THE ROLE OF CERAMIDE IN GROWTH ARREST AND TERMINAL DIFFERENTIATION OF HUMAN LEUKAEMIA CELLS

by

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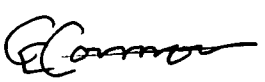
Declaration

This thesis contains no material which has been accepted for a degree or diploma in any University or other institution, and to the best of my knowledge and belief contains no material previously published or written by another person except where due acknowledgement is made in the text.



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Publication List

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Table of Contents

List of Abbreviations

ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
anti-	antibodies directed against
ATM	ataxia telangiectasia gene
bp	base pairs
BSA	bovine serum albumin
CAPP	ceramide-activated protein phosphatase
CD	cluster of differentiation antigen
CDK	cyclin dependent kinase
cDNA	complementary DNA
C_2-ceramide	N-Acetylsphingosine, D-erythro
CKI	cyclin dependent kinase inhibitor
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CO_2	carbon dioxide
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytosine 5'-triphosphate
DD	differential display
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
dsDNA	double-stranded DNA
DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	grams
GADD45	growth arrest & DNA-damage-inducible protein 45
GADD153	growth arrest & DNA-damage-inducible protein 153
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HCl	hydrochloric acid
H_2O	water

HPI	hypotonic propidium iodide solution
HRP	horseradish peroxidase
Ig	immunoglobulin
kb	kilobases
kDa	kilodaltons
L	litre
m	milli (10^{-3})
M	molar (mol/L)
mcl-1	induced myeloid leukaemia cell differentiation protein
MgCl	magnesium chloride
MIC1	Macrophage inhibitory cytokine 1
mol	moles
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	messenger RNA
n	nano (10^{-9})
NaCl	sodium chloride
NaF	sodium fluoride
NaN_3	sodium azide
NHL	non-Hodgkin's lymphoma
NP-40	nonidet P-40 (octylphenoxypolyethoxyethanol)
nt	nucleotides
p	pico (10^{-12})
PBS	phosphate buffered saline
PBS-A	PBS + 0.1% sodium azide + 1% FCS
PBST	PBS + 0.1% Tween-20
PCNA	proliferating cell/cyclic nuclear antigen
PCR	polymerase chain reaction
PE	phycoerythrin
PI	propidium iodide
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PP	protein phosphatase
PRAME	preferentially expressed antigen of melanoma
PVDF	polyvinyldifluoride
Rb	retinoblastoma protein
RHH	Royal Hobart Hospital

RNA	ribonucleic acid
rRNA	ribosomal RNA
RNase	ribonuclease
RPA	ribonuclease protection assay
RT-PCR	reverse transcriptase - polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SM	sphingomyelin
ssDNA	single-stranded deoxyribonucleic acid
ssRNA	single-stranded ribonucleic acid
TAE	tris-acetate EDTA
Taq	*Thermus aquaticus* (DNA polymerase)
TBE	tris-borate EDTA
TEMED	N,N,N',N'–tetra-methyl-ethylenediamine
TNF	tumour necrosis factor
Triton X-100	t-octylphenoxypolyethoxyethanol
TS	tumour suppressor
Tween 20	polyoxyethylene (20) sorbitan monolaurate
UTP	uracil 5'-triphosphate
UV	ultraviolet
V	volts
°C	degrees Celsius
β-ME	β-mercaptoethanol
μ	micro (10^{-6})

Abstract

The processes of proliferation, differentiation, and apoptosis are tightly regulated during haemopoiesis with aberrations resulting in the uncontrolled clonal growth of non-functional haemopoietic cells, a disease state known as leukaemia. Approaches that target cellular growth and differentiation pathways to alter the proliferative potential of leukaemia cells while limiting cytotoxicity to healthy cells have recently undergone a revival in clinical interest. Known as differentiation therapy, the immature cancer cells undergo terminal differentiation culminating in the ultimate removal of these cells by apoptosis.

The sphingomyelin signalling pathway, and its second messenger ceramide, has emerged as an important regulator of cellular growth, differentiation and apoptosis. Whilst numerous studies have addressed the apoptogenic properties of ceramide signalling, very few have investigated the mechanisms underlying ceramide mediated terminal differentiation.

The aim of this study was to investigate the potential for the ceramide signalling pathway to be utilised in differentiation therapy for the treatment of human leukaemia.

A range of haemopoietic cell lines were treated with the synthetic ceramide analogue, C_2-ceramide. Growth inhibition, cell cycle arrest, activation of the retinoblastoma (Rb) tumour suppressor protein, expression of the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ and apoptosis were shown to be common outcomes of ceramide treatment. Myeloid cells exposed to ceramide differentiated into a mature phenotype. Taken together, these results show that ceramide is inducing terminal differentiation in the treated cell lines.

Phosphatases have a well characterised function in Rb activation and their potential as an effector mechanism for ceramide-induced Rb activation and differentiation was examined. Inhibitor studies revealed that ceramide-mediated Rb activation resulted from protein phosphatase activity, and that the contribution of $p21^{Cip1/Waf1}$ was not significant, at least in the early phases of cell cycle arrest. Interestingly, inhibition of protein phosphatase activity did not impact on the ability of ceramide signalling to induce myeloid cell differentiation. This lead to the conclusion that Rb activation and ceramide-induced differentiation occur by two independent pathways.

Gene expression profiling using a cDNA filter array was performed to elucidate the involvement of genetic mechanisms involved in the ceramide response.

Genes that prevent progression through the cell cycle and genes involved in differentiation were upregulated whereas the expression of proliferation-related genes were downregulated. These findings are consistent with the hypothesis that ceramide reduces the proliferative potential of leukaemic cells by inducing terminal differentiation.

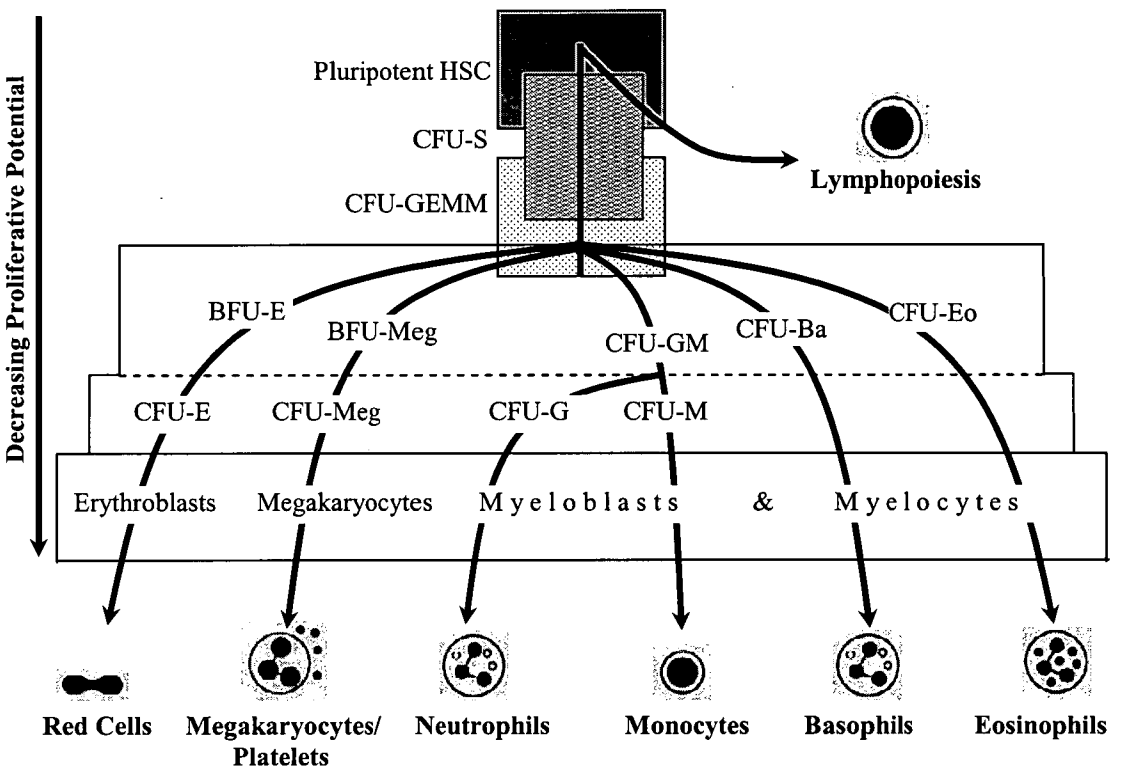
This thesis presents a comprehensive study into the biological effects of the ceramide signalling pathway, and has in part defined the molecular mechanisms responsible for ceramide-induced growth arrest and differentiation. Ceramide was shown to mediate the terminal differentiation of leukaemia cells into mature, non-dividing cells that die by apoptosis. Manipulation of the ceramide-signalling pathway may present a novel therapeutic target for the treatment of diseases that lack control of growth and differentiation processes.

Haemopoiesis

The circulatory system contains a variety of mature blood cells, each with a unique set of specialised properties and functions. However, mature blood cells are destined to live for only a short period of time, ranging from hours (granulocytes) to weeks (erythrocytes) before being destroyed (Testa & Dexter, 1999). To account for this steady-state loss of haemopoietic cells, a new cell must be produced to replace every cell that dies. This process, by which the cellular elements of the blood are produced in order to maintain a homeostatic system, is defined as haemopoiesis. Haemopoiesis not only provides new blood cells throughout adult life, but also provides a remarkable degree of cell specialisation, ultimately originating from a relatively small pool of pluripotent progenitor cells.

Within the bone marrow (the main haemopoietic organ post-birth), various types of blood cells in different stages of development can be identified. These include very primitive cells that act as the precursors for the various mature cell lineages. Each of these precursor cells are originally derived from a common cell, termed the pluripotent haemopoietic stem cell (HSC). Currently, stem cells are broadly defined as any cell capable of reproducing itself, and also possess the ability to differentiate along one or more pathways (Metcalf, 2001, Schwarzenberger *et al.*, 2002). As stem cells differentiate into more mature cells, the self-replicating abilities and differentiation options are increasingly exchanged for functional capacity (Boggs & Boggs, 1984). The exact definition of a true stem cell is still debatable. The status of current research involving haemopoietic stem cells and future implications of this work has been reviewed in detail by Chu & Gage (2001), Lazner *et al.* (2001), Lovell-Badge (2001), Metcalf (2001) and Scwarzenberger *et al.* (2002). Figure 1.1. presents a schematic model of haemopoiesis, illustrating the cell hierarchy from the most primitive precursor cells leading to the trilineage production of the formed elements of the blood (erythroid, myeloid, lymphoid). This seemingly complex system is in fact highly simplified in this diagram.

Figure 1.1.: Representation of the cell hierarchy in the process of haemopoiesis (adapted from Eaves, 1996 and Testa & Dexter, 1999). The progenitor cells [colony-forming units (CFU) and burst-forming units (BFU)] are defined by the following prefixes: E = erythroid; Meg = megakaryocyte; Eo = eosinophil; G = neutrophilic granulocyte; M = macrophage/monocyte; Ba = basophil. Cells which form colonies containing cells from three or more cell lineages are denoted CFU-GEMM (colony-forming units - granulocyte, erythroid, megakaryocyte, and macrophage). HSC = haemopoietic stem cells, CFU-S = colony-forming units - spleen.



As shown in Figure 1.1., the different levels of the haemopoietic hierarchy have been historically defined by functional growth characteristics in specific *ex vivo* culture assays, not by morphologic or cell surface marker characteristics. Furthermore, distinct subsets of haemopoietic precursor cells have been defined by their ability to form specific types of colonies (single lineage versus multilineage) and their ability to give rise to further colonies if serially replated (Pluznik & Sachs, 1965, Bradley & Metcalf, 1966, Senn *et al.*, 1967). The most mature precursor cell subset are those that exhibit lineage restricted colony growth (single lineage) and essentially have no self-renewal capacity and a very limited secondary plating efficiency (e.g. CFU-G, CFU-M, CFU-Meg, and, CFU-E). The more immature precursor cell subset includes CFU-GM, which have more capacity for differentiation (multilineage colony growth) into neutrophilic granulocytes or macrophages (Metcalf, 1977, Till, 1980). An even more primitive level of progenitor cell has the

potential to give rise to several lineages. An example of such a myeloid progenitor is the CFU-GEMM which can differentiate into colonies composed of granulocytic, erythrocytic, megakaryocytic, and macrophage lineages (Fauser & Messner, 1979). CFU-S cells form a population of even more primitive progenitor cells and were the first described candidate stem cell. They were originally shown to be able to generate visible colonies in the spleen of mice (Till & McCulloch, 1961), which can only produce one lineage of cells and do not undergo self-renewal (Magli *et al.*, 1982) as originally proposed (Siminovitch *et al.*, 1963). Pluripotent haemopoietic stem cells comprise the most primitive level of the progenitor cells and are characteristically positive for the cell surface cell marker CD34. The most mature of the blood cells are those that have entered terminal differentiation, that is, they are irreversibly blocked in their ability to proliferate, although they may perform their specialised function for a period of time prior to their eventual destruction. Inhibiting haemopoietic differentiation (i.e. preventing the passage of cells from an immature to mature state) permits an accumulation of immature cells, seen in diseases such as leukaemia and lymphoma, which leads to a dramatic disruption of many physiological processes.

A moderate alteration in the haemopoietic process can be life threatening, which demonstrates that haemopoiesis must be a tightly regulated physiological function that allows the appropriate number and types of blood cells to be produced at any one time. An understanding of the regulatory mechanisms that control the production of new blood cells has progressed dramatically within the past two decades. Diseases such as leukaemia and lymphoma are results of a dramatically disrupted haemopoietic process, leading to clonal overproduction of immature cells that are unable to carry out their normal role. Ironically, inhibition of haemopoiesis represents the most common serious toxicity associated with conventional cytotoxic cancer therapies. Clearly, a better understanding of the haemopoietic system would prove beneficial to improving the therapeutic index of cancer treatments and regaining control of abnormal blood cell populations (Demetri, 1997). By preventing the occurrence of alterations to haemopoiesis, it is possible that haematological neoplastic diseases could be effectively treated, if not prevented.

The complex and essential process of haemopoiesis has captured the interest of clinicians and scientific researchers for many years. An ability to manipulate the haemopoietic system has potential for the treatment of diseases arising from alterations that affect haemopoiesis and have already been achieved for conditions of insufficient haemopoiesis. The cytokine erythropoietin (EPO) is licensed for use as a physiological regulator of erythropoiesis for the treatment of anaemia (Kojima,

1996). Granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) are cytokines that influence the production of neutrophils while mobilising peripheral blood stem cells, and are used in the treatment of chronic neutropaenia following myeloblative chemotherapy (Jones & Millar, 1989, Steward & Scarffe, 1989, Dempke *et al.*, 2000). Consequently, a more complete understanding of the exact mechanisms involved in blood cell proliferation, differentiation, and subsequent death, will provide strategies for reversing excessive haemopoiesis, which will inevitably prove beneficial for potential cancer therapies.

1.1. Human Leukaemia

1.1.1. General Information

Leukaemia is a neoplastic proliferation of cells of haemopoietic origin which arises following somatic mutation in a single haemopoietic stem cell, the progeny of which form a clone of leukaemic cells (Bain, 1999). The term *leukaemia*, meaning *white blood*, was introduced in the 19th century by Rudolf Virchow (Virchow, 1845) because of the enormous accumulation of white blood cells found in a patient with far-advanced chronic myeloid leukaemia and originally reported by Velpeau in 1827. Similar cases were soon reported by Craigie (1845) and Barth (1856). The subsequent disordered differentiation and proliferation of these neoplastic cells leads to their accumulation, thus suppressing the differentiation and proliferation of normal haemopoietic cells. Leukaemia leads to disturbances principally in marrow, blood, and spleen (Lichtman, 1990). Accumulation in the marrow results in the diminished production of normal erythrocytes, granulocytes, and platelets, leading to manifestations of the disease including weakness, fatigue, and pallor as a result of anaemia; infection arising from granulocytopenia; and haemorrhage as a result of thrombocytopenia (Henderson, 1990). Although less common, other manifestations of the disease are produced by infiltration of leukaemic cells into sites other than the marrow, principally leading to disturbances in the lungs (Green & Nichols, 1959), heart (Roberts *et al.*, 1968), bone (Thomas *et al.*, 1961), gastrointestinal tract (Steinberg *et al.*, 1973), kidney (Uno, 1967), skin (Gunz & Baike, 1974), central nervous system (Davies-Jones *et al.*, 1980), or virtually any other organ.

1.1.2. Classification and Diagnosis of Human Leukaemia

The human leukaemias have been historically divided into two categories, determined clinically by the observed duration of patient survival; 1) acute leukaemia, which generally comprises populations of immature proliferating cells and if untreated usually causes death in weeks or months, and 2) chronic leukaemia, which consists of more mature cells and if untreated causes death in months to years (Goasguen *et al.*, 1996, Bain, 1999). They are further divided into lymphoid, myeloid and biphenotypic (both lymphoid and myeloid) leukaemias. This broad classification system has remained in use to the present day, however elaborate methods of sub-classification of the human leukaemias have been introduced, in turn improving the management of this disease.

Leukaemias are a heterogenous group of diseases that vary in aetiology, pathogenesis and prognosis. The need for identification of leukaemic sub-classes is important for three reasons: 1) some leukaemias have clinical features that influence therapeutic approaches; 2) response rates and survival differ, so that classification is necessary for estimating prognosis [for example, curative therapy is well established for acute leukaemias (Gillis & Goa, 1995, DeLoughery & Goodnight, 1996), but remains elusive for chronic leukaemias]; and 3) classification facilitates communication and cooperation throughout the world.

One system, a morphological classification first proposed by the French-American-British (FAB) Cooperative Group in 1976, has stood the test of time and remains in use throughout the world in the present day. The FAB classification system was first applied to the acute leukaemias (Bennett *et al.*, 1976), and was subsequently applied to the pre-leukaemic syndromes (Bennett *et al.*, 1982) and chronic leukaemias (Bennett *et al.*, 1989) and has been under continual revision as knowledge has developed (Nielsen *et al.*, 1984, Bennett *et al.*, 1985, Lilleyman *et al.*, 1986, van Eys *et al.*, 1986, Zafar, 1992, Laurencet *et al.*, 1994, Wong & Chan, 1995, Budde, 1996). The features that are taken into account in the FAB system include the degree of maturation of the abnormal proliferating cells and the lineage involved. The presence of membrane and cytoplasmic antigens or enzymes by cytochemical, immunologic, cytogenetic, and molecular genetic methods provide supplementary information allowing a more precise classification in this system.

Rapid advances in monoclonal antibody production, cytogenetic and molecular biology techniques soon lead to the derivation of a number of new leukaemia and lymphoma classification systems throughout the world with a great deal of controversy, including the Kiel classification (Lennert, 1981, Stansfeld *et al.*, 1988, Lennert & Feller, 1992), the Lukes-Collins classification (Lukes & Collins, 1974, 1975 & 1977), and the Working Formulation (Non-Hodgkin's Lymphoma NCI Working Formulation, 1982).

The International Lymphoma Study Group, comprising of 19 expert haemopathologists, proposed a new classification system in 1993 in Berlin, Germany (published in 1994) known as the Revised European-American Classification of Lymphoid Neoplasms (REAL), in an attempt to arrive at a consensus regarding the categories of lymphoid neoplasia that were readily recognisable at that time, based on morphological, immunological and genetic techniques, and also provides features of lymphoma diagnosis not catered for in previous classification schemes (Harris *et al.*, 1994). The REAL classification has since proven to be the desired system of

lymphoma classification and is used worldwide. Since 1995, minor modifications were made to the REAL system, thus deriving a new system using principles of the REAL system named the World Health Organisation classification (Society for Hematopathology Program, 1997, Jaffe *et al.*, 1998 & 1999, Harris *et al.*, 2000a, 2000b & 2000c).

The following section presents the various human leukaemias, and for the purpose of this thesis are grouped in accordance with the classification system proposed by the FAB Cooperative Group (Bain, 1999). As the content of this thesis deals with chronic and acute leukaemias, the myelodysplastic syndromes and lymphomas will not be discussed in detail.

A) Acute Leukaemia

Acute Myeloid Leukaemia (AML)

Having correctly diagnosed an acute disease from chronic disease and established myeloid involvement, AML can be further classified based on two features: cell attributes that identify the specific myeloid lineage, and the presence of more than 30% blast cells among all nucleated cells in peripheral blood and bone marrow differential counts (Goasguen *et al.*, 1996). These can be supplemented with cytochemical analyses and lysozyme levels. The acute myeloid leukaemias are broadly divided into 8 subtypes according to the FAB system (summarised in Table 1.1.): The first four types (M0, M1, M2, and M3 and M3var) correspond to a predominant granulocytic proliferation; one (M5) is of a monocytic lineage only; M4 represents a mixture of granulocytic and monocytic lineages; M6 and M7 corresponds to dysplastic erythroid and megakaryocytic proliferation respectively. Rare cases in which granulocytic maturation cannot be demonstrated by standard methods, and its myeloid nature is only revealed by exclusive expression of myeloid antigens are now designated AML M0, and is the most recent to be added (Bennett *et al.*, 1991). Interestingly, certain clinical, haematological, and prognostic features correlate with the AML FAB classes. For example, patients with the M0 subtype have a poorer than average response to combination remission induction therapy (Lee *et al.*, 1987, Goasguen *et al.*, 1996), and patients with M3 AML are younger than average, usually have disseminated intravascular coagulation (DIC) (Velasco *et al.*, 1992, Randolf, 2000) and often have a relatively low white cell count. The FAB category with the best prognosis is M2, followed in order by M4, M1, M3 (and M3var), M5, and M6 (Bloomfield *et al.*, 1984).

Table 1.1.: Classification of the Acute Myeloid Leukaemias according to the FAB classification system proposed by the French-American-British (FAB) Cooperative Group in 1976 (Bennett *et al.*, 1976), and still applying today (taken from Bain, 1999).

Cell Clone	Abbrv. Name	FAB Subtype	Common Terms
Myelogenous	AML	M0	Myeloblastic w/o cytologic maturation
		M1	Myeloblastic with minimal maturation
		M2	Myeloblastic with significant maturation
	APL	M3	Acute promyelocytic leukaemia (usual form)
		M3 variant	APL unusual hypogranular form
Myelogenous and monocytic	AMML	M4	Acute myelomonocytic leukaemia
		M4eo	M4 with eosinophilic maturation
		M4baso	M4 with basophilic maturation
Monocytic	AMoL	M5a	Acute monoblastic leukaemia (poorly differentiated)
		M5b	Acute monocytic leukaemia
Erythroid	AEL	M6	Acute erythroid leukaemia
Megakaryoblastic		M7	Acute megakaryoblastic leukaemia

Despite extensive morphological characterisation of these subtypes, there remains only one cytochemical marker in AML, the Auer rod, which demonstrates that a blast cell is definitely myeloid and of leukaemic origin (Auer, 1906, Jain *et al.*, 1987). Auer rods have been shown to be abnormal azurophilic crystalline-like granules that result from the concentration primary peroxidase containing lysosomal granules of myeloid precursors (Ackerman, 1950, Bainton *et al.*, 1977). These structures are demonstrable by Wright-Giemsa staining or with the use of a cytochemical detection technique using benzidine base or 3, 3'-diaminobenzidine (Goasguen *et al.*, 1996).

In addition to the distinctive clinical and morphological features of M3 and its variant (M3var), acute promyelocytic leukaemia (APL) can be characterised by a t(15;17)(q22;q12) translocation (Larson *et al.*, 1984) which fuses portions of the PML gene on chromosome 15 to the retinoic acid receptor alpha (*RARα*) gene on chromosome 17 (Borrow *et al.*, 1990, de The *et al.*, 1990, Alcalay *et al.*, 1991, de The *et al.*, 1991, Kakizuka *et al.*, 1991, Chang *et al.*, 1992a & 1992b, Goddard *et al.*, 1992, Grignani *et al.*, 1993). This specific translocation is present in nearly 100 percent of APL cases (Van Den Berghe *et al.*, 1979, Sheer *et al.*, 1985, Dong *et al.*,

1993), making it a novel target for molecular diagnosis and treatment. Although the PML-RARα comprises the most diagnostically significant translocation, other cytogenetic aberrations have also been reported in AML cases (Le Beau *et al.*, 1983, Heim *et al.*, 1987, Mitelman, 1991, Soekarman *et al.*, 1992).

Acute Lymphoblastic Leukaemia (ALL)

ALL is the most common malignancy in children (Brincker, 1982). It was one of the first malignancies to respond to chemotherapy (Farber *et al.*, 1948) and was later among the first that could be cured in a majority of cases (George *et al.*, 1979). ALL is currently curable in 60-70% of children and 20-35% of adults (Cortes & Kantarjian, 1995). Patients with ALL have lymphadenopathy in up to 80% of cases and hepatomegaly and/or splenomegaly in up to 75% of cases (Henderson, 1990). Other organs have also been shown to be involved including the kidney (Henderson, 1990), lungs (Corbaton *et al.*, 1984), heart (Mancuso *et al.*, 1985), and eyes (Ninane *et al.*, 1980). Diagnosis of ALL is made from a combination of morphological features and with the use of immunophenotyping. The cell surface marker CD10, initially known as the common ALL antigen (cALLA), was identified as one of the earliest markers expressed by leukemic cells of the lymphoblastic lineage, and remains a useful subclassification tool for B-lineage leukemias (Bene & Faure, 1997). Cases of ALL can be categorised into three FAB subgroups (summarised in Table 1.2.): Childhood type ALL (L1), adult type ALL (L2), and Burkitt-like ALL (L3). Recognition of cases of L3 is regarded as generally straightforward, however the distinction of a case of L1 or L2 is considered more difficult (Bain, 1999). Distinction between the ALL subtypes is made by morphological considerations including cell size, nucleocytoplasmic ratio, and nuclear shape. ALL of L3 subtype has a prognosis worse than that of the other ALL subtypes, although it is rapidly improving with the introduction of modern treatment protocols (Bain, 1990).

Table 1.2.: Morphological characteristics of the ALL subtypes according to the FAB classification system (adapted from Bain, 1999).

Feature	L1	L2	L3
Cell size	Small, homogenous	Large, heterogeneous	Large, homogenous
Nuclear shape	Regular	Heterogenous	Regular
Nucleolus	Not visible or small	Usually visible, large	Usually noticeable
Cytoplasm	Scanty	Variable, often abundant	Moderately abundant
Cytoplasmic basophilia	Slight to moderate	Variable	Strong
Cytoplasmic vacuoles	Variable	Variable	Often prominent

B) Chronic Leukaemia

Chronic Myeloid Leukaemia (CML)

The FAB group have not yet proposed a classification for CML. CML cells display more mitotic divisions than normal in the maturing compartment, and the life span of CML cells is longer than normal, and thus these cells have been described as having an inability to die (Goasguen *et al.*, 1991). These two factors combined, result in a characteristic malignant proliferation in the bone marrow of cells of the granulocytic and megakaryocytic series (Strife & Clarkson, 1988). The four recognised forms of CML are chronic granulocytic leukaemia (CGL), atypical CML, chronic neutrophilic leukaemia, and juvenile CML.

CML usually has a biphasic, and sometimes triphasic course. The initial phase is a benign chronic phase, in which the disease is frequently asymptomatic. Patients with symptoms usually have a gradual onset of fatigue, weight loss, increased sweating, and early satiety due to splenic enlargement (Bain, 1990, Cortes *et al.*, 1996). The disease may then progress through a transitional phase called the accelerated phase (Kantarjian *et al.*, 1988 & 1990a) in which some patients develop fever and night sweats, and progressive enlargement of the spleen. However at least 20% of patients progress directly to an acute blastic phase without evidence of an accelerated phase (Kantarjian *et al.*, 1993, Goasguen *et al.*, 1996). It is in this phase that patients are most likely to have symptoms including those described above, and generally also includes bone pains (Hughes & Goldman, 1991). Symptoms of anaemia, infectious complications, and bleeding are also commonly seen in the

blastic phase (Cortes *et al.*, 1996), and signs of CNS leukaemia can be identified (Hughes & Goldman, 1991).

The most distinguishing feature of CML is the Philadelphia (Ph) chromosome (Nowell & Hungerford, 1966, Rowley, 1973), t(9;22)(q34;q11), a shortened chromosome resulting from a chromosomal translocation consequently generating the fusion gene known as *bcr-abl*, that is, a relocation of the *abl* proto-oncogene (which codes for a tyrosine kinase domain) adjacent to the *bcr* gene (Melo, 1996). This juxtaposition greatly enhances the kinase activity of ABL (Goldman, 2000). The resultant protein BCR-ABL, denoted p210, perturbs stem cell function, resulting in the chronic phase of CML (Goldman, 1997), transforming the ABL tyrosine kinase into a constitutively expressed kinase. The Ph chromosome can be found in 95% of typical CGL cases (Bain, 1999). However in approximately 5% of CGL cases in which patients display otherwise typical clinical features of CML, the Ph chromosome is absent (Kantarjian *et al.*, 1990b). Ph-negative CGL must be distinguished from atypical CML which are also frequently Ph-negative.

Chronic neutrophilic leukaemia is a rare form of CML and is predominantly confined to the elderly. It is characterised by a considerable increase in the neutrophil count, with few granulocyte precursors in the peripheral blood, anaemia, splenomegaly and sometimes hepatomegaly (Bain, 1990). This disease is also Ph-negative, and blastic transformation is uncommon.

Juvenile CML is a disease that mainly affects children less than five years of age, and is associated with a reversion to some characteristics of foetal erythropoiesis (Castro-Malaspina *et al.*, 1984). It is generally readily distinguishable from CGL, which is very rare in children. The concentration of haemoglobin F has been proposed as a useful marker in diagnosing juvenile CML (Bain, 1999).

Chronic Leukaemias of Lymphoid Lineage

A classification system for chronic T and B lymphoid leukaemias was first proposed by the FAB group in 1989 (Bennett *et al.*, 1989). Under this proposal, chronic lymphoid leukaemia is separated into B lineage and T lineage leukaemias, and subsequent classification is based on clinical features, cell morphology, membrane markers and histology. The introduction of immunological methods and flow cytometry for detecting membrane antigens has permitted accurate distinction between true chronic lymphoid leukaemia (B-CLL: CD5+, CD20+, κ/λ+) and the appearance of abnormal circulating lymphocytes found in non-Hodgkin's lymphoma cases (CD5-, CD20+), and has enabled the distinction of the abnormal T or B-cell

subpopulations involved in the disease. In addition, cytogenetic techniques have revealed an association between some B-CLL subtypes and particular chromosome translocations, thus even further clarifying these subclassifications (Goasguen *et al.*, 1996). The translocation t(14;18), common to most follicular lymphomas which juxtaposes the IgH gene to the 3' region of bcl-2 (deregulating bcl-2), has been reported to occur in a significant fraction (approximately of 10%) of B-CLL and is proposed to function in leukemogenesis (Adachi & Tsujimoto, 1989, Adachi *et al.*, 1990).

The chronic B-cell leukaemias include B-cell chronic lymphocytic leukaemia (B-CLL), B-cell prolymphocytic leukaemia (B-PLL), hairy cell leukaemia (HCL) and plasma cell leukaemia. CLL is the most common of these and is typically a disease of the elderly, characterised by lymphadenopathy, hepatomegaly and splenomegaly, and eventual impairment of bone marrow function (Bain, 1990). A demonstration of a monoclonal population of B-cells with characteristic markers allows early diagnosis of the disease. B-CLL cells are typically small with more uniform morphology compared with normal peripheral blood lymphocytes (Bennett *et al.*, 1989). Nuclear chromatin is often highly condensed in course blocks, and the leukaemic cells are more fragile than their normal counterparts, therefore the formation of smear cells during blood film preparation can be helpful in diagnosis (Bain, 1999). Another form of CLL, CLL with mixed cell types (Bennett *et al.*, 1989), appears as typical B-CLL but with an increase in prolymphocytes (designated CLL/PL) (Enno *et al.*, 1979), or an increase in a spectrum of cells from small to large lymphocytes. PLL is diagnosed on the basis of a high white cell count, and the presence of more than 55% PLL cells (easily demonstrated due to high surface Ig density surrounding these cells) (Galton *et al.*, 1974). Patients with PLL generally have a shorter survival than that of patients with either CLL or CLL/PL (Goasguen *et al.*, 1996).

Hairy-cell leukaemia (HCL), a rare B-cell disorder affecting males 4-5 times more frequently than females (Golomb *et al.*, 1983), involves cells with distinctive morphological features. They are generally of medium size with an abundant, lightly basophilic cytoplasm of 'fluffy' appearance, and 'hairy' cytoplasmic projections extending from an irregular cytoplasmic border (Kroft *et al.*, 1995). Hairy-cell variant (HCV), also a very rare malignancy, is characterised by cells with morphological features intermediate between hairy-cells and prolymphocytes (Bennett *et al.*, 1989). In contrast to HCL, HCV patients present with a high white cell count and lack monocytopenia (characteristic of HCL).

Chronic leukaemias of the T-cell lineage are uncommon. Abnormal proliferations of mature T-cells are easily identified by immunophenotyping and specific morphology. T-cell morphology can be variable, and may include large granular lymphocytes with an abundant cytoplasm containing azurophilic granules (as in T-CLL), or typical prolymphocytes (found in T-PLL). Twenty percent of PLL cases are reported to be of the T lineage (Catovsky *et al.*, 1974). Adult T-cell leukaemia/lymphoma (ATLL) is a chronic T-cell disease, first reported in Japanese adults (Uchiyama *et al.*, 1977), and is the only human leukaemia to have been linked to infection with the human retrovirus HTLV (Blattner *et al.*, 1982, Bain, 1990).

1.1.3. Diagnosis of Human Leukaemia

Diagnosis of human leukaemia has previously been based on cell morphology and cytochemical staining to provide rapid identification of the involved lineage (Bennett *et al.*, 1976, Shibata *et al.*, 1985). These include the detection of myeloperoxidase and naphthol ASD chloroacetate esterase to demonstrate myeloid involvement and to provide prognostic evaluation significance (Bennett & Begg, 1981, Matsuo *et al.*, 1989), and the Sudan black B and Periodic Acid-Schiff reactions. However, these techniques have been superseded by immunophenotyping and molecular biology procedures.

Current immunophenotyping protocols require the preparation of a viable cell suspension by red cell lysis (AFCG, 1997) and subsequent analysis using monoclonal antibodies directed against cluster of differentiation (CD) antigens found on the cell surface, or intracellular enzymes (MPO and TdT), using flow cytometry. Major progress made in the last decade in flow cytometry and the computer technology used to acquire and process the data, has allowed for simultaneous detection of four or more fluorochromes which include FITC, PE, PerCP and PE-Cy5. Flow cytometry allows the enumeration of leukocyte subsets within the peripheral blood, lymph node and bone marrow aspirate cell populations and is now considered an essential technique in the diagnosis of leukaemias and lymphomas (Macey, 1993). Distinction of cells is based primarily on their forward and 90° light-scattering properties, and subsequently the binding (depending on the cell type) of fluorochrome-labelled monoclonal antibodies to the cell (Carter & Myer, 1994). The main application for flow cytometry in leukaemia diagnosis is in the detection of abnormal patterns of cell surface marker expression. To provide examples, Tables 1.3. & 1.4. have been included to illustrate the patterns of reactivity with antibodies commonly observed in FAB categories of AML and chronic B-cell leukaemias respectively.

Loken *et al.* (1990) established a protocol using a combination of fluorescence associated with CD45/CD14 and forward and side light scatter, to accurately distinguish lymphocytes from other leukocyte populations in peripheral blood. Having identified the cell population of interest based on immunofluorescence, a light scattering window was then drawn to include all of the lymphocytes, a process called 'gating'. The reactivity of monoclonal antibodies on monocytes and granulocytes can be accounted for once the nonlymphocytes have been identified as being within the acquisition gate (Loken *et al.*, 1990). Gating of leukocyte subpopulations is now routinely used for the diagnosis of leukaemia and lymphoma using flow cytometry.

Table 1.3.: Patterns of reactivity with antibodies observed in a selection of AML FAB subtypes (adapted from Bain, 1999).

	Markers of precursor cells			Myeloid Markers				Monocyte Markers	
	TdT	HLA-Dr	CD34	CD13	CD33	CD117	CD15	CD11b	CD14
M0	+ or -	+	+	mainly +	+ or -	often +	mainly -	mainly -	mainly -
M1	+ or -	+	mainly +	mainly +	+	often +	mainly -	+ or -	mainly -
M2	-	+	mainly -	+	+	+	+	+ or -	mainly -
M3*	-	-	-	+	+	+ or -	+ or -	mainly -	mainly -
M4	mainly -	+	+ or -	mainly +	+	+ or -	+	+	often +
M5	mainly -	+	+ or -	+ or -	+	+ or -	+	+	often +
M6**	-	+ or -	+ or -	+ or -	+ or -	+	mainly -	+ or -	mainly -
M7***	-	mainly +	mainly +	mainly -	+ or -	often +	mainly -	-	-
AML overall	10-20% positive	About 70% positive	30-40% positive	60-90% positive	70-90% positive	60-70% positive	40-70% positive	50-60% positive	15-40% positive

* Also CD9 positive
** Also CD36 and glycophorin positive
*** Also CD9, CD36, CD41, CD42a, CD42b, CD61 positive

Table 1.4.: Immunophenotype patterns commonly observed in some chronic B-cell leukaemias according to the FAB classification system (adapted from Bain, 1999).

Marker	B-CLL	B-PLL	HCL	Plasma cell leukaemia
surface membrane Ig	weak	strong	mod.-strong	negative
cytoplasmic Ig	-	-/+	-/+	++
CD5	++	-	-	-
CD19, 20, 24, 79a	++	++	++*	-
CD79b	-	++	-/+	-
CD23	++	-	-	-
FMC7, CD22	-/+	++	++	-
CD10	-	-/+	-	-/+
CD25	-	-	-	-
CD38	-	-	-/+	++
HLA-DR	++	++	++	-

* HCL cells are negative with at least some antibodies of the CD24 cluster

Many significant advances have emerged in the underlying knowledge of cellular and molecular biology of leukaemias. Traditionally, cytogenetic techniques utilising metaphase chromosomal spreads have been used to detect specific deletions, additions and translocations, a number of which are diagnostic. Metaphase spreads are however becoming replaced with fluorescent *in situ* hybridisation (FISH) which employs multicolour nucleotide probes. FISH is superior to traditional techniques in that it provides unambiguous results, in a relatively short period of time (Ekong & Wolfe, 1998, Neri *et al.*, 2000).

Although now superseded because of lengthy protocols, Southern blotting has previously been an important molecular biology technique for the detection of specific gene rearrangements. More rapid techniques however, including the polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), and real-time RT-PCR are now becoming routinely used for leukaemia diagnosis. The list of chromosome changes and gene mutations in leukaemia is now very extensive and include changes that are leukaemia subtype specific. Detection of the PML-RARα translocation which predominates in APL, the *bcr-abl* fusion gene and the Ph gene in CML, and similarly many other reported translocations are now routinely diagnosed by PCR, RT-PCR, and becoming more routinely diagnosed by real-time RT-PCR (Borrow *et al.*, 1992, Castaigne *et al.*, 1992, Hussey *et al.*, 1999, Otazu *et al.*, 1999,

Preudhomme *et al.*, 1999, Kurahashi *et al.*, 2000, Otazu *et al.*, 2000). RT-PCR and real-time RT-PCR are emerging as useful techniques because they allow rapid detection of actively expressed fusion gene transcripts. Other diagnostic alterations reported in leukaemia are more broadly based, including mutations in the *n-ras* proto-oncogene and the *p53* tumour suppressor gene. Although not routinely performed, many flow cytometers have the ability to sort which are proving to be useful in collecting malignant cells for analysis by PCR and obtaining chromosomes for FISH studies (Rasmussen *et al.*, 1998).

A fundamental feature of leukaemia is its clonal nature. Clonal polymorphism of immunoglobulin and T-cell receptor genes have provided valuable markers of clonality in lymphoid neoplasms (Baruchel *et al.*, 1995, Gong *et al.*, 1999, Moreau *et al.*, 1999, Wickham *et al.*, 2000). Other acquired markers linked to the leukaemic process (chromosomal alterations, rearrangements and mutations) have also been exploited as clonal markers. Advances in our understanding of the cellular and molecular biology of leukaemia coupled with progress in the use of stem cell biology and growth factors is likely to contribute to more successful treatment and management of leukaemic patients (Greaves, 1996).

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1.1.4. Treatment of Human Leukaemia

Arsenous oxide (Fowler's solution) was first administered by Lissauer in 1865 to treat a patient with CML, resulting in a complete remission (Lissauer, 1865). Roentgen rays, discovered in 1895, were first applied to the treatment of leukaemia in 1902 (Pusey, 1902) and quickly replaced arsenicals as the treatment of choice for leukaemias. However, both arsenicals and radiotherapy were shown to be ineffective in acute cases, and leukaemia remained incurable until the 1940's. The discovery of the haematological effects of alkylating agents, naturally occurring cytotoxic antibiotics, and the development of antimetabolite therapy has provided the basis for therapeutics used in the current day.

For a chemotherapeutic to be effective, it must have differential cytotoxic or cytostatic effects on the malignant cell. Current chemotherapeutic agents rely on differences between the sensitivity of leukaemic cells and normal cells, or specifically target the genetic apparatus and the cell division mechanism of malignant cells (Creaven *et al.*, 1996).

Conventional Therapies

Folate antagonists, including methotrexate (MTX) and aminopterin have played an important role in treatment of acute leukaemia. These agents act by interfering with biosynthetic pathways involving the folate coenzyme dihydrofolate reductase (Osborne *et al.*, 1958), and specifically target neoplastic cells due to their increased metabolic rate. Dihydrofolate reductase inhibition in turn prevents the synthesis of thymidylate, an essential factor in the DNA replication process (Fridland, 1974, Tattersall *et al.*, 1974). Aminopterin was initially used as a single agent to induce remission in children with acute leukaemia (Farber *et al.*, 1948) and subsequently is now used in combination with MTX to treat this disease (Bertino *et al.*, 1997). In addition to treatment of ALL, MTX used in combination with other chemotherapeutics has been shown to be of value as part of the treatment of AML (Hudson *et al.*, 1990, Rots, 2001).

Having established that nucleic acid synthesis was an essential factor in cell growth control, purine and pyrimidine analogues were developed, which function to inhibit DNA and RNA polymerase enzymes (Pizzorno *et al.*, 1997). These include fluorouracil, cytosine arabinoside, 5-azacytidine (pyrimidine analogues), 6-mercaptopurine, deoxycoformycin, 2-chlorodeoxyadenosine, and 6-thioguanine (purine analogues). Cytosine arabinoside (araC) and 5-azacytidine (5-AC), both cytidine analogues, are the most commonly used of these in human leukaemia treatment. Since its introduction in 1963 (Talley & Vaitkevicius, 1963) araC has remained as one of the most important drugs in the treatment of AML (Bishop, 1997), and is also effective against ALL and, to a lesser extent, CML and non-Hodgkin's lymphoma (NHL) (Chabner, 1990). 5-AC is primarily used in the treatment of AML and myelodysplastic syndrome (MDS) (Saiki, 1978, Pizzorno *et al.*, 1997). The purine analogue deoxycoformycin has been useful in the treatment of CLL, prolymphocytic leukaemia, and ATLL (Dearden *et al.*, 1987), although demonstrating high toxicity in some patients (Pizzorno *et al.*, 1997). Similarly, 2-chlorodeoxyadenosine is beneficial in CLL, NHL, and in children with AML (Piro *et al.*, 1988, Santana *et al.*, 1994). The most successful use of these two purine analogues however has been in the treatment of HCL, where complete remissions of the disease are now available, which raises the issue of detection and significance of minimal residual disease (Piro *et al.*, 1988, Kroft *et al.*, 1995).

In addition to nucleotide analogues, other agents that inhibit biosynthetic reactions leading to nucleic acid precursors have been developed, including acivicin and hydroxyurea (HU). The oral chemotherapeutic HU, which functions to inhibit

DNA synthesis by inhibition of the enzyme ribonucleotide reductase, has been an initial therapy of choice for the treatment of CML to relieve symptoms or retard their progression (in combination with alkylating agents and interferon-α) (Hehlmann *et al.*, 1994). Although HU does not markedly prolong survival (Mughal & Goldman, 1995) it remains a popular choice for CML treatment because of its rapid action, low level of adverse effects (Kennedy, 1972, Simonsson, 1998), and specificity for myeloid cells (Goldman, 1997).

The alkylating agents comprise a large therapeutic component of human leukaemia treatment. These agents form covalent chemical bonds with nucleophiles such as sulphur in proteins and nitrogen in DNA, and appear to employ cytotoxicity primarily by inhibition of DNA replication and cellular division (Colvin, 1997). Sulphur mustard was first recognised as an antitumour agent following its use in weapons in World War II. The closely related, but less toxic, nitrogen mustard was chosen for further study. Five remain in common use in cancer therapies today, and include mechlorethamine, cyclophosphamide, ifosfamide, melphalan, and chlorambucil. Chlorambucil has been used extensively in the treatment of CLL and is well tolerated by patients compared with cyclophosphamide or melphalan, and results in a remission rate higher than that achieved with CHOP (combined therapy which incorporates cyclophosphamide and other chemotherapeutics) (Han & Rai, 1990, Catovsky & Murphy, 1995). The alkyl sulphonate class of drugs include one of the earliest alkylating agents, busulphan, is relatively inexpensive and has selective toxicity for early myeloid precursors (Frei III & Antman, 1997), making it a useful drug in CML therapy. Busulphan however has a higher toxicity profile than HU (Weatherall *et al.*, 1969, Vicariot *et al.*, 1979, Bolin *et al.*, 1982), and therefore is now reserved for CML patients whose disease is resistant to HU (Goldman, 1997). It is now commonly used as a component of bone marrow ablative therapy for bone marrow transplantation (BMT) of AML (Zander *et al.*, 1997).

Anthracyclines are included in a class of drugs which become inserted between successive DNA base pairs (DNA intercalators), and hence alter the function of polymerase enzymes. Actinomycin D was the first anthracycline to be used in cancer treatment, followed by doxorubicin (Adriamycin) and others, most commonly daunorubicin, and idarubucin. Doxorubicin was found to possess very broad antitumour properties, and subsequently has been used in combination with araC in the treatment of AML (Myers, 1997, Bishop, 1999), and in combination with vincristine (a *Vinca* alkaloid) and prednisone (a corticosteroid) for ALL treatment (Cortes & Kantarjian, 1995, Myers, 1997). Daunorubicin, in combination with araC,

has been a standard AML therapy for many years, but is now often replaced by idarubicin (Bishop, 1999). Similar to doxorubicin, daunorubicin and idarubicin have proved useful in the treatment of ALL when combined with vincristine and prednisone (Myers, 1997).

The epipodophyllotoxin etoposide (VP-16) is a semisynthetic derivative of podophyllotoxin, a compound found to exhibit a wide antitumour activity spectrum extracted from the *Podophyllum* plant. These agents have a unique interaction with the DNA topoisomerase II enzyme, leading to DNA damage by preventing DNA resealing (Wozniak & Ross, 1997). Etoposide is a front-line therapy for treatment of a vast array of cancers, including AML, AMML, and NHL (Issell *et al.*, 1984). Etoposide also plays a very important role in ablative protocols for both autologous and allogeneic BMT (Wozniak & Ross, 1997).

Also plant derived, the *Vinca* alkaloids represent one of the most important anticancer drug classes. The most commonly used *Vinca* alkaloids include vinblastine, vincristine, and vinorelbine (Navelbine), and as opposed to the anticancer drug classes discussed previously, do not target DNA. Their anticancer properties arise because of their ability to interact with intact microtubules (components of the cellular cytoskeleton) or microtubule subunit molecules. These agents were first discovered to be useful following studies into the hypoglycaemic properties of the periwinkle plant extract (Johnson *et al.*, 1963). Although unable to show any antidiabetic effects, researchers found that the extract prolonged the life of mice with transplantable lymphocytic leukaemia. *Vinca* alkaloids are mainly used in combination therapies due to their unique mode of action. Although the *Vinca* alkaloids play a crucial role in leukaemia treatment (in particular, VCR in the treatment of ALL and CLL) their role is somewhat limited in comparison to their uses in other forms of human cancers including Hodgkin's lymphoma and NHL (Tesch *et al.*, 1998, El Helw *et al.*, 2000, Takenaka *et al.*, 2000).

First introduced in 1985, Interferon-α (IFN-α) has become a well established first-line treatment for patients with early-stage CML. IFN-α has been reported to improve survival by a median of 20 months in chronic-phase CML patients, compared to those treated with the standard HU or busulphan therapy (Silver *et al.*, 1999). Treatment with IFN-α is demonstrated to lead to a complete, or partial, return of Ph-negative cells in a significant number patients (Giralt *et al.*, 1995). In fact, it has been reported to result in a complete haematological remission (CHR) in 50-80% of CML patients and a complete cytogenetic response (CCR) in up to 60% (Simonsson, 1998). This is of significance as an association has been found between

IFN-α treatment and a 10-year survival of 90% in patients who achieve a CCR, which compares favourably to allogeneic BMT (Kantarjian & Giles, 1999). IFN-α is highly effective in CML patients when used in combination with other agents, most commonly araC, further increasing the number of patients with both a CHR and CCR (Kantarjian *et al.*, 1998). Recently, another combination yielding promising results is the use of IFN-α and homoharringtonine (HHT), another novel plant alkaloid, with or without araC (Kantarjian *et al.*, 1998). Results from these studies suggests that HHT acts in synergy with IFN-α, and even further increases the number of patients with a CHR and remarkably accelerates the time to CCR compared with IFN-α/araC treatment (Kantarjian & Giles, 1999). Unfortunately, IFN-α is known to cause pronounced side effects including adverse neurological symptoms (Quesada *et al.*, 1986), thus limiting the duration and dosage of therapy in some patients (Kantarjian *et al.*, 1993). Another more recent approach to IFN treatment has arisen with the development of pegylated IFN-α (PEG-IFN-α) which allows more sustained serum IFN-α levels by reducing the peak serum levels, which is expected to reduce toxicities, allowing higher and more effective doses to be delivered (Kantarjian & Giles, 1999).

Combination therapies have been derived in part from studies of the development of drug resistance (Law, 1956, Schnipper, 1986). Combination regimens incorporating a mixture of two or more antineoplastic and other drugs are frequently used in patients with leukaemia; the combination of which drugs being varied according to the type of leukaemia being treated. Essentially all curative cancer chemotherapy involves a combination of agents (Frei III & Antman, 1997). The most widely used regimens include chlorambucil with prednisone in CLL treatment (Montserrat & Rozman, 1993), IFN-α and araC or HU in CML treatment (Cortes *et al.*, 1996), COP (cyclophosphamide, vincristine and prednisone) for treatment of CLL (Liepman & Votaw, 1978, Oken *et al.*, 1979, Catovsky & Murphy, 1995), CAP (cyclophosphamide, doxorubicin and prednisone), CHOP (COP + doxorubicin), POACH (COP + doxorubicin and araC), and M2 (COP + BCNU and melphalan). Combination therapy is highly effective in the treatment of cancers because the therapeutic effect of each of the drugs used is additive (Frei III & Antman, 1997). As such, combination regimens allow for achievement of a high Summation Dose Intensity (SDI) (Freireich *et al.*, 1959). Careful selection of drugs must be made, to ensure that each agent has independent non-additive, non-overlapping toxicity, while maintaining a high SDI (Frei III & Antman, 1997). Studies have been performed to investigate the possibility of applying combination

chemotherapy for inhibiting metabolic pathways by blocking sequential or concurrent points in two metabolic pathways such that the final metabolic product is ineffective (Sartorelli & Caresy, 1982), and for simultaneously synchronising cells *in vivo*, and exploiting this with a cell cycle phase-specific agent (Allegra, 1990, Ruiz-Cabello *et al.*, 1995).

Over the past fifteen years there has been increasing interest in the use of bone marrow transplantation for the treatment and potential cure of human leukaemia. BMT relies on intensive cytoreductive therapy to reduce the number of leukaemic cells, incurable by conventional-dose therapies, followed by the administration of healthy cells from the same patient (autologous) or from a matched related or unrelated donor (allogeneic), capable of producing complete trilineage haemopoietic engraftment (Thomas & Storb, 1970). Cytoreductive therapies vary depending on the leukemia subtype being treated, but include the use of cyclophosphamide and busulphan for CML (Cortes *et al.*, 1996), and etoposide combined with busulphan and cyclophosphamide for AML treatment (Zander *et al.*, 1997, Kroger *et al.*, 2000). It is now clear that BMT remains as one of the only curative leukaemia treatments. A better understanding of immunogenetics of histocompatibility and the cellular events leading to graft rejection and graft-vs.-host disease (GvHD), has led to the evolution of allogeneic BMT for leukaemia treatment and has been extensively reported to cure CML (Thomas & Clift, 1989, Litzow & Tefferi, 1994, Mughal & Goldman, 1995), with relapse rates occurring in less than 10-20% of patients (Goldman, 1997). While the chance of GvHD is increased in patients receiving an allogeneic BMT (second leading cause of death in these patients in Australia after relapse [ABMTRR, 2000]),. allogeneic transplants offer an advantage over autologous transplants in that there is resistance towards the leukaemic cells through adoptive immune cell transfer from the healthy donor, a phenomenon termed the graft-vs.-leukaemia (GvL) effect (Antin, 1993). Despite improvements in tissue typing techniques and supportive care, procedure-related mortality rates are increased in allogeneic BMT recipients, especially during the first year after transplantation. However, the long-term outcome for these patients is better than those receiving conventional chemotherapy (Simonsson, 1998). Data collected in Australia by the Australasian Bone Marrow Transplant Recipient Registry (ABMTRR, 2000) over seven years (1992-1999) reveals a survival rate of 65% in related allogeneic transplants and 52% in unrelated transplants for CML patients. The indications for autologous BMT are similar to that for allogeneic transplants in the treatment of leukaemia. Autologous transplants, however, do not possess the added

benefits of GvL effects, but do essentially eliminate the chance of early morbidity due to GvHD. ABMTRR data show a 68% survival rate after seven years in CML patients receiving an autologous transplant (ABMTRR, 2000).

Differentiation therapies which utilise agents known to induce lineage maturation in the immature leukaemia cell have been used previously, although with limited efficacy to date. These agents, which include vitamin D_3 and all-*trans* retinoic acid, will be discussed in detail in Chapter 1.4., and therefore will not be dealt with here.

New and Arising Therapies

Although many patients achieve a complete remission following conventional chemotherapy, a large proportion will eventually relapse with a disease that is resistant to a broad spectrum of chemotherapeutic agents (Nooter & Sonneveld, 1993, McKenna & Padua, 1997). A significant number of studies have been conducted into the mechanisms of multidrug resistance (MDR) in an attempt to establish their prognostic importance and to develop a means to overcome such resistance. The most extensively studied form of MDR is due to the membrane transporter protein P-glycoprotein (PgP), which is capable of actively excluding a range of anticancer drugs including anthracyclines, *Vinca* alkaloids, and epipodophyllotoxins, thus lowering their intracellular concentration to sublethal levels (McKenna & Padua, 1997). A reported 10-50% of ALL patients at diagnosis and 15-60% at relapse express the *PgP* MDR gene (List, 1993). Hence, there is an increasing need for the development of new drugs with the ability to target novel cellular mechanisms.

The cellular immune response is thought to have evolved to recognise and eliminate clones of tumour cells. T cells, NK cells, dendritic cells and activated macrophages have all been proposed as candidates for maintaining tumour surveillance (Karre *et al.*, 1986). However, many types of tumour cells lack the expression of immunogenic cell surface antigens and so are able to evade the immune response. Attention has recently been directed towards the hypothesis that differentiation of immature clones of tumour cells into a mature phenotype will lead to increased immunogenic antigen expression and their subsequent removal from the population by immune surveillance. A significant number of these studies have focussed on the CD80 antigen (the product of the *B7* gene), which is required for co-stimulation through CD28 binding and activation of T cells. Most human leukaemic cell lines, and other types of cancer cells, have been shown to express low or

undetectable levels of CD80 protein, suggesting that CD80 plays a critical role in tumour immunity (Wollenberg *et al.*, 1998, Jia *et al.*, 2002). Increased CD80 expression has been seen following treatment of *in vitro* cell populations with human IFN-α (Hakem *et al.*, 1989) and following gamma-ray irradiation (Vereecque *et al.*, 2000). Transfection of the *B7* gene into human cell lines deficient in CD80 expression increases their antigen presentation potency for activation of anti-tumour T cells and NK cells (Zhao *et al.*, 1998, McCarthy *et al.*, 2000, Jia & Ke, 2002, Ke *et al.*, 2003). Conversely, inhibition of CD80 in CD80-expressing CHO cells using anti-B7 monoclonal antibodies leads to T cell anergy (Wang *et al.*, 2002). Thus, enhanced expression of the CD80 antigen in cancer cells that lack expression of this co-stimulation factor may provide a novel immunotherapeutic approach for treating human haemopoietic malignancies.

Recently, attention has been directed towards the retinoids, in particular all-*trans* retinoic acid (ATRA) as an alternative therapy for patients with APL (Huang *et al.*, 1988, Gillis & Goa, 1995). ATRA has long been known to induce APL cell differentiation and reduce proliferative activity both *in vitro* and *in vivo* (Chen *et al.*, 1991, Fenaux & Degos, 1996, Shiohara *et al.*, 1999, Hansen *et al.*, 2000). Since its introduction into clinical use, ATRA has proved to be highly effective, inducing remission in up to 94% of patients with APL (DeLoughery & Goodnight, 1996), while resolving the coagulopathic problems that arise in conventionally-treated patients (Kawai *et al.*, 1994). Unfortunately, retinoic acid therapy has been strongly linked to an increased leukocyte count due to the release of several cytokines by the maturing blast cells in about 25% of treated APL patients (Fenaux & De Botton, 1998). This phenomenon has been termed the ATRA syndrome and is often fatal in the absence of prophylactic measures. However, with measures in place (dexamethasone and occasionally antineoplastic therapy to relieve the white cell count), the incidence of ATRA syndrome has decreased to about 15% and has reduced mortality to 1% of ATRA-treated patients (Fenaux & De Botton, 1998). ATRA has been used in combination with araC in the treatment of myelodysplastic syndrome, but results from recent retrospective studies suggest that this combination is no more effective than ATRA or araC therapy alone (Nair *et al.*, 1998, Letendre *et al.*, 1999).

Monoclonal antibody therapy has recently emerged as a promising antileukaemia treatment. With the use of Mab Thera, an anti-CD20 therapy, in common use today for the treatment of low grade NHL, new monoclonal antibodies are being developed as a target-specific therapy. Preliminary results using two anti-

CD33 (HuM195 and CMA-676) antibodies conjugated with calicheamicin (combination chemotherapy), recently developed and tested for use against AML for targeted therapy, appear very promising (Bernstein, 2000, Jurcic, 2000, Maslak & Scheinberg, 2000, Radich & Sievers, 2000, Ruffner & Matthews, 2000). HuM195 has also been tested with an IL-2 conjugate (Kossman *et al.*, 1999). While demonstrating antileukaemic activity this combination produced significant toxicity. Also being considered is the use radiolabelled HuM195 for targeted alpha and beta particle therapy which is suggested to minimise radiation exposure to normal tissues (Jurcic, 2000). Another monoclonal antibody which has recently been introduced, Campath-1H (alemtuzumab), has been used with promising results in the treatment of T- and B-CLL (Schulz *et al.*, 2000, Kennedy *et al.*, 2002) and NHL (Khorana *et al.*, 2001, Uppenkamp *et al.*, 2002). Campath-1H is directed against CD52, a cell suface antigen present on the lymphocytes of almost all patients with B and T cell lymphocytic leukaemia (Sorokin, 2001). The most significant response to Campath-1H has been in the treatment of T-PLL (Dearden *et al.*, 2001). A rationale for this has been proposed by Ginaldi *et al.* (1998), who have reported that T-PLL cells show the greatest expression of CD52. The effectiveness of Campath-1H, and the summary of results from its use in clinical trials to date, have been reviewed by Dyer (1999), Flynn & Byrd (2000), Pangalis *et al.* (2001) and Sorokin (2001).

The aberrant *bcr-abl* fusion gene found in nearly every CML cell, and its protein product, a constitutively active tyrosine kinase p210 BCR-ABL, have been successfully targeted recently. By inhibiting BCR-ABL it has been possible to reverse the leukaemic phenotype (Goldman, 1997). Labelled as the most promising agent to emerge to date by leading researchers for the treatment of CML is a specific inhibitor originally known as CGP 57-148B, and recently renamed signal transduction inhibitor 571 (STI_{571}, Gleevec/Glivec - Novartis Pharmaceuticals) (Kantarjian & Giles, 1999, Mauro & Druker, 2001) which competes with the ATP-binding site of the enzyme, preventing subsequent phosphorylation of a substrate (Goldman, 2000). Introduced into clinical trials in 1998, STI_{571} has been given to chronic-phase CML patients who had failed IFN-α therapy. CHRs have been achieved in up to 96% of patients after four weeks, and a CCR was achieved in 33% of patients within two months of treatment (Kantarjian & Giles, 1999). A recent clinical trial by Druker *et al* (2001) demonstrated minimal toxicities with STI_{571}. A long term follow-up is required for a full evaluation of the efficacy of STI_{571}. Another approach to inhibiting BCR-ABL has been with the use of antisense oligonucleotides directed against *bcr-abl* transcripts. Early studies demonstrated that these oligonucleotides could in fact

suppress colony formation of CML cells (and not normal cells) *in vitro*, however later studies have been less encouraging; the main problem being difficulties in delivering oligonucleotides into the cells (Mughal & Goldman, 1995, Kantarjian & Giles, 1999). Of the anti-sense *bcr-abl* mRNA oligonucleotides constructed, none has definite clinical efficacy (Goldman, 2000).

Due to existing problems associated with drug resistance and toxicity as a result of drug non-specificity, there continues to be a need for the development of new leukaemia therapies. Much research is currently being undertaken into novel therapies targeting cellular growth and differentiation pathways in an attempt to induce the immature leukaemia cells to mature, whilst limiting cytotoxic effects to surrounding healthy cells. Current studies into differentiation therapies appear promising, and may prove to be of great clinical significance over the next decade for the treatment of haematological disorders.

Significance of Apoptosis in Leukaemia Treatment

Unregulated apoptosis (programmed cell death) has been associated with a number of diseases. Excessive apoptosis has been linked to neurodegenerative disorders including Alzheimer's disease and Huntington's disease, which demonstrate an increase in caspase activity. Other examples of excessive apoptosis resulting in disease include insulin-dependent diabetes mellitus, Hodgkin's disease, ischaemic disease and transplant rejection (Fadeel *et al.*, 1999). A defective Fas antigen, which is normally responsible for the deletion of autoreactive T cells, has been shown in mice to exist in cells involved in a lymphoproliferative disorder and its associated autoimmune disease, systemic lupus erythematosus (SLE) (Watanabe-Fukunaga *et al.*, 1992).

Overactivation of the pro-survival protein Bcl-2 leads to a failure of cells to die. Overexpression of *Bcl-2* has been reported in B-cell precursor ALL cells, resulting in prolonged survival of leukaemic cells *in vitro* (Campana *et al.*, 1993), and has been shown in all patients with ALL by Gala *et al.* (1994), except those patients with Burkitt's phenotype. Similarly, Bcl-2 protein levels are elevated in primary CML cells (Handa *et al.*, 1997, Maguer-Satta, 1998).

Agents modulating apoptosis at the levels of initiation, mediation, and execution show potential for the treatment of diseases affected by overactive or underactive apoptosis, including leukaemia. Some current strategies in apoptosis-related drug development are discussed below with particular reference to leukaemia and cancer in general. The use of Fas-specific Abs have been shown to induce

apoptosis in cancer cells, and treatment with anti-Fas Abs has been successfully applied to mice bearing human haemopoietic tumours (Debatin *et al.*, 1993). Additionally, Rensing-Ehl and colleagues (Rensing-Ehl *et al.*, 1995) have shown elimination of the Fas expressing lymphoma YAC-1 cells in mice following treatment with a soluble Fas-L.

Treatment with the glutathione-depleting agent buthionine sulfoximine increases the level of reactive oxygen species, thus producing a decrease in mitochondrial transmembrane potential, which has been recently shown to have anti-leukaemic activity in patients (Hedley *et al.*, 1998). It is likely that inhibitors of NF-κB and AP-1 will find application for increasing the pro-apoptotic efficacy of chemotherapeutics and anti-cancer drugs (Deigner & Kinscherf, 1999). Chemotherapeutic-mediated cell death involves the activation of caspase-9 (Hakem *et al.*, 1998), while caspase-3 has been shown to be activated by a number of agents including the protein kinase inhibitor staurospine and the DNA-damaging agent etoposide (Zhivotovsky *et al.*, 1995, Weil *et al.*, 1996).

Cell lines expressing elevated levels of Bcl-2 show an increased resistance to apoptosis-inducing agents including etoposide, methotrexate and doxorubicin, and an increased resistance to γ-irradiation (Zhan *et al.*, 1993, Ascaso *et al.*, 1994, Datta *et al.*, 1995). It has therefore been postulated that agents which restore a normal level of Bcl-2 expression or reduce Bcl-2-mediated effects should enhance apoptosis and improve the anti-tumour effects of conventional chemotherapeutics (Deigner & Kinscherf, 1999). Bcl-2 is constitutively expressed in the promyelocytic leukaemia cell line NB4, and treatment of these cells with As_2O_3 produced downregulation of Bcl-2 protein resulting in cell death (Chen *et al.*, 1996). In addition, human B-cell lymphomas bearing Bcl-2 translocation can be specifically inhibited by antisense oligonucleotides (Reed *et al.*, 1990).

1.1.5. Experimental Models of Human Leukaemia

The study of human leukaemias has been aided greatly by the establishment of immortal human haemopoietic leukaemic cell lines, which are blocked at different stages of maturation. Leukaemic cell lines provide a simplified study of leukaemic cell responses following drug addition (e.g. differentiation), and allow the convenient investigation of a homogenous population of neoplastic cells (Koeffler, 1983). They provide a renewable source of material for characterisation of cell surface markers and receptors, and of mRNA for molecular analysis. Cell lines usually become independent of growth factors during adaptation to tissue culture conditions

(Markham *et al.*, 1984), in some cases due to the constitutive production of autostimulatory factors (Schrader, 1986). It is important to remember that all haemopoietic cell lines, being leukaemic in origin, are abnormal in a variety of ways. Thus responses observed in these cells (e.g. the response to addition of a given substance) may not always result in the acquisition of all the traits of a normal cell response (Lyons & Ashman, 1989). Over the last decade, a majority of the established cell lines have been comprehensively studied for the presence of particular adhesion molecules, differentiation antigens, cytochemical properties, and for growth behaviour, in an attempt to characterise each individual cell line for further research applications. Table 1.5. provides a brief account of common human haemopoietic cell lines used in leukaemia research, with emphasis on those cell lines used in this thesis. Expression profiles and differentiation capacity for the various myeloid cell lines have been extensively reviewed by Lubbert *et al.* (1991).

Leukaemic cell lines have provided the opportunity for studies to be performed into the induction of leukaemia cell differentiation. Many agents possess the ability to induce maturation of these cells from their previously blocked state. These include phorbol diesters (e.g. 12-O-tetradecanoyl-phorbol 13-acetate [TPA]) for induction of differentiation in KG-1, HL60, U-937 and THP-1 cells, teleocidins (indole alkaloids) for HL60 and KG-1 differentiation, polar-planar drugs (e.g. DMSO and acetamide) for HL60 differentiation, and some chemotherapeutic agents including actinomycin-D, methotrexate, 5-AC, bromodeoxyuridine, 6-thioguanine, araC and vincristine, for HL60 cell differentiation (Koeffler, 1983). The use of leukaemic cell lines as a model for research into potential treatments for leukaemia *in vivo* is well accepted in the scientific community, hence the use of leukaemic cell lines in this current study.

Table 1.5.: A summary of commonly used haemopoietic cell lines and accompanying characteristic features (Hb = Haemoglobin, DMSO = dimethyl sulphoxide, IL-2 = Interleukin-2, EBNA = Epstein-Barr virus nuclear antigen).

Cell Line	Diagnosis of Disease	Cell Lineage	Characteristic Features
K562	blast crisis of CML	erythromyeloid, early granulocytic precursor	- strongly glycophorin positive[1] - carry the Ph chromosome[2] - can induce to produce Hb in presence of haemin[1,2] - spontaneously differentiate into erythroid, granulocytic and monocytic precursors[3]
KG-1	AML	myeloblastic and promyeloblastic	- strongly CD34 positive[4] - spontaneously differentiate into granulocytes[3] - differentiate into macrophages in presence of phorbol esters[5] - rely on CSF in soft-gel culture[1]
KG-1a	AML	very young myeloblasts (KG-1 variant)	- strongly CD34 positive[4] - do not spontaneously differentiate[3] or respond to phorbol esters[5] - do not express DR[3] or respond to CSF[6]
HL60	PML	promyelocytic	- highly responsive to a range of differentiation agents - differentiate into granulocytes in presence of DMSO[1], and macrophages in presence of phorbol esters[7]
THP-1	AML	Monocytic	- differentiate into macrophages in presence of phorbol esters[8] - lack prominent chromosomal abnormalities[9]
U-937	Histiocytic Lymphoma	Monocytic	- differentiate into macrophages in presence of phorbol esters[10]
Jurkat	ALL	T lymphocytic	- produce large amounts of IL-2 in presence of phorbol esters[3] - CD3 positive[3]
MOLT-4	ALL	T lymphocytic	- have rosette-forming ability[11]
Raji	Burkitt's Lymphoma	B lymphocytic	- EBNA positive[3]

List of References Provided in Table 1.5.: (1) Koeffler & Golde, 1980, (2) Lozzio & Lozzio, 1975, (3) ATCC®, 2001, (4) Pasternak & Pasternak, 1994, (5) Koeffler *et al.*, 1981, (6) Koeffler & Golde, 1978, (7) Rovera *et al.*, 1979, (8) Tsuchiya *et al.*, 1980, (9) Koeffler, 1983, (10) Sundstrom & Nilsson, 1976, (11) Minowada *et al.*, 1972.

1.2. The Cell Cycle

1.2.1. Role of the Cell Cycle

The cell cycle describes the life cycle of a cell. Essentially, the role of the cell cycle is to ensure that the DNA is accurately replicated once every cycle, and that identical chromosomal copies are distributed equally to the two resulting daughter cells (Heichman & Roberts, 1994).

The cell cycle is depicted in Figure 1.2., and consists of four main phases: Gap 1 (G_1), Synthesis (S), Gap 2 (G_2), and Mitosis (M). On recognition of a growth signal, which usually originates outside the cell and sensed by receptors in the cell membrane, a cell will enter into G_1 phase of the cell cycle. In G_1 phase, RNA and protein synthesis occur but the cellular DNA content remains constant. DNA synthesis, or S phase, begins with replication of DNA and continues until the entire complement of DNA is replicated, and the DNA content has doubled. It is just prior to the transition from G_1 to S phase (known as the Restriction Point) that a number of key regulatory events takes place, and the cell must overcome checkpoints to allow continued progression through the cell cycle. A diagrammatic representation of the events that occur at the Restriction Point of the cell cycle is presented in Figure 1.3., and the key events will be discussed in detail. Having progressed through S phase, the cell then prepares for division as it enters G_2 phase. Division takes place in M phase where the cell splits into two new daughter cells each containing half the DNA content of the parental cell (Fingert *et al.*, 1997).

After completing M phase, a cell can re-enter the cycle in G_1 phase, or can exit the cell cycle and enter a resting state denoted as G_0. Cells may stay in this quiescent state for days, weeks, or years, until the cell is stimulated to divide again. The cell then becomes active, and re-enters the cell cycle in G_1 (Fingert *et al.*, 1997). Because cells in G_0 and G_1 both contain the same amount of DNA, they are indistinguishable and are often noted together as G_0/G_1 (Mangiarotti *et al.*, 1998).

DNA content distribution has long been analysed by staining with a hypotonic solution of propidium iodide, a dye which intercalates into the DNA molecules and is excited at 536nm, fluorescing at 617nm (Krishan, 1975, Nicoletti *et al.*, 1991). Using flow cytometry, cycling cells can be gated and DNA content depicted in a resulting DNA histogram (shown in Figure 1.4.). Analysis of DNA distribution allows the detection of differences in ploidy between cell populations, and detection of the relative proportions of cells in each phase of the cell cycle. This technique is invaluable in analysis of the cell cycle (Crissman & Steinkamp, 1973, Krishan, 1975,

Figure 1.2.: Diagrammatic representation of the cell cycle (illustration by George Eade) Following a proliferative signal, the cell enters the cell cycle at the Gap 1 (G_1) phase and advances through the cycle by activation of cyclin dependent kinases. The cell can progress through the restriction point (R), where key tumour suppressor gene products play a critical regulatory role, into the Synthesis (S) phase, and subsequently through the Gap 2 (G_2) and Mitosis (M) phase of the cell cycle resulting in the formation of a new daughter cell. In the presence of a further proliferative signal the cell may then re-enter the cell cycle at G_1 phase or enter quiescence (G_0 phase).

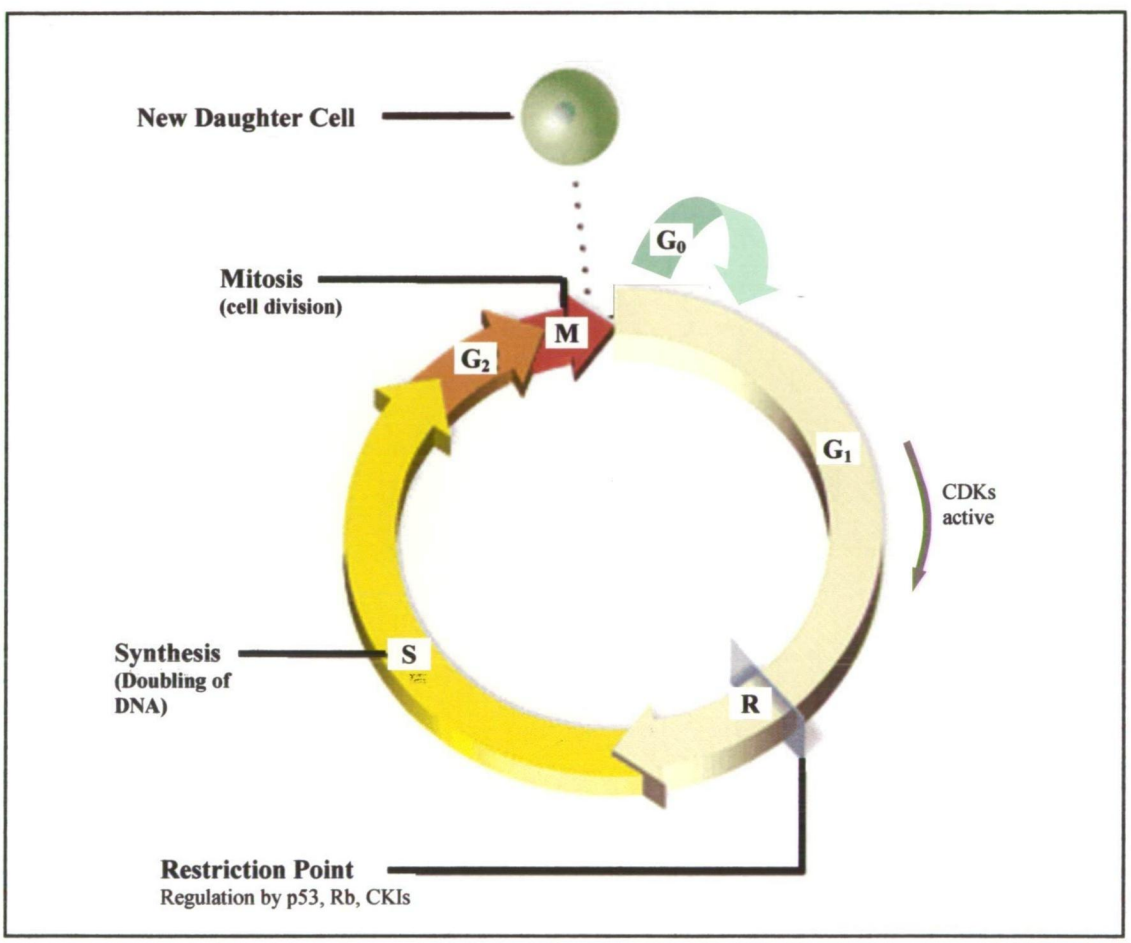


Figure 1.3.: Restriction Point control (taken from Sherr, 1996). Progression through the Restriction Point is regulated by activation of cyclin dependent kinases (CDKs) by binding with their cyclin counterparts, interactions between cyclin dependent kinase inhibitors (CKIs) and CDKs, and the activation of the tumour suppressor proteins p53 and retinoblastoma. The retinoblastoma protein (RB) must be phosphorylated by cyclin D-dependent kinases (specifically CDK4) to permit the release of the transcriptional regulator E2F, which can subsequently transactivate genes whose products are essential for S phase entry. The p53 tumour suppressor protein is crucial in activating expression of CKIs in response to DNA damage and other stress-related stimuli. Once cells progress through the Restriction Point of the cell cycle they become refractory to extracellular signals (become mitogen-independent).

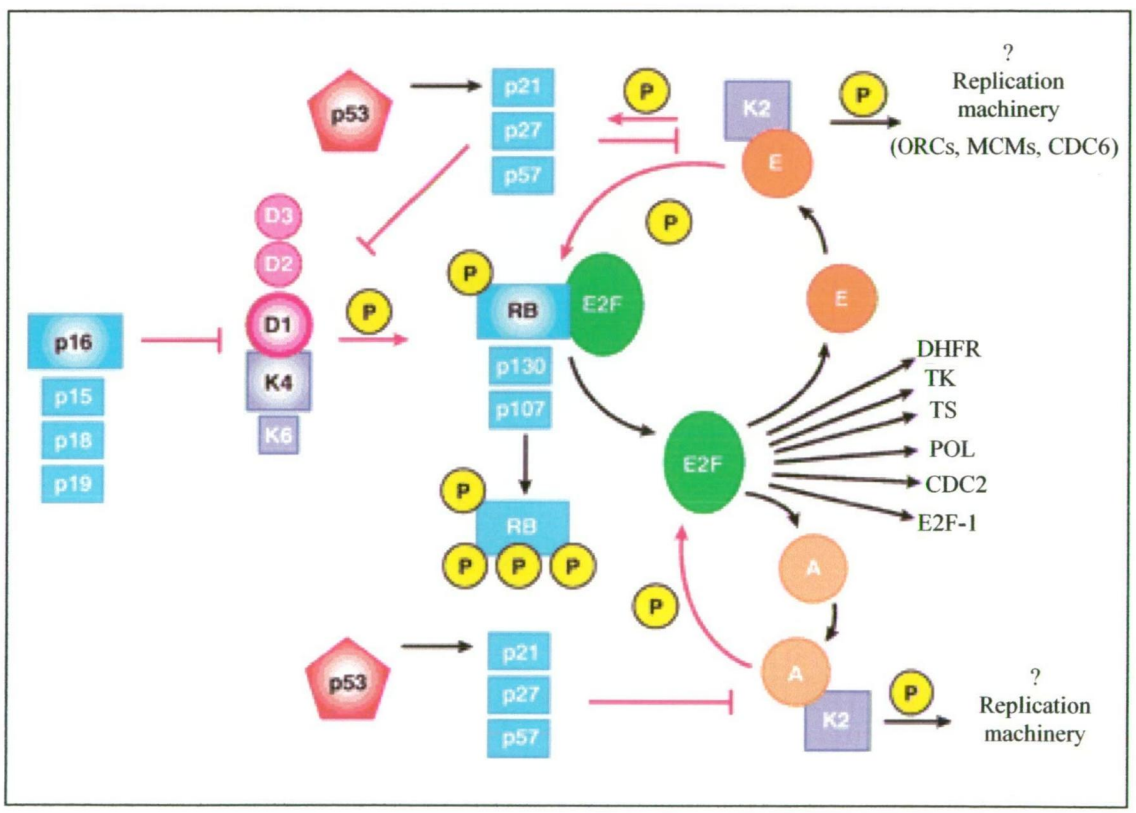
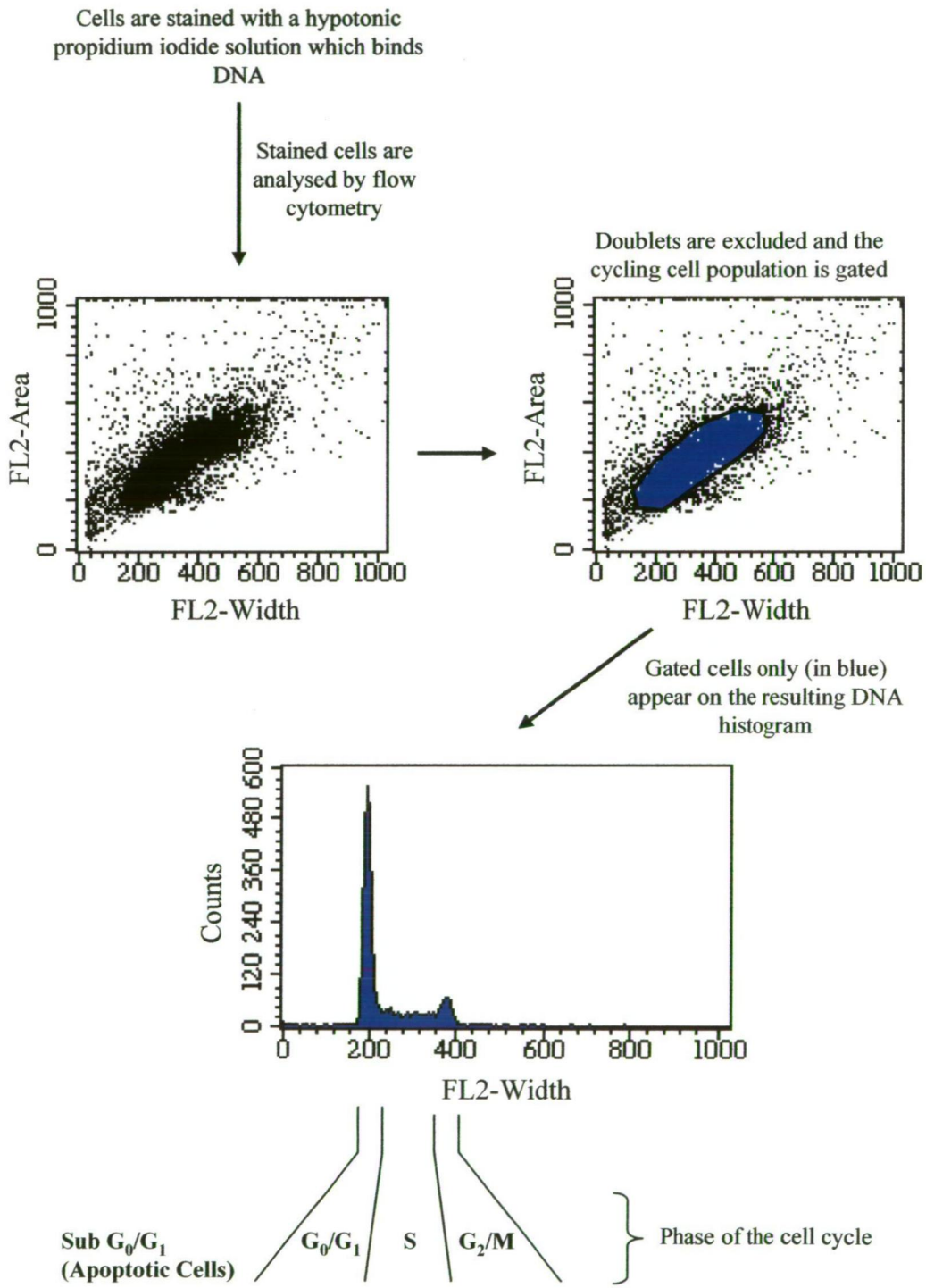


Figure 1.4.: Flow cytometry histograms demonstrating how data is obtained, following the gating of cells in the cell cycle using a dot plot (at top) and subsequent analysis using a DNA histogram (at bottom) to determine the proportion of cells in each phase of the cell cycle.



Fried *et al.*, 1976) and apoptosis (Nicoletti *et al.*, 1991, Ormerod *et al.*, 1993, Belloc *et al.*, 1994, McCloskey *et al.*, 1994) in mammalian cell populations and has proved to be useful in the clinical setting for detecting abnormal aneuploid populations (Valet *et al.*, 1984, Dressler *et al.*, 1988).

1.2.2. Key Regulators and Kinetics of the Cell Cycle

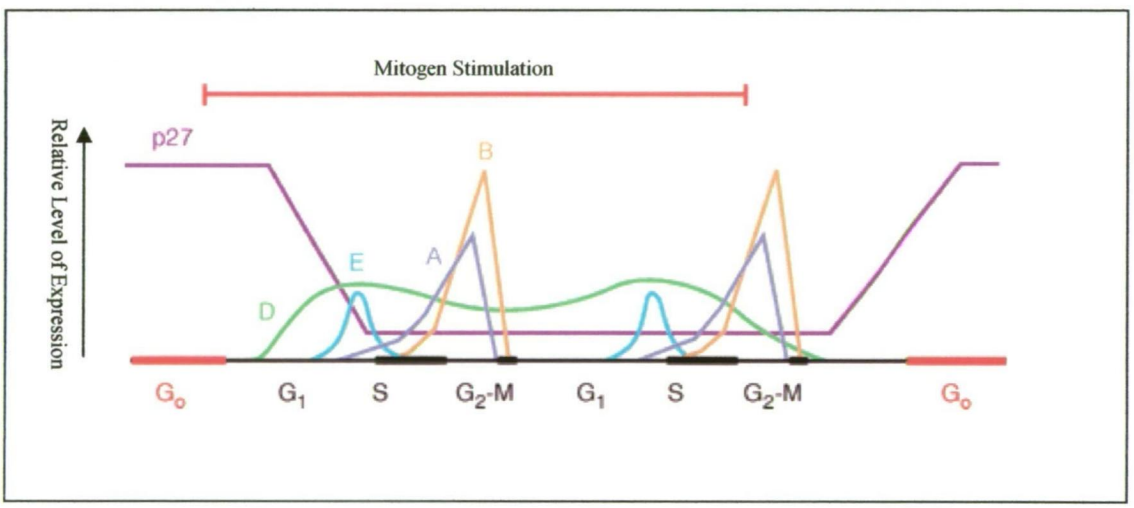
A number of regulatory proteins have been found to be involved in controlling the cell cycle. These involve both proteins that promote cell cycle progression, including cyclin-dependent kinases which are activated by their cyclin protein subunits, and proteins that negatively regulate the cell cycle which include cyclin-dependent kinase inhibitors and tumour suppressor proteins. The important cell cycle regulatory elements are discussed in detail below.

A) Cyclins and Cyclin-Dependent Kinases

Progression through the cell cycle is mediated by a phylogenetically conserved family of protein kinases known as cyclin-dependent kinases (CDKs), whose activity requires interaction with their positive regulatory subunit proteins termed cyclins (Pines, 1995). A vast number of cyclin and CDK members have been identified to play a role in mammalian cell cycles which appear to be conserved among multicellular eukaryotes, but are distinct in yeast. The A-, B-, D- and E-type cyclins have been shown to be critical in cell cycle progression, along with their CDK partners CDK1, CDK2, CDK4, and CDK6. Both are present in *Drosophila* and vertebrates (Edgar & Lehner, 1996). As positive regulation of CDK activity is mediated by the level of cyclin accumulation within the cell (Morgan, 1995, Nigg, 1995), the expression levels of the cyclins vary as the cell progresses through the phases of the cell cycle (as shown in Figure 1.5.)

Passage through the Restriction Point is dependent on levels of cyclins D, E, and A. D-type cyclins are proposed to act as growth factor sensors as their expression and assembly with their respective catalytic CDK partners (CDK4 and CDK6), have been shown to be regulated according to extracellular cues (Sherr, 1993 & 1994). Expression of the D-type cyclins (D1, D2, and D3) is increased as the cell moves through the Restriction Point (Sherr, 1994). Interestingly, a loss in cyclin D1-dependent kinase activity prior to the Restriction Point results in a cell which is unable to enter S phase, while its absence later in the cell cycle is without effect (Baldin *et al.*, 1993). This would suggest that cyclin D-dependent kinases must phosphorylate a key substrate whose modification is essential for G_1 exit.

Figure 1.5.: Diagrammatic representation showing the fluctuations in expression of cyclins, and the cyclin-dependent kinase inhibitor $p27^{Kip1}$, as the cell progresses through the cell cycle (taken from Sherr, 1996). Expression of E-, A-, and B-type cyclins is periodic, while D-type cyclins (D1, D2, and D3) are expressed throughout the cycle following initiation by mitogenic stimuli. The cyclins associate with cyclin-dependent kinase enzymes to regulate their activities (cyclin D forms complexes with CDK4 and CDK6; cyclin E with CDK2; cyclin A with CDK2 in S phase and CDK1 in late S and G_2 phases; and cyclin B with CDK1). $p27^{Kip1}$ levels are high in quiescent cells, and rapidly fall in response to mitogenic stimulation, remaining at low threshold levels in proliferating cells, and increase again when mitogenic stimulation is withdrawn. It appears to be most directly involved in Restriction Point control (G_1 to S phase transition).



Cyclin E expression is maximal at the Restriction Point and complexes with CDK2, which irreversibly commits the cell to entering S phase. The cyclin E-CDK2 complex (in addition to cyclin D-CDK4) has been shown to be essential for the transition from G_1 to S phase (Resnitzky *et al.*, 1994). Expression of cyclins A and B peak as the cell enters S phase, when cyclin E expression begins subsiding. During the early stages of cyclin A expression, cyclin E also associates with CDK2 until late S phase and in G_2 phase where it forms complexes with CDK1 (also known as Cdc2). Cyclin B only complexes with CDK1 (Sherr, 1996), a complex historically known as the maturation promoting factor (MPF). Cyclins A and B in association with CDK1 regulate entry into M phase (Sherr, 1994) where a series of proteolytic reactions are required to trigger transition from metaphase to anaphase (reviewed by Yu *et al.*, 1996). Cyclin A and B expression ceases as the cell moves towards the end of M phase. Expression levels of cyclin A mRNA have been previously proposed as a new marker of proliferation for human haematological malignancies (Paterlini *et al.*, 1993), and has been shown to be a powerful early indicator of clinical response (growth arrest) to tamoxifen therapy in breast cancer patients (Maas *et al.*, 1995).

B) Cyclin-Dependent Kinase Inhibitors

Activity of CDKs that govern cell cycle progression require strict coordination and regulation. As mentioned above, the level of cyclin accumulation within the cell generally mediates positive regulation. Negative regulation can be achieved either by phosphorylation of the catalytic subunit, or via the binding of CDK inhibitory proteins known as cyclin-dependent kinase inhibitors (CKIs) (Morgan, 1995). Increases in the levels of CKIs, which bind in place of the cyclin subunit of cyclin-CDK complexes, render these complexes inactive. Two families of CKIs have been described (Elledge & Harper, 1994, Sherr & Roberts, 1995): the INK4 (inhibitors of CDK4 and CDK6) inhibitors, and the Cip/Kip inhibitors.

INK4 proteins have been shown to directly block cyclin D-dependent kinase activity, causing G_1 cell cycle arrest (Serrano *et al.*, 1993, Kamb *et al.*, 1994, Hirai *et al.*, 1995). The four known INK4 proteins $p16^{INK4a}$ (Sherr & Roberts, 1995), $p15^{INK4b}$ (Hannon & Beach, 1994), $p18^{INK4c}$ (Guan *et al.*, 1994, Hirai *et al.*, 1995), and $p19^{INK4d}$ (Hirai *et al.*, 1995) (15-19kDa) bind and inhibit CDK4 and CDK6 only, revealing that they play a role specifically at the Restriction Point of the cell cycle.

The Cip/Kip family of inhibitors target a broader spectrum of CDKs, including CDK2, CDK4, CDK6 and possibly CDK1 (Harper *et al.*, 1995). The

family consists of three members: $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (Sherr & Roberts, 1995).

Using a subtractive hybridisation approach, el-Deiry *et al.* (1993) identified a gene in rodents which became known as WAF1 (wildtype p53-activated fragment 1), whose expression induction was found to be associated with p53 expression (a tumour suppressor – to be discussed in more detail in Chapter 1.2.2(C)) and could suppress growth of a variety of tumour cells in culture. At the same time, Harper *et al.* (1993) identified a gene in mammalian cells, named CIP1, whose expression was also induced by p53 and whose product inhibited the function of CDKs, specifically CDK2. It is now known that the sequence of WAF1 is identical to that of CIP1 (el-Deiry *et al.*, 1993, Harper *et al.*, 1993). In addition an identical gene sequence was associated with human melanoma cells by Jiang *et al.* (1995) and was named MDA6 (melanoma differentiation-associated gene 6). Being regulated by the p53 tumour suppressor, it is commonly regarded to play its major role at the Restriction Point, but has been shown to accumulate in the cell nucleus at the onset of mitosis (Dulic *et al.*, 1998). Many reports have revealed that $p21^{Cip1/Waf1}$ expression can be induced by DNA damaging agents that trigger G_1 arrest in cells with wildtype p53 (el-Deiry *et al.*, 1994, Akashi *et al.*, 1995, Russo *et al.*, 1995), as well as p53-independent mechanism involving activation by mitogens (Michieli *et al.*, 1994), immunosuppressive agents (Khanna & Hosenpud, 1999), cytokines (Datto *et al.*, 1995, Robson *et al.*, 1999), and by a variety of differentiating agents in fibroblasts, haemopoietic, and hepatoma cells (Jiang *et al.*, 1994, Steinman *et al.*, 1994, Zhang *et al.*, 1995, Liu *et al.*, 1996a, Zeng & el-Deiry, 1996, Brown *et al.*, 1997, Ragg *et al.*, 1998). Additionally, p53-independent expression of $p21^{Cip1/Waf1}$ has been shown in muscle and other terminally differentiating cells (Steinman *et al.*, 1994, Halevy *et al.*, 1995, Parker *et al.*, 1995, Flink *et al.*, 1998), thus emphasising an important link between $p21^{Cip1/Waf1}$ and a proposed role in terminal differentiation. Furthermore, Taniguchi *et al.* (1999) have shown that the expression of $p21^{Cip1/Waf1}$ is regulated in a lineage-specific manner during normal haemopoiesis, and $p21^{Cip1/Waf1}$ protein levels are particularly high in megakaryocytes, leading to the proposal that it must be involved in terminal exit from the cell cycle. These findings further imply that differentiation agents that can activate $p21^{Cip1/Waf1}$ via a p53-independent mechanism may have significant potential for use in cancer therapies (Manfredi *et al.*, 1996). Interestingly, $p21^{Cip1/Waf1}$ is not coupled to apoptosis (Steinman *et al.*, 1994, Freemerman *et al.*, 1997).

Several reports have suggested that $p21^{Cip1/Waf1}$ may also have a role in blocking the action of the proliferating cell nuclear antigen (PCNA), which functions by direct binding both in DNA replication and repair as a subunit of DNA polymerase δ (Dietrich, 1993, Flores-Rozas *et al.*, 1994, Henderson *et al.*, 1994, Li *et al.*, 1994, Waga *et al.*, 1994, Luo *et al.*, 1995). This further potentiates the ability of $p21^{Cip1/Waf1}$ to induce G_1 cell cycle arrest when overexpressed.

$p27^{Kip1}$, first described by Toyoshima & Hunter (1994), appears to be most directly involved in the Restriction Point of the cell cycle, and interacts with CDK4. The level of $p27^{Kip1}$ is high in quiescent cells (antisense inhibition of $p27^{Kip1}$ in cycling cells can prevent them from becoming quiescent [Coats *et al.*, 1996, Rivard *et al.*, 1996]) but fall once the cell enters the cell cycle (Figure 1.4.) (Kato *et al.*, 1994, Nourse *et al.*, 1994). Residual $p27^{Kip1}$ is believed to be sequestered into complexes with excess cyclin D-CDK complexes (Kato *et al.*, 1994, Nourse *et al.*, 1994, Polyak *et al.*, 1994), alleviating $p27^{Kip1}$ repression in cycling cells. $p27^{Kip1}$ is fundamental in regulating both cell number and cell size as mice nullizygous for the $p27^{Kip1}$ gene grow faster and exhibit tissues containing increased numbers of smaller cells than littermate controls (Nakayama *et al.*, 1996).

$p57^{Kip2}$, along with its counterparts $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, inhibits several cyclin-CDK complexes at the Restriction Point, as its overexpression blocks cells in G_1 phase of the cell cycle (Lee *et al.*, 1995, Matsuoka *et al.*, 1995). Like $p21^{Cip1/Waf1}$, $p57^{Kip2}$ is expressed in terminally differentiated cells suggesting an involvement of this CKI in the cell cycle exit of specific cell types (Grana & Reddy, 1995).

C) Tumour Suppressor (TS) Proteins

The existence of tumour suppressors was first suggested in 1969 by Harris and colleagues following experiments using normal cells fused with tumour cells, which resulted in suppression of the neoplastic properties of the tumour cell (Harris *et al.*, 1969). Inactivation of tumour suppressor proteins contributes significantly to the neoplastic phenotype by preventing critical cell cycle checkpoints and DNA repair mechanisms. The transcription factor p53 and the retinoblastoma TS protein are the two TS proteins most worthy of consideration, and will be detailed in this review.

p53 Tumour Suppressor

The *p53* gene, the most commonly mutated tumour suppressor gene in human cancer (Nigro *et al.*, 1989, Levine *et al.*, 1991, Hollstein *et al.*, 1991 & 1994,

Greenblatt *et al.*, 1994), was originally discovered in 1979 by Arnold Levine, David Lane, and William Old. In 1989 Bert Vogelstein, Ray White and colleagues revealed that p53 was actually a TS, and that it was altered in most colon cancers. Since then, many mutant forms of the gene have been reported in a variety of tumour types. In the wildtype form, p53 acts to stop cell division whenever DNA damage is detected in the cell, thus allowing the cell to repair the DNA prior to duplication, and subsequent transfer to daughter cells. This is emphasised by reports identifying the ability of p53 to protect against UV-B radiation-induced skin cancer (Jiang *et al.*, 1999). The p53 protein also becomes stabilised and activated in response to other stressful stimuli including hypoxia, nucleotide depletion, and oncogene activation (reviewed by el-Deiry, 1998). It is normally expressed at low levels and has a rapid turnover mediated by ubiquitination and proteolysis, and is activated by phosphorylation, dephosphorylation, and acetylation to yield a potent sequence-specific DNA-binding transcription factor (Levine, 1997, el-Deiry, 1998).

p53 acts specifically at the Restriction Point. More explicitly, p53 has the potential to induce expression of the $p21^{Cip1/Waf1}$ CKI (as discussed previously), thus resulting in G_0/G_1 cell cycle arrest. In fact, it has been identified that the promoter of the $p21^{Cip1/Waf1}$ gene possesses a binding site for p53, and as such p53 functions as a transcription factor to activate expression of $p21^{Cip1/Waf1}$ (Kim, 1997). p53 expression has also been coupled to activation of a number of other target genes that result in growth controlling endpoints including apoptosis, senescence, differentiation, and antiangiogenesis (el-Deiry, 1998). Specifically, these targets include the growth arrest and DNA-damage inducible gene 45 (GADD45) (Zhan *et al.*, 1998), 14-3-3σ (Hermeking *et al.*, 1997), Bax and Bcl-2 (Miyashita *et al.*, 1994 & 1995), and Fas (Owen-Schaub *et al.*, 1995).

An important negative regulator of p53 at the protein level is the MDM2 oncoprotein, which is itself responsive to p53 levels within the cell (Kubbutat *et al.*, 1997, Kubbutat *et al.*, 1998). MDM2 binding to p53 conceals its transactivation domain thus inhibiting p53-dependent effects (Kubbutat *et al.*, 1998). It is also believed that MDM2 can target the p53 protein for ubiquitin-mediated proteolysis (Haupt *et al.*, 1997, Kubbutat *et al.*, 1997, Kubbutat *et al.*, 1998). MDM2 binding to p53 can be alleviated by phosphorylation of serine residues 15 and 37 on p53 by the ATM and DNA-dependent protein kinases, thus stabilising p53 and reducing its degradation (Shieh *et al.*, 1997, Woo *et al.*, 1998, Banin *et al.*, 1998). This is highlighted by the fact that both ATM- and DNA-PK-deficient cells have defects in p53 activation following DNA damaging ionising radiation (el-Deiry, 1998). The

ability to enhance the function of p53 by phosphorylation modifications to specific target serine residues may have very significant implications in the future treatment of cancer.

Retinoblastoma Tumour Suppressor

Retinoblastoma is a childhood disease that results from the loss of function of the retinoblastoma (*Rb*) gene (Godbout *et al.*, 1983, Sparkes *et al.*, 1983). Given this information, it was later suspected that Rb might play a role in restraining or controlling cell growth, a hypothesis supported by the finding of *Rb* gene mutations in a variety of human cancers including osteosarcomas, and cancers of the lung, breast, bladder and prostate (reviewed by Weinberg, 1991, Budillon, 1995, and Brown, 1997). Abnormalities to the *Rb* gene have been shown to occur in 10 to 30 percent of all types of acute leukaemia, especially those with monocytic character (Ahuja *et al.*, 1991). The widespread tissue distribution of *Rb* mutations, and its involvement in such a wide array of tumours emphasises the biological importance of the *Rb* gene in normal cell proliferation (Ewen, 1994). The *Rb* gene may have an important function in the control of neoplastic growth, which is supported by evidence revealing that *Rb* associates with the transformation proteins of several viruses including E1A of adenovirus (Whyte *et al.*, 1988), large T antigen of SV40 (DeCaprio *et al.*, 1988), and the E7 protein of human papillomavirus type 16 (Dyson *et al.*, 1989). The retinoblastoma tumour suppressor protein was the first tumour suppressor system to be studied in humans (Riley *et al.*, 1994).

The retinoblastoma family of proteins include the retinoblastoma protein (Rb or p105), and other Rb-like proteins (p107, p130) (Riley *et al.*, 1994). These proteins are differentially regulated during the various stages of cell proliferation and differentiation (Garriga *et al.*, 1998) and are considered to serve as mediators for extracellular signals for growth and differentiation (Chen *et al.*, 1995a). The product of the RB1 gene, the Rb protein, is a nuclear phosphoprotein, predominantly regulated by phosphorylation on serine and threonine residues (Ludlow *et al.*, 1989, Shew *et al.*, 1989). It plays an essential role at the Restriction Point of the cell cycle, where it functions to moderate a family of heterodimeric transcription factors, collectively termed the E2Fs (Nevins, 1992, Helin *et al.*, 1993, Lees *et al.*, 1993), which can transactivate genes whose products are important for S phase entry (Neuman *et al.*, 1994, Buchmann *et al.*, 1998). In its hypophosphorylated state Rb is inactive, binding to E2F complexes and converting them to repressors that prevent expression of E2F target genes (Weintraub *et al.*, 1992). When Rb is phosphorylated

(hyperphosphorylated or active state), the bound E2Fs are released and are subsequently able to transactivate their gene targets. It is now well established that Rb is the key target for phosphorylation by cyclin D-dependent kinase complexes (Ewen *et al.*, 1993, Kato *et al.*, 1993, Sherr, 1994). The finding that cyclin D-dependent kinases are dispensable for passage through the G_1 to S phase in cells that lack functional Rb (Lukas *et al.*, 1994) further supports this notion.

The process of E2F release by cyclin D-dependent-mediated phosphorylation of Rb is further accelerated by the formation of the cyclin E-CDK2 complex (Hinds *et al.*, 1992, Mittnacht *et al.*, 1994), because the cyclin E gene is itself E2F-responsive. That is, cyclin E-CDK2 acts through positive feedback, which in turn facilitates progressive rounds of Rb phosphorylation and subsequent E2F release (Weinberg, 1995) (refer to Figure 1.3.). It is at the G_1-S boundary that the cell undergoes irreversible commitment to enter S phase, and Rb inactivation shifts from being mitogen-dependent (cyclin D-driven) to mitogen-independent (cyclin E-driven) (Sherr, 1996). Rb is then maintained in its hyperphosphorylated state by cyclin A- and cyclin B-dependent kinases, and remains dephosphorylated until the cell completes mitosis and re-enters G_0/G_1 phase.

The phosphorylation status of Rb during cell differentiation has been extensively studied in a variety of cell types. Whyte and Eisenman (1992) found that PMA-induced macrophage differentiation of HL60 cells resulted in the reduction of phosphorylated Rb, concurrent with cells undergoing G_0/G_1 cell cycle arrest. Additionally, Juan *et al.* (1998) have reported that terminal differentiation of HL60 cells involves both an increase in content of Rb and dephosphorylation of Rb already present in the cell.

Two mechanisms of Rb dephosphorylation have been noted to date in the literature: indirectly via activation of the CKIs $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ which inhibit cyclin D-dependent kinases from phosphorylating Rb (Harper *et al.*, 1993, Toyoshima & Hunter, 1994), and, by direct removal of phosphate groups by protein phosphatase (PP) activity. A number of reports were initially made by Durfee *et al.* (1993) and Ludlow *et al.* (1993), and more recently by Nelson *et al.* (1997), showing that PPs are involved in Rb dephosphorylation during mitosis and back into G_0/G_1 phase. Schonthal & Feramisco (1993) and Dou *et al.* (1995) were fundamental in advancing the role of PP-mediated Rb dephosphorylation, culminating in G_0/G_1 cell cycle arrest. Many investigators have since reported on the involvement of PPs, and all are in agreement that serine/threonine phosphatases belonging to the PP1 (Schonthal & Feramisco, 1993, Dou *et al.*, 1995, Ludlow & Nelson, 1995, Berndt *et*

al., 1997, Nelson, 1997, Rubin *et al.*, 1998, Kishikawa *et al.*, 1999, Edwards & Thomas, 2000) and PP2A (Schonthal & Feramisco, 1993, Dou *et al.*, 1995, Voorhoeve *et al.*, 1999) sub-families of protein phosphatases are involved. This was determined in each case using specific PP1 and PP2A inhibitors including okadaic acid, calyculin A, and phosphatidic acid (Ishihara *et al.*, 1989, Kishikawa *et al.*, 1999).

Rb has been implicated in apoptosis by a number of investigators who have reported findings implying that Rb is cleaved by Interleukin 1β-converting enzyme-like proteases and other caspases, thus contributing to the activation of the cell-death pathway (An & Dou, 1996, Janicke *et al.*, 1996, Chen *et al.*, 1997, Tan & Wang, 1998, Fan & Steer, 1999, Schrantz *et al.*, 1999). Further to this, Song and colleagues (Song *et al.*, 1992, Song & Lavin, 1993) have reported an involvement of PPs in apoptosis. That is, by inhibiting PP activity, γ-irradiation-induced apoptosis was prevented. More recently, Morana *et al.* (1996) and Wolf *et al.* (1997) have drawn a link between phosphatase activity, Rb dephosphorylation, and etoposide-induced apoptosis in ML-1 human haemopoietic cells, suggesting that Rb dephosphorylation is a critical regulator of apoptosis.

A thorough understanding of the p53 pathway, which controls activation of negative regulatory elements of the cell cycle, and the Rb pathway and its components, forms the basis for understanding how to regain control of the cell cycle in neoplastic cells.

1.2.3. Abnormalities in Cancer Cell Cycles

The hallmark of cancer is uncontrolled proliferation, which typically arises due to acquired damage to genes that directly regulate the cell cycle (Sherr, 1996). The result is an uncoupling of normal proliferative and differentiation events culminating in neoplastic proliferation, that is, cancer. It is now clear that the development of cancer is not the result of a single oncogenic event, but rather a combination of events proceeding in a step-wise fashion, likely to be influenced by the cellular environment (Ho & O'Neill, 1995). Consequently, transformed immature cells can give rise to a spectrum of diseases depending on the growth factors available, and indeed studies by O'Connor *et al.* (1990) and Kurtzberg *et al.* (1989) have shown that growth factors are capable of dictating the lineage commitment of immature ATLL cells.

A myriad of genetic alterations has been reported in cancer. Of the more than 100 proto-oncogenes and TS genes identified, most function to uncouple cells from their environmental controls by mimicking the effects of persistent mitogenic stimulation (Sherr, 1996). Alterations to pivotal cellular components (for example DNA replication and chromosome segregation) are highly debilitating, if not fatal, to cells. The uncontrolled proliferative nature of cancer implies that genes commanding these processes are not 'ideal' candidates for targets of mutation, deletion, or amplification in cancer. Instead, a significant majority of genes altered in cancer are those that govern control of the cell cycle, in particular genes that regulate exit from the G1 phase of the cell cycle. In fact, such alterations are so frequent in human cancers it has been proposed that inactivation of such genes (p53, Rb, CDKs and CKIs) may well be necessary for tumour development.

The *p53* gene is reported to be the most frequently mutated gene in human cancer (Nigro *et al.*, 1989, Hollstein *et al.*, 1991, Hollstein *et al.*, 1994, Greenblatt *et al.*, 1994). Loss of p53 predisposes cells to aberrant chromosomal segregation during mitosis (Fukasawa *et al.*, 1996), leading to changes in chromosome number and ploidy. *Rb* inactivation is of course a feature of retinoblastomas (Knudson, 1971), and is most often targeted in adult cancers, particularly osteosarcomas and small-cell carcinomas of the lung (Hall & Peters, 1996). As it is a significant target protein for p53, loss of Rb function can bypass p53-mediated G_1 arrest. Overexpression of *E2F* genes has not yet been reported in human cancers.

Mutations affecting the cyclins, CDKs and CKIs have been extensively reported in human cancers. Overexpression of cyclin D1 has been noted in a variety of different human cancers including squamous cell carcinomas, oesophageal carcinomas, bladder cancer, breast carcinoma, small-cell lung tumours, hepatocellular carcinomas, colorectal tumours, melanomas, and B-cell leukaemias (Motokura *et al.*, 1991, Hall & Peters, 1996). Alterations in the cyclin E and A genes appear to be rare (Hall & Peters, 1996). In fact, there is only one instance in which the cyclin A gene was found to be altered in a hepatoma (Wang *et al.*, 1990). A mutation in CDK4 preventing its interaction with p16 has been found in melanoma (Wolfel *et al.*, 1995). While $p16^{INK4a}$ mutations have been associated with familial melanomas and occur in high frequency in billiary and oesophageal carcinomas (Hall & Peters, 1996, Elledge & Harper, 1994, Sherr & Roberts, 1995), mutations or deletions of $p15^{INK4b}$, $p18^{INK4c}$, or $p19^{INK4d}$ have not been reported in tumours. Interestingly, inactivation of $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ genes have not been reported either, however a reduction in their levels has been noted (Sherr, 1996). This may

suggest that p53 probably activates additional, critical tumour suppressor pathways (Shiohara *et al.*, 1997).

1.3. The Sphingomyelin Pathway

1.3.1. Introduction

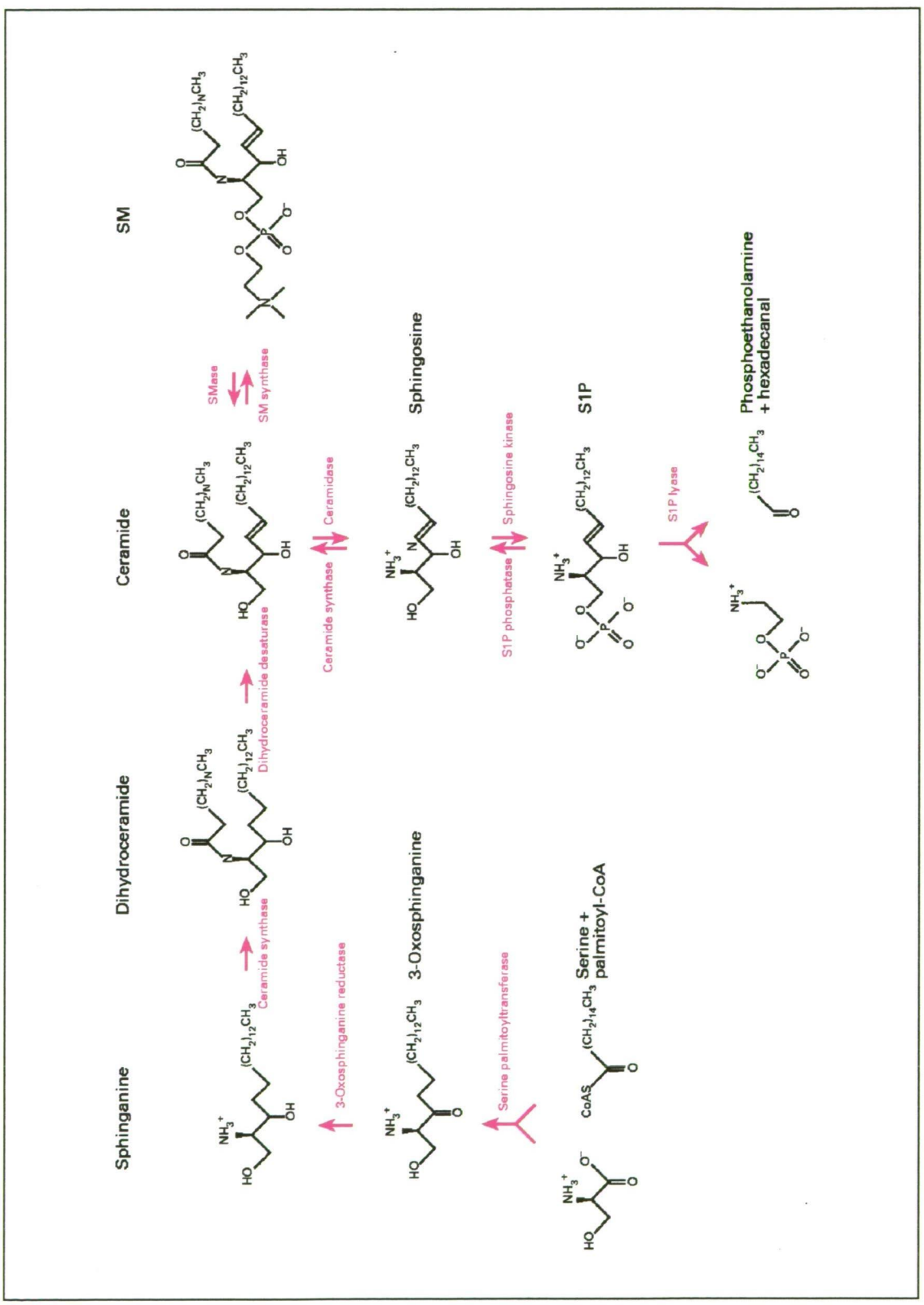
The sphingomyelin (SM) pathway is a ubiquitous, cellular signalling system that is highly conserved from yeast to humans (Hannun, 1996, Spiegel *et al.*, 1996, Mathias *et al.*, 1998) and is responsible for conveying signals throughout the cell in response to cellular stress, cytokines and ligation of a number of receptor types (Hannun & Luberto, 2000). The SM pathway involves a number of different sphingolipids which result from the hydrolysis of the sphingophospholipid, sphingomyelin, which is confined to both the inner and outer leaflets of cell membranes, although there is still uncertainty as to which of these pools is important in signalling (Perry & Hannun, 1998). SM consists of a sphingosine backbone base linked to a fatty acid by an amide bond, and has a hydrophilic headgroup that extends into the aqueous environment (Saba *et al.*, 1996). Figure 1.6. details the key components of the SM pathway. SM and its associated sphingolipid hydrolysis products were long regarded as metabolically inactive, and stable structural components of the membrane, and are considered only minor components of cell membranes (Ghosh *et al.*, 1997) compared with the more familiar glycerolipids which were known to produce active second messengers such as diaglycerol and IP_3 (Igarashi, 1997). The notion that sphingolipids were biologically inactive was first challenged by Okazaki and colleagues (1989), who reported Vitamin D_3-induced turnover of SM in HL60 cells. Sphingolipids are a diverse class of molecules, a number of which have been shown to play an important role as second messengers within the cell (Kolesnick *et al.*, 1994), producing a diverse range of stress-related cellular responses including cell cycle arrest, apoptosis, and cell senescence (Kolesnick and Golde, 1994, Hannun, 1996).

Sphingolipid signal transduction has become a dominant theme in biology today. As more research is conducted into elucidation of the molecular events that occur to elicit an intracellular response, the more it is recognised that sphingolipid signalling is crucial for normal cell behaviour. Furthermore, aberrant sphingolipid signalling consistently results in a diverse range of pathological consequences (Ghosh *et al.*, 1997).

1.3.2. Ceramide Synthesis in the Sphingomyelin Pathway

Ceramide is the central molecule in the SM pathway. Two mechanisms have been shown to generate ceramide: hydrolysis of SM by enzymes termed

Figure 1.6.: Diagram showing the chemical structures of the sphingomyelin signalling pathway members, detailing the various pathways to yield the second messenger ceramide (taken from Mathias *et al.*, 1998)



sphingomyelinases (SMases), and, *de novo* synthesis by the ceramide synthase enzyme (Kolesnick, 1991, Spiegel *et al.*, 1996, Spiegel & Merrill, 1996, Merrill *et al.*, 1997). Both synthesis pathways are presented in Figure 1.6. and are described in some detail below. Interestingly, enzymes involved in ceramide generation are located in different subcellular compartments, and this significantly affects the final ceramide-mediated outcome (Mathias *et al.*, 1998).

The *de novo* synthesis of ceramide is initiated by the condensation of serine and palmitoyl-CoA resulting in the formation of 3-oxosphinganine, which is subsequently reduced to sphinganine. An amide linkage of fatty acyl groups to sphinganine yields dihydroceramide, and the introduction of a *trans*-4,5-double bond results in the formation of ceramide. The early stages of the *de novo* pathway occur at the endoplasmic reticulum and proceeds to the Golgi network where the newly generated ceramide can act as a precursor to other SM members (Perry & Hannun, 1998).

Ceramide generation resulting from hydrolysis of SM at the cell membrane involves the action of one of a number of SMase isoforms, with at least seven having been identified to date (Levade & Jaffrezou, 1999). SMases are classified according to their pH optima and divalent ion dependence (Levade & Jaffrezou, 1999). The most well characterised of the SMases include the following: the lysosomal acid SMase (A-SMase), the acid Zn^{2+}-dependent SMase, the neutral membrane bound Mg^{2+}-dependent SMase (N-SMase), the neutral Mg-independent SMase present in myelin sheath and the cytosol of leukaemic cells, and the alkaline SMase present in bile and the digestive tract (Fowler, 1969, Rao *et al.*, 1976, Nyberg *et al.*, 1996, Schissel *et al.*, 1996, Chakraborty *et al.*, 1997). The best characterised of these SMases is the lysosomal A-SMase, as it has been found to be deficient in patients affected with the autosomal recessive condition, Niemann-Pick disease (NPD), a lysosomal SM storage disorder (Levade *et al.*, 1986, Levade & Jaffrezou, 1999). The properties, tissue distribution, subcellular localisation, and regulation of SMase activity associated with ceramide generation are outside the scope of this thesis, but are reviewed by Liu *et al.* (1997 & 1999a) and Levade & Jaffrezou (1999).

Kinetic studies have shown that ceramide can be generated within minutes or hours, depending upon the extracellular stimuli. The most dramatic change in ceramide generation has been found to occur after deprivation of serum in leukaemia cell cultures (Hannun, 1996). The magnitude of ceramide generation, the site and source of generation, and the phase of the cell cycle and state of activation of transmodulating signals all appear to play a role in the final biological effect

mediated by ceramide (Mathias *et al.*, 1998). As well as having a role in cellular function (discussed below), ceramide serves as a precursor for all other complex sphingolipids such as galactosylceramide, glucosylceramide, acyl ceramides and ceramide phosphate through the action of a direct kinase (Kolesnick & Hemer, 1990).

1.3.3. Inducers of Ceramide Generation

A number of substances can induce hydrolysis of SM by SMases. Okazaki *et al.* (1989) found that addition of the differentiating agent 1α,25-dihydroxyvitamin D_3 to HL60 human leukaemia cells caused early and reversible hydrolysis of SM and subsequent generation of ceramide. TNF-α and γ-interferon have also been shown to induce SM hydrolysis in HL60 cells (Kim *et al.*, 1991, Mathias *et al.*, 1991, Dressler *et al.*, 1992). These studies defined the basic components of the SM pathway, and it was later shown that ceramide generation as a response to treatment with these agents involved activation of the neutral cytosolic SMase (Okazaki *et al.*, 1994). Serum withdrawal has also been shown to induce ceramide production through activation of this SMase, resulting in G_0/G_1 cell cycle arrest (Jayadev *et al.*, 1995). Interestingly, other differentiating agents including retinoic acid, dibutyl cyclic AMP, dimethyl sulfoxide and phorbol esters failed to induce SM hydrolysis (Kiss *et al.*, 1988, Kim *et al.*, 1991). Ceramide levels have been shown to be significantly elevated following retinoic acid treatment, but this has been proposed to be due to *de novo* synthesis and not SMase activation (Kalen *et al.*, 1992). Ligation of the Fas receptor has also been reported to induce SM hydrolysis to increase cellular ceramide levels (Jarvis *et al.*, 1996). Additional inducers of SM hydrolysis include IL-1β (Ballou *et al.*, 1992, Mathias *et al.*, 1993), dexamethasone (Ramachandran *et al.*, 1990), and complement components (Niculescu *et al.*, 1993).

Chan & Ochi (1995) have shown that ligation of the CD28 membrane glycoprotein of T cells with its ligand initiates SM hydrolysis and generated ceramide. Furthermore, treatment of T cells with either exogenous SMase or a ceramide analogue resulted in the proliferation of these cells and transcription of the IL-2 gene, thus mimicking the CD28 signal. They concluded that the ceramide-signalling pathway is a candidate for mediating the costimulatory signal.

Ceramide synthesis can therefore be induced during periods of cellular stress (for example, during serum withdrawal) on the one hand, and during periods of cellular proliferation or differentiation on the other hand. This suggests therefore, that ceramide is a multi-functional molecule within the cell, and its role as a second messenger varies markedly depending upon the extracellular circumstances.

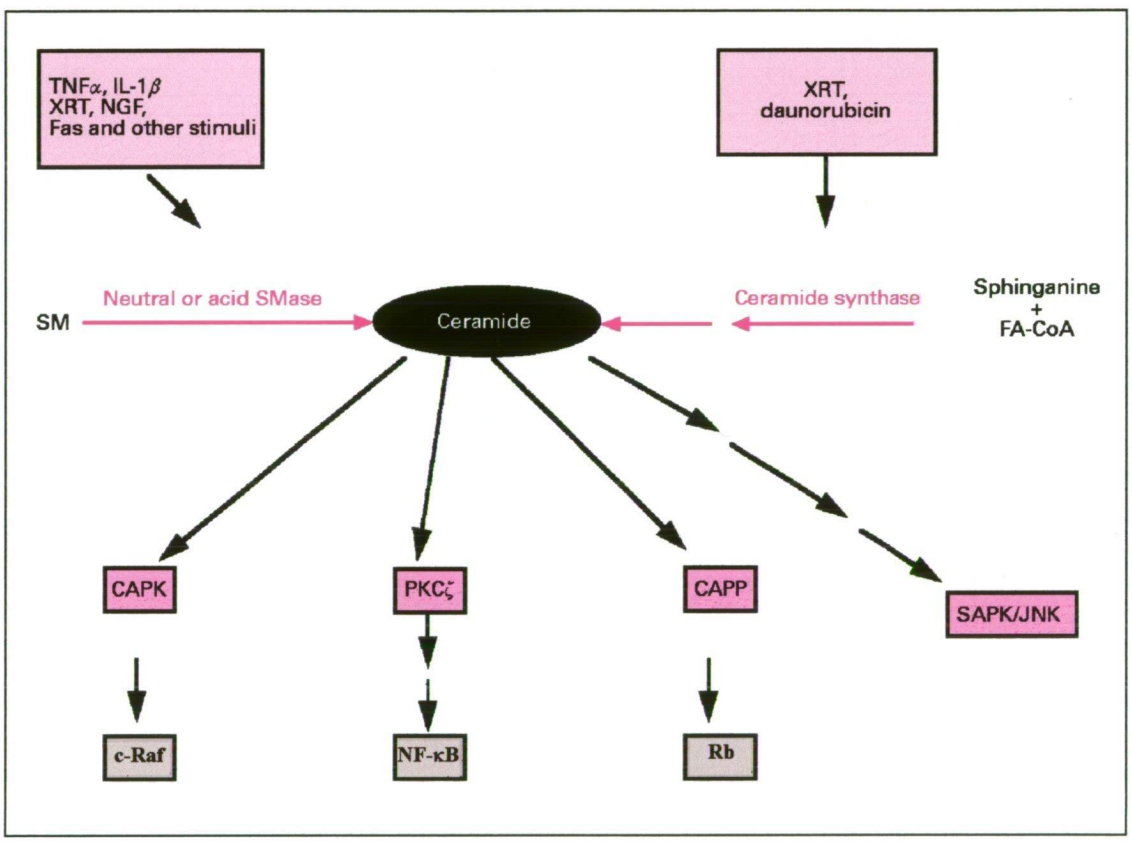
1.3.4. Cellular Targets of Ceramide

Ceramide has been shown to mediate a diverse range of cellular responses. The resulting response depends on the initial stimulus, cell type and the phase of the cell cycle when stimulated (refer to Figure 1.7.). To allow for such a vast array of cellular responses, it has become clear that ceramide has multiple molecular targets within the cell. Studies performed over the last decade to investigate the targets of ceramide signalling and the resulting cellular response have used cell-permeable, biologically active ceramide analogues including C_2-ceramide and C_6-ceramide, or ceramide produced in the plasma membrane by incubation of cells with a bacterial SMase. These models effectively mimic the action of an extracellular stimulus (Levade & Jaffrezou, 1999). Ceramide has been shown to activate a plethora of downstream targets including kinases and phosphatases, members of the stress-response kinase cascade (SAPK/JNK) and components of the mitogen-activated protein kinase cascade (MAPK) (reviewed by Testi, 1996, Riboni *et al.*, 1997 and Gomez-Munoz, 1998).

One such target of ceramide is a Mg^{2+}-dependent proline-directed serine/threonine specific kinase, exclusively confined to the cell membrane, which has been named the ceramide-activated protein kinase (CAPK) (Mathias *et al.*, 1991, Dressler *et al.*, 1992, Joseph *et al.*, 1993). CAPK has been shown to phosphorylate the Raf-1 kinase, coupling the SM pathway with the MAPK cascade. Raf-1 activation in turn activates the extracellular-signal-regulated kinase 2 (ERK-2), a type of MAPK, via phosphorylation of MAPK/ERK kinase (MEK) (Yao *et al.*, 1995). Additionally, a direct target of ceramide is a guanine nucleotide exchange factor, Vav, which is able to activate Ras, indicating that ceramide may interact at different levels with MAPK signalling (Gulbins *et al.*, 1994).

Ceramide activates a cytosolic heterotrimeric protein phosphatase, which has been named the ceramide-activated protein phosphatase (CAPP) (Dobrowsky & Hannun, 1992 & 1993, Dobrowsky *et al.*, 1993, Hannun, 1996). CAPP has been shown to belong to the PP2A subfamily of protein phosphatases (Dobrowsky *et al.*, 1993, Galadari *et al.*, 1998), and is believed to mediate *c-myc* downregulation and apoptosis in HL60 cells (Wolff *et al.*, 1994), c-jun phosphorylation in A431 human epithelial tumour cells (Reyes *et al.*, 1996), PKCα inactivation in MOLT-4 and Jurkat T cells (Lee *et al.*, 1996), and among other effects is also considered a key mediator for dephosphorylation of Rb (discussed previously in Chapter 1.2.2.) thus resulting in G_0/G_1 cell cycle arrest (Schonthal & Feramisco, 1993, Dbaibo *et al.*, 1995, Voorhoeve *et al.*, 1999, Lee *et al.*, 2000). In addition, another protein

Figure 1.7.: Mechanisms of ceramide generation and targets of ceramide action (modified from Mathias *et al.*, 1998). Ceramide is generated by hydrolysis of sphingomyelin (SM) by sphingomyelinases (SMases), or by the *de novo* synthesis pathway. Ceramide is pleiotropic in nature, capable of activating a number of different molecular targets depending on specific circumstances. Downstream effects of ceramide generation include cell cycle arrest, cellular senescence, differentiation, and/or apoptosis.



phosphatase, belonging to the PP1 subfamily of protein phosphatases, is also believed to be activated by long chain ceramides (Chalfant *et al.*, 1999) and is associated with Rb dephosphorylation and cell cycle arrest (Dou *et al.*, 1995, Ludlow & Nelson, 1995, Berndt *et al.*, 1997, Nelson *et al.*, 1997, Rubin *et al.*, 1998, Kishikawa *et al.*, 1999, Liu *et al.*, 1999b, Edwards & Thomas, 2000).

Ceramide has also been shown to activate transcriptional targets including *c-fos* and *c-jun*. In the stress-response kinase cascade, ceramide activates MEK kinase (MEKK) to activate Jun kinase 1 (JNK-1) by phosphorylation via stress-activated protein kinase/ERK kinase (SEK) (Westwick *et al.*, 1995, Verheij *et al.*, 1996, reviewed by Liu *et al.*, 1999a). Corresponding with this, the levels of the transcription factor complex AP-1 increase following ceramide treatment (Okazaki *et al.*, 1998), which is important in ceramide-induced apoptosis in HL60 cells (Sawai *et al.*, 1995). In addition, ceramide has been shown to suppress the expression of the *c-myc* proto-oncogene by interfering with transcription elongation (Wolff *et al.*, 1994).

The protein kinase C ξ isoform (a DAG and phorbol ester-insensitive isoenzyme of PKC) is known to be a target of ceramide signalling (Muller *et al.*, 1995). A number of investigators have suggested that ceramide rapidly induces the translocation and activation of NF-κB (Schutze *et al.*, 1992, Yang *et al.*, 1993, Wiegmann *et al.*, 1994), most likely through phosphorylation of the cytosolic IκBα inhibitor by PKCξ (Lozano *et al.*, 1994, Limatola *et al.*, 1997). The subsequent induction of degradation of NF-κB critically controls the expression of a number of genes that are dependent on this factor (Testi, 1996).

In the same way that ceramide synthesis can be activated during distinctly different extracellular circumstances, the targets of ceramide signalling are markedly varied. This indicates that ceramide synthesis and the interaction of ceramide with its different target molecules, will give rise to a multitude of cellular responses. This further supports the notion that ceramide is multi-functional (although able to initiate a specific response depending on the target activated), as opposed to having a single, defined function within the cell.

1.3.5. Ceramide-Mediated Cellular Effects

The transduction of extracellular signals is mediated by transient molecules termed second messengers (McGovern *et al.*, 1995), of which ceramide is regarded to be an important member. Second messenger molecules are generated from a pre-existing precursor (sphingomyelin, in the case of ceramide) strictly in response to a specific signal, and function to elicit a specific biological response, thus forming a

tight coupling of the intracellular reaction to the extracellular signal (Ghosh *et al.*, 1997).

The second messenger ceramide, the central component of the SM pathway, has a highly pleiotropic nature, capable of instigating a range of anti-proliferative cellular responses. These include proliferation, differentiation, growth arrest and apoptosis (Mathias *et al.*, 1998), depending on the cell type and the initial stimuli presented to the external surface of the cell, and also the amount and location of ceramide generation (Perry & Hannun, 1998). This is due to the fact that ceramide signalling is linked to a variety of cell receptors. Furthermore, Spiegel and colleagues describe the "ceramide/sphingosine-1-phosphate rheostat" to propose that a balance exists between cellular ceramide levels that favour death and sphingosine-1-phosphate levels that inhibit death, and strongly suggest that this is a critical factor that ultimately determines the fate of cells (Van Brocklyn *et al.*, 1998). Some of the downstream cellular functions of ceramide signalling are detailed below.

A) Cell Cycle Regulation and Senescence

Treatment of HL60 with the cell permeable ceramide analogue, C_2-ceramide, was sufficient to induce specific and potent antiproliferative effects even at concentrations as low as 1-10 μM (Okazaki *et al.*, 1990). Ceramide has now been shown to induce these effects in a number of different human leukaemia cells and in normal fibroblasts in logarithmic phase of growth (Hannun, 1994). Similarly, a considerable increase in ceramide levels has been noted in WI-38 human diploid fibroblast cells entering senescence (Venable *et al.*, 1995). Ceramide-induced cell cycle arrest occurs specifically in the G_0/G_1 phase of the cell cycle, thus preventing progression into S phase (Jayadev *et al.*, 1995). Extensive research has been performed into the mechanisms by which ceramide induces cell cycle arrest, and it now appears that ceramide-induced cell cycle arrest certainly involves the dephosphorylation (activation) of Rb (Chao *et al.*, 1992, Jayadev *et al.*, 1995, Dbaibo *et al.*, 1995). Cells partially deficient in Rb or expressing proteins that sequester the Rb protein were resistant to ceramide-induced cell cycle arrest (Mathias *et al.*, 1998). As previously discussed, two mechanisms exist for Rb activation: activation of expression of the CKI $p21^{Cip1/Waf1}$, and activation of protein phosphatases, both of which are shown to occur following ceramide treatment (Dobrowsky *et al.*, 1992, Dobrowsky *et al.*, 1993, Kolesnick & Golde, 1994, Wolff *et al.*, 1994, Hannun, 1996, Ragg *et al.*, 1998, Lee *et al.*, 2000).

Therefore, ceramide has the ability to control significant components of the cell cycle, such as the phosphorylation status of Rb at the G_1 to S phase transition. This in turn implies that manipulation of the ceramide signalling pathway may be beneficial in the treatment of proliferative disorders such as leukaemia, where regaining control of the cell cycle is of extreme importance.

B) Differentiation

Ceramide was first postulated to be an intracellular modulator of differentiation following the finding that treatment of HL60 cells with a ceramide analogue results in differentiation of these cells along the monocytic lineage (Okazaki *et al.*, 1990, Kim *et al.*, 1991). This response mimics the effect of TNFα, 1α,25-dihydroxyvitamin D_3, IL-1β and γ-interferon treatment previously noted in HL60 cells (Okazaki *et al.*, 1989, Ballou *et al.*, 1992, Kim *et al.*, 1991, Mathias *et al.*, 1993, Kolesnick & Golde, 1994). Ragg *et al.* (1998) have demonstrated ceramide-induced terminal differentiation (passage from the G_0/G_1 phase into the S phase of the cell cycle is prevented) accompanied by concomitant cell cycle arrest in U-937 monoblastic cells. Similarly, studies on the rat neuronal T9 cell line have revealed that these cells form neurite processes, a marker of differentiation in T9 cells, four days after ceramide addition (Dobrowsky *et al.*, 1994). In addition, Prinetti *et al.* (1997) have linked ceramide-mediated protein phosphatase activation with differentiation of a neuroblastoma Neuro2a cells.

C) Apoptosis

The involvement of ceramide in inducing apoptosis (programmed cell death) was first demonstrated by Obeid *et al.* (1993) in U-937 cells and has since been extensively studied. Jarvis & Grant (1998) have demonstrated an association between ceramide signalling and chemotherapy-related apoptosis. Ceramide levels are increased in HIV-infected T lymphocytes, which are known to undergo apoptosis (Van Veldhoven *et al.*, 1992). Ligation of the Fas and TNFα receptors, which results in apoptosis, has been associated with SM hydrolysis and the subsequent generation of ceramide (Kim *et al.*, 1991, Mathias *et al.*, 1991, Dressler *et al.*, 1992, Jarvis *et al.*, 1994a, Verheij *et al.*, 1996, Kolesnick & Kronke, 1998). Addition of exogenous ceramide results in apoptosis in a number of cell lines. These two findings were used to postulate the hypothesis that ceramide was directly responsible for Fas- and TNFα-induced apoptosis (Obeid *et al.*, 1993, Cuvillier *et al.*, 1996, Verheij *et al.*, 1996, Mathias *et al.*, 1998).

More recent studies have shown that ceramide is not the primary second messenger responsible for Fas-induced apoptosis. Instead, ceramide generation as a result of Fas receptor ligation may be merely accentuating the apoptotic signal, or may be a coincidental finding, or a redundant secondary pathway of Fas signalling (Gamen *et al.*, 1998, Hsu *et al.*, 1998, Tepper *et al.*, 1999). In fact, a study by Watts *et al.* (1997), and a similar study by Sillence and Allan (1997), has dissociated the link between ceramide signalling and Fas-induced apoptosis, after showing that Fas-induced apoptosis in Jurkat T cells was not accompanied by a detectable increase in ceramide production. The role of ceramide in TNFα-induced apoptosis is also not universally accepted. Some authors suggest that ceramide generation is a consequence of TNFα-mediated apoptosis and that ceramide and TNFα can induce the apoptotic pathway via separate means (reviewed by Liu *et al.*, 1999a). As ceramide-induced apoptosis relies on activation of the transcription factor c-jun, and TNFα-induced apoptosis occurs independent of c-jun activation, Liu *et al.* (1996b) suggest that ceramide production and TNFα-mediated apoptosis are separate events. Although not directly related to the Fas or TNFα studies, Jayadev *et al.* (1995) showed that activators of PKC, known antagonists of ceramide-induced apoptosis (Obeid *et al.*, 1993, Jarvis *et al.*, 1994b), had no effect on ceramide-induced cell cycle arrest. This suggests that ceramide regulates the outcomes of apoptosis and cell cycle arrest independent of one another. Mengubas *et al.* (1999) have shown that ceramide-induced death of malignant cells from patients with B-CLL and normal B lymphocytes occurs via a non-apoptotic mechanism.

The mechanism by which ceramide causes apoptosis has remained elusive, and is still regarded to be somewhat controversial. There is a lack of data demonstrating a direct interaction between ceramide signalling and the primary components (the caspase and Bcl-2 families) of the apoptotic machinery (Perry & Hannun, 1998). There is some, although limited, support for the involvement of ceramide-induced apoptosis by activation of caspases (Smyth *et al.*, 1996, Spinedi *et al.*, 1998), while other authors present conflicting arguments to say that ceramide-mediated apoptosis occurs independently of caspase activation (Belaud-Rotureau, 1999). Recent reports now indicate that the mitochondria may be central in ceramide-induced apoptosis. Modulation of mitochondria function by ceramide in mainly cell-free systems has been shown in a number of studies (Arora *et al.*, 1997, Garcia-Ruiz *et al.*, 1997, Ruvolo *et al.*, 1999, Di Paola *et al.*, 2000). More specifically, ceramide can induce the release of cytochrome *c* from isolated mitochondria (Ghafourifar *et al.*, 1999) and enhances cytoplasmic cytochrome *c* levels prior to caspase activation

(Ito *et al.*, 1999, Cuvillier *et al.*, 2000). These findings provide the link between ceramide signalling and execution caspases. Recent studies have revealed that there is a reduction in the transmembrane potential of the mitochondria during chemically-induced apoptosis (Scarlett *et al.*, 2000), and during ceramide-induced apoptosis in Jurkat T cells (Hearps *et al.*, 2002). A reduction in mitochondrial transmembrane potential is consistent with compromised permeability of the organelle, which allows for the release of cytochrome c and its subsequent activation of effector caspases in coordination with a protein called Apaf-1 (Zou *et al.*, 1997).

As mentioned in Chapter 1.2.2(C)., the retinoblastoma protein, known to be activated by ceramide, has been implicated to play a role in apoptosis. It has been suggested that Rb is cleaved by Interleukin 1β-converting enzyme-like proteases, accentuating the apoptotic response (An & Dou, 1996, Janicke *et al.*, 1996, Chen *et al.*, 1997, Tan & Wang, 1998, Fan & Steer, 1999, Schrantz *et al.*, 1999). There is also some evidence to suggest that ceramide mediates apoptosis via activation of protein phosphatases (Song *et al.*, 1992, Song & Lavin, 1993). Protein phosphatase inhibition has been shown to prevent drug-induced caspase activation (Morana *et al.*, 1996). Some members of the Bcl-2 family are regulated by phosphorylation and it is possible that these are likely targets for ceramide effectors in apoptosis (Perry & Hannun, 1998).

A better understanding of the molecular basis of ceramide action may be useful in providing greater insights into the pathogenesis of disease and may potentially offer novel strategies for therapeutic intervention. Further elucidation of the mechanisms of ceramide-induced cellular effects is paramount to releasing this potential.

1.4. Differentiation Therapy

1.4.1. Current Proceedings in Differentiation Therapy

Current conventional therapies for cancer treatment are not tumour specific and are often associated with considerable toxicity (Mokyr & Dray, 1987, Pai & Nahata, 2000). Furthermore, the emergence of drug resistant cells, despite the use of combined therapies as an attempt to overcome this, is one of the main obstacles for successful chemotherapeutic treatment of haematological malignancies (Nooter & Sonneveld, 1993, Sikic, 1999, Bunn & Kelly, 2000). As such, there is a need for the development of new drugs or treatment strategies for leukaemia and related diseases. A potentially less toxic approach is to target cellular growth and differentiation pathways to modify the state of differentiation and growth of cancer cells, while limiting cytotoxicity to healthy neighbouring cells, a strategy termed 'differentiation therapy' (Sachs, 1978, Fisher *et al.*, 1985, Waxman, 1996, Leszczyniecka *et al.*, 2001). Differentiation therapy is based on the assumption that many neoplastic cells show reversible defects in normal differentiation and cell growth programs, and with the appropriate treatment, these tumour cells can be reprogrammed to result in loss of proliferative capacity, induction of terminal differentiation (Sachs, 1978, Fisher *et al.*, 1985, Waxman 1996), and ultimately apoptosis (Martin *et al.*, 1990).

The three major fates of haemopoietic cells (proliferation, differentiation and death) are closely interconnected. Induction of differentiation is generally associated with reduced proliferative capacity, and cell death regularly accompanies the latter stages of differentiation (Leszczyniecka *et al.*, 2001). Therefore, it is not surprising therefore that in disease states which exhibit haemopoietic deregulation, such as leukaemia, there is a disruption to the processes of cellular proliferation, differentiation and/or apoptosis. To fully appreciate the close inter-relationship between proliferation, maturation, and death, it is necessary to understand the factors affecting haemopoietic cell differentiation, and how deregulation leads to enhanced proliferation and/or a block in differentiation.

Differentiation is initially triggered by the binding of haemopoietic growth factors to their appropriate receptor on the external surface of the cell. Several growth factors have been shown to be involved during the early stages of haemopoietic proliferation and differentiation, including stem cell factor, IL-1 and IL-3, while other growth factors such as GM-CSF and G-CSF have been associated with this process during the latter stages (Leszczyniecka *et al.*, 2001). The binding of growth factors to cell surface receptors activates a variety of signal transduction pathways, and it is the

components of these pathways (in particular, the modulation in activity) that have attracted significant attention in recent differentiation studies. The final cellular response is dependent upon the specific pathway activated. For example, activation of ERK MAPK pathway is associated with proliferation, whereas SEK/JNK activation is associated with an antiproliferative response (growth arrest, differentiation and apoptosis) (Szabo *et al.*, 1994, Xia *et al.*, 1995, Pyne *et al.*, 1996, Verheij *et al.*, 1996). That ERK is hyperexpressed in human breast cancer and may be a critical element in initiation and metastatic potential of this disease (Johnson, 1997, Sivaraman *et al.*, 1997, Mueller *et al.*, 2000) is further evidence that aberrant intracellular signalling can be a causative factor in cancer. Interestingly, the finding that inhibitors of PKC and MEK/MAPK can block phorbol ester- and retinoic acid-induced leukaemic cell maturation (Ragg *et al.*, 1998, Yen *et al.*, 1998, Hu *et al.*, 2000) implies that this pathway also has the potential to regulate terminal differentiation.

Stimulation of signal transduction pathways results in the activation of transcription factors (TFs), which permit the transcription of specific genes, whose protein products promote the differentiation response. TFs triggered by differentiation stimuli in haemopoietic cells include PU.1 (Olson *et al.*, 1995, Simon *et al.*, 1996, Bellon *et al.*, 1997, DeKoter *et al.*, 1998, Anderson *et al.*, 1999, Oikawa *et al.*, 1999), c-jun (Adunyah *et al.*, 1992 & 1995), AP-1 (Liebermann *et al.*, 1998, Rosson & O'Brien, 1998), substance P-1 (Laurenzi *et al.*, 1989, Bost & Pascual, 1992, Pascual *et al.*, 1992), and c-Myc (Lerga *et al.*, 1999, Lin *et al.*, 2000). It is not unexpected to note that TFs that are involved in cell differentiation have also been linked to regulation of apoptosis, emphasising the close relationship that exists between differentiation and cell death (Leszczyniecka *et al.*, 2001).

A crucial prerequisite for haemopoietic cell terminal differentiation is cell cycle arrest (Freytag, 1988, Furukawa, 1997, 1998, & 2002), a process that has been tightly linked with the activation of TFs factors via differentiation-associated signalling. A consequence of cells escaping this regulation process is seen in disease states such as melanoma (Platz *et al.*, 1996) and leukaemia (Drexler, 1998). Myeloid cell exposure to phorbol esters results in an increase in $p21^{Cip1/Waf1}$ expression via MAPK (Jiang *et al.*, 1994, Kharbanda *et al.*, 1994, Zeng & el-Deiry, 1996), a well-noted feature in terminally differentiated cells (Halevy *et al.*, 1995, Liebermann *et al.*, 1995, Parker *et al.*, 1995, Flink *et al.*, 1998). The ability to link activation of the MAPK signal transduction pathway and $p21^{Cip1/Waf1}$ expression is important from a therapeutic perspective, as studies by Park *et al.* (1999), Niibe *et al.* (1999), Tian *et*

al. (2000), and others have shown that $p21^{Cip1/Waf1}$ null cells or cells expressing p21 antisense have increased sensitivity to chemotherapy and radiotherapy-induced apoptosis. Thus the ability to control expression of $p21^{Cip1/Waf1}$ is of dual importance, as upregulated expression is essential for reducing the proliferative capacity via terminal differentiation, whereas downregulated expression may increase susceptibility of the cancer cells to apoptosis-inducing agents. These studies highlight that manipulation of intracellular signalling pathways may represent a potential strategy for the induction of terminal differentiation or the sensitisation of cells to chemotherapeutic agents or radiation.

1.4.2. Differentiation-Inducing Agents

To date, differentiation therapy has had the greatest impact in haematological malignancies, most notably leukaemia (Leszczyniecka *et al.*, 2001). *In vivo*, the majority of tumour cells, regardless of their lineage, appear to be refractory to the most extensively studied inducers of differentiation (Manfredi *et al.*, 1996). Considerable research over the last decade has concentrated on finding suitable differentiation-mediating drugs. Two, in particular, have emerged as showing promise in the clinical setting in their ability to overcome maturation defects in leukaemia cells; vitamin D_3 and all-*trans* retinoic acid, although the use of these agents are associated with clinical limitations. Examples of various differentiation-inducing agents that have been investigated to date are discussed below. While each of these agents have been shown to be effective *in vitro*, only a minority have been applied to the clinical treatment of patients with haematological malignancies.

A) Vitamin D_3

The hormonal form of vitamin D, 1α,25-dihydroxyvitamin D_3 (or vitamin D_3), plays a role in calcium ion homeostasis, and was first demonstrated by Koeffler *et al.* (1984) to induce human normal and leukaemic myeloid cells to terminally differentiate into macrophages/monocytes. Likewise, vitamin D_3 induced macrophage differentiation of leukemic colony-forming cells taken from patients. In fact, 80% of CML and approximately 50% of AML colony-forming cells differentiated to macrophage-like cells. The authors concluded that these findings suggested that vitamin D_3 may play a role in haemopoiesis and that the compound or a related analogue may possibly have a therapeutic role in some leukaemias (Koeffler *et al.*, 1984).

Differentiation has been demonstrated following vitamin D_3 treatment of human promyelocytic cells HL60, and human monocytic leukaemia cells U-937 (Bar-Shavit *et al.*, 1983, Olsson *et al.*, 1983, Mangelsdorf *et al.*, 1984, Koeffler *et al.*, 1985, Taimi *et al.*, 1993). Accompanying differentiation was a noted impediment to cell cycle progression, believed to be due to a G_1, and possibly a G_2/M block (Godyn *et al.*, 1994). This has also been reported following treatment in U-937 cells (Oberg *et al.*, 1991), murine myelomonocytic leukaemia cells WEHI-3 (Abe *et al.*, 1986), and human T lymphocytes activated to proliferate (Rigby *et al.*, 1985). The exact mechanism by which vitamin D_3 induces leukaemic cell differentiation is not known, but it has been related to induction of CKIs including $p21^{Cip1/Waf1}$, $p27^{Kip1}$, 15, and p18 (Liu *et al.*, 1996a, Kawa *et al.*, 1997, Park *et al.*, 2000, Hager *et al.*, 2001, Zenmyo *et al.*, 2001), and perturbations in the subcellular distribution of protein phosphatases (Song & Norman, 1998). This further demonstrates the close link between cell cycle arrest and terminal differentiation, and supports the hypothesis that $p21^{Cip1/Waf1}$ expression is directly coupled with terminal differentiation (Liu *et al.*, 1996a).

Clinical trials using vitamin D_3 are still in their infancy. The use of vitamin D_3 in the clinical setting for the treatment of malignancies has been hampered by its associated toxicity, namely hypercalcaemia, at a concentration required to suppress cancer cell proliferation (Laubenthal *et al.*, 1975, Nagpal *et al.*, 2001, Mehta & Mehta, 2002). In an attempt to overcome the hypercalcaemic effects of vitamin D_3, and exploit the ability of vitamin D_3 to inhibit the growth of neoplastic cells, nearly 400 structural analogues have been synthesised and evaluated for their efficacy and toxicity. Among these are EB1089, RO24-5531, 1 alpha-hydroxyvitamin D_5, and calcitrol, which have been evaluated in Phase I clinical trials for dose tolerance in advanced patients (Smith *et al.*, 1999, Mehta & Mehta, 2002). Vitamin D_3 acts synergistically in a sequence-dependent manner with the cytotoxic drug cytosine arabinoside (araC) to induce apoptosis in human leukaemia cells (Studzinski *et al.*, 1991), and with other drugs in solid tumours (Moffatt *et al.*, 1999). As such, it has been proposed that the role of vitamin D_3 in leukaemia therapy may be as a potentiator of cytotoxic drug action (Leszczyniecka *et al.*, 2001).

B) All-*Trans*-Retinoic Acid

The retinoid All-trans retinoic acid (ATRA) is a vitamin A-related compound, and binds to the retinoic acid receptor (RAR) which belongs to a superfamily of transcription factors. Early *in vitro* studies demonstrated that ATRA induces

granulocytic differentiation of HL60 cells (Drach *et al.*, 1994), and it has been described to be a regulator of macrophage functions and cytokine production (Dillehay *et al.*, 1988). Subsequent studies revealed that treatment of HL60 cells with ATRA also resulted in apoptosis, presumably secondary to the differentiation process (Gianni *et al.*, 2000).

Over the last decade, the molecular basis for ATRA-induced maturation has been elucidated. It was the finding that APL cells exhibit a t(15;17) chromosomal translocation which has lead to the clinical use of ATRA (Borrow *et al.*, 1990, de The *et al.*, 1990, Alcalay *et al.*, 1991, de The *et al.*, 1991, Kakizuka *et al.*, 1991, Warrell *et al.*, 1991, Chang *et al.*, 1992a & 1992b, Goddard *et al.*, 1992, Grignani *et al.*, 1993). This translocation encodes for the PML-RAR fusion promyelocytic leukaemia protein (Degos, 1992, Li *et al.*, 1997). Under normal conditions, retinoids bind to the RAR which leads to its dissociation from a histone deacetylase co-repressor complex, ultimately resulting in transcriptional activation of a variety of genes involved in haemopoietic cell maturation (Grignani *et al.*, 1998). In APL cells, this process is disrupted by the PML-RAR fusion protein, but can be reactivated with supraphysiologic concentrations of ATRA (Grignani *et al.*, 1998).

Since its introduction into clinical trials in 1988 (Chen *et al.*, 1991), ATRA has proved to be a safe and effective agent, inducing complete remission in excess of 80% of APL patients based on the results of several clinical trials (Castaigne *et al.*, 1990, Warrell *et al.*, 1991, Warrell *et al.*, 1993, Warrell *et al.*, 1997, Lo Coco *et al.*, 1998, Slack *et al.*, 2000). ATRA therapy exerted rapid improvement in abnormal haemostatic markers in APL patients without the need for anticoagulant therapies (Kawai *et al.*, 1994), a problem commonly seen in conventionally treated APL patients. Rb has been proposed to be a downstream target or effector of ATRA (Brooks *et al.*, 1996, Dimberg *et al.*, 2002), and this is consistent with the demonstration of G_1 cell cycle arrest (following ATRA treatment) and an increase in expression of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$.

ATRA treatment has however been associated with a potentially fatal increased leukocyte count in a proportion of patients, due to the release of several cytokines by the maturing blast cells (Fenaux & Debotton, 1998). This phenomenon has been termed the ATRA syndrome. The incidence of ATRA syndrome has been reduced however with prophylactic measures such as dexamethasone and occasionally antineoplastic therapy to relieve the white cell count (Fenaux & Debotton, 1998).

Other problems have also previously existed with ATRA therapy. Because ATRA therapy fails to eradicate the malignant clone, a high percentage of patients who were in complete remission from APL following ATRA treatment relapsed within a few months (Castaigne *et al.*, 1990, Chen *et al.*, 1991, Glasser *et al.*, 1994, Elstner *et al.*, 1997). Additionally, secondary resistance to ATRA was seen in all patients who relapsed soon after withdrawal of ATRA, believed to be due to activation of a cytochrome P_{450} (CP450)-dependent enzyme. The use of CP450 inhibitors, which include ketoconazole and liarozole, is being investigated, and the use of interferon-α in combination with ATRA has been proposed because interferon can inhibit the CP450 pathway (Fenaux & Degos, 1996).

Combined therapy with ATRA and chemotherapy (daunorubicin and araC) is now regarded as the standard treatment for the APL variant (Bruserud *et al.*, 2000, Fenaux *et al.*, 1999, Fenaux, 2000). A follow-up of a long-term randomised trial by Fenaux (2000) has revealed that ATRA/daunorubicin/araC combined therapy results in significantly reduced relapse rates, and longer survival times compared with chemotherapy or ATRA alone.

C) Phorbol Myristate Acetate

The discovery that phorbol esters could restore a normal differentiation program in leukaemic cells represented one of the first examples for the potential of differentiation therapy (Huberman & Callaham, 1979, Sachs, 1978, Koeffler *et al.*, 1980). Phorbol myristate acetate (PMA) binds with high affinity to PKC, activating the enzyme and subsequently induces various transcription factors involved in leukaemic cell differentiation, including PU.1 (Carey *et al.*, 1996). PMA exposure is also associated with activation of the MEK/MAPK pathway (Hu *et al.*, 2000), which in turn activates expression of $p21^{Cip1/Waf1}$ (Jiang *et al.*, 1994, Kharbanda *et al.*, 1994, Zeng & el-Deiry, 1996).

The use of PMA in the clinical setting is very limited because of its tumour promoting properties (Hecker, 1968, Baird & Boutwell, 1971). However, studies by Han *et al.* (1998a & 1998b) have demonstrated the feasibility of administering PMA to patients with refractory leukaemia concurrently receiving cytotoxic chemotherapy. In fact, the results from these studies reveal that PMA may attenuate the myelosuppressive effects of conventional chemotherapy, and as such Phase I trials are currently being conducted in the United States (Leszczyniecka *et al.*, 2001). PMA treatment may also have a role as a modulator of cytotoxic drug action in leukaemia

as combined treatment with araC and gemcitabine with PMA results in increased apoptosis of human leukaemia cells (Vrana *et al.*, 1999a).

D) Dimethylsulfoxide

Dimethylsulfoxide (DMSO) is a polar compound that has been shown to induce differentiation in a variety of transformed cell types. DMSO induces granulocytic maturation in HL60 promyelocytic cells (Collins *et al.*, 1978, Santos-Beneit & Mollinedo, 2000) and erythroid differentiation in MEL cells (Yamada *et al.*, 1997). DMSO-induced differentiation is associated with G_0/G_1 cell cycle arrest, and the induction of $p21^{Cip1/Waf1}$ (Jiang *et al.*, 1994). It is postulated that DMSO mediates differentiation via perturbations in PKC activity, intracellular Ca^{2+}, or protein tyrosine phosphorylation (Morley & Whitfield, 1993, Ginestier-Verne *et al.*, 1996). Published evidence for the use of DMSO in the clinical arena is very limited, however peak plasma concentrations of approximately 20 mM have been achieved in patients undergoing peripheral blood stem cell transplantation (Egorin *et al.*, 1998), suggesting the feasibility of use of DMSO as a differentiation-inducing agent in patients with haematological malignancies.

E) Chromatin Remodelling Agents

Interference with DNA methylation and histone deacetylases represents a logical target for therapeutic intervention as this process ultimately influences chromatin structure and chromosomal stability, in addition to gene expression (Hergersberg, 1991). DNA methylation status of genes is maintained by the enzyme cytosine DNA methyltransferase (Leonhardt & Bestor, 1993) and results in transcriptional repression and impaired induction of genes required for differentiation. Hypermethylation of leukaemia-specific tumour suppressor genes including calcitonin and p16 have been reported in leukaemic cells (Baylin *et al.*, 1987, Kamb, 1995). Hypermethylation of the $p21^{Cip1/Waf1}$ gene promoter in bone marrow cells of ALL patients has been correlated strongly with decreased $p21^{Cip1/Waf1}$ mRNA levels, which is highly predictive of a poor clinical outcome (Roman-Gomez *et al.*, 2002). Several DNA demethylating agents have been identified, including the cytidine analogue 5-azacytidine (5-AC) which has been in clinical use since the mid 1980s. Attention has been directed towards the 5-AC analogue, 5-aza-deoxycytidine, which is a potent inhibitor of DNA methylation and an effective inducer of leukaemic cell differentiation (Attadia, 1993). 5-aza-deoxycytidine has been effective

clinically in the treatment of acute leukaemia and MDS (Kantarjian *et al.*, 1997, Wijermans *et al.*, 1997).

Histone deacetylases are enzymes that regulate the deacetylation of core nucleosomal histones, repressing the transcriptional activation of genes (Turner, 1993, Ferrara *et al.*, 2001). Suberoylanilide hyroxamic acid (SAHA) functions as an inhibitor of histone deacetylases, and may be used in the transcriptional activation of genes involved in the maturation process (Richon *et al.*, 1998 & 2000, Licht, 2001), by reversing the aberrant repression of fusion proteins. SAHA is yet to be trailed in the clinical setting, although *in vitro* studies have shown its potential for use as an inducer of differentiation and apoptosis. SAHA-induced differentiation is accompanied by an increase in $p21^{Cip1/Waf1}$ and downregulation in c-Myb (Richon *et al.*, 1996 & 1998, Vrana *et al.*, 1999b) in MEL cells. In studies involving human cells (U-937 and HL60) however, SAHA induces an aberrant differentiation response, acting as a relatively weak inducer of maturation, but a potent inducer of apoptosis (Vrana *et al.*, 1999b).

The use of demethylating agents in combination with histone deacetylase inhibitors provides a promising new approach to differentiation therapy through modulation of gene expression (Chiurazzi *et al.*, 1999).

1.5. Summary and Thesis Aims

1.5.1. Summary of the Literature

Leukaemia cells differ from their normal counterparts in that their cell division and differentiation are aberrantly regulated and the failure of these cells to properly regulate survival, proliferation, differentiation and apoptosis results in an altered phenotype and cancer. Understanding the molecular mechanisms controlling cell proliferation and developing therapeutic strategies to correct non-functional regulatory mechanisms is an emerging area of medical research (Bruserud *et al.*, 2000, Leszczyniecka *et al.*, 2001). Current therapies halt the growth of aggressively multiplying leukaemic cells by killing them through the use of cytotoxic drugs or radiation therapy, a rather toxic and highly non-specific approach. Differentiation therapy represents a potentially less toxic form of cancer treatment involving the use of agents, that alone, or in combination, modify the state of differentiation and growth of leukaemic cells (Fenaux *et al.*, 1999, Bruserud & Gjertsen, 2000, Waxman, 2000). This therapeutic protocol is based on the observation that many leukaemic subtypes display reversible alterations in the normal programs of growth control and that correction by appropriate treatment results in the resumption of both normal differentiation processes and susceptibility to apoptosis (Waxman, 2000). The promising results from laboratory studies that have investigated differentiation-inducing agents are now beginning to show transitional promise in the clinical setting, in particular the use of ATRA in APL therapy. While the effectiveness of use of these agents is encouraging, their use is limited due to relapse (in ATRA-treated patients) or toxic side-effects (hypercalcaemia in vitamin D_3-treated patients), and the narrow scope of sensitive leukaemias that they act upon. Consequently, novel synthetic agents that can overcome these problems are required.

Recent attention has been focussed on the second messenger sphingolipid, ceramide. Activation of the ceramide-signalling pathway by synthetic analogues of this second messenger presents a currently unexplored area of potential therapeutic development. Whilst many studies have addressed the involvement of ceramide in the apoptotic process (reviewed in Jarvis *et al.*, 1996, Haimovitz-Friedman *et al.*, 1997, Hannun & Obeid, 1997, Kolesnick & Hannun, 1999), the differentiation-inducing capability of this signalling pathway has been relatively ignored. Several lines of evidence suggest that signalling by ceramide possesses significant antiproliferative and differentiation-promoting capacity, as evidenced by its ability to induce:

- G_0/G_1 cell cycle arrest via Rb dephosphorylation

(Chao *et al.*, 1992, Jayadev *et al.*, 1995, Dbaibo *et al.*, 1995, Mathias *et al.*, 1998)

- transcriptional modification of cell cycle regulatory genes

(Dobrowsky *et al.*, 1992, Dobrowsky *et al.*, 1993, Kolesnick & Golde, 1994, Wolff *et al.*, 1994, Hannun, 1996, Ragg *et al.*, 1998, Lee *et al.*, 2000)

- activation of the antiproliferative SAPK/JNK pathway

(Westwick *et al.* 1995, Verheij *et al.*, 1996, Pena *et al.*, 1997, Ruvolo, 2001)

- terminal differentiation

(Okazaki *et al.*, 1990, Kim *et al.*, 1991, Dobrowsky *et al.*, 1994, Ragg *et al.*, 1998)

Consequently, manipulation of the ceramide-signalling pathway presents a novel therapeutic target for the treatment of diseases that lack control of growth and differentiation processes, such as leukaemia. However, the processes by which ceramide promotes these potentially therapeutic outcomes occur by mechanisms that are largely undefined.

1.5.2. Thesis Aims

The aim of this thesis is to define the ceramide-activated molecular mechanisms that result in growth arrest and differentiation of human leukaemic cells in order to assess the therapeutic potential of this second messenger and its signalling pathway. More specifically, the findings presented in this thesis aim to characterise the growth arrest and differentiation response of haemopoietic cell lines to ceramide signalling, to determine whether common responses and mechanisms are utilised, and to investigate the molecular mechanisms involved in ceramide-mediated growth arrest and differentiation.

2.1. Materials

The reagents, antibodies, commercial kits, and disposables and equipment commonly used in this study, and the suppliers from which they were obtained, are listed in Tables 2.1.A., 2.1.B., 2.1.C. and 2.1.D. respectively.

Table 2.1.A.: Commonly used reagents and suppliers.

REAGENT	SUPPLIER
7-amino-actinomycin D (7-AAD)	Sigma-Aldrich, USA
Acetic acid (glacial) (AR Grade)	BDH Chemicals, England
40% Acrylamide-bis (19:1)	Amresco, USA
40% Acrylamide-bis (29:1)	BIO-RAD Laboratories, USA
Agarose (Molecular Biology Grade)	BDH Chemicals, England
Ammonium persulphate	BIO-RAD Laboratories, USA
Ampicillin powder (sodium salt)	Sigma-Aldrich, USA
Bacto-agar	Oxoid Ltd., England
Bacto-tryptone	Oxoid Ltd., England
Bacto-yeast extract	Oxoid Ltd., England
β-mercaptoethanol (β-ME)	BDH Chemicals, England
Boric acid	Amresco, USA
Bromophenol blue	BDH Chemicals, England
Bovine serum albumin (BSA) for restriction digests	New England Biolabs, USA
Calyculin A	Sigma-Aldrich, USA
C_2-ceramide (N-Acetylsphingosine, D-erythro)	BIOMOL Research Laboratories, Inc., USA
Chloroform (AR Grade)	BDH Chemicals, England
dATP, dCTP, dGTP, dTTP (100 mM)	Promega, USA
dUTP (20 mM)	Promega, USA
D-glucose	May & Baker, Australia
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, USA
Ethylenediaminetetra acetic acid (EDTA)	ICN Biochemicals, USA
Ethanol (AR Grade)	APS Chemicals Ltd., Australia
Ethidium bromide	Sigma-Aldrich, USA
Extran MA03	Merck Pty. Ltd., Australia
Foetal calf serum (FCS)	CSL Limited, Australia
Formaldehyde (40% w/v)	May & Baker, Australia
Formamide	BDH Chemicals, England
Gentamicin	Pharmacia & Upjohn, Australia
L-Glutamine	JRH Biosciences, USA
Glycerol (AR Grade)	May & Baker, Australia
Glycine	Sigma-Aldrich, USA
Methanol	EM Science, USA
NE Buffers 2 and 3 (for restriction digests)	New England Biolabs, USA
3-(N-morpholino) propane sulfonic acid (MOPS)	Amresco, USA
Nonidet P-40 (NP-40)	Shell, USA
Potassium acetate	Sigma-Aldrich, USA
Polyamp Duofit™ Water For Injections BP (10 mL)	ASTRA Pharmaceuticals Pty. Ltd., Australia
Prestained SDS-PAGE broad range standards	BIO-RAD Laboratories, USA
Propan-2-ol (isopropanol) (AR Grade)	BDH Chemicals, England
Propidium iodide	Sigma-Aldrich, USA

Proteinase K	Roche Diagnostics, USA
Pyroneg	Diversey, Australia
RNA loading buffer	Ambion, USA
RNase A	Roche Diagnostics, Germany
RPMI-1640 powdered media (w/o L-Glutamine, w/o $NaHCO_3$)	JRH Biosciences, USA
Sheared salmon testes DNA	Sigma-Aldrich, USA
Skim milk powder	Bonlac Foods Ltd., Australia
Sodium acetate (CH_3COONa)	Ajax Chemicals, Australia
Sodium azide (NaN_3)	Ajax Chemicals, Australia
Sodium chloride (NaCl)	Univar-Ajax Chemicals, Australia
Sodium citrate ($C_6H_5Na_3O_7.2H_2O$)	May & Baker, England
Sodium deoxycholate ($C_{24}H_{39}O_4Na$)	BDH Chemicals, England
Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	Ajax Chemicals, Australia
Sodium dodecyl sulphate (SDS)	BDH Chemicals, England
Sodium fluoride (NaF)	Merck, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	May & Baker, England
Disodium hydrogen orthophosphate (Na_2HPO_4) anhydrous	Ajax Chemical, Australia
Sodium hydroxide (NaOH)	Spectrum Chemical Mfg. Corp., USA
SPP1/*EcoR I* DNA-molecular weight marker	Bresatec Pty. Ltd., Australia
Sucrose	Spectrum, USA
TEMED (N,N,N',N'–tetra-methyl-ethylenediamine)	BIO-RAD Laboratories, USA
Tris base	Merck, Germany
Tris-HCl	Merck, Germany
Tris-saturated phenol	Amresco, USA
Triton X-100	Sigma-Aldrich, USA
Trypan blue	Sigma-Aldrich, USA
Tween 20	BDH Chemicals, England
Urea	Spectrum, USA
X-vivo 15 serum-free media	Biowhittaker, USA
Xylene cyanole FF	Sigma-Aldrich, USA
20 bp molecular ruler	BIO-RAD Laboratories, USA
100 bp PCR molecular ruler	BIO-RAD Laboratories, USA
[α-^{32}P]dATP dye-free	NEN Life Science Products, Inc., USA
[α-^{33}P]dATP dye-free	NEN Life Science Products, Inc., USA
[α-^{33}P]UTP dye-free	NEN Life Science Products, Inc., USA

Table 2.1.B.: Antibodies used in the study.

ANTIBODY	SUPPLIER
PRIMARY ANTIBODIES	
Goat polyclonal anti-Rb IgG (sc-50-G)	Santa Cruz Biotechnology, USA
Mouse monoclonal anti-$p21^{Waf1/Cip1}$ IgG (sc-817)	Santa Cruz Biotechnology, USA
SECONDARY CONJUGATED ANTIBODIES	
Anti-goat IgG HRP	Santa Cruz Biotechnology, USA
Anti-mouse IgG HRP	Santa Cruz Biotechnology, USA
IMMUNOPHENOTYPING ANTIBODIES	
Anti-CD11b PE	Becton-Dickinson, USA
Anti-CD14 PE	Becton-Dickinson, USA
Anti-CD34 PE	Becton-Dickinson, USA
Anti-glycophorin A PE	Dako Corporation, USA

Table 2.1.C.: Reagent kits used in the study.

NAME OF KIT	SUPPLIER
AmpliTaq® PCR Kit AmpliTaq® DNA polymerase, 10X PCR buffer, $MgCl_2$	Roche Diagnostics, Germany
Annexin V-FITC Apoptosis Detection Kit	Pharmingen, USA
Atlas™ cDNA Expression Array Human Cancer 1.2	Clontech, USA
Atlas™ Pure Total RNA Labelling System	Clontech, USA
Complete™, Mini Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Germany
DNA-free™ DNase treatment and removal Kit	Ambion, USA
Enhanced Chemiluminescence Detection Kit	Amersham Life Science, England
LightCycler capillaries	Roche Diagnostics, Germany
LightCycler RNA Master SYBR Green I real-time RT-PCR kit	Roche Diagnostics, Germany
PowerScript™ Reverse Transcriptase kit	Clontech, USA
RiboQuant™ Multi-Probe RNase Protection Assay System *In vitro* transcription kit and template	PharMingen, USA
RNeasy® Mini spin column kit RNA extraction, RNA cleanup	QIAGEN, Germany
SuperScript™ One-Step RT-PCR kit with SuperScript™ II RT and Platinum® Taq Mix	Invitrogen Life Technologies, USA
T_4 DNA ligase & 5X ligation buffer	Gibco BRL, Australia
QIAquick® Spin Kit PCR purification, nucleotide removal, gel extraction	QIAGEN, Germany

Table 2.1.D.: Commonly used disposables, equipment and software.

DISPOSABLES/EQUIPMENT/SOFTWARE	SUPPLIER
Biological safety cabinet class II	Email Air Handling, Australia
BioMax™ MR film	Eastman Kodak Company, USA
Bottletop filters (0.2 μm)	Nalgene, USA
Cell culture incubator 37°C, 5% CO_2, water-jacketed 3250	Forma Scientific, USA
Cell culture flasks (25 cm^2, 75 cm^2 and 225 cm^2)	Iwaki, Japan
Cell culture plates - 24 wells	Iwaki, Japan
CellQuest software package (V 3.3)	Becton-Dickinson, USA
Centrifuge (GS-15R)	Beckman, Germany
Centrifuge tubes (15 mL and 50 mL)	Iwaki, Japan
Densitometer calibrated (GS-800)	BIO-RAD Laboratories, USA
Dry block heater	Selby, Australia
Electrophoresis apparatus (DNA/RNA)	BIO-RAD Laboratories, USA
Electrophoresis power supply	BIO-RAD Laboratories, USA
FACScan flow cytometer	Becton-Dickinson, USA
FACS tubes (5 mL)	Becton-Dickinson, USA
GenomyxLR™ programmable DNA sequencer	Genomyx Corporation, USA
Heat sealer – Futura-Jr.	Audion Elektro®
Hyperfilm™ autoradiography film	Amersham Life Science, England
Immun-Blot® polyvinyldifluoride (PVDF) membrane for protein blotting	BIO-RAD Laboratories, USA
Inverted light microscopes	Wild Heerbrugg, Switzerland, and Leitz, Switzerland
LightCycler	Roche Diagnostics, Germany
Light microscope	Olympus, Japan
Microfuge centrifuge (Force 7)	Denver Instruments, USA
Micro hybridisation incubator, Model 2000, and glass roller tubes	Robbins Scientific, USA
Micropipettes (2 μL, 10 μL, 20 μL, 200 μL, 1000 μL Pipetman)	Gilson, France
Micropipette tips (10 μL, 20 μL, 200 μL and 1000μL)	Quantum Scientific Plastics, Australia
Microscope camera (Vario-Orthomat)	Leitz, Switzerland
Microfuge tubes (0.2 mL, 0.6 mL, 1.5 mL and 2.0 mL RNase/DNase Free)	Quantum Scientific Plastics, Australia
Microwave oven	Sanyo, Singapore
Milli-Q and Milli-RO water purification system	Millipore Corporation, USA
ModFit software package	Becton-Dickinson, USA
Oligonucleotide synthesis	GeneWorks, Australia
Pipetboy acu	Integra Biosciences, Switzerland
Polaroid black and white film (665)	Polaroid, USA
Polaroid MP-4 land camera	Polaroid, USA
Quantity One® quantitation software package (V4.0)	BIO-RAD Laboratories, USA
Scintillation counter (1214 Rackbeta)	LKB Wallac, Finland
SDS-PAGE electrophoresis apparatus and electrotransfer apparatus (XCell II™ Mini-Cell and Blot Module)	Novex, USA
Spectrophotometer (BioPhotometer) & UVettes	Eppendorf, Germany
Syringe filters – Supor® Acrodisc® 25 (0.2μm)	Gelman Sciences, USA
Thermal cycler	Eppendorf, Germany

Tissue culture flasks (25 cm^2 and 75 cm^2)	Iwaki, Japan
Ultracentrifuge (RC 5C Plus)	Sorvall, USA
UV transilluminator (Spectroline®)	Spectronics Corporation, USA
VacuCap® 90 filters (0.2 μm)	Gelman Sciences, USA
Vacuum evaporator centrifuge (Centrivap Concentrator)	Labconco, USA
Vario MACS magnetic particle separator	Miltenyi Biotech, Germany
Vortex mixer (MS1 Minishaker)	IKA Works, Malaysia
Waterbath	Grant Instruments, England

2.2. General Methods

All solutions were stored and used at room temperature unless otherwise stated. Solutions that were required to be sterile were either autoclaved using a standard liquid autoclave cycle, or were aseptically filtered through a 0.22 μm filter into a clean bottle that had been previously sterilised by autoclaving at 121°C for 15 minutes. Deionised ultrapure water was used to prepare all solutions and was obtained from a Milli-Q water purification system (Millipore Corporation, USA). All biological samples were considered biohazardous and as such were handled in a class II biological safety cabinet. All DNA and RNA extractions, and subsequent manipulation of DNA and RNA, were performed using DNase/RNase-free solutions and filtered pipette tips whilst wearing clean gloves to ensure minimal degradation of the nucleic acids.

2.2.1. Solutions

7-amino-actinomycin D (7-AAD) Solution:

1 mg	7-AAD
0.5 mL	DMSO
0.5 mL	1X PBS

Stock 7-AAD solution was prepared by dissolving the 7-AAD in DMSO and 1X PBS. Gloves and a mask must be worn when preparing this solution due to the toxicity of 7-AAD. A working solution of 25 μg/mL 7-AAD was prepared by adding 975 μL 1X PBS to 25 μL stock 7-AAD solution. The solution was aliquotted into tubes and stored at -20°C. Working tubes were stored at 4°C.

Agarose Gels (for cDNA/DNA):

2.4 g	agarose (for a 1.2% agarose gel)
20 μL	10 mg/mL ethidium bromide (1 μg/mL)

Agarose was added to 200 mL 1X TAE or TBE buffer and carefully heated in a microwave oven. The solution was gently swirled to dissolve the agarose and allowed to cool to handling temperature prior to pouring into a gel tray and allowing to set at room temperature with the appropriate gel comb in position.

25 μM Calyculin A Stock Solution:

10 μg Calyculin A desiccate

396.4 μL ethanol was added to an opened vial of Calyculin A and mixed carefully. Aliquots of 20 μL volumes were dispended into sterile 0.6 mL tubes and were stored at -80°C.

10 mM C_2-ceramide Stock Solution:

3.415 mg C_2-ceramide

C_2-ceramide was carefully weighed out in an analytical balance in a 1.5 mL eppendorf tube and dissolved in 1 mL ethanol by vortexing. The solution was aliquotted into 100 μL volumes in sterile 0.6 mL tubes and stored at -80°C for up to 6 months. Working stocks of the solution were stored at -20°C.

FACS Fixative:

5 mL 20X PBS

2 g glucose

0.67 mL 15% NaN_3

8 mL 40% (w/v) formaldehyde

The solution was adjusted to a final volume of 100 mL with Milli-Q water, filtered and stored at 4°C.

Hypotonic Propidium Iodide (HPI) Solution:

5 mg propidium iodide

0.1 g sodium citrate

0.1 mL Triton X-100

As described previously by Nicoletti *et al.* (1991), propidium iodide and sodium citrate were dissolved in 100 mL Milli-Q water overnight at 4°C in the dark. Triton X-100 was added last to minimise frothing on the following day. The solution was stored at 4°C in the dark.

2 X Laemelli Sample Buffer:

2.5 mL 0.5 M Tris-HCl (pH 6.8)

2 mL glycerol

4 mL 10% SDS

0.5 mL 0.1% bromophenol blue

0.5 mL β-ME

Reagents were combined and volume adjusted to 10 mL with Milli-Q water.

Luria-Bertani (LB) Media and LB Plate Preparation:

10 g	bacto-tryptone
5 g	bacto-yeast extract
10 g	NaCl

The ingredients above were added to 950 mL Milli-Q water and stirred until dissolved. The pH was adjusted to 7.0 with 5M NaOH and final volume of the solution made up to 1 L with Milli-Q water. This solution was sterilised by autoclaving. If LB agar plates were needed, bacto-agar was added to the LB media at 15 g/L and autoclaved to sterilise and to dissolve the agar. The LB media/agar solution was mixed gently to avoid air bubble formation, poured into Petri dishes and allowed to set in the biological safety cabinet (Sambrook *et al.*, 1989).

6X Loading Buffer for DNA:

0.0125 g	bromophenol blue (0.25%)
0.0125 g	xylene cyanole FF (0.25%)
2 g	sucrose (40% w/v)

The sucrose was first dissolved in approximately 4 mL Milli-Q water, followed by the addition of bromophenol blue and xylene cyanole FF. The final volume was made up to 5 mL with Milli-Q water (Sambrook *et al.*, 1989).

Lysis Buffer for Nuclear Protein Extraction:

20 µL	10% SDS
25 µL	1 M Tris (pH 7.4)
50 µL	1M NaCl
50 µL	10% sodium deoxycholate
50 µL	1 M NaF
150 µL	protease inhibitor solution (final = 1 tablet/10 mL lysis solution) (stock = 1 tablet/1.5 mL water, 150 µL aliquots stored at -20°C - Complete™, Mini Protease Inhibitor Cocktail, Roche Diagnostics, Germany)
200 µL	NP-40
455 µL	Milli-Q water

As previously described by Chao *et al.* (1992), reagents were combined fresh on the day that the lysis solution was needed. The solution was stored overnight at 4°C if needed on the subsequent day.

Miniprep Plasmid Extraction (Alkaline Lysis) Solution 1:

0.9 g	glucose (50 mM)
2.5 mL	1 M Tris-HCl, pH 8.0 (25 mM)
2 mL	0.5 M EDTA, pH 8.0 (10 mM)

The reagents were prepared in approximately 85 mL Milli-Q water and once the glucose had dissolved, the pH was checked to ensure it was at 8.0. The solution was autoclaved and stored at 4°C.

Miniprep Plasmid Extraction (Alkaline Lysis) Solution 2:

40 µL	5 M NaOH (200 mM)
100 µL	10% SDS (1%)

The solution was made up to 1 mL with Milli-Q water. Solution 2 was prepared fresh on the day of the miniprep plasmid extraction.

Miniprep Plasmid Extraction (Alkaline Lysis) Solution 3:

29.44 g	potassium acetate (3 M)
11.5 mL	glacial acetic acid

The potassium acetate was dissolved in approximately 70 mL milli-Q water. The acetic acid was added and the final volume adjusted to 100 mL with Milli-Q water.

10X MOPS Buffer:

83.72 g	MOPS (0.4 M)
33.34 mL	3 M sodium acetate (100 mM)
10 mL	1 M EDTA, pH 8.0 (10 mM)

The solution was made up in approximately 900 mL Milli-Q water prior to adjusting the pH to 7 with 5 M NaOH. The final volume was made up to 1 L with Milli-Q water and the solution was autoclaved, changing the colour of the buffer to yellow. The solution was stored at 4°C in the dark.

20X Phosphate Buffered Saline (PBS):

170 g NaCl

21.4 g Na_2HPO_4 anhydrous

7.8 g $NaH_2PO_4.2H_2O$

The above salts were dissolved in approximately 700 mL Milli-Q water by heating and stirring with a magnetic stirrer. The solution was allowed to cool to room temperature prior to adjusting to a final volume of 1 L with Milli-Q water and mixing. The solution was stored at room temperature without the need for sterilisation.

PBS + 0.1% Azide + 1% FCS (PBS-A):

25 mL 20X PBS

3.33 mL 15% NaN_3 (0.1%)

5 mL FCS (1%)

The solution was made up to 500 mL with Milli-Q water, filtered to remove FCS debris and stored at 4°C. Working aliquots were taken as required.

PBS + 0.1% Tween 20 (PBST):

50 mL 20X PBS

1 mL Tween 20 (0.1%)

The 1X PBS solution was prepared (50 mL 20X PBS added to 950 mL Milli-Q water) fresh on the day of use. The tip of a 1 mL disposable micropipette tip was cut off and a 1 mL volume of Tween 20 was dispensed into the PBS solution, tip ejected, and solution stirred using a magnetic stirrer until the tip had emptied of residual Tween 20.

RNase A Solution (10 mg/mL stock):

10 mg RNase A powder

10mM Tris-HCl (pH 7.5)

15 mM NaCl

As described by Sambrook *et al.* (1989), concentrated stock Tris-HCl (pH 7.5) and NaCl solutions were used to prepare a 1 mL solution of 10 mM Tris-HCl (pH 7.5)/15 mM NaCl by adding 10 µL 1M Tris-HCl (pH 7.5) and 15 µL 1M NaCl to 975 µL milli-Q water. The RNase A powder was dissolved in this solution by gentle vortexing and was heated at 100°C for 15 minutes and allowed to cool slowly

to room temperature. The solution was aliquotted into 100 µL volumes and stored at -20°C.

RPMI Media Preparation:

52 g RPMI 1640 powdered media (w/o L-glutamine, w/o $NaHCO_3$)

10 g $NaHCO_3$ (2 g/L)

The RPMI powder and $NaHCO_3$ was added on top of approximately 4.5 L Milli-Q water and stirred with a magnetic stirrer to dissolve. The pH of the solution was then adjusted to 7.0 with concentrated HCl, and final volume made up to 5 L with Milli-Q water. The flask containing the solution was transferred into the biological safety cabinet and was filtered into sterile 500 mL screw cap bottles using a VacuCap® 90 vacuum filtration device. The bottles were stored at 37°C overnight for sterility confirmation. Media showing no signs of contamination were stored in the dark at room temperature until required.

10X Running Buffer (for SDS-PAGE):

29 g Tris base

144 g glycine

10 g SDS

Reagents were combined and dissolved in 1 L Milli-Q water. The pH was checked to ensure it was at 8.3, and was not adjusted. This solution was re-used up to five times.

20% SDS:

100 g SDS was dissolved in 500 mL Milli-Q water and heated to 65°C to dissolve.

20X SSC:

175.3 g NaCl

88.2 g sodium citrate

The salts were dissolved in 900 mL Milli-Q water and the pH adjusted to 7.0 if necessary with 1 M HCl. The solution was adjusted to a final volume of 1 L with Milli-Q water and stored at room temperature without the need for sterilisation.

50X Tris-Acetate Buffer (TAE Buffer):

242 g Tris base
57.1 mL glacial acetic acid
100 mL 0.5 M EDTA, pH 8.0

The Tris base was dissolved in approximately 700 mL Milli-Q water and the remaining reagents added. The final volume was adjusted to 1 L with Milli-Q water. TAE buffer was used as a 1X working solution (Sambrook *et al.*, 1989).

5X Tris-Borate Buffer (TBE Buffer):

54 g Tris base
27.5 g boric acid
20 mL 0.5 M EDTA, pH8.0

Prepared as for Tris-Acetate buffer. Stock TBE buffer can form a precipitate when stored for a long period of time. When this occurred a new batch of TBE buffer was prepared. TBE buffer was used at a working strength of 0.5X for agarose gel electrophoresis, and 1X for polyacrylamide gel electrophoresis (Sambrook *et al.*, 1989).

Tris-EDTA Buffer (TE Buffer):

10 mL 50 mM Tris-HCl, pH8.0 (10 mM)
100 µL 0.5 M EDTA, pH8.0 (1 mM)

Reagents were combined and the volume was adjusted to 50 mL with Milli-Q water (Sambrook *et al.*, 1989).

Transfer Buffer (for protein electrotransfer):

100 mL 10X Running Buffer (for SDS-PAGE)
200 mL methanol
700 mL Milli-Q water

This solution was de-gassed extensively prior to use and was re-used up to ten times.

0.25% (w/v) Trypan Blue:

2.5 mg trypan blue

The trypan blue was dissolved in 10 mL Milli-Q water and filtered through a 0.2 µm syringe filter into a 50 mL centrifuge tube.

Wash Solution 1 (for cDNA Arrays):

100 mL 20X SSC (2X)

50 mL 20% SDS (1%)

To prevent immediate precipitation of the SDS, the 20X SSC stock was diluted in approximately 800 mL Milli-Q water prior to the addition of 20% SDS. The solution was adjusted to a final volume of 1 L with Milli-Q water. If not used on the day of preparation, a precipitate would form which could be redissolved by heating.

Wash Solution 2 (for cDNA Arrays):

2 mL 20X SSC (0.1X)

10 mL 20% SDS (0.5%)

As for Wash Solution 1 (for cDNA Array), but the solution was adjusted to a final volume of 400 mL with Milli-Q water.

2.2.2. Pipettes and Glassware

Graduated glass culture pipettes and Pasteur pipettes were soaked overnight in 2% Extran MA03 (Merck Pty. Ltd., Australia) and washed in tap water for a minimum of 4 hours using a self-dumping pipette washer. Pipettes were then rinsed 5 times in Milli-RO water, followed by 3 rinses in Milli-Q water. Cleaned pipettes were dried in a 50°C oven before being plugged with cotton wool and autoclaved at 121°C for 15 minutes in copper pipette canisters and allowed to dry overnight in the 50°C oven.

All glassware used for storage of solutions or for reagent preparation, and Western blotting and electrophoresis equipment, was soaked in a water containing dissolved phosphate-free Pyroneg (Diversey, Australia) detergent for a minimum of 4 hours and scrubbed. They were then rinsed thoroughly with tap water followed by several rinses with Milli-Q water and were left to dry.

2.2.3. Maintenance and Treatment of Cell Cultures

A) Maintenance Media

RPMI media was prepared as described in Chapter 2.2.1. Supplementary components were added to the RPMI media to support the growth of the human leukaemic cell lines used in this study. These are as follows:

500 mL RPMI media

1.5 mL 40 mg/mL gentamicin (120 μg/mL)

5 mL 200 mM L-glutamine (2 mM)

50 mL foetal calf serum (10%)

Gentamicin was stored in 2 mL aliquots at 4°C, L-glutamine was stored in 5 mL aliquots at -20°C, and FCS were stored in 50 mL aliquots at -20°C and were thawed at 37°C prior to being aseptically added directly to the RPMI media. The media and additives were stored at 4°C between usage, and was pre-warmed to 37°C in a waterbath prior to addition to cell cultures.

B) Cell Line Stocks

All cell lines used in this study were originally obtained from the American Type Culture Collection, ATCC® (University of Boulevard, Virginia, USA) and had been aseptically frozen down in liquid nitrogen in batches at 5 X 10^6/mL - 1 X 10^7/mL and stored in an inventory system in the Department of Pathology, University of Tasmania. All human haemopoietic cell lines used, and their respective ATCC® reference numbers and lineage type are given in Table 2.2.

Table 2.2.: Human haemopoietic cell lines used in this study.

Name of Cell Line	ATCC® Reference	Cell Lineage
K562	CRL 243	Erythromyeloid, early granulocytic precursor
KG-1	CCL 246	Myeloblastic and promyeloblastic
KG-1a	CCL 246.1	Very young undifferentiated promyeloblastic (KG-1 variant)
HL60	CCL 240	Promyelocytic
Jurkat	TIB 152	T Lymphocytic
Raji	CCL 86	B Lymphocytic

The characteristics of each cell line listed above, is provided in Table 1.5.

C) Cell Culture Conditions

Cells were grown in RPMI media described in Chapter 2.2.3(A). in vented capped tissue culture flasks, and were incubated at 37°C in a humidified atmosphere of 5% CO_2. All cells were maintained at a density of 1-10 X 10^5 cell/mL, except KG-1 and KG-1a cells which were maintained at a density of 2-20 X 10^5 cells/mL and 2-10 X 10^5 cells/mL, respectively. All manipulations of cell lines were

performed in a class II biological safety cabinet. All items entering the cabinet were sterilised by swabbing with 70% ethanol.

D) Treatment of Cell Cultures

Experiments were conducted using unsynchronised cells in exponential growth phase. Prior to each experiment utilising cell cultures, cells were enumerated using a haemocytometer to determine the number of cells per millilitre of culture. The appropriate volume of cells for the experiment was transferred to a sterile centrifuge tube and cells collected by centrifugation at 400 x g for 5 minutes at room temperature. All cells were washed in sterile 1 X PBS and resuspended at 5 X 10^5/mL in serum-free RPMI (SF-RPMI), except experiments using the HL60 cell line which were resuspended in SF-RPMI + 2% FCS, and experiments using Raji cells which were resuspended in X-Vivo serum-free medium (Biowhittaker, USA). SF-RPMI was prepared as described in Chapter 2.2.3(A). except that FCS was omitted. Cells were pre-incubated in their respective treatment media (unless otherwise stated) for a further 2 hours, prior to the direct addition of vehicle (absolute ethanol), C_2-ceramide or Calyculin A (concentrations used are stated in individual protocols) to the cell culture media. Treated cells were either plated out into 24-well cell culture plates at 1 mL/well, or transferred into cell culture flasks, depending on the volume of cells used in the experiment, for a specified period of time. Cells were harvested after designated time periods by centrifugation at 400 x g for 5 minutes and processed according to the desired protocol for a given experiment. All experiments included a 0 hour control time point unless otherwise specified. All experimental samples were accompanied by a time-matched, vehicle-treated (absolute ethanol) control sample.

2.2.4. Flow Cytometry

Fluorescent cells were enumerated and mean fluorescence intensity (MFI) of stained cells acquired using a FACScan flow cytometer (Becton-Dickinson, USA). Phycoerythrin (PE) and fluorescein isothiocyanate (FITC) was excited at 488 nm and by an air-cooled argon ion laser (Uniphase corporation, USA) at 15 mW. PE and FITC were collected using a 525 and 575 band-pass filter, respectively. 10,000 events were analysed for each sample and the data processed according to the specific experiment performed, using a Macintosh computer system.

2.2.5. 6.5% SDS-PAGE Gel Preparation for Rb Analysis

The preparation of 6.5% SDS-polyacrylamide separating gels is outlined in Table 2.3.

Table 2.3.: Preparation of 6.5% SDS-polyacrylamide separating gels.

Reagent	**Volume**
40% Acrylamide-bis (29:1)	3.9 mL
1 M Tris, pH8.8	9.4 mL
10% SDS	250 µL
50% sucrose	4 mL
Milli-Q water	6.8 mL
50 mg/mL ammonium persulphate (made fresh)	625 µL
TEMED	5 µL
	Total = 25 mL

All reagents except ammonium persulfate and TEMED were added in the order given in the above table and were extensively de-gassed to facilitate polymerisation of the gel. The ammonium persulphate was added and the solution mixed well but gently, followed by the addition of TEMED and gentle mixing. Approximately 4-5 mL volumes of the separating gel solution was quickly poured into upright empty gel cassettes, until the cassette was approximately ¾ full. The cassette was very gently topped up with a layer of Milli-Q water such that the separating gel set level.

The preparation of 4% SDS-polyacrylamide stacking gels is outlined in Table 2.4.

Table 2.4.: Preparation of 4% SDS-polyacrylamide stacking gels.

Reagent	Volume
40% Acrylamide-bis (29:1)	1.25 mL
0.375 M Tris, pH6.8	4.15 mL
Milli-Q water	6.6 mL
50 mg/mL ammonium persulphate (made fresh)	500 µL
TEMED	5 µL
	Total = 12.5 mL

As for the separating gel, all reagents except ammonium persulphate and TEMED were combined and de-gassed extensively. The Milli-Q water layer was removed from the top of the separating gel and the cassette allowed to drain. Ammonium persulphate was added to the stacking gel solution, followed by TEMED. The solution was mixed gently, but thoroughly, and was layered on top of the polymerised separating gel. A comb was inserted and the stacking gel allowed to set. Once polymerised, the gels could be stored in the gel cassettes for up to one week at 4°C in sealed plastic bags containing a small amount of Milli-Q water.

2.2.6. 5% Polyacrylamide Gel Preparation For RNase Protection Assays

56.25 mL 40% Acrylamide/Bis (19:1)

50 mL 10X TBE buffer

238.8 g Urea

The 5% acrylamide gel solution was prepared by first dissolving the urea in approximately 100 mL Milli-Q water by heating. The remaining reagents were added and final volume adjusted to 500 mL with Milli-Q water. Bromophenol blue powder was added to the solution until the desired depth of colour was achieved. This solution was enough for the preparation of five 5% polyacrylamide gels for use in RNase protection assays.

5% polyacrylamide gels were prepared as outlined in Table 2.5.

Table 2.5.: Preparation of 5% polyacrylamide gels.

Reagent	Volume
5% Acrylamide gel solution	100 mL
10% Ammonium persulphate (made fresh)	700 µL
TEMED	80 µL
	Total = 100 mL

The ammonium persulphate was added to the 5% polyacrylamide gel solution, mixed well but gently, followed by the addition of TEMED and gentle mixing. The solution was immediately poured between two cleaned 61 cm glass plates with 0.4 mm spacers in place on a horizontal surface. The gel was allowed to polymerise with a sharks tooth comb in position.

2.2.7. Extraction and Quantitation of Nucleic Acids

A) Extraction of DNA

Cells (2 X 10^6) were harvested by centrifugation at maximum speed in a benchtop microfuge, supernatant removed, and cells resuspended in 530 µL proteinase K solution. Samples were incubated at 50°C for 2-3 hours and extracted by phenol/chloroform extraction. An equal volume of phenol and chloroform (530 µL of each) was added to the sample and the solution mixed by inversion. Samples were centrifuged at 10,000 x g and the upper aqueous layer removed to a new microfuge tube. A second phenol/chloroform extraction was performed as above. A $1/10^{th}$ volume of 3M sodium acetate and 2 volumes of ice-cold absolute ethanol was added to the resulting upper phase from the second extraction. The samples were left at -20°C for a minimum of 2 hours and the precipitated DNA collected by centrifugation at 10,000 x g for 15 minutes at 4°C. The supernatant was carefully removed and washed with 70% and absolute ethanol respectively and the pellet air-dried in a biological safety cabinet. The dried pellet was resuspended in 50 µL 10 mg/mL RNase buffer and incubated for 15 minutes at 37°C followed by 30 minutes at room temperature.

For some applications, the RNase-treated DNA (or PCR products) was re-precipitated by adding 40 µL deionised water and 160 µL 95% ethanol, vortexing briefly, and incubating at room temperature for 15 minutes. The samples were then

centrifuged at maximum speed for 20 minutes at 4°C, the resulting pellet washed in 250 µL 70% ethanol, and the samples re-centrifuged at maximum speed for 10 minutes at 4°C. The pellets were air-dried as described previously and resuspended in 20 µL deionised water or TE buffer, depending on the application.

DNA was stored at -20°C for an indefinite period of time.

B) Analysis of DNA by Agarose Gel Electrophoresis

A 1.2% agarose gel containing 1 µg/mL ethidium bromide was prepared as described in Chapter 2.2.1. and placed in an electrophoresis tank filled with 1 X TAE or TBE buffer. The DNA or RT-PCR/PCR products were prepared by adding 6X DNA loading buffer (to 1X final), and 20 µL was loaded onto the gel. Additionally, molecular weight markers were run alongside the sample DNA to provide a reference. The gel was run at 60V constant for approximately 2 hours and DNA visualised on a UV transilluminator. Photographic evidence was obtained using a Polaroid MP4 land camera and black and white Polaroid film.

C) Extraction of RNA

Cells (1 X 10^7) were harvested by centrifugation at 400 x g for 5 minutes at 4°C, supernatant removed, and RNA extraction performed using the RNeasy® Mini spin column kit (QIAGEN, Germany). Briefly, cells were lysed in Buffer RLT (600 µL Buffer RLT + 6 µL β-ME) by pipetting up and down and vortexing thoroughly. The lysate was passed through a 20G needle 5 times, 1 volume (600 µL) of 70% ethanol added, and the solution was mixed by pipetting. The sample was loaded onto an RNeasy® Mini spin column sitting in a 2 mL collection tube and centrifuged at 8,000 x g for 15 seconds. 700 µL of Buffer RW1 was applied to the column and the column centrifuged at 8,000 x g for 15 seconds. 500 µL Buffer RPE was loaded into the column and the column centrifuged at 8,000 x g for 15 seconds followed by another addition of 500 µL Buffer RPE and centrifugation at maximum speed for 2 minutes to dry the column membrane. The column was carefully transferred to a 1.5 mL microfuge tube and 50 µL RNase-free water applied directly to the membrane. The column was left at room temperature for 2 minutes and centrifuged at 10,000 x g for 3 minutes to elute the RNA. The resulting RNA was immediately transferred to -80°C for storage.

D) DNase Treatment of RNA

RNA was treated to ensure the removal of contaminating DNA using the DNA-free™ DNase treatment and removal kit protocol (Ambion, USA). Briefly, 3.5 µL 10X buffer and 2 µL DNase 1 was added to 45 µL RNA in a 0.6 mL PCR microfuge tube and incubated at 37°C for 30 minutes in a thermal cycler. The DNase inactivation reagent was resuspended and 5 µL was added to the DNase-treated RNA and incubated for 2 minutes at room temperature. The RNA was stored at -80°C, and was centrifuged at 10,000 x g for 1 minute to pellet the DNase inactivation reagent prior to use.

E) Determination of RNA Integrity

A 1.2% RNA mini denaturing gel was prepared by dissolving 0.6 g agarose in 50 mL 1X MOPS buffer by heating in a microwave. After allowing the gel solution to cool to handling temperature, 9 mL 40% (w/v) formaldehyde was added in a fume cabinet. The solution was poured into a mini gel tray, comb placed into position and the gel allowed to set at room temperature. RNA samples were prepared as follows: a master-mix containing 8 µL 10X MOPS buffer, 40 µL formamide, and 14.4 µL 40% (w/v) was prepared. 7.8 µL of the master-mix solution was added to 2.5 µL RNA (1-2 µg), heated to 65-70°C for 15 minutes, and cooled on ice. 4 µL 0.5 mg/mL ethidium bromide and 4 µL RNA loading buffer (Ambion, USA) was added to the denatured RNA sample and the total volume loaded onto the gel. The gel was run at 50V constant for approximately 2 hours and RNA visualised on a UV transilluminator. Photographic evidence was obtained using a Polaroid MP4 land camera and black and white Polaroid film.

Total human RNA appeared as two distinct bands at approximately 4.5 and 1.9 kb, correlating to 28s and 18s rRNA respectively. The ratio of intensities of the 28s and 18s rRNA bands was estimated and should be 1.5-2.5:1 indicating minimal RNA degradation. Additional bands appearing below the 18s rRNA band could occasionally be seen which corresponded with 5s rRNA and tRNA. If any contaminating DNA was present it could be seen as smear remaining in the well. RNA that demonstrated degradation or DNA contamination was discarded and new RNA extracted and analysed for integrity.

F) Quantitation and Determination of Nucleic Acid Purity

The concentration and purity of DNA and RNA was determined by measuring the absorbance at 260 nm (A_{260}) and at 280 nm (A_{280}) using a spectrophotometer.

Generally, 2 µL DNA or RNA (typically 1-5 µg) was diluted in 58 µL RNase-free water to give a dilution factor of 30 (60÷2). The diluted sample was then transferred into a clean RNase-free cuvette (UVette – Eppendorf, Germany) which was placed into a spectrophotometer (BioPhotometer – Eppendorf, Germany) and absorbance measured. The readings obtained were based on a blanked absorbance reading taken from water.

An absorbance of 1 unit at 260 nm corresponds to 50 µg/mL DNA and 40 µg/mL RNA prior to taking the dilution factor into account. The ratio of A_{260}/A_{280} gives an estimate of purity and should be above 1.8. Nucleic acid demonstrating a ratio below 1.8 was excluded from use in further applications in this study.

$\Rightarrow$ Concentration (µg/mL) = A_{260} x [50 (for DNA) or 40 (for RNA)] x dilution factor

2.2.8. RNase Protection Assays

RNase protection assays were performed as per the protocol and reagents provided in the RiboQuant™ Multi-Probe RNase Protection Assay System (PharMingen, USA), using DNase-treated RNA (refer to Chapter 2.2.7.). A brief summary of the protocol is provided below.

A) Probe Synthesis

Necessary precautions and routines were followed when handling and disposing radioactive substances, as outlined in the National Health and Medical Research Council's regulations (1985, 1995a, 1995b). The radiolabelled RNA probe was synthesised by adding the RiboQuant™ kit components in the order shown in Table 2.6. to a 1.5 mL microfuge tube, gently mixing by pipetting, and incubating for 1 hour at 37°C.

Table 2.6.: Synthesis of radiolabelled RNA riboprobes.

Reagent	Amount/Volume
40 U/μL RNasin	1 μL
GACU pool mix (2.75 mM G, A, and C; 61 mM U)	1 μL
100 mM DTT	2 μL
5X Transcription buffer	4 μL
RPA template set	1 μL
[α-^{33}P]UTP, dye-free (3000 Ci/mmol, 10 μCi/μL)	10 μL
20 U/μL T7 RNA polymerase	1 μL
	Total = 20 μL

Following the probe synthesis incubation, 2 μL RNase-free DNase was added to the reaction and was incubated for a further 30 minutes at 37°C. A phenol/chloroform extraction was performed by adding 26 μL 20 mM EDTA, 25 μL Tris-saturated phenol, 25 μL chloroform/isoamyl alcohol (50:1), and 2 μL yeast tRNA to the DNase-treated probe mixture. The solution was vortexed into an emulsion, and centrifuged for 5 minutes at maximum speed in a bench-top microfuge. The upper aqueous phase was transferred to a new microfuge tube and 50 μL chloroform/isoamyl alcohol solution added and mixed by vortexing, followed by another separation of phases by centrifugation for 2 minutes at maximum speed. 50 μL 4 M ammonium acetate and 250 μL ice-cold absolute ethanol was added to the resulting upper aqueous phase and the tube was inverted to mix, and incubated for 1 hour at -80°C. The tube was centrifuged for 15 minutes at 4°C at maximum speed, the supernatant carefully removed and the pellet washed with 100 μL ice-cold 90% ethanol, followed by centrifugation for 5 minutes at 4°C. All supernatant was removed and the pellet air-dried and solubilised in 50 μL hybridisation buffer by gentle vortexing. The activity of the resulting probe was measured using a Rackbeta liquid scintillation counter (LKB Wallac, Finland) and the probe stored at -20°C until needed.

B) RNA Preparation and Hybridisation

5 μg aliquots of experimental sample RNA were frozen at -80°C for 15 minutes. The frozen RNA samples, plus additional samples of 5 μg HeLa RNA and 4 μg yeast tRNA, included as positive and negative controls respectively for the RPA protocol, were dried using the Centrivap vacuum evaporation centrifuge (Labconco,

USA) at room temperature. Samples were solubilized in 8 μL hybridisation buffer by gentle vortexing. 2 μL probe was added to each RNA sample and the RNA/probe mix overlaid with 60 μL mineral oil. The tubes were placed into a dry block heater pre-warmed to 90°C. The heat block temperature was immediately adjusted to 56°C and the samples incubated overnight. Prior to removing the samples, the heat block temperature was adjusted to 37°C, and having attained this temperature, the samples were incubated for a further 15 minutes.

C) RNase Treatment and Purification of Hybridised Probe

An RNase cocktail was prepared by combining 1.25 mL RNase buffer and 3 μL RNase A + T1 mix into a microfuge tube (enough for 10 samples). 100 μL of this mix was added directly underneath the oil layer into the hybridisation solution for each sample to degrade any unhybridised, and thus single-stranded RNA. Samples were incubated for 45 minutes at 30°C. A proteinase K cocktail was prepared by combining 195 μL proteinase K buffer, 15 μL proteinase K, and 15 μL yeast tRNA in a 1.5 mL microfuge tube.

The RNase digests were extracted from underneath the oil layer, added to an 18 μL aliquot of proteinase K solution, and incubated for 15 minutes at 37°C. The RNA:RNA hybrids were extracted by adding 65 μL Tris-saturated phenol and 65 μL chloroform/isoamyl alcohol (50:1), vortexing into an emulsion, and separation of the phases by centrifugation for 5 minutes at maximum speed. The upper aqueous phase (120 μL) was transferred to a new microfuge tube and 120 μL 4 M ammonium acetate and 650 μL ice-cold absolute ethanol added. The solution was mixed by inversion and incubated for 1 hour at -80°C. RNA:RNA hybrids were pelleted by centrifugation for 15 minutes at 4°C. The supernatant was carefully removed and pellets washed with 100 μL ice-cold 90% ethanol, and collected by centrifugation for 5 minutes at 4°C. Following removal of the supernatant, the pellets were air-dried and solubilized in 2.5 μL 1X loading buffer by vortexing. Loading buffer was also added to 2 μL of an appropriate dilution of undigested probe (usually 1/20). The samples were prepared for loading onto a gel by heating for 3 minutes at 90°C and placed immediately on ice.

D) Gel Resolution of Protected RNA:RNA Hybrids

A 5% polyacrylamide gel was prepared as described in Chapter 2.2.6. Excess gel was scraped away from the glass plates. The gel was positioned in the GenomyxLR™ programmable DNA sequencer (Genomyx Corporation, USA) and

pre-run for 45 minutes at 40W constant with 0.5X TBE in the upper buffer chamber, and 1X TBE buffer in the lower chamber. The 2.5 μL RNA:RNA hybrid samples (including HeLa RNA and yeast tRNA controls) and undigested probe mixed with 1X loading buffer were loaded onto the gel and the gel run at 50W constant until the dye front reached approximately 30 cm. The top glass plate was removed and the gel was dried, rinsed well with Milli-Q water. This was repeated two more times. The gel was dried thoroughly for a final time and was exposed to BioMax MR™ film (Eastman Kodak Company, USA), and developed after a sufficient exposure period.

E) RNase Protection Assay Analysis

Using the undigested probe as a marker, the identity of the "RNase-protected" bands in the experimental samples and HeLa and yeast tRNA control samples was determined. The autoradiographs were scanned using a GS-800 calibrated densitometer (BIO-RAD Laboratories, USA) and analysed using the Quantity One® quantitation software package (BIO-RAD Laboratories, USA). Pixel volumes (optical density/mm^2) data were obtained and normalised between samples using the data obtained from house-keeping gene analysis. Generally, all raw data was normalised using the 0 hr Control sample for GAPDH. Analysis was performed on various exposure times to enable accurate normalisation to the 0 hr Control sample, and to allow a more complete and accurate collection of data for the genes being expressed. Fold changes between the samples were calculated for each gene analysed where a signal was detectable.

2.2.9. cDNA Arrays

cDNA array analysis was performed using the protocol and reagents provided in the Atlas™ Pure Total RNA Labelling System, and Atlas™ Human Cancer 1.2 cDNA Expression Array kits (Cat . # K1038-1 and # 7851-1 respectively, Clontech, USA). A summary of the protocol, including alterations made for the purpose of this study, is provided below.

A) Poly A^+ RNA Enrichment

Extracted RNA was purified for Poly A^+ RNA only, using the protocol and reagents provided in the Atlas™ Pure Total RNA Labelling System kit (Clontech, USA). The streptavidin-conjugated magnetic beads were resuspended and 15 μL per probe synthesis (usually two probes were synthesised per experiment) was aliquotted into a 0.6 mL microfuge tube. The beads were separated using a Vario MACS

magnetic particle separator (Miltenyi Biotech, Germany) and the supernatant removed. The beads were washed in 150 µL 1X binding buffer and separated on the magnetic particle separator. This was repeated three times and the beads were finally resuspended in 15 µL 1X binding buffer per probe, and kept on ice until needed.

Up to 50 µg of RNA was transferred into a 0.6 mL microfuge tube for each sample (Control and Ceramide; 2, 8, or 24 hours post-treatment). RNase-free water was added to the sample to bring the volume to 45 µL. 1 µL biotinylated oligo(dT) was added and mixed with the RNA and the tubes were immediately incubated for 2 minutes at 70°C on a preheated thermal cycler, and allowed to cool at room temperature for 10 minutes. 45 µL 2X binding buffer was then added and mixed by pipetting. The beads were resuspended and 15 µL was added to each Poly A^+ RNA sample. The Poly A^+ RNA/beads solution was continually mixed for 30 minutes at 1500 rpm on a MSI Minishaker vortexer (IKA Works, Malaysia) at room temperature. The beads were separated, supernatant was removed, and the beads were resuspended in 50 µL 1X wash buffer. The beads were separated and resuspended a second time in 1X wash buffer. The beads were separated, supernatant removed, and the beads were resuspended in 50 µL 1X reaction buffer. The beads were again separated, supernatant was removed, and the beads were resuspended in 3 µL RNase-free water and kept on ice until needed.

B) cDNA Probe Synthesis

Necessary precautions and routines were followed when handling and disposing radioactive substances, as outlined in the National Health and Medical Research Council's regulations (1985, 1995a, 1995b). A master mix (Table 2.7.) for synthesis of the radiolabelled cDNA probes was prepared at room temperature (to make 2 probes + 1 extra).

Table 2.7.: cDNA probe synthesis master mix.

Reagent	Volume
5X Reaction buffer	12 µL
10X dNTP mix (for dATP label)	6 µL
[α-^{32}P]dATP, dye-free (3000 Ci/mmol, 10 µCi/µL)	15 µL
100 mM DTT	1.5 µL
	Total = 34.5 µL (11.5 µL/reaction)

The Poly A^+ RNA/beads solution was resuspended. 4 μL CDS primer mix (specific for the human cancer 1.2 array) was added to the beads, mixed well by pipetting, and incubated for 2 minutes at 65°C in a preheated thermal cycler. The temperature was reduced to 50°C and the tubes incubated for a further 2 minutes. During this incubation, 6 μL PowerScript™ Reverse Transcriptase (Clontech, USA) (2 μL per reaction) was added to the master mix at room temperature and was gently mixed by pipetting. After the completion of the 50°C incubation, 13.5 μL master mix was added to each reaction tube and the contents mixed thoroughly. The reaction tubes were immediately returned to 50°C and incubated for 25 minutes, followed by the addition of 2 μL 10X termination mix.

C) cDNA Probe Purification

All procedures performed and reagents used from this point onwards were from the Atlas™ Human Cancer 1.2 cDNA Expression Array kit (Clontech, USA). Radiolabelled cDNA probes were purified by column chromatography. Briefly, the probe synthesis reactions (approximately 20 μL each) were diluted in 180 μL Buffer NT2 and loaded into a NucleoSpin extraction spin column sitting in a 2 mL collection tube. The columns were centrifuged for 1 minute at maximum speed in a bench-top microfuge and the flow-through discarded as liquid radioactive waste. The columns were transferred into new collection tubes and 400 μL Buffer NT3 was added. The columns were centrifuged for 1 minute at maximum speed and the flow-through discarded. This was repeated two more times. The columns were then transferred into labelled 1.5 mL microfuge tubes and 100 μL Buffer NE was added directly to the membrane and allowed to soak for 2 minutes. The tubes with columns in place were centrifuged for 2 minutes at maximum speed to elute the purified probes and the activity of the probes measured by scintillation counting on a Rackbeta liquid scintillation counter (LKB Wallac, Finland). The probes were stored at -20°C until needed.

D) Hybridisation of cDNA Probe to Atlas™ Array

The ExpressHyb hybridisation solution was prewarmed to 68°C to dissolve any precipitates. 100 μL 10 mg/mL (0.5 mg per probe synthesis) sheared salmon testes DNA was heated for 5 minutes at 100°C, and then immediately chilled on ice. A prehybridisation solution was prepared by adding 50 μL (0.5 mg) of the denatured salmon testes DNA to 5 mL of the prewarmed ExpressHyb solution. This was kept at 68°C until used.

Two Human Cancer 1.2 array blots (one for each control and ceramide sample) were placed into separate cleaned glass hybridisation bottles using forceps, ensuring that they were orientated with the correct side facing inwards and that no air pockets were present between the glass surface and the blots. This step was performed quickly to prevent drying of the array membranes. The 5 mL prehybridisation solution was added to the bottles containing the array blots and the arrays were prehybridised for 30 minutes at 68°C with continuous agitation in a Model 2000 Micro Hybridisation Incubator (Robbins Scientific, USA).

The cDNA probes were prepared for hybridisation by adding 5 µL C_0t-1 DNA and incubating for 2 minutes at 100°C, followed by an immediate incubation on ice for 2 minutes. Once the prehybridisation incubation was completed, each of the probe solutions were then pipetted directly into the prehybridisation solution in the bottles containing the array blots. Hybridisation bottles were labelled according to the probe (control or ceramide) they contained. Blots were hybridised with the probe overnight with continuous agitation at 68°C.

The next day, the hybridisation solution was discarded and blots were washed four times with approximately 200 mL prewarmed Wash Solution 1 (2X SSC; 1% SDS) for 30 minutes at 68°C with continual agitation. A 30 minute wash was then performed in prewarmed Wash Solution 2 (0.1X SSC; 0.5% SDS) at 68°C, and a final wash was performed in 2X SSC at room temperature. The array membranes were removed from the hybridisation bottles, excess solution was briefly drained without allowing the blots to dry, and the blots were wrapped in plastic wrap and mounted on a sheet of blotting paper in the correct orientation. The array blots were exposed to BioMax™ MR film (Eastman Kodak Company, USA) using an intensifying screen at -80°C for 6 hours, 24 hours and 3-10 days depending on the activity of the probe and the resulting signal intensity, and the film was developed.

E) Atlas™ Array Analysis

The developed films were scanned using a GS-800 calibrated densitometer (BIO-RAD Laboratories, USA) and analysed using the Quantity One® quantitation software package (BIO-RAD Laboratories, USA). Optical density values per mm^2 were obtained and were normalised between samples using the data obtained from expression analysis of the house-keeping genes included on the array. Fold changes between the samples were calculated for each gene analysed. Analysis was performed on the various exposures to enable a more complete and accurate collection of data for the genes being expressed.

F) Stripping of cDNA Probe From Atlas™ Array

The array blots were reused for subsequent or confirmatory experiments after exposure to the film by stripping the probe from the membrane. Briefly, the blots were placed in a boiling solution of 500 mL 0.5% SDS for 5-10 minutes. The solution was then removed from the heat source and allowed to cool at room temperature for 10 minutes. The blots were then rinsed in Wash Solution 1 (2X SSC; 1% SDS), wrapped in plastic wrap, and exposed to film at -80°C to check the efficiency of the stripping procedure. If no signal was detected, the blots were stored at -20°C until needed in a subsequent experiment.

2.2.10. Primer Design and Standard RT-PCR Testing

A) Primer Design

Oligonucleotides (primers) for use in reverse transcription-polymerase chain reaction (RT-PCR) applications were designed according to the guidelines discussed by Persing (1993), and only amplified intron-spanning regions of the specific target gene as a precaution to prevent amplification of potentially contaminating DNA. It was ensured that the chosen primer sequences were not self-complementary such that interprimer annealing could not occur. Furthermore, a Blast search (National Centre for Biotechnology Information, NIH) was performed on all primer sequences to ensure they were only complementary with the desired human target gene of interest. Primer sequences were designed using the GenBank nucleotide sequence database (National Centre for Biotechnology Information, NIH) with the accession numbers provided in the Atlas™ Human Cancer 1.2 cDNA Expression Array handbook for each specific gene of interest. All primer sequences were designed to have an optimum annealing temperature at 60°C, and amplified a ≈ 400-500 bp RT-PCR product. These conditions were chosen such that the efficiency of amplification of all transcripts was similar for each primer set. All primers used in this study were synthesised by GeneWorks (Adelaide, Australia) and were RPC purified. The primer sequences used for confirmation of the cDNA array results are given in Table 2.8.

Table 2.8.: Details of the primer sets designed for confirmation of cDNA array results.

Target Gene Name	GenBank Accessn No. / cDNA Array Posn.	Sequence (5' to 3')	RT-PCR Product Size
Preferentially expressed antigen of melanoma (PRAME)	U65011 / C08c	Sense: ACTCTTCATGGCAGCCTTTG Antisense: CGCTTCACTTTCTCAATGAGGT	437 bp
Proliferating cell/cyclic nuclear antigen (PCNA)	M15796 / C13e	Sense: CGGATACCTTGGCGCTAGTA Antisense: TGAGTGTCACCGTTGAAGAGA	396 bp
Macrophage inhibitory cytokine 1 (MIC1)	AF019770 / E09e	Sense: CTCAGAGTTGCACACCGAAG Antisense: AGATTCTGCCAGCAGTTGGT	391 bp
Growth arrest & DNA-damage-inducible protein 153 (GADD153)	S40706 / C05d	Sense: GCCTTTCTCTTCGGACACTG Antisense: TCACCATTCGGTCAATCAGA	473 bp
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	X01677 / G27	Sense: TTCATTGACCTCAACTACAT Antisense: GTGGCAGTGATGGCATGGAC	443 bp

B) One-Step RT-PCR and Product Detection

The primer sequences described in Chapter 2.2.10.(A). were tested by RT-PCR using the SuperScript™ One-Step RT-PCR kit (Invitrogen Life Technologies, USA). A standard protocol described in the SuperScript™ One-Step RT-PCR kit package insert was adopted. The reagents and reaction conditions used are listed in Tables 2.9(A). and 2.9(B). respectively.

Table 2.9(A).: SuperScript™ One-Step RT-PCR reagent set-up.

Reagent	Volume per Reaction	Final Concentration/Amount
2X Buffer (containing 400 mM each GACU & 2.4 mM $MgSO_4$)	12.5 µL	1X (200 mM each dNTP & 1.2 mM $MgCl_2$)
3 µM Sense primer	1.7 µL	0.2 µM
3 µM Antisense Primer	1.7 µL	0.2 µM
SuperScript™ II RT enzyme / Platinum® Taq mix	0.5 µL	Unknown (assuming 1U each)
100 µg/µL RNA Sample	2 µL	200 µg
RNase-free water	6.6 µL	(Final volume to 25 µL)
	Total = 25 µL	

Table 2.9(B).: SuperScript™ One-Step RT-PCR reaction conditions.

Temperature	Time	
50°C	20 minutes	
94°C	2 minutes	
94°C	30 seconds	40 cycles
60°C	30 seconds	
72°C	45 seconds	
72°C	5 minutes	
4°C	∞	

All components of the One-Step RT-PCR reaction were stored at -20°C. The SuperScript RT and Platinum Taq enzyme mix was stored at -20°C until needed and kept on ice while out of the -20°C freezer in order to maintain their fidelity. Additionally, the enzymes were only mixed very gently on all occasions. Reagents were combined in a 0.2 mL PCR tubes on ice, and the reaction performed using an Eppendorf thermal cycler (Eppendorf, Germany). Negative control samples (water instead of RNA) were included with each sample reaction. GAPDH was amplified simultaneously with each amplification run to monitor the validity of the reaction.

RT-PCR products were analysed by agarose gel electrophoresis as described in Chapter 2.2.7(B), using a 100 bp PCR molecular ruler to provide a reference size marker. The products were visualised on the UV transilluminator.

2.2.11. Real-Time RT-PCR Optimisation

The parameters optimised in the real-time RT-PCR include primer concentration, $Mn(OAc)_2$ concentration, template concentration, and the temperature for measuring SYBR Green I fluorescence. Primer, $Mn(OAc)_2$, and template concentrations were optimised by performing real-time RT-PCR using various concentration ranges. The optimal conditions were concluded as that which produced the most specific and intense product as determined by real-time RT-PCR analysis and by agarose gel electrophoresis. The optimal temperature for fluorescence detection was determined to be the temperature that was below the product melting point (Tm) and above primer-dimer product if it existed.

The optimal primer concentration was found to 0.3 μM for each of the primers tested. Optimal $Mn(OAc)_2$ concentrations varied between the primer sets tested and are provided in Table 2.10. A template concentration of 1 μg per reaction was found to be optimal for all primer sets tested. The optimal temperature for measurement of SYBR Green I fluorescence was found to be 78°C for the PCNA primer set, and 81°C for all other primer sets tested.

Table 2.10.: Optimised real-time RT-PCR $Mn(OAc)_2$ concentrations for each of the designed primer sets.

Primer Set	Volume per 10μL Reaction (50 mM Stock)	Final $Mn(OAc)_2$ Concentration
PRAME	0.7 μL	3.5 mM
PCNA	0.5 μL	2.5 mM
MIC1	0.5 μL	2.5 mM
GADD153	0.5 μL	2.5 mM
GAPDH	0.7 μL	3.5 mM

The final optimised real-time RT-PCR reaction set-up and conditions are provided in Table 2.11. and Tables 2.12(A).-2.12(E)., respectively.

Table 2.11.: Final optimised real-time RT-PCR reagent set-up.

Reagent	**Volume per Reaction**	**Final Concentration/Amount**
RNase-free water	Final volume to 10 µL	
50 mM $Mn(OAc)_2$	Refer to Table 5.3.	
3 µM Sense and Antisense Primers	1 µL	0.3 µM
2.7X LightCycler mix (containing Tth DNA polymerase, GACU mix & SYBR Green I)	3.75 µL	1X
500 ng/µL RNA Sample	2 µL	1 µg
	Total = 10 µL	

Table 2.12(A).: Program 1: Reverse Transcription - Final optimised real-time RT-PCR reaction conditions.

Program Parameter	**Value**
Cycles	1
Time	25 minutes
Temperature	61°C
Temperature transition rate	20°C/second
Analysis Mode	None
Acquisition Mode	None

Table 2.12(B).: Program 2: Denaturation - Final optimised real-time RT-PCR reaction conditions.

Program Parameter	**Value**
Cycles	1
Time	3 minutes
Temperature	95°C
Temperature transition rate	20°C/second
Analysis Mode	None
Acquisition Mode	None

Table 2.12(C).: Program 3: Amplification - Final optimised real-time RT-PCR reaction conditions.

Program Parameter	**Value**		
Cycles	50		
Analysis Mode	Quantification		
	Segment 1	**Segment 2**	**Segment 3**
Time	5 seconds	7 seconds	13 seconds
Temperature	95°C	60°C	72°C
Temperature transition rate	20°C/second	20°C/second	2°C/second
Acquisition Mode	None	None	Single (78°C for PCNA, 81°C for others)

Table 2.12(D).: Program 4: Melting Curve Analysis - Final optimised real-time RT-PCR reaction conditions.

Program Parameter	**Value**		
Cycles	1		
Analysis Mode	Melting Curve		
	Segment 1	**Segment 2**	**Segment 3**
Time	5 seconds	15 seconds	0 seconds
Temperature	95°C	65°C	95°C
Temperature transition rate	20°C/second	20°C/second	0.1°C/second
Acquisition Mode	None	None	Continuous

Table 2.12(E).: Program 5: Cooling - Final optimised real-time RT-PCR reaction conditions.

Program Parameter	**Value**
Cycles	1
Time	30 seconds
Temperature	40°C
Temperature transition rate	20°C/second
Analysis Mode	None
Acquisition Mode	None

All components of the real-time RT-PCR were stored at -20°C in aliquots and were thawed as required. The LightCycler mix was stored at -20°C until needed and kept on a cool block in the dark when out of the -20°C freezer in order to maintain fidelity of the enzyme and SYBR Green I fluorescence. Reagents were combined in glass capillaries, and the reaction performed using a LightCycler (Roche Diagnostics, Germany). A negative control sample (water instead of RNA) was included with the sample reactions. GAPDH was amplified simultaneously with each amplification run to monitor the validity of the reaction.

Real-time RT-PCR product size could be confirmed by collecting the product from the LightCycler capillary into a 1.5 mL microfuge tube by brief centrifugation and analysis by agarose gel electrophoresis as described in Chapter 2.2.7(B), using a 100 bp PCR molecular ruler to provide a reference size marker. The products were visualised on the UV transilluminator.

3.1. Introduction

The sphingomyelin (SM) pathway has emerged as an important regulator of cellular growth, differentiation and apoptosis in human leukaemia cells. Studies into ceramide action upon human haemopoietic cells have predominantly concentrated on three cell lines: HL60 promyelocytic, U-937 monocytic, and MOLT-4 T lymphocytic cells.

The first noted cellular effect of ceramide was its ability to induce monocytic differentiation of HL60 cells (Okazaki *et al.*, 1989, Okazaki *et al.*, 1990, Kim *et al.*, 1991), thus mimicking the action of TNFα and IFNγ on these cells. In fact it has been shown that ligation of the TNFα and IFNγ receptors causes significant SM hydrolysis, resulting in ceramide generation. Similarly, treatment of HL60 cells with vitamin D_3, an inducer of monocytic differentiation, caused a rapid time-dependent hydrolysis of SM (Okazaki *et al.*, 1989), which led these researchers to believe that the growth inhibitory effects of vitamin D_3 was mediated by the SM cycle. In contrast, agonists such as retinoic acid or DMSO, which induce granulocytic differentiation, or phorbol esters, which promote macrophage-like differentiation, failed to stimulate SM hydrolysis in HL60 cells (Kim *et al.*, 1991, Hannun, 1994). It was concluded from this work that ceramide generation results in a specific differentiation response.

A significant majority of the reports in the literature have focussed on the ability of ceramide to mediate apoptosis. The ability of ceramide to induce apoptosis was first reported in U-937 cells by Obeid *et al.*, 1993. Exposure to C_2-ceramide mimicked the effect seen following the treatment of this cell line with TNFα, leading to the postulation that ceramide may act as a "death messenger" in apoptosis induction. From these and subsequent findings by other researchers it was concluded, rather simplistically, that ceramide generation was an obligatory step in the apoptotic process, a conclusion that became well accepted and which dominated SM signalling research for many years. Doubts as to the role of ceramide in initiating Fas apoptosis originally arose following the finding that ceramide generation occurred downstream of caspase activation, and was indeed blocked by inhibition of CPP32 (Gamen *et al.*, 1996, Sillence & Allan, 1997). Tepper *et al.* (2000) subsequently revealed that ceramide generation occurred specifically during the execution phase of apoptosis, rather than being involved in the initiation events. Recent studies have shown that ceramide causes the release of cytochrome *c* from the mitochondria, with subsequent activation of the executor caspases, thus demonstrating that ceramide has a role in the

amplification, rather than the initiation of apoptosis (Ghafourifar *et al.*, 1999, Ito *et al.*, 1999, Cuvillier *et al.*, 2000, Hearps *et al.*, 2002).

Despite the intense interest in ceramide signalling during the past decade, very few studies have followed up on the original observation that ceramide signalling could induce growth arrest and haemopoietic differentiation.

Serum starvation of MOLT-4 cells caused significant G_0/G_1 cell cycle arrest that was associated with an accumulation of endogenous ceramide, via activation of a magnesium-dependent SMase. Furthermore, the addition of exogenous ceramide (C_6-ceramide) mimicked the dramatic G_0/G_1 cell cycle arrest comparable to the effects observed with serum withdrawal. This cell cycle arrest was subsequently associated with the activation (dephosphorylation) of Rb (Dbaibo *et al.*, 1995), and the addition of exogenous C_6-ceramide (at levels comparable to that achieved with serum withdrawal) or C_2-ceramide (Chao *et al.*, 1992, Pushkareva *et al.*, 1995) resulted in a concentration- and time-dependent dephosphorylation of Rb in MOLT-4 cells (Dbaibo *et al.*, 1995), thus forming the mechanistic link between ceramide production and cell cycle arrest. Interestingly, the addition of exogenous ceramide had little activity in causing growth suppression of cells that lack a functional Rb gene (Dbaibo *et al.*, 1995), whilst cells transfected with either SV40 T antigen or E1A adenoviral protein, which bind and sequester Rb, showed an inability to undergo ceramide-induced cell cycle arrest. Although interfering with Rb activity abrogated ceramide-induced cell cycle arrest, it did not prevent ceramide-induced apoptosis (Dbaibo *et al.*, 1995), which is evidence that ceramide-induced cell cycle arrest and apoptosis are separate mechanisms.

A study by Ragg *et al.* (1998) demonstrated that treatment of U-937 cells with a sub-apoptotic dose of C_2-ceramide induced terminal differentiation and cell cycle arrest with up to 80% of cells in G_0/G_1 phase by 24 hours. Ceramide-induced G_0/G_1 cell cycle arrest was also accompanied by Rb dephosphorylation and increased $p21^{Cip1/Waf1}$ expression. This was the first report of a ceramide-induced effector mechanism linking cell cycle inhibition, Rb activation, and stable expression of $p21^{Cip1/Waf1}$ with ceramide treatment of cells. This study also demonstrated that cell cycle regulatory mechanisms could be activated in a p53-independent manner, as U-937 cells lack a functional p53 gene. In addition to the above findings, C_2-ceramide-treatment of U-937 cells activated the JNK MAP kinase, leading to the postulation that activation of the JNK pathway and its subsequent pathways may control transcription of the $p21^{Cip1/Waf1}$ gene (Ragg *et al.*, 1998).

While extensive effort has been invested in investigating the relationship between ceramide signalling and apoptosis, the growth arrest and differentiation-inducing effects of ceramide are relatively understudied. With the proposed role of ceramide as an initiator of apoptosis now discredited, it is time to direct attention back towards gaining a greater understanding of the ability of ceramide to mediate growth arrest and differentiation, as these outcomes hold significant therapeutic potential. Studies to date have predominantly examined three cell lines, and have only assessed limited outcomes (e.g. cell cycle and Rb status) with no attempt to determine the extent or consistency of responses with other cell lines. A larger screen of cell lines, from different human haemopoietic cell lineages is needed to determine which type(s) of cells respond to ceramide, to characterise the response, and determine the consistency in terms of outcome and magnitude of ceramide-responsive cells.

This chapter will investigate and characterise the response of haemopoietic cell lines to ceramide signalling in terms of viability/growth rates, cell cycle status, Rb activation, $p21^{Cip1/Waf1}$ expression, and phenotype, and seek to determine whether common responses and mechanisms are utilised.

3.2. Methods

3.2.1. Experimental Plan

Preliminary dose-response experiments were conducted to determine the optimal concentration of C_2-ceramide, and the most suitable culture conditions for each cell line. Experiments were conducted using exponentially growing cells. The optimal concentration of C_2-ceramide was defined as being a dose that was high enough to induce maximal growth arrest while minimising the apoptosis observed at higher concentrations. As such, the concentrations of C_2-ceramide used in a majority of the experiments conducted in this study for each cell line were as follows: 5 μM: K562 and Jurkat; 7.5 μM: KG-1, KG-1a, and HL60; 20 μM: Raji. Where the concentration of C_2-ceramide used varies from that provided above, the appropriate concentration will be stated in the individual section protocols of this thesis.

It was critical that the culture conditions used during an experiment were such that cells were only minimally affected (or not at all) by the culture conditions alone, as serum-free conditions have been noted to induce ceramide generation, cell cycle arrest, Rb dephosphorylation, and apoptosis in some cell lines (Dean *et al.*, 1986, Kim *et al.*, 1991, Howard *et al.*, 1993, Shichiri *et al.*, 1993, Dbaibo *et al.*, 1995, Jayadev *et al.*, 1995). The optimal experimental treatment culture conditions determined for this study are stated in Chapter 2.2.3(D). Treatment conditions that vary from those outlined in Chapter 2.2.3(D). are stated in the individual protocols.

Cellular responses were investigated following the treatment of cells with C_2-ceramide. This included the analysis of ceramide-induced cell cycle arrest, growth arrest, and differentiation. Enumeration and viability studies were performed using trypan blue staining to demonstrate whether ceramide causes growth arrest in the various human haemopoietic cell lines. Hypotonic PI staining and flow cytometry was used to investigate the ability of ceramide to induce cell cycle arrest. The phosphorylation status of Rb following C_2-ceramide treatment was studied by Western blotting. RNase protection assays were used to investigate the expression of $p21^{Cip1/Waf1}$ following C_2-ceramide treatment, and changes in cell surface marker expression were analysed by immunophenotyping using flow cytometry.

3.2.2. Determination of Cell Viability

Cell lines were treated with vehicle or C_2-ceramide and were plated at 5×10^5 cells/mL in 24 well culture dishes and incubated at 37°C/5% CO_2. A 10 μL volume of cells were harvested at various timepoints and mixed with an equal volume of

0.25% (w/v) trypan blue by gentle pipetting. The cell suspension was pipetted into a haemocytometer and the number of viable cells enumerated. Cells were considered viable if they had not taken up the trypan blue stain.

3.2.3. Cell Cycle Analysis

All cell lines were treated with C_2-ceramide and were plated at 5 X 10^5 cells/mL (1 mL/well) in 24 well culture dishes and incubated at 37°C/5% CO_2. Time-matched controls were treated with an equivalent volume of the ethanol vehicle. Cells (1 X 10^6 cells/sample) were harvested by centrifugation in 5 mL FACS tubes at predefined timepoints. The supernatant was removed and the cells were resuspended in 300 µL hypotonic propidium iodide (HPI) solution, and left for at least 2 hours at room temperature or overnight at 4°C in the dark (Nicoletti *et al.*, 1991, Ragg *et al.*, 1998).

Raji cells were washed with 1X PBS, fixed with 1.5 mL 70% ethanol/1X PBS for 30 minutes at 4°C, and treated with RNase prior to staining with HPI solution. The removal of RNA from these cells produced a more satisfactory coefficient of variation when analysed.

DNA content data were acquired using a FACScan flow cytometer (Becton-Dickinson, USA) and subsequent cell cycle analysis was performed using ModFit software (Becton-Dickinson, USA) to model the linear red fluorescence signal area (FL2-Area). The proportion of cells in each phase of the cell cycle was noted for each sample analysed and the percentage change calculated between C_2-ceramide-treated and vehicle-treated, time-matched control samples. Apoptosis was identified as a sub-G_0/G_1 peak. Aggregates were excluded from the analysis by use of a doublet discrimination module and subsequent gating on the linear red fluorescence area and width parameters.

3.2.4. Preparation of Total Cellular Protein Extracts

The Rb phosphorylation status was investigated by Western blotting. Cellular protein extracts were prepared from 2 X 10^6 harvested cells by lysis of the pelleted cells in 50-100 µL freshly prepared lysis buffer (described in Chapter 2.2.1.) and cleared by centrifugation at 18000 x g for 15 minutes at 4°C. Lysates were prepared for gel electrophoresis by boiling for 7 minutes in an equal volume of 2X Laemelli sample buffer (also described in Chapter 2.2.1.) followed by incubation on ice.

3.2.5. Western Blot Analysis of Rb Phosphorylation Status

A 6.5% separating gel and a 4% stacking gel were prepared as described in Chapter 2.2.5. Following polymerisation of the stacking gel, the comb was removed and the wells were thoroughly flushed out with Milli-Q water to remove any unpolymerised gel. The gel cassette was placed into position in an XCell II Mini-Cell (Novex, USA) electrophoresis apparatus and the upper and lower compartments filled with 1X running buffer. The wells of the gel were flushed with 1X running buffer.

Prepared cellular protein samples (30 μL), and a prestained, broad-range SDS-PAGE standard (BIO-RAD Laboratories, USA) sample were loaded onto the gel and the proteins run through the stacking gel by electrophoresis at 80V constant until the dye front had progressed into the separating gel. The voltage was then increased to 120V constant until the dye front had reached the bottom of the separating gel. Proteins were transferred to an Immun-Blot® PVDF membrane (BIO-RAD Laboratories, USA) in a Novex electrotransfer blot module for 2 hours at 25V in 1X transfer buffer.

Following protein transfer, the membrane was blocked for 1 hour at room temperature, or overnight at 4°C, in 5% skim milk powder in PBST, washed three times in PBST, and incubated by rocking overnight in 0.5 μg/mL anti-Rb goat antibody in PBST at 4°C. The blot was washed three times in PBST and incubated for 2 hours in 0.2 μg/mL anti-goat HRP-conjugated antibody in PBST at room temperature, followed by another three washes in PBST. Detection of Rb was performed using Enhanced Chemiluminescence and Hyperfilm (Amersham, England).

3.2.6. RNase Protection Assays - $p21^{Cip1/Waf1}$ Expression Analysis

All cell lines were treated with vehicle or C_2-ceramide at 5 X 10^5 cells/mL in serum-free RPMI media, except HL60 cells which were treated in RPMI + 2% FCS, and Raji cells which were treated in X-Vivo media (Biowhittaker, USA) + 2% FCS. The treated cells were transferred to 75 cm^2 cell culture flasks and incubated at 37°C/5% CO_2. Cells (1 X 10^7 cells/sample) were harvested at 0, 4 and 8 hours post-treatment, and RNA extracted, DNase-treated and quantitated as outlined in Chapter 2.2.7.

RNase protection assays were performed using the extracted RNA, and analysed exactly as described in Chapter 2.2.8. using the human stress-1 (hStress-1) template (Cat. # 45351P, PharMingen, USA). The hStress-1 template contains,

among others, riboprobes directed towards human *$p21^{Cip1/Waf1}$* RNA transcripts, and the house-keeping gene transcripts *L32* and *GAPDH*.

3.2.7. Immunophenotyping

Myeloid cell lines (K562, KG-1, KG-1a and HL60) were treated with vehicle or C_2-ceramide, plated at 5 X 10^5 cells/mL in 24 well culture dishes and incubated at 37°C/5% CO_2. Harvested cells (5 x 10^5 cells/sample) were washed twice, resuspended in 100 µL PBSA + 1% FCS and incubated with 10 µL PE-conjugated anti-human CD11b and CD14 antibodies for 15 minutes at room temperature in the dark. K562 cells were also incubated with anti-glycophorin A, and KG-1 and KG-1a with anti-CD34 antibodies, as these represent specific lineage maturation markers for these cell lines. In addition, all cells were simultaneously incubated with 10 µL of a 25 µg/mL isotonic 7-amino-actinomycin D (7-AAD) solution thus providing a means for dead cell exclusion from the final analysis (Gill *et al.*, 1975, Waggoner, 1990). Stained cells were washed twice with PBSA + 1% FCS prior to fixation in 300 µL FACS Fixative. Cells were immediately analysed by flow cytometry (described in Chapter 2.2.4.) using the CellQuest software package (Becton-Dickinson, USA). Immunophenotype profiles were generated and the percentage change in Mean Fluorescence Intensity (MFI) for ceramide-treated cells relative to the time-matched vehicle-treated controls was calculated.

3.3. Results

3.3.1. Effect of Ceramide Treatment on Growth Rate and Viability

Figure 3.1. shows histograms demonstrating the dramatic effect of C_2-ceramide treatment on cell viability. Cell viability was measured using trypan blue staining and the subsequent enumeration of cells at various timepoints. The change in viable cell numbers was determined by calculating the number of remaining viable cells at each timepoint compared with the number of viable cells at the 0 hour timepoint. Compared with the vehicle-treated, time-matched control cells, a considerable reduction in the number of viable cells was noted following ceramide treatment for all cell lines investigated. Similarly, the percentage of viable cells (i.e. the number of viable cells relative to the total cell count) in the ceramide-treated cell populations was shown to decrease over time.

Treatment of K562 cells with C_2-ceramide caused a slight reduction in the number of viable cells during the first 24 hours (Figure 3.1.) which contrasted the observed continued growth of the time-matched control culture at this time point. The rate of loss of viable ceramide-treated cells accelerated after 24 hours of exposure such that only 15% of the original number of viable cells were present after 72 hours. In comparison, the time-matched, vehicle-treated control cells had proliferated to some 300% of the original inoculum, despite serum-free conditions. The changes in the percentage of viable cells (Figure 3.2.) in the ceramide-treated culture reflects the growth rate pattern observed for K562 cells shown in Figure 3.1. During the first 24 hours the percentage of viable cells decreased to approximately 60% and continued to decrease at a similar rate throughout the remainder of the experiment, such that a large majority of cells were dead by 72 hours (13% viability). In contrast, the percentage viability in the time-matched control culture was consistently >90% throughout the timecourse.

Ceramide-treated HL60 cells showed a steady decrease in cell numbers during the first 48 hours, which countered the increase in cell numbers observed in the time-matched control culture during this period (Figure 3.1.). Viable HL60 cell numbers had more than halved by 48 hours following ceramide treatment, compared with a 150% increase in numbers observed in the control cell culture at the same timepoint. Between 48-72 hours the number of viable cells had stabilised, while there was a significant decrease in viable cell numbers during the 48-72 hour period in the control cell culture, bringing the number of viable cells equal to that in original inoculum. Figure 3.2. reveals that at 24 hours, a majority of the ceramide-treated

Figure 3.1.: Human haemopoietic cell viability curves demonstrating the number of viable cells per mL of culture, at various timepoints, using trypan blue staining following treatment with either vehicle or C_2-ceramide. A single representative set of results from at least four repeated experiments is shown.

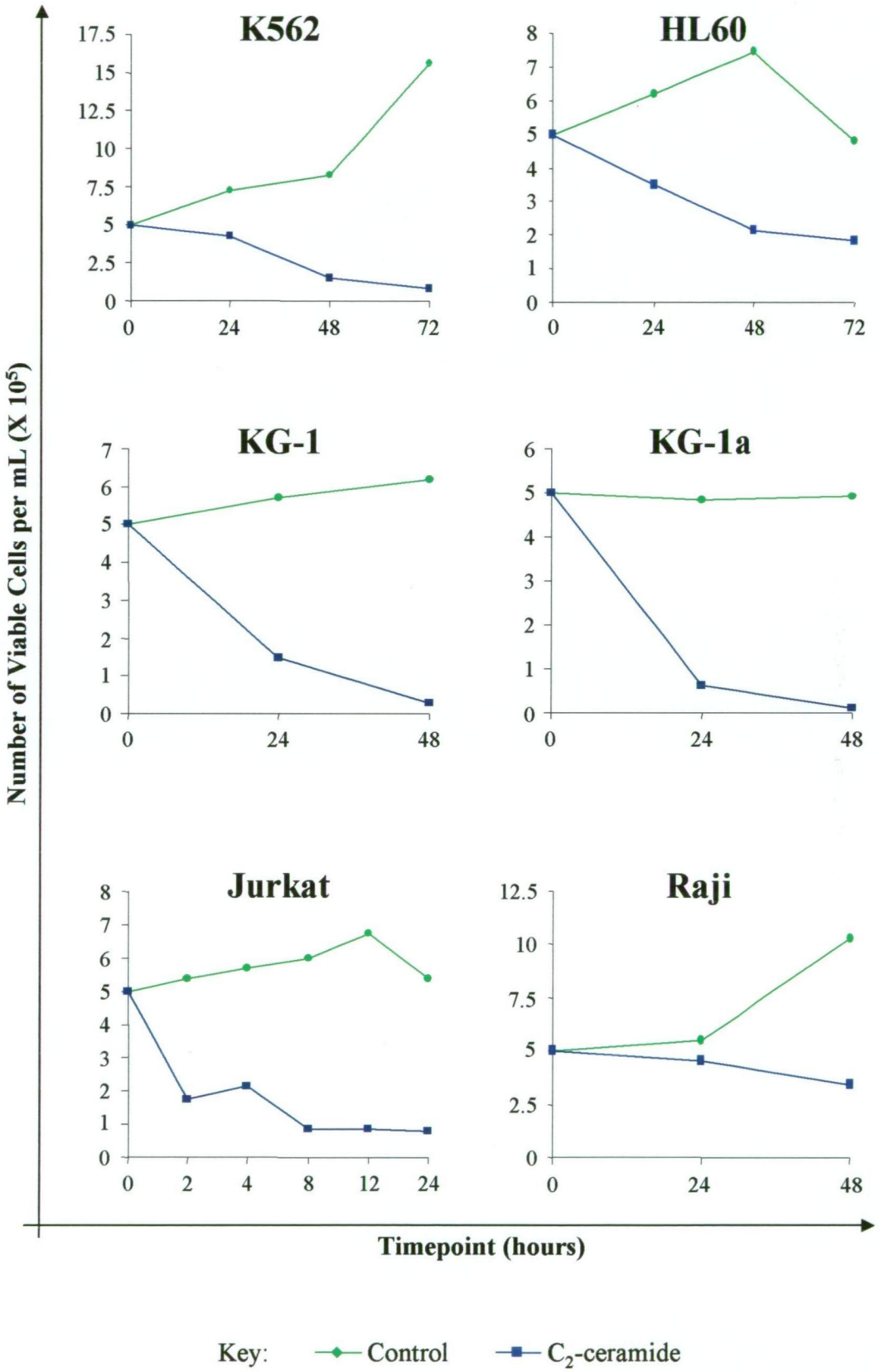
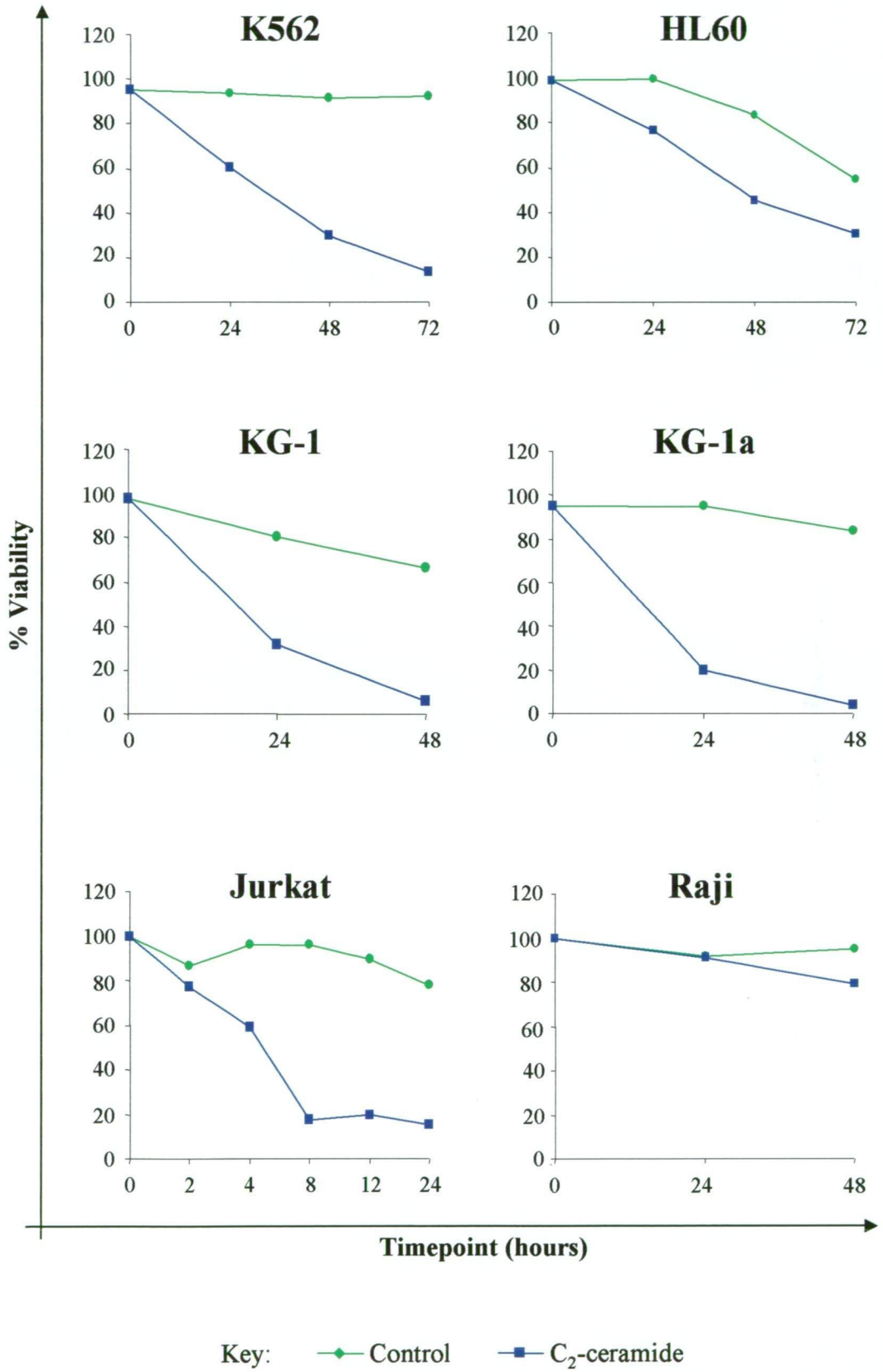

K562
HL60
KG-1
KG-1a
Jurkat
Raji
Number of Viable Cells per mL (X 10^5)
Timepoint (hours)
Key:
Control
C2-ceramide

Figure 3.2.: Human haemopoietic cell viability curves demonstrating the percentage of viable cells at various timepoints, using trypan blue staining following treatment with either vehicle or C_2-ceramide. A single representative set of results from at least four repeated experiments is shown.


K562
HL60
KG-1
KG-1a
Jurkat
Raji
% Viability
Timepoint (hours)
Key:
Control
C2-ceramide

HL60 cells were viable (76% viability), while approximately half of the observed cells were viable by 48 hours, decreasing further to 30% viability by 72 hours. Following the initial 24 hours of culture at which point 99% of cells remained viable, the control culture showed a decreasing percentage viability, although the percentage of viable cells in the control culture was consistently greater than that observed in the ceramide-treated cell population at the remaining matched timepoints.

The KG-1 and KG-1a cell cultures exhibited very similar growth rate patterns over a 48 hour time period (Figure 3.1.). The reduction in viable cell numbers was very dramatic for both cell lines following ceramide treatment. By 24 hours post-treatment, only 30% of the original count of KG-1 cells, and 13% of KG-1a cells remained viable. The number of viable cells continued to decrease during the proceeding 24 hours, and by 48 hours the number of viable cells was almost negligible. The KG-1 control cell culture exhibited a slight increase in proliferation over the 48 hour period, whilst the number of viable cells in the KG-1a control cell culture remained effectively unchanged throughout the experiment. Figure 3.2. shows that the observed decrease in growth rates for both KG-1 and KG-1a cell lines following ceramide treatment was due to increased cell death as these percentage viability curves closely resemble the growth rate curves shown in Figure 3.1. Following exposure to ceramide for 24 hours, the percentage of viable cells had rapidly decreased to 31% and 20% for KG-1 and KG-1a cells respectively. By 48 hours, the percentage viability had further reduced to 6% and 3% for KG-1 and KG-1a cells respectively. The control KG-1 culture showed a slight decrease in percentage viability, with 66% of cells remaining viable by 48 hours. There was negligible change in percent viability in the KG-1a control culture from the 0 hour to 24 hour timepoint (94% viability at both), and only a slight decrease by 48 hours to approximately 84% viability.

Jurkat T lymphocyte cells displayed the most dramatic loss in viable cell numbers (Figure 3.1.), with the rate of cell loss following ceramide treatment far exceeding that observed by the other cell lines investigated. Because of this sudden reduction in viable cell numbers, data could not be obtained for this cell line beyond 24 hours of continuous culture following ceramide treatment. As early as 2 hours post-treatment, the number of viable cells decreased from 5 X 10^5/mL to 1.8 X 10^5/mL. By 8 hours the number of viable cells had decreased further, such that only 18% of the original viable cell number remained in culture, which was sustained up to the 24 hour timepoint. In comparison, the time-matched control cells showed a gradual increase in cell numbers due to proliferation in the first 12 hours, with the

number of viable cells slightly decreasing at 24 hours. The changes in the percentage of viable cells (Figure 3.2.) reflects the observed growth rate pattern, whereby there was a dramatic decrease in the percentage of viable cells during the first 8 hours, such that only 17% of cells remained viable by this timepoint, while the percentage of viable cells in the control population remained >80% throughout the experiment.

Raji cells exhibited a relatively low susceptibility to loss in viability following ceramide treatment over a 48 hour period compared with the other cell lines studied. As seen in Figure 3.1., the number of viable ceramide-treated cells remained relatively constant up to the 24 hour timepoint, similar to that observed in the control culture. At 48 hours, however, a slight decrease in viable cell numbers had occurred. In contrast, the control Raji cell population exhibited a 50% increase in cell numbers during the 24-48 hour period, such that the number of viable cells had more than doubled by 48 hours. The change in the percentage of viable cells (Figure 3.2.) in the ceramide-treated culture paralleled that observed in the control culture by the 24 hour timepoint (approximately 90% viability for both). At 48 hours the percentage viability observed for the ceramide-treated cell population had decreased to 79%, while the control population had increased marginally to 95% viability.

3.3.2. Cell Cycle Analysis

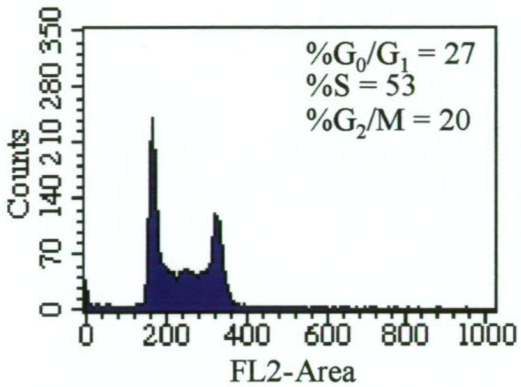
To determine whether C_2-ceramide treatment consistently resulted in withdrawal from the cell cycle, changes in the proportion of cells in each phase of the cell cycle was investigated. Asynchronous cultures of logarithmically growing cells were treated and harvested at various timepoints, stained with a hypotonic solution of propidium iodide, and subsequent flow cytometric analysis performed. DNA content histograms from representative experiments at 24 hours post-treatment are provided in Figures 3.3.A. and 3.3.B. Changes in cell cycle kinetics were evident by 24 hours post-treatment with ceramide. The histograms are accompanied by quantitative data, which identify the proportion of cells in each phase of the cell cycle after gating for only the cycling population of cells.

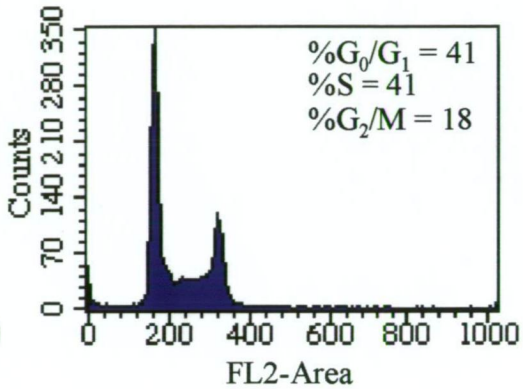
Following ceramide treatment, a 34% increase in the proportion of K562 cells in G_0/G_1 phase of the cell cycle was demonstrated at 24 hours (27% and 41% in the control and ceramide-treated cultures respectively) when compared with time-matched, vehicle-treated control cells (Figure 3.3.A.). Accompanying this increase in G_0/G_1 was a 20% decrease in the proportion of cells in S phase (from 53% in the controls to 41% in the ceramide-treated culture). This is indicative of an inability to cross the G_1-S transition, a hallmark of G_0/G_1 cell cycle arrest. At 48 hours, a 60%

Figure 3.3.A.: DNA histograms demonstrating C_2-ceramide-induced cell cycle arrest in human haemopoietic cells. All histograms represent samples taken 24 hours after C_2-ceramide treatment. Single representative results from at least four repeated experiments are shown.

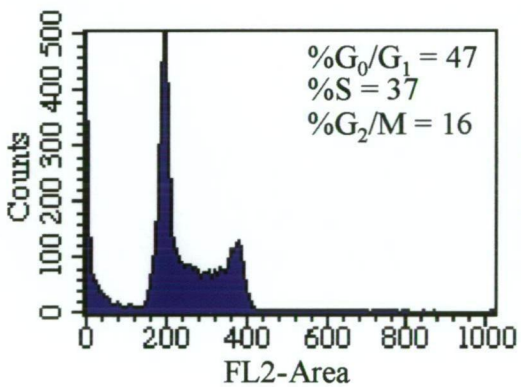
Control, 24 hours **C_2-ceramide, 24 hours**

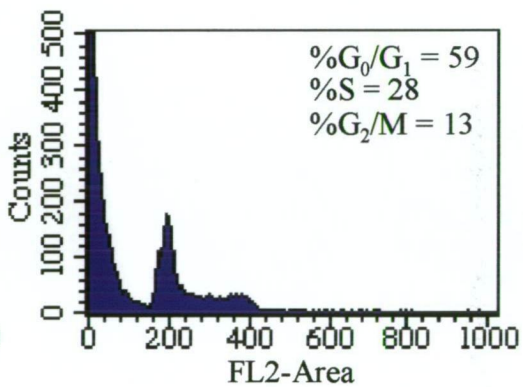
K562



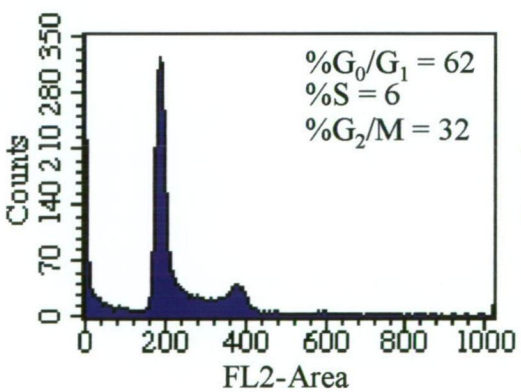


HL60





KG-1



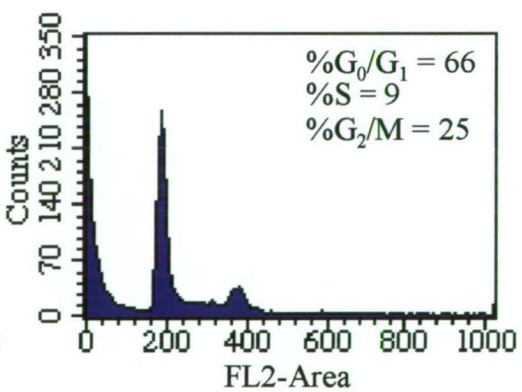
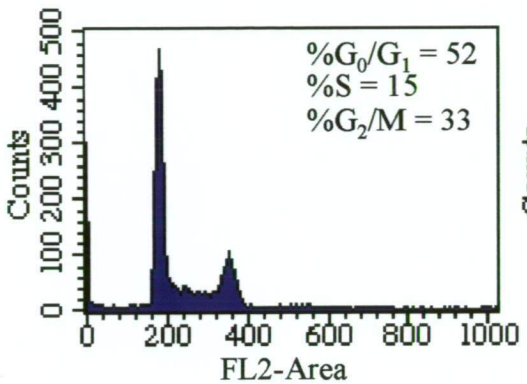
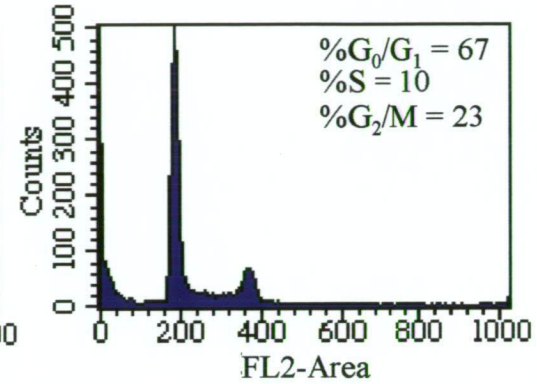


Figure 3.3.B.: DNA histograms demonstrating C_2-ceramide-induced cell cycle arrest in human haemopoietic cells. All histograms represent samples taken 24 hours after C_2-ceramide treatment. Single representative results from at least four repeated experiments are shown.

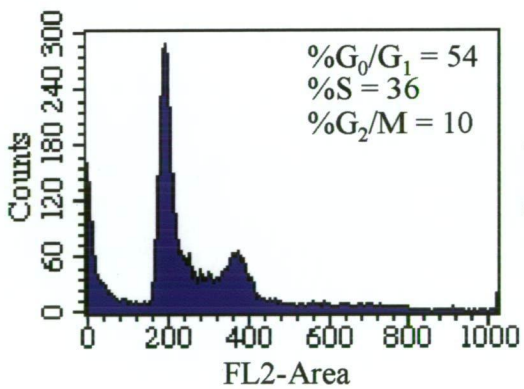
Control, 24 hours **C_2-ceramide, 24 hours**

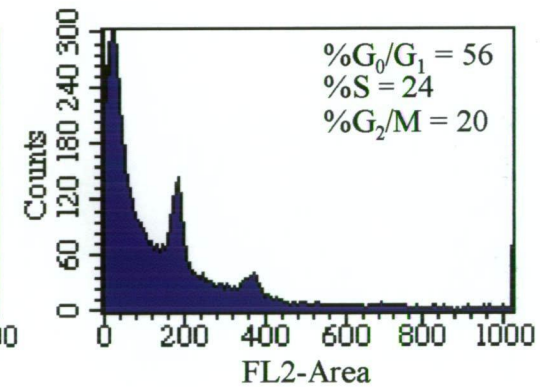
KG-1a



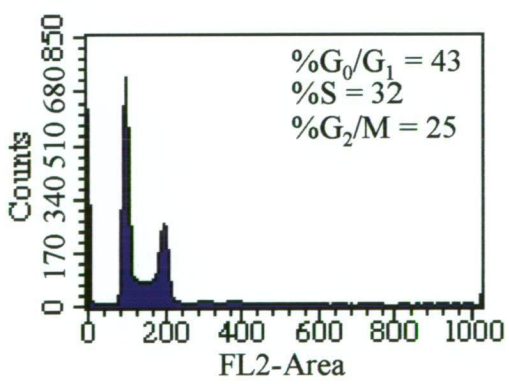


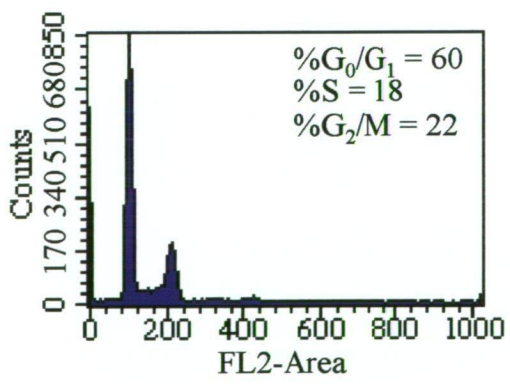
Jurkat





Raji





increase in G_0/G_1 phase cells was seen, which was accompanied by a 34% decrease in S phase. Similarly, a 20% increase in G_0/G_1 phase cells and a 24% decrease in the proportion of cells in S phase was demonstrated in HL60 cells by 24 hours. In contrast to K562 cells however, a sub-G_0/G_1 population was particularly evident by 24 hours in the ceramide-treated HL60 cell preparations.

An early increase in the proportion of KG-1 cells in the G_0/G_1 phase following ceramide treatment is not evident at 24 hours post-treatment. Although a 6-10% increase in G_0/G_1 was consistently demonstrated by 24 hours (Figure 3.3.A.), a 25% increase in the proportion of KG-1 cells in G_0/G_1, accompanied by a 35% decrease in S phase events, was observed at 48 hours (Figure 3.4.). A more significant G_0/G_1 increase was demonstrated in KG-1a cells by 24 hours, increasing from 52% in the control cell population to 67% in the ceramide-treated population (a 22% increase) (Figure 3.3.B.). A 33% decrease in S phase KG-1a cells was shown at 24 hours. By 48 hours, this became a 41% increase in G_0/G_1, and a 61% decrease in S phase (Figure 3.4.). Sub-G_0/G_1 peaks were evident in both KG-1 and KG-1a cells following ceramide treatment.

After 24 hours of ceramide treatment, a significantly large sub-G_0/G_1 population of Jurkat cells was consistently seen. This sub-G_0/G_1 peak, which accounted for an estimated 55%-60% of all events collected in the 24 hour ceramide-treated Jurkat cell population, masked the G_0/G_1 population thus making it difficult to accurately measure the true proportion of cells in the G_0/G_1 phase of the cell cycle.

Raji B cells showed a 28% increase in the proportion of cells in G_0/G_1 phase, and a 44% decrease in the proportion of cells in S phase by 24 hours post-treatment. In contrast to the Jurkat cells, few sub-G_0/G_1 events were noted.

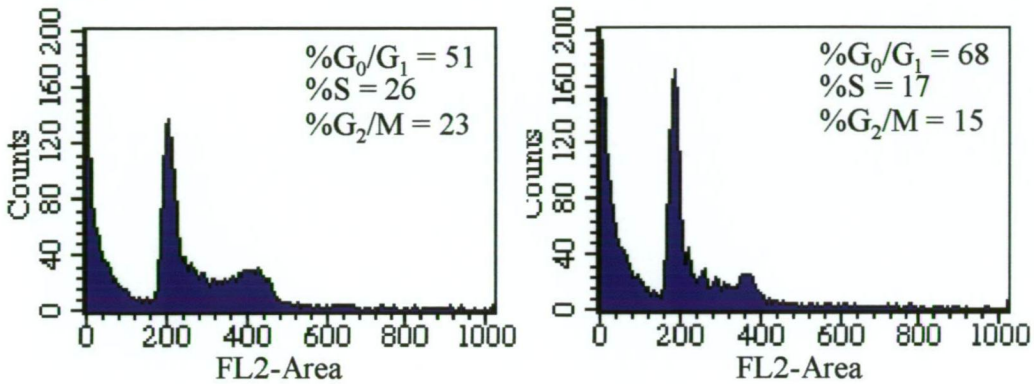
3.3.3. Analysis of Rb Phosphorylation Status

To determine the mechanism for ceramide-induced cell cycle arrest, the phosphorylation status of the Rb tumour suppressor protein was investigated by Western blotting, probing for both the hyper- and hypophosphorylated forms of Rb. Representative Western blot results for K562, HL60, KG-1, KG-1a, Jurkat and Raji cell lines are shown in Figure 3.5. SDS-PAGE and Western blotting takes advantage of the fact that the faster migrating hypophosphorylated (Rb^{hypo}) forms of Rb can be distinguished from the slower migrating hyperphosphorylated (Rb^{hyper}) forms. In all vehicle-treated sample preparations, a band corresponding to ≈105 kDa, representing inactive hyperphosphorylated Rb, was observed. The appearance of the lower M_r hypophosphorylated Rb form was indicative that Rb had become activated.

Figure 3.4.: DNA histograms demonstrating C_2-ceramide-induced cell cycle arrest in KG-1 and KG-1a human haemopoietic cells at 48 hours after C_2-ceramide treatment. Single representative results from at least four repeated experiments are shown.

Control, 48 hours **C_2-ceramide, 48 hours**

KG-1



KG-1a

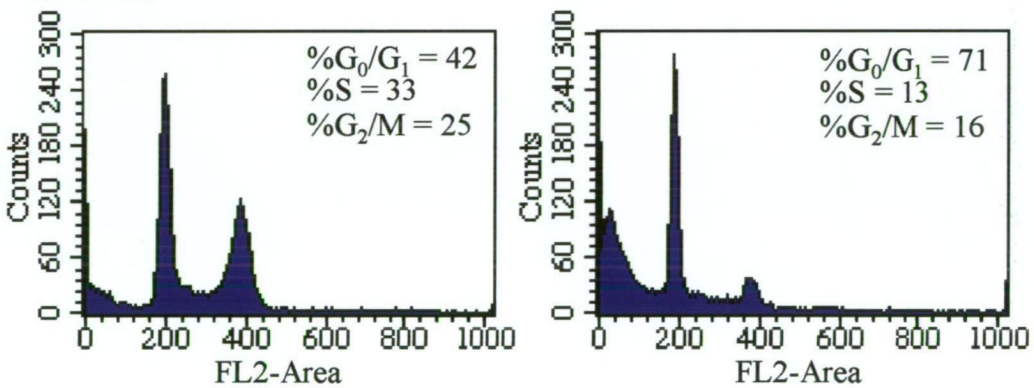
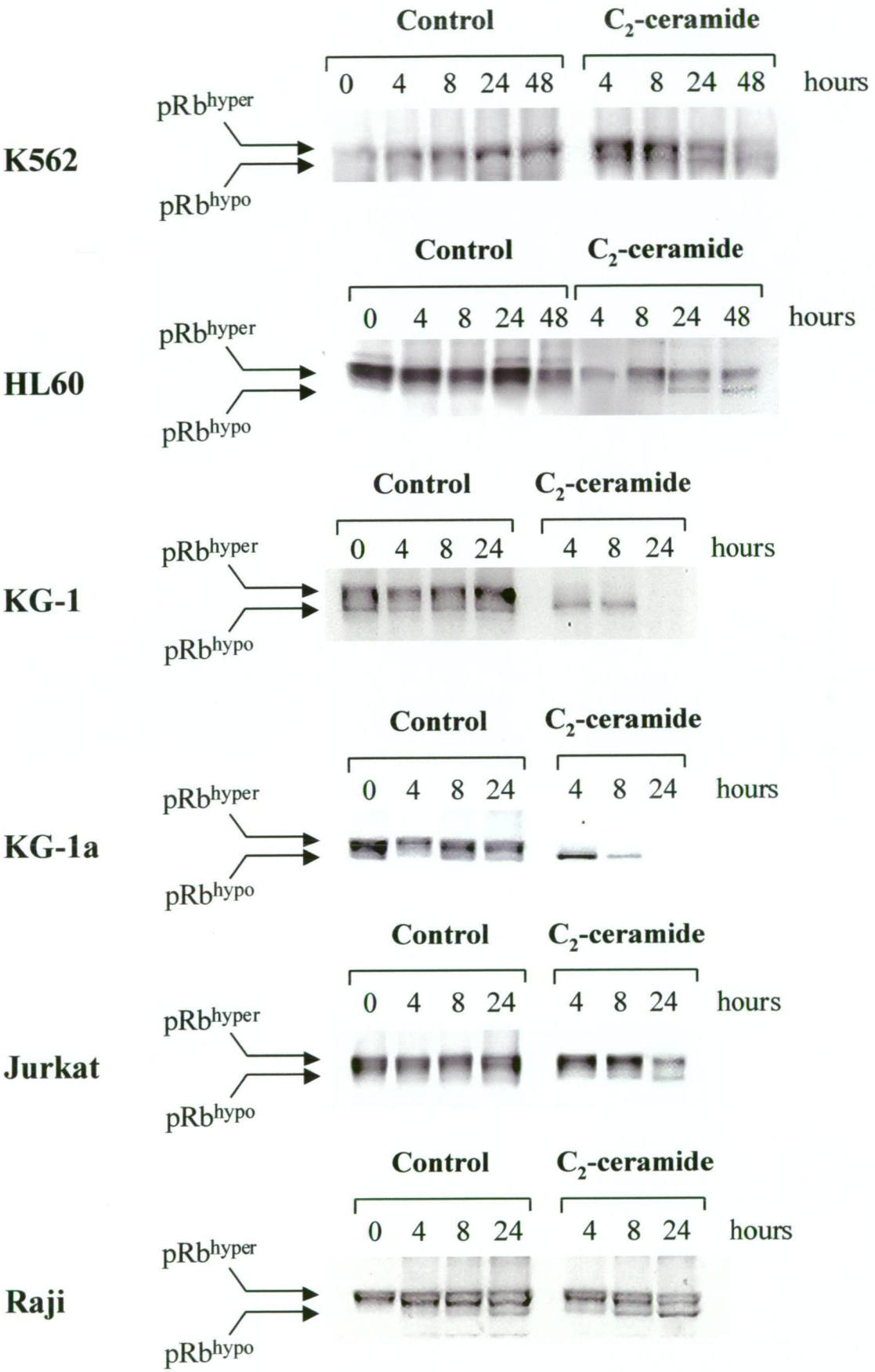


Figure 3.5.: Western blot analysis of the retinoblastoma (Rb) phosphorylation status in human haemopoietic cells following treatment with either vehicle or C_2-ceramide. A single representative set of results from at least four repeated experiments is shown.

pRb^{hyper} = hyperphosphorylated (inactive) Rb, pRb^{hypo} = hypophosphorylated (active) Rb.


Control
C2-ceramide
0 4 8 24 48
4 8 24 48
hours
pRbhyper
pRbhypo
K562
HL60
KG-1
KG-1a
Jurkat
Raji

Activation of Rb was observed as early as 4 hours post-treatment in K562 cells treated with ceramide. The Rb^{hyper} band remained prominent at this timepoint and throughout the experiment. By 24 hours, the intensity of the bands began to decrease presumably due to the reduced number of viable cells harvested. In contrast, Rb^{hypo} was not apparent in ceramide-treated HL60 cells until 24 hours post-treatment and remained visible at 48 hours.

The transformation of Rb^{hyper} into the Rb^{hypo} form by ceramide was very dramatic and an early event in both KG-1 and KG-1a cell lines. By 4 hours post-treatment, only the Rb^{hypo} form of the TS protein could be visualised, while the Rb^{hyper} band was clearly visible in the vehicle-treated control cell preparations at all timepoints during the experiment. No protein bands could be seen by 24 hours, again most likely due to the insufficient numbers of viable cells harvested.

Activation of Rb was not seen in the Jurkat T cell line until 24 hours post-treatment with ceramide, at which time the number of viable cells had diminished greatly, accounting for the lower level of protein seen compared with the earlier timepoints. Although a band corresponding to Rb^{hyper} was seen at all timepoints in the vehicle-treated control Jurkat cell extracts, the lower M_r Rb^{hypo} band was absent in the control extracts throughout the experiment.

Logarithmically growing, vehicle-treated Raji B lymphocytic cells exhibited a slightly different Rb pattern from protein extracts from other cell lines in that more than two phosphorylation isoforms of Rb were observed. Following C_2-ceramide exposure, an additional lower M_r band, representing fully hypophosphorylated Rb, became clearly apparent. This band was visible as early as 4 hours post-treatment with ceramide, and became more intense as the experiment progressed through to the 8 and 24 hour timepoints, increasing incrementally in intensity in proportion to the intensity of the two higher M_r Rb^{hyper} bands. A hypophosphorylated isoform of Rb was identifiable in time-matched, vehicle-treated cell extracts, however an additional lower M_r hypophosphorylated Rb band was observed as early as 4 hours after ceramide treatment. This third isoform was not observed until 24 hours of culture for the time-matched, vehicle-treated controls.

3.3.4. Expression of $p21^{Cip1/Waf1}$ Following Ceramide Treatment

The expression of the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ following treatment with vehicle or C_2-ceramide was investigated at 4 and 8 hours post-treatment in K562, HL60, KG-1, KG-1a, Jurkat and Raji cells by RNase protection assay (RPA) using the RiboQuant™ RPA System and hStress1 template

(PharMingen, USA). An additional 2 hour timepoint was analysed for the Jurkat cell line. Enlarged sections of the un-normalised RPA blots from analysis of each cell line, showing the expression of $p21^{Cip1/Waf1}$ in RNA extracts from ceramide-treated cells and time-matched, vehicle-treated controls is shown in Figures 3.6.A. and 3.6.B. The raw optical density data generated from image analysis were normalised to the expression of housekeeping genes included in the template set (Table 3.1.A.) and fold changes in expression between time-matched, vehicle-treated control cells and C_2-ceramide-treated cells for each of the genes subsequently calculated (Table 3.1.B.). Multiple exposures of varying times were used to overcome pixel saturation for normalisation purposes, and only one exposure time is shown in the results figures in this thesis for representative purposes.

The expression of $p21^{Cip1/Waf1}$ was demonstrated to increase significantly following ceramide treatment compared with the time-matched, vehicle-treated controls for each of the cell lines studied. This was apparent for a majority of the cell lines analysed by observing the raw RPA blots presented in Figures 3.6.A. and 3.6.B., particularly for the K562, HL60 and KG-1 cell lines (Figure 3.6.A.). The increase in expression was however more clearly evident after normalisation of the optical density data to the GAPDH RPA control and presenting the data in numerical form as shown in Table 3.1.A. An increase of approximately 3 fold was calculated by 4 hours post-treatment with ceramide in K562 cells compared with the time-matched controls, and increased further to greater than 4 fold by 8 hours post-treatment. HL60 cells demonstrated an increase in expression of greater than 4 fold by 4 hours post-treatment, compared with the time-matched controls, although the change in expression was relatively negligible by 8 hours post-treatment. Similarly, KG-1a cells demonstrated a significant increase (>6 fold) in $p21^{Cip1/Waf1}$ expression by 4 hours post-treatment, although this increase appeared to have stabilised somewhat by 8 hours post-treatment to less than a 2 fold increase in expression. This increase is not clearly apparent in Figure 3.6.B. for this cell line, due to slight overloading of RNA on the polyacrylamide gel, hence the importance of normalising data to the GAPDH control.

A trend of increasing $p21^{Cip1/Waf1}$ expression was observed in KG-1 cells over the timecourse following ceramide treatment, whereby an increase of approximately 3 fold was evident by 4 hours, and almost 4 fold by 8 hours post-treatment compared with time-matched controls. Less significant changes in $p21^{Cip1/Waf1}$ expression was demonstrated for both Jurkat and Raji cells, although an increase was clearly apparent at the timepoints examined. By 4 hours post-treatment with ceramide no

change was observable for the Raji cell line, and only a relatively slight increase was calculated for Jurkat cells (<1.5 fold increase). By 8 hours post-treatment, an increase of almost 2 fold, and 2.5 fold, was demonstrated in Jurkat and Raji cell RNA extracts, respectively.

Figure 3.6.A.: Enlarged sections of un-normalised RNase protection assay blots for K562, HL60 and KG-1 haemopoietic cell lines, highlighting changes in *p21*$^{Cip1/Waf1}$ RNA expression at 4 and 8 hours following treatment with either vehicle or C_2-ceramide, accompanied by the respective *GAPDH* and *L32* controls for each blot. RPAs were performed using the RiboQuant™ RNase protection assay with the hStress1 template (Pharmingen, USA). C_2-ceramide concentration used were as follows: 5 μM: K562; 7.5 μM: HL60 20 μM: KG-1.

With the exception of the results obtained for the HL60 cell line, all RPA results presented in this figure were obtained in our laboratory by Joanna Burrows and Anna Hearps, and are used with permission.

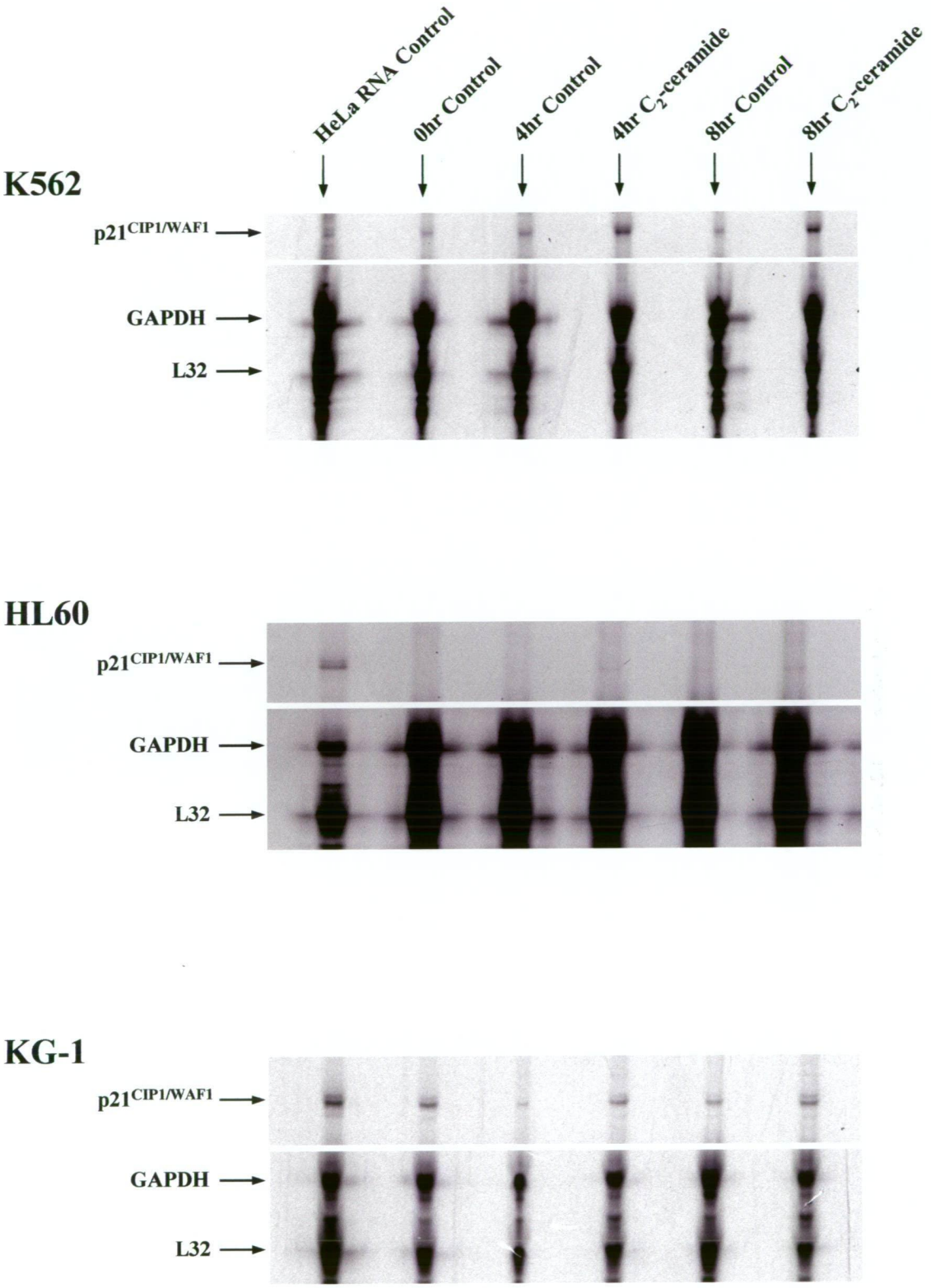

HeLa RNA Control
0hr Control
4hr Control
4hr C2-ceramide
8hr Control
8hr C2-ceramide
K562
p21CIP1/WAF1
GAPDH
L32
HL60
p21CIP1/WAF1
GAPDH
L32
KG-1
p21CIP1/WAF1
GAPDH
L32

Figure 3.6.B.: Enlarged sections of un-normalised RNase protection assay blots for KG-1a, Raji and Jurkat haemopoietic cell lines, highlighting changes in *p21*$^{Cip1/Waf1}$ RNA expression at 4 and 8 hours (2 hour timepoint included for Jurkat cell line) following treatment with either vehicle or C_2-ceramide, accompanied by the respective *GAPDH* and *L32* controls for each blot. RPAs were performed using the RiboQuant™ RNase protection assay with the hStress1 template (Pharmingen, USA). C_2-ceramide concentration used were as follows: 5 μM: Jurkat; 20 μM: KG-1a, and Raji.

All RPA results presented in this figure were obtained in our laboratory by Joanna Burrows and Anna Hearps, and are used with permission.

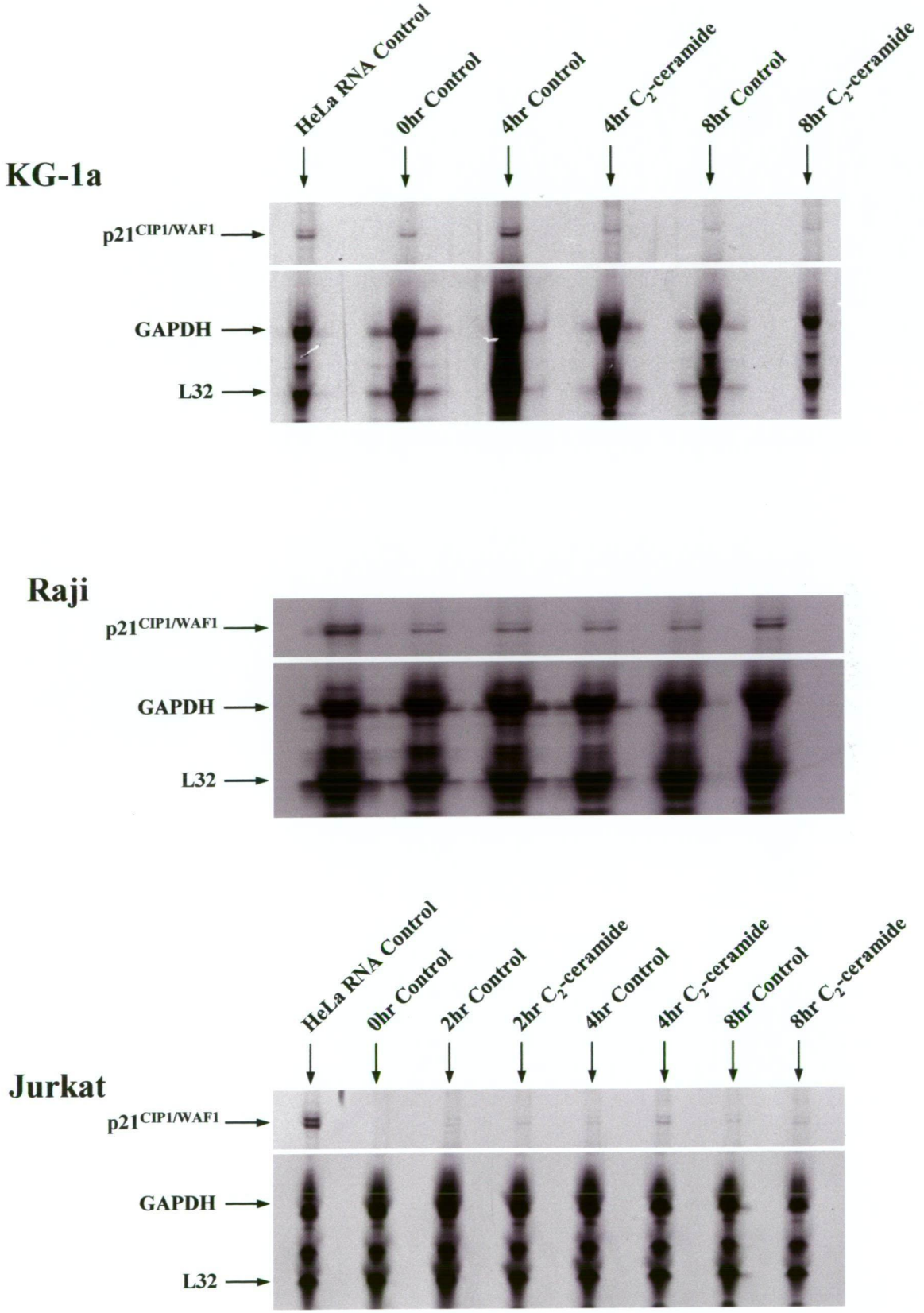

KG-1a
HeLa RNA Control
0hr Control
4hr Control
4hr C2-ceramide
8hr Control
8hr C2-ceramide
p21CIP1/WAF1
GAPDH
L32
Raji
p21CIP1/WAF1
GAPDH
L32
Jurkat
HeLa RNA Control
0hr Control
2hr Control
2hr C2-ceramide
4hr Control
4hr C2-ceramide
8hr Control
8hr C2-ceramide
p21CIP1/WAF1
GAPDH
L32

Table 3.1.A.: Table of $p21^{Cip1/Waf1}$ expression data obtained from image analysis of RPA blots for K562, HL60, KG-1, KG-1a, Raji and Jurkat cells (shown in Figures 3.6.A. and 3.6.B.) using the hStress1 RiboQuant template (PharMingen, USA) at 4 and 8 hours post-treatment with vehicle or C_2-ceramide. All optical density data presented in the table are normalized to the 0 hr Control GAPDH housekeeping gene to allow the determination of fold change in expression of $p21^{Cip1/Waf1}$ at each timepoint. The raw data given in the table has been adjusted to subtract background. With the exception of the results obtained for the HL60 cell line, all data were calculated from RPA experiments conducted in our laboratory by Joanna Burrows and Anna Hearps, and these results are used with permission. The data provided in this table is representative of data obtained from at least three repeated experiments.

	$p21^{Cip1/Waf1}$ Volume Normalised to 0 hour Control GAPDH (optical density/mm^2)				
Cell Line	**0 hr Control**	**4 hr Control**	**4 hr C_2-ceramide**	**8 hr Control**	**8 hr C_2-ceramide**
K562	31.109	27.589	83.262	20.986	89.321
HL60	12.082	24.754	120.546	108.025	84.000
KG-1	268.766	76.587	223.176	78.120	303.084
KG-1a	545.999	106.391	728.733	511.367	929.388
Jurkat	14.450	91.020	117.710	30.930	59.440
Raji	353.273	412.348	434.719	331.267	799.965

Table 3.1.B.: Fold change in $p21^{Cip1/Waf1}$ expression relative to time-matched controls.

Cell Line	4 hours	8 hours
K562	3.02	4.26
HL60	4.87	0.78
KG-1	2.91	3.88
KG-1a	6.85	1.82
Jurkat	1.29	1.92
Raji	1.05	2.42

3.3.5. Changes in Cell Surface Marker Expression in Myeloid Cell Lines

Myeloid cell lines that were responsive to ceramide-induced cell cycle alterations were investigated for changes in cell specific surface marker expression to determine if ceramide was inducing haemopoietic differentiation. Cells were stained with PE-conjugated antibodies, and analysed by flow cytometry in conjunction with the dead cell discriminant dye 7-AAD. Expression profiles representative of each cell line investigated, at 48 hours post-treatment for KG-1 and KG-1a cells, and at 72 hours for K562 and HL60 cells, are shown in Figure 3.7. Histograms of the percentage change in MFI of C_2-ceramide-treated cells relative to vehicle-treated time-matched control cells using results from a series of experiments are shown in Figures 3.8.A. and 3.8.B.

A small increase in CD11b and CD14 expression was observed in all myeloid cell lines studied over the course of the experiments following exposure to C_2-ceramide (Figures 3.8.A and 3.8.B.). Ceramide treatment of K562 cells induced a slight increase in CD11b and CD14 expression, as evidenced by the right shift in the fluorescence profile relative to the time-matched, vehicle-treated controls (Figure 3.7.). After treatment with ceramide, K562 cells exhibited an 11% increase (± SE) in CD11b expression at 24 hours, a 30% increase by 48 hours, and a 46% increase in expression by 72 hours relative to time-matched, vehicle-treated control cells. A 4% increase in CD14 was observed at 24 hours, which increased to a 12% increase by 48 hours and to 20% by 72 hours (Figure 3.8.A.). In contrast to the myeloid markers, expression of glycophorin A dramatically decreased following ceramide treatment (Figure 3.7.), such that a 104% decrease in MFI was readily observed as early 24 hours post-treatment, relative to the time-matched control cells. This became even more apparent at later timepoints, with a 187% decrease in MFI by 48 hours, and a 366% decrease in expression by 72 hours (Figure 3.8.A.).

HL60 cells, however, exhibited a more striking increase in CD11b and CD14 expression as revealed by the right shift of the fluorescence profiles in Figure 3.7. following ceramide treatment. A 34% and 46% increase in MFI for CD11b was observed by 48 and 72 hours respectively relative to the control cells, and an 11%, 55%, and 71% increase in CD14 at 24 hours, 48 hours and 72 hours respectively (Figure 3.8.A.). Interestingly, the CD11b expression profile for the HL60 cell line (Figure 3.7.) identifies a subpopulation of cells responsive to ceramide, which may account for the apparent increase in the proportion of CD11b positive events seen in this cell line.

Figure 3.7.: Immunophenotype expression profiles of human myeloid cells at 48 hours (KG-1 and KG-1a cells) and 72 hours (K562 and HL60 cells) post-treatment with C_2-ceramide demonstrating changes in maturation marker expression. A single representative set of results from at least four repeated experiments is shown.

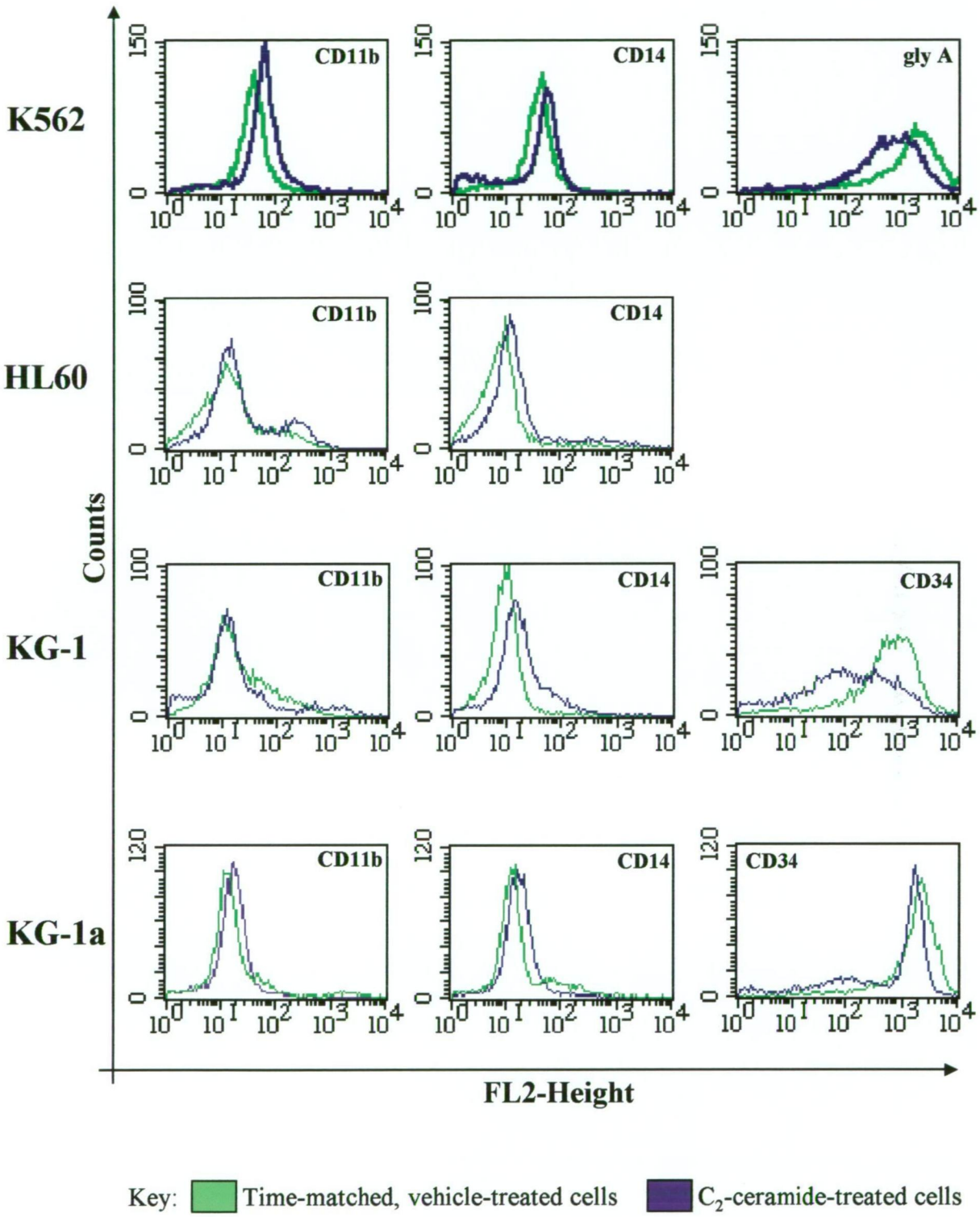
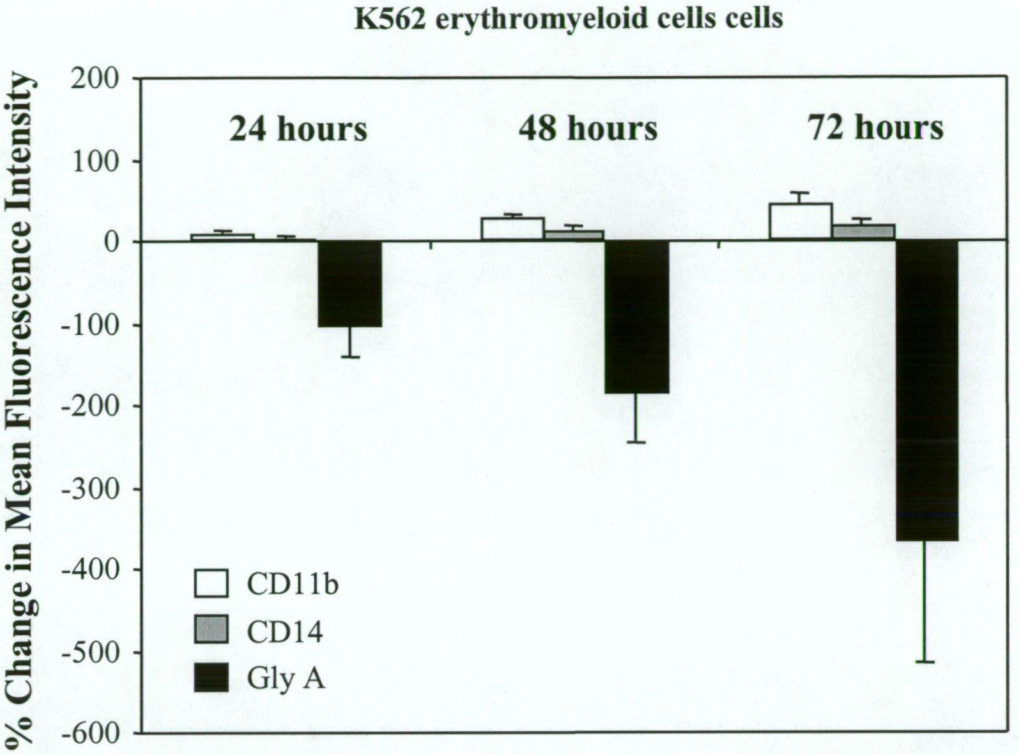

K562
HL60
KG-1
KG-1a
CD11b
CD14
gly A
CD34
Counts
FL2-Height
Key: Time-matched, vehicle-treated cells
C2-ceramide-treated cells

Figure 3.8.A.: Histograms demonstrating changes in maturation marker expression in human myeloid cells at various timepoints post-treatment with C_2-ceramide. Data are expressed as a percentage change in the mean fluorescence intensity, relative to vehicle-treated control cells, and are combined from at least four individual experiments.


K562 erythromyeloid cells cells
% Change in Mean Fluorescence Intensity
200
100
0
-100
-200
-300
-400
-500
-600
24 hours
48 hours
72 hours
CD11b
CD14
Gly A

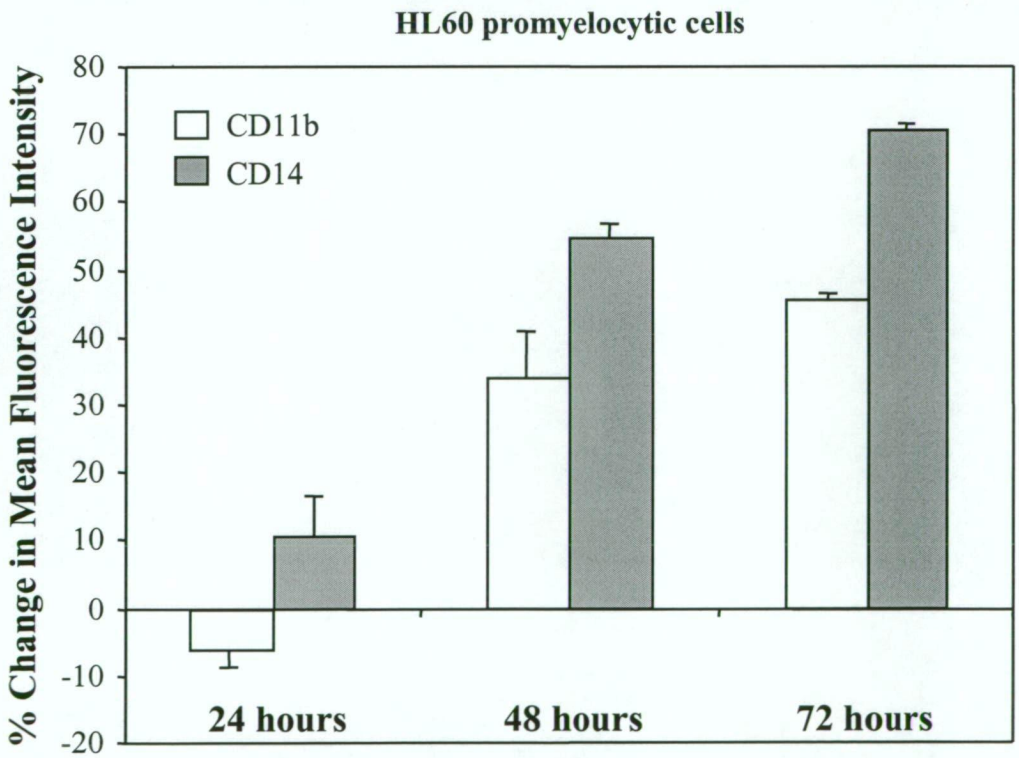
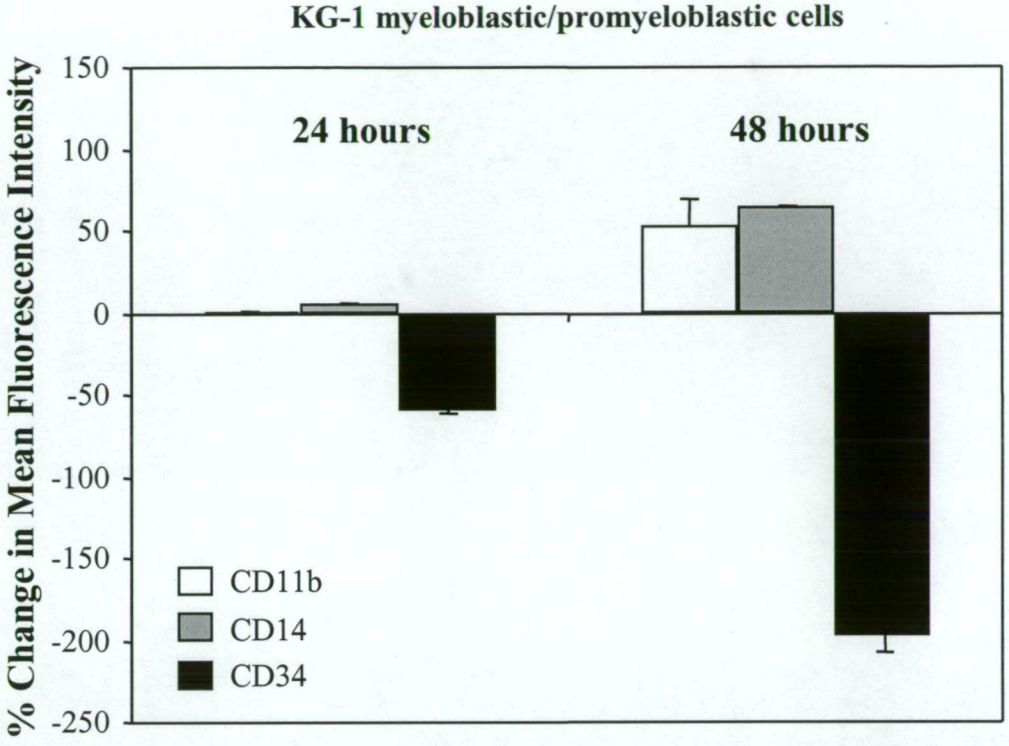

HL60 promyelocytic cells
% Change in Mean Fluorescence Intensity
80
70
60
50
40
30
20
10
0
-10
-20
CD11b
CD14
24 hours
48 hours
72 hours

Figure 3.8.B.: Histograms demonstrating changes in maturation marker expression in human myeloid cells at various timepoints post-treatment with C_2-ceramide. Data are expressed as a percentage change in the mean fluorescence intensity, relative to vehicle-treated control cells, and are combined from at least four individual experiments.


KG-1 myeloblastic/promyeloblastic cells
% Change in Mean Fluorescence Intensity
150
100
50
0
-50
-100
-150
-200
-250
24 hours
48 hours
CD11b
CD14
CD34

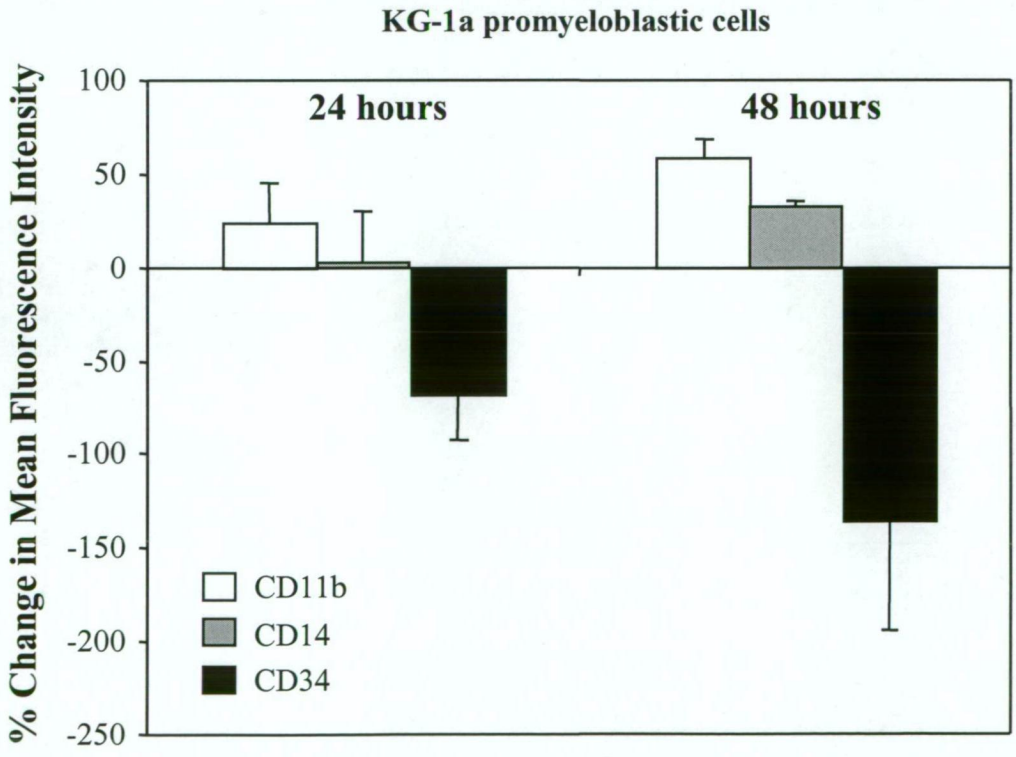

KG-1a promyeloblastic cells
% Change in Mean Fluorescence Intensity
100
50
0
-50
-100
-150
-200
-250
24 hours
48 hours
CD11b
CD14
CD34

Figure 3.8.B. shows that there was a negligible increase in CD11b expression in KG-1 cells at 24 hours post-treatment with ceramide, although this rapidly increased to a 54% increase in MFI by 48 hours, relative to the time-matched, vehicle-treated control cells. Similarly, CD14 expression increased from a 5% increase at 24 hours to a 64% increase by 48 hours (Figure 3.8.B.), relative to the controls. In contrast, a dramatic decrease in CD34 expression was observed in KG-1 cells, as evidenced by a significant left shift in the fluorescence profile for ceramide-treated cells in Figure 3.7. At 24 hours post-treatment, a 60% decrease in MFI for CD34 was observed for ceramide-treated cells, which increased to a 198% decrease in CD34 by 48 hours exposure to ceramide (Figure 3.8.B.).

Similarly, ceramide induced an increase in CD11b and CD14 in KG-1a cells, as evidenced by a right shift in the fluorescence profiles for these markers in Figure 3.7. Following 24 hours of ceramide treatment, a 46% increase in CD11b MFI was observed relative to the time-matched, vehicle-treated control cells. By 48 hours, this was even further amplified to a 60% increase (Figure 3.8.B.). CD14 expression increased during the first 24 hours with a 32% increase in MFI relative to the control cells, and remained stable at this expression level through to the 48 hour timepoint (34% increase) (Figure 3.8.B.). Ceramide-treated KG-1a cells also exhibited a dramatic decrease in CD34 expression shown by the significant left shift in the CD34 fluorescence profile in Figure 3.7. Relative to the time-matched control cells, a 69% decrease in MFI was observed at 24 hours, and a 136% decrease in CD34 MFI was observed at 48 hours, relative to the control cells (Figure 3.8.B.).

3.4. Discussion

Treatment of human leukaemia cell lines with ceramide results in the induction of cell cycle arrest, differentiation, and apoptosis (Okazaki *et al.*, 1989, Okazaki *et al.*, 1990, Kim *et al.*, 1991, Jayadev *et al.*, 1995, Bettaieb *et al.*, 1996, Ragg *et al.*, 1998). To date, research into these ceramide-induced responses has focussed predominantly on three leukaemia cell lines; HL60, U937 and MOLT-4, and a comprehensive study to investigate whether these responses are common to other cell types is lacking in the literature. Instead, a number of individual research papers have been published which investigate these responses rather simplistically and in isolation. The present study has extended these observations by performing a screen of myeloid and lymphoid cell lines, and the capacity for ceramide to induce cell cycle and growth arrest, differentiation and apoptosis was investigated for each cell line in an attempt to highlight whether common cellular responses were elicited. The phosphorylation status of Rb was investigated for those cell lines exhibiting ceramide-induced cell cycle arrest, as a possible effector mechanism for this event.

All cell lines studied were responsive to ceramide, although the responsiveness to ceramide treatment varied in magnitude. The findings presented demonstrate that ceramide commonly induces cell cycle arrest, differentiation, and some degree of apoptosis in a variety of human leukaemia cell lines.

Although one would suggest experiments to study the effect of ceramide on growth rates and viability would be fundamental to the study of other cellular responses to ceramide, comprehensive published data is limited. As such, six cell lines, which broadly represent the myeloid and lymphoid haemopoietic lineages, were treated with C_2-ceramide and the number of viable and dead cells enumerated as part of this study. Cell viability was judged by the ability to exclude trypan blue dye. In the experiments performed, the time-matched, vehicle-treated control cell culture showed continual proliferation or, in the case of KG-1a cells, were maintained at an equal number to that in the original inoculum.

Investigations into the ability of ceramide to cause growth arrest revealed consistent outcomes in all cell lines studied. Following treatment with ceramide, all cell lines underwent a decrease in viable cell numbers, although the degree and rate at which this decrease took place varied considerably between the different cell types studied, demonstrating the pleiotropic nature of ceramide. Cell loss was particularly evident in Jurkat T cells, and also in the myeloblastic and promyeloblastic KG-1 and KG-1a cells. K562, HL60 and Raji cells were somewhat less susceptible to a loss in

viable cells upon treatment with ceramide. By 24 hours post-treatment a majority of the ceramide-treated cells remained viable for these cell lines, particularly in the Raji B cell culture which showed the number of viable cells remained unchanged by this timepoint. Growth arrest was, however, clearly evident by 48 hours in each of these cell lines. The viability results obtained for the HL60 cell line supports the published findings by Okazaki *et al.* (1990), where it was observed that ceramide causes a dose-dependent inhibition of HL60 cell growth. Moreover, this study has demonstrated that a reduction in viable cell numbers, relative to the time-matched, vehicle-treated controls, is a common feature of ceramide treatment.

The observed decrease in viable cell numbers was paralleled by an increase in cell death in all cell lines studied, however the rate and magnitude of cell death varied significantly between each cell type. It is important to note that the number of dead cells may have been underestimated, particularly at the later timepoints, as cells that had been dead for a long period of time could not always be distinguished from the surrounding debris in the cell culture. In most instances, the rate of decrease in percentage viability reflected the rate of viable cell loss. A dramatic rate of cell death was observed in the Jurkat cell line during the first 8 hours of culture, and this has been attributed to apoptotic cell death. Studies in our laboratory have demonstrated this by showing an externalisation of phosphatidylserine residues on the cell membrane within 2 hours of exposure to C_2-ceramide, by double labelling with Annexin V-FITC and Propidium Iodide (Hearps *et al.*, 2002) (see Appendix A). Furthermore, it was revealed that ceramide-treated cells bearing this early apoptotic marker had a reduced mitochondrial transmembrane potential that was preceded by the release of cytochrome *c* from the mitochondria, and subsequent activation of the execution caspase, caspase 3, in Jurkat T cells (Hearps *et al.*, 2002). These findings collectively support the recently accepted notion (Ghafourifar *et al.*, 1999, Ito *et al.*, 1999, Cuvillier *et al.*, 2000, Tepper *et al.*, 2000) that ceramide activates distal caspases via mitochondrial cytochrome *c* release.

Cell death was also observed in the time-matched control cultures, however the absolute number of viable cells continued to increase for all cell lines investigated. That is, the growth rate always exceeded the rate of cell death. This was, however, never observed in ceramide-treated cell populations as the death rate always exceeded the rate of growth. The observed cell death in the vehicle-treated controls was presumably associated with the serum-free culture conditions, as other authors have also noted that culturing in serum-free media can result in cell death (Howard *et al.*, 1993). Regardless, ceramide-treated cells consistently showed a more

significant rate of cell death/loss in viable cell numbers, which was consistently observed earlier than in the time-matched controls at each timepoint, demonstrating that the effects observed were not due to serum-withdrawal alone.

This study has demonstrated that induction of cell cycle arrest, defined as an increase in the proportion of cells in the G_0/G_1 phase of the cell cycle thus preventing progression into S phase (Jayadev *et al.*, 1995), is a consistent outcome of ceramide signalling in all of the cell lines studied. Ceramide-induced G_0/G_1 cell cycle arrest has been previously demonstrated in other cell lines (Dbaibo *et al.*, 1995, Jayadev *et al.*, 1995, Ragg *et al.*, 1998, Lee *et al.*, 2000). Both myeloid and lymphoid haemopoietic cell lines included in this study underwent G_0/G_1 cell cycle arrest following C_2-ceramide treatment, with the degree of arrest varying between cell lines. The degree of apparent responsiveness to ceramide-induced cell cycle arrest reflects the pleiotropic nature of diverse responses associated with the ceramide-signalling pathway. The diversity of responses is most probably a result of ceramide engaging different downstream effectors depending on the cellular microenvironment (Mathias *et al.*, 1998), the phase of the cell cycle in which the signal was initiated, or the magnitude in generation of ceramide metabolites that may enhance or antagonise ceramide-activated signalling mechanisms (Spiegel *et al.*, 1998b).

KG-1 and KG-1a cells showed delayed cell cycle arrest in response to ceramide treatment compared with the other cell lines investigated. Although G_0/G_1 arrest was demonstrable at 24 hours post-treatment in both cell lines, a more significant accumulation of G_0/G_1 events was not clearly apparent until 48 hours after treatment. Thus, despite the extended timeframe, ceramide treatment still initiated G_0/G_1 cell cycle arrest, as observed in the other cell lines examined.

In most cases, it was clearly apparent that the observed accumulation of G_0/G_1 phase cells was accompanied by a reduction in the proportion of cells in both the S and G_2/M phases of the cell cycle. This suggests that cell are able to move through the S and G_2/M phases and into the G_0/G_1 phases, but are prevented from traversing the G_1 to S phase transition, thus supporting the findings by Jayadev *et al.* (1995). These results further support the hypothesis that ceramide signalling plays an important role in regulating significant components of the cell cycle, specifically at the restriction point where expression of cyclin-dependent kinases and regulation of Rb is of extreme importance.

C_2-ceramide-induced cell cycle arrest was usually accompanied by some degree of apoptosis, with the magnitude varying between cell lines and with dosage. A presence of a sub-G_0/G_1 (hypodiploid) peak in a DNA histogram has been

attributed to cells undergoing apoptotic death by many researchers (Nicoletti *et al.*, 1991, McCloskey *et al.*, 1994, Banker *et al.*, 1997, Mangiarotti *et al.*, 1998), although this is still in some dispute by other authors (Qian *et al.*, 1995, Darzynkiewicz *et al.*, 1997). The presence of a sub G_0/G_1 peak was noted in this study to indicate general cell death, whether by an apoptotic or necrotic mechanism, as ceramide-induced non-apoptotic killing has been noted (Mengubas *et al.*, 1999). Regardless, the presence of a hypodiploid peak in ceramide-treated populations is consistent with the results obtained from viability experiments performed in this study, which show cell death in association with ceramide exposure. It is interesting to note that cell lines that had comparatively minimal hypodiploid peaks (K562 and Raji cells) generally showed more pronounced G_0/G_1 cell cycle arrest. This finding may indicate that cell death is occurring primarily in cells that are trapped in the G_0/G_1 phase, hence lowering the apparent number of G_0/G_1 events by selective killing. Therefore, where significant cell death was demonstrated in this study, in particular in the Jurkat cell line, the proportion of cells that have undergone cell cycle arrest in response to ceramide would have been underestimated. Other authors have also demonstrated the selective killing of cells in the G_0/G_1 phase of the cell cycle following treatment with C_6-ceramide (Spinedi *et al.*, 1998) and after drug treatment or activation (Luciano *et al.*, 1996, Vial *et al.*, 1997, Mangiarotti *et al.*, 1998, Mongini *et al.*, 1998). As mentioned previously, ceramide-induced cell death in Jurkat cells has been confirmed as apoptosis in our laboratory using Annexin V-FITC/PI staining (Appendix A) and the demonstration of caspase 3 activation. Apoptotic death was well advanced by 24 hours post-treatment, which accounts for the significant sub-G_0/G_1 population observed in the respective Jurkat DNA histogram.

Research into the mechanisms by which ceramide induces cell cycle arrest has shown that dephosphorylation of Rb is almost certainly always involved (Chao *et al.*, 1992, Jayadev *et al.*, 1995, Dbaibo *et al.*, 1995). Ceramide-induced activation of Rb has been demonstrated in MOLT-4 (Chao *et al.*, 1992, Dbaibo *et al.*, 1995, Jayadev *et al.*, 1995), U-937 (Ragg *et al.*, 1998), and WI38 (Venable *et al.*, 1995) cells. Furthermore, MOLT-4 cells that are partially deficient in Rb, or express proteins that sequester the Rb protein, are resistant to ceramide-induced cell cycle arrest (Mathias *et al.*, 1998). To obtain further insight into the mechanism of C_2-ceramide-induced growth and cell cycle arrest, the phosphorylation status of the Rb protein was determined by taking advantage of the fact that SDS-polyacrylamide gel electrophoresis and Western blotting can resolve the faster migrating hypophosphorylated forms of Rb from the slower migrating hyperphosphorylated

form. The inactive hyperphosphorylated Rb (Rb^{hyper}) was consistently observed in the vehicle-treated, time-matched control protein extracts. However, in the ceramide-treated protein extracts, and additional band of lower molecular weight, corresponding to the active, hypophosphorylated (Rb^{hypo}) form of the Rb protein, was observed. Therefore it has been demonstrated that ceramide can induce Rb activation in all cell lines studied.

Rb activation is shown to occur very rapidly in all cell lines, except HL-60 and Jurkat cells where Rb activation was first observed at 24 hours post-treatment. In fact, the presence of an additional Rb^{hypo} band was observed as early as 4 hours post-treatment with ceramide in extracts from the K562, KG-1, KG-1a and Raji cell lines. It is interesting to note Rb activation in the Jurkat cell line, which exhibited an extreme initial apoptotic response. This would suggest that a significant proportion of the Jurkat cell population undergo apoptosis, leaving a viable sub-population, which are proposed to be resistant to such ceramide-induced cell death. It is reasonable to presume that the remaining sub-population of Jurkat cells would undergo G_0/G_1 cell cycle arrest as a result of ceramide-induced Rb activation. It was not possible to detect Rb in the KG-1 and KG-1a 24 hour post-ceramide-treated samples, presumably due to the loss of cells to apoptosis, which is consistent with the results from viability studies performed in this study. Regardless, activated Rb was clearly demonstrated in earlier timepoints for these cell lines following ceramide treatment, indicating that Rb activation is an early ceramide-induced event. Raji protein extracts displayed a different Rb phosphorylation pattern to that observed in all other cell extracts, in that more than two isoforms of Rb were observed in the vehicle-treated control extracts. However, a third, lower molecular weight band corresponding to fully hypophosphorylated Rb was clearly apparent in the ceramide-treated extracts throughout the experiment.

It is important to mention that although Rb^{hypo} was observed in vehicle-treated control protein extracts of some of the cell lines investigated (presumably due to the serum-free conditions), this form of the protein was always seen in greater proportions to the Rb^{hyper} band in extracts from ceramide-treated cells, compared with the corresponding time-matched control cell extracts. Furthermore, Rb^{hypo} was consistently observed at earlier timepoints for cells treated with ceramide, than in the time-matched, vehicle-treated controls.

Expression of the CKI $p21^{Cip1/Waf1}$ is transcriptionally regulated in a p53-dependent manner in normal cells. As such, $p21^{Cip1/Waf1}$ expression can be induced in response to stimuli that induce activation of the p53 tumour suppressor, including

DNA damaging agents (el-Deiry *et al.*, 1994, Akashi *et al.*, 1995, Russo *et al.*, 1995). Over the last decade a number of investigators have demonstrated an increase in $p21^{Cip1/Waf1}$ expression in a p53-independent manner using mitogenic stimuli (Michieli *et al.*, 1994), immunosuppressive agents (Khanna & Hosenpud, 1999), cytokines (Datto *et al.*, 1995, Robson *et al.*, 1999), and a variety of differentiating agents (Jiang *et al.*, 1994, Steinman *et al.*, 1994, Zhang *et al.*, 1995, Liu *et al.*, 1996a, Zeng & el-Deiry, 1996, Brown *et al.*, 1997, Ragg *et al.*, 1998). The finding that $p21^{Cip1/Waf1}$ is a target for p53 has generated considerable interest in the regulation of its expression, as *p53* mutations and deletions are common in cancers. p53-independent pathways of $p21^{Cip1/Waf1}$ induction could represent alternative approaches to control aberrant proliferation resulting from a nun-functional or absent *p53* gene (Steinman *et al.*, 1994). RNase protection assays were utilised in this study to demonstrate whether ceramide could induce $p21^{Cip1/Waf1}$ expression as part of a coordinated cell cycle arrest response. An increase in $p21^{Cip1/Waf1}$ expression was shown in all cell lines analysed, and is consistent with findings reported by other authors who have shown similar results in other cell lines (Ragg *et al.*, 1998, Lee *et al.*, 2000). The upregulation in $p21^{Cip1/Waf1}$ expression at the transcriptional level was shown to occur as early as 4 hours post-treatment, further confirming that this is an immediate early response to ceramide signalling. Interestingly, the kinetics observed for upregulated $p21^{Cip1/Waf1}$ message parallel those observed for accumulation of activated Rb following ceramide treatment, although the onset of ceramide-mediated $p21^{Cip1/Waf1}$ expression proceeded ceramide-induced Rb activation. Jiang *et al.* (1994) found a close relationship between induction of $p21^{Cip1/Waf1}$ and differentiation, as a delay in $p21^{Cip1/Waf1}$ induction was observed in HL60 cells resistant to TPA induced growth arrest and differentiation. Similarly, Rots et *al.* (1999) have shown that vitamin D_3-induced cell cycle arrest in myeloid cells always precedes differentiation, and involves the induction of $p21^{Cip1/Waf1}$. Taken together, these findings further identify $p21^{Cip1/Waf1}$ as a candidate gene linking differentiation signals to G_0/G_1 arrest and Rb hypophosphorylation in a number of cell lines. This p53-independent induction of expression of $p21^{Cip1/Waf1}$ is of great importance as a future target for leukaemia therapy. Roman-Gomez *et al.* (2002) have recently demonstrated hypermethylation of the $p21^{Cip1/Waf1}$ gene promoter in bone marrow cells of ALL patients, which correlated strongly with decreased $p21^{Cip1/Waf1}$ mRNA levels, and was highly predictive of a poor clinical outcome.

Haemopoiesis is a multistage developmental process in which a pluripotent haempoietic stem cell gives rise to monocytes, granulocytes, lymphocytes,

megakaryocytes, and erythrocytes. As multipotent progenitor cells undergo monocytic development, they sequentially differentiate into proliferating monoblasts, promonocytes, monocytes, and finally macrophages. Granulocytic development involves the differentiation of progenitors into myeloblasts, promyelocytes, myelocytes and then neutrophils.

Specific markers can be used to distinguish monocytic and granulocytic cells at particular stages of maturation. Changes in expression of these cell surface markers are used, not only to demonstrate the normal haemopoietic process, but also to show how haemopoietic cells differentiate in response to treatment with various agents, such as ceramide analogues. The CD11b leukocyte integrin subunit is initially expressed at the monoblastic and myelocytic stages of development but expression increases during monocytic and granulocytic differentiation (Griffin *et al.*, 1981, Rosmarin *et al.*, 1989, Lubbert *et al.*, 1991, Grande *et al.*, 2001). It comprises part of the adhesion molecule CD11b/CD18 (also called Mo1 or Mac-1), and is involved in binding a complement component as well as promotion of homotypic granulocyte adhesion and adhesion of granulocytes and monocytes to endothelial cells during host defence and immune response (Lubbert *et al.*, 1991). Expression of CD11b mRNA has been shown to be sharply upregulated during induction of granulocytic or monocytic differentiation of HL60 cells. This included an increase in the number of CD11b molecules per cell as well as the number of CD11b-positive cells (Hickstein *et al.*, 1989, Rosmarin *et al.*, 1989). The CD14 cell surface antigen is expressed in mature monocytes (Lubbert *et al.*, 1991).

Haemopoietic differentiation was only assessable in the myeloid cell lines. The ability of ceramide to induce myeloid differentiation has been noted by several other authors (Okazaki et *al.*, 1990, Bielawska et *al.*, 1992, Ragg *et al.*, 1998), although all studies to date have been confined to HL60 and U-937 cells. The results presented in this thesis confirm that ceramide has the ability to induce myeloid maturation, and extends these studies by providing evidence of ceramide-induced differentiation in other myeloid cell lines. Haemopoietic differentiation was demonstrated by changes in the expression of the myeloid cell surface markers CD11b and CD14, and the cell-specific surface markers glycophorin A for K562 cells, and CD34 for KG-1 and KG-1a cells.

Treatment of erythromyeloid K562 cells with ceramide resulted in a small increase in CD11b and CD14 expression by 24 hours, and continued to increase over the proceeding 48 hours, whereby a 46% increase in CD11b expression and a 20% increase in expression were noted by 72 hours post-treatment, relative to the time-

matched, vehicle-treated controls. In order to further characterise ceramide-induced differentiation in K562 cells, changes in expression of a third surface marker, glycophorin A (glyA), was assessed. It has been well documented in the literature that parental K562 cells strongly express the glyA cell surface marker (Andersson *et al.*, 1979, Koeffler & Golde, 1980). Differentiation of K562 cells along the erythroid line has been demonstrated in the presence of sodium butyrate (Hoffman, 1979, Andersson *et al.*, 1979), and these cells produce embryonic haemoglobin when cultured with hemin (Koeffler & Golde, 1980). The dramatic downregulation in glyA expression, taken together with the increase in CD11b and CD14 expression, is consistent with ceramide-induced differentiation of this cell line towards the granulocytic/macrophage lineage, and away from the erythroid lineage.

An increase in CD11b and CD14 expression was more clearly evident in HL60 cells, particularly at the 48 and 72 hour timepoints. These results confirm those published by Okazaki *et al.* (1990) and Bielawska *et al.* (1992). Okazaki *et al.* (1990) found that ceramide alone (at 1-6 μM concentrations) was sufficient to induce HL60 monocytic differentiation. Further, the differentiation response noted mimicked that observed when HL60 cells were treated with vitamin D_3 alone, a well-known inducer of HL60 cell maturation. The present study identified a subpopulation of HL60 cells staining positive for CD11b that are responsive to ceramide treatment, which is suggestive that a heterogeneous subpopulation exists within the homogeneous cell line. It is proposed therefore that the cellular response to ceramide treatment is dependent on a number of factors, including the state of the microcellular environment, the presence of co-stimulatory factors, and most likely the phase of the cell cycle, thus further confirming the pleiotropic nature of ceramide as a second messenger.

Ceramide-treated KG-1 cells showed a delayed increase in both CD11b and CD14 expression relative to the other cell lines studied. An increase in expression of these surface markers was not demonstrated until 48 hours post-treatment, although the change in expression by this timepoint was very convincing. Similarly, a significant change in CD11b expression was not observed in KG-1a cells until 48 hours post-treatment, although an increase in CD14 was clearly apparent from the 24 hour timepoint onwards. Changes in expression of the CD34 surface antigen was also investigated for ceramide-induced differentiation studies involving the KG-1 and KG-1a cell lines as this surface marker is expressed on parental KG-1 and KG-1a cells (Satterthwaite *et al.*, 1990, Pasternak & Pasternak, 1994). CD34 is expressed on very immature cells (e.g. stem cells and myeloid progenitor cells in the bone

marrow), and expression of CD34 decreases as these cells differentiate (Berenson *et al.*, 1988 & 1991). CD34 mRNA downregulation associated with induced macrophage differentiation has been shown previously in KG-1 cells by Satterthwaite *et al.* (1990) using TPA and ionomycin. A significant decrease in CD34 expression was observed in both these cell lines by 24 hours post-treatment with ceramide. Downregulation in expression of this very primitive cell surface marker is a convincing indication of cell maturation towards the granulocytic lineage in these two cell lines.

Together with the immunophenotyping results presented here, it is possible to conclude that ceramide can induce haemopoietic differentiation in K562, HL60, KG-1 and KG-1a cells, thus further extending the published findings by other investigators in this field of research.

Treatment of the various cell lines included in this study with the ceramide analogue, C_2-ceramide, has resulted in a number of common responses. All cell lines showed a decrease in the number of viable cell numbers, which was accompanied by an increase in cell death, presumed to be apoptotic in nature. All cells underwent concomitant cell cycle arrest in the G_0/G_1 phase of the cell cycle, presumably via activation of the Rb tumour suppressor protein. In parallel with Rb activation, induction of $p21^{Cip1/Waf1}$ expression was demonstrated which in turn was associated with haemopoietic differentiation into a more mature phenotype for each of the myeloid cell lines studied. Taken together, this chain of events is consistent with an alteration in the balance within the cell from being strongly proliferative to anti-proliferative as a result of ceramide-signalling. Although common outcomes are described, it is interesting to note that the kinetics of the responses observed varied quite considerably from one cell line to the next, seemingly depending upon the cellular context, thus confirming the pleiotropic nature of ceramide.

The rate of loss in cell viability, and the kinetics involved in the decrease of percent viable cells observed in this study, indicates an initial apoptotic response. However, not all cells are initially killed, or the apoptotic response is not completed in a subset of the cell population. This is particularly true in the Jurkat cell line, whereby a significant majority of cells initially underwent apoptosis, although a remaining small subpopulation remained viable, presumably entering G_0/G_1 arrest via Rb activation. Many explanations might be plausible for this phenomenon. It is feasible to suggest that within the homogenous cell populations used as a model for this study, exists heterogenous cell subsets, in a similar way that subpopulations are

observed *in vivo*. Since asynchronous culture were used for the current study, a more feasible postulation might be that the rate of response to ceramide varies significantly depending upon the phase of the cell cycle the cells are in at the time of initial exposure. Delivery of a differentiation signal by ceramide at an inappropriate stage of the cell cycle, or in conflict with an existing proliferative signal may result in apoptosis being initiated. Okazaki *et al.* (1990) suggest that the loss of viable cells may be partly due to the induction of cell differentiation by ceramide, rather than to simple toxicity. Regardless, ceramide-induced apoptosis has been extensively demonstrated by a number of authors (Obeid *et al.*, 1993, Sakakura *et al.*, 1996).

The type of response a cell demonstrates following exposure to exogenous ceramide appears to be entirely dependent upon the stage of differentiation it is at, at the time of ceramide addition. Again, this is particularly evident in the Jurkat cell line, which are fully differentiated T lymphocyte cells. The addition of ceramide to this cell line appears to force the cells into terminal differentiation, the final outcome of terminal differentiation being cell death. Because these cells are already at such a late stage of differentiation, the immediate ceramide-induced response is apoptotic death.

Cells that survived the initial apoptotic response underwent changes consistent with the imposition of an anti-proliferative phenotype. Cell cycle arrest was demonstrated in all cell lines, and activation of the Rb tumour suppressor protein and upregulation of the CKI $p21^{Cip1/Waf1}$ are potentially common mechanisms for this event. Since the cell lines used in this study are $p53^{null}$, the results presented here have demonstrated the activation of cell cycle regulatory elements in a p53-independent manner. Because the inactivation of p53 in cancer has been associated with poor survival, refractory disease and chemoresistance (Lowe *et al.*, 1993, Soini *et al.*, 1993, Marks *et al.*, 1996, Dive 1997), it is reasonable to assume that restoring the function of p53 in cancer cells may have considerable implications in cancer treatment. Thus, manipulation of the ceramide-signalling pathway may be beneficial in the treatment of proliferative disorders such as leukaemia, where regaining control of the cell cycle in the absence of a functional p53 gene is fundamental. Therefore, further elucidation into the mechanisms by which Rb is activated by ceramide is warranted.

This study presents what is believed to be the first demonstration of ceramide-induced differentiation in K562, KG-1 and KG-1a cells. Ceramide-induced HL60 differentiation has been confirmed in the current study. Since $p21^{Cip1/Waf1}$ is considered to play a significant role in terminally differentiating cells such as muscle,

and in cells treated with differentiating agents to force them to become fully matured (Halevy *et al.*, 1995, Parker *et al.*, 1995, Flink *et al.*, 1998), and $p21^{Cip1/Waf1}$ expression is induced by ceramide in all cell lines investigated in this study, it is feasible to conclude that ceramide is inducing terminal differentiation in K562, HL60, KG-1 and KG-1a cells. Ceramide-induced differentiated myeloid cells have a lowered proliferative capacity and eventually die of apoptosis, presumably as a result of terminal differentiation. If cellular differentiation is blocked at a proliferative stage, then blastic leukaemia may eventuate (Testa *et al.*, 1993). Based on the present study, it is possible to hypothesise that ceramide signalling can push leukaemia cells past this blockage, resulting in a non-proliferative, terminally differentiated cell that undergoes normal apoptosis. The ability to change aggressively growing cancer cells into mature, less proliferative cells that then die by programmed cell death forms the fundamental qualities of a potential cancer therapy.

Ceramide-mediated myeloid cell differentiation is most likely to involve a transcriptionally-activated pathway as this study has demonstrated alterations in the expression profiles of CD11b, CD14 and the cell-specific glycophorin A and CD34 cell surface markers. Many authors have shown activation of the protooncogenes *c-fos* and *c-jun* following treatment with ceramide (Kim & Kim, 1998, Ragg *et al.*, 1998, Ruvolo, 2001), whose protein products can heterodimerise to form the AP-1 transcription factor complex. AP-1 has been previously shown to have a role in ceramide-mediated apoptosis (Sawai *et al.*, 1995) but as yet has not been linked to ceramide-induced differentiation. As an increase in CD14 expression following ceramide treatment has been demonstrated, and the CD14 gene promoter has recently been shown to have multiple AP-1 binding sites (Liu *et al.*, 2000), it is suggested that ceramide-induced AP-1 activation is a potential mechanism for ceramide-induced differentiation.

In summary, activation of the sphingomyelin signalling pathway by exogenous addition of a synthetic ceramide analogue causes common outcomes in a variety of cell types, which are mediated via common mechanisms. The overall outcome is growth arrest, Rb activation, $p21^{Cip1/Waf1}$ upregulation, terminal differentiation and apoptosis. Each of these processes results in a reduction in the number of aggressively growing cancer cells, which is the primary aim of cancer therapies. Since maturation and proliferation are linked events, it is proposed that the growth of aggressively proliferating leukaemia cells is greatly diminished by ceramide-initiated cellular maturation and Rb activation. The results presented in this chapter, demonstrate the potential for ceramide in initiating cell cycle regulatory

mechanisms and terminal differentiation of haemopoietic cells into a mature, less proliferative phenotype. Further investigations into the molecular basis of growth arrest and differentiation mechanisms mediated by the ceramide-signalling pathway and their manipulation for therapeutic purposes are warranted.

Chapter 4

Mechanisms of Ceramide-Mediated Cell Cycle Arrest and Differentiation

4.1. Introduction

Despite the retinoblastoma (Rb) tumour suppressor protein being one of the few identified downstream effectors of ceramide signalling, very little is known as to how ceramide activates Rb. The relationship, if any, between ceramide-induced Rb activation and differentiation remains unexplored.

Rb is a nuclear phosphoprotein that is regulated by phosphorylation during normal progression through the cell cycle (Mihara *et al.*, 1989, Ludlow *et al.*, 1990, Riley *et al.*, 1994, Chen *et al.*, 1995a, Weinberg, 1995). It plays an essential role at the Restriction Point of the cell cycle, where it functions to modulate members of the E2F transcription factor family (Nevins, 1992, Helin *et al.*, 1993, Lees *et al.*, 1993). The E2F proteins in turn transactivate genes whose products are important for S phase entry (Neuman *et al.*, 1994, Buchmann *et al.*, 1998). When Rb is in its inactivated form (hyperphosphorylated), the bound E2Fs are released and progression through the S phase is permitted. Hypophosphorylation of Rb prevents the release of E2F complexes, converting them to repressors that prevent expression of E2F target genes (Weintraub *et al.*, 1992).

Rb remains in an inactivated form during cell cycle progression via phosphorylation by cyclin-dependent kinases, in particular by cyclin D-dependent kinase complexes (Ewen *et al.*, 1993, Kato *et al.*, 1993, Sherr, 1994). Ludlow *et al.* (1990) demonstrated that in normal proliferating cells Rb is found in its dephosphorylated form in G_1 phase of the cell cycle, and becomes phosphorylated at the beginning of S and remains phosphorylated through S and G_2 phases. Rb is then completely dephosphorylated between the end of G_2 and the beginning of G_1 phases of the cell cycle. Rb has been shown to be in its activated form in non-cycling, terminally differentiated cells (Furukawa *et al.*, 1990). An understanding of the molecular mechanism or mechanisms that enable Rb to remain in the dephosphorylated/activated form is becoming more apparent.

To date, two effector mechanisms for Rb activation have been determined. The first is via an indirect method, whereby cyclin-dependent kinase (CDK) complexes are inhibited from phosphorylating Rb. CDKs are catalytic proteins whose activity requires interaction with regulatory subunits termed cyclins (Pines, 1995). The Rb protein is specifically phosphorylated by cyclin D-complexed CDK4 and CDK6 (Sherr, 1996). Cyclin-dependent kinase inhibitors (CKIs) function to prevent this cyclin-CDK complex from forming by binding to the CDK in place of the cyclin subunit. The INK4 CKIs ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) have been

shown to directly block cyclin D-dependent kinase activity and cause G_1 arrest (Serrano *et al.*, 1993, Kamb *et al.*, 1994, Hirai *et al.*, 1995). In addition, $p27^{Kip1}$, $p57^{Kip2}$, and $p21^{Cip1/Waf1}$ inhibit cyclin D-dependent kinases. $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ are involved in cell cycle control at the Restriction Point of the cell cycle (Harper *et al.*, 1993, Harper *et al.*, 1995, Toyoshima & Hunter, 1994, Niculescu *et al.*, 1998). Of particular interest, however, is $p21^{Cip1/Waf1}$ as extensive studies have revealed that this CKI is the critical determinant in blocking cell cycle progression by inactivation of Rb (Harper *et al.*, 1993, Harper *et al.*, 1995, Niculescu *et al.*, 1998).

The best-defined mechanism for Rb activation, however, is by direct removal of phosphate groups from the Rb protein via activation of protein phosphatases (PP) (Alberts *et al.*, 1993, Durfee *et al.*, 1993, Ludlow *et al.*, 1993, Schonthal & Feramisco, 1993, Dou *et al.*, 1995, Berndt *et al.*, 1997, Nelson *et al.*, 1997). Mammalian protein phosphatases are represented by four distinct prototypes: PP1, PP2A, PP2B, and PP2C (Ingebritsen & Cohen, 1983). To date, two protein phosphatases have been attributed to the enzymatic dephosphorylation of Rb, PP1 (Durfee *et al.*, 1993, Ludlow *et al.*, 1993, Nelson *et al.*, 1997) and PP2A (Dobrowsky & Hannun, 1992, Dobrowsky *et al.*, 1993, Dobrowsky & Hannun, 1993, Hannun, 1996). Both classes of protein phosphatases direct their activity towards the serine and threonine amino-acid residues of the retinoblastoma protein (Cohen, 1989).

Ceramide has been shown to activate a cytosolic serine/threonine protein phosphatase, which has been named the ceramide-activated protein phosphatase (CAPP) (Dobrowsky & Hannun, 1992, Dobrowsky *et al.*, 1993, Dobrowsky & Hannun, 1993, Hannun, 1996) belonging to the PP2A subfamily of protein phosphatases (Dobrowsky *et al.*, 1993, Galadari *et al.*, 1998). CAPP is believed to be involved in a range of cellular activities, but it has been postulated that one of its major roles is to dephosphorylate Rb via CDK2 inhibition, thus leading to G_0/G_1 cell cycle arrest (Lee *et al.*, 2000). Another protein phosphatase, belonging to the PP1 subfamily of protein phosphatases, is also believed to dephosphorylate Rb and can be activated by long chain ceramides (Chalfant *et al.*, 1999). PP1 appears to play a major role in Rb dephosphorylation during exit of the mitosis phase of the cell cycle (Alberts *et al.*, 1993, Durfee *et al.*, 1993, Ludlow *et al.*, 1993, Berndt *et al.*, 1997, Nelson *et al.*, 1997), as opposed to PP2A, which has been shown to play a role in preventing entry into mitosis (Kinoshita *et al.*, 1990, Lee *et al.*, 2000). The role of protein phosphatases in cell cycle regulation via Rb activation has been studied using PP1 and PP2A inhibitors including okadaic acid, Calyculin A, and phosphatidic acid (Ishihara *et al.*, 1989, Kishikawa *et al.*, 1999). The phosphatase inhibitor Calyculin A

has been previously identified as a potent inhibitor of the catalytic subunit of both type 1 and 2A protein phosphatase families (Ishihara *et al.*, 1989, Song and Lavin, 1993) and has been reported to be equally effective against both PP1 and PP2A (Ishihara *et al.*, 1989, Li *et al.*, 1993), as opposed to other protein phosphatase inhibitors including okadaic acid and cantharidin which are more effective against PP2A (Morana *et al.*, 1996). The discovery that inhibition of these protein phosphatases causes changes in cell growth regulation led to the suggestion that PP1 and PP2A could function as tumour suppressors in mammalian cells (Cohen & Cohen, 1989, Haystead *et al.*, 1989).

The exact role of $p21^{Cip1/Waf1}$ and protein phosphatases in ceramide-mediated cell cycle regulation and differentiation is still largely debatable. Other authors have demonstrated that expression of $p21^{Cip1/Waf1}$ is increased following treatment with exogenous ceramide (Ragg *et al.*, 1998, Lee *et al.*, 2000), and it is accepted that $p21^{Cip1/Waf1}$ plays a crucial role in both Rb dephosphorylation and terminal differentiation (Harper *et al.*, 1993, Harper *et al.*, 1995, Halevy *et al.*, 1995, Liebermann *et al.*, 1995, Parker *et al.*, 1995, Steinman *et al.*, 1994, Flink *et al.*, 1998, Niculescu *et al.*, 1998). Similarly, it is well established that ceramide can activate cytosolic protein phosphatases (Dobrowsky & Hannun, 1992, Dobrowsky *et al.*, 1993, Dobrowsky & Hannun, 1993, Hannun, 1996), and these appear to be important in mediating Rb dephosphorylation (Lee *et al.*, 2000). There is, however, limited knowledge of whether these two cellular factors play equally important roles or whether one is most important in regulating cell cycle control, and the other in terminal differentiation. In the previous chapter, ceramide has been shown to induce both Rb activation and terminal differentiation that is associated with $p21^{Cip1/Waf1}$ transcriptional activation. It is yet to be determined whether Rb activation and terminal differentiation utilise the same or independent upstream ceramide-signalling pathways, and/or whether they are dependent upon each other.

4.2. Methods

4.2.1. Experimental Plan

Effector mechanisms for Rb activation by ceramide were investigated in representative ceramide-responsive cell lines. K562, KG-1, KG-1a and Raji cells were treated with C_2-ceramide and with the potent protein phosphatase inhibitor Calyculin A to determine the role of protein phosphatases in ceramide-induced Rb activation. The phosphorylation status of Rb was determined by Western blotting. Additionally, the role for protein phosphatases in ceramide-induced myeloid differentiation was determined. This was achieved with K562 cells by measuring changes in glycophorin A cell surface marker expression following Calyculin A treatment by flow cytometry. The relative toxicity associated with Calyculin A treatment of KG-1 and KG-1a cells, however, required that another more sensitive technique be utilised to allow an early timepoint to be analysed in these cell lines. As such, an RNase protection assay (RPA) using a custom template was adopted for analysing changes in cell marker RNA levels in KG-1 and KG-1a cells.

4.2.2. Rb Phosphorylation Analysis Following Protein Phosphatase Inhibition

K562, KG-1, KG-1a, and Raji cell lines were treated with the C_2-ceramide concentrations outlined in Chapter 3.2.1., using treatment culture media as previously discussed in Chapter 2.2.3(D)., to induce Rb activation. Additional cells were also treated with C_2-ceramide + 25 nM Calyculin A. Cells were plated out at 5 X 10^5 cells/mL (1 ml/well) in 24 well cell culture dishes and were incubated at 37°C/5% CO_2. Cells (2 X 10^6 cells/sample) were harvested (described in Chapter 2.2.3(D)). At various timepoints, nuclear protein extractions were performed (described previously in Chapter 3.2.4.). Proteins were separated on 6.5% SDS-polyacrylamide gels and Western blotting was performed to demonstrate the phosphorylation status of Rb, as described in Chapter 3.2.5.

4.2.3. Immunophenotyping Following Protein Phosphatase Inhibition

K562 cells were treated with 5μM C_2-ceramide or with C_2-ceramide + 25 nM Calyculin A in serum-free RPMI media and plated out at 5 X 10^5 cells/mL in 24 well culture dishes and incubated at 37°C/5% CO_2. Cells (5 X 10^5 cells/sample) were harvested at 0 and 12 hours post-treatment, washed, stained with PE-conjugated anti-human glycophorin A antibodies, washed again, fixed as described previously in Chapter 3.2.8. Cells were analysed by flow cytometry (described in Chapter 2.2.4.)

using CellQuest software (Becton-Dickinson, USA). Immunophenotype profiles were generated and changes in MFI values between vehicle-treated, C_2-ceramide-treated, Calyculin A-treated, and C_2-ceramide + Calyculin A-treated cells calculated.

4.2.4. RNase Protection Assays – *CD11b, CD14, CD34* Expression Analysis

KG-1 and KG-1a cells were treated with 7.5μM C_2-ceramide or with C_2-ceramide + 25 nM Calyculin A in serum-free RPMI media and transferred to 75 cm^2 cell culture flasks and incubated at 37°C/5% CO_2. Cells (1 X 10^7 cells/sample) were harvested at 0 and 12 hours post-treatment, and RNA extracted, DNase-treated and quantitated as outlined in Chapter 2.2.7.

RPAs were performed using the protocol and reagents provided in the RiboQuant™ Multi-Probe RNase Protection Assay System (PharMingen, USA) as described in Chapter 2.2.8., with a custom template designed to include riboprobes directed towards human *CD11b*, *CD14* and *CD34* RNA transcripts and the house-keeping gene transcripts *L32* and *GAPDH* (Cat. # 559721-16464, PharMingen, USA). Statistical analyses of the change in expression levels were performed using the Students' two-tailed paired t-test.

4.3. Results

4.3.1. Effect of Calyculin A on Ceramide-Induced Rb Activation

The role of protein phosphatases in ceramide-induced Rb activation was investigated by inhibiting protein phosphatase activity with the use of Calyculin A in K562, KG-1, KG-1a and Raji cell lines. Cells previously shown to undergo ceramide-induced Rb activation were treated with C_2-ceramide and with a combination of C_2-ceramide and Calyculin A. SDS-PAGE and Western blotting investigated the phosphorylation status of the Rb tumour suppressor protein in cell extracts at various timepoints after the treatment. Representative Western blot results from this study are provided in Figure 4.1.

Each of the blots presented in Figure 4.1. confirm the results observed in Chapter 3.3.3. of this thesis. That is, following C_2-ceramide treatment, Rb is rapidly activated as indicated by the presence of a lower molecular weight band corresponding to hypophosphorylated Rb. In fact, this event can be seen as early as 2 hours post-treatment with ceramide in the KG-1 and Raji cells. However, only the hyperphosphorylated Rb band can be seen following co-treatment with C_2-ceramide and Calyculin A for all cell lines studied, demonstrating that Calyculin A prevented ceramide-induced Rb activation.

Due to the biological toxicity of Calyculin A, and the synergistically detrimental effects of combined C_2-ceramide and Calyculin A treatment, protein concentration was considerably lower for the later timepoint of 24 hours post-treatment, except for K562 cells which appeared to be more resistant to these toxic effects. Therefore Rb could not be detected by Western blotting for KG-1, KG-1a or Raji cell lines at the 24 hour timepoint following combined C_2-ceramide and Calyculin A treatment.

4.3.2. Effect of Calyculin A on Ceramide-Induced Differentiation

The role of protein phosphatases in ceramide-induced differentiation was investigated by analysing changes in cell surface marker expression of K562, KG-1 and KG-1a cells. Cells were treated with vehicle, C_2-ceramide, Calyculin A, or with a combined treatment of C_2-ceramide and Calyculin A over a 12 hour timecourse.

K562 cells were stained with anti-glycophorin A PE-conjugated antibodies and analysed by flow cytometry. The resulting immunophenotype expression profiles are shown in Figure 4.2. The percentage change in mean fluorescent intensity (MFI) was calculated and the results show that vehicle-treated cells exhibit a strongly

Figure 4.1.: Western blot analysis of the retinoblastoma (Rb) phosphorylation status in human haemopoietic cells following treatment with either C_2-ceramide alone, or C_2-ceramide + Calyculin A. A single representative set of results from at least four repeated experiments is shown.

pRb^{hyper} = hyperphosphorylated (inactive) Rb, pRb^{hypo} = hypophosphorylated (active) Rb.

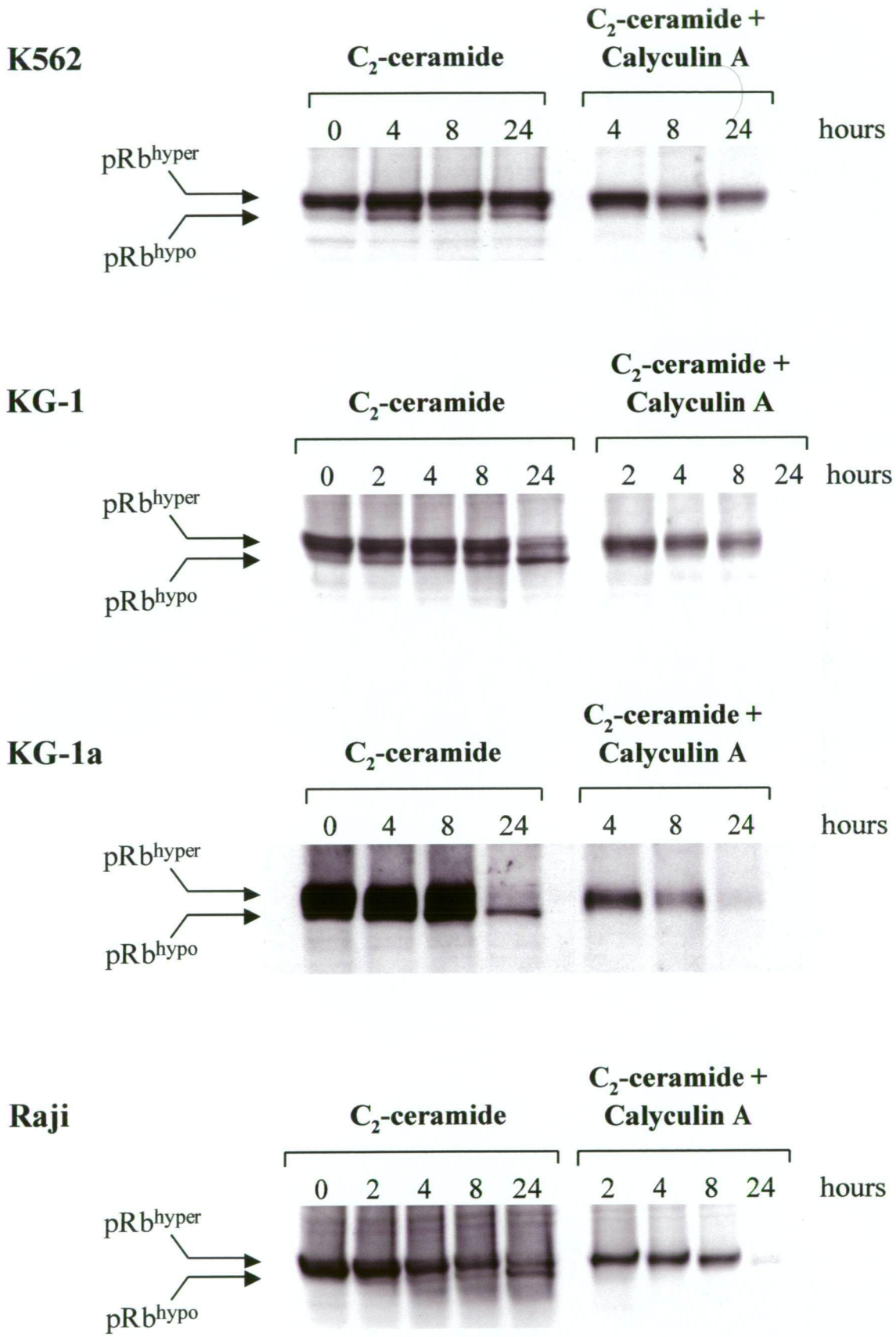

K562
C2-ceramide
C2-ceramide + Calyculin A
0 4 8 24 4 8 24 hours
pRbhyper
pRbhypo
KG-1
C2-ceramide
C2-ceramide + Calyculin A
0 2 4 8 24 2 4 8 24 hours
pRbhyper
pRbhypo
KG-1a
C2-ceramide
C2-ceramide + Calyculin A
0 4 8 24 4 8 24 hours
pRbhyper
pRbhypo
Raji
C2-ceramide
C2-ceramide + Calyculin A
0 2 4 8 24 2 4 8 24 hours
pRbhyper
pRbhypo

Figure 4.2.: Immunophenotype expression profiles of K562 erythromyeloid cells at 12 hours post-treatment with vehicle, C_2-ceramide alone, Calyculin A alone, or C_2-ceramide + Calyculin A, demonstrating changes in glycophorin A expression. A single representative set of results from at least four repeated experiments is shown.

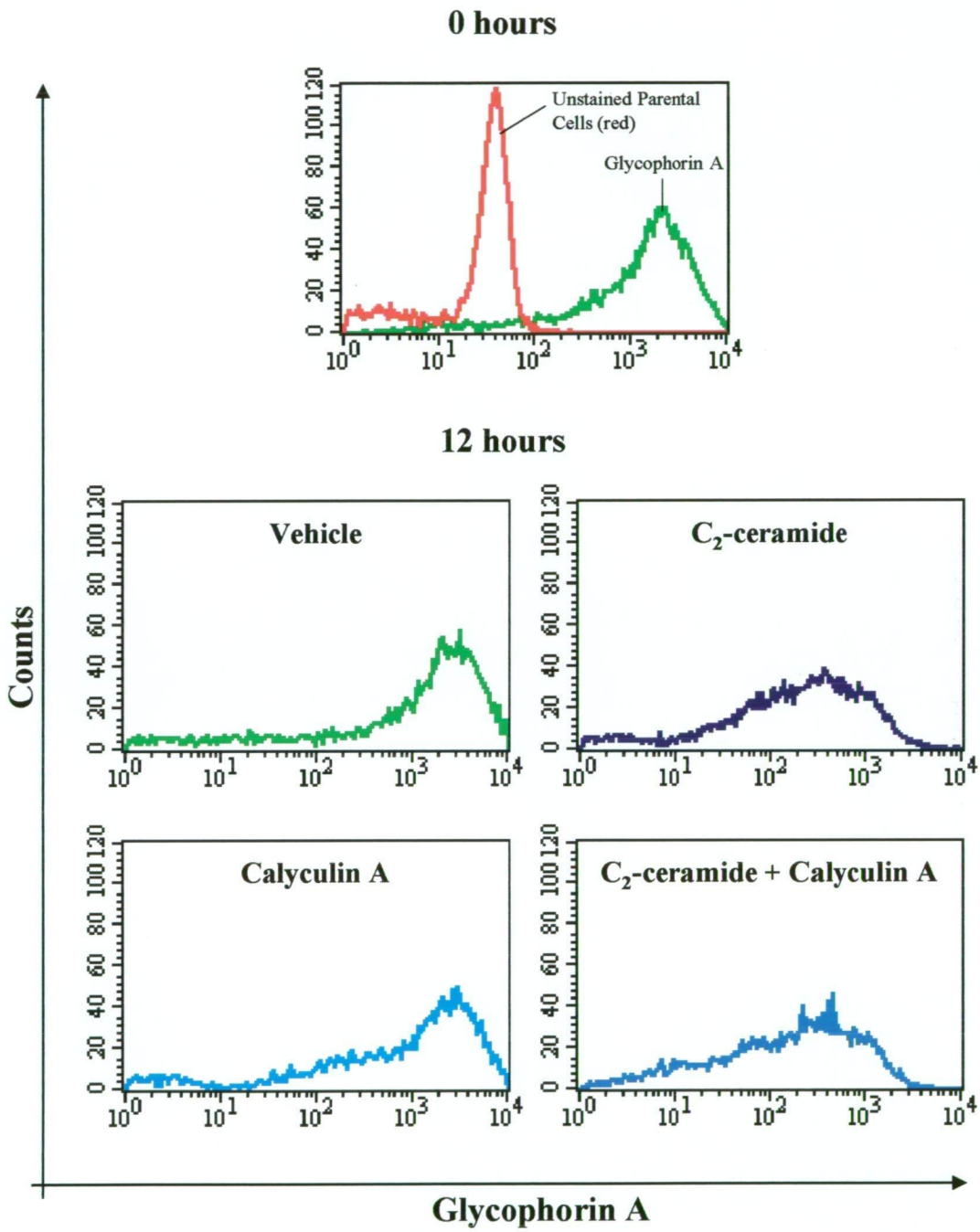
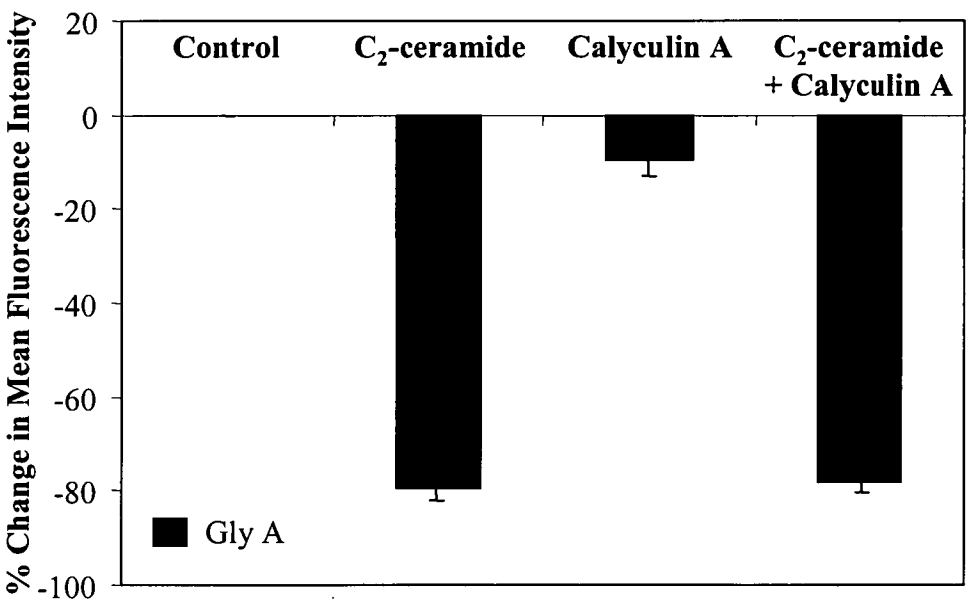

0 hours
Unstained Parental Cells (red)
Glycophorin A
12 hours
Vehicle
C2-ceramide
Calyculin A
C2-ceramide + Calyculin A
Counts
Glycophorin A

Figure 4.3.: Histogram demonstrating changes in glycophorin A expression for K562 erythromyeloid cells at 12 hours post-treatment with vehicle, C_2-ceramide alone, Calyculin A alone, or C_2-ceramide + Calyculin A. Data are expressed as a percentage change in the mean fluorescence intensity relative to the 12 hour vehicle-treated control sample, and are combined from at least four individual experiments.


% Change in Mean Fluorescence Intensity
20
0
-20
-40
-60
-80
-100
Control
C2-ceramide
Calyculin A
C2-ceramide + Calyculin A
Gly A

positive glycophorin A expression profile, while a left-shift in the expression profile is identified in the ceramide-treated cells. K562 cells treated with Calyculin A alone gave an expression profile that very closely resembled that exhibited by the vehicle-treated cells. Treatment with both ceramide and Calyculin A caused a decrease in glycophorin A expression, similar to that shown in cells treated with ceramide alone, indicating that inhibition of phosphatase activity had very little effect.

The combined flow cytometry data histograms from four replicate immunophenotyping experiments are presented in Figure 4.3. This graph verifies the significant down-regulation in glycophorin A expression in both the ceramide-treated and the ceramide + Calyculin A-treated K562 cell populations by a dramatic negative percentage change in MFI relative to the time-matched control. In contrast, cells treated with Calyculin A alone did not exhibit a significant change in MFI compared with the vehicle-treated control cells. These findings indicate that Calyculin A alone does not affect glycophorin A cell surface marker expression in K562 cells, and combined treatment with C_2-ceramide and Calyculin A does not inhibit the downregulated glycophorin A expression indicative of ceramide-induced K562 differentiation.

The significant toxicity of Calyculin A in KG-1 and KG-1a cells by 12 hours post-treatment precluded an immunophenotype analysis of differentiation, hence a novel approach for analysing the role of protein phosphatases in ceramide-induced differentiation was required in these cell lines. KG-1 and KG-1a cell lines were treated as described above and at 12 hours post-treatment, RNA was extracted and analysed for changes in *CD11b*, *CD14* and *CD34* expression levels using a RPA. Representative RPA blots from these experiments are shown in Figures 4.4. and 4.6., respectively. The raw data from image analysis of the blots presented in Figures 4.4 and 4.6. were normalised to the expression of housekeeping gene transcripts for each sample, and fold changes in *CD11b*, *CD14* and *CD34* expression were calculated. These are presented in Tables 4.1. and 4.2. for KG-1 and KG-1a cells, respectively. The expression of *CD11b* for KG-1 cells had not altered by 12 hours post-treatment with ceramide. *CD11b* or *CD14* expression was extremely weak and meaningful data could not be extracted for either KG-1 or KG-1a cells.

Analysis of *CD34* RNA expression showed that at 12 hours post-treatment, a significant downregulation in *CD34* expression was clearly visible for both KG-1 and KG-1a cells (2.6 and 1.8 fold decrease in expression relative to the 12 hour control for KG-1 and KG-1a cells respectively) following C_2-ceramide treatment alone. For both cell lines, the intensity of the bands representing *CD34* essentially remained

unchanged between the 0 hour vehicle-treated control and the 12 hour control. Calyculin A treatment alone resulted in *CD34* expression levels that paralleled those seen in the 12 hour vehicle-treated controls ($p = 0.369$ and 0.382 for KG-1 and KG-1a cells, respectively). Combined treatment with C_2-ceramide and Calyculin A, however, induced a significant downregulation in *CD34* expression, similar to that seen following treatment with C_2-ceramide alone ($p = 0.088$ and 0.010 for KG-1 and KG-1a cells, respectively). The normalised *CD34* data from image analysis of a minimum of four separate RPA experiments are represented in histogram form in Figures 4.5. and 4.7. to show the fold change in *CD34* band volume, relative to the 12 hour vehicle-treated control, following ceramide and/or Calyculin A treatment for KG-1 and KG-1a cells respectively. These histograms demonstrate that Calyculin A alone does not induce a significant change in *CD34* expression, whereas ceramide treatment alone, and ceramide + Calyculin A treatment results in a significant downregulation in *CD34* RNA levels by 12 hours post-treatment.

Figure 4.4.: Analysis of CD11b, CD14 and CD34 RNA expression in KG-1 myeloblastic/promyeloblastic cells at 12 hours post-treatment with either vehicle, C_2-ceramide alone, Calyculin A alone, or C_2-ceramide + Calyculin A. Results were obtained by performing RNase protection assays using a custom RiboQuant™ template set (Pharmingen, USA). The RPA blot is provided in its entirety for comparison of expression of housekeeping genes between each sample. A single representative set of results from four repeated experiments is shown.

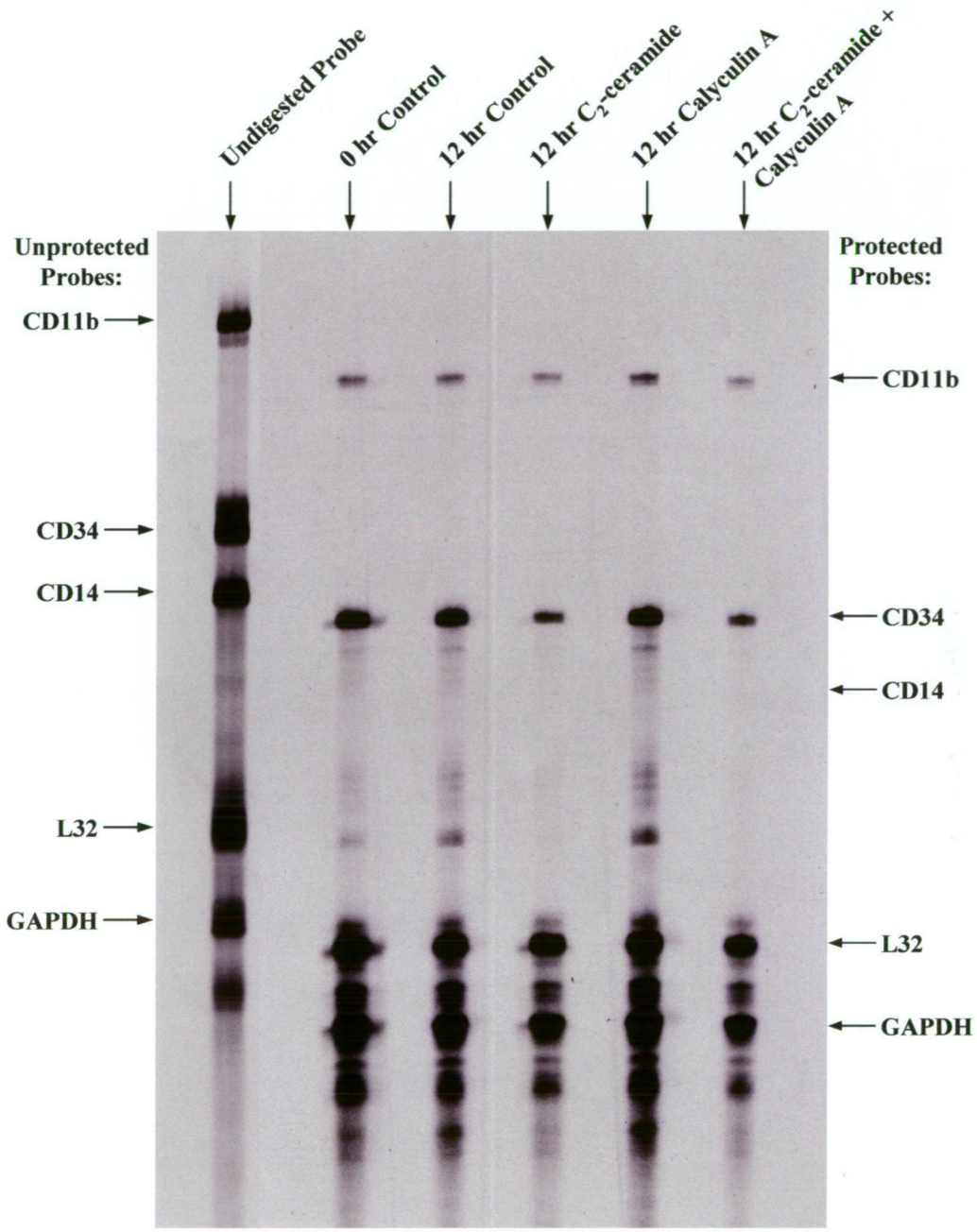

Undigested Probe
0 hr Control
12 hr Control
12 hr C_2-ceramide
12 hr Calyculin A
12 hr C_2-ceramide + Calyculin A
Unprotected Probes:
CD11b
CD34
CD14
L32
GAPDH
Protected Probes:
CD11b
CD34
CD14
L32
GAPDH

Figure 4.5.: Histogram demonstrating changes in CD34 expression for KG-1 promyeloblastic cells at 12 hours post-treatment with vehicle, C_2-ceramide alone, Calyculin A alone, or C_2-ceramide + Calyculin A. Data are expressed as a fold change in the CD34 band volume relative to the 12 hour vehicle-treated control, and are combined from four individual experiments.

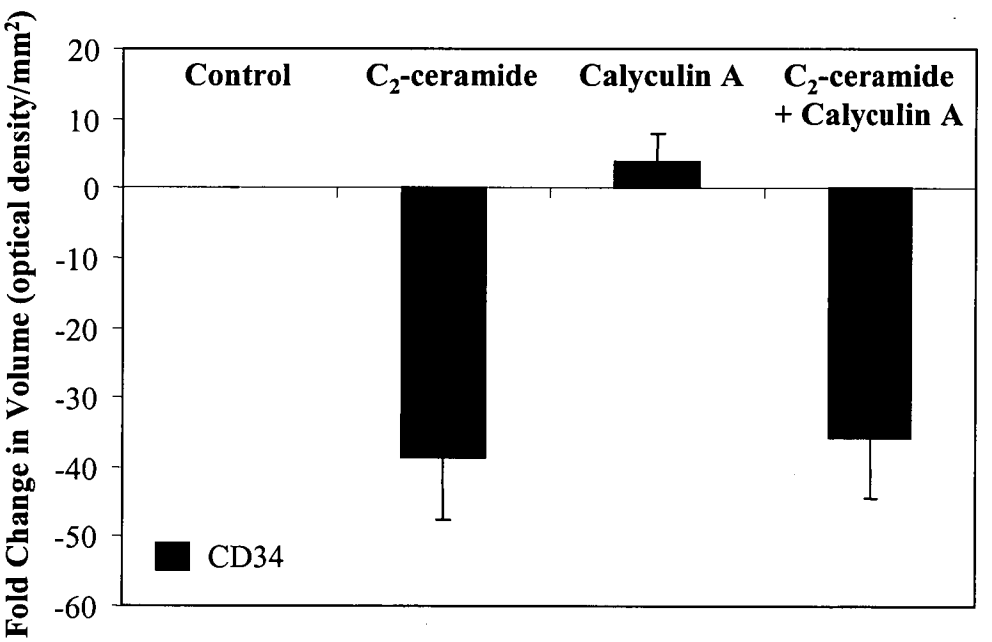

Fold Change in Volume (optical density/mm²)
20
10
0
-10
-20
-30
-40
-50
-60
Control
C2-ceramide
Calyculin A
C2-ceramide + Calyculin A
CD34

Figure 4.6.: Analysis of CD11b, CD14 and CD34 RNA expression in KG-1a promyeloblastic cells at 12 hours post-treatment with either vehicle, C_2-ceramide alone, Calyculin A alone, or C_2-ceramide + Calyculin A. Results were obtained by performing RNase protection assays using a custom RiboQuant™ template set (Pharmingen, USA). The RPA blot is provided in its entirety for comparison of expression of housekeeping genes between each sample. A single representative set of results from three repeated experiments is shown.

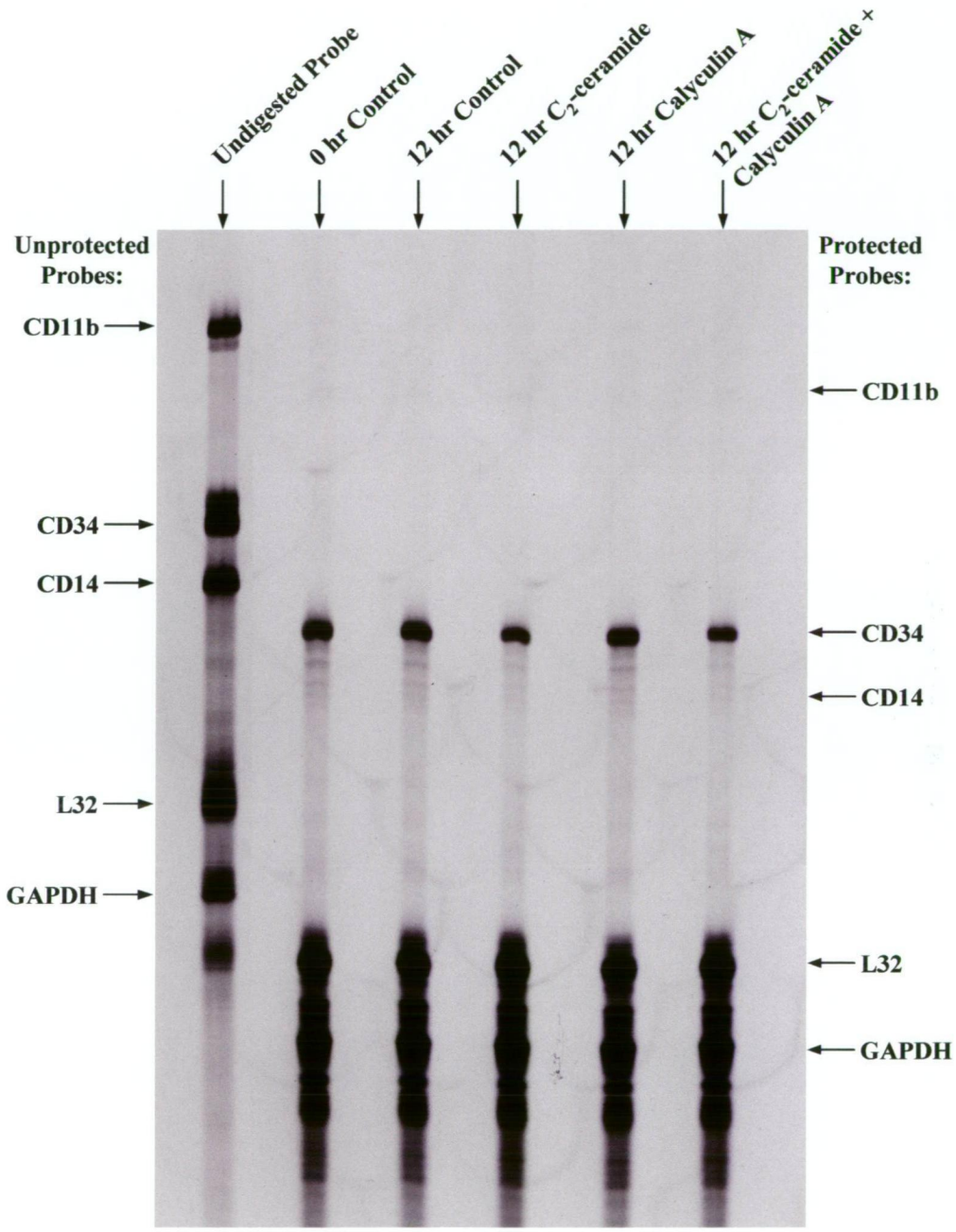

Undigested Probe
0 hr Control
12 hr Control
12 hr C_2-ceramide
12 hr Calyculin A
12 hr C_2-ceramide + Calyculin A
Unprotected Probes:
CD11b
CD34
CD14
L32
GAPDH
Protected Probes:
CD11b
CD34
CD14
L32
GAPDH

Figure 4.7.: Histogram demonstrating changes in CD34 expression for KG-1a promyeloblastic cells at 12 hours post-treatment with vehicle, C_2-ceramide alone, Calyculin A alone, or C_2-ceramide + Calyculin A. Data are expressed as a fold change in the CD34 RPA band volume relative to the 12 hour vehicle-treated control, and are combined from three individual experiments.

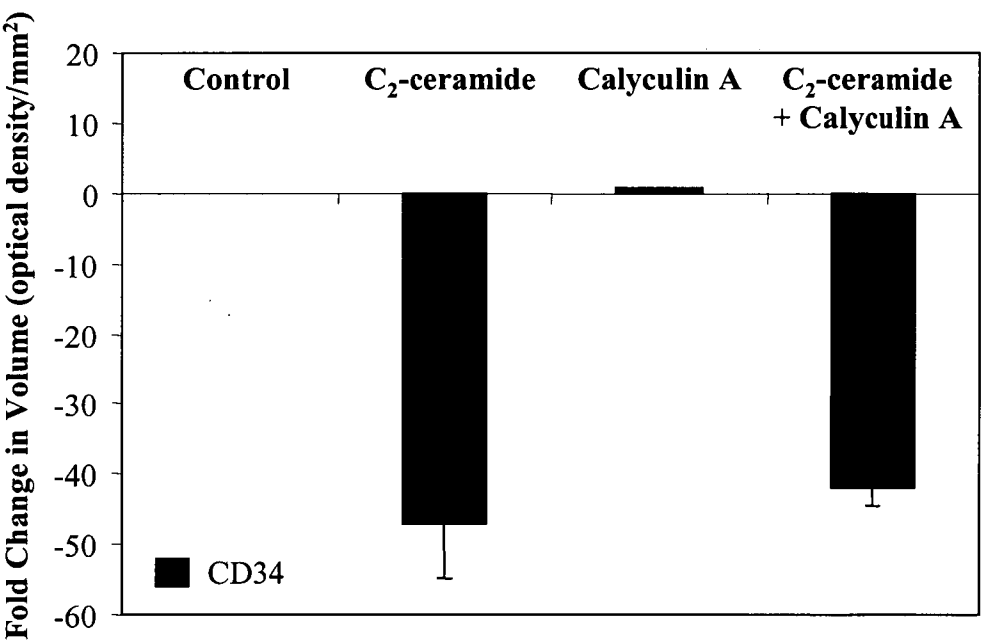

Fold Change in Volume (optical density/mm²)
20
10
0
-10
-20
-30
-40
-50
-60
Control
C2-ceramide
Calyculin A
C2-ceramide + Calyculin A
CD34

Table 4.1.: Table of raw data and final normalised results obtained from image analysis of the RPA blot shown in Figure 4.4. for KG-1 myeloblastic/promyeloblastic cells, showing the calculated fold changes in expression of *CD11b*, *CD14* and *CD34*. The raw data given in the table has been adjusted to subtract background. The data provided in this table is representative of data obtained from four repeated experiments.

N.R. = No result obtained (signal absent or too weak for quantitation).

* Normalised Volume = Raw volume data normalised to the 0 hour Control *GAPDH* raw volume value.

		Volume (optical density/mm^2)				
Gene		**0 hr Control**	**12 hr Control**	**12 hr C_2-ceramide**	**12 hr Calyculin A**	**12 hr C_2-ceramide + Calyculin A**
GAPDH	Raw Volume (OD/mm^2)	66.350	51.916	38.061	63.649	31.191
	*Normalised Volume (OD/mm^2)	1.000	0.783	0.574	0.959	0.470
CD11b	Raw Volume	1.575	1.431	1.021	1.889	0.737
	*Normalised Volume	1.575	1.829	1.779	1.969	1.568
CD14	Raw Volume	N.R.	N.R.	N.R.	N.R.	N.R.
	*Normalised Volume	N.R.	N.R.	N.R.	N.R.	N.R.
CD34	Raw Volume	11.181	10.137	2.867	12.279	2.419
	*Normalised Volume	11.181	12.946	4.995	12.804	5.147

Fold change in expression relative to the 12 hr control:

CD11b		1.0	1.0	1.08	0.86
CD14		N.R	N.R	N.R	N.R
CD34		**1.0**	**0.4**	**1.0**	**0.4**

p = 0.369

p = 0.088

Table 4.2.: Table of raw data and final normalised results obtained from image analysis of the RPA blot shown in Figure 4.5. for KG-1a promyeloblastic cells, showing the calculated fold changes in expression of *CD11b*, *CD14* and *CD34*. The raw data given in the table has been adjusted to subtract background. The data provided in this table is representative of data obtained from three repeated experiments.

N.R. = No result obtained (signal absent or too weak for quantitation).

* Normalised Volume = Raw volume data normalised to the 0 hour Control *GAPDH* raw volume value.

	Volume (optical density/mm^2)					
Gene		**0 hr Control**	**12 hr Control**	**12 hr C_2-ceramide**	**12 hr Calyculin A**	**12 hr C_2-ceramide + Calyculin A**
GAPDH	Raw Volume (OD/mm^2)	163.079	154.562	167.395	154.921	169.410
	*Normalised Volume (OD/mm^2)	1.000	0.948	1.027	0.950	1.039
CD11b	Raw Volume	N.R.	N.R.	N.R.	N.R.	N.R.
	*Normalised Volume	N.R.	N.R.	N.R.	N.R.	N.R.
CD14	Raw Volume	N.R.	N.R.	N.R.	N.R.	N.R.
	*Normalised Volume	N.R.	N.R.	N.R.	N.R.	N.R.
CD34	Raw Volume	16.215	15.782	9.522	16.170	9.690
	*Normalised Volume	16.215	16.651	9.277	17.022	9.325

Fold change in expression relative to the 12 hr control:

CD11b		N.R	N.R	N.R	N.R
CD14		N.R	N.R	N.R	N.R
CD34		**1.0**	**0.56**	**1.0**	**0.56**

p = 0.382

p = 0.010

4.4. Discussion

The purpose of this chapter was to identify whether protein phosphatase activity could account for ceramide-induced Rb activation and to determine if a link existed between ceramide-induced Rb activation and the induction of myeloid differentiation. K562, KG-1, KG-1a and Raji cell lines were used as representative ceramide-responsive cell lines to investigate whether ceramide-induced Rb activation could be prevented by inhibiting protein phosphatase activity. These cell lines gave the most profound Rb activation responses in experiments in the previous chapter of this thesis. The findings presented demonstrate that Rb activation is dependent, at least initially, on phosphatase activity. However differentiation, as evidenced by alterations in cell surface marker expression, is independent of phosphatase activity.

The phosphatase inhibitor Calyculin A is a well characterised inhibitor of the catalytic subunit of both type 1 and 2A protein phosphatase families (Ishihara *et al.*, 1989, Song & Lavin, 1993) and has been used extensively in studies of haemopoietic differentiation and apoptosis (Song & Lavin, 1993, Morana *et al.* 1996, Kishikawa *et al.*, 1999, Lee *et al.*, 2000). A number of investigators have shown that a ser/thr phosphatase (CAPP), belonging to the type 2A subfamily, is a direct target of ceramide signalling (Dobrowsky & Hannun, 1992, Dobrowsky *et al.*, 1993, Wolff *et al.*, 1994). CAPP was first identified by Dressler *et al.* (1992) as a result of initial studies into the sphingomyelin cycle in T9 glioma cells, as protein phosphorylation was known to be a key component of cell signalling. Since these early studies, other investigators have shown that CAPP may serve to mediate the cellular action of ceramide. Wolff *et al.* (1994) suggest that CAPP appears to be partly responsible for ceramide-mediated downregulation of c-*myc* in HL60 cells. Further, there is evidence to show that CAPP plays a role in protein kinase Cξ-induced translocation of preformed nuclear factor-κB from the cytosol to the nucleus (Dressler *et al.*, 1992, Dbaibo *et al.*, 1993, Lozano *et al.*, 1994, Muller *et al.*, 1995), and in ceramide-mediated activation of JNK and the subsequent expression of c-*jun* (Verheij *et al.*, 1996, Reyes *et al.*, 1996).

It has been shown in this thesis that ceramide can rapidly induce activation of the Rb tumour suppressor protein in the absence of a functional *p53* gene. Ceramide-treated K562, KG-1, KG-1a and Raji cells all exhibited the presence of a hypophosphorylated Rb band as early as 4 hours post-treatment, and even as early as 2 hours in the KG-1 and Raji cell lines. Thus ceramide-induced Rb activation is a rapid and early event. On treatment with the protein phosphatase inhibitor Calyculin

A, however, ceramide-induced Rb activation was abrogated even at the earliest timepoints analysed. To date, a clear link between ceramide-induced phosphatase activation and subsequent intracellular events had not been established. The results presented here provide a link between ceramide-induced phosphatase activity and the activation of the retinoblastoma protein and subsequent cell cycle arrest.

Supporting the notion that PPs are primarily responsible for Rb dephosphorylation, are the findings presented by Dou *et al.* (1995) who demonstrated that treatment of HL60 cells with araC resulted in the activation of Rb, although $p21^{Cip1/Waf1}$ could not be detected. However, araC-induced Rb dephosphorylation was inhibited by the addition of Calyculin A or okadaic acid, and therefore it was concluded from these experiments that protein phosphatases are predominantly responsible for Rb dephosphorylation, rather than induction of $p21^{Cip1/Waf1}$ expression. Interestingly, addition of these protein phosphatase inhibitors reproduced the growth arrest-specific Rb dephosphorylation seen following araC treatment, under cell-free conditions.

What remains to be determined is the exact type and species of protein phosphatase that is involved in ceramide-induced Rb activation. Although the substrates of CAPP remain largely undetermined, this phosphatase has been demonstrated to differentially modulate activity of cyclins and CDKs, namely CDK2 (Lee *et al.*, 2000). The possibility exists that Rb is also a likely candidate as a direct substrate for CAPP due to the rapidity of action demonstrated in this study, a notion that is further supported by other investigators (Voorhoeve *et al.*, 1999, Rubin *et al.*, 1998 & 2001). As Calyculin A is capable of inhibiting both PP1 and PP2A protein phosphatase family members, subsequent investigations into the characterisation of the identity of the protein phosphatase induced by C_2-ceramide are warranted, perhaps with the use of Okadaic acid which specifically inhibits PP2A phosphatase members only. It is worthy of mentioning however, that Lee *et al.* (2000) demonstrated that ceramide activates both protein phosphatases 1 and 2A, which provided the reasoning for the choice of Calyculin A used in the present study.

Cyclins associate with their respective catalytic partners, CDKs, and function to regulate the passage of cells through the cell cycle via phosphorylation of their substrate, which in the case of CDK4 and CDK6 is the Rb protein (Ewen *et al.*, 1993, Kato *et al.*, 1993, Sherr, 1994 & 1996). CDK activity is in turn regulated by phosphorylation and dephosphorylation events, as well as interactions with specific inhibitors, named cyclin-dependant kinase inhibitors (CKIs), which include $p21^{Waf1/Cip1}$. This CKI is crucial in inhibiting cyclin-CDK4 and CDK6 complex

formation and thus regulation of the phosphorylation status of Rb (Harper *et al.*, 1993, Harper *et al.*, 1995, Niculescu *et al.*, 1998).

An increase in $p21^{Waf1/Cip1}$ expression was also demonstrated as a ceramide-mediated event in all cell lines investigated. While Rb activation via induction of $p21^{Waf1/Cip1}$ expression requires transcriptional activation and subsequent translation, phosphatase-mediated Rb activation is transcription-independent and is therefore expected to occur much more rapidly than $p21^{Waf1/Cip1}$-mediated Rb activation. This is consistent with the findings presented by Zeng & el-Deiry (1996), and the results demonstrated in the present chapter, where distinct Rb activation is shown to occur as early as 2 hours post-treatment with C_2-ceramide. However, only a weak increase in $p21^{Waf1/Cip1}$ expression was observable by 4 hours, becoming more evident by 8 hours post-treatment with ceramide. Thus the ability of Calyculin A to prevent ceramide-induced Rb activation suggests that ceramide-induced activation of Rb is primarily mediated via protein phosphatase activity and not by transcriptional activation of $p21^{Waf1/Cip1}$. Therefore it is postulated that ceramide-induced expression of $p21^{Waf1/Cip1}$ is most likely a coincidental finding in relation to Rb activation, and is instead perhaps part of the differentiation response noted by others (Liu *et al.*, 1996a, Ragg *et al.*, 1998), or part of a redundant pathway.

The previous chapter used flow cytometry to assess changes in surface marker expression that arise from ceramide-induced myeloid differentiation, however, most cell lines did not exhibit changes measurable by flow cytometry until at least 24-48 hours post-treatment. The notable exception was K562 cells, where a significant downregulation in glycophorin A expression was observed as early as 12 hours post-treatment. The toxicity of Calyculin A confounded attempts to analyse CD34 downregulation by immunophenotyping, as CD34 surface marker downregulation in KG-1 and KG-1a cells is not significant until 24 hours post-treatment. As such, a different approach for analysing these early changes in CD34 downregulation was necessary. A custom designed RNase protection assay permitted the examination of changes in *CD34* RNA levels at a 12 hour timepoint.

On treatment with vehicle alone, the resulting immunophenotype expression profiles at 0 and 12 hours post-treatment demonstrated that K562 cells strongly express the glycophorin A cell surface marker, as reported by other authors (Andersson *et al.*, 1979, Koeffler & Golde, 1980), and confirming the results obtained in the previous chapter of this thesis. A significant left-shift in the expression profile was demonstrated following treatment with ceramide, also confirming the finding that ceramide induces glycophorin A downregulation in K562

cells which is consistent with the induction of a myeloid differentiation response. Calyculin A treatment alone resulted in an expression profile paralleling that produced by treatment with the vehicle alone. This demonstrates an important finding in this study, that Calyculin A treatment alone is not sufficient to induce any degree of differentiation in K562 cells, as determined by alterations in glycophorin A surface marker expression. Combined treatment with both ceramide and Calyculin A caused a significant left shift in the expression profile, equivalent in proportion to that seen after treatment with ceramide alone. Thus, the inhibition of protein phosphatase activity, and hence ceramide-induced Rb activation, has little or no effect on ceramide-induced K562 differentiation.

With the use of a custom designed RPA template set, which included riboprobes directed towards *CD11b*, *CD14* and *CD34* RNA transcripts, the same overall findings as that observed in K562 cells was demonstrated in KG-1 and KG-1a cells. Although conclusive results could not be obtained for *CD11b* and *CD14* analysis, the inclusion of the *CD34* riboprobe in the RPA template set proved to be very useful in demonstrating the effect of Calyculin A treatment on KG-1 and KG-1a differentiation. Expression of *CD11b* and *CD14* is a late maturation event, which would account for the absence in RNA signals at the 12 hour timepoint. A band corresponding to *CD34* was observed at both 0 and 12 hours in the vehicle-treated cell RNA extracts, thus confirming that parental KG-1 and KG-1a cells strongly express the CD34 surface marker as noted by other authors (Satterthwaite *et al.*, 1990, Pasternak & Pasternak, 1994). Treatment with ceramide reduced *CD34* expression compared with the time-matched Calyculin A-treated control, thus indicating differentiation towards a more mature phenotype (Berenson *et al.*, 1988 & 1991). Calyculin A treatment alone did not alter the expression of *CD34* compared with the vehicle-treated control, and combined treatment with ceramide and Calyculin A resulted in a lower intensity *CD34* band that paralleled that seen after treatment with ceramide alone. It is evident, therefore, that inhibiting protein phosphatase activity in KG-1 and KG-1a cells does not prevent ceramide-induced differentiation.

Although it was not observed in the present study using C_2-ceramide, it is interesting to note that inhibition of ser/thr protein phosphatases has been shown to augment myeloid differentiation in other models. Uzunoglu *et al.* (1999) have demonstrated that inhibition of PP1 and PP2A using Calyculin A and okadaic acid augmented methylprednisolone-induced granulocytic differentiation of HL60 cells and monocytic differentiation of K562 cells. Yuksel *et al.* (2002) also demonstrated

improved methylprednisolone-induced granulocytic and monocytic differentiation of HL60 and K562 cells, respectively. Morita *et al.* (1992) showed that treatment of HL60 cells with Calyculin A and okadaic acid lead to enhanced ATRA-induced granulocytic differentiation, although TPA-induced differentiation toward the macrophage lineage was unchanged. Similarly, no change was noted in arsenic trioxide-induced differentiation of HL60 and K562 cells. Downregulation of PP2A activity has been reported during ATRA-induced granulocytic differentiation of HL60 cells (Tawara *et al.*, 1993).

Although such reports of enhanced differentiation responses using a variety of differentiation agents (methylprednisolone and ATRA) following inhibition of protein phosphatase activity exist, this does not appear to be the case for ceramide. On its own, Calyculin A treatment was unable to induce myeloid maturation in the cell lines used in this study. In addition, Calyculin A did not prevent, or even appear to alter, the ceramide-induced differentiation response in K562, KG-1 or KG-1a cells. Therefore it can be concluded that ceramide and methylprednisolone/ATRA are involved in different signalling pathways.

In contrast to Rb dephosphorylation, the addition of the protein phosphatase inhibitor, Calyculin A, does not prevent ceramide-mediated differentiation in K562, KG-1 and KG-1a cells. These findings suggest that although Rb activation is essential for initiating cell cycle arrest, it is not required for ceramide-mediated myeloid differentiation. Furthermore, it can be concluded that ceramide-induced differentiation occurs via an independent signalling pathway to ceramide-induced Rb activation. Other authors (Rots *et al.*, 1999, Dimberg *et al.*, 2002) have suggested that cell cycle arrest and differentiation occur simultaneously and must therefore be linked to a common regulatory mechanism. Rots *et al.* (1999) report that a number of genes are upregulated by vitamin D_3 during myeloid differentiation, including the CKIs $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. However, the authors did not examine Rb activation, and thus failed to discuss the interdependence of Rb activation and differentiation. Rots *et al.* assumed that the expression of CKIs was entirely responsible for cell cycle arrest, and did not consider CKI-independent mechanisms such as protein phosphatase activation. The results from the present study clearly indicate that Rb activation and myeloid differentiation are independently regulated by ceramide. Regardless of the interdependence of these two cellular responses, it is important to remember that both cell cycle arrest and differentiation are crucial in the process of regaining control of immature leukaemia cells. Cell cycle arrest reduces the rate of

appearance of new cancer cells, and terminal differentiation increases the rate of removal of these cells via eventual apoptosis.

Taken together, the results from the present study, and those from the previous chapter of this thesis suggest that ceramide-signalling in haemopoietic cells simultaneously activates two independent pathways; the phosphatase-dependent activation of Rb, and the phosphatase-independent initiation of myeloid differentiation involving $p21^{Cip1/Waf1}$. As Rb activation is an early event following ceramide treatment (as early as 2 hours post-treatment in KG-1 and Raji cell lines), it is feasible to suggest that the direct removal of phosphate groups, or dephosphorylation of Rb via CDK2 inactivation (Lee *et al.*, 2000), by protein phosphatase activity are more likely to be mechanisms for Rb activation than transcriptional activation of $p21^{Cip1/Waf1}$. The subsequent indirect activation of Rb by inhibition of CDKs by CKIs would take a considerably greater period of time to occur than the rapid activation observed here. It is proposed therefore that protein phosphatases are primarily responsible for ceramide-induced Rb activation, and that CDK inactivation by $p21^{Cip1/Waf1}$ is most likely to be part of the terminal differentiation response, and/or perhaps plays a secondary role in ceramide-induced Rb activation. It is possible that protein phosphatases provide the initial early response to activate Rb and halt the cell cycle, and the expression and subsequent translation of $p21^{Cip1/Waf1}$ occurs later as part of the terminal differentiation response to lock the cell into a non-proliferative state.

Supporting the notion that PPs are primarily responsible for Rb dephosphorylation, are the findings presented by Dou *et al.* (1995) who demonstrated that treatment of HL60 cells with araC resulted in the activation of Rb, although $p21^{Cip1/Waf1}$ could not be detected. However, araC-induced Rb dephosphorylation was inhibited by the addition of Calyculin A or okadaic acid, and it was also therefore concluded from these experiments that protein phosphatases are predominantly responsible for Rb dephosphorylation, rather than induction of $p21^{Cip1/Waf1}$ expression. Interestingly, addition of these protein phosphatase inhibitors reproduced the growth arrest-specific Rb dephosphorylation seen following araC treatment, under cell-free conditions.

In conclusion, ceramide-signalling initiates at least two signalling pathways that independently regulate Rb activation and differentiation. As such, ceramide-induced Rb activation and myeloid differentiation are not dependent events. Inhibition of protein phosphatases 1 and 2A completely abolished ceramide-induced activation of the Rb protein, but did not alter the ability of ceramide to induce

myeloid differentiation. This highlights a key role for protein phosphatases in ceramide-mediated regulation of the cell cycle, and presents a novel target for directed therapy of leukaemia.

5.1. Introduction

The three major fates of haemopoietic cells, proliferation, differentiation and death, are closely intertwined. For example, cell proliferation and cell death are carefully balanced under normal circumstances. Similarly, induction of differentiation is usually associated with a loss in proliferative capacity, and cell death is associated with the latter stages of haemopoietic cell differentiation (Leszcyniecka *et al.*, 2001). A crucial prerequisite for haemopoietic cell terminal differentiation is cell cycle arrest (Freytag, 1988, Furukawa, 1997, 1998, & 2002), and a number of genes must be tightly regulated for correct progression through each phase. Given the complexities of the interrelations between haematopoietic cell proliferation, differentiation and death, it is not surprising that leukaemic transformation is related to deregulation of these processes. A greater understanding of these specific changes in gene expression that give rise to cancer may present an opportunity for future therapies.

The factors that regulate haemopoietic cell differentiation remain incompletely understood. It has become clear, however, over the last decade that changes in the expression levels of a plethora of genes occur in a programmed manner during the differentiation process. Early studies investigated intracellular signalling following the binding of haemopoietic growth factors to their appropriate receptor on the external surface of the cell. Since then, many single-gene studies have been performed to investigate the role of cell cycle regulators, transcription factors, and signal transduction pathways in the differentiation process.

Increased expression of $p21^{Cip1/Waf1}$ following differentiation induction by phorbol esters has been reported by a number of authors (Jiang *et al.*, 1994, Kharbanda *et al.*, 1994, Zeng & el-Deiry, 1996), and this is now an accepted feature of terminally differentiated cells (Halevy *et al.*, 1995, Liebermann *et al.*, 1995, Parker *et al.*, 1995, Flink *et al.*, 1998). Cell cycle arrest has been linked with the activation of TFs factors via differentiation-associated signalling. TFs shown to be triggered by differentiation stimuli in haemopoietic cells include PU.1 (Olson *et al.*, 1995, Simon *et al.*, 1996, Bellon *et al.*, 1997, DeKoter *et al.*, 1998, Anderson *et al.*, 1999, Oikawa *et al.*, 1999), c-jun (Adunyah *et al.*, 1992 & 1995), AP-1 (Liebermann *et al.*, 1998, Rosson & O'Brien, 1998), substance P-1 (Laurenzi *et al.*, 1989, Bost & Pascual, 1992, Pascual *et al.*, 1992), and downregulation in c-myc (Nguyen *et al.*, 1995a, Lerga *et al.*, 1999, Lin *et al.*, 2000).

The results from the studies presented in the previous chapter showed that ceramide-induced myeloid differentiation and Rb activation utilise independent pathways, and protein phosphatases are primarily responsible for ceramide-mediated Rb activation. Haemopoietic cell differentiation requires specific changes in gene expression, and to date very few studies have investigated the effect of ceramide on gene expression in relation to the differentiation process. As with the majority of studies that have investigated gene expression changes following treatment with various differentiating agents, research into ceramide-induced haemopoietic cell differentiation has primarily focussed on single gene studies, with particular focus on $p21^{Cip1/Waf1}$ expression (Ragg *et al.*, 1998, Lee *et al.*, 2000), *c-myc* and *c-jun* expression, and activation of the JNK/SEK MAPK pathway members (Sawai *et al.*, 1995, Westwick *et al.*, 1995, Verheij *et al.*, 1996, Ragg *et al.*, 1998, reviewed by Okazaki *et al.*, 1998). A comprehensive screen of genes known to play a role in the three major cellular fates (proliferation, differentiation, and cell death), genes that are often deregulated in malignancies such as leukaemia, is clearly absent from the literature.

There are a number of techniques available for conducting small-scale gene expression screening in a cell population. The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection and quantitation of mRNA species. This assay utilises a DNA-dependent RNA polymerase from the T7 bacteriophage in constructing RNA probes from a DNA template containing the T7 promoter. PharMingen has developed a series of multi-probe RPA template sets, which have been assembled into biologically relevant groups, to be used in conjunction with their RiboQuant™ Multi-Probe RPA System (PharMingen, USA). Within each set are a number of DNA templates, each of distinct length and each representing a sequence in a distinct mRNA species. Having synthesised radiolabelled RNA probes from the DNA template set, the probes are hybridised in excess to target RNA from experimental samples. Any free probes and single-stranded RNA that has not hybridised to the probes are digested with RNases and the remaining "RNase-protected" probes are purified, resolved on denaturing polyacrylamide gels, and the quantity of each mRNA species is determined by autoradiography of by phosphorimaging with normalisation to housekeeping gene expression (included in each template set). The distinct advantage of the RPA approach is that several mRNA species can be simultaneously quantitated in a single sample of total RNA (PharMingen, USA), in contrast to other methods such as Northern blotting and semi-quantitative RT-PCR.

There has been an explosive increase in the knowledge of genes at the sequence level over the last decade, especially since the commencement of the human genome project (Aaronson *et al.*, 1996, Hillier *et al.*, 1996). The rapid increase in sequence information has required corresponding improvements in the following steps of genetic analysis. Existing approaches to genetic analysis focus on individual analysis of particular genes and do not provide a means for high-throughput implementation. There are numerous other methods available to detect and quantify differential gene expression. Differential display (Liang & Pardee, 1992) and its variants provide an efficient way to identify genes that are differentially expressed between two tissues or cell types, or between a control and experimental sample. It does not, however, give quantitative expression information. Systematic cDNA sequencing (Okubo *et al.*, 1992) will provide quantitative results, but is a very tedious technique when investigating genes with low expression levels. The SAGE (serial analysis of gene expression) method (Velculescu *et al.*, 1995) diminishes sequencing requirements but the method is complicated and statistical limitations still apply (Jordan, 1998).

Recently, a hybridisation technique has become available for simultaneous expression analysis of hundreds to tens of thousands of genes, a method known as cDNA expression array analysis. In contrast to previous techniques available for gene expression analysis, hybridisation methods use complex probes and large arrays of targets, and derive their power from the fact that each individual experiment provides a very large amount of information. They appear unrivalled for large-scale measurement of gene expression (Jordan, 1998) and provide an accurate and sensitive evaluation of the magnitude of gene expression changes occurring between two target cell populations (Leszcyniecka *et al.*, 2001). Quantitative use of cDNA arrays for expression measurement using high-density membranes was pioneered by Hans Lehrach's group (Gress *et al.*, 1992), and full implementation of this technique was later published by several authors (Nguyen *et al.*, 1995b, Zhao *et al.*, 1995, Pietu *et al.*, 1996). Three main types of cDNA arrays have been developed (high-density nylon membranes, glass slide microarrays, and oligonucleotide chips), and differ primarily in the way they are analysed and more significantly in the number of genes they permit analysis of. In each design type, plasmid DNA, PCR products, or oligonucleotides are immobilised to a solid support, and complex cDNA probes derived from poly A^+ RNA labelled with radioactive or fluorescent nucleotides are hybridised.

High-density membrane cDNA arrays, or nylon filter arrays, have been used by a number of groups for RNA expression analysis (Dunne *et al.*, 1992, Maier *et al.*, 1994, Nguyen *et al.*, 1995b, Bernard *et al.*, 1996, DeRisi *et al.*, 1996, Gress *et al.*, 1996, Lanfranchi *et al.*, 1996, Pietu *et al.*, 1996, Sehgal *et al.*, 1998). Robotics have made possible the generation of high-density arrays capable of producing results for analysis of nearly 60,000 genes in a 22 X 22 cm^2 area. Generally, high-density nylon arrays keep a niche for relatively small-scale work, for investigating specific sets of genes of interest (Jordan, 1998), and are usually limited to 500 - 2,000 known genes. Blots are hybridised with ^{33}P or ^{32}P-labelled cDNA isolated from test and control tissue or cell samples. Data can be captured on phosphorimager screens or by exposure to X-ray film and subsequent image analysis is performed to determine the relative optical intensity for each gene producing a signal. Hybridisation conditions must give low background (since signals are quite small) and ensure equivalent hybridisation rate over the whole membrane. Long hybridisation times are often used to maximise the signal intensity that increase linearly with probe concentration and hybridisation duration. This approach has been found to yield highly reproducible results by many investigators (Baldwin *et al.*, 1999), although it is accepted that semi- or fully-quantitative methods such as RT-PCR, Northern blotting or real-time RT-PCR should be used to confirm candidates identified on filter arrays. The aims of this chapter were firstly to investigate the changes in gene expression of nine genes involved in cell cycle regulation, DNA damage and apoptosis in cells induced to differentiate using ceramide using an RPA, incorporating the hStress 1 RiboQuant™ template set (PharMingen, USA). This preliminary genetic screen was then expanded with the application of Atlas™ Human Cancer 1.2 cDNA Expression Arrays (Clontech, USA) as a method of mass screening for changes in expression of genes that are known to be involved in a variety of malignancies to investigate the response of other cancer-related genes following ceramide treatment.

5.2. Methods

5.2.1. Experimental Plan

A screen of cell cycle-, DNA damage-, and apoptosis-related genes was performed in a ceramide-responsive cell line using the hStress 1 RiboQuant™ template and the RiboQuant™ Multi-Probe RNase Protection Assay System (PharMingen, USA). HL60 cells were used for this preliminary screen, as this was the first cell line shown to differentiate in response to ceramide treatment (Okazaki *et al.*, 1989, Okazaki *et al.*, 1990, Kim *et al.*, 1991) and this model has been utilised extensively to investigate aspects of ceramide-mediated changes at the morphological and protein level. HL60 cells were treated with C_2-ceramide and RNA was extracted at various timepoints and analysed by RPA. This allowed for a rapid screen of the involvement of various human genes known to play a role in cell cycle regulation, DNA damage repair, and apoptosis, following ceramide treatment in this cell line, and this provided the proof of principle for a more comprehensive study to follow. These genes include the *p53* tumour suppressor, the *growth arrest and DNA-damage-inducible protein 45* (*GADD45*), the differentiation- and proliferation-related transcription factor *c-fos*, and the CKI $p21^{Cip1/Waf1}$. Included in the template set are the anti-apoptotic members *bcl-2* and the closely related *bcl-*X_L, and *mcl-1*, and the pro-apoptotic members *bax* and *bcl-*X_S (Oltvai *et al.*, 1993, Lincz, 1998, Minn *et al.*, 1998, Pellegrini & Strasser, 1999).

Clontech has produced high-density nylon membrane arrays, such as the Atlas™ Human Cancer 1.2 cDNA Expression Array, which includes 1,176 genes that are known to be involved in human cancers. The various DNA targets are arrayed onto nylon membranes into functional classes including tumour suppressors and oncogenes, cell cycle regulators, signal transduction modulators and effectors, apoptosis effectors, DNA damage repair proteins, transcription factors, growth factors and chemokines and their receptors, stress response proteins, proteins involved in metabolic pathways, and cytoskeleton and motility proteins. Plasmid and bacteriophage DNA is included as negative controls to confirm hybridisation specificity, along with DNA for several housekeeping genes, which act as positive controls and provide a means for normalising mRNA abundance. GenBank (NCBI, USA) accession numbers are provided for all gene targets included on all Atlas™ arrays.

The Atlas™ Human Cancer 1.2 cDNA expression array system (Clontech, USA) was used in the current study to investigate the changes in RNA expression of

1,176 genes known to be involved in human cancer. As only purified cDNA is spotted onto Atlas™ arrays, the sensitivity is regarded to be higher than commercially available filter arrays spotted with lysed bacterial colonies available from other sources (Bowtell, 1999). Atlas™ arrays have been extensively used for a range of applications including the demonstration of differentially expressed genes following stimulation of B and T lymphocytes (Ollila & Vihinen, 1998), and differentially expressed genes in nasopharyngeal carcinoma (Xie *et al.*, 2000). Negative controls consisting of plasmid and bacteriophage cDNA spots are included to confirm hybridisation specificity, along with several housekeeping cDNAs which act as positive controls for normalising mRNA abundance. cDNAs for the genes of interest are arrayed into functional classes that represent specific areas of research, in the case of the 1.2 Human Cancer array, genes that are known to be involved in human cancers. The known genes on this array are divided into 25 main categories: cell surface antigens, transcription, cell cycle, cell adhesion receptors/proteins, immune system proteins, extracellular transport/carrier proteins, oncogenes and tumour suppressors, stress response proteins, membrane channels and transporters, extracellular matrix proteins, trafficking/targeting proteins, metabolism, post-translational modification/protein folding, translation, apoptosis associated proteins, RNA processing turnover and transport, DNA binding and chromatin proteins, cell receptors (by ligands), cell signalling/extracellular communication proteins, intracellular transducers/effectors/modulators, protein turnover, cell receptors (by activities), cytoskeleton/motility proteins, functionally unclassed, and DNA synthesis recombination and repair.

The results from previous chapters have shown that the erythromyeloid K562 cell line was the most responsive cell line (in terms of magnitude and rate) to ceramide-induced growth arrest and differentiation, while resulting in only minimal cell death relative to the other cell lines studied. As the focus of the final section of the current study was to be directed towards the growth arrest and differentiation-inducing ability of ceramide in cells of the myeloid lineage, the K562 cell line was chosen to be utilised for cDNA array analysis in order to perform a mass screen of "cancer-related" gene expression following ceramide-induced differentiation. Cells were treated with vehicle or C_2-ceramide and RNA was extracted at early (2 hours post-treatment), intermediate (8 hours post-treatment) and late (24 hours post-treatment) timepoints. Radiolabelled cDNA probes were generated from this RNA and were hybridised to Atlas™ Human Cancer 1.2 cDNA Expression Arrays (Clontech, USA). A comparison was then made between the expression of genes

following treatment with C_2-ceramide and gene expression in the time-matched, vehicle-treated control cells.

5.2.2. RNase Protection Assays

HL60 cells were treated with vehicle or C_2-ceramide (7.5 μM) at 5 X 10^5 cells/mL in RPMI + 2% FCS. The treated cells were transferred to 75 cm^2 cell culture flasks and incubated at 37°C/5% CO_2. Cells (1 X 10^7 cells/sample) were harvested at 0, 4 and 8 hours post-treatment, and RNA extracted, DNase-treated and quantitated as outlined in Chapter 2.2.7.

RNase protection assays were performed on the prepared RNA, and were analysed as described in Chapter 2.2.8., using the human stress-1 (hStress-1) template (Cat. # 45351P, PharMingen, USA). The hStress-1 template contains riboprobes directed towards human *bcl-X_L*, *bcl-X_S*, *p53*, *GADD45*, *c-fos*, *$p21^{Cip1/Waf1}$*, *bax*, *bcl-2* and *mcl-1* RNA transcripts, and the house-keeping gene transcripts *L32* and *GAPDH*.

5.2.3. cDNA Array Analysis

K562 cells were treated with vehicle or 5 μM C_2-ceramide in serum-free RPMI media at 5 X 10^5 cells/mL, and were not pre-incubated prior to their addition. Cells were transferred to 75 cm^2 cell culture flasks and incubated at 37°C. Cells (1 X 10^7 cells/sample) were harvested at 2, 8 and 24 hours post-treatment and RNA was extracted, DNase-treated and quantitated as detailed in Chapter 2.2.7.

cDNA array analysis was performed using the protocol and reagents provided in the Atlas™ Pure Total RNA Labelling System, and Atlas™ Human Cancer 1.2 cDNA Expression Array kits (Cat . # K1038-1 and # 7851-1 respectively, Clontech, USA). Refer to Chapter 2.2.9. for an outline of the cDNA array experimental procedure performed.

5.2.4. Confirmation of Array Results

The results obtained from cDNA array analysis were confirmed by Sandrine Chopin in our laboratory by performing real-time RT-PCR analysis on four representative genes that were included on the Atlas™ Human Cancer 1.2 cDNA Expression Array. These genes only were chosen because they represent different expression patterns and can be taken as specific 'random' examples to confirm the reliability of the Atlas™ cDNA array hybridisation. The genes chosen for confirmation purposes were *PRAME* (*preferentially expressed antigen of melanoma*),

PCNA (*proliferating cell/cyclin nuclear antigen*), *MIC1* (*macrophage inhibitory cytokine 1*), and *GADD153* (*growth arrest and DNA-damage-inducible protein 153*). A primer set directed towards *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) (an endogenous house-keeping control gene that is in abundant levels and remains constant at all timepoints and following treatment) was also designed. *GAPDH* was amplified simultaneously with the amplification of the confirmatory genes, to ensure the validity of each run, and to enable calculation of concentrations in each of the RNA samples. The same RNA that was extracted from vehicle- and C_2-ceramide-treated K562 cells at 2, 8, and 24 hours and used in the array experiments was also used for all confirmation experiments.

Primer sets were designed for each of the gene sequences, and conditions were optimised for amplification of the genes using standard RT-PCR as outlined in Chapter 2.2.10. Primer sequences and optimised standard RT-PCR conditions can be found in Tables 2.8. and 2.9(A). and (B), respectively.

Real-time RT-PCR was performed to confirm the cDNA array results using the LightCycler system, and LightCycler RNA Master SYBR Green I real-time RT-PCR kit and capillaries (Roche Diagnostics, Germany). Optimisation of the conditions for real-time RT-PCR was performed for each primer set as outlined in Chapter 2.2.11.

Amplification curves and melting curves were generated using the software included with the LightCycler system (Roche Diagnostics, Germany) for each confirmatory gene. The amplification curve and the corresponding crossing point (the point at which SYBR Green I fluorescence was detected above background fluorescence) for each gene was compared for each sample to enable the determination of their relative expression levels between vehicle- and C_2-ceramide-treated samples. The melting curve was generated to check the specificity of the primer sets and to ensure the SYBR Green I fluorescence was being measured at a temperature that was below the Tm and above primer-dimer (if present).

5.3. Results

5.3.1. Expression of Cell Cycle-, DNA-Damage- and Apoptosis-Related Genes Following Ceramide Treatment

The RNase protection assay blot obtained for HL60 promyelocytic cells following treatment with vehicle and C_2-ceramide at 4 and 8 hours post-treatment is presented in Figure 5.1. Multiple exposures of varying times were used to overcome pixel saturation for normalisation purposes, and only one exposure time is shown in the results figure in this thesis for representative purposes. The raw optical density data generated from image analysis was normalised to the expression of housekeeping genes included in the template set and fold changes in induced gene expression between time-matched, vehicle-treated control cells and C_2-ceramide-treated cells were calculated and are presented in Tables 5.1.A and 5.1.B.

A significant change in expression was considered to be a 1.5 fold increase/decrease after normalisation of the data, as suggested by the manufacturer of the RPA kit. A significant change in expression was exhibited for *bcl-X_L*, *GADD45*, *c-fos*, *$p21^{Cip1/Waf1}$*, and *bcl-2* in HL-60 cells following ceramide treatment. A 1.7 fold increase in *bcl-X_L* was demonstrated by 4 hours post-treatment with ceramide, and this increased expression continued up to the 8 hour timepoint (1.9 fold change in expression). Although a strong *p53* band was easily observable in the HeLa control lane, no bands were detectable in any of the HL60 RNA extracts, presumably as HL60 cells lack a functional p53 gene. Strong basal expression of *GADD45* was identified in HL60 cells, and a 1.6 fold increase in expression was demonstrated at 4 hours following ceramide treatment, however, no change in expression was noted at 8 hours. A 1.5 fold increase in *c-fos* expression was seen at 4 hours post-treatment with ceramide, which decreased to near basal levels by 8 hours for both the vehicle- and ceramide-treated cells.

$p21^{Cip1/Waf1}$ expression showed the greatest degree of change, with a 4.9 fold increase in expression at 4 hours, rapidly returning to an expression level slightly above that of basal levels in ceramide-treated cells by 8 hours. A 1.5 fold increase in expression was seen for both *c-fos* and *bcl-2* at 4 hours post-treatment, however, no significant changes was noted by 8 hours for either gene. Although *bax* and *mcl-1* were shown to be strongly expressed in HL60 control cells at 0 hours, only a slight increase in expression was observed for these genes at 4 hours post-treatment (1.3 fold increase), although these changes were not considered significant. No significant

change in expression was seen at 8 hours post-treatment with ceramide for either *bax* or *mcl-1*.

No bands were observable on the autoradiograph in the yeast tRNA negative species sensitivity control lane, while the full complement of genes were seen in both the undigested probe and the HeLa positive control.

Figure 5.1.: Analysis of cell cycle, DNA damage, and apoptosis-related gene expression in HL60 promyelocytic cells at 4 and 8 hours post-treatment with either vehicle or C_2-ceramide using the RiboQuant™ RNase protection assay with the hStress1 template (Pharmingen, USA). The RPA blot is provided in its entirety for comparison of expression of housekeeping genes between each sample. A single representative set of results from at least three repeated experiments is shown.

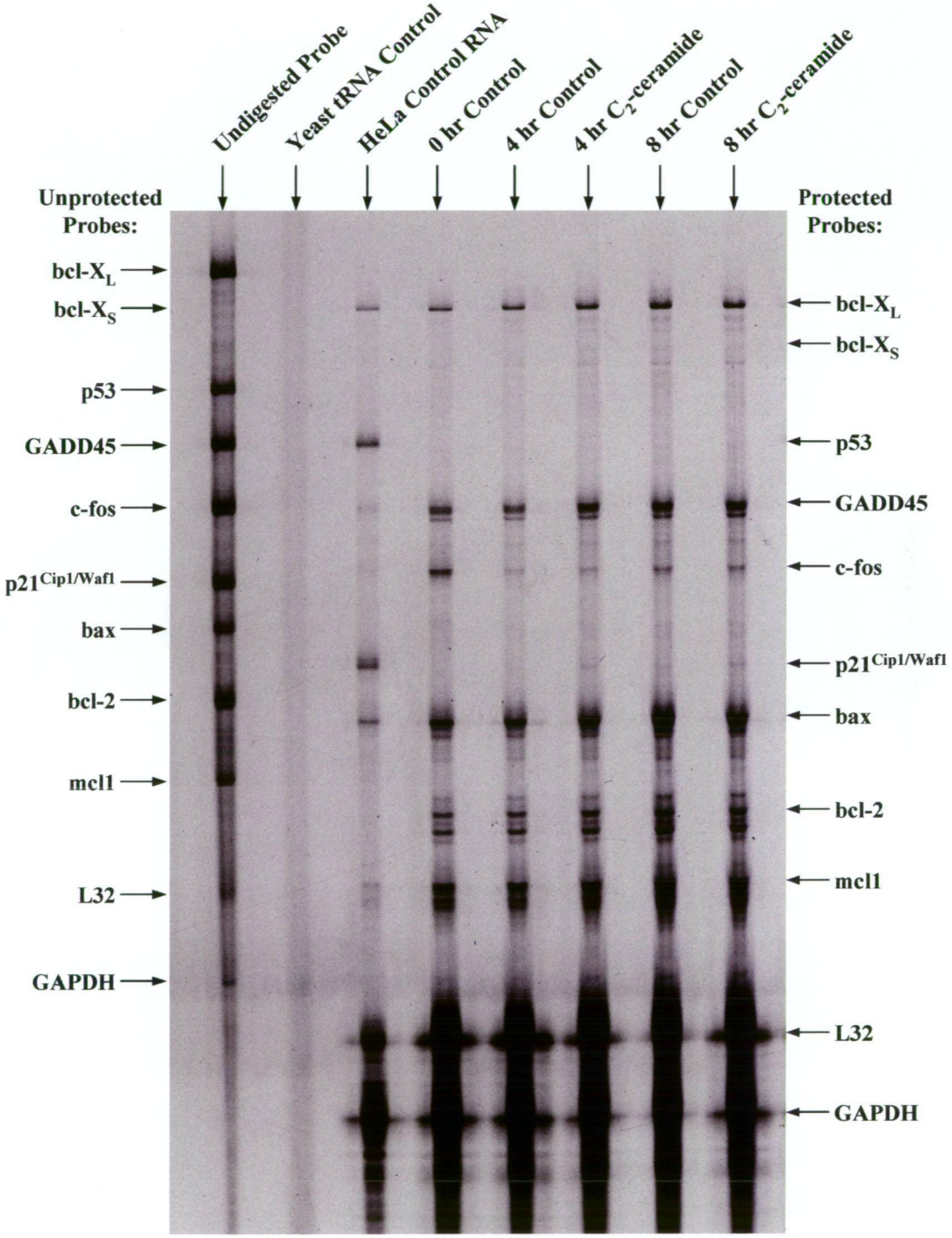

Undigested Probe
Yeast tRNA Control
HeLa Control RNA
0 hr Control
4 hr Control
4 hr C2-ceramide
8 hr Control
8 hr C2-ceramide
Unprotected Probes:
bcl-XL
bcl-XS
p53
GADD45
c-fos
p21Cip1/Waf1
bax
bcl-2
mcl1
L32
GAPDH
Protected Probes:
bcl-XL
bcl-XS
p53
GADD45
c-fos
p21Cip1/Waf1
bax
bcl-2
mcl1
L32
GAPDH

Table 5.1.A.: Table of final normalised data obtained from image analysis of the RPA blot for HL60 promyelocytic cells (shown in Figure 5.1.) using the hStress1 RiboQuant template (PharMingen, USA) at 4 and 8 hours post-treatment with vehicle or 7.5 μM C_2-ceramide. All optical density data presented in the table are normalized to the 0 hour Control *GAPDH* housekeeping gene to allow the determination of fold change in expression of the various genes investigated at each timepoint (Table 5.1.B.), where a signal was detectable. The raw data given in the table has been adjusted to subtract background. The data provided in this table is representative of data obtained from at least three repeated experiments.

N.R. = No result obtained (signal absent or too weak for quantitation).

	Volume Normalised to 0 hour Control *GAPDH* (optical density/mm^2)				
Gene	**0 hr Control**	**4 hr Control**	**4 hr C_2-ceramide**	**8 hr Control**	**8 hr C_2-ceramide**
GAPDH	1.000	1.020	1.084	1.120	1.138
bcl-X_L	156.749	256.786	438.054	530.663	997.732
bcl-X_S	N.R.	N.R.	N.R.	N.R.	N.R.
p53	N.R.	N.R.	N.R.	N.R.	N.R.
GADD45	546.589	579.095	935.688	1034.631	1104.791
c-fos	436.146	132.583	193.520	263.639	219.854
p21$^{Cip1/Waf1}$	12.082	24.754	120.546	108.025	84.000
bax	1142.935	1191.150	1550.615	2169.471	1854.850
bcl-2	755.740	845.926	1271.414	1873.553	1567.238
mcl1	1588.427	1584.709	2052.006	2781.280	2453.099

Table 5.1.B.: Fold change in gene expression relative to time-matched controls.

Gene	4 hours	8 hours
bcl-X_L	1.71	1.88
bcl-X_S	N.R.	N.R.
p53	N.R.	N.R
GADD45	1.62	1.07
c-fos	1.46	0.83
p21$^{Cip1/Waf1}$	4.87	0.78
bax	1.30	0.86
bcl-2	1.50	0.84
mcl1	1.30	0.88

5.3.2. Screen of Cancer-Related Genes by cDNA Array Analysis

K562 cells were treated with vehicle or C_2-ceramide and RNA extracted at 2, 8 and 24 hours post-treatment and hybridised to Atlas™ Human Cancer 1.2 cDNA Expression Arrays. Figures 5.2.A., 5.3.A. and 5.4.A. show the resulting blots that were obtained from cDNA array hybridisation experiments at 2, 8 and 24 hours post-treatment respectively. Differential expression is shown in Figures 5.2.B., 5.3.B. and 5.4.B. by false colouring the blot images (control blot performed in the red channel, and C_2-ceramide blot performed in the green channel), and overlaying both blots from each respective timepoint. As such, genes that are more highly expressed on the control array will appear as red spots (downregulated following C_2-ceramide treatment), and genes that are more highly expressed on the C_2-ceramide array will appear as green spots (upregulated following C_2-ceramide treatment). Those genes that do not change in expression appear as yellow spots. This technique provides a means for rapid screening of the array to determine which genes are differentially expressed.

Genes demonstrating a 1.3 fold change or greater in relative pixel density were considered differentially expressed, as suggested by the manufacturer of the array and a figure that has been adopted in previously published studies using this filter array kit (Ollila & Vihinen, 1998, Xie *et al.*, 2000). Genes that were shown to be significantly differentially expressed at one or more timepoints following C_2-ceramide treatment are compiled in Table 5.2. Image analysis was performed on the cDNA array blots shown in Figures 5.2.A., 5.3.A. and 5.4.A. to generate optical density data which was then normalised to the expression of housekeeping genes included on the array. Included in Table 5.2. are the individual images of spots for each respective gene, taken from the blot overlays (Figures 5.2.B., 5.3.B. and 5.4.B.) at each timepoint for direct qualitative comparison.

While spot saturation is a common feature of nylon filter arrays, this problem was overcome using multiple exposure times ranging from 5 hours up to 11 days. Only one exposure time is shown in the results figures in this thesis for representative purposes. Image analysis of the results was conducted using densitometry and specialised pixel volume image analysis software. This technique was somewhat subjective, although it was sufficient for the determination of general trend in gene expression changes. As such the investigator suggests that that fold changes calculated and presented in this thesis for each gene should be considered approximate only, and not necessarily an absolute reflection of the exact result, hence

the need for further confirmation experiments using techniques such as real-time RT-PCR.

A large number of genes were shown to be altered in expression following ceramide treatment. The categories of genes that were generally upregulated include those involved with negative cell cycle and growth control, DNA damage, differentiation, and cytoskeletal genes, while downregulated genes include those that are involved in cell proliferation and regulating metabolic pathways. A significant increase in the transcription factor-related oncogene *c-jun* (*AP-1 TF complex*) expression was seen at 8 and 24 hours (6.1 and 5.2 fold increases, respectively) while a decrease in *c-myc* expression was seen at both 2 (3.3 fold decrease) and 24 (1.8 fold decrease) hours. An increase of 1.5 fold at 8 hours and 3.9 fold at 24 hours was demonstrated for *c-jun N-terminal kinase 2* (*JNK2*), which is consistent with the increase in *c-jun* expression. However, a decrease in expression of the jun activation domain binding protein was demonstrated at the later timepoints analysed, with a 2.2 fold decrease seen at 8 hours and a 1.6 fold decrease at 24 hours.

There was an increase in expression of other transcription factors including the *TIS11B protein* (*EGF response factor 1*), the *early growth response protein 1* (*hEGR1*), and the *cAMP-responsive element modulator 1 alpha protein* (*HCREM*).

Changes were noted in the expression of cell cycle-related genes including the *G_1/S-specific cyclin D3*, *cyclin H*, the *CDK regulatory subunit 1*, *$p21^{Cip1/Waf1}$* and *$p57^{Kip2}$*. There was a very significant increase in the expression of *growth inhibitory factor* (8.8 fold at 24 hours), and a decrease in cell cycle regulatory *protein D123* (2.3 fold at 2 hours and 2.5 fold at 24 hours) and *cell cycle protein P38-2G4 homolog*. A decrease in expression of *proliferating cyclic/cell nuclear antigen* (*PCNA*) was demonstrated at all timepoints, with a 1.6 fold decrease at 2 hours, extending to a 1.9 fold and 2.6 fold decrease by 8 and 24 hours, respectively. A significant increase in expression (5.2 fold at 8 hours, and 4.1 fold at 24 hours) was seen for the *dual-specificity protein phosphatase 8*, a tyrosine phosphatase.

Changes were noted in genes known to play a role in apoptosis, including an increase in expression of the Bcl family member *induced myeloid leukaemia cell differentiation protein mcl-1*. A significant increase in expression of the *growth arrest and DNA-damage-inducible protein 153* (*GADD153*) was seen at 8 hours (1.5 fold) and increased further to a 9.0 fold change by 24 hours. In addition, an increase in expression of *GADD45* was seen at 24 hours (1.8 fold).

There were changes in expression of signal-transduction-related genes including an increase in expression of the G proteins *transforming protein rho B*, and

ras-related C3 botulinum toxin substrate 1 and 2. A decrease in expression was demonstrated for the calcium-binding proteins *calmodulin 1* and *calvasculin*, while there was an increase in expression of the signal transduction modulators and effectors *zyxin + zyxin-2, junction plakoglobin*, and decrease in expression of other signal transduction modulators and effectors including *guanylate kinase* and the *B-cell receptor-associated protein.* Decreases in a number of stress response proteins, including *cytosolic superoxide dismutase 1* (*SOD1*), *microsomal glutathione S-transferase II* (*microsomal GSTII*), and *GST homolog* were demonstrated.

Increases in expression of the genes translated into DNA-binding and chromatin proteins, including *high mobility group protein 1* (*HMG-1*) and *DNA-binding protein CPBP* (8.2 fold at 8 hours, and 9.1 fold at 24 hours) were seen, while a decrease was demonstrated for *HMG-2*, *HMG-17*, and the *chromatin assembly factor 1 p48 subunit* (also known as the *retinoblastoma-binding protein 4* [*RBBP4*]).

An initial decrease in expression (2.2 fold) was seen for the cell surface antigen *preferentially expressed antigen of melanoma* (*PRAME*), while only a slight increase (1.5 fold) in expression was noted by 8 hours, and no significant change was seen by 24 hours. An increase in expression was demonstrated for the cell adhesion receptors and proteins *integrin alpha E precursor* and *integrin beta 8 precursor.* Changes (an increase in expression by 24 hours) were seen in expression levels of various growth factors, cytokines, and other extracellular communication proteins, including the *macrophage inhibitory cytokine 1* (*MIC1*). Contrary to this was the expression of *macrophage migration inhibitory factor* (*MIF*), which was consistently shown to be downregulated at all timepoints (1.6 fold).

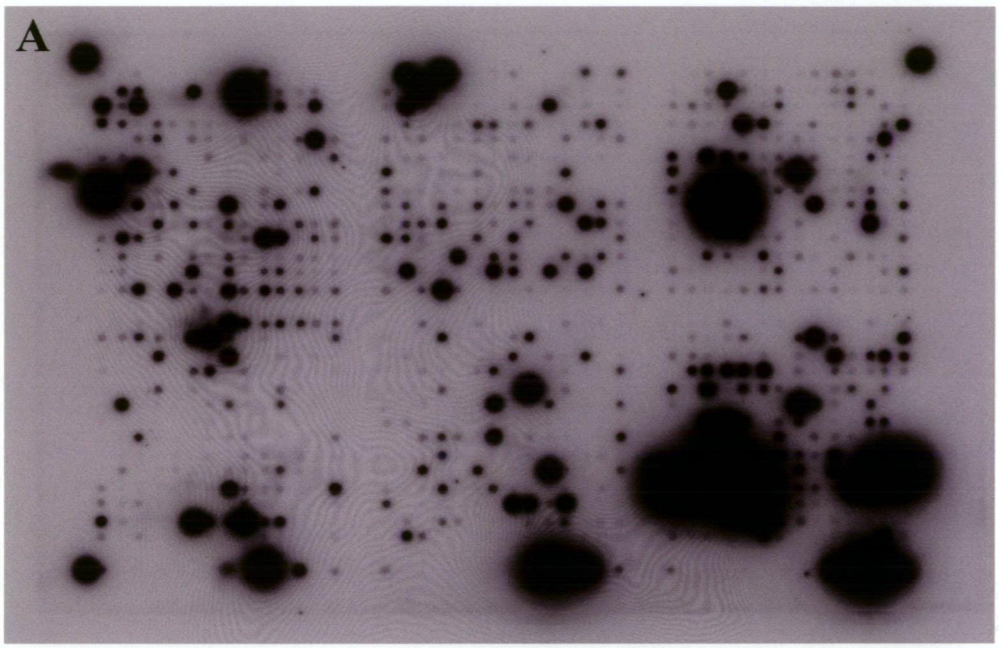
There was a decrease in expression of a number of genes known to be involved in regulation of metabolic pathways, including *nucleoside-diphosphate kinase*, *inosine-5'-monophosphate dehydrogenase 2*, *thymidylate synthase*, *L-lactate dehydrogenase M subunit*, and *ornithine decarboxylase.* Each of these genes demonstrated little or no change at the initial 2 hour timepoint. *Fatty acid synthase* was the only member of this family of genes that demonstrated an increase in expression.

There was initial downregulation in expression of a number of cytoskeletal proteins including *vimentin*, *desmin*, and two *cytokeratin* genes, which was followed by an increase in expression at the 8 and 24 hour timepoints.

A downregulation in expression of the *haemoglobin alpha subunit* was demonstrated at both the 8 and 24 hour timepoints. An initial decrease in another

trafficking protein, the *TRAM protein*, changed to an increase in expression by 24 hours.

Figure 5.2.A.: Demonstration of differential expression of "cancer-related" genes using the Atlas™ Human Cancer 1.2 cDNA Expression Array (Clontech, USA) for K562 erythromyeloid cells at 2 hours post-treatment with vehicle (**A**) and 5 μM C_2-ceramide (**B**).


A

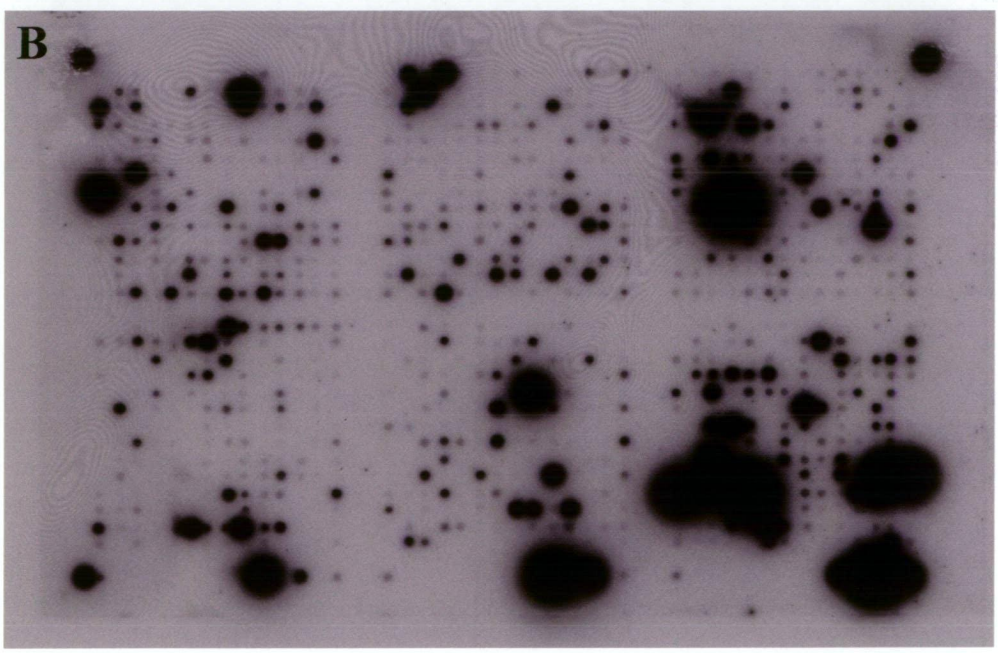
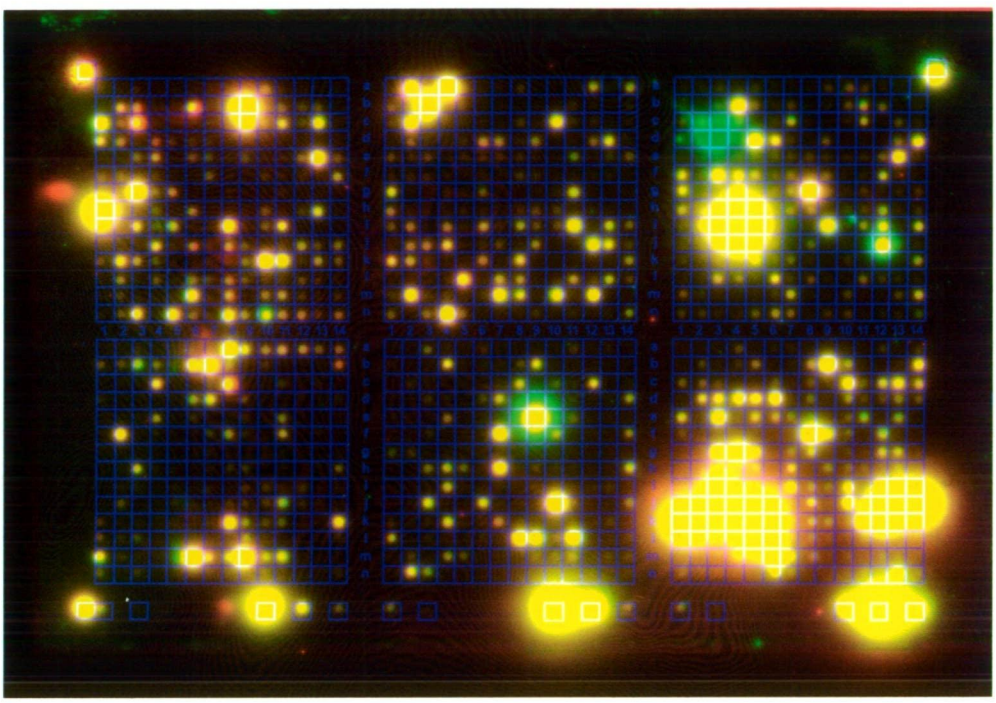
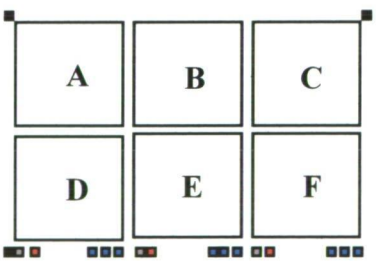

B

Figure 5.2.B.: K562 2 hour cDNA array blots from Figure 5.2.A., overlaid and false-coloured. Genes that are upregulated in expression following C_2-ceramide treatment are represented as green spots, while genes that are downregulated are represented by red spots. Genes that have no change in expression appear as yellow spots.

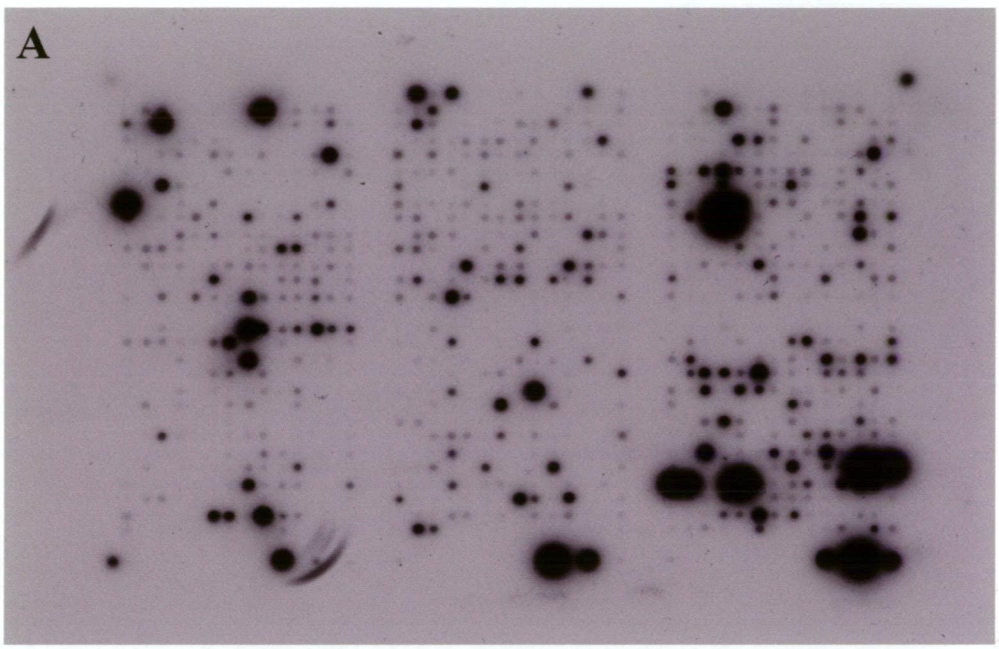


Key:



- Orientation Marks
- Human Genomic DNA
- Negative Controls
- House-Keeping Genes (Positive Controls)

Figure 5.3.A.: Demonstration of differential expression of "cancer-related" genes using the Atlas™ Human Cancer 1.2 cDNA Expression Array (Clontech, USA) for K562 erythromyeloid cells at 8 hours post-treatment with vehicle (**A**) and 5 μM C_2-ceramide (**B**).


A

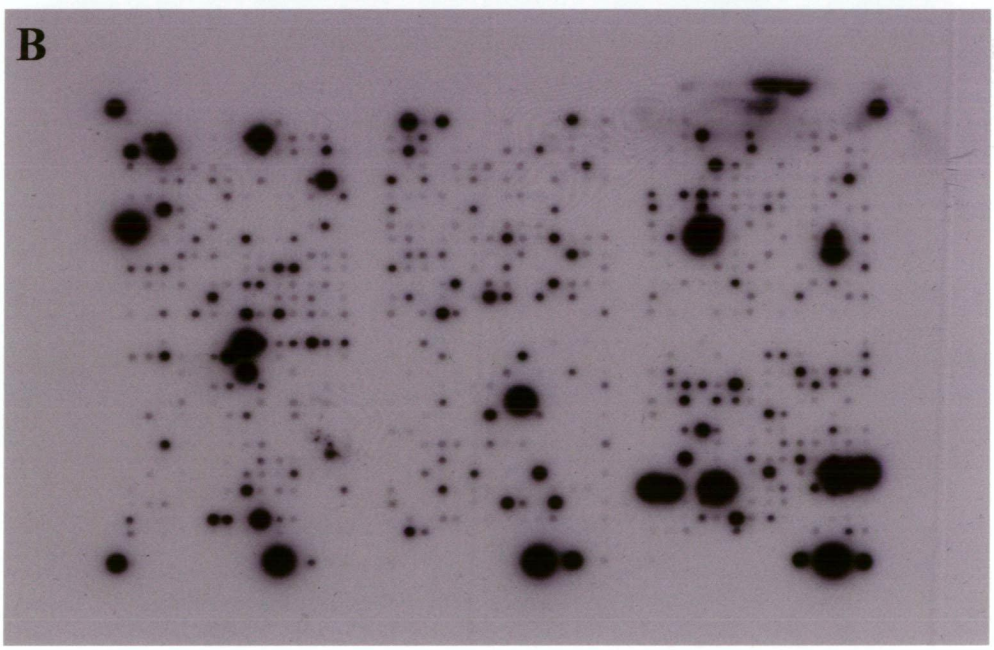
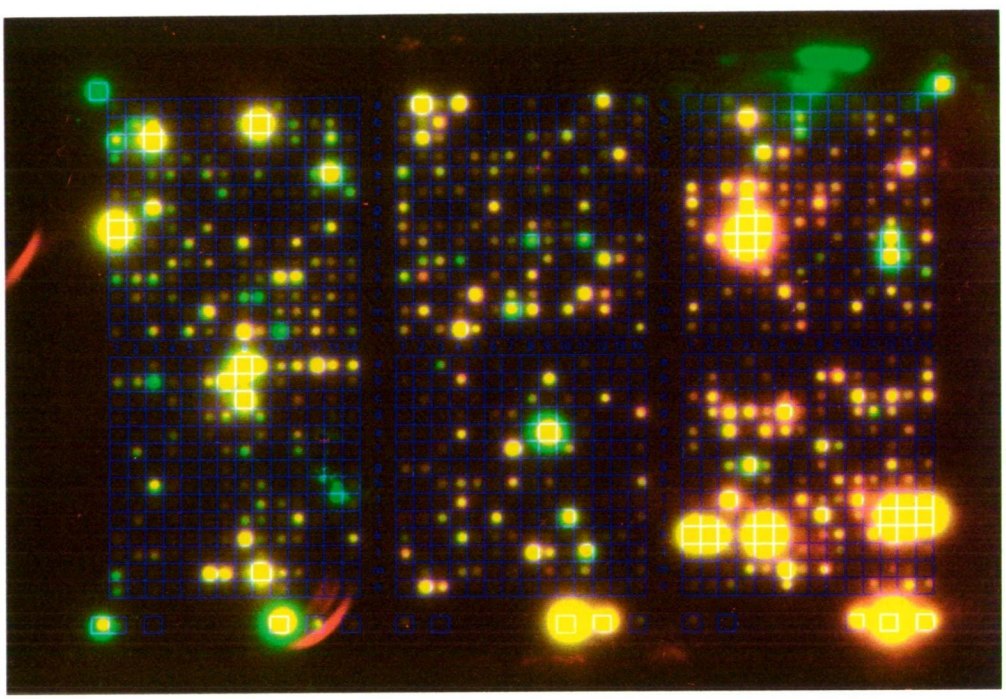

B

Figure 5.3.B.: K562 8 hour cDNA array blots from Figure 5.3.A., overlaid and false-coloured. Genes that are upregulated in expression following C_2-ceramide treatment are represented as green spots, while genes that are downregulated are represented by red spots. Genes that have no change in expression appear as yellow spots.



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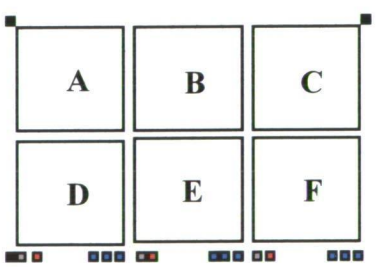


- Orientation Marks
- Human Genomic DNA
- Negative Controls
- House-Keeping Genes (Positive Controls)

Figure 5.4.A.: Demonstration of differential expression of "cancer-related" genes using the Atlas™ Human Cancer 1.2 cDNA Expression Array (Clontech, USA) for K562 erythromyeloid cells at 24 hours post-treatment with vehicle (**A**) and 5 μM C_2-ceramide (**B**).

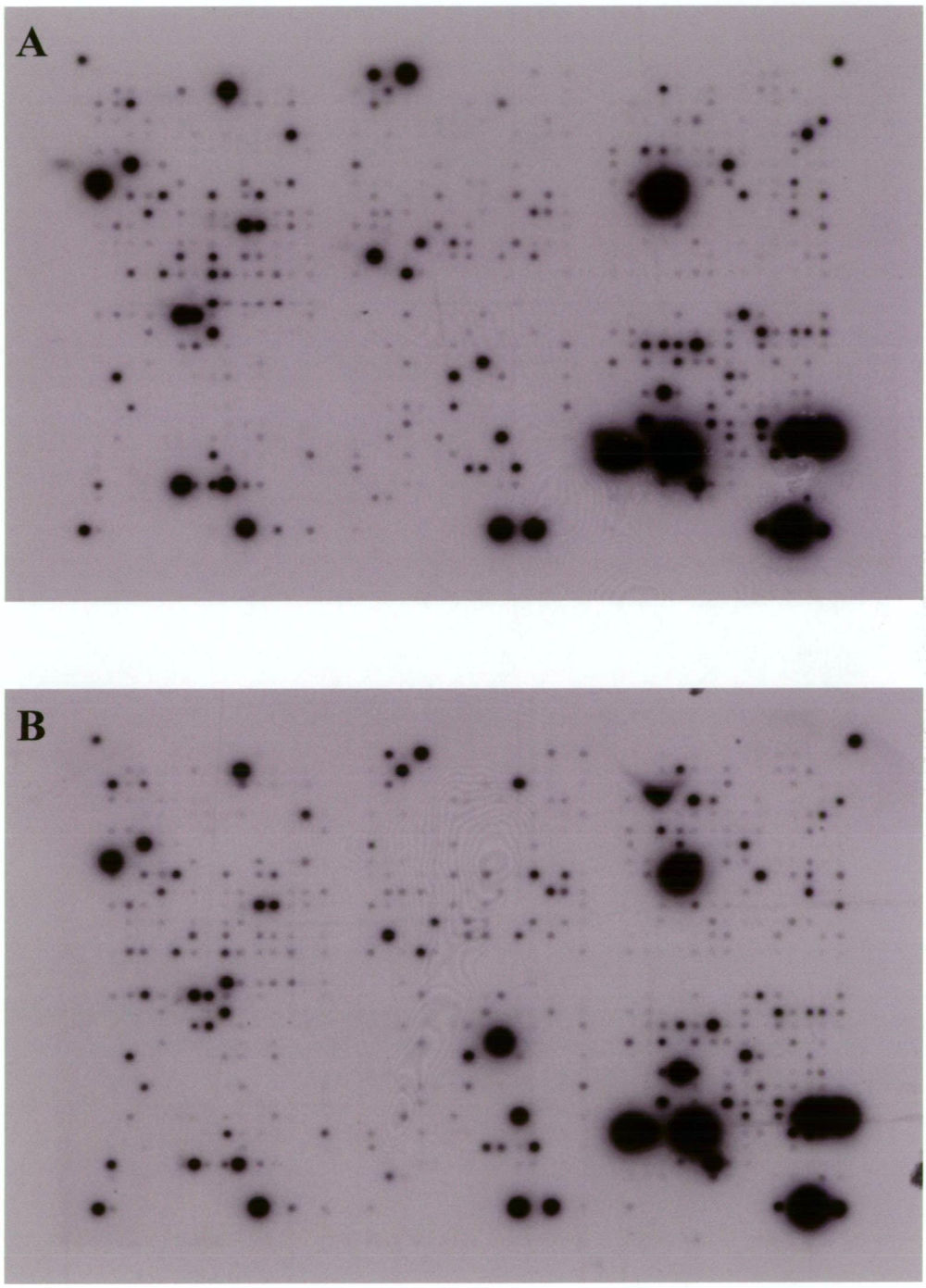
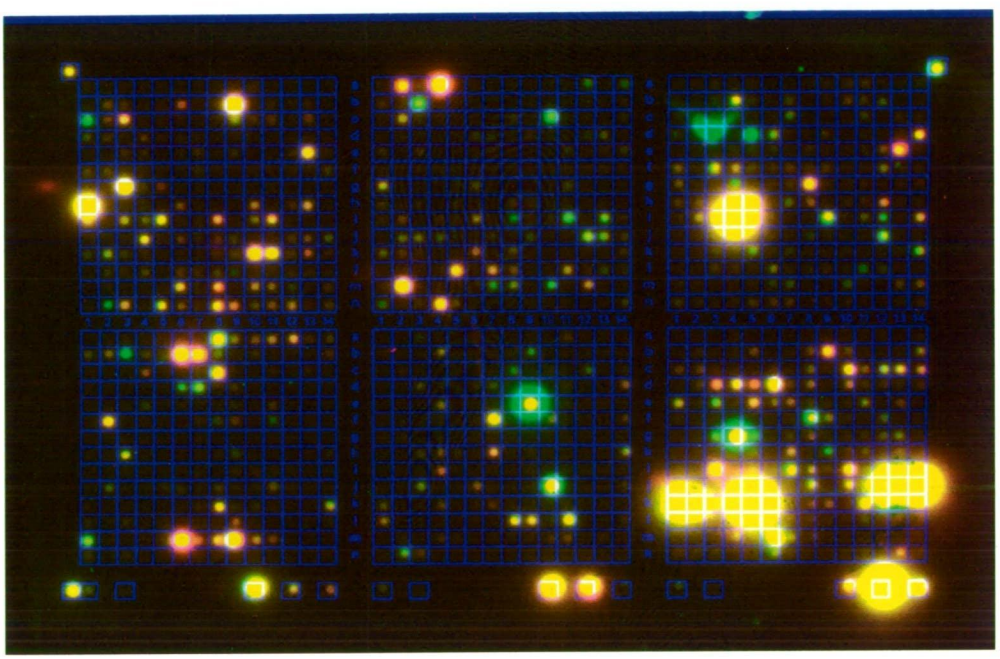
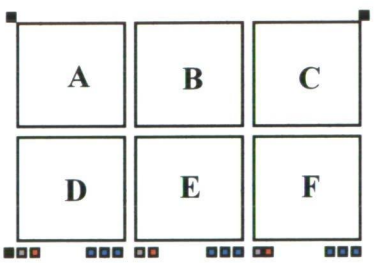

A
B

Figure 5.4.B.: K562 24 hour cDNA array blots from Figure 5.4.A., overlaid and false-coloured. Genes that are upregulated in expression following C_2-ceramide treatment are represented as green spots, while genes that are downregulated are represented by red spots. Genes that have no change in expression appear as yellow spots.



Key:



- Orientation Marks
- Human Genomic DNA
- Negative Controls
- House-Keeping Genes (Positive Controls)

Table 5.2.: Table of qualitative (and final normalised semi-quantitative) results for significantly (≥1.3 fold change at any timepoint) differentially expressed genes in K562 erythromyeloid cells at 2, 8 and 24 hours post-treatment with 5 μM C_2-ceramide, using the Atlas™ Human Cancer 1.2 cDNA Expression Array (Clontech, USA). The individual spots for each respective gene, taken from the array overlay analysis (from Figures 5.2.B., 5.3.B. and 5.4.B.), is provided for direct qualitative comparison between timepoints. Genes that are upregulated in expression following C_2-ceramide treatment are represented as green spots, while red spots represent genes that are downregulated. Genes that have no change in expression appear as yellow spots. Fold changes were obtained by normalising the original optical density data to the housekeeping genes on each of the arrays (following initial background subtraction). Final normalised changes in gene expression between vehicle- and C_2-ceramide-treated cells is provided (numerical value shown below qualitative results) for those genes with ≥1.3 fold change at one or more timepoints. Where no change in expression was demonstrated (≤1.3 fold change), a numerical value has not been provided.

Gene **(Position on Atlas™ Human 1.2 cDNA Array)**	**Timepoint Post-Treatment**		
	2 hrs	**8 hrs**	**24 hrs**
TUMOUR SUPPRESSORS & RELATED PROTEINS:			
prohibitin (PHB) **(A06b)**	0.39	0.77	0.28
TRANSCRIPTION FACTOR-RELATED ONCOGENES:			
c-jun proto-oncogene; transcription factor AP-1 **(A01c)**		6.1	5.2
c-myc oncogene **(A03c)**	0.30		0.56
INTRACELLULAR SIGNAL TRANSDUCTION-RELATED ONCOGENES:			
transforming protein rhoA H12 (RHO12; ARH12; ARHA) **(A13e)**	0.59	1.7	

CYCLINS:			
G1/S-specific cyclin D3 (CCND3) **(A08i)**	 0.59		 0.42
cyclin H (CCNH); MO15-associated protein **(A11i)**	 0.56		 0.63
CELL CYCLE REGULATING KINASES:			
cyclin-dependent kinase regulatory subunit 1 (CKS1) **(A10k)**	 0.63		 0.77
CDK INHIBITORS:			
cyclin-dependent kinase inhibitor 1C (CDKN1C); p57-KIP2 **(A08l)**	 0.37	 3.1	 0.71
cyclin-dependent kinase inhibitor 1 (CDKN1A); melanoma differentiation-associated protein 6 (MDA6); CDK-interacting protein 1 (CIP1); WAF1 **(A09l)**	 0.59	 5.7	
OTHER CELL CYCLE REGULATORS:			
protein D123 **(A08m)**	 0.44		 0.4
growth inhibitory factor; metallothionein-III (MT-III) **(A02n)**		 1.4	 8.8
cell cycle protein P38-2G4 homolog; HG4-1 **(A08n)**	 0.71		 0.67
SYMPORTERS & ANTIPORTERS:			
ADP/ATP carrier protein **(B02a)**	0.4	 1.3	 0.53

ATPase TRANSPORTERS:			
ATP synthase coupling factor 6 mitochondrial precursor (F6) **(B04a)**	 0.63		 0.67
RECEPTOR-ASSOCIATED PROTEINS & ADAPTERS:			
alpha-2-macroglobulin receptor-associated protein precursor (alpha-2-MRAP; A2MRAP); low density lipoprotein receptor-related protein- associated protein 1 (LRPAP1) **(B03b)**		 0.63	 2.6
INTRACELLULAR KINASE NETWORK MEMBERS:			
c-jun N-terminal kinase 2 (JNK2); JNK55 **(B10c)**		 1.5	 3.9
TYROSINE PHOSPHATASES:			
dual-specificity protein phosphatase 8; hVH5 **(B08i)**		 5.2	 4.1
G PROTEINS:			
transforming protein rhoB; ARHB; ARH6 **(B11i)**		 5.2	 2.8
ras-related C3 botulinum toxin substrate 1; p21-rac1; ras-like protein TC25 **(B13i)**		 0.59	 1.9
ras-related C3 botulinum toxin substrate 2; p21-rac2; small G protein **(B12j)**		 1.4	 1.7
guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-10 subunit **(B13j)**		 0.59	 1.6

CALCIUM-BINDING PROTEINS:			
calmodulin 1; delta phosphorylase kinase **(B05l)**	 0.44	 0.67	 0.59
placental calcium-binding protein; calvasculin; S100 calcium-binding protein A4; MTS1 protein **(B07l)**	 0.63		 0.56
KINASE SUBSTRATES & INHIBITORS:			
hint protein; protein kinase C inhibitor 1 (PKCI1) **(B02m)**	 0.56	 1.3	 0.77
OTHER INTRACELLULAR SIGNAL TRANSDUCTION MODULATORS & EFFECTORS:			
zyxin + zyxin-2 **(B07m)**	 0.77	 2.7	 2.1
junction plakoglobin (JUP); desmoplakin III (DP3) **(B10m)**			 2.7
guanylate kinase (GMP kinase) **(B12m)**	 0.77	 0.56	 0.59
B-cell receptor-associated protein (hBAP) **(B04n)**	 0.63		 0.67
Bcl FAMILY:			
induced myeloid leukaemia cell differentiation protein mcl-1 **(C07c)**		 3.1	 1.7

OTHER APOPTOSIS-ASSOCIATED PROTEINS:			
growth arrest & DNA-damage-inducible protein 153 (GADD153); DNA-damage-inducible transcript 3 (DDIT3); C/EBP homologous protein (CHOP) **(C05d)**		 1.5	 9.0
PDCD2 **(C14d)**	 0.71	 0.29	 0.67
DNA POLYMERASES, REPLICATION FACTORS & RECOMBINATION PROTEINS:			
proliferating cyclic/cell nuclear antigen (PCNA); cyclin **(C13e)**	 0.63	 0.53	 0.39
DNA DAMAGE REPAIR PROTEINS, LIGASES & HELICASES:			
mutL protein homolog; DNA mismatch repair protein MLH1; COCA2 **(C08g)**	 0.56	 0.35	 0.63
RAD51C truncated protein **(C12h)**			 0.33
OTHER DNA SYNTHESIS, REPAIR & RECOMBINATION PROTEINS:			
growth arrest & DNA-damage-inducible protein (GADD45); DNA-damage-inducible transcript 1 (DDIT1) **(C09i)**			 1.8
TRANSCRIPTION FACTORS:			
TIS11B protein; EGF response factor 1 (ERF1) **(C12i)**	 1.4	 2.6	 2.0
jun activation domain binding protein **(C14i)**		 0.46	 0.63

early growth response protein 1 (hEGR1); transcription factor ETR103; KROX24; zinc finger protein 225; AT225 **(C12j)**	 1.9	 2.7	 2.0
cAMP-responsive element modulator 1 alpha protein (HCREM) **(C14k)**		 1.7	 1.7
major histocompatibility complex enhancer-binding protein MAD3 **(C11l)**			 3.3
TAX1-binding protein 151 (TXBP151) **(C10m)**		 0.63	 1.5
DNA-BINDING & CHROMATIN PROTEINS:			
high mobility group protein (HMG-I) **(D08a)**		 2.7	 1.4
chromatin assembly factor 1 p48 subunit (CAF1 p48 subunit); retinoblastoma-binding protein 4 (RBBP4); RBAP48; msi1 protein homolog **(D12a)**	 0.44		 0.56
DNA-binding protein CPBP **(D03b)**	 1.3	 8.2	 9.1
nonhistone chromosomal protein HMG17 **(D06b)**	 0.5		 0.67
high mobility group protein HMG2 **(D07b)**	 0.4	 0.71	 0.71
CELL SURFACE ANTIGENS:			
preferentially expressed antigen of melanoma (PRAME) **(D08c)**	 0.46	 1.5	

CELL ADHESION RECEPTORS & PROTEINS:			
integrin alpha E precursor (ITGAE); mucosal lymphocyte-1 antigen; hml-1 antigen; CD103 antigen **(D06d)**			 2.1
integrin beta 8 precursor (ITGB8) **(D07d)**			 2.1
OTHER RECEPTORS:			
Transmembrane protein sex precursor **(D01m)**		 3.1	 2.7
STRESS RESPONSE PROTEINS:			
cytosolic superoxide dismutase 1 (SOD1) **(D06m)**	 0.63		 0.63
microsomal glutathione S-transferase II (microsomal GSTII) **(D08m)**	 0.59		 0.5
glutathione-S-transferase (GST) homolog **(D09m)**	 0.59		
GROWTH FACTORS, CYTOKINES & CHEMOKINES:			
vascular endothelial growth factor precursor (VEGF); vascular permeability factor (VPF) **(E09b)**		 1.3	 2.5
platelet-derived growth factor A subunit precursor (PDGFA; PDGF1) **(E08c)**		 1.4	 3.2
delta-like protein precursor (DLK) **(E12c)**	 0.67	 0.77	 1.8

macrophage inhibitory cytokine 1 (MIC1) **(E09e)**	 2.7	 3.5	 3.9
OTHER EXTRACELLULAR COMMUNICATION PROTEINS:			
macrophage migration inhibitory factor (MIF); glycosylation-inhibiting factor (GIF) **(E07h)**	 0.63	 0.63	 0.63
PROTEIN TURNOVER:			
metalloproteinase inhibitor 1 precursor (TIMP1); erythroid potentiating activity (EPA); fibroblast collagenase inhibitor **(E10j)**	 0.71	 1.8	 1.7
IMMUNE SYSTEM PROTEINS:			
78-kDa glucose regulated protein precursor (GRP 78); immunoglobulin heavy chain binding protein (BIP) **(E02n)**	 0.71		 2.4
METABOLIC PATHWAYS:			
nm23-H4; nucleoside-diphosphate kinase; nucleoside 5'-diphosphate phosphotransferase (NDK) **(F09b)**		 0.35	 0.5
inosine-5'-monophosphate dehydrogenase 2 (IMP dehydrogenase 2; IMPD2) **(F10c)**		 0.77	 0.77
thymidylate synthase (TYMS; TS) **(F03d)**	 0.67	 0.56	 0.4
L-lactate dehydrogenase M subunit (LDHA) **(F05d)**	 0.56	 0.46	 0.44
fatty acid synthase **(F03e)**			 1.7

ornithine decarboxylase **(F05e)**	 0.63	 0.46	 0.67
CYTOSKELETON & MOTILITY PROTEINS:			
type I cytoskeletal 18 keratin; cytokeratin 18 (K18) **(F08f)**			 2.4
type II cytoskeletal 8 keratin (KRT8); cytokeratin 8 (K8; CK8) **(F03g)**	0.59	 1.4	 2.4
vimentin (VIM) **(F04g)**	 0.77	 1.8	 1.5
desmin (DES) **(F05g)**	 0.5		 2.5
TRAFFICKING PROTEINS (ENDOCYTOSIS & EXOCYTOSIS):			
TRAM protein **(F09h)**	 0.56	 0.4	 1.9
haemoglobin alpha subunit **(F03i)**		 0.71	 0.77
RNA PROCESSING, TURNOVER & TRANSPORT:			
arginine/serine-rich splicing factor 7; splicing factor 9G8 **(F10i)**	 0.67	 0.42	 0.71

hVH5	-	5.2	4.1	Increased in K562 cells following PMA-induced differentiation (Johnson *et al.*, 2000). Highly expressed in regions of the body where you would expect to find differentiated cells, including the brain, heart, and skeletal muscle (Martell *et al.*, 1995, Ogata *et al.*, 1995).
rac1	-	0.59	1.9	*rac1* increased in HL60 cells induced to differentiate along the neutrophil lineage, while differentiation induction in both HL60 and U937 cells results in an increase in *rac2* transcript (Didsbury *et al.*, 1989).
rac2	-	1.4	1.7	
zyxin	0.77	2.7	2.1	Zyxin exhibits three copies of the LIM motif, a double zinc-finger domain found in many proteins that play a central role in the regulation of cell differentiation (Sadler *et al.*, 1992, Macalma *et al.*, 1996, Beckerle, 1997). Involved in pathways important for cell differentiation (Beckerle, 1997). An increase in expression of cytoskeletal-related genes is an indication of cellular stabilisation and changes in the mechanical properties of the cell, which is consistent with cell maturation and tumour suppression (Dellagi *et al.*, 1983, Sadler *et al.*, 1992, van der Ven *et al.*, 1992, Macalma *et al.*, 1996, Witcher *et al.*, 1996, Beckerle, 1997).
plakoglobin	-	-	2.7	
cytokeratin 8	0.59	1.4	2.4	
cytokeratin 18	-	-	2.4	
vimentin	0.77	1.8	1.5	
desmin	0.5	-	2.5	
nucleoside-diphosphate kinase	-	0.35	0.5	A reduction in metabolic pathway gene transcripts can be correlated with a reduced growth rate.
IMP dehydrogenase	-	0.77	0.77	
thymidylate synthase	0.67	0.56	0.4	
L-lactate dehydrogenase M subunit	0.56	0.46	0.44	
haemoglobin alpha subunit	-	0.71	0.77	Decrease suggests differentiation of erythromyeloid cells away from the erythroid lineage and towards the granulocytic/macrophage lineage.

5.3.3. Confirmation of cDNA Array Results by Real-Time RT-PCR

The cDNA array results were confirmed by a semi-quantitative real-time RT-PCR method. RNA (2, 8 and 24 hours post-treatment) used for the cDNA array analysis was amplified using designed primer sets targeted towards transcripts of four representative genes included in the Atlas™ Human Cancer 1.2 cDNA Expression Arrays. Sandrine Chopin performed all confirmation work, and the results from these experiments are provided in Appendix B & C. Melting curves were generated to ensure that the real-time RT-PCR products were specific and to determine the temperature at which fluorescence of SYBR Green I would be measured. The resulting melting curves for each primer set for a single representative real-time RT-PCR run are presented in Appendix B.

The concentrations of transcript present in the original sample for each of the confirmatory genes at each timepoint was calculated based on the determined crossing points from the amplification curve (the point at which SYBR Green I fluorescence was detected above background fluorescence), relative to a 10 pg *GAPDH* control RNA sample that was amplified simultaneously. Appendix B shows the amplification curves for RNA from vehicle and ceramide-treated cells, and the corresponding negative control (water) that were generated from representative real-

time RT-PCR assays for each gene analysed. The raw data from the real-time RT-PCR assays are presented in Appendix C, which shows the calculated concentrations determined from a GAPDH standard curve (generated from amplification curves provided in Appendix D). The fold change in expression for each gene at 2, 8, and 24 hours post-treatment with ceramide was then calculated relative to *GAPDH* expression in the same RNA samples and these are also provided in Appendix C.

A left shift was demonstrated in the *PRAME* amplification curve for RNA from ceramide-treated cells at 24 hours, which identifies an increase in expression following ceramide expression. Although the amplification curves at 2 and 8 hours show neither a noticeable left or right shift following ceramide treatment, significant changes in expression were calculated relative to *GAPDH* expression. A 1.8 fold decrease in *PRAME* expression was seen at the 2 hour timepoint following ceramide treatment. By 8 hours, a 2.8 fold increase in expression was determined, and a 3.2 fold increase was calculated for the 24 hour timepoint.

The *PCNA* amplification curves show a right shift of the ceramide curve at 8 and 24 hours, which demonstrate a decrease in *PCNA* expression on treatment with ceramide. Negligible shift is shown between the control and ceramide curves at 2 hours, although relative to *GAPDH* expression, a 3.3 fold decrease in expression was calculated by this early timepoint. At 8 hours, a 1.5 fold decrease in *PCNA* expression was calculated, which was reduced slightly to a 1.2 fold decrease in expression by 24 hours post-treatment with ceramide.

A 1.3 fold decrease in *MIC1* expression was calculated at 2 hours post-treatment with ceramide relative to *GAPDH* expression. This was further increased to a 2.3 fold decrease in expression by 8 hours, however, by 24 hours a 2.9 fold increase in *MIC1* expression was demonstrated. The increase in expression at 24 hours is accompanied by a significant left shift in the amplification curve for the RNA from ceramide-treated cells.

A fold change in expression of *GADD153* could not be calculated at the 2 and 24 hour timepoints, presumably due to insufficient amplification of the target *GADD153* template. Although very low amounts of *GADD153* product were amplified, it was however possible to calculate a 10.8 fold increase in *GADD153* at the 8 hour timepoint relative to *GAPDH* expression. This result may be somewhat unreliable however due to the low amount of amplicon produced.

Table 5.4. provides a summary of the results obtained from gene expression analysis using both cDNA array and real-time RT-PCR methods for comparison

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purposes. The results obtained from the real-time RT-PCR assays support the results obtained from cDNA array analysis for each of the four selected genes.

Table 5.4.: Summary table comparing the fold change in expression by cDNA array analysis (shown in Table 5.2.) with the fold change in expression as determined by real-time RT-PCR (shown in Appendix C) for the following representative genes using RNA from K562 erythromyeloid cells at 2, 8 and 24 hours post-treatment with 5 μM C_2-ceramide: *PRAME*, *PCNA*, *MIC1* and *GADD153*.
N.C. = No change in expression - ≤1.3 fold change (as determined from cDNA array analysis).
N.R. = No result obtained - unable to determine fold change (as determined from real-time RT-PCR analysis).

Gene	Timepoint (hours)	Normalised Fold Change (cDNA array analysis)	Normalised Fold Change (real-time RT-PCR)
PRAME	2	0.46	0.53
	8	1.5	2.78
	24	N.C.	3.24
PCNA	2	0.63	0.31
	8	0.53	0.65
	24	0.39	0.83
MIC1	2	2.7	0.77
	8	3.5	0.44
	24	3.9	2.90
GADD153	2	N.C.	N.R.
	8	1.5	(10.78)
	24	9.0	N.R.

5.4. Discussion

Studies over the last decade have revealed that haemopoietic differentiation engages a complex pattern of changes in expression of numerous genes. The previous chapters in this thesis demonstrate that a number of gene products are involved in ceramide-induced cell cycle arrest and the subsequent maturation of haemopoietic cells including the Rb tumour suppressor protein, protein phosphatases, and the CKI $p21^{Cip1/Waf1}$. A number of studies have concentrated on the changes in expression of individual genes during haemopoietic differentiation. However, comprehensive studies, simultaneously examining the expression of multiple genes, including genes known to have oncogenic potential, and discussions on the inter-relationships to one another are lacking from the literature.

Further elucidation of the signalling pathway utilised by ceramide to induce differentiation, in particular the changes in gene expression, is required to gain a better understanding of the key molecular mechanisms involved. RNase protection assays (RPAs) provide a rapid and relatively simple approach to genetic screening and provide a "proof-of-principle" for justifying the application of larger, more involved techniques. RPAs incorporate a set of gene templates for the simultaneous investigation of changes in expression of a number of key genes of interest. The RPA method, incorporating the hStress1 multi-probe template, was adapted in this study to provide an efficient way of investigating the ceramide-signalling pathway.

The hStress1 set contains probe templates for eleven genes (including two housekeeping genes), and permits expression analysis of cell cycle regulatory elements, DNA-damage repair genes, and apoptosis-related genes. The cell cycle regulatory genes included in the set include *p53* which is known to be either absent or non-functional in most leukaemia cell lines, and $p21^{Cip1/Waf1}$ whose protein product plays a crucial role in inhibiting cyclin D-dependent kinases, thus preventing dephosphorylation of the Retinoblastoma tumour suppressor protein (Harper *et al.*, 1993, Harper *et al.*, 1995), and has been reported to play a role in ceramide-mediated terminal differentiation (Ragg *et al.*, 1998). The transcription factor c-fos is a critical component of the stress-activated protein kinase pathway, activation of which is proposed to be a necessary event in the induction of apoptosis (Verheij *et al.*, 1996). The hStress1 template set also includes riboprobes for studying the mRNA expression of the apoptosis-related genes *bcl-X_L*, *bcl-X_S*, *bcl-2*, *bax* and *mcl-1*, and the *growth arrest and DNA-damage-inducible protein 45* (*GADD45*).

In this study, RPAs using the hStress1 template were performed on the promyelocytic HL60 cell line at 4 and 8 hours post-treatment with ceramide. The HL60 cell line was chosen for the preliminary RPA study, as it was with the use of these cells that the first link was made between ceramide and differentiation (Okazaki *et al.*, 1989, Okazaki *et al.*, 1990, Kim *et al.*, 1991). Subsequently this model has been used extensively for investigations into ceramide-mediated growth regulation and differentiation by a number of authors. A significant change in expression was determined to be 1.5 fold of greater for the purposes of this study, as suggested by the manufacturer of the RPA kit. Greater than 1.5 fold changes were demonstrated for five of the nine genes included in the template set. As expected, no band was observable for *p53* in any of the experimental RNA samples, as HL60 cells lack a functional *p53* gene (Wolf & Rotter, 1985). A band corresponding to *p53* was, however, clearly seen in the HeLa control sample. The most significant change in expression was demonstrated for $p21^{Cip1/Waf1}$, with almost a 5-fold increase at 4 hours, returning to basal expression levels by 8 hours. This further confirms the findings presented in previous chapters of this thesis, and results published by other authors (Ragg *et al.*, 1998, Lee *et al.*, 2000). Furthermore, it supports the hypothesis that ceramide treatment results in the terminal differentiation of immature leukaemia cells (Steinman *et al.*, 1994, Halevy *et al.*, 1995, Liebermann *et al.*, 1995, Parker *et al.*, 1995, Flink *et al.*, 1998).

The significant increase in *GADD45* expression at both timepoints following ceramide treatment is consistent with cell cycle arrest and differentiation in HL60 cells, as shown previously in this study. *GADD45* is a DNA-damage-inducible gene that is translated into a nuclear protein and is expressed in a cell cycle-regulated manner (Carrier *et al.*, 1994, Kearsey *et al.*, 1995) and appears to play a role in co-ordinating aspects of DNA repair and replication. *GADD45* expression is elevated in response to DNA damaging agents such as ionising radiation, nutrient deprivation, and during growth arrest and differentiation (Fornace *et al.*, 1988 & 1989, Papathanasiou *et al.*, 1991, Price & Calderwood, 1992, Zhan *et al.*, 1994). It is transcriptionally activated via p53 (Kastan *et al.*, 1992, Zhan *et al.*, 1993, Carrier *et al.*, 1994, Gujuluva *et al.*, 1994) and interacts with $p21^{Cip1/Waf1}$ and PCNA (Fornace *et al.*, 1989, Smith *et al.*, 1994, Hall *et al.*, 1995, Kearsey *et al.*, 1995). Hence, a p53-independent mechanism for the expression of *GADD45* in HL60 cells has been demonstrated in the current study.

It seems likely that c-fos contributes to ceramide-mediated regulation of the cell cycle and differentiation as the expression of the *c-fos* gene was shown to be

upregulated following ceramide treatment, and has been shown in previous studies to be involved with negative regulation of the cell cycle (Balsalobre & Jolicoeur, 1995). Okada *et al.* (1999) have shown that overexpression of *c-fos* prevents progression of primitive stem cells through the cell cycle, rendering them dormant in the G_0/G_1 phase. Similarly, overexpression of *c-fos* in splenic B cells leads to enhanced $p27^{Kip1}$ expression, and thus cell cycle arrest (Kobayashi *et al.*, 1997). However, it is now well accepted that c-fos in association with c-jun comprise the transcription factor complex AP-1 which is responsible for regulating the transcription of genes involved in both cell survival and death (Okada *et al.*, 1999). Sawai *et al.* (1995) propose that AP-1 is a requirement for apoptosis in HL60 cells, and that this TF complex is activated via ceramide signalling. *c-fos* knockout mice display a greatly reduced rate of apoptosis to stress stimuli such as ionising radiation (Pruschy *et al.*, 1997) indicating the importance of *c-fos* in the apoptotic pathway. *c-fos* has also been shown to be associated with numerous other physiological responses, including mitogenesis (Okada *et al.*, 1999), and differentiation (Sawai *et al.*, 1995). Studies conducted in our laboratory have demonstrated that c-fos and c-jun expression is a pre-requisite for ceramide-induced myeloid differentiation (McQuestin, 2001). Taken together, the findings from each of these studies are consistent with the increase in *c-fos* expression seen in ceramide-treated HL60 cells in the current study, in association with the G_0/G_1 cell cycle arrest, myeloid differentiation and viability results presented in the previous chapters of this thesis.

Expression of a number of Bcl-2 protein family members was also investigated at the transcriptional level as these genes play a crucial role in the regulation of cell death, and function in coordination with each other to determine the susceptibility of a cell to undergo apoptosis (Pellegrini & Strasser, 1999). The expression of bcl-2 is high in myeloid cells, and is regulated in a differentiation-linked manner such that levels of *bcl-2* mRNA decrease dramatically and rapidly during differentiation of HL60 cells induced by PMA and other agents, including vitamin D_3 and RA (Delia *et al.*, 1992, Xu *et al.*, 1993, Park *et al.*, 1994, Naumovski & Cleary, 1994, Blagosklonny *et al.*, 1996). Surprisingly, although HL60 cells did demonstrate strong *bcl-2* basal expression levels, a 1.5 fold increase in *bcl-2* expression was exhibited at the early timepoint following ceramide exposure. However, a slight decrease in *bcl-2* expression was shown at 8 hours, although this was not considered to be significant (<1.5 fold change). The significant increase in the anti-apoptotic *bcl-X_L* demonstrated at 4 and 8 hours, the slight increase in expression of *mcl1* at 4 hours (<1.5 fold change), and the slight increase (<1.5 fold

change) in the pro-apoptotic member *bax*, tend to suggest that ceramide treatment leads to the protection of HL60 cells from apoptosis due to the significant induction of expression of anti-apoptosis genes such as *bcl-2* and *bcl-X_L*. This is in contrast to the findings from previous chapters where HL60 cell death was clearly demonstrated following ceramide treatment. It is likely that post-translational interactions, rather than transcriptional mechanisms, between Bcl-2 family members are responsible for the increased sensitivity to apoptosis as indicated by the study conducted by El-Assaad *et al.* (1998). In this study it was demonstrated that over-expression of *bcl-X_L* did not protect MCF7 breast carcinoma cells from ceramide-induced apoptosis, and the fact that *bcl-2* mRNA levels are not necessarily predictive of bcl-2 protein levels (Blagosklonny *et al.*, 1996). It is the ratio of anti-apoptotic to pro-apoptotic members that is considered to be one of the determining factors as to whether survival or death will occur following an apoptotic signal (Korsmeyer *et al.*, 1993, Oltvai *et al.*, 1993).

The activation of expression of cell cycle regulatory and differentiation-associated genes including *$p21^{Cip1/Waf1}$*, *GADD45* and *c-fos* demonstrated from RPA analysis, together with the results from protein studies presented in previous chapters, suggest a myriad of genes are playing a role in ceramide-mediated cell cycle arrest and subsequent terminal differentiation of haemopoietic cells. As such, it was considered justified to perform a large-scale screen of ceramide-induced genes, particularly as studies of this nature are absent from the literature, in order to further elucidate the relevant genes and potential mechanisms involved in regulating growth, differentiation and death following ceramide treatment.

RNA from K562 cells were used for the cDNA array experiments, as K562 cells were considered to be representative of the other myeloid ceramide-responsive cell lines, and distinctly demonstrated ceramide-induced cell cycle arrest and differentiation, while demonstrating only a small amount of cell death relative to the other cell lines investigated. The focus of this section of the study was to be directed towards the growth arrest and differentiation-inducing ability of ceramide in cells of the myeloid lineage. As such, the K562 cell line was chosen as the model for use in cDNA array analysis to investigate the expression changes in "cancer-related" genes following ceramide treatment.

For the purpose of this study, genes demonstrating a 1.3 fold change or greater in relative pixel density were considered differentially expressed as this figure is suggested by the manufacturer of the array, has been adopted in previously published studies using this filter array kit (Ollila & Vihinen, 1998, Xie *et al.*, 2000),

and provides a reasonable starting point for estimating which genes are differentially expressed as a basis for further investigative work.

In the categories of cell cycle and growth regulators, the downregulation of *G_1/S-specific cyclin D3*, *P38-2G4* and *protein D123* following ceramide treatment indicates negative regulation of the cell cycle and is consistent with cell cycle arrest at the G_0/G_1 phase of the cell cycle. The cell cycle protein P38-2G4, a homolog of murine p38-2G4 (Lamartine *et al.*, 1997, Radomski & Jost, 1995), is a cell cycle-specifically modified and proliferation-associated nuclear protein in mammals (Radomski & Jost, 1995). Protein D123 has been proposed as being necessary for phase entry into the cell cycle, and a decrease in this protein results in G_1 arrest (Onisto *et al.*, 1998). Downregulation of each of these genes further supports the roles for ceramide in cell growth inhibition and G_0/G_1 cell cycle arrest.

A significant increase in expression was demonstrated for $p21^{Cip1/Waf1}$ at 8 hours post-treatment with ceramide, while other timepoints were difficult to analyse due to low signal levels. This is consistent with the results obtained from RPA studies on all cell lines investigated in this thesis, and further confirms ceramide-induced p53-independent G_0/G_1 cell cycle arrest and terminal differentiation in K562 cells. Overexpression of PCNA has been related to tumour size and poor prognosis, and PCNA is inhibited by $p21^{Cip1/Waf1}$ expression during cell cycle arrest (Flores-Roses *et al.*, 1994, Waga *et al.*, 1994, Chen *et al.*, 1995b, Luo *et al.*, 1995, Warbrick *et al.*, 1995, Zhou *et al.*, 1998). Thus, overexpression of $p21^{Cip1/Waf1}$ inhibits PCNA, resulting in the inhibition of PCNA-dependent DNA replication and prevention of cell proliferation (Flores-Roses *et al.*, 1994, Li *et al.*, 1994, Waga *et al.*, 1994). Downregulation of *PCNA*, as demonstrated in the array experiments in the current study, is therefore consistent with an upregulation in $p21^{Cip1/Waf1}$ expression, which would result in a reduction in growth rate, cell cycle arrest and subsequent terminal differentiation.

In the categories of apoptosis and DNA synthesis and repair-related proteins, significant increases in expression were demonstrated for *GADD153* and *GADD45*. The increase in *GADD45* expression also further confirms the results from the RPA study presented in this thesis. *GADD45* transcript is elevated in cells that have been growth arrested or after DNA damage. (Fornace *et al.*, 1988 and 1989), and furthermore, has been shown to bind PCNA *in vivo* (Smith *et al.*, 1994), further assisting in the $p21^{Cip1/Waf1}$-induced inhibition of PCNA-driven cell proliferation.

An upregulation was seen in *mcl-1* expression, which is consistent with haemopoietic cell cycle arrest, differentiation and apoptosis. *mcl1*, a homologue of

Bcl-2, was isolated from the ML-1 human myeloblastic cell line (Kozopas *et al.*, 1993), and is rapidly induced in response to signals for cell differentiation and death, but not to signals for cell proliferation. ML-1 cells induced to differentiate into monocyte/macrophage cells demonstrated an early increase in expression of *mcl1* (Kozopas *et al.*, 1993, Yang *et al.*, 1996). It is considered a potential target for inhibiting cancer development and inducing differentiation and apoptosis in tumour cells (Craig, 2002). Interestingly, mcl1 has also been shown to play a role in cell cycle progression, and has a binding site for PCNA. A mutant of *mcl1* that lacks PCNA binding was not capable of inhibiting cell cycle progression as effectively as wild-type *mcl1* (Fujise *et al.*, 2000). Taken together, an increase in of $p21^{Cip1/Waf1}$, *c-fos*, and *mcl1* expression is consistent with a decrease in PCNA levels and subsequent cell cycle arrest.

In the category of transcription factor-related oncogenes, *c-jun* was found to be highly upregulated, while *c-myc* was downregulated in response to ceramide treatment. c-jun is a key member of the SAPK/JNK pathway (reviewed by Karin *et al.*, 1997) and has been shown to be upregulated in HL60 cells (Sawai *et al.*, 1995), and activated by phosphorylation in U937 cells upon treatment with C_2-ceramide (Ragg *et al.*, 1998). In association with c-fos (to form the transcription factor AP-1), c-jun is known to elicit a variety of cellular responses including proliferation, differentiation and apoptosis, depending on the original stimulus, cell type and microenvironment of the cell. The level of *c-jun* transcript has been reported to increase during HL60 cell differentiation along the macrophage lineage (Lord *et al.*, 1993). It is interesting to note also that c-jun has been implicated as being a downstream target for CAPP in A431 cells (Reyes *et al.*, 1996), which correlates with the results presented in a previous chapter of this thesis. Consistent with an increase in *c-jun* mRNA levels is an upregulation in expression at both the 8 and 24 hour timepoints for *c-jun N-terminal kinase 2* (*JNK2*).

The product of transcription factor and proto-oncogene *c-myc* strongly stimulates cellular proliferation, inducing cells to exit G_0/G_1 and enter the cell cycle via transcriptional regulation of myc-target genes. *c-myc* expression is rapidly suppressed by p53 upon induction of growth arrest and apoptosis (Guillouf *et al.*, 1995). Therefore a decrease in *c-myc* expression, as demonstrated in the current study following treatment with ceramide, is consistent with cells undergoing G_0/G_1 cell cycle arrest. *c-myc* expression has also been shown by various authors to be dramatically suppressed after the induction of myeloid terminal differentiation in HL60, U937, HEL, ML-1 and K562 cells (Collins, 1987, Larsson *et al.*, 1988 &

1994, Liebermann & Hoffman-Liebermann, 1989, Gomez-Casares *et al.*, 1993, Nguyen *et al.*, 1995a, Lerga *et al.*, 1999). Kim *et al.* (1991) have reported early and specific ceramide-induced downregulation of *c-myc* in HL60 cells, resembling results from an earlier study seen after treatment with TNFα (Kronke *et al.*, 1987). Further work has led this group of researchers to speculate that *c-myc* downregulation by ceramide in HL60 cells is a result of activation of CAPP (Wolff *et al.*, 1994). Several lines of investigation have revealed that there is also a close association between c-myc and GADD45, and c-myc and GADD153. Results from these studies identify that c-myc is capable of suppressing the induction of both these DNA damage and growth arrest-related proteins, suggesting that a coordinate downregulation of *GADD45* and *GADD153*, and other *GADD* genes, is one mechanism by which c-myc can circumvent growth arrest (Marhin *et al.*, 1997, Amundson *et al.*, 1998). Thus an increase in *GADD45* and *GADD153* in consistent with a downregulation in *c-myc*, as demonstrated from the array analysis results in the present study, which further correlates with cells undergoing cell cycle arrest.

The primary aim of this study was to investigate the ceramide-induced changes in expression of differentiation-associated genes in a representative myeloid cell line. CDNA array analysis provides a very effective means for conducting a study of this kind. However, use of the Atlas™ Human Cancer 1.2 array also provides supportive data to indicate the involvement of other genes reported to be involved in human cancer. A selection of the remainder of genes analysed, which are categorised into a variety of different gene families contained within the cDNA array, are discussed further. These genes were chosen for discussion, as they appear to be of relative significance in the process of ceramide-induced differentiation. One such gene is the *ADP/ATP carrier* which demonstrated a decrease in expression at both the 2 and 24 hour timepoints following ceramide treatment, and has been shown also to decrease in HL60 cells during the differentiation response to phorbol ester and retinoic acid treatment (Battini *et al.*, 1987).

mRNA levels for the *dual-specificity protein phosphatase 8* (*hVH5*) gene were shown in the present study to be significantly increased at the later timepoints by ceramide, which is consistent with a study recently conducted by Johnson *et al.* (2000), who found upregulation in expression of *hVH5* in K562 cells following PMA-induced differentiation. This demonstrates activation of a protein phosphatase other than the CAPP, which may be playing a regulatory role in the ceramide-signalling pathway. Interestingly *hVH5* is highly expressed in regions of the body

where you would expect to find differentiated cells, including the brain, heart, and skeletal muscle (Martell *et al.*, 1995, Ogata *et al.*, 1995).

An increase in expression of the genes for both ras-like proteins (G proteins), *rac1* and *rac2*, were demonstrated in the array experiments at the 24 hour timepoint. Overexpression of these genes is also consistent with differentiation into a mature phenotype. mRNA levels of *ras* increase with the terminal differentiation of haemopoietic cells into granulocytes, and this is specific to cells of the haemopoietic lineage (Shirsat *et al.*, 1990). *rac1* transcript has been shown to be increased in HL60 cells induced to differentiate along the neutrophil lineage, while differentiation induction in both HL60 and U937 cells results in an increase in *rac2* transcript (Didsbury *et al.*, 1989). Interestingly, neither *rac* mRNAs are present in Jurkat cells, which suggests that rac proteins are only important in myeloid differentiation.

Zyxin is a component of adhesion plaques that are proposed to perform regulatory functions at the plasma membrane (Macalma *et al.*, 1996), and displays features of an intracellular signal transducer (Beckerle, 1997). This protein exhibits three copies of the LIM motif, a double zinc-finger domain found in many proteins that play a central role in the regulation of cell differentiation (Sadler *et al.*, 1992, Macalma *et al.*, 1996, Beckerle, 1997). Together with its partners, zyxin is capable of directing nuclear proteins to the cytosol (Nix & Beckerle, 1997), and is involved in the spatial control of actin filament assembly as well as in pathways important for cell differentiation (Beckerle, 1997). *zyxin* expression was found to be upregulated following ceramide treatment at the later timepoints. Together with an increase in expression of other cytoskeletal-related genes such as *plakoglobin*, *cytokeratin 8* and *18*, *vimentin* and *desmin*, an increase in expression of *zyxin* is an indication of cellular stabilisation and changes in the mechanical properties of the cell, which is again consistent with cell maturation and tumour suppression (Dellagi *et al.*, 1983, Sadler *et al.*, 1992, van der Ven *et al.*, 1992, Macalma *et al.*, 1996, Witcher *et al.*, 1996, Beckerle, 1997).

All of the genes coding for components of metabolic pathways that demonstrated differential expression were consistently downregulated in expression. It is justifiable to propose that a reduction in metabolic pathway gene transcripts can be correlated with a reduced rate of growth as demonstrated in growth curves presented in a previous chapter of this thesis. Finally, it has been proposed that ceramide treatment results in the differentiation of K562 cells away from the erythroid lineage and towards the granulocytic/macrophage lineage due to the demonstrated decrease in glycophorin A cell surface marker expression. This is in

fact consistent with the downregulation in *haemoglobin alpha subunit* expression shown in the array experiments in the present study.

Real-time PCR is used to monitor the fluorescence emitted during an amplification reaction as an indicator of amplicon production during each PCR cycle as opposed to endpoint detection by conventional quantitative PCR methods. It is possible to monitor the amplification reaction during the exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template (known as the crossing point). Real-time RT-PCR is considered a very suitable technique for validation of array results (Rajeevan *et al.*, 2001), and was utilised to support the expression profile obtained for four genes at each of the timepoints investigated in the cDNA array experiments. These genes only were chosen as they represent different expression patterns and can be taken as examples to demonstrate the reliability of array hybridisation. As shown in Table 5.4., these genes showed similar expression patterns using real-time RT-PCR compared with the expression profiles obtained from array hybridisation experiments. The only exception being *GADD153*, for which real-time RT-PCR analysis was not possible due to inefficient Sybr Green I intercalation into the amplified product, resulting in apparent low levels of *GADD153* target amplification. Inefficient Sybr Green I addition resulting in weak signals have been associated with amplicons of long length (Dorak, 2001), although the *GADD153* product (473 bp) is not especially large relative to the product sizes of the other genes tested. In spite of the absence in *GADD153* expression confirmation, the results of the differential array hybridisation can be considered reliable.

The use of a cDNA filter array has provided a simple, relatively non-expensive, and high-throughput approach to comparing differential expression of genes that are known to be involved in cancer following ceramide treatment in the ceramide-responsive myeloid cell line K562. From the results presented in this chapter, it may be deduced that ceramide signalling activates a highly complex, but coordinated, set of changes in gene expression. Furthermore, the overall pattern of changes in expression of the various gene families following ceramide treatment in K562 myeloid cells is indicative of cells undergoing growth and cell cycle arrest, induction of terminal differentiation, and evidence of death by apoptosis (as summarised in Table 5.3.). These results provide important information with regards to communication between genes involved in the ceramide-signalling pathway, and

provide a basis for which further confirmation work should be done. Subsequent follow-up investigations into the roles these differentially expressed genes are playing in ceramide-signalling pathway is well justified, and could potentially provide target genes for the successful treatment of leukaemia.

6. General Discussion

It has become apparent that the regulation of normal development and tissue function is the result of mechanisms regulating cell division, differentiation and cell death. Proliferation, differentiation, and apoptosis are tightly regulated during haemopoiesis, allowing amplification along specific lineages while preventing excessive proliferation of immature cells. Inhibition of apoptosis, disruption of growth regulatory mechanisms, and the inability of non-functional clones of immature cells to differentiate into a mature phenotype are all characteristics of cancer, including leukaemia. Current treatment protocols aim to halt the growth of aggressively multiplying cells using non-selective cytotoxic drugs or radiation therapy. This approach is, however, associated with considerable toxicity (Mokyr & Dray, 1987, Pai & Nahata, 2000). Advances in the understanding of the biochemical events involved in cell growth regulation, differentiation and apoptosis may lead to the development of selective pharmacological interventions to improve the outcome in the treatment of leukaemia. Recently, significant attention has been directed towards a less toxic approach that targets cellular growth and differentiation pathways to alter the proliferative potential of cancer cells, while limiting cytotoxicity to healthy neighbouring cells, a strategy known as 'differentiation therapy' (Sachs, 1978, Fisher *et al.*, 1985, Waxman, 1996, Leszczyniecka *et al.*, 2001, Spira & Carducci, 2003, Gomez-Vidal *et al.*, 2004). The interest in differentiation therapy lies in the fact that differentiated tumour cells can be reprogrammed to result in the loss of proliferative capacity and induction of terminal differentiation into mature non-dividing cells (Sachs, 1978, Fisher *et al.*, 1985, Waxman, 1996), which ultimately results in removal of these cells by apoptosis (Martin *et al.*, 1990) as part of a normal haemostatic mechanism.

The search for suitable differentiation-mediating drugs has come under intense investigation over the last decade. Many drugs have been trialled, but only a small number, including vitamin D_3 and all-*trans* retinoic acid, have exhibited potential in the clinical setting to overcome maturation defects found in leukaemia cells. But even the use of these compounds has been associated with some clinical limitations, including associated toxicity (hypercalcaemia in vitamin D_3-treated patients), disease relapse and fatality (Laubenthal *et al.*, 1975, Castaigne *et al.*, 1990, Chen *et al.*, 1991, Glasser *et al.*, 1994, Elstner *et al.*, 1997, Fenaux & Debotton, 1998, Nagpal *et al.*, 2001, Mehta & Mehta, 2002). Consequently, only a minority of differentiation-inducing drugs are still in clinical use today.

The failure to utilise a number of these differentiation agents has meant the search for suitable alternatives has to be widened. New differentiation agents will inevitably arise from studies that gain a greater understanding of normal cellular processes linked to cellular maturation. One such pathway is the sphingomyelin (SM) pathway, which is an ubiquitous, evolutionarily conserved signalling system initiated by hydrolysis of the plasma membrane phospholipid sphingomyelin to generate the second messenger ceramide (Pena *et al.*, 1997). Okazaki and colleagues (1989) first proposed that sphingolipids were biologically active after demonstrating Vitamin D_3-induced turnover of SM in HL60 cells. Sphingolipids have now been shown to be a diverse class of molecules, that play an important role as second messengers within the cell (Kolesnick *et al.*, 1994), producing a diverse range of stress-related cellular responses including cell cycle arrest, apoptosis, and cell senescence (Kolesnick and Golde, 1994, Hannun, 1996). The central component of the SM pathway, the second messenger ceramide, is highly pleiotropic in nature and is capable of instigating a range of anti-proliferative cellular responses including proliferation, differentiation, growth arrest and apoptosis (Mathias *et al.*, 1998). The extent of such responses depends on the cell type and the initial stimuli presented to the external surface of the cell, and also the amount and location of ceramide generation (Perry & Hannun, 1998). Multiple direct targets of ceramide have been identified, and include a proline-directed ceramide-activated protein kinase (Mathias *et al.*, 1991, Dressler *et al.*, 1992, Joseph *et al.*, 1993), a serine/threonine ceramide-activated protein phosphatase (Dobrowsky & Hannun, 1992 & 1993, Dobrowsky *et al.*, 1993, Hannun, 1996), members of the stress-response kinase cascade (SAPK/JNK) and components of the mitogen-activated protein kinase cascade (MAPK) (reviewed by Testi, 1996, Riboni *et al.*, 1997 and Gomez-Munoz, 1998). Therefore, ceramide is a multi-functional molecule that does not have a single defined function within the cell.

A greater understanding of the events that take place following ceramide treatment will be of therapeutic benefit in the treatment of neoplastic diseases such as leukaemia. By exposing the individual components of the SM pathway it is possible that novel therapeutic targets will be found. A synthetic ceramide, available as a cell permeable, biologically active analogue (C_2-ceramide), has been shown to effectively mimic the action of an extracellular stimulus (Levade & Jaffrezou, 1999). C_2-ceramide provides an ideal model molecule for investigating ceramide-mediated effects in the laboratory setting. To evaluate the biological effects of ceramide *in vitro*, immortal human haemopoietic leukaemic cell lines, which are blocked at different stages of maturation, were utilised as a model for investigating biological

responses following ceramide treatment. They allow the study of a homogenous population of neoplastic cells (Koeffler, 1983), as well as providing a renewable source of material for characterisation of cell surface markers and receptors and mRNA for analysis of gene expression.

To date, research into ceramide-induced responses has predominantly focussed on only a few human leukaemic cell lines, primarily HL60 (Okazaki *et al.*, 1989, Okazaki *et al.*, 1990, Kim *et al.*, 1991, Raines *et al.*, 1993, Hannun, 1994), U-937 (Obeid *et al.*, 1993, Ragg *et al.*, 1998) and MOLT-4 (Chao *et al.*, 1992, Dbaibo *et al.*, 1995, Pushkareva *et al.*, 1995). A number of individual research papers have been published which investigate these responses in isolation, although a thorough study has not been undertaken. The results presented in this thesis provide the outcomes of the first comprehensive study to investigate whether responses to ceramide are consistent in both lymphoid and myeloid cell lineages.

As shown in Chapter 3, the biological effects exhibited by all myeloid and lymphoid cell lines investigated (K562, KG-1, KG-1a, HL60, Jurkat and Raji) following ceramide treatment were generally universal. Exposure to ceramide consistently resulted in activation of the Rb tumour suppressor protein, as determined by Western blotting. This was accompanied by a reduction in total cell numbers and G_0/G_1 cell cycle arrest. An interesting result was shown for Jurkat T cells, which underwent rapid apoptosis before G_0/G_1 arrest could be demonstrated. These cells are fully differentiated along the T cell lineage, and exposure to ceramide forced them into terminal differentiation, the final outcome of this being cell death, thus supporting the proposal that the cellular response at the time of ceramide addition is highly dependant on the stage of differentiation of the treated cell population.

Cell cycle arrest was accompanied by a degree of apoptosis in all cell lines examined, which was comparatively lower in cells showing greater G_0/G_1 phase accumulations in cell cycle studies, suggesting that apoptosis is occurring primarily in cells that are trapped in the G_0/G_1 phase, hence lowering the apparent number of G_0/G_1 events by selective killing. It can be concluded that ceramide-induced Rb activation and cell cycle arrest are linked events and are key events in the repression of uncontrolled cellular growth. Of significance is the fact that this response occurred in a p53-independent manner, as the cell lines examined in this study lack a functional p53 gene. This demonstrates the ability of ceramide treatment to regain control of the cell cycle in the absence of genes that function to regulate progression through the cell cycle and subsequent proliferation in normal, healthy cell populations. Manipulation of this important regulatory mechanism will be beneficial

in future treatment of cancer, as the *p53* gene is recognised as the most commonly mutated tumour suppressor gene in human cancer (Nigro *et al.*, 1989, Levine *et al.*, 1991, Hollstein *et al.*, 1991 & 1994, Greenblatt *et al.*, 1994).

Interestingly, although the ceramide-mediated effects were consistent, the degree to which ceramide was able to elicit cellular responses varied between cell lines, thus reflecting the range of responses associated with the ceramide-signalling pathway. This further confirms that the final outcome of signals transmitted by ceramide could depend on the phase of the cell cycle in which signalling was initiated, the engagement of different downstream effectors in different cell types (Mathias *et al.*, 1998), or the generation of ceramide metabolites that may enhance or antagonise ceramide-activated signalling mechanisms.

Another common finding as a result of ceramide treatment was the induced expression of the cyclin-dependent kinase inhibitor gene $p21^{Cip1/Waf1}$, as demonstrated in Chapter 3 using RNase protection assays. It has been previously proposed (Steinman *et al.*, 1994) that p53-independent pathways of $p21^{Cip1/Waf1}$ induction could represent alternative approaches to control aberrant proliferation resulting from a non-functional or absent *p53* gene. An increase in $p21^{Cip1/Waf1}$ expression was shown in all cell lines analysed, and is consistent with findings reported by other authors who have shown similar results in other cell lines (Ragg *et al.*, 1998, Lee *et al.*, 2000). The upregulation in $p21^{Cip1/Waf1}$ expression was shown to be an immediate early response to ceramide signalling. Interestingly, the kinetics observed in the detection of $p21^{Cip1/Waf1}$ message paralleled that observed for accumulation of activated Rb following ceramide treatment. It would therefore appear that activation of the Rb tumour suppressor protein and upregulation of the CKI $p21^{Cip1/Waf1}$ are common mechanisms for ceramide-induced cell cycle arrest.

Induction of myeloid haemopoietic differentiation was demonstrated by a general increase in the maturation markers CD11b and CD14, and a decrease in glycophorin A in K562 cells and a decrease in CD34 in KG-1 and KG-1a cells using immunophenotyping experiments. Investigation of differentiation was only assessable for the myeloid cell lines, as cells of this lineage possess cell-specific surface markers, which make this study possible. An increase in $p21^{Cip1/Waf1}$ expression and the subsequent induction of cell differentiation is consistent with an alteration in the balance within the cell from being strongly proliferative to anti-proliferative as a result of ceramide-signalling. Although immunophenotyping was not possible for investigation of ceramide-mediated lymphoid differentiation, an

increase in $p21^{Cip1/Waf1}$ expression in the lymphoid cell lines studied indicates these cells are undergoing differentiation in response to ceramide treatment.

Expression of $p21^{Cip1/Waf1}$ is considered to be conclusive evidence that cells are differentiating into a mature phenotype. Rots et *al.* (1999) have shown that vitamin D_3-induced cell cycle arrest in myeloid cells always precedes differentiation, and involves the induction of $p21^{Cip1/Waf1}$. These findings identify $p21^{Cip1/Waf1}$ as a candidate gene linking differentiation signals to G_0/G_1 arrest and Rb hypophosphorylation in a number of cell lines. The upregulated expression of $p21^{Cip1/Waf1}$ in response to ceramide-treatment in the current study indicates terminal differentiation induction, since $p21^{Cip1/Waf1}$ is considered to play a significant role in terminally differentiating cells such as muscle and in cells treated with differentiating agents to force them to become fully matured (Halevy *et al.*, 1995, Parker *et al.*, 1995, Flink *et al.*, 1998). Jiang *et al.* (1994) found a close relationship between induction of $p21^{Cip1/Waf1}$ and differentiation, as a delay in $p21^{Cip1/Waf1}$ induction was observed in HL60 cells resistant to TPA induced growth arrest and differentiation.

Taken together with the cell cycle analysis results, it can be concluded that ceramide-treated cells have a lowered proliferative capacity and consequently die of apoptosis, as a result of terminal differentiation. The significance of this can be appreciated from the findings that the presence of blastic leukaemic cells arises as a result of a blockage in a differentiating ability (Testa *et al.*, 1993). The results presented in Chapter 3 of this thesis support the hypothesis that ceramide signalling halts the growth of proliferating leukaemic cells at the G_0/G_1 phase of the cell cycle, and are forced past a differentiation blockage, resulting in a non-proliferative, terminally differentiated cell that undergoes apoptosis and subsequent removal from the population.

To further characterise the SM pathway, the mechanism by which ceramide could mediate Rb activation was investigated. Two possible effector mechanisms for Rb activation have been postulated in the literature thus far; the inhibition of CDK complexes by CKIs such as $p21^{Cip1/Waf1}$ (Serrano *et al.*, 1993, Kamb *et al.*, 1994, Hirai *et al.*, 1995, Harper *et al.*, 1993, Harper *et al.*, 1995, Toyoshima & Hunter, 1994, Niculescu *et al.*, 1998), and, the direct removal of phosphate groups from the Rb protein via activation of protein phosphatases (Alberts *et al.*, 1993, Durfee *et al.*, 1993, Ludlow *et al.*, 1993, Schonthal & Feramisco, 1993, Dou *et al.*, 1995, Berndt *et al.*, 1997, Nelson *et al.*, 1997).

From the results presented in Chapter 4 together with the results from the preceding chapter, it was determined that the primary mechanism for ceramide-

induced activation of Rb was via activation of protein phosphatases in the cell [presumably the ceramide-activated protein phosphatase (CAPP)], which is consistent with the findings published by Dou *et al.* (1995). Treatment with the protein phosphatase inhibitor Calyculin A totally eliminated the presence of dephosphorylated Rb resulting from ceramide treatment, even at the earliest timepoints analysed. The results from these protein-phosphatase inhibition experiments provide a clear link between ceramide-induced phosphatase activation and subsequent activation of the retinoblastoma protein, leading to cell cycle arrest.

Ceramide-induced Rb activation was demonstrated in Chapter 3 to be a rapid and early event, and was shown to precede induction of $p21^{Cip1/Waf1}$ expression in all cell lines investigated. Rb activation via induction of $p21^{Waf1/Cip1}$ expression would require transcriptional activation and subsequent translation of the $p21^{Cip1/Waf1}$ protein. Conversely, phosphatase-mediated Rb activation is transcription-independent and would therefore occur much more rapidly than $p21^{Waf1/Cip1}$-mediated Rb activation, a notion that is consistent with the findings presented by Zeng & el-Deiry (1996). Thus, the kinetics involved in ceramide-mediated Rb activation, and the demonstration that Calyculin A prevents ceramide-induced Rb activation suggests that ceramide-induced activation of Rb is primarily mediated via protein phosphatase activity and not by transcriptional activation of $p21^{Waf1/Cip1}$. These results therefore imply that ceramide-induced expression of $p21^{Waf1/Cip1}$ is, instead, most likely involved in the differentiation response noted by others (Jiang *et al.*, 1994, Liu *et al.*, 1996a, Ragg *et al.*, 1998).

Although Calyculin A completely prevented ceramide-induced Rb activation, it did not prevent ceramide-induced myeloid differentiation. This would suggest that ceramide-induced cell cycle arrest (presumably via Rb activation) and ceramide-induced differentiation occur by two independent but simultaneous pathways: the phosphatase-dependent activation of Rb and subsequent cell cycle arrest and the phosphatase-independent initiation of myeloid differentiation involving activation of $p21^{Cip1/Waf1}$ expression. The activation of Rb by protein phosphatase activity functions to halt the cell cycle, and transcriptional activation of $p21^{Cip1/Waf1}$ occurs later as part of the terminal differentiation response to lock the cells into a non-proliferative state. Further elucidation as to the type/s of protein phosphatases activated by ceramide may present a novel target for directed therapy of leukaemia.

RNase-protection assays provided a preliminary means for examining transcriptional activation of a variety of cell cycle regulatory and apoptosis-associated genes. RPA experiments were conducted using the promyelocytic HL60

cell line treated with ceramide as the first link between ceramide and differentiation was first discovered using these cells (Okazaki *et al.*, 1989, Okazaki *et al.*, 1990, Kim *et al.*, 1991) and provided an ideal model for subsequent genetic screening for investigations into ceramide-mediated growth regulation and differentiation. As demonstrated in Chapter 5, expression of *p53* was not detected as HL60 cells lack a *p53* gene (Wolf & Rotter, 1985). An upregulation of $p21^{Cip1/Waf1}$ was demonstrated, which further confirms the findings presented in Chapter 3 and results published by other authors (Ragg *et al.*, 1998, Lee *et al.*, 2000), and supports the hypothesis that ceramide treatment results in the terminal differentiation of immature leukaemia cells (Steinman *et al.*, 1994, Halevy *et al.*, 1995, Liebermann *et al.*, 1995, Parker *et al.*, 1995, Flink *et al.*, 1998). An increase in both *GADD45* and *c-fos* expression following ceramide treatment is consistent with cell cycle arrest (Balsalobre & Jolicoeur, 1995, Kobayashi *et al.*, 1997, Okada *et al.*, 1999) and also differentiation (Sawai *et al.*, 1995) in HL60 cells.

These experiments clearly identified that the ceramide-signalling pathway involves a complex pattern of changes in gene expression that were consistent with cell cycle arrest, differentiation and apoptosis, and justified the need for a large-scale study of the genes involved in ceramide signalling. A comprehensive screen of 1,176 genes known to be involved in cancer was conducted on the ceramide-responsive erythromyeloid cell line, K562, using a commercially available cDNA filter array.

The demonstrated downregulation in the cell cycle-regulated *G_1/S-specific cyclin D3*, *P38-2G4* and *protein D123* genes following ceramide treatment illustrates that ceramide-treated K562 cells are undergoing cell cycle arrest, specifically at the G_0/G_1 phase of the cell cycle, which is consistent with the results shown in Chapter 3. Similarly, a downregulation in *PCNA* expression indicates an anti-proliferative response following ceramide treatment (Flores-Roses *et al.*, 1994, Li *et al.*, 1994, Waga *et al.*, 1994), and is consistent with an increase in $p21^{Cip1/Waf1}$ expression (Flores-Roses *et al.*, 1994, Waga *et al.*, 1994, Chen *et al.*, 1995b, Luo *et al.*, 1995, Warbrick *et al.*, 1995, Zhou *et al.*, 1998). The increase in *mcl-1* expression is also strongly indicative of cells undergoing cell cycle arrest, differentiation and apoptosis (Kozopas *et al.*, 1993). c-jun was shown to be upregulated, which is consistent with an increase in c-fos expression demonstrated in the HL-60 cells, as these transcription factors form the AP-1 complex, which is known to be involved in differentiation and apoptotic responses (Lord *et al.*, 1993, Sawai *et al.*, 1995, Pruschy *et al.*, 1997). The downregulation in *c-myc* expression also demonstrates cell cycle arrest, as this gene is rapidly suppressed by p53 upon induction of growth arrest and

apoptosis (Guillouf *et al.*, 1995) in normal, healthy cells. Furthermore, *c-myc* expression is known to be dramatically reduced during myeloid terminal differentiation in a variety of cell types (Collins, 1987, Larsson *et al.*, 1988 & 1994, Liebermann & Hoffman-Liebermann, 1989, Gomez-Casares *et al.*, 1993, Nguyen *et al.*, 1995a, Lerga *et al.*, 1999).

cDNA array analysis also revealed some novel findings that are worthy of mentioning here, including the upregulation in expression of a protein phosphatase (*hVH5*) other than the CAPP. Also, expression of genes whose products are known to be involved in forming and co-ordinating assembly of the components of the cytoskeleton of the cell (zyxin, plakoglobin, cytokeratin 8 and 18, vimentin and desmin) were upregulated following ceramide treatment. This is an indication of cellular stabilisation and demonstrates that changes are occurring in the mechanical properties of the cell. Features that support the hypothesis that ceramide can induce cell maturation and tumour suppression (Dellagi *et al.*, 1983, Sadler *et al.*, 1992, van der Ven *et al.*, 1992, Macalma *et al.*, 1996, Witcher *et al.*, 1996, Beckerle, 1997).

While an enormous amount of data was generated from the cDNA array analysis of ceramide-treated K562 cells, the results from these experiments can be summarised in the following general terms. Genes that are known to be involved in suppressing progression through the cell cycle and genes involved in the differentiation response were upregulated, while the expression of proliferation-related genes were downregulated. These findings are consistent with the hypothesis that ceramide lowers the proliferative potential of leukaemic cells, and induces terminal differentiation of these cells into a mature phenotype.

Supplementary investigation of the genes shown to be differentially expressed in this cDNA array analysis was outside the scope of the current study, but is to be examined as part of a future study for the purpose of further characterising the ceramide-signalling pathway. Results from the array experiments were extremely valuable in confirming changes in genes involved in cell cycle arrest and differentiation, but also revealed unexpected changes in other novel genes. It is feasible to suggest that differentially expressed genes represent potential targets for defining cancer susceptibility to specific treatment protocols, for the development of new drugs for improved therapy, and for the development of genetic reagents for gene-based therapy (e.g. modulation of gene promoter activity) of cancer. Specifically, it may be possible to determine the responsiveness of a patient to a ceramide-related therapy by investigating the pattern of gene expression after treatment *in vitro* from a sample of circulating leukaemic cells. The identification of

sets of genes that change during differentiation therapy would create a molecular profile, and represents an important diagnostic tool. A high-density microarray containing a complete or relevant repertoire of genes, whose expression change as the cells revert to a more normal state by differentiation therapy with ceramide of a component of the SM pathway, could be used to define the effectiveness of such therapy and to monitor disease progression.

With the use of cDNA expression arrays, it is possible to monitor the differential expression in human genes, allowing changes in expression to be detected as a function of cell type, tissue source, physiological state or genetic background. cDNA arrays could also serve as a rapid method for identifying changes in expression that accompany treatment of human cells with drugs, hormones, inhibitors, elicitors and a host of other molecules (Schena, 1996). Extensive patient screening with cDNA arrays may allow the identification of informative patterns in gene expression in subsets of human populations, and thus it may also be possible in the future that cDNA arrays will be utilised in the diagnosis of human disease.

Although filter arrays are limited to the number of genes that can be investigated at any one time, this technology was considered novel at the time this study was performed, and provided an immense amount of useful data. More comprehensive arrays, which utilise similar methods to those used for filter array analysis, are now available for large-scale gene expression investigations, including glass slide microarrays and oligochip arrays. Glass slide microarray analysis of gene expression is a considerably more expensive method than nylon filter array analysis, and requires specialised equipment and software packages. Furthermore, multiple exposure times are possible with nylon filter arrays which utilise radioactive detection techniques such as that used for Atlas™ arrays for obtaining results for less abundant transcripts or preventing spot saturation. In general, applications utilising detection of radioactive sources also provide greater sensitivity over fluorescent-based assays, and Atlas™ arrays use low complexity and high purity arrayed PCR amplified DNA as opposed to lysed bacterial colonies which is thought to further increase the sensitivity of these filters (Bowtell, 1999).

Differentiation therapy relies on the fact that neoplastic cells display alterations in normal programs of differentiation and growth control that can be overcome by appropriate treatment (Leszczyniecka *et al.*, 2001). For an agent to be successfully used in differentiation therapy it must possess certain fundamental qualities. It must be capable of tumour reprogramming by modifying the state of differentiation, leading to a concomitant loss in proliferative capacity of cancer cells

by apoptosis. Furthermore, as is the case with any therapeutic, it must be well tolerated, capable of being delivered via a suitable administration route, biologically available to the target cell population, and, ideally, its effects must be stable and prolonged.

Two differentiation-mediating drugs have shown promise as differentiation agents. All-*trans* retinoic acid (ATRA) has the ability to reduce the proliferative activity of APL cells, and induce granulocytic differentiation and subsequent apoptosis of these neoplastic cells (Chen *et al.*, 1991, Drach *et al.*, 1994, Fenaux & Degos, 1996, Shiohara *et al.*, 1999, Gianni *et al.*, 2000, Hansen *et al.*, 2000). ATRA continues to be used today in the clinical treatment of APL, and overcomes many side effects commonly seen with conventional therapies used in treating APL (Kawai *et al.*, 1994). ATRA treatment is commonly used in combination with conventional chemotherapeutics (daunorubicin and araC) as the standard treatment for APL (Bruserud *et al.*, 2000, Fenaux *et al.*, 1999, Fenaux, 2000) as ATRA/daunorubicin/araC combined therapy results in significantly reduced relapse rates, and longer survival times compared with chemotherapy or ATRA alone (Fenaux, 2000). Restricting its use however, is the fact that it is only effective in cells displaying the t(15;17) chromosomal translocation, which encodes for the PML-RAR fusion promyelocytic leukaemia protein (Degos, 1992, Li *et al.*, 1997), which can be overcome with supraphysiologic concentrations of ATRA (Grignani *et al.*, 1998). Additionally, ATRA treatment is associated with a potentially fatal syndrome (ATRA syndrome) involving a significant increase in leukocyte count (Fenaux & De Botton, 1998).

Vitamin-D_3 (1α,25-dihydroxyvitamin D_3), like ATRA, exhibits the fundamental features necessary for its use as a differentiation therapy. Koeffler and colleagues (1984) were first to publish findings that this agent was capable of inducing myeloid differentiation and mediate cell cycle arrest at the G_0/G_1, and possible the G_2/M phases of the cell cycle. Clinical trials involving vitamin-D_3 are still in their infancy, although its use appears to be severely hampered by its associated toxicity, namely hypercalcaemia, at a concentration required to suppress cancer cell proliferation (Laubenthal *et al.*, 1975, Nagpal *et al.*, 2001, Mehta & Mehta, 2002).

The current study has demonstrated that ceramide also displays the fundamental qualities of a differentiation therapeutic, at least in the *in vitro* model. Treatment with the ceramide analogue, C_2-ceramide, results in a reduction in viable cell numbers, halts cellular proliferation via the induction of G_0/G_1 cell cycle arrest,

and initiates terminal differentiation in myeloid cells which is followed by cell death by apoptosis. What remains to be demonstrated is how circulating leukaemic cells respond to ceramide treatment *in vivo*. Being of lipid composition, it could be assumed that ceramide would be sufficiently available to, and easily taken up by, the target leukaemic cells. In fact, a study published by Venable *et al.* (1995) demonstrated that 16% of total radiolabelled ceramide is taken up by WI-38 fibroblast cells within the first hour of treatment, 44% by 24 hours, and 63% of total ceramide is taken up by the cells by 48 hours. A point considerably worthy of mention is that all ceramide treatments performed in this study were conducted in serum-free culture conditions. Bielawska *et al.* (1992) have reported that serum attenuates the effect of ceramide in HL60 cells, resulting in a 10-fold increase in the IC50% due to the presence of lipid-binding proteins. As such, further investigations into the effect of ceramide uptake in the presence of serum are warranted to ascertain the validity of administering ceramide *in vivo*. Although *in vitro* results are promising, limited data is available in the literature as to how well ceramide is tolerated *in vivo*, and to address this concern, and the concerns of administration route and the longevity of its effects *in vivo*, future experiments using an animal model are well justified.

It must be considered that ceramide itself may not be a suitable therapeutic, for reasons including bioavailability and possible intolerance *in vivo*. This study has aimed to provide a greater understanding into the molecular mechanisms involved in the ceramide-signalling pathway, and has revealed a number of potential key molecules that are worthy of consideration for the target-directed therapy of leukaemia. Thus, it may be more feasible to target a component of this signalling pathway, than focus on ceramide itself as a differentiation therapeutic. Additionally, as is the case with ATRA and its combined use with araC and daunorubicin, it is important to consider that a novel therapy utilising one or more components of the ceramide-signalling pathway could be used in combination with conventional chemotherapeutics, perhaps as a means of sensitising neoplastic cells through induction of terminal differentiation to overcome their differentiation blockage.

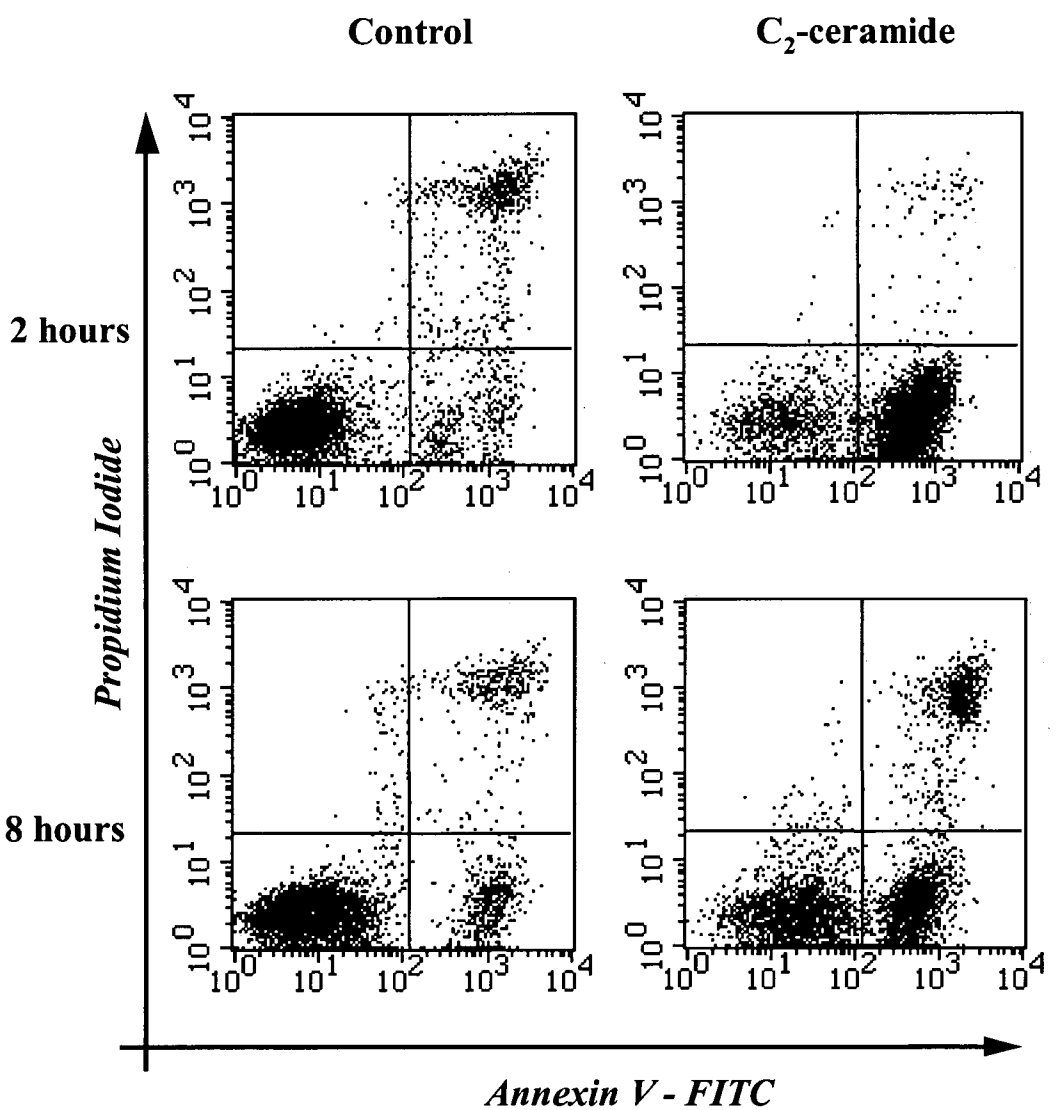
To further investigate the ability of ceramide to induce terminal differentiation, and in the search for potential new therapeutic targets, investigations into the expression of transcription factors (TFs) known to be differentially expressed in terminally differentiated cells are warranted. Examples include PU.1, GATA-1 & -2, SCL, and Id2. Expression of the *Spi-1/Spf-1* gene (PU.1) is required for terminal differentiation of myeloid cells (Chen *et al.*, 1995c, Olson *et al.*, 1995, Simon *et al.*,

1996), but is not expressed in mature erythroid cells (Galson *et al.*, 1993, Hromas *et al.*, 1993). Similarly, *Id2* expression is markedly increased in terminally differentiated myeloid cells (Ishiguro *et al.*, 1996). GATA-1, GATA-2, and SCL expression is high in totipotent precursor cells before they undergo commitment and remains high as cells mature towards the erythroid lineage, and is specifically repressed during differentiation to the monocytic lineage (Green *et al.*, 1991, Mouthon *et al.*, 1993, Shimamoto *et al.*, 1994, Murrell & Green, 1995, Shimamoto *et al.*, 1995). Thus, it is proposed that examination of these TFs during ceramide treatment of myeloid cells would confirm induction of terminal differentiation by ceramide, and would be useful in confirming the lineage-specific differentiation of K562 cells.

In summary, the results presented in this thesis demonstrate that the sphingomyelin-signalling pathway plays a fundamental role in regulating cell growth, differentiation and death. The central component of this signalling pathway, ceramide, is capable of activating cellular regulatory elements in a p53-independent manner, which is of particular interest, as p53 is the most disrupted gene in cancer (Nigro *et al.*, 1989, Levine *et al.*, 1991, Hollstein *et al.*, 1991 & 1994, Greenblatt *et al.*, 1994). The ability to change aggressively growing leukaemic cells into mature, less proliferative cells that then die by programmed cell death are the fundamental attributes of a potential candidate for use as a differentiation therapeutic. It is therefore proposed that components of this ubiquitous, highly conserved signalling pathway, or its downstream effectors, may be manipulated for the purpose of inducing terminal differentiation in abnormally proliferating cell populations, and thus potentially becoming a novel therapy in the treatment of leukaemia.

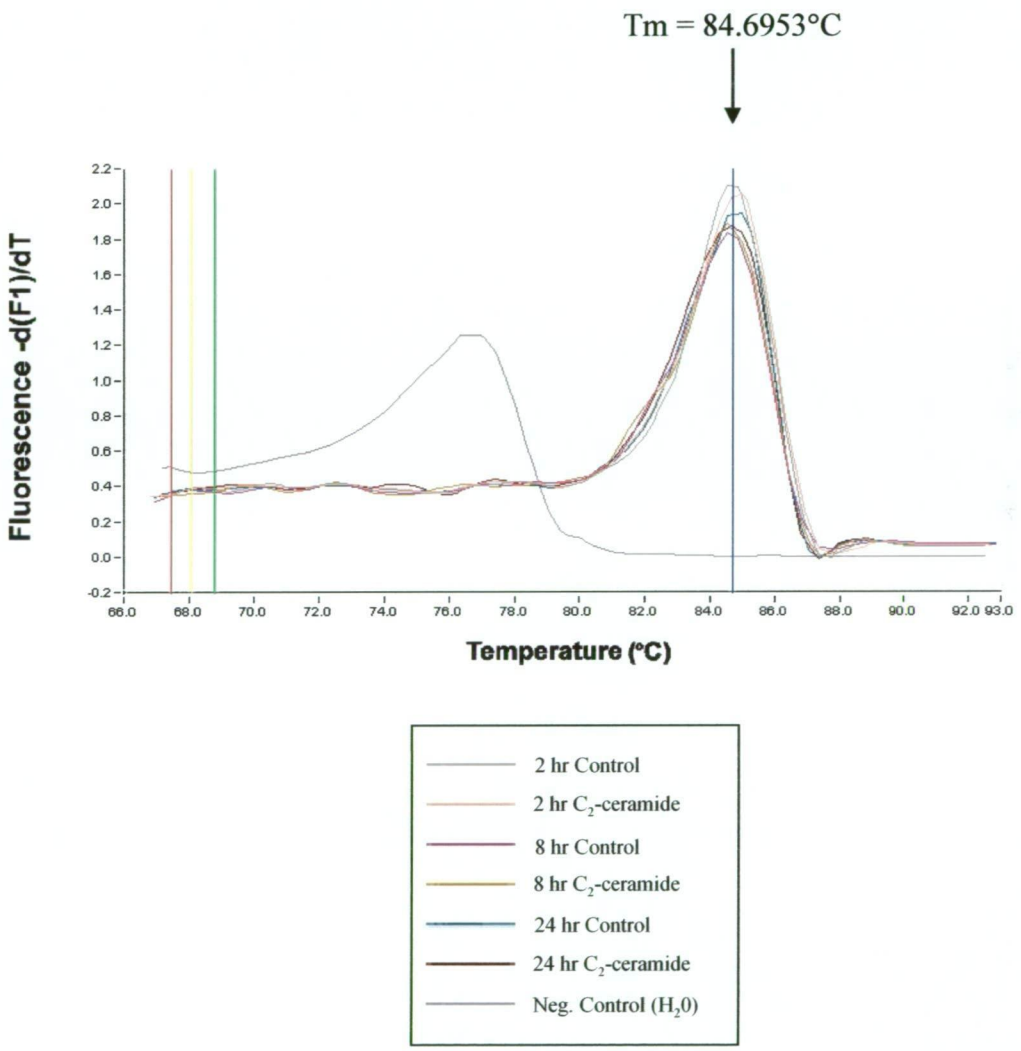
Appendix A

Annexin V-FITC/propidium iodide (Annexin V/PI) analysis to confirm C_2-ceramide-induced apoptosis in Jurkat T lymphocytic cells at 2 and 8 hours post-treatment with 5 μM C_2-ceramide. The live (Annexin V-/ PI-), apoptotic (Annexin V+/PI-) and dead (Annexin V+/PI+) populations can be readily identified. Taken from Hearps *et al.*, 2000.



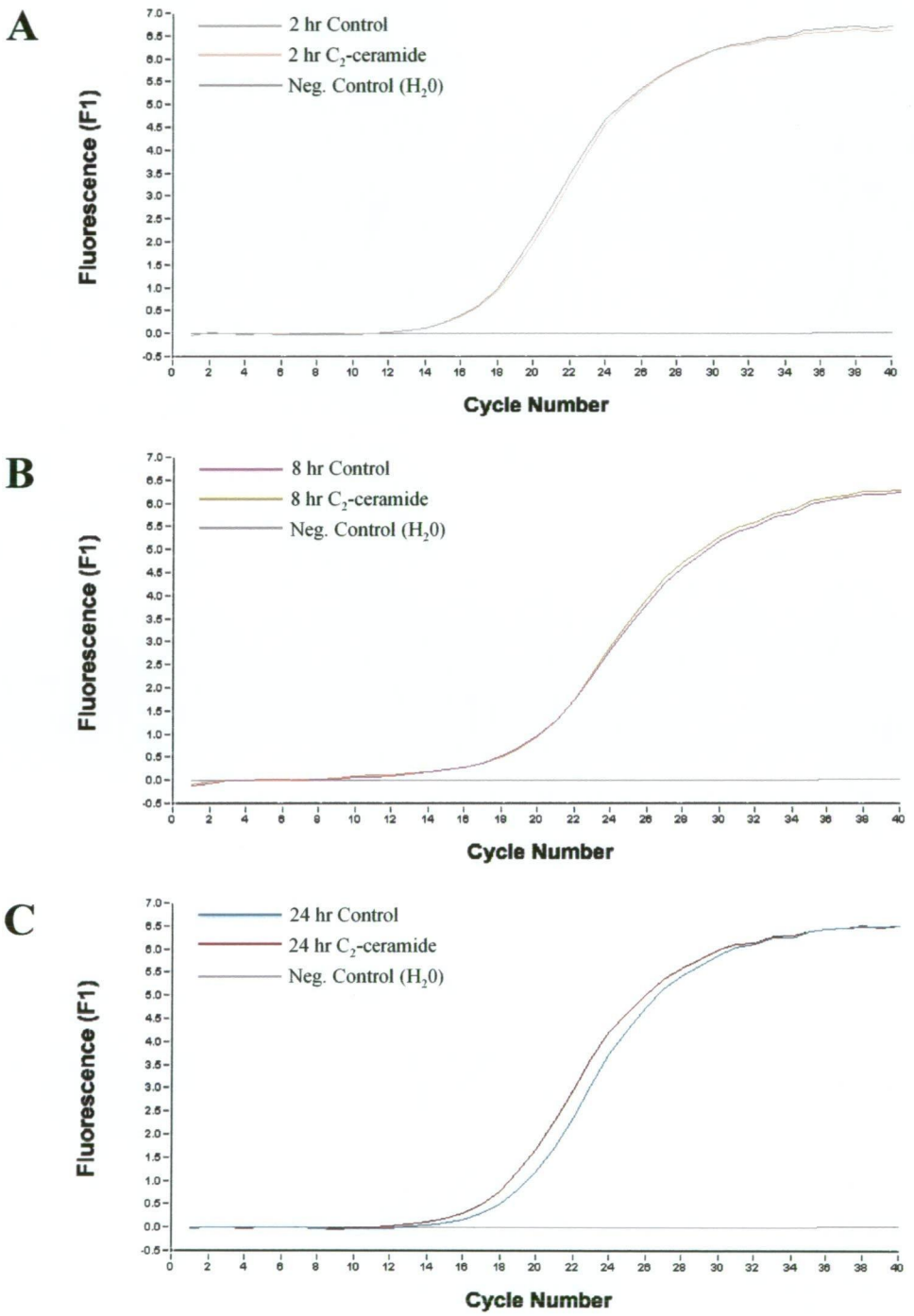
Real-time RT-PCR melting curves generated using the LightCycler software package (Roche Diagnostics, Germany) for the PRAME (preferentially expressed antigen of melanoma) primer set at 2, 8 and 24 hours post-treatment with vehicle or 5 μM C_2-ceramide, to demonstrate the specificity and Tm of the primer set.

Appendix B


Tm = 84.6953°C
Fluorescence -d(F1)/dT
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
66.0
68.0
70.0
72.0
74.0
76.0
78.0
80.0
82.0
84.0
86.0
88.0
90.0
92.0
93.0
Temperature (°C)
2 hr Control
2 hr C2-ceramide
8 hr Control
8 hr C2-ceramide
24 hr Control
24 hr C2-ceramide
Neg. Control (H20)

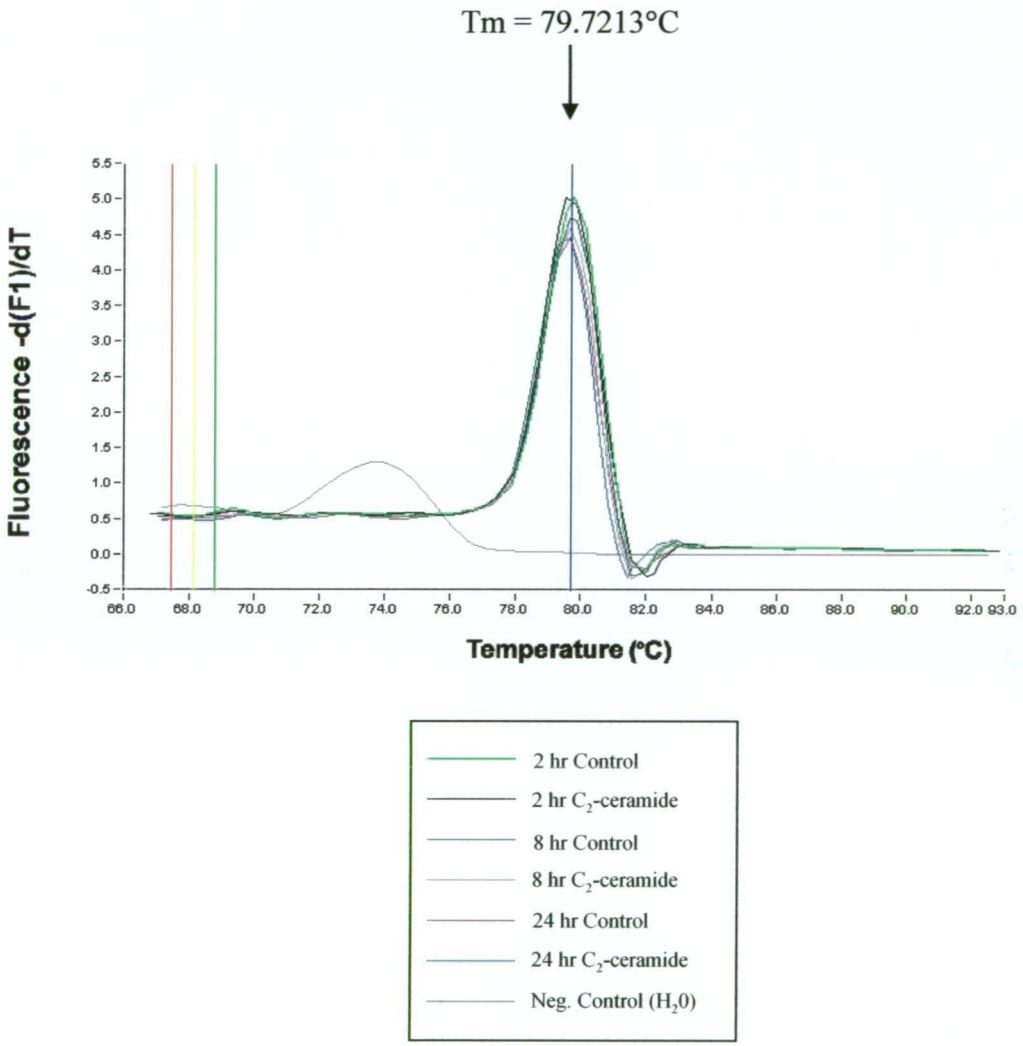
Real-time RT-PCR amplification curves generated using the LightCycler software package (Roche Diagnostics, Germany) for the PRAME (preferentially expressed antigen of melanoma) primer set at 2 hours (**A**), 8 hours (**B**) and 24 hours (**C**) post-treatment with vehicle or 5 μM C_2-ceramide, using the same RNA as that used in the cDNA array experiments with K562 erythromyeloid cells.

Appendix B (continued)



Real-time RT-PCR melting curves generated using the LightCycler software package (Roche Diagnostics, Germany) for the PCNA (proliferating cell/cyclic nuclear antigen) primer set at 2, 8 and 24 hours post-treatment with vehicle or 5 μM C_2-ceramide, to demonstrate the specificity and Tm of the primer set.

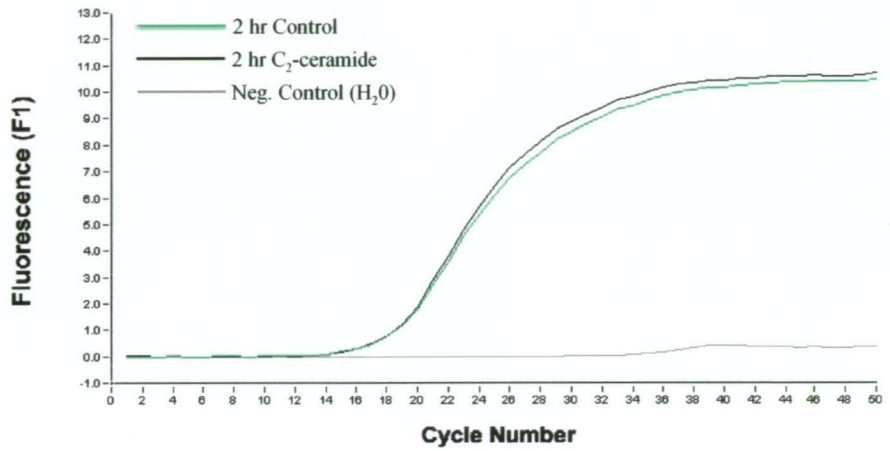
Appendix B (continued)



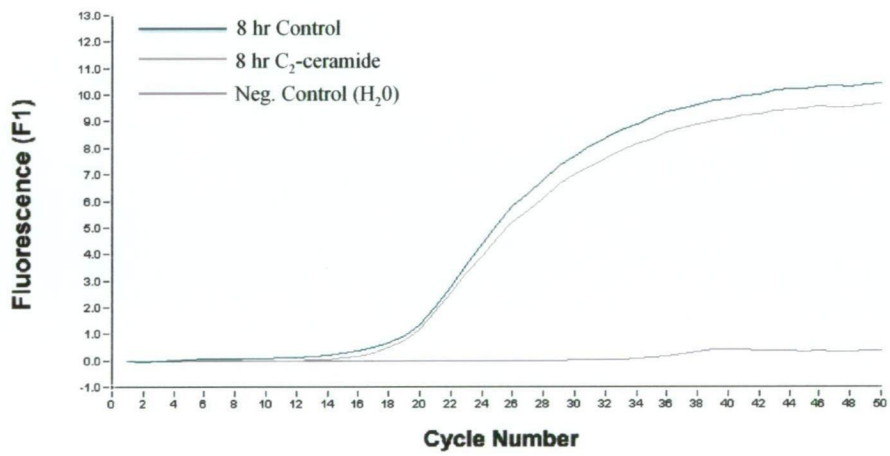
Real-time RT-PCR amplification curves generated using the LightCycler software package (Roche Diagnostics, Germany) for the PCNA (proliferating cell/cyclic nuclear antigen) primer set at 2 hours (**A**), 8 hours (**B**) and 24 hours (**C**) post-treatment with vehicle or 5 μM C_2-ceramide, using the same RNA as that used in the cDNA array experiments with K562 erythromyeloid cells.

Appendix B (continued)

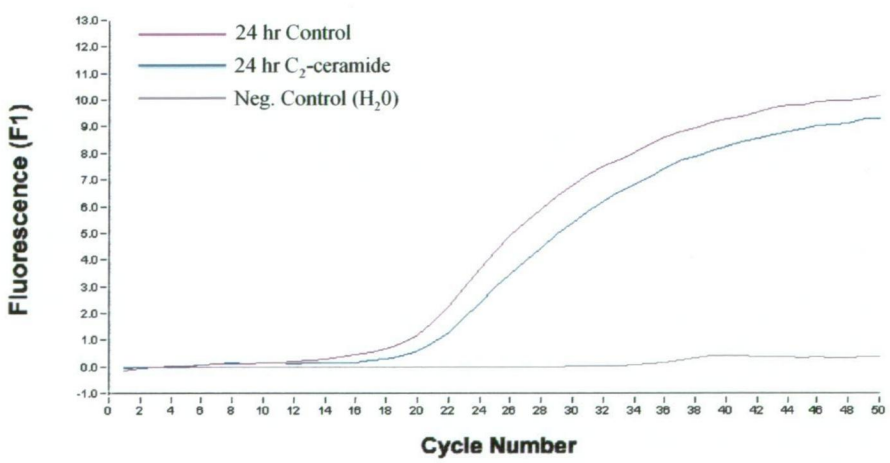
A



B

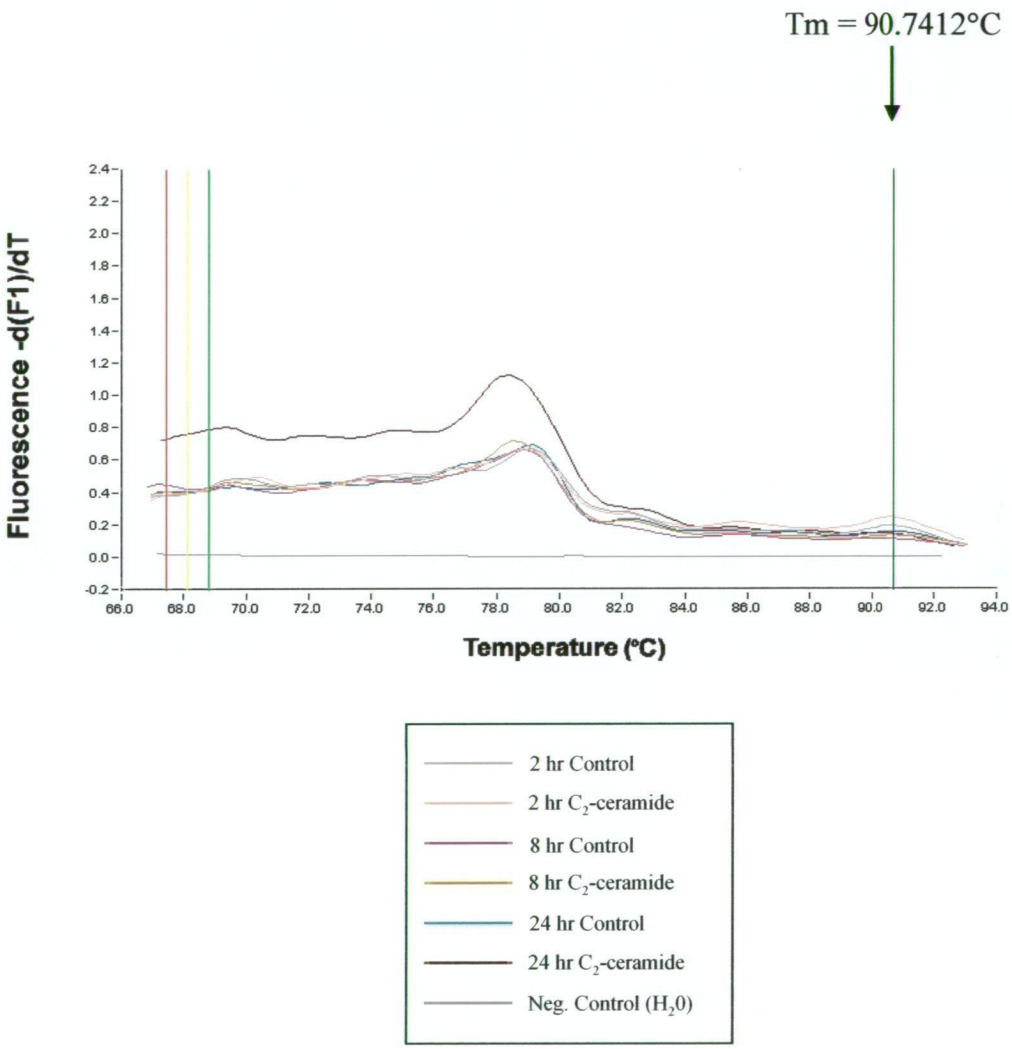


C



Real-time RT-PCR melting curves generated using the LightCycler software package (Roche Diagnostics, Germany) for the MIC1 (macrophage inhibitory cytokine 1) primer set at 2, 8 and 24 hours post-treatment with vehicle or 5 μM C_2-ceramide, to demonstrate the specificity and Tm of the primer set.

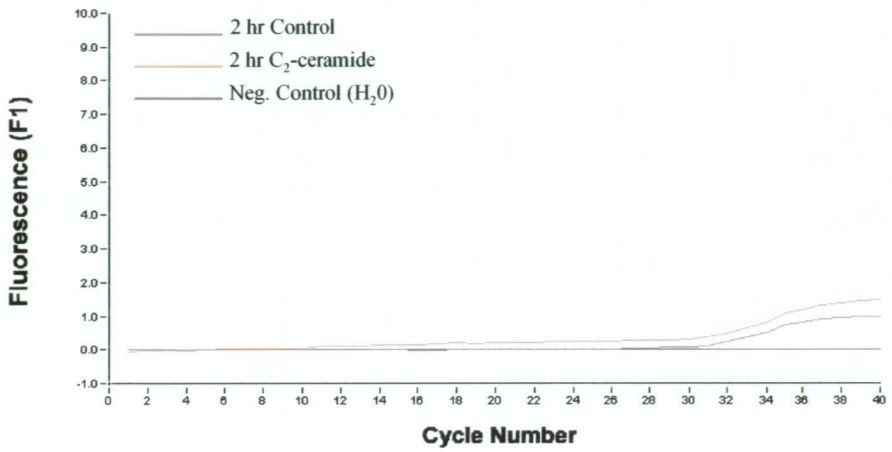
Appendix B (continued)



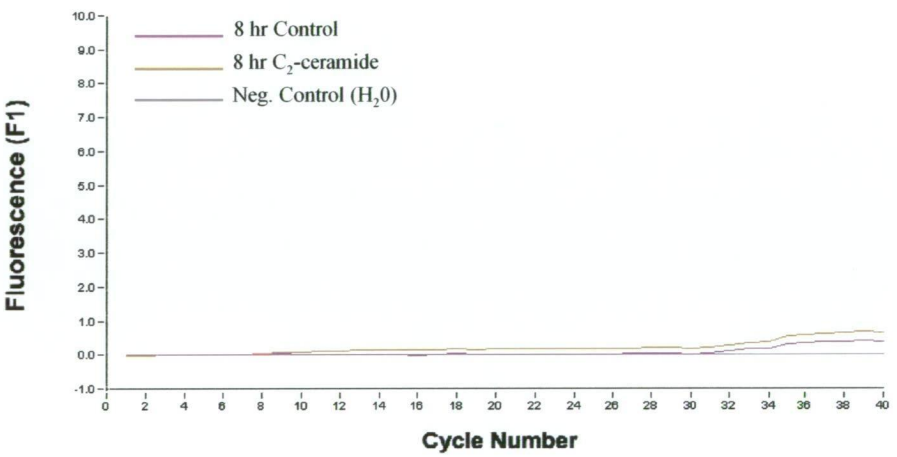
Real-time RT-PCR amplification curves generated using the LightCycler software package (Roche Diagnostics, Germany) for the MIC1 (macrophage inhibitory cytokine 1) primer set at 2 hours (**A**), 8 hours (**B**) and 24 hours (**C**) post-treatment with vehicle or 5 μM C_2-ceramide, using the same RNA as that used in the cDNA array experiments with K562 erythromyeloid cells.

Appendix B (continued)

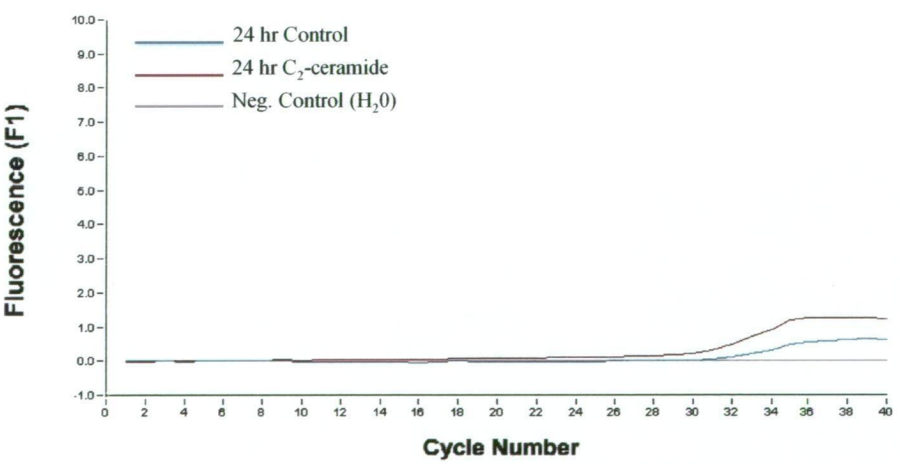
A



B

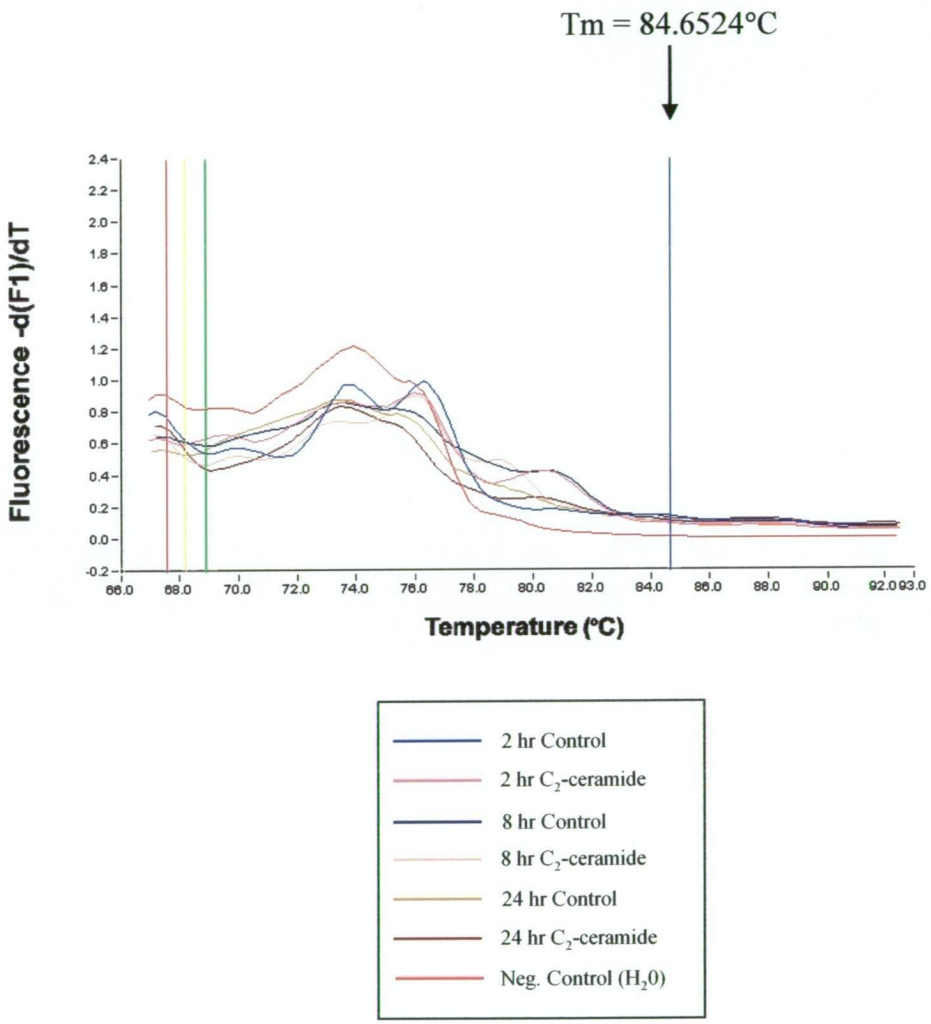


C



Real-time RT-PCR melting curves generated using the LightCycler software package (Roche Diagnostics, Germany) for the GADD153 (growth arrest & DNA-damage-inducible protein 153) primer set at 2, 8 and 24 hours post-treatment with vehicle or 5 μM C_2-ceramide, to demonstrate the specificity and Tm of the primer set.

Appendix B (continued)



Real-time RT-PCR amplification curves generated using the LightCycler software package (Roche Diagnostics, Germany) for the GADD153 (growth arrest & DNA-damage-inducible protein 153) primer set at 2 hours (**A**), 8 hours (**B**) and 24 hours (**C**) post-treatment with vehicle or 5 μM C_2-ceramide, using the same RNA as that used in the cDNA array experiments with K562 erythromyeloid cells.

Appendix B (continued)

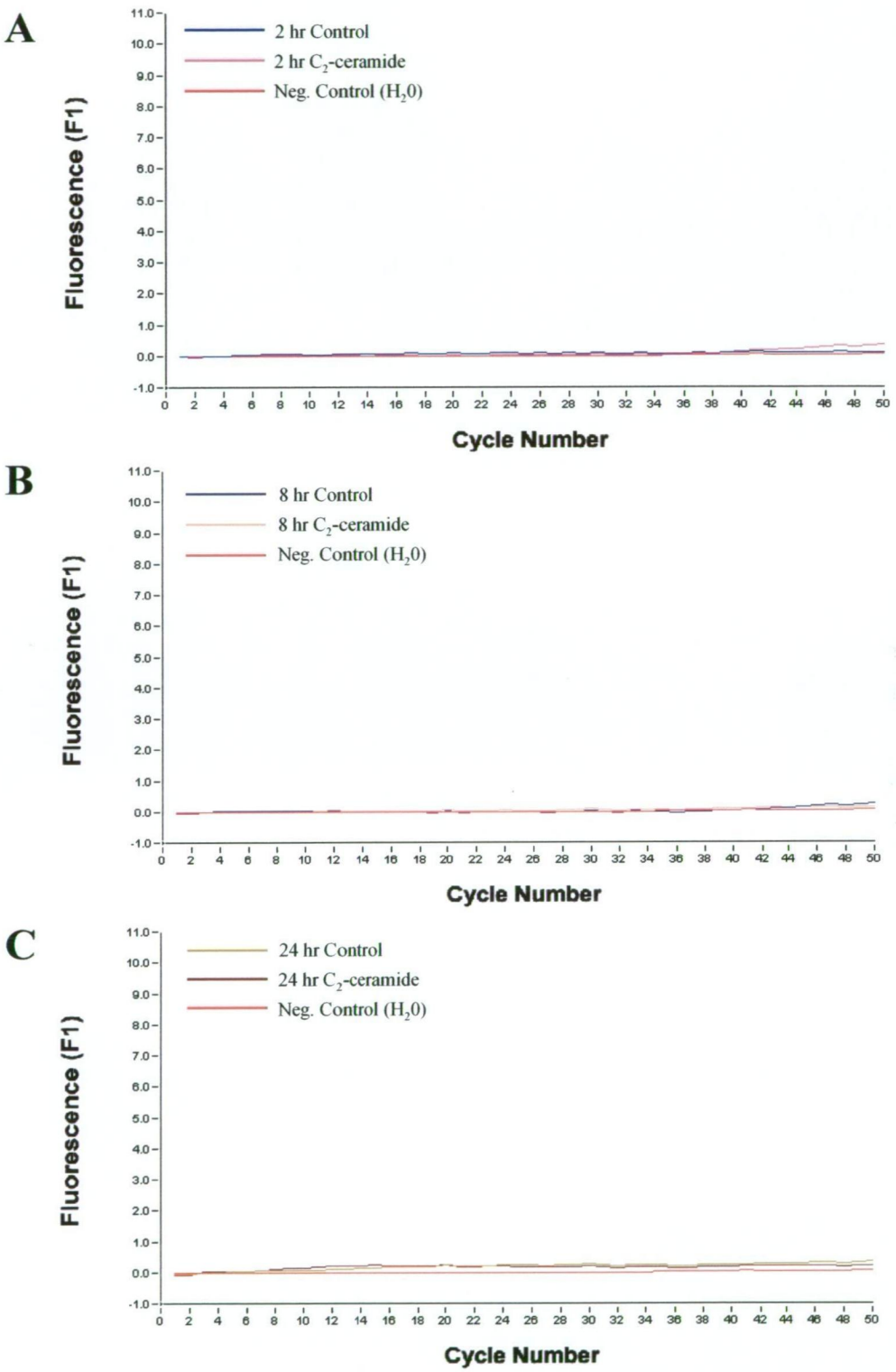


Table of real-time RT-PCR array confirmation data for PRAME, PCNA, MIC1 and GADD153 using K562 RNA used in the cDNA array analysis experiments. GAPDH was co-amplified at each individual timepoint for normalisation purposes. All data presented in the table was obtained by Sandrine Chopin in our laboratory.

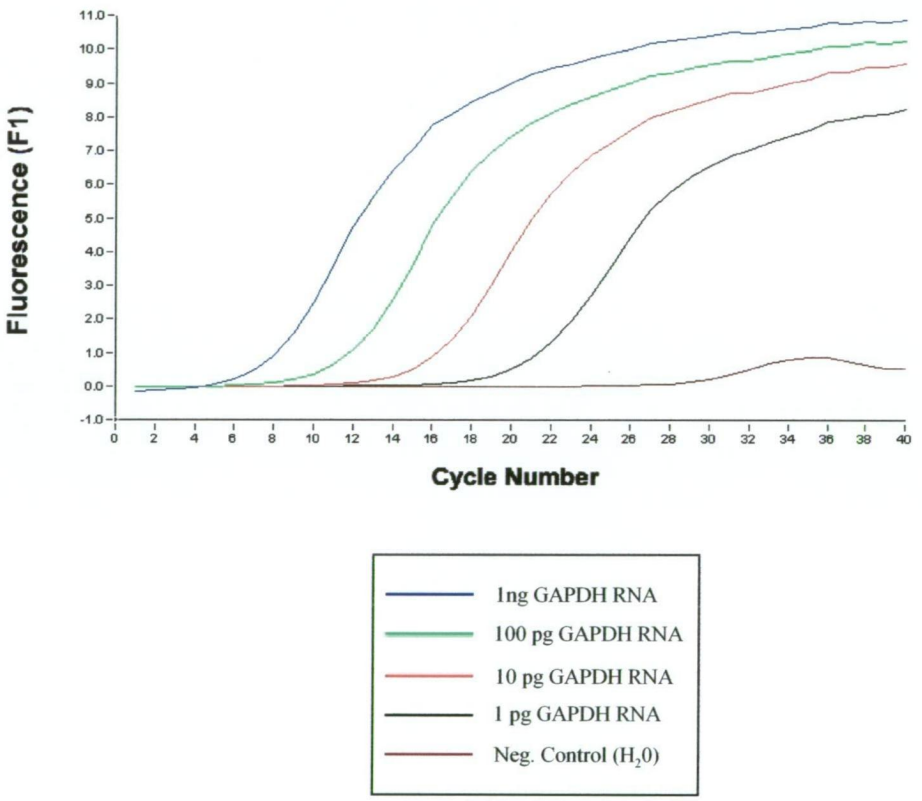
Appendix C

Gene	Timepoint	Crossing Point	Calculated Conc. (ng)	Normalised Fold Change (relative to GAPDH)
PRAME	Vehicle 2hr (GAPDH Vehicle 2hr)	16.82 (19.09)	5.896×10^{-2} (7.049×10^{-3})	0.53
	C_2-ceramide 2hr (GAPDH C_2-ceramide 2hr)	16.29 (17.36)	4.963×10^{-2} (1.115×10^{-2})	
	Vehicle 8hr (GAPDH Vehicle 8hr)	16.92 (18.26)	1.293×10^{-2} (5.827×10^{-3})	2.78
	C_2-ceramide 8hr (GAPDH C_2-ceramide 8hr)	17.31 (18.07)	1.270×10^{-2} (2.057×10^{-3})	
	Vehicle 24hr (GAPDH Vehicle 24hr)	17.06 (17.97)	2.351×10^{-2} (2.165×10^{-3})	3.24
	C_2-ceramide 24hr (GAPDH C_2-ceramide 24hr)	16.53 (22.89)	4.055×10^{-2} (1.149×10^{-3})	
PCNA	Vehicle 2hr (GAPDH Vehicle 2hr)	18.54 (16.85)	1.528×10^{-3} (4.077×10^{-3})	0.31
	C_2-ceramide 2hr (GAPDH C_2-ceramide 2hr)	18.51 (14.78)	1.556×10^{-3} (1.350×10^{-2})	
	Vehicle 8hr (GAPDH Vehicle 8hr)	19.28 (17.73)	9.956×10^{-4} (2.439×10^{-3})	0.65
	C_2-ceramide 8hr (GAPDH C_2-ceramide 8hr)	18.85 (16.56)	4.826×10^{-3} (9.956×10^{-3})	
	Vehicle 24hr (GAPDH Vehicle 24hr)	19.76 (17.65)	7.501×10^{-4} (2.557×10^{-3})	0.83
	C_2-ceramide 24hr (GAPDH C_2-ceramide 24hr)	20.30 (17.86)	5.492×10^{-4} (2.259×10^{-3})	
MIC1	Vehicle 2hr (GAPDH Vehicle 2hr)	30.63 (21.34)	7.162×10^{-6} (1.047×10^{-3})	0.77
	C_2-ceramide 2hr (GAPDH C_2-ceramide 2hr)	30.80 (21.00)	6.551×10^{-6} (1.258×10^{-3})	
	Vehicle 8hr (GAPDH Vehicle 8hr)	30.86 (20.28)	6.319×10^{-6} (1.845×10^{-3})	0.44
	C_2-ceramide 8hr (GAPDH C_2-ceramide 8hr)	33.30 (21.28)	1.714×10^{-6} (1.081×10^{-3})	
	Vehicle 24hr (GAPDH Vehicle 24hr)	30.83 (22.04)	6.443×10^{-6} (7.191×10^{-4})	2.90
	C_2-ceramide 24hr (GAPDH C_2-ceramide 24hr)	30.12 (23.30)	9.420×10^{-6} (3.649×10^{-4})	
GADD153	Vehicle 2hr (GAPDH Vehicle 2hr)	NR (6.93)	NR (1.465×10^{-1})	NR
	C_2-ceramide 2hr (GAPDH C_2-ceramide 2hr)	37.98 (9.50)	1.719×10^{-9} (3.230×10^{-2})	
	Vehicle 8hr (GAPDH Vehicle 8hr)	40.47 (7.57)	3.967×10^{-10} (1.003×10^{-1})	(10.78)
	C_2-ceramide 8hr (GAPDH C_2-ceramide 8hr)	37.30 (8.44)	2.566×10^{-9} (6.017×10^{-2})	
	Vehicle 24hr (GAPDH Vehicle 24hr)	NR (18.50)	NR (1.672×10^{-3})	NR
	C_2-ceramide 24hr (GAPDH C_2-ceramide 24hr)	5.424 (5.424)	3.551×10^{-1} (3.551×10^{-1})	

N.R. = No result obtained (unable to determine fold change).

Real-time RT-PCR amplification curves generated using the LightCycler software package (Roche Diagnostics, Germany) for the GAPDH primer set using a range of GAPDH RNA template concentrations for generation of a standard curve for determining relative concentrations of the confirmatory genes.

Appendix D


Fluorescence (F1)
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Cycle Number
1ng GAPDH RNA
100 pg GAPDH RNA
10 pg GAPDH RNA
1 pg GAPDH RNA
Neg. Control (H_20)

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Cell Cycle and Control

Cell Cycle Arrest of Hematopoietic Cell Lines After Treatment With Ceramide Is Commonly Associated With Retinoblastoma Activation

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Background: Leukaemia cells differ from their normal counterparts in that their ability to properly regulate survival, proliferation, differentiation, and apoptosis is aberrant. Understanding the molecular mechanisms controlling cell proliferation and developing therapeutic strategies to correct nonfunctional regulatory mechanisms are emerging areas of medical research. Ceramide, a metabolite of membrane sphingomyelin hydrolysis, has recently emerged as a key regulator of cellular proliferation, differentiation, and apoptosis in leukaemia cells.
Methods: Leukaemia cell lines were treated with a biologically active analogue of ceramide, C_2-ceramide. Cell cycle status was assessed flow cytometrically using propidium iodide. Induction of apoptosis was confirmed by annexin V staining of externalised phosphatidylserine and retinoblastoma activation was determined by Western blotting.
Results: C_2-ceramide induced activation of retinoblastoma tumour suppressor protein, G_0/G_1 cell cycle arrest, or apoptosis in leukaemia cell lines. In addition, these effects differed depending upon cell type, thus confirming the pleiotropic nature of the ceramide signalling pathway. Most cells studied responded to exogenous C_2-ceramide by entering growth arrest, evidently resulting from activation of retinoblastoma protein, and by displaying some degree of apoptosis.
Conclusions: Taken together, these findings suggest that signalling via ceramide has novel therapeutic applications for treatment of leukaemia. Cytometry 43:164-169, 2001. © 2001 Wiley-Liss, Inc.

Key terms: ceramide; retinoblastoma; cell cycle arrest; apoptosis; leukaemia

Defects in cell cycle regulatory mechanisms can result in uncontrolled proliferation and, when this occurs in haematopoietic cells, cancers such as leukaemia or lymphoma may arise. Halting the growth of aggressively multiplying leukaemic cells is the primary aim of therapies designed to treat this cancer, and most treatment protocols achieve this by killing the leukaemic cells through the use of cytotoxic drugs or by radiation therapy. Another, as yet underused approach to halting the growth of leukaemic cells is to promote their development into a more mature cell type that does not divide, a process called differentiation therapy (1). The interest in differentiation therapy lies in the fact that the differentiated cells lose their proliferative capacity; hence, the abnormal growth of leukaemic cells can be inhibited by inducing them to develop into mature nondividing cells.

In recent years, the sphingomyelin pathway has emerged as an important regulator of cellular growth control mechanisms (2). The sphingomyelin pathway is a ubiquitous, evolutionary conserved signalling system that is initiated by hydrolysis of the plasma membrane sphingolipid, sphingomyelin, to generate the second messenger ceramide. As such, the sphingomyelin pathway is analogous to conventional signalling systems such as cyclic AMP and phosphoinositide pathways. Sphingomyelin hydrolysis results from action of acidic or neutral sphingomyelinase isoforms (3), or ceramide can be generated de

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novo by ceramide synthase (4). Multiple direct targets of ceramide have been identified, including a proline-directed ceramide-activated protein kinase (5), a serine/threonine ceramide-activated protein phosphatase (CAPP) (6), protein kinase Cζ (7), and the proto-oncogenes Vav (8) and c-Raf-1 (9). In addition, ceramide interacts with several signalling cascades including mitogen-associated protein kinase, JNK, caspase and mitochondrial signalling systems (reviewed in 10).

Ceramide is pleiotropic in its effects and, depending upon the cell type, can modulate differentiation, growth arrest, proliferation, and apoptosis. This diversity of response is most probably a result of ceramide engaging different downstream effectors depending upon the cellular microenvironment, the phase of the cell cycle, and the magnitude of ceramide generation and its source (10). In a recent study we demonstrated that ceramide caused terminal differentiation of the U937 monoblastic leukaemia cells that was characterised by cell cycle arrest and activation of the retinoblastoma nuclear tumour suppressor phosphoprotein (Rb). We postulated that this induction of tumour suppressor genes could be exploited for therapeutic purposes (11). This present study further investigates the therapeutic potential of ceramide by investigating whether cell cycle arrest and Rb tumour suppressor protein activation are common features of ceramide-induced growth arrest of human haematopoietic cell lines.

MATERIALS AND METHODS

Reagents

The synthetic, cell-permeable ceramide analogue C_2-ceramide was purchased from BioMol Research Laboratories (Plymouth Meeting, PA, USA), dissolved in absolute ethanol, and stored at −80°C.

Cell Lines and Culture Conditions

The human leukaemia cells used in this study were from the monocytic U937, KG1, KG1a, promyelocytic HL-60, erythromyelocytic K562, and lymphocytic Jurkat and Raji cell lines. All cells were maintained at a density of 1-10 × 10^5 cells/ml, except KG1 cells, which were maintained at 2-20 × 10^5 cells/ml. Cells were cultured in RPMI-1640 medium (JRH Biosciences, Lenexa, KS, USA), supplemented with 2 mM L-glutamine (JRH Biosciences), 5-10% fetal bovine serum (CSL Limited, Parkville, Victoria, Australia), and 100 U/ml gentamicin (Pharmacia & Upjohn, Perth, WA, Australia) and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Experiments were conducted using exponentially growing cells in serum-free medium, except experiments using HL-60 cell lines, which were conducted using RPMI +2% foetal bovine serum and Raji cells treated in X-Vivo serum-free medium (Biowhittaker, Walkersville, MD, USA). Before treatment, cells were washed in phosphate buffered saline (PBS) and resuspended at 5 × 10^5 cells/ml in medium, and incubated in serum-free medium for another 2 h before the addition of C_2-ceramide. Cells were treated with the following concentrations of C_2-ceramide: 5.0 μM: U937, K562, and Jurkat; 7.5 μM: HL-60; 20 μM: KG1, KG1a, and Raji. These doses of C_2-ceramide were selected for each cell line based upon maximal growth arrest and minimising the apoptosis observed at higher concentrations.

Cell Cycle Analysis

Cells (1 × 10^6) were harvested by centrifugation, resuspended in hypotonic propidium iodide (PI) solution (0.1%[w/v] sodium citrate, 0.1% [v/v] Triton X-100, 0.05 mg/ml PI), and left overnight at 4°C in the dark (11,12). Because the coefficients of variation were unsatisfactory with the method described in the previous section, Raji cells were washed with PBS, fixed with 70% ethanol/PBS, and treated with RNase before staining with PI. Cell cycle data were acquired using a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA, USA) and subsequent cell cycle analysis was performed using ModFit software (Becton-Dickinson) to model the linear red fluorescence signal area. Apoptosis was identified as a sub-G_0/G_1 peak and confirmed in separate experiments by annexin V staining of exposed phosphatidylserine groups (Annexin V-FITC Apoptosis Detection Kit, BD Pharmingen, San Diego, CA, USA). Aggregates were excluded from the cell cycle analysis by use of the doublet discrimination module and subsequent gating on the linear red fluorescence area and width parameters.

Western Blot Analysis of Rb Phosphorylation Status

Cellular protein extracts were prepared from 2 × 10^6 harvested cells by lysis of the pelleted cells in 50-100 μl freshly prepared lysis buffer (25 mM Tris [pH 7.4], 50 mM NaCl, 50 mM NaF, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulphate [SDS], Complete protease inhibitor [Roche Diagnostics, Mannheim, Germany]) and cleared by centrifugation at 18,000*g* for 15 min at 4°C. Lysates were prepared for gel electrophoresis by boiling for 7 min in an equal volume of 2× Laemelli sample buffer (0.125 M Tris-HCL [pH 6.8], 20% [v/v] glycerol, 4% SDS, 0.005% bromophenol blue, 5% [v/v] 2-mercaptoethanol). Samples (30 μl) were loaded onto a 6.5% SDS-polyacrylamide gel and proteins were separated by electrophoresis. Proteins were transferred to Immun-Blot polyvinyldifluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA), and the membrane was blocked for 1 h at room temperature in 5% skim milk in PBST (PBS + 0.1% Tween 20), washed 3× in PBST, and incubated overnight at 4°C with 0.5 μg/ml anti-Rb-goat antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBST. The blots were washed 3× in PBST and incubated for 2 h at room temperature in 0.2 μg/ml anti-goat-horseradish peroxidase antibody (Santa Cruz Biotechnology, Inc.), followed by another three washes in PBST. Detection of Rb was performed using Enhanced Chemiluminescence and Hyperfilm (Amersham Life Science, Buckinghamshire, UK).

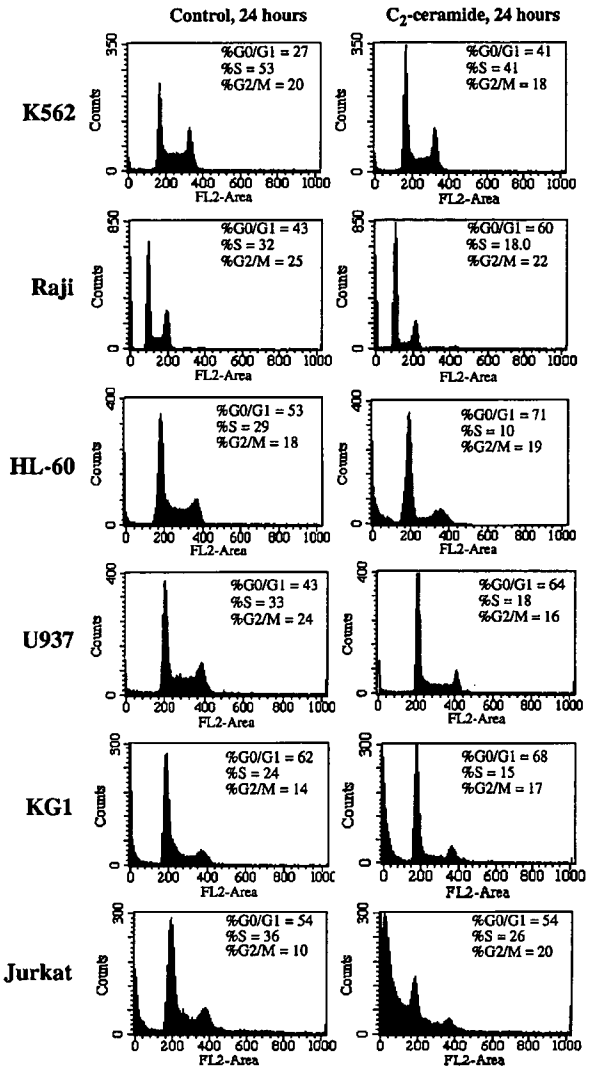

FIG. 1. DNA histograms (propidium iodide, FL2 area) demonstrating C_2-ceramide-induced cell cycle arrest in K562 erythroleukaemia cells, Raji B cells, HL-60 myeloid cells, U937 myeloid cells, KG1 myeloid cells, and apoptosis in Jurkat T cells. All histograms represent samples taken 24 h after C_2-ceramide treatment. Single representative results from at least four repeat experiments are shown.

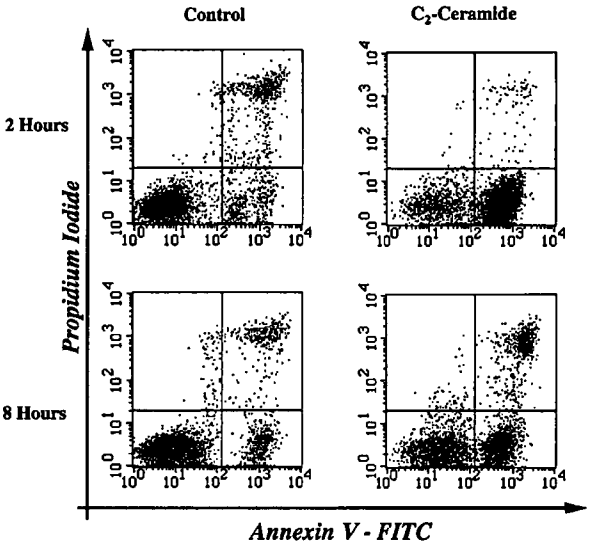

FIG. 2. Confirmation of the rapid onset of apoptosis in C_2-ceramide-treated Jurkat T cells by demonstration of externalised phosphatidylserine residues. Apoptotic (annexin+/propidium iodide−), dead (annexin+/propidium iodide+), and live (annexin−/propiodium iodide−) populations are readily identified. A single representative result from four repeat experiments is shown.

RESULTS

Induction of Cell Cycle Arrest by C_2-Ceramide

A variety of haematopoeitic cell lines were examined to determine whether C_2-ceramide treatment consistently resulted in withdrawal from the cell cycle. Asynchronous cultures of logarithmically growing cells were treated and harvested at various times for cell cycle analysis. Cell cycle arrest was determined by examining for an increased proportion of events in G_0/G_1 and a concurrent decrease in the proportion of events in S phase when compared with time-matched, vehicle-treated controls. This is indicative of an inability to cross the G_1-S transition that is a hallmark of cell cycle arrest. Figure 1 shows DNA histograms depicting the results obtained for cell lines K562, Raji, HL-60, U937, KG1, and Jurkat after 24 h of treatment with C_2-ceramide. Th K562, Raji, HL-60, and U937 cells showed evidence of substantial cell cycle arrest (increased G_0/G_1/decreased S phase), whereas KG1, Jurkat, and KG1a (not shown) cells exhibited slight to negligible cell cycle arrest. Interestingly, although Jurkat cells did not undergo evident cell cycle arrest, their histograms indicated the presence of large amounts of broken DNA and debris indicative of apoptosis. Apoptosis of Jurkat cells was confirmed by annexin V-fluorescein isothiocyanate/PI staining (Fig. 2) and was evident as early as 2 h after adding ceramide. This apoptosis and associated DNA degradation were well advanced by 24 h, accounting for the left shift of the Jurkat sub-G_0/G_1 apoptotic peak in Figure 1. Many cell lines that entered cell cycle arrest in response to C_2-ceramide also exhibited slight to moderate levels of apoptosis as determined by a similar sub-G_0/G_1 apoptotic peak.

Activation of Rb Tumour Suppressor Nuclear Phosphoprotein by C_2-Ceramide

Rb is a 110-kDa nuclear phosphoprotein that is considered to be a tumour suppressor because loss or inactivation of both copies of the RB1 gene results in unrestrained malignant growth (13). Hypophosphorylated Rb is the active form, and dephosphorylation of the Rb protein has been associated with growth arrest, the G_0/G_1 phase of the cell cycle, and the differentiated phenotype of haematopoietic cells (14-16). To obtain further insight into the mechanism of C_2-ceramide-induced growth and cell cycle arrest, the phosphorylation status of the Rb protein was

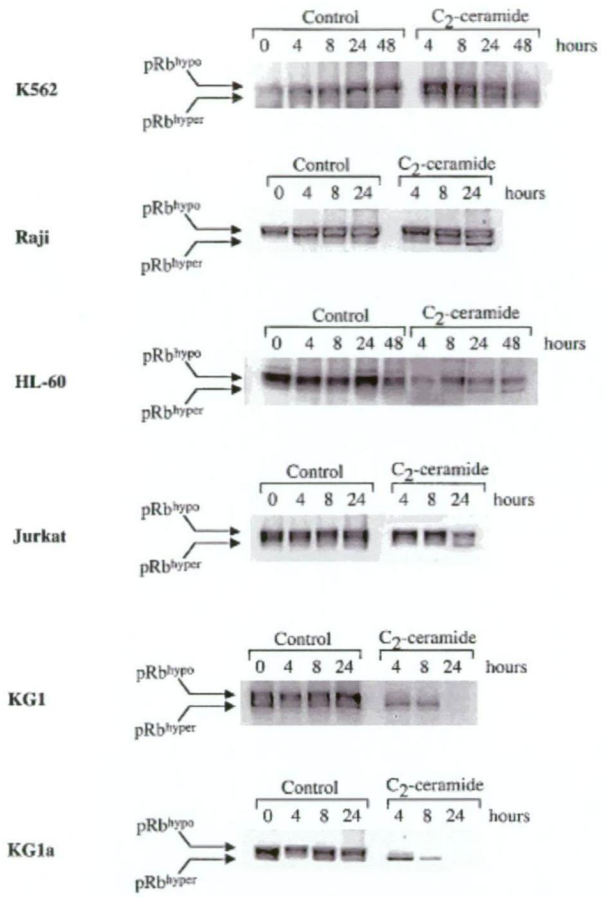

FIG. 3. Western blot analysis of retinoblastoma phosphorylation status after C_2-ceramide treatment of K562 erythroleukaemia cells, Raji B cells, HL-60 myeloid cells, Jurkat T cells, KG1, and KG1a myeloid cells. Single representative results from at least four repeat experiments are shown.

determined by taking advantage of the fact that SDS-polyacylamide gel electrophoresis and Western blotting can resolve the faster migrating hypophosphorylated forms of Rb from the slower migrating hyperphosphorylated forms. Cells were treated with C_2-ceramide and harvested at different times, and lysates were subjected to SDS-polyacylamide gel electrophoresis and Western blotting (Fig. 3). In vehicle-treated samples, a band of $M_r \sim 110$ kDa, representing inactive hyperphosphorylated Rb (Rb^{hyper}), was observed. However, in K562, Raji, HL-60, Jurkat, KG1, and KG1a cells treated with C_2-ceramide, additional, faster migrating bands of $M_r \sim 105$ kDa were noted as soon as 4 h posttreatment. The lower M_r band represented the appearance of hypophosphorylated (active) forms of Rb (Rb^{hypo}), thus demonstrating that C_2-ceramide induced Rb activation. Logarithmic growing, untreated Raji cells exhibited a slightly different Rb pattern from other cells in that two hyperphosphorylated bands were observed; after ceramide exposure, an additional lower M_r band, representing hypophosphorylated Rb, was apparent. In KG1 and KG1a cell lines, it was not possible to detect Rb in 24-h post-C_2-ceramide samples, presumably due to the loss of cells to apoptosis; however, activated Rb was clearly demonstrated in earlier points. Interestingly, Jurkat T cells exhibited a Rb^{hypo} band only at 24 h posttreatment.

DISCUSSION

We investigated cell cycle arrest and Rb nuclear tumour suppressor phosphoprotein activation in haematopoietic cell lines treated with the synthetic lipid analogue C_2-ceramide. Rb activation was a common feature of all ceramide-responsive cell lines, and this was accompanied by G_1 cell cycle arrest or apoptosis. These findings also suggested that modulation of the sphingomyelin pathway and the resultant inactivation of cellular proliferation can be exploited for therapeutic purposes in cancer.

The majority of haematopoietic cells tested exhibited G_1 cell cycle arrest, with the degree of arrest varying between cell lines. Similar growth arrest after exposure of cell lines to synthetic ceramide analogues has been reported (11,17,18), some of which have been confirmed in the present study. The degree of apparent responsiveness to ceramide-induced cell cycle arrest may reflect the pleiotropic nature of responses associated with this signalling pathway. The final outcome of signals transmitted by ceramide could depend on the phase of the cell cycle in which signalling was initiated, the engagement of different downstream effectors in different cell types (10), or the generation of ceramide metabolites that may enhance or antagonise ceramide-activated signalling mechanisms (19). With the exception of Jurkat T cells, which underwent apoptosis before any G_1 arrest could be noted, this study showed that induction of cell cycle arrest in leukaemic cells is a consistent outcome of ceramide signalling.

C_2-ceramide-induced cell cycle arrest was usually accompanied by a degree of apoptosis, the extent of which differed between cell lines and with dosage. Cells lines that had comparatively lower or minimal apoptosis generally showed greater G_1 phase accumulations in cell cycle studies. This observation may indicate that the apoptosis is occurring primarily in cells that are trapped in the G_1 phase, hence lowering the apparent number of G_1 events by selective killing. If this is the case, then the proportion of cells that have undergone cell cycle arrest in response to C_2-ceramide will have been underestimated in this study. This possibility is supported by the finding that C_6-ceramide-induced apoptosis occurred primarily in the G_1/S phases of human neuroepithelioma cells (20). Other studies have reported similar selective apoptosis of G_1-arrested cells after drug treatment or activation (21-24). There is presently great controversy as to the role, if any, of ceramide in directly initiating apoptosis because of the lack of data demonstrating a direct link between the effectors of ceramide and the primary components of the apoptotic machinery (caspases and Bcl-2 family members). Although these direct links remain to be established, studies on the kinetics of ceramide formation (25,26) and modulation of ceramide levels using synthetic

analogues have provided supporting evidence of a close relationship between ceramide and apoptosis.

Rb has a well-documented role in regulating the cell cycle at the G_1-S phase transition (27), with its activity regulated by phosphorylation state, the hypophosphorylated form being active. Rb activation was the common feature of C_2-ceramide responsive cells in this study, indicating that Rb may be a key target or effector of ceramide signalling. Other studies have similarly reported Rb activation after addition of synthetic ceramide analogues (11,18,28), and the present studies strengthen and extend these findings. Rb activation was an early event after ceramide treatment and most likely was the effector of the observed C_2-ceramide-induced G_1 cell cycle arrest. The fact that Rb was activated by dephosphorylation makes it a likely candidate for a target of ceramide signalling because CAPP, a type 2A protein phosphatase (6), is one of the few recognised effectors of the sphingomyelin pathway. Rb has been well documented as a substrate for phosphatases, and its activity can be modulated by these enzymes (28). Rb activity can also be affected by a family of cell cycle regulatory proteins termed cyclin-dependent kinase inhibitors (CKIs) (27). Rb is maintained in the phosphorylated state by the actions of cyclin-dependent kinases that in turn are negatively regulated by CKIs. We recently demonstrated that cell cycle arrest and Rb activation in C_2-ceramide-treated U937 cells was associated with the rapid induction of expression of $p21^{CIP1}$ (11), a CKI with a key role in mediating cell cycle arrest during differentiation (29) and whose expression has been associated with Rb dephosphorylation and growth arrest of leukaemia cells in response to differentiation-inducing agents (30,31). Future studies will concentrate on defining the roles of phosphatase activity and CKI expression in activating Rb after ceramide treatment.

The ability of ceramide to promote differentiation and maturation of different cell types is often overlooked because of the recent intense scrutiny upon its role in apoptotic signal transduction. Signals transduced by ceramide may in fact be maturation/differentiation in nature rather than directly apoptotic, and this notion has been supported by several lines of investigation including the findings that synthetic ceramides induce morphologic changes characteristic of maturation and terminal differentiation in HL-60 (32), U937 (11), K562 (unpublished observations), and T9 glioma cells (33). The recent finding that C_2-ceramide induces expression of the CKI $p21^{CIP1}$ is further evidence (11), as expression of this protein has a well-characterised role in mediating cell cycle arrest during terminal differentiation (29,34). In haematopoietic cells, terminal differentiation culminates in apoptosis and removal from the circulatory pool. A failure to maintain the balance between cytogenesis in the bone marrow and peripheral terminal differentiation results in a disease state such as leukaemia or lymphoma. Delivery of a differentiation signal by ceramide at an inappropriate stage of the cell cycle or in conflict with an existing proliferative signal may result in apoptosis being initiated. If cellular differentiation is blocked at a proliferative stage, then blastic leukemia may eventuate (35), and we are currently examining the hypothesis that ceramide signalling can push leukaemic cells past this blockade, resulting in a nonproliferative phenotype or a terminally differentiated cell that undergoes normal apoptosis.

The common outcome from treatment of leukaemic cell lines with synthetic ceramide analogues is growth arrest or apoptosis. Both processes result in a reduction in the number of aggressively proliferating cancer cells, which is the primary aim of cancer therapies. Ceramide has already been shown to induce apoptosis in vitro in non-dividing B chronic lymphocytic leukemic cells (36), highlighting the potential of ceramide as a novel treatment strategy. However, further investigations into the molecular basis of cell growth mechanisms controlled by the sphingomyelin pathway and their manipulation for therapeutic purposes are warranted.

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Mitochondrial cytochrome *c* release precedes transmembrane depolarisation and caspase-3 activation during ceramide-induced apoptosis of Jurkat T cells

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Whilst the role of ceramide, a second messenger of the sphingolipid family, in the initiation of receptor-mediated apoptosis is controversial, a growing body of evidence is emerging for a role of ceramide in the amplification of apoptosis via mitochondrial perturbations that culminate in the activation of execution caspases. Treatment of Jurkat T cells with the cell-permeable analog, C_2-ceramide, resulted in the rapid onset of apoptosis as evidenced by Annexin V-FITC staining of externalised phosphatidylserine residues. Cells bearing this early apoptotic marker had a reduced mitochondrial transmembrane potential ($\Delta\Psi m$) that was preceded by the release of cytochrome *c* from mitochondria. Subsequent activation of caspase-3 provides the link between these ceramide-induced mitochondrial changes and execution caspases that ultimately result in the physical destruction of the cell. Collectively these results demonstrate that ceramide signalling results in caspase-mediated apoptosis via mitochondrial cytochrome *c* release and are further supportive of the role of ceramide in the amplification of apoptosis.

Keywords: apoptosis; caspase-3; ceramide; cytochrome *c*; mitochondrial transmembrane depolarisation.

Introduction

Apoptosis is an evolutionary conserved mechanism of programmed cell death that has a critical role in the maintenance of homeostasis and the removal of obsolete cells. Defects in its induction and execution form the basis of many diseases, in particular, cancers such as leukaemia and lymphoma. Understanding the mechanisms and regulation of apoptosis is an area of research that has the potential to result in novel and improved therapies for these diseases.

The sphingolipids are a diverse class of eukaryotic lipids that have a structural function in cell membranes as well as playing a role as second messengers within the cell.[1] Ceramide, a second messenger generated from sphingomyelin by the activation of a sphingomyelinase, has been implicated in apoptosis induced by a broad range of apoptotic stimuli, in particular by the ligation of the Fas or TNFα receptors.[1,2] Whilst numerous studies have highlighted the potential role of ceramide in the regulation of Fas-mediated apoptosis[3–5] the proposal that ceramide is a primary initiator has been recently under strong scrutiny. Doubts as to the role of ceramide in initiating Fas apoptosis were initially cast by the findings that ceramide generation occurred downstream of caspase activation and was blocked by inhibition of CPP32.[6,7] Subsequent findings that Fas treatment resulted in apoptosis without any detectible changes in cellular ceramide species[7–9] and that a number of antagonists of apoptosis attenuated or prevented ceramide, but not Fas, induced apoptosis[7,8,10–12] has lead a number of authors to conclude that ceramide is not involved in the initiation of Fas apoptosis but rather may have a role in the amplification or execution phases.[10,11,13]

Nevertheless, the fact remains that exogenous short chain synthetic ceramides, such as C_2-ceramide, initiate apoptosis in a number of cell lines[14,15] and recent reports have indicated that the mitochondria may be central in this phenomenon. Modulation of mitochondria function

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by ceramide in mainly cell-free systems has been detailed in a number of studies.[16–19] However, the findings that ceramide induced the release of cytochrome *c* from isolated mitochondria[20] and enhanced cytoplasmic cytochrome *c* levels prior to caspase activation[21,22] raises the possibility that cytochrome *c* released to the cytoplasm can activate caspases (in particular caspases-9 and -3) during C_2-ceramide-induced apoptosis. Such a mechanism would provide the link between ceramide signalling and execution caspases.

The present study investigated alterations in mitochondrial transmembrane potential ($\Delta\Psi m$), cytochrome *c* content and caspase-3 activation that arise during C_2-ceramide-induced apoptosis of Jurkat T cells, using intact cells and direct visualisation in contrast to previously reported isolated organelles and subcellular fractions.[20–22] We report that cells in the early phase of apoptosis have decreased $\Delta\Psi m$ that is preceded by mitochondrial release of cytochrome *c* with subsequent caspase-3 activation and conclude that the apoptogenic properties of C_2-ceramide are primarily due to its interaction with the mitochondria.

Materials and methods

Cell culture and induction of apoptosis

The Jurkat human T cell leukaemia cell line was maintained in RPMI 1640 media supplemented with 10% foetal calf serum, 2.0 mM L-glutamine and 120 μg/mL gentamicin at 37°C in a humidified, 5% CO_2 atmosphere. Experimental procedures were performed in serum-free conditions at a cell density of 1×10^6/mL. Apoptosis was induced either via the addition of 5 μM C_2-ceramide (BioMol) dissolved in ethanol with controls treated with an equivalent volume of the vehicle, or by addition of 100 ng/ml anti-Fas antibody (CH-11, Upstate Biotechnology).

Confirmation of apoptosis by Annexin V/PI staining

Externalised phosphatidylserine residues were detected using the reagents and protocols from the Annexin V-FITC Apoptosis Detection Kit II (PharMingen). Briefly, 1×10^6 cells were pelleted, washed in cold PBS and resuspended in 500 μL Annexin binding buffer. A 100 μL aliquot (2×10^5 cells) was taken, 5 μL each of Annexin V-FITC and PI added and cells were incubated for 15 minutes at RT in the dark. 400 μL of binding buffer was added and samples were acquired on a FACScan flow cytometer (Becton Dickinson) and analysed using CELLQuest software within 1 hour.

Detection of cytochrome *c* release

Cytochrome *c* release was detected via immunohistochemistry as previously described[23] with minor modification. Briefly, treated cells were harvested, washed once in PBS and resuspended in PBS at a density of 2×10^6/mL. 100 μL of cell suspension (2×10^5 cells) plus 50 μL FCS was cytospun onto slides, fixed in 1:1 methanol/acetone for 2 minutes and air-dried. Fixed slides were incubated in 1:50 dilution of mouse anti-cytochrome *c* Ab (clone 6H2.B4, PharMingen) in PBS/3% BSA for 2 hours at 37° in a humidified chamber. Slides were washed 3 times in PBS for 5 minutes prior to incubation in 1:100 dilution of Alexa-488 conjugated goat anti-mouse secondary Ab (Molecular Probes) for 1 hour at RT in the dark. Slides were washed 3 times in PBS for 5 minutes and mounted using Permafluor aqueous mounting solution. Slides were analysed on a Leitz Dialux 22EB fluorescent microscope fitted with a 488 nm laser. A diffuse, homologous staining pattern was determined to be indicative of cytoplasmic cytochrome *c*. Quantitative data was obtained by calculating the percentage of cells exhibiting cytochrome *c* release from 8 randomly selected fields per sample at 50× magnification. Confocal microscopy images were also captured from the above mentioned slides at 100× magnification via the use of an OptiScan Omnichrome Series 43 confocal microscope.

Measurement of $\Delta\Psi m$ alterations

Alterations to the $\Delta\Psi m$ were detected using the $\Delta\Psi m$-sensitive dye MitoTracker Red (Molecular Probes) and flow cytometry. 20 minutes prior to the conclusion of the incubation period, 500 μL (5×10^6 cells) was extracted and volume made up to 1 mL with serum free medium. 50 nM MTR was added and cells returned to 37°C for the remaining 20 minutes. At the conclusion of the incubation period, cells were washed once in PBS and resuspended in 150 μL Annexin V binding buffer. 5 μL Annexin V-FITC was added and cells incubated for 10 minutes at RT prior to the addition of 100 μL binding buffer and analysis via flow cytometry.

Analysis of caspase-3 activation

Intracellular detection of caspase-3 activation was performed using the IntraStain Fix and Perm kit (Dako). Briefly, 1×10^6 cells were resuspended in 100 μL of fixing reagent and incubated for 15 minutes at RT prior to the addition of 2 mL of PBS and centrifugation at 300 g for 5 minutes. The cell pellet was resuspended in 100 μL permeabilising reagent plus 20 μL of PE-conjugated anti-active caspase 3 Ab (Pharmingen) and

incubated for 15 minutes at RT in the dark. 2 mL PBS was again added and cells centrifuged as above. 300 μL of FACSFix was added and cells acquired immediately on a FACScan flow cytometer and analysed as describe above.

Results

C_2-ceramide induces apoptosis of Jurkat T cells

Externalisation of membrane phosphatidylserine residues is an early event in apoptosis and was visualised by double labelling of cells with Annexin V-FITC and Propidium Iodide (PI) with the stepwise transition from Annexin$^-$/PI$^-$ (viable) to Annexin$^+$/PI$^-$ (early apoptotic) to Annexin$^+$/PI$^+$ (dead) demonstrating apoptosis. Annexin V/PI staining of C_2-ceramide treated Jurkat cells shows that a significant population of these cells become Annexin V$^+$ within 2 hours of treatment (Figure 1). This population peaked at 4 hours post-treatment and, by 8 hours, many of these early apoptotic cells had progressed to PI$^+$ and had thus died. Anti-Fas mAb treated cells demonstrated a more dramatic and rapid transition to apoptosis whilst the majority of vehicle-treated cells remained viable.

Reduced mitochondrial $\Delta\Psi$m occurs only in cells with externalised phosphatidylserine residues following C_2-ceramide induced apoptosis

MitoTracker Red stains mitochondria in a $\Delta\Psi$m-dependent manner and when combined with Annexin V-FITC and flow cytometry, the $\Delta\Psi$m of the apoptotic population can be independently assessed. Following treatment with C_2-ceramide, flow cytometry plots were generated which depicted viable (Annexin V$^-$) or apoptotic (Annexin V$^+$) populations of cells (Figure 2). After 2 hours of C_2-ceramide treatment, approximately 10% of cells were Annexin V$^+$ and this population of apoptotic cells was readily observable by 3 and 4 hours post treatment. Analysis of the MitoTracker Red fluorescence of this apoptotic population revealed that it was significantly reduced compared to the viable cells in that sample ($p = 0.002$, paired t-test). This reduction was evident as early as 2 hours post addition of C_2-ceramide and the statistical significance increased with the later timepoints. Importantly, apoptotic cells in C_2-ceramide-treated samples always had a significantly reduced $\Delta\Psi$m compared to the spontaneous apoptotic population in the time-matched controls. Thus cells in the execution phase of C_2-ceramide

Figure 1. Confirmation of the rapid onset of apoptosis in Jurkat T cells following treatment with ethanol vehicle, C_2-ceramide or anti-Fas antibody by demonstration of externalised phosphatidylserine residues. Apoptotic (Annexin$^+$/PI$^-$), dead (Annexin$^+$/PI$^+$) and alive (Annexin$^-$/PI$^-$) populations are readily identified. A single representative result from 4 repeat experiments is shown.

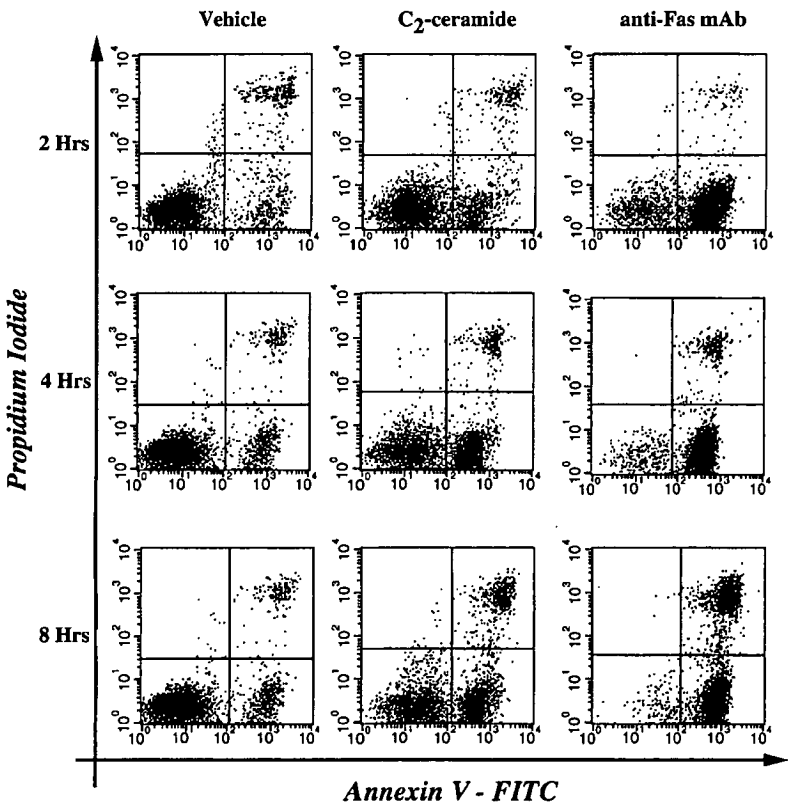
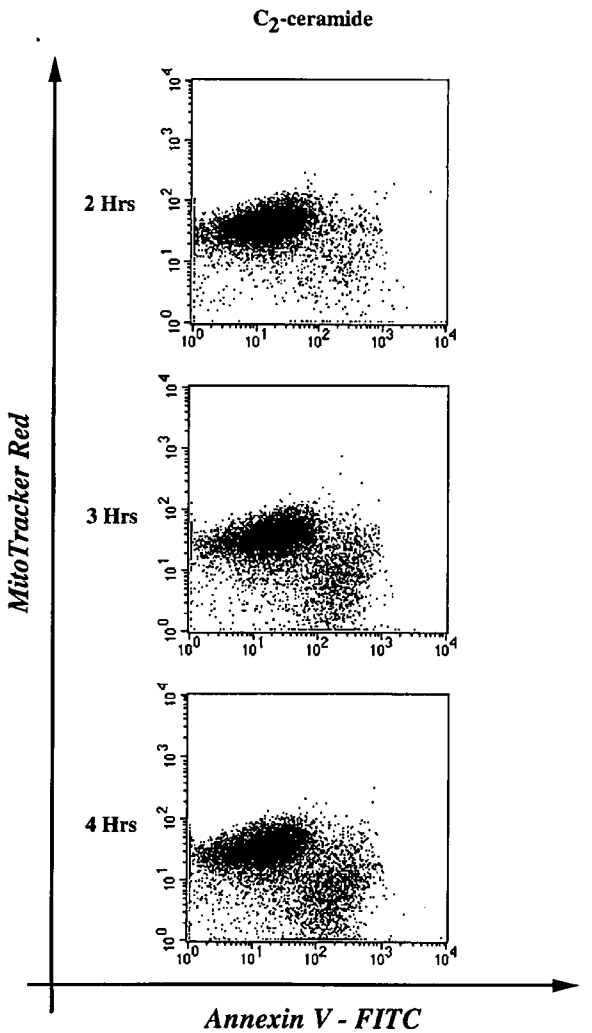


Figure 2. Determination of changes in $\Delta\Psi m$ following treatment with C_2-ceramide. Cells were loaded with the mitochondrial permeability dye, MitoTracker Red and apoptotic cells identified by Annexin V staining of externalised phosphatidylserine residues. A decrease in MitoTracker Red fluorescence is indicative of reduced $\Delta\Psi m$. A single representative result from 6 repeat experiments is shown.



induced apoptosis had reduced $\Delta\Psi m$ relative to both internal and external controls.

C_2-ceramide induces release of cytochrome *c* from mitochondria

To investigate whether C_2-ceramide caused the release of cytochrome *c* from the mitochondria to the cytoplasm, the cellular localisation of cytochrome *c* was examined by confocal immunofluorescence microscopy (Figure 3). In untreated and vehicle-treated Jurkat cells, cytochrome *c* displayed intense punctuate cytoplasmic staining which is in agreement with its localisation in the mitochondria. After apoptosis initiation by C_2-ceramide, the majority of cells exhibited homogenous and diffuse cytochrome *c* staining that is consistent with its translocation from the mitochondria to the cytoplasm. Quantitation of cells exhibiting cytochrome *c* release following treatment with either vehicle or C_2-ceramide demonstrated that over 60% of C_2-ceramide treated cells had released cytochrome *c* from their mitochondria to the cytoplasm as early as 2 hours post-treatment compared to less than 10% of control cells (Figure 4). Taken together, these results clearly demonstrate that release of mitochondrial cytochrome *c* into the cytoplasm is an early event in C_2-ceramide mediated apoptosis of Jurkat T cells.

Activation of caspase-3 by C_2-ceramide

Detection of effector caspase activation in Jurkat T cells during ceramide-mediated apoptosis was performed by probing cells that had been treated with either vehicle, C_2-ceramide or anti-Fas mAb with a phycoerythrin-conjugated antibody to the active form of caspase-3. C_2-ceramide treatment caused activation of caspase-3 in Jurkat T cells as evidenced by a right shift in fluorescence (Figure 5). Activation of caspase-3 was very weak to negligible at 2 hours post-treatment with C_2-ceramide, however more than 30% of C_2-ceramide-treated cells had activated caspase-3 by 4 hours post-treatment. In comparison, negligible caspase-3 activation was observed in time-matched, vehicle- treated cells whereas Fas ligation, which is known to execute apoptosis via caspase-3, produced significant caspase-3 activation. This data clearly shows that activation of the effector caspase, caspase-3, occurs during C_2-ceramide-induced apoptosis.

Discussion

The present study demonstrates that C_2-ceramide initiated apoptosis of Jurkat T cells is associated with an early release of mitochondrial cytochrome *c* with subsequent mitochondrial depolarisation and activation of effector caspases. This report strengthens the hypothesis that ceramide signalling initiates apoptosis by directly targeting the mitochondria, leading to activation of distal caspases and hence cellular destruction.

Recent investigations have highlighted the critical role of mitochondria in the apoptogenic processes (reviewed by [24,25]). The release of cytochrome *c* from mitochondria into the cytoplasm is widely accepted as a critical and decisive initial event of chemically-induced apoptosis.[26] This study has demonstrated, using direct visualisation by confocal immunofluorescence microscopy, that release

Figure 3. Cytochrome *c* release visualised by confocal immunofluorescence microscopy. Immunolocalised cytochrome *c* (green) is shown in untreated, vehicle-treated and C_2-ceramide-treated Jurkat cells. Cells were incubated for 2 hours with the indicated reagents followed by harvesting and staining. A single representative result from 5 individual experiments is shown.

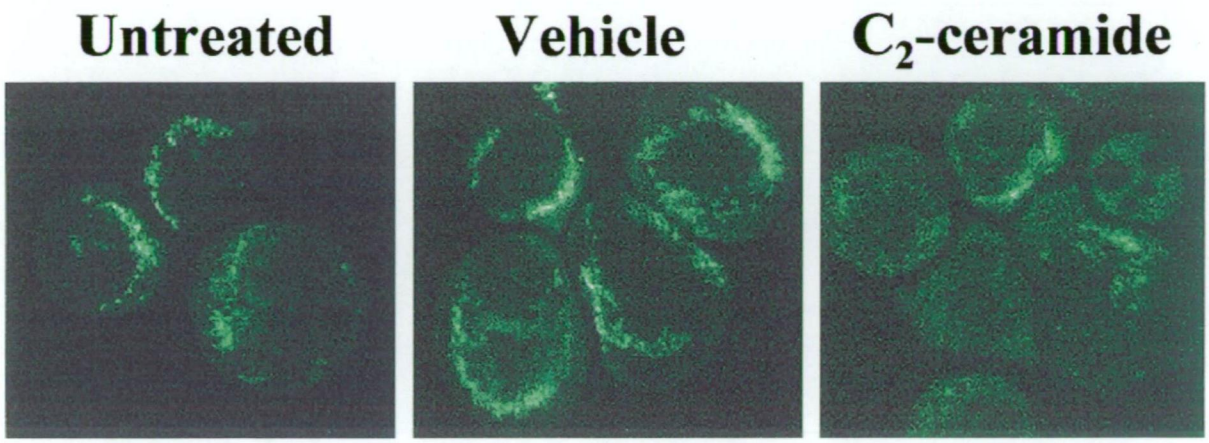
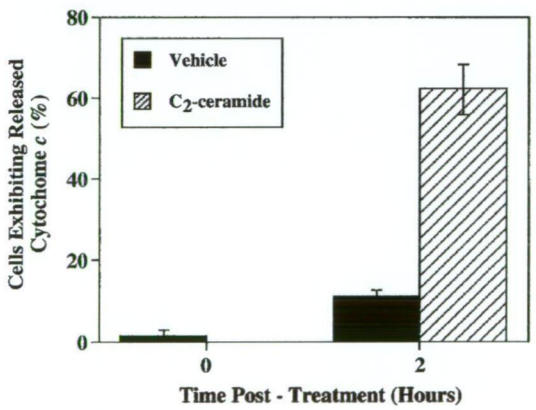


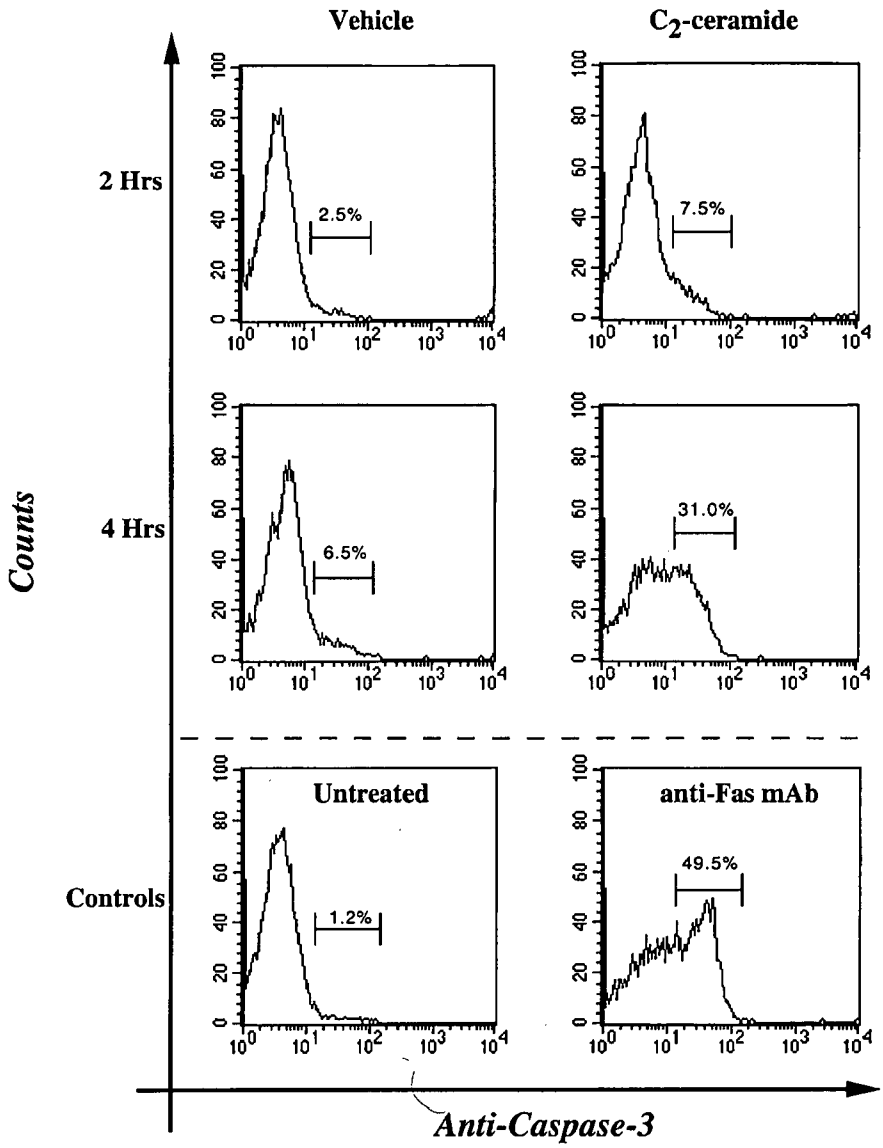
Figure 4. Quantitation of cytochrome *c* release in Jurkat T cells after 2 hours treatment with vehicle or C_2-ceramide. A change in the staining pattern from intense punctuate to diffuse homologous was determined to be indicative of release of cytochrome *c* from the mitochondria to the cytoplasm. Data was enumerated from 8 randomly selected fields per sample. Result shown is the Mean ± SE of 5 individual experiments.



of mitochondrial cytochrome *c* into the cytoplasm occurs in the very early stages of apoptosis induction. More than 60% of cells had released cytochrome *c* within 2 hours of C_2-ceramide treatment, at which timepoint phosphatidylserine externalisation and caspase-3 activation were non-existent or only weakly present in around 10–20% of cells. This finding of ceramide-induced cytochrome *c* release using intact cells confirms recent studies that used isolated mitochondria[20] or subcellular fractionation[21] to demonstrate this effect. Earlier studies[27,28] demonstrated that a 6 hour incubation with synthetic ceramide analogues increased the cytoplasmic concentration of cytochrome *c*, however, as apoptosis is well advanced by this timepoint it is impossible to state with certainty whether such accumulation is a cause, or result, of apoptosis. By examining earlier timepoints and demonstrating cytochrome *c* release in cells yet to show signs of apoptosis, the present study overcomes the deficiencies of this previous work by showing that cytochrome *c* release from the mitochondria is an early event in C_2-ceramide induced apoptosis.

Reductions in $\Delta\Psi m$ suggest that mitochondrial permeability has been compromised and that intermembraneal proteins such cytochrome *c* are released, however the relationship between $\Delta\Psi m$, mitochondrial release of cytochrome *c* and initiation of apoptosis is controversial with studies showing that cytochrome *c* release can occur before, coincidentally, or independently of $\Delta\Psi m$.[19,20,23,26] The present study utilised a flow cytometry based assay that allowed the $\Delta\Psi m$ of cells that were in the early stages of apoptosis (*i.e.* Annexin V^+) to be assessed independently of non-apoptotic cells, thus excluding the confounding effects of viable cells from the analysis. Externalisation of phosphatidylserine is characteristic of the execution phase of apoptosis and results from changes to plasma membrane phospholipid symmetry.[29] Our data showed that apoptotic cells had significantly reduced $\Delta\Psi m$ compared to viable cells, however, it is apparent that reduced $\Delta\Psi m$ accompanies phosphatidylserine externalisation as decreased MitoTracker Red fluorescence was observed only in Annexin V^+ cells. Thus decreased $\Delta\Psi m$ is observed only in cells committed to the execution phase, which occurs post-activation of caspase-3.[30] Furthermore, given that more than 60% of cells exhibited released cytochrome *c*, but only 10–20% had reduced $\Delta\Psi m$, it can be concluded that cytochrome *c* release occurs prior to the collapse in $\Delta\Psi m$ and phosphatidylserine externalisation during C_2-ceramide induced apoptosis, placing C_2-ceramide-mediated cytochrome *c* release upstream of

Figure 5. Activation of caspase-3 in Jurkat T cells after treatment with C_2-ceramide. The active form of caspase-3 was detected using a monoclonal antibody and flow cytometry. A right shift in PE fluorescence is indicative of active caspase-3. A PE positive region (bar) was established from the 0 hour control histogram and this region was transposed to all other histograms. The percentage of cells staining positive for active caspase-3 PE was established by determining the number of cells which exhibited PE fluorescence within this region. Cells incubated with anti-Fas antibody were used as a positive control to confirm the position of positive events. These histograms are representative of 4 separate experiments.



the execution phase. This contradicts a recent study which showed that reduced $\Delta\Psi$m occurred without cytochrome *c* release in C_2-ceramide treated Rat-1 fibroblasts[31] despite evidence of PARP cleavage. However, given the lengthy exposure to high doses of ceramide (50 μM for 6 hours), and the somewhat low levels of apoptosis observed in that study (<50% after 12 hours), the sensitivity of the Rat-1 cell line to ceramide-mediated apoptosis must be questioned. Our finding that C_2-ceramide induced cytochrome *c* release occurs prior to $\Delta\Psi$m reduction is supported by others who utilised isolated mitochondria in cell-free systems.[19,20]

Cytochrome *c* was released from coupled mitochondria, as the decrease in $\Delta\Psi$m was observed after the event, a finding that is in keeping with other models of apoptosis. Reduced $\Delta\Psi$m was not observed until after DNA fragmentation in staurosporine-treated HeLa cells[23] whereas exposure of cells to etoposide resulted in cytochrome *c* release that also preceded losses in $\Delta\Psi$m.[31] Moreover, it is evident that C_2-ceramide-mediated cytochrome *c*

release does not require the opening of the mitochondrial permeability transition (MPT) pore, a dynamic multiprotein complex that is located at the contact site between the outer and inner mitochondrial membranes whose opening has been implicated in cell death induced by reactive oxygen species, hepatotoxins, calcium and anoxia.[32] It is more likely that interaction between ceramide signalling and the Bcl-2 family is mediating the mitochondrial effects given the ability of Bcl-2 to inhibit C_2-ceramide mediated cytochrome *c* release[20] and the resistance of Bcl-2 overexpressing cells to ceramide-induced apoptosis.[28,33] The recent finding that cell-permeable exogenous ceramides induced downregulation of Bcl-2, increased the Bax/Bcl-2 ratio followed by release of cytochrome *c* and activation of caspases-9 and -3 in C6 glioma cells[34] further supports this notion.

Ceramide signalling appears to result in activation of distal caspases, such as caspases 9 and 3, but not the proximal caspases (2, 8 and 10) thus placing sphingomyelinase activation downstream of the proximal caspases with subsequent ceramide generation leading to activation of the distal caspases.[35,36] In the present study, C_2-ceramide resulted in activation of the distal caspase-3, placing ceramide signalling upstream of the effector caspases. Such a finding is supported by other studies[22,36,37] and provides the link between ceramide signalling and the mechanical destruction of the cell as a result of apoptosis. It is well established that released cytochrome *c* activates caspase-9 in concert with the cytoplasmic factors ATP and Apaf-1, and as a result, consequently activates caspase-3.[38] In the present study, caspase-3 activation occurred at timepoints later than cytochrome *c* release and hence the caspase activation can be considered to be distal to the mitochondrial events.

Conclusion

Early studies into the mechanism of receptor-mediated apoptosis highlighted generation of ceramide as the second messenger linking receptor activation and the initiation of cell death.[2–5] However, that ceramide formation was dependent upon activation of proximal caspases,[39] the failure of ceramide signalling to activate proximal caspases[40] and the elucidation of the FADD/MORT1 death domain signalling pathways[41] lead to the demise of this notion with several authors postulating that ceramide generation was either an artefact or consequence of apoptosis rather than an initiator.[8–11,42]

This study clearly demonstrates that ceramide activates the distal executioner caspases by causing the release of cytochrome *c* from the mitochondria to the cytoplasm of intact cells. When taken in concert with other studies which showed that Bcl-2 prevented apoptosis whilst not inhibiting ceramide generation[39] and that Fas-mediated ceramide generation occurred independently of the effector stage of apoptosis,[43] ceramide accumulation and signalling is clearly prior to the effector phase of apoptosis and proximal to the distal caspases. The generation of ceramide, whether by receptor-associated sphingomyelinase,[2] or membrane changes induced by chemotherapeutic agents,[44,45] causes the activation of distal caspases, thus amplifying the response to the initial apoptotic signal. Ceramide-induced cytochrome *c* release is therefore an additional mechanism for caspase-3 activation, complementary to the proximal caspases, which acts to ensure the death of the cell by distal caspases.

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