Université de Sherbrooke

BIOLOGICAL ACTIVITIES OF PHTHALOCYANINES :
Effects of human serum components on the in vitro uptake
and photodynamic activity of zinc phthalocyanine.

By

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"All men by nature desire to know"

(Aristotle, 384-322 B.C.)

To :

Romanus, Ben-Basil, Marcel;

for their support.

and Mum;

for her love.

COMMUNICATIONS/PUBLICATIONS

1. **Obochi, M.O.K.**, R.W. Boyle and J.E. van Lier (1992) Biological Activities of Phthalocyanines XIII. Effects of human serum components on the in vitro uptake and photodynamic activity of Zinc Phthalocyanine. *Photochem. Photobiol.* (in press, accepted June, 1992).

2. Ross W. Boyle, Jacques Rouseau, **Modestus O.K. Obochi** and Johan E. van Lier (1992) Biological Activities of Phthalocyanines XVI. Fluorinated zinc phthalocyanines - photodynamic activity, mechanism of action and biodistribution in tumor-bearing Balb/c mice. *Cancer Res.* (Communicated).

3. **Obochi, M.O.K.**, H. Ali, S.D. Watts and J.E. van Lier (1992) Biological Activities of Phthalocyanines. The effects of halogenation on the photodynamic activity of sulfonated metallophthalocyanines in V-79 cells and tumor-bearing Balb/c mice. (in preparation).

4. **Obochi, M.O.K.**, S.D. Watts, R.W. Boyle and J.E. van Lier (1992) Biological Activities of Phthalocyanines. Photosensitization pathways of hydrophobic, amphiphilic and hydrophilic phthalocyanines used in the photodynamic therapy of cancer. (in preparation).

5. **Obochi, M.O.K.**, B.P. Ruzsciska and J.E. van Lier (1992) Fluorescence quenching of phthalocynines by amino acids and nucleic acid bases. (in preparation).

6. Margaron, P., **M.O.K. Obochi**, R. Ouellet, S.D. Watts and J.E. van Lier (1992) Influence of central metal ligand on the phototoxicity, cell uptake and in vitro fluorescing properties of BSA-bound phthalocyanines. (in preparation).

PRESENTATIONS

1.   Effect of human serum components on the _in vitro_ cell uptake and photodynamic activity of zinc phhtalocyanine. M.O.K. Obochi, R.W. Boyle and J.E. van Lier. Presented at the 20th Annual Meeting of the American Society for Photobiology, June 20-24,1992, Marco Island, Florida, U.S.A.

2.   Mechanisms of actions of phthalocyanines in photodynamic therapy of cancer. Modestus O.K. Obochi, Ross W. Boyle, Sean D. Watts and Johan E. van Lier. Presented at the Free Radicals and Oxygen Reaction Forum, University of Nigeria, Nsukka, Nigeria, August 28, 1992.

3.   L'Effet des composantes du sérum humain sur l'accumulation cellulaire _in vitro_ et sur l'activité photodynamique du zinc phtalocyanine. M.O.K. Obochi, R.W. Boyle et J.E. van Lier. Presented at the 21st Edition of "Journée Scientifique" of the Faculty of Medicine, University of Sherbrooke, Sherbrooke, Québec,Canada, May 20, 1992.

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Abbreviations and Symbols.

ZnPc	Zinc phthalocyanine
$[^{65}Zn]$ZnPc	Gamma-emitting Zn-65 labeled zinc phthalocyanine.
PDT	Photodynamic therapy.
VLDL	Very Low Density Lipoprotein.
LDL	Low Density Lipoprotein.
HDL	High Density Lipoprotein.
HSA	Human serum albumin.
HG	Human globulins.
α MEM	Alpha minimum essential medium.
PBS	Phosphate buffered saline.
DMF	N,N-Dimethyl formamide.
Hp	Hematoporphyrin.
HpD	Hematoporphyrin Derivative.
DHE	Dihematoporphyrin ether (or ester).
HPLC	High pressure liquid chromatography.
SAlPc	Sulfonated aluminium phthalocyanine.
EDTA	Ethylene diamine tetra acetic acid.
LD_{90}	90 % lethal dose
λ_{max}	Maximum absorption wavelength.

ε	molar absorption coefficient.
τ_T	lifetime of triplet state of sensitiser.
ϕ_T	quantum yield of triplet state of sensitizer.
$^1O_2$	singlet oxygen.
Sen^*	excited state of sensitizer.
S_1	singlet excited state of sensitizer.
T_1	triplet excited state of sensitizer.

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Résumé

Nous avons étudié l'effet des composantes du serum humain sur l'activité photodynamique de la phtalocyanine de zinc (ZnPc) sur des fibroblastes de hamster chinois (lignée V-79). Nous avons d'abord montré que les activités photodynamiques sont correlées à l'accumulation cellulaire de ZnPc marquée au ^{65}Zn, ce qui nous a permis d'estimer la quantité de sensibilisateur présent dans les cellules au moment de l'irradiation et d'exprimer les efficacités photodynamiques sur la base de la concentration intracellulaire du pigment. Toutes les composantes sériques, à l'exception des HDL (lipoprotéines de haute densité), inhibent la pénétration de ZnPc dans les cellules V-79 en comparaison avec l'accumulation dans les même cellules de ZnPc délivrées dans du milieu sans sérum. Les HDL ont pour effet d'augmenter de 23 % l'accumulation de ZnPc, sans affecter cependant l'efficacité photodynamique calculée à partir de la concentration cellulaire. Les VLDL (lipoprotéines de très faible densité) et les globulines ont diminué l'accumulation cellulaire également sans affecter l'efficacité photodynamique du produit. En revanche, les lipoprotéines de faible densité (LDL) et l'albumine, tout en inhibitant l'accumulation cellulaire de ZnPc, ont augmenté l'efficacité photodynamique cellulaire de ZnPc, ce qui suggère que ces protéines facilitent la localisation du produit vers des cibles subcellulaires vitales sensibles aux dommages photodynamiques. A partir de ces resultats, nous concluons que l'association de ZnPc avec les composantes sériques peut entraîner des effets importants et largement variés sur le degré de pénétration et la distribution cellulaire du photosensibilisateur.

Abstract

The effect of human serum components on the photodynamic activity of zinc phthalocyanine (ZnPc) towards Chinese hamster fibroblasts (line V-79) was studied. Photodynamic activities were correlated with cellular uptake of radiolabeled [^{65}Zn]ZnPc which allowed corrections to be made for the amount of sensitizer present in the cells at the time of irradiation and to express photodynamic efficiencies on a cellular dye concentration basis. All serum components, with the exception of high density lipoproteins (HDL), inhibit uptake of ZnPc by V-79 cells, when compared to incubation of ZnPc with the same cells in serum free medium. HDL increased ZnPc uptake by 23%, but the photodynamic efficiency corrected for the cellular ZnPc concentration was unaffected. Very low density lipoprotein (VLDL) and globulins decreased ZnPc cell uptake, but likewise did not affect the cellular photodynamic efficiency of the dye. In contrast low density lipoprotein (LDL) and albumin, while inhibiting ZnPc cell uptake, increased the cellular photodynamic efficiency of ZnPc, suggesting that these proteins facilitate localization of the dye at cellular targets sensitive to photodynamic damage and vital to cell survival. We conclude from these results that association of ZnPc with serum components can have important, and widely differing, effects on both degree of uptake and cellular distribution of the photosensitizer.

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

Why is grass green?

Why not black, red or any other color?

Come to think of it, why is blood red?

The answer to these puzzles hovers over one family of pigments. They are called tetrapyrrolic macrocycles - examples of which are at the core of haemoglobin, the red pigment of blood, and of chlorophyll, which puts the green in plants. "Without tetrapyrrolic macrocycles, life, as we know it, would be impossible. Plants use chlorophyll to collect sunlight, which they harness for the conversion of carbon dioxide into carbohydrates. All animal life is ultimately dependent on this process. And without the haem in haemoglobin, there could be no oxygen transport round the human body. So where there is life, there are tetrapyrrolic macrocycles. As if to emphasize our utter dependence on these pigments of life, strange disorders, known as porphyrias, afflict those with faulty tetrapyrrole metabolism" (Milgrom,1984). These same pigments are used in photodynamic therapy for the transduction of photon energy into chemical and biochemical manifestations ultimately leading to the destruction of cancer cells. These dyes can sensitize an organism, cell or tissue to the influence of light in the presence of oxygen. They are called photosensitizers and this is the basis of photodynamic therapy of cancer.

1.1 Historical background of PDT

The principles of photodynamic therapy were established long time ago. Phototherapy is attributed to the ancient Egyptians (Dougherty,1990) and to the ancient cultures of India and China (Spikes and Straight, 1990). But in terms of mordern history, we can certainly trace the discovery of photodynamic effects to 1900 when Oscar Raab and others in Germany laid the foundation for the science of photobiology by demonstrating the photodynamic cell killing of Paramecia using acridine orange and sunlight in the presence of oxygen (Raab, 1900). Few years later, von Tappenier and Jesionek (1903) treated skin cancer with eosin and sunlight. In 1942, Auler and Banzer described uptake of hematoporphyrin (HP) in neoplastic tissue. Figge et al. later confirmed this by fluorescence (Figge et al.,1948). The recent impetus began in the 1960's with the pioneering work of Lipson using hematoporphyrin derivative (HpD) (Lipson et al.,1961). Lipson not only recognized the potential therapeutic benefit of the photodynamic action of HpD but, indeed, treated a patient with metastatic chest wall breast cancer (Lipson,1966). Unfortunately, the significance of this report was not recognised at the time. Interestingly, around 1972 two groups, Dougherty (Dougherty,1974) and Diamond (Diamond et al.,1972), independently picked up this topic again. Though their results were not particularly successful from day one, it began the process to the present state of PDT. Since the mid 1980's, comparative trials and clinical trials with HpD as a photosensitizer have been receiving serious attention. Many people have also become interested in developing new photosensitizers for PDT, improving light delivery to tumor cells and exploiting PDT's unique aspects in other clinical endeavours (Dougherty,1990).

1.2 PHOTODYNAMIC THERAPY

Photodynamic therapy is a new cancer treatment modality that selectively destroys malignant cells by an interaction between absorbed visible light and retained photosensitizing agent (Manyak et al., 1988). This therapy requires the presence of three components : light, sensitizer and oxygen (Kongshaug, 1992) for chemical destruction of cellular components. Though a new modality for therapy of neoplastic disease, it shows promise in other therapeutic methods as well (Kessel, 1990) such as inactivation of viruses. Tumor cell kill depends on both a degree of selective retention of the excited photosensitizer within or around malignant tissue and an ability to deliver light to this tissue (Manyak et al., 1988). An efficient light delivery may be achieved by use of laser light. A systematic explanation of the modality of PDT using HpD is shown in figure 1.

The photosensitizer is administered intravenously. The tumor mass is illuminated after a waiting period of 24-48 h (to allow for selective retention of the dye by cancer cells). Light delivery is facilitated by coupling the argon-dye laser light source to an optical fibre which allows for entry into areas which are not easily accessible. Excitation of the dye leads to oxidative destruction of the cellular components. PDT has an edge over chemotherapy and radiotherapy in that it is safe and effective with no significant adverse effect and it may be used before, during or after chemotherapy, radiotherapy or surgery (Dougherty et al., 1990; Moan and Berg, 1992).

Photodynamic Therapy of Cancer

(Laser & Hematoporphyrin Derivative)

Cancer

Inject HPD(drug)
in vein

Drug selectively
retained by
cancer cells

Argon Laser   Dye Laser

Fiber optic
bundle

514nm
488nm
(blue-green light)

625-635nm
(red light)

$sens + h\nu \longrightarrow {}^1sens^*$

${}^1sens^* \longrightarrow {}^3sens^*$

${}^3sens^* + {}^3O_2 \longrightarrow {}^1O_2 + sens$

${}^1O_2 + substrate \longrightarrow oxidation$

sens = HPD

${}^1O_2$
${}^1O_2$
${}^1O_2$
${}^1O_2$
${}^1O_2$
${}^1O_2$
${}^1O_2$
${}^1O_2$
${}^1O_2$

${}^1O_2$ kills cells
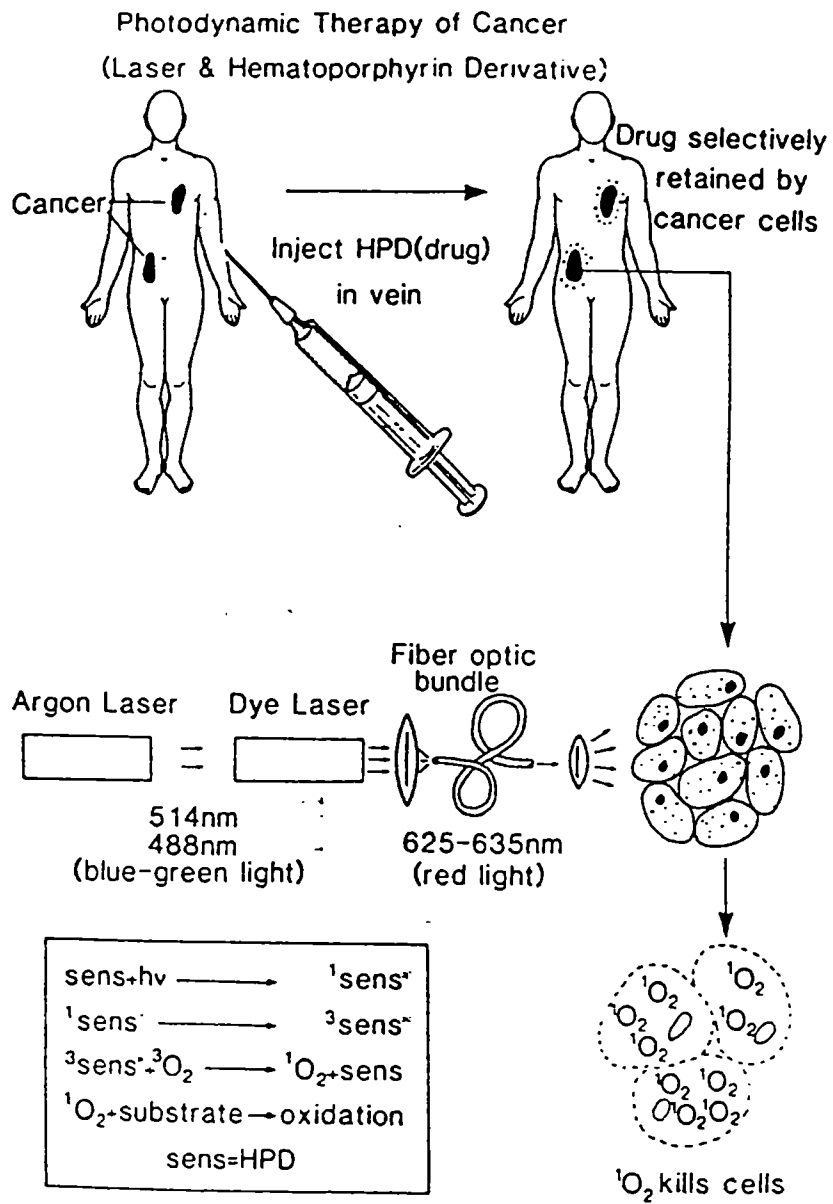
Figure 1. Schematic diagram illustrating the main steps

involved in PDT (Adapted from M.W. Berns (ed), 1984).

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1.3 Photosensitizers: First & Second Generation

The ideal photosensitizer for photodynamic therapy should have certain basic properties namely:

a). it should be a single, non toxic, stable compound of known chemical

structure.

b). it should be retained with high degree of selectivity in malignant tumors in

comparison with the adjacent normal tissues in which the tumor arose.

c). is should have a strong absorption peak in the part of the spectrum where light

penetrates living tissue best and where the photon energy is still high enough to

produce singlet oxygen (600-1100nm)

The first generation photosensitizer preparation widely used in clinical trials of

photodynamic therapy of cancer (Dougherty,1987) consists of mixtures of hematoporphyrin

derivatives (HpD) obtained via alkaline hydrolysis of hematoporphyrin acetate (Lipson et al.,

1961; Bonnet et al.,1981). The active components of HpD consist of a mixture of

dihematoporphyrins and oligomers containing 2-5 hematoporphyrin (Hp) units (Kessel,1987)

linked via ether and ester bonds (Dougherty et al., 1984; Kessel,1986a) (Fig.2). HpD, like the

parent molecule Hp, absorbs weakly above 600 nm (Fig.2) (Dougherty, 1987; van Lier,1988; van

Lier, 1990a) and may be retained in the skin for a period of 1-2 months. This consequently leads

to an increased risk of skin photosensitization (Dougherty et al., 1984). The selectivity of HpD

for malignant tissues as well as its tumor retention is generally weak (Traulau et al.,1990).
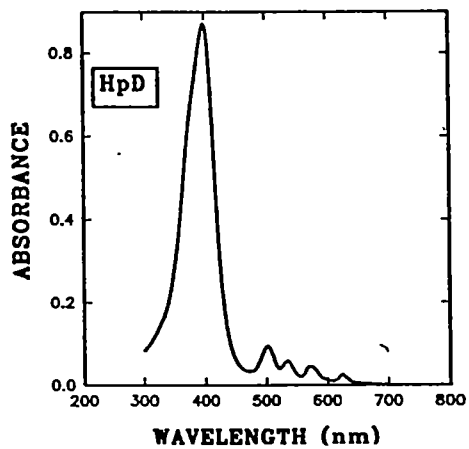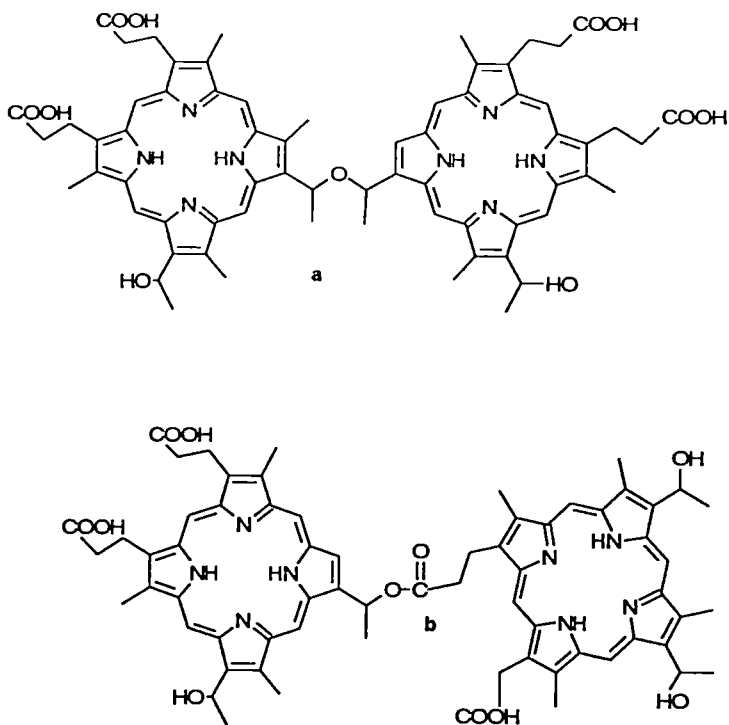
Figure 2. Structure and absorption spectra of HpD

a = Dihematoporphyrin ether, b = Dihematoporphyrin ester

Although its main absorption is around 400 nm, for therapy the dye is activated
by red light (λ = 630 nm) where a minor absorption exists, because of the increased
transparency of tissues to red light.

Above all, HpD, as well as HpD fractions enriched in the biologically active dimers and oligomers, are not ideally compatible with the laser systems presently available for light delivery in PDT (Cassen, 1991). As a result, a number of porphyrin and porphin analogues with improved photophysical properties within the therapeutic light range have been advanced over the years (van Lier, 1988). Figure 3 shows those analogues that are potential second generation photosensitizers.

Protoporphyrin (λ_{max}= 630nm; ε=1x10^{3}M^{-1}cm^{-1})

Phaeophytin a (λ_{max}= 665nm; ε=8x10^{4}M^{-1}cm^{-1})

Bacteriophaeophytin a (λ_{max}= 780nm; ε=9x10^{4}M^{-1}cm^{-1})

Zinc tetraazaporphine (λ_{max}= 610nm; ε=4.6x10^{4}M^{-1}cm^{-1})

Zinc phthalocyanine (λ_{max}=670nm; ε=2x10^{5}M^{-1}cm^{-1})

Zinc naphthalocyanine (λ_{max} = 776nm; ε=3x10^{5}M^{-1}cm^{-1})
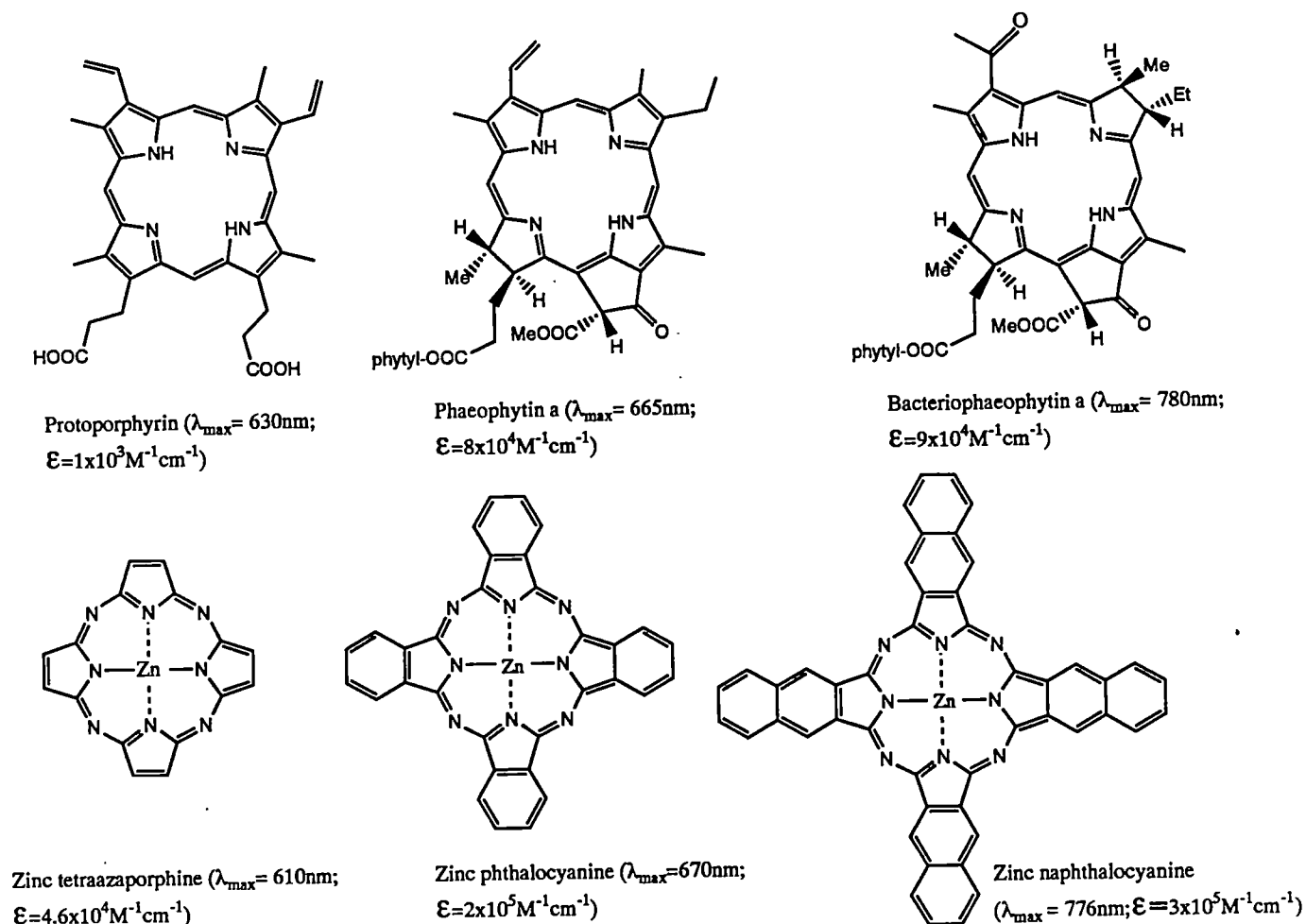
Figure 3. Structures of some 2nd generation photosensitizers.

The top row of structures presented in figure 3 are analogues of natural occuring tetrapyrrolic

macrocycles: porphyrin, chlorophyll a and bacteriochlorophyll a. Removal of their central metals

and the exocyclic double bonds results in a progression from lower to higher values of λ_{max} and

ε thereby leading to improved photophysical and photochemical properties. The bottom row of

structures (Fig. 3), on the other hand, are synthetic analogues of porphyrin in which the carbon

bridges have been replaced by nitrogen atoms. Aza substitution and fusion of the pyrrole units

with benzene rings result to higher λ_{max} and ε values thus improving the capacity to absorb light.

Phthalocyanines are included in these second generation photosensitizers. They have received a

great deal of attention and their potential use has been reviewed from time to time (Spikes, 1986;

Ben-Hur, 1987; van Lier et al., 1988; Rosenthal & Ben-Hur, 1989; van Lier & Spikes, 1989; van

Lier,1990a,b).
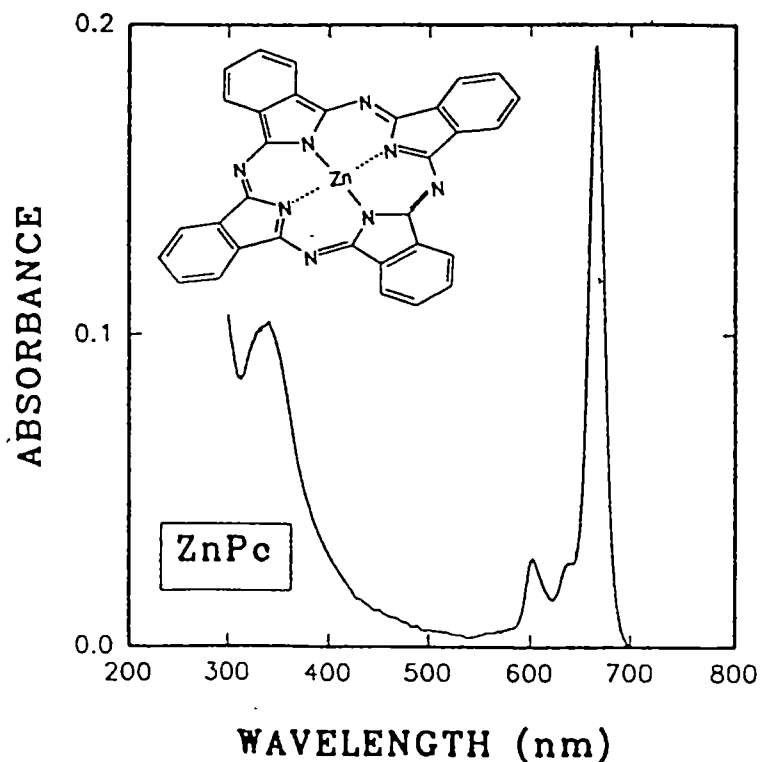
Figure 4. Structure and absorption spectra of zinc pthalocyanine.

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Phthalocyanines have attractive photophysical and chemical properties including strong absorption

maxima at wavelengths (650-860 nm) where tissue provides optimal light transmission (Wilson,

1989) (Fig.4), good capacity to generate singlet oxygen and facile chemical accessibility (van

Lier,1990a). Phthalocyanines have higher molar absorption coefficient ($\varepsilon \approx 10^5$ $M^{-1}cm^{-1}$) and thus

better capacity to absorb light compared to HpD ($\varepsilon \approx 10^4$ $M^{-1}cm^{-1}$).

1.4 PHOTOPHYSICS AND PHOTOCHEMISTRY

OF PHTHALOCYANINES
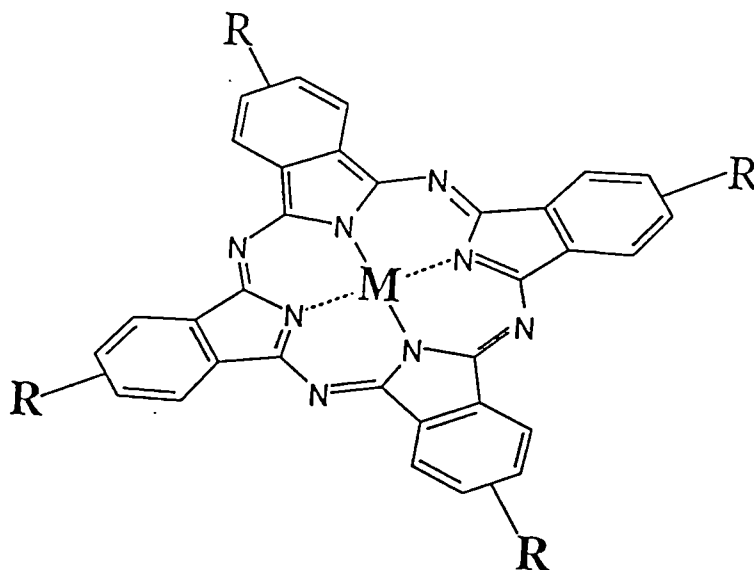
Figure 5.   Structure of phthalocyanine. M = metal, R = substituents.

A schematic diagram of the general structure of phthalocyanine is shown in figure 5.

Different kinds of metals or metalloid atoms can be inserted into the central ring of

phthalocyanines in place of the two hydrogens present in non-metallo phthalocyanines yielding dark blue or green dyes with absorption maxima around 670 nm ($\varepsilon \sim 10^5$ $M^{-1}cm^{-1}$) (Paquette and van Lier, 1992). Most phthalocyanines are usually stable chemically and photochemically (Spikes,1986). Their photophysical properties are mainly determined by the nature of the central metal ion, and particularly diamagnetic ions, such as Al^{3+} and Zn^{2+}, give complexes with both high triplet yields ($\phi_T > 0.4$) and long lifetimes ($\tau_T > 0.1$ msec) (Darwent et al., 1982) whereas ring substituents and axial ligands modulate solubility, tendencies to aggregate or associate with biomolecules, cell penetrating properties and the pharmacokinetics of the dyes (van Lier, 1990b). Most metal free and metallo phthalocyanines are insoluble in water and the usual organic solvents. But several types of water soluble derivatives can be prepared by adding substituents such as amino, carboxylic acid, nitro and sulfonic acid groups to phthalocyanines (Darwent et al., 1982; van Lier et al., 1984).

The only flaw in the derivitization of phthalocyanines is that this may yield complex mixtures of products. The metallo sulfophthalocyanines are prepared either via direct sulfonation of the non-sustituted macrocycle, or by condensation of phthalic/sulfophthalic acid mixtures. Purifaction yields fractions containing isomeric products (Fig. 6) with varying degrees of homogeneity (Ali et al., 1988; Margaron et al., 1992). In general, underivatized phthalocyanines can be obtained with a high degree of purity and display a good efficiency in the generation of activated oxygen species (Maillard et al.,1980; Wu et al., 1985). Such water-insoluble dyes can be solubilized by incoporating them into carriers and emulsions and thus an efficient targeting of experimental tumors may be achieved (Kessel, 1986b; Korbelik and Hung, 1991).
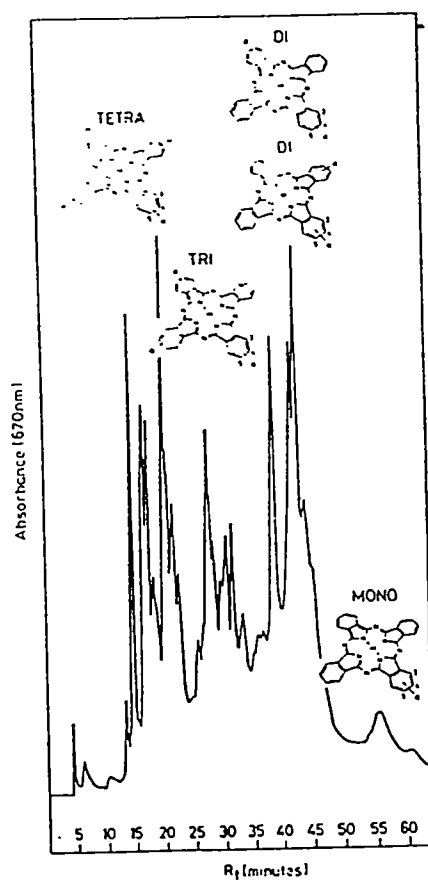
Figure 6. Reverse-phase HPLC profiles of the mono- through tetrasulfonated AlPc

1.5 PHOTODYNAMIC ACTION.

MECHANISMS AND PHOTODYNAMIC DAMAGE

The major components of cellular systems - amino acids, pyrimidine and purine bases and

phospholipids - do not absorb electromagnetic radiation of wavelengths longer than ca 350 nm.

Visible light in itself, therefore, has no biochemical significance in photodynamic cell killing (Rodgers, 1985). Other molecules, containing chromophores that are excitable by visible (400-700 nm) light, must also be present to transduce the photon energy into chemical and biochemical manifestations. In photodynamic therapy, this role is played by porphyrin-like molecules.

Photodynamic action in biological systems refers to the induction of cell death or disfunction by visible light in the presence of a photosensitizer and oxygen (Rodgers, 1985). Initiation of photodynamic activity is caused by excitation of the sensitizer by light that falls within its absorption band. Although the cytotoxic mechanisms of photodynamic action are not completely clear, photodynamic therapy does require the presence of oxygen (Lee et al., 1984; Gibson and Hilf, 1985; Manyak et al., 1988; Mitchell et al., 1985; Moan and Sommer, 1985).

Singlet oxygen, a reactive and short-lived excited state of oxygen produced during photodynamic therapy by irradiation of photosensitizers, is postulated to be responsible for cytotoxicity (Langlois et al., 1986) and this assumption is supported by both in situ chemical trapping and direct detection of singlet oxygen in tissue (Weishaupt et al., 1976; Parker and Stanboro, 1984)

The first step of photodynamic action is absorption of light by a sensitizer (Sens) to produce an excited state (Sens*). The electronically excited molecule formed by photon absorption has a high tendency to lose its energy. Several intramolecular pathways such as fluorescence, internal conversion, intersystem crossing, exist for this and are illustrated in a Jablonski diagram (Fig. 7) (Rodgers,1985). These photophysical processes in an isolated chromophore dispersed in a fluid medium are in a kinetic equilibrium.

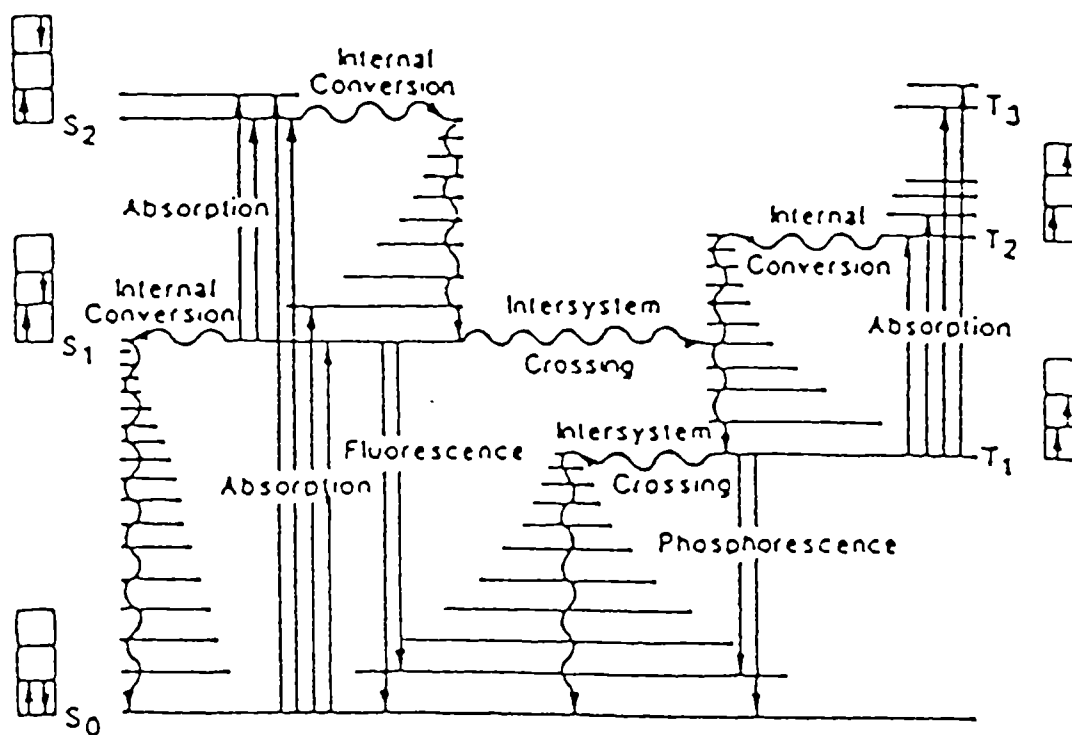13

Figure 7.   Jablonski Diagram showing excited state levels and transitions (S_o, S_1, S_2 = ground state, first excited singlet state and second excited singlet state; T_1, T_2, T_3 = first, second and third triplet state).

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On absorbing light of the appropriate wavelength, the sensitizer is converted from a stable

electronic structure (S_o, the ground state) to an excited state known as the singlet state (S_1), which

is short-lived and may undergo a conversion to a longer-lived excited state known as the triplet

state (T_1). A summary of the competing processes for S_1 are outlined below (MacRobert et al.,

1989) :

1. $S_o + h\nu \rightarrow\rightarrow S^*_1$: **Absorption**

2. $S^*_1 \rightarrow\rightarrow S_o + h\nu$: **Fluorescence**

3. $S^*_1 \rightarrow\rightarrow S_o + heat$: **Internal conversion**

4. $S^*_1 + Q \rightarrow\rightarrow S_o + Q$: **Physical quenching**

5. $S^*_1 \rightarrow\rightarrow T^*_1$: **Intersystem crossing.**

The lifetime of the singlet state (S_1) is generally less than 1 μs and the main role of this

state in the photosensitization mechanism is to act as a precursor of the longer-lived triplet state.

However, its involvement cannot be overlooked because if quenching occurs (equation 4), the

overall excitation efficiency from the ground to triplet state is correspondingly reduced and the

quenching reaction may lead to sensitized damage. The lifetime of the excited triplet states can

be several hundred microseconds in the absence of quenching co-solutes and thus are much more

efficient in sensitizing damage to substrate species than the corresponding excited singlet states.

Interaction of the triplet state with tissue components may proceed either via a Type I or II

mechanism or a combination of both (Schenck,1960, Gollnick 1968 and Foote, 1976). The Type

I reaction results in either proton or electron transfer, yielding radicals or radical ions. Transfer

can occur in either direction, but more commonly, the excited sensitizer acts as an oxidant. The

Type II reaction leads mainly to singlet molecular oxygen ($^1O_2$) by energy transfer. Electron

transfer from sensitizer to molecular oxygen can also occur in some cases giving oxidized

sensitizer and superoxide anion (O_2^-) (Lee and Rodgers, 1987) but is far less efficient (van Lier,

1991). Fig. 8 shows a schematic representation of the two types of reaction.
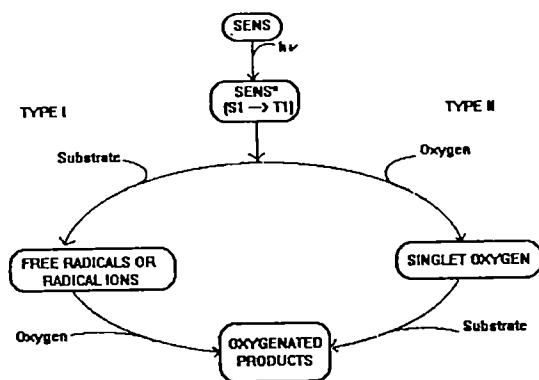
TYPE I (FREE RADICAL OR REDOX)
PATHWAY

$$S(T_1) + SUB \Rightarrow\Rightarrow\Rightarrow S^{+\cdot} + SUB^- \quad OR$$

$$S(T_1) + SUB \Rightarrow\Rightarrow\Rightarrow S^{-\cdot} + SUB^{+\cdot} \quad OR$$

$$S(T_1) + O_2 \Rightarrow\Rightarrow\Rightarrow S^{+\cdot} + O_2^{-\cdot}$$
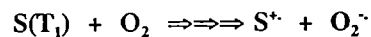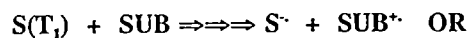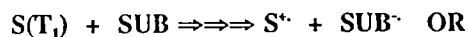
TYPE II (ENERGY TRANSFER)
PATHWAY

$$S(T_1) + SUB \Rightarrow\Rightarrow\Rightarrow S + SUB(T_1) \quad OR$$

$$S(T_1) + O_2 \Rightarrow\Rightarrow\Rightarrow {}^3S + {}^1O_2$$
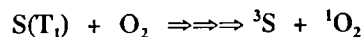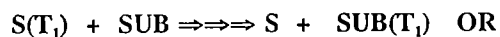
Figure 8. Mechanisms of photodynamic action.

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In some systems, Type I and II can occur simultaneously and the contribution of each pathway depends on the concentrations of oxygen and substrates as well as the characteristics of the sensitizers.

1.6 HYPOTHESIS OF
TUMOR DESTRUCTION IN VIVO

The mechanism of PDT-induced tumor destruction in vivo is complex and may include damage to the neoplastic cells, microvasculature and non-vascular stroma in the tumor. It seems that, in most cases of PDT with HpD, direct tumor cell damage is secondary to the perturbation of tumor microvasculature. The vascular endothelium is thus probably the main target of tumor photosensitization by HpD (Henderson et al., 1984; Nelson et al., 1988; Zhou, 1989). PDT-induced tumor necrosis may be due to an acute inflammatory reaction. The early injury to endothelial cells, circulating platelets and erythrocytes causes physicochemical changes in the vascular wall, reducing the rate of blood flow and initiating the processes of hemostasis and thrombosis. The increased permeability of endothelial cells leads to escape of serum proteins and fluid from blood and to the ready appearance of edema around the injured vessels. The rapid reduction of blood supply coupled to the onset of edema and hemorrhage in the tumor leads to hypoxia or even anoxia of the photoinjured neoplastic cells which eventually undergo necrosis. The overall damaging process is further enhanced by the release of some vasoactive or tissue-

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lysing substances such as histamine, proteases, thromboxanes and acid phosphatases from photodamaged mast cells and neutrophils in the stroma (Fingar et al., 1990; Fingar et al. 1991). A schematic diagram correlating the processes involved in PDT destruction of tumor is shown in figure 9 .
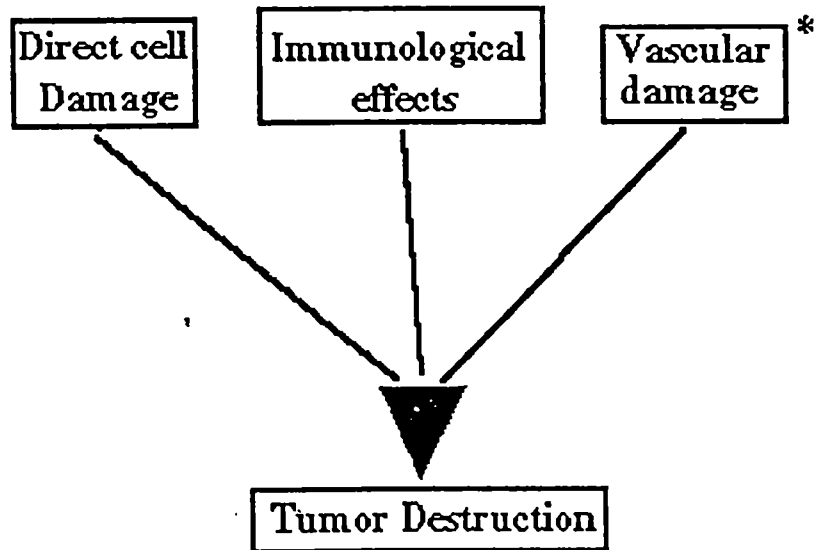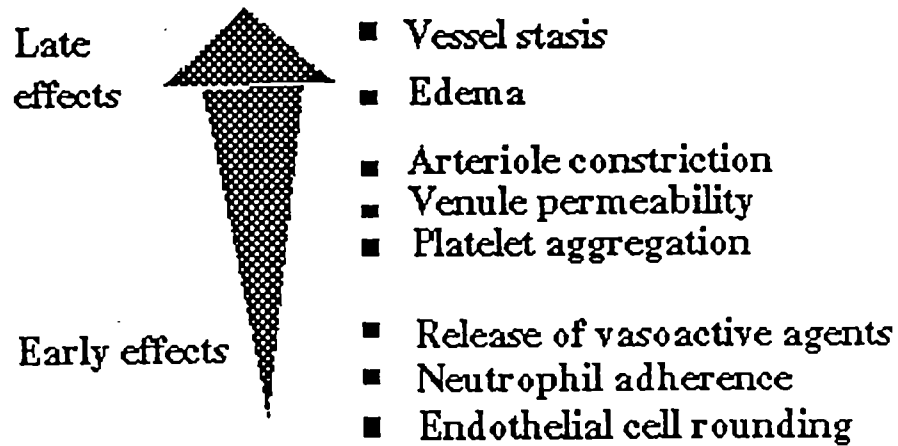
Figure 9.   Mechanisms of tumor destruction in vivo.

1.7 BLOOD TRANSPORT OF DRUGS

Several binders may be involved in blood transport of drugs. These include drug interactions with the main plasma proteins, red cells, specific carriers (e.g. transcortin), and sometimes specific circulating carriers (Tillement et al., 1991). All of these complexes act as drug carriers from blood to tissues. Some drugs may bind exclusively to one plasma protein but many drugs bind to several proteins in the blood. In general, the association and dissociation rates of drug-protein complexes are very high.

Human plasma contains about 100 different proteins of which 13 have a concentration higher than 1g/liter. Among these, five macromolecules can bind noticeable amounts of drugs: serum albumin (HSA), α_1 acid glucoprotein (AAG), and very low-density, low-density and high-density lipoproteins (VLDL, LDL, HDL, respectively). HSA makes up about 60 % of total plasma proteins. Drug binding to human serum albumin occurs in plasma as well as other tissues where albumin exists. The conformational structure of HSA (Figure 10) is flexible owing to its 17 disulfide bonds, which cause the formation of six large loops. This property may explain why HSA is able to bind many drugs.
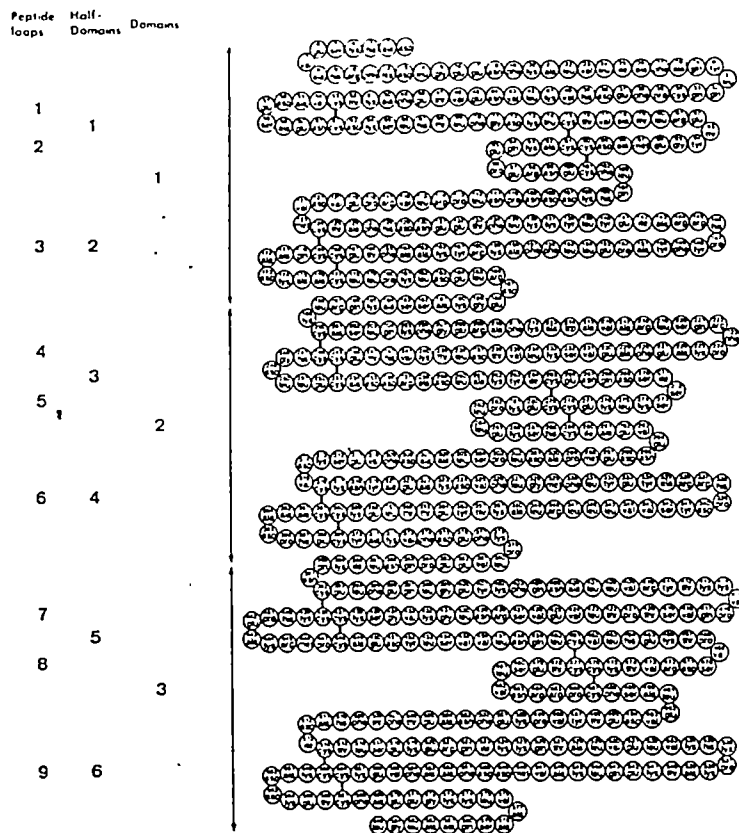
Figure 10. Structure of human serum albumin. (Modified from Pharmacology & Toxicology Supplement, vol. 66 (II), 1990. © Nordic Pharmacological Society. All rights reserved).

Plasma lipoproteins, which carry fatty acids, triglycerides, phospholipids, and cholesterol, may also be responsible for the binding of drugs. Nearly all types of drugs are able to bind to lipoproteins provided they exhibit a certain degree of lipophilicity (Innerarity, 1991). Lipoproteins can be divided into 6 major classes and two specialized classes (Table 1). Figure 11 shows a schematic drawing of plasma lipoproteins.

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Table 1. Characteristics of Plasma Lipoproteins. (From Encyclopedia of Human Biology, vol. 6, 1991. © Academic Press, Inc. All rights reserved).

Apoprotein	Plasma concentration (mg/dl)	Molecular weight (number of amino acids of the mature protein)	Major sites of synthesis	Chromosomal location of the gene	Functions	Clinical disorders due to genetic variants or mutations
A-I	100–130	28,100 (243)	Small intestine, liver	11q	Structural protein of HDL; LCAT activator; tissue cholesterol efflux	Apo A-I-C-III deficiency Apo A-I deficiency A-I multiple mutants Tangier disease
A-II	30–50	17,400 (77)	Small intestine, liver	1q	Structural protein of HDL	
A-IV	15	43,000 (376)	Small intestine	11q	Associated with triglyceride transport in chylomicrons	
B-100	80–120	550,000 (4536)	Liver	2p	Necessary for VLDL biosynthesis and secretion; ligand for the LDL receptor	Abetalipoproteinemia Familial hypobetalipoproteinemia Familial defective apoB-100
B-48	<5	250,000 (2152)	Small intestine	2p	Necessary for chylomicron biosynthesis and secretion	Abetalipoproteinemia Chylomicron retention disease
C-I	5–7	6,600 (57)	Liver	19q	Modulates LCAT activation	
C-II	3–7	8,000 (79)	Liver	19q	Activates LPL	Familial hyperchylòmicronemia
C-III	9–13	8,750 (79)	Liver	11q	Modulates receptor uptake of chylomicron remnants	
D	6–7	33,000 (169)	Adrenal, kidney, brain, liver, small intestine, etc.	3q	Unknown	
E	3–6	34,200 (299)	Liver, macrophages in various organs, astrocytes in the brain	19q	Ligand for lipoprotein receptors	Type III hyperlipoproteinemia Isoforms correlated with plasma cholesterol levels
(a)	0–100	350,000–750,000 (4,529)	Liver	6q	Binds to plasminogen receptors on endothelial cells	Levels and phenotypes of Lp(a) are correlated with coronary heart disease
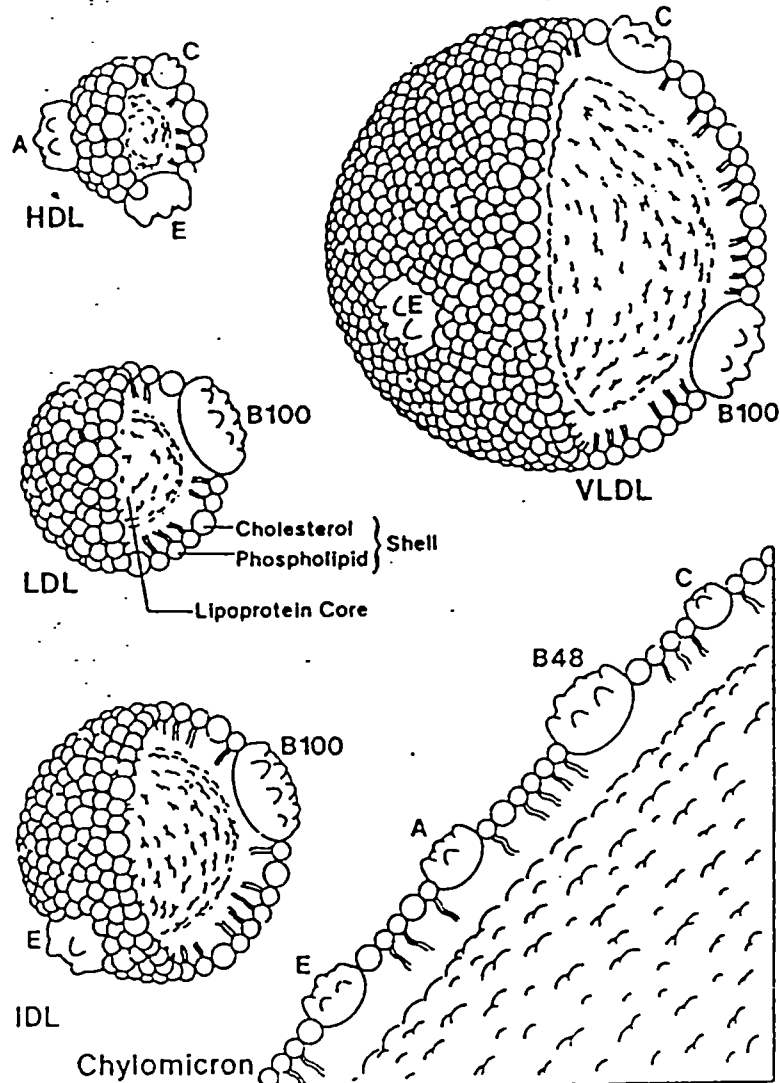
Figure 11. Schematic drawing of the plasma lipoproteins.
(Modified from *Scientific American Medicine*, Section 9,
Subsection 11. © Scientific American, Inc. All rights reserved)

Globulins represent another class of serum proteins. Some, especially γ-globulins (composed

almost entirely of immunoglobulins) show high specificity as carrier systems for drugs.

1.8 DRUG-CARRIER COMPLEXES FOR THE

DELIVERY OF PHTHALOCYANINES INTO TUMOR CELLS

Many investigators have studied the possibilities of associating anti-tumor drugs with carriers which selectively recognize tumor cells, thus minimizing their interaction with normal cells (Jori et al., 1984; Moan et al., 1985; Barel et al., 1986; Zhou et al., 1988; Cohen and Margalit, 1990; Jori and Reddi, 1990; Reddi et al., 1990; Sinn et al., 1990; Vitols et al., 1990; Korbelik and Hung, 1991). This selectivity may enhance direct cell kill and minimize inflammatory reactions and breakdown of normal and tumor tissue microvasculature, as reported by Zhou (1989) and Fingar et al. (1991) all of which affect neighbouring tissue integrity. In both cell culture system and experimental tumor models, certain carrier-linked drugs have been found to exert enhanced chemotherapeutic effects relative to the free drugs (Rudling et al.,1983), however, no drug-carrier system has yet proven to be of definite clinical value in PDT. There are many problems involved in this approach, e.g. stability of the drug-carrier complex and rapid clearance by the reticuloendothelial system of exogenous macromolecules and particles when used as carriers. In addition immunological reactions may result (Vitols et al., 1990) with drugs acting as haptens in drug-carrier interactions. Many of these problems could be reduced by the use of endogenous carriers. Chromatographic and ultra-centrifuge separations of serum-associated photosensitizers suggest that the majority of sensitizers tested for an application in PDT associate with HDL and albumin, while there is generally less association with LDL and VLDL. The affinity of serum albumin and serum lipoproteins for porphyrins indicates a role for these proteins

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as endogenous carriers of photosensitizers for PDT (Kongshaug et al., 1989; Manyak et al., 1988; Cohen and Margalit, 1990). It has been suggested that the binding of photosensitizers to certain plasma proteins may enhance their tumor localization (Reyftman et al., 1984; Barel et al., 1986; Kessel, 1986b; Kessel et al., 1987; Korbelik and Hung, 1991). Two main modes of photosensitizer localization in tumor tissues have been proposed depending on the nature of the carrier protein (Kessel, 1987). In the first, albumin delivers bound drugs primarily to the vascular stroma, while for the second, lipoproteins, especially LDL, efficiently transport the dye to malignant cells. The high concentration of serum albumin in whole serum (Rotenberg et al., 1987) coupled with its affinity for porphyrins is an added advantage in exploiting the use of albumin as a carrier of drugs for PDT. The rationale for using LDL as a carrier system of photosensitizers stems from the fact that neoplastic cells express a particularly large number of receptors at the level of the cytoplasmic membrane, which selectively recognize the apoprotein B and E moieties of LDL (Brown et al., 1980; Gal et al., 1981). Hydrophobic photosensitizers readily partition in the lipid region of LDL (Reyftman et al., 1984; Candide et al., 1986; Beltramini et al., 1987) and do not perturb the endocytotic process of internalization of LDL (Reddi et al., 1990). Hence, associating photosensitizers with different serum proteins may enhance tumor cell localization (Korbelik and Hung, 1991), increase direct tumor cell damage upon light exposure (Milanesi et al., 1990), and improve the retention of hydrophobic drugs. Allison et al. (1990) found that LDL and HDL influence the accumulation of benzoporphyrin derivative in tumor cells.

AIM OF RESEARCH

In order to study the relative contributions of serum components to uptake and photosensitized cell kill by ZnPc, we have associated this dye with different human serum proteins (VLDL, LDL, HDL, albumin and globulins) and assayed the effect of each on the clonogenicity of V-79 Chinese hamster cells following incubation and irradiation. Using ZnPc labeled with gamma-emitting ^{65}Zn, the uptake of [^{65}Zn]ZnPc by cells was determined and expressed in molar units. This latter technique is especially advantageous as it is independent of aggregation state and physical quenching of fluorescence by cellular components, both of which are problems which can complicate the measurement of cellular uptake of photosensitizer by extraction and fluorescence measurement. Using these data, intracellular molar photodynamic efficiencies were calculated. From these values inferences concerning the relative contribution of uptake and intracellular distribution of sensitizer to PDT were drawn. Effects of varying the concentrations of unfractionated serum on the photodynamic efficiency of ZnPc were also investigated.

CHAPTER TWO

MATERIALS

AND METHODS

MATERIALS AND METHODS

Cell culture. Chinese hamster lung fibroblasts (line V-79) were used throughout this experiment. Cells were maintained in regular growth medium (α MEM) supplemented with 10% human serum, 1% L-glutamine, and 1% MEM vitamin solution (Gibco Laboratories, N.Y., USA).

Photosensitizers

Zinc phthalocyanine (ZnPc). ZnPc was purchased from Eastman Kodak Company (N.Y., USA) and used as such.

$[^{65}Zn]ZnPc$. $^{65}ZnCl_2$ ($t_{\frac{1}{2}}$ = 245 days, γ = 1.115 MeV) (300 μCi) (Amersham, Oakville, Ontario, Canada) in 0.3 ml 1 M HCl was dried under a stream of nitrogen; aqueous sodium acetate buffer (1 ml; 10 mM, pH 7.0) and zinc acetate dihydrate (0.35 mmol) were added and the solution was again dried under nitrogen. 1,2-Dicyanobenzene (224 mg; 1.75 mmol) was added to the resulting solid and the mixture was heated at 180°C for 3.0 h to give a blue/green product, which was purified by dissolving it in concentrated sulfuric acid (10 ml) and reprecipitated by pouring into water (20 ml). The phthalocyanine was recovered by centrifugation, washed with water (30 ml), then ethanol (30 ml), and dried to a purple/blue powder. The washings removed any trace of free ^{65}Zn from the product.

Drug formulation

ZnPc. A solution was prepared by first dissolving the drug (30 mg; 52 μmol) in pyridine (20 ml). 1 ml Cremophor EL (Sigma) and 0.3 ml propan-1,2-diol (Sigma) were added to the

solution and the solvent was removed by evaporation in vacuo. Finally, sterile aqueous NaCl

(8.7 ml; 0.154 M) was added and the mixture sonicated. Precipitated ZnPc was removed by

centrifugation followed by filtration of the supernatant through a 0.45 μm filter (Millipore) to

give a homogeneous emulsion. The final ZnPc concentration was estimated by serial dilution of

sample in DMF and reading the absorbance of the monomeric ZnPc at 670 nm, $\varepsilon = 2.5 \times 10^5$

$M^{-1}cm^{-1}$. The absorption spectrum (Fig. 4) was measured with a Varian UV-Vis

spectrophotometer (Techtron Pty. Ltd, Springvale, Australia).

[^{65}Zn]ZnPc. The radioactive ZnPc was formulated as described above for ZnPc but the

concentration was adjusted with the non-radioactive ZnPc to achieve a specific activity of 0.5

μCi/μmol. Concentrations were determined spectrophotometrically as described above.

Serum components. Human serum components : very low density lipoprotein (VLDL),

low density lipoprotein (LDL), high density lipoprotein (HDL), human serum albumin (HSA) and

human globulins (HG, Cohn fraction III) were purchased from Sigma Chemical Company (St.

Louis, USA). They were adjusted to 1 mg/ml in buffer solution containing 0.154 M NaCl and

0.1% EDTA; pH 7.4-7.5 except for VLDL where a concentration of 0.46 mg/ml was used. Dye

emulsions were diluted in growth medium supplemented with 1 % of the selected serum

component and incubated at room temperature for 0.5 h before use. Unfractionated human serum

was prepared from blood sample collected from the author. The blood was centrifuged for serum

at 600 g for 10 min. The serum was stored at -20°C before use. Dye emulsions were diluted in

growth medium supplemented with 1 % of the unfractionted serum and incubated at room

temperature for 0.5 h before use.

Photocytotoxicity test

Cell survival of Chinese hamster lung fibroblasts (line V-79) was determined using colony forming assay as described by Brasseur et al. (1985). Cells (1 ml) were plated in 60 mm petri dishes containing 4 ml regular growth medium and incubated in a dark humid atmosphere containing 5% CO_2 for three hours at 37°C to allow for cell attachment. Later the cells were rinsed with 2-3 ml PBS and incubated for 1 h in the dark at 37°C with 1 ml of growth medium containing 1% human serum and dye concentrations of 5-200 nM. The dye solution was aspirated off and the cells washed with 2-3 ml PBS and exposed at room temperature for 4 min to red light resulting in a fluence of 2.4 J/cm^2 (Appendix 2) (Paquette, 1990). After the phototreatment, the cells were refed with 5 ml regular growth medium supplemented with 0.28% insulin and incubated at 37°C in a dark humid atmosphere containing 5% CO_2 for 6 days to allow colony formation.

Experiments were repeated 3-4 times using 3 dishes per concentration point. Control plates were set out to test for the dark toxicity of the drug and serum and possible phototoxicity of serum without drug.

Effect of serum components on photocytotoxicity

To test for the effects of VLDL, LDL, HDL, HSA and HG on the photocytotoxicity of ZnPc on V-79 cells, drug dilutions were made with growth medium supplemented with 1% of the serum component in question. The effect of increasing unfractionated human serum was

assayed by using drug dilutions in growth medium supplemented with 1 % or 10% human serum or without added serum.

Light source

The illumination of the cells was performed using red light from two 500 W tungsten/halogen lamps (GTE Sylvania, Drummondville, Quebec, Canada) fitted with a circulating refrigerated filter containing aqueous Rhodamine B (Sigma) (OD_{580} = 1.25), and a red filter (λ > 590 nm) (26-4390, Ealing, St-Laurent, Québec, Canada). The fluence rate was measured as 100 W/m^2.

Cellular uptake

V-79 cells in log phase were incubated in disposable, sterile polypropylene round bottom culture tubes (with caps) (Becton Dickinson labware, Lincoln Park, New Jersey, USA) for 5 h in a dark, humid atmosphere containing 5% CO_2 until ~10^6 cells were obtained. The cells were trypsinized and counted using a hemacytometer counting chamber (American Optical, Buffalo, N.Y., USA). [^{65}Zn]ZnPc (0.08 μmol; specific activity, 0.5 μCi/μmol) diluted in α MEM regular growth medium supplemented with 1% human serum, 1% L-glutamine and 1% MEM vitamin solution (Gibco Laboratories, N.Y., USA) were added to the tubes. They were incubated at 37°C in dark humid atmosphere containing 5% CO_2 for 0-6.0 h. After different times, the tubes were centrifuged (600 g x 5 min) and the drug dilution aspirated off. The cells were carefully washed

three times using 2 ml PBS for each washing. Both the cells and the washings were counted for radioactivity using an automatic microcomputer controlled universal gamma counter, the 1282 Compu Gamma(LKB-Wallac, Turku, Finland). The radioactivity (μCi) was converted to concentration units using the specific activity of the stock solution and a standard formula (Saha, 1984). The experiment was repeated three times using three tubes for each time interval. [^{65}Zn]ZnPc uptake by the cells was expressed as the total amount in nmoles/10^6 cells, the average cellular concentration in molarity based on a V-79 cell volume of 1.2×10^{-12} l (Paquette, 1990), and the distribution ratio (DR) between the cells and the incubation medium based on the respective concentrations (Appendix 1).

Effect of serum on dye uptake

The effects of VLDL, LDL, HDL, HSA and HG on the uptake of [^{65}Zn]ZnPc by V-79 cells were studied using drug dilutions in growth medium supplemented with 1% of each of the serum components under study. The experiments were also carried out using serum-free growth medium and growth medium supplemented with 10% human serum.

CHAPTER THREE

RESULTS

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RESULTS

Control V-79 cells incubated in growth medium without added dye, but containing complete human serum, serum components, or no serum, showed no significant effect on growth at the fluence used (2.4 J/cm^2). Their plating efficiencies were taken as 100% cell survival. The dye showed no toxicity in the dark at the concentrations used for this experiment.

Effects of unfractionated human serum on cell uptake and survival.

Fig. 12 shows the effect of unfractionated human serum on the cell uptake of [^{65}Zn]ZnPc as a function of incubation time (0-6 h). Cellular radioactivity was counted after removing the medium at each time point. The uptake of labeled dye was reduced when unfractionated serum was present in the growth medium used to incubate cells. The inhibition of the dye uptake was proportional to the percentage of unfractionated serum in the growth medium. This inhibition of uptake was accompanied by a diminution in the phototoxicity of the dye (Fig. 13). Thus based on the extracellular dye concentration, ZnPc in serum-free medium (LD$_{90}$ = 22 nM) gives greater photodynamic efficiency than medium containing 1 % serum (LD$_{90}$ = 60 nM) or 10 % serum (LD$_{90}$ = 110 nM).

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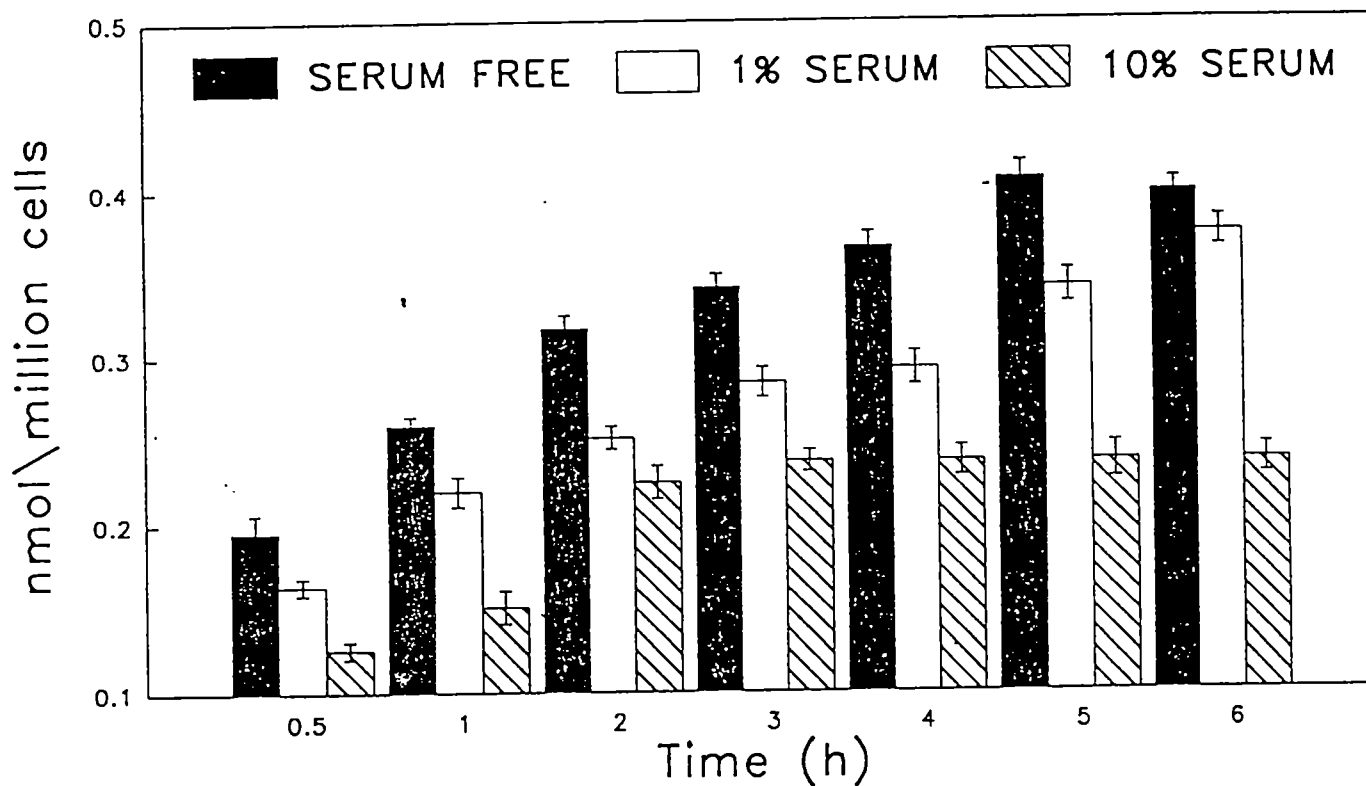
Figure 12. Influence of unfractionated human serum on the uptake by V-79 cells of [^{65}Zn]ZnPc as a function of incubation time. 10^6 V-79 cells were exposed for 0-6 h to 10 μM of the labeled dye in growth medium supplemented with varying concentrations of serum. The medium was removed at each time point, cells washed 3 times with PBS and the uptake determined by the radioactivity associated with the cells. Uptake is expressed in nmoles/10^6 cells. Experimental points represent single determination and these data are representative of 3 independent experiments.
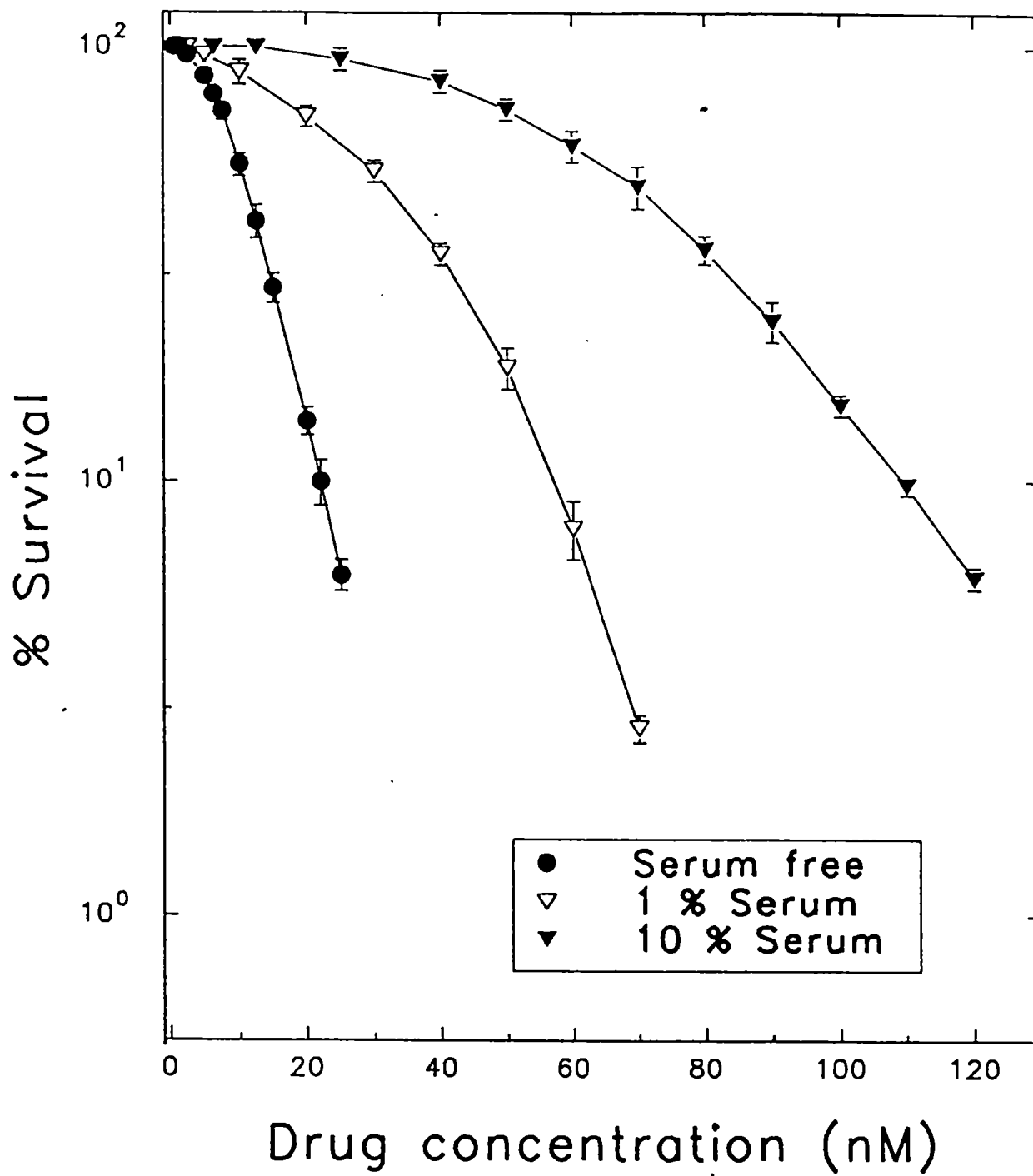
Figure 13. Survival of V-79 cells as a function of ZnPc concentrations after exposure for 4 min to red light

(λ > 590 nm; 100 W/m^2). 5-200 nM of Zn-Pc in growth medium supplemented with varying concentrations

of unfractionated human serum were exposed to cells for 1 h. The cells were washed with PBS prior to

illumination with red light. Error bars represent standard deviations for three different experiments using

3 dishes per concentration point.

Effect of human serum components on cell uptake and survival .

Figure 14. Uptake of [⁶⁵Zn]ZnPc as a function of time.
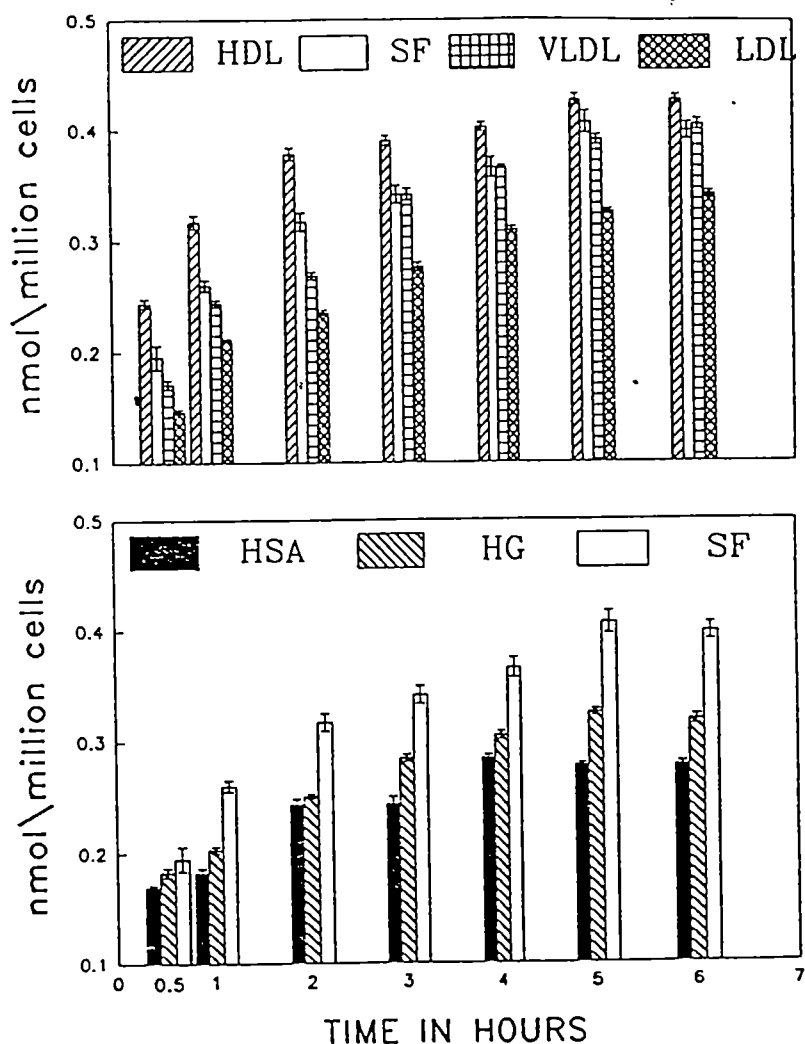
Figure 14. Uptake of $[^{65}Zn]$ZnPc as a function of time. 10^6 V-79 cells were exposed for 0-6 h to 10 μM labeled dye dilution supplemented with 1% pure human serum components. The cells were washed 3 times with PBS prior to counting of radioactivity. Uptake is expressed as nmoles/10^6 cells. These data are representative of 3 different experiments.

The V-79 cell uptake of $[^{65}Zn]$ZnPc in growth medium containing 1 % of different pure serum components is presented in Fig. 14. The activity was counted after the cells were exposed

for 0-6 h to 10 μM solution of [^{65}Zn]ZnPc and expressed in terms of uptake of dye in nanomoles

per million cells. VLDL, LDL, HSA, HG inhibited dye uptake by the cells relative to serum-free

conditions. Conversely, HDL promoted ZnPc uptake by the cells. The effect of ZnPc in medium

containing 1 % of different pure serum components on the survival of V-79 cells after exposure

to red light is presented in Fig. 15.
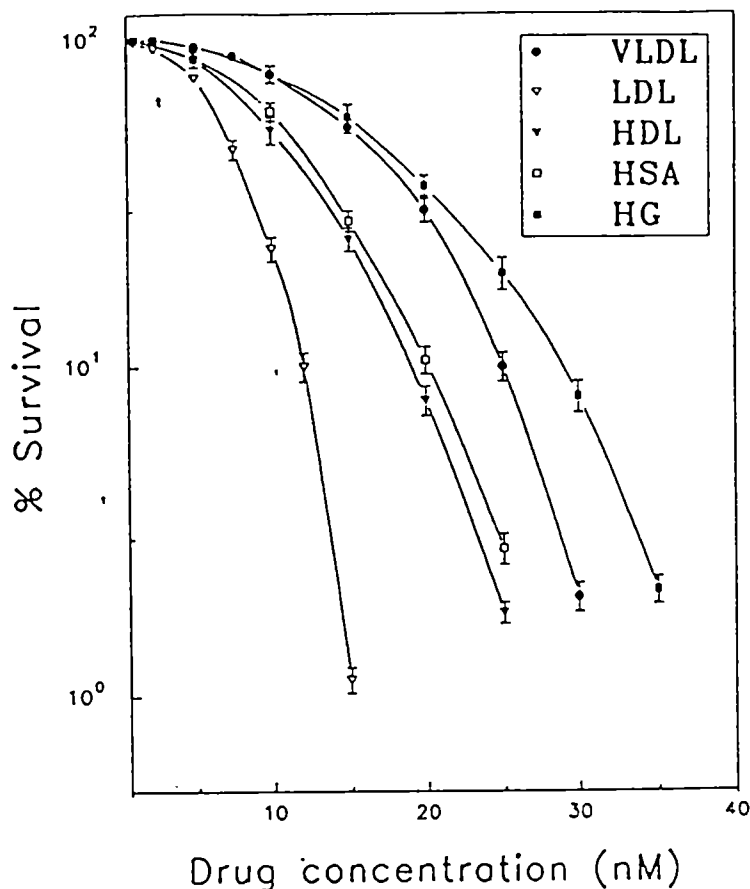
Figure 15. % survival of V-79 cells as a function of ZnPc concentration after exposure for 4 min to red

light ($\lambda > 590$ nm; 100 W/m^2). Cells were exposed for 1 h to 5-200 nM ZnPc in growth medium supplemented

with 1% pure human serum components. The cells were washed with PBS and exposed to red light for 4 min.

Error bars represent standard deviations for three different experiments using 3 dishes per concentration

point.

The presence of VLDL and HG resulted in increased drug concentration required to achieve 90% mortality (extracellular LD_{90}'s of 25 and 29 nM respectively) when compared to serum-free conditions (LD_{90} 22 nM). The promotion of cellular uptake by HDL resulted in enhanced photosensitized cell kill, with an extracellular LD_{90} value of 19 nM. However, association of LDL and HSA with ZnPc which resulted in reduced dye uptake, also resulted in increased cell photosensitivity with extracellular LD_{90}'s of 12 and 20 nM respectively. LDL, therefore, exhibits the largest enhancement of phototoxicity of all the components studied.

Cell/medium distribution ratio and photodynamic efficiency corrected for dye uptake

Table 2 shows the calculated cell/medium distribution ratio and the effect on photodynamic efficiency of each of the pure serum components based on the dye concentration in the incubation medium (extracellular LD_{90}) and corrected for dye uptake by V-79 cells (cellular LD_{90} based on the average concentration of the dye at the cellular level). ZnPc associated with LDL results in a cellular LD_{90} of 0.22 μM and thus is photodynamically more efficient than all other human serum components, and is more than twice as efficient as ZnPc incubated with cells in the absence of serum. HSA has a similar effect although to a lesser extent (cellular LD_{90} = 0.3 μM). Added HDL results in the highest ZnPc uptake and cell/medium distribution ratio of all the serum components, yet the addition of HDL, VLDL or HG has no effect on the cellular LD_{90} (≈ 0.5 μM). Addition of whole serum not only inhibits cell uptake of the dye, but also substantially diminishes the photodynamic efficiency of the ZnPc associated with the V-79 cells (Table 2).

Table 2. Cell uptake and photodynamic efficiency of ZnPc in the presence of various serum components.

Serum[1] components	LD_{90}[2] extracell. nM	Cell uptake[3]			LD_{90}[4] intracellular μM
		$\dfrac{nmol}{10^6 cells}$	μM	DR	
VLDL	25	0.24	200	20	0.50 ± 0.020
LDL	12	0.21	175	18	0.22 ± 0.018
HDL	19	0.32	267	27	0.51 ± 0.022
HSA	20	0.18	150	15	0.30 ± 0.015
HG	29	0.20	167	17	0.49 ± 0.015
10% Serum	110	0.15	125	13	1.43 ± 0.016
1% Serum	60	0.22	183	18	1.08 ± 0.022
Serum-free	22	0.26	217	22	0.48 ± 0.026

[1] Chinese hamster fibroblasts (line V-79) were incubated for 1 h with various concentrations of ZnPc in the presence of 1% of the different serum components.

[2] After removal of the dye solution cells were exposed to red light and survival was measured via colony formation. The extracellular LD_{90} is the ZnPc concentration in the incubation medium required for 90% cell kill. Standard deviations represented by error bars are as shown in figures 13 & 15.

[3] ZnPc uptake per 10^6 V-79 cells. Cells were incubated with 10 μM [^{65}Zn]ZnPc for 1 h, washed and counted for radioactivity. Uptake is expressed as nmol/10^6 cells, μM, taking a single cell volume of 1.2 x 10^{-12} l, and as the distribution ratio (DR) representing the ratio between cellular and extracellular ZnPc concentrations. Standard deviations represented by error bars are as indicated in figures 12 & 14.

[4] The intracellular LD_{90} is the ZnPc concentration associated with the V-79 cells required for 90% cell kill, i.e. the extracellular LD_{90} times the DR. Standard deviation was calculated using error propagation formula as described in appendix 3.

CHAPTER FOUR

DISCUSSION

DISCUSSION

The effect of different human serum components on the distribution of photosensitizers and their implications for different mechanisms of action of phthalocyanines in PDT are not fully understood. We anticipated that the use of different pure serum components would give important information regarding the specific contributions of each component to the photo-inactivation of V-79 cells. This data may help in understanding better the mechanisms of drug delivery and photodynamic inactivation of cancer cells in vivo. The solubilising agent, Cremophor EL, was used in our drug formulation to promote the dispersion of the photosensitizer in the growth medium (Kessel et al., 1991). In this study, cells were washed prior to illumination therefore any phthalocyanine not taken up by the cells could not participate in photodynamic cell killing. Also, the use of ZnPc labeled with gamma-emitting ^{65}Zn gave an absolute value of sensitizer concentrations in the cells, obviating the problems commonly associated with fluorescence measurement of sensitizer uptake, namely aggregation, physical quenching and inefficient extraction techniques. Centrifugation prior to washing of cells exposed to [^{65}Zn]ZnPc prevented any significant loss of cells during the course of the assay.

In relation to serum-free samples, we found, with the exception of HDL, that associating ZnPc with complete human serum or pure serum components resulted in a reduction in the amount of dye taken up by the cells. Based on the extracellular ZnPc concentration, the decreased ZnPc uptake found with human serum, VLDL, and globulins resulted also in

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diminished photodynamic activity, whereas HDL enhanced photosensitized cell kill. However, it is interesting to note that associating ZnPc with either LDL or HSA led to greater cell kill than with ZnPc in serum-free medium. This suggests facilitated selective transport by LDL and HSA to sensitive cellular targets essential for the survival of the cells.

Serum

The addition of 1% or 10% serum inhibited ZnPc uptake by the V-79 cells and strongly reduced the photosensitized cell killing potential of ZnPc. The substantial increase in the cellular LD_{90} upon the addition of whole serum (Table 2) suggests either that :

i). unfractionated serum contains components which promote binding of ZnPc to photodynamically insensitive sites; or

ii). unfractionated serum contains components which inhibit cellular uptake resulting to reduced photosensitized cell kill.

Furthermore, the effect of unfractionated serum on uptake and photoefficiency of ZnPc may also be due to other factors not considered in this work. Such factors as intracellular pH, retention/rate of efflux, other plasma components, differences in the physiological level of the serum components and differences in affinity for ZnPc may contribute to the uptake and photodynamic efficiency of the dye.

VLDL

In relation to serum-free samples, VLDL inhibited the uptake of [^{65}Zn]ZnPc by V-79 cells and consequently decreased photodynamic efficiency based on the extracellular dye concentration.

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However, after correcting the LD_{90} for the amount of dye taken up by the cells, the photodynamic potential of ZnPc associated with VLDL or without serum was found to be the same (cellular LD_{90} ≈ 0.5 μM) as described in Table 2.

LDL

There was no increase in dye uptake seen despite the hypothesis of selective accumulation of photosensitizers associated with LDL (Kessel, 1986b). Lipids contained in LDL may, upon incoporation into the cell membrane, change the membrane fluidity thus affecting the uptake of ZnPc (Salzberg et al., 1985). This finding is supported by similar findings by Korbelik et al. (Korbelik et al.,1990; Korbelik and Jung, 1991). However, the ZnPc uptake increased steadily with time of exposure (0-6 h). Added LDL results in a dye uptake value similar to that obtained with drug dilutions containing 1% serum. However, the presence of LDL enhanced the photosensitized cell kill of V-79 cells with ZnPc more than any other serum component. Hence, both the cellular and extracellular LD_{90} were the lowest measured (Table 2). ZnPc associated with LDL was also photodynamically more efficient than serum-free dye dilutions. The enhanced cellular photodynamic efficiency of ZnPc suggests that LDL delivers ZnPc to cellular targets which are both sensitive to photodynamic damage and vital to the survival of the cell.

The mechanism of delivery of ZnPc associated with LDL could be via attachment to specific receptors located at the cell surface (Shireman et al., 1977). This is because :

i). animal cells obtain cholesterol for synthesis of cell membrane by the receptor-mediated endocytosis of the cholesterol-carrying LDL (Goldstein et al.; 1979); and

ii). hydrophobic drugs readily partition in the lipid region of LDL (Beltramini et al., 1987)

without perturbing the endocytic process of internalization of LDL (Reddi et al., 1990).

When cells are in need of cholesterol, they synthesise cell surface receptors, a transmembrane protein, that bind the protein component of LDL (Innerarity, 1991). Bound LDL are internalized via coated pits/coated vesicles which are degraded at the lysosome level. On the other hand, LDL may be trapped in plasmalemmal vesicles which are taken up through the invagination of noncoated region of cell membrane (Goldstein et al., 1979). This suggests that the primary targets of ZnPc internalized by LDL may be lysosomes and the cell membrane. On phototreatment, the excited ZnPc initiates chain reaction of oxidative destruction at the levels of lysosomes and cell membrane (lipid peroxidation). This destruction may spread rapidly to other intracellular organelles such as mitochondria, nucleus and golgi apparatus. This hypothesis, however, is open to question; further experiments such as use of intracellular organelle markers and laser scanning confocal microscopy may be needed to test it.

HDL

Association of the dye with HDL led to an enhanced uptake of ZnPc by V-79 cells and increased photosensitized cell kill based on the extracellular dye concentration. However, the LD_{90} corrected for the amount of ZnPc taken up by V-79 cells (Table 2) is only slightly different from those obtained for either VLDL, HG or without added serum. This suggests that HDL delivers ZnPc to cellular sites which are either relatively resistant to photodynamic damage or of lesser importance to cell viability compared to the same dye internalized with LDL or HSA. The mechanism by which HDL internalises ZnPc is not clear. HDL receptor is yet to be isolated or characterised (Innerarity, 1991).

<u>Albumin</u>

Association of the dye with HSA resulted in a marked reduction in the uptake of the ZnPc throughout the period of study. This is in line with the strong binding of photosensitizers to albumin which in turn inhibits cellular uptake. Cozzani <u>et al.</u> (1984) have shown that association of porphyrin with bovine serum albumin (BSA) interferes with the uptake by cells <u>in vitro</u>. However, HSA enhanced the cellular photodynamic efficiency of ZnPc when compared to all other serum components except LDL (Table 2). HSA, as is the case with LDL, thus seems to promote the distribution of the dye in cells to photodynamically sensitive sites.

However, the mechanism for the internalization of albumin-bound photosensitizers <u>in vitro</u> is not fully understood. Weisiger <u>et al.</u>, (1981) found that receptor for albumin on the liver surface may mediate uptake of fatty acids and other albumin-bound substances. It may be possible that albumin receptor may participate in the uptake of wide variety of albumin-bound substances, including drugs, hormones and carcinogens. Whether V-79 cells contain similar receptors for albumin is not yet known. This may be a point to be addressed in future experiments.

But, a contribution to increased photodynamic potency resulting from inefficient transfer of ZnPc from Cremophor to albumin, thus causing pseudo serum-free conditions cannot be ruled out. Ginevra <u>et al.</u> (1990) reported that in whole serum only a minor fraction of liposome-delivered ZnPc was transfered to nonlipoproteins such as albumin. In spite of that, had enhanced cell killing efficiency been caused solely by poor dye to albumin transfer , we would have expected sensitizer uptake values similar to serum-free conditions. On the contrary, HSA decreased dye uptake by approximately 30 % relative to serum-free conditions. More detailed studies are

however required to determine the efficiency of the transfer of Cremophor-bound dyes to albumin.

Globulins

The cell uptake of ZnPc was reduced in the presence of HG relative to serum-free conditions, and the extracellular LD_{90} increased accordingly. The cellular LD_{90} of HG-associated ZnPc was, however, identical to that found for the serum-free samples suggesting that localization of the dye at the cellular level is not affected by HG.

Generally, our findings underline the contributions of different individual serum components to the uptake and distribution of ZnPc in V-79 cells, and emphasize the influence of both these parameters on the overall phototoxicity of this dye. The concentration of the serum components used for this experiment is solely for comparative purposes as it does not reflect their physiological levels. The concentration of albumin in human plasma (40-50 g/l; MW = 66,290) is by far more than that of other major plasma proteins such as γ-globulins (8-18 g/l; MW IgG = 150,000), HDL-Apo A-I (1.3 g/l; MW = 28,016), LDL-Apo B (0.8 g/l; MW B-48 = 264,000; MW B-100 = 550,000) (Gotto et al., 1986; Kongshaug, 1992). The variation in photodynamic efficiencies of ZnPc caused by these serum components is presumably not only an indication of their different affinities for the dye, but may also be due to differences in cellular distribution or the uptake of the different serum components may change properties of cell membranes and other vital targets. Alternatively, it may also reflect differences in the aggregation state of the photosensitizer, thus influencing the primary photophysics of the dye. The use of distribution

ratio (DR) obtained at 10 μM to calculate the cellular LD_{90} (Table 2) is based on the work of Remsen and Shireman (1981) who found that the incorporation of benzo(a)pyrene by cultured cells was linear with respect to concentration of the carcinogen in culture medium. We therefore, assumed that DR, in the presence of serum or individual serum components and in serum-free conditions, is independent of the total drug concentration added to the medium. The possibility of V-79 cells having high affinity but low capacity receptors for the serum components is acknowledged. However, as the concentration of the separated components used for the assay were constant, we believed this factor did not affect our results. HDL enchanced the uptake of ZnPc more than any other serum component studied, but has one of the lowest cellular photodynamic efficiencies suggesting an inefficient distribution mechanism to vital targets for cell survival. Conversely VLDL, which exhibited a lower extracellular LD_{90}, cell uptake, and distribution ratio in comparison to HDL, differed only slightly from the values of cellular LD_{90} (photodynamic efficiency) obtained for HDL. Both VLDL and HDL may, therefore, have similar cellular distribution patterns. Globulins give the same cellular photodynamic efficiency as for serum-free samples, suggesting little or no binding of ZnPc to the protein. LDL and HSA enchanced the photodynamic efficiency of ZnPc. These proteins seem to deliver the dye to vital cellular targets, thereby increasing the photoinactivation of V-79 cells even with reduced cellular uptake of the dye.

However, in vivo, the effects of ZnPc preincubated with serum proteins, especially LDL, may be reduced. This is because endogeneous LDL receptors in the circulation will compete for LDL receptors on malignant cells and consequently reduce the anti-tumoral effect of the complex. Above all, there may be problems of redistribution of the pre-complexed dye to other blood

components such as plasma proteins and erythrocytes. To overcome this,

i). the serum proteins may be modified/derivatized prior to complexation. Sinn et al., (1990) found that albumin linked compounds, especially those derivatized with a deoxysorbitol residue, showed considerable accumulation in neoplastic tissue.

ii). the complexation techniques of phthalocyanines with plasma proteins need to be improved. Covalently linking phthalocyanines to plasma proteins may reduce their dissociation in vivo.

In conclusion, the association of photosensitizers to serum proteins, especially LDL and HSA improves their photodynamic efficiency for the inactivation of V-79 Chinese hamster cells. Attachment of the dyes to such proteins might prove to be an interesting, simple, and efficient system of administering phthalocyanines for photodynamic therapy of cancer.

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CHAPTER FIVE

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APPENDIX

APPENDIX 1

FORMULAE

1. Plating Efficiency (PE) = (# Cells in control petri dishes) x (# Cells seeded)$^{-1}$ x 100.

2. % Survival = (# Cells in test petri dishes) x (# Cells in control dishes)$^{-1}$ x 100.

3. Specific activity (SA) = Radioactivity (cpm or μCi) per μmol of labeled sensitizer.

4. Total cell uptake = Net radioactivity in cells/SA.

5. Uptake per unit cell = Total cell uptake/ (# Cells x volume* of cell).

   * Volume of V-79 cell = 1.2 x 10^{-12} liter (Paquette, 1990).

6. Distribution Ratio (DR) = Uptake per unit cell/Dye concentration in medium.

7. Cellular LD$_{90}$ = Extracellular LD$_{90}$ x DR

   LD$_{90}$ = Concentration of dye required for 90 % mortality of the cells after PDT.

   Extracellular LD$_{90}$ = Concentration of dye in the growth medium required for 90 % mortality of the cells after PDT.

   Cellular LD$_{90}$ = Concentration of dye within the cells required for 90 % mortality of the cells after PDT.

APPENDIX 2

LIGHT SOURCE (ILLUMINATION "BLUE" BOX)

Calculation of Light dose (Fluence or Energy)

Fluence rate (Power) = 100 W/m^2 = 100 W/10^4 cm^2.

Time of exposure to light = 4 min = 240 sec

But, Power (W/cm^2) = Energy (J/cm^2) / Unit time (sec).

Hence, Energy or Light dose = Power/Unit time

Note that : 1 W = 1 J/s

Thus, Light dose (J/cm^2) = 100 (J/s) / 10^4 cm^2 multiplied by 240 sec

= 2.4 J/cm^2

Therefore, exposure of the cells for 4 min to light at a fixed fluence rate of 100 W/m^2 yields a light dose of 2.4 J/cm^2.

APPENDIX 3

ERROR PROPAGATION

The standard deviation for the calculated intracellular LD_{90} was obtained using error propagation formula as described by Knoll (1989)[1].

In error propagation, if x, y, z.... are directly measured quantities or related variables for which we know σ_x, σ_y, σ_z, ..., then the standard deviation for any quantity u derived from these directly measured quantities can be calculated from :

$$\sigma_u^2 = (\delta_u/\delta_x)^2.\sigma_x^2 + (\delta_u/\delta_y)^2.\sigma_y^2 + (\delta_u/\delta_z)^2.\sigma_z^2 + ... \qquad \text{(Equation 1)}$$

where u = u(x, y, z, ...) represents the derived quantity.

The variables, x, y, z, ..., however, must be chosen so that they are truly independent in order to avoid the effects of correlation. For multiplication of independent variables, x, y :

$$u = xy \qquad \text{(Equation 2)}$$

$$\delta_u/\delta_x = y \ \text{ and } \ \delta_u/\delta_y = x \qquad \text{(Equation 3)}$$

Hence, $\sigma_u^2 = y^2\sigma_x^2 + x^2\sigma_y^2$ (Equation 4)

Dividing both sides by $u^2 = x^2y^2$ we have,

$$(\sigma_u/u)^2 = (\sigma_x/x)^2 + (\sigma_y/y)^2 \qquad \text{(Equation 5)}$$

Therefore,

$$\sigma_u = [u^2\{(\sigma_x/x)^2 + (\sigma_y/y)^2\}]^{\frac{1}{2}} \qquad \text{(Equation 6)}$$

In our experiment (Table 2) ,

Intracellular LD$_{90}$ (ILD-90) = Extracellular LD$_{90}$ (ELD-90) x Distribution Ratio (DR)

$$\textbf{ILD-90 = ELD-90 x DR} \qquad \text{(Equation 7)}$$

Substituting equation 7 in equation 6, we obtain,

$$\sigma_{\text{ILD-90}} = [(\text{ILD-90})^2\{(\sigma_{\text{ELD-90}}/\text{ELD-90})^2 + (\sigma_{\text{DR}}/\text{DR})^2\}]^{\frac{1}{2}}$$

$$\text{(Equation 8)}$$

[1] G.F. Knoll (1989) Radiation Detection and Measurement. 2nd Edition. John Wiley and Sons, New York, pp.87-94.