THE MODULATION OF VARIOUS SIGNAL TRANSDUCTION PATHWAYS IN COLORECTAL CARCINOMA CELLS BY DOCOSAHEXAENOIC ACID

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety of in part submitted it at any university for a degree.

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SUMMARY

Introduction: The ability of different polyunsaturated fatty acids (PUFAs), especially n-3 PUFAs, to prevent the development of cancer has been under intense investigation the past three decades. Numerous studies have shown that these fatty acids can kill cancer cells *in vitro* as well as *in vivo* whilst normal cells remain unaffected. Unfortunately, the cellular and molecular mechanisms responsible for this phenomenon are still poorly understood. This study investigated the signalling pathways modulated by docosahexaenoic acid (DHA) in an adenocarcinoma cell line, in order to shed some light on these unknown mechanisms.

Materials & Methods: NCM460 (normal colon epithelial) and CaCo2 (colon adenocarcinoma) cells were cultured and treated with low doses of palmitic acid (PMA), oleic acid (OA), arachidonic acid (AA), and DHA. The effects of these fatty acids on the proliferation of the cells were measured with the MTT assay. The composition of membrane phospholipids of CaCo2 cells was determined after 48h supplementation with different fatty acids by gas chromatography. Also, CaCo2 cells were treated with DHA (10 μM) only and proteins were harvested at fixed time points ranging from 2 minutes to 48 hours. The protein inhibitors wortmannin (PI3 kinase inhibitor), PD 98059 (MEK inhibitor) and SB 203580 (p38 inhibitor) and also RNA interference (RNAi) of the p38 MAPK protein were used to investigate cross-talk between signalling pathways. ERK, p38 MAP kinase, Akt, and p53 were then analysed by Western blotting using phospho-specific and total antibodies. The cleavage of the apoptotic proteins, caspase-3 and PARP were also analysed.

Results and discussion: MTT assays revealed that none of the fatty acids were toxic to normal cells. In addition, DHA was shown to be most effective to kill CaCo2 cells whilst protecting NCM460 cells and a subsequent dose response

experiment revealed that lower concentrations are most suitable for this purpose. DHA was also shown to be readily incorporated into phospholipids, along with AA. This is associated with increased membrane fluidity, which could affect the localisation, and downstream effects, of various signalling proteins within the membrane. Western blot analysis revealed a rapid increase in activity in most proteins under investigation, especially ERK and Akt (Ser473). Long-term DHA supplementation suppressed the full activation of Akt. This down regulation of survival signalling could lead to cell death in CaCo2 cells. In addition, it was shown that after 48h, DHA induced the cleavage of caspase-3 and PARP, which is indicative of apoptosis. RNAi experiments suggested a possible role for p38 MAPK in the phosphorylation of p53 at Ser15, a site which is associated with DNA damage.

Conclusion: DHA exerts its effects by means of cellular signal transduction pathways, particularly by suppression of the important survival-related kinase, Akt. This could have implications for future therapeutic interventions in cancer patients, as fatty acids are safe to use and do not interfere with the functionality of normal tissue.

OPSOMMING

Inleiding: Die vermoë van verskillende poli-onversadigde vetsure (POVSe), veral n-3 POVSe, om die ontstaan van kanker te voorkom, is intens nagevors die afgelope drie dekades. Menigte studies het aangevoer dat hierdie vetsure kankerselle *in vitro* asook *in vivo* kan doodmaak, terwyl normale selle nie daardeur beïnvloed word nie. Ongelukkig word die sellulêre and molekulêre meganismes onderliggend tot hierdie verskynsel nie goed begryp nie. Hierdie studie het verskeie seintransduksie-paaie wat deur dokosaheksaenoësuur (DHS) in 'n adenokarsinoom sellyn gemoduleer word, ondersoek.

Materiale & Metodes: NCM460 (normale kolonepiteel) en CaCo2 (kolon adenokarsinoom) selle is onderhou in 'n selkultuur-laboratorium en behandel met lae dosisse palmitiensuur (PMS), oleïensuur (OS), aragidoonsuur (AS), en DHS. Die invloed van hierdie vetsure op die proliferasie van die selle is d.m.v. die MTT toets bepaal. The samestelling van membraan-fosfolipiede van CaCo2 selle is na 48h behandeling met die verskillende vetsure bepaal deur middel van gaschromatografie. Die CaCo2 selle is ook met DHA (10 μM) alleenlik behandel en teen vaste tydpunte wat wissel van 2 minute tot 48h, waarna proteïene geëkstraeer is. Die proteïen-inhibitore wortmannin (PI3 kinase inhibitor), PD 98059 (MEK inhibitor), en SB 203580 (p38 inhibitor) asook RNA-interferensie (RNAi) teen die p38 MAPK proteïen is ingespan om oorvleueling tussen seintransduksie–weë te ondersoek. ERK, p38 MAPK, Akt, en p53 is geanaliseer deur middel van die Western–klad metode met fosfo–spesifieke en totale antiliggame. Die kliewing van die apoptotiese proteïene caspase-3 en PARP is ook bepaal.

Resultate en bespreking: MTT toetse het ontul dat geen vetsure toksies was vir die normale selle nie. Daar is ook gevind dat DHS die mees effektiewe vetsuur was om CaCo2 selle te dood, terwyl NCM460 selle beskerm word. Gevolglik

het ‘n dosis-respons eksperiment getoon dat laer konsentrasies die beste geskik is vir hierdie doel. Daar is ook gevind dat DHA maklik in fosfolipiede geïnkorporeer word, tesame met AS. Dit word geassosieer met verhoogde membraan-vloeibaarheid, wat die ligging, en ook stroom-af werking, van verskeie seintransduksie proteïene in die membraan, kan beïnvloed. Western-klad analises het ‘n vinnige verhoging in die aktiwiteite van die meeste proteïene onder die soeklig, getoon, veral ERK en Akt (Ser473). Langdurige DHS behandeling het die maksimale aktiwiteit van Akt onderdruk. Hierdie afname van oorlewing-gerigte seine kan lei tot seldood in CaCo2 selle. Daar is boonop geving dat DHS die kliewing van caspase-3 en PARP geïnduseer het na 48, wat dui op apoptose. Uit die RNAi eksperiment kon daar ook ‘n moontlike rol vir p38 MAPK in die fosforilering van p53 by Ser15, wat geassosieer word met DNS-skade, getoon word.

Gevolgtrekking: DHS beoefen sy effekte deur middel van seintransduksie paaie, veral deur die oorlewing-geassosieerde kinase, Akt, te onderdruk. Dit kan implikasies hê vir toekomende terapeutiese ingrypings in kankerpasiënte, aangesien vetsure veilig is om te gebruik en nie skadelik is vir normale weefsel nie.

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ABBREVIATIONS

AA	Arachidonic acid
ACF	Aberrant crypt foci
AGC	PKA/PKG/PKC
AIF	Apoptosis-inducing factor
AIP	Apaf-1-interacting protein
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
AP1	Activator protein 1
Apaf	Apoptotic protease activating factor
APC	Adenomatous polyposis coli
ASK1	Apoptosis signal-regulating kinase 1
ATF2	Activating transcription factor 2
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related
Bad	Bcl-associated dimer
Bak	Bcl2 homologous antagonist/killer
Bax	Bcl-associated partner containing six exons
BBOT	2,5-bis(5'-tertbutylbenzoxazolyl-(2')thiophene
Bcl2	B-cell lymphoma 2
BHT	Butylated hydroxytoluene
Bid	Bcl2-interacting death agonist
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CARD	Caspase-associated recruitment domain
Caspase	Cysteine aspartate-specific protease
CDC	Centres for disease control and prevention
Chk	Checkpoint kinase
CK2	Casein kinase 2
CML	Chronic myeloid leukaemia
COX	Cyclooxygenase
cPLA$_2$	Cytosolic phospholipase A_2
CREB	cAMP-response element binding protein
CSBP	Cytokine-suppressive anti-inflammatory drug-binding protein
CVD	Cardiovascular disease

DAG	Diacylglycerol
DCC	Deleted in colorectal carcinoma
DED	Death effector domain
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	Double-stranded DNA-dependent protein kinase
DR	Death receptor
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FADD	Fas-associated death domain
FAME	Fatty acid methyl ester
FAP	Familial adenomatous polyposis
FasL	Fas-ligand
FBS	Foetal bovine serum
FCS	Foetal calf serum
FLICE	FADD-like interleukin-1β-converting enzyme
FLIP	FLICE-like inhibitory protein
GADD	Growth arrest and DNA damage
GLA	Gamma-linolenic acid
GLC	Gas-liquid chromatography
GPCR	G-protein coupled receptor
GSK3	Glycogen synthase kinase 3
h	Hour
HM	Hydrophobic motif
HNPCC	Hereditary nonpolyposis colon cancer
HPV	Human papilloma virus
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IAP	Inhibitor of apoptosis protein
IARC	International agency for research on cancer
ICAD	Inhibitor of caspase-activated DNAase

ICE	Interleukin-1β-converting enzyme
IHD	Ischaemic heart disease
IKK	IκB kinase
ILK	Integrin-linked kinase
KRAS	Kirsten rat sarcoma
LA	Linoleic acid
LCFA	Long chain fatty acid
LH	Luteinising hormone
LOX	Lipooxygenase
MAP3K	MAPK kinase kinase
MAP4K	MAPK kinase kinase kinase
MAPKAPK	MAPK-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MCFA	Medium chain fatty acid
MD	Mean difference
MDM2	Murine double minute 2
MEF2	Myocyte enhancer factor 2
MEK	MAP/ERK kinase
MEKK	MEK kinase
MEM	Eagle's minimum essential medium
min	Minutes
MITF	Microphthalmia transcription factor
MKP	MAPK phosphatase
MSI	Microsatellite instability
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	Monounsaturated fatty acid
NAD^+	Nicotinamide adenine dinucleotide
NFAT	Nuclear factor of activated T cells
NHE1	N^+/H^+ exchanger 1
NK	Natural killer
NLK	Nemo-like kinase
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
OA	Oleic acid
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered-saline

PC	Phosphatidylcholine
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependent kinase 1
PE	Phosphatidylethanolamine
PFK2	6-Phosphofructo-2-kinase
PI	Phosphatidylinositol or phosphoinositide
PI 3 kinase	Phosphatidylinositol 3 kinase or phosphoinositide 3 kinase
PI(3,4)P_2	Phosphatidylinositol 3,4 bisphosphate
PI(3,4,5)P_3	Phosphatidylinositol 3,4,5 triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PMA	Palmitic acid
PMSF	Phenylmethylsulphonylfluoride
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
PtdIns	Phosphatidylinositol
PTP	Permeability transition pore
PUFA	Polyunsaturated fatty acid
Rb	Retinoblastoma
RIP1	Receptor interacting protein 1
Ripa	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNAi	RNA interference
RTK	Receptor tyrosine kinase
SAP1	Stress-activated protein 1
SAPK	Stress-activated protein kinase
SBTI	Soybean trypsin inhibitor
SCFA	Short chain fatty acid
SCR-1	Steroid receptor coactivator-1
SDA	Stearidonic acid
SDS	Sodium dodecyl sulphate
SDS PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFA	Saturated fatty acid
SH2	Src homology domain
siRNA	Short interfering RNA
SKK	SAPK kinase

SMAC	Second mitochondria-derived activator of caspases
STAT	Signal transducer and activator of transcription
STR	Short tandem repeat
TAF	TATA-binding protein-associated factor
TAK1	TGFβ-activated kinase 1
TAO	Thousand and one kinase
TBS	Tris-buffered saline
TFA	Trans fatty acids
TGF	Transforming growth factor
TLC	Thin layer chromatography
TNFR	TNF receptor
TNFα	Tumour necrosis factor α
TPL2	Tumour progression locus 2
TRAF2	TNFR-associated factor 2
USF1	Upstream transcription factor 1
V	Volts
v/v	Volume per volume
w/v	Weight per volume
WHO	World health organization

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CHAPTER 1
INTRODUCTION AND OBJECTIVES

In recent years, numerous publications have reviewed the area of dietary fatty acids and cancer. The immense interest in this field stems from early epidemiological studies on the relation between the intake of fish and marine fatty acids and the risks of various cancers. Also, it is known that at least one third of human cancers may be associated with diet, and lifestyle (Bartsch *et al.*, 1999). The concept of a dietary intervention to decrease one's risk of developing certain cancers thus seems reasonable, and the notion that certain fatty acids have anti-tumourigenic properties, is now widely accepted.

Researchers mostly agree that a high dietary intake of n-6 polyunsaturated fatty acids (PUFAs) could lead to increased risks for cancers of the breast, colon, and prostate. On the other hand, the n-3 PUFAs can inhibit the growth of tumours, and it is this class of fatty acids that has received the most attention. It is now believed that the balance between the intakes of n-6 and n-3 fatty acids are the key to an increased or decreased cancer risk (Kang, 2005).

Although there are numerous studies on this topic, ranging from epidemiological studies, studies involving experimental animals as well as those done in cell cultures, the mechanisms are poorly characterised. It is known that fatty acids do not harm normal cells (Begin *et al.*, 1985), and this should make fatty acids an attractive adjuvant, or perhaps even a replacement, for conventional cancer therapies. Sadly, fatty acids have not received much attention in the clinic, and it is hoped that insights into their molecular actions in cancer cells could change this.

Therefore, the main objective of the present study was to shed some light on

the mechanisms of n-3 PUFAs, particularly docosahexaenoic acid (DHA).

The aims of the current study were threefold:

1. To test the effects of different fatty acids on the proliferation of both normal and cancer cells
2. To assess the effects of the different fatty acids on the membrane phospholipid composition of cancer cells
3. To investigate the modulation of different signalling molecules by fatty acids in cancer cells in an attempt to uncover a possible signalling-dependent mechanism.

CHAPTER 2
LITERATURE REVIEW

2.1. CANCER

2.1.1. Introduction

According to the World Cancer Report, a 351-page study released in 2003, cancer rates could increase by 50% to 15 million new cases by 2020 (World Cancer Report, 2003). The International Agency for Research on Cancer (IARC), a branch of the World Health Organization (WHO) conducted the study described in the report.

In 2000, 12% of the 56 million deaths worldwide were due to cancer and more than 22 million people were treated for cancer worldwide. In many countries, especially industrialized nations, more than 25% of deaths were attributable to cancer, a rate more than twice as high as developing countries in 2000 (Laurier, 2003). However, according to the report cancer is now also a major health problem in developing nations, matching its effect in industrialized nations for the first time. Industrialized countries with the highest overall cancer rates are: the United States of America, Italy, Australia, Germany, the Netherlands, Canada and France (Laurier, 2003).

According to the report the most common cancers worldwide are lung, breast and colorectal cancer. Table 1 shows the incidences of cancer worldwide, as was reported in 2003.

Table 2.1 Most common cancers worldwide, according to the World Cancer Report (World Cancer Report, 2003).

Cancer site	New cases annually
Lung	1,2 million
Breast	1 million
Colorectal	940 000
Stomach	870 000
Liver	560 000
Cervical	470 000
Oesophageal	410 000
Head and neck	390 000
Bladder	330 000
Malignant non-Hodgkin's lymphomas	290 000
Leukaemia	250 000
Prostate and testicle	250 000
Pancreas	216 000
Ovarian	190 000
Kidney	190 000
Endometrium	188 000
Nervous system	175 000
Melanoma	133 000
Thyroid	123 000
Pharynx	65 000
Hodgkin's disease	62 000

2.1.2. Biology of cancer

2.1.2.1. Introduction

Cancer can be described as a disorder of the balance between cell proliferation and cell death. During the progression of cancer, the tumour cells acquire a variety of phenotypic properties that allow them to proliferate both swiftly and

uncontrollably and spread from their original site to other locations in the body, often leading to the death of cancer patients (Hanahan and Weinberg, 2000).

2.1.2.2. Nomenclature of tumours

The tissue and cell type from which cancer has arisen, and whether it is benign or malignant, determines its classification. Malignant tumours arising from epithelium are termed carcinomas, whereas benign tumours arising from epithelium are known as papillomas or adenomas, depending on the tissue type or appearance of the tumour (Franks, 1997). For example, an adenoma has a glandular organization. The corresponding type of malignant tumour is known as an adenocarcinoma, implicating a similar glandular structure (Alberts *et al.*, 2002). Malignant tumours originating in muscle or connective tissue are known as sarcomas. The various leukaemias are derived from haemopoietic cells. In the nervous system, malignant tumours from neurons are termed blastomas; those derived from supporting cells such as astrocytes or oligodendrocytes are called cytomas. Each of the broad categories has various subdivisions, usually depending on cell type. For example, a cytoma of oligodendrocytes is called oligodendrocytoma. Very seldom, tumours that contain a mixture of different tissues may be found. Such tumours of mixed tissues are known as teratomas (Franks, 1997). Approximately 90% of human cancers are carcinomas, perhaps because epithelium is the most proliferative tissue in the body or because epithelial tissues are most frequently exposed to physical damage and harmful chemical agents (Alberts *et al.*, 2002).

2.1.2.3. Genetics and carcinogenesis

Cancer is considered to be a genetic disorder at the somatic cell level, due to the apparent link between carcinogenesis (the genesis of cancer) and mutagenesis (the production of a mutation in DNA). In the human body which is comprised of more than 10^{14} cells, billions of cells experience mutations each day. Mutations associated with cancer can involve small-scale changes, such

as a point mutation (the substitution of a single nucleotide), or large scale abnormalities, including rearrangement of chromosomes, gain or loss of chromosomes, or even the integration of viral DNA or RNA (Hanahan and Weinberg, 2000; Klug and Cummings, 2003). Large-scale genomic alterations are a common feature of cancer and most tumours are characterized by visible changes in chromosomal structure. Certain chromosomal abnormalities are so characteristic that they are used to diagnose a particular type of cancer and make predictions about the severity and progression of the disease, such as the classical example of the Philadelphia chromosome, an unusually small chromosome, observed in chronic myeloid leukaemia (CML) (Wasan and Bodmer, 1997; Klug and Cummings, 2003).

It is estimated that at least 50% of all cancers are environmentally induced (Klug and Cummings, 2003). Environmental agents known to increase the likelihood of tumour formation are known as carcinogens (or cancer-causing agents) and include chemical carcinogens (for example tobacco and asbestos), radiation (such as X-rays and ultraviolet light exposure), and viruses (for example *Helicobacter pylori* and *human papilloma virus* (HPV), that are known to induce stomach and cervical cancer, respectively) (Alberts *et al.*, 2002; World Cancer Report, 2003). For cancers that have a discernible external cause, the disease does not usually become apparent until long after exposure to the causal carcinogen. The occurrence of lung cancer, for example, only starts to increase steeply after 10 to 20 years of heavy smoking (Alberts *et al.*, 2002). The majority of carcinogens are also mutagens.

Carcinogenesis is not an immediate event, because a single mutant cell that does not proliferate abnormally does no significant damage to its surrounding microenvironment, no matter what other confounding properties it may have acquired. Instead, carcinogenesis is a multi-step process, involving a series of successive genetic alterations following the initial exposure to the carcinogen.

The primary step in carcinogenesis is initiation, which involves DNA damage or genomic changes of some sort. Although cells do possess mechanisms to repair damaged DNA, repair cannot be done if cells are in the process of replicating DNA while the mutation is induced, and thus the mutation is left intact in the replicated DNA (Tennant *et al.*, 1997). Following initiation, tumour promotion by promoting agents is necessary for the development of a tumour, because initiated cells remain latent until acted on by promoting agents (Franks, 1997). Promoting agents (for example inflammation) are not inherently carcinogenic, but they do induce cell division in initiated cells. It has been suggested that promoting agents may interfere with the process of differentiation that normally takes place when cells move from the dividing population into a population of functioning, usually post-mitotic, cells (Franks, 1997). Once such a cell proliferates uncontrollably, it will give rise to a tumour, or neoplasm – a relentlessly growing mass of mutant cells. This is the third step: tumour progression. Cancer is thus caused by genetic defects within somatic cells, enabling them to prosper at the expense of their neighbouring cells and ultimately destroy the organism.

As long as the neoplastic cells remain clustered together in a single group, the tumour is said to be benign. Benign tumours usually resemble their tissue of origin and may still function indistinguishably from their normal counterparts. For example, benign skin tumour cells are likely to continue the production of skin pigments. Benign tumours are usually separated from the neighbouring normal tissue by a connective tissue capsule and do not invade its surroundings (Franks, 1997). During this stage, a complete cure is usually attainable by surgical removal of the tumour. However, once such a tumour features more severe cellular abnormalities and becomes invasive, it is considered to be malignant and the patient is only then said to have cancer. Such invasiveness implies that the tumour cells break away from the primary tumour and enter the bloodstream or lymphatic system to form secondary tumours elsewhere in the

body, a process known as metastasis (Alberts *et al.*, 2002). Malignant tumours are not encapsulated like benign tumours. The standard cellular criteria for the diagnosis of a malignant tumour include a local increase in cell number, loss of the normal regular arrangement of cells, variation in cell shape and size, an increase in nuclear size and total DNA content, increased cell division, and abnormal chromosomes (Franks, 1997). By the time it is detected, a typical tumour usually contains about a billion cells or more, often including normal cells such as fibroblasts in the supporting tissue (Alberts *et al.*, 2002).

Genes such as *fos, raf, ras,* and *myc* that promote cell division under normal circumstances are known as protooncogenes. Because malignant cells have acquired the ability to divide seemingly limitlessly, mutations in these protooncogenes are often implicated, resulting in their permanent activation. The mutant form of a protooncogene is known as an oncogene. Oncogenic DNA viruses (such as the adenoviruses and Epstein-Barr virus) as well as a number of retroviruses (i.e. viruses with RNA genomes such as hepatitis C virus) also act by inserting viral oncogenes into the host genome, which can lead to the transformation of somatic cells, resulting in neoplasms. Those genes that normally function to suppress cell division such as *p53* and *retinoblastoma* (*Rb*) are known as tumour suppressor genes and are also often mutated in cancer, rendering them inactive (Nigro *et al.*, 1989; Teich, 1997; Klug and Cummings, 2003). A single mutation in an oncogene is usually sufficient to induce cancer, whereas two mutations in a tumour suppressor gene are generally required (the so-called “two-hit” model of tumourigenesis) (Knudson, 1971; Teich, 1997). According to this model, tumour suppressor genes become tumourigenic only once both alleles have been inactivated by separate mutations or chromosomal deletions (Knudson, 1971; Pannett and Thakker, 2001) Also, the gain of function associated with an oncogene is dominant, whilst the loss of function associated with a mutated tumour suppressor gene is recessive (Teich, 1997).

2.1.3. Colorectal cancer

2.1.3.1. Introduction

Colorectal cancer (cancer originating in either the colon or rectum) is the third most prevalent cancer worldwide after lung and breast cancer (World Cancer Report, 2003). More than 90% of all cases of colorectal cancer are diagnosed in men and women over the age of 50 according to the Centres for Disease Control and Prevention (CDC) in the USA (Colorectal Cancer, 2006). There has been little change in death rates over the last 50 years because the improvement in survival rates has been masked by the increase in incidence (Macdonald *et al.*, 2004). Death rates in the world's major industrialized countries are shown in Table 2.2.

Table 2.2 Age-adjusted death rates for colorectal cancer in the major industrialised countries of the world (Macdonald *et al.*, 2004).

Country	Age-adjusted death rates per 100 000 population	
	Male	Female
Germany	21,7	17,0
United Kingdom	18,7	13,8
France	18,3	12,1
Canada	16,4	11,6
United States of America	15,9	12,0
Japan	17,6	11,0
Russian Federation	17,5	12,7

Regular screening of the colon is crucial for the early detection of benign tissue masses known as polyps that can be removed before they become cancerous. However, screening rates are low and less than 40% of colorectal cancer cases are detected early. Colonoscopy is considered to be the gold standard for colon cancer screening, and the American Cancer Society recommends that everyone should be screened regularly after the age of 50 to detect polyps

(Harding, 2006). Polyps and colorectal cancer do not always cause symptoms. Nevertheless, symptoms do sometimes appear, such as bloody stools, unexplained stomach aches or cramps, and unexplained weight loss (Colorectal Cancer, 2006).

2.1.3.2. Aetiology of colorectal cancer

A small proportion of cases of colorectal cancer are due to inheritance of certain mutations, although the majority of cases of colorectal cancer are sporadic (i.e. due to mutations arising in somatic cells and not due to inherited mutations) (Key *et al.*, 1997; Macdonald *et al.*, 2004). A host of genes that are implicated in the genesis and progression of colorectal carcinomas have already been identified, including *MYC, ras, akt, p53, APC, Smad* and others. Approximately 85% of colorectal tumours have some sort of chromosomal instability, whereas the remaining 15% usually exhibit microsatellite instability or MSI (Macdonald *et al.*, 2004). Microsatellites (or short tandem repeats; STRs) are sequences of repeated nucleotide motifs, between 2 and 9 base-pairs in length, that are inserted into the normal DNA sequence. Histological observations have led researchers to believe that most colorectal carcinomas are preceded by precancerous epithelial polyps or adenomas, and do not necessarily develop directly from normal epithelium, although such colorectal tumours also exist.

The progression of colon carcinogenesis from benign adenomas or polyps is known as the adenoma-carcinoma sequence (Morson, 1974). As time progressed, some oncogenes and tumour suppressor genes involved were assigned to the various steps of the adenoma-carcinoma sequence in a proposal for the progression of colorectal tumourigenesis (Fearon and Vogelstein, 1990). Although it seems from this model as though mutations occur in a precise order, it is the accumulation of mutations which is more important than the order. *p53* was one of the first genes to be studied intensively in colorectal carcinogenesis, although it is mutated late in the progression from adenoma to carcinoma. Additional lesions, the so-called

aberrant crypt foci (ACF) are believed by some researchers to be the intermediate between normal tissue and early adenoma (Macdonald *et al.*, 2004). ACF were first described in 1987 and often contain the *KRAS* or *APC* mutations (Pretlow and Pretlow, 2005). *KRAS* and *APC* mutations are thus the "gatekeepers" or initiators for colorectal carcinogenesis.

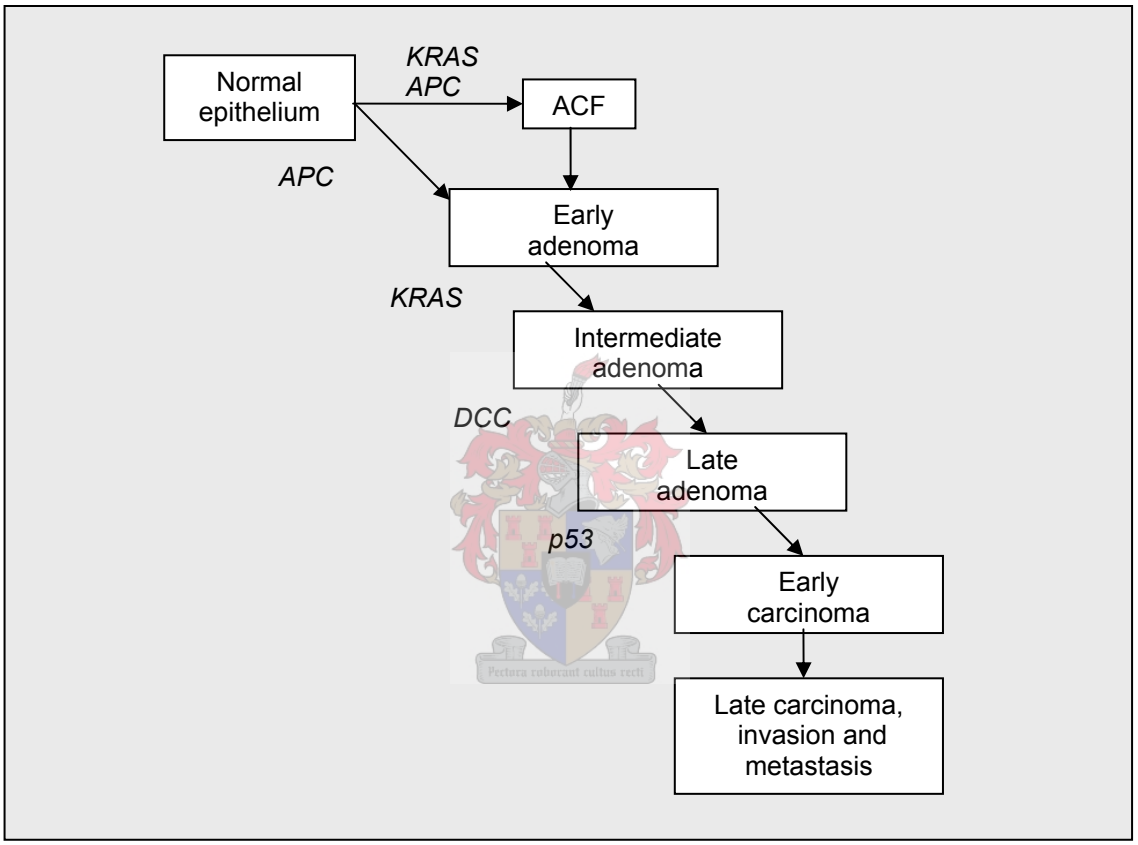

Figure 2.1 The paradigm for colorectal carcinogenesis together with the relevant genetic markers (Macdonald *et al.*, 2004; Pretlow and Pretlow, 2005). *Abbreviations:* ACF: aberrant crypt foci; APC: adenomatous polyposis coli; DCC: deleted in colorectal carcinoma; KRAS: Kirsten rat sarcoma.

Other factors involved in colorectal carcinogenesis include the loss of control over transforming growth factor (TGF) β as well as deregulation of the cell cycle. More than 75% of cases of colorectal cancer show a loss of responsiveness to TGFβ, which inactivates the TGFβ signalling cascade. This

could be due to either mutation of the *TGFβRII* gene or of the receptor. Mutations in genes involved in cell cycle control such as *p16* and *p27* have also been identified in a minority of colorectal cancer cases (Macdonald *et al.*, 2004).

In 10-15% of the cases of colorectal cancer, a hereditary component is at work. Although the underlying mechanisms are not fully understood yet, they have been uncovered for two distinct conditions, these being hereditary nonpolyposis colon cancer (HNPCC) and familial adenomatous polyposis (FAP). FAP accounts for approximately 1-2% of all cases of colorectal cancer and is characterized by the occurrence of hundreds to thousands of benign polyps throughout the colon and rectum. The polyps usually appear in early adulthood. Without timely intervention by the third or fourth decade of life, the probability that a FAP patient will develop adenocarcinoma, is close to 100% (Rose and Connolly, 1999; Macdonald *et al.*, 2004).

Interest in the role of nutrition in the aetiology of colorectal cancer stems largely from the wide variation in disease rates between populations with different diets. This led to the hypothesis that the contents of the colon and rectum could affect the risk of cancer. It was originally suggested that starch and dietary fibre may protect against colorectal cancer, whilst meat or animal fat could increase the risk. Results from analytical studies are in general consistent with these hypotheses that meat and animal fat are high-risk foods for colorectal cancer whereas fibre-rich foods are protective (Key *et al.*, 1997).

In addition to the effects of diet, evidence also suggests that the risk for colorectal cancer is reduced by physical activity as well as long-term use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs). This effect of aspirin and NSAIDs probably involves inhibition of prostaglandin synthesis which would inhibit tumour growth and spread (Key *et al.*, 1997).

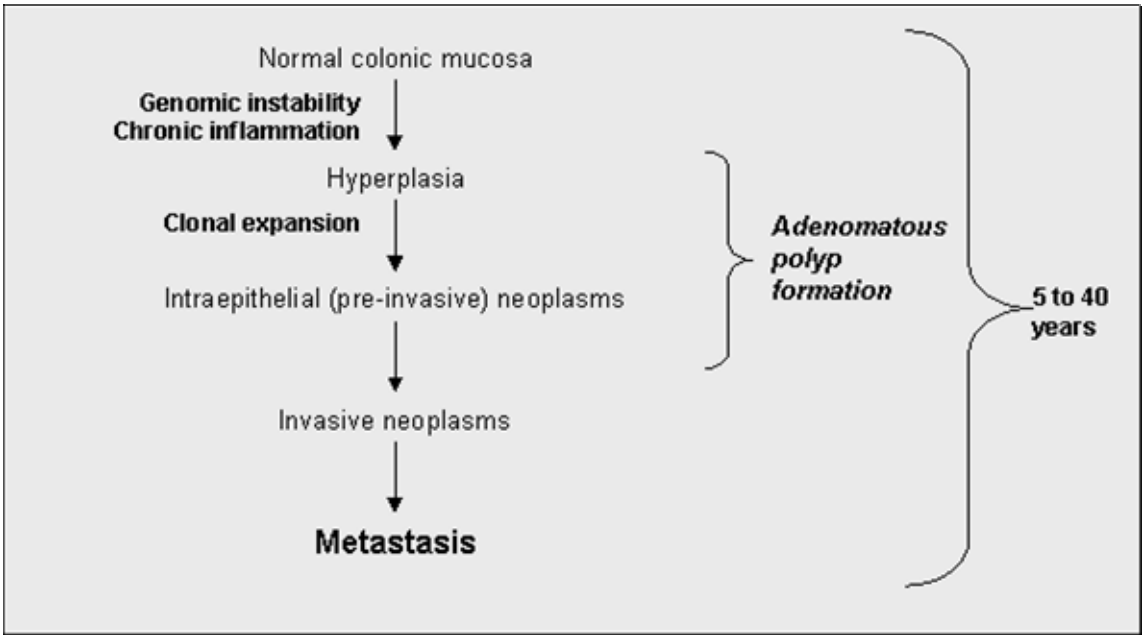

Figure 2.2 The stages of initiation and progression of colorectal cancer (Roynette *et al.*, 2004).

2.2. FATTY ACIDS AND CARCINOGENESIS

2.2.1. Introduction

The notion that fatty acid intake may influence the incidence of tumourigenesis and cancer, has been studied intensively *in vivo* and *in vitro* for more than 30 years and seems to be widely accepted. Ranging from epidemiological studies to those conducted using cell culture models, most studies provide evidence that n-3 fatty acids, especially the long-chain PUFAs eicosapentaenoic acid (EPA) and DHA, found abundantly in fish oil, are able to inhibit the development of cancer or slow down tumour growth (Hardman, 2004; Larsson *et al.*, 2004). However, the cellular mechanisms responsible for these effects have not yet been clarified, although various hypotheses exist, including modifications to eicosanoid metabolism and activation of different cellular signalling pathways (Kinsella *et al.*, 1990; Hwang and Rhee, 1999).

2.2.2. Characteristics and nomenclature of fatty acids

Lipids are molecules with a strong tendency to associate with each other through non-covalent interactions. These non-covalent interactions are responsible for the characteristic behaviour of lipid molecules in an aqueous environment, causing them to clump together. The interaction of lipid molecules with water has vast biological importance in the formation of membranes and micelles. There are two stabilizing mechanisms at work: the hydrophobic interactions between the non-polar tails as well as Van der Waals interactions between hydrocarbon regions. The head groups, on the other hand, are polar and hydrophilic and tend to associate with water (Mathews and van Holde, 1990). The general structure of lipids is shown in figure 2.3.

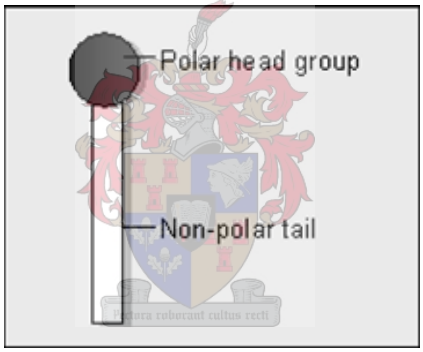

Figure 2.3 The general structure of a lipid molecule (Voet and Voet, 1995).

The simplest lipids are carboxylic acids known as fatty acids. The structure of fatty acids also reflects the general structure of all lipids: a hydrophilic carboxylate group is attached to a chain of hydrocarbon groups (Mathews and van Holde, 1990). Naturally occurring fatty acids usually consist of an even number of carbon atoms (Voet and Voet, 1995).

Fatty acids are weak organic acids, with pK_a values averaging at approximately 4.5. At physiological pH, fatty acids thus exist in an anionic form:

$$RCOOH \Leftrightarrow RCOO^- + H^+$$

When ionized, solubility in water is promoted, due to the charged carboxyl group which is extremely hydrophilic (Mathews and van Holde, 1990). In figure 3 the structure of stearate, the ionized form of stearic acid, is shown.

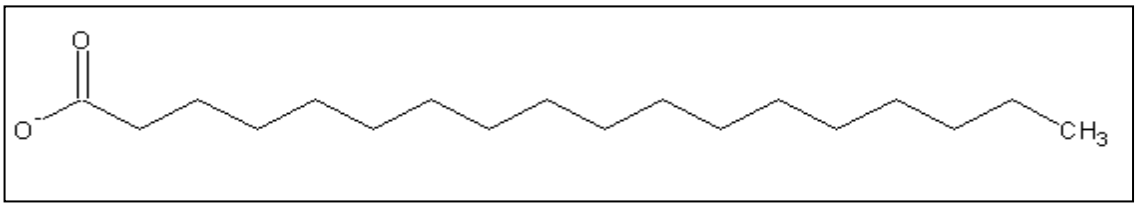

Figure 2.4 The structure of a single stearate ion (Voet and Voet, 1995).

Stearic acid is an example of a saturated fatty acid (SFA). The carbons of the hydrocarbon tail of SFAs are all coupled to hydrogen atoms, and there are no double bonds in such tails. SFAs form straight chains due to their lack of double bonds, and can thus be packed very tightly together. Other important SFAs include lauric acid and palmitic acid. SFAs with chain lengths of 4 and 6 are known as short chain fatty acids (SCFAs), whereas SFAs with chain lengths of 8, 10, and 12 are medium chain fatty acids (MCFAs). Long chain fatty acids (LCFAs) consist of more than 12 hydrocarbons (Mathews and van Holde, 1990; Fatty acid, 2006). A list of important SFAs is given in table 2.3.

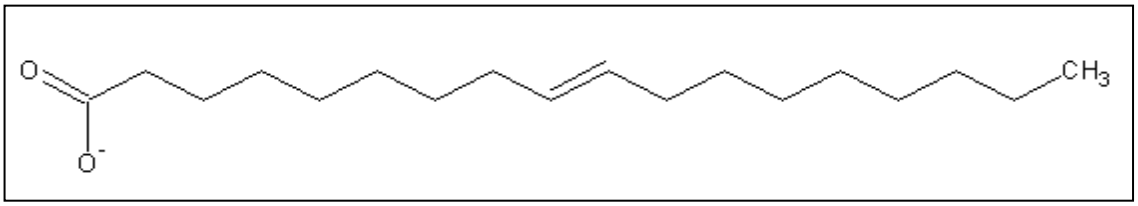

Figure 2.5 The structure of a single oleate ion (Voet and Voet, 1995).

Many important fatty acids are however unsaturated (see oleate ion in figure 2.5). In these fatty acids, the hydrocarbon tails contain one or more double bonds and are thus not “saturated” with hydrogen atoms. Fatty acids with only a single double bond are known as monounsaturated fatty acids (MUFAs), whereas those with two or more double bonds are so-called polyunsaturated

fatty acids (PUFAs). Important MUFAs and PUFAs are also given in table 2.3.

Table 2.3 A summary of some biologically important fatty acids (as adapted from Mathews and van Holde, 1990; Bartsch et al., 1999; Larsson et al., 2004). *Abbreviations:* MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Common Name	IUPAC Name	Numerical Abbreviation	Structural Formula	Source
SFAs				
Lauric Acid	*n*-Dodecanoic Acid	12:0	$CH_3(CH_2)_{10}COOH$	Coconut oil
Mystiric Acid	*n*-Tetradecanoic Acid	14:0	$CH_3(CH_2)_{12}COOH$	Palm kernel oil
Palmitic Acid	*n*-Hexadecanoic Acid	16:0	$CH_3(CH_2)_{14}COOH$	Beef fat, palm oil
Stearic Acid	*n*-Octadecanoic Acid	18:0	$CH_3(CH_2)_{16}COOH$	Beef fat, cocoa butter
MUFAs				
Palmitoleic Acid	*cis*-9-Hexadecenoic Acid	16:1 n-7	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	Macadamia nut oil
Oleic Acid	*cis*-9-Octadecenoic Acid	18:1 n-9	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	Olive oil, canola oil
PUFAs				
Linoleic Acid	*cis*-9,*cis*-12-Octadecadienoic Acid	18:2 n-6	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	Sunflower oil, soybean oil
α-Linolenic Acid	*all-cis*-9,12,15-Octadecatrienoic acid	18:3 n-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	Canola oil, walnut oil
γ-Linolenic Acid	*all-cis*-6,9,12-Octadecatrienoic acid	18:3 n-6	$CH_3(CH_2)_4(CH=CHCH_2)_3(CH_2)_3COOH$	Borage oil, Evening Primrose oil
Arachidonic Acid	*all-cis*-5,8,11,14-Eicosatetraenoic acid	20:4 n-6	$CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2COOH$	Pork fat, peanut oil
Docosahexaenoic Acid	*all-cis*-Docosa-4,7,10,13,16,19-hexaenoic acid	22:6 n-3	$CH_3CH_2(CH=CHCH2)_6CH_2COOH$	Fish oil
Eicosapentaenoic Acid	*all-cis*-5,8,11,14,17-Eicosapentaenoic acid	20:5 n-3	$CH_3CH_2(CH=CHCH_2)_5(CH_2)_2COOH$	Fish oil

The numerical abbreviations of fatty acids allow for insight into their chemical structures. Such an abbreviation designates the length of the hydrocarbon chain, the number of double bonds, as well as the position of the double bond closest to the methyl end of the molecule (Roynette *et al.*, 2004). The carbon atom in this methyl group is known as the "omega" (ω) carbon, whereas the second carbon from the carboxyl group is referred to as the α carbon (Rose and Connolly, 1999). n-3 and n-6 PUFAs are therefore also sometimes referred to as ω-3 and ω-6, respectively.

Another system in use to assign abbreviations to fatty acids, involves the indication of the positions of all double bonds (as from the carboxyl end) as a superscript together with a Greek letter delta (Δ). According to this nomenclature system, oleic acid would thus be abbreviated as $18{:}1^{\Delta 9}$ and arachidonic acid as $20{:}4^{\Delta 5,8,11,14}$ (Mathews and van Holde, 1990). This method allows for easy identification of the IUPAC name along with the chemical structure, although it is not widely used.

In most naturally occurring fatty acids, all double bonds are *cis*, which means that the hydrogen atoms bound to double-bonded carbons are on the same side of the chain. In the *cis* configuration, the hydrogen atoms would repel each other, causing the hydrocarbon chain to bend. The more *cis* double bonds in a chain, the more bent it appears (Voet and Voet, 1995).

Fatty acids with a *trans* configuration (so-called trans fatty acids; TFAs) do not usually occur naturally. Such MUFAs and PUFAs are produced during industrial processes to harden edible oils to stable products for storage and transportation convenience and are most often found in fast food, confectionaries, and hard margarines (Bartsch *et al.*, 1999; Stender and Dyerberg, 2004). Although many believe that an increased intake of TFAs is a health hazard and could increase the risk of developing cancer, allergies, type 2

diabetes and other diseases (Stender and Dyerberg, 2004), evidence to support this is ambiguous and TFAs will therefore not be discussed further.

2.2.3. Fatty acid metabolism

2.2.3.1. Metabolic synthesis and conversion of polyunsaturated fatty acids

Most n-6 and n-3 PUFAs are synthesized from the metabolic precursor fatty acids, linoleic acid (LA; 18:2 n-6) and α-linolenic acid (ALA; 18:3 n-3), respectively. Unlike plants, mammals do not possess the particular enzymes necessary for the synthesis of LA and ALA. These fatty acids are therefore considered to be essential fatty acids, because they have to be consumed in the diet in order to maintain an adequate pool. Vegetable seeds and oils, such as soybean, coconut, and sunflower oil, contain high proportions of LA, whereas dark green leafy vegetables as well as linseed, canola, walnut, and blackcurrant seed oils are good sources of ALA (Bartsch *et al.*, 1999; Roynette *et al.*, 2004).

In order to yield more n-6 unsaturated fatty acids from LA, it is first desaturated to γ-linolenic acid (GLA; 18:3 n-6) by the enzyme Δ6 desaturase. Thereafter, the molecule is elongated by 2 carbon atoms to dihomo-γ-linolenic acid (DGLA; 20:3 n-6). DGLA is subsequently desaturated by the action of Δ5 desaturase to yield arachidonic acid (AA; 20:4 n-6) (Whelan and McEntee, 2004).

The metabolism of the precursor ALA to produce more n-3 PUFAs is fairly similar to the process just described and involves the same enzymes. The enzyme Δ6 desaturase firstly catalyzes the conversion of ALA to stearidonic acid (SDA; 18:4 n-3), which is subsequently elongated and desaturated to EPA (20:5 n-3). Following a series of steps including elongation, desaturation and β-oxidation, DHA (22:6 n-3) is produced (Whelan and McEntee, 2004).

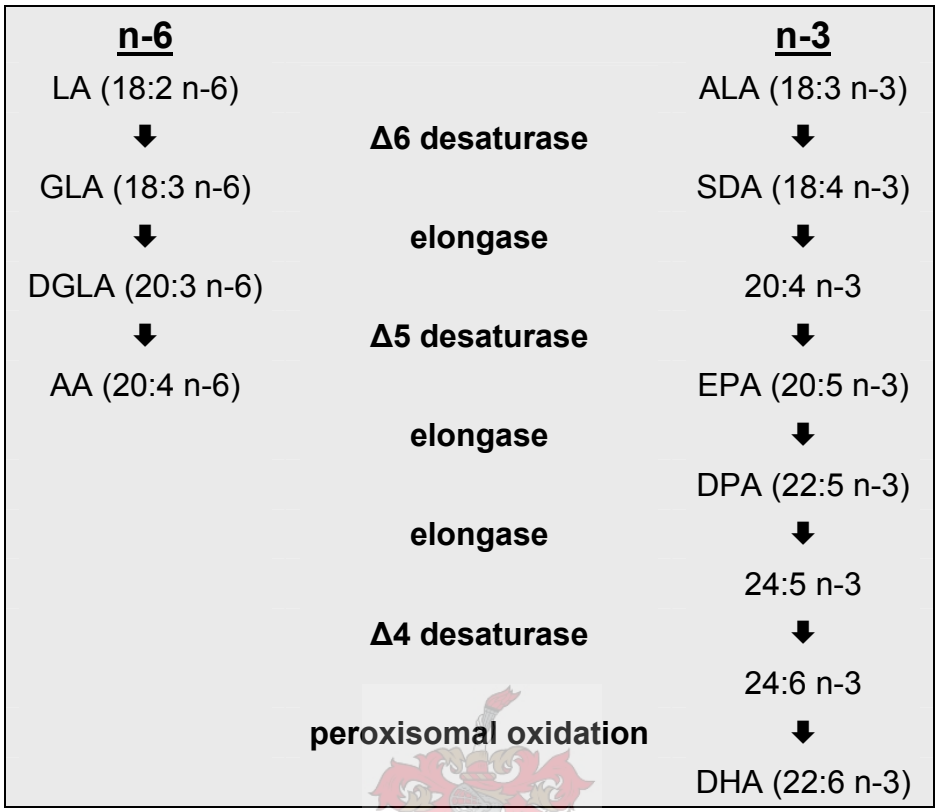

Figure 2.6 Metabolism of linoleic acid and α-linolenic acid to yield arachidonic acid and docosahexaenoic acid, respectively (Rose and Connolly, 1999; Whelan and McEntee, 2004). *Abbreviations:* AA, arachidonic acid; ALA, α-linolenic acid; DGLA; dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid; SDA, stearidonic acid.

There exists competition between n-6 and n-3 PUFAs as substrates for the desaturase and elongase enzymes that are common to both metabolic pathways (Rose and Connolly, 1999). The enzymes have a greater affinity for n-3 PUFAs, which implicates the preferential metabolism of those fatty acids, given that the dietary intake of n-3 PUFAs is high. This leads to a "competitive inhibition" of n-6 PUFA metabolism (Roynette *et al.*, 2004).

2.2.3.2. Eicosanoid metabolism

n-6 and n-3 PUFAs have crucial functions in the body, especially as

phospholipids that comprise biological membranes. They also modulate membrane fluidity (an increased number of PUFAs in the membrane increases fluidity), cell signalling pathways, and cellular interaction. However, their most vital role is arguably immune regulation, because certain PUFAs, both n-6 and n-3, are metabolic precursors for the synthesis of eicosanoids (Roynette *et al.*, 2004).

Eicosanoids are biologically potent, short-lived, hormone-like lipids with chain lengths of 20 carbon atoms. These molecules are crucial in the modulation of immune responses, especially inflammation, platelet aggregation, cellular growth, and differentiation. The fatty acids that are the precursors for eicosanoid synthesis are DGLA, AA, and EPA. Before conversion of these fatty acids can take place, the fatty acids need to be liberated from phospholipids in biological membranes by the so-called phospholipase enzymes. Then, the fatty acids are converted by cyclooxygenase (COX) or lipoxygenase (LOX) to different types of eicosanoids. COX gives rise to prostaglandins (PGs) and thromboxanes (TXs), collectively known as prostanoids. There are two known isoforms of the COX enzyme: COX-1, which is constitutively expressed in most tissues and considered to generate PGs for tissue homeostasis, and COX-2, an inducible enzyme which is up regulated in response to inflammatory cytokines, growth factors and tumour promoters (Larsson *et al.*, 2004; Sinicrope and Gill, 2004). The eicosanoids produced by the LOX enzyme, which also has different isoforms, are known as leukotrienes, hydroxy fatty acids, and lipoxins (Larsson *et al.*, 2004).

2.2.3.2.a) Arachidonic acid-derived eicosanoids

Because AA is the major PUFA in cell membranes, most eicosanoids are AA derivatives. These eicosanoids comprise the 2-series prostanoids and 4-series leukotrienes. As the numerical prefixes denote the number of double bonds, these molecules thus have two and four double bonds, respectively.

COX firstly converts AA to PGG_2 (the subscript also indicates the number of double bonds present), which is subsequently reduced to PGH_2 by the peroxidase activity of COX. PGH_2 is thereafter the precursor for various prostanoids: PGD_2, PGE_2, PGF_2, TXA_2, and PGI_2. The PGs have a wide range of biological functions, although specific cells are usually highly selective regarding the prostanoids formed following the production of PGH_2. For example, vascular endothelial cells produce mainly PGI_2, which inhibits platelet activation and aggregation (Rose and Connolly, 1999).

LOX inserts molecular oxygen into AA to produce 5-, 12-, or 15-hydroperoxyeicosatetraenoic acid (HPETE), depending on the corresponding LOX-isoform responsible for the conversion (i.e. 5-, 12-, or 15-LOX). HPETE is thereafter reduced to the corresponding hydroxyeicosatetraenoic acid (HETE). The HETEs' functions include immune responses, ion transport, and hormone secretion. However, 5-HPETE is also converted to leukotriene A_4 (LTA_4), which undergoes further reactions to produce LTE_4 and LTB_4. Leukotrienes are believed to be involved in the pathogenesis of asthma, cystic fibrosis, and pulmonary hypertension (Rose and Connolly, 1999; Larsson *et al.*, 2004).

Generally, AA-derived eicosanoids are pro-inflammatory, although PGE_2 has been suggested to have anti-inflammatory properties. AA-derived eicosanoids have also been positively linked to carcinogenesis (Larsson *et al.*, 2004).

2.2.3.2.b) Eicosapentaenoic acid-derived eicosanoids

EPA-derived eicosanoids are the 3-series prostanoids and 5-series leukotrienes, thus having 3 and 5 double bonds in their structure, respectively. The production of these EPA-derived molecules involves many of the same enzymes involved in AA-derived eicosanoid metabolism.

COX converts EPA to PGH_3, which is then further metabolized to PGE_3, PGI_3, and TXA_3. PGI_3 and TXA_3 are thereafter converted to the inactive metabolites

Δ^{17}-6-keto-$PGF_{1\alpha}$ and TXB_3, respectively (Rose and Connolly, 1999).
When the 5-LOX enzyme acts upon EPA, LTA_5 is produced. This is thereafter metabolized to LTB_5. However, LTB_5 has only 5-10% of the activity of its AA-derived counterpart, LTB_4. All EPA-derived eicosanoids are considered to be anti-inflammatory (Rose and Connolly, 1999).

EPA competes with AA as the substrate of both the COX and LOX enzymes. As with the desaturases and elongases, EPA is once again the preferred substrate. Thus, increased dietary intake of EPA leads to decreased generation of AA-derived eicosanoids as well as an elevation in the generation of EPA-derived mediators (Calder and Grimble, 2002; Roynette *et al.*, 2004). Following supplementation with ALA, EPA or DHA, phospholipid-AA concentrations are significantly decreased, which has implications for eicosanoid production, since n-3 PUFAs would be incorporated into membrane phospholipids at the expense of AA (Calder and Grimble, 2002). This effectively decreases the availability of AA as eicosanoid precursors. Supplementation with those fatty acids also leads to the inhibition of LA desaturation and subsequently decreased AA concentrations (Roynette *et al.*, 2004). Thereby the production of EPA-derived eicosanoids is favoured at the expense of AA-derived eicosanoids (Larsson *et al.*, 2004).

Furthermore, n-3 PUFAs also enhance the breakdown of eicosanoids. The generation of AA-derived eicosanoids is not inhibited only by n-3 PUFAs, but also by the eicosanoids derived from them, and some of these eicosanoids have an even stronger inhibitory effect than that of EPA (Larsson *et al.*, 2004).

2.2.4. The anti-tumourigenic and anti-carcinogenic effects of n-3 polyunsaturated fatty acids

2.2.4.1. The n-6/n-3 imbalance

Omega-3 (n-3) fatty acids are known to be protective against a host of diseases

such as cardiovascular disease (CVD), inflammation, neurodegenerative diseases and especially cancer (Akbar *et al.*, 2005; Kang, 2005). However, the typical Western diet consumed today seems to be deficient in these important PUFAs, resulting in an increased risk for modern diseases such as those mentioned previously. In addition to the apparent n-3 PUFA deficiency, the Western diet has elevated n-6 PUFA content, especially LA and AA (Rose and Connolly, 1999; Kang, 2005). It is believed that the foods available to our ancestors (before agricultural practices and animal domestication were taken on) were rich in n-3 PUFAs and contained them in a ratio with n-6 PUFAs of approximately 1:1. Such a fatty acid profile in food led the human body to establish a genetic pattern without genes that would enable it to synthesize fatty acids or convert them to another form. It seems that the n-6:n-3 PUFA ratio has increased over time, as the Western diet today contains a ratio of 15–20:1. Unfortunately, the human body cannot adjust its genome to suit such a lipid profile in such a short time, making modern man susceptible to modern, devastating disorders. It is thus necessary to supplement our diets to enrich tissues with n-3 fatty acids and correct for the n-6/n-3 imbalance. Lower organisms such as plants, microorganisms, and the roundworm *C. elegans* are able to convert n-6 to n-3 PUFAs, and certain genes responsible for this conversion (such as *fat-1*) have already been successfully cloned and introduced into mammalian cells and animal models (Simopoulos, 1991; Bartsch *et al.*, 1999; Kang, 2005).

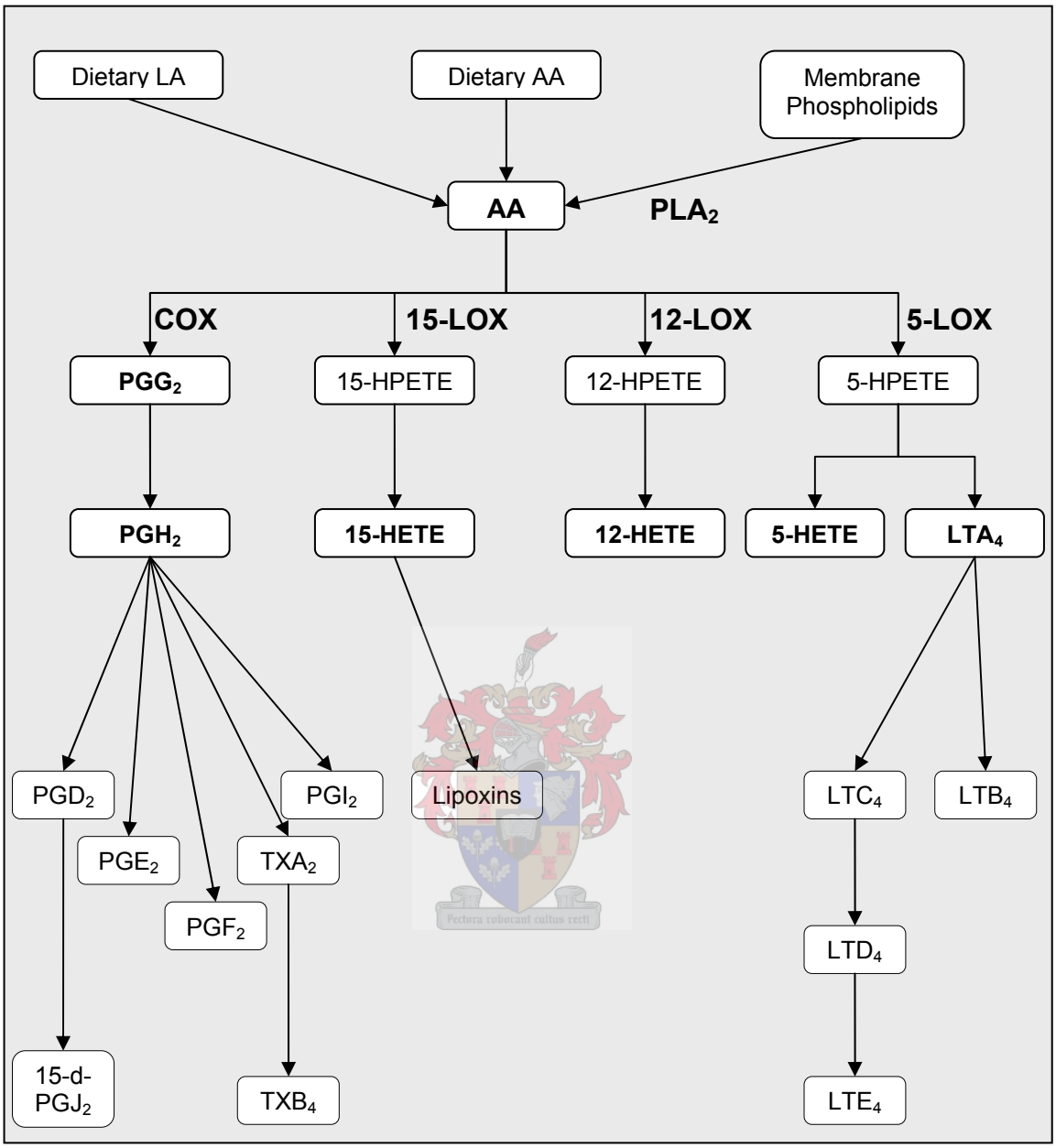

Figure 2.7 An overview of the metabolism of arachidonic acid-derived eicosanoids (Larsson *et al.*, 2004). *Abbreviations*: LA, linoleic acid; AA, arachidonic acid; PLA_2, phospholipase A_2; COX, cyclooxygenase; LOX, lipoxygenase; PG, prostaglandin; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosa-tetraenoic acid; LT, leukotrienes; TX, thromboxane.

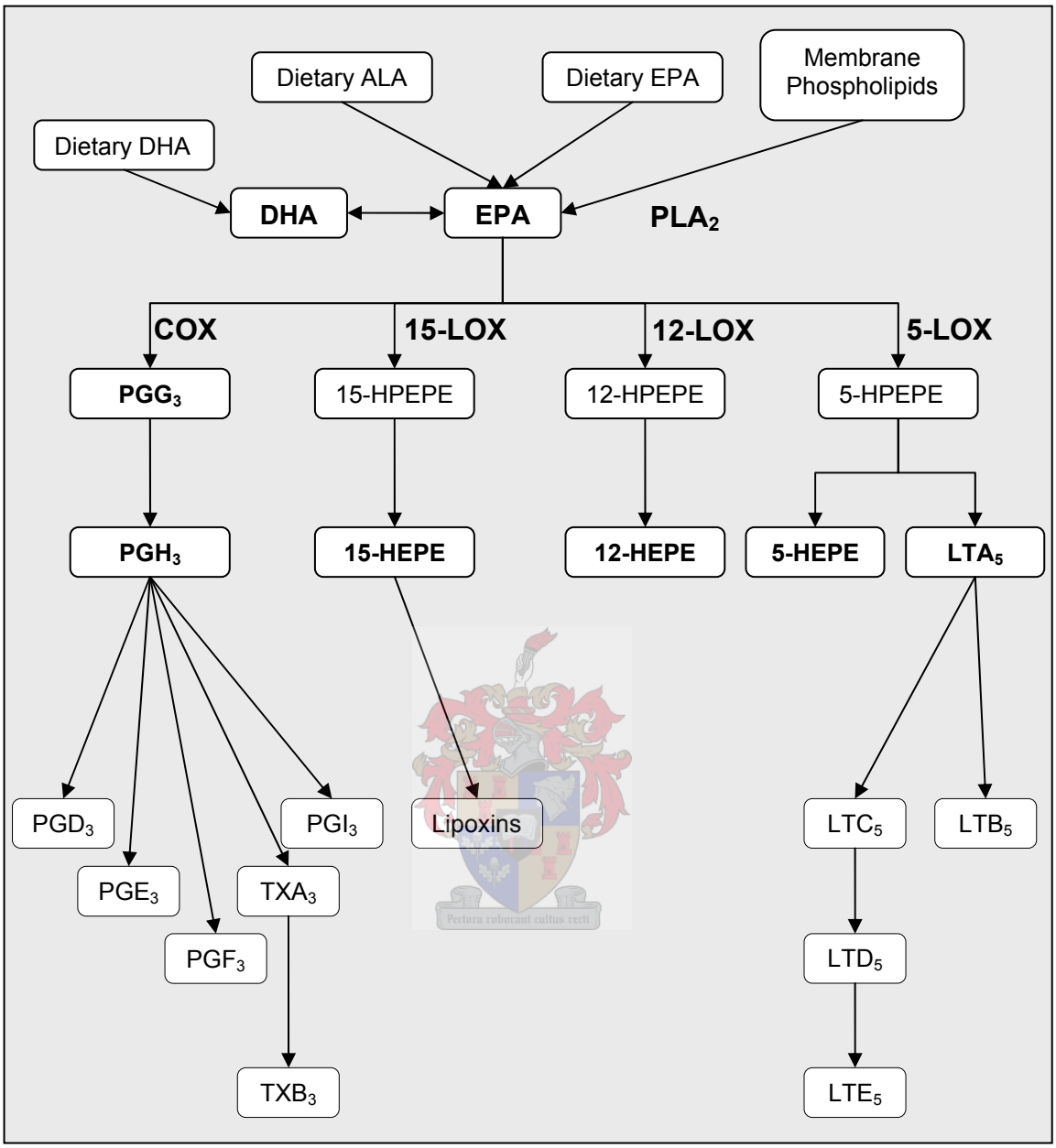

Figure 2.8 An overview of the metabolism of eicosapentaenoic acid-derived eicosanoids (Larsson *et al.*, 2004). Abbreviations: DHA, docosahexaenoic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; PLA2, phospholipase A2; COX, cyclooxygenase; LOX, lipoxygenase; PG, prostaglandin; HPEPE, hydro-peroxyeicosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; LT, leukotrienes; TX, thromboxane.

2.2.4.2. The role of fatty acids as constituents of membrane phospholipids in normal and tumour cells

The fatty acids contained in membrane phospholipids are essential in determining certain membrane physical properties such as fluidity and flexibility. They also regulate cellular functions, including the movement of ions and metabolic products across the membrane, receptor binding and eicosanoid production (Spector and Burns, 1987). Mammalian cells *in vitro* readily take up lipids such as fatty acids from their culture medium (Spector and Yorek, 1985), especially the serum component. Supplementation of media with different fatty acids is thus a simple method to manipulate the fatty acid composition of membrane phospholipids in cultured cells.

Although the cells are then often cultured in serum-free medium to abolish interference from serum lipids (Nano *et al.*, 2003), extensive changes in the phospholipid composition can also be achieved in the presence of serum. The type of serum is also of importance. For example, foetal calf (a.k.a. bovine) serum (FCS or FBS) contains approximately 65% less PUFAs than horse serum. Modification of membrane phospholipids can also be achieved by supplementation of media with intact phospholipid vesicles, sphingolipids, or even cholesterol (Spector and Yorek, 1985).

When membrane phospholipid composition is modified, so are various cellular processes and responses. These include carrier-mediated transport, activities of membrane-bound enzymes, binding properties of membrane receptors, cytotoxicity, growth, modulation of cellular signalling events, and eicosanoid synthesis, amongst others (Spector and Yorek, 1985; Spector and Burns, 1987; Kang, 2005).

2.2.4.3. In vitro studies on the effects of fatty acids on cancer cell lines

The research group of Begin and colleagues performed some of the earliest

studies showing that PUFAs kill human tumour cells *in vitro*. These tumour cells were derived from breast, lung, and prostate carcinomas. Their studies also investigated the effect of various fatty acids on co-cultures of tumour cells and normal fibroblasts or other normal cells. In these co-culture systems, supplementation with EPA, GLA, or AA led to the selective death of malignant cells. Although the normal cells were not killed and outgrew the malignant cells, their rate of division was lowered. In contrast with PUFAs, SFAs and MUFAs did not have any cytotoxic effects on malignant cells (Begin *et al.*, 1985; Begin *et al.*, 1986; Das *et al.*, 1987).

The validity of these studies was later often questioned because the "normal" cell lines used were either human fibroblasts or derived from different tissues and different species. The possibility of tissue and species specific effects were therefore not taken into consideration when it was concluded that PUFAs exert their cytotoxic effects only on tumour cells (Diggle, 2002). Nevertheless, the cancer-specificity of PUFAs' cytotoxicity was shown in 1998 when GLA was tested in astrocytoma cells (Vartak *et al.*, 1998). Although GLA killed the astrocytoma cells, "normal" astrocytes were protected. Both cell lines were also of murine origin, thus concurrently eliminating possible species specific as well tissue specific effects.

As these studies found that both n-3 (such as EPA) and n-6 (such as AA) PUFAs killed cancer cells, the researchers speculated that the number of double bonds was the determining factor of the cytotoxic potential of particular fatty acids. They also suggested that those PUFAs with three, four, or five double bonds were most effective, whereas DHA (which contains six double bonds) was least effective (Begin and Ells, 1987; Begin *et al.*, 1988). Today, however, it is believed that the class of the PUFA, i.e. whether it is n-3 or n-6, as well as chain length are the determining factors of cytotoxicity, rather than the number of double bonds. This could explain the dramatic effects observed

in most *in vitro* studies with the use of DHA throughout the years of research in this field (Rose and Connolly, 1991; Sagar *et al.*, 1992; Siddiqui *et al.*, 2001; Nano *et al.*, 2003).

It has already been speculated in early studies that the cytotoxic effects of certain fatty acids such as EPA might be, at least partially, due to enhanced free radical generation (Das *et al.*, 1987a; Das *et al.*, 1987b). In fact, the addition of vitamin E to cancer cell cultures reduced the efficacy of PUFAs (Begin *et al.*, 1988; Falconer *et al.*, 1994; Chen and Istfan, 2000; Nano *et al.*, 2003). It therefore seems quite likely that oxidative stress is a major role-player in PUFA-induced cytotoxicity in tumour cells. Still, the question whether a similar mechanism is at work in normal cells and why they tend to be unharmed by it, remains unanswered.

Although these early studies uncovered the cytotoxic abilities of PUFAs *in vitro* and aimed to explore the effects of using different concentrations on a variety of cell lines, a much-needed shift occurred in the late nineties. The objective of research was thereafter to uncover the underlying mechanisms responsible for the PUFAs' effects. Numerous malignant cell lines were supplemented with different fatty acids and efforts made to attribute the findings to cellular events such as apoptosis, cellular signalling, alterations in eicosanoid production, increased oxidative stress, cell cycle arrest, and changes in membrane phospholipid composition (Conklin, 2002; Hardman, 2002). However, despite all these attempts, an understanding of the precise cellular events modulated by exogenous fatty acid treatment is still elusive. It is likely that it is not an isolated mechanism at work, but a collection of processes including those mentioned earlier and particularly signalling pathways.

2.2.4.4. Experimental evidence from animal models

Although *in vitro* models are arguably the most useful to gain insight into the selective cytotoxicity of certain fatty acids and their underlying mechanisms,

animal models are better suited for the study of cancer incidence and progression and how it could be influenced by fatty acid intake. Animal models have been described for a host of cancer subtypes, including breast, colon, and prostate cancer. The studies have already shown convincingly that n-3 PUFAs are effective agents to inhibit or suppress tumour growth, whereas n-6 PUFAs stimulate the growth of tumours. The *in vitro* studies, on the other hand, deliver more conflicting results, as it is often found that n-6 PUFAs are equal to n-3 in their ability to kill tumour cells. In general, though, the n-6 PUFAs are associated with the enhancement of tumourigenesis, whereas long-chain n-3 PUFAs such as DHA have profound inhibitory effects (Rose, 1997; Bartsch *et al.*, 1999).

There exist numerous protocols to induce cancer in experimental animals, ranging from exposure to known carcinogens to genetic engineering. The use of nude mice injected with cancer cells to induce malignancies is arguably the most popular way to produce models for cancer, because they cannot reject any grafts.

A recent study from the laboratories of Hardman and colleagues found that a diet rich in n-3 PUFAs could reduce the growth rate of breast tumours, as well as tumour angiogenesis (Hardman *et al.*, 2005). In this study, nude mice received mammary injections of MDA-MB-231 cells, a human breast cancer cell line. Following tumour establishment, half the mice were fed a diet high in n-3 PUFAs and half were fed an n-6 PUFA-rich diet. When the rate of tumour growth was analyzed, the n-3 diet effectively decreased tumour growth and also suppressed tumour angiogenesis. The n-6 diet, on the other hand, dramatically increased the tumour growth rate. In fact, the effect of the n-3 diet on tumour growth rate was equal to that produced with gamma irradiation.

In a much earlier study, also done in nude mice receiving breast cancer cell

grafts, the researchers found that DHA and EPA also effectively suppress the metastases of tumour cells in addition to reducing tumour size (Rose and Connolly, 1993; Rose *et al.*, 1995). It was also found that an EPA supplemented diet seemed superior to a DHA supplemented diet in limiting metastasis at a low concentration, whereas no differences occurred between the diets when containing higher concentrations of either fatty acid (Rose *et al.*, 1995).

n-3 PUFAs are also able to increase the efficiency of chemotherapeutic drugs. In a study done on nude mice bearing MDA-MB-231 tumour cell grafts, tumour growth was more effectively decreased in mice receiving fish oil supplementation together with doxorubicin chemotherapeutic treatment than in those treated with doxorubicin alone (Hardman *et al.*, 2001). The amount of fish oil fed to the experimental animals was equivalent to the amount fit for human consumption without intolerable gastrointestinal side-effects (Burns *et al.*, 1999).

The effect of fatty acid supplementation on the progression of colon cancer in rats is often studied with the use of azoxymethane, a potent carcinogen that induces colon tumours. When F344 rats were treated with azoxymethane and fed different diets, tumour incidence was dramatically reduced in those animals fed a fish oil supplemented diet rich in EPA and DHA. This diet also reduced tumour multiplicity, i.e. the number of tumours formed per rat. Both incidence and multiplicity of tumours were increased in rats fed a corn oil diet (Reddy and Sugie, 1988), reaffirming the tumour enhancing effects of the n-6 series PUFAs. In a more recent study it was confirmed that a diet rich in n-6 PUFAs led to higher incidence and multiplicity of colonic tumours in azoxymethane-treated F344 rats (Rao *et al.*, 2001).

The metastasis of colonic tumour cells to secondary sites is also inhibited by n-

3 PUFAs. This was shown in F344 rats bearing colon tumours from ACL-15 cell grafts. In rats receiving EPA treatment, metastasis of the cancer cells to the liver was inhibited, compared with a group receiving LA treatment (Iwamoto *et al.*, 1998).

However, the question is not only whether n-3 PUFAs are able to reduce the appearance or metastasis of malignant tumours, but also whether it could reduce the risk for tumourigenesis to start with. This would be denoted by a reduced incidence of those biomarkers known to show an increased risk, such as the appearance of aberrant crypt foci (ACF) in the colon as a definite sign of high risk to develop colon cancer. In fact, a fish oil rich diet was indeed effective in reducing the appearance of ACF in both the proximal and distal colon regions of azoxymethane-treated F344 and Sprague-Dawley rats, respectively (Takahashi *et al.*, 1997; Coleman *et al.*, 2002). Importantly, when the fish oil diet was combined with the ingestion of α-cellulose, the ACF-reducing effect of the fish oil was augmented (Coleman *et al.*, 2002). This amplification effect could be ascribed to the beneficial effect of a high fibre diet on a reduced risk of colon cancer (Howe *et al.*, 1992).

ACF formation and growth was also successfully reduced by treatment with DHA alone. When DHA were administered intragastrically during as well as after treatment with a specific carcinogen, the number of ACF formed as well as the mean number of aberrant crypts per focus, were significantly reduced (Takahashi *et al.*, 1993; Takahashi *et al.*, 1997).

From the evidence given it thus seems that, in animal models, an EPA or DHA rich diet is able to reduce the formation of tumours, as well as retard their growth. The efficacy of n-3 PUFAs in this regard can be investigated by transferring animals to experimental diets either before or during carcinogen treatment or following tumour induction. However, the majority of animal

studies on this subject involve the assignment of animals to experimental diets before the onset of carcinogen treatment. Nevertheless, in 1991 Reddy *et al.* published their findings on the effect of n-3 and n-6 rich diets during the initiation (two weeks before first treatment with carcinogen) and post-initiation (three days after final treatment) stages of colon tumour induction. It was found that an n-3 rich diet either during initiation or post-initiation stages significantly reduced tumour incidence as well as multiplicity, whereas the n-6 rich diet during post-initiation significantly elevated tumour incidence and multiplicity. The n-6 diet had no effect during the initiation phase (Reddy *et al.*, 1991). However, this study failed to show that n-3 PUFAs are able to reduce the size of established tumours.

In the study by Hardman *et al.* in 2005 (referred to in an earlier paragraph) animals were indeed assigned to different experimental diets five weeks after final treatment with MDA-MB-231 cells. Tumour growth was monitored to ensure that the breast tumours were in fact established before the effects of either an n-3 or n-6 rich diet were investigated. The study then showed that the n-3 rich diet significantly reduced mammary tumour growth rate and size, whereas the n-6 rich diet accelerated tumour growth and caused increased tumour size (Hardman *et al.*, 2005). Although this study convincingly showed the effects of fatty acids on already established tumours, more such studies are required, because the scenario of either a change in diet or n-3 PUFA supplementation following the diagnosis of cancer would be much more relevant.

2.2.4.5. Epidemiological evidence for link between diet and cancer incidence

Epidemiological studies have demonstrated a lower incidence of certain malignancies amongst certain populations. This phenomenon was most notably described in the Eskimo population of Greenland (Blot *et al.*, 1975; Lanier *et al.*, 1976; Lanier *et al.*, 1980; Lanier *et al.*, 1982). The typical

Greenland Eskimo diet is high in lipids and contains predominantly large quantities of seal and fish and is rich in PUFAs, especially EPA and ALA (Bang *et al.*, 1976; Bang *et al.*, 1980). When the plasma lipids of native Greenland Eskimos were analyzed and compared to that of Danes and of Eskimos living in Denmark, the native Eskimos had higher plasma lipid concentrations of LA of the n-6 series, and ALA, EPA, and DHA of the n-3 series (Dyerberg *et al.*, 1975). During this time, much attention was given to the proposal that such a plasma lipid profile was favourable towards a decreased incidence of atherosclerosis and ischaemic heart disease (IHD) (Dyerberg *et al.*, 1975; Dyerberg and Bang, 1979). However, other research groups were focusing on the decreased cancer risk among Eskimos and other Alaskan natives, such as the Aleuts. These cancers included those of the lung, larynx, bladder, prostate, breast, and other sites (Lanier *et al.*, 1976). Yet as time passed the cancer profile of these populations changed markedly to resemble that of the American Caucasian population (Lanier *et al.*, 1982; Lanier *et al.*, 2001), which might be due to the adaptation of a Western lifestyle or the fact that the average age of the Alaskan population is increasing. Nevertheless, those early studies of the dietary habits of the Greenland inhabitants and the once rarity of cancer amongst them, led to the first suggestions of possible anti-carcinogenic effects of PUFAs.

A related phenomenon was also seen amongst the population of the Mediterranean countries such as Greece, where olive oil is an important part of the diet (Berrino and Muti, 1989; Kushi *et al.*, 1995; Trichopoulou and Lagiou, 1997; Trichopoulou *et al.*, 2000). This oil is essentially composed of oleic acid (OA), a long chain MUFA. However, olive oil also contains high levels of antioxidants (Visioli *et al.*, 1998; Wahle *et al.*, 2004) which may be accountable for its beneficial effects, rather than the OA content.

2.2.4.6. Clinical trials on n-3 fatty acid supplementation

Since fatty acids are safe for human consumption and appropriate dosages are

known, it can be concluded from animal studies that n-3 supplementation would be a beneficial addition to conventional cancer therapy, although more clinical trials need to be carried out.

Some clinical trials to investigate the effect of PUFA supplementation have been done on cancer patients and subjects with a high cancer risk. In one of these, high doses of GLA were administered in the form of evening primrose oil to cancer patients. The GLA successfully prolonged the lives of patients suffering from liver, breast, brain and oesophageal cancers without any side-effects (Van der Merwe *et al.*, 1987; van der Merwe *et al.*, 1990).

Nonetheless, despite evidence from various models supporting the anti-tumourigenic effect of PUFAs, few studies have investigated the molecular mechanisms responsible for these effects. This is surprising, as it is well established that fatty acids can modulate the activities of various enzymes involved in signal transduction, such as protein kinase C (PKC) and phospholipases (Sumida *et al.*, 1993; Murray *et al.*, 2002) as well as ion channels (Chow and Jondal, 1990; Ordway *et al.*, 1991).

2.3. SIGNAL TRANSDUCTION AND CANCER

2.3.1. Introduction

Signal transduction concerns the mechanisms whereby external influences determine cellular activities and enable the exchange of information between the cell membrane and the cytosol. Such information exchange mechanisms are crucial in higher organisms to regulate and coordinate cellular processes such as division and differentiation, and conveying sensory information such as stress, vision etc. (Krauss, 2003). Since the deregulation of these intracellular signalling pathways is often implicated in the development of cancer and other diseases, an understanding of the signalling mechanisms involved in normal cellular activities such as proliferation and differentiation is important for greater

insight into the abnormal growth of tumour cells.

Specialised mechanisms to relay external information into the cell are crucial since the plasma membrane presents a barrier to the extracellular environment to alter cellular response directly. This is because most “signals” (or so-called first messengers), such as growth factors and many hormones, are hydrophilic and will therefore not pass through the phospholipid bilayer of the plasma membrane. In contrast, steroid hormones, eicosanoids, etc. are hydrophobic and will cross membranes to interact with intracellular receptors. To overcome the problem, hydrophilic substances bind to receptors located on the surfaces of their target cells. A mechanism by which first messengers can direct and control intracellular physiological changes thus evolved together with the appearance of cell-surface receptors. (Gomperts *et al.*, 2003).

2.3.2. Activation of cell-surface receptors

The activation of receptors on the cell surface can elicit a variety of cellular responses including proliferation, migration, differentiation, changes in metabolism, and apoptosis (Schlessinger and Ullrich, 1992), depending on the nature of the extracellular ligand and the receptor. In order to bring forth physiological changes within a cell, the activated receptor firstly interacts with another protein such as a tyrosine kinase, a GTP-binding protein (G-protein), or an ion channel, depending on the nature of the receptor.

2.3.2.1. Tyrosine kinase receptors

Many receptors, such as those for platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), have an intrinsic tyrosine kinase activity. Upon ligand interaction, these so-called tyrosine kinase receptors (TKRs) cannot function as monomers to initiate signalling pathways and therefore the receptors will form either dimers or oligomers (Hwang and Rhee, 1999; Gomperts *et al.*, 2003). Following dimerization (or oligomerisation), TKRs will autophosphorylate different tyrosine amino acid residues firstly in their

cytoplasmic domains and thereafter in their kinase domains, leading to activation. The activated TKRs will then also phosphorylate tyrosine residues on other transducing proteins (Carpenter, 1987). The autophosphorylated tyrosine residues serve as docking sites for proteins with *Src* homology 2 (SH2) domains, which are highly conserved regions present in many transducing and adaptor molecules (Hwang and Rhee, 1999). Following the recruitment of certain molecules, a cascade of protein interaction and activation by means of phosphorylation (or other types of modification) ensues, which conveys the information to the nucleus in order to elicit a physiological response such as gene expression (see figure 2.9).

2.3.2.2. G-protein coupled receptors

G-protein coupled receptors (GPCRs) represent the largest group of transmembrane receptors and interact with ligands such as adrenaline, noradrenaline, luteinising hormone (LH), and bradykinin. The GPCR typically consists of seven transmembrane helices in close proximity to a G-protein, hence the name of these receptors (Krauss, 2003). Activation of GPCRs is often followed by the rapid generation of so-called second messengers such as diacylglycerol (DAG), cyclic AMP (cAMP), calcium ions, nitric oxide (NO), and phosphoinositides (PIs) (Berridge, 1984; Berridge, 1988; Krauss, 2003) by specific effector enzymes. The second messengers subsequently activate a cascade of kinases, ultimately leading to different cellular responses.

2.3.3. Major cellular signalling pathways

For the purposes of this dissertation, the following pathways will be discussed: ERK1/2, p38 MAPK, Akt, and p53, as well as the mechanisms of apoptosis.

Both ERK (extracellular regulated kinase)1/2 and p38 are members of the mitogen-activated protein kinase (MAPK) family, together with JNK (c-jun NH2-terminal kinase; also known as stress-activated protein kinase or SAPK), ERK3, ERK4, ERK5, NLK (nemo-like kinase) and other lesser known kinases (Pearson

et al., 2001). The MAPKs are closely related Ser/Thr kinases activated by a cascade downstream of both TKRs (by e.g. insulin) and GPCRs (by e.g. angiotensin II) (Lopez-Ilasaca, 1998; Pearson *et al.*, 2001).

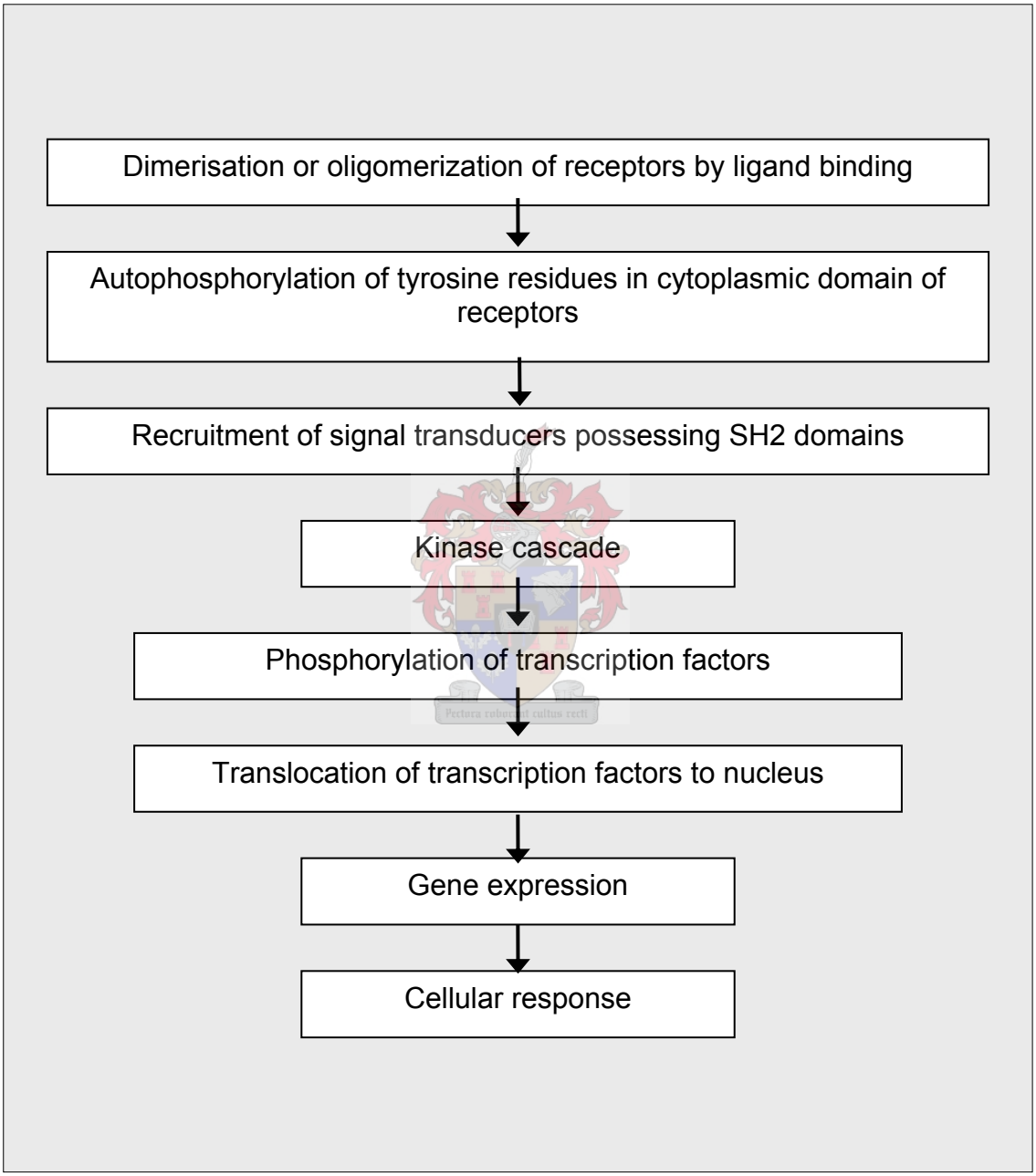

Figure 2.9 General steps in receptor-mediated signal transduction pathways via tyrosine kinase receptors (Hwang and Rhee, 1999). *Abbreviations:* SH2: *Src* homology 2.

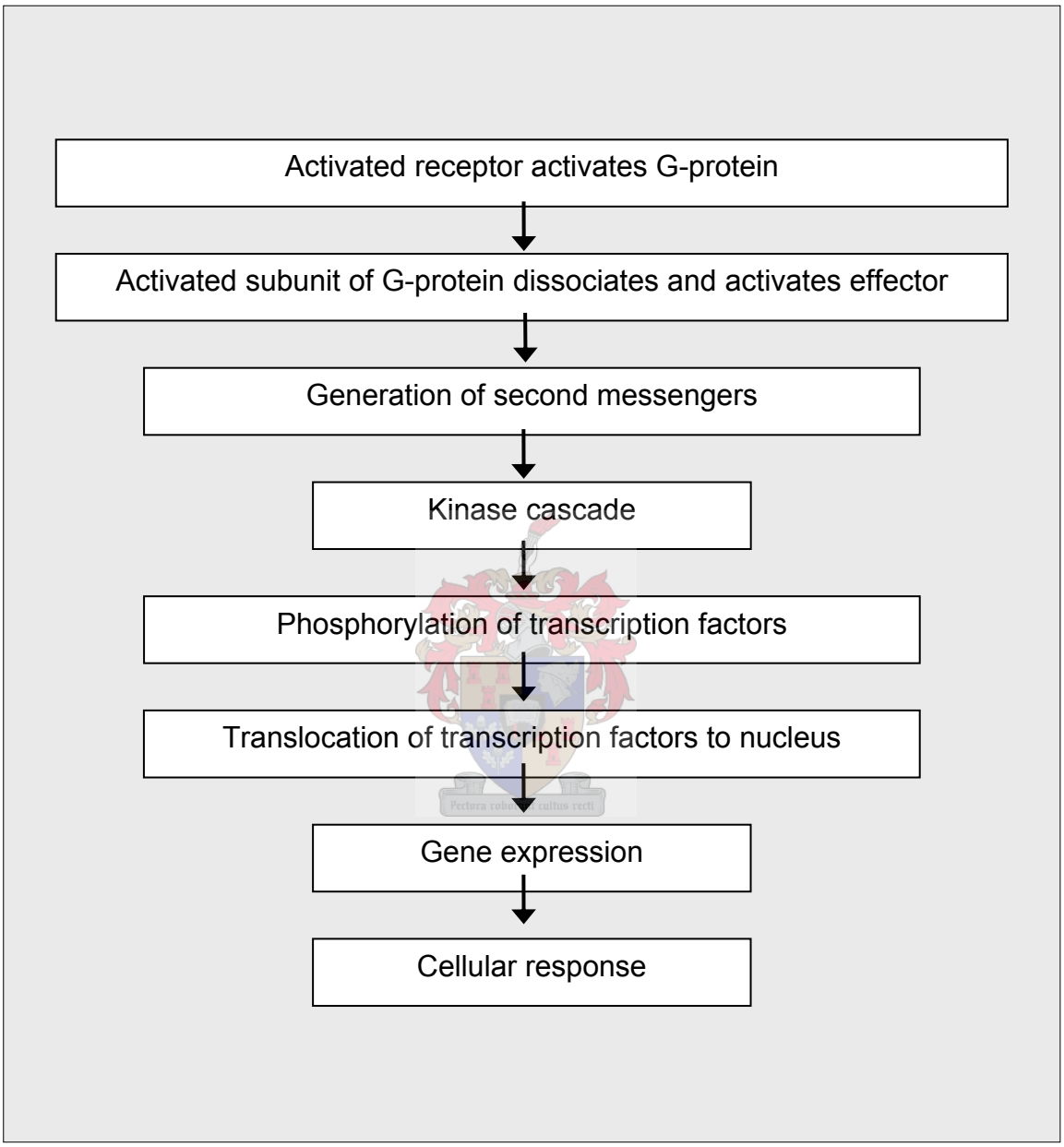

Figure 2.10 General steps in receptor-mediated signal transduction via G-protein coupled receptors.

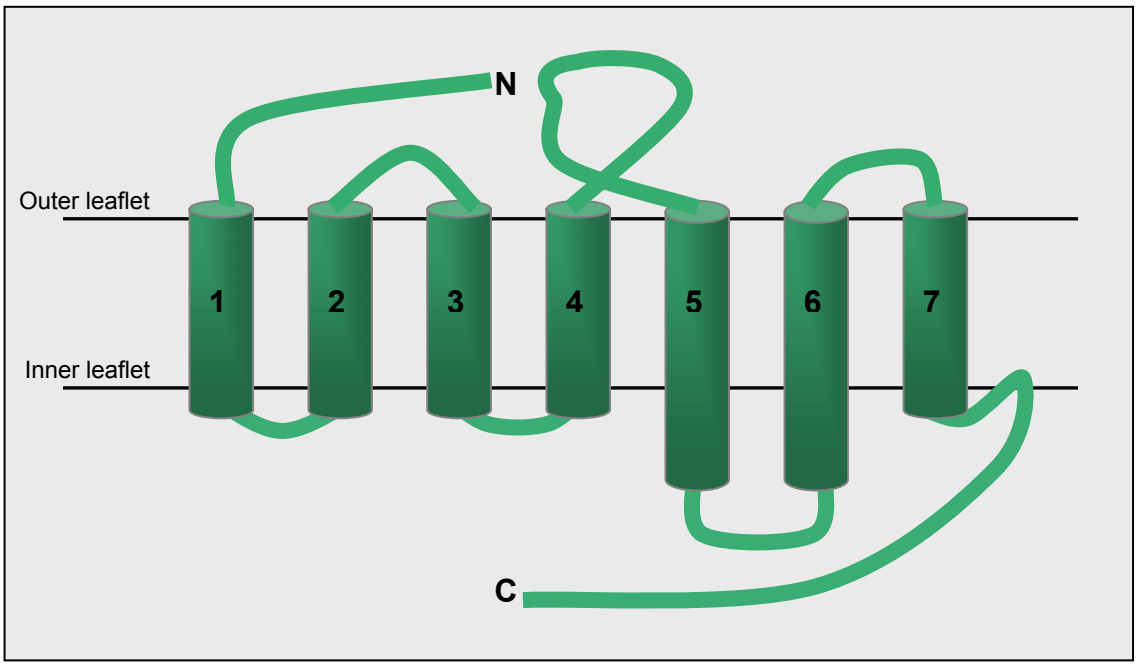

Figure 2.11 Structure of a transmembrane G-protein coupled receptor (Krauss, 2003)

Akt, also known as protein kinase B (PKB), is also a Ser/Thr kinase. It is activated downstream of both TKRs and GPCRs via a specific enzyme known as phosphoinositide 3-kinase (PI3K) (Krauss, 2003; Scheid and Woodgett, 2003). The Akt pathway is involved in cellular processes including proliferation, differentiation, and metabolism, and is fundamental to cellular survival by means of the suppression of apoptosis (Nicholson and Anderson, 2002).

p53 was mentioned earlier when it was stated that the *p53* gene is often mutated in cancer cells. In fact, more than 50% of human tumours bear a mutated form of p53 (Nigro *et al.*, 1989). This was the first indication that p53 must serve critical functions within cells to ensure normal control of division. Overall, p53 serves to protect the cell from the potentially damaging consequences of various stresses, e.g. hypoxia and irradiation, which is why it is a normal mechanism of defence against tumour induction (Krauss, 2003).

Apoptosis (a.k.a. programmed cell death) is a type of cell death which has been defined both morphologically and biochemically. The morphological features of this process have been well described and can be observed in fixed and stained tissues, or in cultured cells. With regard to its biochemical characteristics, numerous apoptosis-related genes and their products have been uncovered (Kiechle and Zhang, 2002). Although apoptosis is essential for normal physiological functioning, it is also deeply involved in the pathogenesis of many diseases, such as cancer and cardiovascular diseases (Cho and Choi, 2002).

2.3.3.1. ERK 1/2

MAPK pathways are organized in cascades consisting of three core signalling modules which transmit signals by sequential phosphorylation events (Kyriakis and Avruch, 2001; Pearson *et al.*, 2001). Incoming signals (such as the activation of a receptor by a particular ligand) are received by an initial upstream protein kinase. From there, the signal is subsequently transmitted to another kinase which in turn activates the terminal protein kinase(s). In the ERK pathway, ERK1 and ERK2 (also known as p44 and p42 MAPK, respectively) are the terminal kinases (Pearson *et al.*, 2001; Krauss, 2003).

ERK1 and ERK2 are approximately 85% homologous, with even greater homology in their core regions involved in substrate binding (Pearson *et al.*, 2001). Because ERK1 and ERK2 are believed to have overlapping, and perhaps redundant, signalling capabilities, they are frequently simply referred to as ERK1/2 (Cobb and Goldsmith, 2000). ERK1 and ERK2 are both ubiquitously expressed, although their relative abundance varies across different tissue types (Pearson *et al.*, 2001). Although they were historically believed to be essentially interchangeable due to their seemingly overlapping functions, knock-out studies have proven this to be incorrect. Whereas ERK1 deficient mice are viable with only minor defects in T cell maturation, ERK2 deficient mice die early in development, proving that ERK1 cannot compensate

for ERK2 during embryogenesis (Saba-El-Leil *et al.*, 2003; Pages and Pouyssegur, 2004).

The kinase immediately upstream of ERK1/2 in the cascade is a member of the MEK (MAP/ERK kinase, also known as MAPK-kinase or MAPKK) family of kinases. These are dual specificity protein kinases able to phosphorylate their substrates on tyrosine as well as serine or threonine residues. MEK phosphorylates ERK on a TEY motif (threonine and tyrosine residues separated by a single glutamate residue) in the activation loop of subdomain VIII of the kinase domain (Payne *et al.*, 1991; Kyriakis and Avruch, 2001), with the tyrosine residue being phosphorylated first (Ferrell and Bhatt, 1997). In fact, phosphorylation of threonine takes place only after the accumulation of a certain quantity of tyrosine-phosphorylated ERK1/2 protein, known as the accumulation threshold. Once it is reached, the threonine residues are phosphorylated and ERK molecules are subsequently activated. It is generally believed that this mechanism of activation of ERK (and the other MAPKs) serves to enhance the cooperativity of MAPK activation and to allow modulation by other signalling events, in addition to amplification of the signal from MEK (Pearson *et al.*, 2001). Several members of the MEK family can also be phosphorylated by kinases in other pathways, enabling crosstalk (Mansour *et al.*, 1994).

However, the entry point of the ERK pathway is at a kinase which precedes MEK. MAP3K (also known as MEK kinase, MEKK, or MAPKKK) phosphorylates members of the MEK family at two specific serine residues in a conserved NSXANS motif (where X represents any amino acid) (Krauss, 2003) following the formation of MAP3K homo-oligomers (Kyriakis and Avruch, 2001). Representatives of the MAP3K family include Raf, Mos, and MEKK1-3. The proteins that recruit and phosphorylate MAP3K are most often small GTPases such as Ras or members of the Rho/Rac family, although other protein kinases

can also deliver the entry point signals. If another protein kinase is indeed responsible for the phosphorylation of MAP3K and the commencement of the kinase cascade, it is referred to as a MAP4K (thus a MAP3K kinase) (Hagemann and Blank, 2001; Krauss, 2003).

ERK was the first mammalian MAPK to be identified. This pathway is largely regulated by Ras, a monomeric GTPase, which recruits isoforms of Raf (the MAP3K). The Raf family of kinases is composed of the three isoforms: A-Raf, B-Raf, and the ubiquitously expressed Raf-1 (Kyriakis and Avruch, 2001; Pearson *et al.*, 2001). Raf activates MEK1 and MEK2 (MAPKKs) in turn, which activates ERK1 and ERK2 (Kyriakis and Avruch, 2001). At this time, it seems that Raf has no other substrates apart from MEK1 and MEK2, and that MEK1/2 have no other substrates than ERK1/2, which places Raf and MEK1/2 exclusively in the ERK pathway (Pearson *et al.*, 2001). Following activation, ERK delivers signals to a variety of substrates, both cytosolic and nuclear (Krauss, 2003). All MAPKs phosphorylate substrates on Ser and Thr residues which are followed by either a Pro or Gly residue.

Phosphorylation by MEK causes ERK2 to dimerise. This dimerization is brought on by a conformational change induced by phosphorylation that favours tight interactions between phosphorylated ERK2 proteins. It is believed that this dimerization could promote nuclear localization of ERK2, which occurs by means of active transport (Cobb and Goldsmith, 2000). Nuclear substrates include the transcription factor Elk-1, which is positively regulated by ERK. Elk-1 regulates the transcription of genes for proteins such as c-fos, which forms part of the transcription factor AP1 (activator protein 1) (Krauss, 2003). Other important substrates include:

- Cytosolic phospholipase A_2 ($Cpla_2$)
- p90 ribosomal S6 kinases (p90RSK) 1-3
- MAPK-interacting kinase (MNK) 1 and MNK2

- Mitogen- and stress-activated protein kinase (MSK)
- Steroid receptor coactivator-1 (SRC-1)
- c-Jun
- c-Fos
- Activating transcription factor 2 (ATF2)
- Signal transducer and activator of transcription 3 (STAT3) (Pearson *et al.*, 2001).

2.3.3.2. p38 MAPK

As with ERK1/2, the p38 MAPK proteins are terminal kinases in a cascade consisting of three signalling modules that act in a sequential fashion. However, stimuli associated with activation of the p38 pathway are mostly stressful, such as heat and osmotic shock, UV irradiation, proinflammatory cytokines e.g. tumour necrosis factor (TNF) α and ischaemia (Hagemann and Blank, 2001; Matsukawa *et al.*, 2004).

There are at least four known isoforms of p38: p38α, p38β, p38γ (a.k.a. ERK6 or SAPK3), and p38δ (a.k.a. SAPK4) (Pearson *et al.*, 2001). The α-isoform was the first to be purified and identified and is presently the best characterized isoform of p38 (Kyriakis and Avruch, 2001; Shi and Gaestel, 2002). It was discovered as the target of a pyridinyl-imidazole drug (known as SB203580) that inhibited TNFα transcription and was consequently named cytokine-suppressive anti-inflammatory drug-binding protein (CSBP) (Lee *et al.*, 1994; Cuenda *et al.*, 1995). p38α (CSBP) was also independently identified as a tyrosine phosphoprotein in cytokine-treated cell extracts (Han *et al.*, 1994). The other three isoforms were then identified by means of cloning strategies (Pearson *et al.*, 2001). Both p38α and also p38β are inhibited by the pyridinyl-imidazole type compounds, but p38γ and p38δ are apparently insensitive to them (Kumar *et al.*, 1997).

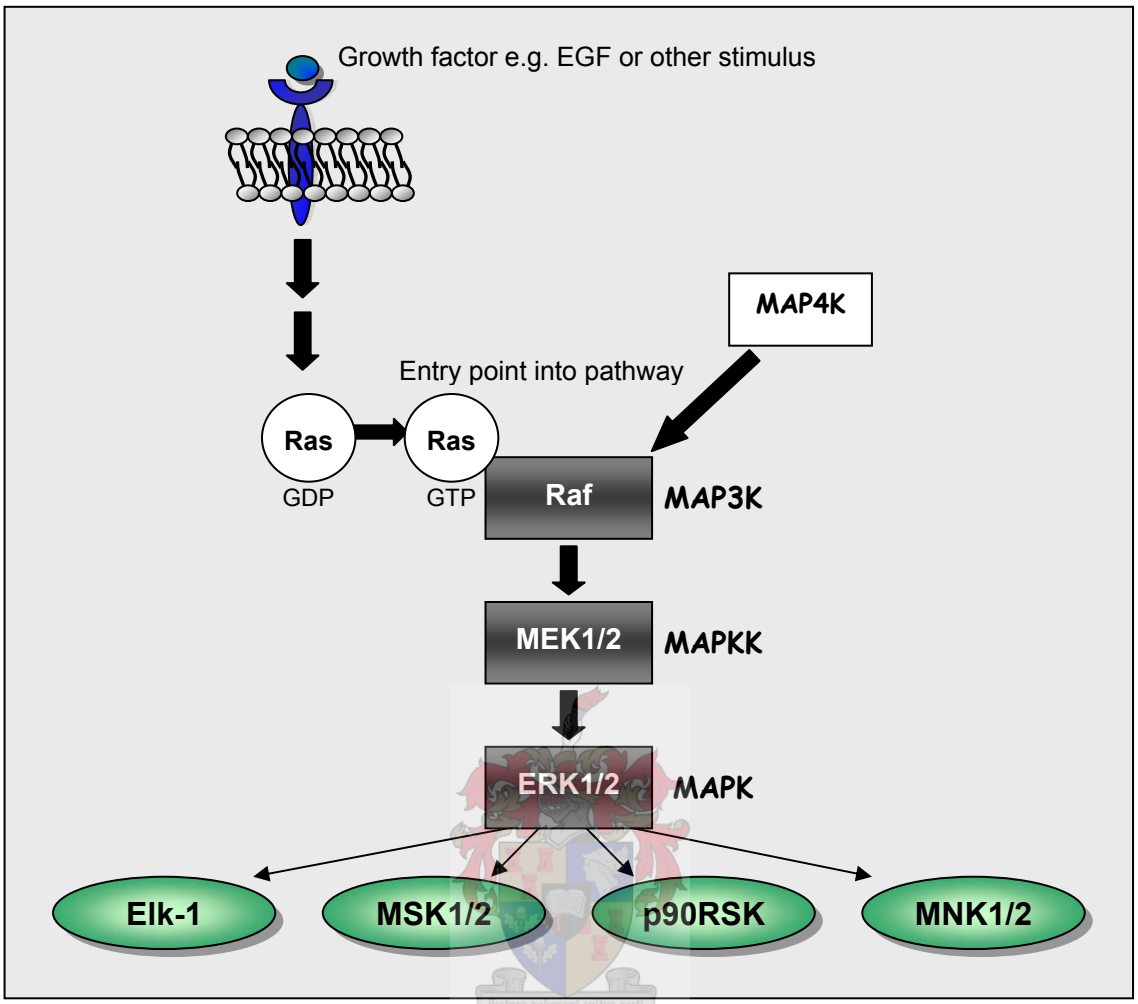

Figure 2.12 The ERK1/2 pathway and a number of substrates: Pathway entry, steps in the cascade, and examples of ERK substrates (modified from Pearson *et al.*, 2001 and Krauss, 2003). *Abbreviations:* EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAP3K, MAPKK kinase; MAP4K, MAP3K kinase; MEK, MAP/ERK kinase; MEKK, MEK kinase; ERK, extracellular regulated kinase; MSK, mitogen- and stress-activated protein kinase; p90RSK, 90 kDA-ribosomal S6 kinase; MNK, MAPK-interacting kinase.

Gene-targeting (knockout) studies have shown that mice deficient in p38α die during embryonic development (Allen *et al.*, 2000), due to defective angiogenesis of the placenta and decreased erythropoietin production (Kuida and Boucher, 2004). On the other hand, knockouts of the β- and γ-isoforms do not cause any obvious phenotype (Kuida and Boucher, 2004), suggesting dispensable functions of these isoforms.

In the p38 MAPK pathway (figure 2.13), the MAP3Ks at the top of the cascade are a complex and diverse group of proteins, with many members also signalling to JNK. They can be subdivided as follows: (1) MEKKs and (2) the thousand and one kinases (TAOs). The mammalian MEKKs capable of delivering signals to p38 are MEKK2-4 (though little is known about their regulation), TGFβ-activated kinase 1 (TAK1), apoptosis signal-regulating kinase 1 (ASK1), and tumour progression locus 2 (TPL2) (Kyriakis and Avruch, 2001). TAO1 and TAO2 of the TAO family are also distinctly specific activators of p38 (Hagemann and Blank, 2001; Kyriakis and Avruch, 2001; Pearson *et al.*, 2001).

Downstream of the MAP3Ks, a group of MKKs are activated by means of Ser/Thr phosphorylation. In the p38 MAPK pathway, these MKKs are MKK3 (a.k.a. MEK3, SAPK-kinase 2, or SKK2) and MKK6 (a.k.a. MEK6 and SKK3) , which are both highly selective for p38 (Derijard *et al.*, 1995; Han *et al.*, 1996; Hagemann and Blank, 2001). Both MKK3 and MKK6 activate p38 by means of dual phosphorylation within a TGY-motif in the activation loop of subdomain VIII of p38's kinase domain (Shi and Gaestel, 2002). Although MKK3 prefentially activates the α- and β-isoforms, MKK6 activates all known isoform equally strongly (Kyriakis and Avruch, 2001).

The downstream targets of p38 include a wide range of regulatory proteins including protein kinases, transcription factors, $cPLA_2$, and the Na^+/H^+ exchanger NHE1. These substrates act to fulfil the functions of the p38

pathway, which are currently known to be the regulation of gene transcription, posttranslational regulation of gene expression, cell cycle control (by means of p53), cell growth, differentiation, apoptosis, and cytoskeletal remodelling (Shi and Gaestel, 2002). Precisely which substrates are responsible for the different effects of p38 activation, has not yet been fully elucidated, although targeted deletions of different substrates have been employed to at least partially address this. A summary of known substrates and their roles in p38 signalling is given in table 4. The p38 pathway specifically regulates the expression of the "early genes" *c-fos*, *fosB*, *c-jun*, *junB*, and *junD* in response to stress stimuli (Shi and Gaestel, 2002). The expression of these genes is possibly mediated by means of the transcription factor Elk-1 (though results have been conflicting), which can also be regulated by ERK1/2 (Pearson *et al.*, 2001; Shi and Gaestel, 2002).

The inactivation of p38 is also of physiological relevance and is mediated by dephosphorylation via specific MAPK phosphatases (MKPs). The MKPs are dual specificity phosphatases acting on the super-family of MAPKs and are thus also involved in the inactivation of ERK, JNK, and other MAPKs. Among the family of MKPs, at least MKP1, 5, and 7 specifically dephosphorylate p38 MAPK (Pearson *et al.*, 2001; Shi and Gaestel, 2002).

2.3.3.3. Akt

Akt (a.k.a. protein kinase B or PKB) is a 57 kDa Ser/Thr kinase with similarities to both PKA and PKC (Coffer and Woodgett, 1991) and belongs to the "AGC" (for protein kinases A, G, and C) kinase super-family (Scheid and Woodgett, 2003). The Akt protein was discovered when two genes, *Akt1* and *Akt2*, were identified as the human homologues of the viral oncogene *v-akt* (Staal, 1987; Bellacosa *et al.*, 1991). Presently, there are three known isoforms of Akt: Akt1 (a.k.a. PKBα), Akt2 (PKBβ), and Akt3 (PKBγ). Although the isoforms are encoded by different genes and are expressed differentially (Akt2 and Akt3 are

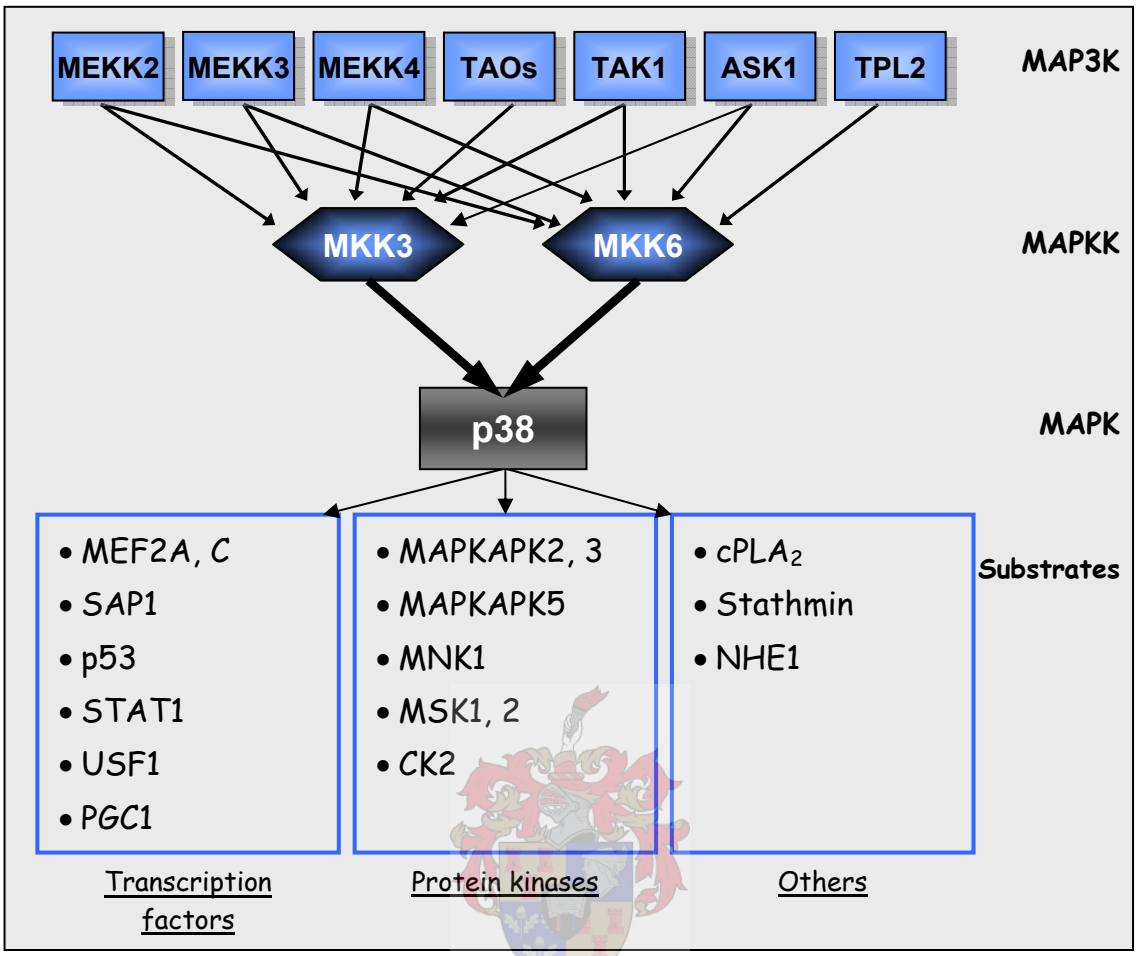

Figure 2.13 The p38 MAPK pathway and selected substrates (modified from Kyriakis and Avruch, 2001 and Shi and Gaestel, 2002). *Abbreviations*: ASK1, apoptosis signal-regulating kinase 1; CK2, casein kinase 2; $cPLA_2$, cytosolic phospholipase A_2; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAP3K, MAPKK kinase; MAPKAPK, MAPK-activated protein kinase; MEF2, myocyte enhancer factor 2; MEKK, MEK kinase; MKK, MAPK kinase; MNK, MAPK-interacting kinase; MSK, mitogen- and stress-activated protein kinase; NHE1, N^+/H^+ exchanger 1; PGC1, peroxisome proliferator-activated receptor (PPAR) co-activator 1; SAP1, stress-activated protein 1; TAK1, TGFβ-activated kinase 1; TAO, thousand and one kinase; TPL2, tumour progression locus 2; USF1, upstream transcription factor 1.

Table 2.4 Substrates of p38 MAPK and their roles, where known, in p38 MAPK related signalling (compiled from Shi and Gaestel, 2002 and Kyriakis and Avruch, 2001). *Abbreviations*: C/EBPβ, CCAAT/enhancer-binding protein β; CHOP/GADD153, cAMP response element-binding protein-homologous protein/growth arrest DNA damage 153; CK2, casein kinase 2; $cPLA_2$, cytosolic phospholipase A_2; MAPKAPK, MAPK-activated protein kinase; MEF2, myocyte enhancer factor 2; MITF, microphthalmia transcription factor; MNK, MAPK-interacting kinase; MSK, mitogen- and stress-activated protein kinase; NHE1, N^+/H^+ exchanger 1; NFATp, nuclear factor of activated T cells; PGC1, PPAR co-activator 1; SAP1, stress-activated protein 1; STAT1, signal transducer and activator of transcription 1; USF1, upstream transcription factor 1.

Substrate		Function					
Type	Name	Apop-tosis	Cell cycle control	Growth and/or different-tiation	Gene expression/ transcript-tion	Trans-lational control	Cyto-skeletal remodel-ling
Kinases	MAPKAPK2, 3					✔	✔
	PRAK/ MAPKAPK5						✔
	MNK1					✔	
	MSK1,2				✔		
	CK2			✔			
Transcription factors	MEF2C, 2A			✔	✔		
	SAP1	✔			✔		
	CHOP/GADD153			✔	✔		
	C/EBPβ			✔	✔		
	p53	✔	✔		✔		
	STAT1				✔		
	MITF			✔	✔		
	USF1				✔		
	NFATp				✔		
	CDX3			✔	✔		
	MAX	✔		✔	✔		

Substrate		Function					
Type	Name	Apop-tosis	Cell cycle control	Growth and/or different-tiation	Gene expression/ transcript-tion	Trans-lational control	Cyto-skeletal remodel-ling
	PGC1				✔		
Other	$cPLA_2$						
	Stathmin	✔					
	NHE1						
	Cdc25B		✔				

more tissue specific, whereas Akt1 is expressed broadly), they share more than 80% amino acid homology (Nicholson and Anderson, 2002).

Structural examination of Akt has revealed an N-terminal pleckstrin homology (PH) domain (figure 2.14). This was an important discovery, since the PH domain as a lipid-binding module is one of the major ways in which signal transduction molecules in the cytosol can be influenced by membrane-bound lipids (Lietzke *et al.*, 2000; Vanhaesebroeck and Alessi, 2000). Subsequent *in vitro* experiments confirmed that, just as with other signal transduction molecules with PH domains, Akt is capable of interaction with phosphoinositides via its PH domain (Klippel *et al.*, 1997; Lietzke *et al.*, 2000). In fact, the binding

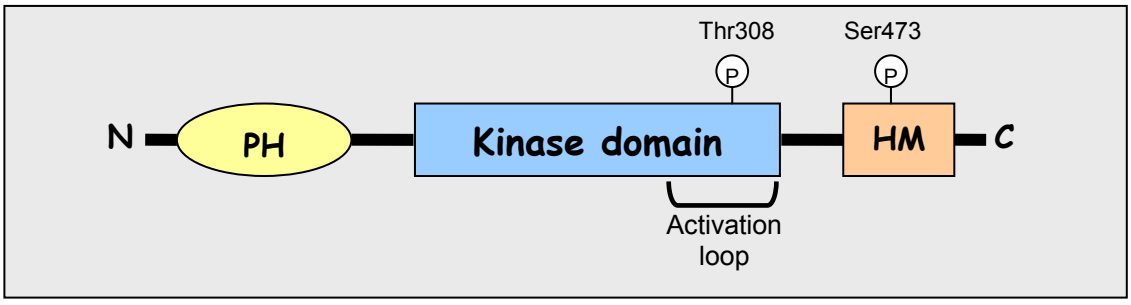

Figure 2.14 Protein structure of Akt (Vanhaesebroeck and Alessi, 2000). *Abbreviations:* HM, hydrophobic motif; PH, pleckstrin homology.

of the phosphoinositides $PI(3,4)P_2$ and $PI(3,4,5)P_3$, to the PH domain of Akt is

a key step for activation, although it does not activate Akt directly (James *et al.*, 1996; Franke *et al.*, 1997; King *et al.*, 1997; Klippel *et al.*, 1997).

Phosphoinositides are the phosphorylated derivatives of phosphatidylinositol (PtdIns), a minor constituent of cell membranes (Vanhaesebroeck and Alessi, 2000). There are eight different phosphoinositides and their levels are regulated by PtdIns kinases that phosphorylate free –OH groups at the 3, 4, or 5 position of the inositol ring in different combinations (Vanhaesebroeck and Alessi, 2000; Toker, 2002). The super-family of PtdIns 3 kinases (or PI3 kinases), with its three different subclasses, phosphorylates the 3-OH position of the inositol ring of phosphoinositides to yield 3'-Pis. The subclasses of the PtdIns 3 kinase family differ by their phosphoinositide substrate preference. For example, the Class I PtdIns 3 kinases utilise PtdIns, PI(4)P, and $PI(4,5)P_2$ as substrates *in vitro*, to yield PI(3)P, $PI(3,4)P_2$, and $PI(3,4,5)P_3$, respectively (Krasilnikov, 2000; Toker, 2002). However, the preferred substrate of PI3 kinase *in vivo* seems to be $PI(4,5)P_2$, which is converted to $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ is then thought to be dephosphorylated by a 5'-inositol phosphatase to generate $PI(3,4)P_2$ (Vanhaesebroeck and Alessi, 2000; Scheid *et al.*, 2002). The first class of PtdIns 3 kinase is often simply referred to as phosphoinositide 3 kinase (PI3 kinase), which reflects its substrate specificity more accurately than the super-family name, phosphatidylinositol 3 kinase (Krasilnikov, 2000). The nature of the lipid products generated by Class I PI3 kinase places PI3 kinase upstream of Akt. However, as previously mentioned, interaction between phosphoinositides and Akt does not amount to activation. Instead, the activation of Akt is mediated by two distinct Ser/Thr phosphorylation events following phosphoinositide interaction at the plasma membrane.

Phosphorylation of Akt occurs within its activation loop (at Thr308 of Akt1) and also within the hydrophobic motif (HM; at Ser473 of Akt1) (Scheid and Woodgett, 2003). For Akt, the resting level of Thr308 phosphorylation is very

low, but increases rapidly upon stimulation. The kinase responsible for activation loop phosphorylation is the constitutively activated phosphoinositide-dependent kinase 1 (PDK1). Like Akt, PDK1 possesses a PH domain that can bind $PI(3,4)P_2$ and $PI(3,4,5)P_3$. According to studies on the interplay between PDK1 and Akt, the binding of phosphoinositides to the PH domain of Akt induces a favourable conformation of Akt which allows phosphorylation by PDK1. Phosphoinositides may also serve an additional function towards Akt activation by promoting the co-localisation of Akt and PDK1 at the plasma membrane inner leaflet via PH domain interaction (Leevers *et al.*, 1999). Besides Akt, PDK1 also phosphorylates the activation loops of numerous other AGC kinases (Scheid and Woodgett, 2003).

Additional regulation of the activation of Akt is provided by an additional phosphorylation target in the HM (Ser473). However, the mechanism of this phosphorylation is as yet not fully understood, and neither has the kinase responsible for it been identified with absolute certainty. Although there are some reports suggestive of autophosphorylation (Toker and Newton, 2000) at the Ser473 site, others believe that a distinct kinase is at work. Although it is not yet clear whether this is a novel protein or not, it is often referred to as PDK2, which implies a functional similarity to PDK1. In fact, this is quite feasible, as it is known that both PI3 kinase activity as well as localisation of Akt to the plasma membrane is necessary for Ser473 phosphorylation (Scheid *et al.*, 2002; Scheid *et al.*, 2002), possibly implicating a dependence upon phosphoinositide interaction. Numerous candidate kinases serving as PDK2 have been suggested, including MAPKAPK2, integrin-linked kinase (ILK), p38 MAPK, PKC, and DNA-PK (double-stranded DNA-dependent protein kinase) (Dong and Liu, 2005).

Phosphorylation of both the Thr308 and Ser473 residues is essential for full activation of Akt (Alessi *et al.*, 1996). It has been speculated by some that

Thr308 phosphorylation causes a charge-induced change in the tertiary structure of Akt, which promotes a suitable conformation of the HM for Ser473 phosphorylation (Scheid and Woodgett, 2003). According to this hypothesis, phosphorylation at Thr308 occurs prior to Ser473 phosphorylation. However, other hypotheses state that Ser473 phosphorylation takes place before the phosphorylation of Thr308 (Scheid and Woodgett, 2003). Either way, research has shown that phosphorylation of these residues occurs independently from one another (Alessi *et al.*, 1996).

Other recent reports have even claimed that multiple Tyr phosphorylations are necessary for full Akt activation. According to one such study, Tyr315 and Tyr326 in the catalytic domain are phosphorylated and assist in activation, since mutation of these residues abolished Akt's enzymatic activity (Chen *et al.*, 2001). Another study also identified Tyr474 as a site of phosphorylation, based on phosphopeptide mapping, and showed that phosphorylation of Ser473 and Tyr474 are mutually exclusive (Conus *et al.*, 2002). Interaction of Akt with Hsp90 is apparently also necessary to maintain activation (Brazil *et al.*, 2002).

Upon activation, Akt phosphorylates numerous substrates and thereby regulates important biological processes: cellular survival, cell-cycle progression, growth, and metabolism (Shaw and Cantley, 2006). These substrates are listed in figure 2.15. Although it is not yet possible to link all substrates (partly because more substrates are bound to be discovered) with the different functions of Akt that they control, some are known. For example, phosphorylation of Bad (Bcl-associated dimer), a pro-apoptotic member of the Bcl (B-cell lymphoma) 2 family, at Ser136 promotes its sequestration in the cytoplasm by 14-3-3 proteins. Its interaction with other Bcl2 proteins at the mitochondrial membrane, which leads to the release of cytochrome c and subsequently apoptosis, is thereby prevented (del Peso *et al.*, 1997). The phosphorylation of Bad is therefore associated with cell survival. Another

survival mechanism controlled by Akt is the phosphorylation of members of the Forkhead family of transcription factors such as FKHR (a.k.a. FOXO). Phosphorylation by Akt inhibits its transcription of pro-apoptotic genes (such as Fas-ligand; FasL) by facilitating nuclear export towards the cytoplasm by 14-3-3 proteins, inhibition of DNA binding, and disruption of cofactor interaction necessary for transcriptional activity (Burgering and Kops, 2002). Other substrates associated with cellular survival include IκB kinase (IKK), caspase-9, ASK1, murine double minute 2 (MDM2), and cAMP-response element binding protein (CREB) (Nicholson and Anderson, 2002; Song *et al.*, 2005). However, Akt also plays a role in regulation of cell cycle progression by phosphorylation of the downstream targets glycogen synthase kinase 3 (GSK3), and p21. Some substrates serve multiple purposes, such as MDM2, whose regulation is also implicated in cell cycle progression via p53 (Song *et al.*, 2005). Another dual purpose substrate is GSK3, which is inactivated by Akt to promote the storage of glucose as glycogen, in the presence of insulin. Hereby, Akt is also implicated in the regulation of metabolism. The glycolytic enzymes 6-phosphofructo-2-kinase (PFK2) and hexokinase are also regulated by Akt. (Nicholson and Anderson, 2002; Shaw and Cantley, 2006). In fact, the involvement of Akt in the regulation of glycolysis via mitochondrial hexokinase serves to couple the availability of glucose as a metabolic fuel to the inhibition of apoptosis (Gottlob *et al.*, 2001). The ways by which Akt mediates its effects are thus complex, as its substrates are mostly involved in more than one cellular process. This possibly makes the Akt signalling pathway one of the most vital links between different events in the same cell.

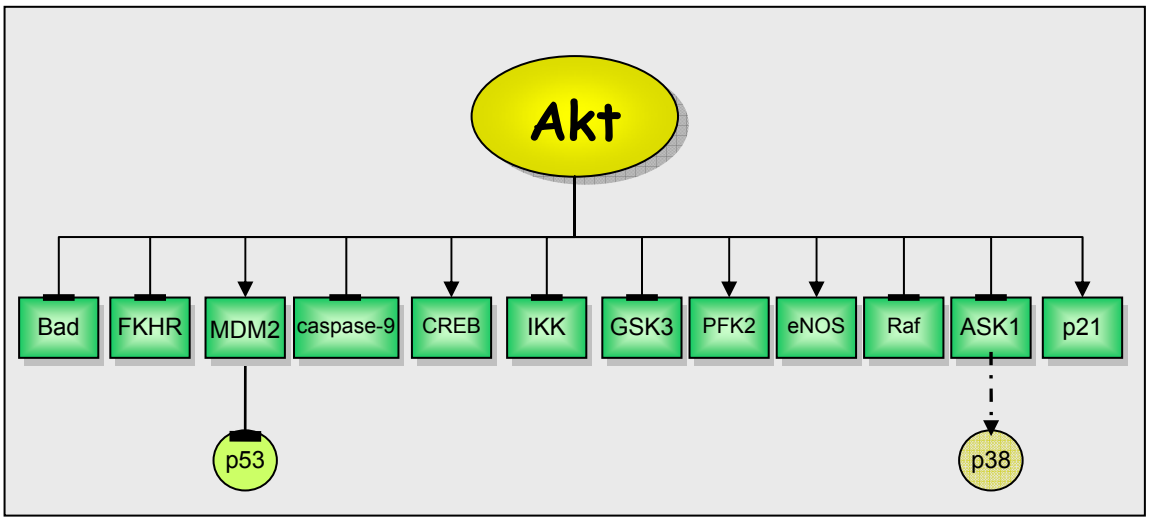

Figure 2.15 The substrates of Akt (Nicholson and Anderson, 2002; Song *et al.*, 2005; Shaw and Cantley, 2006). *Abbreviations:* ASK1, apoptosis signal-regulating kinase 1; CREB, cAMP-response element binding protein; eNOS, endothelial nitric oxide synthase; GSK3, glycogen synthase kinase 3; IKK, IκB kinase; MDM2, murine double minute 2; PFK2, 6-phosphofructo-2-kinase.

2.3.3.4. p53

The *p53* gene is a tumour-suppressor with a decisive role in the prevention of human cancer. Its protein product, known as p53, is a sequence-specific transcription factor (Oren, 2003). The close correlation between tumourigenesis and mutations in the *p53* gene has pressed researchers to investigate the structure and functions of this gene. It is now known that p53 has a network of regulatory functions, both transcription-related and transcription independent, within the cell.

The following are the central biological functions that have been ascribed to p53 (Krauss, 2003):

- It is an activator of the transcription of various genes that are involved in cell cycle control, apoptosis, angiogenesis, and the stress response.
- It is part of a control mechanism that couples cell cycle progression to DNA integrity. When the DNA is damaged, p53 halts the cell cycle at G_1

or at another phase whilst necessary maintenance is performed on the genetic material.

- It is involved in the initiation of apoptosis.

p53 is present in almost all tissues but is almost undetectable under normal circumstances due to its rapid degradation (Vousden, 2002; Krauss, 2003). This is because it resides in a latent state in the absence of cellular stressors. The few p53 molecules that do exist at this point are also relatively incapable of DNA binding and transcriptional activation. However, p53 is rapidly activated upon stressful conditions. The activation is marked by both a dramatic increase in the number of p53 molecules, and certain post-translational modifications including phosphorylation, methylation, ubiquitination, acetylation, and sumoylation (Krauss, 2003; Oren, 2003). It appears that different types of damage induces different modification patterns via different enzymes, which transmit the nature of the stress signal to p53 (Harris and Levine, 2005). The modifications appear to alter p53 in two ways: firstly, its half-life is increased from 6-20 minutes to hours, which results in a 3–10-fold increase in concentration. Secondly, its DNA binding ability is enhanced, and thereby its transcriptional activity (Harris and Levine, 2005). There are generally three types of stressors that are able to induce p53 accumulation (Krauss, 2003; Harris and Levine, 2005):

- Genotoxic stress (DNA damage) induced by e.g. γ-irradiation, UV, X-rays, carcinogens, oxidative free radicals etc.
- Oncogenic stress (the aberrant activation of growth factor-signalling cascades
- Non-genotoxic stress e.g. hypoxia or ribonucleotide depletion

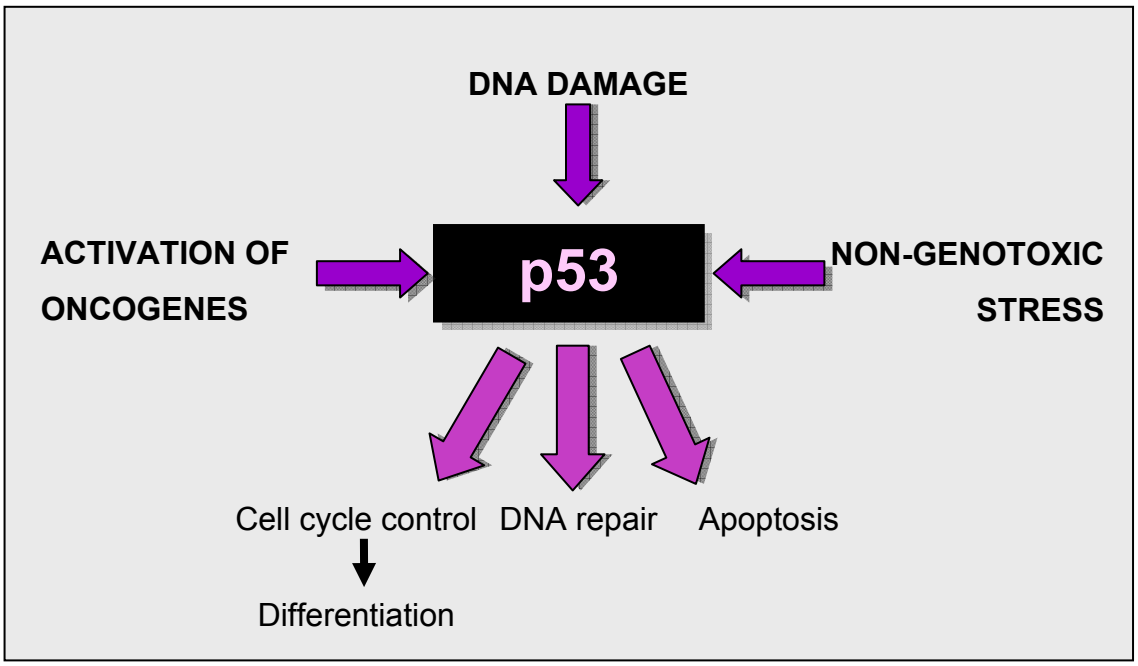

Figure 2.16 Overview of the effects of the p53 pathway, and the three main signals for its activation (Krauss, 2003).

Activation of p53 in response to genotoxic stress (DNA damage) is almost always accompanied by the stabilisation of p53. One of the major components regulating p53 stability is MDM2. MDM2 regulates p53 in two ways: by blocking its transcriptional activity (in the nucleus), and by shuttling it to the cytoplasm to be degraded.

MDM2 forms a complex with p53 in the nucleus and thereby blocks its activation (Momand *et al.*, 1992). The subcellular localisation of p53 is also influenced by interaction with MDM2, as nuclear export of p53 is enhanced by MDM2 (Mayo and Donner, 2002). The amount of p53 available for transcription is thereby diminished (Krauss, 2003). Nuclear export of p53 also underlies the degradation of the p53 protein. MDM2 functions as an E3 ubiquitin ligase for p53, which mediates its ubiquitination and subsequent degradation in the 26S proteasome (Honda *et al.*, 1997). Interestingly, the *MDM2* gene is a transcriptional target of p53, thus establishing an autoregulatory negative

feedback loop in which p53 controls the expression of its own regulator (Haupt *et al.*, 1997). The fate of p53, as directed by MDM2, is directed by the level of MDM2 activity. Whereas low MDM2 activity induces mono-ubiquitination and the nuclear export of p53, high MDM2 activity promotes poly-ubiquitination and p53 degradation (Li *et al.*, 2003).

In 2001, MDM2 was identified as a substrate of the Ser/Thr kinase Akt (Mayo and Donner, 2001; Zhou *et al.*, 2001). Akt phosphorylates MDM2 on Ser166 and Ser186 in a domain containing a nuclear localisation motif (Mayo and Donner, 2002), thus facilitating its entry into the nucleus where it interacts with p53. Phosphorylation of MDM2 is also associated with enhanced degradation of p53 in the proteasome. The role of Akt-mediated MDM2 phosphorylation could be to suppress p53 activity during periods of proliferation in normal cells, and may also contribute to the ability of Akt to protect against p53-induced apoptosis, and thus enhanced cell survival (Vousden, 2002; Krauss, 2003). Apart from Akt, other signalling proteins such as Src and AP1 family members (e.g. c-Jun) are able to suppress *p53* expression and also to inhibit the binding of p53 to target promoters. These may also contribute in allowing normal cellular growth and proliferation and to reverse p53-mediated growth arrest following a stress response (Vousden, 2002). Research has also shown that Akt can form a complex with the MDM2 protein, and that this interaction stands independent from the Akt-mediated phosphorylation of MDM2. The significance of this association is not yet clear, although an *in vitro* role in the degradation of androgen receptors in prostate cells have been suggested (Brazil *et al.*, 2002).

Phosphorylation of p53 in the MDM2-binding region can impair the interaction between the two proteins. Kinases such as chk (checkpoint kinase) 1 and 2, ATM (ataxia telangiectasia mutated) kinase, and ATR (ATM and Rad3-related) kinase are responsible for phosphorylation of these sites. Other kinases such as JNK2 (in the face of non-genotoxic stress) have also been shown to

phosphorylate p53 and thereby regulate its stability. ATM is also able to phosphorylate MDM2, although it occurs on a site distant from the p53- binding region (Maya *et al.*, 2001). Phosphorylation of the p53-binding region on MDM2 by DNA-PK may also inhibit the interaction between p53 and MDM2 and inhibit the degradation of p53 (Mayo *et al.*, 1997). There are also many other mechanisms that allow for the stabilisation of p53, such as down-regulation of MDM2 expression, down-regulation of free ubiquitin levels, or competition between MDM2 and other proteins such as the TATA-binding protein-associated factor (TAF) II31 and Strap that bind to the same region of p53 (Vousden, 2002).

Once p53 is activated, p53 tetramerises and initiates the transcription of genes that reflect the nature of the stress signal, the modifications to p53, and proteins associated with the p53 protein. The activated p53 protein binds to a specific DNA sequence, termed the p53-responsive element. The genes with this sequence initiate one of three programs that result in cell cycle arrest (G_1 or G_2 blocks), cellular senescence, or apoptosis (Harris and Levine, 2005). G_1-arrest is largely mediated by p21, which inhibits cyclin E-cdk2. G_2-arrest, on the other hand, is partly mediated by the synthesis of 14-3-3σ, which sequesters the CDC25C phosphatase in the cytoplasm. Very little is known concerning the genes regulated by p53 to bring about senescence. The protein products of p53 regulated genes involved in apoptosis (*bax, nox, puma*) enhance the leakage of cytochrome c from the mitochondria into the cytoplasm, which sets off the intrinsic apoptotic programme (Miyashita and Reed, 1995; Schuler *et al.*, 2000). There is also support for the notion that p53 can trigger apoptosis prior to transcription when it translocates to the mitochondria and interacts with pro-apoptotic proteins such as the Fas death receptor and Bax (Bennett *et al.*, 1998; Erster *et al.*, 2004). p53 also regulates genes of which the products initiate the extrinsic pathway (FasL, killer/DR receptor) (Harris and Levine, 2005). Other proteins from genes regulated by p53, including maspin and

thrombospondin, are secreted by cells into the extracellular matrix to communicate the stress response to neighbouring cells (Komarova *et al.*, 1998). A list of target genes is given in table 5.

2.3.3.5. Apoptosis

Apoptosis is the highly regulated process by which cells undergo programmed death and was first described in the nematode *C. elegans*. It serves to eradicate undesired cells and is indispensable to the development and function of an organism (Cohen, 1997; Krauss, 2003).

Apoptosis is characterized by a series of explicit changes in cellular morphology and biochemistry which include chromatin condensation, DNA cleavage (a.k.a. DNA laddering), fragmentation of the nucleus, compaction of cell organelles, cell shrinkage, blebbing, and the formation of membrane-bound cytoplasmic vesicles (apoptotic bodies) (Cohen, 1997; Krauss, 2003; Wang *et al.*, 2005). Apoptotic cells eventually lose contact with the extracellular matrix and collapse, without ever releasing their contents into the environment. The apoptotic debris is scavenged by neighbouring cells or phagocytes (macrophages and dendritic cells) without causing any inflammatory response. This type of cell death is clearly discernible from the other major form of cell death termed necrosis, in which damaged cells lose cell membrane integrity and release their contents into the environment, leading to an inflammatory response. Necrosis is also a passive process, whereas apoptosis requires energy (Gomperts *et al.*, 2003; Vermeulen *et al.*, 2005).

The conditions under which apoptosis is initiated are diverse and include:

- Tissue and organ homeostasis: Increased cell numbers due to mitotic division in an organ or tissue are counter-balanced by apoptosis, which eliminates aged or defective cells. The cell number in an organ or tissue is thereby kept within a suitable limit.

Table 2.5 Examples of genes regulated by the transcriptional activity of p53 (Yu *et al.*, 1999; Krauss, 2003). *Abbreviations*: AIP, Apaf-1-interacting protein; COX, cyclooxygenase; GADD, Growth arrest and DNA damage; MDM2, murine double minute 2; NOS, Nitric oxide synthase; TFIIH, TATA-binding protein-associated factor IIH.

Target gene	Function
14-3-3σ	Cell cycle arrest
AIP1	Apoptosis
Bax	Apoptosis
Bcl2	Apoptosis
COX2	Stress response
Cyclin A	Cell cycle arrest
DR5/Killer	Apoptosis
Fas	Apoptosis
GADD45	Cell cycle arrest
Maspin	Communication with neighbouring cells
MDM2	Negative feedback control
NOS1	Stress response
Noxa	Apoptosis
p21	Cell cycle arrest
Puma	Apoptosis
Rad51	DNA repair
TFIIH p62 subunit	DNA repair
Thrombospodin	Angiogenesis
XPB and XPD	DNA repair

- Development and differentiation: Especially during embryonic development, cells that are no longer needed are eliminated by apoptosis.
- Immunity: T-lymphocytes eliminate target cells (e.g. virally-infected cells) by means of apoptosis. Also, autoreactive T- or B-lymphocytes undergo apoptotic death.
- Cell damage: Damaged cells can be destroyed by apoptosis. In the case of cells with DNA damage or viral infection, apoptosis is especially important to eliminate these cells before they can accumulate mutations and become neoplastic (Krauss, 2003; Vermeulen *et al.*, 2005).

Central to the apoptotic programme is the family of proteolytic enzymes known as caspases (a.k.a. cysteine aspartate-specific proteases, because they contain a Cys residue in their catalytic site and cleave substrates at specific peptide sequences containing an Asp residue) (Gomperts *et al.*, 2003). When the first caspase enzyme was discovered, it was named the interleukin-1β-converting enzyme (ICE). Enzymes similar to ICE were later discovered, and found to be involved in apoptosis. A collective name was thereafter assigned to this group of enzymes, now known as caspases, and today ICE is known as caspase-1 (Cohen, 1997). To date, fourteen members of the caspase family have been identified and cloned in mice, of which seven are involved in apoptosis (Vermeulen *et al.*, 2005). Of these, all but caspases-11 and -13 have human counterparts (Ho and Hawkins, 2005).

Caspases share similarities in sequence, structure, size (30–50 kDa) and substrate specificity. They are all expressed as single-chain, inactive pro-enzymes with three domains: an N-terminal pro-domain, a large subunit, and a small subunit. Inactive caspases undergo conformational changes and are cleaved at their internal proteolytic site to yield the active form of the enzyme (Ho and Hawkins, 2005; Wang *et al.*, 2005).

The two caspase sub-families, initiator (caspase-2, -8, -9, -10, and -12) and effector caspases (caspase-3, -6, -7, and -14), are involved in the initiation and execution of apoptosis, respectively (Ho and Hawkins, 2005). Initiator caspases have long N-terminal prodomains that contain recognisable protein-protein interaction motifs (Wang *et al.*, 2005). These motifs enable initiator caspases to cleave and thereby activate themselves following dimerisation. The effector caspases, on the other hand, possess short or no prodomains, and are cleaved and activated by the initiator caspases (Cohen, 1997; Gomperts *et al.*, 2003). The initiator caspases-8 and -10 contain a death effector domain (DED) within their prodomain, which is involved in interactions with adaptor molecules, which will be discussed later. Caspases-2 and -9 contain a caspase-associated recruitment domain (CARD) which is also involved in binding with adaptor molecules, but also in the activation of downstream effector caspases (Ho and Hawkins, 2005; Vermeulen *et al.*, 2005).

It is believed that the activation of initiator caspases is suppressed under normal conditions through binding of inhibitory proteins that inhibit caspase dimerisation and subsequent activation. Another mechanism for the inhibition of initiator caspases is the binding of Bcl2 to Apaf (apoptotic protease activating factor), an adaptor molecule for initiator procaspase dimerisation (Gomperts *et al.*, 2003).

Usually, activated initiator caspases will activate downstream effector caspases in a cascade fashion (Slee *et al.*, 1999). Effector enzymes, the true executioners of the apoptotic programme, will then cleave their substrates, such as poly(ADP-ribose) polymerase (PARP), lamins (the major structural proteins of the nuclear envelope), DNA-PK, inhibitor of caspase-activated DNAase (ICAD), actin, and a host of others. The cleavage of these substrates would ultimately result in the stereotypical morphological changes associated with apoptosis (Vermeulen *et al.*, 2005; Wang *et al.*, 2005). PARP is possibly the

best characterised proteolytic caspase substrate. Under normal conditions, PARP plays an important role in DNA repair, a process which can easily deplete stores of nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP). It has been suggested that the cleavage of PARP serves to conserve NAD^+ and ATP to be utilised for the execution of apoptosis (Cohen, 1997; Vermeulen *et al.*, 2005). The caspase-mediated cleavage of signalling proteins such as Akt and Raf as well as pro-apoptotic proteins Bid (Bcl2-interacting death agonist) and Bax (Bcl-associated partner containing six exons) and certain transcription factors have also been reported (Vermeulen *et al.*, 2005).

Overall, the functions of the caspases in apoptosis can be summarised as follows (Wang *et al.*, 2005):

- to arrest cell cycle progression
- to inactivate the DNA repair and replication systems
- to inactivate the inhibitor of apoptosis proteins (IAPs)
- to dismantle the cytoskeleton

To date, four major pathways leading to the activation of caspases have been suggested (Wang *et al.*, 2005). Of these, the two best characterised pathways are the so-called death receptor-dependent (extrinsic) and mitochondrial (intrinsic) pathways. Other pathways are the endoplastic reticulum (ER)-mediated and the granzyme B-mediated pathways.

2.3.3.5.a) The death receptor-dependent pathway of apoptosis

Death receptors (DRs) belong to the TNF receptor (TNFR) superfamily. They include Fas, TNFR1, TNFR2, and DR3-6. These transmembrane receptors are characterised by the presence of a cytoplasmic death domain. Interactions between the death domains of these receptors lead to the recruitment of adaptor proteins and also initiator procaspases (Ho and Hawkins, 2005).

In the Fas-mediated pathway, Fas is activated by its ligand FasL in order to initiate the caspase cascade. In the absence of FasL, inactive complexes of Fas are formed by the pre-ligand-binding assembly domains of the receptors. Interaction with FasL causes the reassembly of these complexes and allows the formation of a death-inducing signalling complex (DISC). DISC contains the adaptor protein Fas-associated death domain (FADD) and also procaspases-8 and -10. The FasL-induced clustering of Fas, FADD, and caspase-8 or -10 within the DISC brings the caspases in close proximity, resulting in mutual cleavage and activation. The activated caspases are then released from the cluster. Activated caspase-8 will in turn directly activate effector caspases to induce apoptosis (Cho and Choi, 2002; Wajant, 2002).

The caspase-8–activating capacity of the DISC is mainly regulated by FADD-like ICE (FLICE)-like inhibitory protein (FLIP). FLIP can be integrated into the DISC of death receptors (such as Fas or TNF receptors), thereby inhibiting DISC-mediated recruitment, processing and release of active caspase-8. In addition, Fas-mediated apoptosis is controlled by numerous regulators of the mitochondrial pathway, such as members of the Bcl2 (Wajant, 2002).

Besides Fas, other DRs activate initiator caspases in a similar fashion. The binding of the extracellular signalling protein TNFα to its receptor TNFR1 leads to the formation of TNFR1 trimers and also recruits adaptor molecules TNFR-associated death domain protein (TRADD), receptor interacting protein (RIP) 1, and TNFR-associated factor 2 (TRAF2). During receptor endocytosis the receptor cluster is modified, promoting the dissociation of TRADD and the other adaptor molecules (Ho and Hawkins, 2005). The death domain of TRADD then interacts with the death domain of FADD, which recruits procaspase-8 and causes its autoproteolytic activation. Active caspase-8 then activates downstream effector caspases (Vermeulen *et al.*, 2005).

2.3.3.5.b) The mitochondrial pathway of apoptosis

The mitochondrial pathway is activated from within the cell and does not involve any extracellular mediators. In this pathway, apoptotic signals are relayed by the pro-apoptotic proteins of the Bcl2 family, such as Bax and Bak (Bcl2 homologous antagonist/killer). Normally, these pro-apoptotic proteins are regulated by the anti-apoptotic Bcl2/BclX_L family members, with which they form heterodimers. However, upon receipt of the appropriate signals, these pro-apoptotic proteins translocate from the cytoplasm to the outer membrane of the mitochondria where the prompt it to release different proteins from the intermembranous space between the mitochondrial membranes. These proteins include cytochrome c, apoptosis-inducing factor (AIF), endonuclease G, and second mitochondria-derived activator of caspases/direct IAP-binding protein with low PI (SMAC/Diablo) (Krauss, 2003). Although the mechanism of the release of these proteins has not yet been completely elucidated, it is likely that the mitochondrial permeability transition pore (PTP) at the contact site between the inner and outer mitochondrial membranes plays a fundamental role (Vermeulen *et al.*, 2005). Of these mitochondrial proteins, cytochrome c is arguably the most important, because it promotes the assembly of the apoptosome, which is also known as the mitochondrial DISC (Krauss, 2003). The apoptosome is a multiprotein complex, consisting of seven Apaf-1 molecules in association with cytochrome C and ATP, surrounding seven procaspase-9 molecules in its core (Acehan *et al.*, 2002). It is believed that Apaf-1 plays a structural role in the apoptosome, enhancing allosteric conformation of procaspase-9 rather than driving its activation by autoproteolysis. In accordance with this hypothesis, it has also been shown that the cleavage of procaspase-3 is in fact mediated by the procaspase-9/Apaf-1 complex (Rodriguez and Lazebnik, 1999; Krauss, 2003).

2.3.3.5.c) The ER-mediated pathway

In this pathway, procaspase-12, specifically localised on the cytoplasmic face of the ER, is thought to be an initiator caspase. Several possible mechanisms for

the cleavage of procaspase-12 have been postulated, such as m-calpain-mediated processing or by association with Apaf-1. Downstream of caspase-12, JNK is then translocated to the mitochondria, where it indirectly stimulates cytochrome c release (Wang *et al.*, 2005).

2.3.3.5.d) The granzyme B-mediated pathway

This pathway is involved in the cytotoxicity of natural killer (NK) cells and cytotoxic T cells. Granzyme B can induce caspase-dependent apoptosis in target cells by initiating caspase cleavage, although it cannot fully activate caspase-3. Other mediators such as cytochrome C, SMAC/Diablo, and Bid are also involved (Wang *et al.*, 2005).

2.4. PURPOSE OF RESEARCH

It is clear from several *in vivo* studies that the incidence and progression of cancer can be influenced by certain fatty acids. *In vitro,* certain fatty acids have also been shown to be cytotoxic, whilst normal cells remain unaffected. However, the underlying mechanisms by which fatty acids exert these differential effects have not yet been elucidated in cancer cells *in vitro*. Therefore, the objective of the present study was to uncover some of these mechanisms, especially those related to signal transduction.

CHAPTER 3
MATERIALS AND METHODS

3.1. MATERIALS

NCM460 cells were purchased from InCell Corporation (San Antonia, Texas, USA). CaCo2 cells, Eagle's minimum essential medium (MEM), FCS, trypsin and penicillin/streptomycin were obtained from Highveld Biological (Lyndhurst, RSA). Cell culture plastics were purchased from Greiner Bio-one (Frickenhausen, Germany). Arachidonic acid (n-6, 20:4; AA), palmitic acid (16:0; PMA), oleic acid (n-9, 18:1; OA), docosahexaenoic acid (n-3, 22:6; DHA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and protease inhibitors were obtained from Sigma Chemicals (St. Louis, Missouri, USA).

Silica gel plates and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Megabore DB225 chromatography columns were obtained from J&W Scientific (Folsom, California, USA). Fatty acid methyl ester standards were obtained from Nu-Chek-Prep Inc. (Elysian, Minnesota, USA).

2'-Amino-3'-methoxyflavone (PD 98059; MEK inhibitor), and water-soluble 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole, HCl (SB 203580; p38 MAPK inhibitor) were obtained from Calbiochem (La Jolla, California, USA). Wortmannin (PI3 kinase inhibitor), rabbit polyclonal antibodies, rabbit monoclonal antibodies, and the SignalSilence® p38 MAPK short interfering RNA (siRNA) kit (which includes transfection reagent and p38 MAPK siRNA) were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). All electrophoresis equipment was from Bio-Rad (Herculus, California, USA). Polyvinylidene fluoride (PVDF; 0.45 µm) membranes for Western blots were obtained from Millipore (Bedford,

Massachusetts, USA). Prestained protein markers were obtained from PeQLAB Biotechnologie (Erlangen, Germany) and Bio-Rad (Herculus, California, USA). The chemiluminescence system (ECL Plus and ECL), autoradiography film as well as anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (from donkey) were obtained from Amersham Biosciences (Arlington Heights, Illinois, USA). Other standard chemicals were obtained from commercial sources.

3.2. METHODS

3.2.1. Preparation of fatty acid stock solutions

Fatty acid stock solutions (100 mM) were prepared in 99% ethanol. The stock solutions were stored under nitrogen gas in opaque microcentrifuge tubes at -80°C to prevent their degradation.

3.2.2. Cell culture

NCM460 and CaCo2 cells were maintained at 37°C in a humidified 5% CO_2 atmosphere in MEM supplemented with 10% FCS and 1% penicillin/streptomycin (standard medium). Cells were routinely subcultured before reaching confluency and maintained for up to 20 passages. Cell numbers were determined using a haemocytometer following trypsinization. For MTT assays, CaCo2 and NCM460 cells were seeded in 35 mm Petri dishes (120 000 cells/dish and 350 000 cells/dish, respectively). For total phospholipid analysis and Western blots, CaCo2 cells were seeded in 100 mm (360 000 cells/dish) and 60 mm Petri dishes (240 000 cells/dish), respectively. CaCo2 cells were seeded in six-well plates for RNAi experiments (120 000 cells/well).

Prior to fatty acid treatment, standard media were replaced by MEM supplemented with 2% FCS and 1% penicillin/streptomycin (serum-poor medium). Working fatty acid solutions were also prepared in serum-poor medium (1 mM). Cells were treated with the working fatty acid solutions at a

final concentration of 10 µM. Cells treated with the ethanol vehicle only were used as controls.

3.2.3. Treatment of cells with kinase inhibitors

Stock solutions of kinase inhibitors were prepared in DMSO (wortmannin and PD 98059) or in water (SB 203580) and stored at -20°C. CaCo2 cells were treated with the inhibitors wortmannin (500 nM), PD 98059 (10 µM), or SB 203580 (10 µM) in standard medium for 30 min. Media were then replaced with serum-poor medium, treated with DHA for 30 min, and prepared for Western blot analysis.

3.2.4. Cell proliferation assessment

The MTT assay was employed to determine the effect of fatty acids and the different vehicles on proliferation (or cell functionality). The MTT assay is a colorimetric technique based on the ability of the mitochondria of living cells to convert the tetrazolium compound, MTT, into blue formazan crystals (Campling *et al.*, 1988). Following treatment with fatty acids or vehicle for 48 h, cells were washed with phosphate buffered-saline (PBS) and incubated with the MTT (0.2% in PBS) for 2 h at 37°C. Then the crystals formed were dissolved in 0.002% Triton X-100 in 1% HCl-isopropanol. Each sample was assayed spectrophotometrically (540 nm). Proliferation of samples is expressed relative to control values (100%).

3.2.5. Determination of fatty acid composition of phospholipids

To examine the incorporation of supplemented fatty acids in the CaCo2 cells' phospholipid fraction after a 48 h incubation period, cells were rinsed twice with ice cold saline (0.9% NaCl w/v), and scraped into 500 µl of cold saline. The lysates were sonicated for short bursts and centrifuged at 4°C for 15 minutes at 6 000 g. Phospholipids extracts were prepared in chloroform/methanol (2:1, v/v) according to the method of Folch and colleagues (Folch *et al.*, 1957). All extraction solvents contained butylated hydroxytoluene (BHT; 0.01% v/v) as an

antioxidant. Heptadecanoic acid (C17:0) was used as internal standard to quantify the individual fatty acids. Neutral lipids were separated from phospholipids by thin layer chromatography (TLC) on pre-coated 10x10 cm silica gel plates without a fluorescent indicator using the solvent system petroleum benzene (boiling range 40-60°C)/diethyl ether (peroxide free)/acetic acid (90:30:1 v/v). Lipid bands were visualized with longwave ultraviolet light after spraying the plates with chloroform/methanol (1:1 v/v) containing 2,5-bis(5'-tertbutylbenzoxazolyl-(2')thiophene (BBOT; 10 mg/100ml). The total phospholipids band was scraped off and its fatty acid composition was analysed as described previously (van Jaarsveld *et al.*, 2000). Lipids were transmethylated using 5% H_2SO_4/methanol at 70°C for 2h. After cooling, the resulting fatty acid methyl esters (FAMEs) were extracted with 1 ml water and 2 ml *n*-hexane. The top hexane layer was evaporated to dryness, redissolved in CS_2 and analysed by gas-liquid chromatography (GLC) using a Varian 3300 Gas Chromatograph on 30 m fused silica DB225 columns of 0.53 mm internal diameter. Gas flow rates were: hydrogen 25 ml/min, air 250 ml/min, and hydrogen (carrier gas), 5-8 ml/min. Temperature programming was linear at 4°C/min: initial temperature 160°C, final temperature 220°C, injector temperature 240°C, and detector temperature 250°C. The FAMEs were identified by comparison of the retention times to those of a standard FAME mixture.

3.2.6. siRNA knockdown of p38 MAPK

CaCo2 cells were plated in six-well plates in standard medium. The following day, siRNA was freshly prepared in serum-free medium containing the transfection agent. The cells were then transfected with 15 nM siRNA in standard medium and agitated vigorously to disperse siRNA evenly. After 24h, the siRNA-containing medium was taken off and cells were treated with DHA in serum-poor medium for 30 min. Cells were then lysed, and prepared for Western blot analysis.

3.2.7. Western blot analysis

Cells were washed twice with icy cold PBS and lysed on ice with 120 µl modified radioimmunoprecipitation assay (Ripa) buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P40 substitute, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM phenylmethylsulphonylfluoride [PMSF], 1 µg/ml leupeptin, 4 µg/ml soybean trypsin inhibitor [SBTI], 1 mM benzamidine, 1 mM Na_3VO_4, and 1 mM NaF). The lysates were sonicated for short bursts and centrifuged at 4°C for 15 min at 6000g. Protein content of the supernatants was determined by the method according to Bradford (Bradford, 1976), using bovine serum albumin (BSA) as a standard. The supernatants were diluted 1:1 with sample buffer (59.5 mM Tris-HCl pH 6.8, 23.8% glycerol, 5% β-mercaptoethanol, 1.9% sodium dodecyl sulphate [SDS], 0.05% bromophenol blue) and heated to 95-100°C for 4 min prior to electrophoresis. Proteins (20 µg per lane) were resolved on 7, 10, or 12% SDS-polyacrylamide gels at 200 V for 50 minutes. Resolved proteins were then transferred onto PVDF membranes in a Bio-Rad Trans-Blot SD semi-dry transfer cell (15V, 1 h). Residual binding sites on the membrane were blocked in 5% non-fat milk in TBS-T (1 x tris-buffered saline [TBS], pH 6.8, 0.1% Tween-20) for 1 h. Blots were then incubated (overnight at 2-4°C) with anti-rabbit primary antibodies (1:1000) in TBST (for polyclonals) or 5% milk (for monoclonals). Blots were washed in TBS-T (3 x 5 min) and incubated with the HRP-conjugated secondary antibody (1:8000) in TBS-T at room temperature for 1 – 2 h and washed (3 x 15 min). Bands were finally visualized with ECL or ECL Plus. Densitometric data of autoradiographs were determined with the Un-Scan-It software package (version 5.1, Silk Scientific Corporation, Orem, Utah, USA).

3.2.8. Statistical analysis

All experiments were carried out in triplicate, except where stated otherwise. Results were analysed by one-way analysis of variance (ANOVA) for comparison of multiple groups, followed by a post-hoc test with the Bonferroni

correction using Prism version 2.01 (GraphPad Software Inc, San Diego, California, USA). Unless specified otherwise, data are presented as mean ± standard error of the mean (SEM).

CHAPTER 4
RESULTS I

In this chapter, results are shown from the experiments in which four different fatty acids were tested on NCM460 and CaCo2 cells. The effects of these fatty acids on proliferation of the cells were assessed by MTT assays. The incorporation of these fatty acids into the phospholipid fraction of total lipids was also assessed in CaCo2 cells by means of GLC.

4.1 THE EFFECTS OF DIFFERENT FATTY ACIDS ON PROLIFERATION

NCM460 and CaCo2 cells were cultured to 70-80% confluency and then supplemented for 48h with AA, PMA, OA, or DHA (10 μM) in serum-poor medium. MTT assays were performed to evaluate the effects of the fatty acids on proliferation (or cell functionality; figure 4.1). DHA and PMA significantly increased the viability of NCM460 cells compared with control cells (156 ± 5%, $p<0.001$ for DHA and 140 ± 7% for PMA). Furthermore, DHA caused a significant decrease in the functionality of the CaCo2 cells compared with the control cells (89 ± 0.577%, $p<0.01$).

4.2. THE FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS OF CACO2 CELLS SUPPLEMENTED WITH DIFFERENT FATTY ACIDS

The membrane phospholipids of CaCo2 cells supplemented for 48h with different fatty acids were isolated and analysed. Fatty acid content was determined as percentages of total phospholipids, which when combined yields 100%. The relative fatty acid composition of total phospholipids in the experimental groups is shown in table 4.1. In figure 4.2, the total saturated, monounsaturated, and polyunsaturated fatty acids as percentages of the total

phospholipids in experimental groups are summarised.

Regarding the mean profiles of individual fatty acids, all experimental groups except the PMA group had significantly altered fatty acid content compared to the control. As could be expected, AA, OA, and DHA supplementation resulted in a significantly higher AA (mean difference [MD] 2.46%, $p<0.05$), OA (MD 3.62%, $p<0.001$), and DHA (MD 1.09%, $p<0.01$) content in those groups, compared to controls, respectively. However, AA supplementation also resulted in a decreased content of OA (MD 2.76%, $p<0.01$) together with an increased content of 22:4 n-6 (MD 1.07%, $p<0.001$). Compared with the control, AA supplementation resulted in an overall lower total MUFA index (MD 4.41%; $p<0.05$) in those cells. The AA group also had a significantly lower total MUFA index in comparison with the PMA (MD 5.47%; $p<0.01$) and OA (MD 7.27%; $p<0.001$) groups, but a higher total SFA index compared with the OA group (MD 3.78%; $p<0.05$). Supplementation with OA, on the other hand, decreased the palmitoleic acid (16:1 n-7; MD 1.26%, $p<0.05$) content of that group, whilst also resulting in an increased content of eicosenoic acid (20:1 n-9; 0.33%, $p<0.05$). However, the total MUFA index of the OA group was significantly higher than that of the DHA group (MD 5.07%, $p<0.01$), even though it did not differ significantly from the control or any other group. DHA supplementation did not significantly modulate the phospholipid content of any fatty acid other than itself in that group. No further statistically significant differences were observed in other groups with regard to total SFA, MUFA, or PUFA indices.

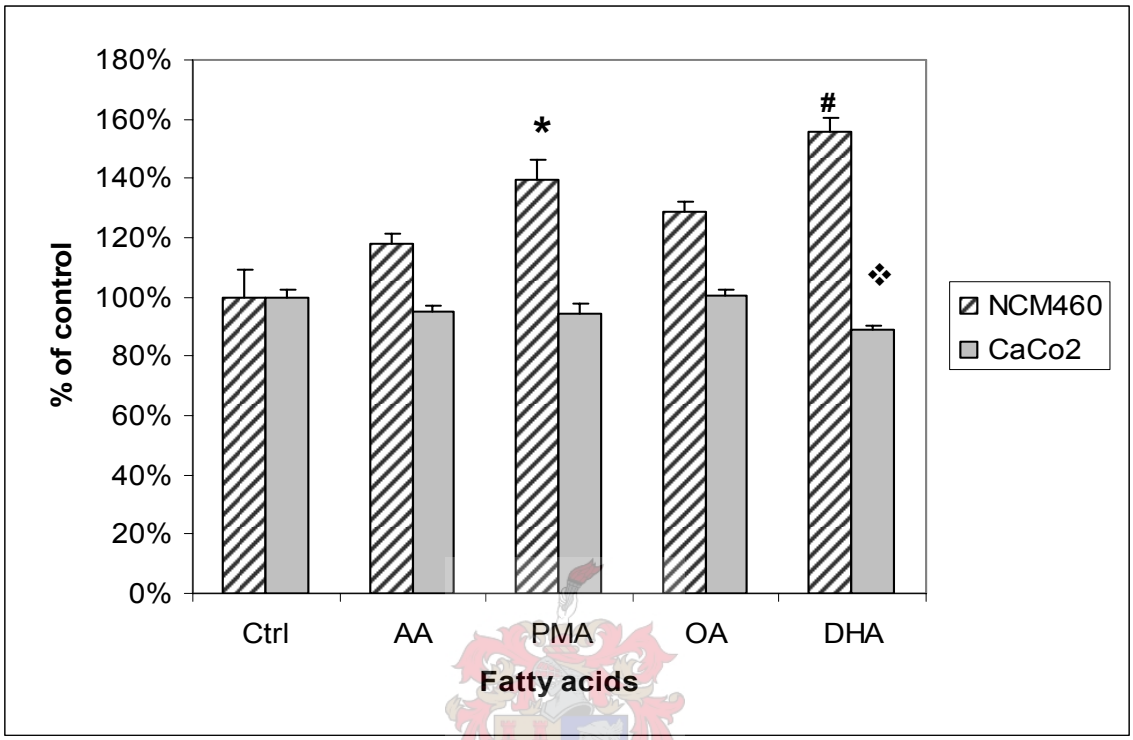

Figure 4.1 Effect of supplementation with different fatty acids on proliferation of NCM460 and CaCo2 cells. Cells were cultured in standard medium and supplemented with fatty acids (10 µM) in serum-poor medium for 48h. The MTT assay was performed as described in the Methods section. Values are expressed as percentages of controls (100%). Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; OA, oleic acid; PMA, palmitic acid; * $p<0.05$ versus NCM460 control; # $p<0.001$ versus NCM460 control; ❖ $p<0.01$ versus CaCo2 control.

Table 4.1 Fatty acid composition of total phospholipids of CaCo2 cells supplemented with various fatty aids. Cells were supplemented with different fatty acids (10 μM) for 48h in serum-poor medium. Total phospholipids were isolated and their fatty acid profiles determined as outlined in the Methods section. *Abbreviations:* AA, arachidonic acid; DHA, docosahexaenoic acid; OA, oleic acid; PMA, palmitic acid.

Fatty acid	Ctrl	AA	PMA	OA	DHA	ANOVA (vs. Ctrl)
14:0	1.54±0.24	1.76±0.09	1.47±0.51	1.20±0.39	1.70±0.17	ns
16:0	22.76±0.37	24.02±0.21	23.00±0.34	21.10±1.26	23.16±0.31	ns
18:0	11.60±0.17	11.93±0.19	11.60±0.13	11.68±0.43	12.13±0.31	ns
20:0	0.21±0.00	0.20±0.00	0.22±0.01	0.21±0.01	0.22±0.01	ns
24:0	0.52±0.04	0.46±0.01	0.53±0.03	0.42±0.01	0.47±0.02	ns
16:1 n-7	4.79±0.23	3.78±0.12	4.55±0.05	3.53±0.47*	4.39±0.05	p<0.05
18:1 n-7	15.62±0.61	14.08±0.11	16.48±0.10	15.24±0.24	14.53±1.14	ns
18:1 n-9	23.78±0.15	21.02±0.09*	24.20±0.23	27.40±0.48#	22.87±0.47	* p<0.01; # p<0.001
20:1 n-9	1.18±0.03	1.06±0.06	1.21±0.03	1.51±0.06*	1.02±0.07	p<0.05
24:1 n-9	0.34±0.34	1.38±0.10	0.34±0.34	0.91±0.47	0.71±0.71	ns
18:2 n-6	3.77±0.23	3.44±0.06	3.51±0.12	3.46±0.13	3.88±0.15	ns
20:2 n-6	0.41±0.02	0.39±0.01	0.41±0.01	0.47±0.04	0.42±0.01	ns
18:3 n-6	0.50±0.04	0.48±0.02	0.53±0.04	0.48±0.05	0.50±0.02	ns
20:3 n-6	1.04±0.04	1.02±0.01	1.02±0.02	0.98±0.02	1.05±0.06	ns
20:4 n-6	6.88±0.58	9.35±0.14*	6.19±0.14	6.80±0.45	6.95±0.49	p<0.05
22:4 n-6	0.93±0.09	2.00±0.10*	0.84±0.04	0.90±0.04	0.86±0.06	p<0.001
20:5 n-3	0.20±0.02	0.16±0.01	0.18±0.01	0.16±0.00	0.23±0.03	ns
22:5 n-3	1.13±0.10	1.00±0.04	1.20±0.17	1.04±0.05	1.08±0.07	ns
22:5 n-6	0.14±0.02	0.14±0.03	0.12±0.01	0.13±0.01	0.13±0.01	ns
22:6 n-3	2.31±0.20	2.05±0.07	2.07±0.08	2.12±0.11	3.40±0.25*	p<0.01

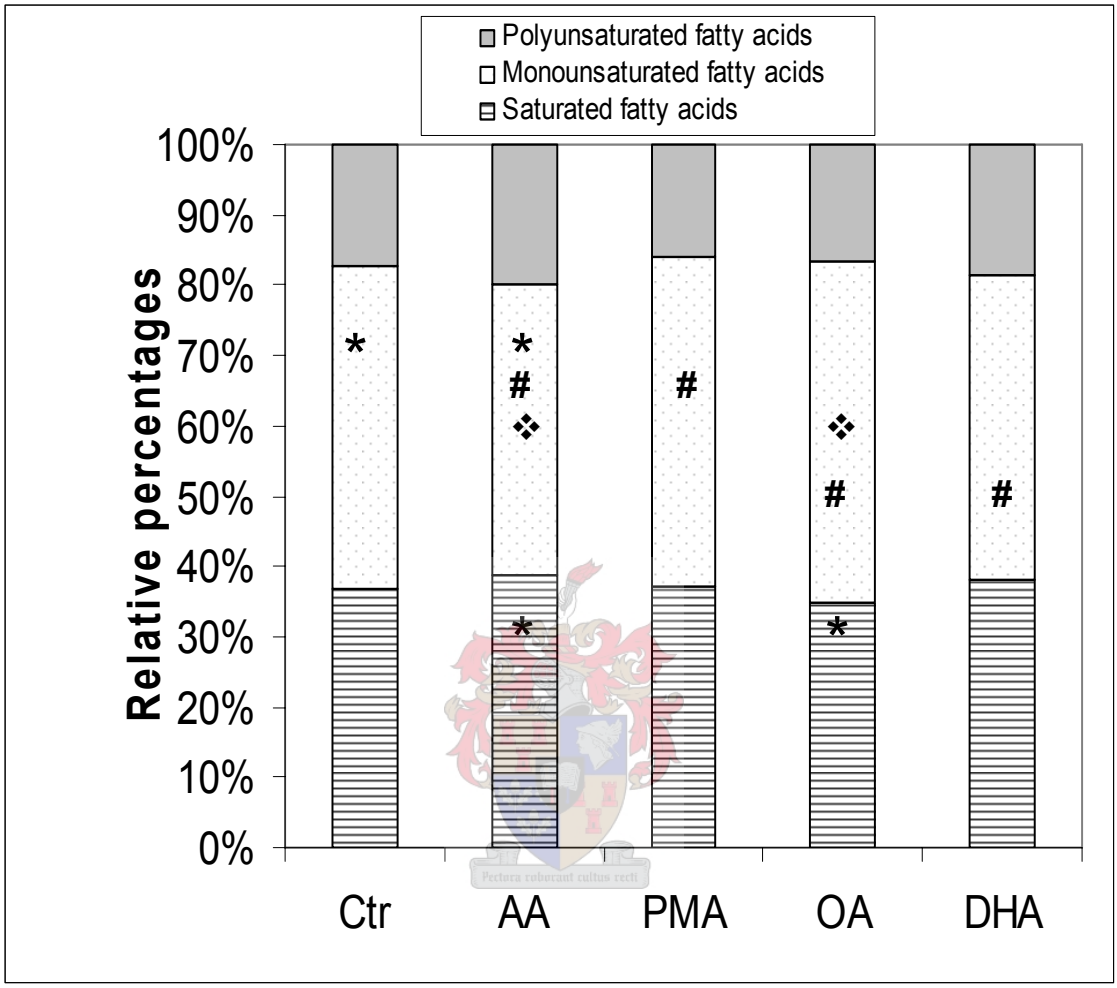

Figure 4.2 Comparison of the relative mean percentages of total saturated, monounsaturated, and polyunsaturated fatty acids in total phospholipids of CaCo2 cells treated with different fatty acids. Cells were cultured, supplemented with fatty acids (10 μM) for 48h and total phospholipids were analysed as outlined in the Methods section. *Abbreviations:* AA, arachidonic acid; DHA, docosahexaenoic acid; OA, oleic acid; PMA, palmitic acid; * $p<0.05$; # $p<0.01$; ❖ $p<0.001$.

CHAPTER 5
RESULTS II

The results presented in this chapter involve the use of DHA only. In light of the results shown in the preceding chapter, DHA was selected as the most suitable fatty acid to use in the subsequent experiments.

5.1. THE CYTOTOXICITY OF VARIOUS CONCENTRATIONS OF DHA IN NORMAL AND CANCER CELLS

To confirm the effect of DHA, different concentrations were tested on both cell lines. In CaCo2 cells, decreased proliferation was observed with increased concentrations of DHA (figure 5.1), although the dose efficacy can only be evaluated in conjunction with its effect on NCM460 cells. Low and medium concentrations (5-50 μM) seem to be most efficient, because NCM460 functionality is not compromised. The high dose (100 μM) was toxic to both cell lines.

5.2. SHORT- AND LONG-TERM DHA SUPPLEMENTATION INFLUENCES PHOSPHORYLATION OF VARIOUS SIGNALLING MOLECULES

The short-term as well as long-term effects of DHA on CaCo2 cells were assessed by Western blotting. CaCo2 cells were cultured until 70-80% confluence and then supplemented with DHA (10 μM) in serum-poor medium for varying time periods ranging from 2 min – 6h (short-term treatment) and from 6h – 48h (long-term treatment). The phosphorylation status of Akt (Ser473 and Thr308), ERK (Thr202/Tyr204), p38 (Thr180/Tyr182), and p53 (Ser15) was then measured with phospho-specific antibodies. Changes in total protein level

were also measured with antibodies that recognise the proteins of interest regardless of their phosphorylation status.

5.2.1. Phosphorylation of Akt at Ser473

Short-term DHA supplementation induced phosphorylation of Akt at Ser473 as early as 2 minutes following treatment (figure 5.2A). The increased intensity of phosphorylation continued up to 10 min after addition of the fatty acid, but declined thereafter to its basal intensity. Although statistically significant differences between the different time points could not be seen, this trend was well established in three successive experiments. Long-term DHA supplementation seemed to have a strong suppressive effect on Ser473 phosphorylation which continued up to 48h (figure 5.2B). Once again, this trend was well established despite the lack of statistical significance. Total protein levels of Akt did not change (figure 5.2B).

5.2.2. Phosphorylation of Akt at Thr308

Short-term DHA treatment induced a modest increase in phosphorylation of Thr308 of Akt which was present up to 1h (figure 5.3A). After 6h, phosphorylation intensity had returned to its basal state. Unlike with the Ser473 residue, long-term treatment did not have any effect on the intensity of Thr308 phosphorylation (figure 5.3B). Although statistical significance could not be shown with three successive experiments, this trend was well established for short- and long-term DHA supplementation.

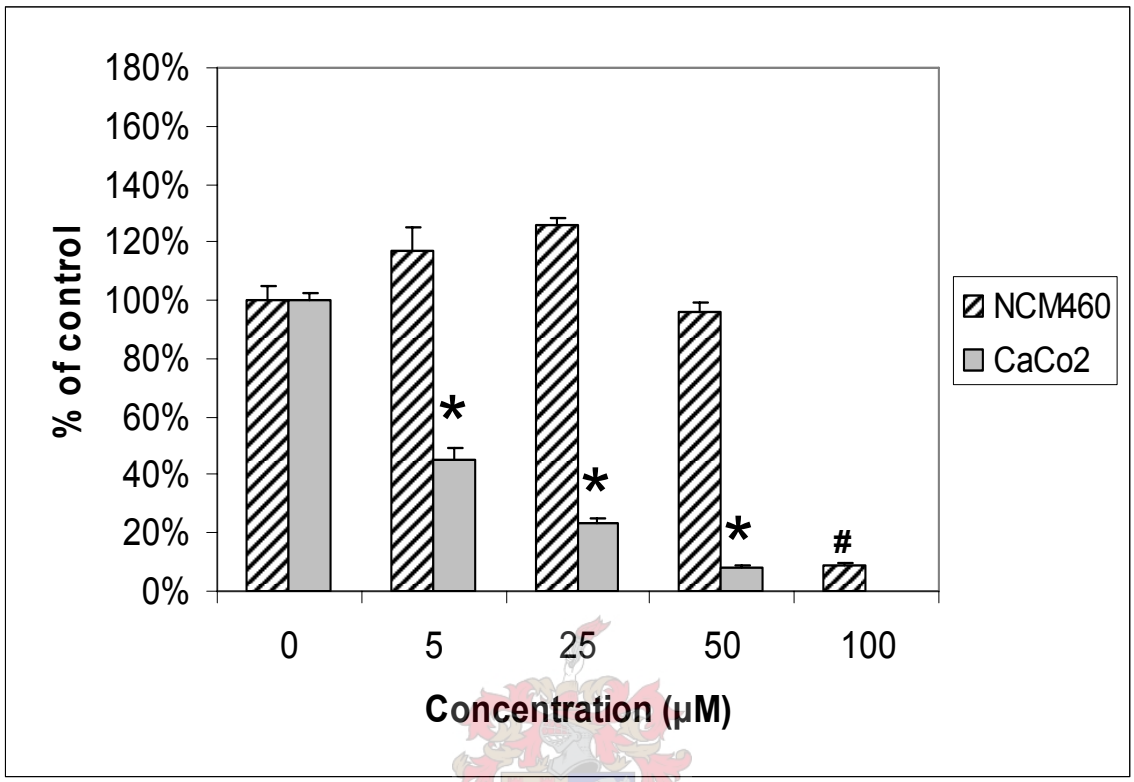

Figure 5.1 Effect of supplementation with different concentrations of docosahexaenoic acid (DHA) on NCM460 and CaCo2 cell proliferation. Cells were cultured in standard medium and supplemented with fatty acids (10 µM) in serum-poor medium for 48h. The MTT assay was performed as described in the Methods section. Values are expressed as percentages of controls (100%); * $p<0.001$; # $p<0.05$ versus control.

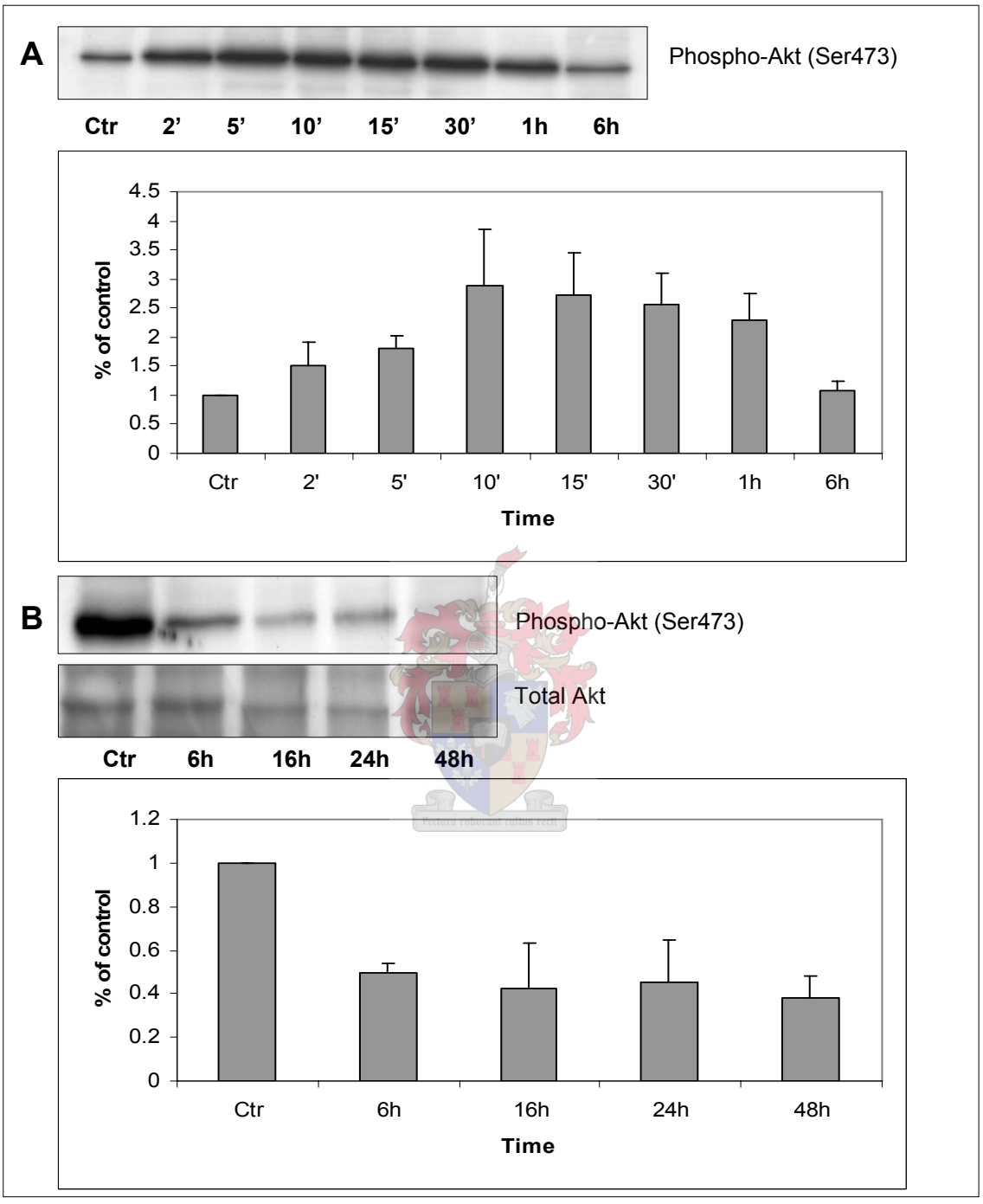

Figure 5.2 **A**: Effect of short-term (2' – 6h) DHA supplementation on phosphorylation of Akt at Ser473 in CaCo2 cells. **B**: Effect of long-term (6h – 48h) DHA supplementation on phosphorylation of Akt at Ser473 in CaCo2 cells. Graphs represent densitometric data of phosphorylated proteins; $p>0.05$ versus control.

5.2.3. Phosphorylation of ERK1/2 and p38 MAPK

With short-term DHA supplementation, a distinct trend was observed towards increased phosphorylation of ERK1/2 which decreased towards its basal intensity before 1h (figure 5.4A). Long-term supplementation, however, yielded conflicting results in three successive experiments with regard to ERK1/2 phosphorylation (figure 5.4B), and no meaningful conclusions could be made. Regarding the phosphorylation of p38 MAPK, short-term DHA supplementation did not seem to have any effect, although an increase was observed after 48h (figure 5.4A and B). The total protein levels of ERK1/2 and p38 MAPK did not change.

5.2.4. Phosphorylation of p53 at Ser15

Short-term DHA supplementation seemed to induce phosphorylation of p53 at Ser15, which was maximal at 10 – 15 minutes (figure 5.5A). After 6h, phosphorylation intensity had returned to its normal state. This trend was well established in three successive experiments, even though statistical significance could not be shown. With long-term supplementation, increased phosphorylation was observed after 48h. The total protein level of p53 did not change.

5.2.5. Cleavage of apoptotic proteins procaspase-3 and PARP

Procaspase-3 can be cleaved by an initiator caspase into 20, 19, or 17 kDa active fragments (Uetsuki *et al.*, 1999). A monoclonal antibody which detects the 17 and 19 kDa subunits was used. In normal conditions, equal amounts of these subunits were detected, which was taken to represent the basal level of apoptosis for CaCo2 cells. Long-term DHA supplementation significantly altered the cleavage pattern of caspase-3 after 16h, when the level of the larger subunit decreased significantly ($p<0.01$), though this was not accompanied by an increase of the smaller subunit (figure 5.6). Forty-eight hours following the addition of DHA, the 17 kDa subunit had nearly disappeared ($p<0.01$),

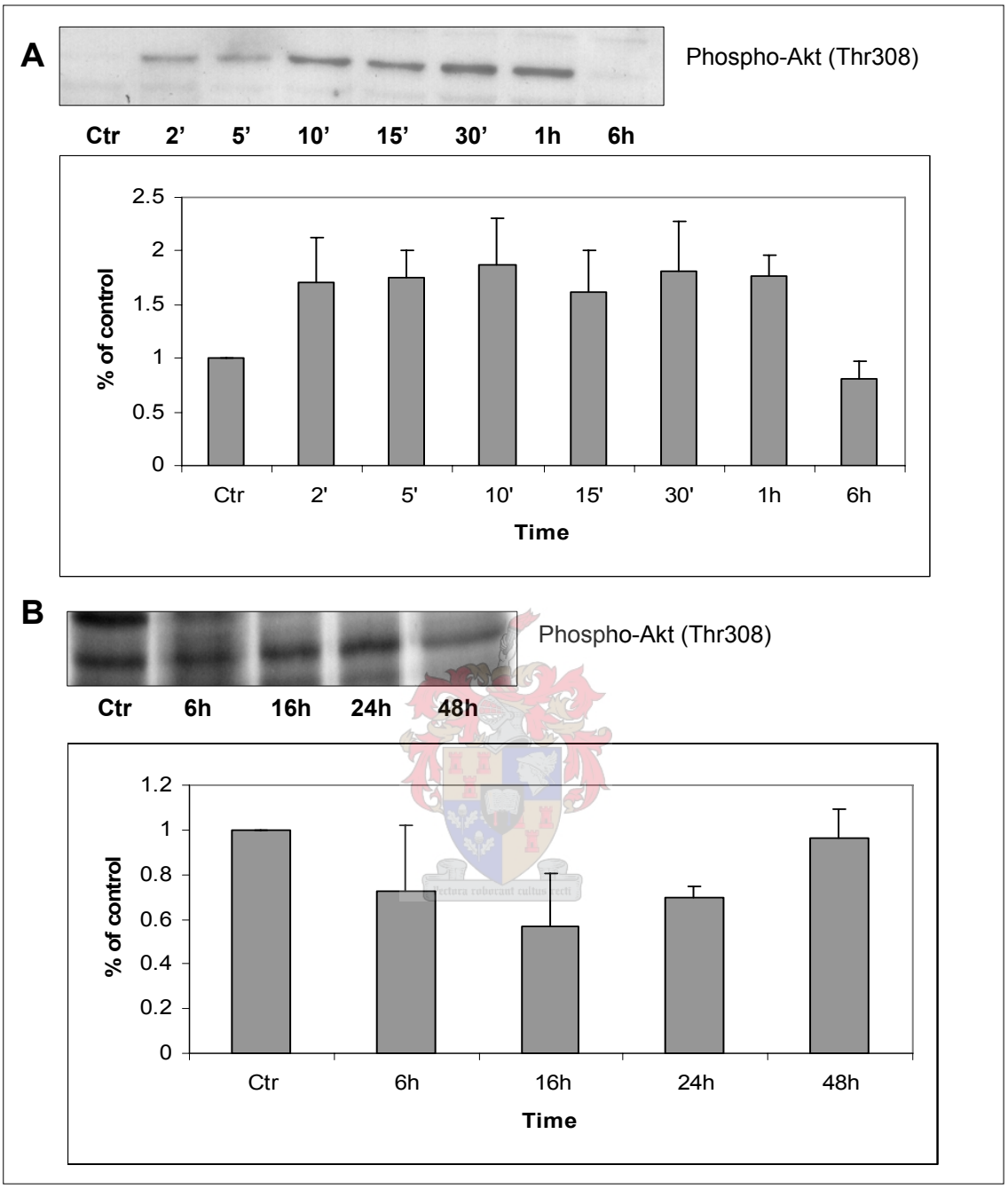

Figure 5.3 **A**: Effect of short-term (2' – 6h) DHA supplementation on phosphorylation of Akt at Thr308 in CaCo2 cells. **B**: Effect of long-term (6h – 48h) DHA supplementation on phosphorylation of Akt at Thr308 in CaCo2 cells. Graphs represent densitometric data of phosphorylated proteins; $p>0.05$ versus control.

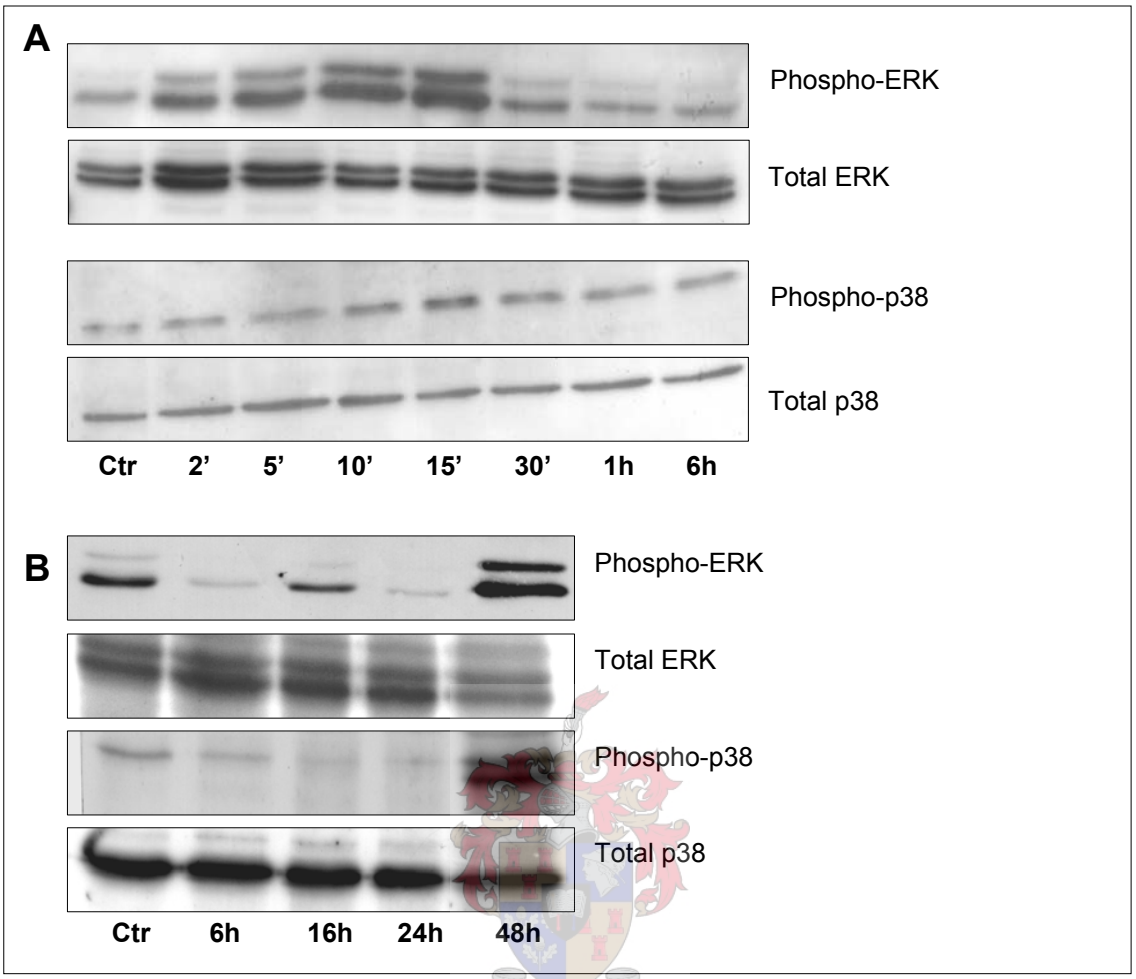

Figure 5.4 **A**: Effect of short-term (2' – 6h) DHA supplementation on phosphorylation of ERK and p38 MAPK (first and third row) in CaCo2 cells. **B**: Effect of long-term (6h – 48h) DHA supplementation on phosphorylation of ERK and p38 MAPK (first and third row) in CaCo2 cells; $p>0.05$ versus control.

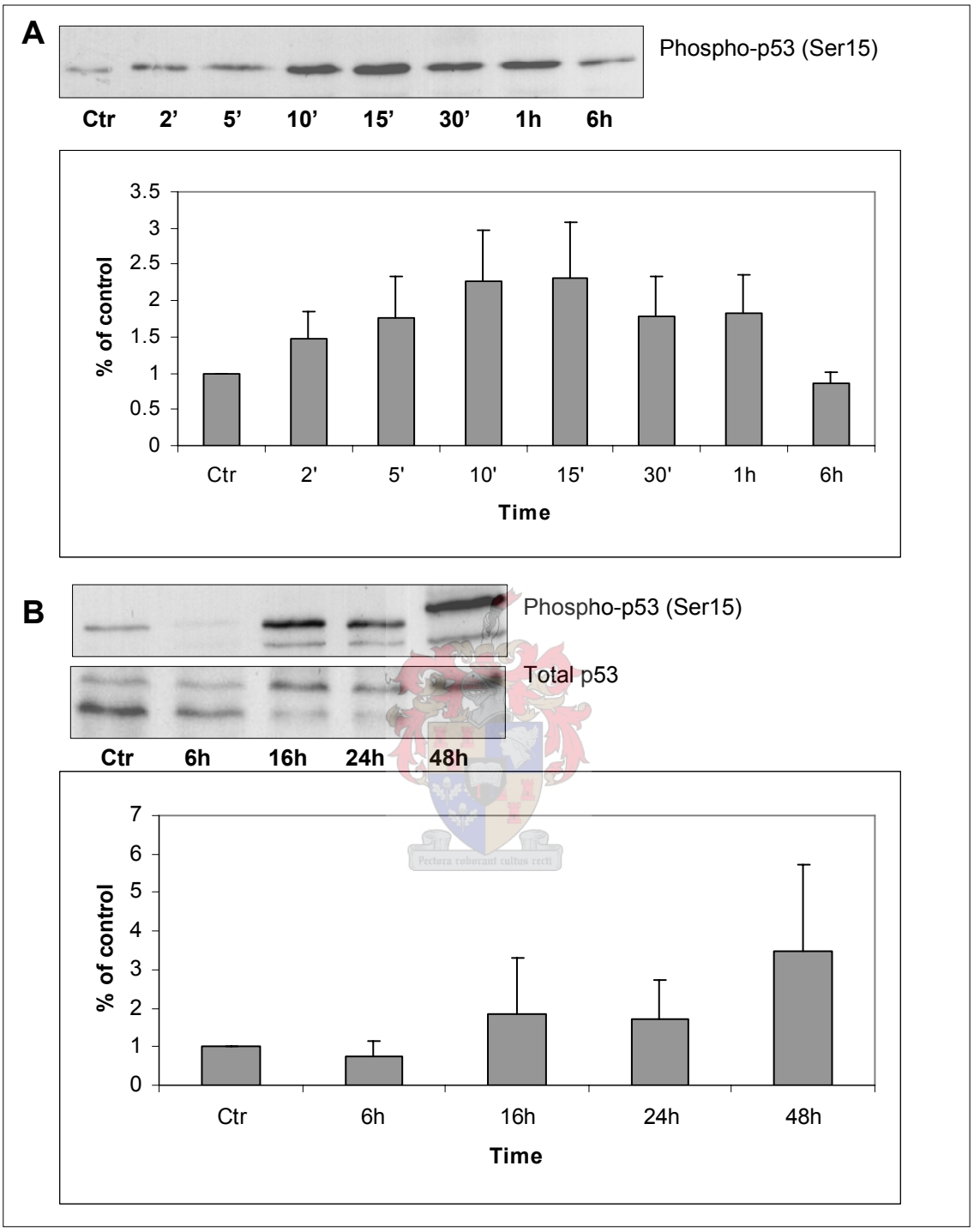

Figure 5.5 **A**: Effect of short-term (2' – 6h) DHA supplementation on phosphorylation of p53 in CaCo2 cells. **B**: Effect of long-term (6h – 48h) DHA supplementation on phosphorylation of p53 in CaCo2 cells. Graphs represent densitometric data of phosphorylated proteins; $p>0.05$ versus control.

together with a return of the 19 kDa subunit to its normal intensity. The disappearance of the smaller fragment of caspase-3 at 48h was coupled with the cleavage of PARP to yield an 89 kDa fragment. This was detected with the use of a monoclonal antibody which detects uncleaved PARP (116 kDa) as well as its subunits following cleavage (89 and 24 kDa), although the smaller fragment could not be detected together with the larger one in these blots.

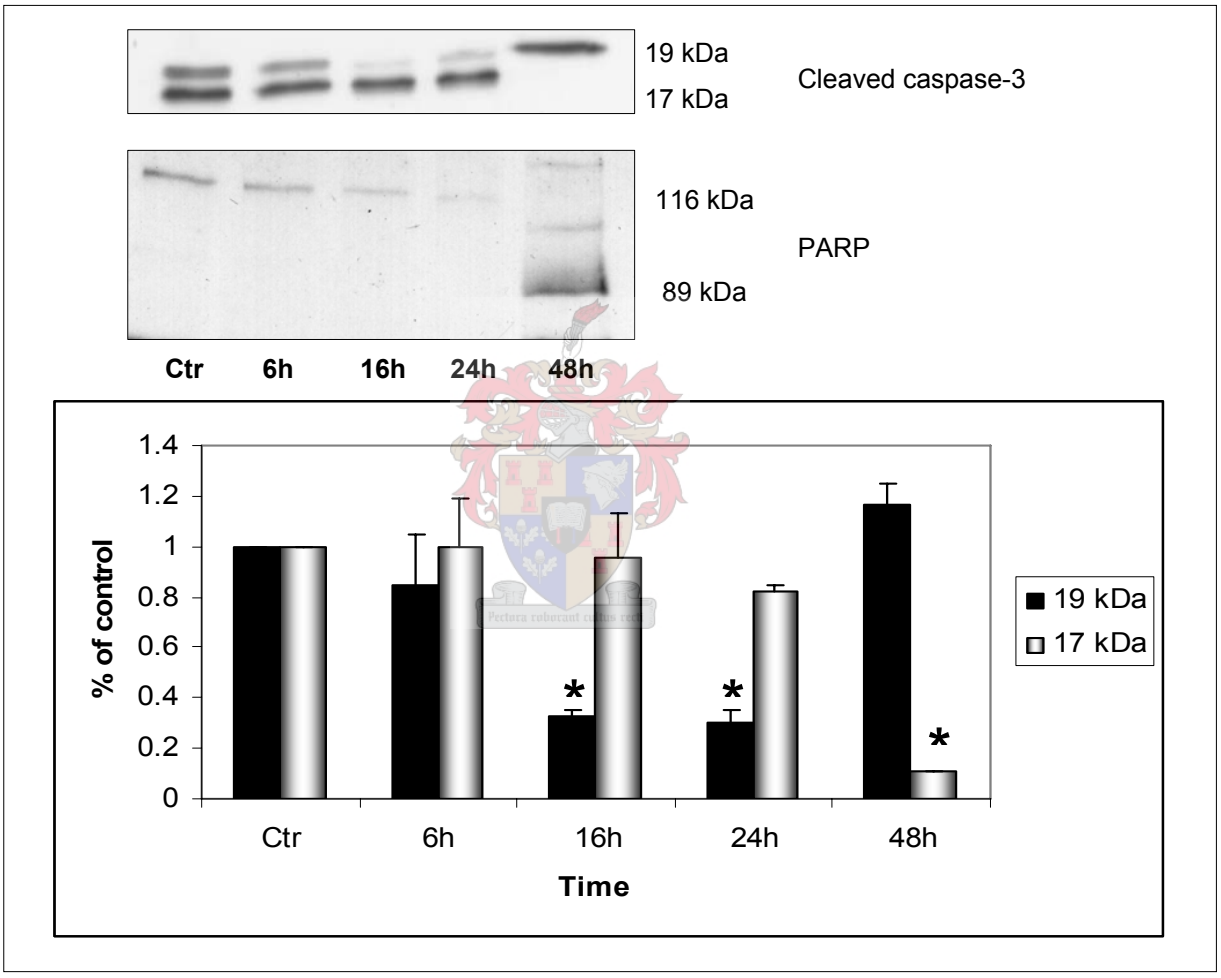

Figure 5.6 The effect of long-term DHA supplementation on the cleavage of procaspase-3 and PARP in CaCo2 cells. The graph represents densitometric data of caspase-3 subunits; * $p<0.01$ versus control.

CHAPTER 6
RESULTS III

In this chapter, the results presented were obtained from experiments investigating possible crosstalk between the components of the various signalling pathways shown to be modulated by DHA. This was accomplished by using three pharmacological kinase inhibitors and also RNAi directed against p38 MAPK (figure 6.1). The pharmacological inhibitors were wortmannin (an inhibitor of PI3 kinase), PD 98059 (an inhibitor of MEK), and SB 203580 (a p38 MAPK inhibitor).

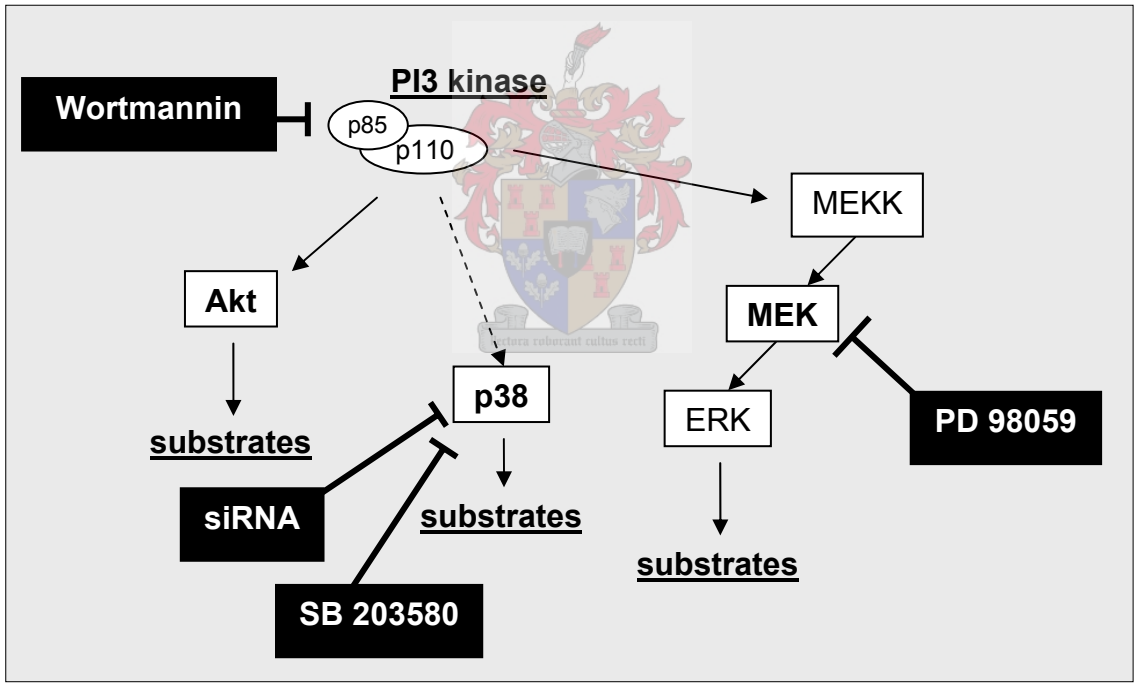

Figure 6.1 The inhibition of signalling kinases by pharmacological inhibitors and short interfering RNAs. *Abbreviations*: ERK, extracellular regulated kinase; MEK, MAP/ERK kinase; MEKK, MEK kinase; PI3 kinase, phosphatidylinositol 3 kinase; siRNA, short interfering RNA.

6.1. THE EFFECTS OF DIFFERENT KINASE INHIBITORS ON AKT, ERK, P38 AND P53 PHOSPHORYLATION

CaCo2 cells were cultured until 70-80% confluence and treated with the inhibitors (wortmannin, 500 nM; PD 98059, 10 μM; SB 203580, 10 μM) for 30 min prior to 30 min DHA supplementation (10 μM) in serum-poor medium. The phosphorylation status of Akt (Ser473 and Thr308), ERK (Thr202/Tyr204), p38 (Thr180/Tyr182), and p53 (Ser15) was then measured with phospho-specific antibodies. Changes in total protein level were also measured with antibodies that recognise the proteins of interest regardless of their phosphorylation status. In an MTT assay, the possibility of any vehicle effects (DMSO and ethanol) was ruled out (figure 6.2).

6.1.1. Phosphorylation of Akt

Treatment with wortmannin reduced the phosphorylation of Akt at Ser473, in both the presence and absence of DHA (figure 6.3). Whereas PD 98059 and SB 203580 did not have any effect on Ser473 phosphorylation when given in the absence of DHA, DHA could not increase phosphorylation in the presence of PD 98059. On the other hand, SB203580 did not have an inhibitory effect on the ability of DHA to increase Ser473 phosphorylation ($p<0.05$). DHA supplementation increased Thr308 phosphorylation slightly ($p>0.05$), but not in the presence of inhibitors. The inhibitors by themselves did not seem to have any effects on Thr308 phosphorylation.

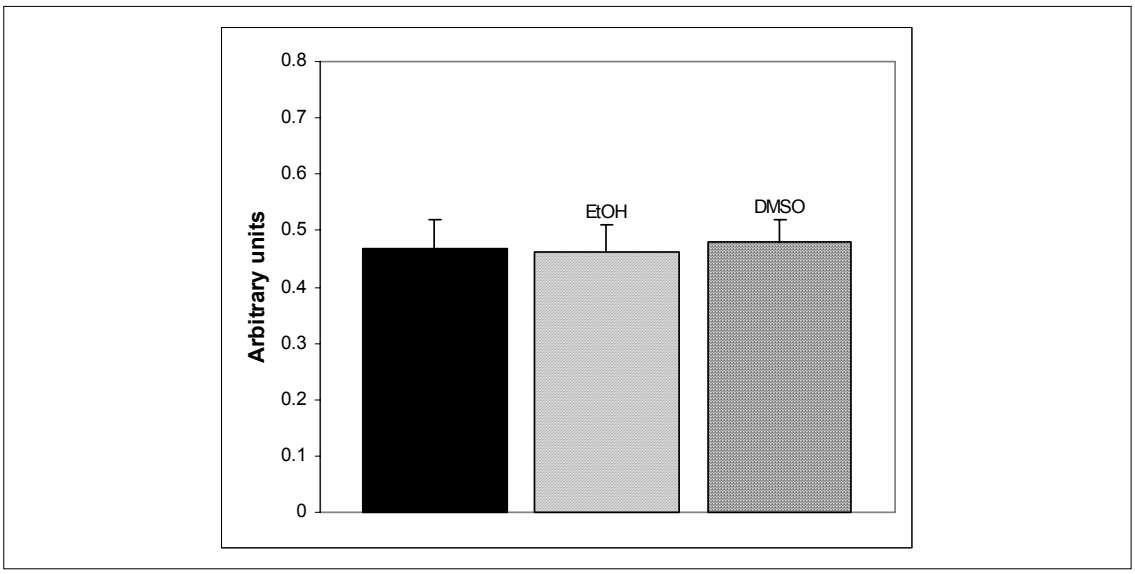

Figure 6.2 The effects of ethanol (fatty acid vehicle) and DMSO (kinase inhibitor vehicle) on the functionality of CaCo2 cells; $p>0.05$.

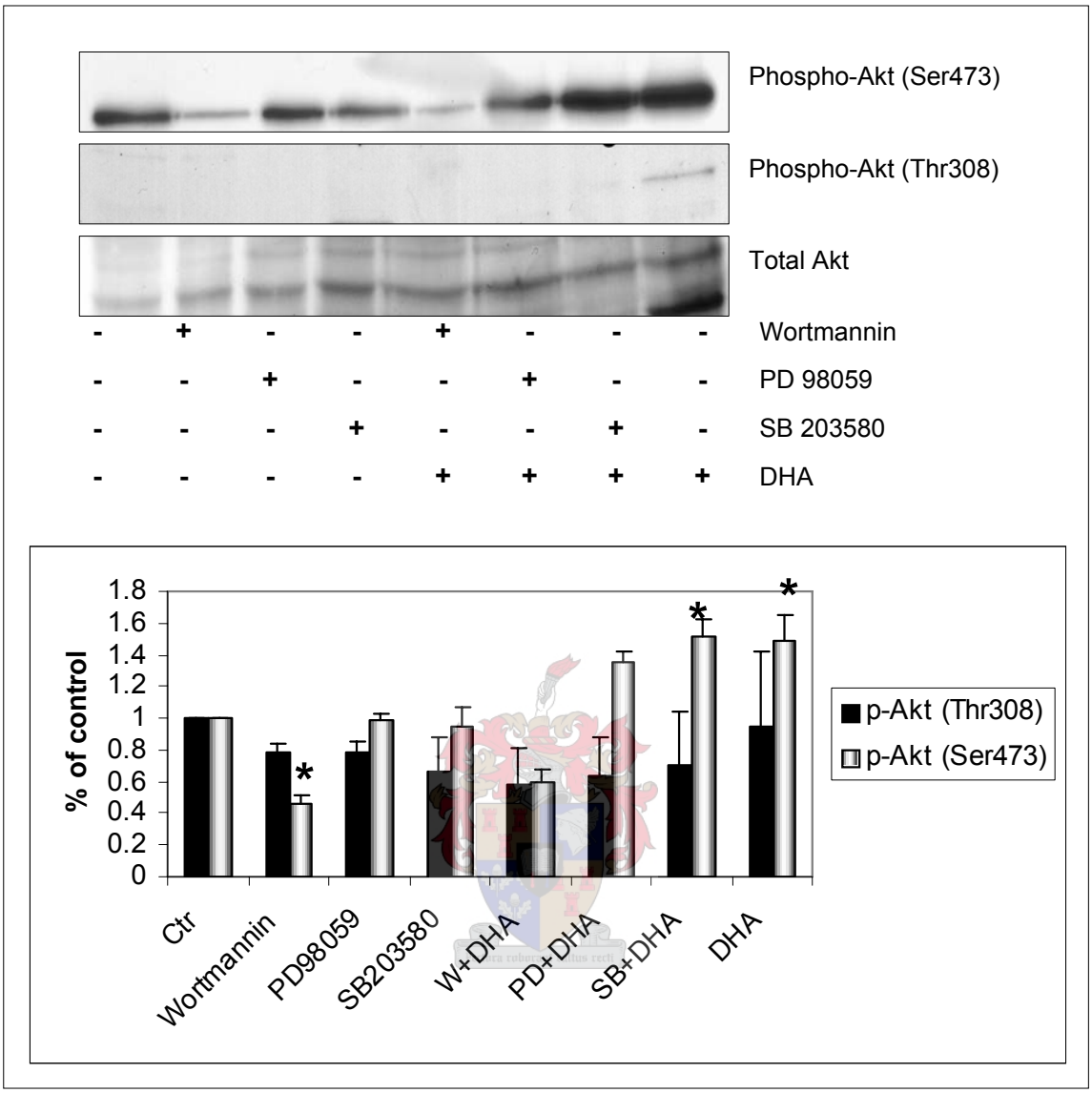

Figure 6.3 The effect of three kinase inhibitors on phosphorylation of Akt at Ser473 and Thr308 with or without 30 min DHA supplementation. CaCo2 cells were treated with either wortmannin (500 nM), PD98059 (10 μM), or SB203580 (10 μM) for 30 min. Then, three groups were also treated with DHA (10 μM) for 30 min. The graph represents densitometric data of phosphorylated proteins; * $p<0.05$ versus control.

6.1.2. The phosphorylation of ERK

The MEK inhibitor, PD 98059, suppressed the phosphorylation of ERK1/2, in the presence and absence of DHA (figure 6.4). Wortmannin also reduced ERK1/2 phosphorylation in both cases. Although SB 203580 did not have an inhibitory effect in the absence of DHA, DHA seemed unable to increase ERK1/2 phosphorylation following SB 208350 treatment. However, from short-term experiments it can be concluded that ERK1/2 phosphorylation had already returned to a basal state by 30 minutes following DHA addition.

6.1.3. Phosphorylation of p38 MAPK

The inhibitors did not seem to have any effect on p38 MAPK phosphorylation (figure 6.5). DHA did not intensify or repress the effects of any inhibitors, and also failed to modify phosphorylation by itself.

6.1.4. Phosphorylation of p53 at Ser15

In these experiments, 30 min DHA supplementation suppressed Ser15 phosphorylation significantly ($p<0.01$), in contrast to the results from short-term supplementation experiments (figure 6.6). This suppression was also seen in the presence of SB 203580 ($p<0.05$), although wortmannin and PD 98059 attenuated this.

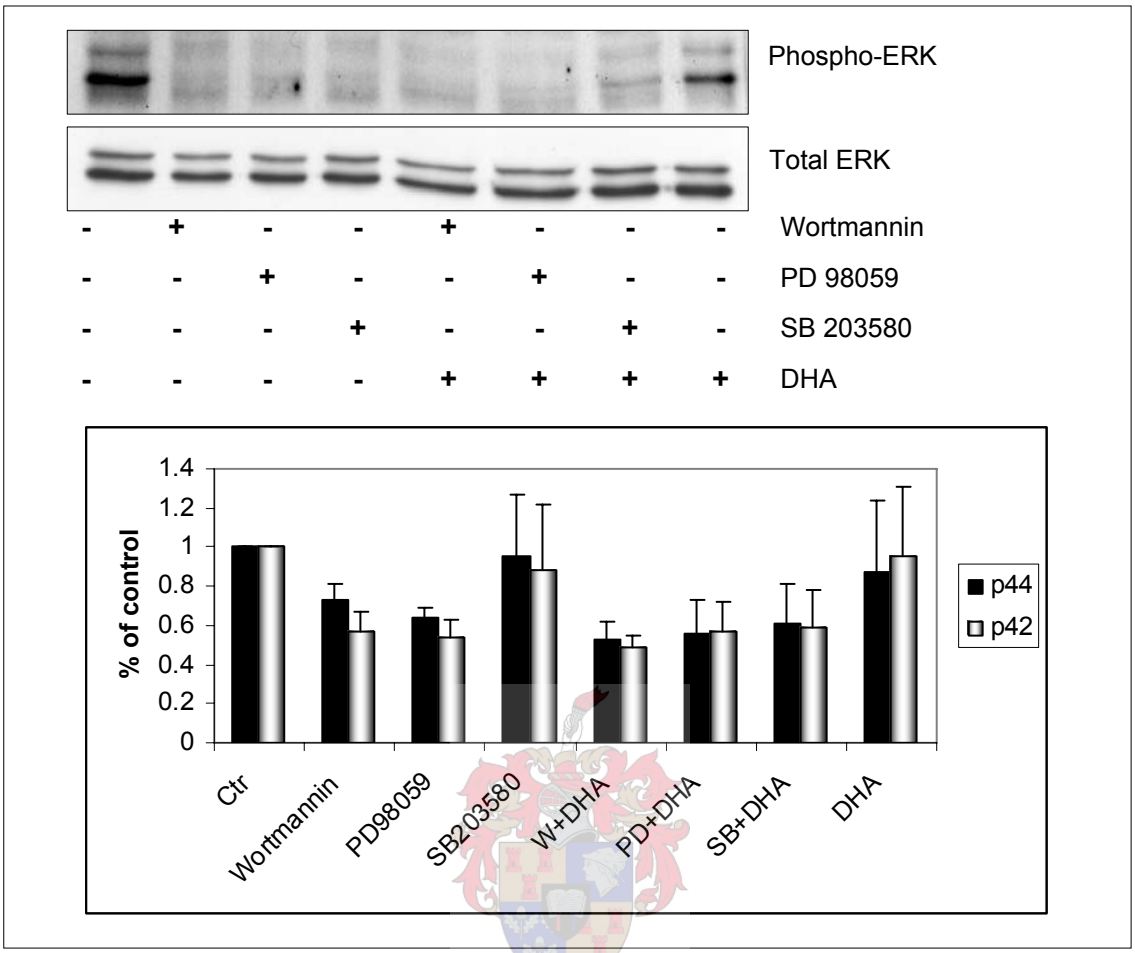

Figure 6.4 The effect of three kinase inhibitors on phosphorylation of ERK with or without 30 min DHA supplementation. CaCo2 cells were treated with either wortmannin (500 nM), PD98059 (10 μM), or SB203580 (10 μM) for 30 min. Then, three groups were also treated with DHA (10 μM) for 30 min. The graph represents densitometric data of the phosphorylated protein; $p>0.05$.

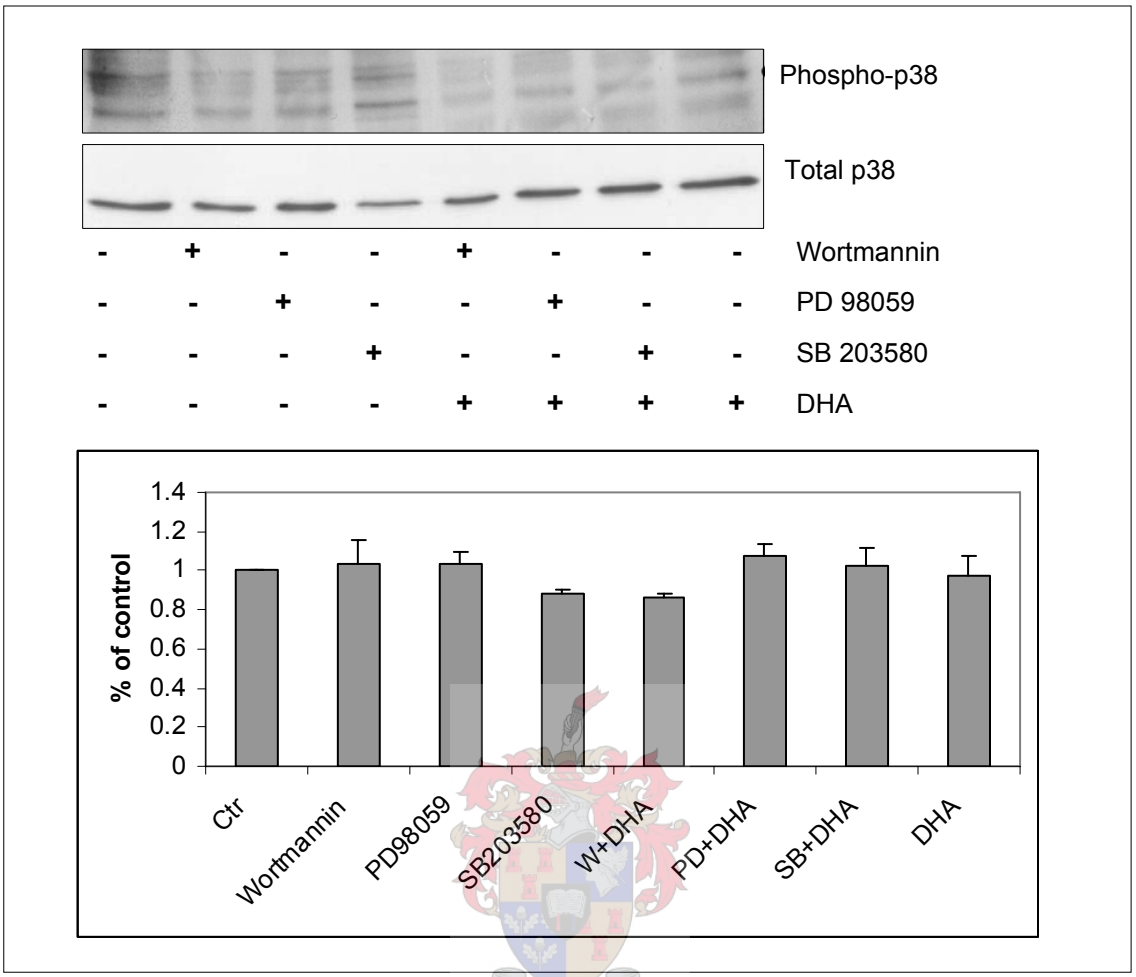

Figure 6.5 The effect of three kinase inhibitors on phosphorylation of p38 MAPK with or without 30 min DHA supplementation. CaCo2 cells were treated with either wortmannin (500 nM), PD98059 (10 μM), or SB203580 (10 μM) for 30 min. Then, three groups were also treated with DHA (10 μM) for 30 min. The graph represents densitometric data of the phosphorylated protein; $p>0.05$ versus control.

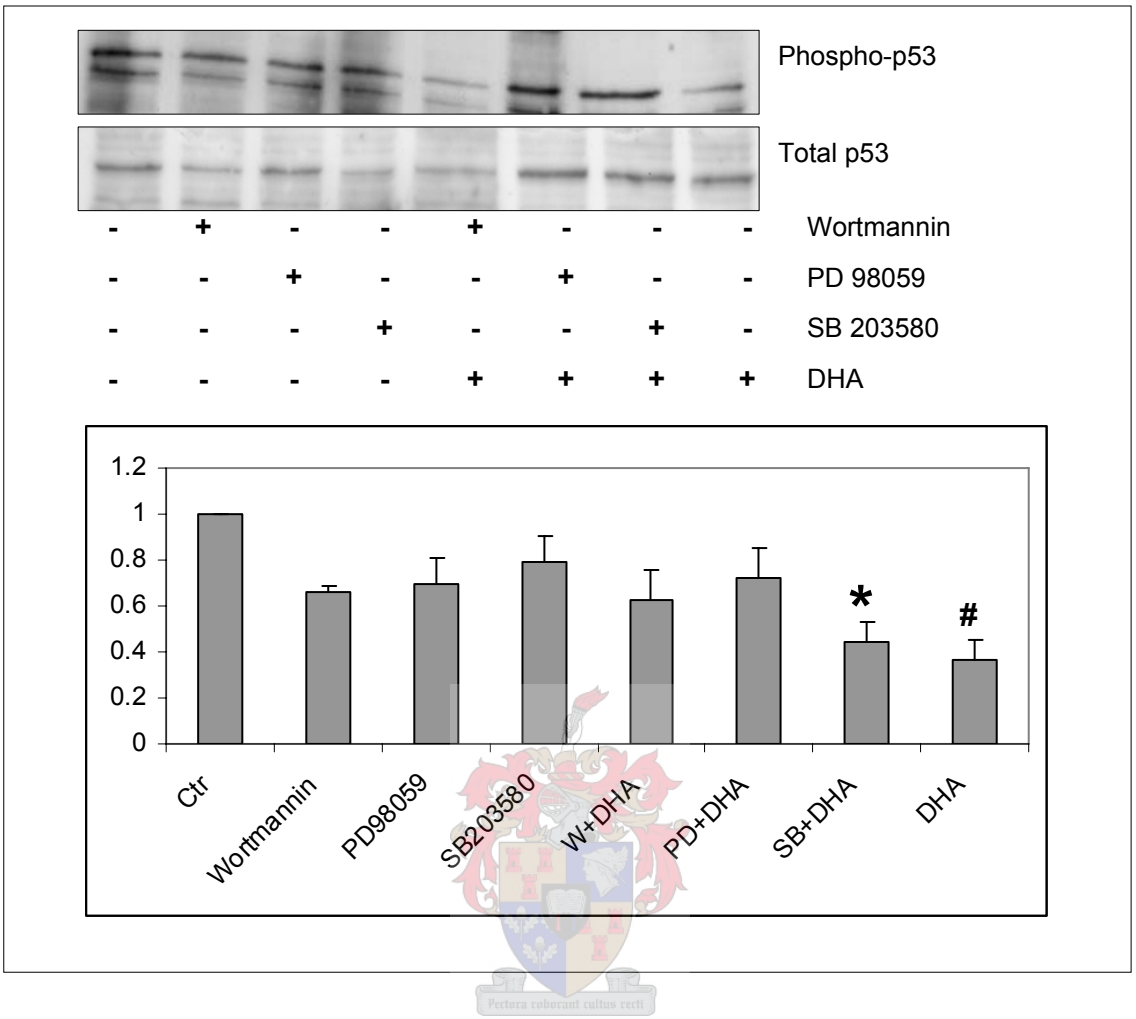

Figure 6.6 The effect of three kinase inhibitors on phosphorylation of p53 at Ser15 with or without 30 min DHA supplementation. CaCo2 cells were treated with either wortmannin (500 nM), PD98059 (10 μM), or SB203580 (10 μM) for 30 min. Then, three groups were also treated with DHA (10 μM) for 30 min. The graph represents densitometric data of the phosphorylated protein;* $p<0.05$; # $p<0.01$ (n=2).

6.2. RNAi AGAINST P38 MAPK

RNA interference is a powerful tool to prevent the protein expression of a certain gene. In this experiment, CaCo2 cells were transfected with siRNAs against p38 MAPK in standard medium for 24h. Thereafter, cells were supplemented with DHA (10 μM) in serum-poor medium for 30 minutes prior to lysis and preparation for Western blotting.

Samples treated with siRNA are in lanes 3 and 4 (figure 6.7). These lanes show decreased band intensity when probed for total p38 MAPK. Phosphorylation of p53 at Ser15 was suppressed by DHA when p38 MAPK was knocked down, although the knockdown by itself did not affect phosphorylation. Phosphorylation of Akt at Ser473 was not suppressed by the knockdown of p38 MAPK.

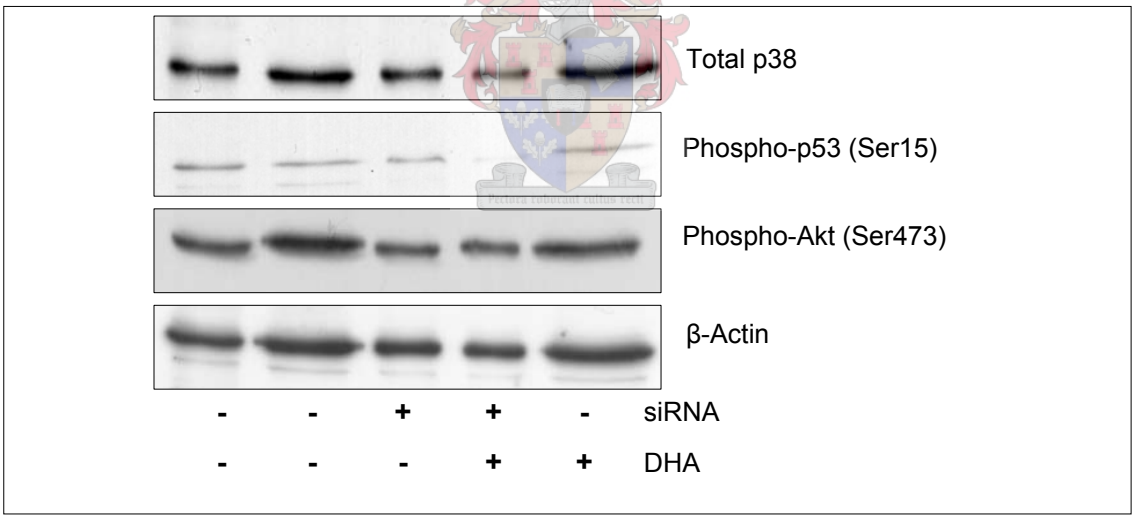

Figure 6.7 The effect of siRNA directed against p38 MAPK on total p38 protein, and the phosphorylation of p53 at Ser15 and Akt at Ser473. Lanes 1 and 2 contain controls with 10% and 2% foetal calf serum, respectively. The total protein level of β-actin was used to control for equal sample loading.

CHAPTER 7
DISCUSSION

7.1. THE EFFECTS OF FATTY ACIDS ON PROLIFERATION OF NORMAL AND CANCER CELLS

In order for a therapeutic agent to be truly effective, it should be toxic to tumour cells without harming normal cells. From the literature, it seems as though fatty acids fulfil this criterion. Also, fatty acids have been shown to augment standard cancer therapeutics in two ways: (1) by enhancing efficacy of chemotherapeutic drugs, and (2) by reducing the side effects associated with chemotherapy (Hardman, 2002).

In the current study, both cell lines were representative of the colon, and a tissue-specific effect was thereby ruled out. This is important, as many early *in vitro* studies are currently disregarded due to different origins of the cell lines (Diggle, 2002). None of the fatty acids used in the current study were toxic to the normal cells, although they had differential effects on the cancer cells (figure 3.1). As many published studies have already shown the effectiveness of DHA on cancer cells, our results were not surprising and encouraged us to use DHA in the subsequent experiments.

Our results have also shown that lower fatty acid concentrations are more appropriate, as normal cell proliferation is not compromised. A high dose, on the other hand, is cytotoxic to both normal and cancer cells (figure 4.1). Even though many similar studies have been published over the years on the effect of fatty acids on cancer cells, in some of these the fatty acid concentrations that were used (Chen and Istfan, 2000) would have been cytotoxic to normal cells as well. Therefore the appropriateness of those studies to encourage the

potential use of fatty acids in cancer therapy can now be questioned.

7.2. THE INCORPORATION OF FATTY ACIDS INTO MEMBRANE PHOSPHOLIPIDS

It is known that the fatty acid composition of membrane phospholipids can influence the function of certain membrane proteins such as receptors, transporters, and enzymes (Spector and Yorek, 1985). Such changes would also have downstream signalling effects, and could ultimately change the profile of gene expression. It is also known that cancer cells tend to have a low content of PUFAs within membrane phospholipids and thus rigidity of cell membranes (Dianzani, 1989). In the present study, the low content of PUFAs in cancer cells was confirmed (table 4.1). Also, it was shown that a SFA such as PMA is extremely poorly incorporated in cell membranes, which is in accordance with previous studies in CaCo2 and also other cell lines (Nano *et al.*, 2003). MUFAs (such as OA in this study) were also poorly incorporated and revealed only 1.2-fold and 1.3-fold increases (versus control) in OA and 20:1 n-9 content, respectively, together with a 1.4-fold decrease in 16:1 n-7. However, OA was found to be the most abundant fatty acid in the CaCo2 cells, followed by PMA. Because the tumour cells are PUFA deficient, cells may synthesise OA from stearic acid (18:0) to compensate for this (Kinsella *et al.*, 1990), thereby attempting to counteract membrane rigidity. High OA content is also associated with the rapid growth and division of cancer cells (Ntambi, 1999).

Supplementation with AA was able to decrease the OA content of the cells, and although this was not a major change, a longer treatment period could have lowered this further. This is probably due to the displacement of OA by AA within the phospholipid, rather than the metabolism of OA in the presence of AA. AA also increased the AA content of the phospholipids by 1.4-fold. The elongated form of AA, 22:4 n-6, also increased 2.2-fold. It has already been shown much earlier that AA supplementation results in a large increase in 22:4

n-6 in cells of endothelial origin (Spector and Yorek, 1985), and this was therefore to be expected. It may also suggest that the additional AA was not employed for eicosanoid synthesis. Although 22:4 can be converted to 22:5 (docosapentaenoic acid), this did not occur during this time of supplementation (48h), which could indicate a lowered Δ4 desaturase activity in the CaCo2 cells.

DHA was also incorporated effectively, and did not have such complex effects on the CaCo2 membranes as AA or OA, as the content of no other fatty acids apart from DHA was altered (1.5-fold increase after 48h). It is known that DHA is preferentially incorporated into the phosphatidylethanolamine (PE), phosphatidylserine (PS) and to a lesser extent phosphatidylcholine (PC) fractions of phospholipids, and preferentially accumulates in the inner leaflet of membranes (Stillwell and Wassall, 2003).

Taken together our results confirm how effectively PUFAs are incorporated into phospholipids of membranes. The enrichment of tumour cell membranes with PUFAs has important therapeutic potential, as it had been shown to enhance the cytotoxicity of therapeutic agents (Burns and Spector, 1987). This could easily be ascribed to the fact that the increased membrane fluidity that accompanies PUFA enrichment, enhances the uptake of such agents and may also reverse resistance to certain therapeutics (Burns and North, 1986; Liu and Tan, 2000; Conklin, 2002). Numerous reports have also ascribed increased membrane permeability and increased flip-flop rates to DHA enrichment (Stillwell and Wassall, 2003), which could also enhance the uptake of therapeutics.

Many mechanisms have been proposed to explain the tumour suppressive effect of n-3 PUFAS. It is likely that this phenomenon is mediated by a combination of these mechanisms, rather than the activity of a single mechanism. Some of these mechanisms are as follows:

- If n-3 PUFAs are available, they will inhibit eicosanoid synthesis from AA, especially reducing the growth-promoting prostaglandin E2 which is often found in higher concentrations in tumour cells (Rose and Connolly, 1999; Hardman, 2004).
- The n-3 PUFAs can inhibit PKC, thereby suppressing mitosis and multiplication of tumour cells (McCarty, 1996). PKC activation has been associated with the onset of colon cancer (Rose and Connolly, 1999).
- The induction of COX-2 is associated with many cancers and is associated with maintaining tumour integrity, angiogenesis, and metastasis (Whelan and McEntee, 2004). This can be suppressed by n-3 PUFAs (Rose and Connolly, 1999).
- The n-3 PUFAs may be peroxidised to generate free radicals that may be involved in the initiation of apoptosis and growth arrest (Larsson *et al.*, 2004).
- Some n-3 PUFAs, particularly DHA, when incorporated in phospholipid membranes, induce changes in the lateral organisation of membranes which may influence the location of signalling proteins and have downstream signalling effects (Wassall *et al.*, 2004).

7.3. DHA MODULATES SIGNALLING MOLECULES

DHA has been shown to modulate the activities of various signalling enzymes and transcription factors in various cell types. These include ERK1/2 in human T lymphocytes (Denys *et al.*, 2001), Akt in neurons (Akbar *et al.*, 2005), p38 MAPK in vascular smooth muscle (Diep *et al.*, 2000), adenylyl cyclase in liver cells (Flier *et al.*, 1985), nuclear factor (NF) κB in macrophages (Novak *et al.*, 2003), and Ras and PKC in colon cells (Holian and Nelson, 1992; Collett *et al.*, 2001). However, these studies were not done in the context of cancer research. An exception is PKC, which has been studied extensively in tumour cells and is believed to be involved in the beneficial effects of PUFAs (Holian and Nelson, 1992; Blobe *et al.*, 1994).

7.3.3. Akt

At this point in time, the mechanism of the activation of Akt is still controversial, even though it is a widely researched field. It is clear that the sites required for activation are Ser473 and Thr308 (in Akt1), and it is mostly believed that phosphorylation occurs at Thr308 before phosphorylation at Ser473 takes place (Scheid and Woodgett, 2003).

Our results have shown that following short-term DHA supplementation, an increase in phosphorylation occurs at both these residues and returns to a basal state within less than 6h (figures 5.2A and 5.3A). Whether this transient heightened phosphorylation, which is indicative of activation, has any effects on gene expression is questionable. However, long-term DHA supplementation has suppressed Ser473 phosphorylation, while Thr308 phosphorylation remained unaltered (figures 5.2B and 5.3B). Very low Thr308 phosphorylation is considered to be normal in resting cells (Scheid and Woodgett, 2003) and it was thus not expected that DHA should suppress Thr308 phosphorylation as well. However, the decreased Ser473 phosphorylation can be associated with decreased activity of Akt, as the phosphorylation of this residue is associated with full activation.

Because interaction with membrane phospholipids is essential for Ser473 phosphorylation (Scheid and Woodgett, 2003), the way in which DHA modifies phospholipids could be responsible for the decreased Ser473 phosphorylation observed. Moreover, as Akt signalling is associated with cell survival (Datta *et al.*, 1999), the restriction of full Akt activity could lead to apoptosis (Kennedy *et al.*, 1997).

Wortmannin, an irreversible inhibitor of PI3 kinase by means of covalent modification (Wymann *et al.*, 1996), suppressed the phosphorylation of Akt at Ser473 and also inhibited the increased phosphorylation induced by DHA

(figure 6.2). With the use of the MEK inhibitor (PD 98059) DHA was also unable to induce the heightened phosphorylation that was seen with the use of DHA (alone or following SB 203580 treatment). This supports a possible upstream role for MEK or perhaps ERK1/2 in the phosphorylation of Akt at Ser473.

Because PDK1 requires interaction with phosphoinositides (Dong and Liu, 2005), it was not surprising to see that Thr308 phosphorylation was abolished by wortmannin. Interestingly, DHA was also unable to induce phosphorylation at Thr308 following treatment with either PD 98059 or SB 203580. This could suggest upstream signalling roles of MEK, ERK1/2, or p38 MAPK in the activation of PDK1, the kinase responsible for Thr308 phosphorylation.

Although it has been suggested that p38 MAPK could be responsible for this phosphorylation following receptor stimulation by certain agonists (Dong and Liu, 2005), our results did not reflect this (figure 6.6). The use of siRNAs to inhibit the expression of p38 MAPK did not diminish phosphorylation at the Ser473 residue, whether treatment with DHA followed or not.

7.3.3. ERK1/2

The increased phosphorylation of ERK1/2 observed with DHA supplementation (figure 5.4) could actually reflect activation of PKC, as it is known that PKC can activate the ERK MAPK cascade via Raf-1 (Kolch *et al.*, 1993; Hausser *et al.*, 2001). Numerous studies have shown that both EPA and DHA can actually reduce PKC activity (May *et al.*, 1983; Holian and Nelson, 1992). If this was the case, it may well have been reflected in ERK1/2 activity. However, conflicting results were obtained regarding ERK phosphorylation in three successive experiments with long-term DHA supplementation, and whether DHA could suppress this crucial survival-associated pathway cannot be stated with certainty.

In the experiments with inhibitors it was found that PD 98059 successfully inhibited the phosphorylation of ERK1/2, in the presence and absence of DHA (figure 6.3). Wortmannin also reduced ERK1/2 phosphorylation in both cases. Previous reports have stated that PI3 kinase is an upstream mediator of ERK1/2 activity (Fuller *et al.*, 2001), which would explain this effect. Although SB 203580 did not have an inhibitory effect in the absence of DHA, DHA seemed unable to increase ERK1/2 phosphorylation following SB 208350 treatment. However, the specificity of this inhibitor has recently been questioned (Godl *et al.*, 2003). Nonetheless, short-term experiments have shown that ERK1/2 phosphorylation had already returned to a basal state by 30 minutes following DHA addition, which is a partial explanation for the lack of phosphorylation in those groups that were treated with an inhibitor and DHA.

7.3.3. p38 MAPK

Although fatty acids have been shown to induce rapid p38 MAPK phosphorylation in other systems (Diep *et al.*, 2000), it was unable to do so in the CaCo2 cells (figure 5.4). A change in phosphorylation following DHA supplementation was only observed at 48h. This could be indicative of a delayed stress response, and might also contribute, together with suppression of Akt signalling, to apoptosis via ASK1 (Matsukawa *et al.*, 2004). It has been speculated repeatedly that the cytotoxicity of PUFAs in cancer cells can be ascribed to their peroxidation and the generation of free radicals (Sagar and Das, 1995; Conklin, 2002). Oxidants are known to activate ASK1 upstream of p38 MAPK, and this pathway can be associated with apoptosis (Matsukawa *et al.*, 2004). It is possible that ASK1 is not activated by DHA supplementation directly, but instead by other apoptotic proteins such as Fas and TRAF2 (Kyriakis and Avruch, 2001). This could explain the delayed response that was observed in p38 MAPK activation following DHA supplementation and why no short-term effects were observed.

The kinase inhibitors did not affect p38 MAPK phosphorylation (figure 5.4).

Also, the effects of the inhibitors were not intensified or repressed by DHA, which was to be expected since DHA failed to induce p38 MAPK phosphorylation by itself.

7.3.4. p53 (Ser15)

Numerous residues at which p53 can be phosphorylated have been described. Similarly, p53 has been described as a substrate for many kinases *in vitro*. Nevertheless, relatively little is known concerning which sites are phosphorylated by which kinases (Ashcroft *et al.*, 1999). The current study has specifically examined phosphorylation at Ser15, which is primarily associated with DNA damage (Shieh *et al.*, 1997). This site is known to be phosphorylated by ATR and DNA-PK *in vivo* (Bulavin *et al.*, 1999).

Short-term DHA seemed to increase p53 (Ser15) phosphorylation transiently, as this effect has already been abolished in less than 6h (figure 5.5A). However, phosphorylation at Ser15 was strongly increased within 16h and remained elevated (figure 5.5B). This increase can be associated with a stress response associated with loss of DNA integrity. Once again, increased oxidative stress that could be associated with PUFA supplementation could be responsible for damage to DNA and Ser15 phosphorylation. However, Ser15 phosphorylation alone was found to be insufficient to block the interaction between p53 and its regulator, MDM2 (Dumaz and Meek, 1999). Therefore, although it is possible, it cannot be stated with absolute certainty that p53 can mediate apoptosis following long-term DHA supplementation.

In the kinase inhibitor experiments, Ser15 phosphorylation was lower with DHA than the control value after 30 minutes (figure 6.6). This effect was also observed with SB 203580 and DHA treatment, whereas it was attenuated by both wortmannin and PD 98059.

A possible role for p38 MAPK in the phosphorylation of p53 at Ser15 was

investigated with RNAi (figure 6.7). It was shown that following knockdown of p38 MAPK, DHA is unable to induce phosphorylation of Ser15. This implicates a role for p38 MAPK in the phosphorylation of p53 at Ser15 and also for p53 activation.

7.3.5. Caspase-3 and PARP

Caspase-3 is an important effector caspase in the apoptotic pathway. It is responsible for the cleavage of various substrates, including PARP, a DNA repair enzyme. We have demonstrated that DHA significantly induced caspase-3- and PARP cleavage in the CaCo2 cells (figure 5.6). However, differential cleavage of the inactive enzyme was apparent. At 48h, the cleaved caspase-3 subunit was 19 kDa in size, which was also when cleavage of PARP first became apparent. Although it is known that caspase-3 can be cleaved at more than one site to yield active enzymes of different molecular masses (Uetsuki *et al.*, 1999), our results implicate a possible differential role for these different active enzymes. Furthermore, it was also previously demonstrated in HT-29 cells (also a colon cancer cell line) that a low dose of another long chain n-3 PUFA, EPA, could induce caspase-3 activity after 24 and 48h of exposure (Latham *et al.*, 2001).

CHAPTER 8
CONCLUSION

This was the first complete study, to our knowledge, to investigate the range of effects of fatty acids on different cellular processes such as proliferation, membrane composition, and signal transduction in the context of colorectal cancer. Our results have clearly shown that DHA, the longest chain and most unsaturated fatty acid in biological systems (Stillwell and Wassall, 2003), has multiple cellular effects (figure 8.1). These effects include changes in membrane properties, decreased proliferation of cancer cells whilst normal cells remain unaffected, and also, the modulation of different signalling molecules. It is probable that the down regulation of survival-related signalling observed can be a direct consequence of the changes to membrane structure and that this would ultimately decrease the functionality of cancer cells.

A schematic representation of the proposed mechanisms of the action of DHA on CaCo2 cells, based on the findings of the present study, is given in figure 8.1. Firstly, the incorporation of DHA into membrane phospholipids may result in increased overall membrane fluidity. This could lead to changes in the lateral organisation of membranes, which in turn affects signalling proteins associated with the membranes, such as PKC and PDK1. A possible down regulation of PKC activity due to membrane reorganisation, would lead to decreased signal transduction to ERK, a kinase associated with cell survival and proliferation. Thereby, the activity and downstream signalling of ERK is suppressed. Membrane reorganisation could also decrease the activity of the kinase responsible for Ser473 phosphorylation of Akt, another kinase central to cell survival and proliferation. Thereby the full activation of Akt is suppressed. Thus, DHA is able to reduce proliferation of CaCo2 cells by the down regulation of survival-associated kinase activity. We propose this as the primary anti-

tumourigenic mechanism of DHA.

Furthermore, we also propose a secondary anti-tumourigenic mechanism of DHA. This mechanism involves the ability to up regulate the activity of proteins associated with apoptosis. It is a secondary mechanism because it only becomes apparent more than 16h following DHA supplementation, whereas the primary mechanism is evident much earlier. The secondary mechanism can possibly be ascribed to DHA peroxidation and the subsequent generation of free radicals. Free radicals are able to activate both ASK1, a kinase upstream of p38 MAPK (which is associated with stress and apoptosis), and p53, a transcription factor that can also be connected to stress (especially DNA damage) and apoptosis. It is proposed that p38 MAPK and p53 is involved in the induction of the apoptotic cascade, as the cleavage of the apoptotic proteins, procaspase-3 and PARP, was observed.

From the use of pharmacological inhibitors and siRNA, we can also propose other novel findings. Firstly, our results suggest a role for ERK in the activity of PDK1, the kinase responsible for Thr308 phosphorylation in Akt. Secondly, we propose that p38 MAPK is responsible for the phosphorylation of Ser15 in p53.

Our results confirmed the anti-tumourigenic efficacy of n-3 PUFAs in cancer cells, together with their safety with regard to normal cells. However, why normal cells are not harmed, remains an open question. Nevertheless, the clinical use of n-3 fatty acid therapy, whether as a precautionary agent or as an adjuvant to conventional therapy, is long overdue.

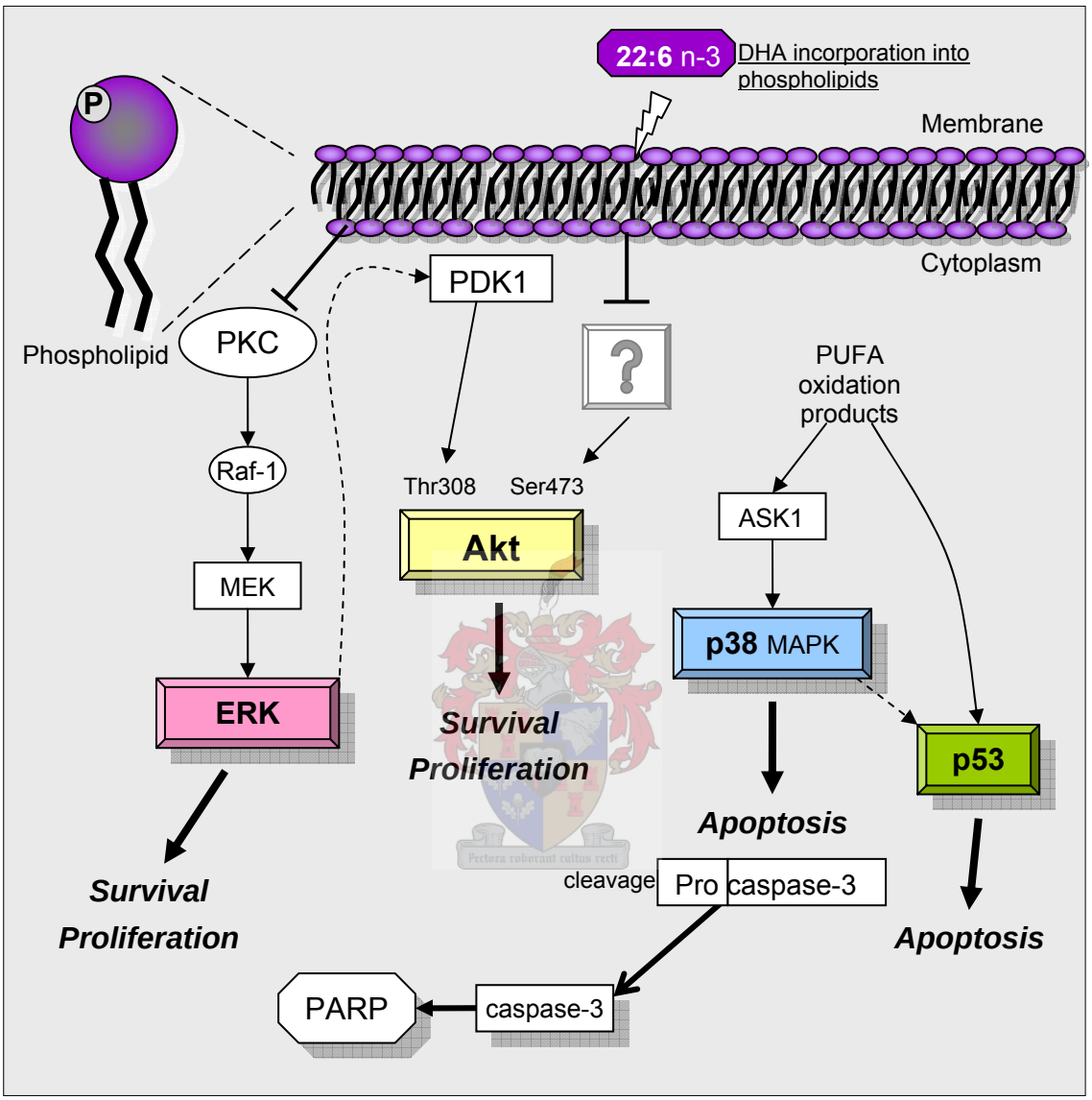

Figure 8.1 Proposed signalling mechanisms of DHA in CaCo2 cells. *Abbreviations:* ASK1, apoptosis signal-regulating kinase 1; ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; PARP, poly(ADP-ribose) polymerase; PDK1, phosphoinositide dependant kinase 1; PKC, protein kinase C; PUFA, polyunsaturated fatty acid.

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