007;43:358–65.

119. Etminan N, Peters C, Ficnar J, Anlasik S, Bünemann E, Slotty PJ, Hänggi D, Steiger HJ, Sorg RV, Stummer W. Modulation of migratory activity and invasiveness of human glioma spheroids following 5-aminolevulinic acid-based photodynamic treatment. Laboratory investigation. J Neurosurg. 2011;115:281–8.
120. Wang CP, Lou PJ, Lo FY, Shieh MJ. Meta-tetrahydroxyphenyl chlorine mediated photodynamic therapy inhibits the migration and invasion of a nasopharyngeal carcinoma KJ-1 cell line. J Formos Med Assoc. 2014;113:173–8.
121. Jiang Y, Leung AW, Wang X, Zhang H, Xu C. Effect of photodynamic therapy with hypocrellin B on apoptosis, adhesion, and migration of cancer cells. Int J Radiat Biol. 2014;90(7):575–9.
122. Rottenberg S, Jonkers J. Modeling therapy resistance in genetically engineered mouse cancer models. Drug Resist Updat. 2008;11:51–60.
123. Politi K, Pao W. How genetically engineered mouse tumor models provide insights into human cancers. J Clin Oncol. 2011;29:2273–81.
124. Momma T, Hamblin MR, Wu HC, Hasan T. Photodynamic therapy of orthotopic prostate cancer with benzoporphyrin derivative: local control and distant metastasis. Cancer Res. 1998;58:5425–31.

Part III
Specific Strategies to Sensitize Tumor Cells to PDT

Chapter 6
GRP78-targeting Sensitizes Cancer Cells to Cytotoxic Effects of Photodynamic Therapy

Malgorzata Firczuk, Magdalena Gabrysiak and Jakub Golab

Abstract Photodynamic therapy (PDT) induces cytotoxic effects against tumor cells by triggering photochemical reactions leading to the production of singlet oxygen and reactive oxygen species. Intracellular proteins have been shown to undergo oxidation-related damage in response to PDT. A number of cytoprotective mechanisms have been demonstrated to relay on mechanisms associated with removal or re-folding of these proteins or leading to the induction of unfolded protein response. The latter is regulated by GRP78, a member of the heat shock protein family that undergoes up-regulation in tumor cells in response to PDT. The most selective GRP78-targeting compound is subtilase cytotoxin (SubAB) originally isolated from Shiga toxigenic *Escherichia coli* strains. We observed that a fusion protein consisting of the cytotoxin catalytic A subunit (SubA) with a human epidermal growth factor (EGF) designed to selectively target EGFR-positive tumor cells increases the cytotoxic effects of PDT. Although the combination treatment activated apoptotic pathways, tumor cell death occurred in cells resistant to apoptosis and was not inhibited by inhibitors of necrotic cell death or autophagy-associated death pathways. Instead, tumor cells undergo an atypical form of cell death, which is characterized by cellular vacuolization originating from the endoplasmic reticulum.

Keywords Photodynamic therapy · GRP78 · Heat shock proteins · Unfolded protein response · Endoplasmic reticulum stress

Abbreviations

ATF	activating transcription factor
AlPc	aluminum phthalocyanine
C/EBP	CCAAT/enhancer-binding protein
CHOP	C/EBP homologous protein
EGF	epidermal growth factor

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EGFR	EGF receptor
ER	endoplasmic reticulum
ERAD	ER-associated degradation
GRP	glucose-regulated protein
HSP	heat shock protein
IRE1	inositol-requiring enzyme 1
PDT	photodynamic therapy
PERK	protein kinase RNA-like ER kinase
PS	photosensitizer
ROS	reactive oxygen species
STEC	Shiga toxigenic *Escherichia coli*
Sub	subtilase cytotoxin
UPR	unfolded protein response

Introduction

Proteins constitute roughly 2/3 of a dry cellular mass and frequently form complex structures composed of multimeric components. Most drugs, including some photosensitizers (PS) used in PDT, either bind to or are localized in a close vicinity to proteins. Therefore, proteins become immediate targets for the action of singlet oxygen and reactive oxygen species (ROS) generated during PDT. A number of protein modifications has been reported to occur in response to PDT including their fragmentation, multimerization, unfolding or aggregation. These lesions result in structural alterations and functional inactivation of proteins or lead to changes in their mechanical properties, binding of co-factors and metal ions or accelerated degradation [1]. A typical biomarker for oxidative protein damage is carbonylation, a largely irreversible process which leads to a surface exposure of hydrophobic residues that are normally hidden in the interior of soluble proteins. The formation of hydrophobic patches within proteins also leads to their unfolding followed by ubiquitination and degradation in proteasomes. Accumulation of excess of unfolded and ubiquitinated proteins favours formation of large protein aggregates. These 'aggresomes' can be to some extent removed in a process of autophagy, but when these processes become saturated cells usually fail to cope with accumulated misfolded, damaged or aggregated proteins and trigger cell death. A significant accumulation of ubiquitinated proteins has been observed in tumor cells in response to PDT, and proteasome inhibitors potentiated antitumor effects of PDT [2].

Build-up of unfolded proteins, especially in the endoplasmic reticulum (ER), triggers cytoprotective mechanisms, collectively referred to as unfolded protein response (UPR), which is aimed at re-establishing protein homeostasis. Similar responses are elicited in other cellular organelles, such as the mitochondria.

Altogether, oxidative protein damage is usually non-repairable and has deleterious consequences for the cell survival. To survive, cells must activate protective

mechanisms that either repair damaged protein structure or eliminate physiologically useless proteins. The ubiquitin-proteasome system of protein degradation and autophagy is used to eliminate damaged proteins, while UPR and molecular chaperones are involved in the repair of misfolded proteins. Robust, high dose PDT results in the massive oxidative damage to intracellular macromolecules and leads to cell death. However, due to the limited penetration and unhomogeneous light distribution in tissues, not all tumor cells experience lethal damage. The low fluence PDT in these tumor regions triggers various cytoprotective mechanisms such as the unfolded protein response (UPR), and induces the expression of proteins which promote tumor survival and contribute to PDT resistance. One of the proteins up-regulated in response to PDT is the glucose regulated protein 78 (GRP78).

GRP78 and Unfolded Protein Response (UPR)

GRP78 belongs to the heat shock protein (HSP) family. HSPs are involved in protein folding. Usually, they act through repeatable cycles of "client" protein binding and release. Although initially described in cellular response to hyperthermia, HSPs help cells in dealing with diverse stress conditions. They also play a role in "fixing" or re-folding of oxidatively damaged proteins and protein aggregates by binding to exposed surfaces of damaged proteins. PDT has been shown to induce the expression of a variety of HSPs including HS1, HSP27, HSP60, HSP70, and HSP90. Some of these proteins seem to play a protective role in PDT-treated cells. For example, overexpression of HSP27 increased survival of tumor cells exposed to PDT [3]. Cellular levels of HSP60 and HSP70 negatively correlate with tumor cells sensitivity to PDT-induced damage [4–5]. Combination of PDT with geldanamycin that interferes with binding of client proteins to HSP90, leads to a higher apoptosis rate and increased cytotoxicity [6].

The role of HSPs in cellular physiology is far beyond just protein re-folding and involves regulation of the cellular signaling mostly due to stabilization or sequestration of various factors. Indeed, some of the "client" proteins for HSPs are involved in survival promoting pathways [7]. Therefore, it is possible that HSPs protect cells not only by enabling a repair of oxidatively damaged proteins but also by less specific inhibition of apoptosis [8].

Glucose regulated proteins (GRPs) share homology with heat shock proteins. GRPs are induced under stress conditions such as glucose deprivation, disturbances of Ca^{2+} homeostasis, increased demand for protein folding, and oxidative stress. GRPs mainly reside in the endoplasmic reticulum and mitochondria, where they contribute to protein quality control machinery. One of the members of GRPs is the glucose-regulated protein 78 (GRP78), also known as the immunoglobulin heavy chain-binding protein (BIP). It is an ER-resident chaperone, a key regulator of the ER stress response signaling. GRP78 plays multiple roles. It assists in the folding of extracellular and membrane proteins, prevents protein aggregation and contributes to Ca^{2+} homeostasis.

GRP78 is also a master regulator of the signaling program triggered by the accumulation of unfolded proteins in the ER, known as unfolded protein response (UPR). Under normal conditions, GRP78 is localized in the ER lumen, where it binds to the ER transmembrane sensor proteins: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), and prevents their downstream signaling. When the unfolded proteins accumulate in the ER, they compete for GRP78 binding, which results in the sensor protein release and triggers UPR [9]. The initial effects of UPR include: translational attenuation, transcriptional activation of chaperones, enzymes degrading unfolded proteins, and ERAD (ER-associated degradation) components facilitating retrotranslocation of misfolded proteins from the ER to the cytoplasm. Hence, UPR is an adaptive response facilitating the cell to deal with ER stress and supporting cell survival. However, if ER homeostasis is not restored, UPR leads to cell death [10]. The mechanisms of UPR-induced cell death are still not completely understood. It has been recently discovered that the major death-promoting arm of UPR is PERK-eIF2α. The two downstream transcription factors of PERK: (i) C/EBP (CCAAT/enhancer-binding protein) homologous protein (CHOP) and (ii) activating transcription factor 4 (ATF4), induce the transcription of genes responsible for increased protein synthesis [11]. Moreover, PERK-eIF2α down-regulates the anti-apoptotic protein XIAP which leads to cell death [12].

GRP78, apart from indirect, UPR-mediated regulation of cell fate, also controls programmed cell death directly. GRP78 binds and renders some of the pro-apoptotic proteins like caspase-7 [13], Bik and Bax [14] inactive. Thus, in stressed cells, GRP78 represents one of the major cytoprotective components of UPR that promotes cell survival.

GRP78 in Tumors and its Role in Anti-tumor Therapies

Extensive data indicate that GRP78 is overexpressed in various cancer cell lines and contributes to the invasion and metastasis in many human tumors. The tumor microenvironment is characterized by increased glucose and oxygen demands and higher protein folding capacity. These conditions cause the up-regulation of various UPR signaling pathway components, among them GRP78 [15–16]. Increased GRP78 levels usually correlate with higher pathologic grade, recurrence, and poor survival in human prostate, breast, liver, colon, and gastric cancers [17]. Likewise, elevated levels of GRP78 were detected in tumor metastases and knockdown of GRP78 suppressed metastasis formation in xenograft models [18]. Moreover, the undesirable effect of various anticancer therapies is the induction of GRP78 and selection of chemoresistant cell populations [19]. GRP78 overexpression correlated with adriamycin resistance in breast cancer patients [20]. Furthermore, increased GRP78 levels were observed in malignant gliomas refractory to standard chemotherapy treatment with etoposide or camptothecin and to radiotherapy [21].

Importantly, the chemoresistance could be mitigated by GRP78 inhibitors or its knockdown [21–22].

Photodynamic therapy also leads to UPR induction and GRP78 up-regulation [2]. It was demonstrated in various *in vitro* and *in vivo* tumor models that a wide spectrum of stress proteins is up-regulated in response to PDT, including GRP78 [23–26]. The role of GRP78 up-regulation in the cellular response to PDT depends on the photosensitizer and the PDT protocol used. Examples include both enhanced resistance and increased sensitivity to PDT due to the GRP78 up-regulation. Radiation-induced fibrosarcoma (RIF) cells, when pre-incubated with GRP78-inducing Ca^{2+} ionophore A23187, were more resistant to PDT in comparison with untreated cells [27]. In another study, a combination of the same [28] or a different [29] Ca^{2+} ionophore with PDT produced quite opposite results. The latter work by Xue et al. reported on synergistic effects of the treatment modality combining Ca^{2+} ionophore nigericin and aluminum phthalocyanine (AlPc)-PDT. The observed sensitization of cells to PDT was accompanied by the induction of GRP78 [29]. Another study, with GRP78-overexpressing CHO cells, demonstrated that GRP78 itself is able to modulate cellular sensitivity to PDT. Surprisingly, the increased GRP78 levels sensitized CHO cells to VBBO-PDT, while they conferred resistance to Photofrin-PDT. The authors suggested that the subcellular localization of the photosensitizer is a major factor, which contributes to the observed opposite effects in the cellular response [28].

SubAB5 Cytotoxin, a Specific GRP78-downregulating Agent

Because GRP78 is overexpressed in various tumors and is associated with malignant and treatment-resistant phenotypes, attempts leading to GRP78 suppression are under consideration and are evaluated in many pre-clinical studies. The vast majority of agents reported to down-regulate or to inhibit GRP78, including genistein, epigallocatechine gallate, salicylic acid, are all characterized by a wide spectrum of activities. A notable exception is a bacterial cytotoxin SubAB, that has an unusual ability to specifically cleave GRP78.

The subtilase cytotoxin, SubAB, is a member of the toxin family AB5 produced by some Shiga toxigenic *Escherichia coli* (STEC) strains and constitutes the key virulence factor and most likely the reason for hemolytic uremic syndrome, a serious clinical complication associated with STEC infection [30]. The holotoxin is composed of one A subunit with proteolytic activity and five non-covalently linked B subunits with an affinity to cellular glycan receptors, which are responsible for eukaryotic cell entry [31]. It was demonstrated that SubAB specifically cleaves and inactivates GRP78 (Fig. 6.1), which results in massive ER stress and ultimately triggers ER stress-mediated apoptosis [32, 33]. The subtilase-like serine protease activity is fundamental to GRP78 cleavage and the cellular cytotoxicity, as

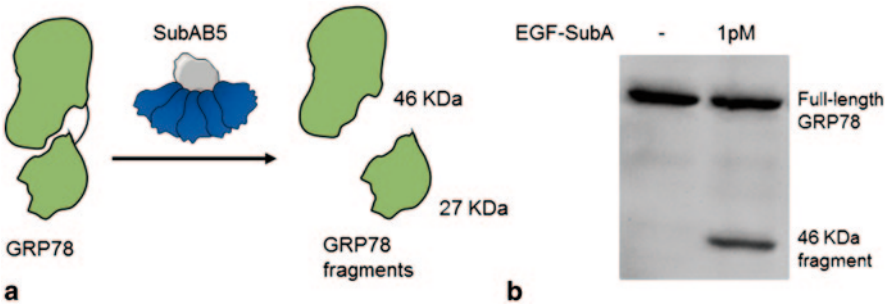

Fig. 6.1 Structure and mechanism of action of SubA-EGF fusion molecules. **a** Construction of the fusion *EGF-SubA* molecules. Targeting subunit B was exchanged for EGF. **b** *EGF-SubA* induces cleavage of *GRP78* at picomolar concentrations. This Figure shows immunoblotting of tumor cell lysates incubated with *1 pM EGF-SubA*

the toxin variant carrying a single mutation of the active site catalytic Ser residue (Ser 272 Ala) is neither capable of GRP78 cleavage nor cytotoxic. SubAB cleaves GRP78 between two consecutive leucine residues (Leu416-Leu417) located in the hinge region linking N-and C-terminal domains and is absent in other Hsp70 family members, consistent with their resistance to SubAB cleavage. Surprisingly, no other SubAB substrates have been detected and the exclusive substrate specificity for GRP78 is explained by the location of the active site residues in an unusually deep groove revealed by the toxins' crystal structure [34].

Despite the fact that GRP78 is a well-defined target for SubAB, the exact molecular mechanisms which trigger SubAB-mediated cell death are not fully elucidated. However, the unquestionable event which follows SubAB treatment is the induction of a severe ER stress response, suggesting that cleaved GRP78 dissociates from its ER luminal partners. The activation of all three UPR signaling cascades upon SubAB treatment have been documented in various cell lines, although the details of the contribution of particular branches vary [33, 35, 36]. The proteins known to be up-regulated include GRP94, ATF4, CHOP, GADD34, the spliced variant of XBP-1, and others. GRP78 is also transcriptionally induced, but in most cell lines it is undetectable at the protein level due to concomitant degradation. Some of these proteins link UPR with apoptosis. The investigations in Vero or HeLa cells suggested that SubAB triggered Bax and Bak conformational changes, which caused cytochrome c to be released from the mitochondria and thus launched a classical intrinsic apoptotic signaling [37]. Other studies in the rat renal epithelial NRK-52E cells revealed that SubAB activated MAP kinases pathways in the PERK and IRE-1-dependent manners [36]. However, no matter what the exact upstream events are, clear evidence of apoptosis has been demonstrated for incubation times exceeding 24 h, manifested by caspase activation, cytochrome c release, and DNA fragmentation [38, 39].

Fusion of a Catalytic Subunit SubA of the Cytotoxin with EGF (EGF-SubA) for Selective Tumor Cell Delivery

The SubAB holotoxin non-specifically targets various cells expressing glycoproteins with N-linked glycans, so it is highly toxic and lethal to mice. Therefore, the fusion of the cytotoxin catalytic A subunit (SubA) with a human epidermal growth factor (EGF) was designed to selectively target EGFR-positive tumor cells. The epidermal growth factor receptor (EGFR) is a cell surface receptor with intrinsic tyrosine kinase activity. Mutations affecting its expression levels and activity are detected in various tumors, and have been associated with tumor progression. Therefore, EGFR is considered to be an oncogene. The conjugations of EGF or EGF-derived peptides with drugs, toxins or nano-carriers have been validated in pre-clinical experiments and have been shown to be efficient in selective drug delivery to the EGFR over-expressing cells [40].

The EGF-SubA fusion protein selectively killed EGFR-positive tumor cells in picomolar concentrations *in vitro* and attenuated growth of human prostate cancer xenografts in a mouse model after intraperitoneal injection. Moreover, the EGF-SubA synergized with thapsigargin, a potent ER stress-inducing agent [41].

Combination of EGF-SubA Cytotoxin with Photodynamic Therapy

In a recent study carried out by our group, we aimed to investigate the antitumor effects of a treatment combining PDT with a fusion protein EGF-SubA and to elucidate cell death mechanisms induced by such therapy [42]. As a photosensitizer, we used the porphyrin oligomer Photofrin. The study was performed using five different EGFR-expressing human cancer cell lines including prostate cancer (DU-145 and PC-3), colorectal carcinoma (HCT-116 and LoVo) and squamous cell lung carcinoma (SW-900) cells. The mechanism of cell death induced by combination treatment was assessed in the BAX-deficient, apoptosis-incompetent DU-145 cell line and apoptosis-competent SW-900 cells.

As PDT is known to induce ER-stress and unfolded protein response [2] with subsequent induction of various chaperone proteins, including GRP78 [23], we investigated whether altered expression of GRP78 could contribute to the PDT outcome. After confirming the strong up-regulation of GRP78 in our models, we aimed to define the role of GRP78 in PDT harnessing two different approaches. Using a lentiviral system, we over expressed GRP78 in DU-145 cells and noted significantly increased resistance of such modified cells to PDT as compared to cells treated with a mock vector. In line with this finding was the observation that cells with transiently silenced GRP78 are slightly more susceptible to cytotoxic effects of PDT. All these experiments brought us to a conclusion that GRP78 plays a

cytoprotective role in cellular response to PDT. It provided a rationale to combine PDT with GRP78-targeting compounds, which could improve the PDT effect.

The most selective compound with an ability to cleave and inactivate GRP78 is EGF-SubA. Therefore, we assessed the cytotoxic/cytostatic effects of PDT after the addition of EGF-SubA to the treatment scheme and observed potentiation of these effects in most of the tested cell lines. The enhanced cytotoxicity in combination groups was accompanied by time-dependent accumulation of cleaved GRP78 and decreased level of full-length GRP78. As GRP78 is perceived as a key regulator of ER-stress response and UPR, we hypothesized that lowering its level could influence PDT-induced UPR. Strong activation of both PERK and IRE1 branches was observed as early as one hour (PERK) and 3 h (IRE1) post PDT. Comparing to PDT, the combination treatment resulted in a slight attenuation of the level of P-eIF2α, which is a commonly used marker of PERK branch activation. As phosphorylation of eIF2α leads to translational block, its decrease may suggest that in combination groups protein synthesis is less impaired or restored faster. In contrast, significant increase in the spliced form of XBP1, a widely used indicator of activation of IRE1 branch, was observed in groups treated with EGF-SubA and PDT as compared with PDT alone.

Prolonged or too severe ER-stress often leads to induction of the transcription factor CHOP, which is an important step leading to apoptotic cell death [43]. CHOP induction has been also found to play a significant role in increasing protein translation [11]. In our models, we observed a significant increase in the levels of CHOP both at the mRNA and protein levels, however, there was no correlation between CHOP induction and apoptosis. Surprisingly, despite enhancing cytotoxicity, the combination treatment resulted in a decreased level of apoptosis as compared with PDT alone, both in apoptosis-competent and deficient cell lines. Although silencing of CHOP resulted in complete blockade of PARP cleavage, it did not influence the cytotoxic/cytostatic effects of the combination.

As apoptosis was found not to play a significant role in cell death induced by the combination therapy, we aimed to determine the mechanism responsible for its cytotoxicity. Although the PI3K inhibitor, 3-methyladenine, was able to significantly delay the cytotoxic effects of therapy, no other signs of autophagy were observed. What is more, there were no differences in LDH release between the groups, which excluded necrosis as a dominant cell death modality. Data obtained from the electron and fluorescence microscopy revealed that upon PDT cells undergo an atypical form of cell death, which is characterized by cellular vacuolization originating from endoplasmic reticulum. This effect was further enhanced by an addition of EGF-SubA, which may suggest the important role of GRP78 and UPR in this process (Fig. 6.2).

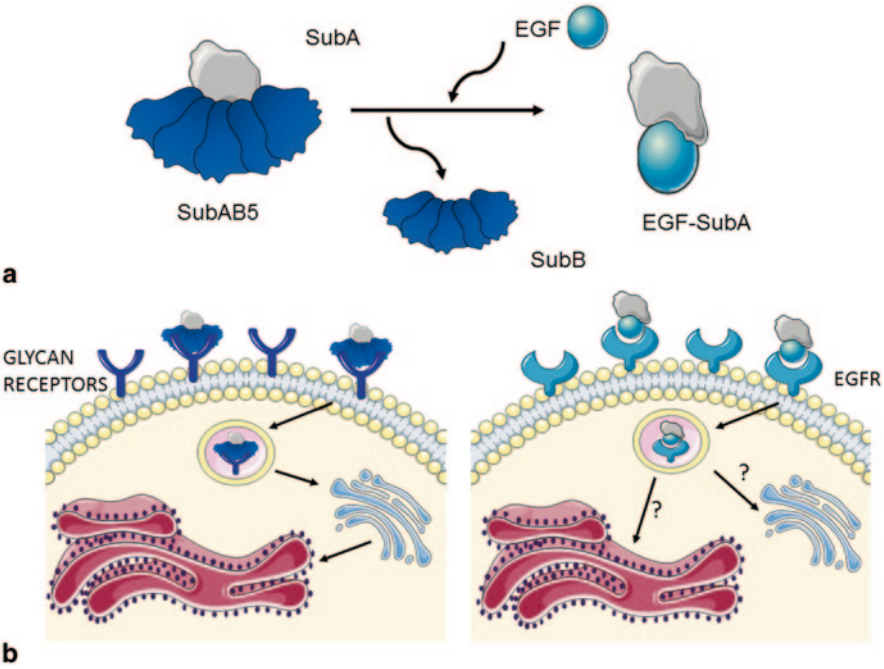

Fig. 6.2 Targeting mechanism of *EGF-SubA* molecules. **a** *SubAB* binds to *glycan receptors* on cell surface, enters the cell via clathrin-dependent endocytosis and is transported to the ER. **b** *EGF-SubA* binds to *EGFR* receptor, which allows its endocytosis and transport to the ER

Conclusions

To summarize, our findings indicate that the combination of photodynamic therapy with GRP78-targeting fusion protein EGF-SubA is an effective strategy that could be used to target EGFR-expressing tumors of various origins. The combination treatment results in a formation of massive vacuoles, which derive from the dilated endoplasmic reticulum, probably due to the enhanced ER-stress and UPR (Fig. 6.3). What is more, the cytotoxic/cytostatic effects of the therapy are independent of the apoptotic competence of the cells. It is an important finding, considering that in many types of cancers, dysregulation of the apoptotic machinery is observed, which often contributes to increased resistance of these cells to apoptosis-inducing agents.

No conflict statement "No potential conflicts of interest were disclosed."

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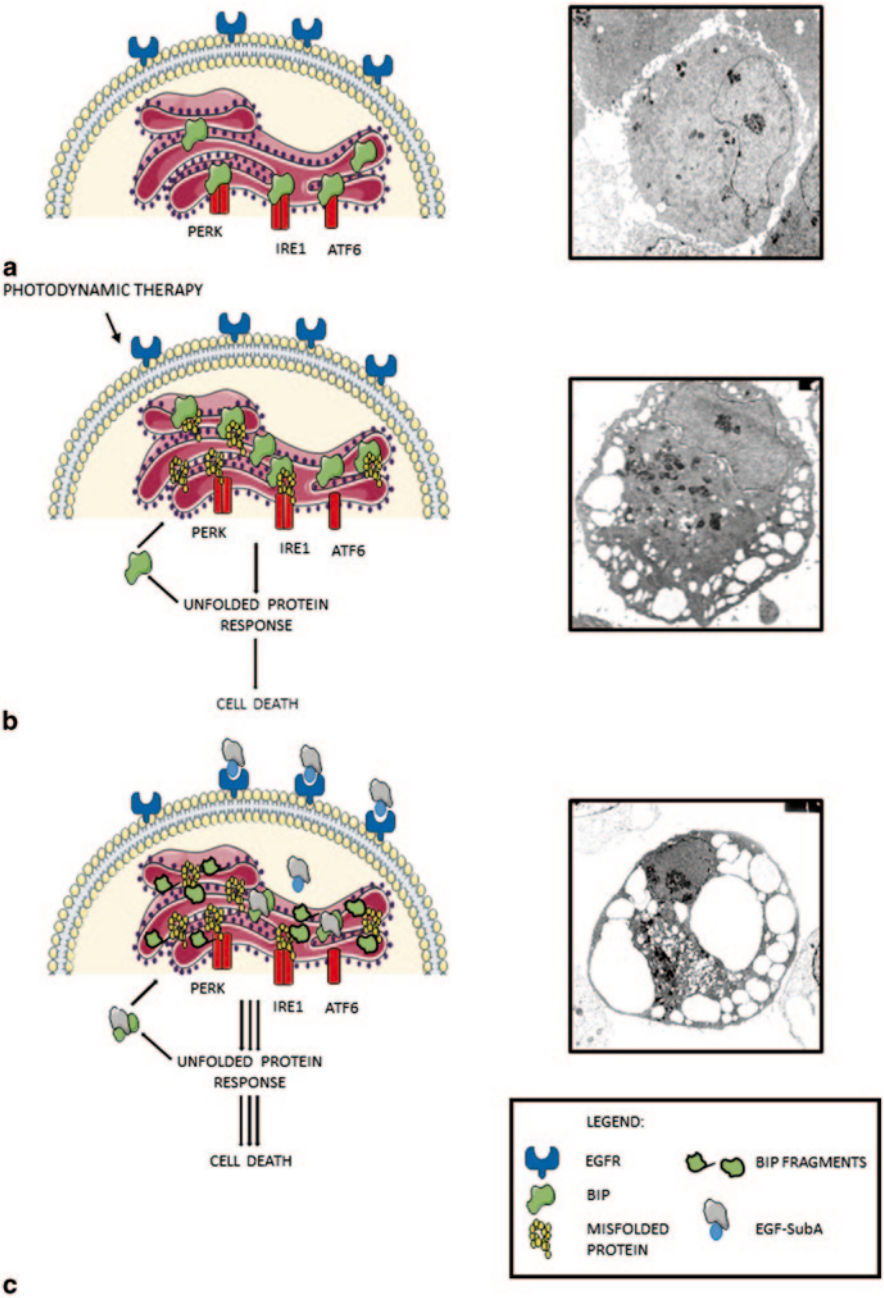

Fig. 6.3 Atypical form of cell death induced by PDT+SubA-EGF fusion protein, characterized by cellular vacuolization. Scheme representing differences in the cellular response between non-treated (**a**), PDT-treated (**b**) and PDT+EGF-SubA-treated (**c**) cells. In control cells, as GRP78 is bound to its three sensors: *PERK*, *IRE1* and *ATF6*, no activation of UPR occurs (**a**, *left panel*).

References

1. Davies MJ. Singlet oxygen-mediated damage to proteins and its consequences. Biochem Biophys Res Commun. 2003;305:761–70.
2. Szokalska A, Makowski M, Nowis D, Wilczynski GM, Kujawa M, Wojcik C, Mlynarczuk-Bialy I, Salwa P, Bil J, Janowska S, Agostinis P, Verfaillie T, Bugajski M, Gietka J, Issat T, Glodkowska E, Mrowka P, Stoklosa T, Hamblin MR, Mroz P, Jakobisiak M, Golab J. Proteasome inhibition potentiates antitumor effects of photodynamic therapy in mice through induction of endoplasmic reticulum stress and unfolded protein response. Cancer Res. 2009;69:4235–43.
3. Wang HP, Hanlon JG, Rainbow AJ, Espiritu M, Singh G. Up-regulation of Hsp27 plays a role in the resistance of human colon carcinoma HT29 cells to photooxidative stress. Photochem Photobiol. 2002;76:98–104.
4. Hanlon JG, Adams K, Rainbow AJ, Gupta RS, Singh G. Induction of Hsp60 by Photofrin-mediated photodynamic therapy. J Photochem Photobiol. 2001;64:55–61.
5. Nonaka M, Ikeda H, Inokuchi T. Inhibitory effect of heat shock protein 70 on apoptosis induced by photodynamic therapy in vitro. Photochem Photobiol. 2004;79:94–8.
6. Ferrario A, Rucker N, Wong S, Luna M, Gomer CJ. Survivin, a member of the inhibitor of apoptosis family, is induced by photodynamic therapy and is a target for improving treatment response. Cancer Res. 2007;67:4989–95.
7. Brodsky JL, Chiosis G. Hsp70 molecular chaperones: emerging roles in human disease and identification of small molecule modulators. Curr Top Med Chem. 2006;6:1215–25.
8. Takayama S, Reed JC, Homma S. Heat-shock proteins as regulators of apoptosis. Oncogene. 2003;22:9041–7.
9. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol. 2000;2:326–32.
10. Rao RV, Peel A, Logvinova A, del Rio G, Hermel E, Yokota T, Goldsmith PC, Ellerby LM, Ellerby HM, Bredesen DE. Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. FEBS Lett. 2002;514:122–8.
11. Han J, Back SH, Hur J, Lin YH, Gildersleeve R, Shan J, Yuan CL, Krokowski D, Wang S, Hatzoglou M, Kilberg MS, Sartor MA, Kaufman RJ. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. Nat Cell Biol. 2013;15:481–90.
12. Hiramatsu N, Messah C, Han J, Lavail MM, Kaufman RJ, Lin JH. Translational and post-translational regulation of XIAP by eIF2alpha and ATF4 promotes ER stress-induced cell death during the unfolded protein response. Mol Biol Cell. 2014;25:1411–20.
13. Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS. Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. J Biol Chem. 2003;278:20915–24.
14. Fu Y, Li J, Lee AS. GRP78/BiP inhibits endoplasmic reticulum BIK and protects human breast cancer cells against estrogen starvation-induced apoptosis. Cancer Res. 2007;67:3734–40.
15. Li J, Lee AS. Stress induction of GRP78/BiP and its role in cancer. Curr Mol Med. 2006;6:45–54.

Moreover, no vacuoles can be observed by electron microscopy (**a**, *right panel*). PDT treatment results in the dissociation of GRP78 from its three sensors, leading to activation of UPR and subsequent cell death (**b**, *left panel*). In electron microscopy, emerging vacuoles can be observed (**b**, *right panel*). The addition of *EGF-SubA* to PDT results in a cleavage of GRP78, enhanced UPR and increased cell death (**c**, *left panel*). This is accompanied by the formation of large, empty vacuoles (**c**, *right panel*)

16. Romero-Ramirez L, Cao H, Nelson D, Hammond E, Lee AH, Yoshida H, Mori K, Glimcher LH, Denko NC, Giaccia AJ, Le QT, Koong AC. XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. Cancer Res. 2004;64:5943–7.
17. Zhang LH, Zhang X. Roles of GRP78 in physiology and cancer. J Cell Biochem. 2010;110:1299–1305.
18. Chiu CC, Lin CY, Lee LY, Chen YJ, Kuo TF, Chang JT, Liao CT, Wang HM, Yen TC, Shen CR, Liao SK, Cheng AJ. Glucose-regulated protein 78 regulates multiple malignant phenotypes in head and neck cancer and may serve as a molecular target of therapeutic intervention. Mol Cancer Ther. 2008;7:2788–97.
19. Dong D, Ko B, Baumeister P, Swenson S, Costa F, Markland F, Stiles C, Patterson JB, Bates SE, Lee AS. Vascular targeting and antiangiogenesis agents induce drug resistance effector GRP78 within the tumor microenvironment. Cancer Res. 2005;65:5785–91.
20. Lee E, Nichols P, Spicer D, Groshen S, Yu MC, Lee AS. GRP78 as a novel predictor of responsiveness to chemotherapy in breast cancer. Cancer Res. 2006;66:7849–53.
21. Pyrko P, Schonthal AH, Hofman FM, Chen TC, Lee AS. The unfolded protein response regulator GRP78/BiP as a novel target for increasing chemosensitivity in malignant gliomas. Cancer Res. 2007;67:9809–16.
22. Virrey JJ, Dong D, Stiles C, Patterson JB, Pen L, Ni M, Schonthal AH, Chen TC, Hofman FM, Lee AS. Stress chaperone GRP78/BiP confers chemoresistance to tumor-associated endothelial cells. Mol Cancer Res. 2008;6:1268–75.
23. Jalili A, Makowski M, Switaj T, Nowis D, Wilczynski GM, Wilczek E, Chorazy-Massalska M, Radzikowska A, Maslinski W, Bialy L, Sienko J, Sieron A, Adamek M, Basak G, Mroz P, Krasnodebski IW, Jakobisiak M, Golab J. Effective photoimmunotherapy of murine colon carcinoma induced by the combination of photodynamic therapy and dendritic cells. Clin Cancer Res. 2004;10:4498–508.
24. Mak NK, Li KM, Leung WN, Wong RN, Huang DP, Lung ML, Lau YK, Chang CK. Involvement of both endoplasmic reticulum and mitochondria in photokilling of nasopharyngeal carcinoma cells by the photosensitizer Zn-BC-AM. Biochem Pharmacol. 2004;68:2387–96.
25. Marchal S, Francois A, Dumas D, Guillemin F, Bezdetnaya L. Relationship between subcellular localisation of Foscan and caspase activation in photosensitised MCF-7 cells. Br J Cancer. 2007;96:944–51.
26. Wong S, Luna M, Ferrario A, Gomer CJ. CHOP activation by photodynamic therapy increases treatment induced photosensitization. Lasers Surg Med. 2004;35:336–41.
27. Gomer CJ, Ferrario A, Rucker N, Wong S, Lee AS. Glucose regulated protein induction and cellular resistance to oxidative stress mediated by porphyrin photosensitization. Cancer Res. 1991;51:6574–9.
28. Morgan J, Whitaker JE, Oseroff AR. GRP78 induction by calcium ionophore potentiates photodynamic therapy using the mitochondrial targeting dye victoria blue BO. Photochem Photobiol. 1998;67:155–64.
29. Xue LY, Agarwal ML, Varnes ME. Elevation of GRP-78 and loss of HSP-70 following photodynamic treatment of V79 cells: sensitization by nigericin. Photochem Photobiol. 1995;62:135–43.
30. Paton AW, Srimanote P, Talbot UM, Wang H, Paton JC. A new family of potent AB(5) cytotoxins produced by Shiga toxigenic Escherichia coli. J Exp Med. 2004;200:35–46.
31. Byres E, Paton AW, Paton JC, Lofling JC, Smith DF, Wilce MC, Talbot UM, Chong DC, Yu H, Huang S, Chen X, Varki NM, Varki A, Rossjohn J, Beddoe T. Incorporation of a non-human glycan mediates human susceptibility to a bacterial toxin. Nature. 2008;456:648–52.
32. Paton AW, Beddoe T, Thorpe CM, Whisstock JC, Wilce MC, Rossjohn J, Talbot UM, Paton JC. AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. Nature. 2006;443:548–52.
33. Wolfson JJ, May KL, Thorpe CM, Jandhyala DM, Paton JC, Paton AW. Subtilase cytotoxin activates PERK, IRE1 and ATF6 endoplasmic reticulum stress-signalling pathways. Cell Microbiol. 2008;10:1775–86.

34. Paton JJ, Belova MA, Morrison SE, Salzman CD. The primate amygdala represents the positive and negative value of visual stimuli during learning. Nature. 2006;439:865–70.
35. Yamazaki H, Hiramatsu N, Hayakawa K, Tagawa Y, Okamura M, Ogata R, Huang T, Nakajima S, Yao J, Paton AW, Paton JC, Kitamura M. Activation of the Akt-NF-kappaB pathway by subtilase cytotoxin through the ATF6 branch of the unfolded protein response. J Immunol. 2009;183:1480–7.
36. Zhao Y, Tian T, Huang T, Nakajima S, Saito Y, Takahashi S, Yao J, Paton AW, Paton JC, Kitamura M. Subtilase cytotoxin activates MAP kinases through PERK and IRE1 branches of the unfolded protein response. Toxicol Sci. 2011;120:79–86.
37. Yahiro K, Morinaga N, Moss J, Noda M. Subtilase cytotoxin induces apoptosis in HeLa cells by mitochondrial permeabilization via activation of Bax/Bak, independent of C/EBF-homologue protein (CHOP), Ire1alpha or JNK signaling. Microb Pathog. 2010;49:153–63.
38. Lass A, Kujawa M, McConnell E, Paton AW, Paton JC, Wojcik C. Decreased ER-associated degradation of alpha-TCR induced by Grp78 depletion with the SubAB cytotoxin. Int J Biochem Cell Biol. 2008;40:2865–79.
39. Matsuura G, Morinaga N, Yahiro K, Komine R, Moss J, Yoshida H, Noda M. Novel subtilase cytotoxin produced by Shiga-toxigenic Escherichia coli induces apoptosis in vero cells via mitochondrial membrane damage. Infect Immun. 2009;77:2919–24.
40. Oh S, Stish BJ, Vickers SM, Buchsbaum DJ, Saluja AK, Vallera DA. A new drug delivery method of bispecific ligand-directed toxins, which reduces toxicity and promotes efficacy in a model of orthotopic pancreatic cancer. Pancreas. 2010;39:913–22.
41. Backer JM, Krivoshein AV, Hamby CV, Pizzonia J, Gilbert KS, Ray YS, Brand H, Paton AW, Paton JC, Backer MV. Chaperone-targeting cytotoxin and endoplasmic reticulum stress-inducing drug synergize to kill cancer cells. Neoplasia. 2009;11:1165–73.
42. Firczuk M, Gabrysiak M, Barankiewicz J, Domagala A, Nowis D, Kujawa M, Jankowska-Steifer E, Wachowska M, Glodkowska-Mrowka E, Korsak B, Winiarska M, Golab J. GRP78-targeting subtilase cytotoxin sensitizes cancer cells to photodynamic therapy. Cell Death Dis. 2013;4:e741.
43. Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol. 2011;13:184–90.

Chapter 7
Optimization of Photodynamic Therapy Response by Survivin Gene

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Abstract Cancer is typically a consequence of imbalance between cell death and proliferation in a way favorable to cell proliferation and survival. Most conventional cancer therapies are based on targeting rapidly growing cancerous cells to block growth or enhance cell death, thereby, restoring the balance between these processes. In many instances, malignancies that develop resistance to current treatment modalities, for example photodynamic therapy (PDT), often present the greatest challenge in subsequent management of the patient. In this context, the role of survivin in resistance to anti-cancer therapies has become an area of intensive investigation. Survivin is a member of the inhibitor of apoptosis (IAP) family that correlates inversely with patient prognosis. The application of PDT resulted in an over-expression of survivin in tumor cells and, moreover, survivin has a specific role in modulating PDT-mediated apoptotic response. Tumor cells which present downregulated survivin and then are treated with PDT exhibit increased apoptotic indexes and cytotoxicity when compared to single agent-treated cells. There are several strategies under investigation to target survivin that include of molecular antagonists, small molecules and immunotherapy. The translation of these findings to the clinic is currently ongoing with a number of phase I/II clinical trials targeting survivin that are in progress. Therefore, combining PDT with a survivin inhibitor may attribute to a more favorable clinical outcome than the use of single-modality PDT.

Keywords Apoptosis · Cancer · Immunotherapy · Molecular antagonist · Photodynamic therapy (*PDT*) · Survivin

Abbreviations

CPC Chromosomal passenger complex
IAP Inhibitor of apoptosis protein

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IC	Irradiated cells
MAL	Delta-aminoleavulinic acid methyl ester hydrochloride
PDT	Photodynamic therapy
pSil_1	Plasmid silencer against survivin

Introduction

The resistance of human tumors to cancer therapies is attributed to mutations, amplifications of genetic and epigenetic changes that influence in the take, transport and metabolism of the drug and a great network of survival and proliferation mechanisms. In this context, PDT-mediated oxidative stress induces a transient increase in the downstream early-response genes (*c-fos, c-jun, c-myc and egr-l*) and stress genes (coding for heat shock *proteins [Hsp], glucose-regulated proteins* and *heme oxygenase*) in mammalian cells [1–6]. The early-response genes function as transcription factors and act by regulating the expression of a variety of genes via specific regulatory domains. PDT appears to stimulate several different signaling pathways, some of which lead to cell death, by caspase-dependent [7] and –independent [8] apoptosis, whereas others mediate cell survival such that the ultimate survival of a given cell results from the combined action or interaction (or both) of these different pathways [9]. Therefore, survival cells may cooperate in tumor recurrences following PDT and underline the need to more fully understand the molecular responses initiated by PDT. In this context, there have been reports that showed that PDT induces the expression of heat-shock proteins (HSPs) such as HSP70 [10], HSP47 [11] and HSP60 [4], as well as other stress-inducible proteins [12]. HSPs are abundant. HSP70 and HSP90 correlate with a poor prognosis in acute myeloid leukemias and myelodysplastic syndromes [13, 14].

Conversely, it was observed that PDT induces overexpression and phosphorylation of a protein called “Survivin” in human cancer cells and tumors [15, 16]. Recently, there has been increasing clinical interest in this protein, because it possesses inherent properties that make it an ideal tumor marker and a potential therapeutic target [17–19].

Overview of Survivin

Survivin is the smallest member of the inhibitor of apoptosis protein (IAP) family [20], containing a single Baculovirus IAP Repeat (BIR), which is the hallmark of these molecules. The survivin gene encodes a 16.5 kDa protein consisting of an N-

terminal Zn^{2+}-binding BIR domain linked to a 65 Å amphipathic C-terminal-helix [21]. Survivin can function as a monomer for at least some protein–protein interactions, as well as mechanisms of subcellular localization [22]. The control of survivin gene expression at the tip of chromosome 17 in humans (17q25) is complex, and involves scores of positive and negative regulators. Several oncogenic transcription factors stimulate expression of the survivin gene, whereas multiple tumor suppressors actively repress the survivin gene [23]. A survivin protein is extensively post-translationally modified by degradative and non-degradative cycles of ubiquitylation and de-ubiquitylation, as well as phosphorylation [24], which control protein stability, binding to molecular partners and trafficking to various subcellular compartments.

Alternative splicing of survivin pre-mRNA from chromosome 17q25 produces five different mRNAs, which potentially encode distinct proteins, survivin, survivin DEx3 [25], survivin 3B [26] and survivin 2α [27]. Full-length survivin is derived from exons 1–4. Survivin 2B is also derived from exons 1–4 but retains an additional 69 bp from intron 2 as a cryptic exon. Survivin DEx3 is derived from exons 1, 2 and 4, as a frameshift read-through leads to exclusion of exon 3 [25]. Survivin 3B is derived from exons 1, 2, 3 and 4, and includes a new sequence of 165 bp from intron three [25]. Acquisition of an in-frame TGA stop codon within the novel exon 3B results in an open reading frame of 363 nucleotides, predicting a truncated 120 amino acid protein [25]. Survivin 2α comprises exons 1 and 2 of the survivin gene as well as a 30,197 bp region of intron 2 [26]. The acquisition of an in-frame stop codon within intron 2 results in an open reading frame of 225 nucleotides and predicts for a truncated 74 amino acid protein. The survivin 3B protein is predicted to have 120 aa, while survivin DEx3, full length survivin and survivin 2B are predicted to have 137, 142 and 165 aa, respectively.

There are on-going interests in identifying the molecular functions of survivin since it is an interesting molecule that interferes with a variety of cellular process. It is known that survivin is a multifunctional protein and is essential, in that constitutive or conditional deletion of the survivin gene is incompatible with tissue or organism viability [24]. Orthologs of survivin have been found in lower organisms, such as yeast, worms, and flies, suggesting evolutionary conservation of this pathway. In mammalian cells, survivin participates in at least three homeostatic networks: the control of mitosis (1), the regulation of apoptosis and autophagy (2), and the cellular stress response (3) (Fig. 7.1). This classification is not restrictive, as novel functions of survivin are continuously proposed, as well as new roles for known properties. Even within the same network, survivin plays multiple roles.

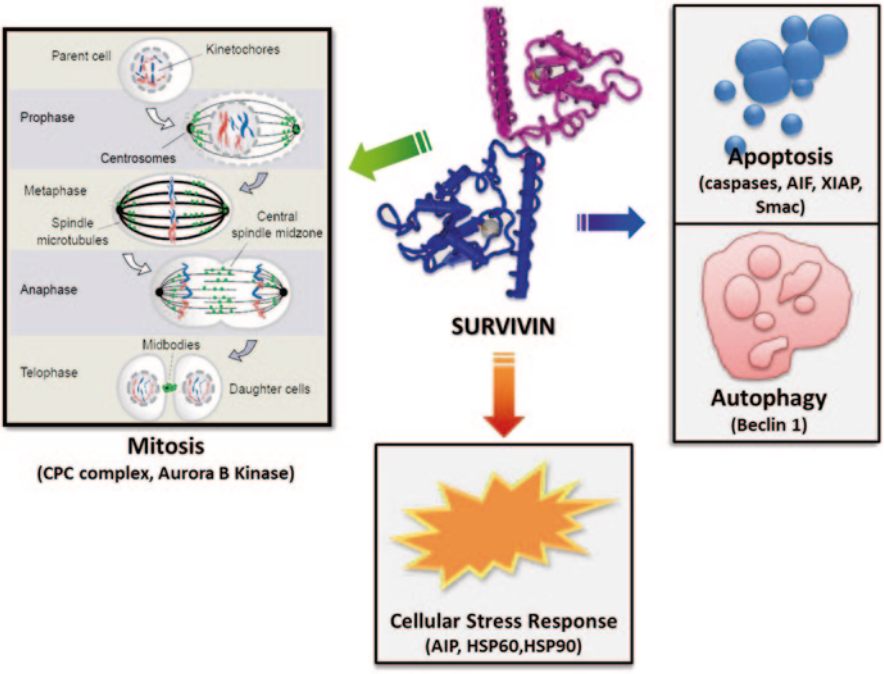

Fig. 7.1 Functions of survivin. Survivin is implicated in the control of mitosis, the regulation of apoptosis and autophagy, and the cellular stress response

The Role of Survivn in Cell Division

Some investigators have suggested that the primary function of survivin is in controlling cell division, rather than apoptosis inhibition [28–30]. During mitosis, survivin exists as a multi-protein complex, known as the chromosomal passenger complex (CPC) [31–33]. The CPC is a key regulator of mitosis, and this complex is composed of survivin, Borealin, and INCENP and Aurora B kinase. Structurally, the BIR domain of survivin can bind to the phosphorylated Thr3 site of histone H3 [34]. Upon CPC complex formation at the G2/M phase of cell cycle, survivin reads phosphorylated histone H3 and subsequently activates the mitotic kinase Aurora B [35–37] (Fig. 7.2). The formation of CPC and the interaction between CPC and Aurora B kinase through survivin's BIR domain are both crucial for the completion of mitosis. In fact, it has been shown that survivin-depleted cells could exit mitosis prior to completion of sister chromatid segregation. An alternative possibility is that survivin promotes mitosis by acting as an interphase between the centromere/central spindle and the CPC [32, 33]. Coincident with its role in cell proliferation, survivin expression is predominantly regulated by a cell cycle-dependent and cell cycle homology region within the promoter, which leads to maximum expression during the G2/M phase of the cell cycle [38]. Interestingly, survivin may interfere with the dynamic formation of microtubules. Over-expression of survivin has been

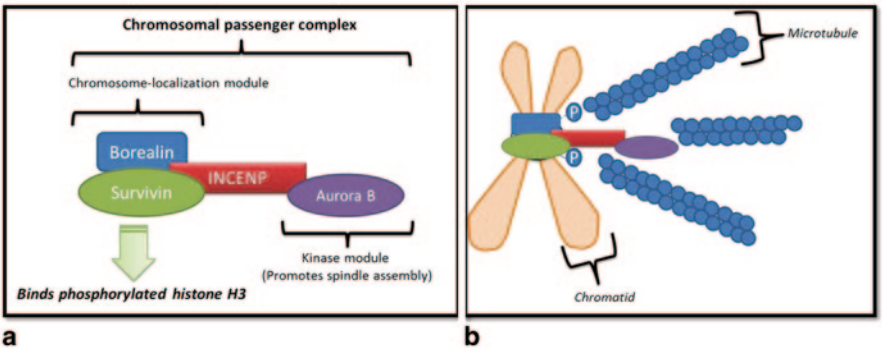

Fig. 7.2 Function of suvivin in cell division. **a** Structure of the chromosomal passenger complex (CPC). **b** Survivin regulates microtubule dynamics at kinetochores via the CPC. Aurora B phosphorylation promotes spindle formation

shown to reduce centrosomal microtubule nucleation and suppress both microtubule dynamics instability in mitotic spindles and bidirectional growth of microtubules in midbodies during cytokinesis [39]. It has been proposed that the splice variants function to modulate the function of full-length survivin [40]. While this may be true for apoptosis inhibition, where survivin and survivin DEx3 interact within the mitochondria to inhibit mitochondrial-dependent apoptosis [40], recent evidence suggests that the splice variants cannot modulate survivin's function during cell division [41]. Structural comparison studies have supported this finding [22].

The Role of Survivin in Apoptosis and Autophagy

Multiple *in vitro* and *in vivo* studies have shown that survivin inhibits cell death, especially apoptosis [42–45]. Survivin interferes with the process of apoptosis by inhibiting the activity of different caspases in cancer cells [43–46]. It is not surprising that survivin can interfere with the activity of caspases, given that survivin contains a single BIR domain and that the presence of the BIR domain was widely shown to be important in targeting caspases in various IAP family members [21]. Survivin, like all other IAPs except XIAP [20], does not directly inhibit caspases, but interacts with protein partners, most notably XIAP [47]. This complex promotes increased XIAP stability against degradation, activates multiple signaling pathways, including NF-κB, synergistically inhibits caspase-3 and -9, suppresses apoptosis, and accelerates tumor progression, *in vivo* (Fig. 7.3). Other mechanisms of survivin cytoprotection have been proposed, including the ability of mitochondrial localized pool of survivin to sequester pro-apoptotic Smac away from XIAP [48], or altogether prevent its release from the mitochondria [49] (Fig. 7.3).

Furthermore, survivin play a role in inhibiting the caspase-independent apoptosis of cancer cells. Translocation of the apoptosis-inducing factor (AIF) from the cytoplasm to the nucleus is a molecular indicator of the caspase-independent apoptosis

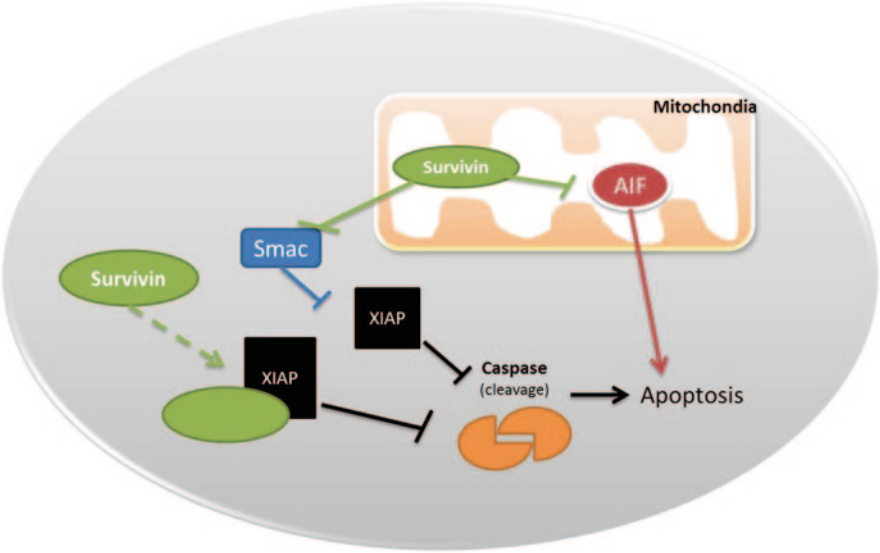

Fig. 7.3 Survivin's function in apoptosis. Survivin binds to XIAP, this complex promotes increased XIAP stability against degradation and synergistically inhibits caspase-3 and -9 and suppressing apoptosis. Also, a pool of survivin localized in the mitochondrial sequesters pro-apoptotic Smac away from XIAP. Moreover, survivin prevents AIF release from the mitochondrial intermembrane space and inhibiting the caspase-independent apoptosis

of cells. Down-regulation of survivin by siRNA induces the translocation of AIF from the cytoplasm to the nucleus in various cancer cells [50]. A study carried out by Pavlyukov et al. further showed that monomeric survivin (not dimeric survivin) prevents AIF release from the mitochondrial intermembrane space, protecting human fibrosarcoma HT1080 cells from caspase-independent apoptosis [51]. On the other hand, AIF knockout was shown effective in reversing the pro-apoptotic effect caused by the dominant-negative survivin in acute lymphoblastic leukemia (ALL) cells *in vitro* [52]. Taken together, these studies suggest that AIF plays critical roles in survivin-mediated caspase-independent apoptosis (Fig. 7.3).

Conversely, multiple evidences indicate that survivin interferes with the process of cell autophagy and down-regulation of survivin may induce apoptosis through autophagy-dependent mechanisms. First of all, an interaction between the autophagy regulator, Beclin 1, and survivin has been shown in human glioma cells in response to TRAIL [53]. Second, results from Roca et al.'s study showed that CCL2 (Chemokine (C–C motif) ligand 2) protected human PC3 prostate cancer cells from autophagic cell death via the phosphatidylinositol 3-kinase/Akt/survivin pathway [54]. Induction of autophagy-dependent apoptosis has further been shown in prostate cancer cells treated by the survivin suppressant YM155 [55]. Taken altogether, these studies showed that up-regulation of survivin inhibit autophagy, whereas downregulation of survivin promotes cell autophagy. However, the mechanisms by which survivin regulates autophagy remains to be determined.

The Role of Survivin in the Cellular Stress Response

A third function of survivin in the cellular stress response is beginning to emerge, and involves the association of survivin with various molecular chaperones, including the aryl hydrocarbon receptor-interacting protein (AIP) [56], Hsp60 [57], and Hsp90 [58]. These interactions may promote adaptation under conditions of cellular stress by maintaining survivin protein stability, folding, and subcellular trafficking.

For instance, HSP90 associated with survivin is overexpressed in cancers with roles in mitotic control and apoptosis inhibition. The cytoprotection mechanism of survivin–HSP90 association is centred on the mitochondrial pathway, where survivin has a role in the regulation of mitochondrial apoptosis specifically in tumors [58]. However, the possible disruption of the survivin–HSP90 complex destabilizes survivin leading to mitochondrial apoptosis and ultimately cell growth suppression [58]. HSP90 interaction with survivin enables stabilization of cofactors such as AKT, human epidermal growth factor receptor (Erb-2) and HIF-1a, which can lead to tumor progression [59].

As we saw so far, because of its nodal properties, and over-expression in virtually every human tumor, survivin has been intensely pursued as a drug target for novel cancer therapeutics [24, 60, 61]. Moreover, the ability of survivin to counteract apoptotic stimuli enhances cell survival, which in turn facilitates cell proliferation, including the proliferation of mutant cells. This proliferation may ultimately give rise to malignancy. The failure to execute apoptosis also renders malignant cells resistant to various forms of therapy including photodynamic therapy [15].

Photodynamic Therapy and Survivin Expression

PDT uses non-toxic dyes and harmless visible light in combination with oxygen to produce highly reactive oxygen species that kill cells. Our laboratory studies conducted on PDT-treatment using delta-aminoleavulinic acid methyl ester hydrochloride (MAL), such as a photosensitizer in T47D breast cancer cells, revealed increased both expression of survivin and its phosphorylated form [15]. Our results were in accordance with another study that observed increased survivin expression after PDT [62]. This suggests the possibility of interfering with the cellular response to photochemical therapy. Moreover, there are many signaling molecules up-regulated by PDT, including phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase, hypoxia inducible factor-1a, activator protein-1, and nuclear factor-κB, all of which are inducers of survivin expression [63]. Similarly, inflammatory cytokines, vascular endothelial growth factor, vascular injury, and hypoxia are associated with increased expression and/or stability of survivin and these responses are also increased following PDT.

PDT stimulate several different signaling pathways, some of which lead to cell death, whereas others mediate cell survival such that the ultimate survival of a given cell results from the combined action or interaction (or both) of these different

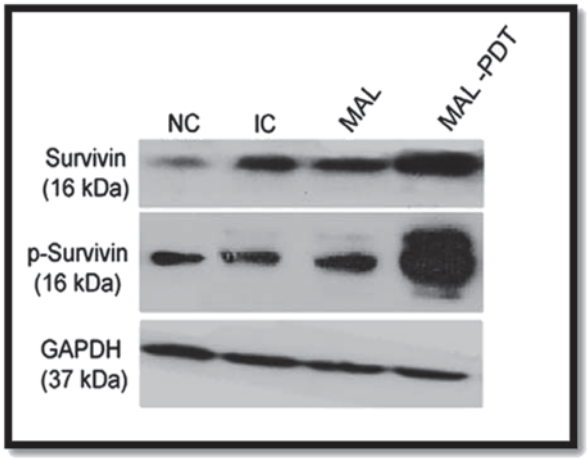

Fig. 7.4 Survivin and phosphorylated survivin expression after PDT treatment. Tumor cells were incubated with MAL and then exposed to ligth. After 24 h of light treatment, whole extracts from non-treated cells (*NC*), irradiated cells (*IC*), cells treated with MAL alone (*MAL*) and PDT-treated cells (*PDT*) were prepared and analyzed by Western blot to determine survivin, phosphorylated survivin (Phospho-Survivin), and *GAPDH* (internal control) levels

pathways. Our experience demonstrates that before treatment, the population of T47D cells has a level of survivin expression that is similar in all cells (Fig. 7.4). We argued that the increased expression observed on irradiated cells (IC) and cells treated with MAL alone (MAL) is attributed to some stressing condition given to the cells. But it is clear that only after treatment, a certain percentage of these cells could trigger signaling pathways which lead to cell survival involving survivin overexpression (Fig. 7.4).

Enhanced survivin after PDT in T47D cells led us to hypothesize that this anti-apoptotic protein would be potentially relevant to the PDT outcome in sensitized cells. In an attempt to demonstrate the crucial role of survivin on moderate PDT response in metastatic breast cancer cells, we targeted specifically mRNA survivin by siRNA technology (pSil_1). Indeed, growth-inhibitory effects in T47D cells in the absence of any further cytotoxic stimulus was observed when survivin was downregulated, and this agrees with results previously reported [50]. Moreover, we performed a dose–response curve by combining treatment of T47D cells with MAL-PDT and survivin downregulation. We found the condition to sensitize T47D cells to PDT synergistically, suggesting a survivin specific role in modulating PDT. The synergistic combination increased apoptosis and cytotoxic effect when compared with single treatments (Fig. 7.5). We could observe that this procedure also led to enhanced PARP- and caspase-3 cleavage, a strong decrease in the Bcl-2/Bax ratio and activation of caspase-8. Furthermore, to confirm the specific role of survivin in the modulation of PDT, we overexpressed survivin. We observed the increase of cell viability and the reduction of cell death in breast cancer cells treated with PDT. Therefore, we suggest that survivin plays an important role in modulating cancer cell survival by PDT treatment during cancer therapy.

It has been proposed that survivin may inhibit apoptosis through suppression of caspase activity [43], but we have previously observed that silencing survivin in T47D cells by siRNA triggered apoptosis in a caspase-independent manner,

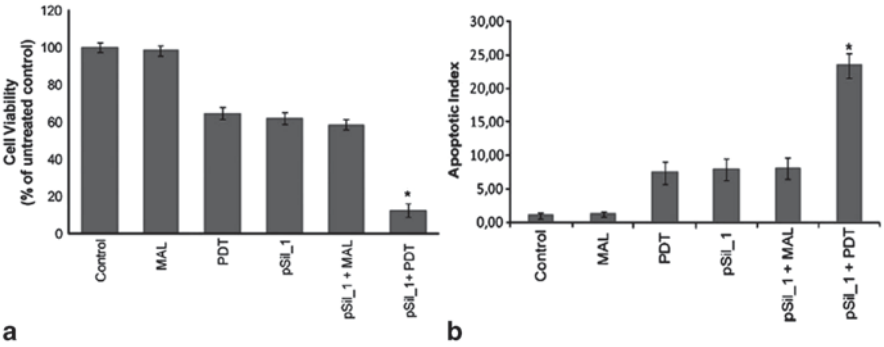

Fig. 7.5 Evaluation of cytotoxicity and apoptosis rate on PDT-treated tumor cells with survivin down-regulated. Cells were pre-transfected with pSil_1 for 72 h and then incubated with MAL and exposed to light. **a** Tumor cells viability was measured using MTT assay 24 h after MAL-PDT. Values are expressed as means ± SDs of eight separate experiments. A statistically significant difference in the level of viability between cells treated with pSil_1/PDT combination therapy and pSil_1 or PDT monotherapy is denoted by "*" ($p<0.05$). **b** Apoptotic indexes were measured 24 h after MAL-PDT using the Cell Death Apoptosis Detection ELISA Plus kit. Values are expressed as means ± SDs of two separate experiments. A statistically significant difference in the level of apoptosis between cells treated with pSil_1/PDT combination therapy and pSil_1 or PDT monotherapy is denoted by "*" ($p<0.05$)

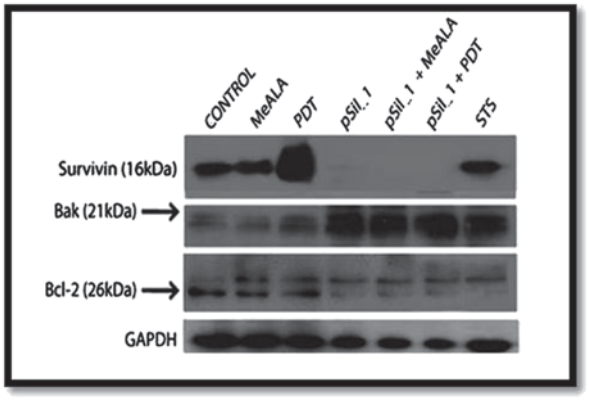

Fig. 7.6 Analysis of Bcl-2/Bak relation in MAL-PDT apoptotic cells. Cell lysates from control (Control), photosensitizer alone (*MAL*), pSil_1 alone (*pSil_1*), and PDT-treated cells in the absence (*PDT*) or presence of pSil_1 (*pSil_1 + PDT*) were collected 24 h after light exposure. Expressions of survivin, Bcl-2, Bak, and GAPDH (internal control) were determined by Western blot analysis. Staurosporine (*STS*)-treated tumor cells were used as positive control of apoptosis

involving nuclear translocation of mitochondrial AIF [50]. Interestingly, when the combined treatment was applied, apoptosis was triggered in a caspase-dependent manner. Therefore, our results demonstrated that in PDT protocols survivin directly or indirectly could interfere with caspase-3 activity.

Curiously, as a consequence of survivin downregulation, with or without PDT, a diminished Bcl-2/Bax ratio was observed (Fig. 7.6). It has been suggested that an

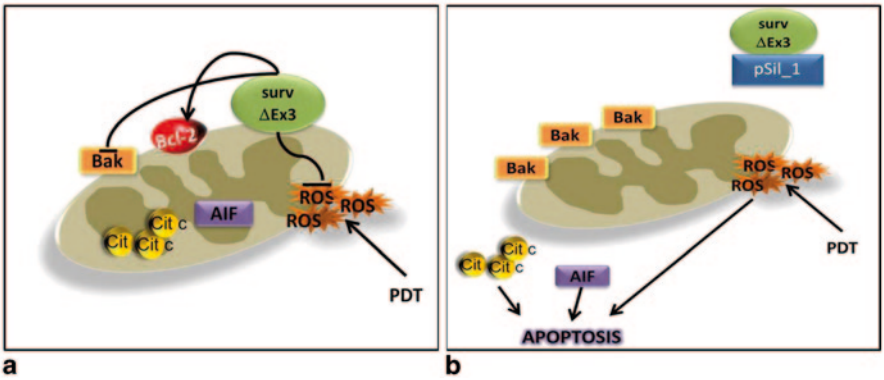

Fig. 7.7 Role of survivin in modulating PDT-mediated apoptotic response. **a** An alternatively spliced survivin variant, called survivin-DEx-3 which localizes in mitochondria, interacts with Bcl-2 and Bax. Survivin-DEx-3 hyperphosphorylated Bcl-2, and reduced activation of proapoptotic Bax. Furthermore, survivin-DEx-3 maintains mitochondrial membrane potential and controls the production of reactive oxygen species in response to cell-death stimuli, like in PDT. **b** Absence of survivin-DEx-3 mitochondrial permeability change. The expression of Bak and ROS increases, proapoptotic proteins are released from the mitochondria and all these manifestations produce apoptosis

alternatively spliced survivin variant, called survivin-DEx-3, which localizes in the mitochondria, interacts with Bcl-2 [64]. Since anti-apoptotic Bcl-2 proteins function as inhibitors of mitochondrial permeability transition, this recognition would position survivin, or at least one of its spliced variants, in the regulation of mitochondrial membrane integrity. Alternatives of this pathway have been suggested, involving hyperphosphorylation of Bcl-2, and a reduced activation of proapoptotic Bax by survivin, potentially upstream of caspase activation [65], thus, further dampening mitochondrial permeability. Furthermore, survivin-DEx-3 was recently shown to maintain mitochondrial membrane potential and to control the production of reactive oxygen species in response to cell-death stimuli [66]. Since the siRNA that we used for our experiments targets exon 1 of the human survivin mRNA, survivin-DEx-3 was blocked that would explain how survivin modulates the response of cancer cells to PDT (Fig. 7.7).

In summary, the intricate relationship between programmed cell death and cell survival may directly or indirectly be dependent on survivin. Since this protein is overexpressed in MAL-PDT treated cells, this could result in aggressive tumor behavior yielding a poor survival rate and resistance to cancer therapies. Moreover, down-regulation of survivin sensitizes breast cancer cells to PDT-induced cytotoxicity. Since a number of different strategies are now employed to treat metastatic breast cancer, it is promising to demonstrate that a combined modality and sequential therapy can prove beneficial to treatment. Therefore, our data suggest that emerging strategies in targeting protective proteins may increase the clinical effectiveness of cancer treatments.

Targeting Survivin: Which is the best option?

As survivin is not a cell surface protein and does not have an intrinsic enzymatic activity, targeting of survivin for therapeutic purposes might be expected to be difficult. In addition, crystallographic data have revealed few potential drugable sites on the survivin protein [21]. Despite these problems, several research groups have attempted to target survivin using different strategies (Table 7.1), which are discussed below.

Molecular Antagonists

The first described molecular antagonist of survivin is a phosphorothioate antisense oligonucleotide [67], which suppressed survivin mRNA and protein expressions, and produced a strong anticancer activity in preclinical models. Sponsored by Ely Lilly and Co., this agent designated LY2181308 showed a favorable toxicity profile with evidence of survivin downregulation in a phase I trial in patients with advanced cancers [68]. LY2181308 (also called ISIS23722 and Gataparsen) is a survivin-specific second generation antisense oligonucleotide with 2′-O-methoxyethyl modified 18-mer structure (5′-TGTGCTATTCTGTGAATT-3′) [69, 70]. The major mechanism of action of LY2181308 is relatively straightforward: it selectively binds to the 3′-untranslated region of the survivin transcript by Watson–Crick base pairing, resulting in the destruction of survivin RNA by RNase H [69–71]. In the first-in-human dose (FHD) study performed by Talbot et al. [72], patients with advanced solid tumors (gastrointestinal, melanoma, lung and breast cancers)

Table 7.1 Ongoing clinical trails targeting survivin

Type of agent	Compound name	Developed by/at	Clinical trail phase
Molecular antagonist	LY2181308	Elli Lilly and company	Phase II
	SPC3042(or ENZ3042)	Santaris pharma; ENZON pharmaceuticals	Phase I
	siRNA		Preclinical studies
Small molecules	YM155	Yamanouchi pharmaceuticals; astellas pharma	Phase II
	Terameprocol	Erimos pharmaceuticals	Phase I
Immunotherapy	Survivin peptide	Julius-Maximilians University	Phase I Phase II
	Mage-3, MelanA and surviving Peptide vaccine	Dermatologische Klinilk MIT Poliklinik	Phase I Phase II
	Survivin peptide vaccine +IL-2	Herlev hospital	Phase I Phase II

were treated with LY2181308 using the following schedule: daily i.v. infusion on day 1–3, then weekly infusion. The most common symptoms observed in patients treated with LY2181308 were shown to be fatigue, fever, vomiting and thrombocytopenia, and the recommended dose of LY2181308 was suggested as 750 mg [72]. In another study, Japanese patients with advanced solid tumors (lung, pancreatic, and breast cancers) were administered with the drug, and the maximum tolerated dose (MTD) of LY2181308 determined was shown to be 750 mg (daily i.v. infusion on day 1–3, then weekly infusion), which is similar to the MTD reported in Talbot et al.'s study [72]. Pharmacokinetic analysis of LY2181308 in the same clinical study revealed that the terminal half-life, distribution clearance and Vss were 21 days, 2.0 L/h and 2.05–105 L, respectively. Thrombocytopenia and fatigue were reported as common reversible grade ½ toxicities related to the therapy, similar to those reported in Talbot et al.'s study [72]. It is worth noting that a study carried out by Gurbuxani et al. [73] has found that murine survivin plays a role in hematopoietic cell development [73]. Therefore, human survivin may also play a role in erythropoiesis. However, further investigations are needed to determine whether thrombocytopenia is a mechanism-based toxicity induced by LY2181308.

Santaris Pharma, and, more recently, ENZON Pharmaceuticals [74] have also developed an agent that showed excellent safety in a phase I clinical, called SPC3042 (or ENZ3042 [75]. SPC3042 is an antisense 16-mer locked nucleic acid (LNA) oligonucleotide (5′-CTCAATCCATGGCAGC-3′) that targets exon 4 of the survivin transcript [74]. A study published in 2010 revealed that treatment with SPC3042 alone induced 60% down-regulation of survivin mRNA in tumors and 37–45% tumor growth inhibition (TGI) in the A549 and Calu-6 lung xenograft models [76].

Additional strategies to acutely lower survivin levels in tumor cells involved small interfering RNA (siRNA) or hammerhead ribozymes [19]. In preclinical studies, these agents consistently showed anticancer activity, alone or in combination with chemotherapy, with no detectable systemic toxicity [19]. The delivery of siRNA *in vivo* is challenging, but the apparent success of recent studies [77] suggests that survivin-directed gene silencing may at some point be evaluable in cancer patients.

Small Molecules

Small molecules that directly target survivin have been developed [24], and several clinical trials with these agents have been completed, with more underway. YM155 monobromide(1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1Hnaphtho [2,3-d] imidazolium bromide) is a small molecule survivin gene suppressant, and it is the most functionally evaluated survivin inhibitor in both pre-clinical and clinical studies so far [29]. Originally developed by Yamanouchi Pharmaceuticals, and, more recently, by Astellas Pharma [78]. At the molecular level, YM155 binds to the Sp1 rich region of the promoter of survivin, and inhibits the transcription of survivin in cells [79, 80].

Two phase I studies of YM155 in heavily-pretreated cancer patients have been published. The trial conducted in the US reported impressive responses, with tumor shrinkage and durable remissions in patients with advanced prostate cancer, large cell non-Hodgkin's lymphoma and non-small cell lung cancer [81]. The Japan phase I trial of YM155 also provided evidence of disease stabilization in nine patients [78]. Importantly, both studies showed a favorable toxicity profile with minimal and rapidly reversible side effects.

Despite various pre-clinical and phase I clinical studies indicating that YM155 could be an effective anti-cancer reagent [78], phase II clinical trials showed disappointing results. In a phase II clinical trial, 34 patients with un-resectable stage III or IV melanoma were infused with YM155 according to the following schedule: 168 h (7 days) continuous infusion at a dosage of 4.8 mg/m2/day, followed by a 14 rest period, for 6 cycles [19]. The most common adverse events of YM155 monotherapy reported in this study were fatigue, nausea, pyrexia, headache, arthralgia and back pain. The same study also reported that one patient developed Grade 3 acute renal failure during Cycle 1 of therapy and four patients (11.8%) developed cardiac adverse events (AE). However, the development of cardiac adverse events was not likely related to YM155. The objective tumor response rate (ORR) of patients with the YM155 treatment was approximately 3% in such study [19]. On the other hand, Giaccone et al.'s study[82] reported that YM155 only exhibited modest single-agent activity in patients, and the ORR to YM155 treatment (similar dosage schedule as the abovementioned study) was approximately 5.4% in patients with advanced refractory nonsmall-cell lung carcinoma [82]. Notably, a recent study showed that YM155 is a substrate of the multi-drug resistant protein (MDR1/ABCB1/P-gp), suggesting that YM155 treatment may not be useful in treating cancer patients with MDR1-related drug resistance after prolonged chemotherapy [83].

Another direct small molecule inhibitor of survivin is tetra-O-methyl nordihydroguaiaretic acid (M(4)N), which also acts as a transcriptional repressor of the survivin promoter, potentially by antagonizing Sp1-dependent gene expression [84]. This compound, designated Terameprecol (EM-1421) [85], has shown good preclinical activity with an impressive 88% bioavailability, *in vivo* [86]. Terameprecol has been formulated for systemic delivery to cancer patients, and a phase I study in patients with advanced solid malignancies has shown favorable safety and disease stabilization in 8 out of 25 evaluable patients [85]. Another phase I study of Terapremecol in 16 heavily pretreated patients with adult myelogenous leukemia (AML) has also shown a favorable safety, one partial response and disease stabilization in five patients [87]. In addition, Terameprecol has been formulated as a 1% or 2% vaginal ointment for local application in women with papillomavirus- or herpes simplex virus-associated carcinogenesis. Two phase I studies with Terameprecol ointment have shown excellent safety, no adverse events and no systemic absorption of the agent [88, 89].

Cancer Vaccine/Immunotherapy

Immunotherapy is the use of the immune system to either cure a disease or to avoid the development of a disease. Several studies have reported successful application of cell-based immunotherapy as cancer treatment [90–92]. Cell-based cancer immunotherapy involves the use of immune cells such as the natural killer cells, dendritic cells, and cytotoxic T lymphocytes, which are isolated from the patient, activated *in vitro* and transfused back to the patient to target cancer cells. Because of its differential expression in cancer, as opposed to normal tissues, it has been hypothesized that cancer patients may recognize survivin as a non-self protein, and mount an immune response to it [93]. This concept has been validated in the clinic, and sera from cancer patients contained antibodies [94], and cytolytic T cells against survivin [95]. This immune recognition has been mapped in detail [96, 97], and dendritic cells pulsed with survivin peptides, survivin-containing tumor lysates or transduced/transfected with survivin, elicit cytolytic T cell responses and MHC-restricted anticancer activity *in vitro* [98, 99] and in preclinical models [100]. Several phase I/II trials of survivin-directed immunotherapy have been reported [101]. In these studies, survivin-based vaccination was invariably safe, devoid of significant side-effects, and associated with antigen-specific immunologic responses. Although no objective responses were noted, two phase I/II trials with infusion of dendritic cells pulsed with survivin showed durable (>6 months) disease stabilization in 24% of melanoma patients [102], and 50% of renal cell carcinoma patients [103]. A phase I/II trial of systemic delivery of protamine-protected survivin mRNA in melanoma was also safe, produced detectable T cell responses, and achieved one complete response out of seven evaluable patients [104]. Anecdotal reports also suggest that survivin-based vaccination may be effective against metastatic disease [105]. Based on these encouraging findings, several additional phase I and II clinical trials using survivin-based immunotherapy are ongoing (Table 7.1). Currently, studies are focusing on the specific epitopes that elicit the most potent immunodominant, immunoprevalent T cell responses against survivin, with the likelihood that those inducing both a CD8+ and CD4+ response will be most effective [106].

Conclusion

PDT induces considerable stress within the tumor microenvironment. This includes both oxidative stress produced by the photochemical generation of reactive oxygen species and hypoxia resulting from the rapid vascular damage produced by PDT and/or by the photochemical consumption of oxygen [107]. A consequence of PDT-mediated stress is the induction of a survival phenotype associated with increased expression of angiogenic growth factors, cytokines, proteinases, and antiapoptotic molecules. Our increasing knowledge of PDT responses at a molecular level provides significant opportunities to further improve the therapeutic effectiveness of

PDT. In particular, over the last period, it has become increasingly clear that survivin may have an important role in the survival phenotype observed in PDT treatments [15–108]. Survivin has many functions involved in cell survival including complex intracellular signaling, stabilizing mitosis and facilitating cellular adaptation. So, it is clinically relevant that inhibitors of survivin expression may enhance PDT responsiveness.

Survivin antagonists may function not as single protein inhibitors, but rather as global pathway inhibitors that may disable multiple signaling circuits in tumors. Clinical trials have highlighted the problems with attempts to correlate survivin expression with clinical outcome. Small sample numbers, nonuniform treatments, the presence of multiple alternatively spliced survivin mRNAs with differing effects on apoptosis and the different methods of detection of survivin, all lead to difficulties in trial interpretations. Further efforts are required to achieve a greater understanding of the biology of survivin and the other IAPs and more effectively exploit strategies that target this protein in cancer.

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References

1. Gomer CJ, Ferrario A, Rucker N, Wong S, Lee AS. Glucose regulated protein induction and cellular resistance to oxidative stress mediated by porphyrin photosensitization. Cancer Res. 1991;51:6574–9.
2. Gomer CJ, Luna M, Ferrario A, Rucker N. Increased transcription and translation of heme oxygenase in Chinese hamster fibroblasts following photodynamic stress or Photofrin II incubation. Photochem Photobiol. 1991;53:275–9.
3. Luna MC, Gomer CJ. Isolation and initial characterization of mouse tumor cells resistant to porphyrin-mediated photodynamic therapy. Cancer Res. 1991;51:4243–9.
4. Hanlon JG, Adams K, Rainbow AJ, Gupta RS, Singh G. Induction of Hsp60 by Photofrin-mediated photodynamic therapy. J Photochem Photobiol B. 2001;64:55–61.
5. Tong Z, Singh G, Rainbow AJ. Sustained activation of the extracellular signal-regulated kinase pathway protects cells from photofrin-mediated photodynamic therapy. Cancer Res. 2002;62:5528–35.
6. Tong Z, Singh G, Valerie K, Rainbow AJ. Activation of the stress-activated JNK and p38 MAP kinases in human cells by Photofrin-mediated photodynamic therapy. J Photochem Photobiol B. 2003;71:77–85.
7. Alvarez MG, Prucca C, Milanesio ME, Durantini EN, Rivarola V. Photodynamic activity of a new sensitizer derived from porphyrin-C60 dyad and its biological consequences in a human carcinoma cell line. Int J Biochem Cell Biol. 2006;38:2092–101.
8. Vittar NB, Awruch J, Azizuddin K, Rivarola V. Caspase-independent apoptosis, in human MCF-7c3 breast cancer cells, following photodynamic therapy, with a novel water-soluble phthalocyanine. Int J Biochem Cell Biol. 2010;42:1123–31.

9. Moor AC. Signaling pathways in cell death and survival after photodynamic therapy. J Photochem Photobiol B. 2000;57:1–13.
10. Castano AP, Mroz P, Hamblin MR. Photodynamic therapy and anti-tumor immunity. Nat Rev Cancer. 2006;6:535–45.
11. Verrico AK, Haylett AK, Moore J V. In vivo expression of the collagen-related heat shock protein HSP47, following hyperthermia or photodynamic therapy. Lasers Med Sci. 2001;16:192–98.
12. Korbelik M, Sun J, Cecic I. Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response. Cancer Res. 2005;65:1018–26.
13. Duval A, Olaru D, Campos L, Flandrin P, Nadal N, Guyotat D. Expression and prognostic significance of heat-shock proteins in myelodysplastic syndromes. Haematologica. 2006;91:713–4.
14. Thomas X, Campos L, Mounier C, Cornillon J, Flandrin P, Le Q-H et al. Expression of heat-shock proteins is associated with major adverse prognostic factors in acute myeloid leukemia. Leukemia Res. 2005;29:1049–58.
15. Cogno IS, Vittar NB, Lamberti MJ, Rivarola VA. Optimization of photodynamic therapy response by survivin gene knockdown in human metastatic breast cancer T47D cells. J Photochem Photobiol B. 2011;104:434–43.
16. Ferrario A, Rucker N, Wong S, Luna M, Gomer CJ. Survivin, a member of the inhibitor of apoptosis family, is induced by photodynamic therapy and is a target for improving treatment response. Cancer Res. 2007;67:4989–95.
17. Zaffaroni N, Pennati M, Daidone MG. Survivin as a target for new anticancer interventions. J Cell Mol Med. 2009;9:360–72.
18. Pennati M, Folini M, Zaffaroni N. Targeting survivin in cancer therapy: fulfilled promises and open questions. Carcinogenesis. 2007;28:1133–39.
19. Pennati M, Folini M, Zaffaroni N. Targeting survivin in cancer therapy. Expert Opin Ther Targets. 2008;12:463–76.
20. Srinivasula SM, Ashwell JD. IAPs: what's in a name?. Mol Cell. 2008;30:123–35.
21. Chantalat L, Skoufias DA, Kleman JP, Jung B, Dideberg O, Margolis RL. Crystal structure of human survivin reveals a bow tie-shaped dimer with two unusual alpha-helical extensions. Mol Cell. 2000;6:183–89.
22. Jeyaprakash AA, Klein UR, Lindner D, Ebert J, Nigg EA, Conti E. Structure of a Survivin-Borealin-INCENP core complex reveals how chromosomal passengers travel together. Cell. 2007;131:271–85.
23. Guha M, Altieri DC. Survivin as a global target of intrinsic tumor suppression networks. Cell Cycle. 2009; 8:2708–10.
24. Altieri DC. Survivin, cancer networks and pathway-directed drug discovery. Nat Rev Cancer. 2008;8:61–70.
25. Mahotka C, Wenzel M, Springer E, Gabbert HE, Gerharz CD. Survivin-deltaEx3 and survivin-2B: two novel splice variants of the apoptosis inhibitor survivin with different anti-apoptotic properties. Cancer Res. 1999;59:6097–102.
26. Badran A, Yoshida A, Ishikawa K, Goi T, Yamaguchi A, Ueda T, Inuzuka, M. Identification of a novel splice variant of the human anti-apoptopsis gene survivin. Biochem Biophys Res Commun. 2004;314:902–7.
27. Caldas H, Honsey LE, Altura RA. Survivin 2alpha: a novel Survivin splice variant expressed in human malignancies. Mol Cancer. 2005;4:11–20.
28. Yang D, Welm A, Bishop JM. Cell division and cell survival in the absence of survivin. Proc Natl Acad Sci USA. 2004;101:15100–105.
29. Okada H, Mak TW. Pathways of apoptotic and non-apoptotic death in tumor cells. Nat Rev Cancer. 2004;4:592–603.
30. Wolanin K, Piwocka K. Role of survivin in mitosis. Postepy Biochemii. 2007;53:10–8.
31. Vader G, Kauw JJW, Medema RH, Lens SMA. Survivin mediates targeting of the chromosomal passenger complex to the centromere and midbody. EMBO Rep. 2006;7:85–92.
32. Ruchaud S, Carmena M, Earnshaw WC. The chromosomal passenger complex: one for all and all for one. Cell. 2007;131:230–31.

33. Gassmann R, Carvalho A, Henzing AJ, Ruchaud S, Hudson DF, Honda R, Nigg EA, Gerloff DL, Earnshaw WC. Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle. J. Cell Biol. 2004;166:179–91.
34. Jeyaprakash AA, Basquin C, Jayachandran U, Conti E. Structural basis for the recognition of phosphorylated histone h3 by the survivin subunit of the chromosomal passenger complex. Structure. 2011;19:1625–34.
35. Bolton MA, Lan W, Powers SE, McCleland ML, Kuang J, Stukenberg PT. Aurora B kinase exists in a complex with survivin and INCENP and its kinase activity is stimulated by survivin binding and phosphorylation. Mol Biol Cell 2002;13:3064–77.
36. Lens SM1, Wolthuis RM, Klompmaker R, Kauw J, Agami R, Brummelkamp T, Kops G, Medema RH. Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. EMBO J. 2003;22:2934–47.
37. Kelly AE, Ghenoiu C, Xue JZ, Zierhut C, Kimura H, Funabiki H. Survivin reads phosphorylated histone H3 threonine 3 to activate the mitotic kinase Aurora B. Science. 2010;330:235–9.
38. Li F, Altieri DC. Transcriptional analysis of human survivin gene expression. Biochem J. 1999;344Pt 2:305–11.
39. Rosa J, Canovas P, Islam A, Altieri DC. Doxsey SJ. Survivin modulates microtubule dynamics and nucleation throughout the cell cycle. Mol Biol Cell. 2006;17:1483–93.
40. Caldas H1, Jiang Y, Holloway MP, Fangusaro J, Mahotka C, Conway EM, Altura RA. Survivin splice variants regulate the balance between proliferation and cell death. Oncogene. 2005;24:1994–2007.
41. Noton EA, Colnaghi R, Tate S, Starck C, Carvalho A, Ko Ferrigno P, Wheatley SP. Molecular analysis of survivin isoforms: evidence that alternatively spliced variants do not play a role in mitosis. J Biol Chem. 2006;281:1286–95.
42. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med. 1997;3:917–21.
43. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res. 1998;58:5315–20.
44. Zaffaroni N1, Pennati M, Colella G, Perego P, Supino R, Gatti L, Pilotti S, Zunino F, Daidone MG. Expression of the anti-apoptotic gene survivin correlates with taxol resistance in human ovarian cancer. Cell Mol Life Sci. 2002;59:1406–12.
45. Grossman D, Kim PJ, Schechner JS, Altieri DC. Inhibition of melanoma tumor growth in vivo by survivin targeting. Proc Natl Acad Sci USA. 2001;98:635–40.
46. Shin S, Sung BJ, Cho YS, Kim HJ, Ha NC, Hwang JI, Chung CW, Jung YK, Oh BH. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry. 2001;40:1117–23.
47. Dohi T, Xia F, Altieri DC. Compartmentalized phosphorylation of IAP by protein kinase A regulates cytoprotection. Mol Cell. 2007;27:17–28.
48. Song Z, Yao X, Wu M. Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. J Biol Chem. 2003;278:23130–40.
49. Ceballos-Cancino G, Espinosa M, Maldonado V, Melendez-Zajgla J. Regulation of mitochondrial Smac/DIABLO-selective release by survivin. Oncogene. 2007;26:7569–75.
50. Croci DO, Cogno IS, Vittar NB, Salvatierra E, Trajtenberg F, Podhajcer OL, Osinaga E, Rabinovich GA, Rivarola VA. Silencing survivin gene expression promotes apoptosis of human breast cancer cells through a caspase-independent pathway. J Cell Biochem. 2008;105:381–90.
51. Pavlyukov MS, Antipova N V, Balashova M V, Vinogradova T V, Kopantzev EP, Shakhparonov MI. Survivin monomer plays an essential role in apoptosis regulation. J Cell Biochem. 2011;286:23296–307.
52. Okuya M, Kurosawa H, Kikuchi J, Furukawa Y, Matsui H, Aki D, Matsunaga T, Inukai T, Goto H, Altura RA, Sugita K, Arisaka O, Look AT, Inaba T. Up-regulation of survivin by the E2A-HLF chimera is indispensable for the survival of t(17;19)-positive leukemia cells. J Biol Chem. 2010;285:1850–60.

53. Niu T-K, Cheng Y, Ren X, Yang J-M. Interaction of Beclin 1 with survivin regulates sensitivity of human glioma cells to TRAIL-induced apoptosis. FEBS Lett. 2010;584:3519–24.
54. Roca H, Varsos Z, Pienta KJ. CCL2 protects prostate cancer PC3 cells from autophagic death via phosphatidylinositol 3-kinase/AKT-dependent survivin up-regulation. J Biol Chem. 2008;283:25057–73.
55. Wang Q, Chen Z, Diao X, Huang S. Induction of autophagy-dependent apoptosis by the survivin suppressant YM155 in prostate cancer cells. Cancer Lett. 2011;302:29–36.
56. Kang BH, Altieri DC. Regulation of survivin stability by the aryl hydrocarbon receptor-interacting protein. J Biol Chem. 2006;281:24721–27.
57. Ghosh JC, Dohi T, Kang BH, Altieri DC. Hsp60 regulation of tumor cell apoptosis. J Biol Chem. 2008;283:5188–94.
58. Fortugno P, Beltrami E, Plescia J, Fontana J, Pradhan D, Marchisio PC, Sessa WC, Altieri DC. Regulation of survivin function by Hsp90. Proc Natl Acad Sci USA. 2003;100:13791–96.
59. Cheung CHA, Chen H-H, Cheng L-T, Lyu KW, Kanwar JR, Chang J-Y. Targeting Hsp90 with small molecule inhibitors induces the over-expression of the anti-apoptotic molecule, survivin, in human A549, HONE-1 and HT-29 cancer cells. Mol Cancer. 2010;9:77–89.
60. Ryan BM, O'Donovan N, Duffy MJ. Survivin: a new target for anti-cancer therapy. Cancer Treat Rev. 2009;35:553–62.
61. Mita AC, Mita MM, Nawrocki ST, Giles FJ. Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. Clin Cancer Res. 2008;14:5000–5.
62. Ferrario A, Rucker N, Wong S, Luna M, Gomer CJ. Survivin, a member of the inhibitor of apoptosis family, is induced by photodynamic therapy and is a target for improving treatment response. Cancer Res. 2007;67:4989–95.
63. Gomer CJ, Ferrario A, Luna M, Rucker N, Wong S. Photodynamic therapy: combined modality approaches targeting the tumor microenvironment. Lasers Surg Med. 2006;38:516–21.
64. Wang H-W, Sharp T V, Koumi A, Koentges G, Boshoff C. Characterization of an anti-apoptotic glycoprotein encoded by Kaposi's sarcoma-associated herpesvirus which resembles a spliced variant of human survivin. EMBO J. 2002;21:2602–15.
65. Vogel C, Hager C, Bastians H. Mechanisms of mitotic cell death induced by chemotherapy-mediated G2 checkpoint abrogation. Cancer Res. 2007;67:339–45.
66. You R-I, Chen M-C, Wang H-W, Chou Y-C, Lin C-H, Hsieh S-L. Inhibition of lymphotoxin-beta receptor-mediated cell death by survivin-DeltaEx3. Cancer Res. 2006;66:3051–61.
67. Li F1, Ackermann EJ, Bennett CF, Rothermel AL, Plescia J, Tognin S, Villa A, Marchisio PC, Altieri DC. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. Nat Cell Biol. 1999;1:461–66.
68. Molckovsky A, Siu LL. First-in-class, first-in-human phase I results of targeted agents: highlights of the 2008 American society of clinical oncology meeting. J Hematol Oncol. 2008;1:20.
69. Hayashi N, Asano K, Suzuki H, Yamamoto T, Tanigawa N, Egawa S, Manome Y. Adenoviral infection of survivin antisense sensitizes prostate cancer cells to etoposide in vivo. Prostate. 2005;65:10–9.
70. Olie RA, Simões-Wüst AP, Baumann B, Leech SH, Fabbro D, Stahel RA, Zangemeister-Wittke U. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res. 2000;60:2805–09.
71. Kim KW, Mutter RW, Willey CD, Subhawong TK, Shinohara ET, Albert JM, Ling G, Cao C, Gi YJ, Lu B. Inhibition of survivin and aurora B kinase sensitizes mesothelioma cells by enhancing mitotic arrests. Int J Radiat Oncol Biol Phys. 2007;67:1519–25.
72. Talbot DC, Ranson M, Davies J, Lahn M, Callies S, André V, Kadam S, Burgess M, Slapak C, Olsen AL, McHugh PJ, de Bono JS, Matthews J, Saleem A, Price P. Tumor survivin is down-regulated by the antisense oligonucleotide LY2181308: a proof-of-concept, first-in-human dose study. Clin Cancer Res. 2010;16:6150–58.
73. Gurbuxani S, Xu Y, Keerthivasan G, Wickrema A, Crispino JD. Differential requirements for survivin in hematopoietic cell development. Proc Natl Acad Sci USA. 2005;102:11480–85.

74. Hansen JB, Fisker N, Westergaard M, Kjaerulff LS, Hansen HF, Thrue CA, Rosenbohm C, Wissenbach M, Orum H, Koch T. SPC3042: a proapoptotic survivin inhibitor. Mol Cancer Ther. 2008;7:2736–45.
75. Walker K, Padhiar M. AACR-NCI-EORTC-21st International Symposium. Molecular targets and cancer therapeutics-Part 1. IDrugs. 2010;13:7–9.
76. Sapra P, Wang M, Bandaru R, Zhao H, Greenberger LM, Horak ID. Down-modulation of survivin expression and inhibition of tumor growth in vivo by EZN-3042, a locked nucleic acid antisense oligonucleotide. Nucleosides Nucleotides Nucleic Acids. 2010;29:97–112.
77. Davis ME1, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD, Ribas A. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature. 2010;464:1067–70.
78. Satoh T1, Okamoto I, Miyazaki M, Morinaga R, Tsuya A, Hasegawa Y, Terashima M, Ueda S, Fukuoka M, Ariyoshi Y, Saito T, Masuda N, Watanabe H, Taguchi T, Kakihara T, Aoyama Y, Hashimoto Y, Nakagawa K. Phase I study of YM155, a novel survivin suppressant, in patients with advanced solid tumors. Clin Cancer Res. 2009;15:3872–80.
79. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer. 2003;3:401–10.
80. O'Connor DS, Schechner JS, Adida C, Mesri M, Rothermel AL, Li F, Nath AK, Pober JS, Altieri DC. Control of apoptosis during angiogenesis by survivin expression in endothelial cells. Am J Pathol. 2000;156:393–8.
81. Tolcher AW, Mita A, Lewis LD, Garrett CR, Till E, Daud AI, Patnaik A, Papadopoulos K, Takimoto C, Bartels P, Keating A, Antonia S. Phase I and pharmacokinetic study of YM155, a small-molecule inhibitor of survivin. J Clin Oncol. 2008;26:5198–203.
82. Giaccone G, Zatloukal P, Roubec J, Floor K, Musil J, Kuta M, van Klaveren RJ, Chaudhary S, Gunther A, Shamsili S. Multicenter phase II trial of YM155, a small-molecule suppressor of survivin, in patients with advanced, refractory, non-small-cell lung cancer. J Clin Oncol. 2009;27:4481–86.
83. Rödel C, Haas J, Groth A, Grabenbauer GG, Sauer R, Rödel F. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor. Int J Radiat Oncol Biol Phys. 2003;55:1341–47.
84. Chang C-C, Heller JD, Kuo J, Huang RCC. Tetra-O-methyl nordihydroguaiaretic acid induces growth arrest and cellular apoptosis by inhibiting Cdc2 and survivin expression. Proc Natl Acad Sci USA. 2004;101:13239–44.
85. Smolewski P. Terameprocol, a novel site-specific transcription inhibitor with anticancer activity. IDrugs. 2008;11:204–14.
86. Park R, Chang CC, Liang YC, Chung Y, Henry RA, Lin E, Mold DE, Huang RC. Systemic treatment with tetra-O-methyl nordihydroguaiaretic acid suppresses the growth of human xenograft tumors. Clin Cancer Res. 2005;11:4601–09.
87. Zhu X, Ma Y, Liu D. Novel agents and regimens for acute myeloid leukemia: 2009 ASH annual meeting highlights. J Hematol Oncol. 2010;3:17.
88. Khanna N, Dalby R, Connor A, Church A, Stern J, Frazer N. Phase I clinical trial of repeat dose terameprocol vaginal ointment in healthy female volunteers. SexTransm Dis. 2008;35:577–82.
89. Khanna N, Dalby R, Tan M, Arnold S, Stern J, Frazer N. Phase I/II clinical safety studies of terameprocol vaginal ointment. Gynecol Oncol. 2007;107:554–62.
90. Knorr DA, Bachanova V, Verneris MR, Miller JS. Clinical utility of natural killer cells in cancer therapy and transplantation. Semin Immunol. 2014;26:161–72.
91. Saji H, Song W, Furumoto K, Kato H, Engleman EG. Systemic antitumor effect of intratumoral injection of dendritic cells in combination with local photodynamic therapy cancer therapy. Clin Cancer Res. 2006;12:2568–74.
92. Sanlorenzo M, Vujic I, Posch C, Dajee A, Yen A, Kim S, Ashworth M, Rosenblum MD, Algazi A, Osella-Abate S, Quaglino P, Daud A, Ortiz-Urda S. Melanoma immunotherapy. Cancer Biol Ther. 2014;15:665–74.

93. Andersen MH, Sørensen RB, Schrama D, Svane IM, Becker JC, Thor Straten P. Cancer treatment: the combination of vaccination with other therapies. Cancer Immunol Immunother. 2008;57:1735–43.
94. Rohayem J, Diestelkoetter P, Weigle B, Oehmichen A, Schmitz M, Mehlhorn J, Conrad K, Rieber EP. Antibody response to the tumor-associated inhibitor of apoptosis protein survivin in cancer patients. Cancer Res. 2000;60:1815–17.
95. Schmidt SM, Schag K, Müller MR, Weck MM, Appel S, Kanz L, Grünebach F, Brossart P. Survivin is a shared tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells. Blood. 2003;102:571–76.
96. Casati C, Dalerba P, Rivoltini L, Gallino G, Deho P, Rini F, Belli F, Mezzanzanica D, Costa A, Andreola S, Leo E, Parmiani G, Castelli C. The apoptosis inhibitor protein survivin induces tumor-specific CD8+ and CD4+ T cells in colorectal cancer patients. Cancer Res. 2003;63:4507–15.
97. Idenoue S1, Hirohashi Y, Torigoe T, Sato Y, Tamura Y, Hariu H, Yamamoto M, Kurotaki T, Tsuruma T, Asanuma H, Kanaseki T, Ikeda H, Kashiwagi K, Okazaki M, Sasaki K, Sato T, Ohmura T, Hata F, Yamaguchi K, Hirata K, Sato N. A potent immunogenic general cancer vaccine that targets survivin, an inhibitor of apoptosis proteins. Clin Cancer Res. 2005;11:1474–82.
98. Pisarev V, Yu B, Salup R, Sherman S, Altieri DC, Gabrilovich DI. Full-length dominant-negative survivin for cancer immunotherapy. Clin Cancer Res. 2003;9:6523–33.
99. Schmitz M, Diestelkoetter P, Weigle B, Schmachtenberg F, Stevanovic S, Ockert D, Rammensee H, Rieber EP. Generation of Survivin-specific CD8+ T Effector Cells by Dendritic Cells Pulsed with Protein or Selected Peptides. Cancer Res. 2000;60:4845–49.
100. Siegel S, Wagner A, Schmitz N, Zeis M. Induction of antitumor immunity using survivin peptide-pulsed dendritic cells in a murine lymphoma model. Br J Haematol. 2003;122:911–14.
101. Friedrichs B, Siegel S, Andersen MH, Schmitz N, Zeis M. Survivin-derived peptide epitopes and their role for induction of antitumor immunity in hematological malignancies. Leuk Lymphoma. 2006;47:978–85.
102. Trepiakas R, Berntsen A, Hadrup SR, Bjørn J, Geertsen PF, Straten PT, Andersen MH, Pedersen AE, Soleimani A, Lorentzen T, Johansen JS, Svane IM. Vaccination with autologous dendritic cells pulsed with multiple tumor antigens for treatment of patients with malignant melanoma: results from a phase I/II trial. Cytotherapy. 2010;12:721–34.
103. Berntsen A, Trepiakas R, Wenandy L, Geertsen PF, thor Straten P, Andersen MH, Pedersen AE, Claesson MH, Lorentzen T, Johansen JS, Svane IM. Therapeutic dendritic cell vaccination of patients with metastatic renal cell carcinoma: a clinical phase 1/2 trial. J Immunother. 2008;31:771–80.
104. Weide B1, Pascolo S, Scheel B, Derhovanessian E, Pflugfelder A, Eigentler TK, Pawelec G, Hoerr I, Rammensee HG, Garbe C. Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients. J Immunother. 2009;32:498–507.
105. Wobser M, Keikavoussi P, Kunzmann V, Weininger M, Andersen MH, Becker JC. Complete remission of liver metastasis of pancreatic cancer under vaccination with a HLA-A2 restricted peptide derived from the universal tumor antigen survivin. Cancer Immunol Immunother. 2006;55:1294–98.
106. Wang R, Wang X, Li B, Lin F, Dong K, Gao P, Zhang HZ. Tumor-specific adenovirus-mediated PUMA gene transfer using the survivin promoter enhances radiosensitivity of breast cancer cells in vitro and in vivo. Breast Cancer Res Treat. 2009;117:45–54.
107. Macdonald IJ, Dougherty TJ. Basic principles of photodynamic therapy. J Porphyrins Phthalocyanines. 2001;5:105–29.
108. Ferrario A, Rucker N, Wong S, Luna M, Gomer CJ. Survivin, a member of the inhibitor of apoptosis family, is induced by photodynamic therapy and is a target for improving treatment response. Cancer Res. 2007;67:4989–95.

Chapter 8
Cellular Targets and Molecular Responses Associated with Photodynamic Therapy

Marian Luna, Angela Ferrario, Natalie Rucker, Emma Balouzian, Sam Wong, Sophie Mansfield and Charles J. Gomer

Abstract The effectiveness of photodynamic therapy (PDT) for treating solid tumors remains variable. Our research team has examined cellular and tissue responses associated with the use of PDT and we have observed increased expression of several prosurvival molecules that can modulate treatment efficacy. Specifically, angiogenic growth factors, inflammatory proteins, and anti-apoptotic molecules are often overexpressed following PDT-mediated oxidative stress. The relevance of PDT-induced expression of vascular endothelial growth factor (VEGF), cyclooxygenase 2 (COX-2), matrix metalloproteinases (MMPs), and survivin will be reviewed. In addition, our team has had a long-standing interest in the application of PDT to treat retinoblastoma (Rb), an intraocular pediatric eye tumor. We describe our initial preclinical and clinical ocular studies as well as our recent cellular and tissue responses to PDT in Rb cells and tumors. These data provide new information on possible reasons why earlier PDT procedures were only partially effective in treating Rb. We conclude with suggestions on how combined modality approaches using targeted therapy together with fractionated PDT may enhance outcomes for treated ocular tumors.

Keywords Cyclooxygenase · Cytotoxicity · Photodynamic therapy · Pro-survival · Retinoblastoma · Survivin · VEGF

Abbreviations

COX-2	Cyclooxygenase 2
HIF-1α	Hypoxia inducible factor-1 alpha
MMP	Matrix metalloproteinase
PDT	Photodynamic therapy
PH	Photofrin
Rb	Retinoblastoma
VEGF	Vascular endothelial growth factor

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Introduction

Understanding the basic mechanisms associated with therapeutic protocols usually increases the likelihood the procedure can be improved. Laboratories from around the world have focused their research on examining cellular and tissue responses following PDT in an effort to better identify the targets of treatment-induced cytotoxicity [1]. Other laboratories have focused their efforts on documenting photosensitizer pharmacokinetics and pharmacodynamics, evaluating light delivery and dosimetry parameters, as well as measuring reactive oxygen generation [1]. All of these studies have proven to be extremely valuable in understanding how cells and tissues respond to PDT. A growing number of research groups have also concentrated on analyzing the molecular responses associated with PDT. In this regard, our research team has examined PDT-inducible genes and proteins with a goal of better understanding possible mechanisms of treatment response and treatment-associated resistance [2]. This chapter will provide an overview of PDT research performed to treat Rb, our examination of PDT effects on the tumor microenvironment, and subsequent studies examining potential reasons for the sub-optimal clinical Rb PDT responses we observed. The chapter will conclude with suggestions on how Rb-mediated PDT may be more effective when combined with other targeted therapies.

Retinoblastoma and PDT

Retinoblastoma is the most common intraocular malignancy in childhood, affects 1 in 15,000 children, and accounts for 12% of infant cancers and 3% of all childhood malignancies [3]. There are approximately 8000 new cases of Rb diagnosed worldwide each year. Sixty percent of these ocular tumors occur as sporadic (unilateral) lesions and 40% are hereditary (bilateral) lesions. Children with hereditary Rb have a predisposition for additional primary cancers [3]. Therapy decisions for Rb depend on the size and location of the lesion(s) at the time of diagnosis and treatment options include enucleation for unsalvageable lesions and various localized procedures including external beam radiation therapy, scleral plaque radiation brachytherapy, combination chemotherapy including carboplatin, vincristine, and etoposide, focal laser therapy, single agent chemotherapy using melphalan, and cryotherapy [4]. Current procedures focus on maintaining useful vision, increasing cytotoxic tumor responses, and reducing side effects. Unfortunately, unacceptably high rates of tumor recurrences are still observed following treatment especially in children with bilateral disease [4].

Our ocular oncology research team was among the first to systematically evaluate PDT for the treatment of primary and recurrent intraocular Rb lesions. We hypothesized that PDT might be uniquely effective for treating Rb due in part to: (a) Rb lesions being visible to the ophthalmologist during treatment using standard ophthalmic equipment (b) Rb lesions being uniformly non-pigmented and this would enhance light penetration and light distribution within the lesion and (c) Rb lesions being amenable to standard laser treatments by ophthalmologists.

The effectiveness of our original research program was due, in large part, to the diverse and complementary skills and experience of the individuals assembled for the project. Experienced and enthusiastic ophthalmologists, biomedical engineers, laser specialists, and translational radiation biologists interacted in a collaborative effort to move a novel concept from the laboratory bench to the bedside.

A variety of preclinical studies were performed using Hematoporphyrin Derivative, a precursor to Photofrin (PH), as the photosensitizer. The central goal of these studies was to move ocular PDT into the clinic. Initial experiments were performed using an orthotopic human Rb ocular tumor model in athymic mice to document the photosensitizer localization properties [5]. The administered photosensitizer was found to be taken up in the transplanted ocular lesions. The mutagenic potential of PDT was directly compared to ionizing radiation since ocular radiation therapy was known to play a major role in the induction of secondary malignancies of the orbit, which were within the treatment field [6]. Experiments were also performed to compare the efficiency of ionizing radiation and PDT to induce malignant cell transformation [7]. In these two studies, PDT was observed to be an extremely weak mutagen and carcinogen when directly compared to ionizing radiation. Additional studies examined acute normal ocular tissue toxicity in a rabbit eye model and confirmed that the ocular PDT response was very localized and predictable [8]. Ocular toxicity was next examined in rabbit eyes with transplanted tumors and again PDT-associated toxicity was localized to treatment sites [9]. Long-term ocular toxicity, and photosensitizer distribution within normal ocular structures and malignant tumors transplanted to the rabbit eye, were also evaluated [10]. Ocular tissue toxicity remained within the treatment field and significant photosensitizer levels accumulated within the transplanted tumor tissue. Results of these preclinical studies allowed us to move our program forward and a limited clinical study was performed on children with Rb. The initial ocular tumor responses following PDT were positive but recurrences were observed in all cases [11]. At the same time that our PDT studies were being performed, several ocular oncology groups, including ours, were also examining the use of a combined chemotherapy protocol for Rb. These Rb chemotherapy studies resulted in improved long-term responses when compared to other procedures, including PDT [12]. Therefore, our ocular PDT studies were discontinued at that time. However, mechanistic PDT studies continued in our laboratory with goals of understanding treatment response at a molecular and tissue levels, and of determining how to improve PDT responsiveness. Our initial ocular PDT studies had an important clinical spinoff. Specifically, the clinical application of laser-mediated PDT for treating macular degeneration used our preclinical ocular toxicology data and ocular tissue response data in moving this non-malignant PDT treatment forward [13].

PDT and the Tumor Microenvironment

Our research team and other laboratories have demonstrated that PDT-mediated oxidative stress induces expression of a variety of angiogenic and pro-survival molecules within the tumor microenvironment [14]. These molecules include

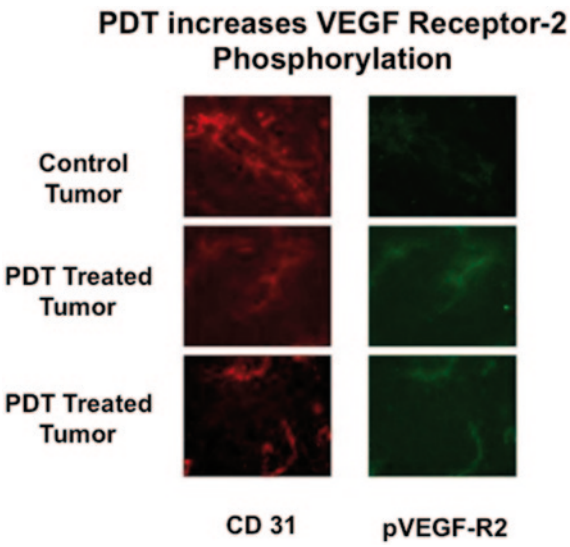

Fig. 8.1 *PDT* induces increased expression of the phosphorylated (activated) form of the VEGF receptor-2 (VEGFR2). Dual staining using antibodies to *CD31* and *pVEGFR2* show mouse mammary tumor vessels (CD-31, *red* staining) and phosphorylated VEGF receptor-2 (*pVEGFR2*, *green* staining). Increases in *green* staining are observed following PDT. Tumor samples were obtained 1 h after Photofrin mediated PDT (48 J/sq.cm)

vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), matrix metalloproteinases (MMPs), and survivin. Preclinical data from our laboratory also indicated that pharmacologic inhibitors targeting these molecules could improve treatment efficacy [2]. Results from studies described below led us to reexamine our ocular PDT studies in the context of possible mechanisms for the tumor recurrences we observed.

Our research team demonstrated that PDT treatments induce the expression of vascular endothelial growth factor (VEGF), a molecule that stimulates blood vessel growth [15]. This molecule plays an important role in tumor recurrences following therapy. Our team also showed that PDT induces expression of the transcription factor HIF-1α in treated tumors and this factor is known to be a primary inducer of VEGF expression [15]. VEGF is known to originate from both tumor cells and the host stromal cells. We used a human mammary carcinoma transplanted subcutaneously in mice to demonstrate that VEGF expressed following PDT originated primarily from the tumor since human VEGF was detected within the tissue lysates [16]. Interestingly, we also observed that PDT induced an increase in the expression of the activated (phosphorylated) form of the VEGF receptor-2 on blood vessels within the treatment field (Fig. 8.1). In an additional study, we documented that tumor-bearing mice treated with a combination of PDT and non-specific anti-angiogenic agents, IM862 and EMAP-II, exhibited improved tumoricidal responses as measured by increased time to recur and increased cures when compared to individual treatment regimens [15]. We also used a therapeutic VEGF specific antibody, bevacizumab or Avastin, in order to examine how a clinically relevant targeted anti-angiogenic inhibitor interacts with PDT [16]. Using a human Kaposi's sarcoma tumor growing in athymic mice we were able to demonstrate the effectiveness of this combined modality approach in improving PDT responsiveness. These discoveries

have led to the current examination of combined PDT and VEGF inhibitor therapy to treat ocular macular degeneration and solid tumors.

COX-2 is a rate-limiting enzyme in the metabolism of arachidonic acid to prostaglandins [17]. Expression of COX-2 in the tumor microenvironment is associated with a poor prognosis. We documented that PDT is a strong inducer of COX-2 expression within tumor cells grown in culture and in solid tumors growing in mice [18]. PDT activates COX-2 at a transcriptional level. A variety of experiments, using COX-2 promoter reporter constructs with mutated transcription elements, transcription factor binding assays, kinase phosphorylation analysis, and cell exposure to signaling molecule inhibitors demonstrated that multiple protein kinase cascades were activated by PDT and that the p38 MAPK signaling pathway and CRE-2 binding were involved in COX-2 expression following PDT [19].

COX-2 induced by PDT was found to be biologically active. Prostaglandin E_2 was up regulated in PDT-treated cells and tumors overexpressing COX-2 and inhibitors of COX-2 (NS-398 and celecoxib) attenuated PGE_2 levels [20]. A major portion of our COX-2 research examined the tumoricidal effects of combining PDT with COX-2 inhibitors. We found that PDT combined with celecoxib increased cytotoxicity and apoptosis in tumor cells. We also observed enhanced long-term survival in tumor-bearing mice treated with the combination of PDT and celecoxib compared to responses for single agent treatments. This combination of PDT and celecoxib resulted in decreased levels of PGE_2, VEGF, IL-1β and TNF-α within tumor lysates [21]. Normal tissue responses, defined as skin photosensitization, were not altered when PDT was combined with a COX-2 inhibitor.

MMPs are important modulators of the tumor microenvironment and are involved in tumor angiogenesis, growth, invasion and metastasis. We reported that PDT-treated tumors exhibit strong expression of MMPs and the extracellular matrix metalloproteinase inducer (EMMPRIN) along with concomitant decreases in expression of the tissue inhibitor of matrix metalloproteinase (TIMP-1) [22]. Immunohistochemical analysis indicated that infiltrating inflammatory cells and endothelial cells were the source of MMP-9 expression. Administration of a broad-spectrum MMP inhibitor, Prinomastat, improved PDT-mediated tumor response without affecting normal skin photosensitization.

Additional studies from our laboratory demonstrated that PDT induced the expression and phosphorylation of survivin [23]. This molecule is a member of the inhibitor of apoptosis family of proteins and inhibits caspase 9, blocks apoptosis, and modulates the cell cycle. We examined whether PDT efficacy was reduced by treatment-mediated expression and phosphorylation of survivin and if targeting the survivin pathway could increase PDT responsiveness. Proper maturation of survivin requires that this protein binds to the 90-kDa heat shock protein. A geldanamycin-based HSP-90 inhibitor was used to block survivin maturation and function. We observed decreased expression of phospho-survivin and increased apoptosis in cells when PDT was combined with the geldanamycin derivative [24]. Results from these experiments confirmed that targeting survivin, and possibly other hsp-90 client proteins, improves PDT responsiveness.

Mechanisms of action for PDT include increased expression of regulators of angiogenic pathways including growth factors, metalloproteinases, cytokines, prostanoids, and signaling molecules. As with chemotherapeutic regimens, it is likely that PDT effectiveness can be improved by employing combined modality approaches involving pharmaceuticals targeting the tumor microenvironment and/or tumor cell death pathways [2, 21].

PDT Effects on Rb Cells and Tumors

Experiments examining the tumor microenvironment have identified a variety of pro-survival molecules that may have played a role in the suboptimal responses associated with our initial clinical Rb-PDT study. We, therefore, have initiated studies to evaluate the responses of Rb cell and tissues following PDT. Figures 8.2 and 8.3 show that pH-mediated PDT induces increased expression of both HIF-1α and VEGF in human Rb tumors growing in nude mice. The second-generation photosensitizer, benzoporphyrin derivative (Visudyne), also is associated with increased expression of the VEGF and HIF-1α in Rb tumors. Figure 8.3 also shows that the majority of PDT-induced VEGF originates from the tumor (human VEGF) and not from the host stromal cells (mouse VEGF). These results suggest that single PDT treatments could activate an angiogenic response in Rb tumor tissue and that anti-VEGF or other anti-angiogenic procedures might be beneficial when PDT is used to treat this ocular tumor [15, 16].

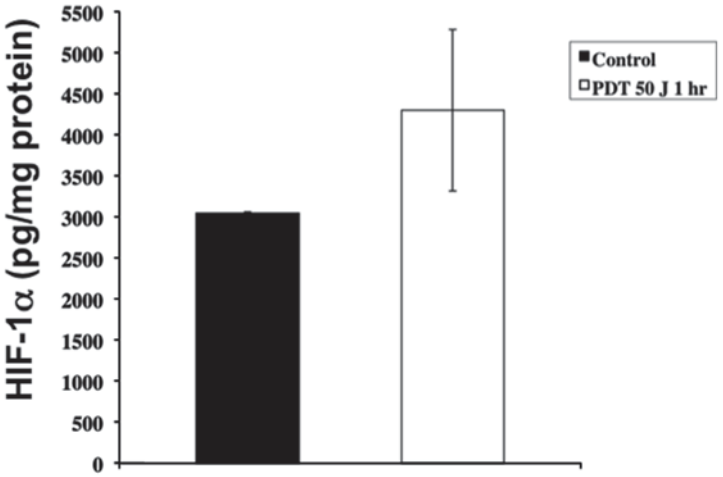

Fig. 8.2 PDT treatment of human Y-79 retinoblastoma tumors transplanted in the flank of athymic mice induces *HIF-1α* expression. Tumors were collected 1 h after PDT (5 mg/kg PH, 50 J/sq.cm) and evaluated by Western immunoblot. $N = 2–3$

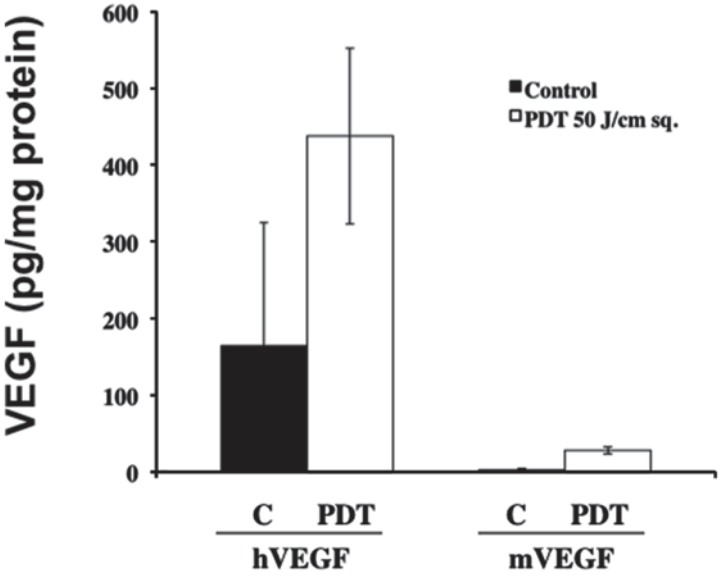

Fig. 8.3 Differential expression patterns of human VEGF (*hVEGF*) and mouse VEGF (*mVEGF*) for PDT-treated human Y-79 Rb tumors growing in athymic mice. Tumor lysates were collected from non-treated control mice and 24 h after PDT (5 mg/kg PH, 50 J/sq.cm.). Tumor lysates were evaluated using commercial VEGF ELISA kits. $N = 4–6$

Basal levels of COX-2 have been reported in Rb tumors but over expression of this enzyme in untreated clinical Rb samples is not common [17]. We measured the enzymatic product of COX-2 enzymatic activity (metabolic synthesis of PGE_2) in control and PDT-treated human Rb tumors subcutaneously transplanted in nude mice. Figure 8.4 shows PDT resulted in 3–4 fold increases in PGE_2 levels in Rb lysates within 24 h of treatment. The strong tumoricidal enhancement of celecoxib in our published tumor microenvironment studies suggests clinical Rb-PDT may benefit including a COX-2 inhibitor with PDT [18–20].

Matrix metalloproteinases play multiple roles within the tumor microenvironment and over expression of members of this family of proteinases can have negative consequences for therapeutic procedures used to treat malignancies [22]. Experiments were performed to determine if Rb tumors treated with PDT exhibited effects related to expression of MMPs. Specifically, we examined whether EMMPRIN levels with Rb tumor tissue were modified. Figure 8.5 shows that human Rb tumors growing in nude mice have a detectable background level and that EMMPRIN levels more than double within 24 h of PDT. Additional experiments will be needed in order to confirm that MMPs can play a role in RB-mediated PDT responses. However, our observation that PDT induces EMMPRIN expression in Rb tumors strongly suggests that MMPs may be involved in modulating Rb tumors following PDT.

Survivin is a pro-survival molecule expressed in multiple human malignancies. Our studies, described in the previous section, show that attenuating survivin

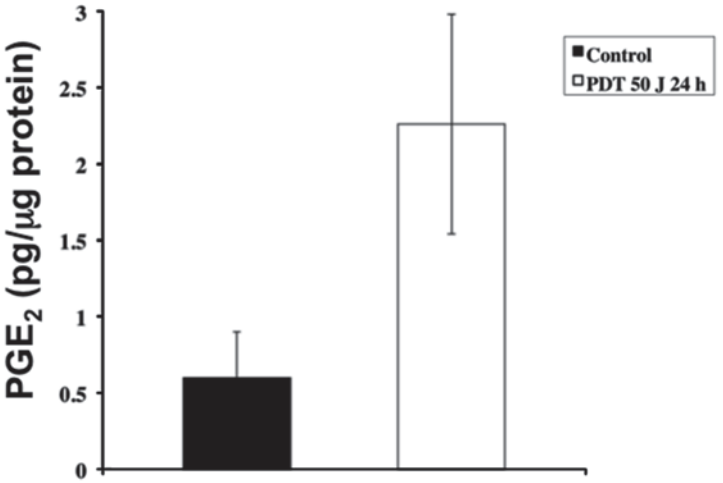

Fig. 8.4 PDT induces increased expression of PGE_2 in treated human Y-79 Rb tumors. Tumor lysates were collected from non-treated control tumors and 24 h after PH-PDT (5 mg/kg PH, 50 J/sq.cm). Expression levels of PGE_2 were determined using a commercial EIA kit. $N=3$

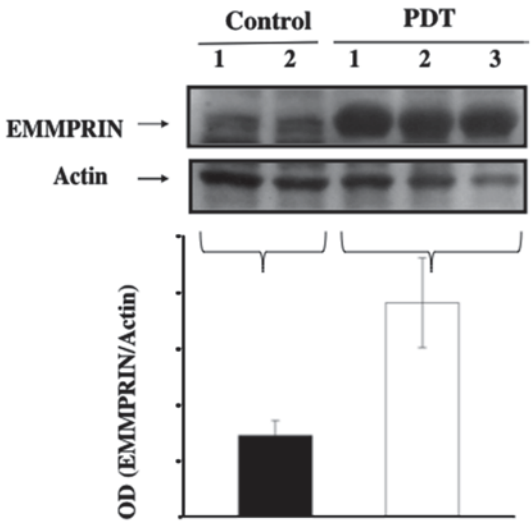

Fig. 8.5 PDT induces overexpression of EMMPRIN in human Y-79 Rb tumors. Tumor lysates were collected 24 h following PDT (5 mg/kg PH, 50 J/sq.cm) and EMMPRIN levels were determined by Western immunoblot. $N=2–3$

expression can improve PDT responses in mouse tumors [23]. We have now observed that PDT treatment of human Rb cells can result in an increase in survivin expression. These results suggest that Rb-PDT may be improved by targeting survivin. Additional experiments are examining the Rb tumor response to PDT and whether survivin is upregulated following treatment. Studies are also being performed to determine whether a combined approach with PDT and anti-survivin therapy can improve tumoricidal activity.

Conclusion

A detailed understanding of the mechanisms of actions for PDT is needed in order to maximize therapeutic results. Previous studies demonstrated that PDT can induce the expression of multiple genes and proteins having pro-survival activity. We also documented that ocular PDT for Rb was safe but that long-term tumoricidal activity had not been obtained. New attempts to use PDT in the armamentarium of therapeutic options for Rb may prove to be successful if past lessons can be incorporated into new PDT treatment approaches. In order for PDT to have a future in treating Rb there will need to be engaged ophthalmologists working with tumor biologists and biomedical engineers. A more efficient and rapidly clearing photosensitizer, such as Visudyne, could provide for same-day procedures, minimal side-effects associated with skin photosensitization, and a greatly decreased need for young patients to stay indoors for extended time periods. The fact that PDT can be repeated multiple times with minimal or no negative side effects may also be a significant benefit especially when combined with a rapidly clearing photosensitizer. Our on going studies continue to suggest that combined modality protocols, using PDT and inhibitors to pro-survival molecules such as VEGF, COX-2, MMPs or survivin, could also improve overall Rb treatment responsiveness. Finally, one of the more important considerations for moving Rb-PDT forward will be to incorporate a more realistic treatment regimen. Figure 8.6 shows a schematic for ocular PDT involving a fractionated approach. Using multiple PDT treatments could allow for a more deliberate and effective means of decreasing Rb size than the previously used single treatment protocol.

No conflict statement "No potential conflicts of interest were disclosed."

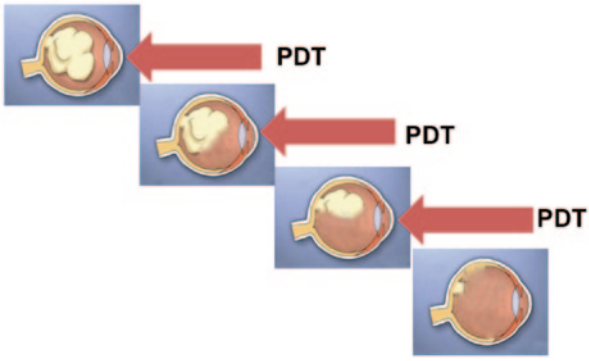

Fig. 8.6 Schematic of possible future ocular PDT treatment regimens for Rb. Large Rb lesions could be treated with fractionated PDT. Instead of trying to complete PDT with one treatment, it may be more efficacious to perform multiple treatments with the goal of reducing the size, not eliminating, the Rb lesions during each procedure. Such a regimen could also decrease potential normal ocular tissue damage within the treatment site and allow for increased tumoricidal activity. This procedure will be most effective using a rapidly clearing photosensitizer

References

1. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M, Moan J, Peng Q. Photodynamic therapy. J Natl Cancer Inst. 1998;90:889–905.
2. Gomer CJ. Induction of prosurvival molecules during treatment; rethinking therapy options for photodynamic therapy. J Natl Comp Cancer Net. 2012;10:35–9.
3. DiCiommo D, Gallie BL, Bremner R. Retinoblastoma: the disease, gene and protein provide critical leads to understand cancer. Semin Cancer Biol. 2000;10:255–69.
4. Chintagumpala M, Chevez-Barrios P, Paysse EA, Plon SE, Hurwitz R. Retinoblastoma: review of current management. Oncologist. 2007;12:1237–46.
5. Gomer CJ, Rucker N, Mark C, Benedict WF, Murphee AL. Tissue distribution of 3H-hematoporphyrin derivative in athymic "nude" mice heterotransplanted with human retinoblastoma. Invest Ophthalmol Vis Sci. 1982;22:118–20.
6. Gomer CJ, Rucker N, Banerjee A, Benedict WF. Comparison of mutagenicity and induction of sister chromatid exchange in Chinese hamster cells exposed to hematoporphyrin derivative photoradiation, ionizing radiation or ultraviolet radiation. Cancer Res. 1983;43:2622–7.
7. Gomer CJ, Rucker N, Murphree AL. Transformation and mutagenic potential of porphyrin photodynamic therapy in mammalian cells. Int J Radiat Biol. 1988;53:651–9.
8. Gomer CJ, Doiron DR, Jester JV, Szirth BC, Murphree AL. Hematoporphyrin derivative photoradiation therapy for the treatment of intraocular tumors: examination of acute normal ocular tissue toxicity. Cancer Res. 1983;43:721–7.
9. Gomer CJ, Doiron DR, White L, Jester JV, Dunn S, Szirth BC, Razum NJ, Murphree AL. Hematoporphyrin derivative photoradiation induced damage to normal and tumor tissue of the pigmented rabbit eye. Curr Eye Res. 1984;3:229–37.
10. Gomer CJ, Jester JV, Razum NJ, Szirth BC, Murphree AL. Photodynamic therapy of intraocular tumors: examination of hematoporphyrin derivative distribution and long term damage in rabbit ocular tissue. Cancer Res. 1985;45:3718–25.
11. Murphree AL, Cote M, Gomer CJ. The evolution of photodynamic therapy techniques in the treatment of intraocular tumors. Photochem Photobiology. 1987;46:919–23.
12. Murphree AL, Villablanca JG, Deegan WF, sato JK, Malogolowkin M, Fisher A, Parker R, Reed E, Gomer CJ. Chemotherapy plus local treatment in the management of intraocular retinoblastoma. Arch Ophthalmology. 1996;114:1348–56.
13. Augustin A, Puls S, Offermann I. Triple therapy for choroidal neovascularization due to age-related macular degeneration. Retina. 2007;27:133–40.
14. Gomer CJ, Ferrario A, Luna M, rucker N, Wong S. Photodynamic therapy: combined modality approaches targeting the tumor microenvironment. Lasers Surg Med. 2006;38:516–21.
15. Ferrario A, von Tiehl KF, Rucker N, Schwartz MA, Gill PS, Gomer CJ. Anti-angiogenic treatment enhances photodynamic therapy responsiveness in a mouse mammary carcinoma. Cancer Res. 2000;60:4066–69.
16. Ferrario A, Gomer CJ. Avastin enhances photodynamic therapy treatment of Kaposi's sarcoma in a mouse tumor model. J Environ Pathol Toxicol Oncol. 2006;25:251–59.
17. de Souza Filho JP, Correa ZM, Marshall JC, Anteka E, Coutinho AB, Martinis MC, Burnier MN. The effect of a selective cyclooxygenase-2 inhibitor on the proliferation rate of retinoblastoma cell lines. Eye. 2005;20:598–601.
18. Ferrario A, von Tiehl KF, Wong S, Luna M, Gomer CJ. Cyclooxygenase-2 inhibitor treatment enhances photodynamic therapy mediated tumor response. Cancer Res. 2002;62:3956–61.
19. Luna M, Wong S, Ferrario A, Gomer CJ. Cyclooxygenase-2 expression induced by Photofrin photodynamic therapy involves the p38 MAPK pathway. Photochem Photobiol. 2008;84:509–14.
20. Ferrario A, Fisher A, Rucker N, Gomer CJ. Celecoxib and NS-398 enhance Photodynamic therapy by increasing in-vitro apoptosis and decreasing in-vivo expression of inflammatory and angiogenic molecules. Cancer Res. 2005;65:9473–79.

21. Ferrario A, Gomer CJ. Targeting the tumor microenvironment using PDT combined with inhibitors of COX-2 or VEGF. Methods Mol Biol. 2010;635:121–32.
22. Ferrario A, Chantrain CF, von Tiehl KF, Buckley S, Rucker N, Shalinsky DR, Shimada H, DeClerck YA, Gomer CJ. The matrix metalloproteinase inhibitor Prinomastat enhances photodynamic therapy responsiveness in a mouse tumor model. Cancer Res. 2004;64:2328–32.
23. Ferrario A, Rucker N, Wong S. Survivin, a member of the inhibitor of apoptosis family, is induced by photodynamic therapy and is a target for improving treatment response. Cancer Res. 2007;67:4989–95.
24. Ferrario A, Gomer CJ. Targeting the HSP-90 heat shock protein improves photodynamic therapy. Cancer Lett. 2010;289:188–94.

Part IV
Approaches to Reduce the Multi-Drug Resistance of Tumor Cells by PDT

Chapter 9
How Nanoparticles Can Solve Resistance and Limitation in PDT Efficiency

Magali Toussaint, Muriel Barberi-Heyob, Sophie Pinel and Céline Frochot

Abstract PDT efficiency photosensitizers can be improved by different ways: development of targeted photosensitizers that also present rapid clearance from normal tissues, photosensitizers that own better photophysical properties (such as absorption in the red to use light that can better penetrate tissue, limited photobleaching), photosensitizers whose pharmacokinetics matched to the application, improve light equipments and the selective delivery of the activating light. In this chapter, our aim is to address how nanoparticles could be one of the solutions to improve PDT efficiency and to bypass the *phenomena* of resistance and limitations to PDT. We will describe how the use of nanoparticles can be positive for activation system, biodistribution properties, tumor selectivity by selecting judicious molecular and cellular targets.

Keywords Cellular targeting · Nanoparticle · Photodynamic therapy resistance · Photosensitizer delivery

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Abbreviations

5-ALA	5-aminolevulinic acid
AIPc	Aluminium pthalocyanine
AuNP	Gold nanoparticle
BDSA	9,10-bis (4′-(4″-aminostyryl)styryl)anthracene
DOTA	1,4,7,10-tetraazacycloDOdecane-Tetraacetic Acid
DPBF	1,3-DiPhenilisoBenzoFuran
DTPA	Diethylene Triamine Pentaacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor
FDA	Food Drug Administration
FITC	Fluorescein IsoThioCyanate
FRET	Förster Resonance Energy Transfer
HPPH	2-(1-Hexyloxyethyl)-2-devinyl PyroPheophorbide-a
Hy	Hypericin
ICG	IndoCyanine Green
LDL	Low Density Lipoprotein
MB	Methylene Blue
MRI	Magnetic Resonance Imaging
*m*THPC	m-TetraHydroxyPhenylChlorin
MTT	bromure de 3-(4,5-diMethylThiazol-2-yl)-2,5-diphenyl Tetrazolium
NRP-1	NeuRoPilin-1
PAA	PolyAcrylAmide
Pc4	Phtalocyanine
PDT	Photo Dynamic Therapy
PEBBLE	Photonic Explorer for Bianalysis with Biologically Localized Embedding
PEG	PolyEthylene Glycol
PEGDMA	Poly(Ethylene Glycol) Di MethAcrylate
PEG-PCL	Poly (Ethylene Glycol)-block-PyreoPheophorbide a
PUNPS	Photon Up-Converting Nanoparticles
RGD	ArginylGlycylAspartic acid
ROS	Reactive Oxygen Species
SLN	Solid Lipid Nanoparticle
SLPDT	Self-Lighting PDT
TMPyP	5,10,15,20-Tetrakis(1-Methyl 4-Pyridino)Porphyrin tetra(p-toluensulfonate)
TPA	Two Photon Absorption
UCNP	UpConversion NanoPlateform
VEGF	Vascular Endothelial Growth Factor
VTP	Vascular Targeted Photodynamic therapy

Introduction

Photodynamic Therapy (PDT) was the first drug-device combination approved by the US Food and Drug Administration (FDA) almost two decades ago, but even so remains underutilized clinically. In principle, PDT is a simple adaptation of chemotherapy that consists of three essential components: photosensitizer, light, and oxygen. Each of these parameters may cause problems of resistance to treatment. Researchers continue to study ways to improve the effectiveness of PDT in different fields. For example, clinical trials and research studies are under way to evaluate the use of PDT for cancers of the brain, skin, prostate, cervix, and peritoneal cavity (the space in the abdomen that contains the intestines, stomach, and liver). Other research is focused on the development of photosensitizers that are more powerful, especially more specifically targeted to cancer cells, and that can be activated by light that can better penetrate tissue and treat deep or large tumors. Researchers are also investigating ways to improve equipment and the selective delivery of the activating light.

The selectivity of PDT is indeed achieved by an increased photosensitizer accumulation within the tumor as compared to normal tissues and by the fact that illumination is limited to a specified location. Several possible mechanisms of selective photosensitizer retention within tumors include greater proliferative rates of neoplastic cells, a lack of or poor lymphatic drainage, high expression of LDL receptors on tumor cells (many photosensitizers bind to LDL), low pH (which facilitates cellular uptake), increased vascular permeability, and/or tumor infiltration by macrophages that are efficient traps for hydrophobic photosensitizers. Therefore, selectivity is derived from both the ability of a useful photosensitizer to localize in neoplastic lesions and the precise delivery of light to the treated sites. General guidelines were suggested for the properties desired for an ideal photosensitizer such as high absorption for maximum photons penetration in the tissue; stability against rapid photobleaching in order to retain efficacy during treatment or, alternatively, pharmacokinetics matched to the application (*e.g.* rapid clearance for vascular targeting); selective uptake in target tissues/tissue structures and relatively rapid clearance from normal tissues, minimizing phototoxic side effects (ideally, measured in hours and days, not weeks). The tissue or vascular half-life should be amenable to the clinical application.

Considering these guidelines, what are the main limitations of *in vivo* PDT? Resistant cell lines have been studied by several investigators. This has been achieved by looking at either the PDT susceptibility of cells resistant to various other treatment modalities or at the nature of PDT-induced *in vitro* resistance. The use of various cell lines, photosensitizers, irradiation protocols, and light/dark applications has made it difficult to reach general conclusions. Finally, the information and reflexion available regarding PDT-induced *in vivo* resistance is somewhat more limited.

In this chapter, our aim will be to address how nanoparticles could be one of the solutions to bypass the *phenomena* of resistance and limitations to PDT by improving the activation system, biodistribution properties, tumor selectivity and by selecting judicious molecular and cellular targets (Fig. 9.1).

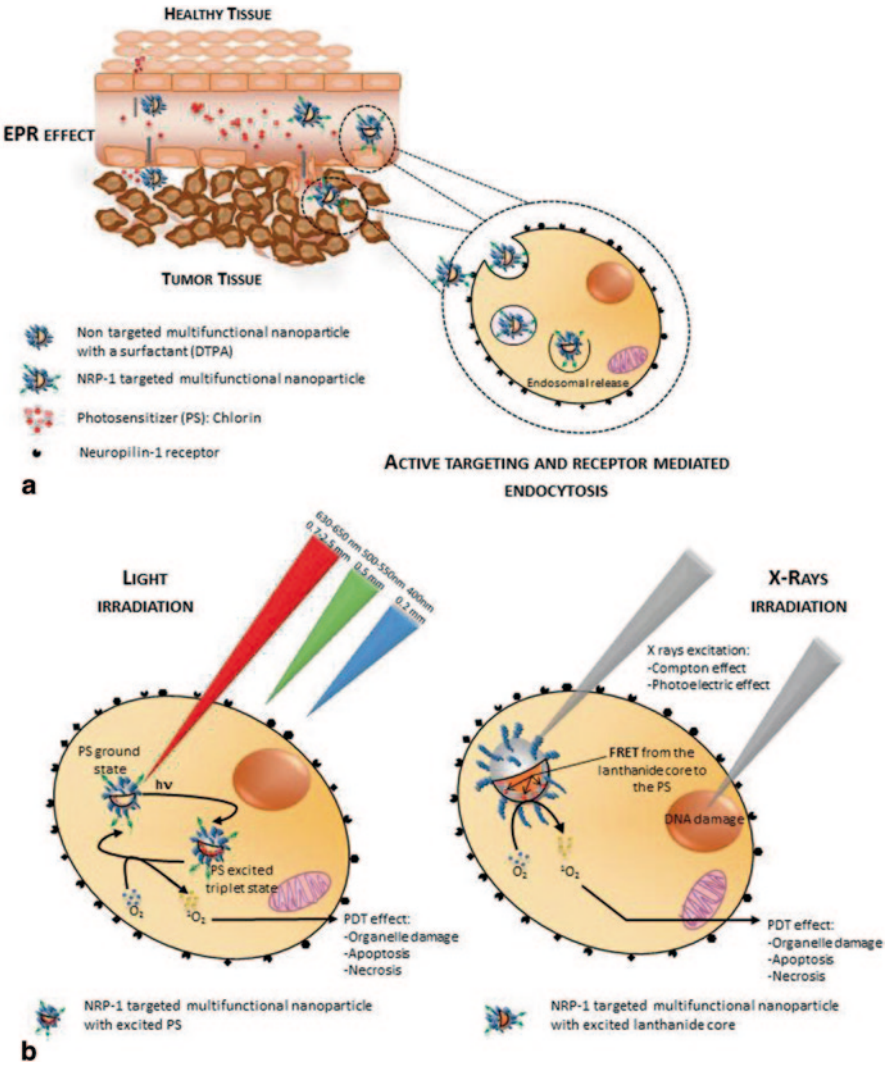

Fig. 9.1 **a** *Left*. EPR effect-mediated infiltration of PS alone and non targeted nanoparticle in the interstitial cell space of healthy and tumor cells. *Right*. Active targeting-mediated receptor endocytosis of NRP-1 targeted nanoparticle in tumor cells and neoangiogenic cells overexpressing NRP-1 receptor. **b** *Left*. Light irradiation activates the PS into nanoparticles, the PS changes from a ground state to an excited triplet state. An energy transfer to O_2 or an oxidoreduction reaction with cell substrate leads to the formation of ROS and PDT effects. *Right*. Thanks to X rays excitation of the lanthanide core and FRET to the PS, classical PDT effects are triggered

Improvement of Activation System by Using Nanoparticles with Specific Photophysical Properties

PDT is a non or minimally invasive treatment therapy relying on the ability of a photosensitizer to generate, upon activation with light, free radicals or singlet oxygen. Usually, laser light with an optical fiber with an adapted light diffuser are used to irradiate the tumor tissue. Compared to radiotherapy by ionizing radiation, the light irradiation used in PDT is less energetic, harmless and non-mutagenic. When activated by light irradiation, the photosensitizer interacts with molecular oxygen to produce a cytotoxic, short-lived species. PDT has already shown great promise to improve treatment options for cancer patients. Unfortunately, only near infrared light in the range of 700–1100 nm can penetrate deeper into the tissue because most tissue chromophores, absorb weakly in the near infrared window and most available photosensitizers have absorption bands at wavelengths shorter than 700 nm. Thus, one of the most relevant remaining limitations and resistance of PDT effect is the limited light penetration of tissues. Two-photon absorption (TPA)-induced excitation of the photosensitizer appears as a promising approach for increasing light penetration by using two photons of lesser energy (higher wavelength) to produce an excitation that would normally be produced by the absorption of a single photon of higher energy (lower wavelength). TPA-induced excitation of molecules is one of the promising approaches to increase light penetration as it avoids wasteful tissue absorption or scattering and allows a deeper penetration of light into the tissue. It is a nonlinear process involving the absorption of two photons whose combined energy is sufficient to induce a molecular transition to an excited electronic state. In this aim, various compounds and molecules with increased TPA cross-sections have been suggested. Various molecules with relatively large TPA cross-sections have been designed mainly in a nanoplatform design; Gao et al. described the elaboration of 5,10,15,20-tetrakis(1-methyl 4-pyridino)porphyrin tetra(p-toluenesulfonate) (TMPyP), encapsulated in a polyacrylamide-based nanoparticles. Infrared two-photon nanoplatform phototoxicity was demonstrated *in vitro* by modulating the time exposure to light [1]. Kim et al. also described the synthesis of organically modified silica nanoparticles co-encapsulating pyropheophorbide and an excess amount of 9,10-bis (4′-(4″-aminostyryl)styryl)anthracene (BDSA), a highly two-photon active molecule as a donor [2]. Pyropheophorbide absorption in nanoparticles had significant overlap with the fluorescence of BDSA aggregates which enabled an efficient energy transfer through FRET (Förster Resonance Energy Transfer) mechanisms. After indirect two-photon excitation (850 nm), the authors demonstrated that the energy of the near-infra red light was efficiently up-converted by BDSA aggregates to excite HPPH followed by $^1O_2$ formation, leading to drastic changes of the cell morphology [2]

Another approach has been published by Zhang et al. [3] describing a new concept based on photon up-converting nanoparticles (PUNPs). Up-conversion, in which excitation light at a longer wavelength produces emission at a shorter wavelength is very promising since with PUNPs, it becomes possible to excite the

photosensitizers in the near infra-red. The PUNPs used are the most efficient photon-up converting phosphors.

Once irradiated by X-rays, the scintillator core can emit a visible light and activates a photosensitizer that generates singlet oxygen ($^1O_2$) for tumor destruction. Such nanoparticles combine both PDT (high and reactive oxygen species (ROS) generation) and enhanced radiation therapy (high-Z core). Some nanoparticles are based on quantum dots ($Z \approx 40$) and FRET energy transfer to the photosensitizer. In this context, the nanoparticles containing elements with high atomic number Z are capable to intensify the production of secondary electrons suitable for enhancing radiation therapy. Besides their high Z, gadolinium-based nanoparticles offer an innovative approach due to their capacity to act as powerful contrast agents in Magnetic Resonance Imaging (MRI). We should also mention the potential of using nanoparticles for self-lighting PDT (SLPDT) using X-rays as an activation system. The advantages of SLPDT compared to PDT alone are: (i) light delivery is not necessary; (ii) radiotherapy and PDT are combined and activated by a single source, leading to a simple and less expensive system than PDT alone or both therapies used simultaneously; and (iii) similar therapeutic results can be achieved using lower radiation doses. This will allow a reduction of radiation dose, since even when the X-ray source will be off, the PDT will still be active. This approach provides a simple, convenient and inexpensive *in vivo* light source for PDT. Chen and Zhang designed a new PDT agent system combining radiation and PDT, in which scintillation luminescent nanoparticles are attached to the photosensitizers [4]. Upon exposure to ionizing radiation such as X-rays (with no limit in tissue penetration), scintillation luminescence will emit from the nanoparticles and activate a photoactivatable agent. Very recently, we published our results concerning the X-ray excitation of a Tb_2O_3 nanoparticle coated with a polysiloxane layer in which it is covalently linked to a porphyrin [5]. We proved that it is possible to produce singlet oxygen so the PDT effect is achieved after X-ray excitation of the nanoparticles' core. The proposed nanohybrid system could be a good candidate for photodynamic effects in deep tissue. Cooper et al. [6] demonstrated an energy transfer between chlorin e6 and cerium fluoride and cerium doped lanthanum fluoride nanoparticles through steady-state and time-resolved photoluminescencec spectroscopy. The authors claim that the next step will be the excitation of these nanoparticles by ionizing irradiation.

Improvement of Biodistribution Properties

Most photosensitizers used in clinic or in preclinical development are hydrophobic and tend to aggregate in an aqueous environment, while the monomer state is required to maintain their photophysical, chemical, and biological properties for efficient PDT [7]. This limits their delivery and photosensitizing efficiency. Additionally, an insufficient affinity of most photosensitizers to tumor sites also results in some damage to the normal tissue following PDT [8]. In order to circumvent these issues, drug delivery systems for photosensitizers are required to achieve selective

delivery to tumor sites. During the continuous search for improving the efficacy and safety of PDT, nanoparticles with high loading capacity and flexibility to accommodate photosensitizers with variable physicochemical properties came into focus. The design of an efficient photosensitizer delivery system requires the knowledge of the drug physicochemical properties, its specific intended therapeutic application and the characteristics of interaction of the delivery system with the biological structures. One of the most important drug properties to consider is the payload. The use of unreasonably high quantities of the carrier can lead to problems of carrier toxicity, metabolism, elimination, or/and biodegradability. Additional properties such as stability, solubility, size, molecular weight, and charge are also important, as they govern the means to entrap the photosensitizer into a delivery system.

There are several general advantages of using delivery systems for cancer therapy: they can carry a large payload of photosensitizer molecules and protect them from degradation and increase drug water solubility. For example, a 110 nm liposome can contain at about 10,000 *m*THPC molecules [9]. The improvement of pharmacokinetics and biodistribution parameters compared to a free drug is a particular strength using a drug livery system such as liposomes [10]. Generally, the drug clearance decreases, the volume of distribution decreases, and the area under the time *versus* concentration curve increases. For large delivery systems (50–200 nm), the size of the carrier confines it mainly to the blood compartment. Surface modifications such as PEGylation, may dramatically change the circulation time. The main advantage of using nanoparticles is that they can be decorated with appropriates ligands that will modulate the circulation time. Different ligands can be used but the most commonly used is polyethylene glycol (PEG). Derivatization by PEG chains limits the uptake by phagocytes in the liver and in the spleen and limits opsonization. Nevertheless, the positive effect of PEG could be limited if the nanoparticle own charges by itself. Length and ending groups of the PEG polymers have an influence on the biodistribution. Indeed, Faure et al. studied four different PEG coupled to gadolinium oxide nanoparticles embedded in a fluorescence polysiloxane shell, PEG250-COOH, PEG2000-COOH, PEG2000-NH2 and PEG2000-OCH3. Biodistributions were totally different and even if PEG250-COOH is the shortest hydrophilic polymer of the series, it confers the ability to circulate freely in the blood pool. DOTA (1, 4, 7, 10-tetraazacyclododecane-tetraacetic acid) and DTPA (diethylenetriaminepentaacetic acid) are also interesting ligands since they can (1) facilitate the dispersion of the nanoparticles into the biological media (2) provide chemical functions allowing further functionalization [11], and (3) also chelate gadolinium allowing the elaboration of nanoparticles for theranostic [12].

Changes in the biodistribution generally occur through a tumor-specific mechanism known as the enhanced permeability and retention (EPR) effect [13] of the tumor vasculature, also called passive targeting. Alternatively, active targeting may be applied with a particular using drug nanoparticles functionalization with molecular conjugates (*e.g.* conjugation of antibodies, aptamers, peptides, folic acid or transferrin to the nanoparticles surface [14] to focus drug delivery to specific sites of action. Either of the mechanisms is intended to increase the drug concentration at the desired site of action, reduce systemic drug levels and toxicity [15].

Alternatively, active targeting may be applied with particular delivery systems, using functionalization with molecular conjugates in order to restrict the photosensitizer delivery to specific sites of action. The selective targeted delivery of photosensitizers to diseased cells is one of the major limitations in PDT and is still a challenge to take up. Targeted therapy is a new promising therapeutic strategy, created to overcome growing problems of contemporary medicine, such as drug toxicity and resistance. An emerging modality of this approach is targeted PDT with the main aim of improving delivery of photosensitizer to cancer tissue and at the same time enhancing selectivity and efficiency [16]. A study by Hahn et al. in 2006 demonstrated that differences in selectivity of photofrin® for normal and tumor tissues are weak, leading to a narrow therapeutic window [17]. Moriwaki et al. reported that Photofrin® caused long skin photosensitivity after photofrin-PDT (*about 5 weeks*), underlining the importance of a discriminatory approach between healthy and tumor cells. In consequence, selectivity for malignant cells needs to be improved and a proposed solution is to address the photoactivatable agent by a specific ligand. Many ligands were suggested for targeting neoplastic cells. They can be coupled covalently to the photosensitizer by itself, or coupled to the nanoparticles. In this case, the advantage is that it is possible to play on the number of ligands to improve the selectivity, as well as having at the same time the EPR effect due to the nanoparticles by themselves [18]. Recently, [19] we showed that there is a relationship between the number of targeting units and the affinity. Comparing the affinity for neuropilin-1 of three kinds of nanohybrid nanoparticles with 4, 10 or 100 peptides, we proved that the best affinity was obtained for four peptides with a decrease of affinity with the increase of peptides. Moreover, the presence of four peptide moieties leads to a positive cooperatively in binding to the targeted receptor.

Master *et al.* worked on a PEG-PCL (Poly (Ethylene Glycol)-block-Poly (ε-CaproLactone) methyl ether, a biodegradable nanoparticle, which carries Phtalocyanine-4 (Pc4) for head and neck treatment. The nanoparticle is functionalized with a peptide GE11 specific from the epidermal growth factor (EGR) receptor. The authors related a significant uptake and selectivity from functionalized nanoparticles in an SCC-15 head and neck cell line, which overexpressed the EGF receptor [20]. Gary-Bobo et al. reported the improvement of galactose functionalized mesoporous silica nanoparticles carrying fluorescein uptake by colorectal cancer cells. Confocal microscopy shows an improvement of uptake and a new localization in the lysosome and the endosome. The internalization of mesoporous silica nanoparticles binding galactose is mediated by the receptor while endocytosis of mesoporous silica nanoparticles alone is passive. This endocytic mechanism can explain the new localization [21]. Gamal-Eldeen et al. present data on the *in vivo* efficacy of ICG-PEBBLE-anti EGFR (*Photonic Explorer for Bioanalysis with Biologically Localized Embedding*), which is a nanocarrier made with silica, entrapping Indo-Cyanine Green, and links to the anti-EGFR antibody [22]. *In vivo*, a better inhibition of VEGF and activation of caspase-3 were found for ICG-PEBBLE-anti EGFR compared to ICG-PEBBLE, but no other differences were detected. The targeting of nanoplateform brings selectivity, but also it modified some other mechanisms like the internalization mechanism, the localization and has an incidence on the death

pathway. In 2005, our team demonstrated, for the first time, the interest of using folic acid as a targeting unit [23]. Since then, many studies revealed the real potential of folic acid coupled to nanoparticles. For example, Teng et al. used folic acid for targeting Hela cells which are overexpressed by the folic acid receptor. With confocal microscopy, Hela cells show high red fluorescence emitted by Protoporphyrin IX (PpIX), unlike A 549 cells (low folate receptor expression). Cellular uptake followed with a spectrophotometer has elaborated the result. It shows a greater uptake with silica nanocarrier bound to folic acid, than the PpIX free. All these few examples show different strategies that afford to target specifically malignant cells and, thus, limiting side effects. But studies such are made *in vitro* and these results remain to be confirmed *in vivo* [24].

The use of nanoparticles could also improve cell localization of either mechanisms that were intended to increase the drug concentration at the desired site of action, reducing systemic drug levels, potential resistance mechanisms and allowing for lower effective drug dose. Baek et al. present the improvement of sulfonated aluminium phtalocyanine (AlPcs) by a polyethylenimine (PEI) nanoparticle. Uptake in Hela cells is 87 times higher for PEI-AlPcs than free AlPcs. This phenomenon can be explained by the difference in chemical properties of the nanoobject and the photosensitizer alone. AlPcs is hydrophilic because of the sulfonated group and negatively charged, that allows repulsion between it and the cell membrane surface. The following of intracellular localization shows that nanoparticles travel to the endosome [25]. According to the functionalization and chemical composition of the nanoobject, the mechanism of internalization is different. For example, Malatesta et al. [26] showed the intracellular fate of chitosan-FITC nanoparticle. Chitosan nanoparticles are inside invaginations in the plasma membrane, suggesting early endocytosis. This internalization mechanism with the plasma membrane is initiated by the interaction between polycation of chitosan nanoparticle and negative charge of the cell membrane.

The Chitosan nanoparticle undergoes passive endocytosis and goes in the perinuclear cytoplasmic region, while silica nanocarrier bearing folic acid is internalized by active endocytosis mediated by the receptor [24, 27]. Zhou et al. developed also a nanocarrier made in chitosan which carries a targeting peptide RGD. The use of a ligand/receptor system leads to endocytosis mediated by the receptor and a cytosolic localization. Unfortunately, no more information on the intracellular fate is available in these studies [27].

Improvement of Photodynamic Efficiency

Hydrophobicity of photosensitizers, which concerns most of them, causes their aggregation in aqueous media and reduces their photodynamic activity. Encapsulation in a nanocarrier can overcome this issue. Nevertheless, the number of photosensitizers encapsulated or grafted into nanoparticles should be controlled. Indeed, we showed in the case of a chlorin covalently linked to hybrid nanoparticles in different

amounts that the photophysical properties of the chlorin depend on the concentration of the photosensitizers. If the payload is too high, we can observe a decrease in the fluorescence and singlet oxygen quantum yields due to partial quenching linked to FRET and dimers formation [28]. Muehlmann et al. used a water dispersible nanoparticle (Poly methyl vinyl ether co maleic anhydride) to carry an hydrophobic aluminium chloride phtalocyanine (AlPc) [29]. One hour after incubation with the nanoparticles, MCF-7 cells internalize 73.9 % more than the healthy cells MCF-10A. This phenomenon can be explained by the higher endocytic activity of cancerous cells. Generation of oxygen species was found to be better with the nanoparticles (60 %) than with free AlPc (5 %) in aqueous solution. This could be explained by the capacity of nanoparticles to disaggregate AlPc and render them soluble so they became more effective in aqueous media.

Lima et al. elaborated hypericin-loaded solid nanoparticles to tend to decrease aggregation of hypericin to obtain a better photodynamic efficiency. They authors proved that (i) encapsulation of Hypericin (Hy) increases solubility of Hy, certainly due to a decrease of dimer-monomer formation (ii) encapsulated Hy-SLN was less sensitive to photodegradation than free Hy, and (iii) photoactivity of hypericin evaluated by using two chemical probes DPBF (1, 3-Diphenylisobenzofuran) and uric acid is higher when it is encapsulated in the solid nanoparticles due, in all probability, to the decrease of aggregation [30]. Zhao et al. [31] studied the photostability of phtalocyanine (Pc4) encapsulated in a silica nanoparticle. Encapsulation of Pc4 reduced and delayed the photobleaching and, thus, protected its photodynamic effectiveness. As expected, these characteristics have a repercussion on ROS production, which is higher for Pc4 entrapped in silica nanoparticles than for free Pc4, concluding that the use of nanoobject is very promising to overcome limitation emanating from the photosensitizer.

ROS production results also from photophysical capacity of the photosensitizer. We have seen that nanocarrier can overcome aggregation of the photosensitizer and then improve its solubility and photostability. Yoon et al. presented a study on improving ROS production by utilization of nanoobjects. Methylene Blue (MB) is embedded in polyacrylamide (PAA) nanoparticle with a cross-linker in poly(ethylene glycol) dimethacrylate (PEGDMA). The structure of PAA nanoparticle presents a pore, small enough to prevent the embedded MB from reduction into the photoinactive form “leuko MB” due to an isomerization reaction catalyzed by bioenzymes, and large enough to allow $^1O_2$ circulation [32]. The PEGDMA-PAA structure protected MB from aggregation and dimerization thanks to PEGDMA linkers, which keep the same distance between the MB molecules [32]. Yoon et al. assumed that these improvements presumably originate from a reduced amount of a longer cross-linker, which increases the distances between MB, resulting in reduction of self-quenching while also providing a larger pore size, which allows a better oxygen permeation and lower collision probability between the produced ROS [32]. Benito et al. described a study on gold nanoparticles (AuNP) embedding 5-aminolevulinic acid (5-ALA) and their impact on endogenous ROS production, cell viability and death mechanisms involved [33]. Comparison of free 5-ALA and AuNP-5-ALA shows an increase of 21 % of ROS fluorescence. This increase in ROS production is

correlated with a reduction of cell viability between free 5-ALA and 5-ALA-AuNP of around 62 and 37 % for MTT assay, and crystal violet assay respectively. Moreover, early and late apoptosis were studied. The presence of 5-ALA-AuNP on Hela cells *vs.* free 5-ALA increases more than 6 fold the late apoptosis.

Select Vascular Effects

Destruction of the vasculature may indirectly bypass the phenomena of resistance by leading to tumor eradication, following deprivation of life-sustaining nutrients and oxygen. The vascular effect of PDT is thought to play a major role in the destruction of some tumors [34]. Hence, tumor vasculature is a potential target of PDT damage. Generally, anti neovascular strategies present many advantages as they do not target tumor cells themselves but endothelial cells forming tumor vessels; these latter are genetically more stable than tumor cells and less likely to develop resistance. Moreover, endothelial cells involved in tumor angiogenesis describe a different phenotype than endothelial cells from normal vessels that could be suggested in PDT targeting strategies to the tumor endothelium [35]. The first example of the use of a nanoparticle involved in vascular targeted PDT (VTP) strategies was reported by Harrell and Kopelman [36, 37] who synthesized a multifunctional platform capable of diagnosis with an MRI contrast enhancer and with a photosensitizer, as well as the integrin targeting RGD peptide for specific neovessels targeting [38, 39]. The authors synthesized polyacrylamide nanoparticles containing both the photosensitizer photofrin® and MRI contrast enhancing agents with a surface coating of both PEG and RGD peptide. This assembly enhances the controllable particle residence time owing to the presence of PEG and the recognition of the tumor neovasculature. *In vitro* experiments confirmed the production of $^1O_2$ at levels believed to be sufficient to cause cell death. *In vivo*, the MRI contrast agent was useful to monitor changes in tumor diffusion, tumor growth and tumor load. Application of photofrin-containing nanoparticles followed by irradiation of the photosensitizer produced massive regional necrosis, whereas the tumor cells continued to grow in the control sample [38, 40]. Applying a similar principle, Reddy et al. developed another polyacrylamide nanoparticle encapsulating photofrin and imaging agents (fluorescent dye or iron oxide), with an F3 peptide targeting the surface located vasculature [41]. F3 is a 31-amino acid sequence that can accumulate on the cell surface and then translocate to the nucleus of cells of the human breast cancer line MDA-MB-435 both *in vitro* and in xenograft studies that the nanoparticles were internalized into the nucleus. *In vivo* studies demonstrated that the contrast enhancement was increased in both the magnitude and the duration when targeted nanoparticles were injected in comparison with that of controls that were non-specifically targeted. Sixty days after treatment, 40 % of animals treated with F3 targeted photofrin® nanoparticles were found to be tumor free. Diffusion MRI allowed to evaluate changes in tumor diffusion properties [38]. Several publications of the same team have demonstrated the interest of the use of this targeting peptide coupled to nanoparticles of iron oxide

[42–44]. The peptide F3 was also grafted to PAA nanoparticles conjugated with methylene blue [42–44].

Receptors specifically located on angiogenic endothelial cells, such as receptors to vascular endothelial growth factor (VEGF), can also be used as molecular targets. For 10 years, we developed photosensitizers and nanoparticles coupled with peptides that target vascular endothelial growth factor receptors or co-receptors. Non-biodegradable nanoparticles seemed to be very promising careers satisfying all the requirements for an ideal targeted PDT [45–46]. We described the design and photophysical characteristics of multifunctional nanoparticles consisting of a surface localized tumor vasculature targeting heptapeptide (ATWLPPR) specific for neuropilin-1 (NRP-1) [47–48], and encapsulated PDT (chlorin) and imaging agents (gadolinium oxide for RMI). As a proof of concept, nanoparticles functionalized with ~4.2 peptides were elaborated and proved to bind to recombinant NRP-1 protein and conferred photosensitivity to cells overexpressing this receptor [19]. Because no MRI signal could be detected, we elaborated new smaller nanoparticles and proved that by decreased the size of the silica shell, it was then possible to detect an MRI signal, as well as keeping the PDT effect and the selectivity of the nanoparticles [11, 49].

Zhou et al. worked on the RGD peptide which is a specific ligand for $\alpha_5\beta_3$ integrins. This integrin plays a role in the establishment of angiogenesis, and binds extracellular matrix proteins with the exposed RGD tripeptide sequence. The tripeptide is bound to an upconversion nanoplateform (UCNP) which is carried pyreopheophorbide a (Ppa), structure is called UCNP-Ppa-RGD. By fluorescence, they showed that a strong fluorescence is observed in U87 (overexpressing $\alpha_5\beta_3$) incubated with UCNP-Ppa-RGD, and a low fluorescence in U87 incubated with UCNP-Ppa-RGD. In contrast, in MCF-7 (low $\alpha_5\beta_3$ expression) a low level of fluorescence is observed with both UCNP. This experiment showed that by addressing the nanoplateforms, one can improve their internalization in specific cells (which overexpress the target) [27].

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References

1. Gao D, Agayan R, Xu H, Philibert M, Kopelman R. Nanoparticles for two-photon photodynamic therapy in living cells. Nano Lett. 2006;6:2383–6.
2. Kim S, Ohulchanskyy TH, Pudavar HE, Pandey RK, Prasad PN. Organically modified silica nanoparticles co-encapulating photosensitizing drug and aggregation-enhanced two-photon absorbing fluorescent dye aggregates for two-photon photodynamic therapy. J Am Chem Soc. 2007;129:2669–75.
3. Zhang P, Steelant W, Kumar M, Scholfield M. Versatile photosensitizers for photodynamic therapy at infrarted excitation. J Am Chem Soc. 2007;129:4526–7.

4. Chen W, Zhang J. Using nanoparticles to enable simultaneous radiation and photodynamic therapies for cancer treatment. J Nanosci Nanotechnol. 2006;6:1159–66.
5. Bulin AL, Truillet C, Chouikrat R, Lux F, Frochot C, Amans D, Ledoux G, Tillement O, Perriat P, Barberi-Heyob M, Dujardin C. X-ray-induced singlet oxygen activation with nanoscintillator-coupled porphyrins. J Phys Chem. 2013;117:21583–9.
6. Cooper DR, Kadinov K, Tyagi P, Hill CK, Bradforth SE, Nadeau JL. Photoluminescence of cerium fluoride and ceriundoped lanthanum fluoride nanoparticles and investigation of energy transfer to photosensitizer molecules. Phys Chem Chem Phys. 2014;16:12441–53.
7. Konan YN, Gurny R, Allémann E. State of the art in the delivery of photosensitizers for photodynamic therapy. J Photochem Photobiol B. 2002;66:89–103.
8. Brown SB, Brown EA, Walker I. The present and future role of photodynamic therapy in cancer treatment. Lancet Oncol. 2004;5:497–508.
9. Kachatkou D, Sasnouski S, Zorin V, Zorina T, D'Hallewin MA, Guillemin F, Bezdetnaya L. Unusual photoinduced response of mTHPC liposomal formulation (Foslip). Photochem Photobiol. 2009;85:719–24.
10. Vasey PA, Kaye SB, Morrison R, Twelves C, Wilson P, Duncan R, Thomson AH, Murray LS, Hilditchand TE, Murray T, Burtles S, Fraier D, Frigerio E, Cassidy J. Phase I clinical and pharmacokinetic study of PK1 [N-(2-Hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents—drug-polymer conjugates. Clin Cancer Res. 1999;5:83–94.
11. Benachour H, Sève A, Bastogne T, Frochot C, Vanderesse R, Jasniewski J, Miladi I, Billotey C, Tillement O, Lux F, Barberi-Heyob M. Multifunctional Peptide-conjugated hybrid silica nanoparticles for photodynamic therapy and MRI. Theranostics. 2012;2:889–904.
12. Faure AC, Dufort S, Josserand V, Perriat P, Coll JL, Roux S, Tillement O. Control of the in vivo biodistribution of hybrid nanoparticles with different poly(ethylene glycol) coatings. Small. 2009;5:2565–75.
13. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release. 2000;65:271–84.
14. Torchilin VP. Targeted pharmaceutical nanocarriers for cancer therapy and imaging. AAPS J. 2007;9:128–47.
15. Wang J, Sui M, Fan W. Nanoparticles for tumor targeted therapies and their pharmacokinetics. Curr Drug Metab. 2010;11:129–41.
16. Pernot M, Frochot C, Vanderesse R, Barberi-Heyob M. Targeting strategies in photodynamic therapy for cancer treatment. In: Hamblin MR, Huang Y, editors. Handbook of photomedicine. CRC press, 2013.
17. Hahn S, Putt ME, Metz J, Shin DB, Rickter E, Menon C, Smith D, Glatstein E, Fraker DL, Busch TM. Photofrin uptake in the tumor and normal tissues of patients receiving intraperitoneal photodynamic therapy. Clin Cancer Res. 2006;12:5464–70.
18. Moriwaki SI, Misawa J, Yoshinari Y, Yamada I, Takigawa M, Tokura Y. Analysis of photosensitivity in Japanese cancer-bearing patients receiving photodynamic therapy with porfimer sodium (PhotofrinTM). Photodermatol Photoimmunol Photomed. 2001;17:241–3.
19. Couleaud P, Bechet D, Vanderesse R, Barberi-Heyob M, Faure AC, Roux S, Tillement O, Porhel S, Guillemin F, Frochot C. Functionalized silica-based nanoparticles for photodynamic therapy. Nanomedicine. 2011;6:995–1009.
20. Master A, Malamas, Solanki R, Clausen DM, Eiseman JL, Gupta AS. A cell-targeted photodynamic nanomedicine strategy for head and neck cancers. Mol Pharm. 2013;10:1988–97.
21. Gary-Bobo M, Hocine O, Brevet D, Maynadier M, Raehm L, Richeter S, Charasson V, Loock B, Morère A, Maillard P, Garcia M, Durand JO. Cancer therapy improvement with mesoporous silica nanoparticles combining targeting, drug delivery and PDT. Int J Pharm. 2012;423:509–15.
22. Gamal-Eldeen AM, El-Daly SM, Borai IH, Wafay HA, Abdel-Ghaffar AR. Photodynamic therapeutic effect ofindocyanine green entrapped in polymericnanoparticles and their anti-EGFR-conjugate in skin cancer in CD1 mice. Photodiagnosis Photodyn Ther. 2013;10:446–59.

23. Schneider R, Schmitt F, Frochot C, Fort Y, Lourette N, Guillemin F, Müller JF, Barberi-Heyob M. Design, synthesis, and biological evaluation of folic acid targeted tetraphenylporphyrin as novel photosensitizers for selective photodynamic therapy. Bioorg Med Chem. 2005;13:2799–808.
24. Teng IT, Chang YJ, Wang LS, Lu HY, Wu LC, Yang CM, Chiu CC, Yang CH, Hsu SL, Ho JA. Phospholipid-functionalized mesoporous silica nanocarriers for selective photodynamic therapy of cancer. Biomaterials. 2013;34:7462–70.
25. Baek S, Na K. A nano complex of hydrophilic phthalocyanine and polyethylenimine for improved cellular internalization efficiency and phototoxicity. Colloids Surf B: Biointerfaces. 2013;101:493–500.
26. Malatesta M, Pellicciari C, Cisterna B, Costanzoa M, Galimberti V, Biggiogera M, Zancanaro C. Tracing nanoparticles and photosensitizing molecules at transmission electron microscopy by diaminobenzidine photo-oxidation. Micron. 2014;59:44–51.
27. Zhou A, Wei Y, Wu B, Chen Q, Xing D. Pyropheophorbide A and c(RGDyK) comodified chitosan-wrapped upconversion nanoparticle for targeted near-infrared photodynamic therapy. Mol Pharma. 2012;9:1580–9.
28. Sève A, Couleaud P, Lux F, Tillement O, Arnoux P, André JC, Frochot C. Long-distance energy transfer photosensitizers arising in hybrid nanoparticles leading to fluorescence emission and singlet oxygen luminescence quenching. Photochem Photobiol Sci. 2012;11:803–11.
29. Muehlmann LA, Ma BC, Longo JPF, de Fátima Menezes M, Santos A, Azevedo RB. Aluminum–phthalocyanine chloride associated to poly(methyl vinyl ether-co-maleic anhydride) nanoparticles as a new third-generation photosensitizer for anticancer photodynamic therapy. Int J Nanomedicine. 2014;9:1199–213.
30. Lima AM, Pizzol CD, Monteiro FB, Creczynski-Pasa TB, Andrade GP, Ribeiro AO, Perussi JR. Hypericin encapsulated in solid lipid nanoparticles: phototoxicity and photodynamic efficiency. J Photochem Photobiol B. 2013;125:146–54.
31. Zhao B, Yin JJ, Bilski PJ, Chignell CF, Roberts JE, He YY. Enhanced photodynamic efficacy towards melanoma cells by encapsulation of Pc4 in silica nanoparticles. Toxicol Appl Pharmacol. 2009;241:163–72.
32. Yoon HK, Lou X, Chen YC, Lee KYE, Yoon E, Kopelman R. Nanophotosensitizers engineered to generate a tunable mix of reactive oxygen species, for optimizing photodynamic therapy, using a microfluidic device. Chem Mater. 2014;26:1592–600.
33. Benito M, Martin V, Blanco MD, Teijon JM, Gomez C. Cooperative effect of 5-aminolevulinic acid and gold nanoparticles for photodynamic therapy of cancer. J Pharm Sci. 2013;102:2760–9.
34. Madar-Balakirski N, Tempel-Brami C, Kalchenko V, Brenner O, Varon D, Scherz A, Salomon Y. Permanent occlusion of feeding arteries and draining veins in solid mouse tumors by vascular targeted photodynamic therapy (vtp) with Tookad. Plos One. 2010;5:e10282.
35. Ran S, Thorpe PE. Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy. Int J Radiat Oncol Biol Phys. 2002;54:1479–84.
36. Harrell J, Kopelman R. Biocompatible probes measure intracellular activity. Biophotonics Int. 2001;7:22–4.
37. Kopelman R, Koo YEL, Philbert M, Moffat BA, Reddy GR, McConville P, Hall DE, Chenevert TL, Bhojani MS, Buck SM, Rehemtulla A, Ross BD. Multifunctional nanoparticle platforms for in vivo MRI enhancement and photodynamic therapy of a rat brain cancer. J Magn Magn Mat. 2005;293:404–10.
38. Koo YEL, Fan W, Hah H, Xu H, Orringer D, Ross B, Rehemtulla A, Philbert MA, Kopelman R. Photonic explorers based on multifunctional nanoplatforms for biosensing and photodynamic therapy. Appl Opt. 2007;46:1924–30.
39. Ross B, Rehemtulla A, Koo YEL, Reddy R, Kim G, Behrend C, Buck S, Schneider RJ, Philbert MA, Weissleder R, Kopelman R. Nanobiophotonics and biomedical applications (photonic and magnetic nanopexplorers for Biomedical use: from subcellular imaging to cancer diagnostics and Therapy, vol. 5331, Cartwright AN, Éd., Proceedings of SPIE, 2004.

40. Xu H, Aylott J, Kopelman R, Miller T, Philbert M. A real-time ratiometric method for the determination of molecular oxygen inside living cells using sol-gel-based spherical optical nanosensors with applications to rat C6 glioma. Anal Chem. 2001;73:4124–33.
41. Reddy GR, Bhojani MS, McConville P, Moody J, Moffat BA, Hall DE, Kim G, Koo YEL, Croft MJ, Sugai JV, Johnson TD, Philbert MA, Kopelman R, Rehemtulla A, Ross BD. Vascular targeted nanoparticles for imaging and treatment of brain tumors. Clin Cancer Res. 2006;12:6677–86.
42. Moffat BA, Chenevert TL, Meyer CR, Mckeever PE, Hall DE, Hoff BA, Johnson TD, Rehemtulla A, Ross BD. The functional diffusion map: an imaging biomarker for the early prediction of cancer treatment outcome. Neoplasia. 2006;8:259–67.
43. Qin M, Hah HJ, Kim G, Nie G, Lee YEK, Kopelman R. Methylene blue covalently loaded polyacrylamide nanoparticles for enhanced tumor-targeted photodynamic therapy. Photochem Photobiol Sci. 2011;10:832–41.
44. Hah HJ, Kim G, Lee YEK, Orringer DA, Sagher O, Philbert MA, Kopelman R. Methylene blue-conjugated hydrogel nanoparticles and tumor-cell targeted photodynamic therapy. Macromol Biosci. 2011;10:90–9.
45. Bechet D, Couleaud P, Frochot C, Viriot ML, Guillemin F, Barberi-Heyob M. Nanoparticles as vehicles for delivery of photodynamic therapy agents. Trends Biotechnol. 2008;26:612–21.
46. Bechet D, Tirand L, Faivre B, Plénat F, Bonnet C, Bastogne T, Frochot C, Guillemin F, Barberi-Heyob M. Neuropilin-1 targeting photosensitization-induced early stages of thrombosis via tissue factor release. Pharm Res. 2010;27:468–79.
47. Thomas N, Bechet D, Becuwe P, Tirand L, Vanderesse R, Frochot C, Guillemin F, Barberi-Heyob M. Peptide-conjugated chlorin-type photosensitizer binds neuropilin-1 in vitro and in vivo. J Photochem Photobiol B. 2009;96:101–8.
48. Tirand L, Frochot C, Vanderesse R, Thomas N, Trinquet E, Pinel S, Viriot ML, Guillemin F, Barberi-Heyob M. A peptide competing with VEGF165 binding on neuropilin-1 mediates targeting of a chlorin-type photosensitizer and potentiates its photodynamic activity in human endothelial cells. J Control Release. 2006;111:153–64.
49. Benachour H, Bastogne T, Toussaint M, Chemli Y, Sève A, Frochot C, Lux F, Tillement O, Vanderesse R, Barberi-Heyob M. Real-time monitoring of photocytotoxicity in nanoparticles-based photodynamic therapy: a model-based approach. Plos One. 2012;7: e48617.

Chapter 10
Mechanisms of Tumor Cell Resistance to ALA-PDT

Zvi Malik, Abraham Nudelman and Benjamin Ehrenberg

Abstract Cancer cells are considered to express primary drug resistance, yet chemotherapeutics are the front line of cancer treatments and have achieved great successes. In time, most neoplastic cells develop secondary resistance to chemotherapy, which remains the major obstacle to cancer treatment. Progressive multi-drug resistance develops by upregulation of a large family of drug ABC transporters, such as the P-glycoprotein.

ALA-PDT is a non-invasive cancer therapy that is limited to organs that are accessible to light and it is dependent on the biosynthesis of the natural photosensitizer PpIX, largely detached of sensitizer efflux by transporters. The action mechanism of ALA-PDT starts by light irradiation and production of singlet oxygen as the toxic molecule, which hits the tumor at many subcellular targets and induces both necrosis and apoptosis, the Achilles heel of a tumor. Recently, it was suggested that PDT, based on multifunctional ALA-prodrugs, may overcome drug resistance of tumors.

Multifunctional acyloxyalkyl ester prodrugs induce efficient PpIX synthesis due to upregulated PpIX biosynthesis and efficient photodynamic killing of cancer cells. One of these prodrugs, AlaAcBu, releases three active products: ALA, acetaldehyde and butyric acid. They stimulate independent pathways through activation of specific biochemical routes; ALA stimulates PpIX synthesis and PDT, acetaldehyde endorses dark-tumor cytotoxicity and butyric acid inhibits histone deacetylase, leading to gene expression and tumor differentiation. All are targeted to boost anticancer actions and to reduce tumor recurrence.

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We describe here a protocol for boosting PpIX synthesis in Multi Drug Resistant cells by two rounds of ALA exposures. The first induces synthesis of dipyrromethane from ALA, which is essential for PBGD activity, while the second provides the precursor for PpIX synthesis and photodynamic cell killing.

In conclusion, new ALA delivery protocols and novel generations of multifunctional ALA prodrugs may render ALA-PDT into a more potent method in the front line cancer therapy. We believe that the future of ALA-PDT will involve the introduction of combinatory concepts of multifunctional ALA prodrugs to maximize sensitizer biosynthesis and to hit tumors in multiple subcellular targets independent of multi-drug resistance.

Keywords ALA · ALA dehydratase · MDR · PDT · Porphobilinogen deaminase · Prodrugs

Abbreviations

ALA	5-amino levulinic acid
ALAD	ALA dehydratase
DPM	Dipyrromethane
MDR	Multidrug resistance
PBGD	Porphobilinogen deaminase
PpIX	Protoporphyrin IX

Introduction

The main distress in chemotherapeutic management of cancer is the significant population of drug-resistant cells which remain even after most of the drug-sensitive cells have been eliminated. With each bout of chemotherapy, successive, alternative chemotherapies become more likely to fail since the remaining tumor cells evolve resistance even to structurally and mechanistically unrelated drugs; consequently treatment options become more limited [1, 2]. Breast cancer is the second most common cancer to metastasize to the skin, and these metastases are often resistant to chemotherapy [2].

Cancer Resistance to Chemotherapy

In a situation of multidrug resistance (MDR), cancer cells resist a broad spectrum of chemotherapeutic agents and, thus, survive high intracellular concentrations of anti-tumor agents and cytotoxic effects. The mechanism underlying this resistance is based on membrane pumps that actively eject specific chemotherapy drugs from within the transformed cells and, thus, protect cancer cells from cytotoxic com-

pounds. These membrane pumps are the main obstacle to successful treatment of cancer. ABC transporters constitute the largest family of transmembrane proteins, comprising 49 transporters which may be divided into subfamilies. Hence, tumor cells avoid the toxic effects of chemotherapy envisioned to affect nuclear or cytosolic activities. The ABC energy-dependent pumps function as drug efflux pumps, extruding structurally diverse lipophilic anions from the cytosol into the extracellular space. The first ABC pumps discovered was the ABCB1/P-glycoprotein (P-gp). [3, 4]

The MDR phenotype is associated with increased drug efflux mediated by an energy-dependent mechanism, and so it has been proposed that MDR can be characterized by over-expression of ATP-binding drug transporters such as P-glycoproteins, multidrug resistance-associated proteins, and breast cancer resistance protein [5]. Recently, it has also been suggested that epigenetic changes to DNA methylation and histone modifications also play a role in breast cancer resistance to chemotherapeutic agents. Evidently, gene expression is also mediated by small regulatory RNA, particularly microRNA (miRNA), but its role in cancer drug resistance remains unexplored [6].

Tumor cell survival and proliferation are attributed, in part, to the shut down of normal mechanisms of programmed cell death. Unconstrained by apoptosis, tumors grow, their cells proliferate unrestrained, and their tissue undergoes neoplastic transformation. The means of suppressing apoptosis may be by products of certain metabolic processes: many cancer cells express heme oxygenase-1 (HO-1), which catabolizes heme to generate biliverdin, Fe^{2+}, and carbon monoxide (CO). These end products

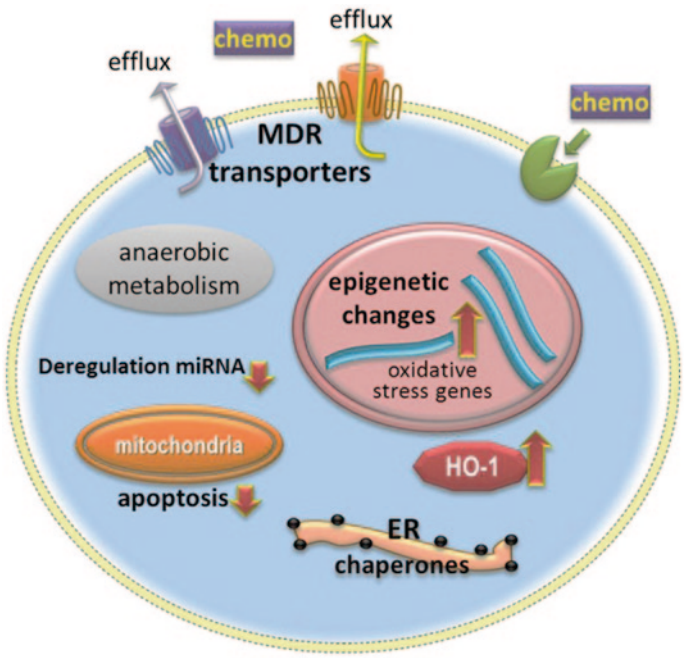

Fig. 10.1 Illustrated summary of resistance mechanisms to cytotoxic anticancer drugs in solid tumors. *Red* arrows point to increase or decrease in the effect

may then, in turn, suppress apoptotic signals [7]. An illustrated summary of the ways in which tumor cells gain resistance to cytotoxic anticancer drugs is shown in Fig. 10.1.

Resistance to Photodynamic Therapy

An alternative treatment for light accessible tumors, such as drug-resistant skin metastases, is photodynamic therapy (PDT) [8]. PDT is based on the local administration of a photosensitizer followed by light activation which leads to production of singlet oxygen and eventually to cell death. Photodynamic therapy can be divided generally into two modes of action, the one using exogenous light-sensitive compounds that are accumulated into neoplastic tissue, while the other uses the natural endogenous heme synthesis pathway which upon supplementation of 5-aminolevulinic acid causes the buildup of proptoporphyrin IX in the mitochondria. In both methods, light exposure activates the sensitizer and energy transfer leads to the formation of singlet oxygen in subcellular compartments in which the sensitizer was accumulated: the plasma membrane, endo-lysosomal compartment, endoplasmic reticulum or mitochondria where PpIX is produced. Thus, exogenous photosensitizers may affect the compartments in which the sensitizers were accumulated, which in turn are dependent on their chemical hydrophobic, hydrophilic or amphiphilic nature. Hydrophobic versus hydrophilic sensitizers are bound differently to blood proteins and complexes and are taken up by cells by divergent mechanisms. Hydrophobic sensitizers tend to accumulate in hydrophobic membrane compartments such as the endoplasmic reticulum while hydrophilic sensitizers mostly enter cells by endocytosis and are found in the endo-lysosomal vesicular system [9, 10]. The differences between these mechanisms are depicted in Fig. 10.2.

Clinical PDT was approved by the FDA for Photofrin (dihematoporphyrin ether), a hydrophobic molecule which was applied successfully in the treatment of bronchial cancers, gastrointestinal diseases and other malignant conditions. A large variety of synthetic photo-sensitizers with variable chemical natures were investigated in tumor-bearing animal models and in *in vitro* using various cancer cell lines; the results revealed the outstanding potential of PDT to eradicate transformed cells [11]. The alternative treatment to overcome MDR resistance after prolonged chemotherapy treatments by photosensitization was a driving force in PDT research. One of the first studies showed that ex vivo purging of residual acute myelogenous leukemia cells by benzoporphyrin derivative (BPD-MA) and Photofrin revealed that PDT is independent of MDR mechanisms [12]. Acridine orange photodynamic treatment of MDR osteosarcoma cells resulted in a strong cytotoxic effect [13]. Another study showed the PDT susceptibility of MCF-7/DXR human breast cancer cells expressing multidrug resistance to doxorubicin, and the wild-type MCF-7 parental cell line, when treated by mTHPC. The results indicated that mTHPC PDT was efficient in killing cells that express MDR doxorubicin resistance [14]. Photodynamic therapy of human R-HepG2 hepatocellular carcinoma using pheophorbide *a* indicated that PDT could inhibit the MDR activity by down-regulating the ex-

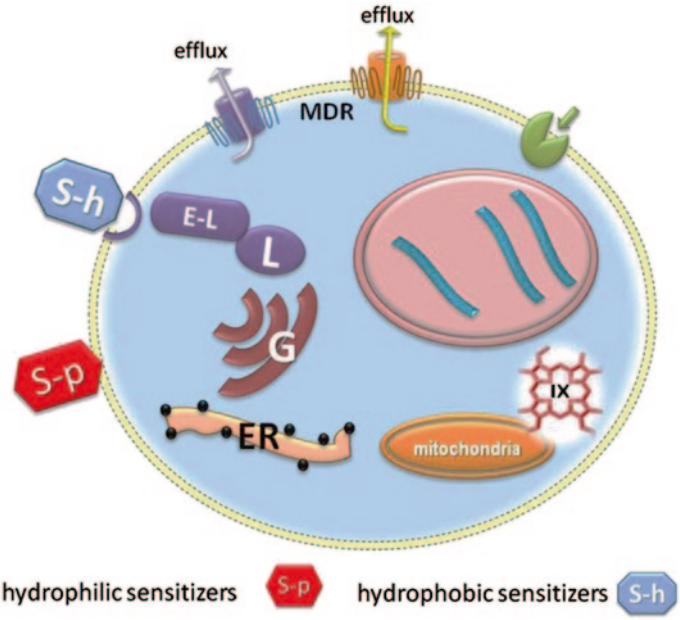

Fig. 10.2 Uptake pathways and/or sub-cellular localization of hydrophobic and hydrophilic sensitizers which affect cellular damage during light irradiation. MDR -drug transporters; ER -endoplasmic reticulum, L-lysosomes; PpIX -protoporphyrin IX synthesized in mitochondria [10]

pression of P-glycoprotein via the JNK signaling pathway leading to activation of intrinsic apoptotic caspases cascade [15].

Another extensive study demonstrated that ABCG2 expression can limit the retention of certain photosensitizing agents, specifically, the chlorin-based drugs, pheophorbide a, pyropheophorbide a methyl ester and chlorin e6. Overexpression of ABCG2 expression decreased PDT cytotoxicity accompanied by reduced accumulation of these photosensitizers in an ABCG2-expressing cell line. Indeed, experimental overexpression of ABCG2 induced resistance to photodynamic therapy by lowering intracellular concentrations of hydrophobic and hydrophilic sensitizers [16].

ALA-PDT and Chemotherapy Resistance

ALA-based PDT is dependent on the administration of the pro-drug ALA, the cytosolic natural precursor for the synthesis of PpIX in neoplastic cells [18, 19]. PpIX levels may reach more than 3 fold increase in tumors in comparison to the non malignant surrounding tissue. PpIX production and accumulation depends on the enzymatic activity of both cytosolic and mitochondrial enzymes. ALA dehydratase (ALAD), the first cytosolic enzyme, condenses two ALA molecules to form

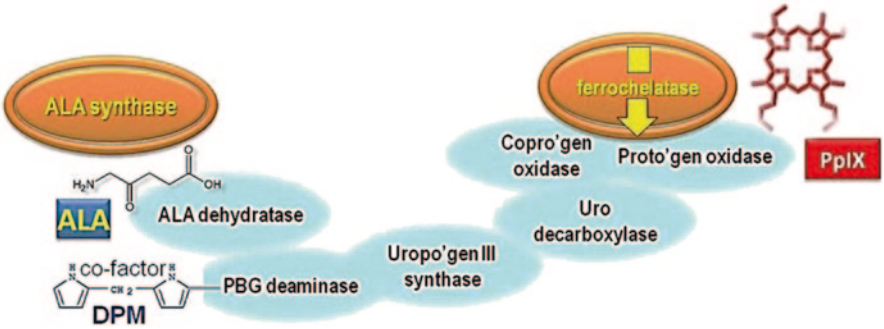

Fig. 10.3 Enzymatic pathway of PpIX synthesis induced by 5-aminolevulinic acid treatment. *DPM* dipyrromethane

a pyrrole ring porphobilinogen (PBG), which is the substrate of the rate-limiting enzyme—porphobilinogen deaminase (PBGD). Two main products are synthesized by PBGD: dipyrromethane (DPM) which is the internal cofactor of the enzyme, and uropophyrinogen, which is a linear tetrapyrrole formed by covalent binding of four PBG molecules on its cofactor anchor DPM. The porphyrin ring is then closed to create PpIX by several enzymatic steps (Fig. 10.3). Normally, the final event is catalyzed by the mitochondrial ferrochelatase (FECH) which inserts Fe^{+2} into PpIX to produce heme. However, cancer cells express low FECH activity that results in PpIX accumulation in the mitochondria [19].

Light irradiation at PpIX's main absorption bands, mainly 410 or 630 nm, stimulates energy transfer to dissolved oxygen, to generate singlet oxygen which is highly toxic to the treated cells. ALA-PDT was intensively studied during the last 25 years yielding hundreds of articles covering the basic sciences of ALA-induced porphyrin synthesis, major regulatory key enzymes of this metabolic pathway as well as stimulating agents to enhance PpIX accumulation in the inflicting cancer cell.

Theoretically, multi-drug resistance may affect ALA-PDT by efflux of ALA combined with up-regulated anti-oxidative defenses, defective apoptosis and other general resistance mechanisms as illustrated in Fig. 10.4. Surviving cells post ALA-PDT may regrow independent of MDR mechanisms since resistance to apoptosis, alternatively being dormant G_0 or non proliferative cells producing insufficient PpIX to be photoactivated. It should be taken into consideration that breakdown of heme by heme oxygenase 1, will upregulate higher PpIX accumulation and elevated photo-destruction. It was shown that ALA-PDT of MDR leukemic cells had no cross resistance with chemotherapy, thus P-glycoprotein-dependent drug resistance did not interfere with the fluorescence kinetics of ALA-induced PpIX production in leukemic cells [20].

A well-established MDR cell line of human breast cancer MCF-7 was studied extensively as a basic model for ALA-PDT. To date, there is inconsistent evidence in regard to the cross resistance of cells to drugs plus PDT [20]. Tsai et al. [21] reported that ALA-PDT of human breast cancer MCF-7/MDR cells were less sensitive to ALA-PDT in comparison to MCF-7 WT cells.

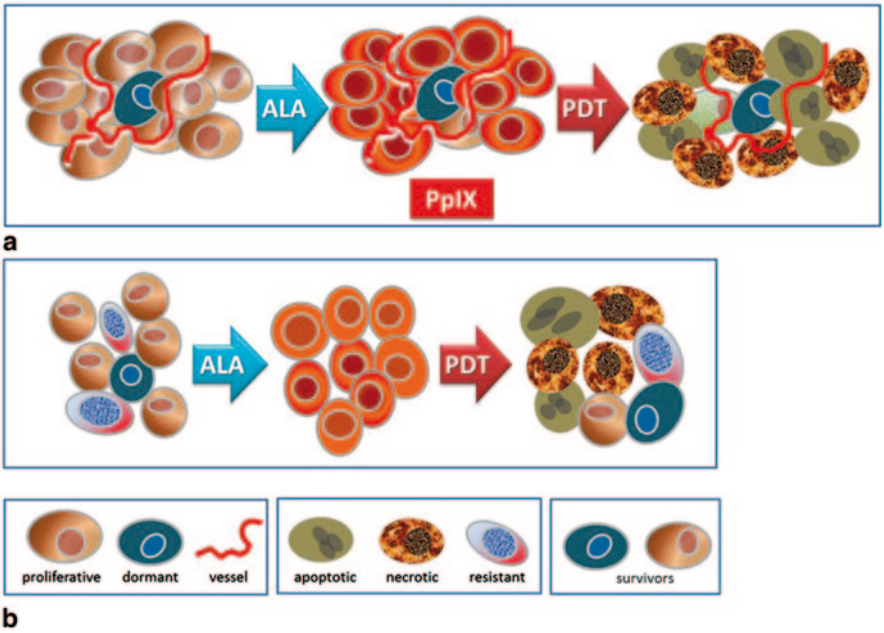

Fig. 10.4 Virtual outline of the ways cancer cells survive ALA-PDT. Frame A depicts the first cycle of treatment; B depicts the outcome at a second round of ALA-PDT

The central conclusion of these studies was that ALA-PDT resistance is of relatively lower efficacy of ALA-PDT due to the capacity of PpIX synthesis which may be attributed to low accumulation of ALA [23], dependence on the differentiation status of the tumor cells [23, 24], low activity of a key enzyme in the pathway, especially of ferrochelatase [25, 27] and anti-oxidative mechanisms including high activity of heme oxygenase. Figure 10.5 displays the potential molecular mechanisms that affect ALA-PDT efficacy and resistance.

Conventional chemotherapy is the mainstay of adjuvant systemic treatment for most breast cancer patients where Doxorubicin (Dox) is a remarkably beneficial substance, although Dox development of resistance reduces treatment efficacy [28]. As discussed above, the major resistance mechanism to chemotherapy depends on overexpression of the ABC transporters which increase drug efflux and decrease drug cellular concentration, a process applicable to ALA-PDT. Feuerstein et al. [22] demonstrated the higher dark toxicity of ALA for wild type MCF-7 cells in comparison to the resistant sub-line MCF-7/DOX due to the efflux of ALA. The toxic concentrations of ALA for the resistant DOX cells were significantly higher, similarly to DOX toxicity reported for the same cells [23]. The higher IC_{50}'s for ALA and DOX of the MCF-7/DOX subline probably reflects increased activity of the P-gp transporter. Nevertheless, PpIX production by MCF-7/DOX cells was still high.

ALA-PDT of primary tumor cells that were not exposed to chemotherapy results in 2 or more orders of magnitude of survival due to necrosis and apoptosis. The sur-

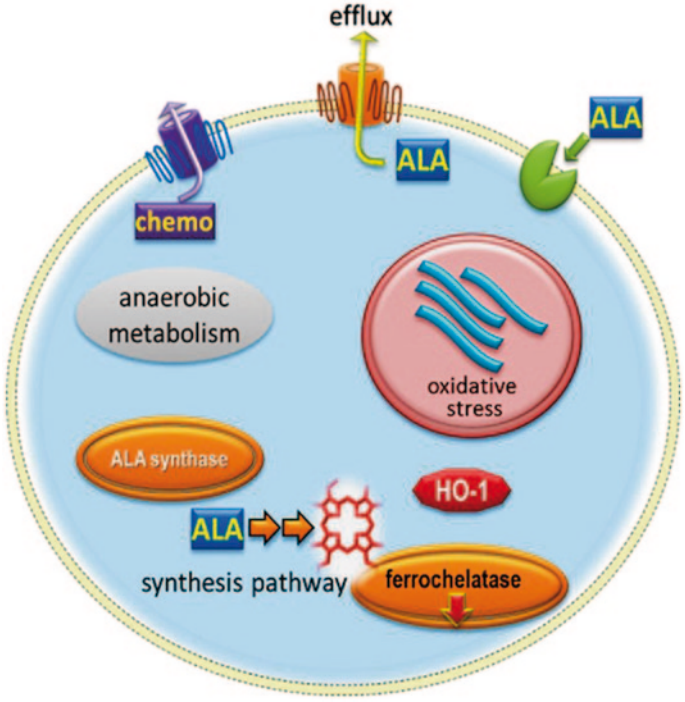

Fig. 10.5 The cellular mechanisms that affect PpIX accumulation, PDT efficacy and resistance. ALA efflux that reduces cellular ALA is regulated by drug transporters, PpIX synthesis is dependent on synthesis pathway, ferrochelatase activity and heme breakdown by HO-1. The level of anti-oxidant defense will affect death outcome

viving cell fraction escapes death due to low PpIX synthesis as a result of three apparent effects: low PBGD enzymatic activity, high ferrochelatase activity combined with elevated HO-1 activity (Fig. 10.6).

Experimental Solutions to Reduce Resistance: ALA Multifunctional Prodrugs

We have described recently a series of multifunctional ALA prodrugs for combined PDT and PDT-independent toxic effects. We synthesized several multifunctional ALA acyloxyalkyl ester prodrugs which are more hydrophobic in comparison to other ALA derivatives. The ALA prodrugs, upon metabolism by unspecific cellular esterases, release the active metabolites ALA, aldehydes and short chain carboxylic acids; ALA is transformed into PpIX [23, 29, 31]. Four of these prodrugs are (1)

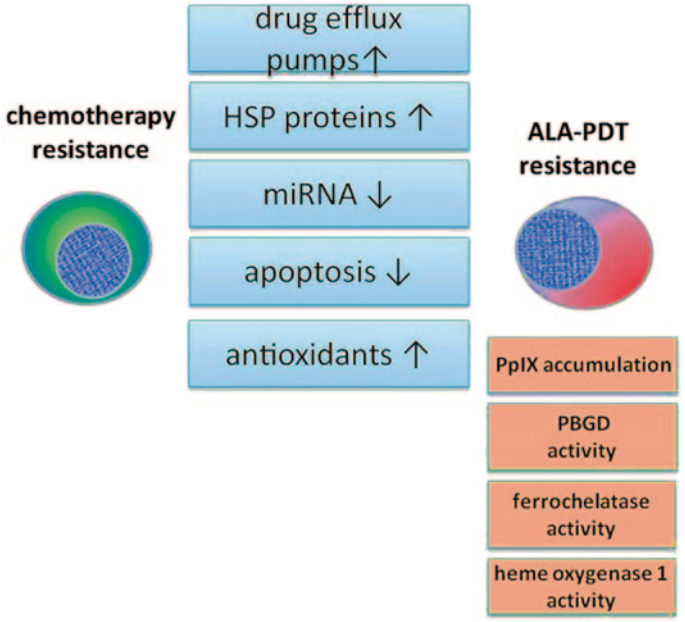

Fig. 10.6. Molecular mechanisms involved in resistance to chemotherapy versus ALA-PDT. Resistance to chemotherapy treatments is due to drug efflux and protection mechanisms acquired by upregulated heat shock proteins and antioxidants' expression. Resistance may develop in conjunction with specific miRNA down regulation and failure to carry on apoptosis. Additionally, ALA-PDT resistance has specific characteristics essentially low PpIX synthesis as a result of low PBGD enzymatic activity, high errochelatase activity combined with elevated HO-1 activity

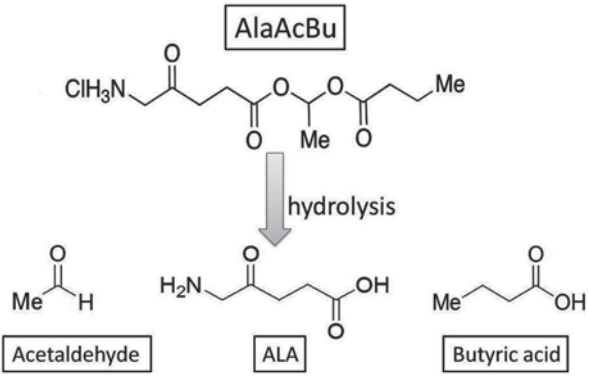

Fig. 10.7 Chemical structure of AlaAcBu an acyloxyalkyl ester prodrug and its hydrolytic metabolites. AlaAcBu undergos enzymatic intracellular hydrolysis releasing ALA, butyric acid and acetaldehyde

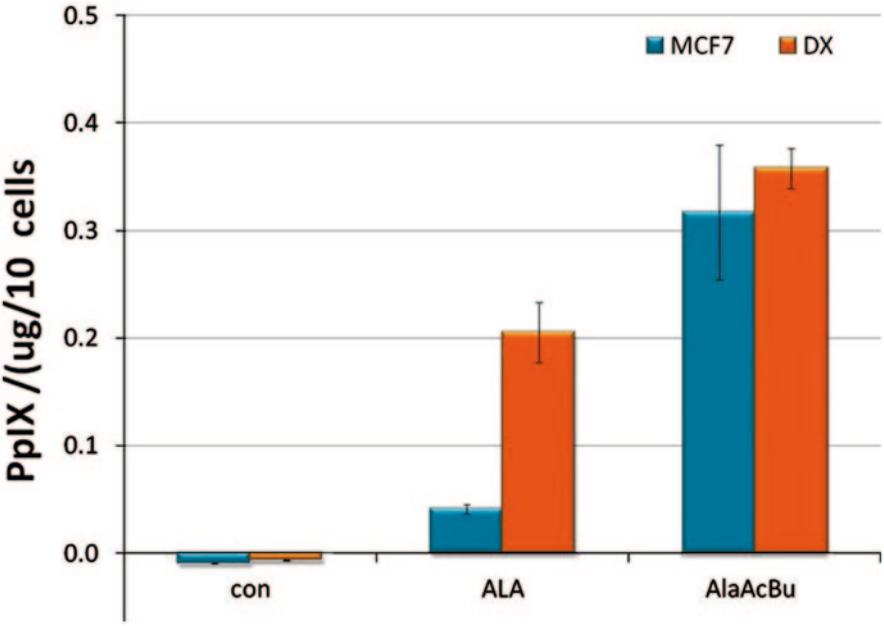

Fig. 10.8 Porphyrin synthesis is elevated using ALA-AcBA in MCF-7/WT and MCF-7/DOX cells. MCF-7/WT and MCF-7/DOX cells were incubated in serum-free medium containing 200 mM ALA or AlaAcBu for 4 h. Relative cellular porphyrins (5×10^8 cells) were measured by spectrophotometericaly (ex. 400 nm, em. 630 nm)

AlaAcBu, that releases ALA, acetaldehyde, and butyric acid (2) AlaFaBu, that releases ALA, formaldehyde, and butyric acid (3) AlaFaPi, that releases ALA, formaldehyde and pivalic acid and (4) AlaAcPi that releases ALA, acetaldehyde and pivalic acid, after hydrolysis by unspecific hydrolases.

AlaAcBu (Fig. 10.7) was found to be the superior prodrug for PpIX synthesis due to its ability to induce the highest PpIX synthesis in MCF-7/WT as well as MCF-7/DOX cells (Fig. 10.8). In addition, acetaldehyde and butyric acid released from AlaAcBu have other independent biological effects. Butyric acid is a strong inhibitor of histone deacetylase (iHDAC) affecting gene activation and cellular differentiation [31]. The active moiety butyrate unregulated porphobilinogen deaminase (PBGD) activity and accelerated the biosynthesis of PpIX [29, 30].

PpIX accumulation in MCF-7/WT and MCF-7/DOX cells treated by ALA or AlaAcBu for 4 h was measured by FACS and spectroscopic analyses. We found that MCF-7/DOX cells are competent to produce higher quantities of PpIX possibly as a result of low ferrochelatase activity as shown in Fig. 10.8. More importantly, AlaAcBu treatment doubled PpIX production in both cell lines.

The relative accumulation of heme *versus* PpIX is dependent on the expression and activity of mitochondrial ferrochelatase. Two possible mechanisms are respon-

sible for low heme synthesis, the first one owing to low mitochondrial ferrochelatase and the second one due to low iron availability. Both will increase PpIX levels.

PpIX production is directly related to the activity of two cytosolic heme biosynthesis enzymes ALAD and PBGD, both shown to be crucial to the PpIX production [31, 32]. Unexpectedly, the expression level of PBGD and ALAD were found to be much higher in MCF-7/WT compared to MCF-7/DOX cells [23]. However, ferrochelatase expression was found to be very low and nearly undetectable in the DOX resistant MCF-7/DOX cells [23] while MCF-7/WT cells expressed high levels of ferrochelatase. Thus, the conclusion undoubtedly is that ferrochelatase is fully responsible for the PpIX accumulation in the MDR cells.

Heme oxygenase-1 (HO-1) is a stress-responsive enzyme that catabolizes heme into carbon monoxide (CO), biliverdin and iron. Both CO and biliverdin/bilirubin have been shown to exert anti-oxidative stress effects and protective effects against programmed cell death stimuli. The expression levels of HO-1 is highly dependent on heme cellular levels and, thus, on ferrochelatase expression, cells with low levels of heme will express low levels of HO-1 and thus will be less protected against oxidative stress. We have shown that both lines, MCF-7/WT and MCF-7/DOX, undergo apoptosis following ALA-PDT. We may speculate that the MDR resistant cells are less protected against PDT in comparison to the wild type line expressing high levels of ferrochelatase. Apoptotic and necrotic cell death were evident to a similar extent induced by ALA and AlaAcBu. However, the marked advantage of the multifunctional prodrug AlaAcBu in killing both MCF-7/WT and MCF-7/DOX cells was depicted.

Centrality of Porphobilinogen Deaminase in ALA-PDT and Resistance

The understanding that PBGD, the third enzyme, is central and rate limiting in tetrapyrrole synthesis should be considered with a biochemical perspective. Activation of PBGD enzymatic activity is totally dependent on self-synthesis of the cofactor DPM (di-pyrro-methane) from two molecules of PBG, followed by covalent binding to the active site of the enzyme (Fig. 10.3). Thus, PBGD may exist in a tumor cell in its inactive form, lacking DPM, and in its active DPM bound conformer. The transition from inactive to the active conformer is dependent on the supply of PBG by the second enzyme of the pathway, ALAD. It is conceivable that the ALA supply is essential for the synthesis PBG by ALAD, moreover, PBG synthesis is a prerequisite in the synthesis DPM by dimerizing and activation of PBGD, and a condition for continuing synthesis of coproporphyrin.

Self-activation of PBGD by DPM synthesis following pre-incubation of cells with ALA prior to ALA-PDT may well accelerate PpIX synthesis in neoplastic cells (Fig. 10.9). Thus, it is conceivable that an activation interval of 4–24 h with ALA followed by a second round of ALA supplementation will produce higher amounts of PpIX for standard ALA-PDT even by MDR resistant cancer cells.

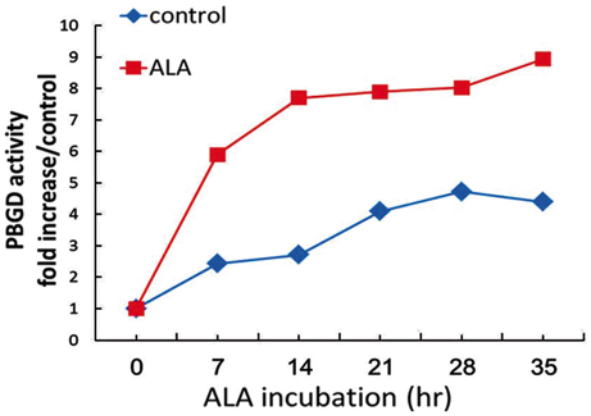

Fig. 10.9 Activation of PBGD enzymatic activity by pre-incubation with ALA. The cells were incubated with ALA (0.1 mg/mL) for the indicated times and enzymatic activity was determined as described [32]

MCF-7/WT and MCF-7/DOX cells were incubated with ALA 0.7 mM for 4 and 24 h, and then a second dose of ALA was supplemented to the medium. PpIX fluorescence was measured by FACS analysis. Figure 10.10, reveals that PpIX synthesis was markedly increased in both cell lines.

Since ALA pre-treatment increased thePpIX levels in all investigated cell lines, we anticipated an increased cell death following ALA pre-treatment and ALA-PDT. MCF-7/WT and MCF-7/DOX cells were treated with ALA for 4, 24 and 48 h followed by another dose of ALA for 4 h. After the two rounds of ALA incubations the cells were irradiated and LDH leakage was measured as a marker of cell death. As can be seen in Fig. 10.11, the MCF-7/WT cells reached their maximal LDH leakage after a single 4 h period of ALA incubation, and did not increase further following ALA pre-treatment, although PpIX level was higher. However, in the

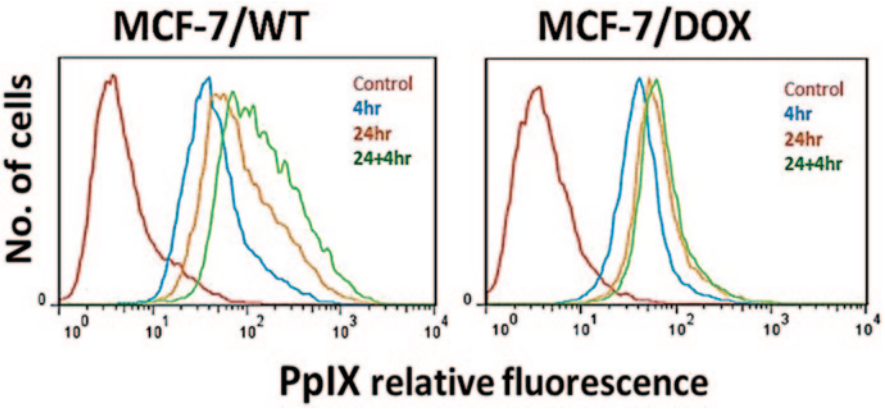

Fig. 10.10 ALA pre-incubations to stimulate PpIX synthesis in MCF-7/WT and MCF-7/DOX cells. MCF-7/WT and MCF-7/DOX cells were incubated with 200 mM ALA for indicated times: 4, 24, and 24 + 4 h (2 ALA incubations). Porphyrin fluorescence was measured by FACS analysis

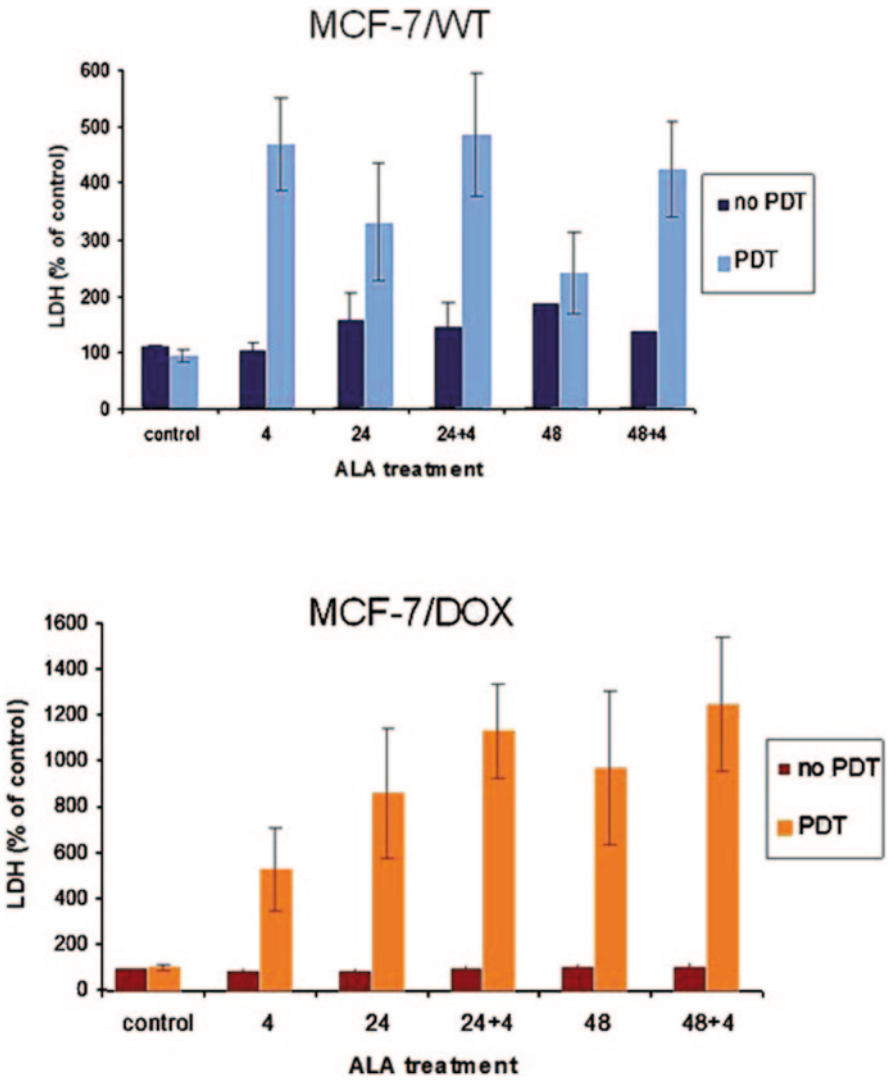

Fig. 10.11 ALA-PDT of MCF-7/WT and MCF-7/DOX stimulated by ALA pre-incubations. MCF-7/WT and MCF-7/DOX cells incubated with 200 mM ALA for indicated times; 4, 24, 48 or 24+4, 48+4 h followed by 410 nm irradiation [31]. LDH leakage was measured 24 h after PDT

MCF-7/DOX sub-line a significant increase in ALA-PDT efficacy is revealed as a result of the activation of PBGD by ALA pre-incubations up to 48 h. While ALA incubation for 4 h increased LDH leakage following PDT by approximately five folds, compared to control cells, 24 and 48 h incubation resulted in an eight and nine folds of increase, respectively, and ALA addition for 4 additional hours further

increased LDH leakage by more than ten folds. This result indicates of a possible way to improve ALA-PDT.

Conclusions and Perspective

First and foremost, ALA-PDT provides an alternative method to the range of cancer therapies, although it should be noted that there are other therapeutic options for these disorders. Of particular interest is the possible combined effect of ALA-PDT with chemotherapy. Yu and Yu [33] reported recently that ALA-PDT impairs tumor-initiating and chemo-resistance properties of head and neck cancer-derived cancer stem cells. We have reviewed here the novel ALA multifunctional derivatives that may alter cancer cell drug resistance. In addition, we showed that improved timing of ALA delivery may maximize sensitizer biosynthesis and PDT results. Moreover, the current concept of enhancing anticancer therapy using multifunctional prodrugs to target different cellular sites simultaneously is central in pharmacology.

Conflict of Interest No potential conflicts of interest were disclosed.

References

1. Hurtig J. Managing patients with advanced and metastatic breast cancer. Clin J Oncol Nurs. 2010;14:313–23.
2. Moore S. Cutaneous metastatic breast cancer. Clin J Oncol Nurs. 2002;6:255–60.
3. Gillet JP, Gottesman M. Mechanisms of multidrug resistance in cancer. Methods Mol Biol. 2010;596:47–76.
4. Gottesman MM. Mechanisms of multidrug resistance. Annu Rev Med. 2002;53:615–27.
5. Wong ST, Goodin S. Overcoming drug resistance in patients with metastatic breast cancer. Pharmacotherapy. 2009;29:954–65.
6. Kovalchuk O, Filkowski J, Meservy J, Ilnytskyy Y, Tryndyak VP, Chekhun VF, Pogribny IP. Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. Mol Cancer Ther. 2008;7:2152–9.
7. Al-Owais MM, Scragg JL, Dallas ML, Boycott HE, Warburton P, Chakrabarty A, Boyle JP, Peers C. Carbon monoxide mediates the anti-apoptotic effects of heme oxygenase-1 in medulloblastoma DAOY cells via K+ channel inhibition. J Biol Chem. 2012;287:24754–64.
8. Capella MA, Capella LS. A light in multidrug resistance: photodynamic treatment of multidrug-resistant tumors. J Biomed Sci. 2003;10:361–6.
9. Mroz P, Yaroslavsky A, Kharkwal GB, Hamblin MR. Cell death pathways in photodynamic therapy of cancer. Cancers (Basel). 2011;3:2516–39.
10. Haupt S, Malik Z, Ehrenberg B. Comparative kinetics of damage to the plasma and mitochondrial membranes by intra-cellularly synthesized and externally-provided photosensitizers using multi-color FACS. Photochem Photobiol Sci. 2014;13:38–47.
11. Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, Hahn SM, Hamblin MR, Juzeniene A, Kessel D, Korbelik M, Moan J, Mroz P, Nowis D, Piette J, Wilson BC, Golab J. Photodynamic therapy of cancer: an update. CA Cancer J Clin. 2011; 61:250–81.

12. Lemoli RM, Igarashi T, Knizewski M, Acaba L, Richter A, Jain A, Mitchell D, Levy J, Gulati SC. Dye-mediated photolysis is capable of eliminating drug-resistant (MDR+) tumor cells. Blood. 1993;81:793–800.
13. Kusuzaki K, Minami G, Takeshita H, Murata H, Hashiguchi S, Nozaki T, Ashihara T, Hirasawa Y. Photodynamic inactivation with acridine orange on a multidrug-resistant mouse osteosarcoma cell line. Jpn J Cancer Res. 2000;91:439–45.
14. Teiten MH, Bezdetnaya L, Merlin JL, Bour-Dill C, Pauly ME, Dicato M, Guillemin F. Effect of meta-tetra(hydroxyphenyl)chlorin (mTHPC)-mediated photodynamic therapy on sensitive and multidrug-resistant human breast cancer cells. J Photochem Photobiol B. 2001;62:146–52.
15. Tang PM, Zhang DM, Xuan NH, Tsui SK, Waye MM, Kong SK, Fong WP, Fung KP. Photodynamic therapy inhibits P-glycoprotein mediated multidrug resistance via JNK activation in human hepatocellular carcinoma using the photosensitizer pheophorbide a. Mol Cancer. 2009;8:56–68.
16. Robey RW, Steadman K, Polgar O, Bates SE. ABCG2-mediated transport of photosensitizers: potential impact on photodynamic therapy. Cancer Biol Ther. 2005; 4:187–94.
17. Malik Z, Lugaci H. Destruction of erythroleukaemic cells by photoactivation of endogenous porphyrins. Br J Cancer. 1987;56:589–95.
18. Malik Z, Lugaci H, Hanania J. Stimulation of friend erythroleukemic cell cytodifferentiation by 5-amino levulinic acid; porphyrins, cell size, segregation of sialoglycoproteins, and nuclear translocation. Exp Hematol. 1988;16:330–5.
19. Ponka P. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. Blood. 1997;89:1–25.
20. Li W, Zhang WJ, Ohnishi K, Yamada I, Ohno R, Hashimoto K. 5-Aminolaevulinic acid-mediated photodynamic therapy in multidrug resistant leukemia cells. J Photochem Photobiol B. 2001;60:79–86.
21. Tsai T, Hong RL, Tsai JC, Lou PJ, Ling IF, Chen CT. Effect of 5-aminolevulinic acid-mediated photodynamic therapy on MCF-7 and MCF-7/ADR cells. Lasers Surg Med. 2004;34:62–72.
22. Feuerstein T, Berkovitch-Luria G, Nudelman A, Rephaeli A, Malik Z. Modulating ALA-PDT efficacy of mutlidrug resistant MCF-7 breast cancer cells using ALA prodrug. Photochem Photobiol Sci. 2011;10:1926–33.
23. Schoenfeld N, Mamet R, Nordenberg Y, Shafran M, Babushkin T, Malik Z. Protoporphyrin biosynthesis in melanoma B16 cells stimulated by 5-aminolevulinic acid and chemical inducers: characterization of photodynamic inactivation. Int J Cancer. 1994;56:106–12.
24. Ickowicz Schwartz D, Gozlan Y, Greenbaum L, Babushkina T, Katcoff DJ, Malik Z. Differentiation-dependent photodynamic therapy regulated by porphobilinogen deaminase in B16 melanoma. Br J Cancer. 2004;90:1833–41.
25. Hanania J, Malik Z. The effect of EDTA and serum on endogenous porphyrin accumulation and photodynamic sensitization of human K562 leukemic cells. Cancer Lett. 1992;65:127–31.
26. Ohgari Y, Nakayasu Y, Kitajima S, Sawamoto M, Mori H, Shimokawa O, Matsui H, Taketani S. Mechanisms involved in delta-aminolevulinic acid (ALA)-induced photosensitivity of tumor cells: relation of ferrochelatase and uptake of ALA to the accumulation of protoporphyrin. Biochem Pharmacol. 2005;71:42–9.
27. Joensuu H, Gligorov J. Adjuvant treatments for triple-negative breast cancers. Ann Oncol. 2012;23:40–5.
28. Berkovitch-Luria G, Weitman M, Nudelman A, Rephaeli A, Malik Z. Multifunctional 5-aminolevulinic acid prodrugs activating diverse cell-death pathways. Invest New Drugs. 2012;30:1028–38.
29. Berkovitch-Luria G, Yakobovitch S, Weitman M, Nudelman A, Rozic G, Rephaeli A, Malik Z. A multifunctional 5-aminolevulinic acid derivative induces erythroid differentiation of K562 human erythroleukemic cells. Eur J Pharm Sci. 2012;47:206–14.

30. Berkovitch G, Doron D, Nudelman A, Malik Z, Rephaeli A. Novel multifunctional acyloxyalkyl ester prodrugs of 5-aminolevulinic acid display improved anticancer activity independent and dependent on photoactivation. J Med Chem. 2008;51:7356–69.
31. Feuerstein T, Schauder A, Malik Z. Silencing of ALA dehydratase affects ALA-photodynamic therapy efficacy in K562 erythroleukemic cells. Photochem Photobiol Sci. 2009;8:1461–6.
32. Schauder A, Feuerstein T, Malik Z. The centrality of PBGD expression levels on ALA-PDT efficacy. Photochem Photobiol Sci. 2011;10:1310–7.
33. Yu CH, Yu CC. Photodynamic therapy with 5-aminolevulinic acid (ALA) impairs tumor initiating and chemo-resistance property in head and neck cancer-derived cancer stem cells. PLoS One. 2014;9:e87129

Chapter 11
Melanoma Resistance to Photodynamic Therapy

Sulbha K Sharma, Ying-Ying Huang and Michael R Hamblin

Abstract Melanoma is a dreaded form of skin cancer caused by the malignant transformation of skin melanocytes and can be highly aggressive and has a rapidly growing incidence and elevated mortality and a poor prognosis at an advanced stage. Because melanomas are intrinsically resistant to conventional radiotherapy and chemotherapy, many alternative treatment approaches are being developed. Photodynamic therapy (PDT) has shown promising results for the treatment of different types of cancer. This therapy involves administration of a photosensitizer (PS), which on excitation after suitable irradiation generates singlet oxygen and other cytotoxic reactive oxygen species (ROS), thus, killing the cancer cells. Unfortunately, melanoma is considered to be resistant to PDT. There are many different reasons for this resistance including: (1) optical interference by melanin; (2) the anti-oxidant effect of melanin (3) sequestration of PS inside melanosomes (4) efflux of PS by multi-drug transporters and (5) errors in apoptotic pathways. Various approaches to overcome this PDT resistance of melanoma are being evaluated such as the use of agents that overcome the apoptotic defects, or hinder the efflux of PS, or use of methods to reduce the quantity or the pigmentation of the melanin. The introduction of highly active PS absorbing in the 700–800 nm near infrared (NIR) spectral region, and new advances in two-photon excitation of PS, together with PS linked to upconverting nanoparticles may overcome the optical interference of melanin. Finally, employing immunotherapy and, thus, exploiting the ability of PDT to activate the host immune system against the treated tumor, may also play a role in allowing PDT to overcome resistant melanomas.

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Abbreviation

ABC	ATP-binding cassette
APAF-1	apoptosis protease-activating factor-1
MDR	multidrug resistance
MAPK	mitogen-activated protein kinase
NIR	near infrared
PDT	photodynamic therapy
PS	photosensitizers
UV	ultraviolet

Introduction

Melanoma is a rapidly spreading malignant tumor of the skin arising from the melanocytes which are transformed into malignant melanoma. Melanoma accounts for around 13,000 deaths with an overall survival of 8–18 months [1]. The main factors responsible for the malignant transformation in melanoma are environmental as well as genetic. Though ultraviolet (UV) exposure intermittently is known to cause tanning that protects the skin from chronic DNA damage, intense exposure can cause sunburn and can lead to sudden DNA damage and genetic alterations in melanocytes. Melanin pigment produced by melanocytes plays a critical role in camouflage, mimicry, social communication, and protection against harmful effects of solar radiation [2]. Melanin is known to protect the skin against UV-induced skin damage through its optical and chemical filtering properties. The treatment options available for metastatic melanoma are chemotherapy alone or in combination with other therapy, immunotherapy based on cytokines such as interferon and interleukin-2, or monoclonal antibody targeted therapy [3]. Unfortunately, melanoma due to its intrinsic resistance mechanisms is poorly responsive to standard therapies. Thus, various experimental therapies are being explored [4]. Though some new therapies show hope for melanoma treatment such as targeted therapy [5] and immunotherapy, [6] and in recent years photodynamic therapy (PDT) has also shown some encouraging results in the management of melanoma [7].

Different Resistance Mechanisms of Melanoma to Standard Therapies

Most of the available anticancer treatments are not effective for melanoma, due to a complex array of resistance mechanisms compared to many other cancer types. Various reasons for this multi-faceted resistance are explained below.

Dysregulation of the Apoptotic Pathways

Inactivation of programmed cell death pathways is not unique to melanoma, but is a 'hallmark' of cancer [8]. Various genetic, functional and biochemical studies suggest that melanoma cells become 'bullet proof' against a variety of chemotherapeutic drugs by exploiting their intrinsic resistance mechanism and by reprogramming their proliferation and survival pathways during melanoma progression [9]. New insights have been developed into melanoma resistance to chemotherapy by identifying the molecules involved in the regulation and execution of apoptosis, and their alteration in melanoma. The main pathways involved are the inactivation of pro-apoptotic factors or the over-expression of anti-apoptotic factors. In the case of melanoma, the gene responsible for the cancerous transformation of melanoma is *apoptosis protease-activating factor-1 (APAF-1),* a critical downstream effector of the p53-dependent mitochondrial apoptotic pathway which is deleted or inactivated by methylation in metastatic melanomas. Defects in Apaf-1 in cells significantly reduces p53-dependent apoptosis and promotes oncogenic transformation [10]. Moreover, anti-apoptotic factors such as Bcl-2 can be activated or upregulated and there may be defects in a pro-apoptotic pathway or a pro-apoptotic factor like BAX, which would lead to the failure of the treatment regimen that induces apoptosis [9]. With this understanding, the challenge now is to develop new approaches to overcome or bypass these cell death defects and improve the actual poor prognosis of patients.

Melanogenesis-mediated MDR

Melanogenesis is the process of synthesis of melanin by the melanosomes which are subcellular organelles related to the lysosome. These melanosomes play a key role in melanin synthesis and are also concerned with drug trapping and export [11]. The presence of these melanosomes and melanin in the melanoma are responsible for the multidrug resistance (MDR) in melanoma. For this reason, melanoma is resistant to many conventional therapies such as chemotherapy and radiotherapy while non-melanoma cancers are relatively sensitive. Melanogenesis is involved in the regulation of drug sensitivity. The process of melanogenesis involves first the biogenesis of melanosomes, synthesis of melanin and homeostasis-associated endogenous melanogenic cytotoxicity (EMC). There are four stages of melanosome

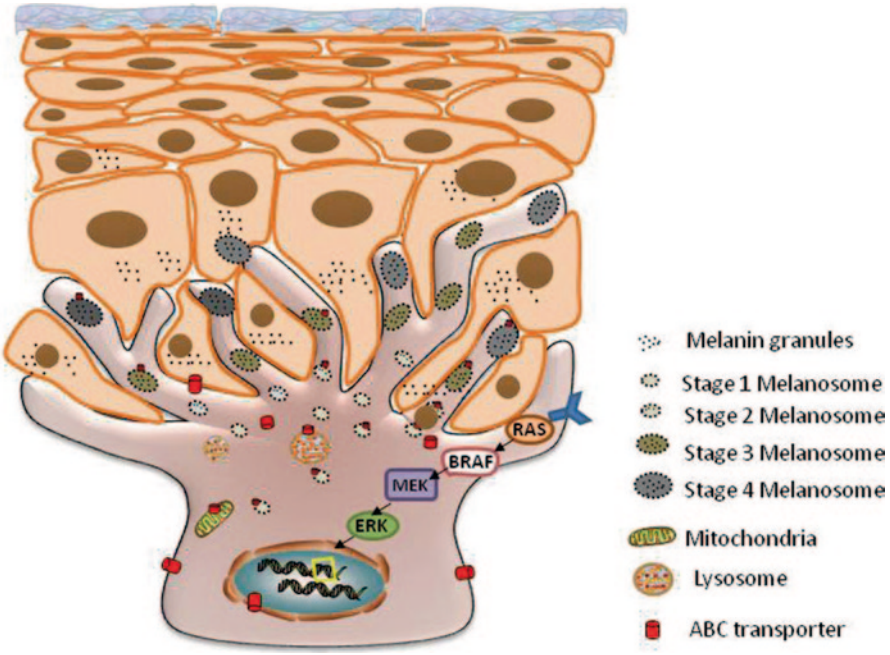

Fig. 11.1 Process of melanogenesis and resistance mechanisms of melanoma. Melanosomes mature through stages 1–4 and are finally transferred to keratinocytes where they release melanin granules. Defects in apoptosis (BCL2), activation of BRAF, and ABC-transporter drug efflux pumps contribute to melanoma resistance

biogenesis which are completed in three phases of melanogenesis (Fig. 11.1). In Phase I the melanosomes are at an early stage I and II and are termed as "premelanosomes" and these premelanosomes are responsible for trapping and exporting the drugs such as cisplastin. In Phase II, the melanosomes are in stage III of melanosome biogenesis. Stage III is marked with the active synthesis of melanin and this nascent melanin has the highest capacity to entrap cytotoxic drugs and, thus, these melansosomes are likely to be concerned with drug resistance. In Phase III, the melanosomes are at stage IV where they are known to produce endogenous melanogenic cytotoxic by-products resulting in an autophagic effect on damaged melanosomes. This effect causes the melanosomes and melanoma cells to be more susceptible to cytotoxic drugs. Finally, these fully formed melanosomes are transferred to the keratinocytes through the melanocyte dendrites and they form melanin granules which act as the defense against the ultraviolet from the sun. Some enzymes involved in melanogenesis are also responsible for resistance in melanoma cells. One such enzyme is the tyrosinase-related protein-2 [11].

DNA Repair as a Resistance Mechanism

Since many anti-cancer drugs act on the DNA, some tumor cells have developed mechanisms that work against the DNA damage by repairing it. This is done by hyper-activation of DNA repair mechanisms or by upregulating mismatch repair genes or by activating enzymes that restore DNA-alkylation damage [9]. The upregulation of DNA repair mechanism was demonstrated in cisplastin resistant human melanoma cells [12]. Again, reports in the melanoma literature showed conflicting data. For instance, mismatch repair genes can be found upregulated [13] or downregulated in melanomas [14].

ABC Transporter Mediated MDR

The ATP-binding cassette (ABC) transporters are responsible for the transporting of various molecules across the biological membranes in an ATP-dependent manner. These ABC transporters are present both on the cell membrane and in subcellular organelles. Forty-eight different types of these transporters are known in the human genome. ABC transporters that are present in human cancer cells are the root cause of MDR as they are responsible for the efflux of anticancer drugs [11]. Different groups of ABC transporters are found in melanoma but the most commonly expressed is ABC5. It was reported that on transfection of cell lines with this ABC transporter, melanoma showed increased drug resistance [15]. Chen et al. concluded that over-expression of ABC5 transporter is possibly the main cause of MDR in melanoma cells. It was also shown that the ABCB5 positive subpopulation of cells when injected in a mouse were more tumorigenic than the ABCB5 negative cells. As mentioned above, melanoma cells exploit the additional mechanisms of using melanosomes for entrapping cytotoxic drugs as compared to other non-melanoma cancer cells. The increased expression of these ABC transporters in subcellular organelles would prevent importation of cytotoxic drugs, protecting the cells from late-stage melanogenic cytotoxicity

Mechanism of Resistance to MAPK Pathway Inhibitors

Kinase pathways are important in regulating the cell cycle progression. Among these kinases, the mitogen-activated protein kinase (MAPK) pathway plays an important role particularly in melanoma [16]. This pathway is known to be activated in human tumors through a cascade of events. This cascade involves RAF, MEK (MAPK kinase), and ERK (extracellular signaling regulated kinase). RAF kinase ((ARAF, BRAF, and CRAF/RAF1) is activated by RAS and GTPase protein. RAF on activation initiates a cascade of phosphorylation of MEK and ERK. Phosphorylated ERK goes on to further phosphorylate transcription factors which aid in cell

proliferation [17]. Fifty percent of melanomas have BRAF mutation and, thus, modern targeted therapies exploit this in the form of BRAF inhibitors [18].

PDT and Melanoma

PDT is a minimally invasive clinically approved therapy that causes selective destruction of cancer cells. PDT involves the administration of a photosensitizing agent followed by irradiation with appropriate wavelength light corresponding to an absorbance band of the sensitizer resulting in production of ROS. The ROS generated by the PS are responsible for the selective tumor destruction, tumor-associated vascular damage, and activation of antitumor immune responses [19]. PDT is an effective treatment for several different types of cancers [7] and has also become an established treatment modality for dermato-oncologic conditions like actinic keratosis, Bowen's disease, in situ squamous cell carcinoma and superficial basal cell carcinoma [20]. The first study to evaluate the efficacy of PDT for melanoma was done in the year 1988. A comparison study of the effects of PDT with Photofrin II on human xenografts of amelanotic and melanotic malignant melanoma in the athymic nude mouse model was performed. The results indicated that human xenograft melanotic melanoma, as compared to amelanotic melanoma, is far less responsive to PDT. The authors reported that the resistance of malignant melanotic melanoma to PDT was due to the melanin pigment that competed with the PS for the absorption of photons [21]. Thus, the presence of melanin, a stable protein-complex with a broad absorption spectrum, in the same tissue, competes with PS for available photons resulting in lower phototoxicity. Thus, there was perceived to be a need to work with different PS with absorption wavelengths at longer wavelengths than those absorbed by the melanin pigment. Thus, the second generation PS were used such as Si(IV)-naphthalocyanine, bacteriochlorin(a) and Lu(III)-texaphyrin, characterized by absorption at the 750–800 nm spectral band which enhanced the efficacy of PDT on experimentally implanted melanotic melanoma [22–24]. It was demonstrated by Busetti et al. that melanosomes when preirradiated with high peak power pulsed laser radiation at 1064 nm, PDT efficacy was enhanced [25]. Phase I clinical trials were performed in 14 patients with chlorin(e6) on skin melanoma metastases in humans. PDT with chlorin(e6) for skin metastases from pigmented melanoma was well tolerated and effective, especially in cases of isolated melanoma skin metastases. [26]. A transgenic model of skin melanoma was developed. These results showed that, even though metallothionein-I/ret (MT-ret) melanoma cells are vulnerable to 5-ALA PDT *in vitro*, malignant metallothionein-I/ret (MT-ret) melanomas *in vivo* were quite resistant to this type of therapy at doses which are highly effective *in vitro* [27].

PDT is a potentially effective therapy for themanagement of melanoma and much understanding has been developed during these years of extensive research; still, additional research will be needed to conquer the resistance of melanoma to PDT. There remains the need for the development of novel and effective approaches

to treat melanoma via PDT, and this therapy could also be applied as an adjuvant therapy alone or in combination with current therapeutics to combat melanoma [28]. To better understand the cause of resistance of melanoma to PDT different resistance mechanisms of melanoma to PDT are discussed below followed by the approaches to overcome them.

Different Resistance Mechanisms of Melanoma to PDT

Resistance to PDT Due to Presence of Melanin

It has been reported that the melanin pigment is responsible for the resistance of melanoma towards PDT [29]. This could be due to the reason that melanin could act as an optical shield preventing the light from penetrating the lesion. Besides this, melanin can also scatter or absorb the incident light [30]. Melanin is known to be the dominant absorber at wavelengths between 500–600 nm and transmittance in melanoma with melanin only occurs above 700 nm [31]. With a few PS, such as hypericin, competitive absorbance is observed [30]. This is shown even with PDT with Photofrin. One way to avoid the interference of melanin is by using the second generation PS which absorb in the range of 750–800 nm where the absorption of melanin is minimal. Some of these PS are Si(IV)-naphthalocyanine, bacteriochlorins(a) and Lu (III)-texaphyrin [25, 32].

Anti-oxidant Defense Mechanisms of Melanin

Another important role that melanin plays is as an ROS scavenger and it can act as an intracellular antioxidant [33]. A direct correlation is seen between the amount of melanin in melanoma and resistance to damage by ROS [30]. Suzukawa et al showed that both types of melanin (eumelanin and pheomelanin) led to DNA breakage in the absence of light irradiation, and that eumelanin was more harmful than pheomelanin. Interestingly, melanins were found to bind to the minor grooves of DNA, guaranteeing close proximity to DNA and potentially causing the observed high levels of strand breaks. They also showed that the interaction of melanin with DNA could impair the access of repair enzymes to lesions, contributing to the perpetuation of DNA damage. Moreover, after melanins interacted with singlet oxygen they exhibited a lower ability to induce DNA breakage which was proposed to be part of the melanoma PDT-resistance mechanism [34].

The mechanism of action of PDT on the cells is by generation of oxidative stress and cytotoxicity through generation of ROS. Oxidative stress is known to stimulate internal antioxidant systems, which could be another limitation to PDT efficacy. Thus, inhibition of melanin synthesis when combined with PDT could be a good therapeutic approach to treat melanoma. Melanin synthesis involves a series

of enzyme reactions and tyrosinase (TYR) is the rate-limiting enzyme in the synthesis of melanin. Phenylthiourea (PTU) is an inhibitor of the tyrosinase enzyme. Hypericin-mediated PDT when combined with PTU increased the susceptibility of melanoma cells to HYP-PDT. When PTU was removed and melanin formation was allowed, the pigmented melanoma cells showed an increased resistance to PDT-induced cell death. Another important aspect could be the concentration of thiobarbituric acid-reacting substances (TBARS), which are important markers of PDT damage, and melanin is known to keep the concentration of TBARS constant. It was demonstrated in one study that melanin could play a protective role in the hypericin-mediated photodamage of membrane lipids [30].

Melanosomes Protect Melanocytes and Melanoma Cells Against Harmful Effects of Toxic Intermediates

Melanosomes are responsible for defending the cells from harmful toxic materials that arise as by-products of melanin biosynthesis. Melanosomes are known to capture the toxic molecules and prevent them from spilling into the cytoplasm [33, 35]. These melanosomes are also a target for PDT and are responsible for the difference in the type of cell death in pigmented and non-pigmented melanomas. This association has been observed in one study where different modes of cell death were induced in melanotic and amelanotic cells; necrosis in case of pigmented and apoptosis in case of non-pigmented cells [36]. However, both types of melanoma cells demonstrated a similar cytoprotective mechanism through the initiation of autophagy, which is a cell survival program of cells experiencing environmental stress [35].

ABCG2 Transporters Acting as Efflux Pumps

The ATP-binding cassette (ABC) transporters are responsible for transporting various molecules across biological membranes and are present both on the cell membrane and the membranes of subcellular organelles and are the root cause of multidrug resistance as they are also responsible for the efflux of anticancer drugs [11]. δ-aminolevulinic acid (ALA) (one of the most common clinically used PS) induces the cell synthesis of the PS, protoporphyrin (PPIX [37]. PPIX is normally synthesized in cells and is important for normal cell function as a precursor of heme, but if it accumulates in more than normal amounts it is toxic and phototoxic for the cells. Thus, expulsion of the extra amount of PPIX is required, which is the role of ABCG2 transporter heme efflux system. This role of the ABCG2 also renders the cancer cells to be multidrug resistant and resistant to PDT. Methotrexate, a substrate of ABCG2 transporter, when combined with ALA-PDT enhances the PDT effect [38]. This could be due to the interference of methotrexate with the porphyrin transport by ABCG2. ABCG2 inhibitors such as Ko-134 are also known to increase the sensitivity of cancer cells to PDT [37].

Overcoming Melanoma Resistance to PDT

Different strategies have been adopted to overcome the PDT resistance of melanoma such as using PS absorbing in the far-red and near-infrared regions where the absorption and scattering of melanin is negligible. Decreasing the amount of melanin in the cells is another approach. Besides these, immunotherapy and various other therapies can be combined to offer a new hope in advanced melanoma.

Near Infrared Absorbing Photosensitizers

Melanin absorbs and scatters in the full visible range (400–700 nm). Most of the clinically approved PS absorb below 700 nm and, thus, there is a need for newer PS absorbing in the near infrared region (NIR) (Fig. 11.2). One new family of PS that absorb in this 2 region is that of bacteriochlorins. Bacteriochlorins were

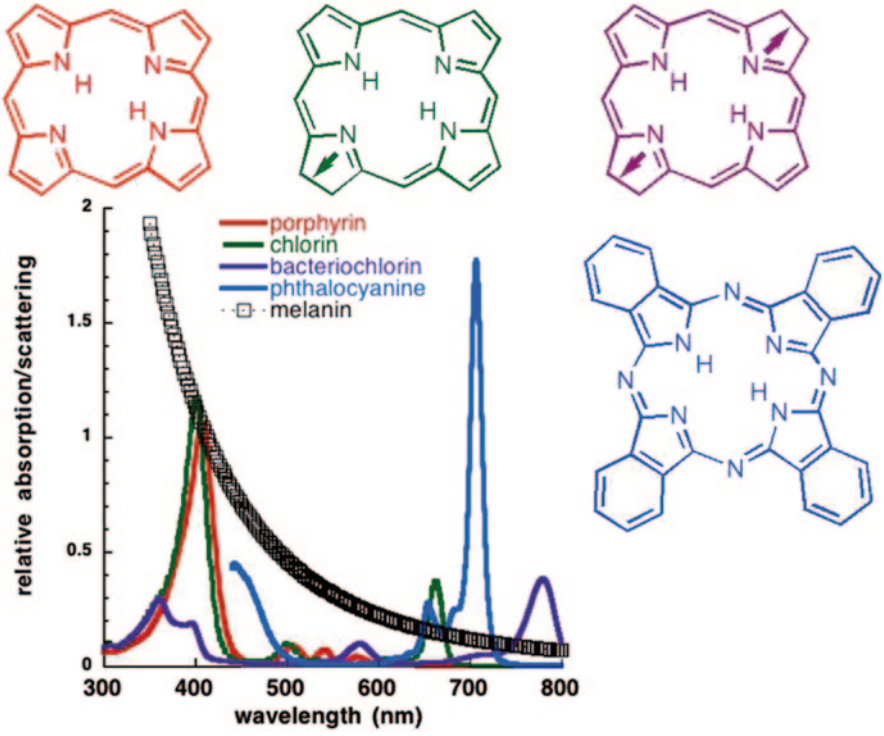

Fig. 11.2 Absorption/scattering spectra of melanin and 1st and 2nd generation photosensitizers**.** PS with substantial peaks in the NIR spectral region (>660 nm) such as chlorins, bacteriochlorins and phthalocyanines avoid optical interference by melanin that inhibits porphyrin PDT

originally isolated from natural products, but were found to be rather unstable and especially easily photobleached. Recently, newer stable synthetic bacteriochlorins have been prepared which demonstrate good photophysical properties and have proved to be potential photodynamic PS [39, 40]. In one study, tumor growth inhibition was observed in S91 melanoma cells with a water-soluble bacteriochlorin, 5,10,15,20-tetrakis(2-chloro-5-sulfophenyl) bacteriochlorin (TCBSO3H) [41]. In another study, [42] a group of synthetic bacteriochlorins stabilized from re-oxidation with gem-dimethyl groups was studied in different pigmented mouse melanoma cell lines and it was shown that all bacteriochlorins, were localized in melanosomes which led to the destruction of these melanosomes and death of the melanoma cells. Among these bacteriochlorins, the one with best *in vitro* results (bacteriochlorin 3) was evaluated *in vivo* against a B16F10 mouse melanoma model that expressed green-fluorescent protein resulting in a marked reduction in tumor size and significant survival advantage with 20% of cures as shown in Fig. 11.3. Several non-bateriochlorin PS absorbing in the NIR region have also been evaluated for melanoma treatment. A diamagnetic water-soluble lutetium texaphyrin (PCI-0123) was evaluated for the treatment of heavily pigmented metastatic B16F10 melanoma. This PS caused tumor apoptosis with a considerable tumor regrowth decay and enhanced the survival time [24].

Upconverting Nanoparticles

PDT based on upconversion nanoparticles (UCNPs) has received increasing attention in recent years. Excited with NIR light, UCNPs are able to emit higher-energy lower wavelength visible light, which can activate surrounding PS molecules to produce cytotoxic singlet oxygen. The rare-earth (erbium, yttrium and ytterbium) containing nanoparticles are able to absorb two NIR photons via a long-lived metastable intermediate state that then emits a single high-energy photon, so they work with relatively low power CW light. Since NIR light has a higher penetration in melanin-containing tissue, NIR-excited UCNPs can be used to activate PS molecules in much deeper tissues compared to traditional PDT excited with visible or ultraviolet (UV) light. Recently, Idris et al. [43] demonstrated for the first time *in vivo* tumor-targeted PDT using UCNPs. UCNP-based PDT has been successfully demonstrated *in vivo* at first time, showing an encouraging therapeutic effect upon either local injection or systemic administration of UCNP-PS nanocomplexes. NaYF4 (Yb:Er) UCNPs were coated with mesoporous silica and co-loaded with zinc phthalocyanine (ZnPc) and merocyanine 540 (MC540). UCNPs excited by 980 nm laser had two main peaks, green (~540 nm) and red (~660 nm) emissions, which matched well with the absorption of two types of PS molecules for fully utilization of upconverted energy to maximize the PDT efficiency (Fig. 11.4). ZnPc/MC540 co-loaded UCNPs resulted in a high rate of generation of $^1O_2$ in solution as well as in live cells under the NIR light treatment. They confirmed the PDT efficacy in C57BL/6 mice bearing B16F10 melanoma tumors. A good tumor regression effect was observed after the tumors were exposed to the NIR laser after intratumorally

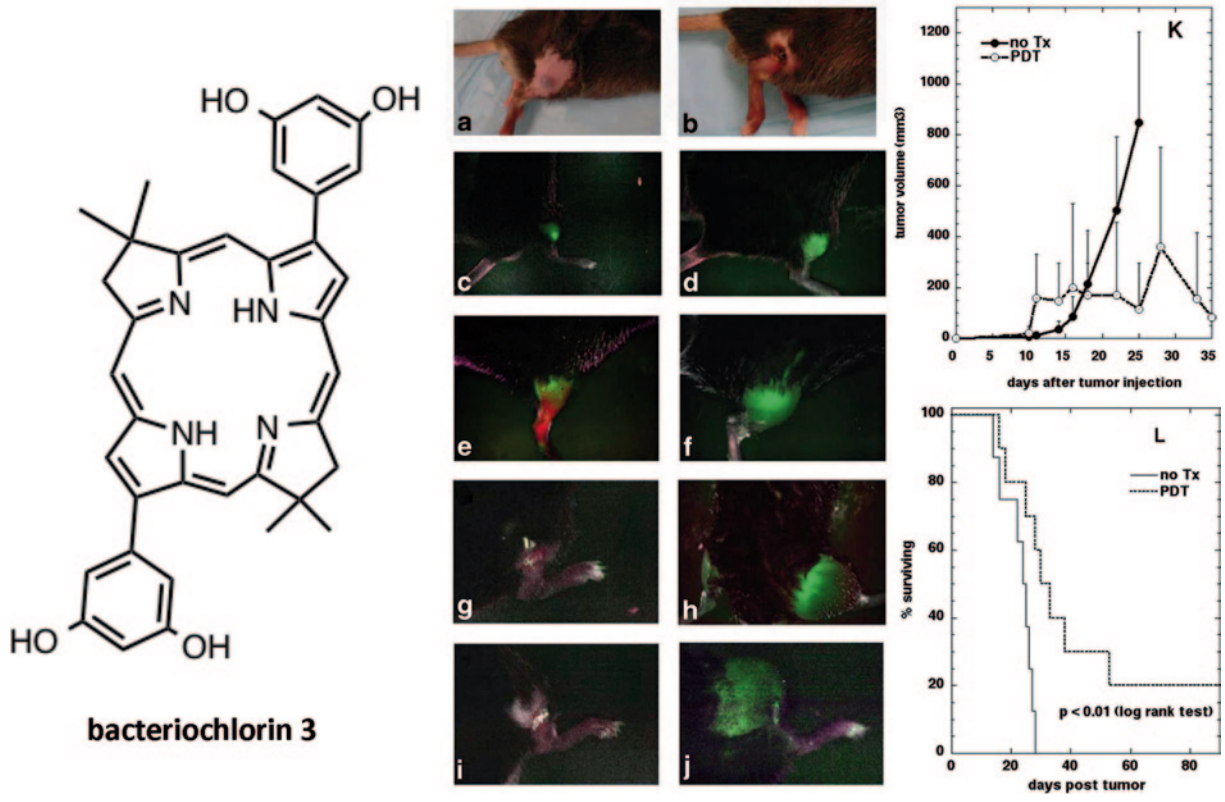

Fig. 11.3 The PDT effects of bacteriochlorin 3 on GFP positive B16F10 melanoma tumors [34]. **a** B16F10-GFP subcutaneous tumor on day 7 before PDT treatment. **b** B16F10-GFP tumor on day 13 after PDT treatment. **c** In vivo fluorescence imaging of B16F10-GFP tumor immediately after inoculation. **d** GFP signal from tumor on day 7 before PDT. **e** Two-color imaging of 3 (*red*) and B16F10-GFP tumor (*green*) 15 min post IV injection on day 9. **f** Control tumor on day 13. **g** PDT-treated tumor on day 13. **h** Control tumor on day 23. **i** PDT-treated tumor on day 23. **j** Regrowth of a PDT-treated tumor on day 26. **k** Survival analysis of in vivo PDT effectiveness with bacteriochlorin 3 on B16F10-GFP tumors; solid line no treatment ($n=8$), dotted line PDT ($n=10$). $p<0.01$ (log rank test). Reprinted with permission from [34]

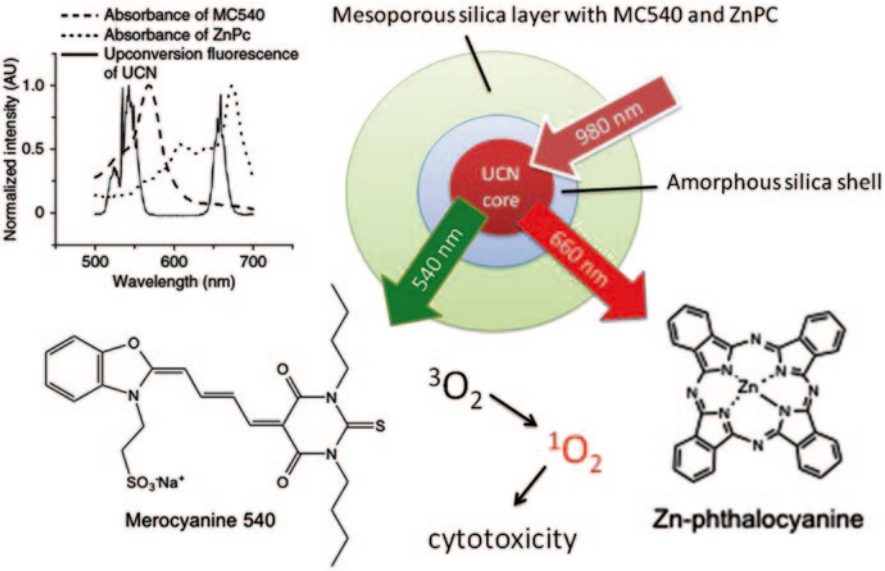

Fig. 11.4 Mesoporous-silica–coated NaYF4:Yb, Er UCN containing MC540 and ZnPC as a photosensitizer for melanoma [26]. The fluorescence emission spectra of the UCN after 980 nm NIR laser excitation and the structure and absorption spectra of ZnPc and MC540 photosensitizers. Schematic of mesoporous-silica–coated UCN coloaded with ZnPc and MC540After excitation by 980 nm light, the UCN can upconvert NIR light to visible light of different wavelengths. Spectral overlap between the emitted light and the maximum absorption wavelengths of the respective loaded photosensitive drugs activates the dual combination of photosensitizers to generate an elevated amount of cytotoxic singlet oxygen

injecting ZnPc/MC540 co-loaded UCNPs. They further examined the targeted PDT efficacy using PEGylated UCNPs conjugated with folic acid. A significant reduction was observed in B16F10 tumor growth of the treatment group with a 980 nm laser at 4 h post-injection of ZnPc/MC540 co-loaded FA-PEG-UCNPs, compared to control mice treated with PBS. Further efforts in this field may enable a new photodynamic therapeutic approach with greatly improved tissue penetration, suitable for treatment of relatively large or internal tumors.

Two-Photon PDT

Since one cause of this resistance is the interference with PS optical absorption by the black pigment melanin, an alternative approach to overcoming the resistance is the use of NIR-absorbing PS, that use light no longer absorbed by melanin [42]. Two photon absorption (2PA) is a nonlinear optical process, in which simultaneous absorption of two photons at wavelength practically in the NIR region to promote a molecule from one state (usually the ground state) to a higher energy electronic excited state with the sum energy of the two-photons. Khurana et al. [44] used

endothelial cells to assess the TPE-PDT efficacy of PS approved for one photon PDT—namely, Photofrin and verteporfin. While utilizing clinically approved PS for TPE-PDT is very attractive, their small two-photon absorption may be limiting in the clinical context. Spangler et al. [45] synthesized a proprietary tetrapyrrole-based PS (MPA79), by attaching two separate 2PA-enhancing functionalities, bis(diphenylamino)distyrylbenzene groups (each containing two triphenylamine groups) and two peptide-targeting moieties to a central porphyrin core (Fig. 11.5). Irradiation of photosensitized tumors was undertaken through the whole bodies of tumor-bearing mice to give a treatment depth of 2 cm. Regressions of all tumor types tested were seen in human small cell lung cancer (NCI-H69), non-small cell lung cancer (A549), and breast cancer (MDA-MB-231) xenografts induced in SCID mice. Although there has been no report for 2PA-PDT on melanoma, it could be a potential application for melanoma in the future since the therapeutic window for 2PA-PDT is between 780–900 nm and this wavelength bypasses melanin.

Depigmentation Strategies

Melanin present in the melanoma cells is responsible for the protection of cells against the PDT induced ROS and causes optical interference with the therapeutic light used in PDT. These two factors are responsible for the development of resistance in melanoma cells against PDT and also for the difference between the treatment outcome of PDT in pigmented and non-pigmented melanomas [42]. Strategies that could remove the pigmentation in melanoma could be used to overcome this drawback [33]. Inhibition of melanogenesis by the tyrosine enzyme inhibitor phenylthiourea (PTU) was applied for this purpose and increased the susceptibility to PDT. Photobleaching of melanin may also gave a better PDT effect. In one study by Ma et al. melanin was photobleached with 420 nm light which increased the susceptibility of melanomas towards ALA-PDT by using red 635 nm light in B16F10 melanoma-bearing mice [46]. Bussetti et al. [47] used a single 650 mJ pulse (10 ns) of 1064 nm light from a Q-switched Nd: YAG laser to bleach the pigmented melanoma, thereby, increasing the effectiveness of PDT. They used a B16 pigmented melanoma subcutaneously transplanted in C57 mice with a single 650 mJ pulse (10 ns) of 1064 nm light from a Q-switched Nd: YAG laser caused instantaneous bleaching of the pigmented tissue. Visual and histological examination of the resulting gray-colored tumor revealed the breakdown of melanosomes with no detectable alteration of the normal and tumor-overlying skin. Histological examination of the irradiated tumor showed some degree of vascular damage; the depth of the photodamage was not affected by the successive delivery of three consecutive light pulses. The bleached tumor grew at a modestly slower rate but the high-peak-power (HPP) laser treatment did not affect the tumor concentration of a treatment of the B16 pigmented melanoma by photodynamic therapy (PDT: 1 mg/kg isoBO-SiNc, 300 mW/cm^2, 520 J/cm^2) from a 774 nm diode laser immediately after the 1064 nm irradiation resulted in a 16 day delay of tumor regrowth, which was markedly longer than the delay (ca 6 days) obtained after PDT under identical conditions without the preirradiation.

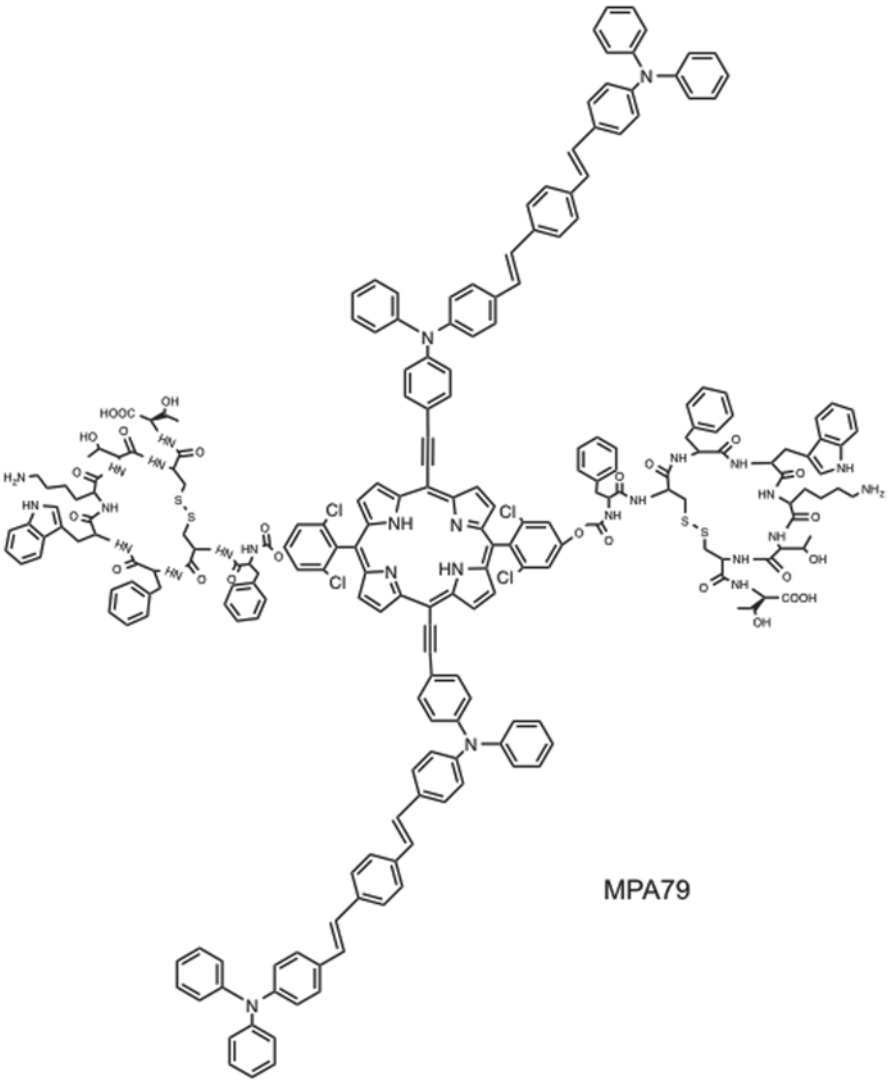

Fig. 11.5 Structure of a porphyrin-based targeted PS with a high two-photon cross—section [49]. The central porphyrin core bears two bis(diphenylamino)distyrylbenzene groups attached to the meso position via ethynyl linkers giving 2-photon cross sections of 2000 GM units. Attached to the other two meso-positions are 2,6-dichlorophenyl groups designed to enhance intersystem crossing to improve the triplet yield. Further attached are two somatostatin receptor (SST2r and SST5r) targeting peptides

Combination with Hyperthermia

Hyperthermia is a treatment regimen in which the cells are exposed to elevated temperatures (40–45 °C). Hyperthermia has been used in combination with radiotherapy

as well as with chemotherapy. Hyperthermia has also been tried in combination with PDT and has been reported to induce lipid peroxidation by a thermal effect and the induction of early apoptosis in the murine melanoma B16F10 [48]. A synergistic effect was also achieved by heating cells after AlPCS-PDT treatment [49].

Immune Stimulation Strategies

PDT when combined with immunostimulatory strategies is known to have a synergistic effect. Different immune stimulatory molecules and substances have been used for this approach eg. imiquimoid (a toll-like receptor 7 agonist) that stimulates dendritic cells. This combination has also been used for the treatment of melanoma and good results have been observed [50]. Indocyanine green when combined with topical imiquimod increased survival up to 70% in human melanoma patients [42]. Besides a local response, metastatic nodules also demonstrated a good immune response. Injection of dendritic cells in the tumor with PDT showed an effective prominent antitumor effect and a strong systemic antitumor immunity. PDT is known to produce acute inflammation, release tumor antigens, and attract and stimulate dendritic cells into the tumors after treatment [19]. The *in vitro* loading of dendritic cells combined with PDT was reported to induce tumor-specific immunity and led to destruction of targeted tumors together with regression of distant tumors. This effect was demonstrated in the B16 tumor model with excellent and long-lasting tumor-specific results [51].

Conclusion

Since the beginning of clinical exploration of anticancer PDT, it has been assumed and/or claimed that pigmented melanomas are particularly resistant to PDT. While there is of course a considerable amount of truth in that generally-held belief, it is by no means the whole story. While some of the resistance mechanisms are common to other cancer therapies as well as PDT (defects in apoptosis, upregulated DNA repair, MDR efflux pumps, drug sequestration in melanosomes) others are much more specific to PDT (optical interference of melanin and antioxidant effect of melanin). Since the whole oncology community at large is devoting considerable efforts towards discovering targeted approaches to overcome cancer resistance to treatment, it is expected that PDT will benefit from these discoveries as they emerge. Moreover specific strategies to overcoming PDT specific resistance are becoming known. Certainly the search for highly active PS absorbing in the 700–800 nm range will continue and 2-photon activation of PS will become more widely studied as high-power femtosecond lasers become more available and affordable. Melanin reduction strategies will also continue to be studied. Finally it is being predicted that the age of immunotherapy for cancer has finally arrived as highly active antibodies can overcome the evasion mechanisms that tumors (especially

melanomas) have developed to avoid the host response [52]. The known activity of PDT in stimulating anti-tumor immunity suggests it would be a very good idea to combine PDT with these newly introduced agents.

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References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics 2010. CA Cancer J Clin. 2010;60:277–300.
2. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev. 2004;84:1155–1228.
3. Tarhini AA, Agarwala SS. Cutaneous melanoma: available therapy for metastatic disease. Dermatol Ther. 2006;19:19–25.
4. Jilaveanu LB, Aziz SA, Kluger HM. Chemotherapy and biologic therapies for melanoma: do they work? Clin Dermatol. 2009;27:614–25.
5. Shah DJ, Dronca RS. Latest advances in chemotherapeutic, targeted, and immune approaches in the treatment of metastatic melanoma. Mayo Clin Proc. 2014;89:504–19.
6. Atkins MB, Lotze MT, Dutcher JP, Fisher RI, Weiss G, Margolin K, Abrams J, Sznol M, Parkinson D, Hawkins M, Paradise C, Kunkel L, Rosenberg SA. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. J Clin Oncol. 1999;17:2105–16.
7. Baldea I, Filip AG. Photodynamic therapy in melanoma–an update. J. Physiol Pharmacol. 2012;63:109–18.
8. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57–70.
9. Soengas MS, Lowe SW. Apoptosis and melanoma chemoresistance. Oncogene. 2003;22:3138–51.
10. Campioni M, Santini D, Tonini G, Murace R, Dragonetti E, Spugnini EP, Baldi A. Role of Apaf-1, a key regulator of apoptosis, in melanoma progression and chemoresistance. Exp Dermatol. 2005;14:811–8.
11. Chen KG, Valencia JC, Gillet JP, Hearing VJ, Gottesman MM. Involvement of ABC transporters in melanogenesis and the development of multidrug resistance of melanoma. Pigment Cell Melanoma Res. 2009;22:740–9.
12. Runger TM, Emmert S, Schadendorf D, Diem C, Epe B, Hellfritsch D. Alterations of DNA repair in melanoma cell lines resistant to cisplatin, fotemustine, or etoposide. J Invest Dermatol. 2000;114:34–9.
13. Rass K, Gutwein P, Welter C, Meineke V, Tilgen W, Reichrath J. DNA mismatch repair enzyme hMSH2 in malignant melanoma: increased immunoreactivity as compared to acquired melanocytic nevi and strong mRNA expression in melanoma cell lines. Histochem J. 2001;33:459–67.
14. Korabiowska M, Brinck U, Dengler H, Stachura J, Schauer A, Droese M. Analysis of the DNA mismatch repair proteins expression in malignant melanomas. Anticancer Res. 2000;20:4499–505.
15. Chen KG, Szakacs G, Annereau JP, Rouzaud F, Liang XJ, Valencia JC, Nagineni CN, Hooks JJ, Hearing VJ, Gottesman MM. Principal expression of two mRNA isoforms (ABCB 5alpha and ABCB 5beta) of the ATP-binding cassette transporter gene ABCB 5 in melanoma cells and melanocytes. Pigment Cell Res. 2005;18:102–12.
16. Eigentler TK, Meier F, Garbe C. Protein kinase inhibitors in melanoma. Expert Opin Pharmacother. 2013;14:2195–201.

17. Nissan MH, Solit DB. The "SWOT" of BRAF inhibition in melanoma: RAF inhibitors, MEK inhibitors or both? Curr Oncol Rep. 2011;13:479–87.
18. Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, Cho H, Spevak W, Zhang C, Zhang Y, Habets G, Burton EA, Wong B, Tsang G, West BL, Powell B, Shellooe R, Marimuthu A, Nguyen H, Zhang KY, Artis DR, Schlessinger J, Su F, Higgins B, Iyer R, D'Andrea K, Koehler A, Stumm M, Lin PS, Lee RJ, Grippo J, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, Chapman PB, Flaherty KT, Xu X, Nathanson KL, Nolop K. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. Nature. 2010;467:596–9.
19. Castano AP, Mroz P, Hamblin MR. Photodynamic therapy and anti-tumor immunity. Nat Rev Cancer. 2006;6:535–45.
20. Babilas P, Schreml S, Landthaler M, Szeimies RM. Photodynamic therapy in dermatology: state-of-the-art. Photodermatol Photoimmunol Photomed. 2010;26:118–32.
21. Nelson JS, McCullough JL, Berns MW. Photodynamic therapy of human malignant melanoma xenografts in athymic nude mice. J Natl Cancer Inst. 1988;80:56–60.
22. Biolo R, Jori G, Soncin M, Rihter B, Kenney ME, Rodgers MA. Effect of photosensitizer delivery system and irradiation parameters on the efficiency of photodynamic therapy of B16 pigmented melanoma in mice. Photochem Photobiol. 1996;63:224–8.
23. Schuitmaker JJ, van Best JA, van Delft JL, Dubbelman TM, Oosterhuis JA, de Wolff-Rouendaal D. Bacteriochlorin a, a new photosensitizer in photodynamic therapy. In vivo results. Invest Ophthalmol Vis Sci. 1990;31:1444–50.
24. Woodburn KW, Fan Q, Kessel D, Luo Y, Young SW. Photodynamic therapy of B16F10 murine melanoma with lutetium texaphyrin. J Invest Dermatol. 1998;110:746–51.
25. Busetti A, Soncin M, Jori G, Rodgers MA. High efficiency of benzoporphyrin derivative in the photodynamic therapy of pigmented malignant melanoma. Br J Cancer. 1999;79:821–4.
26. Sheleg SV, Zhavrid EA, Khodina TV, Kochubeev GA, Istomin YP, Chalov VN, Zhuravkin IN. Photodynamic therapy with chlorin e(6) for skin metastases of melanoma. Photodermatol Photoimmunol Photomed. 2004;20:21–6.
27. Cordoba F, Braathen LR, Weissenberger J, Vallan C, Kato M, Nakashima I, Weis J, von Felbert V. 5-aminolaevulinic acid photodynamic therapy in a transgenic mouse model of skin melanoma. Exp Dermatol. 2005;14:429–37.
28. Davids LM, Kleemann B. Combating melanoma: the use of photodynamic therapy as a novel, adjuvant therapeutic tool. Cancer Treat Rev. 2011;37:465–75.
29. Ando T, Xuan W, Xu T, Dai T, Sharma SK, Kharkwal GB, Huang YY, Wu Q, Whalen MJ, Sato S, Obara M, Hamblin MR. Comparison of therapeutic effects between pulsed and continuous wave 810-nm wavelength laser irradiation for traumatic brain injury in mice. PLoS One. 2011;6:e26212.
30. Hadjur C, Richard MJ, Parat MO, Jardon P, Favier A. Photodynamic effects of hypericin on lipid peroxidation and antioxidant status in melanoma cells. Photochem Photobiol 1996;64:375–81.
31. Kollias N, Sayre RM, Zeise L, Chedekel MR. Photoprotection by melanin. J Photochem Photobiol B. 1991;9:135–60.
32. Busetti A, Soncin M, Reddi E, Rodgers MA, Kenney ME, Jori G. Photothermal sensitization of amelanotic melanoma cells by Ni(II)-octabutoxy-naphthalocyanine. J Photochem Photobiol B. 1999;53:103–9.
33. Sharma KV, Bowers N, Davids LM. Photodynamic therapy-induced killing is enhanced in depigmented metastatic melanoma cells. Cell Biol Int. 2011;35:939–44.
34. Suzukawa AA, Vieira A, Winnischofer SM, Scalfo AC, Di Mascio P, Ferreira AM, Ravanat JL, Martins Dde L, Rocha ME, Martinez GR. Novel properties of melanins include promotion of DNA strand breaks, impairment of repair, and reduced ability to damage DNA after quenching of singlet oxygen. Free Radic Biol Med. 2012;52:1945–53.
35. Davids LM, Kleemann B, Cooper S, Kidson SH. Melanomas display increased cytoprotection to hypericin-mediated cytotoxicity through the induction of autophagy. Cell Biol Int. 2009;33:1065–72.
36. Davids LM, Kleemann B, Kacerovska D, Pizinger K, Kidson SH. Hypericin phototoxicity induces different modes of cell death in melanoma and human skin cells. J Photochem Photobiol B. 2008;91:67–76.

37. Bebes A, Nagy T, Bata-Csorgo Z, Kemeny L, Dobozy A, Szell M. Specific inhibition of the ABCG2 transporter could improve the efficacy of photodynamic therapy. J Photochem Photobiol B. 2011;105:162–6.
38. Anand S, Honari G, Hasan T, Elson P, Maytin EV. Low-dose methotrexate enhances aminolevulinate-based photodynamic therapy in skin carcinoma cells in vitro and in vivo. Clin Cancer Res. 2009;15:3333–43.
39. Ruzie C, Krayer M, Balasubramanian T, Lindsey JS. Tailoring a bacteriochlorin building block with cationic, amphipathic, or lipophilic substituents. J Org Chem. 2008;73:5806–20.
40. Taniguchi M, Cramer DL, Bhise AD, Kee HL, Bocian DF, Holten D, Lindsey JS. Accessing the near-infrared spectral region with stable, synthetic, wavelength-tunable bacteriochlorins. New J Chem. 2008;32:947–58.
41. Dabrowski JM, Krzykawska M, Arnaut LG, Pereira MM, Monteiro CJ, Simoes S, Urbanska K, Stochel G. Tissue uptake study and photodynamic therapy of melanoma-bearing mice with a nontoxic, effective chlorin. Chem Med Chem. 2011;6:1715–26.
42. Mroz P, Huang YY, Szokalska A, Zhiyentayev T, Janjua S, Nifli AP, Sherwood ME, Ruzie C, Borbas KE, Fan D, Krayer M, Balasubramanian T, Yang E, Kee HL, Kirmaier C, Diers JR, Bocian DF, Holten D, Lindsey JS, Hamblin MR. Stable synthetic bacteriochlorins overcome the resistance of melanoma to photodynamic therapy. FASEB J. 2010;24:3160–70.
43. Idris NM, Gnanasammandhan MK, Zhang J, Ho PC, Mahendran R, Zhang Y. In vivo photodynamic therapy using upconversion nanoparticles as remote-controlled nanotransducers. Nat Med. 2012;18:1580–5.
44. Khurana M, Collins HA, Karotki A, Anderson HL, Cramb DT, Wilson BC. Quantitative in vitro demonstration of two-photon photodynamic therapy using photofrin and visudyne. Photochem Photobiol. 2007;83:1441–8.
45. Starkey JR, Rebane AK, Drobizhev MA, Meng F, Gong A, Elliott A, McInnerney K, Spangler CW. New two-photon activated photodynamic therapy sensitizers induce xenograft tumor regressions after near-IR laser treatment through the body of the host mouse. Clin Cancer Res. 2008;14:6564–73.
46. Ma LW, Nielsen KP, Iani V, Moan J. A new method for photodynamic therapy of melanotic melanoma -effects of depigmentation with violet light photodynamic therapy. J Environ Pathol Toxicol Oncol. 2007;26:165–72.
47. Busetti A, Soncin M, Jori G, Kenney ME, Rodgers MA. Treatment of malignant melanoma by high-peak-power 1064 nm irradiation followed by photodynamic therapy. Photochem Photobiol. 1998;68:377–81.
48. Radzi R, Osaki T, Tsuka T, Imagawa T, Minami S, Nakayama Y, Okamoto Y. Photodynamic hyperthermal therapy with indocyanine green (ICG) induces apoptosis and cell cycle arrest in B16F10 murine melanoma cells. J Vet Med Sci. 2012;74:545–51.
49. Glassberg E, Lewandowski L, Halcin C, Lask G, Uitto J. Hyperthermia potentiates the effects of aluminum phthalocyanine tetrasulfonate-mediated photodynamic toxicity in human malignant and normal cell lines. Lasers Surg Med. 1991;11:432–9.
50. Naylor MF, Chen WR, Teague TK, Perry LA, Nordquist RE. In situ photoimmunotherapy: a tumor-directed treatment for melanoma. Br J Dermatol. 2006;155:1287–92.
51. Saji H, Song W, Furumoto K, Kato H, Engleman EG. Systemic antitumor effect of intratumoral injection of dendritic cells in combination with local photodynamic therapy. Clin Cancer Res. 2006;12:2568–74.
52. Gogas H, Polyzos A, Kirkwood J. Immunotherapy for advanced melanoma: fulfilling the promise. Cancer Treat Rev. 2013;39:879–85.

Erratum

Resistance to Photodynamic Therapy in Cancer

Edited by Valentina Rapozzi and Giulio Jori

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Resistance to Targeted Anti-Cancer Therapeutics 5, DOI 10.1007/978-3-319-12730-9

DOI 10.1007/978-3-319-12730-9_12

The publisher regrets to inform you that volume co-editor Giulio Jori is now deceased. Thus, the scientific community has inserted the following Dedication in the Frontmatter of the volume.

"In Memoriam of Dr. Giulio Jori –

The scientific community is sad to learn of your early departure. You have been a leader in the PDT field, and you have contributed significantly to the benefit of mankind.
We also remember you as a great friend, an excellent teacher, and a compassionate collaborator. You will always be remembered, and we are forever indebted to you.
This book is dedicated to you and your family."
Valentina Rapozzi, PhD., Co-Editor
Benjamin Bonavida, PhD., Series Editor

The online version of the original book can be found at
http://dx.doi.org/10.1007/978-3-319-12730-9

V. Rapozzi, G. Jori (eds.), *Resistance to Photodynamic Therapy in Cancer,*
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