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Chapter 1

Photosensitizers Imprinting Intracellular Signaling Pathways in Dermato-Oncology Therapy

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65979

Abstract

This chapter describes the main deregulated intracellular pathways at both genetic and proteomic levels that are found in three main skin cancers: basal cell carcinoma, squamous cell carcinoma and melanoma. In basal cell carcinoma, the main intracellular signaling pathways is the Sonic Hedgehog pathway, while in squamous cell carcinoma, it is the p53 pathway. However, in both nonmelanoma skin cancers, these major pathways trigger cross-activation with other important ones. In melanoma, mitogen-activated protein kinase pathway and PI3K/Akt pathways are deeply deregulated, and moreover due to the disease complexity, BRAF, RAS (N/H/K), NF1 and Triple-WT melanoma subtypes need additional molecular stratification. The stage in which photodynamic therapies' clinical application is in the treatment of these diseases is another subject tackled by the chapter. Thus, if basal cell carcinoma and squamous cell carcinoma possess in their therapeutical armamentarium photodynamic therapies approach, melanoma, with its particularities, still needs thorough molecular investigations to adapt this particular therapy. Based on the accumulated knowledge on pathological intracellular pathways, the chapter describes the molecular details that reside in applying photodynamic therapy. *In vivo* and *in vitro* models of cutaneous malignancy and photodynamic therapies' molecular events are further detailed.

Keywords: skin cancer, intracellular signaling, biology, photodynamic therapy, photosensitizer, proteomics

1. Introduction

Skin cancers, especially cutaneous melanoma, remain a complex therapeutic challenge owing to a multiangle problem such as the emergent incidence in white population, the inefficiency of classical therapies like surgery and owing to transition to the new wave

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treatments like targeted therapies, immunotherapy and alternative therapies like photodynamic therapy (PDT). Skin tumors are classified as melanoma and nonmelanoma type. Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) along with actinic keratosis (AK) are considered nonmelanoma skin cancers (NMSC), being the most frequent skin cancers in comparison with melanoma which is more rare (only 4%) but highly deadly [1]. Similarly, organ transplant recipients (OTR) record an extremely high risk of developing NMSC [2]. All these types of cancers are generated by complex molecular events that favor tumor proliferation and invasion. At the core of this diseases' pathology intracellular pathways deregulation dwell. Therapies that are developed in each cancer type should acknowledge the molecular events particularities. In the therapeutical armamentarium of skin cancer, PDT has already gained its place. As further presented, the intracellular molecular pattern triggered in each type of skin cancer by PDT has both common outlines and particularities.

2. Skin cancer, photodynamic therapy and signaling pathways

2.1. Biology of skin cancer in the light of new therapeutical era

Deepening the biology of skin cancer by unraveling the intracellular mechanisms that trigger the neoplastic transformation could lead to deciphering new therapy targets and new therapeutical approaches. Both cellular and molecular basis of a successful therapy still needs new explorations and additional biomedical technologies in order to manage this high morbidity and mortality group of diseases.

2.1.1. Basal cell carcinoma

As described in the early 1990 [3], basal cell carcinoma (BCC) is the most common malignancy in humans, and although rarely metastatic, accounts for about two-thirds of all skin cancers with a worldwide incidence steady increase [4]. The diversity in the phenotypic appearance of BCCs relies on the fact that the majority of BCCs represent monoclonal tumors and anatomically distinct BCCs may sometimes share the same cellular origin [5]. An extensive genetic study on BCC profiling was published in 2016 investigating around 300 tumor tissue that displayed the highest mutation rate in cancer (65 mutations per mega base). About 85% of all tumors have mutations in the Sonic Hedgehog (Hh) pathway. These genes are PTCH1, SMO, SUFU and TP53. Other mutations were found in MYCN, PPP6C, STK19, LATS1, ERBB2, PIK3CA and NRAS. Loss-of-function and deleterious missense mutations were found in PTPN14, RB1 and FBXW7 genes [6]. Moreover, 2016 studies have shown that genetic predisposition in familial BCC has the most commonly gene mutated, PTCH1, main player in the Hh pathway. Another gene associated with familial BCC is SUFU being involved in the same pathway. This gene is loosing its function and hence inducing BCC predisposition. Understanding the deregulated genes that trigger BCCs can lead to new targeted therapy trials [7]. In the last five years, studies on the biology of BCC have shown that Hh pathway is deeply involved in the initiation of this skin tumor. This pathway cross-talks

with other main intracellular pathways involved in skin's homeostasis. Hence, Wnt pathway was found as having increased levels of beta-catenin, a critical mediator of Wnt signaling in BCCs [8, 9]. Another important pathway involved in BCC is EGFR/MEK/ERK that modulates GLI-dependent transcription in human keratinocytes and drives their oncogenic transformation [10, 11]. As epithelial-stromal interactions are creating a protumoral microenvironment, stromal cells isolated from BCCs, have high levels of gremlin 1. This protein antagonizes with the prodifferentiation factors BMP2 and BMP4, sustaining, therefore, tumor proliferation [12]. If Hh pathway is deregulated at gene and protein level, another deregulated pathway in BCC is the MEK-ERK pathway. Hence, acknowledging that IL-17 (IL-17A) sustains a chronic inflammatory microenvironment with protumoral consequences some important reports were published in 2015. IL-17 binding to its receptor activates the route IL-17R-Act1-TRAF4-MEKK3-ERK5 that directly stimulates keratinocyte proliferation and tumor formation. In the BCC context, this axis sustains the expression of Steap4-p63 through p63-mediated TRAF4 expression that directly enhances keratinocyte proliferation and further tumor formation [13].

2.1.2. Squamous cell carcinoma

Cutaneous squamous cell carcinoma (SCC) is the second most common human skin malignancy after BCC, and opposed to BCC, it can trigger metastasis. SCC originates the premalign lesions actinic keratosis (AK) and it develops from keratinocytes of the spinous layer. The progression of AK to malignancy relies on the sequential DNA mutations in oncogenes and tumor suppressor genes. This multistep process triggered by chronic UV irradiation leads to increased genomic instability and loss of cell cycle control, thus driving the malignant uncontrolled proliferation of keratinocytes. Details of the molecular pathogenesis of SCC are still a subject of intense research [14]. Intracellular signaling deregulated pathways in SCC pinpoint to p53 pathway as mutated p53 is the most common genetic abnormality found in SCC. AK lesions can harbor mutated p53, and remain as such through the malignization process toward SCC [15, 16]. Whole transcriptome analysis published in 2016 has shown in SCC cancer cells, in comparison to normal human epidermal keratinocytes, an overexpression of long intergenic non coding RNA (LINC00162). LINC00162 is upregulated by the inhibition of the p38α and p38δ mitogen-activated protein kinases. Knockdown of LINC00162 inhibited proliferation and migration of SCC cells *in vitro* and inhibited extracellular signal-regulated kinase 1/2 activity, up regulating dual specificity phosphatase 6 (DUSP6) [17]. Other, recently published molecules involved in the complex biology of SCC are Ets2 and Elk3 genes, required for malignant progression from AK lesions to SCC. ETS2-overactivation in epidermal cancer stem cells induces hyperproliferation and SCC superenhancer-associated genes Fos, Junb and Klf5 expression [18]. Epidermal cancer stem cells are characterized by alterations in keratinocyte stem cells (KSC) and survivin gene expression. Silencing survivin reduces the classical expression of stem cell markers (OCT4, NOTCH1, CD133 and β_1-integrin), and increases differentiation markers (K10 and involucrin). Recently published results indicate survivin as a key gene in SCC development [19]. Another recently published protein, involved in SCC, is S100A8, whose overexpression regulates SCC differentiation [20].

2.1.3. Cutaneous melanoma

Cutaneous melanoma is one of the solid tumors that bear the highest rate of mutations. In 2015, The Cancer Genome Atlas Network has proposed a new genomic classification of these tumors divided into genomic subtypes: BRAF, RAS (N/H/K), NF1 and Triple-WT [21]. These subtypes have significant intracellular pathways deregulations. A BRAF mutation is present in the majority of melanomas, and an NRAS isoform is present in 15–30% of melanomas [22]. The mitogen-activated protein kinase (MAPK) pathway (Ras/Raf/MEK/ERK pathway) has several mutated points so that uncontrolled cellular proliferation may occur [23, 24]. Alike to the MAPK pathway, the PI3K/Akt pathway can also be activated by Ras. Once this pathway is activated, cell proliferation and invasion are promoted. Although PI3K mutations are believed to be rare, downstream components of the PI3K/Akt pathway steadily increase during melanoma progression, and are altered in 50–60% of melanomas [25]. CDKN2A is another gene that encodes proteins involved in cell cycle regulation. Approximately 10% of all melanomas have a familial susceptibility linked to the CDKN2A gene. Somatic CDKN2A mutations have been reported in 30–70% of sporadic melanomas [25].

The pathophysiology of each skin cancer can have different gene/protein/intracellular signaling foundation or can share the same molecular pathways. **Figure 1** resumes the main intracellular pathways that trigger the three main skin cancers in humans.

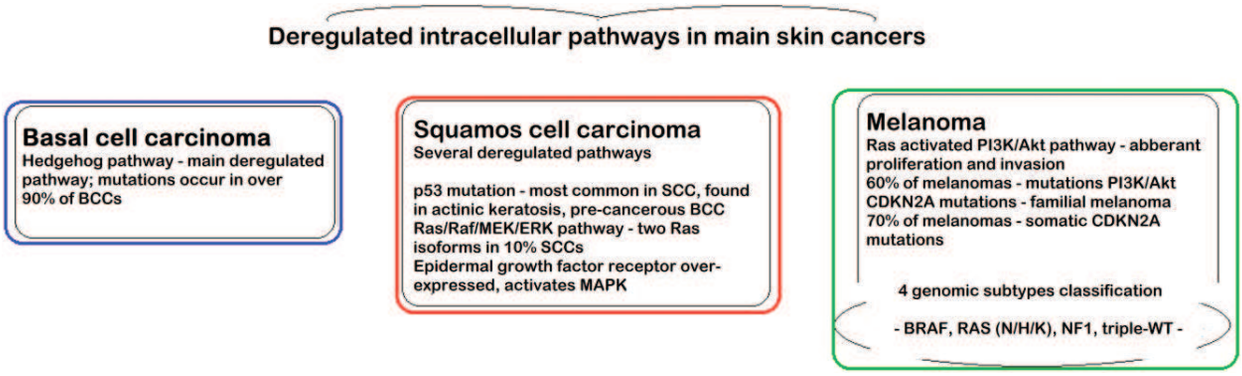


Figure 1. Intracellular signaling pathways that characterize the main skin cancer types: basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma.

2.2. PDT in the clinical management of skin cancers

Owing to a constantly increasing incidence, malignant skin tumors need a multidisciplinary approach regarding their clinical management, comprising specialists and therapeutical lines, which could be personalized for every situation [26]. In this context, PDT is involved in the management of nonmelanoma skin cancers, primary superficial BCCs, low-risk nodular BCCs and superficial SCCs [27]. In recent years, even for melanoma, PDT starts to be considered as an alternative treatment option.

2.2.1. PDT in BCC therapy

Predominantly located in head and neck region, there are three types of BCC: nodular, superficial and morpheic with an increased heterogeneity [28, 29]. Due to this heterogeneity, there

are several therapy lines and several ongoing clinical trials that are thoroughly resumed in Ref. [10]. Among all these therapies, PDT gains its place. Thus, topical PDT with methyl ester-based photosensitizer (PS) is currently accepted for superficial BCC. Accordingly, 16% methyl ester methyl aminolevulinate (MAL) is approved for topical PDT of BCC in Europe (Metvix®) while in the USA the corresponding approved drug is Levulan® (20% of 5-aminolevulinic acid in ethanol solution) [28]. The clinical response for PDT in BCC is not clear-cut. Thus evaluating more than 130 patients treated with MAL-PDT the best response was obtained for superficial BCC (82%) compared to nodular BCC (only 33%). Analyzing the subtypes some predictor factors emerged, nodular infiltrative histotypes, ulceration tumor thickness and localization on limbs were the negative ones [30]. Trying to improve the clinical outcome, two-fold illumination scheme for aminolevulinic acid-photodynamic therapy (ALA-PDT) was investigated and one-year post-treatment clinical evolution was assessed. For small BCC located outside the head and neck area, this activation scheme has proven good clinical outcome [31]. For a three-year follow-up in patients treated with MAL-PDT, fluorouracil and MAL-PDT have proven the same efficacy in the treatment of superficial BCCs [32].

2.2.2. PDT in SCC therapy

SCC and AK represent the same skin disease but in different stages of evolution, as AK is superficial, SCC involves also the dermis. Local PDT is suitable for AK and for *in situ* SCC [33]. Therapeutical protocols in SCC implies surgery (cryosurgery, electrosurgery and radiotherapy), topical treatments with 5-fluorouracil and imiquimod or PDT [34], successful ALA-PDT and blue light being reported several years ago [35]. In this type of cancer, although, surgical excision is the first therapeutical choice, PDT is a noninvasive approach and it can provide optimum cosmetic outcomes. As in SCC, resistant or recurrent tumors can appear, and PDT should be combined with other therapeutic modalities. Hence PDT can be combined with immunomodulatory (Imiquimod) and/or chemotherapeutic agents (5-fluorouracil, methotrexate, diclofenac or ingenol mebutate), and/or inhibitors of molecules involved in tumorigenesis, such as COX2 or MAPK [36].

2.2.3. PDT in melanoma therapy

There is an interesting link between melanoma patients and the subsequent appearance of BCC or SCC. In a study published in 2016, the associations between melanoma diagnosis and SCC were studied. The study showed a clear correlation between age, sex, skin characteristics, sun exposure and the existence of p.R163Q/p.D294H MC1R variants in melanoma patients, parameters that favor the risk of developing a SCC. This study has shown that melanoma patients with increased risk of developing another skin cancer should be further stratified [37].

Although in cutaneous melanoma targeted immune-therapies are the best clinical option, there is a therapeutical niche for PDT with second generation PS, especially as postsurgery adjuvant treatment or even as a preventive approach [38]. PDT exerts specific effects in melanoma cells both upon melanocytic antioxidant system and multidrug resistance cellular machinery decreasing these functions essential for melanoma survival. While successful in other skin cancers, PDT is not effective in pigmented melanoma due to photophysical properties of melanin from melanocyte-transformed cells, as melanin absorbs light over the

entire wavelength region used by PS for PDT (400–750 nm). Amelanotic melanoma with low melanin pigment load has been more approached in PDT studies [38]. Still only few studies are reported in this topic, and clinically there are some promising results for PDT with chlorin e(6) in skin metastases [39] and ocular choroidal melanomas [40, 41]. PDT with verteporfin alone or in combination with bevacizumab may be useful as primary or preoperative procedure for ocular melanoma. There are also recent experimental studies involving amelanotic melanoma, the nonpigmented melanoma type owned to a poor differentiation of melanocytes, which produce less melanin, refractive to classic treatments and for which alternative therapies are taking into account [42]. Thus, in a mouse C57/BL6 model bearing B78H1 amelanotic melanoma, it was tested a novel PS assembly comprising a Zn(II)-phthalocyanine, a polyethylene glycol (PEG) derivative and gold nanoparticles. The deposition of PEG on the nanoparticle surface makes the conjugate hydrosoluble prolonging the retaining in serum, improving thus the PDT efficacy. The nanoparticle conjugates were significantly accumulated and retained in the tumor 3 h postinjection followed by PDT. The experimental approach lead to 40% survival of the treated mice, without tumor relapses. These types of PS functionalized with nanoparticles have good potential in PDT for difficult to treat cancers such as amelanotic melanoma [43]. Another strategy is to use natural compounds as PS in PDT of skin cancer; hence, positive results were obtained with hypericin in human melanoma cells, Where an inhibition of proliferation was registered [44]. In addition, natural compounds are "back in fashion" and hypericin was tested also in NMSC treatment approaches.

2.3. Specific intracellular networks triggered by PDT in skin tumor cells

PDT is an alternative therapy for some type of cancers and several nonmalignant diseases, involving application and preferentially accumulation of a PS in the target cell/tissue, followed by PS photoactivation with a light wavelength fitting the compound's absorption. The actual effect of PDT depends on many factors but the cell/tissue where the PS is accumulating triggers the main process (**Figure 2**).

Typically the structure of many PSs is based on the tetrapyrrole ring—porphyrins and phthalocyanines, including their derivatives (porphycenes)—or natural products such as hypericin, riboflavin or curcumin [45]. Several conditions should be attained by a potential PS, namely: chemical purity, stability, high solubility in water, preferentially loading in the target cell/tissue, low "dark" toxicity and high quantum yield of singlet oxygen ($^1O_2$) generated upon photoactivation [45]. This light activation causes the transition of PS first in a short-lived excited state then in a long-lived energetic state triggering reactive oxygen species (ROS) as main agents of tumor vasculature damage, immune response generation and further tumor eradication [46, 47]. Among relatively innovative approaches in skin tumors treatment, especially in NMSC, PDT could reveal new lines in terms of novel PSs embossing different insights in key intracellular pathways influenced by the PDT treatment. Elucidation of mechanisms in PDT has gained advance within the last decade, data regarding signaling pathways, transcription factors related to cell cycle control, inflammation and cellular death accumulated lately. Nevertheless in a living cell, all these processes are deeply overlapped so

http://dx.doi.org/10.5772/65979

minute understanding should be proceeded step by step [48]. Furthermore, survival mechanisms are activated in PDT-treated cells and some transcription factors have been identified to be involved in cell resistance following PDT, such as AP-1 transcription factor family members, NRF2, hypoxia-inducible factor-1 alpha (HIF-1), nuclear factor-kappa B (NF-κB), HSF1 and unfolded protein response (UPR) protein group [47]. Involvement of a certain signaling pathway could define the response of a treatment. For instance PDT with ALA as PS has been widely used to treat SCC. However, a segment of SCC patients does not respond well to this PDT therapy, the lack of efficacy being evoked at molecular level by the MAPK signaling pathway. *In vitro* studies on SCL-1 human squamous carcinoma cell line revealed that adding inhibitors of MAPK on ALA-treated SCL-1 cells could augment the cytotoxic effects of ALA-PDT. More specific, the addition of inhibitors for key components belonging MAPK, namely ERK1/2, p38 and JNK, induced a more dramatic decrease on cellular viability than induced with ALA-PDT alone [49].

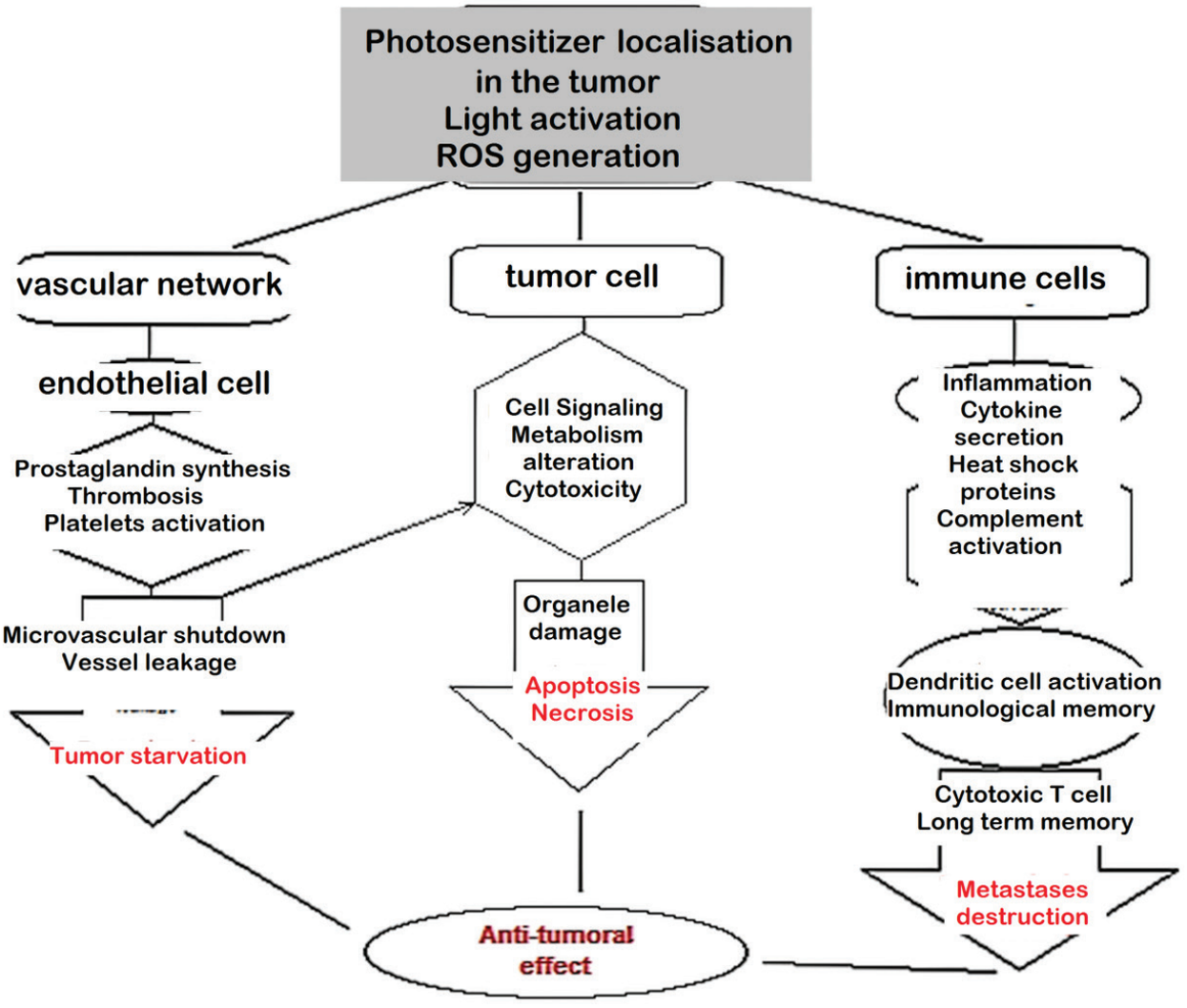


Figure 2. PDT effect inflicted upon localization in different cells and tissues. Regardless of the localization there is an overall antitumoral effect.

Figure 3 resumes intracellular events triggered by PDT in cells that were uptaking PS.

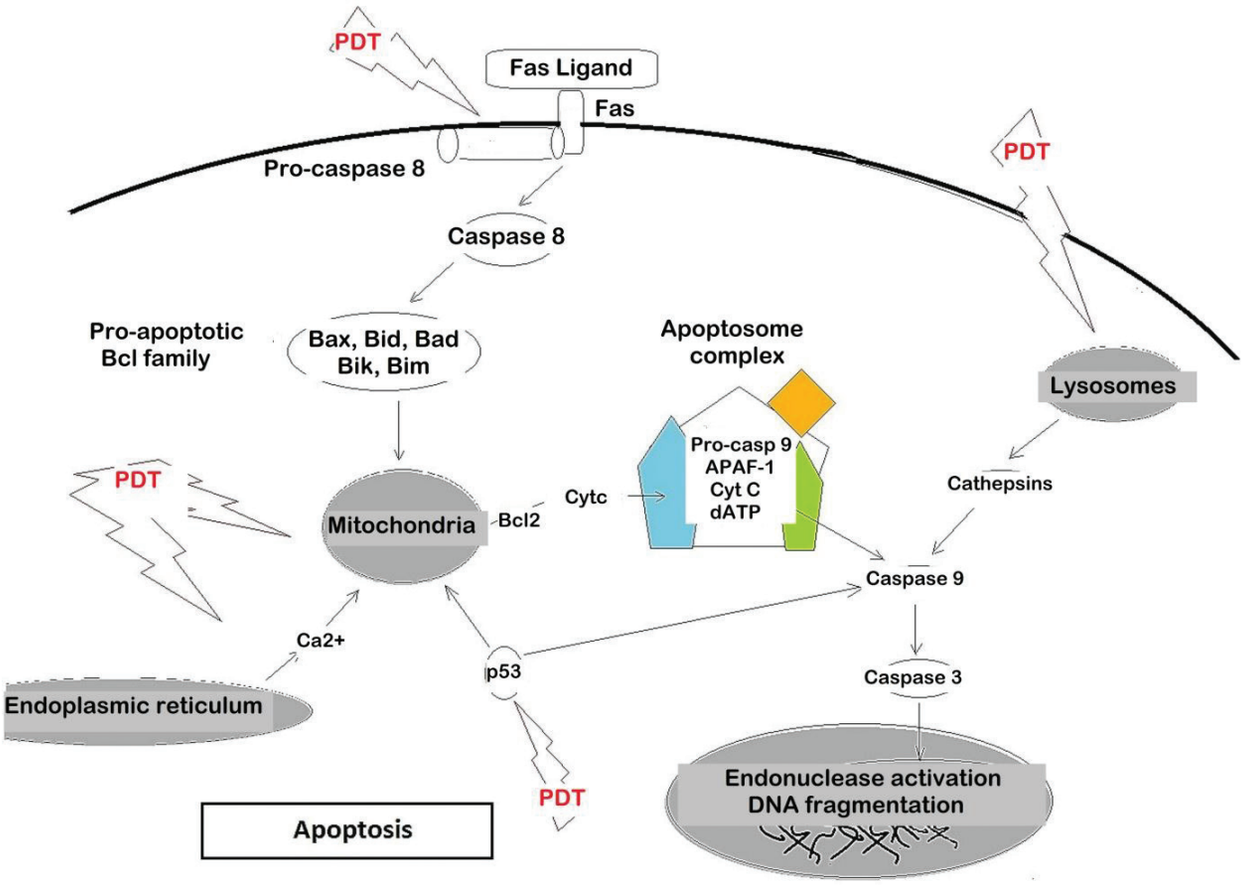


Figure 3. Intracellular events triggered by PDT in cells that were uptaking PS. PDT is inflicting upon cellular membrane an activation of procaspase 8 to caspase 8 that activates the proapoptotic Bcl family of proteins (Bax, Bid, Bad, Bik and Bim). PDT acting on mitochondria induces Bcl-2 that further induces cytochrome C (Cyt C) to form the apoptosome that comprises Procaspase 9, APAF-1, cytochrome C and dATP; the result is the formation of active caspase 9 that through caspase 3 activates intra-nuclear endonucleases and induces hence DNA fragmentation. When PDT acts on lysosomes the cathepsin activation induces activation of caspase 9 with the same fate of DNA fragmentation. When PDT acts on the endoplasmic reticulum it induces Ca^{2+} increase that acts upon the mitochondria to generate Bcl-2 family. PDT can activate directly p53 that will act upon mitochondria and upon caspase 9 activation. The overall effect is the cell death in PDT-treated cells by apoptotic mechanisms.

2.3.1. Pathways in intracellular events triggered by various photosensitizers used in PDT

As a general picture, in skin cancers, the PDT is a topic procedure, especially applied for NMSC while for melanoma could be an adjuvant postsurgery alternative. As PS used in dermatopathology, only two of them are known to be approved by FDA, namely 5-ALA and MAL, which are prodrugs becoming active upon intracellular metabolization [50], while other PS attempts are currently made with novel (bio) structures related to porphyrin skeleton such as porphycenes [51, 52]. Once activated in tumor cell, a PS triggers different pathways depending on intracellular localization of PS, PS's dose and type, light dose, cell genotype, affecting cell fate in terms of death and proliferation. PDT triggers the death of cellular target, occurred primarily by apoptosis, necrosis and, as recently shown, by autophagy [47]. The PS genera-

tions evolve continuously, hence from the first generation of PS, Photofrin, a partially purified form of hematoporphyrin derivative [53], the second generation was developed to overcome disadvantages of the first one, such as tetrapyrrole rings, substituted derivatives of porphyrin, chlorin and bacteriochlorin [54]. Recently the newer, third generation of PSs was put in scene in novel chemical compounds (e.g. fullerenes) or novel platforms such as PS coupled on different carriers [47]. Nowadays, a so called targeted PDT has emerged and in this type of therapy, antibodies, peptides, proteins, liposomes, cholesterol or other ligands are coupled to PS displaying an improved selective accumulation in the tumor [55].

An advanced generation of PS is always defined by longer wavelengths of light (as red as possible) which corroborates with a deeper penetration of target tissues and a decreased photosensitivity [56]. The antitumor PDT addresses two important issues: direct harmful effects on target cells and vascular injury that will limit blood and other nutrients supply to the affected region [57]. There are mainly two ways of cell fate following PDT: cellular death or cellular survival. The border between these two opposite processes is fine-lined by specific intracellular mechanisms development.

2.3.1.1. Cellular death induced by PDT

Once inside the target cell subjected to photoactivation, a variety of intracellular pathways are initiated by the PS. Although at first sight these biochemical pathways look complex, overlapped, multiple and hard to decipher, the target cell will act in one main direction, namely survival as a response to PDT-triggered aggression.

Generation of reactive oxygen species upon PDT: The first molecular steps in ROS generation are related to PSs' chemistry, depending on oxygen supply in the target tissue [58], and comprise the generation of ROS, as main tools for tumor/target cell destruction. Under the specific light action, the PS will absorb a photon moving from a low energetically short-lived singlet state to an excited long-lived triplet state able to react with molecular oxygen to produce superoxide anion $O_2^{\bullet -}$ (low reactivity, long lifetime—type I reaction) or singlet oxygen $^1O_2$ (high reactivity, short lifetime—type II reaction) [59].

What is the fate of ROS in photosensitized cells? Different types of ROS inflict different actions. Singlet oxygen being highly reactive but evanescent, will oxidize various many biomolecules (lipids, nucleic acids, proteins, etc.) at the level of their electron-dense regions [57, 60], while superoxide anion due to its low reactivity on direct biological target, will act mainly as a precursor for other reactive species (e.g. H_2O_2 or $^{\bullet}OH$) that will cause cellular fatal injuries triggering cell death through necrosis or apoptosis [61]. Also it is important to notice that since singlet oxygen is a short-lived species, will act immediately upon intracellular targets close to the site where PS was accumulated, influencing thus the type of response upon intracellular localization [57]. Regardless of ROS type, the outcome is tumor cell eradication by different mechanisms of cytotoxicity operated by PDT [46].

Cellular toxicity induced by reactive oxygen species: Three cytotoxicity modes are induced post-PDT: oxidative stress, hypoxia and antitumor immune response. Oxidative stress it's installed when generated ROS oxidize and irreversible damages nucleic acids (DNA and RNA) [62, 63],

lipids [64] and proteins [65] with consequences upon the whole cellular physiology. Certain particular changes are related to cellular membrane where phospholipid peroxidation leads to alterations of membrane fluidity, permeability and the (photo)oxidation of cellular membrane contributing strongly to cell death [66, 67]. Oxidative stress is linked to locally induced hypoxia in the photosensitized tissue, which abort the ATP production by oxidative phosphorylation [68] thus leading to cellular death (namely necrosis, an ATP-independent process), and further to the antitumor immune response initiation which is the decisive piece for complete removal of photodamaged tissue [69]. Upon PDT-induced cellular death, namely apoptosis, necrosis, necroptosis [70] and/or autophagy [71]), intracellular damage-associated molecular patterns (DAMPs) [72] and tumor-associated antigens (TAAs) are released from the photosensitized cells and subsequently trigger an immune response aimed at removing the PDT-treated tumor [73].

2.3.1.2. Activation of intracellular survival pathways in photodynamic action

Although cellular death conducted by ROS is envisaged, tumor cells subjected to PDT could encounter this stress by triggering survival mechanisms when vascular shutdown was not completed following PDT action. This type of response is primarily mediated by several pathways raising in an interconnected manner where beside classical NF-kB-mediated proinflammatory and proangiogenic activity, is raising also a NRF2-mediated antioxidant response, a HIF-1-mediated hypoxia survival, a proteotoxic stress response interceded by certain transcription factors (HSF1, XBP1, ATF6 and ATF4) corroborated with an acute stress reply where factors from MAPK pathway are being involved. As PDT means an oxidative stress upon target cells, many studies related to signaling pathways were treated through the prism of oxidative stress and therefore extrapolated to PDT. Consequently, recent works refer to signaling pathways in PDT as to signaling pathways activated in cells subjected to oxidative stress [74].

NRF2 is the main transcription factor protecting against the oxidative stress by restoring the intracellular redox balance in a post-PDT–treated cell, promoting the transcription of the genes encoding for antioxidant enzymes, antioxidant proteins as well as for multidrug response proteins. Moreover, NRF2 is likely constitutively active in many cancer types potentially desensitizing these cells to PDT effects, mediated by ROS. Cells from various layers of the skin benefit from NRF2 protective actions, both in abnormal differentiation, wound healing and controlling inflammation. Thus in keratinocytes and melanocytes, NRF2 protects against mutation during keratinization and melanogenesis. Also, in fibroblasts, NRF2 protects against differentiation and fibrosis processes; therefore, NRF2 activity could be modulated in the context of skin diseases pharmacotherapy and in PDT in order to improve the PDT efficacy by impairing adaptation of target cells to oxidative stress. In addition, NRF2 could be a key biomolecule in the searching for new drugs for various skin disorders including vitiligo or even cutaneous melanoma [75].

NF-κB is a family of transcription factors with a crucial role in inflammation, apoptosis, innate immunity and also in cancer initiation. NF-kB interferes with an ample array of signaling pathways, including HIF-1, and certain biomolecules such as ROS [76]. The NF-κB activation

following PDT could initiate the survival of tumor cells by inhibiting apoptosis and facilitating angiogenesis. Also, NF-κB pathway could display equally antitumor and protumor functions in different carcinogenesis processes, for instance in epidermal keratinocytes, NF-κB seems to exert mainly tumor growth inhibitory functions [77]. In melanoma, the NF-κB activity in tumorigenesis was demonstrated in a mouse model where HR as-mediated tumorigenesis onset relies on IKK2-mediated NF-κB activation [78].

HIF-1 is the central modulating pathway for hypoxic conditions in most tumor cells, as well as skin cancers, condition in which it is constitutively activated [79]. Thus, in a hypoxic or even anoxic milieu, HIF-1 becomes hyperactivate as a part of survival actions adopted post-PDT [80].

The ASK1 pathway directs the immediate early stress response, namely the rapid transcription of a set of genes encoding for stress adapting proteins. Classical ASK1 sends its signal *via* MAPKs proteins to the AP-1 transcription factors responsible for the rapid induction of immediate early gene transcription. Nevertheless, the direct ASK1 activation post-PDT is still difficult to demonstrate, so the actual involvement of ASK1 in PDT response can only be assumed from the effects on downstream kinases (MAPKs) and other transcription factors. This kind of indirect proof was reported in a model of PDT with murine PAM212 keratinocytes loaded with a benzoporphyrin derivative, where the activation of JNK and p38MAPK were associated with early stress response mediated by ASK1. In addition, the early survival response upon PDT means a transient JNK and p38MAPK activation triggered by AP-1 transcription factors phosphorylation from ASK-1 pathway. Thus, an approach for improving PDT efficacy could be the AP-1 pharmacological suppressing while preserving the JNK and p38MAPK functions [81].

The proteotoxic stress response arises as well as a survival mechanism in PDT stressed cells triggering certain transcriptional level responses known as the unfolded protein response (UPR), a form of endoplasmic reticulum (ER) stress. These resulted unfolded proteins activate further HSF transcription factors by which an adaptive response comprising chaperones upregulated and protein synthesis inhibition is raising, allowing protein refolding and degradation of those protein aggregates wrongly appeared during stress [82].

However, this UPR process should be analyzed in report with cell, type, PS nature, PS intracellular localization and PDT regimen. It is expected to launch an UPR response for those PS which accumulate preferentially in ER, such as hypericin. For instance, such ER-related oxidative stress was reported for a PDT model with sodium-porfimer photosensitizer, which conducts to protein polyubiquitination, carbonylation and ER lumen enlargement [65]. As a result, the UPR constitutively activated in tumors, as many other protection mechanisms [83], will protect tumor cells against anticancer therapies such as PDT [74].

2.3.2. Different effects triggered by in vitro PDT experimental models

Subtle changes triggered by photodynamic treatment are incompletely revealed, as signaling pathways regulate a myriad of cellular processes starting with genetic ones, such as transcription and translation, and ending with complex cellular behavior such as proliferation, apoptosis, differentiation, metabolism and overall cell survival upon a certain therapeutic regimen.

Such finest inquiries are needed to be explored with various techniques and experimental models that deliver data regarding the best way of PS delivery, the best PDT regimen, cellular and molecular characteristics imprinted by a certain PS, therapeutical outcome and so on. The "practical" history of experimental PDT begun in the mid-1970s when it was discovered that a hematoporphyrin derivative activated with red light "cured" a mammary tumor in a mouse model [84]. The studies are in continuous development as an arsenal of in vitro cellular models were settled for studying PDT effects on various cancers including skin cancers. Experimental *in vitro* models for PDT implying novel PS to be used in skin tumors, are related to cell types or types of cellular cultures (adherent, suspension, 2D or more recently 3D cellular cultures). For instance, three dimensional spheroid culture cells provide very convenient approaches for *in vitro* assessment of new PS and new PDT responses and, in addition, they could mimic many *in vivo* intercellular interactions. Their convenient growth characteristics and exploitable features in imagistic approaches allow *in vitro* PDT multiple parameters studies in an "all in one" manner [85].

2.3.2.1. In vitro models for nonmelanoma skin cancer PDT therapy

Among PS tested for NMSC, hypericin a natural quinone extracted from *Hypericum perforatum*, gain constant attention due to its good photosensitizing properties, an ideal candidate for PDT applied in skin tumors [86]. Hypericin as PS was currently investigated in a recent report where was tested in an *in vitro* system with human normal primary cells (keratinocytes, melanocytes and fibroblasts) mimicking thus the epidermis and dermis of human skin. Fibroblasts were the most susceptible to hypericin-PDT, followed by melanocytes and keratinocytes in terms of viability. The cellular morphologies were affected by PDT for all investigated cell types, keratinocytes being the most unaffected even at highest PS doses. Other results indicate a cytoplasmic localization of hypericin in all investigated skin cell types whereas the intracellular generated ROS were the most elevated in fibroblasts. This study describes the effects induced by *in vitro* PDT using hypericin on different human skin cells, gathering hence data on PS efficacy that could impacts *in vivo* application for NMSC [87].

2.3.2.2. In vitro models for melanoma PDT therapy

The main stream of the studies regarding PDT applied in melanoma encounter a major issue raised by this type of cancer: an evident resistance to this therapy owing to melanin that will compete with the PS for photons in the detriment of molecular oxygen, leading to an impaired phototoxicity upon target cell [47]. Therefore, the attempts regarding PDT in malignant melanoma have tried to combine PDT with a complementary procedure such as magneto-hyperthermia [88], or use an improved PS delivery system, namely a liposome formula including a second generation PS such as metallated phthalocyanines. This *in vitro* model with B16-F10 standard cell line was used for melanoma studies [89].

Although PDT seems not to be an option in melanoma, recent publication has shown that in a B16-melanoma cell line and also in a B16 ectopic tumor model, ALA-SDT had been more

efficacious when compared to ALA-PDT. SDT is sonodynamic therapy in which the activation of a nontoxic sensitizer drug is performed using low-intensity ultrasound to produce cytotoxic ROS. SDT can activate sensitizers at a greater depth within human tissue because of the low tissue attenuation of ultrasound. In PDT for melanoma the low efficacy was attributed to the dark pigmentation of the melanoma that filters the excitation light. In SDT, the sensitizer is activated by ultrasound and it is not hindered by pigmentation. These results suggest SDT as a better approach in comparison to PDT when treating highly pigmented cancerous skin lesions [90].

Nanocarriers for delivering one or even simultaneously two PSs [91] seem to be a good instrument to outcome the recognized melanoma resistance, and have been tested further in a mouse model of xenograft melanoma proving an increased efficacy of treatment and an enhanced accumulation in melanoma cell [92].

2.4. Proteomics technologies in intracellular signaling events triggered by PDT

The complex and intermingled intracellular mechanisms triggered by PDT claim high-throughput proteomic tools to thoroughly quest the signaling events occurred in cancer cell followed PS activation. The cellular signaling events are first triggered by the activation of plasma membrane events [93]. Recently emerged, the proteomics branch focusing on these events is plasma membrane proteomics. From antibody-based techniques to large-scale "precision proteomics" centered on mass spectrometry, posttranslational modifications, protein-protein interactions and changes in protein expression could be analyzed by large scale proteomics. Proteomics in this domain is important as it conveys accurate information concerning (patho) physiological changes in terms of qualitative and quantitative terms of thousands of proteins as response to a certain antitumor therapy.

Skin cancers alterations affect specific genes and thus specific protein mediators from different signaling pathways including the Sonic Hedgehog and NF-kB, targeting these proteins being the trigger for new approaches in skin cancers therapy [94].

Before tackling the proteomic involvement in PDT pattern, it must be underlined that genomic technologies' advancements paved the road to molecular insights in skin cancers and related signaling pathways. In skin tumors, advances in sequencing techniques were driven at the beginning of 2000s in receptor tyrosine kinases studies, and were the tools that identified and indicated the presence of BRAF mutations in 50% of skin melanomas [95]. This imprints a major impact in development of selective BRAF inhibitors (vemurafenib and dabrafenib) triggering impressive remissions in melanoma patients who benefit now from an improved treatment, leading to a new era in targeted therapy [96]. Starting from this crossing-point, it could be further underlined that proteomics is continuing to make major progresses in biological processes discoveries as well as in establishing an "universal" assay platform for measuring proteins status and levels in any biological system subjected to different physiological milieu. A strength of proteomic approach is that it can translate almost in "real-time" fundamental science achievements into clinical practice helping in outlining personalized medicine and precision medicine [97].

2.4.1. Proteomic technologies in skin cancers at a glance

Proteomics includes a number of methods that could be classified depending on several criteria, such as scope (for identification—quality *versus* measurement—quantity; discovery *versus* validation of biomarkers) and detection method (labeling *versus* label-free), and are broadly comprising spectrometry, electrophoresis and array methods. Regardless of classification criteria, for analyzing PDT events, all approaches could be used to obtain important data in comprehending the dynamic biology of malignant transformation, tumor cell behavior and therapy outcome [98].

It is not the chapters' intend to go in depth with proteomic techniques but one should keep in mind that there are several main stream approaches currently in use in different experimental sets in cancer research [97, 99] as resumed in **Table 1**.

Technology type	Characteristics
Two-dimensional gel electrophoresis (2-DE)	Quantitative method allowing extraction and separation in two dimensions (isoelectric point, molecular mass) of proteins from sample of interest
Mass spectroscopy (MS)	Generates peptide mass fingerprints for protein detected on 2-DE; MS has multiple variants—electrospray ionization-liquid chromatography tandem mass spectroscopy (ESI-LC-MS); matrix-assisted laser desorption ionization time of flight (MALDI-TOF); surface-enhanced laser desorption ionization time of flight (SELDI-TOF); MALDI MS imaging (MALDI-MSI); laser capture microdissection-MS (LCM-MS)—optimum for extracting cells from biological specimens preserving the morphologies of captured cells and the nearby tissues
Protein microarray (PM) technology	Proteins/antibodies/other biomolecules covalently attached to a solid support like glass are used to detect various interactions such as protein-protein, enzymatic targets, protein-small molecule (peptide, DNA, etc.), based on the antigen-antibody reaction; it has also many recent variants—cell arrays—it can analyze particular molecular targets expression triggered in in vivo experimental models; tissues array—it can analyze the molecular targets in situ across a panel of primary tissues in order to evaluate their clinical significance

Table 1. Main proteomic technologies applied for intracellular mechanisms investigation in skin cancer.

The future in the proteomic domain relies in multianalyte investigation with different congruent methods based on molecular characteristic evaluation such as 2-DE, MS and protein microarrays. As the proteomic approach is complex, so the future therapeutical approaches in skin cancer need the same multitargeted approach. In this aspect, photo-immune-theranostics reagents are the future compounds that will enter the PDT scene. This future to be therapeutical method combines molecular optical imaging, photodynamic therapy and immunotherapy using SNAP-tag technology which is a derivative of the O(6)-alkylguanine-DNA alkyltransferase (AGT) with the ability to efficiently conjugate to O(6)-benzylguanine (BG) molecules under physiological conditions depending on its folding pattern. An approach like this could

simultaneously monitor and suppress the growth of skin squamous carcinoma and melanoma cells expressing EGFR [100].

2.4.2. Proteomic data for skin cancers

Data for cutaneous tumors were obtained from proteomic studies' involving MS. Referring strictly to cutaneous cancers, ESI-LC-MS was used for investigating in paraffin-embedded metastatic melanomas for comparative proteomic study [101]. This method quantifies peptide spectra that have been sequenced by the MS and can be used for biomarkers discovery through comparing the peak intensities derived from multiple LC-MS data set [102].

Biomarker identification in melanoma along with other type of cancers was also subjected of SELDI-TOF-MS analysis [103]. In SELDI, the protein sample mixture is spotted on a specific solid surface with chemical functionality such as binding affinity where some analytes in the samples would attach while the others will be washed off. The spotted samples on an SELDI surface are analyzed with TOF mass spectrometry [104].

Nevertheless, proteomic studies concerning PDT in skin tumor are still missing and are limited to *in vitro* approaches by assessing various cell lines. Thus a recent study published in 2016 involves the hexyl-aminolevulinate-mediated PDT in the human epidermoid carcinoma cell line A431 [105]. This analysis is another attempt to elucidate the exact trigger mechanisms for various death-pathways induced by PDT which are still unknown. One of the alteration induced by PDT *via* ROS is the reversible oxidation of cysteine thiols groups (–SH), as potential redox switch for protein activity and cellular signaling. Using MS as proteomic tool, the authors found that over 2000 proteins were reversibly oxidized post-PDT, of which 115 of the high confidence proteins were related to the apoptotic mechanisms and 257 have not been reported yet to be reversibly oxidized on –SH group. This study is considered the first complete mapping of reversibly oxidized proteins following PDT, among which ATM, p63, RSK1 p38, APE1/Ref-1 and three 14-3-3 family members represent potential signaling core in apoptosis death. This "core protein" furnished an apoptotic map that can subsequently identify potentially new redox-regulated triggers as well as potential targets for PDT efficacy improvement, demonstrating the benefit of proteomics in PDT [97, 105].

PM methodology is not yet a routine approach in PDT topic, although possess all strengths to become a robust tool in deciphering the protein-pattern of this domain. Many formats have been developed with whole proteomes, peptides, nucleic acids and lectins, although antibodies platforms remain the most popular PM surfaces. High-throughput tools in a miniaturized format, the arrays could perform parallel analysis, interactions and protein function on a large scale for benefit of both basic research and clinical applications [106].

Using antibody microarray, we have assessed the probable intracellular pathways by which PDT with aluminum-substituted disulfonated phthalocyanine trigger apoptosis in dysplastic oral keratinocytes cells (DOK cell line), leading to the tumoral cells eradication. Among the analyzed apoptotic factors, Bcl-2, P70S6K kinase, Raf-1 and Bad proteins were the biomolecules whose expression changes with the greatest amplitude. Until now, the intimate

apoptotic mechanisms activated by PDT with metallated phthalocyanine in this type of keratinocytes are still to be deciphered as well as PDT-related signaling events per se [107]. This complex methodology is a versatile tool allowing investigation in detail of molecular events related to cellular death induced by PDT.

3. Conclusion

PDT procedures have several lines of improvement in skin cancers treatment. For example, BCC superficial lesions, preferentially located on the trunk, have the best therapeutic response when treated with PDT. In SCC, PDT should be combined with immune modulators and chemotherapeutic agents. For melanoma, there is still a huge array of improvement due to its particularities and probably with the prospect of advances in gene discovery and translation, multidisciplinary team has to solve all the emerging issues for introducing PDT in melanoma.

The therapeutic future relies in the homogeneous photo-immune-theranostics reagents combining molecular imaging, PDT and immunotherapy. Using "next generation" proteomic technologies (SNAP-tag) it would be possible to simultaneously monitor and suppress the growth of skin squamous carcinoma and melanoma cells expressing specific markers, like EGFR.

Concluding at a glance, large-scale proteomics-based signaling research will be one of the leaders in future photomedicine by enlarging the basic knowledge regarding (photo)therapy-targeted networks and molecules, and by deciphering new intracellular avenues for future precision medicine in skin tumors. A deeper knowledge regarding signaling mechanisms in PDT could furnish new molecular targets and increase its clinical efficacy.

Acknowledgements

This work was financed by national grants PNII-ID-PCE-2011-3-0918 and PNII-PT-PCCA-2013-4-1407.

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