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## Minireview

Ying-Ying Huang, Daniela Vecchio, Pinar Avci, Rui Yin, Maria Garcia-Diaz  
and Michael R. Hamblin\*

# Melanoma resistance to photodynamic therapy: new insights

**Abstract:** Melanoma is the most dangerous form of skin cancer, with a steeply rising incidence and a poor prognosis in its advanced stages. Melanoma is highly resistant to traditional chemotherapy and radiotherapy, although modern targeted therapies such as BRAF inhibitors are showing some promise. Photodynamic therapy (PDT, the combination of photosensitizing dyes and visible light) has been tested in the treatment of melanoma with some promising results, but melanoma is generally considered to be resistant to it. Optical interference by the highly-pigmented melanin, the antioxidant effect of melanin, the sequestration of photosensitizers inside melanosomes, defects in apoptotic pathways, and the efflux of photosensitizers by ATP-binding cassette transporters have all been implicated in melanoma resistance to PDT. Approaches to overcoming melanoma resistance to PDT include: the discovery of highly active photosensitizers absorbing in the 700–800-nm near infrared spectral region; interventions that can temporarily reduce the amount or pigmentation of the melanin; compounds that can reverse apoptotic defects or inhibit drug-efflux of photosensitizers; and immunotherapy approaches that can take advantage of the ability of PDT to activate the host immune system against the tumor being treated.

**Keywords:** antitumor immune response; depigmentation; drug efflux systems; melanoma; melanosomes; photodynamic therapy; photosensitizers; resistance mechanisms.

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## Introduction

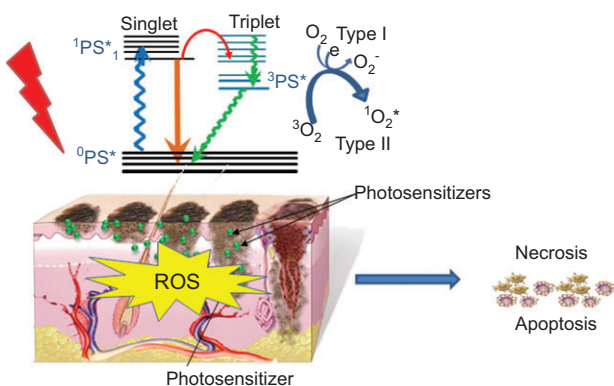
Melanoma is the malignancy responsible for the highest incidence of deaths from skin cancer. Genetic and environmental factors such as ultraviolet (UV) damage can cause the transformation of skin melanocytes into a tumorigenic melanoma (Carlson et al., 2009). Melanocytes are the main cells responsible for the production of melanin, the pigment that protects the skin from sun damage by absorbing UV light (Slominski et al., 2004). Although chronic and intermittent exposure to UV leads to tanning that protects the skin from DNA damage, intense exposure leading to sunburn can lead to DNA damage and genetic alterations in melanocytes. Malignant melanomas can be pigmented (melanotic), characterized by black lesions due to melanin accumulation, or can be unpigmented (amelanotic) if the melanocytes involved are less differentiated and therefore produce less melanin.

It has been claimed that in recent years there has been an ‘epidemic’ of melanoma because it is being diagnosed at more than double the rate it was in 1986, increasing faster than any other major cancer (Burton et al., 1993). There is disagreement on this point, however, as some dermatologists assert (Glusac, 2011) that the increasing numbers represent not an epidemic of melanoma, but an epidemic of melanoma screening, and a study lends support to this view (Aguilar et al., 1991). Melanoma is resistant to most traditional forms of chemotherapy and radiotherapy, and for this reason many alternative treatments have been investigated (Jilaveanu et al., 2009).

## Photodynamic therapy and melanoma

Photodynamic therapy (PDT) is an effective treatment for several different cancers (Agostinis et al., 2011). Its efficacy has been shown in non-melanoma skin cancers, other skin cancers, such as lymphoma, and in dermatologic disorders such as vitiligo and psoriasis (Babilas et al., 2010). PDT involves the systemic or local administration of a photosensitizer (PS), which localizes in the tumor. The photosensitizers are activated by irradiation at a specific wavelength and in the presence of oxygen generate short-lived reactive oxygen species (ROS) (Dougherty et al., 1998). The ROS generated by the photosensitizer are responsible for selective tumor destruction, tumor-associated vascular damage, and activation of antitumor immune responses (Castano et al., 2006). This treatment offers many advantages, such as a low systemic cumulative toxicity, the selectivity and noninvasiveness of the method, and the possibility of repeating the treatment many times without serious effects. Figure 1 shows the generation of ROS from the excited PS (represented by a Jablonski diagram) and the destruction of tumor cells by apoptosis and necrosis.

One of the first studies carried out in 1988 was to verify the efficacy of PDT on malignant melanoma compared to the effect of hematoporphyrin derivate (photofrin II) on melanotic and amelanotic malignant melanoma in athymic nude mice. This study demonstrated that PDT was effective in the treatment of amelanotic cancer but not in melanotic melanoma (Nelson et al., 1988). The authors concluded that the resistance of malignant melanotic melanoma to PDT was due to the presence of the melanin that



**Figure 1** Mechanisms of photodynamic therapy.

The ground state PS is initially excited to an excited singlet state that undergoes a transition to a long-lived triplet state that can interact with oxygen in a type I mechanism to produce hydroxyl radicals or in a type II mechanism to produce reactive singlet oxygen. These reactive oxygen species can cause the death of tumor cells by apoptosis or necrosis and destroy the tumor.

competed with the photosensitizer for the absorption of photons or in the energy transfer process from the excited triplet state of the sensitizer to melanin instead of cellular oxygen. PDT is a photochemical reaction, thus the energy of the photon is absorbed by PS, which can transfer its energy to the target molecule. Usually, PDT induces tumor necrosis by transferring energy from the excited triplet state of the PS to ground state molecular oxygen, producing excited state singlet oxygen, which causes irreversible oxidation of some essential cellular components. The presence of melanin, a stable protein complex with a wide absorption spectrum, in the same tissue led to competition with PS for photons, resulting in inefficient phototoxicity (Nelson et al., 1988).

Subsequent studies aimed to investigate and synthesize new photosensitizers that were able to exert their action after irradiation at different (longer) wavelengths from the melanin absorption spectrum. The employment of selected second-generation photosensitizing agents, such as Si(IV)-naphthalocyanine, bacteriochlorin a and Lu(III)-texaphyrin, characterized by an extended macrocycle and high molar absorptivity in the 750–800 nm spectral interval, improved the efficacy of PDT on experimentally implanted melanotic melanoma (Schuitmaker et al., 1990; Biolo et al., 1996; Woodburn et al., 1998). Ten years after the first study, Busetti et al. showed that if the melanosomes were preirradiated with high peak power pulsed laser radiation at 1064 nm, PDT treatment was improved (Busetti et al., 1999). In 1999, the same group investigated the effect of PDT using a benzoporphyrin derivative monoacid ring A, called verteporfin, against B16 pigmented melanoma, after preirradiation.

Several studies have been carried out to investigate and improve the efficacy of PDT against melanoma. PDT techniques were initially developed in experiments on animals (Kostenich et al., 1994). Some years later, Sheleg et al. (2004) investigated PDT using chlorin (e6) in phase I clinical trials on skin melanoma metastases in humans. The patients in this study received five PDT courses over 18 months. No new skin metastases from melanoma were detected in the patient within 2 years after the treatment. PDT with chlorin (e6) for skin metastases from pigmented melanoma was well tolerated and effective, especially in cases of isolated melanoma skin metastases. The study was limited, with only 14 cases being included, thus further clinical investigation is necessary.

The PS employed is very important in PDT, and some PSs frequently used in clinical practice are not always effective against melanoma skin cancer. Cordoba et al. (2005) analyzed the effects of 5-aminolaevulinic acid, which is widely used in clinical applications in

dermatology, on melanoma cell lines and on experimental melanomas. To mimic the clinical situation, a transgenic model of skin melanoma was developed. The results show that, although MT-ret melanoma cells were vulnerable to 5-aminolaevulinic acid PDT *in vitro*, malignant MT-ret melanomas *in vivo* were quite resistant to this type of therapy at doses that are highly effective *in vitro*.

PDT is a good potential candidate for the treatment of melanoma and much knowledge has been acquired during these years of intense research, but more research will be necessary to overcome the resistance of melanoma to PDT. The need for the development of novel and effective approaches to treat melanoma remains. PDT could be applied as an adjuvant therapy alone or in combination with current therapeutics to combat melanoma (Davids and Kleemann, 2011).

## Mechanisms of melanoma resistance to standard therapy

There are currently only a few US Food and Drug Administration-approved treatments for metastatic melanoma (Tarhini and Agarwala, 2006), including conventional chemotherapy (single agent and combination chemotherapy), cytokine-based therapies (such as interferon and interleukin-2), and recently-developed targeted therapy with monoclonal antibodies and small molecule kinase inhibitors. A large number of anticancer treatments are ineffective at killing melanoma cells, which implies that melanoma has complex resistance mechanisms in melanoma. Even though melanoma is thought to be susceptible novel targeted therapy and immunotherapies, such treatments are only successful in a small subset of patients (Atkins et al., 1999).

Multidrug resistance (MDR) still remains a big problem. Mechanisms of drug resistance in human melanoma are not well understood, and they are likely to depend on the chemotherapeutic agent and the tumor entity. In the first phase, drug effect-specific mechanisms may intervene in the drug-target interaction via drug transport mechanisms and detoxification or target modulation. Other mechanisms reverse and compensate for the drug effects before the cell death cascades are initiated. Another drug-resistance mechanism lies in dysregulation of the apoptotic pathways leading to a deficiency in apoptosis and the prevention of cell death (Helmbach et al., 2001). In this article, the general resistance mechanisms of melanoma will be described before the resistance to PDT is analyzed.

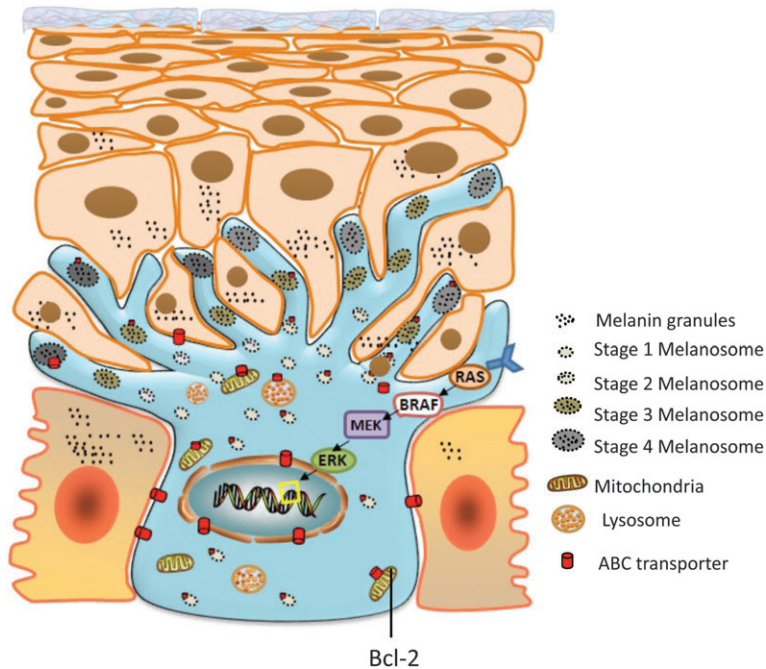
## Melanogenesis-mediated multidrug resistance

The general resistance mechanisms for solid tumors can be applied to explain the MDR of melanoma but do, not explain why melanomas are particularly insensitive to conventional chemotherapy and radiotherapy compared to many other non-melanoma cancers. The major difference between melanoma and non-melanoma cancer cells lies in a unique subcellular organelle termed the melanosome, a lysosome-related organelle modified for melanin synthesis that has been implicated in drug trapping and export (Chen et al., 2009). Chen developed a melanogenesis model theory to better explain how the melanosomes affect drug sensitivity and how they are involved in intrinsic MDR in melanoma.

Melanogenesis includes three major processes: melanosome biogenesis, melanin synthesis, and endogenous melanogenic cytotoxicity-related homeostasis. Melanogenesis is involved in the regulation of drug sensitivity. It has three distinct phases depending on the four stages of melanosome biogenesis. In phase I melanogenesis, the melanosome called “premelanosome”, is at an early stage I and II of melanosome biogenesis without melanin synthesis. Premelanosome possesses the ability to trap and export cytotoxic drugs such as cisplatin. In phase II, melanosomes are predominately in stage III of melanosome biogenesis, with active melanin synthesis. They possess a maximal capacity to trap cytotoxic drugs in the nascent melanin, and thus they are likely to be involved in drug resistance. In phase III, melanosomes can generate endogenous melanogenic cytotoxic byproducts, triggering an autophagic program on damaged stage IV melanosomes. This in turn causes the melanosomes to be more susceptible to cytotoxic drugs. At the end of the melanogenesis process, the mature melanosomes are transferred from the dendrites of the melanocytes into the surrounding keratinocytes, where they form the melanin granules responsible for sun-protection properties. Tyrosinase-related protein-2, a melanogenic enzyme, was shown to confer resistance to cisplatin in melanoma cells (Chen et al., 2009). Figure 2 illustrates the process of melanogenesis and depicts some of the mechanisms of resistance specific to melanomas.

## ATP-binding cassette transporter-mediated multidrug resistance

The most common cause of multidrug resistance in human cancers is the expression and function of one or more



**Figure 2** Process of melanogenesis and the resistance mechanisms of melanoma.

Melanosomes mature through stages 1–4 and are finally transferred to keratinocytes, where they release melanin granules. Defects in apoptosis (BCL2), BRAF mutation-activated mitogen-activated protein kinase/extracellular signaling regulated kinase pathway, and ATP-binding cassette transporter drug efflux pumps contribute to melanoma resistance.

ATP-binding cassette (ABC) transporters that efflux anti-cancer drugs from cells (Chen et al., 2009). ABC transporters (48 have been identified in the human genome) are located at the cell membrane and many subcellular organelles, conveying structurally diverse molecules across biological membranes in an ATP-dependent manner. A cluster of ABC transporters is expressed in melanomas; ABCB5 is the most frequently found here. Moreover, transfected full length of ABCB5 conferred drug resistance to two cell lines (Chen et al., 2005) therefore over-expression of ABCB5 protein is likely to be a major mechanism of MDR in melanoma cells. Furthermore ABCB5 was considered to be marker of melanoma stem cells; isolated ABCB5+ subpopulation cells were shown to have high tumorigenicity in a mouse model compared to ABCB5- control (Schatten et al., 2008).

Chen et al. (2009) also integrated ABC transporters in the melanogenesis model. Melanoma cells utilize additional and specific subcellular organelles (melanosomes) for subcellular drug trapping or sequestration, beside other subcellular compartments (vesicles, lysosomes and endosomes) in non-melanoma cancer cells. The distribution and regional intensity of this transporter network in diverse subcellular organelles constitutes a buffer system that prevents the nuclear or mitochondrial import of cytotoxic drugs, thereby protecting the cells from the

endogenous melanogenic cytotoxicity generated by late-stage melanosomes.

### DNA repair mechanisms mediated multidrug resistance

One mechanism by which to counteract the deleterious effects of DNA-damaging drugs could be hyperactivation of DNA repair mechanisms by up-regulating mismatch repair genes or by potentiating enzymes that remove DNA-alkylation damage (Soengas and Lowe, 2003). Drug-resistant melanoma cell lines were demonstrated in fote-mustine- and cisplatin-resistant human melanoma cells, exhibiting increased repair of DNA (Runger et al., 2000). DNA-mismatch repair deficiency results in drug resistance by impairing the ability of cells to repair DNA damage (Fink et al., 1998).

### Dysregulation of apoptosis pathway

The molecular mechanism for drug resistance is poorly understood; however, it has been proposed that defects in the apoptotic pathway may be a critical event in melanoma progression. One of the genes related to tumorigenesis

and chemoresistance in melanoma is *p53*. Although *p53* mutations are rare in melanoma, apoptosis protease-activating factor-1 (APAF-1), a critical downstream effector of the *p53*-dependent mitochondrial apoptotic pathway, is deleted and inactivated by methylation in metastatic melanomas. Disruption of Apaf-1 in cells dramatically reduces *p53*-dependent apoptosis and facilitates oncogenic transformation (Campioni et al., 2005). Alternatively, activation of an anti-apoptotic factor, such as Bcl-2, impairment of a pro-apoptotic pathway, or loss/inactivation a pro-apoptotic factor like BAX, may lead to resistance of apoptotic-induced agents (Soengas and Lowe, 2003).

### Mechanism of resistance to mitogen-activated protein kinase pathway inhibitors

The mitogen-activated protein kinase (MAPK) pathway is a key regulator of cell progression and is commonly activated in human tumors though mutation of the three-tiered kinase cascade consisting of RAF, MAPK kinase (MEK), and extracellular signaling regulated kinase (ERK). In normal cells, the RAS (K-, N-, and HRAS) small GTPase proteins regulate activation of the RAF kinase (ARAF, BRAF, and CRAF/RAF1). Activated RAF initiates a series of phosphorylation events, including the serial phosphorylation of the MEK and ERK kinases. When phosphorylated, ERK enters the nucleus where it phosphorylates transcription factors, and thus promotes cell cycle progression and proliferation (Nissan and Solit, 2011). BRAF mutation has been found in 50% of melanomas.

The best validated targeted therapies in melanoma by far are the BRAF inhibitors (Bollag et al., 2010). However, responses to RAF inhibitors are transient, resistance to the inhibitors develops, and tumors invariably recur. There are multiple mechanisms involved in the resistance of BRAF inhibitors. In most instances, reactivation of the MAPK pathway is required to circumvent chronic BRAF inhibition and resume proliferation. Melanoma cells initially addicted to BRAF can switch to one of the other RAF isoforms (most CRAF or ARAF isoforms) to continue proliferating (Dummer and Flaherty, 2012). Additionally, overexpression of CRAF or the cancer osaka thyroid (COT) can also lead to MAPK reactivation. Alternatively, treatment with BRAF inhibitors could select for minor, preexisting NRAS mutants clones that do not respond to BRAF inhibitors but paradoxically hyperactivate the MAPK pathway. Moreover, the activation of receptor tyrosine kinases, in particular insulin-like growth factor receptor I and platelet-derived growth factor receptor beta, can cause the BRAF inhibition to be bypassed by activating the parallel

phosphatidylinositol-3-kinase/AKT/mammalian target of rapamycin signaling pathway, thereby modulating survival.

## Melanoma resistance to photodynamic therapy

Due to melanoma's intrinsic resistance to radiation and chemotherapeutic drugs, PDT has been suggested as an alternative therapeutic modality. It involves light-induced destruction of cells or target tissues through sensitization to light by various photosensitizing agents; hypericin (Hadjur et al., 1996), benzoporphyrin derivatives (Busetti et al., 1999) and protoporphyrin IX (PPIX) being just a few examples (Kiesslich et al., 2006). However, several resistance mechanisms cause a reduction in the efficacy of PDT on melanoma cells. Such mechanisms include optical interference (Hadjur et al., 1996; Busetti et al., 1999), the antioxidant defense mechanisms of melanin (Hadjur et al., 1996; Davids et al., 2009), cytoprotective response through the induction of autophagy (Davids et al., 2009), melanosomes protecting melanocytes and melanoma cells against harmful effects of toxic intermediates (Davids et al., 2009) and lastly ABCG2 transporters acting as efflux pumps making cells capable of eliminating toxic amounts of porphyrins (Bebes et al. 2011).

The resistance of pigmented melanomas over their unpigmented counterparts to various therapies including PDT suggests that the presence of melanin plays a role in rendering these cells less susceptible to cell death (Sharma et al., 2011). One explanation for this phenomenon is that melanin may act as a filter by preventing any in-depth penetration of light and shielding certain cellular targets from light as well as absorbing and scattering therapeutic light (Hadjur et al., 1996). It has been shown in a study that in the 500–600 nm interval, melanin is the dominant absorber and the optical penetration depth demonstrated that light transmittance of melanotic melanomas occurs only above 700 nm (Kollias et al., 1991). Competitive absorbance of melanin at certain wavelengths might also reduce the efficiency of photosensitization by certain photosensitizing agents such as hypericin (Hadjur et al., 1996). In heavily pigmented melanomas especially it is known that PDT with Photofrin is ineffective, owing to the strong absorbance of melanin in the 630 nm range, which is the wavelength that is used to activate Photofrin in clinical PDT. However, studies demonstrated that by using second-generation photosensitizing agents characterized by high molar absorbance in the 750–800 nm spectral

interval, melanotic melanoma can be made responsive to PDT (Buseti et al., 1999). Such agents include Si(IV)-naphthalocyanine, bacteriochlorin a and lutetium (III)-texaphyrin. With these agents, melanin exhibits a small residual absorbance, thereby minimizing the optical filtering action (Buseti et al., 1999).

Melanin has a significant role in cytoprotection by acting as an intracellular antioxidant, decreasing high levels of ROS by acting as a ROS scavenger (Sharma et al., 2011). It has been demonstrated that melanocytes with high melanin content were more resistant to ROS than ones containing low melanin (Hadjur et al., 1996). Suzukawa found that melanins bound to the minor grooves of DNA, guaranteeing close proximity to DNA and potentially causing the high levels of strand breaks observed. Moreover, they also found that after melanins interact with  $^1O_2$ , they exhibit a lower ability to induce DNA breakage (Suzukawa et al., 2012). PDT, by contrast, induces photo-oxidative stress and cytotoxicity, where cells generally respond by up-regulating their endogenous antioxidant systems, which in return neutralizes the efficacy of PDT treatment. The formation of melanin is via the melanogenic pathway that comprises a series of reactions driven by a rate-limiting enzyme, tyrosinase. Sharma et al. (2011) recently demonstrated that combining the inhibition of melanogenesis with PDT could be explored as a valid therapeutic target for the management of advanced melanoma. They found that when a reversible tyrosinase inhibitor phenylthiourea is used in conjunction with hypericin-mediated PDT (HYP-PDT), the melanotic phenotype reduced phenylthiourea+hypericin+PDT toxicity. Conversely, the inhibition by phenylthiourea increased the susceptibility of these melanoma cells to HYP-PDT. In addition to this, when phenylthiourea is removed and melanin formation is allowed, the pigmented melanoma cells showed an increased resistance to PDT-induced cell death. Peroxidation of membrane phospholipids is believed to be another principle target for PDT, and this event can be measured by a change in thiobarbituric acid-reacting substances. In the presence of melanin, it has also been found that thiobarbituric acid-reacting substances concentration remains unchanged after HYP-PDT, suggesting that melanin plays a protective role in the hypericin photodamage of membrane lipids (Hadjur et al., 1996).

Melanosomes are membrane-bound organelles found in both melanocytes and melanoma cells. In the process of melanin synthesis, they are responsible for protecting these cells from the harmful effects of toxic intermediate products by compartmentalizing cytotoxic melanin intermediates, preventing them from spilling into the cytoplasm (Davids et al., 2009; Sharma et al., 2011). It has

been assumed that melanosomes serve as targets for PDT, and different modes of cell death in pigmented and unpigmented melanomas are thought to be associated with melanosomes themselves. In a study conducted by Davids et al. (2008), when cells were treated with HYP-PDT it was observed that pigmented cells died by necrosis, whereas unpigmented cells died by apoptosis. However, initially both melanoma cell types showed a similar cytoprotective response through the induction of autophagy, which is a survival program instigated by cells undergoing environmental stress (Davids et al., 2009).

One of the most commonly used photosensitizers is protoporphyrin IX (PPIX), which is synthesized by the target cells from the applied prodrugs plus  $\delta$ -aminolevulinic acid (ALA) or its methyl ester (Bebes et al., 2011). While PPIX and heme (iron-PPIX) are crucial for cellular homeostasis, however, free uncommitted pools of these molecules are extremely toxic. This leads to tight control of the intracellular concentrations of free porphyrins by biosynthetic components and efflux systems. The ABC transporter superfamily member ABCG2 is a member of this heme efflux system, which by extruding a variety of cytotoxic substrates from cancer cells has a significant role in the acquired MDR of tumors. Its expression has been detected in the basal layer of the keratinocytes of the murine and human epidermis (Bebes et al., 2011). Although high-level expression of ABCG2 makes the cells capable of eliminating toxic amounts of porphyrins, however, it also causes increased resistance to PDT. It has been reported that, low-dose methotrexate enhances  $\delta$ -aminolevulinic acid-based PDT in skin carcinoma cells (Anand et al., 2009) and this enhancement might be attributed to methotrexate being a substrate for the ABCG2 transporter and therefore interfering with the porphyrin transport of ABCG2. Bebes and colleagues also investigated the specific inhibition of ABCG2 by Ko-134, a non-toxic fumitremorgin C analog, and demonstrated that pretreatment in combination with Ko-134 significantly and dose-dependently increased the sensitivity of HaCaT keratinocytes to a dose of 1.5 J/cm<sup>2</sup> red light (Bebes et al., 2011).

## Overcoming melanoma resistance to photodynamic therapy

Many efforts have been devoted to the development of new strategies addressing the challenge of treating melanoma with PDT. These efforts have been motivated by the good results of this therapy in non-melanoma skin

cancers and the immune system activation that could minimize the metastatic potential of this type of cancer (Gupta and Ryder, 2003; Sidoroff and Thaler, 2010; Choudhary et al., 2011). The use of near infrared (NIR)-absorbing PS, decreasing the melanin levels, as well as the combination with other therapies, has shown considerable promise.

### Near infrared-absorbing PS

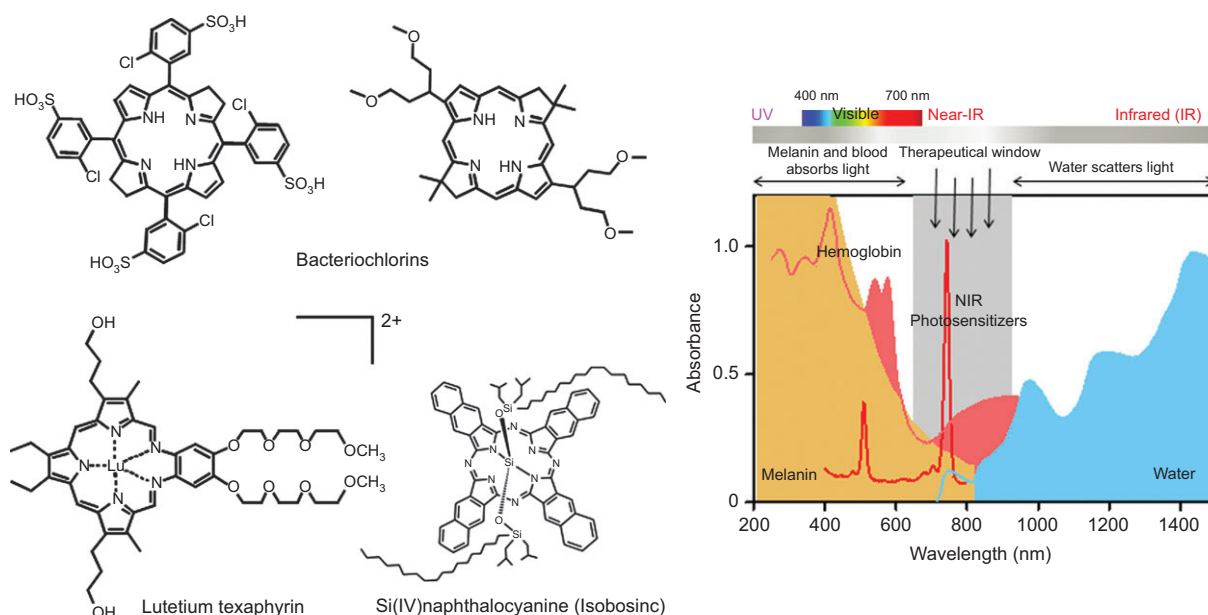
In contrast to other skin cancers, melanomas grow aggressively both in radial and vertical directions, passing through the basement membrane into the deeper layer of the skin. Its high melanin content absorbs light over practically the entire visible spectral range used for PDT (400–750 nm) (Ma et al., 2007). PDT treatment therefore requires a deep penetrating light source through the tissue, and a PS that absorbs in the region bypassing the melanin absorption. The limitations of the clinically approved porphyrin-derived PSs that normally absorb light below 700 nm have prompted the synthesis of NIR-absorbing PS (see Figure 3).

Bacteriochlorins, due to their large absorption peak in the NIR spectrum, are considered one of the most interesting families of PS. The initial unstable naturally-derived bacteriochlorins have given way to stable synthetic molecules with favorable photophysical properties and promising photodynamic results. The water-soluble bac-

teriochlorin, 5,10,15,20-tetrakis(2-chloro-5-sulphophenyl) bacteriochlorin (TCBSO<sub>3</sub>H) showed preferential accumulation in S91 mouse melanoma and long-term tumor growth inhibition (Dabrowski et al., 2011). Mroz et al. (2010) studied a set of three chemically-stable bacteriochlorins (BC1, 2 and 3) in a panel of pigmented mouse melanoma cell lines, B16, with different levels of pigmentation. All of the bacteriochlorins localized in melanosomes, leading to melanosomal destruction after illumination.

Figure 4 shows co-localization studies in pigmented B16F10 cells with probes for mitochondria, lysosomes and melanosomes. The best *in vitro* performing bacteriochlorin (BC3) was tested *in vivo* in a B16F10 mouse melanoma model that expressed green-fluorescent protein, resulting in a marked reduction in tumor size and significant survival advantage with a 20% cure rate, as shown in Figure 5.

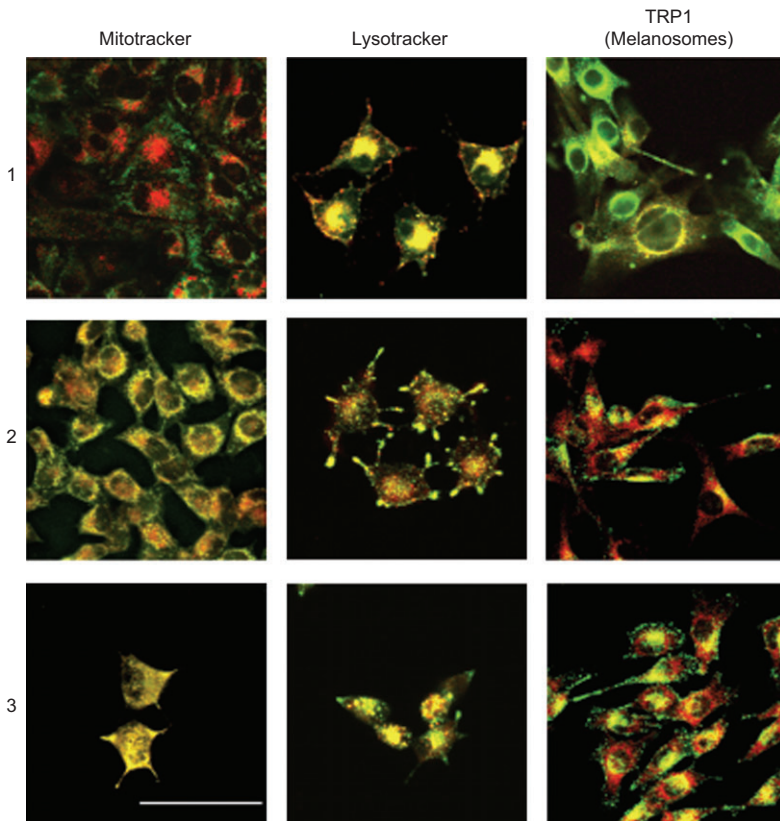
Besides bacteriochlorins, different NIR photosensitizers have been proposed for the treatment of melanoma. PDT of the heavily-pigmented metastatic B16F10 melanoma using a diamagnetic water-soluble lutetium texaphyrin (PCI-0123) resulted in tumor apoptosis with a significant tumor regrowth decay and increase in survival (Woodburn et al., 1998). Biolo et al. (1994) used a liposomal formulation of Si(IV)-naphthalocyanine for the induction of tumor necrosis and to delay tumor regrowth in B16-pigmented melanoma, although no tumor selectivity was observed.



**Figure 3** Chemical structures of near infrared absorbing photosensitizers and the optical window in tissue.

(A) Bacteriochlorin TCBSO<sub>3</sub>H from (Dabrowski et al., 2011). (B) Bacteriochlorin 3 from (Mroz et al., 2010). (C) Lutetium texaphyrin from (Woodburn et al., 1998). (D) Si(IV)-naphthalocyanine (Isobosinc) from (Biolo et al., 1994).





**Figure 4** Fluorescence micrographs of B16F10 cells.

The red fluorescence from BC 1, 2 or 3 is overlaid with green fluorescence from lysotracker, mitotracker or FITC-anti-TRP1 antibody that stains melanosomes. Reprinted with permission from (Mroz et al., 2010).

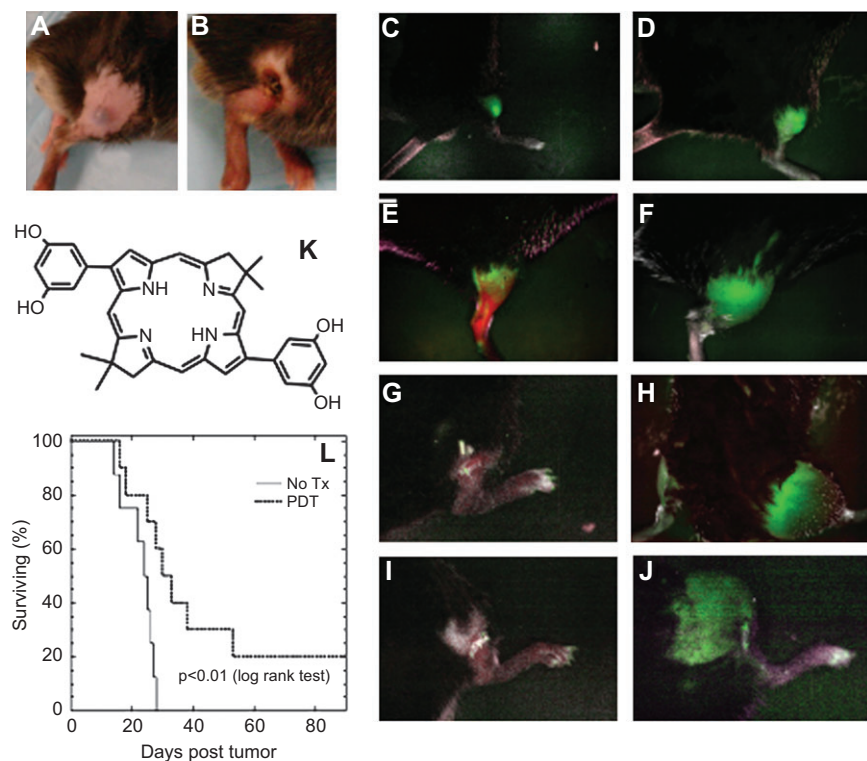
## Depigmentation strategies

Some studies have pointed out differences in photodynamic susceptibility between pigmented and unpigmented melanoma (Mroz et al., 2010; Sharma et al., 2011). The optical interference and ROS photoprotection related to the presence of polymeric melanin can be minimized by using one of the strategies proposed for the depigmentation of melanoma cells. Sharma et al. (2011) suggested a new adjuvant using a known tyrosinase inhibitor, phenylthiourea, for the suppression of melanogenesis. Depigmented melanoma cells showed an enhanced susceptibility to death following PDT, which approached that of unpigmented melanoma cells. This inhibition of melanin formation is reversible and thus, upon removing phenylthiourea, the pigmented cells again showed resistance to PDT-induced cell death. Another approach is the photobleaching of melanin. Ma et al. (2007) used violet light (420 nm) to bleach melanin in melanotic tumors and thereby increased their sensitivity to PDT treatments. B16F10 melanomas were then treated with the topical application of methyl 5-aminolevulinat and irradiated with red light, resulting in greater growth inhibition of tumors. Based on the same principle, Busetti

et al. (1998) used 1064 nm light from a Q-switched Nd: YAG laser to cause instantaneous bleaching of the pigmented tissue. This pretreatment appeared to enhance the susceptibility to conventional PDT without altering the pharmacokinetics of the PS.

## Combination with hyperthermia

Therapeutic hyperthermia is a cancer treatment in which body tissue is exposed to high temperatures (40–45°C). It has been used in combination with radiotherapy and/or chemotherapy for many years, with remarkable success (Rao et al., 2010). Synergistic effects of PDT and hyperthermia in melanoma treatment have also been demonstrated. The photooxidative damage induced by PDT is amplified by free radicals generated by thermal lipid peroxidation. Photodynamic hyperthermal therapy exposes cells to increased temperature (43°C) at the same time as irradiation. This combination induced early apoptosis of murine melanoma B16F10 with a short duration of treatment (Radzi et al., 2011). A synergic effect was also achieved by heating cells after ALPcS PDT treatment (Glassberg et al.



**Figure 5** The photodynamic therapy (PDT) effects of stable synthetic bacteriochlorin on GFP positive B16F10 melanoma tumors. (A) B16F10-GFP [a green fluorescent protein (GFP) expressing subcutaneous tumor] subcutaneous tumor on day 7 before PDT treatment. (B) B16F10-GFP tumor on day 13 after PDT treatment. (C) *In vivo* fluorescence imaging of B16F10-GFP tumor immediately after inoculation. (D) GFP signal from tumor on day 7 before PDT. (E) Two-color imaging of bacteriochlorin 3 (red) and B16F10-GFP tumor (green) 15 min post-IV injection on day 9. (F) Control tumor on day 13. (G) PDT-treated tumor on day 13. (H) Control tumor on day 23. (I) PDT-treated tumor on day 23. (J) Regrowth of a PDT-treated tumor on day 26. (K) Survival analysis of *in vivo* PDT effectiveness with bacteriochlorin 3 on B16F10-GFP tumors; solid line no treatment ( $n=8$ ), dotted line PDT ( $n=10$ );  $p<0.01$  (log rank test). (L) Structure of stable synthetic bacteriochlorin. Reprinted with permission from (Mroz et al., 2010).

1991). A 150% increased selective toxicity and therapeutic ratios were achieved compared to those obtained with PDT alone.

## Immune stimulation strategies

*In situ* photoimmunotherapy is a promising modality for the treatment of metastatic melanoma that combines photodynamic therapy with immunological stimulation (Naylor et al., 2006). A continued local application of topical imiquimod (a toll-like receptor 7 agonist) in combination with indocyanine green PDT treatment increased the 12-month overall survival up to 70% in melanoma patients (Li et al., 2010). *In situ* photoimmunotherapy not only exerted a complete local response but also demonstrated effective immune response against metastatic nodules.

The immune response can also be stimulated by means of intratumoral injection of dendritic cells (DCs),

which combined with local photodynamic therapy induces a striking antitumor effect with potent systemic antitumor immunity (Saji et al., 2006). PDT creates the perfect microenvironment for tumor antigen acquisition and DC activation, alleviating the need to carry out *in vitro* loading of DCs with tumor antigens.

These studies show that these combined treatments induce strong and durable tumor-specific immunity that results in the destruction not only of targeted tumors but also those at distant sites. The potential of this synergy was corroborated using the B16 tumor model, a poorly immunogenic and highly aggressive melanoma with strong and durable tumor-specific results (Saji et al., 2006).

## Conclusion

Although it was thought for many years that melanoma was particularly resistant to PDT, recent insights have

suggested that in fact there actually be feasible approaches to overcome this resistance. The development of highly active PS absorbing in the NIR spectral region (700–900-nm) will allow testing of PDT, even in highly pigmented melanomas. Interventions that can (even temporarily) reduce the amount of or the optical absorption of the interfering melanin may allow better photoactivation of the PS inside the melanoma tumor due to better light penetration. Highly active research efforts into the molecular resistance mechanisms of melanomas to cytotoxic drugs and to targeted therapies may have future applications to PDT as well. For instance, if efflux-pump inhibitors can be developed they may be combined with PSs that are substrates of the efflux pumps. Compounds that overcome overactive kinases and deficiencies in apoptotic pathways may also be combined with PDT. Finally, the realization

that the host immune response can be activated by PDT combined with the known immunogenic potential of melanomas in general may lead to immunotherapy combinations with the hope that even advanced metastatic melanomas could be treated with PDT.

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