EX VIVO BONE MARROW PURGING USING BPD- MEDIATED PHOTODYNAMIC THERAPY

by

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ABSTRACT

Photodynamic therapy (PDT) using the second generation photosensitiser benzoporphyrin derivative monoacid ring- A (BPD, Verteporfin®) offers an attractive alternative to purging of contaminating neoplastic cells during autologous haematopoietic stem cell transplantation. Enhancement of PDT cytotoxicity was attempted using two independent approaches: combination treatment with doxorubicin (Dox) and selective protection of normal haematopoietic cells using the tetrapeptide N-AcSDKP.

The murine leukaemic cell line L1210 was 45 x more susceptible to the sequenced combination regimen of 1 h 2.5 μM Dox incubation followed by PDT mediated by 5.0 ng/ml BPD and 15 J/cm^2 red light (Dox-> PDT) than normal murine haematopoietic cells. The significant enhancement in cytotoxicity was dependent on the concentration of BPD used as well as on the sequence of treatment. Specifically, it was observed with 5.0 ng/ml but not with 2.5 ng/ml BPD and only when Dox was used before PDT. The reverse sequence of PDT-> Dox and simultaneous Dox/PDT treatment were not associated with enhanced killing. Interestingly, L1210 cells were much more susceptible to the combination therapy than normal DBA/2 haematopoietic progenitor cells which offered interesting therapeutic implications. BPD uptake and cellular GSH content, appeared not to be responsible for the potentiation of L1210 killing in the Dox-> PDT sequence.

Next, the potential of selective stem cell protection in BPD- mediated PDT was investigated. Preincubation of DBA/2 bone marrow cells with 100 nM N-AcSDKP for 1.5 h significantly protected resultant colony formation from PDT by a factor of 1.5- 2 over cells that were incubated with control peptides or with tissue culture medium. Interestingly, L1210 cells were not protected by N-AcSDKP and the control peptides. However, N-AcSDKP-mediated photoprotection did not appear to extend to earlier murine haematopoietic cells and stem cells as demonstrated by the long- term bone marrow culture (LTBMC) assay. The same findings of differential photoprotection were also demonstrated in human haematopoietic cells. The mechanism of protection appeared to be mediated by inhibition of progression to S phase of the cell cycle since depletion of cycling DBA/2 bone marrow cells with 50 μM cytosine arabinoside (ara- C) resulted in cells more tolerant to subsequent PDT cytotoxicity. The above two approaches enhanced selective BPD- mediated PDT cytotoxicity and therefore maybe of merit in the clinical use of PDT in purging.

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LIST OF ABBREVIATIONS

4- HC	4- hydroperoxycyclophosphamide
8- MOP	8- methoxypsoralen
ACTH	Adreno- corticotrophic hormone
alBMT	Allogeneic one marrow transplantation
ALDH	Aldehyde dehydrogenase
ALL	Acute lymphoid leukaemia
AlSPc	Sulphonated aluminium phthalocyanine
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
APL	Acute promyelocytic leukaemia
Ara- C	Cytosine arabinoside
ATG	Antithymocyte globulin
ATRA	All- trans retinoic acid
auBMT	Autologous bone marrow transplantation
BCG	Bacillus Calmette- Guérin
BMMNCs	Bone marrow mononuclear cells
BMP	Bone marrow purging
BMT	Bone marrow transplantation
BPD	Benzoporphyrin derivative monoacid ring- A
BSA	Bovine serum albumin
BSO	Buthionine sulphoximine
Bu	Busulphan
CFU- GM	Colony forming unit- granulocyte & macrophage
CFU- L	Colony forming unit- leukaemic cell
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte
CW	Continuous wavelength
CY	Cyclophosphamide

CYA	Cyclosporin A
DHE	Dihaematoporphyrin ether
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's modified Eagle's medium
DTNB	5,5'- dithiobis- (2- nitrobenzoic acid)
Dox	Doxorubicin hydrochloride
EPO	Erythropoietin
EtNBS	5- ethylamino- 9- diethylaminobenzo[a] phenothiazinium chloride
FAB	French- American- British classification
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
G- CSF	Granulocyte- colony stimulating factor
GM- CSF	Granulocyte & macrophage- colony stimulating factor
GSH	Glutathione
GvHD	Graft- versus- host- disease
GvL	Graft- versus- leukaemia
HI- FCS	Heat- inactivated- foetal calf serum
HLA	Human leukocyte antigen
HOCl	Hypochlorous acid
HPC	Haematopoietic progenitor cell
HPD	Haematoporphyrin derivative
HSC	Haematopoietic stem cell
HUC	Human umbilical cord blood cells
ICSBP	Interferon consensus binding sequence protein
IFNα	Interferon α
IMDM	Iscove's Modified Dulbecco's Medium
LAK	Lymphokine activated killer
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
LTBMC	Long- term bone marrow culture

MC540	Merocyanine 540
MDR	Multidrug resistance
MESNA	Sodium 2- mercaptoethanesulfonate
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mHC	minor histocompatibility complex
MIP- 1α	Macrophage inflammatory protein- 1α
MLR	Mixed lymphocyte reaction
MMC	Mitomycin C
MnSOD	Manganese superoxide dismutase
MRD	Minimal residual disease
MTT	3- [4,5- Dimethylthiazol- 2- yl]- 2,5- diphenyltetrazolium bromide
MUD	Matched unrelated donor
N-AcSDKE	N-Acetyl- Ser- Asp- Lys- Asp
N-AcSDKP	N-Acetyl- Ser- Asp- Lys- Pro
NAC	N-Acetyl- cysteine
NCI	National Cancer Institute
NEM	N- ethylmaleimide
NF- κβ	Nuclear factor- κβ
ODN	Oligodeoxynucleotide
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cell
PBSCT	Peripheral blood stem cell transplantation
PCR	Polymerase chain reaction
PDT	Photodynamic therapy
PDTC	Pyrrolidone derivative of dithiocarbamate
pEEDCK	PyroGlu- Glu- Asp- Cys- Lys
PHA- LCM	phytohaemaggultinin stimulated- human leucocyte conditioned medium
PHSC	Pluripotent haematopoietic stem cell
PH′	Philadelphia chromosome

PMT	Photomultiplier tube
PTK	Protein tyrosine kinase
PWM- SCCM	Pokeweed mitogen- spleen cell conditioned medium
qRT- PCR	Quantitative reverse transcriptase- polymerase chain reaction
RAPA	Rapamycin
RB	Retinoblastoma protein
RBC	Red blood cell
RFLP	Restriction fragment length polymorphism
Rh123	Rhodamine 123
rhIL- 2	Recombinant human interleukin- 2
ROIs	Reactive oxygen species
RT- PCR	Reverse transcriptase- polymerase chain reaction
SCA	Sickle cell anaemia
SCID	Severe combined immunodeficiency
SDKP	Ser- Asp- Lys- Pro
SEM	Standard error of the mean
SLT- 1	Shiga- like toxin 1
SOD	Superoxide dismutase
sODN	Phosphorothioate- modified oligodeoxynucleotide
TBI	Total body irradiation
TCD	T cell depletion
TGF- β	Transforming growth factor- β
TIL	Tumour infiltrating lymphocyte
TNFα	Tumour necrosis factor α
TNFr	Tumour necrosis factor receptor
TPO	Thrombopoietin

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Blood is the originating cause of all men's diseases.

The Talmud
Baba Nathra, III.58a

INTRODUCTION

1.1 LEUKAEMIA : HISTORY

a. Clinical observations

The history of leukaemia in the past two centuries says as much about the disease itself as about the scientific advancements and paradigm shifts in the field of medicine. Sir William Osler, in his seminal work *The Principles and Practice of Medicine*, described the three components of disease as manifestations, pathogenesis, and cause. True appreciation of an illness and therefore formulation of rational treatments require understandings of all three of the above. Unfortunately, the extent of our medical knowledge does not allow us to fully comprehend their combined significance. We are often left with the most obvious facet of the disease trilogy, the manifestations, which was never more true than in Osler's days. The manifestations of a disease belong to the domain of signs, symptoms, and the treatments designed to ameliorate them. Symptomatic treatments were universal during Osler's time, some of which are still in use today. In 1893, when the first edition of the textbook was published, physicians already had an idea that leukaemia constituted a distinct entity of the systemic disorders.[1] The first documented case of leukaemia was reported in 1827 by the French physician Alfred Velpeau.

> "The blood was thick, like gruel... resembling in consistency and color the yeast of red wine... One might have asked if it were not rather laudable pus, mixed with blackish coloring matter, than blood."

Ten years later, gross observations were complemented with rudimentary microscopic examination of the blood from leukaemic patients. Donne concluded that the proliferation of "mucous cells" was responsible for the patient's hepatosplenomegaly. At that time, there was still great controversy concerning the aetiology of leukaemia. Many prominent physicians believed that the disease was a severe form of general pyaemia and therefore was suppurative in nature. Virchow correctly defined leukaemia as a distinct disease with "an increase in the number of colorless blood corpuscles to the extent that the red color of blood turns into reddish, yellowish, or greenish white" and was associated with the findings of hepatosplenomegaly and lymphadenopathy. He also attempted to classify leukaemia into two categories: the splenic and lymphatic form.[1] In 1854, Vogel described in detail the signs and symptoms of leukaemia which included lethargy, diarrhoea, pallor, hepatosplenomegaly, and lymphadenopathy in some cases. Significantly, Vogel questioned whether the leukaemic blood profile was caused by enlargements of the spleen, liver, and lymphatics or the other way around. In the late 1800's, Woodward introduced the aniline dyes into diagnostic haematology. Ehrlich later exploited the differential staining ability of these dyes to identify the different blood cells in both normal subjects and leukaemic patients; his most significant contribution to the field was the demonstration that leukaemia could be diagnosed by simple blood smears. Thus entered Sir William Osler's textbook in 1893. The manifestations of leukaemia were fairly well understood and physicians were starting to focus their attention on the pathogenesis and aetiology of the disease. However, leukaemic treatments were still symptomatic in nature. In fact, many were palliative and included "fresh air, good diet, and abstention from mental worry and care...".[2]

b. Diagnostics and Therapeutics

The 20th century has brought significant progress in both the diagnosis and treatment of leukaemia. One important landmark was the discovery of the Philadelphia chromosome (PH′) by Nowell and Hungerford in 1960.[3] PH′ is a product of the t(9; 22)(q34; q21) rearrangement in chronic myelogenous leukaemia (CML). This discovery not only provided a unique and reliable marker for CML, it also paved the path for further discoveries in the molecular pathogenesis and aetiology of this form of leukaemia. Other

chromosomal abnormalities were soon linked to different forms of cancers; ironically, the majority of chromosomal translocations are found to be associated with leukaemias.[4] Great strides were made in elucidating the cause of leukaemia from studies in the viral transmission of leukaemia in animals and from epidemiological studies of the patients exposed to radiation and chemicals. At the same time, development of novel chemotherapy was being conducted at the National Cancer Institute (NCI) under C. Gordon Zubrod's direction.[1] The systemic nature of leukaemia precluded the effective use (except during oncological emergency or in local control) of surgery and radiation treatments; therefore, chemotherapy by default became the therapy of choice. Early use of chemical compounds in the control of leukaemic growth was often empirical in nature. In 1907, Osler mentioned the use of potassium arsenic by his contemporaries in the management of leukaemia; the results, however, were predictably unpredictable. Ehrlich, pessimistic in regard to the future of chemotherapy in cancer management, displayed a sign with the line from Dante's *Inferno*; "Abandon all hope all ye who enter here," prominently at the entrance to his laboratory. Pessimism concerning the success of leukaemia chemotherapy also was shared by Woglom half a century later.

> "Those who have not been trained in chemistry or medicine... may not realize how difficult the problem of cancer treatment really is. It is almost- not quite, but almost- as hard as finding some agent that will dissolve away the left ear, say, yet leave the right ear unharmed: So slight is the difference between the cancer cell and its normal ancestor."[5]

Research into alkylating agents such as busulphan and cyclophosphamide followed rapidly after the initial discovery of the use of nitrogen mustard in leukaemia control. Busulphan, developed by Sir Alexander Haddow, was found to be extremely myelosuppressive and was therefore useful in the control of excessive neutrophils in chronic phase CML.[6] Early observations of the biological activities of corticosteroids had lead to the subsequent use of adreno- corticotrophic hormone (ACTH) and cortisone on patients with lymphocytic leukaemia. At the same time, development of the different antimetabolites was underway. Folic acid plays an important role in haematopoiesis; its deficiency leads to retarded haematopoiesis and its abundance accelerates the growth of leukaemic cells.[7, 8] The therapeutic potential of antimetabolites was realised initially with aminopterin and later with

methotrexate, both folic acid antagonists. In 1948, Hitchings and Elion, who later won the Nobel prize for medicine and physiology, developed the purine analogue 6- mercaptopurine which was subsequently used for the maintenance of patients with acute lymphocytic leukaemia (ALL).[9] Clinical introduction of the pyrimidine analogue cytosine arabinoside (Ara- C) in 1963 signified a milestone in acute myelogenous leukaemia (AML) management.[10] Commonly used in conjunction with anthracycline antibiotics such as daunorubicin or more recently idarubicin, Ara- C has been the mainstay of induction therapy for a variety of AMLs. The next big breakthrough in therapeutics was the discovery of the vinca alkaloid vincristine and its extraordinary efficacy in paediatric ALL when used in combination with prednisone.[11, 12] Discovery of the anthracycline antibiotics and the podophyllotoxins further complemented an already potent arsenal of drugs.

Realisation of the potential of combination chemotherapy in the early 1950's was due primarily to understanding of cellular metabolism.[13, 14] Encouraging results from experiments that used drugs to effect sequential and concurrent blocking of biochemical pathways lead to the initiation of more ambitious clinical trials.[14] The rationale for combination therapy has changed somewhat since then and drugs with different mechanisms of action have been used together to increase the potency of treatment, decrease the combined adverse effects as well as the possibility of developing multidrug resistance.[15, 16] Current treatment protocols often call for combinations of different chemotherapeutic agents at different stages of the disease so as to tailor the pharmacological profiles of the drugs to the unique growth dynamic and peculiarity of the leukaemia.

c. Supportive therapy

Significant improvement in the prognosis of leukaemic patients in the period between 1900- 1970 was also due to the introduction of various ancillary therapies. Advances in transfusion science allowed clinicians to provide "custom" haematological supports in the form of packed red blood cell or platelet transfusions. Early recognition of the relationship between thrombocytopaenia and bleeding diathesis in patients had lead to the clinical introduction of platelet transfusions, initially as whole blood and later as platelet concentrates.[17] Improvement in both purification and storage techniques also contributed to

the universal use of platelet transfusions in leukaemic patients suffering from thrombocytopaenia. However, early recognition of the link between thrombocytopaenia and bleeding diathesis did not lead to increased use of platelet replacements. The delay was mainly due to technical inexperience in the handling of platelets *ex vivo*. In addition, scepticism concerning the merits of the procedure also contributed to delays in its implementation in the clinics. Paradoxically, increased allo- transfusions of platelets lead to immunologically mediated- "transfusion refractoriness".[18] This problem was later ameliorated with the use of partially matched donors.

Ironically, adequate control of haemostasis by platelet transfusions accentuated the problem of infections related to the underlying disease or chemotherapy- induced neutropaenia. The role of neutrophils in nonspecific immunity was clearly established in the 1800's. Correction of neutropaenia not only decreases the incidence of life- threatening infections, it also permits the use of more aggressive chemotherapy. Strumia, in 1934, attempted to reinfuse leukocytes into a patient with unmitigated infection. He described the miraculous effects that "leukocyte cream", a dubious mixture of whole cell and cell debris, had on the patient.[19] Later, "surplus" neutrophils from chronic phase CML patients were used as supplements in neutropaenic patients, a rather adventurous form of replacement therapy.[20] Increased use of neutrophil transfusions exposed the recipient to the problem of cytomegalovirus (CMV) transmission, even from asymptomatic donors with latent CMV infections.[21, 22] Development of CMV infections in leukaemia patients is extremely problematic because of suppressed immunity caused by the disease and the cytotoxic treatments. The appropriate use of antimicrobial therapy later supplanted the use of neutrophil transfusions in patients. Gerald Bodey was an early advocate of the appropriate yet aggressive use of antibiotics in neutropaenic patients.[23] The emerging problem with fatal fungal infections in leukaemic patients mirrored that of bacterial infection. Again, the principal culprit was persistence of the neutropaenic state initiated by the leukaemia which was sustained by the ever more aggressive myelosuppressive chemotherapy regimens. A retrospective review conducted by Baker in 1962 demonstrated the increased incidence of fatal fungal infections and concluded that "some factor of modern therapy is responsible for the fatalities".[24] Liberal and invaluable use of indwelling catheters such as the Hickman line also contributed to the emergence of systemic infections. Amphotericin B was the drug of choice from a small arsenal of antifungal agents. Unfortunately, the side effects were

horrendous and patients often complained of the "bake and shake" effects of "amphoterrible".[25]

d. Bone marrow transplantation

Appreciation of the curative potential of BMT in humans began not long after the dawn of the atomic age. Fear of the consequences of radiation exposures; inadvertently from civilian accidents or deliberately and more sinisterly, as a result of military conflicts, perpetuated early research in human BMT.[26] In 1922 Fabricious- Moeller demonstrated, albeit obliquely, the principles of BMT. He showed that shielding of the legs during lethal irradiation protected guinea pigs from subsequent bone marrow aplasia.[27] Jacobson and colleagues repeated similar experiments in mice with splenic shielding; in addition, they observed that intraperitoneal injections of splenocytes also rescued the irradiated animals.[28] Meanwhile, Morrison and Samwick successfully treated a patient afflicted with idiopathic aplastic anaemia with sternal injections of donated bone marrow. They were, however, mistaken in their interpretation of the result in that they thought the donated marrow contained humoral factors that promoted the maturation of the diseased host haematopoietic compartments.[29] Recognition of a stable population of haematopoietic cells of donor origin or chimerism posttransplantation and its role in the protection of the lethally irradiated host further strengthened the theoretical foundation of BMT.[30] Additional animal experiments also helped to define the relatively novel concepts of graft- versus- host disease (GvHD), also known as secondary or runt disease, and immune tolerance.[31-33] However, it was not until much later that the human equivalents of the mouse major histocompatibility antigens H- 2 were identified and the concept of human leucocyte antigen (HLA) matching was introduced into clinical marrow transplantation.[34-36] In a landmark paper published in 1957, E. Donald Thomas and colleagues demonstrated the feasibility of clinical marrow transplantation in leukaemia and more importantly, they showed evidence of transient marrow engraftment in a patient.[37] With great prescience, the authors foresaw the use of BMT as a form of salvage regimen in high dose radiotherapy,

> "In selected patients with disseminated neoplasia it may be advantageous to use total- body radiation in large dosage and to cover the resultant aplasia by marrow transplantation";

they also predicted the increasing demand for this novel form of medical technology rivalling that of kidney transplantations.

Prior to 1968, allogeneic BMTs were performed without the benefits of HLA matching and the results were dismal. The rare cases of success were later ascribed to fortuitous HLA matching between the host and the sibling donor.[26] In 1968, a patient with congenital severe combined immunodeficiency disease (SCID) received marrow from a HLA- matched sibling, thus heralding in a new era in clinical marrow transplantation, one that is based on the rational selection of donors.[38] To compensate for the paucity of genotypically HLA- matched sibling donors, partly as a result of the limited size of the Western family and also a consequence of the enormous polymorphism of the HLA haplotypes - different matching criteria were used. Complex algorithms were developed for the identification of marrow donors, starting from the best to the least compatible match i.e. from a monozygotic twin donor to a HLA- matched unrelated donor (MUD), respectively.[39] Utilisation of less rigorously matched marrow donors necessitated the use of more efficacious immunosuppressive agents. Introduction of cyclosporin A and later, a more effective cyclosporin A and methotrexate combination significantly decreased the incidence of GvHD and hence improved the outcomes of allogeneic BMTs.[40, 41]

Development of new conditioning regimens not only reflected the paradigm shifts in BMT, but also the newly acquired knowledge in immunobiology and haematology. Originally intended for the rescue of victims of accidental radiation exposure, BMT was later adapted as a form of experimental therapy for endstage leukaemic patients. Subsequently, people with congenital or acquired deficiency of the haematopoietic system also benefited from BMT. The purpose of the preconditioning regimen is to achieve adequate immunosuppression of the host to effect graft acceptance, to "relieve" the marrow microenvironment of diseased host haematopoietic cells so that the donor cells can "take up residence"; and, in the case of a host with malignancy, to destroy a significant number of residual cancer cells.[42] The need for unique preconditioning regimens was evident from the different types of disease indicated above. For example, patients with Fanconi's anaemia, a form of congenital aplastic aplasia, are exceptionally sensitive to alkylating agents therefore

necessitating a less severe round of pretransplant conditioning.[43] The functional status of the host's immune system, in addition to the degree of donor- host disparity, also determines the intensity of the regimen. Obviously, a foetal host is different from an adult host with congenital immunodeficiency and is quite different from a host with an intact immune system, most likely a leukaemic patient. Early BMT recipients, with their restricted range of disease indications, were often preconditioned with radiation (mostly unintentional) or high doses of chemotherapeutic agents. Later, with allogeneic BMT assuming a more "proactive" role in the overall management of diseases such as congenital immunodeficiency, novel and often more effective conditioning regimens were developed. Single dose total body irradiation (TBI) was originally designed to suppress the immune system and ablate the host marrow; later, fractionated TBI was used to reduce extramedullary toxicity.[44] Cyclophosphamide (CY), a nitrogen mustard derivative, and antithymocyte globulin (ATG) are both potent immunosuppressants that do not appreciably ablate the marrow; therefore, regimens that utilise CY or ATG were successful only in patients with intrinsic stem cell dysfunction i.e. severe marrow aplasia. A transplant candidate suffering from T cell- mediated aplastic anaemia would require a regimen that both suppresses host immunity and ablates residual endogenous haematopoiesis. Since busulphan (Bu) has the capability to damage cells that reside in the G_0/G_1 phases of the cell cycle, i.e. the quiescent stem cell compartment, Bu along with TBI became the rational choice for marrow ablation.[42] In 1968, Santos and Haghshenass outlined a new regimen busulphan- cyclophosphamide (BuCy), which was subsequently used for the pretransplant conditioning of various types of diseases.[45, 46] In the same year, Bach and colleagues performed a successful allogeneic BMT on a paediatric patient suffering from Wiskott-Aldrich syndrome; donor marrow was obtained from a HLA- identical sibling.[47] Shortly thereafter, the first detailed study of allogeneic BMT in acute leukaemia was performed by the Fred Hutchinson group; HLA- matched marrows were infused into end stage leukaemic patients conditioned with CY and TBI followed by a two week anti- GvHD regimen of methotrexate.[48]

The history of leukaemia mirrors the progress of medicine in general; it evolved from an area of empirical observations and speculations to more substantive (and often more objective) investigations. Leukaemic therapy also improved from the early use of

alkylating agents to the development and incorporation of BMT into the overall management plan of the disease.

1.2 LEUKAEMIA : THE STATE OF THE ART

a. Clinical advances

Recent developments in the field of leukaemic research have not only significantly contributed to the understanding of the disease but also, more importantly, have led to improvements in both the prolongation and the quality of life of the leukaemic patient.

b. Diagnostics and therapeutics

i. Epidemiology & aetiology

Epidemiological studies of childhood leukaemia in geographical regions exposed to high levels of radioactive fallout from Chernobyl further illustrated the correlation between foetal radiation exposure and an increased incidence of leukaemia.[49] However, the contribution of previous paternal radiation exposure, which implicates mutations in the male germ cell compartment, to the increased incidence of childhood leukaemia is still controversial.[50] From retrospective studies of the occurrence of ALL in British children that were relocated during the Second World War, Mel Greaves proposed that common childhood infections promote leukaemogenesis of haematopoietic precursor cells that have received mutations (first hit but silent ?) *in utero*.[51, 52] The novel fusion gene products *BCR- ABL* and *PML- RARα* are implicated in the initiation and maintenance of chronic myelogenous leukaemia (CML) and acute promyelocytic leukaemia (APL), respectively. In fact, countless chromosomal abnormalities have been attributed to the different types of leukaemia.[4] Unfortunately, the role of many of these genomic events in the actual initiation and progression of leukaemia is not certain. Since leukaemia, and cancer in general, results from multiple genetic and sometimes epigenetic events, finding the proverbial "smoking gun" has proven to be difficult. One must appreciate the combined role of proto-oncogenes, tumour suppressor genes, and the environment in leukaemia pathogenesis.

However, there is little doubt that definitive identification of an aberrant genetic event in the initiation and maintenance of leukaemia provides a unique target for molecular therapy. Technical advances in molecular diagnostics permit the sensitive detection of genetic changes in leukaemic cells, which provide clinicians with invaluable leukaemia- specific markers for the purpose of detecting minimal residual disease.[53] Different mutagenic agents leave behind unique basepair alterations in the p53 tumour suppressor gene which afford the investigator with clues as to the identity the aetiologic agent, thereby providing an important tool in the emerging field of molecular epidemiology.[54] One has to realise that societal and policy changes often come as a result of the positive identification of leukaemogenic agents, especially in the face of "irrefutable scientific proofs". Therefore, the improved understanding of the molecular events that underlie the initiation and maintenance of leukaemia will not only translate into improved diagnostics and therapeutics in the clinic, but also the overall wellness of society.[55]

ii. Novel chemotherapeutics

The search for novel chemotherapeutic agents diverges into two paths: identification of naturally occurring antineoplastic agents and the chemical synthesis of potentially useful cytotoxic compounds. The majority of the anticancer drugs in current clinical use are naturally derived and are usually chemically modified to achieve reasonable pharmacokinetic and pharmacodynamic characteristics. Lately, significant developments in the field of combinatorial libraries have created large databases of potentially useful pharmacological agents.[56, 57] A foreseeable use of this technology in leukaemia therapy would be the production of a large number of compounds which could potentially interact with the signal transduction machinery of the leukaemic cell. Meydan and colleagues have shown that the tyrphostin analogue AG-490, synthesised using traditional chemical techniques, inhibited the growth of lymphoblastic leukaemic cells *in vitro* and *in vivo*. Importantly, the authors showed that the compound selectively inhibited the JAK2 protein tyrosine kinase, a downstream component of the cytokine receptor superfamily signalling cascade; JAK2 is shown to be elevated in relapsing lymphoblastic leukaemic cells, thus providing a rational molecular target for future investigations.[58] Several groups are

working on the development of other compounds which could inhibit the tyrosine kinase activity of the pathognomonic P210 *BCR- ABL* fusion protein in CML. Recently, Druker and colleagues showed that the 2- phenylaminopyrimidine- based compound CGP 57148 selectively inhibited the growth of leukaemic cells from CML patients with no deleterious effects on the colony formation of normal haematopoietic cells.[59] Research into the myriad signal transduction pathways of blood cells will hopefully identify more potential targets for pharmacological intervention.

Screening of naturally derived compounds still remains the backbone of chemotherapeutic drug discovery. Treatment of hairy cell leukaemia was significantly improved with the discovery of the new purine analogues such as 2'- deoxycoformycin, chlorodeoxyadenosine, and fludarabine.[60] Development of chemotherapy protocols based on all- trans retinoic acid (ATRA), in combination with traditional induction therapy, remains one of the major achievements of medical science in the late 20th century and illustrates that mutually beneficial cross talk exists between science and medicine.[61] Leo Sachs initially pioneered the theory that growth of some leukaemic clones could be successfully controlled with differentiation- inducing chemical compounds or cytokines.[62, 63] Later, Breitman and colleagues demonstrated that retinoic acid (RA) could induce the AML cell line HL- 60 to differentiate *in vitro*.[64] Subsequently, AML M3 or acute promyelocytic leukaemia (APL) became the obvious therapeutic candidate for RA- mediated differentiation therapy because of the cumulative *in vitro* data and the therapeutic urgency in its management. The use of oral ATRA successfully controlled life- threatening episodes of haemorrhagic diathesis of APL by forcing the differentiation of the leukaemic promyelocytes into endstage cells of the granulocyte lineage; clinicians also found that a "3+7" regimen of cytosine arabinoside /anthracycline following ATRA therapy was successful in maintaining the remission. Apparently, leukaemic promyelocytes contain vast amounts of tissue factor whose release leads to vicious cycles of clotting and thrombolysis, a condition known clinically as disseminated intravascular coagulopathy or DIC. Subsequent molecular studies demonstrated that the canonical t(15; 17) translocation of APL results in the fusion of the retinoic acid receptor α gene (*rar*α) with the *pml* gene. Ectopic cellular expression of the fusion protein *PML- RAR*α and inappropriate dimerisation with the normal binding partners of *RAR* explained partly the dysregulated growth of APL cells; however, the exact mechanisms of enforced differentiation of the

leukaemic cells by high dose ATRA is still not clear.[61] Reports from China suggested arsenic trioxide (As203) is effective in treating APL patients refractory to ATRA and conventional chemotherapeutic regimens; in addition, Chen and colleagues recently demonstrated that As203 promotes apoptotic cell death in APL cells via the downregulation of the antiapoptotic protein *BCL- 2* as well as modulation of the intracellular localisation of *PML- RARα* and *PML*.[65]

Recent developments in the area of tumour angiogenesis have lead to the discovery of positive and negative regulators of angiogenesis, as well as the appreciation of angiogenesis in tumour growth and metastasis.[66] Leukaemia has traditionally been viewed as "blood tumour" in which angiogenesis is irrelevant; however, many of the haematopoietic growth factors are angiogenic factors and vice versa.[67] Folkman and colleagues recently showed increased microvessel density in leukaemic bone marrow with the capillaries surrounded by cords of leukaemic cells, an observation commonly made in solid tumours; in addition, the levels of urinary basic fibroblastic growth factor, a potent inducer of angiogenesis, were elevated in all the leukaemic subjects studied (ALL).[68] This discovery has lead to clinical trials of the use of angiostatin, a potent angiogenesis inhibitor derived from proteolytic cleavage of fibrinogen, on paediatric ALL patients who have failed initial therapy.

iii. Antibody- based diagnostics & therapeutics

The concept of eradication of neoplasm by the specific targeting of cancer cells first evolved from Ehrlich's "magic bullet" theory of immune recognition. Initial optimism in antibody- directed immunotherapy of leukaemia was tempered by various scientific and technical problems.[69, 70] The majority of the problems associated with immunotherapy is due to the paucity of genuine leukaemia- specific antigens and subsequently the compromised choice of the target antigens. In addition, therapeutically effective eradication of minimal residual disease requires the expression of the target antigen on leukaemic stem cells. Fortunately, judicious selection of the disease and target antigen can sometimes circumvent the above problems. Normal expression of the B cell developmental antigens CD19 and CD20 are tightly regulated; both are not expressed on haematopoietic stem cells

and plasma cells. However, B cell lymphoma and B cell precursor leukaemia express abundant levels of the above two antigens, thus offering unique targets for immunotherapy without significantly affecting haematopoiesis and antibody secretion. Kaminski and colleagues demonstrated the efficacy of ^{131}I- antiB1 (CD20) radioimmunoconjugates on B cell lymphoma patients refractory to chemotherapy in a recent trial.[71] Another approach, using immunoconjugates of the tyrosine kinase inhibitor genistein- antiB4 (CD19), was successfully used by Uckun's group in abrogating the growth of lethal doses of human B cell precursor leukaemia, which constitutes 75% of all adult ALLs, in a SCID mouse model.[72] Using radioimmunoconjugates against the panhaematopoietic antigen CD45, a group at the Fred Hutchinson Cancer Research Centre was able to deplete BMT recipients of host lymphohaematopoietic cells for the purpose of pretransplant conditioning while sparing the radiosensitive organs such as the lungs.[73, 74] Identification of therapeutically useful monoclonal antibodies and the production of humanised mouse antibody will significantly improve the utility of *in vivo* immunotherapy.[75] Monoclonal antibodies have also been used therapeutically in the *ex vivo* manipulation of autologous or allogeneic bone marrow grafts for the purpose of leukaemic cell purging or the removal of GvHD-mediating donor T lymphocytes, respectively.[76] Regardless, widespread use of immunoconjugates in leukaemia treatment is far from reality.

Development of antibody- based diagnostics has made more of an impact in the overall clinical management of leukaemia, much more so than the antibody- based therapeutics discussed above. Haematopoietic maturation is accompanied by the appearance and disappearance of developmentally- restricted cell surface antigens. Leukaemogenesis from normal haematopoietic precursor cells means that leukaemic cells would share many of the lineage antigens present on normal cells, hence the difficulty in the specific targeting of leukaemic cells in therapeutics. Immunophenotyping of peripheral blood and bone marrow aspirates with panels of antibodies against lineage- specific antigens has dramatically improved the specificity of traditional morphologic and cytochemical diagnosis; for example, the definitive diagnosis of FAB M7 acute megakaryoblastic leukaemia using monoclonal antibodies that recognise the platelet antigens glycoprotein Ib and IIb- IIIa.[77, 78] In addition, antibody- based diagnostics also provide information with significant prognostic implications such as the identification of biphenotypic leukaemia, which coexpresses both myeloid and lymphoid markers and may require more aggressive

treatment.[79] Improved detection of leukaemic cells with flow cytometry also permits the sensitive detection of minimal residual disease during and posttherapy, which hopefully should translate into better clinical management of the patient.[80]

iv. Cell- mediated immunotherapy

Bacillus Calmette- Guérin (BCG) and other adjuvants such as *Corynebacterium Parvum* have been used experimentally as immunomodulatory agents for leukaemia, unfortunately with mixed results due to the nonspecific nature of the elicited immune response.[81-83] Several groups were actively involved in therapeutic trials in which patients were vaccinated with allogeneic leukaemic cells (live or irradiated), often coadministered with adjuvants.[84] However, realisations of the immense potential of cellular immunity in leukaemia therapy came only after publication of results from clinical trials conducted by Steven Rosenberg's group at the NCI. Lymphocytes isolated from the draining lymph nodes or biopsy samples of patients with solid tumours were stimulated *in vitro* with recombinant human interleukin- 2 (rhIL- 2). Reinjection of these activated lymphokine-activated killer (LAK) cells or tumour- infiltrating lymphocytes (TILs), often followed with *in vivo* administration of rhIL- 2, was found to have tremendous therapeutic effects on selected patient populations.[85, 86] Development of clinical protocols that take advantage of the above discoveries in leukaemia patients first came about in trials studying the efficacy of *in vivo* administration of rhIL- 2.[87-89] Surface MHC I presentation of antigenic peptides consisting of the fusion region of the translocation product on leukaemic cells, for example *BCR- ABL* in CML, presents the possibility of cell mediated antileukaemia response in the patient. Presence of fusion peptide- specific cytotoxic T lymphocytes (CTLs) has been demonstrated; however, the clinical significance of these cells remains ambiguous.[90, 91] Investigators soon realised that active and adoptive immunotherapy could be incorporated into BMTs for various malignancies. Handling of the autologous or allogeneic graft *ex vivo* offers unique opportunities for the generation of a leukaemia- specific immune response *in vitro* as a form of bone marrow purging in auBMT and *in* vivo to eradicate minimal residual

disease in both alBMT and auBMT patients postgraft reinfusion. These forms of immune manipulation will be discussed in a later section.

v. Murine models of human leukaemia

Basic immunological research principally focuses on the development and effector function of members of the lymphoid compartment. However, one can easily appreciate that information garnered from the study of the immune system can be applied to leukaemia research (and not just restricted to lymphoid leukaemia). One example is the development of the SCID- hu model of human lymphopoiesis by McCune's team.[92, 93] This concept was later extended to the study of human haematopoietic development in a murine host. The SCID- hu system is an invaluable tool for the study of human haematopoiesis because the available *in vitro* assays can only detect progenitor cells with limited proliferating and replating potentials; the murine system also offers an opportunity to test the *in vivo* effects of various cytokines and therapeutic protocols on human blood cell development.[94] The same system later allowed for the identification of a rare population of human myeloid leukaemic precursor cells that behave similarly in the immunocompromised murine hosts as in the original human patients.[95] The above examples illustrate the importance of the murine system for the study of human haematopoiesis; in addition, phylogenetic closeness between the two species means the sharing of genes, developmental pathways, and signalling pathways. Genetic alterations in transgenic mice and gene knockout mice provide numerous clues to the normal and abnormal functioning of the human haematopoietic system. Expression (increased or ectopic) or deletion of genes implicated in human leukaemias in the mouse will often recapitulate the human phenotype. Neoplastic development in *bcr- abl* transgenic mice and in mice reconstituted with haematopoietic stem cells infected with *bcr- abl* carrying retroviruses partially recapitulated the human phenotype.[96-98] However, the occasional unexpected results serve to remind the investigators of the complexity of the biological system and the fact that cancer often results from polygenetic cooperativity. Serendipitous discovery by Tak Mak's group of natural leukaemia resistance in the $TcRV_{\gamma}1.1J_{\gamma}4C_{\gamma}4$ transgenic mice will certainly spur additional

research into the role of the γδ T cell population in immune surveillance.[99] In a separate study, Tak Mak's group deleted the mouse equivalent of the gene coding for the human Hodgkin's Disease antigen CD30; the resulting impairment in lymphocyte cell death signalling and thymic deletion offers an intriguing glimpse into the normal regulatory role of this protein in the organism, and possibly its role in Hodgkin's Disease.[100] Recently, Holtschke and colleagues engineered a knockout mouse strain that faithfully recapitulates the human equivalent of CML.[101] Mice without functional interferon consensus binding sequence protein ($ICSBP^{-/-}$) develop acute leukaemia after an initial prodromal period characterised by benign neutrophilia. Numerous other examples also serve to illustrate the importance of the mouse model for the study of human leukaemia, either in its pathogenesis or in the search for different treatment modalities - *in vivo veritas*.

vi. Gene- based therapeutics

As mentioned in an earlier section, identification of leukaemia- specific genetic changes in patients offers unique targets for molecular therapy. Encouraging results in the development of antisense technology with oligodeoxynucleotides (ODNs), retroviral vectors, or ribozymes serve to propel the discipline forward. Examples include the specific eradication of *bcr- abl*$^{+}$ mRNA and the growth of *bcr- abl*$^{+}$ cells with junction- region specific antisense ODNs.[102] Similar results were obtained with retrovirus expressing antisense *bcr- abl* cDNA and ribozymes.[103, 104] Clearly, one can appreciate the potential applications of this technology in leukaemia therapy, either in bone marrow purging *in vitro* or the eradication of minimal residual disease *in vivo*. Recent technical advances in the design of nuclease- resistant nucleotides and of cationic liposome delivery systems enable greater latitude in antisense- based therapy.[105] The current system is restricted to the inhibition of mRNA transcription without affecting the source of the corrupted genetic code; however, the future of gene- specific therapeutics appears to lie with catalytic ODNs or ribozymes with the capability to effect site- specific DNA hence permanent corrections.[106]

c. Supportive therapy

i. G- CSF and GM- CSF support for chemotherapy- induced neutropaenia

Haematotrophic cytokines, particularly the colony stimulating factors (CSFs) in their current capacity are not considered to be true therapeutic agents. A notable exception is the successful application of α- interferon (IFNα) for the treatment of chronic phase CML and hairy cell leukaemia.[107, 108] CSFs essentially play an ancillary role in leukaemia management; principally in accelerating normal haematopoietic recovery postradiochemotherapy to reduce the incidence of opportunistic infections, thereby fulfilling the same function as Strumia's "leukocyte cream" more than fifty years ago. Utilisation of CSFs in leukaemia management poses the theoretical dilemma of the unintentional stimulation of leukaemic growth. *In vitro* studies have shown that many leukaemic cells upregulate the expression of both CSFs and their corresponding receptors.[109] CSFs are mitogenic for both primary leukaemic cells and cell lines *in vitro* and *in vivo*.[95] In addition, prolonged use of granulocyte- CSF (G- CSF) in patients with severe congenital neutropaenia (Kostmann's syndrome) may augment an already increased risk of AML.[110] A recent study revealed mutations in the domain responsible for the transmission of maturation signals in the G- CSF receptor gene and is implicated in the pathogenesis of congenital neutropaenia and AML, as well as the possible contributory role of therapeutic G- CSF in leukaemogenesis.[111] Nevertheless, at the present time the clinical benefits of exogenous G- CSF in congenital neutropaenia outweigh the elevated risk of subsequent AML development.[112]

Results from two recent multicentre, double- blind, randomised, placebo-controlled clinical trials questioned the efficacy of G- CSF and GM- CSF in elderly AML patients.[113, 114] Both studies showed that use of either cytokine did not lead to stimulation of leukaemic relapse; however, despite improvements in objective parameters such as shortening of the neutropaenic period, overall survival was not enhanced. Treatment-related failures (i.e. intolerance to chemotherapy) and possibly the presence of a biologically distinct form of AML remain the major problems that plague AML management in the elderly.[115] Unfortunately, 60% of AMLs occur in patients 60 years and older. Age-

related intolerance to chemotherapy also extends to the intense preparative regimen for alBMT and the subsequent episodes of GvHD; therefore, most centres refuse to perform alBMT on patients 55 years and older. One can clearly appreciate the urgency in identifying new agents that could protect the patient from the severity of leukaemia treatments.

ii. Infection and haemostasis control

Antibiotics are often used prophylactically or empirically to manage episodes of pyrexia of unknown origin in neutropaenic patients.[116, 117] Fungal infections have proven to be more recalcitrant to therapy; however, introduction of the less toxic fluconazole and liposomal amphotericin B should improve somewhat the clinical exigency.[118, 119] Itraconazole, another antifungal agent, was discovered to have the ability to reverse the multidrug resistance phenotype, possibly via steric interference of the membrane glycoprotein pump.[120] Careful selection of blood product donors contribute to the reduction of viral transmission; in addition, application of flow cytometry improved the rapidity of viral diagnosis and therefore treatment.[121] Nevertheless, infection still remains one of the thorniest problems in the management of leukaemic patients.[122] Limited availability of effective antifungal and antiviral agents has created significant interest in the use of intravenous gamma globulin as prophylaxis against infections, especially in the BMT setting.[123, 124] Recent advances in platelet storage techniques should enable the prolonged and safe storage of platelets.[125] However, the most important development in haemostatic research was the successful identification and cloning of the human platelet growth factor or thrombopoietin (TPO).[126] Preclinical research has demonstrated that TPO, often in combination with other cytokines, has significant mitogenic effects on megakaryocytes and the resultant platelet counts; clearly desirable in patients receiving myelosuppressive chemotherapy.[127]

Understanding of leukaemia and the clinical management of the disease has come a long way since Osler's era. However, except for selected diseases such as paediatric ALL or APL, overall survival of the leukaemic patient is still fairly dismal. Technological advances in molecular diagnosis does not necessarily translate into better outcome.

Nowadays, clinicians have knowledge of the exact location of the chromosomal translocation or the type of basepair alteration. Unfortunately, the majority of treatments are still archaic involving various cytotoxic substances with little selectivity; not much different from Osler's prescription of "clean air and absence of worry" for leukaemic patients. The next hurdle in disease management will be the translation of knowledge and skill gained in molecular diagnosis to molecular therapeutics - i.e. targeting and the corrections of the genetic lesions.

1.3 TRANSPLANTATIONS OF HAEMATOPOIETIC STEM CELLS: CURRENT STATUS

Intravenous infusion of haematopoietic progenitor and stem cells (HPCs & HSCs) into a patient with damaged or defective bone marrow is already an established form of treatment for several benign and malignant haematological conditions. Cells can be harvested from the marrow cavities of the donor, hence the term bone marrow transplantation (BMT); in peripheral blood stem cell transplantation (PBSCT), circulating stem and progenitor cells are harvested from the patient (autologous) or an allogeneic donor through apheresis, an extracorporeal process which physically separates blood into its cellular components. Placental and umbilical cord blood represents a third source of stem cells with unique properties which appear to permit greater margins in donor: recipient HLA disparity. The following section will focus on recent advances in stem cell transplantation.

a. Bone Marrow Transplantation (BMT)

i. Allogeneic bone marrow transplantation (alBMT)

In brief, successful engraftment of allogeneic haematopoietic cells in a foreign host requires a *modus vivendi* between the graft and the host. This is aided by the careful selection of donors based on the matching of major and even minor histocompatibility antigen loci as well as the effective posttransplantation management of the patient in the form of adequate immunosuppression with vigilant infection control and clinical precautions.

1. HLA matching

Limitation in the size of the optimal marrow donor pool i.e. human leukocyte antigen or HLA matched siblings drives the search for other sources of stem cells. Less desirable donors include HLA matched unrelated donors (MUDs) and related donors with partial HLA matches. The science of HLA matching has progressed from phenotyping through the use of the mixed lymphocyte reaction (MLR) and serology to the more precise method of genotyping using the polymerase chain reaction (PCR). Accurate matching of donors and recipients should decrease the incidence of graft rejection and of graft- versus-host disease (GvHD); however, the paucity of potential well- matched donors often necessitates the use of marrows from the suboptimal donor pools mentioned above. DNA typing of HLA genes using analytical techniques such as DNA restriction fragment length polymorphism (RFLP), PCR- sequence- specific oligonucleotide probe typing, and PCR 'fingerprinting' played significant roles in improving the outcome of MUD transplantations by optimising the HLA matching between unrelated donors and recipients.[39] Creation of the national and global registries of volunteer bone marrow donors facilitates the search for potential MUD donors. Nonetheless, GvHD and graft rejection will remain a fact of life in alBMT regardless of the improvements made in HLA matching technology simply because of the enormous polymorphism of the HLA loci and consequently the scarcity of perfectly matched donors. Therefore, improvement of the outcome of alBMT requires developments

of novel immunosuppressive regimens and patient management techniques such as the effective control of infections, in addition to better HLA matching technology.

2. Prevention and the management of graft- versus- host disease (GvHD)

Cyclosporin A (CYA), the venerable antirejection drug, revolutionised the management of graft/ host HLA discordance in BMT. However, CYA is nephrotoxic and requires frequent drug level monitoring. The novel microemulsion formulation of CYA appears to improve the pharmacokinetics of the drug in patients, therefore a lower drug dose is required to maintain the same level of immunosuppression.[128] Several ongoing alBMT trials are being conducted to compare the efficacy of CYA with the macrolide antibiotic FK506, an immunosuppressive agent with a relatively successful history in liver transplantations.[129, 130] Rapamycin (RAPA), another naturally derived macrolide- based immunosuppressant, binds to the same molecular target as FK506 yet mediates different downstream signalling pathways. Preliminary murine studies indicated the potential of RAPA in MHC- mismatched BMT; however, this agent has several unique immunological effects which warrant additional studies prior to any clinical testing on patients.[131] *Ex vivo* T cell depletion (TCD) using α– CD3 (OKT3) or α– CD52 (Campath1) monoclonal antibodies is an effective method of preventing acute GvHD by removing donor T lymphocytes. However, this often comes with the price of increased incidences of leukaemia relapse and graft failure.[132, 133] The expression of other T cell- specific antigens are also exploited in antibody- mediated *ex vivo* and *in vivo* TCD; in fact, several trials have combined both modalities of TCD in the hope of improving the clinical outcome, especially of MUD transplantations. Monoclonal antibodies specific for the TcR αβ chain, LFA1, CD2, and CD5 have all been used singly or in different combinations in TCD.[134-136] *Ex vivo* TCD is typically accomplished by antibody- complement mediated lysis of the target cells while *in vivo* TCD is often carried out by antibody- toxin conjugates. Nonetheless, the clinician must realise that T cells mediate proper engraftment of the donor stem cells, immune surveillance of leukaemic relapses (GvL) as well as viral- associated lymphoproliferative disorders, and unfortunately also GvHD.[137] The graft- versus-

leukaemia effect (GvL) is mediated by donor T cells which recognise residual leukaemic cells in the patient. Studies have demonstrated that GvL is responsible for the lower rates of leukaemic relapses in patients receiving alBMT; in addition, reinfusions of donor- derived "buffy- coat" containing lymphocytes have been shown to suppress leukaemic relapse post-alBMT in CML patients.[138]. Therefore, overzealous and injudicious use of TCD will affect not only the final outcome of the graft but ultimately the welfare of the patient.[139] Better definition of the T cell subset(s) responsible for GvHD should help in the selective removal of this population without affecting the others.[140]

Chronic GvHD, a different form of graft/host conflict than acute GvHD, can still occur despite pharmacological control of the latter; chronic GvHD manifests itself as a multisystem disorder which resembles the collagen vascular diseases.[141] Prolongation of the survival of the transplantation recipient highlights the problems associated with this disorder. Management of chronic GvHD with thalidomide offers an attractive alternative to the traditional regimen of prednisolone and azathiaprine because of the former's effectiveness in treating skin chronic GvHD without myelotoxicity.[142]

3. Identification of minor histocompatibility antigens (mHC)

The minor histocompatibility antigens (mHC) denote transplantation antigens which are not encoded by the major histocompatibility antigen loci (HLA in the human and H- 2 in the mouse). Polymorphisms of these genes in the population contribute to their roles as alloreactive antigens in organ transplantations. The most obvious mHC is the sex- linked male antigen H- Y, which elicits alloreactive T cell responses in male patients receiving MHC- matched female grafts.[143] Since only HLA matching is performed in clinical alBMT, GvHD often can result from mismatches in the mHC even in transplantations between HLA- identical individuals. Recently, two reports highlighted the importance of the non- sex- linked mHC in clinical alBMTs. Goulmy and colleagues demonstrated the importance of HA- 1 matching in HLA- matched alBMT.[144] Interestingly, the authors showed that mismatches in HA- 1 contributes to GvHD only in donors and recipients positive for HLA- A2; therefore, a complex interplay exists between mHC (simple "protein

antigens" presented by the MHC) and the MHC (alloantigens, protein antigens, and binding partners of potential mHC), therefore not all mHCs contribute to GvHD. In another study, direct sequencing of the complementary DNA of the CD31 (PECAM- 1) adhesion molecule revealed a single polymorphism in normal subjects; further investigation in alBMT patients who received bone marrow cells from HLA- identical sibling donors revealed a positive correlation between donor: recipient CD31 polymorphism and the incidence of GvHD.[145]

Unfortunately, the insufficiency of HLA- matched donors precludes the routine matching of mHC in actual clinical practice. However, as indicated in an accompanying editorial, mHC matching may be beneficial in situations where more than one HLA-identical donor is available or when the degree of mHC disparity can be used to tailor the extent of the immunoprophylaxis for the patient.[146]

4. Novel indications for alBMT in nonmalignant and malignant disorders

A recent multicentre trial demonstrated the curative potential of alBMT in paediatric patients symptomatic for sickle cells anaemia (SCA).[147] The authors showed that alBMT was able to halt the various symptoms of SCA and to abolish the need for RBC transfusions. Similarly, patients suffering from another benign haematological disorder, β-thalassaemia, also benefited from alBMT.[148] AlBMT, when used responsibly, is able to improve the quality of life in patients suffering from various forms of benign haematological disorders.[149] This however will increase the demand for an already scarce resource and will probably provide an impetus for utilising novel sources of stem cells such as umbilical cord blood and mobilised peripheral blood stem cells. Finally, several groups are actively pursuing the use of alBMT as a form of adoptive immunotherapy; infusion of donor leukocytes for leukaemic relapse control post- alBMT and the transfer of tumour antigen specific immunity from an immunised allogeneic donor are two such examples.[150]

ii. Autologous bone marrow transplantation (auBMT)

> "waiting for a MUD is like waiting for Godot"- Sometimes, a patient becomes an unwitting player in Beckett's play.[Golstone, 1993 #610]

Autologous BMT (auBMT) offers the only hope for elderly patients or without HLA- compatible donors; elderly patients are especially susceptible to the deleterious effects of GvHD associated with alBMT and therefore most centres do not perform the procedure in patients more than 50 years old.[151, 152] Theoretically, bone marrow harvest should be performed when the probability of neoplastic cell contamination is at its lowest i.e. during remission. This period usually follows successful intensive chemotherapy, i.e. at first complete remission postinduction therapy in AML and during chronic phase in CML. The patient then receives high- dose chemotherapy and radiation therapy followed by reinfusion of the harvested marrow cells. The lack of GvHD in auBMT unfortunately means the absence of the GvL effect; therefore, the higher rate of leukaemic relapses essentially balances out the lower incidence of BMT procedure- related mortality (i.e. GvHD) in auBMT patients.[152] In addition, reinfusion of contaminating tumour cells also increase the chance of relapse. Two independent studies, indirectly and directly, demonstrated the contributory role of unpurged autografts in subsequent relapses. The tumour- specific translocation t(14; 18), which joins together the *bcl- 2* gene with the immunoglobulin heavy chain promoter, is common to a majority of follicular non-Hodgkin's lymphoma (NHL) and 30% of B- cell NHL and allows the use of PCR to detect rare lymphoma cells. PCR positivity of the reinfused purged autograft correlated with the incidence of subsequent lymphoma relapse.[153] Brenner and colleagues unambiguously and directly demonstrated, via *ex vivo* retroviral marking of the harvested product, that neoplastic cells in the reinfused graft contribute to subsequent relapses in AML and neuroblastoma patients.[154, 155] However, this does not discount the presence of endogenous neoplastic cells that have survived the conditioning regimen. Several groups have tried to improve the odds of auBMT by the administration of low dose cyclosporin A, which appears to disrupt immune tolerance and thereby provokes a form of GvL but also GvHD in the patient.[156]

1. auBMT and chronic myelogenous leukaemia (CML)

AlBMT is currently the sole curative regimen for chronic myelogenous leukaemia (CML) possibly because of the role of GvL.[140] CML can strike at all ages yet the median age of incidence is 50 years, which renders most patients unsuitable for alBMT for reasons discussed above.[157] Therefore, intensive chemotherapy coupled with autologous bone marrow rescue becomes an alternative regimen for those patients in which alBMT is not indicated.[158, 159] Contamination of the harvested marrow by CML cells and the absence of GvL effects combine to increase the incidence of relapse in patients receiving unpurged autologous marrow grafts.[160] Presence of the unique molecular lesion *bcr- abl* in CML cells allows clinicians to assess the extent of minimal residual disease (MRD) in the patient's marrow and circulation using sensitive reverse transcriptase- polymerase chain reaction (RT- PCR), which can detect a single *bcr- abl*$^{+}$ cell in 10^5 to 10^6 normal cells.[161] Newer quantitative RT- PCR (qRT- PCR) methodology based on the competitive amplification of exogenous competitor templates permits the quantitation of *in vivo* MRD or the degree of autologous graft contamination as well as the efficacy of the graft purging procedure. Serial monitoring of the patient posttransplantation using qRT- PCR facilitates the early detection of relapse.[162] However, Miyamura and colleagues have shown that *bcr-abl* PCR positivity post- alBMT does not necessary correlate with clinical relapses.[163] Therefore, clinical remission may exist as a state of equilibrium between the residual leukaemic cells and the immune system.[164] Regardless, PCR technology allows for the rapid evaluation of *ex vivo* purging efficacy and is invaluable for the comparison of the different purging systems.

Without significant improvements in the management of GvHD and the development of other forms of therapies, auBMT will remain a feasible alternative in leukaemia treatment. Development of novel *ex vivo* techniques such as leukaemic cell purging and immune modulation will make auBMT a more viable option than alBMT.[165, 166]

b. Peripheral blood stem cell transplantations

Peripheral blood stem cell (PBSC) transplantation can be performed in either autologous or allogeneic setting. Advantages and problems associated with alBMT and auBMT are also applicable respectively to allogeneic and autologous PBSC grafts. However, transplantation of PBSCs in general has several advantages over BMT - the former procedure does not require general anaesthesia and PBSC grafts mediate faster haematological recovery than marrow grafts, possibly because of an abundance of committed progenitor cells in the circulation.[167, 168] The procedure is especially useful in paediatric patients because of the risks associated with bone marrow harvests in children.[169] The harvesting of PBSC can be accomplished by leukapheresis, an extracorporeal procedure in which the person' s blood is circulated through a blood cell separator such as the Fenwall 3000CS and cells within a certain range of specific density (mainly mononuclear cells) are isolated via centrifugal elutriation. Lymphocytes and monocytes are collected along with the committed progenitor and stem cells, which can be selected positively using monoclonal antibody specific for the haematopoietic stem cell antigen CD34. Most of the experience in PBSC transplantation has been with autologous grafts; however, allogeneic PBSC transplantation is gaining more acceptance simply because of the relative ease of the harvesting procedure. "Mobilisation" is necessary to increase the number of stem and progenitor cells in the circulation prior to the actual harvests.

Recent advances in the functional characterisation of PBSC and the development of novel mobilisation and stem cell expansion protocols have established PBSC transplantation as an integral part of the standard treatment regimen in oncology. For example, flow cytometric analysis of $CD34^+$ cells in the PBSC harvests enables direct and rapid enumeration of progenitor and stem cells which provides an index of the reconstitutive potential of the harvested product without resorting to the labour- intensive and time- consuming biological assays (CFU- GM assays, delta assays, and long- term culture- initiating cell assays).[170] Furthermore, Brugger and colleagues recently showed that *ex vivo* expansion of positively selected $CD34^+$ cells from a single session of leukapheresis was sufficient to effect rapid and sustained haematopoietic engraftment.[171] The above findings coupled with enhanced design of the cell separator and better

haemodynamic support during leukapheresis significantly increased the universality of PBSC transplantation.

i. Autologous transplantation of PBSC in cancer patients

PBSC transplantation in this setting bypasses problems associated with the autologous marrow such as inadequate specimen size due to marrow fibrosis (from pelvic irradiation or disease process) and tumour contamination of the marrow space. To obtain enough PBSC, mobilisation of the oncology patient can be accomplished by myeloablative chemotherapy in conjunction with the administration of recombinant haematopoietic growth factors such as G- CSF. For obvious reasons, only growth factors are used for allogeneic donor mobilisation. This procedure increases the number of PBSC to a level which is practical for harvesting; usually three leukapheresis sessions are required to obtain the $2X10^6$ $CD34^+$ PBSC/kg of recipient needed for adequate haematological engraftment.[172] Clinicians, however, must be vigilant in dealing with mobilised autologous harvests from these patients because the operation also increases the circulatory tumour load.[173, 174] Therefore, additional steps such as $CD34^+$ positive selection coupled with tumour cell purging are necessary in autologous PBSC transplantations involving leukaemic patients; whereas $CD34^+$ positive selection alone would suffice for patients with solid tumours because of the absence of CD34 expression on these tumour cells.[175-177] In allogeneic PBSC transplantation, positive selection of $CD34^+$ cells also reduces the possibility of reinfusion of donor lymphocytes, which can contribute to GvHD.[178]

c. Umbilical cord blood transplantation

Human umbilical cord blood (HUC), normally discarded after delivery of the foetus, is an unique source of haematopoietic stem and progenitor cells for transplantation. Most studies have focused on HUC transplantations in the paediatric setting because of the fear of insufficient cells for adult recipients, even though HUC contains approximately the same concentration of stem cells as adult bone marrow but only at a typical harvest volume

of 100 ml.[179] The unique developmental state or immunological naiveté of the HUCs endows them with the capability to engraft across disparate HLA barriers without resulting in significant GvHD.[180] HUCs also appear to be unusually robust, possibly due to their developmental status, in their ability to effect haematopoietic engraftment; therefore adult patients may benefit from HUC transplantations.[181] Standardisation of HUC collection, storage, and characterisation should increase the use of this form of stem cell transplantation.[182]

d. Future directions

The raison d' etre of stem cell rescue in leukaemia therapy is the reconstitution of the haematopoietic system damaged by the high dose chemotherapeutic agents and radiation treatment needed to eradicate leukaemic cells from the host. The relative nonselectivity of the current generation of treatments results in "collateral" damage to normal tissues such as cells of the haematopoietic system and other rapidly dividing cells. Development of more specific antineoplastic agents such as gene- based or signalling pathway specific antagonists will make this particular indication redundant.[152] In the future, stem cell transplantation probably will be used for the correction of intrinsic genetic defects or in customised modifications to the host immune system.

1.4 EXPLOITABLE DIFFERENCES BETWEEN NORMAL AND LEUKAEMIC CELLS

a. Leukaemogenesis

Oncogenesis in cells of haematopoietic origin is often a multistep event and involves the acquisition of oncogenic changes as well as the loss or inactivation of normal antiproliferative control mechanisms.[183, 184] Animal viruses, especially those belonging to the family of retroviridae, can cause leukaemia in a variety of mammalian hosts. Recently,

Gross speculated that dormant oncogenic viruses may play a even bigger role in animal leukaemogenesis than previously thought.[185] Some oncogenic events are so potent that no other genetic changes are necessary for leukaemic transformation; however, successful evolution of the leukaemic clone in the host often needs more than one genetic "hit" in the guise of additional oncogenic changes or abrogation of normal growth control.[186]

Biochemical and physiological differences between normal and leukaemic cells form the basis of antileukaemic therapy; however, the differences can be quite subtle since leukaemogenesis arises from normal haematopoietic precursors. The therapeutic window of opportunity between normal and malignant cells relies on the differential sensitivity to treatment which results from a range of dissimilarities.[187] The following is a brief description of selected leukaemia- specific changes resulting from the *bcr- abl* translocation in CML, and their implications in therapy.

b. Genetic differences

Gene- based differences form the foundation of subsequent deviations in signal transduction, adhesion properties, and response to extracellular stimuli in leukaemic cells.[55] Acquisition of transforming oncogenes for example, through point mutations and chromosomal translocations, in conjunction with the loss of tumour suppressor genes conceive the script for the ensuing leukaemic transformation. These changes, which are absent in normal cells, are responsible for the initiation as well as maintenance of the leukaemic phenotype. Mentioned previously, *bcr- abl* and *pml- rarα* are respectively unique to CML and APL; both translocation events result in a plethora of changes which endow the cells with growth autonomy. Absence of oncogenic changes in normal haematopoietic cells therefore allows specific targeting of the leukaemic clone.

Complementary binding of specific antisense oligodeoxynucleotides (ODNs) or ribozymes can target leukaemic- specific sequences at the mRNA level.[105] Szczylik and colleagues have demonstrated the specificity of the *bcr- abl* junction- region antisense ODN in the inhibition of *bcr- abl*$^+$ cells by both RT- PCR as well as colony assays.[102] Therefore, identification of genetic aberrations which are pathognomonic for leukaemia remains important in not only the diagnosis, but also the treatment of the disease.

c. Differences in intracellular signalling and cell death pathways

Signal transduction is paramount in the conveyance of extracellular information to the nucleus or executive centre of the cell; it is also important in the regulation of cellular development. Aberrant signal transduction events can lead to dysregulated growth or oncogenesis, therefore such pathways have been deemed appropriate targets in cancer therapy.[188] Tyrosine phosphorylation, which consists of less than 2% of total cellular protein phosphorylation, plays an extremely important role in the mediation of cellular signalling. Dysregulation of the expression and regulation of protein tyrosine kinases (PTK) can result in catastrophic changes in cells and the organism.[189] For example, genetic knockout of the *CSK* tyrosine kinase in mice, which negatively regulates the *SRC* PTK, results in substantial upregulation of *SRC* kinase activity. A phenotype of embryonic lethality with significant neural tube defects was elicited from the *CSK* knockout mice.[190] The *BCR- ABL* fusion protein in CML has significantly upregulated tyrosine kinase activity in comparison to the wild- type *ABL* protein in normal haematopoietic cells.[191] In addition, *BCR- ABL* localises to the cytoplasm whereas wild- type *ABL* expression is restricted to the nucleus where it interacts with growth regulatory proteins such as the retinoblastoma protein.[192, 193]. Since tyrosine kinase activation of *BCR- ABL* appears to be crucial in the initiation and maintenance of the leukaemic phenotype in CML, signal transduction events mediated by *BCR- ABL* therefore become logical targets for therapeutic interventions.[194] Several examples of the successful use of tyrosine kinase inhibitors have been mentioned previously.[58, 59]

Transformed cells, in addition to changes which promote proliferation and growth autonomy, also are deficient in regulatory pathways resulting in resistance to normal homeostatic controls. For example, loss of the wild- type p53 phenotype results in the abrogation of intrinsic senescence control and sensitivity to apoptosis- inducing anticancer agents.[195, 196] Restoration of the wild- type p53 phenotype, via retrovirus- or adenovirus-mediated gene transfer, can therefore increase sensitivity to chemotherapeutic agents or effect cellular differentiation.

d. Response to extracellular regulatory signals

Leukaemic cells exhibit heightened responses to cytokine stimulation or may even become independent of survival or mitogenic factors. *BCR- ABL* endows cytokine independence to the interleukin- 3 (IL- 3) dependent cell line FDCP- 1 by substituting for normal signal transduction events mediated by IL- 3 binding to its receptor; in addition, *BCR- ABL*, via its Src- homology 2 domain (SH2), induces the secretion of IL- 3 to effect paracrine stimulation.[197, 198] Furthermore, Shuai and colleagues have demonstrated that STAT5, a cytoplasmic protein which mediates GM- CSF signalling, is constitutively activated by *BCR- ABL* in the absence of GM- CSF.[199] Therefore, *BCR-* ABL, through its exaggerated PTK activity and the presence of various adapter or binding regions such as the SH2 and SH3 domains, can substitute for mitogenic and survival cytokines which normally maintain homeostasis.

At the other extreme, evidence has shown that leukaemic cells do not respond to the growth inhibitory effects of a family of cytokines loosely termed haematopoietic or stem cell inhibitors.[200] One such molecule, the macrophage inflammatory protein- 1α (MIP- 1α), is a member of the chemokine family but was originally characterised as an inhibitor of haematopoietic stem cell proliferation.[201, 202] Eaves and colleagues reported that CML cells are unresponsive to the regulatory effects of MIP- 1α.[203] Another example is the lack of response to the tetrapeptide N-Acetyl- Ser- Asp- Lys- Pro (N-AcSDKP) by CML but not by normal haematopoietic cells.[204] N-AcSDKP and pyroGlu- Glu- Asp- Cys- Lys (pEEDCK) belong to a new class of low molecular weight haematopoietic inhibitors which block cell cycle progression from the quiescent or G_0 phase; normally, quiescent cells are not susceptible to the cytotoxic effects of cycle- specific chemotherapeutic agents and to ionising radiation.[205] Differential sensitivity to the above molecules can be exploited in therapeutics. Selective cycle inhibition of normal cells by AcSDKP protects them from chemotherapy, hyperthermia, radiation, as well as PDT treatments.[206-209] Selective inhibition of normal haematopoietic cell cycling by N-AcSDKP, pEEDCK, and MIP- 1α should permit dose intensification in the *in vivo* treatment of leukaemia patients or during *ex vivo* purging.[210]

e. Cell surface markers, adhesion properties and the haematopoietic microenvironment

Haematopoiesis is a tightly regulated process and depends a great deal on the physical interactions between the haematopoietic cells and the stromal element in the bone marrow. This is controlled by the expression and activity of multiple adhesion molecules and their counter receptors. Engagement of selected receptors on the surface of progenitor cells and stromal ligands results in the transduction of growth inhibitory signals.[211] Gordon and colleagues first demonstrated that CML progenitor cells exhibit attenuated adhesion to the marrow stroma, even though CML progenitors express similar numbers of integrin adhesion receptors as than normal counterparts.[212, 213] Subsequent studies showed that treatment with interferon- α (IFN- α) restores normal β1 integrin- dependent adhesion in CML progenitor cells, possibly via mediation by MIP- 1α.[214, 215] Reestablishment of stromal contact and transduction of growth inhibitory signals by IFN- α may explain its clinical effects in CML management.[108] Since PTKs are implicated in the mediation of integrin signalling, abnormality in PTK activation could therefore affect normal cellular responses to integrin- mediated cell adhesion.[216] In a recent review article, Verfaillie and colleagues speculated the *BCR- ABL* fusion protein in CML interfered with integrin- mediated signal transduction events and brings about abnormal adhesion, trafficking and as well as proliferation observed in CML.[213]

f. Susceptibility to cytotoxic agents

In theory, the sum of the above differences should lead to a comfortable therapeutic window of opportunity in leukaemia treatment. Unfortunately, most practical experience in therapy does not give credence to the scientific evidence. In addition, most neoplastic cells have evolved sophisticated protective mechanisms that confer resistance to various radiochemotherapeutic regimens. For example, expression of the P170 ATP- dependent membrane glycoprotein pump, encoded by the *mdr1* gene, is significantly increased in cells which express the multidrug resistance (MDR) phenotype.[217] In one study of adult acute leukaemias, heightened *mdr1* expression was documented in 50% of leukaemic relapses but only 19% of newly diagnosed leukaemia; in addition, an inverse correlation was made

between the complete remission rate and the amount of *mdr1* expression.[218] Successful treatment of leukaemia will therefore depend on the application of newly acquired knowledge in the molecular biology of leukaemia to the clinic.[219]

1.5 PURGING IN AUTOLOGOUS STEM CELL TRANSPLANTATIONS

a. Introduction

Bone marrow purging (BMP) is deemed beneficial in certain situations involving the reinfusion of autologous marrow cells because of contamination of the harvested product by neoplastic cells.[220] Purging assumes that exploitable differences exist between normal and leukaemic cells such that selectivity can be ensured in the removal or destruction of the undesirable population. Clinically effective BMP presents a challenging dilemma because the clinician must achieve a balance between sufficient eradication of the leukaemic cells with preservation of enough of the normal cells for haematopoietic reconstitution. Overzealous purging will lead to eventual graft failure or a delay in engraftment; on the other hand, inadequate purging may contribute to leukaemic relapses in the patient. BMP has been studied extensively in CML autografts and provides an excellent model system for the study of the different purging strategies because of the availability of *bcr- abl*$^{+}$ cell lines and the sensitivity of the PCR detection assay.[221, 222] Accumulating evidence also points to the effectiveness of BMP in AML patients receiving autografts. A report from a recent conference showed significant survival advantage in patients receiving purged compared to unpurged autografts and led the coauthor of the report, Elizabeth J. Shpall to declare "These results suggest that autotransplantation of acute myelogenous leukaemia should use purged bone marrow for restoration of blood production".[223] Lastly, purging is also being considered for autologous PBSC transplantations in order to reduce the level of tumour cell contamination of the mobilised leukapheresed product.[224]

b. Purging strategies

The four chief modalities of purging; physical, immunological, biological, and pharmacological, are used singly or in combinations to effect clinically beneficial results.[222, 225] Differences in biophysical characteristics are exploited in physical purging. Examples include counterflow elutriation, density gradient separation, filtration, and heating. For example, clusters of neuroblastoma cells can be removed from the autograft via filtration through a 40μm multilayer mesh filter which results in the elimination of 0.5 log of the cancer cells. Several groups have discovered that leukaemic cell lines are much more susceptible to hyperthermia than normal haematopoietic cells.[226, 227]

Surface expression of differentiation markers permit recognition of cells by antibodies. However, as mentioned earlier, leukaemic and normal haematopoietic cells share many of the same markers since the former population derives from neoplastic transformation of the latter.[228] For example, many AML cells and essentially all AML stem cells express the haematopoietic stem cell antigen CD34; therefore, procedures which involve only the positive selection of $CD34^+$ cells are applicable solely for solid nonhaematopoietic tumours, which do not express CD34. Nevertheless, several commercial ventures have been started to take advantage of CD34 positive selection technology in the *ex vivo* manipulation of stem cells. CD34- specific monoclonal antibodies conjugated to a solid- phase support, either in the form of ferromagnetic beads or the surface of tissue culture flasks, are in various stages of clinical trials for stem cell purification and such approaches afford promise for conditions like neuroblastoma and breast cancer.[229] Positive selection of $CD34^+$ cells from a leukaemic sample will therefore require additional steps such as negative selection or pharmacological purging to eradicate CD34- expressing leukaemic cells. Negative selection based on the expression of mature lineage markers provides greater latitude in purging since normal committed progenitor cells and haematopoietic stem cells do not express such markers. For example, presence of CD2, 3, 5, 7, and CD11a on leukaemic cells targets them for destruction by immunotoxins and radioisotope immunoconjugates, without significant reduction in the viability and clonogenicity of normal haematopoietic cells.[69, 70] Biological purging operates on the basis that normal and leukaemic cells behave differently in culture due to unique expression profiles of haematopoietic growth factors, their receptors, as well as adhesion molecules. Scheffold and associates showed that CML- specific cytokine- induced killer cells can be

generated from the peripheral circulation of CML patients; furthermore, they showed that these $CD56^+$ cells can be used as a form of bone marrow purging in CML.[230] Growth kinetics in response to cytokines also differentiate normal cells from their malignant counterpart, as mentioned in a previous section. *In vitro* culture of bone marrow cells harvested from selected CML patients resulted in the gradual disappearance of Philadelphia-chromosome positive leukaemic cells with a concomitant expansion of normal haematopoietic cells.[231, 232] Established chemotherapeutic agents such as 4-hydroperoxycyclophosphamide (4- HC) and its congenor mafosfamide (or ASTA- Z®) are both analogues of the commonly used alkylating agent cyclophosphamide; the two former drugs have been modified such that *in vivo* hepatic activation is not required. ASTA- Z® and 4- HC remain the two most commonly used pharmacological purging agents.[233-235] In addition, other chemotherapeutic agents are also being considered for *ex vivo* purging.[236] Alkyl- lysophospholipids, analogoues of the platelet- activating factors, also appear to be promising candidates in various purging applications.[237]

c. Combination purging

As shown in clinical pharmacology, combination therapy in cancer management is often superior to the single drug approach. Therefore, research in purging which combines the different modalities, such as immunological and pharmacological purging, or different drugs, such as mafosfamide with etoposide, are being actively pursued.[225] The *ex vivo* nature of the treatment affords the clinician with greater latitude of graft manipulation without much fear of systemic toxicity; the singular goal of purging is to effect maximal log reduction of the contaminating neoplastic cells while preserving an adequate number of normal haematopoietic cells for rapid yet durable engraftment. Other examples of combination purging include combinations of 4- HC or ASTA- Z® with antibody-mediated- complement lysis, immunotoxins, and *bcr- abl* junction specific antisense oligodeoxynucleotide (ODN).[238-240]

d. Purging outside the traditional paradigm

Novel purging modalities are constantly being developed to exploit the preferential killing of leukaemic cells. Gene- based strategies are used to target leukaemia- specific oncogenes such as the *bcr- abl* translocation sequence in CML. Some examples include the *bcr- abl* junction region- specific antisense ODN and retroviral expression vector.[103, 241] Enforced reexpression of the wild- type tumour suppressor gene p53 can lead to a reversion of the tumour phenotype or even apoptosis in cells with mutated or deleted p53 sequence. Seth and colleagues used an adenoviral vector in a human breast cancer purging model to effect the transfer of the wild- type p53 tumour suppressor gene, which induced apoptosis preferentially in the breast cancer cells.[242] Often, aberrant signal transduction events are implicated in the initiation and maintenance of the leukaemic phenotype; therefore, specific targeting of signalling pathways can lead to the selective destruction of the cells dependent on their survival. Novel compounds which disrupt the signalling pathways of neoplastic cells or promote apoptosis will increasingly find their way into purging.[58, 59, 72] LaCasse and colleagues described the successful purging of the human B- cell lymphoma cell line Daudi with the protein synthesis inhibitor Shiga- like toxin 1 (SLT- 1), which binds to CD77, a cell surface glycolipid.[243] Restricted expression of CD77 to a subset of activated B cells and lymphoma cells means that normal haematopoietic stem and precursor cells are spared from SLT- 1- mediated killing. A similar strategy utilises a fusion GM- CSF/ diphtheria toxin which selectively targets and destroys GM-CSF dependent leukaemic cells (as well as normal granulocyte- macrophage progenitors) *in vitro*.[244] The above examples illustrate some of the novel methods of purging which show promise for future clinical success.

1.6 PHOTODYNAMIC THERAPY (PDT) AND ITS ROLE IN PURGING

a. Reactive oxygen intermediates (ROIs) in biology

Molecular oxygen is essential for the survival of the vast majority of terrestrial biological organisms. It, however, can behave deleteriously if not for the elaborate protective mechanisms that are evolutionarily conserved in the different species. Reactive oxygen intermediates (ROIs) such as superoxide anions (O_2^-) and hydrogen peroxide

(H_2O_2) are generated during oxidative respiration and are normally deactivated by superoxide dismutase (SOD) and catalase, respectively. The family of glutathione (GSH)-dependent enzymes are extremely important in the detoxification of a variety of ROIs and lipid peroxides.[245]

In addition to their role as byproducts of mitochondrial respiration, ROIs are also implicated in a myriad of intracellular and intercellular signal transduction pathways. [246] Activated phagocytic cells produce O_2^- that is bactericidal for intracellular pathogens. Phagocytes from patients suffering from chronic granulomatous disease are defective in the generation of O_2^-, which predisposes these patients to persistent and severe infections. Neutrophils contain the enzyme myeloperoxidase, which catalyses the oxidation of chloride ion by H_2O_2 into the highly reactive hypochlorous acid (HOCl). Therefore, the body utilises ROIs for various aspects of nonspecific immune defence.[247] Oxidative injury to cells can occur at the different levels of cellular constituencies. Lipid peroxidation, oxidative damage to the thiol groups of structural proteins as well as enzymes, and direct damage to DNA are observed in cells exposed to oxidative stress.[248, 249] Increasing evidence from different experimental systems also demonstrate the role of ROIs in the induction of apoptotic cell death.[250] In addition, some studies have shown that the antiapoptotic protein *BCL- 2* has antioxidative capability which can inhibit the formation and actions of ROIs.[251, 252] However, contradictory findings also suggest that ROIs are not necessary for the induction of apoptosis and that *BCL- 2* retains its antiapoptotic ability even in anoxic conditions.[253]

In summary, ROIs are generated during normal cellular metabolism and are also implicated in the various signal transduction pathways. Phagocytes use ROIs as part of the nonspecific defence against pathogens. All cellular components are potential targets of oxidative damage; in addition, oxidative stress can effect its cytotoxicity via apoptosis.

b. PDT: cytotoxicity and biological responses

Photodynamic therapy (PDT) exploits the biophysical characteristics of a group of photoreactive compounds that, when activated by light of the appropriate wavelengths, enter into a higher energy state and will subsequently excite either cellular biomolecules (type I photosensitised reaction) or ground state oxygen (type II photosensitised reaction);

singlet oxygen ($^1O_2$), which is extremely reactive, is generated as a result of the type II reaction.[254, 255] The relatively short life span of $^1O_2$ (4 μs) in aqueous solution means that the sites of oxygen- dependent photodamage are dictated by the initial localisation of the photosensitiser.[256] Both apoptosis and necrosis have been observed after PDT and this appears to be dependent on the type and the dose of the photosensitiser. Since these agents are inert until activated by light, the specificity of the PDT cytotoxic response can be fine-tuned by manipulating the time and the site of photoirradiation.

Neoplastic tissues and other rapidly proliferating tissues such as angiogenically stimulated endothelial cells readily take up the photosensitising compound, one of the mechanisms is mediated by the upregulation of low density lipoprotein (LDL) receptors.[257] Various porphyrin- based photosensitisers associate with plasma lipoproteins; therefore, upregulation of the LDL receptors (LDLr) will increase the accumulation of the photosensitiser- LDL complex, and hence subsequent PDT cytotoxicity.[258] Other factors such as the local milieu of the cell and the chemical characteristics of the compound also determine the uptake profile and susceptibility to PDT.[259] Oxidative damage to cellular components results in various changes to the cells, tissues, and the organism [260]. The degree of PDT cytotoxicity is dependent on the amount and activity of the various cellular protective enzymes mentioned previously.[261] Recently, much research has been devoted to the role of apoptosis in PDT cytotoxicity, which appears to be mediated by phospholipase-C in L5178Y lymphoma cells.[262] He and colleagues showed that overexpression of antiapoptotic protein *BCL- 2* in the chinese hamster ovary cell line effected protection from PDT- mediated cytotoxicity.[263] Several established stress signal transduction pathways are also initiated by PDT; increased expression of specific heat shock proteins and various early response genes as well as the activation of the NF- κβ, JNK/SAPK pathways as well as increased production of ceramide are observed post- PDT.[264-268] These pathways may be involved either as a normal protective response to PDT- mediated stress or as part of the cell death pathways initiated by PDT.

Numerous photosensitising compounds with different biophysical and biochemical characteristics have been proposed for PDT. Two members of the porphyrin family, Photofrin® (porfimer sodium) and the benzoporphyrin- derivatives, are in current clinical use.[269] Other photosensitisers such as sulphonated aluminium phthalocyanine (AlSPc) and Merocyanin 540 (MC 540) being considered as well. The "personality" of each

photosensitiser, which includes hydrophobicity, extinction coefficient, absorption spectra, and singlet oxygen yield, is determined by the unique chemical structure of the compound. These factors, in conjunction with the biological attributes of the cell (*in vitro* and *in vivo* PDT) and the organism (*in vivo* PDT) determine the overall effectiveness of PDT.[270]

c. In vivo uses of PDT

Accessibility of the skin to photoirradiation and the high occurrence of cutaneous tumours combined to provide an ideal indication for PDT. In addition, PDT has shown promise in the treatment of tumours of the brain, lung, oesophagus, and of the bladder.[249, 271, 272] Damage to the tumour- associated vasculature by PDT also contributes to the destruction of the tumour.[273] However, limited photopenetration remains one of the major impediments in the application of *in vivo* PDT for the treatment of large tumours; this limitation was evident in the first generation haematoporphyrin derivative- based photosensitiser Photofrin® (porfimer sodium) with an activation wavelength at 630nm, which coincides with tissue absorption.

d. The Benzoporphyrin derivatives- second generation photosensitisers

To further improve the efficacy of PDT, various second generation photosensitising compounds with absorption peaks between 650- and 700 nm are under investigation. Some of these include the benzoporphyrin- derivatives, phthalocyanines, purpurins, and chlorin e_6.[274-276] The benzoporphyrin derivatives absorb maximally at approximately 690 nm, a wavelength with at least twice the tissue penetration of Photofrin®.[277, 278] One analogue in current clinical testing, benzoporphyrin- derivative monoacid ring A (BPD- MA or BPD), possesses rather high singlet and triplet oxygen quantum yields which contribute to its potential as an efficient photosensitiser.[279] In addition, BPD has a pharmacokinetic profile which favours tumour accumulation and therefore optimal tumour- to- tissue ratios in DBA/2 mice with the implanted rhabdomyosarcoma tumour M1.[280, 281] Even though BPD persists for up to 72 h in the mouse skin, photosensitivity only lasts for 24 h postinjection possibly as a result of inactivation rather than clearance of the photosensitiser.[282] Furthermore, Logan and colleagues have shown *in vivo* PDT mediated by BPD increased

the immunogenicity of M1 tumour in the murine host.[283] In summary, the advantages of BPD make it an effective and ideal photosensitiser for *in vivo* applications.[284]

e. Ex vivo uses of PDT

The *ex vivo* use of PDT, principally in the manipulation of blood products, bypasses the many constraints of *in vivo* PDT; namely, interference of photoactivation by the tissues and blood surrounding the target site, and concerns with achieving a therapeutic concentration of the photosensitiser in the target without eliciting significant skin photosensitivity and other side effects. Many of the techniques developed for preclinical *in vitro* studies of PDT can be adapted into clinical *ex vivo* applications. In addition, whole blood can be separated into its different components prior to PDT. The concentration and purification of leukocytes through apheresis eliminates most of the red blood cells, which may interfere with both the uptake of the photosensitiser and subsequent light activation. Essentially, *ex vivo* PDT simplifies the pharmacokinetics and pharmacodynamics by restricting the potential target populations. For example, leukapheresis of a patient with autoimmune disease will yield both activated (proliferating) and nonactivated (quiescent) lymphocytes; the former population has been shown to be more susceptible to PDT because of its proliferative state.[285] Gasparro's group has completed several promising clinical trials on the extracorporeal UVA irradiation of blood cells pretreated with 8-methoxypsoralen (8- MOP), for the management of cutaneous T- cell lymphoma and several other autoimmune diseases.[286] Photophoresis usually takes place two hours after the ingestion of 8- MOP with minimum plasma drug level at 50 ng/ml. A specially designed photophoresis unit is used to separate the patient's blood into different components followed by photoactivation of the leukocytes in recirculating clear plastic tubings sandwiched between banks of UVA lamps; the treated cells are then returned to the patient's systemic circulation. Another use of PDT is the extracorporeal inactivation of enveloped viruses in blood products; BPD has shown promise in the inactivation of FeLV and HIV1.[287, 288]

Another emerging use of the technology is in the immunomodulation of organ grafts prior to transplantation. Obochi and colleagues have observed that sublethal doses of PDT using BPD was sufficient to prolong the survival of allogeneic skin grafts in mice,

which was attributed to the reduction of the expression of immunostimulatory cell surface molecules in the Langerhan cells, professional antigen presenting cells which reside in the skin.[289]

f. Bone marrow purging

PDT- mediated bone marrow purging (BMP), which requires the extracorporeal photoirradiation of bone marrow cells preincubated with photosensitiser, naturally progressed from the other *ex vivo* PDT applications discussed above.[290, 291] Preferential uptake of the photosensitiser by leukaemic cells in comparison to normal nonproliferating cells provides a window of opportunity for this modality in BMP. The feasibility of using BPD- mediated PDT in purging has been investigated exhaustively in both cytotoxicity assays and *ex vivo* purging systems.[292] Fluorescent microscopy and flow cytometry have demonstrated that leukaemic cell lines and primary leukaemic cells preferentially take up more BPD than normal blood cells. Specifically, Jamieson et al. reported that the human leukaemic cell line K562 took up 10- fold and primary CML clinical samples took up 7-fold more of BPD than normal human bone marrow cells.[293] Furthermore, short- term colony assays and long- term bone marrow cultures as well as murine bone marrow purging experiments all demonstrated the utility of BPD in BMP.[294, 295] Lemoli and colleagues found that multidrug resistant (MDR) leukaemic cell lines that express the P170 membrane glycoprotein are refractory to BPD- mediated but not to dihaematoporphyrin ether (DHE)- mediated PDT; in addition, other cellular protection mechanisms may also affect the effectiveness of purging.[261, 296] Therefore, the heterogeneity of the cytotoxic response to PDT depends on the photosensitiser, its formulation (eg. liposome-encapsulated), and the type of cells targeted. Active drug efflux as well as defence mechanisms against oxidative stress can be attenuated pharmacologically in order to enhance PDT cytotoxicity. Recently, Gluck and colleagues demonstrated the effectiveness of BPD- mediated PDT purging of multiple myeloma cells *ex vivo*.[297] Photosensitisers other than BPD are also being considered for purging, some notable examples include the phthalocyanines and merocyanine 540 (MC540).[298-300] PDT purging has also been shown to be effective in the eradication of cancers other than leukaemia; Sieber and colleagues demonstrated the effectiveness of MC540 in purging contaminating

neuroblastoma cells.[301] Therefore, PDT purging can be considered for auBMTs in neoplasms other than the leukaemias.

g. Clinical trials

Clinical bone marrow purging using PDT is still relatively uncommon compared to the other methodologies such as monoclonal antibody- mediated negative selection. Sieber and associates have conducted phase I clinical testing of MC540- mediated PDT- purging of autologous bone marrow cells from lymphoma and leukaemia patients.[302] An ongoing clinical trial of BMP in acute leukaemia undergoing auBMT using the photosensitiser BPD is being conducted in Montreal. Initial findings from the small group of patients are extremely encouraging. Purging at 10 and 20 ng/ml of BPD with 15J/cm^2 photoactivation resulted in the prolonged survival in five of seven patients with the longest disease- free-survival interval at 349 days posttransplantation. One patient relapsed on day 84 and another patient required infusion of the unmanipulated backup marrow and subsequently relapsed on day 122. BPD dose escalation will be incorporated into future trials in order to increase the efficacy of the purging. A phase I trial studying the feasibility of using BPD to purge CD34$^+$ cells isolated from the autologous PBSC harvests of patients with Non-Hodgkin's Lymphoma is in its final planning stage.

OBJECTIVES AND RATIONALE

In this thesis, we attempted to improve the efficacy of BPD- mediated PDT in an *in vitro* setting. This work is based on previous findings by Jamieson that BPD- mediated PDT was selective in the destruction of leukaemic cells.[291, 292] We decided to approach the problem via two independent angles.

There exists a considerable amount of literature on the various forms of PDT combination therapy.[303-306] Several groups have already performed studies on the combination of PDT and Dox, albeit with different results.[307, 308] We therefore decided to

study the combined effects of BPD- mediated PDT and Dox for the purpose of *in vitro* PDT, i.e. for stem cell purging. Furthermore, we were interested on the effects of treatment sequence on the cytotoxic outcome and selectivity of the regimen. Treatment sequence is an important parameter in PDT combination therapy which could potentially affect the efficacy of the therapy.[303, 309]. BPD, a recently developed second generation porphyrin- based photosensitiser, is much more potent than Photofrin® and is considered for various clinical applications.[269, 284] BPD, unlike its predecessor Photofrin®, is a relatively pure photosensitiser with characterised active components.[278] Therefore, PDT combination therapy using BPD is a novel area of research with relevant clinical potentials especially for stem cell purging because of its *in vitro* nature. Cincotta *et al.* recently demonstrated the advantage of a PDT regimen combining BPD with another photosensitiser.[305] We were also interested in the possible interactions, physical and biochemical, between BPD and Dox. Murine haematopoietic cells, normal and leukaemic, were used throughout the experiment. The standard agar colony assay allows the convenient and reproducible measurement of clonogenicity of cells as a result of the various permutations of treatment parameters. We therefore employed the agar colony assay as a primary assessment tool of the effectiveness of the different combinations. We found that the murine leukaemic cell line L1210 was much more susceptible to the combination of Dox-> PDT than normal DBA/2 haematopoietic progenitors (Chapter 3). Therefore, we decided to further explore some of the possible mechanisms behind the findings (Chapter 4).

The second approach involves the incorporation of the haematopoietic inhibitory peptide N-AcSDKP into BPD- mediated PDT of normal and leukaemic cells. N-AcSDKP has been demonstrated to effect the selective protection of normal haematopoietic cells from various forms of cytotoxic treatments and therefore was considered to be an ideal protector of normal bone marrow cells during radiochemotherapy.[205, 210] Coutton and colleagues showed that N-AcSDKP also selectively protected normal human haematopoietic cells from Photofrin®- mediated PDT in a purging setting.[209] We therefore wanted to extend their findings to the second generation compound BPD. We also wished to extend the experimental system to the murine setting such that, if needed, bone marrow purging and transplantation experiments could be performed to assess the protective effect of N-AcSDKP. Again, we were able to determine the effectiveness of the scheme by using the standard agar colony assay. We found that a preincubation period of 1.5 h with 100 nM N-

AcSDKP significantly and selectively protected DBA/2 late haematopoietic progenitors but not the leukaemic cell line L1210 from BPD- mediated PDT (chapter 5). Furthermore, we attempted to study some of the potential mechanisms responsible for N-AcSDKP-photoprotection (chapter 6). Our findings suggested that cell cycle inhibition mediated by the peptide was partly responsible for the observed protective effect.

Therefore, the overall theme of this thesis is to improve the efficacy of BPD-mediated PDT. In addition, we hope that in the process we will reveal findings beneficial to the clinical applications of PDT as well as conducive to further understanding of the molecular mechanisms of PDT.

EXPERIMENTAL PROCEDURES

2.1 EXPERIMENTAL REAGENTS

a. Benzoporphyrin derivative monoacid ring A (BPD, Verteporfin®)

The monoacid ring- A analogue of benzoporphyrin derivative in a liposomal formulation (BPD, Verteporfin®) was obtained from QLT PhotoTherapeutics Inc. (QLT, Vancouver, BC, Canada). Lyophilised BPD was reconstituted to a concentration of 1.5 mg/ml with sterile double- distilled water (ddH_20) every two weeks and stored at 4°C until use. Further dilutions were carried out during the experiment with tissue culture medium in a reduced light environment.

b. Chemical reagents

Doxorubicin hydrochloride (Dox) and cytosine arabinoside (Ara- C) were purchased from the Sigma Chemical Co. (St. Louis, MO) and were supplied in powder form. Dox was reconstituted in sterile ddH_2O to a concentration of 10 mM and stored as 50 µl aliquots at -20°C. Ara- C was reconstituted in sterile ddH_2O to a concentration of 500 mM and stored as 50 µM aliquots at - 20°C. All other chemical reagents, except where specifically noted, were purchased from Sigma Chemical Co. (St. Louis, MO).

c. Tissue culture reagents

Tissue culture media Dulbecco's Modified Eagles Medium (DMEM) and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from Gibco/BRL Life Tech. Inc. (Grand Island, N.Y.) and were prepared according to the manufacturer's instructions. Fetal calf serum (FCS) was purchased from the Sigma Chemical Co. (St. Louis, MO) and was heat- inactivated at 57°C for 15 min. DMEM was further supplemented with streptomycin

(final 100 μg/ml) and penicillin (final 100 U/ml) (Gibco/BRL), 1 mM sodium pyruvate (Gibco/BRL), and 25 mM of (N- [2- Hydroxyethyl] piperazine- N'- [2- ethanesulfonic acid]) (HEPES, Sigma). Supplemental glutamine in the form of GlutaMAX I (Gibco/BRL), a L- Alanyl- L- Glutamine dipeptide, was added to a final concentration of 2 mM to DMEM that had been in storage for more than one month. All tissue culture reagents were stored at 4°C.

d. Reagents for colony assays and long- term marrow cultures

Reagents for performing primary haematopoietic colony assays and long- term bone marrow medium were purchased from StemCell Technologies (SCT, Vancouver, B.C., Canada). They were pokeweed mitogen- spleen cell conditioned medium (PWM- SCCM, HemoStim M2100) which contained interleukin- 3 (IL- 3) and granulocyte- macrophage colony stimulating factor (GM- CSF), and murine myeloid long- term culture medium (MyeloCult M5300). Tissue culture grade bovine serum albumin (BSA) in IMDM was purchased from Boehringer Mannheim Canada. (Laval, Quebec, Canada).

e. Fluorescence activated cell sorting (FACS) reagents

Antibodies and fluorochromes used for FACS analysis were purchased from Pharmingen, Inc. (San Diego, CA) and are listed below (Table 2.1). FACS washing buffer consisted of 1% heat- inactivated FCS (HI- FCS, Sigma Chemical Co., St. Luois, MO) and 0.1% sodium azide (Sigma) in phosphate buffered saline (PBS). A 4% paraformaldehyde (Sigma) double- strength FACS fixation buffer was prepared in PBS.

Table 2.1 Reagents used for FACS analysis

Specificity	Antibody	Clone	Isotype	Form	Reference Number
mouse haematopoietic progenitor cells	CD34	RAM34 (49E8)	Rat IgG_{2a}, κ	Biotin	09432D
isotypic control		R35- 95	Rat IgG_{2a}, κ	Biotin	11022C
Biotin	-	-	-	Steptavidin-FITC	13024D

* FITC, fluorescein isothiocyanate

f. Synthetic peptides

The tetrapeptides acetylated- serine- aspartate- lysine- proline (N-AcSDKP), acetylated- serine- aspartate- lysine- glutamate (N-AcSDKE), and serine- aspartate- lysine- proline (SDKP) were synthesised at the Microsequencing Centre of the University of Victoria (Victoria, B.C., Canada). The peptides were further purified to homogeneity by high performance liquid chromatography (HPLC) and characterised by amino acid analysis, capillary electrophoresis, and fast atom bombardment mass spectroscopy. Peptides were supplied as lyophilised powders and were stored at -80°C in a desiccated environment. Peptides were reconstituted to 5 mM with 10% HI- FCS/IMDM and stored as single- use aliquots at -80°C.

2.2 LIGHT SOURCE

A specifically constructed light box was used for photoactivation of cells. The unit consisted of upper and lower banks each of eight GE F15T8- R red fluorescent tubes (Sylvannia, Drummondville, Quebec, Canada) which emit between 600- 900 nm. In addition, two electric fans were incorporated into the unit to ensure adequate cooling of the samples during exposure. Cell samples were placed on a clear plexiglass platform at

equidistant between the two banks of light. The unit was warmed up for thirty minutes before irradiation and photometric measurements in three axes (x, y, z) were made with an IL 1350 Radiometer/Photometer power meter (International Light, Inc., MA) immediately before exposure. Exposure time (Y) was calibrated to provide 15 J/cm^2 of light energy using the following formula:

$$Y\ (\text{min}) = \frac{15\ \text{J/cm}^2}{X\ \text{mW/cm}^2 \times 0.06\ (\text{W s/mW min})}$$

The average of the six photometric measurements of the three axes constituted the variable X.

2.3 EXPERIMENTAL ANIMALS

a. Mice

DBA/2 (H- 2^d) mice (6- 8 weeks of age) were purchased from Charles River Breeding Laboratories Canada (Montreal, Quebec, Canada) and maintained under pathogen- free conditions in the animal facility of the Department of Microbiology & Immunology at the University of British Columbia (Vancouver, B.C., Canada). All animals were housed in microisolator units and were given standard laboratory rodent diet (Ralston Purina) and HCl- acidified water (pH 2.5) *ad libitum*. Newly arrived animals were kept in quarantine for two weeks prior to introduction into the core facility. Experiments were conducted on animals that had been in the facility for two weeks or more to ensure acclimatisation. The protocol for animal experimentation was approved by the animal care committee of UBC.

2.4 CELL PREPARATION

a. Mouse bone marrow cells

Bone marrow mononuclear cells (BMMNCs) were obtained from the femurs of CO_2- euthanised 8- 12 week old DBA/2 mice. Briefly, the mouse was rinsed with 70% ethanol and a straight line vertical incision was made on the ventral surface followed by manual separation of the skin exposing the abdomen and the legs of the mouse. The muscles surrounding the femur were removed and the femur was cut superior to the patella and inferior to the acetabulum. The marrow cavity of the femur was then flushed with IMDM supplemented with 10% HI- FCS delivered by a 22 gauge needle connected to a 5 cc syringe (Becton- Dickinson, Rutherford, NJ). The marrow was then dispersed into a single cell suspension via gentle manipulation with the syringe without the needle. The process was repeated for the other femur. One mouse normally yielded 1 x 10^7 BMMNCs from its two rear femurs. The harvested cells were then washed twice and resuspended in IMDM. Cells intended for FACS analysis underwent an additional step of erythrocyte lysis in which 5 x 10^7 bone marrow cells were mixed with 5 ml of Tris/NH_4Cl buffer (pH 7.3) and incubated at 37°C for 10 min followed by two washes with IMDM.

b. Mouse leukaemic cell lines

The L1210 murine lymphocytic leukaemia cell line (ATCC CCL 219) was purchased from the American Type Culture Collection (ATCC, Rockyville, Maryland) and maintained in DMEM supplemented with 10% heat- inactivated FCS (HI- FCS). L1210 was originally derived from the DBA/2 mouse strain following skin paintings with 0.2% methylcholanthrene in ethyl ether and has been used extensively in the screening of new chemotherapeutic agents.[310] The cells were grown at 37°C and maintained in a 5% carbon dioxide (CO_2, Praxair, Mississauga, ON, Canada) - room air aerated, fully humidified incubator (Forma Scientific, Marietta, Ohio). Ongoing cultures of L1210 cells were routinely replaced at two month intervals with frozen stocks from liquid nitrogen storage to ensure that the cells did not acquire any mutations *in vitro* which could affect their behaviour.

c. Primary human haematopoietic cells

Normal human bone marrow cells were obtained during sternotomy and were kindly supplied by Dr. Lawrence Burr at the Vancouver General Hospital (VGH, Vancouver, B.C., Canada). Cell samples were maintained in IMDM supplemented with 200 units of sterile sodium heparin (Fisher Scientific, Fair Lawn, NJ) at room temperature and processed on the same day of extraction. Chronic myelogenous leukaemic (CML) cells were obtained from venupuntures of newly diagnosed, untreated CML patients and were kindly supplied by Dr. Noel Buskard at the Monroe outpatient clinic of VGH. The leukaemic blood samples were stored in 10 ml Vacutainers containing 143 USP of lithium heparin (Becton Dickinson) and were processed on the day of extraction. To isolate the fraction containing mononuclear cells, the blood samples were mixed with IMDM in a 1: 1 ratio and 7 ml of the resultant mixture was then layered on top of 3 ml of room temperature Ficoll- Paque Plus (Pharmacia Biotech AB, Uppsala, Sweden) and centrifuged at 300 RCF for 15 min. The interface was harvested with a sterile Pasteur pipette and the cells were washed twice with IMDM.

d. Human leukaemic cell lines

The human chronic myelogenous leukaemic cell line K562 (ATCC CCL 243), originally derived from the pleural effusion of a CML patient in blast crisis, was obtained from ATCC and was maintained in 10% HI- FCS/RPMI 1640. The cells were grown at 37°C and maintained in a 5% carbon dioxide (CO_2, Praxair, Mississauga, ON, Canada) - room air aerated, fully humidified incubator (Forma Scientific, Marietta, Ohio).

2.5 CYTOTOXIC TREATMENT OF CELLS

a. PDT treatment of murine bone marrow cells and L1210 cells for short term evaluations (combination experiments)

Each mouse provided approximately 1 x 10^7 BMMNCs. Freshly extracted BMMNCs were washed twice with warm IMDM and resuspended in the same medium. A

small aliquot of the cells was then stained with the vital stain Eosin Y (0.3% in PBS) and the cell concentration and viability were determined followed by dilution with IMDM into 2.2 x 10^6 cells/ml. At the same time, BPD and Dox were diluted into the appropriate working concentrations in IMDM and 100 µl of the 10 x drug was dispensed into the sterile 5 ml polystyrene test tubes (Falcon brand, Becton Dickinson) along with 900 µl of the BMMNCs. The tubes were then incubated at 37°C for 60 min after gentle mixing. The cells were washed once with IMDM and resuspended in 1.0 ml of IMDM supplemented with 10% HI- FCS, dispensed into a 24 well tissue culture plate (Linbro brand, Flow Laboratories, Inc., Hamdesn, Connecticut) and were photoirradiation with red light at 15J/cm^2. Clonogenicity of the treated cells was then assessed (section 2.6a). The L1210 cells were processed in the same manner (section 2.6b).

Experiments of combined simultaneous BPD and Dox treatments were conducted with both normal and leukaemic cells. Samples were incubated concomitantly with both BPD and Dox in different combinations for one hour at 37°C. Subsequent manipulations followed the same protocol as for the single agent- treated cells. Experiments which involved sequential BPD/ Dox or Dox/ BPD treatments involved pretreatment of cells with PDT followed by a one hour incubation period with Dox, or preincubation of cells with Dox followed by BPD incubation and photoirradiation. Cells were washed after the first treatment and handling protocols were identical to those for single agent treatment.

b. PDT treatment of normal and leukaemic murine haematopoietic cells preincubated with peptides (short- term colony assay)

Murine BMMNCs were obtained from DBA/2 mice as described previously (section 2.4a). BMMNCs or L1210 cells at 2.2 x 10^6 cells/ml in 900 µl of IMDM were mixed with 10 µl of N-AcSDKP or the control peptides N-AcSDKE and SDKP to a final concentration of 100 nM. The cells were incubated at 37°C for 1.5 h followed by the addition of 100 µl of BPD at 10 x the final concentrations. The cells were then incubated for an additional 1 h at 37°C and were washed 1 x with IMDM. Cell pellets were resuspended gently with 1 ml of 10 % HI- FCS/IMDM and dispensed into wells of a 24

well tissue culture plate and photoirradiated at 15 J/cm^2. Clonogenicity of the treated cells was then assessed (section 2.6a and 2.6b).

c. PDT treatment of murine bone marrow cells for long- term bone marrow cultures

Murine BMMNCs were obtained from DBA/2 mice as described previously (section 2.4a). BMMNCs at concentration of 2.2 x 10^6 cells/ml in 900 µl of IMDM were mixed with 10 µl of N-AcSDKP to a final concentration of 100 nM. The cells were incubated at 37°C for 1.5 h followed by the addition of 100 µl of BPD at 10 x the final concentrations. The cells were then incubated for an additional 1 h at 37°C and were washed 1 x with IMDM. Cell pellets were resuspended gently with 1 ml of 10 % HI-FCS/IMDM and dispensed into wells of a 24 well tissue culture plate and photoirradiated at 15 J/cm^2. The treated cells were then assessed using the LTBMC assay (section 2.6d).

d. PDT treatment of human normal haematopoietic and leukaemic cells preincubated with peptides (short- term colony assay)

Normal or leukaemic cells at 2.2 x 10^6 cells/ml in 900 µl of IMDM were mixed with 10 µl of N-AcSDKP or the control peptides N-AcSDKE and SDKP to a final concentration of 100 nM. The cells were incubated at 37°C for 1.5 h followed by the addition of 100 µl of BPD at 10 x the final concentrations. The cells were then incubated for an additional 1 h at 37°C and were washed 1 x with IMDM. Cell pellets were resuspended gently with 1 ml of 10 % HI- FCS/IMDM and dispensed into wells of a 24 well tissue culture plate and photoirradiated at 15 J/cm^2. Clonogenicity of the treated cells was then assessed (section 2.6e and 2.6f).

e. ara- C treatment of DBA/2 bone marrow cells preincubated with peptides

Murine BMMNCs were obtained from DBA/2 mice as described previously (section 2.4a). BMMNCs at concentration of 2.2 x 10^6 cells/ml in 900 µl of IMDM were

mixed with 10 µl of N-AcSDKP to a final peptide concentration of 100 nM. The cells were incubated at 37°C for 1.5 h followed by the addition of 100 µl of ara- C at 10 x the final concentrations. The cells were then incubated for an additional 1 h at 37°C and were washed 1 x with IMDM. Cell pellets were resuspended gently with 1 ml of 10 % HI-FCS/IMDM and 140 µl (2.8 x 10^5 cells) was mixed with the other ingredients of the standard agar colony assay for clonogenicity determination (section 2.6a).

f. PDT of DBA/2 bone marrow cells preincubated with 50 µM ara- C

Murine BMMNCs were obtained from DBA/2 mice as described previously (section 2.4a). BMMNCs at concentration of 2.2 x 10^6 cells/ml in 900 µl of IMDM were mixed with 100 µl of ara- C to a final concentration of 50 µM. The cells were incubated at 37°C for 1 h, washed once with IMDM, and resuspended in 900 µl of IMDM. Next, 10 x solutions of BPD (100 µl) were then added to the appropriate sample tubes to a final volume of 1.0 ml. The cells were then incubated for an additional 1 h at 37°C and were washed 1 x with IMDM. Cell pellets were resuspended gently with 1 ml of 10 % HI-FCS/IMDM and dispensed into wells of a 24 well tissue culture plate and photoirradiated at 15 J/cm^2. Clonogenicity of the treated cells was then assessed (section 2.6a).

2.6 CYTOTOXICITY ASSAYS

a. Agar colony assay of treated murine bone marrow cells

Standard agar- based colony assays were used to assess cytotoxicity on normal murine bone marrow mononuclear cells (BMMNCs). The protocol for the murine haematopoietic granulocyte- macrophage progenitor assay has been described elsewhere [294, 311]. 140 µl of treated DBA/2 bone marrow cells was added to a plating mixture which consisted of 2235 µl IMDM, 750 µl of pretested HI- FCS, 375 µl of 100 mg/ml (w/v) bovine serum albumin (BSA) in IMDM (Boehringer Mannheim, Laval, Quebec), and 100 µl of Hemostim M2100 (see section 2.1d) as a source of cytokines. Finally, 400 µl of prewarmed 3 % agar Noble solution (w/v) in sterile ddH_2O (Difco Laboratories, Detroit,

Michigan) was dispensed into the cell mixture to achieve a final agar concentration of 0.3% and the resulting mixture was dispensed immediately in 1.0 ml volumes to triplicate 35 mm tissue culture dishes (Sarstedt, Newton, NC) with approximately 70000 cells dispensed per tissue culture dish. Two of the agar containing plates were then placed inside a 100 mm petri dish (Fisher Scientific, Edmonton, Alberta, Canada) along with a 35 mm dish containing 5 ml ddH_2O. The plates were then placed inside a 37°C incubator for seven days prior to colony enumeration. A Zeiss Axiovert 35 inverted microscope (Carl Zeiss Canada, Don Mills, Ontario, Canada) was used for colony counting and a cluster consisting of forty or more cells was counted as a granulocyte- macrophage colony forming unit (CFU- GM). Cloning efficiency for DBA/2 CFU- GM was approximately 0.126 %.

b. Colony assay of L1210 cells

Agar colony assay of the leukaemic cell line L1210 was slightly different from the previously described assay. A 334 µl aliquot of diluted cell suspension was mixed with 2247 µl of IMDM, 800 µl of pretested HI- FCS, 400 µl of BSA, and finally 400 µl of 3.0 % Agar- Noble. To fully appreciate to range of L1210 log reduction, seeding numbers of cells was customised for individual drug concentrations. Again, 1.0 ml was dispensed into triplicate 35 mm tissue culture dishes. Colonies consisting of forty or more cells were enumerated on day 6 using the inverted microscope. Cloning efficiency of L1210 CFU- L in this system was approximately 36 %.

c. Short-term cytotoxicity assay (MTT assay)

Cells in log phase growth were washed twice with DMEM to remove residual serum and 900 µl of cells at 1.4 x 10^6 cells/ml were dispensed to 5 ml polystyrene test tubes (Falcon). BPD and other chemotherapeutic agents at 10 x final concentrations were then added to each tube in volumes of 100 µl. The tubes were then gently agitated and allowed to incubate for 1 hour. The treated cells were then washed once and resuspended in 900 µl of DMEM. The content of a single test tube was then distributed into eight wells of a 96 well tissue culture plate (Falcon) in 100 µl aliquots followed by 15 J/cm^2 of red light photoirradiation as described in section 2.2. The plate was then incubated overnight at 37°C in a 5% CO_2 incubator. The next day, 10 µl of 5 mg/ml 3-[4,5-Dimethylthiazol-2-yl]-2,5-

diphenyltetrazolium bromide (MTT, Sigma) in PBS was then dispensed into the wells with an Eppendorf Repeator Pipette dispenser (Brinkmann Instruments, Mississauga, Ontario, Canada) and allowed to incubate for 60 min at 37°C before fixation with 150 µl of 0.05 N HCl/ i. Mitochondrial dehydrogenase in viable cells convert the yellow MTT tetrazolium salt to purple formazon crystals, which could be quantified spectrophotometrically at 595 nm (OD_{595}).[312, 313] The plate was read on a SpectraMax 250 scanning multiwell spectrophotometer (Molecular Devices, Sunnyvale, CA). OD_{595} measurements of control cells were averaged and arbitrarily set as 100% survival and viability of the treated cells were calculated based on their relative values.

d. One step long- term bone marrow culture (LTBMC) of murine haematopoietic cells

Long- term bone marrow culture (LTBMC) was initiated to determine the fitness of earlier haematopoietic progenitors and stem cells. Murine LTBMC culture medium (MyeloCult M5300, StemCell Technologies Inc.) was used to initiate and maintain the growth of PDT- treated DBA/2 BMMNCs for six weeks. A one step LTBMC protocol was adapted in which the inoculum was used to start the stromal layer as well as to initiate haematopoiesis.[314] Hydrocortisone (Sigma Chemcial Co.) dissolved in 100 % ethanol was added to the LTBMC medium to a final concentration of 10^{-6} M before use. The PDT-treated cells were divided into two equal portions of 1 x 10^6 cells and dispensed into wells of a 24 well tissue culture plate (Corning Costar Corp., Corning, NY) in volume of 1 ml. The plates were incubated at 33°C during the first week and moved to a 37°C 5 % CO_2 incubator for the subsequent duration of the experiment. Cells were harvested at weekly intervals during which the entire content of the appropriate well was used for cell number determination followed by incorporation of the cells into the short- term agar colony assay (section 2.6a). The rest of the cells were demidepopulated, i.e. gently agitated followed by aspiration of 500 µl of the cells (50 %), and replenished with 500 µl of murine LTBMC culture medium containing 10^{-6} M. The cells were then returned to the incubator (37°C, 5 % CO_2, fully humidified) until the next harvest seven days later.

e. Colony assay of treated normal and leukaemic human haematopoietic cells

Short- term colony assays of human haematopoietic cells were initially described by Messner *et al.*[315] PDT- treated or untreated cells at 400 µl volume were mixed with 400 µl phytohaemagglutinin- stimulated leucocyte conditioned medium (PHA- LCM), 1200 µl pretested HI- FCS, 1600 µl IMDM, and 400 µl 10 % methyl cellulose dissolved in IMDM (Methocel MC, Fluka Biochemika, Switzerland) to a total assay volume of 4.0 ml. PHA-LCM was harvested from the peripheral blood of a healthy volunteer. Briefly, buffy coat (mononuclear cells and plasma), which settled to the upper layer of "rested" whole blood containing 50 U sodium heparin (Fisher) per ml, was removed by aspiration. The mononuclear cells were then separated from the plasma by centrifugation for 10 min at 400 x RCF. The cells were washed 2 x with IMDM and the resuspended at 1 x 10^6 cells/ml in 1 % PHA (v/v)/ IMDM and incubated at 37°C for 7 days. The conditioned medium (PHA-LCM), separated from the cells, was dispensed and stored at -80°C. PHA- LCM contains a variety of haematopoietic growth factors and supports the growth of different colonies. The mixture was then combined and 1 ml each was dispensed into triplicate 35 mm dishes (Sarstedt) using a 16 gauge needle connected to a 3 cc syringe (Becton- Dickenson). Normal human BMMNCs and primary leukaemic cells from CML patients were plated at 1 x 10^5 cells/ dish. Colonies consisting of forty or more cells were counted after 14 days of culture at 37°C using an inverted microscope (Carl Zeiss Canada).

f. Colony assay of the human leukaemic cell line K562

PDT- treated or untreated K562 cells were assayed similarly as the primary human haematopoietic cells described above (section 2.6e) with the following modifications. K562 cells were plated at a final concentration of 1 x 10^4 cells/ dish and PHA- LCM was replaced with IMDM in the plating mixture.

2.7 PHOTOMETRIC AND SPECTROSCOPIC ANALYSES

a. Spectrophotometric analysis

Stock BPD and Dox were dissolved in phosphate buffered saline (PBS) to concentrations of 1 μg/ml and 10 μM, respectively. Equal amounts of BPD and Dox were dispensed into a spectrophotometric cuvette and then analysed with the HP 8452A spectrophotometer (Palo Alto, CA).

b. Spectrofluorimetric analysis of the interaction between lBPD and Dox

The protocol for BPD uptake analysis has been described elsewhere [285]. Briefly, L1210 cells were incubated with BPD alone, BPD in the presence of Dox, and BPD after one hour preincubation with Dox. Cells were washed twice with PBS and then lysed in the presence of 500 μl of 2% Triton X- 100 (Sigma) /PBS. Samples were snap frozen in dry ice/ methanol bath and stored at -80°C if readings were not planned on the same day of the experiment. Prior to spectrofluorimetric readings, the lysates were put through three rounds of freeze/ thawing. An additional volume of 500 μl PBS was added to the rest of the cell lysate and the resultant mixture was used for analysis with the Aminco SLM 500 C sprectrofluorimeter (SLM instruments Inc., Urbana, IL). All measurements were performed using the continuous wave (CW) setting and at excitation and emission bandpass of 0.5 nm. Determination of BPD concentration was achieved using excitation reference at 440 nm and emission reference at 700 nm. Mean fluorescence intensity (MFI) was measured at 694 nm for each sample. Emission reference was adjusted to 600 nm for Dox concentration determination and MFI was measured at 590 nm. All experiments were done in duplicates.

2.8 CELL ANALYSIS BY FLUORESCENCE ACTIVATED CELLS SORTING (FACS)

The protocol for the determination of cellular content of BPD in murine splenocytes has been described elsewhere.[285] Essentially the same protocol has been adopted for analysis of DBA/2 BMMNCs. One million of freshly isolated BMMNCs in 1.0 ml IMDM

were preincubated with N-AcSDKP or SDKP (negative control) to a final concentration of 100 nM. Tissue culture medium was used in place of peptide in a second negative control. The cells were incubated at 37°C for 1.5 h followed by 1 x wash with IMDM. Cell pellets were resuspended in 900 µl IMDM and mixed with 100 µl of 10 x BPD (100 ng/ml). The cells were incubated with an additional 0.5 h followed by 2 x washes with FACS buffer and transferred to a round bottom 96 well plate (Costar, Cambridge, MA). The cells were labelled with 10 µl of biotin- CD34 antibody (clone RAM34) or 2 µl of biotin- isotypic control for 0.5 h at 4°C. The secondary FITC- strepavidin reagent was added after 2 washes with FACS buffer. An additional incubation period of 0.5 h was followed by 2 final washes with FACS buffer. The cell pellets were then transferred to 5 ml polystyrene tubes (Falcon) in 500 µl of FACS buffer and 500 µl of 4% paraformaldehyde/PBS and stored at 4°C until analysis.

A Coulter EPICS XL® flow cytometry system (Coulter Corp., Miami, FL) was used for dual colour FACS analysis of BPD uptake and surface antigen expression. The excitation wavelength employed for FACS analysis involving BPD and FITC was 488 nm while a 690 nm emission (longpass) filter was utilised to detect BPD (red) fluorescence and a 525 nm emission (longpass) filter was used to detect FITC (green) fluorescence. One hundred thousand cells were analysed for each sample. Data from three independent experiments are presented and source of error is derived from the standard error of the mean.

2.9 FLUORESCENT MICROSCOPY

K562 Cells were resuspended at a concentration of 1 x 10^6 cells/ml in DMEM and incubated at 37°C with 10 µg/ml of BPD for 60 min. Cells were then washed once and resuspended in 1 ml of DMEM. A 50 µl drop (5 x 10^4 cells) was placed on a glass slide (Fisher Scientific, Edmonton, Alberta, Canada) and subsequently covered with a cover slip (Fisher). The cells were observed under an Olympus New Vanox microscope with the AH2- FL transmitted light fluorescence attachment on "Blue" setting (excitation @ 380-490 nm, observation @ 515 nm +). Pictures were taken with the attached camera using Fujichrome 400 ISO film at automatic settings.

3.0 ANALYSIS OF INTRACELLULAR GLUTATHIONE (GSH) CONTENT

a. Tietze enzymatic assay

Cellular glutathione (GSH) was determined using enzymatic cycling and Ellman's reagent (5,5'- dithiobis- (2- nitrobenzoic acid), DTNB) as described by Eyer and Podhradsky.[316] Briefly, cells were lysed in 0.6 % sulphosalicyclic acid/ 0.5 mM EDTA (Sigma) at a final concentration of 100 µl lysis buffer per 1 x 10^6 cells for 1 h at 4°C. The supernatant was then separated after centrifugation at 16000 x RCF for 10 min (maximum speed setting on the Beckmann microcentrifuge) at 4°C. The clarified lysates were dispensed into a 96 well microtitre plate (Falcon) and the volume made up to 180 µl with PBS. A mixture containing 4 µg/µl NADPH (Calbiochem, La Jolla, CA) and 1.2 µg/µl DTNB (Sigma) was dispensed into each well using a Eppendorf repeating pipetter dispensing at 10 µl (Brinkmann). Lastly, 10 µl of yeast- derived glutathione reductase at 0.012 U/µl (Calbiochem) in PBS was dispensed into each well. The plate was then read at $OD_{412\,nm}$ for 6 min in 1 min intervals using a SpectraMax 250 scanning multiwell spectrophotometer (Molecular Devices, Sunnyvale, CA). The difference between the 1 min and 6 min readings was calculated and the concentration of GSH was determined in comparison to known GSH standards (Sigma).

3.1 STATISTICAL ANALYSIS

a. Analysis of colony assay data from PDT/Dox combination experiments

Data were inputted into the NCSS 6.0.21 statistical analysis package (NCSS Statistical software, Kaysville, Utah). The various treatment and control group means were compared using the Student's *t*- test with paired sample for means. The data were further analysed using 2- way analysis of variance (2- way ANOVA) with the two BPD concentrations (2.5 and 5.0 ng/ml) and the three different combinations (Dox/PDT, PDT-> Dox, Dox-> PDT) set as the two parameters. Additional statistical analyses were carried out with the term significant at alpha (ρ value) = 0.05 and intragroup differences were examined using the Bonferroni (all- pairwise) multiple comparison test.

b. Analysis of colony assay data from the inhibitory peptide pretreatment experiments

Data were inputted into the NCSS 6.0.21 statistical analysis package (NCSS Statistical software, Kaysville, Utah). The various treatment and control group means were compared using the Student's *t*- test with paired sample for means. The data were further analysed using two- way analysis of variance (two- way ANOVA) with the parameters assigned to the three peptides (N-AcSDKP, SDKP, N-AcSDKE) as well as the no peptide control groups. Additional statistical analyses were carried out with the term significant at alpha (ρ value) = 0.05 and intragroup differences were examined using the Bonferroni (all-pairwise) multiple comparison test.

CHAPTER 3: COMBINED TREATMENT OF MURINE NORMAL HAEMATOPOIETIC AND LEUKAEMIC CELLS WITH DOXORUBICIN AND PDT: CYTOTOXICITY STUDY

3.1 ABSTRACT

Benzoporphyrin derivative monoacid ring A (BPD), a porphyrin based photosensitiser, shows ideal properties for the purpose of photodynamic therapy (PDT)-based bone marrow purging and is currently undergoing clinical testing for stem cell purging in Canada. To enhance the efficacy of BPD- mediated PDT, a combination approach with Doxorubicin (Dox) was pursued. Short term colony assays were used to measure the frequency of colony forming unit- granulocyte/macrophage progenitors (CFU-GM assay) of normal DBA/2 bone marrow cells and colony forming unit- leukaemia (CFU- L) of the murine leukaemic cell line L1210 after drug treatment. BPD alone in the absence of light resulted in no reduction of CFU- GM; however, 10 ng/ml of BPD followed by 15 J/cm^2 of red light activation resulted in the elimination of 0.5 logs of the CFU- GM and 4 logs of CFU- L. Dox at 5.0 μM resulted in one log reduction of both normal as well as leukaemic L1210 colony forming units; in addition, the cytotoxicity of Dox was neither enhanced nor diminished in the presence of 15 J/cm^2 of red light exposure. Simultaneous treatment of normal DBA/2 bone marrow cells or the L1210 cell line with PDT and Dox (PDT/Dox) resulted in the predicted log reductions. However, when cells were preincubated with Dox for one hour prior to PDT (Dox-> PDT), the resultant cytotoxicity was 1.5- 2.5 fold more than that observed in the simultaneous treatment (PDT/Dox) and in the reverse sequence (PDT-> Dox). Significantly, L1210 cells were more susceptible to the Dox-> PDT treatment than normal CFU- GM resulting in enlargement of the therapeutic window. This augmentation is treatment sequence dependent as well as drug dose dependent.

The above findings suggest that care must be taken when PDT is used in conjunction with other chemotherapeutic agents in the clinical setting and that judicious use

of PDT combination therapy can lead to the augmentation of the therapeutic window between normal and neoplastic cells.

3.2 INTRODUCTION

High dose radiochemotherapy coupled with haematopoietic stem cell rescue is an effective form of treatment for various kinds of human malignancies. Bone marrow from allogeneic human leukocyte antigen (HLA)- matched donors is an ideal source of haematopoietic stem cells for leukaemic patients because of the associated yet beneficial graft- versus- leukaemia (GvL) effect. Unfortunately, the dearth of HLA- matched donors restricts the use of allogeneic bone marrow transplantation (alBMT). In addition, many older patients exhibit a high incidence of age- related intolerance to graft- versus- host-disease (GvHD). Therefore, autologous bone marrows are used in older cancer patients or those without HLA- matched donors. Cancer patients undergoing autologous bone marrow transplantations (auBMT) have higher relapse rates than those receiving allogeneic bone marrow.[152] Through retroviral marking studies, Brenner and colleagues have unequivocally demonstrated the presence of contaminating neoplastic cells in autologous bone marrow harvests and the contribution of these cells to relapses posttransplantation.[154] Bone marrow purging therefore attempts to address this problem by the selective removal or destruction of the contaminating cancer cells while preserving enough of the normal haematopoietic progenitor and stem cells to effect haematopoietic reconstitution.

Bone marrow purging using photodynamic therapy (PDT) represents an ideal application of this technology. The *ex vivo* nature of purging eliminates problems associated with light delivery and *in vivo* pharmacokinetics. Various photosensitisers have been proposed for purging including the benzoporphyrin derivatives, phthalocyanines and Merocyanine 540 (MC540).[290, 291] In this laboratory, the mono- acid ring A analogue of benzoporphyrin derivative (BPD) was shown to be a promising purging agent because of preferential accumulation by leukaemic cells in addition to their higher susceptibility to PDT mediated by BPD.[292] Jamieson has also demonstrated the effectiveness of BPD purging in an *ex vivo* murine purging model.[294] In addition, BPD has an unique absorption peak at

690 nm which is outside the absorption range of haemoglobin; therefore, photoactivation of BPD is not affected by the presence of erythrocytes, a common occurrence in haematological samples.[317]

Cancer therapies which combine different drugs have been in use for many years. Combination therapy is especially effective in the management of leukaemia and lymphoma. PDT has been used in combinations with traditional chemotherapeutic agents or other photosensitisers to achieve improved killing of cancerous cells.[305, 318] Ideally, combination therapy aims to utilise drugs with different mechanisms of action such that the probability of the cancer developing drug resistance to all the drugs in the regimen is minimised. In addition, drugs used in combination therapy should have additive anticancer cytotoxicity but not adverse effects against normal tissues.

In this chapter, BPD- mediated PDT was combined with Dox in various concentration as well as sequence combinations. The relative sensitivities of the murine leukaemic cell line L1210 and its normal counterpart, haematopoietic progenitor cells from DBA/2 mice, were determined using standard agar based colony assays. This chapter addresses the question as to whether a combination approach using Dox and BPD can be applied to PDT purging and whether the differential susceptibility of normal and leukaemic cells can be altered with such a combination approach.

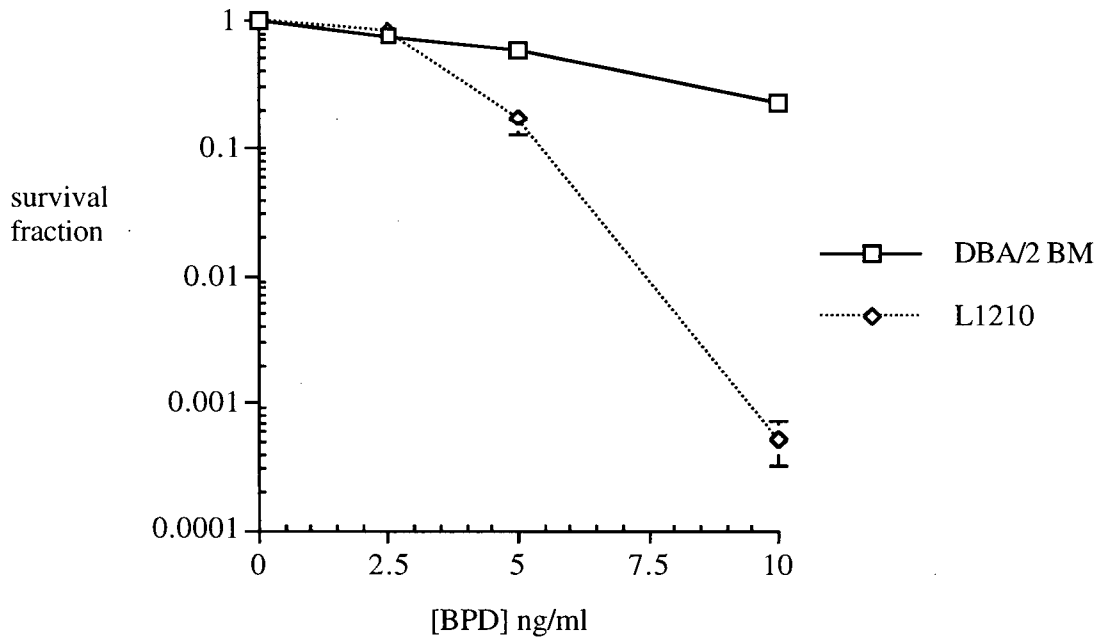
3.3 RESULTS

Single agent cytotoxicity

In order to establish baseline susceptibility profiles to single- agent treatments. L1210 cells and haematopoietic progenitor cells derived from DBA/2 mice were treated singly with either BPD- mediated PDT or Dox (with red light irradiation). In figure 3.1, normal and leukaemic cells were incubated with various concentrations of BPD followed by red light irradiation at 15 J/cm^2. Clonogenicity of the cells was then examined using standard agar based colony assays, which measured colony forming units- leukaemic cell (CFU- L) and colony forming units- granulocyte/ macrophage (CFU- GM) of L1210 and the DBA/2 haematopoietic progenitors, respectively. The number of colonies which consisted of 50 or more cells were enumerated at day 6 (leukaemic cells) or day 7 (normal progenitor cells) with an inverted microscope. Results are expressed as survival fraction relative to control cells that were exposed to red light in the absence of BPD or Dox. Under identical conditions, both L1210 cells and DBA/2 haematopoietic cells responded to treatment in a dose- dependent manner. However, the L1210 cells were much more susceptible to BPD- mediated PDT cytotoxicity than the DBA/2 haematopoietic cells. BPD at a dose of 10 ng/ml BPD and red light (15 J/cm^2) resulted in 4- log reduction of L1210 clonogenic cells while the same dose of BPD only reduced DBA/2 progenitors by 0.5 log. Similar differential susceptibility between L1210 and DBA/2 progenitor cytotoxicity was observed, albeit to a lower degree, at 5 ng/ml of BPD. Nevertheless, the data (figure 3.1) clearly showed that the leukaemic cell line L1210 was much more susceptible to BPD-mediated PDT than normal murine haematopoietic cells. BPD alone in the absence of direct light exposure was not cytotoxic as determined by the MTT assay, which measures mitochondrial dehydrogenase activity in viable cells (figure 3.2). Doxorubicin hydrochloride (Dox), unlike BPD and light, showed essentially no selective killing of L1210 cells over normal progenitor cells (figure 3.3). In addition, Dox cytotoxicity on L1210 cells was unaltered with or without direct light exposure (figure 3.4).

Figure 3.1 The effect of BPD- mediated PDT on the clonogenicity of normal DBA/2 haematopoietic progenitors and the leukaemic cell line L1210

Differential susceptibility of L1210 leukaemic cells and DBA/2 haematopoietic progenitors to PDT mediated by BPD. Cells received different doses of BPD and were exposed to 15 J/cm^2 of red light (600- 900 nm). Colony forming units of normal and leukaemic cells were determined with standard assays and colonies were scored on day 6 (CFU- L) and day 7 (CFU- GM) of culture using an inverted microscope. Only colonies with 50 or more cells were counted. Untreated L1210 cells and DBA/2 progenitors yielded 137.85 $\pm$ 9.57 and 97.35 $\pm$ 7.78 colonies, which translated to clonogenicity of 41 % and 0.126 % respectively. Survival fractions of treated cells were calculated based on the number of colonies generated divided by the number of colonies from the untreated control. Furthermore, dilution or correction factors were incorporated into the calculations for L1210 cells in order to cover the 4- log survival fraction range. Data obtained from 12 (L1210) and 11 (DBA/2 progenitors) independent experiments is presented. Error bars are derived from standard errors of the mean.


survival fraction
1
0.1
0.01
0.001
0.0001
0
2.5
5
7.5
10
[BPD] ng/ml
DBA/2 BM
L1210

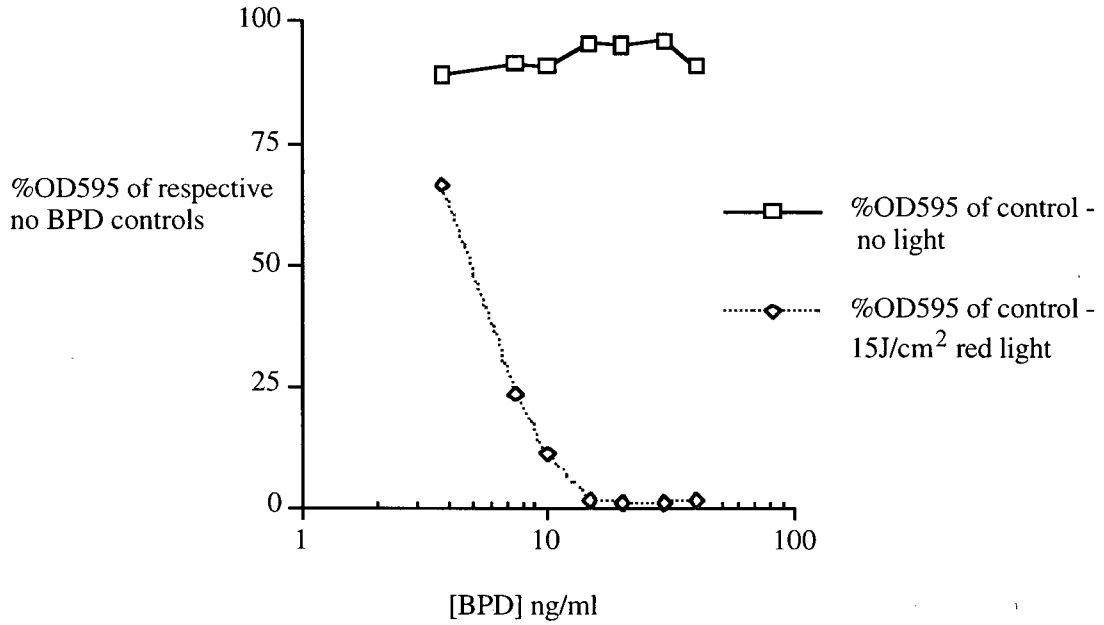
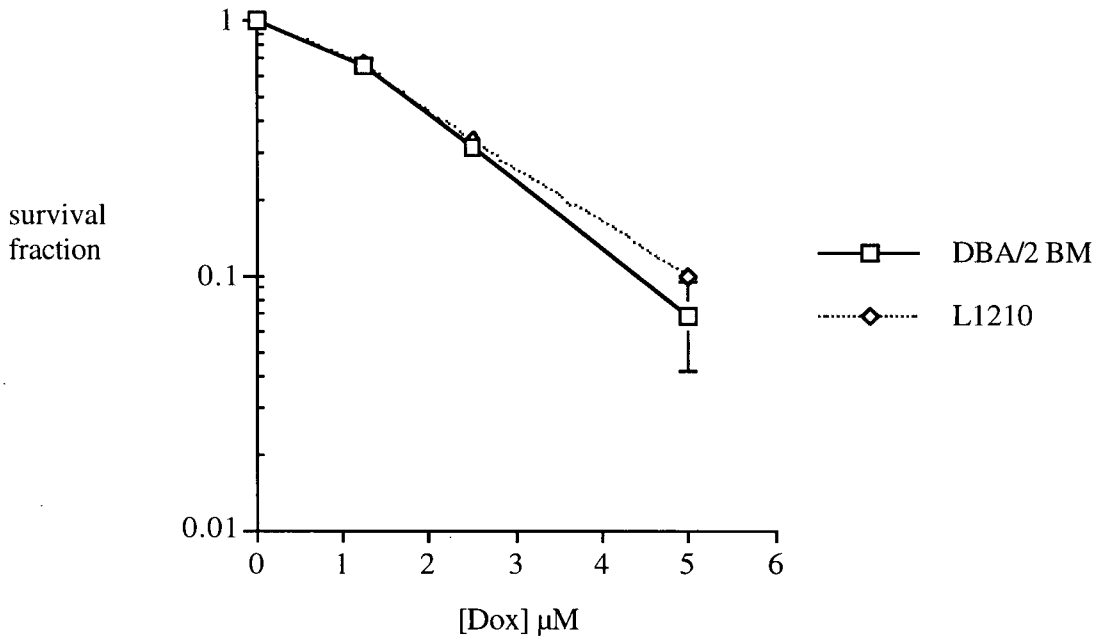


Figure 3.2 Cytotoxicity of BPD in the absence or presence of 15 J/cm^2 red light irradiation on L1210 cells as determined by the MTT viability assay

L1210 cells were incubated with different doses of BPD for 1 hr followed by red light exposure at 15 J/cm^2 or no illumination. Viability of the cells was determined after overnight incubation at 37° C using the MTT viability assay. OD_{595} of cells that received no BPD was arbitrarily designated to be 100 %. BPD- mediated PDT is only cytotoxic when activated by light and inhibited 50 % of the L1210 cells ($IC50_{L1210}$) at 6 ng/ml. Cells which had received BPD but no light remained viable. Data from a single representative experiment is presented.

Figure 3.3 The effect of Dox and 15 J/cm^2 red light on the clonogenicity of DBA/2 haematopoietic progenitor cells and L1210 leukaemic cells

Differential susceptibility of L1210 leukaemic cells and DBA/2 haematopoietic progenitors to Dox and light is minimal. Cells received different doses of Dox and exposed to 15 J/cm^2 of red light (600- 900 nm). Colony forming units of normal and leukaemic cells were determined with standard assays and colonies were scored on day 6 (CFU- L) and day 7 (CFU- GM) of culture using an inverted microscope. Untreated L1210 cells and DBA/2 progenitors yielded 119.33 $\pm$ 9.67 and 97.35 $\pm$ 7.78 colonies, which translated to clonogenicity of 36 % and 0.126 % respectively Survival fractions of treated cells were calculated based on the number of colonies generated divided by the number of colonies from the untreated control. Data obtained from 5 (L1210) and 6 (DBA/2 progenitors) independent experiments is presented. Error bars are derived from standard errors of the mean.


survival
fraction
1
0.1
0.01
0
1
2
3
4
5
6
[Dox] μM
DBA/2 BM
L1210

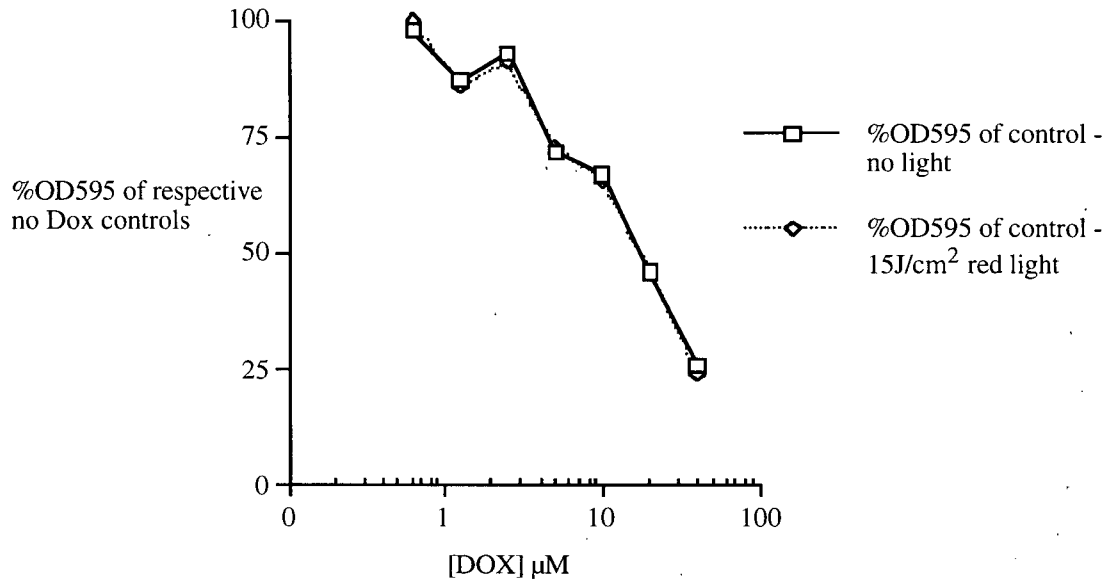


Figure 3.4 Cytotoxicity of Dox in the absence or presence of 15 J/cm^2 red light irradiation on L1210 cells as determined by the MTT viability assay

L1210 cells were incubated with different doses of Dox followed by red light exposure at 15 J/cm^2 or no illumination. Viability of the cells was determined after overnight incubation at 37°C using the MTT viability assay. OD_{595} of cells that received no Dox was arbitrarily designated to be 100 %. Light activation subsequent to Dox incubation did not alter the viability of L1210 cells. The concentrations of Dox which inhibited 50 % of L1210 cells therefore were similar at 20 μM, in the presence or absence of 15 J/cm^2 red light exposure. Data from a single representative experiment is presented.

Combination experiments with BPD- mediated PDT and Dox: simultaneous and sequenced treatments of DBA/2 haematopoietic progenitor cells

To examine the possible cytotoxic interactions between BPD- mediated PDT and Dox, DBA/2 bone marrow cells were treated with various combinations of the above two agents, simultaneously and sequentially. Coincubation of DBA/2 haematopoietic progenitor cells with 5.0 ng/ml BPD and 2.5 μM Dox followed by 15 J/cm^2 red light exposure (Dox/ PDT) resulted in a modest increase in cytotoxicity compared to PDT or Dox single agent treatments (Figure 3.5). Cytotoxicity did not change appreciably from the simultaneous regimen when the cells were treated first with PDT followed by one hour incubation with 2.5 μM Dox (PDT-> Dox). When DBA/2 haematopoietic progenitors were incubated with Dox for one hour prior to PDT treatment (Dox-> PDT), a moderate reduction of the survival fraction of normal CFU- GM from 0.47 $\pm$ 0.08 to 0.15 $\pm$ 0.04 or a 3.2- fold increase in killing compared to reverse sequence was observed (PDT-> Dox). Therefore, preincubation with Dox appeared to presensitise cells to the subsequent PDT treatment. As shown in figure 3.6, similar findings were made when 5.0 ng/ml BPD was combined with 1.25 μM Dox in the different combinations. Figures 3.7 and 3.8 show that the sequence of Dox-> PDT using 2.5 ng/ml of BPD in combinations with 2.5 or 1.25 μM Dox effected superior killing compared to PDT-> Dox and the simultaneous use of Dox/ PDT. It should be noted that a less than one log reduction in cell numbers was mediated by the treatments shown in figures 3.6 and 3.7 since a lower dose of BPD was used.

Combination experiments with BPD- mediated PDT and Dox: simultaneous and sequenced treatments of L1210 leukaemic cells

The clonogenicity of the leukaemic cell line L1210 was similarly affected by the combination treatments as normal haematopoietic progenitors. As demonstrated in figure 3.5- 3.8, preincubation of L1210 cells with either 1.25 or 2.5 μM of Dox for one hour

increased the efficacy of subsequent PDT- mediated cell killing. The resultant cytotoxicity was significantly greater than the reverse sequence PDT-> Dox and simultaneous treatment. In addition, L1210 cells were more susceptible to the Dox-> PDT combination regimen than DBA/2 haematopoietic progenitors. As shown in figure 3.5, the survival fraction of L1210 cells was significantly reduced from 0.10 ± 0.07 to 0.0035 ± 0.005, or a 28.8- fold increase in killing, when the cells were exposed to 2.5 μM Dox prior to PDT mediated by 5.0 ng/ml PDT (Dox-> PDT) compared to the reverse sequence PDT-> Dox.

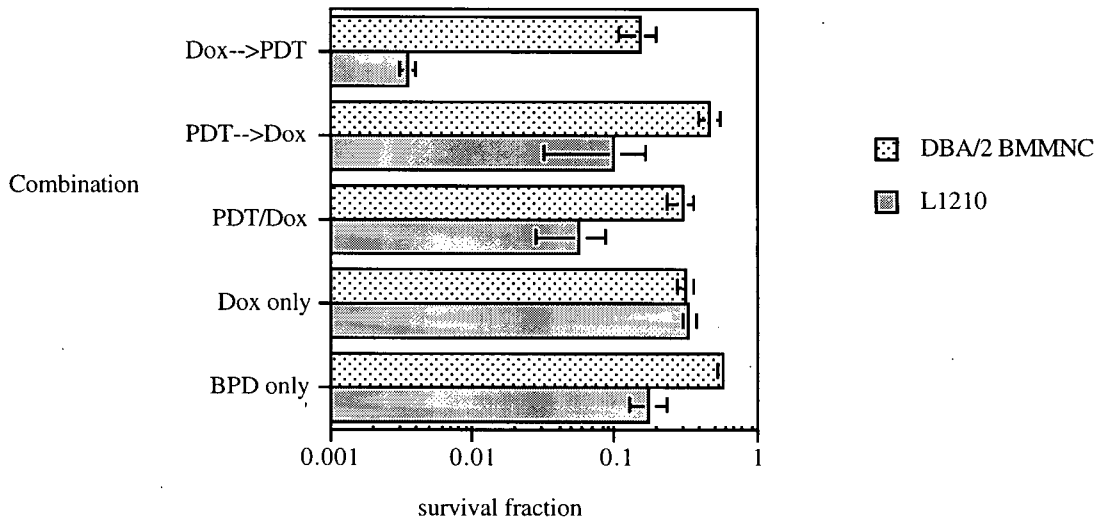


Figure 3.5 Survival fraction of DBA/2 haematopoietic progenitors and L1210 cells to combinations of 5 ng/ml BPD and 2.5 μM Dox in conjunction with 15 J/cm^2 of red light exposure.

Simultaneous Dox/ PDT and PDT-> Dox treatments using 5 ng/ml of BPD and 2.5 μM of Dox yielded similar reductions in the clonogenicity of DBA/2 CFU- GM and L1210 CFU- L. However, L1210 cells were more susceptible to the Dox-> PDT treatment than its nonmalignant counterpart, resulting in the enlargement of the therapeutic window. Data obtained from 6 (Dox/PDT of L1210 and DBA/2 cells) and 3 (Dox-> PDT and PDT-> Dox of L1210 and DBA/2 cells) independent experiments is presented. Error bars are derived from standard errors of the mean.

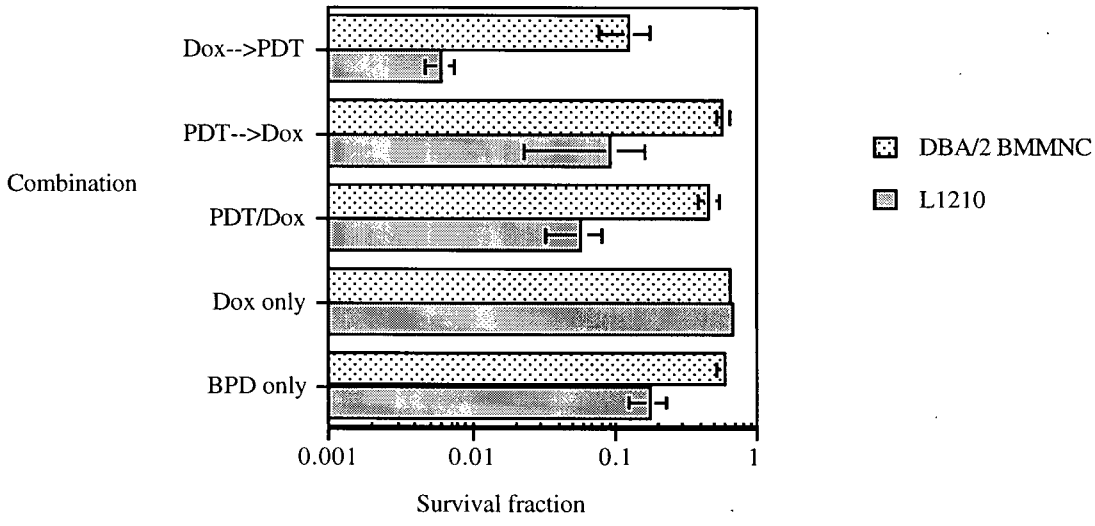


Figure 3.6 Survival fraction of DBA/2 haematopoietic progenitors and L1210 cells to combinations of 5 ng/ml BPD and 1.25 μM Dox in conjunction with 15 J/cm^2 of red light exposure

Simultaneous Dox/ PDT and PDT-> Dox treatments using 5 ng/ml of BPD and 1.25 μM of Dox yielded similar reductions in the clonogenicity of DBA/2 CFU- GM and L1210 CFU- L. However, L1210 cells were more susceptible to the Dox-> PDT treatment than its nonmalignant counterpart, resulting in the enlargement of the therapeutic window. Data obtained from 6 (Dox/PDT of L1210 and DBA/2 cells) and 3 (Dox-> PDT and PDT-> Dox of L1210 and DBA/2 cells) independent experiments is presented. Error bars are derived from standard errors of the mean.

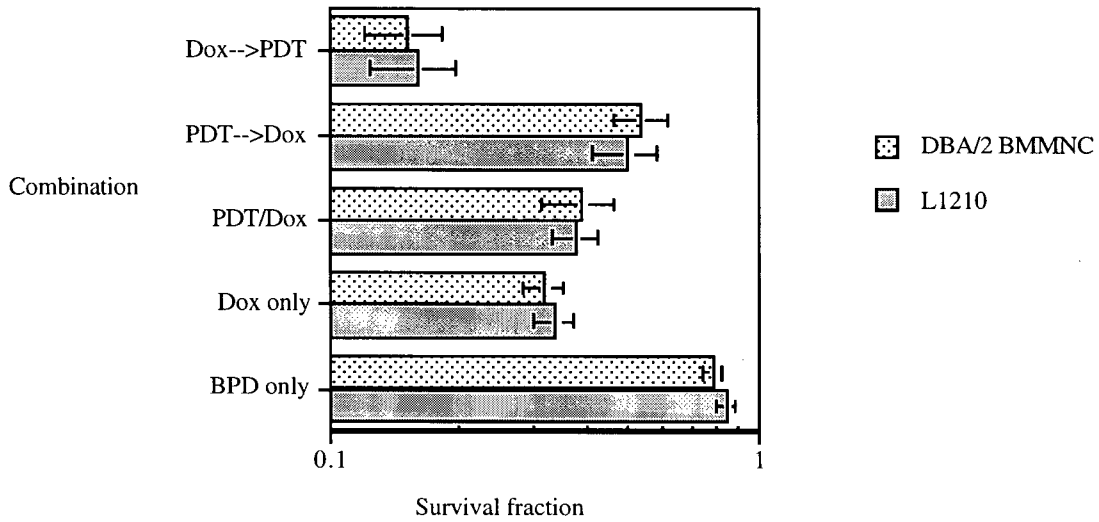


Figure 3.7 Survival fraction of DBA/2 haematopoietic progenitors and L1210 cells to combinations of 2.5 ng/ml BPD and 2.5 μM Dox in conjunction with 15 J/cm^2 of red light exposure

Preferential killing of L1210 cells of the Dox-> PDT treatment protocol disappeared when a lower dose of BPD was used. Clonogenicity of normal CFU- GM and leukaemic CFU- L were reduced by the same amount when 2.5 ng/ml of BPD was used with 2.5 μM of Dox. Data obtained from 6 (Dox/PDT of L1210 and DBA/2 cells) and 3 (Dox-> PDT and PDT-> Dox of L1210 and DBA/2 cells) independent experiments is presented. Error bars are derived from standard errors of the mean.

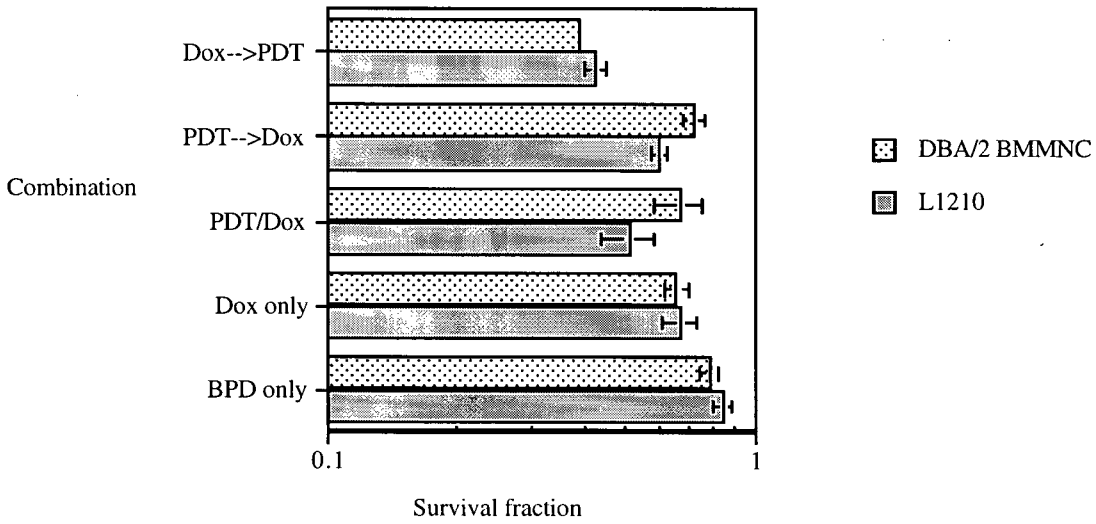


Figure 3.8 Survival fraction of DBA/2 haematopoietic progenitors and L1210 cells to combinations of 2.5 ng/ml BPD and 1.25 μM Dox in conjunction with 15 J/cm^2 of red light exposure

Preferential killing of L1210 cells of the Dox-> PDT treatment protocol disappeared when a lower dose of BPD was used. Clonogenicity of normal CFU- GM and leukaemic CFU- L were reduced similarly when 2.5 ng/ml of BPD was used with 1.25 μM of Dox. Data obtained from 6 (Dox/PDT of L1210 and DBA/2 cells) and 3 (Dox-> PDT and PDT-> Dox of L1210 and DBA/2 cells) independent experiments is presented. Error bars are derived from standard errors of the mean.

Differential susceptibility of L1210 cells and DBA/2 haematopoietic progenitors to Dox-> PDT sequenced combination treatment

To further investigate the effects that treatments which combine Dox and BPD-mediated PDT had on the cytotoxicity therapeutic window between DBA/2 haematopoietic progenitors and L1210 cells, data from the above combinations were represented as ratios of L1210 CFU- L survival fractions over DBA/2 CFU- GM survival fractions (Figure 3.9). A therapeutic ratio of 1 implies equivalent killing of normal and leukaemic cells. On the other hand, a ratio higher than 1 denotes preferential killing of the leukaemic cells in lieu of normal haematopoietic cells. Single agent PDT treatment using BPD at 5.0 ng/ml was moderately effective in the selective eradication of L1210 clonogenic cells with a therapeutic ratio of 3.30. Dox, however, was not selective in its killing of leukaemic cells with therapeutic ratios of 0.94 and 0.98 at 2.5 μM and 1.25 mM, respectively. The advantage of treating cells with Dox prior to PDT (Dox-> PDT) was clearly demonstrated in combinations which utilised 5.0 ng/ml BPD with 2.5 μM or 1.25 μM Dox; however, preferential killing of L1210 cells was not realised when the lower dose of BPD at 2.5 ng/ml was used. Combination treatments which involved the simultaneous administration of PDT and Dox (Dox/PDT) as well as PDT before Dox (PDT- Dox) yielded similar therapeutic ratios. Again, advantageous therapeutic ratios were achieved only at the higher BPD dose of 5.0 ng/ml but not at 2.5 ng/ml.

Statistical analysis of combination treatments

The significance of results from the cytotoxicity assays was determined using the two- way analysis of variance (ANOVA) test with term significant at alpha (ρ- value) = 0.05. Data from combination treatments of DBA/2 haematopoietic progenitor cells and L1210 leukaemic cells were independently analysed by arbitrarily setting the concentration of Dox (1.25 and 2.5 μM) as the constant and testing the significance of the resultant survival fractions of the two different doses of BPD (2.5 and 5.0 ng/ml) and the three

combination treatment regimens were used (Dox/PDT, PDT-> Dox, Dox-> PDT). As shown in table 3.1a, there were significant differences between 2.5 and 5.0 ng/ml BPD as well as amongst the three combination treatment regimens when 2.5 μM Dox was used in cytotoxicity assays involving DBA/2 haematopoietic progenitor cells; however, at the lower dose of Dox (1.25 μM), only the combination regimens were significantly different from each other. Next, intergroup differences were compared with the Bonferroni (all- pairwise) multiple comparison test, a more powerful analysis tool which provided a more detailed picture of the significance of differences between the groups. As shown in table 3.1b, cytotoxicity results from combination treatments which incorporated the higher dose of Dox of 2.5 μM were significantly different from each other in terms of the dose of BPD used (2.5 and 5.0 ng/ml). More importantly, Bonferroni analysis revealed that the regimen Dox-> PDT was significantly different from the other combination regimens, PDT-> Dox and Dox/PDT. At the lower Dox concentration of 1.25 μM, significant intergroup difference was only demonstrated between Dox-> PDT and PDT-> Dox.

The same analyses were performed on clonogenicity data of L1210 cells treated with the different combinations. In table 3.2a, two- way ANOVA demonstrated significance of the results of combination experiments which utilised the higher Dox dose of 2.5 μM. Experiments which used the lower Dox dose of 1.25 μM were only significant in terms of treatment sequence but not in the dose of BPD used. Again, Bonferroni (all-pairwise) multiple comparison test was used to provide more statistical details. Table 3.2b shows that the Dox-> PDT combination was significantly different from PDT-> Dox and Dox/PDT when 2.5 μM of Dox was used. However, the three combinations were not significantly different when a lower dose of Dox was used.

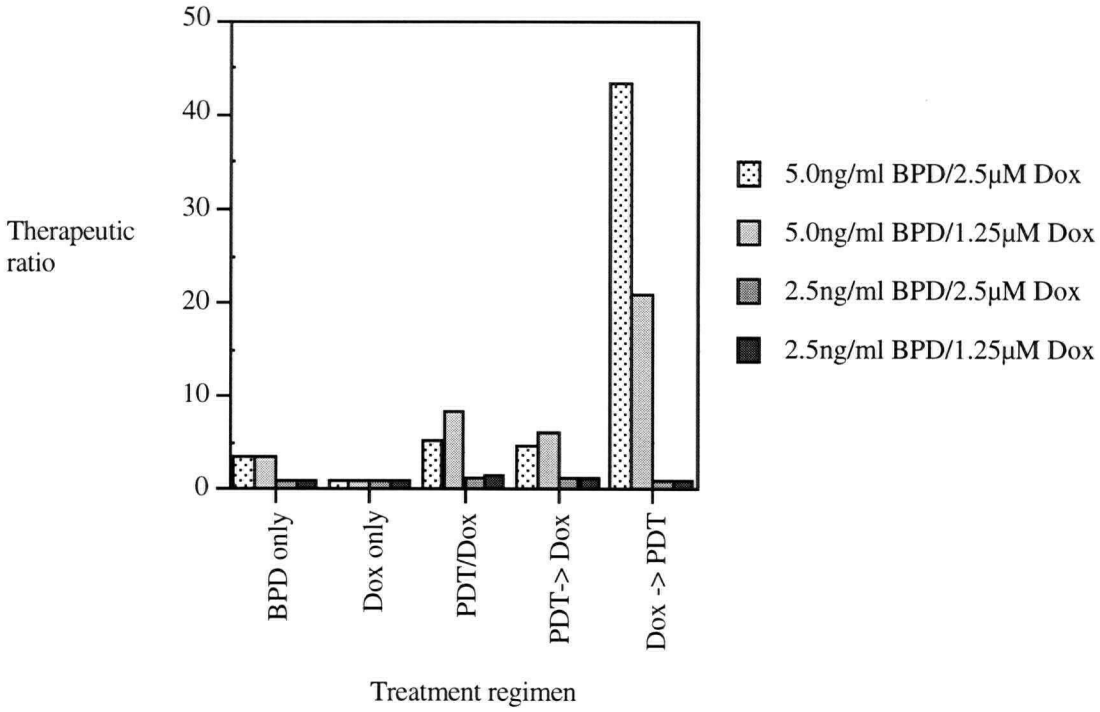


Figure 3.9 Differential killing of L1210 cells over DBA/2 progenitors in the different PDT/ Dox combinations

Preferential killing of L1210 cells in the various PDT/Dox combinations are expressed as therapeutic ratios and are derived from the ratios of survival fractions of DBA/2 CFU- GM over the survival fractions of L1210 CFU-L. Therefore, a therapeutic ratio of 1 denotes equal killing of normal and leukaemic cells. Ratios over 1 suggest preferential killing of the L1210 cells over normal DBA/2 bone marrow cells. Preferential killing of L1210 cells in the Dox-> PDT regimen was dependent on the BPD concentration since the lower BPD dose of 2.5 ng/ml effected no enhanced cytotoxicity.

Table 3.1a Two- way analysis of variance (ANOVA) of cytotoxicity data from DBA/2 haematopoietic progenitor cells treated with the three different drug combinations involving Dox and BPD-mediated PDT: significance of treatment sequence and BPD dose

	BPD dose (2.5 vs 5.0 ng/ml)	Treatment sequence (Dox/PDT, PDT-> Dox, Dox-> PDT)
2.5 μM Dox	+	+
1.25 μM Dox	-	+

Two- way analysis of variance (ANOVA) was used to determine the significance of the different experimental groups by arbitrarily setting the Dox concentrations (2.5 and 1.25 μM) as the constants and examining the significance of BPD doses (2.5 vs 5.0 ng/ml) as well as the three treatment regimens (Dox/ PDT, PDT-> Dox, Dox-> PDT). The plus sign (+) within shaded cell indicates significant difference with probability level less than a predetermined α value of 0.05 ($p< 0.05$).

Table 3.1b Statistical analyses of data from the different Dox/PDT combinations from DBA/2 haematopoietic cells: Bonferroni (all- pairwise) multiple- comparison testings of BPD concentrations and treatment sequences

	BPD dose (2.5 vs 5.0 ng/ml)	Treatment sequence
2.5 μM Dox	ϕ	
1.25 μM Dox		

2.5 μM Dox	Dox/ PDT	PDT-> Dox	Dox-> PDT
Dox/ PDT			
PDT-> Dox			
Dox-> PDT	++	++	

1.25 μM Dox	Dox/ PDT	PDT-> Dox	Dox-> PDT
Dox/ PDT			
PDT-> Dox			
Dox-> PDT		++	

Bonferroni (all-pairwise) multiple comparison analysis was performed on cytotoxicity data from DBA/2 haematopoietic progenitor cells treated with the different combinations of BPD- mediated PDT and Dox. Again, the concentration of Dox was arbitrarily designated the constant in the analysis. The ϕ sign (ϕ) within the shaded cell in the middle column indicates an significant intergroup difference between the two BPD concentrations of 2.5 and 5.0 ng/ml within a constant Dox concentration. The plus sign (++) within the cells in the rightmost column indicate intergroup significant difference between the represented treatment regimen within a constant Dox concentration of either 1.25 or 2.5 μM.

Table 3.2a Two- way analysis of variance (ANOVA) of cytotoxicity data from L1210 leukaemic cells treated with the three different drug combinations involving Dox and BPD- mediated PDT: significance of treatment sequence and BPD dose

	BPD dose (2.5 vs 5.0 ng/ml)	Treatment sequence (Dox/PDT, PDT-> Dox, Dox-> PDT)
2.5 μM Dox	+	+
1.25 μM Dox	+	-

Two- way analysis of variance (ANOVA) was used to determine the significance of the different experimental groups by arbitrarily setting the Dox concentrations (2.5 and 1.25 μM) as the constants and examining the significance of BPD doses (2.5 vs 5.0 ng/ml) as well as the three treatment regimens (Dox/ PDT, PDT-> Dox, Dox-> PDT). The plus sign (+) within shaded cell indicates significant difference with probability level less than a predetermined α value of 0.05 ($p < 0.05$).

Table 3.2b Statistical analyses of data from the different Dox/ PDT combinations from L1210 leukaemic cells: Bonferroni multiple- comparison testings of BPD concentrations and treatment sequences

	BPD dose (2.5 vs 5.0 ng/ml)	Treatment sequence
2.5 μM Dox	ϕ	
1.25 μM Dox	ϕ	

	Dox/ PDT	PDT- > Dox	Dox- > PDT
Dox/ PDT			
PDT- > Dox			
Dox- > PDT	++	++	

	Dox/ PDT	PDT- > Dox	Dox- > PDT
Dox/ PDT			
PDT- > Dox			
Dox- > PDT			

Bonferroni (all- pairwise) multiple comparison analysis was performed on cytotoxicity data from L1210 leukaemic cells treated with the different combinations of BPD- mediated PDT and Dox. Again, the concentration of Dox was arbitrarily designated as the constant in the analysis. The ϕ sign (ϕ) within the shaded cells in the middle column indicate an intergroup significant difference between the two BPD concentrations of 2.5 and 5.0 ng/ml within a constant Dox concentration. The plus sign (++) within the cells in the rightmost column indicate intergroup significant difference between the represented treatment regimen within a constant Dox concentration of either 1.25 or 2.5 μM.

DISCUSSION

The utility of benzoporphyrin derivative- mediated photodynamic therapy (PDT) in autologous bone marrow purging was extensively explored by Jamieson and colleagues.[292-294] These investigators found that the monoacid ring- A analogue of benzoporphyrin derivative (BPD) was selectively taken up by leukaemic cells of human and mouse origin. On average, a six- fold increase in photosensitiser uptake over normal haematopoietic cells was observed. Further studies examining the cytotoxicity of BPD-mediated PDT on the clonogenicity of normal haematopoietic cells and leukaemic cells also demonstrated the selectivity of this modality of treatment. In addition, using the long- term bone marrow assay (LTBMC), which measures the growth of less developed cells of the haematopoietic system, these authors demonstrated that BPD- mediated PDT had minimal toxicity on human early haematopoietic progenitor cells. Photosensitisers other than BPD, such as merocyanine 540 (MC540) and the phthalocyanines are also being considered for PDT- mediated purging of contaminated bone marrow [275, 291].

PDT has also been used in conjunction with different treatment modalities such as heat and ionising radiation for *in vivo* applications.[303, 319, 320] In addition, various groups have combined PDT with chemotherapeutic agents, cytokines, and even multiple photosensitisers in an effort to improve the efficacy and selectivity of treatment.[305, 318, 321-323] Doxorubicin or Dox is an anthracycline- based antibiotic derived from the Streptomyces species and has been used in the management of various forms of cancer.[324] Daunorubicin and idarubicin are also members of the anthracycline family with extensive uses in antileukaemia therapy. Dox was chosen because its photophysical profile and cytotoxic mechanisms were unique from those of BPD. The *ex vivo* nature of pharmacological purging bypasses many of the constraints imposed by systemic pharmacokinetics *in vivo* and therefore permits greater latitude in dose and sequence experimentation in combination therapy.

The principal goal of the present project was to increase the efficacy of BPD-mediated photodynamic purging. In this system, when either normal DBA/2 haematopoietic progenitors or L1210 leukaemic cells were treated simultaneously with PDT and Dox (Dox/PDT), the resultant cytotoxicity was roughly additive compared to PDT or Dox single agent

treatment of the same dose. The same observations were made when cells were treated first with PDT followed by one hour incubation with Dox (PDT-> Dox). Interestingly, preincubation of the cells with Dox prior to PDT (Dox-> PDT) significantly increased the cytotoxicity of the treatment in both normal and leukaemic cells. More significantly, the L1210 cell line was much more susceptible to the Dox-> PDT regimen than normal DBA/2 haematopoietic progenitor cells with a resultant enlargement of the therapeutic window. As demonstrated in figure 3.9, the enhancement of differential killing of L1210 cells was only evident when the higher BPD dose of 5.0 ng/ml was used and the cells were exposed to Dox before PDT treatment. The results for DBA/2 haematopoietic progenitors and L1210 leukaemic cells treated with different combination regimens incorporating the higher Dox concentration of 2.5 μM were significantly different from each other as demonstrated by two- way ANOVA with $p< 0.05$. Importantly, there was significant intergroup difference between the Dox-> PDT sequence and the other two combination regimens of PDT-> Dox and Dox/PDT in both normal and leukaemic cells exposed to 2.5 μM of Dox. This phenomenon could be attributed to several events which will be the subject of chapter 4 of this thesis.

It is possible that pretreatment of cells with Dox could increase the subsequent uptake of the photosensitiser or reduce the amounts of cellular antioxidants such as glutathione. Daunorubicin, a congenor of Dox, increases ceramide production through its modulation of the enzyme ceramide synthase.[325] Ceramide is a sphingolipid which mediates several signal transduction pathways such as those downstreams of the tumour necrosis factor type I receptor (TNFr) and FAS; furthermore, addition of exogenous short chain ceramide analogue induced apoptotic cell death.[326] Separovic and colleagues recently demonstrated ceramide production in response to PDT mediated by the phthalocyanine photosensitiser Pc 4.[268] Pretreatment of cells with Dox could therefore raise intracellular ceramide to a critical level with exacerbation of subsequent PDT cytotoxicity. Recently, several groups have implicated the transcriptional factor NF- κβ in anthracycline- mediated cytotoxicity. Inhibition of NF- κβ nuclear translocation enhanced the cytotoxicity of daunorubicin, TNFα, and γ- radiation in HT1080 human fibrosarcoma cells which implicated NF- κβ activation as part of the normal response to environmental stresses which threaten cellular survival.[327] However, contradictory studies have shown that NF- κβ activation precedes cell death therefore the exact role of this ubiquitous transcriptional

factor in cellular demise could be quite idiosyncratic.[328] Das and White demonstrated that both doxorubicin and daunorubicin effected NF- κβ activation via protein kinase C.[329] Since PDT has been shown to activate NF- κβ, pretreatment of cells with Dox could conceivably alter the activation threshold of the transcriptional factor and therefore the resultant cytotoxicity.[264]

In summary, combination treatment of Dox and BPD- mediated PDT preferentially affected the leukaemic cell line L1210 over normal DBA/2 haematopoietic cells. Selective killing was only realised when Dox was used prior to PDT (Dox-> PDT) and at a dose of 5.0 ng/ml BPD and 15 J/cm^2 red light.

CHAPTER 4: COMBINED TREATMENT OF MURINE NORMAL HAEMATOPOIETIC AND LEUKAEMIC CELLS WITH DOXORUBICIN AND PDT: MECHANISTIC STUDY

4.1 ABSTRACT

Benzoporphyrin derivative monoacid ring- A (BPD) is a porphyrin- based photosensitiser with potential in the clinical application of photodynamic therapy (PDT). Previously, we have demonstrated the effectiveness of BPD in PDT purging of contaminated leukaemic cells from autologous haematopoietic stem cell harvests. In order to improve its efficacy, we combined BPD- mediated PDT with doxorubicin (Dox) and found that the sequence of Dox-> PDT was superior to simultaneous Dox/PDT and the reverse sequence of PDT-> Dox. In addition, the murine leukaemic cell line L1210 was much more susceptible to the Dox-> PDT regimen than normal DBA/2 haematopoietic progenitor cells. In this chapter, we studied some of the interactions between Dox and BPD and specifically the possible mechanisms responsible for the augmentation of PDT cytotoxicity effected by Dox preincubation. The unique photophysical and biological properties of BPD and Dox permit their cytotoxic cooperativity in the simultaneous Dox/PDT regimen. Fluorescent microscopy showed that BPD was restricted to the cytoplasm and the plasma membrane whereas Dox localised to both cytoplasmic and nuclear compartments. Dox- mediated inhibition of BPD photobleaching was observed at the excitation wavelength of 440 nm but not at 630 nm. Since a red light source emitting between 600- 900 nm was used in the cytotoxicity assays described in chapter 3, the presence of Dox should not affect BPD photoactivation. Spectrofluorimetric measurements of L1210 cell lysates revealed that uptake of BPD was reduced in the presence of Dox; however, preincubation of L1210 with Dox restored BPD accumulation to levels of control cells incubated with only BPD. Next, we proceeded to examine the cellular level of glutathione (GSH), an ubiquitous protective thiol. L1210 cells incubated with 5 μM of Dox for 1 h resulted in slight but reproducible reduction of cellular GSH. The above factors, in

conjunction with other mechanisms, could be responsible for the presensitisation of L1210 cells by Dox.

4.2 INTRODUCTION

Numerous factors can affect the efficiency of photodynamic therapy (PDT). Considerably more variables are involved when PDT is used *in vivo*; for example, the extent of first pass hepatic metabolism and the degree of binding to various serum proteins affect the delivery of the photosensitiser to the target site. Other factors of particular importance in oncology include tissue oncotic pressure, regional blood flow, and the hypoxic state of the tumour, which are governed by tumour size. The above concerns are not unique to PDT and are likewise of importance in the delivery of other forms of therapies such as chemotherapeutic agents and antibodies.[330] At the cellular level, photosensitiser uptake is also governed by similar factors which affect the uptake of other chemotherapeutic compounds. For example, molecular size and partition coefficient of the photosensitiser influence the cellular accumulation of the compound. Environmental factors such as the rate of cellular proliferation, intracellular and extracellular pH, cell size, and serum concentration all influence the uptake of the photosensitiser.[259, 331] The photosensitiser initially associates with the cell membrane then gradually relocalises to the different intracellular compartments such as the mitochondria and lysosomes.[332] In addition, private attributes such as charge characteristics and presence or absence of sidechain moieties further contribute to the intracellular localisation of the compound.[333] Cationic dyes such as merocyanine 540 (MC 540) and rhodamine 123 (Rh 123) preferentially accumulate in the mitochondria as a result of the electrochemical gradient whereas the porphyrin- based photosensitiser benzoporphyrin derivative monoacid ring- A (BPD), which is lipophilic, initially localises to the plasma membrane and then rapidly to various cellular organelles with longer incubation. In addition to photosensitiser accumulation, other factors also determine the effectiveness of PDT cytotoxicity. Of particular importance is the ratio of cellular pro- and antioxidants since PDT cytotoxicity is

primarily mediated by reactive oxygen species (ROIs) and radical formations.[255] Therefore, the amount of cellular antioxidants such as glutathione (GSH) and α- tocopherol can influence the extent of PDT damage and their modulations can alter PDT susceptibility.[261, 334, 335]

PDT combination therapy, through the incorporation of PDT with other treatment modalities or therapeutic agents, attempts to effect modulation on the cell or the organism in order to maximise cytotoxic effects on the target cell population. Manipulations of the timing, chemical and physical characteristics of the photosensitiser and drug dose, light fluence rate, as well as the activation wavelength can substantially affect the efficiency of PDT- mediated cytotoxicity. Therefore, introduction of another form of treatment into a PDT regimen can significantly increase its complexity and also the interpretation of data. Hyperthermia and ionising radiation have similar cytotoxic mechanisms as PDT and were naturally used in different variations of combinations with PDT. Several groups have demonstrated the beneficial effects of PDT prior to hyperthermia treatment and sequence specificity of this regimen was a result of tumour and vascular effects of PDT.[303, 309, 320] Chemotherapeutic agents such as etoposide and doxorubicin are also used in conjunction with PDT.[306, 308] Ma *et al.* observed that presence of mitomycin C (MMC) during Photofrin®- mediated PDT significantly increased its antitumour effects *in vitro* and *in vivo*.[304, 318] Some of the most innovative regimens involved combinations of different photosensitisers and activation wavelengths; for example, Cincotta and colleagues reported synergistic antitumour effects of PDT *in vivo* mediated by BPD and 5- ethylamino- 9- diethylaminobenzo[a] phenothiazinium chloride (EtNBS).[305] Coadministration of cytokines such as GM- CSF and TNFα potentiated the antitumour efficacy of PDT *in vivo*.[322, 336] Myers *et al.* observed that the addition of the immunostimulant, *C. parvum*, significantly enhanced PDT efficacy in an animal bladder tumour model.[337] These experiments highlighted the participatory role of the immune system in *in vivo* antitumour action mediated by PDT.

Various mechanisms have been proposed for the different forms of PDT combination therapy. In regimens involving combinations with other photosensitisers, two groups have noted that synergistic killing of tumour cells *in vitro* and *in vivo* was achieved with differential localisations of the agents.[305, 338] Combining photosensitisers or chemotherapeutic agents with unique photophysical and biochemical characteristics can

invariably ensure an increase in cellular targets. Ma *et al.* reported that cell cycle arrest followed by S- phase accumulation was responsible for the increased uptake of Photofrin® in the presence MMC.[304] Therefore, one can understand why simultaneous incubation of cells with MMC and Photofrin® was superior to administration of MMC post- PDT (P->light-> MMC). In this chapter, we attempted to study the interactions between BPD and Dox and specifically, to investigate the mechanisms responsible for the enhanced killing of L1210 cells when Dox was administered prior to BPD- mediated PDT (Dox-> PDT).

4.3 RESULTS

Photophysical properties of BPD and Dox

The molecular structure of benzoporphyrin derivative monoacid ring A (one regioisomer, BPD) is given in figure 4.1. BPD, a member of the porphyrin family, is a second generation photosensitiser which is more potent and purer than its predecessor Photofrin®. The structure of the anthracycline antibiotic doxorubicin (Dox, adriamycin) is shown in figure 4.2. Dox is a 14- hydroxy analogue of daunorubicin isolated from *Streptomyces peucetius* (var. *caesius*) and is used extensively in anticancer chemotherapy. The absorption spectra of BPD and Dox in 10 % HI- FCS/ PBS are given in figure 4.3 and 1 μg/ml (1.376 μM) of BPD and 10 μM of Dox were used during the analysis. The presence of the unique BPD absorption peak at 690 nm, in addition to several other peaks above 600 nm, permits BPD photoactivation at higher wavelength and is an unique feature of this photosensitiser. The spike observed at 656 nm, which is present in both Dox and BPD spectrum, is an artefact of the deuterium lamp used for the spectrophotometric scan. Dox has a prominent broad absorption peak at 500 nm and a smaller peak at 579 nm. Fluorescence emission spectra (λ_{ex} = 440 nm) of 10 ng/ml (13.76 nM) BPD and 5 μM Dox are presented in figure 4.4a. Note that this is the same BPD: Dox molar ratio that was used in the cytotoxicity assays described in chapter 3. At this ratio, Dox fluorescence emission

virtually concealed almost all of the BPD profile save the unique BPD emission peak at 693 nm, which remained detectable even in a solution containing 363.5 fold more of Dox. This is of particular significance for the interpretation of the photobleaching data in a subsequent section as well in the appreciation of the general distinctiveness between BPD and Dox. Figure 4.4b, which restricts the emission spectra to 650- 700 nm, highlights this unique property of BPD.

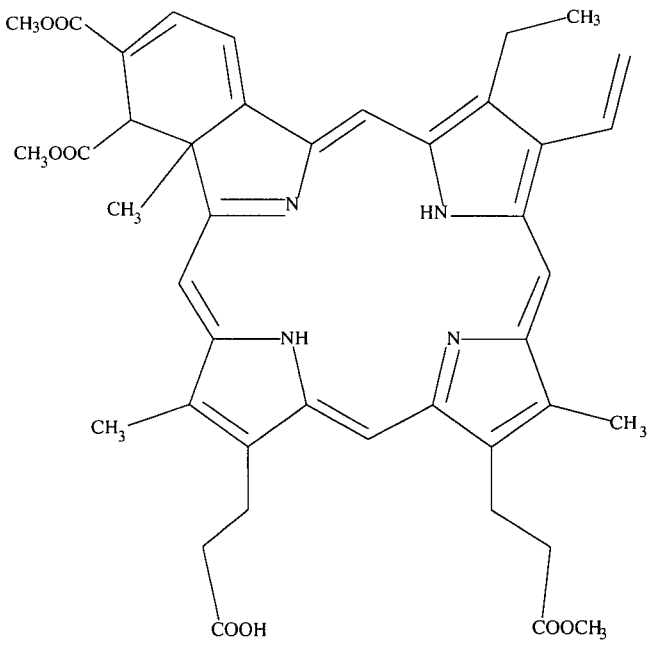


Figure 4.1 Chemical structure of the benzoporphyrin derivative monoacid ring- A (BPD, Verteporfin)

The molecular structure of one regioisomer of benzoporphyrin derivative monoacid ring A (BPD, Verteporfin) is shown.

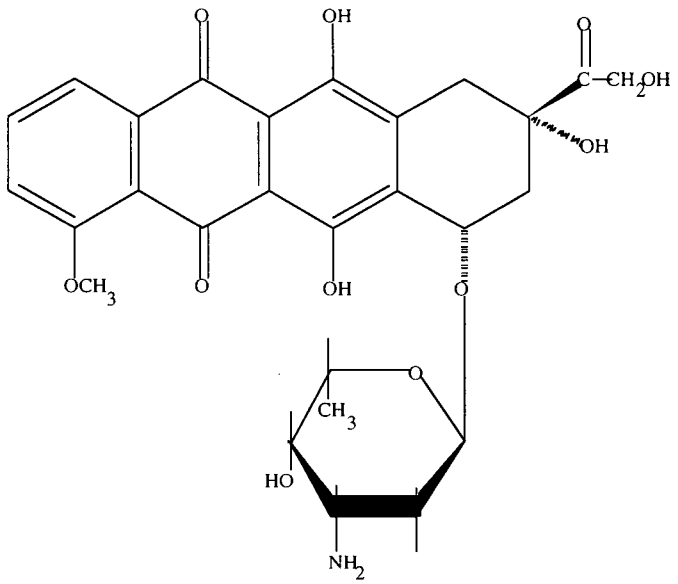


Figure 4.2 Chemical structure of Doxorubicin (Dox)

The molecular structure of Doxorubicin (Dox) is shown.

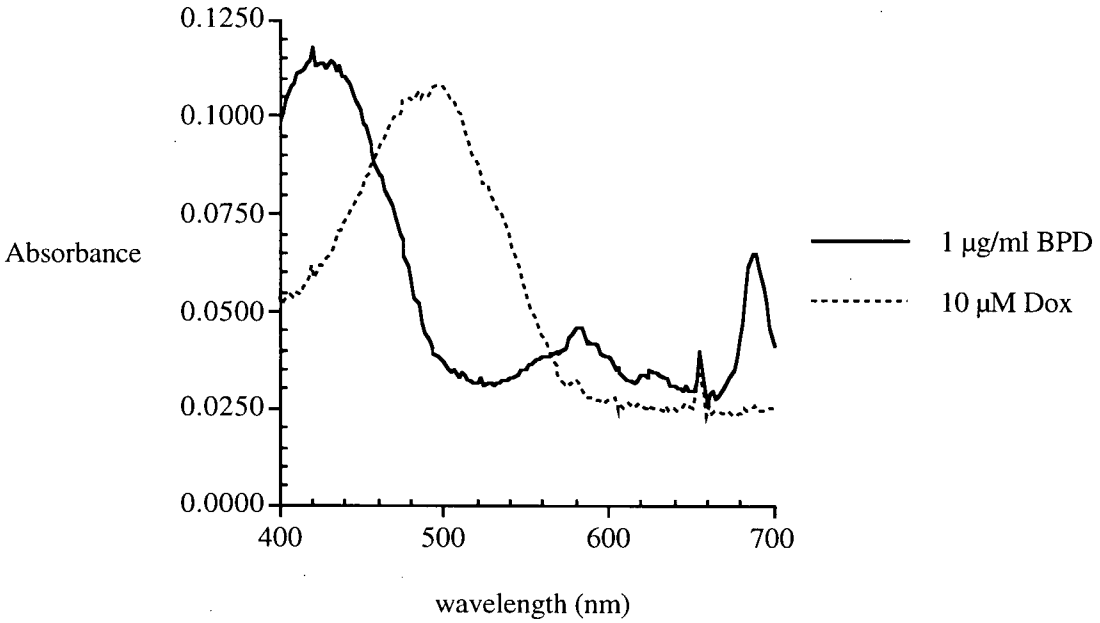


Figure 4.3 Absorption spectra of 1 µg/ml (1.376 µM) BPD and 10 µM Dox in 10 % HI- FCS/ PBS

The absorption spectra of 1 µg/ml (1.376 µM) BPD and 10 µM Dox in PBS containing 10% HI- FCS are presented. The 656 nm spike observed in both samples was an artefact of the deuterium lamp from the spectrophotometer.

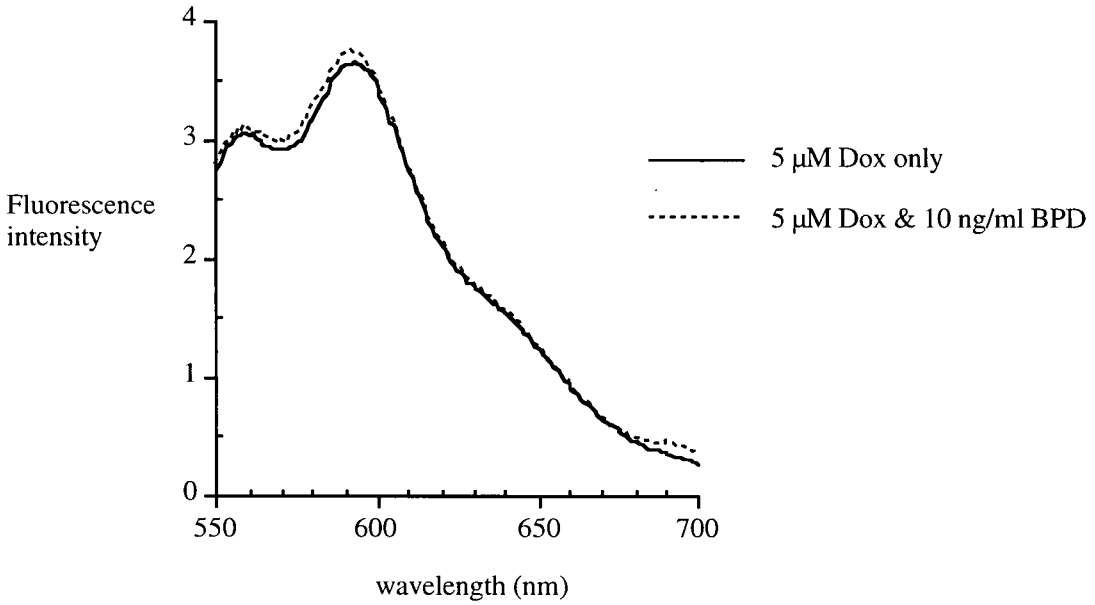


Figure 4.4a Fluorescence emission spectra of BPD in the presence of Dox (550 - 700 nm)

Superimposition of the fluorescence emission profile of 5 μM Dox and a mixture consisting of 5 μM Dox with 10 ng/ml BPD in PBS containing 10% HI- FCS. BPD has an emission peak at 693 nm which permits its activation even in the presence of Dox. Note that the molar ratio of BPD to Dox (1: 363.5) is the same that was used in the cytotoxicity assays. Settings for the spectrofluorimeter: continuous wavelength (CW) excitation at 440 nm with bandpass of 4 nm, emission at 700 nm with bandpass of 4 nm, 1 nm/s scan rate, photomultiplier voltage (PMT) was set at 650 V.

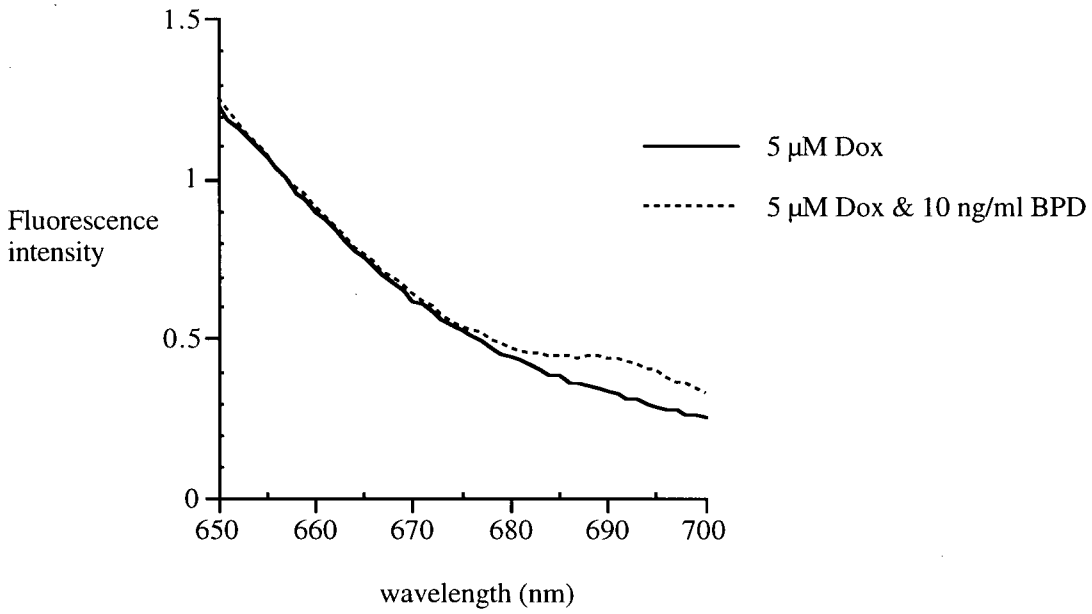


Figure 4.4b Fluorescence emission profile of BPD in the presence of Dox (650 - 700 nm)

Superposition of the fluorescence emission profile of 5 μM Dox and a mixture consisting of 5 μM Dox with 10 ng/ml BPD in PBS containing 10% HI- FCS. BPD has a emission peak at 693 nm, accentuated in this emission scan between 650- 700 nm, which permits its activation even in the presence of Dox. Note that the molar ratio of BPD to Dox (1: 363.5) is the same that was used in the cytotoxicity assays. Settings for the spectrofluorimeter: continuous wavelength (CW) excitation at 440 nm with bandpass of 4 nm, emission at 700 nm with bandpass of 4 nm, 1 nm/s scan rate, photomultiplier voltage (PMT) was set at 650 V.

Intracellular localisations of BPD and Dox in the human leukaemic cell line K562

To further delineate the differences between BPD and Dox, intracellular localisations of the two compounds were established by fluorescent microscopy. The cell line K562, originally derived from the pleural effusions of a chronic myelogenous leukaemia (CML) patient in blast crisis, was used for this study. Fluorescence was visualised with an Olympus New Vanox microscope attached to the AH2- FL transmitted fluorescence unit, a blue range barrier filter was used for both BPD and Dox observations. Pictures were taken with the attached camera using Fujichrome 400 ISO film. As presented in figure 4.5, K562 cells that were incubated with 10 μg/ml BPD for 60 min showed intense yet diffused cytoplasmic fluorescence around but not within the nuclear poles. In addition, discrete and punctate fluorescence patterns, suggesting BPD accumulation in cytoplasmic organelles, were also observed. This is consistent with the findings of Cincotta *et al.* using the murine mammary sarcoma cell line EMT- 6.[305] Dox, at 10 μM, demonstrated both nuclear and cytoplasmic fluorescence in a diffused pattern (figure 4.6). Interestingly, nuclear fluorescence of Dox was much stronger than cytoplasmic fluorescence which implies significant nuclear accumulation of Dox. The fluorescence pattern of Dox is in agreement with known properties of this compound which targets the lipid membrane bilayers, various cellular enzymes, and the genetic materials of the cell.[324] In figure 4.7, K562 cells were incubated simultaneously with 10 μg/ml BPD and 10 μM Dox for 60 min and observed under the fluorescent microscope at the same settings as before. All the cells demonstrated the red cytoplasmic fluorescence pattern of BPD while Dox nuclear fluorescence was observed in some of the cells. Differential localisations of BPD and Dox to the different compartments of the K562 cell line, in addition to their unique but parallel cytotoxic mechanisms of action, support their use in combination PDT therapy.

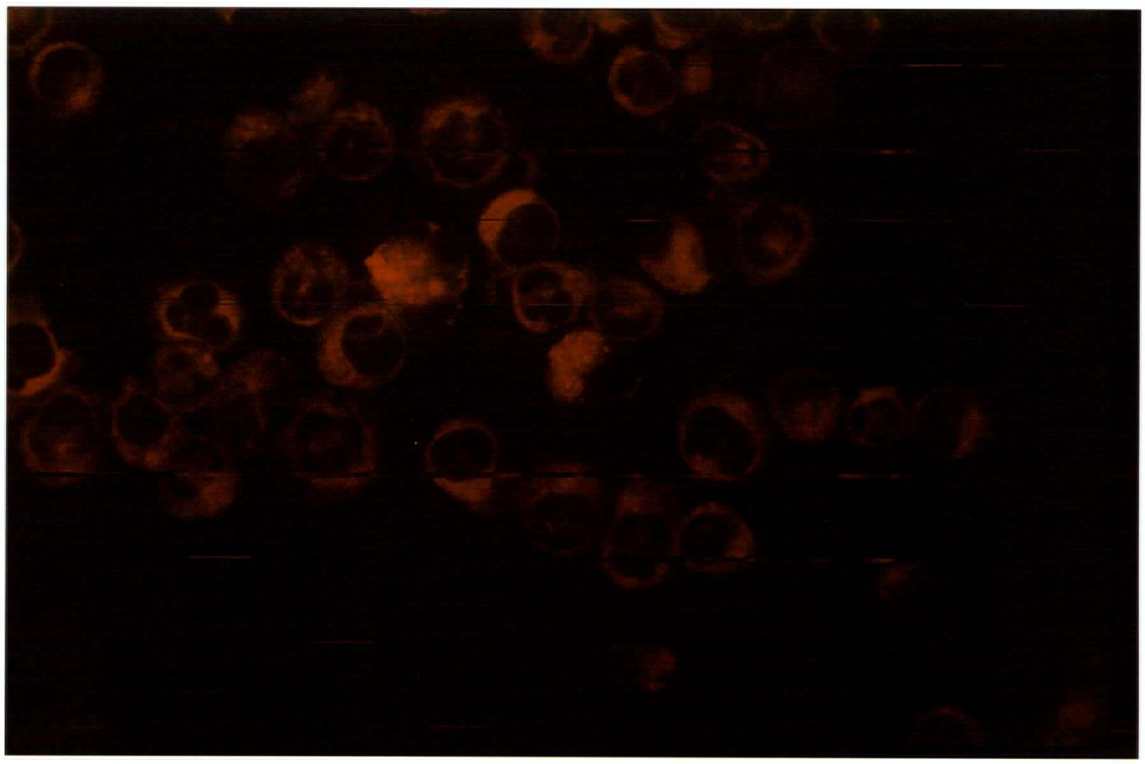


Figure 4.5 Fluorescence micrograph of K562 leukaemic cells incubated with BPD

K562 Cells were resuspended at a concentration of 1 x 10^6 cells/ml in serum- free DMEM and incubated with 10 µg/ml of BPD for 60 min. Cells were then washed once and resuspended in 1 ml of medium. A drop containing 50 µl or 5 x 10^4 of cells was placed on a glass slide and subsequently covered with a cover slip. The cells were observed under an Olympus New Vanox microscope with the AH2- FL transmitted light fluorescence attachment on "Blue" setting (excitation @ 380- 490 nm, observation @ 515 nm +).

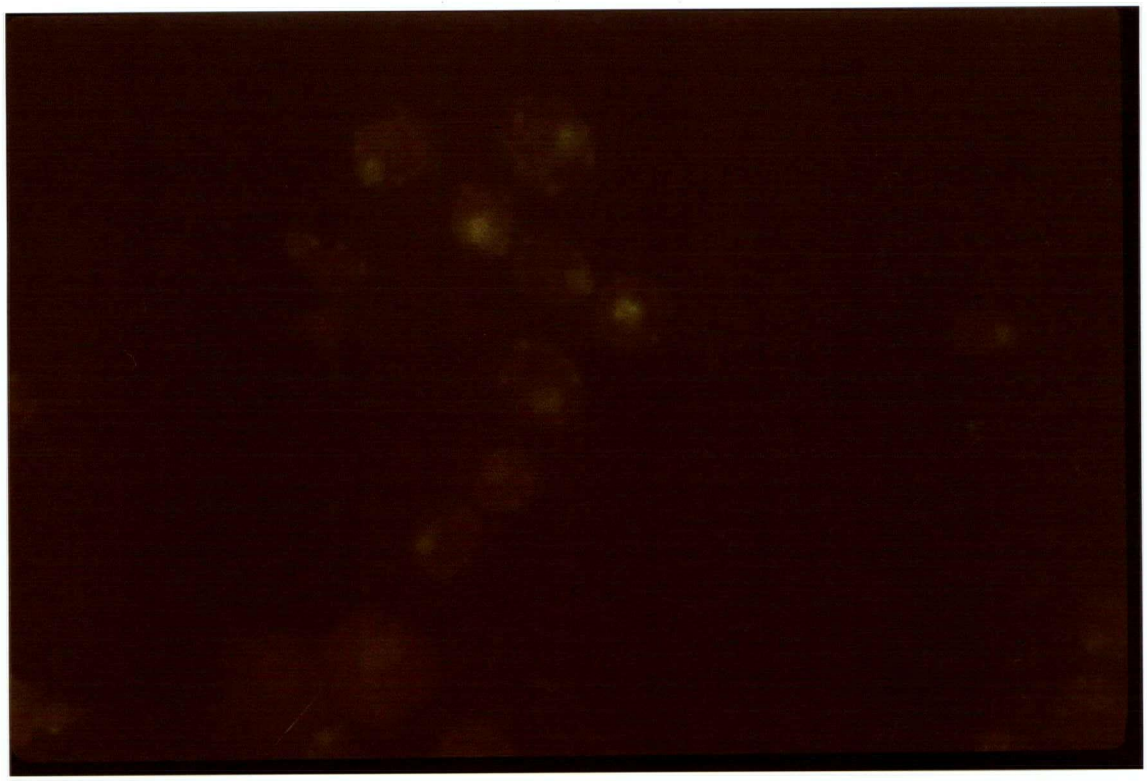


Figure 4.6 Fluorescence micrograph of K562 leukaemic cells incubated with Dox

K562 Cells were resuspended at a concentration of 1 x 10^6 cells/ml in serum- free DMEM and incubated with 10 μM of Dox for 60 min. Cells were then washed once and resuspended in 1 ml of medium. A drop containing 50 μl or 5 x 10^4 of cells was placed on a glass slide and then covered with a cover slip. The cells were observed under an Olympus New Vanox microscope with the AH2- FL transmitted light fluorescence attachment on "Blue" setting (excitation @ 380- 490 nm, observation @ 515 nm +).

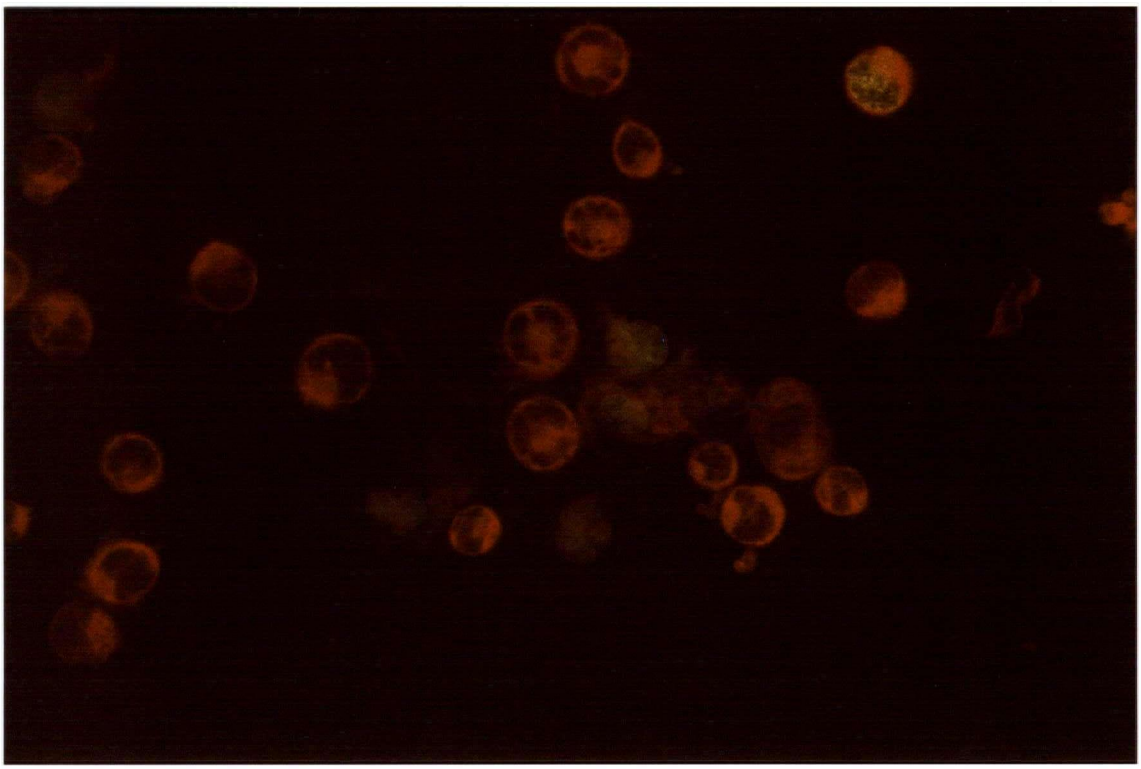


Figure 4.7 Fluorescence micrograph of K562 leukaemic cells incubated with BPD and Dox

K562 Cells were resuspended at a concentration of 1 x 10^6 cells/ml in serum- free DMEM and incubated with 10 μg/ml (13.76 μM) BPD and 10 μM Dox for 60 min. Cells were then washed once and resuspended in 1 ml of medium. A drop containing 50 μl of cells was placed on a glass slide and thereafter covered with a cover slip. The cells were observed under an Olympus New Vanox microscope with the AH2- FL transmitted light fluorescence attachment on "Blue" setting (excitation @ 380- 490 nm, observation @ 515 nm +).

The role of excitation wavelength (λ_{ex}) has on the photobleaching of BPD in the presence of Dox

To further investigate the interactions between BPD and Dox, the two compounds were mixed together in a solvent consisting of 10 % fresh human plasma in phosphate buffered saline (10 % HP/ PBS) and photobleaching of BPD in the presence of different concentrations of Dox was measured. Photobleaching is the process of irreversible autodegradation of the excited photosensitiser and originates from the triplet excited state of the compound as a result of intersystem crossing from the activated singlet state. However, it is inherently a complex phenomenon and can also occur from the singlet state. BPD does not photobleach readily in organic solvents such as methanol or dichloromethane; however, photobleaching is significantly enhanced in the presence of human plasma or foetal calf serum (data not shown). This is consistent with findings by Aveline *et al.*[279] In the present study, BPD photobleaching was used as a surrogate marker of photosensitiser activation. As demonstrated in figure 4.8, the mean fluorescence intensity of 10 ng/ml of BPD at 693 nm ($MFI_{693\ nm}$) decreased steadily when the compound was subjected to continuous light exposure at the excitation wavelength (λ_{ex}) of 440 nm (0 μM Dox, bottommost curve). In fact, the $MFI_{693\ nm}$ at the end of a 15 min exposure period was 43.2 % of the starting level. Interestingly, BPD photobleaching was ameliorated when increasing concentrations of Dox were introduced into the reaction cuvette. To control for any contributions of Dox fluorescence to the observed reduction of BPD photobleaching, different concentrations of Dox were subjected to the same photoactivation protocol and Dox fluorescence emissions at 593 nm (fluorescence emission peak unique to Dox) and 693 nm (fluorescence emission peak unique to BPD) were monitored for 15 min. In both cases, MFI was not significantly altered at the end of the observation period suggesting Dox itself was not affected by the light exposure and the reduction of BPD photobleaching in the presence of Dox was caused by some form of molecular interaction between Dox and BPD. Figure 4.9 summarises the results of three independent BPD photobleaching experiments in terms of percent reductions of $MFI_{693\ nm}$ at the end of the 15 min exposure period compared to starting $MFI_{693\ nm}$. In the experiments described above, photobleaching or photoactivation of BPD was found to be inversely correlated with the concentration of Dox present when λ_{ex} of 440

nm was used. Since a red light source emitting between the range of 600- 900 nm was used for BPD photoactivation in the cytotoxicity assays described in chapter 3, the photobleaching experiments were repeated with λ_{ex} set at 630 nm. Figure 4.10 compares the effects of Dox had on BPD photobleaching between λ_{ex} of 440 nm and 630 nm. The data show that at λ_{ex} of 630 nm, reduction of BPD photobleaching by Dox was much less obvious than at λ_{ex} of 440 nm. In fact, photobleaching of BPD was dramatically lower at the former wavelength. Figure 4.11 illustrates that 1 mM of Dox in 10 % HI- FCS/ PBS does not appreciably interfere with light transmission above 600 nm. At 630 nm, light transmittance was 89.39 % and reached a plateau at 720 nm (97.83 %). Since a much lower concentration of Dox (5- 10 μM) was used in the cytotoxicity assays (chapter 3) and in the spectrofluorimetric analyses described in this section, the presence of Dox should have minimal negative effect on light transmission to the BPD molecules in the solution when λ_{ex} of above 600 nm was used. The results, in sum, suggest that the presence of Dox did not affect the efficiency of red light activation of BPD during the cytotoxicity assay and the observed attenuation of BPD activation by Dox at 440 nm was most probably secondary to shielding of light by the abundance of Dox in solution.

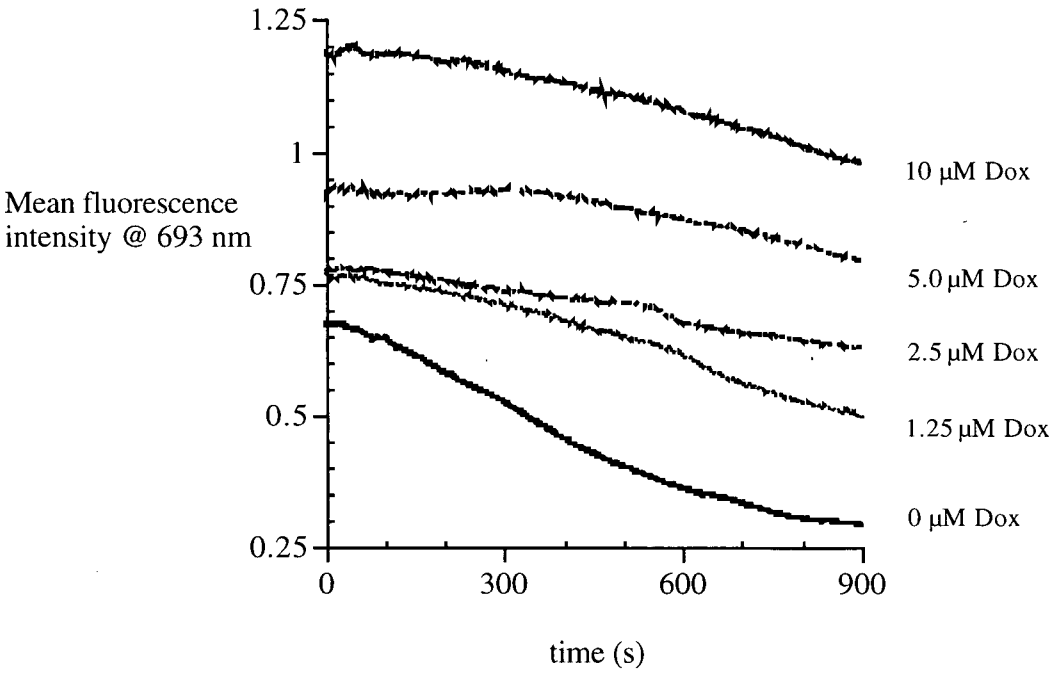


Figure 4.8 Photobleaching of 10 ng/ml BPD in the presence of different concentrations of Dox (λ_{ex} = 440 nm)

BPD fluorescence intensity at 693 nm was monitored for 15 min while the sample was photoactivated at 440 nm in a solvent consisting of 10 % human plasma/ PBS. The presence of increasing concentrations of Dox in the reaction significantly reduced the degree of BPD photobleaching. Settings for the spectrofluorimeter: continuous wavelength (CW) excitation at 440 nm with bandpass of 16 nm, emission at 700 nm with bandpass of 4 nm, MFI at 693 nm continuously monitored for 900 s, photomultiplier voltage (PMT) was set at 650 V

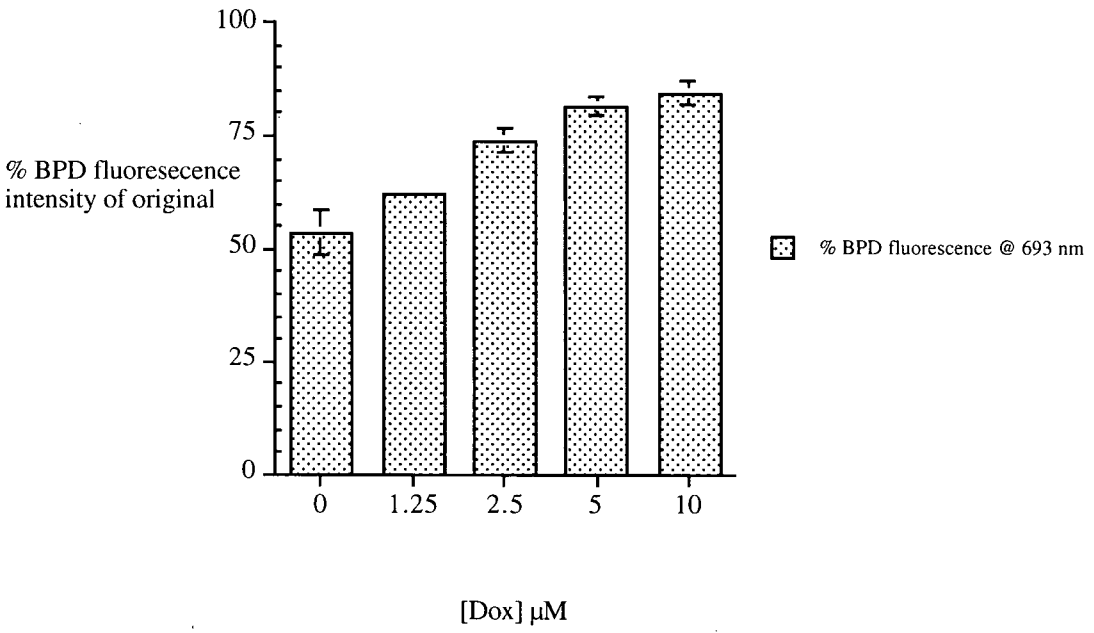


Figure 4.9 Summary of BPD photobleaching in the presence of different concentrations of Dox (440 nm excitation)

Photobleaching of BPD as measured by its fluorescence intensity at 693 nm was proportionally decreased in the presence of increasing concentrations of Dox. Mixtures consisting of 10 ng/ml of BPD and various concentrations of Dox were mixed in 10 % human plasma/ PBS and were subjected to continuous wavelength (CW) activation and fluorescence emission at 693 nm was monitored for 900 s. Percent photobleaching of BPD was obtained by dividing BPD fluorescence at the end of the 900 s exposure period by BPD fluorescence at the beginning of the monitoring period. Data from 3 independent experiments is presented. Error bars are derived from standard errors of the mean.

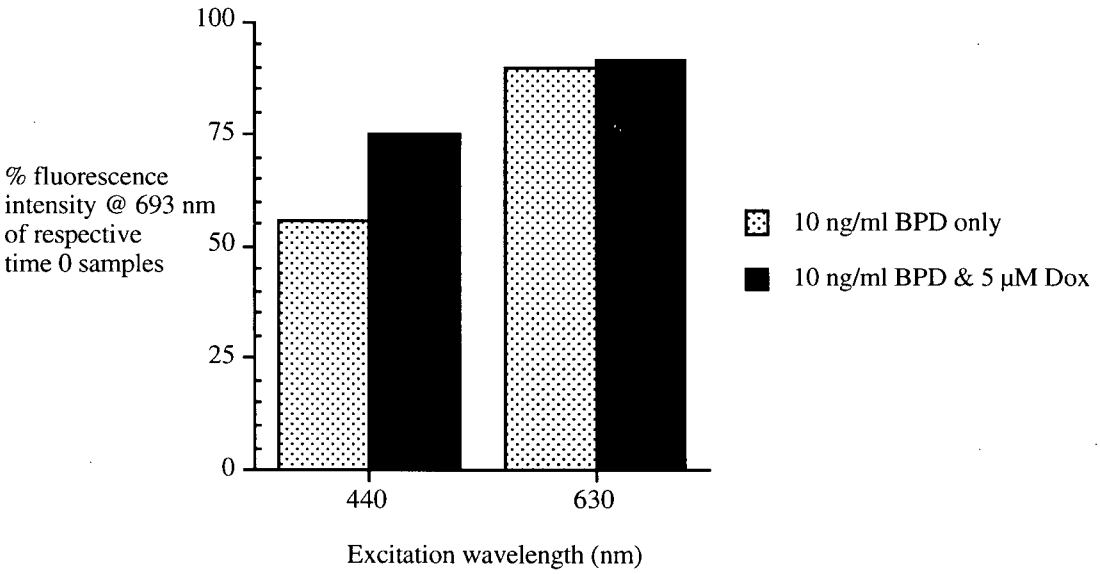


Figure 4.10 The effect of λ_{ex} on BPD photobleaching in the presence and absence of 5 μM Dox

The inhibitory effect of Dox had on BPD photobleaching was observed at the lower excitation wavelength (λ_{ex}) of 440 nm but was not evident at the higher λ_{ex} of 630 nm. BPD photobleaching was assessed by comparing $MFI_{693\ nm}$ at the end of 900 s of exposure to that at the beginning of the monitoring period. Results from a single representative experiment are shown.

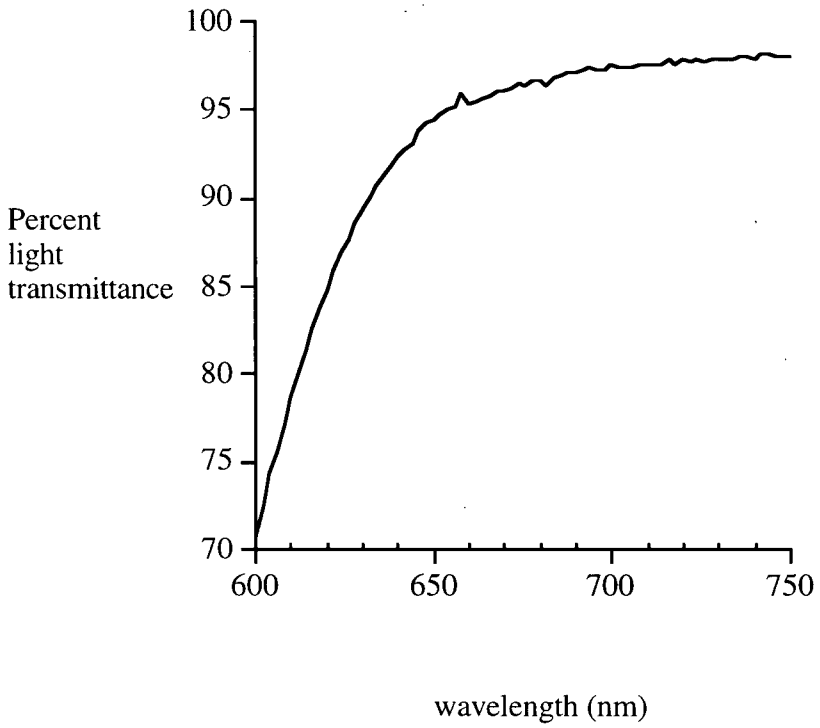


Figure 4.11 Light transmittance of 1 mM Dox in 10 % HI- FCS/ PBS

1 mM of Dox does not appear to significantly interfere with light transmittance in a solution of 10 % HI- FCS/ PBS. Therefore, the presence of 5- 10 μM of Dox in a solution containing BPD should have no adverse effects on the photoactivation of the photosensitiser with λ_{ex} above 600 nm.

Uptake of BPD by L1210 cells as affected by the presence of Dox in different combination regimens

Spectrofluorimetric measurements of lysates from L1210 cells that were incubated with either BPD and Dox are presented in figures 4.12a and 4.12b. Sensitive detection of the two compounds was accomplished by measuring BPD and Dox mean fluorescence intensity (MFI) at their unique fluorescence emission peaks (λ_{em}) at 593 nm (Dox) and 693 nm (BPD). All experiments were performed using 1 x 10^6 cells as described in the *experimental procedure* section. A linear correlation was observed between MFI at λ_{em} of 693 nm and the concentration of BPD (figure 4.12a). Interestingly, fluorescence intensities of L1210 cell lysates were similar between samples that were incubated with the photosensitiser for 10 min and 60 min. Figure 4.12b shows that a linear relationship also existed between MFI at λ_{em} of 593 nm and the concentration of Dox; in addition, uptake of the drug was also quite rapid because of the similarities between the MFI measurements from the 10 min and 60 min cell lysate samples. Figure 4.13 illustrates the effects that 10 μM Dox coincubation (Dox/ BPD) or 10 μM Dox preincubation (Dox-> BPD) had on the uptake of BPD. L1210 cells that were coincubated with Dox (Dox/ BPD) appeared to take up less BPD than control cells incubated with only BPD; this phenomenon became more prominent with increasing concentrations of the photosensitiser. In summary, BPD uptake by L1210 cells was not affected by prior incubation with Dox; however, presence of Dox (10 μM) during BPD incubation appeared to reduce the uptake of the photosensitiser.

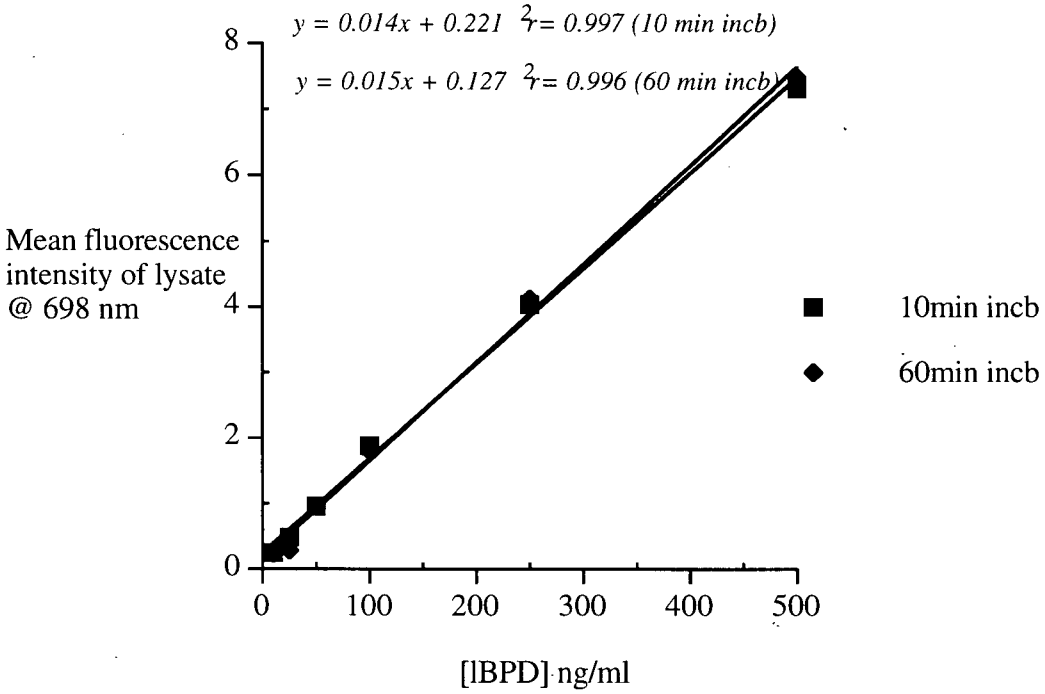


Figure 4.12a Uptake of BPD by L1210 cells as determined by spectrofluorimetric measurements of cell lysates

$MFI_{698\ nm}$ of lysates from 1 x 10^6 L1210 cells was directly correlated with the concentration of BPD. Uptake of BPD was rapid since $MFI_{698\ nm}$ of lysates from cells incubated with BPD for 10 min were similar to those of the 60 min group.

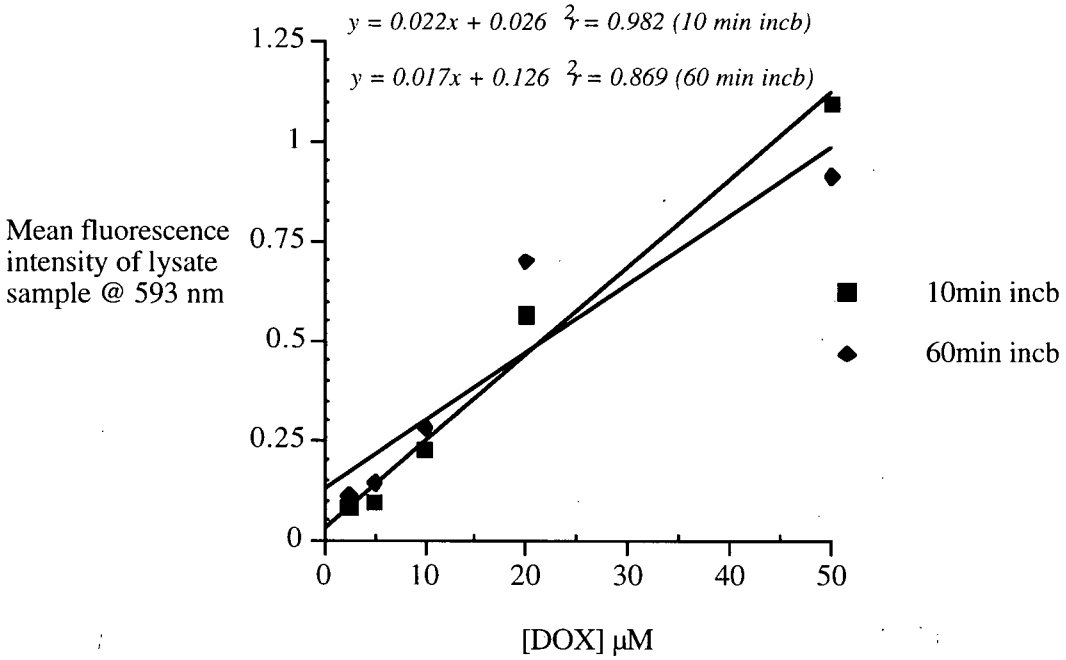


Figure 4.12b Uptake of Dox by L1210 cells as determined by spectrofluorimetric measurement of cell lysates

$MFI_{593\ nm}$ of lysates from 1 x 10^6 L1210 cells was directly correlated with the concentration of Dox used during the incubation. Uptake of Dox was rapid since $MFI_{593\ nm}$ of lysates from cells incubated with Dox for 10 min were similar to those of the 60 min test group.

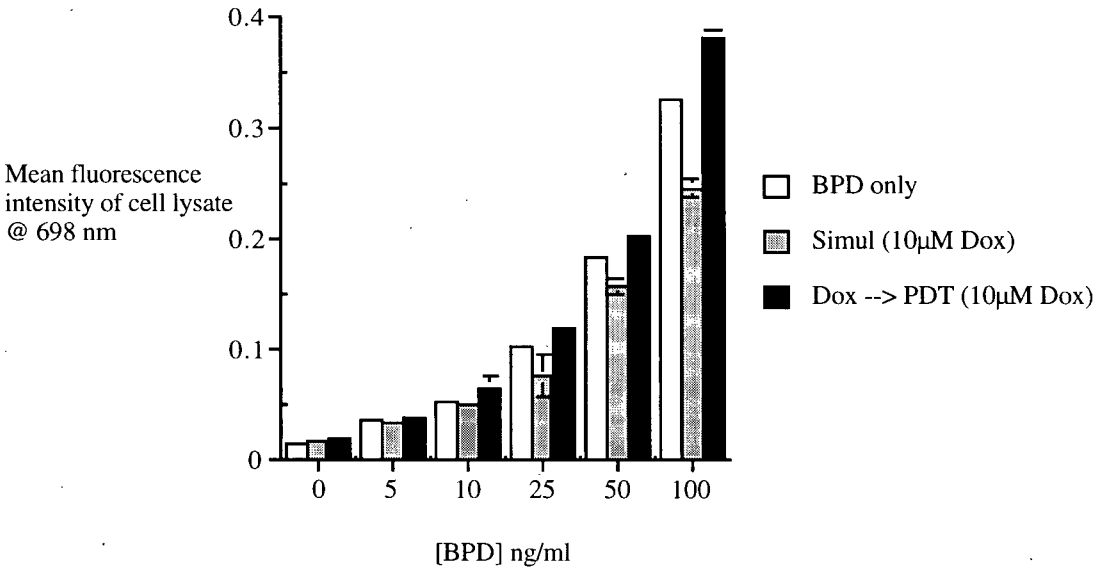


Figure 4.13- Uptake of BPD by L1210 cells when incubated simultaneously (BPD/ Dox) or after Dox (Dox-> BPD)

L1210 cells appeared to take up less BPD in the presence of 10 μM of Dox compared to the respective BPD only controls. Reduction of BPD mean fluorescence intensity at 698 nm ($MFI_{698\ nm}$) was more prominent at higher BPD doses. Preincubation of cells with 10 μM of Dox appeared to have no inhibitory effects on the subsequent uptake of the photosensitiser. Data obtained from 3 independent experiments is presented. Error bars are derived from standard errors of the mean.

Depletion of cellular glutathione (GSH) in L1210 cells by preincubation with Dox

To further investigate the effects of Dox preincubation had on subsequent PDT cytotoxicity on L1210 cells, cellular glutathione (GSH) levels were measured after 1 h incubation with Dox. Again, the same experimental conditions as the cytotoxic assays described in chapter 3 were used for this set of experiments in order to ensure relevancy of the data. Results are summarised in figure 4.14. Preincubation of L1210 cells with Dox resulted in a dose- related, albeit insignificant (student's t- test, $p > 0.05$), reduction of cellular GSH. For example, 11.93 ± 0.76 μM GSH/ 1 x 10^6 cells was obtained after 1 h incubation with 5 μM Dox compared to 13.54 ± 1.51 nmole GSH/ 1 x 10^6 cells from the untreated control group or 88.16 % of the untreated control. Using the Tietze enzymatic method, that preincubation of L1210 cells with 5 μM of Dox did not significantly reduced the amount of cellular GSH. Therefore, this ruled out the role of GSH in the enhancement of PDT cytotoxicity via Dox preincubation.

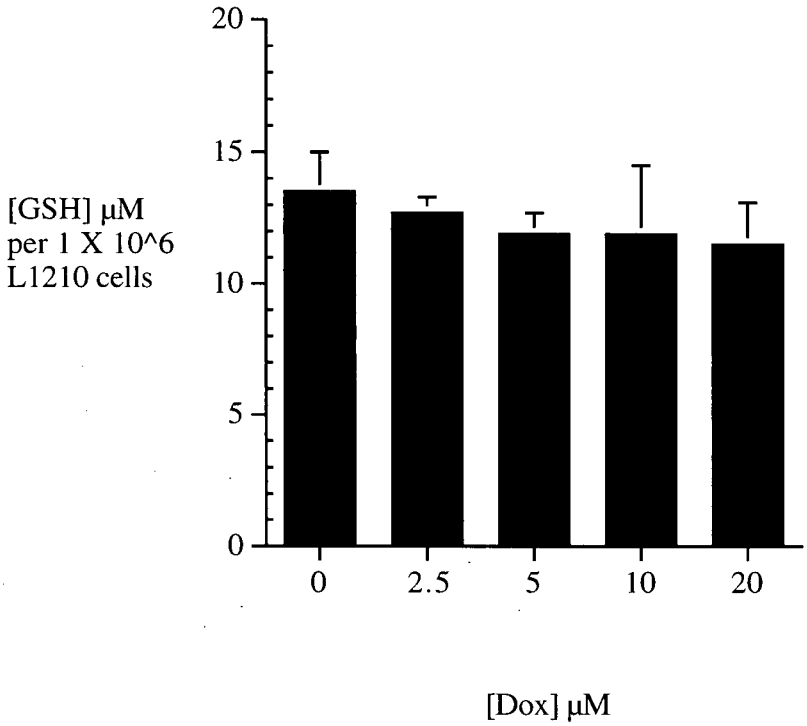


Figure 4.14 Cellular glutathione levels in L1210 cells after 1 h incubation with different doses of Dox

Incubated with Dox for 1 h resulted in a dose- related reduction of cellular glutathione (GSH) in L1210 cells. GSH was determined using the Tietze enzymatic assay. Data from a three independent experiments is presented. Error bars are derived from standard errors of the mean.

4.4 DISCUSSION

The efficiency of PDT is influenced by multiple intrinsic and extrinsic factors. Obviously, variables such as first- pass hepatic metabolism and relative tissue distribution are of concern for *in vivo* applications of PDT; however, the picture is no less complicated for *in vitro* PDT. Biophysical and biochemical properties of the photosensitiser such as hydrophobicity, extinction coefficient, singlet oxygen yield, as well as its absorption profile are some of the examples of the determinants of PDT behaviour *in vitro* and *in vivo*.[333] Susceptibility to PDT cytotoxicity is additionally established by cellular growth rate, expression of surface receptors such as the receptor for low density lipoprotein (LDLr), and relative concentrations of prooxidants and antioxidants in the cell.[255] Combination therapy has been in use for the management of various forms of medical conditions; in addition, it is indispensable in the treatment many oncologic diseases. The principal goals of combination therapy are to use agents with different mechanisms of action so as to reduce the probability of drug resistance development; furthermore, the cytotoxic potentials of the drugs should complement and not nullify each other. Ideally, the side effects of the drugs should not be additive such that the patient but not the tumour remains tolerant to the drug combination. A successful example of such therapy is the use of corticosteroid with vincristine for the treatment of paediatric acute lymphoblastic leukaemia (ALL).[11] Since the advent of the clinical use of PDT, numerous groups have attempted to combine PDT with other treatment modalities such as hyperthermia, ionising radiation, and surgery, with different chemotherapeutic agents or with different combinations of photosensitisers. Henderson and colleagues initially documented that the combination of Photofrin®- mediated PDT and hyperthermia was synergistic in tumour killing only when PDT was used before hyperthermic treatment (PDT-> heat); the authors further demonstrated that pretreatment with hyperthermia resulted in extensive haemorrhage of the tumour site which interfered with the subsequent delivery of the photosensitiser as well as reduced the amount of light reaching the target site.[303] Additional studies supported the early findings of the influence that treatment sequence had on the efficacy of PDT/ hyperthermia combination therapy.[309] Significantly, Chen *et al.* noticed that PDT resulted in tumour acidity and hypoxia which sensitised the tumour to subsequent hyperthermia.[339]

The experiments described above were performed on implanted tumours in murine hosts, which introduced *in vivo* factors affecting pharmacokinetics in addition to unique determinants such as tumour vasculature and oxygenation status. Paradoxically, the reverse sequence of heat-> PDT was found to be more cytotoxic than PDT-> heat on L1210 cells *in vitro*.[340]

PDT was also used in combinations with chemotherapeutic agents including etoposide VP16, taxol, vincristine, and ET-18- OCH_3, an alkyl- lysophospholipid to effect additive antitumour effects.[306, 341, 342] The bioreductive alkylating agent mitomycin C (MMC) has also been extensively studied in PDT combination therapy. Ma *et al.* reported that simultaneous administration of MMC and Photofrin® followed by light exposure 16 h later (MMC/P-> light) resulted in synergistic killing of the human colon adenocarcinoma cell line WiDr *in vitro* and *in vivo* in nude mice; the authors also concluded that MMC effected cell cycle inhibition which increased the tumour S- phase fraction and consequently the uptake of Photofrin®.[304, 318] This is consistent with earlier findings by Christensen *et al.* that susceptibility to PDT in several cell lines (NHIK 3025, HeLa S_3, NHIK 1922, and V79) is correlated with S- phase.[343] Baas *et al.* repeated the experiment on the EO9 mouse tumour model and confirmed the sequence- specific synergy of the MMC/P-> light regimen since MMC given after PDT did not enhance tumour killing.[344] The sequence combination of MMC/P-> light was later successfully used in a limited clinical trial on patients with skin metastases of primary mammary carcinomas.[323] Clearly, numerous parameters can affect the interpretation of the different variations of PDT combination therapy, chief among them include the photosensitiser and drug used, the sequence of treatment, and the experimental system (tumour type, *in vitro* or *in vivo*).

In this study, Dox was observed to effect sequence- specific potentiated killing of the murine leukaemic cell line L1210 but not normal DBA/2 haematopoietic progenitors when used in combination with BPD- mediated PDT. The sequence of Dox-> PDT was found to be superior to simultaneous Dox/PDT, the reverse combination sequence of PDT-> Dox, as well as single agent PDT or Dox treatments. In addition, effectiveness of the Dox-> PDT sequence was found to be BPD dose dependent since enhancement was not observed at 2.5 ng/ml but at 5.0 ng/ml BPD (chapter 3 of this thesis). Several other groups have also studied the use of Dox in PDT combination therapy. Niahabedian and colleagues successfully used a combination of haematoporphyrin derivative- mediated PDT with Dox

to treat BALB/c mice carrying the EMT- 6 tumour.[308] They attributed the potentiation of cytotoxicity to photochemical activation of Dox by the red laser light (630 nm) used for PDT or alternatively to hyperthermic enhancement of Dox as a result of laser irradiation. The first explanation was dubious because Dox with an absorption peak of 579 nm absorbs light minimally beyond 600 nm.[345] Interestingly, Lanks *et al.* was able to demonstrate photodynamic enhancement of Dox cytotoxicity in the L929 cell line *in vitro* at the λ_{ex} of 514.1 nm and 488 nm.[346] One year earlier, Cowled *et al.* published a report citing the discrepancy between *in vitro* and *in vivo* results of haematoporphyrin derivative (HPD)-mediated PDT combination therapy involving Dox.[307] The authors found that the simultaneous PDT/Dox regimen was superior to PDT-> Dox sequence treatment on subcutaneously implanted Lewis lung carcinoma *in vivo*; however, they found that Dox inhibited PDT cytotoxicity when the same regimen (PDT/Dox) was tested *in vitro* on the same cell line. In addition, they showed that Dox inhibited the uptake of HPD *in vitro*.

In this study, fluorescence microscopy was used to establish the intracellular localisations of BPD and Dox. Using the human leukaemic cell line K562 as a model, BPD was found to localise to the cytosol and the cell membrane whereas Dox was found in both cytoplasmic and nuclear compartments. We believe that differential localisations of the two compounds coupled with their slightly different cytotoxic mechanisms contributed to the observed additivity in cell killing of the PDT/Dox regimen. We also performed a series of spectrofluorimetric experiments to further study the molecular interactions between these two compounds. BPD photobleaching, monitored as mean fluorescence intensity at 693 nm ($MFI_{693\ nm}$), was used as a surrogate marker of the degree of its photoactivation. Using physiologically relevant concentrations of BPD and Dox, we determined that Dox inhibited BPD activation when λ_{ex} of 440 nm was used (figure 4.9- 4.11). This was probably caused by interference of BPD light absorption by Dox which was present at 363.5 fold molar excess of BPD. When an λ_{ex} of 630 nm was used, the presence of Dox had a negligible effect on BPD photobleaching. This is expected because Dox absorbs weakly beyond 600 nm and BPD, even at a concentration 363.5 fold less than that of Dox, has three distinct absorption peaks above 600 nm. The findings from the photobleaching experiments suggest that in PDT combination therapy, the photophysical characteristics of the compound and their possible interactions must be investigated thoroughly. Since a red light source emitting between 600- 900 nm was used for the cytotoxicity experiments in

chapter 3, the presence of Dox should not affect the photoactivation of BPD in the Dox/PDT regimen. Cowled *et al.* found that Dox at 200 μg/ml (345 μM) completely inhibited the uptake of HPD in the Lewis lung carcinoma and Raji cell lines *in vitro*.[307] We observed that 10 μM of Dox decreased the accumulation of BPD in the Dox/BPD regimen. However, L1210 cells that were preincubated with 10 μM Dox followed by 1 h incubation with BPD (Dox-> BPD) displayed normal levels of the associated photosensitiser. Mild inhibition of BPD uptake by the presence of Dox could explain the additive but not synergistic killing of L1210 cells. On the other hand, "normalised" BPD accumulation in Dox-> PDT probably accounted for the significant improvement in cytotoxicity of this regimen. Nevertheless, one must note that the difference in BPD accumulation between the two regimens (Dox/BPD vs Dox-> BPD) was only evident at the higher BPD doses which therefore questions the relevancy of uptake modulation in the combination experiments. Also, we believe that the moderate effect on BPD uptake by Dox preincubation could not, by itself, account for the improvement in cell killing seen at 5 ng/ml BPD in the Dox-> PDT sequence.

To explain the superiority of the Dox-> PDT sequence compared to Dox/PDT and PDT-> Dox regimen, we postulated that pretreatment of L1210 cells might result in the depletion of cellular glutathione (GSH) which would then predispose the cells to subsequent oxidative damage mediated by PDT. GSH, an abundant tripeptide sulphydryl, is involved in a variety of cellular processes.[347] Alone or in conjunction with the enzymes glutathione S- transferase and selenium- glutathione peroxidase, GSH scavenges free radicals, reduces H_2O_2 and organic peroxides. Xenobiotics and other chemotherapeutic agents such as cisplatin can cause oxidative stress to tumour cells which is buffered by cellular GSH and numerous studies have shown an inverse correlation between the concentration of GSH and susceptibility to chemotherapy. Cancer cells which exhibit the multidrug resistance (MDR) phenotype often have higher amounts of GSH or demonstrate heightened GSH metabolism.[348] Conversely, artificial depletion of cellular GSH is achievable via the use of buthionine sulphoximine (BSO) which specifically inhibits γ-glutamylcysteine synthase, a rate- limiting enzyme in the GSH synthesis cascade. BSO has been successfully used, in conjunction with verapamil, to overcome the MDR phenotype in the MCF- 7 breast carcinoma cell line.[245] Furthermore, doxorubicin and cisplatin cytotoxicity are enhanced by BSO.[349, 350] Similarly, GSH would be expected to play a

significant role in determining cellular susceptibility to PDT because of the central role of oxidative stress in PDT cytotoxicity.[261, 334, 351] In this study, superior killing of L1210 by the Dox-> PDT sequence could not be related to modulation of cellular GSH by Dox. Using the Tietze enzymatic assay, pre- incubation of L1210 cells with 2.5 μM Dox did not effect a significant reduction of cellular GSH. Nevertheless, GSH reduction was observed in a dose- dependent manner with respect to the concentrations of Dox used. In summary, the current assay method (Tietze assay) did not show a significant alteration of cellular GSH which could potentially be secondary to the low resolution of the assay itself. A more sensitive method of measurement, such as one that is based on the fluorescence detection of GSH- bound monochlorobimane or monobromobimane, could possibly reveal any alterations of cellular GSH as a result of Dox preincubation.[411]

The unique photophysical characteristics of BPD and Dox, in addition to their distinct intracellular localisations, are responsible for the additivity in cytotoxicity when L1210 cells were treated simultaneously with BPD- mediated PDT and Dox (PDT/Dox). The apparent synergistic killing of L1210 cells in the Dox pretreatment regimen (Dox-> PDT) did not effect apparent changes in the uptake of BPD nor reduction in GSH; therefore, other factors such as the generation of ceramide and alteration of activation threshold of the nuclear transcription factor NF- κβ could explain the biological findings. Alternatively, small changes in the above measured parameters, in sum, could potentially affect PDT cytotoxicity in the Dox-> PDT regimen. Nevertheless, more sensitive detection assays are needed in order to ascertain the role of GSH in Dox pretreatment and other possible mechanisms as list above must be considered.

CHAPTER 5: SELECTIVE PREPROTECTION OF NORMAL HAEMATOPOIETIC COMMITTED PROGENITIOR CELLS FROM PDT WITH N-ACSDKP: CYTOTOXICITY STUDY

5.1 ABSTRACT

Several studies have demonstrated that the tetrapeptide N-AcSDKP (Seraspenide) selectively inhibits cell cycle progression of normal haematopoietic cells thereby protecting them from the cycle- specific cytotoxicity of chemoradiation. It was found that N-AcSDKP, but not the control peptides N-AcSDKE and SDKP, protected DBA/2 bone marrow cells from BPD- mediated PDT cytotoxicity. When DBA/2 bone marrow cells were incubated with 100 nM of N-AcSDKP for 1.5 hours prior to PDT, subsequent CFU- GM survivals were improved by 1.5 - 2 folds over cells that were preincubated with the control peptides or with medium. The leukaemic cell line L1210 was not protected from PDT with N-AcSDKP preincubation. Similar results were observed in normal and leukaemic human haematopoietic cells and in the leukaemic cell line K562. That is, N-AcSDKP effected selective photoprotection of normal human CFU- GM formation but not leukaemic CFU- L formation from patient isolates or from the K562 cell line. Therefore, results from the *in vitro* short- term colony assays suggest that N-AcSDKP could be used for the selective protection of the normal committed progenitor cells during PDT purging. We then used the one step long- term bone marrow culture (LTBMC) assay to assess whether the photoprotective effect of N-AcSDKP also extends to earlier haematopoietic progenitor and stem cells. Direct quantification of cell numbers and CFU- GM production over a five-week period showed no difference between DBA/2 bone marrow cells treated with PDT after preincubation with 100 nM of N-AcSDKP or with medium. We therefore concluded that the photoprotective effect of N-AcSDKP as used here applied only to committed progenitor cells which give rise to CFU- GM colonies. However, this level of protection, albeit limited, could still be of clinical advantage in the improvement of the early phase of engraftment mediated by the committed progenitor populations.

5.2 INTRODUCTION

Haematopoiesis is a highly dynamic yet coordinated process controlled by a plethora of positive and negative regulators; the bone marrow, site of principal haematopoiesis in humans, must be able to function in a steady state equilibrium in order to replace mature blood cells that are lost to turnovers for the lifespan of the organism. In addition, the bone marrow must be able to support and maintain heightened haematopoiesis in the course of increased demands such as during infections or blood loss.[200, 352] The soluble factors which positively regulate haematopoiesis are also termed growth factors, cytokines, or colony stimulating factors (CSFs). The first CSF identified was erythropoietin (EPO) by Reissmann and colleagues; however, murine granulocyte-macrophage colony stimulating factor (GM- CSF) was the first haematopoietic cytokine to be cloned.[353, 354] Identification of CSFs was also aided by the maturation of the hierarchical paradigm of haematopoiesis and technical developments in the isolation of stem cells as well as their *in vitro* propagation.[352, 355, 356] Industrial- scale productions of various recombinant human CSFs have revolutionised clinical medicine, especially in the management of cancer patients.[357] EPO, G- CSF and GM- CSF are such examples and are indicated for the amelioration of anaemia and leukopaenia, respectively.[358] In addition, thrombopoietin (TPO), a recently isolated cytokine, shows great promise in managing the thrombocytopaenic condition in cancer patients undergoing myeloablative therapy.[359] Rational use of CSFs in the clinic is still far from realisation, however. Recently, several multicentre trials have demonstrated the abilities of G- and GM- CSFs to improve objective criteria without significant improvements in the clinical endpoints of the patients.[113, 114, 360] Hoelzer suggested that CSFs can play important and relevant roles in the clinic, given the appropriate indications.[361]

Maintenance of a dynamic equilibrium in haematopoiesis also requires the activities of multiple feedback mechanisms which negatively modulate the system, some of which include soluble regulatory or inhibitory factors, adhesion molecules, and intrinsic programming within the cells.[200] Inhibitory factors such as tumour necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), and interleukin- 1 (IL- 1) have been well characterised.[362] Macrophage inhibitory factor- 1α (MIP- 1α) is a member of the C-C chemokine family and was originally identified as a stem cell inhibitor by Graham and

colleagues.[201] MIP- 1α also mediates the inhibitory effects of interferon- α (IFN- α) on CML progenitors, through the restoration of β1 integrin mediated adhesion of the leukaemic progenitor cells to the bone marrow stroma, hence effecting restitution of the growth inhibitory signals from the stroma.[215]

A new class of peptide- based haematopoietic inhibitors which include N-AcSDKP, pEEDCK, and SK&F108636 were identified with various methods.[363, 364] The biochemical as well as the biological properties of N-AcSDKP has since then been characterised in detail.[205] Numerous studies have shown that the peptide as well as cytokine inhibitors selectively affect only normal haematopoietic cells, a fact of clinical significance.[204, 365, 366] Haematopoietic inhibitors act principally through the prevention of stem cell cycle entry from G_0/G_1 to S phase; therefore, these factors can protect haematopoietic cells from cycle- specific chemotherapy.[210, 367, 368] Furthermore, the inhibitors also effect other cellular changes that result in the protection of normal haematopoietic cells from γ- radiation, and hyperthermia, in addition to chemotherapy. For example, IL- 1 and TNF- α induce the expression of protective enzymes.[369, 370] N-AcSDKP has been demonstrated to selectively protect normal human haematopoietic progenitors from PDT- mediated phototoxicity by the first generation photosensitiser Photofrin®.[209]

In this chapter, the photoprotective effects of N-AcSDKP or Seraspenide was further investigated on PDT mediated by the second generation photosensitiser BPD. Relative susceptibility of DBA/2 haematopoietic progenitors and L1210 leukaemic cells to PDT was studied after preincubation with N-AcSDKP, or the control peptides SDKP and N-AcSDKE. In addition, the long- term bone marrow culture (LTBMC) assay was used to study whether the photoprotective effect also extended to earlier normal haematopoietic progenitors. To complement the murine studies, N-AcSDKP was used in PDT experiments involving normal and leukaemic human haematopoietic cells.

5.3 RESULTS

Photoprotective effect of N-AcSDKP on DBA/2 haematopoietic progenitors

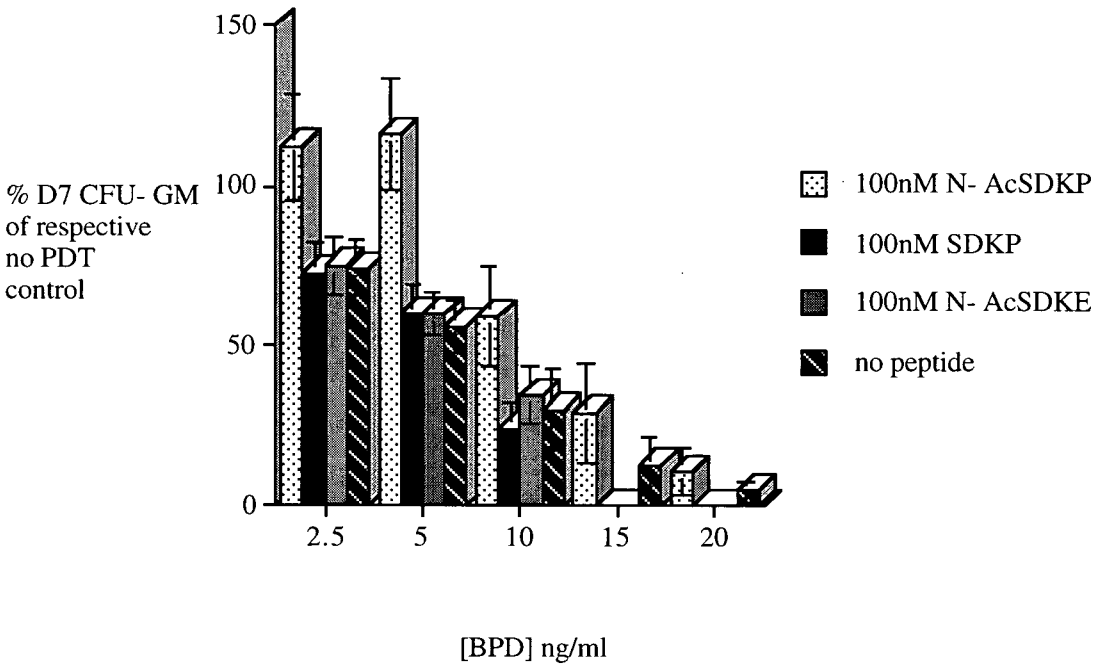
Figure 5.1 demonstrates that preincubation of DBA/2 bone marrow cells with 100 nM of N-AcSDKP for 1.5 hr protected the haematopoietic progenitors from subsequent BPD- mediated PDT, as measured by the short- term colony assay. In addition, 100 nM of the control peptides N-AcSDKE and SDKP, as well as tissue culture medium, did not offer protection. The photoprotective effect mediated by 100 nM N-AcSDKP was especially prominent at BPD concentrations of 2.5, 5, and 10 ng/ml with approximately 1.5 - 2 fold higher survival of DBA/2 CFU- GM compared to cells preincubated with 100 nM of control peptides or medium. Control peptides were not tested at the highest BPD concentrations of 15 and 20 ng/ml; however, N-AcSDKP was still able to offer approximately 2- fold protection of the progenitors in comparison to medium preincubation. Unlike its effects on human haematopoietic cells, N-AcSDKP did not suppress murine CFU- GM formations.[204] We showed that preincubation for 1.5 hr with 100 nM of N-AcSDKP, SDKP, and N-AcSDKE did not decrease the numbers of day 7 colonies in standard agar based colony assays (figure 5.2).

Absence of N-AcSDKP mediated photoprotection of L1210 cells

The murine leukaemic cell line L1210 was not protected from subsequent PDT by 1.5 hr of preincubation with 100 nM N-AcSDKP. As shown in figure 5.3, cells that were preincubated with N-AcSDKP did not show any survival advantage post- PDT in comparison with cells that were preincubated with the control peptides N-AcSDKE and SDKP or in the absence of any peptides. Figure 5.4 shows that neither the haematopoietic inhibitory peptide N-AcSDKP nor the control peptides had any inhibitory effects on the number of day 6 L1210 colonies. The above results suggest that N-AcSDKP has no photoprotective effects on L1210 cell line.

Figure 5.1 Selective cytoprotective effects of N-AcSDKP on DBA/2 haematopoietic progenitor cells subjected to BPD- mediated PDT

DBA/2 haematopoietic progenitors were preincubated with 100 nM of N-AcSDKP (Seraspenide), 100 nM of the control peptides SDKP or N-AcSDKE, or medium for 1.5 hr followed by PDT treatment. Clonogenicity was determined with the standard agar- based colony assay and colonies (CFU- GM) were scored on day 7 of culture using an inverted microscope. Only colonies with 40 or more cells were counted. Percent survival of treated cells was calculated based on the number of colonies generated divided by the number of colonies from the no BPD control within the same peptide group Average number from the 0 ng/ml BPD control samples of the four pretreatment groups is 100.815 day 7 colonies from 70000 cells plated. Data obtained from five independent experiments (n= 5) is presented. Error bars are derived from standard errors of experimental means. Results are significant with $p< 0.05$ as determined by two way-analysis of variance test (ANOVA).


% D7 CFU- GM of respective no PDT control
150
100
50
0
2.5
5
10
15
20
[BPD] ng/ml
100nM N- AcSDKP
100nM SDKP
100nM N- AcSDKE
no peptide

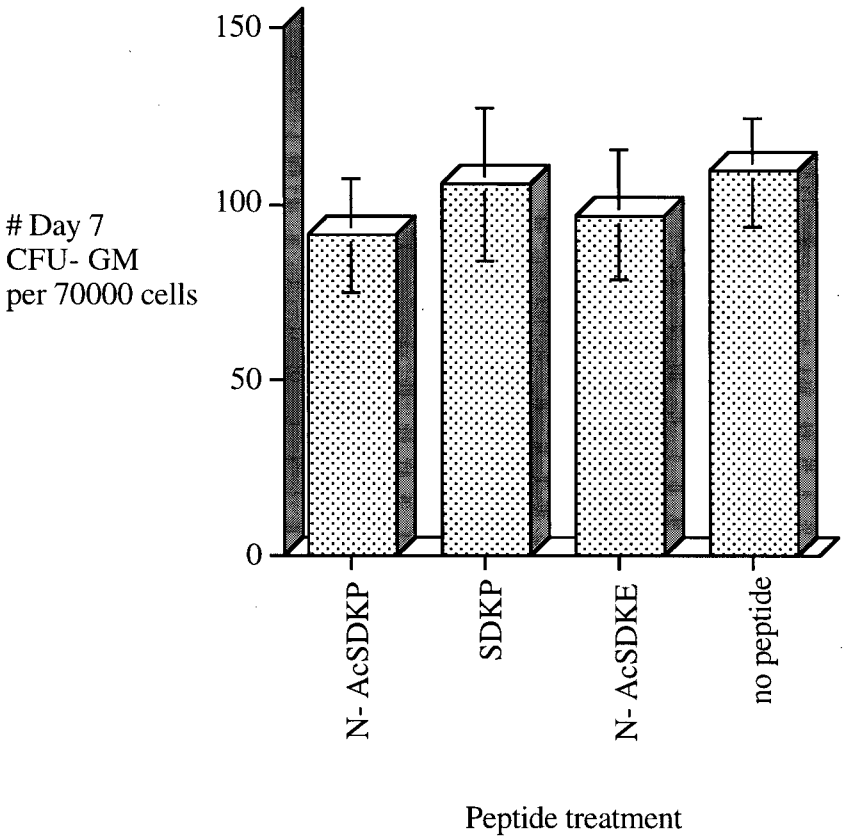
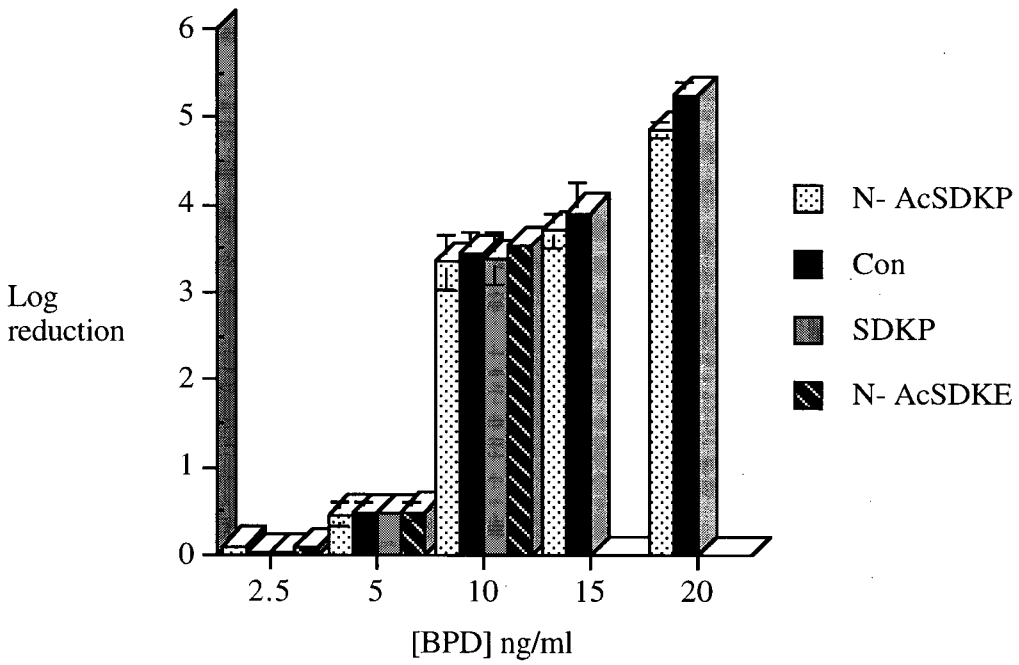


Figure 5.2 Effects of N-AcSDKP, control peptides N-AcSDKE or SDKP, and medium on the clonogenicity of DBA/2 haematopoietic progenitor cells

DBA/2 haematopoietic progenitor cells were incubated with 100 nM of N-AcSDKP, 100 nM of the control peptides N-AcSDKE and SDKP, or medium prior to mock PDT treatment, i.e. exposure to 15J/cm^2 red light in the absence of the photosensitiser BPD. Day 7 CFU- GM formations in each group were assessed using the standard agar- based colony assay. An inverted microscope was used to enumerate colonies consisting of 50 or more cells. The number of day 7 CFU- GM was not significantly altered in each of the peptide- treated group. Data from five independent experiments is presented (n= 5). Source of error is derived from the standard errors of experimental means.

Figure 5.3 Absence of cytoprotective effects of N-AcSDKP on L1210 leukaemic cell line subjected to BPD- mediated PDT

L1210 leukaemic cells were preincubated with 100 nM of N-AcSDKP (Seraspenide), 100 nM of the control peptides SDKP or N-AcSDKE, or medium for 1.5 hr followed by PDT treatment. Clonogenicity was determined with the standard agar- based colony assay and colonies (CFU-L) were scored on day 6 of culture using an inverted microscope. Only colonies with 50 or more cells were counted. Log reductions of treated cells were calculated with respect to the no BPD control within the same peptide group. Data obtained from three independent experiments (n= 3) is presented. Error bars are derived from standard errors of experimental means. Results are significant with $p< 0.05$ as determined by two way-analysis of variance test (ANOVA).


Log
reduction
6
5
4
3
2
1
0
2.5
5
10
15
20
[BPD] ng/ml
N- AcSDKP
Con
SDKP
N- AcSDKE

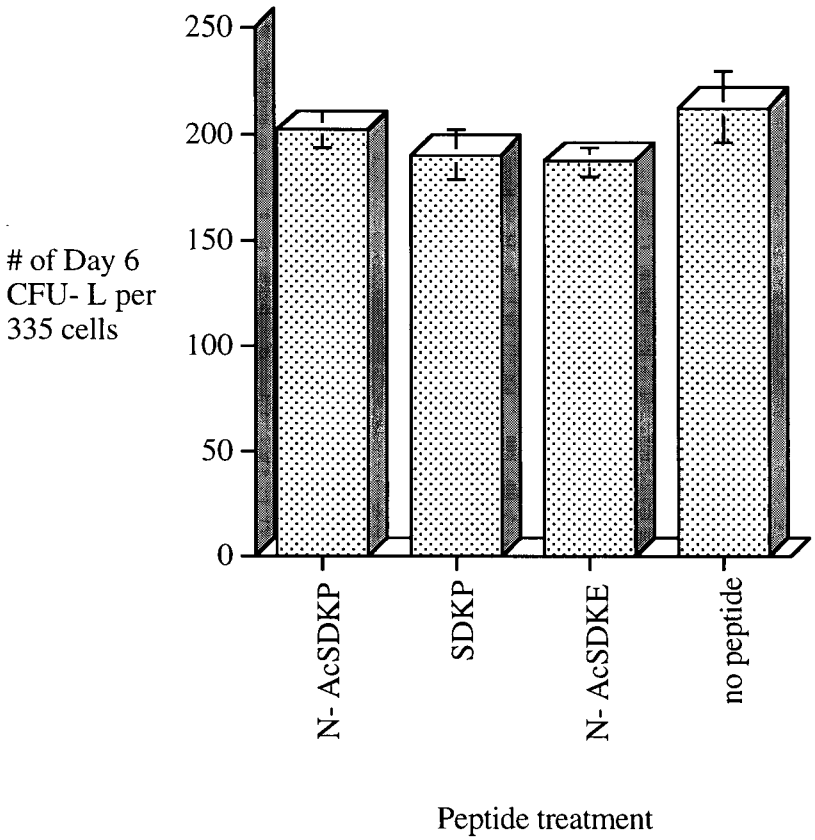


Figure 5.4 Effects of N-AcSDKP, control peptides N-AcSDKE or SDKP, and medium on the clonogenicity of L1210 leukaemic cells

L1210 leukaemic cells were incubated with 100 nM of N-AcSDKP, 100 nM of the control peptides N-AcSDKE and SDKP, or medium only prior to mock PDT treatment, i.e. exposure to 15J/cm^2 red light in the absence of BPD. Day 6 CFU- L formation in each group were assessed using the standard agar- based colony assay. An inverted microscope was used to enumerate colonies consisting of 50 or more cells. The number of day 6 CFU- L was not significantly altered in each of the peptide- treated group. Data from three independent experiments is presented (n= 3). Source of error is derived from the standard errors of experimental means.

Statistical analyses of progenitor assay data

Two- way analysis of variance (ANOVA) and Bonferroni (all- pairwise) multiple comparison tests were employed to test for the significance of data from the progenitor assays. Two- way ANOVA demonstrated significant differences in colony survivals (p <0.05) between the three BPD doses of 2.5, 5, and 10 ng/ml in both DBA/2 and L1210 cells (data not shown). More importantly, the ANOVA test demonstrated that the PDT survival data of DBA/2 cells preincubated with the different peptides were significantly different from each other yet there was no significant difference amongst PDT- treated L1210 cells preincubated with the different peptides. This suggested that selective photoprotective action was being exerted on the normal DBA/2 haematopoietic cells, but not on the leukaemic cell line L1210. To further delineate the difference between the various groups, a more powerful statistical analytical tool was used. The Bonferroni (all-pairwise) multiple comparison test was used to reveal whether intergroup differences exist between the different peptide (N-AcSDKP, SDKP, N-AcSDKE, and medium) and BPD (2.5, 5, 10 ng/ml) groups in both DBA/2 haematopoietic cells and L1210 leukaemic cells. Results from normal cells are presented in tables 5.1a and 5.1b. Table 5.1a shows that a significant intergroup difference exists between 10 ng/ml BPD and the two lower doses of 5 and 2.5 ng/ml. There was no significant difference between 5 and 2.5 ng/ml. Table 5.1b shows that the haematopoietic inhibitory peptide N-AcSDKP acted selectively on the normal haematopoietic cells since progenitor assay data of N-AcSDKP preincubated cells treated with PDT was significantly different from PDT- treated cells that were preincubated with the control peptides N-AcSDKE, SDKP, as well as with medium control. Data from the L1210 experiments are presented in tables 5.2a and 5.2b. All three BPD doses are significantly different from each other (table 5.2a). More importantly, there was no significant intergroup difference in PDT survival data amongst L1210 cells preincubated with the different peptides (table 5.2b). Therefore, N-AcSDKP selectively photoprotected normal DBA/2 haematopoietic progenitor cells but not L1210 leukaemic cells from BPD-mediated PDT.

Table 5.1a- Bonferroni (all- pairwise) multiple comparison test of clonogenicity data of DBA/2 haematopoietic progenitors: significance of BPD doses

[BPD] ng/ml	10	5	2.5
10	--	++	++
5	++	--	--
2.5	++	--	--

Table 5.1a Bonferroni (all- pairwise) multiple comparison test of absolute numbers of day 7 CFU- GM of DBA/2 haematopoietic progenitor cells treated with 10, 5, and 2.5 ng/ml of BPD and 15J/cm^2 of red- light irradiation. The plus signs (++) within the shaded cells indicate intergroup significant differences between the represented BPD concentration regardless of the type of peptide used during the preincubation.

Table 5.1b- Bonferroni (all- pairwise) multiple comparison test of clonogenicity data of DBA/2 haematopoietic progenitors: significance of peptides

Peptide	N-AcSDKP	SDKP	N-AcSDKE	no peptide
N-AcSDKP	--	++	++	++
SDKP	++	--	--	--
N-AcSDKE	++	--	--	--
no peptide	++	--	--	--

Figure 5.1b Bonferroni (all- pairwise) multiple comparison test of absolute numbers of day 7 CFU- GM of DBA/2 haematopoietic progenitor cells preincubated with 100 nM of N-AcSDKP, 100 nM of control peptides SDKP or N-AcSDKE, or medium only for 1.5 hr followed by BPD- mediated PDT. The plus sign (++) within the shaded cells indicate intergroup significant differences in the survival of DBA/2 haematopoietic progenitor cells between the represented peptide used regardless of the concentration of BPD used.

Table 5.2a- Bonferroni (all- pairwise) multiple comparison test of clonogenicity data of L1210 leukaemic cells: significance of BPD doses

[BPD] ng/ml	10	5	2.5
10	++	++	++
5	++	++	++
2.5	++	++	++

Table 5.2a Bonferroni (all- pairwise) multiple comparison test of absolute numbers of day 6 CFU- L of L1210 leukaemic cells treated with 10, 5, and 2.5 ng/ml of BPD and 15J/cm^2 of red- light irradiation. The plus sign (++) within the shaded cells indicate intergroup significant differences between the represented BPD concentration regardless of the type of peptide used during the preincubation.

Table 5.2b- Bonferroni (all- pairwise) multiple comparison test of clonogenicity data of L1210 leukaemic cells: significance of peptides

Peptide	N-AcSDKP	SDKP	N-AcSDKE	no peptide
N-AcSDKP	--	--	--	--
SDKP	--	--	--	--
N-AcSDKE	--	--	--	--
no peptide	--	--	--	--

Table 5.2b Bonferroni (all- pairwise) multiple comparison test of absolute numbers of day 6 CFU- L of L1210 leukaemic cells preincubated with 100 nM of N-AcSDKP, 100 nM of control peptides SDKP or N-AcSDKE, or medium only for 1.5 hr followed by BPD-mediated PDT. There was no significant difference in cytotoxic response of L1210 cells preincubated with N-AcSDKP, the control peptides, or medium.

N-AcSDKP protection of DBA/2 haematopoietic cells does not extend to earlier progenitors and stem cells

A one step long- term bone marrow culture (LTBMC) assay was used to assess whether N-AcSDKP- mediated photoprotection also extends to earlier DBA/2 haematopoietic progenitors or even stem cells. In this LTBMC assay, freshly harvested bone marrow cells were again preincubated with 100 nM N-AcSDKP or tissue culture medium for 1.5 hr prior to BPD- mediated PDT. The cells were then mixed with LTBMC culture medium and plated into duplicate wells of 24 well tissue culture plates and assessed as described in the *experimental procedures* section. During the course of *in vitro* culture, stromal precursors adhered to the bottom surface of the wells and established a stromal microenvironment similar to that found in the bone marrow; haematopoietic progenitor cells and stem cells undergo maturation in close contact with the stromal layer (figure 5.5). The one- step LTBMC assay uses cells from a single harvest for both the establishment of the stroma as well as for subsequent haematopoiesis. Colony formations from long- term progenitors are apparent at week four and five of the assay whereas colonies from week one to three harvests are derived from the more mature progenitor cell populations.[371]

In table 5.3, results in the form of cell concentrations from weekly harvestings of a five week LTBMC assay are presented. Cell numbers of LTBMC suspension harvests from both peptide- treated and control groups inversely correlated with BPD concentrations and was most obvious in cultures from week one to three (figure 5.6). Later time points (week 4 and 5, data not shown) showed less definitive difference; the lack of correlation between harvested cell number and BPD dose later in culture was caused by dehiscence of cells from the stromal layer. In addition, contributions of cells from the dehisced stromal layer greatly inflated the cell counts in the control as well as the peptide- treated group in week four and five. N- AcSDKP does not significantly affect suspension cell numbers in the PDT- treated cells, especially in samples harvested at week one to three, suggesting that 100 nM N-AcSDKP did not appear to offer photoprotection to the population(s) of cells measured by the LTBMC assay.

Suspension cells from the weekly harvests were subsequently put into short- term colony assay which measures the late haematopoietic progenitors such as those that form CFU- GMs. As shown in table 5.4, there was again an inverse correlation between the numbers of colonies from the harvested cells and the dose of BPD. However, the numbers were not significantly different between the peptide- treated and the control group; therefore, results from the LTBMC assay suggest that N-AcSDKP- mediated photoprotection seen in late haematopoietic progenitors does not extend to earlier progenitor cells.

Table 5.3- Suspension cell numbers of weekly harvests of long- term bone marrow culture of DBA/2 haematopoietic cells preincubated in the presence or absence of 100 nM N-AcSDKP followed by BPD- mediated PDT

	No peptide control				100 nM N-AcSDKP			
[BPD] ng/ml	20	10	5	0	20	10	5	0
week 1	15000 ± 300	17300 ± 6300	23000 ± 800	27400 ± 3400	5600 ± 500	15600 ± 2700	21600 ± 2500	28300 ± 6900
week 2	3900 ± 300	5400 ± 800	8000 ± 1100	9800 ± 300	1600 ± 200	7500 ± 2000	6600 ± 400	9500 ± 1200
week 3	5600 ± 1600	6700 ± 3000	12000 ± 400	14000 ± 5900	3900 ± 800	2700 ± 1400	6100 ± 600	23600 ± 900

Table 5.3 Freshly isolated bone marrow cells from DBA/2 mice were preincubated with 100 nM N-AcSDKP or medium for 1.5 hr prior to BPD- mediated PDT. Treated cells were resuspended in LTBMC medium and plated into 24 well tissue culture plates in duplicates. At weekly intervals, nonadherent cells from each well were harvested by gentle agitation and aspiration using an Eppendorf pipetter. The number of cells from each sample was counted under a haemocytometer by Eosin Y staining of an aliquot of cells from each well. The concentrations of cells from the samples were then calculated. Each number, rounded off to two significant figures, represent an average of two independent samples (n= 2) and source of error is standard error of the mean.

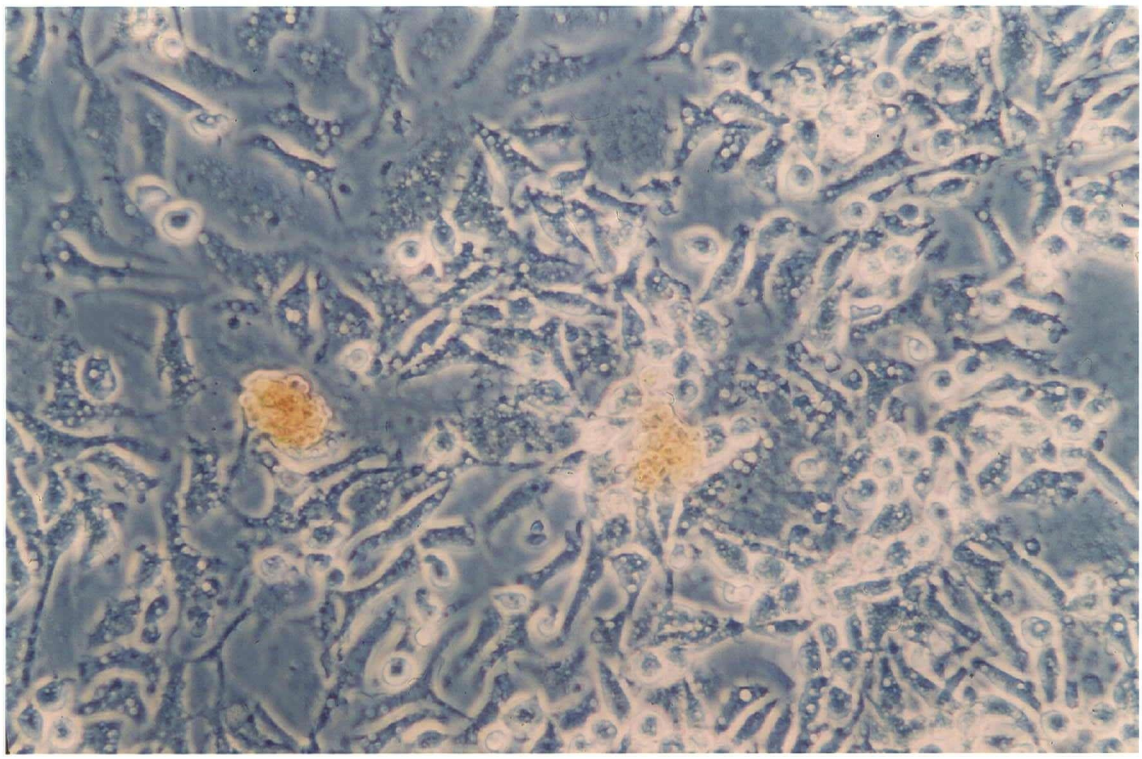


Figure 5.5 One step long- term bone marrow culture (LTBMC) of DBA/2 bone marrow cells

Freshly harvested bone marrow cells from DBA/2 mice were resuspended in long- term bone marrow culture (LTBMC) medium (MyeloCult 5300) containing 10^{-6} M hydrocortisone and dispensed into 24 well tissue culture plates in 1.0 ml volumes containing 1 x 10^6 cells. Establishment of the stromal layer by stromal precursor cells was followed by initiation and maintenance of haematopoiesis. The nonadherent cells which contain haematopoietic cells at various phases of maturation were harvested weekly via gentle agitation and aspiration of the media, leaving the stroma behind and intact. This picture was taken at week 2 of a five week LTBMC culture using a Nikon F3 camera connected to the Zeiss Axiovert 35 inverted microscope. The 10x objective with phase contrasting setting 1 (P1) was selected. Note the confluent stromal layer as well as the clusters of cells that appeared to rest on top of the it; active haematopoiesis occurs at these clusters.

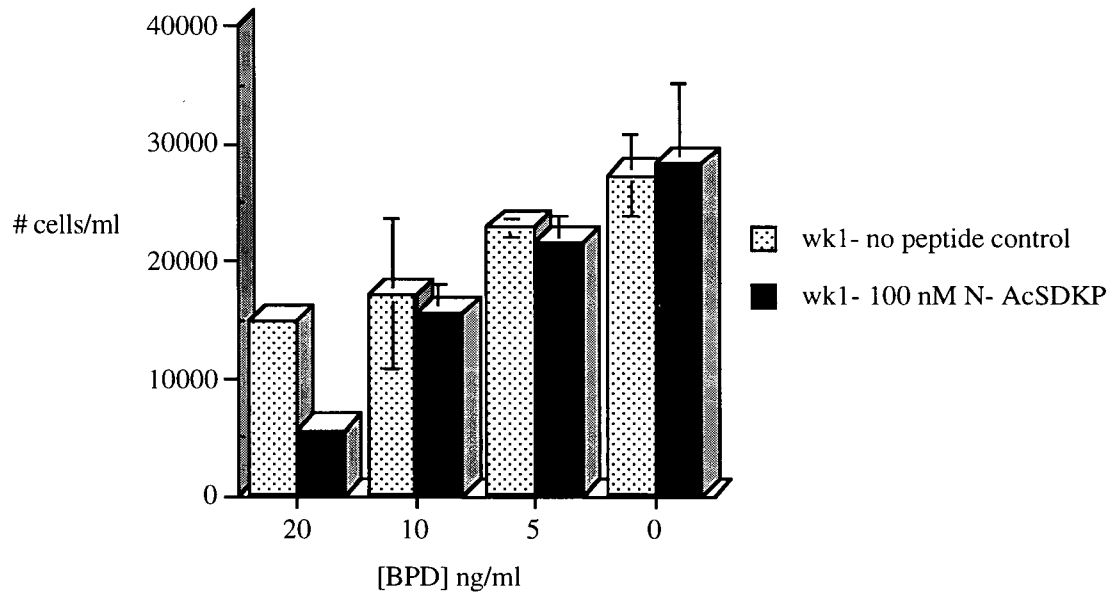


Figure 5.6 Comparison of week 1 long- term bone marrow culture (LTBMC) harvests of nonadherent cells from samples preincubated with 100 nM N-AcSDKP or medium followed by PDT

Graphic representation of the first row of table 5.3 compares the cell numbers of the harvested nonadherent populations of week 1 LTBMC. Cell numbers were similar between the peptide- treated and control groups that were exposed to 0, 5, 10 ng/ml of BPD and 15 J/cm^2 red light. The difference observed at 20 ng/ml did not extend to later harvests (week 2- 5).

Table 5.4- Numbers of CFU- GM progenitor colonies generated from nonadherent cells harvested weekly from long- term bone marrow culture of DBA/2 haematopoietic cells preincubated in the presence or absence of 100 nM N-AcSDKP followed by BPD- mediated PDT

	No peptide control				100 nM N-AcSDKP			
[BPD] ng/ml	20	10	5	0	20	10	5	0
week 1	6.80 ± 1.20	12.30 ± 2.30	16.00 ± 1.70	19.80 ± 2.20	2.20 ± 0.50	11.20 ± 0.20	14.50 ± 0.50	13.00 ± 1.00
week 2	1.80 ± 0.80	2.80 ± 0.20	5.70 ± 3.00	7.20 ± 3.50	0.70 ± 0.70	5.80 ± 3.80	7.50 ± 3.80	11.50 ± 0.20
week 3	7.70 ± 0	7.30 ± 1.00	23.50 ± 15.20	15.70 ± 3.00	2.30 ± 0.30	5.80 ± 4.20	19.70 ± 13.70	32.50 ± 2.80

Table 5.4 Cells from the weekly harvestings of nonadherent populations of LTBMC were resuspended at a standard volume and grown in standard short- term colony assays. The numbers of day 7 CFU- GM consisting of 40 or more cells were enumerated using an inverted microscope. Each number, rounded off to one significant figure, represent an average of two independent samples (n= 2) and source of error is standard error of the mean.

Selective photoprotection of normal human bone marrow cells but not leukaemic cells from PDT by N-AcSDKP

Human bone marrow cells or primary leukaemic cells from a newly diagnosed CML patient were preincubated with 100 nM N-AcSDKP or tissue culture medium for 1.5

hr prior to PDT mediated by 10 ng/ml BPD and 15 J/cm^2 red light. The treated cells were then put into short- term colony assays which measured late human haematopoietic progenitors which give rise to CFU- GM or CML leukaemic precursor cells which give rise to CFU- L. In addition, the human CML cell line K562 was also examined. Numbers of colonies from the peptide group were divided by the number of colonies in the tissue culture medium group to obtain a photoprotection ratio. A ratio of more than 1 signifies photoprotection and the results is shown in figure 5.7. Cells from two independent human bone marrow samples benefited from preincubation with 100 nM N-AcSDKP with photoprotection ratios of 4.5 and 8. However, the leukaemic cell line K562 and cells from the primary leukaemic cell sample did not benefit from N-AcSDKP because both samples demonstrated photoprotection ratios of 1. Results from the human experiments mirrored those of the earlier murine assays; that is, N-AcSDKP selectively protected late haematopoietic progenitors but not leukaemic cells from BPD- mediated PDT.

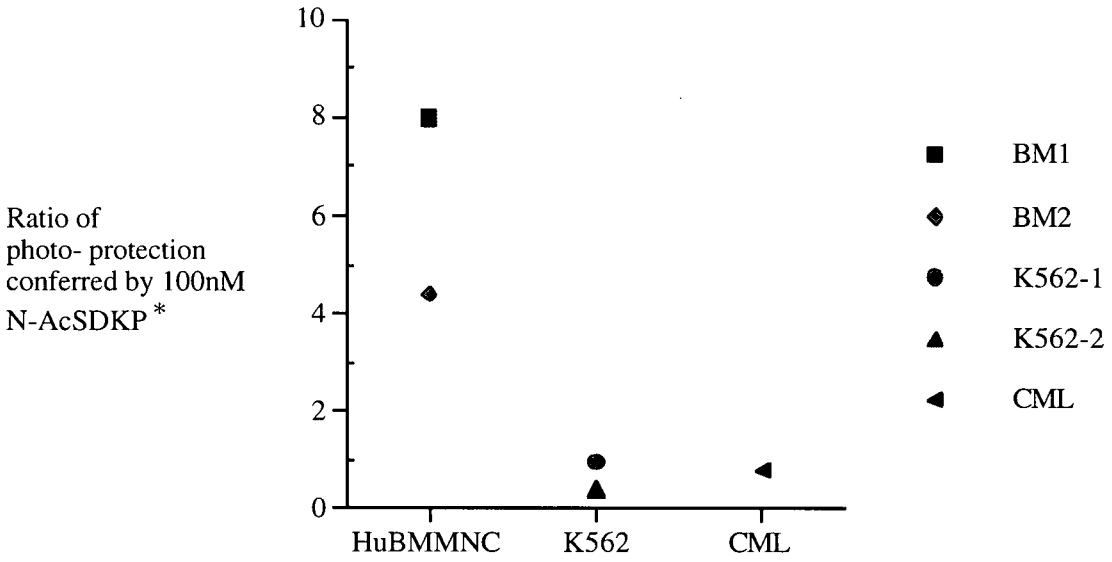


Figure 5.7- Cytoprotection conferred by 100 nM of N-AcSDKP against subsequent BPD- mediated PDT was specific for normal human bone marrow cells

Preincubation of normal human bone marrow cells with 100 nM of N-AcSDKP for 1.5 hr selectively protected them from subsequent PDT treatment mediated by 10 ng/ml of BPD and 15 J/cm^2 of red light. The human myelogenous leukaemic cell line K562 and primary untreated human CML cells did not respond to the photoprotective effects of N-AcSDKP. The ratios of photoprotection of the respective cell type were derived from the ratio of the percentage of colonies from PDT- treated cells that were preincubated with 100 nM N-AcSDKP over the percentage of colonies of PDT- treated cells that were preincubated with medium.

DISCUSSION

The process of haematopoiesis is a highly regulated process involving both positive and negative signals.[200] Various colony stimulating factors or cytokines have been identified and cloned and some of these factors show promise in the clinical management of the cancer patient.[357] Functional haematopoiesis also depends on its efficient control via negative regulators such as IL- 1, TGF- β, and chemokines such as MIP- 1α.[362] A new class of oligopeptide haematopoietic inhibitors which include the tetrapeptide N-Acetyl-Ser- Asp- Lys- Pro (AcSDKP) and pyroGlu- Glu- Asp- Cys- Lys (pEEDCK) was recently characterised.[205] N-AcSDKP is derived from proteolytic processing of the N- terminus of thymosin β4, a protein which binds F- actin.[372, 373] Serum level of N-AcSDKP changes during chemotherapy in leukaemic patients and in mice after treatment with cytosine arabinoside (ara- C).[374, 375] Numerous studies have demonstrated that N-AcSDKP selectively inhibits cell cycle progression of normal haematopoietic cells, preventing the entry from G_0/G_1 to S- phase.[204, 365] Selective cycle inhibition of normal cells by N-AcSDKP protects them from chemotherapy, hyperthermia, radiation, as well as PDT treatments.[206-209] However, other mechanisms mediated by N-AcSDKP could be involved as well. Large scale production of the peptide for proposed trials for human applications prompted subsequent human pharmacokinetic studies.[376] N-AcSDKP therefore joins the other haematopoietic inhibitory cytokines and peptides and becomes part of the armamentorium in the new field of stem cell protection, in which biological factors are used to effect selective protection of normal haematopoietic cells from chemotherapy and radiation therapy.[210, 367, 368, 377] In addition, other candidates such as the thiol- based compound amifostine showed promise in ameliorating the harmful side effects of chemotherapy on normal haematopoietic cells.[378] For example, incorporation of amifostine in the *ex vivo* purging of autologous bone marrow from breast cancer patients with 4-hydroperoxycyclophosphamide significantly improved subsequent haematological recovery.[379]

In this study, N-AcSDKP was used to effect selective photoprotection on normal haematopoietic cells. A previous study has shown that N-AcSDKP could be used to protect normal human bone marrow cells from PDT mediated by the first generation

photosensitiser Photofrin®.[209] Since BPD is a much more potent and pure preparation than Photofrin®, this study aimed to investigate whether N-AcSDKP also extended its protective potential to BPD. Freshly isolated bone marrow cells from DBA/2 mice or L1210 leukaemic cells were incubated with 100 nM of N-AcSDKP for 1.5 h prior to exposure to PDT mediated by BPD. Colony forming units of granulocyte/ macrophage (CFU- GM) and colony forming units of leukaemic cells (CFU- L) were determined using standard colony assays. Neither the tetrapeptide N-AcSDKP nor the control peptides SDKP and N-AcSDKE inhibited the growth of CFU- GM and CFU- L. The absence of inhibitory effect on late murine progenitors supported earlier experimental findings using CBA/H derived haematopoietic cells.[380] N-AcSDKP protected DBA/2 late haematopoietic progenitors from PDT, especially at the lower doses of BPD of 2.5 and 5.0 ng/ml. The control peptides N-AcSDKE and SDKP as well as the medium control had no protective effects on the cells. The same protective effect was not observed in L1210 cells preincubated with N-AcSDKP or the control peptides. In addition, Bonferroni (all- pairwise) multiple comparison test of the data from the progenitor assays demonstrated significant intergroup differences between PDT- treated DBA/2 haematopoietic cells that were preincubated with N-AcSDKP and the PDT- treated cells that were preincubated with the control peptides or medium. No significant intergroup difference was observed in PDT- treated L1210 cells preincubated with the different peptides and one therefore can conclude that N-AcSDKP selectively protected DBA/2 haematopoietic progenitor cells but not L1210 leukaemic cells from BPD-mediated PDT. Furthermore, one step long- term bone marrow culture (LTBMC) assay revealed that the photoprotective effect on the late progenitors measured by the colony assays did not extend to earlier progenitors. Cell numbers of weekly harvests as well as the numbers of CFU- GM produced from the harvests were not significantly different between the peptide and control group treated with PDT at different doses. Both harvested cell number and colony number correlated inversely with the dose of BPD and this correlation was more obvious in the earlier harvests of week 1 to 3. The loss of adherence of the stromal layer during harvesting of the suspension cells contributed to the inflated cell numbers and therefore colony numbers in the harvests of weeks 4 and 5 (data not shown), which also contributed to the loss of correlation between the BPD doses and the results of the LTBMC assay. An apparent contradiction exists between the results in Figure 5.1 and table 5.4. Specifically, short- term agar colony assay of DBA/2 bone marrow cells

demonstrated the photoprotective effect of 100 nM N- AcSDKP (figure 5.1); however, the numbers of CFU- GM derived from week 1 LTBMC harvests of control and peptide treated cells showed no sigificant difference. In fact, the same trend was noted in the direct cell counts of the week 1 harvests (table 5.3).

N-AcSDKP at 100 nM was able to selectively protect normal human bone marrow cells from BPD- mediated PDT. This confirms earlier results by Coutton and colleagues using Photofrin®- mediated PDT.[209] The procedures described here differed somewhat from the ones used in the above mentioned paper. Specifically, we were able to achieve photoprotection of the normal human haematopoietic progenitors by 1.5 h incubation with 100 nM of N-AcSDKP whereas a 20 h incubation period was used in Coutton' s paper. We also observed inhibitory activity against human *in vitro* CFU- GM in which human bone marrow cells incubated with 100 nM N-AcSDKP for 1.5 h resulted in the production of 54- 67 % of day 14 colonies compared to the no peptide controls. In addition, the inhibitory effects of N-AcSDKP were not uniform in the different human bone marrow samples which suggested idiosyncratic susceptibility to the peptide. Photoprotection against BPD was not observed when N-AcSDKP was used during preincubation of K562 cells or primary CML cells.

The utility of selective photoprotection mediated by N-AcSDKP is still obvious even though it does not extend to earlier haematopoietic progenitor and stem cells. Normal haematopoietic cells, especially the earlier progenitor and stem cells, already exhibit a great resistance to BPD- mediated PDT as demonstrated in the LTBMC assay performed here and in earlier results of the human LTBMC assays.[292] Pretreatment of murine bone marrow cells effected significant protection of the CFU- GM population from PDT. This permits BPD dose escalation in purging without significant damage to the haematopoietic cell compartment responsible for long- term reconstitution (early progenitors and stem cells) and cells that are responsible for short- term and rapid engraftment (CFU- GMs).

CHAPTER 6: PREPROTECTION OF NORMAL HAEMATOPOIETIC CELLS WITH N-ACSDKP: MECHANISTIC STUDY

6.1 ABSTRACT

Preincubation of DBA/2 bone marrow cells with 100 nM of N-AcSDKP selectively protected of the committed granulocyte/macrophage progenitors (CFU- GM) but not cells of earlier lineage from PDT. Peptide pretreatment, however, did not protect the murine leukaemic cell line L1210. FACS analysis was used to determine BPD uptake in the cells responsible for short- term haematopoietic reconstitution. Double labelling of DBA/2 bone marrow cells preincubated with control medium or 100 nM of N-AcSDKP with anti- CD34 antibody and 10 ng/ml BPD demonstrated no significant difference in mean fluorescent intensity (MFI) of BPD in the $CD34^+$ population. In addition, peptide incubation did not alter the proportion of $CD34^+$ cells. The bioactivity of N-AcSDKP was verified by standard ara- C suicide assay. Incubation of DBA/2 bone marrow cells with 100 nM N-AcSDKP for 1.5 h or 5.0 h resulted in substantial protection of CFU- GM formation, via prevention of S- phase entry, from subsequent exposure to 50 or 100 μM of ara- C. Since 1.5 h incubation with N-AcSDKP was able to effect cycle inhibition in CFU- GM progenitors, the observed photoprotective effect could therefore be the result of N-AcSDKP- mediated cycle inhibition. This possibility was proven by pretreating the bone marrow cells with 50 μM Ara- C which resulted in the destruction of S- phase cells. The surviving cells were more tolerant to subsequent PDT-cytotoxicity, suggesting that quiescent CFU- GM progenitors are more resistant to PDT than their active counterpart. Examination of cellular glutathione content revealed no significant difference between the peptide- treated cells and the control cells. Therefore, the specific mechanisms responsible for N-AcSDKP- mediated photoprotection are still not clear. However, it is possible that N-AcSDKP- mediated cell cycle inhibition could affect multiple pathways (i.e. drug uptake, levels of protective thiols, subcellular localisation of target proteins, etc) that together result ultimately in protection from PDT.

6.2 INTRODUCTION

Selective protection of normal haematopoietic stem cells and progenitor cells during *in vivo* and *ex vivo* antineoplastic radiochemotherapy is a therapeutic goal long sought after by clinicians (and patients alike). Other normal tissues, such as the regenerating epithelium of the gastrointestinal tract and hair follicular cells, are also susceptible to the side effects of therapy; in addition, organ- specific toxicities from potent yet indispensable drugs such as doxorubicin and vincristine limit the utility of these agents. Nevertheless, bone marrow toxicity encourages the development of life- threatening episodes of thrombocytopaenia, anaemia, as well as immunosuppression and therefore remains one of the major limiting factors in the use of therapeutically efficient doses of radiochemotherapy. Therefore, one ideally would like to selectively protect normal haematopoietic cells during therapy so as to enlarge the therapeutic window. *In vivo* bone marrow protection can be effected through the use of positive haematopoietic regulators, such as granulocyte and granulocyte- macrophage colony stimulating factor (G- CSF, GM-CSF) and erythropoietin (EPO), which stimulate the proliferation plus differentiation of normal haematopoietic cells to counteract therapy- induced myelosuppression.[200] The usefulness of these factors in the clinical setting, especially in effecting myeloprotection from dose and schedule intensification of radiochemotherapy, is controversial.[114, 115, 360] Nevertheless, judicious and rational use coupled with improving clinical and scientific knowledge will increase the applicability of these factors in cancer therapy.[361] Conversely, cytokines such as tumour necrosis factor- α (TNFα) and interleukin- 1 (IL- 1), the chemokine macrophage inflammatory factor- 1α (MIP- 1α), and small peptides such as acetyl- N- Ser- Asp- Lys- Pro (N-AcSDKP) and pGlu- Glu- Asp- Cys- Lys (pEEDCK) can also effect stem cell protection through the inhibition of cell cycling and possibly other means.[210, 368] The phosphorylated sulphydryl compound amifostine (WR- 2721) has been demonstrated to afford haematopoietic protection to murine hosts exposed to γ- radiation or cytotoxic agents, probably via the modulation of cellular thiol concentrations.[381] Amifostine, in combination with G- CSF, can significantly accelerate myelopoietic recovery in lethally irradiated mice.[382, 383]

Haematopoietic stem cell transplantation is essentially a form of *ex vivo* bone marrow protection and the transplantation of bone marrow- derived stem cells constituted one of the earliest application of such therapy.[37, 384] Autologous haematopoietic stem cell transplantations (bone marrow or peripheral blood stem cells) eliminate the need for HLA-compatible allogeneic donors and hence reduce the risk of graft- versus- host disease.[152] Nevertheless, contamination of autologous stem cell harvests by neoplastic cells leads to an increase in the incidence of disease relapse posttransplantation.[154] *Ex vivo* manipulation or purging of the harvests with pharmacological agents attempts to eradicate a significant number of the contaminating cancer cells while preserving enough of the normal haematopoietic cells for reconstitution. The same principle that is applicable for *in vivo* stem cell protection is also suitable for *ex vivo* purging in that the procedure attempts to enlarge the therapeutic window via the selective protection of normal haematopoietic cells, which permits dose escalation of the purging regimen.[205, 210]

Mechanistically, the current paradigm of stem cell protection mediated by inhibitory molecules revolves around the cell cycle. Moser and Paukovits, in their review article, conceded that cytostatic drug- induced haematopoietic damage is effected on the proliferating compartments of the bone marrow which contain the mitotically active haematopoietic progenitor and precursor cells, whose depletions result in the recruitment of normally quiescent stem cells into cycle.[367] Unfortunately, the cycling stem cells, in an effort to replenish the progenitors, in turn become vulnerable to damage and repeated rounds of chemotherapy can lead to the irreversible impairment or quantitative impairment of the stem cell pool.[385] Gardner *et al.* recently demonstrated that chemotherapy causes the exhaustion of the pluripotent primitive haematopoietic stem cell (PHSC) pool, resulting in prolonged marrow reconstitutive defects.[386] Stem cell inhibitors such as MIP- 1α, N-AcSDKP and pEEDCK selectively prevent cell cycle entry of normal haematopoietic stem cells and precursor cells. N-AcSDKP was originally characterised to prevent the recruitment of murine PHSCs into the DNA synthetic or S- phase after ara- C chemotherapy as determined by spleen colony forming units (CFU- S) and administration of the peptide protected mice from lethal doses of ara- C.[363, 387] Furthermore, N-AcSDKP was shown to inhibit the S- phase entry of human haematopoietic progenitor and precursor cells *in vitro* in short- term progenitor assays and long- term bone marrow culture.[388, 389] Leukaemic cells do not appear to respond to the regulatory effects of N-AcSDKP and

therefore are not afforded protection by the peptide.[204, 365] MIP- 1α was found to be another mediator of stem cell protection with similar target populations and mechanisms as N-AcSDKP.[201, 202, 366] Interestingly, Cashman *et al.* presented evidence that the N-AcSDKP- mediated inhibitory effect could be abrogated by the simultaneous addition of MIP- 1β, an antagonist of MIP- 1α therefore suggesting that MIP- 1α is a downstream mediator of N-AcSDKP bioactivity.[204]

A different form of stem cell protection described by Zucali and colleagues concerns the cytoprotective effects of TNFα and IL- 1 on human and murine haematopoietic cells from ionising radiation and the purging agent 4- hydroperoxycyclophosphamide (4-HC).[390, 391] Furthermore, they presented evidence that cytokine induction of the enzymes manganese- superoxide dismutase (MnSOD) and aldehyde dehydrogenase (ALDH) was responsible for their cytoprotective effects.[369, 370, 377] MnSOD detoxifies the highly reactive superoxide anion, generated during cellular exposure to ionising radiation or the drug doxorubicin, via its transmutation into hydrogen peroxide.[392] ALDH, whose transcription in human marrow cells is increased three- fold with IL- 1 or TNFα, detoxifies 4- HC.[369]

Stem cell protection can be mediated by cell cycle inhibition, induction of enzymes, and augmentation of the cellular thiol pool or, conversely through the use of positive regulators which numerically expand blood cells to counteract the deleterious effects of therapy. In this chapter, we examined some of the mechanisms responsible for the selective photoprotection of normal haematopoietic cells by N-AcSDKP; specifically, the effects of N-AcSDKP on cell cycling, photosensitiser uptake, and cellular glutathione were investigated.

6.3 *RESULTS*

BPD uptake by DBA/2 bone marrow cells preincubated with 100 nM N-AcSDKP

Freshly isolated femoral bone marrow cells from DBA/2 mice were incubated in the presence or absence of 100 nM N-AcSDKP in serum- free IMDM for 1.5 h followed by incubation with 10 ng/ml BPD for 0.5 h. FACS analysis was then used to assess for the percentage of CD34- positive cells. CD34 is a surface sialomucin expressed by populations of haematopoietic progenitor and stem cells.[393] Next, the level of BPD uptake or association in the peptide- treated population was determined by analysing BPD mean fluorescence intensity (MFI) in $CD34^+$ cells (described in the *Experimental procedure* chapter). As shown in table 6.1, preincubation with N-AcSDKP did not alter the proportion of CD34- expressing cells in the peptide- treated group (16.13 ± 0.94 %) compared to the culture medium- treated control group (16.83 ± 0.43 %). Furthermore, there was no significant difference in BPD MFI between the peptide- treated group (4.67 ± 0.25) and the control group (4.84 ± 0.18). Therefore, *in vitro* preincubation of DBA/2 bone marrow cells with 100 nM N-AcSDKP for 1.5 h did not appreciably alter the proportion of $CD34^+$ cells nor did it change the degree of BPD association within the $CD34^+$ population. Nevertheless, as demonstrated in chapter 5, the same peptide preincubation protocol was able to effect cytoprotection of DBA/2 CFU- GM progenitors from PDT.

Figure 6.1a-c Representative FACS histograms of BPD uptake in control and N- AcSDKP- treated CD34- expressing murine bone marrow mononuclear cells (BMMNC)

Preincubation of DBA/2 bone marrow cells with 100 nM N-AcSDKP for 1.5 h did not significantly alter the relative proportion of $CD34^+$ cells nor BPD uptake in these cells. Two colour FACS analysis of cells labelled with biotin- CD34 antibody (clone RAM34)/ FITC- strepavidin and 10 ng/ml BPD. RAM34, a monoclonal antibody of rat origin, is specific for the mouse haematopoietic stem cell antigen CD34. FACS analysis was based on 100000 events per sample. N-AcSDKP does not significantly alter the percentage of $CD34^+$ cells (Figure 6.1a) and the peptide does not affect BPD association and uptake in BMMNCs (Figure 6.1b). In addition, N-AcSDKP does not alter BPD association and uptake in $CD34^+$ cells (Figure 6.1c).

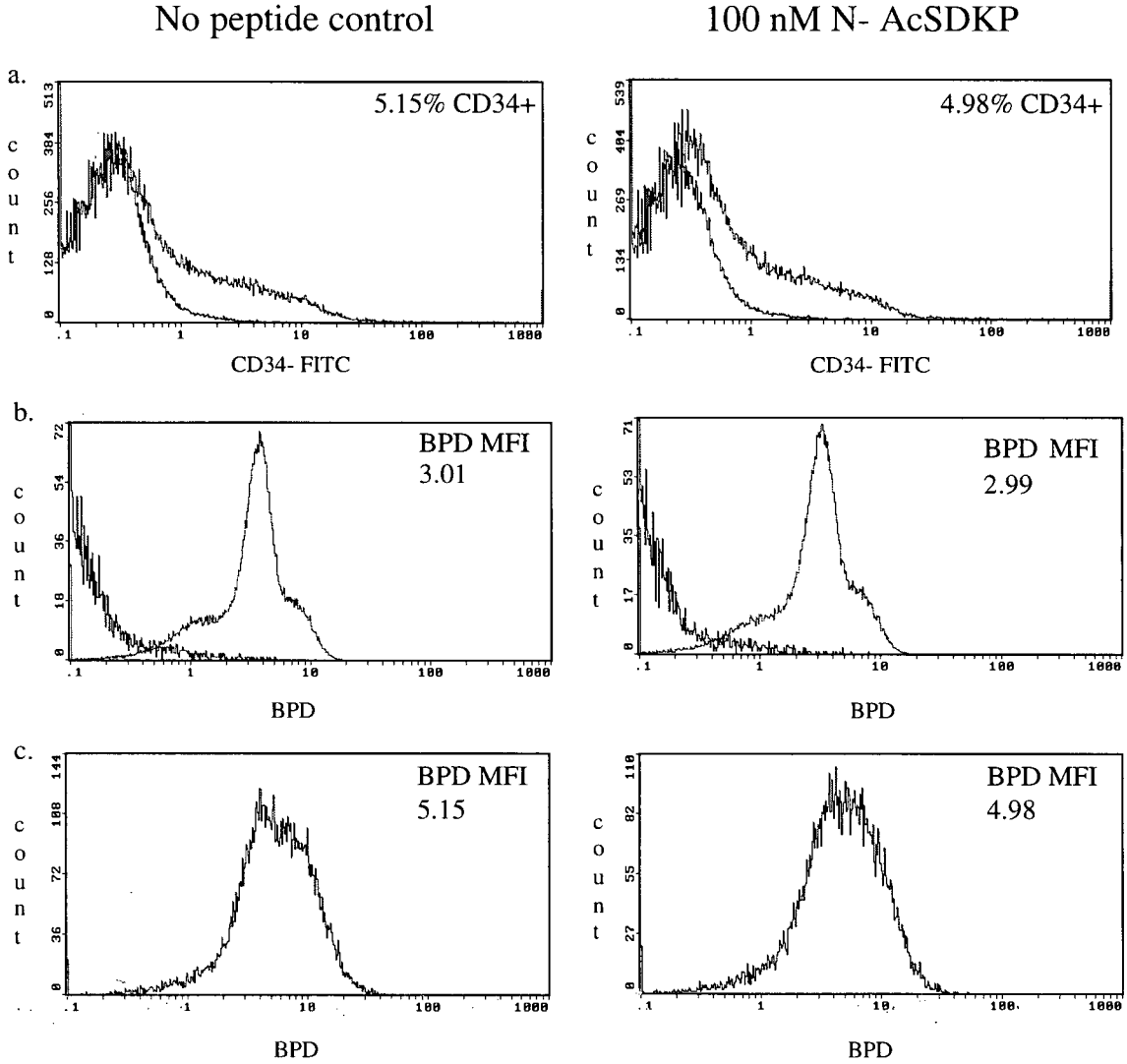

No peptide control
100 nM N- AcSDKP
a.
5.15% CD34+
4.98% CD34+
count
CD34- FITC
b.
BPD MFI
3.01
BPD MFI
2.99
BPD
c.
BPD MFI
5.15
BPD MFI
4.98

Table 6.1 BPD uptake in the CD34 expressing subpopulation of DBA/2 bone marrow cells

	% CD34 expressing bone marrow cells		*BPD MFI in $CD34^+$ cells*	
inhibitor treatment	**no peptide**	**100 nM N-AcSDKP**	**no peptide**	**100 nM N-AcSDKP**
expt. 1	15.30	17.10	4.33	4.48
expt. 2	18.00	17.40	5.15	4.98
expt. 3	15.10	16.00	4.52	5.05
Mean	16.13	16.83	4.67	4.84
S.E.	0.94	0.43	0.25	0.18

Table 6.1 Preincubation of DBA/2 bone marrow cells with 100 nM N-AcSDKP for 1.5 h did not significantly alter the relative proportion of $CD34^+$ cells or BPD uptake in these cells. Two colour FACS analysis of cells labelled with biotin- CD34 antibody (clone RAM34)/ FITC- strepavidin and 10 ng/ml BPD. RAM34, a monoclonal antibody of rat origin, is specific for the mouse haematopoietic stem cell antigen CD34. FACS analysis was based on 100000 events per sample. Data from three independent experiments are presented and source of error is derived from the standard error of the mean.

Inhibition of the proliferative activity of murine CFU- GM progenitors by 100 nM N-AcSDKP

Figure 6.2 shows that incubation of freshly harvested DBA/2 bone marrow cells with 100 nM N-AcSDKP for 1.5 h resulted in the inhibition of S- phase entry of the CFU-GM progenitors. Cells that were exposed to N-AcSDKP became resistant to the cytotoxic activity of ara- C, an S- phase specific toxin. Inhibition of S- phase entry was also demonstrated in cell samples that were incubated with N-AcSDKP for 5.0 h (figure 6.3). Interestingly, the absolute numbers of day 7 colonies were not appreciably reduced by N-AcSDKP preincubation. We therefore demonstrated that the fraction of S- phase DBA/2 CFU- GM progenitors could be reduced with *in vitro* exposure to 100 nM N-AcSDKP for 1.5 h. In addition, our data suggests that the observed photoprotective effect mediated by N-AcSDKP could be due to its ability to inhibit cell cycle progression into the DNA synthetic or S- phase. We proceeded to examine the relationship between cell cycling and PDT susceptibility in DBA/2 CFU- GM progenitors.

Cell cycle inhibition of DBA/2 haematopoietic progenitor cells mediated by 100 nM N-AcSDKP

Exposure of freshly harvested DBA/2 bone marrow cells to 100 nM N-AcSDKP for 1.5 h effected cell cycle inhibition of the CFU- GM fraction, rendering the cells resistant to the S- phase specific cytotoxicity of ara- C. The observation also suggest that the photoprotective effect reported in chapter 5 was mediated via the inhibition of cycle entry. Figure 6.4 further illustrates the relationship between cell cycling and susceptibility to BPD- mediated PDT in the CFU- GM progenitor population. Harvested marrow cells were incubated with 50 μM ara- C for 1.0 h which, as shown in figure 6.2, resulted in the destruction of approximately 50 % of the CFU- GM progenitor population. Ara- C effected

selective cytotoxicity to cells in S- phase which resulted in the enrichment of quiescent cells (G_0/G_1). Subsequent exposure to BPD- mediated PDT revealed differential susceptibility to PDT cytotoxicity. Even though 50 μM ara- C caused a reduction of the absolute number of day 7 CFU- GM, the surviving fraction was significantly more resistant to PDT than the control cells not pretreated with ara- C (figure 6.4).

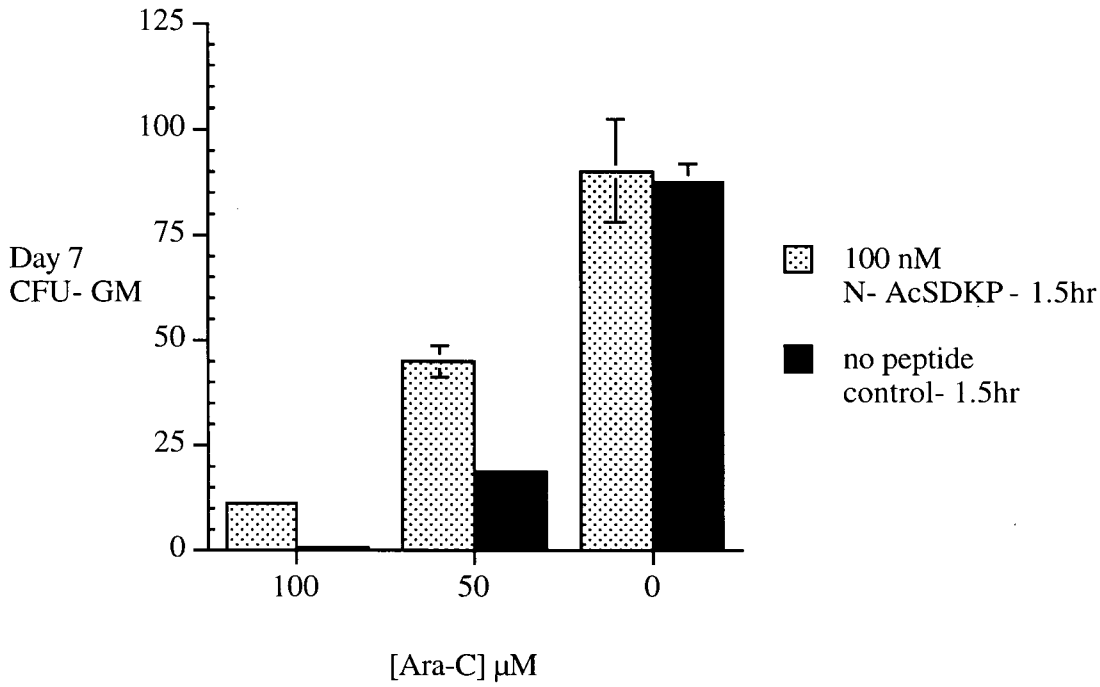


Figure 6.2 Effect of 1.5 h preincubation with 100 nM N-AcSDKP had on the cycling status of CFU- GM from DBA/2 mice

CFU- GM formation is protected from ara- C cytotoxicity by N-AcSDKP; however, exposure to the peptide alone had no effects on the number of CFU- GM. DBA/2 bone marrow cells were incubated with 100 nM N-AcSDKP for 1.5 h in serum free IMDM at 37°C. The cells were washed and incubated with 0, 50, or 100 μM of ara- C for 1 h and put into standard agar- based colony assay in triplicates. Colonies, which consisted of 50 or more cells, were counted on day 7 of the experiment with an inverted microscope. Approximately 70000 cells were seeded per plate which translated to a plating efficiency of 0.129 %. Data obtained from 3 independent experiments is presented. Error bars are derived from standard error of the mean (SEM).

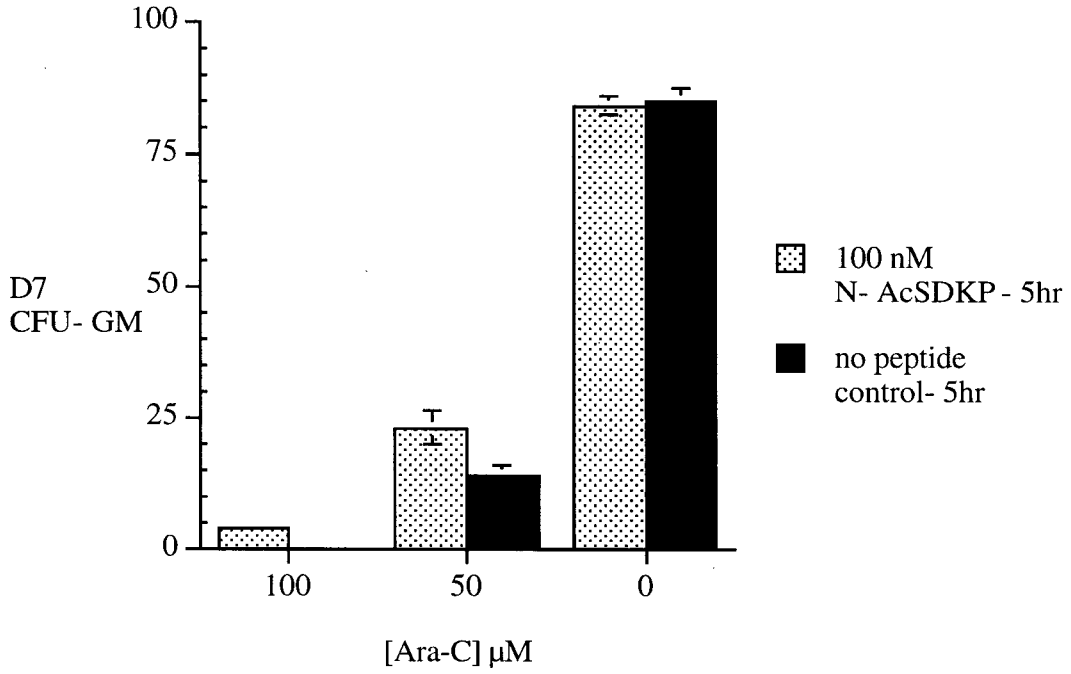


Figure 6.3 Effect of 5.0 h preincubation with 100 nM N-AcSDKP had on the cycling status of CFU- GM from DBA/2 mice

CFU- GM formation is protected from ara- C cytotoxicity by N-AcSDKP; however, exposure to the peptide alone had no effects on the number of CFU- GM. DBA/2 bone marrow cells were incubated with 100 nM N-AcSDKP for 5.0 h in serum free IMDM at 37°C. The cells were washed and incubated with 0, 50, or 100 μM of ara- C for 1 h and put into standard agar- based colony assay in triplicates. Colonies, which consisted of 50 or more cells, were counted on day 7 of the experiment with an inverted microscope. Approximately 70000 cells were seeded per plate which translated to a plating efficiency of 0.129 %. Data obtained from 3 independent experiments is presented. Error bars are derived from standard error of the mean (SEM).

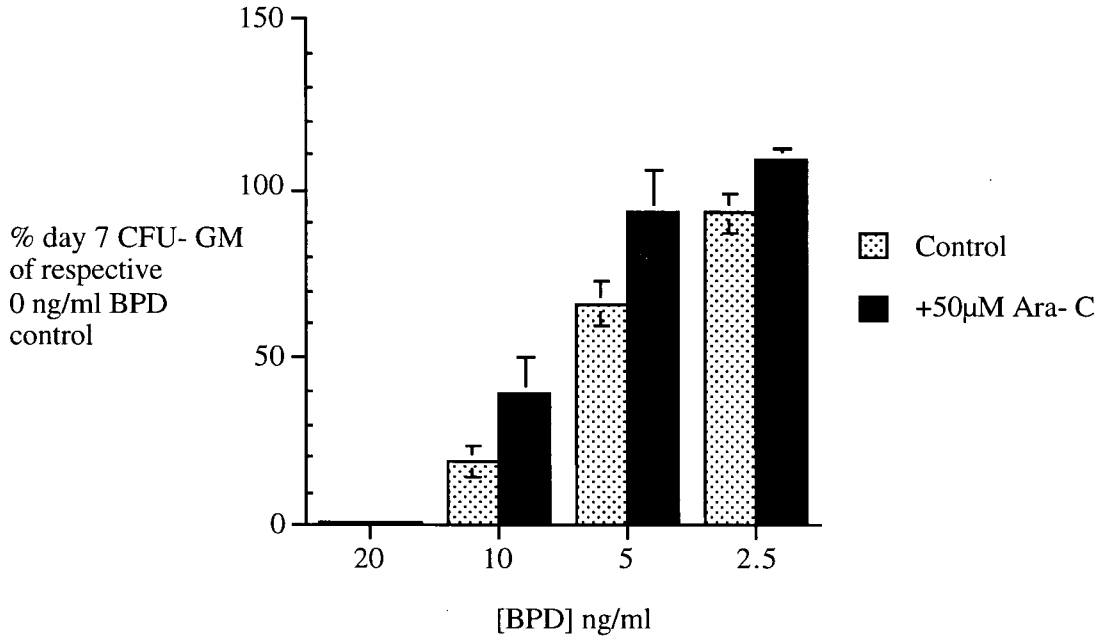


Figure 6.4 Correlation between cell cycling and susceptibility to BPD-mediated PDT in DBA/2 CFU- GM progenitors

CFU- GM progenitors preincubated with 50 μM were less susceptible to BPD- mediated PDT. Even though ara- C resulted in a reduction of the absolute number of day 7 CFU- GM, cells that were preincubated with 50 μM ara- C showed higher relative survival from subsequent PDT treatment than control cells preincubated with medium. Control DBA/2 progenitors yielded 185.67 ± 14.57 colonies from 70000 cells plated (0.264 % cloning efficiency) whereas progenitors cells treated with 50 μM ara- C yielded 169.33 ± 9.07 colonies from 140000 cells plated (0.121 % cloning efficiency). Results from 3 independent experiments are presented. Error bars are derived from standard errors of the mean (SEM).

Glutathione level in DBA/2 bone marrow cells treated with 100 nM N-AcSDKP

The amount of intracellular glutathione (GSH) was established using the Tietze method as described in the *Experimental procedure* chapter. The GSH content from cell extracts derived from 2 x 10^6 DBA/2 bone marrow cells incubated with 0 (51.67 $\pm$ 11.46 μM GSH), 10 (47.01 $\pm$ 12.59 μM GSH), or 100 nM (51.77 $\pm$ 15.54 μM GSH) N-AcSDKP for 1.5 h were not significantly different from one another (figure 6.5). We therefore could not attribute the photoprotective effect of N-AcSDKP to an increase of the intracellular antioxidant GSH. However, a more sensitive method of measurement, such as one that is based on the fluorescence detection of GSH- bound monochlorobimane or monobromobimane, could possibly reveal any alterations of cellular GSH as a result of N-AcSDKP incubation. In addition, we did not consider other cellular antioxidants which could mediate photoprotection in this experiment.

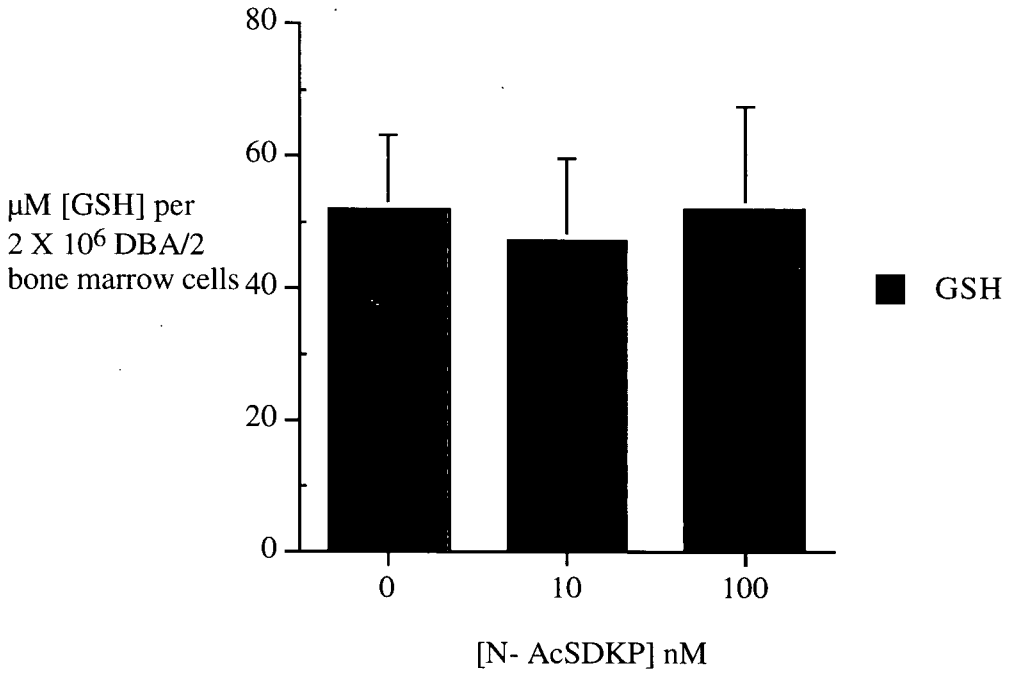


Figure 6.5 Cellular glutathione content in DBA/2 bone marrow cells incubated with 0, 10, or 100 nM N-AcSDKP

Incubation of DBA/2 bone marrow cells with N-AcSDKP for 1.5 h had no effect on cellular glutathione (GSH) content as determined by the Tietze method. Cell extracts from 2 x 10^6 cells incubated with 0, 10, 100 nM N-AcSDKP were processed as described in the *Experimental Procedure* chapter and GSH content in μM was measured. Results from two independent experiments are shown. Standard errors of the means (SEM) are used as source of error.

6.4 DISCUSSION

Bone marrow toxicity is one of the major limiting factors in the effective use of radiochemotherapy in cancer control, and dose escalation in therapy is invariably accompanied by life- threatening episodes of marrow aplasia and end- organ toxicities. Interruptions of treatment regimens are frequent occurrences in the clinic and ultimately contribute to inefficient control of cancer. Bone marrow toxicity is especially problematic in allogeneic and autologous haematopoietic stem cell transplantations but it is, however perverse, a necessary part of the therapy. High dose radiochemotherapy is required during the conditioning regimen to prepare the host for the acceptance of donor (allogeneic) haematopoietic cells and the destruction of a significant number of neoplastic cells *in vivo*. Long- term defects in marrow reconstitutive capability as a result of the intensive therapy are evident in human patients and could be due to irreversible damage to the marrow microenvironment which remain of host origin.[385] In fact, Novitzky and Mohammed documented functional aberrations in the haematopoietic progenitor populations of patients 8 years after their transplantations.[394] In addition, the problem of iatrogenic damage to normal haematopoietic cells is also encountered *ex vivo* in the purging of autologous stem cell harvests from cancer patients. Overzealous purging will result in effective destruction of the contaminating neoplastic cells, at the expense of normal haematopoietic cells in the harvest. This can result in faulty engraftment manifested either by a temporal delay in achieving haematopoietic normalisation or an absence of permanent engraftment.[220] Haematopoietic stem cell protection attempts to selectively preserve normal haematopoietic cells from cytotoxic damage, which theoretically permits dose escalation of therapy to effect efficient destruction of the neoplastic cells *in vivo* or *ex vivo*.[210] At the present moment, toxicities to other normal tissues such as the lining of the gastrointestinal tract and hair follicular cells are not obviated by stem cell protection. However, improvements in chemotherapeutic agents and treatment protocols in addition to developments of novel protective compounds will help to ameliorate the iatrogenic damage to normal tissues and alleviate organ- specific toxicity. For example, sodium 2- mercaptoethanesulfonate (MESNA), in conjunction with prophylactic hydration, is used to reduce the incidence of acute haemorrhagic cystitis in patients receiving cyclophosphamide treatment.[395]

Stem cell protection effected by negative regulators of haematopoiesis is a promising area of research. Of special significance are two low molecular- weight oligopeptide inhibitors, the pentapeptide pGlu- Glu- Asp- Cys- Lys (pEEDCK) and the tetrapeptide acetyl- N- Ser- Asp- Lys- Pro (N-AcSDKP).[205] The acknowledged principal mechanism of protection is the inhibition of cell cycle entry into S- phase, where the cell becomes vulnerable to a plethora of cytotoxic agents.[367] Another apparently unique feature of these inhibitors is in the ability to preferentially affect normal haematopoietic cells hence specificity in their protective action.[204, 365] Already, several groups have demonstrated that N-AcSDKP mediated stem cell protection *in vivo* in mice as well as monkeys and dogs.[205, 387, 396]

In this study, we showed that exposure of freshly harvested DBA/2 bone marrow cells to 100 nM (10^{-7} M) N-AcSDKP for 1.5 h in serum free IMDM resulted in the inhibition of S- phase entry of CFU- GM progenitors, manifested by resistance to cycle-specific toxicity of ara- C. N-AcSDKP, however, did not alter the total number of CFU-GM. Together, this demonstrated that inhibition of S- phase entry was achieved without toxicity to the CFU- GM progenitors. Significantly, the incubation protocol was identical to the one used in the cytotoxicity experiments described in chapter 5, suggesting a possible explanation for the observed photoprotection of DBA/2 CFU- GM. Our results are in agreement with those published by Wierenga and Konings.[397] Using a slightly different protocol, they showed that incubation with 10^{-7} to 10^{-12} M of N-AcSDKP for 8 h in the presence of 30 % foetal calf serum and 1 μM captopril resulted in protection of CFU- GM from the S- phase- specific toxin hydroxyurea. The authors rationalised, that due to random distribution of cells over the cell cycle, an incubation period of 8 h was necessary to realise optimal inhibitory effect on the CFU- GM progenitors; this in turn necessitated inclusion of serum in the medium and the addition of 1 μM captopril. Grillon *et al.* showed that the bioactivity of N-AcSDKP is significantly reduced by serum proteases, whose proteolytic activities could be abrogated by the addition of captopril, a metalloproteinase inhibitor.[398] Since we were able to achieve cycle inhibition with an incubation period of 1.5 h, we did not include serum or captopril in the tissue culture medium. In addition, we observed no adverse effects on CFU- GM numbers resulting from our incubatory condition up to 5 h. Other groups could not demonstrate N-AcSDKP- mediated inhibition of the proliferative activity of murine CFU- GM *in vitro*.[380, 399] We, however, believe that the discrepancies

could be due to the different experimental conditions as well as the source of bone marrow cells.

The observed photoprotection of DBA/2 CFU- GM progenitors could therefore be a result of N-AcSDKP- mediated cell cycle inhibition. We showed data supporting this hypothesis by depleting the S- phase fraction from harvested bone marrow cells with 50 μM of ara- C followed by PDT. Overall, the absolute numbers of day 7 CFU- GM in the group that was pretreated with ara- C were approximately 50 % of the control group; nevertheless, the relative percent survival in response to different doses of BPD were significantly higher in the experimental group pretreated with ara- C. Cells in S- phase were susceptible to ara- C cytotoxicity which resulted in the enrichment of non S- phase cells in the survival fraction and the CFU- GM progenitors in ara- C- enriched fraction were found to have higher tolerance to BPD- mediated PDT cytotoxicity (figure 6.4). Studies have shown that cells in S and G_2/M phases are more susceptible to PDT than cells that are in the quiescent G_0/ G_1 phase.[343, 400, 401] Furthermore, Gantchev and colleagues have documented that PDT cytotoxicity from low dose Photofrin® and light exposure resulted in preferential damage to cells in S- phase; whereas high photosensitiser load resulted in general cytotoxicity without cell cycle selectivity.[402] Obochi *et al.* recently demonstrated that activated murine splenic lymphocytes take up more BPD than resting lymphocytes, which subsequently translated to increased susceptibility to PDT.[285]

In addition, previous studies in this laboratory have shown that primary leukaemic cells and leukaemic cell lines take up more BPD than normal haematopoietic cells.[293] Increased uptake of photosensitiser by proliferating cells could be related to cell volume and the surface expression of low density lipoprotein receptors.[257, 259, 403] We therefore proceeded to evaluate the uptake of BPD by DBA/2 bone marrow cells preincubated with 100 nM N-AcSDKP using two colour fluorescent activated cell sorting (FACS) analysis. A rat monoclonal antibody, RAM34, was used to label cells which express the murine equivalence of the human haematopoietic stem and progenitor cell antigen CD34.[404] Morel *et al.* isolated CD34- expressing murine haematopoietic cells from femoral bone marrow labelled with RAM34 which resulted in significant enrichments of various types of primitive haematopoietic cells.[393] *In vitro* incubation of DBA/2 bone marrow cells with 100 nM N-AcSDKP did not alter the proportion of $CD34^+$ cells (16.83 $\pm$ 0.43 % of the peptide-treated group vs 16.13 $\pm$ 0.94 % of medium control). This was expected since the known

characteristics of N-AcSDKP do not include the alterations of the percentage of $CD34^+$ cells nor the surface expression of the CD34 glycoprotein. In addition, it is doubtful whether the short incubatory period of 1.5 h has any effects on these parameters. Unfortunately, we also showed no significant difference in the level of BPD mean fluorescence intensity (MFI) in $CD34^+$ cells between the N-AcSDKP- treated cells (4.67 ± 0.25) and the control cells (4.84 ± 0.18). The absence of demonstrable difference in BPD uptake between the two groups, however, does not diminish the significance of our earlier findings. We have shown in chapter 4 that uptake of BPD is rapid and reaches an equilibrium quickly; therefore, it is not surprising that BPD uptake or association was similar between the two groups. In the experiments described by Obochi and colleagues, murine splenocytes were incubated with 2.5 μg/ml of concanavalin A (Con A) for 72 h which resulted in blast cell formation and increased expressions of the activation markers IL- 2R (5- fold x control) and CD71 (2.67- fold x control).[285] Bohmer and colleagues demonstrated a direct correlation between cell size and photosensitiser uptake.[259] Con A was used to effect maximal stimulation and to highlight the difference between resting and activated lymphocytes in terms of BPD uptake and susceptibility to BPD- mediated PDT. In our system, BPD uptake or association was evaluated between two populations of CFU- GM progenitors which were much closer in their biophysical and biochemical characteristics. Furthermore, Hunt *et al.* have shown discordance between BPD uptake and PDT susceptibility in purified DBA/2 peritoneal macrophages subjected to different forms of cell activation.[405] Specifically, they reported the highest BPD uptake in cells stimulated with 10 μg/ml lipopolysaccharide (LPS); interestingly, cells activated with interferon- γ (IFN- γ, 100 U/ml for 72 h) or medium control took up equivalent levels of the photosensitiser. Susceptibility to PDT, however, was highest in the IFN- γ stimulated cells followed by resting cells (medium control) and lastly, LPS- stimulated cells. The authors postulated that the dichotomy between uptake and cytotoxicity could be due to a multitude of factors including quenching of reactive oxygen species, enhancement of PDT cytotoxicity from nitric oxide generated via IFN- γ stimulation, and distinct cellular targets in the differentially stimulated cells. Of the last point, FACS analysis does not distinguish between cells with different intracellular localisations of photosensitiser and therefore cells with similar BPD MFIs could have different intracellular distributions of the photosensitiser. The group of Wilson *et al.* at the Ontario Cancer Institute noticed similar total cellular uptake of

Photofrin® by the PDT- resistant cell line RIF- 8A and the parental line RIF- 1; they, however, found significant reduction in the mitochondrial accumulation of the photosensitiser in the PDT resistant cell line.[406, 407] We believe that the unique intracellular milieu during the S- phase of the cell cycle presents novel targets for PDT. The expressions of S- phase specific protein products such as cyclin D and the S- phase specific assembly of the DNA replication complexes are such examples.[408, 409] Berg and Moan, in their recent review article, postulated that microtubules are cycle- specific targets of PDT cytotoxicity.[410]

Another possible mechanism responsible for N-AcSDKP- mediated photoprotection is through the modulation of the cellular thiol pool, which could effect defence against oxidative damage. We could not detect any difference in the intracellular concentrations of glutathione (GSH) between N-AcSDKP- treated cells and control cells using the Tietze method. Another assay technique using either the GSH- specific fluorescent probes monochlorobimane or monobromobimane in conjunction with FACS analysis could provide a more sensitive method of single cell measurement of GSH.[411]

N-AcSDKP- mediated protection of DBA/2 bone marrow progenitor cells from PDT cytotoxicity is correlated with the ability of the peptide to prevent cell cycle progression. Artificial depletion of S- phase cells with ara- C resulted in an enrichment of cells which are significantly more resistant to PDT than their proliferating counterpart. Attempts to elucidate the mechanisms responsible for N-AcSDKP- mediated photoprotection included the examination BPD uptake and GSH content in cells after peptide incubation. However, neither of the two parameters showed significant difference between the N-AcSDKP and the control group. We believe that other cell cycle- specific factors such as the expression of novel proteins and assembly of phase- specific protein complexes and therefore novel targets may contribute to the observed heightened PDT cytotoxicity in proliferating cells.

CHAPTER 7: DISCUSSION

Purging of autologous haematopoietic stem cell harvests with light- activated drugs is gaining increasing exposure in the clinical setting as a result of the explosive growth of the field of photodynamic therapy (PDT) and the need for new and effective purging modalities. Purging is sometimes necessary in autologous haematopoietic stem cell transplantations because of the probable presence of contaminating neoplastic cells in the harvest, which can contribute to posttransplantation relapses.[154] Development of the second generation photosensitiser benzoporphyrin derivative monoacid ring- A (BPD), which has many biophysical and biochemical characteristics conducive to effective PDT, has significantly improved the efficacy as well as broadened the indications of this treatment modality.[269, 284] In addition, BPD- mediated PDT has been shown to effect significant yet selective destructions of murine and human leukaemic cells *in vitro* and in an *ex vivo* murine bone marrow purging model.[291, 292, 294]

In this thesis, augmentation of BPD- mediated PDT cytotoxicity was described using two independent approaches: combination of PDT with doxorubicin (Dox) and selective preprotection of normal haematopoietic cells with the stem cell inhibitory peptide N-AcSDKP. The findings from the above experiments will help to advance our understanding of the apparent selective cytotoxicity of BPD has on leukaemic cells. In addition, factors which could influence *in vitro* PDT cytotoxicity will be discussed.

PDT COMBINATION THERAPY

PDT combination therapy attempts to optimise photocytotoxicity through the interaction of the light- activated photosensitiser with another drug or treatment modality such as hyperthermia or ionising radiation. However, one cannot discount the possibility that PDT also can endow the other partner of the regimen with enhanced cytotoxic activity. Clearly, combination therapy requires mutual cooperativity between the different participants within the regimen and the effectiveness of a particular regimen is predicated on multiple factors. Chief among them are the type of photosensitiser or drug and the form of

treatment modality selected, the temporal order of drug administration i.e. simultaneous or sequenced, and the type of model system and tumour (*in vitro* vs *in vivo*, murine vs human) used in the experiment. Adequate delivery of the chemotherapeutic agent or photosensitiser to the desired target organ remains one of the chief obstacle in *in vivo* drug therapy. Parenteral and nonparenteral administration present two different routes of drug delivery; in addition, binding to various serum protein components and hepatic metabolism further complicate the delivery of drug in an organism. As mentioned in a previous chapter, bone marrow purging, being an *ex vivo (*or *in vitro*) procedure, bypasses many of the constraints encountered *in vivo*. In addition, *ex vivo* PDT eliminates the problems of ensuring adequate light delivery to the appropriate organ.

In studies performed for this thesis, the combination involving Dox treatment prior to BPD- mediated PDT (Dox-> PDT) effected significant cytotoxicity on L1210 cells whereas the reverse sequence PDT-> Dox and simultaneous Dox/PDT treatment were not as efficacious as the first regimen (chapter 3). The enhancement in cytotoxicity was dependent on the concentration of BPD; specifically, enhanced killing of L1210 cells was achieved with 5.0 but not 2.5 ng/ml BPD. Additionally, even though Dox-> PDT also effected heightened cytotoxicity against DBA/2 haematopoietic progenitors, L1210 cells were found to be much more susceptible to the Dox-> PDT sequence than normal haematopoietic cells. Subsequent investigations revealed that the photophysical properties of Dox and BPD are unique such that Dox did not interfere with the photoactivation of BPD at λ_{ex} above 600 nm (chapter 4). The presence of Dox appeared to reduce the uptake of BPD whereas preincubation of L1210 cells with Dox did not affect the subsequent uptake of BPD (Dox/PDT). In addition, preincubation with 2.5 μM or 5 μM Dox did not significantly reduce cellular glutathione (GSH). The factors behind the superiority of the Dox-> PDT regimen are still not clear and multiple mechanisms are responsible.

N-ACSDKP- MEDIATED PHOTOPROTECTION FROM BPD

Haematopoietic stem cell protection attempts to selectively protect normal blood cell progenitors and precursors from treatment- related cytotoxicity. Many of mediators of

protection are the negative regulators of haematopoiesis which effect negative inhibition on the growth of haematopoietic cells.[210, 368] For some of the negative mediators, specifically the peptides N-AcSDKP and pEEDCK, the protective mechanism appeared to rely on the selective inhibition of cell cycling in normal haematopoietic cells which therefore results in the sparing of above population from cycle- specific toxicity of treatment.[367] Coutton and colleagues later demonstrated that N-AcSDKP also effected selective protection of normal human CFU- GM progenitors from Photofrin® - mediated PDT.[209]

In chapter 4, preincubation of DBA/2 bone marrow cells with 100 nM of the tetrapeptide N-AcSDKP resulted in statistically significant photoprotection from BPD-mediated PDT. The control peptides N-AcSDKE and SDKP, as well as tissue culture medium control, did not alter sensitivity to PDT. The protective effect, however, was restricted to the late committed progenitor population which was measured by the CFU-GM assay. Cells of earlier lineages, as measured by the LTBMC assay, were not protected by N-AcSDKP. The L1210 murine leukaemic cell line was not responsive to N-AcSDKP as well as the two control peptides and therefore was not protected from PDT. Findings from the murine experiments were extended into human cells. Specifically, normal human late haematopoietic progenitors were photoprotected by preincubation with N-AcSDKP whereas the leukaemic cell line K562 and primary leukaemic cells from CML patients were not protected by N-AcSDKP preincubation. To further examine the mechanisms responsible, I measured BPD uptake (or association) in a subpopulation of DBA/2 bone marrow cells responsible for short- term as well as long- term haematopoiesis. Interestingly, BPD uptake was not statistically different between CD34- expressing cells from the N-AcSDKP preincubated and the control sample. In addition, no significant difference in cellular GSH between peptide- treated and control cells was noted. Using the traditional ara- C suicide assay, I showed that preincubation of DBA/2 cells with 100 nM N-AcSDKP for 1.5 h did prevent the progression of cell cycle into S- phase. Therefore, the observed photoprotective effect can be attributed to cell cycle inhibition. I was able to prove this by depleting the harvested DBA/2 bone marrow sample of S- phase cells with 50 μM ara- C followed by PDT. S- phase depletion resulted in cells with higher survival to PDT which showed that susceptibility to PDT was related to cell cycle and N-AcSDKP effected its photoprotective effect via the inhibition of cell cycling. However, the exact downstream

mechanisms are still not clear still we were not able to show significant difference in the uptake of BPD and in the amount of the cellular antioxidant GSH.

FACTORS AFFECTING PDT CYTOTOXICITY

Thiols, α- tocopherol, and haeme oxygenase

Cellular GSH is correlated with susceptibility to a variety of chemotherapeutic agents, possibly via detoxification of reactive oxygen species (ROIs) and lipid peroxides.[245, 412, 413] *In vitro* and *in vivo* PDT cytotoxicity also appeared to be modulated by the amount of GSH and the degree of its metabolism.[334, 351] Another important cellular antioxidant is α- tocopherol, a form of vitamin E. Wells and colleagues have demonstrated a correlation between α- tocopherol level and resistance to Dox.[414] Gomer and colleagues initially described the induction of haeme oxygenase 1 (HO1) transcription after Photofrin® - mediated PDT.[415] Lin *et al* recently described the induction of HO1 expression and PDT resistance (MC540) after prolonged incubation of L1210 cells with haemin (ferriprotoporphyrin IX); the role of HO1 in the induction of PDT resistance is not clear, however.[416]

NF- κB and its role in PDT cytotoxicity

A variety of stimuli, including cycloheximide, double- stranded RNA, calcium ionophore, TNF- α, active phorbol ester, interleukin- 1, lipopolysaccharide and lectin, can mediate the induction of the transcriptional factor NF- kappa B (NF- κβ).[417] In addition, Boland *et al.* showed the anthracycline daunorubicin also activates NF- κβ in the HL- 60

promyelocytic and Jurkat T lymphoma cell lines.[418] Activation of NF- κβ involves separation from its cytoplasmic binding partner, Iκβ, followed by nuclear translocation the induction of κβ- dependent gene expression.[419] Ryter and Gomer initially reported that Photofrin- mediated PDT causes NF- κβ activation in the L1210 murine leukaemic cell line.[264]. Their observation was not surprising in light of findings that reactive oxygen species (ROIs) are essential as signalling intermediates in the activation of NF- κβ by a variety of stimuli.[420-422] Schreck and colleagues also demonstrated that the radical scavengers N-Acetyl- cysteine (NAC) and the pyrrolidone derivative of dithiocarbamate (PDTC) effectively inhibited NF- κβ activation upon cellular stimulation. ROIs are also important intermediates mediating the activation of NF- κβ in response to stimulation of the T cell surface receptor CD28.[423] Through their studies on human B cell lines, Schieven *et al.* have implicated tyrosine kinase in the ROI- mediated activation of NF- κβ.[424]

Recently, several reports have demonstrated that NF- κβ plays an important role in determining cellular response to a variety of cytotoxic agents. Significantly, Wang and colleagues found that activation of the transcription factor was paramount in the cytoprotection of the human sarcoma cell line HT1080 from TNF- α, ionising radiation, and the daunorubicin.[327] Paradoxically, several papers have reported conflicting roles of NF- κβ in *in vitro* excitotoxic neurodestruction.[328] With respect to the role of NF- κβ in determining cellular response to PDT, Anderson *et al.* initially reported that the presence of 0.1- 10 mM salicyclic acid (SA) during MC540- mediated PDT significantly enhanced cytotoxicity to L1210 and K562 cell lines.[425] In addition, SA and sodium salicyclate (ASA or aspirin) are known inhibitors of NF- κβ activation.[426, 427] Therefore, one can assume that the modulation of NF- κβ activation can also alter cellular response to PDT. Unfortunately, Traul *et al.* later showed that SA induced nonselective enhancement of PDT killing of L1210 cells and normal haematopoietic stem cells but more importantly, they found that SA displaces MC540 from serum albumin which resulted in the increase of free photosensitiser available for binding.[428] However, the question of the role that NF- κβ plays in PDT remain opened. We used the proteasome inhibitor MG132 (carbobenzoxyl- leucinyl- leucinyl- leucinal), which inhibits the proteasome degradation of Iκβ and hence nuclear translocation of NF- κβ.[327] Preliminary results showed that 1 h preincubation of Jurkat cells with 40 μM MG132 resulted in moderate yet consistent enhancement of BPD-mediated PDT cytotoxicity (data not shown). I found that the same experimental protocol

also potentiated doxorubicin killing in Jurkat cells. Interestingly, MG132 preincubation protected the human CML cell line K562 from BPD- mediated PDT. I am in the process of examining the amount of Iκβ in cell lysates from the above two cell lines and will continue to investigate the role of NF- κβ in PDT- mediated cell killing.

I also tried to introduce MG132 into the PDT combination experiments described in chapter 3. Since Wang *et al.* reported that MG132 protected the cell line HT1080 from daunorubicin cytotoxicity, I was interested in whether the peptide aldehyde could affect the killing of L1210 cells in the Dox-> PDT combination.[327] Obliquely, I was curious of the role of NF- κβ activation during the Dox pretreatment phase of Dox-> PDT, which resulted in significant enhancement of cytotoxicity of the regimen. Unfortunately, the L1210 cell line was found to be extremely sensitive to MG132 and prevented the incorporation of the inhibitor into the existing PDT experiments in L1210.

Summary

As long as there is a need for the therapeutic transfusions of autologous haematopoietic stem cells in the oncologic setting, there is a need for purging. The overall efficiency of the different established purging modalities is similar to each other. Therefore, enhancements to a proven modality, such as BPD- mediated PDT purging, should increase its attractiveness to the clinician. On the other hand, new and exciting technologies are emerging and may offer a quantum leap in purging performance.[213, 429]

The underlying theme of this project is the improvement of PDT cytotoxicity via two independent approaches. Dox treatment prior to PDT (Dox-> PDT) resulted in a significant improvement in the killing of the leukaemic cell line L1210 but not of DBA/2 haematopoietic progenitor cells. The reverse sequence (PDT-> Dox) and simultaneous treatment (Dox/PDT) only effected moderate improvement in killing. Therefore, one must be cautious in combining PDT with other drugs or treatment modalities in the clinic. Specifically, the sequence of the combination could significantly affect the outcome of the treatment. The second approach involved the selective protection of normal haematopoietic cells with N-AcSDKP prior to PDT. Murine and human leukaemic cells did not respond to

the tetrapeptide and therefore were not afforded subsequent photoprotection. The above findings should also aid in the continuing understanding of the mechanisms of PDT cytotoxicity.

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