The Menace of Melanoma: A Photodynamic Approach to Adjunctive Cancer Therapy

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Additional information is available at the end of the chapter

1. Introduction

Metastatic malignant melanoma (MMM) remains one of the most dreaded skin cancers worldwide. Numerous factors contribute to its resistance to hosts of treatment regimes and despite significant scientific advances over the last decade in the field of chemotherapeutics and melanocytic targets, there still remains the need for improved therapeutic modalities. Photodynamic therapy (PDT), a minimally invasive therapeutic modality has been shown to be effective in a number of oncologic and non-oncologic conditions. Using second-generation stable, lipophillic photosensitizers with optimised activation wavelengths, PDT may be a promising tool for adjuvant therapy and even pre-treatment in combating melanoma. Potential targets for PDT in melanoma eradication include cell proliferation inhibition, activation of cell death and reduction in pro-survival autophagy, a decrease in the cellular melanocytic antioxidant system and a disruption in the endogenous multi-drug resistant (MDR) cellular machinery. This chapter highlights the current knowledge with respect to these characteristics and suggests that PDT be considered as a good candidate for adjuvant treatment in post-resected malignant metastatic melanoma. Furthermore, it suggests that primary consideration must be given to organelle-specific destruction in melanoma specifically targeting the melanosomes – the one organelle that is specific to cells of the melanocytic lineage that houses the toxic compound, melanin. We believe that using this combined knowledge may eventually lead to an effective therapeutic tool to combat this highly intractable disease.

1.1. Melanoma clinical statistics

Melanoma accounts for 4% of all dermatologic cancers but remains responsible for 80% of deaths from skin cancer with the average patient diagnosed with disseminated metastases

surviving for an average of 5 years (Cancer facts and figures, 2003, Atlanta, American Cancer Society, 2003]. According to the World Health Organization (WHO) melanoma skin cancer has been increasing over the past decades with a global estimation of 132 000 melanoma-related skin cancers reported to occur each year. Over the past 50 years, melanoma incidence has risen by 3–8% per year in most people of European background, with the greatest increases in elderly men [1]. In Europe, the current estimates at 15-20 per 100 000 people predominating in the 20-35 year old age group in Caucasians [2]. South Africa, next to Australia, has one of the highest incidences of malignant melanoma in the world. Reliable statistics for South Africa are lacking, however currently an estimate figure for the South African Cape region is 69 new cases per year per population of 100 000 Caucasians (Australia is 65 per 100 000). This means that 1 in 1429 people will develop malignant melanoma. The age-standardised incidence of melanoma was 27.2 per 100 000 for males and 22.2 per 100 000 for females from 1990-1999 but this increased to 36.9 for males and 33.5 per 100 000 for females (2000-2003) (CANSA association of South Africa www.melanoma.co.za/D_doccnr_MFS.asp) (Table 1).

	Age-standardised incidence(10^5 /yr)	Lifetime risk (incidence)	Incidence trend over 10 years	Mortality trend over 10 years	Most common cancer (ranking)
Australia (2001)					
Men	41.4 (world)	1 in 25	22% increase	2% increase	4th
Women	31.1 (world)	1 in 35	12% increase	0% increase	3rd
South Africa (2000)					
Men	36.9 (world)	1 in 29	33% increase	1.5% increase	4th
Women	33.5 (world)	1 in 40	27% increase	1% increase	3rd
USA (2001)					
Men	21.4 (world)	1 in 53	31% increase	0% increase	5th
Women	13.8 (world)	1 in 78	25% increase	1% decrease	7th
UK (2000)					
Men	9.7 (world)	1 in 147	59% increase	20% increase	12th
Women	11.2 (world)	1 in 117	41% incraese	3% increase	7th

Table 1. Melanoma statistics in 2 southern and northern hemisphere countries

Despite extensive research and clinical trials, the prognosis and survival of metastatic mela-noma remains dismal. Early detection of localized melanoma may be cured through surgery however there is no therapy for metastatic melanoma or melanoma with metastatic poten-tial. In addition, recurrence rates of resected melanoma remain high. Because melanoma is inherently resistant to traditional forms of chemotherapy and radiotherapy [3], various strat-egies have been developed for treatments which include immunotherapy eg. interleukin-2 (IL-2) [4], radiotherapy [5] biochemotherapy [6-9] and gene therapy [4,10]. A limited number of these therapies have progressed to human clinical trials but their outcomes remain negli-gible. One promising therapy is high-dose interferon (IFN) alpha-2b therapy which has just recently been approved as the only adjuvant therapy for melanoma approved by the US Food and Drug Administration [11]. The other is the use of BRAF kinase inhibitors such as vemurafenib [12]. Despite convincing evidence of improved disease-free survival associated with this therapy, the overall survival remains negligible or very small [13-15]. In addition, a number of melanoma-specific and melanoma-associated tumor antigens such as gp100, MART-1 and MAGE3 have been cloned [16] and the hope is that these potential antigens may be developed to stimulate tumor-specific T cells to eliminate melanoma cells [17]. De-spite these advances, there remains the need for the development of novel and effective ap-proaches to treat melanoma and this review explores the possibility of using photodynamic therapy (PDT) as an adjunctive therapy alone or in combination with current therapeutics to combat melanoma.

1.2. Melanoma origins

Melanoma represents the malignant phenotype of a skin melanocyte. Melanoma occurs most frequently after intermittent exposure to UV radiation and in people with chronic sun-burns. Epidemiologic data suggest that chronic or low-grade exposures to UV induce pro-tection against DNA damage, whereas acute, intense UV exposure leads to DNA damage and concomitant genetic alterations in the melanocyte genome [18]. It develops as a result of accumulated abnormalities in genetic pathways within the melanocyte which give way to increased cell proliferation and prevent normal pathways of apoptosis in response to DNA damage. Furthermore, this damage results in the selection for genetic mutations that allow all aspects of the malignant phenotype, including stimulation of blood vessel growth, eva-sion of the immune response, tumour invasion, and metastasis [19]. Although the mecha-nisms of differential cancer cell killing are poorly understood [20], selection of cells that are resistant to apoptotic mechanisms might contribute to the resistance of melanoma cells to the cytotoxic effects of chemotherapy, radiotherapy, and immunotherapy, especially through the expression of apoptosis inhibitors such as B-cell lymphoma derived protein 2 (Bcl-2) and BclxL [21].

Melanocytes progress through a series of steps toward malignant transformation by the ac-quisition of various phenotypic features. The particular histological features characterising each step of progression are the visible manifestations of underlying genetic changes [22].

Originating from a benign nevus, melanocytes undergo aberrant growth within the lesion subsequently displaying irregular borders, a change in colour and often an associated allergic response. At this stage the lesion is considered dysplastic. At a molecular level, these changes are associated with abnormal activation of the mitogen-activated protein kinase (MAPK) signalling pathway resulting in somatic mutations in the N-RAS and BRAF genes which are associated with about 15 and 50% of melanomas, respectively [23,24]. There is complementarity between the presence of NRAS and BRAF mutations in any individual melanoma since each has the same effect of causing unrestrained cell proliferation.

In addition, mutations in both the cyclin-dependent kinase inhibitor 2A (CDKN2A) and the phosphatase and tensin homologue (PTEN) gene increases the probability of dysplastic naevi becoming malignant [25]. This genetic locus is frequently targeted for disruption in melanomas [26]. When defective, p16 is unable to inactivate CDK4 and CDK6, which phosphorylate Rb, releasing the transcription factor E2F and leading to cell cycle progression [27].The molecule that is usually central to protection against DNA damage, p53, is rarely mutated early in melanoma, which is possibly one of several adaptations to permit survival of cells responsible for generating sun-protective pigment, melanin [28]. Interestingly, by-products of melanin biosynthesis can themselves cause oxidative stress and contribute to malignant change.

Further progression of melanoma is associated with decreased differentiation and clonal proliferation leading to the radial growth phase (RGP). Clinically, RGP presents as patches or plaques which can measure up to 2.5cm. Superficial spreading melanoma lesions are slightly raised and show striking variations of red, blue, white, brown, and black coloration. In RGP, melanoma mitoses are frequently seen in the epidermis but rarely in the dermis. After complete surgical excision of the tumor, RGP melanomas are usually associated with longterm metastasis-free survival [29-33]. RGP cells can progress to vertical growth phase (VGP) cells which breach the basement membrane and invade the dermis as nodules or nests of cells. Vertical growth phase (VGP) melanomas usually present as gray-black, blue-black, or even amelanotic nodules. In late or developed VGP, melanomas form expansile nodules in the dermis with cytology different from melanoma cells in the overlying epidermis. Mitotic figures are variably present, and tumor aggregates may extend into the reticular dermis or even subcutaneous fat. Dermal tumoral nests are larger in VGP than in RGP. Moreover, these cells are considered to have metastatic potential. Interestingly, not all melanomas pass through each of these individual phases – RGP and VGP can both develop directly from melanocytes or naevi and both can progress directly to metastatic malignant melanoma [34]. Moreover, the transition from RGP to VGP in cutaneous melanoma isassociated with the loss of c-KIT expression and the gain of the melanoma cell adhesion molecule (MCAM/MUC18) [35].

Increased proliferation and survival, chemoresistance, the ability to resist apoptosis, the induction of autophagy and the presence of the pigment melanin have all been listed as rea-

sons contributing to the high mortality rates associated with cutaneous melanoma. Each of these topics will be dealt with in the context of targeting them with PDT.

1.3. Photodynamic Therapy (PDT) as a cancer treatment

PDT is a minimally invasive therapeutic modality which has been shown to be effective in several types of cancer including non-melanoma skin cancer (NMSC) and other skin tumors such as lymphoma as well as non-oncological conditions such as psoriasis vulgaris, acne vulgaris and human papilloma virus-induced skin disease [36,37]. The basis of PDT is the systemic or topical application and preferential uptake of a photosensitizer (PS). The PS is then activated at a specific wavelength of light and in the presence of oxygen, produces reactive oxygen species (ROS). The accumulative presence of these cytotoxic photoproducts start a cascade of molecular and biochemical events resulting in cell death via apoptotic or necrotic mechanisms [38,39].

The main advantage of PDT over conventional cancer treatments are i) it has a very low systemic cumulative toxicity allowing repeated dosing, ii) its ability to destroy tumors selectively (this seems to be related to the lipophillic nature of photosensitizers). Due to this selectivity, damage to normal surrounding cells is minimal. Finally, iii) PDT can be applied alone or in combination as an adjuvant therapeutic modality with chemotherapy, surgery, radiotherapy and immunotherapy [40,41]. These properties have led to PDT receiving increased support from preclinical research [42,43]. PDT requires three elements to be efficacious - a good PS, a coherent light source and the presence of molecular oxygen. A large amount of data with regard to these three elements over the last few years have resulted in the development of more naturally-derived, efficacious, second-generation photosensitizers.

1.3.1. Photosensitizers and melanoma-PDT

Photosensitizers are critical to the successful eradication of malignant cells and numerous first and second-generation photosensitizers have been tested both clinically (in vivo) and in vitro over the past years (for a detailed summary of melanoma-PDT research see Table 2). The structure of many PS is based on the tetrapyrrol ring eg. protoporphyrin IX, Photofrin and chlorines related to it eg. phthalocyanines. Newer, more stable second-generation PS include natural hydroxyquinone chromophores such as hypericins and porphycenes [44-47]. It is now accepted that a good PS for PDT is – i) chemically pure with good stability, ii) preferentially accumulated and retained by target tissue, iii) minimal toxicity in the absence of light with maximal efficacy upon activation, iv) high quantum yield of $^1O_2$ with an associated high molecular extinction coefficient [40]. Due to these properties, a number of synthetic or natural compounds have thus far been studied for a variety of cancers however these have been limited to porphycenes (structural isomers of porphyrins) such as aminolevulinic acid (ALA, trade name, Levulan®) and methylaminolevulinic acid (MAL, trade name, Metvix®) for the treatment of squamous cell (SCC) and basal cell carcinomas (BCC) as well as actinic keratoses [48-50] (Table 2).

Type of study	Tumour/Cell line	Photosensitizer	Ref
in vitro	B16-F10 melanoma cells	magnetoliposomes (MLs) loaded with zinc phthalocyanine (ZnPc) complexed with cucurbituril (CB) (CB:ZnPc-MLs)	[51]
in vivo	mice carrying B16-F10 melanoma xenografts	butadiyne-linked conjugated porphyrin dimer (Oxdime)	[52]
in vivo	subcutaneous amelanotic melanoma transplanted in C57/BL6 mice	pheophorbide a (Pba) and monomethoxy-polyethylene glycol-Pba	[53]
in vitro	A375, UCT Mel-1 human melanoma cells	hypericin and kojic acid (depigmenting agent)	[54]
in vitro/ in vivo	B16F10 mouse melanoma cells and lung melanomas in C57BL/6 mice	aminolevulic acid, gaussia luciferase, and its' substrate coelenterazine; murine neural stem cells (NSCs) and rat umbilical cord matrix-derived stem cells (RUCMSCs) with a plasmid expressing gaussia luciferase	[55]
in vitro	C32 human melanoma cells	Ficus carica L. cultivar Dottato extracts	[56]
in vitro	Melanoma, keratinocyte and fibroblast cells	aluminum tetrasulfophthalocyanines	[57]
in vivo	B16-F1 and Cloudman S9 melanoma-bearing mice	chlorin e(6) and modular nanotransporters targeted to α-melanocyte-stimulating hormone (αMSH) and epidermal growth factor (EGF) receptor	[58]
in vivo	malignant melanoma mouse model	methylene blue	[59]
in vitro	A549 and S91 melanoma cells	halogenated sulfonamide bacteriochlorins	[60]
in vitro	A375 melanoma cells	carotenoids (neoxanthin, fucoxanthin and siphonaxanthin)	[61]
in vitro	melanoma cells	2 cationic octanuclear metalla-cubes dual photosensitizers and chemotherapeutics	[62]
in vitro	A375 melanoma cells	Cachrys pungens Jan extracts from Italy	[63]
in vitro	B16F10 murine melanoma	indocyanine green (ICG) and hyperthermia	[64]
in vitro	B78-H1 murine melanoma cells	pheophorbide a	[65]
in vitro	S91 Cloudman melanoma cells and DBA mice	synthetic chlorin derivative (TCPCSO₃H)	[66]
in vitro/ in vivo	Melanoma cells and xenograft melanoma model	cis-Dichlorobis [3,4,7,8-tetramethyl-1,10-phenanthroline) rhodium(III) chloride (OCTBP)	[67]
in vitro	M21 human melanoma cells	Hedyotis corymbosa extracts	[68]
in vitro	A375, UCT Mel-1 human melanoma cells	hypericin and phenylthiourea (depigmenting agent)	[69]
in vitro	melanoma, keratinocyte and fibroblast cells	zinc tetrasulfophthalocyanines (ZnTSPc)	[70]

Type of study	Tumour/Cell line	Photosensizer	Ref
in vitro	melanoma cells	PDT and Lycopene, β-carotene, vitamin C, N-acetylcysteine, trolox, N-tert-butyl-α-phenylnitrone and HO-1 activity inhibitor zinc protoporphyrine IX (ZnPPIX)	[71]
in vivo	mice bearing mouse melanomas	verteporfin	[72]
in vitro/ in vivo	S91 mouse melanoma cells and DBA mice	5,10,15,20-tetrakis[2-chloro-5-sulfophenyl)bacteriochlorin (TCPBSO3H)	[66]
in vitro	WM451LU melanoma cells	photosensitizers and heme oxygenase I (HO-I) and poly(ADP-ribose) polymerase (PARP) inhibitors	[73]
in vitro	A375 melanoma cells	5-aminolevulinic acid [5-ALA) and novel metallophthalocyanine (MPc)	[74]
in vivo	transplanted B16 melanoma	novel derivatives of chlorin e[6]	[75]
in vitro/ in vivo	melanoma bearing mice	C(60)-(Glc)1 (D-glucose residue pendant fullerene) and C(60)-(6Glc)1 (a maltohexaose residue pendant fullerene)	[76]
in vitro	SK-MEL-188 (human melanoma) cells	chlorin and bacteriochlorin derivatives of 5,10,15,20-tetrakis[2-chloro-5-sulfophenyl)porphyrin	[77]
in vitro	B16 melanoma cells	IPL and IPL plus 5-ALA	[78]
in vitro	melanoma cells	bacteriochlorins and photofrin	[79]
in vitro	C57 mice bearing a sub-cutaneously transplanted melanoma	Zn(II)-phthalocyanine disulphide (C11Pc)	[80]
in vitro	WM 1552C human melanoma cells	liposomes (LP) and nanocapsules (NC) containing Chloroaluminum phthalocyanine (ClAlPc)	[81]
in vitro	A375 melanoma cells	5,15-Diarylporphyrins (1-5) and Photofrin	[82]
in vivo	B16 melanoma tumours on mice	2 doses of photosensitizer	[83]
in vitro	B16 mouse melanoma cells	chlordiazepoxide (CDZ)	[84]
in vitro/ in vivo	B78H1 amelanotic mouse melanoma cells and C57BL/6 mice bearing a subcutaneously transplanted B78H1 amelanotic melanoma.	octabutoxy-naphthalocyanines	[85]
in vitro	G361 human melanoma cells	zinc-5,10,15,20-tetrakis(4-sulphonatophenyl) porphyrine (ZnTPPS(4)), chloraluminium phtalocyanine disulfonate (ClAlPcS(2)) and 5-aminolevulinic acid (ALA)	[86]
in vitro	A375 human melanoma cells B16F10 mouse melanoma cells	Pc4 encapsulated in silica nanoparticles	[87]

Type of study	Tumour/Cell line	Photosensitizer	Ref
in vitro	Sk-Mel-28 human skin melanoma cells	indocyanine green	[88]
in vitro	UCT Mel-1 and A375 human melanoma cells	hypericin	[89]
in vitro	G361 human melanoma cells	chloroaluminum phthalocyanine (ClAlPc) and ultrasound	[90]
in vitro	A375 human melanoma cells	5-aminolevulinic acid (ALA)	[91]
in vitro/ in vivo	B16F1 mouse melanoma cells and C57BL6 mice bearing a subcutaneously injected B16F1 melanoma.	methylene blue	[92]
in vitro/ in vivo	B16F1 mouse melanoma cells and C57BL6 mice bearing a subcutaneously injected B16F1 melanoma.	carboranyl-containing chlorin (TPFC)	[93]
in vitro/ in vivo	human malignant melanoma cells (MMCs)	porfimer sodium	[94]
in vivo	B57BL/6 mice bearing a B16BL6 melanoma	porfimer sodium and antibodies neutralizing decay-accelerating factor (DAF), complement-receptor-1-related protein y (Crry), and protectin	[95]
in vitro	G361 human melanoma cells	porphyrines (TPPS4, ZnTPPS4 and PdTPPS4	[96]
in vitro/ in vivo	B-16 mouse melanoma cells and subcutaneous B-16 melanoma-bearing C57BL/6 mice	5,10,15,20-tetraphenylporphin-loaded PEG-PE micelles	[97]
in vitro	UCT Mel-1 and UCT Mel-3 human melanoma cells	hypericin	[98]
in vitro	Me300 human melanoma cells	Five 5,10,15,20-tetra[4-pyridyl)porphyrin (TPP) areneruthenium(II) derivatives and a p-cymeneosmium and two pentamethylcyclopentadienyliridium and -rhodium analogues	[99]
in vitro	B16F10 melanotic melanomas transplanted to nude mice	methyl 5-aminolevulinate (MAL) and depigmentation with violet light	[100]
in vitro	S-91 mouse melanoma cells	titanium dioxide modified with platinum(IV) chloride complexes ($TiO_2/PtCl_4$)	[101]
in vitro	G361 human melanoma cells	zinc-5,10,15,20-tetrakis(4-sulphonatophenyl) porphyrine (ZnTPPS4) and atomic force microscopy	[102]
in vitro	WM451Lu metastatic human melanoma cells	5-aminolevulinic acid (ALA)	[103]
in vitro	B16F1 mouse melanoma cells	meso-tetra[4-nido-carboranylphenyl)porphyrin (H2TCP)	[104]

Type of study	Tumour/Cell line	Photosensitizer	Ref
in vitro	B16F10 mouse melanoma cells	solketal-substituted phthalocyanine (Si(sol)2Pc in mPEG-b-p(HPMAm-Lac2) micelles	[105]
in vitro	B19 mouse and G361 human melanoma cells	phthalocyanine ClAlPcS(2)	[106]
in vitro	S91 mouse and SKMEL 188 human melanoma cells	5,10,15,20-tetrakis[2-chloro-3-sulfophenyl)porphyrin (TCPPSO(3)H),	[107]
in vivo	choroidal melanomas in 46 New Zealand albino rabbit eyes	hematoporphyrin monomethyl ether	[108]
in vitro	G361 human melanoma cells	3 porphyrin sensitizers (TPPS(4), ZnTPPS[4] and PdTPPS(4))	[109]
in vitro	YUSAC2/T34A-C4 human melanoma cell line	porfimer sodium	[110]
in vivo	subcutaneous B16BL6 melanoma-bearing C57BL/6 mice	BPD, ce6, Photofrin, and mTHPC and gamma-inulin	[111]
in vitro	A375 human melanoma cells	acridine orange	[112]
in vitro	Cloudman S91/I3 mouse melanoma cells	photofrin II (PfII- porfirmer sodium), verteporfin, and merocyanine 540 (MC540)	[113]
in vivo	A-Mel-3 melanomas implanted in the dorsal skin fold chamber of Syrian Golden hamsters	5-aminolaevulinic acid (ALA)	[114]
in vivo	B-16 melanoma-bearing C57BL/6 mice	ATX-S10 No (II) and intratumoral injection of naïve dendritic cells (IT-DC)	[115]
in vitro/ in vivo	B16F1 mouse melanoma cells and C57BL6 mice bearing a subcutaneously injected B16F1 melanoma.	Zn(ii)-phthalocyanine derivative bearing four 10B-enriched o-carboranyl units [10B-ZnB4Pc]	[116]
in vitro	human Beidegröm Melanoma (BM) cell line	porfirmer sodium (photofrin II)	[117]
in vitro	G361 human melanoma cells	ZnTPPS(4) sensitizer bound to cyclodextrin hpbetaCD	[118].
in vitro	B78H1 mouse melanoma cells	Ni(II)-octabutoxy-naphthalocyanine (NiNc)	[119]
in vitro	G361 human melanoma cells	ZnTPPS(4) sensitizer bound to cyclodextrin hpbetaCD	[120]
in vitro	M2R mouse melanoma cells	O-[Pd-bacteriochlorophyllide]-serine methyl ester (Pd-Bchl-Ser)	[121]
in vitro	B78H1 melanoma cells	liposome-delivered Ni(II)-octabutoxy-naphthalocyanine	[119]
in vitro	human choroidal melanoma (CM) cells	tetrahydroporphyrin tetratosylat (THPTS)	[122]

Type of study	Tumour/Cell line	Photosensitizer	Ref
in vivo	M2R mouse melanoma xenografts	WST11	[123]
in vitro	B16 mouse melanoma cells	5-aminolevulinic acid [5-ALA) ester derivatives	[124]
in vivo	B-16 melanoma-bearing C57BL/6 mice	metal-free sulfonated phthalocyanine (H(2)PcS(2.4))	[125]
in vitro	A375 human melanoma cells	alpha-methylene-gamma-butyrolactone-psoralen heterodimer 2	[126]
in vitro	B16 mouse melanoma cells	5-aminolevulinic acid (ALA)	[127]
in vivo	C57BL6 mice bearing a subcutaneously injected B16F10 melanoma	silkworm excreta (SPbalpha) porfirmer sodium	[128]
in vitro	M3Dau human melanoma cells	silicon-phthalocyanines (SiPc) and chloro-aluminium Pc (ClAlPc),	[129]
in vitro	Me45 human melanoma cells	meso-tetra-4-N-methylpyridyl-porphyrin iodide and 5,10-di-[4-acetamidophenyl)-15,20-di-[4-N-methylpyridyl) porphyrin	[130]
in vitro	G361 human melanoma cells	meso-tetrakis[4-sulphonatophenyl)porphine (TPPS4) and zinc metallocomplex (ZnTPPS4)	[131]
in vitro	G361 human melanoma cells	ATX-S10(Na)	[132]
in vivo	syrian Golden hamsters fitted with dorsal skinfold chambers containing A-Mel-3 melanoma cells	5-aminolaevulinic acid (ALA)	[133]
in vitro	B16 mouse melanoma cells	5-aminolaevulinic acid (ALA)	[134]
in vivo	UB900518 human melanoma cells transplanted on nude (nu/nu) CD-1 mice	Liposomal meso-tetrakis-phenylporphyrin (TPP)	[135]
in vivo	pigmented choroidal melanoma 44 New Zealand albino rabbit eyes	liposomal preparation of benzoporphyrin derivative (BPD), verteporfin	[136]
in vitro	S91 mouse and SKMEL 188 human melanoma cells	indocyanine green (ICG)	[137]
in vitro	B16A45 (B16) mouse melanoma cells	delta-aminolevulinic acid (ALA) and meta(tetrahydroxyphenyl)chlorin or m-THPC	[138]
in vitro	B16 mouse melanoma cells	m-THPC and four apoptosis inhibitors: BAPTA-AM, Forskolin, DSF, and Z.VAD.fmk	[47]
in vitro	Bro, SKMel-23, SKMel-28	5-aminolevulinic acid (ALA)	[139]
In vitro	SKMEL 188 human melanoma cells	tritolylporphyrin dimer (T-D).	[140]
in vivo	Syrian Golden hamsters fitted with dorsal skinfold chambers containing A-Mel-3 melanoma cells	5-aminolaevulinic acid (ALA)	[141]
in vivo	Nude CD1 mice bearing malignant M2R melanoma xenografts	bacteriochlorophyll-serine (Bchl-Ser),	[142]

Type of study	Tumour/Cell line	Photosensitizer	Ref
in vitro	SK-23 mouse melanoma and SK-Mel 28 human melanoma	methylene blue	[143]
in vitro	B78H1 melanoma cells	liposome-incorporated Ni(II)-octabutoxy-naphthalocyanine (NiNc),	[144]
in vitro	B78H1 melanoma cells	Cu(II)-hematoporphyrin (CuHp)	[145]
in vivo	Syrian Golden hamsters fitted with dorsal skinfold chambers containing A-Mel-3 melanoma cells	5-aminolaevulinic acid (ALA)	[146]
in vitro	M6 human melanoma cells	dichlorosilicon phthalocyanine (Cl2SiPc) and bis(tri-n-hexylsiloxy) silicon phthalocyanine (HexSiPc)	[147]
in vivo	C57/BL6 mice bearing a subcutaneously transplanted B1 melanoma	benzoporphyrin derivative monoacid ring A (verteporfin, BPD-MA)	[148]
in vitro	SkMel-23 melanoma cells	5-aminolaevulinic acid (ALA)	[149]
in vivo	C57/BL6 mice bearing a subcutaneously transplanted B16 melanoma	Si(i.v.)-naphthalocyanine (isoBO-SiNc)	[150]
in vivo	C57/BL6 mice bearing a subcutaneously transplanted B16 melanoma	aluminum phthalocyanine (AlpcS4	[151]
in vivo	C57/BL6 mice bearing a subcutaneously transplanted B16F10 melanoma	lutetium texaphyrin (PCI-0123),	[152]
in vivo	Syrian Golden hamsters fitted with dorsal skinfold chambers containing A-Mel-3 melanoma cells	9-acetoxy-2,7,12,17-tetrakis-(beta-methoxyethyl)-porphycene (ATMPn)	[153].
in vivo	C57/BL6 mice bearing a subcutaneously transplanted B16 melanoma	Si(IV)-methoxyethylene-glycol-naphthalocyanine	[154]
in vivo	Syrian Golden hamsters fitted with dorsal skinfold chambers containing A-Mel-3 melanoma cells	5-aminolaevulinic acid (ALA)	[155]
in vivo	10 choroidal melanomas in rabbits	liposomal preparation of benzoporphyrin derivative	[156]
in vivo	M2R mouse melanoma tumors implanted in CD1 nude mice	bacteriochlorophyll-serine (Bchl-Ser),	[157]
in vitro	uveal melanoma cells	hematoporphyrin esters (HPE)	[158]
in vivo	Syrian Golden hamsters fitted with dorsal skinfold chambers containing A-Mel-3 melanoma cells	5-aminolaevulinic acid (ALA)	[159]
in vivo	C57/BL6 mice bearing a subcutaneously transplanted B16 melanoma	Zn(II)-2,3 naphthalocyanine (ZnNc)	[160]

Type of study	Tumour/Cell line	Photosensitizer	Ref
in vivo	32 choroidal tumours in New Zealand albino rabbit eyes.	benzoporphyrin derivative	[161]
in vitro	G361,M18 and M6 human melanoma cells	hypericin	[162]
in vitro	melanoma cell lines	hypericin	[163]

Table 2. Comprehensive update of in vivo and in vitro photodynamic therapy studies from 1996-present.

For melanoma treatment, where PDT will be more effective as a post-operative adjunctive treatment, very few reports highlight its effectiveness even though laboratory studies using melanoma cells show promise. Clinically, PDT has shown promise in the treatment of both ocular amelanotic melanomas [164] and skin metastases [165] however, more extensive clinical studies need to be conducted before PDT is accepted as the adjunctive therapy of choice [166] (Table 3).

Type of study	Tumour	Pigmentary phenotype	Photosensitizer	Outcome of study	Ref
clinical	choroidal melanoma	unpigmented	verteporfin	Dramatic tumor regression over 2 months to a completely flat scar [1.3 mm thickness), and remained stable at 50 months of follow-up.	[167]
clinical	3 choroidal melanomas	pigmented	PDT and intravitreal bevacizumab	The tumors treated with PDT and bevacizumab showed a marked reduction in tumor vascularity. The tumors receiving PDT as a primary treatment were followed by progressive tumor growth that led to enucleation years after.	[168]
clinical	9 posteriorly located choroidal melanomas	unpigmented	verteporfin	Eight tumors demonstrated apparent complete regression over 1 month to 14 months with no recurrence during follow-up of between 34 months and 81 months. One case developed 2 separate local recurrences at 21 months and 34 months.	[169]
clinical	11 late stage melanomas (cutaneous metastases)	ND	indocyanine green and imiquimod (immune modifier)	Complete response was observed in 6 patients. All lesions in the treatment area of the patients responded to photo-immunotherapy, 8 of which achieved complete local response (CLR). CLR was observed in the non-treatment site (regional) lesions in four patients. Five patients were still alive at the time of last follow-up.	[170]

Type of study	Tumour	Pigmentary phenotype	Photosensitizer	Outcome of study	Ref
clinical	1 duodenal metastatic melanoma	ND	porfirmer sodium	Successful treatment.	[171]
clinical	6 brain metastasis of malignant melanoma	ND	porfirmer sodium	All 6 patients [100%] remained free of brain disease till death, 50% died of malignant melanoma elsewhere and 50% died of unrelated causes.	[172]
clinical	melanoma in situ	unigmented	methyl aminolevulinate (MAL)	Recurrence at the original tumour site 4 months after PDT.	[173]
clinical	choroidal melanoma	unpigmented	benzoporphyrin derivative (BPD)	The tumor fully disappeared 1 month after the treatment, the visual acuity improved from 4/16 to 4/4. The disease did not recur during 24-month follow-up.	[174]
clinical	2 late-stage melanoma. Patient 1 had the primary tumour and local metastases on the left arm and metastatic tumours in the lungs. Patient 2 had a head and neck melanoma with multiple local metastases, which had failed repeated attempts at surgical resection and high-dose radiation therapy.	pigmented	indocyanine green (ICG) + imiquimod (toll-like receptor agonist)	Patient 1 free of all clinically detectable tumours (including the lung metastases) "/>20 months after the first treatment cycle. Patient 2 has been free of any clinical evidence of the tumour for over 6 months.	[175]
clinical	4 uveal melanoma, PDT on actual tumour site	mildly to heavily pigmented	benzoporphyrin derivative (BPD)	Vascular occlusion and thrombosis in mildly pigmented melanoma but no response in pigmented ones.	[176]
clinical	25 small and medium choroidal melanomas	ND	indocyanine green and transpupillary thermotherapy	After a mean of 2.4 treatments (range, 1 to 5 treatments), all of the tumors but one showed a significant volume reduction without clinical evidence of recurrences. Complications included retinal vascular occlusions, edema and superficial scarring of the macula, and rhegmatogenous retinal detachment.	[177]

Type of study	Tumour	Pigmentary phenotype	Photosensitizer	Outcome of study	Ref
clinical	14 skin metastasis from malignant melanoma, despite multiple courses of chemotherapy	pigmented	chlorin e_6	Complete regression after the first PDT treatment in eight cases and complete regression after multiple treatments in six cases. 11 of 14 patients died due to the progression of the melanoma, the median survival time after surgery was 883 days.	[165]
clinical	38 choroidal melanomas	ND	indocyanine green (ICG)	Changes in microcirculation 6 months after PDT, as well as significant decrease of tumors thickness in ultrasonography (mean 38%), were detected in all cases. Complete regression of intrinsic vessels was demonstrated by ICGA in 26 cases, and partial regression of pathological vascularization was found in 12 patients.	[178]
clinical	4 choroidal melanomas	ND	benzoporphyrin derivative (BPD)	One tumor decreased in size and remained stable for 18 months. One tumor had no growth for 11 months. Two melanomas continued to grow, necessitating enucleation.	[179]
clinical	3 uveal melanoma, PDT on unaffected areas before enucleation	ND	benzoporphyrin derivative (BPD)	Vascular thrombosis. No damage to the photoreceptors.	[180]
clinical	36 uveal melanomas	various	hematoporphyrin derivative	76 % of tumours were not growing at the end of the first year, 62 % after the second year and 38% after the fifth year. No eyes were lost as a result of PDT. The degree of tumour pigmentation and patient age at therapy significantly influence the tumour response to PDT.	[181]

Table 3. Clinical reports and outcomes of photodynamic effectiveness of photodynamic therapy protocols including the melanoma pigmentary phenotype.

1.3.2. Hypericin, a second generation photosensitizer for PDT

Hypericin, a second generation PS isolated from the plant *Hypericum perforatum*, is a phenan-throperylenequinone with two broad peaks of absorption – 300-400nm (ultraviolet) and 500-600nm (white light) (Figure 1). This may be considered as a disadvantage as a number of current second-generation photosensitizers have absorption peaks beyond 630nm allowing for increased penetration into tissues [182]. However, white light, used to activate hypericin, does penetrate deep into the dermis of the skin. Moreover, activation with ultraviolet light could be a distinct advantage for the use of hypericin in daylight-mediated PDT. This

type of PDT is more convenient for patients and clinicians and causes less pain. It poses a particularly interesting avenue to explore for hospitals in developing countries where space is limited and budgets are inadequate. Daylight-mediated PDT is an effective treatment for thin actinic keratosis, as shown in three randomized controlled clinical studies (reviewed in Wiegell et al., 2011) [183]. The potential of hypericin in clinical practice has been highlighted by reports on its use to treat squamous and basal cell carcinomas [184-187], pancreatic tumors [188], bladder carcinomas [189-193], nasophyrangeal tumors [194,195] and recently melanomas [196].
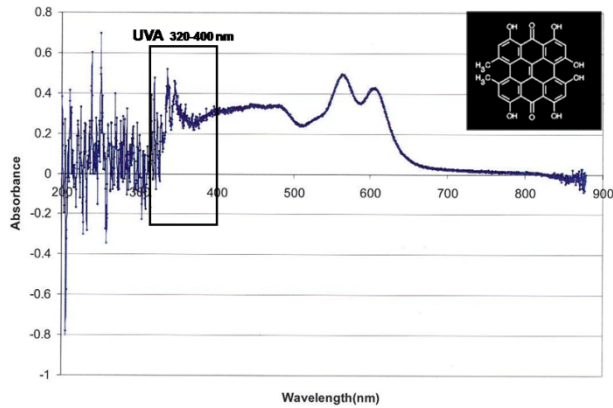
Figure 1. Absorbance spectrum of hypericin. Box, the wavelength of light used in our studies [98,197] representing one of the two activation peaks. Inset, chemical structure of hypericin.

1.3.3. Melanoma cell death and biological mechanisms induced by hypericin-PDT

Despite these promising studies, very few reports have highlighted hypericin's role in targeting melanoma. For the most part, cytotoxicity testing of new photosensitizers are tested on cell lines in vitro using assays such as the 3-[4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is a colorimetric assays for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color [198,199]. Other tests for cytotoxicity include dead cell protease tests. Despite being used as the "gold standard" for cytotoxicity testing, it must be borne in mind that these assays are based on cellular metabolic activity and could result in a false positive result were the treatment to produce a cytostatic effect in cells. Moreover, as these are colorimetric-based tests, the photosensitizer used itself may interfere with the wavelength at which these tests are read.

One of the first few reports testing 1 to 20mg/ml hypericin efficacy on squamous carcinoma, sarcoma and melanoma cell lines found that a combination of activating laser light sources resulted in a reduction in cell viability of 90% [163]. Following this, Hadjur et al. (1996) exposed human pigmented and unpigmented melanoma cell lines to hypericin and showed minimal cytotoxicity on uptake but upon activation with white light, increased cell death in

all three cell lines. Their findings thus suggest that amelanotic melanomas may be more susceptible to hypericin-PDT than pigmented melanomas. Their possible reasons for this related to the presence of melanin and antioxidant status of melanomas [162].

Work from our laboratory has shown a pigmentation dependant susceptibility of melanoma cells to hypericin-PDT, with pigmented cells being less susceptible than unpigmented cells [54,98,200,201]. Upon depigmentation with tyrosinase inhibitors, kojic acid and phenylthiourea, pigmented melanoma cells become more susceptible to hypericin-PDT [54,69]. Moreover, 72 hours after hypericin-PDT the cell viability of the depigmented melanoma cells remained significantly less than the control cells. Over the same time period the cells not treated with kojic acid approached a cell viability similar to the control.

Melanin is a potent antioxidant which could be a reason for the increased resistance of pigmented melanoma cells to PDT due to the scavenging of ROS produced by this therapy. Indeed we have shown that after depigmenting melanoma cells with kojic acid more ROS is produced upon treatment with hypericin-PDT compared to pigmented melanoma cells which were not depigmented [54]. We did not find a difference between the caspase 3, 7 activity after hypericin-PDT for both the depigmented and pigmented melanoma cells, which was lower than control. This suggests that pigmented melanoma cells might induce a caspase-independent mode of cell death such as the activation of apoptosis-inducing factor (AIF). Moreover, these cells might also undergo necrosis, necroptosis or autophagy in response to hypericin-PDT. We have further shown induction of autophagy at 4hours after hypericin-PDT in both pigmented and unpigmented melanoma cells [197]. Interestingly, pigmented melanoma cells (UCT Mel-1) show higher levels of externalisation of Annexin V, an early apoptotic event, compared to mildy and unpigmented melanoma cells [501mel and A375, respectively) after hypericin-PDT (Figure 2). However, the cell death response of pigmented and unpigmented melanoma cells is very complex and does seem to be cell type dependant. A possible explanation for this may be that the cell lines used in our studies are from different genetic origins and they thus might differ in various biochemical characteristics, including their antioxidant systems. The subcellular localisation of the photosensitizer is another factor determining the cell death mode initiated by PDT. Upon activation by light, photosensitizers produce ROS which are short-lived species acting directly in their vicinity of production. Localisation to different cellular compartments thus induces different modes of cell death.

Note: Since the discovery of programmed cell death in the 1960's the cell death field has evolved immensely. Researchers have shifted from morphological classifications to using more biochemical criteria. The increase in cell death studies necessitated a systemic classification of cell death modalities, which led to the formation of the Nomenclature Committee on Cell Death (NCCD). The main mission of this committee is 'to provide a forum in which names describing distinct modalities of cell death are critically evaluated and recommendations on their definition and use are formulated, hoping that a non-rigid, yet uniform nomenclature will facilitate the communication among scientists and ultimately accelerate the pace of discovery' [202-204].
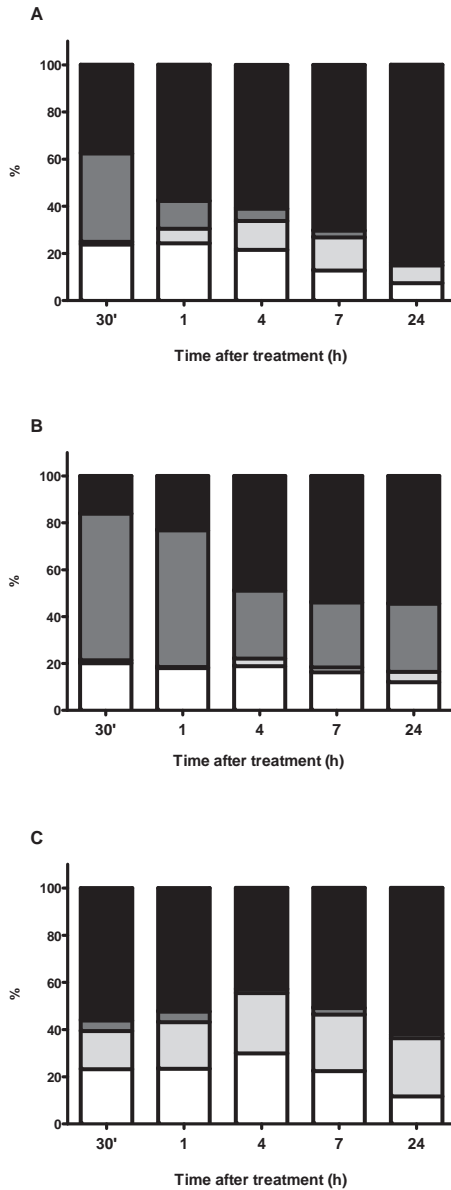
Figure 2. Graphs representing fluorescent activated cell sorting (FACS) analyses of melanoma cells at 30min, 1, 4, 7 and 24h after hypericin-PDT treatment (3µM hypericin with 1 J/cm^2 UVA). A: unpigmented A375, B: mildly pigmented 501mel and C: pigmented UCT Mel-1. Cells were stained for early apoptosis (FITC Annexin V, BD Biosciences) and necrosis (LIVE/DEAD Fixable Violet stain, Invitrogen). Different modes of cell death are represented as proportional percentages normalised to the control, black: late apoptotic/ necrotic, dark grey: necrotic, light grey: apoptotic, white: live; n=3.

1.4. PDT targets to treat melanoma

1.4.1. Cell proliferation and survival

It is now well established that one of the chief characteristics of cancer cells is their ability to overcome cellular control of proliferation [205]. In melanocytes, proliferation is caused by a combination of several mitogenic growth factors such as stem cell factor (SCF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) which cause a sustained extracellular receptor kinase (ERK) activity [206]. In melanoma, the RAS/Raf/MEK/ERK pathway is a key regulating pathway in proliferation with ERK being hyperactivated in up to 90% of human melanomas [207]. BRAF and PTEN mutations (see above) are co-incident in about 20% of cases [208]. The most common mutation in BRAF is a glutamic acid for valine substitution at position 600 (^{V600E}BRAF) [209]. This mutation leads to constitutive ERK signalling resulting in hyperproliferation and cell survival [210]. This pathway, through the EGF receptor as an extracellular ligand, has been a worthwhile target for PDT in that sustained activation of the ERK pathway protected cells from photofrin-based PDT as well as a reduction in the Raf protein levels in treated cells [211].

Nuclear factor kappa beta (NF-κβ) signalling leads to transcriptional regulation of a number of genes involved in responses ranging from proliferation, metastasis, and survival to inflammation. It therefore is an important target in PDT to stop aberrant cell proliferation. PDT-induced oxidative stress through increased ROS production has been shown to activate (NF-κβ) [212] and inactivate its inhibitor (Iκβ). Moreover, Ryter and Gomer showed increased NF-κβ binding in response to PDT stress in mouse cancer cells leading to a reduction in proliferation [213].

1.4.2. Inhibition of apoptosis

Apoptosis, a controlled mode of cell death, is characterised by cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and activation of caspases [214]. It is now well established that activation of the caspase cascade occurs through death receptor activation (extrinsic pathway) or through mitochondrial outer membrane permeabilization (intrinsic pathway). Both of these pathways have been shown to be activated through PDT. Several biochemical studies have established that PDT with different photosensitizers, including hypericin, utilise the mitochondrial-mediated pathway of caspase activation [215,216] although PDT has recently been shown to also engage caspase-independent pathways [217]. It is further known that several anti-cancer agents induce apoptosis and may share common pathways leading to cell killing with Fas/APO-1/CD95 [218,219]. Ali et al. (2002) elegantly showed that hypericin-PDT induces human nasopharyngeal cancer cells to undergo apoptosis through the Fas/FasL system. Moreover, they showed that the upregulation of Fas/FasL results in the release of cytochrome c into the cytoplasm with subsequent caspase induction – results that suggest that although apoptosis is considered a product of either an extrinsic or intrinsic mechanism; the overall response to PDT may be a combination of mechanisms [220].

Cancer cells are known to resist cell death by upregulation of anti-apoptotic proteins, mutations in pro-apoptotic proteins, inhibition of cell senescence or through protective mechanisms such as autophagy. PDT that is targeted for cancer therapy aims to invoke cell cytoxicity through attacking these characteristics – topics of a number of recent reviews [36,221-223].

Intriguingly, the emergence of a defined 'immunogenic apoptosis' seems to be a new 'subset' of apoptosis and autophagic cell death which has been shown to have the ability to release/expose damage-associated membrane proteins (DAMPs) [224-227]. Therapeutically, the immunogenicity of apoptosis is preferable for application rather than necrosis (or for that matter autophagic cell death) since necrosis can lead to harmful immunological reactions [228] (on the other hand, the extent of immunological impact of autophagic cell death is as yet uncharacterized, thereby making it an uncertain modality to use in the context of 'immunochemotherapy') [229]. The cell killing effectiveness is however dependent on parameters such as the PS used, the light dose and most importantly, the subcellular localization of the PS. It is crucial for photosensitizers to effectively enter the cell and accumulate in specific intracellular organelles in order to be efficient in their killing ability. Clearly, the final destination of the PS and its immediate vicinity will lead to different modes of cell death and consequently different efficiencies. Recent reports have highlighted that hypericin not only localises to different subcellular organelles but that this localization is exposure and dose-dependent in addition to being tumour cell-specific [230-234]. As a start however, the lipophillic nature of hypericin dictates its association with cellular membranes [235]. The fact that hypericin has been shown to associate with serum proteins (LDL and HDL lipoproteins [236] ensues that it enters cells quickly and is preferentially taken up by cancer cells in the 3-dimensional milieu as recent reports showed that these cells have high levels of LDL surface receptors [237]. This is further supported by a recent report showing that cholesterol serves as a key determinant for the uptake of hypericin into cellular membranes [238].

Noteworthy however is that even though high levels of hydrophobicity ensues, high levels of intracellular accumulation of the photosensitizer, changes in the physical structure of the PS due to aggregation and other modifications, may lead to reduced PDT efficiency [239]. Overall, the consensus emerging is that hypericin localises to three intracellular organelles namely, the endoplasmic reticulum (ER)-Golgi network [230,231,240,241], mitochondria (Mt) [242-245] and lysosomes [237,246] where through synergistic action, apoptosis is induced. More recent work by the Agostinis group show that hypericin-based PDT would produce photo-oxidative ER (p-ox ER stress) stress while 5-ALA (localizes in the mitochondria)-based PDT would produce photo-oxidative mitochondrial stress [36,247]. They also observed that Hyp-PDT induces 'pre-apoptotic' active exo-ATP secretion and late stage passive release of DAMPs like HSP70, HSP90 and CRT [223]. Overall they suggest that the potential of Hyp-PDT in causing exposure/secretion of 'critical' DAMPs add to the apoptotic cell death modality in a rather 'small club' of anti-cancerous therapeutic agents/modalities capable of exposing immunogenic signals like ecto-CRT [227,248].

1.4.3. Induction of autophagy

A recent finding is the induction of the cytoprotective programme of autophagy in melanomas in response to PDT-induced oxidative stress [89]. In addition, recent reports showed that cancer cells may respond to chemotherapeutics or other forms of oxidative stress such as PDT, through the induction of autophagy initially but continued stress leads to an overwhelming of the endogenous antioxidant enzymes along with a shift from autophagy to a possible senescent phenotype in an attempt to prolong cellular survival. Consequently however, the cell enters an apoptotic or necrotic mode of cell death [249-251]. Autophagy, defined as a cellular response to nutrient deprivation with consequent organelle breakdown, could converge with PDT at a number of cellular locations. Although more work relating to this aspect in melanomas is needed, reports on other cancer cells have shown that autophagy can be induced if the lysosomal system, needed for the clearance of ROS-damaged organelles, is affected by PDT [252]. Another cellular location is the mitochondria, where the PDT-induced loss of anti-apoptotic protein Bcl-2, may lead to an initation of autophagy [253].

1.4.4. Chemoresistance due to increased antioxidants

Cancer cells are considered to be under continuous oxidative stress which has been suggested to aid in tumor progression [254]. In support, several studies have shown tumor cell lines producing higher levels of ROS compared to their normal counterparts [255,256]. Due to this increased level of ROS and hence constitutive increased level of oxidative stress, it is not surprising that cancer cells have an extensive and advanced intracellular antioxidant network – a characteristic which further increases their chemoresistant property. Interestingly, the antioxidant status of melanomas differs from that of other skin cancers such as basal and squamous cell carcinomas in that their antioxidant activity levels (i.e. catalase, glutathione peroxidise, superoxide dismutase) are much higher [257]. In contrast, melanocytes, their normal untransformed phenotype, have lower levels of antioxidant activities and associated lower levels of resistance to oxidative stress [258]. It is therefore reasonable to postulate whether breaking this tolerance to oxidative stress may increase therapeutic efficacy in targeting melanoma. A number of studies have therefore suggested that treating melanoma by inhibiting cellular antioxidants may be efficacious [259-261]. One example of this was the addition of the superoxide dismutase (SOD) activity inhibitor, 2-methoxyestradiol (2-ME$_2$), to a mouse transplant model which induced growth arrest of melanoma cells after injection [262]. Paradoxically, several studies have suggested that antioxidants can enhance the action of cancer chemotherapeutics drugs in their in vitro models through inhibition of a variety of factors which contribute to the malignant phenotype [263,264]. A recent study however, using six different combinations of antioxidants and chemotherapeutic drugs in combination, failed to identify a single combination in which an antioxidant reduced the survival of malignant breast carcinoma cells [265]. To our knowledge the use of PDT as an inhibitor of antioxidants has not been tested in cancer cells.

1.4.5. Melanin and melanosomes as pro-survival agents

All the potential intracellular organelle targets for PDT mentioned above are consistent with most cancer cells. However, the one aspect that sets melanoma apart from other cancers is the presence of its cell-specific organelle called the melanosome and its associated product, melanin pigment. It is thus not inconceivable to believe that the intractability of this skin disease may in some way be related to this organelle and its function [266,267]. It follows logically then, that treatment regimes need to consider the melanosome as another potential target organelle in the fight against melanoma [268].

Melanosomes are membrane-bound organelles in melanocytic cells which house the pathway that results in the formation of the polymeric pigment, melanin [269,270]. The enzymes which participate in this pathway are translated in the cytoplasm and chaperoned to the melanosomes. Tyrosinase (TYR), the rate-limiting enzyme of the pathway, and its related proteins tyrosinase-related proteins 1 and 2 (TYRP-1 and TYRP-2) act in concert to first convert tyrosine to 3,4-dihydroxy-phenylalanine (DOPA) via tyrosine hydroxylase activity and then convert DOPA to DOPAquinone via dopa oxidase activity. Both of these activities occur via separate tyrosinase catalytic sites. During melanin synthesis toxic intermediates such as 5, 6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid are produced. Structurally, the melanosomes are designed to compartmentalise these cytotoxic melanin intermediates from spilling into the cytoplasm [271]. Melanosomal biogenesis progresses through four distinct stages of maturation where the first two stages contain no melanin and the later stages constitute intermediates required to generate a matrix favourable for the formation of melanin [269,272]. The Pmel17/gp100/Silv/ME20 protein, a product of the Silver locus in melanocytic cells [273], is capable of polymerizing into fibrillar arrays that form the backbone of melanosomes. As a major component of the fibrillar matrix of early stage melanosomes, Pmel-17 serves as the best marker to follow intracellular trafficking steps that regulate melanosomal formation [274].

Moreover, as Pmel-17 facilitates melanin deposition and plays a pivotal role in melanosome biogenesis, it remains a strategic target when trying to combat melanoma through the fact that melanosomes are involved in scavenging endogenous cytotoxic metabolites and storing their waste products - a function that has been suggested to be a key in creating multi-drug resistance [267]. With the premise that melanosomes may be acting as cytotoxic drug "sinks" through the sequestration of chemotherapeutic drugs [267], it would be considered an effective therapy for a PS to enter the melanosomal membrane and damage the wall of the melanosome thus allowing the leakage of toxic melanin intermediates resulting in cell death. The drawback is that melanosomes, which are classified into stages along their biogenesis [269,270] only produce the toxic intermediates during their final maturing stages III and IV [268-270,275-277]. Most pigmented melanomas do however present with a majority of these end-stage melanosomes in their cytoplasm making the melanosomal membrane an attractive target for PDT. On the basis of this information, one may imagine that pigmented melanomas are therefore more susceptible to PDT-induced cell death. In contrast, our work has shown that pigmented melanomas are much less susceptible to hypericin-PDT than unpigmented/amelanotic melanomas despite hypericin readily entering the melanosomes [89].

We hypothesize that the reason for this is due to the presence of the pigment melanin. In support of this, pigmented human xenograft melanotic melanoma in mice, was shown to be far less responsive to PDT than amelanotic melanoma [278].

Melanin has been shown to act as both an oxidant and antioxidant [266,279] and in parallel studies, its presence in melanomas have been linked to chemoresistance. In support, further studies have shown that a lack of pigment in melanomas decreases their resistance to cell death. Our ongoing investigation into susceptibility to PDT-induced cell death in depigmented melanomas supports this hypothesis [54,69].

1.4.6. Cancer stem cells as future PDT targets

The cancer stem cell hypothesis purports the idea that a subset of cancer cells is capable of maintaining and driving disease progression [280,281]. With the identification of cancer stem cell populations in colon, breast and brain tumors [282-285], it is believed that these cells are integrally related to tumor formation, resistance to chemotherapy and escape from remission [286]. While the qualifications for melanoma stem cells have generally been defined as tumorigenicity in xenograft spheroid formation and self-renewal in non-adherent cultures, the markers used to identify these cells from the general tumor population remain debatable. A brief summary of these markers and their potential as targets for novel PDT-based therapy, follow.

ATP-binding cassette (ABC) transporters are a vast family of transmembrane proteins that have been studied for their ability to actively transport cytotoxic substances out of cells [287]. Intriguingly, some of these transporters have been demonstrated to be highly expressed in highly tumorigenic subpopulations of melanoma suggesting that they may be markers of melanoma stem cells [286]. One of these includes the ABCB5 transporter. Known for increased expression during melanoma progression in human tumor samples, ABCB5+ cells were able to resist treatment with doxorubicin [288]. While ABCB5+ cells were not able to renew in culture (a "stemness trait"), a subpopulation of cells that were indeed able to renew expressed the ABC transporter, Multi-drug Resistant-1 (MDR1) [289]. In vitro, MDR1+ cells exhibited less pigmentation than MDR1- cells, possessed the ability to continuously self-renew in soft agar and expressed the pluripotency and self-renewal regulators, human telomerase reverse transcriptase (hTERT) – all characteristics pointing towards "stemness". Interestingly, while MDR+ cells did exhibit cancer stem-cell like properties in vitro, they also co-expressed ABCB5 and ABCC2 mRNAs suggesting that a number of ABC transporters may be expressed in sub-populations [289]. To further add to the complication of delineating melanoma stem cell markers as potential targets for PDT is the fact that a number of recent markers are co-expressed with ABC transporters. These include CD133/prominin-1/AC133, which is co-expressed with ABCB5 and ABCG2 [288,290] and Nestin ([286].

Accumulating evidence suggests that another transporter, ABCG2, has physiological relevance in terms of photosensitivity and hence, PDT [291,292]. It has been shown that clinical photosensitizers and chemotherapeutic drugs have been transported out of cells by ABCG2 whereas this effect was abrogated by co-administration of its inhibitor, imatinib mesylate [293]. It is fascinating to speculate that a PDT protocol using a new, more stable photosensitizer such as hypericin may, through optimized concentrations, inhibit the action of the

ABCG2 transporter and thus create an intracellular pool of ROS resulting in efficacious cell death. This is definitely an avenue for exploration.

Overall, the ability to halt melanoma cancer progression through targeting melanoma stem cells could be extremely advantageous. However, with such a large number of potential markers and their interaction with PDT unknown, it may be a better option to focus on ABC transporters and investigate their susceptibility to second generation PS-based PDT as a means to an end for melanoma progression.

2. Conclusion and future directions

There is no doubt that our understanding of the molecular and cellular basis of melanoma has grown substantially over the past decade. However, due to its multifunctional nature, the nee d for better, improved therapies to combat or target melanoma remain essential. In addition, better understanding of the heterogenous nature of this diverse disease will likely lead to re-evaluation of the basic concepts underlying melanoma therapeutics development and clinical trial design. Till then however, novel adjuvant treatment modalities such as PDT using photostable, second-generation photosensitizers such as hypericin remain an option and need to be investigated further. Moreover, optimization of this type of therapy with regard to subcellular localization and its effect on cell death mechanisms within melanoma cells is needed. Targeting the integrity of melanocytes-specific organelles such as the melanosomes and producing an over-riding increase in ROS with consequent cytotoxicity remains a good therapeutic option but needs a systematic, scientific approach. Intriguingly, as more avenues of therapeutic targets such as melanoma stem cells and ABC transporters become illuminated, the ability to invoke cell death modalities in combination with PDT become more evident. Finally, it is clear that all these factors need to be considered in synergy if progress is to be made toward combating the menace that is metastatic melanoma.

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