Conjugated Linoleic Acid (CLA) Isomers as Anticancer Lipids: Analysis, bioformation and mechanisms of action in the HT-29 human colon cancer cell line

Ph.D. thesis

By

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Declaration

This thesis is submitted in fulfilment of the requirements for Doctor of Philosophy, by research and thesis. Except where otherwise acknowledged, this work was carried out by the author alone, on a full time basis between October 2001 and July 2006 at the School of Biotechnology, Dublin City University.

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Abstract

Title Conjugated Linoleic Acid (CLA) Isomers as Anticancer Lipids Analysis, bioformation and mechanisms of action in the HT-29 human colon cancer cell line

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Conjugated linoleic acid (CLA), a group of polyunsaturated fatty acids occurring naturally in dairy products but also produced by certain strains of human intestinal *bifidobacteria* is known to exhibit potent anticancer effect both *in vivo* and in a range of tumour epithelial cell lines The HT-29 human colon cancer cell line was used in this study as an *in vitro* model to investigate the effects of CLA and *trans*-vaccenic acid (*t*-VA), a putative precursor of *c*9, *t*11 CLA on markers of growth, differentiation and apoptosis Sodium butyrate, which maintains a balance between cell proliferation, differentiation and apoptosis in intestinal epithelium was used as positive control For comparative purposes, parallel experiments were performed with linoleic acid HT-29 cells were sensitive to the growth inhibitory effects of a CLA mixture of isomers and to three of its constituent isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA at physiological levels *t*-VA was cytotoxic to the HT-29 cells at concentrations greater than 70μM and was less inhibitory than CLA treatments The CLA mixture of isomers, *c*9, *t*11 CLA and *t*10, *c*12 CLA showed evidence of apoptosis of HT-29 cells as reflected by annexin binding, measured by flow cytometry All CLA isomers induced carcinoembryonic antigen (CEA) and showed varying levels of reduction in histone deacetylase (HDAC) activity Increased level of ceramide was observed when cells were incubated with the CLA mixture of isomers In this study the gas chromatographic methods for analysis of CLA and *t*-VA in HT-29 cancer cells was validated This study provided evidence for cellular bioconversion of *t*-VA to *c*9, *t*11 CLA in HT-29 cells CLA isomers altered fatty acid composition in HT-29 cells which may be via modulation of fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD) activities This study indicated that the antiproliferative effect of CLA on HT-29 colon cancer cell line may be mediated by differentiation and apoptosis and by modulation of FAS and SCD activities

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Abbreviations

AA	arachidonic acid
ABB	Annexin binding buffer
ACF	aberrant crypt foci
AICR	American Institute for Cancer Research
ALA	α-linolenic acid
ALP	alkaline phosphatase
APC	Adenomatous polyposis coli
ATCC	American type culture collection
ATM	Ataxia-telangiectasia
BF-3	Boron triflouride
BRCA-1	Breast cancer gene 1
BRCA-2	Breast cancer gene 2
BSA	Bovine serum albumein
BSS	Balanced salt solution
c	*cis*
CD	conjugated diene
CDK	cyclin dependent kinases
CDKIs	cyclin dependent kinase inhibitors
CEA	Carcinoembryonic antigen
CHK-2	Cell cycle checkpoint kinase
CLA	conjugated linoleic acid
CO_2	carbondioxide
COX	cyclooxygenase
CYP450	Cytochrome, P450
DAG	diacylglycerol
DGLA	Dihomo-γ-linoleic acid
dH_2O	Distilled water
DHA	docosahexaenoic acid
DMBA	7,12-dimethyl-benz[a]anthracene
DMEM	Dulbecco's Minimum Essential Medium
DMH	Dimethylhydrazine
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbant assay
ENCR	European Network of Cancer Registries
EPA	eicosapentaenoic acid
ER	Estrogen receptor
ERKs	Extracellular signal regulated kinases
FA	Fatty acid
FACS	Fluorescence activated cell sorting
FAME	fatty acid methyl ester

FAP	Familial adenomatous polyposis
FAS	Fatty acid synthase
FCS	Fetal calf serum
FFA	Free fatty acid
FID	flame ionisation detector
FITC	fluorescein
FTIS	Farnesyl transferaseinhibitors
G1	Growth 1 phase
G2	Growth 2 phase
GAPs	GTPase activating proteins
GC	Gas chromatography
GLA	γ-linolenic acid
HCL	Hydrochloric acid
HDAC	Histone deacetylase activity
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HER-1	Human epithelial growth factor receptor 1
HER-2	Human epithelial growth factor receptor 2
HIC-1	Hypermethylated in cancer-1
HNPCC	Hereditary nonpolyposis colorectal cancer
HPLC	high performance liquid chromatography
HRG	heregulin
IGF	insulin-like growth factors
IMS	Industrial methylated spirit
IP3	inositol triphosphate
IQ	2-amino-3-methyl-imidazo[4,5-f]-quinoline
KAI-I	Kangai-1
LA	linoleic acid
LCSFA	Long chain saturated fatty acid
M	Mitosis
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MEK	MAP kinase kinase
MeOH	Methanol
MMR	Mismatch repair
mRNA	messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MUFA	Monounsaturated fatty acid
NaCL	Sodium chloride
$NaHCO_3$	Sodium-bi-carbonate
NaOH	Sodum hydroxide
NL	Neutral lipid
OPA	O-phthalldehyde
PBS	phosphate buffered saline
PBST	PBS containing tween 20
PCNA	Proliferating cell nuclear antigen

PG	prostaglandin,
PGE_2	prostaglandin E_2
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PI	Propidium iodide
PI3K	Phospinositide-3-kinase
PKB	protein kinase B
PKC	protein kinase C
PL	phospholipid
PMS	Phenazine methosulfate
PMSF	Phenyl methyl sulfonyl fluoride
pNP	p-nitrophenol
pNPP	p-nitrophenyl phosphate
PPAR	peroxisome proliferator-activated receptor
pRb	retinoblastoma protein
PS	phosphatidylserine
PTEN	Phosphatase and tensinhomolog
PUFA	polyunsaturated fatty acid
Rb	retinolastoma
RNA	Ribonucleic acid
SCD	Steroyl CoA desaturase
SCID	severe combined immuno deficient mice
SD	Standard deviation
SM	sphingomyelin
SP1	Sphingosin 1
SP2	Sphingosin 2
SPneat	$Sphingosin_{neat}$
TG	triglyceride
TSFA	Total saturated fatty acid
t	*trans*
t-VA	trans-vaccenic acid
WCRF	World Cancer Research Fund
WHO	World Health Organisation

Units

^{0}C	centigrade
g	Gram
h	Hour
L	Liter
mg	milligram
min	Minute
Ml	Milliliter
mM	Milli molar
mmol	Milli mole
mol	mole
mU	Milliunite
ng	nanogram
pg	Picagram
pmol	Picamole
rpm	Revolutions per minute
v/v	Volume per volume
w/v	Weight per volume
μg	Microgram
μmol	Micromol
μM	Micromolar

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CHAPTER 1

General Introduction

1.0 Overview

Colorectal cancer is one of the most common malignancies in the western world Although surgical excision is the best option for treatment, many patients who undergo therapeutic resection will develop tumor recurrences Therefore, there is increasing urgency to develop strategies to prevent this disease The role of diet in the development and prevention of cancer has been the focus of much scientific research during the past decade Evidence suggests that dietary fats are associated with risk of colorectal cancer Fats are adversely implicated in the etiology of many cancers, yet evidence is accumulating that certain fatty acids, such as the highly polyunsaturated n–3 fish oil fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have potential anticancer activity Recent development in the understanding of diet in colon cancer etiology has raised expectations that this increasing knowledge might lead to improved cancer prevention In this regard, the identification of dietary factors that can prevent colon cancer would show particular promise More recently, anticancer activity was demonstrated for conjugated linoleic acid (CLA) in both human tumor cell lines and in well-accepted rodent models of carcinogenesis Conjugated linoleic acid (CLA) is a group of polyunsaturated fatty acids found in diary products, beef, and lamb *In vitro* and experimental animal studies document a growing number of potential health benefits for CLA Not only is CLA a powerful anticarcinogen but it also has been reported to have anti-atherogenic, immunomodulating, growth promoting, anti-diabetic and anti-obesity properties The challenge now is to determine the effects of CLA in human subjects and to identify the specific physiological mechanism(s) by which different CLA isomers exert their unique biological effects Such knowledge will accelerate the development of CLA-enriched dairy foods, such as milk, butter, cheese and yoghurt Consumption of such natural products may produce a natural chemopreventive effect, without the additional cost of oral supplements or the need for disturbing dietary changes

The aim of this chapter is to present the evidence for the anticancer activity of CLA and to provide a comprehensive background to the research work contained in this thesis

1.1 Cancer

Cancer is a group of more than 100 different diseases. They affect the body's basic unit, the cell. Cancer occurs when cells become abnormal and divide without control or order. Normally, cells divide to produce more cells only when the body needs them. If cells keep dividing when new cells are not needed, a mass of tissue forms. This mass of extra tissue, called a growth or tumor, can be benign or malignant. Benign tumors are not cancer. They can usually be removed and, in most cases, they do not come back. Most important, cells from benign tumors do not spread to other parts of the body. Benign tumors are rarely a threat to life. Malignant tumors are cancer. Cancer cells can invade and damage tissues and organs near the tumor. Also, cancer cells can break away from a malignant tumor and enter the bloodstream or lymphatic system. This is how cancer spreads from the original (primary) tumor to form new tumors in other parts of the body. The spread of cancer is called metastasis. When cancer spreads to another part of the body, the new tumor has the same kind of abnormal cells and the same name as the primary tumor. For example, if colon cancer spreads to the liver, the cancer cells in the liver are colon cancer cells. The disease is metastatic colon cancer (it is not liver cancer). [www.medicinenet.com/colon_cancer/index.htm]

At the beginning of the third millennium, cancer remains the second leading cause of death in the developed world (Zhang, 2002). A total of 1,372,910 new cancer cases and 570,280 deaths are expected in the United States in 2005 (Jemal *et al.*, 2005). There were an estimated 2.6 million new cases of cancer in Europe in 1995, representing over one-quarter of the world burden of cancer. The corresponding number of deaths from cancer was approximately 1.6 million. After adjusting for differing population age structures, overall incidence rates in men were highest in the Western European countries (420.9 per 100 000), with only Austria having a rate under 400. Eastern European men had the second highest rates of cancer (414.2), with extremely high rates being observed in Hungary (566.6) and in the Czech Republic (480.5). In contrast to men, the highest rates in women were observed in Northern Europe (315.9) and were particularly high in Denmark (396.2) and the other Nordic countries excepting Finland. Deaths from cancers of the colon and rectum (189 000) ranked second, followed by deaths from stomach

cancer (152 000), which due to poorer survival ranked higher than breast cancer (124 000) (Bray *et al*, 2002)

In Ireland there are over 19000 new cases of cancer reported each year, with over 11000 cancer deaths This excludes 5800 cases of non-melanoma skin cancer, which are rarely life-threatening The risk of cancer increases markedly with age Age-specific rates of incidence for men and women aged between 80 to 84 y are two to three times higher than rates for those aged 60 to 64 y, and rates for men and women aged 60-64 y are roughly four to ten times higher than for the 40-44 y age group Given the fact that more and more people are living well past 65 y, the number of cancer cases is sure to continue to rise However, risk factors other than age are modifiable It is known that, approximately one third of all cancers are caused by tobacco, one third by diet (high fat/low fruit and vegetables), and most of the remaining third by other lifestyle choices such as excessive drinking, lack of regular exercise, sexual and reproductive patterns, and frequent sunburns Occupational exposures account for the remaining cancer risk, while the final and very small-outstanding proportion of risk relates to toxins in the environment (Campo *et al*, 2004)

A recent report by the Irish Cancer Registry revealed that mortality rates are higher for both men and women in Ireland than in the US even though the incidence is lower Men have higher incidence (20%) and mortality (40%) rates than women in Ireland However, while the rates for men are equivalent to those in the EU, for women in Ireland the incidence and mortality rates are significantly higher than in the EU (Campo *et al*, 2004) This highlights the scope for improvement in translational research, interdisciplinary and inter-institutional collaboration and communication promoting a free-flow of information and new treatments from the laboratory bench to the patient's bedside

1.2 Genetic alterations of the cell and development of cancer

It is now widely accepted that carcinogenesis is a multistep process characterized by genetic alterations in cells that influence key cellular pathways involved in growth and development (Fig 1 1) (reviewed in Osborne *et al*, 2004 and Yokota, 2000) For a normal cell to transform to a fully malignant cell, a number of specific genes need to be mutated Each mutation alone or in combination with other mutations render one or more malignant phenotypes (Fig 1 2) (reviewed in Fukasawa, 2005) Upregulation or downregulation of some genes is the basis of tumor initiation and progression

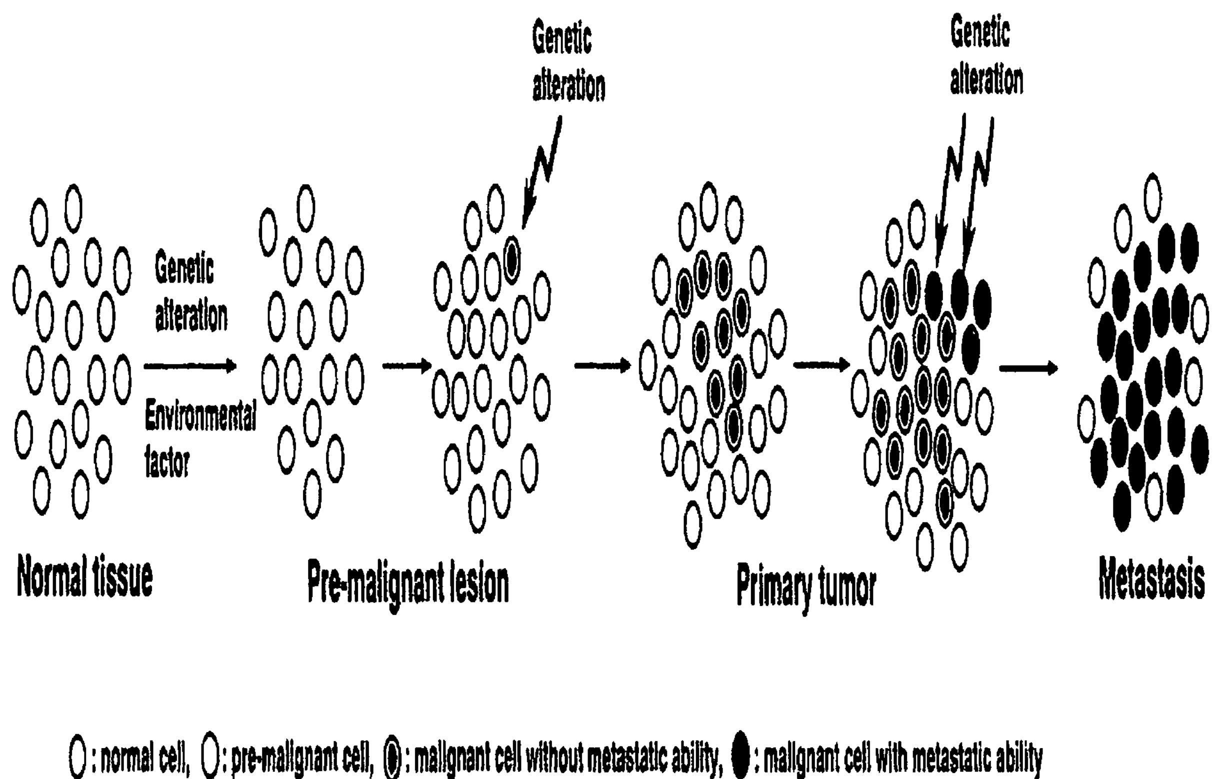

Figure 1.1 Stepwise malignant progression of human cancer in association with accumulation of genetic alterations in cells (Source Yokota, 2000)

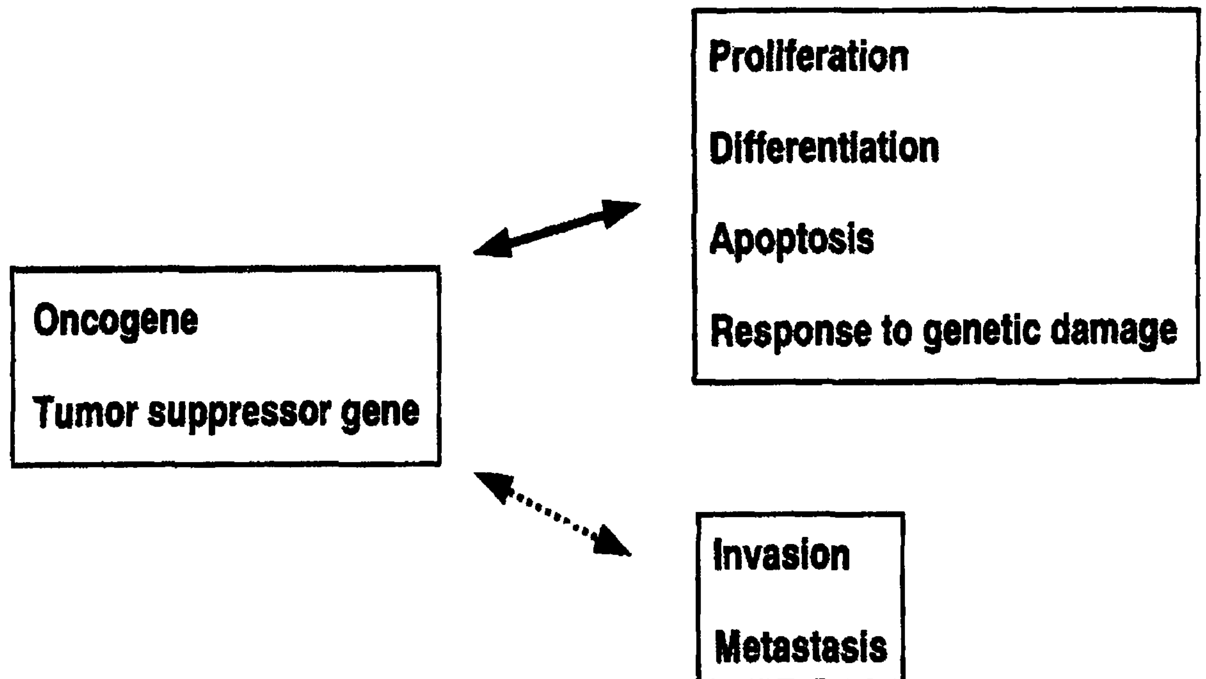

Figure 1 2 Crossroads between genetic and phenotypic alterations in human cancer (Source Yokota, 2000)

Genes commonly mutated in human cancer belong to one of three different classes oncogenes, tumor suppressor genes, and mismatch repair (MMR) genes (Weinberg, 1993, Chung and Rustgi, 1995) (Fig 1 3) Oncogenes and tumor suppressor genes are known to have functions to regulate proliferation, differentiation, apoptosis and responses to genetic damages (Yokota, 2000) Oncogenes refer to those genes whose alterations cause gain-of-function effects, while tumor suppressor genes cause loss-of-function effects that contribute to the malignant phenotype (reviw in Osborne *et al*, 2004) While the latter eliminates cancerous cells via apoptosis, the former enhances cell proliferation (review in El-Aneed, 2004) Oncogenes are normal genes responsible for the stimulation of controlled cellular proliferation (Sherr, 1996)

Numerous oncogenes have been characterized in human cancers. Amplification and overexpression of these oncogenes and oncogene products are the major mechanisms through which these genes participate in carcinogenesis (Reviwed in Osborne *et al.*, 2004).

One of the most prominent oncogenes is *bcl-2* gene, a prototypical inhibitor of apoptosis (Gross *et al.*, 1999). Over-expression of *bcl-2* also increases resistance to chemo- and radiotherapies in cancer cells (Reed, 1999).

Amplification of the *N-myc* oncogene is now a valuable prognostic marker for patients with neuroblastoma (Brodeur *et al.*, 1984 and Seeger *et al.*, 1985), and amplification/overexpression of the erbB-2 (also known as Her-2) oncogene is also a marker for the aggressiveness of ovarian and breast cancers (Slamon *et al.*, 1987 and 1989). The c-*myc* oncogene encodes a nuclear phosphoprotein that acts as a transcriptional regulator involved in cellular proliferation, differentiation and apoptosis. It is amplified and overexpressed in 15%–25% of breast tumors (Nass *et al.*, 1997). In general, oncogene amplification occurs late in tumor progression and correlates well with clinical aggressiveness of tumors (Yokota *et al.*, 1986 and 1988).

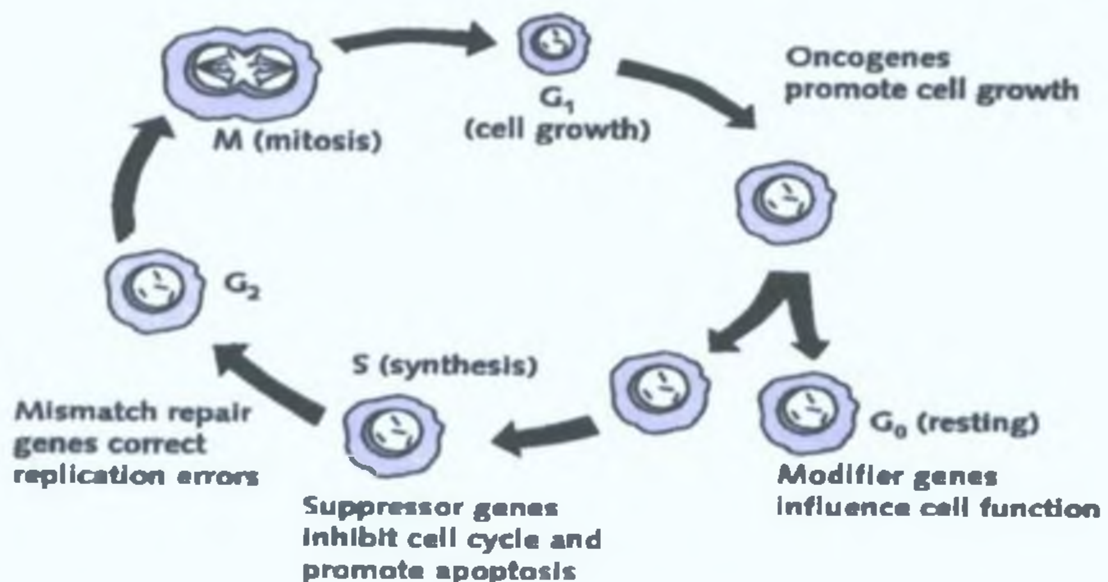

Figure 1.3 The normal function of the different classes of cancer-causing genes according to the cell cycle stage (Source: Calvert and Frucht, 2002).

Point mutations of the *ras* oncogenes, in particular of the K-ras gene, occur in a variety of human cancers, such as pancreatic cancer, colorectal cancer, lung adenocarcinoma and thyroid carcinoma The prognostic significance of *ras* mutation has been documented in lung adenocarcinoma (Rodenhius and Slebos, 1992) Furthermore, alterations in several oncogenes have been also detected in a subset of cancer cells For instance, alterations in the adenomatous polyposis coli (APC), K-ras and p53 genes are common in colorectal cancer (Kinzler and Vogelstein, 1996), while those in the p53, RB/p16, c-myc and K-ras genes are common in lung cancer (Yokota, 1999 and Sekido *et al*, 1998) Ras, a downstream central acting protein, activates the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein (MAP) kinase pathways Farnesyl transferase inhibitors (FTIs) prevent the translocation of Ras to the inner membrane, where it is activated While the HER-2 (human epithelial growth factor receptor 2, also known as HER-2 or erbB-2) membrane receptor tyrosine kinase is the most studied component of the cell signaling system, many other proteins including Ras, are involved in transducing and modulating this signal, which has many end events, including cell proliferation, alterations in drug sensitivity and DNA repair, angiogenesis, apoptosis, protease activity and cell motility (Reviwed in Osborne *et al*, 2004)

The HER-2 gene encodes a 185-kDa transmembrane tyrosine kinase growth factor receptor (Yarden and Sliwkowski, 2001) This leads to multiple transduction cascades acting through a variety of pathways including the MAP kinase and PI3K/Akt pathways, which eventuate in proliferation, angiogenesis, altered cell-cell interactions, increased cell motility, metastases and resistance to apoptosis (Oved and Yarden, 2002)

The epidermal growth factor receptor (EGFR, also known as HER-1), are relevant in breast cancer Expression of *EGFR* has been reported in some studies to be associated with a worse clinical outcome as well as estrogen-receptor (ER) negativity (Witton *et al*, 2003) Growth-factor-mediated signal transduction activates several key kinases that serve as master switches and can control numerous pathways The mammalian target of rapamycin (mTOR) is a pivotal downstream kinase that couples growth stimuli from receptors or cytoplasmic kinases to regulation of the cell cycle Rapamycin and its analogues inhibit phosphorylation of mTOR, thus blocking in translation of key protein

synthesis machinery components and cell cycle regulatory proteins such as c-Myc and cyclin D1 respectively (Mita *et al*, 2003) The c-*myc* gene is a member of the *myc* family of nuclear protooncogenes play roles in cell proliferation, differentiation, and apoptosis Inappropriate expression of c-*myc* genes contributes to the development of many types of cancers in humans It has been proposed that c-*myc* may normally function by coordinating expression of growth-related genes in response to mitogenic signals Deregulated c-*myc* expression may predispose to cancer by enhancing cell growth to levels required for uncontrolled cell division (Iritani and Eisenman, 1999)

The biological activity of oncogenes can be modulated and suppressed either on the RNA or the DNA levels Anti-oncogenes are oligonucleotides (short nucleic acid segments) that can bind to a specific sequence of the RNA (antisense oligonucleotides) or the DNA (antigene oligonucleotides) resulting in the inhibition of the oncogene activity (reviewed in El-Aneed, 2004, Helene, 1994, Zhang and Roth, 1994)

In contrast to the oncogenes discussed above, tumor suppressor genes act as the cell's brakes by encoding proteins that repress biochemical function and cell proliferation Tumor suppressor genes refer to those genes whose loss of function results in the promotion of malignancy Tumor suppressor genes are usually negative regulators of growth or other functions that may affect invasive and metastatic potential, such as cell adhesion and regulation of protease activity (reviwed in Osborne *et al*, 2004) These genes induce apoptosis and/or cell cycle arrest in malignant cells (Opalka *et al*, 2002)

The main representative gene of this family is the *p53* gene which is responsible for the detection of DNA damage followed by repair initiation or apoptosis induction (Sager, 1989) Under normal conditions, p53 acts as a regulating mechanism for cell division When activated, p53 can directly interact with DNA to yield transcription of a number of genes, including the cyclin-dependent protein kinase inhibitors (CKIs), p21 and a temporary arrest of the cell cycle in the G_1 or G_2/M phase, prior to mitosis to allow for DNA repair p53 is also capable of interacting with other cellular pathways to trigger apoptosis or differentiation (Lane *et al*, 1994) It is well documented that p53 gene induces apoptosis and cell cycle arrest in cultured cells (Roy *et at*, 2002, Sauter *et al*,

2002, Mitry *et al*, 1997) Similarly, tumor growth inhibition and tumor regression in animal models were observed after p53 transfection (Dolivet *et al*, 2002, Anderson *et al*, 1998, Hsiao *et al*, 1997) p53 protein interfere in the biochemical pathways of many gene groups which regulate cell growth and differentiation namely, bcl-2 and caspase (Reviewed in Shen and White, 2001) p53 has also been shown to factor in the expression of other proposed tumor suppressors or regulators of angiogenesis and metastasis, including the proteins maspin, hypermethylated in cancer (HIC)-1, and Kangai-1 (KAI-1) (Zou *et al*, 2000, Mashimo *et al*, 1998, Wales *et al*, 1995)

p27 and Skp2 negative regulators of the cell cycle are also considered tumor suppressor genes in that a loss of their function can contribute to malignant behavior p27 belongs to a family of CKIs known as Cip/Kip, whose other members are p21 and p57 In general, CKIs slow the progression of the cell cycle, p27 is capable of binding to a number of unique cyclin/CDK complexes to attenuate their activity, typically directing the cell toward arrest in the G_1 phase (Russo *et al*, 1996) p27 expression has been shown to have prognostic value in a variety of tumors, including lung and colon (Esposito *et al*, 1997, Loda *et al*, 1997)

Cell cycle checkpoint kinase (CHK2) is a serine threonine kinase that is mutated in some families that have a high breast cancer risk (Bell *et al*, 1999) This kinase is activated by the ataxia-telangiectasia mutated (ATM) protein in response to DNA damage and then phosphorylates p53 and BRCA-1 (Vahteristo *et al*, 2002) The ATM gene senses DNA damage and activates checkpoints and DNA repair pathways through rapid phosphorylation of several substrates including p53, BRCA-1 and CHK2 (Shiloh, 2003)

PTEN (phosphatase and tensin homolog) encodes a phosphatase that serves as a negative regulator to Akt Loss of PTEN function augments the Akt cell survival signal (Burke *et al*, 1997)

In addition to oncogenes, tumor suppressor genes and MMR genes, several other genes seem to be important in colon carcinogenesis, although their exact roles and mechanisms of action have not been fully determined (Figure 1 3) Cyclooxygenase (COX)-2 is one of two COXs, the other being COX-1 Although COX-1 is a constitutive component of

cells, COX-2 enzyme probably has a role in programmed cell death (Calvert and Frucht, 2002)

1.3 Colon cancer

Colon cancer is a major cause of cancer mortality and morbidity both in the USA and worldwide (Bailar and Gornik, 1997, WHO, 1997) affecting about one in 20 people over a lifetime (Bleiberg *et al*, 2002) It is considered among the big killers, together with lung, prostate and breast cancer (Labianca *et al*, 2004)

Colorectal cancer is the leading cause of cancer in Europe as well (United European Gastroenterology Federation, 2003) The incidence is slightly higher in the west and north than in south and east Europe Other high risk areas include North America and Australia Central and South America, Asia and Africa are areas of low risk (Parkin *et al*, 2002) An estimated 225,000 European colon cancer cases occurred in 2000, accounting for 8% of all malignant tumours in adults (Parkin *et al*, 2002, Ferlay *et al*, 2001) The incidence in men is about 50% greater than in women (Ferlay *et al*, 2001)

Data from European Network of Cancer Registries (ENCR), about 70% of patients with colon cancer are over 65 years of age Colon cancer is rare under the age of 45 (2 per 100,000 per year) (ENCR 2001) In the age group 45–54 colon cancer incidence is about 20 per 100,000 per year and thereafter increases at a much higher rates (55 per 100,000 per year for aged 55–64, 120 for aged 65–74 and 200 per 100,000 per year for those older than 75 years of age) (ENCR 2001)

Epidemiological studies have shown a significant difference in colon cancer incidence among different ethnic groups The incidence of colon cancer is much higher in the United States and European countries compared with Asian countries (Parkin *et al*, 2002) such as Japan and China, which is believed to be partly attributed to dietary habits (Messina *et al*, 1991) One of the major differences in diet between these populations is that the Japanese and the Chinese consume a traditional diet high in soy products The increased incidence of colorectal cancer in the developed world is suggestive of

environmental and nutritional influences in its pathogenesis (Weisburger and Wynder 1987)

Colon cancer is the result of an abnormal balance in many cellular processes, such as cell growth and differentiation of colonic epithial cells (Navarro *et al*, 1997) It is usually observed in one of three specific patterns sporadic, inherited and familial Sporadic disease, with no familial or inherited predisposition, accounts for approximately 70% of colorectal cancer in the population Sporadic colon cancer is common in persons older than 50 years of age, probably as a result of dietary and environmental factors as well as normal aging (Calvert and Frucht, 2002)

Colorectal cancer is inherited in fewer than 10 out of every 100 cases (Stewart *et al*, 2003 and Calvert and Frucht, 2002) People with inherited colorectal cancer have polyposis or nonpolyposis syndromes In polyposis syndromes, patients develop many polyps in their colons Some of these polyps become cancer The main polyposis syndrome is familial adenomatous polyposis (FAP) which is associated with mutation or loss of FAP (also called the adenomatous polyposis coli (APC) gene (Stewart *et al*, 2003) Colorectal cancer is familial in possibly up to 25 of every 100 cases People in families with familial colorectal cancer have a higher than average risk for colorectal cancer Some patients do not have polyposis but do have inherited genes that put them at very high risk for colorectal cancer but the pattern of inheritance is not consistent with an inherited syndrome (Calvert and Frucht, 2002) Hereditary nonpolyposis colorectal cancer (HNPCC) syndrome is associated with germline mutations in six DNA mismatch repair genes (Stewart *et al*, 2003) HNPCC is a most common known hereditary cause of colon cancer The prevalence of colorectal cancer associated with the HNPCC syndrome is very low as estimated by the Modena Cancer registry slightly more than 7% of the total colorectal cancer prevalence (Gatta *et al*, 1999)

Diet is the most important exogenous factor identified up to now in the aetiology of colon cancer It has been estimated that 70% of colorectal cancers could be prevented by nutritional intervention (Stewart *et al*, 2003) A substantial number of dietary factors and factors related to diet, possibly modify the risk of colon cancer These factors are diets

high in starch, non-starch polysaccharides (fiber) and carotenoids, all of which are found in foods of plant origin, and possibly decrease the risk Evidence that diets rich in vegetables protect against colon cancer is substantial, while the data on fruits are more limited and inconsistent Consumption of non-digestible fructo-oligosaccharides may selectively promote the growth and activity of potentially beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* (WCRF and AICR, 1997) Recently, the role of fibre as a protective factor for colon cancer was confirmed in a large cohort European study on diet (Bingham *et al* , 2003) In populations with low average intake of dietary fibre, an approximate doubling of total fibre intake from food could reduce the risk of colorectal cancer by 40%

1.4 Diet and cancer

The increasing use of functional foods by the public to improve their general health and prevent the incidence of chronic diseases (eg, cardiovascular disease, diabetes, and cancer) has become a major area of interest within the nutrition community Previously epidemiological evidence supports the association of intake of dietary fat with the incidence and mortality of colorectal and prostate cancers (Erickson, 1998, Giovannucci and Goldin, 1997, Willett, 1989, Rose *et al*, 1986)

High fat diets, rich in cholesterol and saturated lipids, may favour colon cancer because of their high caloric content, or they could lead to increased levels of bile acids in the colonic rumen or a disbalance of the essential fatty acids metabolism (Eynard, 1997) But recent epidemiologic studies suggest that high intakes of high-fat dairy foods may reduce the risk of colorectal cancer (Larsson *et al*, 2005) Increasing knowledge about the 20 - 40 year process of human carcinogenesis is providing many new opportunities for early intervention and prevention and specifically for chemoprevention Cancer chemoprevention may be defined as the use of specific chemical substances, many of which occur naturally in foods, to prevent cancer initiation and to inhibit or reverse the development of invasive cancer (Singletary, 2000)

Carcinogenesis is a complex, multi-step process that progresses over many years Since it is exceptionally difficult to cure malignant tumors, cancer prevention may be a more effective strategy to control and, ultimately, overcome cancer A promising and important group of potential cancer preventive agents are those derived from natural products, particularly dietary substances because of their low toxicity and apparent benefit in other chronic diseases (Lim *et al*, 2005) And also, because replicating cancer cells have an increased requirement for lipids for membrane formation and metabolic energy, dietary intervention with fatty acids possessing anticarcinogenic properties may represents a novel, practical and relatively safe approach to reduce the proliferation of colorectal and prostate cancer cells (Palombo *et al*, 2002) It is well established that, beef together with whole milk and dairy derivatives, are almost the only sources for conjugated linoleic acid (CLAs) family Furthermore CLAs are the only natural fatty acids accepted by the National Academy of Sciences of USA as exhibiting consistent antitumour properties at levels as low as 0 25 – 1 0 per cent of total fats (Eynard and Lopez, 2003)

1.5 Introduction to CLA

Conjugated linoleic acids (CLAs) refer to a naturally occurring group of positional and geometric isomers of linoleic acid (18 2n − 6, LA) that are formed by biohydrogenation and oxidation processes in nature (reviewed in Wahle *et al*, 2004, reviewed in Belury, 2002a) Whereas the double bonds in LA are at the 9th and 12th carbon from the carboxyl group in the *cis* configuration, the bonds in CLA are in positions 9 and 11 or 10 and 12 (i e conjugated), each of these bonds may be in the *cis* or *trans* configuration The *cis*-9, *trans*-11 (*c*9, *t*11) and *trans*-10, *cis*-12 (*t*10, *c*12) isomers are considered to be biologically active (Palombo *et al*, 2002) The two predominant isomers of CLA which are found primarily in ruminant meats and milk products and commercial preparation are *c*9, *t*11 CLA and *t*10, *c*12 CLA (Brown *et al*, 2004) Recenlty, *t*9, *t*11 CLA is also available as a commercial preparation

CLA isomers have been studied extensively due to their ability to modulate cancer, atherosclerosis, obesity, immune function and diabetes in a variety of experimental

models (Reviewed in Brown and McIntosh, 2003). It exhibits chemoprotective effects in several tissues in experimental animals, such as chemically induced forestomach neoplasia, skin tumors in mice, mammary and colon carcinogenesis in rats (Belury, 2002b). CLAs were first discovered by Pariza and his group when investigating the carcinogenic components of grilled beef (Pariza and Hargraves, 1985).

1.6 Structure of CLAs

CLAs are a series of positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12-18:2$n-6$) where one or both of the double bonds are either in the *cis* or the *trans* configuration and transposed to different positions along the acyl chain with the bonds separated by a simple carbon–carbon linkage rather than by the normal methylene group (Figure 1.4). A number of *cis–cis*, *cis–trans*, *trans–cis* and *trans–trans* isomers with the double bonds at various locations along the acyl chain, from carbon-6 to carbon-15, have been identified by various chemical reductive, chromatographic and spectroscopic techniques (Adlof, 2003; Christie, 2003; Dobson, 2003).

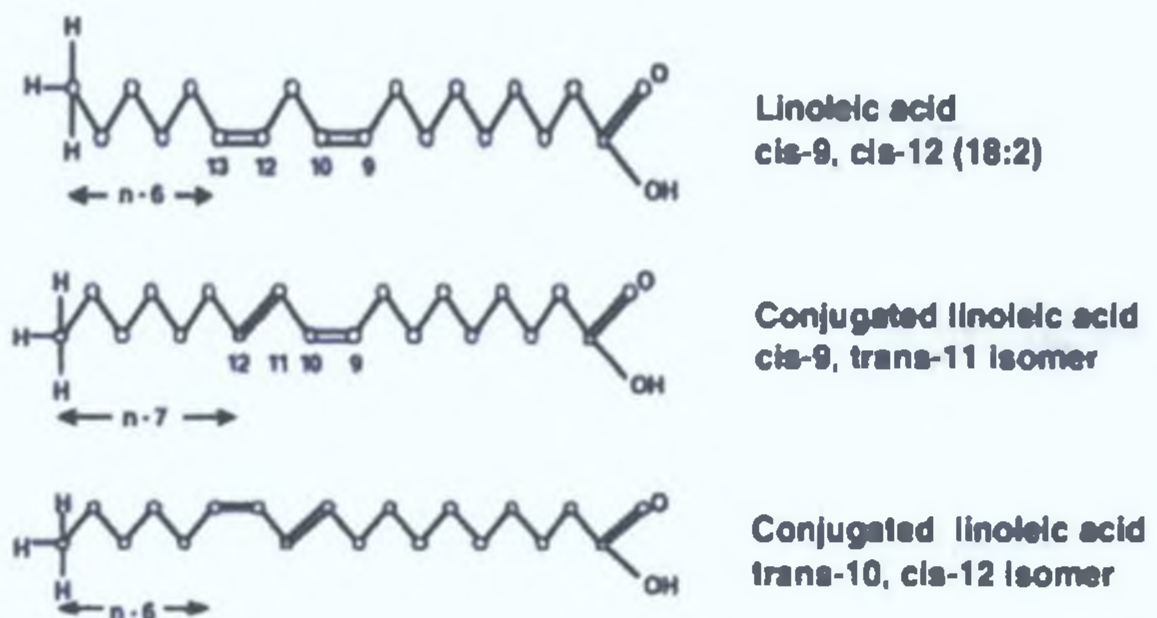

Figure 1.4 Structure of the parent omega-6 fatty acid linoleic acid and its two main conjugated derivatives (source: reviewed in Wahle *et al.*, 2004).

1.7 Sources of CLAs

The major dietary sources of CLAs are foods derived from ruminant animals, in particular dairy products Consequently, the main food source of CLAs in the Western diet is from meat and dairy products derived from cows, sheep, goats and deer (reviewed in Wahle *et al*, 2004, Griinari *et al*, 2000, Ma *et al*, 1999, Chin *et al*, 1992) The rumen of these animals is likened to a large anaerobic fermentation tub which contains microbes capable of biohydrogenating the ingested polyunsaturated fatty acids derived largely from forage but also from other feed sources, natural or otherwise (e g added grain or fish oils) (reviewed in Wahle *et al*, 2004)

The predominant isomer in milk and other dairy products is the *c*9, *t*11 CLA with minor but significant proportions of *t*10, *c*12 CLA (Parodi, 2003, McGuire *et al*, 1999 Parodi, 1997) This contrasts with commercial preparations of CLA where proportions of the two main isomers are usually almost equal, although the chemical method for synthesis will allow a variety of ratios for the two isomers in the final mixture (Parodi, 2003, Saebo, 2003, McGuire *et al*, 1999, Parodi, 1997) The greatest concentrations of CLA in milk are obtained when cows are fed supplemental feed oils, particularly fish oil supplements (Parrish *et al*, 2003, Stanton *et al*, 2003, Chilliard *et al*, 2001 Gulati *et al*, 2000, Parodi, 1997) Interestingly, the highest natural levels of CLA observed to date in nature occur in wallaby milk (Parodi, 1997) Fat associated with meat of ruminant animals, contributes in the region of 25–30% of the total intake in Western populations (Parodi, 2003, McGuire *et al*, 1999, Parodi, 1997)

Ruminant products are the principal source of CLA in human diets with ~70% and 25% coming from dairy products and red meat, respectively (Ritzenthaler *et al*, 2001) The *c*9, *t*11 CLA (rumenic acid, RA) represents 75–90% of total CLA in dairy foods (Lock and Bauman, 2004, Parodi, 2003, McGuire *et al*, 1999, Parodi, 1997)

CLA is found in minor amounts in oils and seafood (0 2–0 8 mg CLA/g fat) but in greater amounts in meats (1 0–4 0 mg CLA/g fat) and dairy products (5 0–7 0 mg CLA/g fat) (Herbel *et al*, 1998) The *c*9, *t*11 CLA is the primary dietary form of CLA in human diets The second most abundant isomer of CLA is the *t*10, *c*12 CLA form initially

identified in grilled beef (reviewed in Wahle *et al*, 2004) However, the relative concentration of *c*9, *t*11 CLA and other isomers including *t*10, *c*12 CLA in dairy and meat products is influenced by the type and amount of vegetable fats fed to ruminants (Pariza *et al*, 2001) The accumulation of CLA isomers and of several elongated/desaturated and β-oxidation metabolites have been reported in tissues of animals fed diets with CLA (reviewed in Belury, 2002a)

CLA is present in natural sources in only minute amounts, which makes it extremely difficult to purify from such sources Furthermore, it is difficult to separate CLA isomers prepared by alkali isomerization in bulk and therefore only CLA mixtures are currently on the market as health supplements (Tsuzuki *et al*, 2004)

CLA is not found in any of the vegetable oils commonly used in the food chain, although fatty acids with conjugated double bonds were observed in various seed oils from a number of plant species (Reviewed in Wahle *et al*, 2004) In Okinawa, Japan, which is in itself one of the leading countries in the world in terms of life expectancy, people often eat bitter gourds (*Momordica charantia*) The seed oil of such gourds contains 60% α-eleostearic acid (α –ESA, 9,11,13-18 3) (w w) Interestingly it was reported that *c*9, *t*11 CLA can be produced in rats from the conversion of conjugated triene α –ESA A significant amount of *c*9, *t*11 CLA was found in the liver and plasma lipids of rats fed a 1% (w/w % of diet) eleostearic acid diet for 4 weeks (Tsuzuki *et al*, 2004)

1.8 Dietary intake of CLAs in humans

The daily intake of CLA in human populations varies from country to country The estimated daily CLA intakes range from negligible to 1500 mg in Australian populations (Parodi, 2003, Fritsche *et al*, 1999), whereas the average CLA intake in the UK is about 400–600 mg/d Interestingly, intakes in women are generally lower than in men due possibly to a lower dairy fat consumption in the former (Parodi, 2003, McGuire *et al*, 1999, Parodi, 1997)

Herbel *et al*, (1998) reported that young men and women living in the United States consumed approximately 127 mg CLA/day Somewhat similar values were obtained in another US study that the dietary intake of CLA in young men and woman was 137 and 52 mg/day, respectively (Ritzenthaler *et al*, 1998) It is interesting to note that college-aged women have extremely low CLA intakes The CLA intake in young Canadians (Ens *et al*, 2001) has been estimated to be 94 mg/day Dietary intake of CLA in Germany was also estimated to be lower in women (350 mg CLA/day) than in men (430 mg CLA/day) (Fritsche and Steinhart, 1998) on the basis of the West German National Consumption Survey In a more recent German study, daily intake was reported to be 246 and 323 mg CLA/day To achieve an intake of 0 1 g/100g diet, the level of CLA that has been shown to significantly reduce tumors in animals (Ip *et al*, 1994), the *c*9, *t*11-CLA intake would need to be 620 and 441 mg/day for men and women, respectively (Ritzenthaler *et al*, 2001)

The average intake of CLA probably may not reflect the total CLA available to an individual because of endogenous conversion of *t*-VA from dairy products to CLA via the Δ-9 desaturase enzyme (Corl *et al*, 2003) It has been estimated that 20% of *t*-VA is converted to CLA in this way (Turpeinen *et al*, 2002) Dietary modifications can increase CLA concentration in human tissues Specific intervention studies have shown that increasing the CLA content of the diet increased the CLA content in human milk (Park *et al*, 1999a), plasma (Huang *et al*, 1994) and adipose tissue (Jiang *et al*, 1999) The amount of *c*9, *t*11-CLA in human adipose tissue was significantly related to milk fat intake (Jiang *et al*, 1999)

1.9 The biosynthesis of CLA in ruminant, rodent and man

CLA has been identified in human blood, milk (Fogerty *et al*, 1988), adipose tissue (Ackman *et al*, 1981), bile and duodenal juices (Cawood *et al*, 1983) with *c*9, *t*11 CLA as the most predominant isomer present The origin of CLA in human tissues is thought to be dietary as the consumption of CLA-containing foods such as cheese has been shown

to increase plasma CLA levels (Huang *et al*, 1994, Britton *et al*, 1992) It was first proposed by Parodi (1994) that *trans*-vaccenic acid (*t*-VA), the predominant *trans* monounsaturated fatty acid in milk fat could be desaturated to *c*9, *t*11 CLA in humans based on the observation that a Δ^9 desaturase enzyme from rat liver microsomes has been shown to produce CLA from *t*-VA (Mahfouz *et al*, 1980, Pollard *et al*, 1981)

There are two pathways for the production of CLA in the dairy cow Firstly, they are formed as intermediates through incomplete biohydrogenation of PUFA from the diet specifically linoleic (18 2n–6) and linolenic acids (18 3n–3) by anaerobic rumen micro organisms (Hughes *et al*, 1982, Kepler *et al*, 1966) Two major groups of rumen bacteria have been identified that isomerize either the *c*12 bond to *t*11, eg, *Butyrivibrio fibrisolvens* (Kim *et al*, 2000, Hughes *et al*, 1982, Kepler *et al*, 1966), or the *c*9 bond to *t*10, eg, *Megasphaera elsdenii* (Kim *et al*, 2002a) The cascade of possible FAs from 18 2n–6 and 18 3n–3 by these 2 groups of rumen bacteria is shown in Figure 1 5

Isomerization followed by biohydrogenation in the normal rumen produces mainly *t*11-containing fatty acids, whereas during dysfunctional states mainly *t*10 fatty acids are produced Metabolites produced in the rumen can pass through the blood into tissues, including milk fat, the transfer of selected fatty acids is shown by dotted arrows *t*11-18 1 is desaturated to *c*9, *t*11-18 2 (*c*9, *t*11 CLA) by Δ^9-desaturase, whereas *t*10-18 1 is not converted to *t*10, *c*12-18 2 (*t*10, *c*12 CLA) in the tissue The underlined *trans* double bond indicates the common *trans* double bond formed by the respective rumen bacteria (Figure 1 5) (reviewed in Kramer *et al*, 2004)

A second pathway for production of *c*9, *t*11 CLA is via Δ9 desaturation of *t*-VA in the mammary gland (Griinari *et al*, 2000) Ip *et al*, (1999a) demonstrated that rats consuming CLA-enriched butterfat accumulated more total CLA in their tissues compared to those consuming either Matreya CLA or Nu-Chek Prep CLA The authors hypothesised that the availability of *t*-VA in the high CLA butterfat may serve as the precursor for the endogenous synthesis of CLA via the Δ9 desaturase reaction Santora *et al*, (2000) reported and quantified the desaturation of *t*-VA to CLA in mice When equal

quantities of *t*-VA and CLA were fed to mice they reported that 12 % of the *t*-VA consumed during a 2-wk feeding period was recovered in the carcass as CLA. Of the proportion of *t*-VA in the tissues that was available for bioconversion, 48.8 % was desaturated. CLA was found in the carcass only when vaccenic acid or CLA was fed. CLA was found in both triglyceride and phospholipids when CLA was fed, but only in triglyceride when *t*-VA was fed, suggesting that bioconversion occurred in the adipose tissue (Santora *et al.*, 2000).

Salminen *et al.*, (1998) provided evidence that CLA in human serum has been derived in part from the diet and in part by conversion of dietary *trans* fatty acids. Serum CLA levels were significantly higher in subjects fed a high-dairy fat diet, rich in CLA and *trans*-fatty acids than when fed a CLA-poor stearic acid diet. Evidently, CLA was formed during consumption of the diet rich in *trans* fatty acids and incorporated into serum lipids. Adlof *et al.*, (2000) showed that *t*-VA was converted into CLA in humans, at a CLA enrichment of approximately 30%.

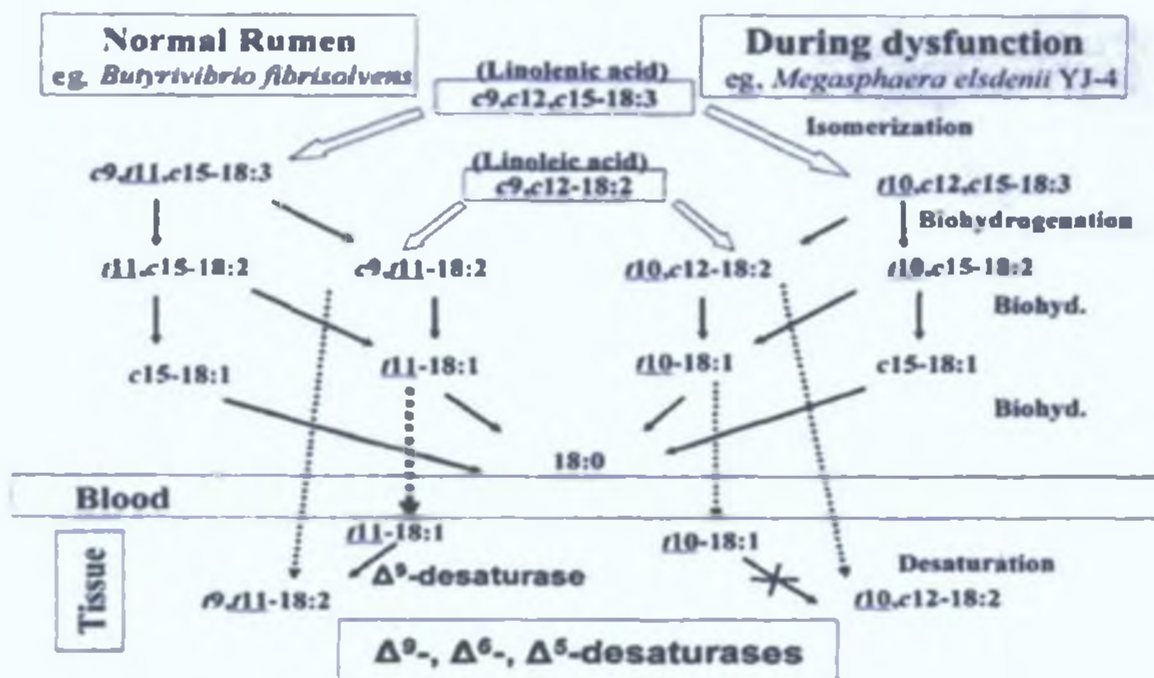

Figure 1.5 Possible metabolic intermediates of linoleic and linolenic acid produced by rumen bacteria (Source: reviewed in Kramer *et al.*, 2004).

Recently Miller *et al*, (2003) observed that, when cells were incubated in the presence of *t*-VA at concentrations of 5 to 20 µg/mL, both *t*-VA and *c*9, *t*11 CLA increased in cellular lipids in a dose-dependent manner After 4 d of incubation of SW480 and MCF-7 cells with VA (20 µg/mL), *c*9, *t*11 CLA increased from undetectable levels to 8 57 and 12 14 g/100 g FAME in cellular lipids, respectively O'Shea *et al*, (2000) also examined the fatty acid composition of total cell lipids of MCF-7 human breast cancer cells, incubated in the presence of pure *c*9, *t*11 CLA (20 µg/ml) and with a CLA-enriched milk fat containing 20 µg/ml CLA CLA uptake was approximately 6 fold more proficient from the milk fat than from the synthetic pure *c*9, *t*11-CLA source, supporting the study by Ip *et al*, (1999a) The study also suggested that CLA could be formed from *t*-VA present in the milk fat by a Δ9 desaturase enzyme present in human breast cancer cells

It has also been proposed that CLA may also be synthesised from LA by intestinal flora or by free radical induced isomerisation of LA In normal rats, dietary linoleic acid gave rise to CLA in various tissues in proportion to the amount of linoleic acid fed, but this conversion was not evident in germ-free animals (Chin *et al*, 1994) However Salminen *et al*, (1998) refuted the concept of production of CLA from linoleic acid in humans because significantly different levels of CLA were found in serum lipids from subjects fed three different dietary regimes that contained the same levels of LA The consumption of LA in triglyceride form in sunflower oil did not increase plasma levels of esterfied CLA in the total lipids of human subjects (Herbel *et al*, 1998) But there is evidence that small amounts of PUFAs are absorved in the large intestine (Adlof *et al*, 2000)

1.10 Health benefits of CLAs

Interest in CLAs has increased recently because of its anticarcinogenic properties In addition to their anticarcinogenic properties (reviewed in Belury, 2002b), CLA isomers have been shown to modulate immune function (reviewed in Wahle *et al*, 2004), as well as markers of atherosclerosis (reviewed in Kritchevsky, *et al*, 2000), diabetes (reviewed in Belury, 2002a), and obesity risk (reviewed in Evans *et al*, 2002b)

They also have reported beneficial regulatory effects on bone formation, lipid and eicosanoid metabolism, cytokine and immuno-globulin production and can modulate the expression of a number of genes, either directly or through specific transcription factors involved in the many metabolic processes they affect (reviewed in Wahle *et al*, 2004, Eggert *et al*, 2002, Park *et al*, 2000b, Li and Watkins, 1998, Belury and Kempa-Steczko, 1997, reviewed in Ip, 1997)

1.11 CLA inhibits carcinogenesis

The most studied bioactivity of CLA is its anticancer effect The development of anticancer research involving CLA began when Ha *et al*, (1987) found that CLA inhibited *in vivo* initiation of mouse epidermal tumors Since then CLA has been shown to inhibit the formation of tumour in numerous animal models of cancer and to inhibit the growth of a large variety of human cancer cells

Experimental studies have shown that, in contrast to LA, CLA is an effective inhibitory agent of human mammary, colorectal and prostate cancer in vitro and in vivo (Park *et al*, 2000a, Cesano *et al*, 1998b, Schut *et al*, 1997, Liew *et al*, 1995, Ip *et al*, 1994b, Rose *et al*, 1993, Shultz *et al*,1992b, Ip *et al*, 1991) CLA behaved as a powerful anticarcinogen in a rat mammary tumor model with an effective range as low as 0 5% in the diet [Liew *et al*, 1995] Interestingly, the protective effect of CLA was expressed at concentrations close to human consumption levels [Ip *et al*, 1994a]

When transplanted into nude mice, growth of mammary (Visonneau *et al*, 1997) or prostate (Cesano *et al*, 1998b) cancer cell lines was significantly reduced if animals were fed a diet with CLA (1 0%) The *c*9, *t*11 CLA and *t*10, *c*12 CLA appear to be equally active in inhibiting mammary carcinogenesis in rats (Ip *et al*, 2002)

Studies in animal models of human prostate cancer that used transplanted DU145 cells have shown clear anti-tumorigenic effects of dietary CLAs similar to those observed with breast cancer models when implanted into SCID mice and these effects were opposite to

those observed with linoleic acid feeding (Cesano *et al.*, 1998b). CLAs were found to be cytotoxic to the rat dRLh-84 hepatoma cells at concentrations as low as 1 μM when compared to control (Yamasaki *et al.*, 2002b).

CLAs also inhibited the growth of a human hepatoma cell line (HepG2) in vitro. These effects were due to alterations of fatty acid metabolism in the cells (Igarashi and Miyazawa, 2001).

Neovascularisation or angiogenesis in tumours is an important mechanism for ensuring the nutrient supply and consequently the growth of the tumour and also in maintaining complex atherosclerotic lesions. Inhibition of angiogenesis would be expected to reduce rapid tumour growth and plaque progression. Evidence that CLAs can inhibit angiogenesis in mammary cancer (Ip *et al.*, 2002). This indicates that these fatty acids may inhibit tumour growth through a reduced blood supply. Recently, a possible role of *c*9, *t*11 CLA in inhibition of angiogenesis has been proposed when *c*9, *t*11 CLA prevented the conversion of mammary stromal stem cells to endothelial cells (Masso-Welch *et al.*, 2002).

Whereas a great deal of evidence demonstrates that dietary CLA inhibits the initiation and promotion stages of carcinogenesis, the role of CLA in the progression stage of carcinogenesis has not been comprehensively addressed (review in Belury, 2002a). It is critical to understand how CLA modulates malignant tumor formation and metastasis because the growth of secondary tumors is the major cause of morbidity and mortality in people with cancer. However a study has demonstrated that CLAs, both main isomers and a mix, at a concentration of 0.5% to 1% (w/w) of the diet, had a significant and dose-dependent inhibitory effect on pulmonary tumour burden, an index of metastasis, in mice with transplantable tumours (Hubbard *et al.*, 2000; Kuniyasu *et al.*, 2006).

The evidence of possible anti cancer activity of CLA against intestinal cancer first arose when CLA was shown to inhibit the formation of 2-amino-3-methyl-imidazo[4,5-f]-quinoline (IQ)-DNA adducts in a number of organs including the large intestine of CFD_1 mice (Zu and Schut, 1992). The heterocyclic amine IQ reacts with DNA to form carcinogen-DNA adducts, leading to mutation and subsequently, to the initiation of the

carcinogenic process Other experimental evidence was the modulation of azoxymethane-induced colonic aberrant crypt foci (ACF) in male rats fed a CLA supplemented diet (Kohno *et al*, 2002) The administration of CLA caused a significant reduction in the frequency of ACF Also, these mixtures of CLA isomers lowered the proliferating cell nuclear antigen (PCNA) index in colonic ACF whereas apoptosis occurred (Kohno *et al*, 2002) Park *et al*, (2001) reported that dietary CLA can inhibit 1 2-dimethylhydrazine-induced colon carcinogenesis by a mechanism probably involving increased apoptosis These authors suggested a possible chemopreventive activity of CLA in the early phase of colon tumorigenesis through modulation of cryptal cell proliferation activity and apoptosis (Park *et al*, 2001)

CLA at 0 5% and 1% of the diet has been shown to significantly reduce the induction of mutations in distal colon of the Big Blue^R rat (a transgenic animal model developed for evaluation of mutagenicity of chemical compounds) (Yang *et al*, 2002) In a study mimicking human dietary supplementation, the effect of timing of CLA feeding on mutagenesis was studied Simultaneous administration of CLA with 2-amino-1-methyl-6-pheny-imidazo[4,5-b] pyridine (PhIP), suppressed PhIP-induced mutations in the distal colon by 23% Consistent with inhibition of PhIP-induced mutation frequency, dietary CLA also inhibited aberrant crypt foci formation in male F344 rats given PhIP in basal diet (Yang *et al*, 2002) In view of the presence of both PhIP and CLA in the typical western human diet, understanding the effects of CLA on mutagenesis and DNA repair will be necessary for development of strategies which can optimally impact on cancer control

Park *et al*, (2004) reported that CLA decreased the incidence of colon cancer by decreasing cellular proliferation and inducing apoptosis of the colonic mucosa of rats These effects may be due in part to decreased PGE_2 levels and increased Bax/Bcl-2 ratios (Park *et al*, 2004) Bcl-2 actively forms heterodimers with Bax to neutralize the latter's proapoptotic activity (Reed, 1994) and that phosphorylation of Bcl-2 functionally stabilizes the Bcl-2-Bax heterodimerization (Deng *et al*, 2000) Therefore, the Bax/Bcl-2 ratio can function as a controller to modulate cellular fate (Buckley, 2001)

Although most of the experiments showed positive effects of CLA as anticarcinogen of colon cancer, contradictory observations have also been reported for the effects of CLAs as well In an Apc^{Min} mouse model (transgenic mouse model, mutation in Apc gene) of colon cancer CLA did not reduce tumour load but omega-3 PUFA from fish did (Petrick *et al*, 2000) A recent study also reported that the *t*10, *c*12 CLA actually promoted colon carcinogenesis rather than inhibiting it in the Apc^{Min} mouse model (Rajakangas *et al*, 2003)

The beneficial effects of CLAs in gastro-intestinal cancer have been observed mainly in chemically induced tumours in animal models But anti-proliferative effects of CLA have also been investigated in cultures of colon cancer cells (Cho *et al*, 2003, Kemp *et al*, 2003, Miller *et al*, 2002, Palombo *et al*, 2002, O'Shea *et al*, 2000, Shultz *et al*, 1992b)

Unlike the *in vivo* experiments, all of which used a mixture of CLA isomers, some of the *in vitro* studies have provided some insight into the activities of specific CLA isomers on colon cancer cell growth Miller *et al*, (2002) and Kim *et al*, (2002b) compared the individual potencies of the *c*9, *t*11 CLA and the *t*10, *c*12 CLA isomers on the growth of the SW480 and Caco-2 colon cell line respectively In a recent study the *t*10, *c*12 CLA isomer (at 14 and 28 μg/ml) exhibited the greatest potency against colorectal cancer proliferation of the HT-29 and MIP-101 cell lines (Palombo *et al*, 2002)

High-fat dairy foods contain many potentially anticarcinogenic factors that might reduce the risk of colorectal cancer including CLA, sphingomyelin and ether lipids (Molkentin, 2000) However, few epidemiologic studies have specifically evaluated high-fat dairy food consumption and none have evaluated CLA intake, in relation to colorectal cancer risk

Recently in an epidemiological study with a cohort design (Swedish Cohort Study) Larsson and her colleagues (2005) demonstrated that high-fat dairy foods (including whole milk, full-fat cultured milk, cheese, cream, sour cream, and butter) may lower the risk of colorectal cancer, particularly cancer of the distal colon Total high-fat dairy food consumption was significantly and inversely associated with the risk of colorectal cancer It has been proposed that the inverse association might in part, be related to CLA intake

in high fat dairy foods (Larsson *et al*, 2005) CLA intake was estimated according to the published data on the concentrations of CLA found in the total fat of various foods (Jiang *et al*, 1997, Chin *et al*, 1992) In this study 60708 women aged 40–76y participated in the Swedish Mammography Cohort The women's consumption of high-fat dairy foods was assessed at baseline, which was from 1987 to 1990, and again in 1997 After 15 years follow-up, 798 incident cases of colorectal cancer were discovered (Larsson *et al*, 2005) Women who consumed ≥4 servings of high-fat dairy foods/d had a multivariate rate ratio of colorectal cancer of 0 59 when compared with women who consumed <1 serving/d Each increment of 2 servings of high-fat dairy foods/d corresponded to a 13% reduction in the risk of colorectal cancer For CLA, the multivariate rate ratio of colorectal cancer in a comparison of the 2 extreme quartiles of intake was 0 71 It was concluded from the experimental data that, CLA intake was significantly and inversely related to colorectal cancer risk

1.12 Proposed mechanisms underlying the anticarcinogenic effect of CLA

Strong evidence for the anticancer abilities of CLA indicates a need to study the mechanisms of chemoprotection by CLA Efforts have been made to elucidate the mechanistic role of CLA in modulating carcinogenesis by determining the effects on the stages of carcinogenesis known as initiation, promotion, and progression (reviewed in Belury and Vanden Heuvel, 1997)

Preliminary studies have revealed some important insights that may start to explain the molecular basis for anti-tumour activity of CLAs In particular, in view of their effects on reduction in cellular proliferation and increased apoptosis, research has focussed on the molecular mechanisms underlying the control of these pathways

In order to elucidate the anticarcinogenic mechanisms of CLA, early work focused on events associated with initiation As an antiinitiator, CLA may modulate events such as free radical–induced oxidation, carcinogen metabolism and/or carcinogen-DNA adduct

formation in some tumor models (reviewed in Belury *et al*, 1995) Current attention is focused on elucidating the mechanisms by which CLA inhibits carcinogenesis during promotion and progression particularly in the mammary, skin and colon carcinogenesis models (Ip *et al*, 1995, Belury *et al*, 1996, and Palombo *et al*, 2002 respectively) The promotion stage involves the clonal expansion of initiated cells to form a benign tumor This stage of carcinogenesis represents a premalignant state in which tumors arise from cells that have increased cell proliferation, reduced programmed cell death (or apoptosis), and/or deregulated differentiation

1.12 1 CLA effects on cell signaling and apoptosis

Data suggest that CLA modulates molecular signaling events that impact on the cell cycle, ultimately regulating cell proliferation In cultured cells, CLA reduced proliferation of mammary tumor cells in vitro (Durgam and Fernandes, 1997, Shultz *et al*, 1992b) and in vivo (Ip *et al*, 1994b) Autonomous cell proliferation is one of the characteristics of cancer cells, driven by activated growth-stimulating oncogenes

The ErbB family of receptor tyrosine kinases includes the epidermal growth factor receptor (EGFR) or ErbB1, -2, -3, and -4 Activation of these receptors regulates a number of processes including cell proliferation, survival, and differentiation Overexpression of ErbB genes, particularly ErbB2, has been observed in several types of human cancer (Hamdy, and Thomas, 2001, Safran *et al*, 2001, Yamauchi *et al*, 2001) In colon cancer, the expression of mRNA for ErbB2 and -3 as well as the corresponding proteins was increased compared with normal mucosa (Porebska *et al*, 2000, Maurer *et al*, 1998, Ciardiello *et al*, 1991)

One of the many initial events that occur after growth factors bind to their cognate growth factor receptor tyrosine kinases is the recruitment and activation of phosphoinositide 3-kinase (PI3-kinase) (Varticovski *et al*, 1994) In many instances of receptor-activated PI 3-kinase signaling, binding of the p85 adaptor subunit is itself, a response to upstream tyrosine kinase activity (Wymann and Pirola, 1998) ErbB3 is

particularly well adapted to mediate PI3-kinase signaling because it contains six consensus-binding sites for p85 (Hellyer, 2001)

The recruitment and activation of PI3-kinase is one of the many initial events that occur after growth factors bind to their growth factor receptor tyrosine kinases (Varticovski *et al*, 1994) PI3-kinase phosphorylates inositol phospholipids at position 3 of the inositol ring and PI3-kinase lipid products interact with certain proteins and modulate their localization and/or activity (Vanhaesebroeck *et al*, 1997) Akt is a downstream target of PI3-kinase and plays a central role in PI3-kinase-mediated protection against apoptosis (Franke *et al*, 1997) which can be activated by a variety of growth factors and cytokines via phosphorylation on serine and threonine residues (Datta *et al*, 1999, Hemmings, 1997, Klippel *et al*, 1997) and may participate in growth factor-stimulated cell cycle (Gille and Downward, 1999, Muise-Helmericks *et al*, 1998) and inhibition of apoptosis (Kulik *et al*, 1997) Disturbance of normal protein kinase B (PKB)/Akt signaling has been reported in several human cancers (Nicholson and Anderson, 2002, Kandel and Hay, 1999)

Cho *et al*, (2003 and 2005) demonstrated that CLA mixtures of isomers and *t*10, *c*12 CLA inhibits cell proliferation and stimulates apoptosis in HT-29 cells and that this may be mediated by its ability to downregulate ErbB3 signaling and the PI3-kinase/Akt pathway

Cho *et al*, (2003 and 2005) observed that HRG [heregulin (HRG) is a ligand that bind to and activates ErbB3 and -4 receptors (Tzahar, 1996)] stimulated the recruitment of PI3-kinase to the ErbB3 receptor in HT-29 cells and CLA decreased ErbB3-associated PI3-kinase protein levels and PI3-kinase activities CLA mixtures of isomers and *t*10, *c*12 CLA inhibited HRG stimulated phosphorylation of ErbB3, recruitment of the p85 subunit of phosphoinositide 3-kinase (PI3K) to ErbB3, ErbB3-associated PI3K activities and phosphorylation of Akt

In addition to the PI3-kinase/Akt pathway, MAPK, also known as extracellular signal-regulated kinases (ERKs), are protein serine/threonine kinases that play a critical role in the regulation of cell growth and differentiation (Hunter, 1995 and Marshall, 1995)

The Ras-Raf-MEK-MAPK (MAPKs) pathway may be involved in apoptosis, in that the MAPKs are signalling pathways critical for the conversion of various extracellular signals to biological responses (Johnson and Lapadat, 2002) In particular, ERK activation is generally related to cell survival, but there are reports indicating that apoptosis may be associated with the suppression of ERK signaling (Koo *et al*, 2002, Jan *et al*, 1999, Nagata and Todokoro 1999) Miglietta *et al*, (2006) revealed that CLA induced apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and occurrence of apoptosis was related to reduction in phosphorylated form of ERK1/2 and induction of upregulation of pro-apoptotic protein Bak

As a counterbalancing event in promotion, apoptosis offers protection against carcinogenesis via programmed death of cancer cells Degeneration of an established tumour may occur because of either a decrease in cellular proliferation, an increase in programmed cell death, apoptosis, or necrosis of the tumour due to nutrient deprivation (inhibition of tumour angiogenesis) (Cho *et al*, 2003) A number of studies have showed the pro-apoptotic effects of CLA in experimental models of colon cancer in culture HT-29 (Cho *et al*, *2003* and 2006, Palombo *et al*, 2002), MIP-101 (Palombo *et al*, 2002) and SW480 (Miller *et al*, 2002)

Inhibition of proliferation and induction of apoptosis by CLA have already been shown in various cell types Dietary CLA induced apoptosis in numerous tissues including mammary (Ip *et al*, 2000), liver (Lu *et al*, 2002), and adipose (Tsuboyama-Kasaoka *et al*, 2000) tissues and in cultured mammary epithelial cells (Ip *et al*, 1999b) In mammary tissue initiated with methylnitrosourea, dietary CLA induced apoptosis of cells in the terminal end bud and in premalignant lesions known as intraductal proliferation lesions (Ip *et al*, 2000) In these studies, CLA induction of apoptosis was associated with a reduction of bcl-2, a signaling protein known to suppress apoptosis Ip *et al*, (1996)

have shown that CLA inhibited proliferation and induced apoptosis of normal mammary epithelial cells

The effects of CLAs on the pro- and anti-apoptotic pathways and their controlling genes have also revealed some interesting key facts The expression of bcl-2, a key anti-apoptotic proto-oncogene, was decreased in rat mammary tumours and tumour cells by feeding or treating with CLAs, other oncogenes involved in apoptosis such as bax or bak (Banni *et al*, 2003, Ip *et al*, 2000 and1999b) were not affected or were not determined (e g p53, p21, bad and bcl-X)

Majumder *et al*, (2002) carried out a detailed evaluation of the effects of a mix of CLAs and individual isomers on the expression of several pro- and anti-apoptotic oncogenes in human breast cancer and prostate cancer cells Oestrogen sensitive MCF-7 and oestrogen insensitive MDA-MB-231 breast cancer cells were studied Expression of some of the major oncogenes involved in cell survival and cell death (p53, p21, bcl-2, bax, bcl-Xs) were determined at the transcriptional (mRNA) and translational (protein) level CLA treatment inhibited proliferation and induced apoptosis which correlated with increased gene expression (mRNA and protein) of pro-apoptotic p53 and p21 WAF1/CIP1 but reduced expression of anti-apoptotic bcl-2 in MCF-7 cells The reduced bcl-2 protein expression supported findings of lower levels of this protein in rat tumour tissue as well (Ip *et al*, 2000, 1999b and 1995)

This information suggest that CLA may inhibit promotion by inducing signaling events leading to enhanced apoptosis

1 12 2 CLA and cell cycle

Because deregulation of numerous cell cycle components has been implicated in tumorigenic processes, cell cycle regulators are potential molecular targets for cancer prevention

The mammalian cell cycle is divided into four separate phases, referred to as G_1, S, G_2, and M phases In late G_1 phase and before entering S phase, cells move across the restriction point (Sherr, 1996) During the G_1 phase, cells respond to extracellular signals by either advancing toward another division or withdrawing from the cycle into a resting state (G_0) (Pardee, 1989, Sherr, 1994)

The mammalian cell cycle progression is controlled by the sequential activation and inactivation of several cyclin-dependent kinases (CDK) (Johnson and Walker, 1999) The cell cycle progression from G_0/G_1 to S-phase requires phosphorylation of the retinoblastoma tumor suppressor protein (Rb), a member of the pocket protein family, by the cyclin D1-cdk4/6 and cyclin E-cdk2 complexes (Nurse, 2000, Weinberg, 1995) In quiescent cells, hypophosphorylated Rb associates with a family of heterodimeric transcriptional regulators, collectively named the E2Fs (Ikeda *et al*, 1996, Moberg *et al*, 1996, Sherr, 1996) Phosphorylation of Rb in early G1 by cyclin D1/cdk4/6 triggers a cascade of events that begins with the dissociation of E2F from Rb and the activation of transcription of cyclin E by E2F, and culminates with the stimulation by E2F of its own transcription and assembly of cyclin E with its catalytic partner Cdk2 The cyclin E-cdk2 complexes promote further phosphorylation of Rb and the release of E2F thus establishing a positive feedback loop that accelerates the irreversible progression through late G1 (Sherr, 1996)

Recently, Cho *et al*, (2006) reported that *t*10, *c*2, CLA inhibited G1-S progression in HT-29 human colon cancer They observed *t*10, *c*12 CLA induced cell cycle arrest at the G_0/G_1 phase An increase in the levels of p21 in *t*10 *c*12 CLA-treated cells led to the inhibition of the CDK activity, which resulted in a decrease in phosphorylated Rb and an increase in hypophosphorylated Rb It was revealed that *t*10, *c*12 CLA upregulates the level of p21 and its interaction with PCNA, which may also contribute to the observed decreased DNA synthesis (Cho *et al*, 2006) Utilizing HCT116 cells, Kemp *et al*, (2003) have shown that CLA increased accumulation of hypophosphorylated Rb These results indicate that the decreased phospho-Rb (or increased hypophosphorylated Rb) contributes to G_1/S arrest observed in CLA-treated cells Utilizing MCF-7 breast cancer cells Kemp *et al*, (2003) have also shown CLA induced cell cycle arrest in G_0/G_1

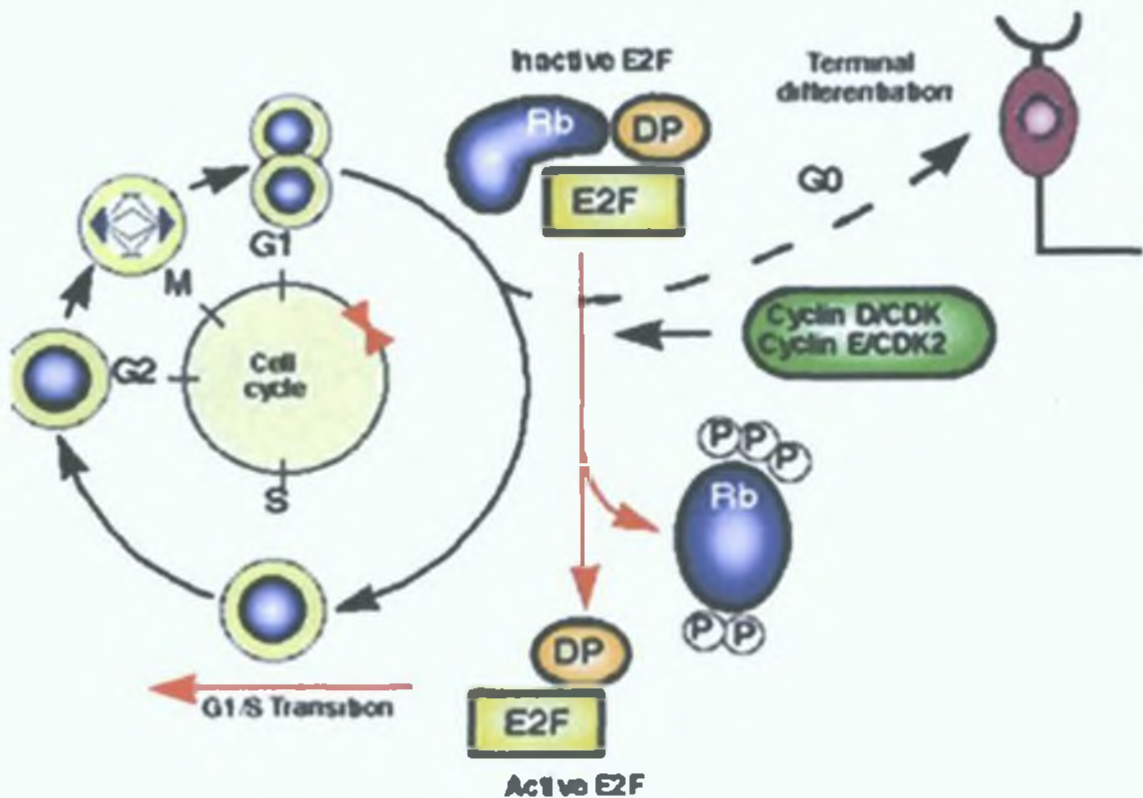

Figure 1.6 Cell Cycle: Simplified G1/S regulation. If a dividing cell is going to proceed through another round of division, the Rb protein (or its related family members) is phosphorylated by cyclin/CDK complexes. This releases the E2F transcription factor and leads to changes in gene expression that are essential for cell cycle progression. Alternately, if a cell is going to exit the cell cycle and terminally differentiate, this phosphylation event is blocked. (Source: http://www.stjude.org/images/en_US/1/3130research-dyer_fig1-0902.jpg, 13/06/06

Lim *et al.*, (2005) examined if physiological levels of CLA alter the cell cycle progression of HT-29 cells and the expression and activities of cell cycle regulatory proteins. They found that CLA induced a G_1/S phase arrest which was accompanied by decreased cyclin A, D1 and E and increased p21 and its interaction with proliferating cell

nuclear antigen (PCNA) PCNA plays an essential role in DNA replication and different types of DNA repair, including nucleotide excision repair, mismatch repair, and base excision repair (reviewed in Tsurimoto, 1999) Lim *et al*, (2005) demonstrated that CLA decreased levels of phospho-Rb in a dose-dependent manner indicating that induction of p21 by CLA leads to inhibition of CDK activity resulting in reduced phosphorylation of CDK substrates and the induction of p21 both by p53-dependent and -independent mechanisms (Majumder *et al*, 2002)

In view of importance of lipid component on cell signaling pathway, the next three chapter of the present study delt with the effects of CLA on growth, apoptosis, differentiation, epigenetic influence of cell death and in modulation of cellular lipid composition of HT-29 human colon cancer cells

The objectives of this study were to investigate the effects of a CLA mixture of isomers, three of its constituent isomers *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA and *trans*-vaccenic acid (*t*-VA), a putative precursor of *c*9, *t*11 CLA on markers of growth, differentiation and apoptosis The HT-29 human colon cancer cell line was used as an *in vitro* model

CHAPTER 2

Effects of fatty acids on growth of HT-29 adenocarcinoma cell line

Abstract

The antiproliferative activity of conjugated linoleic acid (CLA) isomers has been well documented The aim of this study was to compare the growth inhibitory effects of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and trans vaccenic acid (*t*-VA) on HT-29 cells when delivered as complexes with bovine serum albumin (BSA) or as free fatty acids dissolved in ethanol Free fatty acid forms of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA inhibited cells growth in a dose and time dependent manner Relative IC_{50} values were 17±1 6 μM, 59±3 6 μM, 62±2 2 μM and 75±1 7 μM for *t*9, *t*11 CLA, CLA mixture, *t*10, *c*12 CLA and *c*9, *t*11 CLA respectively after 5 days of incubation *t*-VA was inhibitory at higher concentrations and LA showed a stimulatory effect up to 70μM Fatty acid:albumin complexes inhibited growth to a lesser extent than corresponding free fatty acids suggesting that albumin protects cytotoxic effects of CLAs in HT-29 cells The potency of treatments was *t*9, *t*11 CLA > CLA mixture > *t*10, *c*12 CLA> *c*9, *t*11 CLA This study has shown for the first time that *t*9, *t*11 CLA is the most potent cytotoxic CLA isomer in HT-29 cells

2.1 Introduction

New understandings of the nature of colon cancer development have identified metabolic processes as well as endogenous and exogenous factors which, under the influence of the immune system and genetics can modulate the process of carcinogenesis Sodium butyrate, produced in the human colon by bacterial enzymatic breakdown of dietary fibre, undigested starch and non-absorbed simple carbohydrates has emerged as having important structural and physiological effects on the colon It maintains a balance between proliferation, differentiation and apoptosis in both normal and colonic carcinoma cells (McIntyre *et al*, 1991) Other fatty acids of dietary origin that can inhibit growth of colorectal cancer cells include CLA, a collective term for isomers of linoleic acid with conjugated double bonds *c*9, *t*11CLA and *t*10, *c*12 CLA have been observed to be as potent as a CLA mixture of isomers in inhibiting growth in the SW480, HT-29 and MIP-101 colon tumour cell lines (Miller *et al*, 2002, Palombo *et al*, 2002)

t-VA is potentially a very important contributor to tissue levels of CLA It is now clear that several human tissues, in particular the intestine, can convert *t*-VA to *c*9, *t*11 CLA (Duffy *et al*, 2006) *t*-VA a major trans fatty acid in the fat of ruminants, is produced in the rumen and converted in mammary gland to *c*9, *t*11 CLA by Δ^9-desaturase (Turpeinen *et al*, 2002) Miller *et al*, (2003) demonstrated that *t*-VA inhibited the growth of MCF-7 human breast cancer cells and SW480 colon cancer cells by up to 41% and 36 %, respectively Another study has also shown that vaccenic acid in the form of either *cis* or *trans*, significantly reduced growth of HT-29 human colon cancer cells by 23% when compared with control cells (Awad *et al*, 1995)

The effects of CLA, just like those of other PUFAs may be mediated by different mechanisms epigenetic alterations in chromatin structure affecting accessibility to transcription factors, regulation of gene expression, modulation of specific signal transduction pathways through changes in protein kinase expression and activation (Kemp *et al*, 2003), lipid peroxidation (O Shea *et al*, 1999), direct action on gene transcription (Cho *et al*, 2006, 2005 and 2003, Lim *et al*, 2005, Kemp *et al*, 2003),

modulation of eicosanoids (Miller *et al*, 2001) and activation of transcription factors, for example PPARs (Kuniyasu *et al*, 2006)

The involvement of long chain fatty acids in signalling processes is dependent on their interaction with cells Nonesterified fatty acids circulate in plasma of mammals as albumin complexes Albumin solubilises fatty acids in the aqueous environment thus providing a reservoir of bound fatty acids to replenish free fatty acids depleted by cellular uptake A direct role for albumin in cellular uptake of fatty acids has also been proposed (Reviewed in Hamilton, 1998), albumin interacts with cell surface binding sites/receptors from a variety of mammalian cell types (Trigatti and Gerber, 1996) Models for the mechanism of uptake include transcytosis of albumin-fatty acid complexes in a process thought to involve caveolae, diffusion through the lipid bilayer or transfer to a membrane -transport protein apparatus (Hóstmark, 2003) The sequestering of albumin at the cell surface may have major implications for fatty acid-induced cytotoxicity The close association of serum albumin with the cell surface should facilitate the removal of cytotoxic fatty acids from the vicinity of cells and minimise damage to cell membranes

The HT-29 human adenocarcinoma cell line is one of the cell lines of intestinal origin which reversibly displays structural and functional features of mature intestinal epithelial cells Under normal culture conditions they display an undifferentiated phenotype but they can express an 'nterocyte-like' differentiated phenotype in response to sodium butyrate (Schroy, 1994) The collective evidence of antiproliferative effects of CLA suggests that CLA formulations could be developed as dietary adjuvants against to prevent colon cancer However, how the CLA mixture or specific isomers of CLA modulate the interaction of butyrate with its molecular targets is unknown The study therefore examined the effect of co-incubation with a CLA mixture and butyrate on growth prior to examining effects of CLA on specific targets of butyrate action (in Chapter 3)

2.2 Objectives

The objective of this study was to compare the effects of a CLA mixture of isomers, three of its constituent isomers *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA and *trans*-vaccenic acid (*t*-VA) on HT-29 cell growth when delivered as free fatty acids and as complexes with albumin

2.3 Materials and methods

2.3.1 Materials

Cell culture media Dulbecco's Minimum Essential Medium (DMEM) containing glucose (4 5g/L), L-glutamine (0 584g/L), $NaHCO_3$ (3 7g/L) and pyridoxine HCl (0 004g/L), supplements and related solutions were purchased from Sigma-Aldrich, Dublin, Ireland, unless otherwise stated The HT-29 human colon cancer cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA)

Conjugated Linoleic Acid (CLA) mixture of isomers (99% pure, approximately comprising 41% *c*9, *t*11 CLA, 44% *t*10, *c*12 CLA, 10% *c*10, *c*12 CLA and minor amounts of *t*9, *t*11 CLA, *t*10, *t*12 CLA, c9, c11 CLA) (Cat UC-59A) and single preparations (90% pure) of isomers *c*9, *t*11 CLA, *t*10, *c*12 CLA and 99% pure *t*-VA (Cat UC-60A, UC-61A and U48A respectively) were from NuChek-Prep, Elysian, MN, USA and 98% pure *t*9, *t*11 CLA (Cat 1181) from Matreya, Inc , Netherland Linoleic Acid (LA) was purchased from Sigma-Aldrich, Dublin (Cat L1012) All fatty acid preparations were dissolved in sterile filtered ethanol 1 g fatty acid in 10 mL ethanol to yield solutions of 99 mg/mL CLA mixture, *t*-VA and LA, 90 mg/mL *c*9, *t*11 CLA and *t*10, *c*12 CLA and 98 mg/ml pure *trans* 9, *trans* 11- (*t*9, *t*11-) CLA These were then divided into 1 mL aliquots and stored at -20 ^{o}C

Bovine serum albumin (BSA) 35% BSA (lipidated) (Cat A8918) and 10% BSA (delipidated) (Cat A1595) were purchased from Sigma-Aldrich, Dublin, Ireland

All sterile disposable plastic-ware was from Sarstedt Ltd , Wexford, Ireland Phosphate buffered saline (PBS) (Lennox, Cat BR14) was prepared by dissolving five tablets in 500 mL ultra-distilled water (dH_2O) This was then autoclaved at 115 °C for 20 min All water used in cleaning or for maintaining humidity in the incubator was also dH_2O autoclaved PBS and sterile water were both stored at room temperature Trypsin/EDTA solution (T/E) was made up as follows 50 mL of 10X Trypsin (Sigma, Cat T4549) and 10 mL of 1% w/v EDTA (Cat E6511) were added to 440 mL PBS This was aliquoted into sterile universal containers and stored at -20 °C A stock solution of 1% EDTA can be made up in advance and stored at 4 °C

2.3.2 Cell culture

2.3.2 1 Media preparation

Cell culture media was prepared as follows 25 mL (5% v/v) Foetal calf serum (FCS) (Sigma, Cat F7524), 5 mL (1 unit/ml) Penicillin /Streptomycin (P/S) (Cat P0781) and 0 5mL (1 mM) HEPES (Cat H0887) were added to Dulbecco's Minimum Essential Medium (DMEM) (Cat D5796) Complete media was stored at 4 °C for up to two weeks

2.3 2 2 Feeding

HT-29 cells were grown in a ShelLab, IR2424 model CO_2 humidified Incubator at 37°C with 5% CO_2 and 95% room air Cell culture work was carried out in a class II laminar airflow cabinet (Gelaire 85, BSB4 laminar air-flow cabinet) Protocol for maintenance of cell lines was adapted from O'Shea *et al* , (1999) The complete media was incubated at 37 °C for 20 min in a water-bath prior to use Industrial methylated spirits (IMS)

(Lennox, Cat 1170) was used to spray all internal surfaces of the laminar prior to use All bottles, plastics etc brought into the laminar, as well as gloves were also sprayed Waste media was drawn off from the flask with a pipette and transferred to a waste bottle The flask was then rinsed with PBS (Lennox, Cat BR14), 3 mL for T25 flask, 10 mL for T75 flask and again transferred to the waste bottle The appropriate fresh complete media was then added, 5 mL for T25 or 15 mL for T75 The flask was then sprayed with IMS and replaced in the incubator When finished working in the laminar all surfaces was washed down with Virkon solution (Lennox, Cat 222/0154/01) This was then rinsed using tissue paper damped with sterile water Once dry all surfaces were then sprayed down with IMS and allowed to dry again

2.3.2.3 Subculturing

Cells were grown in Falcon T-75 cm^2 flasks, fed every 2nd day and passaged twice a week after exposure to 0 25% (w/v) trypsin/ 0 02% (w/v) EDTA In details Media and trypsin /EDTA (T/E) solution were incubated at 37 °C for 20 min in a water-bath Waste media was drawn off and the flask rinsed with PBS as per feeding method T/E was then added, 2 mL for T25 or 4mL for T75 and incubated until all the cells were detached from the base of the flask (1-3 min) This solution was then transferred to a universal The flask was then rinsed with PBS, 3 mL for T25 or 10 mL for T75 and added to the universal This cell suspension along with a counter balance of another universal containing same volume of liquid was centrifuged at 1000 rcf for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin) The supernatant was removed and the pellet resuspended in 15 mL complete media or appropriate amount Following a cell count the appropriate amount of this cell suspension or stock was then used to re-seed a new flask at the required cell density Two days prior to setting up the each experiment the cell line was passaged and seeded at sufficient density so as to be 70-80% confluent on the day of the setting up the experiment

2.3.2.4 Treatment

Fatty acids used in the cytotoxicity tests on the HT-29 cells, were delivered in three different forms i e 1) as free fatty acids dissolved in ethanol and 2) as complex in two different types of bovine serum albumin (BSA) One was 35% BSA (lipidated), other one was 10% BSA (delipidated) in different molar ratio in DMEM medium

2.3.3 Cell counting and viability assays

2.3.3.1 Determination of cell proliferation by trypan blue exclusion method

A cell suspension is made as per Subculturing method i e a flask of cells was trypsinised, spun and resuspended, 1 mL of this suspension was then transferred to a microtube, into which 200 µL of 0 4% (w/v) trypan blue (Sigma, Cat T8154) was added This was then mixed and 10 µL of this mixture was pipetted to the side of the chamber of the haemocytometer enclosed by a cover slip and was drawn in by capillary motion

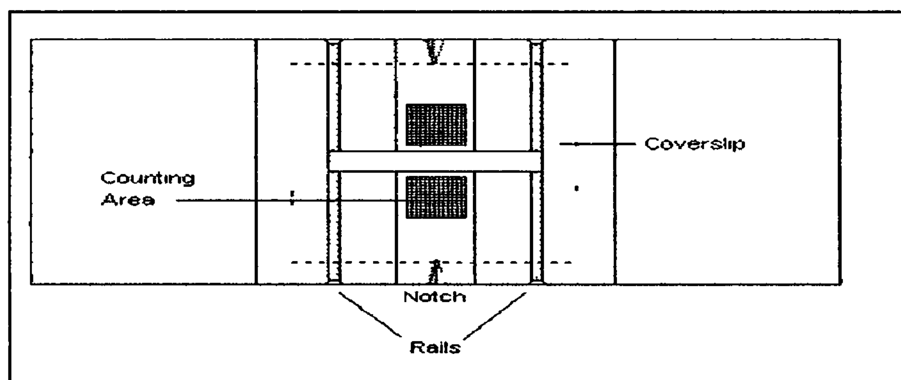

Figure 2.1 Diagram of Haemocytometer with coverslip

Cells were counted from the four large corner quadrants and the centre square as observed under the 10X objective This total number was divided by 5 to give the average cell number per square This was multiplied by the dilution factor of 1 2 and then by 10^4,

which results ın the total cell number per mL Vıable cells appear clear and do not staın, whereas non-vıable cells staın blue from the ınflux of trypan blue across breached membranes The percentage of growth ınhıbıtıon of HT-29 cells were measured related to control and calculated by usıng the followıng equatıon [(Vıable cell number ın control flask - Vıable cell number m sample flask)/Vıable cell number ın control flask x 100]

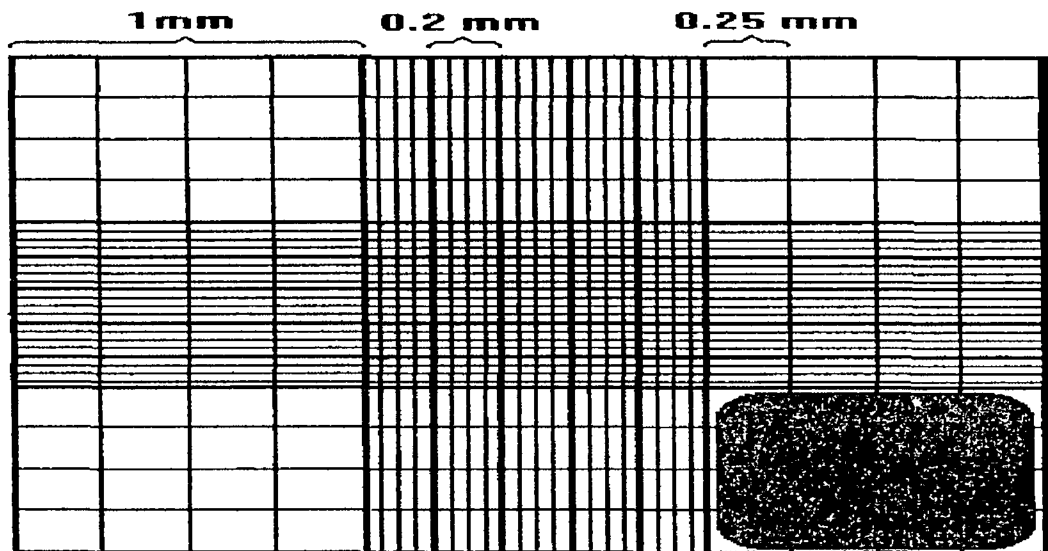

Fıgure 2.2 Illustratıon of squares on a haemocytometer, showıng one of the corner quadrants shaded The volume underneath the coverslıp of thıs area (or one square) ıs 0 1 mm^3 or 10^{-4} ml

2.3.3 2 Determınatıon of growth ınhıbıtıon of HT-29 cells by acıd phosphatase actıvıty assays

Cell growth was also measured usıng a mıcroplate acıd phosphatase actıvıty assay 96 well plates were used for thıs assay The acıd phosphatase (AP) assay ıs based on the abılıty of the AP enzyme ın the lysozomes of cells to hydrolyze the p-nıtrophenyl phosphate (pNPP) yıeldıng p-nıtrophenyl chromophore (Martın and Clynes, 1991)

The procedure in detail A confluent flask was trypsinised and cell suspension made as per Subculturing method A cell count was performed using trypan blue according to the Cell Counting method and a stock solution was made up at a cell density of 1 x 10^4 cells/mL Plates were seeded with 100 µL of cell stock in each well These were then cultured for 24 h following which *100 µL of treatments /media* was added to corresponding wells Cytotoxicity was assessed after specific time points

All assay reagents were prepared in advance PBS was made up as previously listed, as was a 1 M NaOH solution and both were stored at room temperature Sodium acetate buffer was prepared at a concentration of 0 1 M, containing 0 1% Triton X-100 and adjusted to pH 5 5 using glacial Acetic acid This was stored at 4 °C in the dark for up to one month The *p*-nitrophenyl phosphate (pNPP) (Cat P5869) was added immediately prior to performing the assay to yield a 10 mM solution

After the required incubation time all media was removed from the plates by flicking the plates upside-down over a waste container They were then rinsed with 100 µl of PBS 100 µl of freshly prepared 10 mM *p*-nitrophenyl phosphate substrate in sodium acetate buffer solution was added to each well, plates were incubated at 37^{0}C for 2 h Reaction were terminated by addition of 50µl of 1N NaOH each well (this caused an electrophilic shift in the *p*-nitrophenyl chromophore and thus developed the yellow color) After 10-15 minutes the plate were read at 405 nm on a Tecan A-5082 Sunrise microplate reader (Tecan, Austria) (O'Connor, 1998, Martin and Clynes, 1991) The percentage inhibition of cell growth of HT-29 cells was measured related to control and by using the following equation {(OD of control cells - OD of sample cells)/OD of control cells x 100}]

2.3.3.3 Linearity of acid phosphatase assay

To determine the accuracy of the acid phosphatase assay variable numbers of HT-29 cells in the range 1x10^2 to 10x10^2 were aliquots in 96 well plate in triplicate and incubated in a

humidified CO_2 incubator After 1, 3 and 5 days the assay was performed and the linearity of the assay was determined (Figure 2 3)

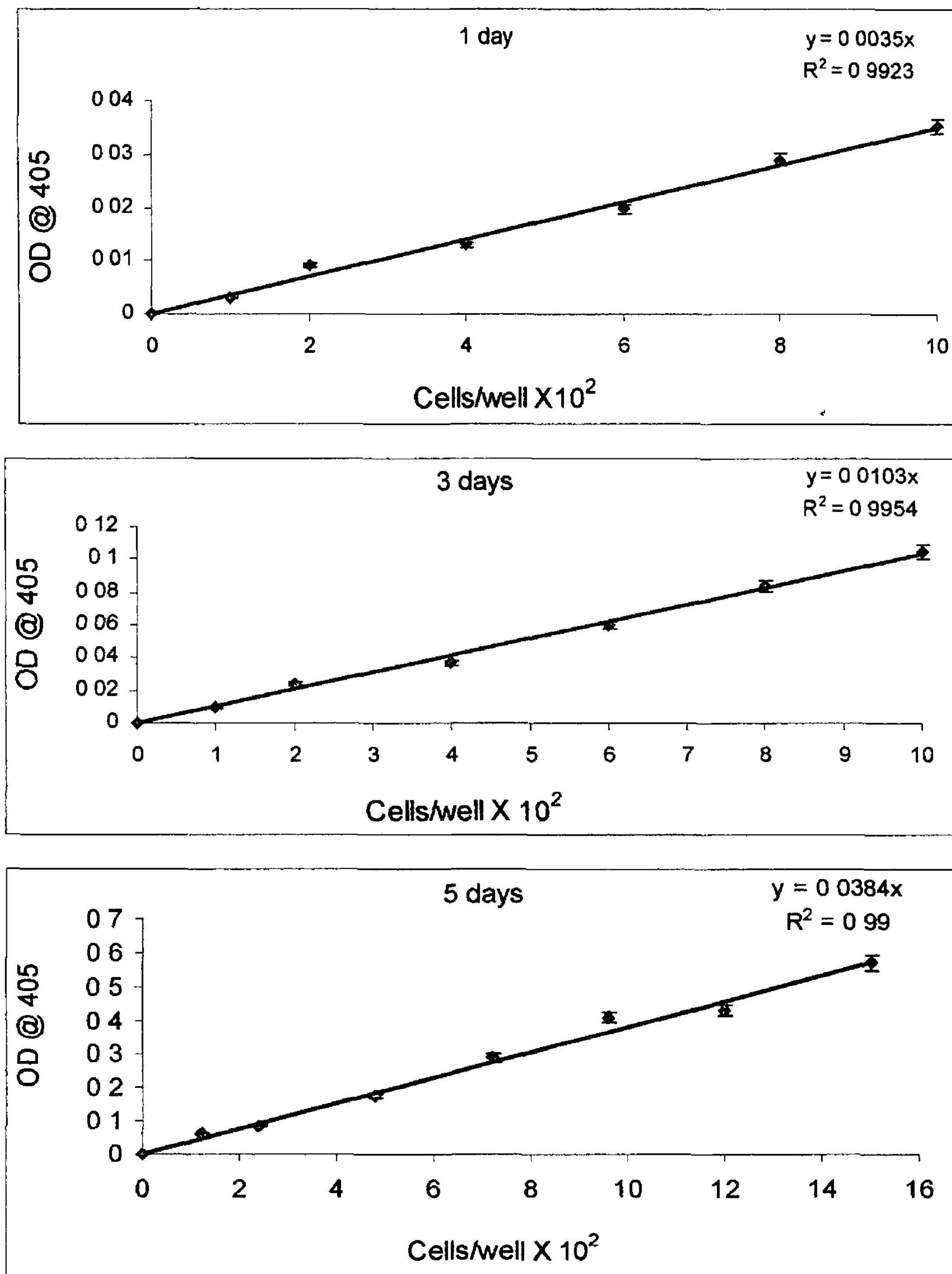

Figure 2.3 Growth of HT-29 cells after a) 1 day, b) 3 days and c) 5 days

2.3.4 Statistical analysis

All data are expressed as mean±SD calculated with Microsoft® Excel 2000 At least three independent experiments were performed in triplicate The statistical significance ($P<0\ 05$) was determined using the Student's t-test and was used to determine significance between treatments

2.4 Results

2.4.1 Effects of CLA isomers on growth of HT-29 cells

The effects of CLA isomers on cell growth were determined by incubating cells for 5 days with various CLA treatments in the range 0-200μM as either free fatty acids or as fatty acid/albumin (2 1 molar ratio) complexes Linoleic acid was included as a control Cell number was determined using a microplate colorimetric assay for cellular acid phosphatase, the activity of which is proportional to cell number (Fig 2 3) It is apparent that free fatty acid forms of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA inhibited growth in a dose-dependent manner after 5 days (Fig 2 4) Relative IC_{50} values were 17±1 6 μM, 59±3 6 μM, 62±2 2 μM and 75±1 7 μM for *t*9, *t*11 CLA, CLA mixture, *t*10, *c*12 CLA and *c*9, *t*11 CLA respectively Linoleic acid stimulated growth in the range 0-75μM and was inhibitory at higher concentrations Trans mono unsaturated vaccenic acid (*t*-VA) at a range of concentration 50-200μM inhibited growth of human HT-29 cancer cells by 10-48%

Prior to complexing fatty acids with albumin, the effects of different types of albumin (delipidated vs non delipidated) on growth of HT-29 cells was investigated over 1, 3 and 5 days Delipidated albumin (5-100μM) had no significant effect on HT-29 cell proliferation after 1 and 3 days treatment (Fig 2 5) After 5 days treatment, modest stimulation of growth by 30-40% was apparant By contrast non-delipidated albumin

exerted a time and dose-dependent growth inhibitory effect on HT29 cells proliferation. Growth was inhibited 10-20% on day 1 following treatment with albumin in the range 5-100μM. Growth was inhibited 15-30% on day 3 over the range 15-100μM and was inhibited 20-80% on day 5. Delipidated albumin was used for complexing fatty acids in the molar ratio 2:1 of fatty acid:albumin.

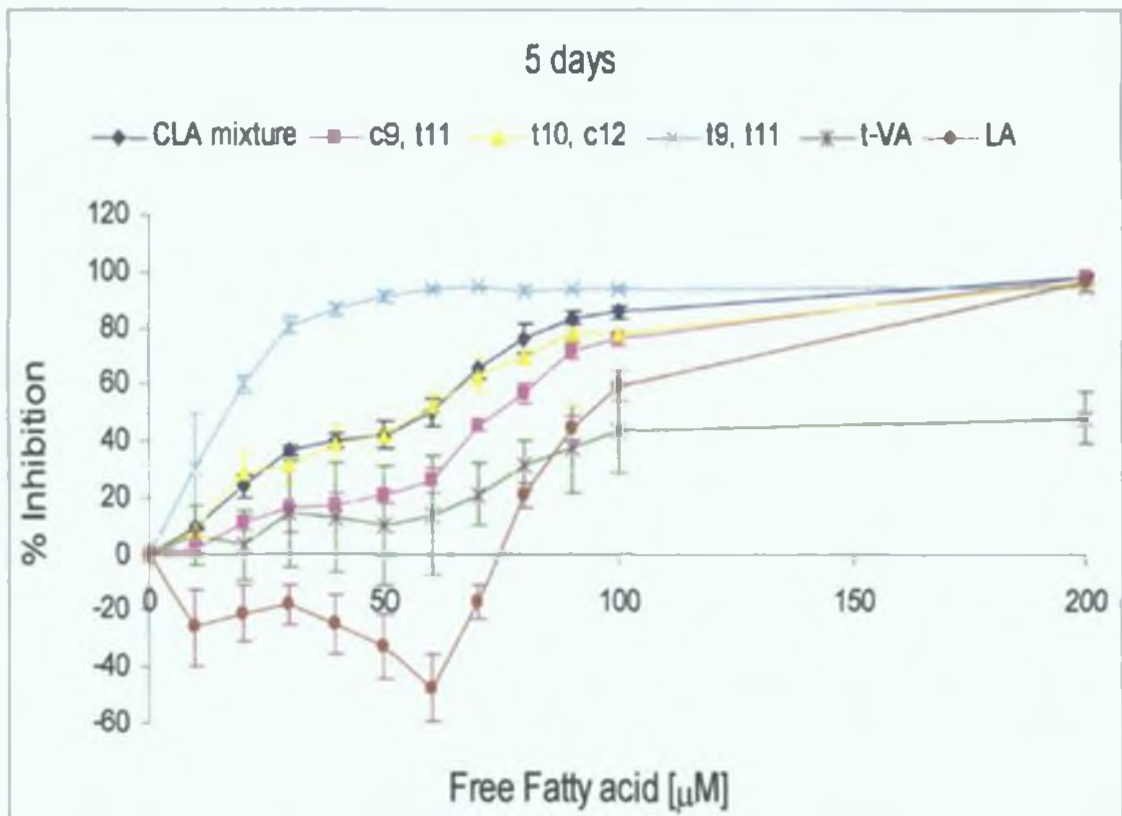

Figure 2.4 HT-29 cells were cultured at a density of 15 x 10^2 cells/well in a 96 well plate in DMEM medium incubated with 0-200 μM of CLA mixture of isomers, *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA, *t*-VA and LA as free fatty acids for 5 days. Growth inhibition was measured by acid phosphatase assay. Results shown are % of control; mean ± SD (n=6).

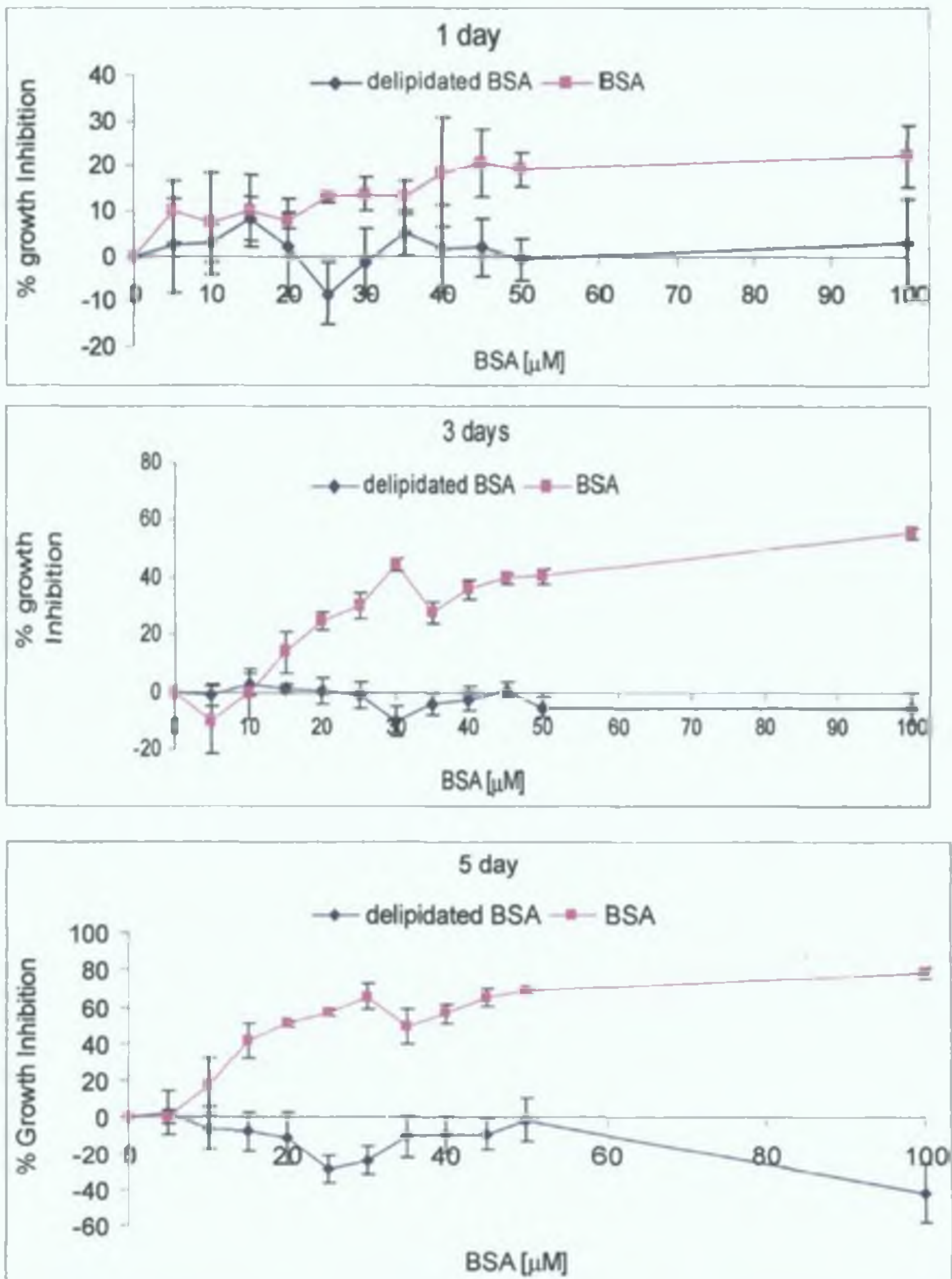

Figure 2.5 Growth of HT-29 cells. Cells were incubated with 0-100 µM of BSA for 1, 3 and 5 days. Results shown are % of control; mean ± SD (n=3).

Fatty acid: albumin complexes inhibited growth to a lesser extent than corresponding free fatty acids. It is apparent from Fig 2.6 that *t*9, *t*11 CLA was the most potent isomer (IC50 24.9±0.1 μM). There was a trend towards greater inhibition the CLA mixture of isomers and *t*10, *c*12 CLA than by *c*9, *t*11 CLA. *c*9, *t*11 CLA inhibited growth by 12-46% over the range 60-200μM. Growth was inhibited 17-56% by CLA mixture of isomers and 21-46% by *t*10, *c*12 CLA over the range 10-200μM. Linoleic acid : albumin complex had negligible effects on growth over the concentration range 0-200μM.

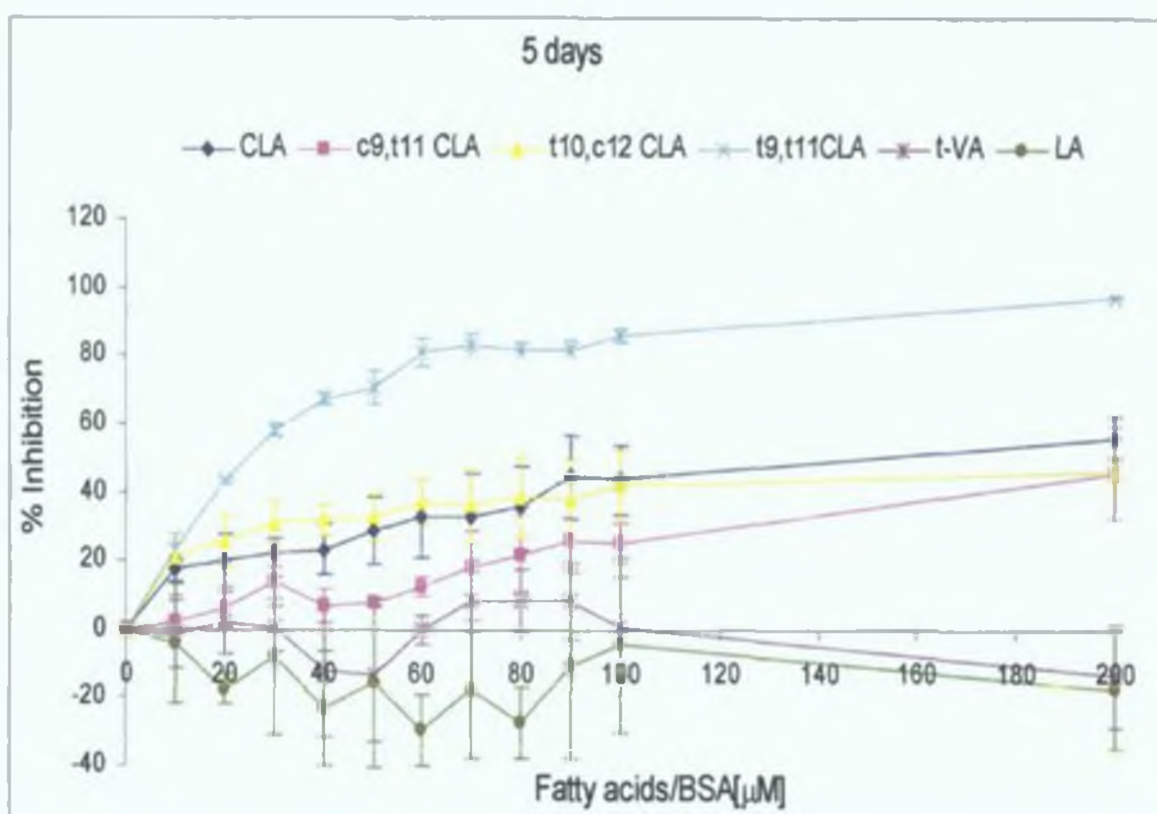

Figure 2.6 HT-29 cells were cultured at a density of 15 x 10^2 cells/well in a 96 well plate in DMEM medium incubated with 0-200 μM of CLA mixture of isomers, *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA; *t*-VA and LA for 5 days. All fatty acids were complexed with BSA prior to treatment at 2:1 ratio. Growth inhibition was measured by acid phosphatase assay. Results shown are % of control; mean ± SD (n=6).

Elevating the CLA mixture: albumin ratio from 2:1 to 8:1 resulted in greater inhibition of cell growth (Fig. 2.7), suggesting that cytotoxicity depends on molar ratio of CLA to albumin.

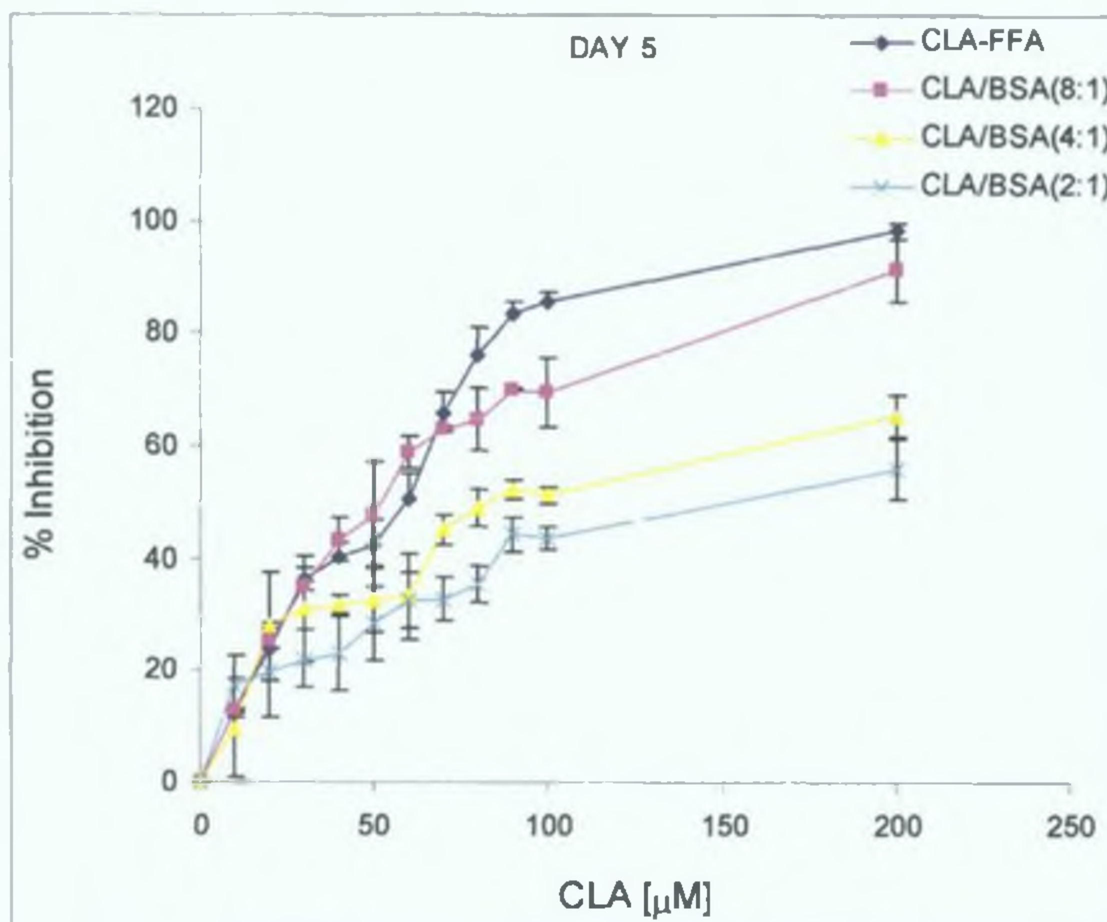

Figure 2.7 HT-29 cells were cultured at a density of 15 x 10^2 cells/well in a 96 well plate in DMEM medium incubated with 0-200 μM of CLA mixture of isomers as free fatty acids and complexed with BSA in the ratios of 2:1, 4:1 and 8:1 for 5 days. Growth inhibition was measured by acid phosphatase assay. Results shown are % of control; mean ± SD (n=3).

When HT-29 cells were incubated for 1, 3 and 5days with the CLA mixture of isomers, *t*10, *c*12CLA and *c*9, *t*11 CLA at 50, 70, 100 and 200μM maximum growth inhibition occurred by day 5 (Fig. 2.8). It was apparent that the various CLA treatments inhibited growth in a time-dependent manner and that the CLA mixture of isomers and *t*10, *c*12 CLA were more potent than equimolar concentrations of *c*9, *t*11CLA (Fig 2.8).

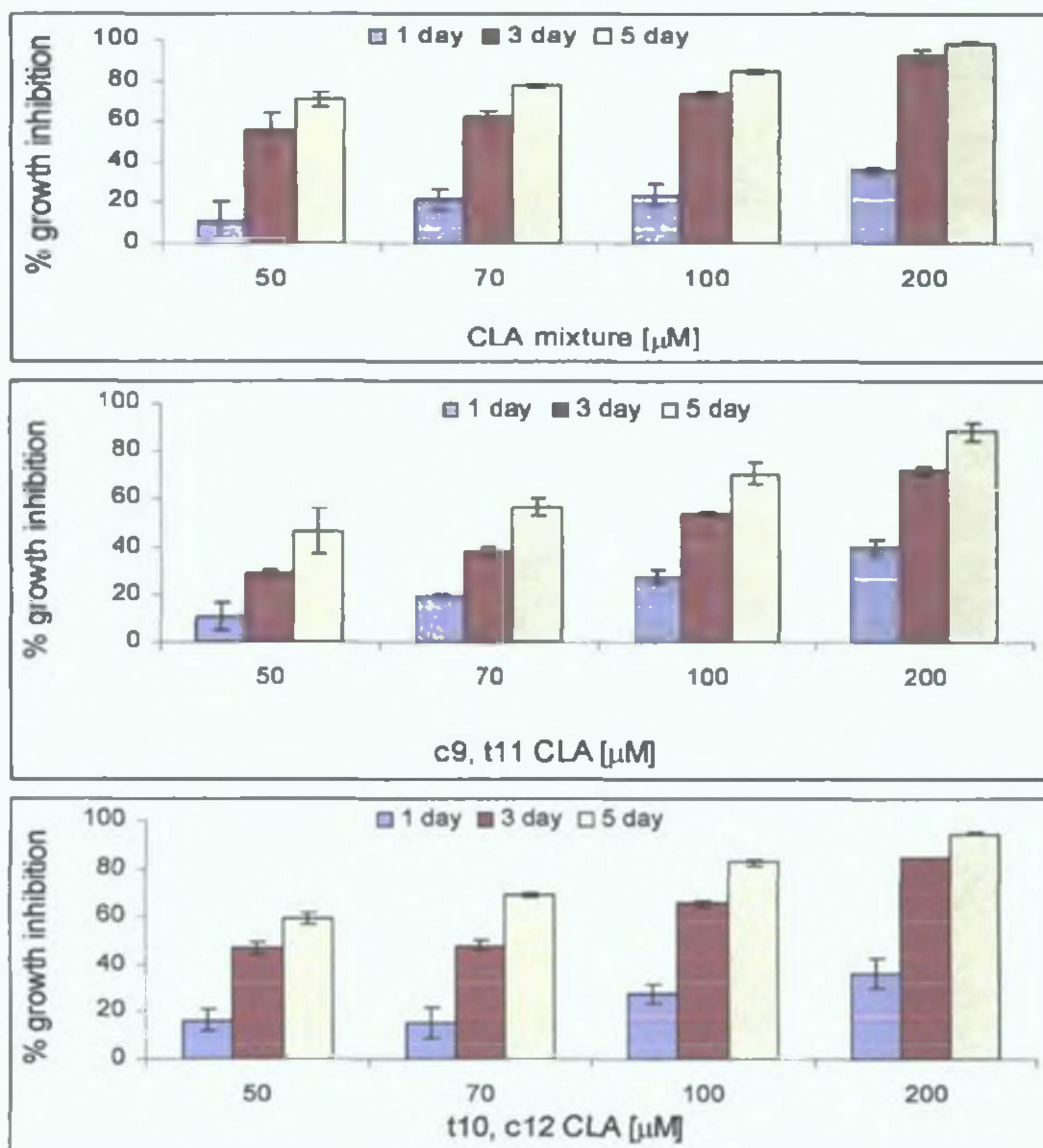

Figure 2.8 % growth inhibition of HT-29 cells after 1, 3 and 5 days when cells were incubated with 50-200 μM of CLA mixture of isomers, *c*9, *t*11 CLA and *t*10, *c*12 CLA. Results shown are % of control; mean ± SD (n=3).

2.4.2 Effect of CLA isomers in combination with sodium butyrate on growth of HT-29 cells

Similar observations of cytotoxicity by CLA isomers were shown by the method of trypan blue dye exclusion. Growth was reduced by 8-27% by various CLA treatments after 1day and by 20-67% after 3 days. The *t*9, *t*11CLA isomer appeared to be more potent than the CLA mixture, inhibiting growth by 27% after 1 day and 62% after 3days. *t*-VA inhibited growth by 6% after 3 days. Linoleic acid had negligible effects on growth. By contrast incubation with 3mM sodium butyrate inhibited growth by 25% after 1day and 66% after 3 days (Fig 2.9).

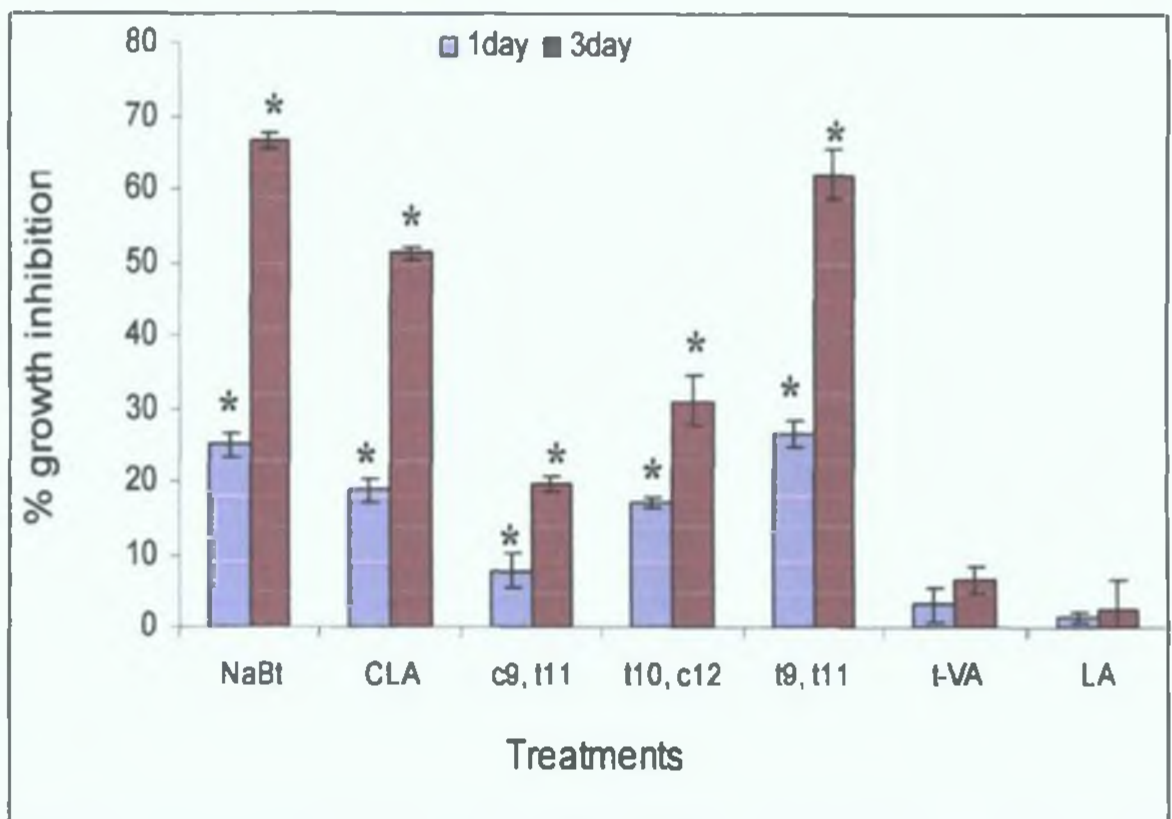

Figure 2.9 Effects of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA isomers, *t*-VA, LA (75μM) and 3mM NaBt on HT-29 cell number, as determined by trypan blue exclution. Results shown are the mean (±SD) of treated cells (n=3). Asterisks (*) denote significant values (P<0.05) relative to control.

A dose and time-dependent inhibitory effect on growth was also observed when HT29 cells were treated with sodium butyrate in the range 0-5mM (Fig 2.10). Cells treated with 1, 2, 3 and 5mM butyrate were harvested after 3, 6 and 9 days of incubation as described in methods. Control cells increased 4.4 fold in number between day 3 and day 9 (Fig 2.10). Butyrate (1mM) had no effect on cell growth over the course of the 9 days. Butyrate at 2mM significantly inhibited growth by approximately 50% on days 6 and 9. Butyrate at 3 mM inhibited growth by 60-70% on days 3, 6 and 9. Similarly, butyrate at 5mM inhibited growth by 56-88%.

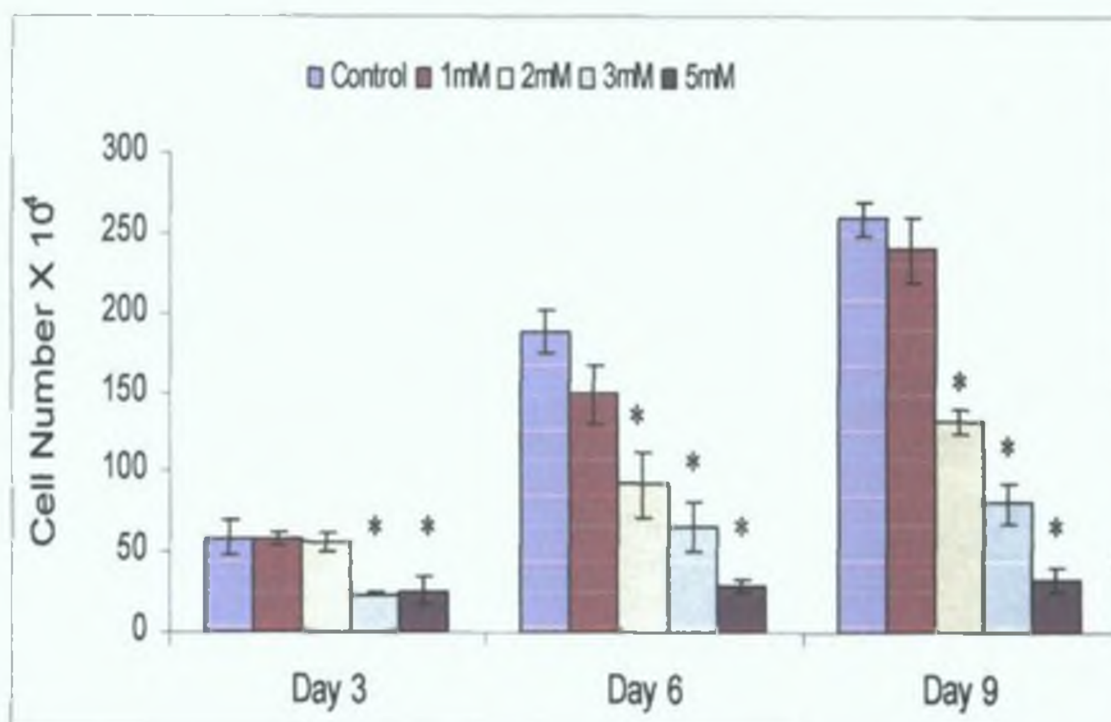

Figure 2.10 Effects of sodium butyrate (1, 2, 3 and 5 mM) on cell growth, as determined by trypan blue exclusion. Growth of HT-29 cells was monitored after 3, 6 and 9 days of treatment. Results shown are mean (±SD) of 3 experiments and compared to control. Asterisks (*) denote significant values (P<0.05) relative to control.

Co-treatment of cells with sodium butyrate (3mM) and either CLA mixture, *c*9, *t*11 CLA or *t*10, *c*12 CLA all at 75μM, inhibited growth by 74%, 67% and 78% compared with butyrate alone (55%) (Fig. 2.11).

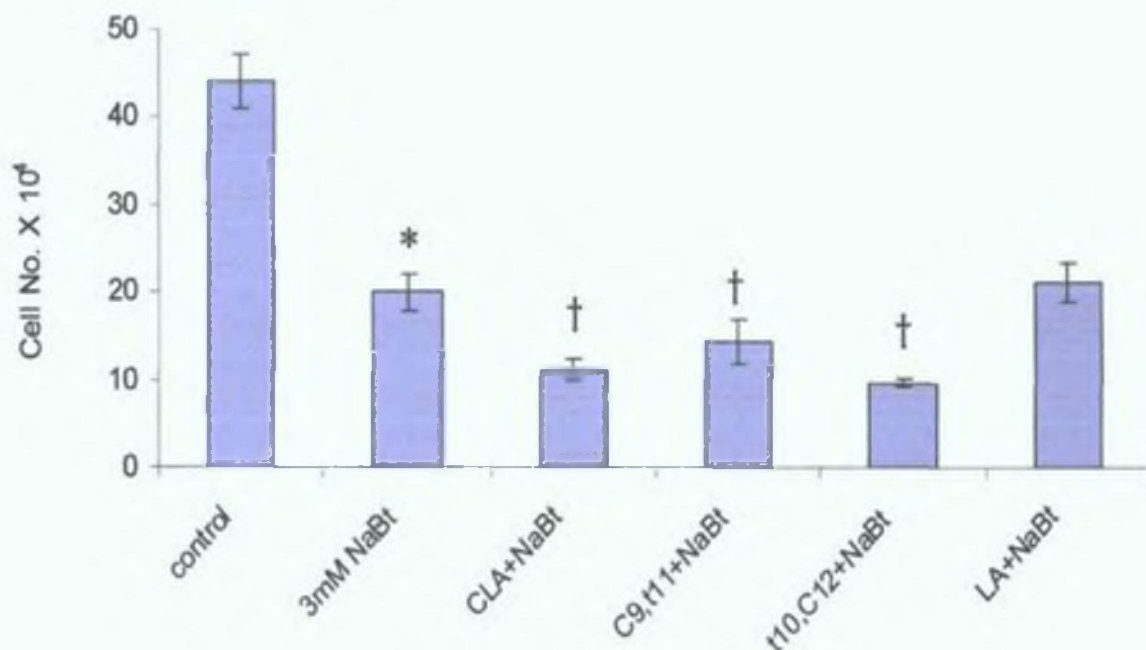

Figure 2.11 HT-29 colorectal cancer cells were cultured at a density of in DMEM medium 0.8 x 10^6 cells/flask in T75 cm^2 incubated with the combination of 3mM NaBt and 75 μM of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA and LA for 3 days. Cell proliferation was measured by trypan blue exclusion. Results shown are the mean (±SD) of treated cells (n=3) expressed as cell number compared to control. Asterisks (*) denote significant values (P<0.05) relative to control. Asterisks (†) denote significant values (P<0.05) relative to 3mM NaBt.

2.5 Discussion

The anticarcinogenic properties of CLA were established by several investigators using cell lines derived from mammary, colon and prostate cancers and animal models of mammary and colon carcinogenesis Shultz *et al*, (1992a) were the first to demonstrate the inhibitory property of a CLA mixture on MCF-7 cell growth Subsequent reports confirmed the inhibitory effect of CLA mixtures containing cis and trans-9,11- and 10,12- isomers in several colon cancer cell lines (SW480, HT-29, Caco 2, MIP and Colo320 cells) Most studies have reported on the inhibitory effects of a CLA mixture with IC50 values ranging between nanomolar up to high micromolar concentrations depending on cell line (Kuniyasu *et al*, 2006, Cho *et al*, 2003, Kemp, *et al*, 2003, Miller *et al*, 2003, Kim *et al*, 2002b, Palombo *et al*, 2002, Roche *et al*, 2001, Igarashi and Miyazawa, 2000) Because specific isomers of CLA have been shown to possess different biological activity in a number of systems we were interested in their potential to inhibit colon cancer growth

Emerging evidence suggests that bioproduction of CLA isomers by various probiotic cultures including lactobacilli and bifidobacteria (Alonso *et al*, 2003) may be a significant source of CLA isomers in addition to bovine milk fat and chemical synthesis The *t*9, *t*11 CLA isomer was identified as an end product CLA isomer in several bifidobacteria strains (Coakley *et al*, 2006 in press) Up to now the action of this type of CLA has not been investigated in cancer cells In light of substantial evidence demonstrating the health promoting properties of bifidobacteria, the possibility that probiotics may be working to inhibit cancer cell growth warrants investigation

This study showed that the HT-29 cell line was sensitive to growth inhibitory effects of not only the CLA mixture but also to three of its constituent isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA following incubation with CLA up to 200µM and including the physiological range (10-75µM) It is of note that levels >100µM have been detected in chronic alcoholics and patients with liver disease (Szebeni *et al*, 1986, Fink *et al*, 1985) and that levels up to 5 times that found in normal serum have been achieved in

humans following long-term supplementation with CLA (Petridou *et al*, 2003) Isomers differed in their range of antiproliferative activity (Fig 2 5), such as that at 75µM, the most potent isomer (*t9*, *t*11 CLA) inhibited growth about 88% while inhibition by the least effective isomer (*c9*, *t*11 CLA) was about 50% As shown in Figure 2 5, the order of potency was (most → least potent) *t9*, *t*11 CLA > CLA mixture > *t*10, *c*12 CLA> *c9*, *t*11 CLA The CLA mixture of isomers at 75µM (yielding a *c9*, *t*11 CLA and *t*10, *c*12 CLA of approximately 8 6µg/ml and 9 2µg/ml each) was equally effective in inhibiting growth as the *t*10, *c*12 CLA added at 75µM This suggests that a plateau effect was reached or that one or more of the other isomers present in the mixture may modulate growth A small number of studies has now revealed that *c9*, *t*11 CLA, the most common naturally occurring isomer in milk fat may not be as potent as other CLA isomers, including *t*10, *c*12 CLA and *c9*, *c*11 CLA (Tanmahasamut *et al*, 2004, Cho *et al*, 2003, Palombo *et al*, 2002) Tanmahasamut *et al*, (2004) showed that *c9*, *t*11 CLA was the least potent isomer in inhibiting growth of a breast cancer cell line However in a HT-29 cell line, *c9*, *c*11 CLA was less potent than *c9*, *t*11 CLA (Palombo *et al*, 2002) It is apparent that such differences may be cell line specific or may even be related to mode of delivery of CLA to cells The growth stimulatory effect of LA at concentrations up to 70µM previously reported in the SW480 cell line was also seen in this study

The short chain fatty acid butyrate, which is derived from the action of anaerobic colonic microflora on undigested polysaccharides is thought to be partially responsible for the anticarcinogenic properties associated with dietary fiber Previous *in vitro* studies using sodium butyrate indicate that it is a potent inducer of differentiation and apoptosis in a variety of cell culture systems (Rouet-Benzineb *et al*, 2004, Vincan *et al*, 2000, Benard and Balasubramanian, 1997, Scheppach *et al*, 1995) A dose and time dependant inhibition of HT-29 cells growth was observed with sodium butyrate The combined effects of CLA and sodium butyrate were greater than the effect of butyrate alone

Albumin is a highly abundant serum protein that serves as a transport vehicle for several endogeneous compounds including fatty acids (FA), hemin, bilirubin and tryptophan, all of which bind with high affinity (Høstmark, 2003) Analysis of literature has revealed

that CLA ıs delıvered to cells ın many forms conjugated to BSA at 20 1 molar ratıo (Palombo *et al*, 2002), 4 1 molar ratıo (Cho *et al*, 2003, Kım *et al*, 2002) or as free fatty acıds (Maggıora *et al*, 2004, Tanmahasamut *et al*, 2004, Mıller *et al*, 2003, O'Shea *et al*, 1999, Shultz *et al*, 1999) Some cell lınes e g Colo320 are extremely sensıtıve to nanomolar concentratıons of CLA (Kunıyasu *et al*, 2006), others are ınhıbıted by mıcromolar concentratıons ın the range low 5μM to 160μM The absence of data evaluatıng mode of delıvery on susceptıbılıty to growth has promted thıs ınvestıgatıon to systematıcally compare the effects of delıverıng a CLA mıxture as free fatty acıds and as complexes to the HT-29 cell lıne Thıs study also ınvestıgated the growth modulatory effects of sıngle CLA ısomers when complexes wıth albumın (2 1 ratıo) Thıs ratıo was chosen on the basıs that under normal phygıologıcal condıtıons up to 2 mol of fatty acıd are bound to albumın, but the molar ratıo of fatty acıd/albumın can rıse to 6 1 or greater ın the perıpheral vasculature durıng fastıng or extreme exercıse or under physıologıcal condıtıons such as dıabetes, lıver and cardıovascular dısease (Høstmark, 2003 and 2005)

Interestıngly all of the CLA-albumın complexes were less effectıve ın ınhıbıtıng growth than the unbound free fatty CLA ısomers The CLA mıxture and *t*10, *c*12 CLA was more potent than the sıngle *c*9, *t*11 CLA ısomer Other reports also ındıcate that CLA mıxture bound to albumın *t*10, *c*12 CLA bound to albumın were more effectıve than *c*9, *t*11 CLA ısomer ın ınhıbıtıng growth of HT-29 cells (Palombo *et al*, 2002), no study has yet compared theır effects wıth those of *t*9, *t*11 CLA It ıs apparent that of the ısomers studıed *t*9, *t*11 CLA was the most potent It ıs ımportant that more basıc research ıs undertaken to determıne the specıfic cellular and molecular effects of thıs and other ısomers present ın the mıxture

Many polyunsaturated fatty acıds protect agaınst colon carcınogenesıs ın part by enhancıng oxıdatıve stress and ınducıng apoptosıs Theır ıncorporatıon ınto mıtochondrıal membrane phospholıpıds enhances membrane lıpıd oxıdatıon and the moderatıon of mıtochondrıal potentıal whıch contrıbutes to the ınductıon of apoptosıs (Ng *et al*, 2005) CLA also prone to oxıdatıon and ıt has been suggested that ıncreased lıpıd oxıdatıon may contrıbute to the antı-tumorıgenıc effects of thıs agent (Mıller *et al*, 2002, O'Shea *et al*,

1999) The relative protection afforded to HT-29 cells by CLA-albumin complexes relative to free fatty acids may relate to the known antioxidant activity of albumin Human and bovine serum albumin afford considerable protection against damage to decarboxyribose and DNA mediated by highly reactive hydroxyl radicals (Smith *et al*, 1992)

t-VA has been shown to elicit a biological response *in vivo*, reducing mammary gland premalignant lesions in carcinogen-treated rats (Banni *et al*, 2001) The present study showed that *t*-VA at 70μM inhibited growth of human HT-29 cancer cells by 21% after 5 days which was much lower than all CLA treatments (45%-94%) Growth inhibition by *t*-VA may be due to its conversion to *c*9, *t*11 CLA by Δ9-desaturase enzyme (Lock *et al*, 2004)

The prospective cohort study in Sweden revealed that high-fat dairy foods (including whole milk, full-fat cultured milk, cheese, cream, sour cream, and butter) may lower the risk of colorectal cancer and suggested that CLA may be the component in high fat dairy foods providing the protective effect (Larsson *et al*, 2005) Although much progress in elucidating mechanisms of action has been made, additional supportive and consistent data is required from more in vitro and in vivo laboratory studies, clinical trials and epidemiology to achieve consensus and sound scientific agreement about the beneficial effects of receiving CLA as dietary supplements To this end appropriate molecular and biochemical markers of CLA exposure and its cellular targets are being sought

CHAPTER 3

Effects of CLA isomers on biological markers of apoptosis, differentiation and epigenetic regulation in HT-29 human colon cancer cells

Abstract

Dietary conjugated linoleic acid (CLA) has been shown to reduce colon tumor incidence in rodents by mechanisms probably involving differentiation and/or apoptosis The aim of this study was to examine the effects of a CLA mixture, *c*9, *t* 11 CLA, *t*10, *c* 12 CLA, *t*9, *t*11 CLA and trans vaccenic (*t*-VA) acid on selective biological markers of apoptosis, differentiation and epigenetic influences in HT-29 colon cancer cells The CLA mixture of isomers, *c*9, *t*11 CLA and *t*10, *c*12 CLA increased annexin V binding to phosphatidylserine suggesting apoptosis after incubation with a physiological level of fatty acid An elevated level of ceramide was observed in HT-29 cells by the CLA mixture of isomers after 3 days incubation All CLA treatments increased carcinoembryonic antigen (CEA) level Histone deacetylase (HDAC) activity was inhibited by *c*9, *t*11 CLA and *t*10, *c*12 CLA suggesting that these two isomers may exert antiproliferative effects in HT-29 cells by modulation of histones The *t*9, *t*11 CLA isomer inhibited HDAC activity and increased CEA level in HT-29 cells but had no effects on ceramide or apoptosis suggesting that cells may have undergone differentiation prior to death by necrosis *t*-VA had no effects on either CEA or HDAC

3.1 Introduction

Colon cancer development is strongly influenced by diet Of the numerous dietary factors that modulate colon cancer incidence in animal models, the amount and type of dietary fats and fibre consumed has received the most attention For example, high fat corn oil diets rich in linoleic acid enhance the development of colon tumors (Reddy and Maeura, 1984, Sakaguchi *et al*, 1984), whereas high fat fish oil diets reduce colon cancer incidence (Calviello *et al*, 1999, Rose and Connolly, 1999, Bartsch *et al*, 1999, Anti *et al*, 1997 and 1992,) Colonic luminal nutrients such as butyrate derived from bacterial fermentation of complex carbohydrates reduced the size and number of tumours (Dzierzewicz, *et al*, 1999, Wolin, 1993, Young and Gibson, 1993) A recent epidemiological study has shown that women consuming a high intake of conjugated linoleic acid (CLA), a type of fat found naturally in dairy products of ruminant origin were predisposed to a significantly lower risk of colon cancer (Larsson *et al*, 2005) Recognition that dietary fats interact with endogenous short chain fatty acids produced in the human colon to modulate colonic cytokinetics in human subjects has stimulated new investigations into how CLA may interact with butyrate at the molecular level

Many pathways may be implicated epigenetic alterations in chromatin structure affecting accessibility to transcription factors, regulation of gene expression, modulation of specific signal transduction pathways through changes in protein kinase expression and activation, lipid peroxidation, modulation of eicosanoids and activation of transcription factors Overall, it is likely that dietary fiber and high CLA diets modulate one or more of the range of genes and signalling pathways known to play a role in colon cancer

Butyrate is a potent modulator of gene regulation Accessibility of gene promoters to transcription complexes is an important level of gene regulation Histone acetylases and deacetylases control accessibility by the addition or removal of acetyl groups to the lysine residues of histones Because DNA is nucleophilic (cation-attracting), histones bearing positively charged deacetylated lysines are more attracted to DNA resulting in the DNA becoming more compacted and less exposed for transcription Conversely, histones

bearing acetylated lysines are less attracted to DNA and are more exposed for transcription and reactivation of tumor suppressor genes which had been silenced by hypoacetylation during tumorigenesis Butyrate permits histones to remain in an acetylated state, and through the resulting alterations in gene regulation, inhibits cell cycle progression and in some cases induces apoptosis and differentiation (Orchel *et al*, 2005, Davie, 2003)

Differentiation is a process by which a cell matures and becomes capable of performing specific functions It can involve both morphological and functional alterations Morphological differentiation emphasises changes within the cellular structure and organisation of the cell while functional differentiation focuses on biochemical and enzymatic function (Rudolph *et al*, 2001)

Butyrate is a potent differentiating agent that promotes the expression of differentiation markers such as alkaline phosphatase (ALP) in colonic cell lines Butyrate has also been shown to influence the morphology and motility of cancer cells *in vitro* Incubation of colonic cell lines with this short chain fatty acid resulted in an increased number of cells progressing to a more differentiated phenotype and subsequent apoptosis, the sequence of events typical for a normal nontransformed crypt cell These events were associated with modulation of activity of c-Jun N-terminal kinases and protein kinase C signal transduction pathways and induction of p21 (Orchel *et al*, 2005) Similarly butyrate is a potent inducer of carcinoembryonic antigen (CEA) expression in colon cancer cell lines differing in their degree of differentiation Increased expression has often been associated with colon carcinomas that have a more differentiated phenotype (Frangsmr *et al*, 1999)

Lampen *et al*, (2005) provided new evidence that the cellular and molecular effects of *c*9, *t*11 CLA may be related to promotion of a more differentiated phenotype in CaCo 2 colonic epithelial cells CLA activated the 5'flanking region of the alkaline phosphatase promoter, increased the expression of alkaline phosphatase mRNA and its specific enzyme activity Other significant effects induced by CLA included downregulation of target genes of the APC-β-catenin-TCF-4 and PPAR δ signalling pathways In particular,

expression of c-myc, c-jun, β-catenin, PPARδ, cyclin D1 and promoter activities of c-myc and AP1 were decreased in a concentration-dependent manner (Lampen *et al*, 2005) Downregulation of the β catenin-TCF-4 pathway is associated with an ability to induce cell differentiation Together with earlier data showing that CLA could downregulate ErbB3 signaling, PI3 kinase Akt signaling, prevent accumulation of hyperphosphorylated Rb and cyclin-cdk complexes (Lim *et al*, 2005, Cho *et al*, 2003, Kemp *et al*, 2003), it would appear that CLA-induced growth arrest and activation of cell apoptosis may be end stages of terminal colonocyte differentiation

Other endogenous biological factors associated with fatty acid-induced differentiation and apoptosis are sphingolipid metabolites such as ceramide and sphingosine Direct evidence for an involvement of sphingolipid signaling in growth arrest by polyunsaturated fatty acids was provided recently when omega-3 polyunsaturated fatty acids attenuated breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway (Wu *et al*, 2005) Sphingomyelinase is an enzyme that catalyzes the hydrolysis of sphingomyelin (SM) to ceramide A variety of studies have shown that ceramide is ubiquitously produced during cellular stress and is associated with apoptosis Furthermore, treating cells with synthetic short-chain ceramide has been shown to induce cell-cycle arrest and apoptosis Ceramide levels also changed during progression through the cell cycle and have been shown to enhance expression of p21, a cellular inhibitor of cdk2 kinase that is involved in cell-cycle arrest via hypophosphorylation of retinoblastoma protein (pRb) The importance of sphingolipid signaling in CLA-mediated induction of differentiation and/or apoptosis of HT-29 cells has not yet been assessed The aim of the study was to investigate the effect of CLA and its isomers on ceramide mass content in cells and on biological markers of apoptosis (Annexin V binding to phosphatidyl serine), differentiation (CEA activity, and alkaline phosphatase activity) and epigenetic regulation (HDAC)

3.2 Objectives

The objective of the present study was to elucidate whether apoptosis and/or differentiation mediate the antiproliferative effects of CLA in the colon HT-29 cell line The effect of 4 different CLA preparations (*c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and the CLA mixture of isomers) on ceramide mass content in cells and on biological markers of apoptosis (Annexin V binding to phosphatidyl serine), differentiation (CEA and ALP) and epigenetic regulation (histone acetylation) were investigated

Specific aims were as follows

- To set up a reproducible method for the assay of ceramide and sphingosine and to determine the effect of fatty acids on the levels of these sphingolipids in HT-29 colon cancer cells
- To determine the proportion of apoptotic HT-29 cells following CLA treatments using fluorescense activated cell sorting analysis with annexin V-PI (propidium iodide)
- To determine the effect of fatty acids on histone deacetylase activity in HT-29 colon cancer cells
- To determine the effect of fatty acids on CEA and on the specific activity of ALP in HT-29 colon cancer cells

3.3 Materials and methods

3.3.1 Materials

HT-29, a colon adenocarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) Culture media and supplements were purchased from Sigma-Aldrich (Dublin, Ireland) HT-29 cells were maintained in Dulbecco's Minimum Essential Medium supplemented with 5% (by volume) fetal calf serum, 1 unit/mL penicillin and streptomycin and 1 mM HEPES Sphingosine, N-acetyl-D-sphingosine (i e ceramide) and O-phthaldehyde (OPA) were purchased from Sigma-Aldrich (Dublin, Ireland) Conjugated Linoleic Acid (CLA) mixture of isomers (99% pure, approximately comprising 44% *trans*10, *cis*12-, 41% *cis* 9, *trans* 11/ *trans* 9, *cis* 11, 10% *cis* 10, *cis* 12 and minor amounts of *trans* 9, *trans* 11, *trans* 10, *trans* 12, *cis* 9, *cis* 11-CLA) (Cat UC-59A) and single preparations (90% pure) of isomers *cis* 9, *trans* 11- (*c*9, *t*11-), *trans* 10, *cis* 12- (*t*10, *c*12-) CLA and 99% pure *t*-VA (Cat UC-60A, UC-61A and U48A respectively) were from NuChek-Prep, Elysian, MN, USA and 98% pure *trans* 9, *trans* 11- (*t*9, *t*11-) CLA (Cat 1181) from Metreya, Inc , Netherland PA All other chemicals and solvents used were of HPLC grade (AGB Scientific Ltd, Dublin 11, Ireland) Vybrant™ Apoptosis Assay Kit #2 was purchased from Molecular Probes, Inc and contained the following components

- **Alexa Fluor 488 annexin V** (Component A), 250 µl of a solution in 25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7 4, plus 0 1% bovine serum albumin (BSA)
- **Propidium iodide** (Component B), 100 µl of a 1 mg/ml (1 5 mM) solution in dH_2O
- **5X Annexin-Binding Buffer** (ABB) (Component C), 15 ml of 50 mM HEPES, 700 mM NaCl, 12 5 mM $CaCl_2$, pH 7 4

Fluometric HDAC assay kits (Cat JM-K330-100) purchased from MBL Med & Biol Lab CO ,LTD, Woburn, MA, USA) CEA colorimetric ELISA kit (Cat BC-1011) purchased from (BioCheck Inc, CA) Alkaline phosphatase assay kit sigma 104

containing Phosphatase substrate (Cat 104-40), p-Nitrophenol standard solution (Cat 104-1) and Alkaline buffer solution (Cat 221) was purchased from Sigma-Aldrich, Dublin, Ireland

3.3.2 Quantitative analysis of cellular ceramide

3.3.2.1 Cell culture

The HT-29 cells were seeded in Falcon T-75 cm^2 flasks at a density of 1 5 x 10^6 cells/flask for 3 days treatments and $3x10^6$ cells/flask for 1 day treatments and allowed to culture for 24 h Cells were maintained at 37°C with medium, pH 7 2-7 4 via a required flow of 95% air and 5% CO_2 Next day the medium was replaced with 15 mL fresh media containing 3 mM NaBt, the CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA or *t*-VA at concentrations of 75 μM (dissolved in 100% ethanol) Control flasks were supplemented with equivalent volumes of ethanol (0 028% v/v) After 1 and 3 days of incubation, both floating and adherent cells were harvested and pooled and counted using trypan blue exclusion method as described in chapter 2 Cellular lipid extraction, sphingolipid extraction by alkaline hydrolysis, deacylation of ceramide, derivatisation of sphingosine with O-phthaldehyde (OPA) and calculation of cellular ceramide were performed as described by Santana *et al* (1996)

3.3.2.2 Cellular lipid extraction

2 mL of lipid extraction solution (chloroform methanol 1 M HCl, 100 100 1, v/v/v) was added to cell pellets and then vortexed 1 minute, 0 6 mL of balanced salt solution (BSS) and 100 mM EDTA (9 1, v/v) solution was added to each cell extract The balanced salt solution contained 135 mM NaCl, 4 5 mM KCl, 1 5 mM $CaCl_2$, 0 5 mM $MgCl_2$, 5 6 mM glucose and 10 mM HEPES, pH 7 2 The samples were vortexed for 30 sec and centrifuged at 1000 rpm for 5 mm using a Labofuge 13 (Heraeus Instruments, Hanau,

Germany) 500 µL of the lower organic phase was transferred to two different sets of tube One set of the tubes was marked 'basal', where an alkaline hydrolysis step was carried out to detect the base level of sphingosine and the other set was marked 'total' and was deacylated to convert ceramide to sphingosine, 'Total' represent ceramide levels plus 'basal' sphingosine levels

3.3.2 3 Sphingolipid extraction by alkaline hydrolysis

Sphingosine (SP_1) standards (0-2000 pmol) dissolved in chloroform:methanol (1 1, v/v) and dried down under nitrogen were prepared alongside the 'basal' samples 500 µL of 0 1 M KOH in methanol was added to the lipid film of both standard and sample tubes, the tubes capped, vortexed and incubated for 1 h in a 37°C water-bath (Grant Instruments, Cambridge, England) 500 µL of chloroform and 270 µL of BSS and 30 µL of EDTA solution were added to all tubes to extract the sphingolipids The tubes were then vortexed and centrifuged (Labofuge 13) at 800 rpm for 5 min 500 µL of the lower organic phase was transferred to eppendorfs where samples were dried down under nitrogen

3.3.2.4 Deacylation of ceramide

Sphingosine (SP_2) and ceramide standards (0-2000 pmol) were dissolved in chloroform methanol (1 1, v/v) to calculate the overall recovery of sphingolipids and the efficiency of the deacylation procedure and dried down under nitrogen alongside the 'total' samples 500 µL of 1 M KOH in methanol was added to the lipid film of all tubes to deacylate the ceramide, the tubes capped, vortexed and incubated at 100°C for 1 5 h in a heating block (COD Reactor purchased from Hach Company, Loveland, Colombia, Canada) The tubes were allowed to cool and were neutralized with 500 µL of 1 M HCl in methanol The sphingoid base was then extracted from each tube by adding 1 mL of chloroform and 900 µL of 1 M NaCl, the tubes were vortexed and centrifuged at 800 rpm for 5 min All of the lower phase was then collected and dried down under nitrogen

3.3.2.5 Derivatisation with O-Phthalaldehyde (OPA)

All samples were derivatised with OPA reagent (99 mL 3% (w/v) boric acid in water, pH 10 5, mixed with 1 mL ethanol containing 50 mg OPA and 50 µL of 2-mercaptoethanol) OPA reagent was prepared fresh daily Neat sphingosine (SP_{NEAT} in the range 0-2000 pmol) dissolved in chloroform methanol (1 1, v/v) and dried down under nitrogen was prepared alongside the other samples and standards to determine the efficiency of the deaceylation procedure of ceramide The sphingoid base in each tube was redissolved in 50 µL of methanol and mixed with 50 µL of OPA reagent and the tubes were incubated at room temperature for 15 min 500 µL of methanol 5 mM potassium phosphate (9 1, v/v), pH 7 0 was added to the tubes, which were then micro-centrifuged at 1600 rpm for 30 s in a Biofuge 13 (Heraeus Instruments, Hanau, Germany) to clarify the samples 500 µL was then transferred to HPLC vials

3 3 2.6 HPLC separation and quantitation

The derivatised sphingosine was separated by HPLC (Varian 9012) fitted with a Dynamix® AI-200 automatic sample injector with a 50 µL injection loop and quantitated using a Varian 9075 fluorescence detector A Nova Pack® C18 column (Waters, Milford, MA, USA) was used for the separation A mobile phase of methanol 5 mM potassium phosphate, pH 7 0 (9 1, v/v) and flow rate of 0 6 mL/min were used to elute the samples An excitation wavelength of 340 nm and an emission wavelength of 455 nm were used

3.3.2.7 Calculations for ceramide cellular content

The method takes advantage of the low basal levels of sphingosine existent in the cells, and the fact that ceramide can be deacylated to generate the free amino group containing sphingosine Sphingosine can, in turn, be derivatized to form flurescent compound,

separated by HPLC from the other sphıngoıd bases, and quantıtated by flurescence detectıon Basal cellular levels of sphıngosıne are also measured ın each sample and thereafter subtracted from the total sphıngosıne accumulated as a consequence of ceramıde deaceylatıon, to obtaın the cell content ceramıde (Santana *et al* , 1996)

Calculatıons for determınıng cellular ceramıde content were carned out accordıng to Santana *et al* , (1996) as follows

pmol ceramıde = [(pmol total x **% recovery of sphıngolıpıd by alkalıne hydrolysıs** x **% efficıency of deacylatıon) – pmol basal]**

Where, **pmol total** = pmol ın the total sample (deacylated) read agaınst the ceramıde standard curve,

efficıency of deacylatıon = averaged peak areas of each poınt ın ceramıde standard curve/peak areas of SPneat standard curve,

efficıency of sphıngolıpıd recovery = averaged peak areas of each poınt ın SP_2 standard curve/peak areas of SP_1 standard curve,

pmol basal = pmol ın the basal sample (alkalıne hydrolysıs) read agaınst the SP_1 standard curve

3 3.2.8 Standard curves

Four standard curves were set up as per "Materıals and Methods" sectıon All standard curves were prepared ın the range 0-2000 pmol Each standard was prepared and analyzed for sphıngosıne and ceramıde (converted to sphıngosıne) content usıng HPLC to obtaın relıable lınear correlatıons between sphıngosıne concentratıon and peak area Below (Table 3 1) ıs a summary of the preparatıon of each standard curve A sphıngosıne standard curve (SP_1) was set up and subȷected to an alkalıne hydrolysıs step ın addıtıon to

derivatisation and was quantified using HPLC. A second sphingosine standard curve (SP_2) was set up and subjected to deacylation, derivatisation and quantified using HPLC. The purpose of these two steps was to calculate the overall recovery of sphingosine from the extraction procedure, which would then be used when quantifying basal sphingosine levels in the HT-29 cell extracts. Another standard curve was constracted in which ceramide was deacylated, derivatised and quantified using HPLC. A sphingosine standard curve (SPneat) was also set up and subjected to derivatisation with OPA and was quantified using HPLC. By comparing the ceramide standard curve with the SPneat standard curve, it is possible to calculate the efficiency of the deacylation procedure. The ceramide is converted to sphingosine via the deacylation procedure and thus a ceramide standard curve allows for quantification of total sphingosine (basal sphingosine levels plus deacylated ceramide levels) in the HT-29 cell samples. A simple subtraction of basal sphingosine levels from total sphingosine levels will subsequently yield cellular ceramide levels.

Table 3.1 Summary of standard curve preparations

Standard	Alkaline Hydrolysis	Deacylation	Derivatisation
SPneat	-	-	√
SP_1	√	-	√
SP_2	-	√	√
Ceramide	-	√	√

3.3.3 Assay of the determination of the apoptotic cell number by fluorescense activated cell sorting (FACS) analysis

Apoptosis is a carefully regulated process of cell death that occurs as a normal part of development. Apoptosis is distinguished from necrosis, or accidental cell death by characteristic morphological and biochemical changes, including compaction and fragmentation of the nuclear chromatin, shrinkage of the cytoplasm and loss of membrane asymmetry. In normal viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human anticoagulant, annexin V, is a 35-36kD Ca2+ dependent phospholipid-binding protein that has a high affinity for PS. Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet. A commercially available 'Vybrant™ Apoptosis Assay Kit #2 containing a recombinant annexin V conjugated to the Alexa Fluor488 dye was used to determine apoptotic cells. Alexa Fluor488 dye is an almost perfect spectral match to fluorescein (FITC), but it creates brighter and more photostable conjugates. In addition, the kit includes a ready-to use solution of the red-fluorescent propidium iodide (PI) nucleic acid-binding dye. PI is impermeant to live cells and apoptotic cells, but stains necrotic cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with Alexa Fluor488 annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. These populations can easily be distinguished using a flow cytometer with the 488nm line of an argon-ion laser for excitation as flow cytometry technology is used to measure properties of cells as they move or flow in liquid suspension.

HT-29 human colon cancer cells were cultured in 6 well plates at a density of 1.2×10^6 cells/well for 6 hour incubation, 1×10^6 cells/well for 1 day treatments and 0.8×10^6 cells/well for 3 days incubation and allowed to culture for 24 hours. Cells were maintained as previously described in the section 3.3.2.1. Next day the medium was

replaced with 5 mL fresh media and treated with fatty acids Control flasks were supplemented with equivalent volumes of ethanol (0 028% v/v) After indicated time of incubation, the media were discarded and cells were washed with ice cold PBS Cells were trypsinised with 200µl trypsin, then 800 µl of fresh media was added to the trypsinised cells and transferred all together in a micro tube (1 5 ml) Cells were centrifuged at 1600 *rpm in* a *Biofuge* 13 for 3 *min* Then FACS analysis was performed using Vybrant™ Apoptosis Assay Kit #2, according to the protocol supplied by the supplier Cells were washed in ice cold-PBS and resuspended in 200 µl of 1X Annexin-Binding Buffer (ABB) which was prepared from 5X Annexin-Binding Buffer 2 5 µl Alexa Fluor488 Annexin V and 0 5 µl of PI (100 µg/ml, prepared from 1 mg/ml with 1X ABB) added to cell suspension and incubated at room temperature for 15 mins A further 200 µl of 1X ABB was then added and placed on ice for 3 hour before read by FACS (FACSCalibur™, BD Indispensibile to human health, UK)

3.3.4 Measurement of histone deacetylase (HDAC) activity

Histone deacetylase activity (HDAC) is implicated in gene expression, affecting transcription of genes regulating apoptosis and differentiation A HDAC activity assay was performed in a 96 well microplate in which a fluorometric substrate (containing an acetylated lysine side chain) was converted into a fluorophore product, which was subsequently read on a fluorescence plate reader This assay (MBL, Woburn, MA USA) eliminates radioactivity, extraction and chromatography as used in traditional assays Briefly, 50µl HT-29 cell lysate was incubated with 10µl of a 10X assay buffer (supplied with kit, MBL, Woburn, MA USA) and 5µl of substrate (4mM) [Boc-Lysine (Ac)-AMC] at 37^0C for 30 minutes After incubation reaction was stopped with 10µl Lysine Developer and incubated at 37^0C for another 30 minutes Samples were read in a fluorescence plate reader with Excitation/Emission=350/460 nm and slit width 10 and 2 5 nm Deacetylase activity was expressed as Relative Fluorescence Units per µg protein in cell lysate

3.3.5 Analyses of cellular carcinoembryonic antigen (CEA) expression

Cellular carcinoembryonic antigen (CEA) level was measured by a colorimetric ELISA kit (BioCheck Inc, CA) using sodium butyrate as a positive control The CEA ELISA test is based on the principal of a solid phase enzyme-linked immunosorbant assay The assay system utilizes a monoclonal antibody directed against a distinct antigen determinant on the intact CEA molecule and goat anti-CEA antibody conjugated to horseradish peroxidase The test sample (cell lysate) was allowed to react simultaneously with the two antibodies, resulting in the CEA molecules being sandwiched between the solid phase and enzyme linked antibodies After 1 hour incubation at room temperature, the wells were washed with water to remove unbound labeled antibodies A solution of tetramethylbenzidine reagent is added and incubated for 20 min , resulting in the development a blue color The color development is stopped with the addition of 1M HCl solution The concentration of CEA is directly proportional to the color intensity of the test sample Absorbance is measured spectrophotometrically at 450 nm

3.3.6 Measurement alkaline phosphatase activity

Alkaline phosphatase activity was measured in cell lysates by the kinetic determination of p-nitrophenol phosphate hydrolysis using a commercially available Sigma diagnostics kit (Sigma, St Louis, MO) 0 5 ml of 2-amino-2-methyl-1-propanol buffer (1 5M) and stock substrate solution (15 mM p-nitrophenyl phosphate disodium) were pipetted in triplicate into each test tube These were incubated in a water bath at 37°C for 5 mins to equilibrate 0 1 ml of sample was then added to each tube, except the blank (to which 0 1 ml water was added) and further incubated in a water bath at 37°C for exactly 30 mins The reaction was terminated by the addition of 10 ml of 0 05 M NaOH Absorbance was measured at 420 nm Standard curves were prepared using stock p-nitrophenol solution (10 mM/L) diluted appropriately In all cases, the specific enzyme activity was expressed as milliunits, where 1 milliunit was equivalent to 1 nmole of p-nitrophenol phosphate (pNPP) hydrolysed per min per mg protein at 37°C

3.3.7 Cell lysate preparation

Cells were harvested after a specific time points using phosphate buffered saline (PBS) containing 0 25% trypsin Cells were spun at 1000rpm for 5 min The resulting pellets were washed in PBS and resuspended in 500 µl lysis buffer [10 mM sodium phosphate buffer (pH 7 2), 100 mM NaCl, 10 mM sodium deoxycholate, 1 mM PMSF, 1% Triton-X 100, 0 1 mM leupeptin and 0 2 mg/ml aprotinin] The samples were then sonicated with a sonicator (Model VC 502, Sonocs & Materials Inc , Newton, CT, USA) on ice

3.3.8 Protein Assay

The protein concentration of cell lysates was estimated by the Bio-Rad dye binding assay BSA standards were prepared from BSA stock (1000 µg/ml) in the concentration range 0-1000 µg/ml BSA Samples were assayed in triplicate in a 96 well plate at 37°C The dye reagent was diluted in distilled water as 1 part Biorad reagent in 4 parts distilled water Absorbance was read in a microplate reader at 620 nm

3.3.9 Statistical analysis

All data are expressed as mean±SD calculated with Microsoft® Excel 2000 At least three independent experiments were performed in triplicate The statistical significance ($P<0.05$) was determined using the Student's t-test and was used to determine significance between treatments

3.4 Results

3.4 1 Ceramide mass assay by HPLC

Figure 3 1 depicts a typical HPLC chromatogram of ceramide (1000 pmol) after deacylation to sphingosine The retention time for derivatised sphingosine was 9 453 min and OPA eluted fully after 3 min Figure 3 2 depicts a typical HPLC chromatogram of total sphingosine in (a) control HT-29 cells and in (b) cells treated with 75µM CLA mixture

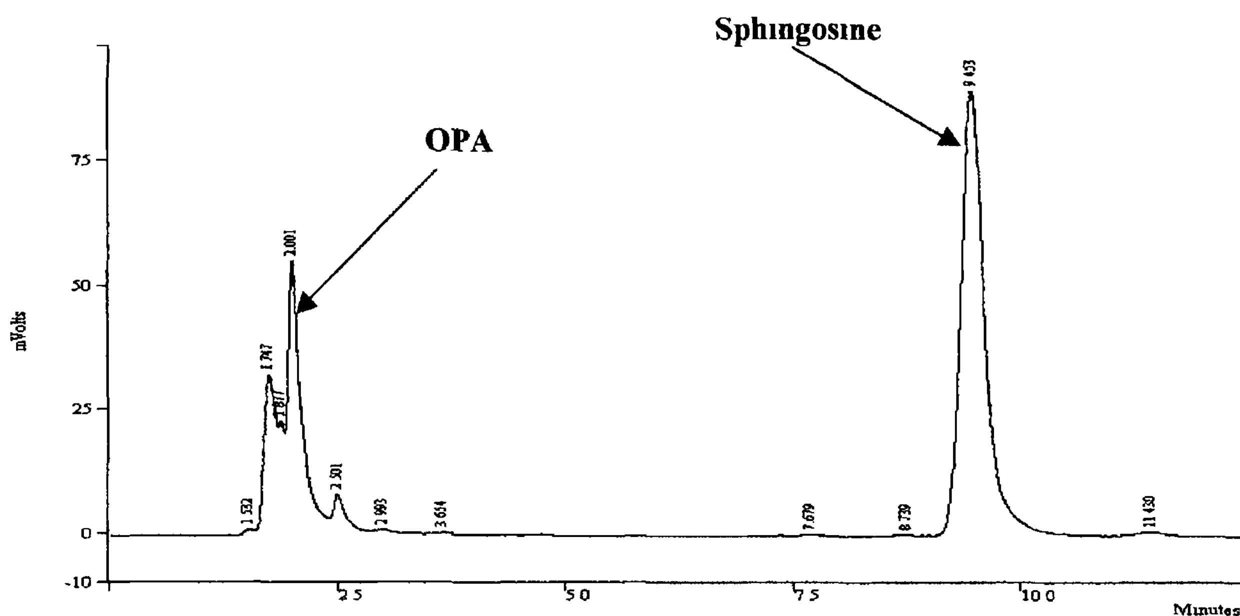

Figure 3.1 Typical HPLC chromatogram of a ceramide standard which is converted to sphingosin after deacetylation procedure The retention time of sphingosine 9 453 min was recorded at a concentration of 1000 pmol The OPA eluted between 1-3 min

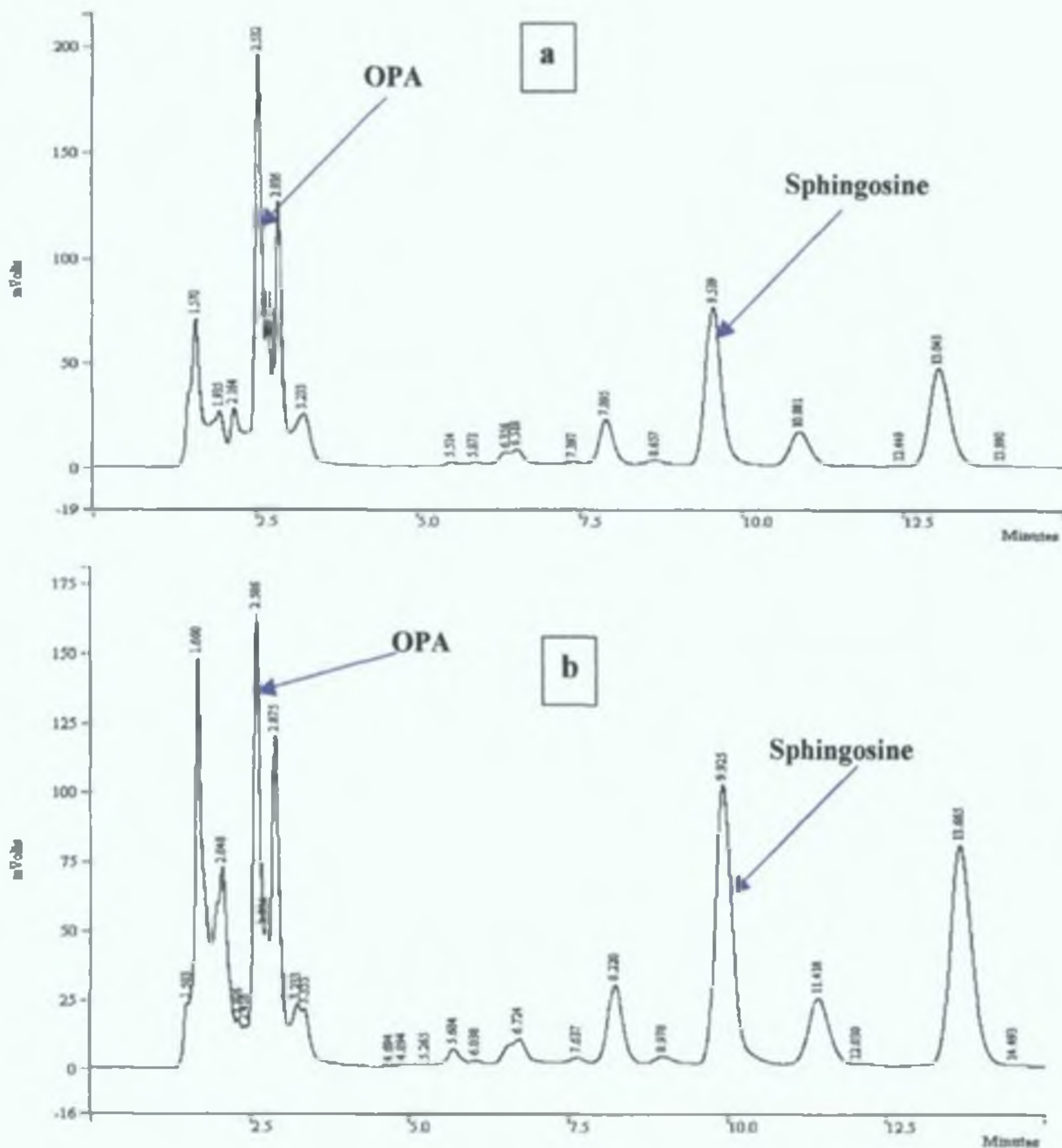

Figure 3.2 Typical HPLC chromatogram of total sphingosine in (a) control HT-29 cells and in (b) cells treated with 75µM CLA mixture.

The linearity of the relationship between peak fluorescence area units and concentration of sphingosine after derivitisation (Fig 3 3, A), alkaline hydrolysis (Fig 3 3, B) and deacylation (Fig 3 3, C) is depicted in Fig 3 3 Figure 3 4 depicts the linearity of the relationship between peak area and concentration of ceramide after deacylation It is apparent that the assay can measure sphingosine in the range 0-2000 pmol after derivatisation with o-phthaldehyde (OPA) as described in Fig 3 3, A

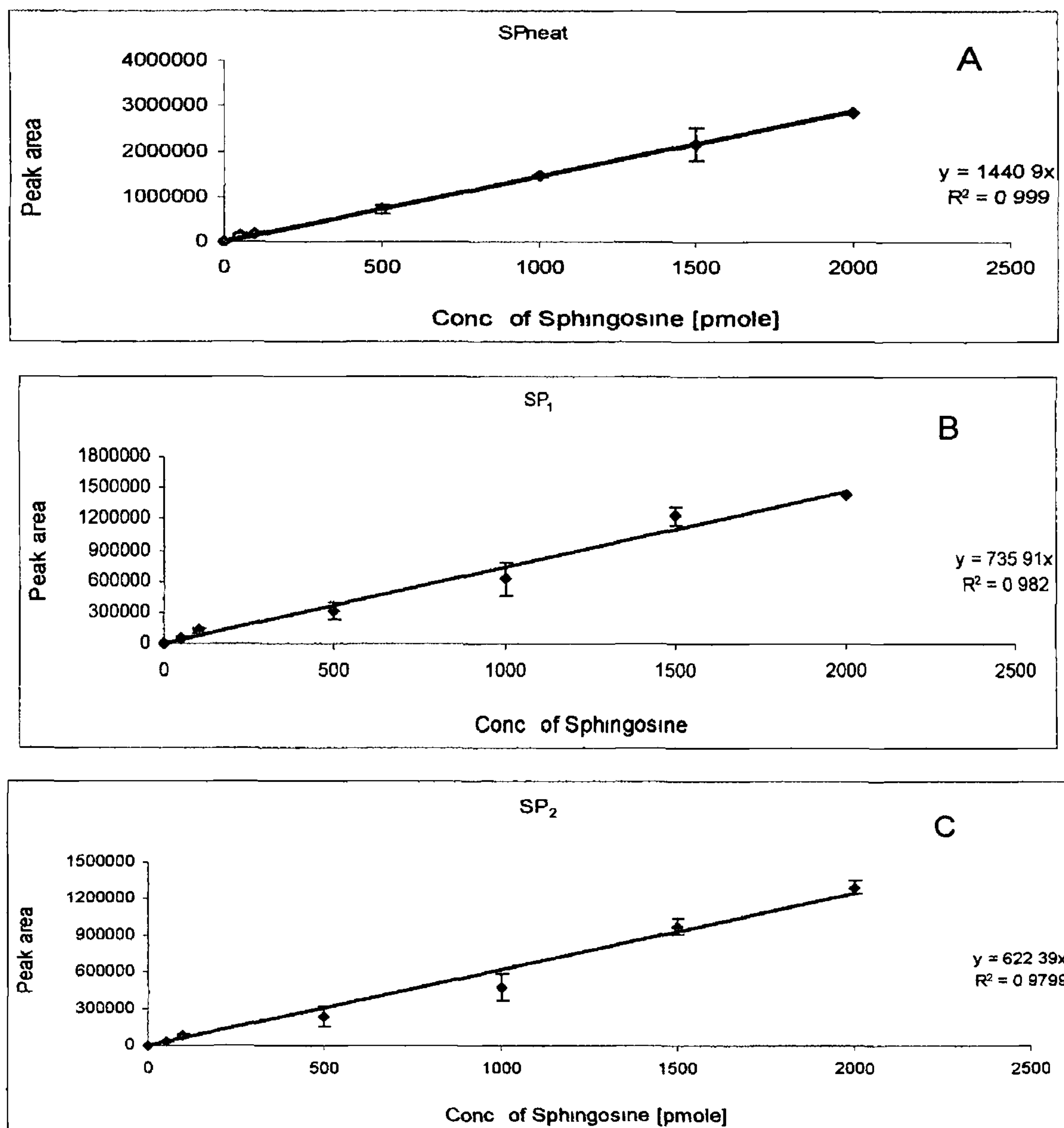

Figure 3 3 (A, B, C) Standard curves of (A) SP_{NEAT} (neat sphingosine, derivatised with OPA), (B) SP_1 (Sphingosine standard, hydrolyzed and derivatised with OPA and (C) SP_2 (Sphingosine standard deacylated and derivatised) and all were quantitated using HPLC (n = 3)

Similarly, known amounts of ceramide in the range 0-2000 pmol can be measured after deacylation to the sphingoid base and derivitization with OPA as described in Fig 3 4 Efficiency of deacylation was approximately 100%, as judged by comparison with Fig 3 3, A Recovery of sphingoid base during deacylation was approximately 82% as judged by comparison of Fig 3 3, B & 3 3, C

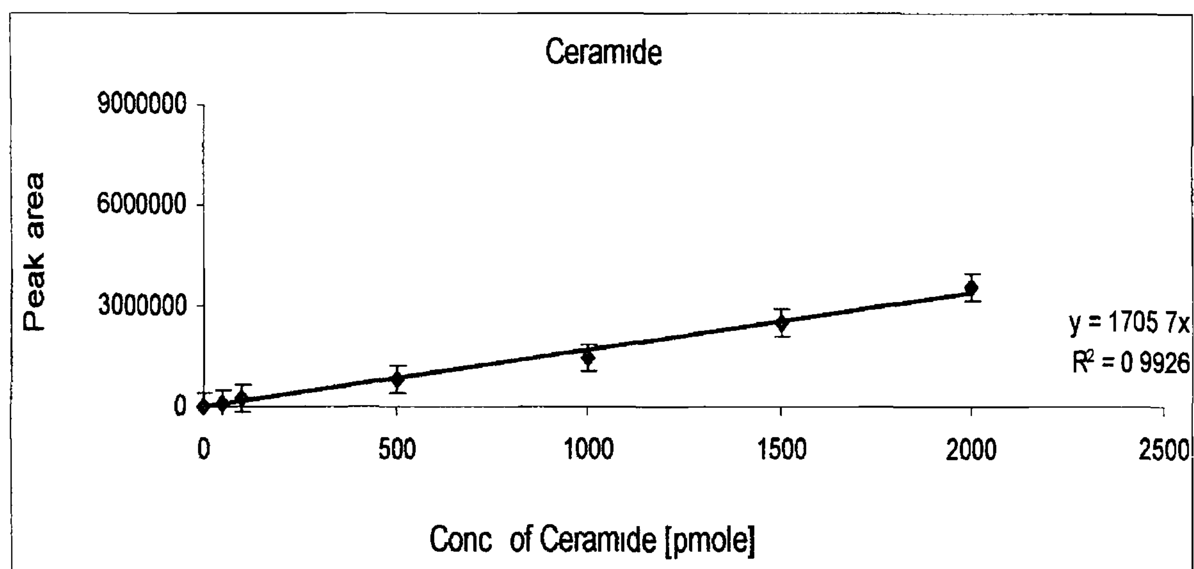

Figure 3 4· Standard curves of Ceramide (0-2000 pmole) was deacylated (to convert ceramide to sphingosine), derivatised and quantitated using HPLC (n = 3)

3.4.2 Effect of CLA isomers on ceramide and sphingosine content in HT-29 cells

Cell content of ceramide was determined by subtraction of level of basal sphingosine from level of total sphingosine as described in the methods section Fig 3 5 show ceramide content of HT-29 cells ($3x10^6$ and $1 5x10^6$) following treatment with ethanol (control) or CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA or *t*-VA all at 75μM for 1 day and 3 day Sodium butyrate at 3mM was used as positive control Data refer to mean ± SD (n=3) ceramide content (pmol/10^6 cells) None of the treatments altered ceramide content after 1day (Fig 3 5) However it is apparent that after 3day the

level of ceramide was significantly higher ($p<0.05$) in cells treated with sodium butyrate (2619 ± 141 pmol/10^6 cells) and the CLA mixture (2106 ± 113 pmol/10^6 cells) compared with control cells (1045 ± 206 pmol/10^6 cells). The single CLA isomers and *t*-VA had negligible effects on cellular ceramide.

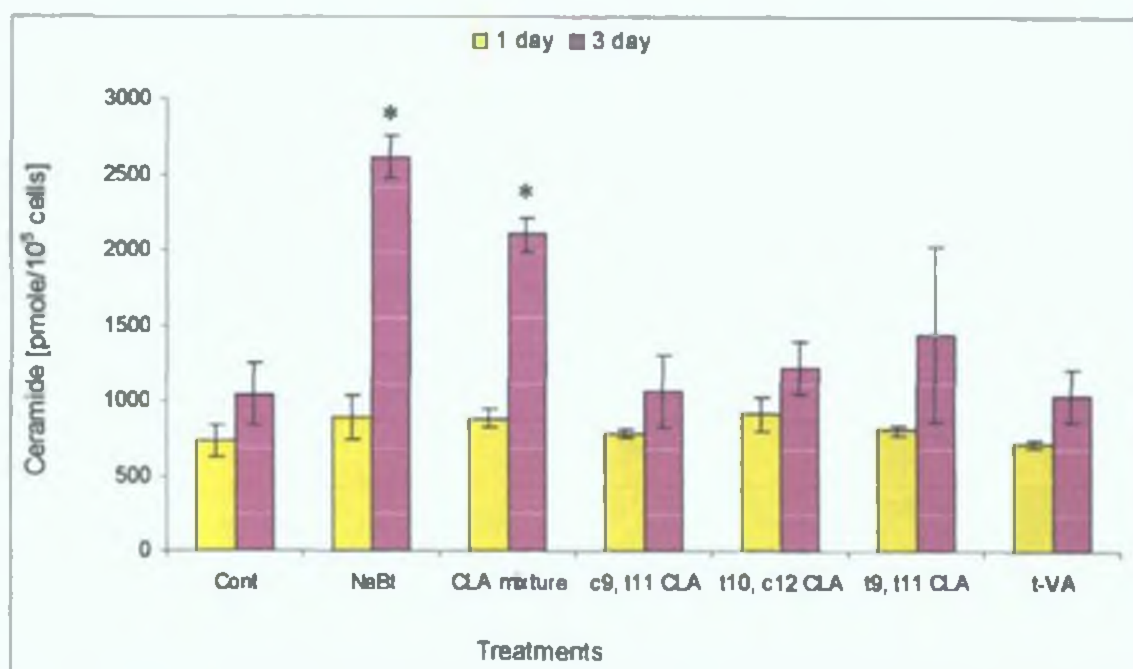

Figure 3.5 Effects of fatty acids on cellular ceramide levels. HT-29 colorectal cancer cells were cultured at a density of 3×10^6 cells/flask for 1 day and 1.5×10^6 cells/flask for 3 day in DMEM medium in T75 cm^2 flask. Cells were incubated with either 3 mM NaBt or 75 μM of CLA mixture of isomers, *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA or *t*-VA for 3 day. Results shown are the mean ±SD (n=3) expressed as pmol ceramide/10^6 cells relative to control.

Basal sphingosine was significantly elevated after treatment with sodium butyrate but not by CLA or *t*-VA treatments (Fig. 3.6).

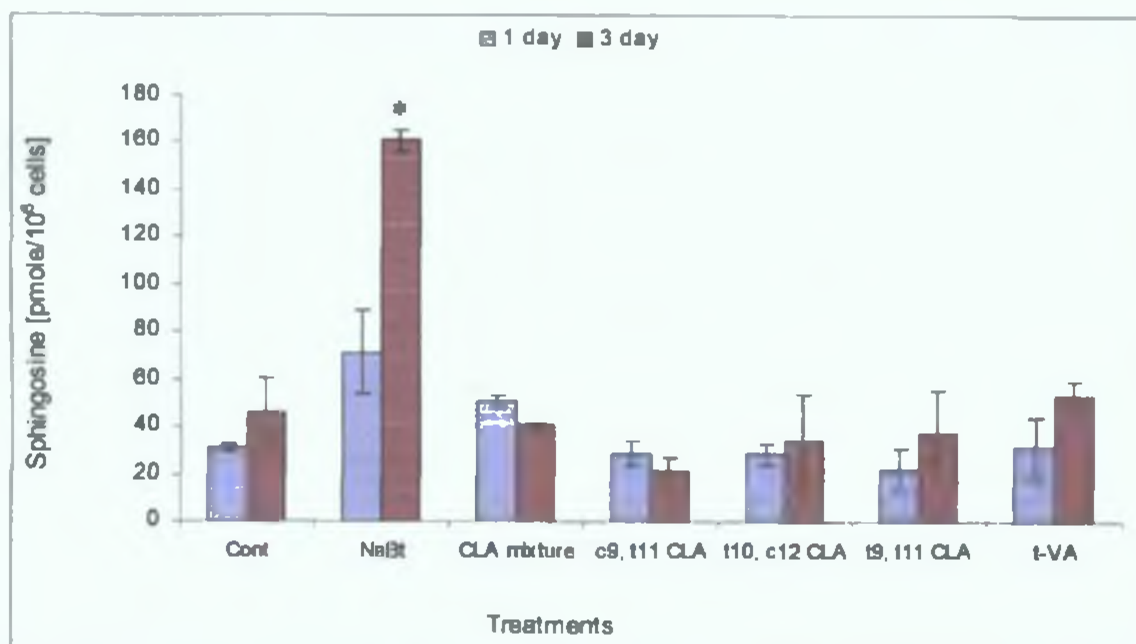

Figure 3.6 Effects of fatty acids on cellular sphingosine levels. HT-29 colorectal cancer cells were cultured at a density of 3 x 10^6 cells/flask for 1 day and 1.5 x 10^6 cells/flask for 3 day in DMEM medium in T75 cm^2 flask. Cells were incubated with either 3 mM NaBt or 75 µM of CLA mixture of isomers, *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA or *t*-VA. Results shown are the mean ±SD (n=3) expressed as pmol sphingosine/10^6 cells relative to control.

3.4.3 Effect of CLA isomers on Annexin V labelling of cells

Annexin V binding to phosphatidylserine exposed on the outer leaflet of plasma membranes was used as an assay for detecting apoptosis. Apoptotic cell number was analysed by flow cytometry and expressed as a % of total cell number. Fig 3.7 shows a typical picture of fluorescence-activated cell sorting in a) control HT-29 cells (0.8 x 10^6 cells/well) and b) cells treated with CLA mixture of isomers (75µM) for 3day. Fig 3.8 shows a typical picture of fluorescence-activated cell sorting in cells treated with 75µM of a) *c*9, *t*11 CLA b) *t*10, *c*12 CLA and c) *t*9, *t*11 CLA for 1 day. The cross wires were

drawn so that the lower left quadrant contains the viable cells that are negative for annexin V and propidium iodide (PI). The upper and lower right quadrants show the apoptotic cells that are positive for annexin V and negative for PI. The upper left quadrant contains the necrotic cells which are positive for PI and negative for annexin V.

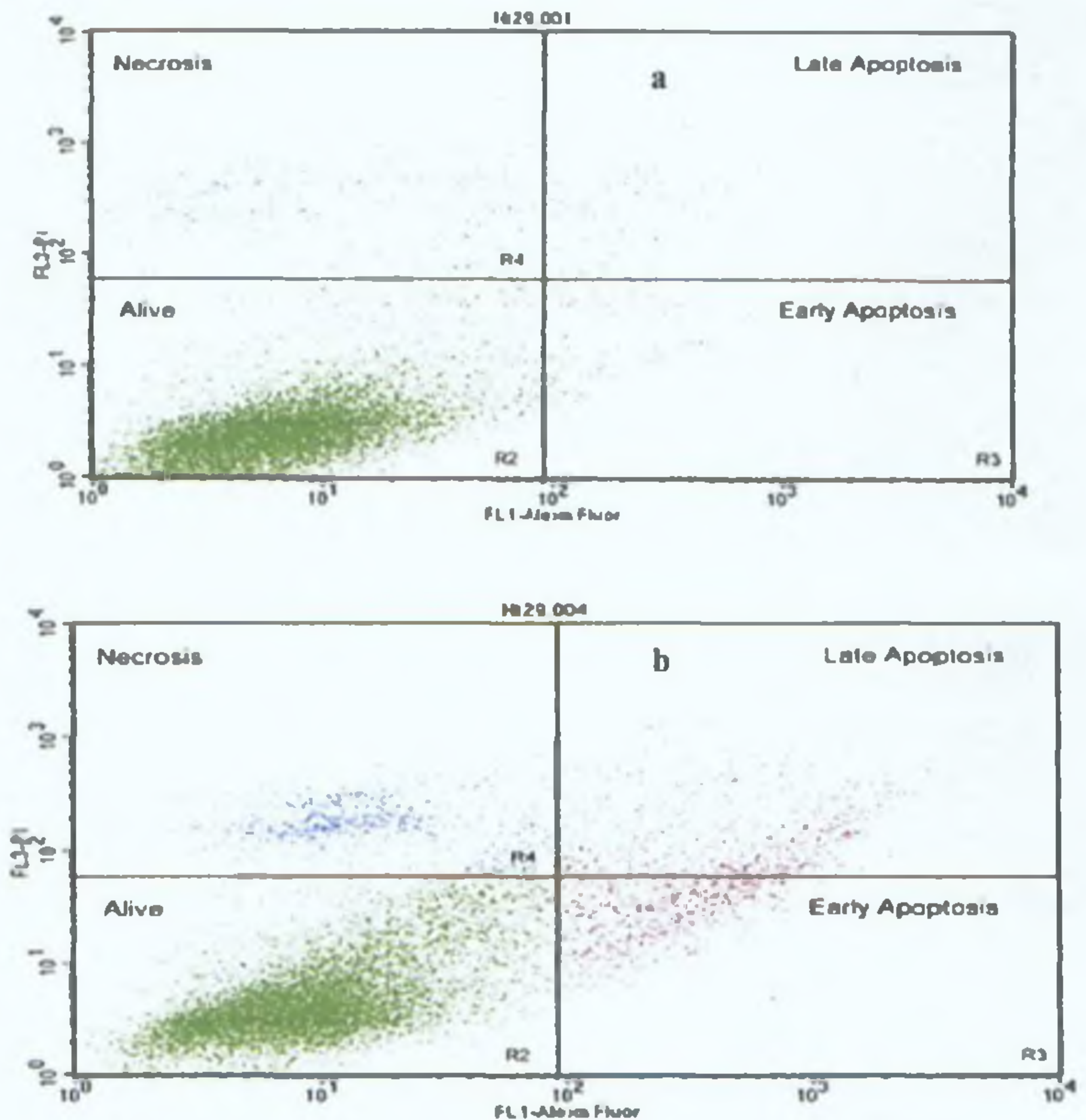

Figure 3.7 Fluorescence-activated cell sorting: shows a typical picture of fluorescence-activated cell sorting in a) control HT-29 cells (0.8 x 10^6 cells/well) and b) cells treated with CLA mixture of isomers (75µM) for 3 day.

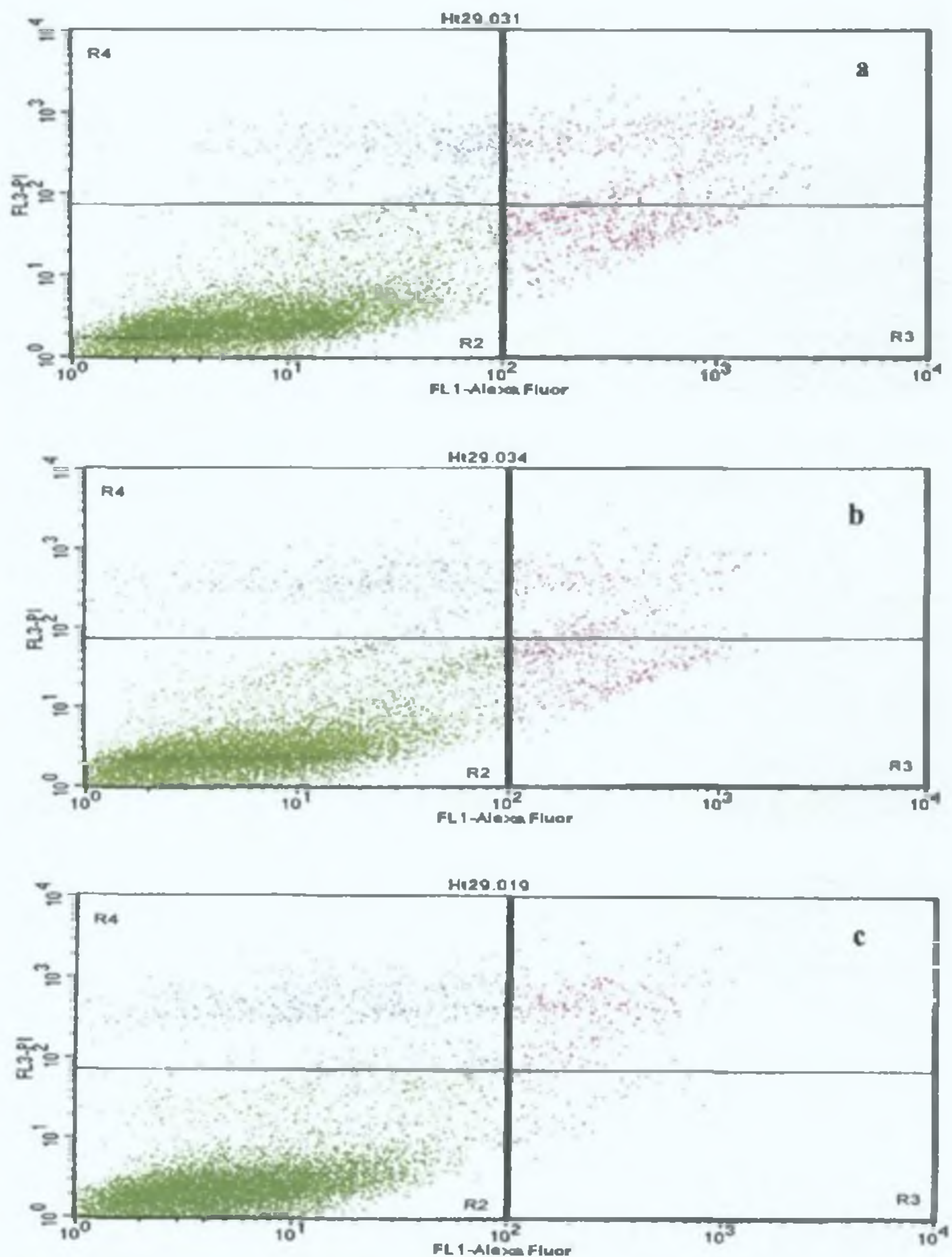

Figure 3.8 Fluorescence-activated cell sorting: shows a typical picture of fluorescence-activated cell sorting in HT-29 cells treated with 75μM of a) *c*9, *t*11 CLA b) *t*10, *c*12 CLA and c) *t*9, *t*11 CLA for 1day.

It is apparent that when HT-29 cells were treated with CLA mixture of isomers for 3 days that there was a 6 fold higher percentage of apoptotic cells relative to untreated control cells (Fig 3.9). Sodium butyrate increased the number of apoptotic cells by approximately 3 fold.

Cells were examined after 6 hrs and 1 day treatment with three different CLA isomers (*c*9, *t*11 CLA, *t*10, c12 CLA and *t*9, *t*11 CLA) (Fig. 3.8) to investigate the apoptosis. The single isomers *c*9, *t*11 CLA and *t*10, c12 CLA at 75μM showed a significant 2-2.7 fold increase in apoptotic cells after 1 day ($p<0.05$) relative to control cells (3.2%±0.2). The *t*9, *t*11 CLA did not show any effect on apoptosis of HT-29 after 1 day. Increasing % of apoptotic HT-29 cells were observed after 6 hour of incubation with *c*9, *t*11 CLA, *t*10, c12 CLA and *t*9, *t*11 CLA but there was no significant difference from untreated control cells (Fig 3.10).

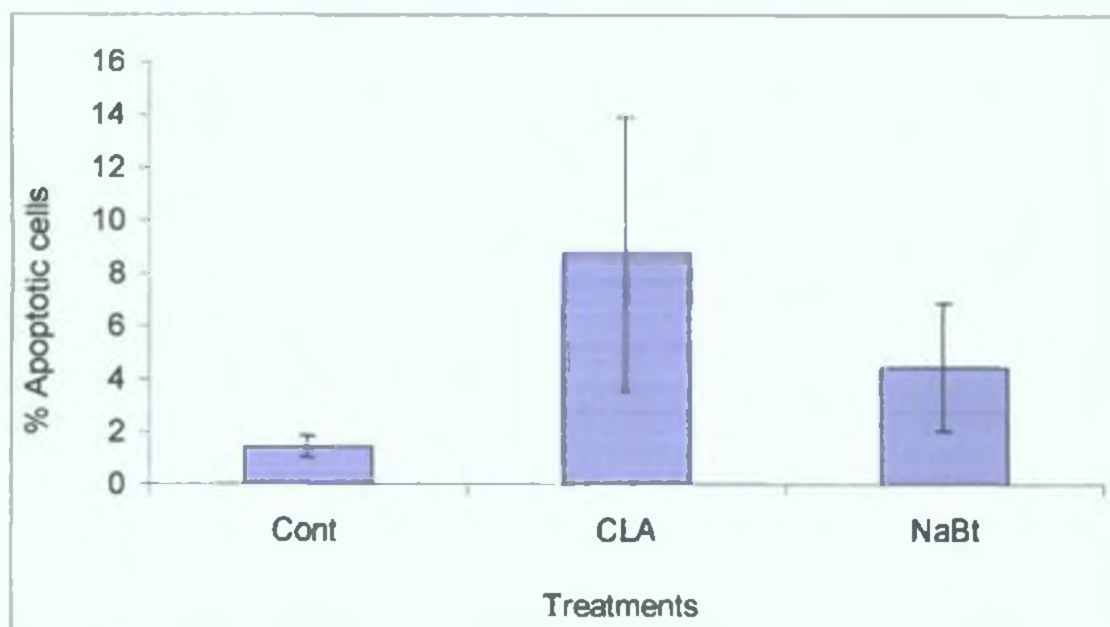

Figure 3.9 Effects of CLA mixture on apoptosis of HT-29 cells. HT-29 cells were cultured and treated with CLA (75μM) and NaBt (3mM) for 3 days. Proportion of apoptotic cell numbers were analyzed by flow cytometry and the number of apoptotic cells is expressed as a percentage of total cell number. Results shown are the mean (±SD) of treated cells (n=3).

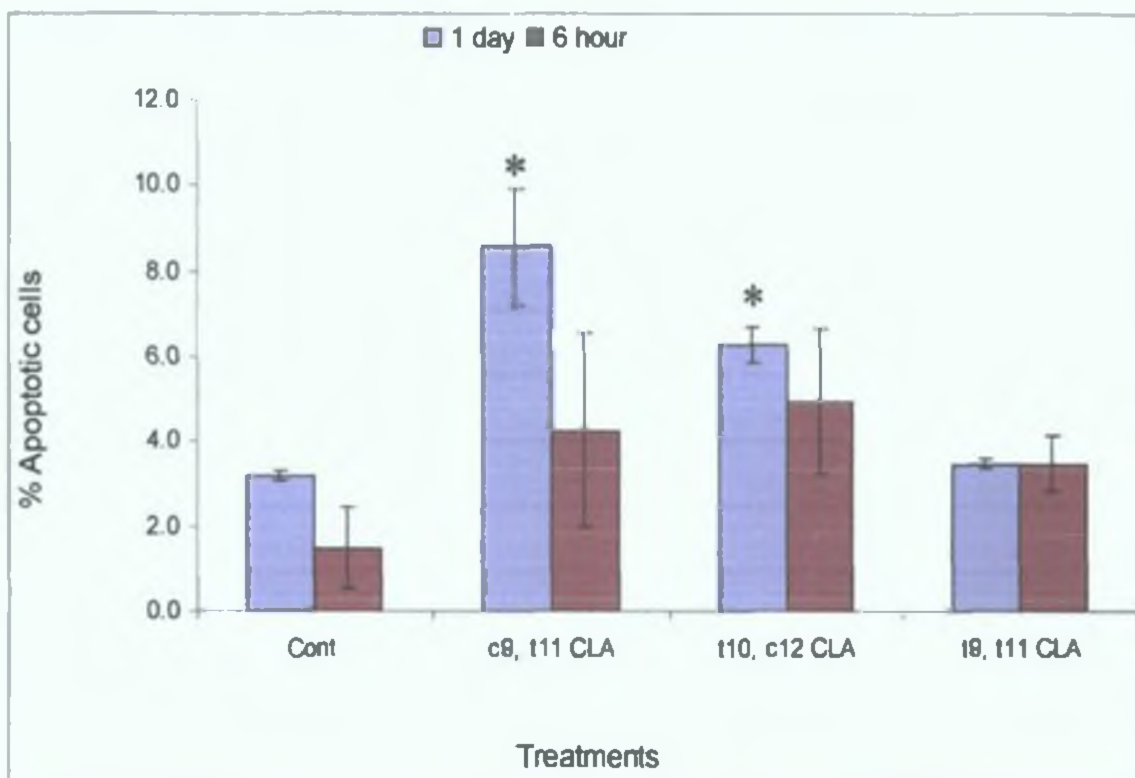

Figure 3.10 Effects of *c*9, *t*11 CLA, *t*10, *c*12 CLA, and *t*9, *t*11 CLA on apoptosis of HT-29 cells. HT-29 cells were cultured and treated with *c*9, *t*11 CLA, *t*10, *c*12 CLA, and *t*9, *t*11 CLA for 6 hours and 1 day. Proportion of apoptotic cell numbers were analyzed by flow cytometry and the number of apoptotic cells is expressed as a percentage of total cell number. Results shown are the mean (±SD) of treated cells (n=4). Asterisks (*) denote significant values (P<0.05) relative to control.

3.4.4 Effect of CLA isomers on histone deacetylase activity

Histone deacetylase (HDAC) activity in HT-29 cells was used as a marker for detecting fatty acid-induced modulation of epigenetics in HT-29 cells. Cells (0.8×10^6 /flask) were treated with the CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA, *t*-VA or LA all at 75μM for 5 days. Sodium butyrate (3mM) was included as a negative control. Level of HDAC was decreased 1.7 fold in sodium butyrate-treated cells relative to control cells (2.0± SD RFU /mg protein) (Fig 3.11).

It is apparent that *c*9, *t*11 CLA and *t*9, *t*11 CLA had significant inhibitory effect on HDAC activity; the reduction in HDAC by the CLA mixture and *t*10, *c*12 CLA did not reach statistical significance. Trans vaccenic acid and linoleic acid had no effect. When adherent cells were analysed, only *t*10, *c*12 CLA and linoleic acid were inhibitory ($p<0.05$) (Fig 3.12), suggesting differential effects of PUFAs on the accumulation of acetylated histones between viable and non viable cells.

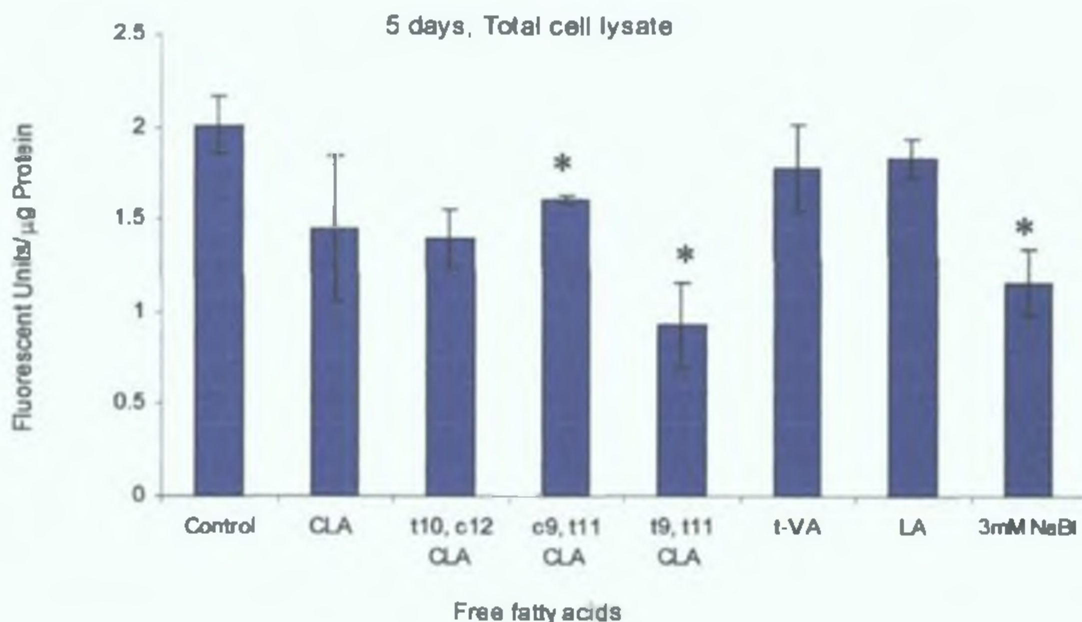

Figure 3.11 Cells were seeded at 0.8 x 10^6 cells/flask in T-75cm2 flask in complete culture media. On the second day media was replenished with fresh media containing 3mM sodium butyrate and 75µM of CLA mixture of isomers; *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA, *t*-VA and LA as free fatty acid. HT-29 cells treated with ethanol (0.028% v/v) served as control. HDAC activity expressed as the Relative Fluorescence Units per µg protein sample. The results represents the mean $\pm$SD (n=3). Asterisks (*) denote significant values ($P<0.05$) relative to control.

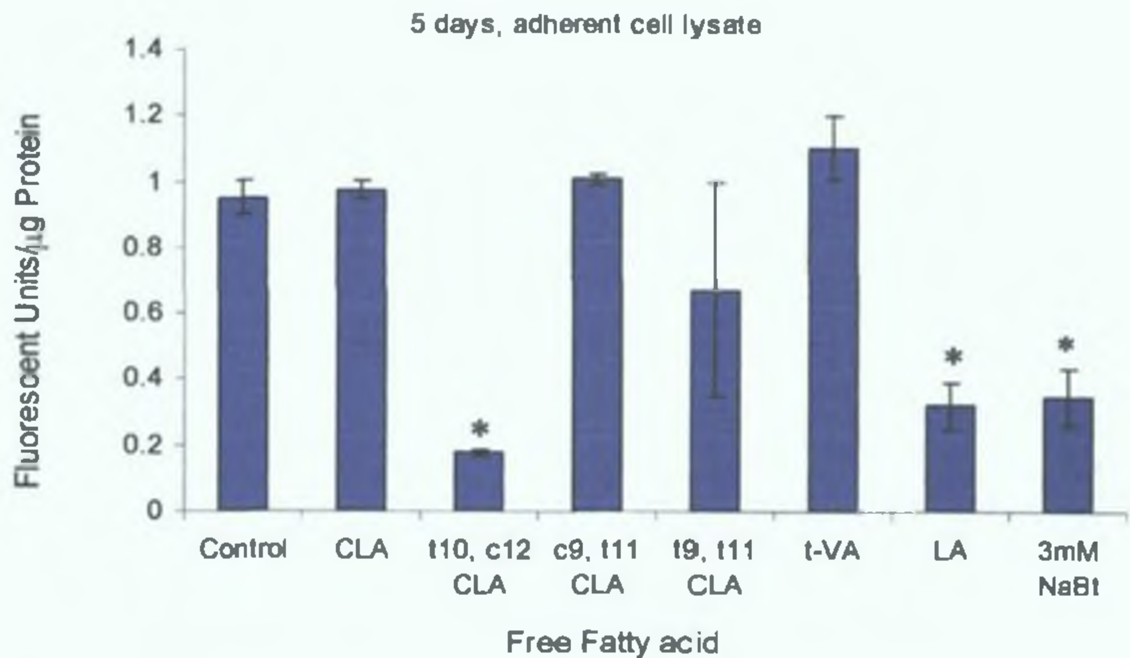

Figure 3.12 Cells were seeded at 0.8 x 10^6 cells/flask in T-75cm2 flask in complete culture media. On the second day media was replenished with fresh media containing 3mM sodium butyrate and 75µM of CLA mixture of isomers; *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA, *t*-VA and LA as free fatty acid. HT-29 cells treated with ethanol (0.028% v/v) served as control. HDAC activity expressed as the Relative Fluorescence Units per µg protein sample. The result represents the mean $\pm$SD (n=3). Asterisks (*) denote significant values (P<0.05) relative to control.

3.4.5 Effect of CLA isomers on CEA expression

CEA expression in lysates from adherent cells was used as a marker for detecting fatty acid-induced differentiation of HT-29 cells. Cells (0.8x10^6 /flask) were treated with the CLA mixture of isomers, *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA, *t*-VA and LA all at 75µM for 5 days. Sodium butyrate (3mM) was included as a positive control.

It is apparent that all of the fatty acids except *t*-VA and LA increased CEA relative to control cells (15.05±0.75 pg/µg protein). Level of CEA was increased 5.6 fold in sodium butyrate-treated cells. Of the CLA isomers studied, the CLA mixture, *t*10, *c*12 CLA and *t*9, *t*11 CLA exhibited the greatest potency, increasing CEA levels by approximately 50% (Fig 3.13). *c*9, *t*11 CLA also showed an increase but it did not attain statistical significance.

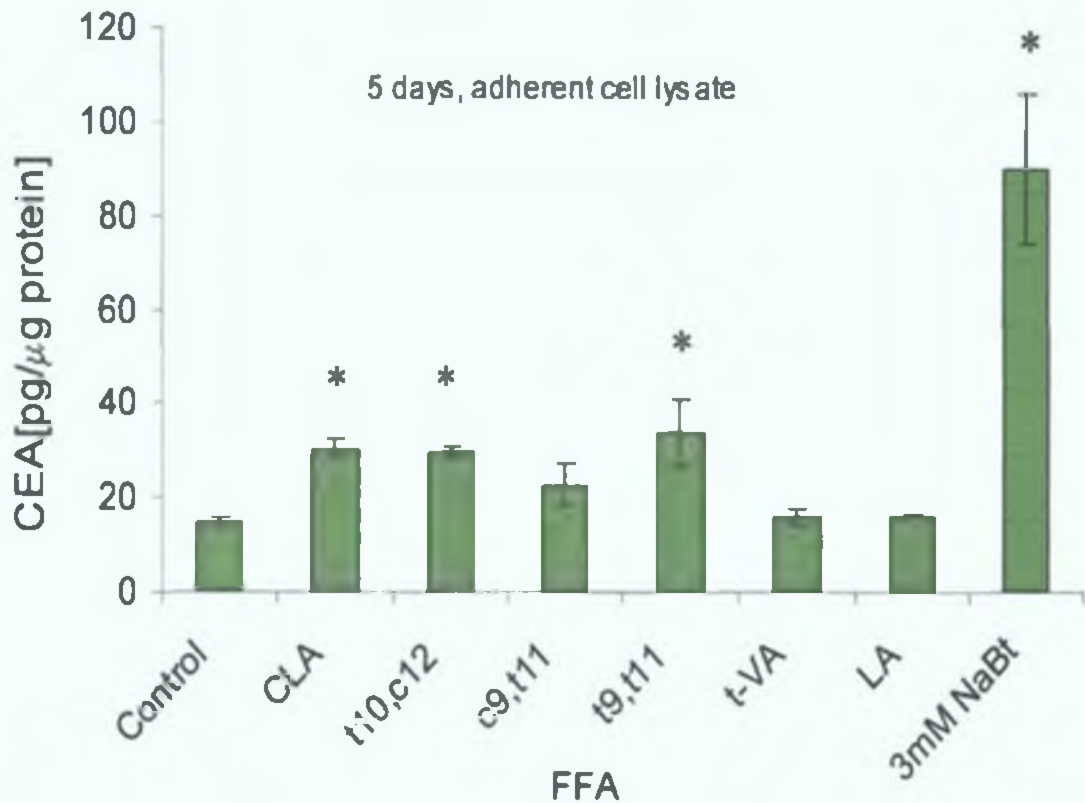

Figure 3.13 HT-29 cells were seeded at 0.8 x 10^6 cells/flask in T-75 cm2 flask in complete culture media. On the second day media was replenished with fresh media containing 3mM sodium butyrate and 75µM of CLA mixture of isomers; *c*9,*t*11-CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA, *t*-VA and LA as free fatty acids. Control flasks were treated with ethanol (0.028% v/v). CEA levels expressed as picagram (pg) per µg protein sample. Results represents mean $\pm$SD (n=6). Asterisks (*) denote significant values (P<0.05) relative to control.

Similar effects were observed when cells were treated with CLA:BSA complexes (2:1 molar ratio). The CLA mixture and *t*10, *c*12 CLA increased CEA levels approximately 2 fold (Fig 3.14). *c*9, *t*11 CLA and *t*9, *t*11 CLA increased CEA by 30-60% ($p<0.05$) relative to control but trans vaccenic acid and LA had no significant effect. When cell lysates from both floating and adherent cells were analysed, only *t*9, *t*11CLA was stimulatory ($p<0.05$) (Fig 3.15), suggesting differential effects of PUFAs on CEA expression between viable and non viable cells.

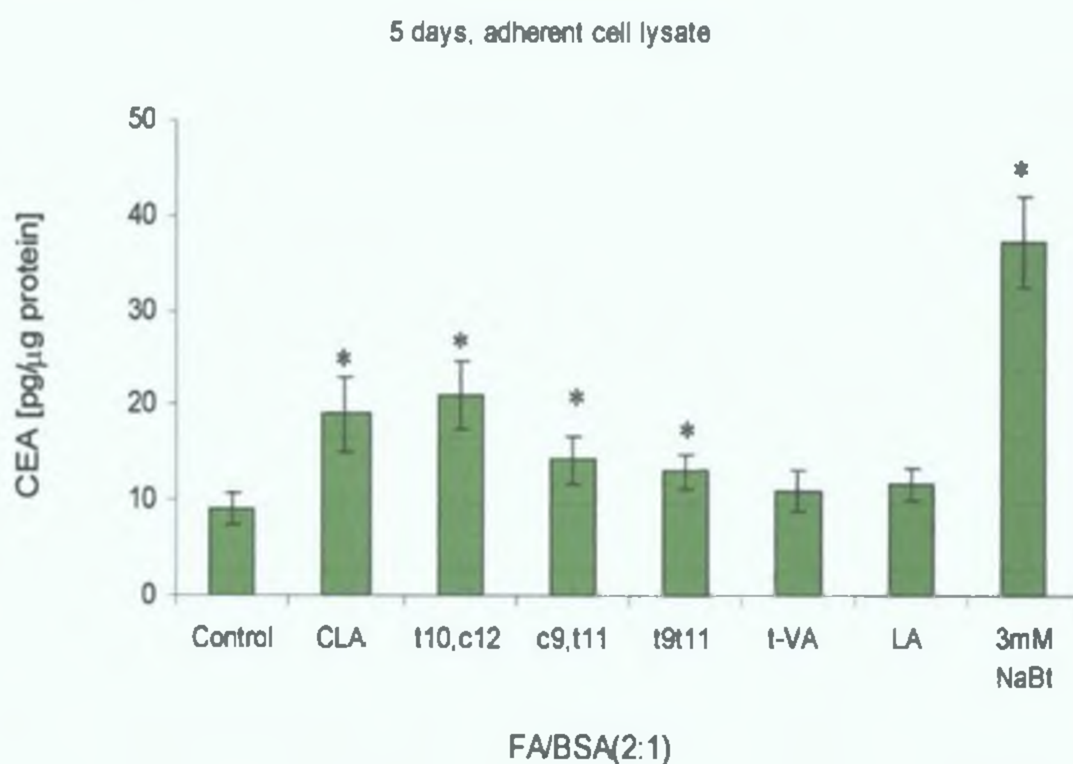

Figure 3.14 HT-29 cells were seeded at 0.8 x 10^6 cells/flask in T-75 cm2 flask in complete culture media. On the second day media was replenished with fresh media containing 3mM sodium butyrate and 75µM of CLA mixture of isomers; *c*9, *t*11-CLA; *t*10, *c*12-CLA; *t*9,*t*11-CLA isomer, *t*-VA and LA. Fatty acids were complexed with BSA at a ratio 2:1 prior to treatment. Control flasks were treated with ethanol (0.028%). CEA levels expressed as picagram (pg) per µg protein sample. Results represents mean $\pm$SD (n=6). Asterisks (*) denote significant values ($P<0.05$) relative to control.

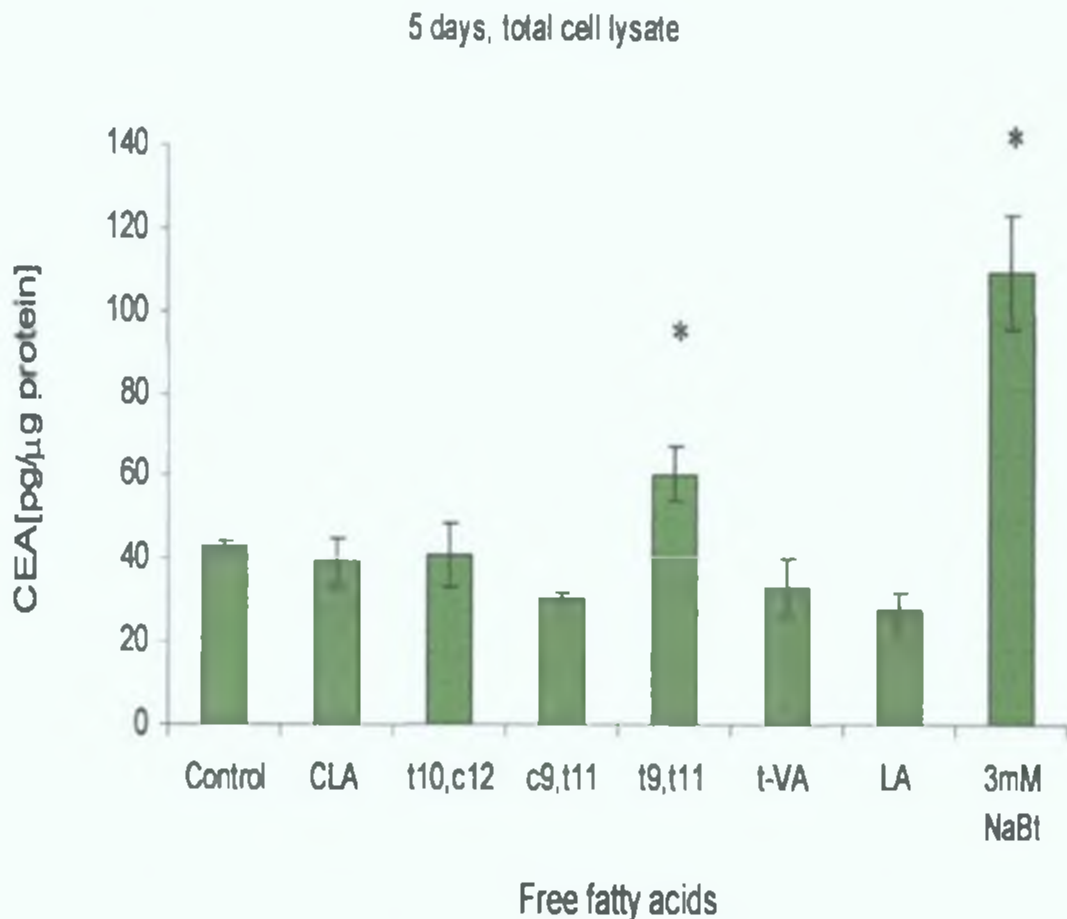

Figure 3.15 HT-29 cells were seeded at 0.8 x 10^6 cells/flask in T-75 cm2 flask in complete culture media. On the second day media was replenished with fresh media containing 3mM sodium butyrate and 75µM of CLA mixture of isomers; *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA isomer, *t*-VA and LA as free fatty acids. Control flasks were treated with ethanol (0.028%). CEA levels expressed as picagram (pg) per µg protein sample. Results represents mean $\pm$SD (n=6). Asterisks (*) denote significant values (P<0.05) relative to control.

3.4.6 Effect of CLA isomers on alkaline phosphatase activity

The specific activity of alkaline phoshatase (ALP) in cell lysates was used as a marker for detecting fatty acid-induced cell differentiation. Cells (0.8×10^6 /flask) were treated with the CLA mixture of isomers, *c*9, *t*11 CLA and *t*10, *c*12 CLA at varying doses between 50-125μM for 2 days. Sodium butyrate (3mM) was included as a positive control. It is apparent that none of the CLA treatments had any effect on ALP activity in adherent cells, with specific activity ranging between 3-4mU. Butyrate increased ALP activity 21 fold ($p<0.05$) relative to control (3 $\pm$ 0.4 mU) (Fig 3.16).

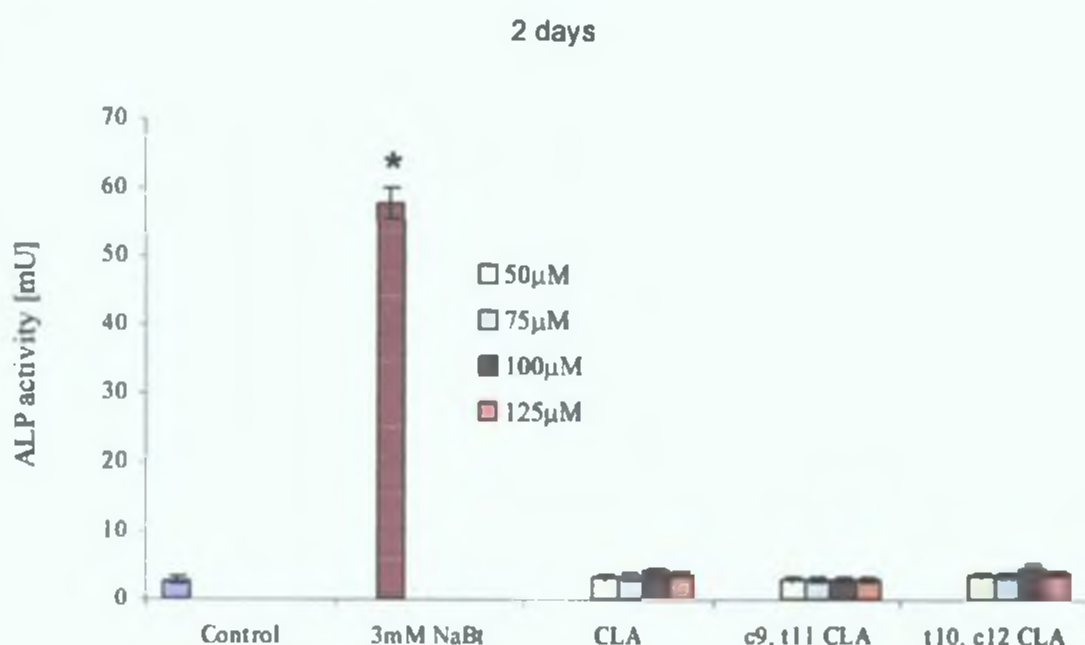

Figure 3.16 Effects of CLA on ALP activity in HT-29 colorectal cancer cells. Cells were cultured at a density of 0.8×10^6 cells/flask in DMEM medium in $75cm^2$ flask with varying concentrations (50, 75, 100 and 125μM) of CLA mixture of isomers, *c*9, *t*11 CLA and *t*10, *c*12 CLA for 2 days. Ethanol (0.028% v/v) was used as control. 3mM NaBt was used as positive control. Results represents mean $\pm$SD (n=3) and expressed as mU where 1 mU = 1nmole product formed/min/mg protein at 37^0C. Asterisks denote significant differences relative to control. Asterisks (*) denote significant values ($P<0.05$) relative to control.

The ALP activity of total cells (both adherent and floating) was also not affected by CLA (Fig 3.16) suggesting that inhibitory effects of CLA on growth are mediated by pathways independent of butyrate. Fig 3.17 shows that when cells are co-treated with sodium butyrate and the various CLA isomers or linoleic acid that sodium butyrate-induced ALP activity was suppressed by 57-65%, suggesting that PUFAs may antagonise the effects of butyrate on expression of the ALP differentiation marker.

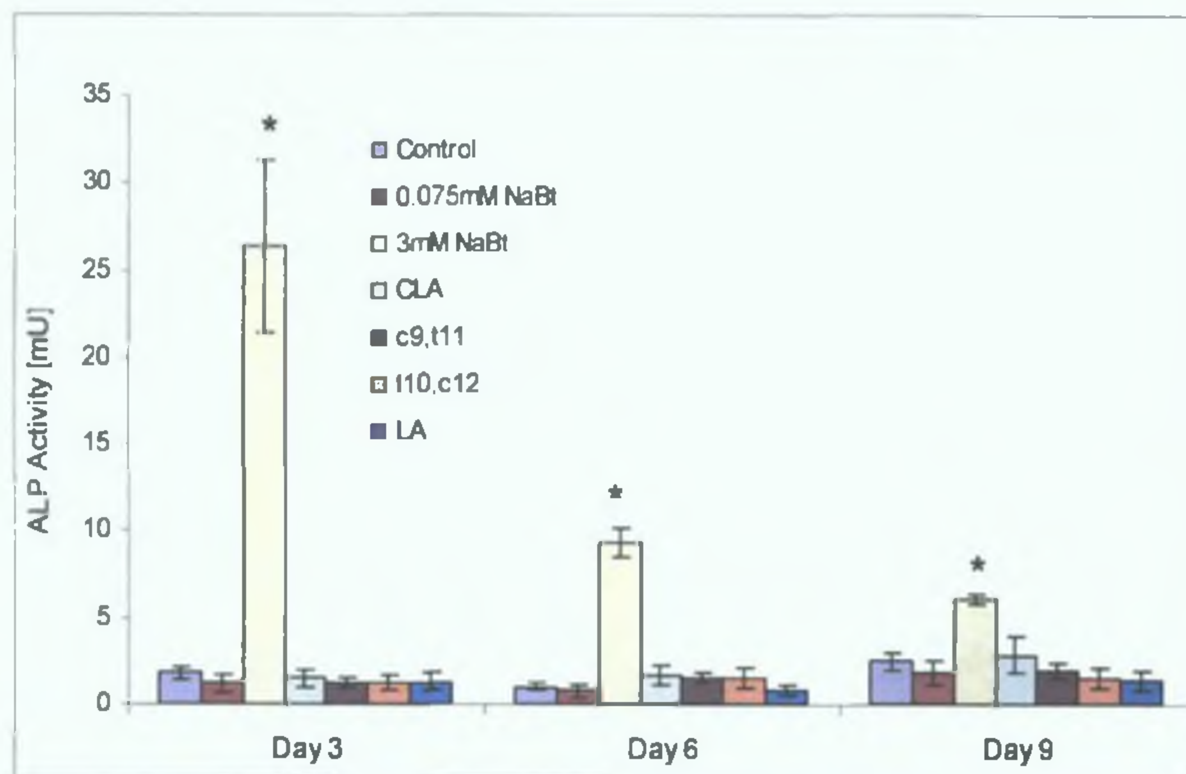

Figure 3.17 Effects of equimolar concentrations of a CLA mixture of isomers (75μM), *c*9, *t*11 CLA (75 μM), *t*10,*c*12 CLA (75 μM), LA (75 μM) and NaBt (75 μM) on alkaline phosphatase (ALP) activity, a commonly employed functional marker of intestinal brush border expression. Ethanol (0.028% v/v) was used as control. ALP activity was measured in total cell lysates (n=3) after 3, 6 and 9 days of continuous treatment. Results shown are the mean (±SD) of treated cells (n=3). ALP activity was expressed as mU where 1 mU = 1nmole product formed/min/mg protein at 37^0C. Asterisks (*) denote significant values (P<0.05) relative to control.

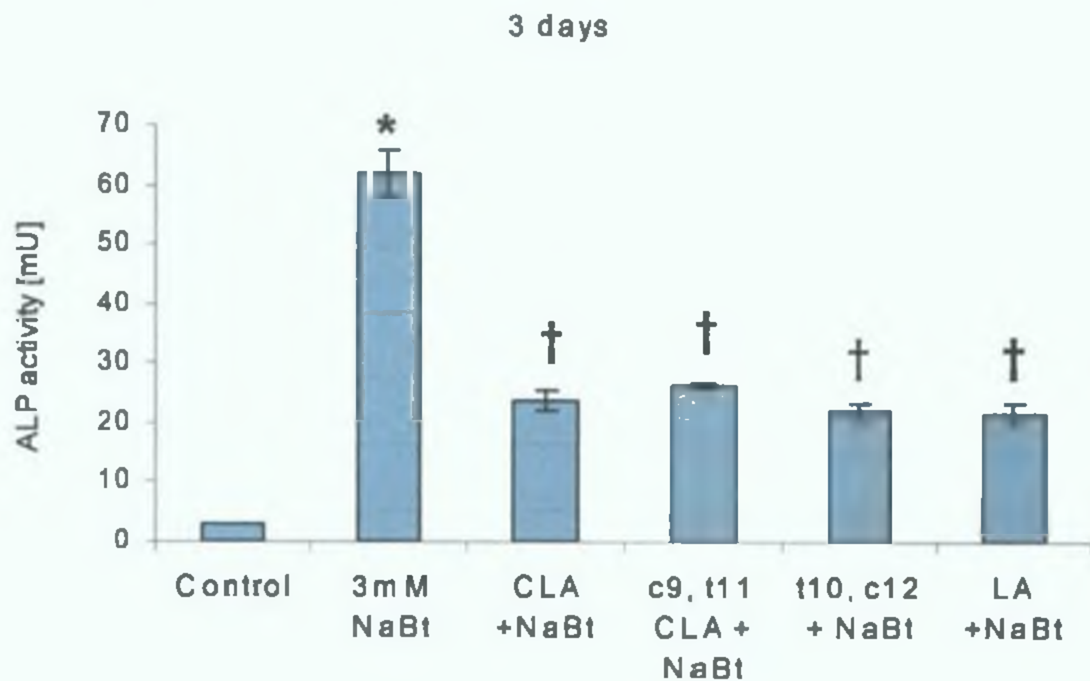

Figure 3.18 Relative alkaline phosphatase (ALP) activity of HT-29 colorectal cancer cells cultured at a density of in DMEM medium 0.8 x 10^6 cells/flask in 75 cm^2 incubated with the combination of 3 mM NaBt and 75μM of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10,*c*12 CLA, and LA for 3 days. Ethanol/BSA (0.028% v/v) was used as control. ALP activity, a commonly employed functional marker of intestinal brush border expression was measured in adherent cell lysates from cultures (n=3) after 2 day of treatment. Result are expressed as mU where 1 mU = 1nmole product formed/min/mg protein at 37^0C. Asterisks (*) denote significant values (P<0.05) relative to control. Asterisks (†) denote significant values (P<0.05) relative to 3mM NaBt.

3.5 Discussion

There is intensive discussion about the potential benefits of a CLA rich diet in prevention of colon cancer A recent longitudinal study that assessed the dietary intake of a large population of woman over 15 years suggested that colorectal cancer risk may be inversely associated with the consumption of high fat dairy foods Although such foods may contain potentially other anticarcinogenic lipids, in particular sphingolipids, much significant research in recent years has turned on their content of CLA It has been previously reported that diets containing a CLA mixture of isomers inhibit colon cancer development in different chemically-induced tumour models (Kohno *et al*, 2004, Yang *et al*, 2002, Park *et al*, 2001, Liew *et al*, 1995, Zu and Schut, 1992) Although primarily absorbed from the small intestine, small amounts of fatty acids can also be absorbed from the large intestine (Caleraro *et al*, 1991, Molina *et al*, 1990) Since growth is a balance between cell proliferation, death and differentiation it is likely that CLA isomers affect not only epithelial cell proliferation but also apoptosis and differentiation in vivo It is noteworthy that cumulative evidence now exists from in vitro studies to show that CLA mixtures of isomers may induce apoptosis and differentiation in colon cancer cells (Lampen *et al*, 2005) Few studies have investigated mechanisms of action of individual CLA isomers such as *c*9, *t*11 CLA or *t*10, *c*12 CLA, the two predominant isomers in the mixture Similarly few studies have investigated the effects of *t*9, *t*11 CLA isomer known to be produced by certain intestinal bifidobacteria (Coakley *et al*, 2006, Rosberg-Cody *et al*, 2004, Coakley *et al*, and 2003) It was shown in Chapter 2 that *t*9, *t*11 CLA was a more potent inhibitor of HT-29 cell growth than *c*9, *t*11 CLA or *t*10, *c*12 CLA This study set out to examine the effects of these isomers on a selection of biological markers that reflect cell death, differentiation and epigenetic influences

In this study, the CLA isomer, *c*9, *t*11 CLA was shown to increase binding of Annexin V to phosphatidylserine exposed on the outer leaflet of plasma membranes as early as 6h after treatment with a concentration physiologically important for humans It is thus likely that *c*9, *t*11 CLA isomer does induce apoptosis of the colon cells investigated here Besides inhibition of proliferation and induction of apoptosis effects on cell

differentiation were also important The increase in CEA level, a molecular biomarker of cell differentiation when cells were treated with *c*9, *t*11 CLA isomer for 5 days confirms a previous report that this CLA isomer may induce differentiation (Lampen *et al*, 2005) The latter showed convincingly that *c*9, *t*11 CLA increased the promoter activity of alkaline phosphatase, its mRNA production and specific activity in Caco 2 cells after 6, 12 and 21 days It is apparent from figure 3 17 that after 3, 6 and 9 days CLA, unlike butyrate did not induce alkaline phosphatase activity in HT-29 cells and even antagonised the effect of butyrate Remarkable differences in the potency of cellular effects of *c*9, *t*11 CLA have been reported between HT-29 cells and Caco 2 colon cells (Lampen *et al*, 2005) The latter were approximately 3 fold more resistant to inhibitory effects of *c*9, *t*11 CLA than HT-29 cells and may therefore be more susceptible to undergo genetic changes associated with cell differentiation before death The observation that CEA levels were increased following treatment with not just *c*9, *t*11 CLA but all CLA treatments suggests CEA may be an earlier onset marker than alkaline phosphatase A comparison of the relative expression of CEA and alkaline phosphatase in three cell lines including HT-29 cells that that are double producers of CEA and alkaline phosphatase revealed that maximux expression of CEA occurred with lower concentration of butyrate that did that of alkaline phosphatase suggesting a differential pattern of expression (Yoshinari *et al*, 1999) It is apparent from this study and in chapter 2 that as early as 6h and 24h that cells are dying and that by 5 days approx 50% have died at this (75μM) concentration The occurrence of higher CEA in CLA-treated cells relative to control suggests that differentiation may be a pre-death cellular characteristics of CLA treatment

Aberrant histone acetylation is believed to be an important etiological factor in several, types of cancer This study showed for the first time that *c*9, *t*11 CLA inhibited the enzymatic deacetylation of DNA-histone complexes to a similar extent as butyrate in total lysates of HT-29 cells Inhibition of HDAC activity, of which there are at least 7 isoenzymes in mammals (Cress and Seto, 2000, Gray and Ekstrom, 2000) permits histones to remain in an acetylated state, which can changes in gene expression and impact on key regulators of apoptosis and the cell cycle such as p21, cyclins (A, E, B1, D1 and D3), apoptosis mediators (Bax and Bcl-2) and transcription factors (c-Myc)

(Louis *et al*, 2004) At present the importance of butyrate-induced HDAC inhibition is believed to be related to its ability to 'reactivate' the expression of epigenetically silenced genes, including those involved in differentiation, cell cycle regulation, apoptosis, angiogenesis, invasion and metastasis Interestingly, much research in recent years has shown that the antiproliferative effects of *c*9, *t*11 CLA are also associated with modulation of HDAC targets such as cyclins, Bcl-2 and p21 in colon cells This study proposes that one of the mechanisms underlying growth arrest and programmed cell death as described by others is that CLA mediates a possible reversal of aberrant epigenetic deacetylation of HT-29 chromatin The consequence of acetylation could be a release of bonds between DNA and histones resulting in an increased accessibility of DNA to various factors involved in upregulation of selected genes eg intestinal alkaline phosphatase (as described by Lampen *et al*, 2005) or CEA In this study CLA-inhibition of HDAC was associated with upregulation of CEA production and membrane flipping of phosphatidylserine Interestingly no effect was observed in adherent cells suggesting that HDAC inhibition is an event associated only with dying cells Further investigations of the physiological function of the different HDAC isoenzymes and their deregulation in human cancer are required in order to devise optimized use of CLA for dietary intervention

The CLA mixture of isomers containing approximately equal proportions of both *c*9, *t*11 CLA or *t*10, *c*12 CLA showed evidence of both differentiation and apoptosis as reflected by CEA and annexin binding It is interesting that all of the CLA treatments investigated, only the CLA mixture increased ceramide content of cells Ceramide belongs to highly bioactive class of molecules known as sphingolipids that are used by cells to regulate growth, differentiation, apoptosis and other cellular functions They are located in lipid-rich structures such as the extracellular leaflet of the cell membrane and are critical for the maintenance of membrane structure, especially that of "microdomains" (such as caveolae) (Harder and Simons 1997), they modulate the behaviour of growth factor receptors and extracellular matrix proteins (Hakomori, 1991) Sphingolipids function as "second messengers" for growth factors, cytokines, differentiation factors and growing list of agonists and toxins (Kolesnick 1998, Merrill *et al*, 1997, Spiegel and Merrill,

1996) Sphingolipid turnover into different bioactive metabolites depends on activation of sphingomyelin hydrolysis to ceramide by sphingomyelinase, which is further metabolized by ceramidase and sphingosine kinase to sphingosine and sphingosine-1-phosphate Agents that activate only sphingomyelinase, which results in ceramide accumulation have profound effects on the behaviour of cells because sphingosine-1-phosphate is potent mitogen and an inhibitor of apoptosis (Cuvillier and Levade, 2003, Olivera and Spiegel, 1993), where sphingosine and ceramide inhibit growth and/or induce apoptosis (Sweeney *et al*, 1998, Hannun, 1994) It would appear that treatment of HT-29 cells with a CLA mixture activates sphingomyelinase to elevate ceramide at the expense of sphingosine (Nikolova-Karakashian *et al*, 1997) or that it inhibits ceramidase Interestingly activation of a neutral sphingomyelinase in tumour tissues by a diet supplemented with fish oils was associated with inhibition of breast cancer growth in nude mice (Wu *et al*, 2005) In parallel in vitro experiments, the latter showed that fish oils inhibited the growth of cultured MDA-MB231 cells while also increasing ceramide formation and neutral sphingomyelinase activity 30-40% Further studies are required to determine if the increase in ceramide levels observed in CLA treated cells may be due to modulation of neutral sphingomyelinase, downregulation of which has been noted to be one of the earliest biochemical changes detected in colon cancer (Dudeja *et al*, 1986)

The *t*10, *c*12 CLA isomer also increased annexin V binding to phosphatidylserine, suggesting apoptosis after incubation with physiological level of fatty acid for 6h and 24h As with *c*9, *t*11 CLA isomer, the *t*10, *c*12 CLA isomer had no effect on alkaline phosphatase but did increase cellular CEA levels in cells It is apparent that isomers of CLA differ in potency as well as in manifestation of cellular and molecular effects Though *c*9, *t*11 CLA significantly inhibited HDAC activity neither *t*10, *c*12 CLA nor the CLA mixture showed a significant effect An investigation of structure-activity requirements of HDAC inhibitors showed that potent HDAC inhibitors such as butyrate fit fully into the active site of the enzyme and that its carboxylate group forms a bidentate ligand with a buried zinc atom (Finnin *et al*, 1999) The inhibition observed with *c*9, *t*11 CLA suggests that this fatty acid must also have gained access to the HDAC active site presumably positioning its terminal carboxylic acid group adjacent to the zinc atom It is

possible that the shape of the fatty acid tail of *t*10, *c*12 CLA and of constituent isomers in the CLA mixture may limit access to the HDAC pocket, thereby diminishing their relative importance in modulating histone-DNA interactions by acetylation

The *t*9, *t*11 CLA isomer, known to be produced by strains of bifidobacteria was observed to be potent inhibitor of HT-29 cell growth (Chapter 2) This study showed that programmed cell death may not be the main mode of death for this isomer as unlike *c*9, *t*11 CLA or *t*10, *c*12 CLA it did not affect annexin V binding to phosphatidylserine Surprisingly, it also increased CEA production but not ceramide suggesting that cells may have undergone differentiation prior to death by necrosis Interestingly, the *t*9, *t*11 CLA isomer (but not C18 1 *t*-11) was also a potent inhibitor of HDAC suggesting it like *c*9, *t*11 CLA may of the desired orientation for the active site of the enzyme The observation that trans vaccenic acid (C18 1 *t*-11) did not inhibit HDAC may be further indication of the specificity of fatty acid binding to HDAC It was apparent from Chapter 2 that *t*-VA was the least potent of the fatty acids in inhibiting cell growth Although a previous study clearly showed that *t*-VA induced DNA fragmentation in SW480 colon cancer cells and that the cellular responses to *t*-VA were likely to be mediated by *t*-VA desaturation to *c*9, *t*11 CLA via Δ9 desaturase (Miller *et al*, 2003), there was no evidence from this study that *t*-VA was a bioactive lipid in HT-29 cells Further studies are required to examine the potential for HT-29 cells to bioconvert *t*-VA to *c*9, *t*11 CLA

Data from this study suggest that there is an association exists between decreased HDAC activity, increased ceramide level, increased CEA level and growth suppression in the HT-29 cells treated with the various CLA isomers

In conclusion this study has identified three novel biological markers (CEA, ceramide and HDAC) by which various CLA isomers may exert antiproliferative effects in HT-29 cells

CHAPTER 4

Modulation of cellular lipids in HT-29 human colon cancer cell line by CLA

Abstract

Modulation of fatty acid synthase (FAS) and stearoyl CoA desaturase (SCD) activities may be an attractive target for inhibition of cancer cell growth The hypothesis that growth inhibition by CLA may be related to modulation of cellular lipids in HT-29 cancer cells was studied CLA treatments and *t*-VA decreased the proportion of palmitate and stearate in HT-29 colon cancer cells compared to untreated control cells suggesting that CLA and *t*-VA treatments may be potent inhibitors of FAS The *c*9, *t*11 CLA and *t*10, *c*12 CLA acted as more potent inhibitors of FAS than the CLA mixture of isomers or *t*9, *t*11 CLA Treatments with CLA and *t*-VA also affected SCD activity in the HT-29 cells Desaturation of stearate to oleate was inhibited with all CLA treatments except *c*9, *t*11 CLA All CLA treatments significantly inhibited Δ-9 desaturation from palmitate to palmitoleate after 120h incubation suggesting that CLA treatments may inhibit SCD activity The *t*-VA also decreased the Δ9 desaturation after 120h at the highest concentration (100μM) suggesting that like CLA, *t*-VA may be an inhibitory regulator of SCD This study also showed that *t*-VA was desaturated to *c*9, *t*11 CLA in HT-29 cells and that conversion was linear with respect to amount of *t*-VA presented to cells and duration of treatment It is conclude that the growth inhibitory effects of CLA may be mediated by changing fatty acid composition through modulating FAS and SCD activities

4.1 Introduction

After numerous clinical and basic studies, it now appears that human cancer cells have the capacity to synthesize their own supply of fatty acids, seemingly independent of the regulatory signals that downregulate fatty-acid synthesis in normal cells (reviewed in Kuhajda, 2000) Fatty-acid synthesis is common to all plants and animals Fatty acids are involved in diverse functions in cells from energy storage and membrane structure to signal transduction cascades and protein acylation (reviewed in Kuhajda, 2000) De novo synthesis of fatty acids by tumor cells accounted for more than 93% of triacylglycerol fatty acids Endogenous fatty-acid synthesis could be a significant source of fatty acids for growth of tumor cells, considering the rates of transport of free fatty acid and plasma triacylglycerol from the host to the tumor cells (Reviewed in Kuhajda, 2000)

The synthesis of malonyl-CoA is the first committed step of fatty acid synthesis and the enzyme that catalyzes this reaction, acetyl-CoA carboxylase (ACC), is the major site of regulation of fatty acid synthesis The synthesis of fatty acids from acetyl-CoA and malonyl-CoA is carried out by fatty acid synthase, FAS (Wakil, 1989, Witkowsk *et al*, 1991) All of the reactions of fatty acid synthesis are carried out by the multiple enzymatic activities of FAS The primary fatty acid synthesized by FAS is palmitate Palmitate is then released from the enzyme and can then undergo separate elongation and/or unsaturation to yield other fatty acid molecules FAS is downregulated in most normal human tissues because of the fat in human diet In contrast, FAS is often highly expressed in human cancers High levels of FAS expression have been found in many human cancers including breast, prostate, colon, ovary, endometrium, thyroid, oral cavity, esophagus, bladder, retinoblastoma and melanoma (reviewed in Guo *et al*, 2004, Camassei, *et al*, 2003, Innocenzi *et al*, 2003, Nemoto *et al*, 2001, Kuhajda, 2000, Alo *et al*, 1996,)

The differential tissue distribution makes FAS an attractive target for cancer cells For example, FAS is highly expressed in colon cancer but not in normal colonic mucosa In this setting, colon cancer could be targeted by a FAS inhibitor, but the proliferating compartment of the colon would be unaffected (reviewed in Kuhajda, 2000) The

association of FAS expression and tumor virulence led to the conception that FAS expression and activity may be vital for the growth and survival of human cancer cells Studies have demonstrated that inhibition of FAS is selectively cytotoxic to human cancer cells in vivo (reviewed in Kuhajda, 2000) There is increasing evidence linking activity of the fatty-acid-synthesis pathway, DNA synthesis, and proliferation in cancer cells (Menendez *et al*, 2005) Inhibition of fatty-acid synthesis could be a means to limit cytotoxic therapy to proliferating cells with high levels of FAS This strategy would likely target cancer cells and leave the normal proliferating cellular compartments in bone marrow, skin, and gastrointestinal tract intact (reviewed in Kuhajda, 2000)

Another important enzyme in fatty acid synthesis is stearoyl-CoA desaturase (SCD) which is the rate-limiting enzyme catalyzing the synthesis of monounsaturated fatty acids, mainly oleate (18 1) and palmitoleate (16 1) These represent the major monounsaturated fatty acids of membrane phospholipids, triglycerides, wax esters (*i e* esters of long-chain fatty alcohols with long-chain fatty acids) and cholesterol esters The ratio of saturated to monounsaturated fatty acids affects phospholipid composition and alteration in this ratio has been implicated in a variety of disease states including cardiovascular disease, obesity, diabetes, neurological disease, and cancer For this reason, the expression of SCD is of physiological significance in both normal and disease states (reviewed in Ntambi and Miyazaki, 2004)

Large numbers of experimental data show that tumour cell growth can be modulated by individual fatty acids (Guthrie and Carroll, 1999, Zhou and Blackburn, 1999) Functional foods contain dietary components that have beneficial properties beyond their traditional nutrient value (National Research Council, 1994) The predominant CLA found in milk fat, the *cis*-9, *trans*-11 isomer (rumenic acid), has been shown to be anticarcinogenic in animal models (McGuire *et al*, 2000) Also trans vaccenic acid has been claimed to have anticarcinogenic properties, probably due to *in vivo* conversion into rumenic acid (Corl *et al*, 2003, Turpeinen *et al*, 2002) Others have also shown endogenous synthesis of *c*9, *t*11 CLA in mice, rats and humans when diets were supplemented with *trans*-11 C18 1 (*t*-VA) (Adlof *et al*, 2000, Santora *et al*, 2000, Ip *et al*, 1999, Salminen *et al*, 1998b)

Different mechanisms of the anticarcinogenic action of CLA have been hypothesized One other possible mechanism for the anticarcinogenic activity of CLA is the alteration of the FA composition of cell membrane phospholipids (PLs) by CLA isomers resulting in reduced synthesis of arachidonic acid (AA) and arachidonate-derived eicosanoids, which are associated with stimulation of cancer cell growth (Park *et al*, 2004, Banni *et al*, 1999) There is some evidence that CLA inhibits the desaturation of LA and the formation of AA and prostaglandin E_2 (PGE_2) (Park *et al*, 2004, Liu and Belury, 1998) Like most other dietary polyunsaturated fatty acids, CLA isomers and their metabolites are readily incorporated into phospholipid and neutral lipid fractions of numerous tissues (Moya-Camarena *et al*, 1999c, Belury and Kempa-Steczko, 1997, Ip *et al*, 1996 and 1991, Ha *et al*, 1990)

There is no evidence to date that CLAs have any effects on FAS activity Therefore one of the objectives of the present study was to examine the relationship between CLA and the FAS products palmitate and stearate

The present study was designed to determine the cellular incorporation of the specific CLA isomers in total lipids and in three major lipid classes phospholipids, neutral lipids and fatty acid fraction in HT-29 cells *t*-VA incorporation in total lipid and its conversion to *c*9, *t*11 CLA in HT-29 cells was also examined In addition this study examined the effects of CLA mixture of isomers, three different single isomers of CLA (*c*9, *t*11 CLA and *t*10, *c*12 CLA and *t*9, *t*11 CLA) and *t*-VA on cellular lipid composition

4.2 Aim and specific objectives

The overall aim of this study was to examine the effect of CLA and *t*-VA on lipid composition of HT-29 human colon cancer cells

The specific objectives were as follows

- To validate a gas chromatographic procedure for analysis of CLA and *t*-VA in HT-29 human colon cancer cells
- To characterize the lipid composition of HT-29 cells
- To determine if cellular lipid composition could be altered by treatment of cells with CLA and *t*-VA, as free fatty acids and as fatty acid–albumin complexes
- To determine the conversion of *t*-VA to *c*9, *t*11 CLA in HT-29 cells

4.3 Materials and methods

4.3.1 Materials

The HT-29 human colon cancer cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) Dulbecco's Minimum Essential Medium (DMEM), supplements and related solutions, boron trifluoride methanol (14% solution), (10%) bovine serum albumin (BSA) essentially fatty acid-free, Chloroform (GC grade), methanol(GC grade), hexane(GC grade), and 2-propanol(GC grade), were purchased from Sigma-Aldrich, Dublin Acetic acid glacial (100%) and HCL (37%) were from BDH Laboratory Supplies England and diethyl-ether (HPLC grade) was from Lab-Scan, Analytical Science GC column WCOT Fused Silica CP-Select CB column- 100m x 0 25 mm ID, 0 2μm film thickness was purchased from Chrompack, Middleburg, The Netherlands Sep-Pak Vac 3cc (500 mg) NH_2 Cartridges were obtained from Waters, Waters Corporation, Ireland, a Vac Elute vacuum elution apparatus with adaptors and Vac Elut sample collector racks were obtained from Analytichem International, Harbor City, CA

All sterile disposable plastic-ware was from Sarstedt Ltd , Wexford, Ireland Phosphate buffered saline (PBS) (Lennox, Cat BR14) was prepared by dissolving five tablets in 500 mL ultra-distilled water (dH_2O)

Free fatty acids. Conjugated Linoleic Acid (CLA) mixture of isomers (99% pure, approximately comprising 44% *t*10, *c*12 , 41% *c* 9, *t*11, 10% *c*10, *c*12 and minor amounts of *t*9, *t*11, *t*10, *t*12, *c*9, *c*11-CLA) and single preparations (90% pure) of isomers *c*9, *t*11 CLA and *t*10, *c*12 CLA were from NuChek-Prep, Elysian, MN, USA Single preparations (98% pure) of *t*9, *t*11 CLA isomer was from Matreya, Inc Netherlands Linoleic Acid (LA), Heptadecanoic acid (C17 0) were purchased from Sigma-Aldrich, Dublin, Ireland

FAME (Fatty acid methyl ester) Standards: FAME of *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and a FAME mixture of 37 fatty acids (Table 1) were purchased from Matreya, Inc Netherland FAME of *t*-VA was from Supelco Sigma-Aldrich Chemical Co (St Louis, MO) A FAME mixture, containing 5 FAME [Methyl octanonate (C8 0) Methyl decanoate (C10 0), Methyl laurate (C12 0), Methyl Myristate (C14 0) Methyl Palmitate (C16 0)] were purchased from Sigma-Aldrich Chemical Co (St Louis, MO) Two more FAME mixture, one (GLC 20A) containing 6 FAME [Methyl Palmitate (C16 0), Methyl Stearate(C18 0), Methyl Oleate (C18 1, *c*9), Methyl Linoleate(C8 2, *c*9, *c*12), Methyl Linolenate (C18 3, *c*9, *c*12 and *c*15) and Methyl Arachidate(C20 0)] and other one (GLC 20A) containing 7 FAME [Methyl Myristate (C14 0) Methyl Palmitate (C16 0), Methyl Palmitoleate (C16 1, *c*9), Methyl Stearate(C18 0), Methyl Oleate (C18 1, *c*9), Methyl Linoleate(C8 2, *c*9, *c*12) and Methyl Linolenate (C18 3, *c*9, *c*12 and *c*15)] were gifted by NuChek-Prep, Elysian, MN, USA

4.3.2 Analysis of fatty acid profiles of HT-29 cells in total cellular lipid

4.3 2 1 Cell culture

Inıtially HT-29 cells were seeded ın 25cm^2 culture flasks at a densıty of 5 x 10^5 cells/flask and cultured for 24 h to allow the cells attach to the substratum The cells were maıntaıned ın a humıdıfied atmosphere The pH of the medıa was maıntaıned at 7 2-7 4 pH by a requıred flow of 95% aır and 5% CO_2 ın a CO_2 ıncubator (Model IR2424)

4.3.2.2 Cell treatment

The medıum was removed after thıs 24 hour ıncubatıon and replaced wıth fresh medıum after washıng the cells wıth phosphate buffered salıne (PBS) Cells were then treated wıth 25-100µM of a CLA mıxture of ısomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA, *t*-VA and LA as eıther free fatty acıds or as fatty acıd-albumın complexes (2 1 molar ratıo) and ıncubated for specıfic tıme poınts Control flasks were supplemented wıth an equıvalent amount of ethanol or ethanol/BSA was used as control (0 028%, v/v)

4 3.2.3 Cell harvestıng

After ıncubatıon, the medıa contaınıng dead cells were collected ın unıversals and centrıfuged at 1000 rpm for 5 mın to collect the floatıng cells The supernatant was dıscarded Adherent cells were harvested usıng phosphate buffered salıne (PBS) contaınıng 0 25% (w/v) trypsın (Sıgma-Aldrıch Ireland Ltd) and collected ın the same unıversal whıch contaıned the floatıng cells and then centrıfuged at 1000 rpm for 5 mın Supernatants were dıscarded and cell pellets saved for lıpıd extractıon

4 3.2.4 Extractıon, methylataıon and gas chromatographıc (GC) analysıs of total cellular lıpıds

Fatty acıd profiles of total cellular lıpıds of HT-29 cells were analysed ın 3 steps lıpıd extractıon, methylatıon and GC analysıs

4.3.2.4 1 Cellular lıpıd extractıon

The procedure used for cellular lıpıd extractıon was a modıfied versıon of the methods of Folch *et al*, (1957) and Blıgh and Dyer (1959) Heptadecanoıc acıd ($C_{17\ 0}$) was used as an ınternal standard to calculate the amounts of fatty acıds ın cellular extracts The ınternal standard was used to correct for varıatıon ın volume ınjectıons from the standard (e g, small dıfferences ın volume, splıt ratıo, dılutıons, etc) Brıefly 3 75 ml chloroform/methanol (2 1, v/v) and 386 4 μg heptadecanoıc acıd ın ethanol ($C_{17\ 0}$) was added to harvested cells Vortexed for 3 mınutes and 1 25 ml chloroform was added followed by vortex another 1 mınute and added 1 25 ml H_2O and vortexed agaın for 1 mm Centrıfuged at 2000 rpm for 8 mm Lower phase was collected ın a GC vıal through the proteın dısk wıth a pasture pıpette Solvent was evaporated wıth N_2 and stored at -20^0 C untıl methylatıon

4 3 2 4 2 Preparatıon of fatty acıd methyl ester

The free fatty acıds were methylated ın 14% BF_3/methanol accordıng to the method descrıbed by Alonso, *et al*, (2004) In brıef, 100μl methanolıc NaOH (1M) was added to the lıpıd extract, mıxed and left ın water bath at 70^0 C for 15 mm Then 200 μl 14% BF_3 ın methanol was added and ıncubated at room temperature for 30 mm 200 μl hexane and 100 μl H_2O were added next followed by vortexed thoroughly Organıc phase (upper layer) was collected ın a GC vıal after 5 mınutes centrıfuge at 1000 rpm and stored at -20^0 C untıl GC analysıs Sample contaınıng FAME ware drıed down under N_2 and 100

µl of hexane was added before injection The injection volume was 1 µl through the experiment

4.3.2.4.3 GC analysis

The methyl esters of fatty acids (FAME) were analyzed with a VARIAN CP-3800 gas chromatograph equipped with a flame ionization detector and a WCOT Fused Silica CP-Select CB column- 100m x 0 25 mm ID, 0 2µm film thickness (Chrompack, Middleburg, The Netherlands) At first the optimal operation conditions of GC were tested with a few different temperature programmes by injecting 1 µl aliquots of a mixture of 41 FAME standards (Matreya, Inc , Netherland) with Hamilton micro syringe Then the following temperature program was utilized for optimal separation of FAMEs The injector and the detector temperature were maintained at 250°C The column temperature was operated isothermally at 190°C for 60 minutes after injection of samples and then raised from 190°C to 225°C at 4°C /min with a final hold of 10 mm at 225°C Nitrogen was used as carrier gas with column flow rate 0 7 ml/min Samples were run in split (1 20) mode

4.3.2.5 Validation of GC Methods

4.3.2.5.1 Recovery of fatty acids

To evaluate the percentage recoveries of fatty acids, 21µg -140µg of fatty acids (CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and *t*-VA) were spiked into 2 x 10^6 HT-29 cells in triplicate The samples were taken through the entire procedure (lipid extraction, methylataion) and analysed by GC Recoveries were calculated by expressing recovered amounts of individual fatty acids as a percentage of the amounts added

4.3.2.5.2 Repeatability

To evaluate the percentage repeatability of the assay, 21μg of fatty acids (CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and *t*-VA) were spiked into 2 x 10^6 HT-29 cells on 5 different days in triplicate The samples were taken through the entire procedure (lipid extraction and methylation) and analysed by GC Repeatability was calculated by determines amounts of individual fatty acids recovered each of the over 5 days

4.3.3 Analysis of phospholipids, neutral lipids and fatty acids fractions of HT-29 cellular lipid

Cellular lipids were fractioned according to the method of Kaluzny *et al*, (1985) Due to the rapidity and high yields (> 95%) of this procedure, it is superior to preparative HPLC or TLC for the separation of lipid mixtures for subsequent analysis (Kaluzny *et al*, 1985)

After cellular lipid extraction liquids were evaporated to dryness under nitrogen and re-dissolved in 0 4 ml chloroform Sep-Pak Vac 3cc (500 mg) NH_2 cartridges (Bond Elut columns) were placed in the Vac Elut apparatus and washed twice under vacuum with 2 ml aliquots of hexane A collection rack with receiving tubes was then placed in the Vac Elut (Fig 4 1) The vacuum was released immediately after the second hexane wash to prevent the columns from becoming completely dry Cellular lipids in chloroform were applied to the column under vaccum and the chloroform was pulled through This left the entire lipid mixture on the column Next, the column was eluted with 4 ml of solvent chloroform 2 propanol (2 1, v/v) to separate the neutral lipid fraction The eluant (neutral lipid) was saved and new collection tubes were placed in the collecting racks The column was then eluted with 4 ml of 2% acetic acid in diethyl ether and the fatty acid fraction of cellular lipids was collected and saved Again another set of new collection tubes was placed in the collecting racks to separate the phospholipids fraction The

column was then eluted with 4 ml of methanol and the eluant (phospholipids) was collected and saved The liquid (solvent) from all three fractions was evaporated with N_2 and 100μL hexane was added to each tube to re-dissolve the lipid fractions and transferred to the GC vial and stored at -20^0 C until methylation The methylation procedure was performed as described in section 4 3 2 4 2

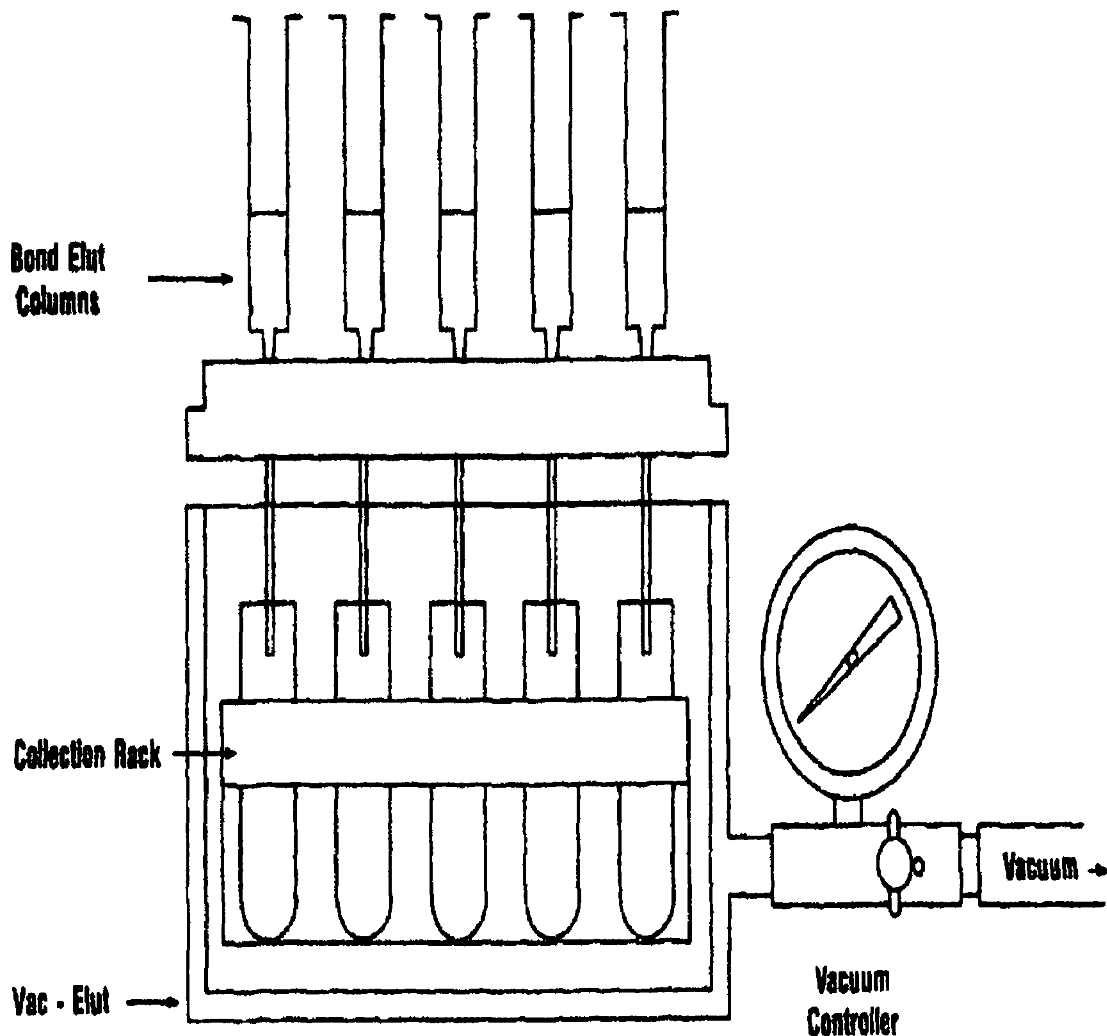

Figure 4.1 Vac-Elut apparatus used to hold Bond Elut columns for isolation of lipid classes The collection rack holding receiving tubes is shown in the 3-dimensional drawing (Kaluzny *et al* , 1985)

4.3.4 Identification and quantitative analysis of FAMEs

Identification of the fatty acids was based on their retention time with reference to a FAME standards mixture (Matreya, Inc., Netherland). Quantification requires several steps. Sample peak areas must be compared with the standard peak areas. Since the concentrations of FAMEs responsible for the standard peaks are known, this comparison permits concentration to be calculated from the sample peak area.

However, direct comparison is not possible because of the likelihood of small differences the in volume, split ratio and dilutions between sample injection and standard injection. An internal standard is required to correct for variations in sample volume; the ratio of all peak areas to the internal standard in both the sample and the standard are determined. Since the internal standard has a known concentration in both sample and standard, it can be used to correct for sample variations. A_x denotes the area of a peak due to compound x in the sample. Ac,x denotes "corrected area" and is determined for each peak in the chromatogram using the equation

$$Ac,x = A_x \times \frac{A\ C_{17:0}\ \text{standard}}{A\ C_{17:0}\ \text{sample}} \times \frac{[C_{17:0}\ \text{sample}]}{[C_{17:0}\ \text{standard}]} \qquad \textbf{Equation 1}$$

The A $C_{17:0}$ standard and A $C_{17:0}$ sample denote the peak area of $C_{17:0}$ standard fatty acid and peak area of $C_{17:0}$ in the sample chromatogram respectively. The [$C_{17:0}$ standard] and [$C_{17:0}$ sample] denote the concentration of $C_{17:0}$ standard fatty acid and concentration of $C_{17:0}$ in the sample respectively (Table 4.1 & 4.2).

From the corrected areas (Ac,x), the concentration of specific fatty acid [x] in cellular lipids can be calculate using the equation 2.

$$[x] = A_{c,x} \times \frac{[x]_{standard}}{A_{x\ standard}}$$ **Equation 2**

The $Ax_{standard}$ and $[x]standard$ denote the peak area and concentration of standard specific fatty acid x respectively (Table 4.1).

An example of how [*t*10, *c*12 CLA] in a lipid extract may be quantified is as follows:
Table 4.1 shows the chemical names, abbreviations, [FAMEs], retention times and peak areas of 41 fatty acid methyl esters of a standard mixture. It is apparent that $C_{17:0}$, the internal standard, at a concentration of 0.6 mg/ml eluted at 21.5 minutes and had a peak area of 182995. By comparison, the *t*10, *c*12 CLA isomer present at 1mg/ml eluted at 36.07 min and had a peak area of 310260.

Table 4.2 shows typical data from GC analysis of a sample. It records peak number, retention times and area counts for all fatty acids present in a sample lipid extract. It is apparent that C17:0, the internal standard, present at a concentration of 3.86 mg/ml eluted at 21.404 min and had an area count of 1176792. *t*10 *c*12 CLA isomer eluted at 35.579 min and had a peak area of 109435.

Using **Equation 1**, Ac,x the corrected peak area for *t*10 *c*12 CLA in the sample is :

$$109435 \times \frac{182995}{1176792} \times \frac{3.86\ mg/ml}{0.6\ mg/ml}$$

= 109479.2

Using **Equation 2,** the [*t*10, *c*12 CLA] in the sample is

$$[t10, c12\ \text{CLA}] = 109479\ 2 \times \frac{1\text{mg/ml}}{310260}$$

$$= 0\ 35\ \text{mg/ml}$$

4.3.5 Identification of the peak of the standard fatty acid methyl ester

A mixture of fatty acids methyl ester (FAME), containing 37 fatty acids was purchased from Matreya, Inc , Netherlands This FAME mixture was analyzed by GC with described GC condition and peaks were identified according to the suppliers specifications Another 2 mixture of FAME [{C14 0, C16 0, C16 1, C18 0, C18 1(cis-9), C18 2(cis-9,12), C18 3(cis-9,12,15)} and {C16 0, C18 0, C18 1(cis-9), C18 2(cis-9,12), C18 3(cis-9,12,15), C20}] got from Nu-Check Prep as a gift (in 2004 AOCS Annual Meeting, Cincinnati), were analyzed and compared with the FAME mixture of Matrya Also another 2 FAME mixture (C8 0-C18 3) from sigma were analyzed and compared with the other FAME mixtured and confirmed with the comparision of the retention time The FAME of *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA were purchased from Matreya, Inc , Netherland and FAME of *t*-VA was purchased from Supelco independently and analyzed by GC independently Then these 4 FAME were mixed into the FAME mixture of Matreya and again analyzed by the same GC programme The peak of *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and *t*-VA in the FAME mixture were identified with the comparision of the retention time in the mixture to retention time of the peak of the individual fatty acids These final mixture of FAME were injected with every experiment and identifications and quantification of fatty acids in the cellular lipids were performed with these mixture Table 4 1 shows the chemical name, abbreviation and retention time of mixture of FAME standard and methyl esters of CLA isomers (*c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA)

Table 4.1 Chemical name, abbreviation, retention time and peak area of standard fatty acid methyl esters mg/ml as determined using Varien CP 3800 gas chromatography

Chemical name	Abbreviation	Amounts mg/ml	Retention time(Min)	Peak Area
Hexane	Solvent		11 37	65816984
Methyl Butyrate	C4 0	1 2	Unidentified	Unidentified
Methyl hexanoate	C6 0	1 2	11 77	156481
Methyl octanoate	C8 0	1 2	12 13	342043
Methyl decanoate	C10 0	1 2	12 76	373759
Methyl undecanoate	C11 0	0 6	13 23	184142
Methyl laurate	C12 0	1 2	13 85	371395
Methyl tetradecanoate	C13 0	0 6	14 67	182420
Methyl myristate	C14 0	1 2	15 75	359816
Methyl myristoleate	C14 1(*cis*-9)	0 6	16 62	182414
Methyl pentadecanoate	C15 0	0 6	17 17	180828
Methyl pentadecenoate	C15 1(*cis*-10)	0 6	18 32	178428
Methyl palmitate	C16 0	1 8	19 06	548625
Methyl palmitoleate	C16 1(*cis*-9)	0 6	20 25	182482
Methyl heptadecanoate	**C17·0**	**0.6**	**21 50**	**182995**
Methyl heptadecenoate	C17 1(*cis*-10)	0 6	23 07	183425
Methyl stearate	C18 0	1 2	24 76	370275
Methyl elaidate	C18 1(*trans*-9)	0 6	25 69	185967
Methyl vaccinate	C18 1(*trans*-11)	**1**	**25 87**	**199579**
Methyl oleate	C18 1(*cis*-9)	1 2	26 47	372318
Methyl linoelaidate	C18 2(*trans*9, *trans*12)	0 6	27 68	182987
Methyl linoleate	C18 2(*cis*9, *cis*12)	0 6	29 48	174958
Methyl gamma-linoleate	C18 3(*cis*6, *cis*9, *cis*12)	0 6	31 83	180502
Methyl linolenate	C18 3(*cis*9, *cis*12, *cis*15)	0 6	33 80	180574
Methyl arachidate	C20 0	1 2	34 58	373062
Methyl conjugated linoleate	**C18 2(*cis*9, *trans*11)**	**1**	**35 19**	**304816**
Methyl conjugated linoleate	**C18·2(*trans* 10, *cis*12)**	**1**	**36 07**	**310260**
Methyl eicosanoate	C20 1(cis11)	0 6	37 31	184172
Methyl conjugated linoleate	**C18 2(*trans*9, *trans* 11)**	**1**	**38 06**	**307955**
Methyl heneicosanoate	C21 0	0 6	41 76	188930
Methyl eicosadienoate	C20 2(*cis*11, *cis*14)	0 6	42 50	184638
Methyl eicosatrienoate	C20 3(*cis*8, *cis*11, *cis*14)	0 6	46 35	173984
Methyl arachidonate	C20 4(*cis*5, *cis*8, *cis*11, *cis*14)	0 6	49 28	166865
Methyl eicosatrienoate	C20 3(*cis*11, *cis*14, *cis*17)	0 6	49 79	177703
Methyl behenate	C22 0	1 2	51 34	379805
Methyl erucate	C22 1(*cis*13)	0 6	55 80	190739
Methyl tricosanoate	C23 0	0 6	58 11	175647
Methyl docosadienoate	C22 2(*cis*13, *cis*16)	0 6	62 97	192641
Methyl eicosapentaenoate	C20 5(*c*5, *c*8, *c*11, *cis*14, *cis*17)	0 6	63 71	189897
Methyl lignocerate	C24 0	1 2	69 55	389994
Methyl nervonate	C24 1(*cis*15)	0 6	71 77	194608
Methyl docosahexaenoate	C22 6(*c*4, *c*7, *c*10, *c*13, *c*16, *c*19)	0 6	75 07	164443

Table 4.2 Typical data from GC analysis of fatty acids in HT-29 cells after incubation with *t*10, *c*12 CLA.

Peak No	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	Status Codes	Group
1		96.7518	11.345	0.000	62383608	0.00	BB	6.0		0
2		0.1018	11.624	0.000	65660	0.00	TF	0.0		0
3		0.1526	11.887	0.000	98363	0.00	TF	0.0		0
4		0.0573	12.170	0.000	36967	0.00	TF	0.0		0
5		0.1037	12.353	0.000	66856	0.00	TF	0.0		0
6		0.0033	12.760	0.000	2096	0.00	TF	0.0		0
7		0.0016	12.891	0.000	1061	0.00	TF	0.0		0
8		0.0049	13.006	0.000	3154	0.00	TF	0.0		0
9		0.0722	13.179	0.000	46567	0.00	TF	0.0		0
10		0.0019	13.779	0.000	1222	0.00	TS	0.0		0
11		0.0410	14.704	0.000	26444	0.00	BB	20.6		0
12		0.0354	15.650	0.000	22849	0.00	BB	5.0		0
13		0.0134	16.132	0.000	8615	0.00	BB	4.2		0
14		0.0053	17.050	0.000	3438	0.00	BV	6.6		0
15		0.0296	17.486	0.000	19082	0.00	VV	9.3		0
16		0.0081	17.883	0.000	5237	0.00	VB	0.0		0
17		0.1962	18.896	0.000	126494	0.00	BB	7.0		0
18	C17:0	0.0057	19.425	0.000	3672	0.00	BV	6.5		0
19		0.0071	19.860	0.000	4588	0.00	VV	7.0		0
20		0.0157	20.073	0.000	10114	0.00	VV	7.2		0
21		0.0055	20.566	0.000	3514	0.00	VB	8.4		0
22		1.8251	21.404	0.000	1176792	0.00	BB	8.6		0
23		0.0060	22.069	0.000	3858	0.00	TF	0.0		0
24		0.0072	22.722	0.000	4632	0.00	TF	0.0		0
25		0.1848	24.504	0.000	119172	0.00	BB	10.2		0
26		0.0363	25.373	0.000	23396	0.00	BB	7.9		0
27		0.0606	26.162	0.000	39084	0.00	BV	9.9		0
28	*t*10, *c*12 CLA	0.0107	26.485	0.000	6901	0.00	VB	9.9		0
29		0.0032	28.665	0.000	2039	0.00	BV	14.6		0
30		0.0113	29.150	0.000	7262	0.00	VB	10.6		0
31		0.0035	30.736	0.000	2233	0.00	BB	13.7		0
32		0.0036	34.119	0.000	2297	0.00	BB	14.7		0
33		0.1697	35.579	0.000	109435	0.00	BB	13.1		0
34		0.0021	37.507	0.000	1369	0.00	BB	14.0		0
35		0.0136	44.064	0.000	8748	0.00	BB	39.9		0
36		0.0031	45.688	0.000	2010	0.00	BB	14.0		0
37		0.0168	48.566	0.000	10805	0.00	BB	17.2		0
38		0.0061	52.751	0.000	3930	0.00	BB	20.9		0
39		0.0044	57.216	0.000	2833	0.00	BB	18.2		0
40		0.0066	73.155	0.000	4285	0.00	BB	10.0		0
41		0.0113	74.631	0.000	7280	0.00	BB	9.9		0
	Totals	**100.0001**		**0.000**	**64477956**					

Figure 4.2 shows the full chromatogram of mixture of FAME standard and methyl esters of CLA isomers[*c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA].

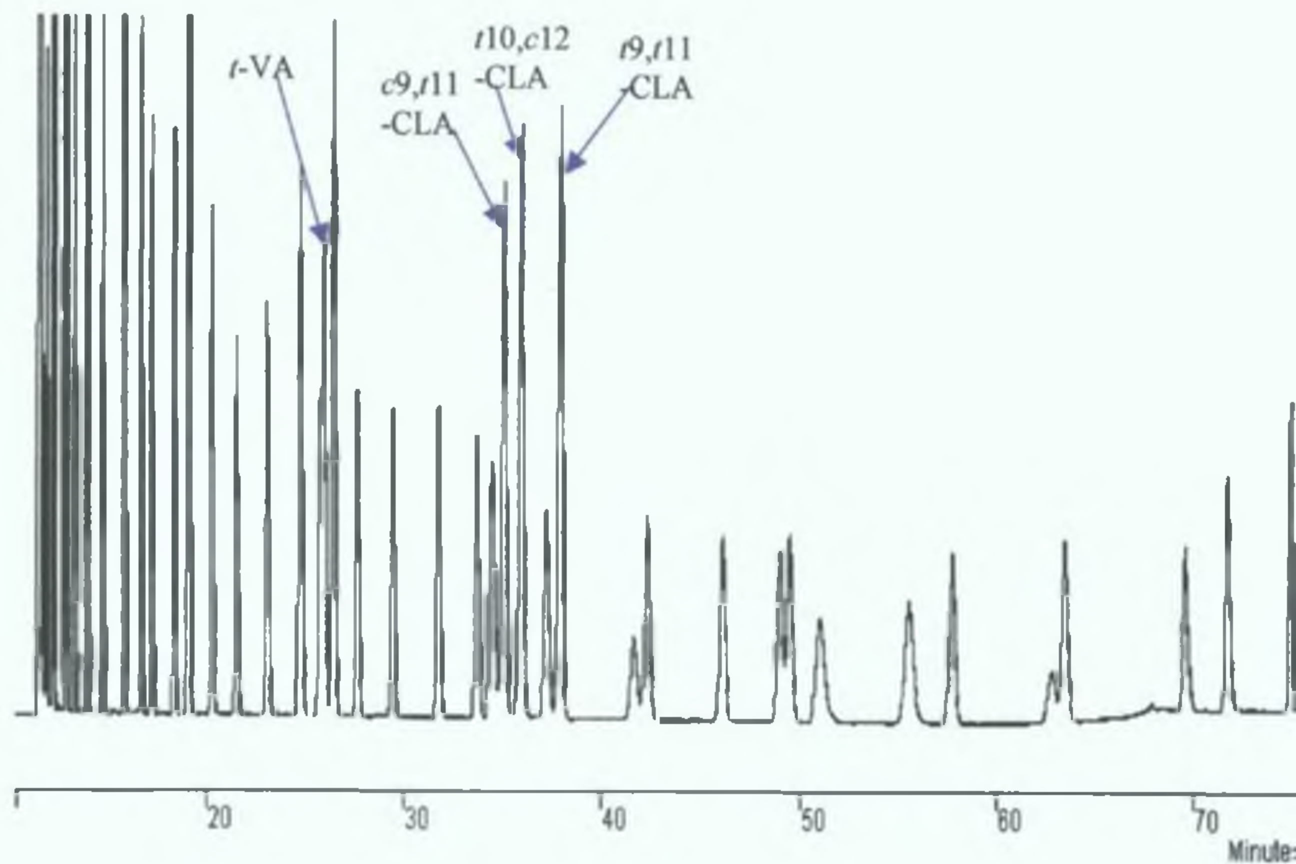

Figure 4.2 Full GC chromatogram of the methyl esters of a 41 standard fatty acid mixture analysed using a 100m CP-Select CB capillary column.

Figure 4.3 shows the partial chromatogram of mixture of FAME standard with C18 (from stearic acid to *t*9, *t*11 CLA) region.

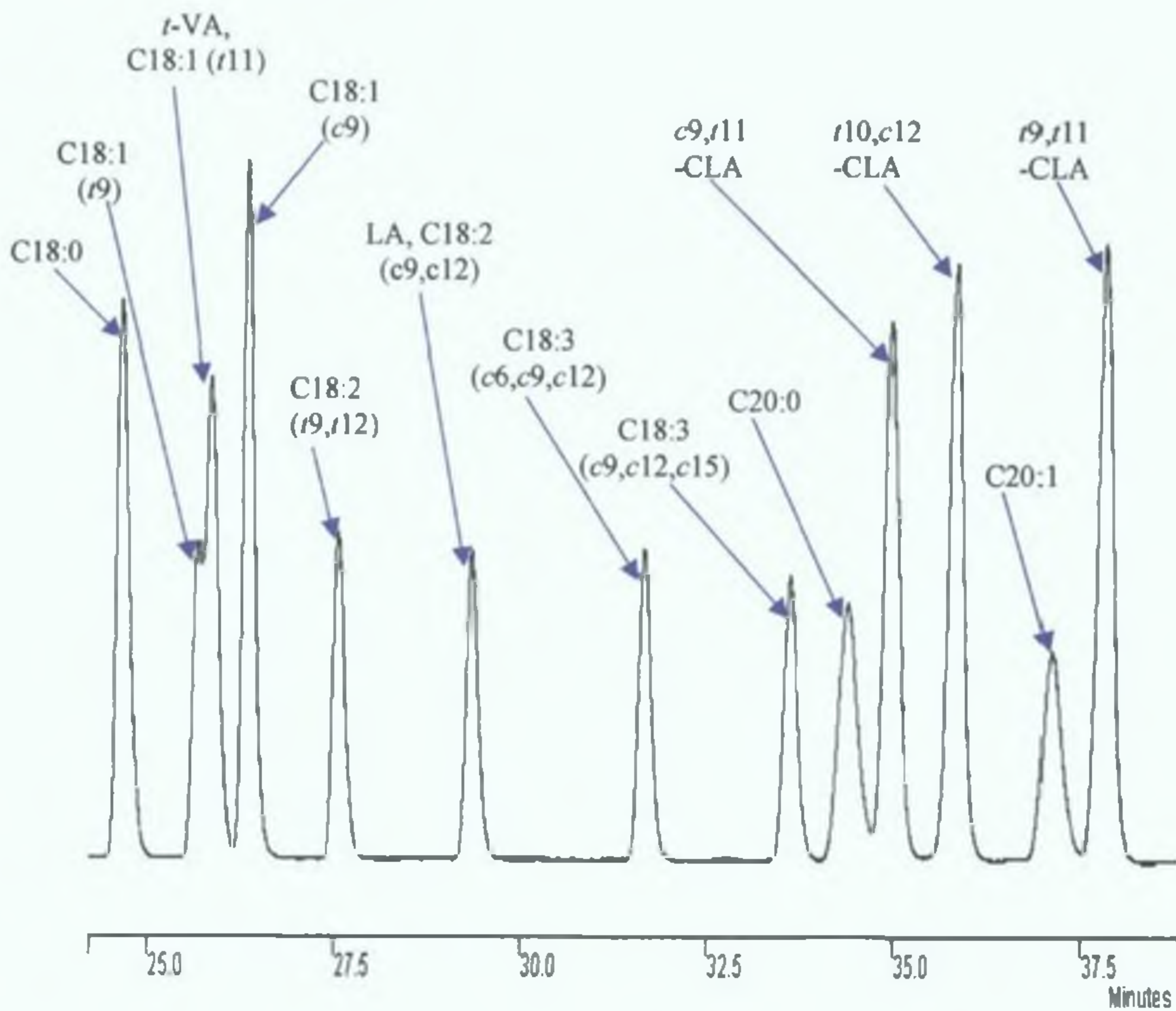

Figure 4.3 Partial GC chromatogram of the FAME C18:0-C20:0 standards analysed using a 100m CP-Select CB capillary column.

4.3.6 Statistical analysis

All data are expressed as mean±SD calculated with Microsoft® Excel 2000 At least three independent experiments were performed in triplicate The statistical significance (P<0 05) was determined using the Student's t-test and was used to determine significance between treatments

4.4 Results

4.4.1 Validation of cellular extraction, methylation and Gas chromatographic separation of CLA isomers and *t*-VA.

HT-29 human adenocarcinoma cell line was cultured exactly as outlined in Chapter 2 Repeatability was assayed over 5 experiment in which 21μg of fatty acid (CLA mixture, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and *t*-VA) was added to cells (2×10^6) Recovery assayed by adding varying amounts (21μg-140μg) of fatty acid (CLA mixture, *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and *t*-VA) to cells The internal standard was added at the same time as fatty acid Cellular lipids were extracted, methylated and analyzed by GC as described above

Figure 4 4 shows the partial GC chromatogram of the fatty acids profile of HT-29 cells spiked with different isomers of CLA, CLA mixture of isomers and *t*-VA for repeatability experiments Chromatograms showed the clear peaks for *c9*, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and *t*-VA

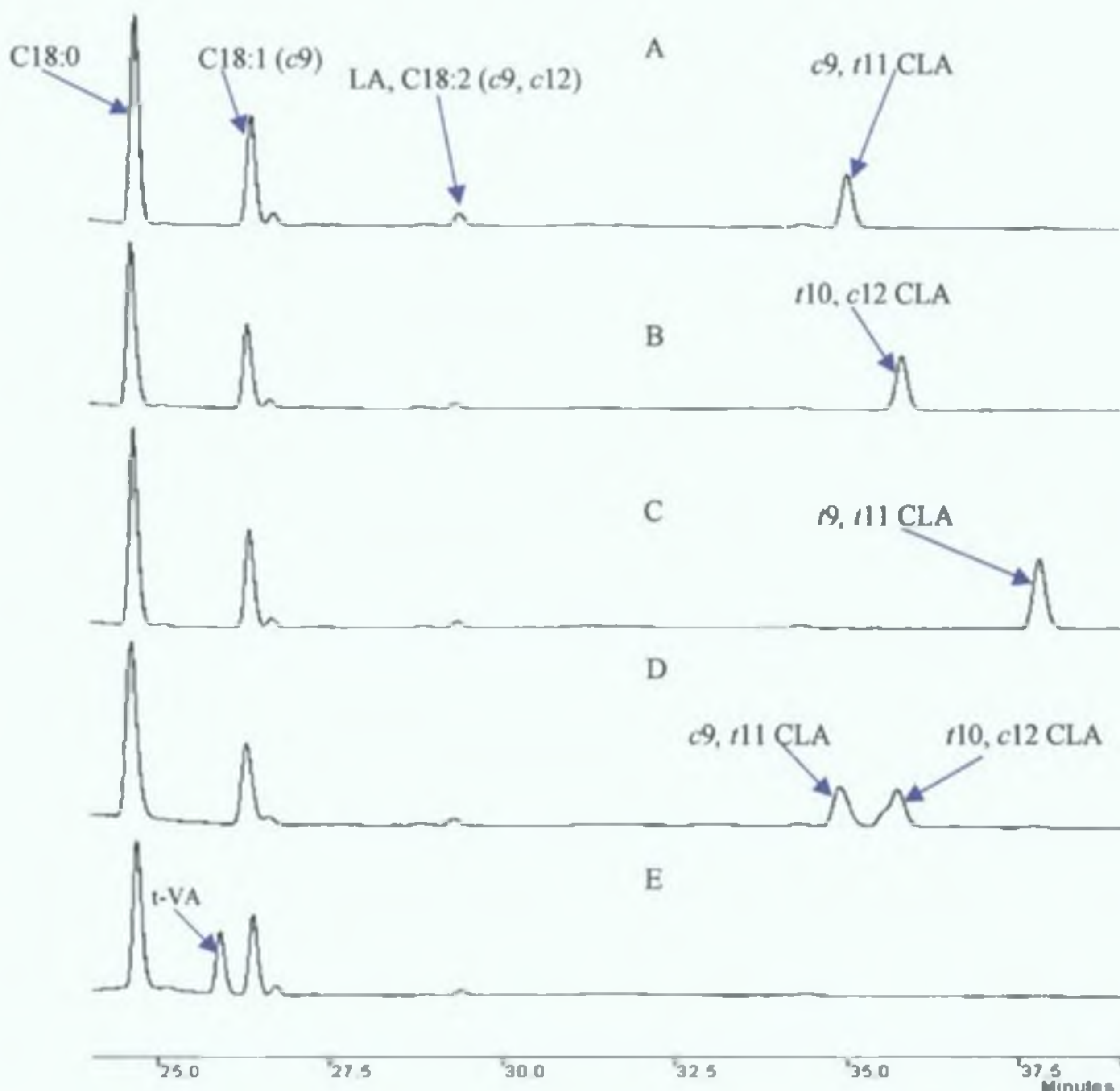

Figure 4.4 Partial gas chromatographic separation of the conjugated linoleic acid region (CLA) after 21μg of (A) *c9*, *t*11 CLA, (B) *t*10, *c*12 CLA, (C) *t*9, *t*11 CLA, (D) CLA mixture and (E) *t*-VA spiked into 2 x 10^6 HT-29 cells before extraction of lipid to do the repeatability of fatty acids.

4.4.1.1 Repeatability analysis

Repeatability of the method for measuring 3 CLA isomers and *t*-VA was assessed with 21μg over 5 days Table 4 3 and 4 4 shows the good repeatability analysis of CLA mixture, 3 single isomers of CLA and *t*-VA The average recovery was 84 8% for *c*9, *t*11 CLA and 84 0% for *t*10, *c*12 CLA, when CLA mixture was analyzed For individual fatty acid spiked the average recovery was 91 4% (*c*9, *t*11 CLA), 90 4% (*t*10, *c*12 CLA), 95 7% (*t*9, *t*11 CLA) and 89 5% (*t*-VA)

Table 4.3 Repeatability of CLA mixtures of isomers spiked in HT-29 cell pellete

CLA mixture	CLA recovered (μg)					Mean ± SD (μg)	% CV	Average % recovery
	1	2	3	4	5			
*c*9, *t*11 CLA (8 6 μg)	7 48	7 28	7 76	7 02	6 93	7 3 ± 0 3	4 6	84 8
*t*10, *c*12 CLA (9 2 μg)	7 97	7 8	8 3	7 45	7 28	7 8 ± 0 4	5 2	84 0

Table 4.4 Repeatability of CLA isomers and *t*-VA spiked in HT-29 cell pellete (n=5)

Fatty acids spiked (21μg)	Fatty acids recovered (μg)					Mean ± SD (μg)	% CV	Average % recovery
	1	2	3	4	5			
*c*9, *t*11 CLA	18 8	20 1	19 0	18 9	19 1	19 2 ± 0 5	2 8	91 4
*t*10, *c*12 CLA	19 6	18 8	19 2	18 6	18 8	19 0 ± 0 4	2 1	90 4
*t*9, *t*11 CLA	20 4	20 1	20 4	19 9	19 7	20 1 ± 0 3	1 6	95 7
t-VA	18 8	19 6	19 0	19 5	17 0	18 8 ± 1 0	5 5	89 5

Figure 4.5 shows the peak for C18:0, C18:1, LA and some other unknown peaks in control cells. The chromatogram of the fatty acids profile of HT-29 cells clearly showed that none of CLA isomers, t-VA and C17 were present in HT-29 cells.

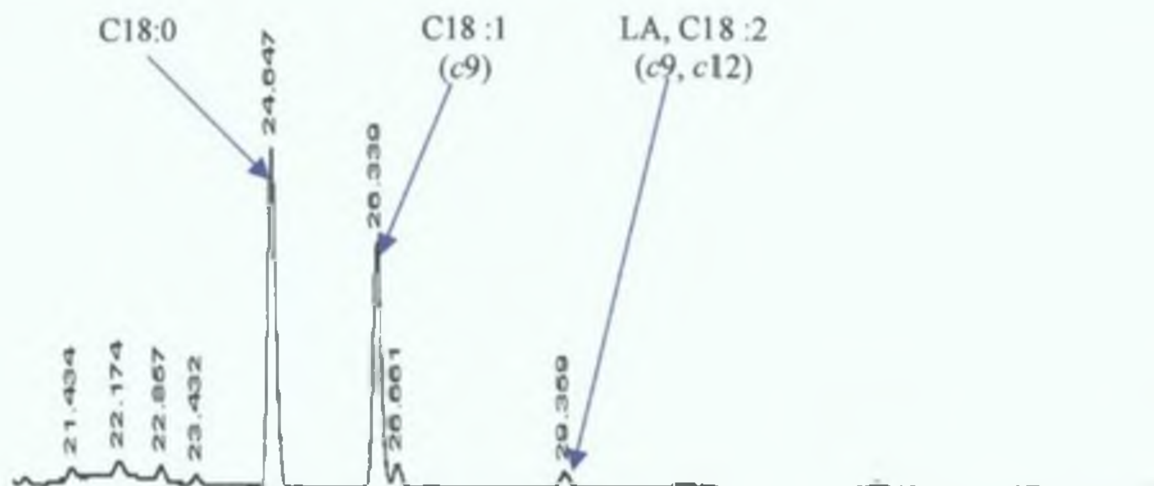

Figure 4.5 Partial GC chromatogram of the Fatty acids profile of HT-29 cells with retention time analysed using a 100m CP-Select CB capillary column.

4.4.1.2 Recovery analysis

For recovery analysis, known amounts in the range 21-140μg of the individual fatty acids (CLA mixture, *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA and *t*-VA) were added to cell pellets. Each experiment was conducted in triplicate.

Tables 4.5, 4.6, 4.7, 4.8 and 4.9 show the mean fatty acid recovery, ± standard deviations (n=3), % coefficient of variation (CV), mean % recovery and the overall mean recovery for *c*9, *t*11 CLA; *t*10, *c*12 CLA; *t*9, *t*11 CLA; CLA mixture and *t*-VA in HT-29 cells.

As can be seen from Table 4 5 when 21μg of *c*9, *t*11 CLA was added to cells 19 2±0 5μg on average was recovered, amountıng to 91 4% mean recovery Sımılar % recovery were observed for 35, 70, 105 and 140μg gıvıng 94 1%, 87 4%, 89 8%, and 91 9% mean recoverıes respectıvely Thıs leads to an overall 90 9% recovery of *c*9, *t*11 CLA, showıng that there could be a loss of 9 1% *c*9, *t*11-CLA through extractıon and methylatıon procedures The coeffıcıent of varıatıon ranged from 2 8-19 6 %

Table 4 6 shows that the mean recovery 90 4%, 81 7%, 79 2%, 80 3% and 90 9% after spıkıng 21, 35, 70, 105, and 140μg spıkıng concentratıons of *t*10, *c*12-CLA respectıvely The coeffıcıent of varıatıon ranged from 2 2-7 8 % The overall 84 5% mean recovery of *t*10, *c*12-CLA was observed

The recovery of *t*9, *t*11 CLA wıth the mean recovery 95 7%, 84 5%, 82 9%, 86 8% and 89 4 when spıkıng 21, 35, 70, 105 and 140μg of *t*9, *t*11CLA The coeffıcıent of varıatıon ranged from 1 6-8 6 % The overall mean recovery was observed 87 9% of *t*9, *t*11 CLA (Table 4 7)

AS can be seen from Table 4 8, spıkıng 21μg of *t*-VA before lıpıd extractıon led to mean recovery of 19 4 μg (92 2% mean recovery) Sımılar mean recoverıes were found after spıkıng wıth 35μg (101 4%), 70μg (98 5%), 105μg (98 9%) and 105μg (88 9%) of *t*-VA Thıs leads to an overall 96 0% mean recovery of *t*-VA, showıng neglıgıble loss of t-VA occurred durıng the lıpıd extractıon, methylatıon and GC procedures The coeffıcıent of varıatıon ranged from 1 7-8 5% for *t*-VA (n=3)

The mean recovery for *c*9, *t*11-CLA was observed 84 8%, 87 9%, 86 9%, 82 8% and 89 1% when 21-140μg CLA mıxture were spıked ın cells For *t*10, *c*12 CLA the mean recovery were 84 0%, 84 4%, 86 9%, 80 9% and 90 8% respectıvely The overall mean recovery was 86 3% and 85 4% for *c*9, *t*11 CLA and *t*10, *c*12 CLA respectıvely (Table 4 9)

Table 4.5 Recovery of *c*9, *t*11 CLA ısomer spıked ın HT 29 cells (n=3)

Fatty acıd spıked (μg)	Mean ± SD (μg)	% CV	Mean % recovery	Overall mean % recovery
21	19 2 ± 0 5	2 8	91 4	
35	32 9 ± 1 3	3 9	94 1	
70	61 2 ± 1 9	3 0	87 4	90 9
105	94 3 ± 8 5	9 0	89 8	
140	128 7 ± 4 6	3 5	91 9	

Table 4.6 Recovery of *t*10, *c*12 CLA ısomer spıked ın HT 29 cells (n=3)

Fatty acıd spıked (μg)	Mean ± SD (μg)	% CV	Mean % recovery	Overall mean % recovery
21	19 0 ± 0 4	2 1	90 4	
35	28 6 ± 2 2	7 8	81 7	
70	55 5 ± 1 3	2 3	79 2	84 5
105	84 3 ± 2 2	2 6	80 3	
140	127 3 ± 2 7	2 2	90 9	

Table 4.7 Recovery of *t*9, *t*11 CLA ısomer spıked ın HT 29 cells (n=3)

Fatty acıd spıked (μg)	Mean ± SD (μg)	% CV	Mean % recovery	Mean % recovery
21	20 1 ± 0 3	1 6	95 7	
35	29 6 ± 2 6	8 6	84 5	
70	58 0 ± 1 5	2 6	83 0	87 9
105	91 2 ± 4 0	4 4	86 8	
140	89 4 ± 4 3	3 4	89 4	

Table 4.8 Recovery of *t*-VA spıked ın HT 29 cells (n=3)

Fatty acıd spıked (μg)	Average recovery (μg)	% CV	Average % recovery	Overall average % recovery
21	19 4 ± 0 3	1 7	92 2	
35	35 5 ± 1 6	4 4	101 4	
70	69 0 ± 2 8	4 1	98 6	96 0
105	103 9 ± 8 6	8 5	98 9	
140	124 4 ± 7 6	6 1	88 9	

Table 4.9 Recovery of CLA mixtures of isomers spiked in HT 29 cells (n=3)

Recovered *c*9, *t*11 CLA				
Fatty acid spiked (μg)	Average recovery (μg)	% CV	Average % recovery	Overall average % recovery
21	7 3 ± 0 3	4 7	84 8	
35	12 6 ± 0 2	1 5	87 9	
70	24 9 ± 0 4	1 7	86 9	86 3
105	35 6 ± 1 5	4 1	82 8	
140	51 2 ± 3 1	6 2	89 1	

Recovered *t*10, *c*12 CLA				
Fatty acid spiked (μg)	Average recovery (μg)	% CV	Average % recovery	Overall average % recovery
21	7 8 ± 0 4	5 3	84 0	
35	13 0 ± 0 4	3 2	84 4	
70	26 8 ± 0 6	2 1	86 9	85 4
105	37 4 ± 0 8	2 2	80 9	
140	55 9 ± 3 6	6 5	90 8	

Figures 4 6, 4 7, 4 8 and 4 9 shows the increasing peak for *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA with spiking the increasing concentration of *c*9, *t*11 CLA, *t*10, *c*12 CLA, *t*9, *t*11 CLA and CLA mixture of isomers in HT-29 cells before extraction of lipid to do the recovery of CLA

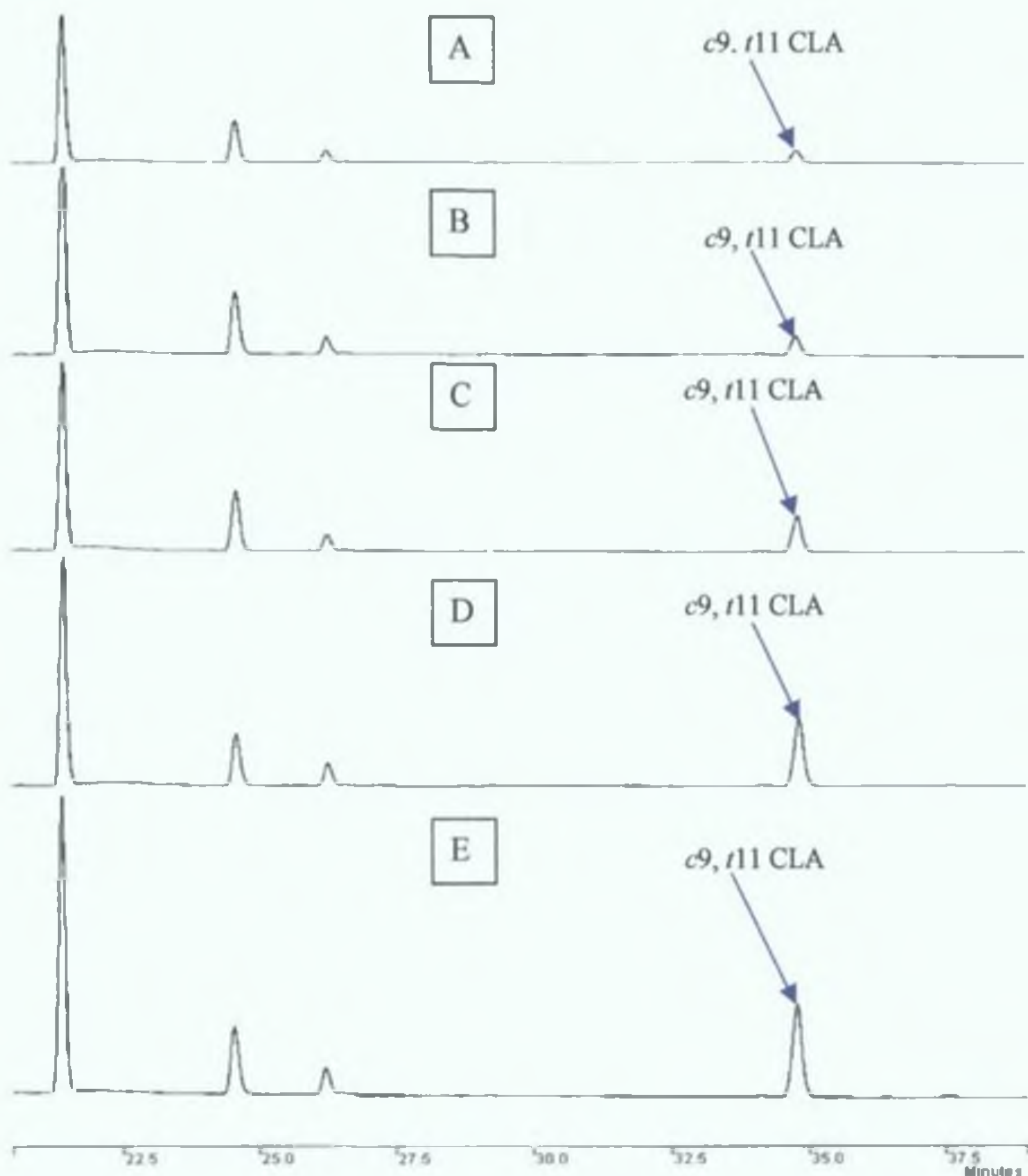

Figure 4.6 Partial gas chromatographic separation of the conjugated linoleic acid (CLA) region of HT-29 cells after (A) 21μg, (B) 35μg, (C) 70μg, (D) 105μg and (E) 140μg of *c*9, *t*11 CLA were spiked into 2 x 10^6 HT-29 cells before extraction of lipid.

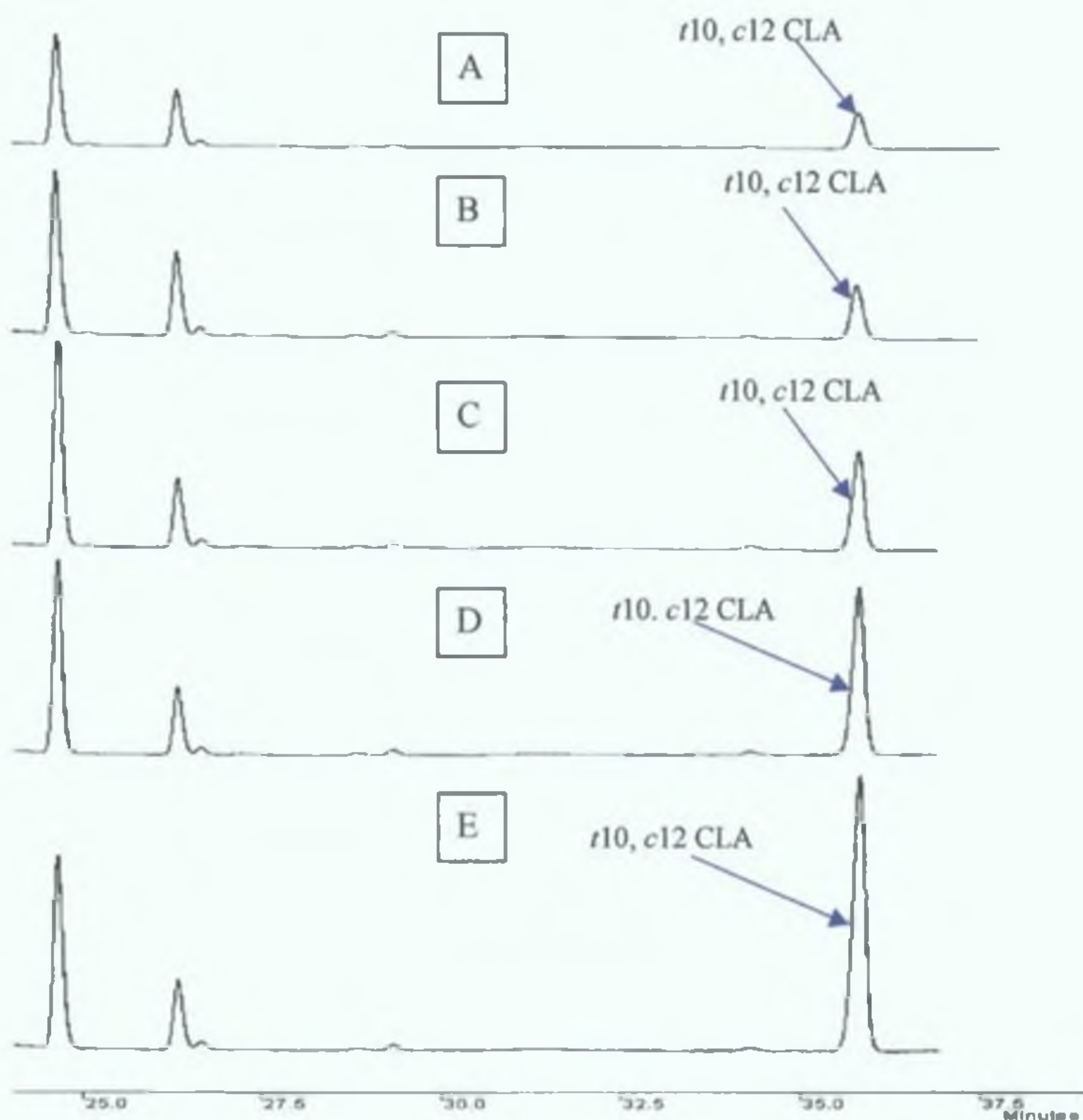

Figure 4.7 Partial gas chromatographic separation of the conjugated linoleic acid (CLA) region of HT-29 cells after (A) 21μg, (B) 35μg, (C) 70μg, (D) 105μg and (D) 140μg of *t*10, *c*12 CLA were spiked into 2 x 10^6 HT-29 cells before extraction of lipid.

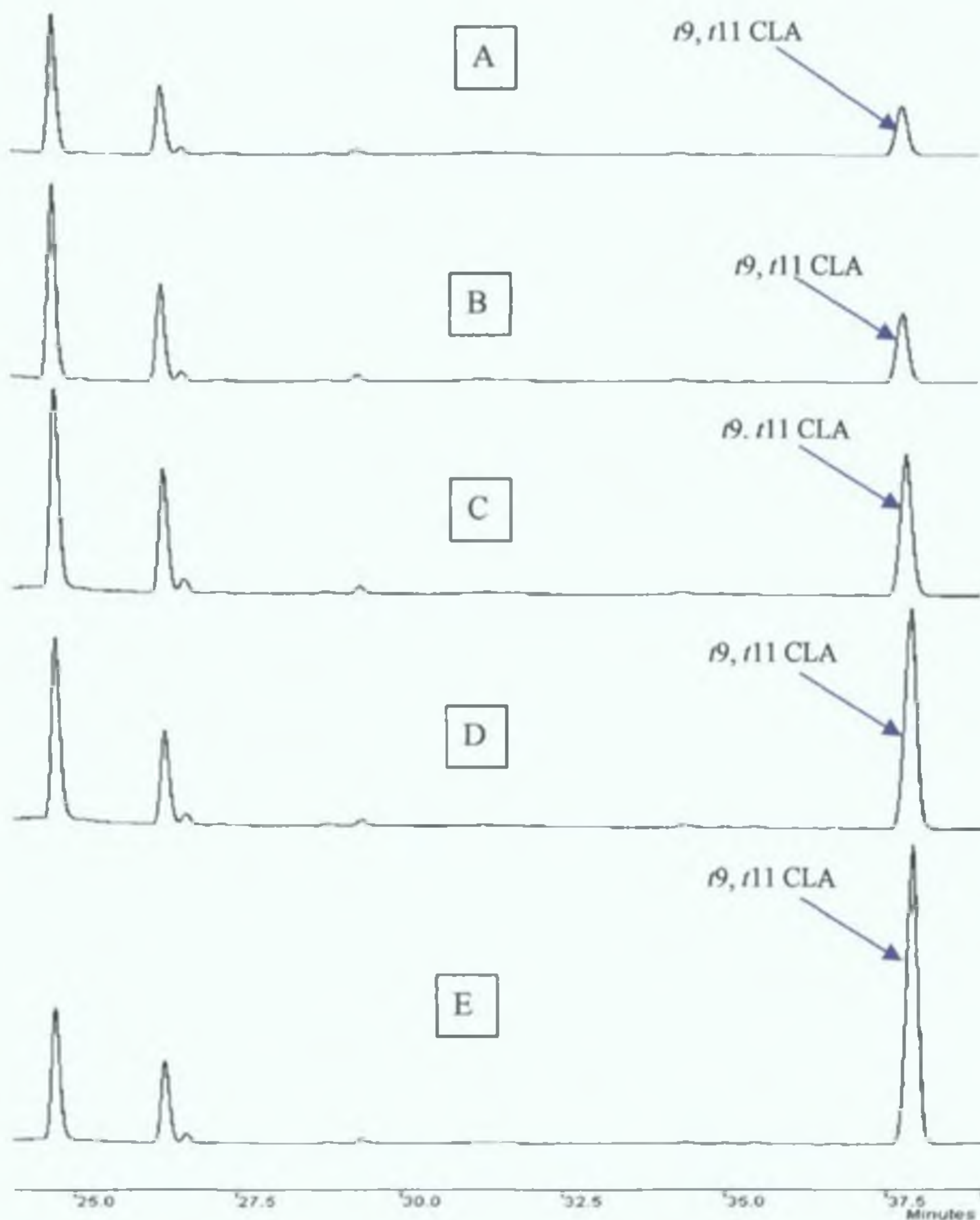

Figure 4.8 Partial gas chromatographic separation of the conjugated linoleic acid (CLA) region of HT-29 cells after (A) 21μg, (B) 35μg, (C) 70μg, (D) 105μg and (E) 140μg of *t*9, *t*11 CLA were spiked into 2 x 10^6 HT-29 cells before extraction of lipid.

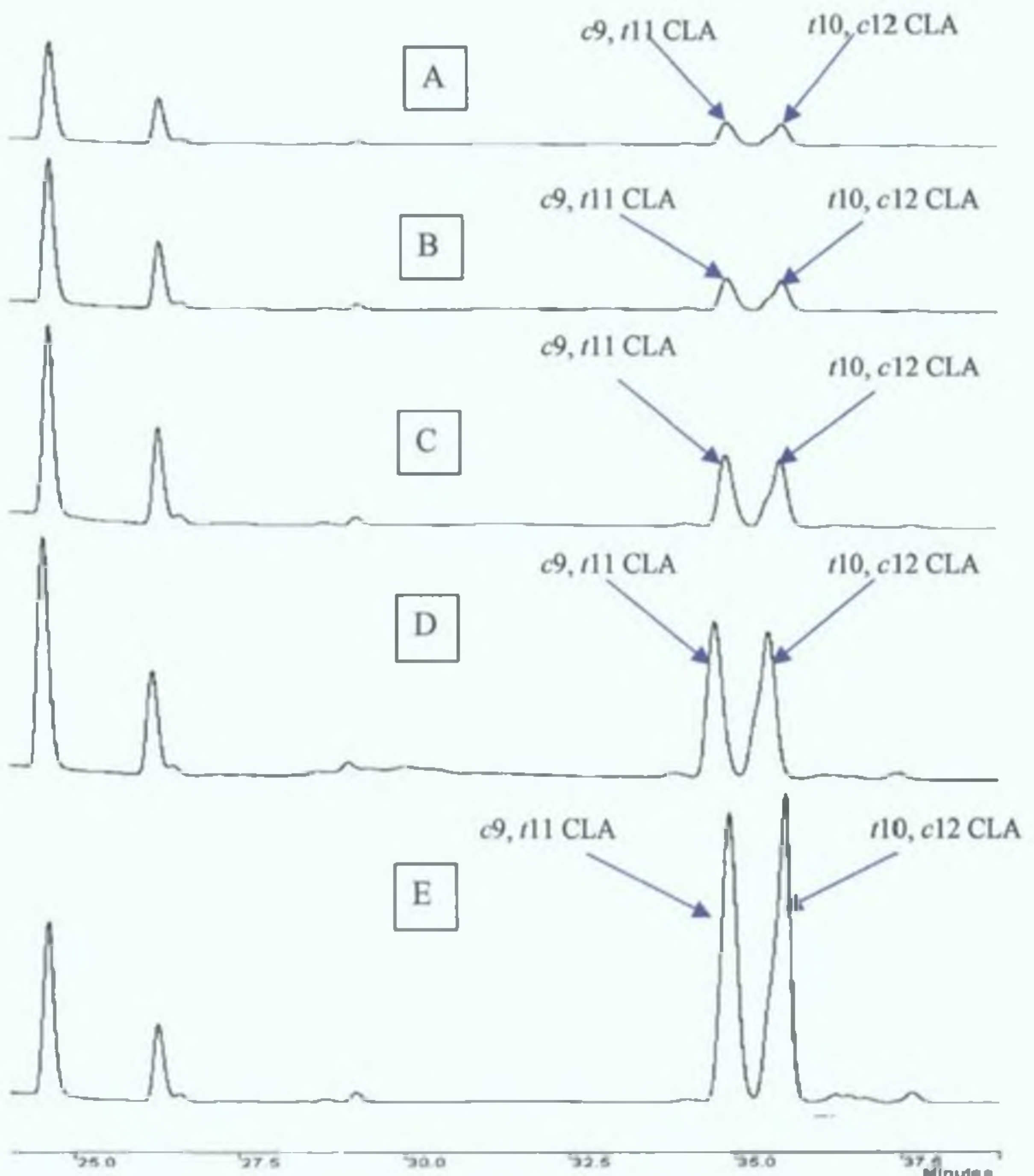

Figure 4.9 Partial gas chromatographic separation of the conjugated linoleic acid region (CLA) after (A) 21µg, (B) 35µg , (C) 70µg, (D) 105µg and (E) 140µg of CLA mixture of isomers containing 41% *c9*, *t*11 CLA and 44% *t*10, *c*12 CLA spiked into 2 x 10^6 HT-29 cells before extraction of lipid.

The fatty acid profiles of untreated (control) HT29 cells maintained in culture for between 24h and 120h are depicted in Table 4 10 It is apparent that long chain saturated fatty acids (LCSFA) constitute the bulk (59-83%) of fatty acids present in the total lipid fractions

Table 4.10 Fatty acids composition of untreated control HT-29 cells after 24, 48, 72 and 120 hours incubation with media

% FAME of cellular lipid				
Fatty acids obtained	24h (n=6)	48h (n=3)	72h (n=6)	120h (n=12)
C14 0	3 2 ± 0 2	4 0 ± 0 28	3 6 ± 0 9	4 3 ± 0 1
C16 0	33 2 ± 2 3	32 3 ± 2 3	30 1 ± 1 4	26 5 ± 0 2
C16 1	2 6 ± 1 2	2 2 ± 1 9	5 0 ± 0 6	10 5 ± 0 8
C18 0	46 6 ± 5 9	45 7 ± 3 1	36 1 ± 2 7	28 3 ± 0 8
C18 1(t-11)	0	0	0	0
C18 1(c-9)	10 3 ± 3 0	11 0 ± 0 7	16 8 ± 0 7	23 1 ± 0 8
LA	0 8 ± 0 9	1 2 ± 1 0	2 5 ± 0 3	1 8 ± 0 2
C20 0	0 2 ± 0 3	0 3 ± 0 5	0	0 3 ± 0 1
*c*9, *t*11 CLA	0	0	0	0
C20 4	1 7 ± 1 6	2 1 ± 1 8	3 4 ± 1 1	3 1 ± 0 5
C22 6	1 2 ± 0 6	1 2 ± 1 0	2 3 ± 0 5	2 1 ± 0 3
LCSFA	83 2 ± 7 1	82 4 ± 5 4	69 8 ± 3 1	59 3 ± 1 0
UFA	16 7 ± 7 1	17 6 ± 5 4	30 2 ± 3 1	40 7 ± 1 0
MUFA/LCSFA	0 2 ± 0 01	0 16 ± 0 03	0 3 ± 0 01	0 6 ± 0 02

Data represent Mean ± SD of n replicates (i e n=3, 6 or 12)

The predominant LCSFA were stearate (28-47% of total FAME) and palmitate (27-33% of total FAME) Minor amounts (<1%) of C20 0 were observed Unsaturated fatty acids comprised the remaining 17-41% of fatty acids in the cell lipid fraction Oleic acid and palmitoleic acid were the predominant *cis*9 monounsaturated fatty acids, present at between 10-23% and 2-11% respectively of total lipid Arachidonic acid, linoleic acid and DHA were each present in similar amounts (0 8-3 4% of total FAME) Rumenic acid (*c*9, *t*11 CLA) and *trans* vaccenic acid were not detectable in control cells

It is apparent that as incubation times increased beyond 48h, the ratio of monounsaturated fatty acids to saturated fatty acids increased suggesting an active delta 9 desaturase activity in HT-29 cells (Table 4 10) After 120h incubation, levels of stearate and palmitate decreased by 39% and 20% respectively compared with 24h incubation, while levels of oleic acid and palmitoleic acid increased 2-fold and 4-fold respectively Levels of linoleic acid remained unaltered, levels of arachidonic acid and DHA were increased almost 2 fold, suggesting active delta 5 / delta 6 desaturase and elongase activities in HT-29 cells Figure 4 10 and 4 11 demonstrate typical chromatograms of fatty acid profiles of HT-29 cells after 24h and 120h The relatively lower amounts of polyunsaturated fatty acids relative to other fatty acids in HT-29 cells is consistent with previous studies showing that membrane lipids of cancer cells are generally lower in polyunsaturated fatty acids, particularly the n6 series

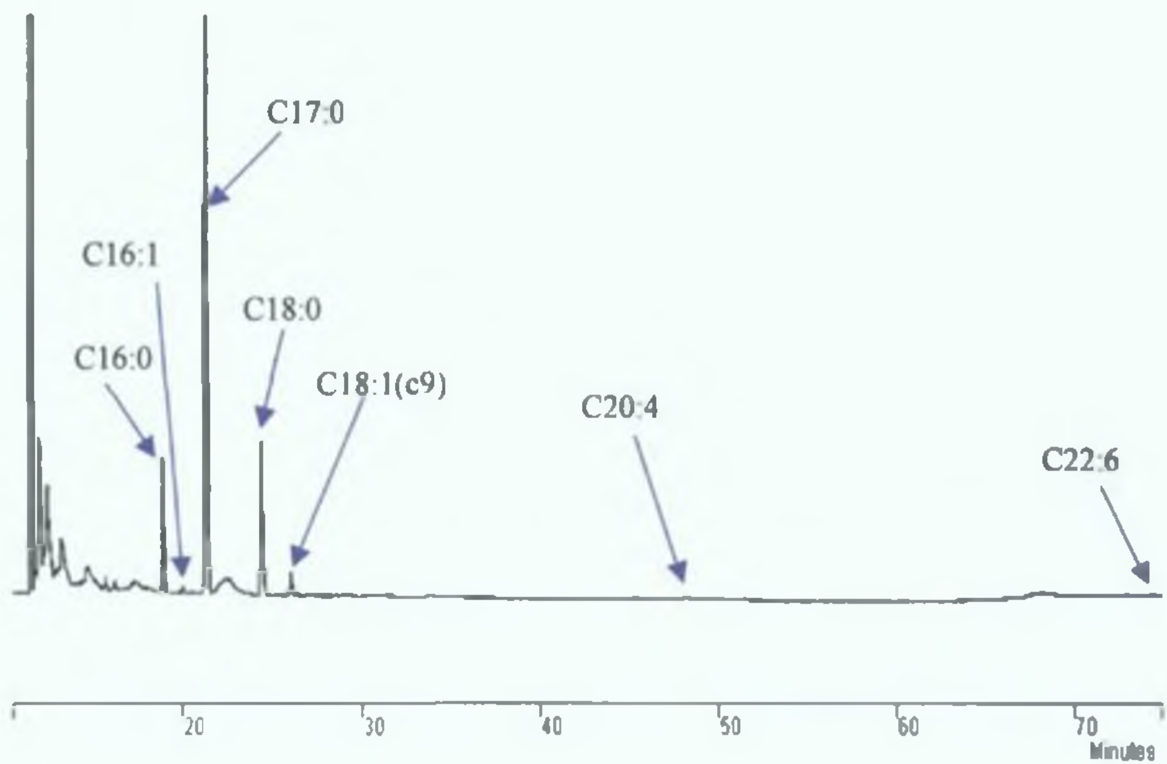

Figure 4.10 Typical chromatogram of fatty acid profiles of HT-29 cells after 24h.

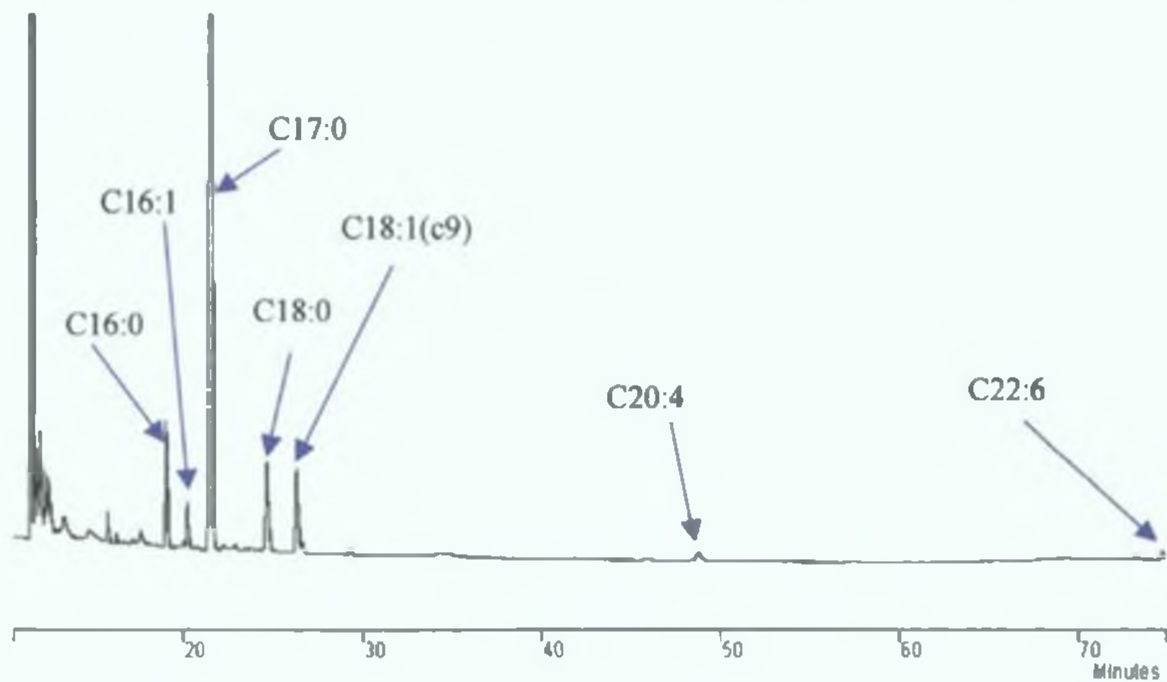

Figure 4.11 Typical chromatograms of fatty acid profiles of HT-29 cells after 120h

4.4.2 Effects of *trans* vaccenic acid (*t*-VA) on fatty acid profiles of total cellular lipids of HT-29 cells

Treatment with *t*-VA in the range 25-100 μM for 48h and 120h altered the fatty acid profile of the total lipid fraction of cells Briefly, levels of LCSFA decreased while total unsaturated fatty acids increased with increasing amounts of *t*-VA in the culture medium The increase in unsaturation was related to increased uptake and to bioconversion of *t*-VA to *c*9, *t*11 CLA by cells As the amount of *t*-VA taken up by cells increased from 8 8 ± 0 7 % of total FAME (n=3) to 37 3± 0 8% of total FAME (n=3) there was a proportionate increase in level of rumenic acid from 1 8±0 1 (n=3) to 5 0± 0 06 % of total FAME (n=3) At 48h, *t*-VA at 100μM decreased ($p<0$ 05) stearic acid and palmitate by 45% and 41% respectively compared with control cells, however oleic acid was decreased by 50% ($p<0$ 05) The ratio of C18 1 Δ 9/C18 0 was decreased by 17% following *t*-VA treatment suggesting inhibition of Δ 9 desaturation of stearate Levels of Arachidonic acid, linoleic acid and DHA (C22 6) were unchanged relative to control (Table 4 11)

Similar effects were observed after treatment with *t*-VA for 120h As the amount of *t*-VA taken up by cells increased from 2 7±0 2 % of total FAME (n=3) to 14 6±0 3 % of total FAME (n=3), there was a proportionate increase in level of *c*9, *t*11 CLA from 3 1±0 07 (n=3) to 9 9±0 3 % of total FAME (n=3) The UFA/LCSFA ratio increased by 35%, *t*-VA at 100μM decreased stearic acid and palmitate by 19% and 14% respectively compared with control cells, oleic acid and palmitoleic acid were decreased by 40% and 32% respectively The ratios of both C18 1 Δ 9/C18 0 and C16 1 Δ 9/C16 0 were significantly lower following 100μM *t*-VA treatment suggesting possible inhibition of Δ9 desaturation of stearate and palmitate Levels of arachidonic acid and linoleic acid were decreased by 35% while DHA remained unchanged compared with control cells (Table 4 12)

Table 4.11 Fatty acids composition of total cellular lipids of HT-29 cells when incubated in the presence of 25 μM-100 μM of *t*-VA as free fatty acid for 48hrs

% FAME of cellular lipid

Fatty acids obtained (μg)	Control (Ethanol)	*t*-VA [25 μM]	*t*-VA [50 μM]	*t*-VA [75 μM]	*t*-VA [100 μM]
C14 0	4 0 ± 0 28	3 7 ± 0 06	3 2 ± 0 1*	2 2 ± 0 2*	2 6 ± 0 1*
C16 0	32 3 ± 2 3	28 5 ± 2 3	24 3 ± 0 3*	22 0 ± 0 6*	19 0 ± 0 1*
C16 1	2 2 ± 1 9	2 6 ± 0 05	1 9 ± 0 1	1 8 ± 0 1	1 6 ± 0 05
C18 0	45 7 ± 3 1	39 8 ± 0 8*	33 1 ± 0 6*	26 4 ± 0 3*	25 3 ± 0 5*
t-VA	0	8 8 ± 0 7*	21 9 ± 1 2*	33 1 ± 0 2*	37 3 ± 0 8*
C18 1(c9)	11 0 ± 0 7	8 5 ± 0 4*	7 0 ± 0 4	7 7 ± 1 1	5 4 ± 0 2*
LA	1 2 ± 1 0	1 3 ± 0 1	1 4 ± 0 4	0 8 ± 0 7	0 9 ± 0 1
C20 0	0 3 ± 0 5	0 9 ± 0 01	0 8 ± 0 1	0	0 4 ± 0 3
c9, *t*11 CLA	0	1 8 ± 0 1*	2 7 ± 0 1*	4 3 ± 0 04*	5 0 ± 0 06*
C20 4	2 1 ± 1 8	2 7 ± 0 3	2 4 ± 0 1	1 1 ± 0 9	1 7 ± 0 1
C22 6	1 2 ± 1 0	1 4 ± 0 1	1 2 ± 0 02	0 5 ± 0 5	1 0 ± 0 02
LCSFA	82 4 ± 5 4	72 9 ± 1 1	61 4 ± 0 9*	50 7 ± 1 0*	47 2 ± 0 8*
UFA	17 6 ± 5 4	27 1 ± 1 1	38 6 ± 0 9*	49 3 ± 1 0*	52 8 ± 0 8*
MUFA	13 2 ± 1 7	19 9 ± 0 7*	30 8 ± 0 8*	42 6 ± 1 4*	44 3 ± 0 9*
UFA/LCSFA	0 22 ± 0 08	0 4 ± 0 02	0 6 ± 0 02*	1 0 ± 0 04*	1 1 ± 0 04*
MUFA/LCSFA	0 16 ± 0 03	0 3 ± 0 01*	0 5 ± 0 02*	0 8 ± 0 03*	0 9 ± 0 03*
C16 1/C16 0	0 07 ± 0 06	0 1 ± 0 02	0 1 ± 0 002	0 1 ± 0 04	0 1 ± 0 02
C18 1(c9)/C18 0	0 24 ± 0 02	0 2 ± 0 01	0 2 ± 0 01	0 3 ± 0 04	0 2 ± 0 01*

*Denotes results which are significantly different to untreated cells (p<0 05) Data represent Mean ± SD of 3 replicates (i e n=3)

Table 4.12 Fatty acids composition of total cellular lipids of HT-29 cells when incubated in the presence of 25 μM-100 μM of *t*-VA as free fatty acid for 120hrs

% FAME of cellular lipid

Fatty acids obtained (μg)	Control (Ethanol)	*t*-VA [25 μM]	*t*-VA [50 μM]	*t*-VA [75 μM]	*t*-VA [100 μM]
C14 0	4 4 ± 0 02	4 8 ± 0 5	4 3 ± 0 1	4 0 ± 0 2	3 9 ± 0 4
C16 0	26 8 ± 0 1	26 2 ± 0 6	25 6 ± 0 4*	24 0 ± 0 3*	23 0 ± 0 3*
C16 1	11 1 ± 0 3	11 2 ± 0 3	9 4 ± 0 6	9 5 ± 0 2	7 5 ± 0 3*
C18 0	29 0 ± 1 7	28 2 ± 1 4	27 9 ± 2 2	23 5 ± 0 8*	23 5 ± 1 5*
t-VA	0	2 7 ± 0 2*	6 1 ± 0 5*	8 9 ± 0 9*	14 6 ± 0 3*
C18 1(c9)	22 0 ± 2 0	18 6 ± 0 9	15 6 ± 0 9*	17 6 ± 0 8*	13 2 ± 0 7*
LA	1 7 ± 0 03	1 4 ± 0 05*	1 3 ± 0 1*	1 3 ± 0 05	1 1 ± 0 1*
C20 0	0 4 ± 0 4	0 5 ± 0 4	0 4 ± 0 3	0 1 ± 0 1	0 3 ± 0 3
c9, *t*11 CLA	0	3 1 ± 0 07*	5 7 ± 0 7*	7 6 ± 0 4*	9 9 ± 0 3*
C20 4	2 8 ± 0 1	1 7 ± 1 5	2 2 ± 0 1*	2 0 ± 0 1*	1 8 ± 0 1*
C22 6	1 9 ± 1 01	1 6 ± 0 04*	1 6 ± 0 1	1 6 ± 0 02*	1 3 ± 0 1*
LCSFA	60 6 ± 2 2	59 7 ± 1 2	58 2 ± 2 7	51 6 ± 1 0*	50 7 ± 1 7*
UFA	39 4 ± 2 2	40 3 ± 1 2	41 8 ± 2 7	48 4 ± 1 0*	49 3 ± 1 7*
MUFA	33 1 ± 2 3	32 4 ± 0 5	31 1 ± 1 9	36 0 ± 0 6	35 3 ± 1 3
UFA/LCSFA	0 65 ± 0 06	0 7 ± 0 03	0 7 ± 0 1	0 9 ± 0 04*	1 0 ± 0 1*
MUFA/LCSFA	0 55 ± 0 06	0 5 ± 0 01	0 5 ± 0 1	0 7 ± 0 02*	0 7 ± 0 05*
C16 1/C16 0	0 4 ± 0 01	0 4 ± 0 02	0 4 ± 0 03	0 4 ± 0 01	0 3 ± 0 01*
C18 1(c9)/C18 0	0 8 ± 0 1	0 7 ± 0 02	0 6 ± 0 1	0 7 ± 0 04	0 6 ± 0 1*

*Denotes results which are significantly different to untreated cells (p<0 05) Data represent Mean ± SD of 3 replicates (i e n=3)

Figure 4.12 and 4.13 are partial gas chromatograms that demonstrate bioconversion of *t*-VA to rumenic acid (*c*9 *t*11 CLA). It was apparent that the *c*9 *t*11 CLA peak increased with increasing concentration of *t*-VA treatments.

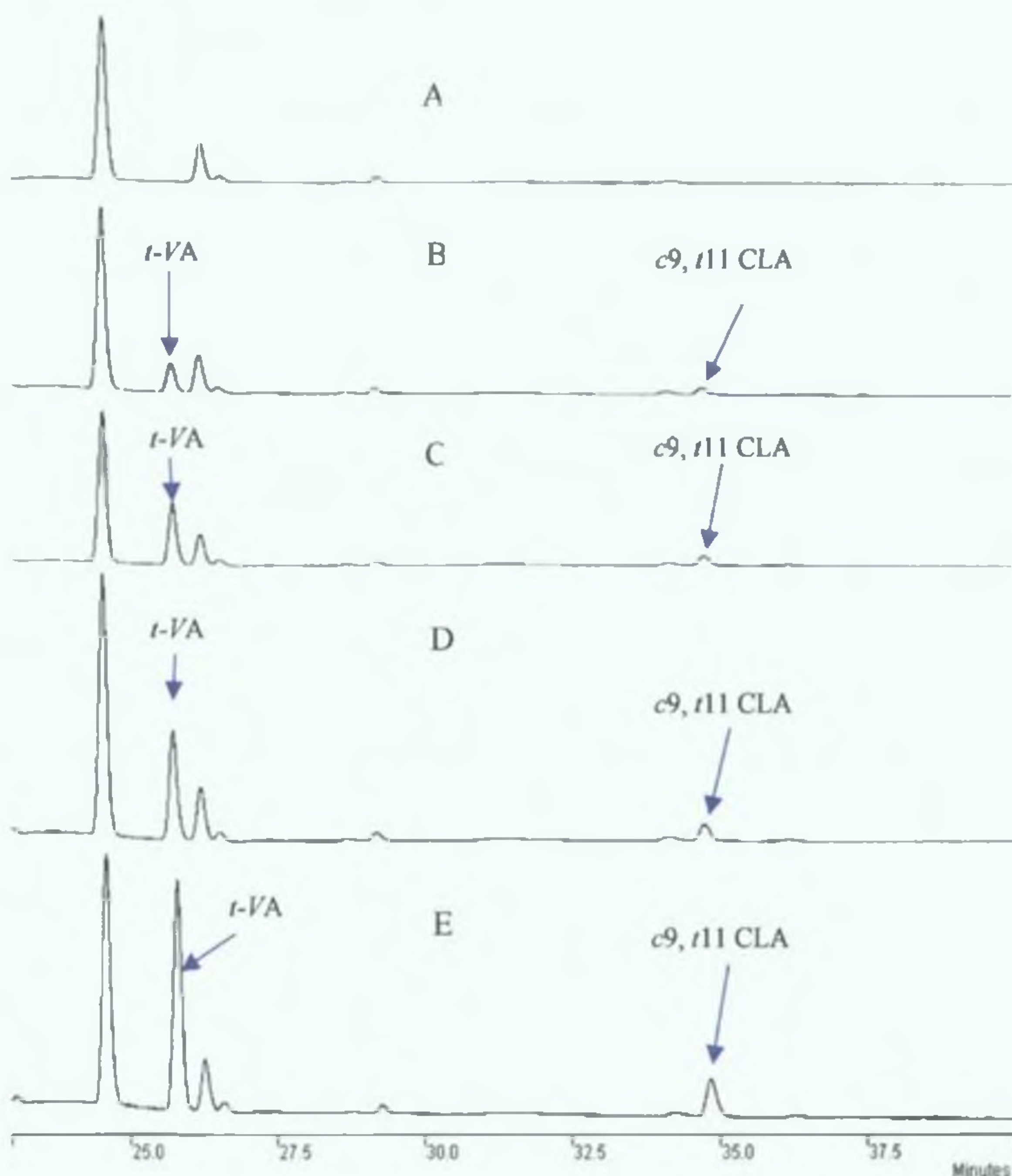

Figure 4.12 Partial gas chromatogram of the conjugated linoleic acid region (CLA) of the fatty acids profile of HT-29 cells, after incubated with different concentrations of *t*-VA for 2 days. (A) ethanol as control (B) 25μM *t*-VA, (C) 50μM *t*-VA, (D) 75μM *t*-VA and (E) 100μM *t*-VA.

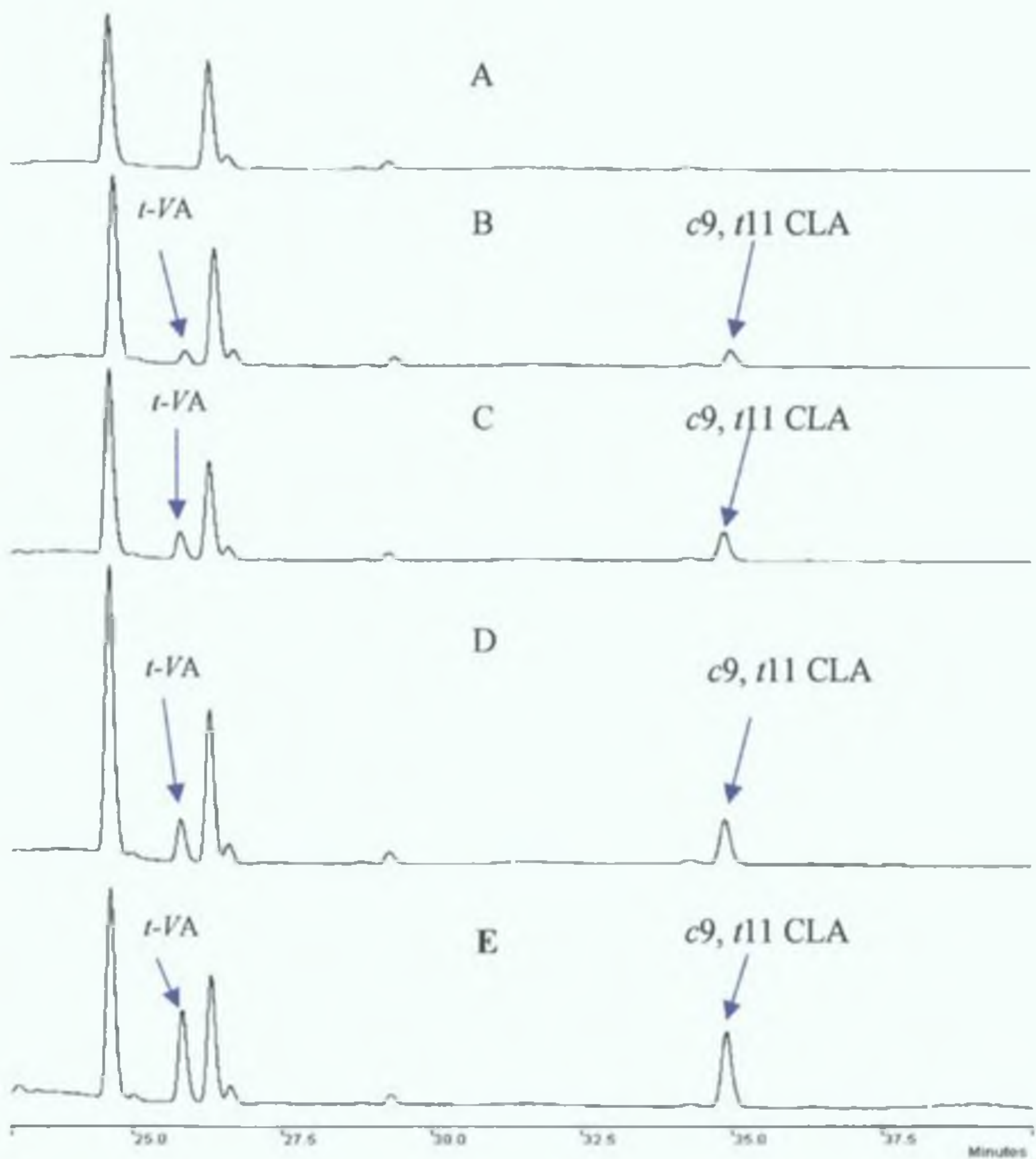

Figure 4.13: Partial gas chromatogram of the conjugated linoleic acid region (CLA) of the fatty acids profile of HT-29 cells, after incubated with different concentrations of *t*-VA for 5 days. (A) ethanol as control (B) 25μM *t*-VA, (C) 50μM *t*-VA, (D) 100μM *t* -VA and (E) 100μM *t*-VA.

Bioconversion of *t*-VA to *c*9 *t*11 CLA in HT-29 cells over 120h was linear with respect to duration of incubation of *t*-VA at 75μM (Fig 4 14)

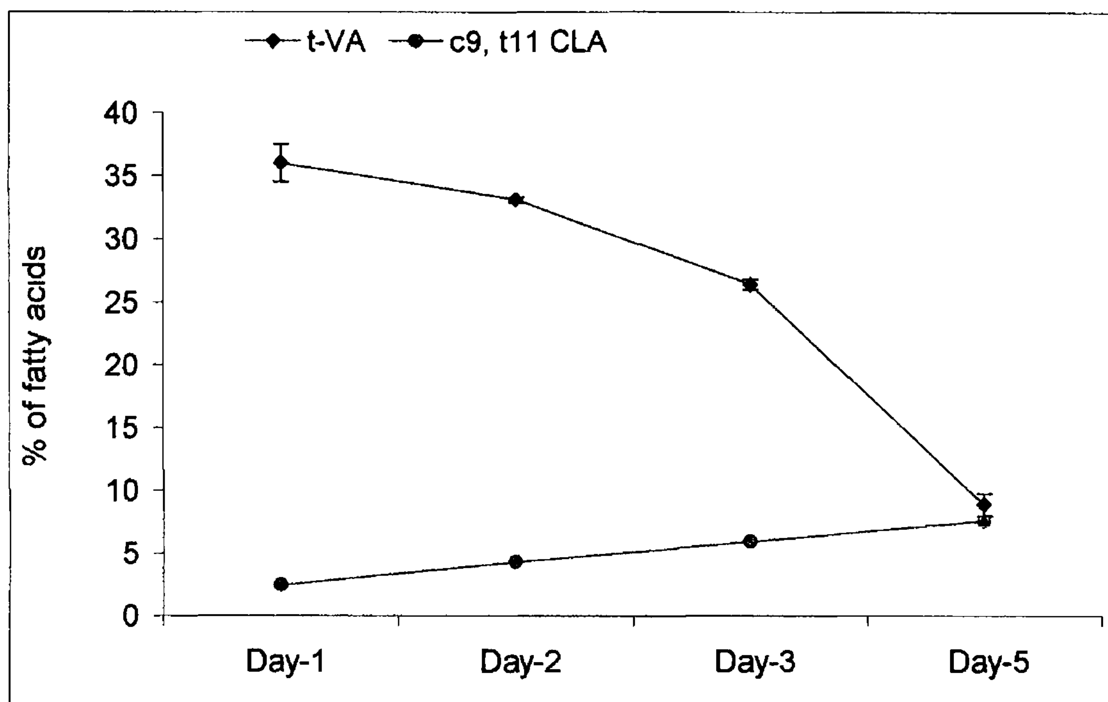

Figure 4.14 % *t*-VA and *c*9, *t*11 CLA in HT-29 cells after 1-5 days incubation with 75μM *t*-VA

4.4.3 Effects of trans vaccenic acid (*t*-VA) when present as fatty acid-BSA complex (molar ratio 2:1)

Fatty acid substrates are often presented to cells growing in culture as their BSA-conjugates The mode of fatty acid presentation however may influence to some extent the cellular response Uptake was therefore examined in HT-29 cells growing in a culture medium supplemented with *t*-VA-BSA complex (molar ratio 2 1) Table 4 13 demonstrates that the fatty acid profile of HT-29 cells is virtually identical when cells are treated with ethanol or with BSA for 120h

Table 4.13 Fatty acid composition of total cellular lipids from HT-29 cells incubated in the presence of 37 5 μM of BSA or ethanol (0 028%, v/v) for 120hrs

% FAME of cellular lipid		
Fatty acids obtained	BSA	Ethanol
C14 0	3 9 ± 0 1	4 1 ± 0 1
C16 0	25 5 ± 0 5	26 1 ± 0 7
C16 1	12 3 ± 0 4	11 7 ± 0 6
C18 0	28 5 ± 1 5	28 3 ± 0 3
t-VA	0	0
C18 1(c9)	24 8 ± 1 5	23 3 ± 0 9
LA	1 0 ± 0 9	1 5 ± 0 05
C20 0	0 4 ± 0 4	0 5 ± 0 4
*c*9, *t*11 CLA	0	0
C20 4	1 7 ± 1 4	2 7 ± 0 2
C22 6	1 8 ± 0 1	1 8 ± 0 03
LCSFA	58 4 ± 1 6	59 0 ± 1 4
UFA	41 6 ± 1 6	41 0 ± 1 4
MUFA	37 1 ± 1 2	35 1 ± 1 5
UFA/LCSFA	0 7 ± 0 05	0 7 ± 0 04
MUFA/LCSFA	0 6 ± 0 02	0 6 ± 0 04
C16 1/C16 0	0 5 ± 0 03	0 5 ± 0 03
C18 1(c9)/C18 0	0 9 ± 0 04	0 8 ± 0 04

Data represent Mean ± SD of 3 replicates (i e n=3)

Treatment wιth *t*-VA-BSA complex for 120h resulted ιn a hιgher level of uptake (10 5±0 3% of total FAME) compared wιth the free fatty acιd (8 9±0 9 % of total FAME) ιnto the total lιpιd fractιon of cells (Table 4 14) However effects on the proportιon of unsaturated fatty acιds to saturated fatty acιds were sιmιlar to those reported for free fatty acιd (Table 4 12) Lιke the free fatty acιd at 100μM, *t*-VA-BSA complex sιgnιficantly ιncreased ($p<0\ 05$) the level of *c*9, *t*11 CLA to 9 1± 0 2 % of total FAME (n=3) whιle ιt decreased ($p<0\ 05$) the levels of LCSFA (52 8 + 0 4 (n=3)) by 10% relatιve to control cells Lιke the free fatty acιd at 100μM, *t*-VA-BSA decreased ($p<0\ 05$) the levels of the two mam Δ9 unsaturated fatty acιds (oleιc acιd and palmιtoleιc acιd) by 40 % and 34 % respectιvely The ratιo of C18 1 Δ 9/C18 0 and C16 1 Δ 9/C16 0 were decreased followιng treatment suggestιng possιble ιnhιbιtιon of Δ9 desaturatιon of stearate and palmιtate Unlιke free *t*-VA treatment, the BSA-fatty acιd complex dιd not alter levels of arachιdonιc acιd and lιnoleιc acιd, levels of DHA were decreased by approxιmately 20% Together the data suggest that the bιoconversιon of *t*-VA to *c*9, *t*11 CLA ιs ιndependent of the manner of ιts uptake by HT-29 cells

Table 4.14 Fatty acids composition of total cellular lipids from HT-29 cells incubated in the presence 37 5 μM of BSA and *t*-VA as fatty acid BSA complexes (2 1) for 120hrs

% FAME of cellular lipid		
Fatty acids obtained	BSA	*t*-VA (75μM)
C14 0	3 9 ± 0 1	4 0 ± 0 1
C16 0	25 5 ± 0 5	24 3 ± 0 3
C16 1	12 3 ± 0 4	8 1 ± 0 4*
C18 0	28 5 ± 1 5	24 2 ± 0 2*
t-VA	0	10 5 ± 0 3*
C18 1(c9)	24 8 ± 1 5	14 8 ± 0 2*
LA	1 0 ± 0 9	1 3 ± 0 02
C20 0	0 4 ± 0 4	0 3 ± 0 3
*c*9, *t*11 CLA	0	9 1 ± 0 2*
C20 4	1 7 ± 1 4	2 1 ± 0 1
C22 6	1 8 ± 0 1	1 4 ± 0 01*
LCSFA	58 4 ± 1 6	52 8 ± 0 4*
UFA	41 6 ± 1 6	47 2 ± 0 4*
MUFA	37 1 ± 1 2	33 4 ± 0 4*
UFA/LCSFA	0 7 ± 0 05	0 9 ± 0 01*
MUFA/LCSFA	0 6 ± 0 02	0 6 ± 0 01
C16 1/C16 0	0 5 ± 0 03	0 3 ± 0 02*
C18 1(c9)/C18 0	0 9 ± 0 04	0 6 ± 0 02*

*Denotes results which are significantly different to BSA treated (BSA control) cells (p<0 05) Data represent Mean ± SD of 3 replicates (i e n=3)

4.4.4 Effects of CLA mixture, *c*9, *t*11 CLA; *t*10, *c*12 CLA and *t*9, *t*11 CLA on fatty acid profiles of total cellular lipids of HT-29 cells

The fatty acid profiles of total cellular lipids from HT-29 cells following treatment with CLA mixture of isomers, *c*9, *t*11 CLA; *t*10, *c*12 CLA and *t*9, *t*11 CLA, all at 75μM for 24h, 72h and 120h are presented in Tables 4.15, 4.16 and 4.17.

The effects of supplementation with CLA in its various isomeric forms on cellular lipid profiles were similar after 24h and 72h. It is apparent that supplementation with all of the CLA preparations for 24h significantly decreased the proportion of LCSFA and increased the unsaturation index (i.e. the ratio of unsaturated fatty acids to saturated fatty acids) of cellular lipid fractions. Levels of C16:0 were decreased by 27 to 42% by the CLA mixture of isomers and by rumenic acid, *t*10, *c*12 CLA and *t*9, *t*11 CLA. Levels of C18:0 were similarly reduced by all treatments.

At 24h all treatments except *c*9, *t*11 CLA significantly decreased oleic acid by 49-56%. Levels of palmitoleic acid were also reduced by CLA treatments. Levels of linoleic acid and archidonic acid were unchanged by CLA treatments after 24h. The decrease in oleic acid and palmitoleic was offset by an increase in the proportion of CLA isomers taken up by cells. It is apparent that after 24h, the CLA content of cells was broadly similar across all treatments, except for *t*10, *c*12 CLA which was the predominant fatty acid present at 41.0±1.2 % total FAME (= 56.6±4.7 μg of the isomer). The amount of rumenic acid (31.2±1.2 μg) and *t*10, *c*12 CLA (37.1±1.2 μg) taken up by cells from the CLA mixture of isomers represented 14.0±0.5 % total FAME and 16.7% of total FAME respectively. Comparable levels of *c*9, *t*11 CLA (30.7±0.6% total FAME) (=75.1±3.2 μg isomer) and *t*9, *t*11 CLA (30.3±1.9% total FAME) (= 62.9 ± 3.3 μg) were observed in the cellular lipid fraction (Table 4.15).

After 72h, all treatments reduced the proportion of LCSFA by 23-29%. Both C16:0 and C18:0 were reduced relative to control cells. All treatments including *c*9, *t*11 CLA reduced oleic acid by 24 to 58% (Table 4.16). Palmitoleic acid was significantly reduced

by the CLA mixture of isomers, *c*9, *t*11 CLA and by *t*10, *c*12 CLA Levels of linoleic acid were also reduced by all treatments Despite the reduction in cis 9 monounsaturated fatty acids and in linoleic and the trend towards reduction in arachidonic acid, the overall unsaturation index (ratio of unsaturated fatty acids to saturated fatty acids) of cellular lipid fractions was higher following each treatment This was attributed to the uptake of CLA by cells After 72h the CLA contents of cells though lower relative to 24h treatments, reflecting metabolism, were similar across all treatments, ranging between 27 and 29% of total FAME (Table 4 16)

After 120h, uptake of various CLA isomers by cells was lower than at 24h, ranging between 17-21% of total FAME (Table 4 17) By contrast with shorter term treatments, the CLA mixture of isomers had negligible effects after 120h on the amount of LCSFA relative to control cells and on the unsaturation index of cellular lipids

The increase in unsaturation due to uptake of CLA mixture was offset by the significant reduction in oleic acid (by 60%) and palmitoleic (by 66%) relative to control The ratios of c18 1 Δ9/C18 0 and c16 1 Δ9/C16 0 were reduced by 63% and 76% respectively relative to control cells Level of linoleic acid was not altered by the CLA mixture while arachidonic acid decreased ($p<0$ 05) by approximately 26%

Both *c*9, *t*11 CLA and *t*9, *t*11 CLA decreased the proportion of LCSFA by 14% and 7% respectively and increased the proportion of unsaturated fatty acids relative to control cells (Table 4 17) Both also decreased the amounts of oleic acid by 19% and 42% respectively The ratios of c18 1 Δ9/C18 0 was not altered by *c*9, *t*11 CLA but was reduced to 38% by *t*9, *t*11 CLA Both decreased the amounts of palmitoleic acid by 30% and 15% respectively, thereby reducing the ratios of c16 1 Δ9/C16 0 by 29% and 11% respectively Neither linoleic acid nor arachidonic acid was significantly altered

The fatty acid profile generated by treating cells with *t*10, *c*12 CLA was similar to that generated by CLA mixture of isomers Uptake of *t*10, *c*12 CLA was 20 9$\pm$1 4 % total FAME The *t*10, *c*12 CLA isomer had negligible effects on LCSFA content reducing it to

55 5$\pm$0 7 % total FAME relative to control cells (59 3$\pm$1 6% total FAME (n=3) though this did not reach statistical significance The levels of C16 1 and C18 1 were reduced by 81% and 65% (p<0 05), the ratios of C16 1 Δ9/C16 0 and C18 1 Δ9/C18 0 were reduced by 76% and 63% respectively suggesting strong inhibition of Δ9 desaturase by *t*10, *c*12 CLA Arachidonic acid level was also decreased (p<0 05) suggesting an inhibitory effect on Δ5/Δ 6 desaturation and elongation activities

A peak corresponding to C18 1 *t*-10 was observed when cells were treated with the CLA mixture of isomers and *t*10, *c*12 CLA only (Fig 4 15, Tables 4 15-4 17), neither *c*9, *t*11 CLA or *t*9, *t*11 CLA treatment produced C18 1 *t*-10 suggesting a possible cellular biohydrogenation reaction occurring on carbon 12

Table 4.15 Fatty acids composition of total cellular lipids from HT-29 cells incubated in the presence of 75 μM of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA as free fatty acid for 24hrs

% FAME of cellular lipid

Fatty acids obtained	Control (Ethanol)	CLA mixture (75μM)	*c*9, *t*11 CLA (75μM)	*t*10, *c*12 CLA (75μM)	*t*9, *t*11 CLA (75μM)
C14 0	2 4 ± 0 2	1 8 ± 0 1*	1 9 ± 0 1*	1 4 ± 0 1*	2 0 ± 0 1
C16 0	34 8 ± 1 2	24 3 ± 0 2*	23 5 ± 0 6*	20 3 ± 0 7*	25 5 ± 0 1*
C16 1	1 8 ± 0 2	1 2 ± 0 1	1 4 ± 0 1	0 7 ± 0 1*	1 5 ± 0 1*
C18 0	50 8 ± 2 1	34 1 ± 1 3*	31 2 ± 0 4*	29 8 ± 1 2*	35 2 ± 0 3*
C18 1[*t*-10]	0	0 6 ± 0 03*	0	3 0 ± 0 4*	0
t-VA	0	0	0	0	0
C18 1(c9)	8 2 ± 0 9	4 2 ± 0 2*	8 5 ± 0 7	3 6 ± 0 6*	3 8 ± 0 7*
LA	0 3 ± 0 5	0 8 ± 0 02	0 7 ± 0 7	0 1 ± 0 4	0 3 ± 0 3
C20 0	0 4 ± 0 7	0 7 ± 0 03	0	0	0 4 ± 0 4
*c*9, *t*11 CLA	0	14 0 ± 0 5*	30 7 ± 0 6*	0	0
*t*10, *c*12 CLA	0	16 7 ± 0 5*	0	41 0 ± 1 2*	0
*t*9, *t*11 CLA	0	0	0	0	30 3 ± 1 9*
C20 4	0 6 ± 1 0	1 3 ± 0 1	1 3 ± 0 1	0 4 ± 0 1	0 5 ± 0 5
C22 6	0 8 ± 0 7	0 4 ± 0 4	0 8 ± 0 03	0 5 ± 0 1	0 6 ± 0 1
LCSFA	88 3 ± 1 9	61 0 ± 1 6*	56 6 ± 0 5*	51 6 ± 2 0*	63 0 ± 0 4*
UFA	11 7 ± 1 9	39 0 ± 1 6*	43 4 ± 0 5*	29 0 ± 2 0*	37 0 ± 0 4*
MUFA (Δ9)	10 0 ± 1 0	5 3 ± 0 1*	9 9 ± 0 5	4 3 ± 0 6*	5 3 ± 0 6*
UFA/LCSFA	0 1 ± 0 02	0 6 ± 0 04*	0 8 ± 0 01*	0 6 ± 0 1*	0 6 ± 0 01*
MUFA/LCSFA	0 1 ± 0 01	0 1 ± 0 004	0 2 ± 0 01*	0 1 ± 0 02	0 1 ± 0 01
C16 1/C16 0	0 05 ± 0 01	0 05 ± 0 002	0 1 ± 0 02	0 03 ± 0 04*	0 1 ± 0 05*
C18 1(c9)/C18 0	0 16 ± 0 02	0 1 ± 0 01	0 3 ± 0 02*	0 1 ± 0 02	0 1 ± 0 02

*Denotes results which are significantly different to untreated cells (p<0 05) Data represent Mean ± SD of 3 replicates (i e n=3)

Table 4.16 Fatty acids composition of total cellular lipids from HT-29 cells incubated in the presence of 75 μM of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA as free fatty acid for 72hrs

% FAME of cellular lipid

Fatty acids obtained	Control (Ethanol)	CLA mixture (75μM)	*c*9, *t*11 CLA (75μM)	*t*10, *c*12 CLA (75μM)	*t*9, *t*11 CLA (75μM)
C14 0	3 0 ± 0 2	2 5 ± 0 2	2 2 ± 0 1*	2 6 ± 0 3	3 1 ± 0 3
C16 0	31 1 ± 0 6	24 3 ± 1 8*	22 7 ± 0 2*	25 1 ± 0 5*	23 3 ± 2 3*
C16 1	4 6 ± 0 3	1 8 ± 0 1*	2 5 ± 0 05*	1 6 ± 0 1*	4 5 ± 0 9
C18 0	38 0 ± 1 0	28 5 ± 1 3*	26 0 ± 0 9*	25 6 ± 2 1*	25 4 ± 3 7*
C18 1[*t*-10]	0	1 9 ± 0 1*	0	7 2 ± 0 4*	0
t-VA	0	0	0	0	0
C18 1(c9)	16 3 ± 0 6	8 1 ± 1 0*	12 4 ± 0 3*	6 9± 0 2*	11 5 ± 2 1
LA	2 3 ± 0 03	1 8 ± 0 1*	1 5 ± 0 1*	1 7 ± 0 1*	1 8 ± 0 2
C20 0	0	0 5 ± 0 4	0	1 2 ± 0 3	0
*c*9, *t*11 CLA	0	14 5 ± 0 9*	29 1 ± 1 4*	0	0
*t*10, *c*12 CLA	0	12 5 ± 2 3*	0	26 8 ± 1 7*	0
*t*9, *t*11 CLA	0	0	0	0	26 6 ± 2 6*
C20 4	2 7 ± 0 5	2 2 ± 0 2	2 1 ± 0 1	1 7 ± 0 1*	2 3 ± 0 3
C22 6	2 0 ± 0 1	1 5 ± 0 3	1 5 ± 0 03*	1 2 ± 0 05*	1 5 ± 0 2
LCSFA	72 0 ± 0 3	55 7 ± 2 9*	50 9 ± 0 9*	54 1 ± 2 1*	51 9 ± 5 7*
UFA	28 0 ± 0 3	44 3 ± 2 9*	49 1 ± 0 9*	42 5 ± 2 1*	48 1 ± 5 7*
MUFA (Δ9)	20 9 ± 0 9	9 9 ± 0 5*	14 9 ± 0 3*	8 5 ± 0 2*	16 1 ± 2 9
UFA/LCSFA	0 4 ± 0 02	0 8 ± 0 1*	1 0 ± 0 03*	0 8 ± 0 1*	0 9 ± 0 2*
MUFA/LCSFA	0 3 ± 0 01	0 17 ± 0 02*	0 3 ± 0 01	0 16 ± 0 02*	0 3 ± 0 1
C16 1/C16 0	0 15 ± 0 01	0 1 ± 0 003*	0 1 ± 0 02*	0 1 ± 0 02*	0 2 ± 0 05
C18 1(c9)/C18 0	0 43 ± 0 03	0 1 ± 0 04*	0 5 ± 0 07	0 3 ± 0 03*	0 5 ± 0 1

*Denotes results which are significantly different to untreated cells (p<0 05) Data represent Mean ± SD of 3 replicates (i e n=3)

Table 4.17 Fatty acids composition of total cellular lipids from HT-29 cells incubated in the presence of 75 μM of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA as free fatty acid for 120hrs

% FAME of cellular lipid

Fatty acids obtained	Control (Ethanol)	CLA mixture (75μM)	*c*9, *t*11 CLA (75μM)	*t*10, *c*12 CLA (75μM)	*t*9, *t*11 CLA (75μM)
C14 0	4 1 ± 0 2	5 4 ± 0 1*	3 7 ± 0 3	4 8 ± 0 2	4 4 ± 0 3
C16 0	26 3 ± 0 6	29 4 ± 0 2*	23 5 ± 0 1*	26 4 ± 0 8	25 1 ± 0 6*
C16 1	11 1 ± 0 8	3 8 ± 0 1*	7 7 ± 0 03*	2 1 ± 0 1*	9 4 ± 0 8*
C18 0	28 8 ± 1 2	27 0 ± 0 2	23 6 ± 0 6*	24 1 ± 0 4	25 7 ± 1 0*
C18 1[*t*-10]	0	2 1 ± 0 003*	0	7 5 ± 0 3*	0
t-VA	0	0	0	0	0
C18 1(c9)	23 4 ± 0 8	9 3 ± 1 1*	18 9 ± 0 3*	8 3 ± 0 4*	13 5 ± 0 9*
LA	1 6 ± 0 1	1 5 ± 0 04	1 1 ± 0 3	1 6 ± 0 01	1 5 ± 0 1
C20 0	0 2 ± 0 3	0 2 ± 0 3	0 2 ± 0 3	0 3 ± 0 2	0
*c*9, *t*11 CLA	0	10 1 ± 0 2*	18 5 ± 1 4*	0	0
*t*10, *c*12 CLA	0	8 0 ± 0 1*	0	20 9 ± 1 4*	0
*t*9, *t*11 CLA	0	0	0	0	16 6 ± 0 7*
C20 4	2 7 ± 0 1	2 0 ± 0 04*	1 4 ± 1 2	2 5 ± 0 04*	2 3 ± 0 1
C22 6	1 9 ± 0 1	1 2 ± 0 3	1 4 ± 0 02*	1 7 ± 0 1*	1 6 ± 0 1*
LCSFA	59 3 ± 1 6	62 1 ± 0 3	51 0 ± 0 7*	55 5 ± 0 7	55 1 ± 1 4*
UFA	40 7 ± 1 6	37 9 ± 0 3	49 0 ± 0 7*	44 5 ± 0 7	44 9 ± 1 4*
MUFA (Δ9)	34 5 ± 1 7	13 2 ± 0 2*	26 6 ± 0 3*	10 4 ± 0 5*	22 9 ± 1 8*
UFA/LCSFA	0 7 ± 0 05	0 6 ± 0 01	1 0 ± 0 02*	0 8 ± 0 02	0 8 ± 0 05
MUFA/LCSFA	0 6 ± 0 04	0 2 ± 0 002*	0 5 ± 0 005	0 3 ± 0 01*	0 4 ± 0 04*
C16 1/C16 0	0 422 ± 0 04	0 1 ± 0 003*	0 3* ± 0 004	0 1 ± 0 001*	0 374 ± 0 04*
C18 1(c9)/C18 0	0 813 ± 0 06	0 3 ± 0 004*	0 804 ± 0 01	0 3 ± 0 02*	0 5 ± 0 1*

*Denotes results which are significantly different to untreated cells (p<0 05) Data represent Mean ± SD of 3 replicates (i e n=3)

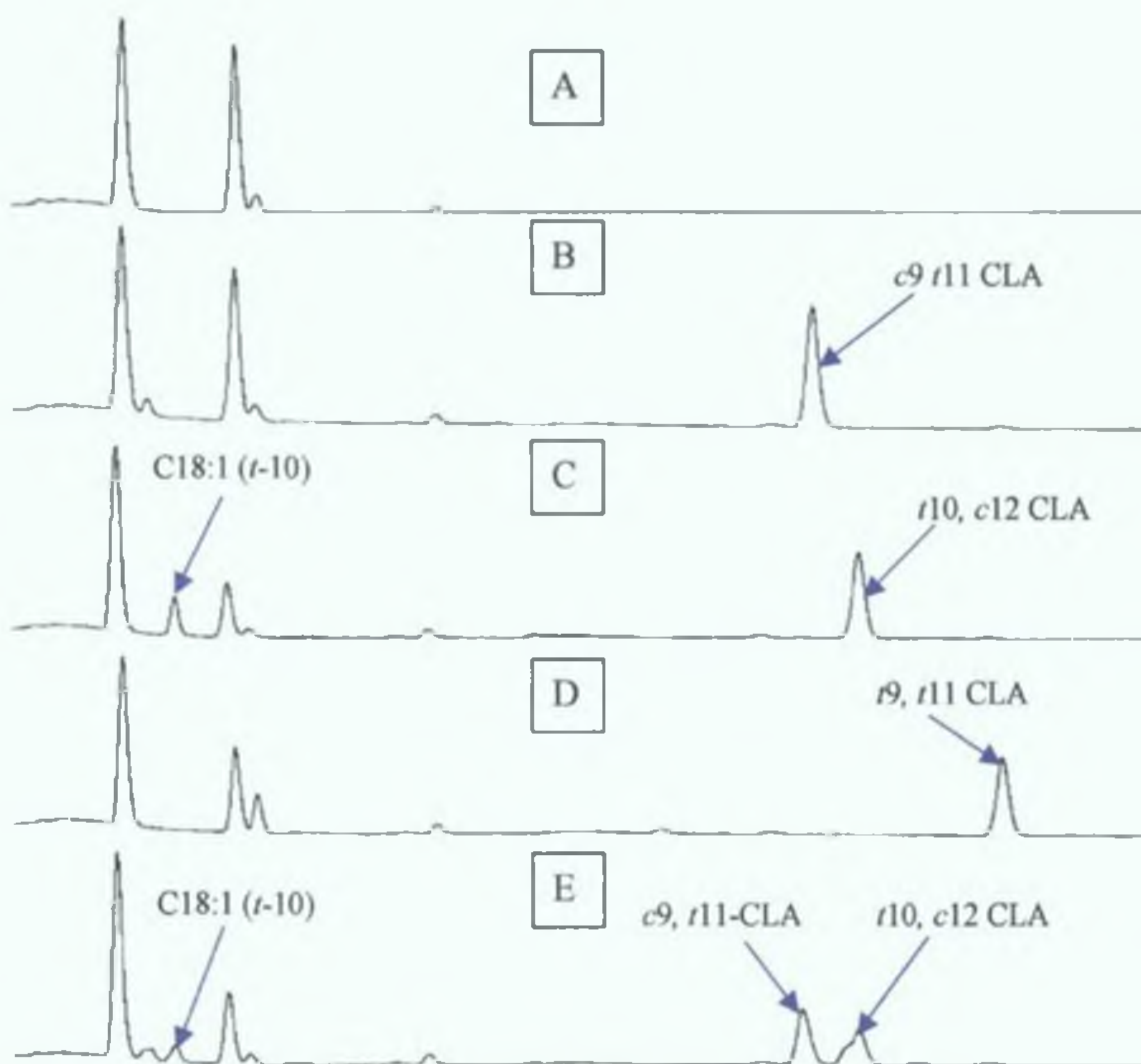

Figure 4.15 Partial gas chromatographic separation of the conjugated linoleic acid (CLA) region of HT-29 cells after 5 days treatment with (A) Ethanol (control), and 75 μM of (B) *c*9, *t*11 CLA, (C) *t*10, *c*12 CLA, (D) *t*9, *t*11 CLA, (E) CLA mixture of isomer.

Figure 4 16 shows that the occurance of C18 1 *t*-10 in cells treated with varying amounts of *t*10, *c*12 CLA was both dose-dependent and time dependent Treatment with 100 μM *t*10, *c*12 CLA for 120h yielded a significantly higher ($p<0\ 05$) level of C18 1 *t*-10 metabolite (9 3±0 1% total FAME) than a similar treatment for 72h (7 7±0 2 % total FAME) and for 24h (2 8±0 1% total FAME)

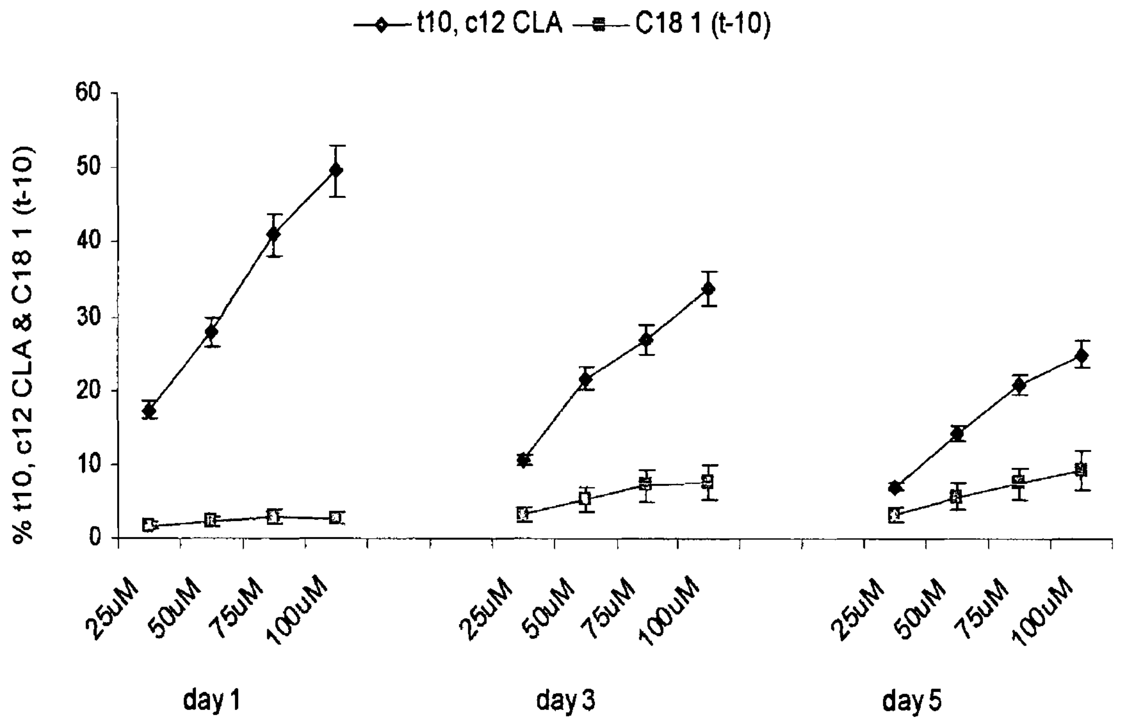

Figure 4.16 Time and dose dependent conversion of *t*10, *c*12 CLA to its metabolite 18 1 *t*-10 trans fatty acids when incubated 25μM, 50μM, 75μM and 100μM CLA

Figure 4.17, summarises the dose and time-dependent effects of *t*10, *c*12 CLA on the lipid profile of HT-29 cells. Interestingly, the fatty acid profile generated by the 100µM *t*10, *c*12 CLA showed a significant reduced proportion of LCSFA (51.3±0.3% total FAME) relative to control cells (58.1±2.9 % total FAME).

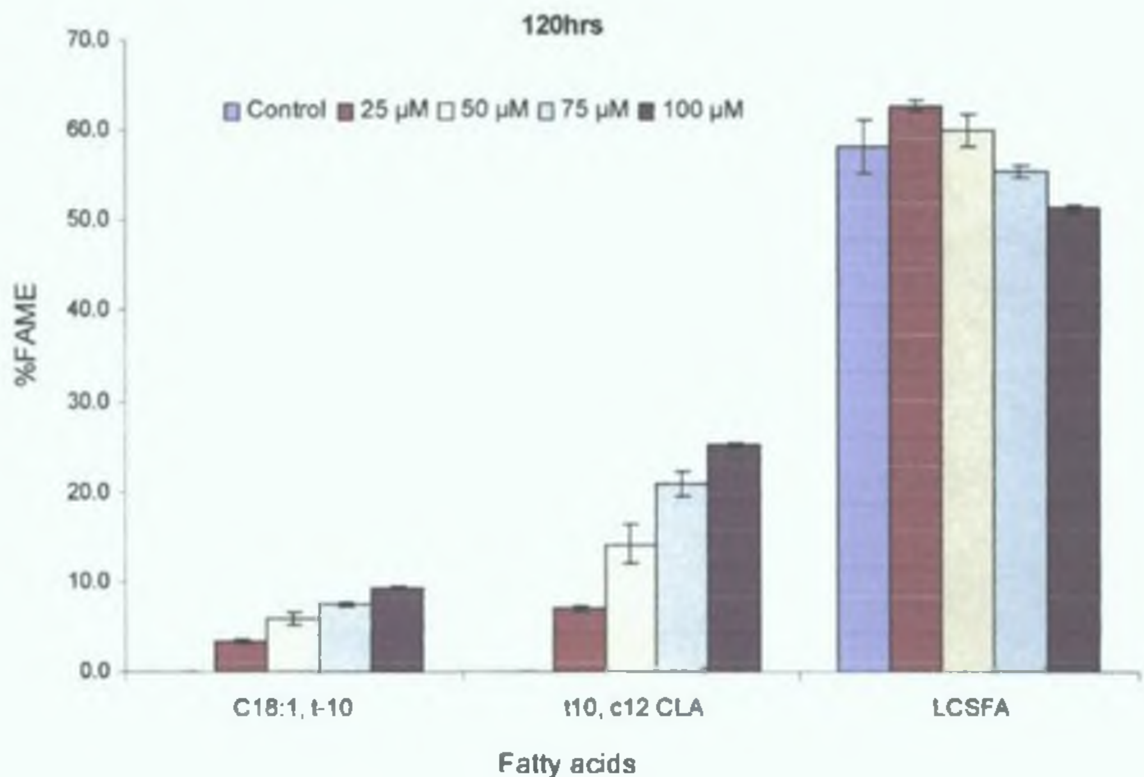

Figure 4.17 Dose dependent effects of *t*10, *c*12 CLA on the lipid profile of HT-29 cells after 120 hrs incubation.

4.4.5 Effects of CLA mixture, *c*9, *t*11 CLA; *t*10, *c*12 CLA and *t*9, *t*11 CLA when present as fatty acid-BSA complexes (molar ratio 2:1)

Table 4 18 demonstrates the lipid profile when cells were presented with fatty acids complexed with BSA (2 1 molar ratio) for 120h The *c*9, *t*11 CLA and *t*9, *t*11 CLA decreased the LCSFA content but only *c*9, *t*11 CLA treatment attained statistical significance, decreasing LCSFA to 51 4±0 4 % total FAME relative to control cells (58 4±1 6 % total FAME) Unlike free CLA isomer, *t*10, *c*12 CLA-BSA significantly (p<0 05) elevated the amount of LCSFA relative to control untreated cells

All fatty acid-BSA complexes reduced oleic acid to a similar extent (by 31-71%) as free fatty acid treatments and decreased the ratio of c18 1 Δ9/C18 0 by 22-67% All except *t*9, *t*11 CLA-BSA reduced palmitoleic acid by 47-81% and reduced the ratio of c16 1 Δ9/C16 0 by 40-80% The *c*9, *t*11 CLA isomer complexes with BSA increased the unsaturation index of cell lipids, similar to free *c*9, *t*11 CLA The *c*9, *t*11 CLA and *t*10, *c*12 CLA components of the CLA mixture were taken up in amounts equivalent to 26 0±0 1μg and 20 1±0 2μg, representing 11 1% and 11 6% of total FAME respectively The *c*9, *t*11 CLA single isomer was taken up in amounts equivalent to 55 7±0 2 μg representing 20 4±0 4 % total FAME The *t*10, *c*12 CLA single isomer was taken up in amounts equivalent to 43 6±2 2 μg representing 17 8±0 5 % total FAME Uptake of *t*9, *t*11 CLA was equivalent to 33 1±2 1 μg representing 14 8±0 6 % total FAME (Table 4 19)

Table 4.18 Fatty acids composition of total cellular lipids from HT-29 cells incubated in the presence of 37 5 μM of BSA, 75 μM of CLA mixture of isomers, *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA as fatty acid BSA complexes (2 1) for 120h

% FAME of cellular lipid					
Fatty acids obtained	BSA	CLA mixture (75μM)	*c*9, *t*11 CLA (75μM)	*t*10, *c*12 CLA (75μM)	*t*9, *t*11 CLA (75μM)
C14 0	3 9 ± 0 1	5 2 ± 0 2*	3 5 ± 0 1	4 6 ± 0 1	5 0 ± 0 1*
C16 0	25 5 ± 0 5	29 1 ± 0 1*	23 9 ± 0 3*	29 3 ± 0 3*	24 5 ± 0 4
C16 1	12 3 ± 0 4	3 8 ± 0 1*	6 5 ± 0 1*	2 3 ± 0 1*	11 7 ± 0 6
C18 0	28 5 ± 1 5	25 2 ± 0 6*	23 8 ± 0 4*	28 4 ± 0 3	25 0 ± 1 2
C18 1[*t*-10]	0	2 4 ± 0 1*	0	5 5 ± 0 2*	0
C18 1(c9)	24 8 ± 1 5	9 6 ± 0 1*	17 2 ± 0 3*	7 3 ± 0 1*	13 6 ± 0 4*
LA	1 0 ± 0 9	1 4 ± 0 1	1 2 ± 0 04	1 2 ± 0 04	1 4 ± 0 1
C20 0	0 4 ± 0 4	0 3 ± 0 3	0 2 ± 0 3	0 5 ± 0 4	0 5 ± 0 04
*c*9, *t*11 CLA	0	11 1 ± 0 3*	20 4 ± 0 4*	0	0
*t*10, *c*12 CLA	0	11 6 ± 0 2*	0	17 8 ± 0 5*	0
*t*9, *t*11 CLA	0	0	0	0	14 8 ± 0 6*
C20 4	1 7 ± 1 4	1 9 ± 0 04	1 9 ± 0 03	1 8 ± 0 02	2 1 ± 0 1
C22 6	1 8 ± 0 1	1 4 ± 0 03*	1 3 ± 0 03*	1 3 ± 0 05*	1 5 ± 0 1*
LCSFA	58 4 ± 1 6	59 8 ± 0 7	51 4 ± 0 4*	62 8 ± 0 4*	55 0 ± 1 5
UFA	41 6 ± 1 6	40 2 ± 0 7	48 6 ± 0 4*	37 2 ± 0 4*	45 0 ± 1 5
MUFA	37 1 ± 1 2	15 8 ± 0 2*	23 8 ± 0 4*	15 1 ± 0 3*	25 2 ± 1 0*
UFA/LCSFA	0 7 ± 0 05	0 7 ± 0 02	0 9 ± 0 01*	0 6 ± 0 10*	0 8 ± 0 05
MUFA/LCSFA	0 6 ± 0 02	0 3 ± 0 005*	0 5 ± 0 01*	0 2 ± 0 01*	0 5 ± 0 03*
C16 1/C16 0	0 5 ± 0 03	0 1 ± 0 004*	0 3 ± 0 01*	0 1 ± 0 003*	0 5 ± 0 03
C18 1(c9)/C18 0	0 9 ± 0 04	0 4 ± 0 01*	0 7 ± 0 01*	0 3 ± 0 003*	0 5 ± 0 04*

*Denotes results which are significantly different to BSA treated (BSA control) cells (p<0 05) Data represent Mean ± SD of 3 replicates (i e n=3)

Table 4.19 Comparison of CLA taken up by HT-29 cells when incubated in the presence of 75 μM of CLA mixture of isomers, 75 μM of *c*9, *t*11 CLA, 75 μM of *t*10, *c*12 CLA and 75 μM of *t*9, *t*11 CLA as free fatty acid and also as complex with BSA (2 1) for 120h

Treatments	*c*9, *t*11 CLA (75μM)	*t*10, *c*12 CLA (75μM)	*t*9, *t*11 CLA (75μM)
CLA mixture (free form)	24 7 ± 0 2	19 6 ± 0 7	0
CLA mixture (complex form)	26 0 ± 0 1	20 1 ± 0 2	0
*c*9, *t*11 CLA (free form)	52 5 ± 3 1	0	0
*c*9, *t*11 CLA (complex form)	55 7 ± 0 2	0	0
*t*10, *c*12 CLA (free form)	0	37 9 ± 2 2	0
*t*10, *c*12 CLA (complex form)	0	43 6 ± 2 2	0
*t*9, *t*11 CLA (free form)	0	0	37 2 ± 3 0
*t*9, *t*11 CLA (complex form)	0	0	33 1 ± 2 1

Data represent Mean ± SD of 3 replicates (i e n=3)

Levels of linoleic acid and arachidonic acids were not altered by the CLA mixture of isomer nor by its two main isomer constituents nor by *t*9, *t*11 CLA As with free fatty acids, a peak corresponding to C18 1 *t*-10 was observed when cells were treated with the CLA mixture of isomers (2 4±0 1% total FAME) and *t*10, *c*12 CLA (5 5±0 2% total FAME) (Figure 4 16)

Together the data suggest that all CLAs are taken up to a similar extent by cells whether presented as free fatty acids or as BSA complexes *t*10, *c*12 CLA and the CLA mixture of isomers generate a lipid profile that is similar, irrespective of being complexed with BSA

4.4.6 Fatty acid composition of HT-29 cells following lipid fractionation

Long chain saturated fatty acids were the predominant fatty acids in the phospholipids (75 4% of total FAME), neutral lipids (65 8% of total FAME) and fatty acids fraction (92 5% of total FAME) of untreated HT-29 cells Monounsaturated fatty acids (C16 1 and C18 1) comprised the bulk of the unsaturated fatty acids in each of the fractions (Table 4 20) Arachidonic acid was predominantly found in the phospholipids fraction (4 4±0 6%) compared with the neutral lipids (0 9±0 04%) and fatty acids fractions (0 2±0 2%)

4 4.7 Incorporation of CLA mixture of isomers, *c*9, *t*11 CLA; *t*10, *c*12 CLA and *t*9, *t* 11 CLA, and into cellular phospholipids neutral lipids and fatty acid fraction

The CLA mixture of isomers was predominantly taken up by the neutral lipids fraction, thereby contributing to a significantly increased ratio of unsaturated fatty acids to saturated fatty acids The monounsaturated fatty acids and arachidonic acid were significantly reduced in the phospholipids and neutral lipids relative to control cells Their reduction in the fatty acids fraction didn't reach statistical significance (Table 4 21)

The two single CLA isomers, *c*9, *t*11 CLA, and *t*10, *c*12 CLA were also predominantly taken up by the neutral lipid fraction thereby increasing the unsaturation index of this fraction All treatments significantly reduced monounsaturated fatty acids in the phospholipids and neutral lipids relative to control cells All treatments except *c*9, *t*11 CLA reduced arachidonic acid in the phospholipids ($P<0 05$), the reduction by *c*9, *t*11 CLA was not significant C18 1 *t*10 was found predominantly in the neutral lipids fraction following treatment with either *t*10, *c*12 CLA or CLA mixture (Table 4 21 and 4 23)

Table 4 20 Fatty acıds composıtıon of HT-29 cells ın three dıfferent classes (phospholıpıds, neutral lıpıds and fatty acıds fractıon) when ıncubated ın the presence of ethanol as control for 120h

% FAME of cellular lıpıd			
Fatty acıds obtaıned	Phospho-lıpıds Fractıon	Neutral lıpıds fractıon	Fatty acıds fractıon
C14	3 1 ± 0 2	4 2 ± 0 3	2 3 ± 0 1
C16	25 7 ± 1 5	30 6 ± 1 5	30 9 ± 0 5
C16 1	5 1 ± 0 3	10 6 ± 1 1	1 6 ± 0 2
C18	46 0 ± 1 8	30 0 ± 3 5	57 6 ± 0 5
C18 1[*t*-10]	0	0	0
C18 1 [c-9]	11 6 ± 0 9	20 2 ± 2 7	3 3 ± 0 6
LA	1 1 ± 0 1	1 3 ± 0 1	0 3 ± 0 3
C20	0 7 ± 0 6	1 0 ± 0 1	1 7 ± 0 1
*c*9, *t*11-CLA	0	0	0
*t*10, *c*12-CLA	0	0	0
*t*9, *t*11-CLA	0	0	0
C20 4	4 4 ± 0 6	0 9 ± 0 04	0 2 ±0 2
C22 6	2 4 ± 0 6	1 2 ± 0 1	2 2 ± 2 0
LCSFA	75 4 ± 2 2	65 8 ± 4 1	92 5 ± 1 0
UFA	24 6 ± 2 2	34 2 ± 4 1	7 5 ± 1 0
MUFA	16 7 ± 1 2	30 7 ± 3 8	4 9 ± 0 8
UFA/LCSFA	0 3 ± 0 04	0 5 ± 0 1	0 0888 ± 0 01
MUFA/LCSFA	0 2 ± 0 02	0 5 ± 0 1	0 05 ± 0 01
C16 1/C16 0	0 2 ± 0 02	0 3 ± 0 05	0 05 ± 0 01
C18 1(c9)/C18 0	0 2 ± 0 03	0 7 ± 0 2	0 1 ± 0 01

Data represent Mean ± SD of 3 replıcates (ı e n=3)

Table 4.21 Fatty acids composition of HT-29 cells in three different classes (phospholipids, neutral lipids and fatty acids fraction) when incubated in the presence of CLA mixture of isomers for 120h

% FAME of cellular lipid

Fatty acids obtained	Phospho-lipids Fraction	Neutral lipids fraction	Fatty acids fraction
C14	2 9 ± 0 3	3 9 ± 0 2	2 4 ± 0 1
C16	27 4 ± 1 1*	24 3 ± 0 5*	30 7 ± 0 3
C16 1	1 3 ± 0 1*	1 8 ± 0 3*	0 9 ± 0 4*
C18	49 0 ± 1 2	26 4 ± 3 2*	57 8 ± 0 2
C18 1[*t*-10]	0 9 ± 0 8	5 8 ± 0 8*	0 6 ± 0 02*
C18 1 [c-9]	4 6 ± 0 3*	7 0 ± 0 7*	1 9 ± 0 2
LA	1 6 ± 0 04	1 2 ± 0 1*	0 3 ± 0 02
C20	0 6 ± 0 5	0 6 ± 0 2*	1 5 ± 0 04
*c*9, *t*11-CLA	5 0 ± 0 4*	14 6 ± 1 0*	2 2 ± 0 2*
*t*10, *c*12-CLA	3 0 ± 0 1*	12 4 ± 0 7*	1 5 ± 0 1*
*t*9, *t*11-CLA	0	0	0
C20 4	2 9 ± 0 2*	0 6 ± 0 01*	0
C22 6	1 3 ± 0 03	1 3 ± 0 1	0 2 ± 0 03
LCSFA	79 9 ± 0 5	55 3 ± 3 6*	92 4 ± 0 4
UFA	20 1 ± 0 5	44 7 ± 3 6*	7 6 ± 0 4
MUFA	6 8 ± 0 5*	14 6 ± 1 8*	3 5 ± 0 4
UFA/LCSFA	0 3 ± 0 01	0 8 ± 0 1*	0 1 ± 0 005
MUFA/LCSFA	0 1 ± 0 01*	0 3 ± 0 05*	0 04 ± 0 005*
C16 1/C16 0	0 001 ± 0 01*	0 1 ± 0 01*	0 04 ± 0 01*
C18 1(c9)/C18 0	0 1 ± 0 05*	0 3 ± 0 1*	0 03 ± 0 004

*Denotes results which are significantly different to untreated control) cells (p<0 05)

Data represent Mean ± SD of 3 replicates (i e n=3)

Table 4.22 Fatty acids composition of HT-29 cells in three different classes (phospholipids, neutral lipids and fatty acids fraction) when incubated in the presence of *c*9, *t*11 CLA for 120h

% FAME of cellular lipid			
Fatty acids obtained	Phospho-lipids Fraction	Neutral lipids fraction	Fatty acids fraction
C14	3 0 ± 0 2	3 5 ± 0 04	2 2 ± 0 05
C16	26 3 ± 0 4	25 5 ± 0 6*	30 1 ± 0 9
C16 1	2 2 ± 0 2*	4 8 ± 0 1*	1 0 ± 0 1
C18	46 0 ± 0 8	22 8 ± 0 8*	56 2 ± 0 9*
C18 1[*t*-10]	0	0	0
C18 1 [c-9]	6 5 ± 0 1*	14 0 ± 0 4*	3 1 ± 0 4
LA	0 9 ± 0 04	1 1 ± 0 04*	0 2 ± 0 02
C20	1 1 ± 0 1	0 5 ± 0 02*	1 5 ± 0 1
*c*9, *t*11-CLA	8 7 ± 0 3*	26 0 ± 0 5*	5 4 ± 1 2*
*t*10, *c*12-CLA	0	0	0
*t*9, *t*12-CLA	0	0	0
C20 4	3 7 ± 0 1	0 5 ± 0 03*	0
C22 6	1 8 ± 0 1	1 3 ± 0 1	0 2 ± 0 05
LCSFA	76 3 ± 0 6	52 3 ± 0 8*	90 1 ± 1 8*
UFA	23 7 ± 0 6	47 7 ± 0 8*	9 9 ± 1 8*
MUFA	8 6 ± 0 1*	18 8 ± 0 4*	4 1 ± 0 5
UFA/LCSFA	0 3 ± 0 01	0 9 ± 0 03*	0 0816 ± 0 02*
MUFA/LCSFA	0 1 ± 0 002*	0 4 ± 0 01	0 05 ± 0 01
C16 1/C16 0	0 1 ± 0 01*	0 2 ± 0 01*	0 03 ± 0 004
C18 1(c9)/C18 0	0 1 ± 0 004*	0 6 ± 0 02	0 1 ± 0 01

*Denotes results which are significantly different to untreated control) cells (p<0 05)

Data represent Mean ± SD of 3 replicates (i e n=3)

Table 4.23 Fatty acids composition of HT-29 cells in three different classes (phospholipids, neutral lipids and fatty acids fraction) when incubated in the presence of *t*10, *c*12 CLA for 120h

% FAME of cellular lipid

Fatty acids obtained	Phospho-lipids Fraction	Neutral lipids fraction	Fatty acids fraction
C14	2 8 ± 0 2	3 4 ± 0 2	2 4 ± 0 1
C16	28 2 ± 6	24 4 ± 0 7*	30 4 ± 0 6*
C16 1	1 0 ± 0 1*	1 4 ± 0 02*	0 4 ± 0 04*
C18	49 7 ± 1 3	28 7 ± 0 3	57 7 ± 0 5
C18 1[*t*-10]	2 8 ± 0 4*	11 3 ± 0 5*	1 5 ± 0 3*
C18 1 [c-9]	4 8 ± 0 6*	5 2 ± 0 2*	1 7 ± 0 1*
LA	1 0 ± 0 2	0 9 ± 0 1*	0 2 ± 0 1
C20	0 5 ± 0 5	0 8 ± 0 04*	1 6 ± 0 1*
*c*9, *t*11-CLA	0	0	0
*t*10, *c*12-CLA	5 1 ± 0 4*	22 2 ± 0 2*	3 9 ± 0 4*
*t*9, *t*11-CLA	0	0	0
C20 4	2 8 ± 0 2	0 6 ± 0 04*	0 2 ± 0 2
C22 6	1 3 ± 0 1	1 2 ± 0 1	0 2 ± 0 01
LCSFA	81 2 ± 0 5*	57 3 ± 0 6*	92 0 ± 1 0
UFA	18 8 ± 0 5*	42 7 ± 0 6*	8 0 ± 1 0
MUFA	8 6 ± 0 4*	17 9 ± 0 7*	3 6 ± 0 2
UFA/LCSFA	0 2 ± 0 01	0 7 ± 0 02*	0 1 ± 0 01
MUFA/LCSFA	0 1 ± 0 01*	0 3 ± 0 01	0 04 ± 0 003
C16 1/C16 0	0 04 ± 0 002*	0 1 ± 0 003*	0 01 ± 0 002*
C18 1(c9)/C18 0	0 1 ± 0 01*	0 2 ± 0 005 *	0 03 ± 0 001*

*Denotes results which are significantly different to untreated control) cells ($p<0\ 05$)

Data represent Mean ± SD of 3 replicates (i e n=3)

Table 4 24 Fatty acids composition of HT-29 cells in three different classes (phospholipids, neutral lipids and fatty acids fraction) when incubated in the presence of *t*9, *t*11 CLA for 120h

% FAME of cellular lipid			
Fatty acids obtained	Phospho-lipids Fraction	Neutral lipids fraction	Fatty acids fraction
C14	2 3 ± 0 1*	2 5 ± 0 1*	1 8 ± 0 1*
C16	30 1 ± 0 7	25 7 ± 0 7	29 3 ± 0 5
C16 1	1 6 ± 0 1*	3 3 ± 0 03*	0 5 ± 0 01*
C18	59 0 ± 0 4*	44 4 ± 1 3*	63 5 ± 0 4*
C18 1[*t*-10]	0	0	0
C18 1 [c-9]	3 6 ± 0 2*	6 6 ± 0 2*	1 3 ± 0 1*
LA	0	0 9 ± 0 01*	0 1 ± 0 01*
C20	0	1 0 ± 0 01	1 5 ± 0 1
*c*9, *t*11-CLA	0	0	0
*t*10, *c*12-CLA	0	0	0
*t*9, *t*11-CLA	1 2 ± 0 05*	13 8 ± 0 3*	2 0 ± 0 1*
C20 4	1 7 ± 0 1*	0 5 ± 0 02*	0
C22 6	0 4 ± 0 01*	1 1 ± 0 04	0
LCSFA	91 4 ± 0 2*	73 6 ± 0 5	96 1 ± 0 1*
UFA	8 6 ± 0 2*	26 4 ± 0 5	3 9 ± 0 1*
MUFA	5 2 ± 0 1*	10 0 ± 0 2*	1 8 ± 0 1*
UFA/LCSFA	0 1 ± 0 003*	0 4 ± 0 01	0 04 ± 0 001*
MUFA/LCSFA	0 1 ± 0 001*	0 1 ± 0 004*	0 02 ± 0 001*
C16 1/C16 0	0 1 ± 0 001*	0 1 ± 0 005*	0 02 ± 0 0002*
C18 1(c9)/C18 0	0 1 ± 0 002*	0 1 ± 0 01*	0 02 ± 0 001*

*Denotes results which are significantly different to untreated control) cells (p<0 05)
Data represent Mean ± SD of 3 replicates (i e n=3)

4.5 Discussion

The hypothesis being tested in this work is that uptake and incorporation of CLA into cells can alter their lipid composition such that cellular processes controlling cancer cell growth are modulated. The overall objective of the work described in this chapter was to determine the extent to which CLA isomers and trans vaccenic acid, its putative precursor can modulate the lipid composition of HT-29 cells, a colon-derived human cancer cell line. To this end, it was important to first develop a validated procedure for separating and quantitating individual CLA isomers (*c*9, *t*11 CLA; *t*10, *c*12 CLA and *t*9, *t*11 CLA) and *t*-VA in cells.

Gas chromatography (GC) has been the method of choice for fatty acid analysis in biological samples for several decades. The GC technique employed here used a long capillary column to separate fatty acids as fatty acid methyl esters (FAME). The method separated FAMEs including CLA *cis/trans*, *trans/cis* and *trans/trans* isomers with good resolution on the basis of chain length, geometric configuration and numbers of double bonds. All FAMEs were quantitated by reference to an internal standard heptadecanoic acid (C17:0) which was added to cell pellets before lipid extraction. Methylation was a two stage procedure involving alkali-catalysed hydrolysis in methanol for derivatising bound fatty acids in lipid extracts and BF_3 /methanol for free fatty acids. This procedure was previously shown to suppress artificial isomerisation of *cis/trans* CLA and *trans/cis* CLA to *trans/trans* CLA (Igarashi *et al.*, 2004; Yurwecz 1997; Koritala and Rohwedder 1972). As presented above, the overall recovery of CLA was in the range 84.5-96.0% with 1.7-9.0%CV for overall reproducibility ranging between 89% and 96%. The data reported here is consistent with those reported in the literature when NaOH-BF3 reagent was used for methylation (Alonso *et al.*, 2004; Kim *et al.*, 2000; Jiang *et al.*, 1996). The former reported 83% recovery of CLA and 6.6% CV for repeatability using capillary GC analysis while the latter reported 89.4% recovery with 3.6% CV. The method was therefore considered advantageous for analysis of CLA isomers in HT-29 cells.

The data presented in Table 4.10 in which HT-29 cancer cells were characterised by higher proportions of long chain saturated fatty acids such as stearate and palmitate

compared with unsaturated fatty acids suggests that these cells were capable of synthesising their own supply of fatty acids via fatty acid synthase (FAS) Palmitate and stearate were also the predominant saturated fatty acids in phospholipids and neutral lipid fractions of HT-29 cells It is likely that FAS may be up regulated in HT-29 cells to meet the demands for oxidising power and/or membrane synthesis in response to cancer related overexpression of growth factors (e g Heregulin) and/or growth factor receptors (e g ErbB) It was also apparent that HT-29 cells possessed stearoyl-CoA desaturase activity by which monounsaturated fatty acids such as oleic acid and palmitoleic acid were produced from corresponding saturated fatty acyl CoAs At 120h control cells showed a 6 fold increase in the $\Delta 9$ desaturation index (the ratio of c9 monounsaturated fatty acids to saturated fatty acids) compared with 24h and was up 2 fold compared with 72h Palmitoleate and oleate were also the major (C16 0 26-30% in PL and 24-26%in NL and for C18 0 246-59% in PL and 23-44% in NL) monounsaturated fatty acids of the phospholipids and neutral lipid fraction The relatively lower amounts of polyunsaturated fatty acids relative to monounsaturated fatty acids suggest a much reduced level of $\Delta 5$ and $\Delta 6$ desaturase activities compared with $\Delta 9$ desaturase

It is apparent that it is possible to influence the lipid composition of cells by controlling the type of lipids added to the culture medium Treatment with *t*-VA for 48h and 120h decreased the proportion of palmitate and stearic acid in cells Similarly, all CLA treatments lowered total LCSFA levels at 24 to 72h suggesting that CLA and TVA treatments may be potent inhibitors of FAS After 24 and 72 h of culture *t*10, *c*12 CLA and *c*9, *t*11 CLA acted as more potent inhibitors than the CLA mixture of isomers or *t*9, *t*11 CLA The effects of CLA treatments on FAS after 120h were not as marked

It has been proposed that FAS activity and/or expression in cancer cells is incapable of being repressed by dietary fatty acids such as linoleic acid and/or arachidonic acid (Menendez *et al*, 2004) However the potential for specific unsaturated fatty acids to modulate FAS expression was recently reported when C18 3 PUFAs, namely GLA and ALA inhibited activity and expression of FAS in SK-Br3 breast cancer cells while supraphysiological levels of PUFAs such as LA and ARA had no effect (Menendez *et al*,

2004) Although the specific mechanisms by which GLA and ALA target tumour associated FAS is not yet known their differential effects suggest that tumour associated FAS does not ignore all dietary fatty acids A hypothetical model for understanding the constitutive upregulation of FAS in cancer cells suggests that FAS regulation occurs through modulation of SREBP1c which is driven by a constitutive hyperactivation of upstream oncogenic cascades such as PI-3K/ AKT and MAPK ERK signalling pathways In light of recent studies showing that CLA downregulated PI-3K/ AKT and MAPK (Miglietta *et al*, 2006, Cho *et al*, 2005) it would be of interest to evaluate if CLA also downregulates FAS via an effect on SREBP1c thus resulting in a CLA sensitivity

Treatments with CLA and *t*-VA also affected SCD activity in the HT-29 cell line Studies on the regulation of stearoyl-CoA desaturase and its role in metabolism have shown how critical this enzyme is in a variety of disease states including cancer The ratio of saturated to monounsaturated fatty acids affects phospholipids composition and has been implicated in the regulation of cell growth, apoptosis and differentiation through effects on membrane fluidity (Ntambi, 1999) and signal transduction (Miyazaki *et al*, 2000)

As index of Δ-9 desaturation, 16 1/16 0 levels were reduced by all CLA treatments revealing that CLA isomers significantly inhibited Δ-9 desaturation from palmitate to palmitoleate Similarly, 18 1/18 0 levels were reduced by all CLA treatments except for *c*9, *t*11 CLA at 120h suggesting that CLA treatments can inhibit Δ9 desaturase The observation that *c*9, *t*11 CLA did not alter the 18 1/18 0 ratio at 120h is a reflection of the similar magnitude in reduction of C18 0 and C18 1 levels Interestingly when cells were treated with CLA isomers complexed with BSA all CLA treatments including *c*9, *t*11 CLA reduced Δ9 desaturation index It was apparent that by 72h of culture both the CLA mixture and *t*10, *c*12 CLA acted as more potent inhibitors of Δ9 desaturation than *c*9, *t*11 CLA or *t*9, *t*11 CLA All CLA treatments also reduced the monounsaturated fatty acid composition of phospholipids and neutral lipid fractions to a similar extent as total lipids Inhibitory effects of *t*10, *c*12 CLA treatments on Δ-9 desaturation are consistent with a previous report using human breast cancer cell lines (Choi *et al*, 2002a), mammary gland of lactating mice (Lin *et al*, 2004) and rat hepatoma cell line (Lee *et al*, 1998) By

contrast the effect of *c*9, *t*11 CLA on inhibition of desaturase activity appears to be cell specific The *c*9, *t*11 CLA isomer was without effect in adipocytes (Bretillon *et al*, 1999) but did inhibit in breast cancer cell lines (Choi *et al*, 2002a) It has been shown that *t*10, *c*12 CLA isomer but not *c*9, *t*11 CLA decreased SCD1 mRNA expression, protein level and enzyme activity in 3T3-L1 preadipocytes The mechanism of *t*10, *c*12 CLA action on SCD gene expression could involve decreased SCD mRNA stability and/or gene transcription Determining whether CLA decreases the expression of the SCD gene in HT-29 cells by reducing mRNA stability and whether the effect is isomer-specific requires further investigation

Oleic acid and palmitoleic acid represent the major monounsaturated fatty acids of membrane phospholipids and triglycerides (neutral lipids) Expression of SCD is highly regulated in response to changes in the cellular environment Amongst the many developmental, dietary, hormonal and environmental factors regulating SCD are PUFAs Many including CLA have been shown to inhibit transcription of one of the main isoforms of SCD mRNA in mice (Choi *et al*, 2000, Park *et al*, 2000 and 1999b, Lee *et al*, 1998) This study showing that long term incubation with CLA mixture for 120h decreased the ratio of oleate stearate and of palmitoleate:palmitate is therefore consistent with in vivo studies showing inhibition of Δ9 desaturase (Lin *et al*, 2004) It is proposed that a future study could verify the strategic importance of a CLA-induced inhibition of Δ9 desaturase if sterculic acid, an inhibitor of Δ9 desaturases was effective in inhibiting colon cancer cell growth

This study also clearly showed that *t*-VA was desaturated to *c*9, *t*11 CLA in HT-29 cells and that conversion was linear with respect to amount of *t*-VA presented to cells and duration of treatment Desaturation normally introduces a single double bond between carbons 9 and 10 into a saturated fatty acyl CoA in a reaction involving NADPH, cyt b5 reductase, cytb5 and molecular oxygen The current hypothesis is that the enzyme removes hydrogen atoms starting with one at the c9 position followed by removal of the second hydrogen atom from the c10 position Desaturation of C18 1 *t*11 was an unusual finding as the preferred substrates for insertion of a double bond are palmitoyl Co A and Stearoyl Co A However it confirms previously reported findings that endogenous

synthesis of *c*9, *t*11 CLA from *t*-VA was dependent on Δ9 desaturase (Gomez *et al*, 2003, Miller *et al*, 2001, Adlof *et al*, 2000, Santora *et al*, 2000, Mahfouz *et al*, 1980, Pollard 1980) The latter quantified the desaturation of *t*-VA to *c*9, *t*11 CLA in humans A recent human intervention study m which desaturation of two different C18 1 trans fatty acids (trans-11-18 1 and trans-12-18 1) was evaluated showed selective endogenous conversion of *t*-VA to *c*9, *t*11 CLA contributed as much as 25% to the human CLA pool

It was also apparent that *t*-VA at the highest concentration (100μM) decreased the ratios of both C18 1Δ9·C18 0 and C16 1Δ9:C16 0, suggesting that like CLA, *t*-VA may be inhibitory regulator of SCD

The mechanism by which CLA and *t*-VA decrease scd1 mRNA is not yet known It may be related to the position and orientation of one of the double bonds present in these PUFAs A polyunsaturated fatty acid responsive region index has been localized in the promoter of stearoyl-CoA de-saturase gene (reviewed in Miyazaki and Ntambi, 2003) Whether this region mediates the action of CLA or *t*-VA on the transcription of the scd1 gene awaits further investigation

The appearance of a peak that putatively corresponded to C18 1 *t*-10 was an unexpected finding in this study It was apparent only when cells were treated with either the CLA mixture of isomers or the *t*10, *c*12 CLA Whether it could represent a metabolite of *t*10, *c*12 CLA in human cells is not yet known Further studies are warranted when and if a purified form becomes commercially available It is of interest however that species of the genus *Propionibacterium* isolated from mouse cecum produced *t*10, *c*12 CLA and C18 1 *t*-10 when cultured in the presence of linoleic acid (Verhulst *et al*, 1987)

In conclusion we report that CLA changes fatty acid composition in HT-29 cells by decreasing saturated and monounsaturated fatty acids The ability to alter the fatty acid composition of tissues by reducing the levels of monounsaturated fatty acids (Lee *et al* 1995) is one of the effects of CLA that has been observed consistently It is proposed that CLA treatments modify cellular fatty acid composition in HT-29 cells by decreasing the

actıvıty of FAS and stearoyl-CoA desaturase enzyme actıvıty The potentıal to alter the ratıo of saturated to monounsaturated fatty acıds may be ımportant ın maıntaınıng membrane fluıdıty, alteratıon of thıs ratıo may underlıe the ınhıbıtory effects of CLA on growth

CHAPTER 5

Final Discussion and Conclusion

Cancer is a leading cause of death in human populations all over the world Food is an important factor in determining cancer incidence in many countries and regions Food can have both positive (carcinogenic) and negative (preventive) effects Improved food, better life styles and developments of functional food are all crucial to cancer prevention (Sugimura, 2002)

Increased knowledge in the nutritional sciences and an improved understanding of the cellular and molecular basis of cancer now make it possible to approach research on nutrient-gene interactions relevant to cancer prevention and treatment Dietary intervention represents an attractive, non-invasive means of providing anticancer preventative and therapeutic benefits to at-risk individuals

Among the macronutrients, lipids have a unique property not shared with other nutrients, the type of lipid ingested modulates the chemical composition of cells to a very significant degree Novel functions for fatty acids and lipid-derived mediators, other than those encompassing membrane structure or provision of energy, have been elucidated Dietary fat has been shown to have profound effects on gene expression, leading to changes in cell metabolism, growth and cell differentiation (Jump and Clark, 1999, Grimaldi, 2001)

Insight into the relationship between CLA and cancer has come in the main from *in vivo* studies Experiments which permit the study, in isolation, of the interactions between specific cell types and dietary components are a powerful tool when conducted in conjunction with animal or human studies The ability to culture epithelial tumor cells *in vitro* has proved very useful in acquiring information on potential mechanisms for the effects of CLA on cancer Immortalised cell lines have genetic alterations that stabilise them for growth in culture but the ability to culture these cells in the presence of fatty acids and to then measure cell behaviour over a relatively short period of time allows for comprehensive studies with reproducible results that permit insight into the effects of these compounds Considerations in the design of cell culture studies include cell line selection, cell culture condition, the vehicle used to deliver the fatty acid, cell seeding densities, timing of measurements, laboratory procedures and selection of biological

endpoints relevant to human cancer Numerous down stream events can be assessed including changes in cellular signalling molecules and gene expression The fatty acid CLA has been shown to be effective at inhibiting carcinogenesis in multiple systems and at several stages including initiation, promotion, progression and metastasis (reviewed in Belury 2002b, reviewed in Scimeca 1999) By way of comparison, fish oil has been shown to exhibit anticancer properties but efficacious levels usually exceed 10 % of diet The ability of CLA to inhibit multiple models of carcinogenesis at much lower dietary levels (0 1 % w/w) appears to be specific for this group of fatty acids and has led to extensive studies being carried out to probe mechanisms and functions that are likely to be unique among PUFAs

The anticarcinogenic effects of a CLA mixture, 3 single isomers of CLA (*c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11 CLA), *t*-VA and LA were examined in this study using HT-29 human colon adenocarcinama cell line Sodium butyrate was used as positive control Results from this study revealed that the HT-29 cell line were sensitive to growth inhibitory effects of not only the CLA mixture but also to *t*10, *c*12 CLA, *c*9, *t*11 CLA and *t*9, *t*11 CLA isomers in a dose and time dependant manner following 1, 3 and 5 days of incubation with 10-200μM concentration

t-VA at 70μM inhibited growth of human HT-29 cancer cells by 21% after 5 days which was much lower than all CLA treatments (45%-94%)and the anticarcinogenic effects of *t*-VA is dependent on its conversion to *c*9, *t*11-Δ9-desaturase enzyme in rats (Lock *et al*, 2004) Another study has also shown that vaccenic acid (8 4 μg/ml), in the form of either *cis* or *trans*, significantly reduced growth of HT-29 human colon cancer cells by 23% when compared with control cells (Awad *et al*, 1995)

It is important that cell culture conditions mimic the *in vivo* environment as best they can Serum albumin is a remarkable protein capable of binding numerous ligands (Peters, 1995, Brown and Shockley, 1982, Peters and Reed, 1978, Foster, 1977) Choi *et al*, (2002b) suggest that albumin has adequate binding capacity for the low plasma levels of very long chain fatty acid (VLCFA) with 20 to 26 carbons, but the protein may not be

able to bind longer chain VLCFA In general, albumin has an important role in regulating the colloid osmotic pressure in blood and serves as a vehicle for the transport of many substances in blood such as hormones, drugs, ammo acids and free fatty acids (Høstmark, 2003) Albumin is the principal carrier of fatty acids that are otherwise insoluble in circulating plasma (Emerson, 1989)

In a separate experiment in the present study, the effect of presenting CLA to cells as a complex with BSA was evaluated and compared with presentation of CLA in free fatty acid form dissolved in ethanol (0 028 % v/v) CLA (50 and 100 μM) complexed with bovine serum albumin reduced growth of HT-29 cells by 28-44 % relative to control but was significantly less toxic ($p<0$ 05) than free CLA which inhibited growth by 42-85 % Similar results were observed with 3 single CLA isomers *c*9, *t*11 CLA (8-25%), *t*10, *c*12 CLA (33-43%) and *t*9, *t*11 CLA (70-86%) showed less growth inhibition as albumin complexes than free *c*9, *t*11 CLA (20-76%), *t*10, *c*12 CLA (42-78%) and *t*9, *t*11 CLA (91-93%) The present study have shown for the first time that *t*9, *t*11 CLA is the most potent cytotoxic CLA isomer in both free fatty acid form and as a complex with BSA *t*-VA didn't show significant effect as with albumin complex

Sodium butyrate was inhibitory in a dose and time dependant manner and the combined effects of CLA and Sodium butyrate were greater than the effect of butyrate alone on the growth inhibition of HT-29 cells CLA mixture of isomers, *c*9, *t*11 CLA and *t*10, *c*12 CLA in combination with Sodium butyrate significantly ($p<0$ 05) increased the inhibition of HT-29 cell growth compared to Sodium butyrate alone

In summary the present study showed that CLA mixture of isomer and three individual isomers *c*9, *t*11 CLA, *t*10, *c*12 CLA and *t*9, *t*11CLA were cytotoxic to HT-29 cells when delivered as complex with BSA or delivered as dissolved in ethanol

Data from Chapter 2 confirmed the anticancer activity of CLA isomers in HT-29 colon cancer cells The CLA-induced cytotoxicity may be related to epigenetic alterations in chromatin structure affecting accessibility to transcription factors, regulation of gene

expression, modulation of specific signal transduction pathways through changes in protein kinase expression and activation, lipid peroxidation, alterations in the distribution of arachidonic acid among cellular lipids, an altered prostaglandin profile, induction of ceramide, CEA and ALP which triggered a cascade of events leading to apoptosis and differentiation Differentiation can involve both morphological and functional alterations Morphological differentiation emphasises changes within the cellular structure and organisation of the cell while functional differentiation focuses on biochemical and enzymatic function (Rudolph *et al*, 2001)

The present study has clearly shown that CLA inhibited HT-29 cell growth via apoptotic pathway The percentage of apoptotic cells was detected by FACS which showed higher % of apoptotic cell numbers by CLA mixture, *c*9, *t*11 CLA, and *t*10, *c*12 CLA compared with untreated control cells Cho *et al*, (2003) also found a 3 fold increase in the percentage of apoptotic cells compared with control after 3 days of incubation of HT-29 cells with CLA The *t*9, *t*11 CLA showed higher % of necrotic cells than apoptotic cells after 24h at 75μM concentration suggesting that IC50 values differ in mode of cell death

This study identified a reduced level of HDAC (epigenetic regulation) and elevation of two possible differentiation markers, ceramide and CEA, whereas ALP level was unchanged The results of this study highlight a possible association between sphingolipids and the reported growth inhibitory properties of CLA Increased levels of ceramide were observed with CLA treatments at concentrations of 75μM after 3 day Recently W-3 PUFAs have been shown to inhibit the growth of breast cancer cells mediated by sphingomyelinase activation (Wu *et al*, 2005) Sphingomyelinase is an enzyme that catalyzes the hydrolysis of sphingomyelin (SM) to ceramide It is a possibility that CLA induced differentiation and apoptosis in this study results in the elevated concentration of cellular ceramide via sphingomyelinase pathway in HT-29 cells Recent studies showed that inhibition of histone deacetylases (HDACs) elicits anti-cancer effects in several tumor cell lines by inhibition of cell growth and inducing cell differentiation (Johnstone, *et al*, 2002) Therefore, the ability of these fatty acids (CLA,

Sodium butyrate, *t*-VA and LA) to induce CEA production and inhibit HDAC activity could be crucial Trans vaccenic acid didn't show any effect

CEA is an intracellular adhesion glycoprotein (Grunt *et al*, 1991) Several studies have shown that the up-regulation of CEA expression is associated with a differentiation induction-like response in human colon cancer cells (Chaakrabarty *et al*, 1992 and 1990, Niles *et al*, 1988, Denk *et al* 1972) It is apparent that all of the fatty acids except *t*-VA and LA increased CEA relative to control cells as free and as fatty acids-albumin complexes Of the CLA isomers studied, the CLA mixture, *t*10, *c*12 CLA and *t*9, *t*11 CLA exhibited the greatest potency *c*9, *t*11 CLA also showed an increase but it did not attain statistical significance However it was elevated in adherent cells incubated with *c*9, *t*11 CLA-BSA complex When cell lysates from both floating and adherent cells were analysed, only *t*9, *t*11 CLA was stimulatory suggesting differential effects of PUFAs on CEA expression between viable and non viable cells

In the present study it was observed that sodium butyrate treatment resulted a in marked increase in the activities of alkaline phosphatase activity in of HT-29 cells But none of the CLA isomers, CLA mixture and *t*-VA showed increased ALP activity Alkaline phosphatase is a well known differentiation marker of colonic epithelium Sodium butyrate -induced differentiation is associated with an increase in the alkaline phosphatase (ALP) activity (Siavoshian *et al*, 1997, Barnard *et al*, 1992)

The mechanisms by which committed cells are allocated to the different cell lineages of the intestine are poorly understood (Velcich *et al*, 1995) HT-29 cells can express, upon exposure to the appropriate inducers, distinct intestinal specific markers, they are, therefore, considered multi-potent, similar to the stem cells of the crypt It was observed that, in HT-29 cells, different inducers (12-O-tetradecanoylphorbol-13-acetate, forskolin, and sodium butyrate) modulate specific sets of markers Forskolin induced the expression of both mucin gene MUC2 and MUC3, whereas 12-O-tetradecanoylphorbol-13-acetate is capable of inducing only MUC2, and sodium butyrate, only MUC3 gene expression Carcinoembryonic antigen, a marker common to enterocytes and goblet cells, can be

induced by all the agents, whereas the alkaline phosphatase gene, the expression of which is characteristic of enterocytes, was responsive solely to sodium butyrate treatment (Velcich *et al*, 1995) of HT-29 cells It was previously reported that ALP activity was not elevated when HT-29 cells treated with tumour necrosis factor alpha (TNF-α) or hexamethylene bisacetamide (HMBA) but elevated by sodium butyrate treatment (Kovaiková *et al*, 2000, Schroy *et al*, 1994) and all those agents inhibited growth of HT-29 cells which suggested that inhibitory effects of HMBA and TNF-α on growth and differentiation of HT-29 cells are mediated by pathways independent of butyrate and ALP activity Therefore the present study suggests that inhibitory effects of CLA on growth and differentiation of HT-29 cells may be mediated by pathways independent of butyrate and ALP activity Recently Lampen *et al*, (2005) provided evidence that the cellular and molecular effects of *c*9, *t*11 CLA may be related to promotion of a more differentiated phenotype in CaCo2 colonic epithelial cells by the induction of the expression of alkaline phosphatase mRNA and its specific enzyme activity But there are no available data for CLA on ALP activity in HT-29 cells in the literature so far

Data from this study suggest that there is an association exists between decreased HDAC activity, increased ceramide level, increased CEA level and growth suppression in the HT-29 cells treated with the various CLA isomers

In summary this study proposed that CEA and ceramide level of HT-29 cells may be regulated by CLA The regulation of apoptosis by means of dietary agents is a novel and promising therapeutic approach for cancer treatment Given the importance of apoptosis in cancer development, apoptosis-inducing lipids could conceivably have an important role in adjunct anticancer therapy The potential clinical usefulness of a CLA based approach to cancer therapy requires further study

Endogenous fatty-acid synthesis could be a significant source of fatty acids for growth of tumor cells, considering the rates of free fatty acid and plasma triacylglycerol transport from the host to the tumor cells (reviewed in Kuhajda, 2000) Fatty-acid synthesis is common to all plants and animals Fatty acids are involved in diverse functions in cells

from energy storage and membrane structure to signal transduction cascades and protein acylation (reviewed in Kuhajda, 2000)

Fatty acid synthesis are carried out by the multiple enzymatic activities of fatty acid synthase (FAS) FAS is downregulated in most normal human tissues because of the fat in human diet In contrast, FAS is often highly expressed in human cancers High levels of FAS expression have been found in many human cancers including breast, prostate, colon, ovary, endometrium, thyroid, oral cavity, esophagus, bladder, retinoblastoma and melanoma ((Shah, *et al*, 2006, Innocenzi *et al*, 2003, Nemoto *et al*, 2001, reviewed in Kuhajda, 2000, Alo *et al*, 1996)

The association of FAS expression and tumor virulence led to the conception that FAS expression and activity may be vital for the growth and survival of human cancer cells Studies have demonstrated that inhibition of FAS is selectively cytotoxic to human cancer cells in vivo (reviewed in Kuhajda, 2000) Inhibition of fatty-acid synthesis by CLA could be a means to limit cytotoxic therapy to proliferating cells with high levels of FAS This strategy would likely target cancer cells and leave the normal proliferating cellular compartments in gastrointestinal tract intact

Uptake and incorporation of CLA into cells can alter their lipid composition such that cellular processes controlling cancer cell growth are modulated Metabolism of CLA by desaturases and elongation enzymes has been well documented now Conjugated metabolites have been identified in numerous tissues This knowledge opens up a new avenue of research which is related to the question of whether the metabolism of CLA is essential for its anticancer activity If purified metabolites become available for cell culture studies, it would be important to conduct studies to delineate whether CLA or one of its metabolites is the proximate effector molecule In the long term, elucidation of the mechanisms by which individual CLA isomers elicit their putative beneficial effects would permit studies to investigate evidence of such effects in cancer patients receiving them as dietary supplements

Stearoyl-CoA desaturase (SCD) which is the rate-limiting enzyme catalyzing the synthesis of monounsaturated fatty acids, mainly oleate (18 1) and palmitoleate (16 1) The ratio of saturated to monounsaturated fatty acids affects phospholipid composition and alteration in this ratio has been implicated in a variety of disease states including cardiovascular disease, obesity, diabetes, neurological disease, and cancer For this reason, the expression of SCD is of physiological significance in both normal and disease states (reviewed in Ntambi and Miyazaki, 2004) Large numbers of experimental data show that tumour cell growth can be modulated by individual fatty acids (Guthrie and Carroll, 1999, Zhou and Blackburn, 1999) Abnormal activation of SCD contributes to the development of many types of cancer, and downregulation of these pathways by CLA could have important therapeutic benefits

This study determined the extent to which CLA isomers and trans vaccenic acid, its putative precursor can modulate the lipid composition of HT-29 cells, a colon-derived human cancer cell line Gas chromatography (GC) has been the method of choice for fatty acid analysis in biological samples for several decades The present study validated a GC methodology for the quantification of CLA in cancer cells Initially the accuracy and separation power of the method was established by the successive analysis of fatty acid standards until satisfactory repeatability and reproducibility was obtained

The GC technique employed here used a long capillary column to separate fatty acids as fatty acid methyl esters (FAME) The method separated FAMEs including CLA *cis/trans*, *trans/cis* and *trans/trans* isomers with good resolution on the basis of chain length, geometric configuration and numbers of double bonds Methylation was a two stage procedure involving alkali-catalysed hydrolysis in methanol for derivatising bound fatty acids in lipid extracts and BF_3 /methanol for free fatty acids This procedure was previously shown to suppress artificial isomerisation of *cis/trans* CLA and *trans/cis* CLA to *trans/trans* CLA (Yurwecz, 1997, Igarashi *et al* 2004, Koritala and Rohwedder, 1972) As presented above, the overall recovery of CLA was in the range 84 5-96 0% with 1 7-9 0%CV for overall reproducibility ranging between 89% and 96% The data reported here is consistent with those reported in the literature when NaOH-BF3 reagent was used

for methylation (Alonso *et al*, 2004, Kim and Liu, 2000, Jiang *et al*, 1996) However a recent review demonstrated that BF3/MeOH derivatization method is not suitable for CLA quantification because *cis*, *trans*-conjugated fatty acids are lost (significant increase in the *t*, *t* CLA isomers occurs) and methoxy artifacts are formed when BF3/MeOH was mixed with lipid extract and heated with high temperature ($100^{\circ}C$) for 2-90 min rather than room temperature (Aldai *et al*, 2005) But in the present study used at room temperature condition for 30 minutes which was shown to suppress artificial isomerisation of *cis/trans* CLA and *trans/cis* CLA to *trans/trans* CLA (Igarashi *et al*, 2004, Yurwecz 1997, Koritala and Rohwedder 1972)

The data presented in this study in which HT-29 cancer cells were characterised by higher proportions of long chain saturated fatty acids such as stearate acid and palmitate compared with unsaturated fatty acids suggests that these cells were capable of synthesising their own supply of fatty acids via fatty acid synthase (FAS) Palmitate and stearate were also the predominant saturated fatty acids in phospholipids and neutral lipid fractions of HT-29 cells It was also apparent that HT-29 cells possessed stearoyl-CoA desaturase activity by which monounsaturated fatty acids such as oleic acid and palmitoleic acid were produced from corresponding saturated fatty acyl CoAs

This study also suggests that it is possible to influence the lipid composition of cells by controlling the type of lipids added to the culture medium Treatment with t-VA decreased the proportion of palmitate and stearic acid in cells Similarly, all CLA treatments lowered total LCSFA levels suggesting that CLA and t-VA treatments may be potent inhibitors of FAS After 24 and 72 h of culture *t*10, *c*12 CLA and *c*9, *t*11 CLA acted as more potent FAS inhibitors than the CLA mixture of isomers or *t*9, *t*11CLA

Data from this study demonstrate that HT-29 cancer cells have the enzymic capability to convert *t*-VA to *c*9, *t*11 CLA and we postulate that the growth suppression and cellular responses of HT-29 cell line are likely to be mediated via *t*-VA desaturation to *c*9, *t*11-CLA via Δ^9-desaturase However, it is impossible to rule out the possibility that *t*-VA may have an independent effect itself Corresponding experiments in which cells are

simultaneously treated with cyclopropene fatty acid (an inhibitor of Δ^9-desaturase) may determine whether the anticancer effect of *t*-VA can be negated Present study revealed that, the levels of C16 1 and C18 1 were reduced by 80% and 64% ($p<0\ 05$), the ratios of C16 1 Δ9/C16 0 and C18 1 Δ9/C18 0 were reduced by 75% and 62% respectively suggesting strong inhibition of Δ9 desaturase by *t*10, *c*12 CLA in HT-29 cells The *t*10, *c*12-CLA isomer has also been shown to inhibit the activity of Δ^9-desaturase in human cultured hepatoblastoma cells (Choi *et al*, 2001) It may be useful to treat HT-29 cells with *t*-VA along with a sub-lethal dose of *t*10, *c*12-CLA capable of inhibiting the activity of Δ^9-desaturase and to subsequently examine the effect on cell viability and CLA-responsive markers To establish the importance of *t*-VA as a precursor of endogenous CLA, it is imperative to conduct studies in humans Both descriptive data (i e the activity of Δ^9-desaturase at various tissue sites) and quantitative studies should be undertaken It may also be useful to determine the levels of FAS and Δ^9-desaturase in normal and tumor cell lines and in tumor biopsies using RT-PCR

It has been hypothesised that FAS activity and/or expression in cancer cells is incapable of being repressed by dietary fatty acids such as linoleic acid and/or arachidonic acid However the potential for specific unsaturated fatty acids to modulate FAS expression was recently reported when C18 3 PUFAs, namely GLA and ALA inhibited activity and expression of FAS in SK-Br3 breast cancer cells while supraphysiological levels of PUFAs such as LA and ARA had no effect Although the specific mechanisms by which GLA and ALA target tumour associated FAS is not yet known their differential effects suggest that tumour associated FAS does not ignore all dietary fatty acids A hypothetical model for understanding the constitutive upregulation of FAS in cancer cells suggests that FAS regulation occurs thro modulation of SREBP1c which is driven by a constitutive hyperactivation of upstream oncogenic cascades such as PI-3K/ AKT and MAPK ERK signalling pathways In light of recent studies showing that CLA downregulated AKT it would be of interest to evaluate if CLA also downregulates FAS via an effect on its upstream regulator, SREBP1c thus resulting in a CLA sensitivity

Because CLA isomers have been identified as having effects on cellular processes such as proliferation, apoptosis and differentiation, it may be opportune to examine if CLA isomers may be possible chemotherapeutic agents, or potential adjuvants to radio-or chemotherapy Resistance to chemotherapy drugs is a significant problem in the treatment of cancer It has been indicated that changes in the fluidity of the membranes due to certain lipids can reduce or completely prevent the efflux of cancer drugs out of cells (Schuldes *et al*, 2000) Plasma membrane fatty acid composition influences how lipophilic drugs diffuse through the membrane The more soluble the drug is m the membrane the more it can diffuse through Increased unsaturation decreases lipid molecular packing Preclinical trials have shown that certain PUFAs may enhance the cytotoxicity of several antineoplastic agents (Conklin, 2002) Polyunsaturated fatty acids such as DHA, eicosapentaenoic (EPA), gamma linolenic acid (GLA) and parinaric acid, have been shown to be cytotoxic to drug-resistant tumour cells by inducing oxidative stress and altering the activity of cell membrane bound enzymes such as sodium-potassium-ATPase and 5'-nucleotidase and the concentration of protein kinase C, central to reduction of intracellular drug levels (Pallares-Trujillo *et al*, 2000, Das *et al*, 1997, Burns and Spector, 1994) Because of enhanced cellular growth rates, certain membrane domains of tumour cells should respond rapidly to circulating fatty acids Altering the physical and functional properties of tumor cell membranes, by enrichment with CLA alone or in combination with other PUFAs (EPA, DHA and GLA), may increase the response to chemotherapy and may, to some degree reverse the resistance of cancer cells to certain chemotherapeutic agents Possible synergism in the action of anticancer drugs and CLA to enhance the intracellular concentration of these drugs warrant investigation A positive outcome from these types of studies could provide a sound scientific basis for combining a lipid based approach with traditional chemotherapy in the treatment of cancer Patients with cancer could ingest defined diets containing CLA and other PUFAs

The possibility that CLA may be considered as a potential dietary component for use in nutritional prevention of colon cancer is an attractive issue However, to date beneficial effects of CLA have been demonstrated in animal experimental models and *in vitro* systems only Even though there is one epidemiological evidence in the literature so far

linking CLA intake and colon tumour prevention in humans, more studies are required to confirm this effect Further studies are needed to identify new mechanisms and to evaluate and verify these mechanisms in humans to gain more understanding of the effects of CLA intake on cancer risk in real-life situations Epidemiologic studies with more detailed information about CLA exposures and improved analytic approaches that take into account the biological interplay between several nutritional factors in cancer development are needed The amount of dietary CLA required, the duration of intervention, as well as the most appropriate stages in life for such an intervention are issues that are not yet known The efficacy of CLA supplementation to inhibit tumour growth in cancer patients needs to be evaluated Analysis of normal and malignant tissues post surgery would give a novel insight into the use of CLA as adjuncts to conventional therapies

Biomarkers of CLA intake need to be identified and validated The physiological consequences of CLA intake throughout the lifespan are currently not understood Evidence suggests that early programming during foetal growth, infancy and childhood might decrease risk for chronic diseases in later life (Lusas, 1991) Thus, a better and more accurate understanding of CLA intakes and factors influencing CLA consumption throughout the lifespan might lend insight into what might be considered appropriate dietary recommendations for this potential nutrient

The goals of future research must therefore be to examine the selectivity of the anti proliferative effect of CLA on a wide variety of cell types including appropriate normal control cells, to examine the selectivity of organ site carcinogenesis intervention by CLA, to carry out epidemiological studies of *c*9, *t*11 CLA exposure and cancer risk and to make use of genomic technology to identify signaling pathways and molecular targets that are relevant to the action of CLA in cancer prevention Cancer-associated surrogate markers may then be investigated in controlled clinical trials to evaluate responsiveness to CLA It is vital that the efficacy of the individual CLA isomers *in vivo* be evaluated and the optimal levels of these isomers required for beneficial effects determined Identification of modulated mechanisms and tangible anti-cancer benefits will give impetus to food

manufacturers to incorporate CLA as a nutraceutical in functional foods which would enhance the health of the general population

The term "functional food" implies that the food has some identified value leading to health benefits, including reduced risk for disease, for the person consuming it (Reviewed in American Dietetic Association, 2004)

According to this definition, unmodified whole foods such as fruits and vegetables represent the simplest form of a functional food Modified foods, including those that have been fortified with nutrients or enhanced with phytochemicals or botanicals, also fall within the realm of functional foods

The scientific evidence for functional foods and their physiologically active components can be categorized into four distinct areas (a) clinical trials, (b) animal studies, (c) experimental in vitro laboratory studies, and (d) epidemiologic studies

Recent review (Reviewed in American Dietetic Association, 2004) demonstrated that, dairy products and meat from ruminant animals containing conjugated linoleic acid (CLA), which may alter carcinogenesis (Belury, 200b and 1995) a fourth category of functional foods For CLA, in vitro, in vivo, or epidemiologic research is available to support their health benefits, however, no health claim exists, partially because of the limited or improperly designed clinical trial data or lack of scientific agreement about the strength of the evidence (Reviewed in American Dietetic Association, 2004) Ideally, the evaluation of the efficacy of individual functional foods must be completed using a scientifically valid risk-benefit model that clearly assesses all physiologic effects, both positive and negative Review of the in vitro, animal, epidemiologic, and clinical data is essential before functional foods or food components are marketed to consumers for their health-promoting qualities (ILSI North American Technical Committee on Food Components for Health Promotion 2002) Therefore to develop CLA enriched functional foods as anticarcinogenic more research has to be done to get adequate data essential for functional foods

CHAPTER 6

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