Polyunsaturated Fatty Acid Requirements of Murine Colon Adenocarcinomas.

by

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Doctor of Philosophy.

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The polyunsaturated fatty acid (PUFA) requirements of three transplantable murine colon adenocarcinomas, the MAC13, MAC16 and MAC26, were evaluated *in vitro* and *in vivo*. When serum concentrations became growth limiting *in vitro*, proliferation of the MAC13 and MAC26 cell lines was stimulated by linoleic acid (LA) at 18μM and arachidonic acid (AA) at 16 or 33μM respectively. This was not demonstrated by the MAC16 cell line. MAC13 and MAC26 cells were found to be biochemically fatty acid deficient as measured by the formation of Mead acid (20:3 n-9), but the MAC16 cells were not. *In vivo* the growth of the MAC26 tumour was stimulated by daily oral administration of LA between 0.4-2.0g/kg. There was a threshold value of 0.4g/kg for the stimulation of MAC26 tumour growth, above which there was no further increase in tumour growth, and below which no increase in tumour growth was observed. This increased tumour growth was due to the stimulation of tumour cell proliferation in all areas of the tumour, with no effect on the cell loss factor.

The growth of the MAC13, MAC16 and MAC26 cell lines *in vitro* were more effectively inhibited by lipoxygenase (LO) inhibitors than the cyclooxygenase inhibitor indomethacin. The specific 5-LO inhibitor Zileuton and the leukotriene D_4 antagonist L-660,711 were less effective inhibitors of MAC cell growth *in vitro* than the less specific LO inhibitors BWA4C, BWB70C and CV6504. Studies of the hyroxyeicosatetraenoic acids (HETEs) produced from exogenous AA in these cells, suggested that a balance of eicosanoids produced from 5-LO, 12-LO and 15-LO pathways was required for cell proliferation.

In vivo BWA4C, BWB70C and CV6504 demonstrated antitumour action against the MAC26 tumour between 20-50mg/kg/day. CV6504 also inhibited the growth of the MAC13 tumour *in vivo* with an optimal effect between 5-10mg/kg/day. The antitumour action against the MAC16 tumour was also accompanied by a reduction in the tumour-induced host body weight loss at 10-25mg/kg/day. The antitumour action of CV6504 in all three tumour models was partially reversed by daily oral administration of 1.0g/kg LA. Studies of the AA metabolism in tumour homogenates suggested that this profound antitumour action, against what are generally chemoresistant tumours, was due to inhibition of eicosanoid production through LO pathways. As a result of these studies, CV6504 has been proposed for stage I./II. clinical trials against pancreatic cancer by the Cancer Research Campaign. This will be the first LO inhibitor entering the clinic as a therapeutic agent.

Keywords: polyunsaturated fatty acids, linoleic acid, arachidonic acid, hydroxyeicosatetraenoic acids, lipoxygenase inhibitors, murine colon adenocarcinoma.

To Nan.

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Abbreviations.

AA	Arachidonic acid (all-cis-5,8,11,14-eicosatetraenoic acid).
aFGF	Acidic fibroblast growth factor.
ALA	α-Linolenic acid (all-cis-9,12,15-octadecatrienoic acid).
ANOVA	Analysis of variance.
AOM	Azoxymethane.
ATP	Adenosine triphosphate.
bFGF	Basic fibroblast growth factor.
BHT	Butylated hydroxytoluene.
BSA	Bovine serum albumin.
c-AMP	Cyclic-adenosine monophosphate
cdk	Cyclin-dependent kinase.
CNS	Central nervous system.
CO	Cyclooxygenase.
CV6504-G	CV6504-Glucuronide.
CV6504-S	CV6504-Sulphate.
DAG	Diacylglycerol (1,2-sn-diacylglycerol).
DHA	Docosahexaenoic acid (all-cis-4,7,10,13,16,19-docosahexaenoic acid).
DMBA	7,12-Dimethylbenzo(a)anthracene.
DMH	Dimethylhydrazine.
DMSO	Dimethyl sulphoxide.
EDTA	Ethylenediaminetetraacetic acid.
EET	Epoxyeicosatrienoic acid.
EFA	Essential fatty acid.
EGF	Epidermal Growth Factor.
EORTC	European Organisation for Research and Treatment of Cancer.
EPA	Eicosapentaenoic acid (all-cis-5,8,11,14,17-eicosapentaenoic acid).
EYTA	Eicosatetraynoic acid (5,8,11,14-eicosatetraynoic acid).
FA	Fatty acid.
FCS	Foetal calf serum.

FLAP	Five lipoxygenase binding protein.
FLM	Fraction labelled mitosis.
GB	Guanidinobenzoatase.
GC	Gas Chromatography.
GLA	γ-Linolenic acid (all-cis-6,9,12-octaecatrienoic acid).
GGTP	γ-Glumtyltransferase.
GR-12	α-Guiconic acid.
HETE	Hydroxyeicosatetraenoic acid.
12(R)HEtrE	12(R)Hydroxy-5,8,11(ZZZ)-eicosatetraenoic acid.
HHT	Hydroxyheptadecatrienoic acid.
HPETE	Hydroperoxyeicosatetraenoic acid.
HPLC	High pressure liquid chromatography.
13(S)HPODE	13-Hydroperoxyoctadecatrienoic acid.
^{3}H-Tdr	[Methyl ^{3}H]thymidine.
IFN	Interferon.
IGF	Insulin growth factor.
IL	Interleukin.
ip	Intraperitoneal.
^{125}I-Udr	[^{125}I]-5'-iodo-2'deoxyuridine.
LA	Linoleic acid (cis,cis-9,12-octadecadienoic acid).
LO	Lipoxygenase.
LT	Leukotriene.
LX	Lipoxin.
MAC	Murine colon adenocarcinoma.
MAPK	Mitogen activated protein kinase.
MDA	Malondialdehyde.
Mead acid	Mead acid (all-cis-5,8,11-eicosatrienoic acid).
MNU	Methyl-nitrosourea.
MSE	Enzyme cleaving 4-methylumbellifeyl-p-guanidinobenzoate.
NCI	National Cancer Institute.

NDGA	Nordihydroguaiaretic acid.
NSAID	Non-steroidal anti-inflammatory drug.
OA	Oleic acid (cis-9-octadecaenoic acid).
ODC	Ornithine decarboxylase.
PA	Phosphatidic acid.
PBS	Phosphate buffered saline.
PD-EFC	Platelet derived endothelial growth factor.
PDGF	Platelet derived growth factor.
PC	Phosphatidyl choline.
PKC	Protein Kinase C.
PL	Phospholipid.
PLA_2	Phospholipase A_2
PLC	Phospholipase C.
PLD	Phospholipase D.
PG	Prostaglandin.
PUFA	Polyunsaturated fatty acid.
PUFA-BP	Polyunsaturated fatty acid binding protein.
SA	Stearic acid (octadecanoic acid).
SMC	Smooth muscle cells.
TAM	Tumour activated macrophages.
TBA	2-Thiobarbituric acid.
TGF	Transforming growth factor.
TD	Actual doubling time.
TLC	Thin layer chromatography.
TNF	Tumour necrosis factor.
TP	Potential doubling time.
TPK	Tyrosine-protein kinase.
VSMC	Vascular smooth muscle cells.
Ø	Cell loss factor.

Chapter 1.
Introduction.

1:0. Introduction.

Cancer, the uncontrolled proliferation and invasion of neoplastic tissue into the surrounding organs, with consequent metastasis to other sites, is the second most common cause of death in the developed world after cardiovascular disease (Murray 1990). A defining characteristic of cancer cells is their ability to divide under conditions where their normal counterparts do not. This unconstrained proliferation can be largely explained by the gain or loss of function in proteins that constitute the cell cycle machinery itself (Clurman and Robert 1995). Cancer cells are inherently disordered, genetically unstable and extremely heterogeneous (Volpe 1988, Fidler and Hart 1982, Sato et al 1991). Tumours though described as organ-like, possess significantly less physiological organisation and function than their organ of origin. As a result tumour characteristics vary largely between individuals and probably within the same host (Gatenby 1995). An altered pattern of metabolism was first observed in cancer cells over 60 years ago (Warburg 1930), where it was found that a preferential use of glycolytic pathways was a fundamental defect in cancer. Though this concept was not supported by the genetic concept of tumourigenesis (Sutherland et al 1988) more recently it was proposed that although metabolic changes are not the fundamental defects that cause cancer, they are the pathways that confer a common advantage on the many tumour types, allowing them to invade and kill the host (Gatenby 1995).

The toxicities associated with conventional cancer chemotherapy arise from the lack of specificity for cancer cells. Most of the currently available chemopreventative drugs are designed to be selectively toxic against rapidly dividing cells (Valeriote and van Puttern 1975). This gives a low therapeutic index, which causes unacceptable damage to normal organs, limiting the drug dose that can be administered (Deonarain and Epenetos 1994).

Historically the National Cancer Institute (NCI) cancer screens have relied heavily on murine leukemias (L1210 and P388) as the index screening tumours (Zubrod et al 1966, Boyd 1989). Therapeutic agents identified through this screen favoured activity against human leukemias and lymphomas (Double and Bibby 1989, Grever et al 1992). The use of these screening systems, together with the increased resistance of solid tumours to cytotoxic agents (Double and Ball 1975, Corbett et al 1984) resulted in the search for an antitumour drug which is clinically responsive against human solid tumours lagging far behind the development of antitumour agents active against leukemias and lymphomas (Double and Bibby 1989). Between 1975 and 1984 the NCI included the additional animal solid tumours and human tumour xenografts in a secondary *in vivo* panel in an effort to enhance the identification of chemotherapeutic agents active against human solid tumours (Boyd 1989), with no impact (Grever et al 1992). In 1985 the NCI screening programme began to use sixty human tumour cell lines derived from seven cancer types (lung, colon, melanoma, renal, ovarian, brain and leukemia) (Boyd 1989, Grever et al 1992). The evaluation of the therapeutic index of a chemotherapeutic agent gives a more realistic representation of the potential of this agent for clinical use, identifying the dose at which antitumour action is achieved, in comparison to the dose at which host toxicity is observed. A requirement for a transplantable mouse tumour model in screening programmes for the development of antitumour agents has been suggested, which would allow the calculation of the therapeutic index (Corbett et al 1987, Double and Bibby 1989).

Most colorectal carcinomas develop from benign adenomas that gradually increase in size, dysplasia and villous (finger like) morphology. The progressive accumulation of genetic alteration of oncogenes (e.g. APC, p53, DCC and ras) governs the transition from normal colon epithelium to adenocarcinomas (Fearson and Vogelstein 1990). Normally tissue homeostasis requires the physiological deletion of cells by activation of apotosis, a genetically determined programme of autonomous cell death (Williams and Smith 1992, Kerr et al 1972). Inhibition of apotosis by the deregulation of certain oncogenes results in clonal expansion (Williams and Smith 1993). Studies of

the mechanism by which genetic changes effect malignant transformation has focused on the deregulation of cell proliferation, but colorectal epithelial homeostasis is dependent not only on the rate of cell production but also on apoptosis (Bedi et al 1995).

An important window for the chemotherapeutic intervention of cancer has been identified as the period during which transition occurs from the hyperproliferative state to the acquisition of the capacity for invasion and metastasis (Kohn and Liotta 1995). In breast cancer the period of transition from the hyperproliferative state of non-invasive disease to metastatic cancer is estimated to average 6 years (Gallager and Martin 1969, Spratt et al 1986). Tumour vascularisation is also required for the growth of tumours beyond microscopic clumps (Folkman 1985). Inhibitors of this neovascularisation are becoming recognised as potential therapeutic agents, especially as the formation of new blood vessels in the adult is usually limited to the reproductive cycle or wound healing (Maione and Sharpe 1990). The rapid advances being made in the molecular pathology of neoplastic cells with the expression of specific proteins, certain oncogenes and inhibition of certain suppresser genes, will provide a basis for the recognition of substantial differences between malignant and normal cells. Furthermore the intracellular signalling pathways that mediate the action of oncogenes and growth factors on proliferation provide additional targets for anticancer drugs (Grever et al 1992). A variety of approaches are under development to improve the effectiveness and specificity of cancer treatment by directing tumour toxic agents such as drugs, radioisotopes, protein cytotoxins, cells of the immune system or enzymes, directly to the tumour by the use of monoclonal antibodies (Deonarain and Epenetos 1994).

1:1. Prevention of Mortality from Colorectal Cancer.

Incidence of colorectal cancer is rated as the fourth commonest cancer worldwide, though the disease is not uniformly fatal (Parkin et al 1993). The large increase in survival observed between treatment during the early stages of disease with treatment at the latter stages of disease, shows the importance of diagnosing these cancers early in their development (Boyle 1995). The simplest form of advice

offered was that any unexpected change in bowel habit or blood in the stool should be investigated (Boyle 1995). The natural history and the role of several risk factors involved in the aetiology of colorectal cancers are becoming more clearly understood (Fearson and Vogelstein 1990), along with genetic information that leads to an increased susceptibility of colorectal cancer (Bodmer et al 1987, Hall et al 1994) and the genetic events associated with the progression of colorectal cancer (Bishop and Thomas 1990, Bishop and Hall 1994). An increased risk of colorectal cancer is also associated with certain lifestyles, with an increased incidence associated with diets high in fat, protein or meat, and a reduced risk in diets with high fruit or vegetable consumption (Potter et al 1993). It was suggested that the increased risk due to these aetiological factors was due to alterations to serum triglycerides and/or plasma glucose (McKeown-Eyssen 1994).

Chemoprevention received a recent major boost when it was shown that supplementation of the diet over a 5 year period with the antioxidants vitamin E, β-carotene or selenium reduced both incidence and mortality of stomach related cancers (Blot et al 1993). Vegetables and fruits contain antioxidants which is consistent with the ability to provide a protective role against colorectal cancer (Boyle 1995). Non-steroidal anti-inflammatory drugs (NSAIDs) have also been shown to have protective roles against colorectal cancers or adenomatous polyps by preventing the progression of familial polyps with the regression of adenomas (Boyle 1995). In laboratory rodents piroxicam, sulindac and aspirin have all been shown to reduce the frequency of colorectal neoplasms (Skinner et al 1991), though the mechanism by which this effect is achieved along with the dose required remains obscure . In the United States a randomised trial using aspirin showed no chemopreventative effect, although the dose was considered to be too low and the intervention period too short (Gann et al 1993). There was still a strong case for a case for a controlled trial of NSAIDs, probably using aspirin in the prevention of colorectal cancer (Farmer et al 1993).

1:2. Tumour Angiogenesis and Metastasis.

Unrestrained growth does not itself result in tumour invasion and metastasis. In addition to loss of growth regulation, an imbalance in the regulation of motility or

proteolysis appears to be required (Liotta et al 1991) along with neovascularisation of the tumour by angiogenesis (Folkman 1990).

Angiogenesis, the development of new capillaries from host blood vessels, is required to provide nutrients for and remove waste products from the interior of the tumour, as well as for tumour growth and development (Folkman 1985). Avascular tumours, with few exceptions, rarely grow beyond 1-2mm^3. The aggressiveness of well vascularised tumours is not reflected in the extensively vascularised but benign adrenal adenocarcinomas, and tumour populations growing as a thin sheet of cells on meninges or within nerve sheaths have also been shown to escape neovascularisation (Folkman 1990). The growth of tumours transplanted subcutaneously into mice was slow whilst avascular, but after neovascularisation was rapid and approached exponential growth (Algire et al 1945). Tumours grown in perfused organs where blood vessels could not form were limited to 1-2mm^3, but when transplanted into mice after neovascularisation growth was rapid and tumour volume reached 1-2cm^3, (Folkman et al 1966). Tumour neovascularisation occurs in parallel with the transition of the tumour from a hyperproliferative state to an invasive tumour capable of host invasion. The new vasculature provides a route by which tumour cells can invade the circulatory and lymphatic system and arrest in distant organs or tissues (Melamed 1992, Liotta et al 1974, Folkman 1971). Markers of angiogenesis are used to predict both the metastatic potential of tumours and relevant course of treatment for cancer patients. These studies use endothelial cell-specific markers to visualise the density of microvessels (Weinstat-Saslow and Steeg 1994). High capillary counts predict nodal metastasis and reduced survival in breast cancer patients, independently of other variables including tumour size, grade, oestrogen receptor, c-erb-B expression, patient age and ploidy (Weidner et al 1991, Horak et al 1992, Bosari et al 1992). Metastasis in melanoma is associated with vascularisation and poor prognosis (Srivastava et al 1988). Preliminary studies have also shown a correlation between microvessel density, metastasis and decreased survival in colon or prostate cancer patients (Wakui et al 1992, Lapertosa et al 1989).

The process of angiogenesis is complex consisting of the follow events: 1. dissolution of the basement membrane, usually a postcapillary venule, 2. migration of endothelial cells to the tumour, 3. proliferation of the endothelial cells at the tailing edge of migration, 4. canalisation, branching and formation of vascular loops and 5. formation of new basement membrane (Ausprunk and Folkman 1977). Other gross changes in vasculature architecture associated with tumour progression can include progressive arteriolisation in which smooth muscle cells proliferate and encase the developing artery. Some of the known angiogenic factors stimulate endothelial cell proliferation, whereas others activate local inflammatory cells to induce angiogenesis (Folkman and Hanahan 1992). The switch from the vascular to the avascular phase may involve the production and/or secretion of angiogenic factors, their release from sequestration in the basement membrane or their conversion from an inactive to an active state (Folkman and Hanahan 1992). A decrease in angiogenesis inhibitors such as thrombospondin may occur when the tumour undergoes malignant transformation and becomes angiogenic (Zajchowski et al 1990). The organisation of the microvasculature in tumours is not as strict as in normal tissues. Tumour vasculature also shows phenotypic changes such as dilated and irregular vessels, a high proliferation rate of endothelial cells and increased permeability (Jain 1985, Heuser and Miller 1986).

Angiogenesis is thought to be initiated by diffusible angiogenic factors either after local activation of genes encoding them or by release from their storage sites. An angiogenic factor is called 'direct' when it is capable of inducing endothelial cell proliferation or migration *in vivo* and stimulating endothelial cell proliferation *in vitro* (Schwartz et al 1982). Direct angiogenic factors include: basic fibroblast growth factor (bFGF) (Folkman and Klagsbrun 1987), acidic fibroblast growth factor (aFGF) (Folkman and Klagsbrun 1987), vascular endothelial growth factor (VEGF) (Leung et al 1989) and platelet derived endothelial growth factor (PD-EGF) (Leung et al 1989). When the *in vitro* action fails an angiogenic factor is considered to be 'indirect'. These factors include: epidermal growth factor (EGF), transforming growth factors alpha or beta (TGF-α or TGF-ß) (Schreiber et al 1986), tumour

necrosis factor alpha (TNF-α) (Fraser-Schroder et al 1987), platelet derived growth factor (PDGF) (Sato et al 1993) series E prostaglandins (Sato et al 1993) and hydroxyeicosatetraenoic acids (Masferer et al 1991).

Rapid endothelial cell division and angiogenesis within tumours, offers a potential therapeutic target, especially as endothelial cells do not possess the multi-resistance gene (Maione and Sharpe 1990). 2-methoxy-oestradiol, an endogenous oestrogen metabolite of previously unknown function, inhibited endothelial cell migration *in vitro.* The compound also prevented neovascularisation of sarcoma A and B16 melanoma tumours in murine models *in vivo* resulting in tumour inhibition (Fotis et al 1994). Fumagilin a product of Aspergillus fumigatus was found to have antiangiogenic properties, but also displayed considerable toxicity. An analogue of fumagilin, TNP-470, was found to have increased antiangiogenic activity but a reduced toxicity (Kusaka et al 1991). Antitumour activity was demonstrated both *in vitro* and *in vivo* on human tumour cell lines (Yanase et al 1993). Osteosarcoma produces highly malignant bone tumours that effects adolescents and young adults. Advances in the therapy of osteosarcoma have greatly improved prognosis and does not involve the amputation of the affected limbs. Approximately 40-50% of osteosarcoma patients develop pulmonary metastasis, which is the major reason for fatality, with a 5 year survival rate remaining at 20-40% (Snyder et al 1991). The murine osteosarcoma LM8, preferentially metastasises to the lungs in C3H mice. Treatment with TNP-470 suppressed pulmonary metastasis in this model (Mori et al 1995). Kaposi's sarcoma is an AIDS related cancer in which the suppression of endothelial cell hyperplasia should significantly reduce the tissue damage associated with lesions (Maione and Sharpe 1990). TNP-470 has been proposed for administration to patients with Kaposi's sarcoma, but careful monitoring especially for neurological toxicity and evidence of bleeding are required as at high concentrations TNP-470 was reported to show neurological toxicity and cause haemorrhages in the lung, heart and brain (Grever et al 1992).

Stimulated endothelial cells can produce degradative proteinases and invade the extracellular matrix in the same manner as tumour cells, with model systems

demonstrating that a finely tuned proteinase/proteinase inhibitor balance regulates vascular morphogenesis as well as tumour invasion (Herron et al 1986, Monteagudo et al 1990). Migrating endothelial cells produced type IV collagenase and other members of the matrix metalloproteinase family as well as serine proteinases. Specific inhibitors of type IV collagenase, general metalloproteinase inhibitors, and serine proteinase inhibition block migration of endothelial cells (Mignatti et al 1989, Moses et al 1990). These inhibitors blocked tumour cell invasion in the same assay (Mignatti et al 1986, Thorgeirsson et al 1982, Thorgeirsson et al 1984).

Tumour metastasis, the spread of tumour cells from a primary tumour to colonise other sites of the body, is a major cause of mortality in cancer patients (Weinstat-Saslow and Steeg 1994). To create a successful metastatic deposit, a tumour cell or group of tumour cells, must leave the primary tumour, invade the local host tissue, enter the circulation, arrest at the distant vascular bed, extravate into the target organ insterstitium and parenchyma, then proliferate as a secondary colony. During all these processes tumour cells must avoid immune recognition and lysis (Liotta et al 1991). Circulating tumour cells can be detected in cancer patients who never succumb to metastasis (Melamed 1992). Less than 0.05% of circulating tumour cells contain the necessary genotypic and phenotypic information to enter the host venous and lymphatic drainage (Nicholson 1991, Liotta et al 1981). Expression of a malignant phenotype is probably not caused by one single gene or protein. Instead, invasion results from an imbalance and deregulation of what are normally stimulatory or inhibitory events (Kohn and Liotta 1995).

At the biochemical level the method of invasion used by tumour cells may parallel the mechanism of invasion used by nonmalignant cells, which transverse membranes under normal physiological conditions (Liotta et al 1991). In contrast to malignant invasion, physiological invasion is tightly controlled and ceases when the stimulus is removed (Wu and Goldberg 1993). A major goal is to understand what signals and signal transduction pathways are perpetually activated or restrained in malignant invasion compared to physiological invasion (Kohn and Liotta 1995).

When a tumour cell invades a three dimensional structure such as a basement membrane, protrusion of cylindrical pseudopodia is the first step prior to the translocation of the whole cell body (Condeelis 1993, Strossel 1993). A series of proteins on the surface of the pseudopodia co-ordinate sensing, protrusion, burrowing and traction. Proteins at the pseudopod tip may locally disrupt the extracellular matrix and permit forward extension. The balance must switch from proteolysis to adhesion for the advancing pseudopod to grip the matrix and pull the cell forward. Consequently to achieve this the invading tumour cell couples proteolysis with co-ordinated and temporally limited attachment or detachment (Liotta et al 1991, Darmsky and Werb 1992). A positive correlation between tumour aggressiveness and protease levels has been observed in all four classes of proteases: serine, aspartyl, cysteinyl and metal atom dependent (Liotta et al 1991, Mignatti et al 1986, Nakajima et al 1990). All these classes of enzyme may be of equal importance at one or more stages of invasion. Recent progress in the field of metalloproteinases serves as an illustrative example of how specific inhibitors can be used to simultaneously test the protease hypothesis and explore therapeutic strategies (Kohn and Liotta 1995).

1:3. Cancer Cachexia

Cachexia is a major cause of mortality amongst cancer patients, though actual recorded deaths range from 10% (Inagaki et al 1974) to 67% (Harnet et al 1952). This difficulty in assessing that the cause of death was due to cachexia can be appreciated when two definitions of cachexia are considered:

'a systemic derangement of host metabolism which results in progressive wasting' (Lindsey 1986),

and

'the sum of those effects produced by neoplasms in the host which are not the immediate result of mechanical interference with recognisable structures (Costa 1963).

Cachexia is derived from the Greek 'kako hexis' meaning bad condition .

The predominant feature of cachexia is the involuntary progressive weight loss associated with muscle wasting and the extensive depletion of adipose tissue (Mays 1969). Weight loss is commonly accompanied by anorexia and nausea (Shamberger 1984), with other clinical manifestations including weakness, muscle atrophy, easy fatigue, impaired immune function, decrease in motor and mental skills, decline in attention span and concentration ability (Lindsey 1986). Electrolyte and water abnormalities (Costa 1963), anaemia, elevated metabolic basal rate, malabsorption and diarrhoea (Shamberger 1984), have also been implicated. The onset of weight loss appears to be independent of tumour size, and in many cases is the sole presenting factor causing patients to seek medical advice, long before detection of a cancerous growth. Extensive weight loss has been observed when tumour mass amounts to less than 0.01% of body weight (just a few grams) (Lindsey 1986). Breast cancer is usually detected early and has a low frequency of weight loss, whereas pancreatic and gastric cancer are associated with a high frequency of weight loss. The presence of gastric and pancreatic tumours often remains undetected until the appearance of secondary symptoms, such as weight loss (DeWys 1986). Weight loss as low as 5-6% of whole body weight has been shown to be significantly deleterious to survival, accompanied by a reduced response to chemotherapy (DeWys 1986, Heber and Tchekmedyian 1992). The perception of this wasting by the patient, leads to further manifestations of cachexia, with the realisation of their terminal state resulting in apathy and loss of enthusiasm for life (Lindsey 1986).

Anorexia caused by a reduction in food intake, is commonly associated with cachexia (Schein et al 1975, Knox 1983). Cancer is often associated with altered taste perception leading to aversions for certain foodstuffs such as meat (DeWys 1978, Schein et al 1975). It was suggested that these changes in taste perception and anorexia is induced by the action of low molecular weight peptide-like substances produced by the tumour, acting directly on the hypothalamus and central nervous system (CNS) (Theologides 1976b). The syndrome induced by cachexia can not be explained by simple anorexia, as weight loss experienced in cancer cachexia is more rapid than that observed due to simple starvation (Brennan 1977).

Tumours not only disturb homeostasis in the host by sequestering nutrients and energy, but may also release compounds into the circulation which amplify any metabolic disturbances induced by these requirements (Argiles and Azcon-Bieto 1988). Hypoglycaemia has been observed in cancer patients and a transplantable murine colon adenocarcinoma inducing cachexia in NMRI mice (MAC16) (McDevitt and Tisdale 1992). Hyperglycaemia has also been observed in other cancer patients. A decreased in glucose tolerance could be either due to a decreased affinity of insulin for its receptor or a decreased insulin secretion (Holrodye and Reichard 1981). Secretion of insulin or insulin growth factors (IGF-I or IGF-II) by the tumour would also lower the circulatory level of glucose (Kahn 1980).

Glycolysis is the dominant pathway of glucose metabolism in tumour cells (Warburg 1930). This leads to an elevated pyruvate level within the tumour cell, which is metabolised to lactate by the action of lactate dehydrogenase (Argiles and Azcon-Bieto 1988). The Cori cycle, producing glucose from lactate, was elevated in the liver of cancer patients with weight loss (Holroyde et al 1975). This process is very energy expensive for the host. Gluconeogensis by the Cori cycle produces a 6-ATP molecule deficit in the host, for every molecule of glucose produced. 8-ATP molecules are required for the conversion of lactate to glucose in the host liver, whilst 2-ATP molecules are produced from the conversion of glucose to lactate in the tumour. The glucose consumption of two transplantable murine tumours, the cachexia-inducing MAC16 tumour and the noncachectic MAC13 tumour was investigated. There was no significant difference in glucose consumption between these tumours, suggesting that glucose consumption, and in particular the requirement of the Cori cycle for gluconeogensis, is not sufficient to explain the wasting observed as a result of cachexia (Mulligan and Tisdale 1991a).

Tumours were described as 'nitrogen traps' with a pronounced avidity for amino acids (Mider 1951). The net host protein metabolism, whether anabolism or catabolism, is dependent on the rate of protein synthesis and protein degradation in both the tumour and peripheral tissues (Hagmuller et al 1995). Studies of protein metabolism in human tumour cells were limited to the fractional synthetic rate

calculated by the quantity of tracer amino acids taken up by biopsy samples (Heys et al 1991, Shaw et al 1991). Whereas there have been many indepth studies of protein metabolism in peripheral tissues, knowledge of uptake and release of amino acids or their metabolites in human colon carcinoma is rudimentary and often conflicting (Hagmuller et al 1995). Human colon tumours take up branched-chain amino acids to a significantly higher degree than healthy or peripheral tissue (Hagmuller et al 1995). Intraoperative tumour leucine/protein metabolism was studied in 15 patients with resectible malignant colonic tumour, both within the tumour and the peripheral tissues . The designation of the tumour as a 'nitrogen trap' was justified by the net protein catabolism demonstrated in peripheral tissues, coupled with the avid uptake of amino acids by the tumour (Hagmuller et al 1995). The relevance of protein degradation to overall aetiology of the tumour remains unknown. The increased requirement for certain amino acids such as leucine (Lazo 1981) and glutamine (Kallinowski et al 1987), produces an amino acid imbalance in the host. This leads to a decrease in protein synthesis as a full complement of amino acids is required for this process (Stein 1978).

Muscle wasting associated with cancer cachexia is an important complication in the management of patients as deletion of cardiac and respiratory muscle will lead to death (Jeevanandam et al 1984). The MAC16 cachexia inducing tumour in NMRI mice showed increased protein degradation as weight loss increased (Beck et al 1991). Serum from these mice increased protein degradation in isolated gastrocnemius muscle, as measured by tyrosine release (Smith and Tisdale 1993). Maximal stimulation of protein degradation was observed at 10-11% weight loss, and was specific to the cachectic state, with no increase in protein synthesis being observed with serum from non tumour-bearing mice or MAC13 tumour-bearing NMRI mice (Smith and Tisdale 1993). This increased protein degradation was found to be associated with an elevated level of PGE_2 in the gastrocnemius muscle. EPA or indomethacin prevented this increased protein degradation, by a circulating proteolysis-inducing factor, and correlated with the prevention of elevated PGE_2 in gastrocnemius muscle (Beck et al 1991). The surface of the MAC16 tumour carried a

guanidinobenzoatase (GB). The serum from MAC16 and MAC13 tumour-bearing NMRI mice contained the proteolytic enzyme MSE, which cleaved the trypsin inhibitor 4-methylumbellifeyl-p-guanidinobenzoate as a true substrate (Smith et al 1993). EPA has an antitumour and anticachectic action on the MAC16 tumour in NMRI mice (Beck et al 1991). PUFAs were found to inhibit MSE at μM concentrations, whilst EPA was found to noncompetitively inhibit both GB and MSE. This effect was specific as other proteolytic enzymes, trypsin, esterase and plasminogen were unaffected by the concentrations of EPA inhibiting MSE, suggesting that GB was likely to be significant in tumour development (Smith et al 1993).

Ubiquitin a 8.6kDa peptide, is involved in the targeting of proteins undergoing cytosolic ATP-dependent proteolysis. In the cell, ubiquitin can be found free or conjugated to other cellular proteins. Proteins with multiple ubiquitins are targeted for degradation by an ATP-dependent protease (Hershko et al 1980). Growth of hepatoma ascites AH-130 in rats elicits an early and conspicuous loss of body weight and skeletal muscle tissue (Tessitore et al 1987), as well as severe perturbation in hormonal homeostasis, elevation of plasma PGE_2 and the presence of circulating TNF (Costelli et al 1993). Recent results using this tumour model have suggested that ubiquitin-dependent proteolytic pathways underlies muscle wastage associated with cancer cachexia (Llovera et al 1995).

Cachexia is characterised not only by muscle wastage but a massive depletion of adipose tissue in response to tumour growth (Mays 1969). This loss of adipose tissue, mainly through neutral lipids, could not be reversed by administration of a high fat diet, suggesting that depletion of adipose tissue was not due to under nutrition but involved disturbances in lipid metabolism homeostasis (Ludholm et al 1981). In Walker 256 tumour-bearing rats hyperlipidaemia correlated with the breakdown of adipose tissue (Frederick and Begg 1956) though no correlation in circulating FA composition and tumour growth was observed. In contrast hypotriglyceridaemia was observed in MAC16 tumour-bearing mice with increasing weight loss (Briddon et al 1991). The importance of hyperlipidaemia in cancer cachexia remains unknown, and has not been found to be a frequent occurrence in cachectic patients (Ludholm et al

1981). Disturbances in fat storage have been observed in a number of tumour models *in vivo* (Kannan et al 1980, Thompson et al 1981) and human cancer patients (Jeevenandam et al 1984) by decreased lipogenesis. Reduction of lipogenesis can be partially explained by the reduction of lipoprotein lipase activity which has been observed in adipose tissue in conjunction with the growth of large tumours. Fatty acids will also be preferentially taken up by these tumours (Thompson et al 1981). Lipogenesis was found to be elevated in liver, kidney and adipose tissue of NMRI mice bearing the cachectic MAC16 tumour and the noncachectic MAC13 tumour, in comparison to non tumour-bearing controls (Mulligan and Tisdale 1991b). In MAC16 tumour-bearing mice further studies showed an elevation of lipoprotein lipase activity until 15% loss of body weight, after which their was a gradual decline in activity to that below non tumour-bearing animals. This peak lipoprotein lipase action was found to coincide with a transient peak in plasma free FAs concentrations (Briddon et al 1991).

It is not clear whether an exogenous supply of FAs is an essential requirement for tumour growth or not. In Ehrilch ascites tumours at least 40-50% of FA uptake by the ascites fluid was directly from host FA supplies, yet when the cells were grown in culture there was no requirement for FA supplementation (Spectre 1967, Spectre and Steinberg 1967). There has also been considerable speculation as to their requirement as an energy source. Mobilisation of FAs from adipose tissue was observed in response to the growth of thymic lymphoma. These FAs were transolcated to other tissues where no increase in oxidation was observed over pair fed controls, but when these pair fed controls were starved FA mobilisation resulted in an increased in FA oxidation (Kitada et al 1980). In the MAC13 tumour in NMRI mice oxidation of palmitate and trolein was decreased in comparison to non tumour-bearing controls. In MAC16 tumour-bearing mice oxidation was increased suggesting that FAs are fuels for the MAC16 cachexia-inducing tumour (Mulligan 1991). An inverse relationship between FA oxidation and glycolysis was observed in Morris hepatoma tumours. In slow growing relatively well differentiated tumours FA oxidation was compatible with that in the liver, whereas in more rapidly growing less

differentiated tumours FA oxidation was low but glycolysis was increased (Bloch-Frankenthal et al 1965). Pyruvate dehydrogenase is a key enzyme in the conversion of pyruvate to acetyl CoA. Oxidation of FAs increases the concentration of acetyl CoA inhibiting pyruvate dehydrogenase, and preventing energy production at the expenses of carbohydrate and protein precursors. Tissues with a low capacity or incapability to oxidise FA have a high pyruvate dehydrogenase activity resulting in the breakdown of glycogen and protein (Fields et al 1982). It was hypothesised that in certain tumours such a hepatoma 777, there was no FA oxidation and the resulting breakdown of carbohydrate and protein stores resulted in tumour induced weight loss (Fields et al 1982).

Tumour necrosis factor (TNF) or cachectin was produced by genetically engineered CHO/TNF 20 cells. These cells were injected intramuscularly into nude mice, where weight loss was observed with a tumour burden as little as 5.0% of body weight (Oliff et al 1987). TNF injected into non tumour-bearing mice induced weight loss which correlated with a decrease in food and water intake through the induction of anorexia (Mahony and Tisdale 1988, Mahony et al 1988). Anorexia has not been observed in weight loss induced by MAC16 tumours in NMRI mice (Beck and Tisdale 1987, Mulligan et al 1992) or C-261VX tumours in CDF1 mice (Strassman et al 1992). No reversal of weight loss was observed when MAC16 tumour-bearing mice were treated with anti-TNF antibodies (Mulligan et al 1992). TNF has been isolated in the plasma of some but not all cachectic cancer patients and it was concluded that although TNF was present it was not an absolute requirement for the development of cachexia (Fearon et al 1991, Oliff 1988).

Interleukin-6 (IL-6) was found to be elevated in the serum of tumour-bearing mice in comparison to non tumour-bearing mice (Strassman et al 1992). Circulating levels of IL-6 were also elevated in cachectic cancer patients in comparison to weight stable controls (Fearon et al 1991). Recently the role of IL-6 and TNF-α has been studied in colon 26 murine adenocarcinoma. In the noncachectic clone 5 no IL-6 was detected, but mRNA TNF-α was detected at tumour sites and in the spleens of tumour-bearing animals. In contrast, IL-6 mRNA was detected in cachectic clone 20, but not

TNF-α mRNA. In this cachectic model anti-IL-6 antibodies partially reversed the weight loss induced, suggesting that a role for IL-6 in cachexia, but not sufficient for the induction of cachexia (Yasumoto et al 1995). No significant differences were observed in IL-6 levels between the MAC16 cachectic inducing and the related noncachectic MAC13 tumour in NMRI mice (Mulligan et al 1992).

The proinflammatory cytokines TNF-α, IL-1 and IL-6 were implicated as potential mediators of metabolic changes associated with cancer cachexia (Fearon 1992, Yoneda et al 1991, Strassman et al 1992). Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) and cyclooxygenase inhibitor, was shown to inhibit the end organ effects of proinflammmatory cytokines (Dinariello and Wolf 1982, Durum et al 1985). Recently an attenuation of both acute-phase response and accelerated whole body protein flux following administration of ibuprofen (1200mg per day) to cachectic patients was observed (Preston et al 1995). However, it remains to be seen whether long-term administration of ibuprofen might alter the course of cachexia in patients with malignant disease.

It was proposed that the metabolic perturbations induced during cancer cachexia are due to the production of low molecular weight compounds capable of regulating the activity of certain enzymes (Theologides 1972b). A lipid mobilising factor was isolated in MAC16 tumour homogenates which induces cachexia in NMRI mice (Beck and Tisdale 1987). When this material was semi-purified and injected into animals-bearing the noncachectic MAC13 tumour, weight loss was observed without a reduction in food or water intake, suggesting that this factor was responsible for the induction cachexia (Beck and Tisdale 1991).

Alteration in serum and urinary lipolytic activity was investigated in cachectic patients with a total loss of body weight ranging upto 50%. A control group of patients with Alzheimer's disease, in which dramatic weight loss was observed with no obvious cause, was used. Lipolytic activity was increased in the serum of cancer patients with weight loss in comparison to both Alzheimer's patients and healthy controls (Groundwater et al 1990). The same relationship was observed between lipolytic activity and weight loss in urine (Groundwater et al 1990). In the serum of

MAC16 tumour-bearing NMRI mice lipolytic activity peaked at 16% weight loss, whereas in cancer patients there was a linear relationship between lipolytic activity and weight loss in both serum and urine until a 20% weight loss (Groundwater et al 1990). Patients who responded to chemotherapy showed a decrease in serum lipolytic activity, whereas patients who were unresponsive showed no change (Beck et al 1990).

Further purification of the lipid-mobilising factor from MAC16 murine tumours using a combination of ion exchange (Mono Q), exclusion and hydrophobic (C_8) chromatography, yielded an active material with an apparent molecular weight (M_r) of 24,000 (McDevitt et al 1995). After transplantation of the MAC16 tumour there is often a delay before tumour induced weight loss is observed. Serum from these animals contained an antibody that was not present in MAC13 tumour-bearing animals, and recognised a band at 24,000 on Western blots (McDevitt et al 1995). These results suggested that this antibody was directed towards the lipid-mobilising factor isolated rather than the tumour itself. Urine from patients with cancer cachexia also contained a lipid mobilising factor with M_r 24,000, which was not present in healthy individuals (McDevitt et al 1995). Serum from MAC16 tumour-bearing mice detected a band in human cachectic patients at M_r 24,000, suggesting that lipid mobilising factors have a high degree of homology and raises the possibility that cachexia in humans may be caused by the same species as in the mouse (McDevitt et al 1995).

1:3:1. The Potential Role for Polyunsaturated Fatty Acids (PUFAs) in Tumour Induced Cachexia.

The effect of EPA and GLA on the growth of the MAC16 tumour and the weight loss induced by this tumour was studied (Beck et al 1991). EPA effectively inhibited both host weight loss and tumour growth in a dose dependent manner between 1.25 and 2.25 g/kg. Host body weight was maintained with a delayed progression of tumour growth to double the survival of EPA-treated animals using the criteria defined by the UK Co-ordinating committee for the welfare of animals with neoplasms (Beck et al 1991). When tumour growth resumed weight loss was not observed. Animals bearing

the MAC16 tumour have an increased protein degradation of skeletal muscle in comparison to non tumour-bearing controls. Treatment with EPA reduced protein degradation with no effect on protein synthesis in these muscles (Beck et al 1991). The effect of GLA on both host body weight and tumour growth was less pronounced than that of EPA, with an effect only being seen at 5g/kg, at which toxicity was also observed (Beck et al 1991). *In vitro* studies showed that whilst EPA was effective in inhibiting tumour induced lipolysis, GLA was ineffective in this respect (Beck et al 1991).

Hormone stimulation of lipolysis in adipocytes is thought to be mediated through the intracellular accumulation of c-AMP by the stimulation of adipocyte adenylate cyclase (Fain 1983). The binding of c-AMP to c-AMP dependent protein kinases activates the phosphorylation and activation of triglyceride lipase, which catalyses the hydrolysis of triglycerides to free FAs and glycerol. This *in vivo* situation can be mimicked by isolated hepatocytes, and lipolysis measured by the production of free FAs or glycerol (Tisdale 1993). In such an *in vitro* system the action of the lipid-mobilising factor purified from MAC16 tumours could be reversed by the addition of the n-3 PUFA, EPA. The effect of other conventional lipolytic stimuli such as ACTH and ß-adrenergic agents, could also be inhibited by EPA (Tisdale and Beck 1991). This effect was specific to EPA and was not demonstrated by other related n-3 or n-6 PUFAs, suggesting that inhibition was not due to a mass action effect on triglyceride hydrolysis. Further studies showed that stimulation of lipolysis was due to elevation of c-AMP levels in the adipocytes which was significantly blocked by EPA (Tisdale and Beck 1991).

The prevention of lipolysis by EPA, could either be through the inhibition of the adenylate cyclase enzyme or an increase in the activity of c-AMP phosphodiesterase. Using plasma membrane fractions prepared from adipocytes the effect of EPA was shown to be due to a direct inhibition of adenylate cyclase activity (Tisdale 1993). This effect was dose dependent with half maximal inhibition being observed at 165μM, close to the half maximal inhibition of lipolysis in intact adipocytes. Again this effect was specific to EPA and was not demonstrated by the structurally similar

PUFAs AA and DHA (Tisdale 1993). This structurally specific action of EPA was found to be due to action with a specific membrane receptor by interaction with a guanine nucleotide binding protein (Gi) that inhibited action of adenylate cyclase in adipocytes (Tisdale 1993).

In vivo studies have shown an enhancement of growth of transplantable mammary tumours by the n-6 PUFA linoleic acid (LA) when administered as a 10% corn oil diet (Gabor et al 1985). Release of LA from adipose tissue stores as occurs in cancer cachexia may therefore lead to a stimulation of tumour growth. Such a release during acute starvation or in streptozotocin-induced diabetes is thought to be responsible for a stimulation of growth of a transplantable rat hepatoma under these nutritional conditions (Sauer and Dauchy 1988).

1:4. Fatty Acids in Carcinogenesis.

Essential fatty acids (EFAs) must be supplied in the diet, as they can not be synthesised, or are synthesised at inadequate rates for their requirements in growth, maintenance and proper functioning of many biological processes (Alfin-Slater and Aftergood 1968). Inadequate dietary intake of EFAs results in a fatty acid (FA) deficiency characterised by weight loss, dermatitis, sterility and increased susceptibility to infection (Alfin-Slater and Aftergood 1968). The potency for the reversal of this syndrome is linoleic acid (LA)< gamma-linolenic acid (GLA)< arachidonic acid (AA), suggesting a requirement for LA metabolism within the body (Horrobin 1992). EFAs have at least 4 essential roles in normal tissues:

1. Modulation of membrane structure.
2. Production of short lived local regulatory substances 'eicosanoids'.
3. The control of skin water permeability, permeability of gastrointestinal tract or blood-brain barrier.
4. The regulation of cholesterol transport and cholesterol synthesis

(Horrobin 1992). The EFA requirement becomes elevated during periods of rapid cell division such as in infancy or pathological conditions such as inflammation and where cellular repair is required (Horrobin 1992). Though alpha-linolenic acid (ALA) has less potency as an EFA than LA, several of the longer chained more highly unsaturated

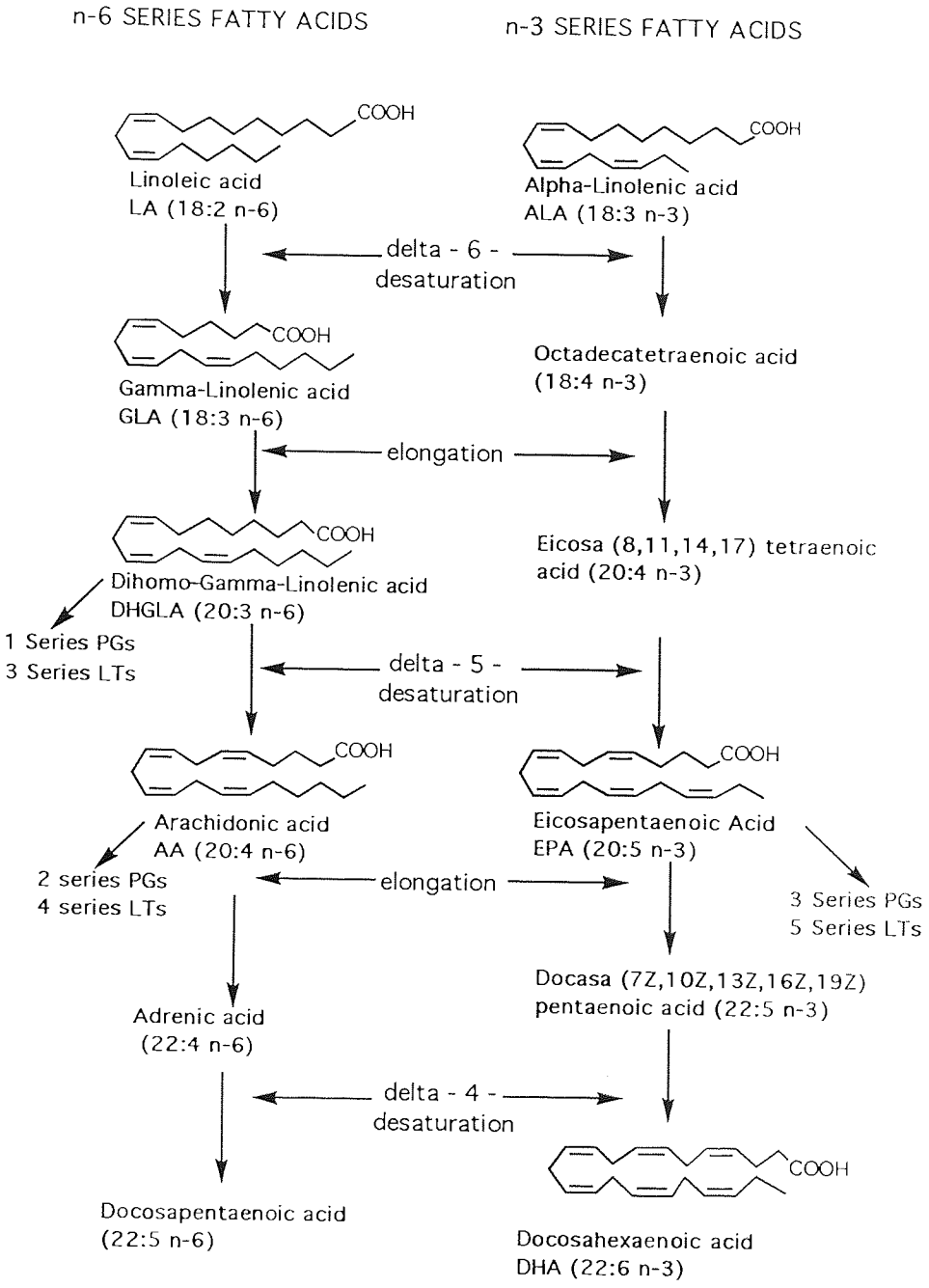

Figure 1.
Desaturation and elongation of n-6 and n-3 polyunsaturated fatty acids (PUFAs).

derivatives such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are required by certain tissues such as the brain, neural tissues or the retina of the eye (Horrobin 1992) and n-3 PUFAs are more loosely termed EFAs. Dietary sources of n-6 PUFAs include: meat, animal fats, sunflowerseed oil, corn oil and safflower oil, whilst dietary sources of n-3 PUFAs include: green leaves, fish oil, and linseed oil. The enzyme involved in the elongation and desaturation of n-6 or n-3 PUFAs (as shown in figure 1.), are assumed to be identical (Horrobin 1992). What has been established, is that the n-3 and n-6 PUFAs compete for the same enzymes, with n-3 PUFAs generally having a higher affinity and being preferentially metabolised (Sprecher 1982).

Epidemiological studies have shown that both the quantity and type of FA in the diet influences the incidence of colonic cancer in humans (Reddy 1994). Cancer of the colon is one of the most common cancers in the Western World, with the United States and Canada exhibiting a more than a tenfold increase in colonic cancers when compared to populations in certain parts of Asia, Africa and North America (Reddy 1994). It is estimated that 35% of all cancer mortality in the United States may be attributed to dietary factors (Wynder and Gori 1977, Doll and Peto 1981). In Greece, Spain and Southern Italy where olive oil is by far the most common dietary fat consumed, mortality from colonic cancer is low (World Health Organisation 1980). Eskimos in Alaska and Greenland with their high ingestion of marine oils have a lower incidence of cancers when compared to other North American and Westernised Populations (Neilsen and Hansen 1980, Blot et al 1975). In Greenland and Iceland a rise in the incidence of breast cancer followed a period when dietary habits became Westernised, with a reduction in n-3 PUFA consumption (Neilsen et al 1980). In Japan an increased risk of prostate cancer was reported amongst individuals whose diet contained little or no seafood (Mishina et al 1985).

Tumour development has two distinct phases tumour: initiation and promotion. Initiation of carcinogensis alters the genetic information of the cell, whereas promotion leads to malignancy by the uncontrolled proliferation of these genetically changed cells. Evidence for this two-stage induction has been observed in colon cancer

(Maskens 1981). The effect of dietary fat on chemically promoted carcinogenesis has been studied in a number of chemically induced rodent colonic tumours (Reddy 1994). Whilst n-6 PUFAs promoted the initiation and promotion of tumour growth, n-3 PUFAs reduced initiation and tumour promotion, in comparison to controls fed a diet rich in saturated FAs (Reddy 1994). An antitumour action and reduction in tumour-induced cachexia was observed by feeding diets rich in fish oil to MAC16 tumour-bearing NMRI mice (Tisdale and Dhesi 1990). This antitumour action could be reproduced by daily administration of 80% pure EPA by gavage (Beck et al 1991).

A comprehensive review by Nicholson et al 1988 proposed 4 possible mechanisms for the influence of PUFAs on carcinogenesis:

1. Direct effect on cell proliferation.
2. Changes in the FA content of the tumour cell membrane leading to increased sensitivity to exogenous stimuli.
3. Suppression of the immune system and the host's defence against the invading tumour.
4. Production of eicosanoids.

1:4:1. The Direct Effect of Polyunsaturated Fatty Acids (PUFAs) on Tumour Cell Proliferation.

The importance of the type of PUFA was demonstrated in breast carcinoma by comparison of human breast carcinoma cell line MCF-7 with the non-cancerous human mammary epithelial cell line MCF-10A (Grammatikos et al 1994). When EPA and DHA was presented to MCF-7 cells bound to albumin cell growth was inhibited in a dose dependent manner between 6-30μM. ALA and AA inhibited proliferation less significantly whilst LA had no effect. In contrast MCF-10A cell growth was not inhibited by any EFAs below 24μM (Grammatikos et al 1994). Desaturation and elongation was observed in MCF-10A cells, whereas no AA was produced from LA, or EPA and DHA from ALA in MCF-7 cells, suggesting a defect in desaturase enzymes in this cell line (Grammatikos et al 1994). In rats, diets containing high levels of n-6 PUFAs enhanced the formation of methyl-nitrosourea (MNU)-induced rat mammary adenocarcinomas (Hu et al 1995). At 30 or 75 days after induction of tumours with

carcinogen, glands from animals fed the high-fat diet had significantly higher fractions of cells containing Ha-ras mutants. This suggested a role for dietary PUFAs in the stimulation of cells harbouring ras mutants (Hu et al 1995). NIH-3T3 cells transfected with the v-Ki-ras oncogene (DT-cells) had a preferential incorporation of n-6 PUFAs into phosphatidyl choline, whereas normal NIH-3T3 cells had a preference for incorporation into the neutral lipid pools particularly triglycerides (de Antueno et al 1994). Since the differences in metabolism can not be explained by the relative growth rate, it was suggested that they were a consequence of the expression of the v-Ki-ras oncogene (de Antueno et al 1994).

PUFAs have been shown to govern nuclear events that control gene transcription (Armstrong et al 1991, Blake and Clarke 1990, Clarke and Abraham 1992). Three potential mechanisms have been proposed by which PUFAs modify this gene expression (Clarke and Jump 1994):

1. Generation of a PUFA regulated signal.
2. Regulation of PUFAs as a transducing factor.
3. By direct interaction of transducing factors on target organs.

PUFAs translocated to the nucleus act as ligands or modifiers of a nuclear-fatty acid binding protein (PUFA-BP). These interactions with PUFA-BP activates a trans-acting element in the target gene governing gene transcription (Clarke and Jump 1994). Alternatively the oxidised product of PUFAs could modify the redox state of a transacting amplifier such as c-fos and c-jun (Abate et al 1990, Hayashi et al 1993). A third possibility is that the PUFA may influence the phosphorylation state of the nuclear protein that governs transcription of genes encoding for enzymatic proteins (Hunter and Karin 1992, Khan et al 1992, Li and Jaiswal 1992). Over the past 25 years several investigators have demonstrated that n-3 and n-6 PUFAs suppress hepatic lipogenesis, whereas saturated and monosaturated FAs have no inhibitory capacity (Armstrong et al 1991). This action is independent of carbohydrate intake (Clarke et al 1984) and occurs at a level of n-6 PUFA intake four or five fold greater than that required to fulfil EFA requirements for optimal cell growth (Clarke and Jump 1993). More recent studies have shown that n-3 PUFAs

expression of h-ras, c-ras, p21ras and myc oncogenes in different mammary tumours (Karmali et al 1989, Fernandes and Venkatram 1991 Torras et al 1992).

Although the mechanism by which n-6 PUFAs promote colonic carcinogenesis in rodents is not fully understood, an increased stimulation of secondary bile acid production has been observed (Reddy 1986). In laboratory animals these secondary bile acids consisting of deoxycholic acid and lithocholic acid along with FAs, are capable of both the stimulation of colonic epithelial cell proliferation (Reddy and Ohmori 1981,) and act as promoters of colonic tumours (Reddy 1986). In human patients at risk from colonic cancer high proliferative rates have been observed in normal appearing colorectal mucosa and is accompanied by a high secretion of secondary bile acids (Reddy 1986).

High levels of secondary bile acids also increase the activity of ornithine decarboxylase (ODC) (Reddy 1994). Colonic tumours induced with azoxymethane (AOM) in rats showed an elevation of ODC activity characteristic of that seen in clinical samples (Rozhin et al 1984). Diets rich in n-3 PUFAs reduced ODC activity (Reddy 1994). Though the mechanism by which PUFAs attenuate ODC production is not fully understood, the corresponding increase or decrease in secondary bile acid secretion suggests a major role for these bile acids (Reddy 1994). Tyrosine-specific phosphorylation is recognised as an important regulatory mechanism of cell proliferation in response to a number of mitogenic stimuli including growth factors, growth factor receptor interaction and stimulation of oncogenes (Cantley et al 1991). Tyrosine-protein kinase (TPK) activity was increased in the colonic mucosa of male F344 rats after treatment with chemical carcinogens (Kumar et al 1990). An increase in TPK activity is associated with increased tumour growth, whilst a decrease in TPK activity is associated with a decreased tumour growth. Diets rich in n-6 PUFAs increase TPK activity, whereas diets rich in n-9 or n-3 PUFAs decrease TPK activity (Reddy 1994). The mechanism of this action remains unclear, though increased secretion of secondary bile acids is thought to increase reactive free oxygen species which in turn activates TPK phosphorylation (Craven et al 1987).

Activation of phospholipase C by various stimuli results in the transient increase

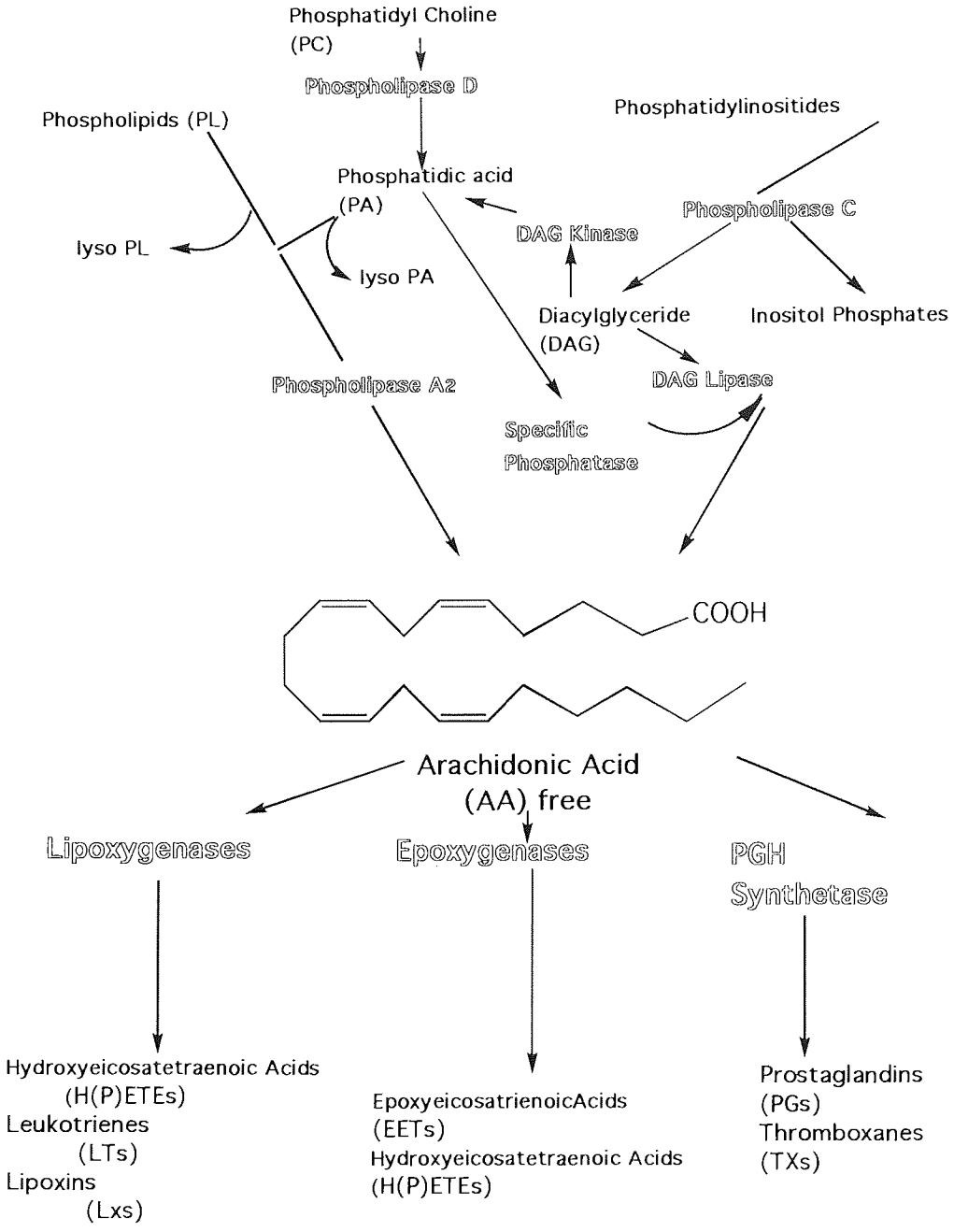

Figure 2. Pathways of arachidonic acid (AA) release and metabolism.
(Adapted from Hecker et al 1989.)

in the cellular 1,2-sn-diacylglycerol (DAG) content derived from the breakdown of inositol phospholipids (see figure 2.) (Nishizuka et al 1984). The secondary bile acid deoxycholic acid increased the accumulation of DAG in colonic epithelial cells (Craven et al 1987). This elevated DAG stimulated protein kinase C (PKC) activity (Craven et al 1987). PKC is a family of enzymes with critical roles in signal transduction, regulating both cellular function and proliferation (Nishizuka et al 1984, Fitzer et al 1987). Activation of PKC can take place through translocation to the cell membrane. In colonic epithelial cells translocation of PKC was activated by DAG, the levels of which were elevated by the secondary bile acid deoxycholic acid (Craven et al 1987, Fitzer et al 1987). The importance of DAG and PKC activation by n-6 PUFAs was also demonstrated in skin tumourigenesis in SENCAR mice (Birt et al 1992). Diets rich in n-6 PUFAs increased the intracellular levels of DAG, which in turn increased the activity of both particulate and soluble forms of PKC, correlating with an increase in tumour growth (Birt et al 1992). Mutations of K-ras was reported to be as high as 50% in some human adenocarcinomas of the colon (Lacal and Tronick 1988, Bos et al 1987). Increased cellular levels of DAG in ras augmented transformation have been observed, suggesting an indirect relationship between dietary PUFAs and ras p21 (Reddy 1994).

In a 12 week study patients with sporadic adenomatous polyps received fish oil containing 4.1g of EPA and 3.6g of DHA in the ethyl ester form, whilst a placebo group received olive oil (Anti et al 1992). Patients receiving fish oil showed a reduction in S-phase cells in the lower part of the colonic crypt (Anti et al 1992,) which is considered to be a reliable marker for a reduced risk of colonic cancer (Bartram et al 1993). The decrease of proliferative activity within the colonic crypts was reduced to that of normal or low risk patients, and correlated with an increased EPA content and a decreased AA content in rectal mucosal cells (Anti et al 1992).

1:4:2. The Influence of Changes in Polyunsaturated Fatty Acid (PUFA) Content of Tumour Cell Membranes on the Sensitivity of Exogenous Stimuli.

Morris hepatoma 5123 and other experimental hepatomas have a reduced PUFA incorporation in their cell membranes which appears to be compensated for by an

increased concentration of OA and SA (Hopewell et al 1993, Dianziani et al 1990). This reduced PUFA content coupled with an increased cholesterol content in tumour cells is present not only in the cytoplasmic membranes but the membranes of cellular organelles including mitochondria (Wood et al 1986). The FA composition of tissues, in particular the cell membrane, can be modified by dietary PUFA intake (Reddy 1994, Baronzio et al 1995). Supplementation of PUFAs are also capable of changing the expression of growth factor receptors, carrier-mediated transporters, membrane-bound enzymes, as well as cytotoxicity, modifying both membrane fluidity and protein interaction (Burns and Spectre 1990, Tritton and Hickman 1990, Hayashi et al 1992, Kinsella 1990). Recently it was observed that the capacity to modify adhesion of colorectal tumour cells to liver macrophages by PUFAs was dependent on the degree of differentiation of these colorectal tumour cells (Meterissian et al 1995). Moderately well differentiated colorectal tumour cells more avidly incorporated PUFAs into their membrane phospholipids suggesting that the successful intervention of colorectal cancer may be dependent on the differential status of the tumour cells (Meterissian et al 1995).

The reduction of PUFAs content in tumour cell membranes renders them more susceptible to products formed by lipid peroxidation (Baronzio et al 1995). In MDA-MB231 breast carcinomas in athymic mice metastasis and tumour growth was reduced by feeding a diet rich in n-3 PUFAs as fish oil (Gonzalez et al 1991). When this fish oil diet was supplemented with antioxidants tumour growth was increased in comparison to fish oil administered alone, suggesting a role for products of lipid peroxidation in the antitumour effect of n-3 PUFAs (Gonzalez et al 1991).

Increasing the PUFA content of membranes increases the fluidity of these membranes (Spectre and Yorek 1988). Membranes from proliferating mammary cells have been shown to have an increased PUFA content in comparison to their quiescent counterparts (Kidwell et al 1982). An increased PUFA content, or fluidity, of cell membranes can also increase the susceptibility of cells to cytotoxic agents. L1210 mouse leukemia cells were more susceptible to cytotoxic agents when their membranes became enriched with OA or in particular LA (Spectre and Yorek 1988).

An increased PUFA content in rat hepatoma cell membranes rendered them more susceptible to both complement-dependent cytolysis (Yoo et al 1980) and spleenocyte mediated cytotoxicity (Yoo et al 1982).

Growth of murine mammary tumours was found to be dependent on hormones and growth factors, though high dietary fat could also enhance the growth and development of these tumours (Aylsworth et al 1985). Evidence suggested that although dietary fat was not a mutagen that initiated permanent genomic changes, once neoplastic transformation had taken place, dietary fat promoted mitogen dependent proliferation and survival of these transformed cells, allowing the expression of sustained cell growth (Alysworth et al 1985, Nandi et al 1992, Welsch et al 1985). *In vitro* studies showed that PUFA supplementation of mammary epithelial cells greatly enhanced their mitogenic responsiveness to hormones and growth factors, but PUFAs alone were unable to stimulate cell proliferation (Bandyopadhyay et al 1987, Bandyopadhyay et al 1993). Recent studies indicated that membrane PUFA content potentiated the epidermal growth factor (EGF) induced activation of PKC and the mitogenic responsiveness of primary cultures of mammary epithelial cells (Sylvester et al 1994). This action was independent of PG synthesis (Sylvester et al 1994).

The desaturation of tumour cell membranes with EFAs to render the tumour more susceptible to cytotoxic agents seems to be a simple and feasible application for the treatment of human cancers (Baronzio et al 1995), though there are many difficulties in its application. The level of EFA required to achieve a biological effect is often unobtainable. Levels of GLA which are effective against tumours in animal models requires the daily ingestion of 6g/kg GLA in humans (Horrobin 1990). This intake is not feasible as the average GLA capsule contains 40-100mg of GLA and a prohibitive number of capsules is therefore required (Baronzio et al 1995). A controlled study with human hepatoma carcinoma patients showed no significant effect of GLA on survival or liver size (Van der Merwe et al 1990). Dosage was considered too low over too short a period of time, coupled with a large tumour burden, which was reported to be as high as 3kg in some patients (Van der Merwe et al 1990). In

another study GLA was given daily at 1.5g/day over many years. There was an increase in patient survival, with a reduction in ascites, pain and cachexia (Van der Merwe et al 1987). Another study on colon cancer with daily administration of 240mg/kg GLA, showed no significant difference in survival when compared to placebo patients (McIllmurray and Turkie 1987).

1:4:3. Suppression of the Immune System and the Host's Defence against the Invading Tumour.

Diets rich in the n-6 PUFA LA contained in corn oil, promoted the growth of transplantable mammary tumours in Balb/c mice (Hillyard and Abraham 1979). This increased tumour growth could not be facilitated by an increased tumour cell proliferation rate alone. Cell kinetic studies showed that promotion of tumour growth by LA was caused by a decrease in cell lysis (Gabor et al 1985). In the same tumour model diets rich in n-3 PUFAs contained in menhaden oil inhibited tumour cell growth and increased tumour cell lysis (Gabor and Abraham 1986). In MAC16 tumour-bearing NMRI mice, inhibition of tumour growth and cachexia induced by the tumour by EPA correlated with an increase in cell loss (Hudson et al 1993). LA when administered alone did not effect the cell loss factor of MAC16 tumours, but when given with EPA, the increase in cell loss observed with EPA alone was reduced and became compatible with that demonstrated in untreated tumours (Hudson et al 1993). EPA administration to MAC16 tumour-bearing mice increased the tissue and tumour levels of EPA (Hudson 1993).

The promotion of breast tumour growth by LA correlated with an increased PG production (Alysworth et al 1987). This increased PG production was thought to suppress the host immune system preventing the immunological rejection of the tumour (Brunda et al 1980). Leukotrienes (LTs) and prostaglandins (PGs) derived from n-3 PUFAs have reduced biological activity in comparison to n-6 derived eicosanoids (Needleman et al 1979, Dyerberg et al 1978, Leitch et al 1985). Consequently n-3 PUFAs can simultaneously reduce inflammation, thrombogenesis and stimulate host immunity against tumour cells, explaining the antitumour action of n-3 PUFAs against certain immunogenic tumours (Torosian et al 1995). The effect

of PUFAs on tumour growth by the suppression or stimulation of the host's immune system can not explain the action of PUFAs in all tumour models, especially as tumour cell proliferation is modified *in vitro* by PUFAs in the absence of any humoral or cell mediated immunity (Rose and Connolly 1989, Rose and Connolly 1990).

LTB_4 actions correlated with the high incidence of immunological deficiencies and inflammatory symptoms associated with head and neck cancer (Papenhausen et al 1979, Bray 1983). In the inflammatory response LTB_4 stimulates vascular permeability and oedema responses particular in the presence of the vasodilator PGE_2 (Wedmore and Williams 1980). LTB_4 significantly inhibits the human mitogen induced lymphocytic proliferation *in vitro* through PGE_2 leading to immuno-suppression (Rola-Pleszcynski and Sirvoto 1983). Patients with head and neck cancer also show a low immune competitance (EL-Hakin 1989). LTs have also been shown to protect tumours against damage from radiotherapy (Hansen 1987).

1:4:4. The Role of Eicosanoids in Carcinogenesis

Eicosanoids are amongst the most potent naturally autacoids and are becoming increasingly recognised as important cell regulatory substances, though their physiological roles are still incompletely understood (Hecker et al 1989). Production of eicosanoids in mammalian cells is usually tightly regulated, but often reaches exaggerated levels in malignant tissues (Karmali 1987). Eicosanoids are not stored in mammalian cells and are synthesised in response to various cellular stimuli. The biological synthesis of eicosanoids is dependent on the availability of free FA precursors. In mammalian cells the more potent and commonly produced eicosanoids are from AA. The mobilisation of AA is therefore an important process in the synthesis of eicosanoids (see figure 2)(Hecker et al 1989). In principle AA can be released by 3 phospholipase pathways (Cockcroft 1992, Billah and Anthens 1990).

1. A phosphatidylinositol-specific phospholipase C (PLC) production of diacyl-glycerol (DAG) which is cleaved by DAG lipase.

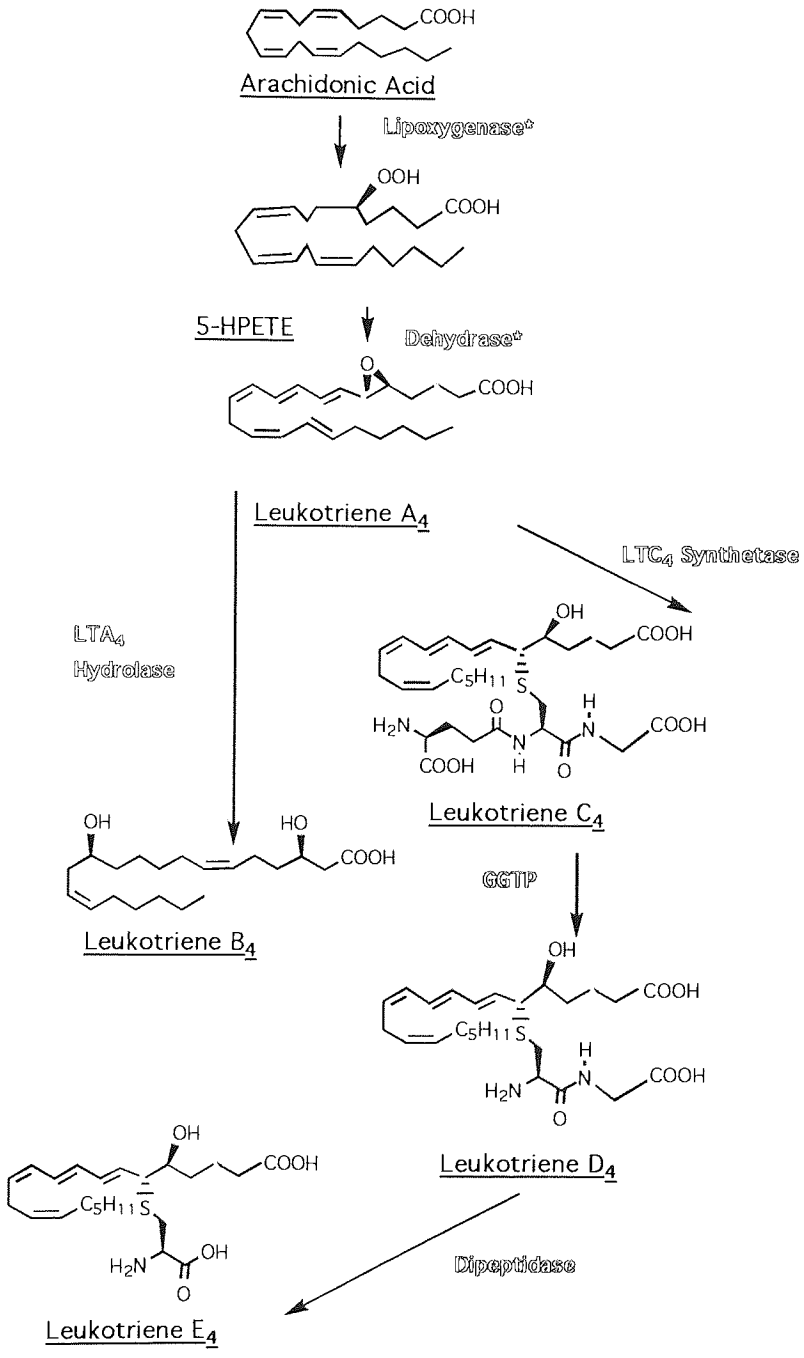

Figure 3a. Leukotriene biosynthesis. * both the lipoxygenase and dehydrase reactions are driven by the single enzyme 5-lipoxygenase.
GGTP= gamma-glutamyl transferase.
(Adapted from Hecker et al 1989.)

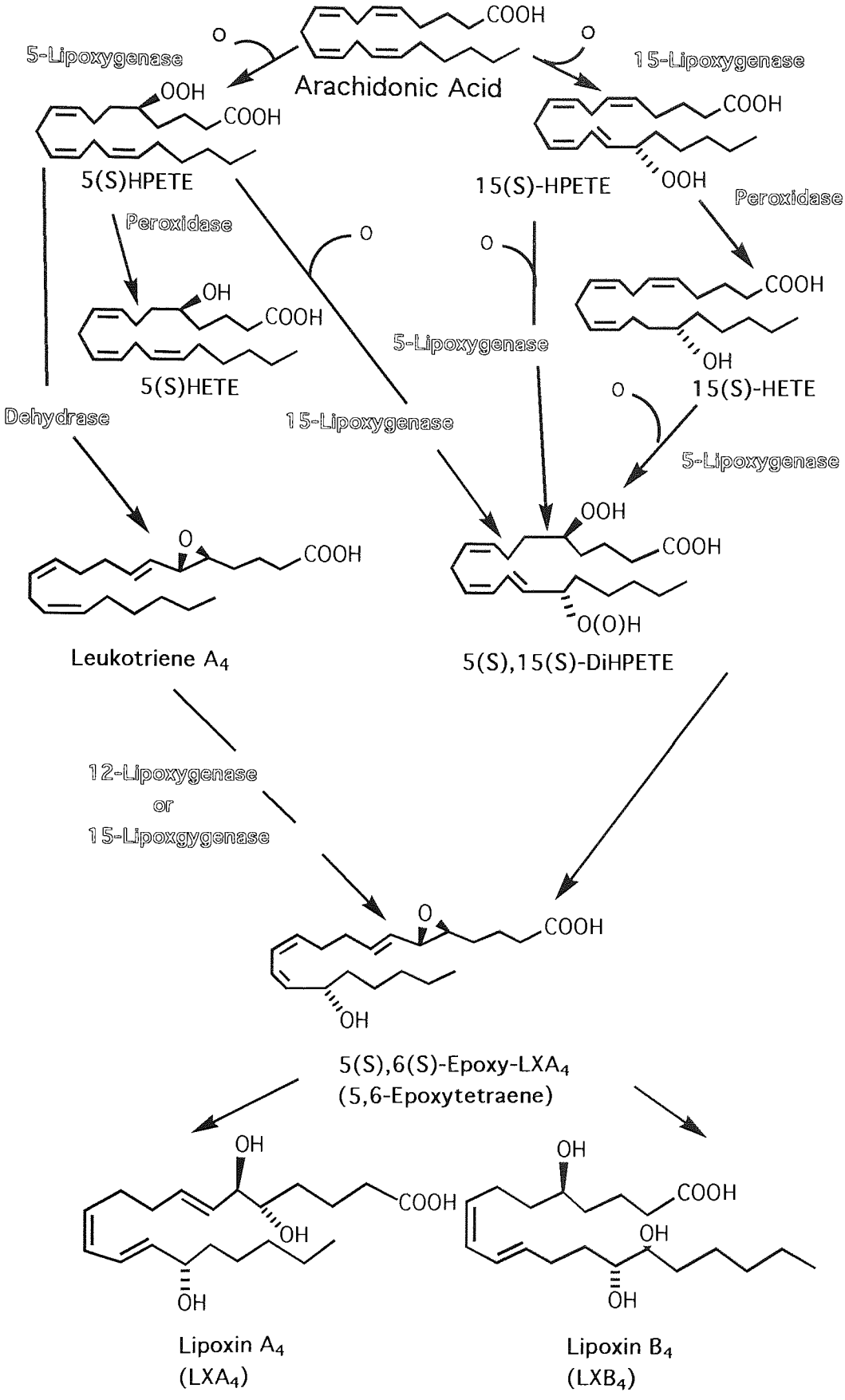

Figure 3B.
The biosynthesis of lipoxins. (Adapted from Heker et 1989).

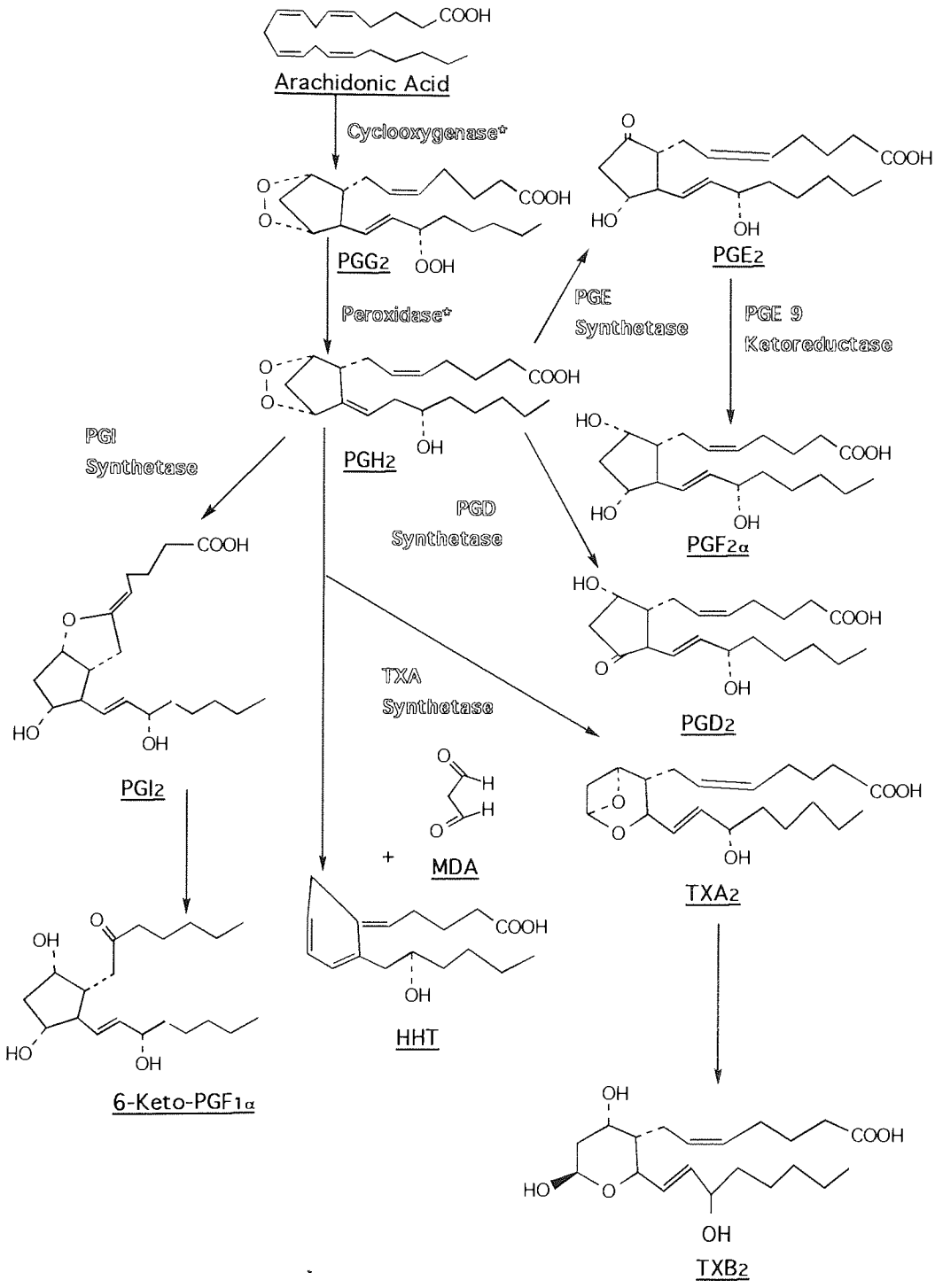

Figure 4. Prostaglandin and thromboxane synthesis. * Both cyclooxygenase and peroxidase are catalyzed by the single enzyme prostaglandin endoperoxide (PGH) synthetase. (HHT Hydroxyheptadecatrienoic acid and MDA Malondialdehyde.) (Hecker et al 1989.)

2. Phospholipase D (PLD) cleaving phosphatidyl choline, with the resulting phoshatidic acid being a substrate for a specific phosphatase, with free FA finally being produced from DAG lipase.

3. Phospholipase A_2 (PLA_2)in a one step reduction that cleaves AA from the 2-position of various phospholipids.

Originally the stimulation of AA release by PLA_2 was proposed to be through a receptor-coupled G protein (Burch et al 1986, Burgoyne et al 1987, Cockcroft and Stutchfield 1989). Recently it has been suggested that rather than a direct coupling of PLA_2 to G protein, the enzyme can be activated by a series of reactions involving PKC (Lin et al 1992, Dyster et al 1992). In neutrophilic HL60 cells, AA mobilisation occurred by overlapping routes involving both G proteins and PKC. G protein activation allowed the mobilisation of AA by activation of PLD/DAG lipase, but in contrast extensive G protein activation involved PKC-regulated activation of PLA_2. In this way much larger pools of AA stored in phosphatidyl choline (PC) could be released (Buhl et al 1995).

Mobilised AA can enter any of three major pathways for eicosanoid metabolism (See figure 2.). The lipoxygenase (LO) pathways produce hydroperoxyeicosa-tetraenoic acid (HPETE), hydroxyeicosatetraenoic acids (HETE), leukotrienes (LTs) (see figure 3A.) and lipoxins (LXs) (see figure 3B.). Free AA can also be metabolised by cytochrome p-450 expoxygenase pathways to yield a variety of epoxyeicosatrienoic acids (EETs) and HETEs (Escalante et al 1991, Frazier and Yorio 1992). Prostaglandins (PGs) and thromboxanes (TXs) are produced by the third pathway, the cyclooxgenase pathway (CO) (see figure 4.). CO activity is contained within the PGH synthetase enzyme along with peroxidase activity. At least two isoforms of PGH synthetase exist within mammalian cells. The first is COX-1 which is constitutively expressed. In contrast COX-2 is expressed in a regulated fashion in response to endotoxin, growth factors, cytokines and phorbol esters (Du Bois et al 1994). Selective inhibition of COX-2 would supress PG synthesis at the site of inflammation such as in response to endotoxin or interleukin-1 (IL-1) in fever, but would spare constitutative PG synthesis in other tissues such as the gastrointestinal

tract (Wallace and Cirino1994). This results in a reduction of gastrointestinal injury by selective COX-2 inhibitors in comparison to conventional NSAIDs (Wallace and Cirino 1994).

The effect of PGs and LTs in the subversion of immune surveillance by the host is discussed in section 1:4:3. Raised PG levels in breast cancer is associated with more clinically aggressive tumours with increased metastic potential (Rolland et al 1980). Elevated PGE_2 and PGF levels in some tumours was found to correlate with both clinically aggressiveness and poor differentiation on histological examination (Folton et al 1981). Recent studies have shown that treatment with chemical carcinogens significantly increased the basal level of PGE_2 and 6-keto$PGF_{1\alpha}$ *ex vivo* production in the colon (Rao and Reddy 1993). The *ex vivo* production of PGE_2 and 6-keto$PGF_{1\alpha}$ was greater in animals fed diets containing high levels of n-6 PUFAs than those containing high levels of n-3 PUFAs (Narisawa et al 1985, Narisawa e al 1991). The inhibitory effect of n-3 PUFAs on colon can be explained by the direct inhibitory action of EPA and DHA on PG synthesis (Culp et al 1979). Animal (Hudson 1993) and human studies (Anti et al 1992) have shown that supplementation of the diet with EPA results in an increased EPA incorporation into tissues at the expense of AA. As well as competing for the same LO and CO enzymes (Rose and Connolly 1990), the eicosanoids produced from EPA have reduced biological activity in comparison to those of AA (Needleman et al 1979, Dyerberg et al 1978, Leitch et al 1985). Any physiological process stimulated within the tumour cell by increased AA eicosanoid production would be less successfully stimulated or inhibited by EPA derived eicosanoids (Torosian et al 1995).

Lipoxygenases (LOs) are dioxygenases which recognise the 1,4-pentadiene structure of PUFAs and incorporate a single molecule of oxygen to a specific carbon atom on the FA molecule. LOs that have been identified in mammalian tissues include, 5-LO, 8-LO, 12-LO and 15-LO, producing 5-HPETE, 8-HPETE, 12-HPETE and 15-HPETE respectively as their major metabolites which undergoes spontaneous reduction due to peroxidase activity contained within the LO enzyme itself to form HETEs (Sigal 1991). The AA is usually oxidised at the major site, though 12-LO

enzymes have been shown to produce 15-HETE, whilst 15-LO has been shown to produce 12-HETE (Sigal 1991). 5-LO metabolites have been shown to play a major role in the pathological processes involved in diseases such as asthma, rheumatoid arthritis and psoriasis (Musser and Kreft 1992) and inhibitors of the 5-LO enzyme have therefore been designed. However, due to the homology which exists between both the action of 5-LO and structures, inhibition of 12-LO and 15-LO is often demonstrated (Yamamoto 1992).

LOs are all non-heam iron containing dioxygenases. Recently studies have shown that Fe(III) LO is the catalytic species, with catalytic rate close to zero when Fe(II) is the main form of the enzyme (Schilstra et al 1994). Inactive 5-LO is located in the cytosol. Both ATP and intracellular free calcium (Ca^{2+}) are required as cofactors (McMillan and Walker 1992). The enzyme is thought to be activated by the presence of hydroperoxides and will undergoe Ca^{2+} dependent translocation to the cell membrane. There is a requirement for an 18kDa membrane bound protein, discovered by Merck-Frost Ltd., for LO activity in intact cells. It is thought that this 5-LO activating protein (FLAP) activates 5-LO enzyme activity when inactive 5-LO binds with membrane bound FLAP (Musser and Kreft 1992).

During recent years novel findings have implicated a role for LTs and LXs as regulators of cell proliferation including a possible impact on human haemopiesis (Stenke et al 1994). LTB_4 and LTD_4 were shown to promote the proliferation of K562 myeloid leukemia cells (Snyder et al 1989) whereas LTD_4 was shown to promote the proliferation of the human leukemia cell line HL60 (Miller et al 1989). The mechanism by which LTs and LXs stimulate the proliferation of leukemia cell lines and other haemopietic cells remains unclear (Wickremasinghe et al 1993). Binding of LTD_4 to its receptor triggers the generation of second messenger molecules via the breakdown of phospholipids which have been implicated in the regulation of cell proliferation (Mong et al 1988). The expression of 5-LO was also shown in two human colonic cancer cell lines HT29 and CaCO2 (Cortese et al 1995). An enzymatic activity was found in mouse epidermis on application of the tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) which catalysed the formation of 8-

hydroperoxyeicosatetraenoic acid (8-HPETE). Unlike 12-LO activity, 8-LO activity was only detected on the application of tumour promoter and was devoid of any peroxidase activity, the major metabolite formed being 8-HPETE (Fürstenburger et al 1991).

The production of cytokines and their various biological functions can be influenced by various factors including eicosanoids (Purasiri et al 1994). The cells of the immune system are the main source of cytokines, though production in other cells such as fibroblasts, keratinocytes and endothelial cells has also been demonstrated (Kennedy and Jones 1991). Recently tumour growth was shown to be influenced by cytokines, and cytokine synthesis within tumours was also demonstrated (Wu et al 1993, Van Meir et al 1990). A notable feature of cytokines is that they act as modulators of both defence mechanisms and pathological processes. When produced in an uncontrolled and prolonged manner, cytokines have been implicated in chronic inflammation, cachexia and fatal shock (Mizel 1989). Cytokine receptors, such as for interleukin-2 (IL-2) have been located in many human tumours (Weldman et al 1992). IL-2, IL-4, IL-6 and tumour necrosis factor-alpha (TNF-α) show differential responses on tumour cells either stimulating or inhibiting cell proliferation (Wu et al 1993, Van Meir et al 1990, Paciotti et al 1988). Addition of LTB_4 to human lymphocytes enhances the production of IL-1, TNF and interferon (IFN), and a reduction in the production of these cytokines was observed when 5-LO activity was inhibited (Dinariello et al 1984, Kunkel and Chensure 1985).

It was suggested that by alteration of dietary EFA intake the production of eicosanoids could influence cytokine action, which in turn could influence the progression of carcinogenesis (Purasiri et al 1994). Prolonged supplementation of GLA, DHA and EPA at 4.8g/day in patients with advanced colorectal cancer suppressed the production of total IL-1, IL-2, IL-6, TNF-α and IFN-γ. All cytokine levels returned to normal three months after discontinuation of EFA intake (Purasiri et al 1994). This study demonstrated that the low cytokine production documented with time was a result not of the malignant process but a consequence of dietary EFA intake.

Reduced cytokine levels had beneficial anti-inflammatory effect, eliminated fever, or reduced malaise, muscle wasting or weight loss in patients with malignancy (Purasiri et al 1994).

Tumour infiltrating macrophages can represent a major component of the cellular infiltrate in some solid tumours, but display a reduced tumouricidal activity at the tumour sites. These tumour activated macrophages (TAMs) may play a sinister role in the promotion and metastasis of some solid tumours (Leek et al 1994). Communications between tumour cells, and tumour cells with the surrounding stromal cells occurs via a complex network of extracellular signals involving not only hormones and antibodies but cytokines, their inhibitors or antagonists, counterparts and cellular receptors (Leek et al 1994). Though the effect of cytokines is not fully established, they can regulate the proliferation and metastasis of tumour cells by suppressing the activity of TAMs, and enhancing the establishment of the tumour in the vascular compartment by both deposition of matrix protein and stimulation of angiogenesis (Lewis and O'Sullivan 1994, Bicknell 1994). (The importance of metastasis and angiogenesis in tumour development is discussed in section 1:2.)

Release of AA by proliferative endothelial cells has been shown to be an intrinsic property and becomes reduced as cells become confluent (Whatley et al 1994). The spectrum of eicosanoids produced amongst endothelial cells types varies, but in microvascular endothelial cells the prominent product is PGI_2 (Gerristen 1987). The secretion of eicosanoids by endothelial cells has been shown to regulate vascular tone and interaction with other blood vessels (Gryglewski et al 1988, Whatley et al 1990). Activated platelets may adhere to vascular endothelial cells increasing cell permeability and releasing growth factors characterised as angiogenic molecules (Folkman and Shing 1992). LTC_4 and LTD_4 promoted angiogenesis in a dose dependent manner whilst LTB_4 was ineffective (Tsopangolou et al 1994). The potent and selective peptidyl LT receptor antagonist SK&F 10453-7 abolished this action, suggesting that stimulation of angiogenesis is through receptor mediated interactions (Tsopanogolou et al 1994). PGE_2 and LTC_4 have been shown to stimulate both

proliferation and tube formation, the initial stages of angiogenesis, in bovine carotid artery *in vivo* (Kanayasu et al 1989), whilst 12(R)HETE was found to be both a potent chemotactic and angiogenic factor in the rabbit micropocket technique (Masferer et al 1991). α-Guiconic acid (GR-12) and GR-01 one of its derivatives inhibited AA metabolism in human vascular cells *in vitro* to prevent both tube formation and cell proliferation (Ito et al 1993). *In vivo* the growth of murine gliomas was also inhibited by the prevention of neovascularisation (Ito et al 1993). 12(S)HETE was metabolised in culture by rat aortic smooth muscle cells (SMC) by β-oxidation to produce 8-hydroxy-4,6,10-hexadecatrienoic acid or by 10-11 reductase to produce 12(R)HETE which was further oxidised to 8-hydroxyhexadecadienoic acid (Berard et al 1994). Serum stimulation of rat aortic SMC increased 10-11 reductase activity and cells passed from the quiescent to the proliferating state. 12(R)HETE became incorporated into phosphatidylcholine and phosphatidylethanolamine where it was considered to influence cellular function (Berard et al 1994).

12(R)Hydroxy-5,8,11(ZZZ)-eicosatetraenoic acid (12(R)HETrE) is produced from the cytochrome P450 metabolism of AA (Murphy et al 1988). 12(R)HETrE had a direct mitogenic action on microvessel endothelial cells (Laniado-Schartzman et al 1994). There was a significant increase in oncogene expression of c-jun, c-myc and c-fos as detected by northern blotting (Laniado-Schartzman et al 1994). NFκβ binding activity was selectively and potently stimulated within minutes of 12(R)HETrE application to endothelial cells, thereby transferring a signal to the nucleus and resulting in the induction of gene expression (Laniado-Schartzman et al 1994).

Mitogen activated protein kinases (MAPKs) are a group of serine/threonine kinases which are activated early in the response to a variety of growth stimuli (Ray and Sturgill 1987, Ahn et al 1990). These MAPKs are thought to phosphorylate and activate gene transcription factors such as c-jun, c-myc and $p62^{TCF}$, which in turn modulates the expression of target genes (Alvarez et al 1991, Marais et al 1993). In vascular smooth muscle cells (VSMCs) AA activated MAPK in a time and dose

dependent manner. The main eicosanoid produced in these VSMCs was 15-HETE (Rao et al 1994). The action of AA or 15-HETE on the stimulation of MAPK in VSMCs was inhibited by the LO inhibitor nordihydroguaiaretic acid (NDGA) but not the CO inhibitor indomethacin, and was regulated by PKC activity (Rao et al 1995). The metabolism of LA by LO to form 13-hydroperoxyoctadecatrienoic acid (13(S)HPODE) in VSMCs was also found to induce c-fos, c-jun and c-myc mRNA expression by activation of PKC (Rao et al 1995).

B16a melanoma cells with low (LM180) and high (HM340) metastic potential were shown to have different profiles of mono-HETE production from AA (Lui et al 1994). LM180 cells produced equal quantities of 12-HETE and 5-HETE, whilst HM340 cells synthesised predominantly 12-HETE and small amounts of 15, 11 and 5-HETE. At equal concentrations of substrate AA, HM340 cells produced four times higher concentrations of 12-HETE than LM180 (Lui et al 1994). The specific 12-LO inhibitor N-benzyl-N-hydroxy-5-phenylpentamide, decreased adhesion of HM340 cells to microvessel endothelial cells *in vitro* and lung colony formation *in vivo* (Lui et al 1994). These results suggest that inhibition of 12(S)-HETE biosynthesis in tumour cells may be a crucial target for intervening in metastasis.

12(S)-HETE production was shown to be important in the metastasis of other tumour models such as Lewis lung carcinoma cells (W256) (Hagman et al 1995). 12(S)-HETE was shown to increase permeability of capillaries by the induction of endothelial cell retardation (Honn et al 1994), stimulate cells spreading and motility on matrix (Timar et al 1993), or stimulate the release of cathepsin a protease associated with tumour invasiveness (Sloane et al 1991). The activation of PKC by 12(S)HETE was found to regulate many of these processes involved in metastasis (Hagman et al 1995). Endogenous application of 12(S)HETE stimulated proliferation of rat W256 cells and mouse B16a melanoma cells *in vitro,* suggesting that the 12(S)HETE produced by the host may also stimulate tumour cell proliferation in addition to facilitating metastasis (Lui et al 1991). Adhesion of W256 cells to rat endothelial monolayers was enhanced by the AA LO metabolite 12(S)HETE but inhibited by the LO LA metabolite 13(S)HPODE (Lui et al 1991). This action was

regulated by PKC with 12(S)HETE stimulating translocation and activation, whilst 13(S)HPODE prevented the translocation of PKC and subsequent activation (Lui et al 1991).

1:5. Aims of The Investigation

Primary tumours of the colon were induced by weekly injections of 1,2-dimethylhydrazine (DMH) in NMRI mice. All visible polyps were removed from the colon and transplanted in recipient NMRI mice. Tumours developed from subcutaneous transplantation of induced tumour fragments no larger than 1-2mm^3 by trocar (Double et al 1975). These tumours produced a series of transplantable murine adenocarcinomas (MACs) or MAC cells lines with varying degrees of differentiaition and histology similar to human colorectal cancer (Cowen et al 1980). MAC tumours and cell lines have also been shown to be refractive to cytotoxic agents (Double and Bibby 1989). Cachexia is frequently associated with malignant disease in humans, but in animal models weight loss is usually only induced by large tumour burdens, when the animal is close to death caused by anorexia (Tisdale 1991). The MAC16 tumour induces weight loss at relatively small tumour burdens and is not accompanied by a reduction in either food or water intake in NMRI mice (Bibby et al 1987, Beck and Tisdale 1987). The MAC13 tumour is of the same histological type but grows without the expression of cachexia in NMRI mice (Bibby et al 1987).

The role of PUFAs in MAC16 tumour-induced cachexia has been extensively studied (Hudson 1993) along with the change in FA composition of tissues, plasma and tumour, with and without treatment with EPA, which is weight stabilising with antitumour action (Hudson and Tisdale 1994, Hudson 1993, Hudson et al 1993, Beck et la 1991). The mechanism by which PUFAs alter the course of carcinogenesis remains unclear especially the mechanism by which the dietary n-6 PUFA LA promotes tumour development. The aim of this study was to investigate the requirements of MAC cells *in vitro* and MAC tumours *in vivo*, for PUFAs, in particular any promotional effects of LA.

The second part of this study was to establish whether there was a role for eicosanoids in the proliferation of MAC cells *in vitro*, or the growth and development

of MAC tumours in NMRI mice. The effect of LO inhibitors designed to act via different mechanisms, and the CO inhibitor indomethacin was investigated in these MAC models. Many currently available chemotherapeutic drugs are cytotoxic and have relatively limited action against human solid tumours. Identification of an eicosanoid inhibitor against these chemoresistant tumours may have important implications for use in the clinic, as not only will it be directed towards a specific process required for tumour growth, but drugs would have a reduced toxicity and a larger therapeutic index. The final part of this investigation looks at the metabolism and mechanism of action of CV6504 on these tumour models. CV6504 is a bioreductive 5-LO inhibitor, which also inhibits thromboxane synthetase action and the generation of lipid peroxides, that has a profound antitumour action against these tumour models.

Chapter 2.
Materials.

2:0. Materials

All chemicals used for tissue culture were tissue culture grade. Chemicals used for analytical techniques were ANALAR grade, whilst solvents (unless otherwise stated e.g. for HPLC,) were GPR.

2:1. Animals

Pure strain NMRI mice were obtained from Aston University breeding colony.

2:2. Tissue Culture

Bovine Serum Albumin, fatty acid free (BSA)	Sigma
Foetal bovine serum (FCS)	Gibco
Dulbecco's Modified Eagle Medium (DMEM) without sodium pyruvate, with 4500mg/l glucose, with nonessential amino acids.	Gibco
Dimethyl sulphoxide (DMSO)	Sigma
EDTA (disodium salt)	Sigma
Phenol red	Sigma
Phosphate Buffered Saline (PBS) without calcium and magnesium x10 strength	Gibco
Nunc 50ml (T25) flasks	Gibco
Nunc 250ml (T70) flasks	Gibco
Nunc 24-well multi-dishes	Gibco
Nutrient Mixture F-12 HAM with glutamine	Gibco
RMPI1640 with 1mM Glutamine and 25mM Hepes Buffer	Gibco
0.5% Trypsin : 0.25% EDTA x 10 strength	Gibco

2:3. Chemicals

Acetone	Fisons
Arachis oil (Peanut oil)	Sigma
14% Boron trifluoride methanol	Fisons
n-Butanol	Fisons
Butylated hydroxytoluene (BHT)	Sigma

Ethanol	Fisons
Ethyl acetate	Fisons
Evans Blue	Sigma
Chloroform	Fisons
Glacial acetic acid	Fisons
ß-Glucuronidase (Helix Pomatia)	Fisons
n-Hexane	Fisons
Halothane	ICI Chemicals Ltd.
Hydrochloric acid	Fisons
Insulin	Sigma
Kodak D19 Developer	Sigma
Kodak Fixer	Sigma
Liquid paraffin	Pharmacist
Methanol	Fisons
NTB-3 High Sensitivity Autoradiation Emulsion	Kodak Chemicals Ltd.
Optiphase HiSafe II	Fisons
Optiphase HiSafe III	Fisons
Paraffin wax	Fisons
PBS tablets (with calcium and magnesium)	Gibco
Phosphotungstic acid	Sigma
Rat and mouse breeding diet	Pilsbury Ltd.
Sodium chloride	Fisons
Sodium hydroxide	Fisons
Sodium sulphate	Fisons
Sulphuric acid	Fisons
1,1,1-Tetramethoxypropane	Sigma
2-Thiobarbituric acid (TBA)	Sigma
Transferrin (human)	Sigma

2:4. Gases

Helium, Carbon Dioxide, Hydrogen, and Nitrogen (Oxygen free)	BOC. Ltd
Nitrous Oxide and Oxygen	Medical Gases BOC Ltd

2:5. GC. Analysis

Column:30M DB-23 Narrow Bore Capillary Column	Jones Chromatography Ltd.
Alpha -linoleic methyl ester	Sigma
Arachidonic acid methyl ester	Sigma
Docosahexaenoic acid (DHA) methyl ester	Sigma
Eicosapentaenoic (EPA) methyl ester	Sigma
Mead acid methyl ester	Sigma
Gamma-linolenic acid (GLA) methyl ester	Sigma
Margaric acid (heptadecaenoic acid)	Sigma
Margaric acid (heptadecaenoic acid) methyl ester	Sigma
Oleic acid methyl ester	Sigma
Palmitic acid methyl ester	Sigma
Stearic acid methyl ester	Sigma
Standard 1. Palmitic acid methyl ester	Sigma
Stearic acid methyl ester	
Oleic acid methyl ester	
Linoleic acid methyl ester	
Alpha-linolenic acid methyl ester	
Standard 2. Octadecatetraenoic acid methyl ester	Sigma
Arachidonic acid methyl ester	
DHA methyl ester	
EPA methyl ester	

2:6. HPLC Analysis

Column: μBondapak C18 (3.9mm x 300mm)	Waters
Hydroxyeicosatetraenoic acids (HETEs)	

HPLC Standard 1:(±)5-HETE (±)12-HETE (±)15-HETE	Sigma
HPLC Standard 2:(±)8-HETE (±)9-HETE (±)11-HETE	Sigma
(±)5-HETE	Sigma
(±)8-HETE	Cascade Biochem Ltd.
(±)9-HETE	Sigma
(±)11-HETE	Cascade Biochem Ltd.
(±)12-HETE	Sigma
(±)15-HETE	Sigma
Acetic acid (HPLC grade)	Fisons
Acetonitrile Far UV (HPLC grade)	Fisons
Methanol (HPLC grade)	Fisons
Water (HPLC grade)	Fisons

2:7. Inhibitors

BWA4C and BWB70C were kindly provided by Dr L. G. Garland, The Wellcome Research Laboratories (Kent, UK).

CV6504 and Metabolites were kindly provided by Takeda Chemical Ltd. (Japan)

Indomethacin — Sigma

L-660,711 was kindly provided by Merck Frost Laboratories

Zileuton was kindly provided by Abbot Laboratories

2:8. Polyunsaturated Fatty Acids (PUFAs)

Arachidonic acid (99%)	Sigma
Docosahexaenoic acid (DHA) (99%)	Sigma
Eicosapentaenoic acid (EPA) (99%)	Sigma
Gamma-linolenic acid (GLA) (99%)	Sigma

Hydroxyeicosatetraenoic acid (HETEs) see HPLC analysis

Hyroperoxyeicosatetraenoic acids (HPETEs)

5(S)-HPETE	Cascade Biochem. Ltd.
12(S)-HPETE	Cascade Biochem. Ltd.
15(S)-HPETE	Cascade Biochem. Ltd.
Linoleic acid (LA) 99% for *In Vitro* studies	Sigma
Linoleic acid (LA) 95% for *In Vivo* studies	Sigma

2:9. Radioactive Chemicals

[^{3}H]-Arachidonic acid (specific activity 184.60 Ci/mmol)	Du Pont
[^{14}C]-CV6504 (specific activity 41.9 μCi/mg)	kindly donated by Takeda Chemicals Ltd.
[^{125}I]-5-Iodo-2'deoxyuridine (specific activity 2000 Ci/mol)	Amersham International Ltd.
methyl[^{3}H]thymidine (specific activity 5Ci/mmol)	Amersham International Ltd

2:10. Name and Addresses of Suppliers

Aston University

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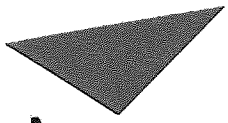

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Chapter 3.
Methods.

3:1. *In Vitro* Methods

3:1:1 Subculture and Maintenance of Cell Lines

MAC13, MAC16 and MAC26 cell lines were provided by Prof. J. A. Double and Dr. M. C. Bibby (University of Bradford, UK). The cell lines were originally derived from colon tumours induced by 1,2,-dimethylhydrazine DMH in NMRI mice (Double et al 1975). Cells were stored under liquid nitrogen, at 2 x 10^6 cells per ml in RPMI1640 culture media supplemented with 20% foetal calf serum (FCS) and 10% dimethyl sulphoxide (DMSO). On resurrection from liquid nitrogen storage, cells were kept at a relatively low passage number (<20), to minimise any differentiation that may occur from continuous culture.

MAC13 and MAC26 cell lines were maintained in RPMI1640 culture medium supplemented with 10% FCS. Doubling times were every 18 and 20 hr respectively. Cells were initially seeded at 0.5 x 10^4 cells per ml and subcultured at 3-4 day intervals as they approached confluence. Cells grow as a monolayer and trypsinisation was required prior to subculture. Cells were washed in calcium and magnesium free phosphate buffered saline (PBS), then trypsinised in 0.05% trypsin: 0.025% EDTA diluted in calcium and magnesium free PBS. MAC16 cells grow as a suspension culture with a doubling time of 32 hr. Cells that attach to the flask can be removed by a firm tap. Cells were seeded at 2 x 10^4 cells per ml and subcultured in RPMI1640 supplemented with 5% FCS every 4-5 days..

The RPMI used for all cell lines contained 1mM glutamine and 25mM Hepes Buffer. Cells were gassed with 5% CO_2:95% air and incubated in a humid atmosphere at 37°C, either in Nunc 50ml or 250ml flasks. Cell suspensions were counted using a ZM Coulter Counter with settings determined by particle sizing and remaining constant for each cell line throughout. Experiments requiring results as cell counts were carried out in triplicate on three cell pools. All experiments were carried out in 24 well multi-dishes, unless otherwise stated, each well containing 1ml of cell suspension. MAC13 and MAC26 cells in normal serum were seeded at 0.5 x 10^4 cells per ml, whilst MAC16 cells were seeded at 2 x 10^4 cells per ml. Cells were incubated

for 72 hr prior to counting.

In reduced serum a level of serum was required that allowed cell viability but not optimal cell growth. This was determined by carrying out growth curves over 144 hr in reduced serum levels. Serum levels appeared to fit these criteria between 1.0 % and 2.5 % for MAC26 cells or 0.5% and 1.0% for MAC13 cells. MAC16 cells are quiescent in serum free medium, but have been shown to be stimulated by mouse serum in serum free medium containing 10μg/ml transferrin and 20μg/ml insulin. 0.1% FCS is capable of sustaining suboptimal MAC16 cell growth. 0% FCS, 0% FCS supplemented with 10μg per ml transferrin:20μg per ml insulin and 0.1% FCS were all used as conditions for the administration of polyunsaturated fatty acids (PUFAs) to MAC16 cells. MAC13 and MAC26 cells were seeded at 2 x 10^4 cells per ml, whilst MAC16 cells were seeded at 4 x 10^4 cells per ml in reduced serum. Cells were incubated for 144 hr.

3:1:2. The effect of Polyunsaturated Fatty Acids (PUFAs) on the Growth of the MAC13, MAC16 and MAC26 Cells *In Vitro.*

Free fatty acids (FA) form a complex with serum albumin in the blood for transport to target organs. PUFAs were given to MAC cells *in vitro* complexed with fatty acid free bovine serum albumin (BSA) as it was thought to be more representative of transport to the tumour *in vivo* . Linoleic acid (LA), arachidonic acid (AA), γ-linolenic acid (GLA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were dissolved gram:gram in double distilled water with BSA. This was neutralised by equimolar sodium hydrogen carbonate ($NaHCO_3$) and sonicated so the complexed PUFAs formed micelles. Cells were seeded and incubated for 2 hr prior to the administration of PUFAs. 10μl of PUFA solution was added to the wells and control wells received BSA that had been neutralised and sonicated in the same way as the complexed PUFAs. In normal serum cells were incubated for 72 hr, whilst in reduced serum cells were incubated for 144 hr prior to counting.

Hydroxyeicosatetraenoic acids ((±)-HETEs) and hydroperoxyeicosatetraenoic acids ((S)-HPETEs) are more susceptible to peroxidation than PUFAs (Spectre and Yorek 1988). (±)-HETEs and (S)-HPETEs were therefore dissolved in ethanol so

5μl aliquots per ml of cells gave concentrations of 0 to 6.4μM, with control wells receiving 5μl of ethanol. Cells were seeded in reduced serum and incubated for 2 hr prior to the administration of (±)-HETEs or (S)-HPETEs. Cells were incubated for 144 hr and then counted.

3:1:3. The Effect of Indomethacin and Lipoxygenase Inhibitors on the Growth of the MAC13, MAC16 and MAC26 Cells *In Vitro*

Indomethacin was selected as a cyclooxygenase inhibitor. BWA4C, BWB70C, CV6504, L-660,711 and Zileuton were selected as lipoxygenase inhibitors as they were considered to be both representative of the potential inhibitory mechanisms (McMillan and Walker 1992) and were at advanced stages in their development, entering clinical trials for such pathological conditions as asthma and nephritis (Musser and Kreft 1992). Cells were seeded in normal levels of serum and incubated for 2 hr. Indomethacin and Zileuton were dissolved in ethanol, BWA4C and BWB70C were dissolved in DMSO, whilst CV6504 and L-660,711 were dissolved in water. A 5μl aliquot of inhibitor gave a concentration range of 0.5μM to 100μM. Control wells received a 5μl aliquot of inhibitor diluent. Cells were counted 72 hr after the administration of inhibitor.

The more potent inhibitors of BWA4C and CV6504 along with indomethacin were tested for inhibitory action against MAC13 and MAC26 cells stimulated by optimal concentrations of LA and AA (see section 4:1:2.). PUFAs were complexed with BSA and neutralised as previously described (3:1:1.). Inhibitors were dissolved as described above. Cells were seeded in reduced serum levels and incubated for 2 hr. PUFAs were added in 5μl aliquots and the cells were incubated for a further 2 hr. Inhibitors were added in 5μl aliquots to give a concentration range of between 0.5μM to 100μM. Cells were incubated for 144 hr prior to counting.

(±)-HETEs and (S)-HPETEs were added to MAC26 cells inhibited by BWA4C and CV6504 to see if it was possible to reverse the action of these inhibitors. Cells were seeded in normal serum and incubated for 2hr. 5μl aliquots of BWA4C and CV6504 were given per ml of cells to give a final concentration of inhibitor of 5μM and 10μM respectively. Cells were incubated for a further 2hr. (±)-HETEs and (S)-HPETEs,

as previously described, were dissolved in ethanol. Each well received a 5μl aliquot of (±)-HETE or (S)-HPETE to give a concentration range in cells inhibited by 5μM BWA4C or 10μM CV6504 of 0-6.4μM. Cells were counted 72 hr after the administration of (±)-HETEs and (S)-HPETEs.

3:1:4. Determination of the IC_{50} Value of CV6504 for A549 and Caco-2 Cells *In Vitro*.

A549 cells were grown in Nutrient Mixture F-12 HAM with glutamine, supplemented with 10% FCS. Cells grew as a monolayer and were trypsinised every 4-5 day with seeding at 0.5 x 10^4 cells per ml. Trypsinisation was in versene buffer. (A x10 stock solution containing 10 PBS tablets (with calcium and magnesium,) 371mg/kg EDTA disodium and 50mg phenol red, was prepared in 100ml of double distilled water. The pH was adjusted 7.4. 10ml of this solution was diluted to 100ml with double distilled water containing 0.05% trypsin and 0.025% EDTA.) The IC_{50} value of CV6504 was calculated by seeding the cells at 0.5 x 10^4 cells per ml in 24 well multi-dishes and incubating for 2 hr. A 5μl aliquot of CV6504 dissolved in water was given per ml of cells to give a concentration range of between 0.5μM and 100μM. Cells were incubated at 37°C for 72 hr prior to counting.

Caco-2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) without sodium pyruvate, with 4500mg/l glucose and nonessential amino acids, supplemented with 10% FCS. Cells were subcultured every 4-5 days by trypsinisation and seeded at 2 x 10^4 cells per ml in an atmosphere of 10% CO_2:90% air. Cells were seeded at 2 x 10^4 cells per ml in 24 well multi-dishes and incubated for 2 hr. CV6504 was dissolved in water and a 5μl aliquot per ml of cells gave a concentration range of 0.5μM to 100μM. Cells were incubated for 72 hr in a humid atmosphere of 10% CO_2:90% air prior to counting.

3:1:5. The Effect of CV6504 Metabolites on the Growth of MAC13, MAC16 and MAC26 Cells *In Vitro*.

In mammalian cells CV6504 is rapidly metabolised to form sulphates and glucuronides (Takeda Chemicals Ltd. unpublished results). The major metabolites are

CV6504-1-sulphate, CV6504-4-sulphate, CV6504-1-glucuronide and CV6504-4-glucuronide. These metabolites were tested for cytotoxicity against the MAC13, MAC16 and MAC26 cells *in vitro*. Cells were seeded in normal serum levels and incubated for 2 hr. CV6504 and CV6504 metabolites were dissolved in water so a 5μl aliquot added to 1ml of cells gave a concentration range of between 0.5μM and 200μM. Cells were counted 72 hr after administration of CV6504 and CV6504-metabolites.

3:2. *In Vivo* Methods.

3:2:1. Transplantation of Tumours.

Tumours were originally supplied for transplantation by Prof. J. A. Double and Dr. M. C. Bibby (University of Bradford, UK.). Transplantation of tumours in NMRI mice was performed by Mr. M. P. Wynter and Mr. W. A. Fleary at Aston University. Tumours were excised from donor animals into sterile saline, where they were cut into 1-2mm^3 fragments. These fragments were transplanted subcutaneously by trocar into the right flank of recipient animals. MAC13, MAC16 and MAC26 tumours were transplanted into male NMRI mice weighing between 20-25g. MAC16 tumours were transplanted into female mice between 18-22g. Unless stated otherwise tumour-bearing animals were male. After transplantation mice were fed on rat/mouse breeding diet and water *ad libitum.*

The initiation of experiments was dependent on tumour volume. The dimensions of the tumour were measured by callipers and the tumour volume calculated from the formula:

$$\text{Volume} = \frac{\text{length} \times (\text{width})^2}{2}$$ (measurements in mm, volume in mm^3)

Initial tumour volumes for all experiments were between 72mm^3 and 128mm^3. This was between 12-14 days after transplantation in MAC26 tumour-bearing mice and 9-11 days after transplantation for either MAC13 or MAC16 tumour-bearing mice. Body weight was monitored in all tumour-bearing animals and recorded daily in MAC16 tumour-bearing mice due to the tumour-induced cachexia produced.

Experiments were continued until tumour volumes reached 1000mm^3 where the tumour became neither ulcerative or necrotic. Animals were sacrificed if they lost 20% of their body weight due to cytotoxicity or 30% of their body weight due to cachexia. Experiments were also terminated if the animals became moribund. Each experimental group contained at least 9 animals.

3:2:2. The Dosing of Tumour-Bearing Mice with Linoleic Acid (LA).

LA was administered daily by gavage in the 'free acid' form from when the tumours became palpable. LA was diluted in arachis oil (peanut oil). Arachis oil consists of the following FAs in the triglyceride form: 2% palmitic acid, 10% stearic acid, 50%

oleic acid and 25% LA. Doses of LA were between 0.04 g/kg and 2.0 g/kg administered in a glass syringe to minimise the generation of lipid peroxides which may occur with plastic syringes.

3:2:3. The Dosing of Tumour-Bearing Mice with Indomethacin and Lipoxygenase Inhibitors.

Treatment began when tumours became palpable (72-128mm^3). Indomethacin was dissolved in 0.1% ethanol and given daily as a 0.1ml intraperitoneal injection (ip) giving a dose of 5mg/kg. BWA4C and BWB70C were suspended in liquid paraffin and given every 12 hr orally at doses of 5mg/kg to 25 mg/kg from when tumours became palpable. CV6504 was dissolved in water and given orally to mice with palpable tumours between 1mg/kg and 50mg/kg daily by gavage. CV6504-metabolites were given to tumour-bearing animals with palpable tumours at 50 mg/kg daily by gavage. LA dosed in conjunction with inhibitor was given at 1.0 g/kg orally, dissolved in arachis oil.

3:2:4. The Effect of Linoleic Acid (LA) on the Growth Kinetics of the MAC26 Tumour.

The cell loss and potential doubling times of the MAC26 tumour, with and without daily administration of 2.0g/Kg LA were calculated by the method outlined by Gabor et al 1985

When MAC26 tumours became palpable, 12 days after the transplantation of the tumours, animals were split into two groups and maintained on potassium iodide in their drinking water. This saturates iodine metabolism in the thyroid and other organs enhancing the uptake of [^{125}I]-5-iodo-2'-deoxyuridine (^{125}I-Udr) into the tumour (Gabor et al 1985). The control group received 50μl of saline daily by gavage whilst the LA treated group received 50μl of LA (2.0 g/kg). LA was given at 2.0g/kg rather than 1.0g/kg so a third group of mice did not have to be introduced into the experiment, dosed with arachis oil. Animals were dosed and tumour volume measured for 3 consecutive days. On the fourth day prior to dosing each mouse received and an ip injection (0.1ml) of 20μCi of ^{125}I-Udr (specific activity 2000 Ci/mmol) in saline. Mice were then dosed with LA. 2 hr later 4 mice from each group were sacrificed by

cervical dislocation, the tumour was removed weighed, and stored at -70°C for 24 hr prior to freezing. On the next 4 consecutive days 4 mice from each group were sacrificed 2 hr after dosing, tumours were removed and treated as described above.

To determine the tumour cell DNA-radioactivity tumours were minced into 1-2mm^3 pieces. Acid soluble material was removed by 3 washes over 72 hr in of 3:1 methanol:acetic acid (volume:volume). The radioactivity within the tumour was determined using a Pakard Tricarb Gamma-Scintillation Counter. The half-life of the tumour radiolabelled DNA (T 1/2) was determined by plotting cpm/g of tumour against time on semi-log paper. T 1/2 is the time it would take the tumour to double in size if there is no cell loss and is referred to as the potential doubling time (TP). The actual doubling time is the actual time it took for the tumour to double in size as measured by callipers. This was calculated by plotting the tumour volume against time and is referred to as the actual doubling time (TD). From calculation of TP and TD it is possible to calculate the cell loss within the tumour (Ø):

$$\% \text{ Ø} = 1 - \frac{TP}{TD} \times 100$$

(Steel 1977).

3:2:5. The Determination of the Region of the MAC26 Tumour Stimulated by Linoleic Acid (LA) using Autoradiography.

MAC26 tumour-bearing mice were divided into two groups when tumours became palpable 10 days after transplantation. Control mice received 50μl of saline daily by gavage whilst LA treated animals received 50μl of LA (which is 1.0g/kg LA). This concentration was chosen as it stimulates maximum cell growth but can be dosed as neat LA, neither requiring dilution in arachis oil or introduction of another control group to the experiment. Mice were dosed daily and tumour volume measured for 7 consecutive days. On day 8 tumour volume was measured but mice received an IP injection (0.1ml) containing 50μCi of [methyl-^{3}H]thymidine (^{3}H-Tdr) (specific activity 5Ci/mmol) in sterile saline. Mice were dosed and 4 hr latter 3 mice were sacrificed by cervical dislocation, tumours removed and fixed in Bouins Fluid. On day 9, mice were dosed with LA and 24 hr after the initial injection with ^{3}H-Tdr, were killed by cervical dislocation and tumours dissected out.

Tumours were fixed for 24 hr in Bouins Fluid and transferred to 70% alcohol for 1 week. They were dehydrated, cleared in chloroform and embedded in paraffin wax. Sections were cut 10 microns thick and dipped in NTB-3 high-sensitivity autoradiography emulsion, which records all charged particles. The sections were left for 3 weeks in light proof boxes prior to development with Kodak D19 developer and fixer. Sections were then examined under the microscope.

3:2:6. The Determination of the Blood Volume of the MAC26 Tumour by Evans Blue.

Angiogenesis has been reported to be a requirement for tumour growth when tumour volume exceeds 1-2mm^3 (Folkman 1986). When MAC26 tumours become palpable at 72-128mm^3 it seems likely that neovasculisation may have already been established. To investigate the effect of LA on the vasculature of the MAC26 tumour, treatment was initiated the day after the transplantation of the tumour. As LA was to be administered over a longer period of time it was given at a lower dose of 1.0 g/kg in arachis oil. There were two control groups one receiving 100μl of saline and the other 100μl of arachis oil, which also gave the opportunity to see whether LA in the triglyceride form can influence the growth of MAC26 tumours when given over a prolonged period of time. Animals received either 100μl of saline, 100μl of arachis oil or 1.0 g/kg LA dissolved in arachis oil daily by gavage for 21 days. Tumour volume was measured from day 14 when they became palpable. The blood volume of the tumours was calculated by Evans Blue dye when tumour volumes were <400mm^3. Before the animal was sacrificed 1mg of Evans Blue dye contained in 0.1ml of saline was injected into the tail vein. 1 min latter 100μl of whole blood was collected by cardiac puncture under anaesthesia, with Boyles apparatus using a halothane, oxygen and nitrous oxide mixture. The blood was diluted in 30:70 acetone:5% aqueous sodium sulphate (volume:volume). The mouse was sacrificed by cervical dislocation and the tumour removed immediately, weighed and minced in 70:30 actone:5% aqueous sodium sulphate. Samples were left 24 hr to extract.

A calibration curve between 0-20μM Evans Blue was constructed from the absorption at 620nm on a Beckman DU-70 Spectrophotometer. Samples were

A calibration curve between 0-20μM Evans Blue was constructed from the absorption at 620nm on a Beckman DU-70 Spectrophotometer. Samples were centrifuged at 1500g for 5 min then read at 620nm. From these readings it was possible to evaluate the blood volume and the blood volume per gram of tumour.

$$\text{Blood Volume (ml)} = \frac{\text{Dose of Evans Blue}}{\text{Evans Blue } \mu\text{g/ml of blood}} - \text{Injection volume}$$

$$\text{Blood Volume per gram of tumour (ml)} = \frac{\text{Evans Blue } \mu\text{g/g of Tumour}}{\text{Evans Blue } \mu\text{g/ml of Blood}}$$

3:2:7. The Measurement of Lipid Peroxide Levels in Blood.

Lipid peroxide levels were determined in the blood of control and CV6504 treated tumour-bearing mice. Animals were terminated by the criteria described in section 3:2, and 200μl of blood was taken by cardiac puncture, as previously described in section 3:2:6., for the determination of lipid peroxide levels. This 2-thiobarbituric acid (TBA) method was devised by Yagi (1982) to isolate lipid peroxides in biological samples from TBA reacting substances. The principle of this method is to isolate lipid peroxides by co-precipitating them with protein using phosphotungstic acid and sulphuric acid, then determining their amounts by TBA reacting substances in acetic acid.

The 200μl of whole blood was diluted in 9.8ml of saline. This was centrifuged at 15000g for 10 min. 100μl of the supernatant was added to 4ml of 12N sulphuric acid (H_2SO_4) and mixed gently. 0.5ml of 0.5% phosphotungstic acid was added and mixed. The samples were left to react at room temp for 10 min, then centrifuged at 3000g for 10 min. The supernatant was discarded and the pellet redissolved in 2ml of 12N H_2SO_4 and 0.3ml of 0.5% phosphotungstic acid. The samples were again centrifuged at 3000g for 10 min, and the sediment dissolved in 4ml of distilled water. TBA reagent was prepared by dissolving 0.67% TBA in aqueous solution and diluting 1:1 with glacial acetic acid, 1ml of this reagent was added to each sample. A standard was prepared of 0.5nM tetramethoxypropane in 4ml of distilled water. 1ml of TBA reagent was added. Both samples and standard were heated to 90°C for 1 hr, and cooled rapidly under running water. They were then extracted by shaking vigorously with

Spectrophotometer. Excitation was at 515nm with emission at 553nm. Serum lipid peroxide levels were calculated by the formula:

$$\text{Serum Lipid Peroxides} = \frac{f}{F} \times \frac{1.0}{0.02} = \mu M \text{ in Serum}$$

Where f is the fluorescence of the sample and F is the fluorescence of the standard.

3:3. Analytical Methods

3:3:1. The Determination of the Fatty Acid (FA) Content of Plasma, Tissues and Cells by Gas Chromatography (GC).

3:3:1:1. Preparation of Plasma, Tissues and Cells for Fatty Acid (FA) Extraction.

MAC16 tumour-bearing mice were treated daily by gavage for 7 days after the tumour became palpable with either 0.1ml saline, 1.0 g/Kg LA , 1.0 g/Kg LA + 10 mg/Kg CV6504, 25 mg/Kg CV6504 or 10 mg/Kg CV6504. Animals were anaesthetised with a halothane, oxygen and nitrous oxide mixture using Boyles apparatus. Blood was taken by cardiac puncture into heparinised microfuge tubes. Plasma was separated from blood cells by centrifugation at 15000g for 5 min. A 0.01 volume of butylated hydroxytoluene (BHT) in 50% ethanol was added to each sample to prevent oxidation during manipulation and extraction. Samples were stored under nitrogen at -70°C prior to extraction. Livers and tumours were dissected out of the animal on sacrifice by cervical dislocation. The tissues were snap frozen and stored under nitrogen at -70°C prior to extraction.

MAC13, MAC16 and MAC26 cells were grown in normal serum in Nunc flasks until they approached confluence. MAC13 and MAC26 cells were trypsinised (as described in 3:1:1) to detach the cells. The cells were counted and enough cell suspension was used to form a pellet containing 5 x 10^6 cells. The cell pellet was washed twice in PBS. The cells were sonicated and FAs extracted immediately as described below.

3:3:1:2. The Extraction of Fatty Acids (FAs) from Plasma, Tissues and Cells.

The extraction of fatty acids from plasma, tissues and cells was based on the method of Folch et al (1957). Tissue samples were homogenised in 10 volumes of 2:1 chloroform:methanol (volume:volume) containing an internal standard of 50μg margaric acid and 0.01 volume of BHT dissolved in 50% ethanol. Cell pellets consisting of 5 x 10^6 cells were also suspended in 10 volumes of 2:1 chloroform:methanol (volume:volume) with 50μg of internal standard and 0.01 volume of BHT. Cells were sonicated for 3 pulses of 15 sec with 10 sec intervals between each pulse. 200μl of plasma sample was extracted in 2:1 chloroform:methanol (volume:volume) containing 50μg of internal standard.

Extraction of FAs from cells, tissues and plasma then followed the same protocol outlined below.

Organic and inorganic layers were separated by addition of 0.2 volumes of distilled water and 1ml of methanol, followed by centrifugation at 1500g for 5 min. The organic layer was transferred to a boiling tube for saponification by heating to 100°C in 2.5ml of 5% sodium hydroxide (NaOH) in 50% methanol under nitrogen for 45-60 min. The samples were cooled and acidified to pH 2.0 with concentrated hydrochloric acid (conc. HCl). The extracted FAs were then methylated by heating to 90°C for 5 min with 2.5ml of 14% boron trifluoride in methanol. The cooled mixture was then extracted with 2 washings each containing 2ml of 4:1 hexane:chloroform (volume: volume). The volume was reduced to dryness by rotary evaporation and the extracted FA methyl esters were dissolved in 200μl of n-hexane. Samples were stored at -70°C under nitrogen until analysed by GC.

3:3:1:3. The Determination of Fatty Acid Methyl Esters by Capillary Gas Chromatography (GC).

Analysis of the FA methyl ester content of extracted samples was determined using a Hewlett Packard 5890 Series II Gas Chromatograph connected to a Hewlett Packard HP3396A Integrator. The GC was fitted with a 30m DB-23 narrow bore capillary column. The injector temperature was set at 190°C and the flame ionisation detector at 240°C. The column head pressure was at 21psi, with a split ratio of between 5 to 8 m/min and the optimal linear velocity lying between 20-25cm. Samples were run on a temperature programmed run with initial temperature at 180°C for 5 min, followed by a 5°C per min rise in temperature to 220°C, which was held for 15 min.

Peaks were identified using authentic standards. Margaric acid was used as an internal standard as it is not usually present in detectable amounts in mammalian tissue. The amount detected by the GC will be the 50μg originally added to the samples before extraction. From this it was possible to work out both the composition of FAs and concentration of these FAs in the original tissues, plasma or cells.

3:3:2. The Determination of the Lipoxygenase (LO) Pathways of the MAC13, MAC16, and MAC26 Cells *In Vitro* and Tumours *Ex Vivo* from the production of Hydroxeicosatetraenoic Acids (HETEs).

3:3:2:1 Preparation of Hydroxyeicosatetraenoic Acids (HETEs) from Cells *In Vitro.*

MAC16 cells were suspended in RPMI1640 in normal serum levels at 5 x 10^5 cells per ml. A concentration of 10μM of AA containing 25μcurries of [^{3}H]-AA (specific activity 221 Ci/ mmole) was added and incubated at 37°C for 2 hr. MAC13 and MAC26 cells were incubated in 250ml flasks with 10μM of AA containing 25μcurries of [^{3}H]-AA for 2 hr. Lipoxygenase inhibitors and indomethacin were added 24 hr prior to the administration of AA. The reaction was stopped by the addition of 1N HCl. MAC13 and MAC26 cells were trypsinised (as described in 3:1:1.). All three cells lines were centrifuged to form a cell pellet containing 5 x 10^6 cells. This was washed twice in PBS and resuspended in 0.8ml of ice-cold PBS. Cells were sonicated by 3 15 sec. pulses with 10 sec intervals. HETEs were extracted immediately.

A459 and Caco-2 cells were grown in normal serum levels until they nearly became confluent. 10μM AA containing 25μcurries of [^{3}H]-AA was added for 30 min or 1 hr respectively, as at these time intervals maximum [^{3}H]-AA was incorporated into the cells. The reaction was stopped with 1M HCl. Cells were also incubated with 10μM CV6504 24 hr prior to the administration of 10μM AA and containing 25μcurries of [^{3}H]-AA. Cells were trypsinised and centrifuged to form a cell pellet containing 5 x 10^6 cells. The pellet was washed twice with PBS and suspended in 0.8ml of ice-cold PBS. Cells were sonicated by 3 15 sec pulses with 10 sec intervals. HETEs were extracted immediately.

3:3:2:2. The Preparation of Hydroxyeicosatetraenoic acids (HETEs) from MAC13, MAC16 and MAC26 Tumours *Ex Vivo.*

Tumour-bearing animals were sacrificed by cervical dislocation. Tumours were immediately excised and homogenated in RPMI1640 containing 10% FCS. Cells were washed in RPMI1640 and resuspended at 5 x 10^5 cells per ml. They were incubated at 37°C in a humid atmosphere of 5% CO_2:95% air with 10μM AA containing

25μcurries of ^{3}H-AA. After 1 hr the maximum ^{3}H was recovered from tumour cells. CV6504 was added to suspensions of tumour cells homogenates at 10μM for 30 min prior to the administration of 10μM AA containing 25μcurries of ^{3}H-AA. 100μM of CV6504 metabolites were incubated for up to 2 hr with tumour homogenates. 10μM AA containing 25μcurries of ^{3}H-AA was added at 30 min, 1 hr and 2 hr after the administration of CV6504 metabolites. Cells were centrifuged and the pellet washed twice with PBS. 5 x 10^{6} cells were suspended in 0.8ml ice-cold PBS. Cells were sonicated by x3 15 sec pulses with 10 sec intervals and HETEs were extracted immediately.

3:3:2:3. The Extraction of Hydroxyeicosatetraenoic Acids (HETEs) from Sonicated Cells and Tissues.

The pH of sonicated cells and tumours was adjusted to 3.5 with 1N HCl prior to suspension in 1:2 chloroform:methanol (volume:volume). The solution was vortexed for 1 min and left to extract for 30 min at room temperature. 1ml of chloroform was added and the solution vortexed for a further 10 sec. 1ml of ice-cold 0.001N HCl was added and again the solution was vortexed for 10 sec. The layers were separated by centrifugation at 2000g for 20 min. The HETEs were further extracted by 2ml of chloroform. The chloroform was evaporated under a stream of nitrogen and the HETEs resuspended in 100μl of acetonitrile (far UV HPLC grade).

3:3:2:5. The Determination of Hydroxyeicosatetraenoic Acids (HETEs) by High Pressure Liquid Chromatography (HPLC).

5, 11, 12 and 15-HETEs were determined by HPLC using a method adapted from Lui et al 1994. A μBondapak C18 column (3.9mm x 3000mm) was attached to a Shimazdu System with the UV detector set at 237nm and absorbance at 0.005. There was a constant flow rate of 1.5ml per min. Solution A was 58% containing 20:100:0.05 acetonitrile:water:acetic acid (volume:volume:volume) and solution B was 42% containing 100:0.05 acetonitrile:acetic acid (volume:volume). Resolution of both HPLC Standard Solution 1 ((±)5-HETE, (±)12-HETE and (±)15-HETE) and HPLC Standard Solution 2 ((±)8-HETE, (±)9-HETE and (±)11-HETE) were high

under these conditions. (±)5-HETE, (±)11-HETE, (±)12-HETE and (±)15-HETE were identified using authentic standards.

50μl of extracted HETEs was injected using an auto-analyser, and the fractions corresponding to (±)15-HETE, 11(±)-HETE, (±)12-HETE, (±)5-HETE and AA were collected. The concentration of these fractions was determined by counting the ^{3}H recovered in Optiphase Hisafe III on a 2000-Ca Tri-Carb Liquid Scintillation Analyser.

3:3:3. The Evaluation of CV6504 Metabolism in MAC16 Tumour-Bearing NMRI Mice.

[^{14}C]-CV6504 (sp. act. 41.9 μCi/mg) was administered at 10 mg/Kg orally in water to MAC16 tumour-bearing NMRI mice with tumour volumes above 240mm^3. Samples of plasma were taken at 15 min, 30 min, 2 hr and 24 hr after dosing by cardiac puncture (as described in 3:3:1:1.); animals were killed immediately by cervical dislocation, and the tumours, livers and kidneys were dissected out. A further group of mice were treated with 6 doses of 10 mg/Kg [^{14}C]-CV6504 daily orally in water when the tumour became palpable (72-128mm^3). Plasma, liver, tumour and kidney samples were taken as for the other time points 24 hr after the final dose.

Plasma samples from each time point were pooled. Tumour, liver and kidney samples were homogenised in ice-cold distilled water to form a 20% (weight:volume) homogenate. The pH of plasmas and homogenates was adjusted to 6.0 with 1N HCl. Glucuronide metabolites can be metabolised to free CV6504 by ß-glucuronidase, where as sulphate metabolites are resistant (Takeda Chemicals Ltd. unpublished results). Hydrolysis of CV6504-glucuronides, but not CV6504-sulphates by ß-Glucuronidase, to CV6504 was used to determine the concentration of the respective metabolites as they are not resolvable by TLC. Unchanged CV6504 or hydrolysed CV6504-glucuronides separated from CV6504-sulphates. Samples were run before and after treatment with ß-Glucuronidase to determine the concentration of the metabolites to unchanged CV6504. The samples were split into two halves. One set was treated with equal volumes of 10% ß-glucuronidiase (from Helix Pomatia) and incubated for 16 hr at 37°C. All samples were extracted by addition of 5 volumes of

methanol. Then centrifuged at 3000g for 10 min. The supernatant was removed and evaporated to dryness <40°C and redissolved in 100μl of methanol.

Metabolites and unchanged CV6504 were separated by thin layer chromatography (TLC). Standards of normal metabolites formed in mammalian cells (see figure 31) and unchanged CV6504 were run alongside the samples on general purpose silica gel plates containing a 254nm UV indicator. The solvent used was 50:10:1 ethyl acetate:methanol:acetic acid (volume:volume:volume). Spots were visualised under a UV lamp at 254nm. The concentration of unchanged CV6504 and metabolites were determined by counting the [^{14}C] recovered from TLC in Optiphase HiSafe II on a 2000 CA Tri-Carb Liquid Scintillation Analyser.

Chapter 4.

The Requirements of Polyunsaturated Fatty Acids (PUFAs) and Evaluation of Lipoxygenase (LO) Pathways in Murine Adenocarcinoma Cells (MACs) *In Vitro.*

4:1. The Effect of Polyunsaturated Fatty Acids (PUFAs) on the Growth of the MAC13, MAC16 and MAC26 Cells *In Vitro.*

4:1:1. Introduction

PUFAs have regulatory functions in both the proliferation and differentiation of mammalian cells (Obermeier et al 1995). The n-6 PUFAs LA and AA generally promote tumour cell proliferation (Buckman et al 1991), whilst n-3 series PUFAs such as DHA and EPA are inhibitory (Rose and Connolly 1989). Blackcurrent and evening primrose oil containing high levels of the n-6 PUFA GLA have been shown to suppress mammary tumour growth (Karmali et al 1989, Abou-El-Ela et al 1988). The FA composition of tissues, in particular cell membranes, can be modified by the PUFA content of the diet (Rose et al 1995, Hudson 1993). Cells in culture have relatively low phospholipase activities and any PUFAs added to the culture medium are avidly taken up to replenish their phospholipid pools (Obermeier et al 1995). The rate and the degree to which these PUFAs are taken up is dependent on the cell type (Tiwari et al 1988). The culture of MAC13, MAC16 and MAC26 cells in differing levels of PUFAs provided a useful model to look at the FA requirements of these cells.

Lipid peroxidation involves the oxidative breakdown of lipids (L) as shown below:

<u>Initiation</u>

$$LH \text{ --------- } L^{\cdot} + H^{\cdot}$$

<u>Propagation</u>

$$L^{\cdot} + O_2 \text{ --------- } LOO^{\cdot}$$

$$LOO^{\cdot} + LH \text{ --------- } LOOH + L^{\cdot}$$

<u>Termination</u>

$$LOO^{\cdot} + L^{\cdot} \text{ --------- } LOOL$$

$$LOO^{\cdot} + LOO^{\cdot} \text{ --------- } LOOL + O_2$$

It consists of a self propagating process initiated through the formation of a free radicals able to accept a hydrogen atom from a FA. The residual FA becomes a lipid radical that in turn can react with molecular oxygen. The products obtained react with other FAs forming hydroperoxides, a primary product of oxidation. The donating FA becomes a second lipid radical, which inturn reacts with oxygen, leading to a chain reaction. This process is continual as long as there are FAs available. The majority of primary peroxidation products are unstable, but aldehydes such as malonaldehyde,

which are secondary products, are stable and have an inhibitory effect on cell proliferation (Gonzalez 1992). Though originally thought as a dangerous and destructive process causing cell death, lipid peroxidation is now thought of as a useful process for controlling cell division and possibly being involved in apotosis (Massotti et al 1988, Buttke and Sandstrom 1994).

Peroxidation products increase membrane fluidity at their site of reaction, reducing the lateral movements of lipids and creating localised pore-like structures provoking modifications in water and ion concentration within the cell. The influx of calcium that ensues can result in cell death (Stubs and Smith 1984). The inhibitory effect of PUFAs has been shown to increase with the degree of desaturation, and hence the susceptibility to lipid peroxidation (Begin et al 1988). This peroxidation can be partially counteracted by complexing the PUFAs with bovine serum albumin (BSA), due to binding to the BSA and the antioxidant activity of the BSA (Hostmark and Lydstad 1992). In these experiments PUFAs added to cultures were complexed with BSA, as it was thought to be more representative of the *in vivo* situation as well as offering protection against peroxidation, which may inhibit any stimulatory effects.

4:1:2. Results.

The effect of LA on the growth of MAC13 and MAC26 cells in reduced serum levels of 0.5% and 1.0% respectively, over 144 hr is shown in figure 5A. Stimulation of cell growth for both cell lines was apparent from 0.02μM, becoming significant at 2.0μM. Maximum stimulation of cell growth was at 18μM, and this effect was more pronounced for the MAC26 cell line.

The effect of AA on the growth of the MAC13 and MAC26 cell lines in reduced serum levels as described above is shown in figure 5B. Stimulation of growth by AA in the MAC26 cell line was apparent from the lowest concentration at 0.02μM, reaching significance at 16μM with maximal stimulation being observed at 33μM. Maximum stimulation of MAC13 cell growth was at a lower concentration of 16μM.

The effect of LA on the growth of the MAC26 cell line in varying amounts of serum is shown in figure 6A. Growth was measured over a 144 hr period in 1.0% and 2.5% FCS. In 10% FCS growth was measured over 72 hr period. As serum levels

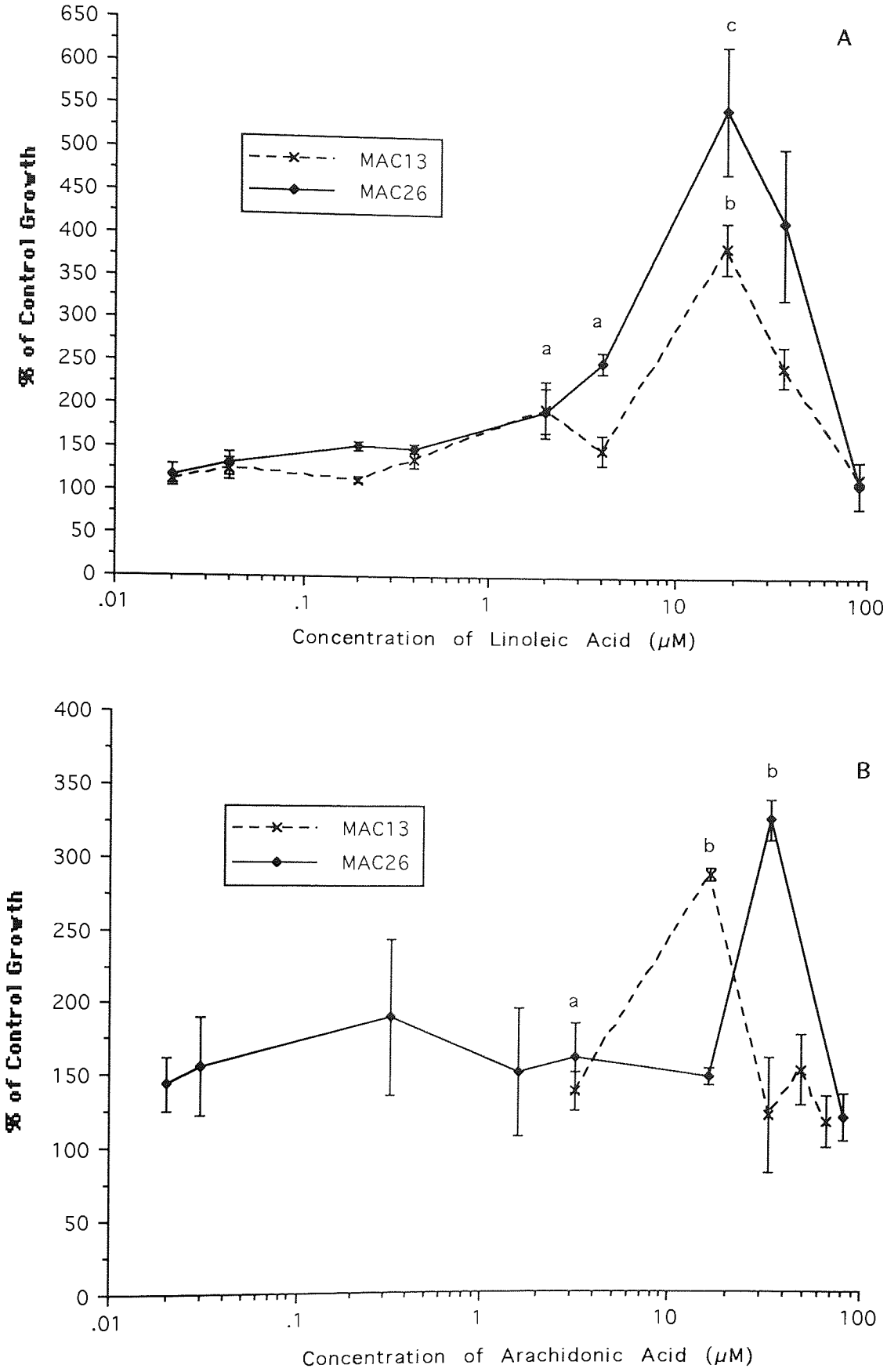


Figure 5. The effect of A. linoleic acid (LA) and B. arachidonic acid (AA) on the growth of the MAC13 and MAC26 cells over 144 hours in reduced serum (n=9).
(a = $p<0.05$, b= $p<0.01$ and c= $p<0.005$ from Control using t-test with Bonferroni Correction).

were increased the stimulation of growth was decreased in magnitude over control growth, although the same pattern of growth stimulation was observed. Significant inhibition of cell growth was observed in 10% FCS at concentration >100μM.

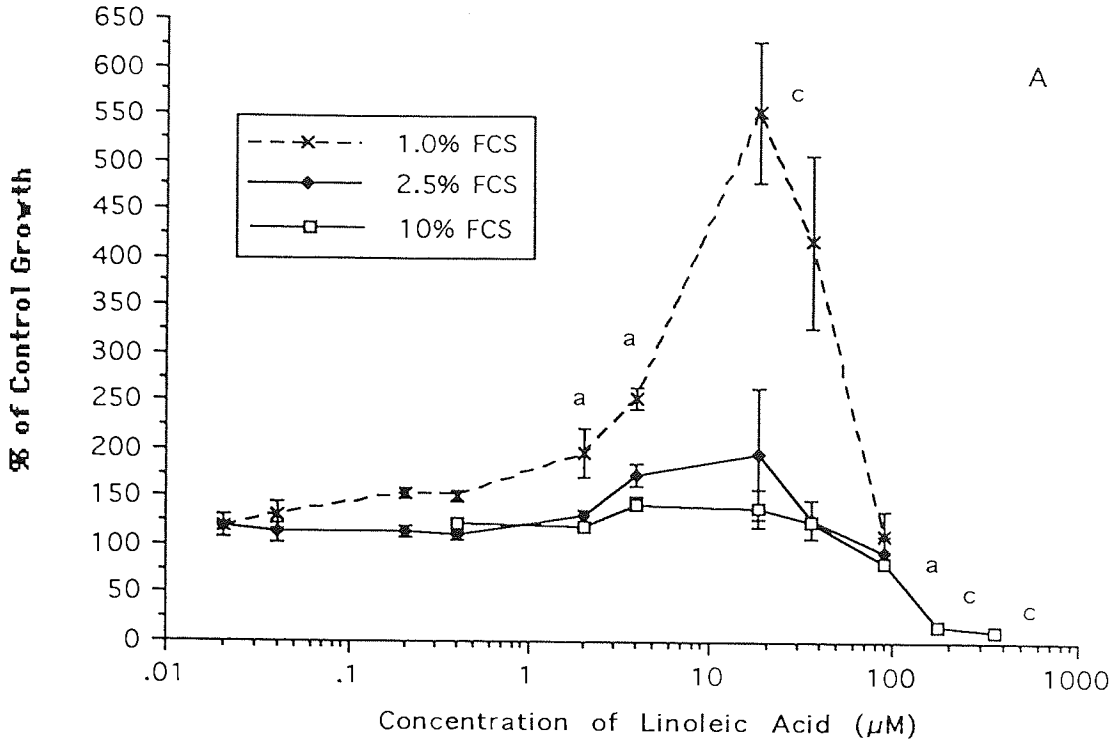
The effect of LA on the growth of the MAC13 cells over 144 hr in 0.5% FCS or 1.0% FCS, and 72 hr in 10% FCS is shown in figure 6B. The same pattern of growth was seen in 0.5% and 1.0% FCS increasing from 0.02μM and reaching a maximum at 16μM. There is no significant stimulation of growth in 10% FCS but growth inhibition commenced at 90μM becoming significant at 178 and 357μM.

The effect of AA on the growth of the MAC26 cells in 1.0% or 2.5 % FCS over 144 hr, and in 10% FCS over 72 hr is shown in figure 7a. The same pattern of growth was again shown in low serum levels, with maximal stimulation being observed at 33μM, which was significant in 1.0% serum. In 10% FCS there was no significant growth stimulation but growth inhibition is observed at concentrations >90μM.

The effect of AA in 0.5% FCS or 1.0% FCS over 144 hr, and 10% FCS over 72 hr is shown in figure 7B. Maximal stimulation of growth was at 18μM, which was again significant in both 0.5% FCS and 1.0% FCS. In 10% FCS there was no significant growth stimulation but growth inhibition was observed at concentrations >90μM.

The effect of AA and LA on the growth of the MAC16 cell line in 0% FCS, 0% FCS supplemented with 10μg/ml transferrin:20μg/ml insulin, and 0.1 % FCS over 144 hr are not shown, as there was no significant stimulation of cell growth between 0.02μM and 90μM. The results of administration of ($\pm$)5-, ($\pm$)12- and ($\pm$)15-HETE on the growth of all 3 cells lines either in normal serum levels or reduced serum levels are not shown, as again there was no stimulation of cell growth.

The effect of GLA on cell proliferation over 144 hr on the MAC13 cell line in 0.5% FCS, MAC16 in 0.1% FCS and the MAC26 in 1.0% FCS is shown in figure 8A. There was no stimulation of cell growth by GLA on the MAC16 cell line between 2μM and 90μM, though there was significant inhibition at 18μM, 33μM and 90μM. Growth was stimulated in the MAC26 cell line between 2μM and 33μM but this was not significant. Stimulation of cell growth was also observed at these concentrations in the MAC13 cell line which was significant at 18μM and maximal at 33μM. Stimulation at 33μM GLA was greater than that achieved by maximal stimulation of



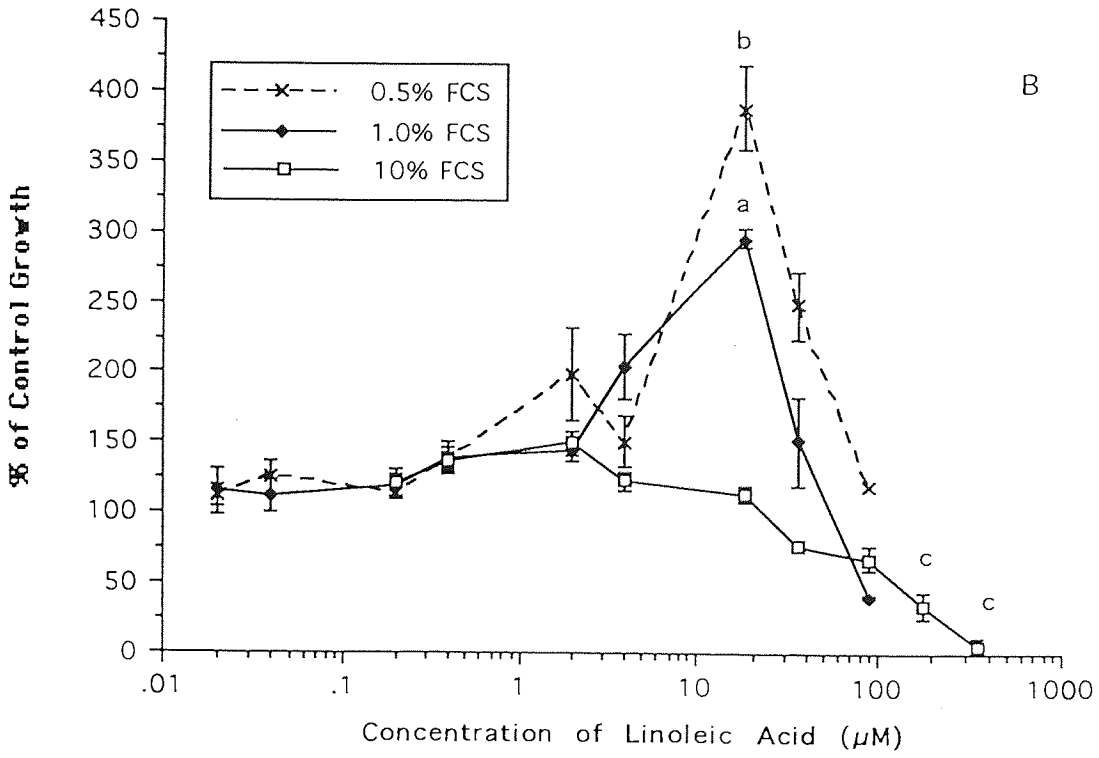


Figure 6. The effect of linoleic acid (LA) on A. MAC26 and B. MAC13 cell growth in varying serum levels (n=9). (a= $p<0.05$, b= $p<0.01$ and c= $p<0.005$ from Control using T-test with Bonferroni Correction.)

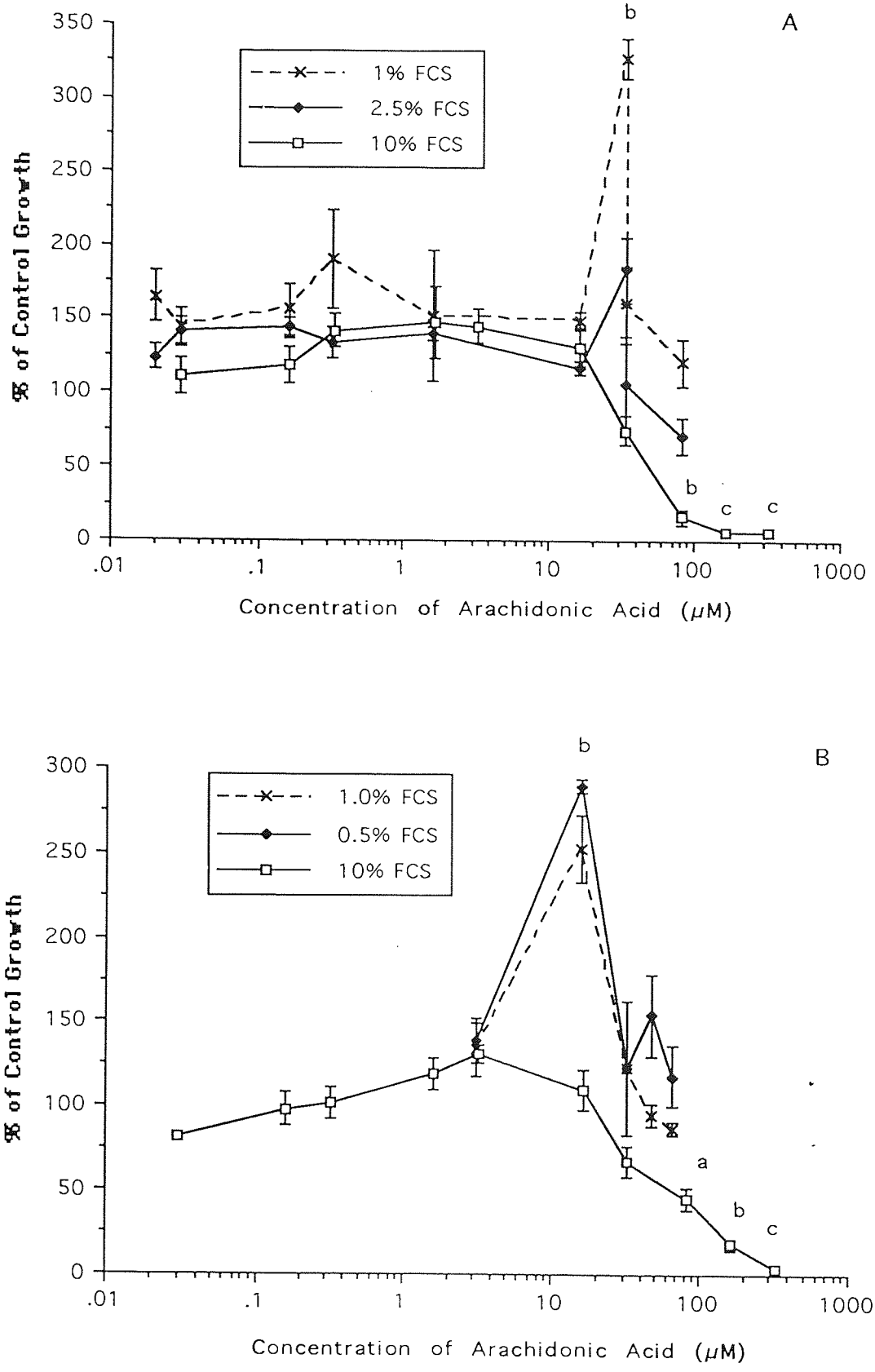


Figure 7. The effect of arachidonic acid (AA) on the growth of a. MAC26 and b. MAC13 cells in varying serum levels (n=9).
(a= $p<0.05$, b= $p<0.01$ and c= $p<0.005$ from Control using T-test with Bonferroni Correction.)

the MAC13 cell line by 18μM AA, and comparable with the stimulation achieved by 18μM LA.

The effect of GLA on cells in normal serum levels over 72 hr between 0.2μM and 90μM is shown in figure 8B. Growth of MAC16 and MAC26 cells was inhibited from 0.4μM and reached significance at 90μM. MAC13 cell growth was stimulated between 0.2μM and 18μM, though not significantly, but significantly inhibited at 90μM.

The effect of DHA over 144 hr on the growth of the MAC13 cells in 0.5% FCS, MAC16 cells in 0.1% FCS and the MAC26 cells in 1.0% FCS is shown in figure 9A. MAC13 cell growth was stimulated by DHA between 0.2μM and 15μM, with maximum stimulation at 3μM. Growth of MAC16 cells was significantly inhibited by DHA at 15μM and 30μM, whilst MAC13 or MAC26 cells were inhibited at 30μM.

The effect of DHA on cells in normal serum levels over 72 hr is shown in figure 9B. MAC16 cell growth was inhibited throughout, whilst MAC26 cells are inhibited from 1.5μM. MAC13 cell growth was slightly stimulated at 0.02μM and 0.3μM . All three cell lines are significantly inhibited at 15μM.

The effect of EPA from 0.03μM to 33μM over 144 hr on the growth of the MAC13 cells in 0.5% FCS, MAC16 cells in 0.1% FCS and MAC26 cells in 2.5% FCS, is shown in figure 10A. MAC13 cells were stimulated from 0.03 to 15μM, where maximum significant stimulation was observed. MAC16 and MAC26 cells were inhibited throughout. Significant inhibition of cell growth was observed for MAC16 cells at 18μM and for MAC13 or MAC26 cells at 33μM.

The effect of EPA on cell growth over 72 hr in normal serum is shown in figure 10B. There is significant inhibition at 83μM for MAC13 and MAC26 cell lines, which is increased at 165μM and 331μM. Significant inhibition of MAC16 cells was achieved at 165μM, increasing at 331μM.

The FA composition of MAC13, MAC16 and MAC26 cell as A. a % of the total FAs recovered and B. μg per 5 x 10^6 cells is shown in figure 11. The total FA concentration of the MAC13 cells was significantly higher than for both MAC16 and MAC26 cells. The MAC26 cell line had a significantly increased total FA content over the MAC16 cell line. The level of stearic acid (SA) was also increased significantly in MAC13 cells relative to MAC16 cells. Levels of EPA and DHA though small were

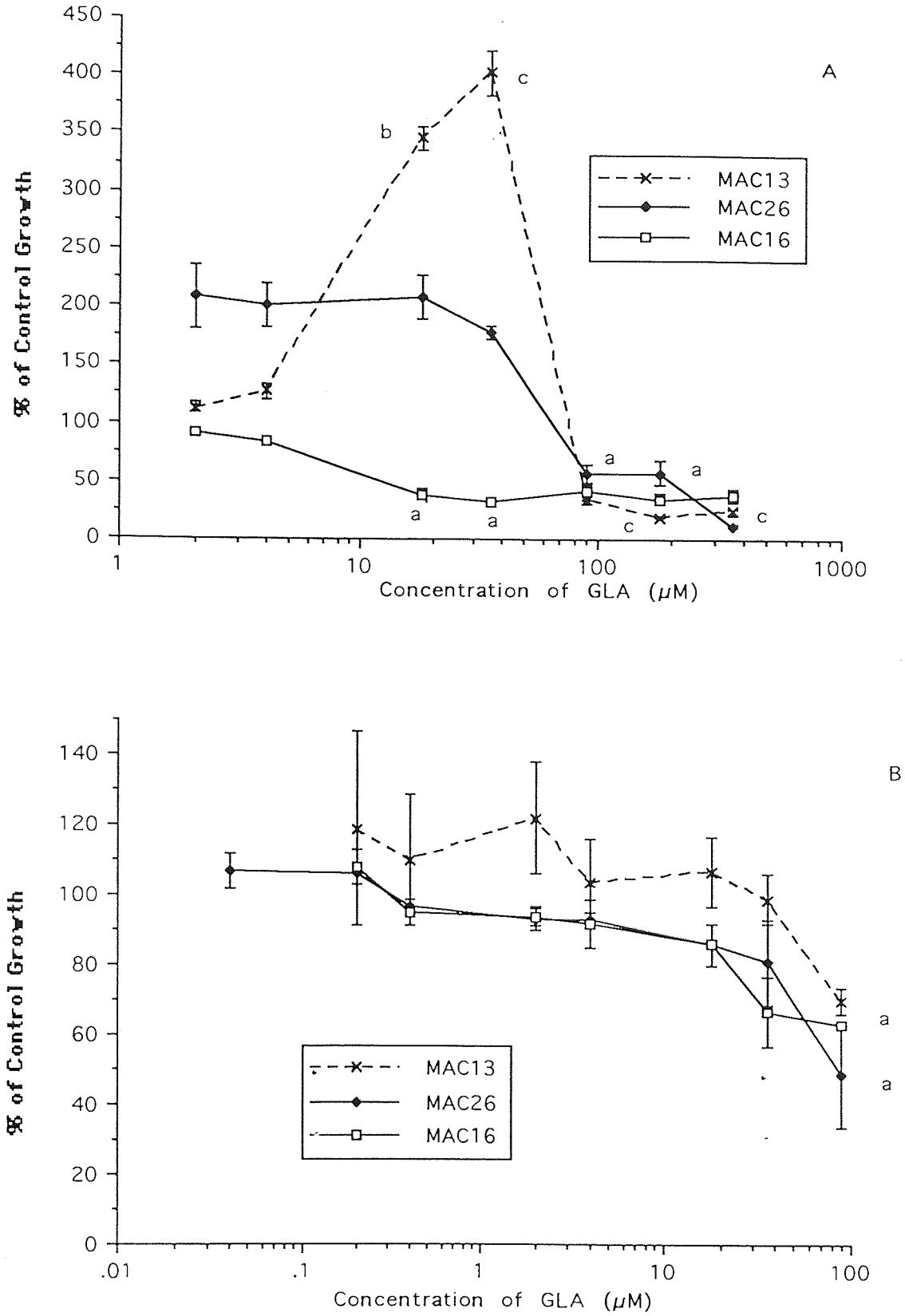
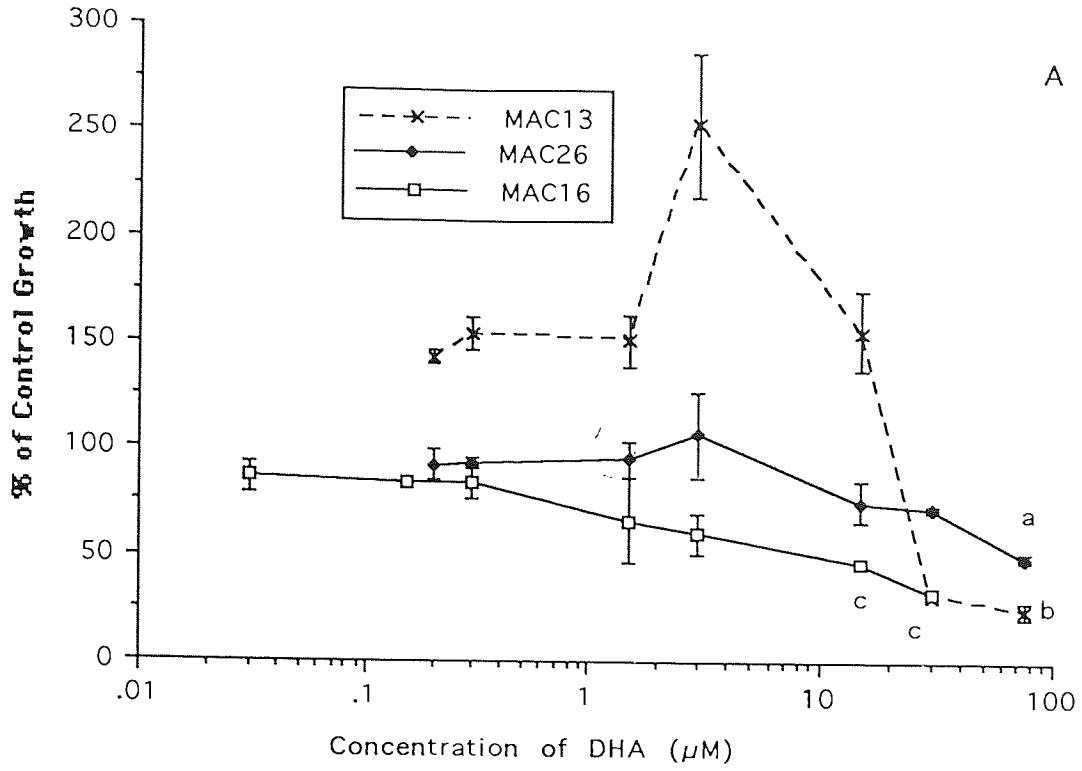


Figure 8. The effect of GLA in A. reduced serum at 144 hours and B. normal serum at 72 hours on MAC cell growth (n=9). (a= $p<0.05$, b= $p<0.01$ and c= $p<0.05$ from Control using T-test with Bonferroni Correction.)



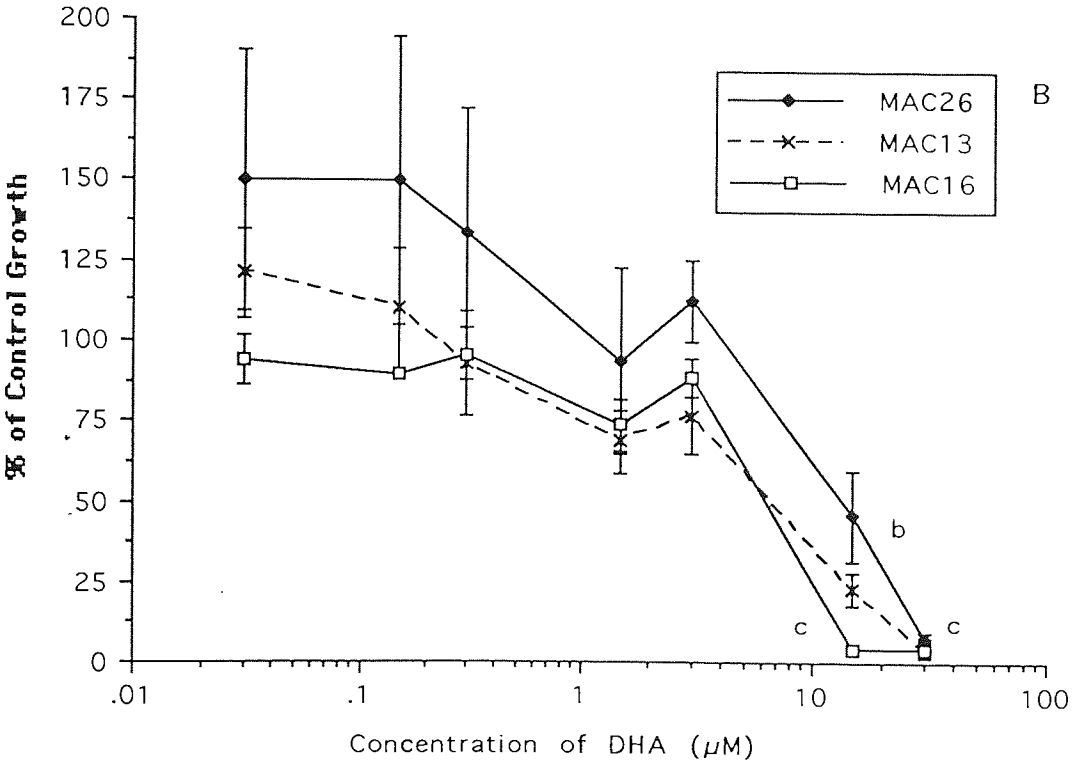


Figure 9. The effect of DHA A. in reduced serum at 144 hours B. in normal serum at 72 hours on MAC cell growth (n=9). (a= $p<0.05$, b= $p<0.01$ and c= $p<0.005$ from Control using T-Test with Bonferroni Correction.)

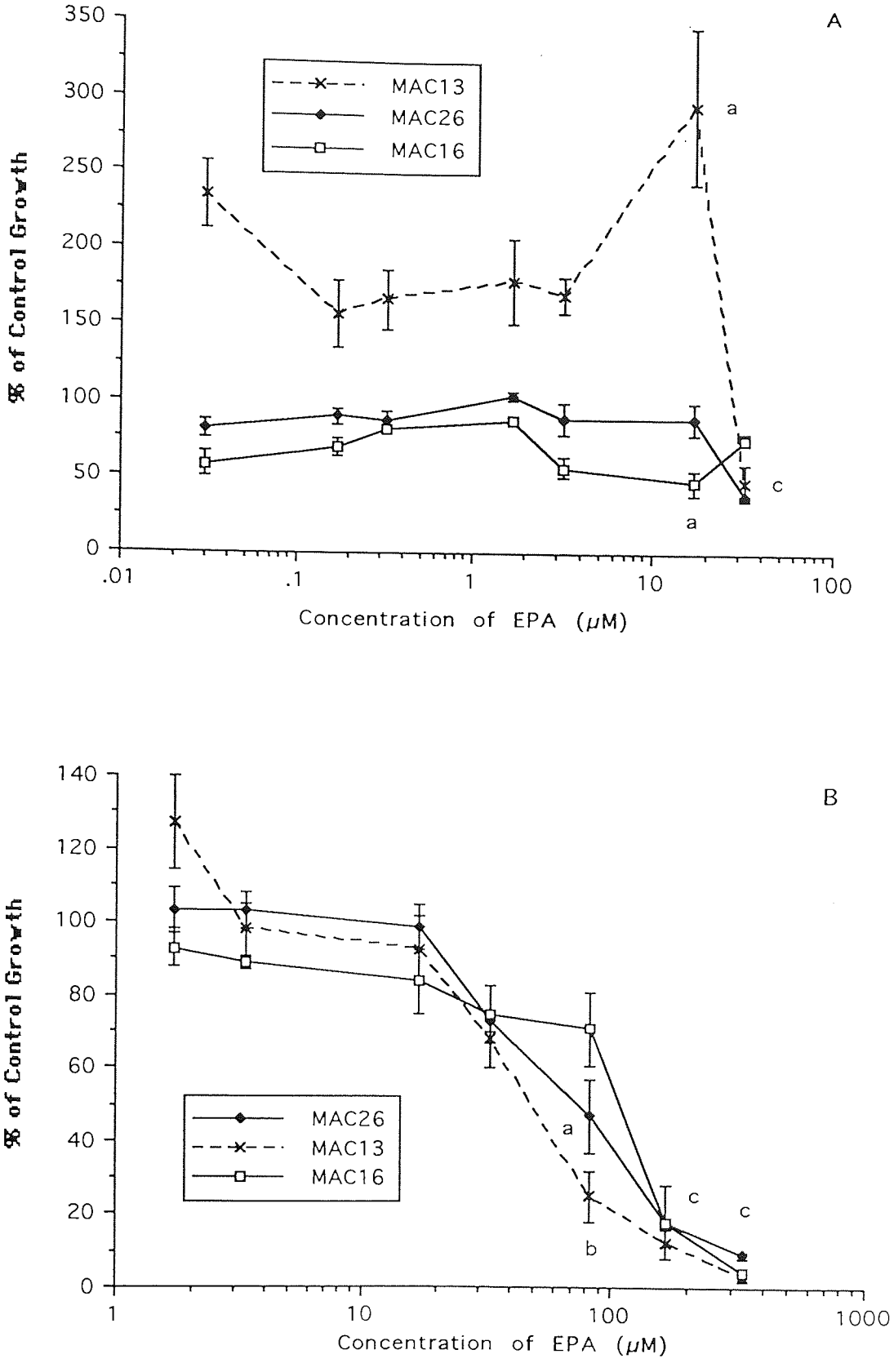


Figure 10. The effect of EPA A. in reduced serum at 144 hours and B. normal serum at 72 hours on MAC cell growth (n=9).
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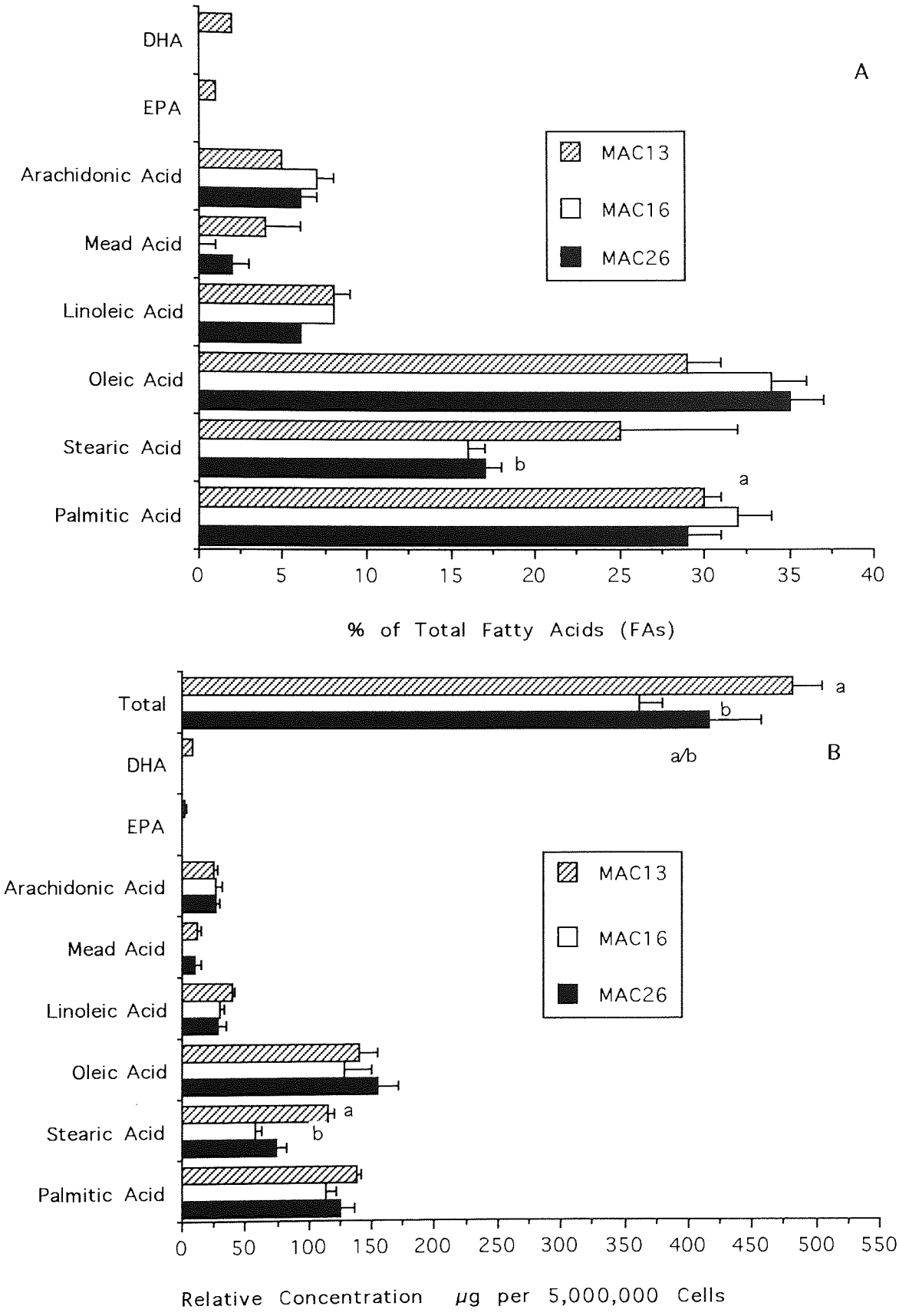


Figure 11. The fatty acid (FA) content of MAC cells A. as % of total FAs recovered and B. μg per 5,000,000 cells (n=9).
(a= $p<0.01$ from MAC16 cells and b= $p<0.01$ from MAC13 cells, using two-way ANOVA followed by Tuckey's Test.)

detected in MAC13 cells by GC. but not in either MAC16 or MAC26 cells. Mead acid was detectable in both MAC13 and MAC26 cells but not MAC16 cells.

4:1:3. Discussion

The traditional method for the measurement of FA deficiency in blood or tissues is the ratio of Mead acid (20:3 n-9) to AA (20:4 n-6). With normal amounts of LA (18:2 n-6) the conversion of oleic acid (OA)(18:1 n-9) to its further metabolites is inhibited at the desaturation stage. As a result the Mead acid to AA ratio is very low. If LA supplies are reduced then inhibition of OA metabolism is reduced and increased Mead acid is formed with a reduction in the formation of AA. (Horrobin 1992). At a ratio of 0.4 Mead acid:AA clinical symptoms of essential fatty acid (EFA) deficiency can be observed, though in normal individuals the ratio is usually less than 0.1, so values from 0.1 upwards are considered to signify a biochemical EFA deficiency (Horrobin 1992). MAC16 cells when grown in normal serum containing medium, though they had a significantly lower FA content than either MAC13 or MAC26 cells, did not have detectable levels of Mead acid, and both relative OA and AA levels were higher, suggesting there is no biochemical EFA deficiency. MAC13 and MAC26 grown in normal medium had Mead acid:AA ratios of 0.40 and 0.53 respectively suggesting that these cells were EFA deficient. This may partially explain why MAC13 and MAC26 cells when grown in reduced serum can be stimulated by AA and LA , whereas MAC16 cells could not even though optimal cell growth was inhibited.

The PUFA content of the cell membrane influences the activity of the cell membrane receptor proteins and modulates the kinetics of ligand receptor binding. Cytokines, such as interferons, interact with specific cell receptors and subsequently elicit secondary signals that result in the transcription and induction of a number of genes (Revel and Chebath 1986). In premonocytic U937 cells n-6 and n-3 PUFAs were rapidly incorporated into membrane phospholipids but there was no effect on cell proliferation or differentiation. When interferon-γ (IFN-γ) was introduced into the culture medium of these PUFA enriched cells, growth stimulation and differentiation was observed (Obermeier et al 1995). In primary culture the proliferation of mammary epithelial cells induced by epidermal growth factor (EGF)

was both enhanced and sustained by LA and its eicosanoid metabolites (Bandyopadhyay et al 1993). The FA content of cells in culture is influenced by the FA content of the medium from which they are avidly taken up to replenish the cellular phospholipid pools (Obermeier et al 1995). In reduced serum levels MAC16 cells may become enriched with FAs such as AA and LA supplemented into the medium, but due to the absence of a growth factor or a cytokine required for signal transduction stimulation of cell growth was not observed.

MAC13 cells are stimulated by 18μM LA, 18-33μM GLA and 16μM AA in reduced serum levels. The degree of stimulation of AA in relation to control growth is reduced in comparison to both LA and GLA. The susceptibility of PUFAs to spontaneous peroxidation in culture is AA>GLA>LA (Begin et al 1988). This may explain why AA is not as successful as LA or GLA in stimulating cell proliferation in MAC13 cells. If LA and GLA are precursors for AA then it appears that desaturation and elongation metabolism in MAC13 cells is efficient, and there is no deficiency in Δ6-desaturase or Δ5-desaturase enzymes as has been reported for other cell lines (Dunbar and Bailey 1974). In MAC26 cells stimulation of cell proliferation by 18μM LA was greater than that produced by 33μM AA. GLA was capable of stimulating cell growth but not significantly. The increased spontaneous peroxidation of AA in comparison to LA may partially explain why AA does not stimulate cell growth as significantly as LA. If LA was elongated and desaturated to form AA which then stimulates cell proliferation in MAC26 cells, then it will be metabolised to GLA, which was not capable of significantly stimulating cell growth. The inability of GLA to stimulate cell growth suggests that Δ5-desaturase activity may be deficient in MAC26 cells. This in relation to the increased susceptibility to peroxidation may prevent a significant stimulation of MAC26 cells by GLA. It also seems likely that LA is stimulating MAC26 cell proliferation by a mechanism independent of AA metabolism which may require the whole molecule such as the formation of 13-hydroperoxyoctactecatrienoic acid (13(S)HPODE) or non-enzymatic metabolism.

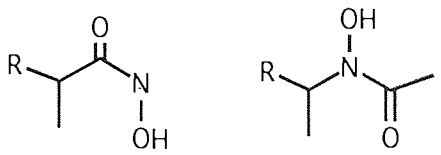
MAC13 and MAC26 cells are stimulated by n-6 PUFAs. This may involve any 3 of the 4 mechanisms proposed by Nicholson et al 1988. Suppression of the immune system and the host's defence against invading tumour was not a plausible mechanism

of action as cells are grown *in vitro* in the absence of any humoral or cell mediated immunity. The PUFAs could have a direct effect on cell proliferation through activation of gene transcription and transduction (Tiwari et al 1991) or the modulation of enzymes involved in proliferation such as the activation of PKC (Blobe et al 1995). Increases in the concentration of PUFAs in the cell membrane increases the fluidity of the cell membrane (Spectre and Yorek 1988). This in turn increases the ligand binding properties and kinetics of protein receptors in these membranes (Revel and Chebath 1986). Modification of membranes by PUFAs leads to increased sensitivity to mitogens (Bandyopadhyay et al 1993, Spectre and Burns 1987, Obermeier et al 1995). MAC13 and MAC26 cells could also be stimulated by eicosanoids produced from AA. Inhibition of eicosanoid synthesis by cyclooxygenase (CO) pathways and/or lipoxygenase (LO) pathways has been shown to inhibit cell growth in certain tumour cell lines (Rose and Connolly 1990, Lee and Ip 1992, Buckman et al 1991).

Tumour cells have a reduced PUFA content especially EFA in relation to normal cells. This appears to be compensated for by increases in saturated FAs and OA (Hopewell et al 1993, Diaziani 1993). MAC13, MAC16 and MAC26 cells all contain high concentrations of palmitic acid, SA and OA relative to EFAs. This will result in decreased fluidity of the membrane (Spectre and Yorek 1988). MAC13 cells, unlike MAC16 or MAC26 cells, have detectable levels of EPA and DHA in their cell membranes. In reduced serum levels 3μM DHA and 3.3μM EPA was capable of stimulating cell growth. It seems likely that this stimulation was due to an increased fluidity of the cell membrane, as induction of gene transcription by EPA or DHA requires a concentration at least 4 times higher than that required to support normal growth and prevent EFA deficiency (Clarke and Jump 1994).

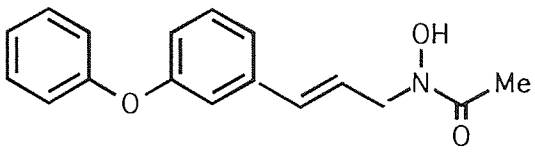
Various experiments in animals with a range of tumours has shown that tumour cells on the whole are more susceptible than normal cells to both spontaneous and induced lipid peroxidation (Massotti et al 1988, Dianziana 1993). The generation of lipid peroxides increases with the degree of desaturation (Begin et al 1988). The cytotoxicity of PUFAs on the MAC13 and MAC26 cell lines is DHA<15μM, EPA<83μM, AA<90μM, GLA<93μM, and LA 178μM in normal serum levels. The degree of toxicity

correlates with the number of double bonds present, and hence the degree of desaturation. It seems likely that the inhibition of MAC13 and MAC26 cell growth by PUFAs is due to the generation of lipid peroxides. MAC16 cells were inhibited by DHA at 5μM, GLA at 90μM and EPA at 165μM, an effect that can not be explained by the generation of lipid peroxides. The effect of EPA on the MAC16 cells *in vitro* was not representative of the situation *in vivo.* EPA has both antitumour and anticachectic action in MAC16 tumour-bearing NMRI mice, an effect which was not displayed by DHA (Beck et al 1991, Hudson 1993).

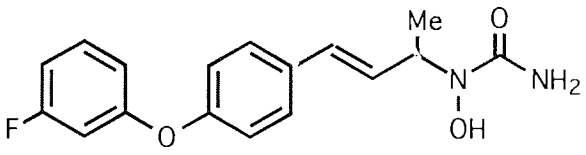


Type A Type B

Hydroxamic Acids



BWA4C



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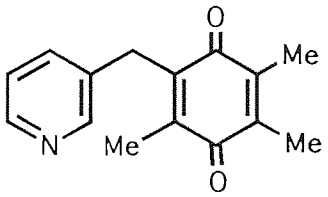

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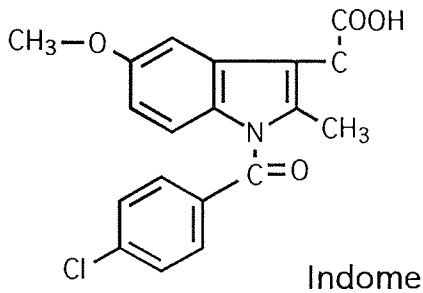
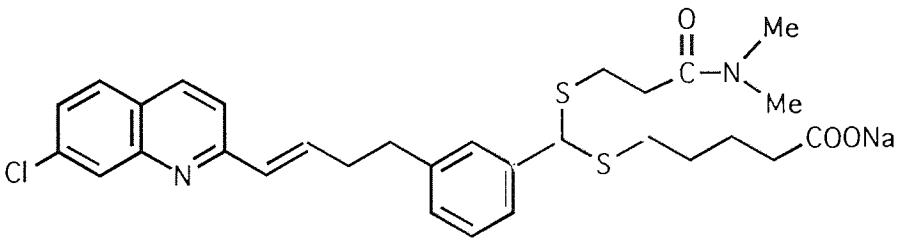


Indomethacin



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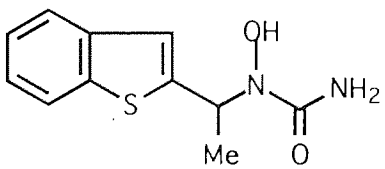


Zileuton

Figure 12.
The structures of indomethacin and lipoxygenase (LO) inhibitors.

4:2. The Effect of Indomethacin and Lipoxygenase (LO) Inhibitors on the MAC13, MAC16 and MAC26 Cells *In Vitro.*

4:2:1 Introduction

Eicosanoids are formed from 'free' AA within the cells either by the cyclooxygenase (CO) pathway leading to the formation of prostaglandins (PGs), and thromboxanes (TXs), or the lipoxygenase (LO) pathways leading to the formation of hydroxyeicosatetraenoic acids (HETEs), leukotrienes (LTs) or lipoxins (LXs). The implication of eicosanoids in the promotion of carcinogenesis has been reviewed extensively in chapter 1 (1:4:4).

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit CO activity preventing the formation of PGs and TXs. Patients with rheumatoid arthritis have a reduced incidence of colonic, liver and stomach cancer, which is thought to be due to the regular use of NSAIDs (Gridley et al 1993). The potency of NSAIDs is medofenate> indomethacin > salicylamide > phenylbutazone > aspirin = salicylic acid (Hial et al 1977). Indomethacin was chosen as a CO inhibitor due the antitumour action reported in other murine tumour models (Fulton 1984, Gabor et al 1985). Indomethacin entered stage III and IV clinical trials in 1981, where it was shown to produce both stabilisation and regression of head and neck derived carcinomas (Panje 1981). A recent clinical trial involving patients with solid metastic tumours demonstrated that indomethacin was therapeutically effective at non-toxic doses, the mean survival of indomethacin treated patients being increased from 250 $\pm$ 28 days in placebo patients, to 510 $\pm$ 28 days (Ludholm et al 1994).

There is a high degree of homology between 5-LO, 12-LO and 15-LO, especially around the non-haem iron containing atom, at the catalytic site of the enzyme (Sigal 1991). Knowledge of both the characteristics and mechanisms of action of LOs have been important in the design of LO inhibitors. The 3 main types of LO inhibitor classified according to their mechanism of action are: i). redox inhibitors, ii). iron chelator inhibitors and iii). non-redox inhibitors (McMillan and Walker 1992).

Redox inhibitors potentially react at a number of places in the LO enzyme mechanism, that may reduce the active Fe^{3+} to Fe^{2+} (the inactive state), or reduce one of the radical intermediates also leaving the enzyme in the Fe^{2+} inactive state.

Although lack of specificity can be a problem with this class of inhibitor, due to the ubiquitous nature of oxidative processes in biological systems, orally active antioxidant 5-LO inhibitors have demonstrated clinical efficacy (Musser and Kreft 1992). CV6504 is a novel, bioreductive, orally active, antioxidant-type 5-LO inhibitor, with added activity against thromboxane synthetase and lipid peroxidation, that has entered clinical trials for glomerular nephritits (Shibouta et al 1991).

Hydroxamic acids have a powerful metal ligand group, with the potential to chelate iron at active sites of LO enzymes (Jackson et al 1988). Hydroxamic acids and their close derivatives are widely used for designing iron chelating 5-LO inhibitors (Corey et al 1984). Abbot Laboratories have defined two distinct types of hydroxamic acid inhibitor, type A and type B, differentiated by the size of the molecular fragment attached to the carbonyl carbon and nitrogen group (see figure 12). Both structures are potent *in vitro* , but *in vivo* type A hydroxamic acids undergo rapid metabolism to the corresponding carboxylic acid. This results in a shorter half-life and reduced potency in comparison to type B (Summers et al 1988). BWA4C, a hydroxamic acid, was evaluated in volunteers where it was well tolerated, absorbed and produced dose dependent inhibition of LTB_4 synthesis *ex vivo* (Salmon and Garland 1991). Further clinical evaluations are precluded by rapid metabolism and the resultant high accumulations of metabolites. Hydroxyureas provide equivalent 5-LO potency, with higher levels maintained after oral administration. BWB70C, a hydroxyurea, was able to maintain both higher levels after oral dosing and prolonged 5-LO activity *in vivo* in comparison to BWA4C (Salmon and Garland 1991). Zileuton, a hydroxamic acid hydroxyurea, is a 5-LO inhibitor very advanced in clinical evaluation. It is well tolerated and absorbed in human volunteers (Carter et al 1991). A double blind clinical trial with rheumatoid arthritis patients given 800mg of Zileuton twice daily, produced significant relief of symptoms (Weinblat et al 1990).

Non-redox inhibitors do not react with the non-haem iron at the active site of the LO enzyme. They inhibit other LO mechanisms of action and include receptor antagonists. Inactive 5-LO is found in the cytosol, from which it must translocate to the cell membrane to become active. It binds to the membrane by a membrane bound

protein called five lipoxygenase activating protein (FLAP) (Musser and Kreft 1992). MK886, a 5-LO inhibitor, designed by Merk and Frost prevents the enzyme binding to this FLAP protein, and becoming active (Bel et al 1990). MK-571, more widely referred to as L-660,711, is an extremely potent and highly selective LTD_4 but not LTC_4 receptor antagonist. The pharmacology profile of L-660,711 indicates that it is a potent, selective, orally active LT receptor antagonist that is suited to the determination of the role played by LTD_4 and LTE_4 in asthma and other pathological conditions (Jones et al 1989).

4:2:2. Results

The IC_{50} values for LO inhibitors and indomethacin on MAC13, MAC16 and MAC26 cells in normal serum over 72 hr, are shown in table 1. BWA4C, BWB70C and CV6504, had lower IC_{50} values than L,660-711, indomethacin and Zileuton. The least effective inhibitor of cell growth in all 3 three cell lines was Zileuton, and even when dosed every 24 hr the IC_{50} values were not reduced.

The IC_{50} values of MAC13, MAC16 and MAC26 cells stimulated with optimum concentrations of AA and LA over 144 hr, compared to values at 72 hr in normal serum levels, for BWA4C, CV6504 and indomethacin are shown in Table 2. IC_{50} values increase when cells were stimulated with AA or LA for both indomethacin and BWA4C, especially when MAC26 cells were stimulated by LA. CV6504 had lower IC_{50} values in MAC13 and MAC26 cells, when stimulated by either AA or LA.

The effect of the addition of exogenous (±)HETEs on MAC26 cells inhibited by A. 5μM BWA4C and B. 10μM CV6504 is shown in figure 13. Addition of (±)12-HETEs in the presence of 5μM BWA4C significantly increased the inhibition of cell growth. Addition of (±)5-HETE slightly increased the inhibition of cell growth over 5μM BWA4C, but this was not significant. (±)15-HETE partially reversed the effect of 5μM BWA4C on MAC26 cells between 0.33μM and 6.61μM, which was significant at 1.65μM. Inhibition of growth of MAC26 cells by 10μM CV6504 decreases by the addition of (±)15-HETE between 0.33μM and 1.65μm, but was not significant. (±)5-HETE increases the inhibition by 10μM CV6504 on the MAC26

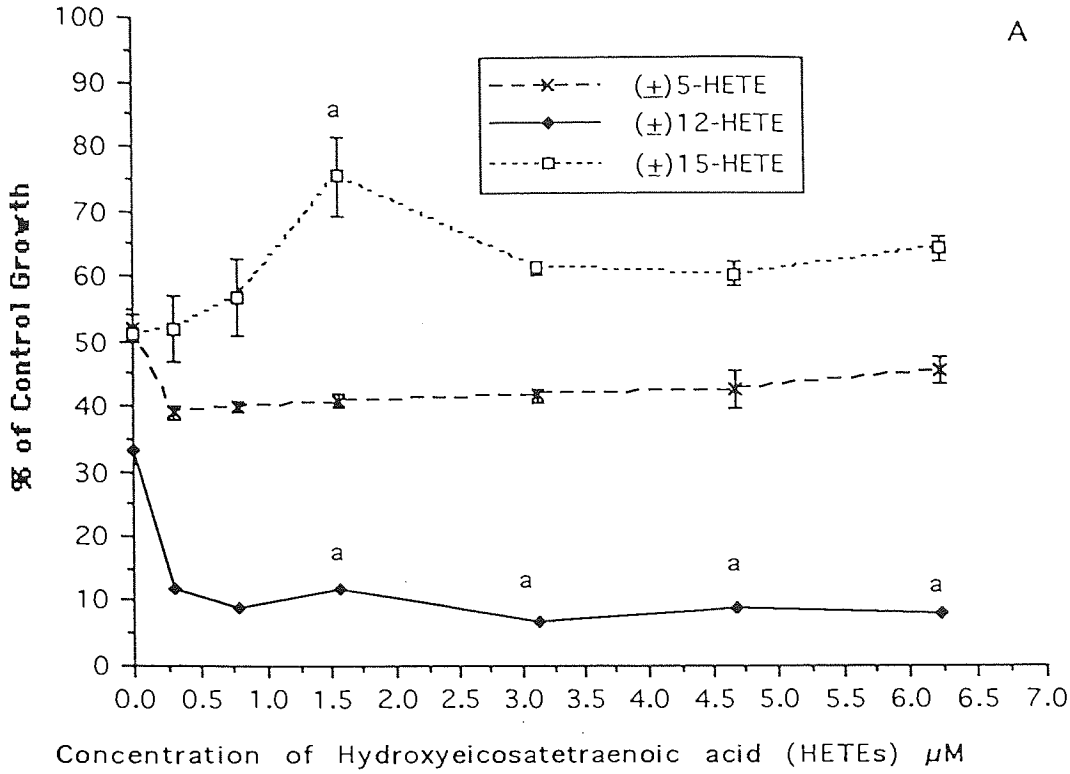
Table 1.
The IC_{50} values of indomethacin and lipoxygenase (LO) inhibitors of normal MAC cell growth over 72 hours (μM) (n=9).

	MAC13	MAC16	MAC26
BWA4C	3 ± 1	4 ± 1	5 ± 1
BWB70C	2 ± 1	5 ± 0	4 ± 1
CV6504	3 ± 1	3 ± 1	7 ± 1
L660-711	22 ± 2	29 ± 7	24 ± 1
Indomethacin	32 ± 3	37 ± 2	34 ± 5
Zileuton	78 ± 12	70 ± 3	44 ± 10
Zileuton (every 24 hr)	43 ± 7	58 ± 15	58 ± 8

Table 2.
The IC_{50} Values of BWA4C, CV6504 and indomethacin on MAC13 and MAC26 cells stimulated by optimal concentrations of arachidonic acid (AA) and linoleic acid (LA) (μM) (n=9).

MAC13	Indomethacin	BWA4C	CV6504
10% FCS	32 ± 3	3 ± 1	3 ± 1
18μM Linoleic Acid	32 ± 3	7 ± 1	2 ± 1
16μM Arachidonic Acid	40 ± 6	6 ± 1	2 ± 1

MAC26	Indomethacin	BWA4C	CV6504
10% FCS	45 ± 1	5 ± 1	7 ± 1
18μM Linoleic Acid	80 ± 21	22 ± 6	1 ± 0
33μM Arachidonic Acid	40 ± 6	6 ± 1	2 ± 1



Where 100% is MAC26 Control cell growth without 5μM BWA4C or 10μM CV6504.

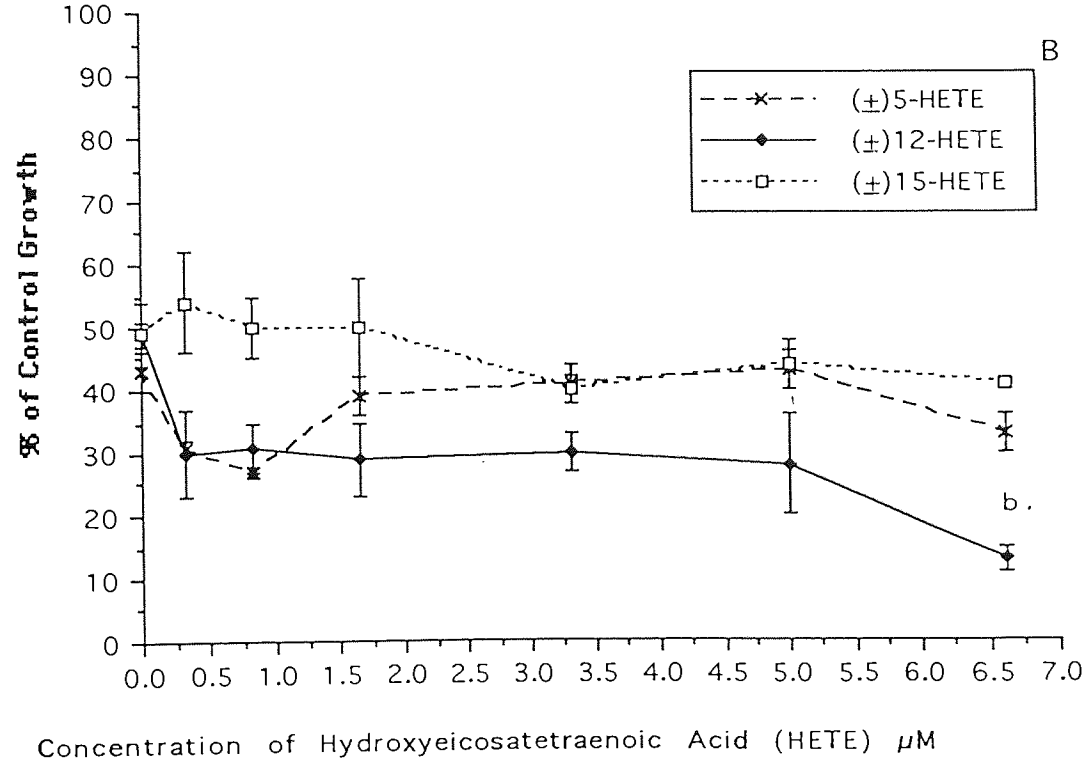
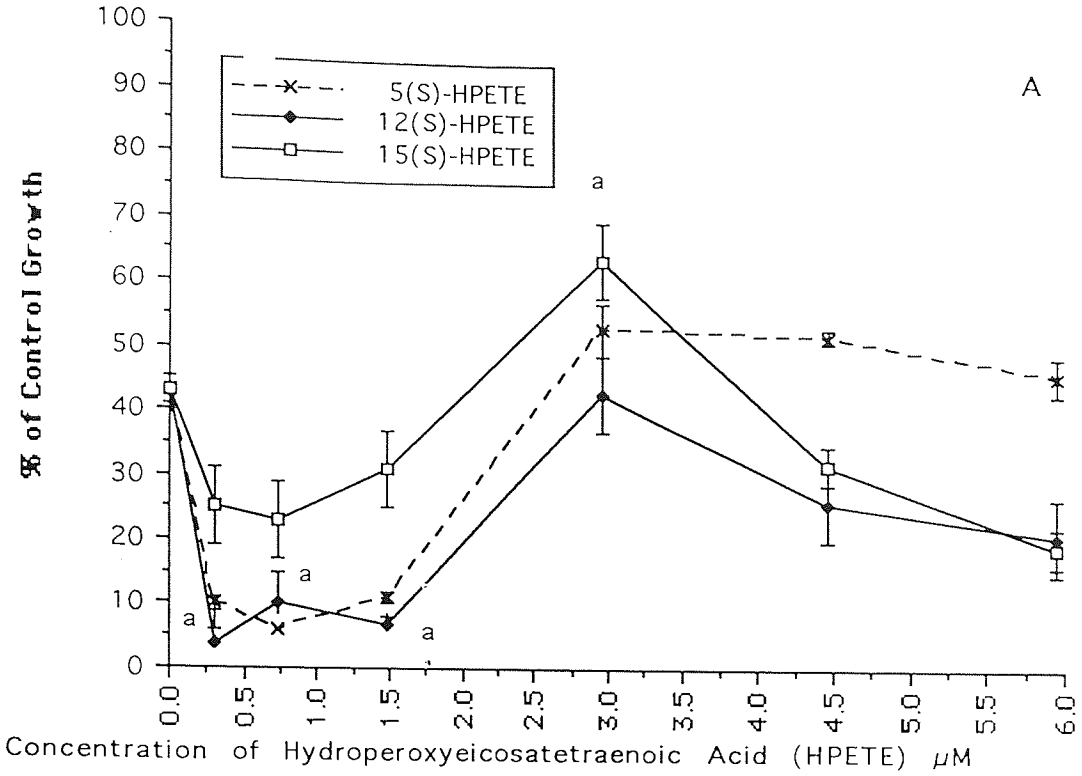


Figure 13. The effect of hydroxyeicosatetraenoic acids (HETEs) on the inhibition of MAC26 cells A. by 5μM BWA4C and B.10μM CV6504 (n=6).
(a= $p<0.01$ from 5μM BWA4C and b= $p<0.05$ from 10μM CV6504 using T-test with Bonferroni Correction.)



Where 100% is MAC26 Control cell growth without 5μM BWA4C or 10μM CV6504.

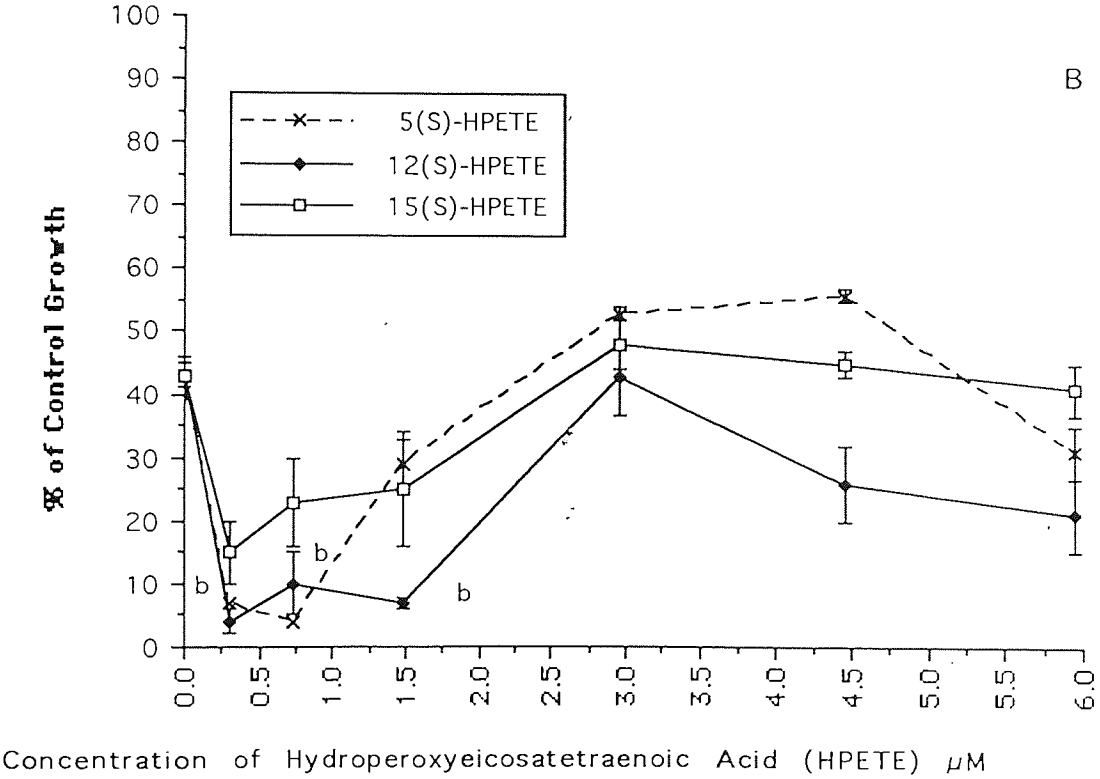


Figure 14. The effect of hydroperoxyeicosatetraenoic acids (HPETEs) on MAC26 cells inhibited by A. 5μM BWA4C and B.10μM CV6504 (n=6).
(a= $p<0.05$ from 5μM BWA4C and b= $p<0.05$ from 10 μM CV6504 using T-test with Bonferroni Correction.)

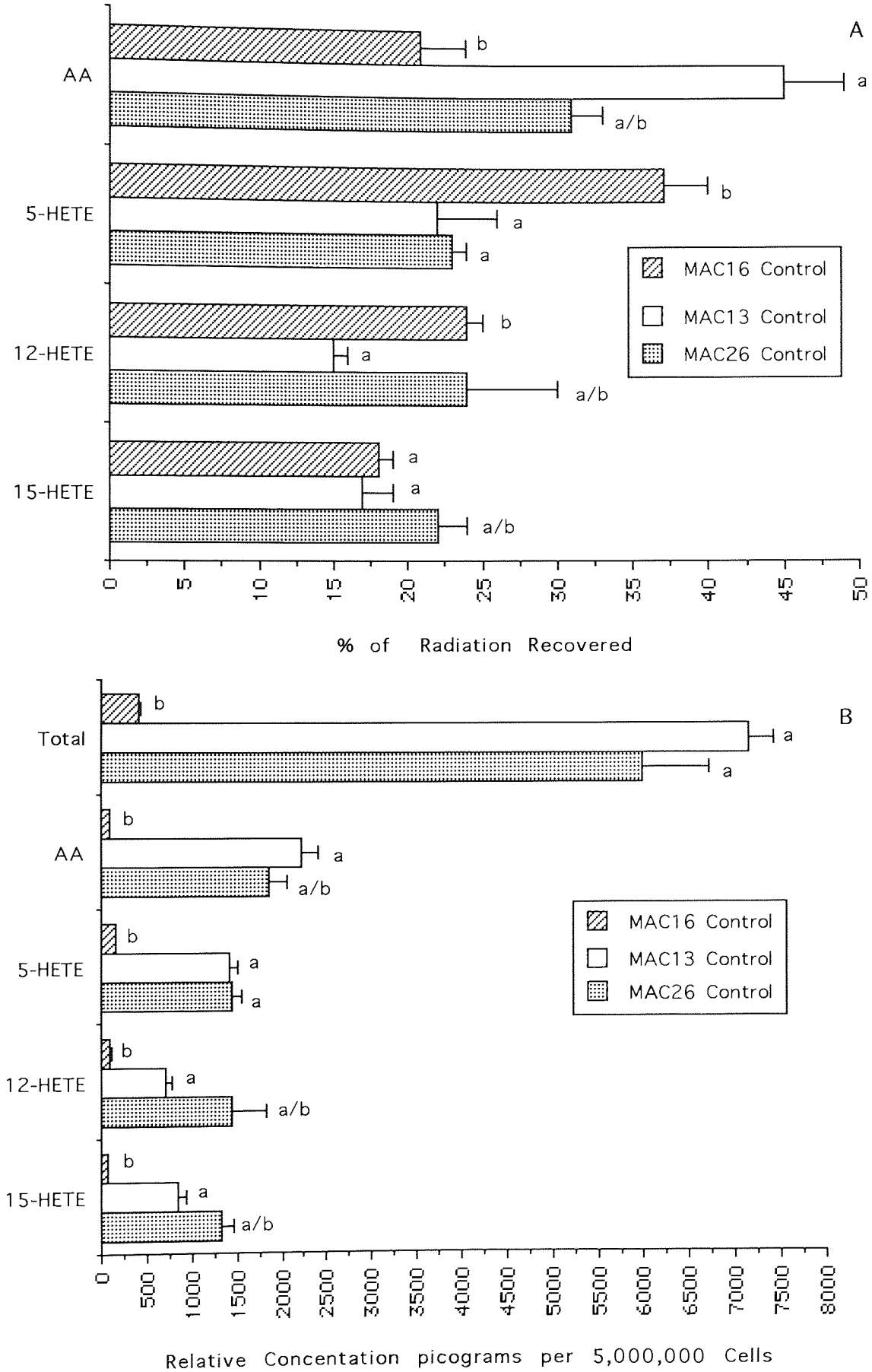


Figure 15. The lipoxygenase (LO) pathways in MAC cells expressed as A. % of total radiation recovered and B. picograms per 5,000,000 cells (n=4).
(a= $p<0.01$ from MAC16 cells and b= $p<0.01$ from MAC13 cells using two-way ANOVA followed by Tuckey's Test).

cell line between 0.33μM and 6.60μM but again this was not significant. ($\pm$)12-HETEs increases the inhibition of MAC26 cell growth by 10μM CV6504 from 0.33μM to 6.60μM, which was significant at 6.60μM.

The effect of 5(S), 12(S), and 15(S)HPETE on the inhibition of MAC26 cells by A. 5μM BWA4C and B. 10μM CV6504 is shown in figure 14. 5(S)HPETE and 12(S)HPETE significantly increased the inhibition of MAC26 cell growth by 5μM BWA4C between 0.30μM and 1.77μM. 15(S)HPETE reversed the inhibitory effect on MAC26 cells by BWA4C at 2.97μM. Cell growth increased, though not significantly at 2.97μM, 4.46μM and 5.94μM. MAC26 cells inhibited with 10μM CV6504 were further inhibited by 5(S)HPETE, 12(S)HPETE and 15(S)HPETE at 0.30μM and 0.74μM respectively. This was significant at 0.30μM for all 3 HPETEs, at 0.74μM for 5(S)HPETE and 12(S)HPETE, extending to 1.49μM for 12(S)HPETE. Inhibition of cell growth by 10μM CV6504 was decreased, but not significantly at 2.97μM and 4.46μM by 5(S)HPETE.

The distribution of metabolised and unmetabolised AA within MAC13, MAC16 and MAC26 cells *in vitro* is shown in figure 15. All three cell lines had detectable levels of 5, 12 and 15-HETE from a pulse of radiolabelled AA ([^{3}H]-AA). There were significant differences in both A. % of the total radiation recovered and B. the concentration passing through these pathways between cell lines. MAC13 cells had significantly higher concentration of unmetabolised AA, whereas MAC16 cells had a higher % of 5-HETE. MAC26 cells form higher concentration of 12-HETE and 15-HETE in comparison to both MAC13 and MAC16 cells. When results are expressed as picgrams of metabolite recovered per 5 x 10^6 cells, MAC16 cells had a significantly lower concentration of total metabolites recovered, as well as for individual metabolites, in comparison to MAC13 and MAC26 cells. The concentration of [^{3}H]-AA passing into the 5-HETE pathways in MAC13 and MAC26 cells was virtually equal. MAC26 cells produced significantly higher concentration of both 12-HETE and 15-HETE.

The effect of 5μM and 10μM CV6504 on the metabolism of [^{3}H]-AA as a % of the total radiation recovered is shown in figure 16. The unmetabolised AA recovered in all

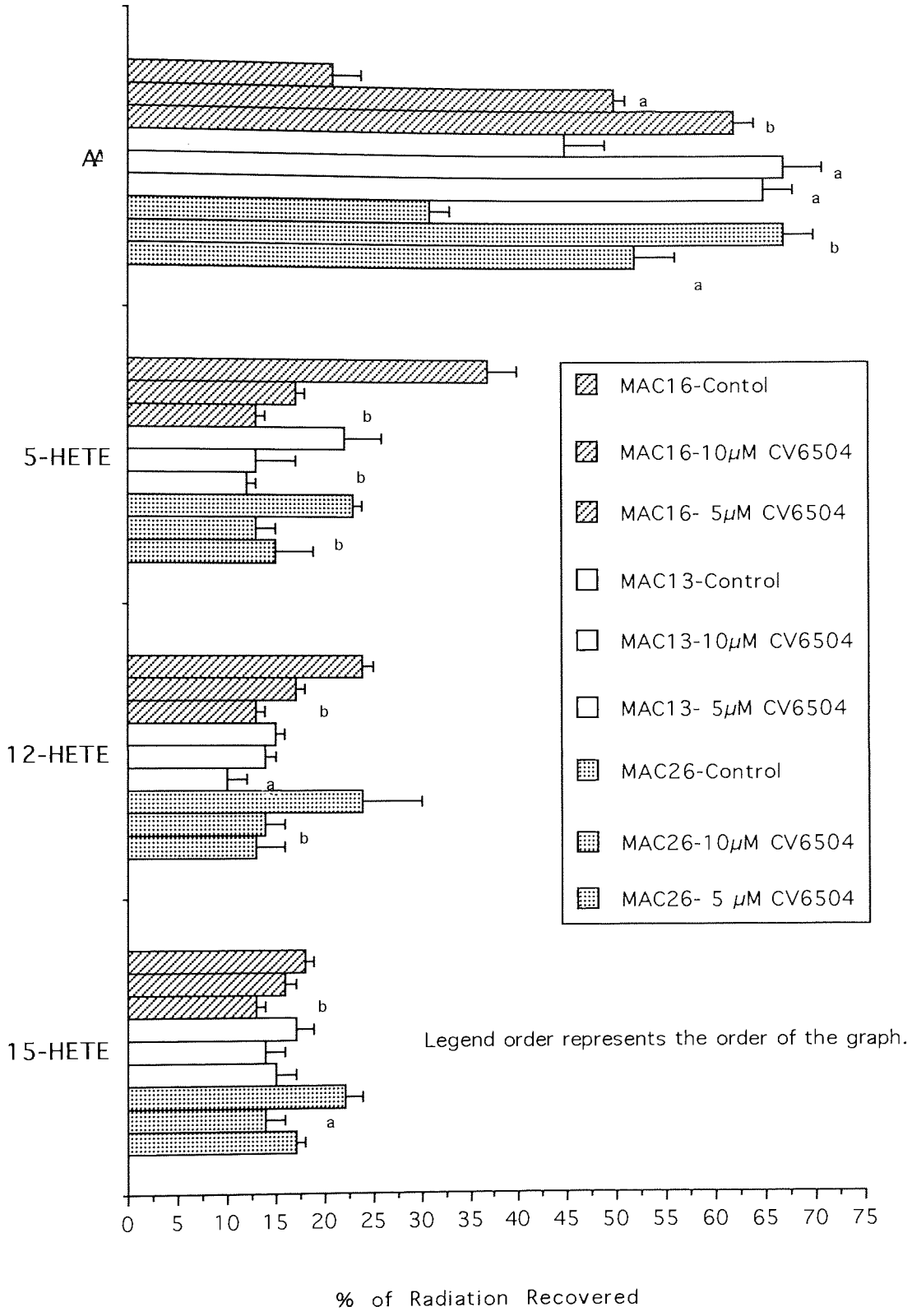
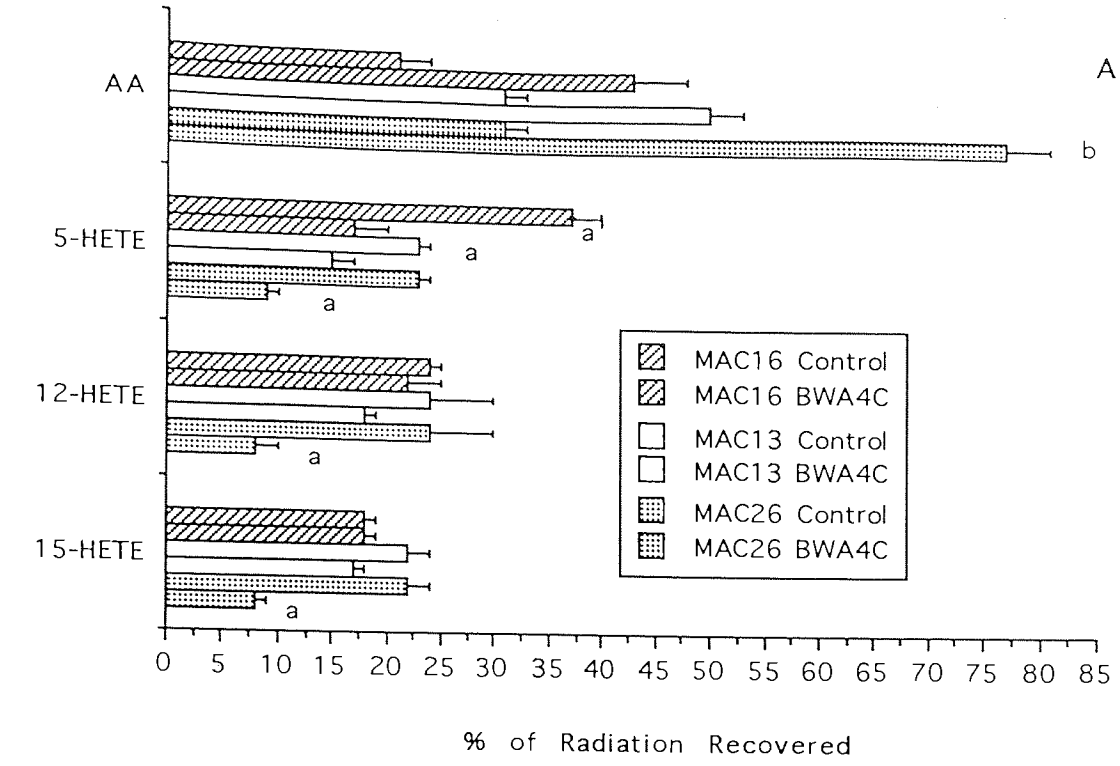


Figure 16. The effect of 5 and 10μM CV6504 on the production of hydroxy-eicosatetraenoic acid (HETEs) from arachidonic acid (AA) in MAC cells (n=4). (a= $p<0.05$ and b= $p<0.01$ from Control pathway using two-way ANOVA followed by Tuckey's Test.)



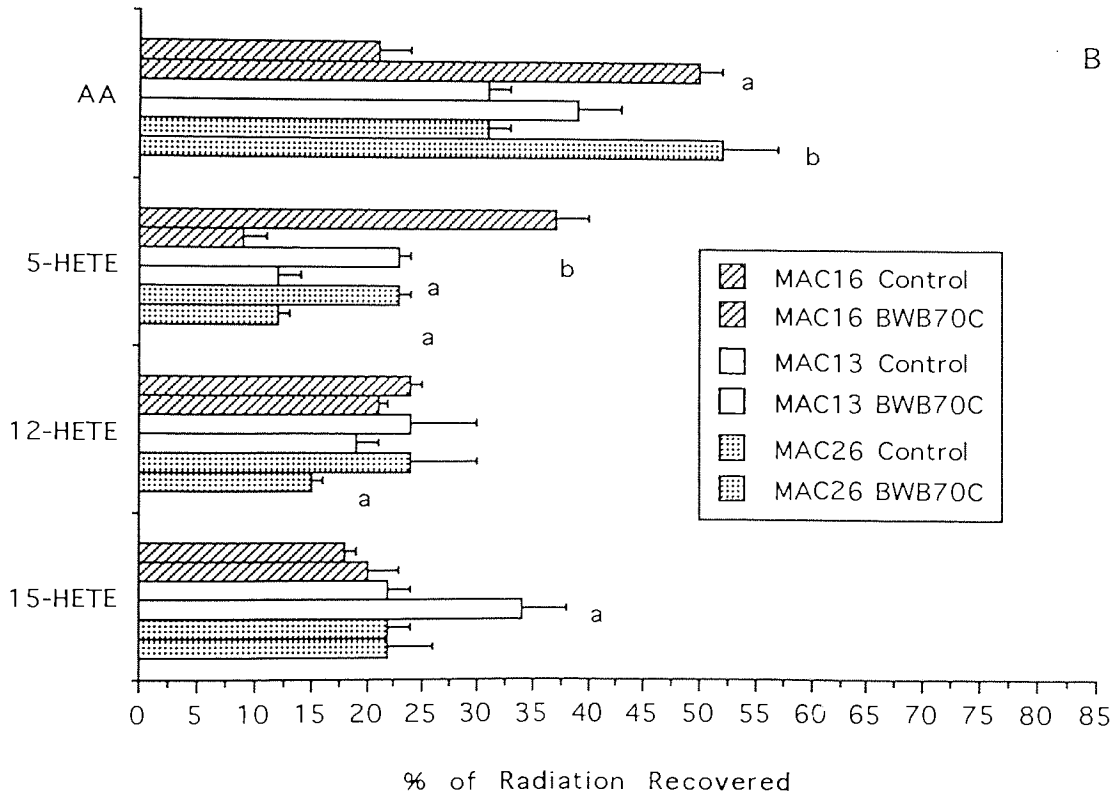


Figure 17. The effect of A. 10μM BWA4C and B. 10μM BWB70C on the hydroxy-eicosatetraenoic acids (HETEs) produced in MAC cells (n=4).
(a =$p<0.05$ and b =$p<0.01$ from Control pathway, using Two-way ANOVA followed by Tuckey's Test.)

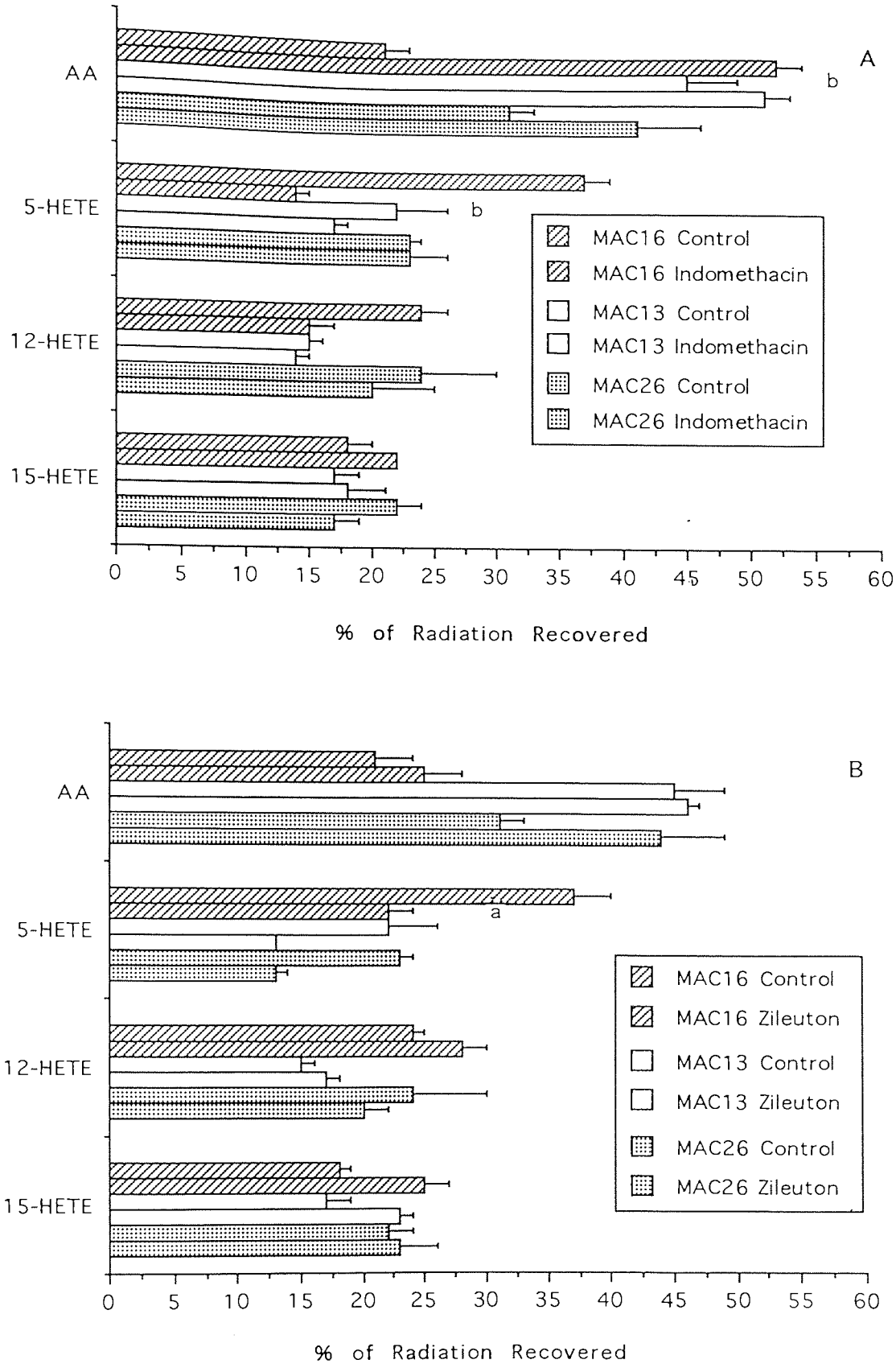


Figure 18. The effect of A. 10μM indomethacin and B. 10μM Zileuton on the production of hydroxyeicosatetraenoic acids (HETEs) by MAC cells (n=4). (a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test.)

3 cell lines at both concentrations was increased, suggesting that AA metabolism was reduced. 5-HETE production was also reduced significantly at both concentrations in all 3 cell lines. 12-HETE was reduced at both concentrations but was only significant at 5μM in MAC13 and MAC16 cells, but both in MAC26 cells. 15-HETE was also reduced in all three cell lines at both concentrations but was only significant for MAC16s at 5μM and MAC26 at 10μM.

The effect of 10μM BWA4C on the metabolism of ^{3}H-AA in MAC13, MAC16 and MAC26 cells expressed as % of total radiation recovered is shown in figure 17A. This reduced AA metabolism as a higher concentration of unmetabolised ^{3}H-AA was recovered from all three cell lines. 5-HETE production was significantly reduced in all three cell lines. 12-HETE and 15-HETE production was also reduced in all three cell lines but is only significant in MAC26 cells.

The effect of 10μM BWB70C on the metabolism of ^{3}H-AA by MAC cells, expressed as a % of the total radiation recovered is shown in figure 17B. The % of unmetabolised ^{3}H-AA recovered in all three cell lines was increased, again suggesting a reduction in AA metabolism. This was only significant for the MAC16 and MAC26 cell lines. 5-HETE production was significantly reduced in all three cell lines, as was the production of 12-HETE but not significantly. 15-HETE production was increased in MAC16 cells, significantly increased in MAC13 cells and remains the same in MAC26 cells.

The effect of 10μM indomethacin on the metabolism of ^{3}H-AA MAC13, MAC16 and MAC26 cells, expressed as a % of total radiation recovered is shown in figure 18A. The unmetabolised AA recovered in all 3 cell lines was increased, though this was only significant in MAC16 cells, again suggesting a reduction in AA metabolism. 5-HETE production was reduced significantly in MAC16 cells, reduced in MAC13 cells and remains constant in MAC26 cells. 12-HETE reduction is reduced in all three cell lines though not significantly. 15-HETE production is reduced in MAC26 cells, but is increased in MAC13 and MAC16 cells.

The effect of 10μM Zileuton on the metabolism of ^{3}H-AA by MAC13, MAC16 and MAC26 cells, expressed as % of the total radiation recovered is shown in figure 18B.

Unmetabolised ^{3}H-AA was increased, though not significantly in all three cell lines suggesting a reduction in the metabolism of AA. 5-HETE production was reduced in all three cell lines and was significant in the MAC16 cell line. 12-HETE and 15-HETE pathways are increased in both MAC13 and MAC16 cell lines. In the MAC26 cell line the 12-HETE pathway is reduced whilst the 15-HETE pathway was increased.

4:2:3. Discussion

In metastic 4526 murine mammary cells growth was inhibited by LO inhibitors in a dose dependent manner. CO inhibitors reduce the formation of PGE_1 and PGE_2 with no effect on cell proliferation. If LA was supplemented into the medium there was no effect on CO inhibition but the IC_{50} values for LO inhibitors were increased several fold. These results suggest that LO metabolites rather than CO metabolites were required for 4526 cell growth (Buckman et al 1991). MAC cells *in vitro* were also inhibited by LO inhibitors, though at higher concentration indomethacin was also capable of inhibiting cell proliferation. These results suggest that MAC13, MAC16 and MAC26 cell growth *in vitro* was dependent on LO metabolites. Indomethacin is a NSAIDs that inhibits the formation of TXs and PGs by the inhibition of CO. In rat hepatoma cells *in vitro* indomethacin has been shown to inhibit cell growth, but the concentration required to achieve this is higher than that required to inhibit CO (Hial et al 1977). When doses of indomethacin exceed 10μM it has been reported to have additional actions other than CO inhibition. Some authors have reported that it inhibits both phospholipase and LO (Rose and Connolly 1990), whilst other have reported that the mechanism of action is through inhibition of a whole host of enzymes (Cornwell and Morisaki 1986). The IC_{50} values for indomethacin against MAC13, MAC16 and MAC26 cells are above 30μM which suggests the mechanism of action will not be entirely due to the inhibition of CO. At 10μM (a three times lower concentration than that required to inhibit cell proliferation), 5-HETE, 12-HETE or 15-HETE production was reduced, and the concentration of unmetabolised AA was increased, suggesting that indomethacin does effect the LO pathways, and this was the mechanism of action involved in the inhibition of MAC cell proliferation.

Zileuton, BWA4C and BWB70C are all chelating inhibitors of 5-LO, (Musser and Kreft 1992), though Zileuton was an ineffective inhibitor of MAC cell proliferation relative to both BWA4C and BWB70C. This may be due to selectivity to the 5-LO pathway, which has been reported to be 15 times higher than to CO activity, and Zileuton was devoid of 12-LO or 15-LO activity (Cater et al 1991). 10μM Zileuton inhibited the 5-LO pathway in MAC cells, but with the exception of 12-HETE in MAC26 cells, the formation of 12-HETE and 15-HETE was increased. There was an increase in unmetabolised AA within the cells, which was able to pass through these pathways, due to the lack of inhibitory effect by Zileuton. BWB70C was capable of inhibiting both 5-HETE and 12-HETE productions, with 15-HETE production either remaining unchanged, or is slightly increased. In contrast BWA4C is capable of inhibiting all three pathways in all three cell lines, apart from 15-HETE production in MAC16 cell lines. There is no doubt that the acetohydroxamic acid moiety confers the ability to bind iron in BWA4C and BWB70C (Salmon and Garland 1991). Although this property was a consideration in the initial design of the 5-LO inhibitors, it is probable that their mechanism of action is more complex. Both compounds have relatively high electrode potentials and are capable of inhibiting lipid peroxidation (Darley-Usmar et al 1989). This action was clearly separate from the iron chelating properties of these compounds, and suggests that inhibition of LO could be by site-directed peroxyl scavenging.

CV6504 inhibited 5-HETE, 12-HETE and 15-HETE production in all three cell lines at 5μM and 10μM. Inhibition of 15-HETE production was the weakest. CV6504 is a redox inhibitor reducing the active Fe^{3+} at the centre of the LO enzyme, or reducing one of the free radical intermediates (McMillan and Walker 1992). CV6504 is a two electron reducing agent (Ohkawa et al 1991b) whereas most of the redox inhibitors tested for 5-LO action are one electron reducing agents (Musser and Kreft 1992). CV6504 was not specific to 5-LO pathways which may account for its low IC_{50} in MAC cells, and suggests an important role for both 12-LO and 15-LO pathways in cell proliferation. CV6504 was capable of reducing lipid peroxide levels,

and hydroperoxides are required for the stimulation of all three enzymes (Sigal 1991).

L,660-711 had a 5 times higher IC_{50} value in MAC cells than BWA4C, BWB70C and CV6504. The mechanism of action of L-660,711 was reported to be through specific antagonism of LTD_4 receptors, with no activity against LTC_4 (Jones et al 1989). This suggested that it was possible to inhibit the growth of MAC cells *in vitro,* by preventing the action of LTD_4, if the action of L-660,711 was still specific above 20μM. In the human erythroleukemia cell line K562, LO derived eicosanoids of 5, 11,12 and 15-HETE have been identified (Valone et al 1983). Inhibition of cell proliferation in this cell line and another human leukemia cell line (HL60), are possible with specific 5-LO inhibitors (Tsukada et al 1986). It appears possible that although cells may have more than one active LO pathway it was possible to inhibit cell proliferation through complete inhibition of the 5-LO pathway. In rat mammary tumour cells it was suggested that inhibition of cell proliferation by both LO and/or CO inhibitors is due to the imbalance of eicosanoids produced (Lee and Ip 1992).

In U-937 cells DNA synthesis was inhibited by the LO inhibitor 5,8,11,14-eicosatetraynoic acid (EYTA), and this could only be partially restored by the addition of LTD_4 to the culture medium (Ondrey et al 1989). The situation was similar to inhibition of MAC26 cells by 5μM BWA4C and 10μM CV6504, whereby addition of ($\pm$)15-HETE and (S)15 or (S)5-HETE could slightly reverse the inhibition. The more effective inhibitors such as BWB70C, BWA4C and CV6504 inhibit the production of 5-HETE, 12-HETE and 15-HETE from AA. It appears that it was necessary to add a balance of HETEs to the inhibited cells to restore cell growth rather than individual HETEs.

When PUFAs from medium supplementation become incorporated in the cell membrane, cells become more sensitive to external stimuli (Spectre and Yorek 1988). When L1210 cells are grown in medium supplemented with LA it becomes incorporated into the cell membranes rendering them more sensitive to cytotoxic agents (Spectre and Burns 1987). MAC13 and MAC26 cell growth stimulated by AA or LA was more resistant to inhibition by BWA4C or indomethacin, suggesting a role

for eicosanoid metabolism in this process rather than simple incorporation into the cell membranes that would render the cells more sensitive. MAC26 cells were exceptionally resistant to inhibition by BWA4C and indomethacin when stimulated with 18μM LA. This again suggests an alternative mechanism for stimulation of cell growth, such as by incorporation of the whole LA molecule, or nonenzymic metabolism. The stimulation of MAC26 cells by LA, was more sensitive to inhibition by CV6504, which suggested a role for lipid peroxidation products.

12(S)-HETE activates PKC in tumour cells by stimulating translocation of the enzyme to the cell membrane (Chen et al 1994 and Honn et al 1994). PKCs are a family of serine/threonine kinases that play a critical role both in signal transduction, tumour promotion and cell regulation (Blobe et al 1995). Friend erythroleukemic cells in log phase metabolise higher concentration of 15-HETE, in comparison to those cells in stationary phase. If this 15-HETE production was inhibited DNA synthesis was also inhibited, suggesting a role for 15-HETE in the proliferation of Friend erythroleukemic cells (Postoak et al 1990). In an oestrogen-independent human mammary cell line LA stimulated growth *in vitro*, was unaffected by CO inhibition but completely blocked by esculetin, a selective 12-LO and 15-LO inhibitor (Rose and Connolly 1990). Mice bearing MDA-MB-231 mammary cell tumours in their fat pads produced relatively high levels of 12-HETE and 15-HETE when fed a diet rich in LA. When these tumour-bearing mice are fed a diet containing high levels of DHA and EPA, along with tumour growth inhibition there was also a 4-5 fold reduction in the level of 12-HETE and 15-HETE produced (Rose et al 1995).

In MAC cells 5-HETE, 12-HETE and 15-HETE are produced from AA when this is supplemented into the medium. Inhibition of normal cell growth was more effective by BWA4C, BWB70C or CV6504 in comparison to indomethacin, L,660-711 or Zileuton. BWA4C, BWB70C and CV6504 were all capable of inhibiting 5-LO, 12-LO and 15-LO pathways in MAC cells at relatively low concentration of 10μM. CV6504 is designed as a free radical scavenger (Ohkawa et al 1991b), whereas BWA4C and BWB70C, were originally designed as iron chelating inhibitors but were also found to be free radical scavengers, (Salmon and Garland 1991). The reduction of MAC cell

proliferation *in vitro* by these inhibitors at such low concentration was due to the inhibition of all three LO pathways, and was a consequence of the less specific mechanism of inhibition. In rat mammary tumour cells it was proposed that tumour growth was dependent on the balance of eicosanoids produced by both LO and CO pathways (Lee and Ip 1992). In MAC13, MAC16 and MAC26 cell *in vitro* growth appeared to be dependent on the balance of eicosanoids produced by 5-LO, 12-LO and 15-LO.

Chapter 5.

The Promotion of Tumour Growth by Linoleic Acid (LA) and the Reduction of Tumour Growth by Lipoxygenase (LO) Inhibitors in Murine Colon Adenocarcinomas (MAC) *In Vivo* .

5:1. The Effect of Linoleic Acid (LA) on the Growth of the MAC26 Tumour in NMRI Mice.

5:1:1. Introduction.

Using a variety of chemically induced tumours both the quantity and type of PUFA in the diet has been shown to influence the initiation, promotion and development of colonic tumours in laboratory animals (Shamsuddin 1983 and Reddy 1986). When comparing a relatively low fat diet with a high fat diet, there will be major changes in the consumption of other dietary compounds unless the content of the diet is changed isocalorically. Laboratory animals have been shown to adjust their food intake so that a similar energy intake is maintained even when diets contain substantially different energy densities (Reddy et al 1985). A diet with a low energy value per unit weight (low fat) will be consumed at a greater rate than a diet with a more concentrated energy level (high fat). Unless diets are reformulated accordingly intake of proteins, minerals, vitamins and fibre will be lower in animals fed a high fat diet in comparison to those fed a relatively low fat diet. LA as a free acid and as a triglyceride in the form of corn oil have been administered to MAC16 tumour-bearing mice at 2.0g/kg orally (Hudson 1993). In the free acid form LA was capable of promoting suboptimal tumour growth, reversing the antitumour effect of EPA and influencing the FA composition of the tumour. In the triglyceride form LA was not capable of stimulating tumour growth when given alone or in combination with EPA, and did not influence FA incorporation into the tumour. The different effects on tumour growth observed by LA was thought to be due to pharmacokinetics, the free fatty acid form being incorporated into the tissues more readily than the triglyceride form (Hudson 1993). In these studies LA was given in the free acid form to circumvent any problems that may arise through the addition of extra calories and being more readily taken up into the tissues of NMRI mice (Hudson 1993). The MAC26 tumour model was used as it is a relatively slow growing tumour in NMRI mice, the growth of which is more likely to be influenced by the availability of FAs.

Tumour growth is dependent on a delicate balance between the generation of new cells and cell death. If proliferation exceeds cell death then tumour growth will be observed (Steel 1977). LA administered in the form of corn oil to Balb/c mice

bearing transplantable mammary adenocarcinomas promoted tumour growth (Gabor et al 1985). This increased tumour growth could not be explained by the stimulation of tumour cell proliferation and, by cell kinetic studies, was found to be due to a reduction in cell loss (Gabor et al 1985). Cell kinetic studies can be carried out quickly and effectively using [^{125}I]-5-iodo-2'deoxyuridine (^{125}IUdr). Most if not all the ^{125}IUdr becomes incorporated into the tumour DNA during replication, where due to the similarity in size between an iodine and a methyl group it forms a stable compound (Steel 1977). The half-life of the labelled tumour DNA can be calculated to give the potential doubling time, the time it would take the tumour to double in size if there is no cell loss. The actual doubling time is the rate of tumour growth observed. From calculating the potential doubling time and the actual doubling time the cell loss factor can be determined. An alternative method for the calculation of cell kinetics consists of a method utilising the incorporation of methyl[^{3}H]-thymidine (^{3}H-Tdr) into tumours and use of computer aided analysis of fraction labelled mitosis (FLM) (Gabor et al 1985). ^{125}I-Udr and ^{3}H-Tdr techniques produce correlating results in relation to the calculation of cell loss (Begg 1977, Franko and Kallman 1980, Gabor et al 1985). The ^{125}I-Udr technique has been reported to produce lower values of cell loss in tumours with high cell loss, implying reutilisation of the label by the tumour (Begg 1977). Despite this the technique is still considered rapid and effective, without the requirement of time consuming microscope work.

AA has been shown to play an essential role in the mechanism by which basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) stimulate the proliferation of both bovine capillary endothelial cells and aortic smooth muscle cells (Dethlefsen et al 1994). AA has also been found to be involved in the migration of and tube formation of human vascular endothelial cells *in vitro* and the neovascularisation of gliomas in mice (Ito et al 1993). Angiogenesis is a requirement for the growth of most tumours (Folkman 1990) and tumour growth can be further enhanced by the production of growth factors produced by rapidly dividing epithelial cells in this process (Liotta et al 1991).

5:1:2. Results

The effect of LA given daily by gavage on the growth of the MAC26 tumour in NMRI mice is shown in figure 19. LA was dissolved in arachis oil and control groups also received 0.1ml arachis oil daily by gavage. Daily dosing of arachis oil alone had no effect on tumour growth in comparison to 0.1ml saline given daily by gavage (results not shown). There was no difference in food consumption between LA treated animals and control animals, control animals consumed 4.95 $\pm$ 0.80g of food per day and LA treated animals consumed 4.60 $\pm$ 0.80g of food per day. LA had a threshold value of 0.4g/Kg for the stimulation of MAC26 tumour growth in NMRI mice, below which no stimulation of tumour growth was observed and above which at 1.0g/kg LA or 2.0g/kg LA there was no further stimulation of tumour growth. 0.2g/kg and 0.04 g/kg LA dosed over 8 days had no effect on tumour volume in comparison to controls. Stimulation of MAC26 tumour growth was such that at 0.4g/kg, 1.0g/kg and 2.0g/kg tumour volume was increased significantly over that of control at 4, 6, 7 and 8 days.

The effect of 2.0g/kg LA on the growth kinetics of the MAC26 tumour in comparison to control is shown in table 3. LA treated animals received 2.0g/kg LA in a 50μl aliquot daily by gavage, whilst control animals received 50μl of saline. 2.0g/kg LA increased both the potential doubling time and the actual doubling time of the MAC26 tumour in comparison to control with no effect on the cell loss factor. This suggests that the increase in tumour growth observed by daily administration of 2.0g/Kg LA in the MAC26 tumour is through an increase in tumour proliferation rather than a decrease in the cell loss factor. The growth stimulatory effect of 2.0g/Kg LA on the MAC26 tumours used in this kinetic study is shown in figure 20.

The autoradiography of MAC26 tumours from control mice and those treated with 2.0g/kg LA daily orally are shown in photographs 1-4 and 5-8 respectively at x400 magnification. Animals were injected with ^{3}H-Tdr and tumours excised 24 hr latter are used in photographs 1-8. The ^{3}H-Tdr incorporated into the tumour DNA of replicating cells was detected as charged particles by autoradiography emulsion. These charged particles caused darkening of the autoradiography emulsion and can be seen on the tumour sections. Photographs were taken under a blue filter so as much

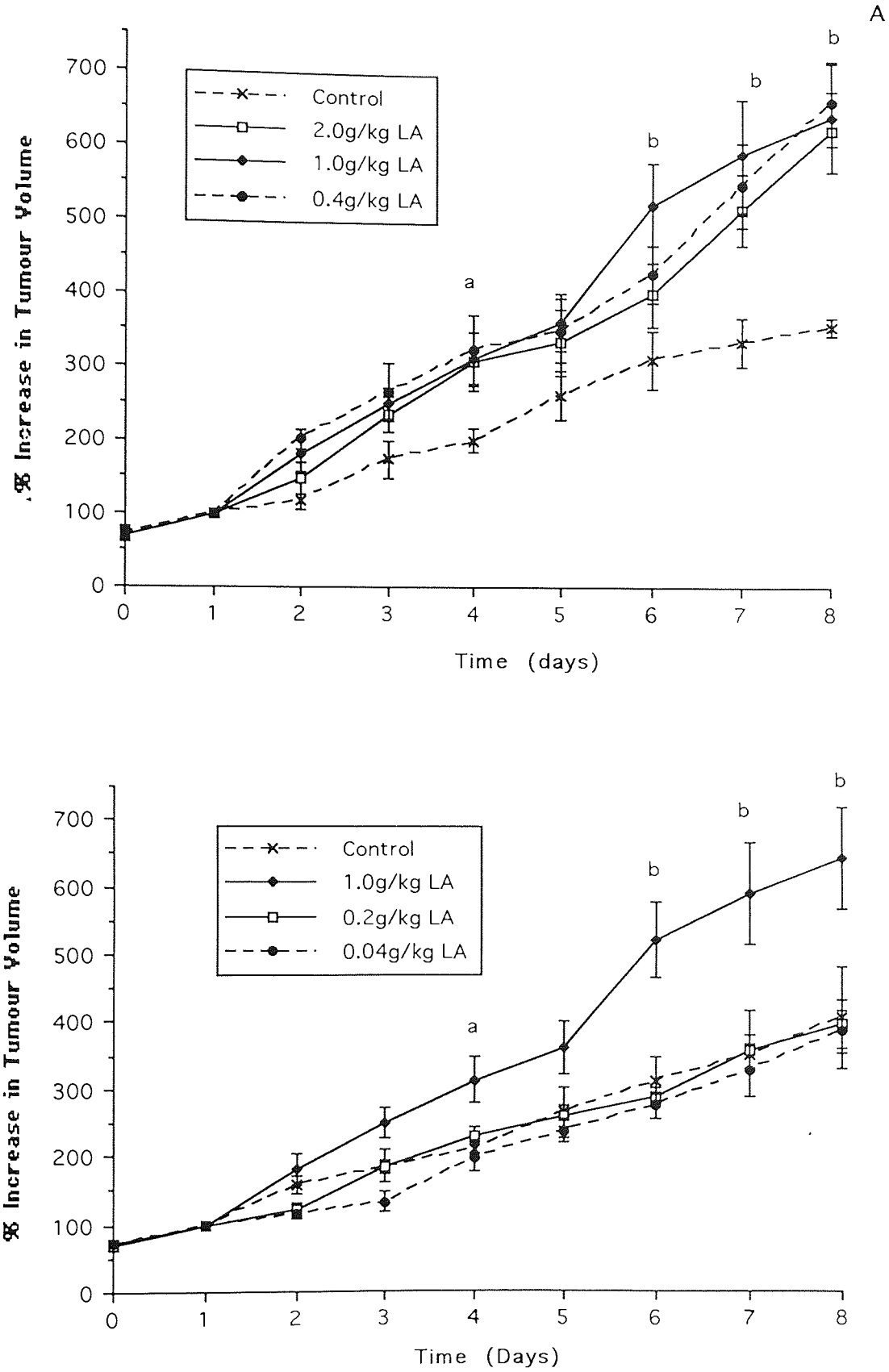


Figure 19 A. and B. The effect of lionleic acid (LA) given by daily oral administration, on the growth of the MAC26 tumour in NMRI mice.
(a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test, (n=9).)

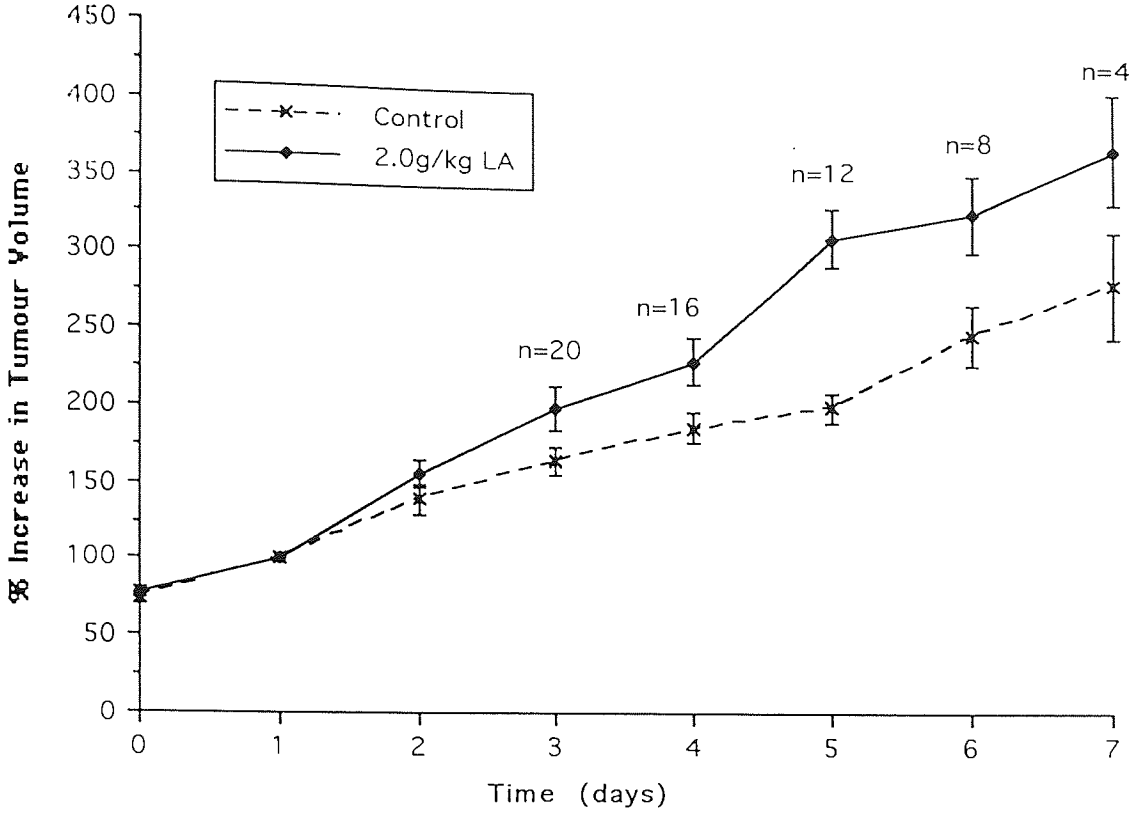


Figure 20. The effect of 2.0g/kg LA on the growth of the MAC26 tumour in NMRI mice. Tumours were used for calculation of cell loss.

Table 3. The growth kinetics of the MAC26 tumour with and without daily oral administration of 2.0 g/kg linoleic acid (LA).

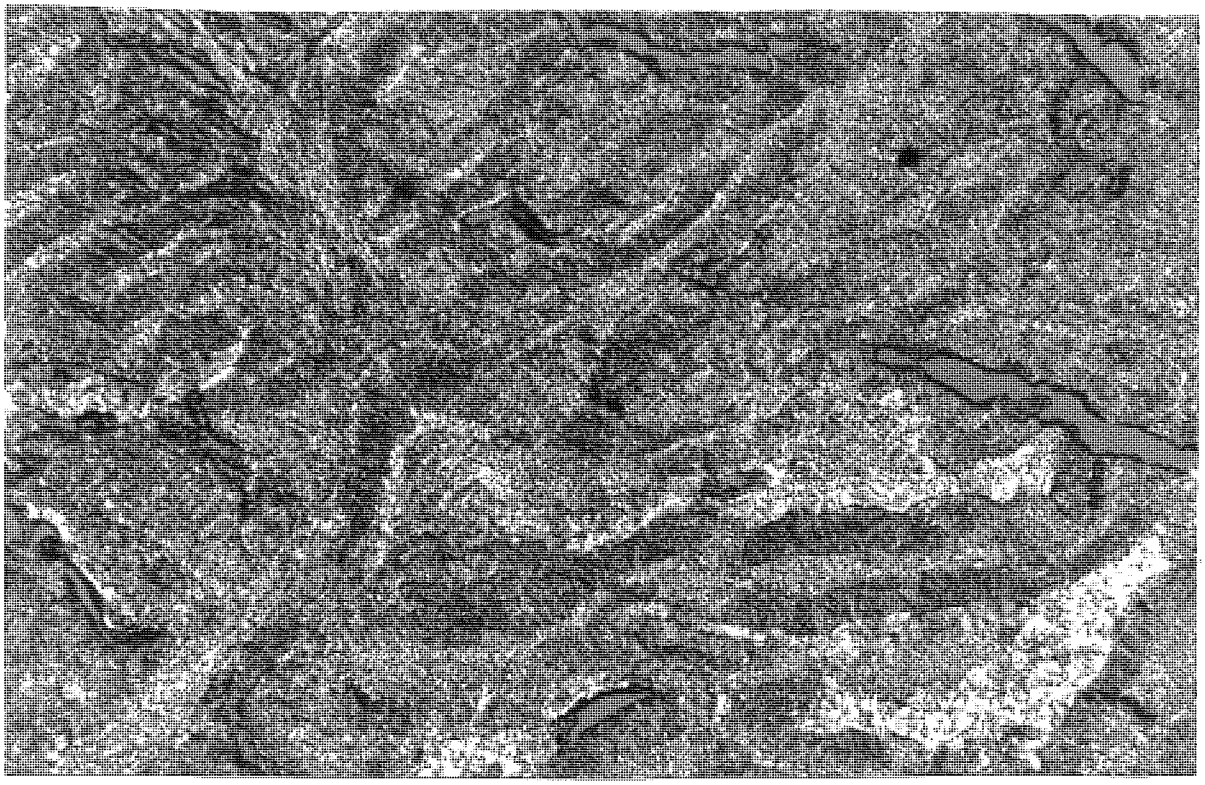
Treatment	Doubling Time (TD) (hours)	Potential Doubling Time (TP) (hours)	Cell Loss (Ø) (%)
Control	130	42	68
2.0g/kg LA	84	28	69

detail in the tumour sections as possible could be observed. Light areas in the sections are due to the presence of wax in which the tumour sections have been embedded. Where the tumour tissue is present the distribution of charged particles is uniform throughout, (though not very clearly), whether the animals were treated with 2.0g/kg LA or not. This suggests that 2.0g/kg LA increased the growth of the MAC26 tumour by increasing cell proliferation in all areas of the tumour.

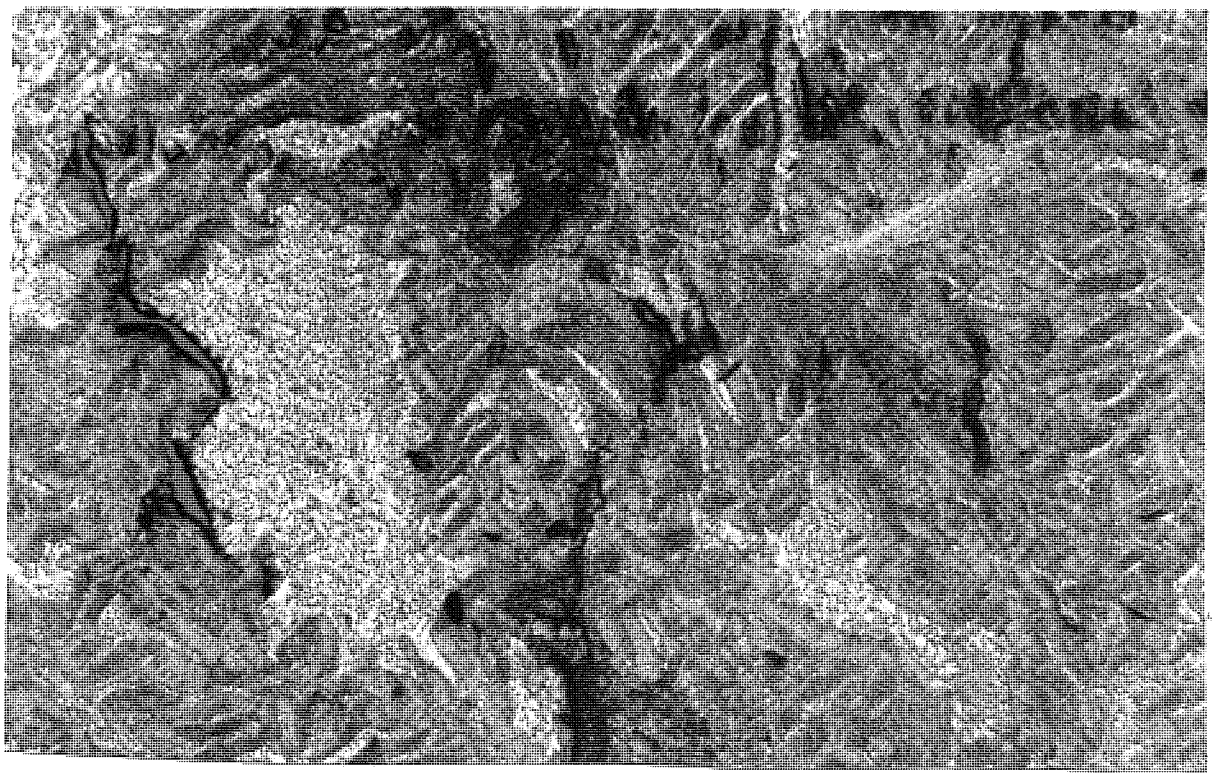
The effect of 0.1ml arachis oil and 1.0g/kg LA dissolved in arachis oil, given daily by gavage from the day after transplantation of the MAC26 tumour is shown in figure 21A. Tumour volumes were recorded 14 days after transplantation when they were smaller than normally measured, at 28-48cm^3. 20 days after transplantation of MAC26 tumour arachis oil and 1.0g/kg LA produced a significant increase in tumour volume over control. On day 21 1.0g/kg LA increased tumour growth to produce a significant increase in tumour volume over both control and arachis oil treated animals. The experiment was terminated on 21 days after transplantation of tumour so tumour growth did not become so rapid that vascularisation became inadequate. The effect of arachis oil and 1.0g/kg LA given daily by gavage from the day after transplantation on the vascular volume of the MAC26 tumour is shown in figure 20B. Both treatments increased the vascular volume per gram of tumour in comparison to that of control but this increase was not significant.

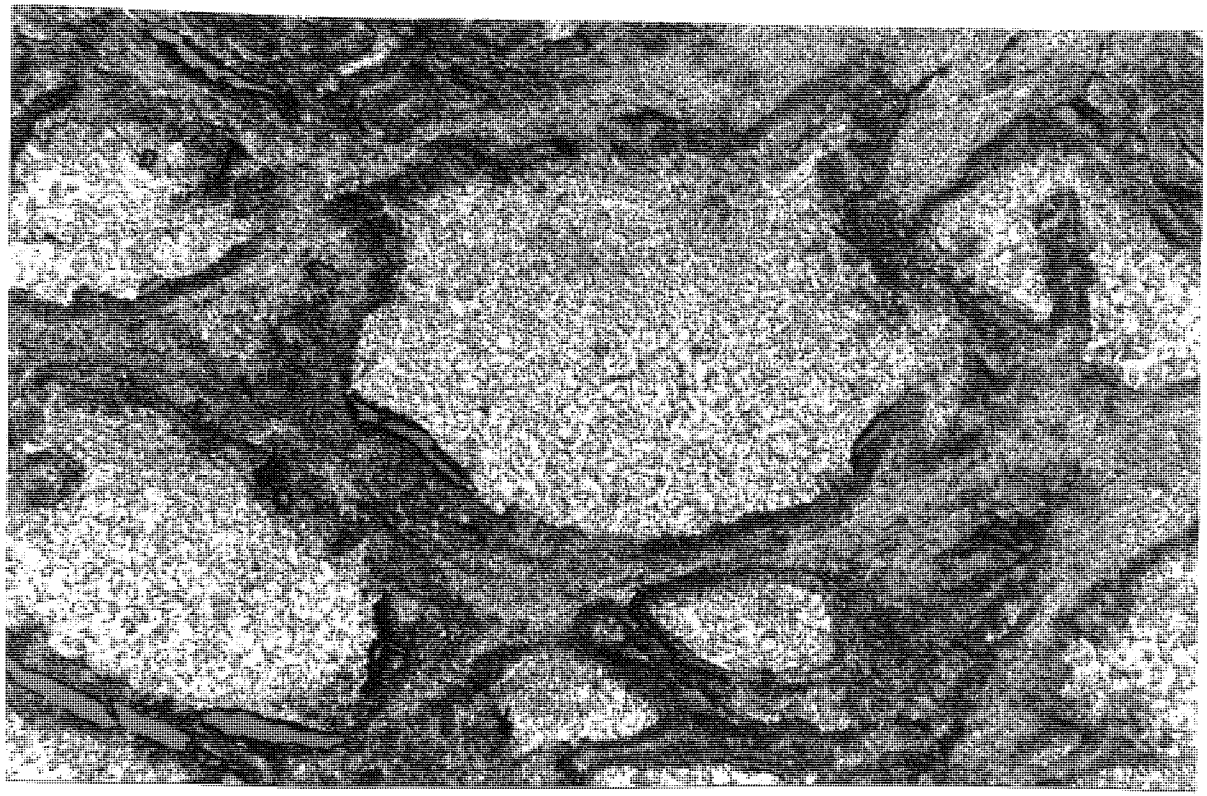
5:1:3. Discussion

LA in the free acid form was capable of increasing the growth rate of the MAC26 tumour in NMRI mice, as was LA in corn oil capable of increasing the growth of transplantable mammary tumours in Balb/c mice (Gabor et al 1985) and MDA-MB-435 human breast cancer cell lines in nude mice (Rose and Connolly 1992). Arachis oil is a mixture of FAs as triglycerides, 25% of which is LA. This means that daily administration of 1.0g/kg LA as a triglyceride over 8 days orally did not stimulate MAC26 tumour growth but 1.0g/kg LA as a free acid did. When arachis oil was given over a more prolonged period of time, 21 days from the day after transplantation of the MAC26 tumour, 1.0g/kg daily as a triglyceride was capable of increasing tumour

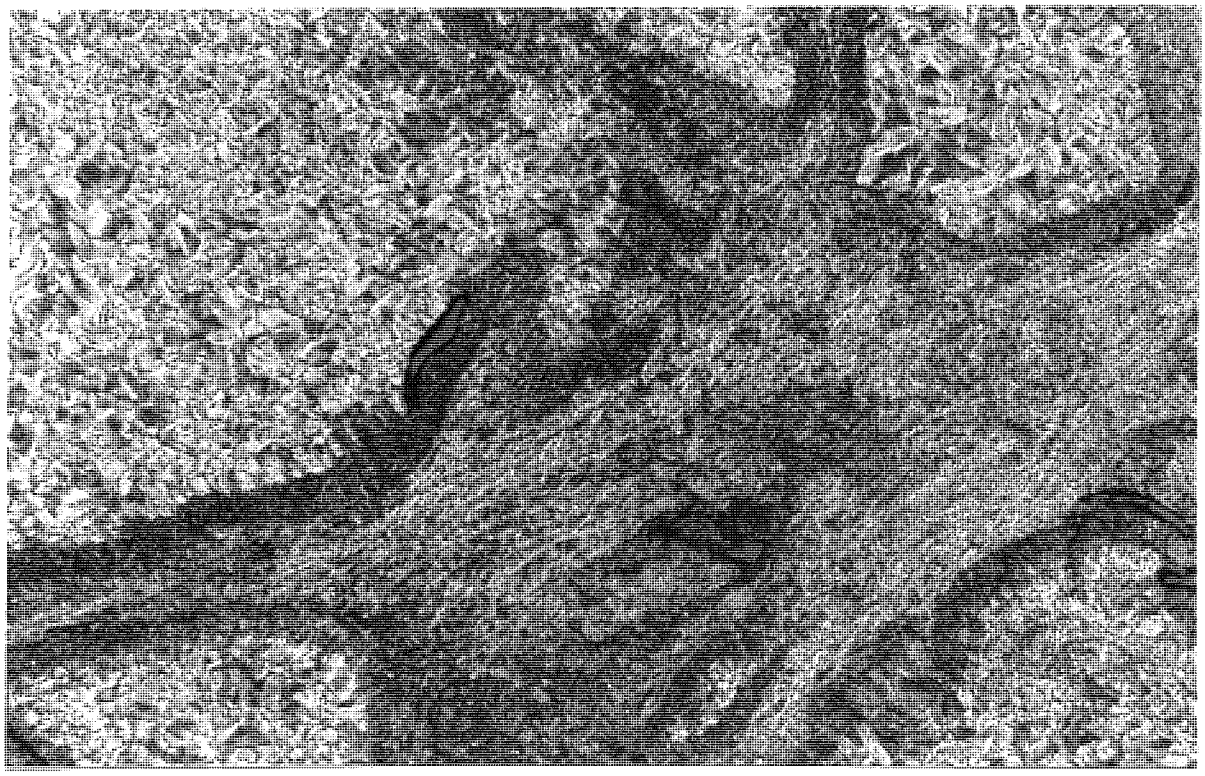


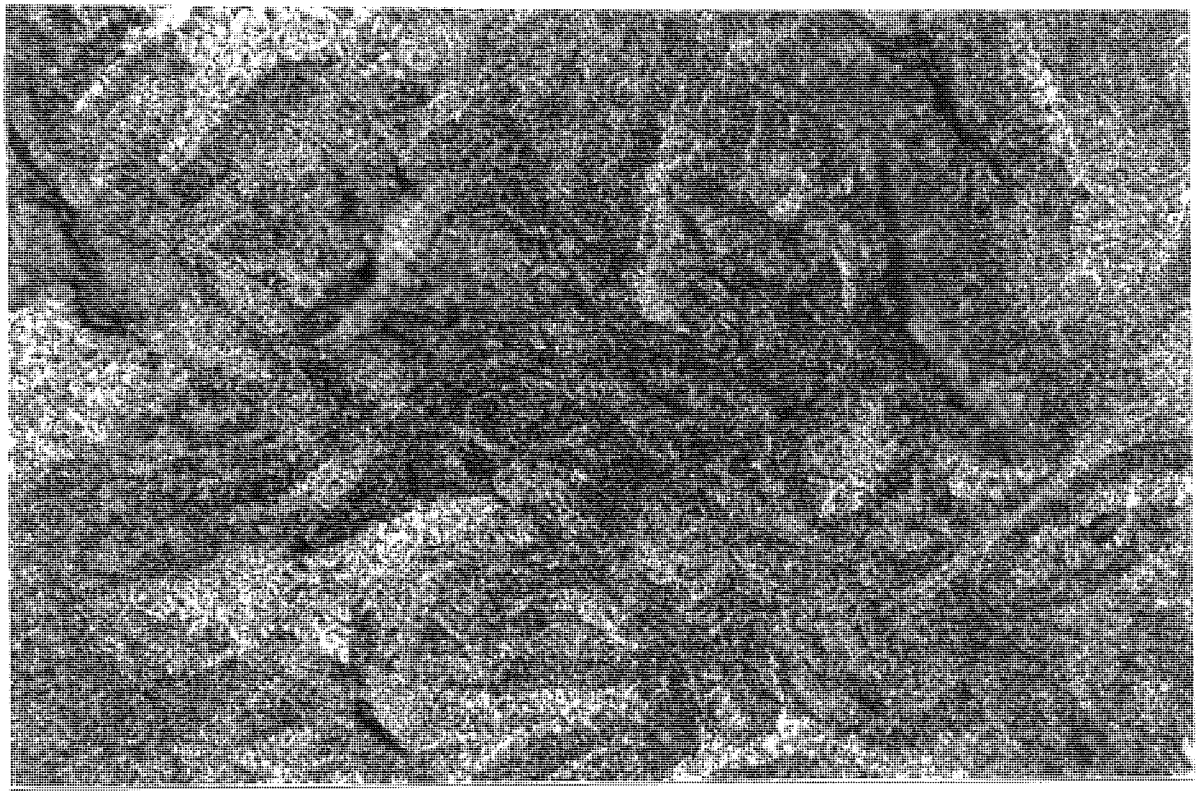
Photograph 1. and 2. Autoradiography sections of Control MAC26 tumour.



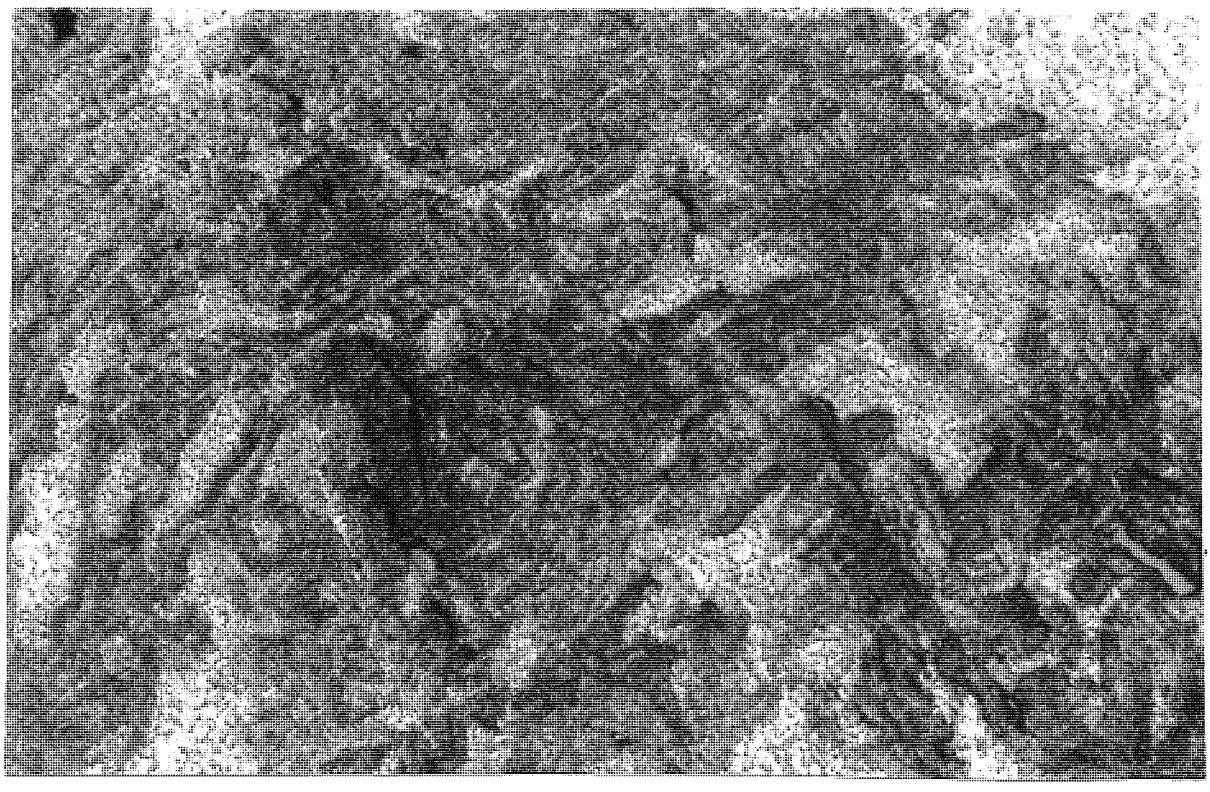


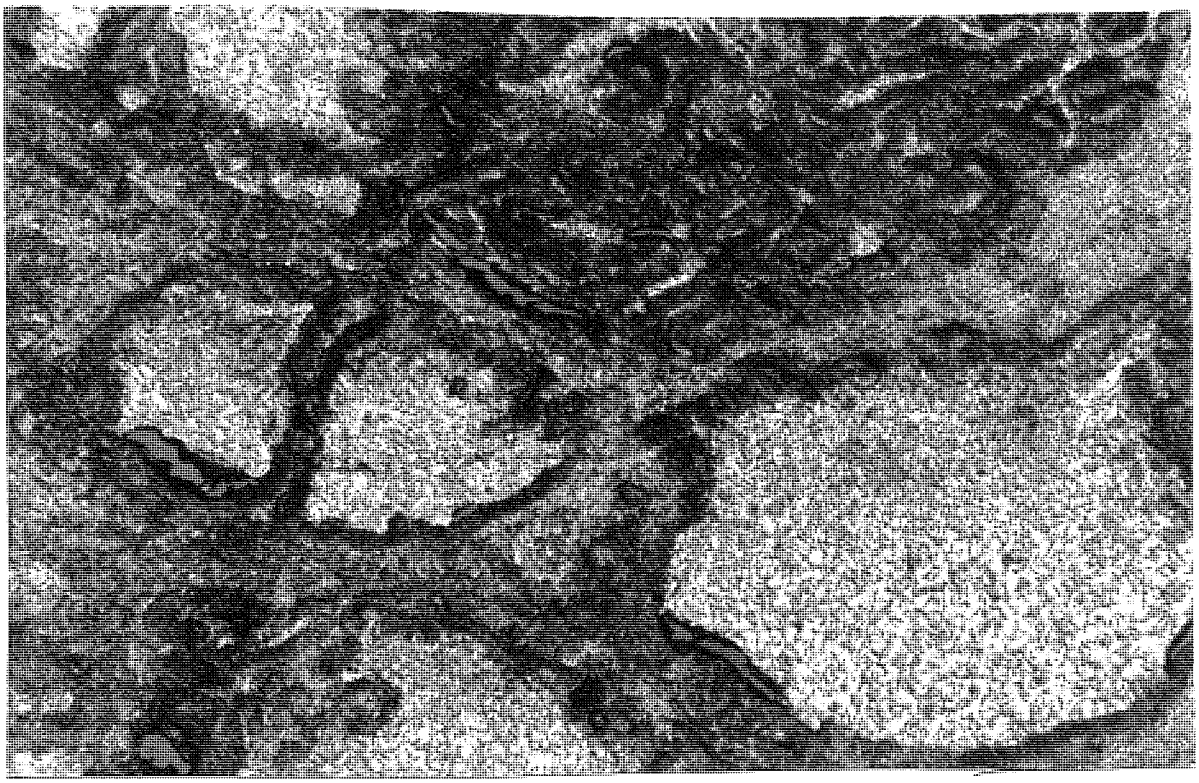
Photograph 3. and 4. Autoradiography sections of Control MAC26 tumour.



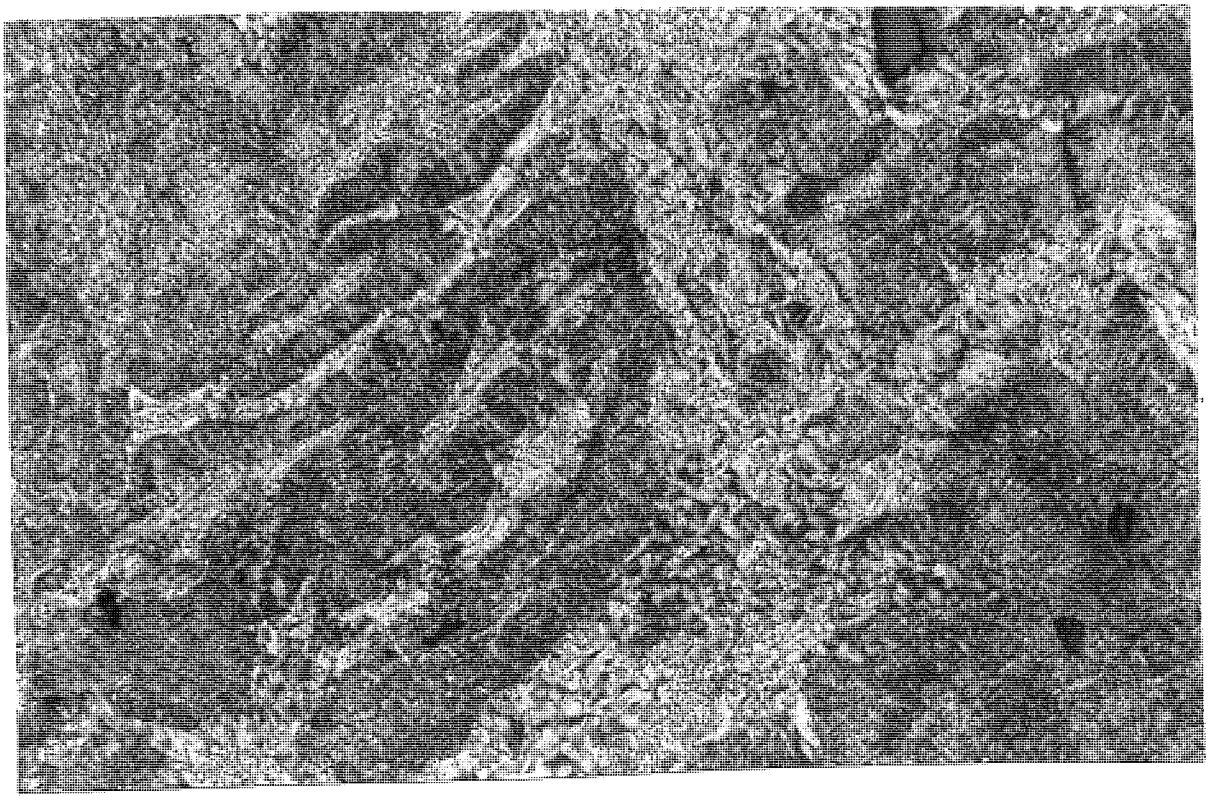


Photograph 5. and 6. Autoradiography sections of MAC26 tumours treated with 1.0g/kg/day linoleic acid (LA).





Photograph 7. and 8. Autoradiography sections of MAC26 tumours treated with 1.0g/kg/day linoleic acid (LA).



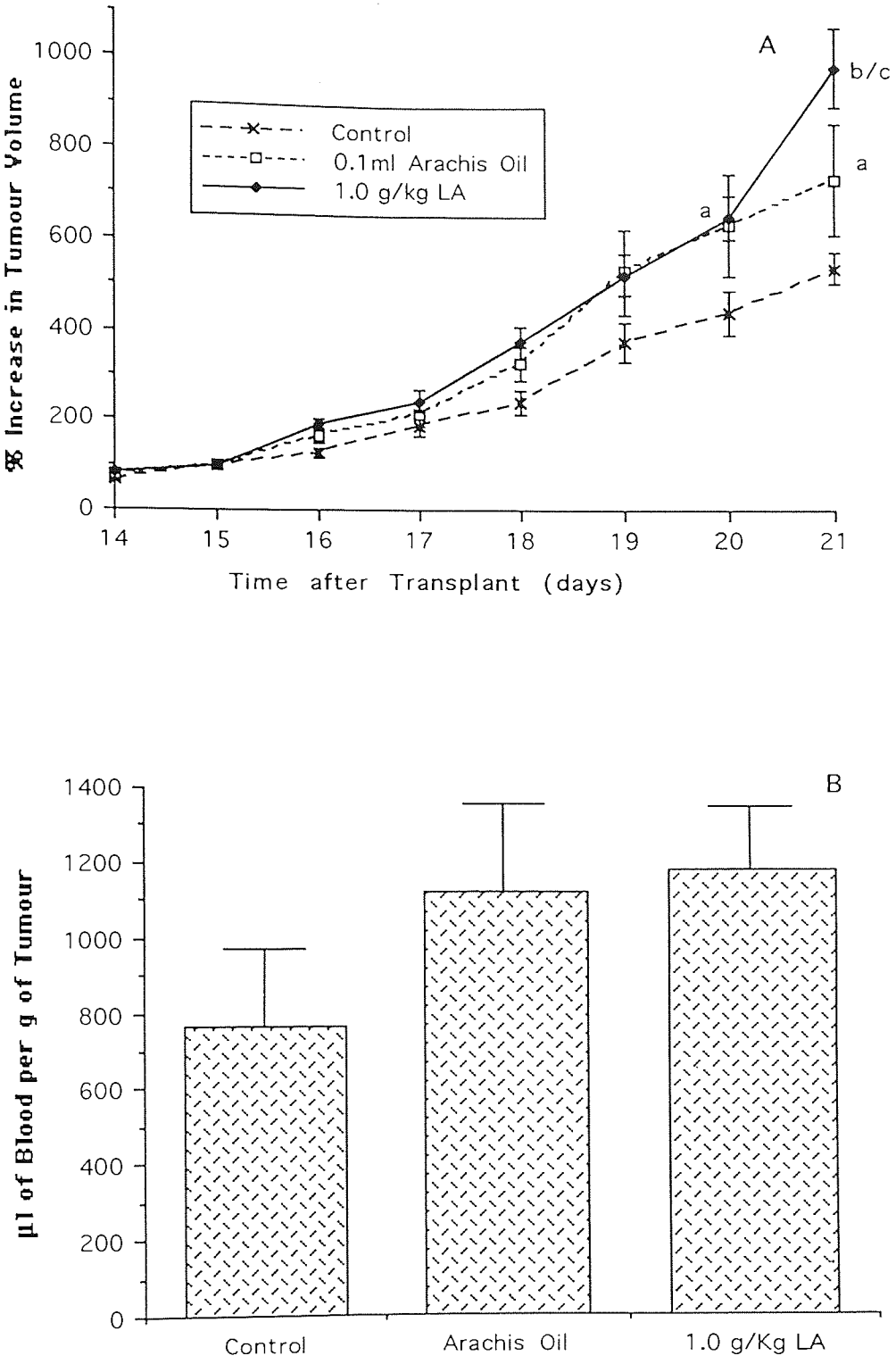


Figure 21. The effect of arachis oil and 1.0 g/Kg linoleic acid (LA) on A. the growth and B. the vasculature of the MAC26 tumour when administered every 24 hours from the day after transplant (n=12).

(a= $p<0.05$ and b= $p<0.01$ from control tumour growth and c= $p<0.01$ from arachis oil treated, using two-way ANOVA followed by Tuckey's Test.)

growth. In the MAC16 tumour-bearing model 2.0g/kg LA given in the free acid form was capable of influencing the FA profile of the tumour, but when given as a triglyceride at 2.0g/kg in the form of corn oil it was not (Hudson 1993). In the MAC26 tumour model prolonged administration of LA as a triglyceride produces suitable pharmacokinetics to increase tumour growth.

The increase in tumour growth produced by LA in MAC26 tumour-bearing NMRI mice had a threshold value of 0.4g/kg LA, above which no further increase in tumour growth was observed. The individual concentration of FA in rat/mouse breeding diet was determined by gas chromatography (GC) of extracted FA methyl esters (Hudson et al 1993). The daily consumption of LA from the diet by MAC26 tumour-bearing mice *ad libitum* was 45 mg per day, which together with 0.4g/kg LA given orally was the equivalent of 3.5% calorific intake. This is close to the threshold value (4% total energy) calculated for the promotion of mammary tumours in rats (Ip et al 1985). The report of the British Nutrition Task Force 1992, recommended for humans a 6% of total calories for PUFAs, where according to these results, maximal stimulation of tumour proliferation will be experienced.

Cell loss in the MAC26 was much higher than that calculated for the MAC16 tumour (Hudson et al 1993). LA administered alone had no effect on the cell loss factor in MAC16 tumour-bearing mice, whereas cell loss was increased by the administration of EPA which had both antitumour and anticachectic action on the MAC16 tumour. LA was capable of reversing the antitumour effect of EPA, and a decrease in the cell loss factor was observed in comparison to that calculated by administration of EPA alone when LA was administered in conjunction with EPA (Hudson et al 1993). LA had no effect on the cell loss factor in MAC26 tumours, so the actual increase in tumour growth observed by oral administration of LA was due to an increase in cell proliferation. Autoradiography showed that cell division was manifested throughout the tumour. LA increased MAC26 tumour growth in NMRI mice by increasing cell proliferation in all areas of the tumour.

LA could stimulate the growth of the MAC26 tumour in NMRI mice by 3 of the 4 possible mechanisms proposed by Nicholson et al 1988. The whole LA molecule may

have been involved in the activation of gene transcription or transduction (Tiwari et al 1991) or the activation of enzymes involved in proliferation such as PKC (Blobe et al 1995). LA may have become incorporated into tumour cell membranes, increasing the fluidity of the membrane and sensitivity to mitogenic stimuli (Spectre and Yorek 1988) produced either by the tumour cells or the host. LA is the dietary source of AA, from which eicosanoids can be metabolised. Eicosanoids have been found to stimulate both tumour cell proliferation (Buckman et al 1991, Lee and Ip 1992) and vascularisation of the tumour (Ito et al 1993 and Dethlefsen et al 1994).

The fourth proposed mechanism of action of PUFAs on tumour growth (Nicholson et al 1988) was the suppression of the immune system and the host's defences against the invading tumour. This was the mechanism of action by which corn oil, rich in LA, was able to increase the growth of a transplantable mammary tumour in Balb/c mice. The increase in tumour growth could not be accounted for by an increase in cell proliferation alone and there was a reduction in the cell loss factor within the tumours (Gabor et al 1985). In MAC26 tumour-bearing mice the increase in tumour growth produced by LA was not accompanied by a decrease in the cell loss factor, suggesting that suppression of the immune system was not a mechanism by which tumour growth was increased.

Tumour growth is accompanied by neovascularisation since all solid tumours require stroma if they are to grow beyond 1 to 2mm^3 (about 10^6 cells) (Folkman 1991). This suggests that when MAC26 tumours became palpable and could be measured by callipers intense angiogenic activity and vascularisation had already taken place. The vascular volume of the MAC26 tumour was increased per gram of tumour if LA is given at 1.0g/kg in the free acid form or at 1.0g/kg as a triglyceride in the form of arachis oil, from the day after transplantation of the tumour every 24 hr orally. Organisation of the microvasculature in tumours was not as strict as in normal tissues. Tumours show phenotypic changes like dilated and irregular vessels (Denekamp 1982) and increased permeability (Jain 1985, Heuser and Miller 1986). MAC26 tumour growth was increased by both arachis oil and LA, so the rate of neovascularisation of the tumour was also increased. This may increase the

phenotypic changes mentioned above and increase the permeability of the vasculature in relation to the control, and could account for the increased tumour volume calculated with LA treatment in the MAC26 tumour. Angiogenesis is initiated and sustained by diffusible angiogenic factors either after local activation of genes or release from their storage (Diaz-Flores et al 1994). Stimulation of both aortic epithelial cell and vascular smooth muscle proliferation is activated through bFGF and PDGF, a process with an essential role for AA. The additional AA released from LA and arachis oil may facilitate a more rapid vascularisation of the MAC26 tumour, supporting increased tumour growth in comparison to control tumour growth rate.

A role for LA in the growth of two murine breast cancer cell lines in nude mice has been suggested at the neovascularistion stage as well as subsequent cell growth (Rose et al 1995). LA was capable of increasing the growth rate of the MAC26 tumour in NMRI mice. Stimulation of the proliferation of tumour cells was throughout the tumour, and includes the vascular cells, with a slight increase in vascular volume per gram of tumour.

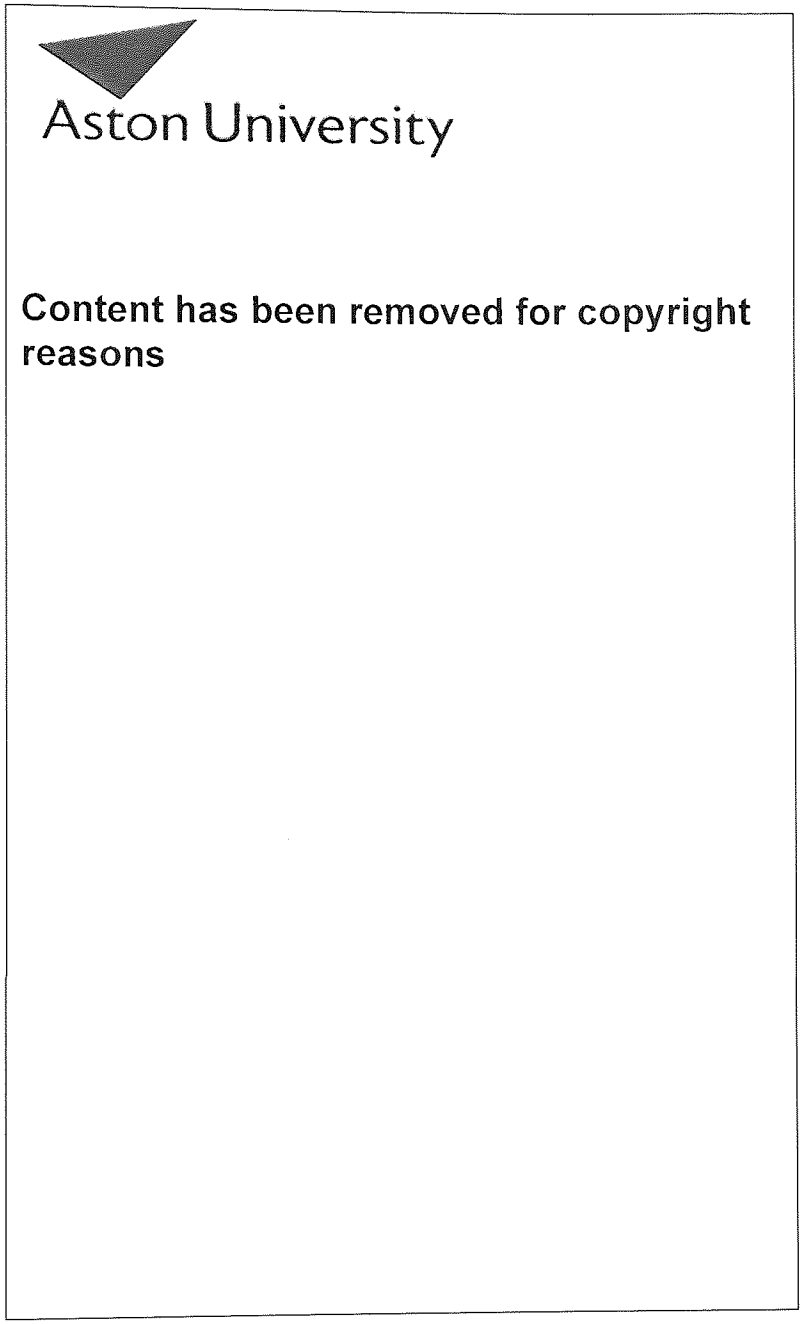


Figure 22.
The redox cycle of CV6504 in mammalian cells.
R represents free radicals such as hydroxy radicals or hydroperoxy radicals.
ROOH and ROH represent a lipid hydroperoxide and lipid hydroxide respectively (taken from Ohkawa et al 1991b).

5:2. The Inhibition of Murine Colon Adenocarcinoma Growth in NMRI Mice by Lipoxygenase Inhibitors.

5:2:1. Introduction.

Eicosanoid production is controlled in most normal cells but often reaches exaggerated levels in malignant tissues (Karmali 1987). The promotion of breast tumour growth by dietary LA was found to correlate with increased PG production (Alysworth et al 1987). It was proposed that these increased PG levels allowed tumours to avoid immunological rejection by suppression of the host immune system (Brunda et al 1980). *In vitro,* in the absence of any humoral or cell mediated immunity, CO derived PGE_2 (Lee and Ip 1992) and LO derived LTB_4 or LTC_4 (Snyder et al 1989) have been shown to directly stimulate the proliferation of malignant cells. Esculetin, a LO inhibitor, has been shown to inhibit 7,12-dimethyl-benzo(a)anthracene (DMBA) induced rat mammary tumour development and growth *in vivo,* whilst the CO inhibitor piroxicam lacked any such activity (Kitagawa and Noguchi 1995). Two LO inhibitors esculetin and nordihydroguaiaretic acid (NDGA) suppressed MDA-MB-231 human breast cancer cell line proliferation *in vitro* but again no action was demonstrated by piroxicam (Rose and Connolly 1990). It has been suggested that LO inhibitors are more active as tumour suppressive agents than CO inhibitors (Noguchi et al 1995). Inhibition of tumour growth by either CO or LO inhibitors can not be reversed by individual eicosanoids, suggesting that the balance of eicosanoids may be a critical determinant of tumour cell proliferation (Lee and Ip 1992, Snyder et al 1989).

The MAC13, MAC16 and MAC26 cell lines *in vitro* were more sensitive to inhibition by the LO inhibitors BWA4C, BWB70C and CV6504, than the CO inhibitor indomethacin (see Chapter 4). BWA4C was tested for inhibitory activity against 5-LO in humans, but as in animal models it had a relatively short half-life (approximately 2hr) (Salmon and Garland 1991). There are three metabolic transformations common to all acetohydroxamic acids in animal studies: i. oxidation of the carbon adjacent to the hydroxamic acid moiety leading to the formation of the corresponding carboxylic acid, ii. glucuronidation of the N-hydroxy group and subsequent excretion into bile or urine, or iii. reduction of acetamide analogue (Salmon and Garland

1991). Hydroxyureas provided equivalent 5-LO potency and selectivity *in vitro* to that of acetohydroxamic acids. When BWB70C, a hydroxyurea, was tested *in vivo* higher levels of unmetabolised drug were maintained after oral administration with an extended 5-LO inhibitory activity *ex vivo* (Salmon and Garland 1991). The difference in pharmacokinetic profile between BWA4C and BWB70C can be explained by the reduced formation of glucuronide metabolites from hydroxyureas in comparison to acetohydroxamic acids (Salmon and Garland 1991). BWA4C and BWB70C were insoluble in water and were given to tumour-bearing NMRI mice orally every 12 hr suspended in liquid paraffin.

CV6504 given orally between 3 to 20 mg/kg/day inhibited TXA_2 synthetase, 5-LO activity and lipid peroxidation dose dependently in rat puromycin aminonucleoside (PAN) nephritis (Shibouta et al 1991). The redox cycle of CV6504 is shown in figure 22. and is the proposed mechanism by which CV6504 inhibits 5-LO and lipid peroxidation (Ohkawa et al 1991b). LOs are non-haem iron containing enzymes that require the iron in the ferric (Fe^{3+}) state for the enzyme to be activated and functional (Schilstra et al 1994). Hydroperoxides oxidise the ferrous (Fe^{2+}) resting state of the LO enzyme to the active Fe^{3+} state (Musser and Kreft 1992). By contrast antioxidants reduce Fe^{3+} at the active site of the enzyme to Fe^{2+} thereby inhibiting the action of the LO enzyme (McMillan and Walker 1992). CV6504 has a redox cycle whereby in mammalian cells CV6504 quinone is reduced to a hydroquinone by a two-electron donating enzyme such as DT-diaphorase. In cycling back to a semiquinone the Fe^{3+} at the active site of the enzyme is reduced to Fe^{2+} by reduction of hydroperoxide levels thereby inhibiting the activity of the 5-LO enzyme. Hydroperoxide levels are further reduced as quinone is reformed from semiquinone. The reformed quinone is susceptible to reduction by a two-electron reducing agent and the cycle can be initiated again (Ohkawa et al 1991b).

5:2:2. Results

The effect of indomethacin with and without concurrent administration of 1.0g/kg LA on the growth of the MAC26 tumour in NMRI mice is shown in figure 23. Indomethacin reduced the stimulatory effect of LA, reducing tumour growth to below

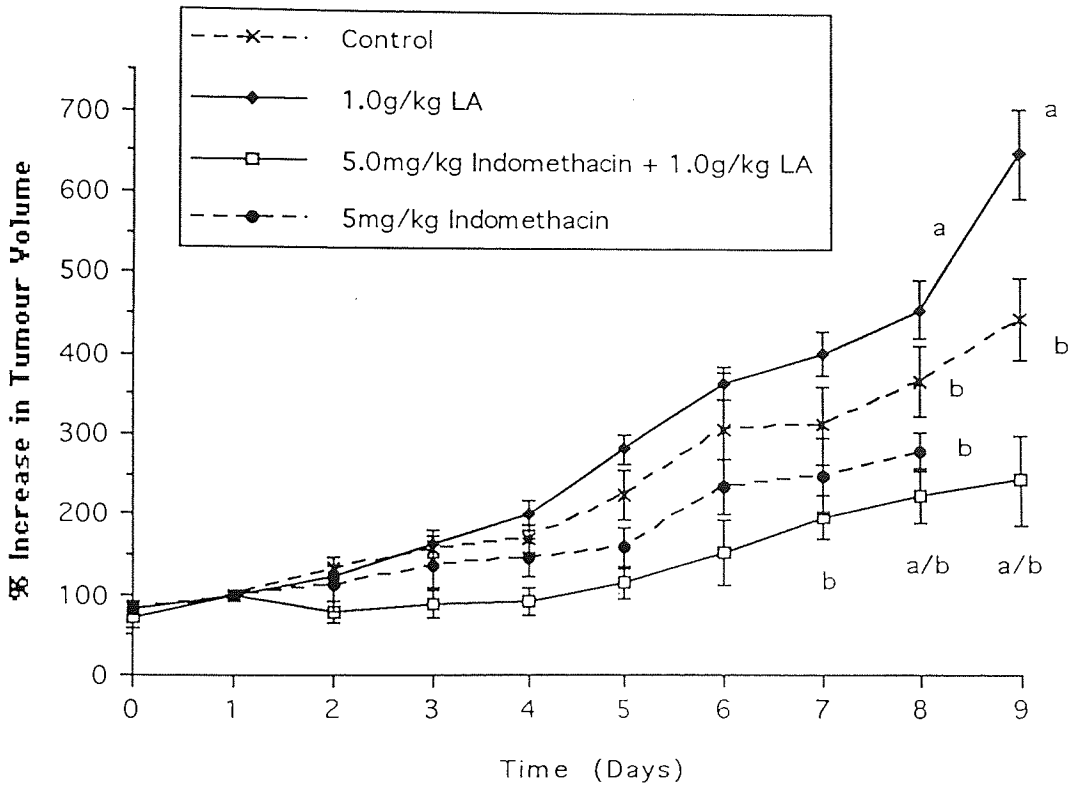


Figure 23. The effect of 5mg/kg indomethacin with and without 1.0g/kg linoleic acid (LA) on the growth of the MAC26 tumour in NMRI mice (n=9).
(a= $p<0.01$ from Control and b= $p<0.01$ from 1.0g/kg LA, using two-way ANOVA followed by Tuckey's Test.)

that of the controls. Antitumour action was such that a significant reduction in tumour volume was observed in comparison to administration of 1.0g/kg LA alone at days 7 to 9, and in comparison to controls at days 8 and 9. Indomethacin administered alone reduced tumour growth to give a significant reduction in tumour volume over control at day 8. Unfortunately when administered at 5mg/kg in 0.1ml of 0.1% ethanol by ip injection indomethacin was toxic to the tumour-bearing mice and the experimental group had to be terminated at day 8.

The effect of BWA4C, dosed orally every 12 hr in liquid paraffin, on the growth of the MAC26 tumour in NMRI mice is shown in figure 24. Administration of 0.1ml liquid paraffin orally every 12 hr had no effect on MAC26 tumour growth over controls given 0.1ml saline orally every 12 hr (results not shown). BWA4C inhibited MAC26 tumour growth in a dose independent manner between 5-25mg/kg, with a significant reduction in tumour volume at 25mg/kg on days 7, 8, and 9, and at 5mg/kg on days 8 and 9. A specific growth delay at the first doubling of 0.46, 0.23 and 0.46, or at the second doubling of 0.17, 0.17 or 0.57, was observed for 5, 10 and 25mg/kg BWA4C respectively. These specific growth delays were sufficient to allow the MAC26 tumour-bearing animals to survive for an extra 2 days compared to controls, to reach the maximum permitted tumour volume of 1000mm^3. LA in this experiment failed to significantly promote MAC26 tumour growth over control (liquid paraffin). Kinetic studies have shown that LA promotes MAC26 tumour growth by a reduction in tumour doubling time from 130 hr to 72 hr (section 5:1.). During the initial period of this experiment a small promotion of MAC26 tumour growth was observed, tumour doubling time was reduced by 1.0g/kg LA from 113 hr to 83 hr. From day 4 the doubling time of both 1.0g/kg LA and control animals was reduced to 53 hr. The inability of LA to stimulate tumour growth could be explained by the increased growth rate of the tumours used in this experiment. Concurrent administration of 1.0g/kg LA with BWA4C produced an antitumour effect which was dose dependent. The antitumour effect at 25mg/kg with 1.0g/kg LA produced a significant reduction in tumour volume at days 6, 7, 8 and 9. The specific growth delay produced at the first doubling with concurrent administration of 1.0g/kg LA was

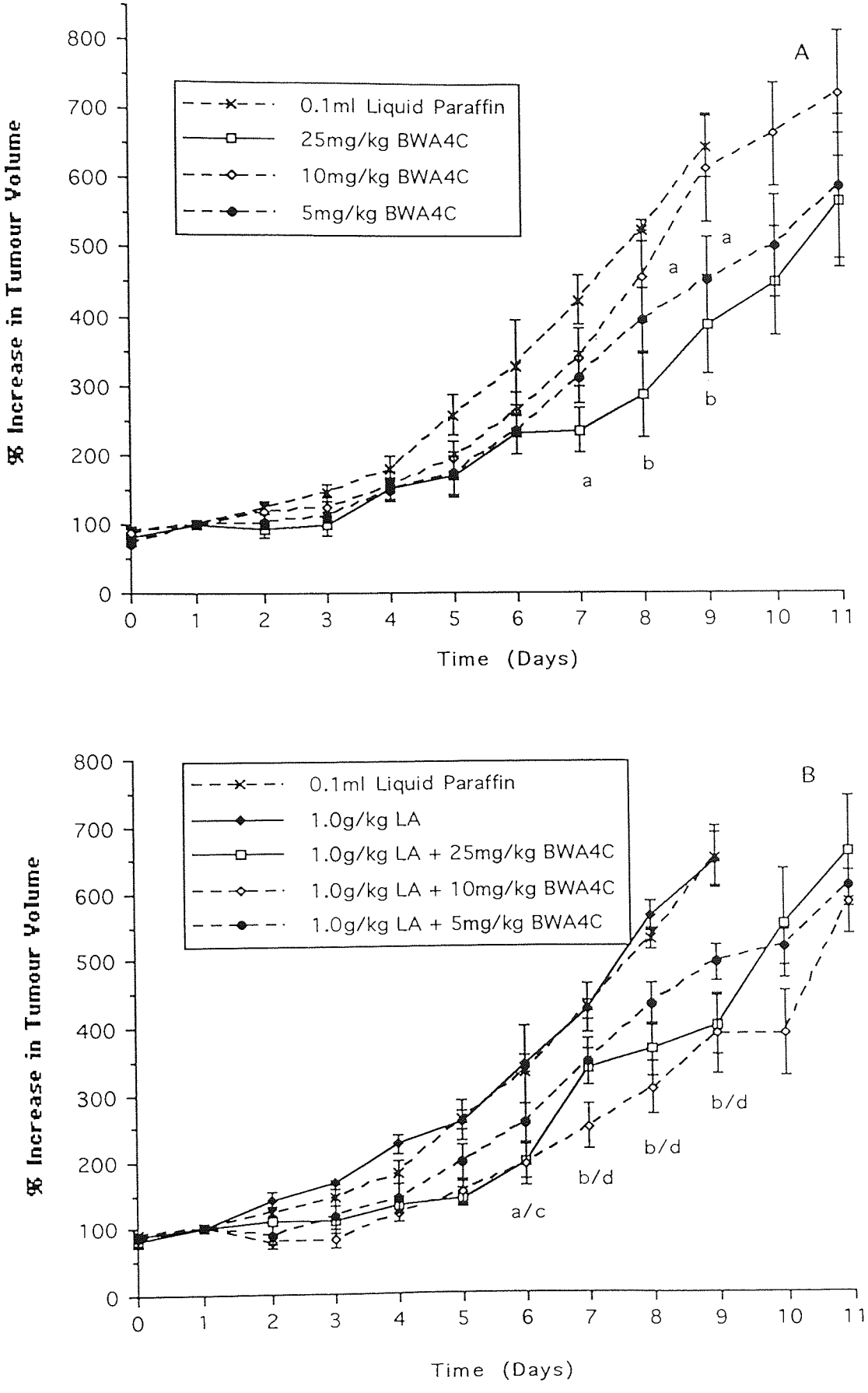


Figure 24. The effect of A. BWA4C every 12 hours in liquid paraffin and B. with 1.0g/kg linoleic acid (LA) daily on the growth of the MAC26 tumour in NMRI mice (n=9).

(a= $p<0.05$ and b= $p<0.01$ from Control, whilst c= $p<0.05$ and d= $p<0.01$ from 1.0g/Kg LA, using two-way ANOVA followed by Tuckey's Test.)

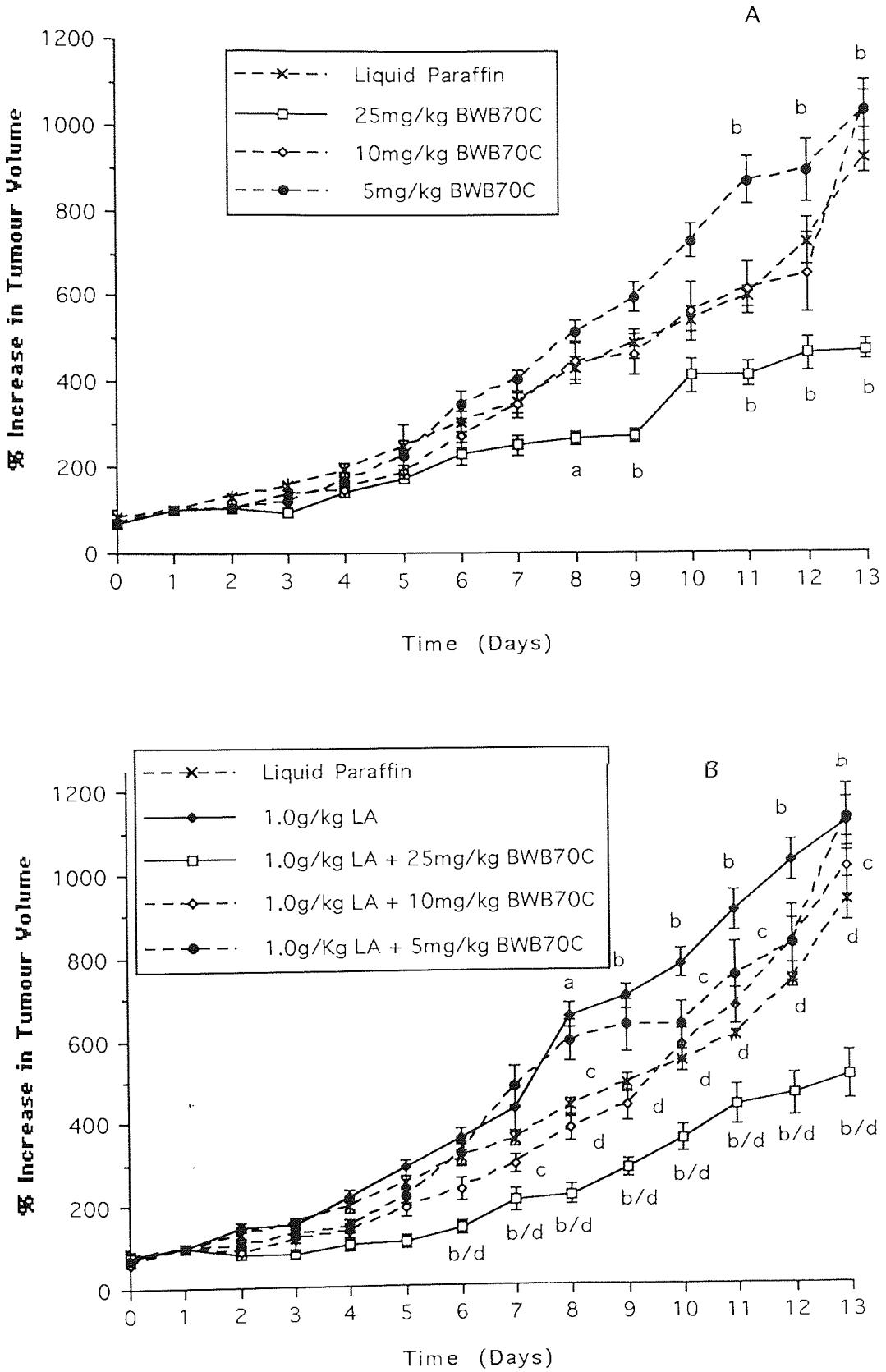


Figure 25. The effect of A. BWB70C every 12 hours in liquid paraffin and B. with 1.0g/kg linoleic acid (LA) daily on the growth of the MAC26 tumour in NMRI mice (n=9).

(a= $p<0.05$ and p= $p<0.01$ from control, whilst c= $p<0.05$ and d= $p<0.01$ from 1.0g/Kg LA, using two-way ANOVA followed by Tuckey's Test.)

0.55, 0.82 and 0.90, for 5, 10 and 25 mg/kg respectively, which was increased in comparison to those observed by the administration of BWA4C alone. At the second doubling there was no specific growth delay at 5 and 10mg/kg, and at 25mg/kg there was a reduction to 0.25. Despite this, 1.0g/kg LA did not completely reverse the antitumour effect of BWA4C and the growth delay produced was sufficient to allow the survival of tumour-bearing animals for an extra 2 days before the criterion for the maximum tumour burden permitted of 1000mm^3 was reached.

The effect of BWB70C given every 12 hr in liquid paraffin on the growth of the MAC26 tumour in NMRI mice is shown in figure 25A. 25mg/kg BWB70C inhibited MAC26 tumour growth to give a significant reduction in tumour volume at day 8 and 9, then day 11 to 13. At 10mg/kg there was no effect on tumour growth, but at 5mg/kg tumour growth was promoted with a significant increase in tumour volume from day 11 to 13. The effect of BWB70C given orally every 12 hr concurrently with 1.0g/kg LA orally every 24 hr on the growth of the MAC26 tumour is shown in figure 25B. 1.0g/kg LA promoted MAC26 tumour growth with a significant increase in tumour volume from day 8 to 13. This promotion of tumour growth was partially reversed by 5mg/kg and 10mg/kg BWB70C with a significant reduction in tumour volume over 1.0g/kg LA from 7 to 13 days and 10 to 11 days respectively. At 25mg/kg BWB70C reduced tumour growth to give a significant reduction in tumour volume from day 6 to 13 over both control (liquid paraffin) and 1.0g/kg LA treated animals.

The effect of 0.1ml liquid paraffin compared to 0.1ml saline (control) dosed every 12 hr orally on A. the growth of the MAC16 tumour and B. the decrease in body weight of MAC16 tumour-bearing NMRI mice is shown in figure 26. Liquid paraffin did not influence tumour growth but reduced weight loss induced by the tumour through cachexia. The reduction in weight loss permitted in these experiments was 30% of initial body weight. The reduction in weight loss produced by liquid paraffin was such that tumour-bearing animals survived 4 days longer than control animals, before reaching the criterion of 30% weight loss. The effect of BWA4C administered every 12 hr orally suspended in liquid paraffin on A. the growth of MAC16 tumour and B.

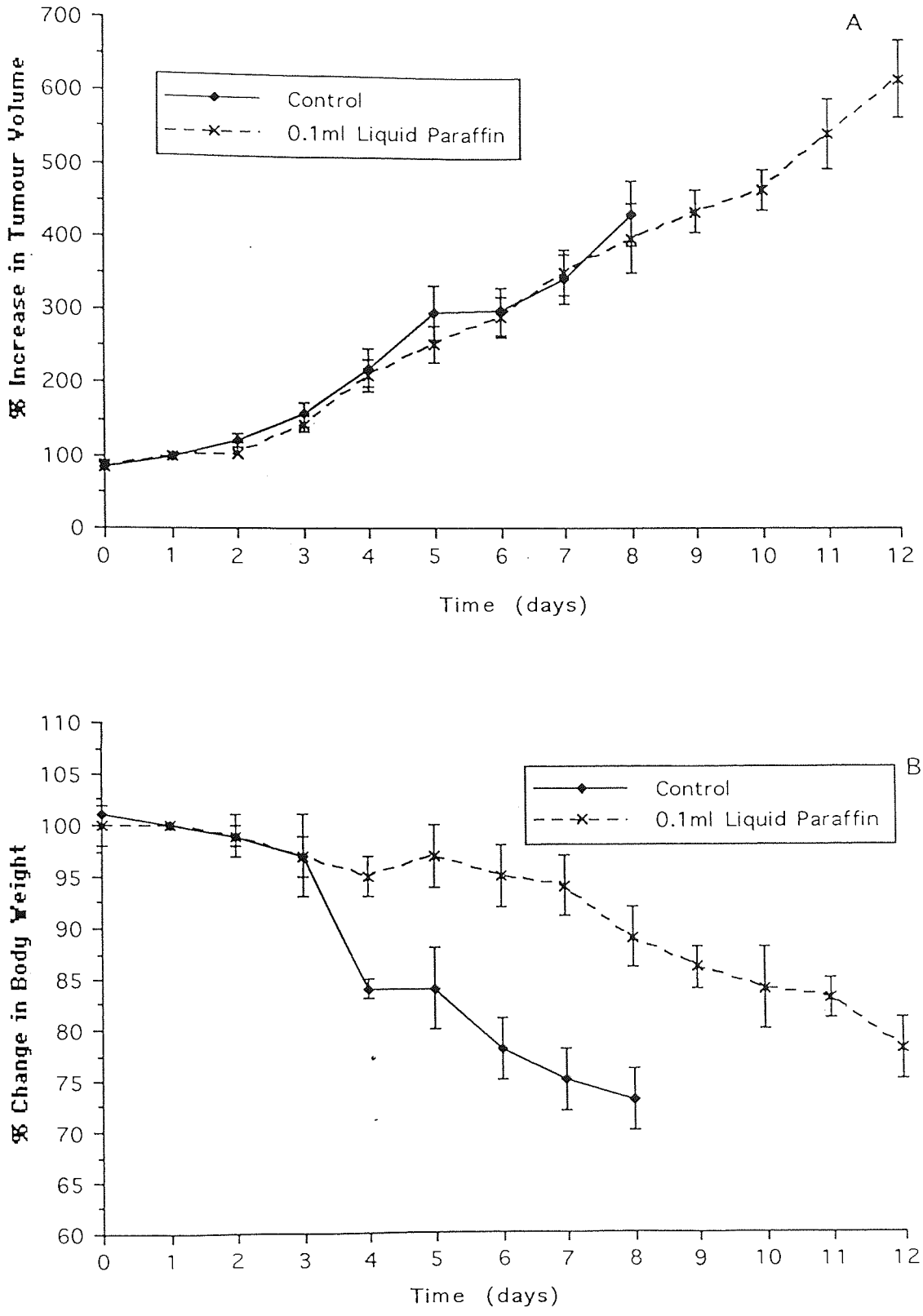


Figure 26. The effect of liquid paraffin given every 12 hours orally on A. the growth the of the tumour and B. body weight of MAC16 tumour-bearing NMRI mice (n=9).

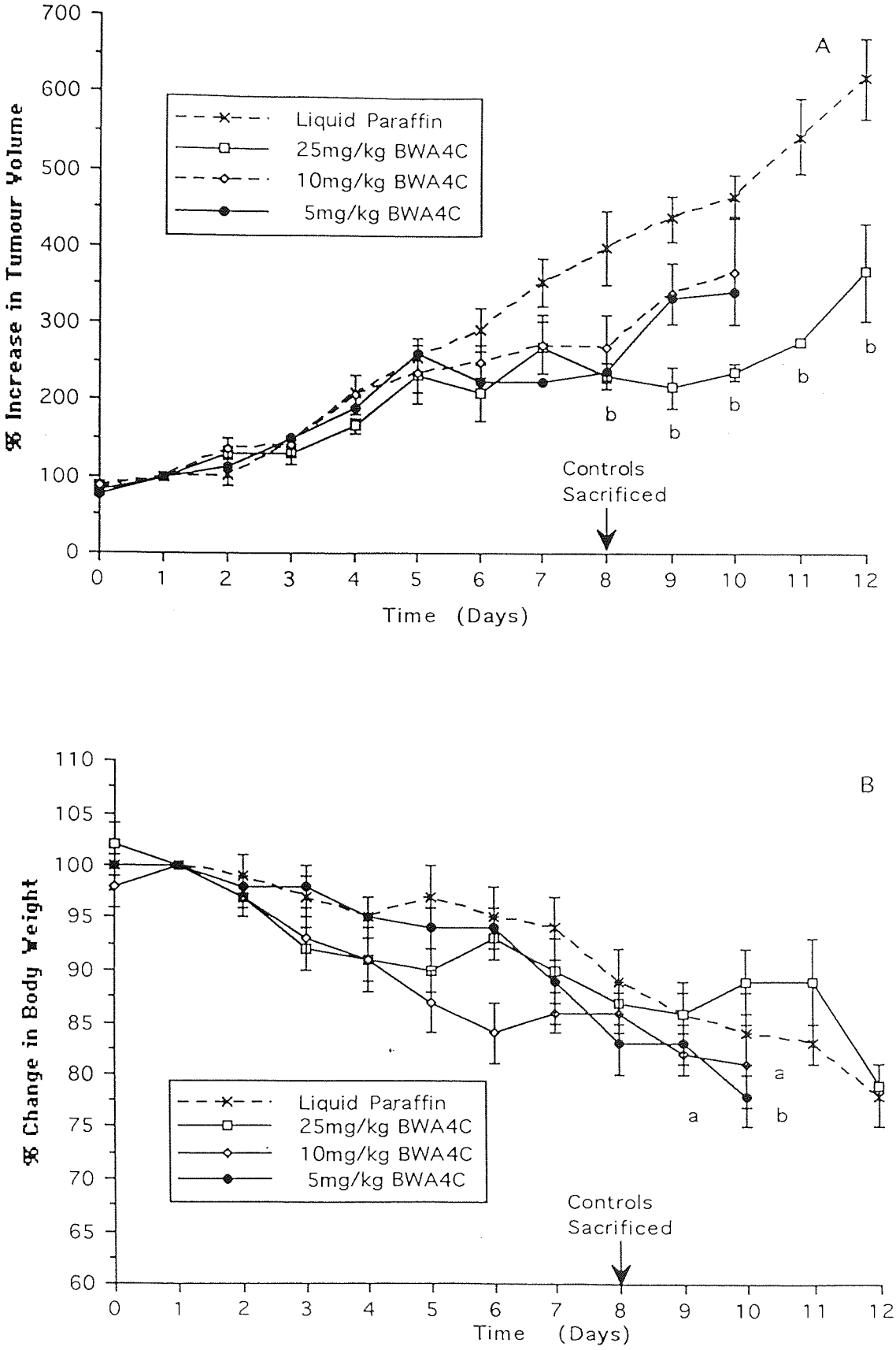


Figure 27. The effect of BWA4C administered every 12 hours in liquid paraffin on A. the growth of the tumour and B. the body weight of MAC16 tumour-bearing NMRI mice (n=9).
(a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test.)

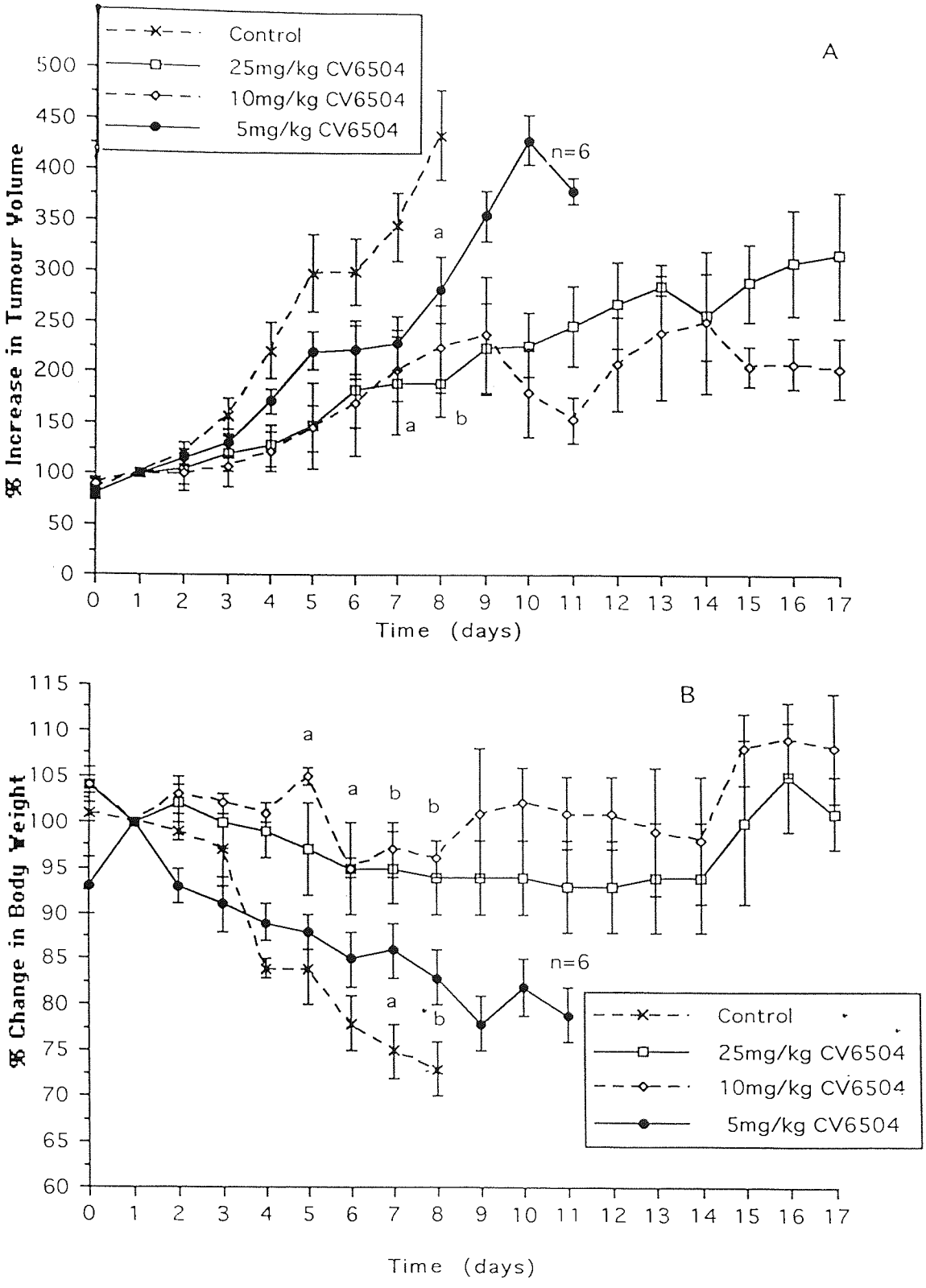
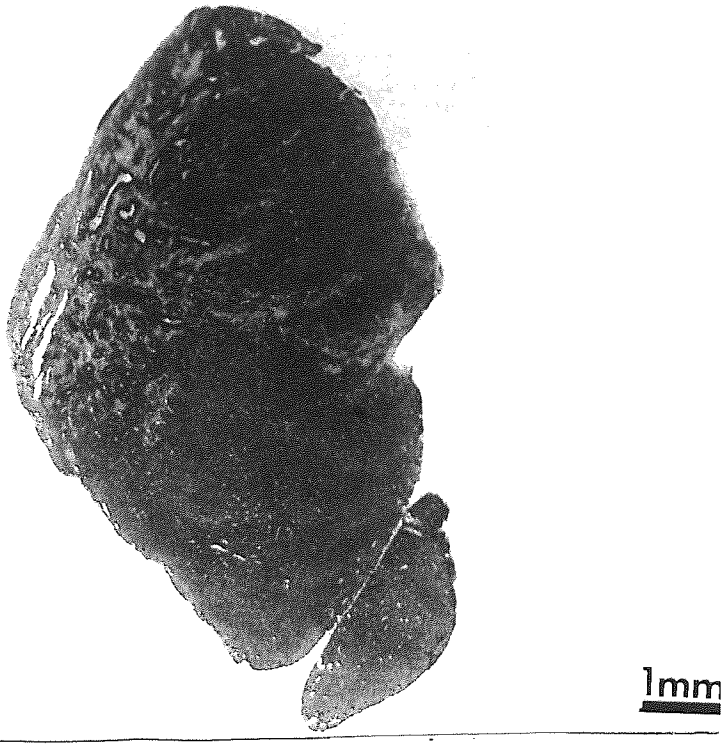


Figure 28. The effect of CV6504 given daily orally on A. the tumour growth and B. the body weight of MAC16 tumour-bearing NMRI mice (n=9).
(a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test.)

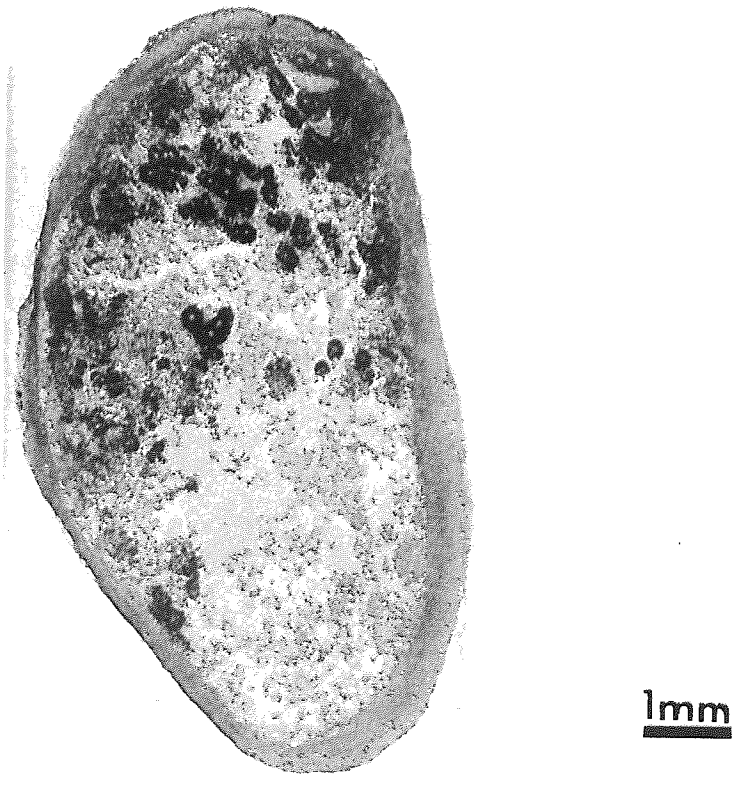
the weight loss of MAC16 tumour-bearing NMRI mice is shown in figure 27. At 5 and 10mg/kg BWA4C produced a limited antitumour activity which did not produce either a significant reduction in tumour volume, or a reduction in weight loss. Weight loss was actually increased over liquid paraffin controls, with a significant decrease in body weight at days 8 or 9. This reduced survival by 2 days in comparison to liquid paraffin treated controls as the criterion of 30% weight loss was met. At 25mg/kg BWA4C reduced the growth of the MAC16 tumour, with a significant reduction in tumour volume from day 8 to 12. This antitumour effect was again not accompanied by a reduction in weight loss, and there was no increased survival over liquid paraffin treated controls as both reached the criterion of maximum permitted weight loss at day 12.

The effect of CV6504 given orally daily on A. the growth of the MAC16 tumour and B. the weight loss in MAC16 tumour-bearing NMRI mice is shown in figure 28. There was an antitumour effect at 5mg/kg CV6504, that produced a specific growth delay of 0.40 after the first doubling, which was increased to 0.70 by the second doubling and a significant reduction in tumour volume at day 8. This was accompanied by a reduction in weight loss through tumour-induced cachexia with a significant increase in body weight in comparison to controls at days 7 and 8. The delay in tumour growth and reduction in weight loss produced by 5mg/kg CV6504 was sufficient to increase the survival by 3 days over control animals to reach the criteria of 30% weight loss and/or tumour burden of $1000mm^3$. At 10 and 25mg/kg CV6504 reduced MAC16 tumour growth to produce a significant reduction in tumour volume from day 7 to 8. This was accompanied by a specific growth delay after the first doubling of 1.40 and 2.00 respectively. At the second doubling specific growth delay was increased to 2.20 at 25mg/kg CV6504 but was never reached by 10mg/kg CV6504 treated tumours. This antitumour effect was accompanied by a reduction in tumour-induced cachexia, with a significant increase in body weight over controls from days 5 to 8. When the experiment was terminated at 17 days there was an increased survival over control animals of 9 days, even though the criteria of 30% weight loss or a $1000mm^3$ tumour burden had not been reached. A section of control MAC16 tumour and a section



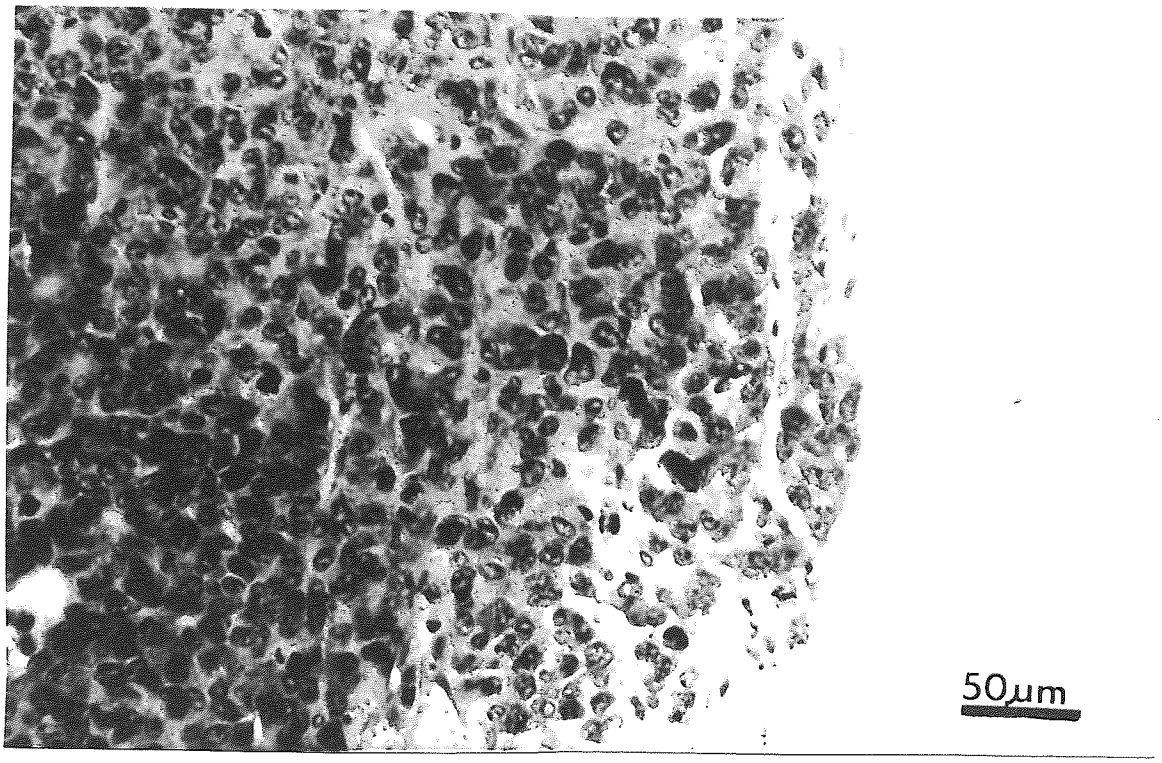
Photograph 9.

Low power section of MAC16 tumour, showing few areas of necrosis, with no fibrous capsule.



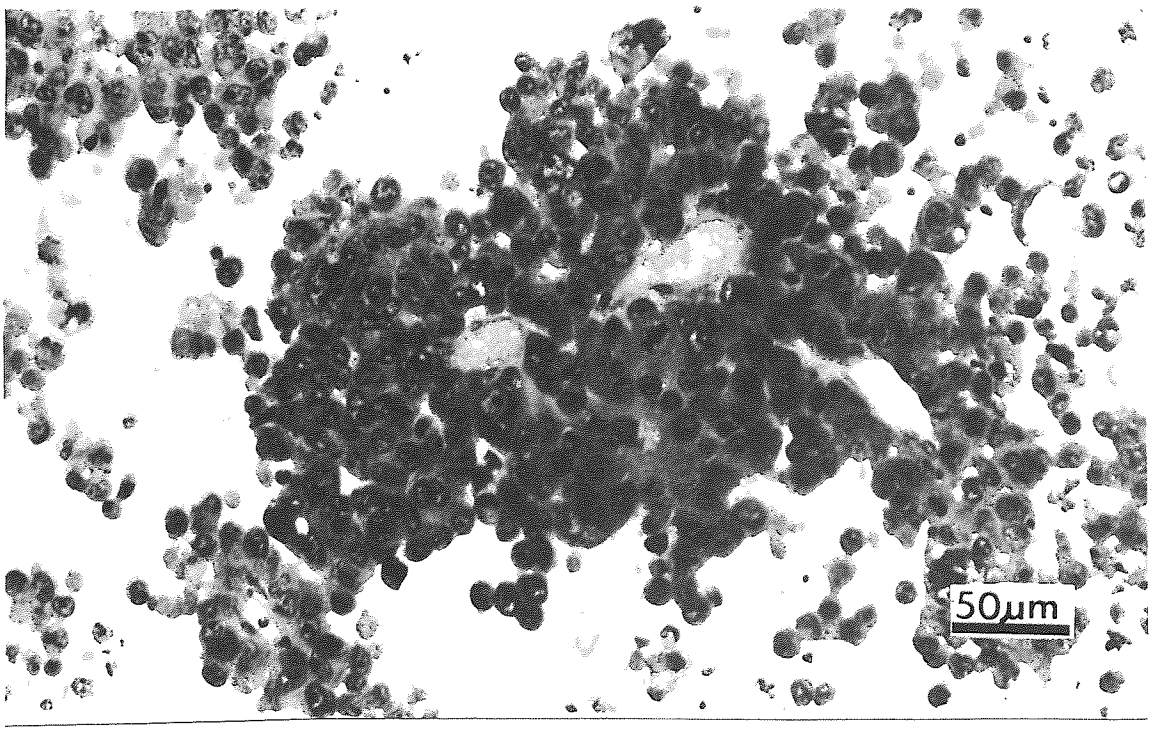
Photograph 10.

Low power section of MAC16 tumour after treatment for 17 days with 10mg/kg/day CV6504, showing large areas of necrosis and fibrous capsule.



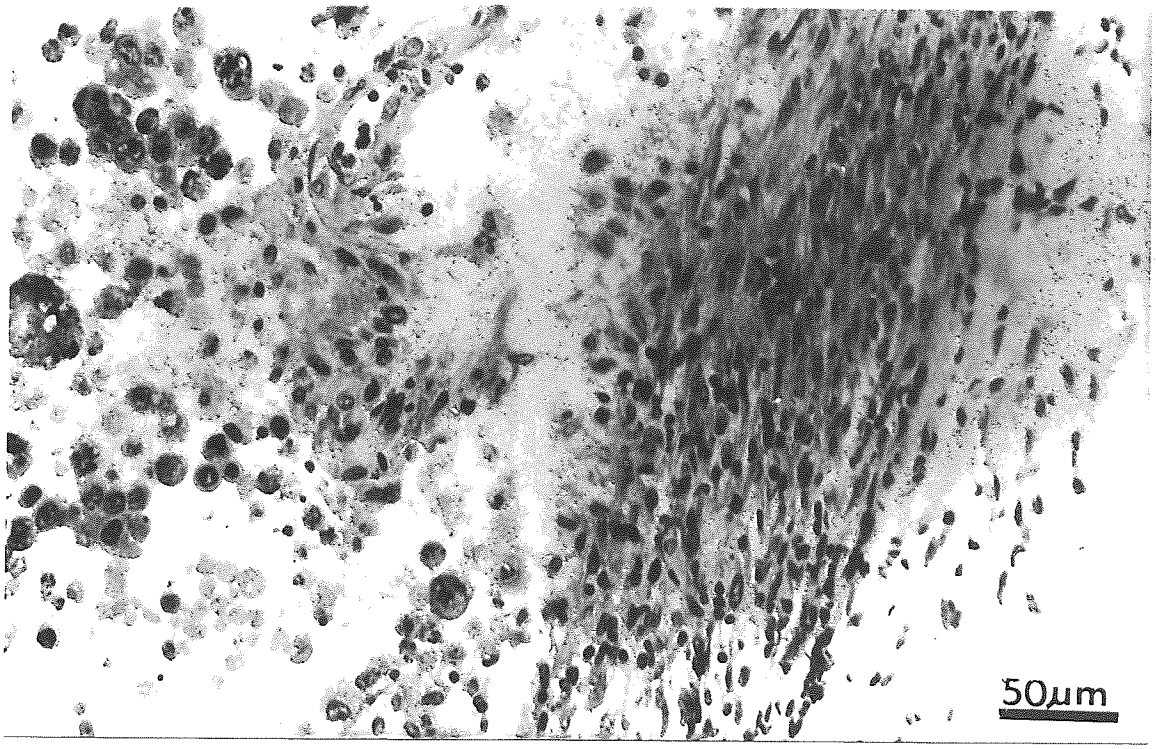
Photograph 11.

High power section of MAC16 tumour showing tumour structure.



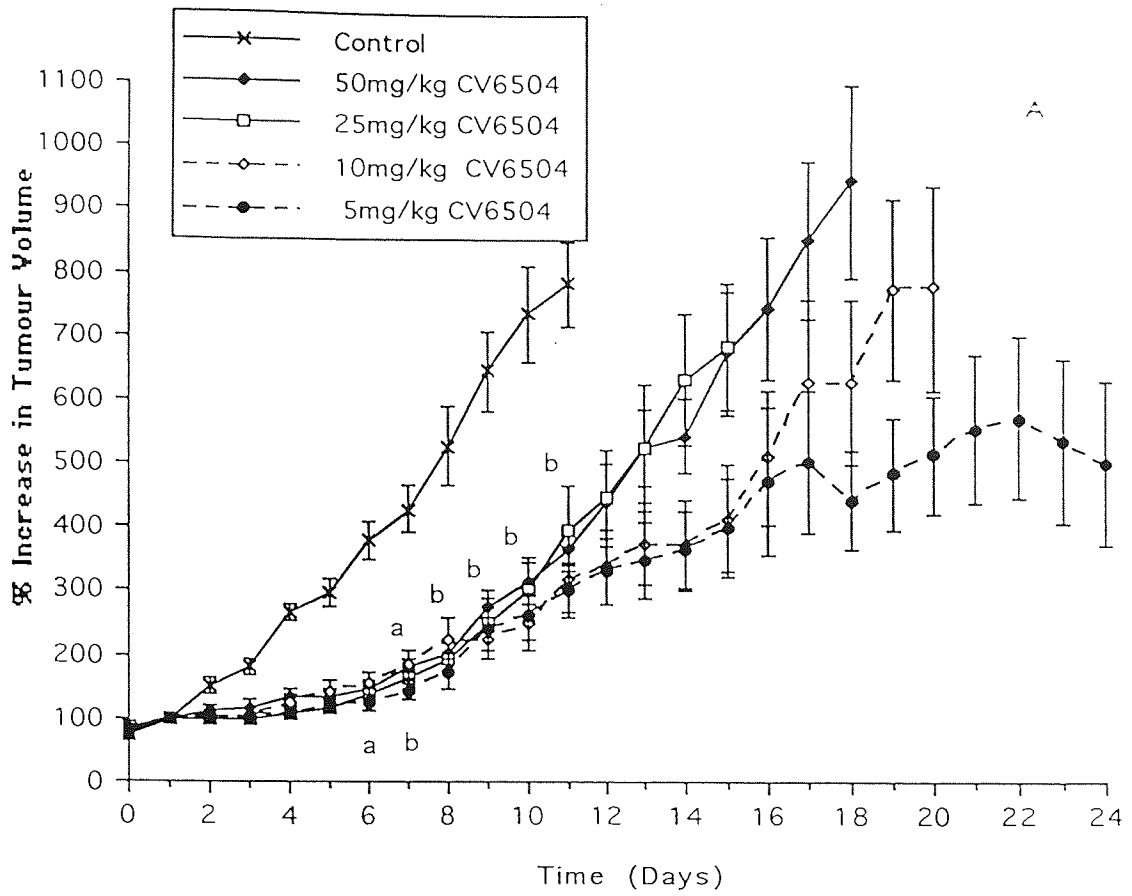
Photograph 12.

High power section of MAC16 tumour treated with 10mg/kg/day CV6504 for 17 days, showing areas of necrosis induced and viable cells around the intact vasculature.



Photograph 13.

High power section of MAC16 tumour treated for 17 days with 10mg/kg CV6504 showing fibrous capsule and lymphocytic infiltration.



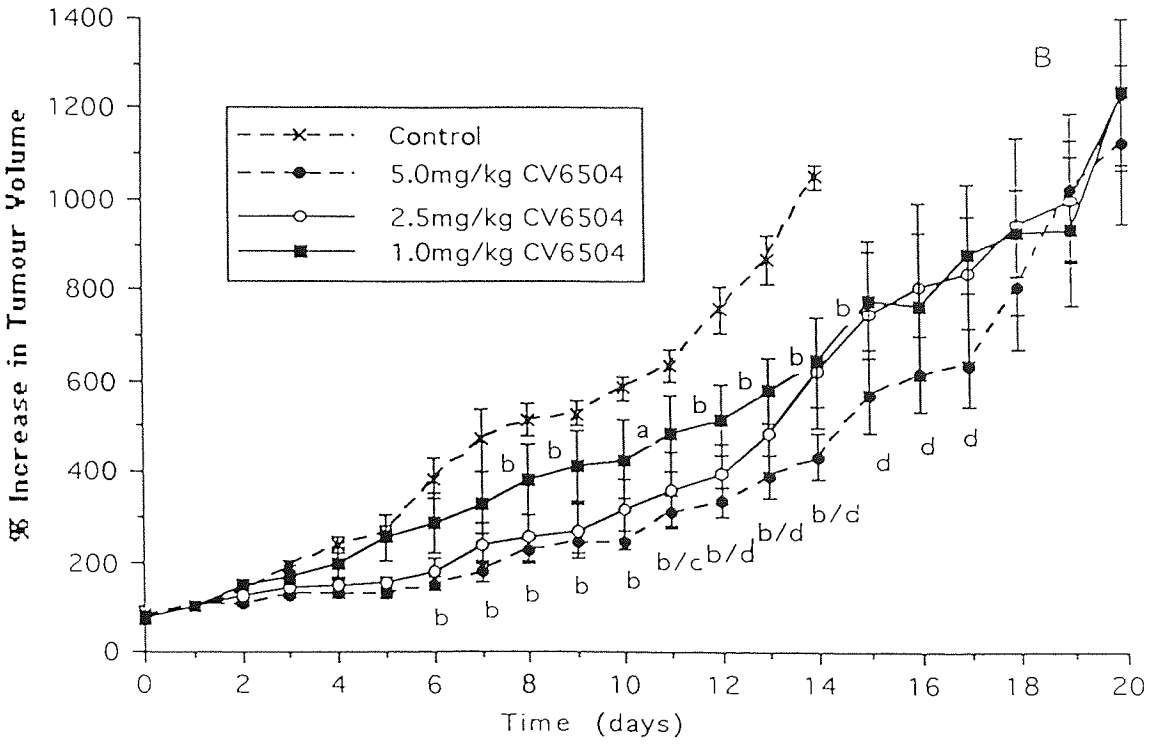
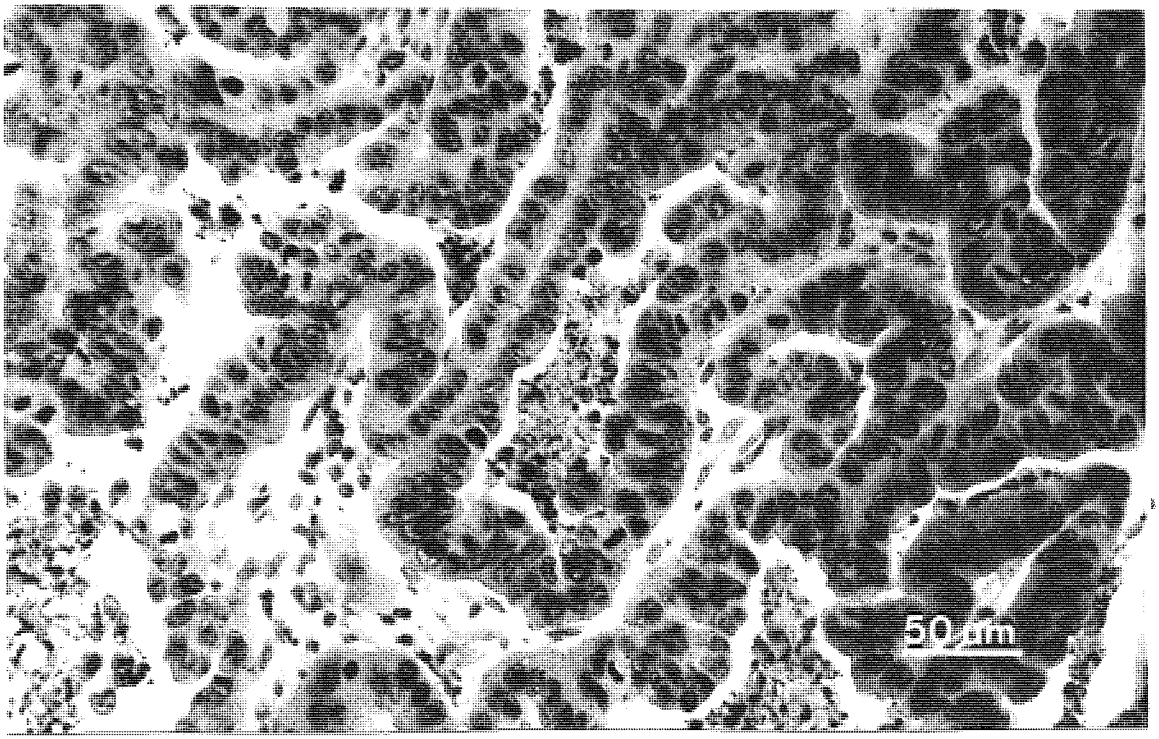
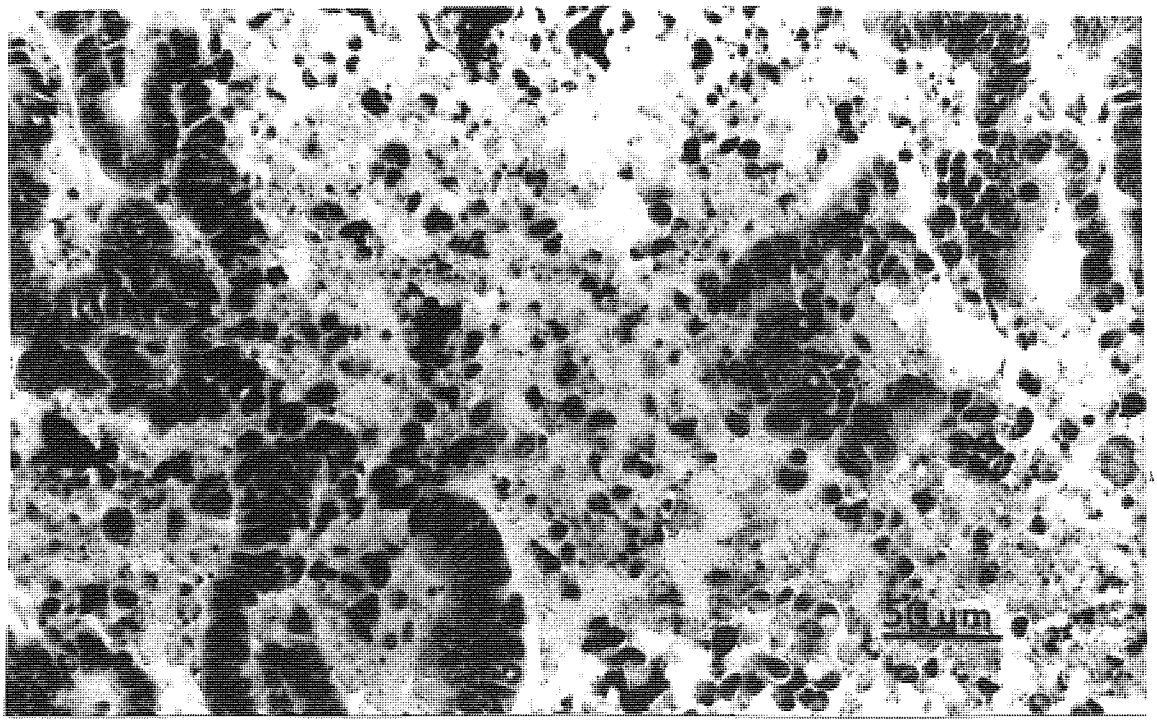


Figure 29 A. and B. The effect of CV6504 given daily orally on the growth of the MAC13 tumour in NMRI mice (n=9).
(a = $p<0.01$ and b= $p<0.05$ from Control growth, c= $p<0.05$ and d= $p<0.01$ from 1.0mg/kg CV6504 using two-way ANOVA followed by Tuckey's Test.)



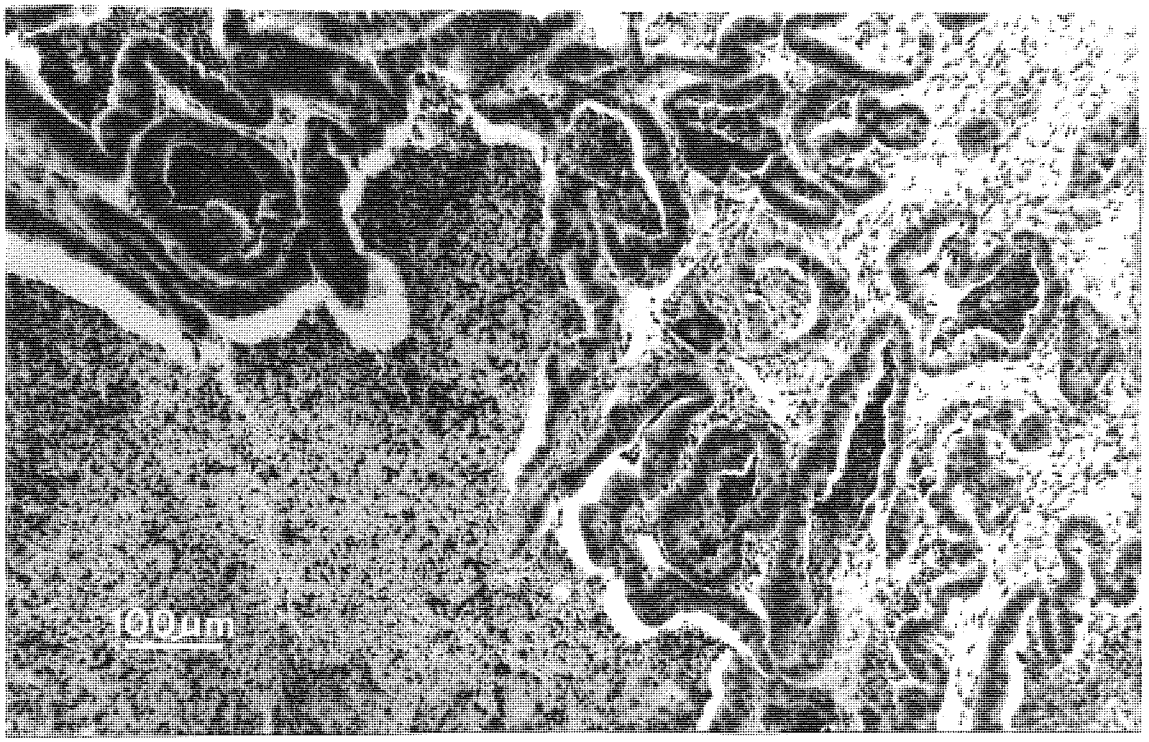
Photograph 14.

High power section of MAC13 tumour showing typical glandular structure.



Photograph 15.

High power section of MAC13 tumour after treatment for 15 days with 25mg/kg CV6504, showing areas of necrosis induced by treatment.



Photograph 16.

Low power section of MAC13 tumour treated for 15 days with 25mg/kg CV6504. Shows the large areas of necrosis, fibrosis and lymphocytic infiltration induced by treatment.

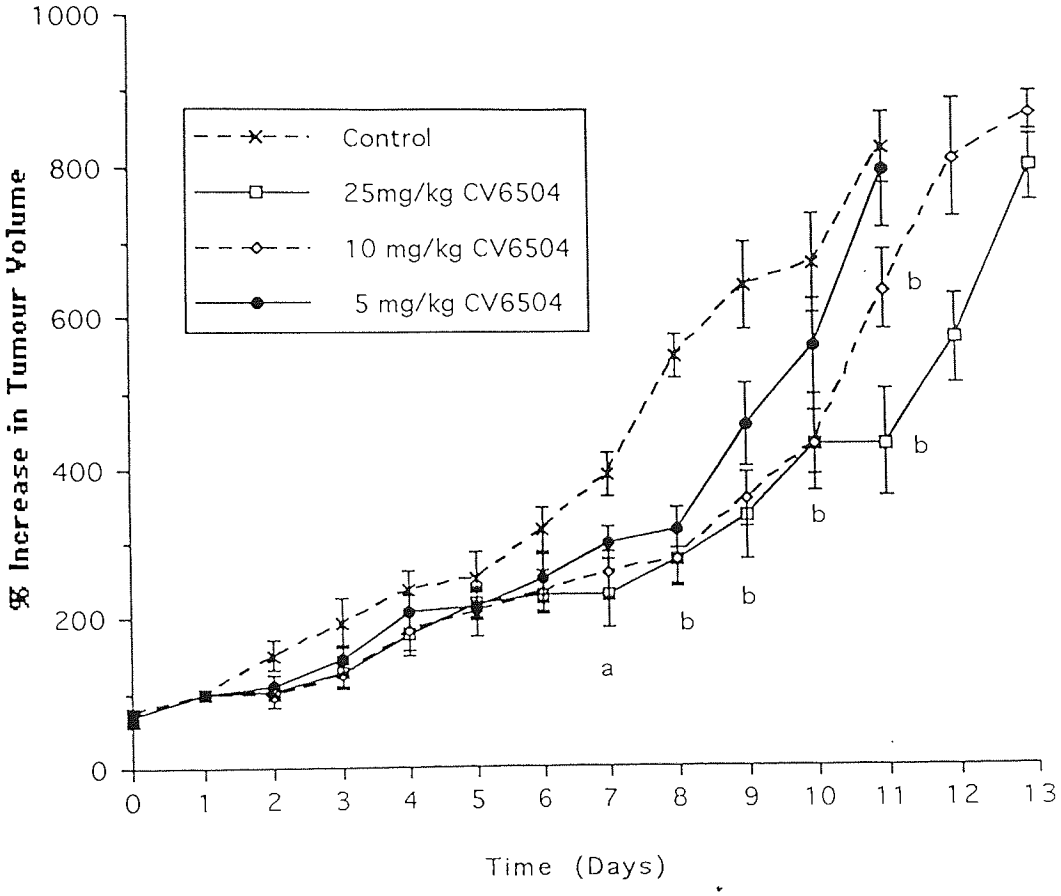


Figure 30. The effect of CV6504 given daily orally on the growth of the MAC26 tumour in NMRI mice (n=9).
(a= $p<0.05$ and b= $p<0.01$ from Control growth using two-way ANOVA followed by Tuckey's Test.)

of MAC16 tumour treated for 17 days with 10mg/kg CV6504 are shown in photographs 9 and 10 respectively. The latter shows the large areas of necrosis comparative to controls induced by CV6504 treatment. The necrotic areas of tumour became walled-in along with a few viable cells by a fibrous capsule, the presence of which would have prevented complete tumour regression. A higher magnification of the MAC16 tumour structure is shown in photograph 11. Areas of necrosis are small in comparison to those shown in photograph 12, which is a section of MAC16 tumour treated with 10mg/kg CV6504. Tumour vasculature remained intact and there was evidence of viable tumour cells around the functional vasculature. The large areas of necrosis induced by 10mg/kg CV6504 in the MAC16 tumour can again be seen in photograph 13, along with the formation of a fibrous capsule and lymphocytic infiltration.

The effect of daily oral administration of CV6504 to MAC13 tumour-bearing NMRI mice is shown in figure 29. At higher doses the antitumour effect was not dose related. A significant reduction in tumour volume was observed from 7-11 days for 25 or 50mg/kg, and 6-11 days for 5 or 10mg/kg. The specific growth delay after the second doubling was 1.20 for 5 or 10mg/kg and 1.00 for 25 or 50mg/kg. This reduction in tumour growth allowed an increased in survival of 13 days at 5mg/kg, 9 days at 10mg/kg, 6 days at 25mg/kg and 7 days at 50mg/kg, before tumour volume reached 1000mm^3, the criterion used for the termination of control animals. A section of MAC13 control tumour demonstrating the typical glandular structure is shown in photograph 14. Treatment with CV6504 (15 days at 25mg/kg) induced large areas of necrosis accompanied by fibrosis and lymphocytic infiltration as shown in photographs 15 or 16.

In another set of MAC13 tumours shown in figure 29B. At 1.0mg/kg, 2.5mg/kg and 5.0mg/kg CV6504 inhibited the growth of the MAC13 tumour initially in a dose dependent manner. At a dose of 1.0mg/kg CV6504 reduced tumour growth with a significant reduction in tumour volume from day 8 to 14. At 2.5 and 5.0mg/kg the antitumour effect of CV6504 produced a significant reduction in tumour from day 6 to 14. Specific growth delays after the first, second and third doubling were 0.33, 0.67

and 0.25 at 1mg/kg, 1.75, 0.83 and 0 at 2.5mg/kg, or 2.25, 1.17 and 0 at 5mg/kg respectively. These reductions in tumour growth permitted an extended survival of CV6504 treated animals of 6 days using the criterion that 1000mm^3 was the maximum tumour burden permitted. The overall antitumour effect of 1 and 5mg/kg CV6504 was the same, even though from day 13 to 17 the tumour volume was significantly reduced at 5mg/kg in comparison to 1mg/kg. The specific growth delays at the first, second and third doubling suggest that initially antitumour action was reduced in comparison to 5mg/kg CV6504, but was still present at the third doubling, unlike 5mg/kg treated tumours that initially had a high specific growth delay but was reduced to 0 by the third doubling.

The effect of CV6504 given orally every 24 hr on the growth of the MAC26 tumour in NMRI mice is shown in figure 30. Tumour growth was inhibited in a dose-dependent manner between 5 and 25mg/kg, with a significant reduction in tumour volume on day 11 at 10mg/kg or 7 to 11 at 25mg/kg. The specific growth delay for 10 and 25mg/kg after the first doubling was 0.60, but was reduced to 0.29 after the second doubling. These growth delays were, however, sufficient to increase the survival of the MAC26 tumour-bearing animals treated with 10 or 25mg/kg CV6504 by 2 days over control using the criterion of the maximum permitted tumour volume of 1000mm^3.

5:2:3. Discussion

Indomethacin had antitumour action against the MAC16 tumours when given at 5-10mg/kg orally in DMSO (Hudson 1993) though at 10mg/kg toxicity was observed. DMBA-induced mammary tumours in rats were promoted by increasing the LA content of the diet. This promotional effect of LA could be reversed by indomethacin, though there was no inhibition of tumour growth below that of animals fed diets with low contents of LA (Carter et al 1983). *In vivo* the growth of murine mammary tumours of varying metastic potential were inhibited by indomethacin. This could not be repeated on the same tumours as cell lines *in vitro,* suggesting an indirect mechanism of action for indomethacin on these tumours (Foulton 1984). Growth of the MAC26 tumour with and without LA stimulation was significantly inhibited by

indomethacin when administered as an ip injection concurrently with LA orally. When administered alone by ip injection indomethacin was toxic to MAC26 tumour bearing-mice. Indomethacin entered stage III/IV clinical trials for head and neck derived carcinomas where its toxicity could be minimised by concurrent administration of the drug with food (Panje 1981). This suggests that administration of indomethacin with LA offers a protective mechanism against indomethacin toxicity in the MAC26 tumour model.

More recently the antitumour effect of indomethacin has been evaluated in undernourished patients with metastic solid tumours (Ludholm et al 1994). No serious complications were observed due to indomethacin treatment, even though the age span was representative of a population where gastric ulcers and gastrointestinal bleeding were likely to occur. Patient survival and quality of life was increased, with both a reduction in pain experienced and requirement for analgesics (Ludholm et al 1994). The mechanism by which indomethacin achieved these effects was unclear. Proposed mechanisms included, a direct effect on tumour proliferation, attenuation of neovascularisation at both primary and secondary tumour sites, or preservation of the host's defence against the invading tumour (Ludholm et al 1994). A decrease in PG production by the tumour in response to treatment with indomethacin, increasing the tumour susceptibility to the host immune system has been observed in a number of animal tumour models (Gabor et al 1985, Fulton 1984). *In vitro* , in the absence of both cell mediated and humoral immunity, indomethacin was shown to inhibit MAC13, MAC16 and MAC26 cell proliferation (see Chapter 4. table 1.). It was concluded that this inhibition of tumour cell proliferation was due to the imbalance of eicosanoids produced (see Chapter 4.). LA stimulates MAC26 tumour growth directly by an increase in tumour cell proliferation, with no effect on the cell loss factor (see section 5:1.). This suggests that indomethacin may act directly on MAC26 tumour cell proliferation, possibly by causing an imbalance in the eicosanoid production required for tumour cell proliferation.

BWA4C administered orally in liquid paraffin at 25mg/kg/12hr had an antitumour action on both MAC16 and MAC26 tumour-bearing mice. The weight loss

induced by MAC16 tumours has been shown to increase with tumour growth (Hudson 1993). This suggests that a reduction in weight loss would accompany any antitumour effect. The lack of anticachectic action displayed by BWA4C can be explained by toxicity, which was shown more clearly at 5mg/kg and 10mg/kg. Any reduction in weight loss produced by tumour growth inhibition was counteracted by weight loss due to toxicity of BWA4C. Liquid paraffin had the opposite effect with no effect on MAC16 tumour growth but a reduction in weight loss. Liquid paraffin is a mixture of long chain hydrocarbons with BHT and α-tocopherol added as antioxidants (British Codex). α-Tocopherol is a small lipid soluble antioxidant molecule which was shown to suppress FA oxidation by contributing a hydrogen atom from its phenolic group to a lipid-derived peroxy and/or alkoxyl radical (Burton and Ingold 1981, McCay 1985). This prevents the peroxy radical from attacking the adjacent lipid membranes, thereby inhibiting the propagation phase of lipid peroxidation (Kelley et al 1995). These results also implicate lipid peroxidation in the cachexia induced by the MAC16 tumour, especially as liquid paraffin demonstrated an anticachectic effect rather than an antitumour action.

BWB70C administered orally in carboxymethyl cellulose or methylcellulose had low antitumour action on the MAC16 tumour *in vivo* (Hudson 1993). BWB70C administered orally in liquid paraffin every 12 hr had antitumour action on the MAC26 tumour in NMRI mice at 10 and 25mg/kg, but at 5mg/kg stimulated tumour growth. *In vitro* BWB70C inhibited MAC13, MAC16 and MAC26 tumour growth, but inhibition appeared to be more specific to the 5-LO pathway than 12 or 15-LO pathways (see section 4:2.). If inhibition of LO pathways shows the same pattern *in vivo*, at lower doses such a 5mg/kg the 5-LO enzyme may be inhibited, whereas 12-LO and 15-LO remain unaffected. This may result in the redirection of eicosanoid metabolism through 12-LO and 15-LO producing a balance of eicosanoids capable of stimulating tumour cell proliferation. This suggests a role for 12-LO and 15-LO in the control of MAC26 tumour growth, and again introduces the importance for the balance of eicosanoids produced as a requirement of tumour growth.

MAC tumours have been shown to be generally refractive to cytotoxic agents, with MAC13 tumours being the most responsive (Double and Bibby 1989). Three established MAC tumours, MAC13, MAC16 and MAC26, were sensitive to inhibition by CV6504. Optimal antitumour activity was observed on the MAC13 tumour at 5-10mg/kg/day, MAC16 at 10mg/kg/day and MAC26 at 25mg/kg/day , doses which are comparable with those required to inhibit puromycin aminonucleoside nephritis in rats (Shibouta et al 1991). For many antitumour agents, antitumour action is only demonstrated at the maximum tolerated dose. In the chemoresistant tumours such as MAC13 the therapeutic index of standard nitrosoureas was <1, and even against L1210 leukemia cells no higher than 7 (Double and Bibby 1989). The chronic toxicity of CV6504 in male mice was reported to be above 100mg/kg (Takeda Chemicals Ltd). This will give a therapeutic index in the MAC tumour models of at least 4-20, even though these tumours are usually chemoresistant.

An increased response of the MAC16 tumour comparative to the MAC26 tumour has also been demonstrated with mitomycin C and doxorubicin (Bibby and Double 1989). These are also quinone containing drugs that can undergo metabolic reduction, which for mitomycin C has been shown to be via DT-diaphorase (Siegal et al 1990). DT-diaphorase was the two electron reducing agent proposed to reduce CV6504 quinone to hydroquinone in the redox cycle shown in figure 22. (Ohkawa et al 1991b), suggesting a correlation between ability to inhibit LO and antitumour activity in MAC16 and MAC26 tumours. The ability to inhibit LO pathways *in vitro* was also found to correlate with the inhibition MAC13, MAC16 and MAC26 cell proliferation (see Chapter 4.).

Histological sections suggested that CV6504 drug toxicity in MAC16 tumours was manifested at regions distant from the vascular supply. Recent studies utilising multicellular spheroids indicate that the gene expression of DT-diaphorase and activity are elevated close to necrotic centres (Phillips et al 1994). This would allow the proposed mechanism of CV6504 action (Ohkawa et al 1991b) in hypoxic areas generally relatively inaccessible to drugs and predominantly non-cycling. MAC13 tumours have an intermediate level of DT-diaphorase activity comparative to MAC16

and MAC26 tumours (Collard 1994). The increased areas of necrosis induced by CV6504 and the relative increased DT-diaphorase activity reported (Phillips et al 1994) may partially explain why MAC13 tumours were as sensitive as MAC16 tumours to CV6504 even though DT-diaphorase levels were reduced. CV6504 was shown to be an excellent substrate for human and mouse DT-diaphorase with kcat of 6 x 10^4 min^{-1} and Km of 50μM. These results are comparable with the classic DT-diaphorase substrate menadione which has the same kcat, but a Km of 3.1μM (R. J. Knox personal communication).

Quinones form the second largest class of cytotoxic agents used as anticancer drugs after chloroethyl alkylating agents and are approved for clinical use in the USA (Driscoll et al 1974). In rapidly dividing cells such as tumour cells the mode of action is by DNA modification. The molecular basis of quinone activity in quiescent cells is through alkylation of essential protein thiols or amine groups and the oxidation of essential protein thiols (O'Brien 1991). Studies with reduced glutathionine (GSH) depleted hepatocytes has suggested that quinone induced cytotoxicity may be modulated by GSH in at least 4 different ways (O'Brien 1991). Cytotoxicity of quinones decreases with increasing methyl substitution of the nucleus, with the higher redox potential benzoquinones being more cytotoxic. The fully substituted benzoquinone 2,3,5,6- tetramethylbenzoquinone (duroquinone) does not form glutathione conjugates or alkylate proteins and is only very weakly cytotoxic (O'Brien 1991). CV6504 would also be expected to show relatively low reactivity which would explain the lack of GSH activity on the cytoxicity of CV6504 *in vitro* (results not shown).

Further experiments are required to completely evaluate the mode of action of CV6504, especially with regard to how LA metabolism may be related to any antitumour action. The wide spectrum of antitumour activity against what are generally considered to be chemoresistant tumours combined with a high measure of safety identifies CV6504 as a potential agent for clinical investigation.

Chapter 6.
The Metabolism and Mechanism of Action of CV6504 in Murine Colon Adenocarcinomas (MACs).

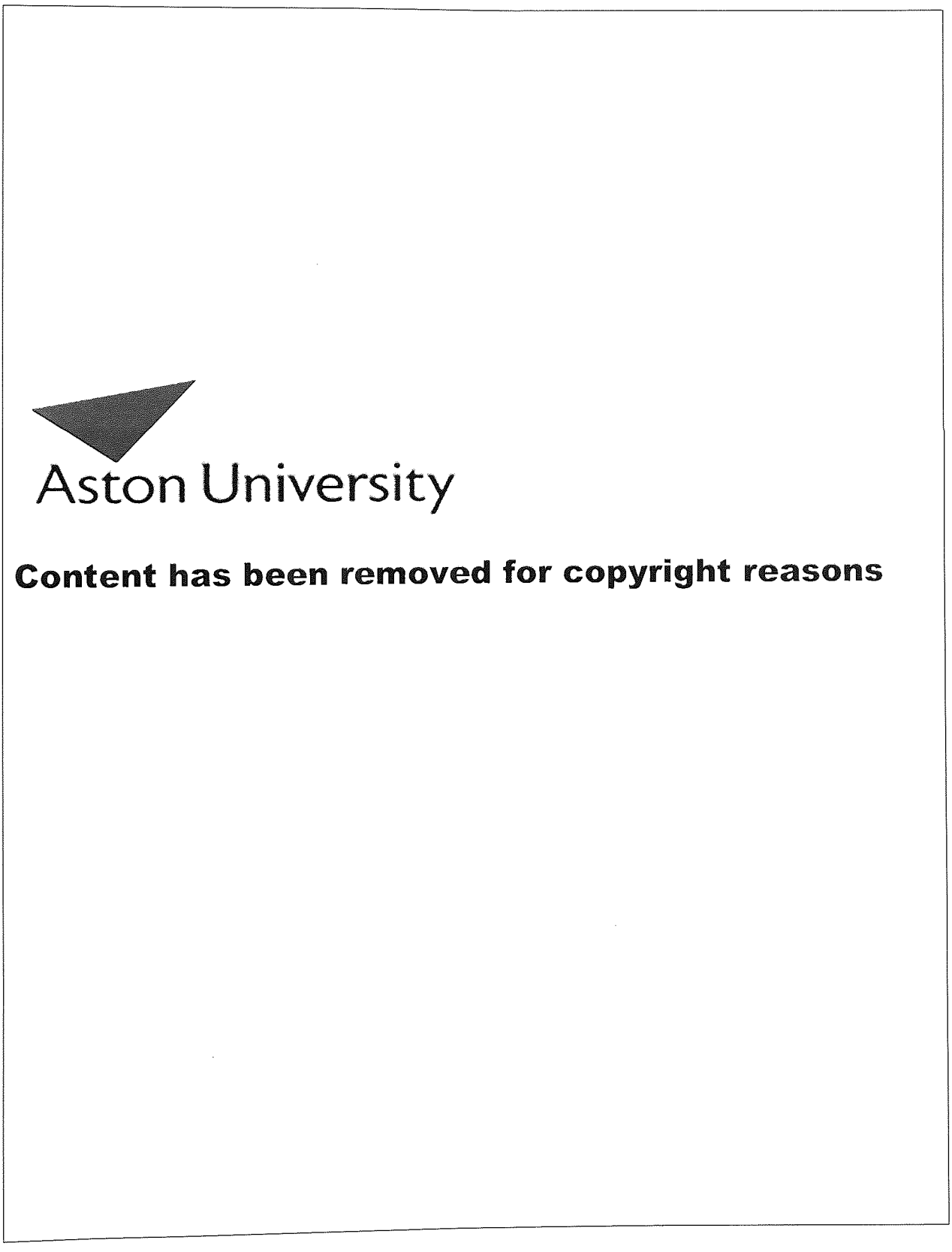
6. The Metabolism and Mechanism of Action of CV6504 on Murine Colon Adenocarcinomas (MACs).

6:1. Introduction.

The effect of CV6504 on the AA cascade enzymes has been extensively studied by Takeda Chemicals Ltd. Microsomes prepared from horse platelets were incubated with CV6504 and the production of TXB_2 the stable metabolite of TXA_2, was measured by radioimmunoassay. An IC_{50} value of 40μM was reported (Takeda Chemicals Ltd. unpublished results). The inhibitory effect on PGI_2 synthetase was studied in bovine aortic cells, by the measurement of the stable metabolite of 6-keto-$PGF_{1\alpha}$ and showed an IC_{50} of >100μM (Takeda Chemicals Ltd. unpublished results). The IC_{50} value for the inhibition of 5-HETE production in rat basophilic leukemia cells was 2.1μM, and 12-HETE production in rat platelets >10μM (Takeda Chemicals Ltd. unpublished results). Inhibition of lipid peroxidation in rat brain homogenates was found to be dose-dependent with an IC_{50} value of 1.8μM (Takeda Chemicals Ltd. unpublished results). In human whole blood CV6504 inhibited LTB_4 production at 0.45μM and TXA_2 synthetase activity at 0.34μM. At these concentration the production of PGI_2 was enhanced (Takeda Chemicals Ltd. unpublished results). CV6504 given orally between 3 to 20mg/kg/day inhibited TXA_2 synthetase, 5-LO activity and lipid peroxidation in a dose dependent manner in puromycin aminonucleoside (PAN) nephritis in rats (Shibouta et al 1991).

50 human tumour cell lines were tested by Takeda Chemicals Ltd. for the activity of CV6504 against cell proliferation *in vitro* (see table 4.). Sensitivity varied from that of MAC cell lines to resistant with IC_{50} values above 100μM. Cell lines derived form colon, breast and pancreas were sensitive to inhibition by CV6504, with IC_{50} values below 10μM. However other cell lines derived from these tissues were resistant to CV6504, suggesting no correlation between the origin of the tumour cell and CV6504 sensitivity. The effect of CV6504 metabolites commonly formed in mammalian cells (as shown in figure 31), on the inhibition of growth of 2 tumour cell lines and a normal cell line is shown in table 5. CV6504 metabolites had no effect on the growth of either tumour cell line or normal cells. Sensitive cell lines such as

Table 4. The IC_{50} values of CV6504 on 50 human tumour cell lines of varying tissues determined by cell counts. (Results supplied by Takeda Chemicals Ltd.)



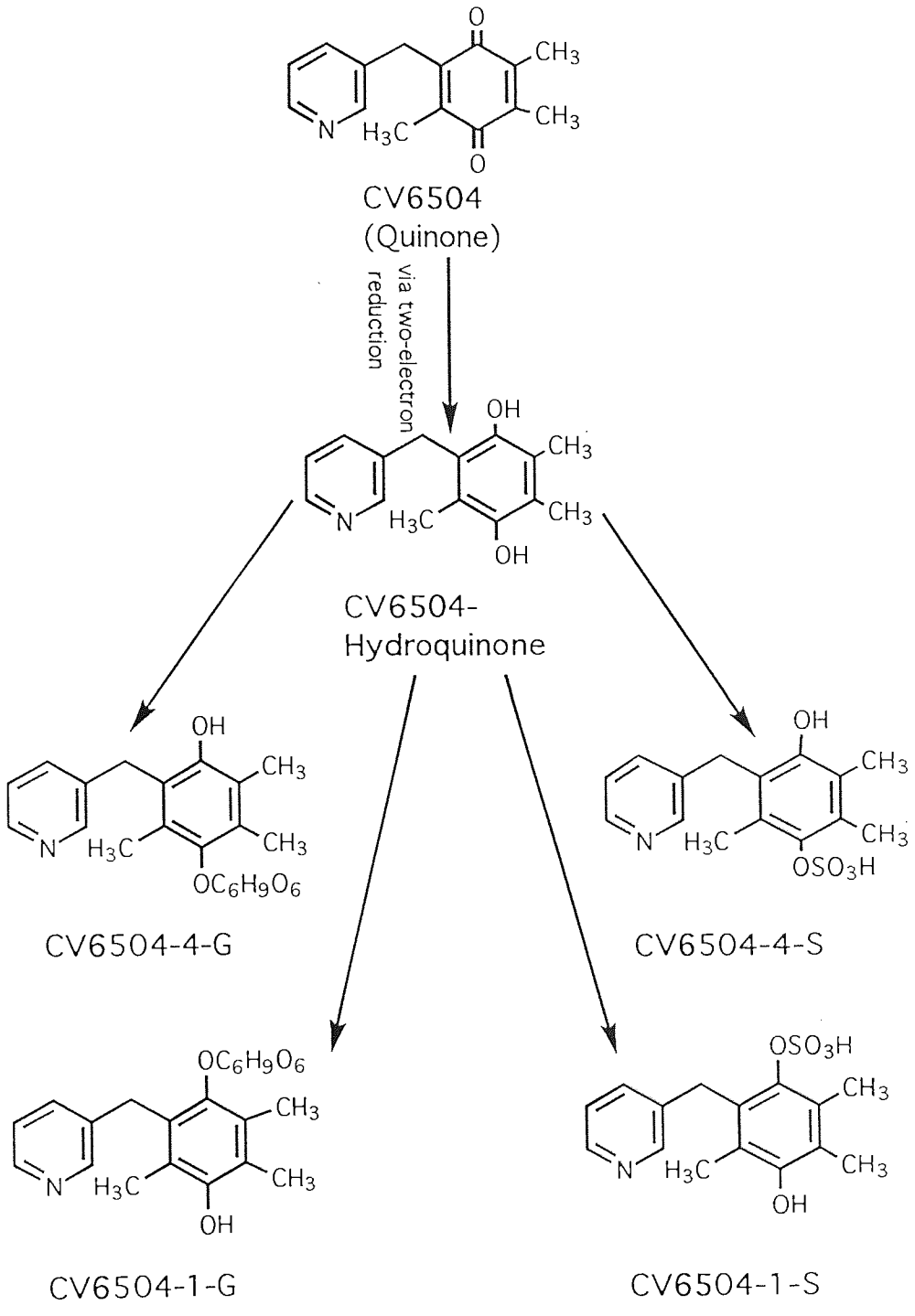


Figure 31.
Metabolism of CV6504 by mammalian cells

Table 5.
The effect of CV6504 in comparison to CV6504 metabolites on the IC_{50} values of two tumour cell lines and a normal cell line *in vitro.* (Results supplied by Takeda Chemicals Ltd.)

Metabolites	Colo320DM (μM)	WiDr (μM)	MRC-5 (μM)
CV6504	3.60	43	93
CV6504-1-Glucuronide	>2500	>2500	>2500
CV6504-4-Glucuronide	1800	>2500	>2500
CV6504-1-Sulphate	>2500	>2500	>2500
CV6504-4-Sulphate	>2500	>2500	>2500

prostate carcinoma PC-3, colon carcinoma Colo320DM and pancreatic carcinoma PANC-1 were also tested for antitumour action of CV6504 as xenograft models in mice, but only marginal antitumour activity was observed (Takeda Chemicals Ltd. unpublished results).

The effect of LA on the promotion of MDA-MB-231 and MDA-MD-435 human breast cancer cell lines both *in vitro* and *in vivo* has been extensively investigated by Rose and Connolly. LA was found to stimulate proliferation of both the MDA-MB-231 oestrogen independent human breast cancer cell line and the MCF-7 human oestrogen dependent human breast cancer cell line *in vitro* in a dose dependent manner between 5 and 750ng/ml (Rose and Connolly 1989). LA was further found to have no activity in three human tumour cell lines derived from sites other than the breast or transformed 3T3 cells (Rose and Connolly 1989). *In vitro* studies with MDA-MB-231 cells showed that proliferation was inhibited by indomethacin at high concentrations where both CO and LO pathways would be inhibited. Further experiments with piroxicam, NDGA and esculetin suggested that MDA-MB-231 cell growth was dependent on LO eicosanoid production rather than CO eicosanoid production (Rose and Connolly 1990). PC-3 a human prostate cancer cell line was shown to be stimulated by LA in a dose dependent manner, whereas another human prostate cancer cell line DU-145 was unresponsive (Rose and Connolly 1991). MDA-MB-435 human breast cancer cells injected into the thoracic mammary fat

pads of female nude mice showed increased growth at the primary site and increased metastasis when diets were enriched with the n-6 PUFA LA (Rose and Connolly 1992). When MDA-MB-231 or MDA-MB-435 cell lines were transplanted into nude mice, diets rich in n-3 PUFAs reduced both tumour growth and metastasis (Rose and Connolly 1993). This reduction in tumour growth and metastasis was latter found to correlate with a 3 to 4 fold decrease in 12-HETE and 15-HETE production (Rose et al 1995).

MDA-MB-231 and MDA-MB-435 cell lines were tested for sensitivity to CV6504 *in vitro* (see table 4.) and in there had IC_{50} values of 9.9μM and 28μM respectively. In PC-3 and DU-145 cell lines, it had IC_{50} values of 17μM and 70μM respectively. The sensitivity of these four cell lines appears to correlate with the stimulation of cell proliferation produced by LA (Rose and Connolly 1989, Rose and Connolly 1991). MAC13, MAC16 and MAC26 cell growth was also sensitive to inhibition by CV6504 *in vitro* and the production of 5-HETE, 12-HETE and 15-HETE was found to be decreased (see chapter 4). The inhibition of both tumour growth and metastasis of MDA-MB-231 and MDA-MB-435 *in vivo* was also found to correlate with a 3 to 4 fold reduction in 12-HETE and 15-HETE production (Rose et al 1995).

6:2. Results

The inhibition of A. tumour growth and B. weight loss by 10mg/kg CV6504 in MAC16 tumour-bearing females is shown in figure 32. There was a significant reduction in tumour volume from day 11 to 15, accompanied by a reduction in the tumour-induced weight loss through cachexia. There was a specific growth delay of 0.55 after the second doubling, which due also to the accompanying reduction in weight loss meant that the criteria of 1000mm^3 maximum tumour burden and the maximum permitted 30% weight loss were not reached even after an increased survival of 2 days. The effect of CV6504 on the growth of MAC16 tumours in female NMRI mice was investigated as the weight loss induced is often more aggressive than that observed in males.

The effect of concurrent administration of CV6504 with 1.0g/kg LA on A. the growth of the MAC16 tumour and B. the weight loss in these tumour-bearing NMRI

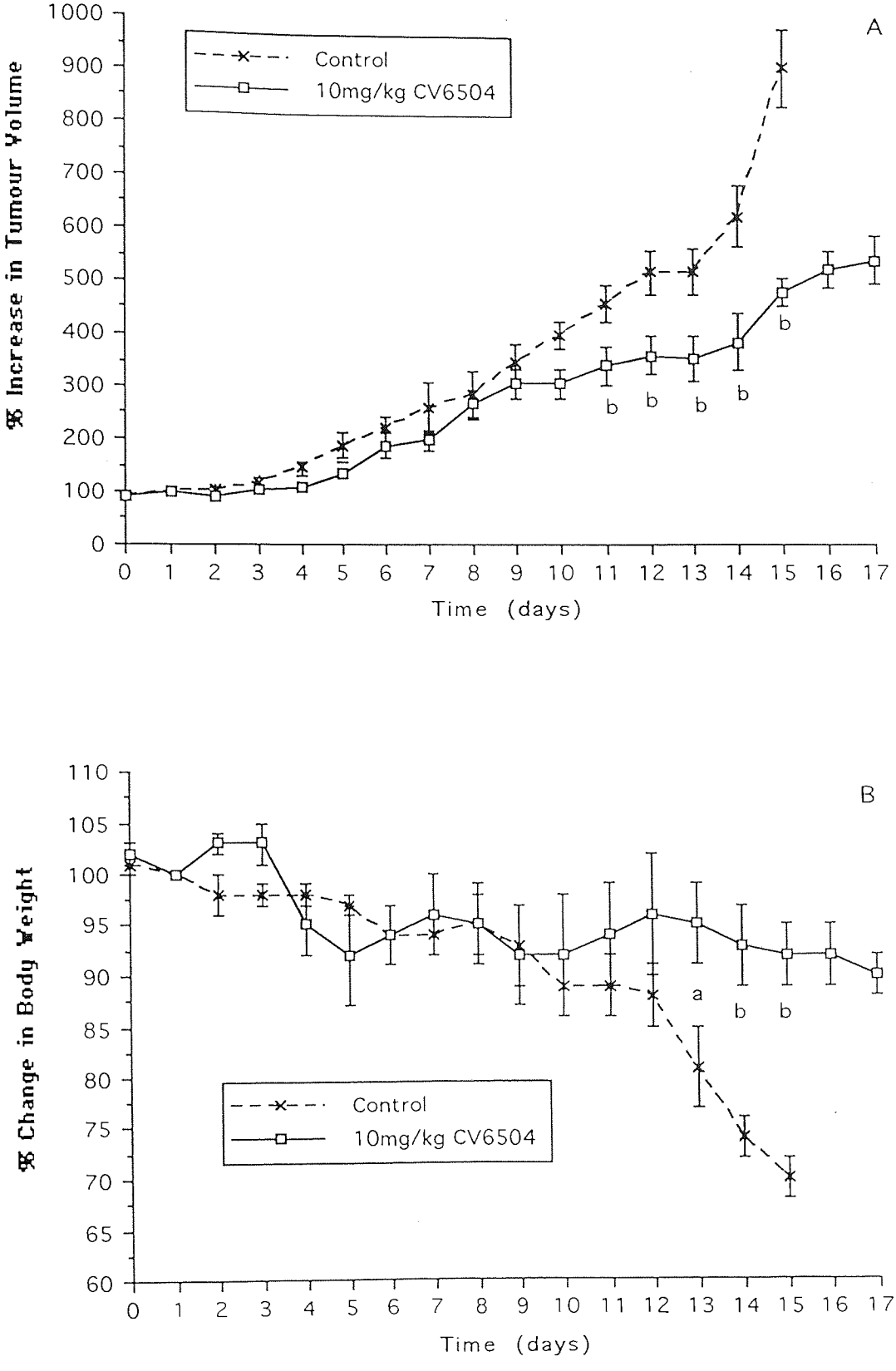


Figure 32. The effect of CV6504 on A. tumour growth and B. weight loss in MAC16 tumour-bearing female NMRI mice (n=9).
(a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test.)

mice is shown in figure 33. Daily oral administration of 1.0g/kg LA was poorly tolerated by the MAC16 tumour-bearing mice. There was no promotion of tumour growth but an increase in weight loss. By day 7, 3 animals had to be sacrificed as they had reached the criterion of a 30% weight loss, with the remaining animals being sacrificed due to weight loss on day 8, one day before the termination of the controls. This increased weight loss was reduced by the concurrent administration of 10mg/kg CV6504, with an increased survival of 3 days in comparison to animals treated with 1.0g/kg LA alone and 2 days in comparison to controls before the criterion of a 30% weight loss was observed. The specific growth delay after the second doubling was 0.60 for 10mg/kg CV6504, 0.40 for 25 mg/kg and 0.40 for 10mg/kg CV6504 with 1.0g/kg LA. The antitumour action of 10mg/kg CV6504 was therefore partially reduced by the concurrent administration of 1.0g/kg LA but specific growth delay was still sufficient to produce a significant reduction in tumour volume from day 6 to 9. The antitumour effect of 10mg/kg CV6504 administered alone produced a significant reduction in tumour volume from day 4 to 9 over control, accompanied by a reduced weight loss. This antitumour and anticachectic effect produced an increase in survival of 3 days to reach the criteria of a 30% weight loss or 1000mm^3 tumour burden used to terminate the control animals.

The effect of dosing 1.0g/kg/ LA with A. 5mg/kg CV6504 and B. 10mg/kg CV6504 on the inhibition of MAC13 tumour growth is shown in figure 34. At 5mg/kg CV6504 again showed a profound antitumour activity against the MAC13 tumour with a significant reduction in tumour volume from day 6 to 11. The specific growth delay after the second doubling was 1.4 which was sufficient to increase the survival of the tumour-bearing animals by 14 days before the criterion of a maximum tumour burden of 1000mm^3 used to terminated the control group was reached. The antitumour action of 5mg/kg CV6504 was reduced by the concurrent administration of 1.0g/kg LA. Specific growth delay after the second doubling was reduced to 0.20, with a significant increase in tumour volume in comparison to 5mg/kg CV6504 administered alone from day 10 to 14. Though reduced by concurrent administration

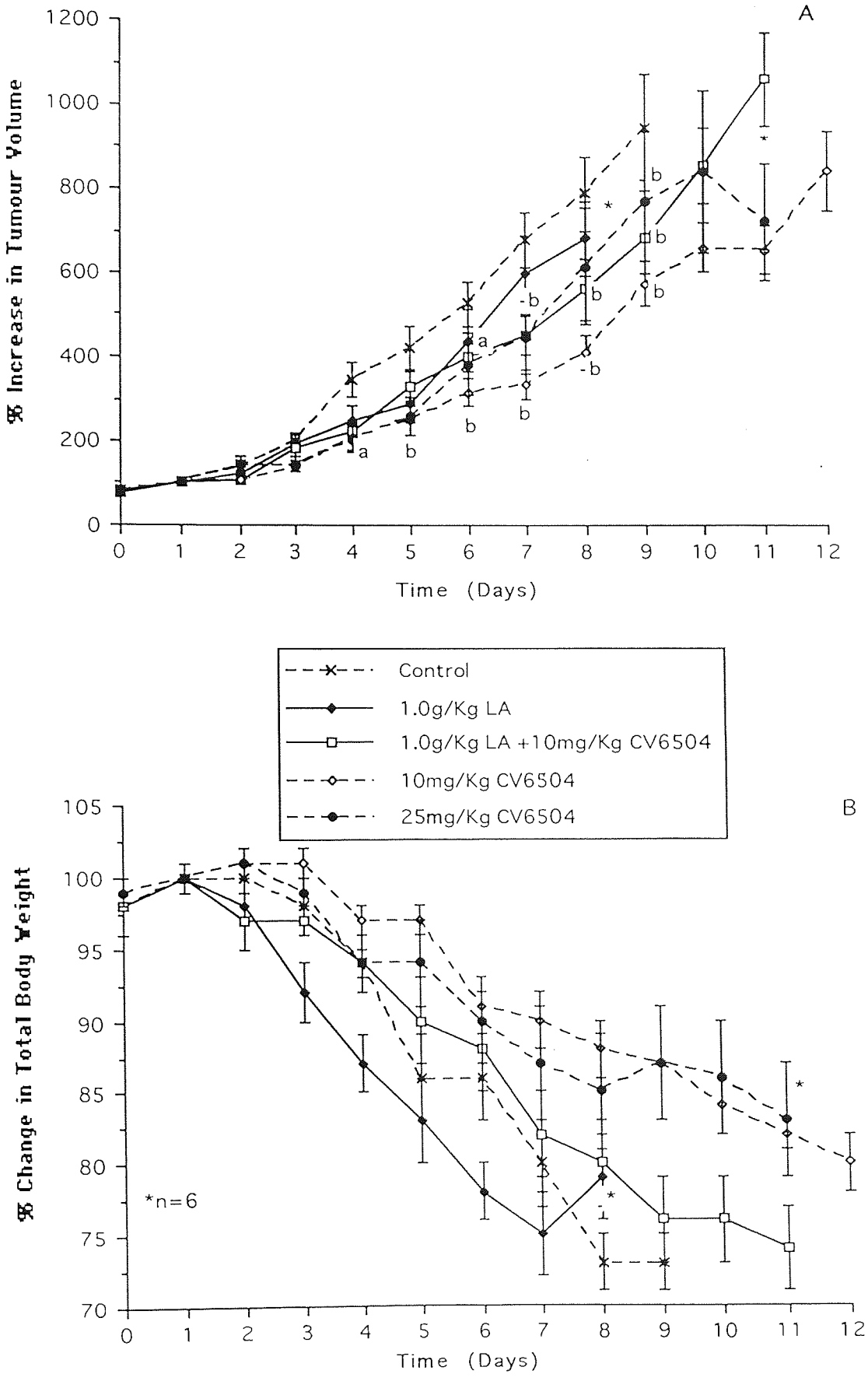


Figure 33. The effect of CV6504 with and without 1.0g/kg linoleic acid (LA) on A. tumour growth and B. decrease in body weight of MAC16 tumour-bearing NMRI mice (n=9).
(a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test.)

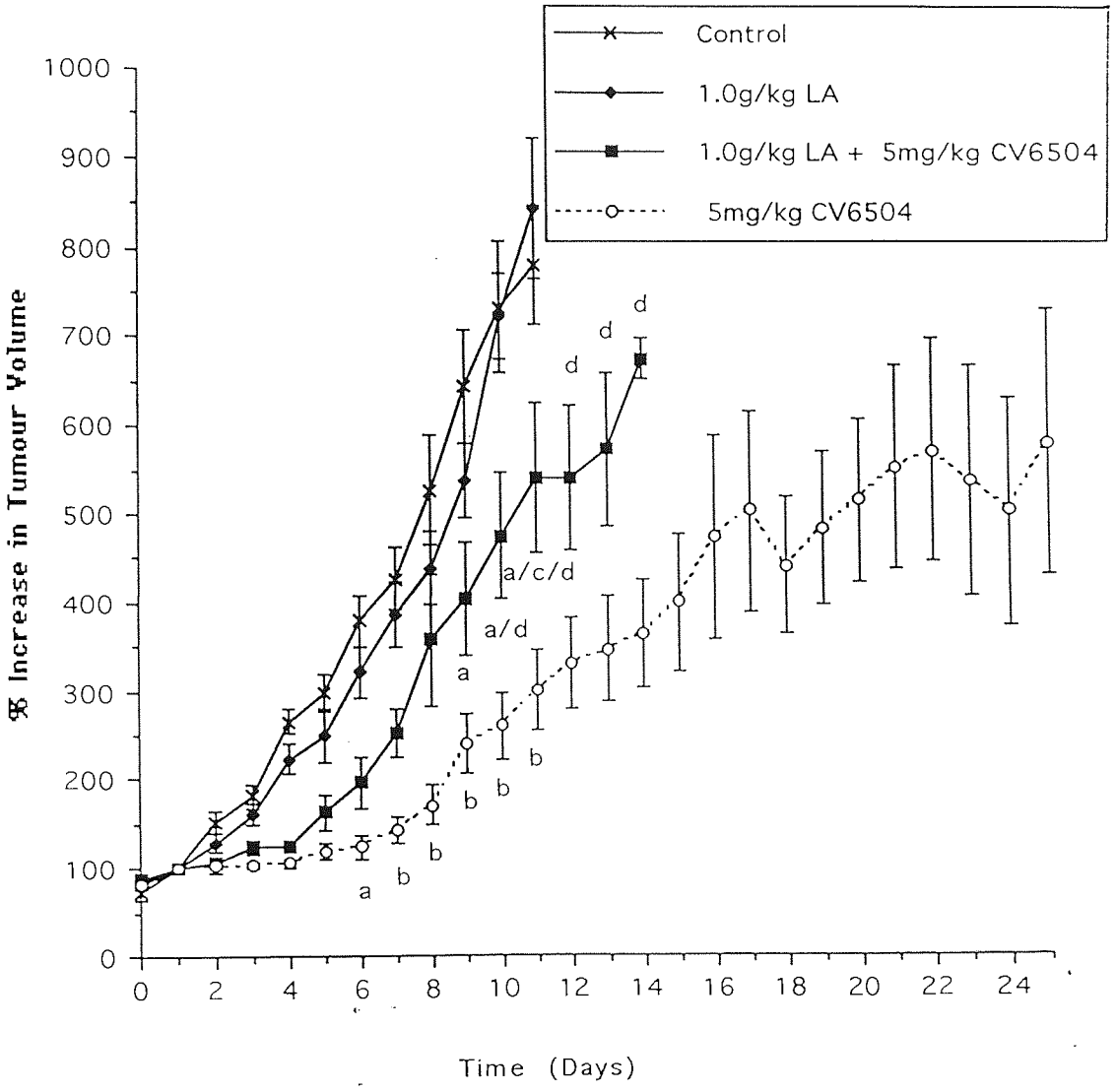


Figure 34A. The effect of 5mg/kg CV6504 with and without 1.0g/kg linoleic acid (LA) on the growth of the MAC13 tumour in NMRI mice (n=9).

(a= $p<0.05$ and b= $p<0.01$ from Control, c= $p<0.05$ from 1.0g/kg LA, and d= $p<0.01$ from 5mg/kg CV6504, using two-way ANOVA followed by Tuckey's Test.)

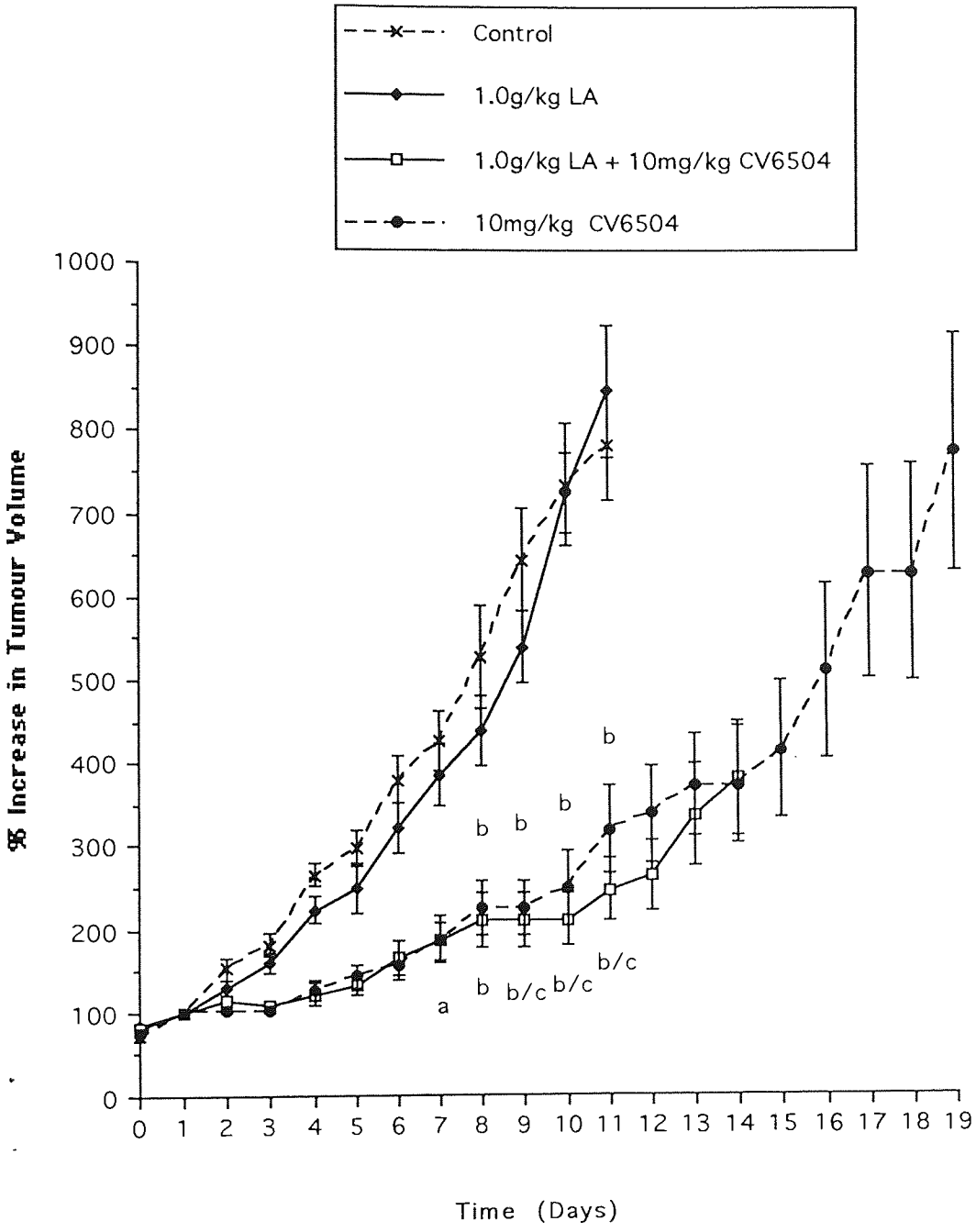


Figure 34B. The effect of 10mg/kg CV6504 with and without 1.0g/kg linoleic acid (LA) on the growth of the MAC13 tumour in NMRI mice (n=9).

(a= $p<0.05$ and b= $p<0.01$ from Control, c= $p<0.01$ from 1.0g/kg LA, using two-way ANOVA followed by Tuckey's Test.)

of 1.0g/kg LA, the antitumour action of 5mg/kg CV6504 was still sufficient to produce a significant reduction in tumour volume over control tumours from day 9 to 11, with an increased survival of 3 days still being required to meet the criterion of maximum permitted tumour volume of 1000mm^3. The partial reversal of the antitumour action of CV6504 by 1.0g/kg LA was prevented by increasing the dose of CV6504 to 10mg/kg, as shown in figure 34 B.

The effect of CV6504 with and without concurrent administration of 1.0g/kg LA on the growth of the MAC26 tumour is shown in figure 35. CV6504 at 25 and 50 mg/kg had an antitumour action on the MAC26 tumour which produced a specific growth delay after the first doubling of 1.5 and 2 respectively, accompanied by a significant reduction in tumour volume from day 5 to 11. The specific growth delay after the first doubling was reduced to 1.00 by concurrent administration of 1.0g/kg LA with 25mg/kg CV6504, but antitumour action was still sufficient to produce a significant reduction in tumour volume over both control and 1.0g/kg LA treated animals at days 7, 9, 10 and 11. The partial reversal of antitumour action of CV6504 by LA produced a significant reduction in tumour volume by 25mg/kg CV6504 administered alone in comparison to concurrent administration with 1.0g/kg LA at days 8 to 11.

The effect of CV6504 on the lipid peroxide levels of the A. MAC13, B. MAC16 and C. MAC26 tumour-bearing NMRI mouse serum is shown in figure 36. CV6504 reduced lipid peroxide levels in MAC13 tumour-bearing mouse serum dose-dependently from 5-25mg/kg/day, with a significant reduction at 25mg/kg/day. Lipid peroxide levels in MAC16 tumour-bearing mouse serum was significantly reduced in a dose dependent manner at 25mg/kg and 10mg/kg. In MAC26 tumour-bearing mouse serum lipid peroxide levels were increased by 1.0g/kg LA. This was significantly reduced by concurrent dosing of 25mg/kg CV6504. The decrease in lipid peroxide levels observed at 10mg/kg and 25mg/kg CV6504 were dose dependent and significantly lower than controls. The decrease in lipid peroxide levels in either of the three tumour models did not correlate with the relative antitumour activity produced by CV6504.

The effect of CV6504 at 10mg/kg with and without 1.0g/kg LA on the FA profile of

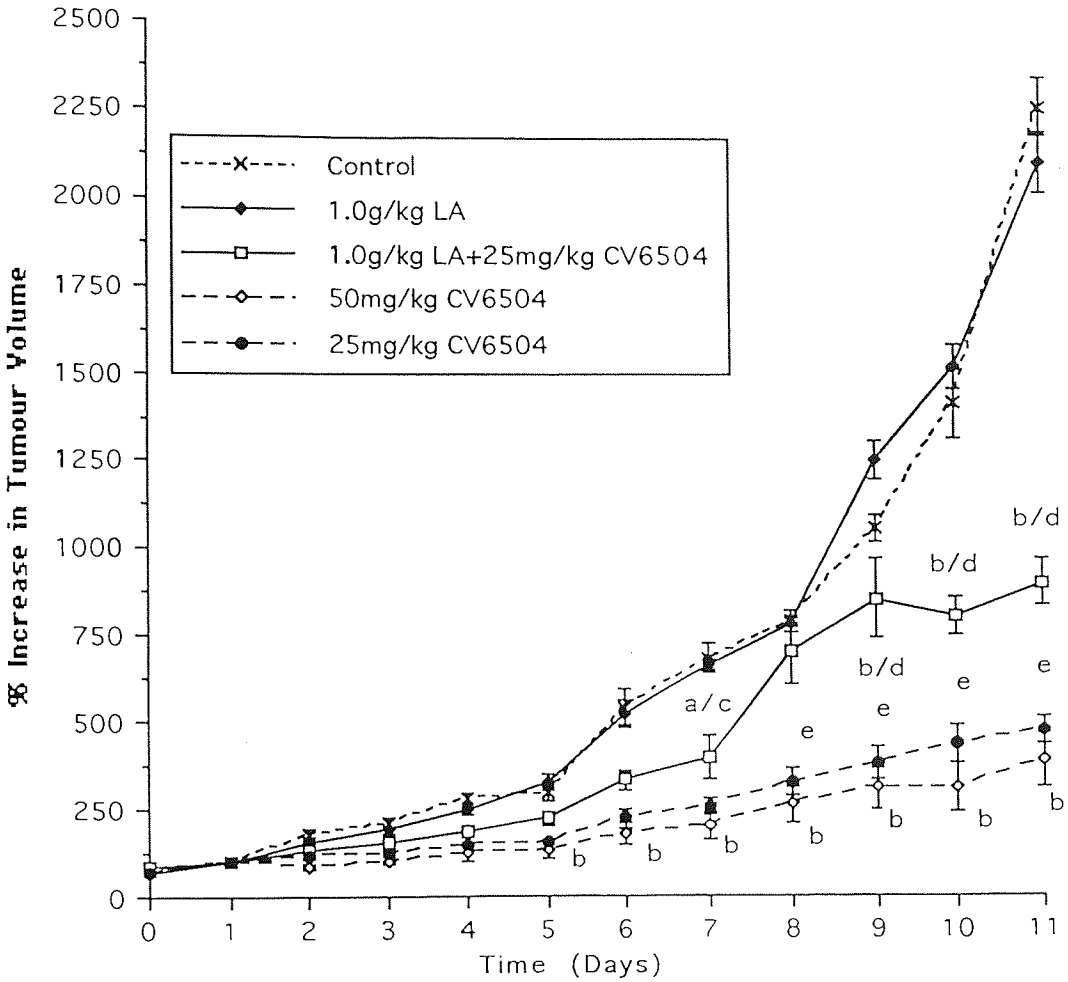
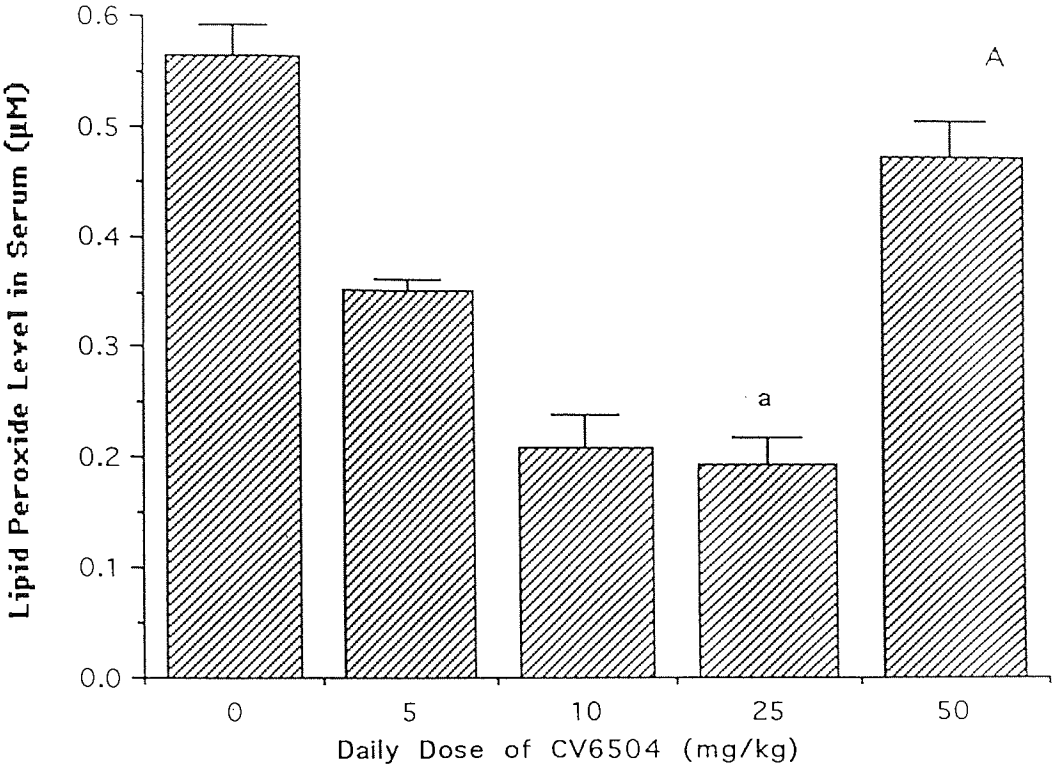


Figure 35. The effect of CV6504 with and without 1.0g/kg linoleic acid (LA) on the growth of the MAC26 tumour in NMRI mice (n=9).

(a= $p<0.05$ and b= $p<0.01$ from Control, c= $p<0.05$ and d= $p<0.01$ from 1.0 g/kg LA, e= $p<0.01$ from 1.0 g/kg LA + 25 mg/kg CV6504 using two-way ANOVA followed by Tuckey's Test.)



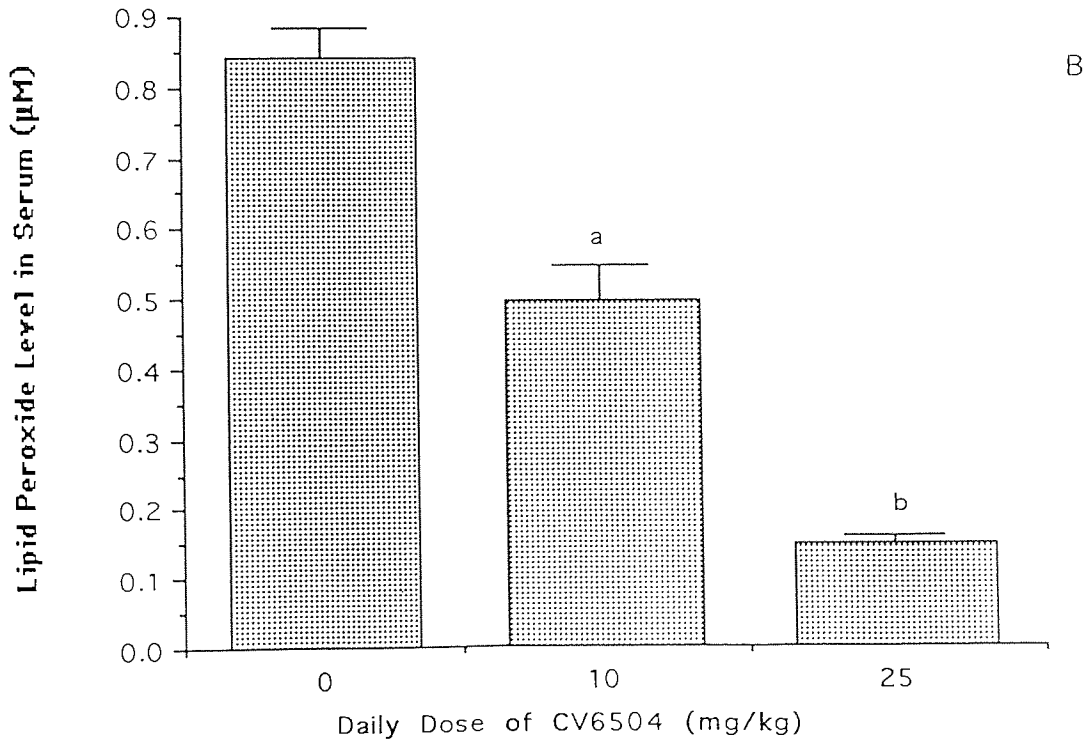


Figure 36. The effect of daily oral aministration of CV6504 on the lipid peroxide levels in the serum of A. MAC13 and B. MAC16 tumour-bearing NMRI mice (n=9). (a= $p<0.05$ and b= $p<0.01$ from untreated animals using T-test with Bonferroni Correction.)

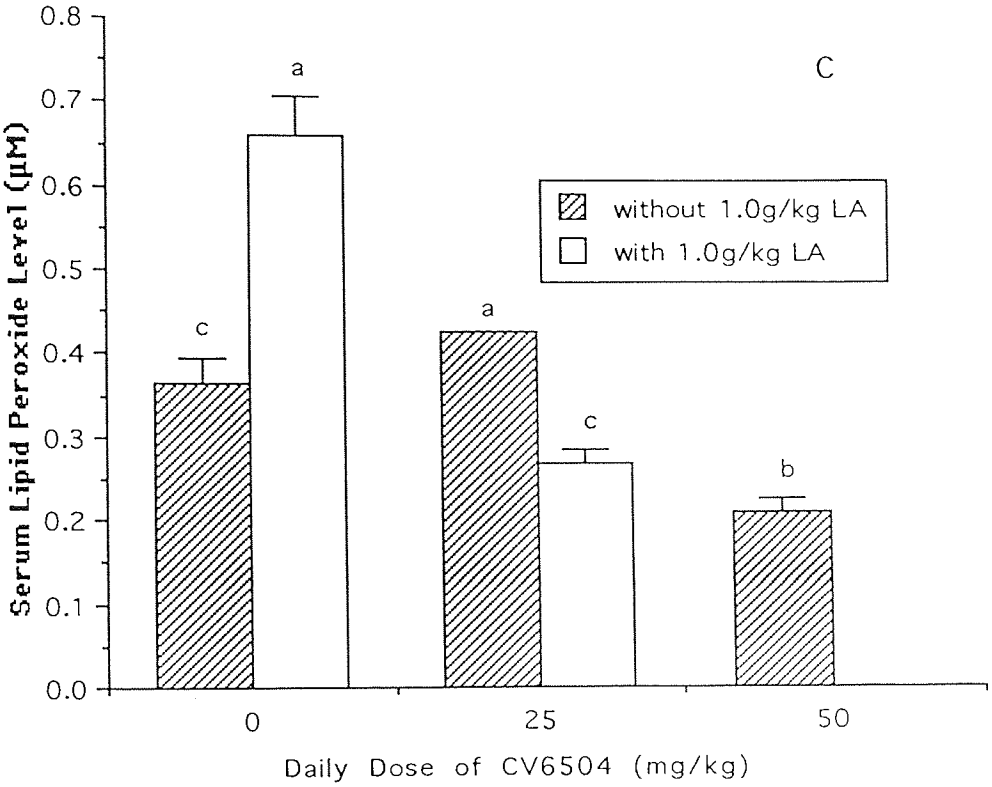


Figure 36C. The effect of daily oral admnisitration of CV6504 on the lipid peroxide levels in MAC26 tumour-bearing NMRI mice (n=9).

(a= $p<0.05$, b= $p<0.01$ and c= $p<0.005$ from untreated animals using T-test with Bonferroni Correction.)

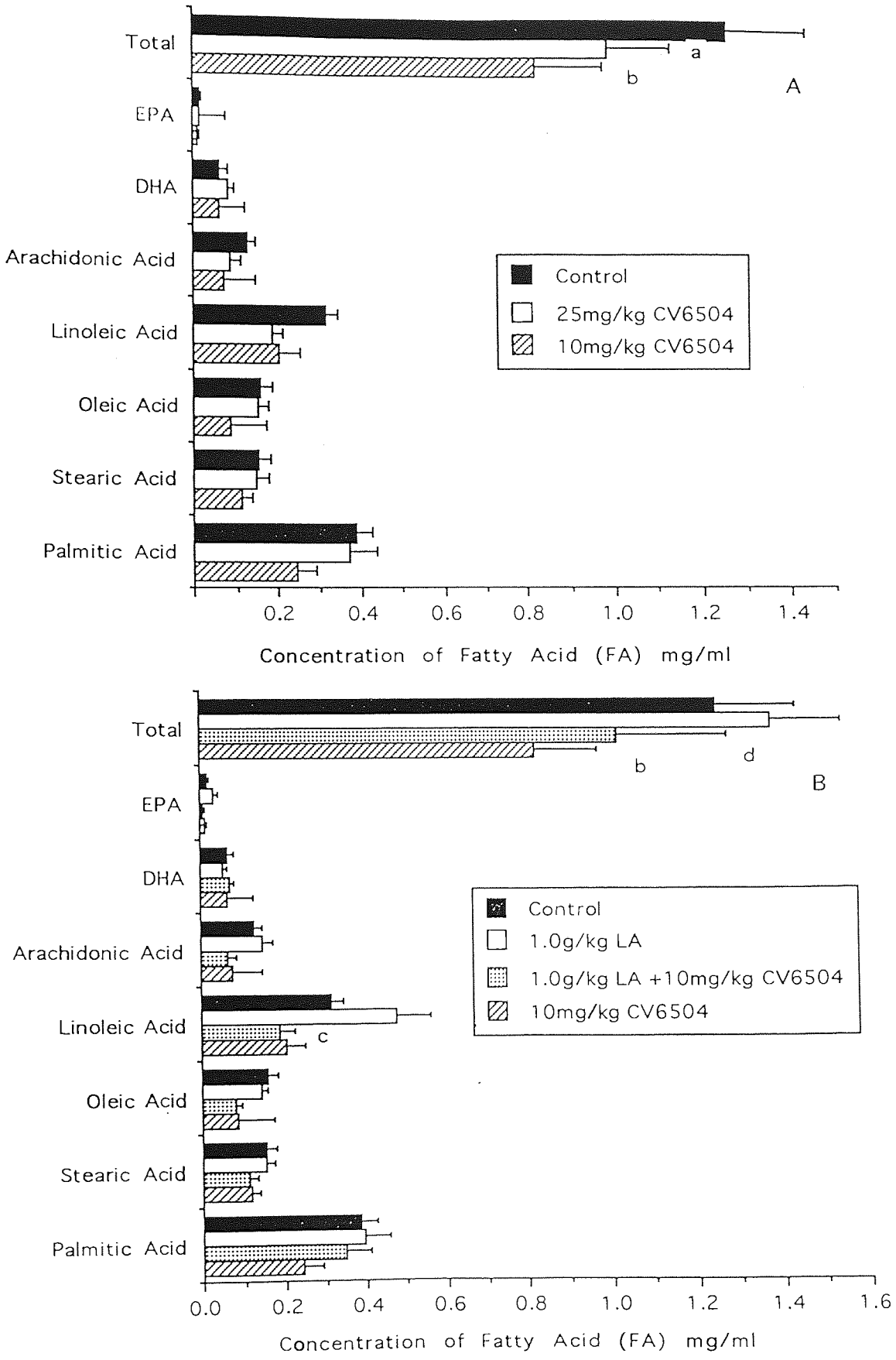


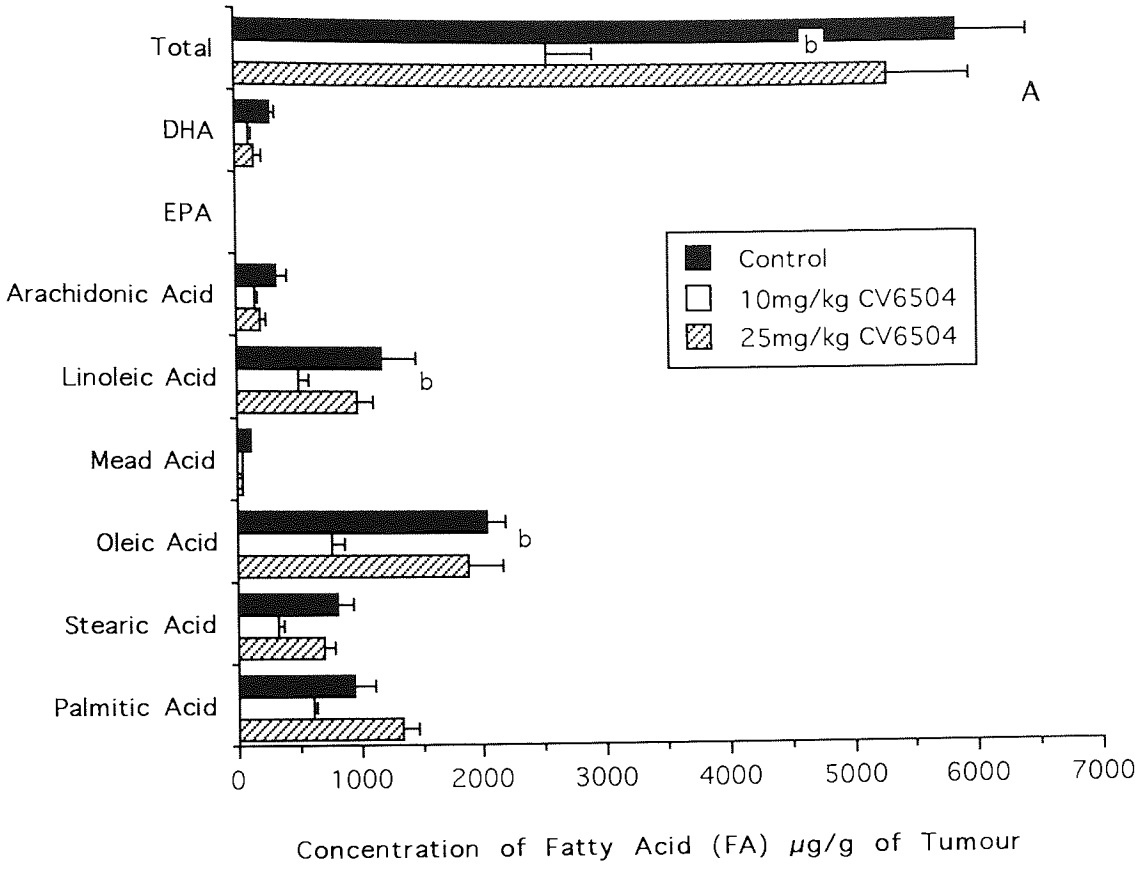
Figure 37. The effect of CV6504 A. without and B. with linoleic acid (LA) on the fatty acid (FA) profile of plasma in MAC16 tumour-bearing NMRI mice (n=10).

(a= $p<0.05$ and b= $p<0.01$ from Control, c= $p<0.05$ and d= $p<0.01$ from 1.0g/kg LA using two-way ANOVA followed by Tuckey's Test.)

MAC16 tumour-bearing mouse plasma is shown in figure 37. The total FA composition was significantly reduced by 10 and 25mg/kg CV6504 though not in a dose dependent manner. This reduction was also nonspecific to any given FA. When 1.0g/kg LA was administered to MAC16 tumour-bearing mice there was a significant increase in the LA concentration of the plasma, which was reversed when 1.0g/kg LA was administered together with 10mg/kg CV6504. Treatment with CV6504 and LA was also capable of significantly reducing the total FA content of the plasma specifically the elevated LA level.

The effect of 7 consecutive daily doses of 10mg/kg or 25mg/kg CV6504 on the FA composition of A. the tumour and B. the liver in MAC16 tumour-bearing NMRI mice is shown in figure 38. At dose levels of 10 and 25mg/kg CV6504 reduced the total FA composition of the tumour, though again this was not in a dose dependent manner. The reduction in total FAs was significant at 10mg/kg. At this dose level the content of tumour LA and oleic acid (OA) was also significantly reduced, along with AA, stearic acid (SA), palmitic acid, Mead acid, and DHA . This resulted in an increase in the SA:OA ratio and Mead acid:AA ratio. The total FA composition of the liver was reduced significantly, nonspecifically and not in a dose dependent manner. There was a significant reduction in AA, LA, OA, SA and palmitic acid levels in the liver after treatment with 10 or 25mg/kg CV6504.

The metabolism of CV6504 in MAC16 tumour-bearing NMRI mice is shown in table 6., and was determined by the recovery of [^{14}C]-CV6504 from tissues and plasma. Unknown CV6504 metabolites recovered from the tumour and plasma at 0.25 0.50 and 2.00 hr were significantly lower than the liver. Unknown metabolites recovered from the kidney were also significantly lower than those recovered from the liver at 0.50 and 2.00 hr. When CV6504 was dosed for 6 consecutive days (24^{6}), 24 hr after the final dose unknown metabolites recovered from the tumour were significantly higher than those recovered from the liver. CV6504-sulphates recovered from the tumour and kidney were significantly lower than the liver at 0.25, 0.50 and 2.00 hr. This was extended in the plasma to 24 hr. The sulphate-metabolites recovered in the tumour and the kidney after 24 hr and the 6 consecutive



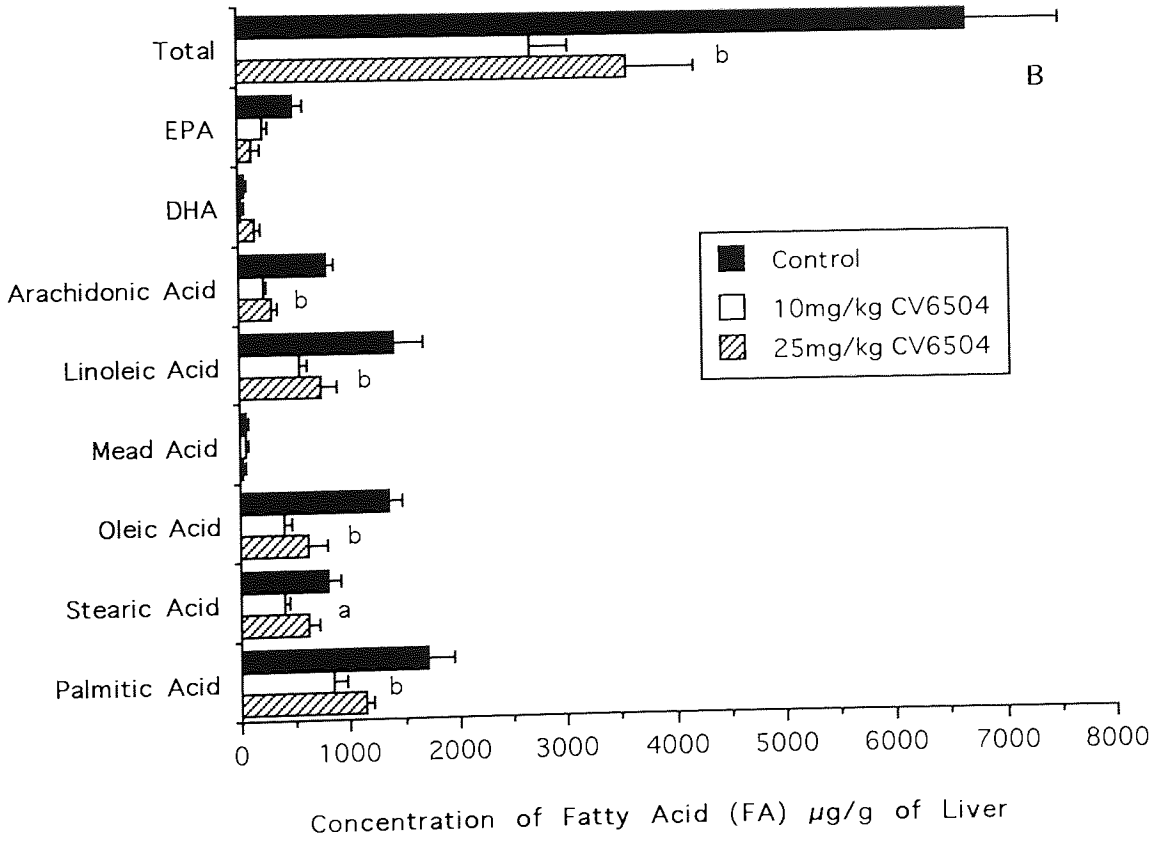


Figure 38. The effect of CV6504 on the fatty acid (FA) profile of A. the tumour and B. the liver, of MAC16 tumour-bearing NMRI mice (n=9).
(a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test.)

Table 6.
The Metabolism of CV6504 by MAC16 Tumour-Bearing NMRI Mice. Results μg/ml plasma (n=4), and μg/g of tissue (n=20). Results in brackets % of total radiation recovered. (*= $p<0.05$ and **= $p<0.01$ from liver samples refers to the concentration in μg/gof tissue and μg/ml of plasma using two-way ANOVA followed by Tuckey's Test.)

	CV6504-Unknown	CV6504-Sulphate	CV6504-Glucuronide	CV6504-Unchanged	Total
Liver					
0.25	0.75 ± 0.20 (4 ± 1)	1.70 ± 0.25 (9 ± 2)	11.25 ± 0.38 (61 ± 3)	4.80 ± 0.30 (26 ± 3)	18.50 ± 0.29
0.50	1.17 ± 0.37 (6 ± 2)	2.38 ± 0.36 (12 ± 3)	15.27 ± 0.38 (69 ± 4)	2.97 ± 0.66 (14 ± 4)	21.58 ± 0.69
2.00	0.57 ± 0.07 (15 ± 3)	0.57 ± 0.07 (18 ± 2)	1.73 ± 0.18 (55 ± 5)	0.37 ± 0.04 (13 ± 4)	3.12 ± 0.10
24.00	0.02 ± 0 (25 ± 4)	0.03 ± 0 (28 ± 2)	0.04 ± 0 (28 ± 2)	0.04 ± 0.00 (30 ± 2)	0.14 ± 0.04
24.00[6]	0.21 ± 0.09 (16 ± 6)	0.20 ± 0.03 (15 ± 2)	0.67 ± 0.08 (50 ± 6)	0.25 ± 0.88 (19 ± 6)	1.33 ± 0.32
Tumour					
0.25	0.22 ± 0.04 (4 ± 1)**	0.32 ± 0.04 (7 ± 1)**	2.36 ± 0.04 (74 ± 1)**	0.28 ± 0.08 (9 ± 2) **	3.18 ± 0.42**
0.50	0.60 ± 0.01 (14 ± 1)**	0.50 ± 0.01 (14 ± 1)**	2.00 ± 0.20 (47 ± 4)**	1.20 ± 0.10 (28 ± 3)**	4.20 ± 0.80**
2.00	0.26 ± 0.02 (23 ± 2)**	0.22 ± 0.02 (22 ± 2)	0.35 ± 0.03 (31 ± 3)	0.28 ± 0.05 (25 ± 3)	1.12 ± 0.15
24.00	0.10 ± 0.01 (29 ± 1)	0.12 ± 0.02 (31 ± 1)	0.10 ± 0.01 (25 ± 1)	0.55 ± 0.13 (21 ± 5)**	0.87 ± 0.10
24.00[6]	0.42 ± 0.13 (16± 5)*	0.44 ± 0.08 (17 ± 3)	1.20 +_0.13 (46 ± 5)	0.56 ± 0.12 (21 ± 5)**	2.60 ± 0.10
Kidney					
0.25	0.66 ± 0.10 (5 ± 1)	0.42 ± 0.13 (7 ± 2)**	8.28 ± 0.13 (80 ±2)	0.66 ± 0.10 (8 ± 1)**	10.07 ± 1.04**
0.50	0.65 ± 0.12 (6 ± 1)**	0.94 ± 0.06 (6 ± 2)**	13.03 ± 0.07 (83 ± 2)	1.06 ± 0.06 (7 ± 2)**	15.64 ± 0.83**
2.00	0.16 ± 0.03 (9 ± 1)**	0.29 ± 0.03 (13 ± 2)	1.95 ± 0.07 (70 ±3)	0.23 ± 0.04 (11 ± 2)	2.61 ± 0.17
24.00	0.04 ± 0.00 (25 ± 4)	0.05 ± 0.00 (28 ± 2)	0.05 ± 00 (31 ± 1)	0.02 ± 0 (15 ±0)	0.16 ± 0.02
24.00[6]	0.33 ± 0.04 (11 ± 2)	0.55 ± 0.08 (13 ± 2)	2.90 ±0.17 (64 ± 4)	0.46 ± 0.04 (8± 2)	4.20 ± 0.10
Plasma					
0.25	0.16 ± 0.01 (4 ± 1)**	0.14 ± 0.08 (4 ± 2)	5.09 ± 1.55 (90 ± 3)	0.20 ± 0.05 (3 ± 1)**	6.72 ± 3.08**
0.50	0.09 ± 0.05 (1 ± 1)**	0.38 ± 0.20 (6 ± 3)	4.85 ± 1.19 (90 ± 6)	0.14 ± 0.07 (2 ± 1)**	5.47 ± 1.43**
2.00	0.02 ± 0.00 (4 ± 1)**	0.11 ± 0.07 (7 ± 3)*	0.73 ± 0.20 (90 ± 2)	0.01 ± 0.00 (1 ± 0)**	0.85 ± 0.26
24.00	0.02 ± 0.00 (3 ± 1)	0.03 ± 0.02 (7 ± 3)	0.31 ± 0.07 (89 ± 3)	0.01 ± 0.00 (1 ± 0)	0.35 ± 0.09
24.00[6]	----------	0.13 ± 0.00 (6 ± 0)	1.87 ± 0.25 (97 ± 3)	0.01 ± 0.01 (1 ±1)*	1.94 ± 0.09

Table 7.
The pharmacokinetics of CV6504 in plasma (μg/ml of plasma (%)) of MAC16 tumour-bearing NMRI mice in comparison to B6C3F1 mice after a single dose of 10mg/kg CV6504. For NMRI mice n=4, results for B63C3F1 mice were supplied by Takeda Chemicals Ltd.

Time (hr)		MAC16 Tumour-Bearing NMRI Mice	B63C3F1 Mice
0.25	CV6504	0.20 ± 0.05 (3 ± 1)	0.06 (1)
	CV6504 -Metabolites	6.52 ± 1.03 (97 ± 3)	5.62 (99)
0.50	CV6504	0.14 ± 0.07 (2 ± 1)	0.02 (1)
	CV6504 -Metabolites	5.33 ± 0.48 (98 ± 3)	2.68 (99)
2.00	CV6504	0.01 ± 0.00 (1 ± 0)	0.01 (1)
	CV6504 -Metabolites	0.84 ± 0.09 (99 ± 2)	0.85 (99)

doses was increased in comparison to the liver, although not significantly. CV6504-glucuronide levels recovered from the tumour, kidney and plasma were significantly reduced in comparison to the liver at 0.25 and 0.50 hr, as were the levels of unmetabolised CV6504. Tumour levels of unmetabolised CV6504 were significantly higher in the tumour than in the liver 24 hr after a single dose and 24 hr after the 6 consecutive daily doses. The total CV6504 recovered, both metabolised and unmetabolised, was significantly higher in the liver compared to tumour, kidney and plasma at 0.25 and 0.50 hr.

The CV6504 and CV6504 metabolites recovered in B6C3F1 mouse plasma in comparison to those in recovered in MAC16 tumour-bearing NMRI mouse plasma, are shown in table 7. [^{14}C]-CV6504 was given at 10mg/kg in both strains of mice and plasma levels detected by recovery of [^{14}C]. Peak plasma levels were observed in both strains at 0.25 hr. This level was halved in B6C3F1 mice by 0.50 hr, but still remained high in MAC16 tumour-bearing NMRI mice. The level of unchanged CV6504 and metabolised CV6504 recovered from the plasmas after 2 hr was similar in concentration.

The activity of CV6504 metabolites commonly produced in mammalian cells in comparison to the activity of CV6504 *in vitro* on growth inhibition of the MAC13, MAC16 and MAC26 cell lines are shown in figures 39, 40 and 41 respectively. CV6504-1-G, CV6504-4-G CV6504-1-S and CV6504-4-G were all inactive as growth inhibitors against all three cell lines at concentrations below 100μM. The effect of these metabolites against the growth of the MAC13 in NMRI mice is shown in figure 42. Antitumour action of metabolites given orally at 50mg/kg/day was compatible, though not as profound as 5mg/kg/day CV6504 orally. Antitumour action was slightly reduced with 50mg/kg CV6504-4-G and CV6504-1-S in comparison to 50mg/kg CV6504-1-G and CV6504 -4-S, although this was not significant.

The metabolism of AA by MAC13, MAC16 and MAC26 tumours *ex vivo* using [^{3}H]-AA is shown in figure 43. Total AA and HETEs recovered were significantly higher in MAC16 tumours compared to MAC13 and MAC26 tumours. MAC13 tumours also had higher levels of 11-HETEs and AA than MAC26 tumours, whilst 15-HETE production was significantly higher in MAC16 tumours.

The effect of CV6504 on the AA metabolism of the tumours *ex vivo* after incubation with 10μM CV6504 for 30 min is shown in figure 44. The total unmetabolised AAand HETEs produced recovered from MAC13 or MAC16 tumour was significantly decreased, whilst in MAC26 tumours there was a significant increase. The unmetabolised AA recovered from MAC16 and MAC26 cells was significantly increased, whilst in MAC13 cells it was significantly reduced. The production of 5-HETE, 11-HETE 12-HETE and 15-HETE was significantly reduced in MAC13 and MAC16 tumour cells *ex vivo*, whilst only a significant reduction in 5-HETEs was observed in MAC26 tumour cells.

The effect of CV6504 metabolites on AA metabolism on the MAC16 tumour *ex vivo* is shown in figure 45. CV6504-1-sulphate (CV6504-1-S) significantly inhibited 5-, 11, 12- and 15-HETE production at 2hr at a concentration of 100μM. There was a significant increase in unmetabolised AA within the tumour cells at this time point, whereas a significant reduction was observed with 10μM CV6504 over a 30 min

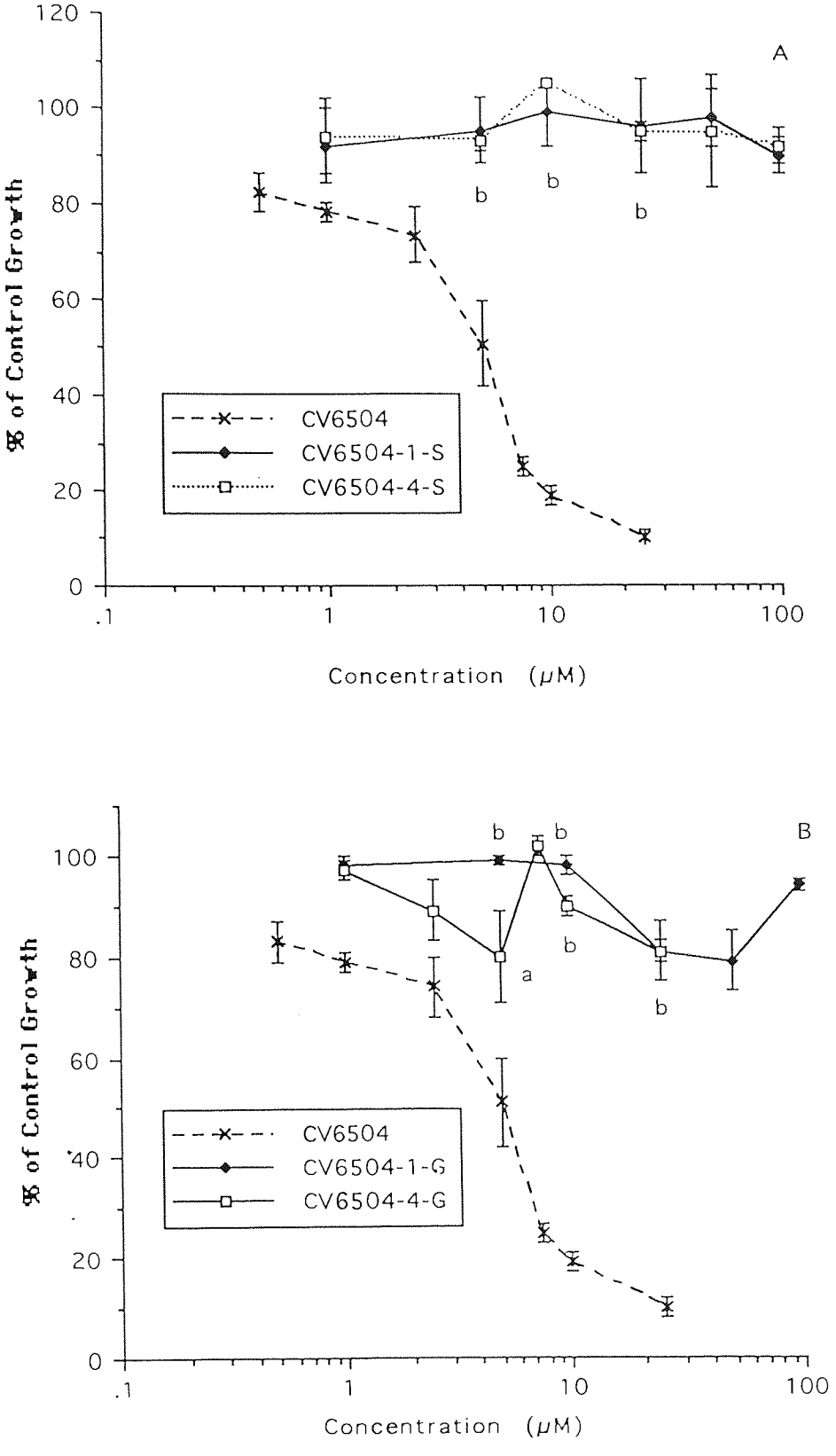


Figure 39 A. and B. The effect of CV6504 and CV6504 metabolites on the growth of MAC13 cells over 72 hours in normal serum (n=9).
(a= $p<0.01$ and b= $p<0.005$ from the effect of CV6504 at the same concentration using T-test with Bonferroni Correction.)

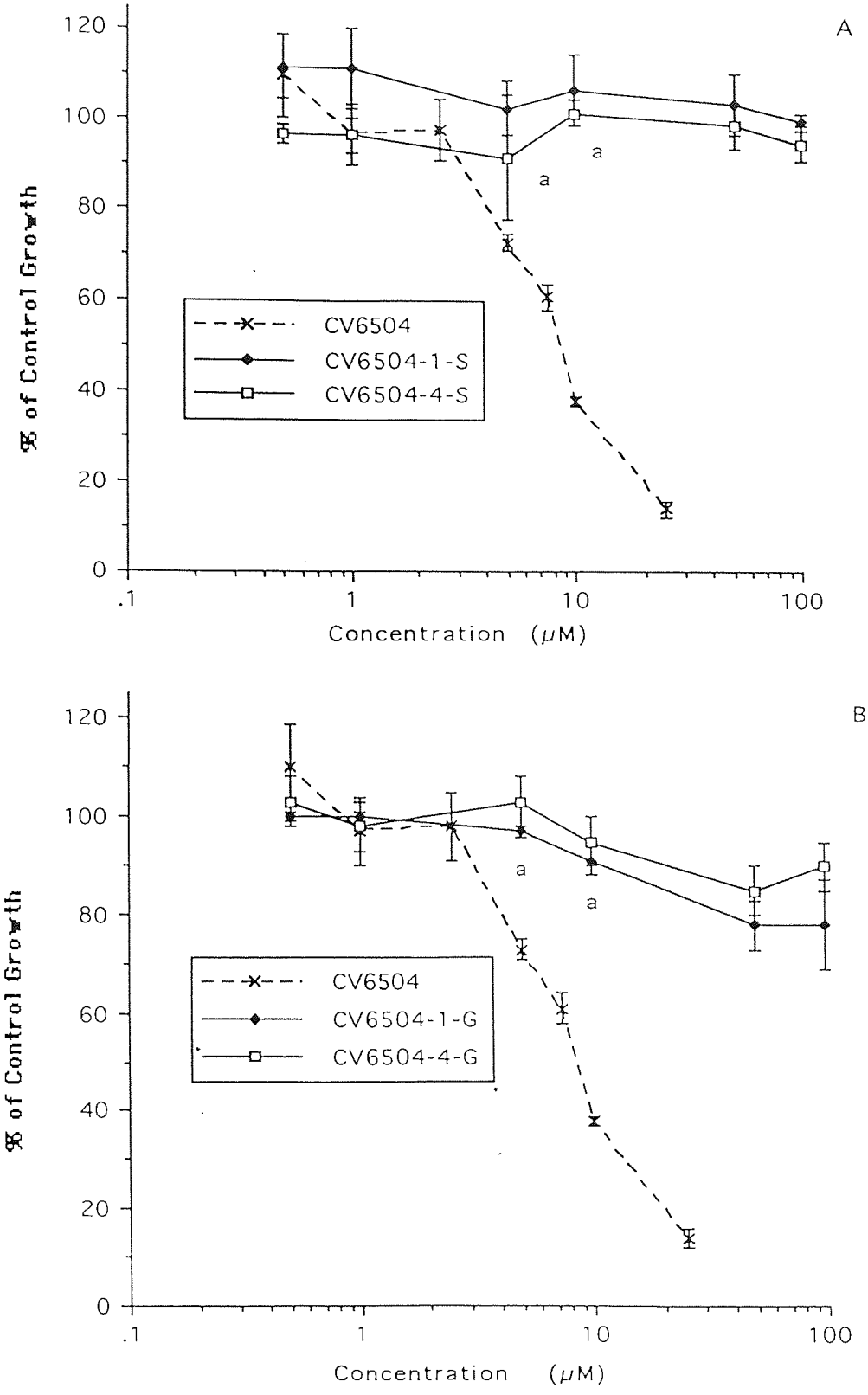
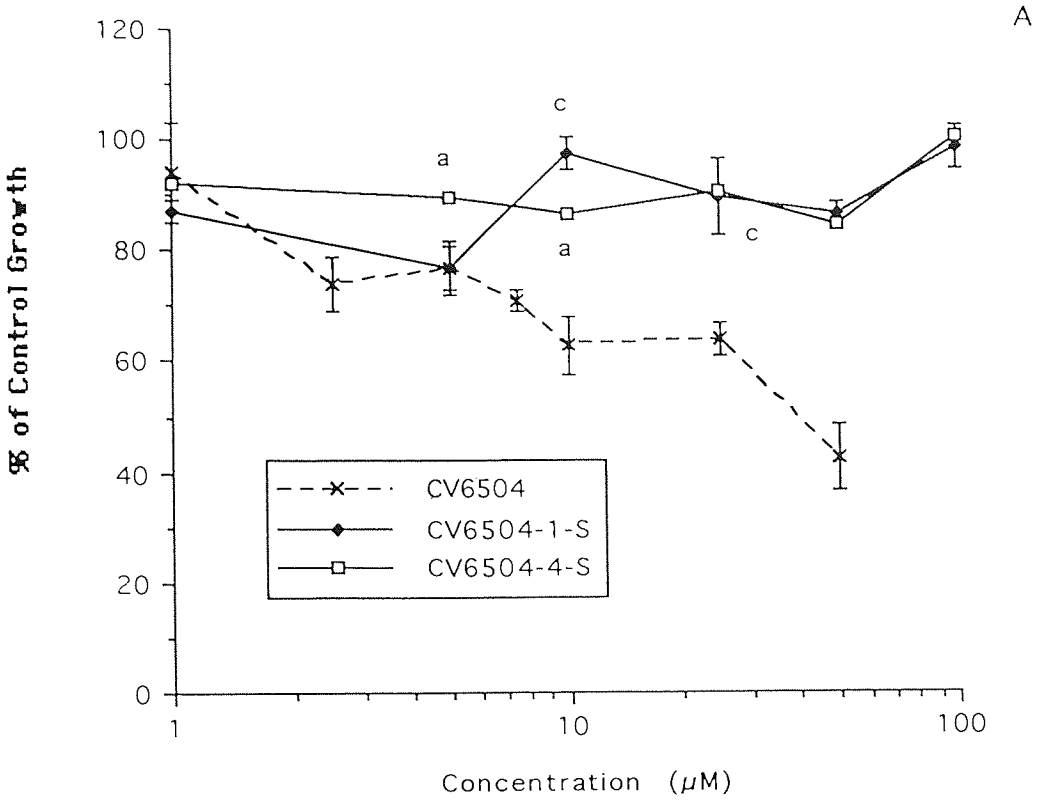


Figure 40 A. and B. The effect of CV6504 and CV6504 metabolites on the growth of MAC16 cells over 72 hours in normal serum (n=9).

(a= $p<0.005$ from the effect of CV6504 at the same concentration using T-test with Bonferroni Correction.)



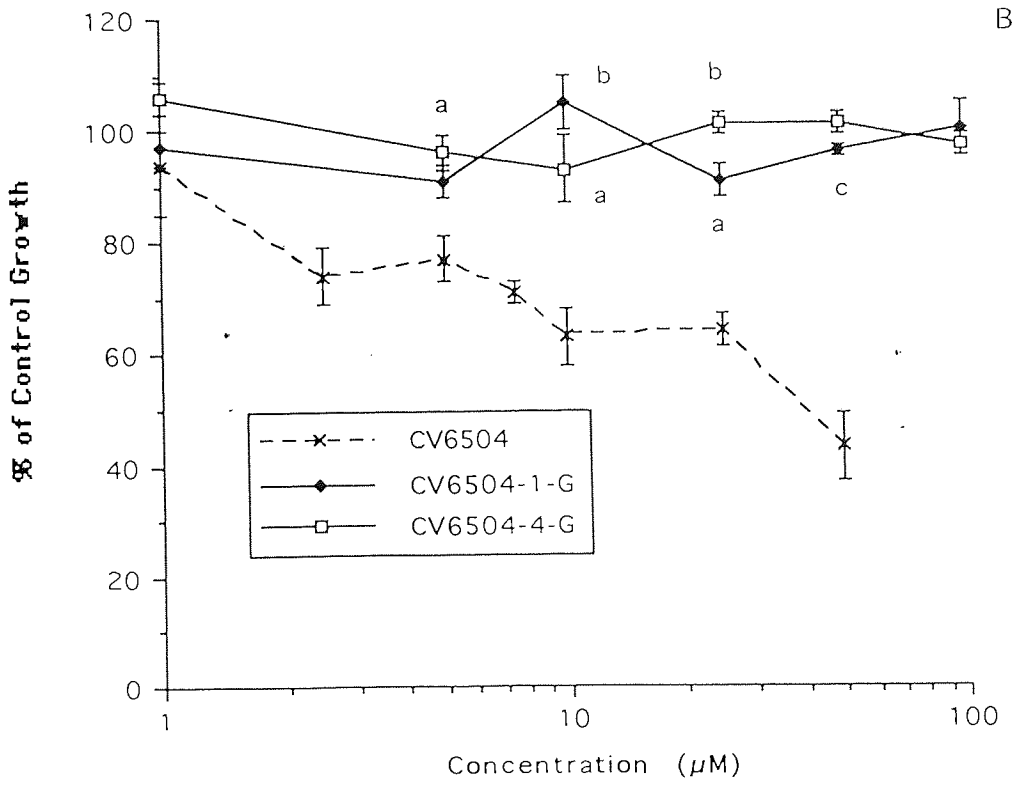


Figure 41 A. and B. The effect of CV6504 and CV6504 metabolites on the growth of the MAC26 cells over 72 hours in normal serum (n=9).

(a= $p<0.05$, b= $p<0.01$ and c= $p<0.005$ from the effect of CV6504 at the same concentration using T-test with Bonferroni Correction.)

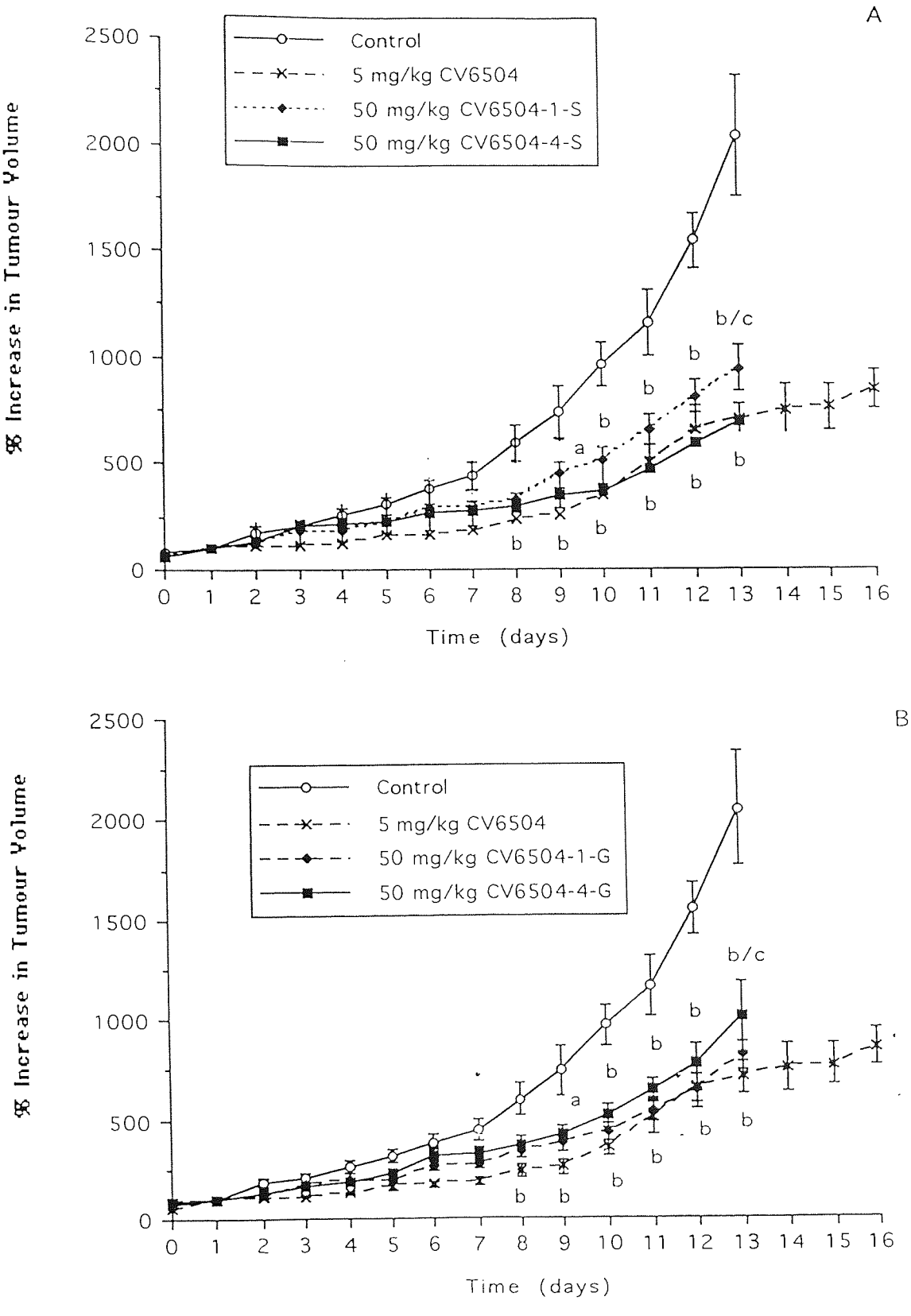


Figure 42. The effect of CV6504 and CV6504 metabolites on the growth of the MAC13 tumour in NMRI mice (n=9).
(a= $p<0.05$ and b= $p<0.01$ from Control, c= $p<0.05$ from 5mg/kg CV6504 treated tumour growth, using two-way ANOVA followed by Tuckey's Test.)

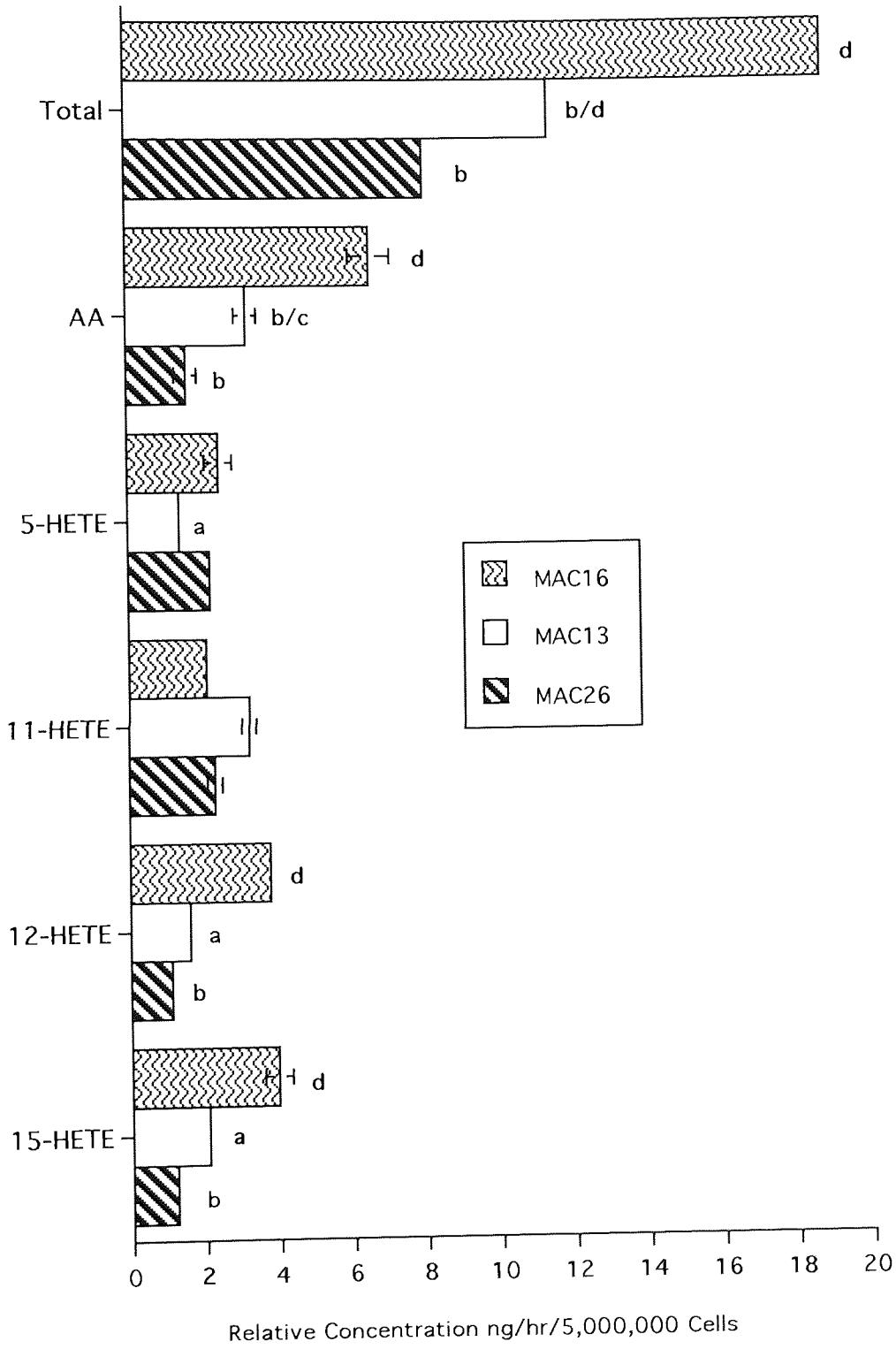


Figure 43. The arachidonic acid (AA) metabolism in MAC tumour homogenates(n=4).
(a= $p<0.05$ and b= $p<0.01$ from MAC16 tumour, or c= $p<0.05$ and b= $p<0.01$ from MAC26 tumour, using two-way ANOVA followed by Tuckey's Test.)

period. At a concentration of 100μM CV6504-4 glucuronide (CV6504-4-G) inhibited the production of 5-HETE, 11-HETE and 15-HETE by 1 hr. This was accompanied by a significant reduction in the unmetabolised AA recovered. After a 2 hr incubation 12-HETE production was also significantly reduced and there was an increased accumulation of unmetabolised AA within the tumour cells.

The effect of CV6504 metabolites on the AA metabolism of the MAC13 tumours *ex vivo* is shown in figure 46. At a concentration of 100μM, CV6504-1-S significantly reduced the production of 5-HETE after 2 hr, 12-HETE or 15-HETE after 30 min and 11-HETE after 1 hr. The unmetabolised AA recovered within the cells at 1 hr was reduced, as was shown by 10μM CV6504, whereas at 30 min and 2 hr it was increased. The total recovery of unmetabolised AA and HETEs was significantly reduced at 1 and 2 hr as was observed for CV6504. The effect of 100μM CV6504-G4 on the AA metabolism in the MAC13 *ex vivo* homogenates is shown in figure 46B. The production of 5, 12 and 11-HETE was reduced significantly from 30 min. 15-HETE production was not reduced until 1 hr. The level of unmetabolised AA recovered from the tumour cells was reduced significantly after 1 hr, as was demonstrated with 10μM CV6504 after 30 min. At 30 min and 2 hr the level of unmetabolised AA detected within the MAC13 tumour cells was significantly increased by treatment with 100μM CV6504-G.

The effect of CV6504 metabolites at a concentration of 100μM on the AA metabolism of the MAC26 tumour *ex vivo* from the recovery of [^{3}H]-AA is shown in figure 47. The production of 5-HETE and 12-HETE was significantly reduced at 2 hr, whereas the production of 15-HETE was increased. The unmetabolised AA recovered within the tumour cells was significantly increased at 30 min, 1 hr and 2 hr, as was demonstrated with 10μM CV6504. The total AA recovered and HETEs produced was significantly increased at 30 min and 1 hr, as was also observed with 10μM CV6504. At a concentration of 100μM CV6504-4-G significantly inhibited the production of 5-HETE after 2 hr, but significantly increased the production of 12-HETE at 30 min, 11-HETE at 1 hr or 15-HETE at 30 min-1 hr. The recovery of unmetabolised AA was significantly increased at 30 min-2 hr as was also demonstrated with 10μM

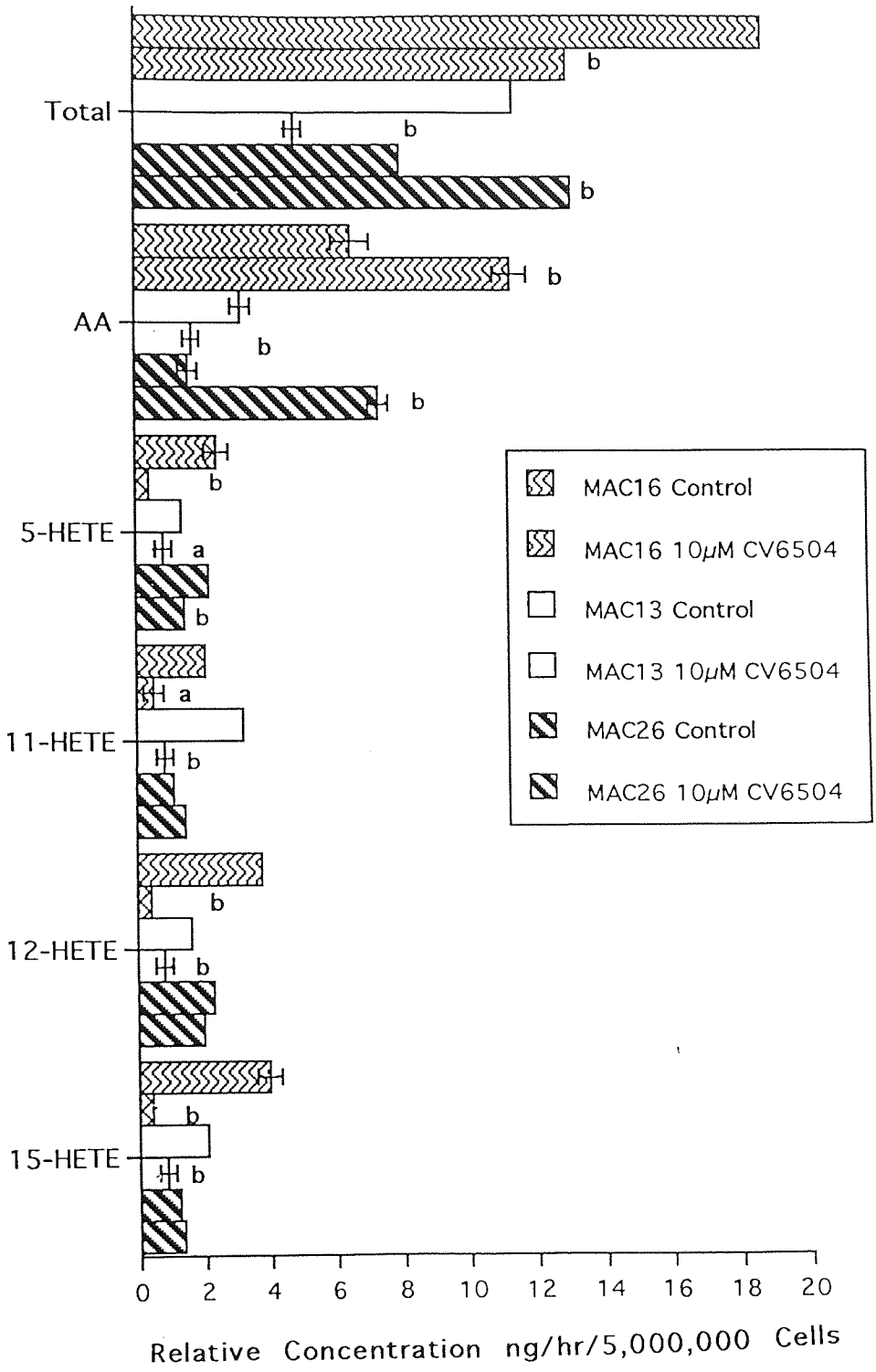


Figure 44. The effect of 10μM CV6504 on arachidonic acid (AA) metabolism in MAC tumour homogenates (n=4). (a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test.)

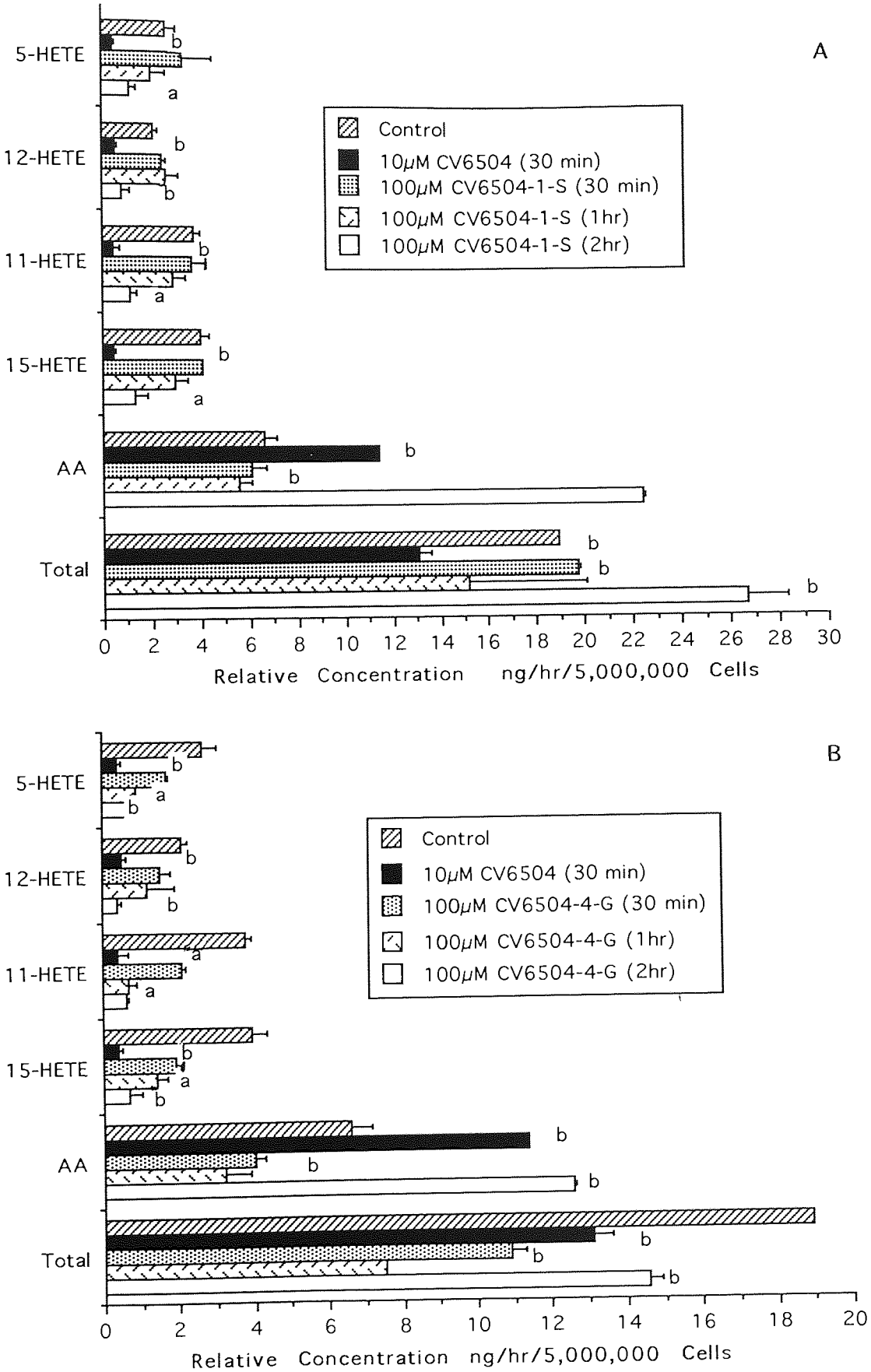


Figure 45. The effect of 10μM CV6504 or 100μM of CV6504 metabolites on the arachidonic acid (AA) metabolism in MAC16 tumour homogenates (n=4).

(a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test.)

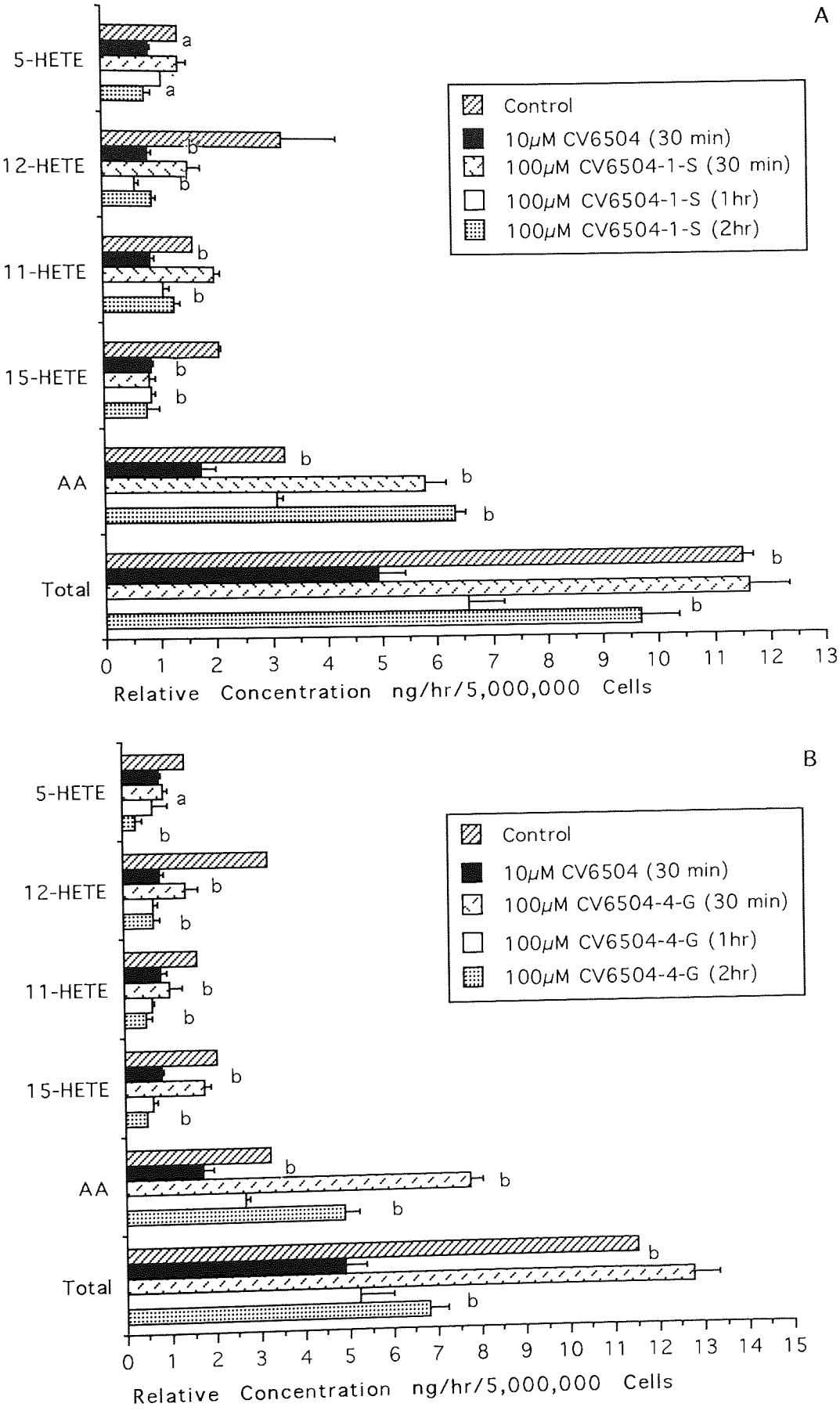


Figure 46. The effect of 10μM CV6504 or 100μM of CV6504 metabolites on the arachidonic acid (AA) metabolism in MAC13 tumour homogenates (n=4).

(a= $p<0.01$ and b= $p<0.05$ from Control using two-way ANOVA followed by Tuckey's Test.)

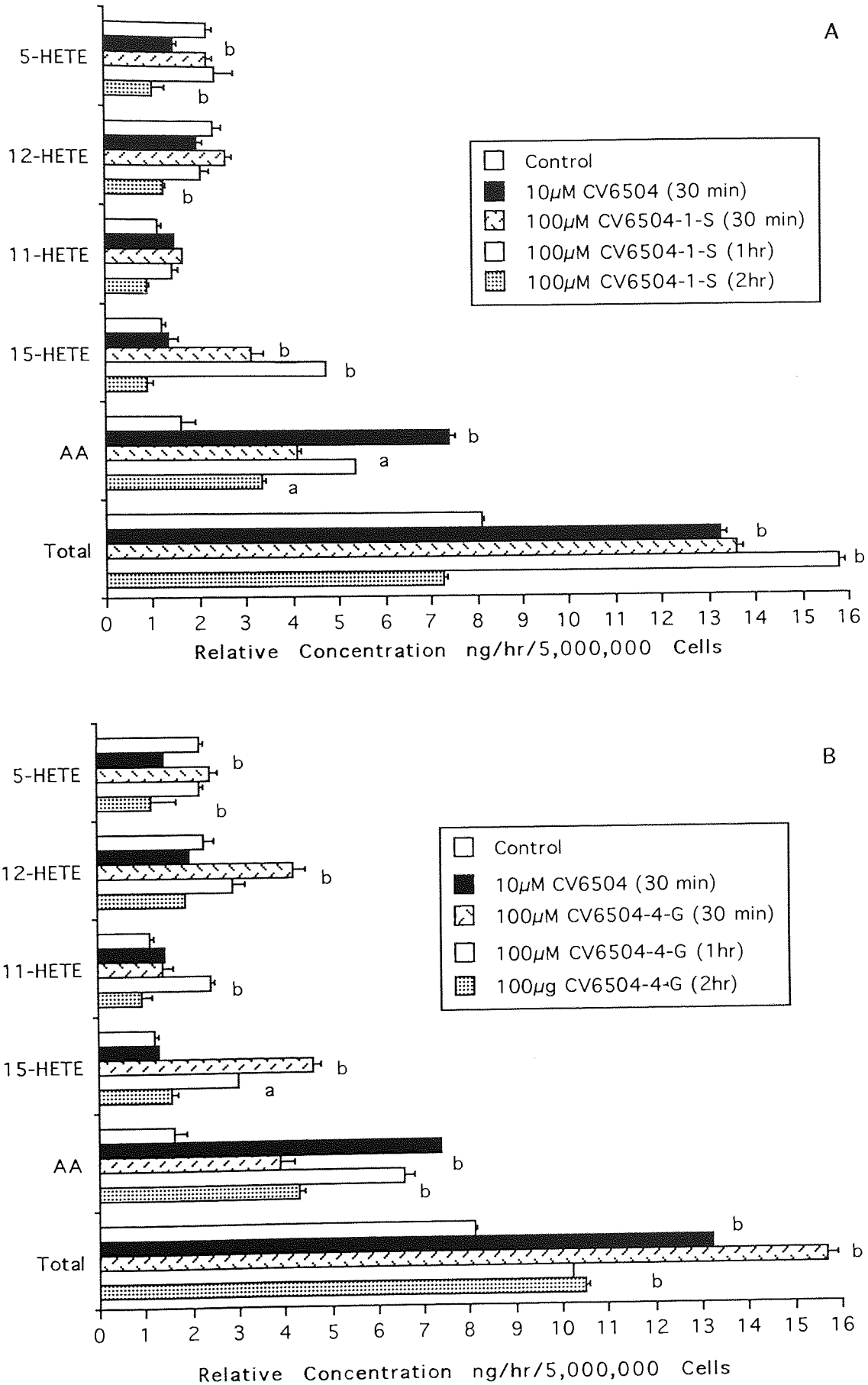


Figure 47. The effect of 10μM CV6504 and 100μM of CV6504 metabolites on the arachidonic acid (AA) metabolism in MAC26 tumour homogenates (n=4).

(a= $p<0.05$ and b= $p<0.01$ from Control using two-way ANOVA followed by Tuckey's Test.)

Table 8.
The IC_{50} values of CV6504 on the MAC13, MAC16, MAC26, A549 and Caco-2 *in vitro* determined by growth inhibition over 72 hr (n=9).

Cell Line	IC_{50} Value (μM)
MAC13	3 ± 1
MAC16	3 ± 1
MAC26	7 ± 1
A549	61 ± 1
Caco-2	5 ± 0

CV6504, as were total AA and HETEs recovered was increased at 30 min-2 hr.

. The IC_{50} values for CV6504 on the growth of MAC13, MAC16, MAC26, A549 and Caco-2 cells over 72 hr in normal serum levels are shown in table 8. The IC_{50} values calculated for A549 and Caco-2 cells were $61 \pm 1\mu M$ and $5 \pm 0\mu M$. Results calculated by Takeda Chemicals Ltd. (as shown in table 4.) were $85\mu M$ and $10\mu M$ respectively. These results suggest that either the cells used in these experiments, or the method of the IC_{50} evaluation, are more sensitive than those used or employed by Takeda Chemicals Ltd., though the relative activities of the cell lines calculated is compatible.

The effect of $10\mu M$ CV6504 on the AA metabolism in MAC13, MAC16, MAC26, A549 and Caco-2 cells measured by the recovery of [^{3}H]-AA is shown in figure 48. Caco-2 cells sensitive to inhibition by CV6504, showed a significant reduction in HETEs produced with a decrease in the unmetabolised AA recovered relative to control. At $10\mu M$ CV6504 had no effect on the relatively low production of HETEs within the A549 cells, or the recovery of unmetabolised AA. In MAC13, MAC16 and MAC26 cells the production of 5-HETE, 12-HETE and 15-HETE was reduced by CV6504. The unmetabolised AA recovered from MAC13 and MAC16 cells was significantly reduced, while in MAC26 cells it was significantly increased. This led to a significant reduction in the recovery of total HETEs produced and unmetabolised AA in MAC13 and MAC16 cells, but in MAC26 cells the concentration remained the same, when incubated with $10\mu M$ CV6504.

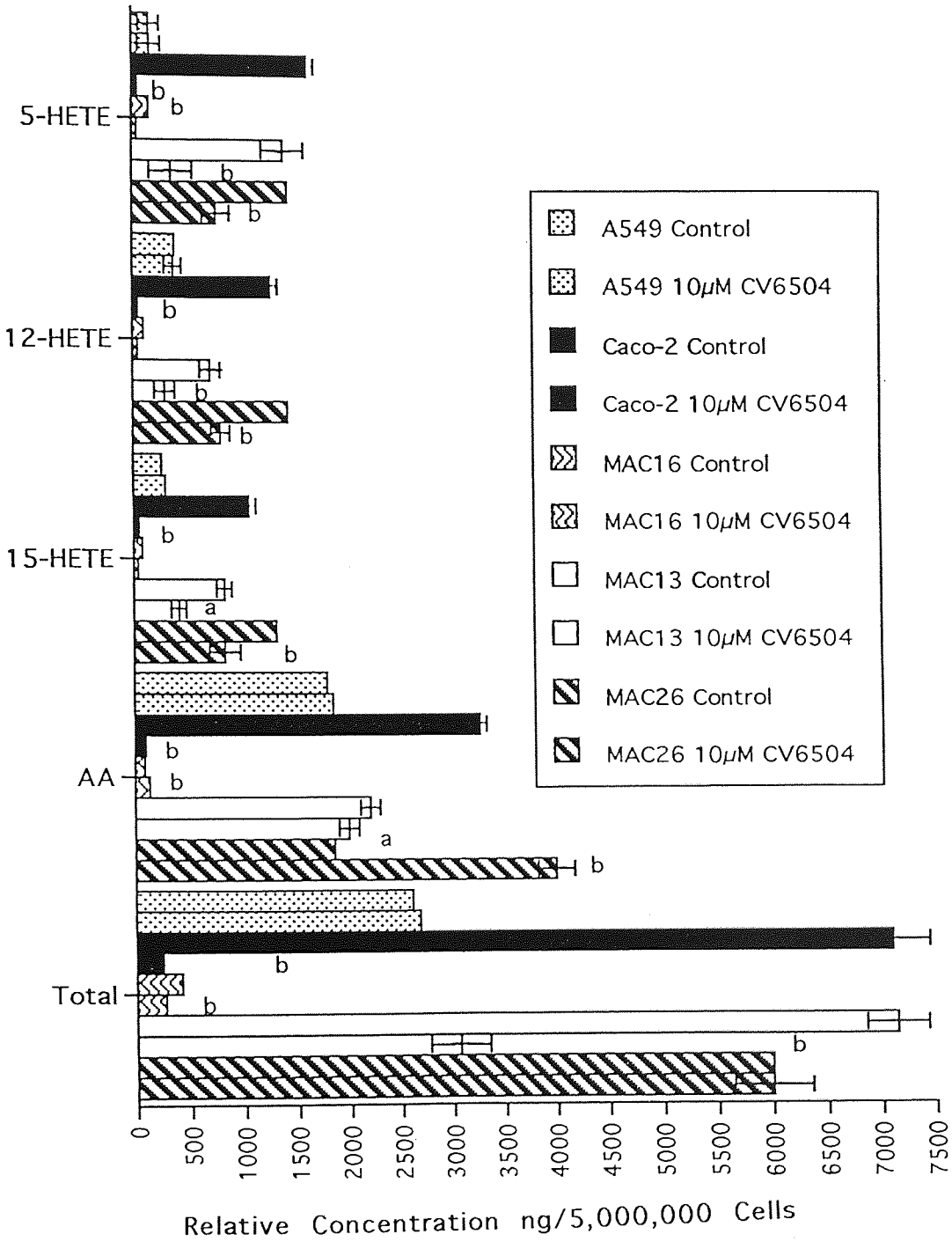


Figure 48. The effect of 10μM CV6504 on the metaobolism of arachidonic acid (AA) in MAC cells, in comparison to A549 and Caco-2 cells (n=4).
(a= $p<0.05$ and b= $p<0.01$ from Control, using two-way ANOVA followed by Tuckey's Test.)

6:3. Discussion.

CV6504 was found to have a profound antitumour action against MAC13, MAC16 and MAC26 tumours in NMRI mice (see chapter 5.), a series of established transplantable murine adenocarcinoma reported to be generally refractive to cytotoxic therapeutic agents (Bibby and Double 1989). The wide spectrum of antitumour action and a degree of safety (see chapter 5.) identified CV6504 as a potential agent for clinical investigation. The metabolism of CV6504 in NMRI tumour-bearing mice was first established, to determine whether this effect was due to metabolism within the tumour or to abnormal metabolism by this particular strain of mouse. Peak levels of CV6504 in both MAC16 tumour-bearing NMRI mice and the B63C3F1 strain resulting from a single oral dose of 10mg/kg CV6504 were reached by 0.25 hr. Though the clearance of this peak plasma level was reduced in MAC16 tumour-bearing mice by 2hr, both the concentration and relative proportion of CV6504 or metabolites were the same in both strains of mice. This reduction in the clearance rate of CV6504 in MAC16 tumour-bearing mice may have been due to the metabolic disturbances observed during cachexia.

Unmetabolised CV6504 and CV6504 metabolites were detected within the tumour after oral administration to MAC16 tumour bearing-mice. The recovery of CV6504 and metabolites from the tumour after 6 consecutive daily doses gave an estimated concentration within the tumour of 14.66μM, with the concentration of unmetabolised drug being calculated at 3.30μM, close to the IC_{50} value calculated for CV6504 in MAC16 cells *in vitro* 3 ± 1 (see chapter 4.). There was an accumulation of CV6504 and its metabolites within the tumour 2 hr after oral dosing or after multiple doses. This was particularly evident in the significantly increased levels of unmetabolised CV6504 recovered per gram of tissue relative to those recovered per gram of liver. At 24 hr after oral dosing and after consecutive daily dosing the relative concentration of unmetabolised drug to metabolised drug was increased, which was especially evident in the reduced % of glucuronide metabolites recovered. CV6504-glucuronides and CV6504-sulphates can be metabolised to unchanged CV6504 by the action of β-glucuronidase and sulphatases respectively (Takeda

Chemicals Ltd. unpublished results,) suggesting a role for these enzymes in the antitumour action of CV6504. *In vitro* the IC_{50} values of MAC13, MAC16 and MAC26 cells was 3 $\pm$ 1, 3 $\pm$ 1 or 7 $\pm$ 1μM respectively. CV6504-sulphates or CV6504-glucuronides had no effect on cell proliferation in these cell lines at concentrations below 100μM, suggesting they were inactive (as shown in table 5.) or activity was reduced compared with the parent drug. However, when these metabolites were dosed daily at 50mg/kg orally to MAC13 tumour-bearing NMRI mice, inhibition of tumour growth was observed. This inhibition was delayed and not as pronounced as that observed with 5mg/kg CV6504. These results again suggest that sulphatase and β-glucuronidase levels within the tumour or the host's tissues such as the liver were of importance in the antitumour action of CV6504. Initial studies into the β-glucuronidase and sulphatase activities of the MAC tumours has suggested levels of these enzymes present in the MAC13 and MAC16 tumours are compatible with those found in the liver (Tisdale 1995 unpublished results).

CV6504 was reported to have three effects *in vivo:* i. TXA_2 synthetase inhibition ii. 5-LO inhibition and iii. reduction of serum lipid peroxidation (Shibouta et al 1991). CV6504 reduced the serum lipid peroxide levels in MAC13, MAC16 and MAC26 tumour-bearing NMRI mice in a dose dependent manner (with the exception of 50mg/kg in MAC13 tumour-bearing mice). This did not correlate with antitumour activity, and due to the large areas of necrosis induced by CV6504 within the tumours, and the detection of lipid peroxide levels by TBA reacting-substances being a fluorometric method, lipid peroxides within the tumour could not be measured.

In the MAC16 tumour-bearing model in NMRI mice a lag period has been suggested whereby FAs are sequestered from plasma and liver of the host causing a drop in FA concentration. Production of lipolytic factors by the tumour then mobilise FA stores and release FAs into the plasma (Hudson et al 1993). A reduction of FA levels in the plasma was observed by treatment with CV6504 and seemed likely to be due to the inhibition of FA mobilisation from host tissue stores. The reduction in FAs correlated with both the antitumour and the anticachectic effect of CV6504. The inhibition of tumour growth would account for this reduction in weight loss, as lipid mobilising

factors produced by the tumour would be reduced. Alternatively CV6504 may interact directly with adipose tissue receptors to prevent the mobilisation of FA stores. Oral administration of 1.0g/kg LA daily to MAC16 tumour-bearing NMRI mice increased weight loss but not tumour growth, with an increased LA content in the plasma. In MAC16 tumour-bearing NMRI mice though increased FA levels associated with cachexia have been reported to be non-specific (Hudson 1993) an increase in the plasma concentration of AA has been reported both in this model and cancer patients at 10-15% weight loss (Hudson 1993). MAC13 and MAC26 tumour-bearing NMRI mice tolerated 1.0g/kg LA given daily orally for more prolonged periods of time than MAC16 tumour-bearing animals. The increased rate of weight loss could have been caused by metabolic disturbances induced in the cachectic state increasing the toxicity of LA, or LA increasing weight loss by increasing these metabolic disturbances. The increased weight loss induced by 1.0g/kg LA given to MAC16 tumour-bearing NMRI mice was reversed by the concurrent administration of 10mg/kg CV6504. This reduction in weight loss also correlated with a reduction in total plasma FAs observed by administration of 1.0g/kg LA alone, especially the elevated LA concentration.

A decrease in liver mass was observed as a result of MAC16 tumour-induced cachexia in NMRI mice (Hudson 1993, Mulligan and Tisdale 1991b). An inverse relationship between liver fatty acids and liver mass was shown, suggesting an ability for the liver to concentrate FAs released into the circulation in a nonspecific manner (Hudson 1993). At 10 and 25 mg/kg CV6504 significantly reduced the FA concentration of the liver in MAC16 tumour-bearing mice, suggesting a reduction in the concentration of FAs within the liver usually associated with cachexia. The levels of AA, LA and OA levels were significantly reduced in liver by 10 or 25mg/kg CV6504, which lead to an increase in both the SA:OA ratio and the Mead acid:AA ratio. The plasma SA:OA ratio has been shown to increase in parallel to the appearance of rodent tumours (Habib et al 1987a). It was suggested that OA became incorporated in the tumour cell membrane increasing the fluidity (Apostolov et al 1985, Habib et al 1987b). In some cachectic patients the SA:OA ratio has been found to decrease (Mosconi et al 1989), whilst in cachectic colonic cancer patients the SA:OA ratio was

increased (Neoptolemos et al 1991). The SA:OA ratio was also found to increase in the MAC16 tumour model (Hudson 1993). The reduction of LA levels within mammalian cells results in an increased Mead acid (20:3 n-9) to AA (20:4 n-6) ratio (Horrobin 1992). Desaturation of OA to form Mead acid is inhibited when levels of LA are normal. However, when LA levels become reduced OA desaturation takes place and Mead acid is formed. When MAC16 tumour-bearing mice were treated with 10 or 25mg/kg CV6504 there was a significant reduction in both AA and LA levels in the liver. Inhibition of OA desaturation and elongation would have been decreased resulting in an increased formation of Mead acid and accompanied by an increase in the SA:OA ratio. FA levels per gram of tumour were also reduced significantly by treatment of MAC16 tumour-bearing mice with CV6504. This reduction of FA levels was nonspecific and suggests that sequestering from host tissues was prevented.

When CV6504 was administered with 1.0g/kg LA there was a reduction in the antitumour action on the MAC13, MAC16 and MAC26 tumours *in vivo,* suggesting a role of LA in the antitumour action CV6504. LA is the dietary source of AA. AA metabolism was studied in tumour homogenates, and the relative antitumour action was found to correlate with the inhibition of 5-HETE, 11-HETE, 12-HETE and 15-HETE. Similar results were also obtained *in vitro* (see chapter 4.). CV6504 metabolites at higher concentrations were also capable of inhibiting the production of HETEs, producing an imbalance of LO eicosanoid products, compared to control tumour homogenates. The metabolites were required at higher concentrations and for prolonged periods of time in comparison to CV6504. These results suggest that either LO enzymes in tumour homogenates are more susceptible to inhibition by CV6504 metabolites in comparison to the LO enzymes contained within the MAC cells *in vitro,* or tumour homogenates contain enzymes such as sulphates or ß-Glucuronidase, capable of metabolising CV6504 metabolites into a more active form.

The inhibition of AA metabolism in MAC13, MAC16 and MAC26 cells is discussed in detail in chapter 4., where activity was found to correlate with the inhibition of AA metabolism and the imbalance of eicosanoids produced. A549 were relatively resistant to inhibition by CV6504 compared to the MAC cell lines, whereas activity in

Caco-2 cells was compatible with that in the MAC cells. AA metabolism studies showed a relatively low production of 5, 12 and 15-HETE in relation to unmetabolised AA in A549 cells. The HETE production and accumulation of unmetabolised AA was not effected by 10μM CV6504 in theses cells. In Caco-2 cells the production of HETEs was compatible to that of MAC cells. At concentrations of 10μM CV6504 was capable of significantly inhibiting 5-HETE, 12-HETE and 15-HETE production with a reduction in the accumulation of unmetabolised AA within these cells. The IC_{50} values of CV6504 on 5 cell lines therefore correlated with the extent of AA metabolism within these cell lines and the ability to inhibit this metabolism, suggesting that LO inhibition is the mechanism by which CV6504 inhibits cell proliferation *in vitro*.

Cell proliferation requires cells to pass through the cell cycle. After an initial gap phase (G_1) DNA replication takes place (S phase), followed by another gap phase (G_2), the DNA is then distributed equally into daughter cells (M-phase). Post transcriptional modification of proteins is mainly phosphorylation of serine, threonine and tyrosine residues mediated by protein kinases. Over 200 protein kinases have been identified amongst these the cyclin-dependent kinases (cdks). Cdks have raised considerable interest due to their essential role in cell proliferation and regulatory role in the cell cycle. Recent evidence has also shown a deregulation of these cdks in human tumour development especially with respect to cyclin D and E (Motokura and Arnold 1993). This frequently observed deregulation of cdks in human tumours has lead to the development of a screening system by the European Organisation for Research and Treatment of Cancer (EORTC). Antimitotic compounds with potential antitumour action are screened using purified cell cycle regulators as molecular targets. CV6504 was screened by EORTC for action against cdc2 kinase or cdc25 phosphatases, where it was found to be inactive. These results along with other evidence presented in this chapter strongly implicate the inhibition of LO and the subsequent imbalance of eicosanoids produced as the mechanism by which CV6504 exerts its antitumour action.

Chapter 7.
Conclusions.

7:0. Conclusions.

The effect of PUFAs on tumour cell proliferation was studied *in vitro* in section 4:1. The n-6 PUFAs AA and LA were found to promote cell proliferation in the MAC13 and MAC26 cell lines in reduced serum, an effect not seen with MAC16 cells. The FA profiles of the MAC13 and MAC26 cells suggested that these cell lines were FA deficient, whereas the MAC16 cells were not. GLA stimulated MAC13 cell growth, and to a lesser extent MAC26 cell growth, in reduced serum. A reduced stimulation of cell proliferation in the MAC13 cells was also supported by low concentrations of DHA and EPA in reduced serum levels, with maximum stimulation being observed at 3μM and 3.3μM respectively. MAC13 cells were very FA deficient, and it was thought that the stimulatory action of these n-3 PUFAs was due to their incorporation into cell membranes and increased fluidity, conferring an advantage on the processes required during proliferation. At higher concentrations PUFAs were cytotoxic to MAC13 and MAC26 cells *in vitro.* The IC_{50} values calculated were DHA 15μM, EPA 83μM, AA 90μM, GLA 93μM and LA 178μM. This correlated with both the degree of desaturation and the ability to generate lipid peroxides (Massotti et al 1988, Dianziana 1993). The IC_{50} values of PUFA on the MAC16 cell line were DHA 5μM, GLA 90μM and EPA 165μM which did not correlate with either the degree of desaturation or the ability to generate lipid peroxides. EPA was shown to have both an antitumour and an anticachectic effect on MAC16 tumour-bearing NMRI mice, an effect which was not displayed by DHA (Beck et al 1991, Hudson 1993). This suggested that the effect of PUFAs on the MAC16 cells *in vitro* was not representative of MAC16 tumours *in vivo.*

LA was found to promote MAC26 tumour growth when given orally as a free acid to NMRI tumour-bearing mice (section 5:1). A threshold value for the stimulation of tumour growth at 0.4g/kg/day was observed, above and below which there was no further stimulation of tumour growth. Maximum stimulation of MAC26 tumour growth was achieved when LA represented 3.8% of total calories, which suggest that at the 6% of total calories recommended by the British Nutrition Task Force in 1992, there may all ready be maximum stimulation of tumour growth. The promotion of the

MAC26 tumour growth by LA, was shown by kinetic studies to be due to an increase in tumour cell proliferation with no effect on cell loss. This increased proliferation was shown by autoradiography to be uniform throughout the tumour sections. Administration of LA in the triglyceride form as arachis oil, stimulated MAC26 tumour growth only when administered over a prolonged period of time, i.e. from the day after transplantation of the tumour rather than from when the tumour became palpable. When LA was given as a free acid or a triglyceride from the day after transplantation of the MAC26 tumour increased growth of the tumour was accompanied by adequate neovascularisation with a small increase in vascular volume per gram of tumour.

The effect of LO inhibitors and indomethacin on the proliferation of the MAC13, MAC16 and MAC26 cells *in vitro* was investigated in section 4:2. The activity of Zileuton a specific 5-LO inhibitor and L-660,711 a LTD_4 receptor antagonist, was low in comparison to the LO inhibitors BWA4C BWB70C and CV6504. 10μM BWA4C or 10μM CV6504 were capable of inhibiting the production of 5-HETE, 12-HETE and 15-HETE, whereas Zileuton had minimal action on the production of 5-HETE in MAC cells *in vitro*. The addition of 5, 12 or 15-HETE alone to MAC26 cells inhibited by 5μM BWA4C or 10μM CV6504 did not restore normal cell growth. These results suggested that there was a requirement for a balance of eicosanoids to be produced through the LO pathways for MAC26 cell proliferation. The effect of BWB70C at 10μM on the inhibition AA metabolism in the MAC cells was investigated and it was found that 5-HETE production was reduced in preference to 12-HETE and 15-HETE. BWA4C, BWB70C and CV6504 are all capable of inhibiting LO by the reduction of the Fe^{3+} at the active site of the LO enzyme to Fe^{2+} (Ohkawa et al 1991b, Salmon and Garland 1991) suggesting that this mechanism of action is also of importance in the inhibition of tumour cell proliferation through LO inhibitors.

Indomethacin was capable of inhibiting MAC cell proliferation *in vitro* (section 4:2) and the growth of the MAC26 tumour *in vivo* (section 5:2). The effect of indomethacin at 10μM on AA metabolism was investigated in MAC cells where it was found to inhibit the production of 5-, 12- and 15-HETE. The IC_{50} value of

indomethacin on these cells lines was 3 times as high as this, suggesting that at the concentrations required to inhibit MAC cell growth indomethacin was inhibiting metabolism through the LO pathways. These results suggest that MAC cell growth *in vitro* was more sensitive to LO inhibitors than CO inhibitors. Indomethacin was capable of reversing the stimulatory effect of LA on the growth of the MAC26 tumour, and inhibiting tumour growth below that of the control. Indomethacin when administered alone inhibited MAC26 tumour growth, but at 5.0mg/kg/day given ip was toxic to tumour-bearing NMRI mice.

The effect of the more active LO inhibitors *in vitro* BWB70C, BWA4C and CV6504 on the growth of the MAC tumour *in vivo* was investigated in section 5:2. BWA4C had antitumour activity on the MAC26 tumour which was not dose dependent between 5-25mg/kg/12hr. When administered with BWA4C, LA did not reverse the antitumour effect, but antitumour action became dose dependent between 5-25mg/kg/12hr. At 25mg/kg/12hr BWB70C administered orally in liquid paraffin inhibited the growth of the MAC26 tumour in NMRI mice, while at 10mg/kg/12hr it had no effect, and at 5mg/kg/12hr it increased the growth rate. LA did not reverse the antitumour action of 25mg/kg/12hr BWB70C, and at doses of 5 or 10mg/kg/12hr tumour growth was increased, but LA stimulation of tumour growth was reduced. BWB70C at 10μM was shown to preferentially inhibit the production of 5-HETE in the MAC cells *in vitro.* If the same mechanism of action was taking place within the MAC26 tumour *in vivo* the production of 5-HETE would be inhibited, whilst the relative concentration of 12-HETE or 15-HETE were increased or remained the same. This imbalance of eicosanoids could have increased tumour growth, again demonstrating the importance of LO eicosanoid balance in MAC tumour cell proliferation.

Liquid paraffin had no effect on the growth of either the MAC16 or MAC26 tumour. There was, however, a reduction in weight loss and an increased survival over saline treated controls when liquid paraffin was administered every 12 hr to MAC16 tumour-bearing NMRI mice. Liquid paraffin consists of a mixture of long chain hydrocarbons with BHT or α-tocopherol added as stabilisers. BHT and α-tocopherol are also antioxidants which were capable of reducing lipid peroxide levels *in vivo.*

This suggested a role for lipid peroxidation in MAC16 tumour induced cachexia. When BWA4C was administered orally in liquid paraffin to MAC16 tumour-bearing mice every 12 hr, there was an antitumour effect. Weight loss in MAC16 tumour-bearing mice has been shown to be increased with tumour growth (Hudson 1993). The antitumour effect of BWA4C was not accompanied by a reduction in tumour induced weight loss suggesting that BWA4C was toxic, as any reduction in weight loss was being counteracted by the weight loss produced by BWA4C toxicity.

The results presented in section 5:2 demonstrated that MAC26 tumour growth is sensitive to LO inhibition. A balance of these LO derived eicosanoids is required for tumour proliferation, with 12-LO and 15-LO derived eicosanoids playing a major role. The potential for LO inhibitors as antitumour agents against these tumour models was further demonstrated by the profound antitumour activity of CV6504. In MAC16 tumour-bearing mice maximum antitumour action was observed between 10-25mg/kg/day, accompanied by a reduction in the tumour induced weight loss. Histological examination of treated tumours showed that CV6504 had induced large areas of necrosis, with only a few viable cells being present around the intact vasculature. This all became encapsulated in a fibrous capsule the presence of which may have prevented further tumour regression. Maximum antitumour action on the MAC13 tumour was observed between 5-10mg/kg. Histological examination of these tumours again showed large areas of necrosis, fibrosis and lymphocytic infiltration induced by CV6504. The optimal concentration for antitumour activity against the MAC26 tumour was observed at 25mg/kg/day CV6504 . Chronic toxicity has not been observed in male mice below 100mg/kg/day (Takeda Chemicals unpublished results) this suggests that CV6504 has a therapeutic index against these generally chemoresistant tumours of at least 4-20.

The mechanism of action and the metabolism of CV6504 was investigated more extensively in chapter 6. The antitumour and anticachectic action of CV6504 was demonstrated in MAC16 tumour-bearing female NMRI mice. The antitumour effect of CV6504 in male MAC16 tumour-bearing mice was partially reversed by LA. However daily oral administration of LA as a free acid at 1.0g/kg was poorly tolerated by

MAC16 tumour-bearing NMRI mice and increased weight loss leading to a decrease in survival. This was due to either the metabolic disturbances induced by the cachectic state increasing the toxicity of LA, or LA itself speeded up the metabolism and metabolic disturbances induced by cachexia. This increased weight loss could be reversed by CV6504. The antitumour effect of CV6504 on the MAC26 tumour at 25mg/kg/day was also partially reversed by LA. In the MAC13 tumour the partial reduction of the antitumour action of CV6504 by LA at 5mg/kg/day was prevented by increasing the dose to 10mg/kg/day. LA is the dietary source of AA. The effect of 10μM CV6504 on the AA metabolism of tumour homogenates was investigated. The inhibition of 5-LO, 12-LO or 15-LO in MAC13 or MAC16 tumour homogenates was very similar, with a reduced inhibitory effect being observed in MAC26 homogenates. This correlated with antitumour action of CV6504 against the tumours *in vivo*. Results obtained in section 4:2, showed the ability to inhibit the 5-LO, 12-LO and 15-LO pathways in MAC13, MAC16 or MAC26 cells correlated with the IC_{50} values of CV6504. Furthermore in two more cell lines , A549 and Caco-2, the sensitivity to CV6504 correlated with both the extent to which the LO pathways were active and the inhibition of these pathways.

Serum lipid peroxide levels were reduced in MAC13, MAC16 or MAC26 tumour-bearing animals in a virtually dose dependent manner by CV6504, though this did not correlate with antitumour activity. MAC16 tumour-bearing animals showed reduced FA levels per ml of plasma, per gram of liver and per gram of tumour after 7 consecutive doses with 10mg/kg or 25mg/kg orally of CV6504. During tumour induced cachexia the liver has been shown to concentrate FAs even though it becomes reduced in size (Hudson and Tisdale 1993). These FAs are sequestered by the tumour causing an elevation in the plasma levels and tumour levels (Hudson 1993). The reduced levels observed after treatment with CV6504 in the liver, tumour and plasma suggest that this process was inhibited. The inhibition of tumour growth and the large areas of necrosis induced by CV6504 would reduce the lipid mobilising factors produced by the tumour leading indirectly to a reduction in weight loss.

Alternatively CV6504 may act directly with host tissues such as adipocytes to prevent the mobilisation of FAs.

One possible explanation for the profound antitumour action of CV6504 was that the compound was metabolised abnormally in tumour-bearing NMRI mice. Peak plasma levels after a 10mg/kg oral dose of CV6504 in MAC16 tumour-bearing mice and B63C3FI mice were at 0.25 hr. Though the clearance of CV6504 and metabolites was slightly inhibited in MAC16 tumour-bearing NMRI mice, by 2 hr both the concentration and relative proportions were the same in both strains of mice. Metabolic studies revealed that within the MAC16 tumour environment *in vivo,* concentrations of unchanged CV6504 could be reached within 6 doses, that were similar to IC_{50} values calculated for the cells *in vitro.* The ratio of unchanged CV6504 to CV6504 metabolites increased in the liver and tumour after 24 hr, and especially after 6 consecutive daily doses. CV6504 metabolites were inactive as growth inhibitors against the MAC13, MAC16 and MAC26 cell lines *in vitro* . *In vivo* CV6504 metabolites had antitumour action against the MAC13 tumour, though this action was delayed and not as pronounced as CV6504. These results strongly suggest a role for ß-glucuronidase or sulphatase, within the tumour and hosts tissues, in the antitumour action of CV6504. The investigation of CV6504 metabolites on the AA metabolism in tumour homogenates showed an imbalance in LO eicosanoids produced, although this was established over a longer period of time and at a higher concentration than those observed for CV6504. This suggested either the tumour LO enzymes were more resistant to inhibition by CV6504 metabolites, or the metabolites were metabolised into unchanged CV6504.

This study has shown a requirement for LO eicosanoids in three transplantable murine colon adenocarcinoma models both *in vitro* and *in vivo.* The study of AA metabolism within these cells and tumour homogenates has shown that antitumour action of more active LO inhibitors is not restricted to the 5-LO enzyme but involves the inhibition of 12-LO and 15-LO enzymes. This suggests that eicosanoids produced by 12-LO and 15-LO pathways are also important in the regulation of tumour cell proliferation. CV6504 a redox LO inhibitor, inhibited both tumour cell proliferation

in vitro and had profound antitumour action *in vivo*, against three tumour models usually refractive to cytotoxic agents. This antitumour activity is achieved with a degree of safety and appears to correlate with the ability to inhibit LO eicosanoid production in tumour homogenates. CV6504 has been proposed for stage I/II clinical trials against pancreatic cancer by the Cancer Research Campaign. Trials are scheduled to start in April 1996. This will be the first lipoxygenase inhibitor entering the clinic as a therapeutic agent.

Chapter 8.
References.

8.0. References.

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Appendix.

9:0. List of Publications.

Papers.

HUSSEY H J, BIBBY M C and TISDALE M J (1996): Novel antitumour activity of 2,3,5-trimehtyl-6-(3-pyridylmethyl)-1,4-benzoquinone (CV6504) against established murine adenocarcinomas (MAC). *Br. J. Cancer* In Press.

HUSSEY H J and TISDALE M J (1994): Effect of polyunsaturated fatty acids on the growth of murine adenocarcinomas *in vitro* and *in vivo. Br. J. Cancer* 70 6-10.

Abstracts.

HUSSEY H J and TISDALE M J (1996): Antitumour action of CV6504 in relation to arachidonic acid metabolism. Accepted for presentation at British Association for Cancer Research, 37TH Annual Meeting (Sunday 31st March-Wednesday 3rd April 1996).

HUSSEY H J and TISDALE M J (1996): Antitumour action of a bioreductive lipoxygenase inhibitor against murine colon adenocarcinomas. Accepted for presentation at 9th NCI-EORTC, Symposium on New Drugs in Cancer Therapy (March 12-15th 1996).

HUSSEY H J and TISDALE M J (1995): *In vivo* antitumour activity of a lipoxygenase inhibitor CV6504. *Br. J. Cancer* 7 1 Supplement XXIV p40.

HUSSEY H J AND TISDALE MJ (1994): Effect of n-6 polyunsaturated fatty acids on tumour growth. *Br. J. Cancer* 6 9 Supplement XXI p35.

HUDSON E A, HUSSEY H J, BECK S A, WYNTER M P and TISDALE M J (1993): Requirements for (n-6) polyunsaturated fatty acids in tumour growth. *Br. J. Cancer* 6 7 Supplement XX p49.

Br. J. Cancer (1994), 70, 6-10

Effect of polyunsaturated fatty acids on the growth of murine colon adenocarcinomas *in vitro* and *in vivo*

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Paper No. 4318

British Journal of Cancer (1996) 73, 0000-0000

Novel anti-tumour activity of 2,3,5-trimethyl-6-(3-pyridylmethyl)-1,4-benzoquinone (CV-6504) against established murine adenocarcinomas (Mac)

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ANTITUMOUR ACTION OF CV6504 IN RELATION TO ARACHIDONIC ACID METABOLISM, H. J. Hussey and M. J. Tisdale, Pharmaceutical Sciences Institute, Aston University, Birmingham.

Antitumour Action of a Bioreductive Lipoxygenase Inhibitor Against Murine Colon Adenocarcinomas

H.J. Hussey and M.J. Tisdale, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET, U.K.

IN VIVO ANTITUMOUR ACTIVITY OF A LIPOXYGENASE INHIBITOR (CV-6504),

H. J. Hussey* and M. J. Tisdale, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET.

EFFECT OF N-6 POLYUNSATURATED FATTY ACIDS ON TUMOUR GROWTH, H.J.Hussey, M.J.Tisdale, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET.

Arachidonic acid (AA)

REQUIREMENTS FOR (n-6) POLYUNSATURATED FATTY ACIDS IN TUMOUR GROWTH.

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CRC Experimental Chemotherapy Group, Pharmaceutical Sciences Institute,
Aston University, Birmingham.