Effect of Photosensitisers on Glioma Cells

By

AU Cheuk-man, Auman

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CERTIFICATE OF ORIGINALITY

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AU Cheuk-man

Abstract

The invasive nature of malignant glioma makes it extremely difficult to be treated by surgery alone. However, the preferential accumulation of photosensitisers in neoplastic tissues suggests that photodynamic therapy (PDT) may be useful as an adjunct therapy following tumour resection. The potential use of three different photosensitisers namely, Photofrin, 5-Aminolevulinic acid (5-ALA) and Calphostin C, in the treatment of glioma was investigated. The uptake, intracellular localisation, cytotoxicity of U87 and GBM6840 glioma cell lines were determined by flow cytometry, fluorescence microscopy and tetrazolium colorimetric reduction (MTT) assay respectively. Their effect on glioma cell invasiveness was evaluated by (1) measuring the levels of matrix metalloproteinase (MMP)-2 and -9 using gelatin zymography, and (2) Matrigel invasion assay. The results showed that uptake of Calphostin C reached saturation within 4 hours, while Photofrin and 5-ALA elevated steadily up to 24 hours. Calphostin C and 5-ALA predominantly localised in the perinuclear region corresponding to the endoplasmic reticulum, whereas Photofrin displayed a lysosomal pattern. The photocytotoxic effect on the two glioma cell lines was similar with LD50 at optimal uptake as follows: 1 μg/mL Photofrin at 1.5 J/cm^2, 1mM 5-ALA at 2 J/cm^2 and 100nM calphostin C at 2 J/cm^2. The inhibition of cell proliferation after Photofrin treatment was similar for both cell lines, which correlated with more cells being arrested in the G0/G1 phase of the cell cycle ($P<0.001$). By contrast, U87 was more sensitive to calphostin C whereas GBM6840 was more susceptible to 5-ALA treatment. The ability of both cell lines to migrate through the Matrigel artificial basement membrane was significantly reduced after PDT

($P<0.001$). This might be due to a decreased production in MMP-2 and MMP-9, together with the reduction of adhesion molecule expression. Photofrin was the most superior in inhibiting cell invasion and calphostin C was the least effective in reducing adhesion molecule expression. Taken together, PDT could be useful in the treatment of gliomas but the choice of photosensitisers must be taken into consideration.

Publication / conference presentation arising from this thesis

1. Cheuk Man AU, Sze Ki LUK, Christopher John JACKSON, Ho Keung NG, Christine Miu YOW, Shing Shun Tony TO. Differential effects of Photofrin, 5-aminolevulinic acid and calphostin C on glioma cells. (J Photochem Photobiol, accepted for publication)

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List of abbreviations

5-ALA	δ-Aminolevulinic acid
aPKCs	Atypical PKCs
BBB	Blood brain barrier
CD	Cluster designation
CNS	Central nervous system
cPKC	Classical PKCs
COX-2	Cyclo-oxygenase-2
DAG	Diacylglycerol
DMSO	Dimethylsulphoxide
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
GBM	Glioblastoma multiforme
HpD	Haematoporphyrin derivative
HVD	Hydroxyethylvinydeuteroporphyrin
ICAM	Intercellular adhesion molecule
IP3	Inositol 1,4,5-trisphosphate
LOH	Loss of heterozygosity
MACE	Monoaspartylchlorin e_6
MAPK	Mitogen-activated protein kinase
MEMα	Minimum essential medium α
MMPs	Matrix metalloproteinases
mPDT	Metronomic photodynamic therapy
MT-MMPs	Membrane type-matrix metalloproteinases
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,-5diphenyltetrazolium bromide
nPKCs	Novel PKCs

p-Akt	Phorphrylated Akt
PBGD	Porphobilinogen deaminase
PBS	phosphate buffered saline
PDGFR	Platelet derived growth factor receptor
PDT	Photodynamic therapy
PI	Propidium iodide
PI3K	Phosphoinositol-3 kinase
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PKC	Protein kinase C
PLD	Phospholipase D
PMA	Phorbol myristate acetate
PRKs	PKC-related kinases
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homologue
Pp	Protoporphyrin
PpIX	Protoporphyrin IX
RT-PCR	Reverse transcription polymerase chain reaction
SnET2	SN (IV)-etiopurpurin
TIMPs	Tissue inhibitors of metalloproteinases
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1. Introduction

Glioblastoma multiforme (GBM), grade IV astrocytoma under the World Health Organisation classification, is the most prevalent and aggressive primary intracranial tumour in adults. Even though advanced surgical techniques are available, the marked infiltration and extensive neovascularisation make complete resection difficult, leading inevitably to tumour recurrence. The partial resistance to radiation and chemotherapy also contributes to its poor prognosis. The median survival time of glioblastoma patients after diagnosis is 4.9 months and the observed mean survival rate is less than 18% beyond one year and less than 4% beyond two years [1,2].

GBM can be classified into primary (*de novo*) and secondary glioblastoma. Most of the primary glioblastomas are found in older patients (mean age 62 years). This form of tumour progresses rapidly, without evidence of less malignant precursor lesions. Secondary glioblastomas occur more frequently in middle-aged individuals (mean age 45 years). This form of tumour develops more slowly by progressing from low-grade or anaplastic astrocytoma. Unfortunately, up to 95% of cases are diagnosed as primary glioblastoma [2].

One of the characteristic features of GBM is the extensive angiogenesis and tissue destruction. The insidious invasiveness of malignant cells plays an important role in the ineffectiveness of current treatment. Residual cells after surgical resection infiltrate the surrounding normal brain tissue causing tumour recurrence. The mechanism of tumour invasion is complicated involving expression of adhesion molecules for local migration, and the production of various kinds of proteolytic enzymes, such as serine and cysteine proteases and metalloproteinases (MMPs) for extracellular matrix (ECM) remodelling [3-5]. Consequently, glioma cells can spread

along the newly formed blood vessels into adjacent normal brain tissues. Among these proteolytic enzymes, matrix metalloproteinase-2 (MMP-2) and MMP-9 (also named gelatinase A and B respectively) expression are significantly higher in GBM as compared with low-grade astrocytomas or normal brain tissue [6-10]. It has been reported that their activities correlate with glioma invasiveness [11-15].

1.2 Genetic alterations in GBM

The most frequent genetic alteration in glioblastoma is loss of heterozygosity (LOH) at chromosome 10q. This occurs in both primary and secondary glioblastoma with similar frequencies, 70% and 63% respectively. However, the frequency of genetic alterations in epidermal growth factor receptor (EGFR) amplification and Phosphatase and tensin homologue (PTEN) mutation are much higher in primary glioblastoma (36% and 25% respectively) than secondary glioblastoma (8% and 4% respectively). By contrast, p53 mutation is more frequently observed in secondary glioblastoma (65%) than in primary glioblastoma (28%) [2].

LOH at chromosome 10q has been found to be associated with reduced survival with regard to glioblastoma [2]. The alteration of mitogenic signalling pathways is thought to play an important role in the development of primary brain tumours, and the cascade of events leading to the progression from low-grade to high-grade glioma has been characterised [16]. Other common genetic aberrations such as EGFR over expression and the deletion of the tumour suppressor gene PTEN are known to promote tumour proliferation by altering signalling pathways, leading to up-regulation of protein kinase C (PKC) resulting in overproduction of MMPs. Therefore, these signalling pathways could be potential targets for therapeutic purposes [17].

1.2.1 EGFR over expression

Over expression of EGFR is responsible for active cellular proliferation including tumour progression, motility, adhesion, invasion and angiogenesis [18]. In GBM, about half of the amplified EGFR gene is mutated. The mutant form of EGFR is constitutively activated and causes low levels of tyrosine phosphorylation. Its mRNA

half-life is extended and escapes from the normal regulatory mechanism. Therefore, therapeutic approaches using antisense RNA to deplete EGFR mRNA shows a marked inhibition of glioma growth [19]. On the other hand, PDT has been demonstrated to down-regulate EGFR expression [20]. Photosensitiser silicon phthalocyanine (Pc4) induces apoptosis in tumour cells both *in vitro* and *in vivo.* Such effect is mediated by the down-regulation of EGFR protein expression.

1.2.2 PTEN mutation

PTEN functions as a tumour suppressor, and is located at chromosome 10q23.3. This region is frequently deleted or mutated in GBM (25% of all cases). Loss of functional PTEN means that the apoptotic cascade of tumour cells is blocked, resulting in tumour proliferation and increased metastatic potential [18,21].

PTEN is a unique protein with a dual-specific phosphatase function mediating both protein and lipid dephosphorylation. It can directly dephosphorylate tyrosine residues on several tyrosine kinases such as focal adhesion kinase (FAK) and Shc-family kinase (the important downstream molecule participating in integrin mediated signalling pathways). In addition, PTEN is able to dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PIP3) and this will reduce the activation of proteins in the signalling pathway downstream of phosphatidylinositol 3-kinase (PI3K) [18].

Akt is over expressed in cancer cells [22]. It requires PIP3 for phosphorylation leading to accumulation of phorphrylated Akt (p-Akt) associated with tumour aggressiveness [21,23]. In physiological conditions, PIP3 and Akt activity are low in the absence of growth factor stimulation. However, PTEN-deficient tumour cell lines exhibit high basal levels of PIP3 and p-Akt. Increased p-Akt levels will block apoptosis

because p-Akt regulates the expression of the antiapoptotic factor Bcl-2 and proapoptotic molecule Bad [24].

Using PI3K inhibitors [21] as well as reintroduction of functional PTEN causes a reduction in glioma invasiveness. This may be associated with a decrease in MMP activity [25]. Furthermore, the effect of PTEN on MMP-2 is unique because it does not alter the MMP-9 expression. This finding implies that regulatory elements in the MMP-2 and MMP-9 promoters are different, and the MMP-2 promoter might be governed by PTEN.

1.3 Matrix metalloproteinases in glioma invasion

1.3.1 Overview of matrix metalloproteinases

Matrix metalloproteinases, the major proteolytic enzymes for ECM remodelling, play important roles in many physiological and pathological processes including wound healing, angiogenesis and tumour invasion [4,26,27]. Based on their substrate specificity and their locations, MMPs can be broadly categorised into 4 general classes: collagenases, gelatinases, stromelysins and membrane type metalloproteinases (MT-MMPs). The former 3 classes of MMP have specific proteolytic actions on their corresponding substrates: collagens, gelatins and laminins respectively [4,28]. MT-MMPs, which exist in association with plasma membranes, and contain a transmembrane domain added on the hemopexin-like C-terminal [29,30]. Most of the MMPs are present in their latent form and need to be activated by proteolytic cleavage. These are mediated mainly by the urokinase/plasminogen-activator/plasmin cascades or the MT-MMP cascades [28,31,32].

1.3.2 Protein Structures of MMP-2 and MMP-9

MMP-2 and MMP-9 are single polypeptides that share the following homologous domains with other MMP family members: (1) pro-domain containing conserved cysteine residue, (2) catalytic domain containing a zinc-binding site, and (3) a hemopexin-like C-terminal domain. However, MMP-2 and MMP-9 are different from others by the inserted gelatin-binding domain between the catalytic domain and zinc-binding site. This domain confers a gelatin proteolytic function on these particular MMPs [26,33,34].

1.4 Interaction between matrix metalloproteinases

1.4.1 Activation

MMP-2 and MMP-9 are secreted in their latent proenzyme form and need to be activated by removing the propeptide extracellularly. The cellular secretion of MMP-2 is associated with a protein molecule called tissue inhibitor of matrix metalloproteinase 2 (TIMP-2), while the secretion of MMP-9 is associated with TIMP-1 molecules. Although MMP-2 and MMP-9 mediate similar biological functions, their activation is controlled by different mechanisms. The activation of MMP-2 is provoked by membrane activation cascade, in which membrane type-1-metalloproteinase (MT-1-MMP) and membrane type-2-metalloproteinase (MT-2-MMP) are able to convert pro-MMP-2 into activated forms [26-29]. However, the activation of MMP-9 is mediated by endogenous proteases such as activated MMP-2, plasmin, stromelysin-1 and exogenous agents such as trypsin and 4-aminophenylmercuric acetate (APMA), but not the MT-MMPs [27,34-36].

1.4.2 Extracellular matrix modulation: Role of TIMPs

TIMP-2 is known to be an inhibitor of activated MMP-2. However, recent data suggest that an appropriate ratio of TIMP-2 together with MT-1-MMP may facilitate the activation of pro-MMP-2 [37]. TIMP-2 may serve as a bridging factor to form a stable pro-MMP-2/TIMP-2/MT-1-MMP tertiary complex on cell surfaces. When the propeptide of MMP-2 molecule is removed, the tertiary complex becomes dissociated and a free TIMP-2 molecule turns into the inhibitor of activated MMP-2. This implies inhibition of MMP-2 by TIMP-2 functions in a concentration dependent fashion. Pro-MMP2 activation is facilitated by the presence of TIMP-2 at low levels; otherwise,

TIMP-2 appears as an inhibitor of activated MMP-2 [33,37-39]. Therefore, MMP inhibitors play an important role in regulating a balance between degradation and remodulation of ECM [36,39-40]. In this manner, interaction between proteolytic enzymes and inhibitors ensure an efficient localised degradation of ECM and may enhance invasion [41,42].

1.5 The protein kinase C

Protein kinase C (PKC) is an important molecule in signal transduction. It plays an important role in the invasiveness of glioma cells and is involved in one of the major regulatory steps in the regulation of MMP-2 and MMP-9 expression. PKC activity has been correlated to the malignancy of gliomas [43].

There are at least 13 isoforms in the PKC family; they are characterised based on calcium, phospholipid and diacylglycerol (DAG) activation. They can be further classified into four subgroups on the basis of their modes of activation [43-46]. All PKC isoforms consist of a single polypeptide with two domains: a regulatory domain at the NH_2 end terminus and a catalytic domain at the COOH end terminus. (Fig.1)

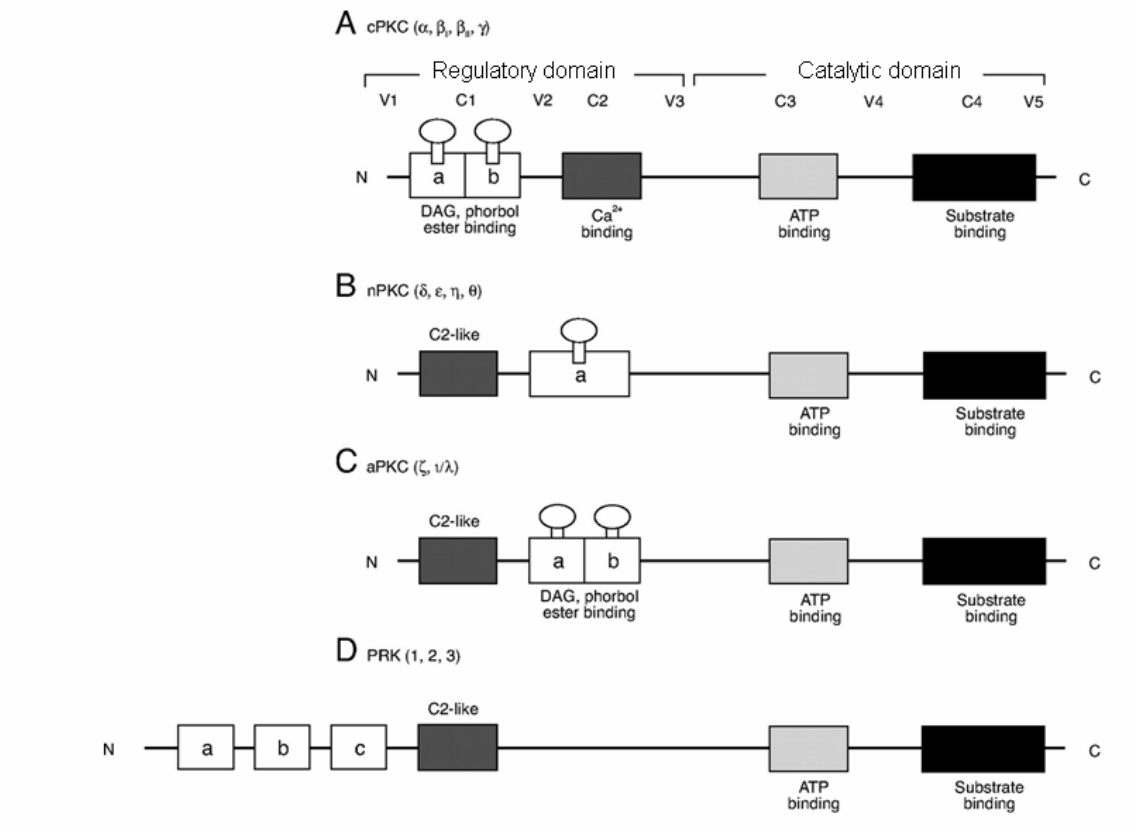


Figure 1. Schematic representation of protein structure showing the various subgroups of the PKC family. PKC can be classified into four subgroups on the basis of their modes of activation including classical PKCs (A), novel PKCs (B), atypical PKCs (C) and PKC-related kinases (D). All PKC isoforms contain a regulatory and a catalytic domain, each isoform consists of four conserved regions (C1-C4) and five variable regions (V1-V5). The regulatory domain is located within the C1 and C2 regions at the NH_2 end terminus (N), and the catalytic domain locates within C3 and C4 regions at the COOH end terminus (C) of the enzyme. (Adapted from Rocha et al. 2002) [47]

The first group of isoforms consists of four classical PKCs (cPKCs): α, β1, β2, and γ isoforms, all of which depend on calcium, diacylglycerol (DAG) and phosphatidylserine (PS) for activation. The second group of isoforms consists of four novel PKCs (nPKCs): δ, ε, η and θ that are independent of calcium but require DAG and PS for activation. The third group of isoforms is the atypical PKCs (aPKCs) λ and ζ. Their activation does not require calcium or DAG, but only PS. More recently, a fourth group of structurally distinct PKCs has been identified. Similar to the aPKCs, these so-called PKC-related kinases (PRKs 1, 2, and 3) only require PS for activation.

The classical PKCs are involved in the plasma-membrane-associated lipid cascades, which require the binding of growth factor receptor, phospholipases C (PLC) and/or phospholipase D (PLD). Activated PLC can catalyse the formation of DAG and inositol 1,4,5-trisphosphate (IP3) from phosphatidyl inositol-3,4-bisphosphate (PIP2). The elevated level of cytoplasmic IP3 triggers the release of calcium from the endoplasmic reticulum, which together with DAG activates the PKCs. Once activated, PKC can transmit growth stimulation signals to the nucleus through different cascades, or trigger the anti-apoptotic pathways (Fig.2).

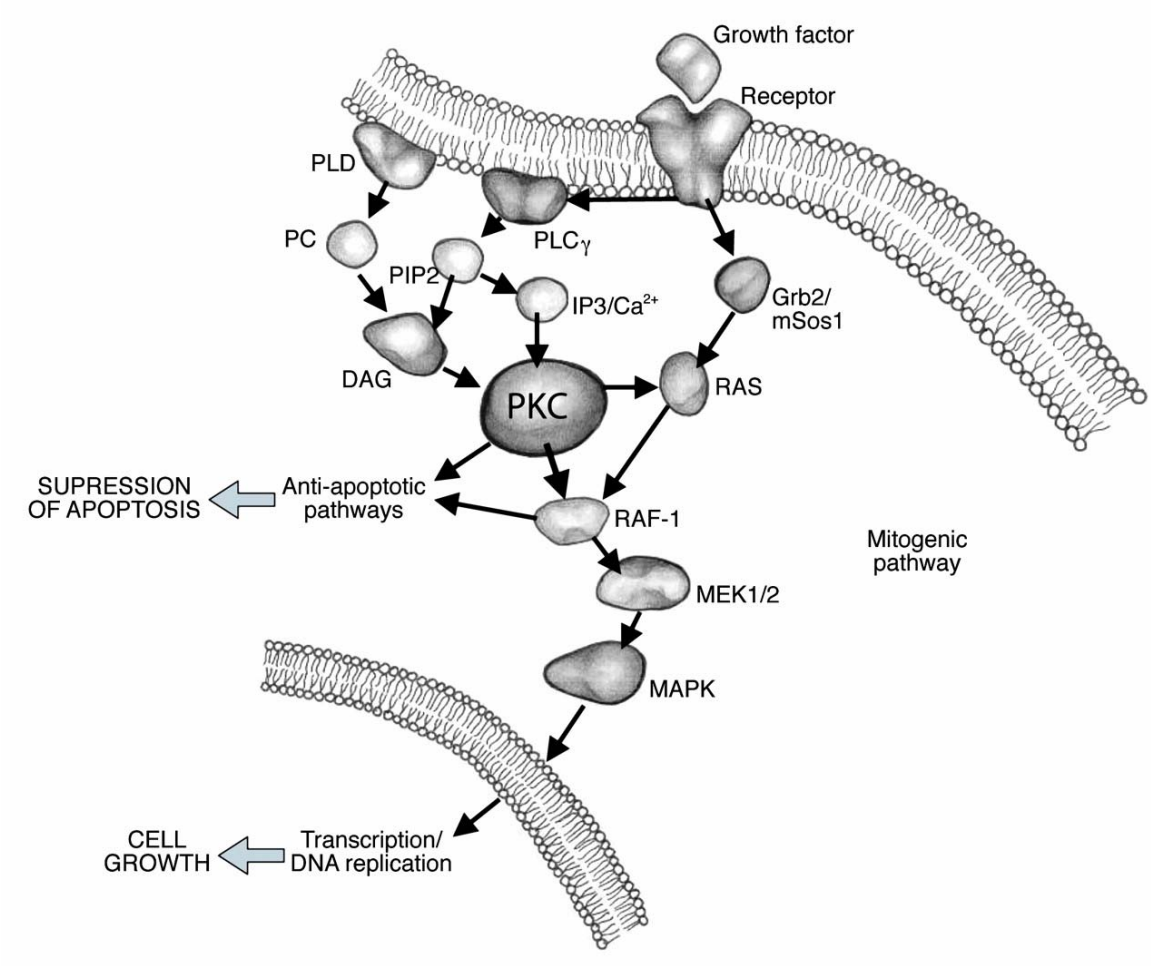


Figure 2. Schematic diagram showing the participation of PKC in the signal transduction pathway. Upon activation by certain up-stream signalling elements (e.g. growth factor receptor, PLD and DAG), PKC can trigger the anti-apoptotic pathways or transmit growth signals to the nucleus via certain downstream proteins. (Adapted from Rocha et al. 2002) [47]

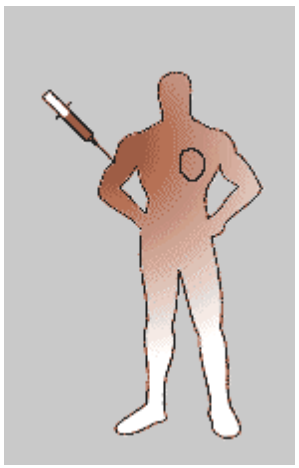
Distribution of different PKC isoforms is organ and cell specific, implying individual PKC isoforms may be responsible for the regulation of different cellular functions [47-51]. Amongst these isoforms, PKCα appears to be more critical in regulating tumorigenesis in human glioma [48,52].

PKC plays important roles in tumorigensis because (1) PKC expression and activation is correlated to tumour growth, (2) PKC is a fundamental component of the signalling pathways of several growth factors and (3) PKC acts as a potent tumour promoter receptor producing a transformed phenotype in a wide range of cell types. Therefore, therapeutic strategies targeting PKC activity significantly arrest tumour proliferation. The application of PKC inhibitors (e.g. Tamoxifen, staurosporine and calphostin C) [47,50,53,54] as well as PKC antisense oligodeoxynucleotides [55-57] in cancer treatment have been studied extensively, and the results are promising and strengthen their potential use in clinical settings.

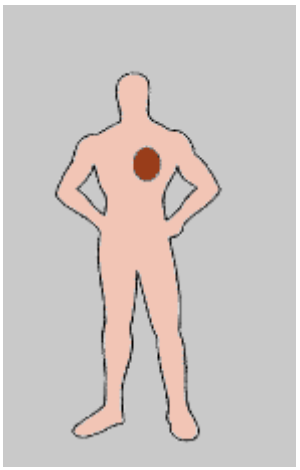
1.6 Photodynamic therapy

1.6.1 Definition of PDT

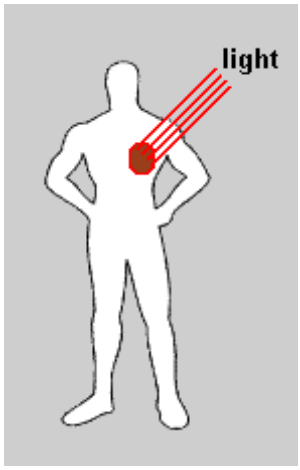
Photodynamic therapy (PDT) is a local form of treatment based on the preferential accumulation of photosensitising drugs in malignant tissue. Subsequent exposure to light of an appropriate wavelength results in the generation of singlet oxygen and causes irreversible photodamage to sensitised cells [58-60]. Singlet oxygen has a very short lifetime (<0.04 μs) and radius of action (<0.02 μm), which limit its migration from the site of formation so that healthy regions of the body are not affected. Furthermore, most photosensitisers do not accumulate in the nucleus, and hence PDT has a low potential for causing DNA damage, mutations and carcinogenesis [58-60].



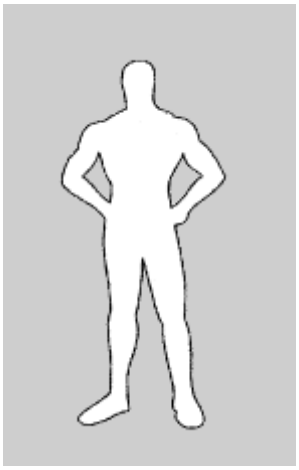
(1) The photosensitiser is given by injection



(2) The photosensitiser concentrates in the tumour



(3) The photosensitiser is activated by light



(4) The tumour is selectively destroyed

Figure 3. The mechanism of how PDT works. (Adapted from http://www.bmb.leeds.ac.uk)

1.6.2 History of photodynamic treatment and photosensitiser development

Making use of light as a therapeutic agent can be traced back to over three thousand years ago in China, India and ancient Egypt. It was used together with the administration of herbs to treat skin diseases, such as psoriasis and vitiligo. The idea of photodynamic treatment was first recognised by Oscar Raab and Von Tappeiner in 1900 [61,62]. They found that protozoa were killed effectively in the presence of acridine red and light, but neither acridine red nor light alone could cause such an effect. Therefore, they concluded that the cause of cell death was the result of energy transfer from light to the chemical, and this was the first report on so-called 'photodynamic treatment'.

Haematoporphyrin is a mixture of porphyrins, first identified by Scherer in 1841 [63]. However, the fluorescence property of this compound was not described until 1867 by Thidichum [64], and it was named 'haematoporphyrin' in 1871 by Hoppe [65]. The first report of haematoporphyrin was published in 1911 when Hausmann [66] reported that the biological effect of haematoporphyrin together with light was such that it could kill paramecium, cause red blood cell lysis and skin photosensitivity in mice. It was not until 1942 when Auler and Banzer [67] reported the selective accumulation of porphyrin in malignant tumours as indicated by the fluorescent signals obtained [67]. This observation prompted many scientists to further investigate the selective localisation properties of porphyrins in cancer therapy (photodynamic therapy) and visual diagnosis (photodynamic detection).

The major disadvantage of haematoporphyrin is the large dosage required and the unacceptable phototoxic effects. In 1955, Schwartz et al. [68] attempted to purify this mixture. Interestingly, it was found that the partially purified product had poor affinity

for tumour tissue, whereas the residue of non-haematoporphyrin fraction had good tumour localisation properties. They further investigated the non-haematoporphyrin fraction and purified the first generation of photosensitiser known as haematoporphyrin derivative (HpD). This product was found to be more efficient than haematoporphyrin in killing tumour cells [69]. HpD consists of a mixture of porphyrin monomers such as haematoporphyrin, hydroxyethylvinyldeuteroporphyrin (HVD) and protoporphyrin (Pp), as well as complex dimeric and oligomeric fractions of porphyrins. It was later in the 1980s that the biological activity residues principally in the oligomeric fraction of porphyrins were found, which became the most widely used commercial product called Photofrin. Photofrin-mediated PDT has proven to be curative in a range of solid tumours including skin, bladder, lung and gynecological cancer [70].

The advantages of this first generation photosensitiser are that it (1) is easy to obtain from readily available substances (haemoglobin), and (2) has a clear historical background with positive results in cancer treatment and has received regulatory approval for PDT application. However, Photofrin has documented drawbacks and the most significant disadvantage is the prolonged subcutaneous retention that may last for several weeks and result in skin photosensitisation. In addition, the presence of several unidentified porphyrins may lead to biological activity not always being reproducible. Therefore, the accuracy of dose-response studies may be affected. Furthermore, it has relatively poor selectivity for tumour tissues. Photofrin has a number of absorption peaks between 400 and 650nm but 630nm, the lowest absorption wavelength, is the most often used wavelength for PDT excitation because of more efficient tissue penetration at this wavelength. Therefore, the application of higher drug doses and higher light doses is required for Photofrin to be effective [70,71].

1.6.3 New generation of photosensitisers

Because of the disadvantages in first generation photosensitisers, other photosensitisers are being investigated for their use in PDT. This “second generation” photosensitisers can be divided into two groups (Table 1) according to the presence or absence of the tetrapyrrole structure [70]. The second generation photosensitisers are more preferred in PDT for many reasons. First, they are single substances rather than complex mixtures, so that a clear formulation and reproducible analytical results can be obtained. Second, they have stronger absorbance from 600nm to 900nm, the region of wavelength where light scattering is low and they allow deep light penetration into tissue. Third, their improved molecular structures may enhance tumour selectivity.

Table 1. Second generation of photosensitisers

Tetrapyrrole group	Non-tetrapyrrole group
(1) Porphyrins and the porphyrin precursor ● Tetra(m-hydroxyphenyl) porphyrins (m-THPP) ● sulphonated tetraphenylporphyrin (TPPSn) ● tetrasulphonic acid ($TPPS_4$) ● δ-aminolaevulinic acid (5-ALA) and its esters **(2) Chlorins** ● Monoaspartylchlorin e_6(MACE) ● tetra-(m-hydrosyphenyl) chlorin (m-THPC) ● Benzoporphyrin derivative monoacid ring A (BPD) ● Sn(IV)-etiopurpurin ($SnET_2$) **(3) Phthalocyanines** ● Aluminium phthalocyanine ● Silicon phthalocyanine	**(1) Texaphyrin** **(2) Perylenequinones** ● Calphostin C **(3) Hypericin**

The recent development of 'third generation' photosensitisers further enhances the effectiveness of PDT. These photosensitisers are formed by conjugating specific molecules such as peptides, polymers (e.g. polyethyleneglycol residues) and lipophilic molecules (e.g. long carbon chain). They may also encapsulate in liposomes or couple with monoclonal antibodies against tumour-associated antigens (e.g. CA125 antibody for ovarian cancer cells). And there is the possibility of identification of novel photosensitisers with stronger affinity for tumour specific molecules as well [72,73]. In general, a good photosensitiser should fulfill the following criteria: (1) exhibit no dark toxicity and only be cytotoxic in the presence of light, (2) have good selectivity for tumours over normal tissue, (3) have rapid systematic excretion after treatment to reduce generalised photosensitivity, (4) be a biologically stable single component compound, (5) have a high triplet quantum yield and efficient energy transfer to produce singlet oxygen, (6) have a peak activation at 600nm-900nm in which transmission of light in tissue is most effective, (7) have low or no photobleaching qualities, and (8) be able to cross an intact blood-brain barrier to reach infiltrated tumour cells such as in gliomas.

1.6.4 Mechanism of photosensitivity

As described previously, the principal cytotoxic product of the PDT photochemical reaction is singlet oxygen, and the effectiveness of the photosensitiser is directly proportional to the total yield of singlet oxygen produced. Photosensitisers are always in the singlet ground state (^1P), the lowest energy level. Once the photosensitiser is illuminated with the appropriate wavelength corresponding to maximum absorbance, electrons of the photosensitiser are excited from the stable ground state to a higher energy stage, called excited singlet state ($^1P^*$). As this is an unstable state, energy is

then released in two possible ways (Fig. 4): the first is in the form of fluorescence and then return to the ground state, and another alternative is through the intersystem crossing pathway into the triplet state ($^3P^*$). Photosensitisers in the triplet state have much longer lifetimes and, thus, the energy can be passed along to other molecules to initiate Type I or Type II reactions (Fig. 5). In Type I reactions, a photosensitiser in the triplet excited state directly draws an electron from surrounding substrate to form a free radical. This radical then reacts rapidly with oxygen resulting in the production of a highly reactive oxygen species (O_2^-). In Type II reactions, the triplet state of the photosensitiser readily transfers its energy down to the ground state molecular oxygen ($^3O_2$) directly, resulting in the production of singlet oxygen ($^1O_2^*$). Such reaction can only take place in oxygenated environments [58]. In PDT, singlet oxygen is the most reactive oxygen species that can oxidise any biological molecule it encounters, and mediate the entire cyototoxic mechanism.

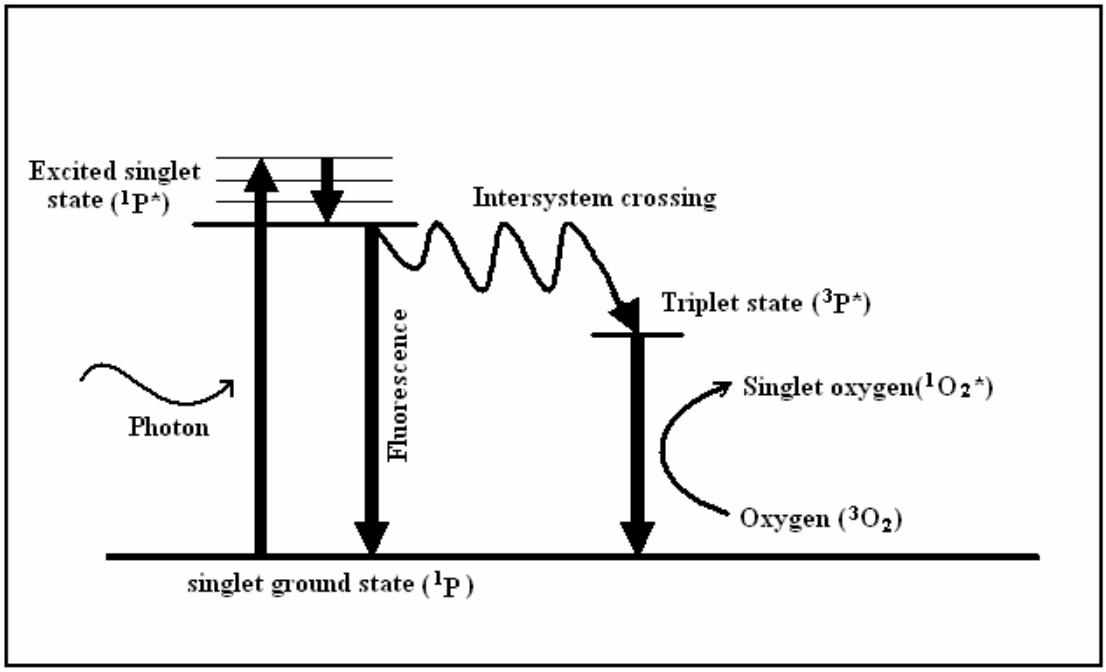


Figure. 4. Diagram illustrating the energy state and interactions of photosensitisers. When light energy (photons) of appropriate wavelength is absorbed, an electron is raised to a higher energy level. The molecule then rearranges to a stable excited state ($^1P^*$). At this point, there are two possibilities: the excited molecule can fluoresce by emitting light energy, and then drop back to the ground state. Alternatively, intersystem crossing can change the spin of an electron. During the lifetime of the resulting triplet state ($^3P^*$), absorbed energy can be passed along to molecular oxygen in tissue, activating oxygen molecules to the singlet state ($^1O_2{}^*$). Singlet oxygen will oxidise any biological molecule that it encounters.

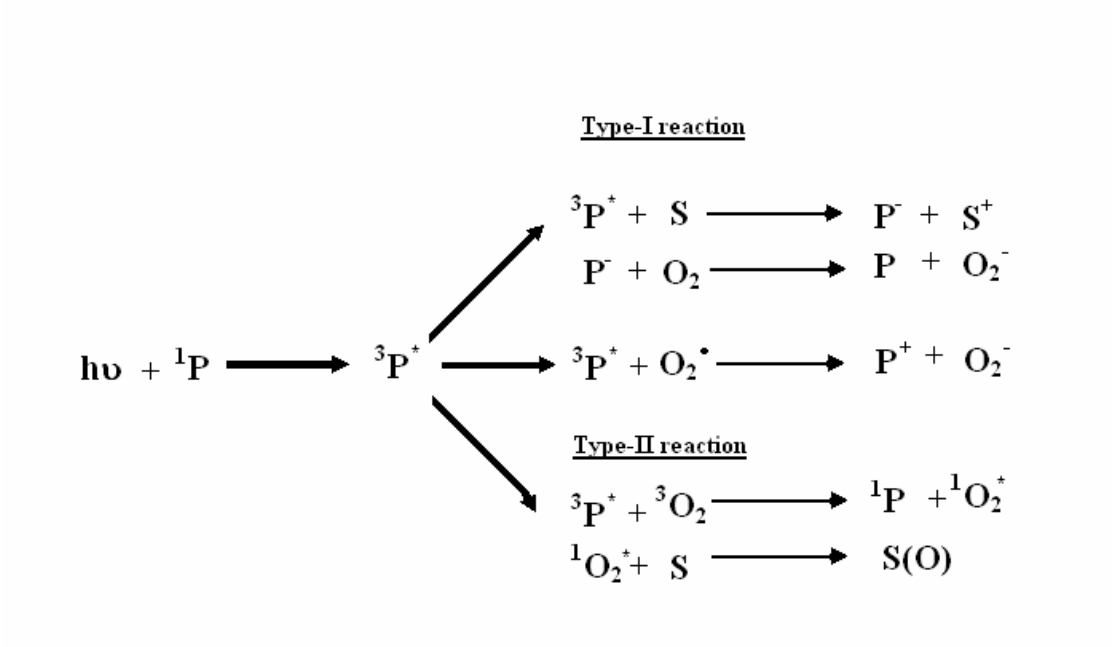


Figure 5. Type-I and Type-II reactions. ^1P is a photosensitiser in a singlet ground state, $^3P^*$ is a photosensitiser in a triplet excited state, S is a substrate molecule, P^- is a reduced photosensitiser molecule, S^+ is an oxidised substrate molecule, O_2 is molecular oxygen, O_2^- is the superoxide anion, $O_2^\bullet$ is the superoxide radical, P^+ is the oxidised photosensitiser, $^3O_2$ is ground state oxygen, $^1O_2^*$ is oxygen in a singlet excited state, and S(O) is an oxygen adduct of a substrate.

1.6.5 History of PDT in brain tumour

In the past 20 years, PDT in brain tumours has been studied extensively in the laboratory as well as in clinical trials. Promising data shows that it is a potentially valuable adjuvant therapy that can be applied in combination with conventional surgical procedures.

In 1980, the first reported clinical trial on glioma was conducted by Perria et al. [74] in which PDT was applied at the residual tumoural bed immediately after resection of the glioma. Although the result was disappointing, their work provided important information for future applications such as dose of light irradiation for brain tumours should be lower than those used in systemic tumours. The development of improved laser delivery systems also help to produce better tissue penetration leading to extensive tumour tissue destruction. Muller and Wilson [75] obtained a more promising result by using a new illumination delivery system. They used a fibre-optic probe attached to an inflatable balloon applicator coupled with an argon-dye pump laser. This device was applied to the cavity after radical tumour resection allowing uniform spherical distribution of light to the cavity. In their clinical trial, the therapeutic light dose was much elevated, which enhanced the survival rate of GBM patients significantly. The median survival was improved to 82% at one year and 57% at 2 years, as compared with the mean survival of patients following standard treatment at less than 12months and the 2-year survival less than 7.5%. In addition, it raised the survival rate of malignant astrocytoma to 44 weeks and astrocytoma-oligodendroglioma to 61 weeks [76].

Although the results are encouraging, recurrence is inevitable because of the residual tumour cells left behind. The infiltrative nature of GBM makes it difficult to

discriminate between normal and tumour tissue, and thus complete glioma resection is impossible. Stummer et al. [77] tried to address this issue by proposing the application of 5-ALA mediated fluorescence as an intraoperative fluorescence-guided resection in glioma surgery. The principle is based on malignant tissue being able to increase endogenous PpIX formation therefore producing a fluorescent image to allow surgeons to remove the tumour mass as much as possible. This technique has been reported to achieve maximum tumour removal safely with 100% specificity and 85% sensitivity [78]. Theoretically, coupling this technique with post-surgical PDT to the resection cavity may improve the prognosis of patients.

Although PDT is a relatively harmless adjuvant treatment for most patients with brain tumours, some limitations should be addressed before its extensive use. Brain oedema and necrosis are the most common side-effects which may lead to permanent neurological deficit. Skin photosensitivity is another issue that needs consideration.

1.7 Characteristics of two different classes of second-generation photosensitisers

The use of traditional photosensitisers is limited by their considerable side effects, such as poor tumour selectivity, that leads to post-operative brain edema and prolonged skin photosensitisation. Recently, several kinds of second-generation photosensitisers targeting different organelles have been investigated, for example, δ-aminolaevulinic acid (5-ALA), precursor of protoporphyrin IX (PpIX) generated endogenously within cells, and Calphostin C, a novel photo-dependent inhibitor of PKC.

1.7.1 5-ALA mediated PDT

5-ALA-induce-PpIX mediated PDT is a novel therapeutic strategy which utilises the haem biosynthesis pathway to produce endogenous protoporphyrin IX (PpIX), and PpIX is the principal substance mediating photoactivity [79]. Under physiological conditions, endogenous production of haem is governed by two mechanisms: substrate availability and the feedback inhibition of enzymes. Two major enzymes control the rate limiting steps in haem formation. The initial step depends on the activity of 5-ALA synthase which mediates the formation of 5-ALA from glycine and succinyl Co-A. Administration of exogenous 5-ALA bypasses this step, leading to a build-up of PpIX. The second step is governed by ferrochelatase which catalyses the haem incorporation of ferrous iron into the porphyrin ring. This process is controlled by enzyme activity as well as iron availability. Certain types of neoplastic cells exhibit insufficient ferrochelatase activity and may also have iron depletion. Therefore, the final conversion rate to haem is reduced, leading to the accumulation of PpIX within the tumour tissue [79,80]. Recently, it was reported that porphobilinogen deaminase (PBGD), the intermediate enzyme in the haem pathway, is elevated in glioma cells.

Thus exogenous administration of 5-ALA may induce PBGD activity which further enhances the localisation of PpIX [81].

1.7.1.1 Application of 5-ALA esters

The efficiency of porphyrin accumulation is proportional to the rate of 5-ALA transport across the plasma membrane. Elevated numbers of low-density protein receptors in neoplastic cells may favour the preferential uptake of lipophilic sensitisers. Liposome-associated or esterification of 5-ALA with long chained carbon has been shown to facilitate their transport across cellular membranes. Consequently, the concentration required to induce PpIX formation is 30 to 150-fold lower with these esters than with 5-ALA [82-86]. Amongst the 5-ALA esters, hexylester of 5-ALA is the most efficient in PpIX formation [82-84]. On the other hand, there is no correlation between lipophilicity of 5-ALA ester and the total cellular PpIX levels, suggesting that drug uptake and ester cleavage are two different processes but both are necessary for efficient PpIX formation [82].

1.7.1.2 Intracellular localisation of PpIX

It was reported that the cytotoxic effect of exogenously supplied PpIX is less than PpIX generated endogenously from 5-ALA [87]. This can be explained by the fact that PpIX produced endogenously accumulates in mitochondria and directly inactivates mitochondrial functions, whereas the exogenous PpIX is localised in the plasma membrane. Although endogenous generated PpIX is located inside mitochondria, prolonged incubation may cause redistribution of PpIX into the endoplasmic reticulum and plasma membrane. Long-term incubation further leads to lysosomal localisation [87]. Thus, mitochondrial photodamage is more effective at short incubation periods

but prolonged treatment with 5-ALA may alter its therapeutic efficiency by allowing PpIX to redistribute to other subcellular organelles.

1.7.1.3 Limitations of 5-ALA mediated PDT

The production of PpIX is mainly dependent on the enzyme activity in the haem pathway. Different cell lines may respond differently in PpIX synthesis and therefore 5-ALA cannot be applied universally [88]. Porphyrin is the principal mediator in 5-ALA photosensitisation. In this reaction, excitation of PpIX occurs at a wavelength of 630 nm. Such wavelength of maximal absorption is similar to HpD and Photofrin, and hence no improvement for the aptitude of tissue penetration. Furthermore, the high affinity of PpIX to albumin may cause it to efflux out of the tumour tissue and redistribute to surrounding non-tumour areas.

1.7.1.4 Use of 5-ALA in fluorescence-guided resection

Owing to endogenous PpIX has higher potential to concentrate in neoplastic tissue, the boundary of tumours can therefore be detected after systemic administration of 5-ALA. Glioma is often very hard to distinguish from normal brain tissue. 5-ALA mediated PpIX accumulation in gliomas may guide the removal of suspicious tissue during operations [77,78,89,90]. 5-ALA can be administrated orally and the fluorescence is sufficiently strong to allow intraoperative identification of malignant tissue by simple illumination systems. However, the limitation in detecting malignant glioma is the bleaching effect after prolonged illumination. Peak fluorescence in malignant tissue is approximately 6-8 hours after oral administration. Therefore, tumour resection should be performed at the 6^{th} hour and final inspection of the tumour cavity for fluorescence detection of residual tumour should be performed within 8 hours after drug administration [91]. Another substantial limitation is the low sensitivity to

display fluorescence in grossly necrotic tissue, a characteristic of glioblastoma multiforme.

1.8 Calphostin C mediated PDT

Perylenequinones, the pigment isolated from fungi, are effective producers of singlet oxygen in the presence of visible light [92,93]. Calphostin C consists of a perylenequinone structure and is regarded as a novel photosensitiser that selectively inactivates protein kinase C (PKC) [94-96]. The photoactivated calphostin C generates a short-lived singlet oxygen species that reacts with the mercapto groups of the PKC cysteine residues present in the regulatory domain, resulting in irreversible oxidative inactivation of intracellular PKC [94]. PKC is an important family of cell regulatory kinases playing a major role in intracellular signal transduction (see section 1.5). Calphostin C is a potent apoptotic inducer and exhibits photocytotoxic effect at nanomolar concentrations with minimal dark toxicity [97,98]. In addition, the formation of calphostin C-PKC complex may further enhance tissue photosensitivity because it has a longer absorption band at 710 nm, a wavelength that facilitates tissue penetration [93,96,99].

1.8.1 Types of PKC inhibitor

Based on the mechanism of inhibition (inhibiting catalytic domain or regulatory domain), PKC inhibitors can be classified into two groups. Staurosporine and tamoxifen, the broad-spectrum protein kinase inhibitors, interact with the catalytic domain of PKC. Hypericin, found in the natural product *hypericum perforatum* (St. John's wort), inhibits the PKC regulatory domain in a photo dependent manner [100,101].

Tamoxifen, already used in adjuvant therapy in malignant tumours, shows moderate potency as a PKC inhibitor and requires relatively high doses to achieve therapeutic levels. Staurosporine is a relatively non-selective protein kinase inhibitor which has unacceptable systemic toxicity at concentrations necessary to achieve optimal PKC inhibition [102,103].

Calphostin C is superior to other PKC inhibitors because its effective photocytotoxic dose is at nanomolar concentrations with little or no dark cytotoxic effect [103]. The ability of calphostin C to induce apoptosis in different neoplastic cell lines is well documented but information such as optimum light dose, the drug uptake pathway and subcellular localisation is limited. Therefore, further studies on this drug may provide insights into its application in photodynamic therapy.

1.8.2 The mechanism of Calphostin C induced apoptosis

Calphostin C exhibits rapid induction of apoptotic changes [104]. It has been reported that activated calphostin C rapidly down-regulates the transcription and translation of anti-apoptotic molecules Bcl-2 and Bcl-xl in glioma cells [97,98,105,106]. In the absence of these molecules, Bax can promote apoptosis. Calphostin C induces

translocation of pro-apoptotic monomeric Bax into mitochondrial membranes, followed by Cytochrome C release into cytosol and subsequent decrease of mitochondrial inner membrane potential before the activation of caspase-3. The apoptosis brought about by calphostin C is redistribution rather than increased levels of Bax. The translocation of Bax from cytosol to mitochondrial membranes may subsequently lead to mitochondrial hallmarks of apoptosis. Apart from apoptosis, calphostin C is able to potently kill drug-resistant breast cancer cells through a mechanism that involves the induction of cytoplasmic vascuolisation [107].

1.9 Project objective

Different photosensitisers act via different pathways to achieve cytotoxicity. In this study, the effect of three photosensitisers (Photofrin representing the first generation drug, 5-ALA the second generation and calphostin C the novel compound) on glioma cells was investigated. The uptake, localisation and cytotoxic effect of these photosensitisers were established. More importantly, their sub-lethal influence on cell cycle, proliferation potential and invasive capacity were evaluated. The difficulty in treating GBM is due to its insidious invasion of surrounding normal tissue. Therefore, a better understanding of how glioma cells respond to sub-lethal photodynamic therapy may provide more information in designing new PDT strategies for the treatment of GBM.

Chapter 2

Materials and Methods

2. Materials and methods

2.1 Cell culture

Human glioblastoma cell line U87, obtained from American Type Culture Collection (Rockville, MD, USA), and GBM6840 cell line [108], derived from a paediatric cerebellar glioblastoma, were grown in Minimum Essential Medium Alpha medium (MEM-α, Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) foetal bovine serum (FBS) with no antibiotics added. Cell cultures were established in 75 cm^2 flasks and kept in humidified atmosphere with 5% CO_2 at 37 °C.

2.2 Drug preparation

Photofrin, a generous gift from Quadra Logic Technologies, Inc. (Vancouver, BC, Canada), was prepared with 5% dextrose to make a 2.5 mg/mL stock solution. 5-Aminolevulinic acid hydrochloride (5-ALA), purchased from Sigma (St. Louis, MO, USA), was reconstituted in deionised water to make a 30 mM stock. Calphostin C, obtained from Sigma, was dissolved in dimethylsulphoxide (DMSO) to make a 1 mM stock. All solutions were kept at -20°C until use. Storage and dilution of all drugs were performed under experimental conditions avoiding light exposure.

2.3 Drug uptake assay

In a preliminary study, the concentration of each photosensitiser to be used was established by incubating glioma cells with various concentrations of the drug. The highest concentration of photosensitisers (1 μg/mL for Photofrin, 1 mM for 5-ALA and 100 nM for calphostin C) that showed no significant dark cytotoxic effect after 24 hours

incubation was chosen for all the experiments in this study. These concentrations are comparable to previously published studies [53,109,110].

One million cells were seeded overnight in 35-mm culture petri dishes. Before addition of drugs, the cells were rinsed with phosphate buffered saline (PBS) and incubated with defined concentration of photosensitisers for various periods of time. For Photofrin and 5-ALA, serum-free medium was used because albumin causes efflux of PpIX from cells [111]. After drugs were added, care was taken to avoid exposure to light. Following incubation with different photosensitisers, cells were trypsinised and then washed twice with PBS to remove any residual drug, and then resuspended in 500 μL PBS. The cellular fluorescence was quantified using a Beckman Coulter Epics® Elite flow cytometer. Fluorescence of all three photosensitisers was initiated by an argon laser excitation at 488 nm and measurement taken by photomultiplier tube PMT4 channel after passing a 610-nm long pass filter. Twenty thousand events were recorded for each sample and the cellular concentration of each drug was expressed as the mean fluorescence intensity per cell.

2.4 Detection of photosensitiser localisation

Similar cell treatment procedures as described in section 2.3 were used, but cells were seeded onto the coverslips placed inside 35-mm culture petri dishes. After incubation (four hours for calphostin C; 24 hours for 5-ALA and Photofrin), the coverslips were removed and washed gently twice with PBS. They were then mounted on glass slides with cell-containing side facing down. Images were captured using a fluorescence microscope (ECLIPSE, Nikon, Japan) with excitation at 488 nm.

To correlate the location of the photosensitisers, organelle probe labeling was performed simultaneously using MitoTracker Red 580 for mitochondria, ER-Tracker Blue-White DPX for endoplasmic reticulum (ER) and acridine orange for lysosome.

MitroTracker Red 580 contains Chloromethyl-X-rosamin, which is a lipophilic cationic fluorescent dye that is selectively concentrated inside mitochondria by their negative mitochondrial membrane potential [112]. ER-Tracker Blue-White DPX contains Dapoxyl dye. This dye is photostable and selective for the endoplasmic reticulum [113]. Acridine orange is a weak basic dye with particular affinity to DNA, RNA and lysosome. Acridine orange can exist as monomer and dimmer forms, which have different absorption spectra and fluorescence properties. Dimerisation will result in quenching the absorption and fluorescence of the dye. Once acridine orange is taken up by the lysosome, the acid pH will cause dimerisation and shifting the colour from green to orange [114,115].

MitoTracker Red 580 and ER-Tracker Blue-white DPX were purchased from Molecular Probes (Eugene, OR, USA), acridine orange was purchased from Sigma. The cells were incubated with probe-containing medium (500 nM MitoTracker or ER-Tracker for 30 minutes; 0.5 μM acridine orange for 15 minutes) at 37°C, then washed and examined by fluorescence microscopy. The following excitation and emission wavelengths were used: MitoTracker and acridine orange, excitation at 581 nm and emission at 600 nm long pass; ER-Tracker, excitation at 374 nm and emission at 430 nm long pass.

2.5 Determination of cell viability

Thirty thousand cells were seeded onto 96-well flat bottom plates. After overnight incubation, the culture medium was removed and cells were rinsed with PBS, followed by incubation with defined concentration of photosensitisers for various periods of time. The cells were then exposed to white light emitted from a 140W quartz-halogen lamp with a 500-nm long pass filter. Cells not incubated with any photosensitiser were treated in the same way as controls. Cells incubated with various drugs but without light irradiation were used as dark controls. The cytotoxic effect was tested by the tetrazolium colorimetric reduction assay (MTT assay). This assay is based on the reduction of a water-soluble tetrazolium salt (3-[4,5-Dimethylthiazol-2-yl]-2,-5diphenyltetrazolium bromide) to a purple, insoluble formazan product by mitochondrial dehydrogenases, and these enzymes are functional only in living, metabolically active cells. The formazan crystals formed are dissolved in DMSO and the optical density measured is proportional to the number of viable cells. One hundred and fifty micro-litre of MTT solution (0.2 mg/mL in PBS) was added to each well of a 96-well plate and then incubated for 4 hours in darkness. The plate was then centrifuged and the supernatant removed. DMSO (150 μL) was then added to each well and the plate was agitated gently for 15 minutes to allow complete dissolution of the formazan crystals. The optical density was quantified by an ELISA plate reader (Magellan TECAN, Austria) at 570nm. Each treatment group is reproduced in fourth for each independent experiment, and the cell viability was calculated as follows:

% cell viable = (mean absorbance of sample / mean absorbance of control) x 100

2.6 Cell cycle study

After PDT treatment, cells were allowed to recover in complete medium for 24 hours, then released by trypsin and washed twice in PBS. The cells were then fixed in cold (-20°C) 70% ethanol for 2 hours. After centrifugation, they were re-suspended in 1 mL PBS containing 20 μL of propidium iodide (PI) staining solution (50 μg/mL PI and 10 μg/mL RNAse A) at 4°C. After 20-minute incubation in the dark, the cells were analysed using a flow cytometer. Approximately 2 x 10^4 events were recorded for each sample. Cell cycle analysis on the nuclear fluorescence signal was performed using the Modfit LT 2.0 software.

2.7 Determination of gelatinase activity

Cells ($1x10^6$ per 35-mm petri dish) undergoing various PDT treatments were subsequently incubated in 2 mL serum-free medium for 24 hours. The supernatant was collected and centrifuged to remove cell debris and their protein contents determined using the Bradford reagent (Bio-Rad, NY, USA). Gelatinolytic activity of MMP-2 and MMP-9 was examined by gelatin zymography as described previously [116]. Briefly, supernatants with equal amounts of protein (1 μg) were mixed with loading dye, then applied onto separating gels containing 10% (w/v) polyacrylamide and 0.1% (w/v) gelatin. Sodium dodecyl sulfate-polyacrylamide gel eletrophoresis (SDS-PAGE) was carried out at 150 mV in a mini-gel apparatus (Hoefer Pharmacia Biotech Inc., CA, USA) until the loading dye (bromophenol blue) reached the bottom of the gel. The gel was then renatured in 0.25% Triton X-100 for one hour at room temperature, then equilibrated in developing buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $CaCl_2$ and 0.02% (w/v) NaN_3, pH 7.5) for 30 minutes with gentle agitation.

Fresh developing buffer was added and the gels were incubated for 18 hours at 37°C. Gelatinase activity was revealed by staining the gel with Coomassie blue dye (0.2% Coomassie Blue R-250 in 50% ethanol and 10% acetic acid) for 15 minutes and then destained in water until clear bands against a blue background was observed.

2.8 Invasion assay

Matrigel stock solution (BD, USA) was first diluted in ice-cold serum-free MEM-α medium to make a final concentration of 3.5 mg/mL. Matrigel solution (150 μL) was spreaded over the entire surface of the transwell insert (BD Falcon, USA; 10.5 mm diameter, 8 μm pores) to form a thin, continuous layer on the top of the filter. This Matrigel-coated insert was allowed to gel at 37°C overnight. Fifty thousand cells in 1 mL serum-free MEM-α medium were seeded into each insert (upper chamber). Complete medium (1 mL) was placed in the lower chamber. The FBS present in the complete medium acted as a chemoattractant. The cells were incubated at 37°C for 18 hours to allow cells to invade through the Matrigel layer. The cells on the upper surface of insert membrane were then removed by wiping with a cotton swab, and the cells migrated to the lower surface were fixed with 10% formaldehyde and stained in crystal violet. The number of control cells (no drug, no light) migrated through the Matrigel was counted and regarded as 100% invasion. The invasion rate of sensitised cells receiving no light (dark control), LD25 and LD50 light dose were calculated as follows:

% Invasion = (no. of cells in treated insert / no. of cells in control insert) x 100

2.9 Adhesion molecule detection

Twenty-four hours after PDT treatment, cells were detached from 35-mm dish with 5 mM EDTA in PBS, washed and collected in centrifuge tubes. Indirect immunofluorescence staining was performed by adding 1 μL of appropriate monoclonal mouse anti-human antibody CD29 (clone 29C02, Lab Vision, CA, USA), CD44 (clone 156-3C11, Lab Vision) or CD49c (clone C3II.1, BD Pharmingen, CA, USA) to $1x10^6$ cells and incubated on ice for 30 minutes with occasional mixing. Cells were washed twice with cold PBS and then incubated with 2 μL FITC-conjugated $F(ab')_2$ fragments of rabbit anti-mouse Ig (Dako, Denmark) for a further period of 30 minutes on ice. After washing twice with PBS, the cells were fixed in 100 μL of 2% paraformaldehyde and stored light protected at 4°C. Direct immunofluorescence staining was also performed where 1 μL of FITC-labelled CD49e (clone SAM 1) or CD54 (clone 84H10) (Immunotech, France) was incubated with the cell suspension for 30 minutes on ice, followed by fixation and storage as described above. Isotype-matched control IgG was used in both immunofluorescence staining procedures. The fluorescence signal, representing the amount of adhesion molecule expression, was quantified by flow cytometry. For each sample, approximately 2×10^4 events were analysed.

2.10 Data analysis

Primary data are presented as mean and standard error. Differences between means were evaluated by two-sided *t*-test when two groups were compared. One-way or two-way analysis of variance (ANOVA) followed by Bonferroni's correction were used when three or more groups were compared. The significance of the difference between two proportions was evaluated by comparison of proportions. P value smaller

than 0.05 ($P<0.05$) was considered significant. All statistical analyses were performed using the GraphPad Prism software (version 4.0).

Chapter 3

Results

3 Results

3.1 Comparison of photosensitiser uptake by glioma cells

Owing to the differences in the physical and chemical nature of these three photosensitisers, it is necessary to determine the optimum uptake of each before PDT investigation. A drug uptake kinetics study on U87 and GBM6840 cell lines was conducted in two phases: (1) verification of the optimal incubation period and (2) investigation of subcellular distribution.

The uptake kinetics was determined by measuring the cellular fluorescence signal using a flow cytometer with a 610 nm long pass filter. The results were expressed as mean fluorescence signal per cell. The rate of fluorescence intensity accumulated in glioma cells was found to be time-dependent, but the time to reach maximum uptake varied between photosensitisers. As shown in Figure 6, the fluorescence signal generated from Photofrin and 5-ALA mediated PpIX elevated gradually reaching a maximum at around 24 hours. Further incubation over 24 hours caused steady drop in fluorescence intensity in both cell lines (data not shown). On the other hand, calphostin C demonstrated quite a different uptake pattern. It was taken up rapidly within 2 hours and remained saturated up to 12 hours.

According to these results, the optimum uptake time for each photosensitiser was selected as follows: 24 hours for Photofrin (1 μg/mL) and 5-ALA (1 mM); 4 hours for calphostin C (100 nM), and these incubation periods were used in the subsequent experiments.

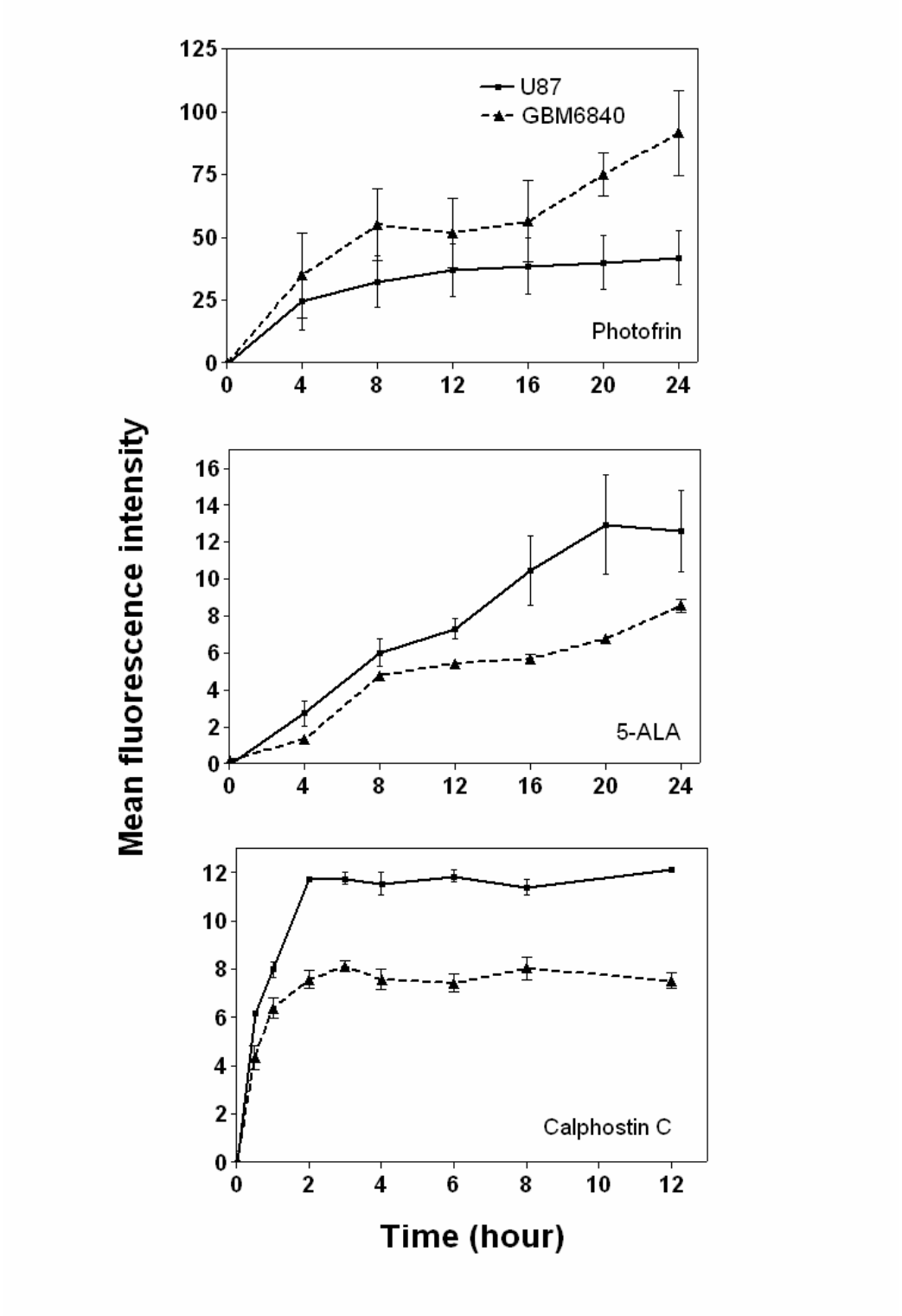


Figure 6. Uptake of photosensitisers by glioma cells. U87 and GBM6840 cells were incubated with 1 μg/mL Photofrin, 1 mM 5-ALA and 100 nM calphostin C respectively. Uptake of all photosensitisers was time dependent. Data is expressed as mean ± SEM of three independent experiments.

3.2 Comparison of intracellular localisation of photosensitisers

The representative fluorescence images of U87 and GBM6840 cells incubated with photosensitisers at their optimum uptake interval are shown in Figure 7. Fluorescence signals were mainly distributed throughout the cytoplasm with little nuclear staining. Fluorescence granules of different sizes was observed in Photofrin treated cells (Fig.7A) whereas the distribution of fluorescence signals in 5-ALA (Fig.7B) and calphostin C (Fig.7C) treated cells were mainly located in the perinuclear cytoplasmic area with a brighter fluorescence signal on one side of the nucleus.

To determine the subcellular localisation of photosensitisers, the distribution of fluorescent probes specific to mitochondria and endoplasmic reticulum were compared. Lysosomes were identified by their selective uptake of the weak basic dye acridine orange [111,112].

Figure 8 shows the distribution of photosensitiser and the organelle specific probe in GBM6840 cells. Similar results were also found in U87 cells (data not shown). Mitochondria, stained with MitoTracker Red, appeared as distinguishable fibrillar like structures that were located mainly in the perinuclear cytoplasmic region (Fig.8A). This pattern did not match with any of the photosensitisers.

The ER fluorescence pattern was diffuse throughout the cytoplasmic region with more concentrated fluorescence intensity located on one side of the perinuclear area (Fig.8A). This coincides with the pattern of 5-ALA induced PpIX and calphostin C (Fig.8B for comparison) pattern.

Acridine orange stained lysosomes appeared as orange fluorescent spots of different sizes distributed in the cytoplasm. The lysosome pattern resembles more that of

Photofrin treated cells in glioma cells (Fig.8A). The green fluorescent background from the cytoplasm and nucleus was caused by the non-specific binding of acridine orange to RNA and DNA.

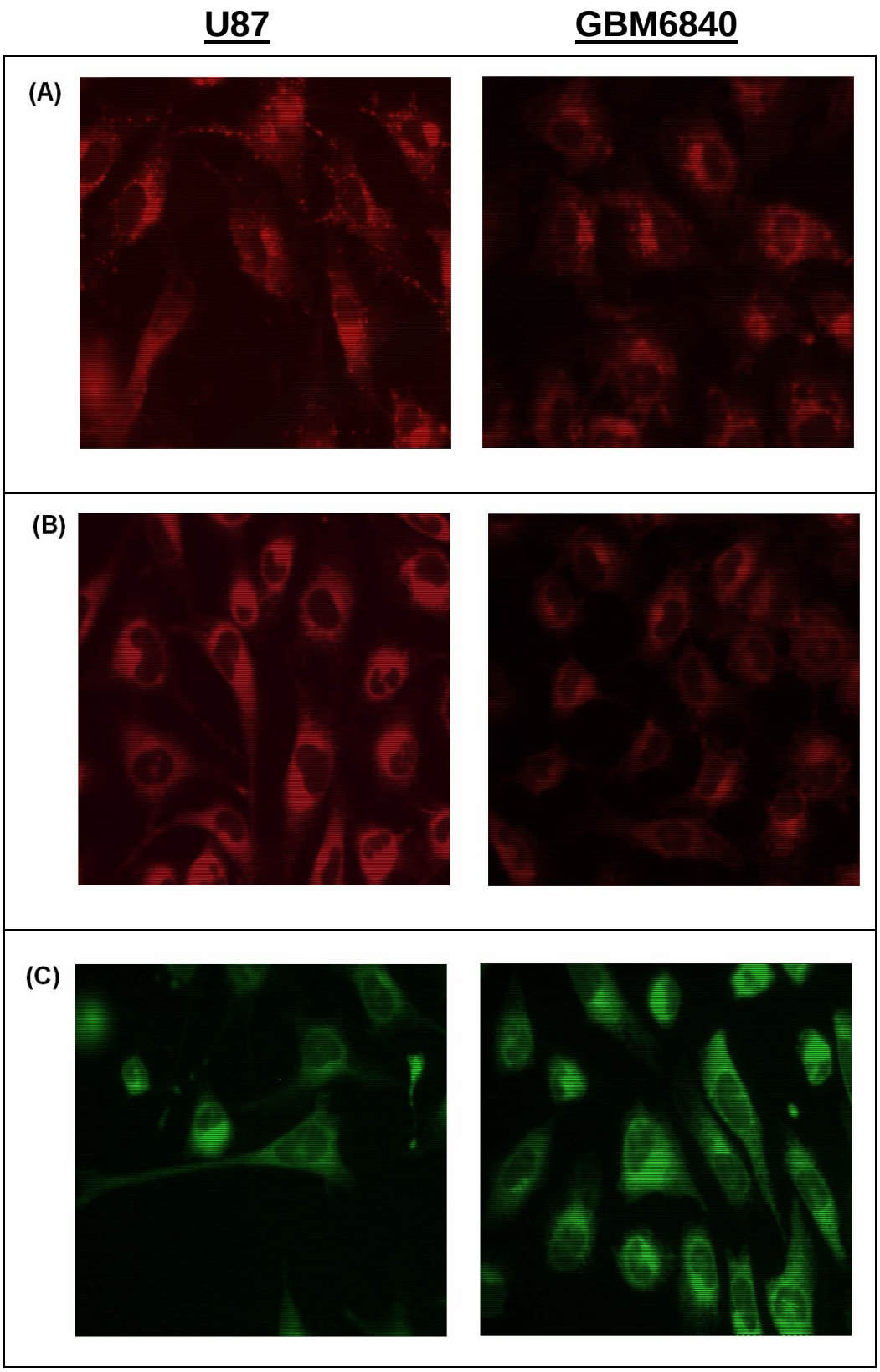


Figure 7. Fluorescence images of photosensitiser distribution in U87 and GBM6840 cells. Cells were incubated to allow maximum uptake of different photosensitisers. Photofrin (1 μg/mL) for 24 hours showed discrete granular fluorescence (A). The staining pattern of (B) 5-ALA (1 mM) and (C) Calphostin C (100 nM) are diffused throughout the cytoplasmic region.

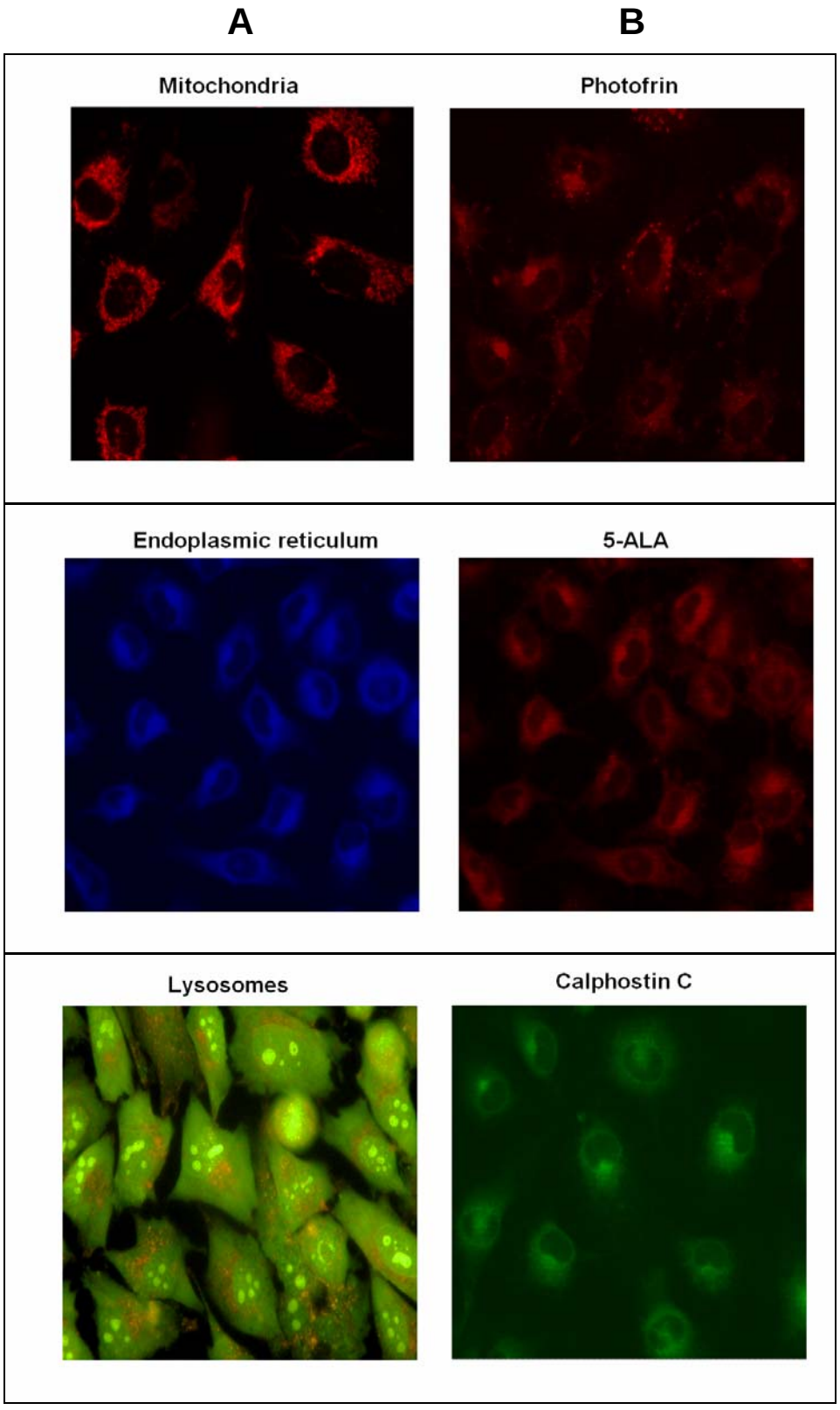


Figure 8. Fluorescence images of sensitised GBM6840 stained with organelle specific probes. Panel A shows glioma cells stained with Mito Tracker, ER Tracker and acridine orange. Panel B shows the staining pattern of Photofrin, 5-ALA and calphostin C for comparison.

3.3 Photocytotoxic effect on glioma cells

After optimal periods of incubation with various photosensitisers (Photofrin and 5-ALA for 24 hours, Calphostin C for 4 hours), cells were exposed to white light passing through a 500 nm long-pass filter with an intensity of 140 W (J/cm^2 = 140W x exposure time in second x 10^{-4}). The selected light doses were 0.5 J/cm^2 to 3 J/cm^2 with 0.5 J/cm^2 increments. Cell viability was determined after 24 hours of incubation by the MTT assay. Figure 9 shows the cytotoxicity data of U87 and GBM6840 cells treated with different photosensitisers at their respective concentration. Dark toxicity was negligible but significant cell death was observed in sensitised cells from as low as a 1 J/cm^2 light dose, and the cytotoxicity increased with increased illumination time.

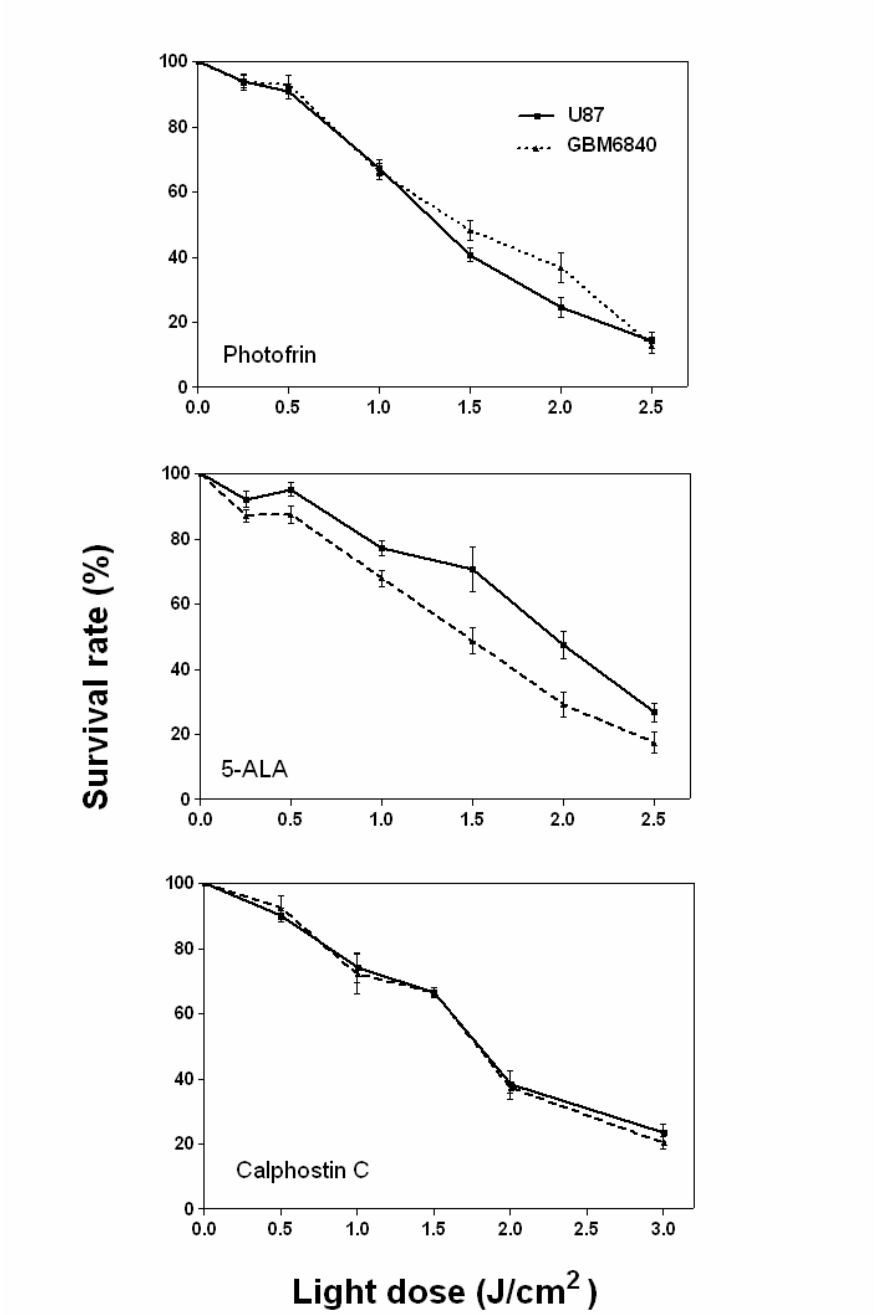


Figure 9. Cytotoxicity of photosensitisers on glioma cells. U87 and GBM6840 cells were incubated with 1 μg/mL Photofrin, 1 mM 5-ALA and 100 nM calphostin C respectively, followed by different light exposure using a 500 nm long-pass filter. Cells without drug treatment were used as a control to compare the percentage of cell death caused by light treatment. Dark control cells were those incubated with drugs but no illumination. The photocytotoxic effect was light dose dependent. Data is expressed as mean ± SEM of three independent experiments.

3.4 Proliferation potential of glioma cells after PDT treatment

To examine the effect of PDT on sub-lethally irradiated glioma cells, their proliferation rate was determined by the MTT assay (Fig.10). In light shielded conditions, Photofrin and calphostin C did not exhibit significant growth inhibitory effects in both cell lines. In contrast, 5-ALA treated glioma cells, even in dark conditions, showed significant growth inhibition ($P<0.01$, ANOVA followed by Bonferroni's correction). Light irradiated cells showed a more pronounced inhibitory effect and significant difference was generally observed at LD25 and LD50 from Day 7 onwards for all photosensitisers except calphostin C on GBM6840. Different cell lines displayed different growth inhibition even when they were treated with the same photosensitiser. For example, GBM6840 was severely affected after 5-ALA treatment no matter what the light dose was, whereas U87 exhibited dose-dependent inhibition. The same glioma cell line also responded differently to different photosensitisers. GBM6840 was more responsive to 5-ALA than Photofrin and to a much lesser extent for calphostin C. The latter was very effective in inhibiting U87.

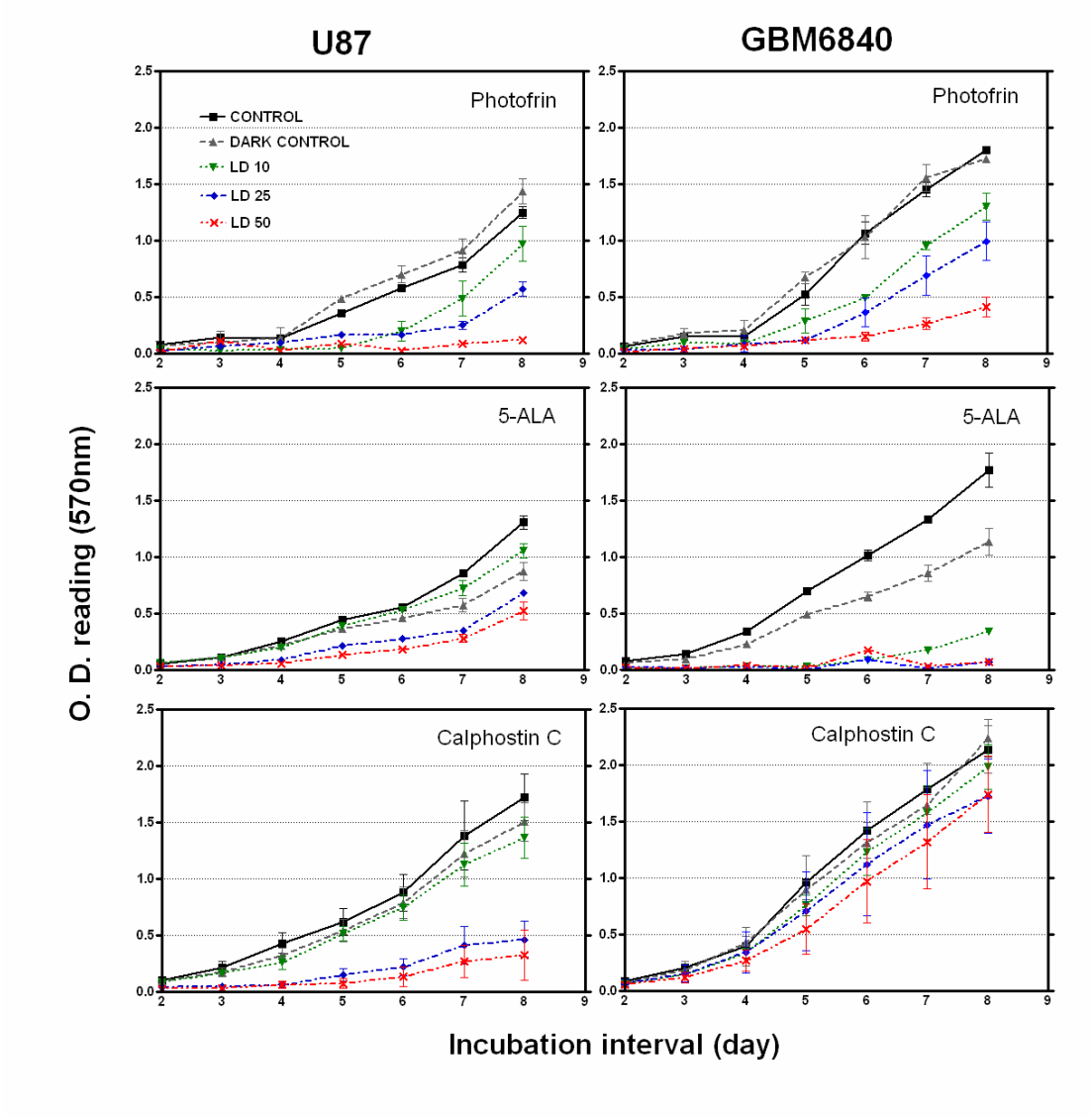
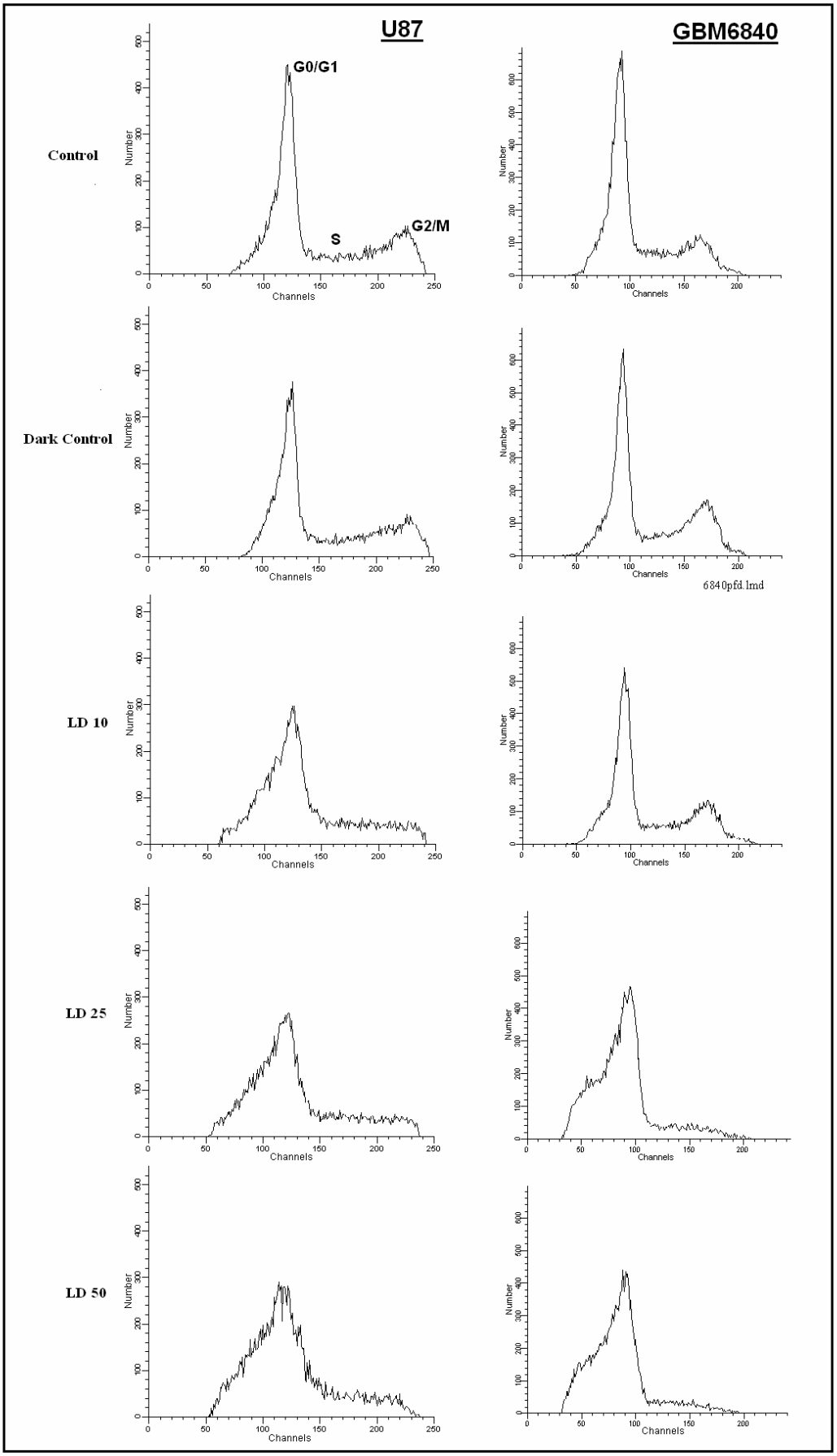


Figure 10. Proliferation rate of glioma cells treated with different photosensitisers. Cells treated with no drug as a control, with drug but light shielded as a dark control, or with various photosensitisers: Photofrin (1 μg/mL), 5-ALA (1 mM) and calphostin C (100 nM) followed by light exposure to achieve LD10, LD25 and LD50 cell toxicity were compared. The proliferation rate was monitored from day 2 to day 8 consecutively. Data is expressed as optical density against incubation time (day) with mean ± SEM of at least three independent experiments.

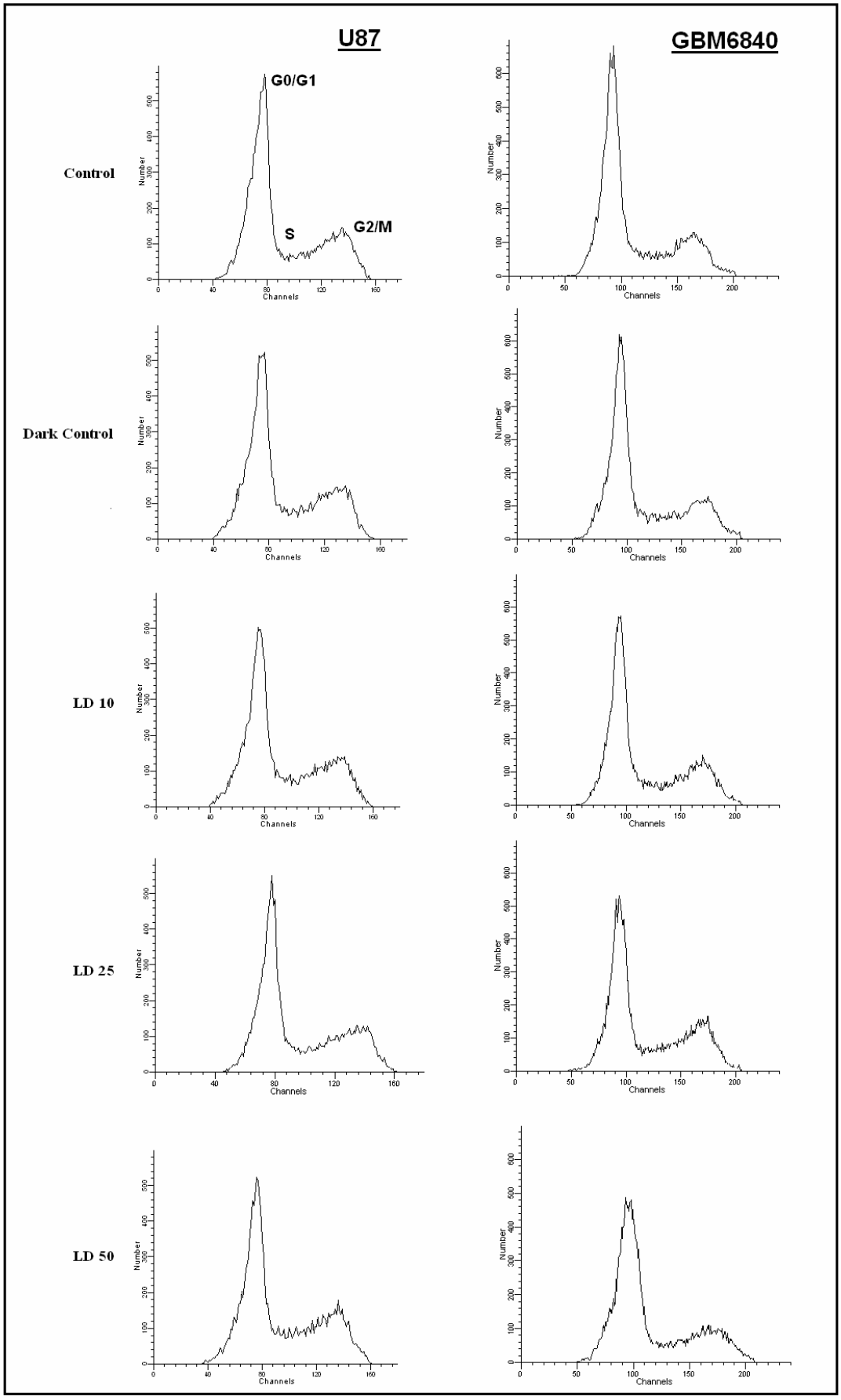
3.5 Influence of PDT on cell cycle

To determine whether cell cycle is affected by photosensitisers, DNA content of sensitised cells was quantitatively measured by flow cytometry. The changes of fraction G0/G1, S and G2/M phase subsequent to sub-lethal light dose were investigated. In light shielded conditions, these three photosensitisers had no significant effect on cell cycle pattern. However, after photo-activation, cells responded differently toward individual photosensitisers. Figure 11 shows the histograms obtained from the flow cytometer and figure 12 summarises the cell cycle changes in response to PDT treatment. In the Photofrin treated group (Fig.12A), about 50% of U87 and 60% of GBM6840 cells were found to be in the G0/G1 phase. The proportion of cells in this phase elevated significantly after photo-irradiation ($P<0.001$, calculated by comparison of proportions) and approximately 80% of cells in both cell lines were arrested. In the 5-ALA treated group (Fig.12B), the percentage of cells in the G0/G1 phase remained at 40-50% for U87 and 50-60% for GBM6840 under all the conditions tested. The percentage of cells at S and G2/M phase were also maintained at a constant level without any significant change. In the calphostin C treated group (Fig.12C), there was no change in cell cycle pattern for both cell lines under all the conditions tested.

(A) Photofrin treatment



(B) 5-ALA treatment



(C) Calphostin C treatment

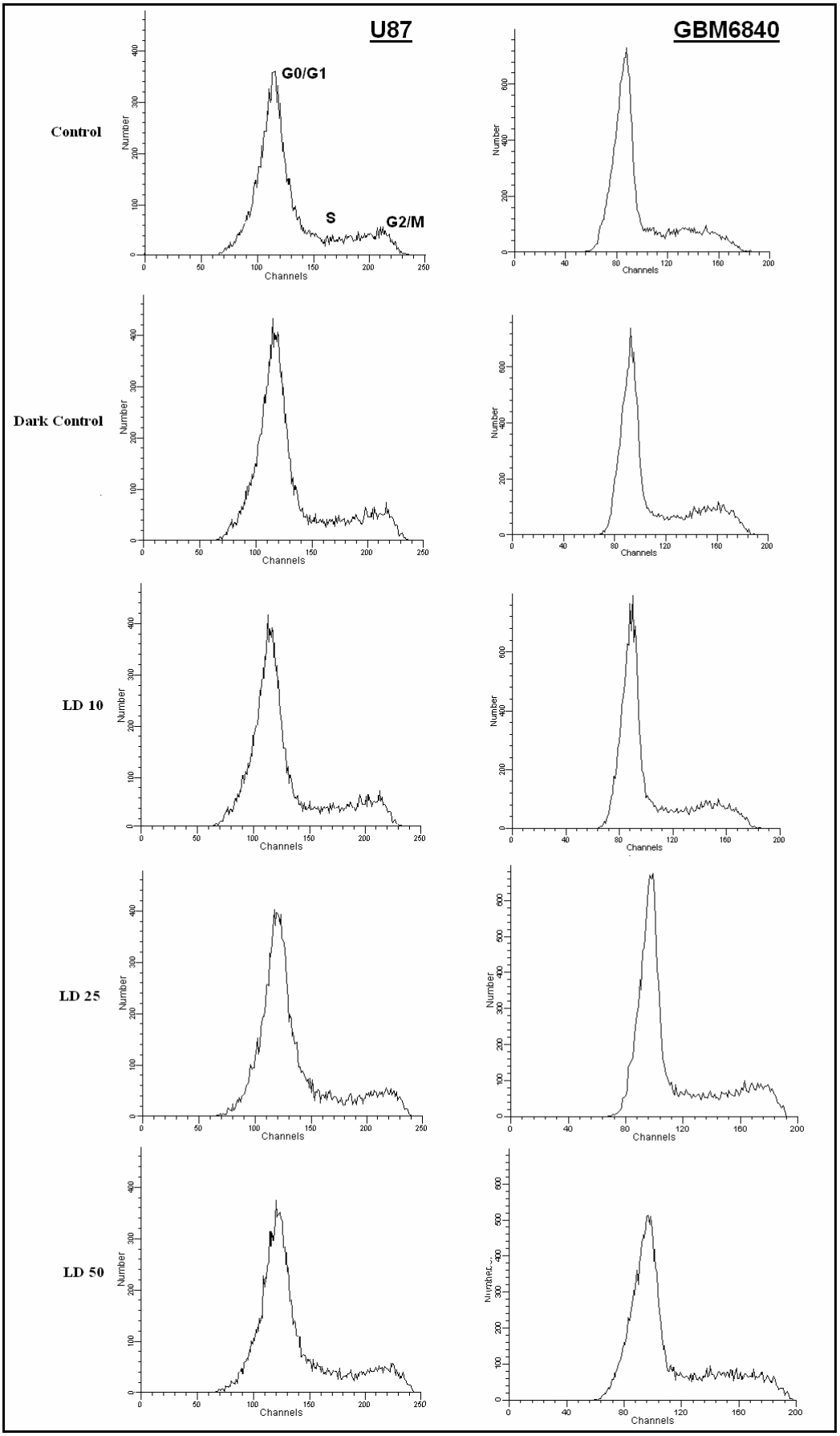


Figure 11. The influence of PDT on cell cycle. The cell cycle response to (A) Photofrin, (B) 5-ALA and (C) Calphostin C PDT on glioma cell lines is shown. The measurement of propidium iodide mediated fluorescence intensity is proportional to the DNA content of the cell. Data shown, expressed as the number of events against fluorescence intensity, is from a representative experiment of at least 2 independent experiments showing similar results.

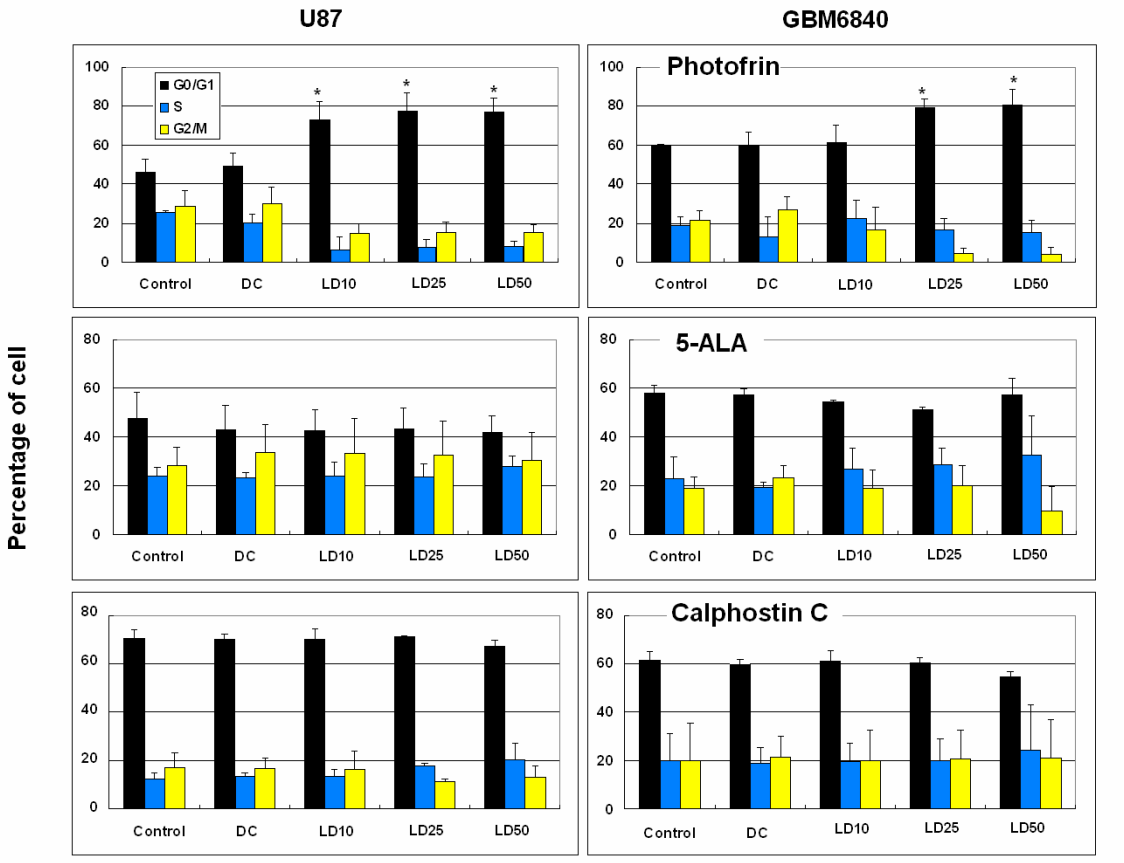


Figure 12. Bar chart showing the influence of PDT on cell cycle. This figure summarises the cell cycle changes in U87 and GBM6804 glioma cells undergoing PDT using Photofrin (1 μg/mL), 5-ALA (1 mM) and calphostin C (100 nM). Data shown is mean ± SEM in percentage of at least two independent experiments. (* indicates P <0.001). P value is calculated by the significance of the difference between two independent proportions.

3.6 PDT inhibits metalloproteinase production

U87 and GBM6840 cells constitutively produced latent and activated forms of MMP-2 (Fig.13, lane 1). However, MMP-9 was present only in the GBM6840 cells. In the presence of photosensitisers alone, there was no difference in the levels of latent and activated MMP-2 between control (lane 1) and dark control (drug in light-shielded conditions, lanes 2, 5 & 8) in U87 cells. On the other hand, there was conversion of the latent MMP-2 to the activated form, with a lower molecular weight, in GBM6840 cells under dark control conditions. After PDT treatment, there was a light dose dependent decrease in both latent and activated forms of MMP-2 in both cell lines, as shown in the zymogram (Fig.13, lanes 3 & 4, 6 & 7, 9 & 10). The intensity of the MMP-9 band in GBM6840 cells exhibited a similar pattern.

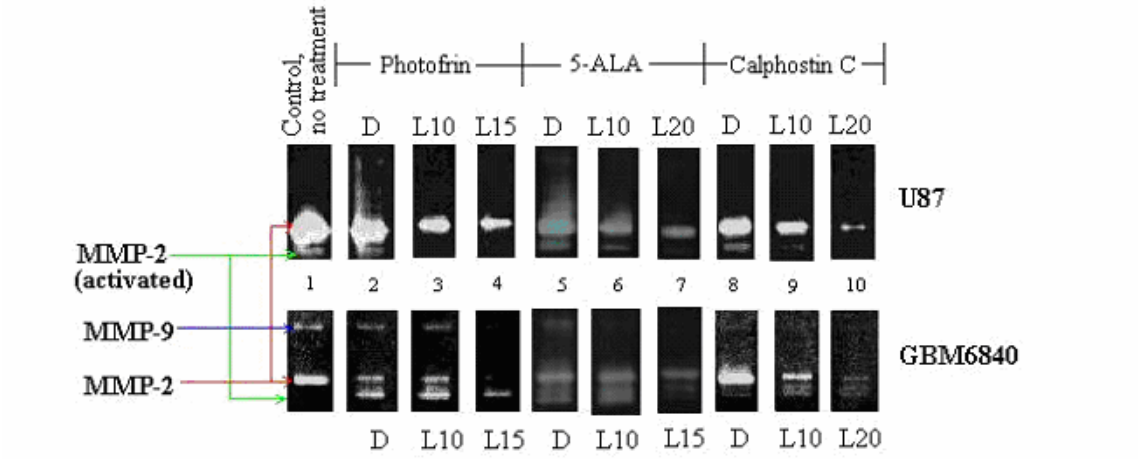


Figure 13. Zymography showing the influence of different photosensitisers on MMP-2 and MMP-9 production in glioma cells. Lane 1: control cells without treatment, lanes 2-4: cells treated with Photofrin (1 μg/mL), lanes 5-7: cells treated with 5-ALA (1 mM) and lanes 8-10: cells treated with calphostin C (100 nM). D = dark control, L10, L15 and L20 = cells receiving 1.0, 1.5 and 2.0 J/cm^2 light dose respectively. Data shown are representative zymographs from three independent experiments.

3.7 PDT retards glioma cell invasion

Invasion ability was indicated by the number of cells that had migrated through the reconstituted Matrigel layer to the bottom surface of the porous membrane. As shown in Figure 14, all there photosensitisers caused a reduction in glioma invading ability, as observed under a phase-contrast microscope, in a light dose dependent manner. Figure 15 summarises the data on the number of cells migrated through the Matrigel. All three photosensitisers caused a significant reduction ($P<0.05$) in invasiveness of both cell lines at LD25, with the exception of calphostin C on GBM6840. At LD50 further reduction in glioma cell invasion was observed where less than 30% of cells invaded through the Matrigel layer ($P<0.001$). Photofrin treatment showed the most potent effect on retarding cell invasion.

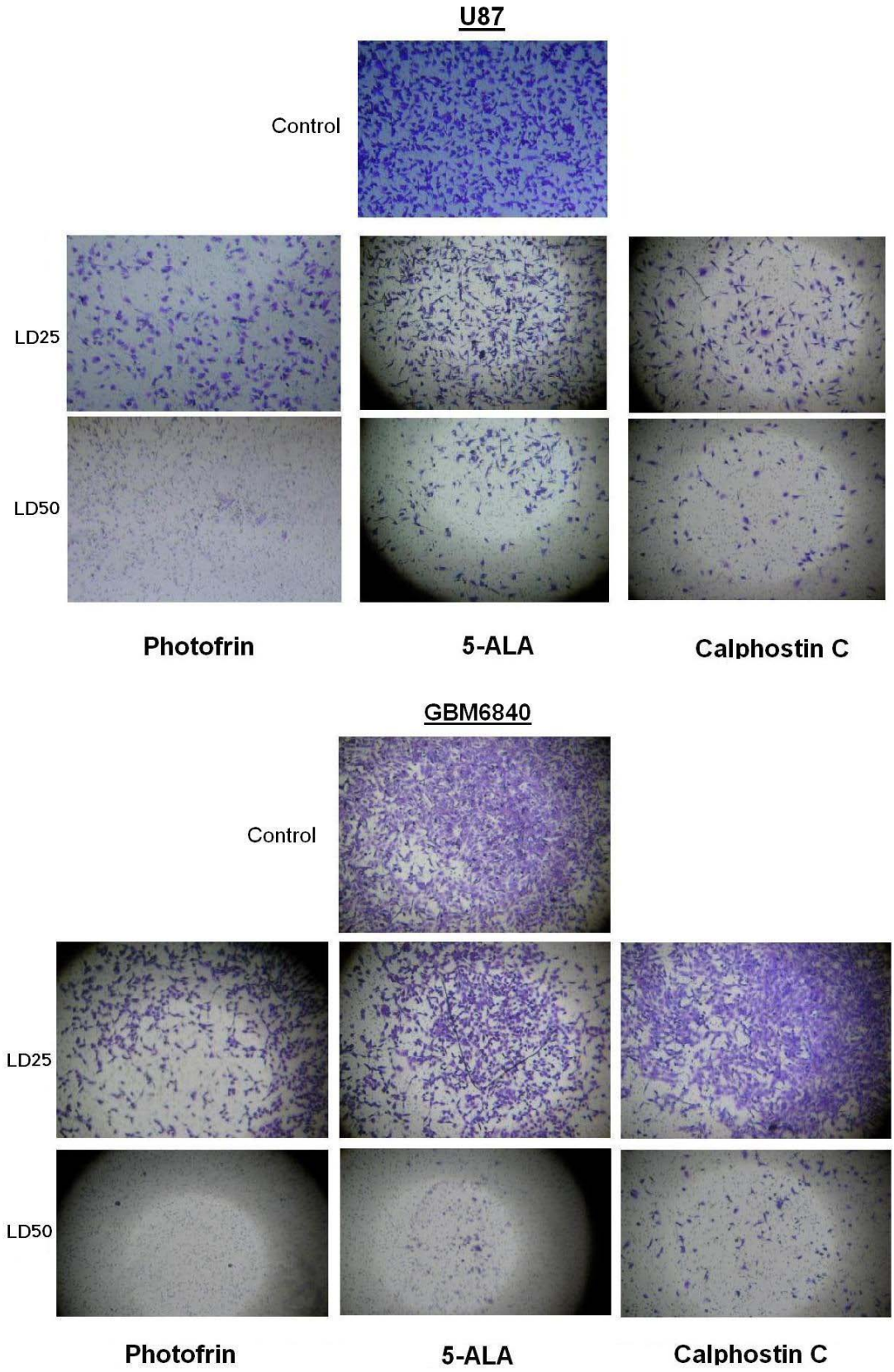


Figure 14. Photomicrographs of glioma cell invasion. The invasion ability of U87 and GBM6840 cells was reduced, compared with the control, after PDT, LD50 light dose influence had a more dramatic reduction in cell invasion as compared with LD25 light dose influence.

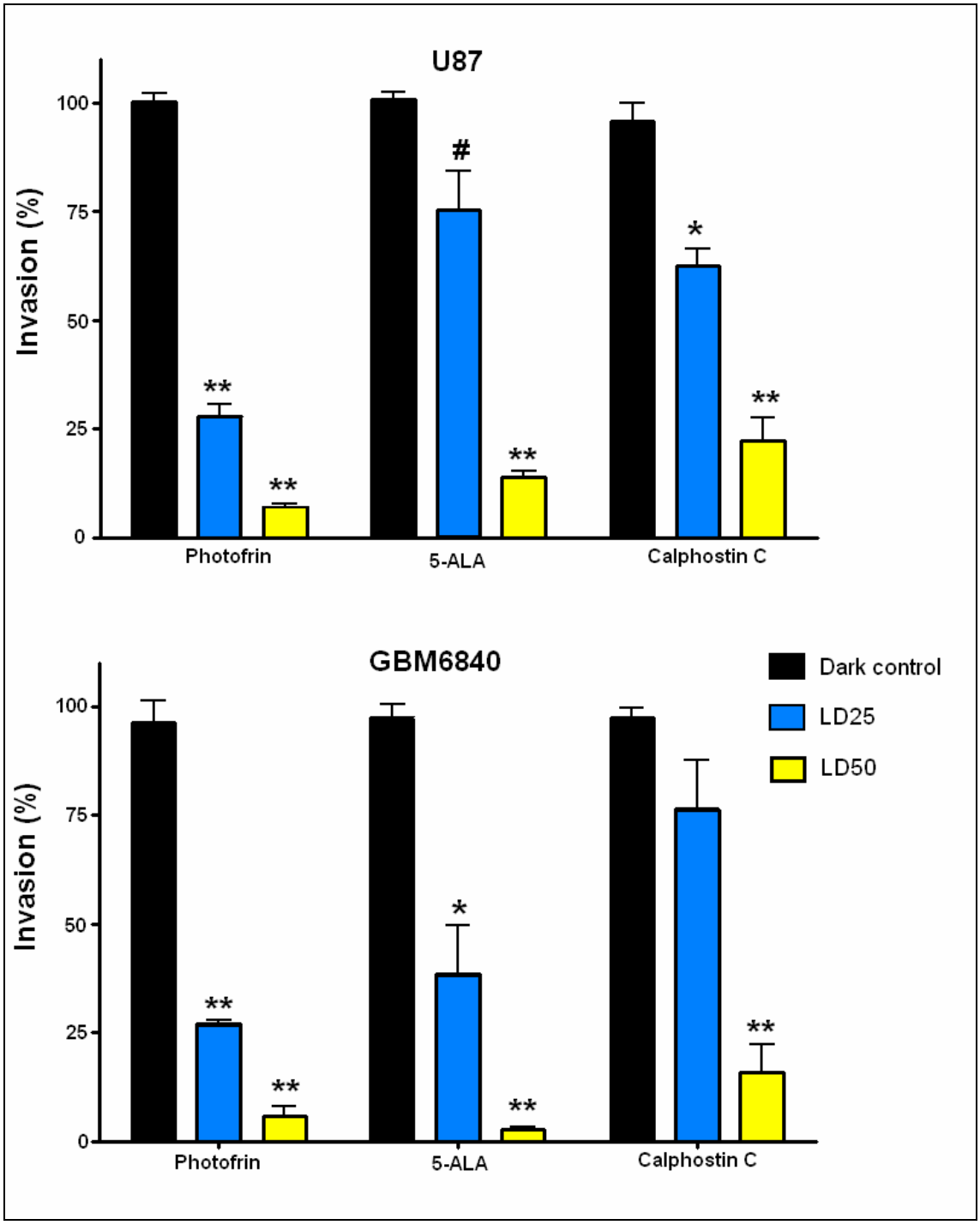


Figure15. Inhibition of glioma cell invasion through the Matrigel membrane. The number of control cells (no drug , no light) migrated though the matrigel membrane was counted as 100% invasion. The invasion rate of sensitised cells receiving no light (dark control), LD 25 and LD 50 light dose were compared. Data are expressed as mean ± SEM of three independent experiments. $^{\#}P<0.05$, *P<0.01, **P<0.001.

3.8 Glioma cell adhesion molecule expression

Cell surface receptors are known to play an important role during tumour invasion. Therefore, an investigation was performed to determine whether cell migration inhibited by PDT was due to the alteration in the levels of cell adhesion molecules on the glioma cells. The expression of CD29, CD44 and CD49c in both cell lines was reduced in sensitised cells as the light dose increased (Figure 16). In the Photofrin treated group, sensitised U87 had 10-20% down regulation in CD29, CD44 and CD49c in light shielded conditions and the level of expression was further reduced after light treatment. On the other hand, sensitised GBM6840 showed a more potent effect on down regulation of CD29 and CD44 expression even in the dark and photo-irradiation only further decreased their expression slightly. The expression of CD49c gradually decreased as the light dose increased and reached significance level only at LD50 light dose ($P<0.05$). In the 5-ALA treated group, CD29 expression in U87 cells was greatly diminished in sensitised cells (40%), and the levels only decreased slightly after light treatment. CD44 and CD49c, on the other hand, showed a light dose dependent decrease in expression. The levels of adhesion molecule inhibition in 5-ALA sensitised GBM6840 cells were much lower than in U87 cells. Light irradiation caused a gradual decrease in expression of all three adhesion molecules. Again, significant reduction was only observed in CD49c expression at LD50 light dose ($P<0.05$). Calphostin C was the least effective photosensitiser in reducing adhesion molecule expression under both light shielded and photo-irradiated conditions (Fig. 16C).

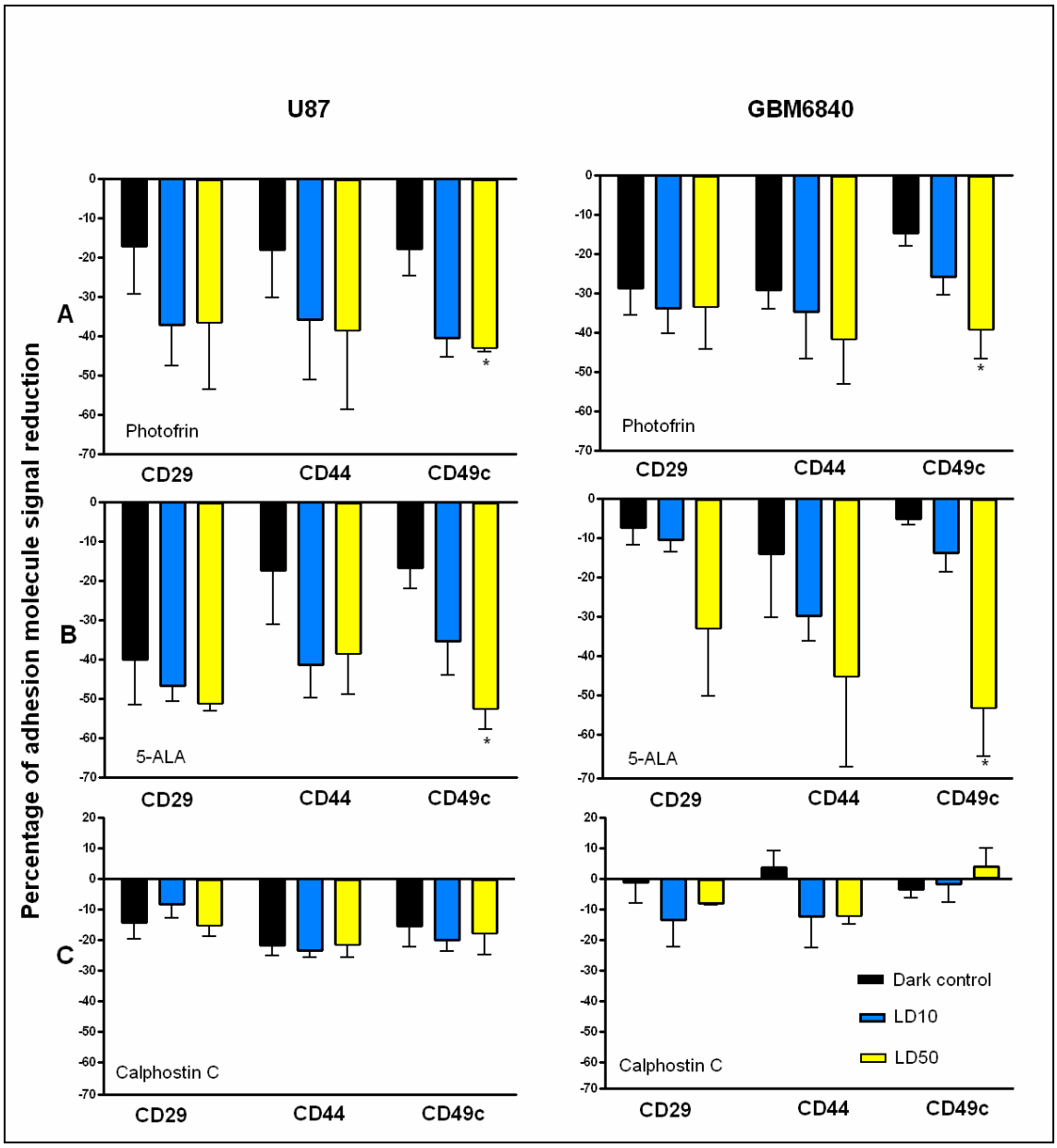


Figure 16. Influence on adhesion molecule expression by PDT. The figures show the expression of adhesion molecules CD29, CD44 and CD49c influenced by Photofrin (1 μg/mL), 5-ALA (1 mM) and calphostin C (100 nM) on glioma cells. Data are expressed as mean ± SEM of 2-3 independent experiments. *P<0.05.

Chapter 4

Discussion

4. Discussion

Photodynamic therapy is a promising technique for the treatment of many forms of tumours, and is generally regarded as an efficient and safe adjuvant therapy. In this study, the uptake kinetics and cellular distributions of three photosensitisers, acting via different cellular pathways, on two glioma cell lines were examined. The cellular responses subsequent to PDT including proliferation, influence on cell cycle, cell invasion and surface receptor expression were also investigated.

4.1 Drug uptake and distribution

Both U87 and GBM6840 cells demonstrated similar patterns of uptake kinetics towards the same photosensitiser, and the accumulation rate was time dependent. However, the rate to reach maximal uptake showed great variations among different photosensitisers. Calphostin C was taken up more rapidly within 2 hours and then maintained at a constant level, whereas a longer incubation period (24 hours) for Photofrin and 5-ALA mediated PpIX was required for optimisation.

The cellular uptake of photosensitiser is a dynamic balance between uptake and efflux of drug. Such equilibrium is determined by many factors, including the nature of drug (lipophilic or hydrophilic), its molecular size, tendency to aggregate, cellular metabolic rate, the availability of enzymes and the amount of cell surface receptors. The rapid uptake of calphostin C can be explained in two ways: firstly, the higher lipophilic nature of calphostin C causes faster penetration of this molecule through the cell membrane; secondly, it is dissolved in DMSO for stock preparation and DMSO may serve as a vehicle to facilitate penetration into the cell.

In the uptake kinetics of Photofrin, GBM6840 cell line exhibited stronger fluorescence signal as incubation time increased. It is probably related to the chemical composition of this drug and the intracellular partitioning of its various components. Photofrin is a mixture of monomeric porphyrins (10-20% of total porphyrin contents) and oligomeric porphyrins with different sizes [117]. The distribution of these porphyrin molecules and the size of their aggregates are strongly dependent on the composition of the culture medium. The oligomeric aggregates are probably internalised by endocytic mechanisms because of their size. Such uptake process is relatively slow [118]. In addition, oligomeric species are weakly fluorescent and inefficient in generating singlet oxygen. Intracellular processing of these oligomers to form monomers is a time consuming process, but is essential for achieving maximal effect. Oligomers of various sizes may distribute to different compartments of the cell during this conversion [117]. The latter may vary between cell lines and may explain the differences in fluorescence intensity observed in U87 and GBM6840 cells.

On the other hand, 5-ALA is a small hydrophilic molecule. Its uptake is through the active transport mechanism, such as pinocytosis [82]. In previous studies, different tumour cell lines were tested for the cellular uptake of 5-ALA by using radioactive ^{14}C-5-ALA [109]. The results indicated that uptake of 5-ALA was concentration dependent and different cell lines accumulated equal amounts of 5-ALA, which was not affected by temperature or the presence of serum in the medium [80]. However, the different rate of porphyrin biosynthesis indicates that the critical step of PpIX formation is determined by the enzyme activity in the haem biosynthesis pathway. Therefore, different cell lines may accumulate different amount of PpIX even though they have been incubated for the same period of time.

Recent studies have indicated that when a 5-ALA molecule is encapsulated in liposomes or esterificated with long chained carbon, the rate of transportation across cell membrane can be greatly enhanced. Therefore, the concentrations required to induce PpIX formation is 30 to 150-fold lower [82-86]. It should be pointed out that even though 5-ALA is an endogenous product under physiological conditions, a high cellular concentration of 5-ALA has been reported to be cytotoxic [119]. High levels of cytoplasmic 5-ALA may induce the efflux of calcium from the mitochondria resulting in calcium-dependent mitochondrial oxidative damage [120]. Therefore, further investigation of 5-ALA ester mediated PDT is required.

4.2 Subcellular localisation of photosensitisers

The intracellular localisation of photosensitisers is assumed to have an important influence on PDT because the generated singlet oxygen molecule has a very short half-life (<0.04 μs) and diffusion radius (<0.02 μm). The site where this molecule is generated is considered to be equivalent to the primary target structure of photoinjury.

Singlet oxygen generated in PDT is the main cause of cell death. In this study, all photosensitisers were found to be located predominantly in cytoplasm. Therefore, it is believed that photoinjury does not take place in the nucleus. Cellular localisation of a photosensitiser is a substantial criterion for selection in PDT. If the photosensitiser is found to be distributed in the nuclear membrane or inside the nucleus, the oxidative action of singlet oxygen could possibly lead to nuclear DNA damage, mutation and carcinogenesis [120]. However, if the photosensitiser is mainly located in the cytoplasm, such as Temoporfin and merocyanine, the chance of DNA strand breakage is much reduced [121].

There are limited data on the intracellular distribution of calphostin C and our results correlate with previous reports [99] that it is mainly localised in endoplasmic reticulum. PpIX, on the other hand, is synthesised from its precursor 5-ALA, inside mitochondrial intermembranous space. However, the fluorescence pattern found in 5-ALA treated glioma cells did not correlate with the mitochondria-staining pattern. This can be due to the PpIX formed inside mitochondria being redistributed rapidly into other cellular compartments and finally released out of the cell by diffusion or transport via the ER system [122]. The rapid redistribution of PpIX may be due to its high affinity for albumin [111]. On the other hand, fast photobleaching could affect image acquisition, and therefore the localisation of PpIX might not be accurately indicated by the

fluorescent images. Similar results have been reported in C6 glioma cells, leukaemia cells and adenocarcinoma cells [88,123-127]. Such redistribution of PpIX indicates that photodamage of sensitised cells may not be acting on targeted organelles only (such as mitochondria), but the multiple sites where sensitisation takes place in the cytoplasm.

The cellular localisation of Photofrin remains controversial because the result may be different in different cell types and for different incubation periods [128,129]. Mitochondria, plasma membrane and Golgi complex have all been reported to be the target sites of Photofrin [128,130]. However, in this study, the preferential organelle was possibly the lysosome. The higher lipophilicity of Photofrin makes it easily aggregate in an aqueous medium, therefore, endocytosis is considered the major route for its uptake. Substances that are not able to penetrate the plasma membrane can still enter the cells via endocytosis - the engulfed substances are preferentially transferred to the lysosome. Geze and coworkers [129], using the quantitative microfluorometric technique, reported that lysosomes are the key target of this hydrophobic photosensitiser.

The limitation of fluorescence microscopy used in this study is that the fluorescence signals from the photosensitiser and the organelle marker were captured separately instead of a single image showing two different colours. The use of a cofocal microscope may solve such limitation and the co-localisation results become more convincing. Furthermore, the amplified signal obtained in cofocal imaging may greatly reduce the light for excitation, therefore, the effect of photobleaching is kept to the minimum.

4.3 Cell death pathway is dependent on photosensitiser localisation

Subcellular distribution is a dynamic interaction between photosensitiser and the intracellular environment. This depends not only on the molecular structure of the photosensitiser and the way it is delivered, but also on the concentration being used and the incubation period. In addition, the cell death pathway may also be determined by the subcellular localisation. Dellinger et al. [130] reported that cells exhibited apoptosis when exposed to lower concentrations of Photofrin with longer periods (1 μg/mL and 24 hours), whereas cells incubated with higher concentrations of Photofrin but shorter periods (10 μg/mL and 1 hour) showed necrotic responses. This can be explained by the plasma membrane localisation of Photofrin after short incubation intervals and subsequent photodamage resulting in leakage of cytoplasmic material. In contrast, a longer incubation period allows subcellular localisation and, therefore, photodamage is mainly targeted at organelles rather than the plasma membrane. Similar findings have been demonstrated by Kessel et al. in their study of membrane targeting photodamage [131,132], in which two structurally related photosensitisers but with different plasma membrane affinities were tested for their cell death mechanism. These reports suggest that photosensitisers with a higher affinity for intracellular organelles may preferably evoke apoptosis rather than necrosis.

4.4 Factors facilitating retention of photosensitiser in tumour tissue

4.4.1 Properties of photosensitisers

The localisation of a photosensitiser in tumour tissue is mainly determined by its lipophilicity. Hydrophilic substances are likely to be transported by albumin and globulins, and localised mainly in the vascular stroma of tumour tissue, whereas lipophilic sensitisers are preferentially carried by lipoproteins, particularly low density lipoproteins (LDL), and accumulate in the parenchymal cells of tumour tissue [133].

In general, when a photosensitiser is administrated intravenously, it will bind to different serum proteins selectively according to its various chemical properties. Such binding determines the subsequent transport in the circulatory system. However, the biodistribution pattern of the photosensitiser may vary with time. Woodburn and Kessel [134] found that the binding of photosensitisers may be a dynamic interaction involving many components within the blood. For example, substances in HpD first bind to albumin and then redistribute rapidly to lipoproteins. Jori et al. [135] showed that HpD could not be found in the serum fraction 48 hours after administration, but lipoproteins became the major reservoir.

It has been shown that Photofrin preferentially locates in the lipophilic region of the cell [118]. A red shift was observed in the emission maxima of Photofrin from 615nm in a buffer solution to 630 nm in lipid. An identical shift pattern occurs in the emission spectrum of Photofrin taken up by tumour cells. This red shift suggests the Photofrin incorporated into cells distributes to lipophilic organelles [118].

Cell damages mediated by lysosomes will result in leakage of lysosomal content which in turn cause cell death via necrosis rather than apoptosis. However, it has been

demonstrated in leukaemia cells that apoptosis may also be induced by lysosomal mediated oxidation [136,137]. This apoptotic response due to lysosomal photodamage is an indirect effect mediated by the release of lysosomal proteases. These enzymes cause mitochondrial degradation and release cytochrome C into the cytosol, triggering the classical apoptotic cascade.

4.4.2 Tumour stroma effects

The reason for the preferential retention of photosensitisers in neoplastic tissues is not fully understood. However, this may be contributed to by two factors: (1) the specific features of tumour cells and (2) the tumour stroma effects. The expression of LDL receptors is enhanced on tumour cell surfaces presumably to compensate the increased demand for cholesterol by its fast proliferative activity. In this way, transport of photosensitisers across the cell membrane is also facilitated by the increased number of LDL-receptors.

Tumour stroma are composed of interstitial connective tissue; the main components include (1) plasma protein-rich interstitial fluid; (2) structural proteins, such as collagens (including gelatin), elastin, fibronectin and laminin; (3) ground substances, such as hyaluronic acid; (4) blood vessels; and (5) tissue cells, such as fibroblasts, macrophages, granulocytes, etc. Furthermore, tumour stroma have extensive highly permeable microvasculature with poor lymphatic drainage resulting in high interstitial fluid pressure. Accumulation of macrophages, neutrophils and abundant tumour cells may cause a low stromal pH due to hypoxia. These factors indicate that structural properties of tumour stroma may account for selective retention of photosensitiser in tumour tissue [62].

Some studies have indicated that the application of PDT in glioma cells is more effective than in malignant tumours in other sites because the presence of an intact blood-brain barrier may reduce the entrance of photosensitiser into normal brain tissue [138]. However, photosensitisers are able to pass through the defective blood-brain barrier at the tumour site and thus facilitate their selective localisation [138].

4.5 Inhibitory effects of PDT on glioma cells

4.5.1 Cell cycle

The cell cycle is responsible for driving cells to proliferate. This is reflected in Photofrin treated glioma cells where the cell cycle was arrested at the G0/G1 phase (Fig.11A) resulting in a decrease in cell proliferation. However, growth arrest is not necessarily governed only by the cell cycle.

The proliferative potential of 5-ALA and calphostin C treated glioma cells was significantly reduced but there was no change in their cell cycle. This suggests other pathways leading to cell proliferation were being affected.

4.5.2 Cell invasion

In general, gliomas infiltrate and spread great distances in the brain. In the majority of cases at the time of diagnosis, tumour cells have already migrated from the primary site. These migrating cells are responsible for local recurrence and tumour progression. Therefore, the invasive nature of malignant glioma plays an important role in the poor prognosis of patients. In this study, PDT appeared to be promising because all three photosensitisers could potently diminish the invasion ability of glioma cells. Even though the glioma cells exhibited different growth inhibition responses towards different photosensitisers, their invading abilities could be significantly diminished after photosensitisation.

Gliomas invade the surrounding brain tissue along blood vessels or white matter. The invading cells must pass through the brain ECM. This process involves at least three major interrelated steps: (1) adhesion and/or disadhesion, (2) enzymatic degradation of the matrix components, and (3) the locomotion through the barrier. The

over expression of MMP-2 and MMP-9 in GBM has been reported to correlate with tumour invasiveness [15,58,60,76,139]. Therefore, it is believed that down-regulation of MMP expression may help to suppress tumour progression. Our zymography results showed that U87 and GBM6840 constitutively produced MMP-2, but the expression of MMP-9 was present only in the GBM6840 cells (Fig. 13). PDT effectively inhibited both MMP-2 and MMP-9 production in a light dose dependant manner. In GBM6840 cells, photosensitisers alone induced the conversion of latent MMP-2 to its activated form. The latter is known to play an important role in the process of extracellular matrix degradation and tumour invasion [14]. However, sensitised GBM6840 cells did not exhibit higher invasive capacity. This contradiction could be explained by the overall reduction of MMPs and consequently the relative increase in activated MMP-2 could not compensate the absolute loss of this proteinase. Therefore, the overall ability to degrade ECM, as demonstrated by the invasion assay, was diminished.

In the gelatine zymography assay, the levels of MMP under different treatment conditions were determined by normalising supernatant proteins to one microgram. However, the reduction of total amount of MMPs might be caused by cell death induced by PDT treatment. To compensate such effect, total cell lysate instead of culture supernatant may provide more reliable results. In addition, Western blotting can be used to quantify the amount of MMP-2 and MMP-9. Quantitative reverse transcription polymerase chain reaction (RT-PCR) can be applied to determine the mRNA levels of MMP so as to correlate the inhibitory effect of PDT on MMP expression at the gene level.

The expression of MMP-9 has been reported to correlate with the invasiveness in numerous peripheral tumours, and the level was found to be markedly elevated in homogenates of resected glioblastomas in comparison with non-invasive tumours and normal brain tissue [10,140]. However, a significant proportion of the over-produced MMP-9 may be contributed to by non-tumour cell populations within the tumour stroma. Endothelial cells, abundantly present in highly vascular glioblastomas, express very high levels of MMP-9 particularly at their basement membrane [81,141]. Although cultured glioma cells express low levels of MMP-9, the fact that their expression can be induced by PMA suggests that in the presence of external stimulation, they could elevate their MMP-9 production. These external stimulants may be produced during the dynamic interaction between glioma and stroma *in situ* [37,142,143].

The majority of investigations correlating to glioma proteolytic activity have focused on the MMPs, the predominant mediator of glioma invasion. However, apart from the secretion of MMPs and their subsequent activation, the simultaneous production of tissue inhibitors of metaloproteinases (TIMPs), which can bind to the MMPs, may inactivate their proteolytic function. An imbalance in the levels of MMPs and their corresponding inhibitors may be a crucial factor during tumour cell invasion. Therefore, degradation of ECM is a complex process involving the interaction between various kinds of matrix modulators. PDT may possibly interfere with ECM modulators other than MMPs.

In this study, the invasion process was determined by a Boyden chamber model in which invasiveness of tumour cells was measured by the rate of migration across an artificial basement membrane (Matrigel) which mimicked the extracellular matrix components. The advantage of using Matrigel gel is to shorten the migration time

required in conventional tissue barrier assays. The short incubation time minimises the serum effect because serum may contain unknown exogenous compounds such as proteases and their inhibitors, which can alter the invasiveness of the tumour cells. The issue of cell doubling, which can interfere with the actual number of cells that invade, could also be eliminated due to the short duration of the assay.

4.5.3 Adhesion molecule expression

The mechanisms responsible for glioma cell migration and invasion are still not fully understood. Interactions between ECM components and the glioma cell surface receptors may probably play an important role during tumour invasion. Photosensitisers act on several membrane targets and induce changes in surface receptor expression. Such induction of cell membrane injury may affect cellular adhesion to ECM and consequently modulate cell adhesiveness. CD29 (β1 integrin) and CD49c (α3 integrin) are common receptors for most of the ECM components and have been shown to be up-regulated in glioma cell lines [144]. Furthermore, α3 and β1 integrins were found to be mainly expressed in the margin between tumour and normal tissue, and their expression correlated with invasiveness [141]. Besides, promoting cell adhesion and migration, adhesion molecules also contribute to intracellular signal transduction. Hyaluronic acid is the major ECM component abundantly present in the brain. The expression of the CD44 molecule (hyaluronic acid receptor) on glioma cells may therefore facilitate their migration and invasion [145-147]. The expression of CD49e (α5 integrin) and CD54 (ICAM-1) were also investigated in this study because their expression is known to influence tumour progression. However, their expression levels in both glioma cell lines were very low and therefore no further investigation was carried out.

Down-regulation of adhesion molecules by photosensitisers may lead to a reduction in tumour cell adhesiveness and invasiveness. Their effectiveness varies between photosensitisers. Photofrin and 5-ALA were more effective but calphostin C only showed minimal effect. Although the mechanism of how PDT affects adhesion molecules is not clear, it is unlikely that they cause direct damage to the adhesion molecules on the cell surface. However, the location of adhesion molecules could change from a focal adhesion plaque form to a diffuse pattern resulting in a non-functional form that fails to bind to ECM proteins [148].

The down-regulation of adhesion molecule expression observed in this study could be verified using quantitative RT-PCR. Besides, the combined results of flow cytometry and RT-PCR may further discriminate whether the PDT effect is targeting at translation or transcription level.

4.6 Further study of PDT on glioma

4.6.1 Detection of apoptosis

Since PDT is very effective in killing glioma cells in a light dose dependent manner, it would be useful to distinguish whether the photosensitisers triggered the apoptotic or the necrotic pathway. In this study, the MTT assay was used to quantify the percentage of cell death. However, this method cannot differentiate apoptotic and necrotic cells. Annexin V-FITC/PI staining [149] as well as terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL) assay [150] are the two appropriate methods to confirm the percentage of apoptotic and necrotic cells after PDT treatment.

4.6.1.1 Annexin V-FITC/PI assay

The principle of Annexin V-FITC/PI assay is that in early apoptosis the membrane phospholipid phosphatidylserine (PS) is externalised from inner to the outer leaflet of the plasma membrane. Annexin V is a protein with strong affinity for PS. When conjugated with fluorochromes such as FITC, it serves as a sensitive probe for flow cytometric analysis of apoptosis. Propidium iodide (PI) is a useful probe for cell viability. Viable cells with intact membrane exclude PI, whereas dead and damaged cells are permeable to PI, which readily binds DNA. Therefore, cells undergoing different cell death pathways can be differentiated as follows: (1) cell that stained positive for Annexin V-FITC and negative for PI are apoptotic, (2) cell that stained positive for both are either in end stage of apoptosis, necrosis or already dead, and (3) cells that stained negative for both are living cells. The stained cells can be analysed by two-color flow cytometry as well as by fluorescence microscopy.

4.6.1.2 TUNEL assay

Other than changes in plasma membrane permeability, fragmentation of nuclear DNA is one of the notable feature that occur in the late phase of apoptosis. Cellular endonucleases cleave nuclear DNA between nucleosomes, producing a mixture of DNA fragments whose lengths vary in multiples of about 200 base pairs. The TUNEL assay involves the addition of fluorescein-labelled dUTP at the free 3'-hydroxyl ends of fragmented DNA. Apoptotic cells exhibit strong nuclear green fluorescence signals which can be detected by flow cytometry or fluorescence microscopy [150,151].

4.6.2 Photodamage on mitochondria

Investigation of subcellular oxidative damage exerted by photosensitisers is also important. It is because cellular localisation of photosensitiser is an important criterion for PDT. Amongst the subcellular photodamge effects, mitochondrial mediated oxidation is considered to be more efficient in triggering the apoptotic cascade [97]. The measurement of changes in mitochondrial membrane potential by Mitosensor is a useful method to determine such mitochondrial cytotoxic effects.

Mitosensor is a cationic dye that fluoresces differently in apoptotic and nonapoptotic cells. This dye is taken up by the mitochondria where it forms aggregates that exhibit intense red fluorescence. In apoptotic cells, this dye cannot accumulate in the mitochondria because of altered mitochondrial membrane potential. As a result, the dye remains as monomeric form in the cytoplasm which gives green fluorescence [152].

4.6.3 Cellular signaling pathway

Over expression of EGFR together with the sequential activation of signaling cascades are key features in glioma [19,20]. The rapid growth of glioma therefore involves the transmission of many mitogenic and oncogenic signals that lead to accelerated proliferation [153,154]. Studying the PDT effect on intracellular kinase cascades such as MAPK and FAK is valuable in clarifying the possible mechanisms that different photosensitisers may act on to reduce cell proliferation.

4.6.4 *In vivo* study

In this study, three photosensitisers efficiently accumulated in glioma cells and killed them in a light dose dependent manner. However, they may also influence normal brain cells such as astrocytes and microvascular endothelial cells. Unfortunately, these

cells are difficult to obtain and astrocytes, in particular, grow very slowly. The use of glioma implanted animal models may provide a much better understanding on the interaction between glioma and normal brain tissue undergoing PDT treatment. Besides, the effects of PDT on vascular endothelial cell in angiogenesis, *in situ* invasion potential of glioma cells as well as the local immune response can be determined. Therefore, *in vivo* studies may help to identify both therapeutic and side effects of PDT using different photosensitisers.

4.7 Effect of PDT on the tumour microenvironment

The induced tumour tissue destruction by PDT is not simply a direct effect on the tumour cells alone. Injury of tumour or adjacent tissue vasculature can lead to oxygen and nourishment depletion. Photodamage also triggers the retention of inflammatory cells to attack the tumour tissue. Interaction between PDT and the tumour microenvironment therefore plays a significant role in determining the therapeutic responsiveness. There is evidence showing that PDT induces angiogenesis in tumour tissue but the actual mechanism is not fully understood although it may be associated with stromal cell response [155-157]. Hypoxia is considered one of the strong stimuli for vascular endothelial growth factor (VEGF) expression in both physiological and pathological conditions. Inflammatory cells in response to PDT produce many kinds of growth factors and cytokines which can enhance inflammation-dependent angiogenesis. Inflammation and oxidative stress promote the expression of cyclo-oxygenase-2 (COX-2) locally. COX-2 is a strong mediator of prostaglandin E2 (PGE_2) production and increased PGE_2 levels in turn up-regulate VEGF expression [156]. In addition, MMPs induced by stromal cells and macrophages assist in the formation of capillary networks and promote the invasion of the ECM by endothelial cells [157-159]. Therefore, understanding the complex relationship between the cytotoxic response and stromal response in PDT is equally important.

4.8 Application of PDT in the future

Treatment strategy focused on tumour selectivity of various photosensitisers, as well as their intracellular localisation, is the popular topic being addressed in many *in vitro* and *in vivo* studies. Attempts have been made to improve the uptake and selectivity of photosensitisers to better control the outcome of PDT [73]. Certainly cell destruction via apoptosis is more preferable in clinical practice because of milder tissue inflammatory response as compared with necrosis.

Recently, sub-lethal light dose effects on brain tumours as well as the tissue adjacent to tumours have been investigated. PDT with high light doses was known to induce haemorrhagic necrosis in tumour tissue as well as damage to the normal brain tissues [139]. This is because normal brain tissues are sensitive to PDT even though only low levels of photosensitiser are present [160]. The term "metronomic photodynamic therapy" mPDT was recently introduced to describe a continuous, low dose administration of photosensitiser and light for an extended period of time [161]. The principle of mPDT is that the fraction of tumour cells killed each time is higher than the regrowth rate, and tumour cell death is initiated by the apoptotic pathway which minimises the photodamage to surrounding normal tissue. mPDT using 5-ALA showed induction of apoptosis in brain tumour cells without causing necrosis in either tumour or normal brain tissue [162].

Although the activation of photosensitiser predominantly takes place at the targeted tumour tissue, the adjacent normal tissue may also receive different magnitudes of light. As a result, VEGF may be produced and angiogenesis occurs within the tumour and some adjacent normal tissue as well. This will facilitate the proliferation and invasion of residual glioma cells and diminish the effectiveness of PDT [162-164].

Therefore, despite the promising results from earlier experimental and clinical trials of PDT, there are considerable investigations needed to be carried out to support the feasibility of this treatment in modern clinical practice.

Conclusion

In this study, the uptake of kinetics and cellular distribution of Photofrin, 5-ALA and calphostin C are found to be different. However, all of them are effective in killing glioma cells in a light dose dependent manner. PDT may be a very useful adjuvant therapy in the treatment of gliomas because it reduces tumour cell proliferation and invasion, possibly via a reduction of MMPs and adhesion molecule expression. The results of this study also highlight the fact that the effectiveness of different photosensitisers can vary considerably, with Photofrin being the most reliable in inhibiting glioma cell function in all the parameters examined. Variation in response by different cell lines suggests that the choice of photosensitiser in clinical practice may depend on the genetic background of the glioma itself.

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