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REVIEW ARTICLE

Emerging Therapeutic Targets in Oncologic Photodynamic Therapy

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Abstract: ***Background***: Reactive oxygen species sustain tumorigenesis and cancer progression through deregulated redox signalling which also sensitizes cancer cells to therapy. Photodynamic therapy (PDT) is a promising anti-cancer therapy based on a provoked singlet oxygen burst, exhibiting a better toxicological profile than chemo- and radiotherapy. Important gaps in the knowledge on underlining molecular mechanisms impede on its translation towards clinical applications.

Aims and Methods: The main objective of this review is to critically analyse the knowledge lately gained on therapeutic targets related to redox and inflammatory networks underlining PDT and its outcome in terms of cell death and resistance to therapy. Emerging therapeutic targets and pharmaceutical tools will be documented based on the identified molecular background of PDT.

Results: Cellular responses and molecular networks in cancer cells exposed to the PDT-triggered singlet oxygen burst and the associated stresses are analysed using a systems medicine approach, addressing both cell death and repair mechanisms. In the context of immunogenic cell death, therapeutic tools for boosting anti-tumor immunity will be outlined. Finally, the transcription factor NRF2, which is a major coordinator of cytoprotective responses, is presented as a promising pharmacologic target for developing co-therapies designed to increase PDT efficacy.

Conclusion: There is an urgent need to perform in-depth molecular investigations in the field of PDT and to correlate them with clinical data through a systems medicine approach for highlighting the complex biological signature of PDT. This will definitely guide translation of PDT to clinic and the development of new therapeutic strategies aimed at improving PDT.

Keywords: cancer, photodynamic therapy, reactive oxygen species, oxidative stress, redox signalling, transcription factor NRF2, inflammation.

1. INTRODUCTION

Cancer remains a huge health issue that puts an important economic burden on healthcare systems, as well as a social and emotional pressure on patients and their families. Significant progress in understanding the multi-factorial nature of cancer was obtained lately from systems approaches that have revealed hidden faces of the complex molecular networks underlining malignancies. Consequently, promising therapeutic targets are emerging that may foster the development of new targeted therapeutic strategies with increased anti-tumor efficacy and acceptable side-effects.

1.1. Fighting Cancer Using the Cytotoxic Potential of Reactive Oxygen Species

A major strategy to fight cancer takes advantage of the cytotoxic potential of reactive oxygen species (ROS). An important paradigm shift in redox biology occurred in the last decade, based on consistent evidence that ROS may act through more subtle mechanisms than a rough and unspecific chemical attack on critical biologic structures which is only relevant in extreme oxidative conditions. Actually, low levels of ROS and an appropriate antioxidant control were shown to be essential for maintaining cellular homeostasis through tightly regulated redox signalling events that are more or less directly connected and control all other known signalling pathways [1]. Consequently, even apparently small deregulations in the redox control of cellular homeostasis may have important health consequences in the long run, if these alterations are persistent. Recently, extensive evidence was generated showing that many chronic diseases, such as cardiovascular diseases, diabetes, neurodegenerative diseases, as well as cancer, are underlined by chronic low-grade oxidative stress and inflammation. These "redox diseases" may benefit from therapies that are boosting the endogenous antioxidant response to counteract, from the inside of cells, the deleterious effects of a chronically enhanced oxidative activity [2].

The dual role of ROS in cancer is highly dependent on the profile, location and magnitude of the ROS burst, as well as on cell-specific responses to the oxidative challenge, as will be described below.

At moderately increased levels, ROS may sustain tumorigenesis, cancer progression and metastasis, whilst high, therapeutically-generated amounts of ROS can promote cancer cells death. More

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specifically, cancer cells have an increased oxidative activity induced by cancer-specific pathologic processes, such as the feed-forward loop connecting ROS generation by NADPH oxidases (i.e. the constitutively activated NOX4 isoform) with oncogenic signalling through Ras, c-Myc or Bcr-Abl [3, 4]. Proliferation of cancer cells is sustained by mitogen-activated kinase (MAPK) signalling through the extracellular receptor kinase (ERK) that is triggered by ROS-induced transactivation of several tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR) [5]. Additionally, the increased ROS levels in the tumor niche were shown to induce metastatic dissemination of cancer cells by activation of metalloproteinases (MMPs), as well as by tumor angiogenesis mediated by ROS-induced release of vascular endothelial growth factor (VEGF) and angiopoietin [6]. The enhanced intricate oxidative activity in cancer cells is correlated with a metabolic shift towards a glycolytic phenotype for coping with their augmented needs for energy and building blocks [7, 8]. Altogether, the chronically enhanced intracellular oxidative activity can sustain tumorigenesis by conferring to cancer cells a survival and proliferation advantage derived from various protective mechanisms activated in response to a low-grade oxidative challenge, such as those mediated by the transcription factor NRF2 which is a key trigger of cytoprotective mechanisms [9].

In turn, a significant burst of cytotoxic ROS, overcoming the endogenous antioxidant protection mechanisms, is able to acutely trigger various forms of cell death [10], depending on ROS levels and the site of generation, as well as the distribution of ROS sensors and detoxifying mechanisms [11, 12]. Profound cell dysfunctions and even cell death may arise in the long run from apparently modest deregulations of the redox balance if oxidative stress is chronic [13].

We emphasize that the very same increased oxidative activity in cancer cells which is driving tumor progression is the one that actually confers to cancer cells responsiveness to therapies acting more or less directly *via* oxidative stress. This is due to the decrease of the oxidative threshold that cancer cells have to overcome for shifting from a redox state that favours survival towards activation of death-inducing mechanisms. Therapeutic efficacy is hence increased, the effective dose could be lowered and consequently unwanted side-effects decreased [14].

1.2. Anti-cancer Therapies Mainly Based on Oxidative Stress: Radiotherapy and Photodynamic Therapy

The main anti-cancer therapies that utilize oxidative stress as the central therapeutic firearm to kill tumor cells are radiotherapy and photodynamic therapy (PDT), albeit chemotherapy efficacy may also rely more or less directly on increased ROS levels.

1.2.1. Radiotherapy

Radiotherapy remains the gold standard therapy in many forms of cancer. It triggers tumor cell death by a sudden burst of ROS that are primarily generated *via* ionizing radiation-induced water radiolysis and subsequent formation of hydrogen peroxide, followed by secondary ROS production from intracellular sources such as NADPH-oxidases (NOX enzymes) and myeloperoxidase (MPO) [15]. Hydroxyl radicals deriving from the chemical reaction of hydrogen peroxide and superoxide anion are particularly harmful for normal and tumor cells. Protection of normal tissues against the deleterious effects of radiation is achieved by precisely targeting radiation beams towards the diseased tissue. Nevertheless, important local and systemic side-effects of radiotherapy are limiting its safety due to the uncontrollable and still elusive out-of-field effects that spread damage beyond the irradiated area, towards neighbouring cells (by-stander effect) and even at systemic level (abscopal effect) [16]. Resistance of particular types of cancer cells to anti-cancer therapies, including radiotherapy, is related to cytoprotective mechanisms developed by cells in response to therapy-induced damages. Intrinsic therapy resistance is due to cancer-specific alterations in critical signalling pathways that confer to cancer cells reduced susceptibility to apoptosis and increased ability to repair DNA damage [17, 18].

1.2.2. Photodynamic Therapy

Photodynamic therapy (PDT) [19], which is also based on a provoked ROS burst, has proved to be very efficient in killing cancer cells. PDT takes advantage of the cytotoxic potential of a particular type of ROS, the singlet oxygen (Figure 1). Singlet oxygen is produced constitutively *via* photosensitizing chlorophylls in plant leaves exposed to light [20]. In turn, it is generated physiologically only in minor amounts in animal cells through various mechanisms [21], comprising MPO-dependent production by neutrophils during microorganisms killing, interaction of peroxyl radicals to form an unstable tetroxide, dismutation of alkoxyl radicals, oxidation of phenolic substances, tryptophan and tyrosine oxidation, conversion of lipid-derived aldehydes by cytochrome c to triplet carbonyls, etc. Because singlet oxygen is not a common type of ROS in animal cells, they are less prepared to detoxify it and to protect themselves against this unusual ROS. It is the reason for which PDT is often more effective in inducing cancer cell death than radiotherapy which is acting mainly *via* radiation-induced hydrogen peroxide against which animal cells are endowed with a powerful armamentarium made of catalase, peroxyredoxins and glutathione peroxidases. It is noteworthy that the anti-tumor action of PDT stems in the direct cytotoxic effect caused by singlet oxygen and ensuing oxidized biomolecules, along with an indirect effect related to local and systemic activation of the anti-tumor immune response, both mechanisms leading to tumor cells death along with the tumor vasculature damage.

PDT is a non-invasive treatment that has been successfully applied in various types of tumors (i.e. head and neck, lung, esophageal and biliary tract cancers, cutaneous T cell lymphoma and high grade dysplasia in Barret's esophagus), as well as in non-malignant conditions (i.e. actinic keratosis and macular degeneration) [22]. The search of the terms "photodynamic therapy" and "active clinical trials" in the ClinicalTrials.gov web page provided 84 ongoing clinical trials in various phases, out of which more than 20 aim at assessing PDT efficacy and safety in various types of solid tumors, either as mono-therapy or in combination with chemotherapy / radiotherapy or biologic therapies. The explosion of clinical trials aimed at demonstrating and expanding clinical applications of PDT in malignant and non-malignant diseases highlights the great promise held by this type of anti-cancer therapy. Preclinical studies and the outcome of clinical practice point out that PDT is able to prolong life expectancy in cancer patients. As PDT is associated with significantly less morbidity than radiotherapy, it is considered a patient-friendly therapeutic approach that does not need prolonged therapeutic follow-ups and can be safely repeated in case of tumor recurrence even to immunosuppressed patients. PDT is also advantageous because it needs relatively inexpensive equipment, unlike the sophisticated infrastructure for radiotherapy. Both radiotherapy and PDT are targeted therapies and therefore metastases cannot be directly addressed, but recent studies highlight the out-of-field effects of these therapies that boost the anti-tumor immune response in particular settings. Although PDT appears to have a safer toxicological profile in comparison with chemo- and radiotherapy, some side-effects should be taken into consideration [23], like pain and local skin reactions (erythema, edema and desquamation) that appear during PDT or in hours/days thereafter.

Results obtained so far in clinical trials offer only a blurred image on PDT effects, mainly because of the lack of standardized treatment guidelines. Moreover, research in the field of PDT has been focused until recently mainly on the physical and chemical components of the therapy (photosensitizers, activating light and singlet oxygen dosimetry – Figure **1**). There is an urgent need to better define, in preclinical and clinical settings, the responses developed by normal and tumor cells exposed to PDT and this will for

sure open new avenues for designing co-therapies aimed at increasing PDT efficacy whilst keeping its side-effects at acceptable levels.

In this context, the review is focused on the knowledge lately gained on the biology standing behind PDT, related to the generation of cytotoxic singlet oxygen and its biological consequences. We will first present PDT, emphasizing existent technologic achievements and research trends for improving critical physical and chemical components of PDT in deep-seated or bulky tumors. Thereafter, the review critically revises the knowledge of molecular mechanisms underlining PDT in solid tumors. Thus, key cellular responses to PDT-triggered cellular stresses (i.e. oxidative stress and associated ER stress) are highlighted from a systems medicine perspective in relation to cell death and cellular repair mechanisms that may limit PDT efficacy. The lessons learned from radiotherapy have guided us in the data mining for molecular mechanisms underlining PDT. More specifically, PDT-induced immunogenic cell death, its out-of-field effects, as well as the impact of PDT on the anti-cancer immune response and existing therapeutic tools will be particularly emphasized. Finally, the transcription factor NRF2, which is a major coordinator of cytoprotective responses, is presented as a promising pharmaceutical target for developing co-therapies designed to increase PDT efficacy by down-regulation of the endogenous antioxidant system and other cytoprotective mechanisms which confer to cancer cells resistance to therapy. This review is not intended to offer an exhaustive image of the biology behind PDT, but to define the main biomedical vocabulary in use in the context of PDT, in relation with underlining molecular mechanisms and potential therapeutic targets.

2. PHOTODYNAMIC THERAPY AND ITS PHYSICO-CHEMICAL COMPONENTS: ACHIEVEMENTS AND CHALLENGES

PDT consists of intravenous, intraperitoneal or topical administration of an inactive and minimally toxic photosensitizer (PS) or PS precursor, followed by PS activation induced by precise tumor illumination with harmless visible light of well-defined wavelength and generation in the presence of molecular oxygen of a localized oxygen burst that inflicts oxidative damage to tumors [19, 24]. PDT is considered a targeted therapy because, even if PS distributes equally in normal and tumor tissues, precise illumination of the diseased area confers to PDT tumor specificity.

Huge research efforts have been initially focused on the development of PDT-tailored PSs based on the following criteria (Table 1): a) well-defined composition and structure, b) low toxicity under "dark" conditions, c) amphiphilic properties for dissolving in body fluids whilst being able to cross membrane barriers, d) preferential retention in diseased tissues, e) activation by visible light in the red spectrum for penetration into tumors without harming normal tissues, f) good photochemical reactivity and generation of cytotoxic amounts of singlet oxygen under illumination in the presence of molecular oxygen.

Preferential accumulation of PSs into tumor cells derives mainly from the increased permeability of tumor neovasculature. PSs are selectively retained here due to the low pH value and the poorly developed lymphatic drainage of tumors. Physical properties of PSs are decisively dictating their tissue distribution and pharmacokinetics. PS transport inside cells can take place by passive diffusion, and, in particular cases, also by active carrier-mediated transport. Lipophilic and amphiphilic PS dissolve well in the lipid bilayer of membranes. Although polar groups in amphiphilic PSs hinder their insertion into the lipid bilayer of membranes, they are required for PS to dissolve in water-based biologic fluids and therefore, fine-tuned amphiphilic properties of PS are a major target for improvement. It is noteworthy that physical, chemical and biological events at the place of PS inoculation, during extravasation and PS diffusion inside tissues, may better focus or divert the PS from its intended target. For instance, intravenously administered PS will first bind to serum proteins such as the low-density lipoprotein (LDL), and these complexes will preferentially accumulate in tumors over-expressing LDL receptors [25]. Much like in the case of the protein corona that coats nano-structures in biological media [26], PS-serum protein complexes will, in fact, dictate how cells "see" the PS, beyond its fine structure. This is the reason why traditional *in vitro* assays could misrepresent the response and cellular-uptake data for PSs. Once arrived inside a cell, the PS preferentially localizes in particular organelles and cell membranes, depending on its physicochemical properties such as charge, amphiphilicity and partition coefficients [27]. Redistribution of PS in a cell may take place due to changes in pH or in PS metabolization within cells. The time-course of PS uptake is highly dependent on the type of PS and cells used in the experiment. The study of Yeh SCA *et al.* (2012) [28] investigated, using two-photon fluorescence lifetime imaging microscopy, the time-lapse fluorescence intensity and distribution of Photofrin in a rat prostate adenocarcinoma cell line. Results indicated that Photofrin accumulated mostly in the cell membrane within the first 4 hrs, than distributed in the cytoplasm and finally labelled mitochondria, starting at 6 hrs until at least 18 hrs.

Preferential accumulation of PS in the tumors confers only limited specificity to PDT. Actually, the precise illumination of the tumor for localized PS activation in the diseased tissue is the mechanism making PDT a highly targeted therapy. The ensuing burst of cytotoxic singlet oxygen confined to a selected area inflicts oxidative damage to particular components of tumor cells, and this dictates PDT outcome in terms of cell death and repair mechanisms. Meanwhile, normal cells seem to be protected in PDT, at least at the intention level, due to precise illumination of tumors with biologically friendly light in the red spectral region.

Tetrapyrrolic structures (porphyrins, chlorins, bacteriochlorins and phthalocyanines) with various functionalizations, along with some natural products (hypericin, hypocrellin and various substituted chlorin derivatives from plants and algae) have been widely investigated as PSs for PDT. The first generation of PSs (Hematoporphyrin derivative and Photofrin) had as major drawbacks significant skin phototoxicity, uncertain composition and low light absorption in the visible range. Substantial improvements regarding compound purity, improved light absorption and lower photosensitization potential in skin were obtained with the second generation of PSs, comprising, for instance, Veteporfin, NPe6 and Foscan, among other PSs. PS currently approved by FDA or EMA for clinical applications [22, 29] are presented in Table **1**. These marketed PSs and the data emerging lately from clinical trials represent valuable tools for in-depth investigation of PDT mechanisms in "real" solid tumors, hence being a huge step forward for overcoming the limited relevance of biologic data that were massively obtained so far in-cell and in-animal studies.

The third generation of PSs is under development for improving tissue specificity mainly by using biocompatible nano-platforms such as gold-nanoclustered hyaluronan nano-assemblies [30], fullerene-based PSs, and biochemically tuned liposomes [31]. Up-conversion nano-platforms were proposed for increased light penetration into tissues [32]. The reasons for using nano-enabled PDT are: 1) to avoid PS self-aggregation in biologically-relevant media [33], which can severely affect PDT efficacy, and 2) to concentrate PS on the surface of nanostructures for decreasing the effective dose. Going further, nano-theranostic approaches [34] for better targeting and attacking cancer cells are under development, combining for instance molecules that specifically target the PS towards the diseased tissues and therapeutic/modulatory agents that are able to sustain PDT or to counteract protective responses of tumor cells to PDT-inflicted damage. From another PDT perspective, nanostructures designed for emitting PS-activating light from the inside of the tumor (nano-scintillators) could replace conventional external

Table 1. Photosensitizers approved for PDT by the European Medicines Agency (EMA) or by Food and Drug Administration (FDA) [22, 29].

Photosensitizer	Compound*	Commercial name / producer	Intended use
Porfimer sodium	Dihematoporphyrin ester	Photofrin / CONCORDIA LABS INC and AXCAN SCANDIPHARM	Approved worldwide for high grade dysplasia in Barret's esophagus and obstructive esophageal or lung cancer (withdrawn in EU for commercial reasons)
5-ALA	5-aminolevulinic acid	Ameluz / BIOFRONTERA Levulan / DUSA	Approved worldwide for mild to moderate actinic keratosis
Methyl aminolevulinate hydrochloride	Methyl aminolevulinate hydrochloride	Metvix (Metvixia) / GALDERMA LABS LP	Approved worldwide for non-hyperkeratotic actinic keratosis and basal cell carcinoma
Temoporfin (mTHPC)	Meso-Tetrahydroxyphenylchlorin	Foscan / BIOLITEC PHARMA LTD	Approved in EU for head and neck cancer
Verteporfin	18-ethenyl-4,4a-dihydro-3,4-bis(methoxycarbonyl)-4a,8,14,19-tetramethyl-23H,25H-benzo(b)porphine-9,13-dipropanoic acid monomethyl ester	Visudyne / VALEANT LUXEMBOURG	Approved worldwide for age-related macular degeneration; used off-label for the treatment of central serous retinopathy
Synthetic hypericin	Hypericin	SGX301 / SOLIGENIX	Orphan status in EU, recommended for cutaneous T-cell lymphoma
Talaporfin (NPe6)	Aspartyl chlorine	Laserphyrin / MEIJI SEIKA PHARMA CO	Approved in Japan for early centrally located lung cancer
Redaporfin	Bacteriochlorin (5,10,15,20-tetrakis(2,6-difluoro-3-N-methylsulfamoylphenyl)	Redaporfin (LUZ11) / LUZITIN SA	Orphan status in EU, recommended for biliary tract cancer

* PubChem, https://pubchem.ncbi.nlm.nih.gov

light beams (lasers) that are often not able to reach deep tumors or to uniformly illuminate bulky ones [35]. Another direction in which nanotechnologies are focused for improving ROS-dependent therapies is related to the development of radical-generating agents that can damage cells by a ROS-type radical mechanism in absence of oxygen, hence being PDT-active even in hypoxic tumor regions [36]. Nanotechnologies have a huge potential to revolutionize medical approaches, despite reasonable concerns regarding toxicity, including ecotoxicity. PDT can take advantage of these emerging technologies, under the condition that the endpoint is well defined in terms of therapy improvement and acceptable toxicity.

Besides PSs, tumor illuminations for PS activation and oxygen availability for singlet oxygen generation are critical components of PDT (Figure 1). They raise significant technological challenges for obtaining an efficient PDT in cancer and for translating PDT into the clinic. Some of these issues are summarized in Table 2, along with emerging solutions for overcoming pitfalls of the actual PDT procedures.

3. THE OXIDATIVE BURST OF SINGLET OXYGEN IN PDT

During PDT a primary burst of intra- and extracellular singlet oxygen is generated as follows: the PS molecule in an inactive singlet state gets transiently activated by absorption of a photon with appropriate wavelength which excites one electron into a higher-energy orbital. The activated PS decays through fluorescent emission of light or through heat production by internal conversion. The excited singlet PS forms by inter-system crossing a more stable excited triplet state with parallel spins and a lifetime of microseconds compared with only nanoseconds for the excited singlet. In this more stable triplet state PS transfers its energy to molecular oxygen, leading to formation of singlet oxygen (Type II photochemical reaction) [37]. Type I photochemical processes may also occur, whereby the excited PS undergoes electron transfer reactions, generating various forms of ROS (superoxide anion, hydroxyl radical), other than singlet oxygen. It is noteworthy that most PS currently used for PDT are acting *via* Type II rather than Type I mechanisms [19].

The highly reactive singlet oxygen generated by PDT can inflict important oxidative damages to critical biomolecules [39]: **a)** unsaturated lipids are particularly oxidized by singlet oxygen and generate allylic hydroperoxides which further propagate the oxidative damage in cellular membranes through the lipid peroxidation chain reaction. Moreover, secondary reactive lipid electrophiles generated by spontaneous cleavage of primary oxidized lipids amplify the deleterious effects of primary oxidized lipids and further expand the range of photo-oxidation in membranes; **b)** proteins are oxidized by singlet oxygen at the level of Cys, Tyr, Trp, His and Met residues, and the ensuing modified amino acids (i.e. Met sulfoxide, cystine and cysteic acid) can alter critical protein functions. Carbonylated proteins are markers of protein oxidations triggered by PDT which induce various cell death scenarios or deprive cells from important protective mechanisms against the PDT-induced proteotoxic stress through alteration of chaperone molecules [40]; **c)** DNA can be oxidized during PDT at the level of deoxyguanosine, generating 8-oxodG which can be further oxidized by singlet oxygen. Most of the PSs in use do not localize in the nucleus, but specifically target mitochondria [19]. Therefore, it is most probable that not genomic but mitochondrial DNA (mtDNA),

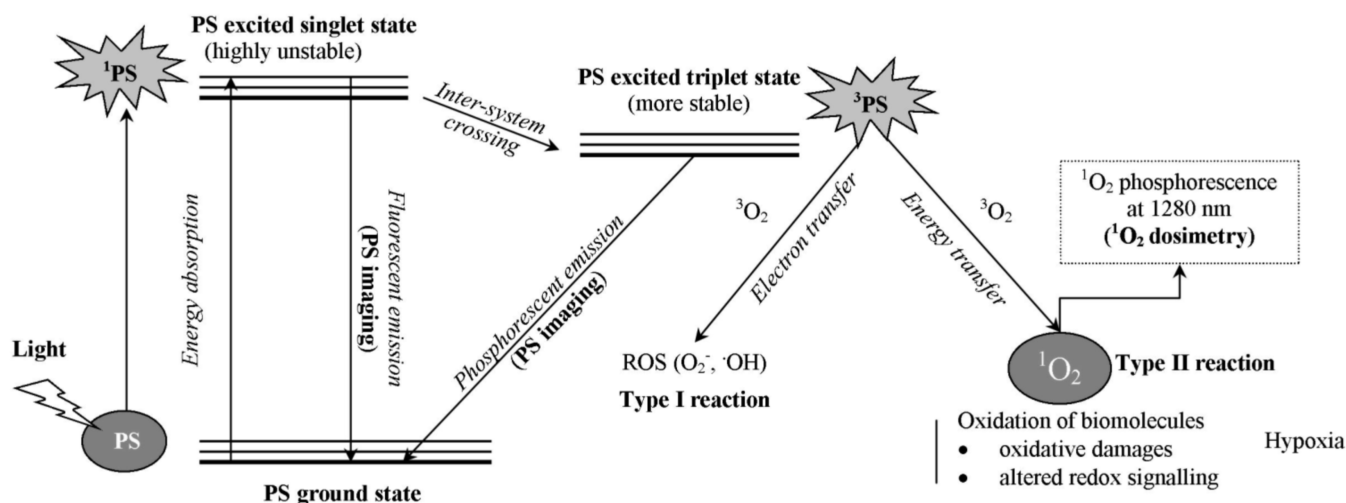

Fig. (1). Schematic representation of PDT. The photosensitizer in the ground energy state (PS) is activated by light with defined wavelength, absorbs a photon and is excited to a highly unstable singlet state (^{1}PS). Relaxation of ^{1}PS to the ground state may occur through loss of energy by fluorescent emission which is used for PS imaging, but part of its energy may be lost by internal conversion (not represented). Of interest for PDT is the relaxation of ^{1}PS by inter-system crossing (reversal of the excited electron spin) to a less energetic but a more stable triplet state (^{3}PS). The excited electron of ^{3}PS may change its orientation, and energy is emitted by phosphorescence that can be used for PS imaging during PDT. In type I photochemical reaction, ^{3}PS reacts with molecular oxygen ($^3O_2$) which is one of the few molecules that are triplet in the ground state, leading through electron transfer to ROS formation (i.e. O_2^- - superoxide anion, $^{\cdot}$OH - hydroxyl radical). Alternatively, ^{3}PS transfers its energy to $^3O_2$ by collision, generating singlet oxygen ($^1O_2$) (type II photochemical reaction) which is the main type of ROS produced in PDT using the currently available PSs. The phosphorescence of $^1O_2$ at 1280 nm can be used for monitoring the PDT reaction. $^1O_2$ is highly reactive and alters various biomolecules, leading to important oxidative damages to critical cell components and alteration of redox signalling pathways, leading to cell death if the PDT reaction is sufficiently intense. Moreover, due to $^3O_2$ consumption by the formation of $^1O_2$ hypoxia is induced locally, inflicts additional cellular damages, but also terminates the $^3O_2$-dependent PDT reaction in the area. Adapted after [38].

Table 2. Main technological issues raised by PDT and emerging solutions.

Critical issues for an efficient PDT	Requirements	Actual drawbacks and proposed solutions
Photosensitizers (PSs)	PS should be a purified compound that allows for good manufacturing practice and quality control (2nd generation PS)	Natural compounds proved valuable photosensitizing properties, but isolation and characterization of their active compounds should be done for further clinical use.
	PS should be minimally toxic in "dark" conditions	Patients have to be protected from light for several hours after PS administration in order to avoid photosensitization. PS that are well retained in tumors but have a rapid clearing from normal tissues are under development.
	PS should specifically accumulate into tumors. This generally occurs due to the leaky tumor vasculature, even in the absence of a targeting agent	PS targeting into tumor cells can be achieved by the following approaches: 1). biocompatible nanoparticles used as nano-carriers that concentrate PSs on their surface and deliver them more efficiently to tumors by improved PS transcytosis across epithelial and endothelial barriers [241, 242]. Additionally, nanostructures can bring in PDT other ROS than singlet oxygen, such as the more cytotoxic hydroxyl radical. Therefore, particular nanostructures (for instance titanium oxide or iron oxide nanoparticles) can sustain PDT-induced cell death by an intensified oxidative stress [243]; 2). carbohydrates, antibodies and peptides that interact with molecules specifically expressed by tumor cells could better target the PS towards the diseased tissue. These targeting biomolecules may be coupled to PS or to functionalized nano-platforms that may greatly improve PDT by facilitating simultaneous co-delivery of targeting agents and/or and various drugs. Nano-architectures used for better targeting PDT should preserve PS ability to generate singlet oxygen when it is activated by light. Supplementary precaution should be taken due to the added organ and systemic toxicity of nanostructures, especially of metal-based ones.

(Table 2) Contd....

Critical issues for an efficient PDT	Requirements	Actual drawbacks and proposed solutions
	PS should remain enough time in circulation to reach and accumulate into tumors	Preferential PS accumulation in tumors is favored by the fenestrated vasculature and reduced lymphatic drainage of most solid tumors (enhanced permeability and retention effect). Meanwhile, sufficiently rapid clearance of PS from normal tissues is needed in order to avoid unwanted photosensitization, especially at the level of skin. Release of PS from cells is mediated by ATP-binding cassette transporters (ABC transporters), some of them being under the control of the transcription factor NRF2 [218]. The pharmakodynamic properties of PSs are essential for planning repeated PDT sessions (drug–light cycles) that are often needed for radical tumor ablation, considering the heterogeneity of cells in the tumor niche and their distinct sensitivities to PDT.
	Accumulation of PS into the tumor and the PDT-generated singlet oxygen should be monitored *in vivo* for personalized therapy (adjustment of PS administration and the PDT plan)	Considering that each tumor has unique topologic and functional characteristics there is an urgent need to develop imaging techniques for real-time *in vivo* monitoring of PDT: 1). PS accumulation, taking advantage of the fluorescent properties of most available PSs. Moreover, binding of PS to iron oxide nanoparticles or to other imagistic tracers can add through MRI or PET new imagistic power [244]; 2). intra-tumor generation of singlet oxygen by PDT, evaluated *in vivo* by near-infrared luminescence measurements at 1280 nm [245]. Singlet oxygen dosimetry involves development of sensitive detection equipment and advanced computational methods [53, 246].
PS activation by light	PS should get activated by visible light at a well-defined wave length. Activating light should easily cross normal tissues with minimal absorption in its way to deep-seated or bulky tumors	For wavelengths in the 625-640 nm spectral region one attenuation length is approximately 3-5 mm. The light dose currently applied in conventional PDT is effective at approximately 0.8-1.0 cm in depth at 630 nm. This limits considerably the treatment of deep-seated or bulky tumors. PS activation by deep-red light, ideally near infrared (NIR) light, is required in order to easily cross normal tissues with low absorption and minimal damage to normal tissues until light is reaching deep-seated or bulky tumors. If PS is activated at lower wavelengths (600 nm and below), PDT can be used to treat only superficial tumors. Efficient light delivery to deep-seated or bulky tumors can be achieved with optical fibers compatible with clinical endoscopes. Intra-luminal irradiation has been used for lung and esophagus, while interstitial illumination has been applied for bulky tumors. Several solutions for uniform and sufficiently intense illumination of tumors were proposed lately: 1). Increased efficiency is achieved by post-operative cavitary PDT, as reviewed by Quirk *et al.* (2015) for intracranial malignancies treated with hematoporphyrin derivative, Talaporfin, Foscan or ALA [247]; 2). Direct PS activation by light can be achieved from the inside of cells using nano-scintillators with high energy transfer efficiency [248]. Another solution might be transfection of cancer cells with firefly luciferase which may locally activate particular PSs *via* the emitted light [249]; 3). PS activation may be obtained using ultrasounds that have a larger penetration depth into tissues than visible light [250]; 4). PS activation in deep-seated tumors can be achieved by high energy photons produced for instance using a Cesium-137 irradiator [251]. A promising perspective to generate high energy photons in very short pulses is opened by the new Extreme Light Infrastructure Nuclear Physics (ELI-NP) which is under construction in Romania as major facility in the Nuclear Physics Long Range Plan in Europe. A very high peak power density provided by the femtosecond pulsed laser could generate deep tissue penetrating NIR-photons that may be sequentially absorbed by particular nanostructures, and lead to the emission of light at shorter wavelength that could activate more efficiently PSs [252].
	PS-activating light should be precisely delivered to the diseased tissue through image-guided techniques	Precise imaging of tumors and computer-guided delivery of PS-activating light will ensure PDT efficacy and minimal lesions of neighboring normal tissues. Magnetic resonance imaging (MRI) with iron oxide nanoparticles (contrast agent) that are loaded with PS could facilitate one step tumor imaging and PDT as theranostic approach [34]. Imaging of tumors that have accumulated fluorescent PS emerges as an alternative of improved intra-operative PDT which proved to be efficacious and safe in glioblastoma [253].

(Table 2) Contd....

Critical issues for an efficient PDT	Requirements	Actual drawbacks and proposed solutions
Oxygen availability	Singlet oxygen generation by light-activated PS needs the presence of molecular oxygen	Hypoxia is drastically limiting PDT efficacy considering that large tumors have a necrotic center beyond the capillary diffusion distance of oxygen from blood vessels, and viable cancer cells exist in an environment of decreasing hypoxia away from this center [254]. For better tumor oxygenation, hyper-oxygenation and a carbogen (30% CO_2 and 70% O_2) proved to be useful for enhancing PDT [255]. Many PSs with promising results *in vitro* failed in animal models due to tumor-specific hypoxic conditions. Nevertheless, it is considered that PDT might be successful even in hypoxic tumors by the damage inflicted to tumor vasculature, if PDT is applied when the PS is still present in the blood vessels [256].

which is highly susceptible to oxidative damage, is the one to be altered by mitochondria-targeted PDT. Oxidatively-altered mtDNA can trigger profound mitochondrial dysfunctions through an "auto-catalytic" loop by which increased production of oxidants cause mtDNA damage which further triggers more mitochondrial dysfunction, finally resulting in higher levels of free radical oxidants [41]. Additionally, PDT-induced oxidative damage to mtDNA impacts cellular responses to therapy, mainly by perturbing mitochondrial ROS production which supports the cytotoxic effects of PDT [42].

PS distributes both inside and outside of the tumor cells and produces distinct PDT-associated singlet oxygen bursts in these areas, as it will be further described. The PDT-triggered intracellular singlet oxygen burst occurs initially in the membranes of particular organelles in which PSs specifically accumulate depending on their physicochemical properties (i.e. charge, amphiphilicity and partition coefficients) [27]. Mitochondria, the endoplasmic reticulum (ER), the Golgi apparatus and lysosomes, along with the cell membrane represent targets of currently available PSs. Massive oxidative damage is inflicted by the singlet oxygen generated in these cellular components by an intense PDT reaction. Specific localization of PSs in particular organelles will dictate the type of ensuing cell death, as will be described later in this review. We emphasize that PDT triggers cell death not only if it generates a sufficiently intense singlet oxygen burst, but also if the local endogenous antioxidant and cell repair mechanisms get plainly overwhelmed by the acute oxidative burst and associated injuries produced by PDT.

Oxidation reactions triggered by PDT may also occur in the extracellular space, near cell membranes where PS is attached to the cell matrix and generates an extracellular burst of singlet oxygen following light activation. PDT-induced damages to matrix proteins and inactivation of extracellular antioxidants sustains the damaging effects of the oxidative burst within cells. As reviewed by Bauer (2016), an extracellular singlet oxygen burst generates a sophisticated auto-amplification cascade, leading to cell death and sustained inter-cellular signalling, as presented below [43]. The first action of PDT-induced singlet oxygen in the extracellular space, near the cell membrane, is inactivation of critical membrane-bound antioxidant enzymes such as catalase which is oxidized at key amino acid residues (Met) positioned in the catalytic center. Hence, a major protective mechanism against extracellular oxidative stress is down-regulated in cancer cells. In the tumor niche cancer cells are continuously under the attack of ROS/RNS, such as hydrogen peroxide and nitric oxide (NO). The membrane-associated catalase confers resistance to oxidative injury by interference with the pathway of hypochlorous acid generation through the decomposition of hydrogen peroxide, and also by interference with NO/peroxynitrite *via* NO oxidation and peroxynitrite decomposition [44]. An increase of hydrogen peroxide levels is generated by PDT outside the cells, and this will spread the oxidative signal towards neighbouring cells considering that hydrogen peroxide is readily crossing membranes through passive or aquaporins-mediated active transport [45]. As reviewed by Sies H (2017), it is now acknowledged that hydrogen peroxide is a critical second messenger of redox signalling in physiologic and stressful conditions [46]. The extracellular hydrogen peroxide that enters into cells can alter their redox homeostasis and therefore the oxidative signals produced in a particular tumor area may affect bystander cells that have not been directly subjected to PDT. Moreover, hydrogen peroxide together with hydroxyl radicals produced in the Fenton reaction will generate high amounts of hydroperoxide radicals that inflict damage to membranes in cells directly exposed to PDT, but can also spread the oxidative injury through a chain reaction in the spatially connected membranes of neighbouring cells.

Through an alternative mechanism, the initial PDT-induced singlet oxygen burst in the extracellular space can activate by oxidative oligomerization the death receptor FAS (CD95, apoptosis antigen 1) in a ligand-independent manner [10]. This results in enhanced activation of the membrane-bound NADPH-oxidase NOX1 [47], complemented by inactivation of superoxide dismutase and consequently sustained production of superoxide anion outside the cells. FAS activation also induces enhanced expression of the inducible form of NO synthase (NOS2) and formation of membrane-diffusible NO. NO can cause an additional inhibition of catalase, at other sites than those directly attacked by the PDT-induced singlet oxygen, hence generating a feed-forward loop for hydrogen peroxide production. The accumulation of hydrogen peroxide, superoxide anion and NO, along with lipid peroxides, leads to the formation of increased levels of peroxynitrite (formed by the chemical reaction of NO with superoxide anion) and to the second wave of singlet oxygen which arises this time from biologic sources through the interaction of hydroperoxides and peroxynitrite.

Despite controversy regarding the succession of events triggered by an extracellular burst of singlet oxygen [43], convincing evidence exists that it exerts an additional attack on cancer cells, hence reinforcing intracellular PDT. The oxidative injury triggered by PDT-derived singlet oxygen is prolonged and disseminated by hydrogen peroxide and NO throughout wider areas than those directly targeted by PDT. In turn, healthy tissues surrounding the tumor seem to be partially protected against the oxidative stress induced by extracellular PDT, considering that significant production of hydrogen peroxide, superoxide anion and NO outside the cells is a characteristic of cancer cells and not of normal ones [48].

It is of utmost importance to mention that singlet oxygen formation by PDT involves consumption of molecular oxygen and therefore transient hypoxia is locally induced and limits further ROS formation due to oxygen unavailability in PDT-targeted areas [49]. Nevertheless, PDT-induced hypoxia, which is adding to the intrinsic tumor hypoxia, can amplify the oxidative injury inflicted by therapy through profound alteration of the energetic metabolism in PDT-targeted cells, going up to significant ATP depletion and cell death. The tumor-specific hypoxic environment alone is apparently

not sufficient to result in cellular death, but it may decisively contribute to cell death in tumor areas affected simultaneously by oxidative stress, glucose deprivation and tumor-specific acidosis [50]. Additionally, hypoxia-oxygenation cycles within the PDT-treated tumor areas can trigger a secondary wave of ROS that will reinforce the PDT-generated oxidative burst-mediated by singlet oxygen and its consequences related to cytotoxicity and altered redox signalling [51].

There is a compelling need to develop sensitive imagistic methods for *in vivo* monitoring of PDT in order to better guide and personalize therapy. One line of action open to molecular imaging is to evaluate PS distribution in the targeted tumor in order to establish if there is sufficient PS and in which regions of the tumor it is distributed. This can be achieved by 3D-measurements of PS fluorescence (Fig. **1**) using fluorescence molecular tomography (FMT) combined with a) X-ray computed tomography, b) positron emission tomography (PET) using molecular markers sensitive to the presence of PS, c) magnetic resonance imaging. The first method is sufficiently sensitive and easily transferable for clinical applications of PDT, but has limitations due to the need for deep-penetrating excitation light in the red to near-infrared (NIR) spectral range for both imaging and PS activation in PDT. PS-targeting with upconversion nanoparticles (i.e. lanthanide-doped nanocrystals), having a broad excitation domain but a narrow emission spectrum, could significantly increase the imaging sensitivity of FMT [52].

Another approach for non-invasive PDT monitoring is to measure *in vivo* the levels and kinetics of singlet oxygen production in the tumor tissue by phosphorescence-based singlet oxygen luminescence measurements (Fig. **1**) [53]. This strategy is more relevant for PDT than PS measurements, considering that PS may accumulate into the diseased tissue, but, if the local light fluence is not sufficient for PS activation, or molecular oxygen is not available due to hypoxia, then the cytotoxic singlet oxygen is generated in too low amounts to inflict cancer cell death. However, measurement of singlet oxygen levels raises important technical issues due to the weak luminescence signal of singlet oxygen and its extremely short lifetime.

Going along the PDT reaction, therapy effects could be monitored in real time by non-invasive measurement of relevant biomarkers, including metabolites that reflect the cytotoxic effect of PDT in the tumor. Tumor-pertaining molecules as well as other endogenous molecules that are sensitive to the levels of free radicals induced by PDT could be detected by the newly-introduced hyperpolarized magnetic resonance [54], with adaptations to the molecular structure to be detected in order to afford sufficient signal lifetime [55]. Sandulache VC *et al.* (2014) [56] evidenced the potential of this technique to asses in real time the metabolic activity of solid tumors exposed to radiotherapy through exogenous conversion of ^{13}C-pyruvate into lactate. This allowed the authors to evaluate the tumor reducing potential, which is inversely correlated with ROS levels and therefore positively correlated with the amount of reducing equivalents. As radiotherapy and PDT rely on massive production of ROS and consequent decrease of reducing equivalents in the tumor, it is reasonable to consider that the experimental approach could be translated for *in vivo* PDT monitoring. Besides needing technologic innovation for *in vivo* measuring low levels of biomolecules reflecting PDT-induced changes, the discovery of reliable biomarkers specific for oncologic PDT, deriving from in-depth mechanistic studies, would greatly help for better orienting and controlling PDT.

4. CELL DEATH TRIGGERED BY PDT

The sub-cellular localization of PS in particular organelles and the intensity of the PDT reaction, along with the intrinsic characteristics of the PDT-exposed tumor cells dictate the signalling networks that govern the responses to therapy developed by cancer and stromal cells in the tumor niche, driving cancer cells either towards death or survival [57]. We should be aware of the new perspective on cell death types that go beyond apoptosis and necrosis, and may involve more sophisticated forms of cell death such as necroptosis, lysosomal and immunogenic cell death [58]. The type of cell death inflicted by PDT is critical for PDT consequences on the anti-tumor immune response, as we will describe later in this review.

4.1. Apoptosis

PS with negative electrochemical potentials accumulates preferentially in mitochondria where PDT initiates the intrinsic pathway of apoptosis [57] involving induction of the mitochondrial permeability transition, consequent release of cytochrome c, formation of the apoptosome and final activation of initiator and executioner caspases [59]. The clearance of apoptotic cells by macrophages is immunologically silent and does not trigger inflammatory reactions. This is an advantage considering that inflammation can sustain tumor progression [60] and may have deleterious effects, for instance in the case of brain tumors. From another point of view, it could also be a disadvantage because the anti-tumor immune response is not activated, and this will limit finally the therapy outcome. In turn, cells deficient in apoptosis may die following PDT by necrosis or necroptosis. In these forms of cell death, the integrity of cell membranes is compromised, various immunogenic factors are released, which further trigger inflammatory reactions and boost the anti-tumor immune response in particular conditions. However, these apoptosis-deficient cells may be spared from photo-killing and account for tumor relapse if PDT is not intense enough to induce necrotic/necroptotic cell death [19].

Direct effects of the PDT-triggered singlet oxygen burst on the apoptotic machinery involve an altered ratio between pro-and anti-apoptotic members of the Bcl-2 family, i.e. Bax/Bcl-2. This occurs through photo-oxidative alteration of the anti-apoptotic Bcl-2 protein, which is found in high levels in tumors [61], along with increased levels of the pro-apoptotic factor Bax [62]. The altered Bax/Bcl-2 ratio found in cancer cells exposed to PDT is also sustained by the increased expression of miR-143 induced by PDT, as demonstrated by Guo Q. *et al.* (2016) on cervical cancer cells exposed to ALA-PDT [63].

A particular death-promoting effect of PDT is related to the oxidative multimerization of TNF (tumor necrosis factor) receptors produced by the singlet oxygen burst in the absence of specific ligands [48, 64]. Activation of death signalling molecules is consequently triggered [65, 66], such as the TNF receptor-associated death domain (TRADD) to which RIPK1 associates together with the TNF receptor-associated factor 2 (TRAF2), the ubiquitin ligases cIAP1/2 (inhibitor of apoptosis protein-1/2) and LUBAC (linear ubiquitin chain assembly complex). This complex promotes the expression of pro-inflammatory genes by activating signalling pathways mediated by MAPKs and the transcription factor NFkB (Nuclear Factor Kappa B). Subsequent formation of a second cytoplasmic complex containing RIPK1 (receptor interacting protein kinase 1), FADD (death domain-containing adaptor) and caspase-8 drives cell death signalling. Auto-processing of caspase-8 initiates the apoptotic demise of the cell, whereas inhibition of caspase-8 activity causes cells expressing RIPK3 (receptor interacting protein kinase 3) and its pseudokinase substrate MLKL (mixed lineage kinase domain-like) to die by necroptosis rather than apoptosis.

As it will be further presented in this review, apoptosis signals are also delivered when other organelles than mitochondria, i.e. endoplasmic reticulum (ER) and lysosomes are targeted and damaged by PDT. Additionally, programmed cell death may occur in PDT-treated cancer cells irrespective of PS localization due to the marked inflammatory response elicited by PDT in cancer and resident immune cells. It is worth mentioning that PDT-triggered inflammation seems to be quite different from a physiologic sterile inflammation [67].

4.2. Necrosis and Necroptosis

PS localized at the level of the cell membrane generally induce cell death by necrosis, but we point out again that necrosis is induced irrespective of PS localization whenever an intense PDT is applied. The fundamental causes of necrosis include Ca^{2+} overload, massive ROS generation, energy depletion and extensive membrane injuries above a threshold that cell can bear. By contrast to apoptosis which is immunologically silent and sometimes even tolerogenic or immunosuppressive, cell rupture during necrosis has a high inflammatory outcome due to the release of intracellular components (i.e. cytokines, damage signals and tumor antigens) that activate the innate inflammatory response and are able to boost the anti-tumor immune response in specific conditions.

A particular type of programmed or regulated necrosis, which is termed as necroptosis [68], may be induced by PDT, most probably in cells with dysfunctional apoptotic machinery. For instance, Miki Y *et al.* (2015) evidenced that Talaporphin-PDT induces necrotic cell death in glioblastoma cells, and this is partly executed by the necroptotic pathway [69]. Necroptosis is generally triggered by death signals (i.e. TNFα, FAS ligand-FASL or TNF-related apoptosis-inducing ligand-TRAIL) which are massively generated in various PDT settings and exert their effects by interacting with specific death receptors that belong to the TNF receptor gene superfamily [70]. As mentioned previously in this review, oligomerization and activation of the death receptors FAS can be induced directly by singlet oxygen-mediated oxidation in absence of their ligands [10]. Activated death receptors further signal downstream *via* RIPK1 / RIPK3 and MLKL, and trigger either necroptosis in cells with dysfunctional caspase-8, or apoptosis in competent cells [68].

Like necrosis, necroptosis has been reported to induce sterile inflammation under stress conditions by disruption of the cell membrane and release of damage-associated molecular pattern (DAMP) proteins and members of the tumor necrosis factor (TNF) superfamily that trigger NFkB-mediated inflammatory signalling through TLR (toll-like receptors) and TNF receptors, respectively. ROS themselves can directly activate NFkB by oxidation of two redox-sensitive cysteines (Cys54 and Cys 347) in the IκB kinase (IKK) complex and further phosphorylation of IKKα/β involving various kinases [71]. As such, oxidative activation of NFkB can be induced directly by the singlet oxygen burst in PDT. Supporting co-activation signals may be delivered to NFkB by PDT-induced hypoxia that arises from oxygen consumption due to singlet oxygen formation [72]. Activation of the inflammatory NFkB signalling pathway triggered by PDT puts a brake on cell death by apoptosis or necrosis, as it up-regulates various anti-apoptotic factors such as Bcl-2 and cIAP [73, 74]. Therefore, pharmacologic inhibition of NFkB could sensitize cancers cells to mild PDT [75], especially in the case of cancer cells exhibiting constitutive translocation of NFkB to the nucleus and enhanced pro-inflammatory activity. In turn, the NFkB pathway can be drastically inhibited by intense PDT through oxidation of critical cysteines in NFkB complexes, such as Cys62 in RelA and Cys17 in IKK, which impairs their DNA binding and transcriptional activity. Accordingly, cancer cells are less protected against the PDT-inflicted injury if PDT intensity is sufficiently high. Moreover, because of cell demise, necrosis and necroptosis are in fact auto-limiting processes that rapidly terminate the robust pro-inflammatory program elicited by the initiating stimuli (i.e. death signals or oxidative stress) [76]. We point out that the transcription factor NFkB in cancer is a double-edged sword. The NFkB signalling network provides to cancer cells protection against death in particular inflammatory settings that sustain tumor progression, but inactivate the local anti-tumor immune response mediated by $CD8^+$ T lymphocytes and NK cells. Meanwhile, NFκB activation is important for initiating efficient anti-tumor immune responses by immune cells recruited into the tumor, hence providing to PDT an additional help to kill cancer cells and to avoid tumor recurrence. A more detailed description of "immunogenic cell death" will be made later in this review.

4.3. Lysosomal Cell Death

PS with positive electrochemical potentials preferentially targets lysosomes and trigger "lysosomal cell death" [77] in which the hydrolases-rich content of lysosomes leaks into the cytosol due to photo-oxidative damage of the lysosomal membrane and its consequent permeabilization (lysosomal membrane permeabilization, LMP). If specific enzyme inhibitors fail to inhibit the cytotoxic lysosomal discharge, then cell death by necrosis or intrinsic mitochondrial apoptosis occurs [78].

It has been shown that cytochrome c release, as early step in the mitochondrial pathway of apoptosis and 'point-of-no-return' for cell death, can be triggered by lysosomal cathepsins through the cleavage of Bid (BH3 interacting domain death agonist) and generation of a truncated form (t-Bid) which induces the relocation of the pro-apoptotic Bax proteins [79]. However, apoptosis induced by lysosomal discharge was shown to evolve with a slower kinetic than apoptosis triggered by mitochondria-targeted PDT [80].

Cathepsins can also increase mitochondrial ROS production which will further sustain the oxidative changes induced by the PDT-generated singlet oxygen [81]. Moreover, after the initial oxidative burst triggered by PDT at the level of lysosomes, secondary ROS might be generated as follows [82]: hydrogen peroxide produced in high amounts by cancer cells enters into the lysosomes and reacts with ferrous iron released after degradation of ferruginous compounds, generating highly reactive hydroxyl radicals *via* the Fenton reaction. Depending on the amount of hydoxyl radical, LMP is triggered by additional lipid peroxidation and destabilization of lysosomal membrane proteins, hence reinforcing the lysosomal cell death induced primarily by PDT. Cathepsin-mediated cleavage of cytosolic proteins can also lead to caspase-independent cell death in case of acute and profound LMP, or may lead to various forms of regulated necrosis in the case of milder LMP.

In particular conditions, PDT itself can switch off the lysosomal cell death through oxidative inactivation of lysosomal enzymes [83, 84], hence limiting PDT efficacy. Another rescue mechanism for maintaining cell homeostasis and organelle quality control is related to lysophagy [85] by which damaged lysosomes are removed. However, because lysosomes may be structurally and functionally damaged by PDT, the generated autophagosomes may become unable to fuse with lysosomes and to get degraded, and this will definitely aggravate the PDT-induced cell death.

4.4. ER Stress-induced Cell Death

ER is the major organelle in eukaryotes that maintain Ca^{2+} homeostasis and is responsible for accurate protein biosynthesis and post-translational processing, including mRNA translation, protein glycosylation and disulfide bonding. These events are highly dependent on the oxidizing environment in ER [86, 87] and therefore oxidative changes induced by PDT may disturb the quality control of proteins in ER.

PSs accumulating in ER, such as hypericin, induce ER stress in response to the singlet oxygen burst that is locally generated by PDT. It is noteworthy that ER stress is generally induced by PDT irrespective of PS localization due to the overload of oxidatively modified and degraded proteins generated by PDT. As reviewed by Moserova (2012) [88], upon accumulation of damaged proteins, the ER chaperone GRP78 (glucose-regulated protein) dissociates from ER membrane sensors [the protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), the activating transcription factor 6 (ATF6) and the inositol-requiring enzyme 1 (IRE1)], leading to their activation. Activated PERK phosphorylates the eukaryotic initiation factor 2 α subunit (eIF2α) to promote translation of the activating transcription factor 4 (ATF4) which subsequently up-regulates the expression of the pro-apoptotic CHOP protein

(C/EBP-homologous protein-10, GADD153). CHOP induction is considered a key element of the switch from pro-survival to pro-death signalling in ER stress [89].

A vicious oxidative cycle is generated by the increased protein-folding load in the ER of cancer cells, as follows [90, 91]: ROS from various sources target ER calcium channels [inositol 1,4,5-triphosphate (IP3) receptor (IP3R)] by the activation of the ER oxidase ERO1α, consequent Ca^{2+} release from ER stores and Ca^{2+} uptake by mitochondria *via* mitochondria-associated ER membranes (MEMs), finally resulting in increased production of mitochondrial ROS. The ER stress sensor PERK, associated with MEMs, is responsible for maintaining the ER-mitochondria juxtaposition and for promoting mitochondrial stress upon ROS-induced ER stress [92]. Additionally, ER stress signalling, involving Ca^{2+} and the calcium/calmodulin-dependent protein kinase II (CaMKII), induces the activation of NADPH-oxidases and consequent oxidative stress inflicted by superoxide anion, which is necessary for ER stress-induced apoptosis. By a feed-forward loop CHOP is amplified following the oxidative activation of a double-stranded RNA–dependent protein kinase (PKR) and triggers apoptosis [93]. As such, significantly high levels of ROS may accumulate and will deepen the oxidative injuries produced by the initial PDT-generated singlet oxygen burst.

ER stress may result in apoptosis through coordinated unfolded protein response (UPR) and Ca^{2+} signalling. Ca^{2+}-dependent cysteine proteases (calpains) are consequently activated and cleave caspases-4/12 [94] and apoptosis is further sustained by the cross-talk between the proteolytic systems of caspases and calpains. A caspase-mediated activation of calpains during apoptotic cell death occurs in absence of Ca^{2+} signals, through the cleavage of the endogenous protein inhibitor calpastatin [95]. Apoptosis can be induced by ER stress through two main mechanisms: one involves CHOP, as presented above, while the other one involves JNK (c-Jun N-terminal kinase) and p38 MAPK. An acute stress response to ROS is mediated by ASK1 (apoptosis signal-regulating kinase 1), downstream activation of JNK and p38 MAPK, along with the transcription factors of the activator protein 1 (AP-1) family [72]. The ER stress-induced apoptosis depends on the crosstalk between mitochondria and ER [96] which is mediated partly by PERK as a critical component of the protein network connecting ER to mitochondria [97]. Moreover, ER has the ability of amplifying or silencing apoptotic signals prior to mitochondria involvement through active Ca^{2+} fluxes that tightly control the physical and functional interaction between mitochondria and ER [98].

4.5. Autophagy

Cells respond to damages inflicted to its organelles either by activating the apoptotic mitochondrial machinery or by autophagy and UPR. Autophagy is a powerful mechanism to repair the damages inflicted by PDT to various organelles [99], therefore drastically limiting PDT efficacy. Additionally, the autophagic machinery can be directly initiated by PDT through the photo-oxidative damage of the anti-apoptotic Bcl-2 protein and consequent alteration of its interaction with the pro-autophagic protein Beclin 1 [100]. Autophagy is occurring independently of apoptosis and has a pro-survival role in mild PDT. It was shown that PDT protocols inflicting sub-lethal damages to lysosomes, ER or mitochondria induce the formation of autophagosomes that further fuse with lysosomes and eventually undergo degradation in the case of PSs that dock to ER or mitochondria. The autophagic flux is less clear in the case of PSs localizing in lysosomes, as PDT may induce in a context-dependent manner either oxidative inactivation of lysosomal enzymes or the discharge of lysosomal content following membrane permeabilization by PDT-induced oxidation.

A pro-death action of autophagy is registered in apoptosis-incompetent cells or in the case of acute cell injuries inflicted by therapy [101]. Autophagic cell death occurs whenever a critical accumulation of autophagosomes occurs under an intense cellular stress that overcomes a cell type-specific threshold. In this scenario, autophagy precedes cell death by necrosis or apoptosis. Alternatively, Buytaert E (2006) showed that following ER photo-damage and consequent disruption of Ca^{2+} homeostasis, the pore-forming Bax and Bak proteins are the ones to model how PDT-mediated cell killing is executed, either by apoptosis in their presence or by an autophagic pathway in their absence [102].

Finally, we emphasize that the crosstalk between cellular organelles makes shallow and imprecise the borders between cell death types inflicted by anti-cancer therapies, including PDT. Therefore molecular events underlining cell death and potential repair mechanisms should be comprehensively analysed for each new PDT setting in relation with PS localization, the cell type, its status and microenvironment. Most important, the type of tumor cell death induced by a particular PDT setting can define not only therapy efficacy, but also potential effects resulting in inflammation and, sometimes, activation of the anti-tumor immune response. These are important PDT consequences in the long run, leading either to complete tumor removal or tumor relapse due to the generation of therapy-resistant tumor cells.

4.6. Combined Therapies for Increasing Cancer Cells Death

As shown before in this review, particular localization of each PS in specific cellular organelles defines the type of death resulting from a sufficiently intense PDT. Some tumor cells may escape to the PDT-induced damages either because PDT was not intense enough or because these cells develop protective responses against the complex web of stresses inflicted by PDT (Fig. **2**).

It is reasonable to assume that a concerted therapeutic attack on several death-inducing pathways could significantly improve PDT outcome in terms of cancer cell death. For instance, by using PDT with well-characterized mixtures of PS which localize in distinct organelles, irreparable injuries could be triggered in cancer cells, making them unable to react to such a concerted attack. In this respect, low-dose photo-damage that sequentially targets first lysosomes and then mitochondria can provide significant advantages over the use of a single PS [103]. Additionally, other studies [104, 105] evidenced that low-intensity PDT protocols using concomitant delivery of two photosensitizers, one targeting lysosomes and the other one targeting the Golgi apparatus, are synergistically killing cancer cells *in vitro*.

Following the same idea, combined anti-cancer therapies applied either concomitantly or sequentially may bring improved efficacy. Various pre-clinical and clinical studies are confined to PDT combined with chemo- or radiotherapy. Besides attacking tumor cells through multiple pathways, such combined therapies significantly enhance ROS levels in cancer cells, especially when PDT and radiotherapy are combined. It is also noteworthy that also chemotherapeutics, such as arsenic trioxide and anthracyclines (doxorubicin, daunorubicin or epirubicin), increase mitochondrial ROS production and may amplify the oxidative damage inflicted by PDT [106]. Moreover, it was shown that particular PSs can be activated with deep-tissue penetrating X rays (5 Gy dose) that allow to perform PDT in deep-seated tumors, to take advantage of the cytotoxic potential of X rays adding to PDT-induced cell death, as well as to increase the radio-sensitivity of tumor cells by the PS [107].

5. OUT-OF-FIELD EFFECTS OF PDT

Singlet oxygen is a very reactive ROS with a short diffusion distance of approximately 20 nm [108]. Therefore, the oxidative damage triggered by PDT occurs in a very narrow area inside the cells exposed to PDT, which is related to PS location in particular membranes and organelles. The initially localized oxidative injury triggered by PDT alters key redox signalling pathways and spreads damage all over the cell due to the cell membrane continuum and the crosstalk between organelles [109]. Additionally, extracellular

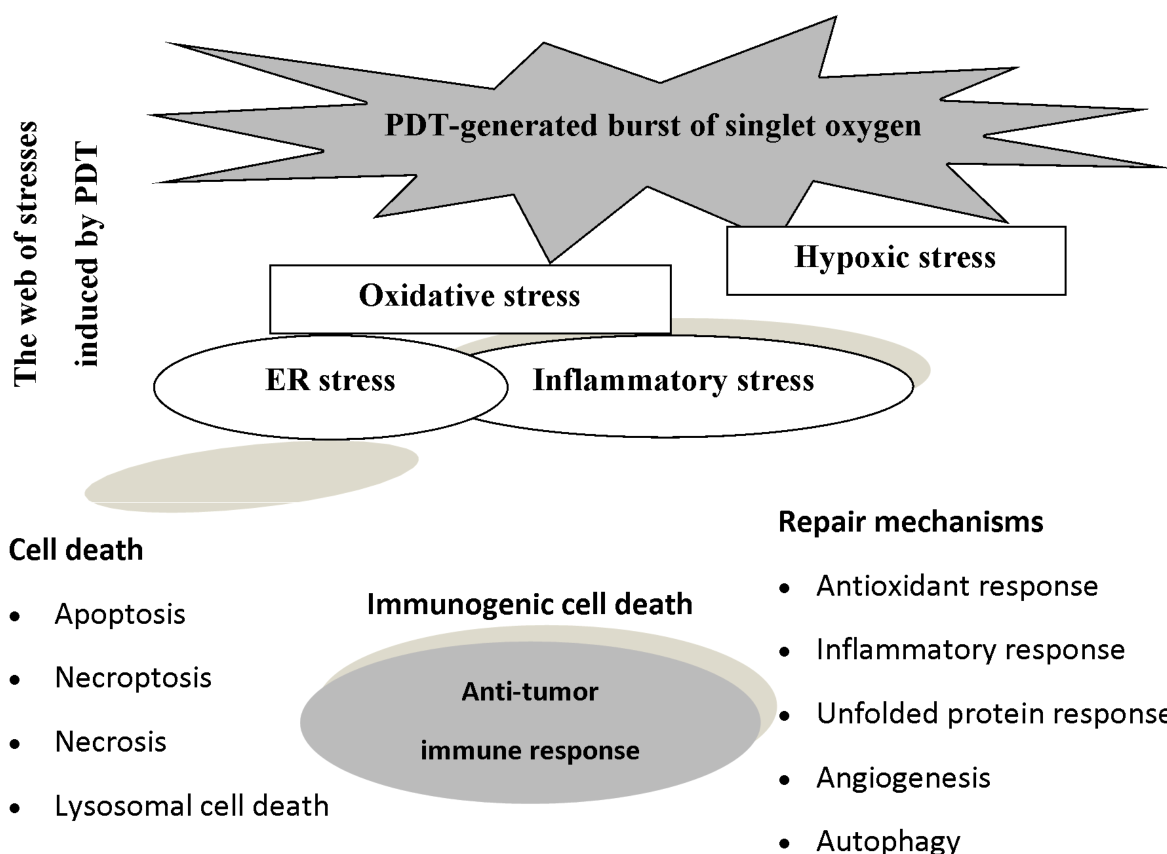

Fig. (2). The web of stressors induced by PDT: cancer cell death *versus* survival in PDT. The singlet oxygen burst triggered by PDT generates a web of inter-connected stresses within exposed cells, comprising: 1) oxidative stress triggered by singlet oxygen and secondary ROS produced following the damages inflicted by PDT, 2) hypoxia induced by local consumption of molecular oxygen due to singlet oxygen formation, c) ER stress arising from direct alteration of ER components or by proteotoxicity of oxidatively modified proteins, and d) inflammatory stress due to damages inflicted by PDT and activation of inflammatory signalling pathways. Depending on the intracellular location of the PS, the intensity of the PDT reaction and its spatial distribution in a "real" tumor, PDT may result in cell death in various forms or may inflict sub-lethal damages to which cells respond by activating various repair mechanisms. The main transcription factors controlling cellular responses to PDT-inflicted injuries are the transcription factor NRF2 which activates endogenous antioxidant and cytoprotective responses, the transcription factor NFkB which governs inflammatory responses, ATF (activating transcription factor) which controls the unfolded protein response, and HIF-1α (hypoxia inducible factor 1 α) which orchestrates cellular responses to hypoxia. Immunogenic cell death triggered by PDT is highlighted for its consequences in boosting the anti-tumor immune response in particular conditions, hence adding to the direct cytotoxicity of the singlet oxygen burst invoked by PDT.

PDT is able to spread the initial oxidative damage to neighbouring cells, as we have shown before in this review [43]. It has been clearly evidenced that PDT can exert out-of-field effects [110], more or less resembling the well-known by-stander effect of radiotherapy [15, 110-112], through which unexposed cells placed in close vicinity to the PDT area are indirectly affected. On one hand, cells within the tumor niche that were not affected directly by the targeted therapy may receive death or danger signals from PDT-damaged cells. By-stander tumor cells may become unstable and the area in which effective PDT occurs is extended. In turn, cells surviving to therapy get shielded against oxidative stress by developing potent cytoprotective mechanisms, hence becoming resistant to a second PDT hit. On the other hand, the normal tumor-neighbouring tissue could be also harmed through the by-stander effect of PDT, leading to unexpected side-effects despite the fact that PDT is intended to be a targeted therapy.

Regardless of extensive evidence on the by-stander effect related to radiotherapy, the underlining mechanisms and mediators are still elusive. It is supposed that ROS, oxidized biomolecules and inflammatory cytokines carry the oxidative and damage signals towards the neighbouring cells through tight junctions and interaction with TNF receptors and TLRs [15], as we will show below in the context of immunogenic cells death. Recently, it was found that various types of vesicles, including exosomes, are released from cells and are stable delivery systems that carry towards the neighbouring cells a complex cargo from the inside of cells (oncogenes, miRNA, membrane-bound signalling molecules, etc.) [113]. Unlike cytokines and other cell-derived soluble factors, these lipid bilayer-encapsulated vesicles protect their cargo and deliver at longer distances the information originating from damaged cells. Exosomes were shown to be critically involved in the initiation, progression and metastasation of tumors, representing therefore a rich source of biomarkers for disease and therapy monitoring [114]. Moreover, massive production and emission of extracellular vesicles occurs in response to anti-cancer therapies, such as radiotherapy and, to a lesser extent, chemotherapy. Exosomes are also released by PDT-treated cancer cells, as shown by Aubertin K. *et al*. (2016) in a prostatic cancer cell line exposed to mild Foscan-PDT [115]. It is worth noticing that a mild PDT stress can trigger reversible apoptosis and substantial extracellular vesicles release, whereas intense PDT induces irreversible cell death accompanied by low vesicle release. The consequences of exosomes release from cancer cells exposed to PDT should be carefully analysed. These cell-derived vesicles can carry the PS across the tumor and improve therefore PDT by enlarging the area around tumor blood vessels in which PS is available for being efficiently uptaken. Moreover, it was shown that PDT can limit tumor aggressiveness and invasiveness by de-

layed exosome-mediated reversion of the mesenchymal profile in head and neck cancer [116]. In turn, uncontrolled dissemination of PS *via* extracellular vesicles and consequent deleterious skin phototoxicity may occur, despite the intended regional limitation of PDT.

5.1. Immunogenic Cell Death and Anti-tumor Immune Responses in PDT

PDT induces a web of interconnected cellular stresses (Fig. **2**), comprising oxidative stress, hypoxia, ER stress and inflammation, that inflict lethal damages to exposed cells if the PDT reaction is sufficiently intense.

Stressed or dying cells release danger/damage signals (damage associated molecular patterns, DAMPs), which are proteins normally retained within cells and are released when cells are injured or are dying. Of note in the context of PDT is that therapy-induced ROS is functioning *per se* as a danger signal that triggers cellular defence responses by modulating redox signalling pathways that use hydrogen peroxide as secondary messenger [117]. DAMPs (Table **3**) warn neighbouring cells within the tumor niche that other cells were injured and, most importantly, alert innate immune cells to react to the therapy-inflicted damage. For more information on this topic, please consult the "danger model" introduced by Matzinger P, which complements the "self-nonself discrimination model" of immunity [118].

Particular forms of cell death have a distinct immunological impact and this is of utmost importance for cancer therapy outcome (Table **4**). Damage / danger signals can boost or suppress particular immune responses, depending on [119]: a) the type of the therapy-inflicted injury; b) the profile of DAMPs released by damaged cancer and normal cells exposed to therapy; c) the immune reactivity of stromal cells in the tumor niche and the competence of the general immune system. The type and efficacy of the immune response elicited by anti-cancer therapies will decide if therapy is further helped by an efficient anti-tumor immune response mediated by T lymphocytes, NK cells or granulocytes, or unwanted inflammation is triggered and provides fuel for residual cancer cells to survive and acquire resistance to therapy [120].

It is obvious that one of the main challenges of the current anti-cancer therapies is to develop strategies to drive cancer cells towards immunologic cell death (ICD) that ultimately results in the activation of a cognate anti-tumor immune response.

ICD is characterized by the release of significant amounts of stimulatory DAMPs, overwhelming immunosuppressive DAMPs that are simultaneously produced by injured cells (Table 3). As reviewed by Garg 2017 [119], the succession of immune events related to ICD could be the following in an ideal scenario: the immunostimulatory DAMPs generated by therapy-induced ICD recruit in the tumor niche and activate innate immune cells like DCs (dendritic cells), macrophages and neutrophils. Thereafter, in the lymph nodes, DCs or other APC (antigen presenting cells) process and present TAAs (tumor-associated antigens) released by dying cancer cells, and prime various immune cells such as $CD4^+$ T lymphocytes that differentiate into pro-inflammatory Th1 or Th17 phenotypes, along with cytolytic $CD8^+$T lymphocytes and $\gamma\delta$ T cells. Antigen-primed T cells then infiltrate the tumor in large numbers, and facilitate the elimination of residual cancer cells. Additionally, neutrophils are recruited within the tumor, get activated by cancer cells undergoing ICD and exert direct cytotoxicity against the remaining viable cancer cells that escaped to therapy. Immunostimulatory DAMPs secreted upon ER stress (i.e. heat shock proteins and calreticulin) activate the immune response, as follows: a) stimulate the pro-inflammatory NFκB pathway [121] and consequent IL-6 and TNFα release; b) directly activate the NLRP3 inflammasome [117] *via* TLRs or other receptors (i.e. purinergic receptors), leading to active generation and release of mature IL-1β and IL-18 which sustain innate and adaptive anti-tumor immune responses; c) activate type I interferon (IFN) responses that boost anti-tumor immunity mediated by NK cells and cytotoxic $CD8^+$ lymphocytes. Moreover, a systemic anti-tumor immune response could be generated by ICD even in highly targeted therapies, which may attack metastatic cells seeded far away from the primary tumor. For instance, radiotherapy combined with immunotherapy (ipilimumab - cytotoxic T-lymphocyte-associated protein 4 inhibitor) were shown to synergize for eliminating metastatic cells at distant sites from the radiation treatment field [122]. The observation that not all cancer patients respond to combined radiotherapy and immunomodulation suggest that a permissive tumor and host environment, along with timely coordinated immunomodulatory events are necessary to trigger systemic immune responses that are clinically meaningful in term of long-term disease control [123-125].

Extensive evidence exists that several anti-cancer therapies are able to induce ICD, i.e. radiotherapy, hyperycin-based PDT and chemotherapy with anthracyclines, cyclophosphamide, oxaliplatin, as well as targeted anti-cancer drugs (cetuximab, bortezomib). Because the ROS-based ER stress is a critical cue for ICD, Garg A *et al* (2015) classified ICD inducers [126] either as class I ICD inducers which do not target directly ER but inflict indirectly ER stress and associated danger signalling, or type II ICD inducers which target ER both for cell death and danger signalling. Type II ICD inducers seem to be more efficient in inducing ICD than Type I due to faster generation of a highly DAMPs-enriched pre-apoptotic stage interfacing dying cancer cells and DCs.

We will further focus on ICD induced by PDT which was demonstrated in various pre-clinical models for Photofrin- and Hypericin-PDT (type I ICD inducer and type II ICD inducer, respectively). The ICD induction ability of PDT adds anti-tumor adaptive immunity to the previously described PDT armamentarium for destroying cancer cells by direct tumor cells killing and vasculature damage through singlet oxygen-mediated oxidative injury. Moreover, from our point of view, it is extremely interesting how the initial singlet oxygen burst triggered by PDT finally results in the activation of an anti-tumor immune response.

The major DAMPs associated with PDT-induced ICD are described below [127].

The heat shock protein HSP70 was shown to be almost instantly exposed on the surface of cancer cells committed towards apoptosis by Photofrin-based PDT, and is thereafter released gradually due to membrane permeabilization upon necrosis [128]. Exposure of HSP70 on the surface of dying glioblastoma spheroids treated with 5-ALA triggers the recruitment and maturation of DCs, as well as antigen uptake by up-regulation of CD83 and co-stimulatory molecules, along with an increase of T cell activity [129]. Moreover, evidence exists that ecto-HSP70 participates in the opsonization of cancer cells by the C3 complement protein, hence increasing PDT significance for tumor cells killing besides the directly-induced oxidative stress [130]. In turn, evidence exists that heat shock proteins (HSPs) may also induce immunosuppression, considering that TLR2 and TLR4, through which HSPs are mainly acting, do not deliver strictly pro-inflammatory signals [131].

HMGB1 release has been demonstrated in Lewis lung carcinoma cells exposed *in vitro* to Photofrin-PDT and also in the serum of tumor-bearing mice treated by this PDT regimen [132]. HMGB1, normally located in the nucleus [133], is released by necrotic, apoptotic and autophagic cancer cells through a complex redox-controlled mechanism [see more on HMGB1 biology in [133]]. The impact of the secreted HMGB1 on cancer and immune cells is dependent on the type of cell death and the microenvironment encountered by the secreted HMGB1. Thus, HMGB1 released by cells suffering primary and secondary necrosis was shown to activate macrophages / DCs, and trigger neutrophil recruitment upon binding to various receptors [127, 134]. In turn, HMGB1 released by γ-irradiated tumor cells activated downstream of the ERK and p38 signalling pathways, promoted the proliferation of living tumor

Table 3. Main types of damps that modulate ICD [adapted after garg AD and agostinis P (2017) [119]].

DAMPs	Receptors	Action leading to ICD modulation
Immunostimulatory DAMPs		
Adenosine triphosphate (ATP) - passively or actively released during primary or secondary necrosis	Purinergic receptors: P2Y2 and P2X7	Immunostimulation: a) recruitment of inflammatory cells *via* P2Y2 receptors [257]; b) NLRP3 inflammasome activation, release of mature IL-18 and consequent increase in $CD8^+$ T lymphocytes [258]. Besides being activated by TLRs and purinergic receptors, NLRP3 inflammasome activation can be triggered directly by ROS [259] or by ER stress in conjunction with ROS through a process independent of the unfolded protein response (UPR) [260]. ROS, including those generated by NADPH-oxidases and mitochondrial ROS, are critically involved in NLRP3 priming by exerting a redox control on the TXN/TXNIP thioredoxin system [261].
Uric acid	?	Uric acid is an inducer of T cell responses towards particulate antigens [262], possibly through the activation of NLPR3 inflammasome [263].
Ecto-calreticulin (ecto-CRT) - surface exposed but sometimes passively released	CD91	These are “eat me” signals that trigger immunogenic phagocytosis [264, 265]. Their receptor, CD91, is involved in clearing proteases in tumors, hence preventing cancer invasion. CD91 may also aid tumor invasion by MAP kinases-regulated focal adhesion disassembly.
Ecto-Heat Shock Protein 90 (ecto-HSP90) - surface exposed and thereafter released by necrotic cells	CD91, TLR2, TLR4, SREC-1 and FEEL-1	
High Mobility Group Box 1 (HMGB1) - passively and sometimes actively released [266]	RAGE, TLR2, TLR4 and TIM3	Immunostimulation: HMGB1 initiates an acute phase response (production of CRP) and release of pro-inflammatory cytokines (TNFα, IL-6, and MIP1α and –β) in human monocytes [267]; HMGB1 can also modulate the adaptive immune response [268]. In cancer, plasma and tissue protein expression correlated with worse overall and progression-free survival [269].
Mitochondrial DNA - passively released by apoptotic and necrotic cells	TLR9	Immunostimulation induced by oxidized mtDNA through activation of the NLRP3 inflammasome [270]; elicits neutrophil-mediated organ injury [271].
Oxidation-associated molecular patterns: reactive protein carbonyls and peroxidized phospholipids - passively released during necrosis	CD36, SR-A, TLR-2/4	Immunostimulation: accumulation of pro-inflammatory Th1 cells, reduced infiltration of immunosuppressive Tregs, and increased ratio of cytotoxic $CD8^+$ T lymphocytes / immunosuppressive Tregs [272].
Peroxyredoxins: PRDX1, PRDX2, PRDX5 and PRDX6 - actively or passively released by necrotic cells	TLR2, TLR4	Immunostimulation: expression of inflammatory cytokines in macrophages and enhanced cytotoxic activity of NK cells [273].
Immunosuppressive DAMPs		
Phosphatidyl serine - surface-exposed by apoptotic cells	Scavanger receptors	“Eat me” signals expressed by apoptotic cells which trigger tolerogenic phagocytosis [274].
Death domain 1α (DD1α) - surface exposed by apoptotic cells [275]		
Annexins (annexin A1) - surface-exposed by apoptotic cells	Formyl peptide receptors	Immunosuppression: inhibition of $CD8^+$ T cell immunity [273, 274, 276].

Table 4. Cell death from the immunological perspective.

Cell Death Defined by Immunological Outcome	Activation *Versus* Inhibition of Immune Mechanisms
Tolerogenic cell death	
Tolerogenic apoptosis	Apoptotic cells are specifically recognized and uptaken by professional and non-professional phagocytes through a “find me” scenario, and are physiologically eliminated through an “eat me” scenario without induction of inflammation (see the critical review [264]). For instance, certain annexins (i.e. annexin 5 and 13) exposed by apoptotic cells were shown to deliver tolerogenic signals to phagocytes [274]. In addition, anti-cancer immunity gets locally and systemically suppressed by release of anti-inflammatory factors such as TGFβ, IL-10, PAF and PGE2 [277], induction of immunosuppressive Tregs [278] and accumulation of functionally polarized M2-macrophages [119]. During apoptosis potent DAMPs that could have boosted inflammatory processes (i.e. HMGB1) are inactivated or degraded by caspase-facilitated oxidation [136]. Nevertheless, abnormally prolonged or incomplete processes for destroying apoptotic cells lead to secondary necrosis that brings about massive release of pro-inflammatory factors [264].
Immunosuppressive necroptosis	Although necroptosis is characterized by permeabilization of plasma membranes and release of pro-inflammatory DAMPs [279], it may also induce immunosuppression by various mechanisms: a) upregulation of chemokines which promote the recruitment of various immunosuppressive cells such as IL-10-producing αβ T cells [278]; b) suppressed production of pro-inflammatory factors such as chemokines (CCL2, CXCL1/2, CCL5) or GM-CSF [119, 280]; c) externalization of the conventional immunosuppressive apoptosis-related “eat me” signal, phosphatidyl serine, before losing plasma membrane integrity [281]. Therefore, it is reasonably to hypothesize that the net balance of immunosuppressive and immunostimulatory signals delivered by cells suffering necroptosis, along with the phenotypic and functional profile of stromal cells will finally dictate the immune outcome of this type of cell death. Nevertheless, necroptosis culminates into typical necrotic cell demise and this may trigger finally a strong inflammatory response (see below immunogenic necroptosis).
Inflammatory cell death	
Accidental necrosis	Necrotic cells loose cell membrane integrity and release their content before phagocytes eliminates them. Consequently, they induce a strong local inflammatory reaction that is exacerbated by active recruitment of inflammatory cells like monocytes and macrophages. Necrosis-induced inflammation superimposes on the tumor niche-specific inflammatory milieu and may either sustain tumor progression by providing pro-tumorigenic factors to remaining viable cells and by inducing carcinogenesis [150]. This impedes on therapy outcome and may account for tumor relapse. In addition, a local massive inflammation may have life-threatening consequences such as in the case of post-therapy inflammation in the brain. Considering that immunosuppressive, immunostimulatory and neutral DAMPs (Table **3**) are released simultaneously by necrotic cells, along with anti-inflammatory cytokines and other immunosuppressive factors, consistent anti-tumor immune responses are not clearly induced by necrosis. Moreover, the pro-inflammatory environment shaped by necrosis induces the differentiation of immunosuppressive CD4+ T cells (Tregs and Th2-polarized T lymphocytes) that tend to resolute inflammation and to limit tumor-directed immune responses. It is worth mentioning that necrotic cancer cells release hidden TAAs, some of them with high immunogenic potential (i.e. melanoma-associated differentiation antigens). If the local load of immunosuppressive factors is low, TAAs may elicit in particular cases threshold levels of anti-tumor immune responses [272].
Immunogenic cell death (ICD)	
Immunogenic apoptosis Immunogenic necroptosis	Major cellular stresses and associated signalling networks that play a critical role in DAMPs trafficking and release are the following: a) ER stress in conjunction with oxidative stress induces a strong ICD through proteo-toxicity, Ca^{2+} imbalance or redox perturbations at the level of ER. ICD may be either dependent (chemo- and radiotherapy-induced ICD) or independent (Hyp-PDT-induced ICD) of the unfolded protein response (UPR) [282]; b) ATP and HMGB1 release by dying, autophagy-competent cancer cells is critical for the induction of anti-tumor immunity [283]. Chaperone-mediated autophagy was shown to modulate danger signalling mainly through heat shock proteins that get expressed on the luminal face of dying/damaged cells and act as “eat me” signals. Meanwhile, macroautophagy generally induces ICD by enhancing chemotherapy-induced ATP secretion, but it may also suppress ICD in particular cases, for instance by dampening ecto-CRT induced by Hyp-PDT [139, 284, 285].

cells [135]. In apoptotic cells, HMGB1 release in conjunction with ROS production was shown to induce immunosuppression through caspase-mediated oxidation [136]. Of note for its relevance to ROS-based therapies, the redox state controls the action of HMGB1 on therapy-induced cell damage. Thus, reduced HMGB1 decreases injuries in cancer cells by autophagy modulation through interference with Beclin-1, whilst oxidized HMGB1 may enhance cell death in response to anti-cancer therapies [137].

Exposure of the ER chaperone calreticulin (CRT) on the surface of dying cells, along with ATP release by these cells seems to be

critically involved in PDT-triggered ICD when the naturally occurring chromophore hypericin (Hyp) was used for photosensitization [138]. Hyp is one of the few type II ICD inducers, along with the oncolytic Coxsackievirus B. It is acting for tumor cell killing by ROS-based ER stress, consequent activation of various signalling pathways related to UPR, and final killing of cancer cells by mitochondrial apoptosis [102]. ICD induced by Hyp-PDT results in the phenotypic/functional maturation of DCs and activation of their phagocytic ability. These activated DCs were shown to exhibit a peculiar functional profile, characterized by increased NO generation, production of high levels of the pro-inflammatory cytokine IL-1β which is required for the polarization of IFNγ-producing cytotoxic $CD8^+$ T cells, paralleled by low production of the anti-inflammatory cytokine IL-10 [58, 138]. The DAMPs signature of Hyp-PDT comprises ecto-CRT, ecto-HSP70 and ATP in early pre-apoptotic stages, before phosphatidylserine exposure. As compared to radiotherapy, the kinetics of DAMPs generation by Hyp-PDT is quicker, and this is possibly translated into the more effective ICD observed in Hyp-PDT. CRT exposure and ATP release were shown to be dependent on singlet oxygen generation, PI3K activity, ER-to-Golgi transport, ER stress-associated PERK, pro-apoptotic Bax and Bak, and the expression of CD91(CRT receptor). Unlike the intensively investigated anthracyclin-induced ICD, Hyp-PDT does not require the ER chaperon ERp57, eIF2α phosphorylation, caspase 8 activity or increased Ca^{2+} concentrations for membrane exposure of CRT. Meanwhile, ATP release in response to Hyp-PDT was shown to be dependent on ER-to-Golgi anteretrograde transport, PI3K and PERK activity, but did not require the pro-apoptotic Bax and Bak proteins [58]. Autophagy induced by Hyp-PDT is seemingly playing a role in suppressing ecto-CRT induction, as demonstrated in LAMP2A-deficient cells [139], but it is not involved in ATP release. This is a Hyp-PDT-specific mechanism aimed at avoiding ICD, that is contrary to the role of autophagy evidenced in chemotherapy-induced ICD [140], and may differentiate therefore type I and II ICD inducers. An important advantage of Hyp-PDT over other anti-cancer therapies consists in the down-regulation of cancer cell-derived pro-inflammatory cytokines, such as GM-CSF, inhibition of tumor-promoting cytokine signalling mediated by NFkB and AP-1, and inhibition of the metastasis-promoting matrix metalloproteinases-9 (MMP9) [141]. In normal cells (macrophages), PDT was shown to induce strong activation of NFκB and AP-1, leading to the release of a plethora of immunoregulatory and pro-inflammatory factors which sustain the anti-tumor immune response [142]. However, particular PDT regimens are able to increase NFkB and AP-1 activity also in cancer cells. For instance, NFκB activation through the IL-1 receptor was evidenced in human colon carcinoma cells that were photo-treated with pyropheophorbide-a methyl ester [143], while AP-1 activation was detected in cervical carcinoma cells photosensitized with Photofrin [144]. This hectic pro-inflammatory response triggered by PDT raises important issues for therapy outcome, because it can positively boost the anti-tumor immune response, but may also trigger unwanted inflammation that, if persistent, may promote carcinogenesis and tumor progression.

For mounting an efficient anti-tumor immune response, ICD has to overcome the suppression of the immune response that is constitutively induced by the tumor itself due to reduced expression of MHC molecules, increased expression of immunosuppressive molecules (Fas ligand) and cytokines (IL-10, TGFβ), along with cancer-induced reprogramming of early myeloid differentiation in the bone marrow to generate immunosuppressive neutrophils [145, 146]. The tumor immune infiltrate is also skewed toward an anti-inflammatory and immunosuppressive functional phenotype due to the expression of the programmed cell death ligand-1 (PD-L1) [147]. Accumulation of myeloid-derived suppressor cells (MDSCs), increased infiltration of T regulatory cells (Tregs), reduced number of effector T cells (e.g., $CD8^+$ cytotoxic T lymphocytes) and a bias of the T $CD4^+$ immune response towards a Th2 functional phenotype greatly contribute to cancer-induced immune suppression [148]. Furthermore, molecular mechanisms triggered by therapy are the ones that could induce immunosuppression through direct interference with immune cells. An alternative mechanism is the inactivation of immunostimulatory DAMPs released by dying cells, such as the oxidative alteration of HMGB1 [149], and consequent shifting of the functional profile of DAMPs towards immunosuppression. It is also noteworthy that DAMPs that underline ICD can be constitutively produced in lower amounts in the tumor niche, and generally exert a tumor-promoting activity [150]. This is the reason why the profile, intensity and spatio-temporal distribution of immunostimulatory and immunosuppressive DAMPs, along with the complex constellation of therapy-induced cytokines and chemokines, will definitely dictate the immune impact of ICD, together with potential tumor-promoting effects in areas sub-lethally hit by therapy. This is critical in PDT considering the non-uniformity of PDT effects within the tumor, deriving from cells heterogeneity in the tumor niche and uneven singlet oxygen generation due to the irregular topological distribution of PS, oxygen and light fluence [151].

Existing data show that the therapy-induced cell death may be complex and pleiotropic, and the DAMPs profile elicited by dying cells within a tumor is not clearly related to the type of cell death and cannot precisely define the immune impact due to the complexity of immune responses. Nevertheless, it is of utmost importance to better define ICD in various pathophysiologic and therapeutic settings for improving stratification of cancer patients regarding disease prognosis and response to particular anti-cancer therapies. It may also instruct therapeutic choices, including combined therapies focused on specific molecular or cellular defects that could be pharmacologically exploited and targeted for improving the final therapy outcome [152].

5.2. Therapeutic Strategies to Boost the Anti-tumor Immune Response

Anti-cancer therapies which promote ICD could be boosted by combination with immunomodulatory strategies. For instance, immune checkpoint blockers could significantly improve anti-tumor immunity by inhibiting auto-regulatory or immunosuppressive pathways at the level of T lymphocytes. These immunosuppressive pathways are mediated by the programmed cell death protein 1 (PD-1) and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which are activated by ligands expressed by tumor-promoting immune cells and help cancer cells to evade from an immune attack [153, 154].

Another strategy for reinforcing the immune impact of ICD is the adoptive T cell transfer therapy (ACT) by which autologous T cells are activated and expanded *ex vivo*, and are thereafter injected back to the patient for enabling TAA-directed immunity, irrespective of the innate immune stimulation produced by PDT [155].

Emerging therapies that target the phagocytic interface of ICD with antibodies against the “don’t eat me” signal CD47, depletion of Tregs by anti-CD25 antibodies or cyclooxygenase-2/arginase inhibitors against MDSCs are under investigation as co-therapies to be combined with chemo- or radiotherapy [119]. Meanwhile, combined PDT and immunotherapy is still in its infancy, albeit showing great promise according to pre-clinical studies that have documented PDT-triggered ICD.

Based on the ability of PDT to release hidden intracellular TAAs with immunogenic potential, and to activate the anti-cancer immunity through ICD of cancer cells (see above Hyp-PDT), an attractive adaptation of PDT is related to the generation of autologous cancer vaccines that are able to orchestrate a strong anti-tumor immune reaction [156]. Briefly, tumor tissues/cells are loaded with PS, are then exposed *ex vivo* to PDT and are further maintained in culture for 16-24 hrs to allow expression of cell death-associated changes in the vaccine cells. Pharmacologic manipulation of cancer

cell death post-PDT using necroptosis inhibitors (necrostatin 1 and 7) or the necrosis inhibitor IM-54, as well as the lethal autophagy inducers STF62247 and Spautin-1, proved to increase the therapeutic efficacy of the PDT-generated cancer vaccine, evidenced by reduced tumor progression in mice bearing squamous cell carcinoma [157]. Moreover, the study indicates that the induction of lethal autophagy closely connected with reticular UPR triggered by ER stress, along with cell death by apoptosis but not by necroptosis, are required in the above presented PDT regimen for generating a good anti-tumor cell vaccine.

6. THE TRANSCRIPTION FACTOR NRF2 AS PROMISING THERAPEUTIC TARGET IN PDT

6.1. Cell Death Versus Cell Repair in PDT

PDT generates in exposed tumors a web of stressors (Figure 2), comprising: **a)** oxidative stress triggered by singlet oxygen and other ROS that are produced consequent to the PDT-generated damage, i.e. ROS produced by altered mitochondria or by hypoxia-oxygenation cycles, **b**) ER stress induced by PDT directly or, indirectly, by accumulation of oxidized proteins, **c)** hypoxia generated due to local oxygen depletion by singlet oxygen formation, **d)** inflammatory stress mediated by cytokines and DAMPs produced by PDT-damaged tumor cells and by resident or recruited immune cells.

The PDT-induced stresses add to the adverse environment of tumor niche where oxidative stress and inflammation, along with acidosis and hypoxia are concurrently generated by tumor and stromal cells [9, 158]. Most probably cancer cells arise from a selection process by which only cells shielded by potent protective mechanisms aimed at counteracting the damaging effects of multiple intra- and extracellular stressors are the ones to survive and proliferate. Exposure of cancer cells to therapy may lead to their death under various forms (necrosis, apoptosis, ferroptosis, necroptosis, etc.), if the therapeutic challenge overcomes a cell-specific threshold of cellular resistance. If cells have enough time until death occurs in response to therapy, or if cells suffer sub-lethal injuries, then additional protective mechanisms are initiated aiming to repair or limit the injuries caused by therapy. Damaged and stressed cells also send signals to neighbouring cells, communicating that a danger appeared in the area and that they are injured (see above the out-of-field effects of PDT).

Cells are endowed with protective mechanisms that, in the case of tumors, give them an exquisite survival and proliferation advantage, and also account for their primary or acquired resistance to anti-cancer therapies [159]. Shielding mechanisms developed by cancer cells against PDT-inflicted injuries have a particular significance, considering that the PDT outcome in terms of lethal and sub-lethal effects is heterogeneous in different areas of the tumor. There are several reasons for this heterogeneity: a) large diversity of both tumor and stromal cells within the tumor niche [160] which makes PS to accumulate differently in these cells; b) non-uniform distribution of PS that diffuses only in a restricted area around the tumor blood vessels. Therefore, cells that are placed away from tumor blood capillaries will have a smaller PS load, or even no PS is up-taken by cells placed in distant areas; c) non-uniform light penetration and fluency in various tumor regions, at least as obtained with the technologies that are currently available; d) uneven oxygen availability, hampering the generation of singlet oxygen by the excited PS in tumor areas with reduced vasculature. All these impede on PDT efficacy and make treatment difficult to control. We presume that within a tumor, one may achieve a gradient of PDT intensities going from mild to intense reactions, while some tumor areas may not be attacked by PDT. For these reasons, in “real” tumors we can expect that acute PDT and massive cell death will be generated only in some regions, while other tumor areas will be exposed to sub-lethal PDT. Accordingly, some cells will die by primary or secondary necrosis, other by apoptosis, whilst other tumor cells may evolve towards a more aggressive phenotype if they are subjected to mild PDT and effective repair mechanisms get initiated for “healing” PDT-induced damages. Moreover, cells escaping PDT may acquire long-lasting resistance against PDT or other anti-cancer therapies, and account for tumor relapse in time and resistance to a second session of therapy. It is worth noticing that therapy-induced resistance complements the intrinsic resistance provided by cancer stem cells which are endowed with an exquisite shielding against various stressors, including anti-cancer therapies [159]. Therefore, both constitutive and induced resistance to PDT should be addressed by in-depth investigations for identifying new therapeutic targets and strategies to increase the sensitivity of tumors to PDT.

6.2. The Transcription Factor NRF2 in Cancer

Considering that the initial type of stress inflicted by PDT is oxidative stress, it is reasonable to assume that an increase in PDT efficacy may be obtained by therapeutically targeting redox signalling networks [161].

In the attempt to prevent or repair oxidative damage, cells respond to PDT by activating their endogenous antioxidant system. Interestingly, human cells are not endowed with sophisticated enzymatic systems for detoxifying the peculiar ROS generated in PDT, and this is because singlet oxygen is not a common ROS in animal cells, as explained before in this review. Singlet oxygen is a highly reactive ROS and has therefore a short life (in the order 40 ns) and an action radius of ~20 nm [108]. Singlet oxygen is actually detoxified through oxidation of biologic molecules (lipids, proteins, nucleic acids) in a very small area around the place of its generation. The ensuing oxidized biomolecules are more stable than singlet oxygen and inflict broader injuries, especially to cellular membranes in which amphiphilic PS localize, and a lipid peroxidation chain occurs. In this context, the endogenous antioxidant system is likely to repair these secondary injuries that propagate inside and outside the PDT-targeted cells, rather than to counteract the initial singlet oxygen burst.

The basic region-leucine zipper (bZip)-type transcription factor NRF2 (Nuclear Factor Erythroid 2-related factor 2) orchestrates the antioxidant response and other cytoprotective mechanisms by targeting the consensus enhancer termed antioxidant response element (ARE) which is located in the promoters of many genes [162, 163]. NRF2 controls the transcription of more than 250 cytoprotective genes involved in stress-mediated induction of many homeostatic responses that include antioxidant regulation, initiation of detoxification mechanisms, adaptation to proteotoxic stress by up-regulation of genes involved in the repair and degradation of damaged biomolecules [164], and the reprogramming of the intermediary metabolism by facilitating flux through the pentose phosphate pathway, increase of NADPH regeneration and purine biosynthesis [165]. The most important NRF2-regulated genes involved in antioxidant defence mechanisms and redox signalling, which are of utmost importance for ROS-based therapies, are presented in the article of Ma Q (2013) [162]. New NRF2 targets continue to be evidenced, pointing out towards the pleiotropic functions of this transcription factor, NRF2 being more or less directly involved in a broad network of processes governing the adaptation of cells to multiple stresses. Therefore, perturbations of the NRF2 interactome have been reported in various chronic diseases underlined by low-grade oxidative stress and inflammation, as well as in cancer [2].

NRF2 behaves in cancer like a proto-oncogene that induces chronic activation of ARE-mediated stress responses, above the physiologic adaptive response against the intricate oxidative and inflammatory environment in which cancer cells evolve in the tumor niche [166]. Up-regulation of the endogenous antioxidant system is a vicious adaptation mechanism of cancer cells in response to increased ROS levels, which allows them to use ROS and redox

signalling for tumor survival and growth, as well as for metastasation [167]. Moreover, constitutive NRF2 activation is critically involved in triggering and maintaining stemness of cancer stem cells which are responsible for tumor relapse and formation of distant metastasis [168]. For instance, the secretome of highly tumorigenic cancer stem cells from human colorectal tumor specimens contains a NRF2-specific protein signature, comprising high transcriptional levels of *GCLC*, *GPX2/3* and *TXNRD1*, that is presumed to provide to these cells exquisite resistance to the stressful microenvironment in the tumor niche and also to anti-cancer therapies [169]. Moreover, the study of Leone A *et al.* [3] established the correlation between oxidative stress gene expression and disease prognosis in various solid tumors. *FOXM1* and *TXNRD1* genes, both involved in ROS metabolism, were found to be highly expressed in poor prognosis patients with pancreatic, colon, lung, prostate and breast cancers. *FOXM1*, encoding the Forkhead box protein M1, plays a key role in proliferation, metastasis, apoptosis and DNA damage repair, as well as in preventing premature senescence induced by oxidative stress. The NRF2 target *TXNRD1* encodes a cytosolic member of the thioredoxin system (thioredoxin reductase 1) that responds to oxidative stress and modulates the redox status by regulating several redox enzymes and signalling proteins. Accordingly, the study emphasizes cytoprotective mechanisms against ROS-induced alterations that are critically involved in tumorigenesis and cancer progression.

Constitutive activation of NRF2-mediated transcription in tumor cells was shown to arise from various cancer-associated alterations: **a)** oncogenic alleles of KRAS, BRAF or *c-MYC*, acting for NRF2 activation *via* oncogene-mediated increase of ROS levels [170], or mutations in the tumor suppressor PTEN (phosphatase and tensin homolog) that lead to hyperactive and oncogenic PI3K/Akt signalling and consequent increase in NRF2 activity [171]. For instance, endometrioid carcinomas lacking PTEN were shown to express high levels of NRF2, and genetic / chemical inhibition of PTEN led to up-regulation of NRF2 as a necessary contributor to tumorigenesis [172]; **b)** somatic mutations or epigenetic changes in the NRF2 repressor KEAP1 or at the level of NRF2 itself, which trigger chronic activation of NRF2 in various types of malignancy [173-177].

6.3. Mechanisms of NRF2 Activation

The mechanisms of NRF2 activation will be presented below for better understanding NRF2 as a therapeutic target for increasing the efficacy of ROS-based therapies and for tailored design of pharmacologic NRF2 modulators.

Under physiological conditions, NRF2 is maintained in inactive form by interaction with a homodimer of KEAP1(Kelch-like ECH-associated protein 1) through the 29-DLG-31 and 79-ETGE-82 motifs contained in the Neh2 domain of NRF2. Both ETGE and DLG bind to similar sites on the bottom surface of the Kelch motif in KEAP1, but with distinct affinities. Thus, while ETGE binds tightly to KEAP1, DLG binding affinity is much weaker and the DLG-KEAP1 complex is quickly dissociated, hence fine-tuning ubiquitination and degradation of NRF2, as will be described below.

KEAP1 is an adaptor protein substrate of the CUL3-RBX1 (Cullin 3-Ring Box Protein 1) E3 ubiquitin ligase which binds to the BTB domain of KEAP1. Consequently, KEAP1 promotes ubiquitination of NRF2 at several lysine residues in the Neh2 domain, finally leading to NRF2 degradation by the 26S proteasome [178]. Altogether, KEAP1 acts as a repressor of NRF2 by promoting its proteasomal degradation. Alternatively, GSK-3 (glycogen synthase kinase 3) phosphorylates NRF2 at the level of the 334-DSGIS-338 sequence in the Neh6 domain, and creates henceforth a recognition motif for the E3 ligase adapter β-transducin repeat containing E3 ubiquitin protein ligase (β-TrCP) which presents NRF2 to a CUL1/RBX1 complex, leading to ubiquitin-dependent proteasomal degradation of NRF2 [179-181].

NRF2 activation *via* the canonical mechanism is carried out by oxidative stress and electrophiles that oxidize sensitive cysteines located in KEAP1, including C151, C273 and C288. KEAP1-NRF2 interaction is disrupted through misalignment of lysine residues within the DLG motif of NRF2. Alternatively, NRF2 is activated by thiol modifications that produce the dissociation of Cul3 from KEAP1 [182].

NRF2 activation can occur also by a non-canonical mechanism in which various proteins can alter the KEAP1-NRF2 interaction. For instance, p21 (Cip1/WAF1) which regulates many cellular processes such as cell cycle arrest, DNA damage repair, cell differentiation, senescence and apoptosis, exert a cytoprotective effect through direct interaction of the p21-154-KRR cluster with the 29-DLG and 79-ETGE motifs in NRF2 [183]. Thus, p21 competes with KEAP1 for NRF2 binding and ubiquitination, NRF2 degradation being therefore compromised. From another perspective, NRF2 prevents cellular processes driven by p21 through physical interaction and inhibition of p21, The autophagy receptor p62/SQSTM1, involved in autophagy and proteasomal degradation of ubiquitinated proteins, competes with NRF2 for binding to KEAP1 [184]. p62 has been identified as a pathogenic target of 5q copy number gains in kidney cancer, and may be responsible for increased NRF2 activation [185-187]. It is noteworthy that p62/SQSTM1 and other autophagy-related genes are transcriptional targets of NRF2 and therefore a regulatory loop is generated for controlling the activation of NRF2 in relation with autophagy. Accordingly, other cellular processes besides direct oxidative alterations in the KEAP1-NRF2 complex, such as autophagy [185], can control the redox status by interference with the antioxidant/cytoprotective signalling mediated by NRF2.

In response to an oxidative or electrophilic attack, the KEAP1/NRF2 interaction is disrupted and newly synthesized NRF2 evades proteasomal degradation and translocates to the nucleus. Here, NRF2 forms heterodimers with Zip (leucine zipper)-containing small Maf proteins, bind to ARE and initiate a broad transcription program of cytoprotective genes [188]. It is noteworthy that AREs overlap with other enhancers that are recognized by Zip proteins, besides NRF2, including AP-1, BACH1, CREB/ATF and Maf homodimers, and may further impinge on the regulation of NRF2-dependent genes [162]. In this respect, BACH1, which has cytoplasmic localization in resting conditions and nuclear localization under stress, is considered to be the main repressor of NRF2 transcriptional activity when excessive antioxidants are formed and the antioxidant response has to be inhibited for restoring homeostasis [189]. Moreover, the antioxidant NRF2 and the pro-inflammatory NFκB transcription factors compete with each other for the transcriptional co-activator CBP (cAMP-responsive element-binding protein (CREB) binding protein) [190]. Hence, a strong pro-inflammatory response restricts the transcriptional activity of NRF2 and promotes enhanced oxidative stress. In turn, NRF2, which is an upstream regulator of cytokine production through interaction with inflammation-responding transcription factors, may attenuate inflammation [191]. Moreover, the study of Beury DW *et al.* (2016) has shown that NRF2 regulates the generation, survival, and suppressive potency of MDSCs which contribute to immune suppression in tumors, and impede on the efficacy of immunotherapies aimed at boosting the immune response [192].

6.4. Therapeutic Strategies Targeting NRF2 in Cancer

A dichotomy appears related to the role of NRF2 in carcinogenesis *versus* constituted tumors [9, 193, 194], which impacts on NRF2-directed therapeutic strategies. The dual roles of both oxidative activity and NRF2-mediated activation of cytoprotective mechanisms might be distinctively revealed in various stages of tumorigenesis and tumor progression. Most probably, there is a

narrow window of therapeutic opportunity regarding ROS levels, redox signalling and antioxidant/cytoprotective mechanisms that sustain or repress malignancy and shape the response to anti-cancer therapies [195, 196].

It appears that carcinogenesis is underlined by chronic oxidative stress that arises from enhanced ROS production by conventional and unconventional ROS sources, which is possibly complemented by the reduced ability of the intricate antioxidant system [9]. For instance, a non-physiologic intracellular oxidative burst, if persistent, was shown to induce activation of oncogenic networks related to EGFR signalling, mainly due to oxidation-induced receptor oligomerization in the absence of ligands, along with oxidative inhibition of phosphatases such as PTEN [197, 198]. Therefore, pharmacologic up-regulation of NRF2 shows great promise for hindering or, at least, for delaying early pre-malignant transformation by counteracting pathologic oxidative changes [199, 200]. A broad panel of NRF2 activators, comprising electrophilic modifiers of KEAP1 and protein-protein interaction (PPI) inhibitors, reached clinical trials, and one is already in the market, namely dimethyl fumarate (DMF) commercialized by Biogen under the trade name Tecfidera [2]. The intended use of these NRF2 activators is generally confined to other diseases than cancer, but these pathologies are underlined by chronic oxidative stress. This is a valid argument for using them for cancer prevention in the frame of the network medicine and pharmacology perspective on diseasomes and drug repurposing [201, 202]. For instance, DMF, the methyl ester of fumaric acid, was shown to alkylate key cysteine residues in KEAP1, hence promoting NRF2 dissociation and stabilization. DMF was approved for treatment of relapsing-remitting multiple sclerosis [203] and it is now in Phase I clinical trial for the treatment of chronic lymphocytic leukemia and small lymphocytic lymphoma (NCT02784834 in ClinicalTrials.gov). Various other therapeutic agents, such as nutraceuticals (sulforaphane, curcumin, resveratrol, lycopene, carnosol, etc.) and synthetic molecules, such as synthetic triterpenoids, were shown to activate NRF2 *via* interaction with KEAP1. They may also target other redox-sensitive biomolecules in addition to KEAP1, and their biologic effects might be therefore more complex than initially thought [204]. Care should be taken regarding the timing and duration of the pharmacologic activation of NRF2 for not providing enhanced cytoprotection to cells engaged in the malignant process.

A different role of NRF2 was highlighted in constituted tumors. In this case, protective antioxidant mechanisms triggered by chronic NRF2 activation sustain disease progression by supporting survival and proliferation of selected malignant cells, especially of cancer stem cells which exhibit an exquisite shielding against tumor–related stressors and therapeutic agents, partly mediated by NRF2 [168]. In the hostile microenvironment of the tumor niche, cancer cells become highly dependent of the cytoprotective mechanisms activated *via* the NRF2 pathway [205], as demonstrated by KEAP1/NRF2 mutations in some tumors or just alteration of signalling pathways that lead to NRF2 up-regulation [173-177]. Compelling evidence has come from the fact that NRF2 knockdown in cancer cells dramatically kills them while having only minor deleterious effects on normal non-dividing cells. As shown by Okano Y *et al*. (2013), a single nucleotide polymorphism (-617C>A) in the ARE-like loci of the human NRF2 gene was able to abolish the self-induction of the NRF2 gene, and this resulted in prolonged survival of lung adenocarcinoma patients [206]. From this perspective, pharmacologic down-regulation of NRF2-mediated cytoprotection appears to be the adjuvant therapy to be applied for increasing the efficacy of anti-cancer therapies, especially of those acting primarily *via* oxidative stress [207, 208].

For the moment no NRF2 inhibitors are available for human therapy, despite a plethora of newly designed chemical structures that proved an inhibitory action on NRF2 activity in preclinical settings. Taking into account the mechanisms of NRF2 activation described above, the areas of opportunity for the development of specific NRF2 inhibitors could be, at least theoretically, the following: **a)** transcriptional down-regulation of NRF2, **b)** enhancement of NRF2 degradation through up-regulation/activation of KEAP1-CUL3 or β-TrCP, **c)** blocking of NRF2 dimerization with small MAF proteins, **d)** pharmacologic manipulation of NRF2 competitors [209]. Singh A *et al*. (2016) conducted a quantitative high-throughput screening on ~400 000 small molecules, and identified ML385 as a promising molecule that binds to the Neh1 domain in NRF2, hence interfering with the binding of the MafG-NRF2 complex to regulatory DNA binding sequences [210]. In preclinical models, ML385 proved to enhance the cytotoxicity of chemotherapeutics (platinum-based drugs, doxorubicin, carboplatin or taxol) in non-small cell lung cancer, especially in forms harbouring KEAP1 mutations. Further work is needed to determine if other bZip transcription factors might be also affected by ML385.

One of the most studied NRF2 inhibitors is brusatol (a component of *Brucea Javanica* seed) that triggers a rapid and transient depletion of NRF2. As demonstrated in a hepatoma cell line, brusatol acts on NRF2 through a post-transcriptional mechanism, independently of KEAP1, proteasomal and autophagic degradation systems [211]. Brusatol was shown to increase the responsiveness of lung cancer cells to radiotherapy [212], but its major drawback for clinical use is related to side-effects generated by global inhibition of protein synthesis [213].

All-trans retinoic acid (ATRA) has shown promise for down-regulating NRF2 in various types of solid tumors and hematologic malignancies [214]. ATRA is needed for NRF2 to make a complex with RARα (retinoic acid receptor alpha) in the nucleus, but the complex is unable to bind to DNA. ATRA was shown to impede on the binding capacity of NRF2 to ARE, hence suppressing its transcriptional activity [215]. Retinoic acid and its analogues are also involved in the inhibition of AP-1 transcription factors which represent the main dimerization partners for NRF2. Thus, PDT with ATRA may exhibit enhanced cytotoxic effects by inhibiting survival signalling mediated by both AP-1 and NRF2 [72].

Increased chemosensitivity in leukemia can be obtained by treatment with the pro-oxidant compound Malabaricone-A (MAL-A) which was shown to decrease NRF2 levels [216]. Moreover, MAL-A can induce the generation of HNE (4-hydroxynonenal) by peroxidation of cardiolipin, and this can drive cancer cells towards apoptosis if high levels of HNE are produced. In turn, lower levels of HNE that are constitutively expressed in particular cancer cells might act as an adaptation mechanism for avoiding apoptosis, hence conferring resistance to anti-cancer therapies [217].

Another strategy to modulate NRF2-mediated effects in cancer cells refers to multidrug resistance transporters. Cancer cells resist to chemotherapy by over-expressing such transporters, some of them being targets of NRF2 [218]. As demonstrated by Gao AM *et al*. (2013), treatment of hepatocellular carcinoma cells with the natural bioflavonoid compound apigenin inhibited NRF2 in a PI3K/Akt-dependent manner, both at transcriptional and translational level [219]. Apigenin down-regulated consequently ABC transporters which are under the transcriptional control of NRF2, and sensitized hepatocellular carcinoma cells to chemotherapy with doxorubicin both *in vitro* and *in vivo*.

It is worth mentioning that, due to the quite long time needed for NRF2 activation and transcription of its target genes, the inhibition of the NRF2 pathway is important not exactly for reinforcing the injuries inflicted by the initial PDT-triggered singlet oxygen burst, but more probably for sensitizing PDT-resistant tumor cells by down-regulation of a main protective mechanism which promotes their survival in the post-PDT microenvironment [72].

6.5. NRF2 In the Context of PDT

As reviewed by Broekgaarden (2015) [72], NRF2 target genes were found over-expressed in various cancer cells exposed to PDT,

including the following genes: **a)** *GCLC* and *GCLM* genes encoding glutamate–cysteine ligase catalytic and modifier subunits, respectively, that are involved in the first steps of glutathione biosynthesis [32], **b)** the *NQO1* gene that encodes NADPH quinone dehydrogenase 1 which is a benzoquinone detoxifying enzyme [32, 220]; **c)** the *ABCG2* gene encoding for an ATP-binding cassette (ABC) transporter [221].

Important "proof of concept" regarding the therapeutic relevance of NRF2 inhibition for increasing the sensitivity of cancer cells to PDT came from studies in which NRF2 was genetically silenced. For instance, NRF2-knockdown in breast carcinoma MDA-MB-231 cells exposed *in vitro* to Pheophorbide a-based PDT increased cell death by apoptosis and necrosis following PDT. in comparison to cells with competent NRF2 pathway [222]. We emphasize herein that most of the data on the involvement of NRF2 in PDT were demonstrated using cellular and animal models in which NRF2 was silenced. These studies have made "proof-of-concept" but do not have clinical and pharmacological relevance. For the moment, there is no clinical evidence that pharmacologic inhibition of NRF2 could trigger similar effects in cancer patients, and this issue will be addressed at the time when clinically approved NRF2 inhibitors will be available.

Other studies have investigated in cancer cells exposed to PDT the expression of particular proteins that are encoded by NRF2 target genes, i.e. heme oxygenase 1 (HO-1) and, to a lesser extent, NQO1 (NADPH quinone dehydrogenase 1). For instance, Hyp-PDT was shown to induce HO-1 in bladder tumor cell lines as a protective mechanism against the PDT-induced oxidative stress [223]. HO-1, encoded by the *HMOX1* gene, is one of the main targets of NRF2 but it is also up-regulated by the transcription factor HIF-1α in response to hypoxia [224]. The NRF2 inhibitor BACH1 might be involved in regulating the expression of *HMOX1* considering that it inhibits a subset of ARE-containing genes, with *HMOX1* being the most important one [225]. The function of HO-1 is to convert mitochondrially-produced heme into CO and biliverdin. Biliverdin is reduced to bilirubin by biliverdin reductases [226, 227]. Bilirubin scavenges peroxidized lipids [228] which are massively produced by PDT-activated lipophilic PS which localize in cellular membranes. Bilirubin may thus contribute significantly to tumor cell survival following PDT by terminating lipid oxidation chain reactions. Additionally, low concentrations of CO have vasodilating, pro-angiogenic, anti-inflammatory and anti-apoptotic properties, and can also contribute to angiogenesis, tumor survival and regeneration *in vivo* [226]. The degradation of heme to bilirubin releases pro-oxidant Fe^{2+} ions, but this does not induce increased production of hydroxyl radicals *via* the Fenton reaction because Fe^{2+} is quickly chelated and neutralized by ferritin which is also generated by PDT in increased levels consequent to NRF2 activation [229]. Kocanova S *et al.* (2007) pointed out that induction of HO-1 by Hyp-PDT was mediated by p38 MAPK in conjunction with PI3 (phosphoinositide 3) kinase, and blocking of these signalling pathways increased additively PDT efficacy in inducing apoptosis [223]. This study showed that HO-1 stimulation by PDT is dependent on gene transcription and *de novo* protein synthesis, and is preceded by p38-regulated accumulation of the NRF2 transcription factor in the nucleus. It is worth mentioning that porphyrinic compounds used as PSs for PDT may induce in absence of PDT the switching of NRF2/small Maf heterodimers, resulting in increased HO-1 generation. Such compounds could bind to the NRF2 competitor BACH1, hinder therefore its DNA binding activity and induce nuclear export, as well as its ubiquitination and degradation. As such, particular porphyrinic compounds may function as NRF2 activators [230] and decrease PDT efficacy by increasing the NRF2-mediated cytoprotection.

Besides HO-1, photo-activation with porphyrins was shown to induce in liver tumor HepG2 cells the expression of ABCG2 (ATP-binding cassette G2) transporters consequent to PDT-induced NRF2 activation [231]. It is a limiting effect in PDT, as porphyrinic PSs may be transported out of cells by these NRF2-induced transporters, hence significantly limiting PDT at its initial stages. The effect on ABCG2 transporters can be abrogated by co-administration of specific inhibitors, such as the tyrosine kinase inhibitor imatinib mesylate. Consequently, PS accumulated better in tumor cells and PDT was significantly more efficient [232]. As such, NRF2 inhibition during the first PDT steps might limit PS accumulation into the tumor, but if NRF2 inhibition is made post-PDT one may expect an increase of the damage inflicted by therapy, as shown above. Therefore, the timing for NRF2 inhibition should be carefully chosen by finding the balance between PS accumulation in cancer cells and further post-PDT events.

Surprisingly, preclinical evidence exists that PDT combined with NRF2 activators can also improve therapy outcome in malignant cells. For instance, sulforaphane (SFN), an isothiocyanate found in vegetables of the *Brassica* family and in broccoli, was shown to enhance PDT cytotoxicity in human head and neck cancer cells, significantly above the effect of PDT alone [233]. This supporting action of SFN at 7.8 μM concentration was attributed to increased ROS formation, particularly of those ROS scavenged by sodium azide. It is not clear how SFN could induce increased ROS generation above the oxidative burst induced by PDT, or why SFN sustained PDT-induced cell death albeit stimulating NRF2-mediated cytoprotection. We may presume that SFN off-target effects may occur, or there is a particular window of opportunity in which NRF2 activity is translated into an increased sensitivity of cancer cells to PDT. Most probably, the cytoprotection conferred by SFN through NRF2 activation may be overwhelmed by synergistic effects with anti-cancer therapies, finally leading to the death of sensitized cancer cells. The above described synergistic action of PDT and SFN is sustained also by other studies. It was shown that SFN may increase in fact PS accumulation into cells, and this accounts for a more efficient PDT reaction in presence of SFN. For instance, pre-treatment of skin with SFN before topical ALA-PDT administration to patients may improve penetration of ALA through the stratum corneum, increase protoporphyrin IX (PpIX) synthesis and consequently boost PDT efficacy [234]. A clear correlation with SFN-induced activation of the NRF2 pathway was not explained, and the above presented mechanism of PDT improvement by SFN might work only in ALA-treated skin. SFN, like other isothiocianates, may generally sustain anti-cancer therapies by sensitizing cancer cells. For instance, SFN (5 μM) was shown to enhance the anti-proliferative activity of lapatinib, an anti-EGFR drug, in HER2-positive breast cancer cells [235]. This action of SFN was connected with an efficient inhibition of pro-survival signalling mechanisms, possibly other than those mediated by NRF2, and induction of apoptosis by increased levels of ROS. Moreover, SFN with concentrations above 10 μM was shown to inhibit the Akt-mTOR survival pathway in breast cancer cells [236], and appears to inhibit more efficiently the growth of these cancer cells than of normal breast epithelial cells [237].

Cytotoxic effects specifically targeted towards cancer cells were evidenced also for DMF, which is generally considered to be a NRF2 activator. The study of Bennett Saidu NE *et al.* (2018) [238] showed that DMF is cytotoxic against KRAS-mutated cancer cells by inducing enhanced ROS formation and glutathione depletion through a process involving profound down-regulation of the DJ-1 NRF2 stabilizer, resulting in marked inhibition of NRF2. In turn, DMF was rather cytoprotective in non-tumorigenic cells, and this was dependent on NRF2 activation by DMF, independently of DJ-1.

These results indicate that particular compounds that are known to trigger NRF2 activation in various settings should be re-tested for their ability to induce cancer cells sensitization to various therapies, deriving probably from NRF-2 mediated limitation of oxidative stress on which cancer cell rely for survival and proliferation.

We do not rule out the hypothesis that their action may be exerted at least partially through other mechanisms than NRF2 activation. For instance SFN or DMF may interact with cysteines in KEAP1 but also with reduced glutathione (GSH), hence depleting the pool of GSH and resulting in oxidative stress that cannot be compensated by NRF2. In this context, we point out that targeting of antioxidant mechanisms down-stream of NRF2 might be a promising therapeutic strategy to specifically target particular pathways that may increase PDT efficacy, whilst avoiding the pleiotropic activities of NRF2. For instance, Theodossiou TA *et al*. (2017) [239] showed that by controlling glutathione metabolism (GSH depletion), and not glutathione biosynthesis which is under the control of NRF2 [240], an increased Hyp-PDT efficacy might be obtained in particular breast cancer cells, partly due to decreased levels of L-cysteine which can bind to Hyp and expel this PS from target tumor cells. Moreover, the study evidenced that glutathione-related enzymes may predict treatment response. Thus, membrane glutathione peroxidise 4 (GPX4) can be used as predictive marker of the cell response to Hyp-PDT, while glutathione S-transferases (GST) can be used as predictive markers for chemoresistance.

Concluding, we emphasize the urgent need to further increase the knowledge on the mechanisms by which NRF2 is activated or inhibited in response to various stressors and interferes with other major signalling pathways in tumors, in order to define new pharmacological targets and approaches that could sensitize cancer cells to therapy. However, care should be taken that a persistent oxidative shift in the redox balance induced by therapy may have uncontrollable consequences in the long run, including *de novo* tumorigenesis in cancer patients.

7. TAKE-HOME MESSAGE

- The tumor niche is under the pressure of concerted stressors, including oxidative stress, hypoxia, acidosis and inflammation, along with metabolic reprogramming, synergizing to provide survival advantage to cancer cells and resistance to therapy.
- Anti-cancer therapies, like radiotherapy and PDT, take advantage of the cytotoxic potential of ROS and of their role in tightly regulating redox signalling in physiologic conditions, and add an acute oxidative burst to the existing increased ROS levels in tumors.
- PDT is a targeted therapy based on the generation of a harmful burst of singlet oxygen coupled with hypoxia, resulting in death of cancer cells and profound deregulation of tumor vasculature in an ideal scenario.
- Cells protect themselves from PDT-inflicted oxidative damages by potent repair mechanisms, involving antioxidant response, autophagy, unfolded protein response and inflammation.
- The intensity and location of the PDT-induced oxidative burst, along with the succession of cellular responses and the microenvironment in the tumor niche are shaping the end-result of PDT, which may be tumor cell death or tumor progression. In PDT both processes may occur in the same bulky tumor due to spatially non-uniform effects of PDT.
- PDT efficacy can be increased by pharmacologic modulation of biologic processes related to specific PS accumulation into tumor cells, sensitization of these cells to therapy and generation of an efficient anti-tumor response.
- The transcription factor NRF2 plays a critical role in cancer by endowing tumor cells with an exquisite shielding against the stressful microenvironment in the tumor niche.
- NRF2 appears as a promising pharmacologic target, as down-regulation of cytoprotective genes that are under NRF2 control may amplify PDT-inflicted oxidative damages. Surprisingly, NRF2 activators may also exert cytotoxic effects preferentially in cancer cells through different mechanisms than those acting in non-tumorigenic cells. NRF2 activators may interact with GSH and consequently increase the oxidative stress on which cancer cells rely for survival and proliferation. Possibly, other protective mechanisms besides the antioxidant response might be repressed by NRF2 activators, as NRF2 interferes with several other signalling pathways and has a pleiotropic action.

8. FUTURE PERSPECTIVES

According to the results obtained so far in preclinical studies and clinical trials, PDT emerges as a promising targeted therapy for several types of solid tumors. It is considered a patient-friendly therapy exhibiting less toxic effects than chemo- and radiotherapy, which can be easily managed, although for the moment there are no data on PDT impact in the long run. Being a targeted therapy, PDT was thought to address only clearly constituted tumors and not metastasis, but recent findings have evidenced its out-of-field effects. This paved the way for boosting the anti-tumor immune response using immunomodulators in combination with PDT, or PDT-generated cell vaccines.

Despite huge progress in improving photosensitizers and illumination devices tailored for oncologic PDT, we face the deficiency of in-depth investigations regarding molecular networks underlining various PDT protocols in connection with particularized tumors. Complex signalling networks seem to be activated by PDT, as demonstrated in various preclinical models. Main difficulties in starting to create the PDT connectome derive from the fact that only limited molecular and biological events were addressed in individual studies so far. Moreover, the evidenced signalling pathways seem to be highly dependent on the PDT setting, the type and status of tumor cells, the succession of biologic events, and the general context in which the investigated cells were evolving. Additional limitations arise from the fact that most of the studies were conducted *in vitro* or in tumor-bearing small animals, and it might be irrelevant to extrapolate these results to a real human tumor. However, molecular networks that underline PDT, related to oxidative stress, hypoxia, ER stress, inflammation and immune responses, have been emphasized by various authors, but they derive in fact from logical connections and not from comprehensive multi-parameter studies. Another important drawback for translation of oncologic PDT into clinical practice resides in the lack of therapy guidelines which, through a vicious cycle, accounts for the reduced amount of clinical and biological data from cancer patients treated with PDT.

Accordingly, there is an urgent need to perform in-depth molecular investigations, to generate “big data” in the field of PDT and to correlate them through a systems medicine approach for highlighting the complex biological fingerprint of PDT. This will definitely guide further clinical translation for PDT and the development of new therapeutic strategies aimed at increasing PDT efficacy.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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LIST OF ABBREVIATIONS

ACT = adoptive T cell transfer therapy
ALA = aminolevulinic acid
AP-1 = activator protein 1
ARE = antioxidant response elements
APC = antigen presenting cell
ASK1 = apoptosis signal-regulating kinase 1
ATF = activating transcription factor
ATRA = all-trans retinoic acid
Bax = BCL2-associated X
Bcl-2 = B-cell lymphoma 2
Bid = BH3 interacting-domain death agonist
CaMKII = calcium/calmodulin-dependent protein kinase II
CD = cluster designation
CHOP = C/EBP-homologous protein-10 protein
cIAP = inhibitor of apoptosis protein-1
CREB = cAMP responsive element-binding protein
CRT = calreticulin
CTLA4 = cytotoxic T-lymphocyte-associated protein 4
CUL3-RBX1 = Cullin 3-Ring Box Protein 1
DCs = dendritic cells
DD1α = death domain 1α
DMF = dimethyl fumarate
EGFR = epidermal growth factor receptor
EMA = European Medicines Agency
ER = endoplasmic reticulum
ERK = extracellular receptor kinase
eIF2α e = eukaryotic initiation factor 2
ERO1 1α = ER oxidase 1α
FADD = death domain-containing adaptor
FAS = apoptosis antigen 1, CD95
FASL = FAS ligand
FDA = Food and Drug Administration
FMT = fluorescence molecular tomography
GCLC = glutamate-cysteine ligase catalytic subunit
GCLM = glutamate-cysteine ligase modfier subunit
GPX = glutathione peroxidise
GRP78 = glucose-regulated protein 78
GSH = reduced glutathione
GSK-3 = glycogen synthase kinase 3
GST = glutathione S-tranferase
HIF = hypoxia inducible factor
HMGB1 = high-mobility-group box chromosomal protein 1
HNE = 4-hydroxynonenal
HO-1 = heme oxygenase 1
HSP = heat shock protein
Hyp = hypericin
ICD = immunogenic cell death
IFN = interferon
IKK = IkB kinase
IP3 = inositol 1,4,5-triphosphate
IP3R = inositol 1,4,5-triphosphate receptor
IRE1 = inositol requiring enzyme 1
JNK = c-Jun N-terminal kinase
LDL = low-density lipoprotein
LMP = lysosomal membrane permeabilization
LUBAC = linear ubiquitin chain assembly complex
MAPK = mitogen-activated protein kinase
MDSCs = myeloid-derived suppressor cells
MEMs = mitochondria-associated ER membranes
MLKL = mixed kinase domain-like
MMPs = metalloproteinases
MPO = myeloperoxidase
NFkB = nuclear factor B
NIR = near infrared light
NK cells = natural killer cells
NO = nitric oxide
NOX = NADPH-oxidase
NOS2 = inducible nitric oxide synthase 2
NQO1 = NADPH quinone dehydrogenase 1

NRF2	=	Nuclear Factor Erythroid 2-related factor 2
PDI	=	disulfide isomerase
PD-L1	=	programmed cell death ligand-1
PDT	=	photodynamic therapy
PERK	=	protein kinase R (PKR)-like endoplasmic reticulum kinase
PI3K	=	phosphoinositide-3-kinase
PPI	=	protein-protein interaction
PpIX	=	protoporphyrin IX
PTEN	=	phosphatase and tensin homolog
RARα	=	retinoic acid receptor alpha
RIPK	=	receptor interacting protein kinase
ROS	=	reactive oxygen species
SFN	=	sulforaphane
TAAs	=	tumor-associated antigens
TGFβ	=	transforming growth factor β
TLR	=	toll-like receptor
TNFα	=	tumor necrosis factor α
TRADD	=	TNF receptor-associated death domain
TRAF2	=	TNF receptor-associated factor 2
TRAIL	=	TNF-related apoptosis-inducing ligand
Tregs	=	T regulatory cells
β-TrCP	=	E3 ligase adapter β-transducin repeat containing E3 ubiquitin protein ligase
TXNRD1	=	thioredoxin reductase 1
UPR	=	unfolded protein response
VEGF	=	vascular endothelial growth factor

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