



Melanoma Resistance to Photodynamic Therapy: New Insights

Citation

Huang, Ying-Ying, Daniela Vecchio, Pinar Avci, Rui Yin, Maria Garcia-Diaz, and Michael R. Hamblin. 2013. "Melanoma Resistance to Photodynamic Therapy: New Insights." *Biological Chemistry* 394 (2). <https://doi.org/10.1515/hsz-2012-0228>.

Published version

<https://doi.org/10.1515/hsz-2012-0228>

Link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:37933094>

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

<https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFIOWI2M2EwYTg>

Accessibility

<https://accessibility.huit.harvard.edu/digital-accessibility-policy>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#)



Published in final edited form as:

Biol Chem. 2013 February 1; 394(2): 239–250. doi:10.1515/hsz-2012-0228.

Melanoma resistance to photodynamic therapy: new insights

Ying-Ying Huang^{1,2,3}, Daniela Vecchio^{1,2}, Pinar Avci¹, Rui Yin^{1,2,4}, Maria Garcia-Diaz^{1,5,6}, and Michael R. Hamblin^{1,2,7,*}

¹Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA 02114, USA

²Department of Dermatology, Harvard Medical School, Boston, MA 02115, USA

³Department of Pathology, Guangxi Medical University, Nanning, Guangxi, 530027, China

⁴Department of Dermatology, Southwest Hospital, Third Military Medical University, Chongqing, China, 400038

⁵Molecular Engineering Group, IQS School of Engineering, Universitat Ramon Llull, Barcelona, Spain

⁶Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, Barcelona, Spain

⁷Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA 02139, USA

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 for melanoma with some promising results, but melanoma is generally considered to also be resistant to PDT. Optical interference by the highly-pigmented melanin, the anti-oxidant effect of melanin, the sequestration of photosensitizers inside melanosomes, defects in apoptotic pathways, and the efflux of photosensitizers by ATP-binding cassette (ABC) 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 the 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 to the treated tumor.

Keywords

melanoma; photodynamic therapy; resistance mechanisms; melanosomes; photosensitizers; drug efflux systems; depigmentation; anti-tumor immune response

Introduction

Melanoma is the malignancy responsible for the highest incidence of deaths from skin cancer. Genetic and environmental factors such as UV damage can cause the transformation of skin melanocytes into a tumorigenic melanoma (Carlson, Ross *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, Tobin *et al.* 2004). Although the

*Corresponding author: hamblin@helix.mgh.harvard.edu.

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, Coates *et al.* 1993). However, there is disagreement on this point 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, Schoendorff *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, Aziz *et al.* 2009).

PDT and melanoma

Photodynamic therapy is an effective treatment for several different cancers (Agostinis, Berg *et al.* 2011). Its efficacy has been shown in non-melanoma skin cancers and other skin cancers such as lymphoma and in dermatologic disorders like vitiligo and psoriasis (Babilas, Schreml *et al.* 2010). PDT involves systemic or local administration of a photosensitizer, which localizes in the tumor. The photosensitizers are activated by irradiation at a specific wavelength and in presence of oxygen generate short-lived reactive oxygen species (ROS) (Dougherty, Gomer *et al.* 1998). The ROS generated by the photosensitizer are responsible for the selective tumor destruction, tumor-associated vascular damage, and activation of antitumor immune responses (Castano, Mroz *et al.* 2006). This treatment offers many advantages such as a low systemic cumulative toxicity; the selectivity and noninvasiveness of the method; 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 to verify the efficacy of PDT on malignant melanoma compared the effect of hematoporphyrin derivate (photofrin II) on melanotic and amelanotic malignant melanoma in athymic nude mice. This study demonstrated effective effect of PDT on amelanotic cancer but not in melanotic melanoma (Nelson, McCullough *et al.* 1988). The authors concluded that the resistance of malignant melanotic melanoma to PDT was due to the presence of the melanin that 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, competed with PS for photons resulting in inefficient phototoxicity (Nelson, McCullough *et al.* 1988).

Thereafter, subsequent studies were directed to investigate and synthesize new photosensitizers 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, van Best *et al.* 1990; Biolo, Jori *et al.* 1996; Woodburn,

Fan *et al.* 1998). Ten years later since 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, Soncin *et al.* 1999). In 1999 the same group investigated the effect of PDT using a benzoporphyrin derivative monoacid ring A (verteporfin, BPD-MA), 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, Zhuravkin *et al.* 1994). Some years later Sheleg *et al.* (Sheleg, Zhavrid *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 during a year and a half. 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 thus more clinical investigation is necessary.

The PS employed is highly important in PDT, and some PS frequently used in clinical practice are not always effective in melanoma skin cancer. Cordoba *et al.* (Cordoba, Braathen *et al.* 2005) analyzed effects of 5-aminolaevulinic acid (5-ALA), 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 even MT-ret melanoma cells were vulnerable to 5-ALA PDT *in vitro*, malignant MT-ret melanomas *in vivo* were quite resistant to this type of therapy at doses which 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. There remains the need for the development of novel and effective approaches to treat melanoma and photodynamic therapy (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 FDA-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. Wide range of anticancer treatments is ineffective at killing melanoma cells, which implies that the resistance mechanisms in melanoma are complex. Even though melanoma is thought to be uniquely susceptible novel target therapy and immunotherapies, such treatments only succeed to benefit a small subset of patients (Atkins, Lotze *et al.* 1999). However, multidrug resistance still remains a big problem.

Drug resistance mechanisms 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 drug–target interaction by drug transport mechanisms and detoxification or target modulation. Other mechanisms reverse and compensate drug effects before the cell death cascades are initiated. Another drug-resistance mechanism may lie in dysregulation of apoptotic pathways leading to apoptosis deficiency and prevention of cell death (Helmbach, Rossmann *et al.* 2001). The general resistance mechanisms of melanoma will be described first, before analyzing the resistance to PDT.

Melanogenesis-mediated MDR

The general resistance mechanisms for solid tumors can be applied to explain the MDR of melanoma. However, it does 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, which has been implicated in drug trapping and export (Chen, Valencia *et al.* 2009). Chen developed a melanogenesis model theory to better explain how the melanosomes affect the drug sensitivity and is involved in intrinsic multidrug resistance (MDR) in melanoma. Melanogenesis include three major processes: melanosome biogenesis, melanin synthesis, and endogenous melanogenic cytotoxicity related homeostasis. Melanogenesis is involved in the regulation of drug sensitivity. Melanogenesis has three distinct phases depending on the 4 stage of melanosome biogenesis. In phase I melanogenesis, the melanosome termed as “premelanosome”, is at an early stage I and II of melanosome biogenesis without melanin synthesis. These premelanosome possesses the ability to trap and export cytotoxic drugs such as cisplatin (CDDP). In phase II, melanosomes are predominately in stage III of melanosome biogenesis with active melanin synthesis. They possess a maximal capacity to trap cytotoxic drug in the nascent melanin, and thus they are likely to be involved in drug resistance. In phase III, melanosomes could generate endogenous melanogenic cytotoxic byproducts causing an autophagic program on damaged stage IV melanosomes. This in turn causes them to be more susceptible to cytotoxic drugs. At the end of 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 the sun-protection properties. Moreover, tyrosinase-related protein-2 (TYRP2), a melanogenic enzyme, was shown to confer resistance to cisplatin in melanoma cells (Chen, Valencia *et al.* 2009). Figure 2 illustrates the process of melanogenesis and depicts some of the resistance mechanisms specific to melanomas.

ABC transporter mediated MDR

The most common cause of multidrug resistance in human cancers is the expression and function of one or more ATP-binding cassette (ABC) transporters that efflux anticancer drugs from cells (Chen, Valencia *et al.* 2009). ABC transporters (48 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 in melanoma. Moreover, transfected full length of ABCB5 conferred drug resistance to two cell lines (Chen, Szakacs *et al.* 2005) therefore over-expression of ABCB5 protein is likely to be a major mechanism of the MDR of 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, Murphy *et al.* 2008).

Chen *et al.* (Chen, Valencia *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 transporters network in diverse subcellular organelles would constitute a buffer system that prevents nuclear or mitochondrial import of cytotoxic drugs, thereby protecting the cells from endogenous melanogenic cytotoxicity generated by late-stage melanosomes.

DNA repair mechanisms mediated MDR

A mechanism to counteract the deleterious effects of DNA-damaging drugs could be a hyperactivation of DNA repair mechanisms, either by upregulating mismatch repair genes or by potentiating enzymes that remove DNA-alkylation damage (Soengas and Lowe 2003). Drug-resistant melanoma cell lines were demonstrated in fotemustine- and cisplatin-resistant human melanoma cells, exhibiting increased repair of DNA (Runger, Emmert *et al.* 2000). DNA-mismatch repair (MMR) deficiency results in drug resistance by impairing the ability of cells to repair DNA damage (Fink, Aebi *et al.* 1998).

Dysregulation of apoptosis pathway

The molecular mechanism for the drug resistance is poorly understood; however, it has been proposed that defects in the apoptotic pathway may present a critical event in melanoma progression. One of the genes related to tumorigenesis and chemoresistance in melanoma is p53. Although p53 mutation are rare in melanoma, apoptosis protease-activating factor-1 (APAF-1), a critical downstream effector of 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, Santini *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 MAPK pathway inhibitors

The mitogen-activated protein kinase (MAPK) pathway is a key regulator of cell progression and is commonly activated in human tumors through mutation of three-tiered kinase cascade consisting of RAF, MEK (MAPK kinase), and ERK (extracellular signaling regulated kinase). 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 initiated 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. By far, the best validated targeted therapies in melanoma are the BRAF inhibitors (Bollag, Hirth *et al.* 2010). However, response to RAF inhibitors are transient, resistance to the inhibitors develops, and tumors invariably recur. Multiple mechanisms involved in the resistance of BRAF inhibitors. In most instances, reactivation of 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) to continue proliferating (Dummer and Flaherty 2012). Additionally, overexpression of CRAF or the COT can also lead to MAPK reactivation. Alternatively, treatment with BRAF inhibitors could select for minor, preexist NRAS mutants clones which do not respond to BRAF inhibitors but paradoxically hyperactivate the MAPK pathway. Moreover, activation of receptor tyrosine kinases (RTKs), in particular insulin-like growth factor receptor I (IGF-IR) and platelet-derived growth factor receptor beta (PDGFR β) can “bypass” BRAF inhibition by activating parallel PI3K/AKT/mTOR signaling pathway and modulate survival.

Melanoma resistance to PDT

Due to melanoma's intrinsic resistance to radiation and chemotherapeutic drugs, PDT has been suggested as an alternative therapeutic modality, which involved light induced destruction of cells or target tissues through sensitization to light by various photosensitizing

agents; hypericin (Hadjur, Richard *et al.* 1996), benzoporphyrin derivatives (Buseti, Soncin *et al.* 1999) or protoporphyrin IX (PPIX) being just a few examples (Kiesslich, Krammer *et al.* 2006). However, several resistance mechanisms such as; optical interference (Hadjur, Richard *et al.* 1996; Buseti, Soncin *et al.* 1999), anti-oxidant defense mechanisms of melanin (Hadjur, Richard *et al.* 1996; Davids, Kleemann *et al.* 2009), cytoprotective response through induction of autophagy (Davids, Kleemann *et al.* 2009), melanosomes protecting melanocytes and melanoma cells against harmful effects of toxic intermediates (Davids, Kleemann *et al.* 2009) and lastly ABCG2 transporters acting as efflux pumps making cells capable of eliminating toxic amounts of porphyrins (Bebes, Nagy *et al.* 2011) cause reduction in efficacy of PDT on melanoma cells.

The resistance of pigmented melanomas over their unpigmented counterparts, to various therapies including PDT suggests that, presence of melanin plays a role in rendering these cells less susceptible to cell death (K Sharma, Dai *et al.* 2011). One explanation for this phenomenon is that melanin may act as a filter by preventing any in-depth penetration of light, shielding certain cellular targets from light as well as absorbing and scattering therapeutic light (Hadjur, Richard *et al.* 1996). It has been showed in a study that in 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, Sayre *et al.* 1991). Competitive absorbance of melanin at certain wavelengths might also reduce the efficiency of the photosensitization by certain photosensitizing agents such as hypericin (Hadjur, Richard *et al.* 1996). Especially in heavily pigmented melanomas, it is already 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 such as Si (IV)-naphthalocyanine, bacteriochlorin a and Lu (III)-texapyrin, where melanin exhibits a small residual absorbance, thereby minimizing the optical filtering action, melanotic melanoma can be made responsive to PDT (Buseti, Soncin *et al.* 1999).

Melanin has a significant role in cytoprotection by acting as an intracellular antioxidant, decreasing high levels of reactive oxygen species (ROS) by acting as a ROS scavenger (Sharma, Bowers *et al.* 2011). It has been demonstrated that melanocytes with high melanin content were more resistant to ROS than ones containing low melanin (Hadjur, Richard *et al.* 1996). Suzukawa found that melanins were found to bind to the minor grooves of DNA, guaranteeing close proximity to DNA and potentially causing the observed high levels of strand breaks. Moreover, they also found that after melanins interact with $^{1}O_2$, they exhibit a lower ability to induce DNA breakage (Suzukawa, Vieira *et al.* 2012). On the other hand, PDT shows its effect on melanoma cells by inducing photo-oxidative stress and cytotoxicity, where general response of cells is to upregulate 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 (TYR). Sharma *et al.* (Sharma, Bowers *et al.* 2011) recently demonstrated that the 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 TYR inhibitor phenylthiourea (PTU) is used in conjunction with hypericin-mediated PDT (HYP-PDT), melanotic phenotype reduced PTU+HYP+PDT toxicity. Conversely, the inhibition by PTU increased the susceptibility of these melanoma cells to HYP-PDT. In addition to this, when PTU 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 change in thiobarbituric acid-reacting substances (TBAR). In the presence of melanin, it has also been found that TBAR concentration

remained unchanged after HYP-PDT, suggesting that melanin could play a protective role in the hypericin photodamage of membrane lipids (Hadjur, Richard *et al.* 1996).

Melanosomes are membrane-bound organelles found both in melanocytes and melanoma cells. In the process of melanin synthesis, they are responsible for protecting these cells from harmful effects of toxic intermediate products, through compartmentalizing cytotoxic melanin intermediates from spilling into the cytoplasm (Davids, Kleemann *et al.* 2009; K Sharma, Dai *et al.* 2011). It has been assumed that presences of 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.* (Davids, Kleemann *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 cell survival program of cells undergoing environmental stress (Davids, Kleemann *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, δ -aminolevulinic acid (ALA) or its methyl ester (Bebes, Nagy *et al.* 2011). On the other hand, while PPIX and heme (iron-PPIX) are crucial for cellular homeostasis, free uncommitted pools of these molecules are extremely toxic. This leads to tightly control of 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 multidrug resistance of tumors, and its expression has been detected in the basal layer of keratinocytes of the murine and human epidermis (Bebes, Nagy *et al.* 2011). As a double-edged sword, while high-level expression of ABCG2 makes the cells capable of eliminating toxic amounts of porphyrins, it also causes increased resistance to PDT. It has been reported that, low-dose methotrexate enhances ALA-based PDT in skin carcinoma cells (Anand, Honari *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 pre-treatment in combination with Ko-134 significantly and dose-dependently increased the sensitivity of HaCaT keratinocytes to a dose of 1.5J/cm² red light (Bebes, Nagy *et al.* 2011).

Overcoming melanoma resistance to PDT

Many efforts have been devoted to the development of new strategies addressing the challenge of treating melanoma with PDT, 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, Tang *et al.* 2011). Use of near infrared (NIR) absorbing PS, decreasing the melanin levels, as well as the combination with other therapies have shown considerable promise.

Near infrared absorbing PS

In contrast to other skin cancers, melanomas grow aggressively both in both radial and vertical directions, invading through the basement membrane into the deeper layer of the skin. Moreover, its high content of melanin absorbs light over practically entire visible spectral range used for PDT (400–750 nm) (Ma, Nielsen *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 prompted the synthesis of near infrared (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 bacteriochlorin, 5,10,15,20-tetrakis(2-chloro-5-sulfophenyl) bacteriochlorin (TCBSO₃H) showed preferential accumulation in S91 mouse melanoma and long-time tumor growth inhibition (Dabrowski, Urbanska *et al.* 2011). Mroz *et al.* (Mroz, Huang *et al.* 2010) studied a set of three chemically stable bacteriochlorins (BC1, 2, 3) in a panel of pigmented mouse melanoma cell lines, B16 with different levels of pigmentation. All bacteriochlorins localized in melanosomes leading to melanosomal destruction after illumination. Figure 4 shows colocalization studies in pigmented B16F10 cells with probes for mitochondria, lysosomes and melanosomes. The best *in vitro* performing bacteriochlorins (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 20% of cures as shown in Figure 5. Besides bacteriochlorins, different NIR photosensitizers have been proposed for melanoma treatments. 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 of survival (Woodburn, Fan *et al.* 1998). Biolo *et al.* (Biolo, Jori *et al.* 1994) used a liposomal formulation of Si(IV)-naphthalocyanine for induction of tumor necrosis and delay of tumor regrowth in B16 pigmented melanoma, although no tumor selectivity was observed.

Depigmentation strategies

Some studies have pointed out differences in photodynamic susceptibility between pigmented and unpigmented melanoma (Mroz, Huang *et al.* 2010; Sharma, Bowers *et al.* 2011). The optical interference and ROS photoprotection related with the presence of polymeric melanin could be minimized using one of the strategies proposed for depigmentation of melanoma cells. Sharma *et al.* (Sharma, Bowers *et al.* 2011) suggested a new adjuvant using a known tyrosinase inhibitor, phenylthiourea (PTU) for suppression of melanogenesis. Depigmented melanoma cells showed an enhanced death susceptibility to PDT, which approached that of unpigmented melanoma cells. This inhibition of melanin formation is reversible and thus, upon removing PTU, the pigmented cells showed again resistance to PDT-induced cell death. Another approach is the photobleaching of melanin. Ma *et al.* (Ma, Nielsen *et al.* 2007) used violet light (420 nm) to bleach melanin in melanotic tumors and therefore increased their sensitivity to PDT treatments. B16F10 melanomas were then treated with topical application of methyl 5-aminolevulinatate (MAL) and irradiated with red light, resulting in a larger growth inhibition of tumors. Based on the same principle, Bussetti *et al.* (Busetti, Soncin *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 alteration of 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). Its combination with radiotherapy and/or chemotherapy has been performed for many years, with remarkable success (Rao, Deng *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 (PHT) exposes cells to increased temperature (43°C) at the same time as irradiation. This combination induced early

apoptosis of murine melanoma B16F10 with short duration treatment (Radzi, Osaki *et al.* 2011). A synergic effect was also achieved by heating cells after AIPcS PDT treatment (Glassberg, Lewandowski *et al.* 1991). A 150% increased selective toxicity and therapeutic ratios were achieved compared to those obtained with PDT alone.

Immune stimulation strategies

In situ photoimmunotherapy (ISPI) is a promising modality for the treatment of metastatic melanoma that combines photodynamic therapy with immunological stimulation (Naylor, Chen *et al.* 2006). A continued local application of topical imiquimoid (a toll-like receptor 7 agonist) in combination with indocyanine green (ICG) PDT treatment increased the 12-month overall survival up to 70% in melanoma patients (Li, Naylor *et al.* 2010). ISPI not only exert a complete local response but also it was demonstrated the effective immune response against metastatic nodules. The immune response can also be stimulated by means of intratumoral injection of dendritic cells (DC), which combined with local photodynamic therapy induces a striking antitumor effect with potent systemic antitumor immunity (Saji, Song *et al.* 2006). PDT creates the perfect microenvironment for tumor antigen acquisition and DCs activation, alleviating the need to do *in vitro* loading of DCs with tumor antigens. The 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 at distant sites. The potential of this synergy was corroborated using B16 tumor model, a poorly immunogenic and highly aggressive melanoma, with strong and durable tumor-specific results (Saji, Song *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 may 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 or reduce 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 also 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 PS 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.

Acknowledgments

This work was supported by US NIH (R01AI050875). Daniela Vecchio was supported by Fondazione MPS, Italy. Rui Yin was supported by National Natural Science Foundation of China (Grant No: 81172495), Maria Garcia-Diaz was supported by a predoctoral fellowship from the Comissionat per a Universitats i Recerca del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya i del Fons Social Europeu.

References

- Agostinis P, Berg K, et al. Photodynamic therapy of cancer: an update. CA: Cancer J Clin. 2011; 61:250–281. [PubMed: 21617154]
- Aguilar A, Schoendorff C, et al. Epidermotropic metastases from internal carcinomas. Am J Dermatopathol. 1991; 13:452–458. [PubMed: 1951982]

- Anand S, Honari G, et al. Low-dose methotrexate enhances aminolevulinic acid-based photodynamic therapy in skin carcinoma cells *in vitro* and *in vivo*. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2009; 15:3333–3343. [PubMed: 19447864]
- Atkins MB, Lotze MT, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol*. 1999; 17:2105–2116. [PubMed: 10561265]
- Babilas P, Schreml S, et al. Photodynamic therapy in dermatology: state-of-the-art. *Photodermatology, photoimmunology & photomedicine*. 2010; 26:118–132.
- Bebes A, Nagy T, et al. Specific inhibition of the ABCG2 transporter could improve the efficacy of photodynamic therapy. *J Photochem Photobiol B*. 2011; 105:162–166. [PubMed: 21911299]
- Biolo R, Jori G, et al. Photodynamic therapy of B16 pigmented melanoma with liposome-delivered Si(IV)-naphthalocyanine. *Photochem Photobiol*. 1994; 59:362–365. [PubMed: 8016217]
- Biolo R, Jori G, et al. Effect of photosensitizer delivery system and irradiation parameters on the efficiency of photodynamic therapy of B16 pigmented melanoma in mice. *Photochem Photobiol*. 1996; 63:224–228. [PubMed: 8657736]
- Bollag G, Hirth P, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*. 2010; 467:596–599. [PubMed: 20823850]
- Burton RC, Coates MS, et al. An analysis of a melanoma epidemic. *Int J Cancer*. 1993; 55:765–770. [PubMed: 8244573]
- Busetti A, Soncin M, et al. Treatment of malignant melanoma by high-peak-power 1064 nm irradiation followed by photodynamic therapy. *Photochemistry and photobiology*. 1998; 68:377–381. [PubMed: 9747592]
- Busetti A, Soncin M, et al. High efficiency of benzoporphyrin derivative in the photodynamic therapy of pigmented malignant melanoma. *Br J Cancer*. 1999; 79:821–824. [PubMed: 10070875]
- Campioni M, Santini D, et al. Role of Apaf-1, a key regulator of apoptosis, in melanoma progression and chemoresistance. *Experimental dermatology*. 2005; 14:811–818. [PubMed: 16232302]
- Carlson JA, Ross JS, et al. New techniques in dermatopathology that help to diagnose and prognosticate melanoma. *Clin Dermatol*. 2009; 27:75–102. [PubMed: 19095155]
- Castano AP, Mroz P, et al. Photodynamic therapy and anti-tumour immunity. *Nat Rev Cancer*. 2006; 6:535–545. [PubMed: 16794636]
- Chen KG, Szakacs G, et al. Principal expression of two mRNA isoforms (ABCB 5alpha and ABCB 5beta) of the ATP-binding cassette transporter gene ABCB 5 in melanoma cells and melanocytes. *Pigment Cell Res*. 2005; 18:102–112. [PubMed: 15760339]
- Chen KG, Valencia JC, et al. Involvement of ABC transporters in melanogenesis and the development of multidrug resistance of melanoma. *Pigment cell & melanoma research*. 2009; 22:740–749. [PubMed: 19725928]
- Choudhary S, Tang J, et al. Lasers in the treatment of nonmelanoma skin cancer. *Dermatol Surg*. 2011; 37:409–425. [PubMed: 21414072]
- Cordoba F, Braathen LR, et al. 5-aminolaevulinic acid photodynamic therapy in a transgenic mouse model of skin melanoma. *Exp Dermatol*. 2005; 14:429–437. [PubMed: 15885078]
- Dabrowski JM, Urbanska K, et al. Biodistribution and photodynamic efficacy of a water-soluble, stable, halogenated bacteriochlorin against melanoma. *ChemMedChem*. 2011; 6:465–475. [PubMed: 21265022]
- Davids LM, Kleemann B. Combating melanoma: the use of photodynamic therapy as a novel, adjuvant therapeutic tool. *Cancer Treat Rev*. 2011; 37:465–475. [PubMed: 21168280]
- Davids LM, Kleemann B, et al. Melanomas display increased cytoprotection to hypericin-mediated cytotoxicity through the induction of autophagy. *Cell Biol Int*. 2009; 33:1065–1072. [PubMed: 19596456]
- Davids LM, Kleemann B, et al. Hypericin phototoxicity induces different modes of cell death in melanoma and human skin cells. *J Photochem Photobiol B*. 2008; 91:67–76. [PubMed: 18342534]
- Dougherty TJ, Gomer CJ, et al. Photodynamic therapy. *Journal of the National Cancer Institute*. 1998; 90:889–905. [PubMed: 9637138]

- Dummer R, Flaherty KT. Resistance patterns with tyrosine kinase inhibitors in melanoma: new insights. *Current opinion in oncology*. 2012; 24:150–154. [PubMed: 22316627]
- Fink D, Aebi S, et al. The role of DNA mismatch repair in drug resistance. *Clin Cancer Res*. 1998; 4:1–6. [PubMed: 9516945]
- Glassberg E, Lewandowski L, et al. Hyperthermia potentiates the effects of aluminum phthalocyanine tetrasulfonate-mediated photodynamic toxicity in human malignant and normal cell lines. *Lasers Surg Med*. 1991; 11:432–439. [PubMed: 1816478]
- Glusac EJ. The melanoma ‘epidemic’, a dermatopathologist’s perspective. *J Cutan Pathol*. 2011; 38:264–267. [PubMed: 21244462]
- Gupta AK, Ryder JE. Photodynamic therapy and topical aminolevulinic acid: an overview. *Am J Clin Dermatol*. 2003; 4:699–708. [PubMed: 14507231]
- Hadjur C, Richard MJ, et al. Photodynamic effects of hypericin on lipid peroxidation and antioxidant status in melanoma cells. *Photochem Photobiol*. 1996; 64:375–381. [PubMed: 8760577]
- Helmbach H, Rossmann E, et al. Drug-resistance in human melanoma. *Int J Cancer*. 2001; 93:617–622. [PubMed: 11477569]
- Jilaveanu LB, Aziz SA, et al. Chemotherapy and biologic therapies for melanoma: do they work? *Clin Dermatol*. 2009; 27:614–625. [PubMed: 19880049]
- Sharma K, Dai ST, et al. Drug discovery of antimicrobial photosensitizers using animal models. *Current pharmaceutical design*. 2011; 17:1303–1319. [PubMed: 21504410]
- Kiesslich T, Krammer B, et al. Cellular mechanisms and prospective applications of hypericin in photodynamic therapy. *Curr Med Chem*. 2006; 13:2189–2204. [PubMed: 16918348]
- Kollias N, Sayre RM, et al. Photoprotection by melanin. *J Photochem Photobiol B*. 1991; 9:135–160. [PubMed: 1907647]
- Kostenich GA I, Zhuravkin N, et al. Experimental grounds for using chlorin e6 in the photodynamic therapy of malignant tumors. *J Photochem Photobiol B*. 1994; 22:211–217. [PubMed: 8014753]
- Li X, Naylor MF, et al. Clinical effects of in situ photoimmunotherapy on late-stage melanoma patients: a preliminary study. *Cancer Biol Ther*. 2010; 10:1081–1087. [PubMed: 20890121]
- Ma LW, Nielsen KP, et al. A new method for photodynamic therapy of melanotic melanoma -- effects of depigmentation with violet light photodynamic therapy. *J Environ Pathol Toxicol Oncol*. 2007; 26:165–172. [PubMed: 18197832]
- Mroz P, Huang YY, et al. Stable synthetic bacteriochlorins overcome the resistance of melanoma to photodynamic therapy. *Faseb J*. 2010; 24:3160–3170. [PubMed: 20385618]
- Naylor MF, Chen WR, et al. In situ photoimmunotherapy: a tumour-directed treatment for melanoma. *Br J Dermatol*. 2006; 155:1287–1292. [PubMed: 17107404]
- Nelson JS, McCullough JL, et al. Photodynamic therapy of human malignant melanoma xenografts in athymic nude mice. *J Natl Cancer Inst*. 1988; 80:56–60. [PubMed: 2963136]
- Nissan MH, Solit DB. The “SWOT” of BRAF inhibition in melanoma: RAF inhibitors, MEK inhibitors or both? *Curr Oncol Rep*. 2011; 13:479–487. [PubMed: 21997758]
- Radzi R, Osaki T, et al. Photodynamic Hyperthermal Therapy with Indocyanine Green (ICG) induces Apoptosis and Cell Cycle Arrest in B16F10 Murine Melanoma Cells. *J Vet Med Sci*. 2011
- Rao W, Deng ZS, et al. A review of hyperthermia combined with radiotherapy/chemotherapy on malignant tumors. *Crit Rev Biomed Eng*. 2010; 38:101–116. [PubMed: 21175406]
- Runger TM, Emmert S, et al. Alterations of DNA repair in melanoma cell lines resistant to cisplatin, fotemustine, or etoposide. *J Invest Dermatol*. 2000; 114:34–39. [PubMed: 10620112]
- Saji H, Song W, et al. Systemic antitumor effect of intratumoral injection of dendritic cells in combination with local photodynamic therapy. *Clin Cancer Res*. 2006; 12:2568–2574. [PubMed: 16638867]
- Schatton T, Murphy GF, et al. Identification of cells initiating human melanomas. *Nature*. 2008; 451:345–349. [PubMed: 18202660]
- Schuitmaker JJ, van Best JA, et al. Bacteriochlorin a, a new photosensitizer in photodynamic therapy. *In vivo* results. *Invest Ophthalmol Vis Sci*. 1990; 31:1444–1450. [PubMed: 2387678]
- Sharma KV, Bowers N, et al. Photodynamic therapy-induced killing is enhanced in depigmented metastatic melanoma cells. *Cell Biol Int*. 2011; 35:939–944. [PubMed: 21542806]

- Sheleg SV, Zhavrid EA, et al. Photodynamic therapy with chlorin e(6) for skin metastases of melanoma. *Photodermatol Photoimmunol Photomed*. 2004; 20:21–26. [PubMed: 14738529]
- Sidoroff A, Thaler P. Taking treatment decisions in non-melanoma skin cancer--the place for topical photodynamic therapy (PDT). *Photodiagnosis Photodyn Ther*. 2010; 7:24–32. [PubMed: 20230990]
- Slominski A, Tobin DJ, et al. Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol Rev*. 2004; 84:1155–1228. [PubMed: 15383650]
- Soengas MS, Lowe SW. Apoptosis and melanoma chemoresistance. *Oncogene*. 2003; 22:3138–3151. [PubMed: 12789290]
- Suzukawa AA, Vieira A, et al. Novel properties of melanins include promotion of DNA strand breaks, impairment of repair, and reduced ability to damage DNA after quenching of singlet oxygen. *Free Radic Biol Med*. 2012; 52:1945–1953. [PubMed: 22401857]
- Tarhini AA, Agarwala SS. Cutaneous melanoma: available therapy for metastatic disease. *Dermatol Ther*. 2006; 19:19–25. [PubMed: 16405566]
- Woodburn KW, Fan Q, et al. Photodynamic therapy of B16F10 murine melanoma with lutetium texaphyrin. *J Invest Dermatol*. 1998; 110:746–751. [PubMed: 9579539]

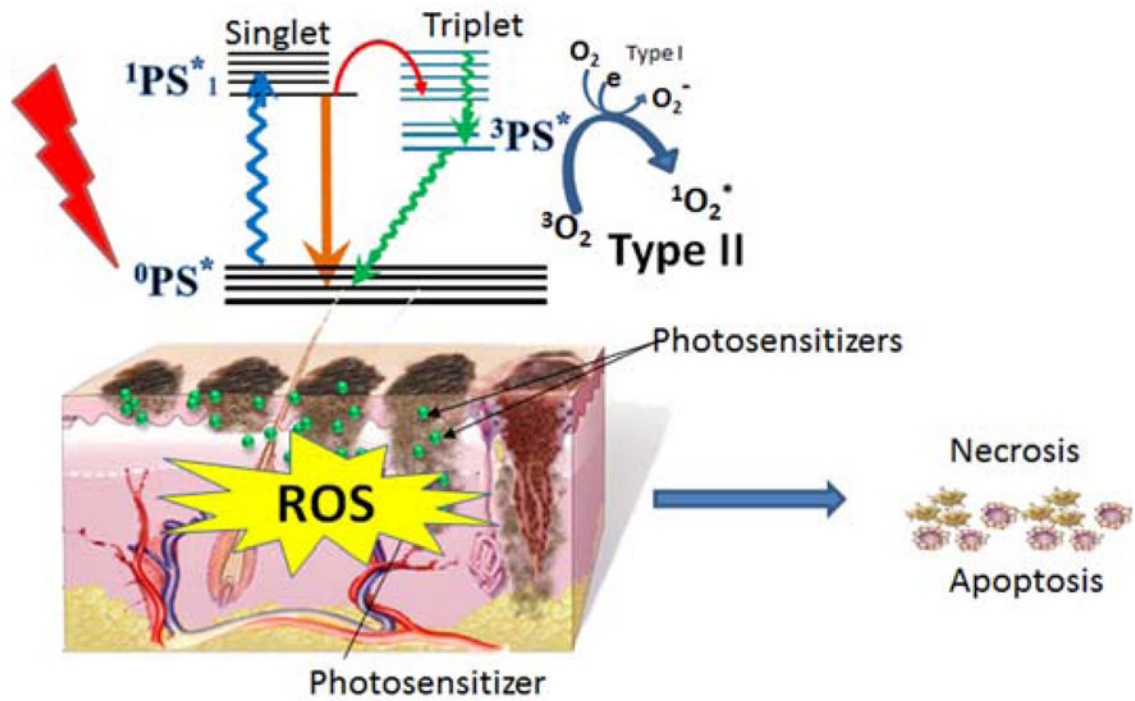


Figure 1. Mechanisms of PDT

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 ROS can cause death of tumor cells by apoptosis or necrosis and destroy the tumor.

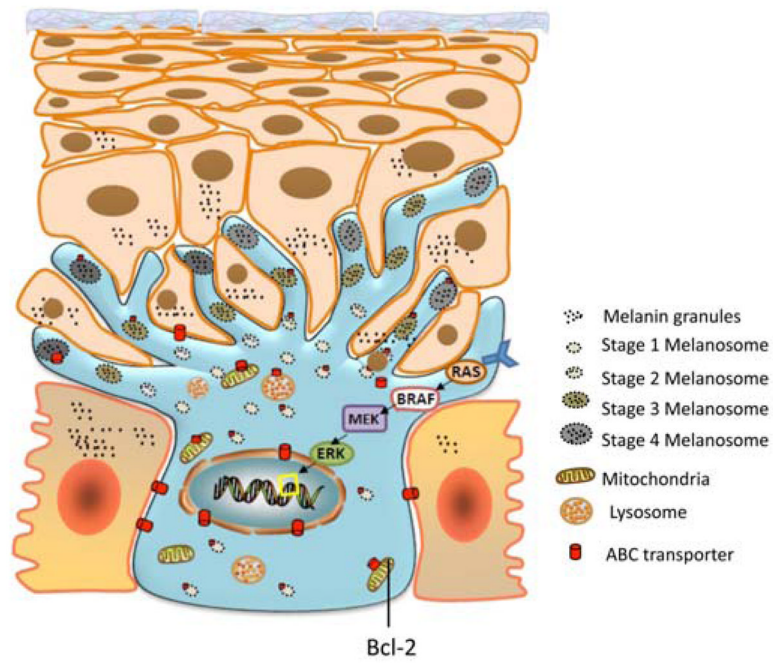


Figure 2. Process of melanogenesis and resistance mechanisms of melanoma

Melanosomes mature through stages 1–4 and are finally transferred to keratinocytes where they release melanin granules. Defects in apoptosis (BCL2), BRAF-mutation activated MAPK/ERK pathway, and ABC-transporter drug efflux pumps contribute to melanoma resistance.

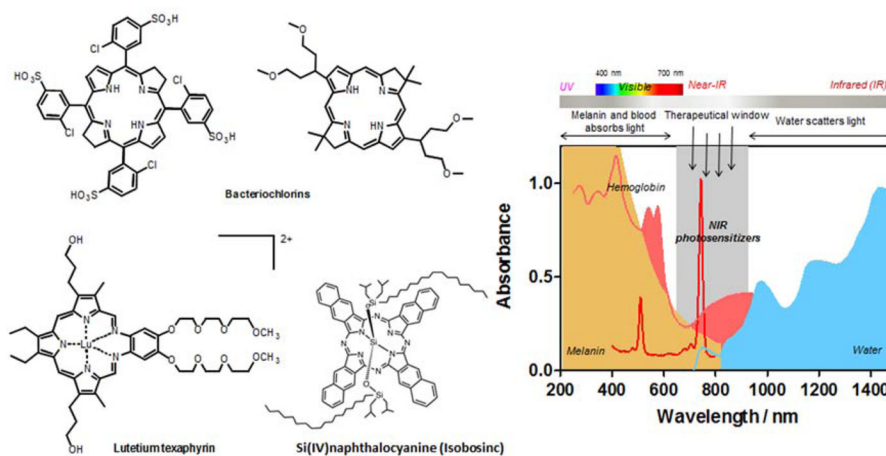


Figure 3. Chemical structures of NIR absorbing photosensitizers and the optical window in tissue

(A) Bacteriochlorin TCBSO₃H from (Dabrowski, Urbanska *et al.* 2011). (B) Bacteriochlorin 3 from (Mroz, Huang *et al.* 2010). (C) Lutetium texaphyrin from (Woodburn, Fan *et al.* 1998). (D) Si(IV)-naphthalocyanine (Isobosinc) from (Biolo, Jori *et al.* 1994).

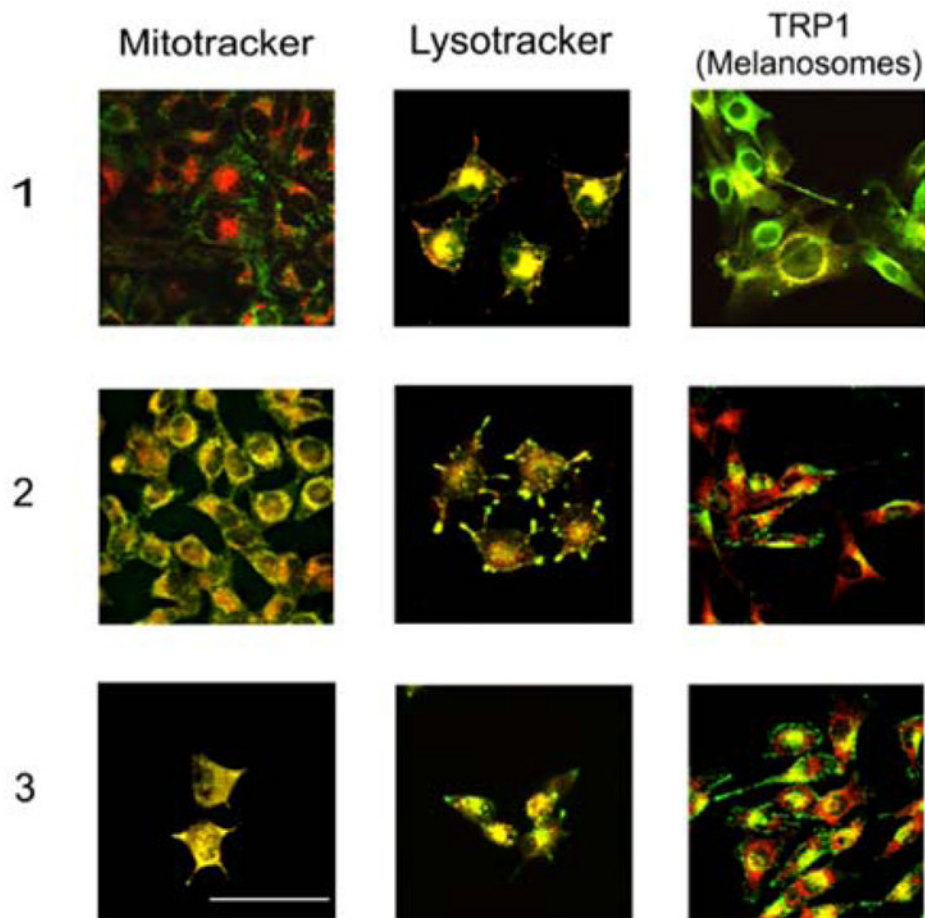


Figure 4. Fluorescence micrographs of B16F10 cells
Showing red fluorescence from BC 1, 2 or 3 overlaid with green fluorescence from lysotracker, mitotracker or FITC-anti-TRP1 antibody that stains melanosomes. Reprinted with permission from (Mroz, Huang *et al.* 2010).

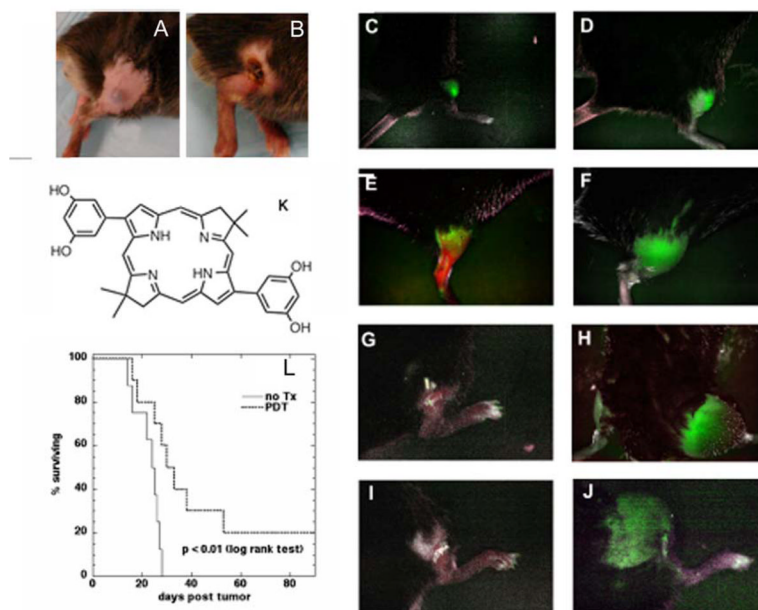


Figure 5. The PDT effects of stable synthetic bacteriochlorin on GFP positive B16F10 melanoma tumors

(A) B16F10-GFP subcutaneous tumor on day 7 before PDT treatment. (B) B16F10-GFP tumor on day 13 after PDT treatment. (C) *In vivo* fluorescence imaging of B16F10-GFP tumor immediately after inoculation. (D) GFP signal from tumor on day 7 before PDT. (E) Two-color imaging of 3 (red) and B16F10-GFP tumor (green) 15 min post IV injection on day 9. (F) Control tumor on day 13. (G) PDT-treated tumor on day 13. (H) Control tumor on day 23. (I) PDT-treated tumor on day 23. (J) Regrowth of a PDT-treated tumor on day 26. (K) Structure of stable synthetic bacteriochlorin. Reprinted with permission from (Mroz, Huang *et al.* 2010).