



UNIVERSITI PUTRA MALAYSIA

**CHARACTERISATION OF TWO PHOTOSENSITIZING COMPOUNDS
FROM *TYPHONIUM FLAGELLIFORME* SCHOTT AND THEIR MODE OF
ACTION IN INDUCING CANCER CELL DEATH**

ANTHONY HO SIONG HOCK

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ANTHONY HO SIONG HOCK

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Chairman: Professor Abdul Manaf Ali, Ph.D.

Faculty: Food Science and Biotechnology

A bio-assay guided approach was used to isolate photosensitizing compounds from *Typhonium flagelliforme Schott*, a plant considered by local communities to possess anticancer properties. Two putative photosensitizers, pheophorbide-a and a possible novel derivative, 13-2, 17-dihydroxyethyl pheophorbide-a were isolated using column chromatography and purified on thin layer chromatography under low light conditions. The phototoxic effect of pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a on the growth of a variety of human cell lines was tested using the MTT assay. Both photosensitizers were inactive in the dark but when activated by light, mediated cell killing with IC₅₀ values ranging from 0.12 to more than 4 µg/ml, with the leukemic cells being the most sensitive. Photodynamic cell killing was dependent on photosensitizer concentration, irradiation dose and drug incubation time. Preliminary characterization showed that both compounds tended to aggregate in aqueous solution and the aggregation was augmented by the presence of serum proteins. This correlated to a decrease in cytotoxic efficacy as the amount of serum in the media was increased suggesting that binding to serum proteins reduced drug

uptake and possibly altered intracellular localization patterns. It has been shown that photosensitizers mediate cell-killing through a free radical (Type I) or singlet oxygen (Type II) dependent reaction. Indirect tests showed that the major species generated by photoactivation using pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a was singlet oxygen molecules with a minor contribution by other radical species. Furthermore, confocal laser scanning microscopy showed that these fluorescent photosensitizers accumulated in lysosomes, suggesting that the release of hydrolytic enzymes may be a common mechanism leading to necrotic injury. However, whereas some cell lines also showed intense membrane and cytoplasmic staining, others showed some mitochondrial accumulation, suggesting that other mechanisms may also contribute to cell-killing. Commercially available PDT compounds, including hypericin, appear to kill cells by inducing apoptosis. The treatment of HL60 cells with pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a resulted in the appearance of typical apoptotic morphology, including membrane blebbing, apoptotic bodies, cell shrinkage and DNA-laddering, suggesting that these compounds also induce apoptosis. Singlet oxygen is thought to be the primary stimulus driving the induction of apoptosis and this was studied by using the singlet oxygen quencher sodium azide. The caspase family of proteases, which are inhibited by the peptide Z-VAD-FMK, are regarded as the main effectors of apoptosis. Zinc ions are inhibitors of endonucleases that cause apoptotic DNA fragmentation. We found that both sodium azide and Z-VAD-FMK effectively reduced the incidence of apoptosis induced by pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a. Zinc did not affect 13-2, 17-dihydroxyethyl pheophorbide-a induced apoptosis but reduced pheophorbide-a induced apoptosis. Cycloheximide, a protein synthesis inhibitor, did not decrease the incidence of apoptosis for either photosensitizer but

actually increased 13-2, 17-dihydroxyethyl pheophorbide-a induced apoptosis. These results support the role of singlet oxygen as primary inducers and caspases as effectors of apoptosis. PDT induced apoptosis does not require the synthesis of new proteins but PDT may, in the case of 13-2, 17-dihydroxyethyl pheophorbide-a, induce the synthesis of proteins that protect cells from further oxidative damage. The results also suggest that PDT with 13-2, 17-dihydroxyethyl pheophorbide-a may activate an endonuclease that is not inhibited by zinc. The progression of apoptosis is highly regulated by pro-(Bax) and anti- apoptotic Bcl-2 family of proteins (Bcl-2, Bcl-X_L). Qualitative detection of these apoptotic marker proteins at time intervals of 4, 8, 12 and 24 hours showed that the ratios of Bax to Bcl-2 and of Bax to Bcl-X_L were markedly increased from 12 hours onwards and this, as has been suggested earlier, is a marker for apoptotic progression. However, because apoptosis can be detected as early as 2 hours post irradiation, Bcl-2 proteins may not play a major role in the initial induction of apoptosis but impacts apoptosis at a much later stage, possibly to ensure the complete demise of the cell. In conclusion, pheophorbide-a and 13-2, 17-dihydroxyethyl pheophorbide-a are effective photosensitizing compounds that kill cells mainly through apoptosis. The possibility of different apoptotic pathways being induced, especially by 13-2, 17-dihydroxyethyl pheophorbide-a, makes the development of these compounds as potential clinical drugs, an exciting prospect.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
untuk memenuhi keperluan untuk ijazah Doktor Falsafah

**PENYIFATAN DUA BAHAN FOTOSENSITIZING DARI SPESIS
TYPHONIUM FLAGELLIFORME SCHOTT DAN MEKANISME
MENYEBABKAN KEMATIAN SEL KANSER**

Oleh

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Teknik pengasingan biocerakinan berpandu digunakan untuk mengasingkan bahan fotosensitizing dari spesis *Typhonium flagelliforme Schott*. Dua fotosensitizer, pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a, satu bahan terbitan baru, diasingkan dengan menggunakan kromatografi kolumn dan TLC di bawah keadaan cahaya rendah. Kesan fototoksik pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a ke atas sel-sel kanser manusia diuji dengan kaedah MTT. Kedua-dua fotosensitizer tidak berkesan dalam keadaan gelap tetapi berjaya membunuh sel dengan IC_{50} dari 0.12 hingga lebih 4 $\mu\text{g/ml}$, dan sel leukemia adalah paling sensitif. Pembunuhan secara fotodinamik bergantung kepada kepekatan fotosensitizer, dos penyingaran dan tempoh pengaraman dengan fotosensitizer. Pencirian awal menunjukkan bahawa kedua-dua kompoun berkumpul bila dilarut dalam larutan akueus dan pengumpulan bertambah dalam kehadiran protein serum. Sifat ini berhubung kait dengan kebolehan serum untuk mengurangkan kesan sitotoksik. Ini mungkin disebabkan serum dapat mengurangkan penyerapan kompoun ke-dalam sel dan juga mengubah lokasi kompoun di dalam sel. Fotosensitizer dapat membunuh sel melalui reaksi bahan radical bebas (Jenis I) atau

'singlet' oksigen (Jenis II). Kajian secara tidak langsung menunjukkan bahawa bahan utama yang diterbitkan melalui pengaktifan sinaran fotosensitizer pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a adalah singlet oksigen dengan penyumbangan yang kurang dari radikal lain. Kaedah mikroskop laser konfokal menunjukkan bahawa fotosensitizer bertempat di dalam organel lysosome dan ini mencadangkan bahawa pembebasan enzim hidrolitik adalah mekanisma yang digunakan untuk membunuh sel. Fotosensitizer juga dikesan pada membran sel, sitoplasma dan mitokondria. Ini menunjukkan bahawa mekanisma lain juga digunakan untuk membunuh sel. Fotosensitizer komersial, termasuk kompoun hypericin, didapati membunuh sel secara 'apoptosis'. Rawatan sel HL60 dengan pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a menghasilkan morfologi 'apoptotic' dan ini termasuk tompok-tompok membran, badan 'apoptotic', pengecutan sel dan fenomena tangga DNA. Singlet oksigen dianggap sebagai rangsangan primer untuk menyebabkan 'apoptosis' dan ini dikaji dengan menggunakan Natrium Azide, salah satu penyah singlet oksigen. Keluarga 'caspase' adalah effektor utama dalam proses 'apoptosis' dan aktiviti mereka dapat disekat oleh bahan peptida Z-VAD-FMK. Aktiviti endonukleas yang menyebabkan fenomena tangga DNA pula, dapat disekat oleh ion-ion Zink. Kami mendapati bahawa Natrium Azide dan Z-VAD-FMK dapat mengurangkan kejadian 'apoptosis' yang disebabkan oleh pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a. Zink tidak berkesan ke atas activity 13-2, 17-dihydroxyethyl pheophorbide-a tetapi dapat mengurangkan 'apoptosis' yang disebabkan oleh pheophorbide-a. Sikloheksimid, sesuatu bahan yang boleh menyekat sintesis protein tidak dapat mengurangkan kejadian 'apoptosis' tetapi sebaliknya 'apoptosis' yang disebabkan oleh 13-2, 17-dihydroxyethyl pheophorbide-a bertambah banyak. Hasil-hasil kajian ini

menyokong pendapat bahawa singlet oksigen adalah rangsangan primer dan enzim-enzim 'caspase' adalah efektor utama dalam proses 'apoptosis'. 'Apoptosis' yang disebabkan oleh terapi fotodinamik tidak memerlukan sintesis protein baru tetapi, terapi ini mungkin merangsangkan sintesis protein yang melindungi sel dari kerosakan yang melanjut seperti yang didapati dengan 13-2, 17-dihydroxyethyl pheophorbide-a. Hasil kajian juga mencadangkan bahawa terapi fotodinamik dengan 13-2, 17-dihydroxyethyl pheophorbide-a menghasilkan endonukleas yang tidak dapat disekat oleh ion zink. Kemajuan proses 'apoptosis' dikawal ketat oleh protein keluarga Bcl-2 yang menyokong (Bax) dan yang menentang (Bcl-2, Bcl-XL) proses ini. Kajian pengesanan kualitatif penanda protein 'apoptosis' pada masa 4, 8, 12 dan 24 jam selepas terapi fotodinamik menunjukkan bahawa nisbah Bax kepada Bcl-2 dan Bax kepada Bcl-XL semakin tinggi dari 12 jam selepas terapi. Ini, sebagaimana yang dianggap terdahulu, adalah penanda yang tepat untuk mengesan kemajuan proses 'apoptosis'. Walaubagaimanapun, 'apoptosis' dapat dikesan seawal 2 jam selepas rawatan dan ini mencadangkan bahawa keluarga Bcl-2 tidak memainkan peranan penting dalam proses induksi 'apoptosis'. Malah, keluarga protin ini lebih menyumbangkan kesan pada peringkat yang lebih lewat, mungkin untuk memastikan kematian sel tersebut. Sebagai kata-kata pengakhiran, pheophorbide-a dan 13-2, 17-dihydroxyethyl pheophorbide-a adalah fotosensitizer yang berkesan dan mereka membunuh sel secara 'apoptosis'. Fotosensitizer yang diperolehi, terutamanya 13-2, 17-dihydroxyethyl pheophorbide-a, didapati merangsangkan pelbagai cara yang berlainan untuk menyebabkan 'apoptosis'. Perkembangan fotosensitizer ini sebagai ubat klinikal merupakan satu prospek yang amat menantik.

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TABLE OF CONTENTS

	Page
ABSTRACT	2
ABSTRAK	5
ACKNOWLEDGEMENTS	8
APPROVAL	9
DECLARATION	11
LIST OF TABLES	15
LIST OF FIGURES	16
LIST OF PLATES	20
LIST OF ABBREVIATIONS	21
 CHAPTER	
I INTRODUCTION	23
II LITERATURE REVIEW	29
Cancer	29
Biology of Tumors	29
Cell Cycle and Cancer	30
Development of Cancer	33
Carcinogenesis: A Molecular Framework	33
Tumor Supressor Genes	34
Oncogenes	35
Current major treatment approaches	37
Established Methods	37
New Approaches to Cancer Treatment	40
Immunotherapy	40
Gene Therapy	40
Photodynamic Therapy (PDT)	41
History of PDT	42
Photophysics of PDT	43
Oxygen Dependence	45
Light Source	46
Uptake and Localization	47
Mechanisms of Tumor Destruction	50
Photosensitizers: past, present and future	54
Historical Development of Hematoporphyrin	54
Clinical Trials	56
Second Generation Photosensitizers	57
Porphyrrins	58
Chlorophylls	64
Reactive Oxygen Species (ROS)	66
Mediators of PDT Cytotoxicity	66
Radical and Non-Radical ROS	67
Oxidative Damage to Membranes	68
Oxidative Damage to Proteins & Nucleic Acids	69
Singlet Oxygen as Modifiers of Gene Expression	70

Cell Death	72
Necrosis	73
Apoptosis	74
Morphological markers of apoptosis	75
Biochemical regulation of apoptosis	76
Caspases	81
Molecular regulation of apoptosis	85
Bcl-2 Family	86
Induction of Apoptosis	92
Death by PDT	98
 III MATERIALS AND METHODS	100
Extraction and Isolation of Phototoxic Compounds	
from <i>Typhonium flagelliforme Schott</i>	100
Preparation of Plant Material	100
Extraction and Fractionation of Crude Sample	102
General Instrumentation	103
Chromatography	103
Isolation of bio-active plant pigments	104
Biological assay	105
Cell Lines	105
Maintenance of Cell Lines	105
Microscopy Analyses of Cell Lines	106
Photosensitizer preparation	106
MTT assay	107
Phototoxicity assay	108
Phototoxicity of Isolated Chlorophyll Derivatives	109
Effect of Serum Content on Phototoxicity	110
Cell Killing Mode of PDT	111
Chemicals	111
Irradiation in the presence of inhibitors	111
Deuterium oxide	114
Induction of Apoptosis by PDT	114
Photodynamic Treatment	114
Effect of Specific Apoptotic Inhibitors	115
Acridine Orange-Propidium Iodide Staining	115
DNA Content Analysis	116
Agarose Gel Analysis of DNA Fragmentation	117
Annexin V-FITC Staining of Membrane	
Phosphatidylserine (PS)	118
Determination of Caspase-3 Activity	120
Preparation of Cell Extracts	120
Assay Protocol	121
Expression of Pro and Anti Apoptotic Bcl-2 Proteins	122
SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot	123

IV	RESULTS AND DISCUSSION	128
	Phototoxicity of Isolated Chlorophyll Derivatives	128
	Screening of Phototoxic activity	128
	Bioassay Guided Fractionation	129
	Structural elucidation of B1	131
	Structural elucidation of G2	137
	Phototoxicity of Isolated Photosensitizers pheophorbide-a and 13-2,17-dihydroxyethyl pheophorbide-a.	135
	Cell Killing Mode of PDT	152
	Intracellular Localization of Pheophorbide-a and 13-2,17-dihydroxyethyl pheophorbide-a	160
	The Induction of Apoptosis	163
	Assessment of Morphology and Quantitative Time Course Analysis by AO/PI	164
	DNA Fragmentation Analysis	172
	Flow Cytometric (FC) Measurement of DNA Content	175
	Flow Cytometric Measurement of Phosphatidylserine redistribution	180
	The Inhibition of PDT induced Apoptosis	185
	Determination of Caspase 3 Activity	188
	Bcl-2, Bax and Bcl-X _{L/S} expression in PDT Induced Apoptosis	190
V	GENERAL DISCUSSION AND SUMMARY	194
	Phototoxicity of Pheophorbide-a and 13-2,17-dihydroxyethyl pheophorbide-a	194
	Type I versus Type II pathways	198
	PDT Induced Apoptosis	201
	Conclusion	210
	Future Work	211
	REFERENCES	216
	APPENDICES	239
	VITA	243

LIST OF TABLES

Table		Page
2.1 Differences between cancerous and normal cells in <i>in vitro</i> culture systems.		30
2.2 Tumor suppressor genes.		34
2.3 Historical development of photodynamic therapy.		43
2.4 Potential photosensitizers of clinical importance.		58
2.5 Reactive Oxygen Species.		68
2.6 Differences between Necrosis and Apoptosis.		75
2.7 The Human Caspase Family.		82
2.8 Division of Caspases based on substrate tetrapeptide specificity.		85
3.1 Addition of cell extracts for the Caspase assay.		122
3.2 Preparation of gels for electrophoresis.		124
4.1 Phototoxicity and intrinsic activity of fractions of <i>T. flagelliforme</i> against CEM-SS and HeLa cells.		129
4.2 Proton chemical shifts of B1 compared with pheophorbide a and Me-pheophorbide a.		134
4.3 Proton chemical shifts of G2 compared with pheophorbide a and 10-hydroxymethyl pheophorbide a.		139
4.4 H-H COSY cross peaks of G2.		141
4.5 IC ₅₀ values of the photosensitizers in a panel of cell lines.		144
4.6 Time course study of HL60 cell populations exposed to PDT with pheophorbide-a and 13-2,17-dihydroxyethyl pheophorbide-a determined from AO/PI staining.		173
4.7 Percentage of cells with a sub-diploid level of DNA (apoptotic cells) after PDT treatment with pheophorbide-a, 13-2,17-dihydroxyethyl pheophorbide-a and hypericin.		176

LIST OF FIGURES

Figure	Page
2.1 Overview of the cell cycle.	32
2.2 Summary of the different classes of proteins encoded by oncogenes.	36
2.3 Simplified energy diagram.	44
2.4 Sequence of events leading to regional tissue hypoxia.	52
2.5 Structure of dihematoporphyrin ester and ether.	55
2.6 Porphyrin skeleton structures.	60
2.7 A typical visible absorption spectra of most porphyrin and chlorin systems.	63
2.8 Chlorophyll structure & degradation chemistry.	65
2.9 Absorption spectra of chlorophyll a and b.	66
2.10 Oxidation of unsaturated lipids by singlet oxygen.	69
2.11 Possible causes of the loss in selective membrane permeability.	74
2.12 The caspase cascade.	84
2.13 Constituents of the death machinery in <i>C.elegans</i> and their corresponding mammalian homologs.	86
2.14 Bcl-2 homology domains (BH) of the Bcl-2 family proteins.	88
2.15 Regulation of apoptosis by Bcl-2 family members.	91
2.16 Components of the mitochondrial permeability transition pore (PTP).	95
2.17 Mitochondria integrates a variety of death signals and regulates the cells response.	99
3.1 Crude sample preparation and solvent partitioning of crude samples into 4 fractions that were screened for phototoxic activity.	102
3.2 Principle of the MTT assay.	107

3.3	Apparatus for the irradiation of micowell plates.	109
3.4	Sample layout for ROS inhibition assay.	113
4.1	Phototoxicity and intrinsic activity of the chloroform fraction of <i>T. flagelliforme</i> against CEM-SS and HeLa cells.	129
4.2	Bioassay guided isolation of G2 and B1 from the chloroform fraction of <i>T. flagelliforme</i> .	130
4.3	Pheophorbide a and phaeoporphyrin a ₅ are both compatible with the molecular formula C ₃₅ H ₃₆ N ₄ O.	131
4.4	Proton NMR (300 MHz) of B1 measured in CDCl ₃ with addition of D ₂ O.	135
4.5	Molecular structure of B1 showing the numbering of the hydrogens and their respective chemical shifts.	136
4.6	Proton NMR (300 MHz) of G2 measured in CDCl ₃ .	140
4.7	H-H COSY of G2.	141
4.8	Molecular structure of G2 showing the numbering of the hydrogens and their respective chemical shifts.	142
4.9	Phototoxicity of A) pheophorbide-a, B) 13-2,17-dihydroxyethyl pheophorbide-a and a control drug C) hypericin was tested against the suspension cell lines Raji, CEM-SS and HL60.	147
4.10	Phototoxicity of A) pheophorbide-a, B) 13-2,17-dihydroxyethyl pheophorbide-a and a control drug C) hypericin was tested against the anchorage dependent cell lines 3T3, HeLa and Chang liver.	148
4.11	Survival curves of CEM-SS cells as a function of photosensitizer concentration and light dose for A) pheophorbide-a, B) 13-2,17-dihydroxyethyl pheophorbide-a and C) hypericin.	149
4.12	The influence of serum content on the phototoxicity of A) pheophorbide-a, B) 13-2,17-dihydroxyethyl pheophorbide-a and C) hypericin in CEM-SS cells.	150
4.13	Phototoxicity of A) pheophorbide-a, B) 13-2,17-dihydroxyethyl pheophorbide-a and C) hypericin in CEM-SS cells as function of photosensitizer incubation time prior to irradiation	151

4.14 The effect of ROS quenchers A) DABCO, B) Sodium azide C) α -tocopherol and D) SOD on the phototoxicity of pheophorbide-a and 13-2,17-dihydroxyethyl pheophorbide-a in Chang liver cells.	155
4.15 The effect of ROS quenchers A) DABCO, B) Sodium azide C) α -tocopherol and D) SOD on the phototoxicity of pheophorbide-a and 13-2,17-dihydroxyethyl pheophorbide-a in HepG2 cells.	156
4.16 Using deuterated media increases the phototoxicity of A) pheophorbide-a and B) 13-2,17-dihydroxyethyl pheophorbide-a against Chang liver cells.	157
4.17 Using deuterated media increases the phototoxicity of A) pheophorbide-a and B) 13-2,17-dihydroxyethyl pheophorbide-a against HepG2 cells.	158
4.18 Quantitative determination of apoptosis using AO/PI double staining.	172
4.19 DNA fragmentation analysis of HL60 cells after PDT using 13-2,17-dihydroxyethyl pheophorbide-a (0.25 μ g/ml), pheophorbide a (0.25 μ g/ml) and hypericin (0.125 μ g/ml).	174
4.20 Flow cytometric analysis of DNA content.	175
4.21 Flow cytometric determination of DNA content of HL60 cells subjected to PDT using pheophorbide-a (0.25 μ g/ml).	177
4.22 Flow cytometric determination of DNA content of HL60 cells subjected to PDT using 13-2,17-dihydroxyethyl pheophorbide-a (0.25 μ g/ml).	178
4.23 Flow cytometric determination of DNA content of HL60 cells subjected to PDT using hypericin (0.2 μ g/ml).	179
4.24 HL60 cells subjected to PDT and stained with annexin V-FITC (X-axis) and PI (Y-axis) were analyzed by flow cytometry.	182
4.25 Quantitation of Annexin V FITC positive (apoptotic) cells by flow cytometry.	184
4.26 The effect of different inhibitors on apoptosis induced by pheophorbide-a, 13-2,17-dihydroxyethyl pheophorbide-a and hypericin.	187
4.27 Specific caspase 3 activity of PDT treated HL60 cells with and without Z-VAD-FMK.	189

4.28	Expression of Bcl-2, Bax and Bcl-X _{L/S} in HL60 cells subjected to PDT with pheophorbide-a (B1), 13-2,17-dihydroxyethyl pheophorbide-a (G2) and hypericin determined by Western Blots.	191
5.1	Possible steps initiated by PDT. Blunt arrows indicate the level at which the inhibitors azide and cycloheximide act.	206
5.2	Diagram of possible pathways to apoptosis activated in response to PDT with pheophorbide-a and 13-2,17-dihydroxyethyl pheophorbide-a.	210

LIST OF PLATES

Plate	Page
3.1 <i>Typhonium flagelliforme Schott.</i>	101
4.1 Morphology of Chang liver (A-C) and HepG2 cells (D-F).	159
4.2 HeLa cells stained with acridine orange (A,B) and rhodamine 123 (C) and viewed with a confocal laser scanning microscope.	160
4.3 HeLa cells incubated with 5.0 µg/ml of (A) pheophorbide-a and (B) 13-2,17-dihydroxyethyl pheophorbide-a for two hours and viewed with a confocal microscope.	162
4.4 HepG2 cells incubated with 5.0 µg/ml of (A) pheophorbide-a and (B) 13-2,17-dihydroxyethyl pheophorbide-a for two hours and viewed with a confocal microscope.	162
4.5 CEM-SS cells incubated with 5.0 µg/ml of (B) pheophorbide-a and (C) 13-2,17-dihydroxyethyl pheophorbide-a for two hours and viewed with a confocal microscope.	162
4.6 Sequence of morphological changes of HL60 cells treated with 13-2,17-dihydroxyethyl pheophorbide a (0.25 µg/ml) PDT.	165
4.7 Further examples of apoptotic morphology of HL60 cells stained with AO/PI.	167
4.8 AO/PI staining of HL60 cells treated with 13-2,17-dihydroxyethyl pheophorbide a (0.25 µg/	169
4.9 Morphology of HL60 cells examined with scanning electron microscopy.	170

LIST OF ABBREVIATIONS

${}^1\text{O}_2$	Singlet oxygen
AIDS	Acquired immune deficiency syndrome
AIF	Apoptosis inducing factor
AMP	Adenosine monophosphate
ANT	Adenine nucleotide translocator
AO	Acridine orange
BH	Bcl-2 homology domains
BPD-MA	Benzoporphyrin derivative monoacid ring A
CAD	Caspase activated deoxyribonuclease
CARD	Caspase recruitment domain
CC	Column chromatography
Cdk	Cyclin dependent kinase
CO ₂	Carbon dioxide
COSY	Correlated spectroscopy
DMSO	Dimethyl sulfoxide
ESI MS	Electro Spray Ionization mass spectrometry
FADD	Fas associated death domain
FBS	Foetal bovine serum
FITC	Flouresceinithiocynate
FT-IR	Fourier transform infrared spectroscopy
GM-CSF	Granulocyte macrophage colony stimulating factor
HMW	High molecular weight
HPD	Hematoporphyrin derivative
IAP	Inhibitors of apoptosis protein
ICS	Intersystem crossing
IFN	Interferon
IL	Interleukin
IMS	Intermembrane space
<i>J</i>	Coupling constant
LDL	Low density lipoprotein
<i>m/z</i>	Mass-to-charge-ratio
M	Molar
mA	Milliampere
mg	Miligram
min	Minutes
ml	Mililiter
MPT	Mitochondrial permeability transition
MS	Mass spectrum
NLS	Nuclear localization signal
nm	Nanometer
NMR	Nuclear magnetic resonance
PARP	Poly(ADP)ribose polymerase
PBS	Phosphate buffered saline
PDT	Photodynamic Therapy
PI	Propidium Iodide
PKA	Protein kinase A
PKC	Protein kinase C

PMSF	:	Phenylmethylsulfonyl fluoride
PS	:	Phosphatidylserine
PTLC	:	Preparative thin layer chromatography
ROS	:	Reactive oxygen species
SDS	:	Sodium dodecyl sulphate
SOD	:	Superoxide dismutase
TEMED	:	N,N,N',N'-tetramethylenediamine
TRADD	:	TNFR1 associated death domain
Tris	:	Tris (hydrosylmethyl) aminoethane
TM	:	Transmembrane
TMS	:	Tetramethylsilane
TNF	:	Tumor Necrosis Factor
TLC	:	Thin layer chromatography
UV	:	Ultraviolet
VDAC	:	Voltage dependent anion channel
μ	:	Micro
%	:	Percentage
δ	:	Chemical shift
λ_{\max}	:	In UV spectroscopy, the wavelength at which maximum absorption occurs

CHAPTER I

INTRODUCTION

Photodynamic therapy (PDT) is a relatively selective method for the destruction of localised solid tumors. Photosensitizers capture the energy from light, producing singlet oxygen and other free radicals that destroy the targeted tissue. Until a decade ago, tumour destruction was thought to occur primarily via cellular necrosis, those of the tumour cells and that of the accompanying vascular supply (Oschner, 1997). The discovery that PDT induced apoptotic cell death raised many questions regarding the mechanisms employed by the affected cell upon PDT (Agarwal, 1991). Many groups have now established the occurrence of apoptosis in parallel with necrosis in both *in vitro* and *in vivo* model systems (Ahmad and Mukhtar, 2000; Dougherty *et al.*, 1998). The distinction of the type of predominant cell death observed is largely dependent on photosensitizer type, intracellular localization, experimental protocol and tumour type (Hassan and Mukhtar, 2000; Agostiniz, *et al.*, 2000). As yet, no universal mechanism of apoptosis can be proposed for PDT because the type of apoptotic pathway affected is dependent on the variables above as well.

Our understanding of the role of mitochondria in apoptosis (Adrain and Martin, 2001) has illuminated a possible unifying starting point for PDT induced apoptosis. Kessel and Luo (1998) showed that photosensitizers that localize in the