Advances in Photodynamic Therapy of Cancer

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Abstract: Photodynamic therapy (PDT) is based on the use of a photosensitizing agent such as porphyrins, although the earliest descriptions of PDT involved the use of eosin. Many of the molecular details of PDT are unclear, while the basic premise of PDT is simple. A photosensitizing agent, either endogenous or exogenous, is exposed to an activating light source and in the presence of oxygen produces activated intermediates, primarily singlet oxygen. Singlet oxygen is a highly reactive molecule that oxidises carbon-carbon bonds and can damage various components of the target cell (e.g., lipids, nucleic acids, etc.) directly leading to cell death. Porphyrins preferentially bind to a receptor located on the outer mitochondrial membrane called the peripheral benzodiazepine receptor. Photoactivation leads to release of mitochondrial cytochrome c and increases levels of caspases 3 and 9. Caspase 3 plays a major role in apoptosis via protein cleavage.

Currently, much research is underway to help identify adjuvant treatments to potentiate the photodynamic response. Active areas of investigation include the use of agents to facilitate cutaneous penetration of photosensitizing agents, amplify the response of macrophages and other inflammatory cells, decrease angiogenesis, inhibit matrix metalloproteinase and cycloxygenase II, and activate the complement cascade. The experimental evidence is that porphyrins accumulate in the tumour tissue. Our results indicate that phenylalanine and tyrosine are the amino acids that have the most affinity in binding HpD. Recently, our investigations *in vitro* have sought to reduce the side effects of PDT and cytostatic therapy by using reduced amounts of compounds with a high rate of toxicity in anti-cancer protocols.

Keywords: Photodynamic therapy, cancer, hematoporphyrins, active transport, LDL, apoprotein receptor, cytostatic drugs.

INTRODUCTION

Photodynamic therapy (PDT) is a new approach to cancer treatment that involves the administration of a tumour-localizing photosensitizing agent (PS) followed by its activation by light of a specific wavelength, resulting in a sequence of photochemical and photobiological processes that cause irreversible photodamage of tumour tissues. The mechanism of photoactivation involves the use of an activated photosensitiser that produces cytotoxic reactive oxygen species (ROS), resulting in cell membrane damage and subsequent cell death (Fig. **1**). At the beginning of the use of this treatment, the potentiality of PDT was not fully appreciated; only recently has the true potential of PDT begun to be fully appreciated; this modality of treating disease can be traced far back in history. The ancient Egyptians used a combination of orally ingested plants (containing light-activated psoralens) and sunlight to successfully treat vitilago over 4000 years ago [1]. The use of ultraviolet light and psoralens for the treatment of psoriasis (psoriasis ultraviolet A, PUVA) has been accepted throughout the world [2]. Contemporary PDT began when Raab described, in 1900, the action of acridine dyes and light on *Paramecia*. Raab showed that these unicellular organisms could be effectively killed with this combination [3]. In 1903, Tappeiner treated a skin cancer with topically applied eosin and light [4]. In 1913, Meyer-Betz injected himself with 200 milligrams of hematoporphyrin derivative and registered no ill effects until he exposed himself to sunlight, whereupon he suffered extreme swelling; this photosensitivity remained for several months [5,6]. In 1925, Policard examined the ability of porphyrins to produce a phototoxic effect [7]. The most recent photoactive drug therapies utilise porphyrin-based chromophores in combination with visible light. Phototherapy research was dormant for several decades, although the idea that light could act as a therapeutic modality was explored extensively. For instance, a book published in 1933 lists over a thousand papers exploring UV light for the treatment of a wide variety of ailments, including arthritis, colitis, lupus, and mental diseases [8]. The usefulness of high-dose light might, at first sight, not seem rational for the treatment of such diseases but in the case of auto-immune disorders, the immunosuppressive nature of UV light is now well established [9].

Nowadays photodynamic therapy (PDT) is based on the use of photosensitizing agents such as porphyrins; although the earliest descriptions of PDT involved the use of eosin, eosin has been replaced by porphyrin as the photosensitiser of choice. The fluorescent properties of hematoporphyrin were first described in the mid-1800s [10], and in 1911, Hausmann published a description of photosensitivity in mice injected with hematoporphyrin and subsequently exposed to light [11]. This work was followed in 1913 by a demonstration by Meyer-Betz that by itself demonstrated a

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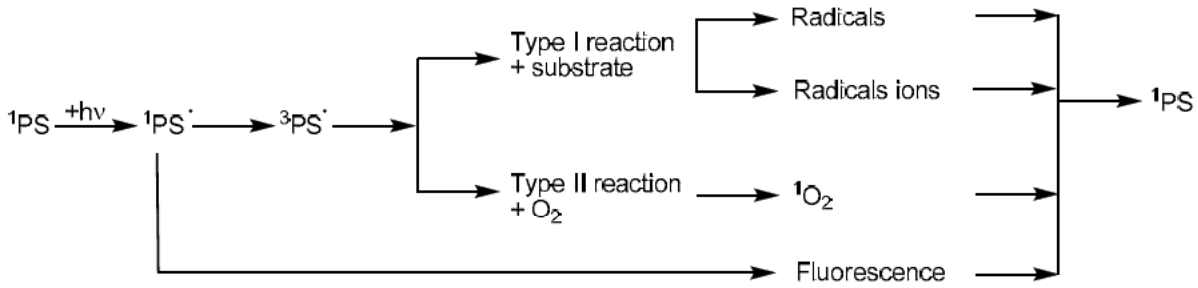

Fig. (1). The principle of photodynamic therapy.

pronounced photosensitisation after injection of hematoporphyrin [5]. In 1924 Policard documented the preferential uptake of hematoporphyrin in malignant tissue as compared with normal tissue [12]. In 1942, Schwartz recognised that the hematoporphyrin previously used was a mixture of porphyrins with different chemical and physical properties [13]. This observation led to the development of hematoporphyrin derivative (HpD), an esterified porphyrin compound with a more pronounced phototoxic effect and a greater affinity for malignant tissue [14,15]. Despite the early work done by von Tappeiner and Jesionek in 1907, the therapeutic potential of PDT was largely neglected until 1972, when Diamond and colleagues [16] found that light activation of hematoporphyrin destroyed rat glioma cells *in vitro* and *in vivo*. However, before that study, many papers focusing on attempts to use fluorescent porphyrins as a means of tumour localisation were published [14,15,17–21]. Finally, in 1975, Dougherty found a complete cure for experimental animal tumours by using, for the first time, systemic HpD activated by red light [22]. This success was followed by human studies, and in 1978, Dougherty described response of primary and secondary cutaneous tumours to HpD activated by xenon arc lamp red light exposure [23]. Numerous described the use of PDT for multiple clinical applications, including the treatment of pulmonary [24–26], bladder [27,28], oesophageal/gastric [29–31], gynaecologic [32], brain [33], and rectal cancers [34] and intraocular disease [35].

Although clearly effective, a major drawback of HpD-PDT is prolonged and pronounced photosensitivity, typically lasting four to six weeks [36]. This limitation prompted the search for alternative photosensitizing agents. HpD (commercially available as Photofrin; Axcan Pharma, Montreal, Canada) remains the most commonly used systemic agent; however, for most dermatologic conditions, a systemic agent is not required.

In 1990, Divaris and coworkers [37] reported that the injection of mice with 5-aminolevulinic acid (ALA), a naturally occurring metabolite involved in heme biosynthesis with no intrinsic photosensitizing effect, resulted in phototoxic damage to the sebaceous glands, hair follicles, and keratinocytes. Soon after, Kennedy and Pottier [38,39] documented peripheral conversion of ALA to protoporphyrin IX (PpIX), a known potent photosensitiser, and the response of human cutaneous tumours to ALA-PDT. ALA-PDT involving blue light activation was initially approved by the US Food and Drug Administration (FDA) for the treatment of actinic keratoses in 1999, and PDT using blue light alone (BLU-U; Dusa Pharmaceuticals, Toronto, Canada) was first approved by the FDA for the treatment of acne in 2003. The ability to develop photosensitizing agents that are selective for malignant or otherwise abnormal tissue and to limit the field of light exposure allows for the treatment of diseased skin with little damage to surrounding normal tissue.

In order to achieve the most efficient photosensitizing effect on tumour cells, the sensitiser must enter the cell and become closely associated with the subcellular structures. Photosensitisers may enter cells either directly through the plasma membrane or by endocytosis. Moreover, uptake over the plasma membrane may occur by simple or facilitated diffusion or by an active transport mechanism. The incubation parameters and mode of delivery as well as the chemical nature of the photosensitiser (molecular size, charge, water-lipid partition coefficient, concentration), the type and physiological state of the cell, the environmental conditions and the nature of the carrier can all influence subcellular localisation, creating a number of potential targets for photodamage [40,41].

Studies on the transport of HpD (hematoporphyrin derivative) by high- and low-density proteins (VLDL, LDL and HDL) have suggested that apparently lipoproteins possess two classes of binding sites for Hp, which are probably located in the apoprotein matrix and the lipid core, respectively. Lipoproteins play an important role in the transport of hematoporphyrins (Hp and HpD) in the bloodstream; it has been suggested that the tumour-localising property of the porphyrin photosensitisers used in photodynamic therapy (PDT) is related to such binding, particularly to low-density lipoprotein. In fact, LDL binds two specific receptors on cellular membranes by arginyl and lysyl residues. Chemical modification of these residues abolishes the interaction between lipoproteins and receptors both *in vitro* and *in vivo*. On the other hand, *in vitro* studies have demonstrated that tumours have affinity binding for Apo A-I. To elucidate the transport mechanism of porphyrins, we have studied the affinity of HpD to proteic amino acids by nuclear magnetic resonance (NMR), using a standard mixture [42].

The aim of this review is to characterise any specific chemical binding between the HpD and a cytostatic drug.

MECHANISM

Although many of the molecular details of PDT are unclear, the basic premise of PDT is simple. A photosensitizing agent, either endogenous or exogenous, is exposed to an activating light source, in the presence of oxygen, which activates intermediates, primarily singlet oxygen. Singlet oxygen is highly reactive (ROS): it oxidises carbon-carbon bonds

and can damage various components of the target cell (e.g., lipids, nucleic acids), leading directly to cell death [43]. Singlet oxygen can also destroy the photosensitizing agent itself, limiting further effect, a process referred to as photobleaching. Further effects include indirect pathways of cellular destruction by recruitment of inflammatory cells [44–48], anti-tumour immune stimulation [46,49], and vascular damage [50,51].

Principle of PDT

PDT involves the administration of a photosensitiser followed by local illumination of the tumour with light of the appropriate wavelength to activate the specific drug. Activation of the photosensitiser upon absorption of the light transforms the drug from its ground state (1PS) into an excited singlet state (1PS*, Fig. **1**). From this state, the drug may decay directly back to the ground state by emitting fluorescence, which is a property that can be used clinically for photodetection. However, to obtain a therapeutic photodynamic effect, the photosensitiser must undergo electron spin conversion to its triplet state (3PS*). In the presence of oxygen, the excited molecule can react directly with a substrate, by proton or electron transfer, to form radicals or radical ions, which can interact with oxygen to produce oxygenated products (type I reaction). Alternatively, the energy of the excited photosensitiser can be directly transferred to oxygen to form singlet oxygen (type II reaction), which is the most damaging species generated during PDT [52].

Histologic examination of tissue after PDT reveals cell necrosis and apoptosis occurring within 1 day after PDT [53–57]. Typically, the type of cellular response is linked to the localisation of photosensitiser within the target cell. Normally, porphyrins preferentially bind to a receptor located on the outer mitochondrial membrane called the peripheral benzodiazepine receptor. In particular, cancer cells show elevated levels of mitochondrial benzodiazepine receptors, which bind porphyrins with high affinity. Consequently, the quantity of porphyrins in cancer cells is higher than in normal cells [58]. Photoactivation leads to release of mitochondrial cytochrome c [59] and increased levels of caspases 3 and 9 [60,61]. Caspase 3 plays a major role in apoptosis via protein cleavage [62]. Such destructive forces may be modified by BCL-2, a protein that is also located on the outer mitochondrial membrane; however, the exact mechanism is unclear [63–65]. Subsequent photoactivation results in endothelial damage and fibrosis, local vascular collapse via platelet activation and thrombus formation, and increased recruitment of polymorphonuclear leukocytes, all of which can lead to ischemic tissue necrosis and tumour regression [66,67].

Endothelial cells selectively absorb certain photosensitizing agents: benzoporphyrin derivative monoacid ring (BPD-MA, verteporfin), porfimer sodium, meta-tetrahydroxyphenylchlorin (temoporfin), and tin ethyl etiopurpurin (SnET2, rostaporfin). The efficacy of PDT also depends on the photosensitiser used, its ability to penetrate diseased tissue selectively, the duration of application, the period of light exposure, the nature of the activating light source, the oxygenation status of the tissue, and the type of cells involved. To have an effect, the damage caused by treatment must surpass cellular repair mechanisms, a phenomenon referred to as *the minimum photodynamic dose*. Currently, much research is being done to help identify adjuvant treatments to potentiate the photodynamic response further. Active areas of investigation include the use of agents to facilitate cutaneous penetration of photosensitizing agents [68–70], in order to amplify the response of macrophages and other inflammatory cells [71–74], decrease angiogenesis [75], inhibit matrix metalloproteinase [76] and cycloxygenase II activity [77], and activate the complement cascade [78].

Mechanism of the Tumour-Localising Effect in PDT

The tumour localisation by PDT occurs as follows:

(i) Cancer cells, similar to other rapidly proliferating cells, may have an increased requirement for cholesterol for membrane biosynthesis. They may therefore upregulate expression of the low-density lipoprotein (LDL) receptor (which recognises the apoB/E lipoprotein) [79]. It is known that lipoproteins are major carriers of lipophilic porphyrins in the bloodstream [80] and may therefore be a means of entry of these compounds into cells.

(ii) Decreased intratumoural pH may affect the ionisation of porphyrin species with weakly acidic pK values, thus retaining them within tumours [81].

(iii) Tumours often contain increased numbers of lipid bodies and particularly neutral lipid droplets; in addition, their cell membranes may be more hydrophobic than those of normal cells. Both phenomena might explain the accumulation of hydrophobic photosensitisers [82].

(iv) A combination of "leaky" tumour vasculature and reduced lymphatic drainage might encourage the build-up of porphyrins (whether as aggregates or protein complexes) in the interstitial space [83].

(v) Tumour cells may have increased capabilities for phagocytosis or pinocytosis of porphyrin aggregates [84].

(vi) Tumour-associated macrophages (TAM) may be largely responsible for the concentration in tumours [85]. Korbelik *et al.* have found that TAM may contain up to nine times the porphyrin levels present in tumour cells [86]. Many experimental tumours can comprise up to 80 % (Inserire unità di misura) TAM [87].

Even in human cancers, TAM can represent 20-50 % of the cellular content. High macrophage content is also a consideration at all the other sites of photosensitiser accumulation listed above. Photodynamic therapy induces a highly complex series of changes in cells. The sequence of events in PDT is shown in the following Fig. (**2**), from which it can be seen that complete establishment of the protocol requires wider study of biochemical and photochemical phenomena (Fig. **3**).

PDT is likely to affect multiple cell targets, of which cell membranes and mitochondria are of particular importance [88]. These cell targets may also include lysosomes, endoplasmic reticulum, DNA and microtubules [76,87,88]. Following exposure, cells experience a rapid increase in calcium concentration accompanied or followed by other electrolyte changes as membrane damage progresses.

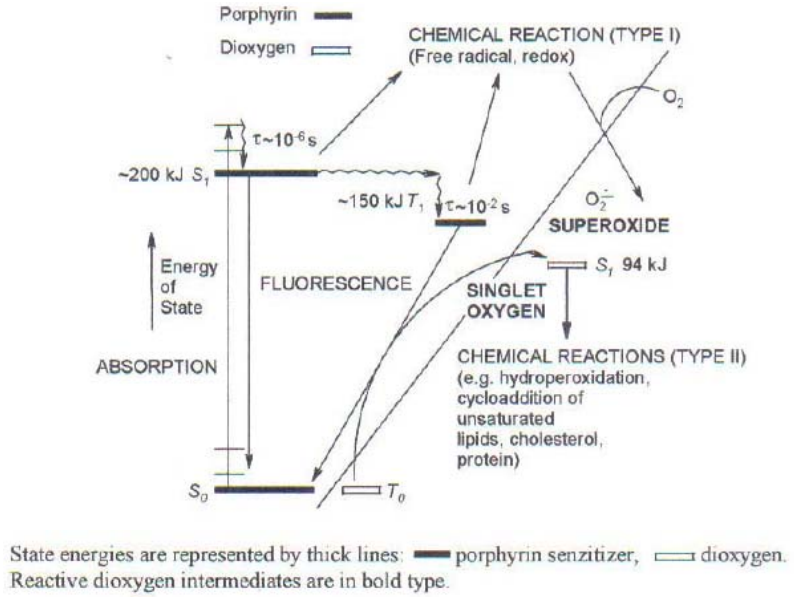

Fig. (2). Light-induced excitation states of porphyrin and formation of singlet oxygen.

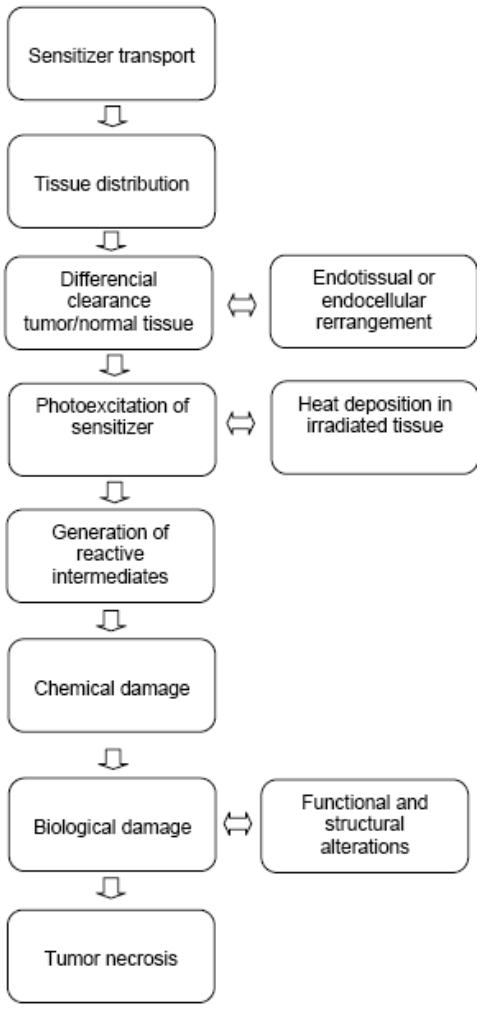

Fig. (3). Sequence of events in PDT.

Sublethal damage may, via various signal transduction pathways, result in apoptosis characterised by a drop in mitochondrial potential, concurrent with a drop in ATP level and a decrease in cell respiration, translocation of phosphatidylserine in the plasma membrane, DNA fragmentation, appearance of apoptotic bodies and eventually loss of plasma membrane integrity [89]. The signalling cascades involved in this process are under investigation. The involvement of components of the signalling network such as cell surface death receptor Fas [90], tumour necrosis factor (TNF) and TNF-related apoptosis-inducing ligand TRAIL [91] as well as downstream molecules such as caspases [92] and Bcl-2 family members [93] have been demonstrated in various PDT-induced models of cell death. Recently, protein phosphorylation has been highlighted as an important regulator of the apoptotic process [94]. The apoptotic signalling cascade in photosensitised human epidermal carcinoma cells was mediated by two-stage activation of the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) [95]. The selective retention of PS in neoplastic tissues is essential for in vivo efficacy of PDT. It is determined, among other factors, by the hydrophobicity and aggregated state of the PS, decreased pH in tumours, tumour neovascularisation, poorly developed tumour lymphatics, stromal cell differences and heterogeneity of the cells within the tumour [96]. The asymmetry of charge distribution has also been suggested as an explanation for the higher uptake of PS [97]. The underlying mechanism reveals a complex interaction of direct and indirect anti-tumour effects triggered by PDT, which may mediate tumour destruction. Tumour cell death results from lethal events initiated by reactive oxygen species. Indirect PDT effects represent necrosis resulting from damage of tumour-associated vasculature with subsequent infarctive death of the tumour cells and initiation of a post-treatment immune response directed against tumour cells [41,98]. The effects of PDT were found to be modulated by dose, or dose rate changes, conjugations of photosensitisers to lipoproteins or liposomes, or by the addition of chemotherapeutic agents. The response of different tumours to PDT is highly variable, ranging from high sensitivity to extreme resistance. Factors such as photosensitiser localisation properties at different

levels (tumour tissue, cellular and intracellular distribution) and tumour oxygenation/vascularity have been identified as the parameters determining tumour sensitivity to PDT. However, a number of other physiological properties characterizing individual tumours may exert a marked influence on the therapeutic outcome. One such property appears to be tumour immunogenicity, since immune reaction induced by PDT against treated tumours can substantially contribute to the cure. The local level of nitric oxide (NO), which directly influences multiple events participating in the anti-tumour effect of PDT, is another important but less recognised parameter [99]. The relevance of this radical, whose production varies considerably in different cancers, to the process of PDT-mediated tumour destruction, has been the subject of recent studies. Many reports in the current literature are confusing and often apparently contradictory. There is clearly the need for much elucidation and future studies should more systematically address phenomena in a range of cell types, photosensitisers, and treatment conditions.

Working Mechanism of PDT *In Vivo*

The efficacy of PDT in the treatment of cancer depends on the type of photosensitiser, drug concentration and intracellular localisation, light dose (fluence) and dose rate (fluence rate), and oxygen availability. Singlet oxygen generated by the photochemical reaction can directly kill tumour cells by the induction of apoptosis and necrosis. It also damages the vasculature of the tumour and surrounding healthy vessels, resulting in indirect tumour death via the induction of hypoxia and starvation. In addition, PDT is able to initiate an immune response against the remaining tumour cells. The outcome of PDT is dependent on all these mechanisms and the relative contribution of each depends on the treatment regimen given [100].

Singlet oxygen is highly reactive and can diffuse only 0.01–0.02 μm during its brief lifetime. Therefore the sensitiser should be localised close to its target at the time of illumination. With the original aim of specific tumour cell death, it was assumed that the optimum response would be obtained for illumination at times when tumour drug levels were high relative to levels in surrounding normal tissues. However, recent animal studies [101, 102] and clinical data [103] have shown that the concentration of sensitiser in the plasma at the time of illumination does not necessarily predict response to PDT. Plasma concentration at the time of illumination is a better indicator of PDT outcome [104–107]. This suggests that tumour cells may not always be direct targets of PDT, but rather indirectly killed as a result of damage to other cell types, for example, vascular endothelial cells.

The relationship between the severity of PDT-induced vascular damage and tumour response has been well documented. Results show that treatment schedules that fail to induce complete shutdown of vessels feeding a tumour will not result in long-term cure [108, 109–113]. Furthermore, inhibition of inflammatory responses and vasoconstriction decrease tumour response to PDT, whereas concomitant pharmacological inhibition of angiogenesis enhances the PDT response [114–117, 118–122]. All these studies demonstrate the importance of vascular-mediated damage in obtaining an effective tumouricidal effect after *in vivo* PDT. The majority of evidence suggests that vascular sensitivity to PDT is a result of physiological factors, such as high local concentrations of both oxygen and photosensitiser in the blood vessels, rather than intrinsic sensitivity of endothelial cells. If the tumour vasculature is the primary target for PDT, the therapeutic benefit results from differences in vascular integrity between tumours and surrounding normal tissues rather than specific drug uptake in the tumour cells.

Applications in Cancer Therapy

The first clinical application of photodynamic therapy (PDT) was described by von Tappeiner and Jesionek in 1903 [100]. The authors applied eosin topically to basal cell carcinomas (BCCs) prior to illumination. von Tappeiner and Jodlbauer later defined PDT as the dynamic interaction among light, a photosensitizing agent, and oxygen resulting in tissue destruction [101]. It took another 70 years, however, before the possibilities of PDT for the treatment of cancer were fully recognised.

In 1975, Dougherty et al. [102] reported that hematoporphyrin derivative (HpD) in combination with red light could completely eradicate mouse mammary tumour growth. Clinical trials were subsequently initiated with HpD to treat patients with bladder cancer and skin tumours [103, 104]. These studies were successful; subsequently, numerous trials were initiated involving a variety of cancers and photosensitisers. This resulted in the approval of PDT, involving the use of porfimer sodium (Photofrin®; Axcan Pharma Inc., Mont-Saint-Hilaire, Canada), for the treatment of bladder cancer in Canada in 1993. Today, three more sensitisers are approved for clinical use—5-aminolevulinic acid (ALA, Levulan®; DUSA Pharmaceuticals Inc., Wilmington, MA), the methyl ester of ALA (Metvix®, Photocure ASA, Oslo, Norway), and *meso*-tetra-hydroxyphenyl-chlorin (mTHPC, temoporfin, Foscan®; Biolitec Pharma Ltd., Dublin, Ireland), and PDT is becoming an established treatment modality for localised cancers (Table **1**).

Fig. (**4**) shows the molecular structures of photosensitisers approved for use in PDT. In Figure 5, the visible spectrum of ALA is compared with that of porfimer sodium. The main characteristics of porfimer sodium, ALA and mTHPC are compared in Table **2**.

The drug (photosensitiser) is the essential part of PDT. An ideal drug should have the following properties:

(i) Proper absorption wavelength: Due to light absorption by endogenous chromophores, mainly hemoglobin and light scattering, the effective light penetration through tissue is very poor in the low-wavelength region of the visible spectrum (Wilson 1989) [124]. As the wavelength increases, effective light penetration increases as well. Experiments indicate that light penetrates effectively through tissue in the red to the near-infrared region (≥650 nm) (Wainwright 1996, Lown 1997) [125,126]. As a result, the ideal drug is one that displays strong absorption in such a region (≥650 nm).

(ii) High preference for accumulation in the tumour: The drug must have a selectivity for enrichment in the tumour tissue as opposed to the normal tissue. Since singlet oxygen is also detrimental to the healthy tissues, a differentiation of drug concentration between biological compartments must

Table 1. Type of Cancer and Approved Drug (*De Rosa et al.)*

Type of Cancer	Photosensitizer	Country
Actinic keratosis	ALA (Levulan®, Metvix®)	U.S., EU
Basal cell carcinoma	ALA (Metvix®)	EU
Barrett's HGD	Porfimer sodium	U.S., Canada, EU, UK
Cervical cancer	Porfimer sodium	Japan
Endobronchial cancer	Porfimer sodium	Canada, Denmark, Finland, France, Germany, Ireland, Japan, The Netherlands, UK, U.S.
Esophageal cancer	Porfimer sodium	Canada, Denmark, Finland, France, Ireland, Japan, The Netherlands, UK, U.S.
Gastric cancer	Porfimer sodium	Japan
Head and neck cancer	Foscan	EU, Norway, Iceland
Papillary bladder cancer	Porfimer sodium	Canada

Abbreviations: ALA, 5-aminolevulinic acid; EU, European Union; HGD, High-grade dysplasia; UK, United Kingdom.

Table 2. Characteristics of Porphyrin Sodium, ALA and mTHPC (*De Rosa et al.*)

Photosensitizer	Porfimer Sodium	ALA	*mTHPC*
Maximum absorption (nm)	630	635	652
Absorption coefficient ($cm^{-1}mol^{-1}l^{-1}$)	1,170	5,000	22,400
Drug dose (mg/Kg)	2	20%[a]	0.10-0.15
Drug-light interval (h)	48-72	3-6	96
Fluence (J/cm2)	100-200	100	10-20
Fluence rate (mW/cm2)	100	100-150	100

[a]Given as topical solution.
Abbreviations: ALA, 5-aminolevulinic acid; mTHPC, meso-tetra-hydroxyphenyl-chlorin.

be achieved before the irradiation. This ensures that the efficient destruction of the diseased tissue takes place, while the healthy tissue remains intact or experiences less ill effect.

(iii) Low dark toxicity and quick metabolisation: The PDT drug itself should be non-toxic in the absence of light. The drug should be excreted or metabolised quickly in a way that does not generate toxic metabolites of any kind after the treatment is complete.

(iv) From the standpoint of chemical synthesis, the drug should be made from readily available materials. The protocol for synthesis should be simple and conducive to use at an industrial scale. The PDT drug should contain groups, such as phenyl groups, that allow easy derivatisation or variation in order to optimise various properties of the drug.

(v) The drug should exhibit some preferred physical or photophysical properties for drug administration, such as good solubility in water and in the body tissue fluid, easy formulation (Woodburn *et al.* 1994) [127], high quantum yield of triple formulation (with a triplet energy greater than 94 kJ/mol) and high singlet oxygen quantum yield.

New insight on HpD Transport by Lipoprotein and Serum Protein

LDL is the major cholesterol carrier in plasma and its uptake is mediated by the LDL-receptor (LDL-R), a glycoprotein present on the surface of most cells. Interaction between lipoproteins and apo-B,E receptors was established by selective chemical modification of specific amino acid residues. The chemical modification of a limited number of arginyl residues by 1,2-cyclohexanedione, and of lysyl residues by acetoacetylation, reductive methylation, and carbamylation, not only established the importance of the apoproteins, but also demonstrated the significance of these particular amino acid residues in the recognition process. The modification of relatively few arginyl or lysyl residues completely prevents LDL or HDL-with apo-E from binding to cell surface receptors on fibroblasts [128].

Regulation of the LDL-R occurs primarily at the transcriptional level and mainly depends on the sterol content of cells [129-131]. In addition to sterol-dependent suppression of LDL-R transcription, other mechanisms of regulating LDL-R synthesis have been reported [132,133]. The LDL-R is upregulated early in the course of growth stimulation in

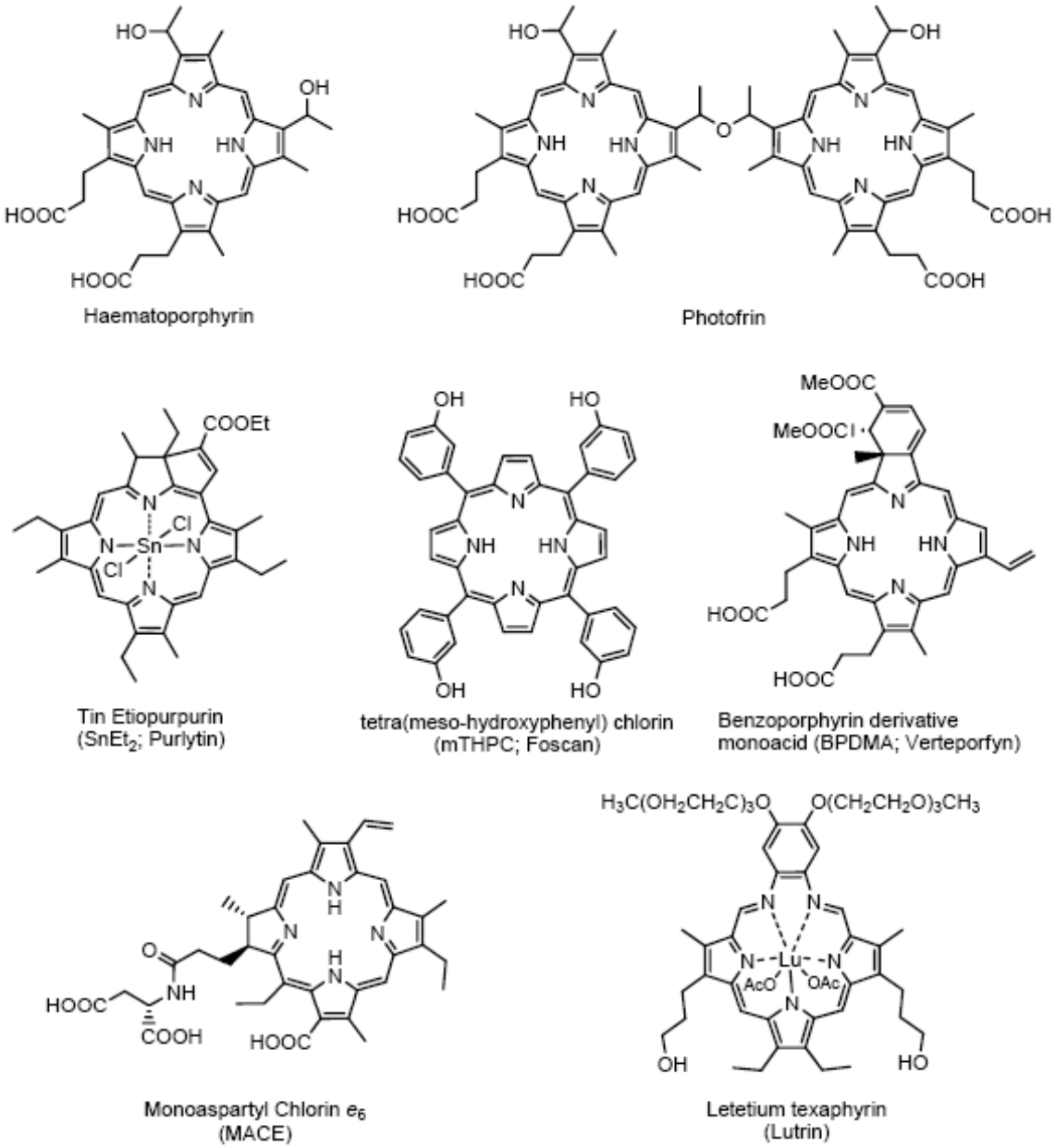

Fig. (4). Molecular Structures of photosensitizers approved for use in PDT.

cell culture [134], and mitogenic activation of human lymphocytes increased LDL-R expression [135]. This type of LDL-R regulation might be important in cancer cells with deregulated proliferation and signal transducing mechanisms. Therefore the number of LDL-R is particularly high in hyperproliferating cells, including tumour cells [136].

Continuously replicating cancer cells have an urgent need for cholesterol, used primarily to build up membranes. Epidemiological studies have shown a relationship between hypocholesterolemia and cancer [137], and certain tumours are known to be associated with low serum cholesterol [138,139]. Increased low-density lipoprotein (LDL)-uptake by tumour cells has also been found to induce hypocholesterolemia [138-140].

Lipoproteins are major carriers of lipophilic porphyrins in the bloodstream [78] and may therefore be a means of entry of these compounds into cells. Tumours often contain increased numbers of lipid bodies and particularly neutral lipid droplets; their cell membranes may be more hydrophobic than those of normal cells. Both phenomena might explain the accumulation of hydrophobic photosensitisers [80]. Tumour cells may have increased capabilities for phagocytosis or pinocytosis of porphyrin aggregates [82]. Tumour-associated macrophages (TAM) may be largely responsible for the concentration in tumours [83]: Korbelik *et al.* found that TAM may contain up to nine times the porphyrin levels present in tumour cells [84]. Experimental tumours can be up to 80 % TAM [85].

The porphyrin skeleton is essentially hydrophobic, and a working hypothesis has emerged that tumour localisation can be improved by the introduction of polar substituents to confer amphiphilicity and improve selectivity [129]. The bind-

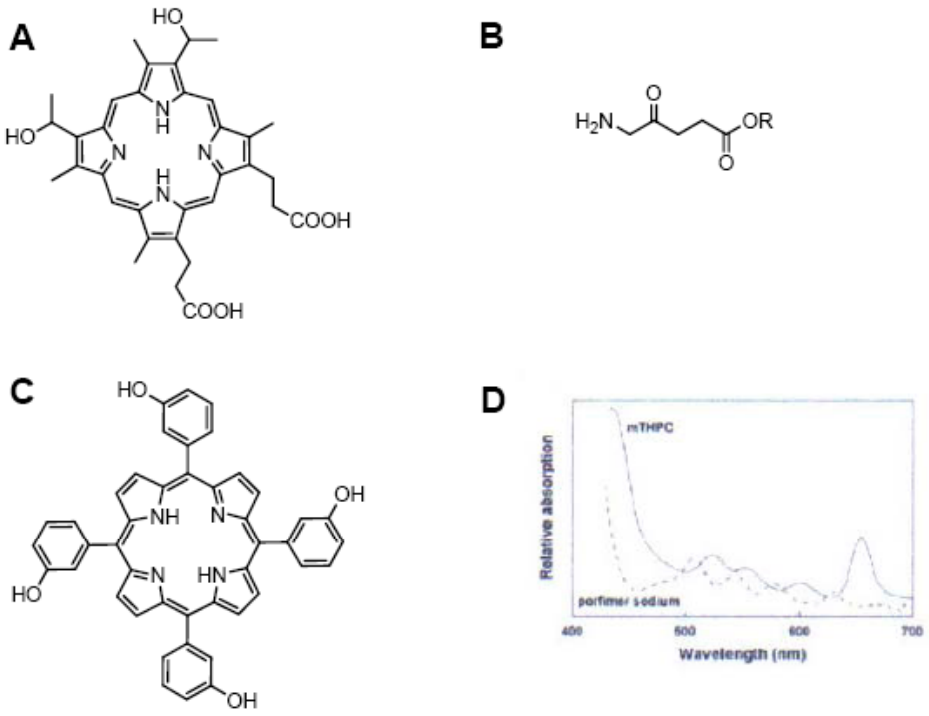

Fig. (5). Comparison of absorption spectrum of ALA with absorption spectrum of porfimer sodium.

ing of a given photosensitiser to LDL seems to be related to its hydrophobicity [82]; however, increasing the number of polar residues in HpD renders the molecule water soluble, allowing it to be transported by serum protein [78].

Porphyrin Platinum Conjugate

Porphyrin platinum conjugates combine platinum fragments and porphyrin systems in the same molecule. The approach is useful for two different approaches in cancer chemotherapy: 'cytostatic therapy' with platinum derivatives and 'photodynamic therapy' with porphyrin compounds. Such porphyrin platinum conjugates should show the cytostatic activity characteristic of the platinum part. Upon irradiation, the photodynamic component of the porphyrin sensitiser should be activated. Furthermore, porphyrin platinum conjugates should exhibit greater tumour selectivity than mixtures of platinum and porphyrin compounds. While porphyrin derivatives enrich in cancer tissues [1,2], platinum complexes such as cis platinum and carboplatinum penetrate non-selectively into all rapidly growing tissues, giving rise to side effects such as nausea, vomiting, myelosuppression and nephrotoxicity.

Thus, selective enrichment of platinum compounds in tumours is expected with porphyrin platinum conjugates. The first papers on this topic have been published [3 /6] Anti-tumour active platinum compounds of type L2PtX2 contain L ligands (non-leaving groups) and X ligands (leaving groups). Typical leaving groups include chloride and carboxylates. Within the cells, they are replaced first by water ligands and finally by DNA nucleophiles (or biomolecules). Typical non-leaving groups include ammonia and amines. Amine ligands should contain NH groups to form hydrogen bonds [10,11], with primary amines serving the purpose most effectively.

Since the discovery of the cytotoxic activity of *cis*-platin (*cis*-diamminedichloroplatinum(II)) [137, 138], research efforts have been directed towards elucidation of the molecular mechanism of its action [2–4] and synthesis of new platinum compounds with improved antitumour activity [5] and reduced side effects. To date, a large library of platinum complexes has been synthesised, and their antitumour activity has been examined, but only *cis*-platin, carboplatin, and oxaliplatin have received worldwide approval and achieved routine clinical use. At present, the cytotoxicity of *cis*-platin and other platinum(II) complexes is thought to originate from their interaction with DNA as well as with non-DNA targets [2, 6–8] and subsequent induction of cell death through apoptosis and/or necrosis [9, 10]. Despite its wide application as an antitumour drug for the treatment of different types of cancer, *cis*-platin has several inherent shortcomings, such as limited solubility and toxic side effects including nausea, nephrotoxicity, vomiting, and so forth [11]. In addition, many tumours display natural resistance to *cis*-platin and others develop resistance after the initial treatment [12]. Thus the application of the drug is restricted to a relatively narrow class of tumours. Research efforts were focused on the design of new platinum compounds with a broader spectrum of antitumour activity and improved pharmacological properties with respect to toxicity and resistance. Several complexes structurally related to *cis*-platin, such as oxaliplatin and carboplatin, nedaplatin (Japan), lobaplatin (China), and heptaplatin (South Korea) [5, 13, 14], are currently in clinical trials and use, but as yet have not demonstrated significant advantages over *cis*-platin. It seems possible that the complex structural analogues of *cis*-platin designed on the basis of structure-activity relationships [15] offer few serious advantages over the existing drugs. Analysing the current status of the platinum-based antitumour drugs

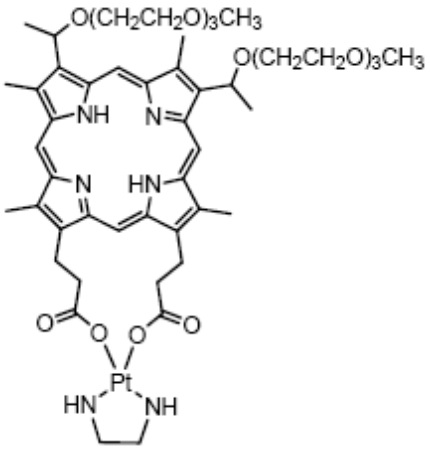

Fig. (6). Platinum (II) complex of pegulated hematoporphyrin.

[5] suggests that the search for improved platinum antitumour agents should continue [16–18] with efforts to modulate the DNA-binding and DNA damage induced by the structure of the platinum drug used.

Improvement of PDT

Our early study sought to elucidate the transport mechanisms of porphyrins to improve photodynamic therapy and suggest a regimen of anti-blastic chemo-therapeutic drugs for use in neoplastic cells. For this purpose, we verified the interaction between HpD and lipoprotein using polyacrylamide gel isoelectric focusing, as shown in Fig. (**6**), which displays the electrophoretic profiles of HpD, A-I and the mixture of HpD and A-I. The HpD pattern was not detectable because the gel was stained with Coomassie G-250, which is specific for proteins. However, many red bands were observed on the gel in acid pH range from 5.0 to 4.0, prior to Comassie G-250 staining (data not shown). The A-I pattern showed a single band in a pH range around 7.0, whereas the mixture pattern showed, in the same pH range, more blue bands (La figura non è a colori. Rivedere il testo o inserire foto a colori) – as many as were observed for the HpD pattern. The remarkable heterogeneity observed in the mixture pattern suggested that the isoelectric point of A-I was modified by different forms of HpD. Notably, the preparation of HpD generates abundant derivative molecules [7]. This result confirmed the interaction between HpD and lipoproteins. To ascertain the nature of this interaction, NMR analysis of a small protein, human α-LA (14.4 kDa), was performed in the presence and absence of HpD, as shown in Fig. (**7**). The NMR spectrum for human α-La in the presence of HpD revealed modified and broad peaks in the resonance field from 6.5 to 8 ppm, characteristic of aromatic protons, and the appearance of two wide peaks in the resonance field from 10 to 11 ppm. These latter peaks were assigned to *meso*-protons of HpD [130; 131]. NMR broadening can be induced by various events such as aggregation or ligand exchange [131]. In our case, electrophoretic results, which suggested a strong interaction between lipoprotein A-I and HpD, verified these events. To elucidate this interaction, NMR analyses of amino acids were performed in the presence and absence of HpD. The most NMR broadening and chemical shift variation in the spectra were observed in the case of tryptophane and HpD mixture, as shown in Fig. (**8**); other aromatic amino acids were observed at secondary levels. The aromatic amino acid involvement confirmed the hydrophobic nature of HpD interactions. This result also suggested a cytostatic molecule with a molecular structure containing an indolic ring can interact with HpD, such that both molecules can be transported by lipoproteins, increasing the effect of photodynamic therapy.

FUTURE PERSPECTIVES

Porphyrins accumulate in the tumour tissue. There are two explanations: according to the older theory, the uptake occurs through low-density lipoproteins (LDL), which play an important role in cholesterol metabolism. Since tumour cells show higher LDL receptor activity than normal cells, a

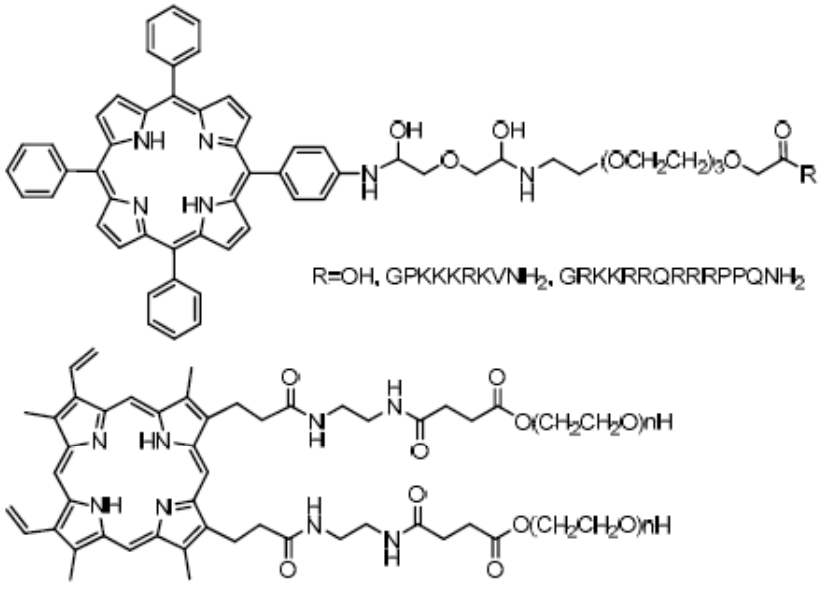

Fig. (7). Pegylated zinc protoporphyrin (PEG-ZnPP).

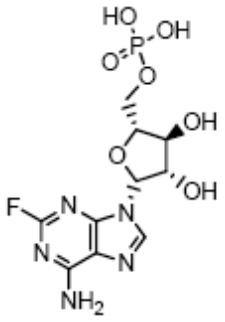

Fig. (8). Structure formula of flurdarabina (F-ara-AMP).

specific enrichment in malignant tissue results [67]. The newer theory ascribes the selectivity to the lower pH value of tumour tissue relative to normal tissue which implies a larger proportion of neutral particles that penetrate the tumour cell membrane [68,69].

Our results indicate that the aromatic amino acids, in particular tyrosine, have the most affinity in binding HpD. These findings recommend the use of cytostatic molecules composed of aromatic rings that interact with HpD and are carried by lipoproteins into cancer cells. The self-assembly of a supramolecular LDL-HpD-drug complex would reinforce the effect of HpD in PDT carrying cytotoxic drug into cells. This would also permit reduction of the quantities of HpD and cytostatic drugs in anti-tumour protocols, with a consequent decrease in the side effects of PDT and cytostatic therapy.

Fludaribina (F-ara-AMP) is an analogue of adenosine arabinoside (vidarabine, Ara-A), which is an antimetabolite whose clinical use is limited by rapid adeno-activation by the enzyme adenosine deaminase (ADA). Fludarabine fluorinates the 2' position of Ara-A (Figure) and is not a substrate for ADA. After intravenous injection, fludarabine is rapidly dephosphorylated in the plasma and enters cells by active transport.

Within cells by the enzyme re-phosphorylated is desossicitidina kinase. The triphosphate form is incorporated into DNA, blocking synthesis and repair. This explains the inhi-

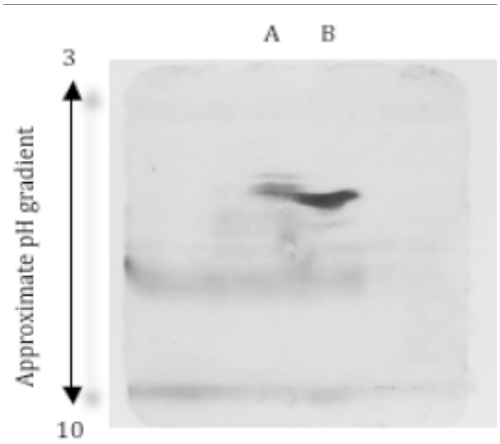

Fig. (8). Structure formula of flurdarabina (F-ara-AMP).

bition of DNA synthesis but does not explain the activity of the drug against non-proliferating cells, which seems to involve apoptosis. The concentrations of fludarabine after intravenous injection follow a triphasic decline curve with a terminal half-life of about ten hours. The drug is excreted mainly through the kidney; it is therefore necessary to reduce the dose in case of significant renal impairment. Fludarabine is currently the first-line treatment for chronic lymphocytic leukaemia and is also used to treat macroglobulinemia Waldenström, cutaneous T-cell lymphoma and hairy cell leukaemia. Our goal is to reduce the toxicity associated with the use of two drugs given individually: fludarabine (F-ara-AMP) and photosensitiser agents (Photosensitisers). Reduced-dosage intravenous infusion of fludarabine-HPD for thirty minutes for five consecutive days every four weeks prevents the decline of fludarabine that follows a three-phase curve with a terminal half-life of approximately ten hours, because the HPD binds four to ten times more tumour cells, remaining in the blood for up to 72 hours. In the chemotherapy protocols, HPD acts as a carrier that allows for reduction of the fludarabine dose used and consequently of related toxicity, resulting in improved patient compliance. Binding HPD (Photosensitisers) with cis-platinum is covalently bound (Fig. **9**), while HPD-fludarabine (F-ara-AMP) is bound by electrostatic interactions and transported by lipo-

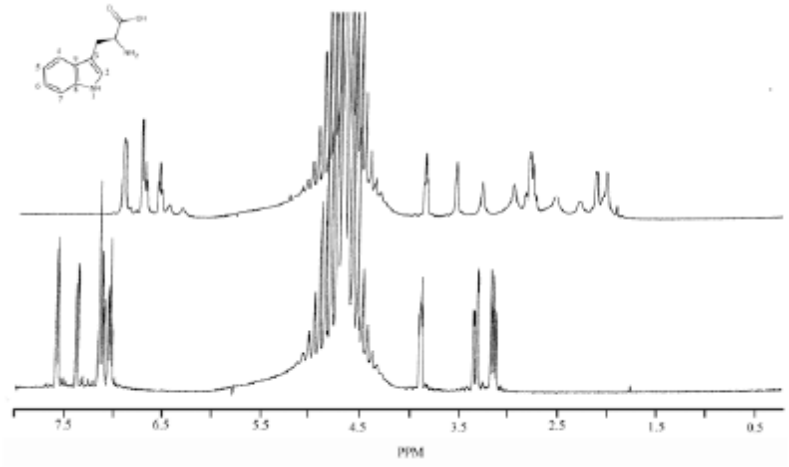

Fig. (10). NMR broadening and chemical shift variation in the spectra were observed in the case of tryptophane and HpD mixture.

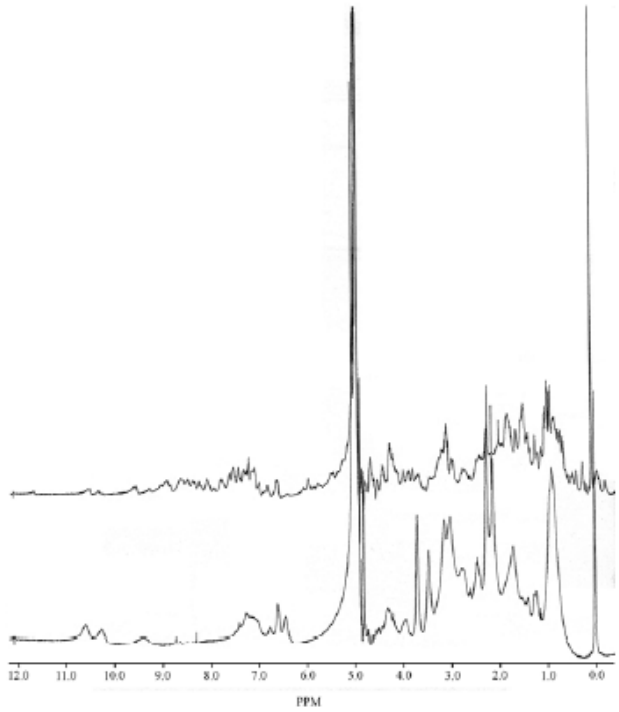

Fig. (11). NMR spectrum of human α-La in presence of HpD.

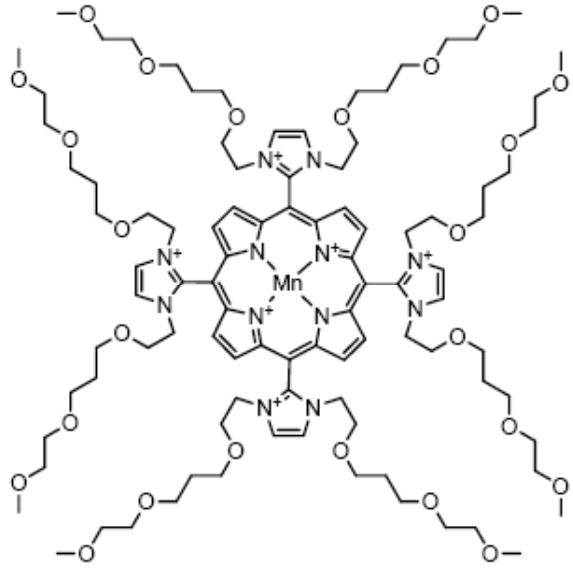

Fig. (12). Tri(ethyleneglycol)-derivatized Mn (III) porphyrin.

proteins in plasma. This allows for increased concentrations in tumour cells due to active transport mechanisms at the level of the neoplastic membrane.

CONCLUSION

In this paper we have demonstrated the potential of PDT and its growth in the context of cancer therapy, emphasizing recent developments such as t he development of new molecules of HpD-conjugated platinum and the possibility of using the cytostatic drug, ARA, non-covalently bound to HpD. The active mechanism of endocytosis, which is based primarily on the uptake of apoprotein present on cell membranes and facilitated by the high number of LDL receptors on tumour cell membranes, justifies the use of those molecules for selective targeting of cancer cells. Our experiments, which aimed to transport cytostatic drug to cancer cells to potentiate the PDT effect, indicated that the indolic structure was the better candidate. Among cytostatic drugs, ARA was chosen and tested *in vitro* to reduce the necessary dose of photosensitiser agent and cytostatic content, thereby reducing individual side effects and increasing the drugs' joint and targeted cytotoxicity, yielding "the minimum photodynamic dose."

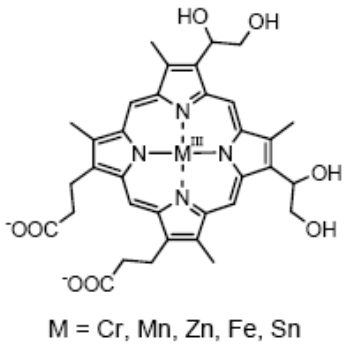

Fig. (13). Metallodeuteroporphyrin IX-2,4-bisethylene glycol.

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