Modulation of Signal Transduction Pathways by Dietary Cancer Chemopreventive Agents

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

Matthew Simon Squires B.Sc. (Hons)

Medical Research Council Toxicology Unit University of Leicester

October 2000

-

UMI Number: U533191

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U533191 Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author. Microform Edition © ProQuest LLC. All rights reserved. This work is protected against unauthorized copying under Title 17, United States Code.



ProQuest LLC 789 East Eisenhower Parkway P.O. Box 1346 Ann Arbor, MI 48106-1346

Modulation of Signal Transduction Pathways by Dietary Cancer Chemopreventive Agents

ABSTRACT

Matthew Simon Squires

The means by which the dietary cancer chemopreventive agents, curcumin, epigallocatechin-3-gallate (EGCG) and indole-3-carbinol (I3C) inhibit growth of human breast cell lines was investigated by examining their effects on various signal transduction pathways.

Curcumin inhibited the growth of HBL 100, T47D and MDA 468 cell lines to a similar extent (IC₅₀ 5 μ M), while EGCG and I3C were most effective against the MDA 468 line (IC₅₀ 10 μ M and 40µM respectively). Curcumin inhibited epidermal growth factor (EGF)-stimulated phosphorylation of the EGF receptor (EGFR) in MDA 468 cells, phosphorylation and activity of extracellular regulated kinases (ERK) 1 and 2 in all three cell lines and Akt phosphorylation induced by several agents in HBL 100 cells. Curcumin inhibited the ability of anisomycin to activate c-jun N terminal kinase (JNK) and MAPK kinase 4 (MKK4), although it did not inhibit activation of p38. Further studies suggested that the target of curcumin upstream of JNK may lie at the MAPK kinase kinase (MEKK) level. EGCG did not inhibit EGFR or ERK phosphorylation. Instead an increase in phosphorylation was observed. Nor did this agent inhibit JNK activity in pre-treated cells, although inhibition was observed when it was added directly to the assay. Similarly EGCG was a potent inhibitor of p38 activity in vitro, but did not affect its ability to activate the downstream kinase MAPK activating protein kinase-2 (MAPKAP K2) when added to cells. I3C had no effect on ERK phosphorylation, JNK or p38 activity. The global effect of curcumin treatment (10µM) on transcription was investigated using cDNA microarrays. Several genes were shown to be affected, including downregulation of a number of matrix metalloproteinases.

Results suggest that the three agents mediate their growth inhibitory effects through very different molecular targets. The mode of action of curcumin may include components of the ERK and JNK pathways, while activation of the ERK pathway by EGCG may be involved in some tumour cells. I3C had no effect on any of the pathways investigated so its inhibitory effects must be exerted through a different mechanism.

ACKNOWLEDGEMENTS

I would like to thank my two supervisors, Dr Maggie Manson and Dr Sally Prigent for their excellent supervision and continuous support throughout this project.

Secondly I am grateful to Dr Martin Dickens (University of Leicester) for his constructive advice as a member of my committee and for providing me with some of the initial materials used during the project. Thankyou to many of my colleagues in CMHT who made my time in Leicester a good one, and especially to the members of TACD who made the lab a pleasure to work in.

A final word for Jane Atkins, whose constant support throughout the final two years of my project and in the preparation of this thesis was all the more appreciated since she was preparing her own at the same time, and for my parents whose financial support and encouragement over the last three years proved invaluable.

CONTENTS

~

TITLE		I
ABSTRACT	I	I
ACKNOWLEDGEMENT	5	I
CONTENTS		1
FIGURES AND TABLES	Χ	Ś
ABBREVIATIONS	XIV	7
CHAPTER 1. INTRODU	2 TION 1	l
1.1 Overview of chemop	evention1	L
1.1.1 Background	1	L
1.1.2 The carcinogenic	process	2
1.1.3 Epidemiological	vidence for the efficacy of dietary chemopreventive	3
compounds		
1.1.4 The design of clin	ical trials for chemopreventive compounds	1
1.1.5 Potential problem	s associated with chemopreventive drugs	5
1.2 The dietary chemop	eventive agents of interest	7
1.2.1 Source and struct	re	7
1.2.1.1 Curcumin.	7	
1.2.1.2 Epigallocate	hin-3-gallate (EGCG). 8	
1.2.1.3 Indole-3-car	inol (I3C). 9	
1.2.2 Epidemiological	vidence for the efficacy of the agents10)
1.2.2.1 Curcumin.	10	
1.2.2.2 EGCG.	11	
1.2.2.3 I3C.	12	
1.2.3 Animal studies of	chemopreventive efficacy12	2
1.2.3.1 Curcumin.	12	
1.2.3.2 EGCG.	15	
1.2.3.3 I3C.	17	
1.2.4 Human studies for	chemopreventive efficacy)
1.2.4.1 Curcumin.	19	
1.2.4.2 EGCG.	19	

1.2.4.3 I3C.	20	
1.2.5 Proposed molecu	lar effects of the chemopreventive agents	20
1.2.5.1 Curcumin.	20	
1.2.5.2 EGCG.	21	
1.2.5.3 I3C.	23	
1.2.6 Metabolism of th	e chemopreventive compounds and identification of the act	ive
molecules		25
1.2.6.1 Curcumin.	25	
1.2.6.2 EGCG.	26	
1.2.6.3 I3C.	27	
1.3 Signal transduction	pathways	
1.3.1 Receptor protein	tyrosine kinases	
1.3.2 The extracellular	regulated kinase (ERK) pathway	
1.3.3 The c-jun N term	inal kinase (JNK) pathway	
1.3.4 The p38 pathway	,	
1.3.5 The phosphatidyl	inositol 3-kinase (PI3K)/Akt pathway	40
1.3.6 Crosstalk betwee	n signalling pathways	43
1.3.7 The cell cycle		
1.3.8 Apoptosis		45
1.3.7 The role of the si	gnalling components in cell growth, apoptosis and carcinog	enesis.
		47
1.4 Breast cancer chemo	oprevention and related signalling pathways	51
1.4.1 Epidemiological	evidence	51
1.4.2 Signalling and br	east cancer	51
1.4.3 Current methods	of breast cancer prevention	53
CHAPTER 2. MATER	IALS AND METHODS	54
2.1 Materials		
2.1.1 General chemical	Is and reagents.	54
2.1.2 Chemopreventive	e agents and inducers of signal transduction.	54
2.1.3 Antibodies		
2.1.4 Radiochemicals		55
2.1.5 Kits		55
2.1.5 Plasmids		55

-

2.1.6 Bacterial strains and growth media.	55
2.1.7 Eukaryotic cells	56
2.1.8 Cell culture media and supplements	56
2.2 Methods	57
2.2.1 Growth and maintenance of cell culture	57
2.2.1.1 Resuscitation of cells from liquid nitrogen. 57	
2.2.1.2 Subculturing of cell lines. 57	
2.2.1.3 Storage of cells. 58	
2.2.2 Analysis of protein	58
2.2.2.1 Protein concentration determination (Biorad assay). 58	
2.2.2.2 Protein concentration determination (Pierce assay)	58
2.2.2.3 One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE)	59
2.2.2.4 Western blot analysis.	61
2.2.3 Isolation and purification of plasmid DNA.	62
2.2.3.1 Buffers and solutions contained in the Promega Wizard™maxiprep kit.	62
2.2.3.2 Protocol for plasmid DNA extraction and purification.	62
2.2.4 Isolation and extraction of RNA from mammalian cells	63
2.2.4.1 Buffers and solutions contained in the Qiagen RNeasy $^{\text{TM}}$ kit. 63	
2.2.5.2 Protocol for RNA extraction and purification. 63	
2.2.5 Quantification of nucleic acid concentration	63
2.2.6 Analysis of nucleic acids (DNA) on an agarose gel	64
2.2.6.1 Gel preparation. 64	
2.2.6.2 Sample preparation and running the gel. 64	
2.2.7 Preparation of the GST-jun fusion protein.	65
2.2.7.1 Bacterial cell transformation. 65	
2.2.7.1 Expression and purification of the GST-jun fusion protein. 65	
2.2.8 Mammalian cell transfection.	66
2.2.9 Determination of cell growth characteristics	66
2.2.9.1 Quantitation of cell growth. 66	
2.2.9.2 Cell cycle analysis. 66	
2.2.9.3 Determination of apoptosis. 66	
2.2.10 Measurement of intracellular ATP.	67
2.2.11 Measurement of EGFR and ERK phosphorylation.	67

2.2.12 Nuclear protein extraction and western	blotting for c-jun and c	:-fos68
2.2.13 Immune-complex kinase assays		69
2.2.13.1 Assays for ERK, JNK, p38 and MA	PKAP K2 activities.	69
2.2.13.2 Assays for MKK4 activity.		70
2.2.13.3 Assays for MEKK1 activity.		70
2.2.14 Protein tyrosine phosphatase assay		71
2.2.14.1 Buffers and assay components.	71	
2.2.14.2 Phosphatase assay protocol.	71	
2.2.15 Microarray analysis.		73
2.2.15.1 Buffers and solutions.	73	
2.2.15.2 Microarray hybridisation protocol.	73	
2.2.17 Statistical analysis.		75
CHAPTER 3. EFFECTS OF CHEMOPREVEN	NTIVE AGENTS ON	CELL GROWTH
AND THE CELL CYCLE.	•••••	76
3.1 Introduction	•••••	76
3.2 Results	••••••	78
3.2.1 Curcumin induced a G2/M cell cycle arre	est and apoptosis	78
3.2.2 EGCG induced apoptosis but not a defin	ite pattern of cell cycle	e arrest82
3.2.4 I3C did not cause cell cycle arrest		85
3.3 Discussion	•••••	
CHAPTER 4. EFFECTS OF CHEMOPREVEN	TIVE AGENTS ON	THE EGFR/ERK
MAPK PATHWAY.	•••••	
4.1 Introduction	•••••	
4.2 Results	••••••	96
4.2.1 EGFR levels in MDA 468, HBL 100 and	T47D cell lines	96
4.2.2 Curcumin inhibits the EGFR/ERK signal	ling pathway	97
4.2.3 Curcumin inhibits PKB/Akt pathway		
4.2.4 EGCG activates components of the EGFI	R/ERK pathway	
4.2.5 I3C does not inhibit the ERK pathway		110

5.1 Introduction	•••••	11
5.2 Results	••••••	12
5.2.1 Curcumin inhibited the JNK pathway.	••••••	12
5.2.1.1 Curcumin inhibited JNK activity.	121	
5.2.1.2 Curcumin inhibited MKK4 activity.	125	
5.2.1.3 Curcumin inhibited MEKK1 activity.	128	
5.2.1.4 Curcumin inhibited MEKK3-induced JNK activity.	131	
5.2.2 Effect of EGCG on the JNK pathway	•••••	13
5.2.3 I3C did not inhibit the JNK pathway		13
5.2.4 Curcumin did not inhibit the p38 pathway	•••••	13
5.2.5 Effect of EGCG on the p38 pathway	••••••	13
5.2.6 I3C did not inhibit the p38 pathway		14
5.2.6 I3C did not inhibit the p38 pathway		

CHAPTER 6. AN INVESTIGATION INTO THE EFFECT OF CURCUMIN ON

GENE TRANSCRIPTION USING cDNA MICROARI	RAYS	
6.1 Introduction		
6.1.1 Gene array technology		146
6.1.2 Applications of gene array technology		147
6.1.2.1 General approaches.	147	
6.1.2.2 Transcriptional programmes.	147	
6.1.2.3 Gene function and drug discovery.	147	
6.1.2.4 Disease diagnosis, prognosis and therapy.	148	
6.1.2.5 Deciphering complex biological systems.	148	
6.1.3 Advantages and disadvantages of microarray te	chnology	148
6.2 Methodology for cDNA microarrays		
6.3 Results		
6.4 Discussion		
6.4.1 Genes related to signal transduction		
6.4.2 Miscellaneous genes with altered expression		
6.4.3 Phase I/II detoxifying enzymes.		164
6.4.4 Matrix metalloproteinases (MMPs)		167

CHAPTER 7. SUMMARY AND CONCLUSIONS	
REFERENCES	
APPENDIX	207

.

-

FIGURES AND TABLES

FIGURES

Chapter 1.

1.1 - Mechanism of carcinogenesis.	2
1.2 - Chemical structure of curcumin.	7
1.3 - Chemical structures of the green tea polyphenols.	9
1.4 - Chemical structure of I3C.	10
1.5 - Chemical structures of curcumoids and curcumin metabolites.	26
1.6 - Chemical structures of the acid condensation products of I3C.	29
1.7 - Structure of the EGFR.	32
1.8 - Simplified scheme of the MAPK signal transduction pathways.	39
1.9 - Proposed mechanism of activation of NF- κ B by the PI3K/Akt pathway.	42
1.10- Simplified scheme of the eukaryotic cell cycle.	44

Chapter 2.

2.1 - Western blotting apparatus.	61
-----------------------------------	----

Chapter 3.

3.1 - Inhibition of cell growth by curcumin.	79
3.2 - Cell cycle analysis of cells treated with curcumin.	80
3.3 - Annexin V staining of curcumin treated cells.	81
3.4 - FACS profiles of cells treated with EGCG.	83
3.5 - Annexin V staining of EGCG treated cells.	84
3.6 - FACS profiles of cells treated with I3C.	85
3.7 - Western blot for CDK6 in cells treated with I3C.	86

Chapter 4.

4.1 - Levels of EGFR protein expression in the breast cell lines of interest.	96
4.2 - The effect of curcumin on EGFR phosphorylation as determined by western	98
blotting.	

4.3 - Effect of curcumin on ERK1/2 phosphorylation as determined by western blotting. 99

4.4 - Western blot for ERK1/2 protein levels in curcumin treated cells.	100
4.5 - The effect of curcumin pre-treatment of cells on ERK1/2 activity.	100
4.6 - The effect of curcumin on TPA-stimulated ERK1/2 phosphorylation.	101
4.7 - The effect of curcumin pre-treatment on the expression of c-fos.	102
4.8 - The effect of curcumin on PKB/Akt phosphorylation as induced by a number of	103
agents.	
4.9 - Western blots showing the effect of EGCG on components of the EGFR/ERK	104
pathway.	
4.10- Effect of EGCG on ERK1/2 activity.	105
4.11- The effect of EGCG on tyrosine phosphorylation in whole cell lysates.	106
4.12- The influence of EGCG on the time course of EGF-induced EGFR	107
phosphorylation.	
4.13- Protein tyrosine phosphatase activity in EGCG treated cells.	109
4.14- In vitro protein tyrosine phosphatase assays in the presence of EGCG.	109
4.15- Western blot showing the effect of I3C on ERK1/2 phosphorylation.	110
4.16- The effect of curcumin pre-treatment on intracellular ATP levels.	111
4.17- The effect of EGCG pre-treatment on intracellular ATP levels.	111

Chapter 5.

5.1 - Effect of curcumin on JNK activity.	
5.2 - Western blot showing expression of JNK in curcumin treated cells.	
5.3 - Direct addition of curcumin to the <i>in vitro</i> JNK kinase assay.	123
5.4 - Western blots showing the expression and phosphorylation of c-jun from curcumin	124
treated cells.	
5.5 - In vitro kinase assays showing the effect of curcumin on MKK4 activity.	126
5.6 - Western blots showing the expression of MKK4 in curcumin-treated cells.	127
5.7 - Direct addition of curcumin to the in vitro MKK4 kinase assay.	127
5.8 - Direct addition of curcumin to the MEKK1 in vitro kinase assay (I).	129
5.9 - Direct addition of curcumin to the MEKK1 in vitro kinase assay (II).	130
5.10- The effect of curcumin on MEKK3-induced JNK and ERK activity.	132
5.11- In vitro kinase assays showing the effect of EGCG on JNK activity.	133
5.12- Direct addition of EGCG to the <i>in vitro</i> JNK kinase assay.	134
5.13- Western blot showing the expression of c-jun in EGCG treated cells.	135
5.14- In vitro kinase assays showing the effect of I3C on JNK activity.	136

5.15- In vitro kinase assays showing the effect of curcumin cell treatment and direct	
addition to the p38 kinase assay.	
5.16- In vitro kinase assays showing the effect of EGCG cell treatment on p38 activity.	138
5.17- The effect of direct addition of EGCG to in vitro p38 kinase assays.	139
5.18- In vitro kinase assays showing the effect of EGCG on MAPKAPK2 activity.	140
5.19- In vitro kinase assays showing the effect of I3C on p38 activity.	141

Chapter 6.

6.1 -Scheme for detection of altered gene expression using microarrays.	151
6.2 -An example of a scatter plot including signals from all the genes in the microarray.	153
6.3 -Microarray analysis Ia.	154
6.4 -Microarray analysis Ib.	156
6.5 - Microarray analysis IIa.	157
6.6 - Microarray analysis IIb.	158
6.7 - Microarray analysis IIIa.	159
6.8 - Microarray analysis IIIb.	160
6.9 - Pathways responsible for the activation and detoxification of B[a]P.	1 6 4

TABLES

Chapter 1.			
1.1 - The EGFR family of receptors and their ligands.	32		
Chapter 2.			
2.1 - Eukaryotic cell media and supplements.	57		
2.2 - Recipes for SDS PAGE gels.			
Chapter 3.			
3.1 - The induction of apoptosis by curcumin as determined by annexin V staining.			
Chapter 6.			
6.1 - Genes affected by curcumin as determined by microarray analysis Ia.	154		
6.2 - Genes affected by curcumin as determined by microarray analysis Ib.	156		

6.3 - Genes affected by curcumin as determined by microarray analysis IIa. 157

6.4 - Genes affected by curcumin as determined by microarray analysis IIb.	158
6.5 - Genes affected by curcumin as determined by microarray analysis IIIa.	159
6.6 - Genes affected by curcumin as determined by microarray analysis IIIb.	160
6.7 - Treeview analysis of microarray data.	161

.

~

.

ABBREVIATIONS.

--

Α	absorbance
AFB ₁	aflatoxin B ₁
Ah	aryl hydrocarbon
AKR	aldoketoreductase
Anti-BPDE	(±)-anti-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene
Anti-HA	anti-hemagglutinin
AOM	azoxymethane
AP-1	activator protein 1
APOB	apolipoprotein B
APS	ammonium persulphate
ASK	apoptosis stimulating kinase
ATBC	alpha-tocopherol beta-carotene
ATF2	activating transcription factor 2
ATP	adenosine 5'-triphosphate
b	bases
B[a]P	benzo[a]pyrene
Bcl-2	B-cell lymphoma/leukaemia-2
BPQ	benzo[a]pyrene-7,8-dione
BSA	bovine serum albumin
CARET	beta-carotene and retinol efficacy trial.
CASP1	caspase 1
CDK	cyclin dependent kinase
CFATE	curcumin-free aqueous turmeric extract
CKI	CDK inhibitor
COX	cyclooxygenase
cpm	counts per minute
CSF-1	colony stimulating factor-1
CTR	5,6,11,12,17,18-hexahydrocyclononal[1,2-b:4,5-b:7,8-b]triindole
Cyp1A1	cytochrome p450 1A1
Cyp1B1	cytochrome p450 B1
DAG	diacylglycerol

DDH1	dihydrodiol dehydrogenase 1
DDX5	dead/H box polypeptide 5
dH ₂ O	distilled water
DIM	diindol-3-ylmethane
DMBA	7,12-dimethylbenz[a]anthracene
DMEM	Dulbecco's modified Eagle's medium
1,2-DMH	1,2-dimethylhydrazine
DMSO	dimethyl sulfoxide
ds	double stranded
DTT	dithiothreitol
EC	epicatechin
ECG	epicatechin-3-gallate
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetracetate
EGC	epigallocatechin
EGCG	epigallocatechin-3-gallate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ENNG	N-ethyl-N-nitro-N-nitrosoguanidine
ER	oestrogen receptor
ERK	extracellular regulated kinase
ETE	ethanolic turmeric extract
FACS	fluorescence activated cell sorting
FADD	Fas associated death domain protein
FCS	fetal calf serum
FGF-2	fibroblast growth factor-2
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
FRK	fos-regulating kinase
G ₁	gap 1 (phase of cell cycle)
G ₂	gap 2 (phase of cell cycle)
GAP	GTPase activating protein
GCK	germinal centre kinase

--

GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GLK	GCK-like kinase
Gln	glutamine
Gly	glycine
GSK3	glycogen synthase kinase 3
GST	glutathione-S-transferase
GTP	guanine triphosphate
HMOX	heme-oxygenase
HPK1	haematopoetic progenitor kinase 1
HPLC	high performance liquid chromatography
4-HPR	N-(4-hydroxyphenyl)retinamide
HPV16	human papillomavirus type 16
HRP	horse radish peroxidase
HSP	heat shock protein
4HT	4-hydroxy tamoxifen
ІкВ	inhibitory kinase B
i.p.	intra-peritoneal
I3C	indole-3-carbinol
IC ₅₀	concentration for 50% inhibition
ICZ	indolo[3,2-b]carbazole
IDI1	isopentenyl diphosphate delta isomerase
IKK	IkB kinase
IL-1β	interleukin-1 ^β
IQ	2-amino-3-methylimidazol[4,5-f]quinoline
JIP	JNK interacting protein
JNK	c-jun N terminal kinase
kb	kilobases
kDa	kilo dalton
LDL	low density lipoprotein
LOX	lipoxygenase
LPS	lipopolysaccharide
LTR	2,3 bis[3-indolylmethyl]indole

~

Μ	mitosis
MAPK	mitogen activated protein kinase
МАРКАР К2	MAPK activating protein kinase 2
MAPKKK	MAPK kinase kinase
MAPKKKK	MAPKKK kinase
MBP	myelin basic protein
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase = MAPKKK
MGSTIL1	mitochondrial glutathione-S-transferase (also known as PIG12)
MKK	MAPK kinase ≡ MEK
МКР	MAPK phosphatase
MLK	mixed lineage kinase
MMP	matrix metalloproteinase
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
MNU	methylnitrosourea
N/D	not determined
NDEA	N-nitrosodiethylamine
NDF	neu differentiating factor
NF-ĸB	nuclear factor kappa B
NGF	nerve growth factor
NHEK	normal human embryonic keratinocyte
NIK	NF-κB inducing kinase
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NRG1	neuregulin (also known as heregulin α)
NTRK1	neurotrophic tyrosine kinase receptor type 1
ODC	ornithine decarboxylase
PAH	polycyclic aromatic hydrocarbons
PAK	p21 activated kinase
PBS	phosphate buffered saline
PCA	perchloric acid
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor

·

SSPE	disodium phosphate EDTA
STAT	signal transducer and activator of transcription
TAE	tris acetate EDTA
TAF2N	TATA box binding protein-associated factor, RNA polymerase II N
TAK1	transforming growth factor-β-activated kinase-1
TBST	tris buffered saline-Tween 20
TCA	trichloroacetic acid
TE	tris/EDTA
TEMED	N,N,N',N'-tetramethylenediamine
Thr	threonine
TIMP	tissue inhibitor of matrix metalloproteinase
TNFα	tumour necrosis factor α
TNFR	tumour necrosis factor receptor
TPA	tetradecanoylphorbol-13-acetate
TRADD	TNFR associated death domain protein
TRAF2	TNFR associated factor 2
Tx-100	triton X-100
Tyr	tyrosine
UV	ultraviolet
+ve	positive
-ve	negative
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein
WEGT	water extract of green tea

CHAPTER 1. INTRODUCTION

1.1 Overview of chemoprevention.

1.1.1 Background.

At the current time four strategies are used to combat cancer: 1) prevention, 2) early diagnosis and intervention, 3) treatment of localised cancer and 4) management of non-localised cancer (Challa et al. 1997). The optimal way of dealing with any disease is by prevention, ideally by individually tailoring the treatment to the problem. This is particularly true of cancer where treatments are often ineffective, or difficult and unpleasant. Chemoprevention may be defined as the use of natural or synthetic compounds to block, reverse or prevent the development of invasive cancers (Hong and Sporn 1997; Shureiqi et al. 2000). Common epithelial cancers such as colon, breast, prostate and lung carcinomas remain the dominant cause of cancer deaths in the United States (Landis 1999) and Western Europe (World Cancer Research Fund 1997). Overall mortality rates for such cancers have not declined in the past 25 years (Hong and Sporn 1997). Cancers arise from interactions between genetic and environmental factors (Hong and Sporn 1997). Correction of genetic mutations that predispose individuals to cancer is not, as yet, possible. The environmental risk factors remain poorly defined for many forms of this disease (Gescher et al. 1998; Osborne 1997) but, for those in which they are identified, avoidance of exposure through changes in working practice and general lifestyle has proved a successful tactic in preventing or delaying the onset of disease. Chemoprevention provides an additional strategy to decrease cancer incidence.

In order to be suitable for human consumption, a chemopreventive agent should, ideally, have the following properties: a) little or no adverse effects, b) high efficacy against multiple sites, c) effectiveness at achievable dose level, d) capability of oral consumption, e) a known mechanism of action, f) low cost, g) history of use by the human population and h) general human acceptance. The use of chemopreventive agents in combination may increase potency and reduce toxicity (Hong and Sporn 1997; Kelloff *et al.* 1996). Preliminary trials are being conducted with combinations of agents that can act to promote differentiation, reduce cell proliferation and induce apoptosis (reviewed in Kelloff *et al.* 1996).

1

1.1.2 The carcinogenic process.

Carcinogenesis is a multistep process that can be divided into three basic phases: initiation, promotion and progression (Figure 1.1).





Initiation of carcinogenesis is rapid and often involves direct binding of the carcinogen to DNA, resulting in mutation. If this damage is not repaired, promotion can follow, where initiated cells proliferate under the influence of mitogens. Progression to a tumour occurs and genotypically and phenotypically altered cells emerge (reviewed in Shureiqi *et al.* 2000). Epithelial carcinogenesis is associated with multiple mutations which occur in a sequential manner, resulting in changes to signal transduction pathways, transcription and apoptosis. This creates an environment in which further mutations occur and large numbers are accumulated throughout the process of carcinogenesis (Shureiqi *et al.* 2000).

Many stages involved in carcinogenesis offer targets for chemopreventive agents to halt, slow down or possibly reverse the process. Compounds that inhibit initiation of carcinogenesis by preventing DNA damage are known as blocking agents. These are particularly effective if administered prior to the carcinogen. Mechanisms of action of blocking agents include alterations to levels of phase I and II drug metabolising enzymes, altered rates of DNA repair and scavenging of reactive oxygen and other free radical species (reviewed in Manson *et al.* 2000). Agents which act at the stages subsequent to initiation are known as suppressing agents. Compounds may suppress the progression of an initiated cell to a tumour through a number of mechanisms: they may scavenge reactive oxygen species, modulate signal transduction pathways, inhibit arachidonic acid metabolism, modulate hormonal/growth factor activity, inhibit polyamine metabolism, inhibit oncogene function or induce terminal differentiation or apoptosis. Indeed individual agents may possess more than one function (Gescher *et al.* 1998; Shureiqi *et al.* 2000).

1.1.3 Epidemiological evidence for the efficacy of dietary chemopreventive compounds.

Diet and cancer are closely linked. There is evidence that around one third of the 500 000 cancer-related deaths that occur annually in the US are due to dietary factors which is equivalent to the number of cancer deaths related to smoking. If cigarette smoking were absent, dietary patterns and physical activity would become the predominant factors in determining cancer risk (Hebert and Miller 1988).

It must be recognised that the risk from dietary factors is not solely due to the nutrient content of the food we eat. The types of food, food preparation, portion sizes, food variety and overall calorific balance can all contribute to the relative risk of developing a tumour. Particular dietary constituents have been shown to have a strong association with cancer causation or prevention (Challa *et al.* 1997).

Animal studies and epidemiological evidence support the role of fat in cancer promotion. Particular correlations are seen between dietary fat and cancers of the breast, prostate, colon and rectum. The type of fats may well be important and it is saturated fats that are most often implicated in this deleterious effect. Some unsaturated fats found in certain fish may actually protect against carcinogenesis. Red meat consumption and colon cancer as well as alcohol intake and liver cancer have also been strongly linked (reviewed in Challa *et al.* 1997).

Many of these dietary associations have been investigated by epidemiological studies in migrant populations which show that those moving from a low risk to a high risk area acquire the cancer risk of their adopted country for a number of different cancers. Migrants from Japan to the USA showed a trend in stomach cancer mortality from high risk in Japan to low risk in the USA. The reverse was true for colon cancer with the originally low risk Japanese population achieving the high rates common in the US within two generations (reviewed in Hakama *et al.* 1996). This strongly suggests that such changes were due to environmental

exposure, which would certainly include dietary habits, since these are very different between the countries. Within countries correlations were observed between consumption of certain foodstuffs and increased cancer risk (reviewed in Challa *et al.* 1997). In some cases these correlations were as high as that between smoking and lung cancer which is one of the strongest dose-response relationships within epidemiology.

In a consideration of the epidemiological evidence for involvement of diet in disease prevention, a review of 200 studies examined the relationship between fruit and vegetable intake and a variety of human cancers. Out of 156 of those studies, which expressed the results in terms of relative risk of developing cancer, 128 showed a statistically significant protective effect of fruit and vegetable consumption. Only 4 demonstrated the inverse relationship (Block *et al.* 1992). Such evidence led to the public health advice that five servings of fruit or vegetables each day would constitute some protection from developing cancer (Helzlsouer *et al.* 1994). It remains unclear, however, which nutrients in particular are responsible for these positive effects and indeed it may well be a case of a number of nutrients which act together resulting in an optimal effect. As studies have moved from investigating the effects of overall diet, to particular foodstuffs and on to individual nutrients the correlations between intake and protective effect are often diminished (Helzlsouer *et al.* 1994).

1.1.4 The design of clinical trials for chemopreventive compounds.

Preclinical models have been developed to assess chemopreventive efficacy. These models have been used in several ways to assess suitability of a chemopreventive compound;

• Biochemical pre-screening assays. These are short-term mechanistic assays, which evaluate a test compound's ability to modulate the process of carcinogenesis. All are *in vitro* assays and include such end points as carcinogen-DNA binding, glutathione-S transferase (GST) induction and tyrosine kinase and ornithine decarboxylase (ODC) inhibition. An example of such a study may be found in Sharma *et al.* (1994).

- *In vitro* efficacy models. These assays test chemopreventive efficacy in 5 epithelial cell lines, which are models for human cancer.
- In vivo short term screening. These assays assess the agent's ability to inhibit the early stages of carcinogenesis in rats and mice.
- Animal efficacy testing. Animal models of chemical carcinogenesis provide reproducible development of tumours upon treatment with the appropriate initiator/promoter regimen. They have been the primary *in vivo* screening tool for chemopreventive agents. Animal models are now available in which genetic mutations result in increased risk of carcinogenesis, as are transgenic models, and these can be used in similar assays (reviewed in Shureiqui *et al.* 2000).

Chemoprevention clinical trials pose a number of challenges in their design. Long term, randomised studies in which the end point is the occurrence of a primary cancer are extremely costly. They also require thousands of subjects who must be studied over a number of years before any useful data is obtained. These factors also lead to problems with recruitment, motivation and compliance of the subjects. To circumvent some of these problems, secondary trials have been conducted in patients cured of a primary tumour and some have yielded valuable data. The reversal or arrest of progression of premalignant lesions or the prevention of second primary tumours are studied in such trials, where patient motivation and compliance is much higher and a meaningful endpoint can be observed much sooner (Hong and Sporn 1997).

Another approach is to study surrogate endpoint biomarkers (SEM). These are the intermediate endpoints of carcinogenesis and can be used to predict future cancer development. In terms of clinical studies, they may be used to predict individuals at risk who can then enter a trial, or to evaluate chemopreventive efficacy directly. (Hong and Sporn 1997; Shureiqi *et al.* 2000). Ideal biomarkers have the following characteristics (Lippman et.al 1990):

- 1) Variable expression between phases of carcinogenesis.
- 2) Detectable early in the carcinogenic process.

- 3) Associated with the risk of developing cancer or the occurrence of precancerous lesions.
- 4) Detectable in body fluids.
- 5) Detectable in body tissue by biopsy.
- 6) Suitable for development of adequate quality control procedures.
- 7) Potential for modification by chemopreventive agents.

1.1.5 Potential problems associated with chemopreventive drugs.

Apart from the problems in the design of effective chemopreventive clinical trials and the search for suitable biomarkers there may be potential problems with the chemopreventive agents themselves. Toxicity is not generally perceived as a problem with dietary derived agents since they have been consumed by the general population over a long period of time, often at high doses, with apparently no detrimental effects. However, the suggestion that caution is necessary came from two large-scale clinical trials in the 1990's. Epidemiological evidence suggested that the consumption of diets rich in carotenoids, selenium and vitamins A, C and E were associated with reduced lung cancer risk. On this basis clinical trials with these agents were planned. In 1994 results of the Finnish alpha-tocopherol, beta-carotene (ATBC) trial were published. This primary prevention trial, in 29,133 smokers, showed that whilst a minimal reduction in lung cancer incidence was seen in men that received vitamin E, an 18% higher incidence of lung cancer and an 8% higher mortality total were observed in subjects taking β -carotene alone or together with vitamin (The Alpha Tocopherol, Beta Carotene Cancer Prevention Study Group 1994). In the USA 18,254 smokers, former smokers and workers exposed to asbestos were included in a trial to test the efficacy of β carotene plus retinyl palmitate. This beta-carotene and retinol efficiency trial (CARET) showed a similar incidence of increased lung cancer and was subsequently suspended (Omenn et al. 1996). These data pointed to β -carotene as being a promoter of human carcinogenesis. Additional analysis of the trial data pointed out the possibility that the increase in lung cancer observed in the cohort taking β -carotene was due to continued smoking throughout the period of intervention in a large proportion of the subjects (Biasco and Paganelli 1999; Hakama et al. 1996). It was postulated that high doses of β -carotene could increase metabolic activation of carcinogens in tobacco smoke or have a promotional effect at later points of carcinogenesis. These trials highlight the problems in trial design and the need to identify the exact

mechanism of action of potential agents that would be suitable to administrate to the population as a whole.

Dr Gladys Block said, in 1994, that 'we ought to be moving towards fortifying the food supply with antioxidant micronutrients' (Helzlsouer *et al.* 1994). The argument for such intervention measures is given weight by the statistics that show only 9% of the US population, on any given day, consume five portions of fresh fruit or vegetables. However, until the questions raised by trials such as the ATBC and CARET studies are answered satisfactorily, such intervention, in the case of chemopreventive agents, is still some way off.

1.2 The Dietary Chemopreventive Agents of Interest.

1.2.1 Source and structure.

1.2.1.1 Curcumin.

Curcumin is the major yellow pigment extracted from the spice turmeric (Figure 1.2). Turmeric is used extensively in a wide range of foodstuffs, including curries and mustards, as a colouring and flavouring agent. Turmeric itself is the powdered rhizome of the perennial herb, *Curcuma longa* Linn, which is grown widely in the tropics of Asia.





1.2.1.2 Epigallocatechin-3-gallate (EGCG).

EGCG is the predominant polyphenol found in tea (*Camellia sinensis*). Next to water, tea is the most widely consumed beverage worldwide. The Chinese used tea as a medicinal drink as early as 3000 BC and as a beverage, by the end of the sixth century (Kuroda and Hara 1999). Tea leaves are processed differently to give green, black or oolong tea. Green tea constitutes about 20% of the tea manufactured in the world, oolong around 2% and black tea the remaining 78%. Green tea is mainly consumed in Japan and China and the enzymes contained in tea leaves that cause oxidation of the polyphenols are inactivated at the start of the production process. In black tea production this oxidation is promoted and in oolong tea the oxidation is partial. Due to this production process it is in green tea that the composition of the chemicals, including the polyphenols, thought to be important for its pharmacological activity, is most comparable to the native tea plant (Kuroda and Hara 1999; Yang and Zhi-Yuan 1993).

Most of the green tea polyphenols are flavanols, commonly known as catechins. The major catechins in green tea are EGCG, epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin (EC), gallocatechin and catechin (reviewed in Yang and Zhi-Yuan 1993). The structures of some of the more important compounds in this list are shown in figure 1.3. A 200ml cup of green tea has been shown to contain 142mg EGCG, 65mg EGC, 28mg ECG and 17 mg caffeine (Yang and Zhi-Yuan 1993).



Figure 1.3. Chemical structures of the green tea polyphenols.

1.2.1.3 Indole-3-carbinol (I3C).

I3C is found in cruciferous vegetables including broccoli, brussels sprouts, cabbage and cauliflower (Figure 1.4). In such vegetables it is present as a glucosinolate complex known as glucobrassican, which undergoes autolysis to form I3C. A further indole, indole-3-acetonitrile can also be formed from the glucobrassican precursor, which can make up 1-3% of the dry weight of these vegetables (Michnovicz and Bradlow 1990). In the UK the mean daily intake of glucobrassican from cooked vegetables is estimated at 1.5-3.1 mg/person (Taioli *et al.* 1997).



Figure 1.4. Chemical structure of I3C.

1.2.2 Epidemiological evidence for the efficacy of the agents.

1.2.2.1 Curcumin.

As well as a common ingredient in foodstuffs, curcumin is used extensively in traditional Indian medicine. It has been used in the treatment of sprains and swellings, biliary and hepatic disorders, rheumatism and tumours (reviewed in Ammon and Wahl 1991). Such biological effects may result from the primary pharmacological actions of curcumin as both an antioxidant and antiinflammatory agent. The antioxidant activity arises from its ability to scavenge active oxygen species such as the hydroxyl radical, superoxide anion and singlet oxygen. It also interferes with lipid peroxidation, xanthine oxidase activity and nitrite/nitrogen oxide production. The antiinflammatory activity may result from its effects on arachidonic acid metabolism including inhibition of phospholipase A_2 , cyclooxygenase and lipoxygenase and NF- κ B (reviewed in Chemoprevention Branch and Agent Development Committee 1996; Ammon and Wahl 1991; Kellof *et al.* 1996). Since oxidant stress and prostaglandin biosynthesis have been linked to both the initiation and postinitiation stages of carcinogenesis, its potential as a chemopreventive compound has been investigated by many groups.

Curcumin has been shown to have little or no deleterious effects when administered orally to rats, dogs or monkeys (Chemoprevention Branch and Agent Development Committee 1996). Since large numbers of people, especially in Asia, consume up to 100mg on a daily basis and have done so over a period of many years it is unlikely to have any toxic side-effects in humans (Ciolini *et al.* 1998). The only evidence of possible toxicity due to chronic exposure

was characterised from respiratory symptoms and dermatitis observed in spice factory workers (reviewed in Kelloff *et al.* 1996).

1.2.2.2 EGCG.

A large number of epidemiological studies have examined the relationship between green tea consumption and the development of various cancers (reviewed in Yang and Zhi-Yuan 1993). Taken as a whole these studies have been largely inconclusive with positive, negative or no effect results being reported for individual tissues in different studies. It is thought that many of the unwanted effects observed for green tea result from the very high temperature at which the tea was drunk. The risk of oesophageal cancer was shown to be increased, by the ingestion of tea at 55-67°C. The opposite was observed at lower temperature. Many of these studies take no account of the temperature of tea so the impact of this parameter cannot be estimated. Notable cases where chemoprevention has been observed include a study in the Shizuoka Prefecture in Japan. The cancer death rate, especially from stomach cancer, in this tea-producing region was lower than the national average. Towns where the lowest incidences were observed were those where the highest levels of consumption of green tea was taking place (reviewed in Yang and Zhi-Yuan 1993). The incidence of gastric cancer was shown to be lower in individuals consuming green tea more frequently or in larger quantities in another Japanese study. A general positive effect was established by studying the mortality rate of Japanese tea ceremony teachers. Mortality rates, at age 50-60, among this cohort were surprisingly low compared to Japanese women in general, indicating that green tea may be a protective factor for a number of diseases (reviewed in Kuroda and Hara 1999).

In the case of breast cancer, a study among Japanese women receiving treatment for breast malignancies showed that long term consumption of >5 cups of green tea per day, prior to clinical onset, was associated with lower recurrence of certain breast cancers (Nakachi *et al.* 1998). However, it was shown to have no effect on the recurrence of breast cancer that had been allowed to progress to an advanced stage.

Taken as a whole the consensus of opinion is that green tea does not have any positive carcinogenic effect and that it may well be beneficial in the prevention of a number of

different cancers (Yang and Zhi-Yuan 1993). Once again there are few worries about possible toxicity, since large numbers of people consume large quantities daily without adverse effect.

1.2.2.3 I3C.

The efficacy of indoles from cruciferous vegetables has been demonstrated in a number of epidemiological studies. Increased consumption of cruciferous vegetables by humans was associated with a decreased risk of colon cancer in case control studies carried out in Buffalo and Wisconsin, USA (Graham *et al.* 1972; Young and Wolf 1988), of lung cancer in a study carried out in Louisiana, USA (Schiffman *et al.* 1988) and of cancers of the colon, rectum and bladder in a further case control study undertaken in Louisiana (Graham 1983).

1.2.3 Animal studies of chemopreventive efficacy.

1.2.3.1 Curcumin.

There is extensive evidence that curcumin can inhibit tumourigenesis in a number of different tissues in a host of animal models. Such studies have been carried out predominantly in rats and mice where curcumin has been shown to be effective against cancer models of the skin (Huang *et al.* 1992), duodenum (Huang *et al.* 1994), colon (Huang *et al.* 1994; Kawamori *et al.* 1999; Pereira *et al.* 1996; Rao *et al.* 1995a; Rao *et al.* 1995b), stomach (Huang *et al.* 1994), tongue (reviewed in Chemoprevention Branch and Agent Development Committee 1996) and breast (Deshpande *et al.* 1998; Pereira *et al.* 1997). It has been shown to be less effective against lung cancer (Huang *et al.* 1997).

Considering some of these studies in more detail, topical application of 3 or 10μ M curcumin, to the skin of female CD-1 mice, 5 minutes prior to the application of benzo[a]pyrene (B[a]P) inhibited the formation of DNA adducts by 39% and 61% respectively. In the same study a two stage tumourigenesis model was used, where weekly, topical application of the initiator (B[a]P) to the backs of mice for 10 weeks was followed by promotion with 15nmol tetradecanoylphorbol-13-acetate (TPA) twice weekly for 21 weeks. This resulted in the

formation of 7.1 skin tumours per mouse and 100% of the mice developed tumours. If 3 or 10 μ M curcumin was applied 5 minutes prior to initiation, then the number of tumours per mouse was reduced by 58% or 62% respectively. The percentage of tumour bearing mice was also reduced by 18% or 25%. In a second two stage model where 7,12-dimethylbenz[a]anthracene (DMBA) was used as the initiator, followed by promotion with TPA, topical application of curcumin also inhibited tumourigenesis (Huang *et al.* 1992). These authors showed that curcumin needed to be applied shortly before the initiating agent for it to be effective.

The effect of curcumin in the diet was investigated on B[a]P-induced cancer of the forestomach in A/J mice, N-ethyl-N-nitro-N-nitrosoguanidine (ENNG)-induced duodenal tumourigenesis in C57BL/6 mice and azoxymethane (AOM)-induced colon tumourigenesis in CF-1 mice (Huang *et al.* 1994). Curcumin was given in the diet during the initiation period, during the postinitiation period or throughout the experiment. The inclusion of 0.5-2.0% curcumin in the diet reduced the number of forestomach tumours by 51-53% if administered in the initiation period and by 47-67% if administered postinitiation. ENNG-induced duodenal tumours were inhibited if curcumin was administered postinitiation and AOM-induced colon tumourigenesis was inhibited by dietary curcumin administered either pre-, or post-initiation or pre- and post-initiation. Curcumin can, therefore, have an inhibitory effect during the initiation and promotion/progression stages of carcinogenesis.

The efficacy of curcumin in the inhibition of colon carcinogenesis was demonstrated in four further studies, detailed as follows. AOM was used to induce colon carcinogenesis in male F344 rats. Groups of animals were fed control diet or diet containing 2000 ppm curcumin for two weeks prior to subcutaneous injection of AOM. The dietary administration of curcumin in this manner inhibited the incidence of colon adenocarcinomas, and the number of adenocarcinomas observed per animal. The colonic tumours that were present were decreased in size by 57% compared to those found in animals receiving the control diet (Rao *et al.* 1995a). Male F344 rats were used in a further study to compare the efficacy of curcumin at the initiation and promotion/progression phases of carcinogenesis. AOM was again used to induce colon tumours. Those rats in the initiation study were fed control diets or diets containing 0.2% curcumin for 2 weeks prior to injections of AOM. Animals destined for the promotion/progression study received control diets for 14 weeks after the second injection of

AOM and were then switched to the curcumin diet. After 52 weeks the body weight of the rats had remained unaffected by curcumin. Dietary curcumin inhibited the formation of tumours when administered at both the initiation and post initiation stages. Animals in the promotion/progression study showed a decrease in both tumour incidence and multiplicity (Kawamori *et al.* 1999). The F344 rat, AOM-induced colon tumour model was used in a long-term feeding study with 2000 ppm curcumin and once again similar inhibition of tumourigenesis was observed (Rao *et al.* 1995b). Similarly Pereira et.al (1996) showed administration of curcumin in the diet of F344 rats prior to induction of colon tumourigenesis with AOM inhibited tumour incidence and multiplicity in a dose-dependent fashion. Tumour incidence of 47% and multiplicity of 0.58 \pm 0.12 adenomas/rat in the AOM treated control, were reduced to 19% and 0.22 \pm 0.08 and 0.06% and 0.08 \pm 0.06 in the low (8g/kg) and high (16g/kg) curcumin dose groups.

These authors also investigated the effect of dietary curcumin in the DMBA-induced model of mammary tumourigenesis in female Sprague-Dawley rats. Minimal effects on tumourigenesis were seen when 10 or 20g/kg curcumin was administered to the rats, in their diet, prior to induction of tumourigenesis with DMBA. While no inhibition of tumour incidence or multiplicity was observed, some latency in the development of tumours was recorded. The lack of effect of curcumin was most likely due to its poor absorption through the gut and hence little active compound actually reached the site of tumourigenesis. In contrast in a second study using the Sprague-Dawley rat and DMBA-induced mammary tumourigenesis, effective inhibition of mammary tumourigenesis by curcumin administered in the diet was observed (Deshpande et al. 1998). These authors took turmeric, an ethanolic turmeric extract (ETE) and a curcumin-free aqueous turmeric extract (CFATE) and examined their effects on the initiation and postinitiation phases of mammary carcinogenesis. Administration of 1% turmeric in the diet of rats during the initiation phases of DMBAinduced tumourigenesis brought about a 47% reduction in tumour multiplicity, an 80% reduction in tumour burden (tumour mass), and a 50% reduction in tumour incidence. Simultaneous addition of 0.05% ETE to the turmeric extract brought about further inhibition. However, simultaneous addition of 1% CFATE did not alter any of the parameters used to measure tumourigenesis. Administration of 1% turmeric in the diet of rats during the postinitiation phase reduced tumour burden by 74% without lowering the tumour multiplicity or incidence. When 0.05% ETE was fed to rats during the postinitiation phase, tumour multiplicity was reduced by 41% and the tumour burden by 70%, without affecting tumour

14

incidence. 1% CFATE was also capable of reducing tumour multiplicity when administered during the postinitiation period. In conclusion the turmeric and ETE extracts, which both contain curcumin, showed chemopreventive activity. The CFATE had only weak chemopreventive activity and these data suggest that it is the curcumin which is the major, active chemopreventive agent in turmeric. Once again positive effects were observed if the chemopreventive extracts were administered either during initiation or afterwards. This study might have been more effective than the first described due to an increased uptake of curcumin in the ethanolic formulation.

1.2.3.2 EGCG.

Green tea extracts or purified components thereof, have been shown to have anticarcinogenic activity in a wide variety of animal models for a number of different tissues. They have been shown to be effective in inhibiting tumourigenesis in the oesophagus, forestomach, stomach, duodenum/small intestine, colon, lung, liver, pancreas, mammary and skin (reviewed in Conney *et al.* 1998).

Examples of such investigations include a study of the effect of EGCG on N-methyl-N-nitro-N-nitrosoguanidine (MNNG) induced cancer of the rat glandular stomach. Male Wistar rats were given MNNG for 28 weeks. The experimental groups were then given 0.05% EGCG in drinking water for a further 15 weeks. Rats were sacrificed and tumours of the glandular stomach characterised. The tumour incidence was 62% in MNNG control rats compared to 31% in those receiving MNNG plus EGCG. EGCG also reduced the number of tumours per animal from 0.88 to 0.43, but had no effect on the size of those tumours which did develop (Yamane *et al.* 1995).

EGCG was shown to inhibit tumour promotion in the gastrointestinal tract of the mouse (Fujita *et al.* 1989). Male C57/BL6 mice were given ENNG orally for four weeks followed by a solution of 0.005% EGCG or tap water, for a further 12 weeks. The mice were sacrificed and the upper digestive tract examined for tumours. EGCG reduced ENNG-induced tumour incidence in the duodenum from 63% to 20%. The total number of tumours per group was also reduced from 19 to 5 and the average number of tumours per mouse from 1.2 to 0.3.

A large study, with 300 Kumming mice, showed that both catechin and EGCG were capable of inhibiting tumours of the large intestine induced by 1,2-dimethylhydrazine (1,2-DMH). Groups of animals received 1,2-DMH by injection throughout the experiment along with catechin (1mg or 2mg/mouse), EGCG (2mg/mouse), or vehicle control by stomach perfusion. Both concentrations of catechin and EGCG reduced the formation of intestinal cancers from an incidence of 80% in the 1,2-DMH control to around 36% for each of the polyphenol treatments (Pingzhang *et al.* 1994).

Lung and forestomach tumourigenesis were investigated in A/J mice. Treatment of mice with B[a]P caused lung tumours in 100% of the animals with an average of 8.7 tumours per mouse. Feeding of a water extract of green tea (WEGT) caused a 24% reduction in lung tumour incidence and a 56% reduction in tumour multiplicity. B[a]P administered in this way also induced forestomach tumours and tumourigenesis of these cancers was also inhibited by feeding of WEGT (Wang *et al.* 1992). N-nitrosodiethylamine (NDEA) was also used as an inducer of tumourigenesis in the same study. Administration of WEGT caused a 30% reduction in lung tumour incidence and a 55% reduction in lung multiplicity. Once again NDEA also induced forestomach tumours and these were inhibited by administration of WEGT.

Lung tumourigenesis was induced by treatment with 4-(methylnitrosamino)-1-(3-pyridyl)-1butanone (NNK) in female A/J mice. Tumours occurred in 100% of mice with 9.6 adenomas per mouse. If 0.6% decaffeinated green tea extracts were given as the source of drinking water, either during initiation or in the period after initiation by NNK, then tumourigenesis was significantly inhibited. In each case tumour multiplicity was more sensitive to treatment with the tea extract than tumour incidence (Shi *et al.* 1994).

As with curcumin these animal studies show EGCG and other tea polyphenols have a wide spectrum of chemopreventive efficacy and are capable of inhibiting both the initiation and the promotion/progression phases of carcinogenesis.

1.2.3.3 I3C.

Large numbers of animal studies have been undertaken to show the efficacy of I3C in models of tumourigenesis. The number of DMBA-induced mammary tumours was decreased when rats were fed diets containing 20% brussels sprouts during the initiation and promotion phases of carcinogenesis (Stoewsand *et al.* 1988). Similar inhibitory effects of diets high in cruciferous vegetables were demonstrated in other studies in rats (Stoewsand *et al.* 1989; Wattenburg 1978). I3C has been shown to inhibit tumourigenesis in the case of spontaneous mammary tumours as well as DMBA and methylnitrosourea (MNU)-induced mammary carcinogenesis in mice, and forestomach cancer in the rat (reviewed in Bradlow *et al.* 1999). I3C was also shown to inhibit tumourigenesis in rat tongue (Tanaka *et al.* 1992) and rat liver (Manson *et al.* 1998).

The chemopreventive properties of I3C on the inhibition of DNA adduct formation by the dietary carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was investigated in female F344 rats. Animals received I3C (0.02% or 0.1%) in their diet for 2 weeks prior to the addition of the carcinogen. DNA adduct levels in mammary epithelial cells, colon, liver and white blood cells were significantly reduced in those animals receiving I3C in the diet (He *et al.* 1997). These observations were confirmed in a similar study which also looked at the induction of DNA adducts by a second dietary carcinogen, 2-amino-3-methylimidazol[4,5-f]quinoline (IQ) in female Sprague Dawley rats. DNA adduct formation was inhibited by the addition of I3C to the diet most effectively in the liver, stomach, mammary gland and spleen. No effect was seen in the lungs, kidney or pancreas (He and Schut 1999).

Mouse skin tumourigenesis was shown to be inhibited by topical application of 250µg of I3C (Srivastava and Shukla 1998). Swiss albino mice were injected with a sub-carcinogenic dose of DMBA to initiate carcinogenesis. The I3C was applied topically to the skin along with 5µg TPA twice weekly for 28 weeks. I3C reduced both the number of animals with tumours and the number of tumours per mouse. Tumour latency was also increased as was the size of tumours which did appear. Fourty four percent of male and 27% of female mice remained tumour free throughout the experiment, whereas a 100% incidence of tumourigenesis was
observed in the control group. This study is an example of inhibition of tumour promotion by I3C.

Mice expressing the transgene for human papillomavirus type 16 (HPV16) develop cervical cancer when given 17β -oestradiol. In a group of mice, fed a diet containing 2000ppm I3C and treated with oestradiol, only 2 out of a group of 24, developed cervical-vaginal cancer, compared to 19 out of 25 in the control group (Jin *et al.* 1999).

Several studies have investigated mammary tumourigenesis. In a long term feeding experiment, over a period of 8 months, C3H/OuJ mice were administered I3C in the diet at 500 or 2000 ppm. Spontaneous mammary tumour incidence and multiplicity were significantly reduced at both dose levels and tumour latency was increased in the high dose group (Bradlow et al. 1991). Mammary carcinogenesis was chemically induced by DMBA in female Sprague-Dawley rats. Doses of 50 and 100mg of I3C per day were highly effective if administered throughout the initiation and promotion phase or solely in the initiation phase. A 91-96% reduction in cancer multiplicity was observed. Similar results were obtained if mammary tumours were initiated by MNU where a 65% reduction in tumour multiplicity was observed (Grubbs et al. 1995). An interesting aside was highlighted in a study by Mallov et.al (1997). These authors confirmed the protective effect of I3C in the appearance of spontaneous mammary tumours in MMTV and Balb/cf3H mice. They also observed a protective effect of standard laboratory chow (food) as opposed to the high fat AIN76A diet, which is used to simulate the western diet in such experiments.

The inhibition of liver carcinogenesis has also been demonstrated. The effect of 0.5% I3C in the diet was investigated on aflatoxin B₁ (AFB₁)-induced hepatocarcinogenesis. I3C inhibited tumour formation in F344 rats, whether it was administered before AFB₁ or 6 weeks post AFB₁ treatment (Manson *et al.* 1998). This is good evidence that I3C can have efficacy at both the initiation and post-initiation phases. Other authors (Bailey *et al.* 1987) have reported that I3C inhibits hepatocarcinogenesis in rainbow trout if it is administered before, or together with, AFB₁. However these authors demonstrated the reverse if I3C was administered during the promotion phase, claiming that an increase in tumour formation was seen in I3C-treated fish.

The picture, in rodents at least, is fairly clear and there is a wealth of evidence to suggest that I3C is an effective chemopreventive agent in a variety of tissues.

1.2.4 Human studies for chemopreventive efficacy.

1.2.4.1 Curcumin.

There have been limited human clinical trials with curcumin as a chemopreventive agent. One uncontrolled trial reported a reduction in lesion size, pain, exudate and itching in 65% of oral cavity carcinoma cases tested (reviewed in Chemoprevention Branch and Agent Development Committee 1996). A phase I, dose escalation, clinical trial is currently underway in Leicester. In this trial patients with advanced colorectal cancer receive an extract of turmeric known as p54, which is mainly curcumin together with some volatile oils from the same plant. The pharmacokinetic part of the study is designed to examine the amount of curcumin and some of the major metabolites in plasma, stool and urine. In terms of pharmacodynamics the chemotherapeutic angle is being studied by measuring a number of tumour markers in blood, including carcinoembryonic antigen and tumour analysis by computer tomography scans, X-ray and magnetic resonance imaging. Quality of life is also assessed by questionnaire. In relation to the identification of chemoprevention biomarkers lymphocyte GST, leukocyte malondialdehyde-guanine adduct levels and the inducibility of cyclooxygenase 2 (COX2) in whole blood are being investigated.

1.2.4.2 EGCG.

There is a need for controlled, human, clinical trials to attempt to verify the wealth of information available from epidemiological, animal and *in vitro* studies. To date a phase I clinical trial has been conducted in Japan with 108 healthy volunteers. In this study the participants took a tablet form of green tea extract over a period of six months and were analysed for adverse side effects. No such side effects were observed and the way is now open for further trials (reviewed in Nakachi *et al.* 1998).

1.2.4.3 I3C.

There are two published phase one clinical trials that have been conducted with I3C. The first showed that oral administration of I3C, to 18 patients with recurrent respiratory papillomatis, caused a cessation of the disease in 33% of cases and a reduction in growth rate in a further 33% (Rosen *et al.* 1998). The second study observed that oral administration of I3C to patients with cervical intraepithelial neoplasia caused complete regression in 4 out of 8 patients who received 200mg/day and 4 out of 9 patients that received 400mg/day. None of the 10 patients receiving the placebo showed any signs of regression (Bell *et al.* 2000). These promising secondary prevention trials open the way for more detailed primary trials that may investigate the prevention of other cancers.

1.2.5 Proposed molecular effects of the chemopreventive agents.

1.2.5.1 Curcumin.

Curcumin has multiple potential chemopreventive mechanisms. As described previously in this chapter (p10) it is a potent antiinflammatory agent and inhibits arachidonic acid metabolism by blocking both the lipoxygenase (LOX) and cyclooxygenase (COX) pathways (reviewed in Kelloff *et al.* 1996). It is a potent antioxidant and has a number of other potential effects, many of which will be discussed in detail later in the thesis. In summary it can inhibit TPA-induced DNA synthesis, modify cytochrome P450 function and enhance GST activity, decrease expression of important early transcription factors such as c-jun, c-fos and c-myc, inhibit ODC activity and inhibit tyrosine kinases. It may also inhibit mutagenesis and upregulate DNA repair and have anti-viral and anti-hormonal activities (reviewed in Kelloff *et al.* 1996).

In further detail turmeric has been shown to have a dose-dependent anti-genotoxic effect as evaluated by the somatic mutation and recombination test (SMART). This assay, which is based on two wing cell markers in *Drosophila melanogaster* showed turmeric to have no genotoxic effect. Furthermore it inhibited the genotoxic effect of the mutagen urethane in this assay (ElHamss *et al.* 1999).

Curcumin has also been shown to be an inhibitor of ODC, a rate limiting enzyme in polyamine biosynthesis. Mouse skin tumours have been shown to exhibit a high level of ODC activity and the induction of this enzyme has been shown to be important for tumour promotion (Lu *et al.* 1993). Curcumin was shown to inhibit TPA-induced ODC mRNA production and activity, when applied topically to the skin of female CD-1 mice. Application of concentrations up to 10µM curcumin, along with TPA, inhibited induced ODC activity by up to 91%. Similar effects were seen if the curcumin was applied up to 60 min before the TPA or within a short time after. *In vitro* addition of curcumin to the ODC assay had no inhibiting effect. Topical application of curcumin also inhibited induced levels of ODC mRNA by up to 82%. An intraperitoneal (i.p) injection of curcumin 60 min prior to TPA administration was also shown to inhibit both ODC activity and mRNA levels. It is plausible that curcumin is inhibiting the synthesis of ODC mRNA and/or stimulating its breakdown.

Curcumin was shown to inhibit LOX and COX activities, dose-dependently, *in vitro* at concentrations from 1-100 μ M (Huang *et al.* 1991). The enzymes were present in extracts from mouse epidermis and their ability to metabolise arachidonic acid was measured in an *in vitro* assay. Arachidonic acid metabolism is thought to be important for TPA-induced inflammation and tumour promotion in mouse skin. These observations were confirmed *in vivo* by Rao et.al (1995a and b), who demonstrated that colon tumours in F344 rats, induced by AOM, showed reduced arachidonic acid metabolism by the COX and LOX pathways if the rats were given a curcumin diet.

1.2.5.2 EGCG.

Tea polyphenols, including EGCG, have been shown to have potent antimutagenic activity both in microbial and mammalian *in vitro* and *in vivo* systems (reviewed in Kuroda and Hara 1999). In terms of the possible biochemical mechanisms for the inhibition of tumourigenesis many authors have attributed their effect to a general antioxidative activity (reviewed in Yang and Zhi-Yuan 1993). Tea polyphenols possess strong antioxidant activity due to three main reasons;

1) The presence of the catechol structure means tea polyphenols are strong metal ion chelators. They can bind and thus decrease the level of free cellular ferric and ferrous ions, which are required for the generation of oxygen radicals (Yang and Zhi-Yuan 1993).

2) They are scavengers of superoxide and hydroxy radicals (Yang and Zhi-Yuan 1993).

3) They can react with peroxy radicals, terminating lipid peroxidation reactions. EGCG and several other catechins were shown to inhibit photo-enhanced lipid peroxidation in mouse liver microsomes (Katiyar *et al.* 1994).

In more specific terms tea polyphenols have been shown to inhibit nitrosation reactions. Nnitroso compounds are formed by the endogenous nitrosation of nitrogenous compounds and are implicated in the occurrence of many cancers worldwide. Tea polyphenols react with the nitrosating species thus inhibiting nitrosation (reviewed in Yang and Zhi-Yuan 1993).

Polyphenols may also act through the modulation of xenobiotic metabolising enzymes. Most carcinogens require activation via cytochrome P450 or other enzymes in order to become metabolically active. The metabolism of NNK to the active carcinogen by mouse lung microsomes was inhibited by EGCG *in vitro* (Shi *et al.* 1994). The inhibition of NNK oxidation and NNK-induced DNA methylation were also observed. At the concentrations of EGCG at which these inhibitions were observed the activities of several P450 enzymes were inhibited. In a second study the feeding of a green tea extract to female SKH-1 mice significantly increased the activities of some antioxidant and phase II enzymes in the small bowel, liver and lungs. Such enzymes are important for the detoxification of carcinogens and those in which the activity was altered in this study included glutathione peroxidase, catalase, quinone reductase and glutahione S-transferase (Khan *et al.* 1992).

The structure of flavanols provides a strongly nucleophilic centre that is capable of trapping the active form of a carcinogen directly before it can cause DNA damage (reviewed in Yang and Zhi-Yuan 1993).

Tea polyphenols have also been shown to inhibit a number of biochemical events linked to tumour promotion and progression. Many of these will be discussed in later chapters, but those, which have been suggested and are not to be investigated in this thesis, include the ODC pathway. ODC activity, induced by MNNG, was decreased in the gastric mucosa of mice receiving EGCG compared with the levels in the control group (Yamane *et al.* 1995). Inhibitory effects of green tea polyphenols have also been described on TPA-induced protein kinase C (PKC) and COX and LOX activities (reviewed in Yang and Zhi-Yuan 1993). The inhibition of TPA-induced activator protein-1 (AP-1) activation in the mouse JB6 cell line has also been suggested as a possible mechanism for the inhibition of carcinogenesis (Dong *et al.* 1997).

1.2.5.3 I3C.

The majority of investigations into the mechanism of action of I3C have centred upon its ability to modulate oestrogen metabolism. Oestradiol is metabolised in the body to 16α -hydroxyoestrone and 2-hydroxyoestrone. 16α -hydroxyoestrone has been shown to be elevated in breast cancer, to be genotoxic, to increase cell proliferation and anchorage independent growth (reviewed in Bradlow *et al.* 1995). As such it is a recognised risk factor in mammary carcinogenesis. 2-hydroxyoestrone has no such risk associated with it. Since there is a finite pool of oestrogen in the body any increase in 2-hydroxyoestrone levels relative to 16α -hydroxyoestrone can be considered as a factor which reduces risk. This oxidative metabolism of oestrogen is regulated by cytochrome P450 enzymes. Cytochrome p450 1A1 (Cyp1A1) is the enzyme responsible for the 2-hydroxylation of oestrogens.

I3C has been shown to modulate oestrogen metabolism and the phase I enzymes in a large number of studies. High levels of a number of P450 enzymes, including Cyp1A1, were induced in the livers of Sprague-Dawley and F344 rats by I3C administered in the diet (Grubbs *et al.* 1995). The induction of several phase II enzymes was also observed. These are responsible for the detoxification of carcinogens. Similarly SW and C3H/OuJ mice fed I3C showed a 5 fold increase in the P450 content of liver microsomes which was accompanied by an increase in oestradiol 2-hydroxylation (Bradlow *et al.* 1991).

Treatment of MCF 7 breast cells with $10^{-11} - 10^{-6}$ M I3C resulted in a five-fold increase in oestradiol 2-hydroxylation (Niwa *et al.* 1994). A study in the human cervical cancer cell line CaSki showed that I3C and 2-hydroxyoestrone inhibited oestradiol induced expression of the HPV oncogene. Furthermore both competed with oestradiol for oestrogen receptor binding

(Yuan *et al.* 1999). As previously described I3C-induced P450 enzymes and increased 2hydroxylation of oestrogen.

Several human studies have confirmed these effects of I3C in the body. A group of both men and women were given 6-7mg/kg/day of I3C over a period of 7 days. The levels of oestrogen metabolites excreted in the urine were compared over this time. The percentage of 2hydroxyoestrone increased from 29.8% before I3C treatment to 44.6% after 7 days and the response of men and women to I3C was indistinguishable (Michnovicz and Bradlow 1991). A similar trial conducted over three months showed again that a substantial increase in 2hydroxyoestrone was observed in the urine of subjects taking 400mg I3C daily. The increase was observed after one month and remained elevated for the entire test period (Bradlow et al. These data were confirmed in a further trial conducted along the same lines 1994). (Michnovicz and Bradlow 1990). A dose-ranging study conducted on 60 women, at risk for breast cancer, used the 16\alpha-hydroxyoestrone/2-hydroxyoestrone metabolite ratio as a surrogate endpoint biomarker. This larger trial showed that the minimum dose required to observe a difference in the biomarker was 300mg I3C daily (Wong et al. 1997). None of the studies described showed any evidence of toxicity and confirm that the effects on oestrogen metabolism, in animal and cell systems, detailed in the literature are relevant in humans.

The induction of phase I and II enzymes has also been implicated in the detoxification of carcinogens by I3C. The feeding of I3C to F344 rats was shown to inhibit AFB_1 -induced hepatocarcinogenesis (Manson *et al.* 1997; Manson *et al.* 1998). This inhibition was shown to be associated with an increase in a number of phase I and II enzymes in liver microsomes. At first glance this was rather contradictory as the metabolism of AFB_1 to an active carcinogen occurs in the liver and is regulated by P450 enzymes. However, the balance between phase I activation of AFB_1 and phase II detoxification of the active metabolite resulted in an overall protective effect (Grubbs *et al.* 1995; Taioli *et al.* 1997; Manson *et al.* 1998).

The tumour suppressive effects of I3C may result from the inhibition of enzyme activities such as ODC and tyrosine kinases. Inhibition of both ODC and tyrosine kinase activity, by I3C, has been observed both in cell lines (Sharma *et al.* 1994) and in extracts from rat liver (Manson *et al.* 1998). Dietary I3C has also been reported to alter the immune function of rats.

Male Sprague-Dawley rats, fed either 150mg/kg or 50mg/kg I3C daily for over a week exhibited inhibited natural killer cell activity. In contrast T-cell activity was increased, but no significant changes in antibody production were observed (Exon and South 2000). The immune system may also play an important role in tumour suppression.

1.2.6 Metabolism of the chemopreventive compounds and identification of the active molecules.

There are two distinct considerations when trying to determine the active constituent(s) in assays of chemopreventive activity: 1) is the compound supplied as a mixture and 2) is it metabolised in some way to the active form once in cells in culture or in the body.

1.2.6.1 Curcumin.

Commercial curcumin, isolated from the rhizome of *Curcuma longa*, contains a mixture of curcuminoids which is approximately 77% curcumin (Figure 1.2), 17% demethoxycurcumin (Figure 1.5a) and 3% bisdemethoxycurcumin (Figure 1.5b) (Conney et al. 1998). Little work has been done so far to determine metabolism of curcumin by cells in culture. Some studies have been carried out, including a recent study in this laboratory, which showed that liver cells from rats and humans reduce curcumin to the same spectrum of metabolites. This biotransformation step was shown to be rapid, but was slower in human cells than in rat cells. This study also showed that the metabolites of curcumin were less effective in inhibiting inducible COX2 expression than the parent compound (Chris Ireson et al., unpublished data). Other published studies have been reviewed in Ammon and Wahl 1991. Curcumin was shown to be rapidly metabolised by liver microsomes and excreted in bile. The major biliary metabolite is tetrahydrocurcumin (Figure 1.5c). The antioxidant activities of these curcuminoids and metabolites is in the order: tetrahydrocurcumin > curcumin > demethoxycurcumin > bisdemethoxycurcumin (Conney et al. 1998; Huang et al. 1997). In animal studies pure curcumin (98%), commercial curcumin and demethoxycurcumin had an equally potent inhibitory effect on TPA-induced tumour promotion in mice initiated with DMBA. Bisdemethoxycurcumin was less active in the same assay (reviewed in Huang et al. 1994; Huang et al. 1997). As far as I am aware there are no similar data for tetrahydrocurcumin, but from the antioxidant and other available data it would be predicted to

be active in the inhibition of tumourigenesis. In all experiments described in this thesis pure curcumin (>98%) has been used.



Figure 1.5. Chemical structures of curcumoids and curcumin metabolites.

1.2.6.2 EGCG.

As described previously in this chapter (p8) there are several green tea polyphenols implicated in the pharmacological activity. Of these EGCG is the most abundant and has been shown in several studies to be the most active. A study of lipid peroxidation in mouse lung microsomes showed EGCG to be a far more potent antioxidant than EGC or ECG (Katiyar *et al.* 1994). A second group showed that the metabolism of the carcinogen NNK to

its active form was inhibited by green tea catechins in the order EGCG>>>ECG=EGC>EC (Shi *et al.* 1994).

There are few data available on the metabolism of EGCG in humans or animals. A review of the available studies showed that three human volunteers given [14 C]catechin excreted 55% of the label in their urine within 2 hours of uptake. The metabolites in the urine were glucuronic and sulphate conjugates of 3-O-methylcatechin in addition to unchanged catechin. These three components constituted 75% of the catechin intake. A study in rats showed that, when administered orally, EGCG moved to the large intestine after 8h. The majority was excreted in faeces and some metabolised by intestinal bacteria. About 20% of the EGCG ingested was estimated to have been absorbed by the digestive organs (reviewed in Kuroda and Hara 1999).

These data suggest that the most active component of green tea extract is EGCG and that of all the catechins it is this component which should be used in its pure form in further studies to elucidate the pharmacological mechanisms of tumour inhibition.

1.2.6.3 I3C.

A number of indoles are present in cruciferous vegetables. As well as I3C, the compounds include indole-3-acetonitrile, indolylacetate, indole-3-carboxaldehyde, indole-2-carboxylic acid and indole-3-carboxylic acid. In studies with MCF-7 cells (Bradlow *et al.* 1995) and in rat hepatic microsomes (Michnovicz and Bradlow 1990) I3C has been shown to be the most active in inducing 2-hydroxylation of oestrogen.

Animal studies have shown that I3C is much more effective as a tumour inhibitor when given orally than when injected (Bradfield and Bjeldanes 1987). It was assumed this difference is due to the conversion of I3C, in the acid conditions of the stomach, to a more active molecule. Under the acidic conditions created by gastric juice a number of oligomers of I3C are formed. These include the dimers: Indolo[3,2-b]carbazole (ICZ) and diindol-3-ylmethane (DIM) and the trimers: 5,6,11,12,17,18-hexahydrocyclononal[1,2-b:4,5-b:7,8-b]triindole (CTR) and 2,3bis[3-indolylmethyl]indole (LTR). The structures of these compounds, which are formed after a 10 minute incubation of I3C in aqueous HCl, are shown in figure 1.6. Such treatment results in a spectrum of metabolites similar to those observed in the gut of rats fed I3C (Dekruif *et al.* 1991; Grose and Bjeldanes 1992). The trend is for more trimer formation as

the pH decreases, while a pH of 1.5 and below significantly reduces the amount of ICZ formed (Grose and Bjeldanes 1992). These compounds have been shown to be capable of inducing P450 activity in rat hepatocytes in vitro (Dekruif et al. 1991). The cyclic trimer, CTR, has recently been shown to be an agonist of the oestrogen receptor and to stimulate the growth of oestrogen responsive cells (Riby et al. 2000). The significance of such an effect in terms of the chemopreventive efficacy of I3C remains to be established, but it could explain some of the tumour promoting effects seen with I3C under certain circumstances. It is plausible that an oestrogenic effect such as this could promote maturation of the mammary gland resulting in a decreased risk of spontaneous mammary tumours as described for the chemopreventive agent genistein (reviewed in Riby et al. 2000). A mixture of acid reaction products of I3C was shown to inhibit the activation of AFB₁ by rat liver microsomes and inhibited binding of the carcinogen to DNA (Fong et al. 1990). There is significant evidence that rapid conversion of I3C to a variety of oligomers occurs under acid conditions. These metabolites have been shown to exhibit chemopreventive activity, at least at the molecular level, and it may well be these products that are responsible for the chemopreventive effects of I3C in vivo. Whether these products are produced when I3C enters cells in culture remains to be seen but there is evidence that conversion of I3C to DIM occurs rapidly and spontaneously in cell culture medium incubated at 37°C (Niwa et al. 1994).



Figure 1.6. Chemical structures of the acid condensation products of I3C.

Thus, all three of the dietary compounds described fit the majority of criteria required of a suitable chemopreventive agent listed on p1. There are still many questions on their mechanisms of action and the suitability of potential surrogate endpoint biomarkers that need to be answered before these compounds can be introduced into everyday life as pharmaceutical compounds.

1.3 Signal transduction pathways.

It is vital for cells to modulate their activities in response to signals from their surrounding environment. In the case of a single-cell organism, the inputs to the signalling pathways are mainly nutrient cues. In the case of a multicellular organism there is a requirement for complex intercellular and intracellular signalling pathways to co-ordinate the activities of one cell with those of its neighbours.

1.3.1 Receptor protein tyrosine kinases.

Receptor protein tyrosine kinases (RPTK) are all transmembrane proteins with a cytoplasmic domain that has intrinsic catalytic activity, which is activated upon ligand binding to the extracellular domain. The N-terminal, extracellular domain has a signal peptide, which targets the protein to the membrane. This is followed by the ligand binding domain which is comprised of several cysteine rich, structural motifs and is often modified by N-linked glycosylation and O-linked sugars. The hydrophobic transmembrane domain is followed by the juxtamembrane region, which precedes the catalytic domain. The catalytic domain is conserved amongst most protein kinases, but sequences either side of this domain and, in some cases, large inserts within it, are not and it is the modulation of these sequences which is important in the regulation of receptor activity and downstream signalling (reviewed by Van der Greer et al. 1994). The activation of a RPTK requires ligand-induced dimerisation of the receptor. This dimerisation juxtaposes the two catalytic domains, which allows mutual phosphorylation of residues contained within the activation loop of the catalytic domain. This activation results in autophosphorylation of residues outside of the catalytic domain which results in the recruitment and activation of downstream molecules (reviewed in Hunter 2000). Recent studies have suggested that dimerisation alone may not be sufficient for receptor activation. A requirement, not only for dimerisation, but also for a correct conformational relationship within a dimer in order to achieve activity has recently been demonstrated (Jiang and Hunter 1999). In some cases pre-existing dimers may occur and ligand binding is required for the active conformation to be achieved.

The phosphorylation of tyrosine residues at the C-terminus of the RPTK provides binding sites for domains of further proteins. The most prevalent phosphotyrosine binding domain in

such systems is the src homology 2 (SH2) domain. SH2 domains bind in a sequence specific fashion, recognising one or more residues in positions 1-6 C-terminal to the tyrosine. Another class of phosphotyrosine binding domain (PTB) is less common and does not always bind in a phosphorylation dependent manner. Proteins containing PTB domains bind to residues up to 5 removed from the N-terminal side of the tyrosine (Van der Greer and Pawson 1995). SH2 and PTB domains are present in proteins with intrinsic enzymatic activity, but also in adapter proteins such as Grb-2, which can recruit effector enzymes to the plasma membrane. There are many other examples of protein interaction domains responsible for the transduction of the signal from a receptor to downstream molecules. All recognise short linear sequences and some require phosphorylation of serine, threonine or tyrosine residues introducing an element of inducibility (reviewed in Hunter 2000).

In summary the function of a RPTK may be governed by three factors. Firstly the expression is restricted to specific cell types within an organism. This is determined by the elements present in the gene promoter and enhancer regions. Secondly where a RPTK is expressed its function is determined by the activating ligands which are present, that are capable of binding to the receptor. Finally the response of a cell to ligand activation depends upon the intracellular proteins which are present to transduce the downstream response.

The EGFR family of RPTKs is a comprehensively studied group of receptors. The subfamily comprises four members: EGFR/Erb-B1 (170kDa) (Ullrich 1984), Erb-B2 (185kDa) (Yamamoto *et al.* 1986), Erb-B3 (160kDa) (Kraus *et al.* 1989) and Erb-B4 (180kDa) (Plowman *et al.* 1993). The EGFR has two cysteine rich extracellular domains and an intracellular tyrosine kinase domain (Figure 1.7). The classical ligand for the EGFR is EGF, which is a monomeric peptide. Several other ligands have been identified that can bind to and activate the EGFR and other family members. These ligands are summarised in table 1.1.



Figure 1.7. Structure of the EGFR.

Receptor	Ligands
Erb-B1/EGFR	EGF, HB-EGF, Betacellulin, TGF α
Erb-B2	No known extracellular ligand
Erb-B3	NDF, Heregulin
Erb-B4	NDF, Heregulin, Betacellulin

Table 1.1. The EGFR family of receptors and their ligands (adapted from GrausPorta et al. 1997). Key: EGF/epidermal growth factor, HB-EGF/heparin binding EGF, TGF α /transforming growth factor α and NDF/neu differentiating factor.

EGFR family receptors can form both homo- and hetero-dimers. As a consequence, although no ligand actually binds Erb-B2, all are capable of inducing its phosphorylation by triggering heterodimerisation, which is followed by transphosphorylation. Erb-B2 increases the affinity of both EGF and NDF for their receptors. The formation of a particular dimer determines the downstream response of the cell (GrausPorta *et al.* 1997). These same authors showed that Erb-B2 is the preferred heterodimerisation partner of all the Erb-B receptors and mediates the transphosphorylation of other receptors. Finally the Erb-B2 receptor was shown to enhance and to prolong MAPK activation induced by a number of stimuli.

1.3.2 The extracellular regulated kinase (ERK) pathway.

The ERK pathway is the best characterised of the mitogen activated protein kinase (MAPK) pathways. This pathway is activated downstream of many of the growth factor RPTKs (Figure 1.8). As is the case for all of the MAPK signalling pathways, the ERK pathway consists of a core signalling module of the MAPK, which is activated by concomitant phosphorylation on a tyrosine and a threonine residue by the MAPK kinase (MEK). The MEK is activated by Ser/Thr phosphorylation by the MAPK kinase kinases (MEKK).

The initial part of the signal transduction cascade involves the recruitment of the Grb2/son of sevenless (SoS) complex to the membrane. This occurs by the phosphorylated receptor directly recruiting Grb2 via its SH2 domain (Van der Greer *et al.* 1994) or indirectly through the recruitment of Shc via its SH2 or PTB domain, followed by its subsequent phosphorylation on Tyr^{317} or Tyr^{239} , which leads to the high affinity binding of Grb2 at its own SH2 domain (Van der Greer *et al.* 1994).

The small G protein Ras, is commonly described as a molecular switch, which relays signals from the receptor to a variety of intracellular effectors. There are three forms of Ras, H-, K- and N-Ras. Ras family proteins are active in the guanine triphosphate (GTP) bound state and inactive in the guanine diphosphate (GDP) bound state. All possess a slow, intrinsic GTPase activity, which is enhanced by GTPase activating proteins (GAP) and a similar slow, intrinsic ability to exchange GDP for GTP which is enhanced by guanine nucleotide exchange factors (GEF) (Avruch *et al.* 1994; Marshall 1995). SoS is a GEF and its recruitment to the membrane by Grb2 is necessary for the activation of Ras, which is membrane bound (Avruch

et al. 1994). Activated Ras can then influence downstream molecules, which interact more strongly with the GTP bound form than the GDP bound form.

The classical effector of Ras is the serine/threonine kinase Raf (Daum *et al.* 1994). Raf family proteins comprise three isoforms, c-Raf-1, B-Raf and A-Raf and bind to Ras in a GTP-dependent manner (Feig and Cooper 1988). Although the binding of Raf to Ras is required for Raf activity, it is not sufficient alone. The function of Ras is to recruit Raf to the plasma membrane and this can be partially replaced by anchoring Raf permanently to the membrane (Wittinghofer and Herrmann 1995). The phosphorylation of Raf on ser/thr residues and on tyrosine residues 340 and 341, possibly by non-receptor tyrosine kinases such as src, are also required for full activity (Marais *et al.* 1995). Raf may also be regulated by oligomerisation since chemically induced oligomerisation can activate Raf (Farrar *et al.* 1996; Luo *et al.* 1996). This leads to the possibility of auto or transphosphorylation events. Raf-1 was also shown to become phosphorylated upon activation of PKC leading to another possible route of regulation (reviewed in Williams and Roberts 1994). Some confusion over the regulation of Raf still remains but from this point on the effectors of Raf are well characterised.

The dual specificity threonine/tyrosine kinases, MEK1 and 2 are activated by phosphorylation on serine and threonine residues and have been shown to be direct substrates of Raf-1 (Dent *et al.* 1992; Howe *et al.* 1992; Kyriakis *et al.* 1992). Activated MEK 1 and 2 phosphorylate ERK 1 and 2 on Thr¹⁸³ and Tyr¹⁸⁵. Upon activation ERK 1 and 2 translocate to the nucleus (Chen *et al.* 1992). Once in the nucleus ERK phosphorylates the Elk1/TCF transcription factor, amongst others, which forms a ternary complex with SRF (serum response element (SRE) – binding factor). This activated complex binds to the SRE in the promoter of genes such as c-fos, activating their transcription. The kinetics of Elk1 phosphorylation is rapid and it can be detected 5 mins after mitogenic stimulation. It may be that the complex of Elk1/SRF is constitutively present and bound to the SRE and requires only phosphorylation for activity (reviewed in Karin and Hunter 1995). C-fos forms an important component of the AP-1 transcription factor. AP-1 activity is enhanced by phosphorylation of c-fos at Thr²³². This phosphorylation is independent of the MAPK and is performed by the Ras activated kinase termed FRK (Fos-regulating kinase) (Deng and Karin 1994).

The non-receptor tyrosine kinase, Src, co-operates with signalling pathways such as the ERK pathway without lying directly within it. Src is capable of phosphorylating the EGFR itself

on Tyr⁸⁴⁵. This phosphorylation enhances its activity in response to growth factor (reviewed in Parsons and Parsons 1997). Src can also phosphorylate downstream molecules such as the previously described phosphorylation of Raf.

1.3.3 The c-jun N terminal kinase (JNK) pathway.

The JNK MAPK family comprises three members, JNK1, 2 and 3. These MAPKs are activated by exposure of cells to environmental stress, or by treatment with pro-inflammatory cytokines. As in the case of the ERK pathway the JNK pathway consists of a core signalling module (Figure 1.8). This core module is well defined for JNK but upstream activators of the pathway are less well characterised (reviewed in Davis 1999). The inflammatory cytokines, capable of activating the JNK pathway, include tumour necrosis factor α (TNF α) and interleukin-1 β (IL-1 β). Such cytokines exert their effects via receptors. A wide variety of cellular stresses including UV light, X-rays, protein synthesis inhibitors, hydrogen peroxide, osmotic shock and withdrawal of growth factors also activate JNK (reviewed in Ichijo 1999).

The activation of the TNF receptor (TNFR) by binding of TNF involves receptor aggregation. The aggregated receptor recruits the cytoplasmic signaling proteins TNFR associated factor 2 (TRAF2) (Rothe *et al.* 1994) and TNFR associated death domain protein (TRADD) (Hsu *et al.* 1995). These molecules bridge the gap between the receptor and the JNK pathway, entering at the MAPK kinase kinase (MAPKKK) level (Ichijo *et al.* 1997). The JNK pathway has also been demonstrated to be activated by EGF in certain cell types. Two recent studies showed the importance of Shc and Grb2 in the regulation of this induction. The first described the interaction of Grb2 with MEKK1 in ER22 cells treated with EGF (Pomerance *et al.* 1998). These authors reported that treatment with EGF caused the Grb2/MEKK1 complex to transiently recruit Shc. Release of Grb2/MEKK1 from this complex coincided with activation of JNK. A second study described this EGF-induced activation of JNK as being Grb2-independent, but Shc-dependent (Hashimoto *et al.* 1999). These studies were carried out by expressing Shc and Grb2 in Grb2/Shc knock-out chicken (DT40 B) cells.

The small G proteins of the Rho family have also been implicated in the activation of JNK. Constitutively active forms of these proteins, especially Rac and Cdc42 activate JNK when overexpressed and inactive mutants of these proteins can inhibit the induction of the MAPK (Bagrodia *et al.* 1995). Proteins at the MAPKKK kinase (MAPKKKK) level, such as p21 activated kinase (PAK), have been shown to bind Cdc42 (Bagrodia *et al.* 1995), as have proteins at the MAPKKK level including MEK kinase 1 (MEKK1) (Fanger and Lassignal Johnson 1997).

At the MAPKKKK level overexpression studies have shown a large number of kinases to be capable of inducing JNK. These include PAK 1, 2, 3 and 4, germinal centre kinase (GCK), GCK-like kinase (GLK) and haematopoetic progenitor kinase 1 (HPK1) (reviewed in Ichijo 1999). The activation of JNK by such kinases is poorly described and there is no direct, biochemical evidence that these kinases activate MAPKKK by phosphorylation. The situation becomes clearer at the MAPKKK level. Once again a number of kinases have been identified at this level, which are capable of activating JNK. They include the mixed lineage kinases (MLK) 1 and 2, transforming growth factor- β -activated kinase-1 (TAK1), apoptosis stimulating kinase (ASK) 1 and 2 and MEKK1, 2, 3 and 4 (reviewed in Ichijo 1999).

Of the MAPKKK proteins described the MEKKs are the best characterised. These activate JNK via the phosphorylation of the MAPK kinases (MKKs) 4 and 7 (reviewed in Davis 1999). MEKK1, 2 and 4 activate both MKK4 and 7, whereas MEKK3 activates MKK7, but not MKK4 (Deacon and Blank 1999). MKK 4 and 7 are dual specificity kinases, which phosphorylate JNK on Thr¹⁸³ and Tyr¹⁸⁵ (Derijard *et al.* 1995).

As with ERK, upon activation JNK translocates to the nucleus. The targets of JNK include the transcription factor, activating transcription factor 2 (ATF2) and c-jun. These transcription factors are components of the AP-1 transcription factor family, which includes the fos proteins (c-fos, fosB, fra1 and fra 2) and the other jun family members, junB and junD. These transcription factors bind as homo- and hetero-dimers to the AP-1 sites of various genes (Curran and Franza 1988). JNK binds to the N-terminal region of c-jun and phosphorylates Ser⁶³ and Ser⁶⁵ within the activation domain of the transcription factor. This phosphorylation leads to increased AP-1 transcriptional activity and increased expression of c-jun (reviewed in Davis 1999). C-jun expression is increased as the gene contains an AP-1 promoter, which is activated by the increased AP-1 activity. The phosphorylation of c-jun increases the half life of the protein as it reduces its ubiquitination and subsequent degradation (Musti *et al.* 1997).

1.3.4 The p38 pathway.

The p38 MAPK pathway is the least well characterised of the three MAPK pathways described here. Four principle isoforms of p38 have been identified and designated α , β , γ and δ (reviewed in Deacon and Blank 1999). It is activated by similar stimuli to JNK i.e. by cytokines and cellular stresses and many components of the pathway, especially the upstream components, are shared with the JNK pathway (Figure 1.8) (reviewed in Ichijo 1999).

As for JNK, the small G proteins of the Rho family, including Cdc42, have been implicated in the activation of p38, and PAK has also been implicated in its upstream activation (Bagrodia *et al.* 1995). At the MAPKKKK level a number of kinases have been implicated and are the same as those described for JNK (see p35-36).

Once again the picture begins to become clearer once the MAPKKK level is reached. There are no known MAPKKKs, which activate p38 alone. Those which do activate p38 are also effective for JNK (Ichijo 1999). Those MAPKKKs which have been shown to be effective for p38 include MEKK 2 and 3 (Deacon and Blank 1999), TAK1 and ASK1 (Ichijo 1999).

The MAPKKs, MKK3 and 6, have been shown to activate p38 by phosphorylation on a tyrosine and threonine residue. MEKK2 and 3 have been shown to activate MKK6 but not MKK3 (Deacon and Blank 1999). The activation of MKK3 must occur via one of the other kinases thought to act at the MAPKKK level. MKK4 has also been shown to activate p38 *in vitro* (Derijard *et al.* 1995). There are, however, three lines of evidence which suggest that MKK4 may not play a role in p38 activation. Firstly dominant negative MKK4 inhibits JNK activity more potently than p38 activity (Davis 1999). Secondly if MEKK1 is transfected into cells it causes selective activation of the JNK signal transduction pathway (Derijard *et al.* 1995). Finally cells with a homozygous deficiency in MKK4 exhibit disregulation of the p38 pathway (Yang *et al.* 1997). This final piece of evidence could be explained by redundancy

37

in the system, with MKK3 and 6 compensating for the loss of MKK4, but no similar redundancy was observed for JNK, which also has MKK7 at the same level as MKK4.

The nuclear targets of p38 are, once again, not well described. p38 has been shown to phosphorylate ATF2 (Kyriakis 1999) and to activate MAPK activated protein kinase (MAPKAPK) 1, 2, 3 and 5 (Ni *et al.* 1998). MAPKAPK1 is also known as p90rsk and can phosphorylate and activate the SRF (Chen *et al.* 1993). Activated MAPKAPK2 has been shown to phosphorylate the small heat shock protein HSP25/27 *in vivo* (Rouse *et al.* 1994).



Figure 1.8. Simplified scheme of the MAPK signal transduction pathways.

1.3.5 The phosphatidylinositol 3-kinase (PI3K)/Akt pathway.

The PI3K/Akt pathway has been shown to be activated by various peptide growth factors and TNF. There has been a great deal of recent interest in this pathway, especially in its link to nuclear factor kappa B (NF- κ B) and possible anti-apoptotic effects. Several recent papers have linked the pathway to the induction of NF- κ B in cells treated with platelet derived growth factor (PDGF) (Romashkova and Makarov 1999) and TNF (Ozes *et al.* 1999). The pathway has also been shown to be activated by EGF (Gibson *et al.* 1999). The proposed pathways of activation are summarised in figure 1.9.

PI3K is a lipid kinase, which phosphorylates the hydroxyl group at position 3 on the inositol ring of phosphoinositides. The products of this phosphorylation include phosphatidylinositol-3,4,5-triphosphate (PIP3). These lipids can then act as second messengers, activating a number of downstream molecules including Akt (reviewed in Downward 1995). PI3K may be recruited directly to the activated receptor, or, in the case of growth factors, activation may occur by Ras. PI3K has been shown to directly bind to Ras (Rubio *et al.* 1997) and the activation of NF- κ B by PDGF was shown to occur via Ras (Romashkova and Makarov 1999). However another study showed the activation of Akt by PDGF to be independent of Ras and Raf, so there may be some cell specificity involved (Boudewijn and Coffer 1995).

Akt lies downstream of PI3K. Akt was first discovered due to it homology to protein kinases A and C (PKA and C). It was also identified as the human homologue to the rodent acutely transforming retrovirus AKT 8 and designated v-akt (reviewed in Downward 1995). PI3K activates Akt through the second messenger PIP3. PIP3 binds to an amino-terminal pleckstrin homology (PH) domain on Akt (Marte and Downward 1997). This binding results in the phosphorylation of Akt on Thr³⁰⁸ and Ser⁴⁷³ by two kinases known as 3-phosphoinositide-dependent kinases 1 and 2 (PDK1 and 2) (Alessi *et al.* 1997;Stokoe *et al.* 1997). This phosphorylation results in fully active Akt.

Akt influences metabolism through the phosphorylation of glycogen synthase kinase-3 (GSK3) and phosphofructokinase, but it also transmits a potent survival signal (reviewed in Downward 1998). Akt is capable of directly phosphorylating and inactivating Bad, a pro-

apoptotic B-cell lymphoma/leukaemia-2 (Bcl-2) family member (Datta *et al.* 1997). Recently it has also been shown to induce NF- κ B activation.

NF- κ B is a transcription factor, which is induced by a myriad of environmental and cellular agents. It plays a critical role in the regulation of cell growth, differentiation, inflammation and apoptosis (reviewed in Mercurio and Manning 1999). It is a dimeric transcription factor that is present in the cytoplasm of most cells in an inactive form. Upon activation it translocates to the nucleus where it activates transcription. The transcription factor is composed of two subunits, which include NF- κ B1 and RelA family members. It exists in the cytoplasm, in an inactive form, associated with a class of inhibitory proteins known as I κ Bs. The I κ B is phosphorylated on serine residues by a kinase complex containing two kinases known as I κ B kinase α and β (IKK α/β). This phosphorylation causes I κ B to dissociate from NF- κ B and it is targetted for degradation by the 26S proteosome (reviewed in Mercurio and Manning 1999) (Figure 1.9) IKK activity is classically induced by NF- κ B inducing kinase (NIK) but other possible inducers have been identified, including Akt.

One study showed PDGF to induce NF- κ B via Akt (Romashkova and Makarov 1999). These authors showed that Akt associates with IKK *in vivo* and induces its activation. In this case they proposed that NF- κ B induced two signals, the first to induce the proto-oncogene c-myc and its proliferative effects, and the second to inhibit the apoptotic effects of this same oncogene. A second study showed Akt to be involved in the TNF-induced activation of NF- κ B (Figure 1.9). This investigation proposed that both Akt and NIK were required for this induction. Akt mediates the phosphorylation of IKK α at Thr²³, whereas NIK causes phosphorylation at Ser¹⁷⁶ and both are required for full activity (Ozes *et al.* 1999).

Chapter 1



Figure 1.9. Proposed mechanism of activation of NF-KB by the PI3K/Akt pathway.

1.3.6 Crosstalk between signalling pathways.

There are many points of crosstalk in the pathways described in this chapter. It is important to understand how signalling specificity can be achieved when many of the same core signalling molecules are activated by receptors that elicit a wide variety of responses.

One proposed method is by anchoring and scaffolding proteins. Anchoring proteins localise signalling molecules and their substrates in a particular part of the cell, increasing the efficiency and specificity of the interaction of the components (Pawson and Scott 1997). Scaffold proteins bring related signalling components together in multiprotein complexes. The best characterised scaffold complex has been identified in the yeast homologue of the MAPK signalling pathways and there are now proteins that function as scaffolds being identified in mammalian cells (reviewed in Hunter 2000). A well-characterised example in the mammalian system is JNK interacting protein (JIP). This selectively binds MKK7, MLK3 and HPK, enhancing the efficiency and substrate specificity of this MAPK module (Whitmarsh *et al.* 1998).

Regulatory domains can localise signalling components at the plasma membrane and receptors may simply use spatial separation, their position within the membrane determining the downstream proteins with which they may interact (Hunter 2000).

Another important consideration in determining specificity is signal thresholds. In certain systems a 2-fold increase or decrease in signal intensity can activate or repress signalling. In other systems the threshold may be higher. In a similar fashion the length of time over which a signal is received may determine the downstream response. In many cell lines sustained MAPK activation over several hours is required to drive DNA synthesis, whereas many growth factors initiate only a transient response (Balmanno and Cook 1999; Cook *et al.* 1999).

The interaction of signalling pathways needs to be determined *in vivo* under conditions where substrate specificity may be increased compared to the *in vitro* situation due to some of the mechanisms described here. Similarly it is important to realise that crosstalk between pathways is an important process and the fate of a cell is determined by the balance of signals it receives from several different sources.

1.3.7 The cell cycle.

The eukaryotic cell cycle is composed of four phases, gap 1 (G₁), DNA synthesis (S), gap 2 (G₂) and mitosis (M). There is also an out of cycle phase, where the cells remain quiescent denoted G_0 (Figure 1.10).



Figure 1.10. Simplified scheme of the eukaryotic cell cycle.

Cell division is controlled by a complex set of biochemical signals that regulate specific cell cycle events. In mammalian cells, extracellular signals control entry into the cycle at G_1 . Once entry has been achieved there is no longer a requirement for external stimulation (Sherr 1996; Van den Heuvel and Harlow 1993). Cell cycle progression is controlled by protein kinases known as cyclin dependent kinases (CDKs). CDKs are primarily activated by binding cyclins, but complete activation requires phosphorylation at a conserved threonine residue. Deactivation may also involve phosphorylation of a tyrosine and a threonine at the amino terminus or the action of a CDK inhibitor (CKI) (reviewed in Morgan 1995). D type cyclins (1, 2 and 3) are important in progression through G_1 phase and into S phase. Their expression is regulated by external mitotic signals. The D type cyclins complex with CDKs 4 and 6 while cyclin E, also expressed at this time, complexes with CDK2. Cyclin A associates with CDK2 during S phase and with CDK1 (cdc2) in late S and G_2 . Cyclin B has also been shown to associate with CDK1 during G_2/M (Sherr 1996). CDK1 was shown to be important in the

progression through G₂/M in studies with dominant negative CDK1 which arrested cells in G_2/M . These cells were rescued by the introduction of wild type CDK1 or various cyclins (Van den Heuvel and Harlow 1993). There are a number of peptide CKIs that have been identified. p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d} inhibit cyclin D dependent kinases, CDK4 and 6. Cyclin D, E and A-dependent kinases are also negatively regulated by a family of inhibitory proteins comprising p21^{CIP1}, p27^{KIP1} and p57^{KIP2} (reviewed in Sherr 1996). An important target of active CDK complexes is the retinoblastoma (Rb) protein. An active cyclin D/CDK4/6 complex phosphorylates Rb protein in the cytoplasm. **Rb** proteins sequester the E2F transcription factor and this phosphorylation releases E2F, which activates transcription of genes involved in DNA synthesis (Qin et al. 1995). The phosphorylation of Rb is initiated by cyclin D/CDK complexes and prolonged by cyclin E/CDK complexes. Cyclin A and B-dependent kinases maintain Rb in a phosphorylated state as the cell cycle moves forward (Sherr 1996). Tumour cells typically acquire damage to genes that directly regulate their cell cycle. Such damage, either to components directly involved in the cycle such as cyclin D and Rb, or to those responsible for regulating the checkpoints and braking the process such as p53, may lead to uncontrolled cell proliferation which is the hallmark of all cancers.

1.3.8 Apoptosis.

Apoptosis, or programmed cell death, is an active, inherently programmed phenomenon and can be initiated or inhibited by a variety of environmental stimuli (Kerr *et al.* 1972). It is important for regulating animal cell populations and is complementary to mitosis. The process contributes to tissue homeostasis as it can result in extensive deletion of cells with little disruption to surrounding tissue.

The execution of apoptosis is associated with characteristic morphological and biochemical changes (Steller 1995). During apoptosis the nucleus and cytoplasm condense and the cell breaks up into a number of membrane bound fragments termed apoptotic bodies. These bodies are rapidly ingested by neighbouring cells, or by macrophages, rapidly removing them from the body and preventing any leakage of potentially harmful substances from within them (Wyllie 1980).

All proteins required for the induction and inhibition of apoptosis are constitutively expressed within a cell (reviewed in Steller 1995). The most important cell surface receptors involved in the modulation of apoptosis are the TNFR superfamily (Yuan 1997). Of these the best described are the TNFR1 (Tartaglia *et al.* 1991) and the Fas receptor (Itoh 1991). These receptors contain a death domain in their intracellular regions. Binding of various adapters to such domains mediates their downstream response (Yuan 1997). Adapters such as TNFR1 associated death domain protein (TRADD) form a complex with TNFR1 upon ligand binding (Hsu *et al.* 1995) and Fas associated death domain protein (FADD) with the Fas receptor (Chinnaiyan *et al.* 1995). The interaction of these receptor/adapter complexes transduce signals downstream which may result in activation of NF- κ B and cell survival (1.3.5) or apoptosis.

Downstream molecules involved in inducing apoptosis include a number of cysteine proteases known as the caspases. To date at least 14 members of the caspase family have been identified. They fall into two categories, those involved in cytokine processing which play a role in sensing apoptotic signals and those involved with the apoptotic cascade itself which effect apoptosis (reviewed in Slee et al. 1999). The majority are sequestered in the cell as inactive pro-caspases. Upon receiving apoptotic signals a proteolytic cascade is activated and the cell is pushed into apoptosis. Caspases 8 and 3 lie at the top of the cascade and integrate signals from the death receptors and other signalling pathways such as the JNK and p38 pathways (reviewed in Cross et al. 2000). The B-cell lymphoma/leukaemia-2 (Bcl-2) family of proteins are also important in the downstream control of apoptosis (reviewed in Alison and Sarraf 1995). Bcl-2 itself is a survival factor that protects cells from apoptosis (Vaux et al. 1988) and an increase in protein levels, in malignant and normal cells, was correlated with increased resistance to apoptotic stimuli (Reed 1994). Another member of the Bcl-2 family is Bax (Veis et al. 1993), which forms homodimers and heterodimers with Bcl-2. When Bax predominates it accelerates apoptosis. Thus the ratio of Bax to Bcl-2 determines survival or death following apoptotic stimuli (reviewed in Alison and Sarraf 1995). The mitochondria has also been implicated in the transduction of pro-apoptotic stimuli. Such stimuli promote changes in mitochondrial membrane permeability that cause the release of certain proteins including cytochrome c. Such proteins play a role in the activation of the caspase cascade. This change in membrane permeability is regulated in a caspase-dependent manner via caspase 8 but can also be regulated in a caspase-independent manner by an, as yet unidentified route. Bcl-2 negatively regulates the release of these proteins thus exerting a protective effect (reviewed in Cross *et al.* 2000; Slee *et al.* 1999).

Apoptosis plays an important role in the growth of most, if not all, solid tumours. The slowing of tumour growth over time is accounted for by increased cell death as blood supply is curtailed, rather than a slowing of proliferation (Alison and Sarraf 1995). Tumour promoters may act by preventing apoptosis in tumour foci (Gerbracht *et al.* 1990) and many cytotoxic anticancer drugs exert their effects through inducing apoptosis (Alison and Sarraf 1992; Anilkumar *et al.* 1992; Dive and Wyllie 1993; Sarraf *et al.* 1993).

1.3.7 The role of the signalling components in cell growth, apoptosis and carcinogenesis.

The activation of ERK 1 and 2, by growth factors, is required for the proliferation of most cell types. Kinase inactive ERK was expressed in CCL39 fibroblast cells and this expression inhibited growth factor-stimulated DNA synthesis (reviewed in Johnson and Vaillancourt 1994). The mutant MAPK competed with endogenous MAPK present in the cell. Other components of the ERK pathway have also been shown to be important for cell growth in similar studies. The overexpression of an inactive form of Src inhibited EGF-induced DNA synthesis and functions as a dominant negative inhibitor of cell cycle progression (Wilson *et al.* 1989). The inhibition of normal Ras proteins by the microinjection of a neutralising antibody, or the expression of dominant negative mutants has shown that Ras signalling is required for growth factor stimulated DNA synthesis (Mulcahy *et al.* 1985).

The role of the JNK pathway in cell growth and apoptosis is somewhat unclear. There are many conflicting reports on its downstream consequences and perhaps the most sensible conclusion is that many of its actions are cell-type specific or dependent upon parallel signalling by other pathways. C-jun null embryos are non-viable in mouse models of embryogenesis. The fibroblasts developed from these embryos have a proliferative defect and accumulate in the G_1 phase of the cell cycle. This cell cycle block was shown to be due to the mutant cells exhibiting a reduced expression of cyclin D1 and D3. There was no change in CDK4 or the cell cycle inhibitory proteins p21 or p27, suggesting that jun regulates cyclin D at a transcriptional level (Wisdom *et al.* 1999). Cyclin D is required for phosphorylation of the retinoblastoma (Rb) protein and entry into G_1 . C-jun was also shown to be the major

component of AP-1 induced upon exposure of these cells to ultraviolet (UV) light. UV treatment induces JNK activity and subsequent c-jun phosphorylation. This response to UV is reported to be the major factor in protecting cells from apoptosis induced in this manner. The c-jun null cells showed an increased sensitivity to UV-induced apoptosis. Through this study these authors concluded that the phosphorylation of jun by JNK is required for the protection of cells from UV-induced apoptosis, but not for c-jun-mediated entry into the cell cycle. The JNK pathway has also been linked with the induction of apoptosis. This has mainly been observed in neuronal cells, which were protected from apoptosis induced by the withdrawal of nerve growth factor (NGF) through the inhibition c-jun by the microinjection of antibodies to the protein or overexpression of dominant negative mutants. The overexpression of c-jun was also shown to drive apoptosis in these cells (reviewed in Leppa and Bohmann 1999). Further studies have shown c-jun overexpression to induce apoptosis in serum-deprived neuronal cells (Bossy-Wetzel et al. 1997). Yet even in neuronal cells the picture is not clear, since c-jun is expressed in response to neuronal damage. The damage of mature axons can lead either to cell death or, conversely, to vigorous regeneration. C-jun is expressed in each case, so the decision on the fate of the cell must be determined downstream of c-jun (reviewed in Herdegen et al. 1997). This review proposed that the final decision on cell fate could be regulated by phosphorylation of c-jun by JNK, or determined by the nature of the AP-1 dimer formed by c-jun.

The role of p38 in apoptosis was investigated in haematopoetic cells. This study showed that growth factor deprivation of these cells lead to apoptosis and that this was related to an enhanced p38 activity (Birkenkamp *et al.* 1999). Inhibition of p38 with SB203580 had a radical inhibitory effect on this apoptosis. This investigation also concluded, however, that some activation of p38 was observed upon treatment with interleukin-1 (IL1), which rescued cells from apoptosis and so cell fate was again decided by the interplay between the signalling pathways.

A recent study showed Akt to be central in the EGF-induced protection of epithelial cells from Fas-induced apoptosis (Gibson *et al.* 1999). In an attempt to dissect the interplay between pathways, the effect of the MAPK and PI3K/Akt pathways on apoptosis in neuronal cells was studied (Marushige and Marushige 1999). These authors showed that activation of JNK by anisomycin, the inhibition of ERK with PD09859 or the blockage of the PI3K pathway by wortmannin or LY294002 individually was not sufficient to induce apoptosis.

However apoptosis was induced if the activation of JNK was coupled to inhibition of PI3K/Akt and enhanced further by inhibition of ERK. The inhibition of p38 was shown to potentiate apoptosis in this case and the inhibition of both ERK and PI3K/Akt caused a slow induction of apoptosis. Once again these effects were observed in neuronal cells and the downstream consequences of the pathways may not hold for other cell types, but it does demonstrate the importance of interplay between the pathways.

It is clear that many of the signalling components described here possess oncogenic potential. Most of the oncogenic effects of tyrosine kinases result from constitutive activation of a pathway, which can occur at a variety of levels. Such constitutive activation may occur by three distinct mechanisms. Firstly a mutation can occur leading to constitutive activity. This is common in many RPTK oncogenes. Such mutations can affect any of the portions of a tyrosine kinase, rendering it constitutively active due to a reduced stringency in substrate recognition, ATP binding or constitutive oligomerisation (reviewed in Kolibaba and Druker 1997). The second mechanism involves the closure of an autocrine loop, when aberrant expression of a receptor occurs in the presence of its ligand or vice versa. This mechanism has been described for the EGFR and colony stimulating factor-1 receptor (CSF-1), among others (reviewed in Kolibaba and Druker 1997). The third category is characterised by alterations in the regulatory machinery of the signalling pathway. These include mutations in proteins that act as negative regulators of a pathway, which may result in disregulation of the pathway as a whole (reviewed in Kolibaba and Druker 1997).

The EGFR and Src have been implicated in carcinogenesis following observations that the overexpression of EGFR and Src in murine fibroblasts correlates with increased tumour formation in nude mice, colony formation in soft agar and DNA synthesis (reviewed in Parsons and Parsons 1997). EGF has been shown to promote migration and invasion in human prostate cancer cells. The Ras oncogene is especially prevalent in human cancer, with around 30% of human tumours having been found to contain an activated allele (Wittinghofer and Nassar 1996). The alteration to the proto-oncogene is a point mutation at Gly^{12}/Gly^{13} or Gln^{61} , resulting in the inability to hydrolyse GTP to GDP, leading to a persistently active state. This situation leads to constitutive activation of the ERKs and cellular transformation (White *et al.* 1995).

ERKs have been shown to be constitutively active in renal carcinomas and this activity was directly correlated with tumour grade. Many of these same tumours overexpressed MEK1. ERKs have also been shown to be overexpressed in non small-cell lung carcinomas and JNK has been shown to be constitutively active in human T-cell leukaemia virus-1-transformed cells and in the leukocytes of patients diagnosed with adult T-cell leukaemia (reviewed in Robinson and Cobb 1997).

EGF-induced activation of NF- κ B has been shown to be important in the cell cycle progression of breast cancer cells which are oestrogen unresponsive (Biswas *et al.* 2000). NF- κ B induces cyclin D and hence entry into the G₁ phase of the cell cycle.

Under normal circumstances the ERK pathway may have a positive effect upon inhibiting carcinogenesis. A recent investigation showed that the induction of a number of phase II detoxifying enzymes by the chemicals *tert*-butylhydroquinone or sulforaphane was mediated via the activation of ERKs (Yu *et al.* 1999). This was shown to occur in a Raf-1 dependent, Ras-independent manner. Since the phase II enzymes are responsible for the detoxification of carcinogenic agents, this pathway offers a mechanism by which the ERK pathway may protect against the damaging effects of carcinogens.

1.4 Breast cancer chemoprevention and related signalling pathways.

1.4.1 Epidemiological evidence.

In a review of 14 studies, which investigated the protective effect of fruit and vegetable consumption on the relative risk of developing breast cancer, 8 were found to have a protective effect (Block *et al.* 1992). This review showed that this protective effect was equal to, although in the opposite direction to, the effect of saturated fat. Dietary fibre was also shown to be significant in protecting against the development of breast cancer. In this case the mechanism of action was postulated to involve lowering the level of circulating oestrogen (reviewed in Challa *et al.* 1997).

1.4.2 Signalling and breast cancer.

The EGFR itself along with the other family members are important in the regulation of normal breast epithelial cell growth. The receptors are commonly expressed at low levels in normal breast tissue. In some breast cancers gene amplification results in an overexpression of the receptor. If this gene amplification is associated with an increased expression of a ligand, such as TGF α , an autocrine loop can be instigated resulting in disregulation of cell growth (Kolibaba and Druker 1997). The particular homo- or hetero-dimers formed may affect the level of dysfunction. Two studies showed the EGFR to be an important prognostic factor in determining the outcome for breast cancer patients (Sainsbury *et al.* 1987; Toi *et al.* 1990). Sainsbury *et al.* (1987) showed that relapse-free survival and overall survival were significantly worse for patients with EGFR positive (+ve) than EGFR negative (-ve) tumours. An inverse relationship was observed with the oestrogen receptor, in that relapse free survival and overall survival were worse in patients with Oestrogen receptor –ve tumours. The worst prognosis was for those patients with EGFR +ve and ER –ve tumours. Toi *et al.* (1990) showed similar findings and both studies concluded that EGFR status was important in predicting the malignancy and relapse potential of the developing tumour.

Several studies have demonstrated the relationship between the gene amplification of erb-B2 and overall survival and time to relapse in breast cancer patients (Helal *et al.* 2000;

Kallioniemi et al. 1992; Merchant et al. 1999; Slamon et al. 1987; Tagliabue et al. 1999). One of these investigations showed that in 30% of breast tumours erb-B2 was amplified from 2-20 fold (Slamon et al. 1987). Others have shown the figure to be up to 40% (Tagliabue et al. 1999). Some investigators have also shown an inverse relationship between the presence of oestrogen receptors and elevated erb-B2, although others have observed no such relationship, so the picture here remains unclear (reviewed by Gullick 1990). A monoclonal antibody to erb-B2 called Herceptin, has been developed and used to successfully treat patients with erb-B2 positive metastatic breast cancer (reviewed in Green et al. 2000; Stebbing et al. 2000). This therapy has been shown to prolong patient survival and has few side effects when used alone, but has demonstrated cardiac toxicity when used in combination with certain chemotherapeutic agents.

Erb-B3 has been shown to be expressed variably within breast tumours and no relationship has been demonstrated between erb-B3 expression and patient survival (reviewed in DeFazio *et al.* 2000). A direct relationship between expression of erb-B3 and the oestrogen receptor was demonstrated in tumour samples removed from 89 patients (Knowlden *et al.* 1998). The same study showed a similar relationship between erb-B4 and the oestrogen receptor. These two members of the erb-B family seem to be associated with the more prognostically favourable oestrogen receptor +ve tumour phenotype. The amplification of erb-B4 has been shown to occur less frequently than erb-B2 in breast carcinomas (Vogt *et al.* 1998). The same authors did demonstrate an inverse relationship between erb-B4 amplification and the expression of the oestrogen receptor. Sawyer et.al (1998) identified two erb-B4 transcripts that possibly differed in their downstream modes of action. These two transcripts were expressed in the same proportions in normal and malignant breast tissue suggesting that they have no relevance in carcinogenesis. As yet there is no clear pattern to the expression and patient survival, so more studies need to be done in order to determine its effect.

The oestrogen receptor has long been implicated in breast cancer development and the loss of oestrogen responsiveness of a tumour is a poor prognostic factor (McGuire 1978). Oestrogen receptor –ve tumour cells do not respond to endocrine therapy and are more aggressive in their growth behaviour. As previously described, the amplification of some of the erb-B family of RPTKs have been associated with the loss of oestrogen-responsiveness of breast tumours, as has constitutive activation of the transcription factor NF- κ B (Nakshatri *et al.*

1997). The constitutive activation of NF- κ B was shown to occur in oestrogen-unresponsive tumours suggesting that this molecule is important in the hormone-independent growth of breast tumour cells.

1.4.3 Current methods of breast cancer prevention.

The major, current methods of breast cancer prevention involve the use of antioestrogens such as tamoxifen (Cristofannilli and Hortbagyi 1998; Osborne 1999). Its use following primary surgery has been associated with delayed relapse and a 20-30% reduction in the risk of death. The drug has also demonstrated a 39% reduction in the incidence of second primary tumours in some studies (reviewed in Cristofannilli and Hortbagyi 1998). Several trials have shown adverse effects of tamoxifen, with an increase in endometrial cancer being observed, but current thinking is that the benefits outweigh the risks (Osborne 1999). A second generation of antioestrogens has been developed which aim to eliminate the negative side effects. These are drugs such as raloxifene and toremifine (Kelloff *et al.* 1999). A third generation of compound is also at an early development stage, with drugs such as droloxifene and idoxifene hoped to improve the situation further (Osborne 1999).

The aim now is to develop chemopreventive agents that are effective against both hormonesensitive and hormone-insensitive tumours and which do not exhibit undesirable side effects.
CHAPTER 2. MATERIALS AND METHODS.

2.1 Materials.

2.1.1 General chemicals and reagents.

All the general chemicals and reagents were purchased from Sigma-Aldrich Company Ltd (Poole. UK), unless otherwise specified.

2.1.2 Chemopreventive agents and inducers of signal transduction.

Curcumin (min 99%), EGCG (min 95%) and I3C (min 99%) were obtained from Sigma-Aldrich Company Ltd (Poole. UK). They were made up as 20mM, 200mM and 1M stock solutions respectively in dimethyl sulfoxide (DMSO). DMSO, anisomycin, 12-Otetradecanoylphorbol-13-acetate (TPA) and tumour necrosis factor α (TNF α), were purchased from Sigma-Aldrich and epidermal growth factor (EGF) from Gibco BRL, Paisley, UK.

2.1.3 Antibodies.

Antibodies against EGFR, phosphotyrosine (PY99), phospho-ERK1/2 (Tyr²⁰⁴), ERK1/2, JNK1, c-jun, c-fos and CDK 6 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz. CA); anti-phospho c-jun (Ser⁷³) and anti-phospho Akt (Ser⁴⁷³) were from New England Biolabs (NEB), Beverly. MA; anti-MAPKAP K2 was from Upstate Biotechnology Inc., Lake Placid, NY; anti-hemagglutinin (anti-HA) monoclonal antibody was purchased from Boehringer Mannheim, Lewes, UK; and anti-p38 and anti-MKK4 polyclonal antibodies were kindly provided by Martin Dickens (University of Leicester). The anti-mouse and anti-rabbit IgG (whole molecule), horse radish peroxidase (HRP) conjugates were obtained from Sigma.

2.1.4 Radiochemicals.

 $[\gamma^{32}P]$ adenosine 5'-triphosphate (ATP) was purchased from Amersham Life Science Ltd, (Little Chalfont, UK).

2.1.5 Kits.

WizardTM maxiprep plasmid preparation kits were purchased from Promega Ltd. (Southampton, UK.). Qiagen RNeasyTM midi-RNA preparation kits were obtained from Qiagen Ltd (Surrey, UK). The bulk GST purification kit was obtained from Amersham Pharmacia Biotech, Uppsala, Sweden. The protein tyrosine phosphatase assay kit was purchased from NEB (Beverly, MA) and the enhanced chemiluminescence (ECL) detection kit from Amersham Life Science.

2.1.5 Plasmids.

The following plasmids were used; pGEX vector and pGEX/jun (1-79) which were gifts from Martin Dickens (University of Leicester), HA-tagged MEKK1 in pcDNA3.1 which was a gift from Johnathan Blank (University of Leicester), pcDNA3.1 purchased from Invitrogen (Carlsbad, CA.) and Δ MEKK3:ER in pBabe puro which was a gift from Simon Cook (Babraham Institute, Cambridge).

2.1.6 Bacterial strains and growth media.

The following *Escherichia coli* strains were used: XL10-Gold and XL-1 Blue, both of which were obtained from Stratagene (Cambridge, UK.). XL10-Gold cells were used as a host for the pcDNA3.1 vectors and XL-1 Blue cells as a host for the pGEX vectors and for the expression of the GST fusion proteins. All bacterial stocks were stored at -80°C in the form of glycerol stocks, made by adding 0.6 ml of an overnight culture to 0.4ml sterilised glycerol. The following bacterial growth media were used: L-Broth, LB agar and YTX (Sambrook *et*

al. 1989) were obtained from core supplies. Supplements added to the media included 50µg/ml ampicillin (Sigma).

2.1.7 Eukaryotic cells.

Cells were obtained as frozen ampoules from core stocks (HBL 100 and HEK 293) or were a gift from Rosemary Walker (Glenfield Hospital, Leicester) (MDA 468 and T47D), or Simon Cook (Babraham Institute, Cambridge) (CCL39). The cell lines used are listed below;

- HBL 100 A human breast cell line derived from normal breast epithelial tissue, but which has been transformed with SV40.
- T47D A human breast tumour cell line.
- MDA 468 An aggressive human breast tumour cell line.
- HEK 293 A human embryonic kidney cell line.
- CCL39 A chinese hamster lung fibroblast cell line.

2.1.8 Cell culture media and supplements.

For eukaryotic cells Dulbecco's modified eagle's medium (DMEM) and RPMI 1640 were obtained from Gibco BRL, Paisley, UK. Phosphate buffered saline (PBS), fetal calf serum (FCS), Glutamax I and trypsin/EDTA were obtained from core stocks in-house. The media used for each cell line is outlined in table 2.1.

All media and solutions for tissue culture were prepared and handled under sterile conditions.

CELL LINES	MEDIA	SUPPLEMENTS
HBL 100 & T47D	DMEM with L-Glutamine, 1000mg/ml D-Glucose & sodium pyruvate.	10% FCS
MDA 468	RPMI 1640 without L- Glutamine.	10% FCS & 2mM Glutamax I
HEK 293	DMEM with Glutamax I, 4500mg/l D-Glucose and without sodium pyruvate.	10% FCS
CCL39	DMEM with L-Glutamine, 1000mg/ml D-Glucose & sodium pyruvate.	7% FCS

Table 2.1. Eukaryotic cell media and supplements.

2.2 Methods.

2.2.1 Growth and maintenance of cell culture.

2.2.1.1 Resuscitation of cells from liquid nitrogen.

Frozen cells were immediately transferred to a 37°C water bath for 1-2 mins until fully thawed. The contents of the ampoule were carefully resuspended in 10ml of pre-warmed media and the cells pelleted by centrifugation at 200 x g for 3 mins. The cells were resuspended in 10ml fresh media and transferred to a 25cm^2 flask. Cells were incubated at 37°C in humidified 5% CO₂. All the cell lines used were tested for mycoplasma contamination and found to be negative (Mycoplasma Experience).

2.2.1.2 Subculturing of cell lines.

Once the cells had reached confluence they were passaged. The media was removed and the cell monolayer washed once with PBS. The cells were detached by adding 2-5 ml 1x Trypsin/EDTA and incubating at 37°C for 3-5 minutes. An equivalent amount of pre-warmed medium was added and the cells pelleted by centrifugation. The pellet was resuspended in

fresh medium and the cells transferred to the required number of culture flasks. Cells were routinely split between 1:4 - 1:10 depending upon the number of cells required. Cells were replenished from liquid nitrogen stocks after 20-25 passages.

2.2.1.3 Storage of cells.

Cells were trypsinised from a 75cm^2 flask and collected by centrifugation. The cell pellet was resuspended in 15ml of fresh medium and the cell number determined by counting on a haemocytometer. The cells were pelleted by centrifugation once more and resuspended in 1ml of freezing medium (7.5% DMSO in medium containing 2mM Glutamax I and 35% FCS), per 1x10⁶ cells. Cells were transferred to 1.5ml freezing vials and frozen slowly overnight at -80°C. They were then transferred to a liquid nitrogen storage tank.

2.2.2 Analysis of protein.

2.2.2.1 Protein concentration determination (Biorad assay).

The Biorad assay is a colorimetric assay for the determination of protein concentration based on the method of Bradford (1976). To obtain a standard linear plot 0, 125, 250, 500, 750 and 1000 μ g bovine serum albumin (BSA) were pipetted into tubes to a final volume of 800 μ l. Protein samples were made up in 1/100 or 1/200 dilutions to 800 μ l. 200 μ l of the biorad assay reagent (Biorad Laboratories Ltd. Hemel Hempstead, UK) was added to each BSA standard and each protein sample and the tubes vortexed. The absorbance at A₅₉₅ was measured against the 0 μ g BSA standard and the concentration of the protein samples determined with reference to the BSA standards.

2.2.2.2 Protein concentration determination (Pierce assay)

The Pierce assay is a colorimetric assay for the determination of protein concentration based on the bicinchoninic acid reaction (Smith *et al.* 1985). This assay was used in preference to the Bradford assay in situations with a significant amount of SDS in a sample, since the Pierce assay is compatible with a high level of SDS whereas the Bradford assay is not. BSA standards were made up, at the same concentrations as the Bradford assay (2.2.2.1), in a total volume of 50µl. The assay reagents A and B (Pierce, Rockford, IL.) were mixed in a ratio of 50 parts A : 1 part B and 1 ml of this working reagent was added to each sample. Samples were mixed and incubated at 37°C for 30 mins, the absorbance at A_{562} was determined for each sample and the standards, against the 0µg standard. The protein concentration of each sample was determined with reference to the BSA standards.

2.2.2.3 One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Gel Solutions

Solution A	-	30% stock solution (w/v) acrylamide and bis-acrylamide (Anachem
		Ltd. Luton, UK.) NB. Acrylamide is a potent neurotoxin.
Solution B	-	1.5M Tris-HCl, pH 8.8
Solution C	-	1M Tris-HCl, pH 6.8
Solution D	-	10% sodium dodecyl sulphate (SDS)
Solution E	-	10% ammonium persulphate (APS)
Solution F	-	N,N,N',N'-tetramethylenediamine (TEMED)

Gel preparation

The choice of acrylamide concentration depends upon the molecular weight range of the proteins being studied. High molecular weight proteins such as EGFR (170kDa) were resolved on 8% gels and the lower molecular weight proteins on 10% (80-45kDa), or 12% (<45kDa) gels. The appropriate volumes (ml) of solutions A-F were mixed to give the percentage gel required (Table 2.2).

Solutions A, B, D and water were mixed and polymerisation was initiated by addition of APS (solution E) and TEMED (solution F). The resolving gel was poured into the gel cassette using a 1ml pipette until the level reached approximately 1.5cm from the top of the glass plate. A small amount of dH_2O was layered over the top to exclude air and ensure the surface of the gel was level. After the resolving gel was set, the dH_2O was poured off. The stacking gel was prepared by mixing solutions A, C, D and water, and polymerisation was initiated by addition of APS and TEMED. The gel was poured onto the resolving gel and a lane-forming comb was inserted.

Sample Preparation

One volume of 5x SDS-PAGE sample buffer (250mM Tris-HCl, pH6.8; 10% (w/v) SDS, 50% (w/v) glycerol, 500mM dithiothreitol (DTT), 0.1% bromophenol blue) was added to 4 volumes of sample to give a final concentration of 1x sample buffer. Samples were boiled for 5 min before loading onto the gel.

Solution	% Acrylamide			
	. 8%	10%	12%	5% (Stack)
A	4.0	5.0	6.0	0.83
В	3.8	3.8	3.8	-
С	-	-	-	0.63
D	0.15	0.15	0.15	0.05
E	0.15	0.15	0.15	0.05
F	0.0009	0.0006	0.0006	0.005
H ₂ O	6.9	5.9	4.9	3.4

Table 2.2. Recipes for SDS PAGE gels.

Gel Running Conditions

The gel was clamped into the Hoeffer gel apparatus, the upper reservoir filled with SDS running buffer (25mM Tris (pH 8.3), 250mM glycine, 0.1% SDS) and the lower reservoir was filled with sufficient buffer to cover the bottom of the gel. Samples were loaded into the wells using round gel tips. The gel was run at a constant voltage of 150V until the bromophenol blue dye front had just run off the end of the gel. The gel was then ready for transfer onto nitro-cellulose membrane (2.2.3.2) or drying down (2.2.13).

2.2.2.4 Western blot analysis.

Solutions.

- Transfer Buffer: 20% methanol (Fisher Scientific Loughborough, UK.), 48mM Tris, 38mM glycine, 0.037% (w/v) SDS.
- Tris buffered saline Tween 20 (TBST) buffer: 50mM Tris (pH 7.5), 150mM NaCl, 0.1% (v/v) Tween-20.

Transfer of proteins onto nitrocellulose membranes.

Prior to blotting, the gel was allowed to equilibrate in transfer buffer for 15mins. At the same time one sheet of nitrocellulose, two sponges and two pieces of blotting paper (Whatman) were also soaked in transfer buffer. The gel, along with the other components, was set up in the Biorad wet-blotting apparatus as in figure 2.1 and run at 100V for 90 minutes.





Visualisation of proteins by ECL.

After transfer, membranes were blocked in 5% BSA in TBST for 1-2 hours at room temperature. Primary antibody was diluted, as required, in 0.5% BSA TBST and the blot incubated for 1-2 hours at room temperature or overnight at 4°C. The blot was washed for 4x 10 mins in TBST followed by incubation with the relevant HRP-conjugated secondary

antibody, diluted 1:2000 in TBST, for 1 hour at room temperature. The blot was washed a further 4x 10 mins in TBST and transferred to ECL reagent (Amersham) for 1 min. The blot was developed on Hyperfilm[™] (Amersham) in the dark. A variety of exposure times were used, usually 1-10 min, to get a usable image.

2.2.3 Isolation and purification of plasmid DNA.

2.2.3.1 Buffers and solutions contained in the Promega Wizard ™ maxiprep kit.

- Cell resuspension solution: 50mM Tris-HCl (pH 7.5), 10mM EDTA, 100µg/ml RNase A.
- Cell lysis solution: 0.2M NaOH, 1% SDS.
- Neutralisation solution: 1.32M potassium acetate (pH4.8).
- Wizard[™] DNA purification resin.
- Column wash solution: 80mM Potassium Acetate, 8.3mM Tris-HCl (pH 7.5), 40μM EDTA, 55% ethanol.

2.2.3.2 Protocol for plasmid DNA extraction and purification.

This kit is designed for the preparation of plasmid DNA from 100 - 400ml bacterial cultures. The minimum plasmid yield is 100 - 500µg depending upon culture size, bacterial host and plasmid. Briefly, 400ml of an overnight culture was pelleted by centrifugation at 5000 x g for 10 minutes at room temperature. The pellet was resuspended in 15ml of cell resuspension buffer and lysed by the addition of 15ml cell lysis buffer. The mixture was neutralised by the addition of 15ml neutralisation solution. The lysate was passed through a column containing the DNA binding resin. The resin was washed several times and the bound DNA eluted with 3ml of pre-heated (65-70°C) Tris/EDTA (TE) buffer. The concentration and purity of the plasmid preparation was assessed as in 2.2.6.

2.2.4 Isolation and extraction of RNA from mammalian cells.

2.2.4.1 Buffers and solutions contained in the Qiagen RNeasy™kit.

- RLT Buffer: Cell resuspension buffer.
- RW1 Buffer: Wash buffer I.
- RPE Buffer: Wash buffer II.
- RNase-free water.

2.2.5.2 Protocol for RNA extraction and purification.

The RNeasyTM midi kit is designed for the extraction of up to 1mg of total RNA from cells. The initial part of the RNA extraction procedure was as per the manufacturer's protocol (RNeasy mid/maxi Handbook, Qiagen). A phenol/chloroform extraction and reprecipitation was then performed to improve purity. Briefly, $3x10^7$ cells were pelleted and resuspended in buffer RLT. The cells were lysed by sonication (6 x 5sec at 10µ) on ice before adding to the RNA binding column. The column was washed once with RW1 buffer and twice with RPE buffer and the RNA eluted in 150µl RNase-free water. 1ml of Tri reagent (Sigma) and 200µl chloroform were added to the RNA and the solution vortexed. The aqueous layer was recovered and the RNA precipitated by addition of 0.5 volumes isopropanol. The RNA was collected by centrifugation at 10 000 x g for 10 min, washed twice with 70% ethanol and resuspended in 200µl RNase-free water. The RNA was re-precipitated with 20µl 3M sodium acetate (pH 5.2) and 600µl ethanol at -70°C overnight. The RNA was collected by centrifugation, washed twice with 70% ethanol and the pellet dried for 5 minutes in a speed vac (Savant Instruments, Farmingdale, NY). The RNA was dissolved in 20µl RNase-free water and the concentration determined as in (2.2.6).

2.2.5 Quantification of nucleic acid concentration.

The concentration of double stranded (ds) DNA or RNA in aqueous solution was determined by measuring the absorbance of a dilution of the nucleic acid solution in a quartz cuvette at 260nm. An aliquot of the nucleic acid solution was diluted using sterile water (usually 100fold, i.e. 5μ was added to 495μ water). The spectrophotometer was zeroed at 260nm using sterile water and the absorbance (A) of the diluted nucleic acid solution was measured. The concentration of DNA or RNA was then calculated using the following equations:-

dsDNA concentration (μ g/ml) = A₂₆₀ × dilution × 50

RNA concentration ($\mu g/ml$) = $A_{260} \times dilution \times 40$

Comparison of absorbance values at 260 and 280nm of a nucleic acid solution provides an estimate of the purity of the preparation. A 260/280 ratio of approximately 1.6-1.8 for DNA and RNA, indicates a protein free preparation, ratios significantly lower than these indicate protein contamination (Sambrook *et al.* 1989).

2.2.6 Analysis of nucleic acids (DNA) on an agarose gel.

2.2.6.1 Gel preparation.

A 1% (w/v) agarose solution was made up in Tris acetate EDTA (TAE) buffer (40mM Tris base, 0.11% (v/v) glacial acetic acid, 1mM EDTA (pH 8.0)). This was melted and allowed to cool. Upon cooling 2μ l of ethidium bromide per 100ml was added to the agarose and the gel solution poured into the gel caster. A gel comb was inserted and the gel allowed to set.

2.2.6.2 Sample preparation and running the gel.

A suitable amount of DNA was taken and made up to 9μ l in sterile water. 1μ l of 10x DNA loading buffer (0.01M EDTA, 0.5% (w/v) sucrose and 0.01% bromophenol blue) was added to each sample and the gel run at 75V until the dye front neared the end. The gel was observed and photographed under ultraviolet light.

2.2.7 Preparation of the GST-jun fusion protein.

2.2.7.1 Bacterial cell transformation.

XL-1-Blue, supercompetent cells were thawed on ice and 40µl aliquots taken. β mercaptoethanol was added to a final concentration of 25mM and the cells mixed gently on ice. 50-100ng of pGEX and of pGEX/jun(1-79) were added to the cells and the mixture placed on ice for 30 mins. The mixture was heat-pulsed for 45 sec at 42°C and placed on ice for 2 mins. Pre-warmed (42°C) SOC media (0.45ml) (20g/l tryptone, 5g/l yeast extract, 0.5g/l NaCl, 2.03g/l MgCl₂, 1.2g/l MgSO₄, 3.6g/l glucose) was added and the mixture incubated for 1 hour at 37°C. The media was diluted 1:1000 and 100µl plated out onto LB-AMP plates. A single colony of this overnight incubation was used to inoculate a 10ml overnight culture in YTA media (YTX media with 50µg/ml ampicillin). Glycerol stocks were created as described previously (2.1.6) and stored at -80°C.

2.2.7.1 Expression and purification of the GST-jun fusion protein.

0.5ml of an overnight culture of the pGEX and pGEX/jun(1-79) plasmid in XL-1 Blue cells was used to inoculate a 400ml culture. This was allowed to grow to an optical density of 0.6-0.8 at A_{600} . Protein expression was induced by the addition of 100nM IPTG and the culture incubated for a further 4 hours. The proteins were purified from the cultures using the GST purification module from Pharmacia Biotech. Briefly, cells were pelleted at 5000 x g for 10 mins and the pellet placed on ice. Cells were resuspended in 8ml ice cold PBS and lysed by sonication (4x 10 sec at 10 μ) on ice. Sonicates were cleared by centrifugation (10 000 x g for 5 min) and 1.6 ml of a 50% slurry of glutathione sepharose added to the lysate. The suspension was mixed on a rotating platform for 30 min at room temperature. The slurry was added to a column and the GST proteins were eluted by the addition of 2x 250 μ l reduced glutathione. The presence of the correct fusion protein and its concentration was determined by running the GST/jun fusion and the pGEX empty vector (GST alone) on a SDS PAGE gel with BSA standards. The gel was stained with coomasie blue and dried down.

2.2.8 Mammalian cell transfection.

The non-liposomal transfection reagent Fugene 6^{TM} (Boehringer Mannheim) was used to transfect HEK 293 cells with pcDNA3.1 and pcDNA3.1/HA-MEKK1. For each transfection 3μ l of Fugene 6^{TM} was added to 97μ l of serum free medium, mixed and allowed to stand for 5 min. This 100µl mixture was added to 2μ g of the DNA to be transfected, mixed and allowed to stand for 15 min. After this time the mixture was added to the culture dish of cells to be transfected and incubated for 72 hours.

2.2.9 Determination of cell growth characteristics.

2.2.9.1 Quantitation of cell growth.

Cells $(1x10^4)$ were incubated in the presence, or absence of the indicated concentrations of chemopreventive agent or DMSO vehicle control. At the indicated times, cells were trypsinised and their number determined using a Coulter ZM electronic cell counter (Beckman Coulter Ltd, High Wycombe, UK).

2.2.9.2 Cell cycle analysis.

For analysis of the cell cycle, 1×10^6 cells were treated with chemopreventive agents, as described in chapter 3, prior to trypsinisation and fixation in 2ml, ice cold, 70% ethanol. Cells were collected by centrifugation and resuspended in 1 ml PBS containing 0.1 mg/ml ribonuclease A (RNase A) and 5mg/ml propidium iodide. The DNA content was measured by flow cytometry (Becton Dickinson FACScan) and the percentage of cells in each phase of the cell cycle estimated using the Cell Quest software programme.

2.2.9.3 Determination of apoptosis.

The number of cells undergoing apoptosis was estimated using binding of annexin V to externalised phosphatidylserine. Cells (1×10^6) were treated as above and harvested at time points from 24 to 48 hrs, washed with PBS, and resuspended in annexin V-binding buffer

(10mM Hepes pH 7.4, 150mM NaCl, 5mM KCl, 1mM MgCl₂ and 1.8mM CaCl₂). Fluorescein isothiocyanate (FITC)-conjugated annexin V (Bender Med Systems, Vienna, Austria) was added to a final concentration of 100ng/ml and cells incubated for 8 min at room temperature. Propidium iodide (50μ g/ml) was added before flow cytometric analysis.

2.2.10 Measurement of intracellular ATP.

Cells (5x10⁵) were treated as indicated and lysed in 2% perchloric acid. The solution was neutralised by the addition of 1M KOH and precipitated protein collected by centrifugation. The precipitate was resuspended in a suitable volume of 1M NaOH and the protein concentration determined using the Pierce BCA protein assay kit (Pierce, Rockford, IL). Five µl of the sample supernatant was added to 70µl of assay buffer, comprising a 5:2:6 ratio of arsenate buffer (100mM sodium arsenate and 40mM magnesium sulphate), phosphate buffer (25mM NaH₂PO₄, 10mM MgSO₄) and H₂O. Twenty five µl of a luciferase preparation (Sigma Aldrich Company Ltd) was added and the number of photons emitted counted using a luminometer. The concentration of ATP was calculated with reference to standards.

2.2.11 Measurement of EGFR and ERK phosphorylation.

To assess the effect of the agents on phosphorylation of both the EGFR and ERKs 1 and 2, cultures, 70-80% confluent, were washed with PBS and incubated in serum-free medium overnight. This medium was changed again 1 hour before treatment.

For the EGFR assays cells were rinsed twice with ice cold PBS and lysed in 500µl Triton lysis buffer, (20mM Tris pH 7.4, 137mM NaCl, 25mM β -glycerol phosphate, 2mM sodium pyrophosphate, 2mM EDTA, 10%(v/v) glycerol, 2mM benzamidine, 1mM sodium vanadate, 1mM PMSF, 5µg/ml pepstatin, 5µg/ml aprotonin, 5µg/ml leupeptin, 1% v/v Triton X-100 (TX-100) and 0.5mM DTT). The lysates were cleared by centrifugation at 1000 x g for 5 min at 4°C, flash frozen and stored at -80°C until required. The receptor was precipitated at 4°C for 4 hours from 500µg cell lysate (determined by Biorad assay), using 2µg of polyclonal anti-EGFR antibody bound to 25µl (equivalent to 1mg) protein A agarose beads. After

centrifugation, the beads were washed twice in PBS supplemented with 350mM NaCl, 0.2% TX-100 and twice in PBS/0.2% TX-100. Samples were analysed by SDS-PAGE (2.2.2.3) on an 8% gel and western blotting (2.2.2.4), using an anti-phosphotyrosine primary antibody (PY99) at 1:2000 dilution, and HRP-coupled secondary antibody. Bound antibody was visualised with an enhanced chemiluminescence detection (ECL) detection system (Amersham Life Science Ltd, Little Chalfont, UK).

For the ERK assays, treated cells, seeded at $3-5 \ge 10^5$ per well, were lysed in 200µl boiling sample buffer, and sonicated for 30 seconds. Twenty to 40 µg of protein per lane (determined by Pierce assay) was run on 10% SDS polyacrylamide gels and analysed as above, by western blotting with an anti-phospho ERK antibody at 1:2000 dilution. Blots were scanned using a densitometer (Molecular Dynamics, Sunnyvale, CA.) and quantified using the Image Quant program.

For each experiment duplicate samples were run out on SDS PAGE and western blotting performed using an antibody for total EGFR (1:2000), or total ERK (1:2000) followed by an HRP-conjugated secondary and visualisation by ECL detection. This showed any changes in protein levels and gave an indication of protein loading consistency.

2.2.12 Nuclear protein extraction and western blotting for c-jun and c-fos.

Cells $(3x10^6)$ were treated as indicated and collected by centrifugation. Nuclear protein was extracted by resuspending cells in 400µl buffer A (10mM Hepes pH 7.8, 10mM KCl, 2mM MgCl₂ 1mM DTT, 0.1mM EDTA, 0.4mM PMSF and 0.2mM sodium orthovanadate), followed by lysis with the addition of 25µl buffer B (10% IGEPAL, Sigma Aldrich Company Ltd., Poole, Dorset, UK). Nuclei were collected by centrifugation and lysed by resuspension in 50µl buffer C (50mM Hepes pH 7.8, 50mM KCl, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 0.4mM PMSF, 10% v/v glycerol, 0.2mM NaF and 0.2mM sodium orthovanadate). Samples were sonicated for 30seconds at 10µ power and protein concentration determined using the Biorad method (2.2.2.1). Forty µg of nuclear protein was resolved on 10% SDS polyacrylamide gels and western blotting performed, as described in 2.2.2.4, for c-jun (1:2000), phosphorylated c-jun (1:1000), or c-fos (1:2000) levels.

2.2.13 Immune-complex kinase assays.

2.2.13.1 Assays for ERK, JNK, p38 and MAPKAP K2 activities.

Cells seeded at 1×10^6 were allowed to recover overnight and then cultured for 60 min in medium with the indicated treatments. Lysates were prepared as for EGFR phosphorylation assays (2.2.11). Clarified lysates were incubated with 15µl (equivalent to 0.6mg) Protein A, or Protein G, agarose to which were bound 5µg of antibodies to ERK, JNK, p38, (protein A), or MAPKAP K2 (protein G). The precipitates were washed twice with Triton lysis buffer and once with kinase assay buffer (25mM Hepes pH 7.4, 25mM β-glycerol phosphate, 25mM MgCl₂, 0.5mM EDTA, 0.5mM sodium vanadate and 0.5mM DTT). Pellets were resuspended in 30µl kinase assay buffer with addition of appropriate substrate [5µg photo, heat and acid stable protein I, PHAS I, (Calbiochem, Nottingham, UK), for ERK assays; 5µg of GST-jun, amino acids 1-79 (2.2.8) for JNK assays; 5µg GST-ATF2 (Martin Dickens, University of Leicester), for p38 assays, or 50µM MAPKAP K2 substrate peptide (Upstate Biotechnology, Lake Placid, NY)], along with 50 μ M ATP containing 2 μ Ci of [γ^{32} P] ATP. Reactions were incubated for 30 min at 30°C. ERK, JNK and p38 assays were terminated by the addition of SDS-PAGE sample buffer. The samples were then subjected to electrophoresis on 10% SDS-PAGE gels. ³²P incorporation into the substrate was determined by PhosphorImager (Molecular Dynamics) analysis of the dried gels. MAPKAP K2 assays were terminated by spotting 30µl aliquots of the reaction mixture on to p81 phosphocellulose squares (Upstate Biotechnology), followed by immersion and repeated washing in 150mM H₃PO₄ with a final wash in acetone prior to drying. Incorporation of 32 P into the substrate was measured by scintillation counting. Background incorporation was determined in reactions lacking substrate peptide.

Forty μ g of the clarified lysates were taken, in the case of JNK, and separated by SDS PAGE as described previously. These gels were subjected to western blotting with anti-JNK1 antibody (1:2000) and visualisation by chemiluminescence, in order to determine any change in protein levels and as a control for protein loading.

69

2.2.13.2 Assay for MKK4 activity.

Five μg of MKK4 antibody was bound to 0.6mg Protein A agarose beads and MKK4 immunoprecipitated as described for the other kinases. The kinase assay itself was a coupled assay with the addition of 1 μg GST-JNK (provided by Martin Dickens) and 5 μg GST-jun as substrates. Samples were again subjected to electrophoresis on SDS-PAGE gels and the incorporation of ³²P into the GST-jun substrate was determined as the measure of activity. Assays in which GST-JNK was excluded were performed as a negative control.

As described for JNK (2.2.13.1), samples of the clarified lysate were analysed by western blotting performed with an anti-MKK4 antibody (1:5000 dilution), as a measure of MKK4 expression and as a control for protein loading.

2.2.13.3 Assays for MEKK1 activity.

Cultures of HEK 293 cells (20% confluent) were transfected (2.2.8) with HA-MEKK1 (2.1.5) or pcDNA 3.1 empty vector (2.1.5) and allowed to recover for 72 hours. Lysates were prepared as for the EGFR assays (2.2.11) and clarified lysates were incubated with 0.6mg Protein G beads, to which was bound 1 μ g high affinity anti-HA monoclonal antibody, for 4 hours at 4°C. The precipitates were prepared and the kinase assay conducted as for ERK, JNK and p38 previously (2.2.13.1) in the presence of the indicated concentration of curcumin. The kinase assay itself was again a coupled assay with the addition of GST-MKK4 (Martin Dickens), or GST-MKK7 (Johnathan Blank, University of Leicester) and GST-JNK and GST-jun as substrates. Samples were subjected to electrophoresis on SDS-PAGE gels and the incorporation of ³²P into the GST-jun substrate was again determined as the measure of activity.

2.2.14 Protein tyrosine phosphatase assay.

2.2.14.1 Buffers and assay components.

- Abl (tyrosine kinase)/Myelin basic protein (MBP) mix supplied in 50mM Tris-HCL (pH 7.5), 100nM NaCl, 0.1mM Na₂EDTA, 1mM DTT, 0.01% Brij 35and 50% glycerol.
- ATP 10mM (pH 7.5).
- 10x Abl buffer 1x is 50mM Tris-HCl (pH 7.5), 10mM MgCl₂, 1mM EGTA, 2mM DTT and 0.01% Brij 35.
- Substrate solubilisation buffer 50mM Tris-HCl (pH 8.5), 0.1mM Na₂EDTA, 2mM DTT and 0.01% Brij 35.
- 10% (w/v) Brij 35.
- 10x Protein phosphatase buffer 1x is 50mM Tris-HCl (pH 7.0), 1mM Na₂EDTA, 5mM DTT and 0.01% Brij 35.
- 10 mg/ml BSA (protease free).

2.2.14.2 Phosphatase assay protocol.

The protein tyrosine phosphatase assay system (New England Biolabs) is designed for the preparation of the phosphotyrosyl protein substrate and the radioactive assay of the protein tyrosine phosphatase activity of a cell extract. The assay was conducted as per the manufacturer's protocol. Briefly, the MBP was prepared by phosphorylation on multiple tyrosine residues with Abl kinase in the presence of $[\gamma^{32}P]$ ATP in the following reaction mixture: 40µl Abl/MBP mix, 20µl 10x Abl buffer, 20µl 10mM ATP, 5µl $[\gamma^{32}P]$ ATP and 115µl dH₂O. This was incubated overnight at 30°C and a sample taken to determine the specific activity of the labelled ATP. The reaction was terminated by the addition of 1/9 volume of 100% trichloroacetic acid (TCA) and incubation on ice for 30 mins. The substrate was collected by centrifugation (12 000 x g for 10 min) at 4°C and the pellet washed with 3x 1ml 20% TCA to remove residual, unbound ³²P. The substrate was dissolved in 1ml substrate solubilisation buffer and dialysed for 16 hours, at 4°C, against 2x 11 of dialysis buffer (25mM Tris-HCl pH 7.5, 0.1mM Na₂EDTA, 2mM DTT, 0.01% Brij 35). The substrate was diluted to a concentration of 25µM phosphotyrosine and stored at 4°C.

MDA 468 cells were seeded at 5×10^5 and allowed to recover overnight prior to treatment as indicated. Cells were washed once with ice cold PBS and lysed in 50µl phosphatase lysis buffer (20mM Tris pH 7.4, 137mM NaCl, 2mM EDTA, 10% v/v glycerol, 2mM benzamidine, 1% v/v protease inhibitor cocktail (Sigma), 1% v/v TX-100, 0.5mM DTT). An aliquot was taken for protein determination by the Biorad assay and the remainder stored at -80°C.

1:40 dilutions of the cell lysate samples were assayed for phosphatase activity as follows: a 10 μ l sample of the cell lysate and 30 μ l of assay buffer (1x phosphatase assay buffer and 1 mg/ml BSA) were pre-incubated at 30°C for 5 min. Blanks were also set up in which the cell lysate was replaced by assay buffer. The reaction was initiated by the addition of 10 μ l of the MBP substrate and the mixture incubated at 30°C for 10 min. After this time the reaction was terminated by the addition of 200 μ l ice cold 20% TCA, vortexed and incubated on ice for 10 min. The substrate was collected by centrifugation and 200 μ l of supernatant mixed with 2ml of scintillation fluid (Ultima Gold, Packard Bioscience, Meriden CT) and counted on a scintillation counter (Packard Bioscience). The phosphatase activity was determined using the following calculation;

Released cpm = Sample cpm – Blank cpm

Activity (unit/ml) = Released cpm/(Total cpm - Blank cpm) x 0.25/10 x 20 x 250/200

Total cpm is the total radioactivity in 10μ l substrate, 0.25 is the number of nanomoles of incorporated phosphate in the assay, 10 is the incubation time in min, 20 converts the results to 1ml rather than 50 μ l of the reaction and 250/200 corrects for the fraction of the TCA supernatant that is counted.

2.2.15 Microarray analysis.

2.2.15.1 Buffers and solutions.

- dNTP mixture 20mM dGTP, 20mM dCTP, 20mM dATP and 8mM dTTP, (all from Pharmacia Biotech)
- 20x Di-sodium phosphate EDTA (SSPE) 3M NaCl, 1mM NaH₂PO₄, 20mM EDTA.
- Pre-hybridisation buffer 48% (v/v) de-ionised formamide, 6x SSPE, 0.5% SDS, 2.5x
 Denhardts buffer (Sigma) and 50µg/ml sonicated salmon sperm DNA (Sigma).
- Hybridisation buffer -71% (v/v) de-ionised formamide, 3.6x Denhardts, 0.7% SDS.
- Cy3-dUTP (green) and Cy5-dUTP (red) dyes (New England Nuclear)

2.2.15.2 Microarray hybridisation protocol.

General remarks

Total RNA was extracted and purified from cells as described in 2.2.4.2. Briefly, the microarray involves the conversion of total cellular mRNA to fluorescently labelled cDNA by reverse transcription polymerase chain reaction (RT-PCR). Control cDNA is labelled with one fluorophore and test cDNA with the other. The RNA is then hydrolysed away and the labelled cDNA purified. Control and test cDNA are mixed and hybridised to a gene array, which is then scanned. Genes equally expressed in both control and test cells show up as one colour which is the result of an equal mixture of the two fluorophores. Those in which expression is elevated or inhibited by the test compound take on the colour of the test or control fluorophore respectively. The array used in these experiments contained 5500 human gene sequences.

Labelling reaction

Fifty μ g of each of the RNA samples was taken and made up to 10 μ l. 0.5 μ l of Oligo dT₂₅ (8 μ g/ml) was added and the mixture annealed at 70°C for 8 min, followed by a reduction in temperature to 42°C over a period of 30 min.

The cDNAs were created and labelled by the addition of the following to the labelling reaction: 0.3μ I RNAsin, 4μ I 5x first strand buffer (1x final, supplied with Superscript II), 2μ I 0.1M DTT (0.01M final), 0.5μ I dNTP mix (dGTP, dATP, dCTP [0.5mM final] and dTTP [0.2mM final]), 2μ I Cy3 or Cy5 fluor-dUTP (0.1mM final) and 0.5 μ I superscript II reverse transcriptase (100 units final). The reaction was incubated for 1 hour at 42°C, a further 0.5 μ I of superscript II reverse transcriptase was added and the reaction incubated for a further hour. The reaction was made up to 41 μ I with the addition of 20.5 μ I water.

Hydrolysis of RNA

The labelling reaction was terminated by the addition of 1µl 0.5M EDTA followed by 1µl of 10% (w/v) SDS and 3µl 3M NaOH. The mixture was incubated at 68°C for 30 min followed by 25°C for 15 min to hydrolyse the residual RNA. The samples were neutralised by the addition of 3µl 2M HCl and 10µl of 1M Tris-HCl pH 7.5. Finally 1µl of 4µg/ml tRNA was added to the labelled probe.

Purification

The labelled probe was passed through a Centristep column (Princeton Separations, Adelphia, NJ) and the DNA eluted in 30μ l of dH₂O by centrifugation ($3000 \times g$ for 2 min).

Pre-hybridisation.

 1μ l of 1μ g/ml polyA and 1μ l of 10mg/ml Cot 1, human placental DNA were added to the control probe. The probes were dried down using a speed vac (Savant Instruments, Farmingdale, NY) and resuspended in 10.5µl hybridisation buffer. 4.5µl of 20x SSPE was added and the probes pooled. The entire probe was denatured by incubation at 100°C for 2 min followed by 42°C for 30 min – 1 hour.

Hybridisation and washing.

The probe was spotted onto the array and a coverslip lowered over the top. The array was sealed in a hybridisation chamber and incubated overnight at 42°C. Slides were washed in 1x

disodium citrate (SSC) containing 0.03% SDS followed by 0.2x SSC and finally 0.05x SSC. The slide was dried by centrifugation at 1000 x g for 5 min.

Scanning

The array was scanned with a Genepix 4000A scanner (Axon, US) and initial analysis undertaken using Genepix software.

<u>Analysis</u>

The data from the array was analysed using Microsoft Excel spread sheets and cluster analysis was performed using Treeview (Mike Eisen, Stanford, US).

2.2.17 Statistical analysis.

Significance of results was determined by balanced ANOVA, followed by Fischer's least significant difference post hoc test in the case of the growth studies. In the case of the microarray analysis see 2.2.15.2. In all other cases significance was determined by one way ANOVA, followed by Tukey's post hoc test. Significant difference from the positive, vehicle control was taken in all cases and a p value of <0.05 was considered significant.

CHAPTER 3. EFFECTS OF CHEMOPREVENTIVE AGENTS ON CELL GROWTH AND THE CELL CYCLE.

3.1 Introduction

A number of recent studies have shown that curcumin, EGCG and I3C have the ability to affect growth via a variety of potential mechanisms. Curcumin has been shown to inhibit proliferation in a variety of cell lines from a number of different tissues (Hanif et al. 1997; Hong et al. 1999; Korutla et al. 1995; Korutla and Kumar 1994; Chen and Huang 1998; Gautam et al. 1998; Simon et al. 1998; Mehta et al. 1997; Khafif et al. 1998. In particular curcumin was shown to have an antiproliferative effect against a panel of human breast cell lines, including the T47D cell line (Mehta et al. 1997). Ramachandran et. al. (1999) observed differential sensitivity to curcumin in two breast cell lines: MCF10A (breast epithelial cell) and MCF 7 (breast carcinoma cell). Two further papers showed inhibition of breast cell proliferation in MCF 7 cells (Hong et al. 1999; Simon et al. 1998), accompanied by a G₂/M arrest which occurred in a dose-dependent fashion over a period of 72hrs (Simon et al. 1998). Curcumin was shown to inhibit the growth of, and induce apoptosis in, NIH 3T3 and mouse sarcoma cells. This was also the case for several human cancer cell lines, but not for a panel of primary cell lines (Jiang et al. 1996). Curcumin induced apoptotic death in promyelotic human leukemia (HL 60) cells in a dose and time dependent manner (Kuo et al. 1996), which was reduced by the addition of antioxidants, suggesting that the cell death was mediated by reactive oxygen species. The expression of the anti-apoptotic protein Bcl-2 was also shown to be inhibited by curcumin treatment. Other studies have shown that curcumin inhibits proliferation of human colon cancer cell lines, HT-29 and HCT-15 (Hanif et al. 1997), rat aortic smooth muscle cells (A7r5) and rabbit smooth muscle cells (Chen and Huang 1998), human epidermoid carcinoma A431 cells (Kortula and Rakesh 1994) and EGFstimulated proliferation of NIH 3T3 cells expressing the human EGFR (Kortula et al. 1995).

Similar data have been obtained for EGCG and other tea polyphenols (Liang *et al.* 1999a; Kennedy *et al.* 1998; Yang *et al.* 1998; Suganuma *et al.* 1999; Lu *et al.* 1998; Khafif *et al.* 1998; Ahn *et al.* 1999; Ahmad *et al.* 2000; Paschka *et al.* 1998; Gupta *et al.* 2000. MCF 7 cells were shown to be growth inhibited and to arrest in the G_0/G_1 phase of the cell cycle (Liang *et al.* 1999a), as were premalignant and malignant human oral epithelial cells (Khafif *et al.* 1998) and a panel of human and murine carcinoma cell lines (Ahmad *et al.* 1997). This last study also reported an induction of apoptosis by EGCG, as did studies in human prostate cancer cells, which also showed a G_0/G_1 arrest (Gupta *et al.* 2000). Three further studies showed induction of apoptosis in human stomach cancer (KATO III) cells (Hibasami *et al.* 1998), a panel of human prostate cell lines (Paschka *et al.* 1998) and lung tumour (H661, H441 and H1299) and HT29 colon cancer cells (Suganuma *et al.* 1999; Yang *et al.* 1998).

I3C has been shown to inhibit the proliferation of breast cell lines in several recent reports. In one study MCF 7 cells were inhibited with an IC₅₀ ten fold lower than that for MDA 231 cells (Tiwari *et al.* 1994). In another the growth of MCF 7 cells was shown to be inhibited by I3C along with induction of arrest in the G_1 phase of the cell cycle (Cover *et al.* 1998). Similar results were reported in a study by Ge *et al.* (1999) where the IC₅₀ for MCF7 cells was shown to be 3-4 times lower than that for T47D cells. The induction of apoptosis was also observed in this study. I3C inhibited the growth of cells derived from mammoplasty which were initiated with a chemical carcinogen or with an oncogene (Telang *et al.* 1997). Such inhibition was compared to that in MDA 231 cells and alterations in oestrogen metabolism were implicated as a causative factor. Many studies with I3C in breast cell lines imply that changes induced in such metabolism may play an important role in the action of this chemopreventive agent.

The ability of curcumin, EGCG and I3C to affect the growth of three breast cell lines (HBL 100, MDA 468 and T47D), in particular, the effects on the cell cycle and induction of apoptosis was investigated to confirm the sensitivity of these lines to growth inhibition by the agents. For each agent potential mechanisms of inhibition of proliferation include the induction of apoptosis or cell cycle arrest. It was hoped to correlate such effects with the observed growth inhibition.

3.2 Results

3.2.1 Curcumin induced a G2/M cell cycle arrest and apoptosis.

In order to establish the relative sensitivity of the cell lines to growth inhibition by curcumin, the cell population grown in the presence of various concentrations of curcumin or vehicle control was determined by cell counting. Curcumin inhibited the growth of all three cell lines to a similar extent (Figure 3.1), with an IC₅₀ of between 1 and 5 μ M. The T47D cell line was slightly less sensitive, with significant inhibition being attained only after 96 hours treatment with 5 μ M curcumin compared to 72 hours in the other cell lines. In each cell line no inhibition was seen at 1 μ M with rapid and significant inhibition occurring when the dose was increased to 5 μ M.

To assay the effect of curcumin on the cell cycle, FACS analysis of cells labelled with propidium iodide following curcumin treatment was used. Curcumin caused an arrest in the G_2/M phase of the cell cycle in MDA 468 cells in a time-dependent fashion over 48 hours (Figure 3.2). Higher doses (40 and 80µM) did not result in a dose-dependent effect as similar levels of arrest were observed (data not shown). The proportion of cells in G_2/M increased from 23% in control to 48% in samples treated with 20µM curcumin for 48 hours. The proportion of cells in G_0/G_1 and S phases were both lowered as a result.

Chapter 3



Figure 3.1. Inhibition of cell growth by curcumin Cells were grown in the presence of increasing concentrations of curcumin or vehicle control (DMSO). Cell number was estimated by trypsinisation of adherent cells and counting on a coulter counter. Each data point represents the mean of 6 experiments \pm standard deviation and statistical significance from the DMSO control was calculated as described in Methods 2.2.17 *= p<0.05.



Figure 3.2. Cell cycle analysis of cells treated with curcumin. After treatment with curcumin or relevant vehicle control (DMSO), for 24 (a) or 48 (b) hours, cells were stained with PI and analysed by flow cytometry. The percentage of cells in each phase of the cell cycle (± standard deviation) was estimated using Cell Quest software and results represent the mean of three separate experiments.

In order to determine the contribution of apoptosis to the growth inhibition of cells by curcumin annexin V staining was used. MDA 468 cells were stained with FITC-conjugated annexin V, which binds to externalised phosphatidylserine, present on the outer membrane of apoptotic cells and propidium iodide which binds to the DNA. They were analysed by flow cytometry to determine the proportions of healthy, apoptotic and necrotic cells. Figure 3.3 shows that treatment with 20µM curcumin for 24 hours induced apoptosis. At this time and dose 61% of cells were apoptotic (Table 3.1). As the concentration and time was increased more cells became necrotic. Much of this will be secondary necrosis resulting from advanced apoptosis. Figure 3.3 shows that after 24h treatment with 80µM curcumin, almost all cells were necrotic. This is reflected in the data in Table 3.1 which show only 9.5% and 2.7% of cells were apoptotic after treatment with 80µM for 24 and 48 hours respectively.



Figure 3.3. Annexin V staining of curcumin treated cells. After treatment for 24 hours with the indicated dose of curcumin, or vehicle control (DMSO), cells were harvested and externalised phophatidylserine stained with FITC-conjugated annexin V. They were then stained with PI and analysed by flow cytometry. The plots shown are representative of a dose response performed for the MDA 468 cell line. Experiments were repeated at least three times. Healthy cells lie in the lower left quadrant in each case, apoptotic cells in the upper left quadrant and necrotic cells appear in the upper right quadrant.

	24 Hours	48 Hours	
Control	1.5% ±0.7	0.9% ±0.76	
20µМ	61.0% ±5.4 47.2% ±5.4		
40µM	60.0% ±3.1	0.0% ±3.1 40.1% ±2.9	
80µM	9.5% ±0.76	2.7% ±0.4	

Table 3.1. The induction of apoptosis by curcumin as determined by annexin V staining. After treatment for 24 or 48 hours with the indicated dose of curcumin, or vehicle control (DMSO), MDA 468 cells were harvested and stained with FITC-conjugated annexin V. They were then stained with PI and analysed by flow cytometry. Cell Quest software was used to estimate the percentage of apoptotic cells \pm standard deviation. Data shown are the result of four separate experiments.

This set of experiments was not repeated in the HBL 100 and T47D cell lines. Since they exhibited similar sensitivity to growth inhibition by curcumin it may be postulated that similar results would have been obtained for these cell lines.

3.2.2 EGCG induced apoptosis but not a definite pattern of cell cycle arrest.

EGCG inhibited the growth of all three breast cell lines but with the MDA 468 line showing much greater (10 fold) sensitivity than the other two lines (see discussion p88).

FACS analysis of cells stained with propidium iodide showed that EGCG did not cause a definite cell cycle arrest in MDA 468 or HBL 100 cells (Figure 3.4). A sub G_1 population, along with a reduction in the number of cells in both the G_1 and G_2 phases of the cell cycle was observed, as indicated in figure 3.4 (c) and (f). This occurred at 50µM in the MDA 468 cell line, but only at 200µM in the HBL 100 after 48 hour treatments. Annexin V staining was used to determine whether this sub G_1 population was due to increased apoptosis. Figure 3.5 shows that as cells were treated with increasing concentrations of EGCG, over a period of 48 hours, apoptosis was induced. At the higher concentrations, especially at 48 hours, high levels of necrosis were also seen. It is possible that this observed necrosis is secondary necrosis occurring once apoptosis was complete. As indicated in table 3.2, significant levels of apoptosis were observed after 24 and 48 hours treatment with 50µM EGCG in MDA 468 cells. In contrast, in order to induce a similar level of apoptosis in the HBL 100, treatment for 48h was required with a much higher concentration (200µM) of EGCG.



Figure 3.4. FACS profiles of cells treated with EGCG. After treatment with EGCG at the indicated concentrations, or vehicle control (DMSO), for 48 hours, cells were stained with PI and analysed by flow cytometry. \checkmark Indicates the sub G₁ population. The plots shown are typical of data from three experiments.



Figure 3.5. Annexin V staining of EGCG-treated cells. After treatment for 48 hours (a), or 24 and 48 hours (b), with the indicated dose of EGCG, or vehicle control (DMSO), cells were harvested and externalised phosphatidylserine stained with FITC-conjugated annexin V. They were then stained with PI and analysed by flow cytometry. The plots shown are representative of a dose response performed for each cell line (a). Experiments were repeated at least three times. Healthy cells lie in the lower left quadrant in each case, apoptotic cells in the upper left quadrant and necrotic cells appear in the upper right quadrant. Cell Quest software was used to estimate the percentage of apoptotic cells \pm standard deviation (b). Data shown are the result of at least three separate experiments. N/D = Data not determined.

3.2.4 I3C did not cause cell cycle arrest.

I3C caused growth inhibition in all three cell lines. The MDA 468 were most sensitive to growth inhibition followed by T47D and finally HBL 100 which where the most resistant (see discussion p89).

FACS analysis of MDA 468 cells stained with propidium iodide showed that I3C did not cause any cell cycle arrest (Figure 3.6). Cover *et al.* (1998) had shown that MCF 7 cells underwent G_1 cell cycle arrest upon treatment with 100µM I3C. Treatment of MDA 468, HBL 100 and MCF 7 cells with concentrations of I3C up to 750µM over time periods of up to 72 hours showed no evidence of this (data not shown). The same study reported that cell cycle arrest was associated with an inhibition of CDK 6 expression. Treatment of cells with I3C followed by western blotting of cell lysates showed that I3C does not inhibit the expression of this important cell cycle regulatory molecule in MDA 468 or HBL 100 cells (Figure 3.7). The concentrations used in these experiments caused significant growth inhibition after 4 days. The 250µM dose of I3C killed all the MDA 468 cells after 24h treatment.



Figure 3.6. FACS profiles of cells treated with I3C. MDA 468 cells were treated for 72 hours with either I3C or vehicle control. They were harvested, stained with propidium iodide and analysed by flow cytometry. The plots shown are representative of at least three experiments.



Figure 3.7. Western blot for CDK6 in cells treated with I3C. After treatment for 24 hours with the indicated concentration of I3C or vehicle control (DMSO) cells were lysed and the proteins separated by SDS PAGE. The proteins were transferred and the resultant filter was immunoblotted with anti-CDK 6 antibody. Bands were visualised by chemiluminescense. Blots shown are representative of a single experiment in each case.

3.3 Discussion

All three cell lines showed similar sensitivity to growth inhibition by curcumin, with significant effects being seen at 5 μ M. Other studies with breast cell lines have shown similar results: For example the T47D cell line was inhibited in the 2-3 μ M range (Mehta *et al.* 1997) and HBL 100 cells responded with an IC₅₀ of around 5 μ M (Hong *et al.* 1999). Ramachandran and You (1999) showed differential sensitivity towards curcumin in two breast cell lines: MCF10A (breast epithelial cell) and MCF 7 (breast carcinoma cell). The MCF7 cells were shown to be 3.5 times more sensitive to inhibition of proliferation than the MCF10A with IC₅₀s of 17.5 and 55 μ M respectively.

Curcumin was demonstrated to induce a G_2/M cell cycle arrest which was accompanied by significant apoptosis at concentrations above 20µM over 48 hours. Concentrations of 20-30 μ M induced a G₂/M arrest after 24 hours treatment in the MDA 231 cell line (Mehta *et al.* 1997). In this case no cell death from apoptosis, nor any changes in the expression of several apoptosis related genes, including Bcl2, p53 and cyclin B were observed in the MCF 7 cell line. A further study demonstrated that 20 and 40µM curcumin induced a G₂ block, and a sub G₁ population after 24 hrs, in MCF7 and MCF 10A cells (Ramachandran and You 1999). Significantly higher levels of apoptosis were observed in the MCF 7 than in the MCF10A cell line after 40µM treatment for 24 hrs. These changes were associated with a down regulation of mRNA for two regulators of cell proliferation, Ki67 and proliferating cell nuclear antigen (PCNA). In addition, in MCF7 cells, there was a reduction in p53 and in mRNA for p21, which can induce apoptosis and inhibit PCNA expression. In the MCF10A cells there was an increase in mRNA for Bax. Bax is often linked with an induction of apoptosis, but there are reports that its dimerisation status is important in determining its downstream effects (Oltavai et al. 1993). In the present study very little difference in sensitivity to growth inhibition between the cell lines was observed. In general the data presented in the literature for breast cell lines, especially where the same lines have been used as in this study, agree closely with those shown here for the three cell lines of interest. There is also a strong implication that a G_2/M arrest and the induction of apoptosis may be important factors in the inhibition of cell proliferation by curcumin.

In contrast to results with curcumin, the cell lines of interest exhibit differential sensitivity to inhibition of growth by EGCG (Lynne Howells, unpublished data). The MDA 468 were inhibited with an IC_{50} of 10µM compared to 100µM for the T47D and HBL 100 cell lines. It may well be significant that growth of the cell line representative of the most malignant cancer phenotype was the most affected by EGCG. Similar differences in sensitivity were observed by other workers in a panel of human and mouse carcinoma cells (Ahmad *et al.* 1997). A recent study reported the effects of EGCG on two breast tumour cell lines, MCF 7 and MDA 231 (Liang *et al.* 1999a), with growth inhibition observed at 30µM, a dose which is within the range used for experiments described in this thesis.

The effect of EGCG on cell cycle progression was investigated in the same manner as that for curcumin. A distinct cell cycle arrest was not observed in either the MDA 468 or HBL 100 cell lines treated with EGCG. At doses which are strongly growth inhibiting (50µM in MDA 468 and 200µM in HBL 100) over 24-48 hours, the cell numbers in both G₁ and G₂ phases of the cell cycle were depleted. It is possible that this effect was due to apoptosis occurring in cells from both the G_0/G_1 and G_2/M phases of the cell cycle. Two groups have reported that EGCG induces a G_0/G_1 cell cycle arrest. EGCG was shown to be less effective in inhibiting the cell cycle progression of premalignant and malignant human oral epithelial cells compared to normal cells (Khafif et al. 1998). Thirty μ M EGCG caused a G₀/G₁ arrest in MCF 7 and MDA 231 breast cells after 24 hours (Liang et al. 1999a). Such a block was observed both in the case of asynchronous cells treated with EGCG and in synchronised cells released in the presence of EGCG. This arrest was shown to be associated with a decrease in Rb phosphorylation and an inhibition of both CDK2 and CDK4 activity. Both CDKs were inhibited by EGCG directly in the reaction mixture and when purified from cells treated with EGCG. The inhibition of these proteins, important in the passage through G_1 phase of the cell cycle, was associated with an increase in protein levels of the cell cycle inhibitors p21 and p27. The actual level of arrest observed was often only in the range of a 10-15% increase and the contrast between these data and that described in this thesis may be attributed to the wide variation generally seen between cell lines, even those derived from the same tissue.

In following up the observation of a sub G_1 population during the cell cycle analysis, EGCG was shown to induce apoptosis in the MDA 468 and HBL 100 cell lines. This induction cccurred at concentrations and times that correlated well with the differences seen in

responsiveness to growth inhibition. The induction of apoptosis by EGCG is documented in the literature. In one study apoptosis induced by EGCG was demonstrated in a panel of human and mouse cancer cell lines. The normal human keratinocyte cell line (NHEK) did not exhibit any apoptosis, even at twice the dose required to induce it in the cancer cell lines (Ahmad *et al.* 1997). EGCG was also shown to induce apoptosis in human lung cancer cells (Suganuma *et al.* 1999). In this study the effects on apoptosis were synergistically enhanced by other chemopreventive agents such as sulindac and tamoxifen. Chemopreventive synergism has also been demonstrated between EGCG and curcumin (Khafif *et al.* 1998). Such an observation suggests that the two agents operate via different mechanisms, which is borne out by the fact that they are reported to cause arrest at different points of the cell cycle. It also raises the question of the use of these agents in combination to enhance the beneficial effects and to reduce any potential side effects.

I3C has also been shown in this laboratory to inhibit the proliferation of the cell lines of interest. The MDA 468 cell line was most sensitive, with an IC_{50} of 40µM, followed by T47D (50µM) and HBL 100 (100µM) (Lynne Howells, unpublished data). It has also been demonstrated that I3C could induce apoptosis in the MDA 468 cell line, when treated with doses of 100µM for 24h or more. The maximum effect was seen at 100µM after 168 hours. No apoptosis was seen in the HBL 100 cells and in this case the predominant form of cell death was necrosis at higher concentrations (Lynne Howells, unpublished data). Significant levels of necrosis were observed after 144 hour treatment with 250µM I3C.

These observations may be compared with the inhibition reported previously in the T47 D cell line (Ge *et al.* 1999). In this case an IC₅₀ of 180 μ M was observed in comparison to that of 50 μ M in the MCF 7 cell line. Apoptosis was also observed in the MCF 7 cell line after 72 hour treatment at the IC₅₀ dosage. This apoptosis was shown to occur in the absence of any changes in the expression of the death promoters, Bax or p53. It was implied by these authors that such differences could be explained by differences in oestrogen responsiveness, the MCF 7 line being oestrogen-responsive and the T47D line being non-responsive. However, it is generally believed that the T47D cell line is responsive to oestrogen, with examples quoted in the literature (Chen *et al.* 1996), and by observations made in our laboratory (Karen Dampier, unpublished observations). The recent identification of a second type of oestrogen receptor throws into question many previous studies of cells' ability to respond to oestrogen based on
the knowledge of only one receptor (Mosselman et al. 1996). The classic oestrogen receptor, designated α , and the new form (β), may be expressed individually or together, or be totally absent depending on the cell line. Preliminary data indicate that the MDA 468 cell line is α receptor positive and β negative, T47D cells are α and β receptor positive with the status of HBL 100 remaining unclear (Karen Dampier, unpublished data). Differences between oestrogen-responsive and non-responsive cell lines were also shown in a paper by Tiwari et al. (1994) where IC₅₀s for growth inhibition of MCF 7 (oestrogen-responsive) and MDA 231 (oestrogen non-responsive) were 50 and 500µM respectively. Such studies are contradicted in a paper by Cover and Firestone (1998) where I3C was shown to inhibit the growth and cause G1 cell cycle arrest through the inhibition of expression of CDK6 irrespective of the oestrogen receptor status of two cell lines (MCF 7 and MDA 231). The inhibition of growth seen in the cell lines in the present study would suggest that the oestrogen non-responsive MDA 468 cell line is more sensitive to I3C than the oestrogen responsive T47D and HBL 100 cell lines. Furthermore it was not possible to replicate the G_1 arrest in any of the cell lines under study or in the MCF 7 cell line. Nor was it not possible to show an inhibition of expression of CDK 6. The techniques used in this study, when compared to those by Cover et al. (1998), varied only in that those authors used I3C which was recrystallised to improve purity. HPLC data from our laboratory indicates, however, that the I3C used in these studies is also free from impurities (Marion Williams, unpublished data). The only remaining difference was a possible variation in the MCF 7 cells which would be difficult to resolve without exchanging cell lines and repeating the experimental work.

Evidence in the literature points to I3C having some kind of modulatory effect on oestrogen metabolism, which is regulated by Cyp450 dependent pathways. Oestrogen can be metabolised in a competitive manner to either antiproliferative 2-hydroxyoestrone or proliferative 16α -hydroxyoestrone. The balance of these two metabolites is therefore relevant to mammary carcinogenesis. It has been shown that in transformed cells this ratio is decreased as compared to normal cells (Telang *et al.* 1997). The same study showed I3C to induce the formation of the antiproliferative metabolite 2-hydroxyoestrone. Similar results carried out in the MCF 7 cell line showed an induction of Cyp1A1 (Tiwari *et al.* 1994). This study confirmed that such metabolism did not occur in the oestrogen-unresponsive MDA 231 cell line.

I3C has also been shown to act synergistically with other chemopreventive agents. The same group that claimed I3C acted independently of oestrogen responsiveness demonstrated that I3C and the antiestrogen tamoxifen co-operated to inhibit the growth of oestrogen-responsive MCF 7 cells (Cover *et al.* 1999). This was again presented as evidence that I3C can act via an oestrogen-independent mechanism.

It is clear from the observed effects of all three agents and from the evidence that they can act in a synergistic manner, that they each have distinct mechanisms of action by which they inhibit growth. There is evidence that they can cause cell cycle arrest; G_2/M in the case of curcumin and G_0/G_1 in the case of the other two agents, although not in all cell types. Each compound is also capable of inducing apoptosis. The aim of the following experimental work was to go on to investigate the signalling pathways for which the observations made in this chapter represent the end points.

CHAPTER 4. EFFECTS OF CHEMOPREVENTIVE AGENTS ON THE EGFR/ERK MAPK PATHWAY.

4.1 Introduction

Signal transduction pathways are central to the cell's ability to transmit signals across the cell membrane and transduce them to the nucleus. In this way they regulate the cells ability to respond to the environment and control cell growth, differentiation and apoptosis. The ability of chemopreventive agents to inhibit growth may lie in their potential to modulate signalling pathways. The EGFR/ERK pathway is one of the best characterised receptor tyrosine kinase pathways. Its activation results in a cascade initiating multiple cellular responses involved in mitogenesis and cell proliferation (Carpenter 1987; Yamane *et al.* 1995; Yarden 1988). Overexpression of this type of receptor has been shown to induce transformed properties in certain cell lines (reviewed in Parsons and Parsons 1997).

Curcumin has been shown to inhibit EGFR tyrosine kinase activity and tyrosine phosphorylation, in a dose- and time-dependent manner, in human epidermoid A431 cells (Korutla and Kumar 1994) and NIH3T3 cells expressing human EGFR (Korutla *et al.* 1995). In both cases EGFR protein levels remained unaffected. A study in the prostate cancer cell lines LNCaP and PC-3 also showed EGFR kinase activity and ligand-induced phosphorylation to be inhibited by curcumin (Dorai *et al.* 2000). In this case a decrease in EGFR protein levels was observed. Another group reported that curcumin could inhibit the autophosphorylation and transphosphorylation of a related receptor, p185^{neu}/erb-B2, *in vitro*. A depletion of erb-B2 protein was also shown *in vivo* (Hong *et al.* 1999). This study was carried out in a large panel of breast cell lines. Further down the signalling cascade curcumin was reported to inhibit TPA-induced ERK activity *in vitro*, in Jurkat cells (Chen and Tan 1998).

There is also evidence that EGCG can inhibit the EGFR pathway. Liang et.al (1997) reported that EGCG inhibited the tyrosine kinase activities of the EGFR (in A431 cells), PDGFR and the FGFR (in NIH 3T3 cells) when added to the assay *in vitro*. This inhibition was shown to be more effective than that seen for the non receptor tyrosine kinases, pp60^{v-src}, PKC and PKA. The same study described inhibition of EGF-induced, tyrosine phosphorylation of the EGFR and PDGF-induced, tyrosine phosphorylation of the PDGFR, without any change in

protein levels. The effects on the EGFR were shown to be associated with EGCG inhibiting binding of EGF to its receptor. The same group also reported that the black tea polyphenol, theaflavin-3,3-digallate, inhibited autophosphorylation of the EGF and PDGF receptors more effectively than EGCG and was also more inhibitory of EGF binding to its receptor (Liang et al. 1999b). Another investigation showed that EGCG selectively inhibited PDGF-BB induced activation of ERKs 1 and 2 and had no effect on EGF- and serum-induced phosphorylation of these molecules (Ahn et al. 1999). Downstream induction of c-fos and egr transcription was also inhibited and upstream, the phosphorylation of the PDGFB receptor was reduced with no effect observed on receptor protein levels. ERK phosphorylation was shown to be unaffected by EGCG when induced by UVB in human keratinocyte cells, although an inhibition of UVB-induced c-fos expression was observed (Chen et al. 1999a). A related tea polyphenol, epigallocatechin (EGC) was shown not to inhibit the transcription of c-fos mRNA or ERK activation stimulated by serum in vascular smooth muscle cells (Lu et al. 1998). As well as the effects described on c-fos, there is evidence that EGCG inhibits the AP-1 transcription factor. A study in the human keratinocyte cell line, HCL14 and in transgenic B6D2 mice, showed that EGCG could inhibit UVB-induced AP-1 activity (Barthelman et al. 1998).

There are no studies in the current literature that have examined the effects of I3C on any of these pathways.

A second growth-regulatory pathway thought to be influenced by curcumin is the NF- κ B pathway. This molecule is important in the transcriptional regulation of proinflammatory gene expression in various cells and is a regulator of genes involved in the cell cycle and apoptosis (reviewed in Mercurio and Manning 1999). EGF-induced activation of NF- κ B was shown to be important for cell cycle progression in oestrogen-insensitive cell lines (Biswas *et al.* 2000). In this study the basal level of active NF- κ B was shown to be elevated, in oestrogen-unresponsive cell lines, by treatment with EGF. This induction was inhibited by anti-EGFR antibodies. The resulting induction of cyclin D1 and the phosphorylation of the retinoblastoma protein was shown to occur via a PI3-kinase-, PKC-, NF- κ B- dependent pathway by using inhibitors of these components to dissect the events observed.

Two studies demonstrated that curcumin can inhibit NF- κ B activation through inhibition of IKK kinase activity (Jobin *et al.* 1999; Plummer *et al.* 1999). IKK α and β phosphorylate the I κ B inhibitory protein which then releases active NF- κ B. Cells which are resistant to apoptosis by curcumin may protect themselves through the activation of NF- κ B. Mouse L929 cells expressing the p65/RelA subunit of NF- κ B showed constitutive NF- κ B binding to DNA and were resistant to curcumin-induced apoptosis, whereas the parental cells were not (Anto *et al.* 2000).

Constitutive NFkB expression was shown to be inhibited by EGCG in carcinoma and normal cell lines (Ahmad et al. 2000), although the normal cell line only showed this inhibition at high doses of EGCG. Similar observations were made for TNF- α - and lipopolysaccharide (LPS)-induced NF-kB in nuclear fractions. The difference in sensitivity to apoptosis observed between normal and carcinoma cell lines could be regulated by differences in the activation of this important survival factor. Recent models of the upstream regulators of NFκB have focussed on the mitogen activated survival factor Akt/PKB. This molecule has been shown to be central in TNF- (Ozes et al. 1999) and PDGF- (Romashkova and Makarov 1999) induced NF-KB activation. All these studies suggested a Ras/PI3K dependent pathway resulting in phosphorylation of IKK by Akt itself. A requirement for IKK phosphorylation by NIK was also demonstrated. It has been implicated in the EGF-stimulated protection of MCF7, T47D and HEK 293 cells from Fas-induced apoptosis (Gibson et al. 1999). EGF was shown to inhibit the Fas-induced activation of caspases and the proteolytic cleavage of downstream molecules such as Akt. The EGF stimulation of Akt protected cells from apoptosis, as did expression of active Akt in MCF 7 cells. Inhibition of ERK activity by PD98059 had no effect on EGF-induced protection of cells from apoptosis but the PI3K inhibitor, wortmannin, inhibited this protection. These authors suggested that, in these cell lines the PI3K/Akt pathway was the most significant in EGF-induced survival signals. Akt has also been linked to the erb-B2 receptor. Overexpression of erb-B2 was shown to constitutively activate the Akt/NF-kB pathway and the action of these pathways conferred resistance in tumour cells to TNF-induced apoptosis (Zhou et al. 2000). There are three forms of Akt, (Akt 1,2 and 3). Hormone-unresponsive breast and prostate cancer cell lines were shown to have a 30-60 fold higher Akt 3 activity, than hormone-responsive cells. This activity was due to increased protein and mRNA levels for the enzyme (Nakatani et al. 1999).

These authors also identified a role for the tumour suppressor PTEN in the regulation of Akt activity.

The effects of curcumin, EGCG and I3C on these pathways were investigated in order to suggest possible mechanisms by which the agents caused the observed effects on cell growth, the cell cycle and apoptosis. It was also hoped to highlight differences between the cell lines which could correlate with differences in the effect seen on these processes.

4.2 Results

4.2.1 EGFR levels in MDA 468, HBL 100 and T47D cell lines.

The levels of the EGFR in each of the breast cell lines were confirmed by western blotting (Figure 4.1). MDA 468 cells express very high levels of the receptor, followed by HBL 100 cells and T47D cells which express the least.



Figure 4.1. Levels of EGFR protein expression in the breast cell lines of interest. Equivalent amounts of protein were taken from a lysate of each cell line and a one in ten dilution made of the MDA 468 sample. The proteins were separated by SDS PAGE and transferred to nitrocellulose. The filter was immunoblotted with anti-EGFR antibody and the resultant band, at 170kDa, was visualised by chemiluminescence. The blot shown is representative of three experiments.

4.2.2 Curcumin inhibits the EGFR/ERK signalling pathway.

In the case of both the EGFR and ERK experiments the dose and treatment times for curcumin were decided with reference to work conducted previously as presented in the literature (Korutla *et al.* 1995; Korutla and Kumar 1994). For stimulation with EGF or TPA optimum treatment times and dosages were determined experimentally with time courses and dose responses.

Upon addition of EGF to cells, which had been starved for 16 hours, a 100 fold increase tyrosine phosphorylation was observed in EGFR immunoprecipitated from cells. This phosphorylation was detected by immunoblotting with PY99, a monoclonal antibody specific for phosphorylated tyrosine (Figure 4.2). Pre-treatment with curcumin for 20 minutes inhibited this activation by 72% (40μ M) and 92% (80μ M) in the MDA 468 cell line (Figure 4.2a and b). No inhibition was seen when the same concentrations were applied to HBL 100 cells (Figure 4.2a). This inhibition of tyrosine phosphorylation was not associated with a decrease in the protein levels of the receptor, which remained unchanged in both cell lines (Figure 4a). In this case filters were immunoblotted with an antibody binding active and inactive forms of the EGFR. The levels of phosphorylation cannot be directly compared between cell lines due to variations in protein levels and exposure times of the films between experiments. Using this technique it was not possible to detect a band in the T47D cell line. This correlates with the observation that the receptor level in this cell line is very low compared to that in the other two.

Treatment of serum-starved cells with EGF resulted in a 3 - 4 fold activation of ERK 1/2 phosphorylation (Figure 4.3). This phosphorylation was detected by western blotting with a monoclonal antibody specific for ERKs 1 and 2 phosphorylated on Tyr²⁰⁴. Pre-treatment with 80µM curcumin followed by EGF stimulation caused significant inhibition of this phosphorylation in all three cell lines. Forty µM curcumin only caused significant inhibition in the T47D and HBL 100 lines (Figure 4.3b). In these two cell lines the highest dose was also capable of inhibiting basal phosphorylation by around 50% (T47D) and 60% (HBL 100). It was confirmed that this inhibition was not a result of decreased levels of ERK protein by western blotting with an antibody recognising all forms of ERK 1 and 2 (Figure 4.4). In order to confirm that inhibition of ERK phosphorylation resulted in loss of activity, the ability of

immunoprecipitated ERK 1/2 to phosphorylate PHAS I was examined (Figure 4.5). The MDA 468 cell line exhibited a high basal level of ERK activity that was inhibited at 40 and 80 μ M. The HBL 100 line showed significant inhibition at 40 μ M, but at 80 μ M this was less pronounced. The T47D cell line showed a dose dependent inhibition of ERK activity from 40 μ M.



Figure 4.2. The effect of curcumin on EGFR phosphorylation as determined by western blotting. Serumstarved cells were treated with the indicated concentrations of curcumin, or vehicle control (DMSO), for 20 minutes followed by stimulation with 15nM EGF for 5 minutes. Cells were lysed in triton lysis buffer and the EGFR was immunoprecipitated from equivalent amounts of protein of each sample. The resulting immunoprecipitates were run on a SDS polyacrylamide gel and transferred to nitrocellulose. The filter was immunoblotted with either anti-phosphotyrosine (a – top panels) or anti-EGFR (a – bottom panels). The bands were detected by chemiluminescence. Blots shown are representative of at least three experiments. Figure 4.2 (b) shows quantitation by densitometry of the data for the MDA 468 cell line. Values represented are the mean of three experiments +/- standard deviation. *=p<0.05.

Chapter 4



Figure 4.3. Effect of curcumin on ERK1/2 phosphorylation as determined by western blotting. After treatment for 20 minutes with the indicated concentrations of curcumin or vehicle control (DMSO), cells were treated with 15nM EGF for 5 mins, lysed and the proteins separated by SDS PAGE. The proteins were transferred and the resultant filters immunoblotted with anti-pERK antibody. Bands were visualised by chemiluminescence. Blots shown are representative of three experiments for each cell line (a). The data from three separate experiments were quantified by densitometry and the means +/- standard deviation plotted (b). *=p<0.05.

Chapter 4



Figure 4.4. Western blot for ERK1/2 protein levels in curcumin-treated cells. Cells were treated for 20 minutes with the indicated concentrations of curcumin, or vehicle control (DMSO), followed by 15nM EGF for 5 mins. Cells were lysed and equivalent amounts of protein separated by SDS PAGE. After blotting onto nitrocellulose, filters were immunoblotted with anti-ERK antibody and bands visualised by chemiluminescence. The blots shown are representative of two separate experiments for each cell line.



Figure 4.5. The effect of curcumin pre-treatment of cells on ERK1/2 activity. Cells were treated for 20 minutes with the indicated concentrations of curcumin or vehicle control, followed by 15nM EGF for 5 minutes. Cells were lysed and ERK 1/2 immunoprecipitated from equivalent amounts of protein. ERK activity was measured by the ability of the purified kinase to phosphorylate PHAS I in an *in vitro* kinase assay. Blots shown are representative of at least 2 determinations.

100

Curcumin was also able to inhibit ERK phosphorylation stimulated by TPA (Figure 4.6). Some inhibition of TPA stimulated ERK phosphorylation was observed when concentrations above 40μ M were used, although it was not as obvious as that seen after stimulation with EGF. The inhibition observed for the MDA 468 was not dose-dependent with an inhibition of 33.9% and 32.1% seen at 40 and 80 μ M respectively. The HBL 100 did exhibit dose dependency with 34.1% and 54.1% inhibition shown after treatment with 40 and 80 μ M curcumin.



Figure 4.6. The effect of curcumin on TPA-stimulated ERK1/2 phosphorylation. Cells were treated with the indicated concentration of curcumin, or vehicle control (DMSO), for 20 minutes followed by stimulation with 0.2µM TPA for 5 mins. Cells were lysed and equal amounts of protein from each sample separated by SDS PAGE. Western blotting was performed with anti-pERK (Tyr²⁰⁴) antibody and the bands detected with chemiluminescence. The blots shown are representative of three determinations. The level of ERK phosphorylation was quantified by densitometry.

Treatment of MDA 468 and HBL 100 cells with 20μ M curcumin significantly inhibited EGFinduced expression of c-fos over a period of 2 hours (Figure 4.7). Treatment with concentrations above 40μ M completely abolished this induced expression.



Figure 4.7. The effect of curcumin pre-treatment on the expression of c-fos. Cells were treated with the indicated concentrations of curcumin, or vehicle control (DMSO), for 30 minutes, followed by stimulation with EGF for 2 hours. Equal amounts of protein were separated on SDS PAGE and western blotting performed with an anti-c-fos antibody. Bands were detected by chemiluminescence and blots shown are representative of two determinations performed for each cell line.

4.2.3 Curcumin inhibits the PKB/Akt pathway

The effect of curcumin on a component of an important survival pathway, PKB/Akt, was investigated by western blotting using an antibody specific for the Ser 473-phosphorylated form of the molecule. Figure 4.8 shows that the phosphorylation of this molecule was induced in the HBL 100 cell line, by treatment with TNF α , anisomycin or EGF. This induction was inhibited completely by 40 μ M curcumin.



Figure 4.8. The effect of curcumin on PKB/Akt phosphorylation as induced by a number of agents. Serum starved cells (untreated (U)), were pre-treated with 40 μ M curcumin (C) or with vehicle control (DMSO (D)) for 30 minutes and were then stimulated for 30 minutes with 7.5ng/ml TNF α , 100nM anisomycin or 15nM EGF as indicated. Equivalent protein from each sample was separated by SDS PAGE and western blotting performed with an anti-phospho Akt (Ser⁴⁷³) antibody. Bands were detected by chemiluminescence and the blot shown is the result of a single experiment.

4.2.4 EGCG activates components of the EGFR/ERK pathway

Western blotting was used to investigate the effect of EGCG on the EGFR/ERK pathway. 50µM EGCG enhanced the EGF-induced phosphorylation of the EGFR and of ERK 1 and 2 (Figure 4.9a and c). The effect in the MDA 468 cell line was more pronounced than in the HBL 100 line. A similar difference in response was seen between the cell lines to the EGF-induced expression of c-fos, which was also enhanced by this concentration over a period of 2 hours (Figure 4.9d). These effects were observed whether the EGCG was added as a 30 minute pretreatment, or at the same time as the EGF. The effects seen on the EGFR were not a result of EGCG affecting levels of EGFR protein in the immunoprecipitate, as these were constant for all treatments (Figure 4.9b). Furthermore 50µM EGCG induced ERK phosphorylation in the absence of EGF as well as c-fos expression (Figure 4.9c and d). There was no evidence of this EGF-independent effect at the level of the receptor. In terms of this response the effects seen were similar for both the MDA 468 and the HBL 100 cell lines.



Figure 4.9. Western blots showing the effect of EGCG on components of the EGFR/ERK pathway. Cells were either pretreated, (p), with the indicated concentration of EGCG, or vehicle control (DMSO), for 30 minutes or had the EGCG/vehicle control added simultaneously (s), with EGF for 5 minutes (a-c) or 2 hours (d). After this time the EGFR was immunoprecipitated out of lysates containing equivalent protein (a and b) or equal amounts of protein from the whole cell lysate was taken (c and d) and the proteins separated by SDS PAGE. Western blotting was performed with anti-phosphotyrosine (a), anti-EGFR (b), anti-phosphoERK (c) or anti-cfos (d) antibodies and bands visualised by chemiluminesence. The blots shown are representative of experiments repeated three times.

In order to confirm that the effect of EGCG on ERK phosphorylation was translated into an effect on activity, *in vitro* kinase assays were performed. The ability of ERK to phosphorylate PHAS I was used as the measure of activity (Figure 4.10). The data shown are representative for the HBL 100 cell line. Similar results were obtained for the MDA 468 cell line. The effects seen on phosphorylation correlated with the ERK kinase assay, with greater activity seen when cells were treated with EGCG plus EGF than with EGF alone. EGCG alone was also capable of inducing ERK activity above basal levels.



Figure 4.10. Effect of EGCG on ERK1/2 activity. Cells were serum-starved for 16 hours prior to treatment with the indicated concentration of EGCG or vehicle control (DMSO) for 30 minutes. They were then treated with 15nM EGF for 5 minutes, followed by lysis and immunoprecipitation of ERK1/2 from equal amounts of protein of each sample. ERK activity was measured by the ability of the purified kinase to phosphorylate the PHAS I substrate in an *in vitro* kinase assay. The blot shown is representative of 2 experiments.

The effect of EGCG on tyrosine phosphorylation in general was examined by taking whole cell lysates treated with EGCG and EGF and western blotting with an anti-phosphotyrosine antibody, which will recognise all proteins phosphorylated on tyrosines (Figure 4.11). Arrows in the same colour pick out proteins behaving in the same fashion. The MDA 468 cell line showed few bands due to the presence of a high molecular weight band (a), which gave a very strong signal making it difficult to expose the blot to detect weaker bands. It is possible that this band is the EGFR which is expressed at a high level in this cell line. This 170 kDa protein was stimulated by EGF, but not by EGCG alone and seemed to be stronger in the presence of EGCG plus EGF, than with EGF alone. Several other bands behaving in the same way were also observed in this cell line. One was a protein with a molecular weight around 120kDa (b) and others at 100kDa (c), 80kDa (d), 65kDa (e) and 33kDa (f). In the case of the HBL 100 cell line many more bands were observed. Bands could be picked out behaving in the same way as that described for those in the MDA 468 line, at; 85kDa (j), 60kDa (o) and 40kDa (t). There were also examples of proteins where EGF-induced phosphorylation was inhibited by treatment with EGCG, at 190kDa (g) and 30 kDa (u). There were proteins whose phosphorylation was induced by EGCG alone after 30 minutes of treatment at 140kDa (h), 75kDa (l) and 70kDa (m). The final set of proteins to note are those where phosphorylation was induced by EGF and remained unaffected by EGCG treatment, at 135kDa (i), 80kDa (k), 60kDa (n), ~50kDa (p/q) and ~45kDa (r/s). This experiment confirmed that EGCG was having multiple effects upon the phosphorylation of a variety of cellular components in addition to those already investigated in detail.

Chapter 4



Figure 4.11. The effect of EGCG on tyrosine phosphorylation in whole cell lysates. Cells were either pretreated, (p), with the indicated concentration of EGCG, or vehicle control (DMSO), for 30 minutes or had the EGCG/vehicle control added simultaneously (s), with EGF for 5 minutes. Equivalent amounts of protein from whole cell lysates were separated by SDS PAGE and western blotting was performed with an anti-phosphotyrosine antibody. Bands were detected by chemiluminescence and blots shown are typical of two experiments.

In order to investigate further the effect of EGCG on EGF-stimulated phosphorylation of the EGFR, a time course for EGF-induced tyrosine phosphorylation of the EGFR in the presence of 50µM EGCG or the DMSO equivalent was conducted. One possible mechanism by which EGCG could appear to increase EGF-induced phosphorylation would be by delaying the dephosphorylation of the receptor. MDA 468 cells exhibited prolonged tyrosine phosphorylation of the receptor in the presence of EGCG or DMSO control (Figure 4.12). This phosphorylation remained at the longest time point, 3 hours, used for EGF treatment. The HBL 100 cell line exhibited a peak of phosphorylation after 5-15 minutes of EGF treatment. This phosphorylation gradually receded as the time course was extended. Although, as expected, the signal was stronger in EGCG treated cells than in DMSO the decrease in phosphorylation showed similar kinetics. Western blotting of the same filters for total EGFR confirmed that EGFR protein levels remained reasonably constant throughout all experiments (Figure 4.12).



Figure 4.12. The influence of EGCG on the time course of EGF-induced EGFR phosphorylation Cells were pretreated with 50µM EGCG, or DMSO equivalent, for 30 minutes followed by 15nM EGF for the indicated times. Cells were harvested and the EGFR immunoprecipitated from equal amounts of the protein samples. Samples were separated on SDS PAGE and western blotting performed with anti-phosphotyrosine antibody or an antibody for total EGFR. Bands were visualised by chemiluminescence and blots shown are representative of three separate experiments.

The apparent increase in EGFR phosphorylation in the presence of EGCG might be due to this compound inhibiting phosphatase activity. The effects of EGCG on the activity of cellular tyrosine phosphatases was investigated using an *in vitro* tyrosine phosphatase assay. Myelin basic protein was labelled with ³²P inorganic phosphate on a tyrosine residue that sits in a recognition site for tyrosine phosphatases. Cellular extracts were incubated with the substrate and released inorganic phosphate was determined by scintillation counting.

Cells treated with EGF showed an increase in specific activity of phosphatases of 0.7 nmol phosphate/min/mg compared to control. Pretreatment with 50µM EGCG, followed by EGF enhanced this induction by a further 0.7 nmol/min/mg. Treatment with EGCG alone increased phosphatase activity by the greatest amount with an increase in specific activity of 1.8 nmol/min/mg over control activity (Figure 4.13). However addition of 50µM EGCG directly to the assay mixture had no effect on phosphatase activity compared to control (Figure 4.14). Tyrosine phosphatase activity was capable of being inhibited in this way by the addition of 1mM sodium orthovanadate to the assay (Figure 4.14). The use of this general tyrosine phosphatase inhibitor was a useful positive control for the assay.

Chapter 4



Figure 4.13. Protein tyrosine phosphatase activity in EGCG-treated cells. Cells were treated with 50 μ M EGCG, or vehicle control (DMSO), for 30 minutes, followed by stimulation with 15nM EGF for 5 minutes. Cells were harvested and the protein concentrations of the samples determined. Lysates were incubated with ³²P tyrosine-labelled myelin basic protein and the release of inorganic phosphate measured by scintillation counting. Data shown are the result of three separate determinations, +/- standard deviation. a – significantly different from DMSO control, b- significantly different from DMSO + EGF, c- significantly different from EGCG + EGF. p=<0.05.



Figure 4.14. In vitro protein tyrosine phosphatase assays in the presence of EGCG. Untreated cells were harvested and the protein concentration of the sample determined. Lysates were incubated with ³²P tyrosine-labelled myelin basic protein in the presence of 50 μ M EGCG, or DMSO equivalent, or 1mM sodium orthovanadate. The release of inorganic phosphate was measured by scintillation counting. Data shown are the result of three separate determinations, +/- standard deviations. *=p<0.05.

4.2.5 I3C does not inhibit the ERK pathway.

The effect of I3C on ERK phosphorylation was investigated as described previously for the other two agents. Figure 4.15 shows a representative blot for the MDA 468 cell line. No inhibition of EGF-induced ERK phosphorylation was seen by treatment of cells with concentrations of I3C up to 1mM, a concentration which would be very toxic to cells at longer time points. T47D and HBL 100 lines gave similar results.

······································	MDA 468										
		•	-	-	<u>ù</u>		-		←	ERK 1/2	2
EGF	-	+	-	+	+	+	+	+			
I3C (µM)	-	-	-	-	50	100	500	1000	I		
DMSO	+	+	-	-	-	-	-	-			

Figure 4.15. Western blot showing the effect of I3C on ERK1/2 phosphorylation. Cells were treated with the indicated concentrations of I3C, or vehicle control (DMSO), for 20 minutes followed by stimulation with 15nM EGF. Cells were lysed and equal amounts of protein from each sample separated by SDS PAGE. Western blotting was performed with anti-pERK (Tyr^{204}) antibody and the bands detected with chemiluminescence. The blot shown is representative of two determinations.

4.2.6 Effect of curcumin and EGCG on intracellular ATP concentration.

During the course of these experiments it became clear that curcumin and EGCG, but not I3C, appeared to be affecting several different kinases. It was important to rule out the possibility that they were non-specific inhibitors of kinases, as well as of many other processes, through a general depletion of intracellular ATP. Such a cytotoxic effect would be unfavourable both in terms of data interpretation in this study and for their more general efficacy as chemopreventive agents. The effect of two of the agents, curcumin and EGCG, on intracellular ATP levels was investigated in order to confirm that they were not exerting a general cytotoxic effect. The effect of I3C on ATP concentration was not determined, since, unlike curcumin and EGCG, it was shown not to affect a number of signalling components, ruling out a general cytotoxic effect. Curcumin treatment for one hour did not significantly deplete the levels of intracellular ATP in any cell line, except at the highest dose in the case of the T47D, where a decrease was observed at 80µM (Figure 4.16). EGCG, at a dose of 50µM

and treatment time of one hour, did not significantly deplete intracellular ATP levels in any cell line (Figure 14.17). This was the highest dose used in any of the experiments to investigate signalling components.



Figure 4.16. The effect of curcumin pre-treatment on intracellular ATP levels. After treatment with the indicated concentrations of curcumin or vehicle control (Ctr), for 60 mins, cells were extracted in 2% PCA. ATP concentration was determined, with reference to a standard curve, by the release of photons upon addition of a luciferase preparation. The protein content of each sample was determined by biorad assay and the amount of ATP measured was normalised to this. Results represent the mean of at least three determinations performed in duplicate. * = p < 0.05.



Figure 4.17. The effect of EGCG pre-treatment on intracellular ATP levels. After treatment with 50µM of EGCG or vehicle control (Ctr), for 60 mins cells were extracted in 2% PCA. ATP concentration was determined, with reference to a standard curve, by the release of photons upon addition of a luciferase preparation. The protein content of each sample was determined by biorad assay and the amount of ATP measured was normalised to this. Results represent the mean of three experiments performed in duplicate.

4.3 Discussion

MDA 468 cells were shown to express very high levels of EGFR, with HBL100 expressing intermediate levels and T47D very low levels. This is in agreement with data in the literature (Daly *et al.* 1997; DeFazio *et al.* 2000; GrausPorta *et al.* 1997).

The basal levels of tyrosine phosphorylation, after serum starvation, were similar in the MDA 468 and HBL 100 cell lines. This low basal phosphorylation was greatly increased in response to EGF, but due to inherent variation between experiments it was not possible to compare levels of response between the two cell lines. The very low levels of EGFR in the T47D cell line meant that it was not possible to immunoprecipitate sufficient receptor to successfully conduct these experiments. Curcumin significantly inhibited EGFR tyrosine phosphorylation in a dose-dependent fashion at 40 and 80µM in the MDA 468 cell line. No similar inhibition was observed in the HBL 100 cell line. The inhibition in MDA 468 cells was shown to occur in the absence of any change in expression of the receptor. These data agree with those from two studies which demonstrated inhibition of EGFR phosphorylation and tyrosine kinase activity in A431 cells (Korutla and Kumar 1994), and NIH3T3 cells expressing human EGFR (Korutla et al. 1995). In these studies curcumin was shown to inhibit the EGFR in a dose and time-dependent fashion from 10µM with no effect on protein The first of these studies showed that curcumin was not a direct inhibitor of the levels. receptor kinase and that its effects were partially reversible. Extracts from curcumin-treated cells were demonstrated to increase dephosphorylation, implicating the involvement of phosphatase activation in the mechanism of action. The second paper described the inability of curcumin to inhibit PDGF-induced tyrosine phosphorylation of several molecules including the PDGFR. Inhibition of EGF-induced serine and threonine phosphorylation of the EGFR was also detected in addition to inhibition of tyrosine phosphorylation. The cell lines in this study were similar to the MDA 468 in that they both expressed high levels of the EGFR. Longer treatment times of 12-24 hours, with 10-30µM curcumin, were shown to deplete EGFR protein levels in prostate cancer cell lines as well as EGFR kinase activity and tyrosine phosphorylation (Dorai et al. 2000). Treatment of breast cell lines with 5-50µM curcumin for 12-24 hours inhibited the autophosphorylation and transphosphorylation of another member of the EGFR family, the p185^{neu}/erb-B2 receptor (Hong et al. 1999). Depletion of the erb-B2 protein in vivo was also observed. In this case direct inhibition of the receptor tyrosine kinase activity was demonstrated, as was an inhibition of the receptor binding to the molecular chaperone GRP94 which stabilises erb-B2 protein *in vivo*.

Curcumin was shown to inhibit EGF-induced ERK phosphorylation from 40µM in the HBL 100 and T47D cell lines, but only at 80µM in the MDA 468. This inhibition of phosphorylation was manifested as an inhibition of ERK activity. TPA-induced ERK activation was also shown to be inhibited by the same concentrations, although to a much lesser extent. The inhibition of TPA-induced ERK activity by curcumin has been demonstrated previously in Jurkat cells where an IC₅₀ of 20µM was obtained. This inhibition of TPA-induced phosphorylation suggests that the inhibition seen at the level of ERK is not a sole consequence of the inhibition seen at the level of the receptor. TPA is a diacylglycerol (DAG) analogue which activates ERK via PKC and Raf and as such is independent of any surface receptor. Two studies have reported that curcumin can inhibit TPA-induced PKC activity in NIH 3T3 cells (Lin et al. 1997; Liu et al. 1993). Both studies used 15-20µM curcumin over short time points and concluded that curcumin inhibits TPA-induced PKC activity without affecting expression of the protein. This inhibition of PKC activity by curcumin could account for the inhibition of TPA-induced ERK phosphorylation and provide an alternate mechanism by which curcumin affects the ERK pathway. Such a mechanism could also explain why ERK is sensitive to inhibition by curcumin in the HBL 100 cells where no inhibition is seen at the level of the receptor.

Downstream of ERK, the inhibition of EGF-induced expression of c-fos was observed at concentrations from 20 μ M. This confirms that inhibition of the upstream components of the EGFR/ERK pathway by curcumin has consequences downstream at the level of transcription. These effects on EGF-induced c-fos expression were shown to occur at lower concentrations than the effects seen at the EGFR and ERK levels. It may be a consequence of time, in that the studies on the ERKs and EGFR were conducted with cells which had been pre-treated with curcumin for 20-30 mins followed by 5 mins EGF stimulation, whereas for c-fos the pretreatment time of 30 mins, followed by EGF for 2 hours resulted in a total curcumin treatment of $2\frac{1}{2}$ hours. It is reasonable to suggest that lower concentrations are more effective over these longer time points. A second possibility is that curcumin is having multiple effects on several pathways which may culminate in the expression of c-fos. In this case the small effects seen on individual pathway components at doses around 20 μ M have a

much greater additive effect on a molecule such as c-fos which lies downstream of all of them. A study by Ahn *et al.* (1999) observed discrepancies in the concentrations at which EGCG inhibited PDGF-BB-induced ERK phosphorylation as opposed to that which inhibited the receptor or other downstream kinases. The kinases further up the pathway such as the PDGFR, PI3K and PLC- γ were less sensitive to inhibition by EGCG than the ERKs. This phenomenon was suggested to be due to the difference in response of the pathway components to the growth factor, with the molecules that were most sensitive to EGCG inhibition being maximally activated by growth factor concentrations which were considerably lower than those required to fully activate those components which were less sensitive to inhibition by EGCG.

Preliminary studies showed 40 μ M curcumin to inhibit the phosphorylation of Akt. This inhibition was observed upon the induction of Akt activity by EGF, TNF or anisomycin. EGF has been shown previously to induce Akt activity (Biswas *et al.* 2000; Boudewijn and Coffer 1995; Gibson *et al.* 1999), as has TNF (Ozes *et al.* 1999), but to our knowledge, this is the first instance in which anisomycin has been shown to induce Akt activity. The inhibition of this pathway suggests an alternative mechanism for the inhibition of the NF- κ B pathway by curcumin. Two studies have previously shown that curcumin (10-100 μ M) inhibits the activation of NF- κ B at the level of the IKK (Jobin *et al.* 1999; Plummer *et al.* 1999). Akt has been shown to feed into the NF- κ B pathway at the level of the IKK (Ozes *et al.* 1999; Romashkova and Makarov 1999) and this inhibition raises the possibility of an Akt-dependent mechanism for the inhibition of NF- κ B by curcumin.

In contrast to curcumin, concentrations of EGCG up to 50μ M did not inhibit EGFR phosphorylation in the breast cell lines studied here. On the contrary, in combination with EGF, EGCG appeared to enhance EGFR phosphorylation in a dose-dependent manner. The only other study where a similar effect has been described previously was conducted on Ehrlich ascites tumour cells. In this case a dose of 100 μ M EGC, but not EGCG, caused an increase in total tyrosine kinase activity within the cells. EGCG at the same concentration was shown to increase the phosphorylation of an unidentified 45 kDa protein (Kennedy *et al.* 1998). The effects described in this study were not due to EGCG prolonging the phosphorylation the EGFR, since the time course of phosphorylation of the receptor was equivalent for cells treated with EGCG, or DMSO in the presence of EGF. In order to

explore this further, the experiment could be modified so that a pulse of EGF was given for a short time after treatment with EGCG. The medium could then be changed and the decay of phosphorylation followed over time. A further possibility is that EGCG might enhance receptor dimerisation in the presence of EGF. Increased dimerisation would lead to increased tyrosine kinase activity and autophosphorylation. An experiment could be designed to study receptor dimerisation using cross-linking agents. Another explanation was that EGCG was inhibiting a phosphatase. This was investigated using an assay for general, cellular, tyrosine phosphatase activity. EGCG was not shown to inhibit phosphatase activity when added directly to an in vitro assay, nor was any inhibition observed when cells were treated with 50µM. Cells treated with EGF showed increased tyrosine phosphatase activity, as indeed, did cells treated with EGCG. Several phosphatases exist which regulate the EGFR/ERK pathway; MAPK phosphatase 1 & 2 (MKP 1 & 2) (reviewed in Brondello et al. 1997), HVH2 (Guan and Butch 1995) and B59 (Shin et al. 1997). All these are dual specificity phosphatases, which act primarily on ERK 1&2. MKP1 & 2 are not expressed in guiescent cells, but their expression is rapidly induced by serum after 30-60 minutes (Brondello et al. 1997). There is also evidence that MKP 1 is transcriptionally induced by stress regulators such as UV and by signalling through the stress activated protein kinase (SAPK) pathway (Shapiro and Ahn 1998). These workers also showed it to positively regulate upstream components of the ERK pathway including MEK1 and Raf-1 in an ERK-independent manner. In this way it may regulate cross talk between signalling pathways and determine the downstream signal. At the receptor level two phosphatases have been shown to regulate EGFR phosphorylation. Protein tyrosine phosphatase 1B (PTP1B) interacts with both the EGFR and the PDGFR (Liu and Chernoff 1997). Upon binding the receptor the enzyme is itself phosphorylated on tyrosine and binds the adapter protein Grb2. This phosphorylation is correlated with a 3-fold increase in phosphatase activity and in this way the enzyme selectively regulates the specific activity of the receptor tyrosine kinases and is itself regulated by them. A second protein tyrosine phosphatase, SHP-1 has also been shown to bind to and dephosphorylate the EGFR (Keilhack et al. 1998). The data presented in this thesis show that both ERK phosphorylation and c-fos transcription are induced by 50µM EGCG alone. This would suggest that it is not the MAPK phosphatases that are being induced by EGCG since these would counteract these effects. Since the assay only measured total phosphatase activity, it is impossible from the data obtained to determine which particular phosphatases are being affected.

The activation of components of the ERK pathway by EGCG was observed in both the MDA 468 and the HBL 100 cell lines. Clearly activation of ERKs and increased fos expression in MDA 468 cells following treatment with EGCG do not cause increased proliferation as both growth inhibition and apoptosis were observed at this concentration. It is possible that activation of this pathway may be growth inhibitory at least in the tumour cell line, since it has been previously reported that EGF inhibited proliferation in cells with very high levels of EGFR. In the particular case of MDA 468 cells, in which growth was inhibited by concentrations of EGF in the nanomolar range (Church and Buick 1988; Filmus et al. 1985), the EGF-mediated growth inhibitory effect was characterised by a reversible G₁ arrest accompanied by a decreased level of mRNA for p53 (Prasad and Church 1991). Α subsequent investigation by the same group demonstrated that in EGF-treated cells, the levels of mutant p53 in the nucleus were inhibited whilst wild type p53 continued to be expressed (Prasad and Church 1997). A recent study in the MDA 468 cell line, showed that treatment with concentrations of EGF (100ng/ml), which would stimulate the growth of many other cells, inhibited proliferation in this cell line. This was attributed to upregulation of p21/WAF1 (Reddy et al. 1999). EGF activated several signalling mechanisms including MAPK and the signal transducers and activators of transcription (STAT) cascades (Van der Geer et al. 1994). Chin et al. (1996) showed that EGF-activated STAT bound to STAT responsive elements in the promoter of p21 enhancing its induction. Reddy et al. (1999) showed that neither the MEK inhibitor PD98059, nor the MAPK inhibitor, apigenin, affected EGF-induced p21 in MDA 468 cells, despite causing a significant reduction in MAPK activity. They showed that STAT1 directly interacts with the EGFR, suggesting that this protein is essential for EGFinduced p21 expression in the MDA 468 breast line. Under certain circumstances the activation of Ras has been shown to be growth inhibitory. Ras is regulated, in part, by the GTPase Rho. If Rho was inhibited in Swiss 3T3 or NIH 3T3 cells then the constitutive activation of Ras resulted in an accumulation of p21 and cell cycle entry was blocked (Olson et al. 1998). If Rho remained active this induction of p21 was inhibited and Ras induced DNA synthesis.

There is evidence that EGCG can inhibit AP-1 activity under certain circumstances. The mouse epidermal cell line, JB6 was transformed with mutant H-ras to mimic a common form of carcinogenesis (Chung *et al.* 1999). These transformed cells showed high levels of AP-1 activity that was inhibited by treatment with EGCG (1-20 μ M). A second investigation

showed that 5μ M EGCG inhibited UVB induced AP-1 activity by 75%, in the human keratinocyte cell line HCL14 (Barthelman *et al.* 1998). The same study showed that AP-1 activity was inhibited by three topical applications of 5mg of EGCG to the skin of transgenic B6D2 mice, engineered to express an AP-1 luciferase reporter.

In contrast to the data presented in this chapter, one study has shown EGCG to inhibit the EGFR/ERK pathway. Liang et al. (1997) showed that EGCG inhibited the tyrosine kinase activities of the EGFR, PDGFR and fibroblast growth factor receptor (FGFR) with an IC_{50} of 0.5-1µg/ml. In the case of the EGFR this inhibition was shown to be due to EGCG blocking the binding of the ligand (EGF) to the receptor. This study was conducted in A431 carcinoma cells which also overexpress the EGFR. A further paper showed that treatment of vascular smooth muscle cells with 10 -50µM EGCG selectively inhibited the PDGF-BB-induced activation of ERK 1/2 (Ahn *et al.* 1999). Concentrations up to 100µM had no effect on ERK activation by EGF, angiotensin II or FCS.

I3C was shown to have no effect on ERK activation at concentrations up to 1mM. Preliminary results with all three of the breast cell lines showed that pretreatment with 1mM I3C inhibited EGF-induced EGFR tyrosine phosphorylation (Barbara Horley, unpublished data.). No inhibition was seen at concentrations up to 500µM. A concentration of 1mM would completely inhibit growth and is cytotoxic to the cell lines described here. This result suggests that I3C exerts its growth inhibitory effects through a mechanism unrelated to the ERK pathway.

The effect of pre-treatment of cells with EGCG or curcumin on possible intracellular ATP depletion was investigated. The fact that no reduction in ATP levels was seen with curcumin in two of the cell lines, and not at all at the lower end of the concentration range, which may be considered more physiologically relevant, gives confidence that the effect of curcumin on the pathways described cannot be attributed to such a phenomenom. Similarly no effect was seen with EGCG up to a dose of 50μ M, the highest concentration used in experiments on signalling components.

The data in this chapter indicate that the three agents have distinct mechanisms of action, which correlate with the differences seen in their effects on cell growth and the cell cycle.

Curcumin was shown to inhibit components of the ERK pathway from the receptor level through to the transcription of c-fos. There is evidence that it has more than one point of intervention in the pathway. EGCG, on the other hand, was shown to have a stimulatory effect on the pathway at the level of the receptor, when added in combination with EGF, while on its own causing activation of ERKs and increased c-fos expression. There was no evidence for inhibition of ERK activation by I3C.

.

CHAPTER 5. EFFECTS OF CHEMOPREVENTIVE AGENTS ON THE JNK/SAPK AND p38 SIGNALLING PATHWAYS.

5.1 Introduction

The JNK and p38 MAPK pathways are activated predominately by stress stimuli and pathogenic insult, although in some cell types, the JNK pathway has been shown to be activated by mitogens such as EGF (Hashimoto *et al.* 1999; Pomerance *et al.* 1998). Both of these studies demonstrated that the adapter molecule Shc is important in this activation. Pomerance et al. (1998) demonstrated that Grb-2 binds to, and activates MEKK1 upon EGF stimulation and that this activation is regulated through binding to Shc in cells overexpressing the EGFR. Hashimoto et al. (1999) used Grb-2 and Shc deficient cells to demonstrate a requirement for Grb-2, but not Shc in the EGF-stimulated activation of ERK and the opposite relationship for the activation of JNK.

Downstream of JNK lies the AP-1 transcription factor component, c-jun. C-jun is regulated both at the protein level and through phosphorylation on Ser 63 and 73 by JNK. p38 regulates ATF-2, another AP-1 component, by phosphorylation (reviewed in Leppa and Bohmann 1999). AP-1 transcription factors have been implicated in phenomena as diverse as cell proliferation, transformation, differentiation, migration and apoptosis.

A study with jun -/- mouse fibroblast cells showed that the presence of c-jun is required for progression through the G_1 phase of the cell cycle, but that the phosphorylation of Ser 63 and 73 is not. The c-jun mediated protection of these cells from UV-induced apoptosis did show a requirement for this phosphorylation (Wisdom *et al.* 1999).

A role for JNK in drug-induced apoptosis was demonstrated in two recent studies. Chen et al. (1999b) observed that the retinoic acid analogue, N-(4-hydroxyphenyl)retinamide (4-HPR), induced apoptosis in several cell types, including LNCaP prostate cancer cells. These authors showed that 4-HPR-induced apoptosis and that such treatment resulted in sustained JNK activation. A dose of 25μ M curcumin was used to inhibit JNK activity and this protected the cells from apoptosis. The second study showed the physiological metabolite methylglyoxal to induce apoptosis in Jurkat cells. This induction was shown to be cell-type specific and the

addition of 20μ M curcumin as a JNK inhibitor reduced apoptosis (Du *et al.* 2000). However, since results presented in this thesis have shown that curcumin has multiple effects on the related ERK MAPK pathway, and evidence in the literature for its effects on other signalling pathways is accumulating, the interpretation of such experiments should be treated with caution.

p38 has also been implicated in both mitogenic and apoptotic processes. The balance of evidence points to p38 activation inducing apoptosis as a default response, in the absence of signals from other pathways such as those involving growth factor receptors and the ERK MAPKs (reviewed in Birkenkamp *et al.* 1999).

Curcumin was previously shown to inhibit the activation of JNK by a variety of agonists in Jurkat cells. A combination of transfection studies showed curcumin not to inhibit the activation of JNK at the MKK4 or MEKK1 levels, or, indeed, to inhibit JNK itself. Some inhibition was seen upstream of MEKK1, but the results were not conclusive enough to suggest the precise molecular target of curcumin in this pathway (Chen and Tan 1998).

There have been very few studies on the effect of green tea polyphenols on the p38 and JNK pathways. EGCG was demonstrated to inhibit UVB-induced expression of c-fos in the human keratinocyte cell line, HaCaT. This inhibition was accompanied by a decrease in activation of p38 MAPK, but not of JNK or ERK. In this case activation of the MAPKs was determined by their phosphorylation (Chen *et al.* 1999a). Another of the catechin derivatives of green tea, EGC, was shown to inhibit JNK phosphorylation and activity, stimulated by TNF α . The level of c-jun mRNA was also reduced at concentrations, and time points, that correlated with JNK inhibition (Lu *et al.* 1998).

The pivotal importance of these two pathways in the determination of the fate of a cell makes them important molecules to study. The effects of the chemopreventive agents upon these pathways could give shed light on their mechanism of action and help to explain the differences seen between the response of the cell lines in terms of growth and apoptosis.

5.2 Results

5.2.1 Curcumin inhibited the JNK pathway.

5.2.1.1 Curcumin inhibited JNK activity.

In vitro kinase assays were used to investigate the effect of curcumin on anisomycinstimulated activity of JNK. The ability of JNK to phosphorylate GST-jun was taken as the measure of JNK activity. Pretreatment of cells with curcumin for 30 mins inhibited JNK activity, in a dose-dependent fashion, in all three cell lines (Figure 5.1a). Quantification of this inhibition showed that the MDA 468 and T47D cell lines were most sensitive with significant inhibition occurring from 10μ M, similar to the growth inhibitory dose for these cell lines. However, in the HBL 100 cell line significant inhibition was only achieved at 40μ M. This inhibition was not due to changes in the protein levels of JNK as western blotting showed these to remain constant for all curcumin treatments (Figure 5.2). Curcumin did not inhibit JNK activity directly, as when concentrations up to 80μ M were added directly into the kinase assay mixture, no inhibition was observed (Figure 5.3). The data shown in figure 5.3 are those for the MDA 468 cell line, but similar results were obtained for T47D and HBL 100.

The downstream consequences of this inhibition were investigated by studying the transcription factor c-jun. HBL 100 cells were found to have a significant basal expression of c-jun, which was not obviously enhanced by anisomycin (Figure 5.4a). Low basal levels of phosphorylated c-jun on the other hand were significantly enhanced by anisomycin within 15 mins (Figure 5.4b), and the increase was inhibited by 40μ M curcumin, which also appeared to decrease basal levels of phosphorylated jun (Figure 5.4c). Similar results were obtained in the MDA 468 cell line (data not shown).

Chapter 5



Figure 5.1. Effect of curcumin on JNK activity. Cells were treated with the indicated concentrations of curcumin, or vehicle control (DMSO), for 1 hour, followed by stimulation with 100nM anisomycin for 30 min. JNK was immunoprecipitated from equivalent amounts of protein from each sample. The activity of JNK was measured by its ability to phosphorylate GST-jun in an *in vitro* assay. The autoradiographs (a) are representative of at least three experiments. These were quantified using a phosphorimager (b), +/- standard deviation and *=p<0.05.



Figure 5.2. Western blot showing expression of JNK in curcumin treated cells. Cells were treated with the indicated concentrations of curcumin, or vehicle control (DMSO), for 1 hour, followed by stimulation with 100nM anisomycin for 30 min. An equivalent amount of protein from each lysate was separated by SDS PAGE and western blotting performed with an antibody specific for JNK. Bands were visualised by chemiluminescence and the blots shown are typical of two experiments.



Figure 5.3. Direct addition of curcumin to the *in vitro* JNK kinase assay. Cells were treated with 100nM anisomycin for 30 min prior to harvest. JNK was immunoprecipitated from equivalent amounts of protein for each sample. Its activity was measured by its ability to phosphorylate GST-jun in the presence of the indicated concentration of curcumin or vehicle control (DMSO). The autoradiograph shown is representative of two separate experiments. Similar results were obtained for the HBL 100 and T47D cell lines.



Figure 5.4. Western blots showing the expression and phosphorylation of c-jun from curcumin-treated cells. Cells were treated with 100nM anisomycin for the indicated times from 15 mins to 3 hrs (a and b). They were then harvested and nuclear proteins were extracted. Equal amounts of nuclear protein were separated by SDS PAGE and western blotting performed with antibodies against total jun (a) or phosphorylated (Ser⁷³) jun (b). Panel c shows results from a 30 minute pretreatment with the indicated concentrations of curcumin, or vehicle control (DMSO), followed by nuclear protein extraction and western blotting with the anti-phospho-jun antibody. Blots shown are typical of at least two experiments. This experiment was carried out by Emily McCann, an undergraduate project student, under my supervision.

5.2.1.2 Curcumin inhibited MKK4 activity.

In vitro kinase assays were used to investigate the effect of curcumin on anisomycinstimulated MKK4 activity. A coupled assay was employed in which GST-JNK was included to couple the phosphorylation through to the GST-jun substrate. The phosphorylation of GST-jun was taken as the measure of activity. Assays where GST-JNK was excluded did not result in any phosphorylation of GST-jun when active MKK4 was included (data not shown). Pretreatment with 20µM curcumin inhibited anisomycin stimulation of MKK4 activity in both the MDA 468 and the HBL 100 cell lines (Figure 5.5). These effects were not due to curcumin affecting the protein level of MKK4 in either cell line (Figure 5.6). Figure 5.7 shows that curcumin was not capable of inhibiting active MKK4 directly in the *in vitro* assay, at concentrations up to 10µM. Limited inhibition was observed at 20-80µM but it is not clear whether this was physiologically significant. The inhibition was not dose-dependent and it was not sufficient to explain the inhibition of JNK activity. Due to time constraints these experiments were not conducted in the T47D cell line.

An attempt was made to assay the activity of MKK7 in a similar way to that of MKK4. Unfortunately a suitable antibody to immunoprecipitate MKK7 was not available and as a result a successful assay could not be set up.


Figure 5.5. In vitro kinase assays showing the effect of curcumin on MKK4 activity. Cells were treated with the indicated concentrations of curcumin, or vehicle control (DMSO), for 1 hour, followed by stimulation with 100nM anisomycin for 30 min. MKK4 was immunoprecipitated from equivalent amounts of protein from each sample. The activity of MKK4 was measured in an *in vitro* assay in which GST-JNK was included to couple the phosphorylation through to GST-jun. The phosphorylation of GST-jun was taken as the measure of activity. The autoradiographs (a) are representative of at least three experiments. These were quantified using a phosphorimager (b) +/- standard deviation and *=p<0.05.



Figure 5.6. Western blots showing the expression of MKK4 in curcumin-treated cells. After pre-treatment with the indicated concentrations of curcumin, or vehicle control (DMSO), for 1 hour, cells were stimulated with 100nM anisomycin for 30 mins. Equal amounts of protein were separated by SDS PAGE and transferred to nitrocellulose. The filter was immunoblotted with an antibody specific for MKK4 and the bands visualised by chemiluminescence. The blots shown are those from a single experiment.



Figure 5.7. Direct addition of curcumin to the *in vitro* MKK4 kinase assay. Cells were treated with 100nM anisomycin for 30 min prior to harvest. MKK4 was immunoprecipitated from equivalent amounts of protein for each sample. The activity of MKK4 was measured in an *in vitro* assay, in which GST-JNK was included to couple the phosphorylation through to GST-jun, in the presence of the indicated concentration of curcumin or vehicle control (DMSO). The autoradiograph shown is representative of three separate experiments.

5.2.1.3 Curcumin inhibited MEKK1 activity.

The effect of curcumin on MEKK1 activity in vitro was investigated using a coupled kinase assay. Full length, HA-tagged MEKK1, or pcDNA3.1 (empty vector) was transfected into human embryonic kidney 293 (HEK 293) cells. In this form MEKK1 is constitutively active. HEK 293 cells were used as they are readily transfectable and, as the assay is conducted in vitro, the properties of the cell used to create the protein have no bearing on the assay. The addition of 10µM curcumin to the assay inhibited the phosphorylation of GST-jun, initiated by MEKK 1 (Figure 5.8). This inhibition was observed whether the assay was coupled through MKK4 (compare lanes e and g), or MKK7 (compare lanes f and h). Several other observations can be made due to the nature of these assays. Since MEKK1 is present at high concentration in the assay it is capable of phosphorylating itself. The phosphorylation of MEKK1 by other MEKK1 molecules, although not a physiological event, can also give a measure of its activity. This self phosphorylation was inhibited by 10µm curcumin compared to control (compare lanes e,f with g,h). Phosphorylated GST-JNK and MKK4 may also be seen. These components of the assay also had their phosphorylation inhibited by curcumin (compare lanes d and f). The final point to note is that GST-MKK7 has some constitutive activity in such assays and is capable of phosphorylating GST-jun in the absence of an upstream signal (lane b). This constitutive activity was also inhibited by 10µM curcumin (compare lanes b and d). This assay was conducted with the help of Karl Deacon (University of Leicester).

The experiment was repeated by myself with some modification (Figure 5.9). This data is also presented as it confirms the observations of the initial experiment and demonstrates an attempt to develop the assay. In this second experiment the assay was either coupled through GST-MKK4 to GST-jun as before (lanes 2) or GST-MKK4 was used as a direct substrate (lanes 1). No band was seen in the assays where GST-MKK4 was used as a direct substrate. Figure 5.9A shows that MEKK1-initiated phosphorylation of GST-jun was inhibited by10µM curcumin (compare lanes g and h). The same two lanes show the inhibition of phosphorylation of the assay components, GST-MKK4 and GST-JNK. The lighter exposure, figure 5.9B, shows that as in the previous experiment the self-phosphorylation of MEKK1 was inhibited by curcumin (compare lanes e and f, and g and h).



Figure 5.8. Direct addition of curcumin to the MEKK1 *in vitro* kinase assay (I). HEK 293 cells were transfected with plasmid to overexpress full length, HA-MEKK1, which was constitutively active, or with pcDNA3.1 (empty vector). Cells were harvested and MEKK1 immunoprecipitated from equal amounts of lysates. The activity of MEKK1 was measured in an *in vitro* kinase assay coupled through either MKK4 (lanes a,c,e and g) or MKK7 (lanes b,d,f and h) and GST-JNK to GST-jun, the phosphorylation of which was taken as the measure of MEKK1 activity. The assay was conducted in the presence of 10µM curcumin or vehicle control (DMSO). The autoradiograph shown was the result of a single experiment in which the kinase assay was conducted with the help of Karl Deacon, University of Leicester.



Figure 5.9. Direct addition of curcumin to the MEKK1 in vitro kinase assay (II). HEK 293 cells were transfected with plasmid to overexpress full length, HA-MEKK1, which was constitutively active, or with pcDNA3.1 (empty vector). Cells were harvested and MEKK1 immunoprecipitated from equal amounts of lysates. The activity of MEKK1 was measured in an *in vitro* kinase assay coupled through MKK4 and GST-JNK to GST-jun, the phosphorylation of which was taken as the measure of MEKK1 activity (lanes indicated by 2), or by using GST-MKK4 as a direct substrate (lanes indicated by 1). The assay was conducted in the presence of 10µM curcumin (lanes b,d,f,h) or vehicle control (DMSO) (lanes a,c,e,g). The autoradiographs shown are from the same experiment (A is a 16 hour exposure and B is a 3 hour exposure). The experiment was repeated once more with similar results.

130

5.2.1.4 Curcumin inhibited MEKK3-induced JNK activity.

CCl39, hamster fibroblast cells expressing Δ MEKK3:ER, which is the activation domain of the oestrogen receptor fused to the kinase domain of MEKK3, were used to investigate the effect of curcumin on MEKK3-induced signalling activity. In this system MEKK3 activates ERK, JNK and p38 upon treatment of the cells with 4-hydroxy tamoxifen (4HT). The advantage of the system is that MEKK3 may be selectively activated in the absence of any other signalling molecules, by treatment with 4HT. Anisomycin did not activate JNK very effectively in this cell line (Figure 5.10a). Treatment of cells with 4HT resulted in a significant activation of JNK activity. This activation was inhibited by pretreatment with curcumin, in a dose-dependent fashion, by concentrations from 10 μ M (Figure 5.10a and b). Conversely, no similar inhibition of ERK activity was observed (Figure 5.10c). The apparent increase in ERK activity at one of the higher curcumin concentrations requires confirmation, but it is not inconsistent with the results obtained in the HBL 100 cell line. In this case ERK activity was inhibited less at 80 μ M than at 40 μ M (Figure 4.5). These experiments were conducted in collaboration with Simon Cook, Babraham Institute, Cambridge.



Figure 5.10. The effect of curcumin on MEKK3-induced JNK and ERK activity. CCl39 cells, stably transfected with Δ MEKK3:ER, were pretreated with the indicated concentrations of curcumin, or vehicle control (DMSO), for 1 hour followed by stimulation with 4HT or anisomycin for 30 minutes. The activity of JNK (a and b) and ERK (c) was determined in an *in vitro* assay using GST-jun (JNK) or MBP (ERK) as a substrate. The results shown are representative of a single experiment in each case. These experiments were conducted by Simon Cook, Babraham Institute, Cambridge.

5.2.2 Effect of EGCG on the JNK pathway.

In vitro kinase assays showed that pretreatment of MDA 468, HBL 100 and T47D cells with concentrations of EGCG up to 50 μ M did not inhibit anisomycin-induced JNK activity (Figure 5.11). When EGCG (20 μ M) was added directly to the immune complex kinase assay, the enzyme was significantly inhibited (76.5% +/- 8.6% for MDA 468, 63.3% +/-12.8% for T47D and 54.7% +/- 12.0% for HBL 100) (Figure 5.12). Despite this inhibition of JNK *in vitro* EGCG did not inhibit c-jun expression induced in cells treated with anisomycin (Figure 5.13).



Figure 5.11. *In vitro* **kinase assays showing the effect of EGCG on JNK activity.** Cells were treated with the indicated concentrations of EGCG, or vehicle control (DMSO), for 1 hour, followed by stimulation with 100nM anisomycin for 30 min. JNK was immunoprecipitated from equivalent amounts of protein from each sample. The activity of JNK was measured by its ability to phosphorylate GST-jun in an *in vitro* assay. The autoradiographs are representative of at least three experiments.

Chapter 5



Figure 5.12. Direct addition of EGCG to the *in vitro* JNK kinase assay. Cells were treated with 100nM anisomycin for 30 min prior to harvest. JNK was immunoprecipitated from equivalent amounts of protein for each sample. JNK activity was measured by its ability to phosphorylate GST-jun in the presence of the indicated concentration of EGCG or vehicle control (DMSO). The autoradiographs shown (a) are representative of three separate experiments. These were quantified by densitometry (b), +/- standard deviation. *= p<0.05.



Figure 5.13. Western blot showing the expression of c-jun in EGCG treated cells. Cells were pretreated with 50µM EGCG for the indicated amount of time followed by stimulation with 100nM anisomycin for 2 hours. Cells were harvested and equivalent amounts of nuclear protein separated by SDS PAGE. Proteins were transferred to nitrocellulose, which was immunoblotted with an antibody specific for c-jun. Bands were visualised by chemiluminescence and the blot shown is typical of two experiments.

5.2.3 I3C did not inhibit the JNK pathway.

In vitro kinase assays were again used to show that concentrations of I3C up to 1mM did not inhibit JNK activity either when cells were pretreated (Figure 5.14a), or when I3C was included directly in the assay (Figure 5.14b). Similar results were obtained for all three cell lines.



Figure 5.14. *In vitro* kinase assays showing the effect of I3C on JNK activity. Cells were pretreated with the indicated concentrations of I3C, or vehicle control (DMSO), followed by stimulation with 100nM anisomycin for 30 mins (a), or treated with anisomycin alone (b). JNK was immunoprecipitated from equivalent amounts of protein. JNK activity was measured by its ability to phosphorylate GST-jun either alone (a) or in the presence of the indicated concentrations of I3C, or vehicle control (DMSO) (b).

5.2.4 Curcumin did not inhibit the p38 pathway.

The effect of curcumin on the ability of p38 to phosphorylate one of its downstream targets, ATF2, was determined. In this case p38 activity was measured using an *in vitro* kinase assay with GST-ATF2 as the substrate. Pretreatment of cells with concentrations of curcumin up to 80µM did not inhibit the ability of anisomycin to induce p38 activity (Figure 5.15a), nor did direct addition of the same concentrations to the assay mixture affect p38 activity, induced by anisomycin (Figure 5.15b).



Figure 5.15. In vitro kinase assays for p38 showing the effect of curcumin treatment on cells or direct addition to the assay. Cells were pretreated with the indicated concentrations of curcumin, or vehicle control (DMSO), followed by stimulation with 100nM anisomycin for 30 mins (a), or treated with anisomycin alone (b). p38 was immunoprecipitated from equivalent amounts of protein for each sample and p38 activity was measured by its ability to phosphorylate GST-ATF-2 either alone (a) or in the presence of the indicated concentrations of curcumin, or vehicle control (DMSO) (b).

5.2.5 Effect of EGCG on the p38 pathway.

In vitro kinase assays showed that EGCG did not inhibit p38 activity, induced by anisomycin, when cells were treated with concentrations up to 50μ M (Figure 5.16). However, significant inhibition was observed when EGCG was added directly to the assay (Figure 5.17). MDA 468 and T47D cells were significantly inhibited from 10μ M (MDA 468 70% +/-16.5%, T47D 74% +/- 10.8%) and HBL 100 from 20μ M (59.3% +/- 33.5%). This apparently contradictory situation could arise, as EGCG used to treat the cells was washed out during the kinase assay. The possible downstream consequences of this inhibition in the cell, were investigated with an *in vitro* kinase assay for MAPKAP K2, a kinase lying downstream of p38 at the ATF2 level. The activity of this kinase was measured by its ability to phosphorylate a peptide substrate. The level of phosphorylation was determined by scintillation counting. Pretreatment of MDA 468 cells with concentrations of EGCG up to 50μ M did not inhibit anisomycin-induced activity of MAPKAP K2 (Figure 5.18). Further experiments showed that neither higher concentrations (200 μ M), nor serum-starving the cells showed evidence of any inhibition (data not shown).



Figure 5.16. In vitro kinase assays showing the effect of EGCG cell treatment on p38 activity. Cells were treated with the indicated concentrations of EGCG, or vehicle control (DMSO), for 1 hour, followed by stimulation with 100nM anisomycin for 30 min. p38 was immunoprecipitated from equivalent amounts of protein from each sample. The activity of p38 was measured by its ability to phosphorylate GST-ATF2 in an *in vitro* assay. The autoradiographs are representative of at least three experiments.

Chapter 5



Figure 5.17. The effect of direct addition of EGCG to *in vitro* p38 kinase assays. Cells were treated with 100nM anisomycin for 30 min prior to harvest. p38 was immunoprecipitated from equivalent amounts of protein for each sample. Its activity was measured by its ability to phosphorylate GST-ATF2 in the presence of the indicated concentration of EGCG or vehicle control (DMSO). The autoradiographs shown (a) are representative of three separate experiments. These were quantified by densitometry (b), +/- standard deviation. *= p<0.05.

139



Figure 5.18. *In vitro* kinase assays showing the effect of EGCG on MAPKAPK2 activity. Cells were pretreated for 60 mins with the indicated concentration of EGCG or vehicle control (DMSO), followed by stimulation with anisomycin for 30 mins. Cells were harvested and MAPKAP K2 immunoprecipitated from equivalent protein for each sample. MAPKAP K2 activity was measured by its ability to phosphorylate a peptide substrate. The extent of this phosphorylation was determined by scintillation counting. The results shown represent the mean of three experiments, +/- standard deviation.

5.2.6 I3C did not inhibit the p38 pathway.

In vitro kinase assays showed that I3C did not inhibit the ability of anisomycin to induce p38 activity at concentrations up to 1mM. This was the case if cells were pretreated with I3C prior to stimulation with anisomycin (Figure 5.19a), or if I3C was added directly to the assay mixture (Figure 5.19b).



Figure 5.19. *In vitro* **kinase assays showing the effect of I3C on p38 activity.** Cells were pretreated with the indicated concentrations of I3C, or vehicle control (DMSO), followed by stimulation with 100nM anisomycin for 30 mins (a), or treated with anisomycin alone (b). p38 was immunoprecipitated from equivalent amounts of protein for each sample. p38 activity was measured by its ability to phosphorylate GST-ATF2 either alone (a) or in the presence of the indicated concentrations of I3C or vehicle control (DMSO) (b).

5.3 Discussion

Curcumin inhibited anisomycin-induced JNK activity, in all cell lines, at doses approaching the IC₅₀ for growth inhibition. The tumour cell lines, MDA 468 and T47D were more sensitive than the non-malignant HBL 100 line, with significant inhibition attained at 10 and 20µM respectively. However this was the only point in the pathway at which this difference in sensitivity was apparent. This inhibition was shown to occur without any change in JNK protein levels and the point of inhibition was upstream of JNK itself, since curcumin was unable to inhibit JNK activity when included in the assay at concentrations up to 80µM. Downstream inhibition of anisomycin-induced c-jun phosphorylation and expression was confirmed at concentrations of curcumin from 20µM. Upstream of JNK the effects of curcumin on MKK4 and MKK7 were investigated. The MKK7 assay did not give any clear result as no suitable antibody could be obtained with which to conduct the immunoprecipitation. Anisomycin-induced MKK4 activity was inhibited in a dose-dependent fashion when MDA 468 and HBL 100 cells were pretreated with curcumin from 20µM. Once again this inhibition was shown to be independent of any change in MKK4 protein levels and curcumin was unable to inhibit MKK4 activity directly in an in vitro assay at concentrations up to 80µM, suggesting that the point of inhibition is upstream of the MKK level. Ten µM curcumin was indeed shown to inhibit constitutively active MEKK1 in vitro. Preliminary investigations also indicate that concentrations of curcumin above 10µM are capable of inhibiting MEKK3-induced JNK activity. ERK activity was shown to be insensitive to such inhibition. These preliminary experiments suggest that MEKK1 could possibly be the target for curcumin in this pathway, although more stringent investigation is required to confirm this since there may also be a component of the inhibition which lies at, or downstream of, MEKK3

These data can be compared to a study by Chen and Tan (1998), in which Jurkat T cells and MCF7 cells were used to investigate the effect of curcumin on the JNK pathway. These authors showed that pretreatment of cells with curcumin inhibited JNK activity induced by a variety of agents. Inhibition induced by the phosphatase inhibitor, sodium orthovanadate was also observed, indicating that the mechanism was not one involving the activation of phosphatases. These authors showed that JNK was inhibited, by curcumin, with an IC₅₀ of 5-10 μ M, compared to the inhibition ERK2 with an IC₅₀ of 20 μ M. This difference in sensitivity

for the two pathways is consistent with the data presented in this thesis. In the published study, in vitro incubation of curcumin, up to 80µM, with purified recombinant proteins did not inhibit the ability of JNK, MKK4 or MEKK1 to phosphorylate their respective substrates. The lack of direct inhibition of JNK and MKK4 also agrees with the data presented in this thesis. The latter, however, were derived from more physiologically relevant conditions. In contrast to the lack of inhibition of MEKK1 seen in the published study, some inhibition of MEKK1 by curcumin was shown in this present study. Experiments in the two studies were conducted in a slightly different manner in that here a more sensitive coupled assay was used. In continuing the investigation these authors co-transfected HA-tagged-JNK along with plasmids encoding the upstream kinases MKK4, MEKK1, TAK1, GCK and HPK1. The cells were then treated with 20µM curcumin for 24 hours and JNK activity assayed. Those cells transfected with JNK alone showed no inhibition of JNK activity, while those transfected with MKK4 actually showed an increased JNK activity. MEKK1-transfected cells showed a slight inhibition in the presence of 20µM curcumin, but those transfected with the upstream kinases TAK1, GCK and HPK1 showed significant inhibition of JNK activity by curcumin. The direct inhibition of these kinases was investigated and no direct inhibition of HPK1 by curcumin was shown. TAK1 and GCK were inhibited directly by curcumin, but only at high concentrations, which cannot account for the inhibition observed of endogenous JNK activation. These authors suggested that curcumin may act at, or proximally upstream of, the MEKK1 level, but the data were inconclusive.

MEKK1 is also involved in the regulation of NF-κB. The inhibition of the NF-κB pathway by curcumin is documented in the literature and described in chapter 4 (p114). Inhibition of MEKK1 by curcumin suggests the possibility that this signalling enzyme could link these observations. Overexpression of MEKK1 in NIH3T3 cells activated κB-Cat reporter expression in a synergistic manner with TNFα and suppressed the inhibitory activity of cotransfected IκBα (Hirano *et al.* 1996). These effects of MEKK1 were shown to be accompanied by the disappearance of IκBα. Overexpression of kinase dead MEKK1 inhibited TNFα-induced reporter expression. These data suggested that MEKK1 is involved in TNFα-mediated induction of NF-κB activation and regulates it through degradation of IκBα. Similar reporter gene expression studies confirmed these observations in Jurkat cells (Meyer *et al.* 1996). This latter investigation also showed that JNK1 synergised with MEKK1 in stimulating reporter expression and that JNK1 binds directly to c-Rel *in vivo*, but is not capable of phosphorylating c-Rel, NF- κ B or I κ B α *in vitro*. The phosphorylation and subsequent degradation of I κ B α was shown to be regulated through the phosphorylation of NIK by MEKK1 (Lee *et al.* 1997). A further investigation suggested the involvement of p38 since overexpression of MEKK1 induced COX-2 expression and this induction was blocked by the inclusion of the pharmacological p38 inhibitor SC68376 (Guan *et al.* 1998). These authors suggested that the induction was via a MEKK1, MKK4, p38 pathway, but the majority of the available evidence suggests that MEKK1 selectively activates the JNK pathway (Derijard *et al.* 1995;Lin *et al.* 1995).

Curcumin did not inhibit p38 either directly or indirectly at concentrations up to 80µM. This lack of inhibition suggests that the chemopreventive agent does not act on a common upstream activator of the JNK and p38 pathways. MEKK1 has, as described above, been shown to be selective for the JNK pathway. MKK4 can activate p38 *in vitro* and in transfection assays (Derijard *et al.* 1995; Lin *et al.* 1995), but dominant negative MKK4 inhibits JNK activity more potently than it inhibits p38 activity (reviewed in Davis 1999). As such it is conceivable that curcumin could inhibit JNK activity via one or other of these molecules without affecting the activity of p38.

EGCG inhibited both JNK and p38 activity directly. No inhibition of the activity of either MAPK was seen when cells were pre-treated with up to 50µM EGCG suggesting that EGCG had no effect upstream of the kinases that could result in an inhibition of their activation. Purified JNK was least sensitive to direct inhibition by EGCG with significant inhibition seen at 20µM. p38 was most sensitive with significance reached at only 10µM. The occurrence of this inhibition *in vivo*, i.e in treated cells, could not be demonstrated for either kinase. In the case of JNK, anisomycin-induced expression of c-jun was not inhibited by pretreatment with up to 50µM EGCG and for p38, this dose of EGCG did not inhibit anisomycin-induced MAPKAP K2 activity, a kinase lying downstream of p38. There was speculation that this may have been either a concentration effect, in that the EGCG concentration achieved in cells was low compared to the known concentration in the media and that added directly to the assay, or that serum, present in the media, sequestered much of the EGCG. These explanations seem unlikely since repeating the MAPKAP K2 experiment, with concentrations of up to 200µM EGCG, used to pretreat cells in serum-free medium, still did not result in any

inhibition. There is very little data in the literature with which to compare the data generated in this study, but 5-10 μ M EGCG has been shown to inhibit phosphorylation of p38, but not JNK, induced by UVB in human keratinocyte cells (Chen *et al.* 1999a). The related catechin derivative, EGC at 10 μ M, was shown to inhibit TNF α -induced JNK phosphorylation and activity in A7r5 vascular smooth muscle cells (Lu *et al.* 1998). Further investigation is required to determine whether either JNK or p38 are direct targets for EGCG *in vivo*.

Once again, as with the ERK pathway, I3C did not have any inhibitory effect on either the JNK or p38 signal transduction pathways. The I3C concentrations used (up to 1mM) were far in excess of those required to observe the effects on cell growth and apoptosis in these cell lines, strongly suggesting that the compound must elicit its effects elsewhere.

The function of the JNK pathway *in vivo* is still relatively poorly understood. It has been implicated in both mitogenesis and in the induction of apoptosis. It is likely to be important in the determination of cell fate, and as a central decision-making molecule, the downstream biological effects are probably determined by its interaction with other, parallel pathways. As such the interaction of curcumin and the other chemopreventive agents with the JNK and p38 pathways could well account for the molecular basis of their effects.

CHAPTER 6. AN INVESTIGATION INTO THE EFFECT OF CURCUMIN ON GENE TRANSCRIPTION USING cDNA MICROARRAYS.

6.1 Introduction.

Microarrays enable the total RNA population of a cell to be analysed in a single experiment. The advent of this technology and access to such a system in the latter stages of this project enabled a broader approach to be taken in the identification of target genes for curcumin. The aim was to discover both novel targets and those which would tie in with work already completed in order to provide validation. Microarray technology allows a different philosophy to be taken in experimental work, in that studies described in previous chapters involved identification of potential targets for the chemopreventive agents and investigation of individual components within the pathway, the microarray experiments allow one to compare overall changes in gene expression, in response to different concentrations and treatment times, as well as differences between cell lines. The only limit to the data obtained is the number of genes on the array in the first place.

6.1.1 Gene array technology.

Nucleic acid array technology is based on hybridisation of labelled RNA or DNA in solution to DNA molecules attached at specific locations on a surface. Arrays of nucleic acids have been used for many years in biological experimentation, with DNA, of often unknown sequence, spotted onto nylon filters. The nucleic acid probe is labelled with a radioactive group to allow detection of recognised sequences. The developments of the use of glass as a substrate and fluorescence detection methods, coupled with the technology to synthesise or deposit nucleic acids onto glass slides at very high densities, have allowed the expansion of this microarray technology (Lockhart and Winzler 2000). Arrays with more than 250 000 different oligonucleotides or 10 000 cDNAs per square centimetre can now be routinely produced. These arrays normally consist of nucleic acids of known sequence.

The array may take one of two basic forms, either a cDNA array where PCR products (0.6 - 2.4 kb) are spotted, robotically, onto a glass slide, or an oligonucleotide array where short (~25b) oligonucleotides are synthesised to be complementary to known genes.

Oligonucletides may be spotted onto an array in the same way as cDNA, or they may be synthesised *in situ* (Duggan *et al.* 1999; Lockhart and Winzler 2000; Young 2000).

6.1.2 Applications of gene array technology.

6.1.2.1 General approaches.

In general there are two ways in which the array may be designed depending upon the question that is being asked. The first is the use of an array with all the available genes for the organism of interest to attempt to obtain a global picture of transcription within the sample. The second is the use of a much more specific targetted array for genes of a particular signal transduction pathway for example.

6.1.2.2 Transcriptional programmes.

The population of RNA within a cell is known as the transcriptome and DNA microarrays may be used to estimate the levels of various RNA species within the transcriptome (Lockhart and Winzler 2000; Young 2000). Such profiles can highlight how global gene expression changes during events such as the cell cycle, or when a particular signal transduction pathway is activated, or a change in environmental conditions occurs. The changes in a gene expression profile of a cell as it ages may be determined and so on. The application of such profiles is limited at the present time by the computational tools available with which to analyse the data obtained (Young 2000).

6.1.2.3 Gene function and drug discovery.

There is evidence that the function of a gene may be accurately predicted by comparison of the expression profile of a wild type cell with that of a cell harbouring a mutation in that gene. The microarray approach is to cluster genes according to their expression behaviour under a range of conditions and to assign function according to a set of known genes that fall into these clusters (Eisen *et al.* 1998). Using a similar hypothesis it is possible to look at the expression profiles of drug-treated cells. Such profiles will be similar to that in cells in which the drug's target is mutated and hence comparison of the profile with panels of profiles

produced by known mutations will suggest a target. In this way many of the stages of the drug discovery process can utilise microarrays, and new therapeutic targets for existing drugs may also be identified.

6.1.2.4 Disease diagnosis, prognosis and therapy.

Current cancer classification techniques rely upon tumour histology. In the future DNA microarrays may be used to classify tumours by their expression profiles, which are valuable both for diagnosis and predicting prognosis (reviewed in Young 2000). Infectious diseases could be tackled in a similar way by identifying the expression profile of a pathogen itself, or the expression profile it elicits in the host cell.

6.1.2.5 Deciphering complex biological systems.

The complex systems that regulate the development and maintenance of a multicellular organism may begin to be unravelled by the use of DNA microarray analysis coupled to more sophisticated computational systems. Such systems may be able to model the interactions of molecules within a signalling pathway under certain environmental conditions and give a clue as to the cross talk occurring between pathways. Ultimately expression profiles may be used to decipher the systems governing the interaction of cells within a tissue since mRNA reflects, albeit indirectly, the functional state of all proteins.

6.1.3 Advantages and disadvantages of microarray technology.

The breadth of array-based experiments almost guarantees surprising findings will be made. The ability to scan thousands of genes in one experiment is a huge advantage over traditional methods for measuring mRNA abundance and gene expression, such as northern blots, polymerase chain reaction following reverse transcription of RNA (RT-PCR), nuclease protection assays, cDNA sequencing, differential display and so on, in which only a limited number of genes can be studied in any one experiment (reviewed in Lockhart and Winzler 2000). The use of mRNA is advantageous over protein based approaches since these are generally less sensitive and not suited to high throughput analyses. However, mRNA is only the intermediate molecule and final expression of the protein product needs to be confirmed

for any altered gene expression that RNA analysis shows up. Post-translational protein modification such as phosphorylation and glycosylation, which affects cell phenotype, can obviously not be interpreted from the mRNA. Inspite of their advantages DNA microarrays do not necessarily replace these other tools, but may allow them to be used in a more targetted fashion.

A major practical problem at the current time is the large amount of RNA, of sufficient purity, that is required for each experiment. Most experiments require $50\mu g$ of total RNA from each sample. Many processes are best studied in a few cells, or even in a single cell and techniques need to be developed to reduce the requirement for such large amounts of starting material (Lockhart and Winzler 2000; Young 2000). A further practical problem is homology between genes, especially between family members, which makes the choice of nucleotide sequence for the array difficult. This is a particular problem in the case of oligonucleotide arrays that use very short sequences, but is a cause for concern in other types of array as well. Once again other techniques need to be employed to confirm that the gene change identified by/ the array is indeed specific for that particular gene.

The biggest challenge in many array experiments is making sense of the vast quantities of data, which they produce. Large amounts of data can be an advantage when looking for patterns in experiments, which may have limited reproducibility, but the data collection and handling in duplicate should not be underestimated and suitable, sophisticated computational software is required to deal with it (Lockhart and Winzler 2000).

6.2 Methodology for cDNA microarrays.

The detailed method for preparing RNA from cells and hybridising the cDNA product to the array is outlined in methods (2.2.15), p73. In a cDNA array many gene-specific polynucleotides, derived from the 3' end of RNA transcripts are individually arrayed onto a single matrix, in this case a glass slide. The targets for the array are labelled representations of cellular RNA pools. Total cellular RNA is purified from test and control cells and labelled cDNA is produced from the RNA by reverse transcription from an oligo-dT primer. The fluorescent labels Cy3-dUTP and Cy5-dUTP are most often used, with one labelling the cDNA derived from control cells and the other labelling cDNA derived from the test cells. For the array experiments described 50µg of total RNA was required, suitably purified, since cellular protein, lipid and carbohydrate contamination can cause high background signals, particularly when using fluorescence detection (Duggan et al. 1999). The labelled cDNA from control and test cells is mixed and hybridised to the array. Following a washing step the array is scanned with dual lasers and the fluorescent signals from the array detected and integrated. The results are presented as yellow coloured spots which are a mixture of the two fluorescent probes if the mRNA is equally expressed, or a spot of the predominant colour of either the control or test fluorescent tag if the mRNA expression is inhibited (green) or induced (red) in the test cells. The method is presented diagrammatically in figure 6.1.

The aim of the array experiments conducted in this study was to investigate the global effects of curcumin on gene transcription in breast cells and to identify potentially interesting and novel targets for the chemopreventive agent. The experiment in MDA 468 cells was carried out using a low dose of curcumin (10 μ M) at two time points (3 and 24 hours). Three hours was chosen as this was the total time of curcumin treatment in investigations into c-fos and c-jun expression described in chapters 4 and 5. Twenty four hours was the time point at which 10 μ M curcumin begins to have an effect on cell growth. In each case these test cells were compared to control cells treated with an equivalent concentration of vehicle control (DMSO). Similar experiments were carried out in HBL 100 cells, using 10 μ M curcumin for 24 hours.





151

6.3 Results

The global effect of curcumin on gene expression was investigated using cDNA microarray analysis. Treatment with 10μ M curcumin caused a significant (two-fold, or greater) change in expression in a relatively small number of genes (Figure 6.2), only a proportion of which were reproducible in duplicate experiments. The scatter plots, along with lists of genes that were at least two-fold up-, or downregulated in individual experiments are shown in figures 6.3 and 6.4 (MDA 468, 3 hour treatment), figures 6.5 and 6.6 (MDA 468, 24 hour treatment) and figures 6.7 and 6.8 (HBL 100, 24 hour treatment). Within the tables, genes are listed in order of decreasing log ratio of Cy5:Cy3 (test:control) fluorescence. The abbreviations for the genes are taken from the National Cancer Institute (NCI) Genecards website (http://nciarray.nci.nih.gov/cards). As a general rule the further away from the red median line and the further towards the top right hand corner of the scatter plots a gene lies, the more relevant the change. Several genes have been highlighted on the scatter plots and these will be discussed in detail later in the chapter.

These same data were analysed using the treeview programme (Mike Eisen, Stanford) (Table 6.7). This is a more sensitive way of analysing the data where in each experiment a coloured block is allocated for each gene depending upon the degree of upregulation (red) or downregulation (green). Unchanged expression compared to control is shown in grey. There are several gradations of each colour to give an idea of the extent of the change. Genes were included in the table if they behaved in the same way in duplicate experiments. They have been grouped into four convenient categories for discussion.

In every case a duplicate experiment was one performed with a fresh RNA preparation from a fresh batch of treated cells.



Figure 6.2. An example of a scatter plot including signals from all the genes in the microarray. This plot is derived from analysis of hybridisation of RNA from MDA 468 cells treated with curcumin for 24 hours (see figure 6.4). The genes upregulated (red points), or downregulated (green points), by at least two-fold, are shown in this and subsequent scatter plots. The yellow points clustered around the diagonal median line represent genes on the array whose expression is not significantly changed.



Figure 6.3. Microarray analysis Ia. MDA 468 cells were treated with 10µM curcumin, or vehicle control (DMSO), for 3 hours. Total RNA was extracted, labelled with Cy5 (red) or Cy3 (green) and microarray analysis performed. Logs of the fluorescence were determined and those genes that were two fold or more upregulated (red points), or downregulated (green points), from the median (represented by red line) were plotted. In this, and subsequent scatter plots those genes which lie within the two-fold change threshold (yellow points) are omitted. Those genes that only gave a reading in one or other channel (grey points) are also omitted.

Genes Upregulated	Genes Downregulated
HMOX1, CSNK1E, APOD, NRAS, SKY	 CDC10, Synaptosomal assoc. protein, P115, HPN, Drosophila fat fac., TTF1, MSX1, PLAB, ADK, LIPC, Human orphan G protein-coupled receptor, CDK9, AKAP7, IDO, ISL1, TAX1BP1, MFAP4, DLC1, ATP1A3, MET, PNMT, SMAP, GPX2, WNT5A, Serum albumin precursor, Myosin heavy chain, ZNF43, PRKCA, ALPI, Tyrosine phosphatase, TIP60, amyloid precursor, GLA, Recipin, NPTX2, ARSD, COL4A4, CHRNA4, CREM, GYPE, E2F-3, CBP2,

	HTR2C, IL-4, Secretogranin precursor,
product sector is a sector of and an and a sector of a sector is a sector of a sector of a sector of a sector is a sector of a	ADD1 CD79A LTB p84 ZNF139
1. 302	SETPA1 MEIS1 Calmodulin CDR2
	Grb2 associated protein BMP8 RASA1
	DRV3 AGL Avonin 1 prequesor TEDI
	MCDS1 CIAL US2ST1 CVD212
	MCK51, GJA1, H55511, CTP2J2,
	NIRKS, C4BPA, FKHR, Coagulation
	factor X precursor, GABPA, Mitotic
	kinesin-like protein, MDCR, ZNF148,
	Alpha-actinin I, Carboxypeptidase Z,
	GS3686, PCNA, CAST, IQGAP1,
	ATP5D, MME, PACE4, GMFB,
	INHBA, AIF1, β -neoendorphin
1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	precursor, FPRL1, SDF1, RGS16,
	THBD, AFP, COX7B, AICL, ATP1G1,
	PLGL, DVL, PCI, HLA-DPB1, CR2,
	survivin, KIAA0737, OZF, ARG1,
	ERDA2, GEF, ATP1B2, CYP1B1,
	RhoE, ITGAV, CASP6, PNLIPRP1,
	Corticosteroid B-dehydrogenase, E2.
	TOP2B THBS2 PK-120 NCBP
	H2BFO_CNCG1_Malate
	ovidoreductase FAP48 M6PR INSI 4
	ETS N-acetylactosamine synthese GRI
	DNA ligana 1 Cyclin T MEE2C E2
	EDE COD CTH ACTDIA Cyclin T20
	CAL CDID DAWD MTD Anti
	CAI, CDID, PAWR, MIR, And-
	microbial LPS binding protein, CYPIAI,
	AVPRIA, CYP2BO, AMPDI, GPLDI,
	APC, Garp, Cyclin C, F11, Iron
	regulatory protein 2, CD8A, COL2A1,
	C1QB, MPP-1, SGCD, CENPE, CYP17,
	ICAM5, hla-2, TPX-1, ACADSB,
	NR1H4, GATM, ZFX, FRXB, TLR3,
	CDK7, MMP13, ATP2B4, GYPB,
	CDH13, PTGER3, Insulin degrading
	enzyme, LMO2, RELB, PECAM1,
	PIK3CA, GC, APOB, LRP2, COPS3,
	DPYD, Transglutaminase, DAB2,
	WNT2, FMO5, CYP1A1, CYP2C9,
	CD36, GEF, FLIP, F2R, COL11A1,
	TRIP, ATP7A, KLRC2CENPC,
	TNFSF9, MYLK

Table 6.1. Genes affected by curcumin as determined by microarray analysis Ia. The genes on the scatter plot (Figure 6.3) are listed in descending order of log ratio of fluorescence. Those genes upregulated are in red and those downregulated are in green. Unidentified genes plotted on the scatter plot were not included in the table.



Figure 6.4. Microarray analysis Ib. This experiment was a duplicate of the previous one (Figure 6.3). MDA 468 cells were treated with 10µM curcumin, or vehicle control (DMSO), for 3 hours. Total RNA was extracted and microarray analysis performed and analysed as before.

Genes Upregulated	Genes Downregulated
HMOX1, Orphan nuclear hormone receptor, Iron regulatory protein, Amyloid A4, CSE1L, Amyloid A4 precursor, Dead box X, Vasopressin activated protein.	SAA1, MDR, MGST2, Antileukoproteinase precursor, Cytoplasmic antiproteinase, Glutathione transferase, S100A8, PIK3CG, POLG, DNA fragmentation factor 4, CYP1A1, GPX3, PRKCZ, IFI56, MMP13, FKBP1.

Table 6.2. Genes affected by curcumin as determined by microarray analysis Ib. The genes plotted on the histogram (Figure 6.4) are listed in descending order of log ratio of fluorescence. Those genes upregulated are in red and those downregulated are in green. Unidentified genes on the scatter plot were not included in the table.

Chapter 6



Figure 6.5. Microarray analysis IIa. MDA 468 cells were treated with 10µM curcumin, or vehicle control (DMSO), for 24 hours. Total RNA was extracted and microarray analysis performed and analysed as before.

Genes Upregulated	Genes Downregulated
CYP1A1 (x2), PKD2, PROS1, CDC25B, IL6, Corticosteroid β-dehydrogenase, EPH-related tyrosine kinase, DDH1, C110RF4, HMR, TAF2N, CASP5, GRIA3, c-Fos, AKR1C3, APOC2, GSTA4.	PLAB, MMP1, YES1, CDKL1, Calmodulin, PCTK3, Inhibitor of apoptosis protein, DNA topoisomerase 2, MMP7, RhoE, IDI1, TAK1 Binding protein, α-tubulin.

Table 6.3. Genes affected by curcumin as determined by microarray analysis IIa. The genes plotted on the histogram (Figure 6.5) are listed in descending order of log ratio of fluorescence. Those genes upregulated are in red and those downregulated are in green. Unidentified genes on the scatter plot were not included in the table.





Figure 6.6. Microarray analysis IIb. This experiment was a duplicate of the previous one (Figure 6.5). MDA 468 cells were treated with 10µM curcumin, or vehicle control (DMSO), for 24 hours. Total RNA was extracted and microarray analysis performed and analysed as before.

Genes Upregulated	Genes Downregulated		
CYP1A1, DDH1, AKR1C3.	EPHA2, PLAB, Br cadherin, PIG6, PRKCZ, MMP1.		

Table 6.4. Genes affected by curcumin as determined by microarray analysis IIb. The genes plotted on the histogram (Figure 6.6) are listed in descending order of log ratio of fluorescence. Those genes upregulated are in red and those downregulated are in green. Unidentified genes on the scatter plot were not included in the table.



Figure 6.7. Microarray analysis IIIa. HBL 100 cells were treated with 10µM curcumin, or vehicle control (DMSO), for 24 hours. Total RNA was extracted and microarray analysis performed and analysed as before.

Genes Upregulated	Genes Downregulated		
NF-ATc, Protein phosphatase,	MMP1, MMP13, PIK3CG, TRADD, KLRC2, Troponin T, ISL1.		

Table 6.5. Genes affected by curcumin as determined by microarray analysis IIIa. The genes plotted on the histogram (Figure 6.7) are listed in descending order of log ratio of fluorescence. Those genes upregulated are in red and those downregulated are in green. Unidentified genes on the scatter plot were not included in the table.





Figure 6.8. Microarray analysis IIIb. This experiment is a duplicate of the previous one (Figure 6.7). HBL 100 cells were treated with 10µM curcumin, or vehicle control (DMSO), for 24 hours. Total RNA was extracted and microarray analysis performed and analysed as before.

Genes Upregulated	Genes Downregulated		
	RALA, ADTB3, TRADD, KIF5B, PI3K, ATP synthese PON1 BFL1 RARA MMP10		
	ISL1, PPY, MDR, CD38, Antileukoproteinase 1 precursor, XDH, CDC25A, IL1R2,		
NONE	pathogenesis related protein, PKG1, Glioma IL8, KNS2, COX10, ECGF1, CSE1L,		
	CDKN2A, KPNB2, RTEF, MAX, HMGCL, Cyclin E, INSM1, IFI56, CALR, ALAS1, NGEB ACP5 LY6F, SLC10A1, CASP1		
	HS3ST1, COX17, NFIX, EV12A, ASP, DNA topoisomerase 1, ASNS, DDX5, HMOX1, MMP1_PPFIA1		

Table 6.6. Genes affected by curcumin as determined by microarray analysis IIIb. The genes plotted on the histogram (Figure 6.8) are listed in descending order of log ratio of fluorescence. No genes were upregulated, those downregulated are in green. Unidentified genes on the scatter plot were not included in the table.

Classification	Gene name	Tree view image			
		M 3h	M 24h	H 24h	
Phase I/II enzymes	CYP1A1 (a)		a file a		
	CYP1A1 (b)				
the set of a burner of a	CYP1B1				
CURRENT OF UP DISCUSSION OF	PIG12/MGSTILI				
	AKR1C3	- Marine			
	AKR1C1/DDH1		E.C.		
nie jaar in het needer in derine en ee	HMOX1				
Matrix metalloproteinases	MMP1	1923211			
	MMP7		The second second	No. 1	
	MMP10				
	MMP12			2.2	
	MMP13				
Signal transduction related	PLAB				
	NTRK1				
	NRG1			and the second second	
	PPFIA1				
	RhoE				
	CASP1				
	РСТК3		NEL SUR		
Misc.	APOB				
	IDI1	-		No fine	
Ly Brown end to n	DDX5				
	TAF2N				

Table 6.7. Treeview analysis of microarray data. The experimental data presented in figures 6.3-6.8 were analysed by the treeview programme to identify patterns of altered expression from all six experiments conducted, MDA 3h (M 3h), MDA 24h (M 24h) and HBL 24h (H 24h), in duplicate. Under each of the three headings there are two experiments, each represented by a coloured block. Green blocks indicate that the gene was downregulated compared to control, red indicates that the gene was upregulated compared to control and grey indicates that there was no change between control and curcumin treatment. The genes were included in the table if they showed the same result in more than one experiment, or because the change in expression fitted with the general pattern of expression seen in related genes. Cyp1A1 was included twice on the array (a and b).
6.4 Discussion

In general the reproducible changes observed in the cells upon treatment with curcumin were modest and small in number considering the number of genes on the array. Of the changes which did occur, the majority were downregulations compared to control and only a few inductions of certain mRNAs were observed upon curcumin treatment. There was little difference in the general gene expression patterns between 3 and 24h treatments in the MDA 468 cells, although some individual genes did show striking differences. In the case of the HBL 100 experiments there were very few genes upregulated compared to the MDA 468 in which several upregulations were observed. More specifically the changes seen in the phase I and II metabolising enzymes observed in the MDA 468 were not seen in the HBL 100. Other than that the downregulated genes were distributed fairly evenly between the two cell lines. Taken together the experiments show that curcumin is not a general inhibitor of gene expression and that there is specificity within the system.

6.4.1 Genes related to signal transduction.

A number of genes related to signal transduction pathways were picked out in table 6.7. The first of these, PLAB, is a member of the TGF β superfamily and is also known as prostate differentiation factor. The expression of this growth factor was inhibited by treatment with 10µM curcumin, for 24 hours, in both cell lines and at both timepoints in MDA 468 cells. There is very little data in the literature on this protein but it has been shown to be expressed at particularly high levels in placenta and can be found at low levels in most tissues of the body (Hromas *et al.* 1997).

A group of genes were also highlighted, whose expression was inhibited by 10 μ M curcumin only in the HBL 100 cell line. These were neurotrophic tyrosine kinase receptor type 1 (NTRK1), which, despite its name is expressed ubiquitously in mammalian cells. The ligands for the receptor include nerve growth factor (NGF) and neurotrophins 3, 4 and 5 and its substrates include shc, PI3K and phospholipase C γ (PLC γ). This receptor belongs to the insulin receptor family and has been shown to activate ERK1 via the pathways described above. NRG1, or heregulin α , is an important growth factor in breast tissue and has been shown to activate erb-B2 through the induction of heterodimerisation of the receptor with other family members. PPFIA1 is a protein tyrosine phosphatase and CASP1, or caspase1, is a cysteine protease with a role in apoptosis.

RhoE expression was inhibited only in the MDA 468 cells. This inhibition was shown in both experiments at 24 hours and in one experiment at 3 hours. RhoE is a Ras homologue and, as a small GTPase, plays a central role in the signal transduction pathways which have been described within this thesis. PCTK3 or PCTAIRE protein kinase 3 is a ser/thr kinase and plays a role in signalling in terminally differentiated cells. It is a member of the cdc2 subfamily. The expression of this kinase was inhibited only in MDA 468 cells at 24 hours and was upregulated in the same cell line, in a single experiment, at 3 hours.

In general it is conceivable that the inhibition of expression of most of these signalling molecules could be beneficial with respect to the chemopreventive effect of curcumin, resulting in an inhibition of growth. The exception to this is the inhibition of caspase 1 which would inhibit apoptosis. It is important to realise that these data, as with all the microarray data presented in this chapter, require confirmation by other methods, such as analysis of protein levels and activity, before solid conclusions may be drawn. The source for these, and subsequent data was the National Cancer Institute (NCI) Genecards website unless otherwise stated.

6.4.2 Miscellaneous genes with altered expression.

Several interesting genes, listed under miscellaneous in table 6.7, were identified. APOB, or apolipoprotein B, expression was inhibited after 3 hours treatment in the MDA 468 cell line. After 24 hours treatment the inhibition was no longer observed so this was an early and transient change. Apolipoprotein B is a major constituent of chylomicrons and of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) and functions as a recognition molecule for the cellular binding and internalisation of LDL by the APOB receptor. IDI1 or isopentenyl diphosphate delta isomerase is an important enzyme in the isoprenoid biosynthetic pathway whose end products include vitamins A, D, E and K, steroid hormones, bile acids and cholesterol. IDI1 expression was downregulated in MDA 468 cells after 24 hour treatment. DDX5, or dead/H box polypeptide 5 is an ATP-dependent helicase whose activity is strongly stimulated by RNA. This enzyme was downregulated in HBL 100 cells after 24 hours. The picture in MDA 468 is less clear since there was downregulation at 24 hours and some upregulation after 3 hours, but both these responses only occurred in a single experiment. Finally the RNA polymerase TAF2N was upregulated in MDA 468 after 24 hours of curcumin treatment.

6.4.3 Phase I/II detoxifying enzymes.

Expression of the phase I cytochrome p450 enzymes, Cyp1A1 and Cyp1B1 was affected by curcumin treatment, in the MDA 468 cells. The levels of mRNA were decreased by curcumin treatment after 3 hours and increased after 24 hours. The aldo-ketoreductases AKR1C1 (DDH1) and AKR1C3, which are dihydrodiol dehydrogenases, were induced after 24h treatments, again only in the MDA 468 cell line. These are phase II enzymes responsible for the detoxification of carbonyl compounds that can react with cellular macromolecules and cause mutagenesis (O Conner et al. 1999). AKR1C3 was shown to be the most dominant isoform of the AKR1C family expressed in the human mammary gland (Penning et al. 2000). These same authors showed AKR1C1 to be expressed in the mammary gland at significant levels. Both the Cyp1A1 and AKR enzymes are components of a pathway responsible for the activation and detoxification of the polycyclic aromatic hydrocarbons (PAHs) (Burczynski et al. 2000). PAHs are carcinogens and include B[a]P amongst their number. B[a]P is converted to B[a]Pdiol by Cyp1A1. AKR1C1 and Cyp1A1 compete for this molecule and turn the carcinogen over to benzo[a]pyrene-7,8-dione (BPQ) or (±)-anti-7,8-dihydroxy-9,10-Both of these epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (anti-BPDE) respectively. compounds are still capable of genotoxicity, but the AKR1C1 route does lead to increased detoxification (Figure 6.9).



Figure 6.9. Pathways responsible for the activation and detoxification of B[a]P.

The sequences for the AKR1C1 and AKR1C3 genes were aligned and the expressed sequence tag (EST) sequences for the PCR products that make up the array identified (see appendix). The alignment was performed using the CLUSTALW programme (European Bioinformatics Institute). There is almost complete homology between AKR1C1 and AKR1C3, although AKLR1C1 is slightly longer. The two ESTs lie within regions of almost 100% homology between the two enzymes. Therefore there is potential for cross hybridisation. These observations mean that curcumin could be affecting the expression of either one or both of these enzymes and results from the array alone cannot distinguish which is correct.

Microsomal glutathione S-transferase (PIG12/MGST1L1), or prostaglandin E synthase (PGE synthase) is another phase II enzyme that was upregulated in one of the duplicates for each of the three experiments. The enzyme is heavily dependent upon glutathione for activity and is induced in response to the pro-inflammatory cytokine IL-1B (Jakobsson et al. 1999). These authors have demonstrated its presence in a number of human tissues, including breast. They also showed that the enzyme is co-regulated with COX2 and that the synthesis of prostaglandin E2 (PGE2) may require the presence of both enzymes. COX2 has been implicated in the development of colon cancer (Dubois et al. 1998) and its expression has been shown to be inhibited by curcumin in human colon cancer cells (Plummer et al. 1999). With relevance to breast cancer, elevated levels of PGE2 have been found in mammary tumours and it is often associated with the loss of oestrogen responsiveness and increased metastatic potential (Rolland et al. 1980; Schrey and Patel 1995). The overexpression of COX2 has also been shown in breast tumour, but not in normal breast tissue and is again associated with poor prognosis (Parrett et al. 1997). If this preliminary microarray result constitutes a real response, then this could be seen to be a negative effect of curcumin and a possible tumour promoting activity.

A number of studies have implicated curcumin in the modulation of phase I and II metabolic enzymes. Ciolini et al. (1998) demonstrated that treatment of MCF7 cells with 1-10 μ M curcumin for 24 hours caused a rapid induction of Cyp1A1 mRNA, but that curcumin competitively inhibited Cyp1A1 activity in DMBA treated cells. These authors also showed that curcumin competed with aryl hydrocarbons (Ahs), including PAHs, for the Ah receptor (AhR). It was suggested that this competition of curcumin with the carcinogen resulted in a reduced formation of the active carcinogenic compound. Curcumin (5-12 μ M) was shown to inhibit both Cyp1A1 and Cyp1A2 and the phase II enzyme glutathione S-transferase (GST) activities when added directly to an *in vitro* assay for their activity (Oetari *et al.* 1996). Administration of 2% curcumin in the diet of female A/J mice inhibited hepatic Cyp1A1 activity and enhanced GST activity (Singh *et al.* 1998). GST activity in rat liver microsomes was shown to be induced by feeding low doses (1-50mg/kg/day), while being inhibited at high doses (50-500mg/kg/day) (Piper *et al.* 1998). Similarly a feeding study in rats where high doses of curcumin (50-200mg/kg) were administered daily, showed no enhancement of GST activity in the liver (Singletary *et al.* 1996). A study in the human leukaemia cell line K562 indicated that the activities of certain GST isoforms were induced by treatment of the cells with 1 μ M curcumin for 16 hours.

A simplistic view of the situation is that an induction of Cyp1A1 and related phase I enzymes is unfavourable, since they are responsible for the activation of pro-carcinogens to their active form. The induction of GST and other phase II enzymes is favourable since they are responsible for detoxification of the carcinogen. In this study there was an initial reduction in Cyp1A1 mRNA in the MDA 468 at the early time point, followed by an induction in curcumin-treated cells after 24 hours. This induction may be considered a negative factor, but it is accompanied by the induction of a number of phase II enzymes. It must be remembered that the level of mRNA does not necessarily equate to protein levels, nor does it indicate levels of activity and further studies require to be done in order to confirm the effects of curcumin on these parameters. Furthermore it is likely that it is the balance achieved between the phase I and phase II enzymes that determines extent of activation/detoxification of a carcinogen.

Heme oxygenase-1 (HMOX1) is a metabolic enzyme involved in the regulation of reactive oxygen and in protecting the cell from potentially damaging oxidative stress. HMOX1 mRNA was induced by curcumin treatment for 3 hours in the MDA 468 cell line. After 24 hours HMOX1 mRNA had returned to basal levels. HMOX1 is the rate limiting enzyme in the breakdown of heme to the biologically active molecules, iron, carbon monoxide (CO) and biliverdin. Iron is a gene regulator, CO is an important second messenger in vasoregulation and biliverdin is converted to bilirubin by biliverdin reductase. Both biliverdin and bilirubin possess potent anti-oxidant activity (reviewed in Dalton *et al.* 1999; Maines 1997). HMOX1 can be induced by a wide array of cellular stresses including heat shock, glutathione

depletion, radiation, hypoxia and a variety of disease states (reviewed in Maines 1997). A very recent paper showed that the treatment of bovine aortic cells with up to 15μ M curcumin resulted in an increase in HMOX1 mRNA, protein expression and activity (Motterlini *et al.* 2000). These authors showed these effects to occur after 1.5 hours treatment and to remain after a total time of 18 hours. Hypoxia was also shown to induce HMOX1 expression and activity and this induction was potentiated by curcumin treatment. Therefore curcumin can increase the antioxidant response to cellular stress through increased levels of HMOX1.

6.4.4 Matrix metalloproteinases (MMPs).

The interaction of cells with the extracellular matrix (ECM) involves the co-ordinated actions of proteolytic systems responsible for the hydrolysis of various ECM components. The breakdown of this regulation results in abnormal development and contributes to the processes of tumour invasion and metastasis. Matrix metalloproteinases (MMPs) are normally required to regulate the processes of inflammation, wound healing and embryogenesis (Baker et al. 2000; Massova et al. 1998). The MMPs are the proteolytic enzymes primarily responsible for ECM degradation in normal and pathological processes. They constitute a large family of zinc-dependent endo-peptidases which consist of an amino terminal propeptide, a catalytic domain and a hemopexin-like domain at the C-terminus. All are produced as an inactive proform and proteolytic removal of the N-terminal peptide results in the active enzyme (Massova et al. 1998). There have been at least 17 MMPs identified in humans and they are grouped according to substrate specificity. The most significant are the gelatinases (MMP2, 9), the collagenases (MMP1, 8, 13), the stromelysins (MMP3, 10, 11), the metalloelastases (MMP12) and the matrilysins (MMP7) (Massova et al. 1998). MMP activity is tightly regulated at the levels of transcription, secretion, activation and inhibition. Inhibition of MMPs is accomplished by members of the tissue inhibitors of MMP (TIMP) family of which there are four members (reviewed in Huppertz et al. 1998). The TIMPS (1-4) can inhibit any of the MMPs and do so by binding to the catalytic domain of the enzyme.

The microarray experiments showed that the mRNA of several of these MMPs was downregulated by curcumin. MMP1, 7, 12 and 13 were downregulated at one or other time point in the MDA 468 cell line and MMP1, 10, 12 and 13 were downregulated in the HBL100

cell line. The gelatinase MMP2, MMP3 and MMP11 as well as the four TIMPs which were included on the array did not show any changes in the levels of mRNA following curcumin treatment. Either their RNA levels were unaffected or they are not expressed in these cell types. It has been previously shown that MMP2 is not expressed in the MDA 468 or HBL 100 cell lines (Louise Jones, personal communication). In order to investigate the possibility of cross hybridisation, the MMP genes shown to be affected by curcumin were aligned using the CLUSTALW programme as for the AKR1C genes (appendix). Alignment of the full length sequences for the affected MMPs showed little homology between them. All the ESTs aligned with their respective gene in regions of no, or very low homology with any of the other MMPs, so we can be confident that the chance of cross hybridisation is very low. These observations give confidence that the changes observed are specific to those particular MMP genes. MMPs 1, 2 and 3 have been detected by immunohistochemistry, in situ hybridisation and northern blotting, in breast carcinomas in two independent studies. In one study the tumour cells were preferentially labelled as opposed to the normal breast tissue (Clavel et al. 1992). The same study also examined the presence of TIMP1 and found this to be more widely expressed in both tumour and normal tissues. Similar results were obtained in a study by Polette et al. (1993). A role for these enzymes in colon cancer has also been suggested. The active forms of MMP2 and MMP9 were detected in significantly greater amounts in colon tumours than in normal colon tissue as were the levels of MMP1, MMP3 and TIMP1. TIMP2 expression was shown to be greater in normal, as opposed to tumour, colon tissue. Total MMP activity in the colon tumours was also greater than in normal colon tissue. A correlation between MMP1 and MMP3 expression and Duke's stage of colon carcinogenesis was also noted (Baker et al. 2000). Immunohistochemistry was also used to demonstrate that MMP2, 7 and 11 expression is greater in pancreatic carcinoma than in normal pancreas (Bramhall et al. 1997). These authors noted that TIMP1 expression was always seen in association with MMP7 and 11, while TIMP2 was expressed in only half the tumours investigated.

The level of MMP1 mRNA in a panel of human breast cells (MDA 231, T47D and MCF7) was investigated (Nutt and Lunec 1996). A higher level of MMP1 mRNA was found in the MDA 231 cells than the other two lines and a two-fold increase in MMP1 transcripts was observed upon treatment with EGF or TGF α . A similar increase was observed upon growth factor deprivation. No similar effects were observed in the other two cell lines. AP-1 and

168

NF- κ B activation were correlated to MMP13 expression in a mouse model of arthritis. In this model the activation of AP-1 and NF- κ B preceded MMP expression. The correlation with NF- κ B was better than that for AP-1 (Han *et al.* 1998). MMP1 and MMP3 were also shown to be transcriptionally regulated by AP-1 in co-operation with members of the Ets family of transcription factors (Buttice *et al.* 1996). A further interesting point, the possible relevance of which is unclear at present, is that MMPs1, 7, 12 and 13 are in the same gene cluster on chromosome 11q22 (Pendas *et al.* 1997). Curcumin has been shown in this, and other, studies to inhibit the expression of components of the AP-1 family and its activity. Transcriptional regulation of the MMPs by AP-1 highlights a possible link between curcumin, the inhibition of AP-1 and subsequent inhibition of MMP transcription.

The MMP family of proteases are obviously important in carcinogenesis and are particularly implicated in the processes of invasion and metastasis. MMP9 was shown to play a role in angiogenesis in a mouse model of multistage pancreatic carcinogenesis (RIP1-Tag2) (Bergers *et al.* 2000). These authors demonstrated that protein levels of MMP9 are upregulated in angiogenic lesions and can induce angiogenesis in normal islet cells. It was proposed that it exerts this effect by making the angiogenic factor vascular endothelial growth factor (VEGF) more available to the surrounding cells. A very recent study described the induction of angiogenesis induced by the implantation of a pellet of fibroblast growth factor-2 (FGF-2) into rabbit corneas. This was shown to activate AP-1, which in turn induced the expression of MMP9. Co-implantaton of a pellet containing curcumoids with an FGF-2 pellet inhibited both the induction of MMP9 and angiogenesis (Mohan *et al.* 2000). This fact, together with the observation that their expression is regulated by pathways we and others have shown to be modulated by curcumin, makes them interesting targets for further investigation.

In order to carry forward the work on this interesting family it will be necessary to carry out further experiments to verify the results obtained in the microarrays. Northern and western blotting could be done to determine that the observed decreases at the level of mRNA are real and are translated through to the protein level. Zymographs may also be employed in order to get an idea of the levels of each protein and also, more importantly, a measure of activity.

CHAPTER 7. SUMMARY AND CONCLUSIONS

It is generally accepted that diet can play an important role in both causing and preventing cancer. There is widespread evidence for a number of foodstuffs acting as chemopreventive agents from epidemiological studies within and between populations, as well as a wealth of data from animal studies showing a number of individual compounds to be effective. The latter have used models of both chemical- and genetically-induced carcinogenesis in a wide variety of different tissues. The difficulties in setting up long-term human primary chemoprevention trials and early scares from studies that suggested the proposed chemopreventive agent, β -carotene may increase cancer incidence and mortality (Omenn et al. 1996; The Alpha Tocopherol, Beta Carotene Cancer Prevention Study Group 1994), have meant that there are limited data available on the efficacy of individual compounds in humans. These concerns, coupled with the need to identify SEMs for use in clinical trials, have resulted recently in extensive investigation into the mechanisms of action of chemopreventive agents with respect to signal transduction pathways and other processes responsible for the initiation and progression of human carcinogenesis. The aims of this project were to investigate the growth inhibitory properties of three chemopreventive agents, curcumin, EGCG and I3C, in a panel of human breast tumour cell lines and to identify mechanisms by which this growth inhibitory effect might be exerted.

The effect of curcumin on the growth of three breast cell lines was determined in the course of this study, while the effects of EGCG and I3C were determined by others in the laboratory. All three cell lines exhibited similar sensitivity to growth inhibition by curcumin, with significant inhibition being obtained at 5μ M in each case. In contrast, both EGCG and I3C exhibited a differential effect on the cell lines. In each case the most malignant cell line, MDA 468, was inhibited most effectively with IC₅₀s of 10 μ M (EGCG) and 40 μ M (I3C) compared to 100 μ M in the T47D and HBL 100 cells for EGCG and 50 μ M (T47D) and 100 μ M (HBL 100) for I3C. Growth inhibitory concentrations of curcumin induced a G₂/M cell cycle arrest in the MDA 468 cell line. This arrest was accompanied by an increase in apoptosis which was maximal at a dose of 20 μ M for 24 hours. EGCG was not shown to induce arrest in a particular phase of the cell cycle, but an induction of apoptosis was observed in the MDA 468 and HBL 100 cell lines that correlated with the differences in sensitivity to growth. Significant apoptosis was observed in the MDA 468 cell line after

treatment with 50μ M EGCG for 24h, but was not seen in the HBL 100 cell line until 48h with a dose of 200 μ M. No obvious cell cycle arrest was observed in any of the cell lines tested following treatment with I3C. It has been shown previously in the laboratory that I3C induces apoptosis after treatment with 100 μ M for 24h in the MDA 468 cell line, but not in the HBL 100 cell line. This correlates well with the data on growth inhibition. The predominant form of cell death in the HBL 100, at higher concentrations of I3C, was necrosis.

The modulation of a number of signalling pathways by the chemopreventive compounds was investigated. In general the treatment conditions used were aimed at identifying early changes in the signal transduction pathways such as phosphorylation, rather than changes in gene expression. Curcumin did not inhibit EGF-induced EGFR phosphorylation in the HBL 100 cell line, but inhibition was observed in MDA 468 cells. Subsequent inhibition of EGFinduced ERK phosphorylation and the transcription of c-fos were seen in both cell lines. Inhibition of TPA-induced ERK phosphorylation was also observed in MDA 468 and HBL 100 and this fact, along with the difference seen at the receptor level between the two cell lines, immediately suggested that curcumin has multiple mechanisms of action within the cell that impinge upon the ERK pathway at different levels. The components of the ERK pathway were inhibited by curcumin at doses from 20-40µM. In vitro kinase assays showed that anisomycin-induced JNK activity was inhibited by curcumin at lower doses, approaching the IC₅₀ for growth (10µM in MDA 468 and T47D and 20µM in HBL 100). This inhibition was seen if the cells were pre-treated with the chemopreventive agent, but not if the agent was added directly to the assay suggesting a point of action upstream of curcumin. Similar results were obtained when the upstream kinase MKK4 was investigated. Upstream of the MKK level, the components become more difficult to assay, especially the endogenous kinases, and transfection studies were used to investigate the effect of curcumin on MEKK1 and 3. Preliminary results indicated that curcumin pre-treatment (10µM) inhibited 4HT-induced JNK, but not ERK, activity in cells expressing Δ MEKK3:ER. This concentration of curcumin was also capable of inhibiting MEKK1 activity in an in vitro assay. These data suggested that one point of action of curcumin might be at level of the MEKKs, possibly on MEKK1 itself. This is given further weight by the observation that anisomycin-induced p38 activity was not inhibited by curcumin either when cells were pre-treated or when curcumin was added directly into the in vitro assay. MEKK1 is not thought to activate p38 and although there may be crosstalk at other points in the pathway, these data are consistent with the hypothesis of

171

MEKK1 being a target for curcumin. Preliminary studies showed that pre-treatment of HBL 100 cells with 40µM curcumin completely inhibited EGF, TNF and anisomycin-induced Akt phosphorylation. The inhibition of this important molecule points towards a further mechanism of action of curcumin, since Akt lies within the PI3K pathway which is activated by a variety of growth factor receptors as well as being central in apoptotic signalling as a survival signal.

The effects of EGCG on the MAPK pathways were investigated in a similar manner. In contrast to curcumin, no inhibition of EGFR or ERK phosphorylation was seen. Treatment of cells with 50µM EGCG, either prior to, or together with, EGF stimulation enhanced the phosphorylation of these signalling components. Treatment with EGCG alone induced both ERK phosphorylation and c-fos protein expression. These effects were much more pronounced in the MDA 468 tumour cell line than in the HBL 100. Pretreatment of cells with EGCG did not inhibit anisomycin-induced JNK or p38 activity, but low concentrations of EGCG were capable of inhibiting the activity of both kinases, directly, in an *in vitro* assay. The downstream consequences of such inhibition *in vivo* were not reflected in MAPKAPK2 activity (downstream of p38) or c-jun expression (downstream of JNK), so the relevance of this inhibition remains unclear.

I3C did not inhibit EGF-induced ERK phosphorylation at concentrations up to 1mM and previous work in our laboratory has shown that EGFR phosphorylation is only inhibited at this very high concentration which is cytotoxic to the cells. Nor did I3C inhibit anisomycin-induced JNK or p38 activity, either when cells were pre-treated, or when added directly to the *in vitro* assay.

It became obvious at an early stage that EGCG, and in particular, curcumin, were having multiple effects within the cell. In order to ensure that the kinase inhibition observed was not due to a general cytotoxic effect on the part of these agents, intracellular ATP levels were measured. This confirmed, in all three cell lines, that the agents were having no effect on intracellular ATP at the concentrations at which kinase inhibition was being observed. Such concerns were further alleviated as not all kinases were inhibited by these agents and indeed EGCG was capable of enhancing certain kinase activities.

The interpretation of these effects on signalling pathways in terms of the observed inhibition of growth and the induction of cell cycle arrest and apoptosis by the chemopreventive agents is not easy. Indeed it is likely that it is the combined effect of an agent on several different pathways that results in the effects observed upon processes such as growth and apoptosis. The EGFR pathway is a classic growth stimulatory pathway, resulting in the activation of AP-1 via ERKs and may also result in the activation of survival signals through components such as NF-KB and Akt, which in turn inhibit apoptosis. This study showed curcumin to act on the EGFR/ERK pathway and preliminary data have shown inhibition of Akt phosphorylation. There is evidence in the literature that curcumin is capable of inhibiting NF-KB (Jobin et al. 1999; Plummer et al. 1999). Thus it is clear that growth inhibition and apoptosis may result from the inhibition of this pathway by an agent such as curcumin. The activation of this pathway by the chemopreventive agent EGCG, in the presence of growth inhibition, may therefore seem contradictory. However, there is evidence in the literature that at nanomolar concentrations of EGF, activation of this pathway may be growth inhibitory, especially in cells such as the MDA 468 in which the EGFR is highly overexpressed (Filmus et al. 1985; Prasad and Church 1991). Other authors have shown that treatment of these cells with EGF can induce apoptosis (Armstrong et al. 1994; Schaerli and Jaggi 1998). This induction of apoptosis was accompanied by an increased expression of c-fos, c-jun and junB, implicating the AP-1 transcription factor in this process.

The role of the JNK pathway in cell growth and apoptosis is somewhat less clear. JNK activity leads to the expression of c-jun, a component of AP-1 that has been shown to be required for progression through G_1 . C-jun has also been implicated in the protection of fibroblast cells from apoptosis, but there is a large amount of evidence in the literature suggesting that JNK and c-jun may play a role in the induction of apoptosis, although much of this work has been in neuronal cells. It is likely that the JNK pathway is important in growth regulation in a cell-type or tissue-specific manner and its ultimate downstream effects depend upon the balance of signals from other pathways. As a central pathway in the processes of cell growth and apoptosis its disruption by agents such as curcumin and EGCG could be responsible for the inhibitory effects observed. The p38 pathway seems less important for the effects of these chemopreventive agents. This pathway is most often implicated in the induction of apoptosis and as such its inhibition by the chemopreventive agents under these circumstances might be counter-productive. I3C was not shown to have an effect on any of

the pathways studied, but it has been shown to induce G_1 cell cycle arrest in certain cell types. As such it must exert its chemopreventive effect elsewhere, such as through its modulation of oestrogen metabolism in the case of oestrogen responsive breast cancer, or more generally through the inhibition of enzymes such as ODC and Akt, whose activities have been shown to be inhibited by I3C previously in the laboratory.

Ultimately changes in cellular signalling will result in changes in gene expression. The preliminary microarray experiments in MDA 468 and HBL 100 cells looked at the global effect of curcumin treatment on mRNA and attempted to identify changes in gene transcription that could be related to previous studies, as well as novel targets for curcumin. The phase I enzymes, Cyp1A1 and Cyp1B1 were downregulated initially, at the early time point (3h), in MDA 468, followed by an upregulation after 24h treatment with 10µM curcumin. These changes were associated with an increase in mRNA levels of several phase II enzymes including AKR1C1, AKR1C3 and PGE synthase. The balance of such drug metabolising enzymes determines the level of activation or detoxification and excretion of carcinogens encountered by the cell. HMOX1 mRNA was shown to be increased in the presence of curcumin. This metabolic enzyme is an important regulator of oxidative stress and plays a role in protecting the cell from potentially damaging reactive oxygen species. Results for MMPs indicated another family of proteins in which the mRNAs of several members were downregulated in response to curcumin in both the MDA 468 and HBL 100 cell lines. These proteases are important in cancer invasion and metastasis, and there is evidence that their expression is regulated by AP-1 and NF-kB activity. The identification of this family as a potential target for curcumin with their relevance in carcinogenesis and their regulation by pathways we have already shown to be modulated by curcumin opens an interesting avenue for investigation. A number of other genes were identified that may be particularly relevant to growth inhibition. These included heregulin- α , a growth factor which is active in breast tissue and has been shown to activate other EGFR family members, including indirectly the erb-B2 receptor, which plays an important role in breast cancer. This growth factor was downregulated by curcumin in the HBL 100 cell line. NTRK1 mRNA was also downregulated in this study. This receptor tyrosine kinase couples to shc, PI3K and PLCy, ultimately resulting in the activation of pathways such as the MAPKs. All these observations, along with the others identified in chapter 6, require verification by another method such as northern or western blotting to confirm their potential involvement in

chemoprevention, but they offer exciting prospects for the investigation of novel targets for curcumin.

REFERENCES

Ahmad, N., Feyes, D. K., Nieminen, A. L., Agarwal, R. and Mukhtar, H. (1997). Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *Journal of the National Cancer Institute* **89**, 1881-1886.

Ahmad, N., Gupta, S. and Mukhtar, H. (2000). Green tea polyphenol epigallocatechin-3gallate differentially modulates nuclear factor kappa b in cancer cells versus normal cells. *Archives of Biochemistry and Biophysics* **376**, 338-346.

Ahn, H. Y., Hadizadeh, K. R., Seul, C., Yun, Y. P., Vetter, H. and Sachinidis, A. (1999). Epigallocatechin-3 gallate selectively inhibits the PDGF-BB induced intracellular signalling transduction pathway in vascular smooth muscle cells and inhibits transformation of *sis*-transfected NIH 3T3 fibrablasts and human glioblastoma cells (A172). *Molecular Biology of the Cell* **10**, 1093-1104.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffeny, P. R. J., Reese, C. B. and Cohen, P. (1997). Characterisation of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Ba. *Current Biology* 7, 261-269.

Alison, M. R. and Sarraf, C. E. (1992). Apoptosis: A gene directed programme of cell death. Journal of the Royal College of Physicians 26 (1), 25-35.

Alison, M. and Sarraf, C. E. (1995). Apoptosis: Regulation and relevance to toxicology. *Human and Experimental Toxicology* 14, 243-247.

Ammon, H. P. T. and Wahl, M. A. (1991). Pharmacology of curcuma longa. Planta Medica 57, 1-7.

Anilkumar, T. V., Sarraf, C. E., Hunt, T. and Alison, M. R. (1992). The nature of cytotoxic drug-induced cell death in murine intestinal crypts. *British Journal of Cancer* 65, 552-558.

Anto, R. J., Maliekal, T. T. and Karunagaran, D. (2000). L-929 cells harboring ectopically expressed relA resist curcumin-induced apoptosis. *Journal of Biological Chemistry* **275** (21), 15601-15604.

Armstrong, D. K., Kaufmann, S. H., Ottaviano, Y. L., Buckley, J. A., Isaacs, J. T. and Davidson, N. E. (1994). Epidermal growth factor mediated apoptosis of MDA-MB-468 human breast cancer cells. *Cancer Research* 54, 5280-5283.

2

Avruch, J., Zhang, X. and Kyriakis, J. M. (1994). Raf meets Ras: completing the framework of a signal transduction pathway. *Trends in Biochemical Science* **19**, 279-283.

Bagrodia, S., Derijard, B., Davis, R. J. and Cerione, R. A. (1995). Cdc42 and PAK-mediated signaling leads to jun kinase and p38 mitogen-activated protein-kinase activation. *Journal of Biological Chemistry* **270**, 27995-27998.

Bailey, G. S., Hendricks, J. D., Shelton, D. W., Nixon, J. E. and Pawlowski, N. E. (1987). Enhancement of carcinogenesis by the natural anticarcinogen indole-3-carbinol. *Journal of the National Cancer Institute* **78** (5), 931-934.

Baker, E. A., Bergin, F. G. and Leaper, D. J. (2000). Matrix metalloproteinases, their tissue inhibitors and colorectal cancer staging. *British Journal of Surgery* 87, 1215-1221.

Balmanno, K. and Cook, S. J. (1999). Sustained MAP kinase activation is required for the expression of cyclin D1, p21^{Cip1} and a subset of AP-1 proteins in CCl39 cells. *Oncogene* 18, 3085-3097.

Barthelman, M., Bair, W. B., Stickland, K. K., Chen, W. X., Timmermann, N., Valcic, S., Dong, Z. G. and Bowden, G. T. (1998). (-)-epigallocatechin-3-gallate inhibition of ultraviolet B-induced AP-1 activity. *Carcinogenesis* **19**, 2201-2204.

Bell, M. C., Crowley-Nowick, P., Bradlow, H. L., Sepkovic, D. W., Schmidt-Grimminger, D., Howell, P., Mayeaux, E. J., Tucker, T. E., Turbat-Herrera, E. A. and Mathis, J. M. (2000). Placebo-controlled trial of indole-3-carbinol in the treatment of CIN. *Gynecologic Oncology* **78** (2), 123-129.

Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z. and Hanahan, D. (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nature Cell Biology* **2**, 737-744.

Biasco, G. and Paganelli, G. M. (1999). European trials on dietary supplementation for cancer prevention. *Annals of the New York Academy of Science* **889**, 152-157.

Birkenkamp, K. U., Dokter, W. H. A., Esselink, M. T., Jonk, L. J. C., Kruijer, W. and Vellenga, E. (1999). A dual function for p38 MAP kinase in haemopoetic cells: involvement in apoptosis and cell activation. *Leukemia* 13, 1037-1045.

Biswas, D. K., Cruz, A. P., Gansberger, E. and Pardee, A. B. (2000). Epidermal growth factor induced nuclear factor kB activation: A major pathway of cell cycle progression in estrogen receptor negative breast cancer cells. *Proceedings of the National Academy of Sciences (USA)* **97 (15)**, 8542-8547.

Block, G., Patterson, B. and Sauber, A. (1992). Fruit and vegetables and cancer prevention; A review of the epidemiological evidence. *Nutrition and Cancer* 18 (1), 1-29.

Bossy-Wetzel, E., Bakiri, L. and Yaniv, M. (1997). Induction af apoptosis by the transcription factor c-jun. *The EMBO Journal* **16** (7), 1695-1709.

Boudewijn, M. and Coffer, P. J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602.

Bradfield, C. A. and Bjeldanes, L. F. (1987). Structure-activity relationships of dietary indoles: A proposed mechanism of action as modifiers of xenobiotic metabolism. *Journal of Toxicology and Environmental Health* **21**, 311-323.

Bradford, M., M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Analytical Biochemistry* 72, 248-254.

Bradlow, H., Michnowicz, J., Telang, N. and Osborne, M. (1991). Effects of dietary indole-3carbinol on estradiol metabolism and spontaneous mammary tumours in mice. *Carcinogenesis* 12, 1571-1574.

Bradlow, H. L., Michnovicz, J. J., Halper, M., Miller, D. G., Wong, G. Y. C. and Osborne, M.
P. (1994). Long-term responses of women to indole-3-carbinol or a high-fiber diet. *Cancer Epidemiology Biomarkers and Prevention* 3, 591-595.

Bradlow, H. L., Sepkovic, D. W., Telang, N. T. and Osborne, M. P. (1995). Indole-3-carbinol - a novel approach to breast cancer prevention. *Annals of the New York Academy of Science* **768**, 180-200.

Bradlow, H. L., Sepkovic, D. W., Telang, N. T. and Osborne, M. P. (1999). Multifunctional aspects of the action of indole-3-carbinol as an antitumour agent. *Annals of the New York* Academy of Science **889**, 204-213.

Bramhall, S. R., Neoptolemos, J. P., Stamp, G. W. H. and Lemoine, N. R. (1997). Imbalance of expression of matrix metalloproteinases (MMPs) and tissue inhibitors of the matrix metalloproteinases (TIMPs) in human pancreatic carcinoma. *Journal of Pathology* **182** (3), 347-355.

Brondello, J. M., Brunet, A., Pouyssegur, J. and McKenzie, F. R. (1997). The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the p42/p44(MAPK) cascade. *Journal of Biological Chemistry* **272**, 1368-1376.

Burczynski, M. E., Lin, H. and Penning, T. M. (2000). Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles and oxidative stress: Implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. *Cancer Research* **59**, 607-614.

Buttice, G., DuterqueCoquilland, M., Basuyaux, J. P., Carrere, S., Kurkinen, M. and Stehelin, D. (1996). Erg, an Ets-family member, differentially regulates human collagenase 1 (MMP1) and stromelysin 1 (MMP3) gene expression by physically interacting with the Fos/Jun complex. *Oncogene* 13, 2297-2306.

Carpenter, G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. Annual Review of Biochemistry 56, 881-914.

Challa, A., Ahmad, N. and Mukhtar, H. (1997). Cancer prevention through sensible nutrition (commentary). *International Journal of Oncology* **11**, 1387-1392.

Chemoprevention Branch and Agent Development Committee. (1996). Clinical development plan: Curcumin. Journal of Cellular Biochemistry 26S, 72-85.

Chen, R. H., Sarnecki, C. and Blenis, J. (1992). Nuclear localisation and regulation of ERK and rsk-encoded protein kinases. *Molecular and Cellular Biology* **12** (3), 915-927.

Chen, R. H., Abate, C. and Blenis, J. (1993). Phosphorylation of the c-fos transrepression domain by mitogen activated protein kinase and 90-kDa ribosomal S6 kinase. *Proceedings of The National Academy of Sciences (USA)* **90**, 10952-10956.

Chen, I., Safe, S. and Bjeldanes, L. (1996). Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. *Biochemical Pharmacology* 51, 1069-1076.

Chen, H. and Huang, H. (1998). Effect of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells. *British Journal of Pharmacology* **124**, 1029-1040.

Chen, Y. R. and Tan, T. H. (1998). Inhibition of c jun N terminal kinase (JNK) signalling pathway by curcumin. *Oncogene* 17, 173-178.

Chen, W., Dong, Z., Valic, S., Timmermann, B. and Bowden, G. T. (1999a). Inhibition of ultraviolet b induced c-fos expression and p38 mitogen activated protein kinase activation by epigallocatechin gallate in a human keratinocyte cell line. *Molecular carcinogenesis* 24, 79-84.

Chen, Y. R., Zhou, G. S. and Tan, T. H. (1999b). C-jun N terminal kinase mediates apoptosic signalling induced by n-(4-hydroxyphenyl) retinamide. *Molecular Pharmacology* 56, 1271-1279.

Chin, Y. E., Kitagawa, M., Su, W. S., You, Z., Iwamoto, Y. and Fu, X. (1996). Cell growth and induction of cyclin dependent kinase inhibitor p21/WAF1/CIP1 mediated by STAT 1. *Science* 27, 719-722.

Chinnaiyan, A. M., O Rourke, K., Tewari, M. and Dixit, V. M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* **81**, 505-512.

Chung, J. Y., Huang, C., Meng, X., Dong, Z. and Yang, C. S. (1999). Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-transformed cells: Structure-activity relationship and mechanisms involved. *Cancer Research* **59**, 4610-4617.

Church, J. G. and Buick, R. N. (1988). G-protein mediated epidermal growth factor signal transduction in a human breast cancer cell line . *Journal of Biological Chemistry* **263**, 4242-4246.

Ciolini, H. P., Daschner, P. J., Wang, T. T. Y. and Yeh, G. C. (1998). Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF7 human breast carcinoma cells. *Biochemical Pharmacology* 56, 197-206.

Clavel, C., Polette, M., Doco, M., Binninger, I. and Birembaut, P. (1992). Immunolocalization of matrix metalloproteinases and their tissue inhibitor in human mammary pathology. *Bulletin Du Cancer* **79** (3), 261-270.

Conney, A. H., Lou, Y. R., Xie, J. G., Osawa, T., Newmark, N., Liu, Y., Chang, R. L. and Huang, M. T. (1998). Some perspectives on dietary inhibition of carcinogenesis: Studies with curcumin and tea. *Dietary Chemicals and Cancer* 1, 234-245.

Cook, S. J., Aziz, N. and McMahon, M. (1999). The repertoire of fos and jun proteins expressed during the G_1 phase of the cell cycle is determined by the duration of mitogen-activated protein kinase activation. *Molecular and Cellular Biology* **19** (1), 330-341.

Cover, C. M., Hsieh, S. J., Tran, S. H., Hallden, G., Kim, G. S., Bjeldanes, L. F. and Firestone, G. L. (1998). Indole-3-carbinol inhibits the expression of cyclin dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signalling. *Journal of Biological Chemistry* **273**, 3838-3847.

Cover, C. M., Hsieh, J., Cram, E., Hong, C., Riby, J. E., Bjeldanes, L. F. and Firestone, G. L. (1999). Indole-3-carbinol and tamoxifen cooperate to arrest the cell cycle of MCF-7 human breast cancer cells. *Cancer Research* **59**, 1244-1251.

Cristofannilli, C. and Hortbagyi, G. N. (1998). Current methods to prevent the development of breast cancer. *In vivo* 12, 659-666.

Cross, T. G., Scheel-Toellner, D., Henriquez, N. V., Deacon, E., Salmon, M. and Lord, J. M. (2000). Serine/threonine protein kinases and apoptosis. *Experimental Cell Research* 256, 34-41.

Curran, T. and Franza, B. R. (1988). Fos and jun: The AP-1 connection. Cell 65, 395-397.

Dalton, T. D., Shertzer, H. G. and Puga, A. (1999). Regulation of gene expression by reactive oxygen. Annual Review of Pharmacology and Toxicology **39**, 67-101.

Daly, J. M., Jannot, C. B., Beerli, R. R., GrausPorta, D., Maurer, F. G. and Hynes, N. E. (1997). Neu differentiation factor induces erbb2 down-regulation and apoptosis of erbb2-overexpressing breast tumor cells. *Cancer Research* 57, 3804-3811.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenburg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231-241.

Daum, G., Eisenmann-Tappe, I., Fries, H. W., Troppmair, J. and Rapp, U. R. (1994). The ins and outs of Raf kinases. *Trends in Biochemical Science* 19, 474-480.

Davis, R. J. (1999). Signal transduction by the c-jun N-terminal kinase. *Biochemical Society* Symposium 64, 1-12.

Deacon, K. and Blank, J. L. (1999). MEK kinase 3 directly activates MKK6 and MKK7, specific activators of the p38 and c-jun NH2-terminal kinases. *Journal of Biological Chemistry* 274, 16604-16610.

DeFazio, A., Chiew, Y. E., Sini, R., Janes, P. W. and Sutherland, R. L. (2000). Expression of c-erbB receptors, heregulin and oestrogen receptor in human breast cell lines. *International Journal of Cancer* 87, 487-498.

Dekruif, C. A., Marsman, J. W., Venekamp, J. C., Falke, H. E., Noordhoek, J., Blaauboer, B. J. and Wortelboer, H. M. (1991). Structure elucidation of acid reaction-products of indole-3carbinol - detection *in vivo* and enzyme-induction *in vitro*. *Chemico-Biological Interactions* **80**, 303-315.

Deng, T. and Karin, M. (1994). c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. *Nature* **371**, 171-175.

Dent, P., Haser, W., Hatstead, A. J., Vincent, A., Roberts, T. M. and Sturgill, T. W. (1992). Activation of mitogen-activated protein kinase by v-Raf in NIH3T3 cells and *in vitro*. *Science* **257**, 1404-1407.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J. and Davis, R. J. (1995). Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* 267, 682-685.

Deshpande, S. S., Ingle, A. D. and Maru, G. B. (1998). Chemopreventive efficacy of curcumin free aqueous turmeric extract in 7,12 dimethylbenz[a]anthracene induced rat mammary tumourigenesis. *Cancer Letters* 123, 35-40.

Dive, C. and Wyllie, A. H. (1993). Apoptosis and Cancer Chemotherapy. (Blackwell Scientific: Oxford.)

Dong, Z., Ma, W. A., Huang, C. and Yang, C. S. (1997). Inhibition of tumour promoterinduced activator protein 1 activation and cell transformation by tea polyphenols, epigallocatechin gallate and theaflavins. *Cancer Research* 57, 4414-4419.

Dorai, T., Gehani, N. and Katz, A. (2000). Therapeutic potential of curcumin in human prostate cancer. Curcumin inhibits tyrosine kinase activity of epidermal growth factor receptor and depletes the protein. *Molecular Urology* **4**, 1-6.

Downward, J. (1995). Signal-transduction - a target for PI(3) kinase. Nature 376, 553-554.

Downward, J. (1998). Signal transduction - lipid-regulated kinases: some common themes at last. Science 279, 673-674.

Du, J., Suzuki, H., Nagase, F., Akhand, A. A., Yokoyama, T., Miyata, T., Kurokawa, K. and Nakashima, I. (2000). Methylglyoxal induces apoptosis in Jurkat leukemia T cells by activating c-jun N-terminal kinase. *Journal of Cellular Biochemistry* **77**, 333-344.

Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van de Putte, L. B. A. and Lipsky, P. E. (1998). Cyclooxygenase in biology and disease. *FASEB* 12, 1063-1073.

Duggan, D. J., Bittner, M., Chen, Y., Meltzer, P. and Trent, J. M. (1999). Expression profiling using cDNA microarrays. *Nature Genetics* **21**, 10-14.

Eisen, M. B., Spellman, P. T., Brown, P. O. and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences (USA)* **95**, 14863-14868.

ElHamss, R., Analla, M., CamposSanchez, J., AlonsoMoraga, A., MunozSerrano, A. and Idaomar, M. (1999). A dose dependent anti-genotoxic effect of turmeric. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis* **446**, 135-139.

Exon, J. H. and South, E. H. (2000). Dietary indole-3-carbinol alters immune functions in rats. Journal of Toxicology and Environmental Health-Part A 59, 271-279.

Fanger, G. R. and Lassignal Johnson, N. (1997). MEK kinases are regulated by EGF and selectively interact with Rac/Cdc42. *The EMBO Journal* 16 (16), 4961-4972.

Farrar, M. A., Alberola-Ila, J. and Perlmutter, R. M. (1996). Activation of the Raf-1 kinase cascade by coumermycin-induced dimerisation. *Nature* **383**, 178-181.

Feig, L. A. and Cooper, G. M. (1988). Relationship among guanine nucleotide exchange, ATP hydrolysis and transforming potential of mutated ras proteins. *Molecular and Cellular Biology* 8 (6), 2472-2478.

Filmus, J., Pollak, M. N., Cailleau, R. and Buick, R. N. (1985). MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. *Biochemical and Biophysical Research Communications* **128** (2), 898-905.

Fong, A. T., Swanson, H. I., Dashwood, R. H., Williams, D. E., Hendricks, J. D. and Bailey, G. S. (1990). Mechanisms of anti-carcinogenesis by indole-3-carbinol - studies of enzymeinduction, electrophile-scavenging, and inhibition of aflatoxin-B1 activation. *Biochemical Pharmacology* **39**, 19-26.

Fujita, Y., Yamane, T., Tanaka, M., Kuwata, K., Okuzumin, J., Takahashi, T., Fujiki, H. and Okuda, T. (1989). Inhibitory effect of epigallocatechin gallate on carcinogenesis with N-ethyl-N-nitro-nitrosoguanidine in mouse duodenum. *Japanese Journal of Cancer Research* **80**, 503-505.

Gautam, S. C., Xu, Y. X., Pindolia, K. R., Janakiraman, N. and Chapman, R. A. (1998). Nonselective inhibition of proliferation of transformed and nontransformed cells by the anti cancer chemopreventive agent curcumin. *Biochemical Pharmacology* **55**, 1333-1337. Ge, X. K., Fares, F. A. and Yannai, S. (1999). Induction of apoptosis in MCF-7 cells by indole-3-carbinol is independent of p53 and bax. *Anticancer Research* 19, 3199-3203.

Gerbracht, U., Bursch, W., Kraus, P., Putz, B., Reinacher, M., Timmermann-Troisiener, I. and Schulte-Hermann, R. (1990). Effects of hypolipidemic drugs nafenopin and clofibrate on phenotypic expression and cell death (apoptosis) in altered foci of rat liver. *Carcinogenesis* 11, 617-624.

Gescher, A., Pastorino, U., Plummer, S. M. and Manson, M. M. (1998). Suppression of tumour development by substances derived from the diet - mechanisms and clinical implications. *British Journal of Clinical Pharmacology* **45**, 1-12.

Gibson, S., Tu, S., Oyer, R., Anderson, S. M. and Johnson, G. L. (1999). Epidermal growth factor protects epithelial cells against Fas induced apoptosis. *Journal of Biological Chemistry* **274**, 17612-17618.

Graham, S. (1983). Results of case-control studies of diet and cancer in Buffalo, New York. *Cancer Research* 43, 2409s-2413s.

Graham, S., Schotz, W. and Martino, P. (1972). Alimentary factors in the epidemiology of gastric cancer. *Cancer* **30**, 927-938.

GrausPorta, D., Beerli, R. R., Daly, J. M. and Hynes, N. E. (1997). Erbb-2, the preferred heterodimerization partner of all erbb receptors, is a mediator of lateral signaling. The *EMBO Journal* 16, 1647-1655.

Green, M. C., Murray, J. L. and Hortobagyi, G. N. (2000). Monoclonal antibody therapy for solid tumours. *Cancer Treatment Reviews* 26 (4), 269-286.

Grose, K. R. and Bjeldanes, L. F. (1992). Oligomerization of indole-3-carbinol in aqueous acid. *Chemical Research in Toxicology* 5, 188-193.

Grubbs, C. J., Steele, V. E., Casebolt, T., Juliana, M. M., Eto, I., Whitaker, L. M., Dragnev,
K. H., Kelloff, G. J. and Lubet, R. L. (1995). Chemoprevention of chemically-induced
mammary carcinogenesis by indole-3-carbinol. *Anticancer Research* 15, 709-716.

Guan, K. L. and Butch, E. (1995). Isolation and characterization of a novel dual specific phosphatase, HVH2, which selectively dephosphorylates the mitogen-activated protein-kinase. *Journal of Biological Chemistry* **270**, 7197-7203.

Guan, Z. H., Buckman, S. Y., Pentland, A. P., Templeton, D. J. and Morrison, A. R. (1998). Induction of cyclooxygenase-2 by the activated MEKK1->SEK1/MKK4- >p38 mitogenactivated protein kinase pathway. *Journal of Biological Chemistry* **273**, 12901-12908.

Gullick, W. J. (1990). The role of the epidermal growth factor receptor and the c-erbB-2 protein in breast cancer. *International Journal of Cancer* 5, s55-s61.

Gupta, S., Ahmad, N., Nieminen, A. L. and Mukhtar, H. (2000). Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. *Toxicology and Applied Pharmacology* **164**, 82-90.

Hakama, M., Bral, V., Buiath, E., Faivre, J. and Parkin, D. M. (1996). Chemoprevention in cancer control. (IARC Scientific Publications: Lyon.), 1-12.

Han, Z. N., Boyle, D. L., Manning, A. M. and Firestein, G. S. (1998). AP-1 and NF- κ B regulation in rheumatoid arthritis and murine collagen-induced arthritis. *Autoimmunity* **28** (4), 197-208.

Hanif, R., Qiao, L., Shiff, S. J. and Rigas, B. (1997). Curcumin, a natural plant phenolic food additive, inhibits cell proliferation and induces cell cycle changes in colon adenocarcinoma cell lines by a prostoglandin-independent pathway. *Journal of Laboratory and Clinical Medicine*. **130**, 576-584.

Hashimoto, A., Kurosaki, M., Gotoh, N., Shibuya, R. and Kurosaki, T. (1999). Shc regulates epidermal growth factor-induced activation of the JNK signaling pathway. *Journal of Biological Chemistry* **274**, 20139-20143.

He, Y. H. and Schut, H. A. J. (1999). Inhibition of DNA adduct formation of 2-amino-1methyl-6- phenylimidazo[4,5-b]pyridine and 2-amino-3-methylimidazo[4,5-f]quinoline by dietary indole-3-carbinol in female rats. *Journal of Biochemical and Molecular Toxicology* **13**, 239-247. He, Y. H., Smale, M. H. E. and Schut, H. A. J. (1997). Chemopreventive properties of indole-3-carbinol (I3C): inhibition of DNA adduct formation of the dietary carcinogen, 2- amino-1methyl-6- phenylimidazo [4,5-b]pyridine (PhIP), in female F344 rats. *Journal of Cellular Biochemistry* **s27**, 42-51.

Hebert, J. R. and Miller, D. R. (1988). Methodologic considerations for investigating the dietcancer link. *American Journal of Clinical Nutrition* **47**, 1068-1077.

Helal, T. E., Khalifa, A. and Kamel, A. S. (2000). Immunohistochemical expression of p53 and c-erbB2 proteins in breast cancer in Egypt. *Anticancer Research* **20** (**3B**), 2145-2150.

Helzlsouer, K. J., Block, G., Blumberg, J., Diplock, A. T., Levine, M., Marnett, L. J., Schulplein, R. J., Spence, J. T. and Simic, M. G. (1994). Summary of the round-table discussion on strategies for cancer prevention - diet, food, additives, supplements, and drugs. *Cancer Research* **54**, S2044-S2051.

Herdegen, T., Skene, P. and Bahr, M. (1997). The c-jun transcription factor - bipotential mediator of neuronal death, survival and regeneration. *Trends in Neuroscience* **20** (5), 227-231.

Hibasami, H., Komiya, T., Achiwa, Y., Ohnishi, K., Kojima, T., Nakanishi, K., Akashi, K. and Hara, Y. (1998). Induction of apoptosis in human stomach cancer cells by green tea catechins. *Oncology Reports* 5, 527-529.

Hirano, M., Osada, S., Aoki, T., Hirai, S., Hosaka, M., Inoue, J. and Ohno, S. (1996). MEK kinase is involved in tumor necrosis factor alpha-induced NF-kappa B activation and degradation of I kappa B-alpha. *Journal of Biological Chemistry* **271**, 13234-13238.

Hong, W. K. and Sporn, M. B. (1997). Recent advances in chemoprevention of cancer. Science 278, 1073-1077.

Hong, R. L., Spohn, W. H. and Hung, M. C. (1999). Curcumin inhibits tyrosine kinase activity of p185^{neu} and also depletes p185^{neu1}. *Clinical Cancer Research* 5, 1884-1891.

Howe, L. R., Leevers, S. J., Gomez, N., Nakielny, S., Cohen, P. and Marshall, C. J. (1992). Activation of the MAP kinase pathway by the protein kinase Raf. *Cell* **71**, 335-342.

Hromas, R., Hufford, M., Sutton, J., Xu, D. W., Li, X. X. and Lu, L. (1997). PLAB, a novel placental bone morphogenic protein. *Biochimica et Biophysica Acta* **1354** (1), 40-44.

Hsu, H., Xiong, J. and Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kB activation. *Cell* **81**, 495-504.

Huang, M. T., Lysz, T., Ferraro, T., Abidi, T. F., Laskin, J. D. and Conney, A. H. (1991). Inhibitory effects of curcumin on *in vitro* lipoxygenase and cyclooxygenase activities in mouse epidermis. *Cancer Research* **51**, 813-819.

Huang, M.T., Wang, Z. Y., Geargiadis, A, Laskin, J. D. and Conney, A. H. (1992). Inhibitory effects of curcumin on tumour initiation by benzo[a]pyrene and 7,12-diethyl benz[a]anthracene. *Carcinogenesis* **13**, 2183-2186.

Huang, M. T., Lou, Y. R., Newmark, H. L., Reuhl, K. R. and Conney, A. H. (1994). Inhibitory effects of dietary curcumin on forestomach, duodenal and colon carcinogenesis in mice. *Cancer Research* 54, 5841-5847.

Huang, M. T., Newmark, H. L. and Frenkel, K. (1997). Inhibitory effects of curcumin on tumorigenesis in mice. *Journal of Cellular Biochemistry* **s27**, 26-34.

Hunter, T. (2000). Signalling-2000 and beyond. Cell 100, 113-127.

Huppertz, B., Kertschanska, S., Demir, A. Y., Frank, H. and Kaufmann, P. (1998). Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. *Cell Tissue Research* **291**, 133-148.

Ichijo, H. (1999). From receptors to stress activated MAP kinases. Oncogene 18, 6087-6093.

Ichijo, H., Nishida, E., Irie, K., Dijkke, P. T., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K. and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signalling pathways. *Science* 275, 90-94.

Itoh, H. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**, 233-243.

Jakobsson, P., Thoren, S., Morgenstern, R. and Samuelsson, B. (1999). Identification of human prostoglandin E synthase: A microsomal, glutathione dependent, inducible enzyme, constituting a potential novel drug target. *Proceedings of the National Academy of Sciences* (USA) **96**, 7220-7225.

Jiang, G. and Hunter, T. (1999). Receptor signalling: When dimerisation is not enough. *Current Biology* 9, R568-R571.

Jiang, M. C., Yang-Yen, H., Yen, J. and Lin, J. (1996). Curcumin induces apoptosis in immortalised NIH3T3 and malignant cancer cell lines. *Nutrition and Cancer* 26, 111-120.

Jin, L., Qi, M., Chen, D. Z., Anderson, A., Yang, G. Y., Arbeit, J. M. and Auborn, K. J. (1999). Indole-3-carbinol prevents cervical cancer in human papilloma virus type 16 (HPV16) transgenic mice. *Cancer Research* **59**, 3991-3997.

Jobin, C., Bradham, C. A., Russo, M. P., Juma, B., Narula, A. S., Brenner, D. A. and Sartor, R. B. (1999). Curcumin blocks cytokine mediated NF-kB activation and proinflammatory gene expression by inhibiting inhibitory factor IkB kinase activity. *Journal of Immunology* **163**, 3474-3483.

Johnson, G. L. and Vaillancourt, R. R. (1994). Sequential protein-kinase reactions controlling cell-growth and differentiation. *Current Opinion in Cell Biology* **6**, 230-238.

Kallioniemi, O. P., Kallioniemi, A., Kurisu, W., Thor, A., Chen, L. C., Smith, H. S., Waldman, F. M., Pinkel, D. and Gray, J. W. (1992). Erb-B2 amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proceedings of the National Academy of Sciences (USA)* **89**, 5321-5325.

Karin, M. and Hunter, T. (1995). Transcriptional control by protein-phosphorylation - signal transmission from the cell-surface to the nucleus. *Current Biology* **5**, 747-757.

Katiyar, S. K., Agarwal, R. and Mukhtar, H. (1994). Inhibition of spontaneous and photoenhanced lipid peroxidation in mouse epidermal microsomes by epicatechin derivatives from green tea. *Cancer Letters* **79**, 61-66. Kawamori, Lubet, R., Steele, V. E., Kellof, G. J., Kaskey, R. B., Rao, C. V. and Reddy, B. S. (1999). Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. *Cancer Research* **59**, 597-601.

Keilhack, H., Tenev, T., Nyakatura, E., Godovac-Zimmermann, J., Nielson, L., Seedorf, K. and Bohmer, F. D. (1998). Phosphotyrosine 1173 mediates binding of the protein-tyrosine phosphatase SHP-1 to the epidermal growth factor receptor and attenuation of receptor signalling. *Journal of Biological Chemistry* **273**, 24839-24846.

Kelloff, G. J., Boone, C. W., Crowell, J. A., Steele, V. E., Lubet, R. A., Doody, L. A., Malone, W. F., Hawk, E. T. and Sigman, C. C. (1996). New agents for cancer chemoprevention. *Journal of Cellular Biochemistry* **s26**, 1-28.

Kelloff, G. J., Crowell, J. A., Steele, V. E., Lubet, R. A., Boone, C. W., Malone, W. A., Hawk, E. T., Lieberman, R., Lawrence, J. A., Kopelovich, L., Ali, I., Viner, J. L. and Sigman, C. C. (1999). Progress in cancer chemoprevention. *Annals of the New York Academy of Science* **889**, 1-13.

Kennedy, D. O., Nishimura, S., Hasuma, T., Yano, Y., Otani, S. and Matsui-Yuasa, I. (1998). Involvement of protein tyrosine phosphorylation in the effect of green tea polyphenols on Ehrlich ascites tumour cells *in vitro*. *Chemico-Biological Interactions* **110**, 159-172.

Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics. *British Journal of Cancer* 26, 239-256.

Khafif, A., Schantz, S. P., Chou, T., Edelstein, D. and Sacks, P. G. (1998). Quantitation of chemopreventive synergism between EGCG and curcumin in normal, premalignant and malignant human oral epithelial cells. *Carcinogenesis* **19**, 419-424.

Khan, S. G., Katiyar, S. K., Agarwal, R. and Mukhtar, H. (1992). Enhancement of antioxidant and phase II enzymes by oral feeding of green tea polyphenols in drinking water to SKH-1 hairless mice: Possible role in cancer chemoprevention. *Cancer Research* **52**, 4050-4052.

Knowlden, J. M., Gee, J. M. W., Seery, L. T., Farrow, L., Gullick, W. J., Ellis, I. O., Blamey, R. W., Robertson, J. F. R. and Nicholson, R. I. (1998). c-erbB3 and c-erbB4 expression is a

feature of the endocrine responsive phenotype in clinical breast cancer. *Oncogene* 17, 1949-1957.

Kolibaba, K. S. and Druker, B. J. (1997). Protein tyrosine kinases and cancer. *Biochimica et Biophysica Acta-Reviews on Cancer* 1333, F217-F248.

Korutla, L., Cheung, J., Mendelsohn, J. and Kurmar, R. (1995). Inhibition of ligand induced activation of EGFR tyrosine phosphorylation by curcumin. *Carcinogenesis* **16**, 1741-1745.

Korutla, L. and Kurmar, R. (1994). Inhibitory efect of curcumin on EGF receptor kinase activity in A431 cells. *Biochimica et Biophysica Acta* 1224, 597-600.

Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. and Aaronson, S. A. (1989). Isolation and characterisation of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: Evidence for overexpression in a subset of human mammary tumours. *Proceedings of the National Academy of Sciences (USA)* **86**, 9193-9197.

Kuo, M. L., Huang, T. S. and Lin, J. K. (1996). Curcumin, an antioxidant and anti-tumour promoter, induces apoptosis in human leukemia cells. *Biochimica et Biophysica Acta* 1317, 95-100.

Kuroda, Y. and Hara, Y. (1999). Anti mutagenic and anticarcinogenic activity of tea polyphenols. *Mutation Research* **436**, 69-97.

Kyriakis, J. M. (1999). Making the connection: coupling of stress-activated erk/mapk (extracellular-signal-regulated kinase mitogen-activated protein kinase) core signalling modules to extracellular stimuli and biological responses. *Biochemical Society Symposium* 29-48.

Kyriakis, J. M., App, H., Zhang, X., Banerjee, P., Brautigan, D. L., Rapp, U. R. and Avruch, J. (1992). Raf-1 activates MAP kinase-kinase. *Nature* **358**, 417-421.

Landis, R. J. (1999). Cancer Statistics 49, 8-31.

Lee, F. S., Hagler, J., Chen, Z. J. and Maniatis, T. (1997). Activation of the IkBα kinase complex by MEKK1, a kinase of the JNK pathway. *Cell* 88, 213-222.

Leppa, S. and Bohmann, D. (1999). Diverse functions of JNK signalling and c-jun in stress response and apoptosis. *Oncogene* 18, 6158-6162.

Liang, Y. C., Lin-Shiau, S. Y., Chen, C. F. and Lin, J. K. (1999a). Inhibition of cyclin dependent kinases 2 and 4 activities as well as induction of CDK inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by epigallocatechin-3-gallate. *Journal of Cellular Biochemistry* **75**, 1-12.

Liang, Y. C., Chen, Y. C, Lin, Y. L., Lin-Shiau, S. Y., Ho, C. T. and Lin, J. K. (1999b). Suppression of extracellular signals and cell proliferation by the black tea polyphenol, theaflavin-3,3-digallate. *Carcinogenesis* **20**, 733-736.

Liang, Y. C., Linshiau, S. Y., Chen, C. F. and Lin, J. K. (1997). Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *Journal of Cellular Biochemistry* **67**, 55-65.

Lin, A., Minden, A., Marineto, H., Claret, F. X., Lange-Carter, C., Mercurio, F., Johnson, G. L. and Karin, M. (1995). Identification of a dual specificity kinase that activates the jun kinases and p38-MPK2. *Science* **268**, 286-290.

Lin, J. K., Chen, Y. C., Huang, Y. T. and Lin-Shiau, S. Y. (1997). Suppression of protein kinase C and nuclear oncogene expression as possible molecular mechanisms of cancer chemoprevention by apigenin and curcumin. *Journal of Cellular Biochemistry* **28/29**, 39-48.

Lippman, S. M., Lee, J. J., Lotan, R., Hittelman, W. Wargovich, M. J. and Hong, W. K. (1990). Biomarkers as endpoints in chemoprevention trials. *Journal of the National Cancer Institute* **82**, 555-560.

Liu, J., Lin, S. and Lin, J. (1993). Inhibitory effects of curcumin on protein kinase C activity induced by 12-O-tetradecanoyl-phorbol-13-acetate in NIH 3T3 cells. *Carcinogenesis* 14, 857-861.

Liu, F. and Chernoff, J. (1997). Protein tyrosine phosphatase 1B interacts with and is tyrosine phosphorylated by the epidermal growth factor receptor. *Biochemical Journal* **327**, 139-145.

Lockhart, D. J. and Winzler, E. A. (2000). Genomics, gene expression and DNA arrays. *Nature* 405, 827-836.

Lu, Y. P., Chang, R. L., Huang, M. and Conney, A. H. (1993). Inhibitory effect of curcumin on 12-O-tetradecanoylphorbol-13-acetate induced increase in ornithine decarboxylase mRNA in mouse epidermis. *Carcinogenesis* 14, 293-297.

Lu, L. H., Lee, S. S. and Huang, H. C. (1998). Epigallocatechin suppression of proliferation of vascular smooth muscle cells: Correlation with c-jun and JNK. *British Journal of Pharmacology* **124** (6), 1227-1237.

Luo, Z., Tzivion, G., Belshaw, P. J., Vavvas, D., Marshall, M. and Avruch, J. (1996). Oligomerization activates c-Raf-1 through a Ras dependent mechanism. *Nature* **383**, 181-185.

Maines, M. D. (1997). The heme oxygenase system: a regulator of second messenger gases. Annual Review of Pharmacology and Toxicology 37, 517-554.

Malloy, V. L., Bradlow, H. L. and Orentreich, N. (1997). Interaction between a semisynthetic diet and indole-3-carbinol on mammary tumor incidence in Balb/Cfc3h mice. *Anticancer Research* **77**, 4333-4337.

Manson, M. M., Ball, H. L., Barrett, M., Clark, H., Judah, D., Williamson, G. and Neal, G. (1997). Mechanism of action of dietary chemopreventive agents in rat liver: Induction of phase I and II drug metabolizing enzymes and aflatoxin B1 metabolism. *Carcinogenesis* 18, 1729-1738.

Manson, M. M., Hudson, E. A., Ball, H. L., Barrett, M., Clark, H., Judah, D., Verschoyle, R. D. and Neal, G. E. (1998). Chemoprevention of aflatoxin B1-induced carcinogenesis by indole-3-carbinol in rat liver - predicting the outcome using early biomarkers. *Carcinogenesis* **19**, 1829-1836.

Manson, M. M., Gescher, A., Hudson, E. A., Plummer, S. M., Squires, M. S. and Prigent, S. A. (2000). Blocking and suppressing mechanisms of chemoprevention by dietary constituents. *Toxicology Letters* **112-113**, 499-505.

Marais, R., Light, Y., Paterson, H. F. and Marshall, C. J. (1995). Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *The EMBO Journal* 14 (13), 3136-3145.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179-185.

Marte, B. M. and Downward, J. (1997). PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends in Biochemical Science* 22, 355-358.

Marushige, K. and Marushige, Y. (1999). Changes in the mitogen activated protein kinase and phosphatidylinositol 3-kinase/Akt signalling associated with the induction of apoptosis. *Anticancer Research* **19**, 3865-3872.

Massova, I., Kotra, L. P., Fridman, R. and Mobashery, S. (1998). Matrix metalloproteinases: Structures, evolution and diversification. *FASEB* 12, 1075-1095.

McGuire, W. (1978). Hormone receptors: their role in predicting prognosis and response to endocrine therapy. *Seminars in Oncology* **5** (4), 428-433.

Mehta, K., Pantazis, P., McQueen, T. and Aggarwal, B. (1997). Antiproliferative effect of curcumin against human breast tumour cell lines. *Anti-Cancer Drugs* **8**, 470-481.

Merchant, W. J., Millis, R. R., Smith, P., Chaudary, M. A. and Barnes, D. M. (1999). Expression of c-erbB2 and p53 protein is similar in breast cancer from British and Japanese women. *International Journal of Cancer* 84 (3), 278-283.

Mercurio, F. and Manning, A. M. (1999). NF-kappa B as a primary regulator of the stress response. Oncogene 18, 6163-6171.

Meyer, C. F., Wang, X. P., Chang, C., Templeton, D. and Tan, T. H. (1996). Interaction between c-rel and the mitogen-activated protein kinase kinase kinase 1 signaling cascade in mediating kappa B enhancer activation. *Journal of Biological Chemistry* **271**, 8971-8976.

Michnovicz, J. J. and Bradlow, H. L. (1990). Induction of estradiol metabolism by dietary indole-3-carbinol in humans. *Journal of the National Cancer Institute* **82**, 947-949.

Michnovicz, J. J. and Bradlow, H. L. (1991). Altered estrogen metabolism and excretion in humans following consumption of indole-3-carbinol. *Nutrition and Cancer* 16, 59-66.

Mohan, R., Sivak, J., Russo, L. A., Pham, B. Q., Kasahara, N., Raizman, M. B. and Fini, M. E. (2000). Curcumoids inhibit the angiogenic response stimulated by fibroblast growth factor-

2, including expression of matrix metalloproteinase gelatinase B. Journal of Biological Chemistry 275, 10405-10412.

Morgan, D. O. (1995). Principles of CDK regulation. Nature 374, 131-134.

Mosselman, S., Polman, J. and Dijkema, R. (1996). ERβ Identification and characterization of a novel human estrogen receptor. *FEBS* **392**, 49-53.

Motterlini, R., Foresti, R., Bassi, R. and Green, C. J. (2000). Curcumin, an antioxidant and antiinflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. *Free Radical Biology and Medicine*. **28** (8), 1303-1312.

Mulcahy, L. S., Smith, M. R. and Stacey, D. W. (1985). Requirement for ras protooncogene function during serum stimulated growth of NIH3T3 cells. *Nature* **313**, 241-243.

Musti, A. M., Treier, M. and Bohmann, D. (1997). Reduced ubiquitin-dependent degradation of c-jun after phosphorylation by MAP kinases. *Science* **275**, 400-402.

Nakachi, K., Suemasu, K., Suga, K., Takeo, T., Imai, K. and Higashi, Y. (1998). Influence of drinking green tea on breast cancer malignancy among japanese patients. *Japanese Journal of Cancer Research* **89**, 254-261.

Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Wiegel, R. J. and Roth, R. A. (1999). Up-regulation of Akt3 in estrogen receptor deficient breast cancers and androgen - independent prostate cancer cell lines. *Journal of Biological Chemistry* **274**, 21528-21532.

Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J. and Sledge, G. W. (1997). Constitutive activation of NF-kB during progression of breast cancer to hormone-independent growth. *Molecular and Cellular biology* **17**, 3629-3639.

Ni, H., Wang, X. H. S., Diener, K. and Yao, Z. B. (1998). MAPKAPK5, a novel mitogenactivated protein kinase (MAPK)- activated protein kinase, is a substrate of the extracellularregulated kinase (ERK) and p38 kinase. *Biochemical and Biophysical Research Communications* 243, 492-496.

Niwa, T., Swaneck, G. and Bradlow, H. L. (1994). Alterations in estradiol metabolism in MCF-7 cells induced by treatment with indole-3-carbinol and related-compounds. *Steroids* **59**, 523-527.

Nutt, J. E. and Lunec, J. (1996). Induction of metalloproteinase (MMP1) expression by epidermal growth factor (EGF) receptor stimulation and serum deprivation in human breast tumour cells. *European Journal of Cancer* **32A** (12), 2127-2135.

O Conner, T., Ireland, L. S., Harrison, D. J. and Hayes, J. D. (1999). Major differences exist in the function and tissue-speific expression of human aflatoxin B_1 aldehyde reductase and the principle human aldo-ketoreductase AKR1 family members. *Biochemical Journal* **343**, 487-504.

Oetari, S., Sudibyo, M., Commandeur, J. N. M., Samhoedi, R. and Vermeulen, N. P. E. (1996). Effects of curcumin on cytochrome P450 and glutathione S-transferase activities in rat liver. *Biochemical Pharmacology* **51**, 39-45.

Olson, M. F., Paterson, H. F. and Marshall, C. J. (1998). Signals from ras and rho GTPases interact to regulate expression of p21(waf1/cip1). *Nature* **394**, 295-299.

Oltavai, Z. N., Milliman, C. L. and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74, 609-619.

Omenn, G. S., Goodman, G. E., Thornquist, M. D., Balmes, J., Cullen, M. R., Glass, A., Keogh, J. P., Meyskens, F. L., Valanis, B., Williams, J. H., Barnhart, S. and Hammar, S. (1996). Effects of a combination of Beta carotene and vitamin A on lung cancer and cardiovascular disease. *The New England Journal of Medicine* **334** (18), 1150-1155.

Osborne, M. P. (1997). Cancer Prevention. The Lancet s111, 27-30.

Osborne, M. P. (1999). Breast cancer prevention by antiestrogens. Annals of the New York Academy of Science 889, 146-151.

Ozes, O.N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R. and Donner, D. B. (1999). NF-kB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **401**, 82-85.

Parrett, M. L., Harris, R. E., Joarder, F. S., Ross, M. S., Clausen, K. P. and Robertson, F. M. (1997). Cyclooxygenase gene expression in human breast cancer. *International Journal of Oncology* **10**, 530-537.

Parsons, J. T. and Parsons, S. J. (1997). Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. *Current Opinion in Cell Biology* **9**, 187-192.

Paschka, A. G., Butler, R. and Young, C. Y. F. (1998). Induction of apoptosis in prostate cancer cell lines by the green tea component, (-)-epigallocatechin-3-gallate. *Cancer Letters* **130**, 1-7.

Pawson, T. and Scott, M. A. (1997). Signalling through scaffold, anchoring and adapter proteins. *Cell* **71**, 359-362.

Pendas, A. M., Balbin, M., Llano, E., Jimenez, M. G. and Lopezotin, C. (1997). Analysis and promoter characterization of the human collagenase-3 gene (MMP13). *Genomics* **40** (2), 222-233.

Penning, T. M., Burczynski, M. E., Jez, J. M., Hung, C., Lin, H., Ma, H., Moore, M., Palackal, N. and Ratnam, K. (2000). Human 3α -hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochemical Journal* **351**, 67-77.

Pereira, M. A., Grubbs, C. J., Barnes, L. H., Li, H., Olson, G. R., Eto, I., Juliana, M., Whitaker, L. M., Kelloff, G. J., Steele, V. E. and Lubet, R. A. (1996). Effects of the phytochemicals, curcumin and quercetin, upon azoxymethane-induced colon cancer and 7,12-dimethylbenz[a]anthracene- induced mammary cancer in rats. *Carcinogenesis* 17, 1305-1311.

Pingzhang, Y., Jinying, Z., Shujun, C., Hara, Y., Qingfan, Z. and Zhengguo, L. (1994). Experimental studies of the inhibitory effects of green tea catechin on mice large intestinal cancers induced by 1,2-dimethylhydrazine. *Cancer Letters* **79**, 33-38.

Piper, J. T., Singhal, S. S., Salameh, M. S., Torman, R. T., Awasthi, Y. C. and Awasthi, S. (1998). Mechanisms of anticarcinogenic properties of curcumin: the effect of curcumin on glutathione linked detoxification enzymes in rat liver. *The International Journal of Biochemistry and Cell Biology* **30**, 445-456.

Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G. and Shoyab, M. (1993). Ligand-specific activation of her4/p180(erbb4), a
4th member of the epidermal growth-factor receptor family. *Proceedings of the National* Academy of Sciences (USA) **90**, 1746-1750.

Plummer, S. M., Holloway, K. A., Manson, M. M., Munks, R. J. L., Kaptein, A., Farrow, S. and Howells, L. (1999). Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kB activation via the NIK/IKK signalling complex. *Oncogene* **18**, 6013-6020.

Polette, M., Clavel, C., Cockett, M., Debentzmann, S. G., Murphy, G. and Birembaut, P. (1993). Detection and localization of messenger-RNAs encoding matrix metalloproteinases and their tissue inhibitors in human breast pathology. *Invasion and Metastasis* **13** (1), 31-37.

Pomerance, M., Multon, M. C., Parker, F., Venot, C., Blondeau, J. P., Tocque, B. and Schweighoffer, F. (1998). Grb2 interaction with MEK-kinase 1 is involved in regulation of jun-kinase activities in response to epidermal growth factor. *Journal of Biological Chemistry* **273**, 24301-24304.

Prasad, K. A. N. and Church, J. G. (1991). EGF dependent growth inhibition in MDA 468 human breast cancer cells is characterised by late G1 arrest and altered gene expression. *Experimental Cell Research* **195**, 20-26.

Prasad, K. A. N. and Church, J. G. (1997). EGF effects on p53 in MDA-468 human breast cancer cells: implications for G(1) arrest. *Cell Proliferation* **30**, 81-94.

Qin, X., Livingston, D. M., Ewen, M., Sellers, W. R., Arany, Z. and Kaelin, W. G. (1995). The transcription factor E2F-1 is a downstream target of Rb action. *Molecular and Cellular Biology* **15** (2), 742-755.

Ramachandran, C. and You, W. (1999). Differential sensitivity of human mammary epithelial and breast carcinoma cell lines to curcumin. *Breast Cancer Research and Treatment*. **54**, 269-278.

Rao, C. V., Rivenson, A., Simi, B. and Reddy, B. S. (1995a). Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally-occurring plant phenolic compound. *Cancer Research* **55**, 259-266.

Rao, C. V., Rivenson, A., Simi, B. and Reddy, B. S. (1995b). Chemoprevention of colon cancer by dietary curcumin. *Annals of the New York Academy of Sciences* **768**, 201-204.

Reddy, K. B., Keshamouni, V. G. and Chen, Y. Q. (1999). The level of tyrosine kinase activity regulates the expression of p21/WAF1 in cancer cells. *International Journal of Oncology* 15, 301-306.

Reed, J. C. (1994). Bcl-2 and the regualtion of programmed cell death. *The Journal of Cellular Biology* **124**, 1-6.

Riby, J. E., Feng, C. L., Chang, Y. C., Schaldach, C. M., Firestone, G. L. and Bjeldanes, L. F. (2000). The major cyclic trimeric product of indole-3-carbinol is a strong agonist of the estrogen receptor signaling pathway. *Biochemistry* **39**, 910-918.

Robinson, M. J. and Cobb, M. H. (1997). Mitogen-activated protein kinase pathways. *Current Opinion in Cell Biology* 9, 180-186.

Rolland, P. H., Martin, P. M., Jaquemeir, J., Rolland, A. M. and Toga, M. (1980). Prostaglandin in human breast cancer: evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. *Journal of the National Cancer Institute* **64**, 1061-1070.

Romashkova, J. A. and Makarov, S. S. (1999). NF-kB is a target of Akt in anti-apoptotic PDGF signalling. *Nature* **401**, 86-89.

Rosen, C. A., Woodson, G. E., Thompson, J. W., Hengesteg, A. P. and Bradlow, H. L. (1998). Preliminary results of the use of indole-3-carbinol for recurrent respiratory papillomatosis. *Otolaryngology-Head and Neck Surgery* **118** (6), 810-815.

Rothe, M., Wong, S. C., Henzel, W. J. and Goeddel, D. V. (1994). A novel family of putative signal transducers associated with the cytoplasmic domain of the 75kDa tumour necrosis factor receptor. *Cell* **78**, 681-692.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A. R. (1994). A novel cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027-1037.

199

Rubio, I., RodriguezViciana, P., Downward, J., and Wetzker, R. (1997). Interaction of ras with phosphoinositide 3-kinase gamma. *Biochemical Journal* **326**, 891-895.

Sainsbury, J. R. C., Farndon, J. R., Needham, G. K, Malcom, A. J. and Harris, A. L. (1987). Epidermal growth factor receptor status as a predictor of early recurrance of and death from breast cancer. *The Lancet* 1398-1402.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). In 'Molecular Cloning: A laboratory manual'. (Cold Spring Harbour Press: New York).

Sarraf, C. E., Ansari, T. W., Conway, P., Notay, N., Hill, S. and Alison, M. R. (1993). Bromodeoxyuridine-labelled apoptosis after treatment with antimetabolites in two murine tumours and in small intestinal crypts. *British Journal of Cancer* **68**, 678-680.

Sawyer, C., Hiles, I., Page, M., Compton, M. and Dean, C. (1998). Two erbB4 transcripts are expressed in normal breasts and in most breast cancer. *Oncogene* 17, 919-924.

Schaerli, P. and Jaggi, R. (1998). EGF induced programmed cell death of human mammary carcinoma MDA-MB-468 cells is preceded by activation of AP-1. *Cellular and Molecular Life Sciences*. **54**, 129-138.

Schiffman, M. H., Pickle, L. W., Fontham, E., Zahm, S. H., Falk, R., Mele, J., Correa, P. and Fraumeni, J. F. (1988). Case-control study of diet and mesothelioma in Louisiana. *Cancer Research* **48**, 2911-2915.

Schrey, M. P. and Patel, K. V. (1995). Prostaglandin E2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators. *British Journal of Cancer* 72, 1412-1419.

Shapiro, P. S. and Ahn, N. G. (1998). Feedback regulation of raf-1 and mitogen-activated protein kinase (MAP) kinase kinases 1 and 2 by map kinase phosphatase-1 (MKP-1). *Journal of Biological Chemistry* **273**, 1788-1793.

Sharma, S., Stutzman, J. D., Kelloff, G. and Steele, V. (1994). Screening of potential chemopreventive agents using biochemical markers of carcinogenesis. *Cancer Research* 54, 5848-5855.

Sherr, C. J. (1996). Cancer cell cycles. Science 274, 1672-1677.

Shi, S. T., Wang, Z. Y., Smith, T. J., Hong, J. T., Chen, W. Y., Ho, C. T. and Yang, C. S. (1994). Effects of green tea and black tea on 4-(methylnitrosamino)-1-(3-pyriyl)-1-butanone bioactivation, DNA methylation and lung tumourigenesis in A/J mice. *Cancer Research* 54, 4641-4647.

Shin, D. Y., Ishibashi, T., Choi, T. S., Chung, E. M., Chung, I. Y., Aaronson, S. A. and Bottaro, D. P. (1997). A novel human ERK phosphatase regulates h-ras and v-raf signal transduction. *Oncogene* 14, 2633-2639.

Shureiqi, I., Reddy, P. and Brenner, D. E. (2000). Chemoprevention: general perspective. *Critical Reviews in Oncology Hematology* **33**, 157-167.

Simon, A., Allais, D. P., Duroux, J. L., Basly, J. P., Durand-Fontanier, S. and Delage, C. (1998). Inhibitory effect of curcumoids on MCF-7 cell proliferation and structure activity relationships. *Cancer Letters* **129**, 111-116.

Singh, S. V., Hu, X., Srivastava, S. K., Singh, M., Hong, X., Orchard, J. L. and Zaren, H. A. (1998). Mechanism of inhibition of benzo[a]pyrene-induced forestomach cancer in mice by dietary curcumin. *Carcinogenesis* **19**, 1357-1360.

Singletary, K., MacDonald, C., WAllig, M. and Fisher, C. (1996). Inhibition of 7, 12dimethylbenz[a]anthracene (DMBA) induced mammary tumourigenesis and DMBA-DNA adduct formation by curcumin. *Cancer Letters* **103**, 137-141.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and Mcguire, W. L. (1987). Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235, 177-182.

Slee, E. A., Adrain, C. and Martin, S. J. (1999). Serial killers: ordering caspase activation events in apoptosis. *Cell Death and Differentiation* **6**, 1067-1074.

Smith, P. K, Krohn, R. I., Hermanson, G. T., Malia, A. K., Gartner, F. H, Provenzano, M. D., Fujimoto, E. K., Goeke, M. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bichinchoninic acid. *Analytical Biochemistry* **150**, 76-85.

Srivastava, B. and Shukla, Y. (1998). Anti tumour promoting activity of indole-3-carbinol in mouse skin carcinogenesis. *Cancer Letters* **134**, 91-95.

Stebbing, J., Copson, E. and OReilly, S. (2000). Herceptin (trastuzamab) in advanced breast cancer. *Cancer Treatment Reviews* **26** (4), 287-290.

Steller, H. (1995). Mechanisms and genes of cellular suicide. Science 267, 1445-1449.

Stoewsand, G. S., Anderson, J. L. and Munson, L. (1988). Protective effect of dietary brussels sprouts against mammary carcinogenesis in Sprague-Dawley rats. *Cancer Letters* **39**, 199-207.

Stoewsand, G. S., Anderson, J. L. Munson, L., and Lisk, D. (1989). Effect of dietary brussels sprouts with increased selinium content on mammary carcinogenesis in the rat. *Cancer Letters* **45**, 43-48.

Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R. J., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. and Hawkins, P. T. (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277**, 567-570.

Suganuma, M., Okabe, S., Kai, Y., Sueoka, N., Sueoka, E. and Fujiki, H. (1999). Synergistic effects of EGCG with epicatechin, sulindac or tamoxifen on cancer chemopreventive activity in the human lung cancer cell line PC-9. *Cancer Research* **59**, 44-47.

Tagliabue, E., Menard, S., Robertson, J. F. R. and Harris, L. (1999). c-erbB-2 expression in primary breast cancer. *International Journal of Biomarkers* 14 (1), 16-26.

Taioli, E., Garbers, S., Bradlow, H. L., Carmella, S. G., Akerkar, S. and Hecht, S. S. (1997). Effects of indole-3-carbinol on the metabolism of 4- (methylnitrosamino)1-(3-pyridyl)-1-butanone in smokers. *Cancer Epidemiology Biomarkers & Prevention* **6**, 517-522.

Tanaka, T., Kojima, T., Morishita, Y. and Mori, H. (1992). Inhibitory effects of the natural products indole-3-carbinol and sinigrin during initiation and promotion phases of 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. *Japanese Journal of Cancer Research* **83**, 835-842.

Tartaglia, L. A., Weber, R. F., Figari, I. S., Reynolds, C., Palladino, M. A. and Goeddel, D. V. (1991). The different receptors for tumour necrosis factor mediate distinct cellular responses. *Proceedings of the National Academy of Sciences (USA)* **88**, 9292-9296.

Telang, N. T., Katdare, M., Bradlow, H. L., Osborne, M. P. and Fishman, J. (1997). Inhibition of proliferation and modulation of estradiol metabolism: novel mechanisms for breast cancer prevention by the phytochemical indole-3-carbinol. *Proceedings of the Society for Experimental Biology and Medicine* **216**, 246-252.

The Alpha Tocopherol, Beta Carotene Cancer Prevention Study Group (1994). The effect of vitamin E and Beta carotene on the incidence of lung cancer and other cancers in male smokers. *The New England Journal of Medicine* **330** (15), 1029-1035.

Tiwari, R. K., Guo, L., Bradlow, H. L., Telang, N. T. and Osborne, M. P. (1994). Selective responsiveness of human breast-cancer cells to indole- 3- carbinol, a chemopreventive agent. *Journal of the National Cancer Institute* **86**, 126-131.

Toi, M., Nakamura, T., Mukaida, H., Wada, T., Osaki, A., Yamada, H., Toge, T., Niimoto, M. and Hattori, T. (1990). Relationship between epidermal growth factor receptor status and various prognostic factors in human breast cancer. *Cancer* **65**, 1980-1984.

Ullrich, A. (1984). Human EGFR cDNA sequence and abberant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* **309**, 418-425.

Van den Heuvel, S. and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262**, 2050-2054.

Van der Geer, P., Hunter, T. and Lindberg, R. A. (1994). Receptor protein tyrosine kinases and their signal transduction pathways. *Annual Review of Cell Biology* **10**, 251-337.

Van der Geer, P. and Pawson, T. (1995). The PTB domain: a new protein module implicated in signal transduction. *Trends in Biochemical Science* **20**, 277-280.

Vaux, D. L., Cory, S. and Adams, J. M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335**, 440-442.

Veis, D., Sorenson, C. M., Shutter, J. R. and Korsmeyer, S. J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys and hypopigmented hair. *Cell* **75**, 229-240.

Vogt, U., Bielawski, K., Schlotter, C. M., Bosse, U., Falkiewicz, B., and Podhajska, A. J. (1998). Amplification of erbB-4 oncogene occurs less frequently than that of erbB-2 in primary human breast cancer. *Gene* **223**, 375-380.

Wang, Z. Y., Agarwal, R., Khan, W. A. and Mukhtar, H. (1992). Protection against benzo[a]pyrene and N-nitrosodiethylamine-induced lung and forestomach tumourigenesis in A/J mice by water extracts of green tea and licorice. *Carcinogenesis* **13**, 1491-1494.

Wattenburg, L. W. (1978). Inhibition of chemical carcinogenesis. Journal of the National Cancer Institute 60, 11-18.

White, M. A., Nicolette, C., Minden, A., Polverino, A., Van Aeist, L., Karin, M. and Wigler,
M. H. (1995). Multiple ras functions can contribute to mammalian cell transformation. *Cell* 80, 533-541.

Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J. and Davis, R. J. (1998). A mammalian scaffold complex that selectively mediates MAP kinase activation. *Science* 281, 1671-1674.

Williams, N. G. and Roberts, T. M. (1994). Signal-transduction pathways involving the raf protooncogene. *Cancer and Metastasis Reviews* **13**, 105-116.

Wilson, L. K., Luttrell, D. K., Parsons, J. T. and Parsons, S. J. (1989). pp60^{c-src} tyrosine kinase, myristylation and modulatory domains are required for enhanced mitogenic responsiveness to epidermal growth factor seen in cells overexpressing c-src. *Molecular and Cellular Biology* **9** (4), 1536-1544.

Wisdom, R., Johnson, R. S. and Moore, C. (1999). c-jun regulates cell cycle progression and apoptosis by distinct mechanisms. The *EMBO Journal* **18** (1), 188-197.

Wittinghofer, A. and Herrmann, C. (1995). Ras-effector interactions, the problem of specificity. *FEBS Letters*. **369**, 52-56.

Wittinghofer, A. and Nassar, N. (1996). How ras-related proteins talk to their effectors. *Trends In Biochemical Sciences* **21**, 488-491. Wong, G., Bradlow, L., Sepkovic, D., Mehl, S., Mailman, J. and Osborne, M. P. (1997). Dose ranging study of indole-3-carbinol for breast cancer prevention. *Journal of Cellular Biochemistry* 28/29, 111-116.

World Cancer Research Fund (1997). Food, nutrition and the prevention of cancer: A global perspective. (American Insitute for Cancer Research: Washington.), chapters 1 and 2.

Wyllie, A. H. (1980). Glucocorticoid-induced thymocyte apoptosis is induced with endogenous endonuclease activity. *Nature* 284, 555-556.

Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T. and Toyoshima, K. (1986). Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* **319**, 230-234.

Yamane, T., Takahashi, T., Kuwata, K., Oya, K., Inagake, M., Kitao, Y., Suganuma, M. and Fujiki, H. (1995). Inhibition of N-methyl-N-nitro-N-nitrosoguanidine-induced carcinogenesis by epigallocatechin gallate in the rat glandular stomach. *Cancer Research* **55**, 2081-2084.

Yang, D., Tournier, C., Wysk, M., Lu, H. T., Xu, J., Davis, R. J. and Flavell, R. A. (1997). Targetted distribution of the MKK4 gene causes embryonic death, inhibition of c-jun NH₂terminal kinase activation and defects in AP-1 transcriptional activity. *Proceedings of the National Academy of Sciences (USA)* **94**, 3004-3009.

Yang, G., Liao, J., Kim, K., Yurkow, E. J. and Yang, C. S. (1998). Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* 58, 1920-1929.

Yang, S. and Zhi-Yuan, W. (1993). Tea and cancer. Journal of the National Cancer Institute 85 (13), 1038-1049.

Yarden, Y. (1988). Growth factor receptor tyrosine kinases. Annual Review of Biochemistry. 57, 443-478.

Young, R. A. (2000). Biomedical discovery with DNA arrays. Cell 102, 9-15.

Young, T. B. and Wolf, D. A. (1988). Case-control study of proximal and distal colon cancer and diet in Wisconsin. *International Journal of Cancer* 42, 167-175.

Yu, R., Lei, W., Mandlekar, S., Weber, M. J., Der, C. J., Wu, J. and Kong, A. N. T. (1999). Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. *Journal of Biological Chemistry* **274**, 27545-27552.

Yuan, F., Chen, D. Z., Liu, K., Sepkovic, D. W., Bradlow, H. L. and Auborn, K. (1999). Antiestrogenic activities of indole-3-carbinol in cervical cells: implication for prevention of cervical cancer. *Anticancer Research* **19**, 1673-1680.

Yuan, J. Y. (1997). Transducing signals of life and death. *Current Opinion in Cell Biology* 9, 247-251.

Zhou, B. P., Hu, M. C. T., Miller, S. A., Yu, Z., Xia, W., Lin, S. Y. and Hung, M. C. (2000). HER2/neu blocks tumour necrosis factor induced apoptosis via the Akt/NF-kB pathway. *Journal of Biological Chemistry* **275**, 8027-8031.

APPENDIX. ALIGNMENT OF MMP AND AKR1C SEQUENCES.

The MMP and AKR1C full length sequences were aligned using the CLUSTALW programme. Areas of homology are designated by stars and the EST sequences used on the array are picked out in colour.

AKR1C alignments

AKR1C1 AKR1C3	CCAGAAATGGATTCGAAATATCAGTGTGTGAAGCTGAATGATGGTCACTTCATGCCTGTC ATGGATTCCAAACAGCAGTGTGTAAAGCTAAATGATGGCCACTTCATGCCTGTA ******* *** * ***********************
AKR1C1 AKR1C3	CTGGGATTTGGCACCTATGCGCCTGCAGAGGTTCCTAAAAGTAAAGCTTTAGAGGCCACC TTGGGATTTGGCACCTATGCACCTCCAGAGGTTCCGAGAAGTAAAGCTTTGGAGGTCACA **********************************
AKR1C1 AKR1C3	AAATTGGCAATTGAAGCTGGCTTCCGCCATATTGATTCTGCTCATTTATACAATAATGAG AAATTAGCAATAGAAGCTGGGTTCCGCCATATAGATTCTGCTCATTTATACAATAATGAG ***** ***** ************************
AKR1C1 AKR1C3	GAGCAGGTTGGACTGGCCATCCGAAGCAAGATTGCAGATGGCAGTGTGAAGAGAGAAGAC GAGCAGGTTGGACTGGCCATCCGAAGCAAGATTGCAGATGGCAGTGTGATGAGAGAAGAC *****************************
AKR1C1 AKR1C3	ATATTCTACACTTCAAAGCTTTGGTGCAATTCCCATCGACCAGAGTTGGTCCGACCAGCC ATATTCTACACTTCAAAGCTTTGGTCCACTTTTCATCGACCAGAGTTGGTCCGACCAGCC *****************************
AKR1C1 AKR1C3	TTGGAAAGGTCACTGAAAAATCTTCAATTGGATTATGTTGACCTCTACCTTATTCATTTT TTGGAAAACTCACTGAAGAAAGCTCAATTGGACTATGTTGACCTCTATCTTATTCATTC
AKR1C1 AKR1C3	CCAGTGTCTGTAAAGCCAGGTGAGGAAGTGATCCCAAAAGATGAAAATGGAAAAATACTA CCAATGTCTCTAAAGCCAGGTGAGGAACTTTCACCAACAGATGAAAATGGAAAAGTAATA *****************
AKR1C1 AKR1C3	TTTGACACAGTGGATCTCTGTGCCACGTGGGAGGCCGTGGAGAAGTGTAAAGATGCAGGA TTTGACATAGTGGATCTCTGTACCACCTGGGAGGCCATGGAGAAGTGTAAGGATGCAGGA ****** ****************************
AKR1C1 AKR1C3	TTGGCCAAGTCCATCGGGGTGTCCAACTTCAACCGCAGGCAG
AKR1C1 AKR1C3	AAGCCAGGGCTCAAGTACAAGCCTGTCTGCAACCAGG - TGGAATGTCATCCTTACTTCA AAGCCAGGACTCAAGTACAAGCCTGTCTGCAACCAGG - TAGAATGTCATCCGTATTTCA ******* *****************************
AKR1C1 AKR1C3	ACCAGAGAAAACTGCTGG-ATTTCTGCAAGTCAAAAGACATTGTTCTGG-TTGCCTATAG ACCGGAGTAAATTGCTAG-ATTTCTGCAAGTCGAAAGATATTGTTCTGG-TTGCCTATAG *** *** *** *** * ****

AKR1C1	- TGCTCTGGG - ATCCCACCGAGAA GAACCATGGG - TGGA CCCGAACTCCCCGG - TG
AKR1C3	-TGCTCTGGG-ATCTCAACGAGACAAACGATGGG-TGGACCCGAACTCCCCGG-TC
	****** *** *** *** *** **** **** ******
AKR1C1	CTCTTGGAGGA CCCAGTCCTTTGTG - CCTTGGCAAAAAAGCACAAG - CGAACCCCAGC
AKR1C3	CTCTTGGAGGACCCAGTCCTTTGTG-CCTTGGCAAAAAAGCACAAG-CGAACCCCAGC
	******** ******************************
AKD1C1	
AKRICI	
ARAICS	CUI-GAITGCCUTGCGUTACCAGCTGCAGCGTGGGGGTTGTGGTCCTGGCCAAGAGCTACA
	*** ***********************************
AKR1C1	ATGAGCAGCGCATCAGACAGAACGTGCAGGTGTTTGAATTCCAGTTGACTTCAGAGGAGA
AKR1C3	ATGAGCAGCGCATCAGACAGAACGTGCAGGTTTTTGAGTTCCAGTTGACTGCAGAGGACA

AKR1C1	TGAAAGCCATAGATGGCCTAAACAGAAATGTGCGATATTTGACCCTTGATATTTTGCTG
AKR1C3	ТСАААСССАТАСАТСССТАСАСАСАААТСТССАСТАТТТОКСССТТОКТИТТТОССС

AKR1C1	GCCCCCTAATTATCCATTATCTCATCAATTAACATCACCAC
AKR1C3	
ARRIES	*** *****************
AKR1C1	CCAGAAGGCCCTGCGTGTGGATGGTGACACAGAGGATGGCTCTATGCTGGTGACTGGACA
AKR1C3	
AKR1C1	CATCGCCTCTGGTTAAATCTCTCCTGCTTGGTGATTTCAGCAAGCTACAGCAAAGCCCAT
AKR1C3	
AKD1C1	<u>ማርሮሮሮ እር አ አ አ ሮር አ አ አ ሮ አ ሮ አ አ ጥ አ አ ምምምምምምምምምም የ ምምምምሮ አ አ አ አ አ አ ምም አ አ አ ምሮሮምሮም</u> ሮም
AKRICI	
UVVIC2	
AKR1C1	CCTAAAGATTCTTCACCTAAAAAA
AKR1C3	

MMP alignments.

MMP12 MMP1 MMP13 MMP7 MMP10	TAG ATATTGGAGTAGCAAGAGGCTGGGAAGCCATCACTTACCTTGCACTGAGAAAGAA
MMP12 MMP1 MMP13 MMP7 MMP10	AAGTT - TACAATGAAGTTTCTTCTAATACTGCTCCTGCAG - GCCACTGCTTCTGGAGCT AGGCCAGTATGCACAGCTTTCCTCCACTGCTGCTGCTGCTG - TTCTGGGGTGTGGTGTCT CATCCAGGGGTCCTGGCTGCCTTCCTTCTTGAGCTGGACTCATTGTCGGGCCCTGCCC TCTCTGGACGGCAGCTATGCGACTCACCGTGCTGTGTGTG

MMP12 MMP1 MMP13 MMP7 MMP10	CTT CCCCTG - AACAGCTCTACAAGCCTGGAAAAAAATAATGTGCTATTTGGTGAGAGA CACAGCTTCCC - AGCGACTCTAGAAACAC AAGAGCAAGATGTGGGACTTAGTCCAGAAA CTTCCCAGTGGTGGTGATGAAGATGATTTGTCTGAGGGAAGACCTCCAGTTTGCAGAGCGC CCTGGCCCTGCCGCTGCCTCAGGAGGCGG GAGGCATGAGTGAGCTACAGTGGGAACA CCTATCCTCTG - AGTGGGGCAGCAAAAGAGGAGGACTCCAACAAGGATCTTGCCCAGCAA * * * * * * * * *
MMD12	
MUD 1	IACIIAGAAAAAIIIIIAGGCCIIGAGATAAACAAACTTCCAGTGACAAAAATGAAATAT
MMPI	TACCTGGAAAAATACTACAACCTGAAGAATGATGGGAGGCAAGTTGAAAAGCGGAGAAAT
MMP13	TACCTGAGATCATACTACCATCCTACAAATCTCGCGGGAATCCTGAAGGAGAATGCAGCA
MMP7	GGCTCAGGACTATCTCAAGAGATTTTATCTCTATGACTCAGAAACAAAAAATGCCAAC
MMPIO	TACCTAGAAAAGTACTACAACCTCGAAAAGGATGTGAAACAGTTTAGAAGAAAGGACAGT * * * * *
MMP12	AGTGGAAACTTAATGAAGGAAAAAATCCAAGAAATGCAGCACTTCTTGGGTCTGAAAGTG
MMP1	AGTGGCCCAGTGGTTGAAAAATTGAAGCAAATGCAGGAATTCTTTGGGCTGAAAGTG
MMP13	AGCTCCATGACTGAGAGGCTCCGAGAAATGCAGTCTTTCTTCGGCTTAGAGGTG
MMP7	AGTTTAGAAGCCAAACTCAAGGAGATGCAAAAATTCTTTGGCCTACCTA
MMP10	AATCTCATTGTTAAAAAAATCCAAGGAATGCAGAAGTTCCTTGGGTTGGAGGTG
	* * * * * *** * * * *
MMP12	ACCGGGCAACTGGACACATCTACCCTGGAGATGATGCACGCAC
MMP1	ACTGGGAAACCAGATGCTGAAACCCTGAAGGTGATGAAGCAGCCCAGATGTGGAGTGCCT
MMP13	ACTGGCAAACTTGACGATAACACCTTAGATGTCATGAAAAAGCCAAGATGCGGGGTTCCT
MMP7	ACTGGAATGTTAAACTCCCGCGTCATAGAAATAATGCAGAAGCCCAGATGTGGAGTGCCA
MMP10	ACAGGGAAGCTAGACACTGACACTCTGGAGGTGATGCGCAAGCCCAGGTGTGGAGTTCCT
	** ** * * * * * * * * * * * * ** **
MMP12	GATCTCCATCATTTCAGGGAAATGCCAGGGGGGCCCGTATGGAGGAAACATTATATCACC
MMP1	GATGTGGCTCAGTTTGTCCTCACTGAGGGGAACCCTCGCTGGGAGCAAACACATCTGACC
MMP13	GATGTGGGTGAATACAATGTTTTCCCTCGAACTCTTAAATGGTCCAAAATGAATTTAACC
MMP7	GATGTTGCAGAATACTCACTATTTCCAAATAGCCCCAAAATGGACTTCCAAAGTGGTCACC
MMP10	GACGTTGGTCACTTCAGCTCCTTTCCTGGCATGCCGAAGTGGAGGAAAACCCCACCTTACA
	** * * * * * * * * * *
MMP12	TACAGAATCAATAATTACACACCTGACATGAACCGTGAGGATGTTGACTACGCAATCCGG
MMP1	TACAGGATTGAAAATTACACGCCAGATTTGCCAAGAGCAGATGTGGACCATGCCATTGAG
MMP13	TACAGAATTGTGAATTACACCCCTGATATGACTCATTCTGAAGTCGAAAAGGCATTCAAA
MMP7	TACAGGATCGTATCATATACTCGAGACTTACCGCATATTACAGTGGATCGATTAGTGTCA
MMP10	TACAGGATTGTGAATTATACACCAGATTTGCCAAGAGATGCTGTTGATTCTGCCATTGAG
11111 1.0	**** ** ** ** ** * ** *
MMP12	AAAGCTTTCCAAGTATGGAGTAATGTTACCCCCTTGAAATTCAGCAAGATTAACACAGGC
MMP1	AAAGCCTTCCAACTCTGGAGTAATGTCACACCTCTGACATTCACCAAGGTCTCTGAGGGT
MMP13	AAAGCCTTCAAAGTTTGGTCCGATGTAACTCCTCTGAATTTTACCAGACTTCACGATGGC
MMP7	AAGGCTTTAAACATGTGGGGCAAAGAGATCCCCCTGCATTTCAGGAAAGTTGTATGGGGA
MMP10	AAAGCTCTGAAAGTCTGGGAAGAGGTGACTCCACTCACATTCTCCAGGCTGTATGAAGGA
IIIII TO	** ** * * * * * * * * * * * * * * * * *
MMP12	ATGGCTGACATTTTGGTGGTTTTTGCCCGTGGAGCTCATGGAGACTTCCATGCTTTTGAT
MMP1	CAAGCAGACATCATGATATCTTTTGTCAGGGGAGATCATCGGGACAACTCTCCTTTTGAT
MMP13	ATTGCTGACATCATGATCTCTTTTGGAATTAAGGAGCATGGCGACTTCTACCCATTTGAT
MMP7	ACTGCTGACATCATGATTGGCTTTGCGCGAGGAGCTCATGGGGACTCCTACCCATTTGAT
MMP10	GAGGCTGATATAATGATCTCTTTCGCAGTTAAAGAACATGGAGACTTTTACTCTTTTGAT
	** ** ** * ** * * * *** * ****

MMP12	GGCAAAGGTGGAATCCTAGCCCATGCTTTTGGACCTGGATCTGGCATTGGAGGGGGATGCA
MMP1	GGACCTGGAGGAAATCTTGCTCATGCTTTTCAACCAGGCCCAGGTATTGGAGGGGATGCT
MMP13	GGGCCCTCTGGCCTGGCTCATGCTTTTCCTCCTGGGCCAAATTATGGAGGAGATGCC
MMP7	GGGCCAGGAAACACGCTGGCTCATGCCTTTGCGCCTGGGACAGGTCTCGGAGGAGATGCT
MMP10	GGCCCAGGACACAGTTTGGCTCATGCCTACCCACCTGGACCTGGGCTTTATGGAGATATT
	** * ** ** * ** ** ** ** **
MMP12	CATTTCGATGAGGACGAATTCTGGACTACACATTCAGGAGGCACAAACTTGTTCCTC
MMP1	CATTTTGATGAAGATGAAAGGTGGACCAACAATTTCAGAGAGTACAACTTACATCGT
MMP13	CATTTTGATGATGATGAAACCTGGACAAGTAGTTCCAAAGGCTACAACTTGTTTCTT
MMP7	CACTTCGATGAGGATGAACGCTGGACGGATGGTAGCAGTCTAGGGATTAACTTCCTGTAT
MMP10	CACTTTGATGATGAAGAAAAATGGACAGAAGATGCATCAGGCACCAATTTATTCCTC
	** ** **** ** *** * ** ** **
MMD12	
MMD1	
MMP12	
MMP13	
MMP 7	
MMPIO	*** ** ** * **** * ** ** ** ** ** ** **
MMP12	GTAATGTTCCCCACCTACAA ATATGTCGACATCAACACATTTCGCCTCTCTGCTGAT
MMP1	TTGATGTACCCTAGCTACACCTTCAGTGGTGATGTTCAGCTAGCTCAGGAT
MMP13	CTCATGTTTCCTATCTACACCTACACCGGCAAAAGCCACTTTATGCTTCCTGATGAC
MMP7	GTGATGTATCCAACCTATGGAAATGGAGATCCCCAAAATTTTAAACTTTCCCAGGAT
MMP10	TTGATGTACCCACTCTACAACTCATTCACAGAGCTCGCCCAGTTCCGCCTTTCGCAAGAT
	* *** ** *** * * ** *
10/010	
MMP12	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA
MMP12 MMP1	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTC
MMP12 MMP1 MMP13	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTC GATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGACCCC
MMP12 MMP1 MMP13 MMP7	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTC GATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGACCCC GATATTAAAGGCATTCAGAAACTATATGGAAAGAGAAGTAA
MMP12 MMP1 MMP13 MMP7 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTC GATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGACCCC GATATTAAAGGCATTCAGAAACTATATGGAAGAGAAGTAA GATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCCTGCCTCTACTGAGGAACCCCTG
MMP12 MMP1 MMP13 MMP7 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTC GATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGACCCC GATATTAAAGGCATTCAGAAACTATATGGAAA
MMP12 MMP1 MMP13 MMP7 MMP10 MMP12	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTC GATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGACCCC GATATTAAAGGCATTCAGAAACTATATGGAAA
MMP12 MMP1 MMP7 MMP10 MMP12 MMP1	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTC GATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGACCCC GATATTAAAGGCATTCAGAAACTATATGGAAGAGAAGTAA GATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCCTGCCTCTACTGAGGAACCCCTG ** * ***** ** ** ** ** ** ** CGCTTGCCAAATCCTGACAATTCAGAACCAGCTCTCTGTGACCCCAATTTG CAGCCCATCGGCCCACAAACCCCAAAAGCATGTGACAGTAAGCTA
MMP12 MMP1 MMP13 MMP7 MMP10 MMP12 MMP13	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAGACATACGTGGCATCCAGGCCATATATGGACGTCCCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGGATGTACAAGGCATTCAGAACTATATGGAGATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCCTGCCTCTACTGAGGAACCCCTG**
MMP12 MMP1 MMP13 MMP7 MMP10 MMP12 MMP1 MMP13 MMP7	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAGACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAGACATTGATGGCATCCAAGCCATATATGGACGTTCCCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGGATGTGAATGGCATTCAGAACTATATGGAGATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCCTGCCTCTACTGAGGAACCCCTG**
MMP12 MMP13 MMP7 MMP10 MMP12 MMP12 MMP13 MMP7 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAGACATACGTGGCATCCAGTCCCTGTATGGAGACCCAAGACATTGATGGCATCCAAGCCATATATGGACGTTCCCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGGATGTGAATGGCATTCAGAACTATATGGAGATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCCTGCCTCTACTGAGGAACCCCTG***
MMP12 MMP13 MMP7 MMP10 MMP12 MMP12 MMP13 MMP7 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAAGACATTGATGGCATCCAGGCCATATATGGACGTTCCCAAAATCCTGTCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGAAGCCCGATATTAAAGGCATTCAGAAACTATATGGAAGAGAAGTAAGATGTGAATGGCATTCAGAACTATATGGAAGAGAAGCCCTGCCCCTGCCTCTACTGAGGAACCCCTG***********************************
MMP12 MMP13 MMP7 MMP10 MMP12 MMP12 MMP13 MMP7 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAAGACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGAACCCCGATGTGAATGGCATTCAGAAACTATATGGAAGAGAAGTAAGATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCTGCCTCTACTGAGGAACCCCTG***CGCTTGCCAAATCCTGACAATTCAGAAGCACGCTTGCCAAATCCTGACAATTCAGAAGCACGCTTGCCAAATCCTGACAATTCAGAAGCACGCTTGGCCACAAACCCCAAAAGCACGCTTAGAACATCCAAAACGCCAGACGCACGCCTAAACATCCAAAAACGCCAGACGCACGCCCACAAAAACGCCAGACAAACGCCCACAAAACCTCCAGGCAAACGCCCACAAAACCTGTCCTCTGGGGATCTGAGATGCCAGCCA
MMP12 MMP13 MMP7 MMP10 MMP12 MMP12 MMP13 MMP7 MMP10 MMP12	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAGACATACGTGGCATCCAGGCCATATATGGACGTTCCCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGGATGTACAAGGCATTCAGAAACTATATGGAGATGTGAATGGCATTCAGAAACTATATGGAGATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCTGCCTCTACTGAGGAACCCCTG****CGCTTGCCAAATCCTGACAATTCAGAACGCTTGCCAAATCCTGACAATTCAGAACGCTTGCCAAATCCTGACAATTCAGAACGCTTGCCAAATCCTGACAATTCAGAACGCTTGCCAAATCCTGACAATTCAGAACGCTTAGCCAAATCCTGACAATTCAGAACGCTTAGCCAAACATCCAAAACGCCAGACCGCTACGGCCCACAAAACCCCAAAACGCCTAAACATCCAAAAACGCCAGACCGCCCACAAAACATCCATTCAGGCAACCCTAAAAAAAAAACTTCAGGCACAACATCCATTCAGTGCCCACAAAATCTGTTCCTTCGGGATCTGAGATGCCAGCCA
MMP12 MMP13 MMP7 MMP10 MMP12 MMP13 MMP13 MMP7 MMP10 MMP12 MMP12 MMP12	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAGACATACGTGGCATCCAGGCCATATATGGACGTCCCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGGATGTACAAGGCATTCAGAAACTATATGGAGATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCCTGCCTCTACTGAGGAACCCCTG******CGCTTGCCAAATCCTGACAATTCAGAAGCCCTACGGCCCACAAACCCCAAAAGCCCTACGGCCCACAAACCCCAAAAGCCCTAAACATCCAAAACGCCAGACCCAAAACATCCAAAAACGCCAGACGGCCCACAAACCCCAAAACCCCAAAAGTGCCCACAAAACCGCCAGAC***AGTTTTGATGCTGTCACTACCGTGGGAAATAAGATCTTTTCTTCAAAGACAGGATCTTCACCTTTGATGCTGTCACTACCGTGGGGAAATAAGATCTTTTTCTTCAAAGACAGGTTCTTC
MMP12 MMP13 MMP7 MMP10 MMP12 MMP13 MMP7 MMP10 MMP12 MMP12 MMP12 MMP13	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAGACATTGATGGCATCCAGGCCATATATGGACGTTCCCGACATTGATGGCATCCAGGCCATATATGGACGTCCCCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGGATGTGAAAGGCATTCAGAACTATATGGAGATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCCTGCCTCTACTGAGGAACCCCTG****CGCTTGCCAAAATCCTGACAATTCAGAAGCCCTACGGCCCACAAACCCCAAAAGCCCTACGGCCCACAAACCCCCAAAAGCCCTACGGCCCACAAACCCCAAAAGCCCTAAACATCCAAAACGCCAGACAACCCTAAACATCCAAAACGCCAGACACCCTAAACATCCAAAAACGCCAGACACCCTAAAAAAATCTGTTCCTTCGGGATCTGAGATGCCAGCCA
MMP12 MMP13 MMP7 MMP10 MMP12 MMP13 MMP7 MMP10 MMP12 MMP12 MMP12 MMP13 MMP13 MMP13 MMP13 MMP13 MMP7	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAAGACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGACCCCGATGTGAATGGCATTCAGAAACTATATGGAAGAGAAGTAAGATGTGAATGGCATTCAGTCTCTCTACGGACCTCCCCTGCCTCTACTGAGGAACCCCTG************************************
MMP12 MMP13 MMP7 MMP10 MMP12 MMP12 MMP13 MMP7 MMP10 MMP13 MMP13 MMP7 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAAGACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTCGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGACCCCGATGTGAAAGGCATTCAGAAACTATATGGAAGAGAAGTAAGATGTGAATGGCAATCCAGTCTCTCTACGGACCTCCCCCTGCCTCTACTGAGGAACCCCTG*********************************
MMP12 MMP13 MMP7 MMP10 MMP12 MMP1 MMP13 MMP7 MMP10 MMP12 MMP13 MMP13 MMP13 MMP7 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAAGACATTGATGGCATCCAGGCCTGTATGGACGTTCCCAAAATCCTGTGGATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAGATGAAGACCCCGATGTGGAATGGCATTCAGAACTATATGGAAGAGAAGTAAGATGTGGAATGGCATTCAGTCTCTCACGGACCTCCCCCTGCCTCTGTGAGGAACCCCTG****CGCTTGCCAAATCCTGACAATTCAGAACCAGCCCTCTGTGGACCCCAATTTGGAGCCCATCGGCCCACAAACCCCAAAAGCACGCTTGCCAAATCCGACACTCCAGGACAAACGCTTGCCAAATCCGGCCAGACAAACGCTTGCCACAAACCTCCTGCGGGACCCCACACTCTGTGGACCCCTACTCATCAGTGCCCACAAAATCTGTTCCTTCGGGGACACTGAGCCAGCC
MMP12 MMP13 MMP7 MMP10 MMP12 MMP1 MMP13 MMP10 MMP12 MMP12 MMP13 MMP13 MMP13 MMP13 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCCAAAATCCTGTC GATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAG
MMP12 MMP13 MMP7 MMP10 MMP12 MMP1 MMP13 MMP10 MMP12 MMP10 MMP13 MMP13 MMP13 MMP13 MMP13 MMP13 MMP13 MMP12 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAA AAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCC AAAATCCTGTC GATGTACAAGGGATCCAGTCTCTCTATGGTCCAGGAG AGAGAAGCCA GATGTGAATGGCATTCAGAACTATATGGA AGAGAAGCCC GATGTGAATGGCATTCAGAACTATATGGA AGAGAACCC GATGTGAATGGCATTCAGAACTATCAGGACCTCCCCCTGCCTCTGTGAGCACCCCAATTTG GACCCTACAGCCCCACAAACCCCCAAAA SCCCCCCCCGCCCCCCCCCCCCCCCCCCCCCCCCCCCC
MMP12 MMP13 MMP7 MMP10 MMP10 MMP12 MMP13 MMP7 MMP10 MMP12 MMP13 MMP13 MMP13 MMP13 MMP13 MMP12 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAA AAGAGAACCAA GACATAGGCATTCAGACCATATATGGACGTTCCC AAAATCCTGTC GATGTGACAAGGCATTCAGAACTATATGGA AGAGAAGCAA GATGTGAATGGCATTCAGAACTATATGGA AGAGAACCCC GATGTGAATGGCATTCAGAACTATATGGA AGAGAACCCC GATGTGCAAATCCTGACAATTCAGAA CCCCTGCCTCTGTGACCACTACGGAC CGCTTGCCAAATCCTGACAATTCAGAA GCCACGCCCCCACAAACCCCAAAA ACGCCTAAACATCCAAAAACGCCAGAC GCCACACAACCCCTCTCTGTGACCACTCCTCT ACCCTAAACATCCAAAAACGCCAGAC AAA TCCAGGAAAAAACTGTTCCTGCGGCACACACGCCAAGTGTGACCCTGCTTCC A ACCCTTGAGAAAAACTGTTCCCTCGGGGAAATAAGATCTTTTTCTTCAAAGACAGGTTCTTC * ACCTTTGATGCTGTCACTACCGTGGGGAAATAAGATCTTTTTTCTTCAAAGACAGGTTCTTC * ACCTTTGATGCCATTACCGTGGGGAAATAAGATCTGTTCTTTAAAGACAGGTTCTTC * ACCTTTGATGCCATTACCGTGGGGAAATAAGATCTGTTCTTTAAAGACAGATTCTCTC * ACCTTTGATGCCATTACCGTGGGGAGAAACAATGATCTGTTCTTTAAAGACAGATTCTCT * * * * * * * * * * * ACCCTTCGATGCCATCAGGACCAAAGGCCAAGGGGAGAATATCTGTTCTTTAAAGACAGATATTT * * * * * * * * * * * *
MMP12 MMP13 MMP7 MMP10 MMP12 MMP1 MMP13 MMP7 MMP10 MMP13 MMP7 MMP10 MMP12 MMP10 MMP13 MMP13 MMP13 MMP13 MMP13 MMP13	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAAAAGAGAACCAA GACATTGATGGCATCCAAGCCATATATGGACGTTCCC
MMP12 MMP13 MMP13 MMP10 MMP10 MMP12 MMP13 MMP10 MMP10 MMP13 MMP7 MMP10 MMP12 MMP12 MMP10 MMP12 MMP10	GACATACGTGGCATTCAGTCCCTGTATGGAGACCCAA

MMP12	ACCTTGCCATCTGGCATTGAAGCTGCTTATGAAATTGAAGCCAGAAATCAAGTTTTTCTT
MMP1	CAACTGCCAAATGGGCTTGAAGCTGCTTACGAATTTGCCGACAGAGATGAAGTCCGGTTT
MMP13	GAACTTCCCAACCGTATTGATGCTGCATATGAGCACCCTTCTCATGACCTCATCTTCATC
MMP7	CTCCTTTTATTGGTTAAACTCCTTTATGGTGTGACTGTCTCTTATTCCATCTATCAC
MMP10	
11111 10	* * * * * * * * *
MMP12	TTTAAAGATGACAAATACTGGTTAATTAGCAATTTAAGACCAGAGCCAAATTATCCCAAG
MMP1	TTCAAAGGGAATAAGTACTGGGCTGTTCAGGGACAGAATGTCCTACACCCCATACCCCCAAC
MMD13	
MMD7	
MMP10	
MMPIU	III AAAGGAAAIGAGITCTGGGCCATCAGAGGAAATGAGGTACAAGCAGGTTATCCAAGA
	* * * * *
MMD12	<u> እርር እ ሞ እ ር እ ሞ ምር ምምምምር ር ምምምር ምር እ እ እ እ እ እ እ እ ምምር እ ምር ር እ ር ር ምር ምምም</u>
MMD1	
MMPI	GACATCTACAGCTCCTTTGGCTTCCCTAGAACTGTGAAGCATATCGATGCTGCTCTTTCT
MMP13	AAAATATCTGAACTGGGTCTTCCAAAAGAAGTTAAGAAGATAAGTGCAGCTGTTCAC
MMP7	GCCATGGTAAATTTACCGAATTCCGCCGATACTGACGGGC
MMP10	GGCATCCATACCCTGGGTTTTCCTCCAACCATAAGGAAAATTGATGCAGCTGTTTCT
	** *
MUD10	
MMP12	AACCACGTITTTATAGGACCTACTTCTTTGTAGATAACCAGTATTGGAGGTATGATGAT
MMPI	GAGGAAAACACTGGAAAAAACCTACTTCTTTGTTGCTAACAAATACTGGAGGTATGATGAA
MMP13	TTTGAGGATACAGGCAAGACTCTCCTGTTCTCAGGAAACCAGGTCTGGAGATATGATGAT
MMP7	
MMP10	GACAAGGAAAAGAAGAAAACATACTTCTTTGCAGCGGACAAATACTGGAGATTTGATGAA
MMD10	
MMP12	
MMPI	татааасдатстатддатссаддттатсесаааа гдатадсаатдасттгсстддаатт
MMP13	ACTAACCATATTATGGATAAAGACTATCCGAGACTAATAGAAGAAGACTTCCCAGGAATT
MMP7	
MMP10	AATAGCCAGTCCATGGAGCAAGGCTTCCCTAGACTAATAGCTGATGACTTTCCAGGAGTT
MMD10	
MMP12	
MMP1	GGCCACAAAGTTGATGCAGTTTTCATGAAAGATGGATTTTTTCTTTCATGGA
MMP13	GGTGATAAAGTAGATGCTGTCTATGAGAAAAATGGTTATATCTATTTTTCAACGGA
MMP7	
MMPIO	GAGCCTAAGGTTGATGCTGTATTACAGGCATTTGGATTTTTCTACTTCTTCAGTGGA
MMP12	TCTAACCAATTTGAATATGACTTCCTACTCCAACGTATCACCAAAACACTGAAAAGCAAT
MMD1	ΔΟΣΔΟΣΟΤΑΟΤΑΣΑΤΤΤΟΣΤΟΟΤΑΣΑΣΑΟΘΑΘΑΘΑΤΤΤΤΟΔΟΤΟΟΑΘΑΒΑΘΟΤΑΑΤ
MMD12	
MMP13	
MMP/	толисловаетичеслении соссолочее соссаствение соста соста соста со соста со соста со соста со соста со соста о сосса со соста со
MMPIO	TCATCACAGITIGAGITIGACCCCCAAIGCCAGGAIGGIGACACACATATTAAAGAGIAAC
MMP12	AGCTGGTTTGGTTGTTAGAAATGGTGTAATTAATGGTTTTTGTTAG
MMD1	ACCTCCTTCAACTCCACCACCAAAAAATTCAACATTACTAATTTCAATGGAAAACAC
MMD12	
MMP15	
MMP/	
WWP10	AGCIGGTIACAIIGCIAGGCGAGAIAGGGGGGAAGACAGAIAIGGGIGIIIII
MMP12	TTCACTTCAGCTTAATAAGTATTTATTGCATATTTG-CTATGTCCTCAGTGT
MMP1	ATGGTGTGAGTCCAAAGAAGGTGTTTTCCTGAAGAACTGTCTATTTTCTCAGTCAT T
MMP13	GGGGTAATACTTCCAGAAGTGCGGGGGAGGGGAAGAAGAGCTATCAGGAGAAAGCTTGGT
MMP7	
MMP10	CTAATAATTATTCATCTAATGTATTATGAGCCAAAATGGTTAATTTTTCCTGCATGT

MMP12	ACCACTACTTAGAGATATGTATCATAAAAATAAAATCTGTAAACCATAGGTAATG
MMP1	TTTAACCTCTAGAGTCACTGATACACAGAATATAATCTTATTATACCTCAGTTTGCATA
MMP13	TCTGTGAACAAGCTTCAGTAAGTTATCTTTGAATATGTAGTATCTATATGACTATGCGTG
MMP7	
MMP10	ΤΟΤΩΤΩΑΟΤΩΑΔΩΑΔΑΩΑΤΩΑΘΟΟΤΤΩΟΑΩΑΤΑ ΤΟΤΟ Ο ΑΤΟΤΟΤΟΑΤΟΑΤΟΑΑΟΑΑ
	TETETENETEMENTEMEETIGERGATA TETE CATGIGICATGAAGAATG
MMP12	ATTATAAAAATACATAATATTTTTCAATTTTGAAAACTCTAATTGTCCATTC
MMP1	TTTTTTTACTATTTAGAATGTAGCCCTTTTTGTACTGATATAATTTAGTTCCACAA
MMP13	GCTGGAACCACATTGAAGAATGTTAGAGTAATGGAAGTGCAGGATCTCTAAAGAGCATCTG
MMP7	
MMP10	TTTCTGGAATTCTTCACTTGCTTTTGAATTGCACTGAACAGAATTAAGAAATACTCATGT
MMD12	
MMD1	
MMP1	AIGGIGGIACAAAAAGICAAGTTTGTGGCT-TATGGATTCATATAGGCCAGAGTTGCAA
MMP13	ATTCTTGTTGCTGTACAAAAGCAATGGTTGATGATACTTCCCACACCACAAATGGGACAC
MMP/	
MMP10	GCAATAGGTGAGAGAATGTATTTTCATAGATGTGTTATTACTTCCTCAATAAAAA
MMP12	- AATTCTATTTGAAGCATGCT - CTGTAA - GTTGCTTCCTAACATCCTTGGACTGAGAAAT
MMP1	AGATCTTTTCCAGAGTATGCAACTCTGACGTTGATCCCAGAGAGCAGCTTCAGTGACAAA
MMP13	ATGGTCTGTCAATGAGAGCATAATTTAAAAAATATATTTATAAGGAAATTTTACAAGGGCA
MMP7	
MMP10	GTTTTATTTTGGGCCTGTTCCTT
MMP12	TATACTTACTTCTGGCATAACTAAAATTAAGTATATATAT
MMP1	CATATCCTTTCAAGACAGAAAGAGACAGGAG - ACATGAGTCTTTGCCGGAGGAAAAGCAG
MMP13	ТАААДТАААТАСАТGCATATAATGAATAAATCATTCTTACTAAAAAGTATAAAATAGTAT
MMP7	
MMP10	
10/010	
MMP12	
MMPL	
MMP13	GAAAATGGAAATTTGGGAGAGCCATACATAAAAGAAATAAACCAAAGGAAAATGTCTGTA
MMP7	
MMP10	
MMD12	
MMD1	ΔΟΔΟΔΔΔΔΤΑΔΟΤΟΤΤΤΤΤΑΤΟΤΤΤΟΤΟΛΑΤΟΔΔΟΓΑΔΟΤΑΔΟΤΤΟΤΟΤΟΤΟΤΟΤΟΤΟΤΟΤΟΤΟΤΟ
MMD12	Α ΤΑ Δ ΤΑ Δ Ο ΤΟ ΤΤΙ ΤΑ ΤΟ ΤΙ
MMP15	
MMP/	
MMPIO	
MMP12	
MMP1	
MMP13	ATGTGCCCTTCTTCACACAGACACTAACGAAATATCAAAGTCATTAAAGACAGGAGACAA
MMD7	
MMD10	
MMPTO	
MMP12	
MMP1	
MMP13	AAGAGCAGTGGTAAGAATAGTAGATGTGGCCTTTGAATTCTGTTTAATTTTCACTTTTGG
MMP7	
MMP10	

MMP12	
MMP1	
MMP13 MMP7	CAATGACTCAAAGTCTGCTCTCATATAAGACAAATATTCCTTTGCATATTATAAAGGATA
MMP10	
MMP12	
MMP1	
MMP13	AAGAAGGATGATGTCTTTTTTTTTAAAATATTTTCAGGTTCTTCAGAAGTCACACATTACAA
MMP7	
MMP10	
MMP12	
MMP1	
MMP13	Α G ΤΤΑ Α Α Α ΤΤ G ΤΤΑ ΤΓΑ Α Α Α ΤΑ G Τ Ο ΤΑ Α Ο G C C Α ΤΟ C C Τ ΤΟ C C ΤΤ ΤΟ ΤΑ Α Α Α ΤΤ Α ΤΤΑ ΤΤ ΤΟ ΤΟ ΤΟ ΤΟ
MMP7	
MMP10	
MMP12	
MMP1	
MMP13	ATTATTTAAGACTAAAAGTTGCATTTTAACCCTATTTTACCTAGCTAATTATTTAATTGT
MMP7	
MMP10	
MMP12	
MMP1	
MMP13	CCGGTTTGTCTTGGATATATAGGCTATTTTCTAAAGACTTGTATAGCATGAAATAAAATA
MMP7	
MMP10	
MMP12	
MMP1	
MMP13	TATCTTATAAAGTGGAAGTATGTATATTAAAAAAGAGACATCCAAATTTTTTTT
MMP7	
MMP10	
MMP12	
MMP1	
MMP13	AGTCTACTAGATTGTGATCCCTTGAGATATGGAAGGATGCCTTTTTTTCTCTGCATTTAA
MMP7	
MMP10	
MMP12	
MMP1	
MMP13	AAAAATCCCCCAGCACTTCCCACAGTGCCTATTGATACTTGGGGGGGG
MMP7	
MMP10	
MMP12	
MMP1	
MMP13	ATTGAATATATGATCGGCCATCAAGGGAAGAACTATTGTGCTCAGAGACACIGIIGAIAA
MMP/	
MMU ()	

MMP12	•••••••••••••••••••••••••••••••••••••••
MMP1	
MMP13	AAACTCAGGCAAAGAAATGAAATGCATATTTGCAAAGTGTATTAGGAAGTGTTTATGTT
MMP7	
MMP10	
MMP12	
MMP1	
MMP13	GTTTATAATAAAAATATATTTTCAACAGAAAAAAAA
MMP7	
MMP10	