Peer reviewed REVIEW

A REVIEW OF THE PHOTODYNAMIC APPLICATION OF 5-AMINOLEVULINIC ACID, HYPERICIN AND PHTHALOCYANINES IN DERMATOLOGY

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ABSTRACT

Ultraviolet radiation can damage human skin leading to photo-aging and cancer. Treatment options for skin cancers are available. Amongst them, there is photodynamic therapy (PDT), the treatment modality that involves a photochemical reaction between a light sensitive compound, visible light and tissue oxygen. In PDT, a photosensitive compound also called photosensitizer (PS) is administered and allowed to accumulate in the cancerous tissue then irradiated with light corresponding to absorption wavelength of the PS, in the presence of molecular oxygen, to produce cytotoxic species that kill the cancerous tissue. PDT is increasingly used and studied globally for the treatment of different classes of cancers because of its selective destruction of diseased tissue or cancer. Newly developed non-invasive imaging technologies including photodynamic diagnosis, may assist with early identification of skin cancer to reduce the rates of morbidity and mortality following treatment. Photodynamic diagnosis (PDD) is a diagnostic modality defined from the PDT principle. It utilizes light and fluorescent PS to highlight tumour cells from normal cells. Excitation of PS by appropriate light source causes them to fluoresce over well-defined spectral regions. Therefore, the fluorescent properties of PSs can serve as an important diagnostic tool to highlight cancer at an early stage of development. In this review, our knowledge about PDT and PDD of skin cancers using 5-aminolevulinic acid (ALA), Hypericin and phthalocyanines as photosensitizers is presented.

KEYWORDS

skin cancer; Photodynamic therapy and diagnosis; fluorescence; irradiation

INTRODUCTION

Skin cancers are named after the type of cells that become cancerous, namely, basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma, Figure 1.[1] These cancers typically form due to excessive exposure of skin to the sun.[2] Malignant melanoma is the rare type of skin cancer which could be very deadly due to its resistance to treatment.[3] The highest incidence of malignant melanoma are recorded in Australia, New Zealand and South Africa. A significant increase in the incidence of melanoma among white women under the age of 35 years has been observed in South Africa.[4, 5, 6]

Ultraviolet (UV) radiation plays a major role in human skin disease and photoaging.[7, 8] Brash *et al.*[9] established that sunlight is a potential carcinogen, especially to those exposed during childhood or teenage years. Biniek *et al.*[10] clearly revealed that excessive exposure of human skin to UV radiation not only cause DNA damage in skin but also alter protective functions of the stratum corneum (SC). The choice of skin cancer treatment varies from surgery, chemotherapy, radiotherapy or PDT, depending on the size and site of the tumour. In chemotherapy the growth and destruction of cancer cells is obtained by use of toxic drugs. The drugs are topically applied on diseased skin or administered intravenously to kill rapidly growing cells. Side effects include damage to healthy cells and organs. Moreover, high energy radiation sources are used in radiotherapy to shrink and kill cancer cells.[11, 12, 13] Unsatisfactory treatment outcomes of skin cancer with chemotherapy and radiotherapy have been reported.[14] In some cases chemotherapy and radiotherapy are administered concurrently to enhance the treatment of head and neck cancers.[11] Therefore, their application for skin cancer remains controversial.

PDT is currently scrutinized for cancer treatment because it exhibits unique treatment possibilities over chemo- and radiotherapy mainly for its selective destruction of targeted abnormal cells while preserving normal tissues. Additionally, it is less invasive and can be repeated many times at the same site if needed, as compared to surgery, chemotherapy and radiotherapy.[12-15]

PHOTODYNAMIC THERAPY

The main components of PDT are PS, light of specific wavelength to the PS selected and molecular oxygen. In PDT, illumination of light on tumour site with PS results in the elevation of PS from its lowest energy to highest energy state followed by intersystem crossing conversion to the tripiet state enabling it to interact with the surrounding molecules and molecular oxygen. The reaction of elevated PS with molecular oxygen produces singlet oxygen species and other free radicals that are destructive to tumour tissue.[16, 17] Neither Ps, light nor molecular oxygen alone causes the destruction of tumour.[15-19] The earliest study of PDT conducted using hematoporphyrin (Hp) as PS combined with light showed that there was massive destruction of porphyrin-containing gliomas in rats.[18] A successful study on the evaluation of hematoporphyrin derivatives (HpD) in combination with xenon arc lamp to destroy tumours was also reported.[20] These studies were repeated in 1978 on 35 patients with subcutaneous and cutaneous tumorous lesions.[21] This work demonstrated that the Hp and its derivative had efficacy for treatment of cancer, leading to an increased interest in PDT globally. Kalka *et al.*[22] presented a review on

potential benefit of PDT for non-malignant skin disorders including vulgaris, viral infections and diseases of epidermal appendages. PDT treatments are greatly accepted globally since they cause destruction of tumour leaving the neighbouring normal cells unharmed.[13, 23]

The equipment used for PDT is user friendly, cost effective and portable, all factors of substantial importance in Africa specifically in terms of deployment of medical diagnostic and therapeutic equipment in remote/rural areas. PDT research has been conducted for decades, and many PSs have been approved for clinical applications in the USA and Europe.[24, 25] However, the strong pain reported in PDT is an issue that may limit the use of this method in dermatology.[23, 26] Nonetheless, the study of Nathali *et al.*[27] demonstrated that treatment-related pain can be minimized using a 2-step irradiance protocol.

Photosensitizers

Photosensitivity is an adverse reaction of a tissue to certain substances, a drug or chemical that makes a tissue more sensitive to light. These substances are called PSs. They are administered either orally, topically, or subcutaneously to the tumour site, allowed to accumulate in tumour, then exposed to light. Porphyrin family PSs were the first generation PSs used in PDT, however, because their side effects includes prolonged cutaneous photosensitivity, studies for potent second and third generation PSs, Table 1, are underway.[17, 25] Porfimer sodium (Photofrin®) was the first US Food and Drug Administration (FDA) approved PS for PDT of oesophageal cancer, followed by 5-aminolevulinic acid (ALA, (Levulan®)) in USA for actinic keratosis (AK); methyl 5-aminolevulinate (MAL (Metvix®) in Europe for AK and BCC; Meso-tetra-hydroxyphenyl chlorine (Foscan/Temoporfin) in Norway and Iceland for neck and head cancer.[24, 25] Although several PSs have been tried in dermatology, this review article reports on the application of ALA, MAL, Hyp and three widely used phthalocyanines (Pcs) on photodynamic treatment of different skin ailments.

1. Aminolevulinic acid

Porphyrins are organic compounds most associated with the red pigment in blood cells. 5-aminolevulinic acid (ALA) is the active reagent regulated via a feedback control mechanism in the biosynthesis of heme, significantly involved in the induction of the endogenously synthesized porphyrin derivative, protoporphyrin IX (PpIX), which when combined with iron, produces heme. Heme gives a red colour to the hemoglobin molecule. The rate of production of PpIX is dependent on the rate at which ALA is synthesized. Exogenously added 5-ALA passes the plasma membrane through amino acid transporters and enters the heme synthesis pathway leading to massive temporary accumulation of PpIX.[17, 28-29] Although ALA and MAL have been approved for PDT of AK and BCC, promising results for their application on other skin lesions (Table 2) have been reported.[28-35] ALA-PDT using red light has been discovered as potential antimicrobial modality to control Pseudomonas aeruginosa infection on chronic skin ulcers.[38] The reliability and safety of using MAL- PDT to control and manage extramammary Paget disease and its associated symptoms was reported.[39] A framework of general guidelines presented by Morten *et al.*[40] on the use of MAL combined with daylight (DL) suggests that the use of MAL-DL-PDT may be a powerful treatment option for multiple grade I and II AK on the face and scalp with little or no pain.

2. Hypericin

Hyp, a lipophilic dianthraquinone, forms red solutions in most organic solvents and precipitates in water. When exposed to ultraviolet or visible light, it becomes toxic.[41, 42, 43] Bublik *et al.*[44] provided *in vitro* evidence that Hyp-PDT using long pulsed laser light may be a powerful tool in the destruction of recurrent head and neck SCC. In addition, Hyp-PDT resulted in caspase-dependent apoptotic modes of cell death in non-pigmented and pigmented melanoma cells.[45] An emulsifying ointment supplemented with Solketal® has been shown to be a suitable delivery agent to enhance penetration of Hyp into mice skin, to

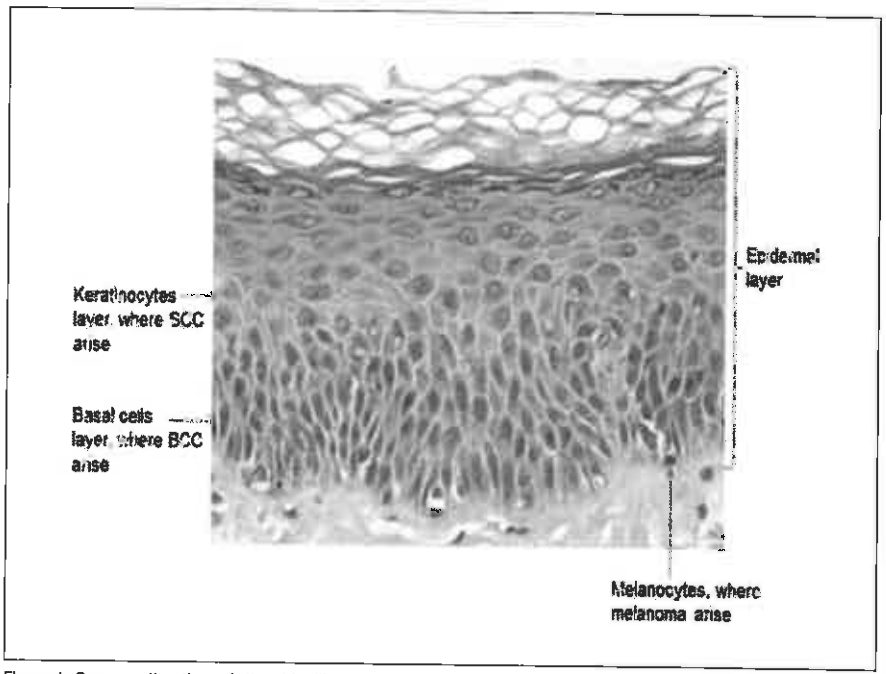

Figure 1: Cross section through the skin, illustrating the epidermal layer and type of skin cells where skin cancer could arise. Modified from[1].

Table 1: First, second and third generation photosensitizers for use in PDT studies.

1ST GENERATION	2ND GENERATION	3RD GENERATION (NON-PORPHYRIN)
• Hematoporphyrin • Hematoporphyrin derivative ([a]Photofrin®)	• Metalloporphyrin • Pheophorbides • Purpurins • Chlorins ([a]Foscan) • Protoporphyrin ([a]Levulan®; [a]Metvix®) • Phthalocyanines	• Psoralens • Anthracyclines • Cyanines • Phenothiazinium dyes • Hypericin

[a]*approved*

improve PDT treatment of psoriasis and other skin lesions.[46] In line with that, a pilot study for topical application of Hyp was conducted on 34 lesions (8 AKs, 5 Bowen diseases (BD) and 21 BCC). Treatment was carried out weekly for six weeks. Hyp was applied on skin lesions, incubated for 2hrs and then irradiated with 75 J/cm² red light. The success rate for treatment was 50% for AKs, 28% for BCC and 40% for BD. Pain and burning sensation was experienced by all patients.[47] Furthermore, a phase II placebo-controlled, double blinded, multicentre clinical study was conducted in 25 patients to evaluate the safety and efficacy of topical Hyp in concentrations of 0.1% and 0.25% under occlusion for 24 hours followed by administration of visible light in doses ranging from 8 to 20 J/cm² twice weekly for 6 weeks for the treatment of psoriasis or mycosis fungoides (MF). Hyp treated lesions had a significantly higher response rate on both psoriasis and MF as compared with placebo-treated lesions. Only mild to moderate phototoxicity at the site of application after exposure to visible light was indicated by majority of patients.[48]

A recent study by Gyenge *et al.*[49] demonstrated that the application of a 1:1 mixture of Hyp and a liposomal mesotetrahydroxyphenyl chlorin (mTHPC) derivative, with the features of reduced dark toxicity and combined apoptotic and necrotic cell death may improve efficacy of PDT of head and neck squamous cell carcinoma. The response of normal primary human keratinocytes (Kc), melanocytes (Mc) and fibroblasts (Fb) *in vitro* to HYP-PDT, suggests that Hyp-PDT has potential damage to normal skin if treatment protocols are not optimized before tumour treatment.[50] Hence, optimization of treatment protocols in Hyp-PDT is highly recommended to avoid significant damage of normal cells at the site of tumour.

3. *Phthalocyanines*

Phthalocyanines (Pcs) are aromatic macrocycles with strong absorption bands at 670-770 nm.[16, 25]

Their ability to generate singlet oxygen upon irradiation with visible light signify a promising group of PSs in photodynamic treatment of skin lesions. Three widely used Pc family PSs in dermatology are derivatives of aluminium, zinc and silicon Pcs. Disulfonated chloroaluminium phthalocyanine ($ClAlPcS_2$) has demonstrated phototoxic properties against human melanoma, human breast adenocarcinoma, mouse fibroblasts and mouse melanoma cell lines.[51] In addition, the study of Krestyn *et al.*[52] also demonstrated a high phototoxic effect with $ClAlPcS_2$-PDT

Table 2: Recently reported studies on investigations of ALA and MAL PDT for skin ailments.

REFERENCE	PS	CLINICAL APPLICATION	NUMBER OF PATIENTS (P), LESIONS (L)	TREATMENT PARAMETERS	RESPONSE RATE (%)
Stebbins and Hanke[30]	MAL	nBCC, SCC *in situ*	10P	Red light, 570-670[a], 75[c]	CRR of 70-90% at 14 months
Dirschka *et al.*[31]	ALA/MAL	AK	600P	Light sources, 630-1400[a], 37-100[c]	CRR of 78.2-90.4% at 3 months
Zhang *et al.*[32]	ALA/PpIX	SCC	1P	Laser, 630[a], 120[c]	CRR of 100% at 15 months
Kim *et al.*[33]	ALA	VW	8P; 41L	Intense-pulsed light (2.5ms pulse duration, 10ms delayed time) 530-750[a], 8-11[c]	100% CRR in 2P and and 50% CRR in 2P at 4 months, 3 or 4 yrs.
Zeitouni *et al.*[27]	MAL	sBCC	25 L	Laser (40 or 50[b]), or light-emitting diode (35[b]), 75[c]	80% in the 50/70[b] and 90% in the 35/70[b] at 24 months
Fernández-Guarino *et al.*[34]	MAL	BCC	191 L	Red light, 630[a], 37[c]	CRR of 74% at 6-72 months
Chen *et al.*[26]	ALA	AV	50P	Red light, 633±10[a], 10[b], 120[c]	TRR of 83.3% at 6 weeks
Cai *et al.*[35]	ALA	BD	11L	Red light + CO_2, 630[a], 100[b], 180[c]	CRR of 72, 32% at 3 months

5-aminolaevulinic acid (ALA); Bowen's disease (BD); superficial basal cell carcinoma (sBCC); actinic keratosis (AK); Complete response rate (CRR), Viral Warts (VW), Acne vulgaris (AV) Total effective rate (TER)

[a] *wavelength (nm)*

[b] *laser power (mW/cm²)*

[c] *dose (J/cm²)*

on melanoma cell-line as compared to zinc-5, 10, 15, 20-tetrakis (4- sulphonatophenyl) porphyrin ($ZnTPPS_4$) and ALA. To gain clinical approval of aluminium phthalocyanine chloride (AlClPc), Kryazi *et al.*[53] investigated the PDT effect, penetration and absorption of AlClPc combined with diode laser in mice tumorous skin as compared to normal skin. They found complete tumour reduction and superb cosmetic effects in 60% of mice treated. Penetration and absorption of AlClPc in tumorous skin was 19 and 40 times, respectively as compared to normal skin.

Three different skin derived cell lines were treated with mono- and tetra-substituted Zn (II)-phthalocyanine (ZnPc). The uptake of tetra- as compared to mono- ZnPc was higher in all cell lines, exhibiting higher photodamage to all cell lines.[54] Pcs tend to form aggregates in aqueous solutions. Therefore, coupling or incorporating them with monoclonal antibodies, emulsifying creams or nanoparticles may enhance their penetration into tumour cell thereby enhancing their PDT effect.[55, 56, 57] Accordingly, a comparative study of the pharmacokinetic and photo-therapeutic properties of a free or bound Zn (II)-phthalocyanine disulphide (C11Pc) to gold nanoparticle in C57 mice bearing a sub-cutaneously transplanted amelanotic melanoma was reported. The C11Pc free or bound to gold nanoparticles was intravenously injected at a dose of 1.5 µmol/kg body weight, using a Cremophor emulsion as a delivery vehicle followed by light treatment 3 h after injection of Pc. Higher PDT destruction of tumour was observed in nanoparticle-bound C11Pc than in the presence of the free C11Pc.[57]

Clinical trials with silicon Pc (Pc 4) for treatment of cutaneous neoplasm have been reported.[58, 59] In the Phase 1 clinical trial of Pc 4-PDT, different topical delivery protocols for Pc 4 on human skin tumours were explored. Successful penetration was obtained with formulation of Pc 4 in an ethanol/propylene glycol vehicle through the basal layer of the epidermis within 1 hr of application. The study was partially complete but promising data was obtained. Therefore, Pc 4-PDT was established as safe and tolerable treatment as no local or systemic toxicities were reported.[58] Subsequently, the potential of topically Pc 4-PDT for treatment of cutaneous neoplasms was reported. Forty three patients with neoplasms including AK, BD, SCC, BCC or mycosis fungoides (MFs) were treated with a single administration of Pc 4-PDT. Fourteen days after treatment, a partial response rate of 37% (16/43) was obtained. 14 patients out of 35 with MF demonstrated a clinical response correlating to Pc 4-PDT induced apoptosis. Results suggest that Pc 4-PDT can effectively trigger apoptosis in MFs.[59] A comparative study with four porphyrins and six Pcs in a radial growth phase melanoma cell line revealed that the most efficient PS for PDT in melanoma cells were Pcs.[3] Many Pcs are currently used for industrial, clinical and biological applications. Therefore, Jančula *et al.*[60] investigated potential health hazards associated with dermal exposure of vertebrates to Pcs. In their study, an immortalized human keratinocyte cell line HaCaT was exposed to 31 Pcs for 2 or 24 h, either with or without irradiation after 60 min of incubating cells with Pcs. Using a neutral red assay, no cytotoxic effects or weak cytotoxic effects were induced by Pc alone but when exposed to light in the presence of Pc, cytotoxic effect were noted. Their results suggest that environmental applications of Pcs at concentrations below 1 mg/L should not represent an acute risk of toxicity for human skin.

MECHANISM OF CELL DEATH

In PDT, PS can transfer energy from the triplet state by two processes: Type I mechanism involving superoxide radical formation or type II mechanism involving PS reaction directly with biological tissue oxygen to generate singlet oxygen.[41, 45] The PS localizes in cell mitochondria, lysosomes, endoplasmic reticulum, Golgi apparatus and plasma membranes. Cell death mechanism in PDT is either via apoptosis, necrosis or autophagy depending on PS concentration and its subcellular localization. Apoptosis, necrosis or autophagy is likely initiated by PSs localized in

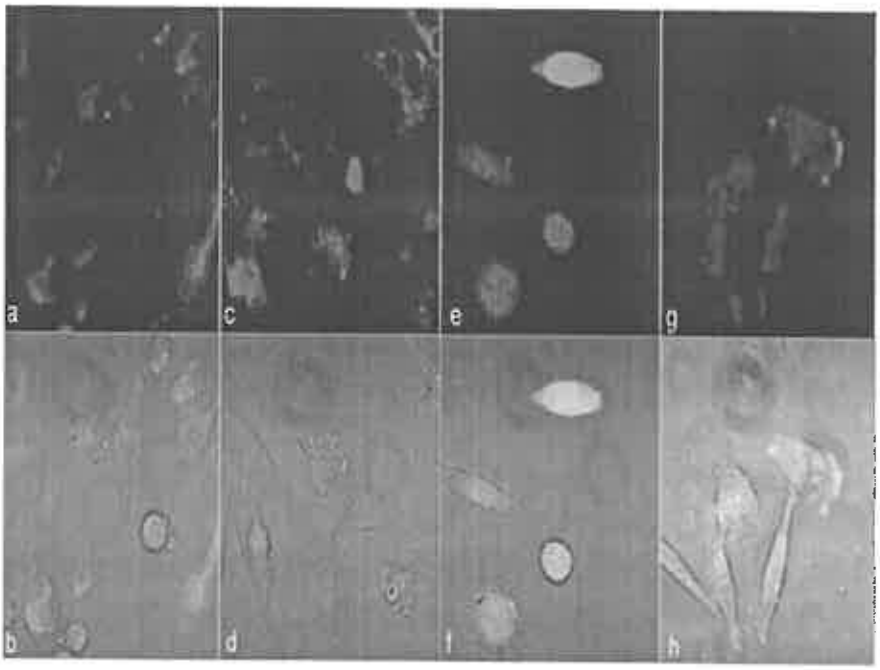

Figure 2: Localization of subcellular markers and $AlPcS_4Cl$. Fluorescent subcellular markers inside HT-144 cells. a: Rhodamine 123; b: LysoTracker®; c: Flou3; d: $AlPcS_4Cl$; localized in the mitochondria, lysosomes, calcium in cytoplasm and $AlPcS_4Cl$ respectively[61].

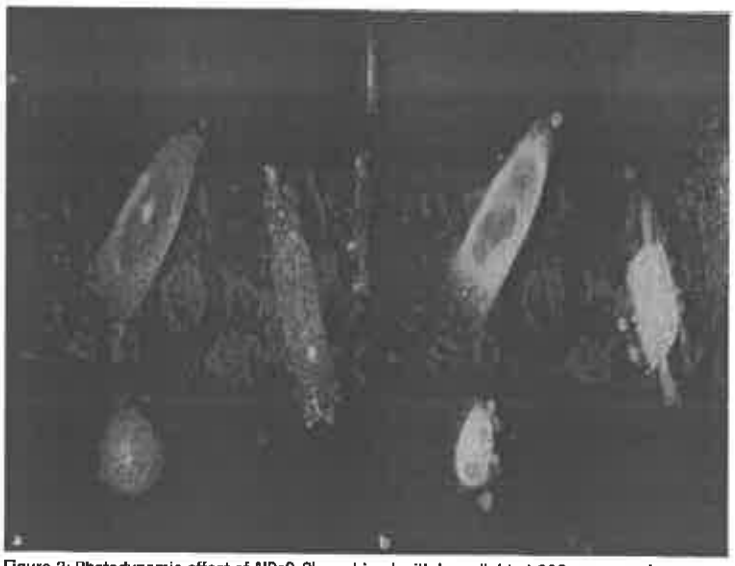

Figure 3: Photodynamic effect of $AlPcS_4Cl$ combined with laser light at 633 nm on melanoma cell line resulting in cell contraction and formation of apoptotic bodies, (a): before irradiation; (b): after irradiation[19].

mitochondria; lysosomal membrane or endoplasmic reticulum, respectively.[17, 61]

The study of Anand *et al.*[62] demonstrated that pre-treatment of epithelial skin tumours *in vivo*, with calcitriol, the active form of vitamin D3, increased the efficacy of ALA-PDT. Enhanced extrinsic apoptotic pathway, with specific cleavage of caspase-8 and increased production of tumour necrosis factor-alpha (TNF-a) in tumours preconditioned by calcitriol before receiving ALA-PDT was observed. Hyp was investigated for its effects on head and neck SCC *in vitro* (HNSCC). Cells incubated with or without Hyp (5-50μM) were irradiated with HQI®-TS-lamp (450-700nm,) for 10-25min. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide- and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay was used to score metabolic and apoptotic activity. Massive reduction of metabolism and excessive apoptosis were observed in almost 100% after Hyp-PDT, on HNSCC cells treated with the combination of Hyp and light. No apoptosis displayed in cells treated with Hyp or lamp alone.[63] Cell death in PDT using aluminium disulphonated phthalocyanine (AlS_2Pc) and Pc-4-PDT has been shown to be via apoptosis whereas ZnPc can induce necrotic or apoptotic cell death.[64, 65, 66]

PHOTODYNAMIC DIAGNOSIS

The current gold standard method of skin cancer diagnosis is the *in vivo* sampling to evaluate status of skin sample as normal or abnormal tissue but this procedure is associated with a number of side effects including false interpretation, multiple biopsies, scarring, pigmentation changes, pain and inflammation. Hence, other skin diagnosis approaches including photodynamic diagnosis (PDD) are explored. PDD is a minimally invasive drug induced fluorescence method currently used for demarcation of malignant tissue, monitoring of residual or recurrent tumours during and after PDT treatment.[67-69] However, photo-bleaching PS, absorption and scattering of light, limit proper fluorescence measurements in skin.[68, 70] Photo-bleaching describes the decrease in PS absorbance and fluorescence caused by light exposure such that the chromophore is decomposed into small fragments that do not absorb visible light.[70] Fluorescence of ALA was observed in 61 of 93 participants in a study conducted using a system named Dyaderm® combined with ALA to detect early NMSC on skin lesions.[71] Additionally, demarcation of BCC margins in PDT was achieved using MAL in conjunction with Wood lamp and by means of ALA/PpIX fluorescence.[12, 72]

Insertion of fibre optics from a KTP532 surgical laser was successfully used to visualize fluorescence of Hyp to destroy SCC in nude mice.[73] Photophysical and photochemical properties of Hyp with absorption peak around 590 nm, and fluorescence peak around 600 nm make this pigment a potent fluorescent marker for clinical tumour diagnosis.[43, 74] High selectivity of tumour targeting properties of zinc (II)-octadecylphthalocyaninato (ZnODPc) incorporated into a Cremophor emulsion in mice bearing an intramuscularly transplanted fibrosarcoma was reported.[75] Aluminium (III) phthalocyanine chloride tetrasulphonate ($AlPcS_4Cl$) has been shown to localize in mitochondrial and lysosomes of human melanoma cell line, Figure 2, and its fluorescence characteristic and potential to induce phototoxic effects in melanoma, Figure 3, suggests it is a potential sensitizer for treatment and possibly diagnostic applications of melanoma skin cancer.[19, 76] Limited reports on the efficacy of Hyp and Pcs for PDD of skin have been published.

CONCLUSION

Sun protection at an early stage in human life can reduce incidences of skin cancer. The decay of the ozone layer poses a threat to human skin diseases. Complete destruction of most skin tumours can be achieved if found and treated early. PDT uses different PS to treat different forms of cancer. Even though during Hyp-PDT several pathways to promote or inhibit cell death process are stimulated, it may be utilized as powerful tumour targeting drug due to its photostability.

Scientists around the world are working on the development of powerful lasers combined with flexible optical fibres; Ps conjugated with nanoparticles, creams and jells as delivery systems that can enhance the effectiveness of PDD and PDT treatments. PDD can possibly be used as an alternative method of skin cancer diagnosis over the current gold standard method of skin diagnosis. Ideally, photostability of PS is desirable for sufficient tumour-visualizing time. The rise in skin cancer cases

globally each year with at least 850 deaths in South Africa from melanoma, calls for an early diagnostic and treatment tools for such a potentially deadly disease.

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