INDUCTION OF APOPTOSIS BY LITHIUM AND MINOCYCLINE IN MAMMALIAN CELLS: ROLE OF GROWTH-RELATED GENES, PROTEIN KINASE C AND CALCIUM

BY

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Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

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April 1998

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DECLARATION

I declare that the dissertation hereby submitted to the University of the North for the degree of Master of Science has not previously been submitted by me for a degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly acknowledged.

Signed: _____

Date: 21/08/98

ACKNOWLEDGEMENTS

"Moreko ga a itekole, gape motho ke motho ka batho (Northern Sotho Proverb). In short, no man is an island". THIS PROJECT IS DEDICATED TO MY FAMILY.

I would like to thank the following:

1. Dr. James M. Wachira for his intelligence, technical skills, understanding of and interest in research, encouragements, constructive criticism and indiscriminating friendship.

2. Dr. Emil K. Abotsi and Dr. Errol M. Tyobeka for their encouragements and appealing managerial and leadership roles.

3. Members of staff in the Department of Biochemistry (Messrs M.A. Tloti, M.J. Mokubebi and L.J. Mampuru, Ms K.C. Lucas, Ms T.G. Kumalo, Ms M.M. Malape, Ms. R. Maleka, Ms. K. Lelope and Dr. R.W. Becker) for their encouragements, invaluable positive and negative criticism and interest shown in the research.

4. BSc Hons and MSc students (classes of 1995-1998) for their unchallengeable warmth and encouragements. To Messrs N.S. Mukhufhi and M.P. Mokgotho, thank you for helping.

5. Members of staff in The Graphics Department for their artistic and photography skills.

6. Mr Ian Campbell (Physiology Department) and Ms Alma Moller (Electron Microscopy Unit) for their skills and assistance.

7. Foundation for Research and Development (FRD) for their continued financial support through the study.

8. My family (mother Caroline, brothers Fourie and Naphtalia, sisters Millicent and Mamoraka, uncle Thiniel and family) for their support, patience, undying love and their trust and belief in me.

9. My girlfriend Matshidiso Kgomotso Euginiah Masebe for her patience, support, unconditional love and encouragements. Thank you for being YOU.

10. THE ALMIGHTY GOD for looking after and guiding me over the years.

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Act. D	-actinomycin D
AP-1	-activator protein-1
ATPase	-adenosine triphosphatase
cAMP	-cyclic adenosine monophosphate
cDNA	-complementary DNA
CHX	-cycloheximide
CSF	-colony stimulating factor
dATP	-deoxyadenosine triphosphate
dCTP	-deoxycytidyl triphosphate
dTTP	-deoxythymidyl triphosphate
dGTP	-deoxyguanidyl triphosphate
DAG	-diacylglycerol
DEX	-dexamethasone
DMC	-demeclocycline
DMSO	-dimethylsulphoxide
DNA	-Deoxyribonucleaic acid
DNase	-deoxyribonuclease
EGTA	-ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
EDTA	-ethylenediamino tetraacetic acid
FBS	-fetal bovine serum
FCS	-fetal calf serum
GSK-3β	-glycogen synthase kinase-3β
IL	-interleukin
IBMX	-3-isobutyl-1-methylxanthine
IP_3	-inositol triphosphate
MIN	-Minocycline
mRNA	-messenger RNA

NBT	-nitroblue tetrazolium
PBS	-phosphate-buffered saline
PCNA	-proliferating cell nuclear antigen
pJMCS	-*c-jun* recombinant pOPRSVI/MCS vector
PKA	-protein kinase A
PKC	-protein kinase C
pMCS	-pOPRSVI/MCS operator vector
RNA	-ribonucleic acid
RNase	-ribonuclease
RT-PCR	-reverse transcriptase polymerase chain reaction
SDS	-sodium dodecyl sulphate
SEM	-standard error of the mean
Ser	-serine
TCA	-trichloroacetic acid
TNF	-tumor necrotic factor
TPA	-12-*O*-tetradecanoyl phorbol-13-acetate
TRE	-TPA respose elements

ABSTRACT

Minocycline is a tetracycline-derived antibiotic and has been referred to as lithium mimetic in rats due to its ability to inhibit accumulation of cAMP in the brain. These studies have demonstrated that MIN like lithium induces apoptosis in HL-60 cells but not in K562, 3T3-L1 and LLC-PK cells. Cycloheximide, a potent inhibitor of protein synthesis, failed to block MIN- and Li^+-induced in HL-60 cells, suggesting that macromolecular synthesis is not essential for apoptosis induced in HL-60 cells by these agents. However, activation of protein kinase C (PKC) by TPA blocked DNA laddering in the presence of MIN and Li^+. Contrary to previous observations with Li^+, chelation of extracellular calcium by EGTA potentiated MIN-induced cytotoxicity, and enhanced DNA fragmentation. Exogenous Ca^{2+}, on the other hand, offered protection against MIN-induced loss of HL-60 cell viability and inhibition of growth.

Induction of expression of AP-1 components by the stress-activated protein kinase (SAPK/JNK) signalling pathway is implicated in cytokines and stress-activated apoptosis. Two of the PKC target genes, *c-fos* and *c-jun* were investigated, and it is reported here that the level of *c-jun* mRNA was not influenced by either MIN or Li^+. *c-fos* mRNA, on the other hand, was not detected in both MIN- and Li^+-treated and untreated control cultures. MIN-induced apoptosis was accompanied by a decrease in *bcl-2* mRNA expression. Lithium, on the other hand, led to downregulation of *bcl-2* mRNA at the growth enhancing concentration of 5 mM. However, 15 mM Li^+ induced apoptosis without causing a decrease in the level of expression levels of *bcl-2* mRNA. Unlike Li^+ that blocks cell cycle progression at the G_2/M phase, MIN-induced toxicity was associated with a steady increase of cells in the G_0/G_1 phase of the cell cycle.

Antisense *c-jun* RNA expression plasmid (pJMCS) inhibited the growth of HL-60 cells and Raji cells in the absence and presence of lithium. Induction of apoptosis in HL-60 cells by lithium could not be reversed by antisense *c-jun* expression. However, plasmid pJMCS abrogated the stimulation of growth by lithium. This study, therefore, demonstrates that MIN, like lithium induces apoptosis in HL-60 cells, but the mechanisms are likely to be different.

CHAPTER 1

INTRODUCTION

1.1. Lithium

1.1.1. Overview

Lithium is the second group IA element with an atomic weight of 6.9. Unlike other alkali metal ions, Li^+ exerts profound effects on both human behaviour and the early embryonic development of diverse organisms. Maintaining a serum Li^+ ion level of approximately 1 mM is known to effectively control manic depressive psychosis by stabilizing the dramatic swings in mood (Berridge *et al.*, 1989).

It is also established that Li^+ causes a wide variety of metabolic effects leading to mitogenicity. It influences morphogenesis in *Xenopus* oocyte embryos and *Drosophilla* (Busa and Gimlich, 1989), enhances granulopoiesis including leucocytosis when administered to manic depressive patients, and it has been shown to inhibit (Zaricznyj and Macara, 1987) and induce (Sokoloski *et al.,* 1993) cellular differentiation in different cell lines.

Lithium has been used extensively to treat manic depressive disorder for many years. However, the administration of lithium is not without side-effects; tremors, nausea, diarrhea, visual disturbances, cardiovascular symptoms, polyuria and leucocytosis are associated with low doses (Lydiard and Gelenberg, 1982; Hetmar, 1988). A lithium overdosage can result in seizure, coma, brain damage and ultimately, death (Appelbaum et al., 1979; Lydiard and Gelenberg, 1982). Nevertheless other uses of lithium such as antiretroviral therapy and stimulation of hematopoiesis during chemotherapy have been suggested (Gallichio *et al.,* 1994).

1.1.2. Effects of lithium on cell culture systems

Lithium induces diverse effects on cells in culture including mitogenicity and

apoptosis in HL-60 cells and blockage of differentiation in 3T3-L1 preadipocytes in addition to the whole organism effect. However, the mechanism of lithium on cells in culture remains poorly understood.

1.1.2.1. HL-60 cells

The human promyelocytic leukemia (HL-60) cell line has provided a useful model for the study of the mechanisms of hematopoietic cell growth and differentiation. HL-60 cells can be induced to undergo differentiation into different lineages upon treatment with different agents (reviewed in Collins, 1987). Treatment of HL-60 cells with the phorbol ester TPA induces differentiation into monocytes (Rovera *et al.*, 1979). The resultant monocytes acquire many phenotypic and functional properties of blood monocytes such as the ability to generate reactive oxygen species, expression of monocyte/macrophage specific α-naphthyl acetate esterase and the ability to perform phagocytosis (reviewed in Collins, 1987). HL-60 cells treated with TPA, like peripheral blood monocytes, have been shown to be slow to undergo apoptosis (McCarthy *et al.*, 1994).

Treatment of HL-60 cells with a wide variety of agents including retinoic acid induces differentiation into neutrophil-like cells with the distinct characteristics typical of peripheral blood neutrophils (reviewed in Collins, 1987). The resulting neutrophil-like cells are functionally similar to neutrophils and are capable of phagocytosis and NBT reduction. Retinoic acid and other inducers of differentiation have shown therapeutic value in the treatment of leukemia since terminally differentiated leukemic cells lose malignancy (reviewed in Sachs, 1990; Fibash and Sachs, 1975; Lotem and Sachs, 1981; Lotem and Sachs, 1984). In addition, these cells undergo apoptosis characterised by internucleosomal DNA fragmentation upon reaching maturity (Martin, 1993). Exposure of HL-60 cells to physiologically permissible levels of dimethylsulphoxide (DMSO) and hypoxanthine induces maturation along the granulocytic pathway and alkaline medium induces eosinophilic differentiation (reviewed in Collins, 1987).

HL-60 promyelocytic leukemia cells are capable of continuously proliferating in suspension cultures with a generation time of between 36 and 48 hours. Surface expression of transferrin and insulin receptors seems to be critical for the proliferative capacity of these cells, as they can grow in serum-free media supplemented with transferrin and insulin (Breitman *et al.*, 1980). The expression of these surface receptors is down regulated upon induction of differentiation, further supporting their role in HL-60 cell proliferation (Tactle *et al.*, 1983).

The *in vitro* proliferation of normal myeloid progenitors is stimulated by the colony-stimulating factor (CSF) family of growth factors (Ruscetti *et al.,* 1981; Metcalf, 1989; Tomonaga *et al.,* 1986). Certain CSFs also stimulate the proliferation of HL-60 cells. HL-60 cells have been shown to produce certain autocrine CSF-like compounds that stimulate their growth *in vitro* (Ruscetti *et al.*, 1981; reviewed in Metcalf, 1983; Tomonaga *et al.*, 1986).

Lithium stimulates the proliferation of HL-60 cells in the 2.5-5 mM concentration range (Becker and Tyobeka, 1990). The enhanced growth of HL-60 cells is associated with some loss of cell viability. These studies supported an earlier finding by Gauwerky and Golde (1982) who showed that at 0.05-5 mM concentration Li^+ was effective in stimulating colony formation by HL-60 cells in the presence of colony stimulating factor (CSF). However, administration of lithium concentrations of 10 mM and above results in inhibition of cell growth and eventual death by apoptosis (Madiehe *et al.,* 1995).

Tyobeka and Becker (1990) conducted morphological studies on HL-60 cells cultured in the presence of cytotoxic concentrations of lithium. Treatment of HL-60 cells with lithium at 10 mM and above caused changes such as the smoothening of the cell surface as a result of loss of microvilli. A decrease in cell volume was observed at very toxic lithium concentrations (20 mM and 50 mM) and was accompanied by cell shrinkage accompanied by the appearence of holes on the cell surface. Following these observations, Madiehe *et al.* (1995) demonstrated

induction of DNA fragmentation following lithium treatment, and arrest of the HL-60 cell cycle in the G_2/M phase.

Lithium is reported to cause a dose-dependent induction of HL-60 cell differentiation along the granulocytic pathway, as indicated by the reduction of NBT and Mo 1 binding in the 10 mM range (Sokoloski *et al.,* 1993). This is in contrast to the observations of Madiehe *et al.* (1995) who reported apoptosis at the same concentration of lithium. The reason for this discrepancy could be that apoptosis induced by lithium follows a differentiation period, after which the cells terminally mature and cannot reenter the cell cycle.

1.1.2.2. K562 cells

K562 human erythroleukemia cells grow continuously and display only a negligible tendency for spontaneous differentiation under standardized conditions (Nagy *et al.,* 1995). K562 cells have been used to study the growth and differentiation of hematopoietic cells along the erythrocytic lineage. When induced to differentiate by Ara-C (Moore *et al.,* 1991), phorbol esters (Pelicci *et al.,* 1983), fagaronine (Comoe *et al.,* 1987), tiazofurin and ribavirin (Olah *et al.,* 1988) and other drugs K562 cells stop dividing and start to synthesise haemoglobin. Interestingly, lithium has been reported to reversibly inhibit DMSO induced differentiation of Friend erythroleukemia cell line 745, and was effective when added in the first 10 hours after DMSO treatment (Zaricznyj and Macara, 1987). The effects of lithium on differentiation were reversed by about 60% with the addition of 15 mM KCl.

A significant enhancement of growth has been reported in K562 cells treated with 3 mM lithium for four days, but a dose response effect was not observed at 1, 2 or 3 mM, respectively (Rosenstock *et al.,* 1979). However, Becker *et al.* (1993) reported that treatment of K562 cells with lithium did not stimulate cell proliferation, but higher concentrations induced reduction in cell viability and inhibition of cell growth. The reduction in cell viability has been attributed to cell death by apoptosis (Madiehe *et al.* 1995).

1.1.2.3. Swiss 3T3-L1 mouse preadipocytes

The Swiss 3T3-L1 cell line was selected on the basis of its ability to differentiate into fully mature adipocytes (Green and Kehinde, 1974). 3T3-L1 cells differentiate spontaneously into adipocytes within 30 days in culture, but this process can be greatly accelerated by both physiological and non-physiological substances (Green and Kehinde, 1976).

Lithium was shown to inhibit dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX) and insulin-induced adipose conversion at concentrations of 2-20 mM (Aratani *et al.,* 1987). The Inhibition of adipose conversion of confluent cells by lithium was accompanied by abrogation of the critical mitosis that occurs before the adipocyte differentiation and was reversible (Aratani *et al.,* 1987). However, lithium stimulated the incorporation of [^{3}H]TdR by 1.3-fold at 5-20 mM concentrations in preconfluent cells (Aratani *et al.,* 1987). Abotsi and Tyobeka (1993), demonstrated that the inhibition of adipose conversion by lithium was reversible only within the first 24-48 hours and was accompanied by accumulation of the 3T3-L1 cells at the G_2/M phase of the cell cycle.

1.1.3. Effects of lithium on early embryo development

In 1970 Nieuwkoop reported that lithium distorts pattern formation during early development, resulting in deformed embryos. This teratogenic effect of lithium has been studied more extensively in amphibians where deformation can be traced back to a respecification of the dorso-ventral axis early in the blastula stage, but it has also been described in simpler organisms such as the sea urchin (Livingston and Wilt, 1989) and slime molds (Peters *et al.*, 1989).

In amphibia the most sensitive period is the 32-64 cell stage during which a brief treatment with lithium results in a dorsalisation of the embryo. The ventral regions are respecified to form characteristic dorsal structures such as notochord and eyes, and in some cases the embryo is radially symmetrical with a broad band of eye pigment and cement gland (Kao and Elinson, 1989).

It has been proposed that the phosphoinositide cycle and its accompanying second messengers may be important in specifying the dorso-ventral axis during normal frog embryogenesis (Busa, 1988; Busa and Gimlich, 1989). This proposal was supported by the finding that the ventral side has a high level of phosphoinositide turnover, which then grades down towards a lower level at the dorsal side. It is unclear how this turnover is achieved, but it is associated with a high density of muscarinic receptors on the pole of the oocyte in animals (Kusano *et al.*, 1982).

Lithium has long been recognised as a mammalian teratogen (Shephard, 1986). The exposure of animals to lithium ions has been linked to cleft palate and spina bilida, both being common abnormalities arising from embryonic teratogens. However, little is known about the possible inductive specification which probably relies on diffusible or tissue-associated growth factors. Although it is listed as a teratogenic agent in humans there is little evidence to support this suggestion when lithium is administered at normal therapeutic doses (Shephard, 1986; Schou *et al.*, 1973). Recently (Klein and Melton, 1996), the teratogenic effects of Li^+ in *Drosophila* and *Xenopus* embryos were attributed to inhibition of GSK-3β although myo-inositol could reverse the dorsalisation by an unclear mechanism.

1.1.4. Clinical Side-effects of lithium administration

Majority of patients on therapeutic doses of lithium experience side effects caused by daily intake and absorption of lithium or by the continuous presence of lithium in the body, and the side effects are categorised as mild as lithium is a useful drug (Amdisen, 1988; Krischel and Jackimczyk, 1991; Mellerup and Plenge, 1990). Obesity and alterations in the immune system and lymphoid cells attributed to lithium treatment have been reported in man and experimental animals (Hart, 1979a). Elevation of lithium concentration to higher levels during treatment could lead to the prevalence of diabetes insipidus (Forrest, 1975; Singer *et al.*, 1971). Diabetes insipidus occurs in at least 40% of patients, often after only a few days on lithium treatment (Forrest *et al.*, 1974).

1.1.5. Mechanisms of Lithium action

Inspite of the intensive research conducted on the effects of lithium on different cellular systems its precise mechanism is incompletely understood. A basic question that has intrigued investigators is whether lithium exerts a direct or an indirect effect on target cells. Results from *in vitro* studies suggest that some effects induced by lithium are indirect, mediated in part by accessory cells that could be the source of important growth factors or cytokines that are responsible for inducing target cell proliferation (Chatelain *et al.*, 1983; Gallichio *et al.*, 1984; McGrath, 1984).

1.1.5.1. Modulation of the Phosphoinositide Second Messenger System by lithium

The phosphoinositol pathway employs the receptor-mediated activation of phospholipase C, which then cleaves phosphatidyl inositol -*bis*phosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). Diacylglycerol is lipophilic and remains in the plasma membrane where it activates a Ca^{2+} -dependent protein kinase C which phosphorylates a number of target proteins, resulting in a cellular response like differentiation. IP_3 is water-soluble and diffuses to intracellular organelles where mobilization of intracellular calcium is activated. IP_3 can be recycled to generate inositol phosphate and PIP_2.

Lithium inhibits the recycling of IP_3 into inositol phospholipids by inhibiting inositol monophosphatases which cleave inositol-1-phosphate and inositol-4-phosphate into inositol and phosphate, leading to the depletion of inositol in the cell (Kofman *et al.*, 1991). Whereas the inositol depletion hypothesis mechanism is well characterised in the brain (reviewed in Jope and Williams, 1994), its role in the mitogenic effects of lithium on cells in culture remains unproven. However, two lines of evidence have suggested that the effects of lithium on cells in culture cannot be attributed to the depletion of inositol. Firstly, the inclusion of *Myo*-inositol in the culture medium did not reverse or change the effects of lithium on the HL-60 cells grown in inositol free RPMI-1640 (Becker and Tyobeka, 1993). Secondly, treatment of HL-60 cells with beryllium did not result in enhanced cell

proliferation although it is known to inhibit inositol-monophosphatase 5-fold more than lithium (Creba *et al.,* 1989). However, lithium mimics *Ha-ras* by enhancing Ca^{2+} oscillations in response to bradykinin but fails to counter growth factor independent growth (Ritter *et al.,* 1997).

1.1.5.2. The effects of lithium on cAMP signalling system

Belmaker (1981) suggested that an imbalance of neuronal cyclic AMP (cAMP) is involved in the etiology of bipolar effective disorder since lithium was found to markedly inhibit agonist stimulated accumulation of cAMP. This effect may be related to inhibition of agonist-stimulated G protein binding (Avissar *et al.*, 1988) or to the inhibition at the catalytic site of adenylate cyclase (Mork, 1990). More recently, lithium has been reported to compete with magnesium for a regulatory site on adenylate cyclase, thereby inhibiting the formation of cyclic AMP (Baraban, 1994). Thus, lithium might function by displacing essential magnesium ions from a regulatory binding site on the catalytic subunit of adenylate cyclase or on a G-protein (Hausman *et al.,* 1989; Newman and Belmaker, 1987). This possibility is yet to be evaluated in HL-60 cells since Sokoloski *et al.*(1993) observed no reversal of lithium effects upon treatment of HL-60 cells in the presence of magnesium chloride (MgCl). Lithium has also been found to affect the activity of the Na^+,K^+ ATPase by competing with K^+ for uptake by the Na^+,K^+ ATPase (Hart, 1979).

1.1.5.3. Modulation of AP-1 by lithium

Activator protein 1 (AP-1) was first identified as a transcriptional factor that binds to an essential *cis*-element of the human metallothionein IIa (hMTIIa) promoter and was found to be required *in vivo* for its optimal basal activity (reviewed in Angel and Karin, 1991). The binding site for AP-1 was later recognised as the 12-*O*-tetradecanyolphorbol-13-acetate (TPA) response element (TRE) found in several cellular and viral genes including collagenase, MTIIa, SV40, stromelysin and interleukin-2 (IL-2) whose transcription is induced in response to treatment of cultured cells with phorbol esters (Angel *et al.*, 1987).

TPA is a potent activator of protein kinase C (PKC) (Nishizuka, 1984) and treatment of cells with TPA activates the AP-1 transcription factor. Furthermore, agents which induce PKC activation such as serum and growth factors have been shown to induce expression of AP-1 dependent genes and inhibition of PKC blocks these induction responses (Brenner *et al.,* 1989).

AP-1 was identified as a protein complex containing the products of *c-jun* and *c-fos* protooncogenes (Bohmann *et al.*, 1987) and many other members of these families have been identified (reviewed in Angel and Karin, 1991). AP-1 exists as either Jun:Jun homodimers or Jun:Fos heterodimers, and since *fos* mRNA is unstable the intronless *jun* mRNA predominates and Jun is the major AP-1 component. Furthermore, Fos:Fos dimers have not been observed as primary components of AP-1 transcription factor, and Fos cannot bind DNA on its own and thus cannot stimulate transcription in the absence of c-Jun. All the Jun family of proteins like JunB, JunD can heterodimerise and homodimerise with any member of the Fos and Jun family of proteins to affect transcription of AP-1 dependent genes by virtue of their ability to efficiently bind and recognise the TRE site of these genes (Sassane-Corsi *et al.*, 1988). This creates great diversity in the responses that are generated by the AP-1 family of transcription factors.

Site-directed mutagenesis experiments indicated that dimerization of Jun and Fos proteins occurs through hydrophobic interactions between the leucine-zipper regions (reviewed in Angel and Karin, 1991). However, hydrophobic interactions alone do not account for the specificity in dimer formation among "leucine-zipper" proteins. For instance, c-Fos dimerizes with the various proteins of the Jun family but not with other "leucine-zipper" proteins like c-Myc, CREB and GCN4 (reviewed in Angel and Karin, 1991).

The Jun:Fos heterodimers were found to be more stable than the Jun homodimers, with dissociation temperatures of 37°C to 42°C for the former, and 25°C to 37°C for the latter. This might explain the enhanced DNA binding of the c-Jun:c-Fos

dimers compared to cJun homodimers; and it can also be explained by the rate of dissociation from the TRE (Rauscher *et al.*, 1988).

Although *c-jun* and *c-fos* genes encode the components of a transcription factor that stimulates the expression of various target genes in response to TPA, their own expression is also subject to PKC activation (Angel *et al.,* 1987; Ryseck *et al.,* 1988; reviewed in Angel and Karin, 1991). Examination of the promoter of the human *c-jun* gene revealed two variant TATA-like sequences, 5'- AGATAAG-3' and 5'-TATTTTA-3', located 24-30 base pairs upstream of a cluster of transcription initiation sites (Hattori *et al.,* 1988). Interestingly, these elements/sequences are perfectly conserved in the chicken *c-jun* gene (Nishimura and Vogt, 1988). The TRE consensus sequence, 5'-GTGACATCAT-3', is located upstream of the TATA-like element (Harroti *et al.,* 1988) and it was confirmed by DNA-binding studies that it is recognised by AP-1complex (Angel *et al.,* 1987). These experiments provided the first direct example of a eukaryotic cellular gene whose transcription can be stimulated by its own protein product. Positive autoregulation is likely to be responsible for signal amplification and conversion of transient signals generated by stimulation of cell surface receptors and PKC into a long term transcriptional response. Furthermore, the ability of cJun to positively regulate *c-jun* transcription stands in marked contrast to the negative effect of cFos and cJun on *c-fos* expression (Sassone-Corsi *et al.,* 1988).

The most common postranslational modification known to modulate protein activity is phosphorylation (Cohen, 1982), and examination of c-Jun in a variety of cell types of human, mouse or chicken origin revealed that it is a nuclear phosphoprotein (reviewed in Angel and Karin, 1991). Two-dimensional peptide maps and phosphoamino acid analysis revealed c-Jun phosphorylation on at least five to seven serine and threonine residues with a pSer:pThr ratio of 5:1 (Boyle *et al.,* 1991).

Surprisingly, bacterially expressed c-*jun* was found to be a substrate of glycogen

synthase kinase-3β (GSK-3β). GSK-3β is known to phosphorylate multiple serine residues in proline-rich regions of glycogen synthase (Woodget and Cohen, 1984) and v-Myb (Boyle *et al.,* 1991). Two serines and one threonine that were identified from peptides of *in vivo* phosphorylated cJun are phosphorylated by GSK-3β *in vitro*, and the precise location of these residues has been mapped to a single tryptic peptide harbouring the sequence: TPPLS^{243}PIDMES (Boyle *et al.,* 1991). These phosphorylation sites are highly conserved in the Jun family of proteins, and Ser-243 is replaced with a Phe residue in v-Jun oncoprotein (Vogt and Bos, 1990, reviewed in Angel and Karin, 1991). However, stimulation of HeLa cells or human fibroblasts with TPA leads to a rapid decrease in phosphorylation of the three residues, such that in only 30 min one of them is completely dephosphorylated. Because this site is adjacent to the DNA binding domain, a phosphorylation/dephosphorylation mechanism is thought to regulate the DNA binding activities of c-Jun (reviewed in Angel and Karin, 1991). Although GSK-3β has not been identified definitively as the kinase that phosphorylates and thus inactivates c-Jun there is a lot of circumstantial evidence. The dephosphorylation of cJun, either by direct inhibition of GSK-3β after TPA treatment or activation of a protein phosphatase, increases the DNA-binding activity of preexisting cJun and results in increased occupancy of the AP-1 site in the *c-jun* promoter, followed by induction of *c-jun* transcription (reviewed in Angel and karin, 1991).

In vivo and *in vitro* studies have demonstrated that c-Jun may be phosphorylated on specific sites by several serine/threonine kinases, such as casein kinase II, protein kinase C, and mitogen-activated protein kinases (MAP kinases) which causes changes in DNA binding and transcriptional activity (reviewed in Angel and Karin, 1991). MAPKs mediate signals from cell membrane receptors to transcription factors by sequential activation of protein kinases (reviewed in Davis, 1994). The Jun NH_2-terminal kinase subgroup of MAPKs, first identified as a protein family that phosphorylate c-Jun at Ser-63 and Ser-73 (reviewed in Davis, 1994), has been implicated in the regulation of apoptosis. Phosphorylation of Ser-243 by casein kinase II or PKC results in the inhibition of DNA binding by AP-1

(reviewed in Angel and karin, 1991), whereas phosphorylation of c-Jun Ser-63 and Ser-73 in the N-terminal domain by MAP kinase (reviewed in Angel and Karin, 1991) and or secondary to activation of H-Ras (Smeal *et al.,* 1989) positively regulates the transactivating activity. Interestingly, purified c-Jun is not a substrate for either PKC or protein kinase A (PKA) phosphorylation, indicating that PKC activation by TPA does not directly lead to modification of c-Jun (Boyle *et al.,* 1991).

Regardless of the exact mechanism, the observed net phosphorylation of c-Jun is a specific response to PKC activation and it does not occur after treatment of cells with forskolin, an agent that activates PKA (Boyle *et al.,* 1991). Forskolin does not induce *c-jun* transcription, thus there is a correlation between dephosphorylation of preexisting c-Jun protein and induction of *c-jun* transcription (Angel and Karin, 1991). Nevertheless, there appears to be multiple signalling pathways that converge at the activation of c-Jun.

The dorsalisation of *Xenopus* embryos by lithium treatment has been attributed to inhibition of GSK-3β (Klein and Melton, 1996). Lithium inhibits GSK-3β *in vivo*, and mimics the biochemical effect of *wnt* signalling by leading to stabilization of β-catenin in *Xenopus* oocyte and embryo. This property is specific for lithium as other monovalent cations showed inability to inhibit purified GSK-3β (Klein and Melton, 1996; Stambolic *et al.,* 1996). Consistent with previous studies phosphorylation of c-Jun by GSK-3β leads to AP-1 inactivation and inefficient binding to the TREs of AP-1 dependent genes (Boyle *et al.,* 1991; de Groot *et al.,* 1992).

The *Drosophila shaggy* gene (*sgg*) product which shares structural homology (83-85% in the central kinase domain) with the mammalian GSK-3β appears to be essential for the imaginal epithelium in *Drosophila* (Woodgett, 1991). The *sgg* protein was found to have the same effect on the activity of c-Jun as GSK-3β suggesting a common mechanism for lithium induced teratogenicity (de Groot *et*

al., 1992).

The relationship between *c-jun* and lithium was formulated from the finding that lithium inhibited GSK-3β *in vivo*. Many cell growth enhancing stimuli like serum, EGF, PGDF and other activators of MAPKs are known to regulate the transcription of AP-1 dependent genes (Brenner *et al.* 1989; reviewed in Angel and Karin, 1991), and as such it is possible to hypothesise that the growth enhancing effects of lithium on HL-60 cells and other cell lines could be mediated by AP-1. The hypothetical mechanism could start with the inhibition of GSK-3β or activation of a protein phosphatase by lithium, and these events could lead to the dephosphorylation of Ser and Thr residues on c-Jun thus activating AP-1 elements.

1. 2. MINOCYCLINE

1.2.1. Introduction

The tetracyclines are a group of antimicrobial agents which were originally isolated from *Streptomyces* species. Tetracyclines are used in the treatment of bacterial infections due to their ability to inhibit the synthesis of proteins. The tetracyclines inhibit protein synthesis by blocking the binding of the aminoacylated tRNA/elongation factor EF-Tu complex to the ribosome (Mathews and van Holde, 1990). Protein synthesis is also inhibited in eukaryotic cells by higher doses of tetracyclines (Mathews and van Holde, 1990). Minocycline, like demeclocycline (demethylchlortetracycline) is a synthetic derivative of tetracyclines but has greater lipophilic properties than other tetracyclines which potentiates its entry into the cells (Kofman *et al.*, 1993).

Figure 1.1: The structure of tetracycline showing the different substituent groups that result in the many derivatives from the frame.

1.2.2. Clinical uses

Tetracyclines are the drugs of choice in the treatment of *Mycoplasma pneumoniae* infections. They are also used in the treatment of chlamydiae and rickettsiae infections (Jawets, 1987). They are employed in many Gram-positive and Gram-negative bacterial infections, including cholera and other *Vibrio* infectious, provided the organism is susceptible. Minocycline has also shown some degree of effectiveness against resistant *Plasmodium falciparum* (Katzung and Goldsmith, 1987).

The side-effects that exist as a result of minocycline treatment are dizziness and nausea, with the frequency of occurrence being dose related (Jawets, 1987). High doses of minocycline and all the other tetracyclines decrease the rate of protein synthesis in host cells, an event that ultimately leads to renal damage and elevated blood urea levels. Long term therapy with these agents may cause bone marrow disturbances. To avoid deposition in growing bones and teeth, tetracyclines are usually not indicated for pregnant women or children less than six years of age.

1.2.3. Minocycline and Lithium

Tetracyclines, especially demeclocycline (DMC), cause pronounced diuresis as a result of inhibition of ADH-sensitive adenylate cyclase (Dousa and Wilson, 1974). The ability of tetracycline antibiotics to attenuate amphetamine-induced hyperactivity and the fact that both DMC and minocycline (MIN) attenuated

agonist-induced accumulation of cAMP suggested that they may share some of the therapeutic properties of lithium (Kofman *et al.*, 1993). Several studies have shown that the hypoactivity induced by drugs that elevate cAMP levels can be attenuated by lithium (Smith, 1990; Kofman *et al.*, 1990). Lithium has also been reported to depress hyperactivity caused by low doses of amphetamine, but not hyperactivity following higher doses of amphetamine (Ebstein *et al.*, 1980). Minocycline and demeclocycline have been referred to as lithium mimetic because of their effects on the accumulation of cAMP and adenylate cyclase.

Since treatment of HL-60 cells with demeclocycline resulted in decrease in viability and inhibition of growth (Becker and Tyobeka, 1993), it was worthwhile investigating the effects of minocycline on these cells with the hope of finding out if they could be of use in leukemia therapy, if not alone, perhaps in combination with other compounds.

1.3. APOPTOSIS

Cell death is an essential event in the development of multicellular organisms and it plays an important role in the pathogenesis of many diseases. It is now well established that there are two modes of cell death, namely necrosis and apoptosis (programmed cell death), that can be distinguished by both morphological and molecular characteristics (Kerr *et al.*, 1972; Tomei and Cope, 1991; reviewed in Hale, 1996). Necrosis is observed as a catastrophic metabolic failure resulting directly from severe molecular or structural damage. It leads to inflammation and damage to surrounding cells because of the leakage of cellular contents. Apoptosis, on the other hand, is associated with the orderly fragmentation of the cells into membrane bound vesicles which are then phagocytosed. Apoptosis plays a crucial role in physiological and pathological processes as distinct as embryo development, haematopoiesis and neoplasia (Giannakis *et al.*, 1991). Besides physiological activation, apoptosis can be triggered or enhanced by various stimuli, including certain toxicants and ionising radiation (Wyllie, 1980). Negative selection of autoreactive T-cell receptors through clonal deletion during T-cell maturation is

thought to occur by apoptosis (McCabe *et al.,* 1993). However, it is unclear how the initial signalling leading to clonal selection differs from signals that induce activation and proliferation of T cells (McCabe *et al.,* 1993). Apoptosis is a highly regulated process involving gene expression, protein synthesis, and activation of specific enzymes including the poorly characterised endonuclease which is thought to be responsible for the characteristic DNA cleavage into oligonucleosomal sized fragments (Giannikis *et al.*, 1991; Arends and Wyllie, 1991). However, some cells die by apoptosis without the characteristic internucleosomal DNA cleavage (Cohen *et al.*, 1992). Apoptosis is also characterised by reduction of cell volume, chromatin condensation and fragmentation of the cell into membrane-encapsulated apoptotic bodies (Perez-Sala and Mollinedo, 1995).

The mechanism of apoptotic cell death remains incompletely understood, although in thymocytes, it is associated with a rise in intracellular calcium (McConkey *et al*, 1989; Spencer *et al*, 1995; Giannakis *et al*, 1991) and cAMP (Kizaki *et al*, 1989). There are a number of events that lead to apoptosis, including serum withdrawal (Jeso *et al.*, 1995), inhibition of N-linked glycosylation in HL-60 cells (Perez-Sala and Mollinedo, 1995) and many drugs used in cancer therapy. It is well documented that cytokines, such as interleukin-1 (IL-1), tumour necrosis factor-α, transforming growth factor-ß and nitric oxide can induce apoptosis (Nishikawa *et al.*, 1995). Induction of apoptosis by TNF-α, FAS ligand and x-rays is mediated by ceramide (Pushkareva *et al.,*1995; Kolesnick and Golde, 1994), which also activates the MAP kinase, stress-activated protein kinase (SAPK/JNK) (Verheij *et al.,* 1996). Intracellular zinc has been shown to prevent the process of apoptosis (McCabe *et al.*, 1993).

It is not clear which enzyme is responsible for the cleavage of DNA into smaller fragments. The activation of Ca^{2+} dependent endonuclease, DNase I, in some cells has been implicated (Peitsch *et al.*, 1993). This stems from the finding that there is an elevation of intracellular calcium during apoptosis. However, the involvement of a pH-dependent endonuclease, DNase II, has been suggested in some cells

(Barry and Eastman, 1993). Perez-Sala *et al.* (1995) observed that intracellular acidification was correlated with severe DNA degradation, possibly by activation of a pH-dependent DNase II which has pH optimum of 5. Treatment of HL-60 cells with lovastatin caused a decrease in pH to about 6.5 which was concomitant with the induction of apoptosis. Agents that induce intracellular alkalination, like PMA were sufficiently active in inhibiting acidification-induced DNA fragmentation, suggesting the involvement of DNase II in apoptosis in the HL-60 cells. The lovastatin-induced acidification is thought to result from the inhibition of PKC which dysregulates the Na^+/H^+ antiporter.

The induction of apoptotic cell death seems to be dependent on the maturation state of the cells and it was reported by McCarthy *et al.* (1994) that TPA-induced monocytic differentiation of HL-60 cells was accompanied by increased resistance to undergoing apoptosis. In addition, improved survival of HL-60 cells induced to undergo apoptosis was observed with retinoic acid treatment (McCarthy *et al.,* 1994). Lithium was also found to induce apoptosis of immature cerebellar granule cells, yet found to have no effect on the viability of mature neurons (D'Mello *et al.,* 1994). These findings associate the onset of apoptosis with cell maturation and cell cycle progression.

1.3.1. Involvement of protooncogenes in apoptosis

1.3.1.1. *p53* proto-oncogene

The biochemical role of p53 phosphoprotein is not fully understood, but it can act as a transcription activator and repressor (reviewed in Hale *et al.,* 1996). Wild-type p53 posseses properties of transcription factor and activates transcription when bound directly to DNA. However, when tethered to the promoter through interactions with TATA binding proteins (TBP), p53 represses transcription (Horikoshi *et al.,* 1995). The mutational inactivation of p53 leads to a genetic lesion that is known to occur in many human cancers (Hollstein *et al.,* 1991).

It is generally accepted that a critical cellular response to DNA damage is the

accumulation of p53 protein, with the subsequent blocking of cell cycle progression at the G_1 phase of the cell cycle to facilitate repair of the damage (Kuerbitz *et al.,* 1992). In the absence of repair mechanism p53 triggers cell death by apoptosis (Cohen *et al.,* 1992). Furthermore, exposure of mammalian cells to ionizing radiation leads to the accumulation of the cells at the G_1 and G_2 phases of the cell cycle (Kuerbitz *et al.,* 1992) and apoptotic cell death (Cohen *et al.,* 1992). Wild-type p53 arrests cells in G_1 phase while mutant p53 gene lacks this checkpoint and arrests in G_2 phase following ionizing radiation (Kuerbitz *et al.,* 1992). It has been suggested that the ***p53*** pathway may have evolved to allow time for the cells to repair DNA damage or to induce apoptosis if the damage cannot be repaired (Lane, 1992).

The mechanism by which p53 induces accumulation of cells in the G_1 phase is attributable to its role as transcription factor (reviewed in Hale *et al.,* 1996). Exposure of cells to ionizing radiation leads to elevation of p53 phosphoprotein levels that induces transcriptional upregulation of a number of proteins including $p21^{WAFI/CIPI}$, GADD45 and Mdm2 (reviewed in Canman and Kastan, 1995). $p21^{WAFI/CIPI}$ is a critical downstream effector in the p53-dependent G_1 checkpoint as it acts as an effective inhibitor of the cyclin-dependent kinases, resulting in the arrest of the cell cycle at the G_1/S border (el-Deiry *et al.,* 1994). Induction of the GADD45 by ionizing radiation is implicated in the supression of the cell cycle progression and is dependent on wild-type p53 (reviewed in Hale *et al.,* 1996). Mdm2 expression is not involved in cell cycle arrest, but it has been implicated in the regulation of p53 transcription (Chen *et al.,* 1994). This would lead to downregulation of p53-dependent genes and the subsequent re-entry into the cell cycle presumably after the DNA damage has been repaired (reviewed in Hale *et al.,* 1996).

Apoptosis caused by DNA damage often occurs through a p53-dependent pathway (Cohen *et al.,* 1992). Immature thymocytes deficient in p53 were found to be resistant to apoptosis induced by ionizing radiation (Lowe *et al.,* 1993) or by the

DNA-damaging topoisomerase II inhibitor etoposide (Clarke *et al.,* 1993). However, p53 deficient cells can be induced to undergo apoptosis by other stimuli, suggesting that p53-dependent and -independent apoptotic pathways exist (reviewed in Hale *et al.,* 1996).

The *p53* gene is a tumour suppressor and is frequently altered, deleted or rearranged in various types of tumour cells. The p53 protein shares some properties with growth associated c-Myc in that both are localised in the nucleus. *p53* oncogene can replace *c-myc* in the *myc-ras* cooperative transformation of primary rodent fibroblasts cultures (Eliyaku *et al.*, 1984; Parada *et al.*, 1984). *p53* is located on the short arm of chromosome 17 (Miller *et al.*, 1986) and this gene is deleted in HL-60 cells resulting in the absence of its protein product (Wolf and Rotter,1985).

1.3.1.2. *bcl-2* oncogene

The *bcl-2* oncogene was initially identified as breakpoint of the t(14;18)(q32;q21), the most common chromosomal translocation in the lymphoid malignancies (Tsujimoto and Croce, 1986; Bakhshi *et al.*, 1985; Cleary and Sklar, 1985). This translocation juxtaposes the Ig heavy chain gene enhancer with the *bcl-2* gene, resulting in the constitutive expression of *bcl-2* transcripts and the subsequent synthesis of the 24-26-kDa Bcl-2 protein. Overexpression of Bcl-2 in lymphoid cell cultures and transgenic mice inhibits apoptosis induced by a wide range of stimuli, including the γ-irradiation, growth factor deprivation and other injurious agents (reviewed in Hale *et al.,* 1996).

The *bcl-2* family of genes is very diverse with certain members inhibiting while others promote the progression of apoptosis (reviewed in Craig, 1995). Some members of the family like *bcl-xs*, which was identified by low stringency hybridisation to *bcl-2* (Boise et al., 1993) and *bax,* which was identified by co-immunoprecipitation with bcl-2 (Oltvai *et al.*, 1993) are known to promote rather than inhibit the onset and progression of programmed cell death (Boise *et al.,*

1993; Krajewski *et al.,* 1994; Oltvai *et al.,* 1993). Other members of the family, *bak* and *bad* promote apoptosis (Chittenden *et al.,*1995; Farrow *et al.,* 1995; Kiefer *et al.,*1995).

Expression of Bcl-2 protein has been shown to decrease with cell maturation and becomes almost undetectable in terminally differentiated neutrophils, which eventually undergo programmed cell death (Martin *et al.,* 1990; Lennon *et al.,* 1990). It has also been found that *Bcl-2* is highly expressed in myeloid cells in the bone marrow (Hockenberry *et al.*, 1991; Savill *et al.*, 1989). Overexpression of Bcl-2 in HL-60 cells seems to cancel G_0/G_1-predominant induction of apoptosis and differentiation (Nauvomski and Cleary, 1994). The downregulation of *bcl-2* mRNA during G_0/G_1 phase might explain the predominant induction of apoptosis and differentiation in HL-60 cells at this stage of the cell cycle (Naumovski and Cleary, 1996).

However, there are circumstances during which *bcl-2* is unable to inhibit cell death (Nunez *et al.*, 1991). For instance cell death caused by cytotoxic T lymphocytes cannot be blocked by Bcl-2 expression (Vaux *et al.*, 1992), and only partial inhibition is observed during exposure to the Apo-1 antibody which recognises the Fas antigen (Itoh *et al.*, 1993). Thus, cell death can be influenced by Bcl-2 in some cases and not in others.

Bcl-2 family members interact with each other, and can form either homo- or heterodimers (Zha *et al.,* 1995; Oltvai *et al.,* 1993). The determining factor for cell viability might therefore be the ratio of the level of the death-inhibiting *bcl-2* family member relative to that of the death promoting *bcl-2* family member (Oltvai *et al.,* 1993). Bcl-2, for example, interacts with Bax, and the high levels of Bcl-2 relative to Bax promotes survival whereas an excess of Bax promotes cell death. Similar observations have been reported for Bcl-2 and Bak (Chittenden *et al.,* 1995), Bcl-x_S (Boise *et al.,* 1993) and Bcl-x_L and Bak (Farrow *et al.,* 1995). These dimerizations are possible due to the three conserved regions within the Bcl-2 related proteins

which have been identified as controlling regions for protein-protein interaction, namely, BH1, BH2 (Yin *et al.,* 1994) and BH3 (Chitteden *et al.,* 1995; reviewed in Kroemer, 1997). A 28 amino acid segment of Bak encompassing BH3 is sufficient for interaction of Bak with Bcl-2. Furthermore, the BH3 segment on its own was found to be sufficient to induce apoptosis (Chittenden *et al.,* 1995). It is not clear whether the BH3 segment of Bak or Bad or other apoptosis inducing members of Bcl-2 family acts by functional sequestration of Bcl-2 activity or as an effector domain to promote apoptosis.

1.3.1.3. *c-myc* oncogene

Malignant transformation seems to require four critical events: acquisition of increased proliferative potential, abrogation or evasion of apoptosis, immortalisation and deletion of access to differentiation programs (reviewed in Meichle *et al.,* 1992).

The human *c-myc* gene was defined by virtue of its homology to *v-myc*, the transforming gene of the avian myelocytoma virus which causes myeloid tumours in chicks. The c-Myc protein is localised in the nucleus (Hann *et al.*, 1983) and amplified expression of the *c-myc* gene is observed immediately after resting lymphocytes or fibroblasts are induced to proliferate (Kelly *et al.*, 1983). Interestingly, c-Myc levels in HL-60 cells are 15-30 fold higher than in normal nonmalignant cells (Land *et al.*, 1983; Collins and Groudine, 1982).

The level of *c-myc* mRNA is decreased when HL-60 cells are induced to undergo terminal differentiation with granulocytic inducers (Westin *et al.*, 1982), and monocytic and macrophage-like differentiation of HL-60 cells is associated with a marked reduction in *c-myc* levels (Reitsma et al., 1983; Sariban et al., 1985). In addition, antisense oligonucleotides to *c-myc* induce granulocytic differentiation in HL-60 cells (reviewed in Dang, 1991).

The relationship between the levels of *c-myc* and the induction of apoptosis was

reported by Wyllie (1987) in which they demonstrated an increased apoptosis rate in Rat-1 fibroblasts transformed with *c-myc*. Despite accelerated proliferation as a result of *c-myc* expression, Screaton *et al.* (1997) reported enhanced progression of apoptosis resulting from overexpression of *c-myc* in L6 myoblasts, and these effects were reversed by overexpression of Bcl-2 protein. This anti-transforming effect of *c-myc*, together with its role in differentiation becomes apparent when growth factors become limiting (reviewed in Meichle *et al.,* 1992). The cell cycle distribution of cells expressing *c-myc* constitutively resembles that of proliferating cells even after growth factor deprivation (reviewed in Meichle *et al.,* 1992). Although the exact mechanisms by which c-Myc promotes proliferation and apoptosis are unknown, Myc is a transcription factor for growth, differentiation and transformation associated genes (reviewed in Meichle *et al.,* 1992; Amati and Land, 1994). More recently (Galaktionov *et al.,* 1996), the levels of cdc25A were shown to be linked with c-Myc expression and ectopic expression of cdc25A could mimic induction of apoptosis by c-Myc, suggesting that c-Myc induced cell proliferation and apoptosis are tightly linked to the control of the cell cycle. This also supports the suggestion that apoptosis is a result of abortive mitosis (reviewed in Pandey and Wang, 1995).

1.3.1.4. The *c-fos* gene

The *c-fos* gene is the homolog of v-fos, the transforming gene of the FBJ-murine sarcoma virus, which induces osteosarcomas in mice (reviewed in Angel and Karin, 1991). The expression of *c-fos* mRNA is rapidly increased in many cell types in response to mitogens such as serum, EGF, TGFα, PDGF and others (Brenner *et al.,* 1989; Angel *et al.,* 1987; Ryseck *et al.,* 1988; reviewed in Angel and Karin, 1991). Microinjection of anti-Fos antibodies or transfection *c-fos* antisense RNA inhibits DNA synthesis and cell proliferation in cultured fibroblasts (Riabowol *et al.,* 1988; Holt *et al.,* 1986). It is paradoxical that a similar increase in *c-fos* mRNA was observed following treatment of HL-60 cells with TPA (Muller *et al.*, 1985; Mitchell *et al.*, 1986; reviewed in Collins, 1987), since TPA induced pronounced inhibition of proliferation (Rovera *et al.*,1979). *c-fos* expression does not appear

to be essential for monocyte differentiation since vitamin D_3 has been shown to induce monocytic differentiation in some HL-60 variant subclones without the induction of *c-fos* expression (Mitchell *et al.,* 1986). Nevertheless, induction of *c-fos* is routinely used as an indicator of cellular activation in many systems.

It has been shown that continuous expression of *c-fos* precedes induction of apoptosis *in vivo* (Smeyne *et al.,* 1993), and furthermore, a number of reports have demonstrated the expression of *c-fos* in distinct cell types induced to undergo apoptosis by growth factor removal and several other agents (Colotta *et al.,* 1992; Mollinedo *et al.,* 1994; Estus *et al.,* 1994). In addition, antisense oligonucleotides directed against *c-fos* have been shown to improve survival of growth factor-deprived lymphoid cells (Colotta *et al.,* 1992), and microinjection of antibodies against the Fos protein family protected nerve growth factor-deprived neurons from apoptosis (Estus *et al.,* 1994). Gajate *et al.*(1996) studied the role of *c-fos* in apoptosis and concluded that Fos is not essential for apoptosis following the observation that *c-fos* deficient mice responded similarly to wild type mice when treated with etoposide, a Topo II inhibitor. They also suggested that the enhanced expression of *c-fos* during apoptosis could be a result of; (a) gratuitous induction by altered activation or inhibition of death specific signal transduction mechanisms, (b) *c-fos* expression may constitute a side effect of the initiation of apoptosis due to the fact that the intracellular signal transduction mechanism involved in apoptosis may result, through crosstalk of signalling processes, in a coincidental induction of *c-fos* without a direct connection between both events in which case, apoptosis may or may not be accompanied by *c-fos* expression and (c) *c-fos* expression may play a crucial role and constitutes a prerequisite for the initiation of apoptosis. However, conclusive evidence on the actual role of *c-fos* in programmed cell death remains to be established.

1.3.1.5. The role *c-jun* in Apoptosis

Following the observation of elevated expression of *c-jun* mRNA during DNA fragmentation in human myeloid leukemia cells induced to undergo apoptosis

(Rubin *et al.,* 1991; Gunji *et al.,* 1991), a direct role of *c-jun* in the induction of apoptosis was suggested. However, taxol-induced apoptosis in HL-60 cells occurred without the induction of enhanced expression of *c-jun* mRNA (Bhalla *et al.,* 1993). It would seem that activation of AP-1 can either induce or suppress cell death depending on the cell type and in a trigger-dependent manner. It is interesting to note that proliferating cells have been shown to express higher levels of *c-jun* and amplified expression of *c-jun* has also been shown to be associated with cell differentiation (reviewed in Angel and Karin, 1991; Wlliams *et al.,* 1990).

A more definitive role of *c-jun* in apoptosis was demonstrated in U937 leukemia cells (Verheij *et al.,* 1996). Activation of SAPK/JNK with ceramide or environmental stress caused apoptosis which was reversed by a dominant negative mutant of *c-jun*, TAM-67. However, induction of apoptosis by ara-C and calphoxin C was not mediated by TAM-67. Increased c-Jun activity is sufficient to trigger apoptosis in NIH 3T3 fibroblasts (Bossy-Wetzel *et al.,* 1997). In these studies, the amino-terminal transactivation domain and the leucine zipper regions were implicated in c-Jun-induced apoptosis, thus suggesting a transcription regulatory mechanism.

Ishikawa *et al.* (1997) reported improved survival of H_2O_2 treated mesangial cells when *c-jun* expression was downregulated with antisense *c-jun* expression vector. The authors further demonstrated elevated expression of *c-jun* mRNA concomitant with DNA fragmentation induced by H_2O_2. Thus this data suggested that c-Jun/AP-1 was involved in oxidant induced apoptosis.

1.3.2. Cytokines and Apoptosis

1.3.2.1. Interleukin 2 (IL-2)

Stimulation of HL-60 cells with interleukin-2 (IL-2) activates at least three pathways leading to the induction of *bcl-2*, *c-myc* and *Lck* (Miyazaki *et al.,* 1995; Ahmed *et al.,* 1997). It has also been established that the induction of Bcl-2 and c-Myc depends on signals transduced from the S region of the IL-2Rβ (Miyazaki

et al., 1995). Signals originating from the S region of IL-2Rβ activate the phosphatidyl inositol 3-kinase, yet it is not clear whether its activation is responsible for the induction of Bcl-2 and c-Myc. Interestingly, 5 mM lithium caused a significant increase in IL-2 production after stimulation of T-cells with PHA (Kucharz *et al.,* 1988). Lithium enhances growth of HL-60 cells and stimulates IL-2 production in T-cells, it would be interesting to demonstrate the role, if any, of IL-2 in lithium-stimulated cell proliferation and apoptosis of HL-60 cells.

1.3.2.2. Interleukin-6 (IL-6)

Many studies have shown that wild-type p53-mediated apoptosis, without prior DNA damage can be effectively suppressed by the cytokine interleukin 6 (IL-6) (Yonish-Rouach *et al.*, 1991; Lotem and Sachs, 1995; Lotem *et al.*, 1996; Lotem and Sachs, 1996). IL-6, also known as BCDF or BSF2, was first identified as a factor required for the final differentiation or growth of B cells into antibody producing cells (Hirano *et al.*, 1986). One of the known major activities of IL-6 was found to be the induction of acute phase proteins in hepatocytes, and it is for this reason that IL-6 was assumed to be able to function as a hepatocyte stimulating factor (HSF) (Gauldie *et al.*,1987).

IL-6 is expressed in various cells of the lymphoid family and non-lymphoid cells such as T cells, B cells, monocytes, fibroblasts, mesangium cells and several tumour cells. The production of IL-6 is subject to strict regulation, for instance in T cells IL-6 production is stimulated by mitogens or antigenic stimulation by direct contact with macrophages (Horii *et al.*, 1988), while in monocytes and fibroblasts it was found that Lipopolysaccharide (LPS) was sufficient to enhance significantly the levels of IL-6 (Helfgott *et al.*,1987). A variety of cytokines, including IL-1, tumour necrosis factor (TNF), platelet-derived growth factor (PDGF), and interferon-β (IFN-β) as well as poly(I)poly(C), and cycloheximide also enhance the expression of IL-6 gene in different cell types (Hirano *et al.*, 1986; reviewed in Kishimoto, 1989). Activation of protein kinase C (PKC) by phorbol esters induces

the production of IL-6 in fibroblasts, as do agents that increase the levels of intracellular cAMP. Glucoccorticoids, on the other hand negatively regulate the production IL-6 in various tissues (Helfgott *et al.*, 1987).

1.4. AIMS AND OBJECTIVES

In many studies it was shown that apoptosis occurs through the alterations in the expression of protooncogenes such as *c-jun, c-myc, c-fos, bcl-2, bax* and, IL-2 was shown to block apoptosis by activating the transcription of *bcl-2.* Since Li^+ induces apoptosis in HL-60 cells, a lithium-mimetic drug that inhibits cAMP accumulation in rats, minocycline was investigated. It then hypothesised that lithium and minocycline induce apoptosis in HL-60 cells by causing changes in the transcription of protooncogens implicated in apoptosis.

Regulation of apoptosis by intracellular second messengers is poorly understood although a pivotal role for known signal transduction pathways such as, cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), cyclic AMP, pH and protein kinase C have been suggested (reviewed in Hale, 1996). In many cells an increase in $[Ca^{2+}]_i$ induced by calcium ionophores can stimulate apoptosis, and a calcium releasing agent thapsigargin has also been shown to promote apoptotic cell death. Furthermore, cell-permeating Ca^{2+} chelating agents have been shown to delay apoptosis (Jiang *et al.,* 1994). The apoptotic inhibitory role of Bcl-2 oncoprotein appears to involve changes of Ca^{2+} compartmentalization (Lam *et al.,* 1995). However, the role of Ca^{2+} seems to differ between cellular systems and an increase in $[Ca^{2+}]_i$ has been shown to prevent rather than induce apoptosis and DNA fragmentation (Edwards *et al.,* 1991). The study also focused on the role of calcium and protein kinase C activation.

The role of c-Jun/AP-I in apoptosis is not clearly defined, but in many reports apoptosis is accompanied by elevated levels of *c-jun* expression. Yet other studies have revealed that downregulation of *c-jun* protects a variety of cell lines against

apoptosis. Furthermore, lithium was shown to activate the AP-1 transcription factor by inhibiting GSK-3β, an enzyme that phosphorylates and inhibit AP-1 binding. The hypothesis tested was whether downregulation of *c-jun* could reverse the effects induced by lithium in HL-60 cells.

Thus the broad aims of this study were to explore the cellular components that are required for the induction of apoptosis by lithium and minocycline in mammalian cell lines. Specifically; 1) To investigate the role played by Ca^{2+} and PKC in lithium and minocycline induced apoptosis. 2) To determine whether minocycline and lithium induced apoptosis requires new protein synthesis. 3) Analyse the expression of *c-myc, c-fos* and *c-jun* and *bcl-2* proto-oncogenes upon exposure of HL-60 cells to lithium and minocycline. 4) Determine the role of c-Jun/AP-1 transcription factor in lithium-induced proliferation and apoptosis.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

All restriction enzymes, λDNA, alkaline phosphatase, proteinase K, cDNA synthesis kit, deoxynucleotide triphophates (dNTPs), DNA gel extraction kit, T4 DNA ligase, DNase-free RNase, RNase-free DNase I and DOTAP transfection medium were purchased from Boeringher-mannheim (Germany). Klenow polymerase was from Amersham Int., Buckinghamshire, UK while *Taq* DNA polymerase was purchased from TAKARA (Japan).

Cycloheximide, minocycline, actinomycin D, EGTA, TPA, LiCl were purchased from Sigma, St.Louis, USA and molecular grade agarose purchased from Promega Corp., Madison, USA.

2.2. METHODS

2.2.1. Cell culture Methods

2.2.1.1 Cell cultures

HL-60 and K562 cells (ATCC, Rockville, USA) were maintained in constant logarithmic growth (1-6 x 10^5 cells/ml) in suspension cultures in RPMI-1640 medium (ATCC, Rockville, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS) (Highveld Biologicals, South Africa), at 37°C in 5% CO_2 humidified atmosphere. The cells were passaged twice or thrice weekly in order to maintain the density of between 1 x 10^5 and 6 x 10^5 cells/ml.

3T3-L1 cells (ATCC, Rockville, USA) were grown in monolayer cultures in DME medium (Sigma, St.Louis, MO.), supplemented with 10% FCS at 37°C. The cells were subcultured at 80-90% confluency by treatment with 0.25% trypsin and

harvested at 1200 rpm for 10 minutes. The cells were then resuspended in fresh DME medium with 10% FCS, and aliqouted in culture dishes to maintain a low density.

Raji cells (ATCC, Rockville, USA) were maintained in suspension cultures in DME medium containing 10% FCS at 37°C. They were passaged twice weekly to ensure a density of between 2-5 X 10^5 cell/ml.

LLC-PK cells (ATCC, Rockville, USA) were grown in monolayer cultures in DME medium supplemented with 5% FCS at 37°C. To maintain a low density of cells LLC-PK cells were subjected to the same treatment as the 3T3-L1 cells (see above paragraph). Experiments were set up with 90% confluent cells.

2.2.2. Cell proliferation studies

2.2.2.1 Cell proliferation

Cells were treated with the respective concentrations of either lithium (Fluka Chemie AG., Buchs, Switzerland) or minocycline (MIN) (Sigma Chemical Co. St Louis, MO, USA) for up to 96 hours, and cell numbers were determined at 24 hour intervals by using Model Z_f Coulter Counter (Coulter Electronics, Inc., Luton, UK).

2.2.2.2. Thymidine incorporation

The cells (200 μl) were aliqouted in triplicates into tissue culture microtiter plates containing 1 μCi of [^{3}H]-thymidine (5 Ci/mmole) (Amersham Int., Buckinghamshire, UK). The microtiter plates were incubated for 2 hours at 37°C in a humidified atmosphere of 5% CO_2/95% air. Thereafter the cells were harvested by centrifugation using a bench-top centrifuge (Beckman Instruments, Inc., Fullerton, CA) for 10 minutes, washed twice with phosphate-buffered saline (PBS) (137 mM NaCl; 2.7 mM Kcl; 1.5 mM KH_2PO_4; 8.1 mM Na_2HPO_4, pH 7.4), and then lysed in lysis buffer (10 mM Tris-HCl, 0.5% SDS and 1 mM EDTA, pH 8.0). The DNA was precipitated with ethanol and recovered by centrifugation at 14 000 rpm. The DNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, Ph 8.0) and

then mixed with aqueous compatible scintillation fluid (Beckman Intsruments Inc., Fullerton, CA). Counts were carried out using a Beckman LS6000IC scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

2.2.2.3. Cell viability assays

Experimental cultures were seeded at 2 X 10^5 cell/ml, unless otherwise stated, and incubated for the duration of the experiment. Cell viability was determined using the Trypan Blue dye-exclusion method. Briefly, cells were treated with specified concentrations of lithium and minocycline and then a 1ml aliquot was stained with trypan blue. The number of cells taking up the dye (appeared blue) were counted on a haemocytometer under a light microscope (Zeiss, Germany). Viability was expressed as percentage of control (untreated) cells.

2.2.2.4. Protein synthesis assays

HL-60 cells were pretreated with minocycline and cycloheximide for 2 hours and then incubated for a further 2 hours in the presence of 20 μCi/ml of [^{3}H]-Leucine (Amersham Int. Buckinghamshire, UK). The labelled cells were harvested by centrifugation at 1000 rpm for 10 minutes, and washed twice in sterile ice-cold PBS, pH 7.4. The cell pellet was lysed in 20 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, pH 8.0, and the cell lysate precipitated with 10% TCA. The precipitate was recovered by centrifugation at 13 000 rpm and resuspended in distilled water. Aqueous compatible scintillation fluid was added in the recommended ratios and the incorporated labelled leucine determined by using a Beckman LS6000IC Scintillation Counter. The results were expressed as percentage of control untreated cells.

2.2.2.5. Analysis of DNA fragmentation

DNA was extracted using a modified version of the method described by Jeso *et al.* (1995). Briefly, about 6 x 10^6 cells were harvested by centrifugation at 1200 rpm for 10 minutes and then washed once with ice-cold sterile phosphate buffered saline (PBS) (58 mM N_2aHPO_4; 17 mM NaH_2PO_4; 68 mM NaCl, pH 7.4). The cell

pellet was resuspended in 200 μl lysis buffer (100 μg/ml proteinase K; 10 mM Tris-HCl pH 8.0; 25 mM EDTA pH 8.0; 0,5% SDS) and then incubated at 50°C for 2 hours. The DNA was extracted once with phenol:chloroform (1:1) mixture and precipitated with 1 volume isopropanol in the presence of 0.1 volume of 3 M sodium acetate (pH 5.2) at -20°C for 4 hours. The precipitated DNA was recovered by centrifugation at 13 000 x g for 15 minutes and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA pH 8.0; 0.1% SDS) and then treated with 5.0 μg/ml DNase-free RNAse for 2 hours at 37°C. The DNA was re-extracted once as described above, precipitated and dissolved in 30 μl of TE buffer. Approximately 40 μg of DNA was analysed on 1.5% agarose gels (Promega Corp., Madison, USA) which were run at 60 V for 3-6 hours.

2.2.2.6. Modulation of extracellular calcium

HL-60 cells were cultured in the presence of minocycline (0 and 20 μM) and varying concentrations (0-500 μM) of the extracellular calcium chelator EGTA. Cell density and viability were determined at 24 hour intervals for four days by using Model Zf Coulter counter and trypan-blue dye exclusion method, respectively. DNA was extracted as outlined above. HL-60 cells were also cultured in calcium free RPMI-1640 and then treated with 20 μM minocycline and varying concentrations of calcium nitrite (Ca_2NO_3) and cell number and viability determined.

2.2.2.7. Inhibition of protein synthesis

Cycloheximide (CHX) was prepared in DMSO and diluted in RPMI-1640 medium before being filter-sterilized. HL-60 cells were treated with either 20 μM MIN or 20 mM Li^+ in the presence of varying concentrations (0, 10, 20, 50 μg/ml) of CHX for 24 hours at 37°C. After 24 hours, the cells were harvested and DNA recovered as outlined above. Cell viability was determined by using the trypan-blue exclusion method.

2.2.2.8. Treatment of other cell lines with lithium and minocycline

The 3T3-L1 cell line was cultured in DMEM supplemented with 10% heat-

inactivated fetal calf serum at 37°C in humidified atmosphere containing 5% CO_2. The cells were allowed to reach confluence before treatment with either lithium (10 and 20 mM) or minocycline (20 and 50 μM). The cells were then incubated for 24 hours and the DNA isolated as described above.

The K562 cell line was maintained in suspension cultures at a density of between 1 and 6 X 10^5 cells/ml in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum at 37°C in 5% CO_2 humidified atmosphere. Experimental cultures were seeded at 3 X 10^5 cells/ml and then treated with minocycline (10-50 μM) for 24 hours before analysis of DNA fragmentation as described above. Cell viability was also analysed by the trypan-blue dye exclusion method. The LLC-PK1 cells were cultured in RPMI-1640 supplemented with 5% heat-inactivated fetal calf serum at 37°C in 5% CO_2/humidified atmosphere. The cells were allowed to reach confluence and then treated with either lithium (20 mM) or minocycline (20 and 50 μM) for 24 hours. DNA fragmentation assays were performed after 24 hours as described above.

2.2.2.9. Activation of protein kinase C

12-*O*-tetradecanoylphorbol-13-acetate (TPA) was prepared in ethanol and diluted in RPM-1640 medium before being filter-sterilized through a 0.2 μm filter (Sartorius, Germany). HL-60 cells were treated with 30 nM TPA for 24 hours and then treated with either minocycline or lithium. DNA fragmentation analysis was performed as described above.

2.2.3. DNA Methodology

2.2.3.1. Recombinant DNA Methods

2.2.3.1.1. Restriction enzyme digests and end filling

pOPRSVI/MCS operator vector (5647 bp) (Stratagene, La Jolla, CA) was linearised at the MCS region by treatment with *Sma*I restriction enzyme. Briefly, 5 μg (5 μl) of the vector was mixed with 5 μl of *Sma*I 10X buffer, 1 μl *Sma*I and sterile distilled H_2O (39 μl) to make a total volume of 50 μl. The mixture was then

incubated at 37°C for 60 minutes after which 5 μl 0.5 M EDTA (pH 8.0) was added to stop the reaction. The efficiency of the linearization reaction was confirmed by electrophoresing a 5 μl aliquot of the digest alongside uncut vector control on a 1% agarose gel at 60 V for 2 hours.

c-jun cDNA fragment (2.6 kb) was excised from the *Eco*RI sites of pGEM-2 recombinant plasmid (ATCC, Rockville, USA), and separated by electrophoresis on a 1% agarose gel. The 2.6 kb band was purified from the agarose gel using agarose gel extraction kit according to the supplier's recommendations (Boehringer-Mannheim, Germany).

The overhangs were subsequently filled in using Klenow DNA polymerase in a reaction mixture containing: 10 μl of an appropriate 10 X buffer, 4 μl of dNTP mixture (2.5 mM each of dATP, dTTP, dCTP and dGTP), the DNA fragment in TE buffer and sterile dH_2O to a total volume of 98μl, and 2 μl of Klenow polymerase. The reaction mixture was incubated at 37°C for 60 minutes. Klenow polymerase was inactivated by heating the mixture to 70°C for 5 minutes, followed by 1X extraction with phenol:chloroform (1:1) mixture. The DNA was precipitated with 2.5 volumes of absolute ethanol and 0.1 volume of 3M sodium acetate (pH 5.2) at -20°C for 4 hours. The DNA was recovered by centrifugation at 14 000 rpm for 10 minutes and then washed once in 75% ethanol, then air-dried and resuspended in a small volume of TE buffer.

2.2.3.1.2. Alkaline phosphatase treatment of vector DNA and Ligation

The *Sma*I linearized operator vector was dephosphorylated by treatment with alkaline phosphatase as recommended by the enzyme supplier (Boehringer-Mannheim, Germany). Briefly, 20 μg of the vector was incubated at 37°C for 60 minutes in a total volume of 100 μl containing; 10 μl of 10 X alkaline phosphatase buffer, 2 μl of alkaline phosphatase and sterile distilled H_2O to make a final volume of 100 μl. After 1 hour incubation at 37°C the reaction was stopped by heating the mixture to 70°C for 10 minutes, followed by 2 X extractions with equal volumes

of phenol:chloroform mixture. The DNA was precipitated from the aqueous phase with 2.5 volumes of absolute ethanol and 0.11 volume of 3 M sodium acetate, pH 5.2 at -20°C. The precipitated DNA was recovered by centrifugation at 13 000 rpm for 10 minutes, washed with 75% ethanol, air-dried at room temperature and resuspended in 50 μl of sterile TE buffer.

The purified *c-jun* cDNA fragment (2.6 kb) was ligated into the MCS region of pOPRSVI/MCS operator vector as follows: 1 μg of *Sma*I digested pOPRSVI/MCS was mixed with 3μg of *c-jun* cDNA fragment in an eppendorff microfuge tube to achieve the ratio of *c-jun*/vector of 3:1. Ligations were carried out at 4°C overnight in a mixture containing 2 μl of T4 ligase 10 X buffer, 1 μl of T4 DNA ligase and distilled water to a total volume of 20 μl.

2.2.3.1.3. Transformation of *E. coli.*

Competent *E. coli* cells were prepared as follows: *E. coli* strain XL-Blue MR (Stratagene, Cambridge, UK) cells were cultured overnight in 10 ml of LB medium at 37°C with vigorous shaking. Approximately 1 ml of cells from an overnight culture was inoculated into 10 ml of LB medium and the culture grown at 37°C with vigorous shaking to an A_{600} of 0.6. The cells were harvested by centrifugation at 4000 rpm at 4°C and then washed in 10 ml of ice-cold 0.1M magnesium chloride ($MgCl_2$). The pellet was then resuspended in 2.5 ml of 0.1M $CaCl_2$ and incubated on ice for at least 1 hour. Transformation of the competent cells was performed as follows: The ligation mixture (200 ng DNA) was added to competent cells and the mixture incubated for 1 hour on ice. The cells were heat-shocked at 42°C for 1/2 minute, and cooled briefly on ice and then 1 ml of prewarmed LB medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, pH 7.2) added and the mixture incubated at 37°C for at least 45 minutes. The cells were plated on LB agar plates (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, 15 g/L bacto-agar) containing 100 μg/ml ampicillin and incubated overnight at 37°C.

2.2.3.1.4. Screening of transformants

The colonies were screened for recombinant plasmid by the mini-plasmid preparation protocol as described in Sambrook *et al.* (1989). A single colony was inoculated in 10 ml of LB broth containing 100 μg/ml ampicillin and incubated overnight at 37°C. Plasmid DNA was isolated as follows: Cells were harvested by centrifuging at 4000 rpm for 10 minutes and the pellet was resuspended in 100 μl of solution I (50 mM Glucose; 25 mM Tris.Cl, pH 8.0; 10 mM EDTA, pH 8.0); 200 μl of freshly prepared solution II (0.2 N NaOH; 1% SDS) was added and the mixture mixed by inversion several times; then 150 μl of solution III (60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml distilled water) was added, followed by vortexing of the mixture to ensure proper mixing. DNA was separated from the proteins and cell debris by centrifugation at 14 000 rpm for 10 minutes at 4°C, and the supernatant was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous layer was mixed with an equal volume of isopropanol and DNA precipitation carried out at room temperature for 30 minutes. Plasmid DNA was recovered by centrifugation at 14 000 rpm and the pelleted DNA was washed once with chilled 75% ethanol. The pellet was air-dried and resuspended in 50 μl TE buffer. The DNA was analysed by restriction enzyme digestion followed by separation on 1% agarose gels in TAE (2 mM EDTA, pH 8.0; 40 mM Tris; 1.142 ml acetic acid) buffer.

2.2.3.1.5. Large scale purification of Plasmid DNA

Large scale purification of plasmid DNA was carried out by CsCl gradient centrifugation method as described in Sambrook *et al.* (1989). *E. coli* cells carrying the desired plasmid DNA were cultured overnight in 500 ml of LB broth at 37°C with vigorous shaking at 200 rpm. The cells were recovered by centrifugation at 7000 rpm for 15 minutes and then resuspended in 20 ml of ice-cold solution I (50 mM Glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0). A few mg of lysozyme (Boehringer-Mannheim, Germany) was added to the cell suspension, then incubated at room temperature for 10 minutes. 50 ml of solution II (0.2 N NaOH; 1% SDS) was added and the lysate mixed thoroughly by inversion. 50 ml of

solution III (60 ml of 5M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml sterile distilled H_2O) was then added and the mixture was shaken vigorously and left on ice for a further 15 minutes. The lysate was centrifuged at 10 000 rpm for 30 minutes to remove the proteins, chromosomal DNA and cell debris. The supernatant containing plasmid DNA was clarified by filtration through four layers of cheese-cloth and then mixed with an equal volume (~100 ml) of isopropanol at room temperature. DNA was recovered by centrifugation at 14 000 rpm for 30 minutes at 4°C. The DNA pellet was dried by sitting the tube in an inverted position on an adsorbent surface for 15 minutes, and then resuspended in 10 ml of sterile TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0). Exactly 11 g of caesium chloride, 400 µl ethidium bromide (10 mg/ml) were added to the DNA and the mixture transferred into quickseal ultracentrifuge tubes (Beckman Instruments Inc., Fullerton, CA). The tubes were centrifuged at 50 000 rpm in a VTi65 rotor (Beckman Instruments Inc., Fullerton, CA) for 20 hours at 25°C. The supercoiled plasmid DNA band was recovered by using a needle and a 5 ml syringe. Ethidium bromide was extracted several times until the pink colour disappeared using water-saturated butanol. To the remaining clean aqueous phase 3 X volumes of sterile distilled water and 2 X volumes of absolute ethanol were added and the mixture incubated at 4°C for 30 minutes. Plasmid DNA was recovered by centrifugation of the mixture at 20 000 rpm in a JA 20 rotor (Beckman Instruments) for 45 minutes, then dried before resuspension in 200 µl of TE buffer. Plasmid DNA was then analysed on a 1% agarose gel in TAE buffer.

2.2.3.2. Transfection protocols

2.2.3.2.1. Transfection protocol for 3T3-L1 cells

Transfection of 3T3-L1 preadipocytes was performed as follows: 20 µg of supercoiled DNA (pOPRSVI/MCS vector and pJMCS) was precipitated in 2.5 volumes of absolute ethanol and 0.1 volume of 3M sodium acetate, pH 5.2 at -20°C for 30 minutes. The DNA was recovered by centrifugation at 13 000 rpm for 10 minutes and then resuspended in 20 µl of sterile TE buffer. The DNA was mixed with the cells at a density of 8 X 10^6 cells/ml in FCS-free DME medium. The

cells were incubated on ice for 10 minutes and then electroporated at 250 V and 960 μF using a Bio-Rad Pulser (Bio-Rad laboratories, Hertfordshire, UK) and held on ice for a further 10 minutes. The cells were diluted in 10% FCS-containing DME medium and seeded into twelve well culture plates at subconfluent density. The cells were allowed to recover for 24 hours at 37°C incubator prior to use in experiments.

Transient transfection of 3T3-L1 cells using the calcium phosphate precipitation method was performed as follows: 3T3-L1 cells were allowed to grow to about 80% confluency in standard 12 well cell culture plates. 20 μg of DNA was precipitated and recovered as described above. DNA was resuspended in 450 μl of sterile 0.1 X TE buffer and gently mixed with 50 μl of 2.5 M $CaCl_2$, while in another tube 500 μl of 2 X HBS (280 mM NaCl; 1.5 mM Na_2HPO_4, pH 7.12) was added. DNA in TE containing $CaCl_2$ was added in dropwise into the tube containing 2 X HBS, pH 7.12, and the transfection mixture left at room temperature for 20 minutes to allow the precipitate to form completely. 200 μl of DNA/$CaCl_2$ per well was added to the cells. The cells were incubated for 24 hours at 37°C to allow full recovery, after which they were shocked by addition of 1 ml of 15% glycerol in PBS for 30 seconds. Fresh DME medium containing 10% FCS was added and the cells incubated at 37°C as described above. Treatment was done immediately.

2.2.3.2.2. Transfection of HL-60 and Raji cells

HL-60 and Raji cells were allowed to grow to a density of 4 X 10^5 cells/ml in RPMI-1640 medium supplemented with 10% FCS and 6 ml aliqouts were split into 10 ml culture flasks. 5 μg of DNA (pOPRSVI/MCS operator vector and pJMCS) was resuspended in 20 mM Hepes buffer, pH 7.4, and in a separate tube 30 μl of DOTAP lipofection reagent was mixed with Hepes buffer to a final volume of 100 μl. The nucleic acid solution (50 μl) was transferred into a reaction tube already containing DOTAP reagent in Hepes buffer (100 μl). The transfection mixture was carefully mixed by gentle pipetting. The mixture was incubated for 10-15 minutes at room temperature. 150 μl of the transfection mixture was added directly to the

cells (6ml) and incubated at 37°C for 6 hours before treatment with lithium. The efficiency of transfection was determined using the CAT ELISA kit according to the supplier's (Boehringer-Mannheim, Germany) recommendations.

2.2.4. RNA Methodology

2.2.4.1. Total RNA isolation

Minocycline or lithium treated cells were harvested in 50 ml aliqouts at the specified time points by centrifugation at 1200 rpm for 10 minutes at 4^0C. RNA was isolated by the method of Chomczynski and Sacchi (1987) as outlined in the Promega Protocols Manual (1997). Briefly, the pellet was washed once in ice-cold sterile PBS, pH 7.4 and lysed in 6 ml of denaturing solution (6 M Guanidium chloride, pH 6.4, containing 50 mM Tris-HCl, 20 mM EDTA). The mixture was homogenised and 2 M sodium acetate (600 μl) pH 4.0 was added and then mixed by inversion. Then 6 ml of phenol:chloroform:isoamyml alcohol (25:24:1) was added and mixed thoroughly without vortexing. The mixture was allowed to stand on ice for 15 minutes and then centrifuged at 8 000 rpm for 30 minutes. The extractions with phenol:chloroform:Isoamylalcohol (25:24:1) were repeated twice and the upper aqueous layer was carefully aspirated into a clean tube and the RNA precipitated with 6 ml of isopropanol at -70^0C for 12 hours. Total RNA was recovered by centrifugation at 14 000 rpm for 15 minutes at 4°C. The RNA pellet was washed twice with 75% ethanol in DEPC-treated water. The pellet was then dried briefly under a vacuum and then resuspended in 50 μl of DEPC-treated water. Contaminating DNA was removed using RNase-free DNase I (Boehringer-Mannheim, Germany) by mixing RNA preparations with 10 μl of 10 X buffer, 2 μl of RNAISIN (RNase inhibitor) (Boehringer-Mannheim, Germany), 2 μl of RNase-free DNase I and sterile DEPC-treated water in a total volume of 100 μl and the mixture incubated at 37°C for 1 hour.

2.2.4.2. Quantitation of RNA and assessment of quality

RNA was quantitated by reading the absorbance at 260 nm with an OD of 1 being considered to be equivalent to 40 μg/ml of RNA (Sambrook *et al.*, 1989).

Contamination by protein was assessed by determining the A_{260}/A_{280} ratio. Samples with A_{260}/A_{280} ratios of greater than 1.7 and A_{260}/A_{230} ratio of greater than 1.5 were considered to be essentially free of protein and guanidine, respectively. However, contaminated samples were subjected to extra extractions with phenol:chloroform mixture.

2.2.4.3. Formaldehyde denaturing gel

The integrity of RNA was assessed by running the RNA on 1.2% agarose gels containing formaldehyde. Briefly, 1.0 g of molecular grade agarose (Promega) was melted in 87ml of DEPC-treated water and cooled to 60°C and 5.1 ml of 35-37% formaldehyde added in the fume hood. 10 ml of 10X MOPS buffer was added to the mixture before pouring in a horizontal gel casting apparatus (Hoeffer Scientific Instruments, San Francisco, USA). Ten μg of RNA samples was prepared for loading by mixing with formaldehyde, formamide, ethidium bromide and gel loading buffer (Sambrook *et al.,* 1989), and then denatured in a 60°C waterbath for 10 minutes. Electrophoresis was performed for 4 hours at 60 V, with frequent mixing of the tank buffer.

2.2.4.4. cDNA synthesis and PCR

cDNA synthesis was performed using MuLV-RT kit according to the manufacturer's instruction (Boehringer-Mannheim, Germany), with minor adjustments. Briefly, 10 μg of total RNA was mixed with the following reagents in the same order and then incubated at 37°C: 4 μl oligo dT primer, 8 μl of 5X MuLV RT buffer (to remove secondary structures in RNA the RNA/oligo dT mixture was incubated at 70°C for 5 minutes and then cooled to room temperature prior to addition of the other reagents), 2 μl of RNase inhibitor (RNAISIN), redistilled H_2O to make the volume to 39 μl, 1 μl of MuLV reverse transcriptase. The mixture was incubated at 37°C for 60 minutes.

Exactly 5 μl of the synthesised cDNA was used in a PCR reaction. A typical PCR reaction mixture consisted of 8 μl of dNTP mixture (2.5 mM each of dGTP, dTTP,

dCTP and dATP)), 10 μl of 10X PCR buffer, 5 μl of template, 200 pmoles of specific primers, 0.25 μl (0,25 units) of *Taq* DNA polymerase and water to make the volume up to 100 μl. The primers were synthesised using Beckman Oligo Synthesiser 1000 as recommended by the manufacturer (Beckman Instruments). The primers were as follows: ***β-actin* sense**: 5'TTC TAC AAT GAG CTG CGT GTG GCT 3' and **antisense**: 5'GCT TCT CCT TAA TGT CAC GCA CGA 3' (373bp product) (Sugimoto *et al.*,1994); ***c-jun* sense:** 5'ACT CAG TTC TTG TGC CCC AA 3' and **antisense:** 5'CGC ACG AAG CCT TCG GCG 3' (64-bp product) (Furukawa *et al.*, 1997); ***Bcl-2* sense**: 5' TGC ACC TGA CGC CCT TCA C 3' and **antisense**: 5'AGA CAG CCA GGA GAA ATC AAA CAG 3' (284-bp product); ***c-myc* sense**: 5'TCC AGT TGT ACC TGC AGG ATC TGA 3' and **antisense:** 5'CCT CCA GCA GAA GGT GAT CCA GAC T 3' (338-bp product) (Sugimoto *et al.*, 1994); A typical PCR reaction involved a 2 minutes denaturation at 94°C, 2 minute annealing at 60°C, 2 minutes exension at 72°C, followed by 30 cycles of 45 seconds at 94°C, 1 minute at 60°C and 2 minutes at 72°C with a final extension of 10 minutes at 72°C. The annealing temperature was adjusted according to the T_m of the individual primers.

2.2.5. Cell analysis methodology

2.2.5.1. Electron microscopy

The cells were centrifuged at 1200 rpm for 5 minutes, rinsed briefly with PBS, and then fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0 until needed. After several rinses in the same buffer the cells were postfixed for 1 hour in 0.1 M cacodylate buffer containing 1% osmium tetroxide. The samples were washed 3 times with buffer, prestained in uranyl acetate and dehydrated in graded series of ethanol (up to 100%). The samples were treated with propylene oxide with increasing amounts of resin. The samples in resin were infiltrated overnight in a rotator and thereafter molded at 60 ^{0}C for 24 hours. The molds were cooled, and sectioned using an ultramicrotome, fixed on grids and stained with lead acetate and uranyl acetate. The samples were then viewed in a Phillips 301 Transmission Electron Microscope.

2.2.5.2. Cell cycle analysis

HL-60 cells were synchronised at the G_1/S interphase by double thymidine block. The cells were cultured in the presence of 5 mM thymidine for 16 hours, harvested and then cultured in the absence of thymidine for a further 8 hours in RPMI-1640/10% FCS. The cultures were once again blocked with 5 mM thymidine for 16 hours, and then the thymidine was removed and the cells resuspended in RPMI-1640/10% FCS. The synchronised cells were treated with increasing minocycline concentrations (0, 10, 20, 50 μM) for 12, 24 and 48 hours. At the specified time points cells were harvested by centrifugation at 1 200 rpm for 10 minutes followed by two washes in sterile PBS, pH 7.4. The cells were fixed in 1ml of 15% methanol and stained in 1ml of 50 μg/ml propidium iodide for 30 minutes. Stained cells were then analysed for cell cycle distribution on a Coulter FacScan (Coulter Corporation Inc., Luton, UK).

2.2.5.3. Analysis of 3T3-L1 cells adipose conversion

3T3-L1 preadipocytes were transfected with pOPRSVI/MCS vector control and pJMCS plasmids with pCMVLacI repressor vector plamid using either electroporation at 960 μF and 250 V or $CaPO_4$ coprecpitation. The cells were then induced to differentiate with the induction medium (10 μg/ml Insulin, 500 μM IBMX, 250 μM DEX) and treated with 0 mM or 5 mM IPTG (to induce the RSVI promoter) in the presence or absence of 20 mM Li^+. The cells were incubated for 48 hours at 37°C, the medium replaced with fresh 10% FCS-containing DME medium, and incubated for a further 96 hours. The cells were washed three times with PBS, then fixed in 50% methanol for 30 minutes, and stained in filtered oil Red O for a further 2 hours. Excess dye was removed by several washes with running tap water. The dye was extracted with isopropanol and the intensity of the colour measured by reading the absorbance at 595 nm. The control uniduced cells were considered 100% undifferentiated.

CHAPTER 3

INDUCTION OF APOPTOSIS BY MINOCYCLINE AND LITHIUM

3.1. Effect of minocycline on growth of mammalian cells *in vitro*

Minocycline is widely used in clinical practice for the treatment of bacterial infections. In addition minocycline has been referred to as "lithium mimetic" because of its ability to inhibit ADH-stimulated accumulation of cAMP in rats (Dousa and Wilson, 1974). The following studies were carried out in order to determine whether minocycline could mimic the effects of lithium on cells in culture.

3.1.1. Effects of minocycline on HL-60 cells

HL-60 cells were cultured in the presence of 0, 1, 5, 10, 20 and 50μM MIN and the cell density determined daily for 96 hours as described in materials and methods. Minocycline induced a time and dose dependent decrease of HL-60 leukemia cell number (Fig. 3.1.1), with 50 μM decreasing the density to less than half of the control untreated cells. Concentrations below 20 μM MIN induced significant decrease in cell numebr as indicated by cell counts. The decrease in cell numebr was associated with loss of cell viability as determined by trypan blue uptake. After 72 hours 50 μM Minocycline induced a drastic loss in cell viability, reaching 30% of control untreated cells (Fig. 3.1.2).

3.1.2. DNA fragmentation

To determine whether the loss in HL-60 cell viability could be attributed to apoptosis, cellular DNA was analysed for the characteristic DNA laddering associated with this mode of cell death (Wyllie, 1980; Wyllie, 1987).

HL-60 and K562 cells were cultured in the presence of 0, 10, 20, and 50 μM minocycline for 24 hours and the DNA isolated and analysed for fragmentation as

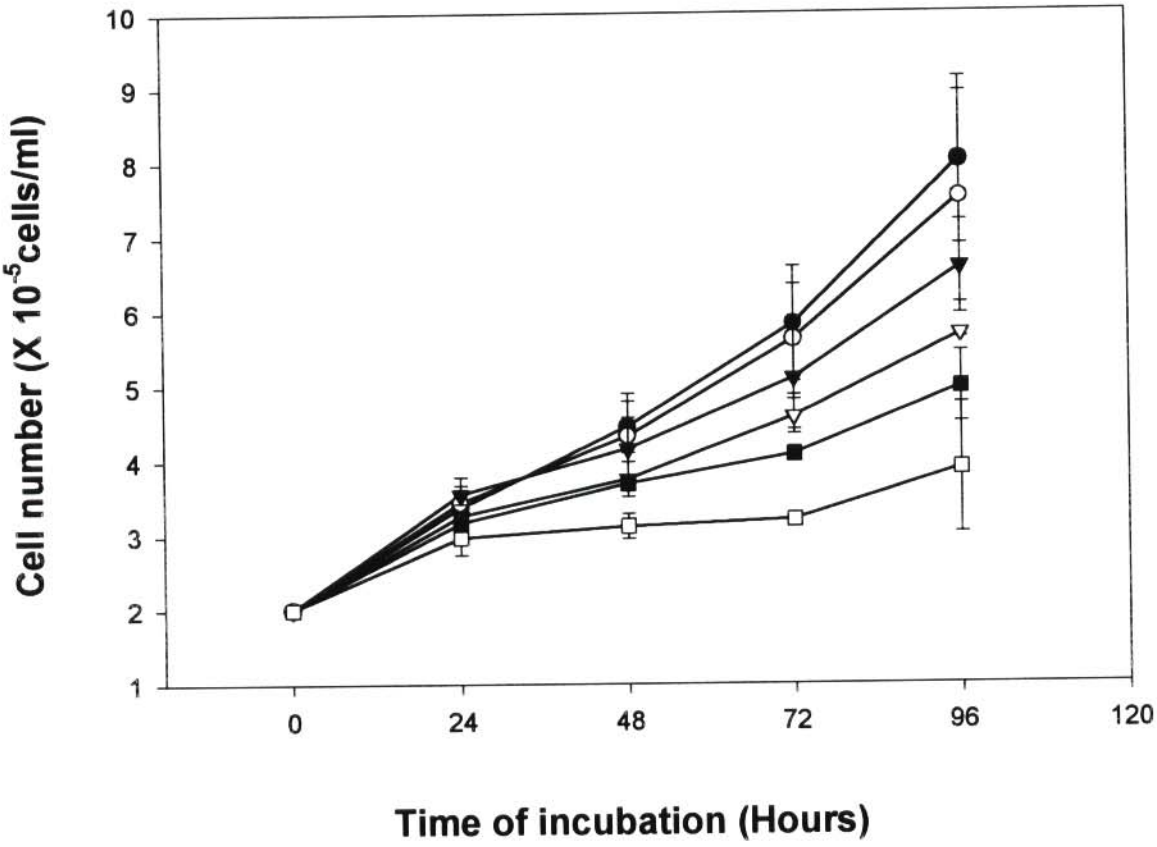

Figure 3.1.1: Cell growth curves of HL-60 cells after treatment with minocycline. HL-60 cells were cultured in the presence of MIN at 0 (●), 1 (○), 5(▼), 10 (▽), 20 (■) and 50 μM (□) and the cell number determined. The results are the mean of 3 independent duplicate experiments ± SEM.

described under meterials and methods. It was demonstrated that MIN induced fragmentation of DNA in HL-60 cells in a concentration-dependent manner (Fig. 3.1.3), with 50 μM MIN resulting in the most laddering within 24 hours of incubation. Surprisingly, all the concentrations of MIN used in the study failed to induce any detectable DNA fragmentation in K562 cells (Fig. 3.1.3), and this resistance to MIN effects was also observed with cell viability assays performed 24 hours post treatment (Table 3.1.1). K562 cells treated with concentrations of up to 50 μM MIN maintained a viability of above 90% while that of HL-60 cells declined to only ~75% after 24 hours in the presence of these concentrations of MIN.

The morphological changes characteristic of apoptosis were observed in HL-60 cells treated with 20 mM Li^+ (Fig. 3.1.4). The condensation of chromatin material into half-moon structures was detected within 24 hours of incubation in the presence of 20 mM Li^+ whereas 20 μM MIN induced the appearance of membrane encapsulated apoptotic bodies, the shrinkage of the cells and the blebbing of the cell membrane (Fig. 3.1.4).

3.1.3. Induction of apoptosis in 3T3-L1 fibroblasts and LLC-PK cells by lithium and minocycline

3T3-L1 and LLC-PK cells were grown in DME medium supplemented with 10% FCS and then treated with minocycline at 0, 10, 20 and 50 μM and lithium at 10 mM and 20 mM for 24 hours. The cells were scrapped off the tissue culture plates using a rubber policeman, and DNA isolated as described above. Apoptosis as determined by DNA laddering was not detected in either 3T3-L1 (Fig. 3.1.5.A) or LLC-PK cells (Fig. 3.1.5.B).

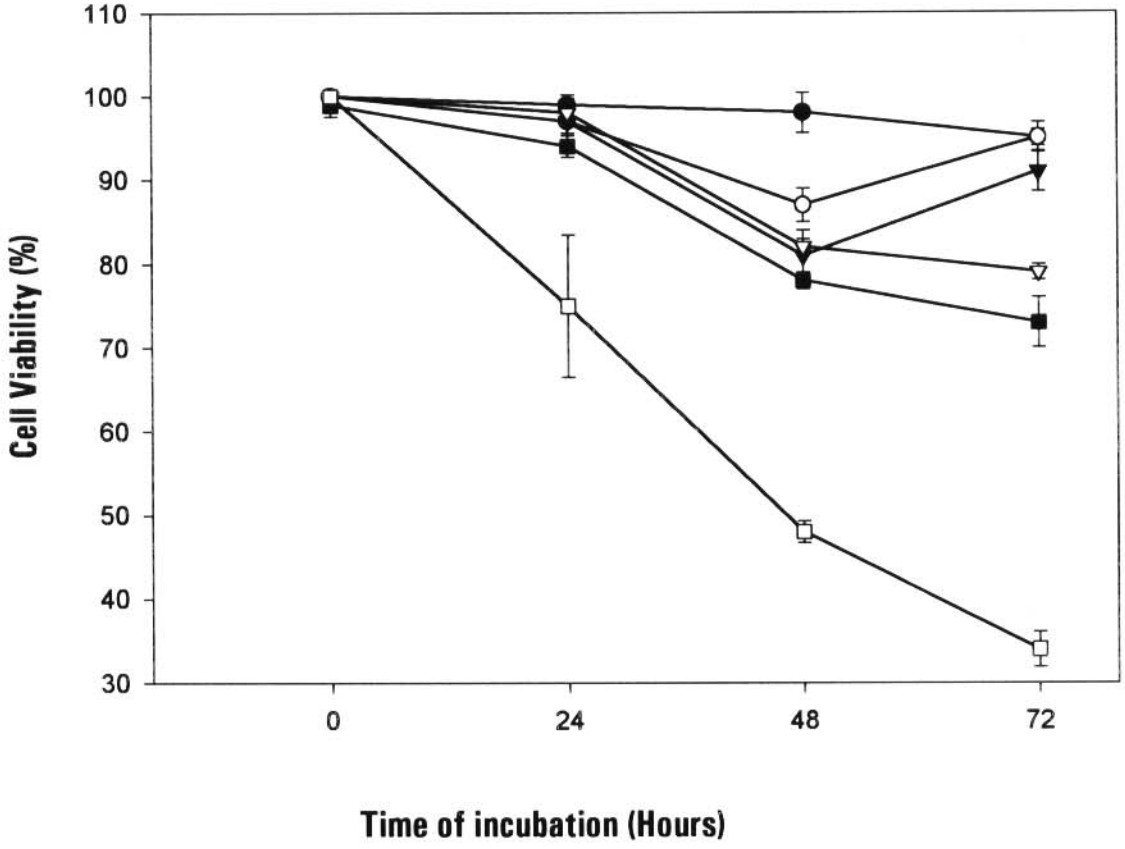

Figure 3.1.2: Cell viability of HL-60 cells treated with MIN. HL-60 cells were treated with increasing concentrations of MIN (μM); 0 (●), 1 (○), 5 (▼), 10 (▽), 20 (■) and 50 (□). Cell viability was determined at 24 hour intervals by trypan blue exclusion method. The results represent the mean of 3 independent duplicate experiments ± SEM.

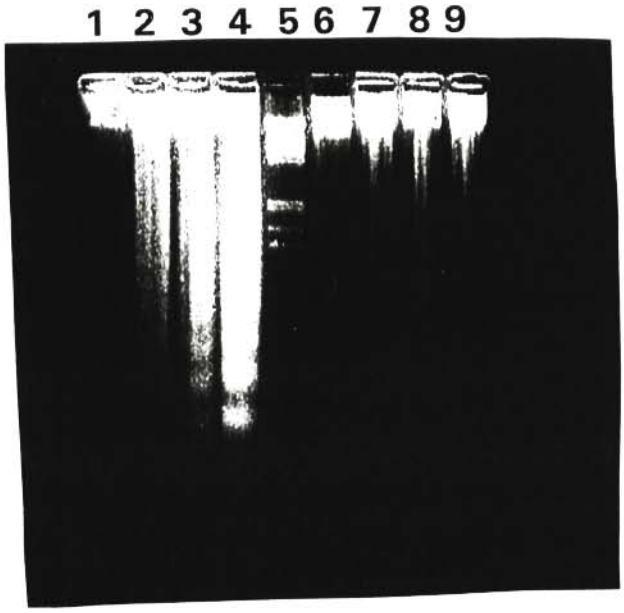

Figure 3.1.3: DNA fragmentation in HL-60 and K562 cells treated with MIN. DNA was isolated from MIN treated cells, separated on a 2% agarose gel. Lanes 1-4) control HL-60 cells; 10 μM; 20 μM; 50 μM; respectively, lane 5, λDNA/*Hind* III markers; lanes 6-9) control K562 cells; 10 μM; 20 μM; 50 μM. Cells were treated with MIN for 24 hours prior isolation and analysis of DNA.

Table 3.1.1: Cell viability of HL-60 and K562 cells treated with minocycline after 24 hours of incubation.

Condition	Cell Viability (%)	
MIN (μM)	HL-60 cells	K562 cells
0.00	98.0 ± 0.85	94.0 ± 1.55
10.00	92.0 ± 0.25	91.0 ± 0.50
20.00	89.0 ± 1.50	89.0 ± 0.50
50.00	75.0 ± 2.00	91.0 ± 1.00

3.1.4. Modulation of MIN- and Li^+-induced apoptosis in HL-60 cells

3.1.4.1. The Role of calcium

Regulation of apoptosis by intracellular second messengers is poorly understood although a pivotal role for known signal transduction pathways such as, cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), cyclic AMP, pH and protein kinase C have been suggested (McKonkey *et al.*, 1994). In many cells an increase in $[Ca^{2+}]_i$ induced by calcium ionophores can stimulate apoptosis, and a calcium releasing agent thapsigargin has also been shown to promote apoptotic cell death. Furthermore, cell-permeating Ca^{2+} chelating agents have been shown to delay apoptosis (Jiang *et al.*, 1994). The apoptotic inhibitory role of Bcl-2 oncoprotein appears to involve changes of Ca^{2+} compartmentalization (Lam *et al.*, 1995). However, the role of Ca^{2+} seems to differ between cellular systems and an increase in $[Ca^{2+}]_i$ has been shown to prevent rather than induce apoptosis and DNA fragmentation (Edwards *et al.*, 1991).

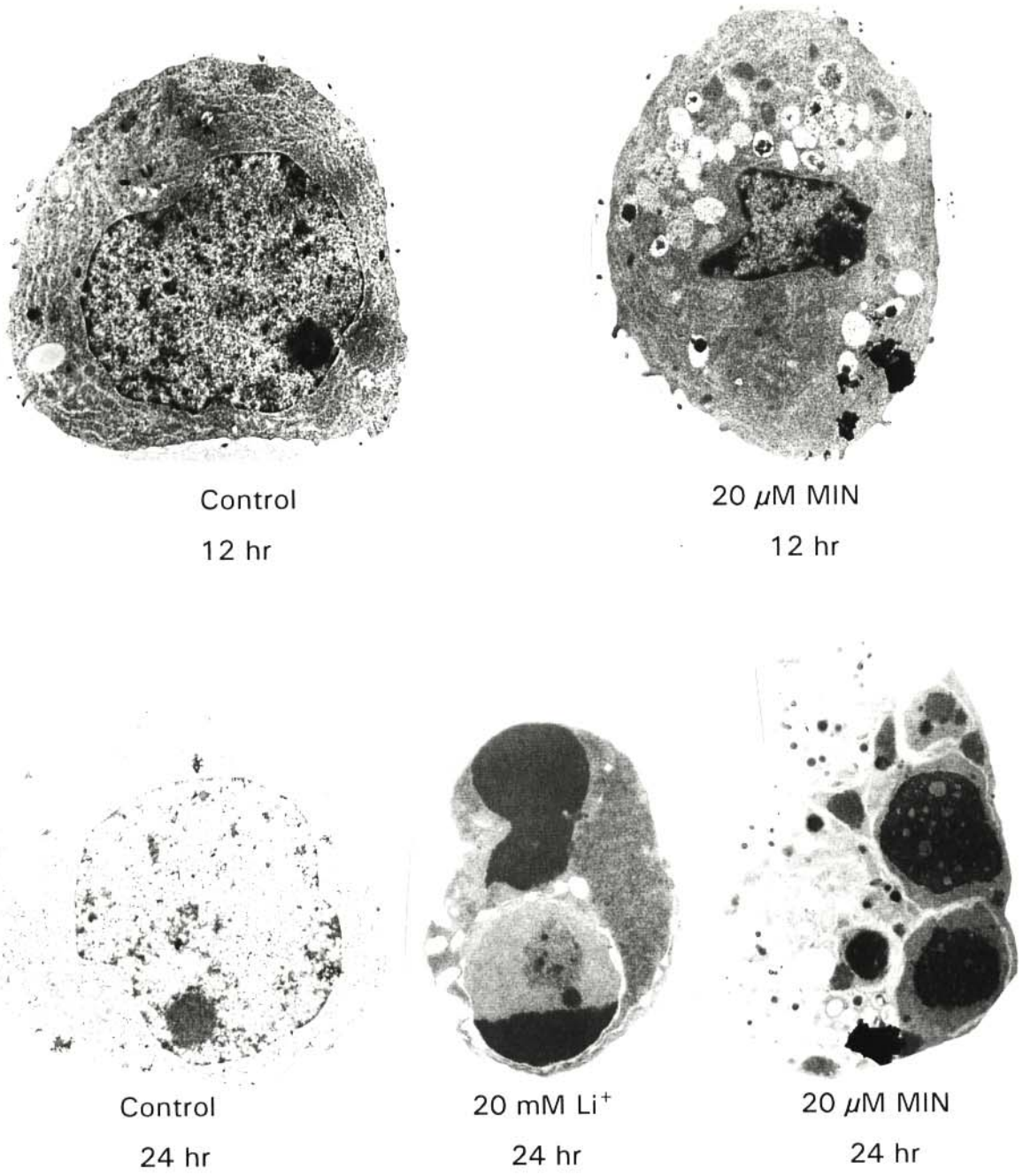

Figure 3.1.4: Scanning electron micrographs of HL-60 cells treated with minocycline and lithium. HL-60 cells were cultured in the presence of 0 and 20 μM MIN and 20 mM Li^+, and aliqouts were analysed for morphological changes using scanning electron microscopy as described under Materials and Methods. The appearence of apoptotic bodies and fragmented nuclear materials are indicative of apoptosis. In lithium-treated cells there is an occurrence of half moon-like structure.

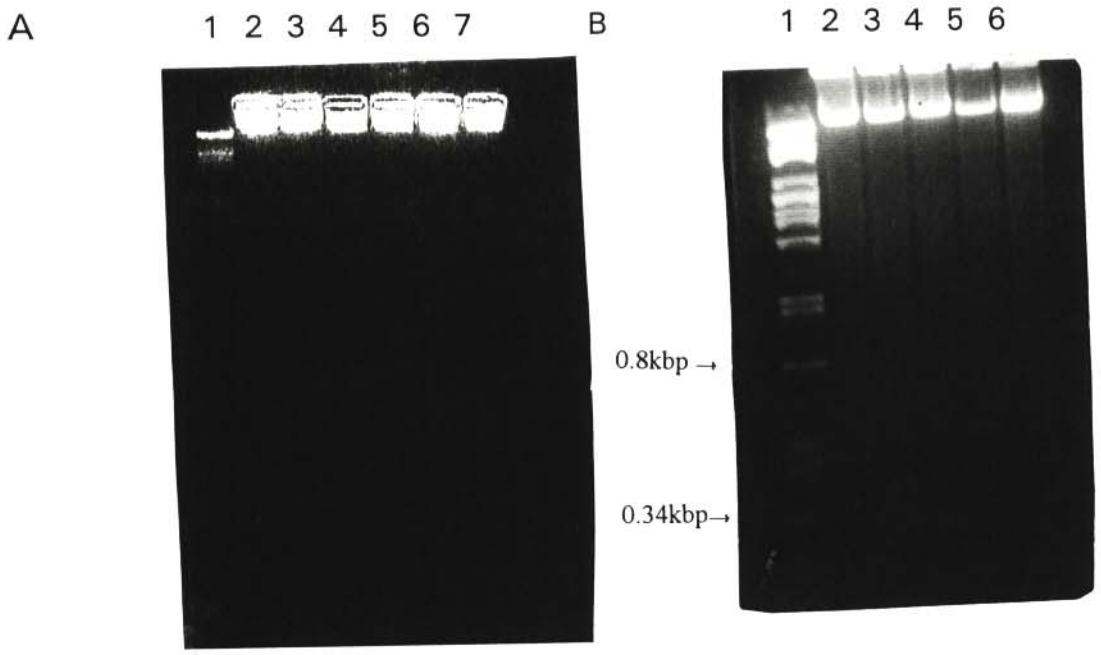

Figure 3.1.5: Agarose gel electrophoresis of DNA isolated from 3T3-L1 (A) and LLC-PK cells (B) cells after 24 hours of incubation. DNA was isolated and analysed on a 2% agarose gel, as described in the previous chapter. (A) Lane 1) *Pst* I/λDNA markers; Lanes 2-7) untreated; 10 μM MIN; 20 μM MIN; 50 μM MIN; 10 mM Li^+; 20 mM Li^+. (B) Lane 1) λ/*Pst* I DNA markers; lanes 2-6) untreated; 10 μM MIN; 20 μM MIN; 10 mM Li^+; 20 mM Li^+, respectively.

EGTA was used in this study to chelate extracellular calcium ions in the medium (RPMI-1640). Minocycline (20 μM) and Li^+ (20 mM) induced apoptosis and EGTA alone failed to induce apoptosis in HL-60 cells at 500 μM (Fig. 3.1.6). However, EGTA appeared to potentiate DNA fragmentation of MIN-treated HL-60 cells.

To investigate the role of extracellular Ca^{2+} in MIN-induced cytotoxicity, HL-60 cells were cultured in the presence of MIN alone or combined with increasing concentrations of EGTA and cell viability was determined. EGTA did not cause a significant reduction in cell viability (Fig. 3.1.7.A). However, increased concentration of EGTA induced a dose dependent reduction of cell viability in 20 μM MIN-treated HL-60 cells. The effects of EGTA on minocycline treated HL-60 cells were concentration dependent.

Treatment of HL-60 cells with 500 μM EGTA alone did not induce decrease in cell number (Fig. 3.1.7B). However, with MIN treated HL-60 cells potentiation of inhibition of cell growth was observed with increasing concentrations of EGTA (up to 500 μM). The effects of EGTA on the growth of MIN-treated HL-60 cells were also time and dose-dependent with increasing concentrations of EGTA causing increased potentiation of the minocycline effects.

HL-60 cells were next cultured in Ca^{2+}-free RPMI-1640 and increasing concentrations of $CaNO_3$ in the presence of 20 μM MIN. The growth of HL-60 cells treated with 20 μM MIN improved with a rise in exogenous Ca^{2+} (Fig. 3.1.8), with 500 μM $CaNO_3$ inducing a 3-fold increase in cell proliferation when compared to cells treated with MIN alone. A similar trend was demonstrated with cell viability assays with cells treated with 20 μM MIN in the presence of 500 μM Ca^{2+} maintaining 75% viability (fig. 3.1.9) as opposed to cells treated with 20 μM MIN without $CaNO_3$ whose viability was ~50% after 72 hours.

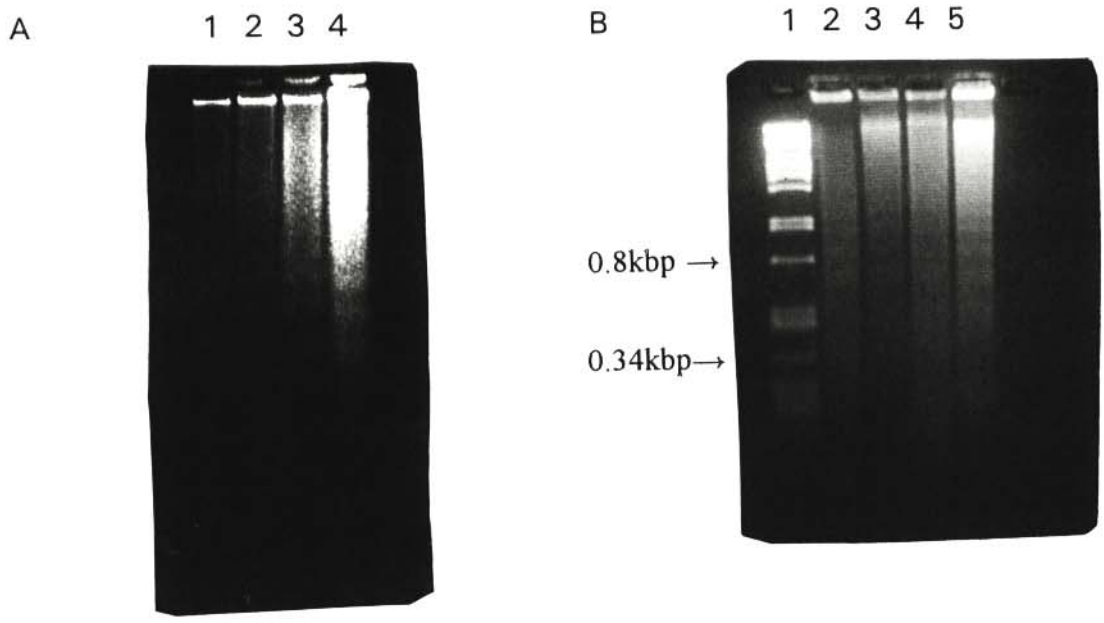

Figure 3.1.6: DNA fragmentation in HL-60 cells treated with either minocycline or lithium in the presence of 0.5 mM EGTA. A) Lane 1, HL-60 cells without any treatment; lane 2, 500 μM EGTA; lane 3, 20 μM MIN; lane 4, 20 μM MIN + 500 μM EGTA. B) Lane 1, λDNA/*Pst*I Marker; lane 2, 20 mM Li^+; Lane 3, 20 mM Li^+ + 500 μ M EGTA; lane 4, 20 μM MIN; lane 5, 20 μM MIN + 500 μM EGTA.

3.1.4.2. Modulation of MIN and Li^+-induced apoptosis by TPA

TPA is an activator of PKC (Nishizuka, 1986) and it has been established that PKC activation can both induce (reviewed in McConkey *et al.,* 1994) and block (McCarthy *et al.,* 1994) apoptosis in a variety of cell lines. This section was aimed at understanding whether PKC activation by TPA would alter the response of HL-60 cells to apoptosis-inducing concentration (20 μM) of minocycline or the documented toxic dose of lithium (20 mM). Since PKC is also Ca^{2+}-dependent, it was also intended to investigate the apoptotic signalling in HL-60 cells when PKC signalling is modulated. HL-60 cells were cultured in the presence of 30 nM TPA for 24 hours and treated with minocycline (20 μM) or lithium (10, 20 mM) for a further 24 hours. The differentiated cells were scrapped from the base of the flask by a sterile cell scrapper, and DNA isolated and analysed for fragmentation as described elsewhere. As expected, 20 μM MIN and 20 mM Li^+ induced DNA fragmentation which was observed in control cells. However, DNA fragmentation was not observed with both substances in the presence of 30 nM TPA (fig. 3.1.10).

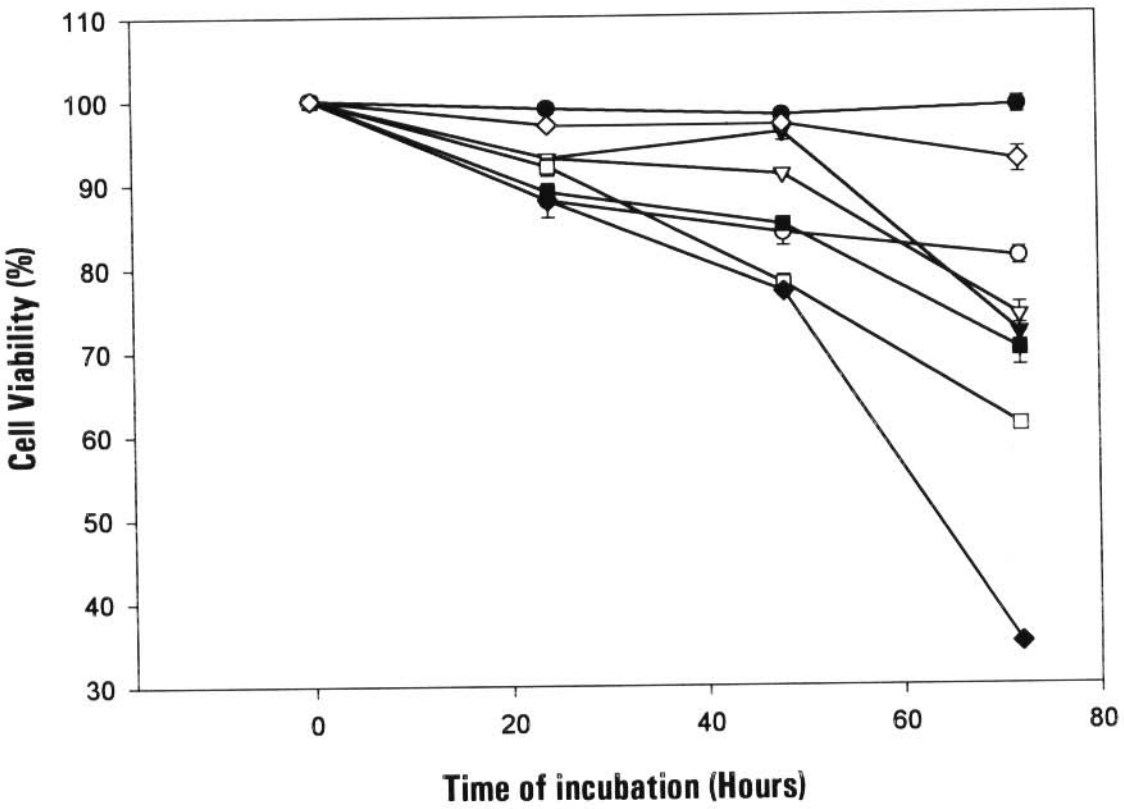

Figure 3.1.7 A: Cell viability of HL-60 cells treated with increasing concentrations of EGTA in the presence 20 μM MIN. Untreated cells (●), 20 μM MIN and EGTA at 0 μM(○), 50μM(▼), 100μM(▽), 200μM(■), 300μM(□), 500μM(♦) and 500 μM EGTA alone (◇). Cell viability was determined daily for 72 hours, as described in materials and methods. The results represent the means of 3 duplicate experiments ± SEM.

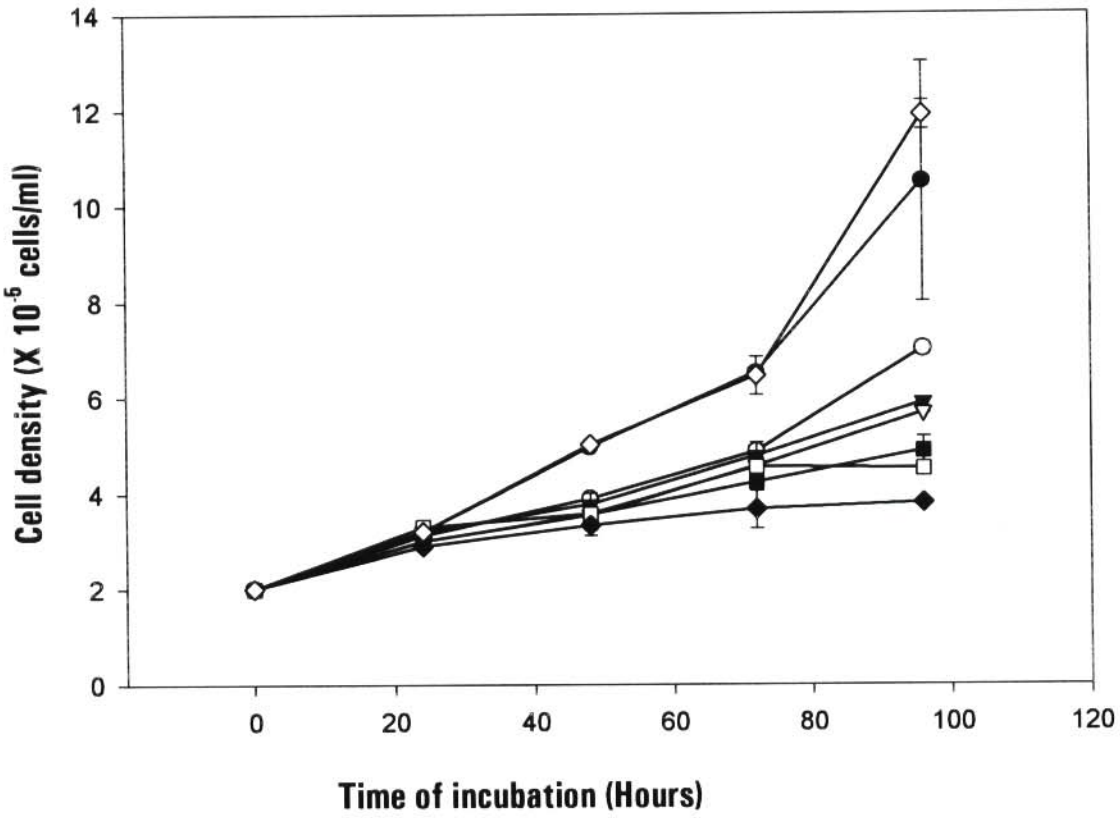

Figure 3.1.7B: Growth of HL-60 cells in the presence of 20 μM MIN alone and in combination with increasing concentrations of EGTA. The cells were cultured for 4 days prior determination of the density. Untreated cells (●), 20 μM MIN(○) or in combination with presence of 50(▾), 100(▽), 200(■), 300(□), 500(♦) μM EGTA and 500 μM EGTA alone(◇). The results represent the mean data of 3 independent duplicate experiments ± SEM.

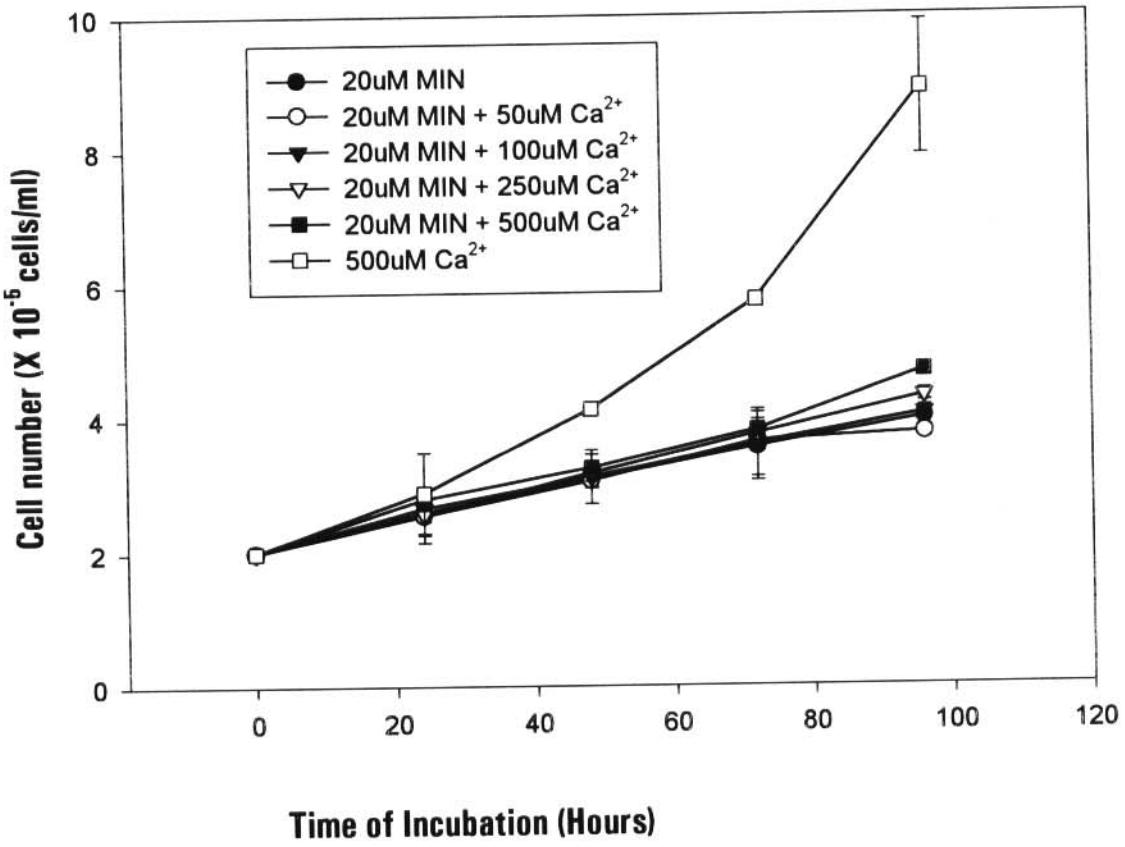

Figure 3.1.8: The effects of exogenous Ca^{2+} on the growth of HL-60 cells treated with 20 μM MIN. Cells were cultured in calcium-free RPMI-1640 and treated with MIN alone or in combination with the indicated concentrations of $CaNO_3$ and cell density determined at 24 hour intervals for 4 days. The results represent the mean data of 3 independent duplicate experiments ± SEM.

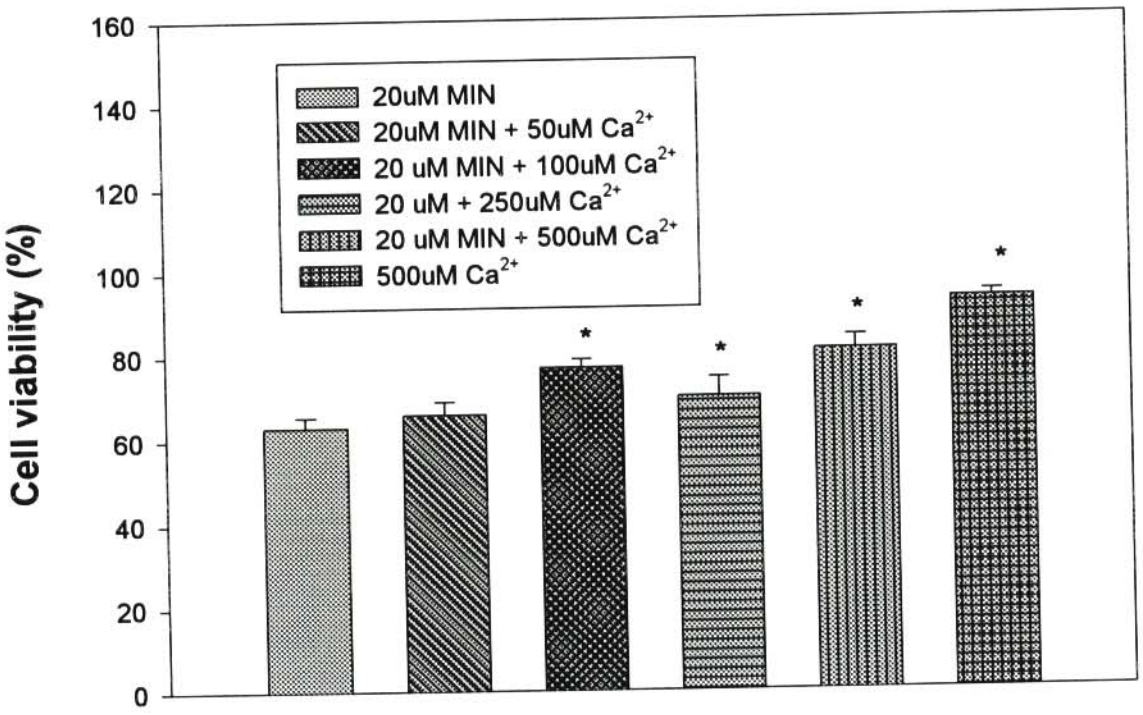

Figure 3.1.9: The effects of exogenous Ca^{2+} ions on the viability of HL-60 cells treated with 20 μM minocycline. Cells were cultured for 72 hours in calcium-free RPMI-1640 medium and treated with 20 μM MIN alone or in combination with 50, 100, 250, 500 μM of $CaNO_3$ after which viability was determined by trypan-blue dye-exclusion method, as described in the previous sections. The results represent mean of 3 independent duplicate experiment ± SEM. $p<0.05$ (*) compared to 20 μM MIN treated control.

Figure 3.1.10: Inhibition of MIN- or Lithium-induced DNA fragmentation in HL-60 cells by TPA. HL-60 cells were pretreated with 30 nM TPA for 24 hours prior to treatment with either lithium (10, 20 mM) or Minocycline (20 μM) for a further 24 hours. Lane 1, untreated controls; lane 2, 10 mM Li^+; lane 3, 20 mM Li^+; lane 4, 20 μM MIN; lane 5, MW markers; lane 6, 30 nM TPA; lane 7, 30 nM TPA + 10 mM Li^+; lane 8, 30 nM TPA + 20 mM Li^+; lane 9, 30 nM TPA + 20 μM MIN

TPA alone did not induce any laddering of DNA indicative of apoptosis in this case (lane 5). However, it was previously shown that 30 nM TPA induces DNA fragmentation in HL-60 cells after 4 days in culture, probably related to cell maturation (Shai, 1996).

3.1.4.3. Requirement of protein synthesis

Several reports have been published on the role of macromolecular synthesis in the induction of apoptosis and DNA fragmentation. It was reported that apoptosis in numerous murine cell types could be abrogated or delayed by inhibition of RNA synthesis with Actinomycin D or protein synthesis with cycloheximide (Wyllie *et al.*, 1984). Similarly Martin *et al.* (1990a) demonstrated that apoptosis induced in HL-60 cells by the calcium ionophore A23187 could be abrogated or delayed by treatment of these cells with actinomycin D or cycloheximide. In the following section, the role of macromolecular synthesis in minocycline and lithium-induced apoptosis of HL-60 cells was studied.

To investigate whether induction of apoptosis in HL-60 cells by lithium or minocycline requires new protein synthesis, cultures of HL-60 cells were treated with either minocycline or lithium in the presence of cycloheximide. It was observed that minocycline- and lithium-induced apoptosis of HL-60 cells could not be prevented by treatment of these cells with 10, 20, 50 μg/ml CHX (Fig. 3.1.11).

However, exposure to CHX appeared to potentiate the loss of cell viability induced by MIN and Li^+ in HL-60 cells in culture. Even CHX (50 μg/ml) alone caused a loss in HL-60 cell viability (Table 3.1.2). CHX did not influence the viability of HL-60 cells in the presence of 20 μM MIN but only caused a marginal decrease in the presence of lithium at 20 mM.

Actinomycin D was used as an inhibitor of RNA synthesis in order to evaluate whether MIN- and Li^+ induced apoptosis requires expression of new genes. Actinomycin D alone resulted in a significant decrease in cell viability to 64%, and

it was apparent that the effect was dose-dependent (Table 3.1.2). Low concentrations of Actinomycin D (0.1, 0.2 μg/ml) resulted in loss of cell viability to ~70%. At 0.3 μg/ml and above, actinomycin D decreased the cell survival of HL-60 cells treated with 20 μM MIN. Culturing of minocycline-treated HL-60 cells in the presence of actinomycin D could not inhibit the fragmentation of DNA characteristic of apoptosis (Fig. 3.1.12). Actinomycin D alone induced DNA laddering in HL-60 cells (Fig. 3.1.12).

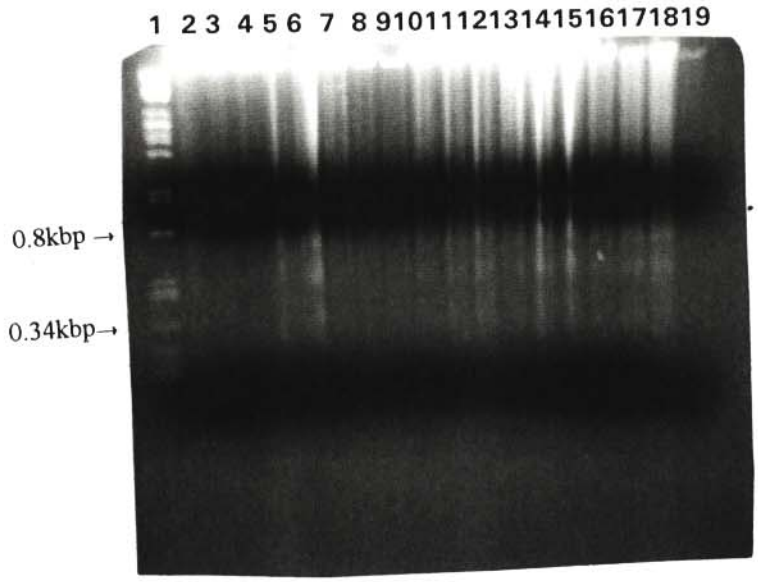

Figure 3.1.11: The effects of Cycloheximide on Lithium- and Minocycline-induced DNA fragmentation in HL-60 cells. Lane 1, *Pst* I λDNA; lane 2, 0.01 μg/ml; lane 3, 0.1 μg/ml CHX; Lane 4, 1.0 μg/ml CHX; lane 5, 2.0 μg/ml CHX ; lane 6, 50 μg/ml CHX; lane 7, 20 μM MIN; lane 8, 20μM MIN + 0.01 μg/ml CHX ; lane 9, 20 μM MIN + 0.1 μg/ml CHX; lane 10, 20 μM MIN + 1.0 μg/ml CHX; lane 11, 20 μM MIN + 2.0 μg/ml CHX; lane 12, 20 μM MIN + 5.0 μg/ml CHX; lane 13, 20 mM Li^+; lane 14, 20 mM Li^+ +0.01 μg/ml CHX; lane 15, 20 mM Li^+ + 0.1 μg/ml CHX; lane 16, 20 mM Li^+ + 1.0 μg/ml CHX; lane 17, 20 mM Li^+ + 2.0 μg/ml CHX; lane 18, 20 mM Li^+ + 5.0 μg/ml CHX; lane 19, untreated HL-60 cells.

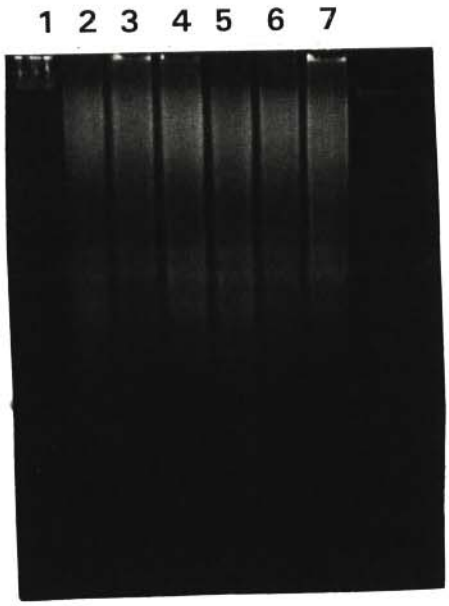

Figure 3.1.12: The role of RNA synthesis on Minocycline-induced apoptosis in HL-60 cells. HL-60 cells were cultured in the presence of the minocycline alone or in combination with increasing concentrations of Act. D. Lane 1, medium alone; lane 2, 20 μM MIN; lane 3, 20 μM MIN + 0.1μg/ml Act D; lane 4, 20 μM MIN + 0.3μg/ml Act D; lane 5, 20 μM MIN + 0.5 μg/ml Act D; lane 6, 20 μM MIN + 1.0 μg/ml Act D; lane 7, 1.0 μg/ml Act D.

Table 3.1.2: The effects of cycloheximide on HL-60 cell viability in the presence of lithium and minocycline after 24 hours of incubation. HL-60 cells were treated with MIN alone or in combination with varying concentrations of CHX for 24 hours and the viability determined by trypan blue dye exclusion method. *$p > 0.05$ compared to untreated control cells.

CHX (μg/ml)	MIN (μM)	Li+ (mM)	VIABILITY (%)
0.00	0.00	0.00	96.5 ± 0.50
0.00	20.00	0.00	88.5 ± 0.50
10.00	20.00	0.00	91.0 ± 0.00
20.00	20.00	0.00	84.5 ± 1.50
50.00	20.00	0.00	84.5 ± 1.50
0.00	0.00	20.00	89.5 ± 0.50
10.00	0.00	20.00	82.0 ± 4.00*
20.00	0.00	20.00	76.5 ± 0.50
50.00	0.00	20.00	71.5 ± 0.50
50.00	0.00	0.00	79.5 ± 2.50

Table 3.1.3: Percentage viability of HL-60 cells treated with either Lithium or Minocycline in the presence of varying concentrations of Actinomycin D. HL-60 cells were cultured in RPMI-1640 containing 20 μM MIN alone or in combination with increasing concentrations of Act. D and cell viability determined 24 hours later as described under materials and methods. $p<0.05$ compared to 20 μM MIN treated controls.

MIN (μM)	ACTINOMYCIN D (μg/ml)	CELL VIABILITY (%)
0.00	0.00	97.0 ± 0.00
20.00	0.00	88.5 ± 1.50
20.00	0.10	72.0 ± 1.00
20.00	0.20	75.0 ± 2.00
20.00	0.30	62.0 ± 1.00
20.00	0.50	67.5 ± 1.50
20.00	1.00	63.5 ± 0.50
0.00	1.00	64.5 ± 5.50

Minocycline is known to inhibit the rate of protein synthesis in microorganisms and eukaryotic cells when used at a higher dose. In eukaryotic cells the inhibition of the protein synthesis may result in renal damage and elevated blood urea levels (reviewed in Jawets, 1987). Further, it has been demonstrated that a majority of protein synthesis inhibitors can induce apoptosis in a variety of cell lines (Martin *et al.,* 1990a). In this section, protein synthesis in HL-60 cells treated with MIN was assayed.

HL-60 cells were cultured in the presence of 20 μM MIN and protein synthesis determined by incorporation of [^{3}H]-Leucine as described under materials and methods. Minocycline resulted in ~20% inhibition of [^{3}H]-Leucine incorporation at the apoptotic dose of 20 μM. On the other hand, 5 μg/ml CHX induced more than 95% inhibition, whereas 50 μM MIN induced a 40% reduction in protein synthesis (Fig. 3.1.13).

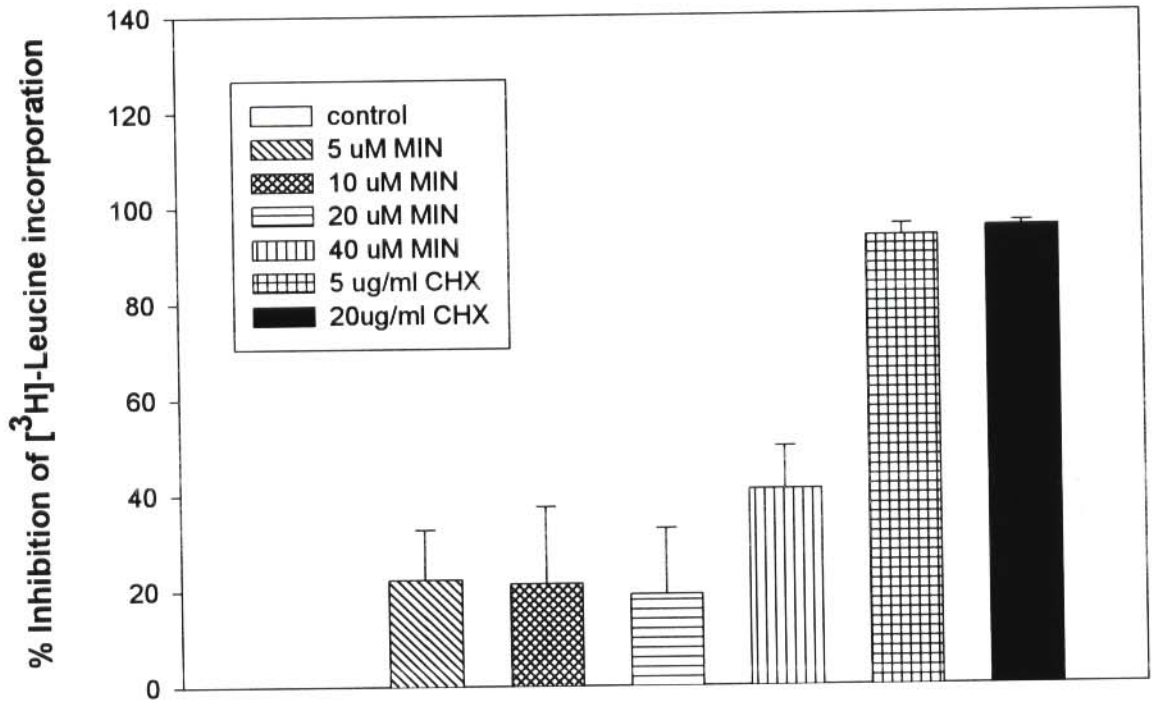

Figure 3.1.13: The effects of MIN on the incorporation of [H]-leucine by HL-60 cells. HL-60 cells were treated with MIN and CHX at the indicated concentration s and then labelled with 20 μCi/ml [H]-leucine for a further 2 hours. The results are expressed as % untreated control cells. The results represent the mean of 3 independent duplicate experiments ± SEM.

3.1.4.4. Cell cycle analysis

Apoptosis is tightly linked with the control of the cell cycle and many substances that induce cell cycle arrest also induce apoptosis (reviewed in Hale *et al.*, 1996). In addition, protocols for detecting apoptosis by determining the ratio of cells with a hypodiploid content of DNA are well documented (reviewed in Darzynkiewicz *et al.*, 1997). Lithium is reported to induce cell cycle arrest at the G_2/M phase accompanied by the onset of apoptosis (Madiehe *et al.*, 1995).

The distribution of MIN-treated HL-60 cells in the different phases of the cell cycle was performed as described above. Table 3.1.4 shows that MIN at 20 μM induced a delay of the cells in the G_1 phase of the cell cycle.

3.2. Effect of Li^+ and MIN on the transcription of apoptosis associated genes

Despite the fact that MIN and Li^+ induced apoptosis did not appearto require new gene transcription, it was decided to investigate whether these two agents could modulate the expression of growth-related genes. RNA was isolated from Li^+ and MIN-treated HL-60 cells and contaminating DNA was removed by treatment with RNase-free DNase I (Sambrook *et al.*, 1989) and reverse transcribed into cDNA with MuLV Reverse transcriptase. Table 3.2.1 shows the quality of RNA isolated 36 hours after treatment, as determined by the ratios A_{260}/A_{280} and $A_{260}/A_{230,}$. The ratios were greater than 1.8, indicating that the RNA was essentially free of contaminating proteins and guanidine, respectively, and therefore suitable for RT-PCR assays.

Table 3.1.4: The effects of minocycline on the cell cycle distribution of HL-60 cells. HL-60 cells were treated with 0, 10 and 20 μM minocycline for 48 hours. Samples were analysed by flow cytometry as described under Materials and Methods.

Condition	Time	Cell cycle phases		
	(hr)	G_0/G_1	S	G_2/M
0 μM MIN	12	60.6	6.6	16.5
10 μM MIN	12	59.2	8.0	19.6
20 μM MIN	12	61.0	5.2	14.1
0 μM MIN	24	45.4	27.7	11.3
10 μM MIN	24	38.8	35.9	14.4
20 μM MIN	24	40.9	31.8	10.0
0 μM MIN	48	47.8	24.3	16.9
10 μM MIN	48	52.5	24.4	14.1
20 μM MIN	48	64.9	16.3	12.0

To asses the integrity, the RNA was separated on formaldehyde-denaturing agarose gels (fig. 3.2.1). 10 μg of RNA was loaded in each well, and the intensity of the bands indicates that roughly equal amounts of RNA were loaded in all the wells and that the RNA was of good quality because the ratio of 28 S band to 18 S band was approximately 2:1.

The results of the RT-PCR reactions carried out with *c-jun, bcl-2* and *β-actin* primers are shown in figure 3.2.2 A, B and C, respectively. *Bcl-2* is expressed in control HL-60 cells, in agreement with published reports. Lithium caused a decrease in the expression of *bcl-2* mRNA at the growth enhancing dose of 5 mM

Li^+ but the levels increased at the apoptosis-inducing dose of 20 mM (fig.3.2.3.A). On the other hand, treatment of HL-60 cells with MIN led to a dose related decrease in *bcl-2* mRNA expression, indicated by the lowered intensity of the 284 bp band. However, 10 μM MIN failed to induce the same effects on the expression of *bcl-2.*

The levels of β-*actin* mRNA (fig. 3.2.3.C) were equal in all the wells indicating equal amounts of cDNA was used in the PCR reactions.

The levels of *c-jun* mRNA remained the same in all treatments with lithium (5, 15 mM) or minocycline (10, 20 μM) after 36 hours of incubation. The 64-bp PCR product maintained the same level in control untreated HL-60 cells (fig. 3.2.3.B). The expression of *c-fos* mRNA was not detected in both lithium and minocycline treated HL-60 cells (data not shown). The amplification of cDNA using *c-myc* primers resulted in a chromosomal DNA-size band that disappeared after DNase I treatment of RNA data not shown).

Table 3.2.1: The integrity of RNA isolated from HL-60 cells treated with minocycline and lithium for after 36 hours of incubation.

	RATIOS	
Condition	A_{260}/A_{280}	A_{260}/A_{230}
Control	1.84	1.69
10 μM MIN	2.00	3.10
20 μM MIN	3.27	2.57
5 mM Li^+	2.40	5.80
15 mM Li^+	1.94	3.10

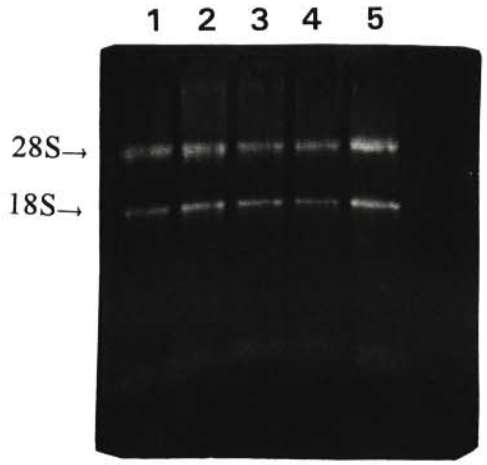

Figure 3.2.1: Formaldehyde-denaturing gel electrophoresis separation of total RNA isolated from HL-60 cells treated with Minocycline and lithium for 36 hours. Lane 1, untreated control cells; lane 2, 10 μM MIN; lane 3, 20 μM MIN; lane 4, 5 mM Li^+; lane 5, 15 mM Li^+.

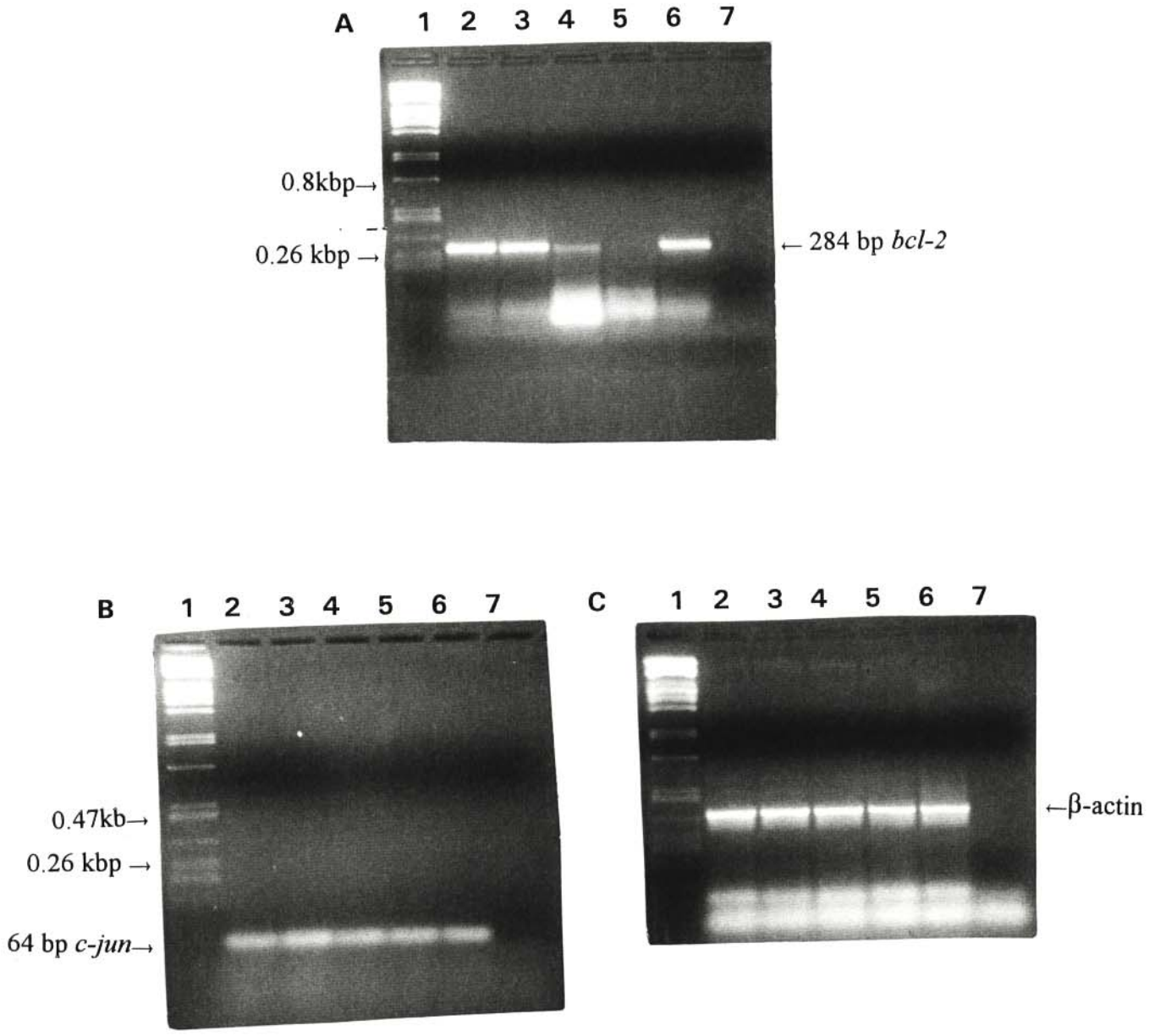

Figure 3.2.2: RT-PCR analysis of the expression of *bcl-2* (A), *c-jun* (B) and *β-actin* (C.) mRNA in HL-60 cells exposed to MIN and Li^+. Lane 1, λDNA digested with *Pst* I; Lane 2, untreated cells; lane 3, 10 μM MIN; lane 4, 20 μM MIN; lane 5, 5 mM Lithium; and lane 6, 15 mM Lithium. Lane 7 represents no template PCR reaction.

CHAPTER 4

THE ROLE OF *c-jun* PROTOONCOGENE IN THE EFFECTS OF LITHIUM IN CELL CULTURES: Studies with antisense *c-jun* expression plasmid

The AP-1 complex is implicated in many cellular responses including cell differentiation, transformation and apoptosis (Karin and Angel, 1991). Lithium shows diverse effects on cells in culture by an unclear mechanism. The proposed mechanisms of lithium actions are inhibition of inositol monophosphatases (Berridge *et al.,* 1989), inhibition of cAMP accumulation (Geissler *et al.,* 1972) and inhibition of GSK-3β (Klein and Melton, 1996). Lithium has been shown to activate AP-1 elements in developing oocytes (Hedgepath *et al.,* 1997) and it alters the phosphorylation pattern of the Tau protein in neurocytes (Munoz-Montano *et al.,* 1997). Although no elevation of *c-jun* mRNA was detected in lithium treated HL-60 cells, it is possible that lithium might function through a mechanism that involves the *c-jun* protooncogenes. To address this possibility, an antisense *c-jun* recombinant plasmid was constructed and introduced into HL-60 promyelocytic cells, Raji lymphoma cells and 3T3-L1 fibroblasts with a view of downregulating the levels of *c-jun* protooncogene expression. The effects of lithium were investigated both in the presence and absence of antisense *c-jun*.

4.1. Construction of antisense *c-jun* recombinant plasmid

4.1.1. Restriction enzyme digests

The restriction maps of both *c-jun* recombinant pGEM-2 plasmid and pOPRSVI/MCS cloning vector are outlined in figure 4.1.1. *c-jun* was excised from pGEM-2 recombinant plasmid using *Eco*RI as described under materials and methods. Figure 4.1.1 shows the *Eco*RI digestion of pGEM-2 recombinant plasmid and the excised 2.6kb *c-jun* fragment that was cut and purified. Plasmid pOPRSVI/MCS was digested with *Sma*I (fig. 4.1.2), dephosphorylated and then

used in the subsequent cloning experiment.

The 2.6 kb fragment of *c-jun* cDNA was end-filled with Klenow DNA polymerase as described elsewhere and ligated into the *Sma* I site of the MCS region of pOPRSVI/MCS operator vector (5647 bp) to generate an 8247 bp recombinant plasmid. A recombinant plasmid with *c-jun* in the antisense orientation to RSVI promoter of pOPRSVI/MCS was identified by restriction enzyme analysis (Fig. 4.1.3) and named pJMCS. The recombinant plasmid generates a 6243 bp fragment which results from the ligation of a 2600 bp *c-jun* cDNA and 3643 bp fragments upon digestion with *Eco*RI (Fig. 4.1.3). The intermediate (1397 bp) and the smaller (607 bp) remained unchanged when digested with *Eco R1*, meaning that the *c-jun* fragment remained ligated to the 3643 bp fragment which confirmed construction of a desired plasmid.

4.1.2.The orientation of the ligated *c-jun* cDNA

c-jun cDNA insert was ligated into the MCS site of pOPRSVI/MCS and the orientation was determined using *Pst*I as follows: *c-jun* cDNA has a unique and assymmetric *Pst*I site at position 1821 and pOPRSVI/MCS contains *Pst*I restriction sites at positions 857, 1054 and 3157. Digestion of pOPRSVI/MCS would result in fragments of sizes 3347, 197 and 2103 bp while *c-jun* would yield fragments of sizes 879 and 1821 bp. *Pst*I digestion of recombinant pOPRSVI/MCS (pJMCS) would yield fragments of sizes 197, 2103, 1825 and 4123 bp if ligated in the sense orientation. If ligated in the reverse orientation the following fragments would result; 197, 2103, 789 and 5164 bp. Figure 4.1.4 shows the *Pst*I digest of pOPRSVI/MCS and pJMCS. *Pst*I digest of pJMCS resulted in fragments of sizes 197, 789, 2103 and 5164 bp, indicating that *c-jun* was ligated in the antisense orientation (Fig. 4.1.4).

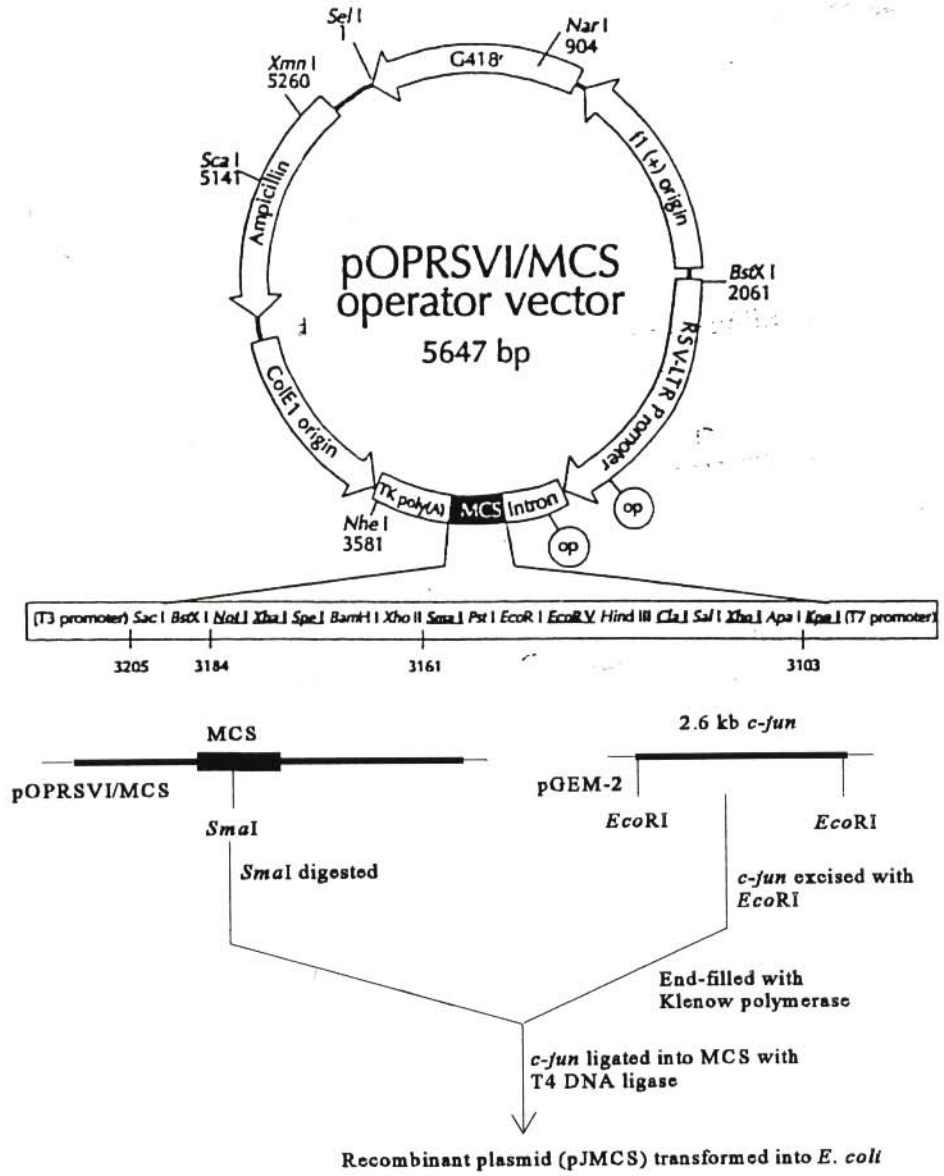

Figure 4.1.1: The restriction maps of pGEM-2 and pOPRSVI/MCS. The recombinant PGEM-2 plasmid, carrying a 2.6kb *c-jun* cDNA and pOPRSVI/MCS operator vector were used in the subsequent cloning experiments. The Rous sarcoma virus (RSV) promoter drives the expression of *c-jun* inserted into the MCS region.

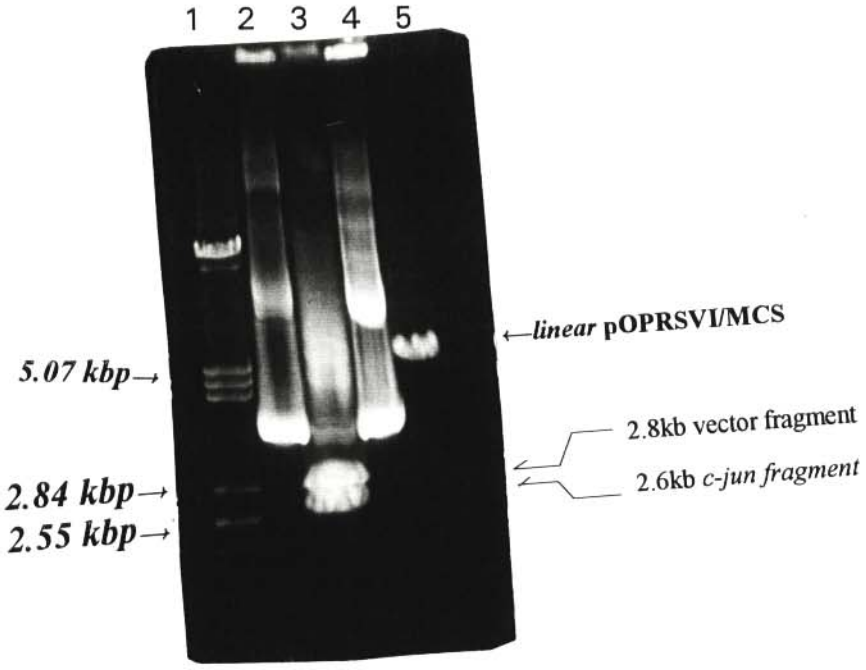

Figure 4.1.2: *Sma* I digest of pOPRSVI/MCS vector and *Eco*RI digest of recombinant *c-jun*/pGEM-2 plasmid. Lane 1, MW markers; lane 2, Undigested *c-jun*/pGEM-2; lane 3, *Eco*RI digested *c-jun*/pGEM-2; lane 4, undigested pOPRSVI/MCS; lane 5, *Sma*I linearised pOPRSVI/MCS.

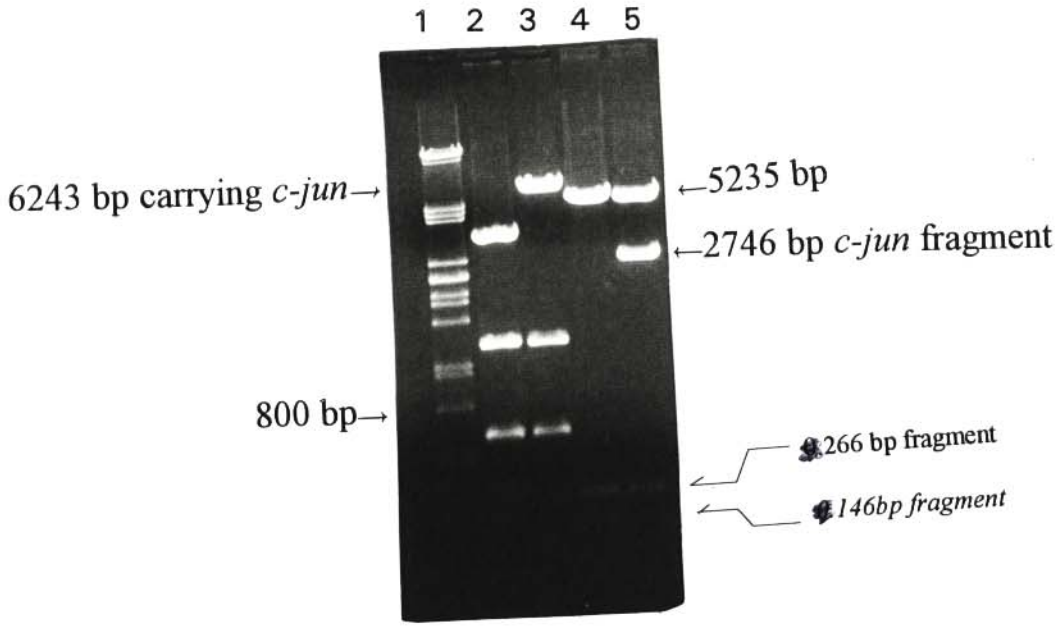

Figure 4.1.3: Restriction enzyme analysis of a recombinant plasmid carrying the 2.6 kbp *c-jun* cDNA fragment and the operator vector pOPRSVI/MCS. lane 1, *Pst*I digested λDNA; lane 2, *Eco* RI digested pOPRSVI/MCS vector; lane3, *Eco*RI digested recombinant plasmid; lane 4, *Bam*HI digested pOPRSVI/MCS vector; lane 5, *Bam*HI digested recombinant plasmid.

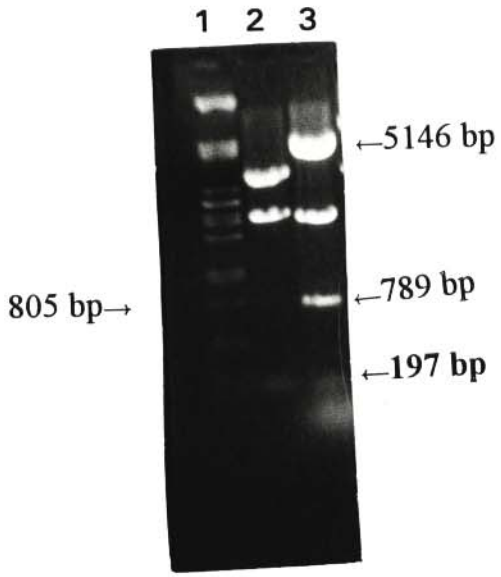

Figure 4.1.4: Determination of the orientation of *c-jun* DNA fragment in pOPRSVI/MCS vector. Plasmids pOPRSVI/MCS and pJMCS were digested with *Pst*I and analysed by agarose gel electrophoresis. Lane 1, *Hind*III digested λDNA; lane 2, *Pst*I digested pOPRSVI/MCS vector; lane 3, *Pst*I digested pJMCS. The 789 bp fragment diagnostic of antisense orientation is shown in lane 3.

4.2. The effects of antisense *c-jun* in different mammalian cell lines in the presence of lithium

4.2.1. 3T3-L1 preadipocytes and differentiation

Lithium blocks inducer-stimulated adipose conversion of 3T3-L1 cells (Aratani *et al.*, 1987). To investigate the role of *c-jun* in this phenomenon, plasmids pJMCS and pOPRSVI/MCS were contransfected with pCMVLacI repressor plasmid into 3T3-L1 preadipocytes by electroporation. Treatment of 3T3-L1 cells with 20 mM Li^+ caused a blockage of inducer-stimulated differentiation as expected. The level of differentiation of pJMCS- and pOPRSVI/MCS transfected cells were comparable in the presence and absence of 20 mM Li^+ (Table 4.2.1). Similar observations were made with $CaPO_4$ mediated transfection with pJMCS alone (Fig. 4.2.1). Antisense *c-jun* failed to influence the differentiation of 3T3-L1 preadipocytes.

4.2.2. Raji lymphoblastic cells

Studies with a mouse acquired immune deficiency syndrome (MAIDS) have shown that lithium greatly reduces the incidence of lymphoma development (Gallichio *et al.*, 1994). Recently, *in vitro* studies have demonstrated toxicity of lithium to Raji cells at concentration below 10 mM (Mukhufhi, 1997). Similarly, antisense oligonucleotides have entered clinical trials for the treatment of lymphomas. In this section, the effects of lithium on Raji cells have been further characterised in the presence of an antisense *c-jun* construct.

Raji cells were transfected with plasmid pOPRSVI/MCS vector control and plasmid pJMCS by lipofection. The cells were allowed to recover for 6 hours and then treated with 5 and 10 mM lithium. Growth and viability were determined after 48 hours and then at 24 hours intervals up to 96 hours (Fig. 4.2.2. and Fig. 4.2.3). pJMCS alone inhibited ($p<0.05$) the growth of Raji cells by 20% compared to pOPRSVI/MCS vector controls (Fig. 4.2.2) although the viability was not significantly ($p>0.05$) different (Fig. 4.2.3). Similar observations were made with both 5 and 10 mM lithium treated cultures. Lithium inhibited the growth of Raji cells both in the presence and absence of pJMCS.

DNA synthesis at the end of the experiment was determined by thymidine incorporation. [^{3}H]-methylthymidine incorporation by pJMCS transfected cells was lowered by above 50% when compared to pOPRSVI/MCS transfected Raji cells (Fig. 4.2.4). In the presence of lithium at 5 and 10 mM, the incorporation of labelled thymidine was dramatically reduced to just above 20% of untreated pOPRSVI/MCS control in both instances, viz. PJMCS and pOPRSVI/MCS-transfected Raji cells in the presence of lithium. Thus, antisense *c-jun* appears to block the proliferation of Raji cells without causing a corresponding reduction in cell viability.

4.2.3. HL-60 promyelocytic leukemia cells

Lithium enhances the proliferation of HL-60 cells in the 2.5-5 mM concentration range and induces apoptosis at 10 mM and above (Becker and Tyobeka, 1990; Madiehe *et al.,* 1995). Similarly, c-Jun/AP-1 may participate in the regulation of apoptosis as an inducer (Yumamoto, 1995; Sawai *et al.,* 1995) or an inhibitor (Butterfield *et al.,* 1997), depending on the inducing agent and cell type. This study was conducted to understand the role of c-Jun/AP-1 factor in growth stimulation and apoptosis induced by lithium in HL-60 cells. Plasmid pJMCS was introduced into HL-60 cells by lipofection method before treatment with lithium (5 mM). Cell number and viability were determined at 24 hour intervals for 96 hours. Constitutive expression of antisense *c-jun* mRNA induced cell death of HL-60 cells (Fig. 4.2.5), reducing percentage survival to approximately 40% of both untreated and 5 mM lithium-treated cultures within the first 48 hours of incubation, while cells transfected with pOPRSVI/MCS operator vector alone maintained a viability of above 95% (Fig. 4.2.5). The pOPRSVI/MCS transfected HL-60 cell cultures still maintained more than 95% cell survival in both untreated and 5 mM Li^+ treated flasks after 96 hours of incubation, while there was hardly any live cell in antisense *c-jun* transfected cultures.

The number of cells in both pOPRSVI/MCS- and pJMCS-transfected HL-60 cultures illustrated the trend exhibited by cell viability, with antisense *c-jun* inhibiting

proliferation of HL-60 cells in both lithium-treated and untreated flasks, while the mitogenic effects were still observed in pOPRSVI/MCS transfected HL-60 cell cultures treated with 5 mM lithium (fig. 4.2.6).

The role of AP-1 in DNA fragmentation in HL-60 cells in the presence of lithium was analysed by agarose gel electrophoresis (fig. 4.2.7). pJMCS induced slight fragmentation of DNA while pOPRSVI/MCS transfection, as expected, did not induce any apoptosis. However, lithium at 20 and 50 mM induced apoptosis in both pJMCS and pORSVI/MCS transfected HL-60 cells.

Table 4.2.1: The effects of antisense *c-jun* on the differentiation of 3T3-L1 preadipocytes. Cells were transfected with pJMCS and pOPRSVI/MCS and then induced to differentiate with the induction medium. After 8 days the cells were washed with PBS and stained in Oil red O. The dye was extracted with isopropanol and the intensity determined by reading the absorbance at 595 nm. IND = induced.

	Condition	**% Differentiation**
pOPRSVI/MCS	IND	100 ± 0.00
	IND + 5 mM IPTG	94.0 ± 4.75
	IND + 5 mM IPTG + 20 mM Li^+	17.0 ± 4.50
	IND + 20 mM Li^+	14.0 ± 3.50
pJMCS	IND	93.0 ± 1.50
	IND + 5 mM IPTG	102.6 ± 0.95
	IND + 5 mM IPTG + 20 mM Li^+	24.15 ± 0.50
	IND + 20 mM Li^+	15.09 ± 1.57

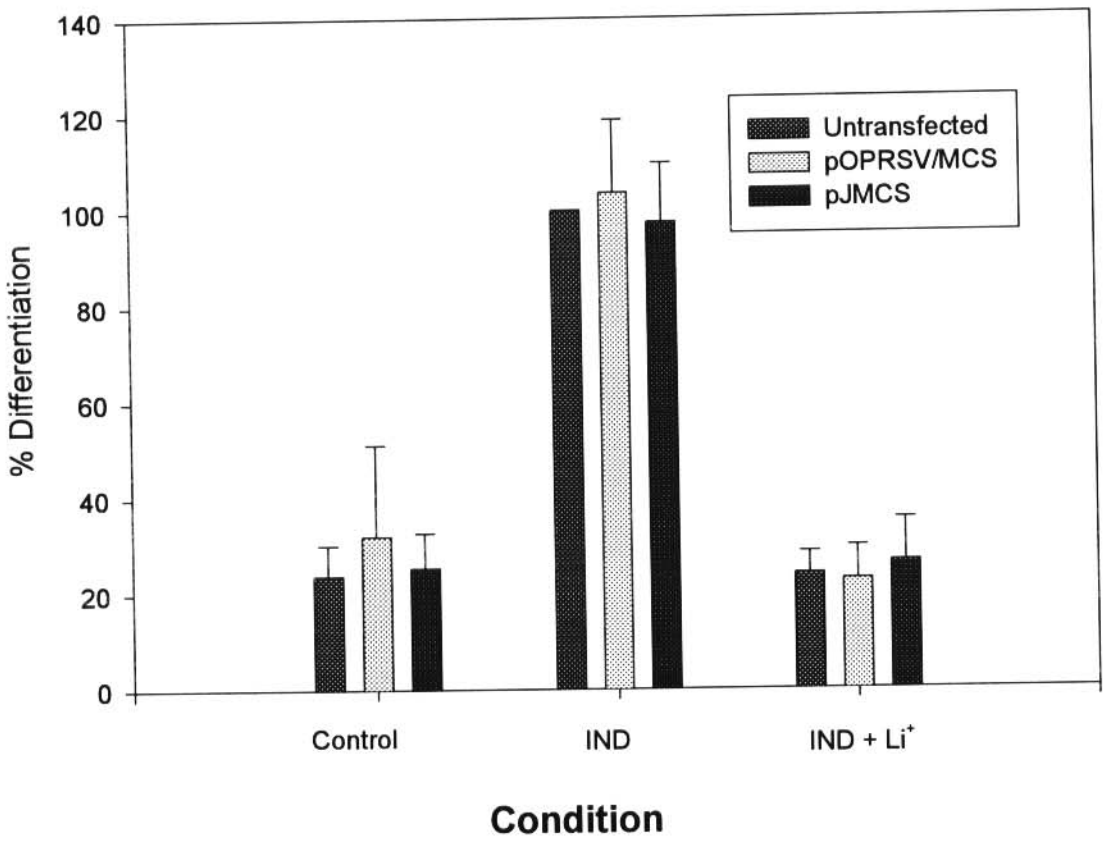

Figure 4.2.1: The effects of antisense *c-jun* on the inducer-stimulated differentiation of 3T3-L1 cells. The cells were transfected with pJMCS and pOPRSVI/MCS vector control plasmid. The cells were induced and stained with Oil red O. The absorbence at 595 nm was determined. The results represent mean data of 2 independent triplicate experiments ± SEM.

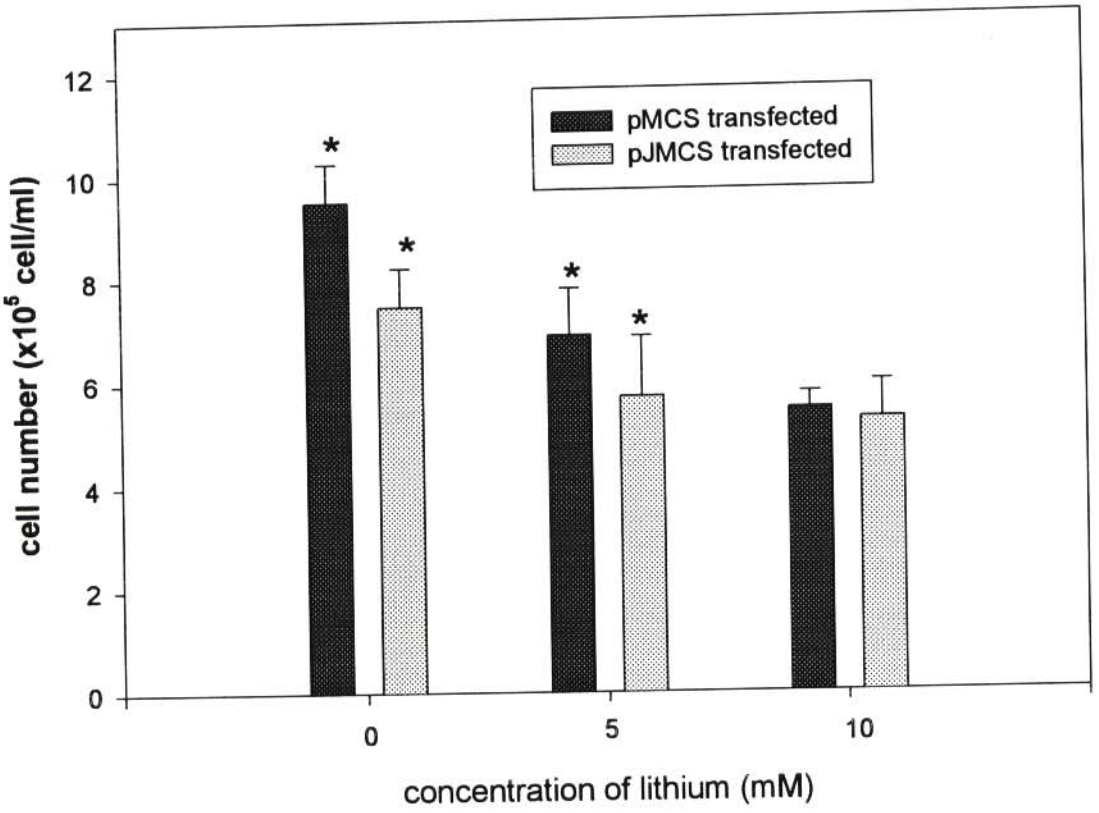

Figure 4.2.2: The effects of antisense *c-jun* on the rate of proliferation of Raji cells in the presence of lithium. Raji cells were transfected with pOPRSVI/MCS vector without *c-jun* antisense and pJMCS. The cells were counted at 72 hours post treatment with lithium as described in Materials and Methods. The results are means of 2 independent triplicate experiments ± SEM. P value < 0.05. * significantly different.

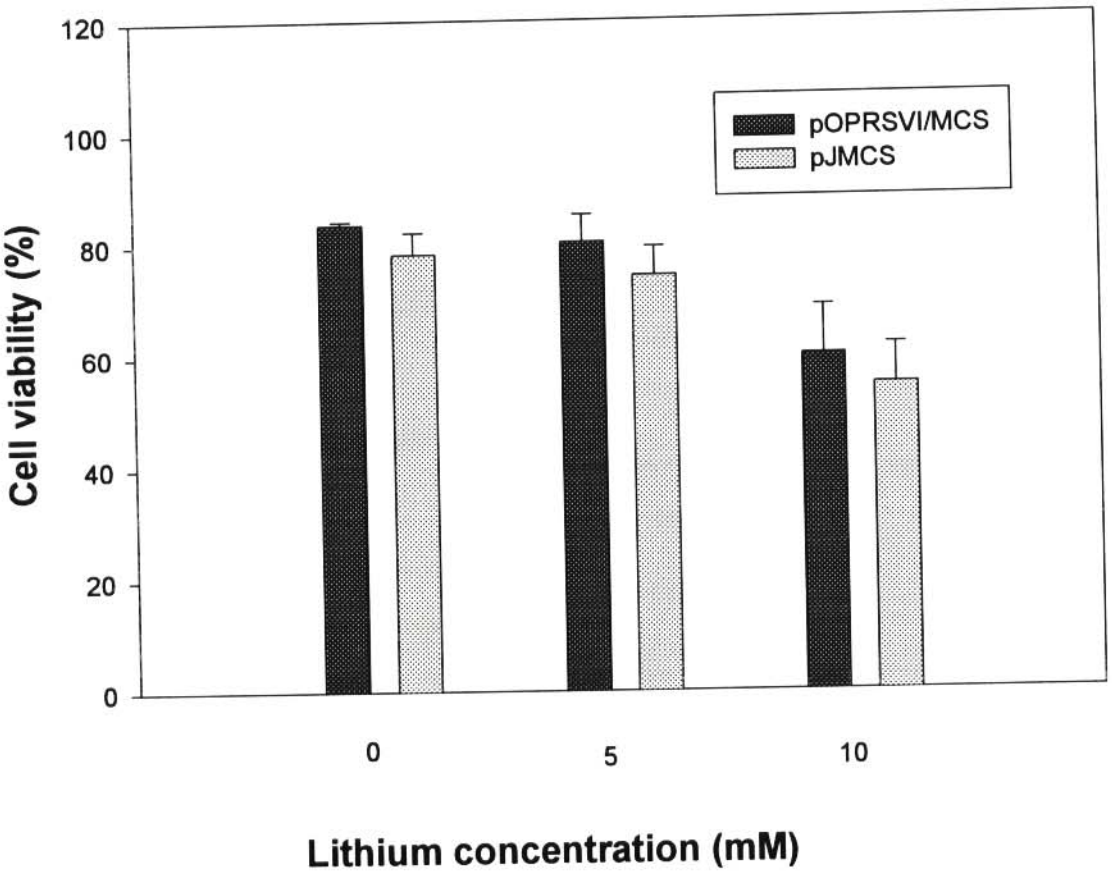

Figure 4.2.3: The effects of antisense *c-jun* on the survival of Raji cells treated with lithium after 72 hours of incubation. Raji cells were transfected as described earlier, with pOPRSVI/MCS vector and pJMCS, and then treated with lithium (0, 5, 10 mM). Cell viability was determined by trypan blue exclusion method, as outlined earlier. Results represent means of 2 independent triplicate experiments ± SEM. P value > 0.05.

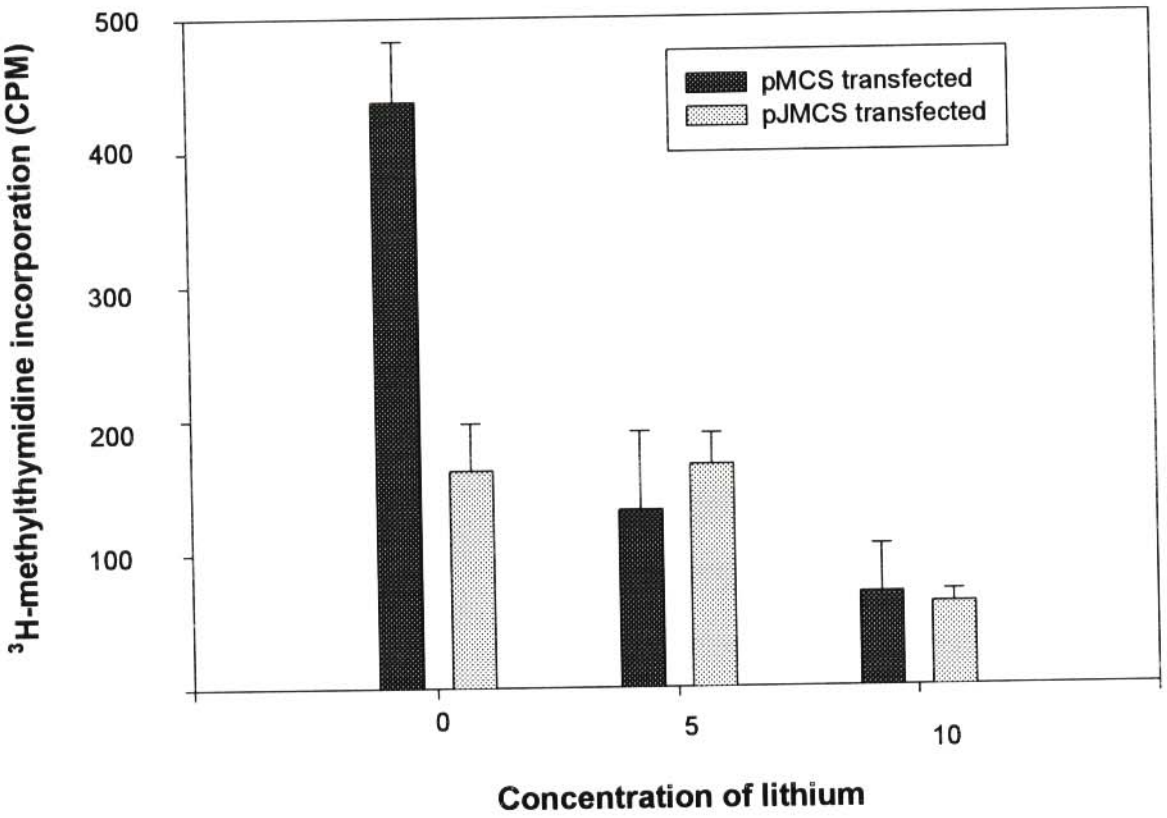

Figure 4.2.4: The effects of antisense *c-jun* and lithium on the rate of [3H]-methylthymidine incoporation by Raji lymphoblastic leukemia cells. Raji cells were transfected as described in Chapter 3, and treated with lithium (0, 5, 10 mM) for 72 hours. Incorporation of labelled thymidine was performed as outlined earlier.

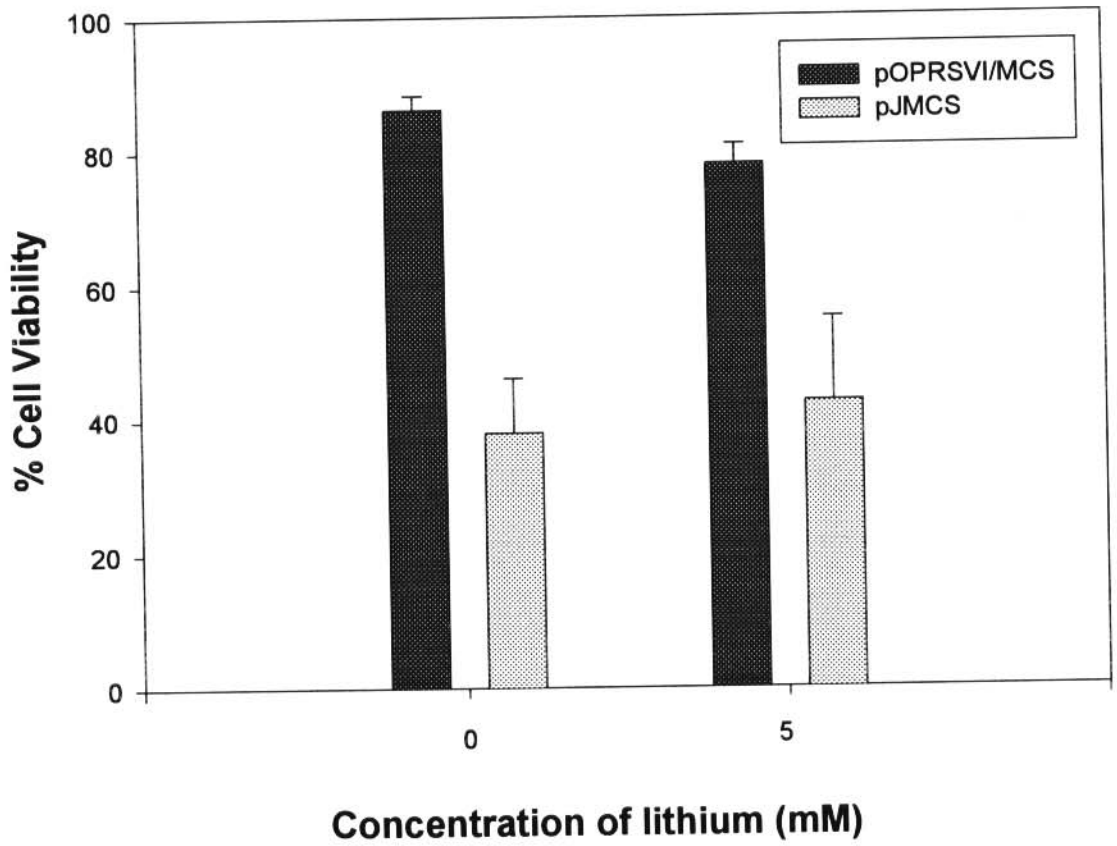

Figure 4.2.5: Cell viability of HL-60 cells at 48 hours post-treatment with lithium. HL-60 cells were transfected with pOPRSVI/MCS or pJMCS for 6 hours and then treated with lithium (0, 5 mM). The percentage of viable cells was determined by trypan-blue exclusion method, as described elsewhere. The results are means ± SEM of 2 triplicate experiments performed independently.

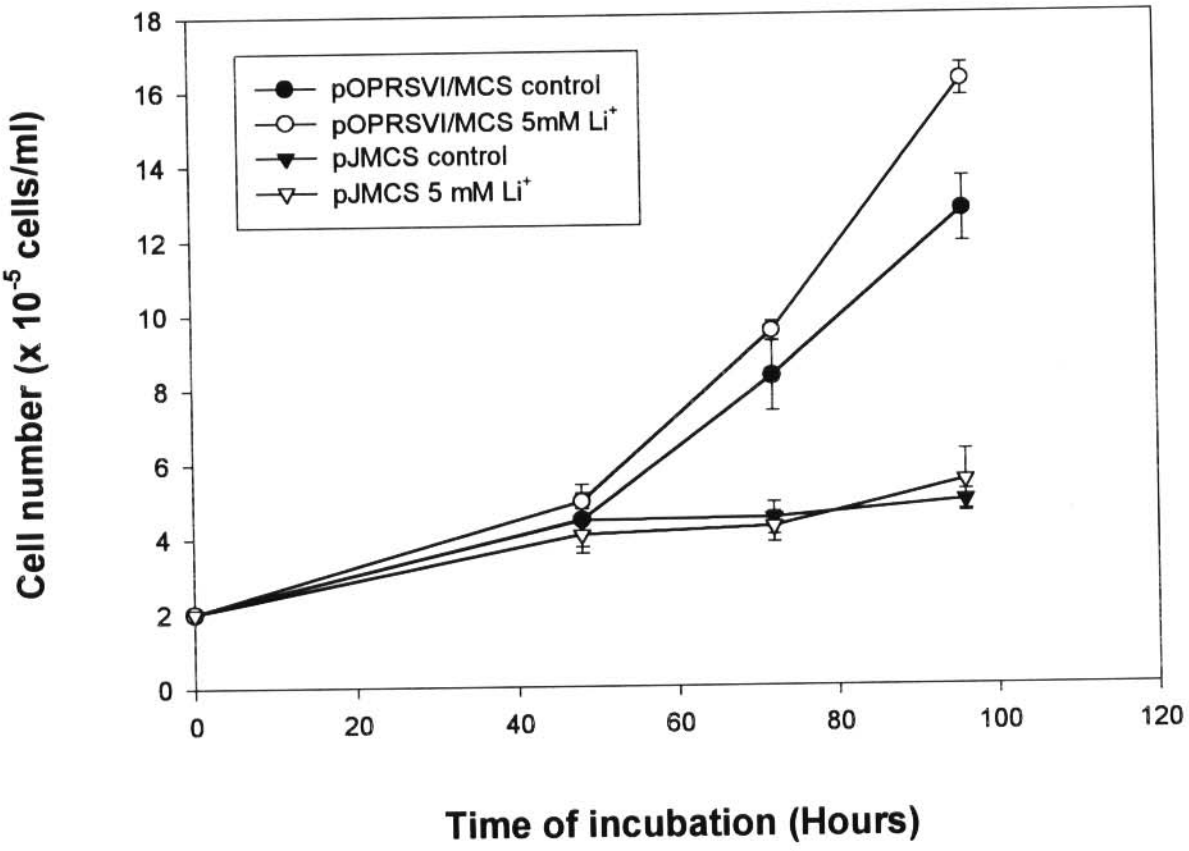

Figure 4.2.6: The effects of antisense *c-jun* on the rate of growth of HL-60 cells treated with lithium. HL-60 cells were transfected with pOPRSVI/MCS or pJMCS, and then treated with lithium (0, 5 mM) 6 hours later for 96 hours. Cell number was determined at 24 hour intervals as described earlier. The results are means ± SEM of 2 independent triplicate experiments

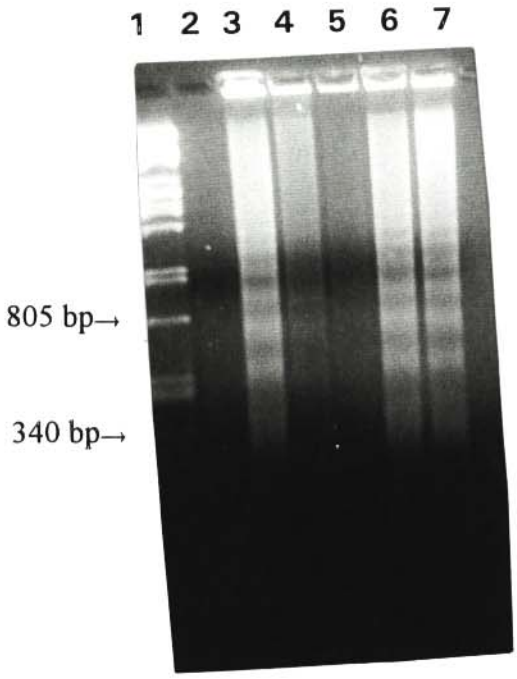

Figure 4.2.7: DNA fragmentation analysis of HL-60 cells transfected with pOPRSVI/MCS vector and pJMCS in the presence of lithium. pOPRSVI/MCS and pJMCS transfected HL-60 cells were treated with lithium for 24 hours and the DNA isolated and analysed. Lane 1, pOPRSVI/MCS control; lane 2, pOPRSVI/MCS 20 mM Li^+; lane 3, pOPRSVI/MCS 50 mM Li^+; lane 4, pJMCS control; lane 5, pJMCS 20 mM Li^+ ; lane 6, pJMCS 50 mM Li+.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1. Cell proliferation and apoptosis

Minocycline belongs to the tetracycline class of antibiotics and it is used clinically for the treatment of diverse bacterial infections (Jawets, 1987). Tetracyclines exhibit their antibiotic action by inhibiting the binding of EFTu-GTP-aminoacyl tRNA to the A site on the ribosome (Brock *et al.,* 1994). Studies have shown that minocycline can mimic the effects of lithium in the nervous system and in the perturbation of renal activity (Dousa and Wilson, 1974; Forrest *et al.,* 1978; Kofman *et al.,* 1993). In addition, minocycline has demonstrated some efficacy in the management of rheumatoid arthritis by an unclear mechanism (Sewell *et al.,* 1996). In these studies, minocycline induced a concentration-dependent inhibition of HL-60 cell growth accompanied by a drastic loss in cell viability (Fig. 3.1.1 and 3.1.2). This study therefore supports previous findings that demeclocycline inhibited the growth of HL-60 cells (Becker and Tyobeka, 1993b).

Two modes of cell death that are distinguished by specific morphological and biochemical characteristics have been described (reviewed in Vaux, 1993). Apoptosis is distinguished from necrosis by the precise regulation of its progression. During apoptosis, DNA is cleaved into fragments of internucleosomal sizes ranging from 180-280 bp (reviewed in Vaux, 1993). While apoptosis is characterised by an active involvement of the target cell in its own demise to the point of triggering the synthesis of effectors of cell death, necrosis is a passive, catabolic and degenerative process (reviewed in Darzynkiewicz *et al.,* 1997). In these studies, minocycline induced a drastic loss in HL-60 cell viability. The loss of viability was attributed to cell death by apoptosis from the observed oligonucleosomal DNA fragmentation (Fig. 3.1.3). Programmed cell death was confirmed by the observation of nuclear DNA condensation and cellular

fragmentation by electron microscopy (Fig. 3.1.4). Madiehe *et al.* (1995) also reported DNA fragmentation and morphological characteristics of apoptosis in HL-60 cells treated with lithium. However, minocycline could not induce apoptosis in K562, 3T3-L1 and LLC-PK1 cells (Fig. 3.1.3 and 3.1.5, Table 3.1.1). In contrast lithium induced apoptosis in K562 cells (Madiehe *et al.,* 1995). Lithium also did not induce DNA fragmentation in 3T3-L1 and LLC-PK cells (Fig. 3.1.5). The reasons for the resistance of some cell lines to undergo apoptosis in the presence of minocycline are not yet established and studies on the uptake of the drug might be useful. It is also possible that the minocycline target in the apoptotic machinery of the HL-60 cells could be modified or absent in these cell lines, making minocycline specific for induction of apoptosis in HL-60 and perhaps other related cell lines. It is also possible that these cell lines express elevated levels of the antiapoptotic genes such as the *bcl-2* family. However, it should be pointed out that in many other cell types DNA degradation does not proceed to nucleosomal sized fragments but rather results in larger 50-300 kb fragments (Oberhammer *et al.,* 1993) which was not assayed in these studies. However, oligonucleosomal DNA fragmentation in K562 cells has been reported (Madiehe *et al.,* 1995). Nevertheless, the K562 cell line has been shown to be resistant to different apoptotic inducers such as colchicine (Martin and Cotter, 1989) protein synthesis inhibitors (Martin *et al.,* 1990) and other agents (Diomede *et al.,* 1993).

Induction of apoptosis in many cell lines by protein synthesis inhibitors is well established (Wyllie *et al.,* 1984). It has also been reported that high doses of minocycline inhibit protein synthesis in eukaryotic cells (Jawets, 1987). In this study minocycline inhibited protein synthesis in HL-60 cells to about 20% of the untreated control cells (Fig. 3.1.13). It is not clear at the moment whether minocycline-induced apoptosis resulted from inhibition of protein synthesis since cycloheximide induced comparative DNA fragmentation despite causing almost 100% inhibition of protein synthesis.

Apoptosis has been linked to a rise in intracellular calcium concentration in many

cell culture systems (reviewed in Hale *et al.,* 1996). Ca^{2+} is believed to activate a Ca^{2+}/Mg^{2+}-dependent DNase I that cleaves DNA into fragments. In thymocytes for instance, EGTA, a chelator of extracellular calcium inhibited or delayed the onset of DNA fragmentation and apoptosis (McConkey *et al.,* 1989) and other investigators have observed enhanced apoptosis in the presence of EGTA (Hampton *et al.,* 1996). In minocycline treated HL-60 cells EGTA did not inhibit DNA fragmentation, instead it potentiated the process (Fig. 3.1.6). A dramatic loss of HL-60 cell viability was observed when the cells were cultured in the presence of minocycline and EGTA (Fig. 3.1.7A). One possibility for this observation might be that EGTA increased effective concentration of minocycline. It is well established that Ca^{2+} and other divalent cations hinder the uptake of minocycline by chelation (Jawets, 1987). EGTA did not influence the induction of apoptosis by lithium (Fig. 3.1.6). These data suggest that induction of apoptosis in HL-60 cells by lithium does not require influx of extracellular calcium whereas that of MIN involves a Ca^{2+} signalling mechanism. However, EGTA was previously reported to abrogate the induction of apoptosis in HL-60 cells by lithium (Madiehe, 1996). Interestingly, it was reported that DNA fragmentation in Chinese hamster ovary cells and HL-60 cells was carried out by a Ca^{2+}/Mg^{2+}-independent DNase II, whose optimum pH is near 5.0 (Barry *et al.,* 1993; Barry and Eastman, 1992; Barry and Eastman, 1993). These studies also suggested that the induction of DNA fragmentation did not result from the rise in intracellular calcium, but rather from intracellular acidification. However, Park *et al.* (1996) further suggested that an increase in intracellular calcium and the lowering of pH were responsible for ionomycin-induced DNA fragmentation in HL-60 cells. Madiehe (1996) observed inhibition of DNA fragmentation in HL-60 cells treated with lithium in the presence of Ca^{2+}, suggesting that a Ca^{2+} dependent DNase was involved. Although the roles of pH and calcium have not been fully described, it can be suggested that apoptotic DNA fragmentation is partly inducible by lowering of pH and a rise in intracellular calcium. Further studies on the role of intracellular calcium in minocycline and lithium-induced apoptosis are required.

The viability of HL-60 cells treated with minocycline in the presence or absence of 500 μM Ca^{2+} was significantly ($p<0.05$) higher than that of the cultures treated with minocycline alone (Fig. 3.1.8). Similarly, the increased toxicity observed in minocycline treated cells in the absence of Ca^{2+} could be attributed to the increased actual concentration of minocycline (Jawets, 1987). However, minocycline was reported to activate the release of intracellular calcium in osteoclasts (Bax *et al.,* 1993) and splenocytes (Sewell *et al.,* 1996). Alternatively, minocycline induced apoptosis in HL-60 cells could result from mobilization of intracellular calcium and activation of Ca^{2+}/Mg^{2+}-dependent endonuclease. In the absence of calcium minocycline induced a drastic inhibition of HL-60 cell growth (Fig. 3.1.9). It is plausible to suggest that an increase in extracellular Ca^{2+} concentration protects HL-60 cells against minocycline-induced cytotoxicity by making minocycline unavailable for uptake by the cells. Alternatively, minocycline could perturb the mobilization of Ca^{2+} which becomes pronounced at low extracellular calcium concentrations.

PKC appears to play an important role in the induction of apoptosis in different cell lines (reviewed in Hale *et al.,* 1996). In this study, the PKC activator TPA completely blocked minocycline-induced DNA fragmentation and apoptosis in HL-60 cells (Fig. 3.1.10). Similarly, the induction of apoptosis by lithium was blocked by TPA. McCarthy *et al.* (1994) reported increased resistance of HL-60 cells to undergo apoptosis with the onset of differentiation induced by either TPA or retinoic acid. It is also interesting to note that inhibitors of PKC such as staurosporine induce apoptosis in numerous cell lines (Bertrand *et al.,* 1994). These findings seem to suggest that the protection could be a result of PKC activation, although it should not be ruled out that maturation of cells could also afford protection against apoptosis. Other activators of PKC can also be used to further demonstrate the role of PKC in the progression of minocycline and lithium-induced apoptosis in HL-60 cells. Madiehe (1996) also reported a concentration-dependent inhibition of lithium-induced apoptosis in HL-60 cells by TPA. However, when HL-60 cells were treated with 30 nM TPA for 96 hours prior to lithium

treatment for a further 24 hours apoptosis was observed (Shai, 1996), suggesting that there is a limited period of time during which TPA protects against lithium-induced apoptosis.

Programmed cell death is an active cellular process which is effected by specific gene products although the signalling mechanism leading to apoptosis remains incompletely understood. In many other cell lines inhibition of protein synthesis and RNA transcription inhibit DNA fragmentation in response to some apoptotic agents, suggesting a requirement of new protein synthesis (reviewed in Martin, 1993). In this study, cycloheximide, an inhibitor of protein synthesis failed to reverse minocycline-induced apoptosis in HL-60 cells (Fig. 3.1.11 and Table 3.1.2). Similarly, actinomycin D, an inhibitor of RNA synthesis failed to inhibit minocycline-induced fragmentation of DNA in the HL-60 cells (Fig. 3.1.12 and Table 3.1.3). Together these data suggest that apoptosis induced in the HL-60 cells by minocycline does not require the synthesis of new proteins. Interestingly, it has been reported elsewhere that induction of apoptosis in HL-60 cells does not seem to require macromolecular synthesis, rather the activation of already existing proteins. Lithium induced apoptosis could not be prevented by incubation of HL-60 cells with cycloheximide and cycloheximide alone induced DNA fragmentation (Fig. 3.1.11). These data further support the suggestion by Wyllie *et al.* (1993), that HL-60 cells represent the prototype of apoptosis-"primed" cells which do not require new protein synthesis but only the activation of effectors of the apoptotic machinery.

The inducers of apoptosis in many cell types seem to be active in specific phases of the cell cycle. For instance, De Bufalo *et al.* (1996) reported that the onset of apoptosis in human breast cell lines, LB9 and MCF-7 ADR^R was preceded by accumulation of the cells in the G_0/G_1 phase, and further suggested that apoptosis occurs through a dissociation of normally integrated cell cycle events. In HL-60 cells it has been previously reported that lithium-induced apoptosis was preceded by arrest of the cells in the G_2/M phase of the cycle (Madiehe *et al.,* 1995). It was

also demonstrated that taxol induces arrest of the cells in the G_2/M phase (Rowinsky *et al.,* 1988). There was a steady increase in the proportion of cells in the G_0/G_1 phase or late cell cycle in 20 μM minocycline treated cultures within a period of 48 hours (Table 3.1.4). G_0/G_1 is marked by synthesis of histone and some of the important enzymes which prepare the cells for entry into the S phase (reviewed in Pardee, 1989). Some of the enzymes that appear late in the G_1 phase include DNA polymerase, ribonucleotide reductase, proliferating cell nuclear antigen (PCNA), thymidine kinase and thymidylate synthase (reviewed in Pardee, 1989). Since there is an increased requirement for protein synthesis in the middle of G_1 phase it is possible that minocycline delays G_1 phase by inhibiting protein synthesis. As a result HL-60 cells treated with 20 μM minocycline accumulate in the G_0/G_1 phase during the induction of apoptosis.

5.2. Gene expression

The induction of apoptosis is controlled by three functional groups of genes. Genes like *c-myc, p53, bax* and *bcl-*X_s drive the process of apoptosis whereas *bcl-2, bcl-*X_L , *Mcl-1, bcr-abl* and *v-abl* inhibit apoptosis (reviewed in Hale *et al.,* 1996). The effectors include the caspase family of proteases. Among the antiapoptotic genes, the *bcl-2* is the best characterised and the protective effects of the Bcl-2 oncoprotein from apoptosis induced by different agents are well established. Bcl-2 is believed to dimerise with Bax, sequestering its apoptosis promoting effects (reviewed in Hale *et al.,* 1996). The induction of apoptosis by minocycline was investigated and the levels of expression of *bcl-2* mRNA determined by RT-PCR. In this study, it was found that minocycline downregulated *bcl-2* mRNA expression at 20 μM (Fig. 3.2.2). This is an important observation since elevated levels of Bcl-2 are associated with cell survival in many human tumors (reviewed in Hale *et al.,* 1996). Surprisingly, the growth enhancing concentration (5 mM) of lithium inhibited the level of expression of *bcl-2* mRNA (Fig. 3.2.2). However, 15 mM Li^+ did not seem to affect the levels of *bcl-2* although this high concentration induces apoptosis (Fig. 3.2.2). The levels of *bcl-2* levels may be elevated or inhibited during apoptosis depending on the stimuli and the cell type (reviewed in Hale *et*

al., 1996). Because the levels of *bcl-2* mRNA were similar in both apoptosis-inducing (15 mM Li^+) treated cultures and untreated control HL-60 cells, it can be suggested that the mechanism of induction of apoptosis by lithium does not involve *bcl-2* expression.

The *c-jun, c-myc* and *c-fos* protooncogenes are tightly linked with cell cycle progression past the G_1 phase (reviewed in Pandey and Wang, 1995), and have been associated with apoptosis and differentiation in a variety of cells (Smeyne *et al.,* 1993). Enhanced *c-jun* expression triggers apoptosis in NIH 3T3 fibroblasts which is more pronounced under growth factor-deprived conditions (Bossy-Wetzel *et al.,* 1997). The levels of expression of *c-jun* mRNA were unaffected by either minocycline or lithium treatment after 36 hours (Fig. 3.2.2). This was unexpected because in this study TPA blocked the induction of apoptosis by both lithium and minocycline. Furthermore, lithium activates AP-1 elements in *Xenopus* oocytes and *Drosophilla* (Hedgepeth *et al.,* 1997; Klein and Melton, 1996). The expression of *c-fos* in both minocycline and lithium treated HL-60 cells could not be detected after 36 hours. It has been demonstrated that *c-fos* mRNA is unstable and is present in the cell for a few hours. After 36 hours of incubation, it could be argued, the composition of AP-1 transcription factor is predominantly c-Jun homodimers. It is also important to note that the effects of Li^+ in HL-60 cells are only observed at 48-72 hours of incubation.

5.3. The role of AP-1

Continuous expression of *c-fos* and *c-jun* has been reported during apoptosis *in vivo* (Smeyne *et al.,* 1993) and during treatment of various cell types with etoposide (Ritke *et al.,* 1994). In addition, antisense oligonucleotides directed against *c-fos* improve the survival of growth factor-deprived cells (Colotta *et al.,* 1992), and antibodies against the Fos family of proteins protect nerve growth factor-deprived neurons from apoptosis (Estus *et al.,* 1994). Lithium is known to activate AP-1 elements possibly through the inhibition of GSK-3β (Hedgepeth *et al.,* 1997; Klein and Melton, 1996). Although the levels of *c-jun* mRNA were not

demonstrated to change in this study, it is possible that the activation of AP-1 could mediate the lithium effects. To investigate this possibility an antisense *c-jun* vector was constructed and transfected into several cell lines to downregulate the levels of c-Jun. In Raji cells antisense *c-jun* inhibited growth while the viability remained high (Fig. 4.2.2, 4.2.3 and 4.2.4). Treatment of Raji cells with Li^+ (5 and 10 mM) blocked their growth, but the viability was only slightly ($p>0.05$) decreased. Surprisingly, the presence of antisense *c-jun* was additive to inhibition of growth observed with 5 mM lithium but not 10 mM lithium. Thus, the effects of antisense *c-jun* in Raji cells resembled those of lithium and no additional toxicity was observed under both conditions.

Plasmid pJMCS was initially constructed to provide controllable levels of antisense *c-jun* when contransfected with the pCMVLacI repressor vector (Stratgene, La Jolla, CA). However, these experiments were carried out with pJMCS alone which would express antisense *c-jun* constitutively. In HL-60 cells the introduction of antisense *c-jun* led to a loss in cell viability and proliferation potential both in the absence and presence of lithium (Fig. 4.2.5 and 4.2.6). It is suggested by the data that contrary to the observations in mesengial cells (Ishikawa *et al.,* 1997), c-Jun expression is required in HL-60 cells for survival and growth. Antisense *c-jun* transfection could not afford any form of protection against apoptosis caused by lithium. It seems unlikely that the removal of a gene required for growth (reviewed in Karin and Angel, 1991) could protect against cell death by apoptosis. However, the loss in cell viability in cells transfected with pJMCS was not associated with DNA fragmentation, indicating that antisense *c-jun* does not trigger an apoptotic cascade culminating in DNA fragmentation (Fig. 4.2.7). Because antisense *c-jun* expression inhibited growth of HL-60 cells even in the presence of the growth enhancing concentration (5 mM) of lithium, it is quite possible that lithium stimulates growth by activating AP-1 transcription factor, and it is possible to speculate that overexpression of *c-jun* would potentiate the growth effects of lithium. However, Ishikawa *et al.* (1997) reported improved survival of mesengial cells in the presence of oxidative stress when *c-jun* was downregulated with the

antisense expression of *c-jun* mRNA.

The level of inhibition of growth and viability by antisense *c-jun* in the HL-60 cells was more pronounced than in Raji lymphoma cells, although the transfection efficiency as determined by the level of expression of chloramphenicol acetyl transferase (CAT) in cells transfected with pOP13CAT plasmid was high (data not shown). This indicates the requirement of *c-jun* and AP-1 in cell survival and growth may differ in different cells.

The involvement of c-Jun/AP-1 in adipose differentiation is implied by the observation that TPA inhibits the adipose conversion of 3T3-L1 cells (Ueda *et al.,* 1991). Further, adipocyte differentiation of 3T3-L1 fibroblasts induced by MIX is associated with temporal elevation of *c-jun* mRNA but not the other AP-1 components (Angel and Karin, 1991). Lithium inhibits the inducer-stimulated differentiation of 3T3-L1 cells (Aratani *et al.,* 1987). The transfection of 3T3-L1 cells with the antisense *c-jun* did not affect their differentiation in the presence or absence of Li^+ (Fig. 4.2.1 and Table 4.2.1). The differentiation was uniform in both pOPRSVI/MCS and pJMCS transfected 3T3-L1 cells and thus, antisense *c-jun* mRNA expression and possible inactivation of AP-1 did not result in the reversal of lithium effects on differentiation of 3T3-L1 cells. However, a role of AP-1 for lithium induced blockage of 3T3-L1 differentiation could not be completely ruled out as the transfection efficiency with calcium phosphate coprecipitation was low. However, when 3T3-L1 cells were made transfected with antisense *c-jun* plasmid (pJMCS) using the DOTAP lipofection reagent (which results in 90-95% transfection efficiency) they died after 72 hours, suggesting that 3T3-L1 cells require *c-jun* expression for growth and survival (reviewed in Angel and Karin, 1991).

Several studies have connected apoptosis with the activation of the interleukin 1^{β}-converting enzyme/Ced-3 (ICE/Ced-3) family (Boldin *et al.,* 1996; Muzio *et al.,* 1996; Gamen *et al.,* 1997). The activation of ICE/Ced-3 family of proteases has

been suggested to represent the first irreversible step in the induction of programmed cell death (Steller, 1995; Oltvai and Korsmeyer, 1994; Fraser and Evan, 1996). For instance, the trimerization of Fas by its ligand FasL recruits several adaptor proteins to the receptor clusters. These proteins are known as the FADD/MORT1 and they bind to Fas intracellular domains to recruit one or several of the cysteine proteases with Asp specificity (caspases). The recruitment of these proteases leads to autocatalytic processing and activation of CPP32/Yama-like proteases, also called apoptotic executioners (Boldin *et al.,* 1996; Muzio *et al.,* 1996). Inhibition of CPP32 activity with DEVD-cho prevented Fas- and doxorubicin-induced apoptosis of Jurkat T cells, suggesting that apoptosis induced by Fas and doxorubicin is mediated by protease activation (Gamen *et al.,* 1997). One substrate that has been identified for the ICE/Ced-3 protease family is the enzyme PARP (Muzio *et al.,* 1996). Inhibitors of ICE/Ced-3 proteases have been shown to inhibit apoptosis in a variety of cell lines Muzio *et al.,* 1996). The involvement of caspases in Li^+- and minocycline-induced apoptosis in HL-60 cells has not been investigated. It would be interesting, based on the finding that some leukemic cell lines do not express functional Fas (Rouvier *et al.,* 1993) to demonstrate the role of caspases in HL-60 cells induced to undergo apoptosis.

This study demonstrated that minocycline and lithium induced cell death in HL-60 cells by the apoptotic pathway and did not require any new gene expression. However, the induction of apoptosis by these substances was not observed in 3T3-L1 and LLC-PK1 cells, and the reasons for the resistance are yet to be established. Furthermore, minocycline failed to induce DNA fragmentation in K562 cells. However, apoptosis induced by lithium was reported in K562 cells (Madiehe *et al.,* 1995). The induction of apoptosis in HL-60 cells by minocycline and lithium is independent of protein synthesis. In minocycline treated cells the removal of extracellular calcium potentiated the fragmentation of DNA in HL-60 cells and addition of exogenous Ca^{2+} conferred protection suggesting a role for extracellular Ca^{2+} in MIN-induced apoptosis. Whether MIN caused apoptosis by perturbation of Ca^{2+} metabolism is unclear. Bax *et al.* (1993) observed enhanced extracellular

Ca^{2+}-stimulated rise in cytosolic Ca^{2+} in osteoclasts treated with minocycline. However, minocycline failed to significantly influence TCR/CD3-stimulated intracellular Ca^{2+} mobilization in T cells (Kloppenburg *et al.,* 1995). The induction of DNA laddering by lithium was not affected by EGTA, suggesting that apoptosis in the presence of lithium is independent of extracellular calcium. However, activation of PKC with TPA led to the inhibition of apoptosis caused by lithium and minocycline. Two of the PKC target genes, *c-fos* and *c-jun* were investigated, and it is reported here that the levels of *c-jun* mRNA were not affected by either lithium or minocycline treatment, while *c-fos* mRNA was not detected. Antisense *c-jun* mRNA expression led to the inhibition of growth of HL-60 and Raji cells in the absence or presence of lithium. Apoptosis induced by lithium could not be reversed or inhibited by the expression of antisense *c-jun,* and even antisense *c-jun* itself induces very slight DNA fragmentation. It is possible that lithium stimulates growth of HL-60 cells by activating AP-1 based on the following: 1) Lithium activates AP-1 (Hedgepeth *et al.,* 1997; Klein amd Melton, 1995), 2) Lithium was shown to stimulate growth in HL-60 cells (Becker and Tyobeka, 1990), 3) and in this study, antisense *c-jun* inhibited lithium induced HL-60 cell proliferation.

Minocycline induced apoptosis is accompanied by a decrease in the level of expression of *bcl-2* mRNA. Lithium, on the other hand led to a decrease in the level of *bcl-2* mRNA at the growth enhancing concentration of 5 mM. However, at 15 mM Li^{+} induced apoptosis without causing a downregulation of *bcl-2* mRNA.

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