The effect of membrane active agents on human leukaemia cells

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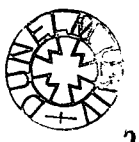
A Thesis submitted to the University of Durham
for the Degree of Doctor of Philosophy
Department of Biological Sciences,
University of Durham
(Graduate Society)

September 1998



I would like to dedicate this Thesis to my parents in appreciation of all their love, help and support over the years

Abstract

This Thesis investigates the effect of membrane-active agents, such as synthetic ether lipids (SEL), local anaesthetics and polyunsaturated fatty acids (PUFAs) on human leukaemia cells. The two cell lines used were human acute myeloblastic leukaemia (HL60) cells and human myelogenous leukaemia (K562) cells.

SEL, local anaesthetics and PUFAs were found to be cytotoxic to both cell lines at certain concentrations. The SEL ET-18-OCH_3 was found to be cytotoxic to both cell lines but the HL60 cells were found to be the more sensitive cell line. HL60 cells were found to be so sensitive to the action of the local anaesthetic dibucaine that a subtoxic concentration that killed $\leq 10\%$ was not determined. However, in K562 cells the combination of a subtoxic dibucaine concentration together with a range of ET-18-OCH_3 concentrations increased the cytotoxicity over that of ether lipid alone.

PUFAs were shown to incorporate into plasma membrane phospholipids at concentrations as low as 1μM after an incubation of 48 hours. PUFAs were shown to be cytotoxic, but the addition of vitamin E reduced the cytotoxicity of arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid in HL60 cells, and of docosahexaenoic acid in K562 cells. This implied that lipid peroxidation was involved in PUFA cytotoxicity. This was, however, not confirmed. PUFA in combination with ET-18-OCH_3 resulted in a slight decrease in cytotoxicity. PUFA combined with dibucaine did not alter cytotoxicity.

Cells were also treated with a combination of PUFA and 1-β-D-arabinofuranosylcytosine (ara-C), which is an agent known to induce cell differentiation. Onset of differentiation was determined by following haemoglobin accumulation in K562 cells. PUFA on their own were found to promote accumulation of haemoglobin. The greatest accumulation of haemoglobin was observed with K562 cells treated with PUFA and ara-C.

Declaration

No part of this Thesis has been previously submitted in support of an application for the degree of Doctor of Philosophy or equivalent qualification at the University of Durham, or any other University or Institute of Higher Education.

Acknowledgements

I would firstly like to thank Ray Manning, my supervisor, for the help and advice during my time in the laboratory and during the writing of this Thesis. Thanks to Judi for her friendship, help and support in the lab. Cell culture would not have been the same without you! Many thanks to John Horton, Ian Gravenor and Trudy Horton for help and advice with the FACS work; to Mirela for statistical advice; to Paddy for help with Chemical figures; to the BBSRC for the funding of this project. Thanks also to Andy Bell for printing the final copies.

I would like to thank all my friends in the lab. for their help, support and nights out over the years: Jenna, Sarah, Karen, Mark, Ian, Judi, Pam and Colin to name but a few.

I would like to thank my parents Nerys and Richard for all their support, encouragement and faith in me, and my brother Ian for being on the end of the 'phone, especially every time my computer crashed! Thanks also to Will for helping me cope with the writing of this thesis, for his constant encouragement and belief in my ability.

Contents

List of Abbreviations

AA	Arachidonic acid
ALA	Alpha-linolenate
ara-C	1-β-D-arabinofuranosylcytosine
BrdUrd	5'-bromo-2-deoxyuridine
BHT	Butylated hydroxytoluene
CT	Cytidylyltransferase
CTP	Choline-phosphate cytidylyltransferase
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulphoxide
DPH	1,6-Diphenyl-1,3,5-hexatriene
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
ET-18-OCH_3	1-*O*-octadecyl-2-methyl-*rac*-glycero-3-phophocholine
FACS	Fluorescence activated cell sorter
FAME	Fatty acid methyl esters
FCS	Foetal calf serum
FL3	flourescence of propidium iodide
FS	Forward scatter
GLA	Gamma-linolenic acid
HL60	Human acute myeloblastic leukaemia cells
HMBA	Hexamethylene bisacetamide
K562	Human myelogenous leukaemia cells
LA	Linoleic acid
NBT	Nitroblue tetrazolium
NDGA	Nordihydroguaiaretic acid
OA	Oleic acid

PAF	Platelet activating factor
PBS	Phosphate buffered saline
PGs	Prostaglandins
PGE_1	Prostaglandin E_1
PI	Propidium iodide
PK-C	Protein kinase C
PUFA	Polyunsaturated fatty acid
SS	Side scatter
TBARS	Thiobarbituric acid-reactive substances
TMA-DPH	1.4-trimethylaminophenyl-6-phenyl-hexa-1,3,5 triene

Materials

All reagents were of analytical grade unless stated otherwise.

Alltech / Applied Science, Carnforth, Lancashire

10% Alltech CS-5, on a chromasorb WAW support (100-120 mesh)

GLC fatty acid methyl ester standards (12:0 - 24:0)

Amersham International plc., Amersham, Buckinghamshire

^{3}H-thymidine

BDH Ltd., Poole Dorset,

Acetic acid

Chloroform

Zinc dibenzyldithiocarbamate

B.O.C. Ltd., Luton, Bedfordshire.

Nitrogen

Air

Hydrogen

Fisons Scientific Apparatus, Loughborough, Leicestershire.

Methanol

Gibco

FCS

L-glutamine

PBS

Penicillin

RPMI 1640 culture media without L-glutamine

Streptomycin

Flow Laboratories Ltd., Rickmansworth, Hertfordshire.

Saponin

Flukka

Propidium iodide

James Burrough (FAD) Ltd., Witham, Essex.

Absolute alcohol

All other reagents were obtained from Sigma Chemical Co., Poole, Dorset.

Chapter I
General Introduction

Leukaemia is characterised by accumulation of abnormal white blood cells in the bone marrow (Hoffband & Pettit, 1985). These abnormal white blood cells cause bone marrow failure and infiltrate other organs. Leukaemia is often thought of as a childhood disease. It can, however, afflict all age groups and there are many different forms of the disease.

Leukaemias are classified into acute and chronic leukaemia (Hoffband & Pettit, 1985). Acute leukaemia is subdivided into acute myeloid (myeloblastic) leukaemia (AML) and acute lymphoblastic leukaemia (ALL). AML is further divided into six variants of French-American-British (FAB) termed M_1-M_6. ALL is subdivided into three variants termed L_1-L_3. Chronic leukaemias comprise two main types, chronic granulocytic (myeloid) leukaemia (CGL) and chronic lymphocytic (lymphatic) leukaemia (CLL).

ALL and many cases of AML may be caused by clonal proliferation by divisions of a single abnormal blast cell. The cells fail to differentiate normally but are capable of further divisions. These abnormal cells accumulate and replace the normal haemopoietic precursor cells of the bone marrow and thus cause bone marrow failure (Hoffband & Pettit, 1985).

In the past, most anti-cancer treatments have attempted to inhibit DNA replication and tumour cell proliferation. However, over the last ten years, the plasma membrane has become a focus for drug development. The plasma membrane forms the interface between the external medium and the cytoplasm. The plasma membrane comprises

of a fluid matrix of membrane lipids containing intrinsic proteins responsible for the maintenance of ionic gradients, nutrient transport and signal transduction. The cell receives information through hormones, growth factors and chemicals that stimulate receptors found on the surface of the cell to start a cascade of events that in turn may alter the growth, secretion and replication of the cells. The receptors are able to translate extracellular stimuli into chemical, ionic and electrical intracellular events (Evans & Graham, 1991). These signal transduction pathways may be used to alter the growth and replication of tumour cells. The disruption of essential signalling pathways may lead to tumour cell death. A number of signalling pathways could be targets for anti-cancer drug development, including inhibition of growth receptor binding, serine and threonine protein kinases, tyrosine protein kinases and phospholipase C (Powis, 1991).

The structure and function of plasma membranes may be modulated either by compounds such as polyunsaturated fatty acids that become incorporated into membrane phospholipids (Wagner *et al.*, 1992) or by lipophilic agents that partition into the membrane lipid matrix, including synthetic ether lipids (Tidwell *et al.*, 1981) and local anaesthetics (Ohki, 1984). In this study the effects of these membrane-active agents on human leukaemia cells will be investigated.

Fatty acids are present as complex lipids (including phospholipids) in mammalian tissues and are obtained from dietary fat or through biosynthesis. There are two classes of polyunsaturated fatty acid that cannot be completely synthesised in mammals and are derived from the diet. These are the n-3 and n-6 fatty acids. Malignant cells derive all of their n-6 and n-3 PUFA from the tumour bearing host

(Burns & Spector, 1994). Therefore the type and quantity of PUFA present is dependant on the host, and the host's diet. By changing the PUFA in the diet the membrane phospholipid structure will be altered and this may modulate membrane function.

Supplementing the culture medium of cells with PUFA alters the fatty acid composition of the cellular phospholipids. HL60 cells supplemented with 32μM DHA for 2 days greatly increased the percentage of 22:6 in the membrane phospholipids, compensated for by a reduction in 18:1 (Wagner *et al.*, 1992). The fatty acids gamma-linolenic acid (GLA) and eicosapentaenoic acid (EPA) inhibited the proliferation of three colon cancers HRT 18, HT 29 and CACO 2 (Mengeaud *et al.*, 1992). The supplementation of these fatty acids into the culture medium at a concentration of 60μM increased membrane fluidity and induced lipid peroxidation of these three cell lines. When the antioxidant vitamin E was present with EPA or GLA membrane fluidity and the amount of lipid peroxidation was reduced (Mengeaud *et al.*, 1992). Membrane fluidity was examined using fluorescence probe and electron spin resonance.

As the membrane contains receptors the increased membrane fluidity could result in receptors not triggering a cascade of events and the loss of some signal transduction or alternatively triggering cascades inappropriately. This could in turn affect the response of the cells to cytotoxic agents.

The effect of different fatty acid supplementation on the cell growth of MDA-MB-231 breast cancer cells was reported by Rose & Connolly (1990). The MDA-MB-231 cells were supplemented with fatty acid for 6 days in serum-free culture medium. At a concentration of 0.75μg/ml LA was found to stimulate cell growth, this was also true for low concentrations of OA (0.25μg/ml). MDA-MB-231 cell growth

was inhibited by DHA (1-2.5μg/ml) and EPA (2.5 μg/ml). The stimulation of cell growth by LA was thought to be dependent on the inhibition of leukotriene biosynthesis. As inhibitors of leukotriene biosynthesis reduced the LA stimulus of cell growth (Rose & Connolly, 1990).

When HL60 cells were supplemented with either AA, EPA or DHA (20μM) for 25 hours over 70% of cells were killed (Hawkins *et al.*, 1998). When HL60 cells were supplemented with EPA (50μM) for 6-12 hours a pattern of chromatin degradation into oligonucleosomes occurred which is characteristic of apoptosis (Hawkins *et al.*, 1998). HL60 cells supplemented with PUFA for 4-5 hours were also shown to induce lipid peroxidation.

In this study HL60 and K562 cells were supplemented with PUFA to determine the effect on cell growth and the incorporation of the fatty acids in to the cell membranes. PUFA have been shown to increase membrane fluidity, lipid peroxidation and induce apoptosis. In this study other agents were combined with PUFA supplementation to determine if the effects of PUFA on leukaemia cells are altered.

Synthetic ether lipids have been the subject of several clinical trials with cancer and leukaemia patients. Initially the ether lipid 1-O-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine, commonly known as ET-18-OCH_3 or edelfosine, was used. Berdel *et al.*, (1987) partially characterised the tolerance to ET-18-OCH_3, eleven patients were treated intravenously with mild toxicity. Patients experienced a variety of side effects including gastrointestinal tract problems, liver toxicity, renal toxicity and a life-threatening interstitial pulmonary edema. All of these side effects were reversible when treatment stopped. ET-18-OCH_3 was also tested on patients with non-small cell lung cancer

(Berdel *et al.*, 1985). Herrman & Neumann (1987) tested another ether lipid, BM41.440, which was given orally in a multi-institutional phase I drug trial. This was then taken on to a phase II drug efficacy trial as a potential treatment for a wide spectrum of neoplastic diseases. Hexadecyl-phosphocholine has been used in phase II trails as a topical treatment of skin metastases in patients with breast cancer (Unger *et al.*, 1988). These clinical studies have shown tumour responses in a small number of patients treated. A cyclic analog of ET-18-OCH_3 , SRI 62-834 (Sandoz Research Institute), was entered into phase I trials and it was found that low doses were tolerated owing to limiting toxicity in the gastrointestinal tract. A clinical phase I/II study was undertaken by Berdel (1990) to assess safety and efficacy of bone marrow autotransplantation after supralethal chemotherapy and radiotherapy in patients with acute leukaemia using remission marrows purged with ether lipids *in vitro*. After clinical trials of patients with acute leukaemia, a dose of 75μg/ml of ET-18-OCH_3 was recommended for bone marrow purging (Vogler *et al.*, 1992). Purified blasts from patients with acute leukaemia were treated with one of two ether lipids, ET-18-OCH_3 and hexadecylphosphocholine (Verdonck & Heugten, 1997). A 4 hour treatment with 50μg/ml of either of the ether lipid effectively killed the leukaemic blasts.

The mechanism of action of ether lipids is unknown but they have been shown to alter various properties of the cell. Ether lipids have an affinity for membrane phospholipids (Noseda *et al.*, 1988a). HL60 and LLC-H61 cell lines treated with ET-18-OCH_3 have increased membrane fluidity (van Blitterswijk *et al.*, 1987). Ether lipids have been shown to increase intracellular free calcium (Lazenby *et al.*, 1990; Lohmeyer & Workman, 1993). Ether lipids have also been shown to be involved in the inhibition of protein kinase-C (Helfman *et al.*, 1983;

Kiss *et al.*, 1987; Shoji *et al.*, 1988). In HT29 colon adenocarcinoma cells, ether lipids progressively arrested the cells in G_1 and G_2 phases of the cell cycle although progression through S and M phases was not altered (Principe *et al.*, 1992). HL60 cells treated with ET-18-OCH_3 have displayed apoptotic cell death (Diomede *et al.*, 1993b; Alonso *et al.*, 1997). In HL60 cells ET-18-OCH_3 also stimulated free radical production but did not stimulate free radicals in K562 cells (Wagner *et al.*, 1998). Cells supplemented with PUFA that are treated with ether lipids can increase lipid peroxidation (Petersen *et al.*, 1992; Wagner *et al.*, 1992).

In this study both leukaemia cell lines were treated with the ether lipid ET-18-OCH_3 to determine its cytotoxic effects. The ether lipid was also combined with other treatments to determine any alterations in its cytotoxicity.

Local anaesthetics have been studied for over one hundred years yet the exact mechanism of action is unknown at a molecular level. Local anaesthetics can alter the physical properties of cellular membranes (Seeman, 1972). Local anaesthetics have been shown to interact with polar head groups of membrane phospholipids (Shimooka *et al.*, 1992) and they have also been shown to increase membrane fluidity (Seeman, 1972). For example, dibucaine has been shown to increase membrane fluidity as monitored by polarisation spectroscopy (Kingston *et al.*, 1993).

Local anaesthetics have been shown to induce cell death in SK-N-MC human neuroblastoma cells (NB cells) in a dose-dependent manner (Kim *et al.*, 1997). At a concentration of 0.1mM dibucaine was shown to increase significantly the membrane fluidity of both the inner

and outer membranes of NB cells at 20°C measured using fluorescence polarisation of DPH and TMA-DPH (Kim *et al.*, 1997). Dibucaine (0.1mM) was also shown to induce apoptosis, shown by the internucleosomal DNA fragmentation. This concentration of dibucaine was also shown to increase intracellular calcium, which was probably due to membrane damage allowing an influx of extracellular calcium (Kim *et al.*, 1997). Dibucaine (30μM) cytotoxicity to NB cells was reduced in the presence of the antioxidants L-ascorbic acid and L-cysteine suggesting that dibucaine-induced NB cell death involves the production of free radicals.

In this study the effect of local anaesthetics on leukaemia cells was determined. The local anaesthetics were combined with other agents to determine if any of their effects were altered.

The purpose of this study was to investigate the effects of three membrane-active agents both alone and in combination on leukaemia cells. These treatments were tested *in vitro* in culture on two human leukaemia cell lines, HL60 and K562 cells. The agents to be investigated were the synthetic ether lipid, ET-18-OCH_3, local anaesthetics (dibucaine, tetracaine and procaine) and polyunsaturated fatty acids, especially docosahexaenoic acid, eicosapentaeonic acid and arachidonic acid.

The two human leukaemia cell lines used in this study were HL60 and K562 cell lines. Human Leukaemia (HL60) cells were obtained in 1976 from the peripheral blood leukocytes of a 35 year old female patient with acute myeloblastic leukaemia with maturation (FAB-M2) (Collins *et al.*, 1977; Dalton *et al.*, 1988). HL60 cells were grown in suspension cultures, firstly in conditioned media and then in

the absence of conditioned media. The HL60 cells consist predominantly of promyelocytes, 5-10% of which spontaneously differentiate into more mature cells including myelocytes, metamyelocytes, and banded and segmented neutrophils (Collins *et al.*, 1980). K562 cells were derived from leukaemic cells obtained in 1970 from a pleural effusion of a 53 year old female who had been suffering from chronic myelogenous leukaemia for about 4 years (Lozzio & Lozzio, 1975). K562 cells were the first permanent cell-line with a persistent positive Philadelphia chromosome after prolonged cultivation *in vitro*. K562 cells have retained meaningful indicators of malignancy including chromosome aberrations, cloning efficiency on agar, and a specific antigen(s) after prolonged cultivation. It therefore represents a unique source of human CML cells for experimental and clinical studies. In 1979, Andersson *et al.* found that K562 cells synthesised and expressed glycophorin. Glycophorin is an integral membrane glycoprotein and is the major sialoglycoprotein of human erythrocytes. K562 cells were found to express the proteins of normal erythrocyte membranes, although they lack the surface expression of the HLA-antigen, which is also compatible with erythroid origin. K562 cells have a Natural Killer cell induced cytotoxicity which is highly compatible with the erythroid origin (Andersson *et al.*, 1979).

Both cell lines have been shown to have the ability to differentiate. HL60 cells can undergo growth arrest and differentiate into one of two functionally and morphologically distinct blood cell types (Collada-Escobar & Mollinedo, 1994). Dimethylsulphoxide (DMSO) and retinoic acid promote differentiation towards neutrophils, whilst vitamin D-3 and phorbol esters induce monocytic differentiation of HL60 cells. K562 cells can differentiate into immature myeloid or

lymphoid cells by hexamethylene bis-acetamide (Green *et al.*, 1993) but also along erythroid differentiation lineage by exposure to haemin, sodium butyrate or 1-β-D-arabinofuranosylcytosine (Andersson, 1979; Horton, 1983; Chen & Wu, 1994).

Chapter 2 will report the cytotoxic effects of the ether lipid ET-18-OCH_3 on HL60 and K562 cells. The cytotoxicity of the three local anaesthetics was also determined separately on both cell lines. Subtoxic doses of the local anaesthetics, defined as a concentration that kills ≤ 10% of cells, were used in combination with a range of ET-18-OCH_3 concentrations to discover if ether lipid cytotoxicity can be increased while using lower concentration of ether lipid with the local anaesthetic.

Chapter 3 will report the effect of PUFAs on both cell lines. The cytotoxicity of the different PUFAs was determined on both cell lines. The incorporation of the PUFAs into the membrane phospholipids was determined by gas-liquid chromatography. Combination experiments were undertaken in which : a subtoxic dose of PUFA was combined with a series of ether lipid concentrations, and subtoxic doses of PUFA were combined with a series of local anaesthetic concentrations.

Chapter 4 will report cell differentiation for K562 cells by a known differentiating agent 1-β-D-arabinofuranosylcytosine (ara-C). The induction of differentiation was monitored and the effects of PUFA on the possible induction of differentiation in the presence or absence of the differentiating agent was investigated. The progression of the K562 cells through the cell cycle will also be monitored after

treatment with PUFA and or differentiating agent using a fluorescence activated cell sorter (FACS).

Chapter II

Effects of Ether Lipids and Local Anaesthetics on Human Leukaemia Cells

2.1 Introduction

The chemical structure of the alkyl phospholipids (ether lipids) is closely related to platelet activating factor (PAF) (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). The general structure of the ether lipid consists of : (a) an ether-linked alkyl moiety at the *sn*-1 position; (b) an apparently nonmetabolisable group at the *sn*-2 position; and (c) a quaternary phosphobase at the *sn*-3 position of the glycerol moiety (Hoffman *et al.*, 1986). The ether lipid used in this study was ET-18-OCH_3 (1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine). Other ether lipids mentioned in this chapter include BM41.440, (1-hexadecylmercapto-2-methyl-*rac*-glycero-3-phosphocholine), a thioanalogue of ET-18-OCH_3 and various thiolysophospholipids. The structures of PAF, ET-18-OCH_3 and BM41.440 are shown in Figure 2.1.

Andreesen *et al.* (1978) discovered that cells from patients with chronic myelocytic leukaemia exhibited sensitivity to alkylphospholipids, one being ET-18-OCH_3. Tidwell *et al.*, (1981) found that two human leukaemia cell lines responded differently to ET-18-OCH_3; HL60 cells were found to be sensitive, whereas K562 cells were more resistant.

Andreesen *et al.* (1978) proposed that tumour cells had an inherently low alkyl cleavage enzyme, and therefore the ether lipid

would reach a concentration sufficient to inhibit vital cell functions and ultimately lead to cell death. This led Hoffman *et al.* (1986) to assay the activity of this alkyl cleavage enzyme in microsomes from HL60, K562 and MDCK cells. The HL60 cells were more sensitive to the ether lipids than the other two cell lines but the activity of their alkyl cleavage enzymes did not differ significantly. Therefore no relationship was found between the response of the cell lines to the ether lipid and the lack of alkyl cleavage enzyme. By using a radiolabelled ET-18-OCH_3 in intact cells, Hoffman *et al.* (1986) showed that ET-18-OCH_3 was associated more with HL60 cells than K562 cells. Autoradiography showed that radiolabelled ET-18-OCH_3 accumulated in the periphery of the HL60 cells, but was more uniformly distributed in K562 cells. Nontoxic PAF was shown to be evenly distributed throughout and rapidly metabolised by both HL60 and K562 cells. Hoffman *et al.* (1986) concluded that the enrichment of ether lipids in the surface membranes of the cell could inhibit the synthesis of 3-*sn*-phosphatidylcholine (Modolell *et al.*, 1979), Ca^{2+}-phospholipid dependent protein kinase (Helfman *et al.*, 1983), and sialyl-transferase (Bador *et al.*, 1983).

The mechanism of cytotoxic action of the ether lipids is unknown but they have been shown to alter various properties of the cell. Tumour cells grown in the presence of 1-*O*-alkyl-2-*O*-methyl-sn-glycero-3-phosphocholine showed alterations in the structural order of the membrane lipids (van Blitterswijk *et al.*, 1987). This treatment increased membrane fluidity in HL60 and LLC-H61 cell lines, the latter being a highly metastatic subclone of the Lewis lung carcinoma cell line. A number of ether lipids were also demonstrated to induce HL60 cells and mouse (M1) myeloid leukaemia cells to differentiate into mature granulocytes and macrophages, while no effect was observed on

normal mouse bone marrow cells (Honma *et al.*, 1991). Ether lipids were shown to have an affinity for membrane phospholipids (Noseda *et al.*, 1988a). Using electron spin resonance spectroscopy, the membrane fluidity of HL60 cells was found to be increased when treated with ET-18-OCH_3 and 1-thiohexadecyl-2-ethyl-*rac*-glycero-3-phosphocholine (ET-16S-OEt). ET-18-OCH_3 and five thio-*lyso*phospholipid analogs (TLP), including BM41.440, were tested on a number of cancer and leukaemia cell lines (Berdel *et al.*, 1983). The TLP revealed strong cytostatic and cytotoxic activity in human leukaemias and solid tumours.

SRI 62 -834 ((±)-2- {hydroxy [tetrahydro-2-(octadecyloxy)-methylfuran-2-yl}-phosphinyloxy}-*N,N,N,*-trimethylethaniminium hydroxide), the cyclic ether lipid analogue of ET-18-OCH_3, also elevated intracellular "free" calcium in HL60 and K562 cells (Lazenby *et al.*, 1990). The rise in calcium was inhibited by TPA which suggested that it may be modulated by protein kinase C. From this Lazenby *et al.* (1990) suggested that PAF receptors may be involved with the toxicity of this ether lipid. In serum free medium HL60 cells treated with subtoxic concentrations of ET-18-OCH_3 or SRI 62-834 were shown to increase the intracellular calcium concentration from internal stores (Lohmeyer & Workman, 1993). This increase in intracellular calcium was thought not to be linked to the antitumour activity of ether lipids. More recently ET-18-OCH_3 was shown to produce apoptosis in HL60 cells but not in DMSO differentiated HL60 cells (Alonso *et al.*, 1997). ET-18-OCH_3 induced an increase in intracellular calcium concentration in differentiated HL60 cells through the PAF receptor. In contrast to Lohmeyer & Workman (1993), undifferentiated HL60 cells do not have a PAF receptor and the intracellular calcium concentration was only slightly increased by ET-18-OCH_3 treatment (Alonso *et al.*, 1997).

When the following ether lipids, ET-18-OCH_3, ET-16S-OEt and 4-aminoethyl-1-[2,3-(di-*n*-decyloxy)-*n*-propyl]-4-phenylpiperidine (CP 46665), were combined with DNA-interactive agents such as adriamycin, 4-hydroperoxycyclophosphamide (4-HC) or cisplatin (CDDP) a marked additive inhibition of growth in BG1cells, a human ovarian adenocarcinoma, was observed (Noseda *et al.*, 1988b). ET-18-OCH_3 had been reported to inhibit the phosphorylation of PKC-substrate proteins in HL60, KG-1 and K562 cells (Helfman *et al.*, 1983; Kiss *et al.*, 1987). Shoji *et al.* (1988) found that BM41.440 was a potent and specific PKC inhibitor. The inhibition of PKC could be a critical factor as a pivotal role is played by this key phosphorylation system in biological processes including transmembrane signalling, cell growth and differentiation (Nishizuka, 1984). BM41.440 inhibited PKC competitively with respect to PS (phosphatidylserine) but noncompetitively with respect to Ca^{2+} (Shoji *et al.*, 1988), indicating that the thioether as well as ET-18-OCH_3 (Helfman *et al.*, 1983) interacted with site(s) on PKC also shared by PS but not Ca^{2+}. ET-18-OCH_3 and BM41.440 have been shown to cause short and long term antiproliferative activity on the colon adenocarcinoma cell line HT29 (Principe *et al.*, 1992). PAF had no similar potential. Flow cytometry showed that HT29 cells treated with ether lipids progressively arrested in G_1 and G_2 phase although progression through S and M phases were not altered. From these experiments Principe *et al.* (1992) suggested that ether lipids may, directly or indirectly, inactivate the complex p34 ^{cdc2}-cyclin that is essential for passage from G_1 to S and G_2 to M phases of the cell cycle.

A range of ET-18-OCH_3 concentrations from 5-100μg/ml significantly inhibited the reproductive ability of HL60 cells when incubated for either 1 hour or 4 hours, but under the same conditions

normal bone marrow cells were unaffected, as determined by clonogenicity (Vogler *et al.*, 1987). When normal bone marrow cells were subjected to cryopreservation and thawing, between 60 % and 79% of the colonies were recovered. This was also true for HL60 cells. However, the combination of cryopreservation after exposure to 50 μg/ml of ET-18-OCH_3 for 4 hours prevented the recovery of HL60. This experiment was repeated on a mixture of normal bone marrow and HL60 cells, resulting in increased killing of HL60 cells while preserving 60-70% of the progenitor cells. As a result of these experiments it was thought that ether lipids may be useful in bone marrow purging. A number of clinical trials have been undertaken with ether lipids. Patients suffering from widespread malignant disease were treated in a phase I pilot study with ET-18-OCH_3 (Berdel *et al.*, 1987) which partly characterised the tolerability to the ether lipid. Cancer patients were also treated with BM41.440, for over nine months, which gave a good indication of their tolerance to, and therapeutic effects of, BM41.440 (Herrman & Neuman, 1987). ET-18-OCH_3 and BM41.440 have undergone phase II clinical trials as reviewed by Berdel (1991). Clinical trials were undertaken on patients with acute leukaemia using remission bone marrows purged with ether lipids coupled with radiotherapy (Berdel, 1991). Vogler *et al.* (1992) recommended a bone marrow purging dose of 75μg/ml of ET-18-OCH_3 for 4 hours at 37°C after a study on patients with acute leukaemia. Koenigsmann *et al.* (1996) purged peripheral blood derived progenitor cells (PBPC) from a variety of cancer patients with ET-18-OCH_3 instead of purging the bone marrow, to reduce the haematological recovery time after high dose tumour therapy. The PBPC were treated with 75μg/ml for 4 hours. The *in vitro* recovery rate for CFU-GM after cryopreservation and purging was significantly reduced compared to cryopreservation alone.

These conditions led to a defined but predictable and tolerable toxicity. Koenigsmann *et al.* (1996) found that haematological recovery times after high-dose therapy were identically short provided similar amounts of PBPC were reinfused. Two ether lipids, ET-18-OCH_3 and hexadecylphosphocholine, were used to treat ten patients with acute leukaemias *in vitro* (Verdonck & Heugten, 1997). Purified blasts of the patients were placed in RPMI 1640 culture medium and treated with 10μg/ml or 50μg/ml of ether lipid for 4 hours. The cytotoxicity of the ether lipids as determined by leukaemic colony forming cells or by incorporation of ^{3}H-thymidine. Leukaemic blasts were effectively killed by both ET-18-OCH_3 and hexadecylphosphocholine. Verdonck *et al.* (1997) also tested the cytotoxic effect of the ether lipids *ex vivo* against multidrug resistance positive leukaemic blasts. ET-18-OCH_3 50μg/ml treatment for 4 hours produced 100% cytotoxicity for clonogenic leukaemia cells and almost 100% cytotoxicity for purified blasts of patients with drug-resistant ALL. The ether lipids were also shown to induce apoptosis within 15 minutes of treatment with 25μg/ml hexadecylphosphocholine. ET-18-OCH_3 has also been shown to produce apoptosis in HL60 cells but not in K562 cells. (Diomede *et al.*, 1993b & 1994). Apoptotic cell death was shown by the percentage of fragmented DNA. HL60 cells treated with 20μM ET-18-OCH_3 for 24 hours increased the fragmented DNA by 40%, the same treatment in K562 cells only increased fragmented DNA by 1.8% (Diomede *et al.*, 1993b).

Kelley *et al.* (1993) investigated the uptake of ET-18-OCH_3 by L1210 cells and found that it was reduced by approximately 50% when human serum was present at levels as low as 0.5% (v/v) serum. When [^{3}H]ET-18-OCH_3 was incubated with freshly obtained human serum, 84% of [^{3}H]-ET-18-OCH_3 was recovered associated with high density

lipoprotein or albumin (Kelley *et al.*, 1993). Therefore only 16% of the ET-18-OCH_3 would have been available to the cells, significantly reducing the concentration of ET-18-OCH_3 given. In L1210 cells, the uptake of ET-18-OCH_3 was nonsaturable, energy-independent and only moderately temperature sensitive, all of which are characteristic of passive diffusion. From this, Kelley *et al.* (1993) concluded that passive diffusion was a feasible mechanism to explain why this compound associates with cell membranes.

As ether lipids bind to serum it was decided to grow cells in serum-free culture medium in the present study so that the concentration in the culture medium reflected the concentration that was available to the cell.

The two human leukaemia cell lines used in this series of experiments were HL60 and K562 cells. The HL60 cells have been shown to be sensitive to synthetic ether lipids (SEL) and were found to contain nearly twice as much naturally-occurring membrane ether lipid than the K562 cells (Chabot *et al.*, 1989). When the membranes of the K562 cells were enriched with naturally-occurring ether lipid the sensitivity to ET-18-OCH_3 was increased, the IC_{50} value being reduced by approximately half. Furthermore, the cholesterol content of the cell membrane may affect the toxicity of ether lipids (Diomede *et al.*, 1992). For example, K562 cells (Tidwell *et al.*, 1981) are richer in cholesterol than the sensitive HL60 cells (Diomede *et al.*, 1990). Furthermore, K562 cells partially depleted of their membrane cholesterol content became sensitive to doses of ether lipid which were originally nontoxic (Diomede *et al.*, 1992). The cholesterol content of the culture medium was also related to the cytotoxic action of ether lipid, suggesting that the cholesterol from serum added to the medium may modulate the biological activities of these drugs (Diomede *et al.*,

1990).

The ether lipid BM41.440 was found to increase the sensitivity of cells, which had been supplemented with a polyunsaturated fatty acid, docosahexaenoic acid (DHA) (22:6, n-3), to lipid peroxidation (Petersen *et al.*, 1992). The chemical structure of ether lipids does not suggest that a free radical would be generated directly as a consequence of the metabolism of the drug. It is possible that free radicals may be generated indirectly as a secondary event of membrane damage and membrane fatty acids with increased numbers of double bonds are more susceptible to this secondary damage. In support of this, cytotoxicity of BM41.440 was found to be increased by prooxidants, such as Fe^{2+} plus ascorbic acid and by glutathione depletion (Petersen *et al.*, 1992). L1210 cells enriched with 22:6 n-3 showed an increase in lipid peroxidation measured by ethane production and TBARS when the cofactors ascorbic acid and Fe^{2+} were present with ET-18-OCH_3 (Wagner *et al.*, 1992). Only trace amounts of ethane and TBARS were generated when ET-18-OCH_3 was present in 22:6 enriched cells without the cofactors.

ET-18-OCH_3 (6μM) induced apoptosis in HL60 cells and U937 human myeloid leukaemia cells but not in DMSO-induced differentiated HL60 cells (Alonso *et al.*, 1997). Apoptosis was determined by degradation of DNA into oligonucleosome-size fragments on gel electrophoresis. The effect of ET-18-OCH_3 on intracellular calcium concentrations was also determined. The apoptotic effect of ET-18-OCH_3 was not related to changes in the intracellular calcium concentration.

In HL60 cells, ET-18-OCH_3 stimulated free radical production at 20μM (Wagner *et al.*, 1998), K562 cells treated with up to 40μM ET-18-OCH_3 did not produce free radical production. Wagner *et al.* (1998)

showed that sensitive HL60 cells had more polyunsaturated fatty acids in membranes than resistant K562 cells. As the fatty acids in the membranes are a target for the production of free radicals, HL60 cells may produce more free radicals after ether lipid treatment than K562 cells.

Local anaesthetics have been shown to affect cells in many different ways. Local anaesthetics act on cell membranes causing membrane expansion (Seeman, 1972), modification of erythrocyte osmotic fragility (Roth & Seeman, 1971), inhibition of cell fusion (Poste & Reeve, 1972), impairment of mitochondrial respiration (Tarba & Cracium, 1990), inhibition of Ca^{2+} influx and prolactin secretion (Wagner *et al.*, 1992), inhibition of $Ca^{2+}Mg^{2+}$ATPase activity (Garcia-Martin & Gutierrez-Merino, 1990), displacement of Ca^{2+} from membranes (Chen, 1974), and inhibition of cell fusion (Poste & Reeve, 1972). Their mode of action is not well understood, but they have been shown to interact with polar head groups of membrane phospholipids (Shimooka *et al.*, 1992) and to increase membrane fluidity (Seeman, 1972; Paterson *et al.*, 1972). Correlation between biological effects and oil : water partition coefficients of local anaesthetics suggest that hydrophobic sites, perhaps involving the membrane lipid matrix, may be an important site of action (Kingston *et al.* ,1993).

In this study the local anaesthetics used were dibucaine, tetracaine and procaine; these tertiary amine local anaesthetics needed to be maintained predominantly in their cationic form at a pH below 7.5 as the pKa values of these anaesthetics are around pH 7.8 (Low *et al*, 1979). Under these conditions, the order of membrane surface adsorption of these cationic forms of local anaesthetics at any given concentration has been shown to be dibucaine > tetracaine > procaine

(Ohki, 1984). The order of toxicity in HTC cells was shown to be dibucaine > tetracaine > procaine (Kingston *et al.*, 1993). In HTC cells, all three anaesthetics enhanced the cytotoxic effects of hyperthermia, and cell survival was decreased as the anaesthetics concentration increased. Dibucaine was shown to affect plasma membrane fluidity, specifically at the acyl region of the membrane, by polarisation spectroscopy, using DPH as a probe (Kingston *et al.*, 1993). Whereas tetracaine caused a smaller concentration-dependent increase in membrane fluidity at 37°C only and procaine did not fluidise the membrane. However, when TMA-DPH was used as a fluorescent probe procaine was found to affect membrane fluidity (Dynlacht & Fow, 1992). The TMA-DPH probe reports fluidity from the polar headgroup region (Mateo *et al.*, 1991). Therefore Kingston *et al.* (1993), concluded that procaine and perhaps tetracaine at 43°C may interact with the polar headgroups.

Local anaesthetics were shown to induce cell death in SK-N-MC human neuroblastoma cells (NB cells) in a dose dependant manner (Kim *et al.*, 1997). The order of cytotoxicity was dibucaine > tetracaine > procaine. NB cells treated with 0.1mM dibucaine for 18 hours induced internucleosomal DNA fragmentation. The fragmentation patterns were consistent with apoptotic cell death. Dibucaine was also shown to increase membrane fluidity using membrane polarisation with DPH and TMA-DPH probes in the dose range which induced apoptosis (Kim *et al.*, 1997). Dibucaine (0.1mM) increased intracellular calcium concentration which resulted partly from intracellular sources (Kim *et al.*, 1997). When the influx of extracellular calcium was prevented NB cell death was reduced. Kim *et al.* (1997) suggested that dibucaine-induced cell death was due to the increase of intracellular calcium due to membrane damage. Apoptotic dibucaine-induced cell death was also

shown to involve the production of oxygen free radicals (Kim *et al.*, 1997).

HL60 cells have been shown to be sensitive to ether lipids whereas K562 cells were more resistant. Both these cells lines were used in the present study to examine the cytotoxic effects of the ether lipid ET-18-OCH_3, and local anaesthetics dibucaine, tetracaine and procaine, alone and in combination with ET-18-OCH_3. Cells were supplemented with ET-18-OCH_3 in serum-free culture medium as ether lipids bind to serum proteins. Cells were supplemented with a local anaesthetic in serum-free culture medium, as experiments combining local anaesthetics with ether lipids were planned.

Figure 2.1 Structures of ether lipids

The structures of three ether lipids that are referred to in this thesis are shown in this Figure.

ET-18-OCH_3

ET-18-OCH_3 (1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine) is the synthetic ether lipid used to treat cells in these experiments.

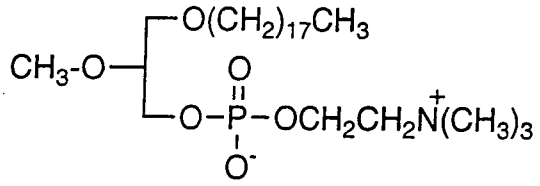
BM41.440

BM41.440 is another synthetic ether lipid is frequently referred to in this study (1-hexadecylmercapto-2-O-methyl-*rac*-glycero-3-phosphocholine).

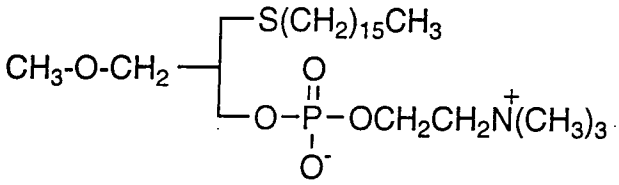
PAF

Platelet activating factor (PAF) (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a naturally occurring biologically active phospholipid. The structure of the synthetic ether lipids are closely related to PAF's structure.

ET-18-OCH$_3$



BM41.440



PAF

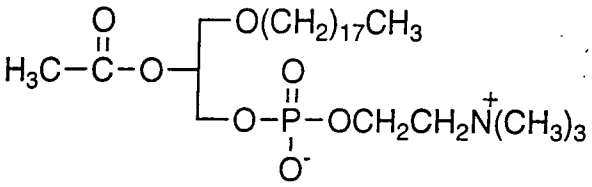
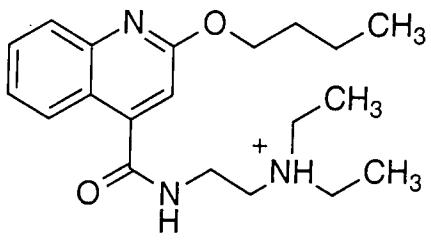


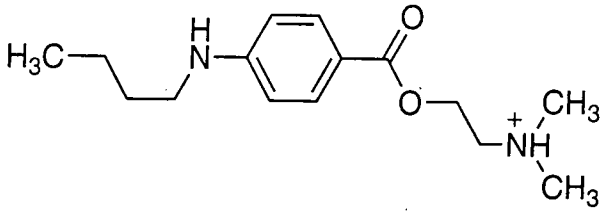
Figure 2.2 Structures of local anaesthetics

Three tertiary amine local anaesthetics were used in this series of experiments. The chemical structures of dibucaine, tetracaine and procaine are shown here.

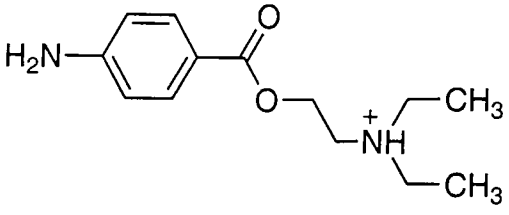
Dibucaine



Tetracaine



Procaine



2.2 Materials and Methods

2.2.1 Culture conditions

The present study utilised HL60 and K562 human leukaemia cell lines. The HL60 cells of unknown passage number were a kind gift from C.M. Bunce at Birmingham University and the K562 cells of unknown passage number were purchased from European Collection of Animal Cell Cultures, Porton Down, Salisbury.

The HL60 and K562 cell lines were grown in suspension culture in plastic flasks and 24-well plates under sterile conditions. Both cell lines were grown in RPMI-1640 medium (without L-glutamine) which was supplemented with heat-inactivated foetal calf serum (10%, v/v), 2mM L-glutamine, penicillin (100IU/ml) and streptomycin (100μg/ml). The culture medium was filter-sterilised before use. The cells were maintained in an humidified incubator at 37°C in air / CO_2 (19:1, v/v). All the cell-handling procedures took place in a class II flow cabinet. Implements used for culturing the cells were purchased sterile or were autoclaved before use.

2.2.2 Culture techniques

Stock supplies of HL60 and K562 cells were grown in 10 ml of culture medium in 25cm^2 flasks, 50 ml of culture medium in 70cm^2 flasks or 100 ml of culture medium in 125cm^2 flasks. Cells were also grown in 1ml of culture medium per well in 24 well plates. For exponential growth, the cell number was kept between 2x10^5 and 1x10^6 cells/ml for both cell lines. The cell lines were seeded at 2x10^{5}cells/ml, either by dilution or pelleting by centrifugation at 300g

for 4 minutes at 20°C, followed by resuspension in fresh culture medium. After pelleting, the HL60 cells were resuspended in 1ml of culture medium by one or two strokes of a syringe fitted with 0.5mm internal diameter hypodermic needle, before adding the required volume of culture medium. HL60 cells were diluted or pelleted every alternate day. After pelleting, the K562 cells were resuspended in a small volume of culture medium using a 5ml or 10ml pipette rather than a syringe and needle. K562 cells had their culture medium changed on the third day and were diluted every second day thereafter.

2.2.3 Estimation of cell number and viability by haemocytometer

Cells were first mixed using a 5ml or 10ml pipette to achieve a homogeneous cell suspension. A 100µl sample of cell suspension was taken and placed into a 1.5ml centrifuge tube. An equal volume of trypan blue solution (0.6%, w/v trypan blue, in phosphate buffered saline (PBS)) was added to the cells and mixed to achieve a homogeneous suspension. A portion of this cell suspension was pipetted into the haemocytometer chamber, the cells were observed on an Olympus inverted microscope at x 10 magnification. The cells lying in the five large squares at the centre of the grid were counted, between 25-75 cells were found in each of the five squares. Viable cells exclude trypan blue, so to obtain the number of viable cells only unstained cells were counted. To obtain the total cell number both stained and unstained cells were counted. A minimum of two counts were performed on each cell suspension.

2.2.4 Cryopreservation

Cells were counted to determine cell number, as described in section 2.2.3, and pelleted by centrifugation at 300g for 4 minutes at 20°C. The cells were resuspended in a freezing mixture, comprising heat inactivated foetal calf serum/glycerol; (92:8, v/v) at a cell density of $1x10^6$ cells/ml for HL60 cells and $8x10^5$ cells/ml for K562 cells. Aliquots (1ml) of the cell suspension were pipetted into plastic freezing vials and placed into a freezing container, containing isopropyl alcohol. The freezing container achieved a freezing rate of -1°C/min when placed at -80°C for a minimum of 5 hours. After this time the vials were stored in liquid nitrogen containers at -196°C.

2.2.5 Thawing

Vials of frozen cells were thawed quickly by semi-immersion in a beaker of warm water. Cells (1ml) were then added to 9ml of culture medium containing 20% (v/v) heat-inactivated foetal calf serum and placed in an humidified incubator at 37°C in air / CO_2 (19:1, v/v). Once the cells had successfully doubled in number they were pelleted by centrifugation, at 300g for 4 minutes at 20°C, and resuspended in culture medium containing 15% (v/v) heat-inactivated foetal calf serum. The percentage of foetal calf serum was reduced in this way until the cells grew in culture medium containing 10% (v/v) serum. The cells were then growing exponentially and were ready for experimentation.

2.2.6 Cell growth

Doubling Time

Cells were grown in culture medium over a period of 14 days, and were kept between $2x10^5$ and $1x10^6$ cells/ml to ensure exponential growth. The cells were counted every 24 hours, as described in section 2.2.3.

Cells were grown in serum-free culture medium for 4 hours when using ether lipids. Cell growth was checked to determine if this length of time in serum-free culture medium affected growth. Cells ($5x10^5$) were seeded in either culture medium or serum-free culture medium and incubated for 4 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). After 4 hours the cells were counted as described in section 2.2.3.

Cell growth in ^{3}H-thymidine

Cells were seeded in culture medium at different cell densities ranging from $0\text{-}1x10^6$ cells/well in a 96-well plate. Culture medium containing ^{3}H-thymidine, 0.2μCi, was added to the cells so that the total reaction volume was 200μl. The 96-well plate was then incubated for 3 hours in an humidified incubator at 37°C in air/CO_2 (19:1, v/v). After 3 hours the cells were harvested using a Skatron AS harvester. The cells were lysed by water onto Whatman glass fibre paper. The fibre paper was dried in an oven, and the discs corresponding to the wells were placed into scintillation vials, 4ml of Betafluor was added, and the radioactivity counted by liquid scintillation counting. The vials

were wiped with methanol to eliminate any counts owing to static. Each sample was counted for 5 minutes.

2.2.7 Ether lipid cytotoxicity experiments

The ether lipid ET-18-OCH_3 was added to the culture medium of HL60 and K562 cells. A stock solution of ET-18-OCH_3 (10mM) was prepared in 100% (v/v) ethanol and filter-sterilised. It was stored for a maximum of two weeks in a glass container at -20°C.

Ranges of ether lipid concentrations were prepared by serial dilution in 100% (v/v) ethanol. The required concentration of ether lipid was added to the serum-free culture medium so that the ethanol was present as 0.1% (v/v) of the culture medium. The concentration range of ET-18-OCH_3 was between 0-10μM for HL60 cells and 0-15μM for K562 cells.

Cells ($5x10^5$) were seeded in 1ml of serum-free culture medium containing a range of ET-18-OCH_3 concentrations and 0.1% (v/v) ethanol final concentration, in 24 well plates, and were incubated for 4 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). The control wells contained 0.1% (v/v) ethanol in the serum-free culture medium. After 4 hours the cells were pelleted by centrifugation at 300g for 4 minutes at 20°C, and resuspended in 1ml of fresh culture medium. The control untreated cells were counted, as described in section 2.2.3. From the control cell suspension a known volume containing $5x10^4$ cells was transferred to a 96-well plate. An equivalent volume was transferred from the ether lipid-treated cell suspension to a 96-well plate. These cells were incubated in culture medium containing ^3H-thymidine as described in section 2.2.6.

2.2.8 Local anaesthetic cytotoxicity experiments

Both cell lines were treated with three local anaesthetics. Stock solutions of dibucaine-hydrochloride (20mM), tetracaine-hydrochloride (100mM) and procaine-hydrochloride (500mM) were prepared in distilled water and were filter- sterilised. They were stored for a maximum of two weeks at 4°C.

Ranges of local anaesthetic concentrations in distilled water were prepared by serial dilution of the stock concentrations. The required volume of local anaesthetic was added to the serum-free culture medium so that the distilled water was present as 0.1% (v/v) of the culture medium. Addition of these local anaesthetics did not affect the pH of the culture medium. The concentration range of the three local anaesthetics on both cell lines was between 0-4mM.

Cells ($5x10^5$) were seeded in 1ml of serum-free culture medium containing a range of local anaesthetic concentrations, in 24-well plates. The control wells contained 0.1% (v/v) distilled water in the serum-free culture medium. The cells were incubated for 4 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v), and then were pelleted by centrifugation at 300g for 4 minutes at 20°C. The cells were resuspended in culture medium, and were incubated with ^{3}H-thymidine as described in section 2.2.6, and counted as described in section 2.2.3.

2.2.9 Combination cytotoxicity experiments with ether lipid and local anaesthetic

A subtoxic dose of local anaesthetic, defined as that concentration which killed ≤ 10% of cells, was determined for dibucaine, tetracaine and procaine. At low concentrations, dibucaine proved to be very cytotoxic to HL60 cells and therefore a subtoxic dose was not used. The HL60 cells were incubated with 0.2mM tetracaine or 4mM procaine. The K562 cells were incubated with 0.1mM dibucaine, 0.15mM tetracaine or 2mM procaine.

Cells ($5x10^4$) were seeded in 1ml of serum-free culture medium in each well, in a 24-well plate. One set of cells was incubated in serum-free culture medium containing ether lipid concentrations ranging between 0-20μM in the absence of local anaesthetic. The other set of cells were incubated in culture medium containing a subtoxic concentration of local anaesthetic plus ether lipid concentrations ranging between 0-20μM. The cells were incubated for 4 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). After 4 hours the cells were pelleted by centrifugation at 300g for 4 minutes at 20°C, and resuspended in culture medium. The cells were incubated with ^{3}H-thymidine as described in section 2.2.6, and counted as described in section 2.2.3.

2.2.10 Statistical analysis

In this study the incorporation of ^{3}H-thymidine into the K562 and HL60 cells was used to determine the cytotoxic effects of the ether lipids and local anaesthetics. The control cells were taken to be a 100% value and the other conditions were determined as a percentage of the control. To ensure the percentage values were normally distributed this

data was transformed using arcsine and then analysed statistically using Oneway ANOVA. In subsequent chapters, when the data is given in percentage form, that data is also transformed using arcsine and then statistically analysed using Oneway ANOVA.

2.3 Results

2.3.1 Growth conditions

HL60 and K562 cells were routinely cultured in RPMI-1640 culture medium containing 10% (v/v) heat-inactivated foetal calf serum, but when the cells were first taken out of liquid nitrogen it proved more successful to grow the cells in culture medium containing 20% (v/v) heat-inactivated foetal calf serum. This was especially true for the HL60 cells. The percentage of serum was then reduced sequentially until the cells grew well in culture medium containing 10% (v/v) serum. The K562 cells responded quickly to the growth conditions and were available for experimentation in one week, whereas the HL60 cells grew much more slowly when thawed and took two weeks before they grew exponentially.

The doubling time of both of the cell lines was established over a period of 14 days. The cell growth over that time is shown in Figure 2.3. The doubling time of the HL60 cells was 24 hours whereas that of the K562 cells was slightly longer at 28 hours.

Initially, the cytotoxic effect of ether lipids differed greatly from one experiment to another when experiments were performed in the presence of serum, probably because ether lipids bind to serum proteins (Kelley *et al.*, 1993). It is possible, therefore, that the majority of the ether lipid remained bound to serum proteins, explaining the variable cytotoxicity of this agent in different experiments.

Subsequently, it was decided to expose cells to ET-18-OCH_3 in serum-free culture medium, in order to avoid the sequestration of the ether lipid by serum proteins. However, it is possible that growth in serum-free conditions could adversely affect cell growth and viability,

Figure 2.3 Cell growth

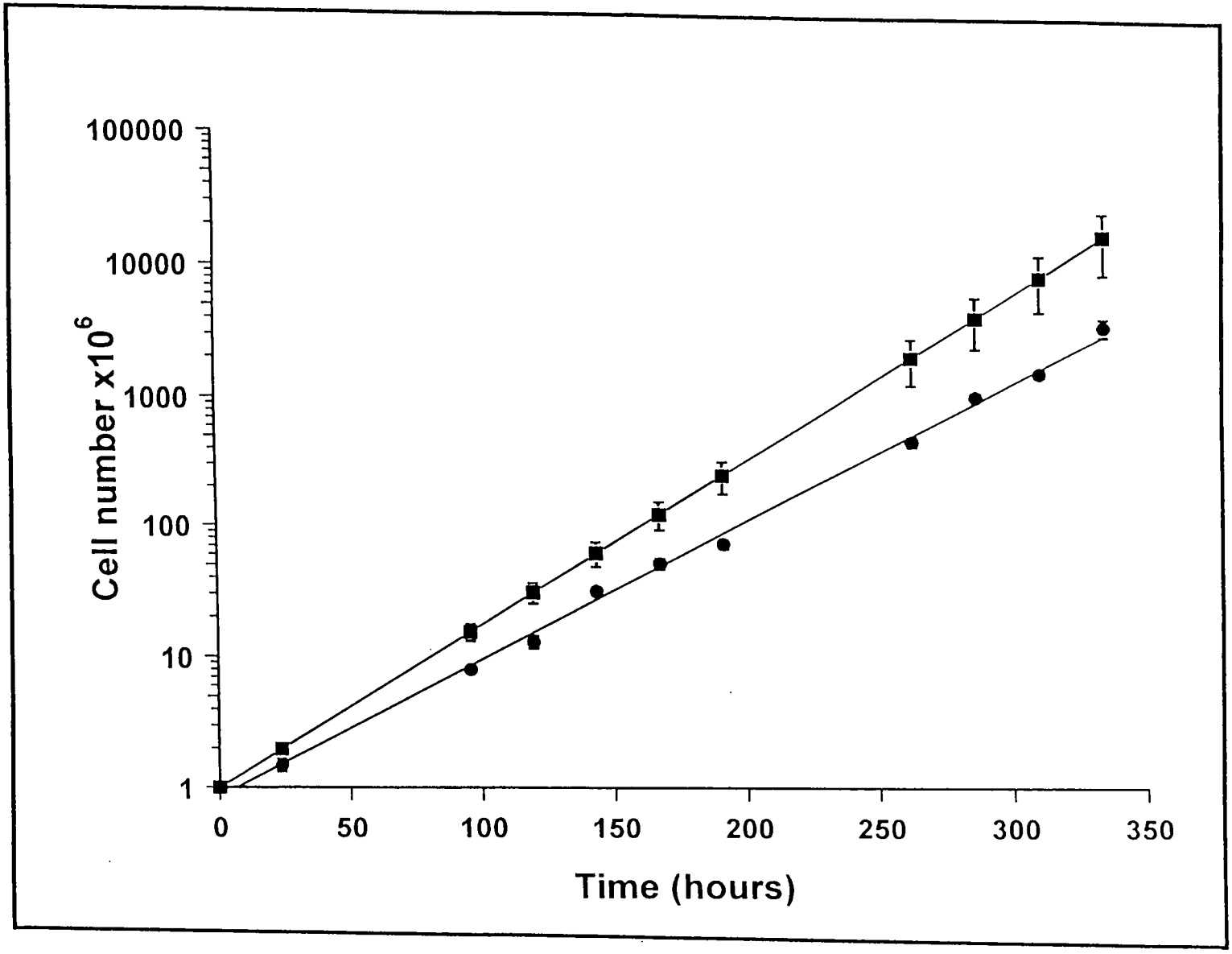
Cells were grown exponentially in RPMI-1640 culture medium. The cell growth over a period of 14 days was determined, as described in section 2.2.6.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

■ HL60 cells

● K562 cells

The HL60 cells doubled in cell number in 24 hours.
The K562 cells doubled in cell number in 28 hours.


100000
10000
1000
100
10
1
Cell number x10^6
0
50
100
150
200
250
300
350
Time (hours)

so it was important to minimise the period of exposure to serum-free conditions. Further experiments demonstrated that both HL60 and K562 cells could be grown in serum-free culture medium for 4 hours with no adverse effects on cell number and viability, as shown in Table 2.1. Therefore, in subsequent experiments, cells were exposed to ether lipid in serum-free conditions for a period of 4 hours.

2.3.2 Cytotoxic effects of ET-18-OCH_3 on HL60 and K562 cell lines

The cytotoxic effects of the ether lipids on both cell lines was initially determined by trypan blue exclusion. A more accurate method of determining the reproductive ability of the cells was required, and the incorporation of ^{3}H-thymidine was used. The seeding density and amount of ^{3}H-thymidine to be used was determined and an incubation of 50,000 cells/well with 0.2μCi ^{3}H-thymidine for 3 hours was chosen, this gave counts in the region of 50,000 - 100,000 dpm .

Routinely, cells were exposed to various concentrations of ET-18-OCH_3 in serum-free medium for 4 hours, then centrifuged and resuspended in fresh medium. A portion of this cell suspension was incubated with ^{3}H-thymidine for 3 hours, as described in section 2.2.6. As results from experiments undertaken on different days needed to be compared, the counts needed to be standardised as they differed between experiments. Therefore, control cells were taken to incorporate 100% ^{3}H-thymidine, and from this value the other conditions were determined as a percentage of control. Table 2.2 shows that the incorporation of ^{3}H-thymidine and the number of viable cells decreased as the concentration of ether lipid was increased. These two methods can be used to show that the ether lipid is inhibiting cell growth. The

Table 2.1 Comparison of cell growth in culture medium and serum free culture medium

Cells ($5x10^5$) were grown in RPMI culture medium of in serum free RPMI-1640 culture medium for 4 hours as described in section 2.2.6. After 4 hours the cells were counted as described in section 2.2.3.

The cell number and viability were determined. The results of three separate experiments are summarised. Each result is the mean value of three replicates from one experiment, the standard deviation for each value was less than 5%.

Cell line and culture medium grown in	Total cell number ($x10^5$ cells/ml)	Viability %
HL60 cells grown in culture medium	5.48	99
	5.42	100
	5.46	99
HL60 cells grown in serum free culture medium	5.47	99
	5.42	98
	5.44	100
K562 cells grown in culture medium	5.35	99
	5.37	99
	5.33	100
K562 cells grown in serum free culture medium	5.34	99
	5.35	100
	5.33	98

Table 2.2 The correlation between ^{3}H-thymidine incorporation by cells and cell viability

HL60 cells were incubated with a range of ET-18-OCH_3 concentrations 0-15μM for 4 hours in serum-free culture medium, as described in section 2.2.7. The incorporation of ^{3}H-thymidine into the control, untreated cells, was taken to be a 100% value and the incorporations in other conditions were determined as a percentage of the control. The viability of the cells was determined as described in section 2.23.

Concentration of ET-18-OCH_3 μM	Number of viable cells x10^5 cells/ml	Incorporation of ^{3}H-thymidine as a percentage of control
0.0	5	100
0.5	4.7	81
1.0	4.2	77
2.5	3.5	74
5.0	2.1	41
7.5	0	0
15.0	0	0

cell counts give a record of the number of cells that do not take up the trypan blue stain at the end of the experiment and are therefore viable cells. The ^{3}H-thymidine shows how many cells within the population are able to replicate. The ^{3}H-thymidine incorporation results were believed to be a more accurate method of determining the effect on cell growth by membrane active agents, as it shows the percentage of cells able to reproduce. This method was used to show the effect of membrane active agents on the K562 and HL60 cells' reproductive ability.

As ether lipid was delivered to the cells in 100% (v/v) ethanol and was present in the culture medium as 0.1% (v/v) ethanol, the control wells contained 0.1% (v/v) ethanol in the serum-free culture medium. From cell counts, viability and ^{3}H-thymidine incorporation this had no effect on cell growth as shown in Table 2.3.

Figure 2.4 shows the effects of ET-18-OCH_3 on both cell lines, determined by the incorporation of ^{3}H-thymidine. On exposure to ET-18-OCH_3 for 4 hours, the reproductive ability of the HL60 cell line was more susceptible to the effects of the ether lipid than the K562 cell line. The reproductive ability of the HL60 cells was markedly reduced at 2.5μM ET-18-OCH_3 and was significantly different ($p < 0.05$) from the K562 cell line at 5μM and 7.5μM. At 7.5μM the ether lipid had completely destroyed the reproductive ability of the HL60 cells compared with 15μM for the K562 cells. This was reflected in the IC_{50} values of 4.11μM for HL60 cells and 8.53μM for K562 cells.

Table 2.3 Effect of 0.1% ethanol in culture medium on cell growth and ^{3}H-thymidine incorporation

Cells ($5x10^5$) were seeded in 1ml of either serum-free culture medium or serum-free culture medium containing 0.1% ethanol, in 24 well plates. The cells were incubated for 4 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). After 4 hours the cells were pelleted as described in section 2.2.7. The cells were then counted as described in section 2.2.3 and the incorporation of ^{3}H-thymidine was determined as described in section 2.2.6. The incorporation of ^{3}H-thymidine into the control, untreated cells, was taken to be a 100% value and the other conditions were determined as a percentage of the control.

The results of three separate experiments are summarised. Each result is the mean value of three replicates from one experiment, the standard deviation for each value was less than 5%.

Cell line and culture medium	Total cell number ($x10^5$cells/ml)	Incorporation of ^{3}H-thymidine as a percentage of control
HL60 serum-free culture medium	5.44 5.48 5.45	100 100 100
HL60 serum-free culture medium + 0.1% ethanol	5.44 5.47 5.46	97 101 99
K562 serum free culture medium	5.34 5.33 5.35	100 100 100
K562 serum-free culture medium + 0.1% ethanol	5.33 5.34 5.34	101 97 98

Figure 2.4 Cytotoxic effects of ether lipids

Cells were incubated with a range of ET-18-OCH_3 concentrations 0-15μM for 4 hours in serum-free culture medium, as described in section 2.2.7. The incorporation of ^{3}H-thymidine into the control, untreated cells, was taken to be a 100% value and the other conditions were determined as a percentage of the control.

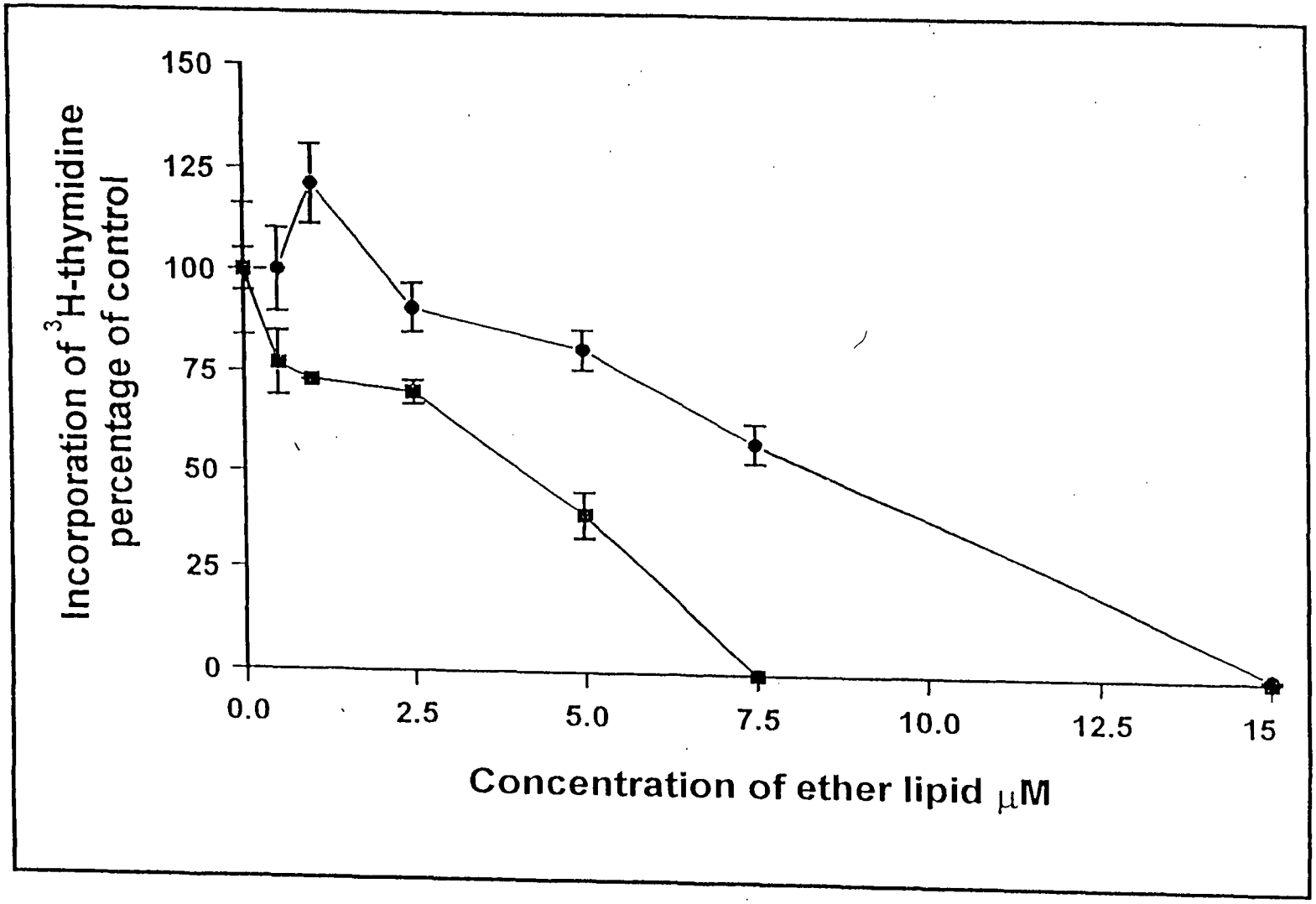
Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

- ■ HL60 cells
- ● K562 cells

The IC_{50} values were 4.11μM for HL60 cells and 8.53μM for K562 cells.

Oneway ANOVA was used to compare the incorporation of ^{3}H-thymidine into HL60 cells treated with ET-18-OCH_3 with the incorporation of ^{3}H-thymidine into K562 cells treated with ET-18-OCH_3.

Significant differences were found between the treatments at 5μM and 7.5μM ($p < 0.05$).


Incorporation of ^{3}H-thymidine
percentage of control
150
125
100
75
50
25
0
0.0
2.5
5.0
7.5
10.0
12.5
15
Concentration of ether lipid μM

2.3.3 Cytotoxic effects of local anaesthetics on K562 and HL60 cell lines

Figures 2.5 and 2.6 show that both the HL60 and K562 cell lines have similar sensitivities to the effects of the three local anaesthetics. The order of cytotoxicity for both cell types was : dibucaine > tetracaine > procaine. The IC_{50} values for dibucaine were very similar for both cells lines; 0.28mM for HL60 cells and 0.22mM for K562 cell line. The initial response to the dibucaine was more severe for the HL60 cells. At a very low concentration of dibucaine (13μM) 28% of the HL60 cell line lost their reproductive ability. As the cells were very sensitive to low concentrations of dibucaine a subtoxic dose was not determined for this local anaesthetic. In the HL60 cell line, the cytotoxic effect of dibucaine increased with concentration but was not as severe as the initial response. Although the IC_{50} value was similar for both cell lines the K562 cell line was affected to a greater extent as the concentration of dibucaine was increased. At a dibucaine concentration of 0.8mM all the K562 cells had lost their reproductive ability compared to 2mM for the HL60 cells. Therefore, although initially the HL60 cells seemed to be affected by a low concentration of dibucaine the K562 cells proved to be more sensitive to higher concentrations. Tetracaine produced a similar effect on the reproductive ability of both cell lines. The IC_{50} value was 0.64mM for K562 cell line and 0.68mM for HL60 cell line. At lower concentrations (0.5mM) of tetracaine the reproductive ability of the HL60 cells was higher than the K562 cells. As the concentration of tetracaine increased both cell lines lost reproductive ability. At 2mM all the K562 and HL60 cells had lost their reproductive ability. The cytotoxic effect of procaine is not fully shown in Figures 2.5 and 2.6.

Figure 2.5 Cytotoxic effects of local anaesthetics on K562 cells

Cells were incubated with dibucaine, tetracaine or procaine (0-2mM) for 4 hours in serum-free culture medium, as described in section 2.2.6, then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine into the controls was taken to be a 100% value and the other conditions were determined as a percentage of control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

■ dibucaine treated cells

● tetracaine treated cells

▲ procaine treated cells

The IC_{50} values for these local anaesthetics were determined from this graph.

Local Anaesthetic	IC_{50} value mM
Dibucaine	0.22
Tetracaine	0.64
Procaine	17.60

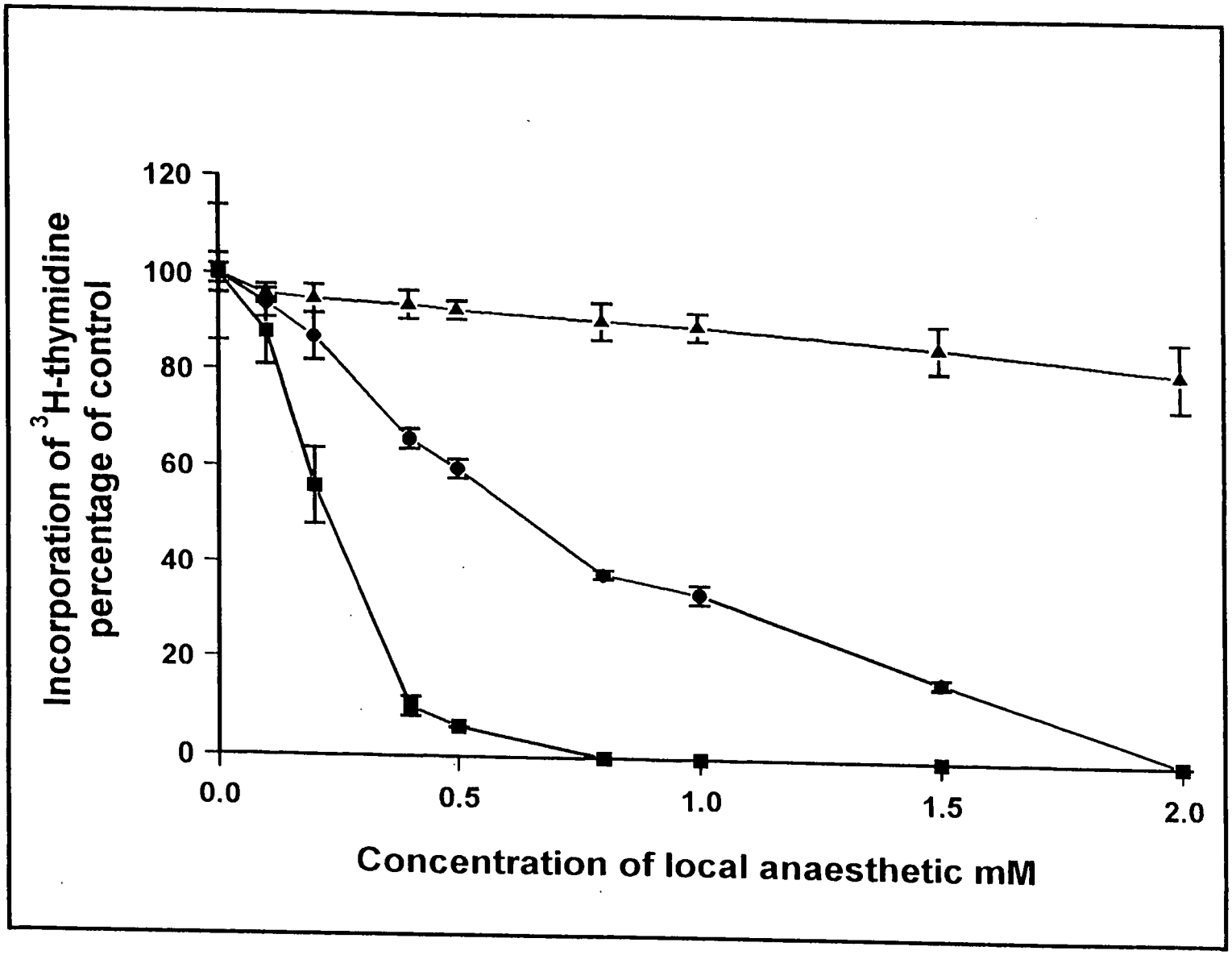

Incorporation of ^{3}H-thymidine
percentage of control
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Concentration of local anaesthetic mM

Figure 2.6 Cytotoxic effects of local anaesthetics on HL60 cells

Cells were incubated with dibucaine, tetracaine or procaine (0-2mM) for 4 hours in serum-free culture medium, as described in section 2.2.6., then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine into the controls was taken to be a 100% value and the other conditions were determined as a percentage of control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

■ dibucaine treated cells

● tetracaine treated cells

▲ procaine treated cells

The IC_{50} values for the local anaesthetics were determined from this graph.

Local Anaesthetic	IC_{50} value mM
Dibucaine	0.28
Tetracaine	0.68
Procaine	16.75

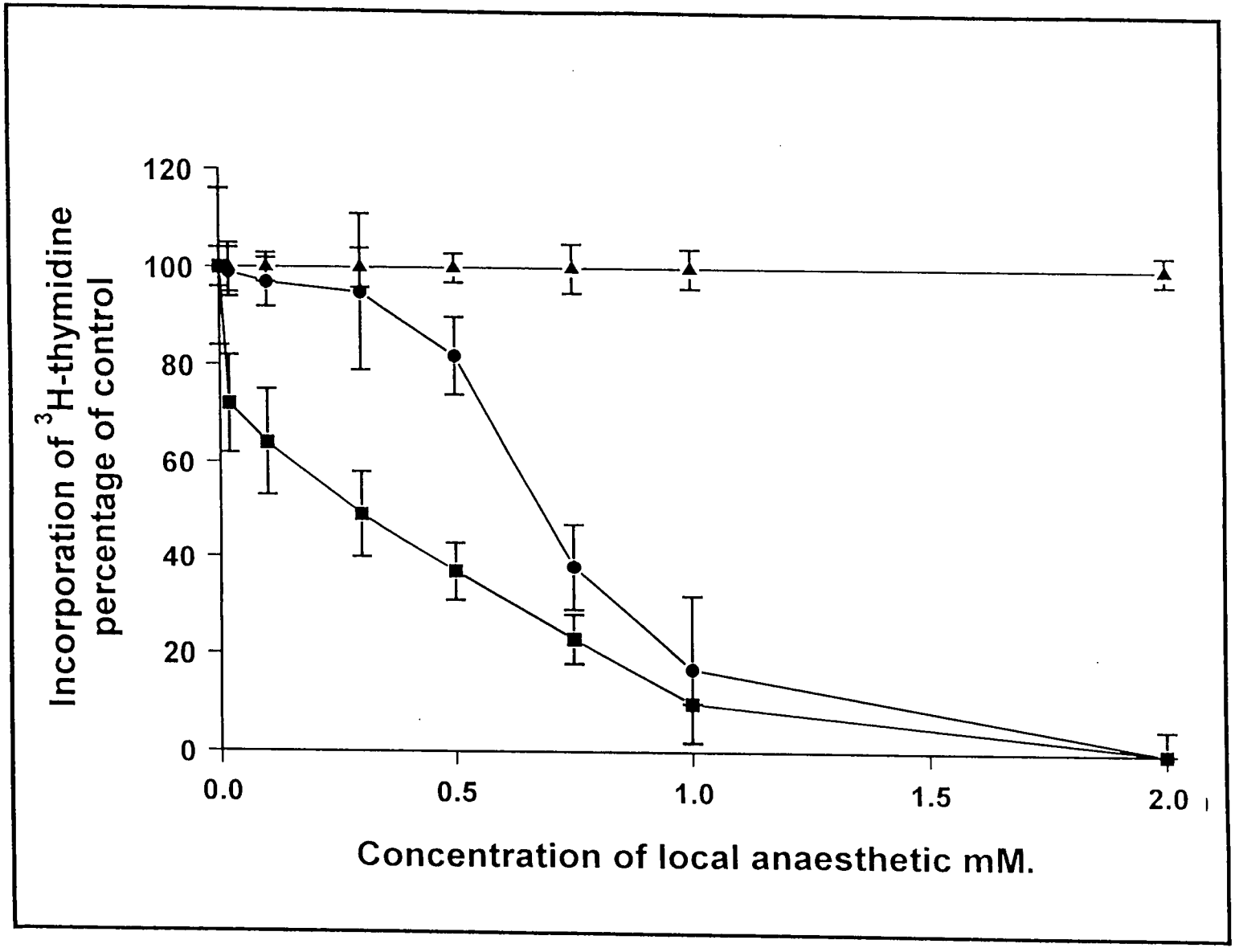

Incorporation of ^{3}H-thymidine
percentage of control
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
Concentration of local anaesthetic mM.

Figure 2.7 demonstrated that the IC_{50} values for procaine were 16.75mM for HL60 cells and 17.6mM for K562 cells. After a procaine concentration of 4mM the HL60 cells started to lose their reproductive ability. Approximately 20% of the HL60 cells lost reproductive ability at 10mM and approximately 80% of them cells lost reproductive ability at 25mM procaine. The K562 cell line started to lose reproductive ability at 1mM. Approximately 20% of the cells had lost their reproductive ability at 8mM procaine concentration and approximately 75% of K562 cells lost reproductive ability at 25mM procaine.

2.3.4 Cytotoxic effects of ether lipid and local anaesthetics in combination on HL60 and K562 cell lines

As both ether lipids and local anaesthetics are membrane-active agents, it was of interest to determine whether they might have additive or synergistic effects on leukaemia cell death. In these combination experiments, it was decided to determine the effect of a subtoxic concentration of local anaesthetic, on the cytotoxicity of ET-18-OCH_3.

Subtoxic doses of the local anaesthetics to be used in combination experiments with ET-18-OCH_3 on K562 cells were determined from Figure 2.5, these were 0.1mM dibucaine, 0.15mM tetracaine and 2mM for procaine. From Figure 2.4 the K562 cells lost their reproductive ability at 15μM ET-18-OCH_3. It was decided to determine the combined effect of ether lipid and dibucaine over the whole ether lipid concentration range, therefore the ether lipid concentrations ranged between 0-20μM. Figure 2.8 shows the cytotoxic effects of 0.1mM dibucaine in combination with 0-20μM ET-18-OCH_3 on K562 cells. The reproductive ability of K562 cells was inhibited by

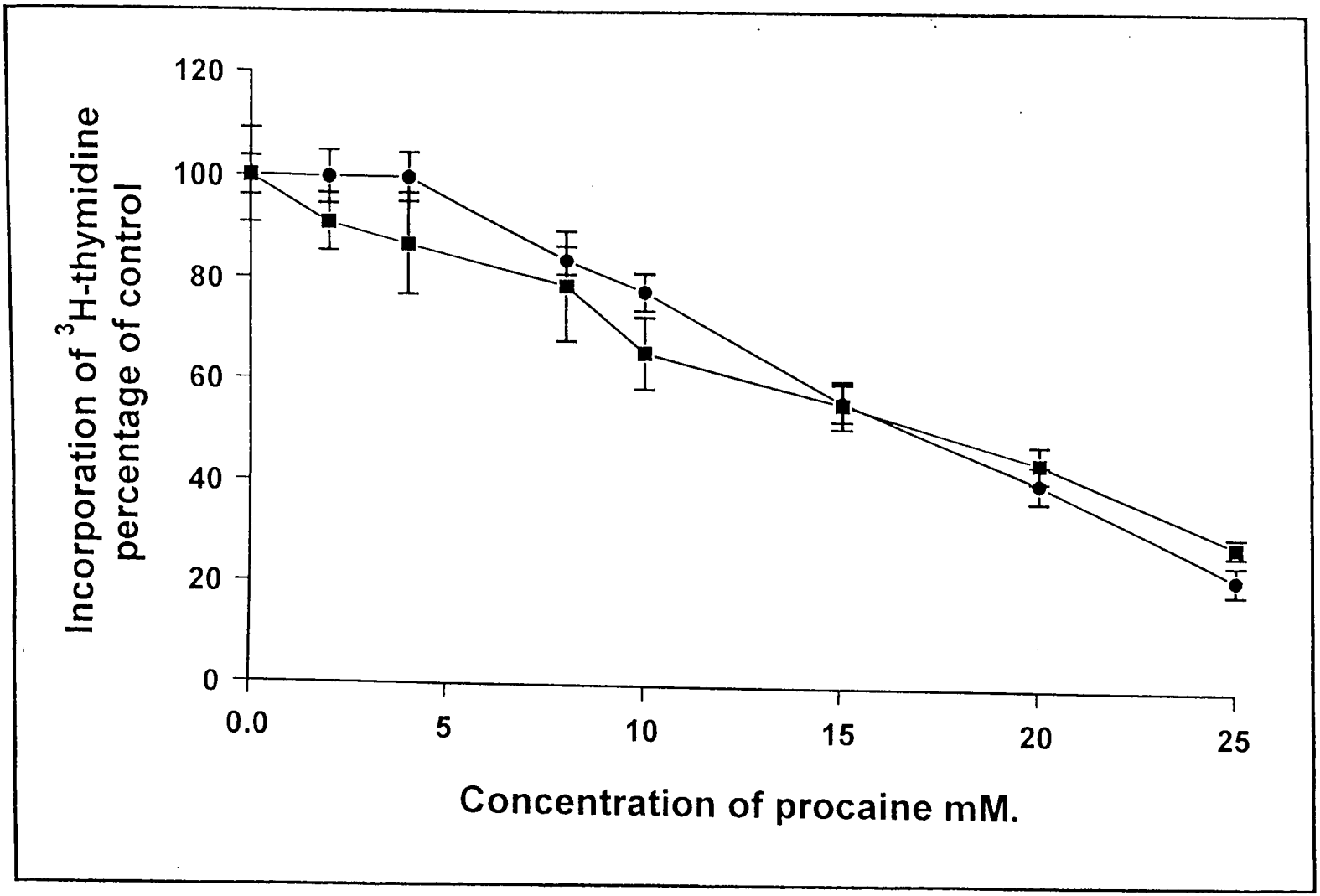
Figure 2.7 Cytotoxic effects of procaine on K562 and HL60 cells

Cells were incubated with procaine (0-25mM) for 4 hours in serum-free culture medium, as described in section 2.2.6, then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine into the controls was taken to be a 100% value and the other conditions were determined as a percentage of control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

- ■ K562 cells
- ● HL60 cells

As can be seen from this graph, the IC_{50} value for procaine was 17.6mM for K562 cells and 16.75mM for HL60 cells.


Incorporation of ^{3}H-thymidine
percentage of control
120
100
80
60
40
20
0
0.0
5
10
15
20
25
Concentration of procaine mM.

ET-18-OCH_3 alone, with an IC_{50} value of approximately 8.5μM. The cytotoxic action of the ether lipid was greatly increased in the presence of 0.1mM dibucaine, giving an IC_{50} value of 3.7μM ET-18-OCH_3. The addition of dibucaine to the ether lipid treated K562 cells significantly decreased the reproductive ability of the cells at ET-18-OCH_3 concentrations of 1μM, 3μM, 5μM and 7.5μM ($p < 0.05$).

Figures 2.9 and 2.10 shows the effect of combining 0.15mM tetracaine or 2mM procaine, respectively, with ET-18-OCH_3. It is clear from these Figures that the combined effect of tetracaine with ET-18-OCH_3 or procaine with ET-18-OCH_3 did not alter the reproductive ability of the K562 cell line compared to the effect of ET-18-OCH_3 alone.

As shown in Figure 2.6 dibucaine proved to be very cytotoxic to HL60 cells and it was not possible to determine a subtoxic dose for combination experiments with ET-18-OCH_3. Subtoxic doses were determined for tetracaine and procaine in the HL60 cells, the concentrations used were 0.2mM and 4mM, respectively. Figure 2.4 showed that the majority of HL60 cells had lost their reproductive ability at 5μM ET-18-OCH_3, from this the ether lipid concentrations to be used for combined experiments of ether lipid and local anaesthetic for HL60 cells ranged between 0-5μM. Figure 2.11 shows the effect of the combination of tetracaine and ET-18-OCH_3. The addition of 0.2mM tetracaine to 0-5μM ET-18-OCH_3 treated HL60 cells did not affect their reproductive ability compared to ET-18-OCH_3 treatment alone. Figure 2.12 shows the effect of combining 4mM procaine and 0-5μM ET-18-OCH_3. No difference in reproductive ability was shown between HL60 cells that had been treated with procaine alone compared to HL60 cells treated with ether lipid and procaine.

Figure 2.8 Cytotoxic effects of ether lipid on K562 cells in the presence/absence of dibucaine

K562 cells were incubated with concentrations of ET-18-OCH_3 ranging between 0-20μM in the presence or absence of dibucaine (0.1mM) for 4 hours in serum-free culture medium. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM ET-18-OCH_3) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

- ● ether lipid treated cells
- ■ ether lipid treated cells in the presence of 0.1mM dibucaine

The IC_{50} values were 8.5μM for ether lipid treated cells and 3.7μM for ether lipid cells in the presence of 0.1mM dibucaine.

Using Oneway ANOVA analysis significant differences were found between the treatments at 1μM, 3μM, 5μM and 7.5μM ether lipid concentrations ($p < 0.05$).

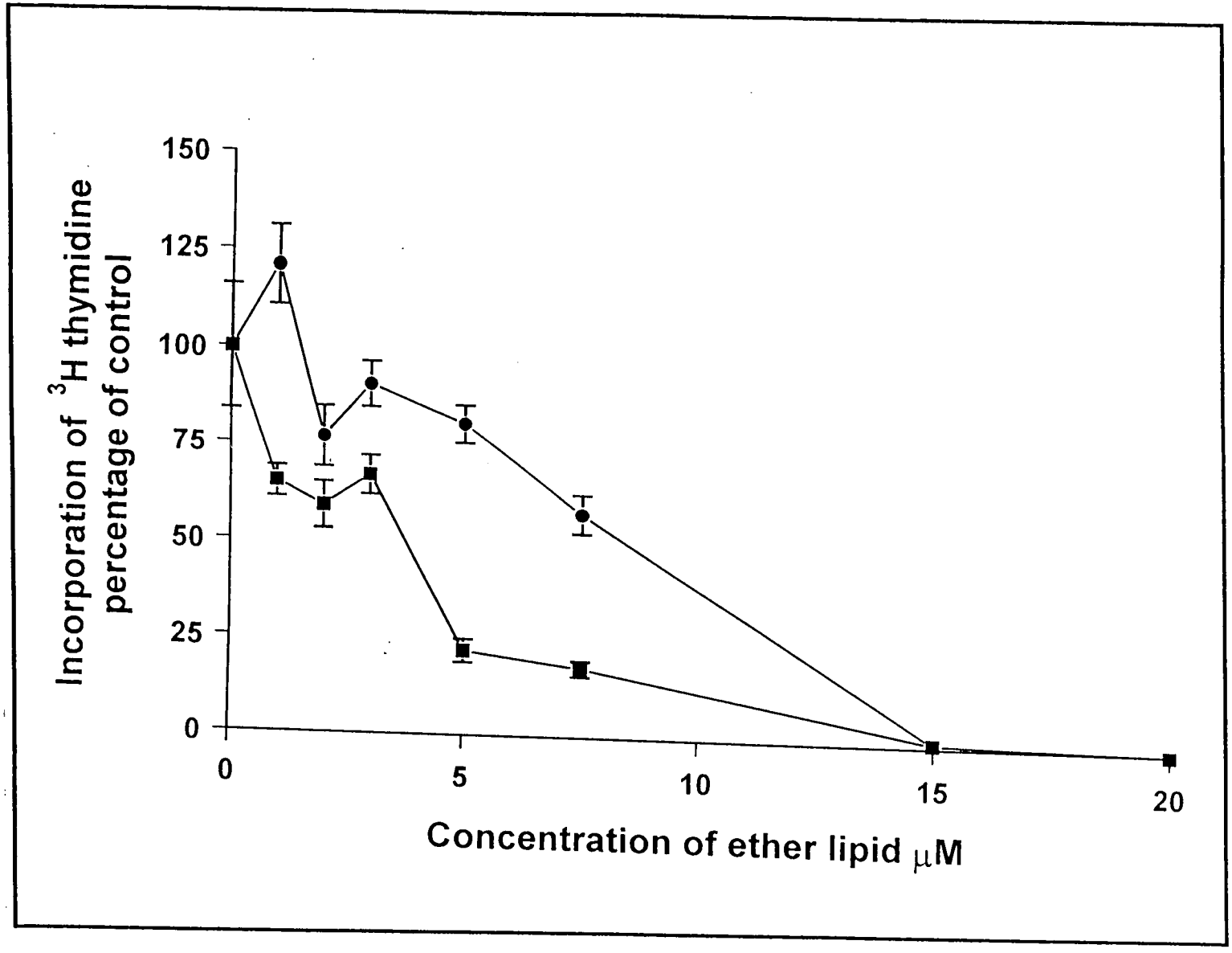

Incorporation of ^{3}H thymidine
percentage of control
150
125
100
75
50
25
0
0
5
10
15
20
Concentration of ether lipid μM

Figure 2.9 Cytotoxic effects of ether lipid on K562 cells in the presence/absence of tetracaine

K562 cells were incubated with concentrations of ET-18-OCH_3 ranging between 0-10μM in the presence or absence of tetracaine (0.15mM) for 4 hours in serum-free culture medium. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM ET-18-OCH_3) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

- ● ether lipid treated cells
- ■ ether lipid treated cells in the presence of 0.15mM tetracaine

The IC_{50} values were 6.2μM for ether lipid treated cells and 5.5μM for ether lipid cells in the presence of 0.15mM tetracaine.

From Oneway ANOVA statistical analysis no significant differences were found between treatments.

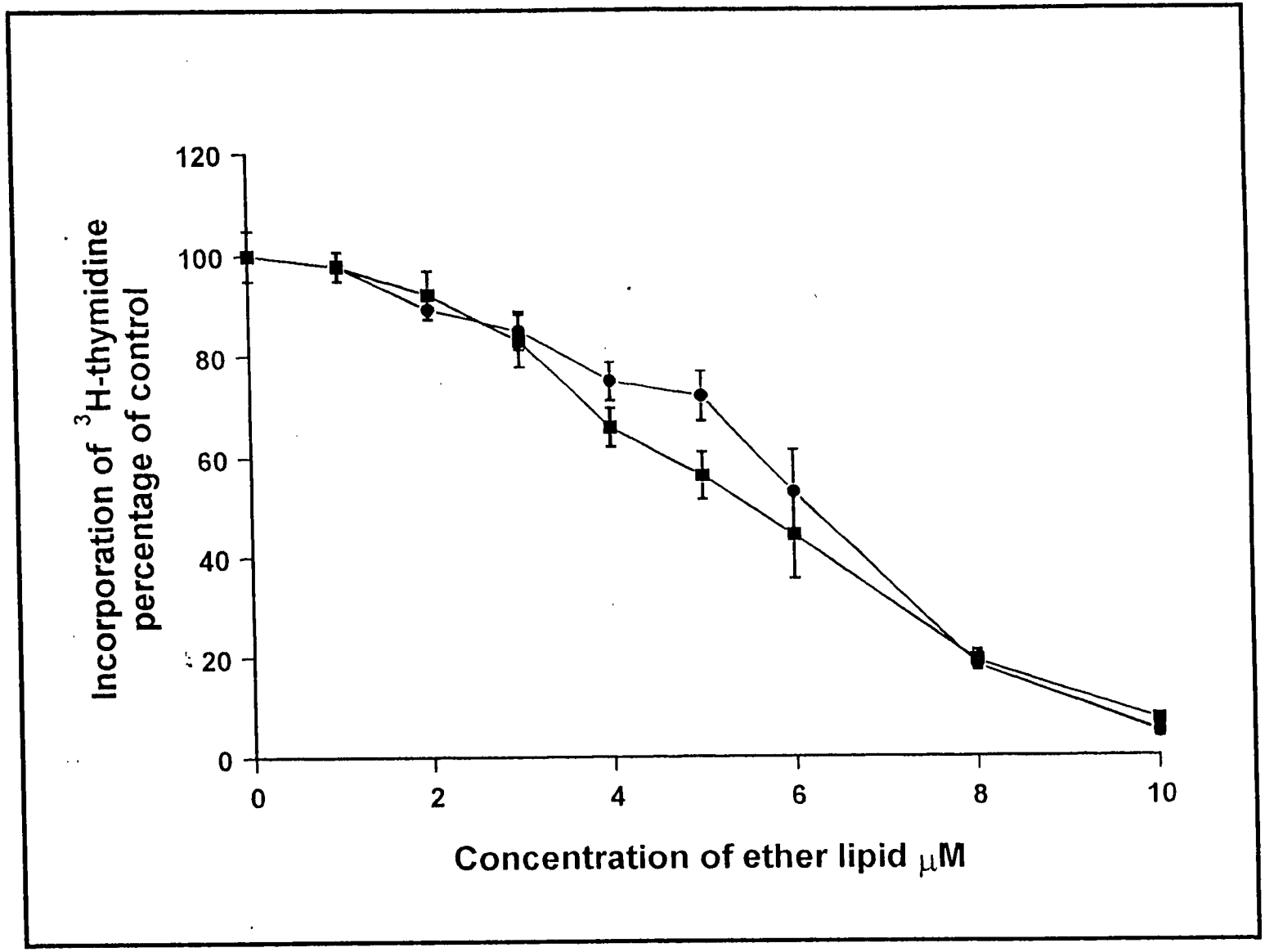

Incorporation of ^{3}H-thymidine
percentage of control
120
100
80
60
40
20
0
0
2
4
6
8
10
Concentration of ether lipid μM

Figure 2.10 Cytotoxic effects of ether lipid on K562 cells in the presence/absence of procaine

K562 cells were incubated with concentrations of ET-18-OCH_3 ranging between 0-10μM in the presence or absence of procaine (2mM) for 4 hours in serum-free culture medium. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM ET-18-OCH_3) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

- ● ether lipid treated cells
- ■ ether lipid treated cells in the presence of 2mM procaine

The IC_{50} values were 5.15μM for ether lipid treated cells and 3.9μM for ether lipid cells in the presence of 2mM procaine.

From Oneway ANOVA statistical analysis no differences were found between treatments.

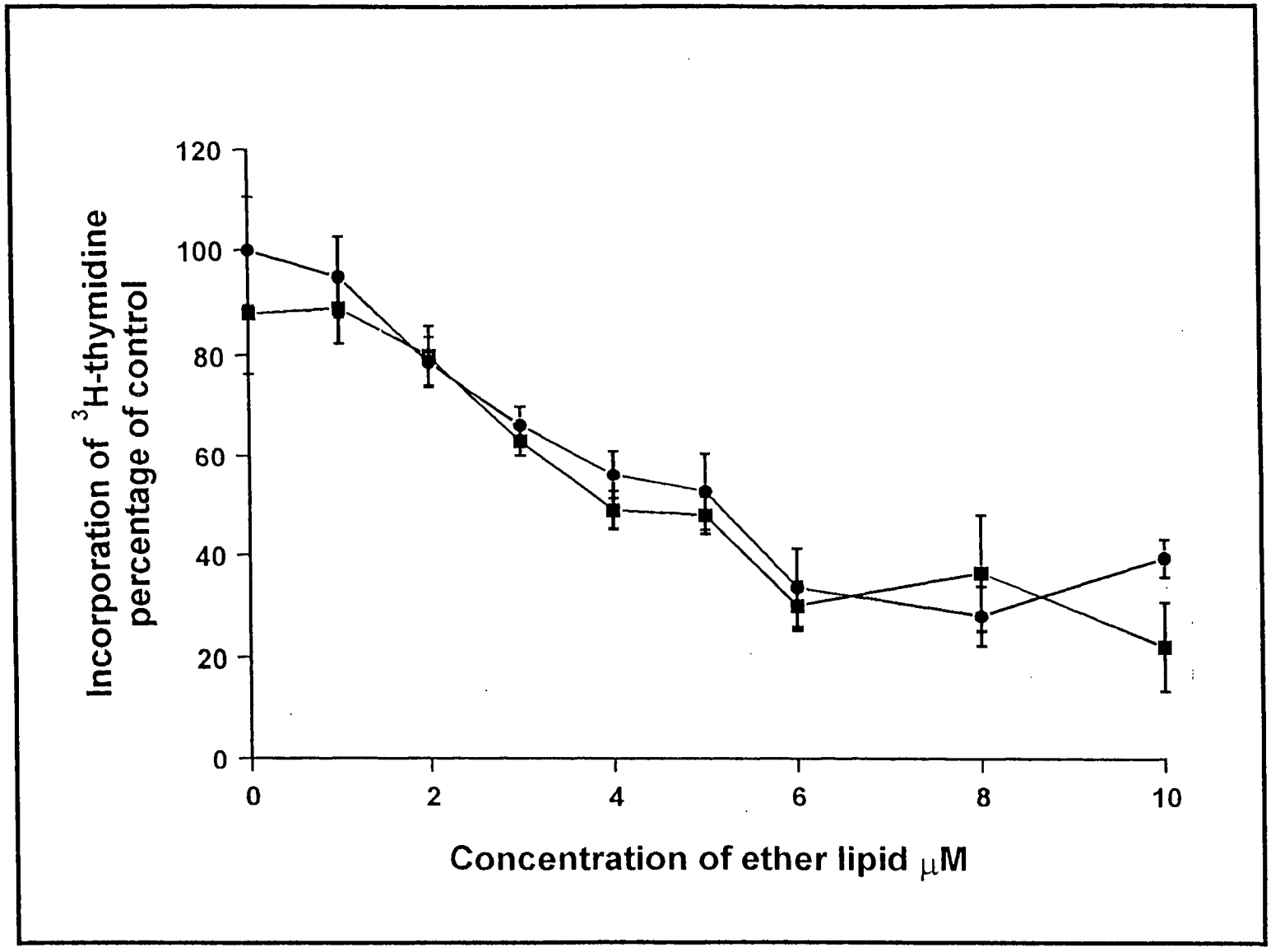

Incorporation of ^{3}H-thymidine
percentage of control
120
100
80
60
40
20
0
0
2
4
6
8
10
Concentration of ether lipid μM

Figure 2.11 Cytotoxic effects of ether lipid on HL60 cells in the presence/absence of tetracaine

HL60 cells were incubated with concentrations of ET-18-OCH_3 ranging between 0-5μM in the presence or absence of tetracaine (0.2mM) for 4 hours in serum-free culture medium. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM ET-18-OCH_3) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

- ● ether lipid treated cells
- ■ ether lipid treated cells in the presence of 0.2mM tetracaine

The IC_{50} values were 3.28μM for ether lipid treated cells and 4.16μM for ether lipid cells in the presence of 0.2mM tetracaine.

From Oneway ANOVA statistical analysis no differences were found between the treatments.

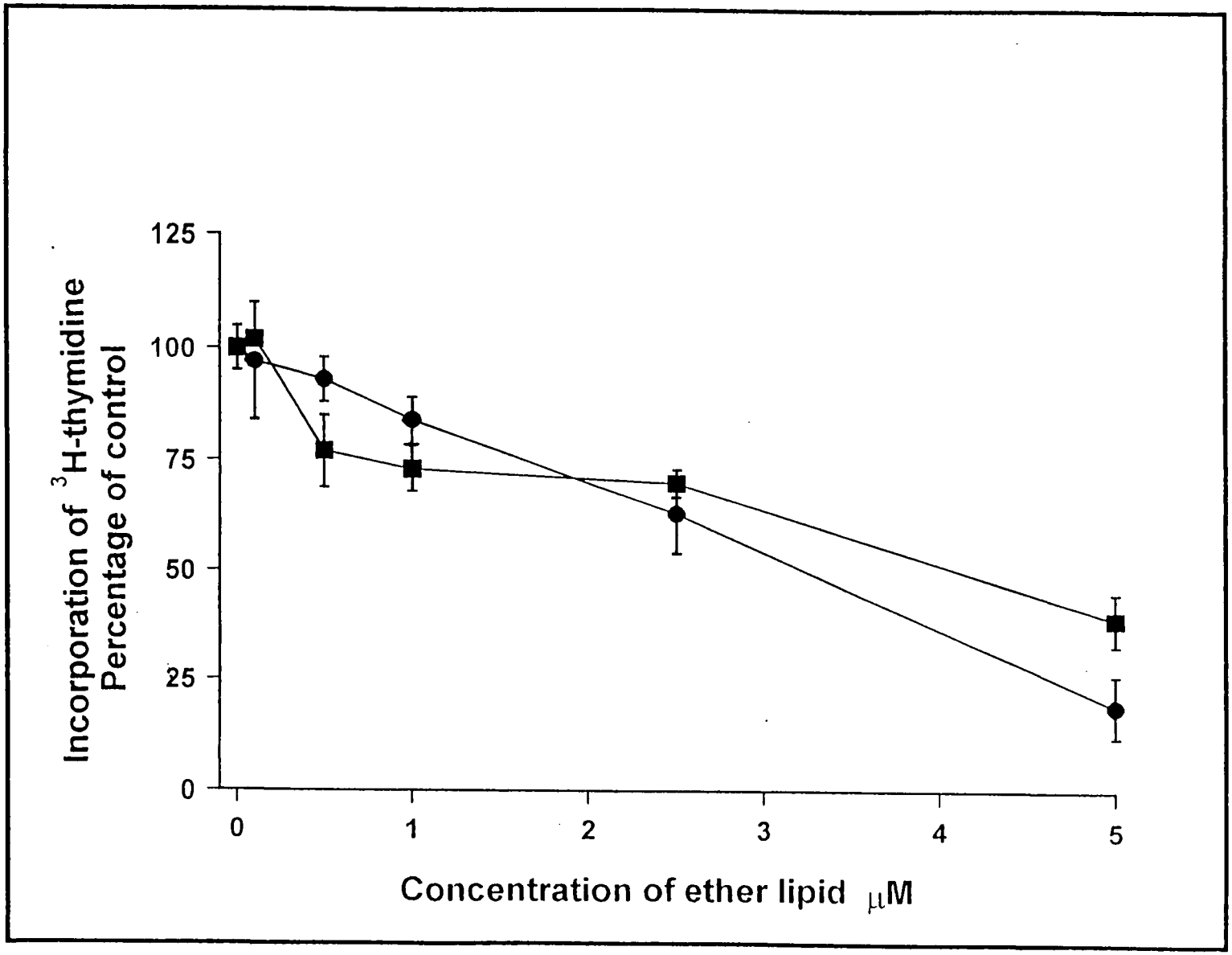

Incorporation of ^{3}H-thymidine
Percentage of control
125
100
75
50
25
0
0
1
2
3
4
5
Concentration of ether lipid μM

Figure 2.12 Cytotoxic effects of ether lipid on HL60 cells in the presence/absence of procaine

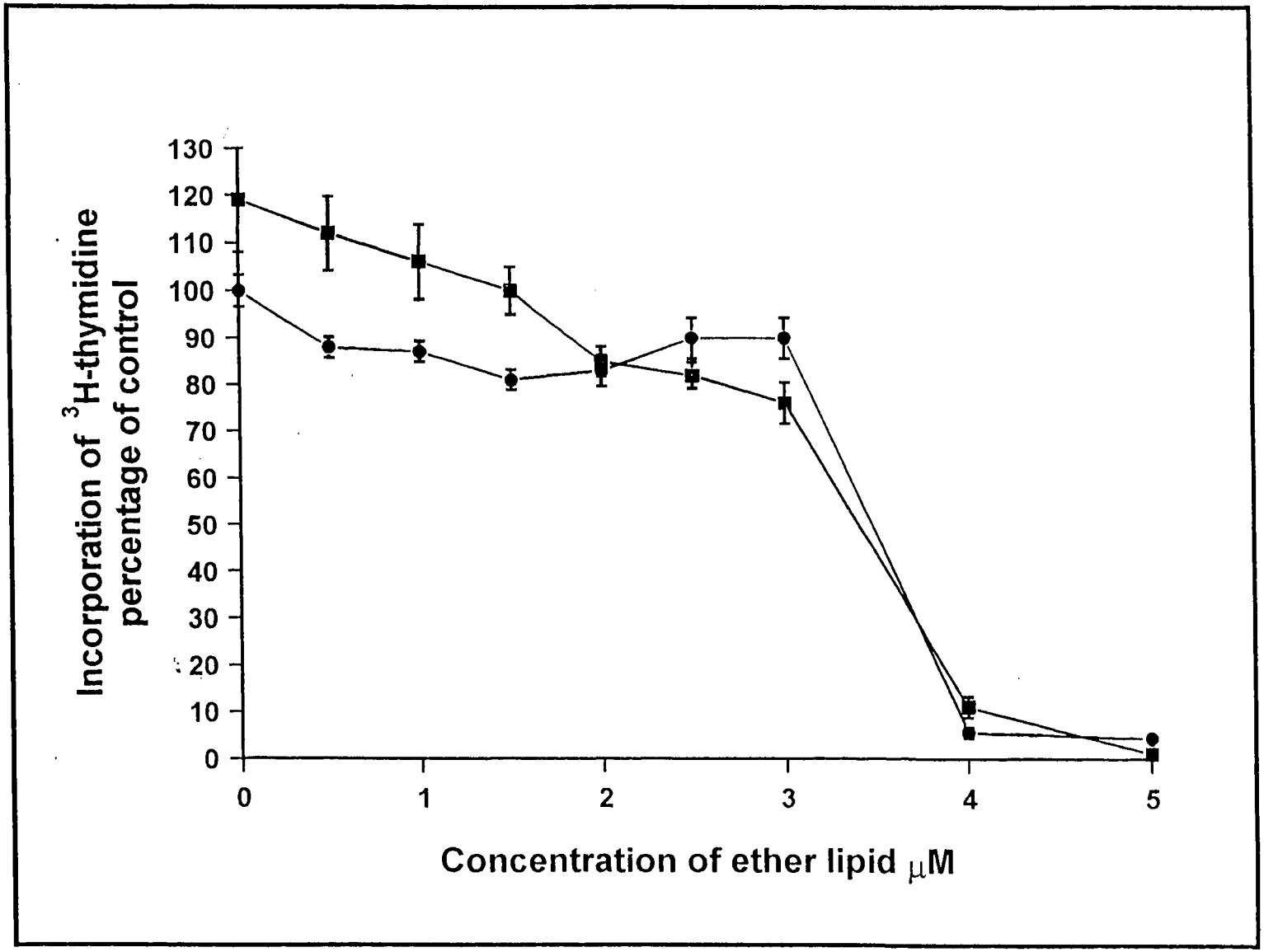
HL60 cells were incubated with concentrations of ET-18-OCH_3 ranging between 0-5μM in the presence or absence of procaine (4mM) for 4 hours in serum-free culture medium. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^3H-thymidine as described in section 2.2.6. The incorporation of ^3H-thymidine was taken to be 100% for control (0μM ET-18-OCH_3) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± S.D. for 3 separate experiments (9 replicates).

- ● ether lipid treated cells
- ■ ether lipid treated cells in the presence of 4mM procaine

The IC_{50} values were 3.49μM for ether lipid treated cells and 3.38μM for ether lipid treated cells in the presence of 4mM procaine.

From Oneway ANOVA statistical analysis no significant differences were found between treatments.


Incorporation of ^{3}H-thymidine
percentage of control
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Concentration of ether lipid μM

2.4 Discussion

Work reported in this chapter examined the cytotoxic effects of two types of membrane active agents on human leukaemia cell lines. The membrane active agents were an ether lipid ET-18-OCH_3 whose structure is closely related to PAF, and three local anaesthetics, dibucaine, tetracaine and procaine. The two cell lines used were human acute myeloblastic leukaemia (HL60) and human myelogenous leukaemia (K562) cells.

The HL60 cell line and K562 cell line were grown in suspension culture. The cells grew exponentially when kept between $2x10^5$ and $1x10^6$ cells/ml and were grown until passage number 40. The time taken for the cell lines to double was found to be 24 hours for HL60 cells and 28 hours for K562 cells. This is similar to that reported by Hoffman *et al.* (1986) who observed generation times of 24 hours for both cell lines.

Ether lipids have been shown to localise in cellular membranes (Andreesen *et al.*, 1978; Tidwell *et al.*, 1981). In L1210 cells the initial unidirectional influx of ET-18-OCH_3 was nonsaturable, energy independent and slightly temperature sensitive (Kelley *et al.*, 1993). From these results Kelley *et al.* (1993) concluded that ET-18-OCH_3 was incorporated into the cell membrane by passive diffusion, and not by endocytosis as was previously thought (Bazill & Dexter, 1990).

In this chapter ether lipids were presented to the cells by including the ether lipid dissolved in 100% ethanol into the culture medium. The ethanol concentration was kept to a minimum of 0.1% (v/v) of the culture medium. Kelley *et al.*, (1993) reported that ether lipids bind to serum proteins and this could reduce their uptake by approximately 50%, even at serum levels as low as 0.5% (v/v). The

culture medium that the HL60 and K562 cell line grew in contained 10% (v/v) heat-inactivated foetal calf serum. Therefore, for a known quantity of ether lipid to be available to the cells the culture medium should not contain serum. A short incubation time of 4 hours was chosen for the cells to grow in serum-free culture medium. Before testing the action of the ether lipid on the cell lines the growth of the cells in serum-free culture medium was established. It was found that a 4 hour incubation in serum-free medium did not alter the growth rate of either cell line.

Initially, the effect of the membrane active agents on cell viability was determined by trypan blue exclusion. From these cell counts total cell numbers and viable cell numbers could be established. The cytotoxic action of the membrane active agents was determined by loss of the cell's reproductive ability, as measured by the incorporation of ^{3}H-thymidine into DNA. Cells (50,000) were incubated in growth medium containing 0.2μCi of ^{3}H-thymidine for 3 hours giving counts in the region of 50,000 to 100,000 dpm .

The effect of the ether lipid ET-18-OCH_3 on cell growth was determined as a percentage of incorporation of ^{3}H-thymidine into control cells. The effect of the ET-18-OCH_3 on HL60 cells reproductive ability was dramatic with an IC_{50} value of 4.11μM. The HL60 cells were much more sensitive to low concentrations of ET-18-OCH_3 than the K562 cells. At a concentration of 1μM ET-18-OCH_3 25% of the HL60 cells lost their reproductive ability, whereas the K562 cells were unaffected. The IC_{50} value for the K562 cells exposed to this ether lipid was 8.53μM ET-18-OCH_3. As the concentration of ET-18-OCH_3 increased the reproductive ability of both cell lines decreased. All of the reproductive ability was lost at an ET-18-OCH_3 concentration of 7.5μM for the HL60 cells and at 15μM for the K562

cells confirming previous observations (Tidwell *et al.*, 1981) that HL60 cells were more sensitive to the ET-18-OCH_3 than the K562 cells. The LD_{50} for HL60 cells was 1.5μM compared to 21μM for K562 cells after a 24 hour exposure to ether lipid (Wagner *et al.*, 1998) determined by clonogenic survival for 14 days. Clonogenic survival was lost totally at 10μM ET-18-OCH_3 for HL60 cells and 100μM for K562 cells. These values corresponding to LD_{50} and total loss of clonogenic survival are higher than the IC_{50} results in the present study. This may be due to the different assay for cytotoxicity, the presence of 10% FBS in the ET-18-OCH_3 incubations or the different exposure time to the ether lipid in the Wagner *et al.* (1998) experiments. The presence of 10% FBS would alter the concentration of ether lipid available to the cells. Heesbeen *et al.* (1995) showed the effect of varying serum levels on the cytotoxic effects of ET-18-OCH_3 in HL60 and K562 cells. The reduction of FCS from 10% to 2% reduced the IC_{50} for the incorporation of ^{3}H-thymidine from approximately 55μM to 10μM, respectively, in HL60 cells and from approximately >96μM to 40μM, respectively, in K562 cells. The reduction of FBS to 2% in the culture medium of HL60 and K562 cells lowered the ET-18-OCH_3 IC_{50} values to nearer those obtained in this study with 0% FCS.

It is unclear why ether lipids are more cytotoxic to tumour cells than to normal cells. In this study the mechanisms of action of ET-18-OCH_3 were not investigated, but there are many different ways in which the ether lipid may affect tumour cells. Soodsma *et al.*, (1970) showed that tumour cells had a low activity of the alkyl cleavage enzyme. This may cause an increase in concentration of the alkyl phospholipids which inhibit cell function, leading to cell death (Andreesen *et al.*, 1978). The activity of the alkyl cleavage enzyme was assayed in microsomes from sensitive HL60 cells and resistant K562

and MDCK cells using 1-hexadecyl-2-methoxy-glycero-3-phosphocholine as a substrate (Hoffman *et al.*, 1986). Alkyl cleavage enzyme activity in HL60 cells did not differ significantly from K562 or MDCK cells. Hoffman *et al.* (1986) concluded that the antineoplastic property of ether lipids cannot be based on a low alkyl cleavage enzyme activity in cancer cells. However, the HL60 cells showed a preferential sequestering of the ET-18-OCH_3 in the plasma membrane, and the incorporation of ether lipids into the plasma membrane has been shown to alter membrane fluidity which may contribute to cell death (Diomede *et al.*, 1990).

Tidwell *et al.*, (1981), also showed that the K562 cells were more resistant to the cytotoxic effect of ET-18-OCH_3 compared to HL60 cells. The growth of HL60 colonies was reduced after a 24 hour pulse of 9.6μM ET-18-OCH_3, whereas K562 colonies showed resistance up to 38μM. The resistance of K562 cells may be related to greater cholesterol content in the plasma membrane compared to HL60 cells (Diomede *et al.*, 1990). In model membranes Diomede *et al.*, (1990), showed that the amount of cholesterol in membranes affected ether lipid uptake. When HL60 cells were supplemented with cholesterol they were found to have a similar level of cholesterol content, and phospholipid to cholesterol ratio, as K562 cells. Over short exposures of 2 hours to ET-18-OCH_3, cholesterol-enriched HL60 cells showed approximately 40% reduction in ether lipid cytotoxicity compared to HL60 cells not enriched with cholesterol. This ether lipid cytotoxicity in the cholesterol-enriched HL60 cells was similar to that found in K562 cells. However, when cholesterol-enriched HL60 cells were exposed for 48 hours to ET-18-OCH_3 the cytotoxicity of the ether lipid was not reduced compared to unmodified HL60 cells, whereas the K562 cells remained more resistant (Diomede *et al.*, 1990).

Other possible effects have been observed that may account for the resistance to ET-18-OCH_3 of the K562 cells compared to HL60 cells. Clonogenic studies (Wagner *et al.*, 1998) showed that the LD_{50} for HL60 cells was 1.5μM compared to 21μM for K562 cells after a 24 hour exposure to ET-18-OCH_3 in culture medium containing 10% FBS. In that study, Wagner *et al.* (1998) showed that HL60 cells generated a lipid derived free radical ($L_d^{\cdot}$) when exposed to ET-18-OCH_3 (20μM and above). The cells were first subjected to oxidative stress of 20μM $FeSO_4 \cdot 7H_2O$ and 100μM ascorbic acid for 5 minutes. After 5 minutes ET-18-OCH_3 was added to the cells. Approximately 3 minutes after the addition of the ether lipid the production of $L_d^{\cdot}$ began in HL60 cells previously initiated to oxidise with Fe^{2+} and ascorbate. K562 cells treated with up to 40μM ET-18-OCH_3 failed to produce the $L_d^{\cdot}$. The production of this radical corresponds to the onset of early cytotoxicity suggesting that these events may be related. A steady state concentration of ascorbate free radical $[Asc^{\cdot -}]_{ss}$ was used to estimate the cellular oxidative stress of the cells. After the addition of ET-18-OCH_3 (> 15μM) HL60 cells previously initiated to oxidise with Fe^{2+} and ascorbate showed a rapid increase in $[Asc^{\cdot -}]_{ss}$. There was no increase in $[Asc^{\cdot -}]_{ss}$ when ET-18-OCH_3 was added to K562 cells except at 40μM ET-18-OCH_3 which compared to the increase in HL60 cells was comparatively small. This indicates that ET-18-OCH_3 results in an increased oxidative state in HL60 cells but not in K562 cells. Ether lipids themselves do not generate oxidative intermediates during metabolism (Magistrelli *et al.*, 1995). Wagner *et al.*, (1998) showed that the HL60 cells had more polyunsaturated fatty acids in membranes than in K562 cells. This makes the HL60 cells membranes more susceptible to oxidation after the addition of ET-18-OCH_3. Wagner *et*

al. (1998) thought that the free radical generation may be related to necrotic cell death or apoptosis.

HL60 cells and HL60 cells induced to differentiate by DMSO for 5 days (dHL60 cells) were used to determine the effect of ET-18-OCH_3 and PAF on cytosolic calcium and apoptosis (Alonso *et al.*, 1997). HL60 cells do not respond to PAF but dHL60 cells express PAF receptors as they differentiate towards granulocytes (Vallari *et al.*, 1990). In dHL60 cells both PAF and ET-18-OCH_3 (6μM) treatments for 12 hours induced an increase in cytosolic free calcium $[Ca^{2+}]_i$ composed of a transient peak and a sustained plateau. This increase was blocked by the PAF antagonist WEB-2170. In dHL60 cells, ET-18-OCH_3 induced a larger increase in $[Ca^{2+}]_i$, as ET-18-OCH_3 had an affinity for the PAF receptor, but did not induce apoptosis (Alonso *et al.*, 1997). In HL60 cells, ET-18-OCH_3, 6μM for 12 hours, only slightly increased $[Ca^{2+}]_i$ and induced apoptosis. Alonso *et al.* (1997) found no correlation between the effects of ET-18-OCH_3 on $[Ca^{2+}]_i$ and apoptosis.

Cytotoxic effects of ether lipids may also be related to effects on cell signalling. The ether lipids ET-18-OCH_3 and BM41.440 have been shown to be specific protein kinase C (PK-C) inhibitors (Shoji *et al.*, 1988). PK-C inhibition would lead to a breakdown in transmembrane signalling, cell growth and differentiation (Nishiguka, 1984). Helfman *et al.*, (1983) and Shoji *et al.*, (1988) showed that BM41.440 and ET-18-OCH_3 inhibited PK-C competitively with respect to phosphatidylserine but noncompetitively with respect to Ca^{2+}. These studies focused on the effects of ether lipids on purified protein kinase C. Heesbeen *et al.* (1994) investigated the effect of ET-18-OCH_3 on PK-C activity while the enzyme was still in its phospholipid environment in the plasma membrane of HL60, dHL60 and K562 cells.

The cytotoxic effect of ET-18-OCH_3 gave an LD_{50} value of 6μg/ml in HL60 cells this was reduced in dHL60 cells to 40μM. The cytotoxic effect of ET-18-OCH_3 on HL60 and K562 cells pretreated with staurosporine, an inhibitor of PK-C activity, was identical to control cells, suggesting that PK-C activity in HL60 and K562 cells is essential for proliferation but not essential for the cytotoxic action of ET-18-OCH_3.

Vogler *et al.* (1996) investigated the effect of ET-18-OCH_3 on the *de novo* synthesis of phosphatidylcholine in HL60 and K562 cells, by examining the activity of the rate-limiting enzyme choline-phosphate cytidylyltransferase. Vogler *et al.* (1996) showed that there was significantly more cytidylytransferase (CT) activity/μg of protein in K562 cells compared to HL60 cells. The enzyme activity was measured by the incorporation of ^{14}C-phosphocholine into CDP-choline in lysates of HL60 and K562 cells. ET-18-OCH_3 inhibited HL60 lysates to a much greater extent than K562 lysates with IC_{50} values of 22μM and 359μM, respectively. CT was partially purified from HL60 and K562 cells, cloned and sequenced. The cDNA of CT from the HL60 and K562 cells were only different at one nucleotide. In K562 cDNA nucleotide number 751 was G, whereas in the HL60 cDNA nucleotide number 751 was A. This corresponded to a change at amino acid number 251 from glutamic acid (negatively charged) in K562 CT to lysine (positively charged) in HL60 CT. Vogler *et al.* (1996) thought that the increase in the positive charge of HL60 CT may contribute to a stronger binding of ET-18-OCH_3, resulting in its greater inhibition of the enzyme.

ET-18-OCH_3 and BM41.440 were shown to significantly inhibit the growth of HT29 cells (Principe *et al.*, 1992). Flow cytometry was used to assess the cell kinetics with bromodeoxyuridine pulse-labelling

experiments (BrdUrd/DNA analysis). These experiments showed that ET-18-OCH_3 and BM41.440 do not affect cell progression through S and M phase but HT29 cells were arrested in the exit from G_1 and G_2 phases. Therefore, ether lipids may directly or indirectly inactivate the complex $p34^{cdc2}$-cyclin essential for passage from G_1 to S and G_2 to M (Principe *et al.,* 1992).

K562 cells (grown in 10% FCS) were treated with ET-18-OCH_3 (48μM) for 2 hours (Botzler, *et al.*, 1996). This treatment was nonlethal but concentrations above 48μM up to 191μM showed a dose-dependent decrease in cell viability and promoted apoptosis. Apoptotic nuclei were detectable in K562 cells treated with 96μM ET-18-OCH_3 for 2 hours as early as 2 hours after the treatment (Botzler, *et al.*, 1996). This was shown by PI staining and FACS analysis. Increasing ET-18-OCH_3 concentrations reduced the number of cells in S and G_2+M. The cytotoxic effect of ET-18-OCH_3 (191μM) on K562 cells was shown by the $G_{1/0}$ peak shift to the left and S and G_2+M peaks disappeared completely. K562 cells treated with the nonlethal dose 48μM ET-18-OCH_3 were shown to be 1.5-fold more sensitive to lysis mediated by an NK enriched effector cell population. Botzler *et al.* (1996) thought that nontoxic ether lipid treatment might induce a major histocompatibility complex-independent modulation in tumour cell membrane increasing immunogenicity.

In the present study the experiments determined the cytotoxic effect of ET-18-OCH_3 on the HL60 and K562 cells reproductive ability. These concentrations were to be used in conjunction with local anaesthetic concentrations. The effect of the local anaesthetics on growth was determined by the incorporation of ^{3}H-thymidine as previously described.

Dibucaine had a profound cytotoxic effect, giving IC_{50} values of 0.22mM for the K562 cells and 0.28mM for the HL60 cells. Although the HL60 and K562 cells had similar IC_{50} values low concentrations of dibucaine had a more severe effect on the HL60 cells than the K562 cells. Tetracaine also proved to be cytotoxic with IC_{50} values of 0.64mM for K562 cells and 0.68mM for HL60 cells. Further experiments showed that procaine had an IC_{50} value of 17mM for both K562 and HL60 cells. Procaine's effect on cell reproductive ability was not as dramatic as the other two local anaesthetics. This order of cytotoxicity of the local anaesthetics was the same for both cell lines, dibucaine > tetracaine > procaine. Kim *et al.*, (1997) found the same order of cytotoxicity in SK-N-MC human neuroblastoma cells, with the IC_{50} values dibucaine 0.035mM > tetracaine 0.1mM > procaine 1.5mM. Ohki (1984) showed this was the order of membrane surface adsorption of the cationic forms of these local anaesthetics at any given concentration. In HTC cells, Kingston *et al.,* (1993), showed this order reflected the linear relationship between oil : water partition coefficients of the local anaesthetics and their IC_{50} values in HTC cells. This implied that their toxicity was related to a tendency to partition into the membrane lipid matrix or adsorb onto other hydrophobic cellular sites (Kingston *et al.,* 1993). When HTC cells were incubated with these local anaesthetics under increased temperatures the membrane fluidity was effected, when measured by DPH fluorescence polarisation spectroscopy. Dibucaine was found to have strong fluidising effects suggesting that it may affect the acyl chain region of membrane phospholipids. Tetracaine's interaction with phospholipids was affected by temperature. Procaine did not fluidise the acyl chain region of the membrane at 37°C or 43°C (Kingston *et al.,* 1993).

In the present study the mechanism of action of the three local anaesthetics was not investigated. The reduction in cell viability may have been related to increased membrane fluidity, increased intracellular calcium concentration or to apoptosis. Increasing dibucaine concentrations (0-1mM) was found to increase significantly membrane fluidity of both inner and outer membranes in SK-N-MC human neuroblastoma cells at 20°C measured using fluorescence polarisation of DPH and TMA-DPH (Kim *et al.*, 1997). A cell suspension of 10^6 cells/ml was mixed with DPH or TMA-DPH and left to equilibrate at 20°C for 30 minutes before the addition of dibucaine which was incubated for 30 minutes at 20°C before membrane polarisation measurements were taken. Dibucaine (0.1mM) was shown to produce apoptosis in SK-N-MC human neuroblastoma cells (Kim *et al.*, 1997). Apoptosis was determined by the production of internucleosomal DNA fragmentation analysed by gel electrophoresis. Internucleosomal DNA fragmentation was apparent after an 18 hour incubation with dibucaine. The resulting fragmentation patterns were consistent with the molecular weight expected from internucleosomal DNA cleavage associated with apoptotic cell death (Gaido & Cidowski, 1991). The percentage of apoptotic cells increased as a function of the log of the dibucaine concentration (Kim *et al.*, 1997).

Local anaesthetics have been shown to increase cytosolic calcium (Grant & Acosta, 1994). In SK-N-MC human neuroblastoma cells dibucaine (0.1mM) was shown to induce a rapid increase in intracellular calcium concentration followed by a sustained increase in concentration (Kim *et al.*, 1997). In calcium free Krebs-Ringer solution the rapid and sustained increase in intracellular calcium was less profound. To determine if the increase in intracellular calcium was a

result of increased membrane permeability by dibucaine resulting in an influx of calcium from extracellular sources or the ability of dibucaine to displace calcium from binding sites calcium release blockers and calcium chelators were used to produce an effect on cell death. A concentration of 45μM dibucaine was used to determine this, this concentration killed approximately 70% of neuroblastoma cells (Kim *et al.*, 1997). The addition of inhibitors of intracellular calcium release and intracellular calcium chelators with dibucaine incubation showed no difference to the percentage of viable cells. However, when extracellular calcium was reduced, neuroblastoma cells treated with dibucaine showed an increase in the percentage of viable cells to approximately 40%. This showed that dibucaine probably induced neuroblastoma cell death by membrane damage allowing an influx of extracellular calcium (Kim *et al.*, 1997).

In SK-N-MC human neuroblastoma cells, the cell viability was significantly increased by the antioxidants L-ascorbic acid, L-cysteine and catalase at 45μM in the presence of 30μM dibucaine (Kim *et al.*, 1997), suggesting that dibucaine induced neuroblastoma cell death involves the production of oxygen free radicals.

In the present study local anaesthetics were used together with the ether lipid, ET-18-OCH_3. Subtoxic concentrations of the local anaesthetics were required for each cell line. At low concentrations (>0.02mM), dibucaine was more cytotoxic to HL60 cells than K562 cells. A subtoxic dose for dibucaine was not determined for the HL60 cells. A subtoxic dose of 0.1mM dibucaine was determined for K562 cells. The IC_{50} values for tetracaine and procaine were similar for the HL60 and K562 cells although at lower concentrations both these local anaesthetics were more cytotoxic to the K562 cells than to the HL60

cells. Subtoxic concentrations of tetracaine used were 0.15mM for K562 cells and 0.2mM for HL60 cells. Subtoxic concentrations of procaine used were 2mM for K562 cells and 4mM for HL60 cells.

Ether lipids and local anaesthetics are considered to be membrane active agents and both affected the reproductive ability of the HL60 and K562 cell lines. A series of experiments were undertaken to discover if combining the ether lipid with a local anaesthetic would alter the cytotoxicity of these agents. A subtoxic dose of local anaesthetic that reduced the reproductive ability $\leq$ 10% was used, in conjunction with a range of ether lipid concentrations (0-20μM). A dose of 0.1mM dibucaine was used for the K562 cells. Statistical analysis by Oneway ANOVA showed a significant difference between cells treated with ether lipid alone and those treated with ether lipid plus 0.1mM dibucaine at ET-18-OCH_3 concentrations of: 1μM, 3μM, 5μM and 7.5μM ($p < 0.05$). The cytotoxic effect was synergistic at ET-18-OCH_3 concentrations of above 3μM and up to 7.5μM. For the K562 cell line, the IC_{50} values were 8.5μM for ether lipid treated cells and 3.7μM for ether lipid plus 0.1mM dibucaine treated cells. As the concentration of ether lipid was increased above 7.5μM the differences between the two treatments became less apparent, and at an ether lipid concentration of 15μM the cells from both treatments lost all of their reproductive ability. Between the ether lipid concentrations of 3μM and 7.5μM, 0.1mM dibucaine significantly reduced the reproductive ability of the K562 cells. As the concentration of ET-18-OCH_3 increased above 5μM the effect of the 0.1mM dibucaine was gradually lost as the cytotoxicity of the ether lipid itself increased.

The effect of ether lipid on K562 cells was determined in the presence or absence of tetracaine. K562 cells were treated with ET-18-CH_3 (0-10μM) only or ET-18-OCH_3 (0-10μM) in the presence of 0.15mM tetracaine, but there was no significant difference between the effect on reproductive ability of these two treatments. The IC_{50} value in the presence of ET-18-OCH_3 alone was 6.2μM and this was reduced slightly to 5.5μM when 0.15mM tetracaine was present with ET-18-OCH_3 for the K562 cell line, but this difference was not statistically significant. The effect of ether lipid on K562 cells in the presence or absence of procaine was also determined. No alteration in the reproductive ability of K562 cells was evident when the cells were grown with ET-18-OCH_3 (0-10μM) alone compared to cells grown with ET-18-OCH_3 (0-10μM) plus 2mM procaine. The IC_{50} values were 5.15μM for ether lipid treated cells, and 3.9μM for ether lipid plus procaine treated cells, but this difference was not statistically significant.

In summary, in these combined ether lipid and local anaesthetic experiments for the K562 cells only the combination of ET-18-OCH_3 and 0.1mM dibucaine produced a synergistic effect on the inhibition of reproductive ability compared to ET-18-OCH_3 alone. In neuroblastoma cells 0.1mM dibucaine increased intracellular calcium and apoptosis. The overall effect of dibucaine on SK-N-MC human neuroblastoma cells was apoptosis (Kim *et al.*, 1997). The apoptotic mechanisms may involve membrane damage, increased intracellular calcium and production of oxygen free radicals. At 0.1mM dibucaine increased intracellular calcium, mainly induced by membrane damage, and induced apoptosis in human neuroblastoma cells. During the present study it is unknown if intracellular calcium was increased, if apoptosis

was induced, or if the membrane was damaged to produce the synergistic effect of dibucaine with ET-18-OCH_3. Tetracaine and procaine when combined with ET-18-OCH_3 showed no alteration in the K562 cell's reproductive ability compared to ET-18-OCH_3 alone.

Combined ether lipid and local anaesthetic experiments were also undertaken on the HL60 cell line. As the HL60 cell line was sensitive to low concentrations of dibucaine, it was decided that a subtoxic dose of dibucaine could not be achieved. Therefore, the combined experiments were undertaken only with tetracaine (0.2mM) or procaine (4mM). The effects of ether lipid (0-5μM) on HL60 cells in the presence or absence of tetracaine (0.2mM) were determined, but no significant differences were observed between HL60 cells treated with ether lipid and those treated with ether lipid plus tetracaine (0.2mM). As the concentration of ET-18-OCH_3 was increased, the reproductive ability of the HL60 cells was reduced with IC_{50} values of 3.3μM for ether lipid plus tetracaine treated cells and 4.16μM for ether lipid treated cells. Although there was a slight decrease in IC_{50} value when using the ET-18-OCH_3 plus tetracaine treatment, no significant difference was observed when statistically analysed using Oneway ANOVA. Furthermore, there was no statistically significant difference observed between HL60 cells treated with ether lipid or with ether lipid plus procaine. As the concentration of ET-18-OCH_3 was increased the reproductive ability of the cells decreased. The IC_{50} values were 3.49μM for ET-18-OCH_3 alone and 3.38μM for ET-18-OCH_3 plus procaine treatment of HL60 cells.

From this series of experiments it is difficult to determine how ET-18-OCH_3 and the local anaesthetics exert their effects on both leukaemia cell lines. It was interesting that only dibucaine in

combination with ET-18-OCH_3 increased toxicity of the ether lipid in K562 cells. This synergistic cytotoxic effect may have been due to dibucaine increasing the membrane fluidity, allowing ET-18-OCH_3 to affect the membrane to a greater extent. Both ether lipids and local anaesthetics are membrane active agents that have a wide variety of effects, and therefore the combination of these agents may alter the activity of the transmembrane signalling enzymes, intracellular calcium, or induce free radicals to produce the synergistic cytotoxicity seen with ET-18-OCH_3 and dibucaine. Unfortunately the HL60 cells were too sensitive to dibucaine to determine any enhancement of cytotoxicity when combined with ether lipid.

Chapter III
Effects of Fatty Acid Supplementation on Human Leukaemia Cells

3.1 Introduction

Fatty acids are present as complex lipids in mammalian tissues and are obtained from dietary fat or through biosynthesis. The n-3 and the n-6 fatty acids are two classes of polyunsaturated fatty acids (PUFAs) that cannot be completely synthesised in mammals and are derived from the diet. Essential fatty acids (EFAs) cannot be synthesised by the body. Fatty acids from the diet are elongated and desaturated to form the EFAs. All EFAs are polyunsaturated fatty acids (PUFAs), but many PUFAs are not EFAs. EFAs are precursors of eicosanoids and are important structural components of cell membranes.

There are two families of EFAs, the n-6 PUFAs derived from linoleic acid $18:2^{\Delta 9,12}$ (LA) and the n-3 PUFAs derived from alpha-linolenate (ALA) $18:3^{\Delta 9,12,15}$. To function fully as EFAs, LA and ALA must have all of their double bonds in the cis-form and be enzymatically transformed by the enzyme delta-6-desaturase (Horrobin, 1982). In the n-6 family, LA is desaturated by this enzyme to form gamma-linolenic acid (GLA) $18:3^{\Delta 6,9,12}$, which is in turn elongated to give rise to dihomogamma-linolenic acid (DGLA) $20:3^{\Delta 8,11,14}$, the precursor of the 1-series prostaglandins (PGs). DGLA can also be desaturated by delta-5-desaturation to form arachidonic acid (AA) 20:4 $\Delta 5,8,11,14$, the precursor of 2-series PGs, thromboxanes and leukotrienes. In the n-3 family, a similar set of enzymes metabolise ALA. ALA is desaturated and elongated to give rise to eicosapentaenoic acid (EPA) $20:5^{\Delta 5,8,11,14,17}$, the precursor of the 3-series PGs. EPA

is further converted to docosahexaenoic acid (DHA) $22:6^{\Delta 4,7,10,13,16,19}$. The delta-6-desaturase enzyme determines the tissue levels of GLA, DGLA and 1-series eicosanoids. Under normal conditions AA, EPA, and DHA can be obtained from the diet.

Several tumours have had their membrane properties and functions modified by fatty acid supplementation. The fatty acid composition of cultured cells can be modified extensively by changing the type and amount of lipid contained in the culture medium (Burns & Spector, 1987). When fatty acids are available in the extracellular fluid, they are utilised preferentially, and *de novo* fatty acid synthesis is suppressed (Spector, 1975 & Spector *et al.*, 1981). L1210 murine leukemia cells or Ehrlich ascites tumour cells were grown in male mice that had been fed on a diet supplemented with 16% saturated fatty acid from coconut oil or 16% polyunsaturated fatty acid from sunflower seed oil for four weeks prior to the inoculation of the tumour and during the growth of the tumour (Burns & Spector, 1987). The fatty acid supplementation altered the membrane fatty acid composition in both the cell treatments. The membranes of L1210 cells grown in mice fed on a diet supplemented with 16% coconut oil contained twice as much monounsaturated fatty acid and half as much polyunsaturated fatty acid, but the overall saturated fatty acid content remained essentially unchanged (Burns & Spector, 1987). A large increase in oleic acid and a large decrease in linoleic acid with little or no change in the content of the other major fatty acids was detected. Similar fatty acid modifications were observed in the phospholipids of the cell membranes of Ehlrlich ascites carcinoma cells grown in mice fed with fatty acid-supplemented diets (Burns & Spector, 1987). In both cases the effects were confined to the membrane fatty acid composition and

there was no change in membrane phospholipid content, cholesterol content or phospholipid head group composition. This suggests a substitution of fatty acyl groups within the same phospholipid of the membrane. Lipid modifications produced in intact tumours have altered the physical properties of the plasma membrane lipid bilayer and produced a change in membrane fluidity (Burns & Spector, 1987). Supplementation of the culture medium of L1210 murine leukaemia cells with 32μM DHA, caused an increase of 250% in polyunsaturated fatty acid content of the cell membrane (Burns & Spector, 1987). This was accounted for by an increase in DHA content, which was compensated for by a decrease of 57% in monunsaturated fatty acid, primarily in 18:1. DHA supplementation also resulted in a 35% increase in the membrane saturated fatty acid content. Similar modifications were found in cultured human Y-79 retinoblastoma cells (Burns & Spector, 1987). Y-79 cells supplemented with AA (30μM) were found to have considerably less 18:1 and 73% more 20:4 in the cell membrane phospholipids compared to cells supplemented with oleic acid. When Y-79 cells were supplemented with DHA (30μM) the cell membranes contained less 18:1 and 270% more 22:6 compared to cells supplemented with oleic acid.

In this study HL60 and K562 cells were supplemented with fatty acids, and the effect on the membrane phospholipids was determined using gas-liquid chromatography. The fatty acids used in this study were OA, LA, AA, EPA and DHA. Their structures are shown in Figure 3.1.

The fatty acid supplementation of L1210 leukaemia cells grown in mice fed a diet of 16% sunflower oil, a rich source of PUFA, was shown to affect carrier-mediated transport (Burns & Spector, 1987). PUFA supplementation reduced the uptake of methotrexate, a

chemotherapeutic drug, by 30% in L1210 leukaemia cells (Burns & Spector, 1987). As the K'_m of the transport process was changed the mechanism probably involved structural lipids altering the conformation of the membrane carrier sufficiently to influence the binding of the substrate. The effects on transport kinetics at 37°C were not uniform for all substances. This suggested a more complex mechanism than a general response to changes in bulk membrane fluidity (Burns & Spector, 1987).

PUFA supplementation of cells has been shown to increase lipid peroxidation and membrane fluidity. GLA, EPA and PGE_1 (between 40 and 100μg/ml) inhibited the proliferation of three colon cancers HRT 18, HT 29 and CACO 2 *in vitro* (Mengeaud *et al.*, 1992). The inhibition of the three cell lines was in the order HRT 18 > HT 29 > CACO 2. Although the IC_{50} values differed between the cell lines the inhibition by the fatty acids was in the order GLA > EPA > PGE_1. GLA or EPA supplementation (60μM) increased the membrane fluidity of all three cells lines, whereas PGE_1 had no effect. GLA and EPA also caused an increase in lipid peroxidation, but PGE_1 had no effect. GLA inhibited the proliferation of the three cell lines more than EPA and also induced lipid peroxidation more than EPA. The presence of 10μM vitamin E totally corrected the increase in membrane fluidity by GLA and EPA and reduced the amount of lipid peroxidation. Mengeaud *et al.* (1992) found that PGE_1 stimulated cAMP synthesis and GLA stimulated cAMP synthesis in the HRT 18 cells only. EPA decreased cAMP synthesis in all three cell lines. This inhibitory effect on tumour cell growth was due to the fatty acids themselves as indomethicin, an inhibitor of PG synthesis, did not modify the inhibition of proliferation caused by EPA and GLA and did not lower the levels of lipid peroxidation induced by these fatty acids (Mengeaud *et al.* 1992).

PUFA-induced lipid peroxidation can alter the effect of other agents on cells and is related to apoptotic cell death. Human breast tumour cells, MDA-MB-231, were treated with a subtoxic concentration of DHA (29μM) in the presence of a cytotoxic concentration of doxorubicin (1μM) for 6 days (Germain *et al.*, 1998). DHA supplementation enhanced doxorubicin toxicity over the 6 days, and this effect was further increased when oxidants were also present with DHA and doxorubicin. The effect of different fatty acids on doxorubicin-induced toxicity in MDA-MB-231 cells was determined (Germain *et al.*, 1998). In the presence of oxidants the fatty acids (LA, α-linolenic acid, γ-linolenic acid, AA, EPA, and DHA) increased doxorubicin toxicity more than the fatty acid and doxorubicin alone. The presence of antioxidants with the fatty acids reduced the enhanced effect of the fatty acids on doxorubicin toxicity. The order of the fatty acid effect of enhancing the toxicity of doxorubicin was in the order DHA > γ-linolenic acid > EPA > AA > α-linolenic acid > linoleic acid. Doxorubicin increased lipid hydroperoxide level in MDA-MB-231 cells 2-fold but the addition of DHA or oxidants did not significantly change the lipid hydroperoxide level. DHA with oxidants significantly increased the lipid peroxide level by 5-fold in MDA-MB-231 cells (Germain *et al.*, 1998). When vitamin E was substituted for the oxidants both cell viability and lipid hydroperoxides returned to baseline levels. An increase in doxorubicin activity may have been due to increased lipid peroxide conditions. Highly unsaturated PUFAs could generate lipid peroxidation products from the primary radicals after the action of cytotoxic drugs (Germain *et al.*, 1998). These products could act as 'second messengers' and enhance the tumour drug sensitivity.

PUFAs were shown to inhibit the growth of pancreatic cancer cell lines and HL60 cells (Hawkins *et al.*, 1998). The PUFA-induced

cytotoxicity increased with the number of double bonds as did the degree of lipid peroxidation, with the exception of *cis*-parinaric acid (CPA) with four conjugated double bonds which was readily peroxidised. HL60 cells supplemented with 20μM fatty acid for 25 hours showed an increase in percentage cell death. The order of the fatty acid cytotoxicity was CPA 100% > DHA 85% = AA 85% > EPA 70% > γ-linolenic acid 35% > α-linolenic acid 7% = linoleic acid 7% > oleic acid 5%. PUFA-induced lipid peroxidation was correlated to the proportion of cell death in pancreatic cells and HL60 cells (Hawkins *et al.*, 1998). Vitamin E (50μM) blocked lipid peroxidation and cell death. HL60 cells treated with EPA (50μM) between 12-30 hours produced a pattern of chromatin cleavage into oligonucleosomes characteristic of apoptosis (Hawkins *et al.*, 1998). In pancreatic cells treated with EPA (50μM) there was a great increase in lipid peroxidation, assayed by the thiobarbituric acid, between 6-24 hours. Hawkins *et al.* (1998) suggested that lipid peroxidation was involved in the PUFA-induced apoptosis in pancreatic cells and HL60 cells.

In this study the effect of different concentrations of PUFA on cell reproducibility was determined. The addition of the antioxidant vitamin E was also used in PUFA supplementation to determine if lipid peroxidation products may be involved with cytotoxicity in HL60 and K562 cells.

The fatty acid supplementation of cells can enhance or inhibit cell growth. The effects of different fatty acids on the cell growth of MDA-MB-231 breast cancer cells in serum free culture medium was investigated by Rose & Connolly (1990). The cells were supplemented with LA, OA, EPA or DHA dissolved in ethanol for 6 days. Over the incubation period LA stimulated growth at an optimal concentration of 0.75μg/ml. OA stimulated cell growth only at low concentrations

(0.25μg/ml). DHA significantly reduced cell growth between 1-2.5 μg/ml. EPA significantly reduced cell growth at 2.5 μg/ml (Rose & Connolly, 1990). Selective inhibitors of prostoglandin and leukotriene synthesis were used to investigate the mechanism for the stimulation of MDA-MB-231 cell growth by LA. Indomethacin reduced the stimulatory effect of LA on the MDA-MB-231 cells but the cells' growth was still above that seen in the absence of LA supplementation. MDA-MB-231 cells were grown in serum-free medium containing 625ng/ml of LA for 6 days with indomethecin, nordihydroguaiaretic acid (NDGA) or esculetin. NDGA and esculetin inhibited cell growth more than indomethicin. NDGA and esculetin inhibited leukotriene biosynthesis. At 16μM NDGA or 225μM esculetin there was approximately a 74% reduction in cell number. These results suggested that MDA-MB-231 cell growth was dependent on inhibition of leucotriene biosynthesis rather than prostaglandin biosythesis, as both NDGA and esculetin were more effective at inhibiting cell growth than indomethecin (Rose & Connolly, 1990).

Some fatty acids are the precursors of prostaglandins so PUFA supplementation may be able to alter the production of prostaglandins. Two murine colon ademocarcinoma cell lines MAC13 and MAC26 were grown in reduced serum concentration for 144 hours supplemented with LA or AA. Both LA and AA enhanced the growth of the two murine colon adenocarcinoma cell lines (Hussey & Tisdale, 1994). The optimum concentration of LA for growth stimulation was 18μM in both cell lines. For cells supplemented with AA the optimum concentration for growth stimulation was 17μM for MAC13 cells and 33μM for MAC26 cells. Higher concentrations of fatty acid inhibited cell growth in both cell lines. To investigate the growth promoting effects of LA and OA the cyclo-oxygenase and lipoxygenase inhibitor

indomethicin and the 5-lipoxygenase inhibitor BWA4C were used on PUFA-stimulated cell growth. MAC26 cells were grown in either 10% FCS or 1% FCS containing 33μM AA. Concentrations above 10μM indomethicin and BWA4C showed a dose-dependent inhibition MAC26 cell growth. Below a concentration of 10μM BWA4C, MAC26 cells supplemented with 33μM AA showed increased cell growth (Hussey & Tisdale, 1994). MAC13 cells supplemented with LA in medium containing 0.5% FCS showed an inhibition of cell growth when treated with indomethacin or BWA4C. BWA4C inhibited the cell growth more effectively than indomethacin in cells supplemented with LA and AA. This suggests that LA and AA stimulate cell growth through a lipoxygenase pathway rather than a cyclo-oxygenase pathway. Neither of the inhibitors decreased LA induced cell proliferation and suggests that growth stimulation may be through another pathway (Hussey & Tisdale, 1994).

Incorporation of PUFAs into membrane phospholipids also modified the metabolism of phospholipids, and especially phosphatidylinositols (Fujiwara *et al.*, 1985). These changes are tolerated by normal cells but are associated with cytotoxicity in cancer cells. L1210 cells supplemented for 48 hours in DHA (32μM) were shown to increase the accumulation of the antitumoural agent adriamycin (Burns & North, 1986). The amplified cytotoxicity was not as a result of increased polyunsaturation.

PUFAs have been shown to enhance differentiation. HL60 cells supplemented with DHA (10μM) for 120 hours have been shown to incorporate into the membrane phospholipids, as shown by gas-liquid chromatography (Burns *et al.*, 1989). HL60 cells supplemented with DHA (10μM) were induced to differentiate with retinoic acid (Burns *et al.*, 1989). During the first 3 days DHA supplementation increased

NBT reduction in retinoic acid treated cells compared to cells supplemented with oleic acid. Cells supplemented with DHA and treated with retinoic acid initiated differentiation, as shown by a proliferative arrest in population size and an increase in the percentage of cells in the $G_{1/0}$ phase of the cell cycle (Burns *et al.*, 1989).

Supplementation of cells with PUFA has also been used to enhance the cytotoxic effect of other agents. L1210 murine leukaemia cells were grown in culture medium supplemented with DHA (32μM) for 2 days. The addition of the ether lipids ET-18-OCH_3 or BM41.440 was shown to be cytotoxic to cells, and to increase membrane lipid peroxidation (Wagner *et al.*, 1992). The addition of vitamin E inhibited the peroxidation and cytotoxicity of ET-18-OCH_3 in a dose dependent manner. In Chapter 2 it was shown that the combination of ET-18-OCH_3 and dibucaine treatment resulted in an increase in cytotoxicity. In this chapter ET-18-OCH_3 was combined with PUFA supplementation to determine if PUFA could alter the cytotoxic effect of this ether lipid. PUFA was also combined with local anaesthetics to determine if the cytotoxic effect can be altered.

Supplementing cells with different fatty acids alters the membranes of tumour cells. In this study human leukaemia cells, K562 and HL60, were supplemented with different fatty acids. The fatty acids used were OA, LA, AA, EPA and DHA. Their structures are shown in Figure 3.1. The incorporation of the fatty acids into the membrane phospholipids was determined by gas-liquid chromatography. PUFAs have been shown to increase lipid peroxidation. In this study vitamin E was used to determine if any of the cytotoxic effects of the PUFAs were due to lipid peroxidation. The products of lipid peroxidation were also

investigated. Combination experiments on cells supplemented with a PUFA and ether lipid treatment and also on cells supplemented with PUFA and local anaesthetics treatment were undertaken to determine any alteration in cytotoxicity.

Figure 3.1 Structure of fatty acids

The structures of five fatty acids that are referred to in this thesis are shown in this Figure.

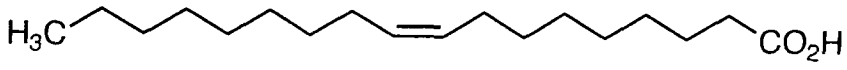
Oleic acid OA (18:1, n-9)

Linoleic acid LA (18:2, n-6)

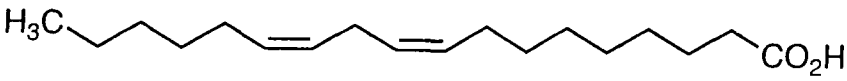
Arachidonic acid AA (20:4, n-6)

Eicosapentaenoic acid EPA (20:5, n-3)

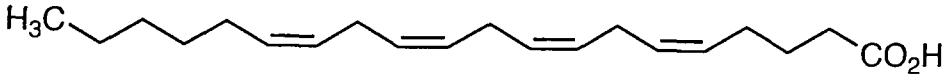
Docosahexaenoic acid DHA (22:6, n-3)



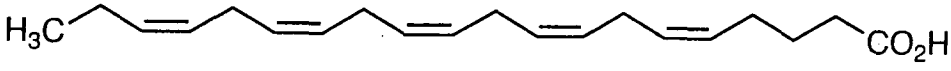
Oleic acid (18:1, n-9)



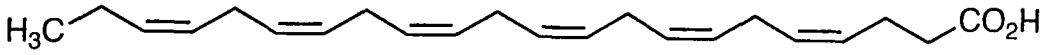
Linoleic acid (18:2, n-6)



Arachidonic acid (20:4, n-6)



Eicosapentaenoic acid (20:5, n-3)



Docosahexaenoic acid (22:6, n-3)

3.2 Materials and Methods

3.2.1 Fatty acid supplementation

The culture medium of K562 cells and HL60 cells was supplemented with one of the following fatty acids in the heat-inactivated foetal calf serum: oleic acid 18:1(n-9) (OA), linoleic acid 18:2(n-6) (LA), arachidonic acid 20:4(n-6) (AA), eicosapentaenoic acid 20:5(n-3) (EPA), or docosahexaenoic acid 22:6(n-3) (DHA).

The fatty acids were obtained either as a free acid or as a sodium salt of the fatty acid in a > 99% pure form. The free fatty acid (76μmoles) was first dissolved in 1ml of 100mM NaOH at 50°C with gentle shaking to form the sodium salt. This represents a 34% molar excess of NaOH over the fatty acid. Otherwise, sodium salts of the fatty acids (76μmoles) were dissolved in 1ml of 20mM NaOH at 50°C. Heat-inactivated foetal calf serum warmed to 40°C was added to achieve a final concentration of approximately 2mM fatty acid. The resulting heat-inactivated foetal calf serum supplemented with fatty acid was sterilised through a 0.22μm filter, aliquoted into glass vials, flushed with nitrogen, sealed and stored at -20°C until required, for a maximum of two months.

Fatty acid-supplemented culture medium was prepared by substituting heat-inactivated foetal calf serum in the culture medium with an appropriate concentration of fatty acid-supplemented heat-inactivated foetal calf serum.

3.2.2 Free fatty acid assay

To determine the concentration of free fatty acid in heat-inactivated foetal calf serum and fatty acid-supplemented heat-inactivated foetal calf serum, a modification of the method described by Antonis (1965) was used. The method is based on the production of the copper salt of the fatty acid in chloroform followed by an estimation of the copper content in the organic phase by reaction with zinc dibenzyldithiocarbamate (ZnDDC). Silicic acid (1.2 ± 0.2g), activated at 110°C for 1 hour prior to use, was slurried with 7.7ml of isopropyl ether in screw cap vials. Previously, the isopropyl ether had been passed through a column of activated alumina to remove peroxides. Heat-inactivated foetal calf serum (0.3ml) or fatty acid-supplemented heat-inactivated foetal calf serum (0.3ml) was added and vortexed for 1.5 minutes. The silicic acid acted as a phospholipid adsorbent, removing phospholipids which were a source of interference, without affecting the levels of free fatty acids. The suspension was allowed to settle and 4ml of the isopropyl ether supernatant was removed and transferred into glass centrifuge tubes which had previously been siliconised using dimethyldichlorosilane (0.5%, v/v) in heptane, then allowed to dry. The isopropyl ether was evaporated to dryness under nitrogen in a 40°C water bath and the lipid was redissolved in 5ml of chloroform. 'Copper reagent' (2.5ml), consisting of 3.8% (w/v) copper sulphate pentahydrate, 0.45M triethanolamine and 0.05M acetic acid, was added and vortexed for 45 seconds. The tubes were then centrifuged at 300g for 10 minutes at room temperature. All of the upper aqueous phase was removed by aspiration with a Pasteur pipette. A 3ml aliquot was taken from the remaining chloroform extract and placed into a clean tube. The colour was then developed by the addition of 0.5ml of ZnDDC (0.3%, w/v) in

chloroform. The tubes were mixed and the absorbance read at 440nm after 15 minutes at room temperature.

Standard curves over the range 0-0.6 μmoles palmitate were produced using 12mM palmitic acid in chloroform and PBS to act as an aqueous phase. Assays were performed in triplicate, with blank assays and standard assays being included in each series of analyses.

The 'copper reagent' and ZnDDC reagent were stored in the dark at 4°C and replaced every 2 months.

3.2.3 Fatty acid toxicity study

The effect of supplementing K562 and HL60 cell lines with fatty acids was determined as follows. Cells (2×10^{5}) were seeded in 1 ml of culture medium supplemented with different concentrations of fatty acid in a 24 well plate, and incubated for 48 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). For K562 cells the fatty acid concentration range was 0-50μM and for HL60 cells it was 0-35μM. The fatty acid concentration in unsupplemented heat-inactivated foetal calf serum was not above 0.117mM. This was reduced to 11.7μM in the culture medium.

After 48 hours the cells were pelleted by centrifugation at 300g for 4 minutes at 20°C. The fatty acid-supplemented culture medium was removed and the cells were resuspended in 1 ml of fresh unsupplemented culture medium. The control cells were counted, using a haemocytometer as described in section 2.2.3. From the control, an appropriate volume containing fifty thousand cells were transferred to a 96 well plate. The same volume of cell suspension was transferred from the other conditions to a 96 well plate. The number of cells in the other conditions were not counted, but the same volume as fifty thousand

control cells was used. Each condition had four replicate wells. These cells were incubated with ^{3}H-Thymidine as described in section 2.2.6.

3.2.4 Fatty acid toxicity in the presence or absence of α-tocopherol

To determine if the effect of PUFA toxicity on the cell lines could be reduced by an antioxidant, α-tocopherol was added to the culture medium of HL60 and K562 cells. A stock solution of α-tocopherol (0.2mM) was prepared in 100% (v/v) ethanol and filter sterilised. It was stored for a maximum of two weeks in a glass container at -20°C. The α-tocopherol was diluted using 100% (v/v) ethanol. The required concentration of α-tocopherol was added to the culture medium so that the ethanol was present as 0.1% (v/v) of the culture medium.

Cells were supplemented with fatty acid for 48 hours, as described in section 3.2.3, in the presence or absence of α-tocopherol (10μM). After 48 hours the cells were pelleted by centrifugation at 300g for 4 minutes at 20°C. The fatty acid-supplemented culture medium ± α -tocopherol was removed and the cells were resuspended in fresh unsupplemented culture medium. The control cells were counted, using a haemocytometer as described in section 2.2.3. From the control, an appropriate volume containing fifty thousand cells was transferred to a 96 well plate and an equivalent volume of cell suspension was transferred from the other conditions to a 96 well plate. These cells were incubated with ^{3}H-Thymidine as described in section 2.2.6.

3.2.5 Fatty acid incorporation into K562 and HL60 cells

The cytotoxicity of the different fatty acids on both cell lines was established in section 3.2.3. From these results a dose which killed ≤ 10% of the cells was chosen to be a subtoxic dose. Cells (2 x 10^5cells/ml) were seeded in culture medium with a subtoxic dose of each fatty acid, and incubated for 48 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). The K562 cells were incubated with 10μM DHA, 20μM EPA, 20μM AA, 20μM OA, and 20μM LA. The HL60 cells were incubated with 1μM DHA, 1μM EPA, 1μM AA, 50μM OA, and 20μM LA. After 48 hours the cells were pelleted by centrifugation at 300g for 4 minutes at 20°C. The culture medium was removed and the cells were resuspended in PBS. The centrifugation was repeated twice and the cells were finally resuspended in 10ml PBS. A cell count was taken to determine the cell number, as described in section 2.2.3. The lipids were then extracted, as described below.

3.2.6 Lipid extraction

Lipid extractions were carried out on serum, fatty acid-supplemented serum and from control and fatty acid-supplemented HL60 and K562 cells by the method of Bligh & Dyer (1959).

All solvents contained 0.005% (w/v) butylated hydroxytoluene (BHT) to minimise oxidation of fatty acids. To 1 volume of sample, 3.75 volumes of chloroform/methanol (1:2, v/v) was added with thorough mixing. Subsequent additions of 1.25 volumes chloroform and 1.25 volumes distilled water were each followed by thorough mixing. To assist the separation of the two phases, a ten minute 300g centrifugation was carried out at room temperature. The bottom phase

was then removed and dried under nitrogen to a small volume which was used for lipid assay procedures.

3.2.7 Separation of phospholipids from neutral lipids

Separation of phospholipids from the lipid extract was achieved by silicic acid column chromatography. As phospholipids are polar molecules they are adsorbed more strongly to silicic acid than neutral lipids. The neutral lipids such as acylglycerols and fatty acids can be washed off the column first with chloroform. Phospholipids are subsequently eluted with methanol.

Silicic acid columns were used to separate phospholipids from the lipid extracts of HL60 and K562 cells (Borgström, 1952; Stein & Hales, 1972). Silicic acid was activated by heating for 1 hour at 110°C. Hyflo Supercel, an inert support, was added to increase the flow rate, using 0.5 g Hyflo Supercel/g silicic acid.

The silicic acid and Hyflo Supercel were suspended in chloroform and poured into a glass column of 0.6cm internal diameter and 7 cm height. The lipid extract from the cells was added to the column in chloroform. The neutral lipids were eluted with 20ml of chloroform; the phospholipids were eluted with 20ml of methanol. When methanol was added the column changed from a cream colour to white. Therefore the cross over point between the two solvents could be seen, and the neutral lipids and phospholipids were kept separate. The phospholipid fraction was then dried under nitrogen and then used to obtain the fatty acid methyl esters (FAME).

3.2.8 Preparation of fatty acid methyl esters (FAME)

The fatty acid composition of the cellular phospholipids was determined using gas-liquid chromatography. The FAME were prepared using a modification of the method of Morrison & Smith (1964). The phospholipid fraction was dissolved in 0.5 ml boron trifluoride/methanol solution, the tubes were flushed with nitrogen, sealed, and incubated for 15 minutes at 100 °C. Cooled tubes were flushed again with nitrogen to remove volatiles created during incubation, and 0.5 ml distilled water and 1ml hexane were added to each tube and vortexed.

To assist the separation of the hexane and aqueous phases the tubes were centrifuged at 500g for 10 minutes at room temperature. The FAME were extracted in the hexane phase which was concentrated to a small volume (10-20µl) under nitrogen. Aliquots of the FAME were either injected onto the GLC column or dried down completely under nitrogen, and then sealed and stored in the dark at -20°C for future use, usually overnight or for 24 hours.

The separation of FAME was carried out using a Shimadzu GC-9A Series gas chromatograph (glass column 2.0 m long, 2.0 mm internal diameter and 6.0 mm external diameter, packed with cyano-silicone stationary phase, 10% Alltech CS-5, on a chromasorb WAW 100-200 mesh support), connected to a Shimadzu C-R6A Chromatopac integrator. Nitrogen was used as the carrier gas and the resolved components were detected by a flame ionisation detector (Hydrogen/air). A temperature programme (30 minutes per sample) was run : initial temperature = 210°C (10 minutes), rate of increase 4°C/minute (10 minutes), final temperature = 250°C (10 minutes), carrier gas flow rate 59 ml/minute. Peaks were identified by comparison

of relative retention times (RRT) of the fatty acids relative to C16:0 with the RRTs of FAME standards.

3.2.9 PUFA in combination with ether lipid toxicity study

K562 cells (2 x 10^5) were seeded in 1ml of culture medium. One set of cells, the controls, had no treatment. The second set of cells had no PUFA treatment. The third set of cells were supplemented with 20μM EPA and all cells were grown for 48 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). The chosen concentration of PUFA was a dose which killed ≤ 10% of the cells, and this was the chosen 'subtoxic' dose determined from section 3.2.3. After 48 hours the cells were counted as described in section 2.2.3, and pelleted by centrifugation at 300g for 4 minutes at 20°C. The culture medium was removed, and the cells were resuspended in 1ml of serum-free culture medium seeded at 5 x 10^5 cells/ml. The control cells had 0.1% ethanol with no ether lipid added. The other two set of cells were incubated with a series of ether lipid concentrations for 4 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v).

A range of ether lipid concentrations were prepared by serial dilution in 100% ethanol, as described in section 2.2.7. The required concentration of ether lipid was added to the serum-free culture medium so that the ethanol was present as 0.1% (v/v) of the culture medium. After 4 hours the cells were pelleted and the cells were incubated with ^{3}H-thymidine as described in section 2.2.6.

3.2.10 PUFA in combination with local anaesthetic toxicity study

K562 cells (2 x 10^5) were seeded in 1ml of culture medium. One set of cells, the controls, had no treatment. The second set of cells had no PUFA treatment. The third set of cells were supplemented with a subtoxic dose of 20μM EPA, then all cells were incubated for 48 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). After 48 hours the cells were counted as described in section 2.2.3, and pelleted by centrifugation at 300g for 4 minutes at 20°C. The culture medium was removed and the cells were resuspended in 1ml of serum-free culture medium seeded at 5 x 10^5 cells/ml. The control cells had 0.1% (v/v) of distilled water added with no dibucaine present. The other two sets of cells were incubated with a series of dibucaine concentrations for 4 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v).

A range of dibucaine concentrations was prepared by serial dilution in distilled water, as described in section 2.2.8. An appropriate volume was then added to the serum-free culture medium to achieve the required concentration of 0.1% (v/v) of distilled water in the culture medium. After 4 hours the cells were pelleted and then incubated with ^{3}H-thymidine as described in section 2.2.6.

3.3 Results

3.3.1 Cytotoxic effects of fatty acids on HL60 and K562 cells

The cytotoxic effect of fatty acids on HL60 and K562 cells' reproductive ability was carried out as described in section 3.2.3. The fatty acids were dissolved in heat inactivated foetal calf serum and following filter-sterilisation the concentration of fatty acid in the stock solution was determined using the free fatty acid assay as described in section 3.2.2 and was found to be in the range of 1.6-1.8mM. The original heat inactivated foetal calf serum contained approximately 0.117mM free fatty acid.

The fatty acids were much more cytotoxic to the reproductive ability of the HL60 cells than the K562 cells. When both cell lines were supplemented with OA or LA a decrease in reproductive ability was observed. However, differences in reproductive ability became apparent between the two cell lines as the number of double bonds and carbon chain length increased in the PUFAs used to supplement the culture medium.

Figure 3.2 shows the effect of supplementing K562 cells with OA, LA, AA, EPA and DHA . The order of cytotoxicity in the K562 cells was OA = LA < EPA < AA < DHA . The concentration at which 50% of the cells were inhibited (IC_{50}) for the K562 cell line was > 50μM OA, > 50μM LA, > 50μM AA, > 50μM EPA, and 22.2μM DHA. EPA was less cytotoxic than AA, even though EPA has a longer carbon chain and more double bonds. DHA was the most cytotoxic fatty acid. This may be related to the double bonds and carbon chain length. DHA has the most double bonds (6) and the longest carbon chain length (22 carbon atoms).

Figure 3.2 The effect of fatty acids on K562 cells

K562 cells were incubated with increasing concentrations of fatty acid for 48 hours, as described in section 3.2.3. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM Fatty acid) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

■ DHA supplemented cells

● EPA supplemented cells

▲ AA supplemented cells

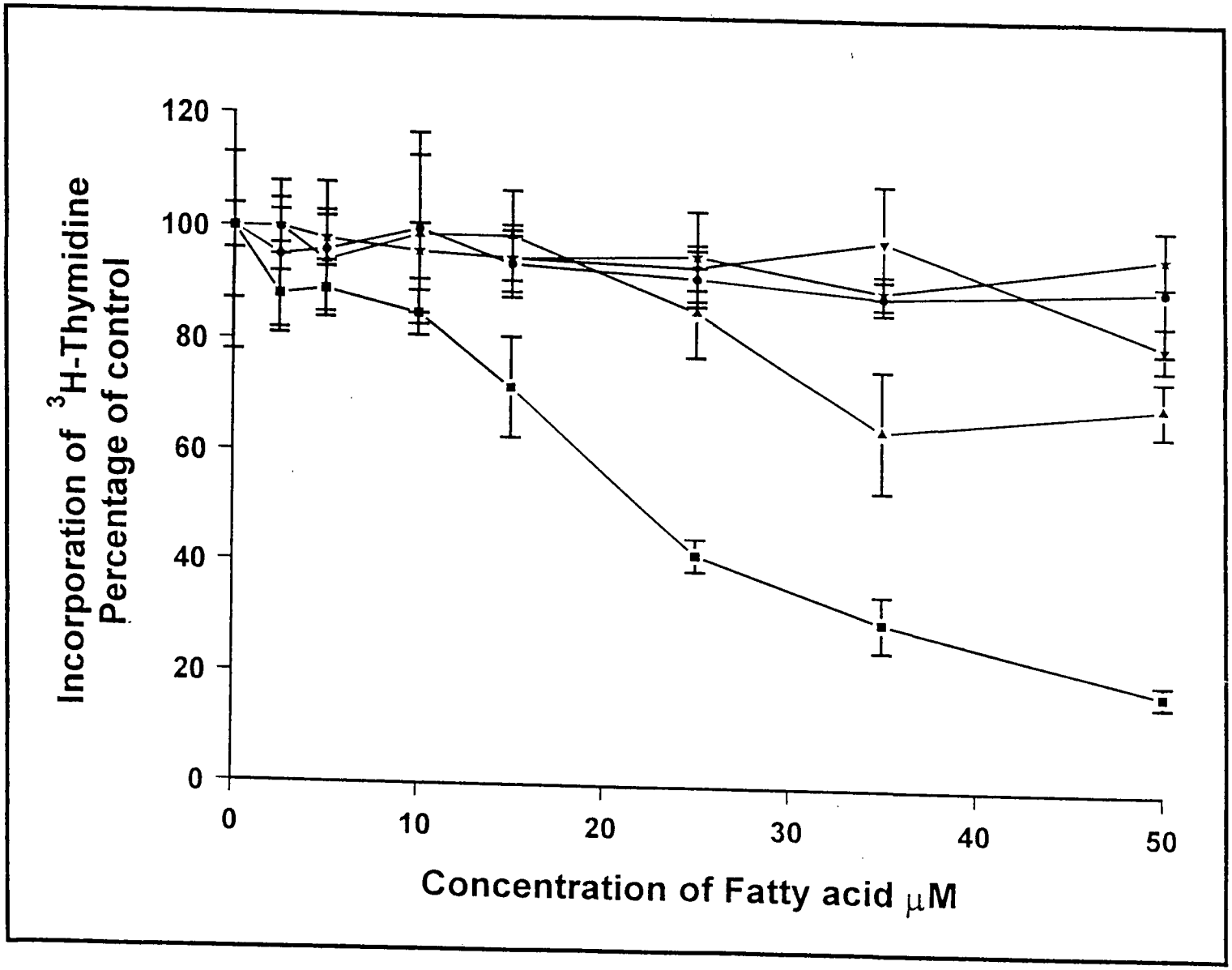
▼ LA supplemented cells

★ OA supplemented cells

The IC_{50} values for the fatty acids were determined from this Figure.

Fatty acid	IC_{50} value μM
DHA	22.2
EPA	> 50
AA	> 50
LA	> 50
OA	> 50

Oneway ANOVA was used to compare the data.
The reproductive ability of cells supplemented with DHA was significantly lower than the reproductive ability of cells supplemented with other PUFAs at 15μM, 25μM, 35μM and 50μM ($p < 0.05$).
The reproductive ability of cells supplemented with AA was significantly lower than the reproductive ability of cells supplemented with EPA, LA or OA at 35μM ($p < 0.05$).
The reproductive ability of cells supplemented with AA was significantly lower than the reproductive ability of cells supplemented with EPA or OA at 50μM ($p < 0.05$).


Incorporation of ^{3}H-Thymidine
Percentage of control
120
100
80
60
40
20
0
0
10
20
30
40
50
Concentration of Fatty acid μM

As the cytotoxicity was determined by the incorporation of ^{3}H-thymidine as a percentage of the control cells, the data had to be transformed by arcsine before statistical analysis. To determine any significant differences between the supplemented fatty acids, Oneway-ANOVA test was performed. The reproductive ability of the DHA-treated cells was significantly lower than that in the presence of other PUFAs at 15μM, 25μM, 35μM and 50 μM ($p < 0.05$). The reproductive ability of the AA-treated cells was significantly lower than EPA, LA and OA at 35μM ($p < 0.05$), and the AA-treated cells was significantly lower than EPA and OA at 50μM ($p < 0.05$).

Figure 3.3 shows the effects of supplementing HL60 cells with OA, LA, AA, EPA and DHA. As the number of double bonds and carbon chain length of the PUFA increased the reproductive ability of the HL60 cells decreased accordingly. The IC_{50} values for the HL60 cell line was > 35μM OA, > 35μM LA, 8.2μM AA, 4.55μM EPA, and 1.6μM DHA. Figure 3.3 shows that DHA supplementation reduced the reproductive ability of the HL60 cells the most, EPA supplementation also reduced the reproductive ability of the HL60 cells considerably. The reproductive ability of cells supplemented with either DHA or EPA were significantly lower than OA supplemented cells at 2.5μM, 5μM, 10μM, 15μM, 25μM, and 35μM ($p < 0.05$). The reproductive ability of cells supplemented with AA were significantly lower than cells supplemented with OA at 5μM, 10μM, 25μM and 35μM ($p < 0.05$).

To obtain a subtoxic dose, which killed ≤10% of the HL60 cells, a very low concentration of PUFA was required. As DHA was the most cytotoxic PUFA the subtoxic dose of 1μM was chosen from its cytotoxicity curve.

Figure 3.4 shows the effect of DHA supplementation in the presence or absence of 10μM α-tocopherol (vitamin E) on the

Figure 3.3 The effect of fatty acids on HL60 cells

HL60 cells were incubated with increasing concentrations of fatty acid for 48 hours, as described in section 3.2.3. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM Fatty acid) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

■ DHA supplemented cells

● EPA supplemented cells

▲ AA supplemented cells

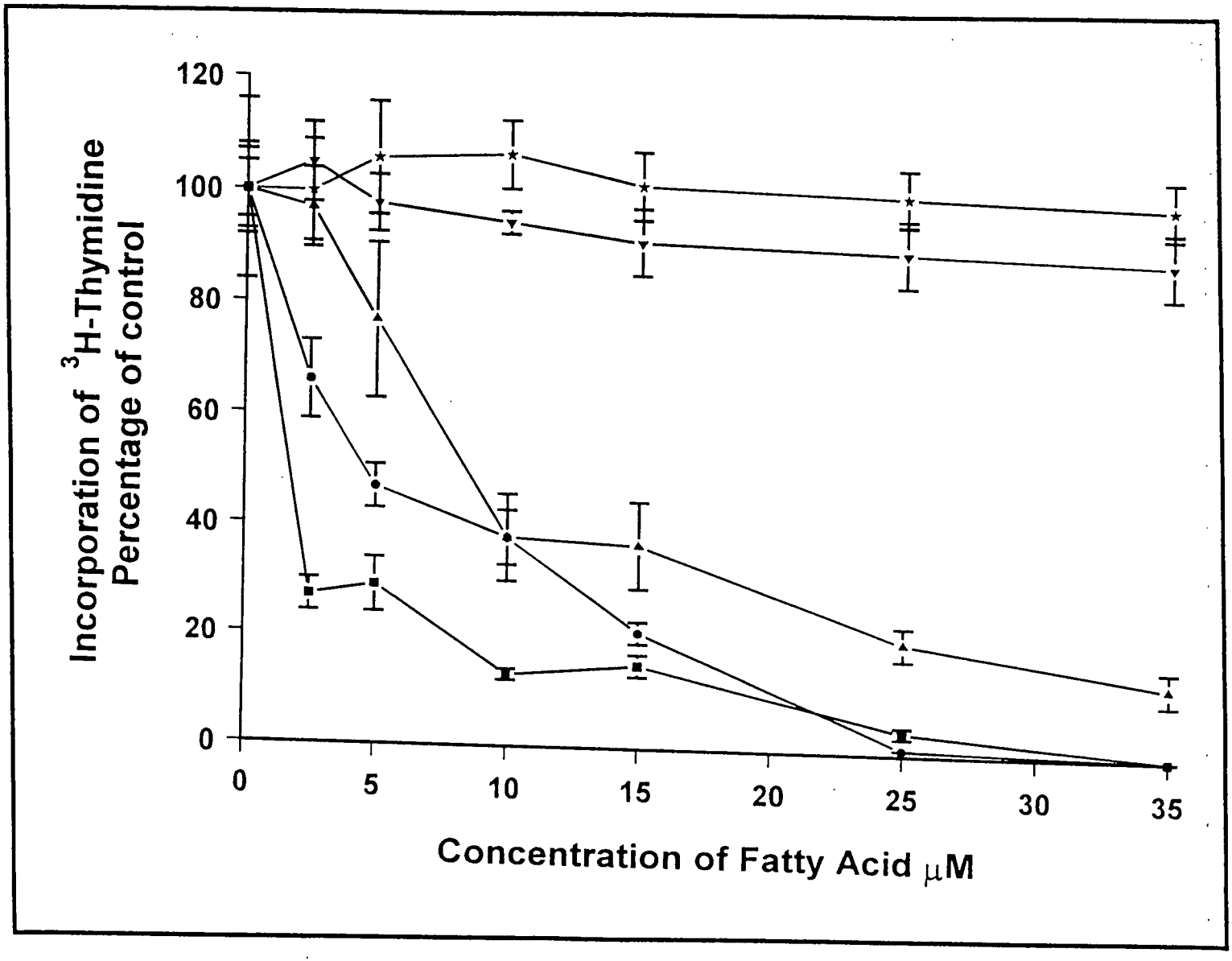
▼ LA supplemented cells

★ OA supplemented cells

The IC_{50} values for the fatty acids were determined from this Figure.

Fatty acid	IC_{50} value μM
DHA	1.6
EPA	4.55
AA	8.2
LA	> 35
OA	> 35

Oneway ANOVA was used to compare the data.
The reproductive ability of cells supplemented with DHA or EPA were significantly lower than the reproductive ability of cells supplemented with OA at 2.5μM, 5μM, 10μM, 15μM, 25μM and 35μM ($p < 0.05$).
The reproductive ability of cells supplemented with AA was significantly lower than the reproductive ability of cells supplemented with OA 5μM, 10μM, 25μM and 35μM ($p < 0.05$).


Incorporation of ^{3}H-Thymidine
Percentage of control
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Concentration of Fatty Acid μM

reproductive ability of the K562 cells. This graph shows that the presence of α-tocopherol considerably reduces the cytotoxic effect of DHA on the cells reproductive ability. The IC_{50} value for DHA supplemented cells was 22.4μM and this was increased to > 50μM in the presence of α-tocopherol. The reproductive ability of DHA supplemented cells in the presence of α-tocopherol was significantly higher than DHA supplemented cells in the absence of α-tocopherol at 25μM, 35μM, and 50μM ($p < 0.05$). When α-tocopherol was present in K562 cells supplemented with either AA or EPA there was no alteration in the cell's reproductive ability from cells that were grown in supplemented fatty acid media alone (summarised in Table 3.1).

The effect of α-tocopherol on fatty acid supplemented HL60 cells was very dramatic, resulting in a considerable reduction in the cytotoxic effects of AA, EPA and DHA on the cells reproductive ability (shown in Figure 3.5). When 10μM α-tocopherol was added to the HL60 cells the IC_{50} of cells supplemented with AA was increased from 8.2μM to > 50μM, the EPA supplemented cells IC_{50} was increased from 4.55μM to 31μM, and the DHA supplemented cells IC_{50} was increased from 1.69μM to 39μM (summarised in Table 3.1).

The reduction in the cytotoxic effects of the fatty acids by the addition of α-tocopherol is believed to be an indication of a reduction in lipid peroxidation. Preliminary experiments were undertaken to determine the onset of lipid peroxidation. No products of lipid peroxidation were discovered. This may have been due to not enough culture medium being examined or that the products of lipid peroxidation had been transformed into untoxic substances.

Figure 3.4 The effect of DHA on K562 cells in the presence or absence of α-tocopherol

K562 cells were incubated with DHA (0-50μM) for 48 hours, in the presence or absence of α-tocopherol (10μM), as described in section 3.2.4. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM DHA) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

- ● DHA supplemented cells in the absence of α-tocopherol
- ■ DHA supplemented cells in the presence of α-tocopherol

The IC_{50} values for K562 cells supplemented with DHA was 22.4μM. The IC_{50} value for K562 cells supplemented with DHA in the presence of α-tocopherol was > 50μM.

Oneway ANOVA was used to compare the DHA supplemented cells in the presence and absence of α-tocopherol.
The reproductive ability of cells supplemented with DHA in the presence of α-tocopherol was significantly higher than cells supplemented with DHA supplemented cells in the absence of α-tocopherol at 25μM, 35μM and 50μM ($p < 0.05$).

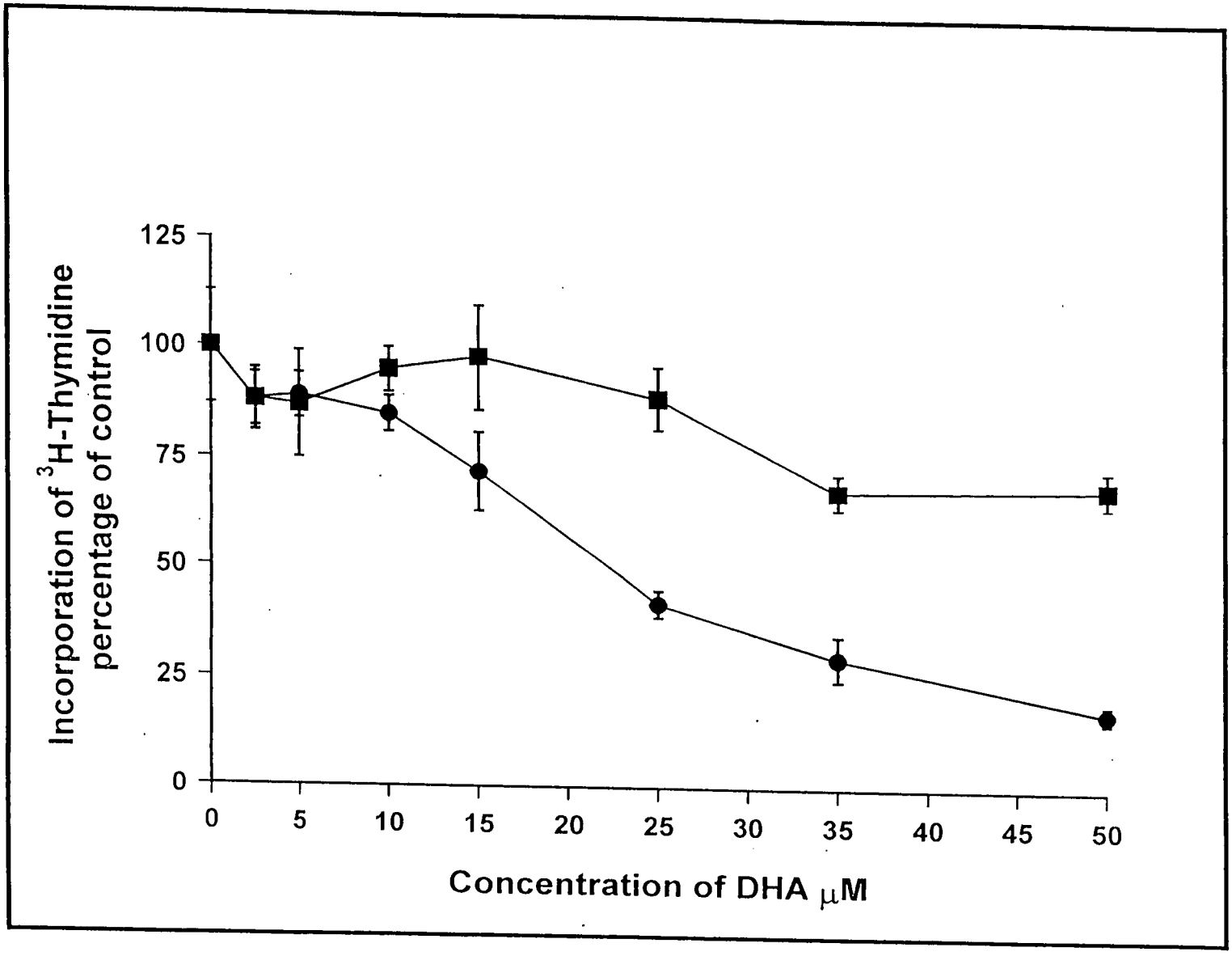

Incorporation of ^{3}H-Thymidine
percentage of control
125
100
75
50
25
0
0
5
10
15
20
25
30
35
40
45
50
Concentration of DHA μM

Figure 3.5 The effect of DHA on HL60 cells in the presence or absence of α-tocopherol

HL60 cells were incubated with DHA (0-50μM) for 48 hours, in the presence or absence of α-tocopherol (10μM), as described in section 3.2.4. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM DHA) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

● DHA supplemented cells in the absence of α-tocopherol

■ DHA supplemented cells in the presence of α-tocopherol

The IC_{50} values for HL60 cells supplemented with DHA was 1.69μM. The IC_{50} value for HL60 cells supplemented with DHA in the presence of α-tocopherol was 39μM.

Oneway ANOVA was used to compare the DHA supplemented cells in the presence and absence of α-tocopherol.

The reproductive ability of cells supplemented with DHA in the presence of α-tocopherol was significantly higher than cells supplemented with DHA in the absence of α-tocopherol at 2.5μM, 5μM, 10μM, 15μM, 25μM, 35μM and 50μM ($p < 0.05$).

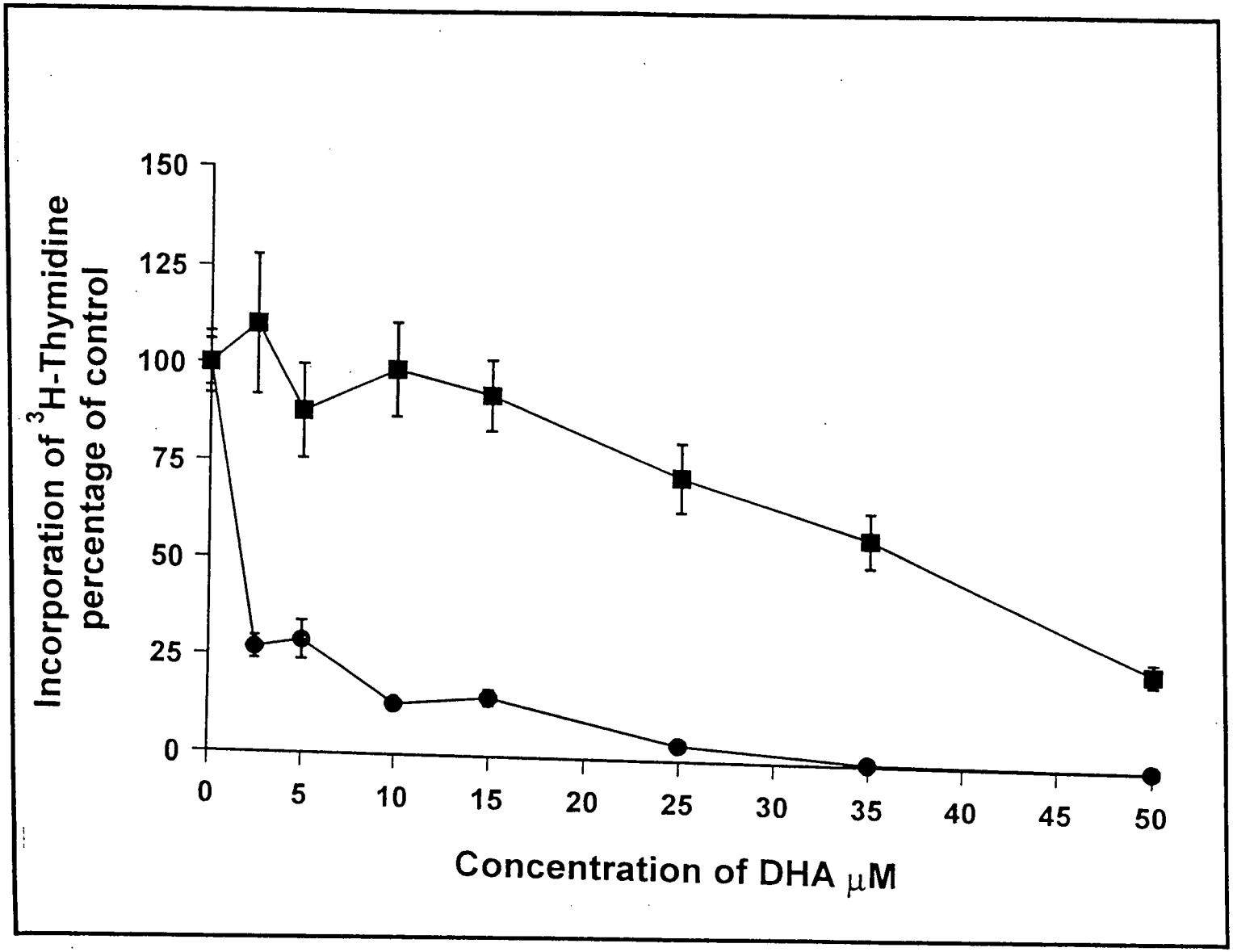

Incorporation of ^{3}H-Thymidine percentage of control
150
125
100
75
50
25
0
0
5
10
15
20
25
30
35
40
45
50
Concentration of DHA μM

Table 3.1 IC_{50} values for the PUFAs in the presence or absence of α-tocopherol in HL60 cells and K562 cells

Cells were incubated with increasing concentrations of fatty acid in the presence or absence of α-tocopherol (10μM), as described in section 3.2.4. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM Fatty acid) and the other conditions were determined as a percentage of the control. The IC_{50} values reflect the concentration of fatty acid that inhibited 50% of the cells from reproducing.

PUFA IC_{50} value	HL60	HL60 + α-tocopherol	K562	K562 + α-tocopherol
AA μM	8.2	> 50	> 50	> 50
EPA μM	4.55	31	> 50	> 50
DHA μM	1.69	39	22.4	> 50

3.3.2 Incorporation of fatty acids into the cell membrane phospholipids

The phospholipid fraction of the cell membranes from both cell lines were separated from the neutral lipid fraction, and the incorporation of the fatty acids into the phospholipid fraction was determined by gas-liquid chromatography. Standards of FAME were injected onto the column to obtain the relative retention times of known fatty acids, then FAME of the phospholipid fraction of the cell membrane of each cell line were injected onto the column. The gas chromatograph (GC) profile, not shown here, gave the incorporation of the fatty acids as a percentage. The retention times of the fatty acids were given from the point of injection. After subtracting the baseline and hexane peak from the profile the retention times were given relative to that of 16:0. Before statistical treatment of the data the percentages were transformed using arcsine.

To identify unknown peaks the $\log_{10}$ of the relative retention times (RRT) with respect to 16:0 and 18:0 were taken from the standards. A graph was drawn of carbon chain length vs $\log_{10}$ of RRT. Two graphs were drawn, one of the saturated fatty acids and the other the monosaturated fatty acids. These graphs are shown in Figure 3.6. From this the chain length of any unknown peak could be determined from the $\log_{10}$ of its RRT. K562 cells supplemented with 20μM AA showed a significant ($p < 0.05$) increase in two peaks, one at 20:4 and an unidentified peak just before 22:6. From the $\log_{10}$ RRT this peak was thought to be 22:5.

Table 3.2 summarises the results of the GC profile from fatty acid supplemented K562 cells. The results show how supplementing

K562 cells with DHA, AA, EPA, LA, and OA differs from control unsupplemented cells. The K562 cells were supplemented with 10μM DHA, 20μM AA, 20μM EPA, 20μM LA, or 20μM OA.

K562 cells supplemented with 10μM DHA were shown to be significantly different ($p < 0.05$) in the 18:0, 18:1 and 22:6 fractions of the phospholipids compared to the corresponding control value. Following supplementation with 10μM DHA, the PUFA content of membrane phospholipids increased substantially, due entirely to the significant increase (10.8%) in DHA. This was accompanied by a significant decrease (15.6%) in 18:1 levels and an increase (10.4%), though not statistically significant, in saturated fatty acids.

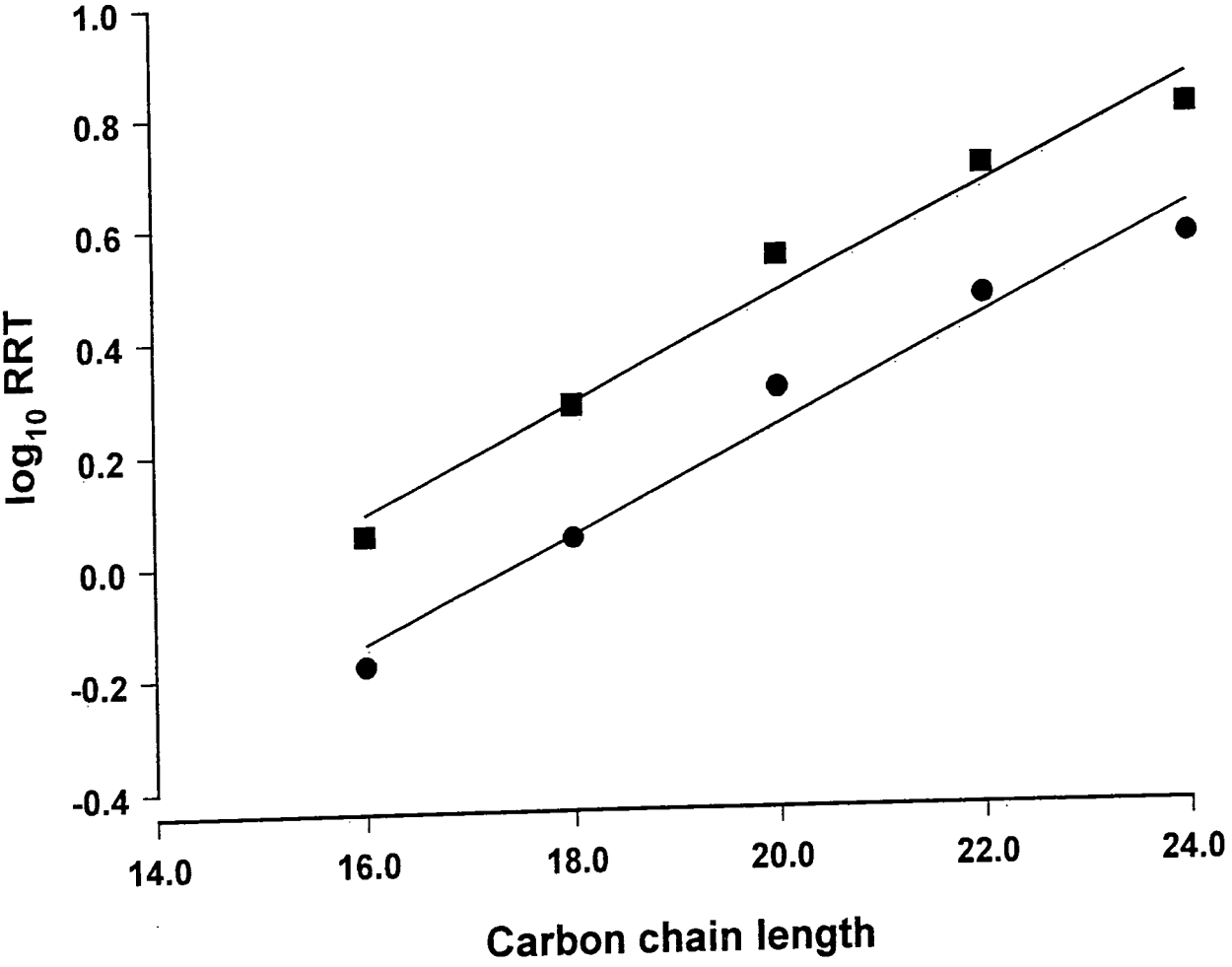
K562 cells supplemented with 20μM AA showed significant differences ($p < 0.05$) from the corresponding control in the 18:0, 18:1, 20:4 and 22:5 fractions of the phospholipids. Supplementation with 20μM AA substantially increased the PUFA content of the membrane phospholipids (20.1%) this was due to the increase (12.6%) in AA accompanied by an increase (7.1%) in 22:5 and by a significant decrease (17.9%) in 18:1 levels and an increase (4.8%) in saturated fatty acids.

K562 cells supplemented with 20μM EPA showed significant differences ($p < 0.05$) from corresponding controls in the 16:1, 18:0, 18:1, 20:5 and 22:6 fractions. EPA supplementation resulted in a substantial increase (20%) in the PUFA content of the membrane phospholipids. This increase was due to the significant increase (10.2%) in EPA and (11.56%) in DHA. This increase was accompanied by slight decrease (3%) in saturated fatty acids and a larger decrease (20%) in monounsaturated fatty acid.

Figure 3.6 The relationship between $\log_{10}$ relative retention time (RRT) and carbon chain length of saturated and unsaturated FAME standards

FAME standards were analysed using gas-liquid chromatography, as described in section 3.2.8. The retention times obtained for saturated FAME standards are represented in Figure A relative to the retention time of 16:0 ● and 18:0 ■. Figure B represents the retention times for monosaturated FAME standards relative to the retention times of 16:0 ● and 18:0 ■.

A


1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
log10 RRT
14.0
16.0
18.0
20.0
22.0
24.0
Carbon chain length

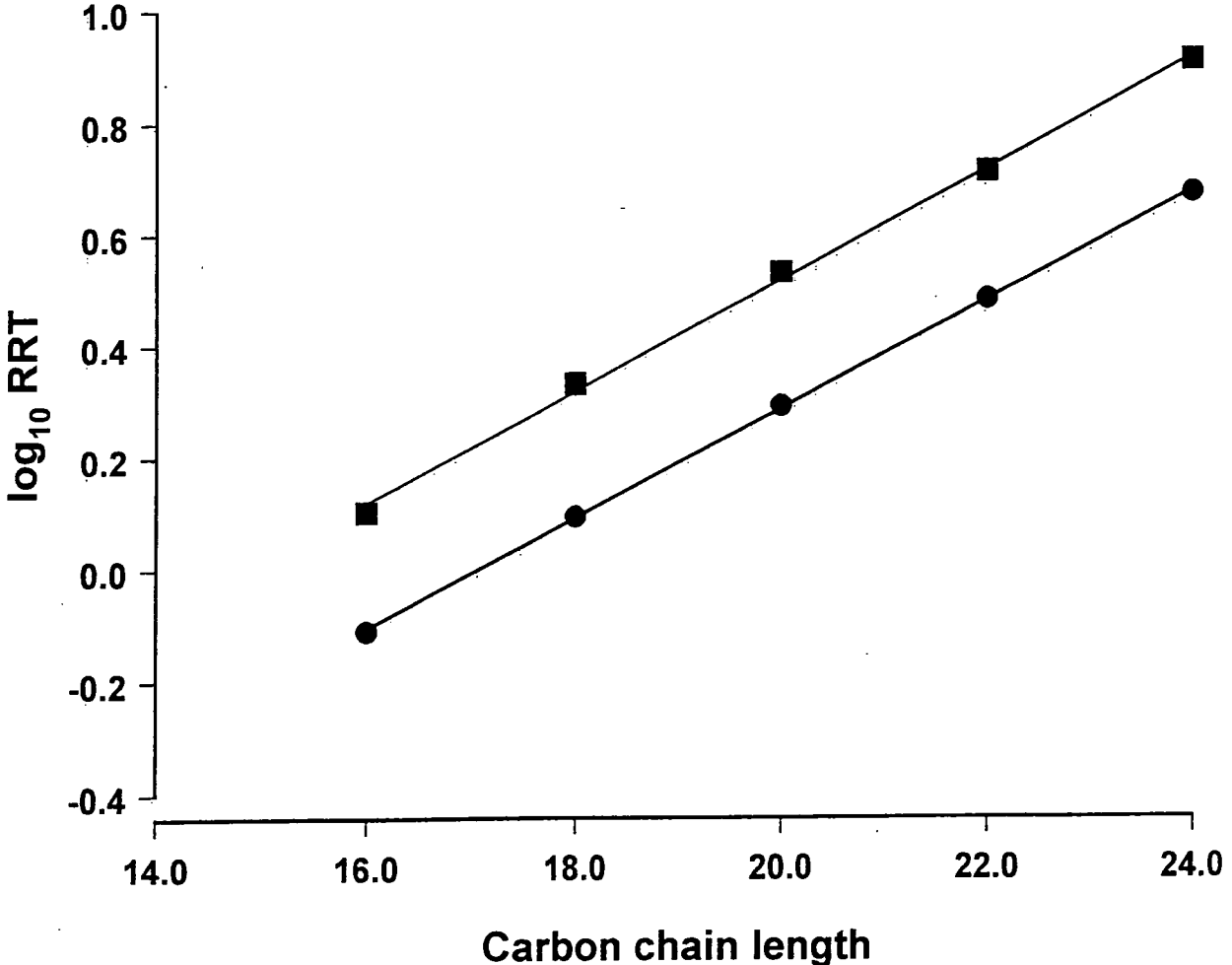

1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
log10 RRT
14.0
16.0
18.0
20.0
22.0
24.0
Carbon chain length

Table 3.2 Incorporation of fatty acids into phospholipids of the cell membranes of K562 cells

K562 cells were incubated for 48 hours with no supplemental fatty acid, 10μM DHA, 20μM EPA, 20μM AA, 20μM LA, or 20μM OA, as described in section 3.2.5. The lipids were extracted and the phospholipids separated, as described in sections 3.2.6-3.2.7. The fatty acid composition of the cellular phospholipids were determined using gas-liquid chromatography, as described in section 3.2.8. The relative concentration for each peak was determined and the mean values ± standard deviations of three separate experiments are shown.

Fatty acid	Control	DHA 10μM	AA 20μM	EPA 20μM	LA 20μM	OA 20μM
16:0	19.18 ± 4.7	27.0 ± 17.4	26.72 ± 4.9	24.9 ± 8.4	28.34 ± 11.9	10.3 ± 3.4
16:1	3.47 ± 2.1	none detected	none detected	0.87 ± 1.7	none detected	none detected
18:0	12.97 ± 1.5	15.53 ± 2.9	10.23 ± 1.3	10.23 ± 1.4	10.27 ± 2.2	10.1 ± 1.24
18:1	49.17 ± 3.8	33.59 † ± 9.1	31.3 † ± 1.7	31.4 † ± 1.2	24.21 † ± 4.1	52.76 ± 5.5
18:2	0.77 ± 1.2	0.27 ± 0.24	0.15 ± 0.22	1.32 ± 1.37	21.94 † ± 2.35	4.17 ± 0
20:4	3.26 ± 3.64	2.13 ± 0.9	15.85 † ± 2.96	2.29 ± 0.49	1.73 ± 1.32	4.34 ± 0.84
20:5	0.98 ± 0.25	1.25 ± 0.73	0.67 ± 0.48	11.18 † ± 2.43	0.39 ± 0.15	1.32 ± 0.2
22:5	0.49 ± 0.14	0.41 ± 0.32	7.56 † ± 1.3	0.32 ± 0.22	0.15 ± 0.006	0.22 ± 0.1
22:6	0.95 ± 0.93	11.78 † ± 6.4	2.34 ± 0.8	12.51 † ± 3.9	1.38 ± 1.3	4.38 ± 1.15
Others	8.76	8.04	5.18	4.98	11.59	12.41

Data in table represent mean value ± standard deviation
† Found to be significantly different ($p < 0.05$) from the corresponding control value using the Oneway ANOVA test.

K562 cells supplemented with 20μM LA showed significant differences ($p < 0.05$) from corresponding controls in the 18:0, 18:1 and 18:2 fractions. After LA supplementation the PUFA content of the membrane phospholipids was greatly increased (19%) this was due to the significant increase (21%) in LA. This was accompanied by a decrease (28%) in monounsaturated fatty acids.

K562 cells supplemented with 20μM OA were found to be significantly different ($p < 0.05$) from the corresponding control cells in 18:0 fraction only. OA supplementation had decreased the 18:0 fraction by 1.3-fold. OA supplementation resulted in lowering (12%) the saturated fatty acid content of the membrane phospholipids the monounsaturated fatty acid content remained the same and the PUFA content was increased (9%), although this was not found to be significant.

Table 3.3 summarises results of the GC profile from fatty acid supplemented HL60 cells. This bar chart shows how supplementing HL60 cells with DHA, AA, EPA, LA, and OA differs from control unsupplemented cells. The HL60 cell line was supplemented with 1μM AA, 1μM EPA, 1μM DHA, 20μM LA or 50μM OA .

Supplementing HL60 cells with 1μM AA showed significant differences ($p < 0.05$) from the corresponding controls in the 18:1 and 20:4 fractions. Supplementation with 1μM AA increased the PUFA content of the membrane phospholipids (4.3%) this was due to mainly the increase (5.05%) in AA accompanied by a decrease (3.8%) in 18:1. There was a slight decrease in monounsaturated fatty acid (2.5%) and a slight increase in saturated fatty acids (4.8%).

Supplementing HL60 cells with 1μM EPA resulted in significant differences ($p < 0.05$) than the corresponding control in 18:1, 20:5 and 22:6 fractions. EPA supplementation resulted in a substantial increase (15.71%) in the PUFA content of the membrane phospholipids. This increase was due to the significant increase in (7%) in EPA and (6.6%) in DHA. This increase was accompanied by a slight decrease (2.5%) in monounsaturated fatty acids and no real difference in saturated fatty acids.

Supplementing HL60 cells with 1μM DHA showed significant differences ($p < 0.05$) from the corresponding control in 18:1 and 22:6 fractions. Following supplementation with 1μM DHA, the PUFA content of membrane phospholipids increased substantially (12.4%), this was mainly due to the increase in DHA (10.1%). This was accompanied by a decrease in monounsaturated fatty acids (9.7%) and a slight increase (0.8%) in unsaturated fatty acids.

Supplementing HL60 cells with 20μM LA showed significant differences ($p < 0.05$) from the corresponding control in the 16:0, 18:0, 18:1 and 18:2 fractions. Following LA supplementation the PUFA content of the membrane phospholipids was greatly increased (27.5%), this was mainly due to the increase in LA (26%). This was accompanied by a significant decrease in monounsaturated fatty acids (33%) and a slight increase in saturated fatty acids (4%).

HL60 cells supplemented with 50μM OA showed significant differences ($p < 0.05$) from the corresponding control in the 18:0, 18:1 and 22:6 fractions. OA supplementation resulted in lowering (7%) the saturated fatty acid content of the membrane phospholipids, the monounsaturated fatty acids were increased (15%) and the PUFA content was slightly increased (2%).

Table 3.3 Incorporation of fatty acids into the phospholipids of HL60 cells

HL60 cells were incubated for 48 hours with no supplemental fatty acid, 1μM DHA, 1μM EPA, 1μM AA, 20μM LA, or 50μM OA, as described in section 3.2.5. The lipids were extracted and the phospholipids separated, as described in sections 3.2.6-3.2.7. The fatty acid composition of the cellular phospholipids were determined using gas-liquid chromatography, as described in section 3.2.8. The relative concentration for each peak was determined and the mean values ± standard deviation of three separate experiments are shown.

Fatty acid	Control	AA 1μM	EPA 1μM	DHA 1μM	LA 20μM	OA 50μM
16:0	17.33 ± 4.2	18.04 ± 5.4	17.51 ± 8.1	19 ± 6.2	28.67 † ± 4.1	21.1 ± 3.9
16:1	3.79 ± 4.4	8.18 ± 4.7	2.5 ± 4.3	2.7 ± 4.8	none detected	none detected
18:0	14.47 ± 1.9	15.25 ± 2.6	14.0 ± 2.4	13.5 ± 0.8	6.9 † ± 4.9	2.93 † ± 1.9
18:1	45.53 ± 3.9	38.66 † ± 2.5	33.84 † ± 2.6	36.9 † ± 5.2	16.6 † ± 2.81	64.66 † ± 5.3
18:2	3.89 ± 3.8	0.38 ± 0.4	4.4 ± 3.4	4.8 ± 4.2	30.32 † ± 4.31	none detected
20:4	2.6 ± 2.3	7.65 † ± 2.6	4.19 ± 2.4	3.5 ± 1.9	3.72 ± 2.2	1.49 ± 1.08
20:5	0.86 ± 0.6	1.28 ± 1.2	7.9 † ± 4.5	1.8 ± 0.4	0.7 ± 0.2	0.82 ± 0.2
22:5	0.26 ± 0.26	0.50 ± 3.5	0.33 ± 0.3	0.29 ± 0.2	0.53 ± 0.2	0.22 ± 0.1
22:6	2.4 ± 1.6	4.63 ± 2.2	9.0 † ± 3.0	12.5 † ± 5.2	1.96 ± 0.86	9.94 † ± 3.0
Others	8.98	5.43	6.33	5.01	10.6	0.0

Data in table represent mean value ± standard deviation
† Found to be significantly different ($p < 0.05$) from the corresponding control value using the Oneway ANOVA test.

monounsaturated fatty acids were increased (15%) and the PUFA content was slightly increased (2%).

K562 cells supplemented with OA showed a similar GC profile to the control cells with slight increases in 18:1, 18:2 and 22:6. There was a decrease by 1.6 -fold in the saturated fatty acids, mainly due to increases in 18:2 and 22:6. Supplementation with PUFAs showed a general trend to slightly increase the saturated fatty acids mainly by increases in the 16:0 fatty acid fraction. Apart from OA supplementation, the monounsaturated fatty acids of the other treatments decreased mainly due to a significant decrease ($p < 0.05$) in 18:1. After fatty acid supplementation the corresponding phospholipid fatty acid fraction was increased.

K562 cells were supplemented with 20μM fatty acid except for DHA which was 10μM. K562 cells supplemented with 20μM EPA or 10μM DHA resulted in increases in the 22:6 fraction. EPA (20μM) supplemented cells increased the 22:6 fraction by 12% and DHA (10μM) supplemented cells increased the 22:6 fraction by 11%. K562 cells could not be supplemented with 20μM DHA, as the cells were too sensitive to this concentration. Therefore it could not be determined if cells supplemented with 20μM DHA would be able to incorporate a higher percentage of DHA into the membrane phospholipids than cells supplemented with 10μM DHA. Similar results were observed with the HL60 cell line. HL60 cells supplemented with EPA (1μM) the 22:6 fraction was increased by 11%; cells supplemented with DHA (1μM) the 22:6 fraction was increased by 10%. The general trend of PUFA supplementation was a significant decrease in the monounsaturated fatty acids, mainly in 18:1, with significant increases in polyunsaturated fatty acids, usually with the supplementing fatty acid, as shown in Table 3.4. Supplementing with 1μM AA showed increases in 20:4 and

Table 3.4 Overall effect of fatty acid supplementation on the phospholipid fraction of the K562 and HL60 cells

Cells incubated for 48 hours with no supplemental fatty acid, the corresponding concentration of DHA, AA, EPA, LA or OA, as described in section 3.2.5. The lipids were extracted and the phospholipids separated, as described in sections 3.2.6-3.2.7. The fatty acid composition of the cellular phospholipids was determined using gas-liquid chromatography, as described in section 3.2.8. This table shows the relative percentages of saturated, monounsaturated and polyunsaturated fatty acids for the following treatments.

Cell line / fatty acid	Saturated fatty acid %	Monounsaturated fatty acid %	Polyunsaturated fatty acid %
K562 cells			
Control	32.15	52.64	7.65
AA 20μM	36.95	31.30	27.79
EPA 20μM	35.13	32.27	27.62
DHA 10μM	42.53	33.59	16.03
LA 20μM	38.61	24.21	26.43
OA 20μM	20.40	52.76	16.74
HL60 cells			
Control	31.69	49.32	10.58
AA 1μM	33.29	46.84	14.86
EPA 1μM	31.51	36.34	26.29
DHA 1μM	32.50	39.60	23.00
LA 20μM	35.57	16.60	38.03
OA 50μM	24.03	64.66	12.59

22:6 fractions but not in 22:5 as in 20μM AA supplemented K562 cells. Only small concentrations of PUFAs were required to alter the phospholipid profile of the cell membranes in HL60 cells.

3.3.3 Combination of PUFA and ether lipid treatment

The cytotoxic effects of the ether lipid ET-18-OCH_3 on the HL60 and K562 cell lines were determined in Chapter 2. As described above PUFAs were incorporated into the membrane phospholipids of the HL60 and K562 cells. As ether lipids are thought to exert their cytotoxic effects by interacting with cell membranes, it was of interest to determine whether PUFA-induced modification of membrane phospholipids might influence the cytotoxic effects of ET-18-OCH_3. Cells supplemented with PUFA were combined with a range of ether lipid treatments to determine if this PUFA plus ether lipid treatment would show any alteration in the cytotoxic effect of the ether lipid alone. The K562 cell line was used for the combination experiments as they were more resistant to the cytotoxic effects of the fatty acids. Therefore higher concentrations of fatty acid could be used to supplement the K562 cells without disrupting the reproductive ability of the cells in combination experiments.

K562 cells were incubated in culture media supplemented with 20μM EPA for 48 hours. This was followed by a 4 hour incubation in serum-free culture medium with a range of ET-18-OCH_3 concentrations (0-8μM). As shown in Figure 3.7, as the concentration of ET-18-OCH_3 was increased the reproductive ability of the K562 cells decreased. Preincubation with 20μM EPA for 48 hours followed by an ET-18-OCH_3 incubation increased the reproductive ability of the

K562 cells at 5μM, 7μM and 8μM ether lipid ($p < 0.05$) compared to cells that had no EPA incubation followed by ET-18-OCH_3.

3.3.4 Combination of PUFA and local anaesthetics treatment

The cytotoxic effects of dibucaine, tetracaine and procaine were determined in Chapter 2. Local anaesthetics have been shown to bind with or partition into the cell membranes. In so doing they may exert some of their cytotoxic effect by interacting with the cell membranes. Therefore experiments to determine whether PUFA supplementation combined with local anaesthetic treatment would alter the cytotoxic effect of the local anaesthetics. Dibucaine was chosen to use in these experiments as it was the most cytotoxic local anaesthetic used and showed an effect in combination with ether lipid, as shown in Chapter 2. Cells supplemented with PUFA were combined with a range of dibucaine treatments to determine if this combined treatment would show any alteration in the cytotoxic effect of dibucaine alone. The K562 cell line was used for the combination experiments as they were more resistant to the cytotoxic effects of the fatty acids. Therefore higher concentrations of fatty acid could be used to supplement the K562 cells without disrupting their reproductive ability.

K562 cells were incubated in culture medium supplemented with 20μM EPA for 48 hours. This was followed by a four hour incubation in serum-free culture medium with a series of dibucaine concentrations (0-0.5mM). As shown in Figure 3.8 as the concentration of dibucaine was increased the reproductive ability of the cells was lost. No

significant difference was observed between K562 cells grown in the presence or absence of EPA (20μM).

Figure 3.7 Effect of PUFA in combination with ether lipid on K562 cells

K562 cells were incubated with a subtoxic dose of PUFA for 48 hours, which was followed by a 4 hour incubation with a series of ether lipid concentrations in serum free medium, as described in section 3.2.9. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM Fatty acid, 0μM ether lipid) and the other conditions were determined as a percentage of the control.

Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

■ no EPA incubation followed by ET18-OCH_3 incubation

● 20μM EPA incubation followed by an ET-18-OCH_3 incubation

The IC_{50} value for K562 cells treated with ET-18-OCH_3 was 6μM. The IC_{50} value for K562 cells supplemented with EPA and treated with ET-18-OCH_3 was 9μM.

Oneway ANOVA was used to compare cells that had no EPA incubation followed by ET-18-OCH_3 incubation with cells that had 20μM EPA incubation followed by an ET-18-OCH_3 incubation.

The reproductive ability of cells exposed to 20μM EPA incubation followed by an ET-18-OCH_3 incubation was significantly higher than cells that had no EPA incubation followed by ET-18-OCH_3 incubation at 5μM, 7μM and 8μM ether lipid ($p < 0.05$).

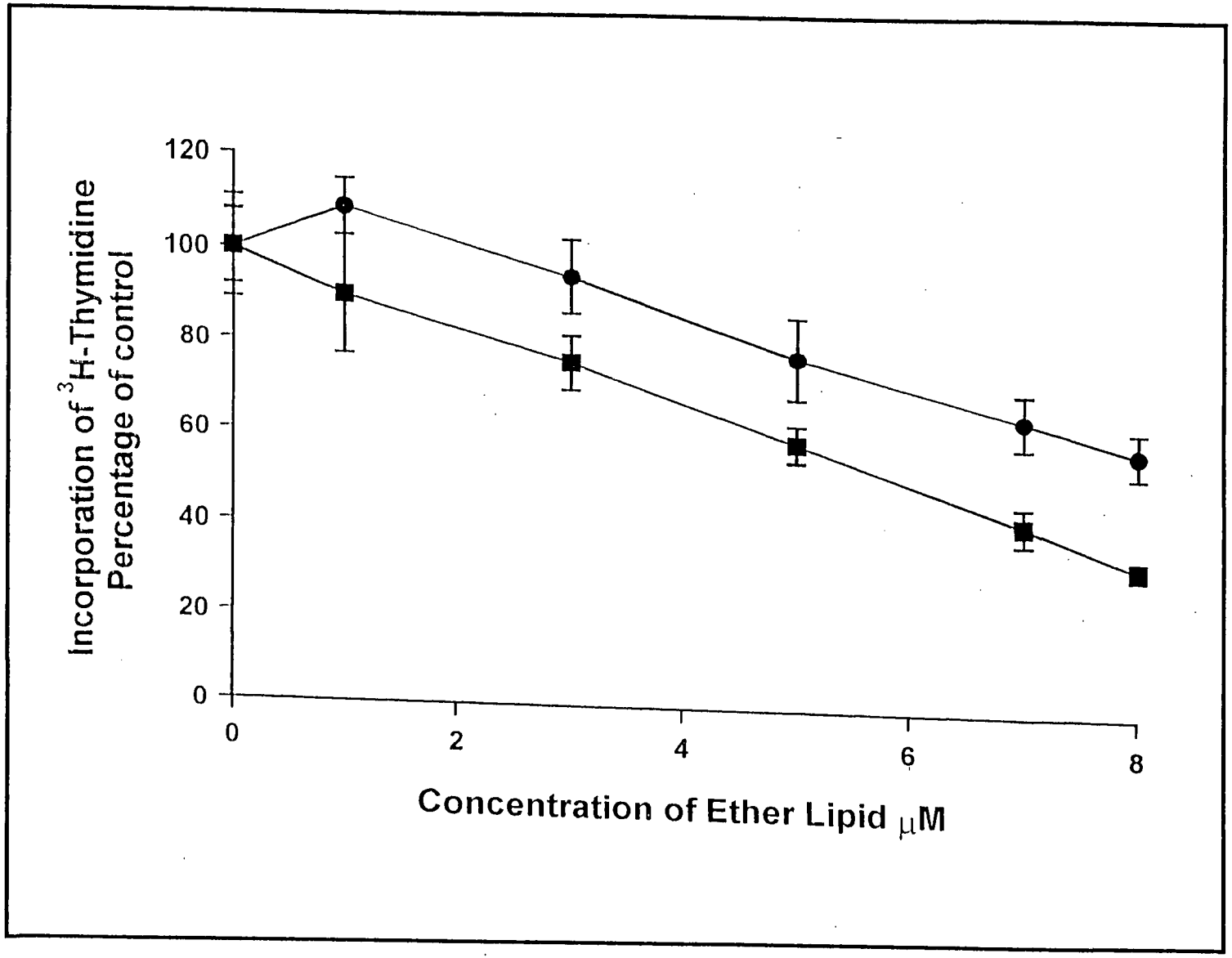

Incorporation of ^{3}H-Thymidine
Percentage of control
120
100
80
60
40
20
0
0
2
4
6
8
Concentration of Ether Lipid μM

Figure 3.8 Effect of PUFA in combination with dibucaine on K562 cells

K562 cells were incubated with a subtoxic dose of PUFA for 48 hours, which was followed by a 4 hour incubation with a series of dibucaine concentrations in serum free medium, as described in section 3.2.10. Then cells were pelleted by centrifugation, resuspended in culture medium and incubated with ^{3}H-thymidine as described in section 2.2.6. The incorporation of ^{3}H-thymidine was taken to be 100% for control (0μM Fatty acid, 0μM ether lipid) and the other conditions were determined as a percentage of the control.

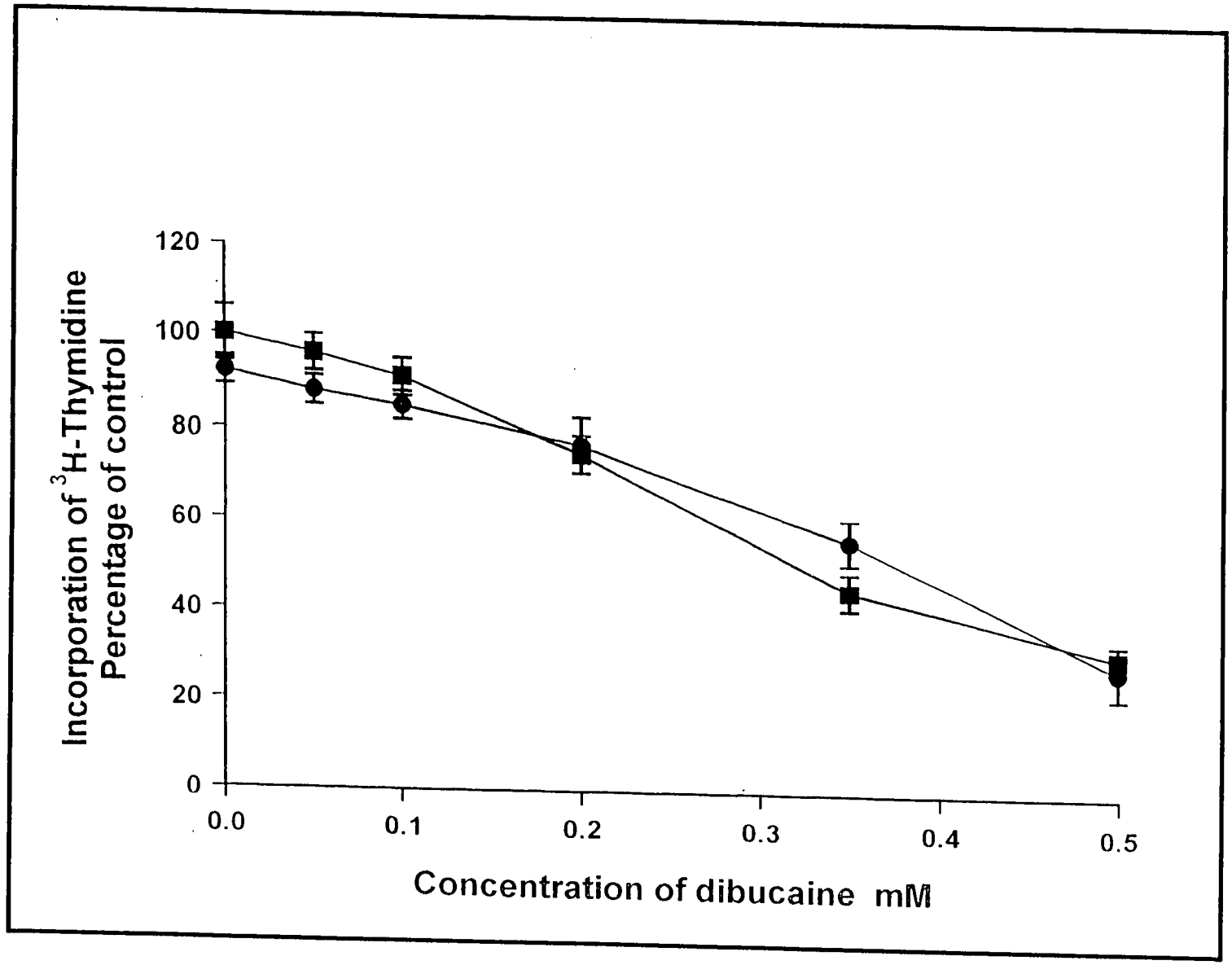
Each point represents the mean value ± standard deviation for 3 separate experiments (9 replicates).

■ no EPA incubation followed by dibucaine incubation

● 20μM EPA incubation followed by dibucaine incubation

The IC_{50} value for K562 cells treated with dibucaine was 0.33mM. The IC_{50} value for K562 cells supplemented with EPA and treated with dibucaine was 0.38mM.

Oneway ANOVA was used to compare the data. No significant difference was found between the treatments at $p < 0.05$.


Incorporation of ^{3}H-Thymidine
Percentage of control
120
100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
Concentration of dibucaine mM

3.4 Discussion

The work reported in this chapter determined the effect of fatty acids supplementation on the reproductive ability of the HL60 and K562 cell lines. The incorporation of fatty acids into the phospholipids of the cell membranes of both cell lines was shown. Combination experiments between PUFA supplemented cells and ether lipid treatment, and between PUFA supplemented cells and local anaesthetic were also undertaken.

The HL60 cell line was found to be more sensitive to the cytotoxic effects of the fatty acid supplementation than the K562 cell line. Both cell lines were supplemented with fatty acids by including the fatty acid dissolved in the heat-inactivated calf serum contained in the culture medium. Initially the K562 and HL60 cells were grown in a range of fatty acid concentrations 0-50μM. OA (18:0 n-9), and LA (18:2 n-6), did not alter the cell growth of K562 or HL60 cell lines even at a concentration of 50μM. Other fatty acids used to supplement the both cell lines were AA (20:4 n-6), EPA (20:5 n-3) and DHA (22:6 n-3).

The order of cytotoxicity for the K562 cells was determined to be OA = LA < EPA < AA < DHA. In K562 cells OA, LA, AA and EPA did not have a cytotoxic effect on the reproductive ability up to a fatty acid concentration of approximately 20μM. Cells supplemented with AA (≥20μM) began to lose their reproductive ability. EPA with 5 double bonds was less cytotoxic than AA with 4 double bonds but DHA with 6 double bonds proved to be the most cytotoxic. The IC_{50} values for K562 supplemented cells were > 50μM for OA, LA, EPA and AA, and 22.2μM for DHA. In contrast the order of cytotoxicity on the reproductive ability of the HL60 cells was in the order OA < LA < AA

< EPA < DHA. PUFA supplementation severely reduced the reproductive ability of the HL60 cells. The IC_{50} values for the HL60 cells were > 35μM OA and LA, 8.2μM AA, 4.55μM EPA and 1.6μM DHA. The IC_{50} values of the HL60 and K562 cell lines were dramatically different showing that the HL60 cells were much more sensitive to PUFA supplementation that the K562 cells. PUFA supplementation of HL60 cells resulted in a sharp decline in the reproductive ability, as reflected by the IC_{50} values. In the HL60 cells the order of cytotoxicity increased as the number of double bonds increased in the PUFAs but this was not true in K562 cells. The effect of various PUFAs on HL60 cells was also examined by toluidine blue staining and light microscopy (Hawkins *et al.*, 1998). HL60 cells were supplemented with 20μM OA, LA, AA, EPA or DHA for 25 hours: the percentage of cell death increased with the number of fatty acid double bonds. In HL60 cells supplemented with OA (no double bonds) and LA (two double bonds) the percentage of cells death was 7%; supplementation with EPA (five double bonds) produced 70% cell death; and supplementation with either AA (four double bonds) or DHA (six double bonds) produced 85% cell death in each case. Surprisingly, HL60 cells supplemented with AA killed more cells than EPA supplementation even though it had one less double bond. This order of fatty acid toxicity is similar to that observed with the K562 cells in this study. Finstad *et al.* (1994) supplemented HL60 cells with 60μmol/l of LA, AA, EPA or DHA and then measured the incorporation of ^{3}H-thymidine after 1,2 and 3 days of treatment. AA and EPA were found to be the strongest inhibitors of ^{3}H-thymidine incorporation. After 3 days of treatment ^{3}H-thymidine incorporation was reduced by 90% by AA, 95% by EPA and 85% by DHA. OA and LA showed no inhibition or stimulation of cell replication.

Mengeaud *et al.* (1992) showed that EPA and GLA increased lipid peroxidation in a dose-dependent manner on three human colon cancer cell lines: HT 29, HRT 18 and CACO 2 cells, but that vitamin E (10μM), an antioxidant, significantly reduced the lipid peroxidation induced by EPA and GLA supplementation. HL60 and K562 cells were supplemented with 0-50μM DHA in the presence or absence of Vitamin E (10μM α-tocopherol) in order to determine if the cytotoxic action of the PUFAs was due to lipid peroxidation. Vitamin E is known to reduce the effects of lipid peroxidation. In K562 cells α-tocopherol had a substantial protective effect on the cytotoxicity of DHA-supplemented cells. The IC_{50} value of DHA supplemented K562 cells was 22.4μM and this was increased to > 50μM in the presence of 10μM α-tocopherol. K562 cells in the presence of α-tocopherol did not show any alteration to the cytotoxicity of AA or EPA supplementation, even though AA supplementation was moderately cytotoxic above 25μM. This may have been because AA and EPA were not as cytotoxic as DHA in K562 cells. When HL60 PUFA supplemented cells were grown in the presence of α-tocopherol the cytotoxic effect of the PUFA was considerably reduced. In the presence of 10μM α-tocopherol the IC_{50} values of HL60 cells supplemented with AA increased from 8.2μM to > 50μM, EPA increased from 4.55μM to 31μM, and DHA was increased from 1.69μM to 39μM. This protective effect of α-tocopherol indicates that PUFA cytotoxicity maybe partially due to lipid peroxidation. Preliminary experiments were undertaken to show the onset of lipid peroxidation but no products of lipid peroxidation were found. There are three possible reasons for this. Firstly, too few cells were examined. Secondly, the assay conditions may have needed more alteration. Thirdly, the products of lipid peroxidation had been transformed into non-toxic substances. Hawkins *et al.* (1998) showed

that HL60 cells and a pancreatic cell line, (Mia-Pa-Ca-2 cells) treated with PUFAs induced lipid peroxidation, in the presence of Fe^{2+}, and that this was correlated to the proportion of cell death caused by PUFA. Antioxidants vitamin E acetate and sodium selenite were able to prevent the cytotoxic effects of 50μM EPA. DNA fragmentation analysis showed that EPA (50μM) treatment of HL60 cells for 24-72 hours induced a specific pattern of chromatin cleavage into oligonucleosomes characteristic of apoptosis.

The incorporation of the fatty acids into the phospholipids of the cell membrane was determined using gas-liquid chromatography. A fatty acid concentration that killed ≤ 10% of the cells was chosen to be subtoxic. Subtoxic concentrations of fatty acids were used to supplement K562 cells. The concentrations used were 10μM DHA, 20μM AA, 20μM EPA, 20μM OA and 20μM LA. K562 cells were supplemented with fatty acid for 48 hours, and then the phospholipid fraction of the cell membrane was isolated and the amount of fatty acids incorporated were determined using gas-liquid chromatography. K562 cells supplemented with DHA (10μM) showed a significant increase ($p < 0.05$) in the 22:6 fraction of the membrane phospholipids compared to control cells, and this was accompanied by significant decreases ($p < 0.05$) in the 18:0 and 18:1 fractions. K562 cells supplemented with AA (20μM) showed significant increases ($p < 0.05$) compared to control cells, in the 20:4 fraction and 22:5 fraction, and this was accompanied by significant decreases ($p < 0.05$) in 18:0 and 18:1. The control FAME used did not include 22:5, and so the 22:5 fraction was determined using a graph of carbon chain length as an x-axis verses a y-axis of $\log_{10}$ of RRT. Two graphs were drawn, one of the saturated fatty acids and the other the unsaturated fatty acids. From these graphs the chain length of any unknown peak could be determined from the

$\log_{10}$ of its RRT. It is clear from K562 cells supplemented with AA (20μM) that some of the 20:4 fatty acid was elongated by 2 carbon atoms and desaturated to form 22:5. EPA (20μM) supplemented K562 cells showed significant increases ($p < 0.05$) compared to control cells in the 20:5 fraction by 11.4 fold, and in the 22:6 fraction by 13.2 fold, and this was accompanied by significant decreases ($p < 0.05$) in 16:1, 18:0 and 18:1. K562 cells supplemented with EPA (20μM) showed that some of the 20:5 fatty acid was elongated and desaturated to form 22:6. LA (20μM) supplemented K562 cells showed a significant increase ($p < 0.05$) in the 18:2 fraction by 28.5 fold compared to control cells and this was accompanied by significant decreases ($p < 0.05$) in 18:0 and 18:1. OA (20μM) supplemented cells showed a significant decrease in 18:0 fraction compared to control cells, and this was accompanied by slight increases in 18:2, 22:6 and the 'other' fractions. From these results it is clear that a 48 hour incubation with fatty acids at the corresponding concentrations was sufficient to alter the fatty acids of the phospholipids in the cell membrane, and that the cells were then able to incorporate the fatty acids into the membrane phospholipids, and use them in some cases to form other essential fatty acids.

Lower concentrations of PUFA had to be used to supplement HL60 cells (that is 1μM AA, 1μM EPA, 1μM DHA, 20μM LA and 50μM OA) because of higher PUFA toxicity in these cells. HL60 cells supplemented with AA (1μM) showed significant increases ($p < 0.05$) in the 20:4 fraction, compared to control cells, and this was accompanied by a significant decrease in 18:1 fraction. HL60 cells supplemented with EPA (1μM) showed significant increases ($p < 0.05$) in 20:5 and 22:6, compared to control cells, and this was accompanied by significant decreases ($p < 0.05$) in the 18:1 fraction. Therefore HL60 cells were able to elongate the 20:5 fatty acid and desaturate it to form

22:6. HL60 cells supplemented with DHA (1μM) showed significant increases ($p < 0.05$) in 22:6 and significant decreases ($p < 0.05$) in 18:1, compared to control cells. HL60 cells supplemented with LA (20μM) showed a significant decrease ($p < 0.05$) in the 18:1, compared to control cells. HL60 cells supplemented with OA (50μM) showed a significant increase ($p < 0.05$) in the 18:1 fraction, compared to control cells. It was clear that a 48 hour incubation was long enough to incorporate the fatty acids into the membrane phospholipids, even at concentrations as low as 1μM. EPA (1μM) showed an increase in the 22:6 fraction as well as in the 20:5, showing that the cells were able to elongate and desaturate the 20:5 fatty acid to form 22:6. Unlike the K562 cells, when HL60 cells were supplemented with AA (1μM) there was no significant increase in the 22:5 fraction. This may have been because the fatty acid concentration was too low. Burns *et al.* (1989) supplemented HL60 cells with 10μM DHA for 120 hours. This concentration was very toxic to the HL60 cells used in the present study. The 10μM DHA significantly increased 22:6 in the membrane phospholipids by over 4-fold (Burns *et al.*, 1989). There was also a smaller but significant increase in 20:5 and decrease in 22:5 compared to unsupplemented cells. Burns *et al.* (1989) showed that there was only a slight increase in the proportion of 22:6 in membrane lipids after an 1 hour incubation with DHA. After a 24 hour incubation the proportion of 22:6 in the membrane lipids was greatly increased.

Fatty acid supplementation in K562 and HL60 cells resulted in altering the phospholipids fatty acid of the cell membranes, with a decrease in monounsaturated fatty acids and an increase in the corresponding PUFA. Even HL60 cells supplemented with concentrations as low as 1μM PUFA showed increases in PUFA fractions and decreases in monounsaturated fatty acids. OA

supplementation resulted in a large increase in monounsaturated fatty acids and an increase in PUFA. PUFAs are incorporated into the cell membrane, so the cytotoxic action could be caused by a variety of membrane functions and physical properties. For example fatty acid supplementation can result in changes in membrane fluidity (Spector & Burns, 1987). GLA and EPA, which contain 3 and 5 double bonds respectively, increased the membrane fluidity of three human colon cancer cell lines, while vitamin E reversed the increased membrane fluidity induced by EPA and GLA supplementation, possibly by interfering with phospholipid rearrangement (Mengeaud *et al.* 1992).

MDA-MB-231 breast cancer cells were supplemented with LA, OA, EPA or DHA dissolved in ethanol for 6 days (Rose & Connolly, 1990). Over the incubation period LA stimulated growth at an optimal concentration of 0.75μg/ml, OA stimulated cell growth only at low concentrations (0.25 μg/ml), DHA significantly reduced cell growth between 1-2.5 μg/ml, and EPA significantly reduced cell growth at 2.5 μg/ml. Inhibitors of prostaglandin and leukotriene synthesis, indomethicin and NDGA or esculetin, were used to investigate the mechanism for the stimulation of MDA-MB-231 cell growth by LA. NDGA and esculetin inhibited cell growth more than indomethicin. As NDGA and esculetin inhibit leukotriene biosynthesis the results suggested that cell growth in MDA-MB-231 cells was dependent on inhibition of leukotriene biosynthesis rather than prostaglandin biosynthesis. The stimulatory effect of LA on the cells could be partly due to an increase in leukotriene biosynthesis (Rose & Connolly, 1990).

After a 144 hour supplementation, LA (18μM) or AA (17μM or 33μM) enhanced the growth of the two murine colon adenocarcinoma cell lines MAC13 and MAC26 (Hussey & Tisdale, 1994). The cyclo-oxygenase and lipoxygenase inhibitor indomethicin and the 5-

lipoxygenase inhibitor BWA4C were used to determine PUFA-stimulated cell growth. BWA4C inhibited the cell growth by LA and AA more effectively than indomethicin suggesting that LA and AA stimulate cell growth through a lipoxygenase pathway rather than a cyclo-oxygenase pathway. Neither of the inhibitors decreased LA induced cell proliferation, and this suggests that growth stimulation may be through another pathway (Hussey & Tisdale, 1994). In the present study LA did not increase cell growth. Cell growth was reduced with AA, EPA and DHA. This may have been due to an alteration in leukotriene biosynthesis or in prostaglandin biosynthesis. Future work could include fatty acid supplementation of HL60 and K562 cells with prostaglandin or leukotriene biosynthesis inhibitors to determine if there is any alteration in cell growth.

When L1210 cells supplemented with 32μM DHA for 48 hours were treated with iron and ascorbic acid, a carbon-centered POBN spin adduct was produced (Wagner, *et al.*, 1993). When 40μM ET-18-OCH_3 was added to these conditions the carbon-centered spin adduct and ascorbate radical was produced. Increasing ET-18-OCH_3 concentration (20-80μM) increased the intensity of the ascorbate radical. The ascorbate radical intensity was shown to peak 40-60 seconds after the addition of ET-18-OCH_3 (Wagner *et al.*, 1993). The production of these lipid radicals shows the involvement of lipid peroxidation with ether lipid cytotoxicity.

PUFA supplementation has also been shown to increase the effect of chemotherapeutic agents. L1210 cells supplemented with 32μM DHA for 2 days followed by 0.4μM adriamycin with no DHA supplementation for up to 5 hours showed a marked decrease in the percentage of cell survival (Spector & Burns, 1987).

As PUFA had been shown to increase the effect of other agents, in this study combination experiments were undertaken to determine if PUFA supplementation could change the effect of other membrane active agents. Firstly PUFA supplementation was combined with ether lipid treatment. Secondly, PUFA supplementation was combined with local anaesthetic treatment. The K562 cells were used for combination experiments as higher concentrations of PUFA could be used, and, as shown in Chapter 2, the K562 cells showed a response to combined experiments between ether lipid and local anaesthetic. EPA (20μM) was the PUFA chosen for combination experiments as it was the PUFA with the largest number of double bonds that could be used at 20μM.

K562 cells were supplemented with a subtoxic concentration of EPA for 48 hours. After this the cells were incubated in serum-free culture medium with a range of ET-18-OCH_3 concentrations for 4 hours. As the ether lipid concentration increased, the reproductive ability of the K562 cells was reduced. The ether lipid IC_{50} value was 6μM for unsupplemented cells and this was significantly decreased ($p<0.05$) to 9μM in EPA-supplemented cells. Therefore PUFA supplementation decreased the cytotoxicity effect of the ether lipid ET-18-OCH_3 in K562 cells.

In contrast, L1210 cells supplemented with 32μM DHA for 2 days followed by ether lipid treatment with BM41.440 (0-30μM) for 8 hours without the fatty acid were shown to increase ether lipid cytotoxicity (Petersen *et al.,* 1992). Petersen *et al.* (1992) repeated this experiment other fatty acids (20μM) and BM41.440 showed that the cytotoxicity of the ether lipid was increased by increasing the number of double bonds in the fatty acids. L1210 cells supplemented with DHA for 48 hours with a prooxidant BSO (to deplete cellular glutathione) added during the final 24 hours increased the cytotoxicity of ET-18-

OCH_3 and BM41.440 at 10μM and 20μM (4 hour incubation). When Fe^{2+} and ascorbic acid was added to DHA supplemented cells treated with 5μM BM41.440 an increase in cytotoxicity was observed. This cytotoxicity was not reduced by the addition of the antioxidants butylated hydroxytoluene nor vitamin E. Vitamin E was also shown not to affect ET-18-OCH_3 cytotoxicity. Petersen *et al.* (1992) suggested that the metabolism of ether lipids may generate free radicals and that membrane fatty acids with increased numbers of double bonds are more susceptible to this secondary damage.

In the present study a different fatty acid, EPA, was used at a lower concentration than that used in Petersen's study. Also a different ether lipid, ET-18-OCH_3, was used and for a 4 hour period, instead of for 8 hours as in Petersen's study. In the present study this lower concentration of EPA protected against the cytotoxic effects of the ether lipid. This may have been due to alterations in membrane fluidity or in membrane targeted pathways in the K562 cells resulting in a protective rather than an additive effect.

Dibucaine was chosen in combination experiment with PUFA and local anaesthetic because it was the most cytotoxic local anaesthetic, and had increased the cytotoxicity of ET-18-OCH_3 in K562 cells, as described in chapter 2. K562 cells were supplemented with EPA (20μM) for 48 hours. The cells were then transferred into serum-free culture medium with a series of dibucaine concentrations. As the concentration of dibucaine was increased the reproductive ability of the cells was decreased but EPA supplementation did not alter the cytotoxic effect of dibucaine on K562 cells. The dibucaine IC_{50} values were 0.33mM for unsupplemented cells, and 0.38mM for EPA-supplemented cells. Therefore EPA did not increase the cytotoxic effect of dibucaine,

suggesting that PUFAs cannot be used to supplement tumour cells to increase the cytotoxic effect of a local anaesthetic.

This study has shown that PUFA concentrations as low as 1μM can be sufficient to produce significant changes in phospholipid fatty acid composition in HL60 cells over a period of 48 hours. The PUFA are incorporated into membrane phospholipids and, in some cases, can be elongated and desaturated to form other PUFAs. Although α-tocopherol was shown to protect HL60 cells from the cytotoxic action of AA, EPA and DHA, α-tocopherol only protected the K562 cells from the cytotoxic action of DHA. The protective action of α-tocopherol indicated that the cytotoxic effect of the fatty acid may be partly due to lipid peroxidation. Unfortunately in this study no lipid peroxidation products were found. Supplementing K562 and HL60 cell lines with fatty acids for 48 hours was sufficient to incorporate the fatty acids into the membrane phospholipids, even at concentrations as low as 1μM in HL60 cells. When the PUFA EPA (20μM) was combined with a ET-18-OCH_3 treatment in K562 cells, the cytotoxic effect was reduced compared to cells treated with ether lipid alone. When EPA (20μM) was combined with the local anaesthetic dibucaine in K562 cells, no alteration in the cytotoxic effect was observed compared to dibucaine treatment alone.

In murine lymphocytic leukemia (L1210) cells the addition of ether lipids to cells previously supplemented with PUFA were shown to increase cytotoxicity and increase lipid peroxidation (Petersen *et al.*, 1992; Wagner *et al.*, 1993). In K562 cells EPA supplementation reduced the cytotoxic effect of the ether lipid ET-18-OCH_3. There was also no alteration of the cytotoxic effect of the local anaesthetic on K562 cells, with or without EPA supplementation. More recent work has shown that prolonged supplementation with PUFA induced

differentiation in HL60 cells, increased lipid peroxidation, and induces apoptosis (Hawkins *et al.*, 1998).

Chapter IV

Effects of Differentiation Agents on Human Leukaemia Cells Supplemented with Fatty Acids

4.1 Introduction

K562 and HL60 cell lines have both been shown to undergo differentiation. K562 cells can be induced to differentiate into erythrocyte-like cells by haemin, (Rutherford & Weatherall, 1979; Dean *et al.*, 1981; Villeval *et al.*, 1983) sodium butyrate, (Andersson, 1979; Chen & Wu, 1994) and 1-β-D-arabinofuranosylcytosine (ara-C) (Luisi-DeLuca *et al.*, 1984; Chen & Wu, 1994). K562 cells have also been induced to differentiate into attachment cells by TPA (Panazis *et al.*, 1981; Villeval *et al.*, 1983). HL60 cells can be induced to differentiate into granulocytes by DMSO, HMBA and retinoic acid, and to macrophage-like cells by TPA (Panazis *et al.*, 1981). As K562 cells differentiate into erythrocyte-like cells, it was thought that the properties of erthythrocytes could be used to show the onset of differentiation in the present study.

K562 cells synthesise the membrane protein spectrin and do so at the same rate, whether non-differentiating or differentiating into haemoglobin synthesising cells in the presence of 0.05mM haemin (Hunt & Marshall, 1981). These workers showed very few differences between the proteins made by non-induced and induced cells, suggesting that K562 cells in culture are already at a late stage of erythroid differentiation and contain erthyrocyte-specific membrane proteins. Although the proteins synthesised by non-induced and induced

cells are similar, induced cells divide asymmetrically to form a nucleus-containing residual cell and an enucleated, smooth-surfaced, reticulocyte-like cell which contains the bulk of the haemoglobin (Andersson, 1979; Hunt & Marshall, 1981). Spectrin and erythrocyte external membrane proteins, including glycophorin, are sequestered into these smooth-surfaced, reticulocyte-like cell protrusion of erythrocyte size. These smooth vesicles are thought to be very similar in composition to that of mature erythrocytes. Although the protein composition of the cell has been greatly altered the only major change during haemin-induced differentiation is the onset of haemoglobin synthesis.

The erythroid origin of K562 cells is shown by the synthesis and surface expression of glycophorin A (Andersson *et al.*, 1979); which is one of the best characterised integral membrane glycoproteins and is the major sialoglycoprotein of human erythrocytes (Marchesi *et al.*, 1976). Glycophorin A is known to be expressed exclusively on basophilic normoblasts and on later stages of the red cell differentiation in human bone marrow (Gahmberg *et al.*, 1978). K562 cells also contain spectrin as seen by indirect immunofluorescence (Andersson, 1979). The erythroid features of K562 cells suggest that during blast crisis, the neoplastic stem cell has the potential to differentiate not only into immature myeloid or lymphoid cells but also along the erythroid differentiation lineage (Andersson *et al.*, 1979).

The distribution of transferrin receptors in bone marrow is restricted to cells of the erythroid cell lineage (Horton, 1983). Transferrin receptors appear at the earliest morphologically identifiable stages of erythropoiesis and are strongly expressed throughout the different maturation stages. The monoclonal antibody, F111/2D1, was used to investigate the transferrin receptors during erythroid maturation

of the K562 cell line during erythroid differentiation. The majority of control K562 cells were found to bind F111/2D1 (Horton, 1983). Exposure to haemin was found to induce haemoglobin accumulation but did not alter transferrin receptor expression: DMSO, not an inducer of differentiation in K562 cells, was also without effect, whereas sodium butyrate resulted in reduction in cell growth but not differentiation, and reduced F111/2D1 binding (Horton, 1983). TPA treatment blocked haemoglobin synthesis, reduced cell growth and also inhibited F111/2D1 expression (Horton, 1983). Therefore, the expression of transferrin receptors in K562 cells, could not be used to determine the degree of erythroid differentiation of K562 cells.

When K562 cells were induced to differentiate by haemin, and synthesise large amounts of haemoglobin, preliminary analysis indicated that this was predominantly in the embryonic form (Rutherford & Weatherall, 1979). The presence of haemoglobin was confirmed by absorption bands at 540, 576, 414nm and quantitated at 414nm. Haemoglobin was analysed and two major haemoglobin components were found in the positions of embryonic haemoglobin, haemoglobin-Portland and haemoglobin-Gower.

K562 cells were also induced to differentiate by culturing with haemin or in glutamine deficient medium, to produce haemoglobin synthesising cells, by Erard *et al.* (1981). Induction with haemin was shown to be reversible. Haemoglobin concentrations were determined using absorption bands at 540, 576 and 414nm. Control K562 cells contained 0.3-0.5pg haemoglobin/cell, whereas glutamine-deficient cells contained 3-5pg haemoglobin/cell after 5 days. Cells treated with haemin in glutamine-deficient medium contained 26-34pg haemoglobin/cell after 5 days. A marked increase in haemoglobin content was also seen with hydroxyurea treatment in the presence of

haemin (Erard *et al.,* 1981). After 4 days of hydroxyurea treatment, the cells contained 7pg haemoglobin/cell, but when haemin was added to the hydroxyurea treatment, the cells contained 22pg haemoglobin/cell. This effect was reversible. The haemoglobins produced were compared by isoelectrofocusing. In untreated cells, embryonic haemoglobins Gower1, Portland and foetal haemoglobin were detectable. When the K562 cells were induced to differentiate by glutamine-deficient medium or by haemin, an increase in the level of the three haemoglobins above was observed (Erard *et al.*, 1981).

Villeval *et al.* (1983) also found that K562 cells exhibit several erythroid features, including production of embryonic and foetal haemoglobin, glycophorin A, spectrin and true acetylcholinesterase, and that three K562 clones showed the above to differing degrees. The effect of haemin, sodium butyrate and TPA on K562 clones were determined. Haemoglobin accumulation was enhanced by exposure to 100μM haemin, without significant modification of the expression of the other erythroid markers. Sodium butyrate greatly increased the activity of acetylcholineserase, slightly enhanced the production of haemoglobin, but did not modify the expression of glycophorin and spectrin. In TPA-induced cells, glycophorin almost disappeared, haemoglobin synthesis was reduced, and the action of haemin on haemoglobin accumulation was nearly abolished (Villeval *et al.,* 1983). All the K562 cell lines were found to exhibit clear erythroid features including acetylcholinesterase. Neither butyrate nor haemin induced terminal differentiation of K562 cells, whereas TPA significantly diminished the erythroid phenotype. Cells treated with sodium butyrate increased in acetylcholinesterase activity (Villeval *et al.,* 1983).

Carbonic anhydrase is the most abundant protein present in human red blood cells, after haemoglobin. Villeval *et al.* (1985)

investigated the expression of carbonic anhydrase as a marker of erythroid differentiation. Studies using the Friend erythroleukemic cell line suggested that carbonic anhydrase synthesis occurred early during the maturation of erythroid cells. This study investigated the expression of carbonic anhydrase isoenzymes, especially carbonic anhydrase I, in human hematopoietic cell lines induced to differentiate with haemin, expression was studied by fluorescence labelling, SDS PAGE and Western blotting. Surprisingly, carbonic anhydrase I was absent from K562 cells, which, considering that glycophorin A is expressed in large amounts at their surface, cannot be explained (Villeval *et al.*, 1985).

Haemin and hydroxyurea both reversibly induce K562 globin synthesis (Erard *et al.*, 1981). Induction by haemin has been shown to take place at the transcriptional level (Dean *et al.*, 1981), increasing the rate and level of accumulation of globin messenger RNAs (Charnay & Maniatis, 1983). Hydroxyurea treatment also results in the increase in the rate of transcription of globin genes (Charnay & Maniatis, 1983).

HL60 and K562 cells were induced to differentiate terminally, and their isoenzyme patterns of lactate dehydrogenase (LDH) in the cells before and after differentiation were determined electrophoretically on agarose gels (Panazis *et al.*, 1981). HL60 cells were induced to differentiate to granulocytes by DMSO, HMBA and retinoic acid, and to macrophage-like cells by TPA. K562 cells were induced to differentiate into haemoglobin-synthesising cells by sodium butyrate and to attachment cells by TPA (Panazis *et al.*, 1981). The treatment of leukemic cells with inducers of differentiation resulted in a quantitative shift of the isoenzyme pattern towards anodic or cathodic forms. This was correlated with the conversion of the chemically treated cells to morphologically more normal cells, as verified by light microscopy and/or synthesis of haemoglobin. The LDH isoenzyme

patterns of the chemically differentiated cells were characteristic for the particular cell type obtained after differentiation rather than for the nature of the inducer used, and was not similar to those of normally differentiated cells of the corresponding lineage (Panazis *et al.*, 1981). This indicated that incomplete differentiation had occurred. When both cell lines were induced to differentiate, the relative levels of each isoenzyme changed. In the K562 cell line, it was difficult to distinguish between control and TPA-treated cells, butyrate-treated cells showed an increase in anodic isoenzymes mainly in LDH-1, and also in LDH-2 and LDH-3. These isoenzymes were predominant in normal erythrocytes, so butyrate-treated cells are similar to normal erythrocytes in that they synthesise haemoglobin and characteristic lactate dehydrogenase isoenzymes (Panazis *et al.*, 1981).

One of the most effective agents in treating human acute myelogenous leukaemia has been shown to be 1-β-D-arabinofuranosylcytosine (ara-C) (Frei *et al.*, 1969), although the reason for its selectivity is still unknown. Ara-C incorporates into the DNA, but not into RNA, of human myeloblasts (Major *et al.*, 1981), and behaves as a relative chain terminator. The incorporation of ara-C into DNA correlates with inhibition of DNA synthesis (Major *et al.*, 1982), causes DNA fragmentation, which results in the loss of clonogenic survival. Previous studies showed that inhibition of eukaryotic DNA replication resulted in an aberrant form of DNA synthesis, with certain segments of DNA being replicated more than once in a single cell cycle (Woodcock & Cooper, 1981; Woodcock *et al.*, 1982). This form of aberrant DNA synthesis occurs after the inhibition of DNA replication by ara-C (Woodcock & Cooper, 1981; Woodcock *et al.*, 1982). The additional copies of certain segments of DNA might result in the accumulation of DNA fragments and an alteration of gene expression.

Ara-C has also been shown to inhibit DNA synthesis in HL60 cells (Griffin *et al.*, 1982). At sublethal doses it induces differentiation, accompanied by the loss of clonogenic survival. It is unclear whether this effect contributes to decreases in the self-renewal capacity of acute myeloblastic leukaemia cells after ara-C treatment (McCulloch *et al.*, 1981).

Luisi-DeLuca *et al.* (1984) showed that ara-C is a potent inducer of K562 haemoglobin expression, which is irreversible. There is also a loss of clonogenic survival suggesting terminal differentiation. When ara-C's cytotoxicity was determined, concentrations of 10^{-8}M and 10^{-7}M slowed cell growth, whereas concentrations of 10^{-6}M and 10^{-5}M resulted in loss of viability. Static cell concentrations without evidence of lethality, determined by trypan blue exclusion, were achieved by exposure to $5x10^{-7}$M ara-C. The differentiated, haemoglobin-containing cells were identified with benzidine staining and there was a progressive increase in the percentage of benzidine-positive cells at concentrations of 10^{-8}M to $5x10^{-7}$M ara-C, with >50% of the cells expressing a differentiated phenotype (Luisi-DeLuca *et al.*, 1984). Higher concentrations of ara-C resulted in a progressive decline in the percentage of benzidine-positive cells. The induction of benzidine-positive cells was linear up to 144 hours of drug exposure. The induction of haemoglobin synthesis by ara-C was confirmed by PAGE (Luisi-DeLuca *et al.*, 1984). Similar globin synthesis was seen in K562 cells cultured with 20μM haemin for 6 days.

K562 cells were exposed to $5x10^{-7}$M ara-C from 12-120 hours. Following exposure the cells were washed, resuspended in drug-free medium and assayed for haemoglobin accumulation at 120 hours. Exposure to ara-C for 12-24 hours was sufficient to result in a significant increase in benzidine-positive cells, while exposures ≥ 48

hours were sufficient to induce haemoglobin synthesis maximally. The irreversible induction of globin synthesis, confirmed by PAGE, could be associated with terminal differentiation (Luisi-DeLuca *et al.,* 1984).

In the presence of the irreversible inducer ara-C (3.6μM), the queuine content of tRNA increased markedly when K562 cells differentiated into benzidine-positive erythroid cells, and cell growth was inhibited (Chen & Wu, 1994). This increase was shown to be an irreversible event during terminal differentiation by ara-C induction. When haemin (0.1mM) was used as an inducer of K562 cell differentiation the increase in the queuine content of tRNA was shown to be a transient event of reversible differentiation.

When the cells undergo terminal differentiation, the cell cycle alters. The relative distribution of a population of cells throughout the cell cycle phases $G_{1/0}$, S, and G_2 plus M can be determined by flow cytometry, using a fluorescence-activated cell sorter (FACS).

Flow cytofluorometric analysis of cell cycle distributions using propidium iodide (PI) allows the rapid determination of relative DNA content (Fried *et al.*, 1976). This gives a quick and accurate means of cell cycle analysis of populations in culture and clinical specimens. The technique utilises a hypotonic solution of sodium citrate to rupture the cell membrane, enabling the dye to reach the nucleus. Most of the cytoplasm is removed in this process, and the remaining cytoplasm is unstained. Flow cytometric analysis of the phases of the cell cycle in K562 cells treated with ara-C (1.8μmol) for 96 hours showed a decrease in the percentage of cells in $G_{1/0}$ and S phases and an increase in cells in the G_2+M phase of the cells cycle (Nagy *et al.*, 1995). These K562

ara-C treated cells also showed a considerable increase in superoxide dismutase activity (SOD) and catalase activity. Nagy *et al.* (1995) suggested that differentation may be induced by increased OH· free radical yield.

Polyunsaturated fatty acids (PUFAs) have been shown to induce differentiation in HL60 cells (Finstad *et al.*, 1994). HL60 cells supplemented with EPA or AA (120μmol/L) for 3 days showed increased NBT reduction and generation of oxidative burst indicating a higher degree of differentiation in cells treated with PUFAs. HL60 cells supplemented with DHA (10μM) accelerated retinoic acid induced differentiation in the first 50 hours (Burns *et al.*, 1989). During the first 50 hours DHA and retinoic acid treatment increased superoxide production and NBT reduction. Cell growth was reduced and the percentage of cells found in $G_{1/0}$ of the cell cycle was increased.

In this Chapter the effect of polyunsaturated fatty acids (PUFAs) on the differentiation of K562 cells induced by ara-C will be described. A concentration of ara-C was required that did not induce total differentiation, so that if the addition of a subtoxic concentration of PUFA had an effect on differentiation, it would not be masked by the effect of ara-C. The onset of differentiation was determined by the production of haemoglobin, which was measured by its absorbance at 414nm. The effect of the onset of differentiation on the cell cycle profile was examined with flow cytometry using propidium iodide to determine the phases of the cell cycle.

4.2 Materials and methods

4.2.1 Differentiation of K562 cells

K562 cells were induced to differentiate into erythrocytes by adding 1-β-D-arabinofuranosylcytosine (ara-C) to the culture medium. As the cells differentiate into red blood cells, the extent of differentiation can be assessed by accumulation of haemoglobin, which can be determined by a spectrophotometric assay. To determine whether the differentiation rate could be altered by the addition of membrane active agents, different fatty acids were used in combination with ara-C.

A stock solution of ara-C (5mM) was prepared in distilled water and was filter-sterilised. It was stored for a maximum of two weeks in a glass container at 4°C. Ranges of ara-C concentrations were prepared by serial dilution in filter-sterilised distilled water. An appropriate volume ≤ 1% of the ara-C solution was then added to the culture medium to achieve the required concentration. When the equivalent volume of filter-sterilised distilled water was added to K562 cells in culture, no adverse effects on cell growth or viability were observed (results not shown).

4.2.2 Ara-C cytotoxicity experiments

a) Cell viability

K562 cells, (2 x 10^5 cells/ml) were incubated in culture medium with different concentrations of ara-C ranging from 0-3.2μM over a period of 0-5 days. At 24 hour periods, the cells were mixed to produce a homogeneous suspension and counted on a haemocytometer to determine cell number and viability as described in section 2.2.3.

b) Propidium Iodide staining of DNA

On day 5, the cells were pelleted by centrifugation at 300 x g at 20°C for 4 minutes. The culture medium was removed and the cells were resuspended in fresh culture medium at a cell density of $1x10^6$ cells/ml. A known volume of the cells, 200µl, was transferred to a small tube, to this 50µl of saponin solution [PBS containing 0.1% saponin (Flow LAbs) and 0.1% BSA, pH 7.4], 500µl of propidium iodide solution (0.25mg/ml propidium iodide (Flukka) in PBS) was added, followed by 500µl PBS and a 50µl aliquot of RNAse (stock solution made up in PBS at 1mg/ml using type I-AS RNAse (Sigma) and stored at -20°C in 200µl aliquots). The samples were thoroughly vortexed and stored in the dark at room temperature for 15 minutes. The samples were analysed immediately on a Coulter Epics XL-MCL flow cytometer.

4.2.3 Effect of EPA and ara-C on cell viability

From previous experiments, (section 3.2.3), subtoxic concentrations of fatty acids were established. These concentrations were used to supplement the cells in the following experiments.

K562 cells were seeded at 2 x 10^5 cells/ml in culture medium. Two sets of K562 control cells had no fatty acid added; another 2 sets of K562 cells were supplemented with a final concentration of 10µM EPA, and all cells were incubated for a period of 48 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). After 48 hours, both sets of cells were counted, as described in section 2.2.3, and pelleted by centrifugation at 300 x g for 4 minutes at 20°C. The culture medium was removed and the cells were resuspended in fresh culture medium at a cell density of 2 x 10^5 cells/ml. One set of the control cells contained

no treatment, the other control set had a final concentration of 0.01μM ara-C added. One set of the EPA-supplemented cells had no EPA supplementation and a final concentration of 0.01μM ara-C, the other EPA-supplemented cells contained a final concentration of 10μM EPA and 0.01μM ara-C. The control cells and the EPA supplemented cells were cultured for up to 5 days at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). To establish that the volume of ara-C solution added did not affect the cells in any way, an equal volume of filter-sterilised distilled water was added to the cells not given ara-C. At 24 hour intervals the cells were mixed, counted, and the viability was determined as described in section 2.2.3.

4.2.4 Determination of differentiation

K562 cells were seeded at 2 x 10^5 cells/ml in culture medium. One set of cells, the controls, had no fatty acid added. A final concentration of 10μM EPA was added to the other set of cells (EPA-supplemented cells). All cells were incubated for a period of 48 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). After 48 hours, both sets of cells were counted, as described in section 2.2.3, and pelleted by centrifugation at 300 x g for 4 minutes at 20°C. The culture medium was then removed and the cells were resuspended in fresh culture medium at a cell density of 2 x 10^5 cells/ml. The control cells were split into two sets. One set contained no treatment, but a final concentration of 0.05μM ara-C was added to the other set. The EPA supplemented cells were also split into two sets. One contained no EPA supplementation and no ara-C, the other contained no EPA supplementation and a final concentration of 0.05μM ara-C. The control cells and the EPA supplemented cells were cultured for up to 5 days at

37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). To establish that the volume of water containing ara-C added did not affect the cells in any way, an equal volume of filter-sterilised distilled water was added to the cells not given ara-C.

Initially this experiment was carried out with a final concentration of 0.01μM ara-C, very little difference was observed between the treatments (results not shown); from then on a final concentration of 0.05μM ara-C was used as described above.

At 24 hour intervals the cells were mixed and counted to determine cell number and viability, as described in section 2.2.3. Haemoglobin accumulation was then tested. Each day for 5 days the culture medium was removed by centrifugation at 300 x g for 4 minutes at 20°C, and the cells were washed twice in cold PBS (Rutherford & Weatherall, 1979) using the same centrifugation conditions at 4°C. The cells were resuspended in lysis buffer, consisting of 0.81% (w/v) NaCl, 0.03% (w/v) Mg-acetate, 0.12% (v/v) Tris-HCl (pH 7.4), and NP-40 was added to 0.5% (w/v), at a cell density of $10x10^6$ cells/ml and cells were lysed by a 15 minute incubation on ice followed by a centrifugation at 300g for 15 minutes at 4°C to remove the nuclei. This method of cell lysis proved to be inconsistent and therefore the following method was used. After the cells had been washed twice in cold PBS, as described above, the cells were resuspended in filter-sterilised distilled water at a cell density of $10x10^6$ cells/ml. The cells were then lysed by three cycles of freezing and thawing (Rutherford *et al.*, 1981). The broken cell suspension was then centrifuged at 500 x g for 10 minutes at room temperature to remove the nuclei. The supernatant was removed and centrifuged at 140,000 g for 45 minutes at room temperature. The pellet was discarded and the haemoglobin

content of the final supernatant was determined from its absorbance at 414nm.

4.2.5 Effect of preincubation and concentration of fatty acid on haemoglobin accumulation

From previous experiments (section 3.2.3) subtoxic concentrations of fatty acids were established. In sections 4.2.3 and 4.2.4 a concentration of 10μM EPA was used to supplement the culture medium. Initially, it was hoped to supplement other batches of K562 cells with culture medium containing 10μM DHA for 48 hours. This concentration was shown to be subtoxic in previous experiments, but when the DHA was removed by centrifugation and replaced with culture medium containing a final concentration of 0.05μM ara-C and no DHA for up to 5 days, the cells clumped together and died after 2 days. Even when the concentration of DHA was lowered to 2μM, the same effect was observed.

It was established from experiment 3.2.3 that 20μM was a subtoxic concentration for EPA, AA, OA and LA. To determine if an increase in fatty acid concentration would increase the accumulation of haemoglobin, both 10μM or 20μM EPA were included in the following experiment.

K562 cells were seeded at 2 x 10^5 cells/ml in culture medium. The first set of cells, the controls, had no fatty acid added; a final concentration of 10μM EPA was added to the second set (EPA1 supplemented cells) and a final concentration of 20μM EPA was added to the third set (EPA2 supplemented cells) for a period of 48 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). After 48 hours, each of the sets of cells were counted, as described in section

2.2.3 and pelleted by centrifugation at 300 x g for 4 minutes at 20°C. The culture medium was removed and the cells were resuspended in fresh culture medium at a cell density of 2 x 10^5 cells/ml. The control cells were split into two sets, one set containing no treatment, and a final concentration of 0.05μM ara-C was added to the other set. The EPA1 supplemented cells were also split into four sets. The first contained a final concentration of 10μM EPA supplementation and no ara-C. The second contained a final concentration of 10μM EPA supplementation and a final concentration of 0.05μM ara-C. The third contained no EPA and no ara-C. The fourth contained no EPA supplementation and a final concentration of 0.05μM ara-C. The EPA2 supplemented cells were also split into four sets. The first contained a final concentration of 20μM EPA supplementation and no ara-C. The second contained a final concentration of 20μM EPA supplementation and a final concentration of 0.05μM ara-C. The third contained no EPA supplementation and no ara-C. The fourth contained no EPA supplementation and a final concentration of 0.05μM ara-C. The control and the EPA supplemented cells were cultured for 3 days at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). To establish that the volume of water containing ara-C added did not effect the cells in any way, an equal volume of filter-sterilised distilled water was added to the cells not given ara-C.

After 3 days, the cells were mixed and counted to determine cell number and viability as described in section 2.2.3. Haemoglobin accumulation was then tested. For each set of cells, the culture medium was removed by centrifugation at 300 x g for 4 minutes at 20°C, and the cells were washed twice in cold PBS (Rutherford & Weatherall, 1979) using the same centrifugation conditions at 4°C. The cells were resuspended in filter-sterilised distilled water, at a cell density of

$10x10^6$ cells/ml. The cells were then lysed by three cycles of freezing and thawing (Rutherford *et al.*, 1981). The broken cell suspension was then centrifuged at 500 x g for 10 minutes at room temperature to remove the nuclei. The supernatant was removed and centrifuged at 140,000 x g for 45 minutes at room temperature. The pellet was discarded, and the haemoglobin content of the final supernatant was determined from its absorbance at 414nm.

4.2.6 Determination of effect of different fatty acids on haemoglobin accumulation

Five sets of K562 cells were seeded at 2 x 10^5 cells/ml in culture medium. The first set of cells, the controls, had no fatty acid added; to the second set, a final concentration of 20μM EPA was added (EPA supplemented cells), to the third set of cells, a final concentration of 20 μM AA was added (AA supplemented cells), to the fourth set of cells, a final concentration of 20μM OA was added (OA supplemented cells) and to the fifth set of cells, a final concentration of 20μM LA was added (LA supplemented cells), then all cells were incubated for a period of 48 hours at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). After 48 hours, each of the sets of cells were counted, as described in section 2.2.3, and pelleted by centrifugation at 300 x g for 4 minutes at 20°C. The culture medium was removed and the cells were resuspended in fresh culture medium at a cell density of 2 x 10^5 cells/ml. The control cells were split into two sets, one set containing no treatment, the other set containing a final concentration of 0.05μM ara-C. Each of the fatty acid supplemented cells were also split into two sets, one containing a final concentration of 20μM of the initial fatty acid supplementation and no ara-C, the other containing a final

concentration of 20μM of the initial fatty acid supplementation and a final concentration of 0.05μM ara-C. The control and the PUFA supplemented cells were cultured for 3 days at 37°C in an humidified atmosphere of air/CO_2 (19:1, v/v). The cells with no ara-C treatment had an equivalent volume of filter-sterilised distilled water added, compared to the volume of ara-C added to the ara-C treated cells.

After 3 days, the cells were mixed and counted to determine cell number and viability as described in section 2.2.3. The set of cells was split into two portions to determine haemoglobin accumulation, as described in section 4.2.5, and DNA was analysed on a Coulter Epics XL-MCL flow cytometer, as described in section 4.2.2.b.

4.3 Results

4.3.1 Effects of ara-C on cell growth

K562 cells were incubated with concentrations of ara-C ranging from 0 - 3.2μM for up to 5 days, in order to determine its effects on cell growth, the results are shown in Figure 4.1. Increasing ara-C concentration caused inhibition of cell growth over 5 days. The concentration of 0.001μM ara-C gave a significant decrease in cell growth ($p < 0.05$) from the control cells at 96 hours and 120 hours. Cells treated with 0.01μM ara-C gave a measurable, intermediate decrease in cell number throughout the 5 day period, this was significantly lower ($p < 0.05$) than control cells at 72 hours, 96 hours and 120 hours. Concentrations of ≥ 0.05μM inhibited cell growth throughout the 5 day period. Although cell growth was inhibited at 0.05μM there was evidence of some cell reproductive ability, cell growth at 0.05μM ara-C was significantly lower ($p < 0.05$) than control cells at 72 hours, 96 hours and 120 hours. Cells treated with 0.1μM ara-C had a significantly lower cell number ($p < 0.05$) than control cells at 72 hours, 96 hours and 120 hours. Concentrations of ara-C at 0.5μM and 3.2μM effectively produced cytostatis, cell growth was significantly lower ($p < 0.05$) at 24 hours with 3.2μM ara-C and at 48 hours, 72 hours, 96 hours and 120 hours at 0.5μM and 3.2μM ara-C. A concentration to be used in conjunction with fatty acids of 0.01μM ara-C was chosen as this produced a decrease in cell number with some proliferation.

Figure 4.1 Effect of ara-C on the cell growth of K562 cells

The cells were incubated with increasing concentrations of ara-C for up to 5 days, as described in section 4.2.2. The effect of ara-C on cell growth is shown in this Figure. Each point represents the mean value for 3 separate experiments, (9 replicates). The error bars have been omitted, but the standard deviation was less than 5%.

■ control cells, no treatment

● 0.001μM ara-C

▲ 0.01μM ara-C

▼ 0.05μM ara-C

★ 0.1μM ara-C

□ 0.5μM ara-C

○ 3.2μM ara-C

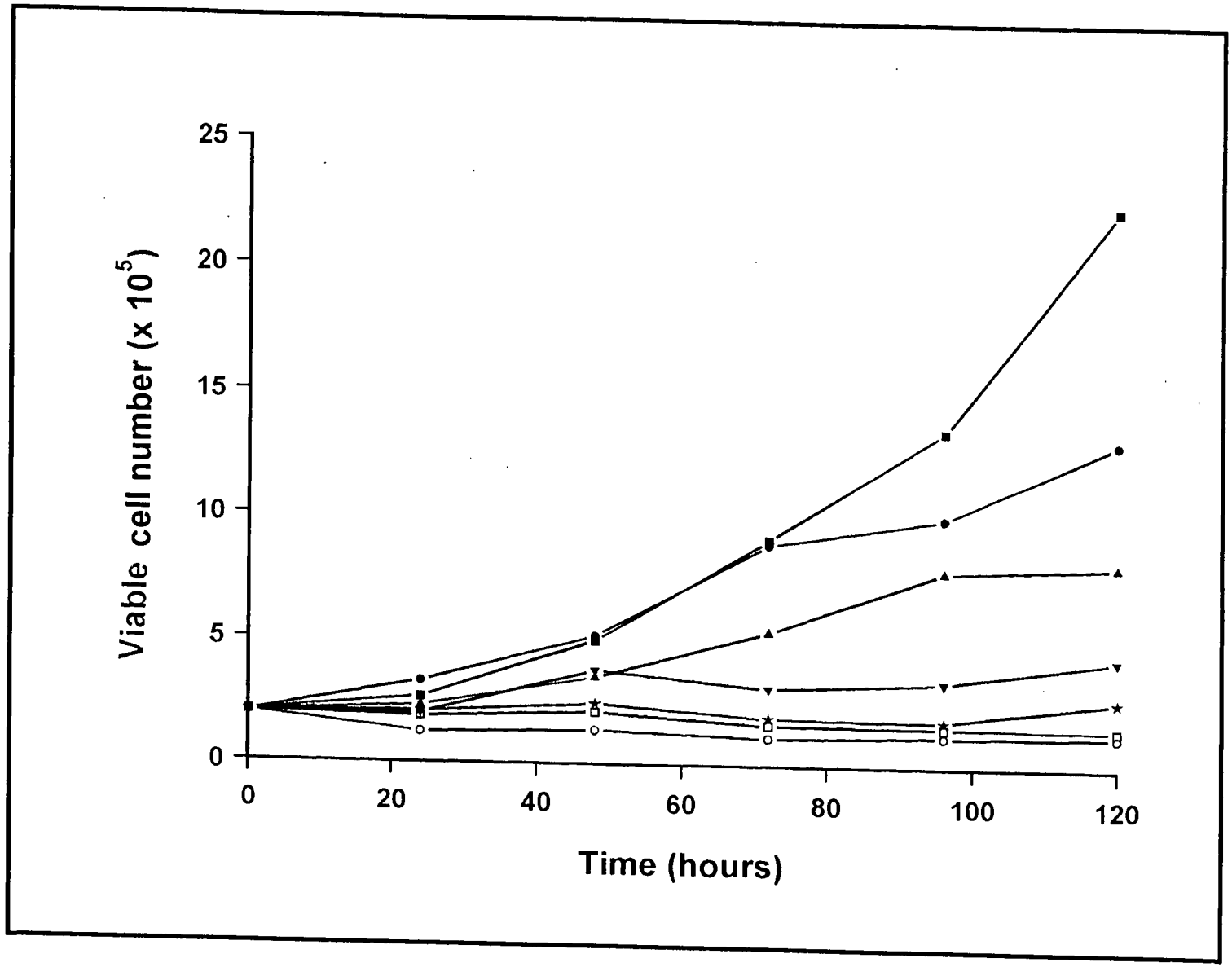
Oneway ANOVA was used to compare cell viability of cells treated with the different concentrations of ara-C with of control cells.

The cell number of cells treated with 0.001μM ara-C was significantly lower ($p < 0.05$) from control cells at 96 hours and 120 hours.

The cell number of cells treated with 0.01μM, 0.05μM and 0.1μM ara-C was significantly lower ($p < 0.05$) from control cells at 72 hours, 96 hours and 120 hours.

The cell number of cells treated with 0.5μM ara-C was significantly lower ($p < 0.05$) from control cells at 48 hours, 72 hours, 96 hours and 120 hours.

The cell number of cells treated with 3.2μM ara-C was significantly lower ($p < 0.05$) from control cells at 24 hours, 48 hours, 72 hours, 96 hours and 120 hours.


Viable cell number (x 10^5)
25
20
15
10
5
0
0
20
40
60
80
100
120
Time (hours)

DNA analysis

The DNA of cells treated with ara-C was analysed on a Coulter Epics XL-MCL flow cytometer to determine the percentage of cells in each stage of the cell cycle. Figure 4.2 shows the typical histograms produced for each sample, 1- 5. Histograms 1-5 are taken from control cells.

Histogram 1- Forward Scatter (FS) vs Side Scatter (SS)

This shows the relative distribution of cells by size and granulation. The x-axis represents SS and the y-axis FS. The further the trace extends along the x-axis, the more granular are the cells; the further the trace extends along the y-axis, the larger the cells. From this control sample, a gate (gate A) is placed around the cells and this gave a true representation of the size and granulation of K562 cells.

Histogram 2 - FL3 vs FL3 Peak (using the FL3 detector)

FL3 is the total flourescence of propidium iodide (PI) associated with the DNA of the cells. FL3 Peak is the brightest intensity of PI flourescence from the sample, as it goes through the beam. This histogram shows the amount of propidium iodide (PI) taken up by the cells. The FL3 vs FL3 Peak histogram ensures that only single cells were analysed. If two cells pass through the analysis point very close together the result would appear to be one cell with twice the DNA, i.e. a cell in G_2+M phase instead of being in $G_{1/0}$ phase. This is a check that doublets are not analysed instead of single cells. A gate (gate B) can be placed around a set of control cells to show how single untreated cells would normally fluoresce PI.

Histogram 3 - FL3 histogram (gated)

This histogram analyses the amount of PI associated with DNA in the cells in both A and B gates. The x-axis represents the amount of PI taken up by the cells and the y-axis the number of cells. In the histograms the letter: C represents the proportion of cells in $G_{1/0}$ of the cell cycle; D represents the proportion of cells in S phase of the cell cycle; and E represents the proportion of cells in G_2+M phase of the cell cycle. When the histogram was produced the mean of the fluorescence was also given and from this the different phases of the cell cycle can be determined. $G_{1/0}$ was determined by its peak and the amount of PI bound reflects the amount of chromosomal DNA. G_2+M is placed where the mean of PI fluorescence was double that of $G_{1/0}$, between these two phases lies the S phase. The percentages of the cell population in each phase of the cell cycle was also determined. Cells found before C, the $G_{1/0}$ phase, were dead cells, and therefore the percentage of dead cells was also determined.

Histogram 4 - FL3 histogram (ungated)

This histogram is identical to histogram 3 except that all the cells are analysed, i.e. including those lying outside gates A and B. In the histograms the letter : F represents the proportion of cells in $G_{1/0}$ of the cell cycle; G represents the proportion of cells in S phase of the cell cycle; and H represents the proportion of cells in G_2+M phase of the cell cycle. When the histogram was produced the mean of the fluorescence was also given and from this the different phases of the cell cycle were determined. In this series of experiments no differences were found between histogram 3 and histogram 4.

Figure 4.2 Effect of ara-C on the cell cycle profile of K562 cells

The cells were incubated with increasing concentrations of ara-C for 5 days, as described in 4.2.2. The effect of ara-C on the cell cycle profile as determined using a FACS machine is shown in this Figure. Histograms 1 - 5 show the typical profiles for each condition. The 3rd histogram from each treatment is used to show the cell cycle profile.

1 Forward scatter vs side scatter. This shows the relative distribution of cells by size and granulation. Gate A represents the majority of the population of cells.
x-axis = side scatter (SS) y-axis = forward scatter (FS)

2 FL3 vs FL3 Peak. This ensures only single cells are analysed. Gate B represents the cells which are fluorescing PI as expected by a population of K562 cells.
x-axis = FL3 (total PI fluorescence)
y-axis = FL3 Peak (brightest intensity of PI fluorescence)

3 FL3 histogram (gated). Shows the cell cycle profile of the cells included in gates A and B.

4 FL3 histogram (ungated). Shows the cell cycle profile of all the cells.

For Histograms **3** and **4**:
C or F = $G_{1/0}$ phase of the cell cycle
D or G = S phase of the cell cycle
E or H = G_2+M phases of the cell cycle
x-axis = fluorescence of PI y-axis = number of cells

5 Time vs FL3. This ensures optimal binding of PI has been achieved.

Treatments:

CON	control cells, no treatment for 5 days
0.001μM	0.001μM ara-C for 5 days
0.01μM	0.01μM ara-C for 5 days
0.05μM	0.05μM ara-C for 5 days
0.1μM	0.1μM ara-C for 5 days
0.5μM	0.5μM ara-C for 5 days
3.2μM	3.2μM ara-C for 5 days

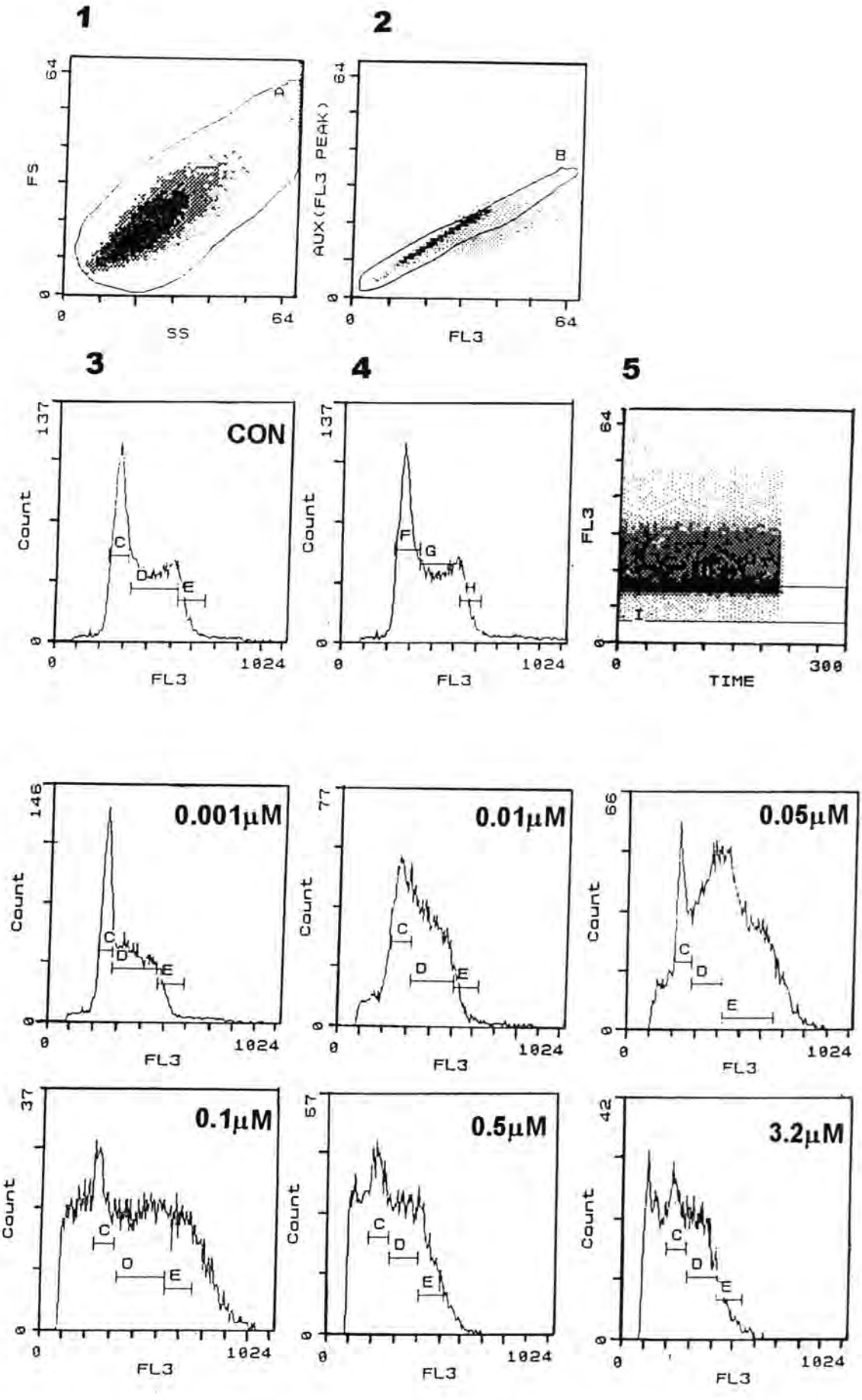

1
2
3
4
5
FS
SS
AUX(FL3 PEAK)
FL3
CON
Count
TIME
0.001μM
0.01μM
0.05μM
0.1μM
0.5μM
3.2μM

Table 4.1 Effect of different concentrations of ara-C on K562 cell cycle profile

The cells were incubated with increasing concentrations of ara-C for 5 days, as described in 4.2.2. The effect of ara-C on the cell cycle profile was determined using a FACS machine. The percentage of cells found in each phase of the cell cycle, was determined from histogram 3 of each treatment, as shown in Figure 4.2. The percentage of dead cells was determined from the percentage of cells found before $G_{1/0}$.

Concentration of ara-C μM	% of cells in $G_{1/0}$ (C)	% of cells in S (D)	% of cells in G_2+M (E)	% of dead cells
0.0 (CON)	37.1	46.1	9.0	7.8
0.001	33.9	50.0	9.4	6.7
0.01	32.0	48.3	6.5	13.2
0.05	15.5	30.5	40.0	14
0.1	16.3	31.0	16.5	36.2
0.5	25.9	29.8	17.6	26.7
3.2	27.2	32.3	8.4	32.1

Histogram 5 - Time vs FL3

This histogram ensures that PI binding has saturated the DNA. This line should remain horizontal for the analysis time. Any deviation either upwards or downwards means that optimal PI binding has not been achieved.

The samples were analysed on low flow rate at a concentration of no greater than 100 events per second for a period of 5 minutes.

The treatment histograms in Figure 4.2 are equivalent to histogram 3 from the control cell five histograms produced on DNA analysis. For each treatment histogram 5 Time vs FL3 was a horizontal line. The control for the K562 cells had no treatment. This is a typical representation of the K562 cell cycle used in this laboratory. The $G_{1/0}$ peak was easily distinguishable, 37.1% percentage of cells were found in $G_{1/0}$, 46.1% were found in S phase and only 9% were found in G_2+M. In control cells, 7.8% of the cells were found before the $G_{1/0}$ peak; cells found in this region were dead. Table 4.1 shows the percentages of cells in each phase of the cell cycle. At 0.001μM and 0.01μM ara-C the percentage of cells in $G_{1/0}$ was reduced accompanied by an increase in percentage of cells in S. At 0.05μM ara-C there was a considerable decrease, compared to control cells, in the percentage of cells in $G_{1/0}$ and S (2.4 -fold and 1.5 -fold respectively), with a large percentage of cells in G_2+M (4.4 -fold increase), while 14% of the cells were dead this was a 1.8-fold increase compared with control cells. Between 0.1μM and 3.2μM ara-C there was a decrease in the percentage of cells in $G_{1/0}$ and S phases compared to control cells with a greater percentage of cells in G_2+M phase and a much higher percentage of dead cells, compared to control cells. At 0.1μM ,0.5μM, and 3.2μM ara-C 36.2%, 26.7% and 32.1% of cells were dead,

respectively. Ara-C altered the percentage of cells in the different phases of the cell cycle. As the concentration of ara-C was increased the percentage of dead cells increased greatly. Up to a concentration of 0.05μM ara-C the majority of the cells were alive. At 0.05μM the main effect of ara-C was to alter the percentage of cells in the phases of the cell cycle. At and above 0.1μM ara-C the percentage of cells in the phases of the cell cycle was altered but the percentage of dead cells was increased considerably. The increasing percentage of dead cells was consistent with the decrease in cell viability in ara-C concentrations of 0.1μM, 0.5μM and 3.2μM, shown in Figure 4.1.

4.3.2 Determination of effect of fatty acids and ara-C on cell growth of K562 cells

The cytotoxic effects of fatty acids alone on K562 cells were reported in Chapter 3. In the present work, a subtoxic dose was chosen to treat the K562 cells in conjunction with ara-C. In previous experiments, the K562 cells had only been grown in fatty acid for 48 hours and it was not known what effect growing the cells for a longer period of time in the presence of PUFA would have on the cells. An experiment was undertaken, therefore, to determine whether PUFA should be present in the 48 hour preincubation period only followed by an ara-C treatment, or in the 48 hour preincubation period followed by continued fatty acid presence during ara-C treatment. Therefore, the cells were grown in the preincubation period of 48 hours in the presence or absence of fatty acid, (10μM EPA), followed by treatment with ara-C (0.01μM) for 5 days, in the presence or absence of 10μM EPA .

As seen in Figure 4.3, the addition of ara-C greatly reduced the cell growth in control and fatty acid-treated cells. As the cell number

was greatly reduced with a 5 day EPA incubation alone, it was decided, initially, that the fatty acid should be present as a 48 hour preincubation followed by the ara-C treatment alone.

Figure 4.3 shows the effect of ara-C on K562 cell growth after the 48 hour preincubation in the presence or absence of EPA (10μM). In the first 24 hours after the addition of ara-C, there was a significant decrease ($p < 0.05$) in the cell viability of all the conditions compared to the no treatment control cells. This was also true for 48 hours, 96 hours and 120 hours. It was evident that the preincubation of EPA (10μM) for 48 hours followed by no EPA incubation decreased the viable cells over the subsequent 48 hour period. Continued growth in EPA after the 48 hour preincubation greatly decreased cell viability throughout the subsequent 5 days. For each of the fatty acid supplementation treatments the addition of ara-C further decreased the viability of the cells compared to fatty acid supplementation alone. After the entire treatment the viable cell number was in the order : control cells, no treatment > treatment with EPA (10μM) for 48 hours, followed by no further treatment > no treatment for 48 hours, then 0.01μM ara-C for a future 120 hours > treatment with EPA (10μM) for 48 hours, then 0.01μM ara-C alone for 120 hours > treatment with EPA (10μM) for 48 hours, then EPA (10μM) for a further 120 hours > treatment with EPA (10μM) for 48 hours, then EPA (10μM) with 0.01μM ara-C for a further 120 hours.

Figure 4.3 Effect of fatty acid and ara-C on K562 cell growth

K562 cells were incubated with fatty acid and ara-C as described in section 4.2.3. The cells were incubated in the presence or absence of EPA (10μM) for 48 hours, followed by incubation in the presence or absence of EPA (10μM) with or without 0.01μM ara-C for a further 120 hours. The effect on cell growth after the 48 hour preincubation is shown in this Figure, time 0 is the start of the 120 hour incubation. Each point represents the mean value for 3 separate experiments, (9 replicates). The error bars have been omitted, but the standard deviation was less than 5%.

■ control cells, no treatment

▼ No treatment for 48 hours, then 0.01μM ara-C alone for a further 120 hours

● Treatment with 10μM EPA for 48 hours, then no further treatment

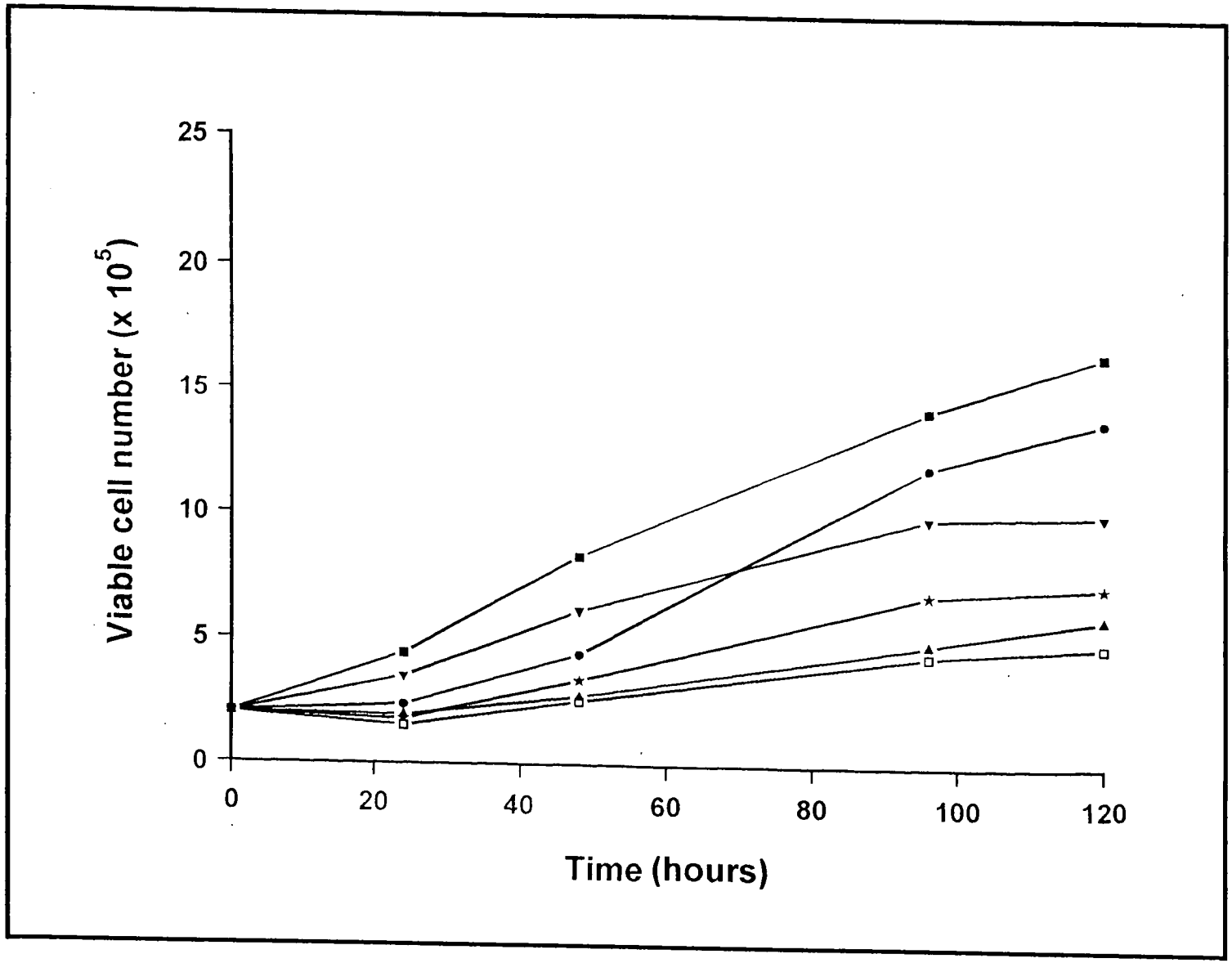
★ Treatment with 10μM EPA for 48 hours, then 0.01μM ara-C alone for a further 120 hours

▲ Treatment with 10μM EPA for 48 hours, then 10μM EPA for a further 120 hours

□ Treatment with 10μM EPA for 48 hours, then 10μM EPA with 0.01μM ara-C for a further 120 hours

Oneway ANOVA was used to compare the different treatments with the control cells.

At 24 hours, 48 hours, 96 hours and 120 hours each of the treatments were significantly different ($p < 0.05$) from the control cells.


Viable cell number (x 10^5)
25
20
15
10
5
0
0
20
40
60
80
100
120
Time (hours)

4.3.3 The effect of fatty acid and ara-C on haemoglobin accumulation in K562 cells

Haemoglobin accumulation was assessed by the absorbance at 414 nm of extracts derived from 10 x 10^6 cells. Two methods were used to lyse the cells and remove the nuclei. The first method involved treating the cells with a 'lysing buffer' followed by an incubation on ice, followed by centrifugation. This method proved to give inconsistent results and the levels of haemoglobin fluctuated greatly for each condition, which suggested that not all cells were being lysed. The second method used three cycles of freeze/thawing, followed by two centrifugations. This method produced consistent results and was used for the haemoglobin accumulation assay.

The culture medium was initially supplemented with 10μM EPA for 48 hours, which was followed by fatty acid free culture medium containing ara-C, (0.01μM) for up to 5 days. The results of this experiment have not been shown as no differences in the accumulation of haemoglobin between the conditions were observed. The ara-C concentration was increased to 0.05μM. Figure 4.1 shows that this concentration of ara-C decreased the cell number with some cell proliferation evident. Figure 4.2 shows that this concentration of ara-C does alter the percentage of cells in each phase of the cell cycle but the majority of cells are still alive.

K562 cells were cultured in the presence or absence of 10μM EPA for 48 hours, followed by ara-C treatment, at 0.05μM for 5 days. The accumulation of haemoglobin was determined using the freeze thaw technique to lyse the cells. Figure 4.4 shows the accumulation of haemoglobin after the preincubation in the presence or absence of EPA (10μM) and after the addition of ara-C, over a period of five days. At

Figure 4.4 Effect of fatty acid and ara-C on haemoglobin accumulation in K562 cells

K562 cells were incubated in the presence or absence 10μM EPA for 48 hours followed by no EPA in the presence or absence of 0.05μM ara-C for 5 days, as described in 4.2.4. The effect on haemoglobin accumulation in cells after the 48 hour preincubation is shown in this Figure; time 0 is the start of the 5 day incubation. In each treatment 10 x 10^6 cells were assayed. Each point represents the mean value ± S.D. for 3 separate experiments, (9 replicates).

■ control cells, no treatment

● Treatment with 10μM EPA for 48 hours, then no further treatment

▲ No treatment for 48 hours, then 0.05μM ara-C alone for a further 120 hours

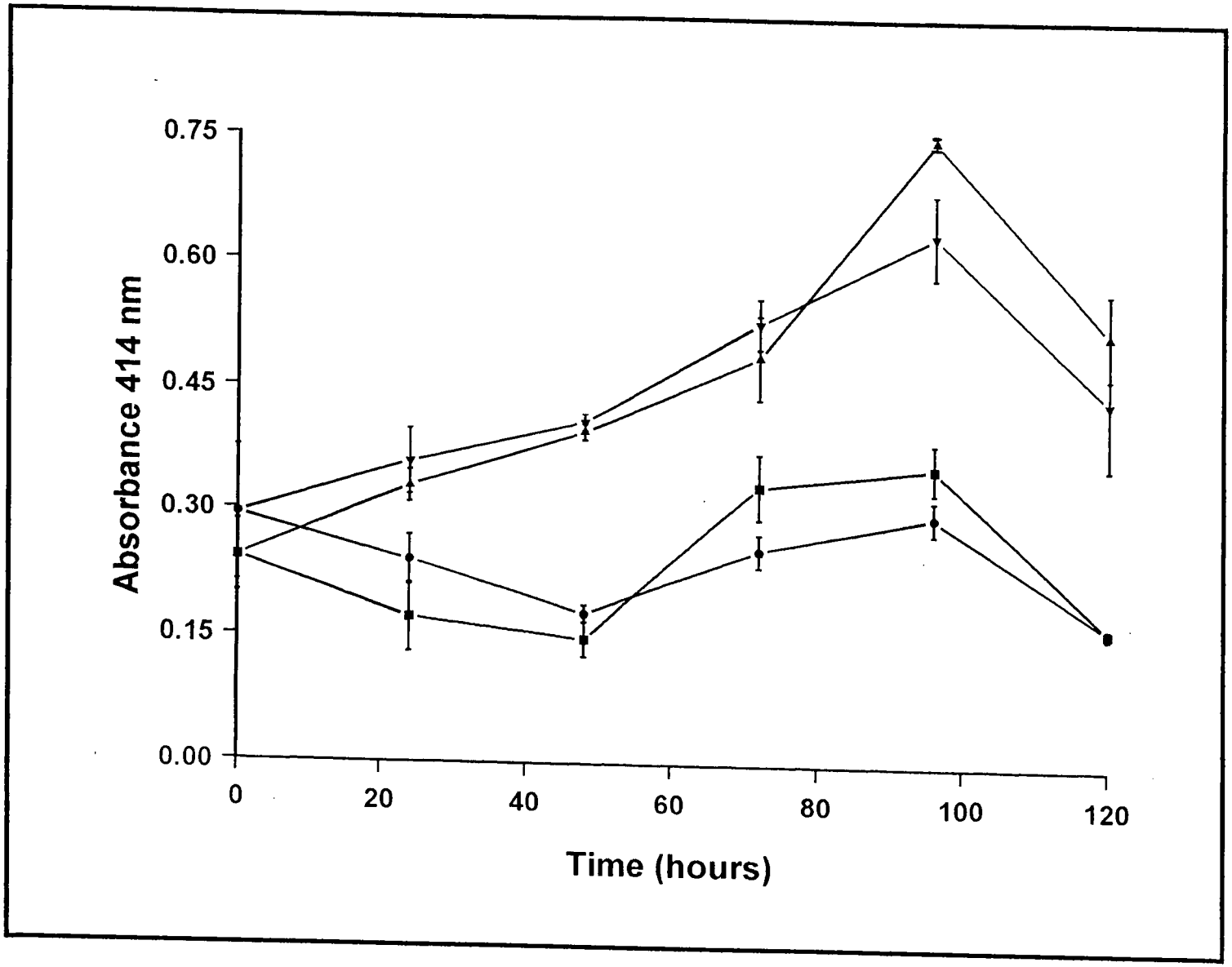
▼ Treatment with 10μM EPA for 48 hours, then 0.05μM ara-C alone for a further 120 hours

Oneway ANOVA was used to compare the data.

At 24 hours, 48 hours, 72 hours and 120 hours the accumulation of haemoglobin in control cell and cells treated with 10μM EPA for 48 hours, then no further treatment were significantly lower ($p < 0.05$) than cells that had no treatment for 48 hours, then 0.05μM ara-C alone for a further 120 hours and cells treated with 10μM EPA for 48 hours, then 0.05μM ara-C alone for a further 120 hours.

At 72 hours and 96 hours the accumulation of haemoglobin in cells treated with 10μM EPA for 48 hours, then no further treatment was significantly lower ($p < 0.05$) than control cells.

At 96 hours the accumulation of haemoglobin in cells treated with 10μM EPA for 48 hours, then 0.05μM ara-C alone for a further 120 hours was significantly lower ($p < 0.05$) than cells that had no treatment for 48 hours, then 0.05μM ara-C alone for a further 120 hours.


0.75
0.60
0.45
0.30
0.15
0.00
Absorbance 414 nm
0
20
40
60
80
100
120
Time (hours)

24 hours, 48 hours, 72 hours, 96 hours and 120 hours cells treated with ara-C accumulated significantly more ($p < 0.05$) haemoglobin than cells without ara-C treatment. At 72 and 96 hours cells preincubated with EPA (10μM) alone accumulated significantly less ($p < 0.05$) haemoglobin than control cells. Only at 96 hours did cells preincubated with EPA (10μM) followed by ara-C treatment accumulate significantly less ($p < 0.05$) haemoglobin than cells treated with ara-C alone. As the effect of EPA (10μM) preincubation had a small effect on haemoglobin accumulation it was decided to increase the EPA concentration to 20μM as this was the highest subtoxic concentration for K562 cells. Therefore a concentration of 20μM was used to determine if increasing fatty acid concentration would increase haemoglobin accumulation.

4.3.4 Effect of different EPA concentrations and ara-C on haemoglobin accumulation in K562 cells

To establish whether different concentrations of fatty acid altered the accumulation of haemoglobin, ten sets of cells were incubated as follows:

con control cells with no treatment for 120 hours; **con+** no treatment for 48 hours, then 0.05μM ara-C alone for a further 72 hours; **p10** treatment with 10μM EPA for 48 hours, then no further treatment for 72 hours; **p10+** treatment with 10μM EPA for 48 hours, then 0.05μM ara-C alone for a further 72 hours; **10** treatment with 10μM EPA for 48 hours, then 10μM EPA for a further 72 hours; **10+** treatment with 10μM EPA for 48 hours, then 10μM EPA with 0.05μM ara-C for a further 72 hours; **p20** treatment with 20μM EPA for 48 hours, then no further treatment for 72 hours; **p20+** treatment with 20μM EPA for 48 hours, then 0.05μM ara-C alone for a further 72 hours; **20** treatment

with 20μM EPA for 48 hours, then 20μM EPA for a further 72 hours; and **20+** treatment with 20μM EPA for 48 hours, then 20μM EPA with 0.05μM ara-C for a further 72 hours. After the total incubation time of 5 days, the haemoglobin accumulation of 10 x 10^6 cells was determined by absorbance of the cell extract at 414nm, this is shown in Figure 4.5.

Only the cells grown with a 48 hour preincubation of EPA alone (**p10** or **p20**) showed no increase in haemoglobin accumulation, which is evident at 10μM and 20μM EPA concentrations. With exposure to EPA alone for 5 days, 10μM or 20μM, (**10** or **20**), a dose dependent increase in haemoglobin accumulation was observed. After a 5 day incubation with 20μM EPA (**20**) the haemoglobin accumulation had increased by 2.3-fold compared to the control; for 10μM EPA (**10**) the increase was by 1.4-fold. Haemoglobin accumulation was found to be EPA dose-dependent.

The addition of ara-C for the final 72 hours of the 5 day incubation showed an increase in haemoglobin accumulation compared to control the cells not incubated with ara-C. Cells with no treatment for 48 hours, then 0.05μM ara-C for a further 72 hours (**con+**) showed a 2.3-fold increase in haemoglobin accumulation; equivalent to that observed with a 5 day exposure to 20μM EPA alone (**20**). When cells were treated with EPA (10μM or 20μM) for 48 hours, then 0.05μM ara-C for a further 72 hours (**p10+** or **p20+**) a decrease of approximately 1.25-fold in haemoglobin accumulation occurred when compared to cells with no treatment for 48 hours, then 0.05μM ara-C for a further 72 hours (**con+**). Cells treated with EPA (10μM or 20μM) for 48 hours, then EPA (10μM or 20μM) with 0.05μM ara-C for a further 72 hours (**10+** or **20+**) showed an increase in haemoglobin accumulation compared to cells with no treatment for 48 hours, then 0.05μM ara-C for a further 72 hours (**con+**). Haemoglobin accumulation

Figure 4.5 Effect of different concentrations of EPA and ara-C on haemoglobin accumulation in K562 cells

K562 cells were incubated in the presence or absence of EPA at 10μM or 20μM for 48 hours, followed by no EPA or 10μM or 20μM EPA in the presence or absence of 0.05μM ara-C for 72 hours, as described in section 4.2.5. The effect of haemoglobin accumulation in cells was measured by absorbance at 414nm. In each treatment 10 x 10^6 cells were assayed. Each point represents the mean value ± standard deviation for 3 separate experiments, (9 replicates).

con control cells, no treatment for 120 hours

con+ No treatment for 48 hours, then 0.05μM ara-C alone for a further 72 hours

p10 Treatment with 10μM EPA for 48 hours, then no further treatment for 72 hours

p10+ Treatment with 10μM EPA for 48 hours, then 0.05μM ara-C alone for a further 72 hours

10 Treatment with 10μM EPA for 48 hours, then 10μM EPA for a further 72 hours

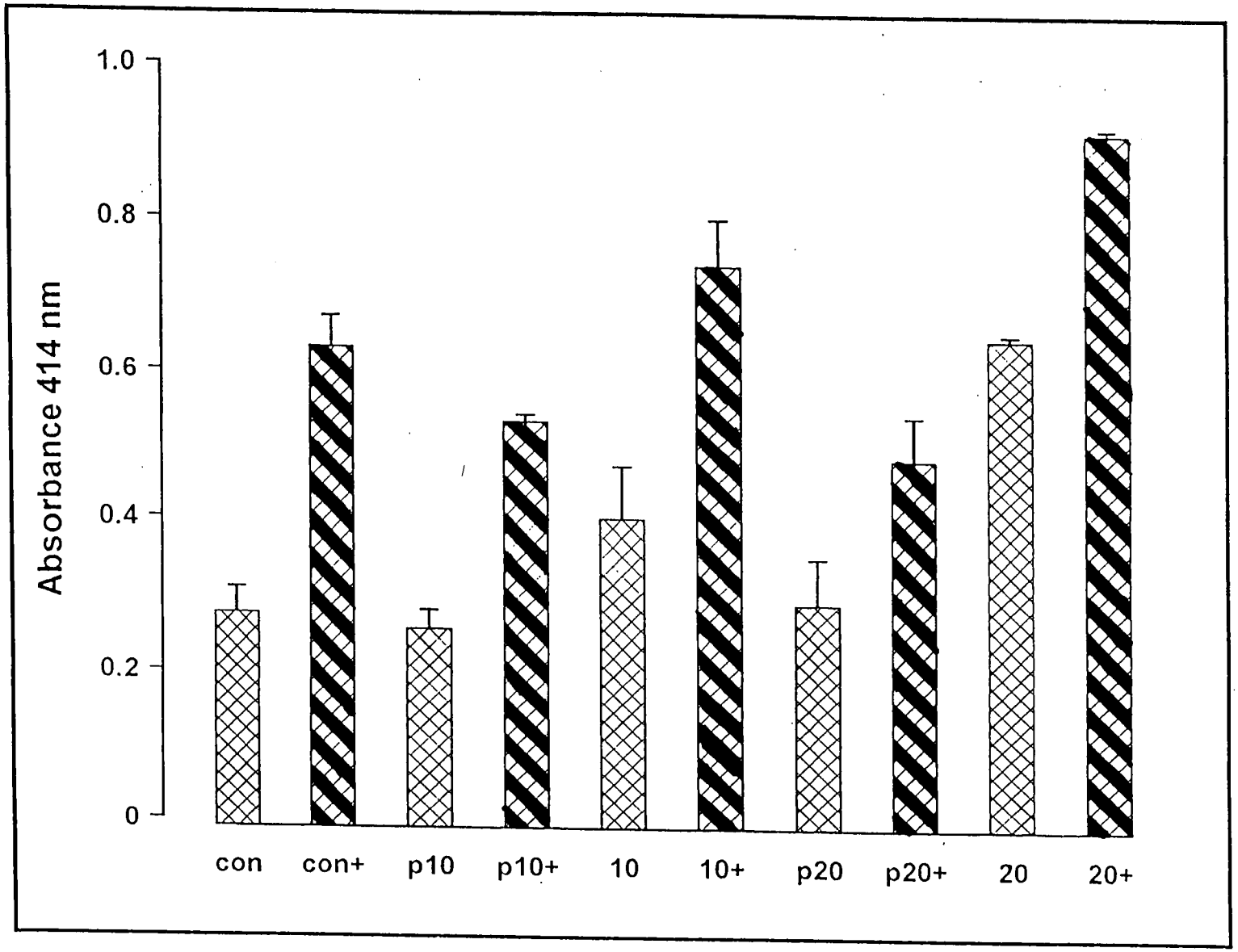
10+ Treatment with 10μM EPA for 48 hours, then 10μM EPA with 0.05μM ara-C for a further 72 hours

p20 Treatment with 20μM EPA for 48 hours, then no further treatment for 72 hours

p20+ Treatment with 20μM EPA for 48 hours, then 0.05μM ara-C alone for a further 72 hours

20 Treatment with 20μM EPA for 48 hours, then 20μM EPA for a further 72 hours

20+ Treatment with 20μM EPA for 48 hours, then 20μM EPA with 0.05μM ara-C for a further 72 hours


Absorbance 414 nm
1.0
0.8
0.6
0.4
0.2
0
con
con+
p10
p10+
10
10+
p20
p20+
20
20+

was 1.12-fold higher with 10μM EPA plus ara-C (**10+**) and 1.45-fold higher in cells treated with 20μM EPA plus ara-C (**20+**) compared to cells treated with ara-C only (**con+**).

This shows that prolonged EPA treatment alone can induce the cells to differentiate and accumulate haemoglobin. EPA in conjunction with ara-C increased accumulation of haemoglobin and differentiation of ara-C alone.

Haemoglobin accumulation was not affected by treatment with EPA (10μM or 20μM) for 48 hours, then no treatment for a further 72 hours (**p10** or **p20**) compared to cells with no treatment (**con**). Haemoglobin accumulation was promoted by ara-C alone for 72 hours (**con+**); EPA alone for 120 hours (**10** or **20**); or EPA (48 or 120 hours) with ara-C treatment for 72 hours (**p10+**, **10+**, **p20+** or **20+**). Treatments with EPA for 48 hours, then for a further 72 hours in the presence or absence of ara-C showed a greater increase in haemoglobin accumulation with 20μM EPA than with 10μM EPA (**20>10**; **20+>10+**). Maximum haemoglobin accumulation was produced by treatment with 20μM EPA for 48 hours, then 20μM EPA with 0.05μM ara-C for a further 72 hours (**20+**). However, haemoglobin accumulation was decreased by treatment with 10μM or 20μM EPA for 48 hours, then 0.05μM ara-C for a further 72 hours (**p10+** and **p20+**), compared to no treatment for 48 hours, then 0.05μM ara-C for a further 72 hour ara-C treatment (**con+**) (**con+>p10**; **con+>p20**). EPA preincubation seemed to decrease the haemoglobin inducing power of ara-C alone.

The effect of the above treatments on cell viability was also determined and is shown in Figure 4.6, the shaded area represents the viable cells and the whole bar the total number of cells.

Cell numbers decreased slightly to 94% of the control when treated with 10μM EPA for 48 hours, then no treatment for a further 72 hours (**p10**). In cells treated with 20μM EPA for 48 hours, then no further treatment for 72 hours (**p20**), there was a further decline in cell number to 60% of the control. This dose-dependent decline in cell number, caused by treatment with EPA for 48 hours, then no treatment for 72 hours, (Figure 4.6), occurred in the absence of changes in haemoglobin accumulation (Figure 4.5).

A more dramatic decrease in cell number was observed when cells were treated with EPA for 48 hours, then EPA for a further 72 hours. Cell numbers decreased to 71% in 10μM EPA (**10**) and to 40.5% in 20μM EPA (**20**), compared to the control (**CON**) (Figure 4.6), and this decline in cell number correlates with an EPA dose-dependent increase in haemoglobin accumulation (Figure 4.5).

Cells with no treatment for 48 hours, then ara-C for a further 72 hours (**CON+**) caused a decline in cell number, to 36% of the control (**CON**) (Figure 4.6), and this correlates with a 2.3-fold increase in haemoglobin accumulation compared to control cells (Figure 4.5).

When cells were treated with EPA (10μM or 20μM) for 48 hours, then ara-C treatment only for a further 72 hours (**p10+** or **p20+**), the cell number decrease to approximately 79% for 10μM EPA (**p10**) and 20μM EPA (**p20**) when compared to cells with no treatment for 48 hours, then ara-C for a further 72 hours (**CON+**) (Figure 4.6). Cells treated with EPA (10μM or 20μM) for 48 hours, then ara-C treatment only for a further 72 hours (**p10+** or **p20+**) decreased haemoglobin accumulation by approximately 48% compared to cells with no treatment for 48 hours, then ara-C for a further 72 hours (**CON+**)(Figure 4.5). The cell number of cells treated with 10μM EPA for 48 hours, then 10μM EPA with ara-C for a further 72 hours (**10+**)

Figure 4.6 Effect of different concentrations of EPA and ara-C on cell growth and viability of K562 cells

K562 cells were incubated in the presence or absence of EPA at 10μM or 20μM for 48 hours, followed by no EPA or 10μM or 20μM EPA in the presence or absence of 0.05μM ara-C for 72 hours, as described in section 4.2.5. The effect on cell growth and viability was determined as described in 2.2.3. Each bar represents the mean value ± S.D. for 3 separate experiments, (9 replicates).

□ total cell number ▨ viable cell number

con control cells, no treatment for 120 hours

con+ No treatment for 48 hours, then 0.05μM ara-C alone for a further 72 hours

p10 Treatment with 10μM EPA for 48 hours, then no further treatment for 72 hours

p10+ Treatment with 10μM EPA for 48 hours, then 0.05μM ara-C alone for a further 72 hours

10 Treatment with 10μM EPA for 48 hours, then 10μM EPA for a further 72 hours

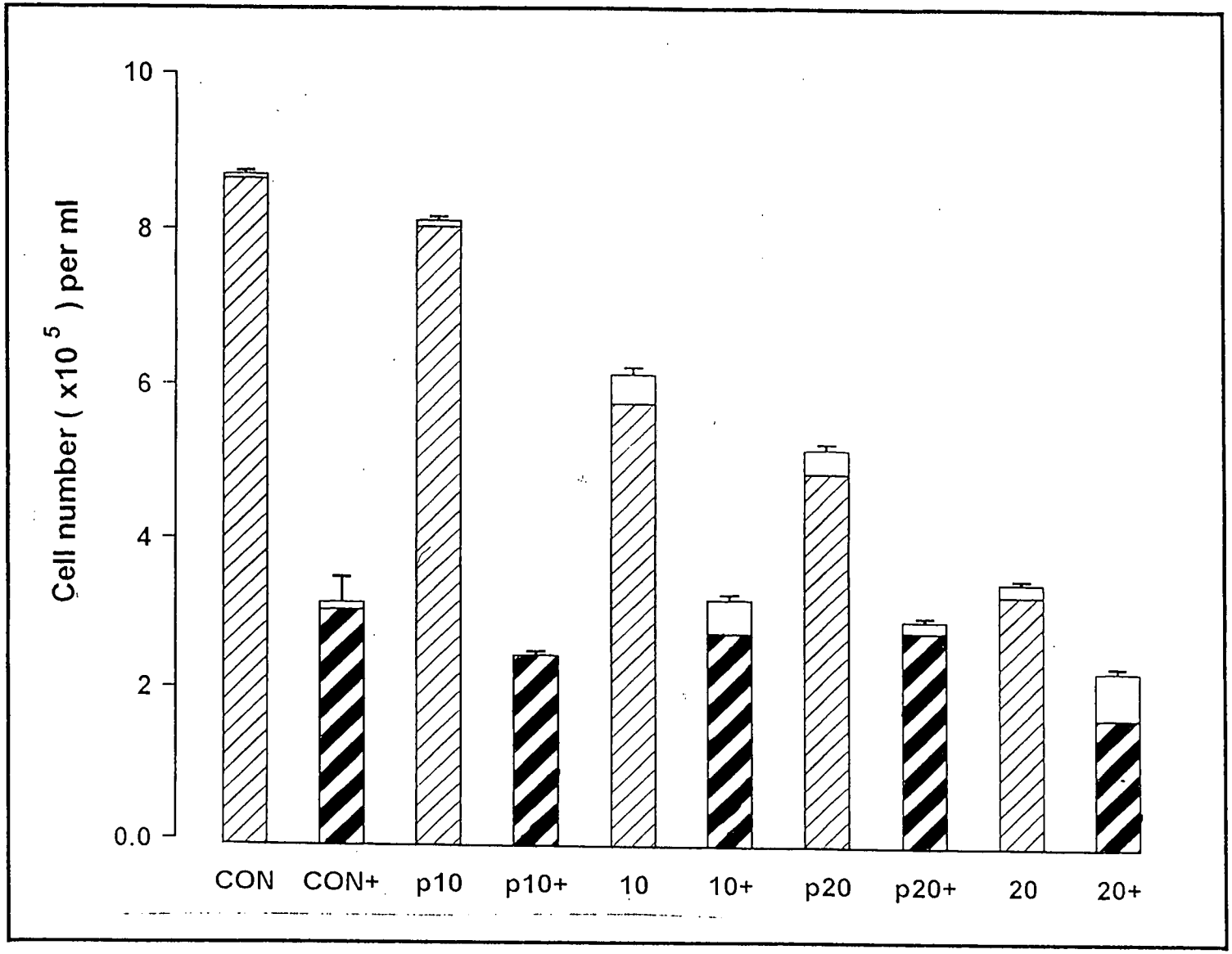
10+ Treatment with 10μM EPA for 48 hours, then 10μM EPA with 0.05μM ara-C for a further 72 hours

p20 Treatment with 20μM EPA for 48 hours, then no further treatment for 72 hours

p20+ Treatment with 20μM EPA for 48 hours, then 0.05μM ara-C alone for a further 72 hours

20 Treatment with 20μM EPA for 48 hours, then 20μM EPA for a further 72 hours

20+ Treatment with 20μM EPA for 48 hours, then 20μM EPA with 0.05μM ara-C for a further 72 hours


Cell number (x10 5) per ml
10
8
6
4
2
0.0
CON
CON+
p10
p10+
10
10+
p20
p20+
20
20+

did not alter compared to cells with no treatment for 48 hours, then ara-C for a further 72 hours (**CON+**), cells treated with 20μM EPA for 48 hours, then 20μM EPA with ara-C for a further 72 hours (**20+**) declined in cell number to 71% of cells with no treatment for 48 hours, then ara-C for a further 72 hours (**CON+**) (Figure 4.6). Under these conditions haemoglobin accumulation was increased by 1.12-fold with 10μM EPA and by 1.45-fold with 20μM EPA compared to cell with an ara-C treatment only for the final 72 hours (Figure 4.5). In general, therefore, when ara-C was present the cell number declined but the magnitude of this decline was not substantially affected by the presence of EPA, at either 10μM or 20μM, during either the 48 hour preincubation or throughout the entire 5 day incubation period.

It is interesting to note that exposure of cells to 20μM EPA for 5 days, including ara-C for the final 72 hour, resulted in the greatest decline in cell numbers, to 25.5% of control, (Figure 4.6) and the greatest accumulation of haemoglobin (Figure 4.5). In general, cell viability was high in most of these conditions, though declined when cells were exposed to EPA for 5 days combined with ara-C for 72 hours (Figure 4.6).

4.3.5 Effect of different fatty acids and ara-C on K562 cells

In this experiment, K562 cells were incubated with one of the following fatty acids EPA, AA, LA or OA. DHA could not be used in this series of experiments as the addition of ara-C to cells supplemented with 10μM DHA for 48 hours caused the cells to adhere to one another forming large clumps and the mortality rate of the cells increased. Reducing the concentration of DHA did not improve the clumping effect during the ara-C treatment. For the other fatty acids the highest

subtoxic concentration was 20μM. Therefore, in this experiment a concentration of 20μM was used for each fatty acid.

K562 cells were incubated with one of four different fatty acids at 20μM, for 48 hours followed by another 72 hours with the fatty acid in the presence or absence of ara-C (0.05μM). The fatty acids used were EPA, AA, LA or OA. At the end of the 120 hours the accumulation of haemoglobin in 10 x 10^6 cells was determined, and the results are shown in Figure 4.7.

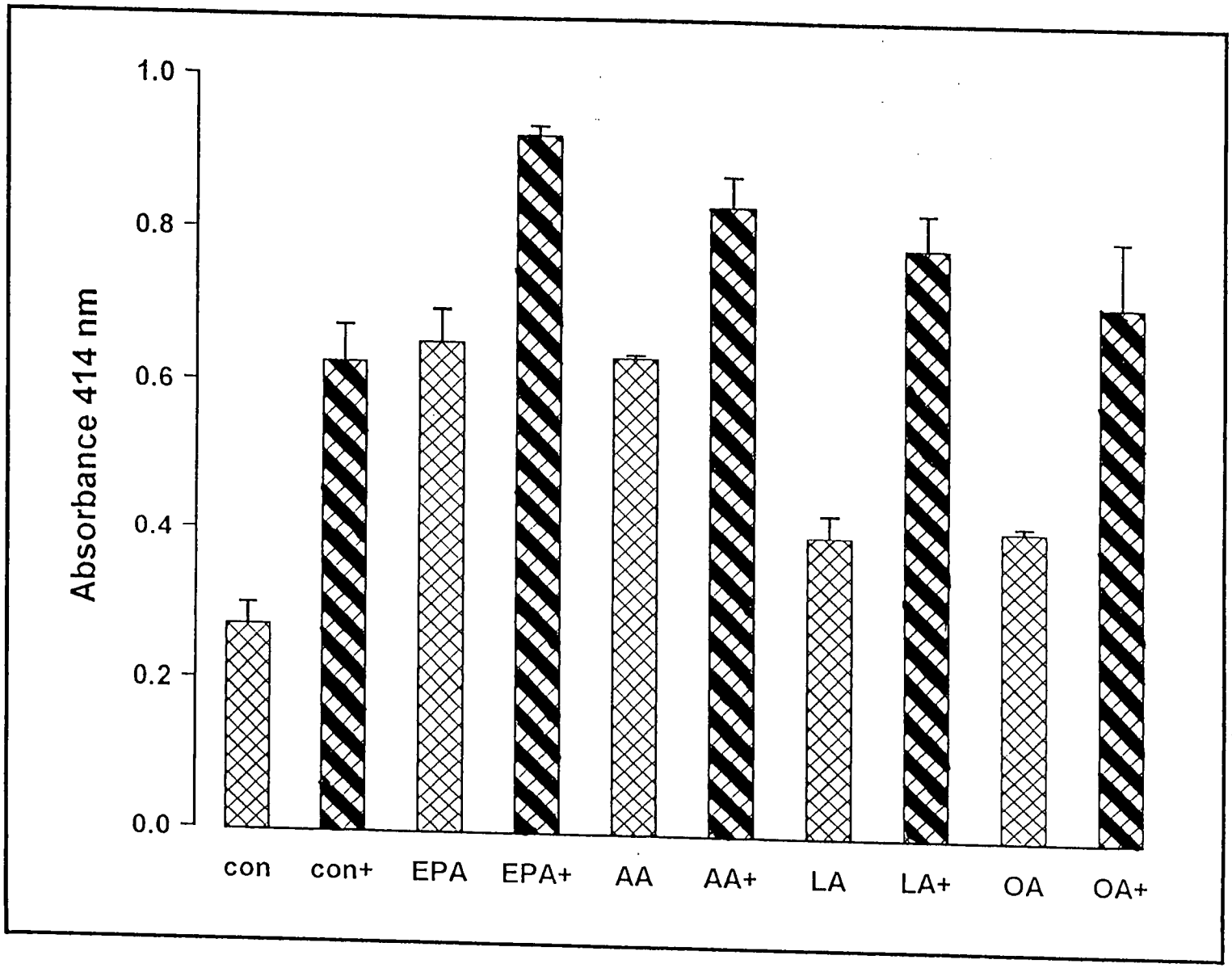
Cells treated with EPA for 120 hours only (**EPA**) accumulated haemoglobin producing a 2.38-fold increase compared to control cells (**con**). Cells treated with ara-C for the final 72 hours only (**con+**) accumulated haemoglobin producing a 2.29-fold increase compared to control cells (**con**), very similar to the response seen in cells treated with EPA for 120 hours (**EPA**). When cells were treated with EPA for 120 hours plus ara-C for the final 72 hours (**EPA+**) haemoglobin accumulation greatly increased, producing a 3.41-fold increase compared to control cells (**con**), a 1.49-fold increase compared to cells with ara-C for the final 72 hours only (**con+**) and a 1.43-fold increase compared to cells treated with EPA for 120 hours (**EPA**).

Cells treated with AA for 120 hours (**AA**) increased haemoglobin accumulation by 2.32-fold compared to control cells (**con**). This increase was very similar to cells treated with EPA for 120 hours (**EPA**) and to cells treated with ara-C for the final 72 hours only (**con+**). The addition of ara-C to the final 72 hours of the 120 hour AA treatment (**AA+**) further increased haemoglobin accumulation, by 3.07-fold compared to control cells (**con**), by 1.34-fold compared to ara-C only for the final 72 hours (**con+**) and by 1.32-fold compared to AA for 120 hours (**AA**).

Figure 4.7 Effect of different fatty acids and ara-C on haemoglobin accumulation in K562 cells

K562 cells were incubated in the presence or absence of fatty acid 20μM for 48 hours followed by fatty acid 20μM in the presence or absence of 0.05μM ara-C for 3 days, as described in 4.2.6. The effect of haemoglobin accumulation in cells was measured by absorbance at 414nm. In each treatment 10 x 10^6 cells were assayed. Each bar represents the mean value ± standard deviation for 3 separate experiments, (9 replicates).

con	No treatment for 120 hours
con+	No treatment for 48 hours, then 0.05μM ara-C alone for a further 72 hours
EPA	Treatment with 20μM EPA for 48 hours, then 20μM EPA without 0.05μM ara-C for a further 72 hours
EPA+	Treatment with 20μM EPA for 48 hours, then 20μM EPA with 0.05μM ara-C for a further 72 hours
AA	Treatment with 20μM AA for 48 hours, then 20μM AA without 0.05μM ara-C for a further 72 hours
AA+	Treatment with 20μM AA for 48 hours, then 20μM AA with 0.05μM ara-C for a further 72 hours
LA	Treatment with 20μM LA for 48 hours, then 20μM LA without 0.05μM ara-C for a further 72 hours
LA+	Treatment with 20μM LA for 48 hours, then 20μM LA with 0.05μM ara-C for a further 72 hours
OA	Treatment with 20μM OA for 48 hours, then 20μM OA without 0.05μM ara-C for a further 72 hours
OA+	Treatment with 20μM OA for 48 hours, then 20μM OA with 0.05μM ara-C for a further 72 hours


Absorbance 414 nm
1.0
0.8
0.6
0.4
0.2
0.0
con
con+
EPA
EPA+
AA
AA+
LA
LA+
OA
OA+

Cells treated with LA for 120 hours (**LA**) accumulated more haemoglobin than control cells (**con**), an increase of 1.32-fold. When ara-C was added for the final 72 hours of the 120 hour LA treatment (**LA+**) haemoglobin accumulation increased by 2.61-fold compared to control cells (**con**), by 1.14-fold compared to cells treated with ara-C for the final 72 hours (**con+**), and by 1.72-fold compared to cells treated with LA for 120 hours (**LA**).

Cells treated with OA for 120 hours (**OA**) also increased haemoglobin accumulation by 1.51-fold compared to control cells (**con**). This increase was similar to that produced by cells treated with LA for 120 hours (**LA**). When ara-C was added to the final 72 hours of the OA 120 hour treatment, haemoglobin accumulation increased by 2.61-fold compared to control cells, by 1.14-fold to cells treated with ara-C for 72 hours (**con+**), by 1.73-fold compared to cells treated with OA 120 hour incubation.

K562 cells were found to accumulate haemoglobin in the presence of 0.05μM ara-C for 72 hours or the presence of fatty acids alone for 5 days. Cells treated with AA or EPA for 120 hours accumulated approximately the same amount of haemoglobin as those treated with ara-C for 72 hours. The maximum haemoglobin accumulation was achieved in the presence of EPA for 120 hours combined with ara-C for the final 72 hours. The potency of the treatments at accumulating haemoglobin were in the order : 120 hours EPA with ara-C for the final 72 hours > 120 hours AA with ara-C for the final 72 hours > 120 hours LA with ara-C for the final 72 hours > 120 hours OA with ara-C for the final 72 hours > 120 hours EPA ≥ 120 hours AA > 72 hours ara-C > 120 hours LA ≥ 120 hours OA **(EPA+> AA+ > LA+ > OA+ > EPA ≥ AA > con+ > LA ≥ OA)**. This order reflects the number of double bonds in the fatty acids.

The effect of these conditions on cell growth and viability is shown in Figure 4.8. The shaded area represents the viable cells/ml and the whole bar the total number of cells/ml.

Cells treated with EPA for 120 hours (**EPA**) produced a decrease in cell number to 67% of the control (**con**). When ara-C was added to the final 72 hours of the EPA 120 hour treatment (**EPA+**) a further reduction in cell number was observed to 28% of the control (**con**). The addition of ara-C to the EPA treatment (**EPA+**) decreased the cell number by 38% compared to EPA treatment (**EPA**). The effect of ara-C alone for 72 hours (**con+**) reduced the cell number to 52% of the control.

Cells treated with AA for 120 hours (**AA**) decreased the cell number to 85% of the control (**con**). Again the addition of ara-C to the final 72 hours of the AA 120 hour treatment (**AA+**) further reduced the cell number to 49% of the control (**con**). The addition of ara-C to the AA treatment (**AA+**) decreased the cell number by 29% compared to AA treatment (**AA**).

When cells were treated with LA for 120 hours (**LA**) an increase in cell growth was observed to 137% of the control (**con**). However the addition of ara-C to the final 72 hours of the LA 120 hour treatment (**LA+**) dramatically decreased the cell number to 49% of control cells (**con**). The same effect on cell number was observed in AA with ara-C treatment (**AA+**). The addition of ara-C to LA treatment (**LA+**) decreased the cell number by 88% compared to LA treatment (**LA**).

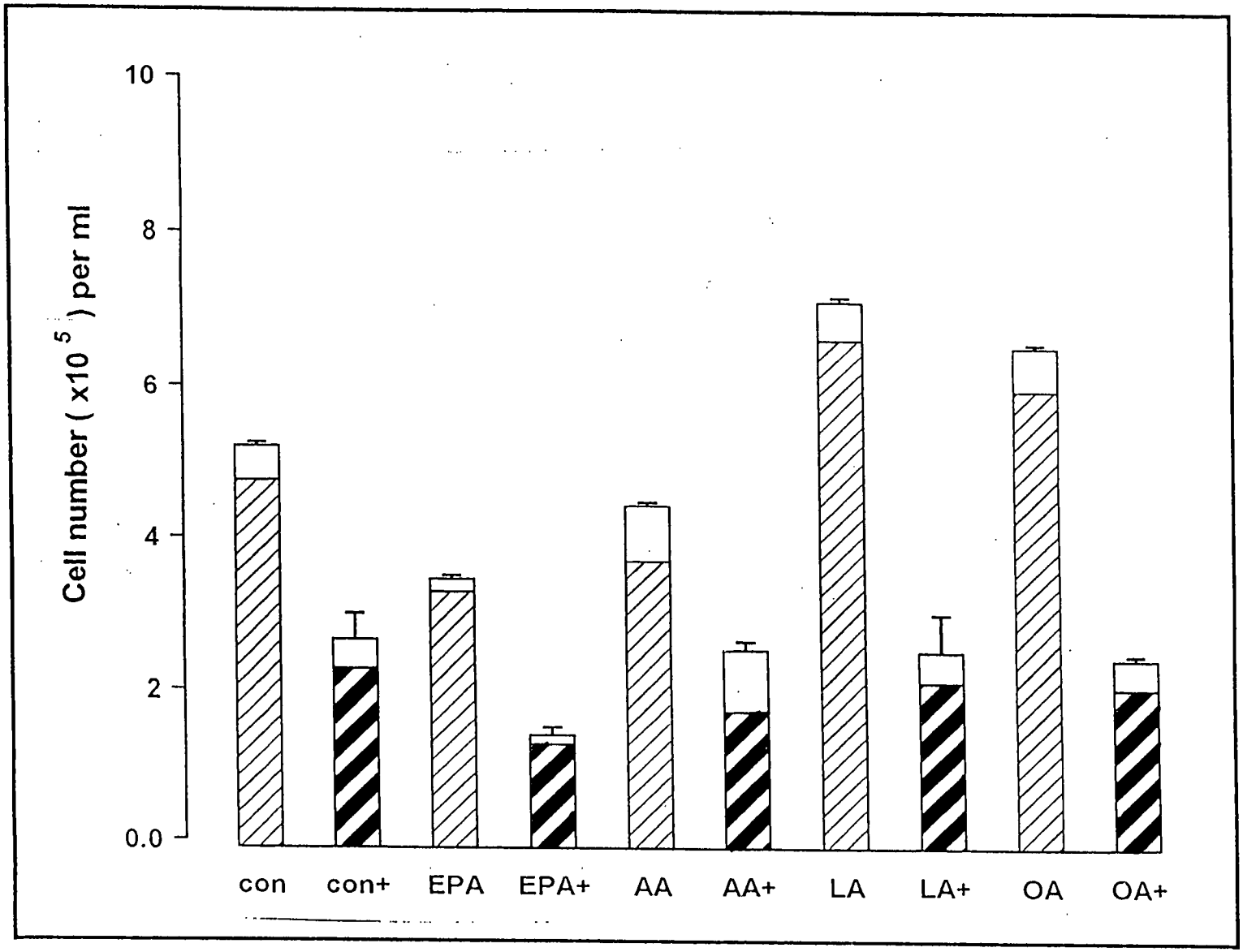
An increase in cell number was again observed when cells were treated with OA for 120 hours (**OA**) to 127% of the control (**con**). The addition of ara-C to the final 72 hours of the 120 hour incubation

Figure 4.8 Effect of different fatty acids and ara-C on cell growth and viability in K562 cells

K562 cells were incubated in the presence or absence of fatty acid (20μM) for 48 hours followed by fatty acid (20μM) in the presence or absence of 0.05μM ara-C for three days, as described in section 4.2.6. The effect on cell growth and viability was measured as described in 2.2.3. Each bar represents the mean value ± standard deviation for 3 experiments, (9 replicates).

☐ total cell number ▨ viable cell number

con	No treatment for 120 hours
con+	No treatment for 48 hours, then 0.05μM ara-C alone for a further 72 hours
EPA	Treatment with 20μM EPA for 48 hours, then 20μM EPA without 0.05μM ara-C for a further 72 hours
EPA+	Treatment with 20μM EPA for 48 hours, then 20μM EPA with 0.05μM ara-C for a further 72 hours
AA	Treatment with 20μM AA for 48 hours, then 20μM AA without 0.05μM ara-C for a further 72 hours
AA+	Treatment with 20μM AA for 48 hours, then 20μM AA with 0.05μM ara-C for a further 72 hours
LA	Treatment with 20μM LA for 48 hours, then 20μM LA without 0.05μM ara-C for a further 72 hours
LA+	Treatment with 20μM LA for 48 hours, then 20μM LA with 0.05μM ara-C for a further 72 hours
OA	Treatment with 20μM OA for 48 hours, then 20μM OA without 0.05μM ara-C for a further 72 hours
OA+	Treatment with 20μM OA for 48 hours, then 20μM OA with 0.05μM ara-C for a further 72 hours


Cell number (x10 5) per ml
10
8
6
4
2
0.0
con
con+
EPA
EPA+
AA
AA+
LA
LA+
OA
OA+

(**OA+**) decreased the cell number to 48% of the control (**con**). The addition of ara-C to the OA treatment (**OA+**) decreased the cell number by 78% compared to OA treatment (**OA**).

Incubations with EPA or AA decreased the cell number whereas OA and LA increased cell number. The addition of ara-C to the fatty acid treatment decreased the cell numbers to below 50% of control cells. Ara-C treatment alone reduced cell number to 52% of control cells. The treatments caused alterations in cells number which were in the following order : LA 120 hours > OA 120 hours > control, no treatment > AA 120 hours > EPA 120 hours > ara-C 72 hours ≥ LA 120 hours with ara-C for the final 72 hours = AA 120 hours with ara-C for the final 72 hours ≥ OA 120 hours with ara-C for the final 72 hours > EPA 120 hours with ara-C for the final 72 hours (**LA > OA > con > AA > EPA > con+ ≥ LA+ = AA+ ≥ OA+ > EPA+**). This order does not correspond to the order of haemoglobin accumulation.

The decrease in cell number produced by EPA or AA was coupled with an increase in haemoglobin accumulation by 2.38 -fold and 2.32 -fold, respectively, compared to control cells. LA or OA treatment alone for 120 hours stimulated both cell growth and haemoglobin accumulation by 1.32-fold and 1.51-fold, respectively, compared to control cells. As shown in Figures 4.5 and 4.6 a reduction in cell number was accompanied by haemoglobin accumulation but in this experiment OA or LA both stimulated cell growth and caused haemoglobin accumulation. EPA or AA decreased cell growth and caused a higher accumulation of haemoglobin than LA or OA.

Cells treated with ara-C for 72 hours (**con+**) caused a decrease in cell number to 52% of the control, this was accompanied by a 2.27-fold increase in haemoglobin accumulation compared to control cells. When

cells were treated with fatty acid for 120 hours and ara-C for the final 72 hours the cell numbers decreased further. The greater decrease in cell number caused by the addition of ara-C to the fatty acid treatment seemed to be accompanied by a further increase in haemoglobin accumulation. Cells treated with EPA for 120 hours with ara-C for the final 72 hours were found to accumulate the largest amount of haemoglobin and the most dramatic decrease in the cell number. Cell number was reduced to approximately 49% when treated with either AA with ara-C, or LA with ara-C or OA with ara-C. However AA with ara-C treatment caused a greater increase in haemoglobin accumulation, whereas LA with ara-C or OA with ara-C accumulated the same amount of haemoglobin.

In Figures 4.5 and 4.6 the decrease in cell number suggests the onset of differentiation. In this experiment, EPA treatment for 120 hours with ara-C for the final 72 hours gave the most dramatic decrease in cell number and caused the largest induction of the onset of differentiation. Even though AA treatment for 120 hours with ara-C for the final 72 hours did not decrease the cell number as significantly as EPA with ara-C, the AA with ara-C treatment did induce a large haemoglobin accumulation and the onset of differentiation. LA with ara-C or OA with ara-C reduced the cell number to approximately the same level as AA with ara-C but the amount of haemoglobin accumulation and onset of differentiation was less than AA with ara-C, this reflects the number of double bonds found in the fatty acids.

The effect of different fatty acids with ara-C on the DNA of K562 cells was determined by FACS. Histogram 3 from the five histograms produced during DNA analysis is shown in Figure 4.9 for each condition of the experiment. Histogram 5 for each condition

showed that PI binding was optimal. The percentage of cells in each phase of the cell cycle is shown in Table 4.2.

The control (**CON**) shows a typical K562 cell cycle profile under current investigation. When ara-C was added to the final 72 hours (**CON+**) an increase in the percentage of cells was observed in S phase and G_2+M phases compared to control cells (**CON**). This was accompanied by a decrease in the percentage of cells in $G_{1/0}$ and an increase in the percentage of dead cells compared to control cells. From Figures 4.7 and 4.8 the addition of ara-C seems to have induced differentiation accompanied by a reduction in cell number and an increase in haemoglobin accumulation. The cell cycle profile seems to reflect this with a reduction in the percentage of cells in $G_{1/0}$ and a much higher percentage of cells in S and G_2+M phase.

The addition of the PUFA EPA for 120 hours (**EPA**) has increased the percentage of cells in $G_{1/0}$ phase, and G_2+M phase and reduced the percentage of cells in the S phase of the cell cycle, the percentage of dead cells was also increased, compared to the control cells (**CON**). From Figures 4.7 and 4.8 the addition of EPA seemed to have induced differentiation accompanied by a decrease in cell number and an increase in haemoglobin accumulation. When ara-C was present for the final 72 hours of the 120 hour EPA treatment (**EPA+**) there was a decrease in the percentage of cells in $G_{1/0}$ phase, S phase and an increase in the percentage of cells in G_2+M phase of the cell cycle, the percentage of dead cells also increased, compared to control cells (**CON**). Figures 4.7 and 4.8 seemed to show that EPA with ara-C treatment induced differentiation, which was accompanied by decreasing the cell number greatly and accumulating the largest amount of haemoglobin. This was reflected in the cell cycle profile with an increase in the percentage of cells G_2+M and a decrease in $G_{1/0}$ and S

Figure 4.9 Effect of different fatty acids and ara-C on the cell cycle profile of K562 cells

K562 cells were incubated in the presence or absence of fatty acid 20μM for 48 hours followed by fatty acid 20μM in the presence or absence of 0.05μM ara-C for three days, as described in 4.2.6. The effect on the cell cycle profile was determined by FACS, the histograms of the cell cycles for the following treatments are shown in this Figure.

CON	No treatment for 120 hours
CON+	No treatment for 48 hours, then 0.05μM ara-C alone for a further 72 hours
EPA	Treatment with 20μM EPA for 48 hours, then 20μM EPA without 0.05μM ara-C for a further 72 hours
EPA+	Treatment with 20μM EPA for 48 hours, then 20μM EPA with 0.05μM ara-C for a further 72 hours
AA	Treatment with 20μM AA for 48 hours, then 20μM AA without 0.05μM ara-C for a further 72 hours
AA+	Treatment with 20μM AA for 48 hours, then 20μM AA with 0.05μM ara-C for a further 72 hours
LA	Treatment with 20μM LA for 48 hours, then 20μM LA without 0.05μM ara-C for a further 72 hours
LA+	Treatment with 20μM LA for 48 hours, then 20μM LA with 0.05μM ara-C for a further 72 hours
OA	Treatment with 20μM OA for 48 hours, then 20μM OA without 0.05μM ara-C for a further 72 hours
OA+	Treatment with 20μM OA for 48 hours, then 20μM OA with 0.05μM ara-C for a further 72 hours

Histograms
x-axis = fluorescence of PI
y-axis = number of cell
C = $G_{1/0}$ phase of the cell cycle
D = S phase of the cell cycle
E = G_2+M phases of the cell cycle

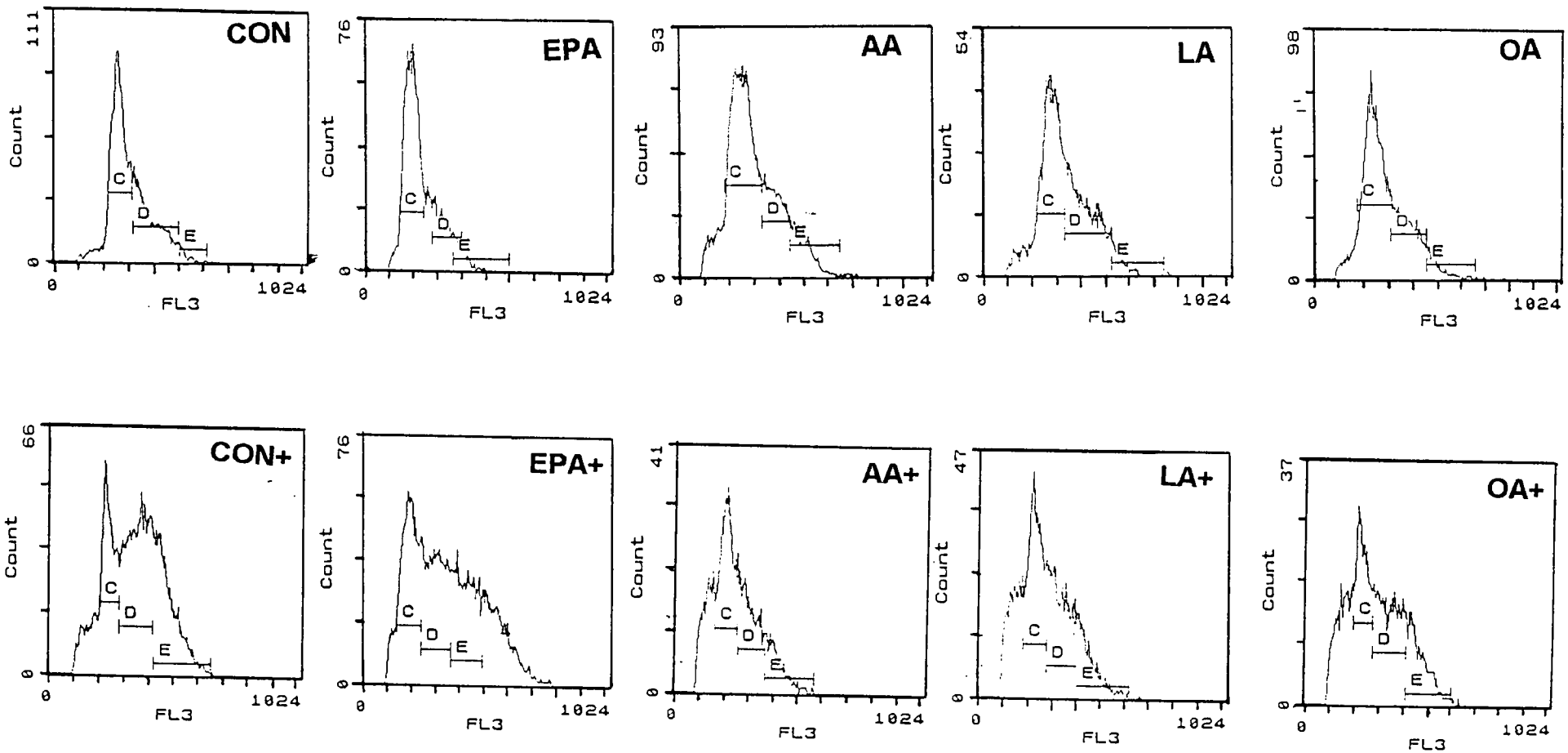

CON
EPA
AA
LA
OA
CON+
EPA+
AA+
LA+
OA+
Count
FL3
0
1024
C
D
E

Table 4.2 Effect of different fatty acids and ara-C on K562 cell cycle profile

K562 cells were incubated in the presence or absence of fatty acid 20μM for 48 hours followed by fatty acid 20μM in the presence or absence of 0.05μM ara-C for three days, as described in 4.2.6. The effect on the cell cycle profile was determined by FACS. This Table shows percentage of cells found in each phase of the cell cycle, this was determined from histogram 3 of each treatment, as shown in Figure 4.9. The percentage of dead cells was determined from the percentage of cells found before $G_{1/0}$.

Fatty acid +/- ara-C	% of cells in $G_{1/0}$ (C)	% of cells in S (D)	% of cells in G_2+M (E)	% of dead cells
CON	57.4	34.7	2.2	5.7
CON+	23.1	41.1	26.4	9.4
EPA	62.5	21.8	6.6	9.1
EPA+	26.8	27.5	24.1	21.6
AA	57.7	22.5	11.2	8.6
AA+	39.6	28.4	12.8	19.2
LA	48.4	39.5	4.6	7.5
LA+	36.3	30.4	13.8	19.5
OA	60.5	28.1	5.4	6.0
OA+	27.6	32.3	17.8	22.3

phases. In both EPA treatments with and without ara-C there was an increase in the percentage of cells in the G_2+M phase of the cell cycle. Cells treated with EPA for 120 hours with ara-C (**EPA+**) for the final 72 hours showed a decrease in the percentage of cells in $G_{1/0}$ compared to cells treated with EPA for 120 hours alone (**EPA**). This was reflected by the 38% reduction in cell number when ara-C was presence in EPA treated cells accompanied by an increase in haemoglobin accumulation. There was a slight increase in the percentage of cells in S phase and an increase in the percentage of cells in G_2+M phase of the cell cycle, there was also an increase in the percentage of dead cells in the EPA 120 hour treatment compared to cells treated with EPA for 120 hours alone (**EPA**). The percentage of dead cells calculated by the DNA analysis, Table 4.2, and the percentage of viable cells as shown in Figure 4.8 do not match. This was due to the different methods used to determine the percentage of dead cells. The trypan blue exclusion method may have underestimated the percentage of dead cells compared PI assosicating with the DNA of the cells.

The addition of the PUFA AA for 120 hours (**AA**) has decreased the percentage of cells in S phase and increased the percentage of cells in G_2+M and dead cells, while the percentage of cells in $G_{1/0}$ remains constant, compared to control cells (**CON**). In Figures 4.7 and 4.8 differentiation was accompanied by the reduction in cell growth and the increase in haemoglobin accumulation. The increase in the percentage of cells in $G_{1/0}$ is surprising but a larger percentage of cells are found in G_2+M compared with control cells. The addition of ara-C for the final 72 hours of the 120 hour AA treatment (**AA+**) caused a decrease in the percentage of cells in $G_{1/0}$ and S phases and an increase in the percentage of cells in G_2+M phase of the cell cycle and dead cells, compared to control cells (**CON**). In the cell cycle profile this was

reflected by the decrease in the percentage of cells in $G_{1/0}$ and the increase in G_2+M. In both AA treatments, with and without ara-C an increase in the percentage of cells in G_2+M phase of the cell cycle occurred, compared to control cells. Cells treated with AA for 120 hours with ara-C for the final 72 hours showed a decrease in the percentage of cells in $G_{1/0}$, a slight increase in the percentage of cells in S and an increase in the proportion of dead cells, the percentage of cells in G_2+M was similar to cells treated with AA for 120 hours. The addition of ara-C for the final 72 hours of the AA 120 hour treatment showed a 29% decrease in cell number accompanied by an increase in haemoglobin accumulation compared to cells treated with AA for 120 hours.

Cells treated with LA for 120 hours showed a decrease in the percentage of cells in $G_{1/0}$ and an increase in S and G_2+M and dead cells, compared to control cells. An increase in cell growth, as shown in Figure 4.8, was not accompanied by an increase in the percentage of cells in $G_{1/0}$ in the cell cycle profile. The accumulation of haemoglobin was however reflected by the increase in the percentage of cells in G_2+M. The addition of ara-C to the LA treatment decreased the percentage of cells in $G_{1/0}$ and S and increased the percentage of cells in G_2+M and dead cells, compared to control cells. The induction of differentiation was accompanied by a reduction in cell number and in the accumulation of haemoglobin, as shown in Figures 4.7 and 4.8, was supported by the cell cycle profile. Cells treated with LA for 120 hours with ara-C for the final 72 hours showed a decrease in the percentage of cells in $G_{1/0}$ and S phases of the cell cycle with an increase in the percentage of cells in G_2+M phase and those dead compared to cells treated with LA for 120 hours. The addition of ara-C for the final 72

hours of the 120 hour LA treatment caused an 88% decrease in cell number accompanied by an increase in haemoglobin accumulation.

The cell cycle profile of cells treated with OA for 120 hours showed an increase in the percentage of cells in $G_{1/0}$ and G_2+M and a decrease in S and dead cells, compared to control cells. This is reflected by the increase in cell number (Figure 4.8) and the increase in haemoglobin accumulation (Figure 4.7). The addition of ara-C for the final 72 hours of the 120 hour OA treatment decreased the percentage of cells in $G_{1/0}$ and S phases and an increase in G_2+M phase and dead cells compared to control cells. This reflects the decrease in cell number as shown in Figure 4.8, and the increase in haemoglobin accumulation shown in Figure 4.7 by this treatment. Cells treated with OA for 120 hours with ara-C for the final 72 hours showed a decrease in the percentage of cells in $G_{1/0}$ of the cell cycle, there was a slight increase in the percentage of cells in S phase and an increase in the percentage of cells in G_2+M and dead cells compared to cells treated with OA for 120 hours. The addition of ara-C for the final 72 hours of the 120 hour treatment resulted in a decrease in cell number by 78%, this was accompanied by an increase in haemoglobin accumulation.

The induction of differentiation by the fatty acids was reflected in the percentage of cells in G_2+M phase of the cells cycle. Although the percentage of cells in G_2+M was high for the EPA with ara-C treatment, it was lower than expected for the AA with ara-C treatment compared to the induction of haemoglobin by this treatment. Cells treated with fatty acid with ara-C show a reduction in the percentage of cells in $G_{1/0}$ and an increase in G_2+M compared to cells treated with the fatty acid alone. Cells treated with ara-C but no fatty acid showed an increase in G_2+M and in S phase. Apart from the LA treatment, the

fatty acid treatment seemed to increase the percentage of cells entering G_2+M.

4.4 Discussion

The work reported in this study focused on the effects of unsaturated fatty acids on differentiation of K562 cells. It has been shown that K562 cells are able to differentiate by exposure to a number of differentiating agents, such as hemin, sodium butyrate and 1-β-D-arabinofuranosylcytosine (ara-C), (Andersson, 1979, Rutherford & Weatherall, 1979; Horton, 1983; Luisi-DeLuca *et al.*, 1984; Chen & Wu, 1994). Ara-C was chosen as the differentiating agent in this study as it induced the cells into terminal differentiation, unlike the other agents which could be removed from the medium and the cells would revert to K562 cells again (Luisi-DeLuca *et al.*, 1984; Chen & Wu, 1994). Chen *et al.* (1994) used a concentration of 3.6μM ara-C to induce differentiation in K562 cells, whereas Luisi-DeLuca *et al.* (1984) determined that 0.5μM was the concentration that produced maximal differentiation in K562 cells.

Ara-C incorporates specifically into DNA and acts as a relative chain terminator which causes DNA fragmentation (Luisi-DeLuca *et al.*, 1984). The extent of ara-C incorporation into DNA correlates with the inhibition of DNA synthesis. Ara-C (0.5μM) was shown to induce differentiation of K562 cells (Luisi-DeLuca *et al.*, 1984) and caused irreversible haemoglobin expression which suggested terminal differentiation. After ara-C inhibition of DNA synthesis certain segments of DNA can be replicated more than once in single cells (Luisi-DeLuca *et al.*, 1984). The accumulation of DNA fragments may alter gene expression leading to differentiation.

Burns *et al.*, (1989) induced HL60 cells to differentiate by supplementing the culture medium with 10μM DHA for 120 hours.

After this time cells were resuspended in fresh culture medium containing DHA and then incubated with a known differentiating agent, retinoic acid (1μM) for up to 5 days. Cells treated with DHA and retinoic acid significantly increased the production of superoxide on days 1 and 2, after this time there was no significant difference (Burns *et al.*, 1989). HL60 cells treated with DHA plus retinoic acid also reduced more NBT at 24 and 48 hours than cells treated with 18:1 plus retinoic acid. In HL60 cells DHA and retinoic acid treatment resulted in the acceleration of differentiation and increased growth arrest was shown in $G_{1/0}$ phase of the cell cycle. Even a short supplementation with DHA for 1 hour produced accelerated differentiation with retinoic acid compared to exposure to 18:1 or no fatty acid treatment (Burns *et al.*, 1989). This short incubation was long enough for the HL60 cells to incorporate the DHA into the plasma membrane. This may have altered the plasma membrane sufficiently to allow the differentiating agent to produce a greater effect.

In the present study K562 cells were treated with fatty acids in the presence or absence of ara-C to ascertain whether induction of differentiation could be accelerated. Differentiated K562 cells were determined by their ability to accumulate haemoglobin. The K562 DNA was also examined using FACS analysis.

Initially K562 cells were grown in a range of ara-C concentrations (0-3.2μM) to determine the effect on cell growth. The effects of ara-C on cell growth was observed every day, over a period of 5 days by trypan blue exclusion. Differences in cell growth were observed after 24 hours. Concentrations of 0.001μM - 0.01μM showed a decrease in cell growth but the cells were still able to replicate over the 5 day period. At a concentration of 0.05μM cells growth was greatly

decreased over the 5 days. Concentrations of 0.1μM - 3.2μM produced cytostatis. The effect of these concentrations on the K562 DNA was determined using FACS, after 5 days. As the concentration of ara-C was increased up to 0.05μM there was an increase in the percentage of cells found in the G_2+M phases of the cells cycle, the percentage of cells found in the $G_{1/0}$ phase and S phase was reduced. At concentrations above 0.1μM ara-C the number of dead cells were greatly increased compared with control cells. The dead cells were shown on the histogram before the $G_{1/0}$ phase of the cell cycle. These dead cells had probably apoptosed as the cells found before C in histogram 3 have fragmented DNA. There was a dose-dependent shift of cells initially from $G_{1/0}$ phase of the cell cycle to S phase then to G_2+M. The shift to S phase is consistent with the suggestion by Major *et al.* (1982) that the ara-C inhibits DNA synthesis. The increase in S phase was probably due to the DNA beginning to fragment with increasing ara-C treatment. With the K562 cells used in this study concentrations of 0.5μM or 3.6μM of ara-C caused a large amount of cell death. If fatty acids were used with the high concentrations of ara-C (3.6μM) used by Luisi-DeLuca *et al.* (1984) and Chen & Wu (1994), the effect of the fatty acid may have been masked by the effect of ara-C. In this study a concentration of 0.05μM ara-C was used in conjunction with fatty acid treatment.

K562 cells supplemented with EPA for 120 hours showed a dose-dependent increase in haemoglobin concentration, this was accompanied by a decrease in cell growth. K562 cells supplemented with EPA for 120 hours plus ara-C for the final 72 hours showed a dose-dependent increase in haemoglobin concentration and this was accompanied by a further decrease in cell growth. The presence of

ara-C increased the effect of EPA alone. The increase in haemoglobin accumulation could have been caused by EPA increasing the membrane fluidity of the K562 cells making ara-C more effective. EPA is a precursor to eicosanoid synthesis. EPA supplementation could have altered eicosanoid synthesis, which may alter ara-C differentiation.

K562 cells were then supplemented with different fatty acids in the presence or absence of ara-C. Cells treated with fatty acid alone for 120 hours accumulated more haemoglobin than control cells and haemoglobin accumulation was in the order EPA > AA > OA ≥ LA. EPA and AA were much more effective at accumulating haemoglobin, and this was accompanied by a reduction in cell number. LA and OA also accumulated haemoglobin, but this was accompanied by a stimulation of cell growth compared to control cells. From these results it appears that the greater the degree of unsaturation in the fatty acid the greater the haemoglobin accumulation. When ara-C was added during the final 72 hours of incubation, haemoglobin accumulation was increased in each condition. Maximum haemoglobin accumulation was achieved by a 120 hour treatment with EPA with ara-C present for the final 72 hours. The order of haemoglobin accumulation in the presence of ara-C was EPA > AA ≥ LA ≥ OA, and this order reflects the unsaturation of the fatty acids. The increase in haemoglobin accumulation was only slight for LA 18:2 n-6 and OA 18:0 n-9 supplemented cells although cell growth was increased. LA stimulation of breast cancer cells growth has previously been shown (Rose & Connolly, 1990). The stimulation in cell growth was linked to an increase in leukotriene biosynthesis. Cells supplemented with either AA 20:4, n-6 or EPA 22:5, n-3 increased haemoglobin accumulation by more than double which was accompanied by a decrease in cell growth.

The cytotoxicity of the n-3 series, EPA and DHA, has been shown in a number of tumours (Mengeaud *et al.*, 1992).

Hawkins *et al.* (1998) showed that PUFA induced apoptosis associated with lipid peroxidation in HL60 cells. The HL60 cells supplemented with 20μM fatty acid (AA, EPA or DHA) for 25 hours induced cell death, as judged by toluidine blue staining and light microscopy and by a crystal violet assay (Hawkins *et al.*, 1998). OA and LA did not alter cell growth, EPA caused 70% cell death, and both DHA or AA produced 85% cell death. Cell death was caused by the induction of apoptosis as EPA (50μM) treatment between 6-12 hours induced a pattern of chromatin cleavage into oligonucleosomes which is characteristic of apoptosis (Hawkins *et al.*, 1998). Furthermore, HL60 cells supplemented with PUFA for 4-5 hours induced lipid peroxidation that was associated with cell kill (Hawkins *et al.*, 1998). The mechanism of peroxidation involves the initial formation of a conjugated diene.

Different PUFAs have also been shown to increase cytotoxicity of a quinone-containing anti-cancer drug doxorubicin which was again related to lipid peroxidation (Germain *et al.*, 1998). PUFA treatment enhanced doxorubicin cytotoxicity in the human breast tumour cell line, MDA-MB-231 (Germain *et al.*, 1998). Cell toxicity of doxorubicin in the presence of subtoxic PUFA concentrations alone or combined with oxidants or antioxidants for 6 days was determined. The order of cytotoxicity was DHA > γ-linolenic acid > EPA > AA > α-linolenic acid > linoleic acid. The effect of PUFA on drug activity was increased with the double bond index with the exception of γ-linolenic acid. Cytotoxicity promoted by doxorubicin with fatty acid was further

increased in the presence of oxidants, while antioxidants abolished the stimulatory action of PUFA on doxorubicin cytotoxicity. Doxorubicin with DHA-induced cell death increased the level of lipid hydroperoxides, suggesting that lipid peroxidation could be involved in increased cytotoxicity (Germain *et al.*, 1998). The antioxidant vitamin E abolished the cell cytotoxicity and lipid hydroperoxide content to baseline levels. This also favours an increase in lipid peroxidation as a possible mechanism of PUFA cytotoxicity.

The DNA of K562 cells treated with the fatty acid in the presence or absence of ara-C was analysed using FACS, to determine the percentage of cells in each phase of the cell cycle. The DNA analysis showed that DNA from fatty acid supplemented cells was very similar to control K562 cells. When ara-C was present with the fatty acid the DNA was similar to K562 cells treated with ara-C alone. K562 cells treated with both fatty acid and ara-C had an increase in the percentage of cells in G_2+M and dead cells. The increase in the percentage of cells found in the G_2+M phase of the cell cycle did not reflect the number of double bonds in the polyunsaturated fatty acids or the sensitivity to PUFA cytotoxicity. The addition of the fatty acids tested had an additive effect on ara-C induced differentiation determined by haemoglobin accumulation.

DNA analysis was able to show the differences in the cell cycle between cells treated with or without ara-C. It would not be able to determine differences in the cell cycle between cells treated with fatty acid and those not. This may result from the different way these two agent produce their effects. Fatty acids are incorporated in to the membrane and have induced haemoglobin accumulation, suggesting the onset of differentiation. Ara-C inhibits DNA synthesis and has been

shown to cause cells to accumulate haemoglobin and to stay in S phase. These two agents effect the cells in different ways and this is also reflected in the DNA analysis.

Future work could include K562 cells treated with fatty acid in the presence or absence of ara-C and determine any effect on the production of lipid peroxide products. The effect of antioxidants on the accumulation of haemoglobin could also indicate if lipid peroxidation was important in differentiation. The effects on eicosanoid synthesis could also be determined. Inhibitors of eicosanoid biosynthesis could be used to determine if there was any alteration in haemoglobin accumulation in K562 cells.

From the results in this study it has been shown that K562 cells can be induced to differentiate along the erythroid lineage by supplementing cells with 20μM PUFA for 120 hours and/or with ara-C for the final 72 hours. The extent of the differentiation seems to be related to the degree of unsaturation of the fatty acid; the higher the degree of unsaturation the higher the induction of differentiation, as shown by haemoglobin accumulation. The degree of differentiation could not be determined from DNA analysis. PUFAs (20μM) were also shown to produce an additive effect on ara-C induction of differentiation. These results suggest that the onset of differentiation may not be due to DNA inhibition alone but may also be modulated by plasma membrane events in K562 cells, as the incorporation of the fatty acids into the membrane produced haemoglobin in the K562 cells, and accelerated the effect of ara-C, a known differentiating agent. This was reflected by DNA analysis. The increase in the percentage of cells in G_2+M phase of the cells cycle compared to the ara-C only treated cells may be related to the acceleration of differentiation in cells treated with

fatty acid and ara-C. The PUFA may affect differentiation by membrane fluidity altering transmembrane signalling pathways, or by altering the production of eicosanoids or by inducing lipid peroxidation, while ara-C inhibits the synthesis of DNA.

Chapter V
General Discussion

Until recently most cancer treatments involved DNA inhibition but now the plasma membrane has become a target for the development of new drugs. The plasma membrane forms the interface between extracellular and intracellular environments and is responsible for the maintenance of ionic gradients, nutrient transport and signal transduction. The structure and function of the cell membrane may be modulated by membrane-active agents such as polyunsaturated fatty acids (PUFAs), synthetic ether lipids (SEL) and local anaesthetics which affect the viability of tumour cells. The aim of this study was to investigate the action and interaction of three membrane-active agents in promoting tumour cell death: polyunsaturated fatty acids (PUFAs), synthetic ether lipid (SEL) and local anaesthetics. Human acute myeloblastic leukaemia (HL60) cells and human myelogenous leukaemia (K562) cells were used *in vitro* as model systems.

PUFAs may influence tumour cells in various ways : (i) they are incorporated into membrane phospholipids and consequently alter membrane fluidity, thereby influencing the activity of membrane-bound proteins including those involved in signal transduction; (ii) n-6 PUFAs (especially arachidonate) are important precursors of eicosanoids and promote metastatic spread in several animal models, while n-3 PUFAs inhibit these processes (Mengeaud *et al.*, 1992); (iii) elevated levels of PUFAs in membranes may lead to enhanced lipid peroxidation, which has been implicated both in tumour promotion (Bull *et al.*, 1988) and cell death (Ip *et al.*, 1991).

SELs, such as ET-18-OCH_3, inhibit proliferation of leukaemic and other tumour cells (Berdel, 1990) and are known to partition into cell membranes (Dietzfelbinger *et al.*, 1992) and to inhibit the phosphoinositide signalling system (Diomede *et al.*, 1990). At low concentrations, SELs inhibit the proliferation of leukaemic and other tumour cells, while normal bone marrow stem cells are affected only at higher concentrations (Honma *et al.*, 1991). This has led to attempts to 'purge' tumour cells from human leukaemic bone marrow samples by treatment with SEL *in vitro* (Berdel *et al.*, 1992).

Local anaesthetics such as dibucaine, tetracaine and procaine also kill tumour cells (Kingston *et al.*, 1993) and partition into the membrane lipid matrix which increases membrane fluidity (Seeman, 1972).

In Chapter 2, HL60 cells and K562 cells were treated with a range of ET-18-OCH_3 concentrations (0-15μM) for 4 hours in serum free culture medium. The results showed that the HL60 cell line was more sensitive to ET-18-OCH_3 than the K562 cell line, with IC_{50} values of 4.11μM and 8.53μM respectively. Cell reproductive ability was determined by incorporation of ^{3}H-thymidine into DNA. HL60 cells had previously been shown to be more sensitive to ET-18-OCH_3 than the K562 cells (Tidwell *et al.*, 1981). The cells were treated in serum free culture medium because ether lipids bind to serum proteins (Kelley *et al*, 1993). In culture medium containing 2% FBS the IC_{50} values for ET-18-OCH_3 were 5.3μg/ml and 21μg/ml in HL60 and K562 cells respectively (Heesbeen *et al.*, 1995). These values are nearer to those obtained in this study. In 1990, Diomede *et al.* showed that the resistance of K562 cells to ET-18-OCH_3 was correlated with a greater amount of cholesterol in membranes compared to that of HL60 cells.

The mechanism of ET-18-OCH_3 cytotoxicity is unknown but it has been shown to affect cells in different ways. HL60 cells treated with 20μM ET-18-OCH_3 have been shown to generate a lipid derived free radical (Wagner *et al.*, 1998). The production of the free radical began approximately 3 minutes after the addition of the ether lipid in the presence of Fe^{2+} and ascorbate. K562 cells treated with 40μM ET-18-OCH_3 did not generate a free radical. Wagner *et al.* (1998) thought that as HL60 cells have more PUFAs in the cell membranes than K562 cells this may make the HL60 cells more susceptible to oxidation after the addition of ET-18-OCH_3. In serum free medium HL60 cells showed evidence of membrane damage at concentrations above 10μM ET-18-OCH_3 (Wagner *et al.*, 1998). This was thought to be related to necrosis or apoptosis. In K562 cells (grown in 10% FCS) treated with ET-18-OCH_3 (50μg/ml) apoptotic nuclei were detectable after 2 hours of treatment (Botzler *et al.*, 1996). A higher concentration of 100μg/ml ET-18-OCH_3 in K562 cells produced an increase in the $G_{1/0}$ phase of the cell cycle and the S and G_2+M phases disappeared completely (Botzler *et al.*, 1996). HT29 cells treated with ET-18-OCH_3 or BM41.440 arrested in the G_1 and G_2 phases of the cell cycle but progression though S and M phases were not affected (Principe *et al.*, 1992). Ether lipids may therefore indirectly affect the passage of cells from the G_1 to S and G_2 to M phases of the cell cycle.

Ether lipids have been shown to alter phospholipid turnover (Diomede *et al.*, 1990). Synthetic ether lipids inhibit inositol (1,4,5)-trisphosphate mediated [Ca^{2+}] signalling (Seewald *et al.*, 1990), which may be as a result of the inhibition of phosphatidylinositol-specific phospholipase C itself (Powis *et al.*, 1992), or by the depletion of phosphatidyl inositol-4-5 bisphosphate as a substrate for PLC, or the increased breakdown of inositol (1,4,5)-trisphosphate. The cytotoxic

effect of ET-18-OCH_3 on HL60 and K562 cells which were pretreated with staurosporine, an inhibitor of PK-C activity, was identical to control cells, suggesting that PK-C activity in HL60 and K562 cells is not related to the cytotoxic action of the ether lipid. (Heesbeen *et al.*, 1994).

Chapter 2 also determined the effect of local anaesthetic on cell growth of both cell lines. The local anaesthetics used in this study were dibucaine, tetracaine and procaine. IC_{50} values for HL60 cells were 0.28mM for dibucaine, 0.68mM for tetracaine and 16.75mM for procaine. IC_{50} values for K562 cells were 0.22mM for dibucaine, 0.64mM for tetracaine and 17.6mM for procaine. Therefore the order of cytotoxicity in both cell lines was dibucaine > tetracaine > procaine. This was the same order of cytotoxicity of the local anaesthetics in SK-N-MC human neuroblastoma cells (Kim *et al.*, 1997). This order also reflects the linear relationship between oil : water partition coefficients (Kingston *et al.*, 1993). From the IC_{50} values it seemed that both cell lines had a similar cytotoxicity to the local anaesthetics, but the HL60 cell line was more sensitive than K562 cells to very low concentrations of dibucaine. Kingston *et al.* (1993) showed that the IC_{50} values of the local anaesthetics in HTC cells reflected the linear relationship between oil : water partition coefficients of the local anaesthetics in HTC cells. This implied that the toxicity of these local anaesthetics may be related to the tendency to partition into the membrane lipid matrix or adsorb onto other hydrophobic cellular sites (Kingston *et al.*, 1993). This may also be true for local anaesthetic cytotoxicity in the HL60 and K562 cells in the present study. In this study dibucaine was more cytotoxic than tetracaine and procaine. This may have been due to the ability of dibucaine to partition into the cell membrane more than tetracaine or procaine. Local anaesthetics have been shown to fluidise the membrane

of HTC cells and SK-N-MC cells (Kingston *et al.*, 1993; Kim *et al*, 1997). At 0.1mM dibucaine was shown to induce apoptosis of, and to increase intracellular calcium levels in, SK-N-MC cells (Kim *et al.*, 1997). At a higher concentration of dibucaine (45μM) the involvement of oxygen free radical in cell death was suggested.

HL60 cells treated with ether lipid in the presence of local anaesthetic showed no difference to the cytotoxicity of cells treated with ether lipid alone. In K562 cells a significant difference was found between cells treated with dibucaine and ether lipid compared to those treated with ether lipid alone. The significant decrease ($p < 0.05$) in ^{3}H-thymidine incorporation was found at 1μM, 3μM, 5μM and 7.5μM ether lipid concentrations in combination with 0.1mM dibucaine in K562 cells. The cytotoxic effect was synergistic at ET-18-OCH_3 concentrations above 3μM and up to 7.5μM. The greatest difference was found at an ether lipid concentration of 5μM, with a 4-fold decrease in ^{3}H-thymidine incorporation in cells treated with ether lipid plus dibucaine compared to ether lipid alone. No differences were observed when tetracaine or procaine was combined with ET-18-OCH_3 in K562 cells.

When K562 cells were treated with dibucaine together with ET-18-OCH_3 the reproductive ability was significantly decreased when compared to K562 cells treated with ether lipid alone. This may be because dibucaine partitions more into the cell membrane than tetracaine and procaine. Kim *et al.* (1997) studied the effect of 0.1mM dibucaine on human neuroblastoma cells (SK-N-MC). This was the same concentration as dibucaine used in this study in combination with ET-18-OCH_3 in K562 cells, which was found to significantly decrease the reproductive ability compared to cells treated with ET-18-OCH_3 alone. The presence of dibucaine may have increased the membrane

fluidity of the K562 cells, allowing ET-18-OCH_3 to affect the cell membrane to a greater extent. Kim *et al.* (1997) found that 0.1mM dibucaine increased membrane fluidity of both inner and outer membranes in SK-N-MC cells. Future work from this study could include fluorescence polarisation of DPH and TMA-DPH at 20°C on K562 cells treated with a range of ether lipid concentrations in the presence or absence of 0.1mM dibucaine. This would determine if the addition of dibucaine increased membrane fluidity in K562 cells treated with ether lipid more than in cells without ether lipid. Dibucaine (0.1mM) also increased the intracellular calcium concentration in SK-N-MC cells (Kim *et al.*, 1997). This increase was probably due to an influx of extracellular calcium as a result of membrane damage. In future work it would be interesting to determine if dibucaine with ET-18-OCH_3 could increase intracellular calcium concentrations. This could be determined using a calcium fluorescence probe. Inhibitors of intracellular calcium and intracellular calcium chelators would show if any increase was from intracellular sources or extracellular sources. Extra cellular calcium could be chelated or depletion prevented to determine if the influx of extracellular calcium affected the degree of cytotoxicity. Again 0.1mM dibucaine has been shown to induce apoptosis in SK-N-MC cells (Kim *et al.*, 1997). ET-18-OCH_3 (50 μg/ml) has also caused apoptosis in K562 cells (Botzler *et al.*, 1996). This concentration would have killed all the K562 cells in the present study. As both of the membrane-active agents used have been related to the induction of apoptosis, future work could investigate this further by the determination of internucleosomal DNA fragmentation. K562 cells treated with ET-18-OCH_3 in the presence or absence of 0.1mM dibucaine would demonstrate if the synergistic cytotoxic effect was due to the induction of apoptosis.

Future work could also include the effect of ET-18-OCH_3 on the arrest of K562 cells in the cell cycle. FACS analysis could also be used to determine the percentage of cells in each phase of the cell cycle when cells are treated with ether lipid in the presence or absence of dibucaine. ET-18-OCH_3 has been shown to arrest cells in G_1 and G_2 phases of the cells cycle (Principe *et al.*, 1992). ET-18-OCH_3 generated a lipid derived free radical in HL60 cells but not in K562 cells (Wagner *et al.*, 1998). Higher concentration of dibucaine (45μM) was thought to involve the production of free radicals, as antioxidants significantly increased SK-N-MC viability. Although the synergistic effect was shown in K562 cells in this study, the study of the generation of free radicals could be an avenue of future work to determine if the combination experiments produced free radicals.

In Chapter 3, fatty acids OA, LA, AA, EPA and DHA were used to supplement both the K562 and HL60 cell lines. These treatments were shown to increase the incorporation of fatty acids into the membrane phospholipids. Burns *et al.* (1989) were able to supplement HL60 cells with 10μM DHA for 5 days. This concentration of DHA would have been very toxic to the HL60 cells used in this study. Burns *et al.* (1989) showed an incorporation of 22:6 in the membrane phospholipids. HL60 cells supplemented with DHA for 1 hour showed a very slight increase in 22:6 membrane phospholipids. A 24 hour incubation with DHA was shown to greatly increase the incorporation of 22:6 in the membrane phospholipids. The incorporation of fatty acids into the membrane phospholipids of HL60 and K562 cells was established in this study. It was found that even when cells were supplemented with very low concentrations of PUFA as used with HL60 cells the fatty acid became incorporated into the cell membrane

phospholipids after 48 hours. The incubation time of 48 hours was chosen as the cells had time to replicate at least once, allowing PUFA to become extensively incorporated into the membrane phospholipids. Both the K562 cells and HL60 cells incorporated the fatty acid into the membrane phospholipids. In the K562 cells AA and EPA (20μM) supplementation resulted in some of the fatty acid being elongated and desaturated. In the HL60 cells EPA (1μM) supplementation was incorporated into the membrane phospholipids and some of the fatty acid was elongated and desaturated.

The order of cytotoxicity for both cell lines was DHA > EPA > AA > LA ≥ OA. The HL60 cell line was found to be more sensitive to the fatty acids than the K562 cell line. When an antioxidant α-tocopherol (vitamin E) was added during fatty acid supplementation some cytotoxicity was lost in K562 cells supplemented with DHA and in HL60 cells supplemented with DHA, EPA or AA. Vitamin E is known to reduce lipid peroxidation and from these results it would seem that especially in HL60 cells the cytotoxicity was partly due to lipid peroxidation. PUFAs have been shown to increase lipid peroxidation. EPA and GLA increased lipid peroxidation in three human colon cancer cell lines (Mengeaud *et al.*, 1992). Hawkins *et al.* (1998) showed that vitamin E considerably reduced the cytotoxic effects of DHA. PUFAs were shown to induce lipid peroxidation of HL60 cells and a human pancreatic cell line in the presence of oxidants (Hawkins *et al.* 1998).

In this study vitamin E reduced the cytotoxic effect of the PUFA on HL60 cells supplemented with DHA, EPA or AA and K562 cells supplemented with DHA. Future work could detect the appearance of lipid peroxidation products in K562 cells supplemented with DHA and

HL60 cells supplemented with DHA, EPA or AA, using the thiobarbituric acid assay. Lipid peroxidation can occur by enzymic methods involving, the synthesis of eicosanoids, or by nonenzymic methods. These two methods can be distinguished by the presence of eicosanoid synthesis. Indomethecin is an inhibitor of cyclooxygenase activity. If the presence of indomethecin reduced the cytotoxic effect and the lipid peroxidation products, this would suggest that the enzymic method and eicosanoid synthesis are involved in lipid peroxidation. However, if indomethecin did not reduce cytotoxicity or lipid peroxidation products then the enzymic method would not be involved in lipid peroxidation. Metals such as iron are known to stimulate lipid peroxidation. If metals stimulate cell death in conjunction with PUFA then this implies that cell death is related to lipid peroxidation.

PUFAs have been shown to increase membrane fluidity. EPA and GLA increase membrane fluidity in human colon cancer cell lines (Mengeaud *et al.*, 1992). Future work could include the treatments used in the present study in order to assess for any increases in membrane fluidity. Membrane fluidity can be assessed by fluorescence-polarisation measurements.

ET-18-OCH_3 and BM41.440 have been shown to increase lipid peroxidation in L1210 murine leukaemia cells supplemented with DHA (Petersen *et al.*, 1992; Wagner *et al.*, 1992). In Chapter 2, the reproductive ability of K562 cells treated with ET-18-OCH_3 plus dibucaine was significantly decreased compared to cells treated with ether lipid alone. Therefore, PUFAs were used in combination experiments with ether lipids or local anaesthetics. EPA was the PUFA chosen for combination experiments as it can be used at the highest

subtoxic concentration and has 5 double bonds. K562 cells were firstly supplemented with EPA for 48 hours followed by a 4 hour incubation with a series of ether lipid concentrations. The IC_{50} value of ET-18-OCH_3 was significantly increased when the K562 cells were supplemented with EPA. This contradicts the work of others. L1210 cells treated with DHA for 48 hours followed by an 8 hours BM41.440 treatment displayed an increase in ether lipid cytotoxicity (Petersen *et al.*, 1992). This cytotoxicity was shown to increase with the number of double bonds in the fatty acid, and cytotoxicity was not reduced by the addition of antioxidants. Petersen *et al.* (1992) suggests that the metabolism of ether lipid may generate free radicals and that membrane fatty acids with increased numbers of double bonds would be more susceptible to secondary damage. In this study EPA had a protective effect on ET-18-OCH_3. This may have been due to different experimental conditions from those of Petersen *et al.* (1992). EPA may have altered eicosanoid biosynthesis in K562 cells, which may have had a protective effect on ET-18-OCH_3. Future work could include the study of the influence of inhibitors of eicosanoid synthesis on this protective effect of EPA on ET-18-OCH_3.

PUFA was also combined with local anaesthetic treatment. Again K562 cells were supplemented with EPA (20μM) for 48 hours, followed by 4 hour dibucaine treatment. Dibucaine was chosen as it was the most cytotoxic local anaesthetic. In this study no difference in ^3H-thymidine was observed between cells supplemented with EPA and control cells. Further study would not be advisable on the strength of these results.

In Chapter 3 PUFAs were shown to be incorporated into the cell membrane phospholipids. In chapter 4, PUFAs were used to determine whether the combination of PUFA with known differentiation agent ara-C would enhance differentiation. The induction of differentiation was monitored by the accumulation of haemoglobin and the decrease in cell growth.

PUFAs have been shown to induce differentiation. HL60 cells were induced to differentiate with retenoic acid (Burns *et al.*, 1989). The presence of 10μM DHA significantly increased differentiation during the first 50 hours, as shown by NBT reduction. After this time the amount of differentiation remained constant with and without the addition of DHA.

In this study the effect of EPA on ara-C differentiation was determined in K562 cells. The accumulation of haemoglobin was used as an assay of differentiation. The presence of EPA for 5 days significantly increased the haemoglobin accumulation, and implied that EPA alone could induce differentiation. EPA (20μM) with ara-C increased the induction of differentiation. As EPA has 5 double bonds other fatty acids were used to determine how varying the number of double bonds would alter the induction of differentiation in the presence or absence of ara-C. K562 cells treated with EPA (20μm) or AA (20μM) for 5 days showed a similar accumulation of haemoglobin to cells treated with ara-C for 72 hours only. When ara-C was added for the final 72 hours of the PUFA 5 day treatment a further increase in haemoglobin accumulation in the order EPA > AA > LA > OA was observed. The addition of LA or OA also increased haemoglobin accumulation above the level observed when using ara-C alone. Cells

treated with fatty acid for 5 days, with ara-C treatment for the final 72 hours, displayed decreases in cell growth.

From this work it has been shown that PUFA, ara-C and PUFA plus ara-C treatments result in haemoglobin accumulation in K562 cells. Luisi-DeLuca *et al.* (1984) found that ara-C (0.5μM) exposures of 48 hours were long enough to demonstrate that ara-C induced reversible differentiation.

Both EPA and AA are precursors of eicosanoids but OA and LA are not. Future work could include supplementing cells with fatty acids with or without ara-C in the presence or absence of an inhibitor of eicosanoid biosynthesis, such as NDGA or indomethecin. If the inhibitors of eicosanoid biosynthesis inhibited the influence of the fatty acids this would suggest that conversion to eicosanoids was an important step in the induction of PUFA differentiation. If no inhibitory effect was determined this may suggest that PUFAs exert their effects via membrane fluidity changes.

OA and LA have both been shown to promote tumour growth (Ip *et al.*, 1985; Rose & Connolly, 1990). OA and LA supplemented cells showed increases in cell growth over the 5 day treatment without ara-C. A decrease in cell number was usually accompanied by increase in haemoglobin accumulation, which implied the onset of differentiation. With LA or OA treatment stimulation of cell growth was also accompanied by haemoglobin accumulation.

PUFA supplementation of cells showed an increase in haemoglobin accumulation. From the DNA analysis PUFA supplementation alone does not appear to cause any great differences from the control cells. K562 cells only accumulate haemoglobin when differentiated, therefore PUFA would seem to be inducing differentiation after a 5 day incubation. Ara-C treatment increased the

accumulation of haemoglobin : the percentage of cells in $G_{1/0}$ was decreased and the percentage of cells in S and G_2+M phase increased. The increase in S phase is consistent with ara-C treatment. Ara-C has been shown to inhibit S-phase DNA replication by causing an accumulation of strand breaks in the replicating DNA (Fram & Kufe, 1982). PUFA supplementation plus ara-C treatment showed a further increase in haemoglobin accumulation compared to cells treated with ara-C alone. The DNA analyses for fatty acid plus ara-C treatment is similar to ara-C treatment alone with a decrease in the percentage of cells in $G_{1/0}$ and an increase in G_2+M but the percentage of cells in S phase is nearer that for the control cells. From the DNA analysis fatty acid supplementation has not greatly altered the percentage of cells in the phases of the cell cycle compared to control cells or control cells plus ara-C. The fatty acids may be causing the increase in haemoglobin by eicosanoid biosynthesis, membrane fluidity or lipid peroxidation. Future work would need to investigate how PUFA supplementation is causing the accumulation of haemoglobin in K562 cells.

The present study has shown that the cytotoxicity of the ether lipid ET-18-OCH_3 was increased by combination with dibucaine in K562 cells. PUFAs were incorporated into cell membranes at concentrations as low as 1μM, with an incubation time of 48 hours. PUFA cytotoxicity was reduced by vitamin E, indicating that cytotoxicity may be partly due to lipid peroxidation, although this was not confirmed. Experiments which combined PUFA with ET-18-OCH_3 showed a decrease in cytotoxicity. The combination of PUFA and local anaesthetics did not enhance cytotoxicity. When K562 cells were incubated with PUFA for 5 days, both haemoglobin accumulation as well as a decrease in cell growth were observed, together with

alterations in cell cycle profile, indicating that PUFA supplementation may induce differentiation. K562 cells supplemented with PUFA for 5 days plus ara-C for the final 72 hours showed the greatest accumulation of haemoglobin, decrease in cell growth, and alteration in cell cycle. Further work is required to determine the mechanisms of the action of the membrane active agents on human leukaemia cells.

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