The effect of nicotine and prostaglandin A_2 on the lung cancer cell line NCI-H157

by

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DECLARATION

I, the undersigned, declare that ***The effect of nicotine and prostaglandin A_2 on the lung cancer cell line NCI-H157*** is my own work and has not previously in its entirety, or in part, been submitted at any university. All the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

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C. Willemse **Date**

DEDICATION

This thesis is dedicated to my mother Jean and father Bernard.

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ABSTRACT

Lung cancer is the most common fatal cancer in terms of both incidence and mortality in the world. The most important cause of lung cancer is exposure to tobacco smoke through active or passive smoking. Nicotine which is a major component of tobacco could be assumed to be a tumour promoter since it had been indicated to stimulate tumour growth. Over expression of Bcl-2 in human lung cancer cells blocked the induction pathways (type I and II) of apoptosis. The increase in Bcl-2 in patients with lung cancer had also been linked to nicotine. In recent years nicotine replacement therapy has become a therapeutic method to treat smoker's withdrawal symptoms and to advise cancer patients to stop smoking because, numerous cancer patients continue to smoke after their diagnosis. Non small cell lung carcinomas constitutes for approximately 80% of lung cancer cases. However, even with the development and improvement in conventional treatments of surgery, radiation and chemotherapy, the 5 year survival rate for these patients remains less than 15%. Chemoprevention, an approach to control cancer, is the use of specific natural or synthetic substances with the objective of delaying, reversing, suppressing or preventing carcinogenic progression to invasive cancer. A promising tool for chemoprevention against lung cancer could be prostaglandin A_2 (PGA_2), since it had been shown to have inhibitory effects on various cancer cell growth. The search for more effective agents, or combination therapies that could induce apoptosis in lung cancer are currently under investigation as a therapeutic target for the treatment of lung cancer. In order to elucidate the effect of nicotine and PGA_2 on lung cancer cell proliferation in this study, an over view of the following was given; the cell cycle, tubulin, nucleoli, apoptosis, lung cancer, the etiology of cancer with reference to tobacco smoke and nicotine, the nutritional influence on carcinogenesis with reference to essential fatty acids and prostaglandins and chemoprevention.

The supplements nicotine and PGA_2 were administered to the NCI-H157 lung cancer cell line at the concentrations of 1 mM, 1 µM and 1 nM for nicotine and 5, 10 and 20 µg/ml PGA_2. The effect of combinations of nicotine and PGA_2 on the proliferation and survival was also tested. 5 µg/ml PGA_2 was added to 1 mM, 1 µM and 1 nM nicotine respectively. This was also done for 10 and 20 µg/ml PGA_2. These concentrations were administered to the cell culture and exposed for three

different time exposures, namely 24, 48 and 72 hours. The objectives were: 1) To determine the effect of nicotine and PGA_2 and combinations thereof on the growth (proliferation) of the NCI-H157 cells, where early results indicative of apoptosis lead to the investigation of the influence of nicotine and PGA_2 on apoptosis. The effect of nicotine and PGA_2 and their combinations on the morphology of interphase and dividing cells, as well as on the morphology of the dying cells were compared and quantified. 2) To study the effects of nicotine and PGA_2 and their combinations on the nucleolar organizer region using silver stain. 3) To study the effects of nicotine and PGA_2 or combinations thereof on the cytoskeleton (α-tubulin) of the cancer cells with aid of indirect immunofluorescence and to identify apoptotic cells using Hoechst 33342. 4) To determine the effect of nicotine and PGA_2 and their combinations on cell cycle progression and apoptosis induction in the transformed cells using flow cytometry (DNA propidium iodide stain, Annexin V and caspase-3). In order to verify the effects of nicotine and PGA_2 and their combinations on protein synthesis, SDS-PAGE and immunoblotting was employed.

This study indicated the anti-apoptotic effects of nicotine. It maintained and stimulated cell proliferation of the NCI-H157 cell line. PGA_2 demonstrated that it has a pro-apoptotic effect. The concentrations of 10 and 20 µg/ml PGA_2 decreased cell proliferation and demonstrated its pro-apoptotic effects more effectively than 5 µg/ml PGA_2. The combination of 10 and 20 µg/ml PGA_2 and nicotine (1 mM, 1 µM and 1 nM) also showed a more pronounced induction of apoptosis than 5 µg/ml PGA_2 and nicotine (1 mM, 1 µM and 1 nM). PGA_2 therefore demonstrated that it blocked the mitogenic and anti-apoptotic effects of nicotine. With its pro-apoptotic effects, PGA_2 could therefore be assumed to be a chemopreventive agent. However, it was evident that apoptotic induction was stimulated via both a dependent and an independent caspase-3 pathway and therefore further investigation is needed to indicate which pathway was activated. This study identified PGA_2 as a chemopreventive agent for *in vitro* conditions; however, further studies are also needed to investigate the effect of *in vivo* conditions.

Key words: apoptosis, chemoprevention, lung cancer, nicotine and prostaglandin A_2 (PGA_2)

List of Abbreviations

53BP1	:	p53 binding protein 1
5-HT	:	5-hydroxytryptamine
AA	:	arachidonic acid
ACh	:	acetylcholine
ADO	:	adenocarcinoma
ADP	:	adenosine diphosphate
AgNOR	:	silver nuclear organizer region
AI	:	adequate intake
AIF	:	apoptotic inducing factor
ALA	:	alpha linolenic acid
AMP	:	adenosine monophosphate
ANDS	:	alternative nicotine delivery systems
ANT	:	adenine nucleotide translocator
Apaf-1	:	apoptotic protease activating factor-1
APC/C	:	anaphase promoting complex or cyclosome
APUD	:	amine precursor uptake and decarboxylation
Asp	:	aspartic acid
ATCC	:	American type culture collection
ATM	:	ataxia-telangiectasia mutated
ATP	:	adenosine triphosphate
ATR	:	ataxia-telangiectasia mutated and Rad3 related
ATRIP	:	ATR interacting protein
Bax	:	Bcl-2 associated x protein
Bcl-2	:	B cell leukaemia-2
BEC	:	bronchial epithelium cells
BLM	:	Bloom's syndrome helicase
BRCA1	:	breast cancer 1
Ca^{2+}	:	calcium
CAK	:	Cdk activating kinase
cAMP	:	cyclic adenosine monophosphate
CARD	:	caspase activation and recruitment domain
caspase	:	cysteine dependent aspartate specific proteases

Cdc	:	cell division cycle
Cdh1	:	cadherin 1
Cdk/s	:	cyclin dependent kinase/s
CENP-E	:	centromere protein E
Chk1	:	check 1
Chk2	:	check 2
CIP	:	Cdk inhibitor protein
CKI/s	:	cyclin dependent kinase inhibitor/s
cm^2	:	centimeter squared
CNS	:	central nervous system
CO_2	:	carbon dioxide
COPD	:	chronic obstructive pulmonary disease
COX	:	cyclooxygenase
CPT	:	camptothecin
CRS	:	cytoplasmic retention sequence
CV	:	crystal violet
CY	:	cytochrome
d-6-d	:	delta-6-desaturase
DA	:	dopamine
DAG	:	diacylglycerol
dATP	:	deoxyadenosine triphosphate
DD	:	death domain
DED	:	death effector domain
DFC	:	dense fibrillar component
DGLA	:	dihomogammalinolenic acid
DHA	:	docosahexaenoic acid
DHFR	:	dihydrofolate reductase
dH_2O	:	distilled water
DISC	:	death inducing signaling complex
DMSO	:	dimethyl sulphoxide
DNA	:	deoxyribonucleic acid
DNA-PK	:	DNA activated protein kinase
DNases	:	deoxyribonuclease
DPA	:	docosapentaenoic acid

E2F	:	elongation factor 2 complex
ECL	:	enhanced chemiluminescence
EET	:	epoxyeicosatrienoic acid
EFAs	:	essential fatty acids
EGF	:	endothelial growth factor
endo G	:	endonuclease G
EOP/s	:	endogenous opiod peptides
EPA	:	eicosapentaenoic acid
ERK2	:	extracellular signal regulated kinase
Fas	:	CD95 or APO-1
FADD	:	Fas-associated death domain
FBS	:	fetal bovine serum
FC	:	fibrillar centre
FDA	:	Food and Drug Administration
FEN-1	:	flap structure specific endonuclease 1
FITC	:	fluorescein isothiocyanate
g/d	:	gram per day
G_0	:	Gap zero
G_1	:	Gap 1
G_2	:	Gap 2
GABA	:	gamma aminobutyric acid
Gadd45	:	growth arrest and DNA damage inducible gene 45
GC	:	granular component
GDP	:	guanosine diphosphate
GIRK	:	G-protein inwardly rectifying potassium channel
GLA	:	gamma linolenic acid
gr	:	gram
GSH	:	glutathione
GTP	:	guanosine triphosphate
GTPase	:	guanosine triphosphatase
H & E	:	haematoxylin and eosin
Hela	:	human epitheloid cervical carcinoma cell line
HETE	:	hydroxyeicosatetraenoic acid
HO342	:	Hoechst 33342

IAP	:	inhibitor of apoptosis
ICAD	:	inhibitor of caspase-activated DNase
INK4	:	Inhibitor of Cdk4
IR	:	infra red
JAK2	:	Janus kinase 2
KAP	:	Cdk associated protein phosphatase
kDa	:	kilodalton
KIP	:	kinase inhibitor protein
KT/s	:	kinetochore/s
LA	:	linoleic acid
LCC	:	large cell carcinoma
LOX	:	lipooxygenase
LTs	:	leukotrienes
M	:	mitosis
MAP	:	mitogen activated protein
MAT1	:	ménage a trios
MCC	:	mitotic checkpoint complex
MCF-7	:	human breast adenocarcinoma cell line
MCM	:	minichromosome maintenance
MDC1	:	mediator of DNA damage checkpoint protein 1
MDM2	:	mouse double minute gene number 2
MI	:	mitotic index/indices
MIAs	:	microtubule interfering agents
mg	:	milligram
ml	:	millilitres
mM	:	millimolar
MMP	:	mitochondrial membrane permeabilitation
MMS	:	methyl-methanesulfonate
MOX	:	monooxygenase
MPF	:	mitosis/maturating promoting factor
MRN	:	Mrell-Rad50-Nbs1 protein complex
MT	:	microtubule
MTOC	:	microtubule organizing center/centrosome
NA	:	noradrenaline

nAChR/s	:	nicotinic acetylcholine receptor/s
$NaCl_2$	:	sodium chloride
NAD	:	nicotinamide adenine dinucleotide
NaOH	:	sodium hydroxide
NCI	:	National Cancer Institute
NCI-H157	:	lung squamous carcinoma cell line
NEB	:	neuroepithelial bodies
NEBD	:	nuclear envelope breakdown
nm	:	nanometre
nM	:	nanomolar
NMK	:	normal monkey kidney
NNK	:	4-methylnitrosamino-1-(3 pyridyl)-1-butanone
Non-SCLC/ NSCLC	:	non-small cell lung carcinomas
NOR	:	nuclear organizer region
NPM	:	nucleophosmin
NRT/s	:	nicotine replacement therapy/s
PARP	:	poly ADP ribose polymerase
PBS	:	phosphate buffer saline
PC	:	phospholipase C
PCD	:	programmed cell death
PCNA	:	proliferating cell nuclear antigen
PG	:	prostaglandin
PGA_2	:	prostaglandin A 2
PGHS	:	PG endoperoxide H synthase
PGI_2	:	prostacyclin
PI	:	propidium iodide
PI3K	:	phosphoinositide-3 kinase/ phosphatidylinositol-3 kinase
PIP_2	:	phosphatidylinositol-4-5-bisphosphate
pKa	:	index of ionic dissociation
PKB	:	protein kinase B
PKC	:	protein kinase C
PNEC	:	pulmonary neuroendocrine cells
pol	:	polymerase

pre-RC	:	pre-replicative complex
pre-rRNA	:	pre-ribosomal RNA
PTP	:	permeability transition pore
PUFAs	:	polyunsaturated fatty acids
RAIDD	:	receptor interactive protein associated ICH/CED-3 homologous protein death domain
R point	:	restriction point
Rb	:	retinoblastoma tumour gene
RFB/s	:	replication fork barrier/s
RF-C/RFC	:	replication factor C
RIP	:	receptor interactive protein
RNA	:	ribonucleic acid
RPA	:	replication protein A
rRNA	:	risbosomal RNA
S	:	synthesis
SCLC	:	small cell lung carcinomas
SDS	:	sodium dodecyl sulphate
SDS PAGE	:	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	:	standard error of the mean
Ser	:	serine
SPB	:	spindle pole bodies
SQC	:	squamous cell carcinoma
Symbols		
α	:	alpha
β	:	beta
γ	:	gamma
δ	:	delta
ε	:	epsilon
ζ	:	zeta
η	:	eta
TBS	:	tris buffered saline
TEMED	:	N,N,N',N-tetramethylethylenediamine
Thr	:	threonine
Tk	:	thymide kinase

TK	:	tyrosine kinase
TNF	:	tumour necrotic factor
TNFADD	:	TNFR 1-associated death domain
TNFR	:	tumour necrotic factor receptor
TNP	:	transdermal nicotine patch
TNRF1	:	tumour necrosis factor-α receptor-1
TRADD	:	TNFR 1-associated death domain
TRAILR	:	tumour necrosis factor related apoptosis inducing ligand receptor
TXs	:	thromboxanes
Tyr	:	tyrosine
Ub	:	ubiquitin
UFA	:	unsaturated fatty acid
µg	:	microgram
UK	:	United Kingdom
µl	:	microlitre
µm	:	micrometre
µM	:	micromolar
USA	:	United States of America
UV	:	ultraviolet
VDAC	:	voltage dependent anion channel
WHCO1 or 3	:	oesophageal carcinoma cell lines
WHO	:	World Health Organization
XIAP	:	inhibitor of apoptosis

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CHAPTER I

Literature Review

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Literature Review

1.1 Introduction

Lung cancer is the leading cause of cancer related deaths in the world (Alberg and Samet, 2003; Guessous *et al*, 2007; Herbst *et al*, 2008; Lau *et al*, 2006; Molina *et al*, 2008; O'Mahony *et al*, 2005; Osada and Takahashi, 2002; Ranga *et al*, 2005; Zhou *et al*, 2006) and emerging as the most commonly occurring cancer worldwide (Hecht, 2002; Nikliński *et al*, 2001; Sozzi and Carney, 1998), with around 1.1 million new cases each year (O' Mahony *et al*, 2005). Despite all the publicity about breast and prostate cancer, lung cancer kills far more men (Ruckdeschel, 1999) and has now surpassed breast cancer as the leading cause of cancer death in women (Alberg and Samet, 2003; Lau *et al*, 2006; McLoud and Swenson, 1999).

According to Sitas (1998) the incidence of lung cancer in South Africa is very similar to that in the United States of America (USA). Most people die within one year of diagnosis, with approximately 150 000 deaths per year, therefore, making lung cancer the leading cause of death among the tumour entities (Fleischhacker *et al*, 1999; Nikliński *et al*, 2001; Osada and Takahashi, 2002).

The most important cause of lung cancer is exposure to tobacco smoke through active or passive smoking (Albreg and Samet, 2003; Herbst *et al*, 2008; Molina *et al*, 2008; Osada and Takahashi, 2002). An estimation of 90% of all lung cancers are caused by smoking (Fox *et al*, 2004; Guessous *et al*, 2007; Ozlu and Bulbul, 2005), and approximately 90% of men and 79% women lung cancer's are attributed to cigarette smoking (Ozlu and Bulbul, 2005). Worldwide it is estimated that nearly two billion people use tobacco on a regular basis (George and O'Malley, 2004). Tobacco could therefore be seen as the leading contributor to lung cancer (Alberg *et al*, 2005; Molina *et al*, 2008). It is also the most common avoidable contributor to cancer deaths (Houston and Kaufman, 2000). According to Bernhard and colleagues (2004), compared to non-smokers, smokers have been found to lose up to 7.2 years of their life span and increasing the risk of a variety of cancers with lung cancer being the most common.

Environmental tobacco smoke alone causes lung cancer deaths of 3 000 annually as was estimated in 1992, by the United States Environmenal Protection Agency (De Andrade *et al*, 2004). According to the World Health Organization (WHO), four million people die annually from tobacco-related diseases (Vainio *et al*, 2001), one death every eight seconds (WHO Pres Release, 1999). The WHO estimated that there were 1.1 billion smokers in the world at the beginning of the 1990's, 300 million in developed countries and 800 million in developing countries (WHO Pres Release, 1999). According to Da Costa e Silva and colleague (2004) estimates of global mortality attributable to smoking have shown that 4.83 million deaths world wide were due to smoking in the year 2000. From this, approximately 2.41 million deaths were from developing countries and 2.43 million from industrialized countries. If the current trends continue, the WHO estimates that the death toll will rise to ten million by 2030, one death every three seconds (WHO Pres Release, 1999).

Since 1950 it has been scientifically proven that smoking is an extraordinarily important cause of premature mortality and disability around the world (Da Costa e Silva and Fishburn, 2004). Cigarette smoking is one of the major risk factors for premature death (Andreoli *et al*, 2003) and the cause of several groups of diseases, such as cancer of a number of organs, cardiovascular disease, chronic lung diseases and peptic ulcer disease (Da Costa e Silva and Fishburn, 2004; Rigotti *et al*, 2008). Not only does these diseases occur at the sites contacted directly by tobacco smoke, the mouth, throat and the lungs but also, at sites reached by tobacco smoke components and metabolites such as the heart, blood vessels, kidney and bladder (Da Costa e Silva and Fishburn, 2004). In populations where cigarette smoking has been customary for several decades, approximately 90% of lung cancer cases, 15%-20% of other cancer cases, 75% of chronic bronchitis and emphysema and 25% of cardiovascular deaths in people between the ages of 35 to 69 years are attributable to tobacco (Da Costa e Silva and Fishburn, 2004). Da Costa e Silva and colleague (2004) have also indicated that half of all long term smokers will die of a tobacco related disease and of this 50% will die before the age of 65.

In order to decrease the mortality statistics of lung cancer, a promising tool for chemoprevention against lung cancer in humans could be prostaglandin A_2 (PGA_2),

as it has been shown to have inhibitory effects on various cancer cell growths (Choi *et al*, 1992; Joubert *et al*, 1999, 2003, 2005, 2006; Kim *et al*, 2002; Seegers *et al*, 2000) including non-small cell lung carcinoma cells (Lin *et al*, 2000). In order to elucidate the effect of nicotine and PGA_2 on lung cancer cell growth, information on the following was needed; the cell cycle, tubulin, nucleoli, apoptosis, lung cancer, the etiology of cancer with reference to tobacco smoke and nicotine, the nutritional influence on carcinogenesis with reference to essential fatty acids and prostaglandins and chemoprevention.

1.2 The cell cycle

The cell cycle (figure 1.1) is the sequence of orderly events by which a cell duplicates its genetic material and divides into two identical daughter cells (Jiang, 2006; Poon, 2005; Tessema *et al*, 2004). Two periods, the interphase and mitosis constitute the organized sequence of events of the cell cycle. The interphase encompasses the following stages (phases) G_1 (Gap 1), S (synthetic) and G_2 (Gap 2) (Rangarajan and Shaw, 2004; Santella *et al*, 2005; Sridhar *et al*, 2006). Mitosis is nuclear division plus cytokinesis (Alberts *et al*, 1994; Tessema *et al*, 2004), and produces two identical daughter cells during the following stages, prophase (and prometaphase), metaphase, anaphase and telophase (Boydston-White *et al*, 2005; Pollard and Earnshaw, 2002; Rangarajan and Shaw, 2004).

The cell cycle's length varies in time from one cell type to another. The cell cycle in culture, generally exhibit a 24 hour period (Boydston-White *et al*, 2005). However, in contrast to the different types of cells of a multicellular organism, the cell cycle times may vary as little as 8 hours to more than 100 days (Voet and Voet, 1995). The G_1 phase is highly variable, ranging from almost nonexistant in rapidly dividing cells to days, weeks or years. However, it typically takes 8 to 10 hours (Pollard and Earnshaw, 2002). The G_1 phase determines the length of the cell cycle (Voet and Voet, 1995).

The length of the S phase of about 7.5 hours (Pollard and Earnshaw, 2002) within any particular type of cell is remarkably constant (Laskey *et al*, 1989). Widrow and colleagues (1998) found that the G_2 period is much shorter than was expected and

may in some cells be nonexistent. The M phase takes approximately an hour, which is only a small fraction of the total cell cycle time (Alberts *et al*, 1994).

The G_0 (Gap zero) phase is the resting or dormant (quiescent) phase in which the cell performs all activities except those related to proliferation (Boydston-White *et al*, 2005; Mattson, 1990). Often G_0 cells are terminally differentiated, and will never re-enter the cell cycle but instead will carry out their function in the organism until they die (Lodish *et al*, 1986; Pollard and Earnshaw, 2002). It is believed that some special growth signal is needed to move cells that have been dormant in G_0 back into the cell cycle (Boydston-White *et al*, 2005; Mattson, 1990).

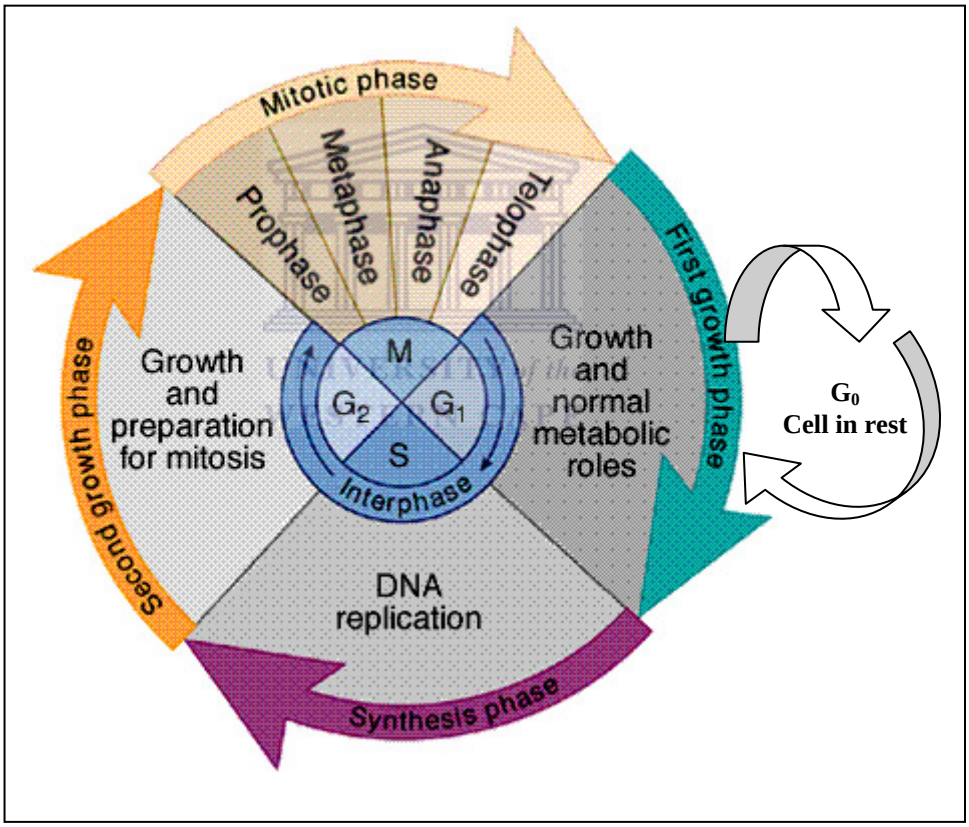

Figure 1.1: The cell cycle as adapted from Boydston-White *et al*, 2005 and Karp, 1996.

During the G_1 phase DNA synthesis ceases whilst gene expression and protein synthesis takes place (Mattson, 1990). Growth factors act throughout this phase by binding to specific cell surface receptors, which in turn trigger signalling cascades that ultimately regulate the transcription of both immediate and delayed early response genes (Nigg, 1995; Sherr, 1993). This phase of the cell cycle is regulated

by extracellular factors such as growth and adhesion factors which ultimately determine the fate of the cells (Van Opstal *et al*, 2005). The extracellular factors thus determine whether a cell in G_0 will begin to proliferate and also whether a normal proliferating cell in G_1 will continue to cycle or will revert to the G_0 phase (Pardee, 1989; Tessema *et al*, 2004). This decision depends on the restriction (R) point within G_1 which determines whether a cell becomes committed to cell cycle progression, once it goes beyond this point (McLaughlin *et al*, 2003; Tessema *et al*, 2004).

The G_1 phase is followed by the S phase (Boydston-White *et al*, 2005). The replication of the DNA could be initiated at multiple origins of replication that are activated either during early, mid or late S phase (Lambert and Carr, 2005). Giving rise to two separate sets of chromosomes (Tessema, *et al*, 2004) and the duplication of centrioles (Doxsey *et al*, 2005).

The G_2 phase is the premitotic phase (Mattson, 1990; Tessema *et al*, 2004). During this phase as in G_1, the DNA synthesis ceases while synthesis of RNA and protein continues (Alberts *et al*, 1994). The cells also "proofread" the DNA structure and make preparations for mitosis (Pollard and Earnshaw, 2002). The cell then finally enters the fourth and final phase of the cell cycle, the M phase (Tessema *et al*, 2004).

1.2.1 The cell cycle control

The eukaryotic cells ensure survival and propagation of accurate copies of the genome on to subsequent generations, by responding to damaged or abnormally structured DNA. This occurs by means of a multifaceted response that coordinates cell cycle progression with DNA repair, chromatin remodelling, transcriptional programs and other metabolic adjustments or cell death (Lukas *et al*, 2004). The arrest or the delay of the cell cycle progression that provides time for DNA repair, or that may permanently prevent cell proliferation if the damage is beyond repair is known as the cell cycle checkpoint (Liu and Greene, 2001; Lukas *et al*, 2004). The cell cycle checkpoint serves as a molecular switch that slows or arrests cell cycle progression, in that way allowing time for appropriate repair mechanism to correct genetic lesions before they are passed on to the next generation of daughter cells (Abraham, 2001; Liu and Greene, 2001). These processes are mediated by a network of signalling pathways referred to as cell cycle checkpoints (Lukas *et al*, 2004).

Checkpoints could be viewed as a location (a border) and a process (examination or proof reading) that represents a feedback mechanism that monitors execution of a process and delays initiation of subsequent events until the process is completed successfully (Elledge, 1996; Lopes and Sunkel, 2003). However, the cell cycle is also responsive to extracellular conditions. Extracellular signals, such as hormones and growth factors, usually control cell proliferation exclusively during the G_1 phase of the cell cycle (Abraham, 2001; Heldin and Purton, 1997).

The cell cycle is composed of a network of components (figure 1.2) and the regulatory proteins that play important roles in controlling cell cycle progression are the cyclins, cyclin dependent kinases (Cdks), their substrate proteins, the Cdk inhibitors (CKIs) and the tumour suppressor gene products p53 and pRb. These proteins comprise the basic regulatory machinery responsible for catalysing cell cycle transition and checkpoint traversation (Golias *et al*, 2004). However, there are also other proteins involved in the regulation of the cell cycle. They are the sensors, mediators and effectors (table 1.1) (Gottifredi and Prives, 2005: Lukas *et al*, 2004).

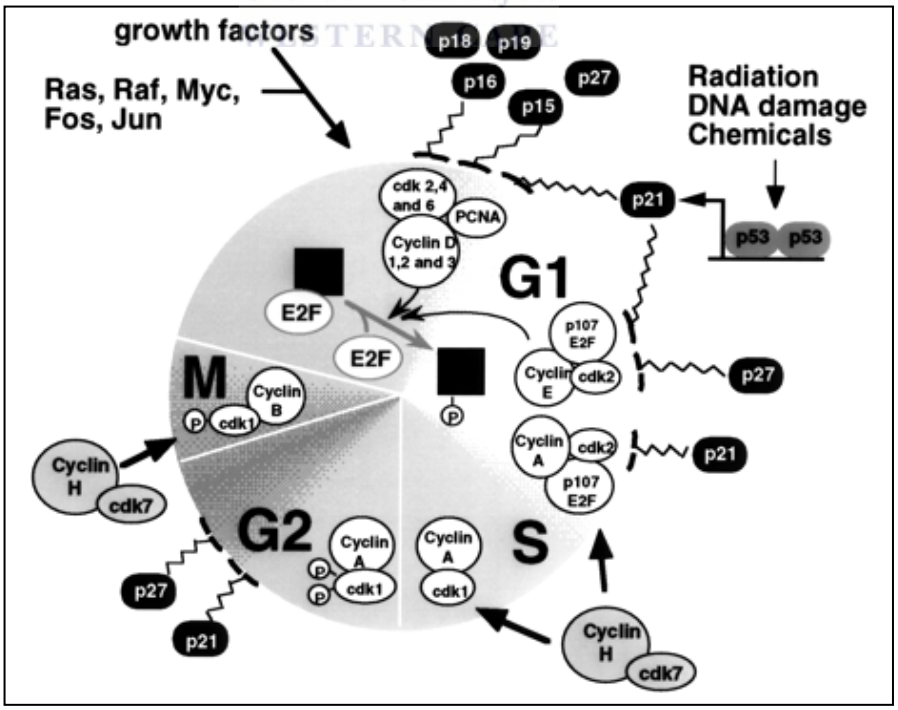

Figure 1.2: The cell cycle indicating the various of components (Senderowicz and Sausville, 2000). E2F = elongation factor 2 complex; PCNA = proliferating cell nuclear antigen.

Table 1.1: Mammalian checkpoint proteins (Gottifredi and Prives, 2005).

Sensors	Mediators	Effectors
Rad17	BRCA1, 53BP1	Chk2
Rad9	Claspin	Chk1
Hus1		
Rad1		
ATR		
ATRIP		
ATM		

1.2.1.1 The cyclin dependent kinases (Cdks)

Cdks are catalytic subunits of a family of heterodimeric serine/threonine protein kinases. Their key function is the phosphorylation of substrates required for cell cycle progression (Collins and Garrett, 2005; Malumbres and Barbacid, 2005; Senderowicz, 2004). By this means, Cdks control the transitions between successive phases of the cell cycle in all eukaryotic cells (Collins and Garrett, 2005; Nasmyth, 1993; Norbury and Nurse, 1992; Tessema *et al*, 2004). Cdks may be the most highly regulated enzymes characterized by multiple mechanisms existing to regulate activity (Lee and Yang, 2001).

Table 1.2: The mammalian Cdk family and their associated cyclins as adapted from Malumbres and Barbacid, 2005.

Symbol	Synonym	Identity to Cdk1 (%)	Main activating cyclin (other cyclins)	Cellular function
Cdk1	Cdc2, Cdc28	100	A1, A2, B1, B2 (E, B3)	Cell cycle (G_2 – M)
Cdk2		65	A1, A2, E1, E2 (D1, D2, B1, B3)	Cell cycle (G_1 – S)
Cdk3		63	E1,E2, A1,A2, C	Cell cycle (G_0 -G_1 –S)
Cdk4	PSK-J3	42	D1, D2, D3	Cell cycle (G_1 – S)
Cdk5		56	p35, p39 (D-, E- and G-type)	Senescence, postmitotic neurons
Cdk6		43	D1, D2, D3	Cell cycle (G_1 – S)
Cdk7	MO15, CAK, STK1	38	H	Cdk activating kinase, transcription
Cdk8	K35	28	C (K?)	Transcription
Cdk9		34	T1, T2, K	Transcription
Cdk10		37	Unknown	Transcription, Cell cycle (G_2 – M)
Cdk11	Cdc2L1, Cdc2L2	26	L1, L2 (D)	Transcription, Cell cycle (M)

Different cell cycle transitions require distinct Cdks (table 1.2) (Malumbres and Barbacid, 2005; Sridhar *et al*, 2006). There are eleven members of the Cdk family (Malumbres and Barbacid, 2005; Sridhar *et al*, 2006) which all are structurally related to each other and each contains the amino acid motif "PSTAIRE" (Hanley-Hyde, 1992). Cdk1 through to Cdk7 are involved in cell cycle control, whereas Cdk8 and Cdk9 are important transcriptional regulators (Senderowicz and Sausville, 2000; Sridhar *et al*, 2006). One defining characteristic of the Cdks is that they are inactive as monomers and become active only after binding to a cyclin partner (Clement *et al*, 2001). Thus, as their name suggest the Cdks must assemble into a holoenzyme with a cyclin subunit to become catalytically active (Heldin and Purton, 1997; Sridhar *et al*, 2006). The holoenzyme however, becomes active by phosphorylation of specific residues in the Cdk catalytic subunit (Senderowicz and Sausville, 2000).

1.2.1.2 The cyclins

There are at least 15 cyclins, cyclin A to cyclin T (Senderowicz and Sausville, 2000; Sridhar *et al*, 2006). However, only 14 had been described. Four of the 14 had been characterised as primary components of the cell cycle. They are cyclins A, B, D and E. Both type D and E cyclins are fundamental in the G_1 phase, while type A and B allow cells to navigate the S and G_2/M phase of the cell cycle respectively (Gemin *et al*, 2005). Different cyclins therefore mediate different cell cycle transitions (table 1.2) (Kaelin and Tae-You, 2001; Malumbres and Barbacid, 2005). The cyclin expression varies during the cell cycle and the periodic expression of different cyclins defines the start of each new phase of the cell cycle, and also marks the transitions between the various phases (Golsteyn, 2005; Senderowicz and Sausville, 2000). All cyclins contain a highly conserved region of 100-150 amino acid residues called the "cyclin box" (Bowen *et al*, 1998), which is required for their interaction with Cdks (Heldin and Purton, 1997). Different cyclins can bind to the same Cdks. This flexibility allows the Cdks to perform distinct functions during progression through the cell cycle as well as in processes that do not relate to proliferation (Clement *et al*, 2001). Cyclins can be divided into two classes, mitotic and interphase cyclins (Murray, 2004). The mitotic cyclins have an amino terminal sequence near their N-terminal, the "destruction box" (Bowen *et al*, 1998) that permits ubiquitin-dependent degradation during anaphase (Glotzer *et al*, 1991). Whilst the interphase cyclins lack this amino terminal sequence, but instead, have a

carboxyl terminal extension rich in serine, proline, threonine and glutamate that serves the same purpose (Hanley-Hyde, 1992). The cyclic accumulation of cyclins and disappearance during the cell cycle phases provides the rational for naming these proteins "cyclins" (Tessema *et al*, 2004).

1.2.1.3 Cyclin dependent kinase inhibitor (CKI) proteins

The cyclin dependent kinase inhibitor (CKI) is known to be an inhibitory cell cycle control element (Bowen *et al*, 1998) that regulates the enzymatic activity of the Cdk negatively (Collins and Garrett, 2005). CKIs act as brakes to stop cell cycle progression in response to regulatory signals (Lee and Yang, 2001). CKIs have been reported to associate with either the Cdk complex, the cyclin or the catalytic subunit (Clement *et al*, 2001). A unique feature of CKIs is that their expression can be induced by or is correlated with a wide range of cell growth inhibitory signals, that include mitogen starvation, cell to cell contact inhibition, DNA damage, anti-proliferative cytokine treatment, terminal cell differentiation and cellular senescence (Watanabe *et al*, 1998). In addition, some CKIs were found to be tumour suppressors or are regulated by tumour suppressors and loss of their function could lead to tumourigenesis (Harper, 1997; Van de Putte *et al*, 2003). Thus far, seven CKIs have been described in the animal cell, and it can be divided into at least two structurally distinct CKI families or classes (Harper, 1997; Obaya and Sedivy, 2002). These two classes are designated as the Cdk inhibitor protein/kinase inhibitor protein (CIP/KIP) and inhibitor of Cdk4 (INK4) family (Agami and Bernards, 2002; Collins and Garrett, 2005).

The CIP/KIP family are composed of $p21^{CIP1/WAF1/CAP20/SD1}$, also known as WAF1, $p27^{KIP1}$ and $p57^{KIP2}$ (Arellano and Moreno, 1997; Clement *et al*, 2001; Hengst *et al*, 1994; Lee and Yang, 2001). The members of this family are general inhibitors, targeted to a broad spectrum of Cdks (Vogt and Reed, 1998) during all phases of the cell cycle (Deane *et al*, 2005; Pietenpol and Stewart, 2002) and are commonly referred to as "universal" CKIs (Harper, 1997). These CKIs are modular in structure and are sometimes referred to as dual specificity inhibitors (Harper, 1997). Whereas, the INK4 CKIs consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ (p15, p16, p18 and p19) (Clement *et al*, 2001; Lee and Yang, 2001). Unlike the CIP/KIP CKIs the INK4 family are specific for Cdk4 and Cdk6 (Bringold and Serrano, 2000; Renner *et*

al, 1998). For example p16 binds to Cdk4/6 and causes G_1 arrest of the cell cycle by competing with cyclin D. p16 also inhibits phosphorylation of Cdk4/6 on threonine 161 by Cdk activating kinase (CAK) (Donjerkovic and Scott, 2000). The CKIs p15 and p16 are associated with tumour suppression rather than development, whereas $p18^{INK4c}$ and $p19^{INK4d}$ (distinct from $p19^{ARF}$) are associated with development (Coskun and Luskin, 2001; Sharpless, 2004). ARF encodes $p14^{ARF}$ (in humans) and $p19^{ARF}$ (in mouse) which inhibits growth of abnormal cells by indirectly activating p53 (Agrawal *et al*, 2006; Rocha *et al*, 2003). $p14^{ARF}$ binds to mouse/human double minute gene number 2 (MDM2/HDM2) and inhibits its activity, preventing p53 degradation (Agrawal *et al*, 2006; Sharpless, 2004). MDM2 binds directly to the p53 gene to elicit degradation of p53, however, inhibition of MDM2 prevents p53 degradation and leads to the activation of the $p14^{ARF}$-MDM2-p53 pathway stimulating the increase expression of p21 (Agrawal *et al*, 2006; Sharpless, 2004). ARF regulation of p53 depends on the cell cycle status (Agrawal *et al*, 2006).

1.2.1.4 The regulation of the cyclin-Cdk complexes

The cell cycle is driven by complexes formed by the catalytic subunit, the Cdk and the regulator subunit, the cyclins (Molinari, 2000; Shimotohno *et al*, 2003). The sequential activation and inactivation of members of the Cdk subfamily of protein kinases are needed to control the progression through the cell cycle. The activities of these enzymes are regulated by activating and inactivating phosphorylation, binding to their regulatory subunits cyclins, subcellular localization, and association with inhibitory proteins, CKIs (Boxem, 2006; Malumbres and Barbacid, 2005; Tessema *et al*, 2004).

Phosphorylation controls the activity of Cdks both negatively and positively, depending on the phosphorylation sites (Van Opstal *et al*, 2005). There are three major amino acid sites of phosphorylation (Heldin and Purton, 1997; Nigg, 1995; Obaya and Sedivy, 2002). These sites are threonine (Thr 160 of Cdk2, Thr 161 of Cdk1, and Thr 172 of Cdk4/6), tyrosine (Tyr 15 of Cdk2 and Cdk1 and Tyr 17 of Cdk4 and Cdk6) and threonine 14 (Thr 14). The phosphorylation of threonine (160, 161 or 172) activates the cyclin-Cdk complex whereas tyrosine (15 or 17) and threonine 14 phosphorylations are dominant and inactivate the complex (Heldin and Purton, 1997; Tessema *et al*, 2004).

Figure 1.3 shows the regulatory mechanism of the cell cycle Cdks. The monomeric Cdk is unphosphorylated and inactive (Coqueret, 2002). The assembly of a Cdk with its corresponding cyclin yields only a partially active complex. For the complex to be completely active, it requires the phosphorylation of the Cdk on the conserved threonine (Thr 160, 161 or 172) residue proximal to the ATP binding cleft (Obaya and Sedivy, 2002). This amino acid (Thr 160, 161 or 172) site is located within a domain (the T-loop) that is present in many protein kinases and is implicated in controlling the access of substrates to the catalytic site (Coqueret, 2002). The interaction of cyclin with Cdk causes a conformational change in the Cdk, making the T-loop more accessible for the activating phosphorylation. The phosphorylation causes a further conformational change in the T-loop, making the catalytic cleft fully accessible to ATP (Obaya and Sedivy, 2002). In addition, the activating threonine phosphorylation has been suggested to enhance the stability of the cyclin-Cdk complex (Arellano and Moreno, 1997).

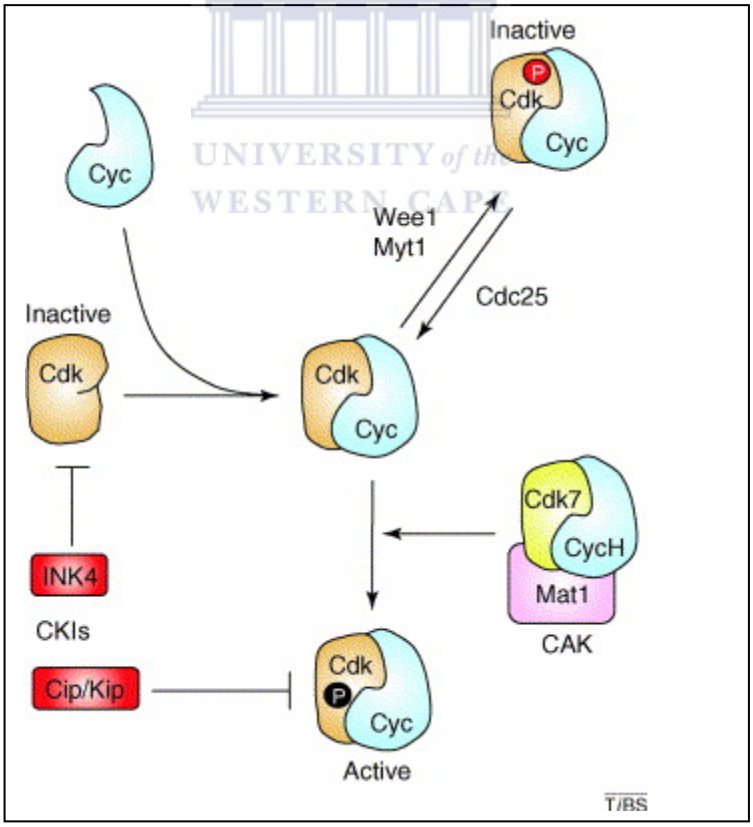

Figure 1.3: The regulation of Cdk (Malumbres and Barbacid, 2005). P = phosphorylation.

The kinase responsible for catalysing the activating threonine phosphorylation has been designated Cdk-activating kinase (CAK) (Boxem, 2006; Lolli and Johnson,

2005). The catalytic subunit of CAK was found to be itself a member of the Cdk family. This kinase was originally designated as MO15, but has since been renamed Cdk7 (Malumbres and Barbacid, 2005). CAK itself is composed of three subunits: a Cdk subunit Cdk7, cyclin H and "ménage a trios" (MAT1) (Lolli and Johnson, 2005). The function of MAT1 may be as an assembly factor that stabilizes interactions between the cyclin and the Cdk (Arellano and Moreno, 1997). Thus, CAK phosphorylates the threonine residue to increase the activity of the cyclin-Cdk complex (Clement *et al*, 2001). The phosphorylation of Cdks by CAK is antagonized by the action of a phosphatase known as Cdk associated protein phosphatase (KAP) (Obaya and Sedivy, 2002). Thus the catalytic subunit can therefore be inhibited through dephosphorylation by KAP at the activating threonine residue (Clement *et al*, 2001).

However, phosphorylation can also negatively regulate the activity of Cdks. The phosphorylation occurs near the N termini of all Cdks. The phosphorylation occur on Tyr 15 (Cdk2 and Cdk1), Tyr 17 (Cdk4 and Cdk6) and Thr 14 (Cdk2 and Cdk1) (Obaya and Sedivy, 2002). The kinases responsible for the phosphorylation of the inhibitory sites on Cdk2 and Cdk1 have been identified as Wee1 and Myt1. Both these kinases are bifunctional, thus both phosphorylate the tyrosine and threonine residues (Malumbres and Barbacid, 2005; Obaya and Sedivy, 2002). However, Wee1 shows a preference for Tyr 15 (Obaya and Sedivy, 2002), and Myt1 for Tyr 15 and Thr 14 whith a preference for Thr 14 (Chow *et al*, 2003; Pagano, 1998). The kinase responsible for phosphorylating Cdk4 and Cdk6 on Tyr 17 has not been identified (Boxem, 2006; Obaya and Sedivy, 2002). Dephosphorylation of the inhibitory sites is performed mainly by Cdc25 a dual specificity phosphatase (Pagano, 1998). Thus Cdc25 phosphatase removes the phosphate from Thr 14 and Tyr 15 and activates the complex with Thr 161 still in its phosphorylation state (Bowen *et al*, 1998; Malumbres and Barbacid, 2005).

Thus, the activity of Cdk is positively regulated by association with cyclins. Also by phosphorylation of Thr by CAK in the T-loop and by dephosphorylation of Thr 14 and Tyr 15 residues, mediated by the Cdc25 phosphatase. On the other hand, the activity of the Cdks are negatively regulated by association with CKI and by dissociation from cyclin. Also the dephosphorylation on a conserved T-loop Thr

residue by KAP, by negative phosphorylation of Thr 14 and Tyr 15 residues mediated by Wee1 and Myt1 (Lee and Yang, 2001; Malumbres and Barbacid, 2005; Obaya and Sedivy, 2002).

1.2.2 The regulation of the cell cycle

Chronological activity and inactivation of Cdks is the primary means of cell cycle regulation (Golias *et al*, 2004). A Further means of control is by the various checkpoints. The following checkpoints have been identified, in the cell cycle (I) the restriction point (mammalian) (Bartek and Lukas, 2001a) or START (yeast), which integrates internal and environmental signals early in G_1 and decides if the cell will replicate (Arellano and Moreno, 1997; Laird and Shalloway, 1997), (II) the DNA damage checkpoint, which arrests cells after DNA damage, to allow repair to occur before DNA is replicated in the S phase (Elledge, 1996), (III) a DNA damage independent, p53 mediated G_1 checkpoint (Khan and Wahl, 1998), (IV) S phase DNA damage checkpoint, which prevents initiation at DNA replicons following DNA damage, without affecting the DNA synthesis which has already started, (V) S/M DNA replication checkpoint which ensures that cells do not enter mitosis if all chromosomes are not replicated fully, (VI) activated Ras G_2 checkpoint, which arrests some type of cells when activated Ras is present (Laird and Shalloway, 1997), (VII) the G_2 DNA repair checkpoint, which surveys DNA replication for faults (Weinert, 1997) and (VIII) the mitotic spindle assembly checkpoint, which prevents chromosome segregation before spindle formation and chromosomal attachment have been accomplished (Elledge, 1996).

1.2.2.1 G_1 phase

With completion of the M phase, cells respond to extracellular signals that determine whether cells can enter G_1 phase again or exit the cell cycle to G_0 phase (Senderowicz and Sausville, 2000; Sherr, 2000). Cells in G_0 have a diploid amount of DNA, which represents the differentiated functioning cell not committed to the cell cycle (Senderowicz and Sausville, 2000). When G_0 cells are stimulated by mitogens to re-enter the cell division cycle, active cyclin D-Cdk complexes progressively accumulate as cells progress through G_1 (Foijer and Te Riele, 2006; Sherr and Roberts, 2004).

Cells entering the G_1 phase is dependent on mitogenic growth factors (Bartek and Lukas, 2001a) which drives cell cycle progression in an orderly sequential activation of cyclins and their associated Cdks (Liu and Greene, 2001; Sherr and Roberts, 2004). The functions of G_1 cyclins and Cdks in mammalian cells are closely analogous to those in budding yeast, although the mechanisms that link their activation to mitogenic signaling are significantly more elaborate. The relevant proteins include three D-type cyclins (D1, D2, and D3) that, in different combinations, bind to, and allosterically regulate, their catalytic partners Cdk4 and Cdk6 (Sherr and Roberts, 2004). These combinations yield at least six possible holoenzymes that are expressed in tissue specific patterns. The D-type cyclins are unstable, short lived and are synthesized in response to mitogenic stimuli acting as growth factor sensors, whereas the Cdks are relatively long lived (Sherr, 2000). Once synthesized the cyclins associate with Cdk4/Cdk6, forms active cyclin-Cdk complexes early in G_1 (Liu and Greene, 2001). Therefore type D cyclin plays an important role in the initiation of a new cell cycle (Gemin *et al*, 2005).

The mitogen induced signal transduction pathways promote the activation of cyclin D-Cdk complexes at many levels. These include gene transcription, cyclin D translation and stability, assembly of D cyclins with their Cdk partners, and import of the holoenzymes into the nucleus, where they ultimately phosphorylate their substrates. Once activated by the phosphorylation of CAK (Stewart and Pietenpol, 1999) the accumulation of cyclin D-Cdk complex promotes cell division by inactivating cell cycle inhibitor proteins (Sherr and Roberts, 2004) by changing the balance between the cyclin-Cdk complexes and CKIs as well as to initiate retinoblastoma protein (Rb) posphorylation (Molinari, 2000; Stewart and Pietenpol, 1999) which is a tumour suppressor gene active in the control of G_1 phase (Senderowicz and Sausville, 2000).

Given that mitogens are available and the cellular environment is favourable for proliferation, a decision to enter S phase is made at the so called ‘restriction point’ in mid-to-late G_1. Cells that are unstressed are committed to replicate DNA and division seems irreversible until the next G_1 phase. Checkpoints activated by genotoxic stress can delay cell cycle progression even when cells have already passed the restriction point (Bartek and Lukas, 2001a). It has been suggested that the

restriction point switch, from the growth factor dependency early in G_1 to the mitogen independent phases in mid-to-late G_1. This therefore reflects the induction of broad transcriptional programmes regulated by the analogous Rb and Myc pathways, which regulate genes critical for G_1/S transition and coordination of S-G_2-M progression (Bartek and Lukas, 2001a).

In phosphorylating the Rb protein, the cyclin-Cdk complexes assist in cancelling its growth repressive functions (Senderowicz and Sausville, 2000; Yamasaki and Pagano, 2004) (figure 1.4). The transcription of genes whose products are required for DNA synthesis are repressesed by Rb. Rb binds to transcription factors such as the elongation factor 2 complex (E2F) family (Sherr, 2000; Yamasaki and Pagano, 2004). The phosphorylation of Rb by the cyclin D-Cdk complexes disrupts these interactions enabling unbound E2Fs to function as transcriptional activators (Sherr, 2000). The E2F family function by activating a group of genes involved in cell progression through G_1 phase as well as DNA replication, cyclin A and E, dihydrofolate reductase and thymidine kinase (Ren *et al*, 2002; Sherr and McCormick, 2002; Stewart and Pietenpol, 1999). The E2F family and Myc jointly activate the cyclin E gene (Bartek and Lukas, 2001b) and E2Fs induce the A genes as well (Sherr, 2000). As cyclin E accumulates it forms its cyclin-Cdk complex with Cdk2. This complex activity increases the phosphorylation of Rb (Foijer and Te Riele, 2006).

The loss of dependency on extracellular growth factors at the restriction point is brought about in part by the shift in Rb phosphorylation from mitogen dependent cyclin D-Cdk complexes to mitogen independent cyclin E-Cdk2 (Sherr, 2000). The cyclin E-Cdk2 complexes are key promoting enzymes which are both rate limiting and essential for S phase entry (Bartek and Lukas, 2001a; Liu and Greene, 2001). Activity peaks at the G_1/S transition after which cyclin E is degraded and replaced by cyclin A (Sherr, 2000). Cyclin A activity appears parallel with the onset of DNA synthesis (Liu and Greene, 2001).

As a common rule, the G_1 Cdk delivers mitogenic signals to the core cell cycle machinery by impairing the functions of the cell cycle inhibitory proteins. Both the D, E and A type cyclins and their associated kinases, had been thought to be essential

and "rate-limiting" for entry into and progression through the G_1 phase of the cell cycle (Bartek and Lukas, 2001a; Sherr and Roberts, 2004).

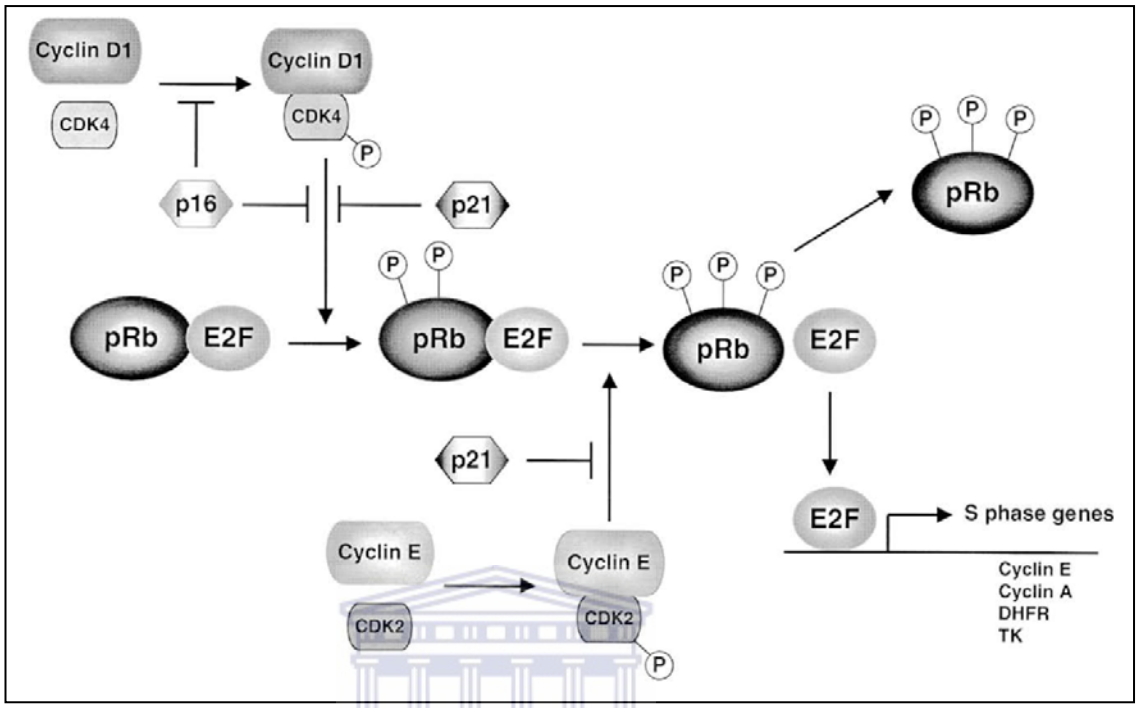

Figure 1.4: The G_1/S transition (Stewart and Pietenpol, 1999). DHFR = dihydrofolate reductase; P = phosphorylation; TK = thymidine kinase.

1.2.2.1.1 DNA damage response pathways of G_1 phase

The G_1 arrest induced because of DNA damage will cause a delay in cell cycle progression to facilitate DNA repair, thereby avoiding the replication and subsequent propagation of potentially harmful mutations. Inhibition of entry into S phase is associated with a p53 dependent and independent pathway, the Cdc25A pathway (Bartek and Lukas, 2001a and b). These pathways share the same key upstream regulators, ataxia-telangiectasia mutated (ATM)/ataxia-telangiectasia mutated and Rad3 related (ATR) and checkpoint kinase 1/2 (Chk1/Chk2) and target Cdc25A and p53 simultaneously within minutes after DNA damage. However, not all DNA damage leads to the activation of both pathways, thus it would relay information to activate both or either one or the other (Bartek and Lukas, 2001a and b).

1.2.2.1.2 The p53-p21 pathway

The p53 gene product is an important cell cycle checkpoint regulator at both the G_1/S and G_2/M check points but does not appear to be imperative at the mitotic

spindle check point since gene knockout of p53 did not alter mitosis (Golias *et al*, 2004). p53, a tumour suppressor, is a homotetrameric transcription factor that is activated in response to many forms of cellular stress, including irradiation, hypoxia, drug induced genotoxic damage, and even oncogene activation (Sherr, 2004), and is the most frequently mutated gene in human cancer (Golias *et al*, 2004; Senderowicz, 2004).

This indicates its vital role in conservation of normal cell cycle progression. One of the key functions of p53 is to arrest cells in G_1 after genotoxic damage, to allow for DNA repair prior to DNA replication and cell division (Golias *et al*, 2004). p53 regulate a transcriptional response that either inhibits cell proliferation or induces apoptosis in response to major DNA damage (Sherr, 2004). The transcription of a number of genes can be affected by activation of p53. The anti-proliferative effect of wild type p53 is mediated by stimulation of a CKI, the p21 that inhibits Cdk activity (Golias *et al*, 2004). p21 induced by p53 dependent transactivation elevates, binds and inactivates cyclin D-Cdk4/6 as well as cyclin E-Cdk2 complexes resulting in Rb protein hypophosphorylation and cell cycle arrest (Pietenpol and Stewart, 2002). Thus p21 is capable of inhibiting the Cdks which are essential for S phase entry (Bartek and Lukas, 2001a).

Before cell cycle progression becomes blocked, the p21 protein has to accumulate to levels sufficiently high to inhibit the cyclin-Cdk complexes (Bartek and Lukas, 2001b). The molecular mechanics of the p53 pathway indicates an early activation (within a few minutes after DNA damage) of ATM/ATR. The choice between the two depends on the precise nature of the DNA lesion. ATM/ATR then phosphorylate a range of substrates, including p53 (on serine 15) and the checkpoint kinases Chk1 and Chk2 (again dependent on the type of damage) which become activated and subsequently propagate the signal to downstream effectors (Bartek and Lukas, 2001a and b). According to Bartek and Lukas (2001a), the activated Chk1 and Chk2 phosphorylate p53 on serine 20, an event which leads to decreased protein turnover and thus accumulation of p53. The high lability of the p53 protein in non-damaged cells depends on MDM2, which binds to the N-terminus of p53 around serine 20 and targets p53 for ubiquitination and proteasome-mediated degradation (Bartek and Lukas, 2001a). Phosphorylation of serine 20 prevents efficient interaction of p53

with MDM2 and appears essential for stabilisation of p53 after DNA damage, but other modifications of p53 itself and phosphorylation of MDM2 by the ATM/ATR and Chk1/Chk2 kinases are likely to be involved as well. MDM2 is itself transcriptionally activated by p53, thereby creating a negative feedback loop with p53. This interplay is further complicated by the dynamic changes of their subcellular localisation. The nuclear import and export of p53 may depend on its modifications, and it had been shown that p53 uses its N-terminus to interact with the microtubules and exploit these structures to aid its nuclear localisation (Bartek and Lukas, 2001a).

Thus DNA damage generates a signal that can activate p53 by post-translational modification (figure 1.5, page 20). Increased p53 activity up regulates p21, which prevents activation of Cdks, required for the G_1/S transition (Golias *et al*, 2004). This protein is up regulated by the p53 tumour suppressor gene in response to DNA damage, and it is also up regulated during replicative cellular senescence (Obaya and Sedivy, 2002). Thus, over expression of this inhibitor leads to cell cycle arrest (Harper, 1997), particularly in G_1 and it also plays an important role at the G_2/M phase transition (Lee and Yang, 2001). p21 was initially identified in normal human fibroblasts as a quaternary cylcin D-Cdk complex that contained proliferating cell nuclear antigen (PCNA) (Watanabe *et al*, 1998). The cyclin-Cdk complex binding domain resides in the N-terminal half of p21 and the PCNA binding site resides in half the C-terminal (Arellano and Moreno, 1997). PCNA is a subunit of DNA polymerase which is essential for both replication and repair of DNA synthesis (Vogt and Reed, 1998). PCNA is also required for nucleotide excision repair of DNA. The interaction of p21 with PCNA facilitates a proper balancing of the DNA replication and repair machinery throughout the cell cycle (Gramatieri *et al*, 2003). Since ternary complexes can be formed with cyclin-Cdk and p21, PCNA seems not necessary for interaction of p21 with Cdks (Bowen *et al*, 1998). This negative cell cycle controller effect may explain why the wild type p53 gene can suppress the transformation of cells by activated oncogenes, thereby inhibiting the growth of malignant cells *in vitro* and suppressing the tumourigenic phenotype *in vivo* (Golias *et al*, 2004). The cell cycle checkpoints may be further prolonged by an additional mechanism (figure 1.5, page 20). A gradual accumulation of p16, a protein that can selectively disrupt the cyclin D-Cdk4/6 complexes, and could lead to the release of

the existing pool of p21. When the threshold level of p21 is achieved, p21 can bind and inhibit the S phase promoting cyclin E-Cdk2 complexes, and thereby secure the maintenance of the G_1 arrest. The inhibition of both the cyclin-Cdk complexes leads to the dephosphorylation of Rb which therefore causes the inhibition of the E2F dependent transcription of S phase genes (Bartek and Lukas, 2001b).

1.2.2.1.3 The Cdc25A pathway

The Cdc25 family consists of Cdc25A, Cdc25B and Cdc25C, which appear to have specificity for diverse cyclin-Cdk complexes. Cdc25A promotes entry into S phase by acting on cyclin A-Cdk2 and cyclin E-Cdk2, both Cdc25B and Cdc25C play a role in the commencement of mitosis (Smits and Medema, 2001).

Preventing entry into S phase with damaged DNA, cells traversing G_1 activate the checkpoint transducing kinases ATM/ATR and Chk1/Chk2 which, in turn, is p53/p21 independent (figure 1.5, page 20) (Bartek and Lukas, 2001a; Lukas *et al*, 2004). This leads to a decrease in the total cellular activity of the Cdc25A phosphatase when cells are exposed to ultraviolet (UV) light or γ-radiation, reflecting DNA damage induced ubiquitination and accelerated turnover of the Cdc25A protein by proteasome (Bartek and Lukas, 2001a). Chk1 and Chk2 phosphorylates Cdc25A on multiple serine residues which leads to enhanced ubiquitination and proteasome-mediated degradation of Cdc25 (Lukas *et al*, 2004). The destruction of Cdc25 and the activation of Wee1 by Chk1/Chk2 might contribute to Thr 14/Tyr 15 phosphorylation of Cdk2 (Chow *et al*, 2003).

The pathway leads to a persistent inhibitory phosphorylation of Cdk2 on Thr 14/Tyr 15, and thus inhibition of cyclin E-Cdk2 and cyclin A-Cdk2 complexes and G_1/S arrest (Bartek and Lukas, 2001a). The inhibition of Cdk2 activity blocks the association of chromatin and Cdc45, a protein required for recruitment of DNA polymerase α, into assembled pre-replication complexes (Nakajima and Masukata, 2002), thus preventing initiation of DNA synthesis (Lukas *et al*, 2004). The expression of Chk1 is also synchronized by E2F, and the basal activity of Chk1 in undamaged cells is required for the constitutive turnover of Cdc25A during late G_1, S and G_2 phases, a mechanism that keeps Cdc25A labile and poised to respond by accelerated degradation upon DNA damage. This pathway that targets Cdc25A is

implemented rapidly, and operates independently of the p53 status, and it is relatively transient, able of delaying cell cycle progression for only few hours. On the other hand, the complementary mechanism depend on p53 responsible for the prolonged maintenance of the G_1 cell cycle arrest in response to DNA damage reflects the other pathway of the G_1 checkpoint as discussed earlier in the text (Lukas *et al*, 2004).

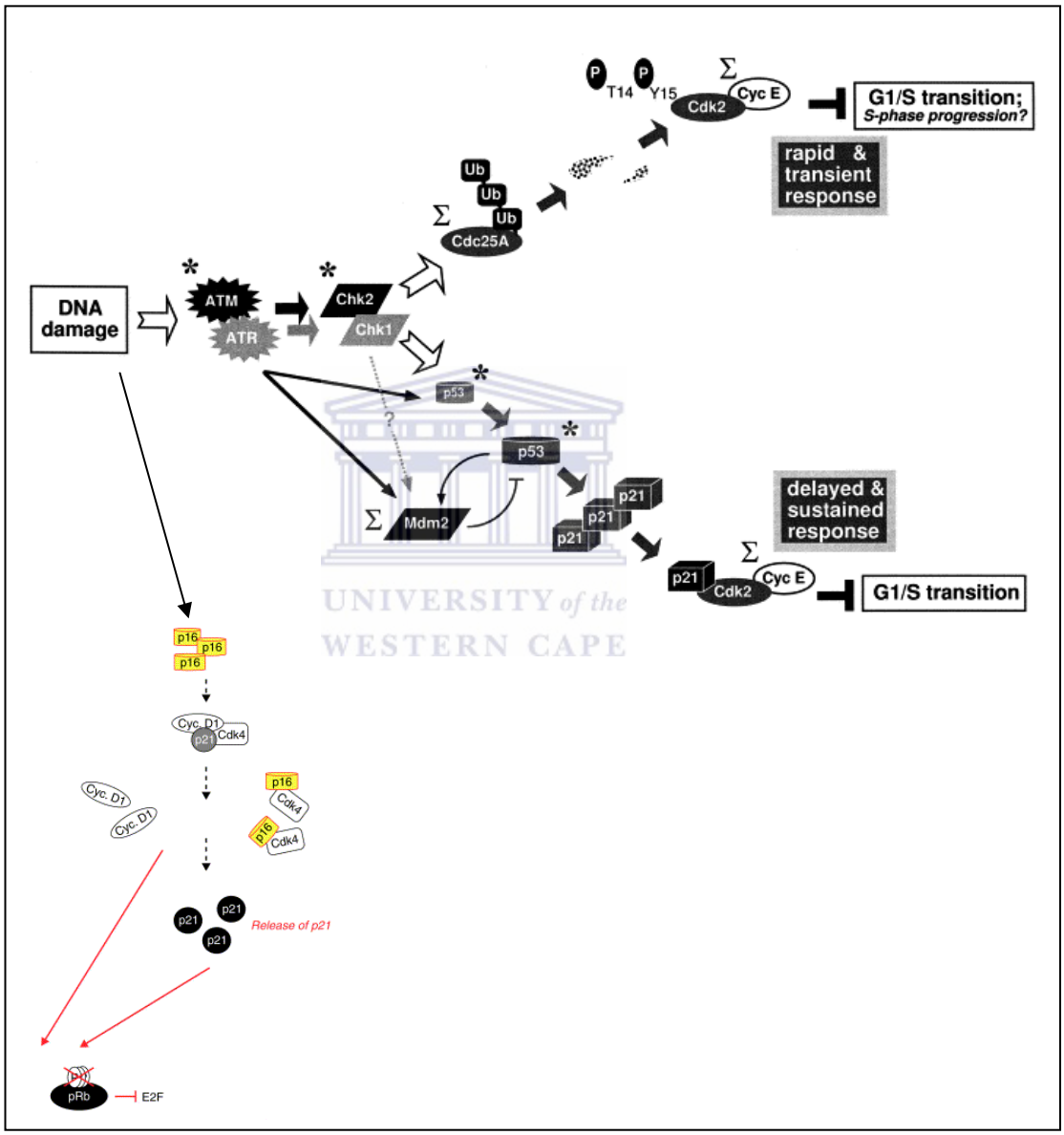

Figure 1.5: The G_1 checkpoint pathways inducing both rapid and delayed G_1/S responses to acute genotoxic stress as adapted from Bartek and Lukas (2001a and b). P = phosphorylation.

1.2.2.2 S phase

DNA synthesis takes place in the S phase (Senderowicz, 2004). During this highly regulated phase all the genetic materials are faithfully duplicated (Masai *et al*, 2005).

The activity of cyclin A is essential for DNA replication and rate limiting to the cell cycle progression (Bartek and Lukas, 2001a). It activates two diverse Cdks and functions in both S and M phase (Yam *et al*, 2002). There are two type A cyclins (A1 and A2). The A1 type is only expressed during meiosis, and in a number of tumour cells, whereas type A2 is present in all proliferating somatic cells. Cyclin A starts to accumulate during late G_1 phase and continue through S phase and G_2 phase (Fung and Poon, 2005). Formation and activation of the prereplication complex requires coordinate actions of G_1 and S phase cyclin-Cdk complexes. Cyclin E-Cdk2 and cyclin A-Cdk2 phosphorylate numerous components of the prereplication complex and replication machinery (Woo and Poon, 2003).

In the S phase, phosphorylation of components of the DNA replication machinery such as Cdc6 by cyclin A-Cdk complexes is believed to be important for initiation of DNA replication (Ayad, 2005) and to restrict the initiation to only once per cell cycle (Yam *et al*, 2002). The synthesis of cyclin A is controlled during the late G_1, involving E2F release from Rb and other transcription factors (Yam *et al*, 2002). However, cyclin A is destroyed slightly ahead of cyclin B and starts to degrade during prometaphase and degradation is completed at metaphase (Fung and Poon, 2005).

Numerous replication proteins are potential substrates for cyclin A-Cdk complex, suggesting that initiation, elongation and checkpoint control of replication could all be regulated by Cdk2.

1.2.2.2.1 DNA replication

Transmission of genetic information from one cell generation to the next requires the accurate duplication of the DNA during the S phase of the cell cycle (Stillman, 1996). Assisting with this is the DNA polymerases. DNA polymerase (pol) α synthesize primers required for replication; DNA pol β is thought to be primarily involved in repair; DNA pols ε and δ are essential for replicative DNA synthesis and DNA pol γ is required for mitochondrial DNA replication (Mozzherin *et al*, 1997).

The first step during DNA replication is structural distortion of the DNA double helix at the replication fork. This is brought about by the protein helicase, which

interacts with the DNA (Bowen *et al*, 1998). DNA replication is semi-discontinuous having a leading strand which is synthesized continuously, from a single initiation event at the replication origin (MacNeil, 2001).

Proliferating cell nuclear antigen (PCNA) is a homotrimeric sliding clamp which encircles the DNA duplex (Lehmann, 2005; MacNeil, 2001). However, PCNA functions at various levels of DNA metabolic pathways. When bound to DNA, PCNA is involved in DNA replication, DNA repair, DNA modification, and chromatin modeling (PCNA not binding to DNA, promotes localization of replication factors with a consensus PCNA binding domain to replication factories) (Majka and Burgers, 2004). PCNA was identified as a specific auxiliary factor of pol δ however PCNA also interacts specifically with several other cellular proteins. These include replicating factor C (RFC), flap structure specific endonuclease 1 (FEN1), p21, Gadd 45 and cyclin D (Mozzherin *et al*, 1997). Once PCNA has been loaded, pol δ assembles with RFC and PCNA into its holoenzyme form (Bowen *et al*, 1998; Uhlmann *et al*, 1996) and synthesis begins (figure 1.6) (MacNeil, 2001).

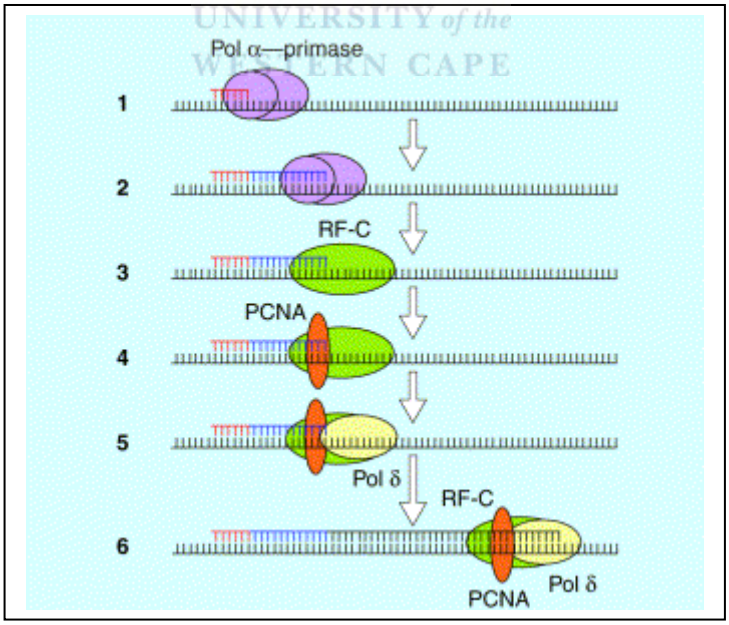

Figure 1.6: A schematic representation of the Okazaki fragmentation synthesis (MacNeil, 2001). Pol α = polymerase alpha; Pol δ = polymerase delta; PCNA = proliferating cell nuclear antigen; RF-C = replication factor C.

Synthesis then continues at least until the 5′ end of the previous Okazaki fragment is reached, at which point Okazaki fragment processing occurs to join the newly

synthesised fragment to the lengthening lagging strand (MacNeil, 2001). DNA pol δ is essential for both leading and lagging strand DNA synthesis (Maga *et al*, 2001). The pol δ complex thus continues replication of the leading strand of DNA and the elongation of the Okazaki fragments complementary to the lagging strand. DNA replication is completed when the RNA primers are removed and the Okazaki fragments are connected (Bowen *et al*, 1998). An error free DNA replication and chromosome segregation will ensure that the cell progress through the cell cycle (Abraham, 2001, Lukas *et al*, 2004).

1.2.2.2.2 S phase DNA damage checkpoints

Replication progression can be compromised by exogenous stress such as direct DNA damage or inhibitors of DNA replication. These exogenous stresses are known as replication fork barriers (RFBs) which slow down or stall fork progression. Replication progression could be compromised by different kinds of RFBs (Lambert and Carr, 2005). Some examples of RFBs include; (1) DNA protein complexes, that lead to natural "pause sites" that are active in every S phase, (2) alternative DNA metabolism, (3) secondary DNA structure, (4) DNA damage which could be produced by endogenous metabolism or induced by exogenous carcinogens including UV, methyl-methanesulfonate (MMS), camptothecin (CPT) and inter strand cross linking agents, and (5) replication inhibitors which competitively inhibits pol α (Lambert and Carr, 2005). Because of the genetic instability caused by the RFB, the nature of the cellular response could lead to DNA structure checkpoint pathways. The intra-S phase checkpoint prevents the firing of new replication forks by inhibiting initiation at licensed but unfired origins (Lambert and Carr, 2005), and result in a reduction in the rate of progression through the S phase rather than an arrest (Oakley and Hickson, 2002). Thus it delays the cell cycle. The DNA replication (S/M) checkpoint is responsible for monitoring the progression of DNA replication and ensuring that mitosis does not occur before replication is complete (Oakley and Hickson, 2002).

1.2.2.2.3 The intra-S phase checkpoint

The S phase checkpoint activated by genotoxic insults causes only temporary, reversible delay in cell cycle progression, mainly by inhibition of new replicon initiation and thereby slowing down DNA replication (Bartek and Lukas, 2001b) but

it can not be permanently arrested (Oakley and Hickson, 2002). Therefore, unlike the G_1 or G_2/M checkpoints, the intra-S phase response to DNA damage lacks the sustained maintenance phase of the cell cycle arrest by the key role player p53 (Bartek and Lukas, 2001b) and is thus independent of p53 (Lukas *et al*, 2004). The initial pathway described so far and the only one understood mechanistically is the ATM/ATR-Chk1/Chk2-Cdc25A-cyclin E(A)/Cdk2-Cdc45 cascade (Lukas *et al*, 2004).

The ATM/ATR-Chk1/Chk2-Cdc25A-cyclin E(A)/Cdk2-Cdc45 cascade emerges as a key mechanism of not only the rapid prevention of S phase admission in the G_1 checkpoint, but also in the transient intra-S phase response. Activation of this pathway affects both the early and late firing origins of DNA replication (Bartek and Lukas, 2001b). Inhibition of Cdk2 activity through Cdc25A degradation leads to a several hour delay of S phase progression. ATM phosphorylates several checkpoint components including Chk2, which then targets not only Cdc25A, but also BRCA1 and Nbs1, a component of the Mre11-Rad50-Nbs1 protein complex (MRN). However, it is not clear whether or not all these regulators feed into the Cdc25A pathway, or whether parallel mechanisms co-operate to inhibit DNA replication (Bartek and Lukas, 2001b).

1.2.2.2.4 The DNA replication (S/M) checkpoint

Disturbance of replication leads to four cellular responses. (I) The inhibition of late origin firing, (II) stabilization of active replication forks, (III) a delay into mitotic entry and (IV) slowing down of replication fork progression on damaged templates. The first three responses are fully dependent on the S phase checkpoint proteins whereas the later is not (Lambert and Carr, 2005).

Figure 1.7 illustrates signal propagation at the slowed down fork replication. Stalled forks produce single stranded DNA that activates the replication checkpoint. This checkpoint functions to protect the stability of the fork until the replication can resume (Branzei and Foiani, 2005). At the onset of DNA replication there is an association of checkpoint complexes with chromatin. The association complexes consist out of the initiation factors of minichromosome maintenance (MCM), replication protein A (RPA) and Cdc45. They bind to the pre-RC (pre-replicative

complex) and onto the chromatin and unwind the DNA (Nakajima and Masukata, 2002; Osborn *et al*, 2002; Sánchez and Dynlacht, 2005). ATR the primary S phase checkpoint kinase binds to ATR interacting protein (ATRIP). ATR is important in preventing replication of damaged DNA and inhibits entry into mitosis (Gottifredi and Prives, 2005; Shechter *et al*, 2004). The ATR/ATRIP complex is recruited onto the chromatin after DNA unwinding (Gottifredi and Prives, 2005).

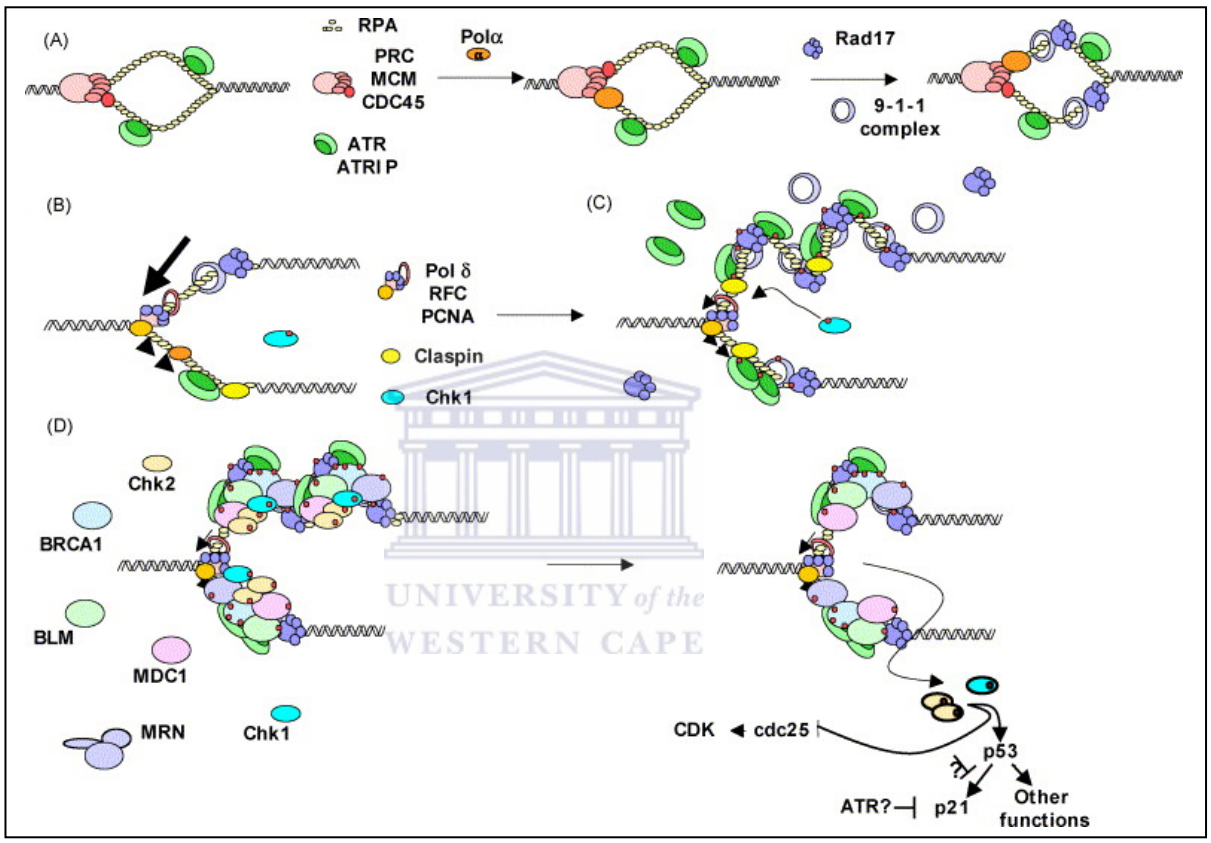

Figure 1.7: An illustration of the signal propagation of a stalled fork replication (Gottifredi and Prives, 2005). The red dots represent phosphorylation. BLM = Bloom's syndrome helicase; MDC1 = mediator of DNA damage checkpoint protein 1; MRN = Mre11-Rad50-Nbs1 protein.

Cdc45 is important for DNA polymerase recruitment (Sánchez and Dynlacht, 2005). The ATR/ATRIP complex associates with RPA independently without being recruited by pol α (Gottifredi and Prives, 2005). Full activation of the ATR/ATRIP complex however needs the association of the replication factor C (RFC) and the PCNA like proteins to this complex (Osborn *et al*, 2002). DNA pol α is however required for the recruitment of Rad17-RFC and the sliding clamp (Parrilla-Castellar *et al*, 2004). Rad17-RFC complex loads a PCNA like trimeric checkpoint sliding clamp onto DNA. The PCNA like trimeric checkpoint sliding clamp bound DNA polymerases. The PCNA like trimeric complex resembles PCNA structurally and are

composed of Rad9, Hus1 and Rad1 (9-1-1 complex) and are required for the activation of the checkpoint signalling pathway (Lambert and Carr, 2005; Osborn *et al*, 2002; Parrilla-Castellar *et al*, 2004). The DNA pol δ complex extends the leading strand and pol α synthesizes the lagging strand (Gottifredi and Prives, 2005). The chromatin bound 9-1-1 complex assist in facilitating the ATR/ATRIP complex in phosphorylating Check (Chk) 1 (Parrilla-Castellar *et al*, 2004). Chk1 has a role in the maintenance of fork stability and claspin bound to the fork (Gottifredi and Prives, 2005). Chk1/Chk2 transfer the signal of DNA damage to the cell division cycle proteins Cdc25A, Cdc25B and Cdc25C (Houtgraaf *et al*, 2006) and perhaps p53 where p53 transcriptional activity might be modulated to guarantee the reversibility of the arrest (Gottifredi and Prives, 2005). Phosphorylation of these proteins by Chk1/Chk2 inactivates the transition of G_1/S and G_2/M (Houtgraaf *et al*, 2006). The above recommend that the foremost function of the S phase checkpoint is to maintain fork integrity (Gottifredi and Prives, 2005).

1.2.2.3 G_2/M transition phase

The transition of G_2/M is regulated by the cyclin A-Cdk1 and cyclin B-Cdk1 complexes (Malumbres and Barbacid, 2005). During the S phase, cyclin A binds to Cdk2 and in G_2 and M phase to Cdk1. Cyclin A is considered to be involved in the regulation of S phase entry and is important in G_2 and M phase (Smits and Medema, 2001), therefore activating both Cdk2 and Cdk1 as mentioned earlier in the text, while cyclin B only activates Cdk1 (Fung and Poon, 2005).

The entry into mitosis is controlled by cyclin B which is also known as a mitotic cyclin (Fung and Poon, 2005; Smits and Medema, 2001). There are three B-type cyclins (B1, B2, and B3). Cyclin B1 and cyclin B2 are co-expressed in the bulk of dividing cells, but are differentially localized within the cell (Fung and Poon, 2005). Cyclin B1 co-localizes with microtubules during interphase and cyclin B2 is associated with the Golgi apparatus (Ohi and Gould, 1999). Cyclin B3 expression is restricted to developing germ cells and in the adult testis (Fung and Poon, 2005) and is constitutively nuclear bound throughout the cell cycle (Ohi and Gould, 1999). The protein levels of cyclin changes cell progression, thus entery into mitosis is dependent on the synthesis of cyclin B (Golsteyn, 2005; Le Breton *et al*, 2005). However, mitosis is initiated by increased activity of the cyclin B-Cdk1 complex,

known as mitosis promoting factor or maturation promoting factor (MPF) (Fung and Poon, 2005; Ohi and Gould, 1999; Smits and Medema, 2001).

The cyclin B-Cdk1 complex levels rise during G_2 and peak in mitosis. Before mitosis, cyclin B-Cdk1 complexes are held in an inactive state by phosphorylation of Cdk1 at Thr 14 and Tyr 15, which is catalyzed by the protein kinases Wee1 (which phosphorylates Tyr 15 only) and Myt1 (which phosphorylates both Thr 14 and Tyr 15) (Stewart and Pietenpol, 1999). The dephosphorylation of Cdk1 of the complex is the rate limiting step for entry into mitosis (Molinari, 2000). Two Cdc25 phosphatases (B and C) play an important role in the onset of mitosis. Cdc25B is thought to regulate centrosomal microtubule nucleation during mitosis. While the Cdc25C phosphatase is responsible for triggering activation of cyclin B-Cdk1 complexes by dephosphorylating the inhibitory Cdk1 sites Thr 14 and Tyr 15 (Golsteyn, 2005; Karlsson-Rosenthal and Millar, 2006; Smits and Medema, 2001; Stewart and Pietenpol, 1999; Takizawa and Morgan, 2000). The activation is also associated with the phosphorylation of Cdk1 on Thr 162 by CAK (figure 1.8) (Malumbres and Barbacid, 2005; Stewart and Pietenpol, 1999).

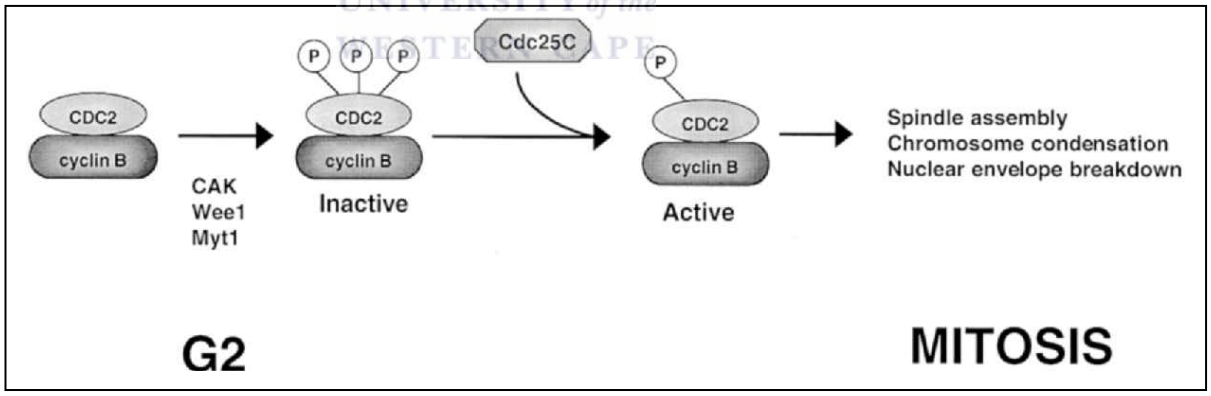

Figure 1.8: The G_2/M transition (Stewart and Pietenpol, 1999). P = phosphorylation.

Cyclin B is initially localized in the cytoplasm during S phase and G_2 phase, and translocated to the nucleus at the start of mitosis (Smits and Medema, 2001). During interphase, cyclin B-Cdk1 is found entirely in the cytoplasm, either in a soluble form or associated with the microtubule network and centrosomes. During late prophase, the active cyclin B-Cdk1 complex translocate to the nucleus to begin phosphorylating nuclear substrates. Most cyclin B-Cdk1 complexes are translocated rapidly from the cytoplasm to the nucleus, after which the nuclear envelope breaks

down. A small amount of the cyclin B-Cdk1 complexes remains in the cytoplasm during prophase and is associated with the duplicated centrosomes as they separate (Takizawa and Morgan, 2000).

The cytoplasmic localization of cyclin B during interphase is attributed to a 42 residue region in the amino-terminal of cyclin B, which is known as the cytoplasmic retention sequence (CRS). The mitotic onset requires phosphorylation of cyclin B within the CRS region (thus inactivating the region), thereby enhancing import and inhibiting export of the complex in the nucleus (Porter and Donoghue, 2003; Takizawa and Morgan, 2000).

1.2.2.3.1 G_2 (G_2/M) checkpoint

The G_2 (G_2/M) checkpoint prevents cells from entering mitosis when DNA damage occurs during G_2, or if they progress into G_2 with some unrepaired damage inflicted in the previous G_1 and S phase (Andreassen *et al*, 2003; Lukas *et al*, 2004). The biochemical pathways involved in the DNA damage induced G_2 checkpoints are thought to involve signaling cascades that unite to inhibit the activation of Cdk1 (Weinert, 1997).

Following DNA damage, members of the phosphoinositide-3 kinase (PI3K) family become activated and initiate signal transduction pathways that regulate DNA repair and cell cycle progression. Numerous members of the PI3K family can directly phosphorylate p53, including DNA activated protein kinase (DNA-PK), ATM and ATR (Pietenpol and Stewart, 2002; Stewart and Pietenpol, 1999). Activation of cyclin B-Cdk1 is prevented through ATM/ATR and Chk1/Chk2 mediated subcellular sequestration and or inhibition of the Cdc25C phosphatase that normally activates Cdk1 at the G_2/M boundary subsequent of DNA damage (Lukas *et al*, 2004). The other two members of the Cdc25 phosphatases, Cdc25A and Cdc25B are also influenced. In response to DNA damage or incomplete replicated DNA, Cdc25A becomes degraded in G_2, probably via the same mechanism as in the G_1 and S phases. However, the fate of Cdc25B after most genotoxic damage is less clear (Lukas *et al*, 2004). DNA damage also induces ATM dependent signaling which results in activation of the Chk1 and Chk2 kinases resulting in phosphorylation of p53. After ATM dependent activation, Chk1 and Chk2 furthermore phosphorylate

Cdc25C on Ser 216. Phosphorylation of Ser 216 on Cdc25C generates a consensus binding site for 14-3-3 proteins (Karlsson-Rosenthal and Millar, 2006; Molinari, 2000; Pietenpol and Stewart, 2002; Stewart and Pietenpol, 1999). Binding of 14-3-3 proteins to Cdc25C results in the nuclear export of Cdc25C impounding of the phosphatase to the cytoplasm and thus inhibition of cyclin B-Cdk1 complex activity (figure 1.9 (A)) (Boutros *et al*, 2006; Pietenpol and Stewart, 2002; Stewart and Pietenpol, 1999).

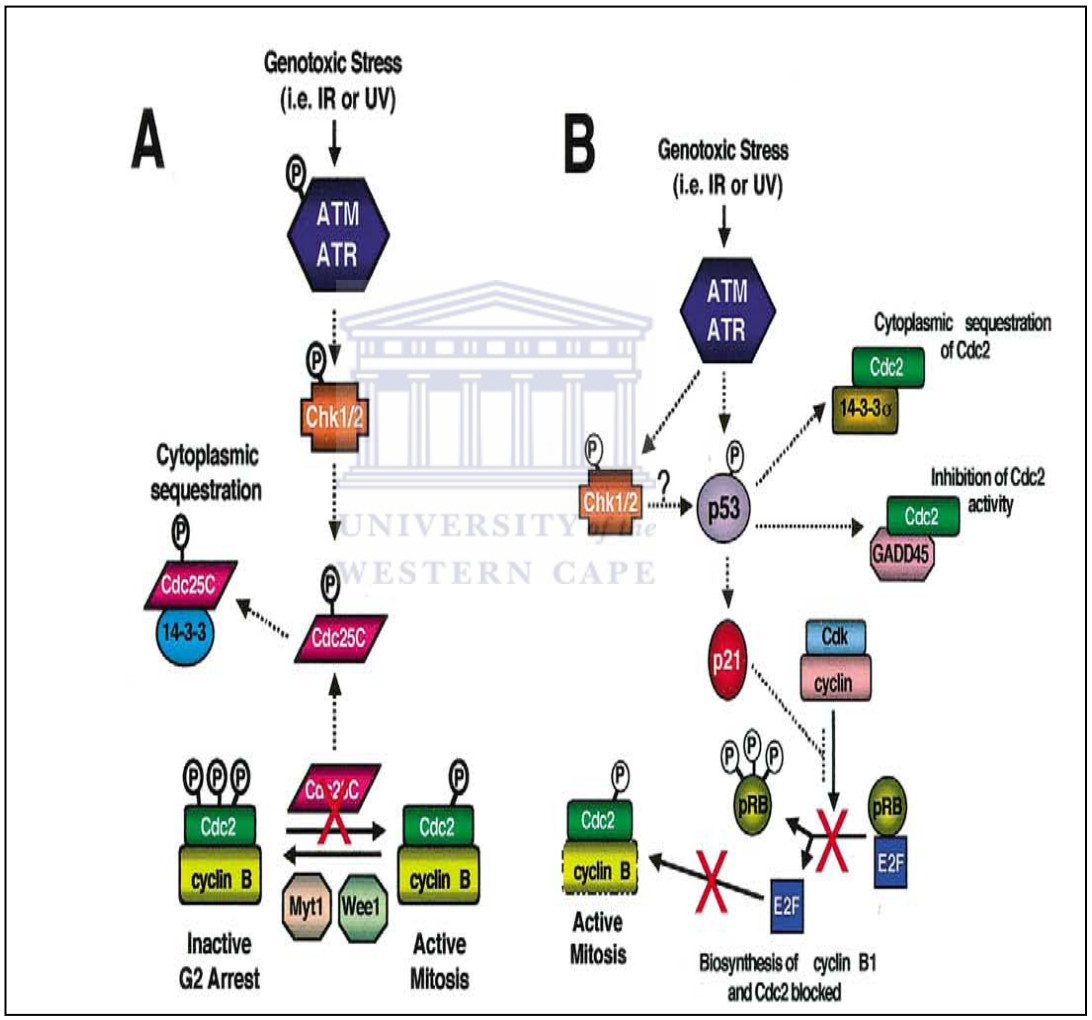

Figure 1.9: G_2 (G_2/M) checkpoints (A) inactivation of Cdc25C (B) p53 pathway (Pietenpol and Stewart, 2002). IR = infra red; P = phosphorylation; UV = ultraviolet.

The mechanism that contributes to long term silencing of cyclin B-Cdk1 is the p53 pathway (figure 1.9 (B)) (Lukas *et al*, 2004). However, to maintain the G_2 arrest following DNA damage, p53 and p21 are required (Pietenpol and Stewart, 2002). In addition to the p53 regulated CKI p21 (which is critical for the G_1 checkpoint), the

G_2 arrest appears to also require additional transcriptional targets of p53. These include the GADD45 and 14-3-3σ proteins (Lukas *et al*, 2004).

The mechanism of p53 dependent G_2 arrest involves an initial inhibition of cyclin B-Cdk1 activity by p21 and a successive reduction of cyclin B and Cdk1 levels. The reduced expression of cyclin B-Cdk1 is mediated in part by p53 dependent repression of the cyclin B and Cdk1 promoters. The 14-3-3σ can modulate the subcellular localization of the cyclin B-Cdk1 complex, as the binding of 14-3-3σ to Cdk1 results in retention of the kinase in the cytoplasm. Loss of 14-3-3σ also results in abolishment of the DNA damage G_2 checkpoint and premature mitotic entry. GADD45 expression in normal fibroblasts induces a G_2 arrest that is abolished by the over expression of cyclin B or Cdc25C (Pietenpol and Stewart, 2002).

1.2.2.4 Progression into and out of mitosis

The commencement of mitosis is brought about by the activation of Cdk1 (Karlsson-Rosenthal and Millar, 2006; Malumbres and Barbacid, 2005). However, both Cdk1 and the anaphase promoting complex or cyclosome (APC/C) plays key roles in controlling chromosome behaviour and its co-ordination with cell division (Zachariae, 1999).

Cdk1 triggers cellular processes required for chromosomal separation. These processes include the phosphorylation of histones for chromatin condensation, a 13S condensing complex for DNA supercoiling, phosphorylation of lamin B for the breakdown of the nuclear envelope, phosphorylation of nucleolin and NO38 for nucleolar disappearance and a formation of a mitotic spindle that can capture microtubules (Malumbres and Barbacid, 2005; Zachariae, 1999). The activation of the cyclin-Cdk1 induces chromatin condensation and centrosome separation (Boutros *et al*, 2006).

Mitosis constitutes two non-reversible transitions, both of which are guarded by checkpoints. The first is the prophase-to-prometaphase transition, which culminates with nuclear envelope breakdown. This defines the G_2/M transition, and it is regulated by those checkpoints that operate during late G_2. The subsequent transition is the metaphase-to-anaphase transition, which leads to the disjunction of sister

chromatids and exit from mitosis. This transition is regulated by pathways operated during mitosis that are defined first and foremost by the spindle assembly checkpoint (Morrison and Rieder, 2004). The spindle assembly in somatic cells begins during prophase while the nuclear envelope is still intact (Kline-Smith and Walczak, 2004). Several stages need to occur to create the spindle assembly. Microtubule nucleation from the centrosomes, microtubule dynamics must be regulated, motor proteins are key players, both in the assembly of the spindle and in the segregation of chromosomes and finally, accurate movement of chromosomes requires a complex integration of motor-protein action and microtubule dynamics (Walczak, 2000).

Cells employ a multi-step cascade to initiate chromosome segregation during mitosis (Tang *et al*, 2004). As the cell enters mitosis, microtubules become the key structural component of the mitotic spindle on which the chromosomes are segregated to the two daughter cells (Walczak, 2000; Wang *et al*, 2003). The spindle consists of microtubules, polar dynamic fibers that polymerize from tubulin subunits, as well as hundreds of other proteins that function together to orchestrate chromosome segregation (Gadde and Heald, 2004). Microtubules must be arranged into a bipolar array or also known as an amphitelic attachment such that each half spindle contains uniformly oriented microtubules (Kapoor, 2004). With their minus ends at the spindle pole [centrosomes function as the spindle poles by virtue of their ability to nucleate and anchor microtubules and often act as the sole microtubule organizing centre (MTOC) (Fisk *et al*, 2002; Rieder *et al*, 2001)] and their plus ends extending away from the poles (Gadde and Heald, 2004). The bipolarity of the spindle is defined by centrosomes in vertebrates and spindle pole bodies (SPB) in yeast (Taylor *et al*, 2004). With the exception to chromosomes, centrioles are the only organelles that duplicate precisely once per cell division (Marshall and Rosenbaum, 1999). The duplication of the centrosome is one of the earliest structural changes that can be observed directly in a cell that qualifies for mitosis (Uzbekov *et al*, 2002). Duplication of the centrosome occurs during the G_1/S phase transition (Rieder *et al*, 2001) and usually starts at the end of G_1 with the replication of the centrioles (Uzbekov *et al*, 2002).

Every duplicated chromosome has a pair of specialized structures at its centromere, called kinetochores, [the kinetochores play a key role in regulating the spindle

checkpoint (Taylor *et al*, 2004)] which function to attach sister chromatids to microtubules from opposite spindle poles, to allow for directed translocation of chromosomes within the spindle (Gadde and Heald, 2004). A segment of the microtubule plus ends attaches to sister chromatids at the kinetochore. Kinetochore microtubules form morphologically distinct bundles called K fibers. They maintain attachment of chromosomes to the spindle and allow them to align and segregate. The remainder of the spindle is made up of interpolar microtubules, some of which extend into the spindle midzone, where they overlap with microtubules from the opposing pole. The interpolar microtubules assist in stabilizing the bipolar spindle through prometaphase and metaphase and enable spindle pole separation during the later stages of mitosis (Kline-Smith and Walczak, 2004). An increase in the frequency of microtubule shrinkage events, known as catastrophes, and a decrease in events rescuing growth contribute to the dismantling of the interphase array, thus allowing interaction between dynamic microtubule plus ends and the condensed chromosomes (Gadde and Heald, 2004). When the microtubules and kinetochore encounter one another, the microtubules becomes stabilized or 'captured", binding the chromosome to that pole. When the sister kinetochore is captured by microtubules emanating from the opposite pole, the now bi-oriented chromosomes congresses to the metaphase plate. As a result in order to maintain genome stability, anaphase must be delayed until the chromosomes are correctly bi-oriented (Taylor *et al*, 2004).

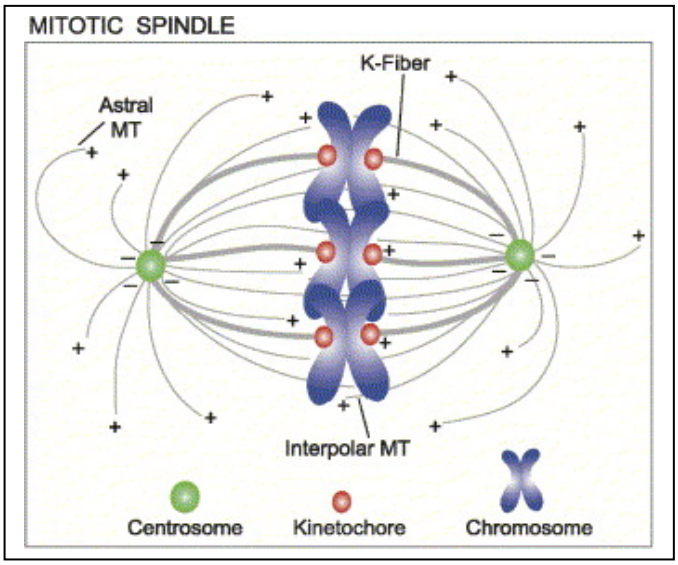

Figure 1.10: Components of the mitotic spindle (Kline-Smith and Walczak, 2004). MT = microtubules.

When all the chromosomes are bi-oriented and aligned, the cell is in metaphase. Thus the kinetochore fibers, the interpolar microtubules that overlap to form an antiparallel array, and the astral microtubules, that extend from each centrosome away from the spindle where they can interact with the cell cortex (figure 1.10) all contribute to the bipolar structure. When the chromosomes are aligned and oriented, the cellular checkpoint will therefore be content (Gadde and Heald, 2004).

Chromosome movement occurs in two steps (figure 1.11): (I) anaphase A, in which chromosomes move toward the spindle and (II) anaphase B in which the spindle poles move away from each other (De Gramont and Cohen-Fix, 2005). Telophase marks the reformation of the nuclear envelopes around daughter cell nuclei as the cytokinetic furrow pinches the cell into two (Gadde and Heald, 2004).

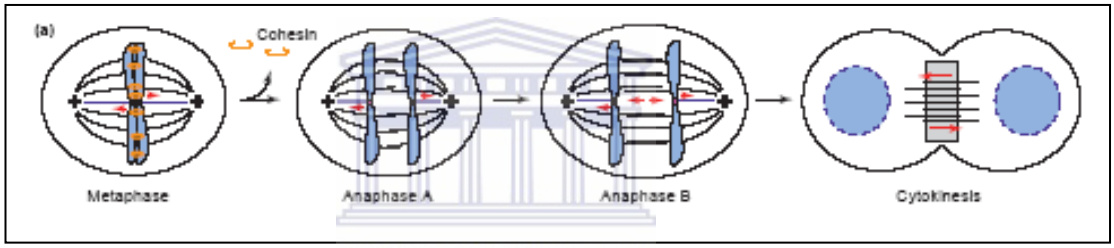

Figure 1.11: The anaphase forces (De Gramont and Cohen-fix, 2005).

Anaphase promoting complex or cyclosome (APC/C) was discovered as E3 which is responsible for degradation of mitotic cyclins. However, APC/C also plays an important role in the ubiquitination of the anaphase inhibitor securing. Securin is an inhibitory subunit of separase, a protease that destroys sister chromatid cohesion at the metaphase to anaphase transition. Therefore it seems as if APC/C has two essential functions, the initiation of sister chromatid segregation and resetting the cell cycle clock by achieving and maintaining a 'low Cdk activity' state (Vodermaier, 2004). However, it also triggers the destruction of many other proteins, such as polo kinases and spindle associated protein (Irniger, 2002). APC/C is a high molecular mass complex consisting of at least 11 subunits (Irniger, 2002; Peters, 2002) but it is only fully active as a ubiquitin ligase once it has bound to Cdc20, Cdh1 or related activators (Peters, 2002). The mitotic form is APC^{Cdc20} and the non-mitotic form is APC^{Cdh1} (Ayad, 2005). Once activated APC/C target securin, mitotic cyclins and other cell cycle regulatory proteins for proteasomal degradation (Wasch

and Engelbert, 2005). In yeast and in somatic animal cells, Cdc20 is degraded during exit from mitosis in an APC^{Cdh1} dependant manner. Cdc20 therefore has to be resynthesized during S phase and G_2 when APC^{Cdh1} is inactive and Cdc20 transcription is upregulated. On the other hand, despite Cdc20 accumulation, APC^{Cdc20} is not activated until mitosis, implying that additional components or modifications are required for APC^{Cdc20} activation or that inhibitors suppress its activity. Evidence exists supporting these possibilities even though their contribution is still poorly understood. Cdh1 is phosphorylated by Cdks throughout most of mitosis and thereby kept inactive. At the end of mitosis, Cdh1 is dephosphorylated and thus able to bind and activate the APC. APC^{Cdh1} then ubiquitinates Cdc20 and thereby inactivates APC^{Cdc20} (Ayad, 2005; Peters, 2002).

Understanding the spindle checkpoint to a great extend originates from genetic studies with the budding yeast *Saccharomyces cerevisiae* (figure 1.12). The protein complex, cohesion, links the two chromatids of a chromosome (Gorbsky, 2001). Cohesin is, however, essential to ensure chromosome bi-orientation. After chromosomes bi-orientation on the spindle, cohesin resists the tendency of microtubules to pull the sister chromatids apart. This protein is thus essential for proper chromosome segregation in at least two ways: (I) cohesin identifies identical chromosomes as sister chromatids, thereby dictating their segregation to the two daughter cells and (II) cohesin creates a physical link that counteracts the pulling forces of the spindle thereby preventing the premature separation of sister chromatids (De Gramont and Cohen-Fix, 2005).

Subsequently its final destruction by a protease (called separase) triggers the segregation of sister chromatids at the metaphase/anaphase transition. Separase is maintained in an inactive state by securin, (securins are proteins that bind to and inhibit the activity of separase) its inhibitory association partner. The eventual activation of separase shortly before the onset of anaphase depends on the proteolysis of securin (Pan and Chen, 2004; Tanaka, 2002; Tang *et al*, 2004). The APC/C ubiquitinates and degrade securing leading to the activation of separase, which cleaves Scc1 a subunit of cohesin (Cimini and Degrassi, 2005; Tang *et al*, 2004). In budding yeast all the Scc1 is cleaved at anaphase onset whereas in mammalian cells, the majority of the cohesin falls off the chromosomes during

prophase and prometaphase. Only a small amount remains concentrated between the kinetochores (Gorbsky, 2001).

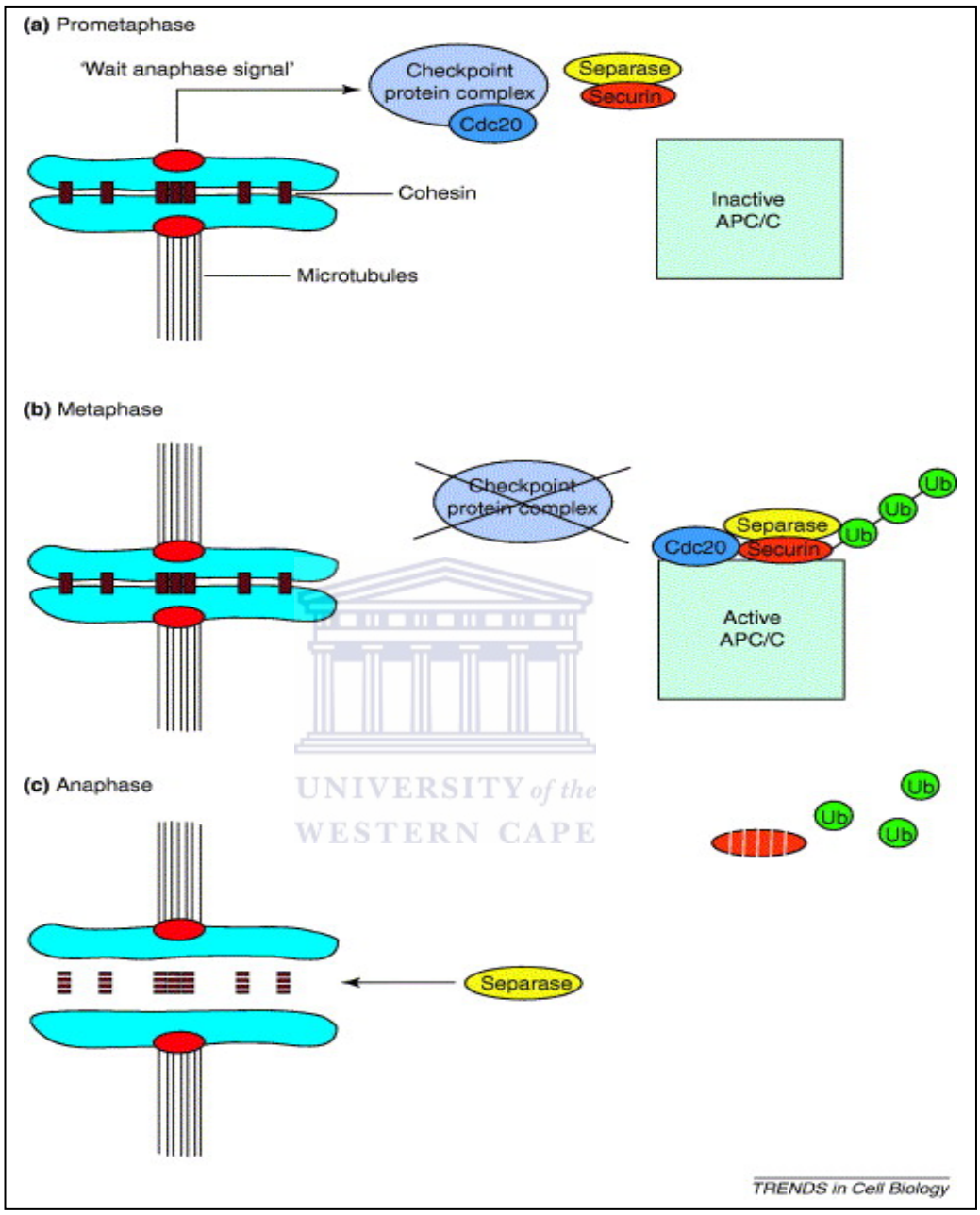

Figure 1.12: The spindle checkpoint (Cimini and Degrassi, 2005). Ub = ubiquitin.

Mitotic cyclins are exclusively destroyed at mitosis (Fung and Poon, 2005), resulting in the inactivation of the mitotic cyclin-Cdk complexes which are regulated by the mitotic ubiquitin ligase APC/C (Gorbsky, 2001). The cyclin A is destroyed in metaphase whereas cyclin B is destroyed when cells enter anaphase when the proteolytic activity of separase initiates anaphase (Gorbsky, 2001; Smits and Medema, 2001). At anaphase onset, APC/C targets securin for ubiquitin-mediated

proteolysis, thus releasing separase. At about the same time, the APC/C also begins to induce the degradation of cyclin B. At entry to mitosis cyclin B binds and activates Cdk1. Cdk1 is the primary kinase that maintains the mitotic state. The degradation of the cyclin B inactivates Cdk1 and leads to exit from mitosis (Gorbsky, 2001; Fung and Poon, 2005; Stewart and Pietenpol, 1999)

Another essential trait would be the forces applied to the kinetochore. The two poles would collapse together, if they were not compensated for by opposing forces originating either from a pole-pole physical connection independent of kinetochore microtubules and able to withstand compression or from a connection of the poles to a rigid cell cortex via astral microtubules (Nédélec *et al*, 2003; Pinksy and Biggins, 2005). Tension could either directly or indirectly influence the spindle checkpoint, through its ability to promote stable microtubule attachments (Cimini and Degrassi, 2005), however, the lack of tension prevents anaphase (Taylor *et al*, 2004). Other secondary traits, which might not be fundamental to all cells, include well-focused poles, aligned chromatids, or kinetochores that form a narrow metaphase plate (Nédélec *et al*, 2003; Pinksy and Biggins, 2005).

1.2.2.4.1 The spindle assembly checkpoint

Making sure that segregation does not occur before all chromosomes make proper attachments, the spindle assembly checkpoint (also known as the spindle checkpoint or mitotic checkpoint) acts in mitosis by delaying the onset of anaphase when chromosomes fail to align completely at the metaphase plate (equator) (Pan and Chen, 2004; Vigneron *et al*, 2004). Based on a negative feedback loop this pathway causes the kinetochores to release an inhibitor of anaphase until they are properly attached to spindle microtubules (Rieder and Cole, 1998). The spindle checkpoint like all checkpoints consists of three functional units, the signal, a signal transduction cascade and an effector (Hardwick, 1998; Wells, 1996).

This checkpoint can detect a multitude of spindle defects, ranging from minor unattached kinetochore to major defects induced by drugs causing microtubule depolymerisation (Amon, 1999; Hardwick, 1998). The kinetochores are the points of attachment for microtubules and directly regulate the interaction with the spindle (Hauf and Watanabe, 2004). Activating the spindle checkpoint leads to two models:

(I) the absence of tension at kinetochores or (II) the presence of an unattached kinetochore (Amon, 1999; Morrison and Rieder, 2004). This will signal and inhibit the onset of anaphase. When the last chromosome however, achieves stable attachment to microtubules, the kinetochore will cease its distress signal. Following the delay for chromosomal alignment for metaphase, anaphase ensues (Gorbsky, 2001).

Kinetochores have multiple microtubule attachment sites. When all kinetochores are attached to the spindle microtubules and have achieved full occupancy and are under tension, the spindle checkpoint is turned off (Cimini and Degrassi, 2005). Even though bipolar kinetochore microtubule interactions are crucial for the commitment of chromosome segregation, achieving the correct attachment is complicated. The flawed process could result in several kinetochore microtubule arrangements that lead to chromosome miss segregation (Pinksy and Biggins, 2005). This could occur due to the absence of microtubule assembly necessary to form a bipolar spindle, in the absence of microtubule dynamics sufficient to maintain tension on sister kinetochores. But it could also be due to the absence of kinetochore function required for the capture of microtubules (Andreassen *et al*, 2003). Thus the cells become aneuploid, which predisposes multicellular organisms to develop cancer and birth defects (Hardwick, 1998; Howell *et al*, 2004; Pinsky and Biggins, 2004). However, amphitelic or bioriented kinetochores satisfy the mitotic checkpoint requirements and enables normal segregation (figure 1.13) (Cimini and Degrassi, 2005).

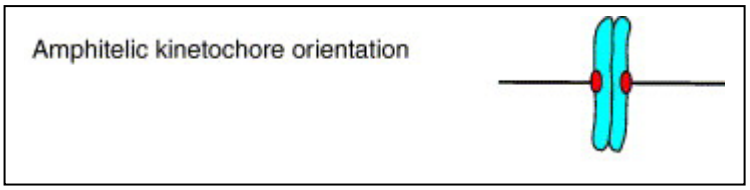

Figure 1.13: Amphitelic microtubule kinetochore orientation (Cimini and Degrassi, 2005).

The spindle checkpoint proteins are confined to kinetochores during mitosis in all organisms that have been examined consistent with the study that the spindle checkpoint is triggered from kinetochores that lack microtubule attachment or are not under tension (Pan and Chen, 2004). The checkpoints major function is to inhibit

APC/C (Morrison and Rieder, 2004). The pathway by which unattached or weakly attached kinetochores inhibit the APCs is intricate, and involves checkpoint proteins such as mitotic arrest deficit 1, 2 and 3 (Mad1, 2, and 3), budding uninhibited by benzimidazole 1, 2 and 3 (Bub1, 2 and 3), monopolar spindle 1 (MPS1) and microtubule interacting proteins centromere protein E (CENP-E) as well as other proteins (Chan *et al*, 2005; Lopes and Sunkel, 2003; Morrison and Rieder, 2004).

Mad and Bub are important proteins in a mitotic arrest responding to microtubule damage in all organisms (Malmanche *et al*, 2006). Homologs of Bub and Mad genes have also been identified in higher eukaryotes (table 1.3) (Lopes and Sunkel, 2003; Yu, 2002). Bub2 however, is the exception since it is located at the spindle pole body and monitors the position of the mitotic spindle. It can therefore regulate late mitotic exit and cytokinesis whereas the remaining proteins are localized at kinetochores during mitosis (Lopes and Sunkel, 2003). An accurate function is required from these components to maintain a mitotic arrest when spindle microtubule assembly is inhibited (Morrison and Rieder, 2004).

Table 1.3: The kinetochore spindle checkpoint proteins (Chan *et al*, 2005; Yu, 2002).

Budding Yeast	**Vertebrates**	**Localization and roles in mitosis**
Bub1	Bub1	Mitotic checkpoint kinase Localises to KTs
Bub2	-	Negatively regulates the mitotic exit Localises to KTs
Bub3	Bub3	Binds to Bub1 and BubR1 Localises to KTs Part of the MCC complex
Mad1	Mad1	Required for kinetochore assembly of Mad2 Localised to nuclear pores and unattached KTs
Mad2	Mad2	Binds to Cdc20 Part of the MCC complex Localised to nuclear pores and unattached KTs
Mad3	BubR1	Mitotic checkpoint kinase Localises to KTs Part of the MCC complex
MPS1	MPS1	Mitotic checkpoint kinase Localised to nuclear pores, centrosomes and KTs

Checkpoint active kinetochores inhibit anaphase by blocking Cdc20 activation of APC/C (Howell *et al*, 2004). Mad2 and BubR1 have key roles in preventing APC/C^{Cdc20} activity at the checkpoint. Both Mad2 and BubR1 bind directly to Cdc20 *in vitro* and either independently or collectively inhibits APC/C^{Cdc20} substrates. In

yeast, *Xenopus*, and mammalian tissue cells, checkpoint activation induces various Cdc20-containing complexes including Cdc20-Mad2, Cdc20-BubR1-Bub3, and Cdc20-Mad2-BubR1-Bub3, also known as the mitotic checkpoint complex (MCC) (Howell *et al*, 2004; Malmanche *et al*, 2006). According to Yu (2002) CENP-E might be the tension sensing mechanism. It physically interacts with BubR1 at the kinetechores and activates the protein to form BubR1-Bub3-Cdc20 complex however, it is unknown how it affects the function of this protein (Chan *et al*, 2005; Yu, 2002).

Figure 1.14 highlights the importance of the complexes that stimulates the spindle assembly checkpoint in response to delay the mitotic progression. However, the formation of the mitotic checkpoint complex (MCC) does not require unattached kinetochores, because this complex is present before the nuclear envelop breakdown (Malmanche *et al*, 2006). Mad2 is recruited to the kinetochore by Mad1, and Cdc20 is brought to the kinetochore by BubR1–Bub3. Mad1 also triggers a conformational change of Mad2, however the mechanism by which Mad2 dissociate from Mad1 is unknown (Yu, 2002). Mad1 is phosphorylated by Bub1 or MPS1 (Lopes and Sunkel, 2003; Taylor *et al*, 2004). Mad2 dissociated from Mad1 might retain a conformation more suitable for binding to Cdc20 that is already bound to BubR1–Bub3, resulting in the formation of the MCC. The complex, or its sub-complexes, might then diffuse away from the kinetochores to associate with APC and block its activity. When the kinetochores are captured by microtubules and all kinetochores achieve bipolar attachment to the mitotic spindle, the checkpoint is inactivated. Therefore Mad1 and Mad2 will no longer localise to the kinetochores (Yu, 2002). BubR1–Bub3 concentration and other checkpoint proteins at the kinetochores will thus decrease and the MCC cease to form. The inability of the formation of the MCC leads to the activation of APC^{Cdc20} complex. The active complex then ubiquitinates securin and allows the activation of separase, which cleaves cohesin. Loss of cohesin triggers chromosome segregation and the onset of anaphase (Malmanche *et al*, 2006; Yu, 2002).

When the spindle assembly checkpoint is satisfied, APCs become active and ubiquitinate several proteins, including cyclin B. The destruction of securin allows the chromatids to separate, while proteolysis of cyclin B allows the cell to exit the

mitotic state (Glotzer, 1995; Morrison and Rieder, 2004). The ubiquitin proteolysis of cyclin B is associated with the inactivation of Cdk1 which allows the cell to escape the mitotic condition and leads to the hallmark features of telophase including chromosome decondensation, nuclear envelope reformation, and cytokinesis (Rieder and Cole, 1998).

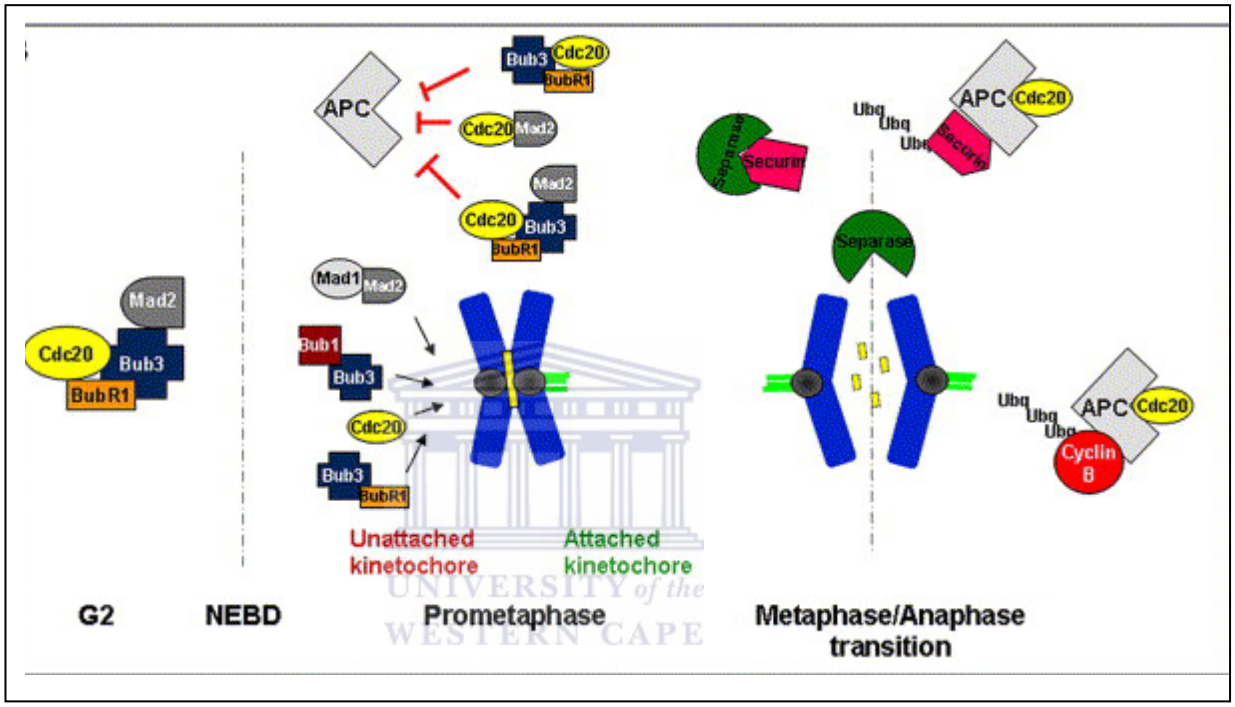

Figure 1.14: The formation of the spindle assemble checkpoint (Malmanche *et al*, 2006). NEBD = nuclear envelope breakdown; Ubq = ubiquitinates.

Chromosome segregation in mammalian cells normally takes less than an hour. However, the spindle checkpoint can delay the onset of anaphase for many hours, and varies among cells derived from different species and among individual cell lines from the same species, even though the delay is rarely permanent. In a process termed adaptation, cells can override the spindle checkpoint and exit mitosis. Normally a mechanism induces apoptosis in cells that have passed through mitosis and had not segregated their chromosomes. However, in cancer cells this mechanism for eliminating cells with abnormal numbers of chromosomes may be ineffective (Gorbsky, 2001).

1.3 Tubulin

Microtubules are dynamic components of the cell cytoskeleton (Waterman-Storer, 1998). They play diverse roles in the eukaryotic cells (Walczak, 2000). Thus they are centrally involved in numerous essential functions (Margolis and Wilson, 1998). The primary importance of microtubules is cell growth and division, where the cell enters mitosis, and they become the key components. They form the mitotic spindle on which the chromosomes are segregated to the two daughter cells (Jordan and Wilson, 1998; Kreis and Vale, 1993; Walczak, 2000). Thus it seems certain that mitotic microtubules are essential for all dividing cells and they appear to be involved in the control of cell cycle progression (Jordan and Wilson, 1998; Margolis and Wilson, 1998). Secondly, the general function of the microtubules is in organizing the cytoplasm (Kreis and Vale, 1993). During interphase they serve as tracks on which motor proteins transport vesicles and other components throughout the cell (Margolis and Wilson, 1998; Walczak, 2000). Furthermore, they are important for the control of the cell morphogenesis, the development and maintenance of the cell shape and signaling (Jordan and Wilson, 1998; Margolis and Wilson, 1998).

Microtubules are tubular structures of 25 nm diameter which may be several μm long (Mollinedo and Gajate, 2003), with the structural subunit of a 100 kDa protein, tubulin (Luduěna, 1998). Each microtubule is assembled of thirteen protofilaments which are arranged parallel to a cylindrical axis (Kline-Smith and Walczak, 2004) which is the most common type *in vivo* (Downing and Nogales, 1998a and b). A protofilament is made up of heterodimers (Hamel, 1996; Kline-Smith and Walczak, 2004). These heterodimers are self-assembeled (Downing and Nogales, 1998a and b) and found in the cytoplasmic pool (Waterman-Storer and Salmon, 1998). Each heterodimer consists of two 50 kDa subunits designated α and β tubulin (Chaudhuri *et al*, 2000; Hamel, 1996). These subunits share approximately 40% sequence homology and their structures are very similar (Downing and Nogales, 1998a and b; Li *et al*, 2002).

The α and β tubulin dimers are 5 nm wide and 8 nm long. The dimers are oriented head to tail at 8 nm spacing along the 13 protofilaments that comprise the 25 nm

diameter cylindrical wall of a microtubule. Microtubules grow by dimeres associating with their ends (Waterman-Storer and Salmon, 1998). These two ends have different assembly and disassembly characteristics creating a polar structure, and hence are designated plus and minus ends (Kline-Smith and Walczak, 2004; Oakley, 2000). Immunofluorescent localization of tubulin has shown that microtubules in interphase tissue cells are for the most part arranged in a radial array with the minus end oriented toward a central microtubule organizing center, the centrosome and the plus end radiating toward the cell periphery (Waterman-Storer, 1998). The plus end, is generally oriented away from the centrosome and is the primary site of growth. In living cells microtubules plus ends grow toward the periphery of the cell (Oakley, 2000). The microtubules are in constant assembly and disassembly. A multitude of agents are able to interact with microtubules, promoting either disassembly (microtubule disruption) or assembly (microtubule stability). Therefore blocking microtubule dynamics and leading to apoptosis. However, the process of apoptosis occurring by disruption of microtubules is unclear (Mollinedo and Gajate, 2003).

β tubulin are at the plus end and α tubulin are at the minus end (Oakley, 2000). The α-β tubulin subunit addition or loss from the ends results in its growth or shrinkage (Peters and Kapoor, 2004). Even though both ends alternately grow or shorten the net growth occurs at the plus and the net shortening at the minus end. The plus ends are free in the cytoplasm or located near the plasma membrane, whereas the minus ends are associated at a single site in the cell named the microtubule organizing centre or centrosome (MTOC). The MTOC represents the location from which microtubules are nucleated (Mollinedo and Gajate, 2003). The dynamics of tubulin depends on four parameters, growth rate, shrinkage rate, catastrophe frequency and the rescue frequency (Mollinedo and Gajate, 2003). The transition from a state of growth to one of shrinkage is called a ‘catastrophe’, whereas a transition from a state of shrinkage back to growth is termed a ‘rescue’. This is because the α and β tubulin heterodimers are inherently dynamic polymers that transduce energy derived from nucleotide hydrolysis accompanying their polymerization into polymer dynamics (Walczak, 2000).

The microtubule dimers form protofilaments that extend along the length of the microtubule and that are slightly offset, forming that is known as the 'B' lattice (Oakley, 2000). The microtubule α tubulin molecules interact laterally with other α tubulin molecules and β tubulin molecules interact laterally with other β tubulin molecules (i.e α-α and β-β) (Oakley, 2000). The most common type microtubules consisting of 13 protofilaments *in vivo*, have the 'seam' along their length in which the lateral contacts are reversed (i.e α-β and β-α contacts occur), resulting in loss of helical symmetry (Amos, 2000; Walczak, 2000).

The α and β tubulin monomers have basically identical structures (Downing and Nogales, 1998a and b). Each monomere can be divided into three functional domains; amino-terminal, an intermediate and the carboxy-terminal domain (Nogales *et al*, 1998). The amino-terminal domain contains the nucleotide binding region (Nogales *et al*, 1998). Each monomer binds to a guanine nucleotide (GTP), which is nonexchangeable in α tubulin (the nucleotide binding site in α tubulin is known as the N-site) and exchangeable in β tubulin (the binding site in β tubulin is known as the E-site). GTP at the E-site is required for microtubule assembly and its hydrolysis follows addition of a dimer to the microtubule end, upon which it becomes non-exchangeable. The stability of the system is maintained by a cap of tubulin-GTP at the ends (Löwe *et al*, 2001) and when this cap is lost the microtubule can come apart. This property is the basis for the dynamic instability of microtubules. It has been shown that a cap of one single tubulin-GTP per protofilament is sufficient to stabilize the microtubule end (Walczak, 2000). GTP hydrolysis thus has an important influence on the microtubule dynamics. When GTP binds to β tubulin of the free heterodimeric tubulin where after it is incorporated into the microtubule structure where GTP is hydrolyzed to GDP, which remains bound to the tubulin (D form). The GTP cap of subunits favours growth thus the T form favours assembly, whereas the GDP containing polymers disrupt microtubules and thus the D form favours disassembly (Mollinedo and Gajate, 2003). The nucleotide position in the amino-terminal domain of the structure is right at the longitudinal interface between monomers along the protofilament. Therefore each subunit interacts with the next monomere along the protofilament; both at the interdimer and at the intradimer interfaces. The N-site in α tubulin is shown to be found at the

intradimer interface and will always be occluded in the dimer, regardless of the state of assembly. The E-site, on the other hand, will be partially exposed in the dimer. Where it can exchange with the solution but will become occluded upon polymerization, resulting in its loss of exchangeability (Löwe *et al*, 2001; Walczak, 2000).

Tubulin differs significantly from other GTPases. This is true because it binds and hydrolysis GTP and acts as its own GTPase-activating protein through interaction at the interdimer interface during polymerization (Downing and Nogales, 1998a and b). Thus these units are different in nucleotide bound states (GTP is bound to α tubulin and GDP and taxol are bound to β tubulin). Therefore in the tubulin research field researchers like to speak about two tubulin conformations a 'straight' conformation of tubulin with bound GTP at the E-site in β tubulin (tubulin-GTP) and a 'curved' conformation containing tubulin-GDP (Walczak, 2000).

The intermediate domain is formed by three sequential alpha helices followed by a mixed beta sheet and two more helices and contains the taxol-binding site (Margolis and Wilson, 1998; Nogales *et al*, 1998). Taxol a drug, which specifically and powerfully suppresses microtubule dynamics, can induce a radical reorientation of microtubules both in the interphase array and in the mitotic spindle. When high concentrations of the drug are applied during anaphase in mitosis, it causes the microtubules of the mitotic spindle to elongate by shifting the assembly equilibrium. As a consequence chromosomes that had been moving pole ward reverse their direction of motion and move away from the spindle poles (Margolis and Wilson, 1998).

The carboxy-terminal domain is all alpha helical and overlaps the two previous domains, making the 'crest' of the protofilament on the outside surface of the microtubule where microtubule associated proteins (MAP) and motor proteins (kinesins and dyneins) bind (Downing and Nogales, 1998a and b). These proteins regulate microtubular dynamics and can be divided into two classes, (I) proteins that stabilize microtubules and (II) those that destabilize microtubules. MAPs are thought to bind along the length of the microtubule polymer and enhance their stability

(Walczak, 2000), where they lower the critical concentration for assembly (Amos, 2000). Motor proteins carry vesicular and other cargo along the microtubular surfaces and some of their binding sites seem to overlap with that of MAPs (Amos, 2000).

Thus, it could be concluded that the assembly of microtubules from α-β tubulin heterodimers and their dynamic behaviour are controlled by a variety of factors, including an exchangeable nucleotide bound to β tubulin and specific MAPs that lower the critical concentration for assembly (Amos, 2000). However, data also have revealed that the tubulin superfamily of proteins is much larger than was thought previously. Genetic analyses and database searches have added four new members of the tubulin superfamily, which adds up to seven and includes now α, β, γ, δ, ε, ξ and η tubulin (Dutcher, 2001). The variety of cellular events in which microtubules are involved encouraged speculation that the functional differences between microtubules might be the result of the assembly of different tubulin gene products (Kreis and Vale, 1993). However, δ, ε, ζ and η tubulin do not appear to have a ubiquitous distribution in eukaryotic organism (Dutcher, 2001). However, γ tubulin is necessary to initiate the assembly of α, β tubulin heterodimers into microtubule polymers (Oakley, 2000) and was discovered as a suppressor of a temperature sensitive mutation in β tubulin in *Aspergillus nidulans*, and it is found at the minus end of microtubules (Dutcher, 2001). Where, δ tubulin were discovered as a flagellar assembly mutant in the green alga, *Chlamydomonas reinhardtii* η tubulin was discovered as a result of a mutation in *Paramecium* that resulted in a basal body duplication defect. The final two new tubulins, ε and ζ tubulin, were discovered by database searches and their cellular functions however, need to be establish (Dutcher, 2001).

1.3.1 Tubulin and cancer

There are four main reasons for developing tubulin binding drugs to achieve improvements in (I) anti-tumour activity, (II) toxicity profile, (III) pharmacology and (IV) drug formulation. Failure of alignment of the daughter chromosomes and their bipolar attachment to the mitotic spindle is caused by drugs that interfere with the microtubule function. This cause the cell to fail to pass through the checkpoints that

exist to ensure that mitosis proceeds appropriately, leading to mitotic arrest at the metaphase/anaphase transition, followed by apoptosis. This has been proposed as the primary anti-neoplastic mechanism of action of tubulin binding drugs (Attard *et al*, 2005).

Tubulin has become the major target for a diversity of anti-mitotic drugs (Chaudhuri *et al*, 2000). This occurrence started ever since the identification and use of taxols for the treatment of lung, ovarian, and breast cancers (Carvalho *et al*, 2005). Taxol (paclitaxel) binding to microtubules, stabilizes the microtubules, resulting in an arrest in cell division and eventual cell death (Carvalho *et al*, 2005), by blocking the cell cycle in the G_2/M phases (Achiwa *et al*, 2003). Vinorelbine binds to the tubulin dimers, preventing them from polymerizing, whereas docetaxel binds to the microtubules, preventing them from depolymerizing. The rationale for combining microtubule-destabilizing agents like vinorelbine with microtubule stabilizing agents like docetaxel is not well established. However, both drugs cause microtubule dysfunction during mitosis. This phenomenon is associated with inhibition of serine/threonine protein phosphates 2A (PP2A). Inhibition of PP2A arrests cells in mitosis, leading to Bcl-2 phosphorylation and apoptosis (Sánchez *et al*, 2002)

Compounds identified more recently with a comparable action to taxol are the epothilones and discodermolides. There are also anti-cancer compounds that destabilize microtubules. The most prominent are vincristine, vinblastine and related compounds. They have been used for several decades in the treatment of childhood neoplasms and adult lymphomas (Carvalho *et al*, 2005). Most tubulin binding anti-cancer agents, such as taxanes and vinca alkaloids, bind to β tubulin and exhibit anti-cancer effects (Achiwa *et al*, 2003). Photoaffinity however, indicated that paclitaxel binds to both α and β tubulin thus confirming that both α and β tubulins contribute to the paclitaxel binding site, although crystallography analysis showed that paclitaxel binds to a single site on β tubulin rather than α tubulin (Kyu-Ho Han *et al*, 2000). For vinblastine, the binding site, or sites appear to consist of α and residues 175 to 213 of β. Rhizoxin is thought to bind to β. There have also been reports that the binding site for colchicines is located on α or β, or on both α and β (Chaudhuri *et al*, 2000). However, most of the entire binding sites of the different drugs can not be

identified (Chaudhuri *et al*, 2000) and the exact mechanism of action of paclitaxel and probably most of the other drugs remains unknown (Achiwa *et al*, 2003).

1.4 The nucleolus with reference to cancer

The nucleolus is a (Chen and Huang, 2001) large, highly organised, non-membrane-bound subcompartment (Gerbi *et al*, 2003) of the interphase cell nucleus (Derenzini *et al*, 1998; Horky *et al,* 2002). It resides next to chromosomes in the interphase nucleus (Pederson, 2002). The number, the size and shape of the nucleoli, vary significantly from cell type, cell cycle and culture conditions (Thiry and Lafontaine, 2005).

"Each nucleolus is the cytological manifestation of the activity of a genetic locus on the chromosome called the nucleolar organizer, which consists of repeated genes for ribosomal RNA (rRNA)" (Pederson, 2002). Thus the nucleolar organizer region (NOR) are chromosomal segments in which ribosomal RNA is encoded. NOR related proteins are acidic non-histone proteins stained by silver, and are known as the silver nucleolar organizer region (AgNOR). AgNOR is accepted as a marker of cellular activity (Hucumenoglu *et al*, 2002). There is approximately 271 nucleolar proteins identified which are involved in rRNA synthesis, processing and in ribosome assembly (Pederson, 2002).

The two major proteins of the AgNOR are C23 and B23 (Derenzini *et al*, 1998). Nucleolin (initially known as protein C23) (Olson *et al*, 2000) and B23 [also known as NO38, numatrin or nucleophosmin (NPM)] (Olson *et al*, 2000; Zimber *et al*; 2004) are both involved in ribosome assembly (Chen and Huang, 2001). The primary function of C23 could be to facilitate the early stages of preribosomal RNA (pre-rRNA) processing. The activities of B23 include nucleic acid and nuclear localization signal binding, stimulation of nuclear import and ribonuclease as well as molecular chaperone activities. Lastly, the protein may also be important in suppressing protein aggregation in the highly concentrated nucleolar environment (Olson *et al*, 2000). B23 is a nucleolar phosphoprotein which is more abundant in tumour and actively dividing cells than in normal resting cells (Yung *et al*, 1990).

In the functional mammalian interphase nucleolus, the structure has been identified in three nucleolar domains (Olson *et al*, 2000; Zimber *et al*, 2004). The three domains are as follow (figure 1.15); (I) the fibrillar centre (FC), a lightly stained region; (II) dense fibrillar component (DFC), a densely stained material surrounding the FC; (III) granular component (GC). The FC and DFC is embedded within a large granule rich region (Thiry and Lafontaine, 2005; Zimber *et al*, 2004).

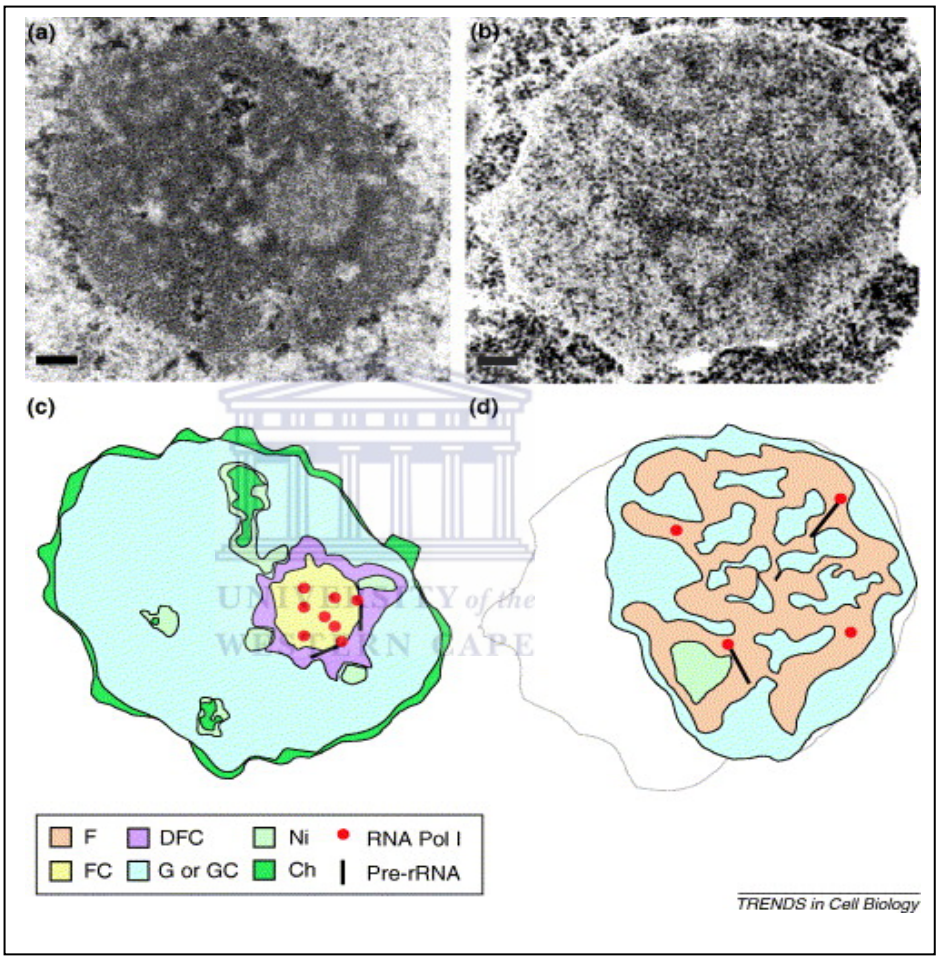

Figure 1.15: The nucleolus (Thiry and Lafontaine, 2005). Electron microscope analysis on cultured human larynx epidermoid carcinoma **(a)** and **(b)** yeat cells and **(c)** " blueprint" illustration of the human nucleolus, and the **(d)** yeast nucleus. **Key**: **F**, fibrillar component; **FC**, fibrillar center; **DFC**, dense fibrillar component; **G or GC**, granular component; **Ni**, nucleolar interstices; **Ch**, condensed chromatin. The RNA polymerase I (**RNA Pol** I) and the developing (nascent) pre-rRNAs (**Pre-rRNA**).

The FC and DFC (DFC carries the primary unprocessed transcripts) house the enzymatic apparatus for transcription (Laurincik *et al*, 2000) and contains newly synthesized pre-rRNA and a collection of proteins (Olson *et al*, 2000). The FC and DFC (fibrillar components) are thus the transcriptional machinery in the nucleolus

(Hernandez-Verdun *et al*, 1998). The GC represents processed transcripts associated with proteins in the form of preribosomal particles that in their final form represent the ribosomal subunits (Laurincik *et al*, 2000) not only this but, GC is involved in maturation and storage of the preribosomal subunits (Masson *et al*, 1990). The proteins B23 and C23 are co-localized to the same nucleolar compartments (Biggiogera *et al*, 1989). Studies by Biggiogera and colleagues (1998) indicated the association of the two proteins with the DFC and GC. Protein C23 is present in the FC as well as in the DFC of the nucleolus, whereas protein B23 appeared in both the granular and the fibrillar RNP nucleolar components (Biggiogera *et al*, 1989).

Apart from being a ribosome factory, additional functions have been attributed to the nucleolus (Pederson, 2002). This includes cell cycle regulation, telomerase activity, p53 metabolism, mRNA transport (Chen and Huang, 2001), nucleotide modifications of several small RNAs, a possible role in the biosynthesis of the signal recognition particle, and the phased sequestration and release of proteins involved in gene silencing, senescene and cell division (Pederson, 2002).

The nucleoli of tumour cells are morphologically distinct from non-tumourous cells this has been attributed to a very high level of nucleolar activity such as rRNA synthesis and the assembly of pre-ribosomal particles (Reddy *et al*, 1989). A number of nucleolar proteins present in proliferating normal cells and constitutively in tumour cells are not found in most normal resting cells. Their presence may be related to the function of the hyperactive and highly pleomorphic nucleoli in malignant cells compared to the small, spherical nucleoli of normal non-dividing cells (Larson *et al*, 1990). Many of the nucleolar proteins identified are involved in rRNA synthesis and processing, and in ribosome assembly (Pederson, 2002).

1.5 Apoptosis

Apoptosis is a descriptive name given to the process programmed cell death (Dlamini *et al*, 2004; Dupont-Versteegden, 2006Rupinder *et al*, 2007) which refers to the genetically regulated form of cell suicide (Afford and Randhawa, 2000; Lv *et al*, 2006; Rogalinska, 2002). This term was introduced by Kerr and colleagues in

1972 (Dlamini *et al*, 2004; Kiechle and Zhang, 2002; Kunapuli *et al*, 2006; Lu *et al*, 2005; Mollineda and Gajate, 2006).

Apoptosis is an essential physiological cell suicide that plays a critical role in the maintenance of homeostasis in embryonic, fetal and adult tissue (D'Agostini *et al*, 2005; Danial and Korsmeyer, 2004; Dupont-Versteegden, 2006; Zimmermann *et al*, 2001) controlling the number of cells in development (Wilson, 1998) and thus ensuring a functional organism (Oliver and Vallette, 2005). During embryonic and fetal growth it is essential in structuring the developing organism (Danial and Korsmeyer, 2004; Lv *et al*, 2006). Examples, being the formation of limb development by the separation of the fingers and toes. The formation of cavities by the transformation of cords into ducts and tubes and the removal of the tail of the tadpole during metamorphosis (Erickson, 1997; Saikumar *et al*, 1999). The evolutionary introduction of differentiated cell types may thus have obligated controlling death as well as division in order to keep neighbouring cells interdependent and insure the proper balance of each cell lineage (Danial and Korsmeyer, 2004). Apoptosis is thus required for development (Dupont-Versteegden, 2006; Saikumar *et al*, 1999).

Apoptosis in adult organisms maintain normal cellular homeostasis (Danial and Korsmeyer, 2004; Zhang *et al*, 2003). In humans, apoptosis is responsible for balancing cell proliferation with cell death and for maintaining constant cell numbers in tissues (Ricci and Zong, 2006; Zhang *et al*, 2003). Balancing cell division, maintaining the constancy of tissue mass (Saikumar *et al*, 1999) through apoptosis, approximately 100 000 cells die every second and are replaced by new cells of the same number in humans (Takahashi *et al*, 2004), such as the blood cell population and unwanted reproductive tissue (Erickson, 1997).

Besides maintenance of normal cellular homeostasis, apoptosis provides a defence mechanism (Zhang *et al*, 2003). Removal of cells injured by genetic defects, aging, disease, or exposure to noxious stimuli, such as heat, irradiation or toxins is made possible by apoptosis. Moreover, the normal immune response requires regulated elimination of specific cell populations by this mode of cell death. Apoptosis has important biological roles in the development and homeostasis of cell populations,

and in the pathogenesis and expression of disease processes (Kunapuli *et al*, 2006; Mollinedo and Gajate, 2006; Saikumar *et al*, 1999). Evidence indicates that insufficient apoptosis can contribute to various pathological processes including tumour development, chronic inflammatory diseases, immunological disorder (Kleibl *et al*, 2002; Mollinedo and Gajate, 2006) while accelerated cell death is evident in acute and chronic degenerative diseases, immunodeficiency, and infertility (Danial and Korsmeyer, 2004; Kunapuli *et al*, 2006; Mollinedo and Gajate, 2006).

1.5.1 Morphological features of apoptosis with reference to necrosis

In general two types of cell death, apoptosis and necrosis occur (figure 1.16) (Kunapuli *et al*, 2006; Rupinder *et al*, 2007; Zhang *et al*, 2003; Zimmermann and Green, 2001). Necrotic cell death or pathological cell death (Maslinska, 2003) is considered as an accidental death (Kunapuli *et al*, 2006; Zimmermann and Green, 2001). This cell death occurs usually as a result of acute injury (Zhang *et al*, 2003) of severe physical or chemical extracellular factors (Zlender, 2003). Therefore resulting in gross cell injury, and results in the death of groups of cells within a tissue (Zimmermann and Green, 2001) accompanied by inflammatory reactions of tissue (Zhang *et al*, 2003; Zlender, 2003).

In contrast, apoptosis is a physiological process where a cell itself actively induces its own death (Zlender, 2003). Since the process of apoptosis is mediated by specific proteins encoded in the host's genome, it is a programmed cell death (Maslinska, 2003), by which an individual cell may be eliminated from the living tissue (Zimmermann and Green, 2001). Cells in apoptosis may be characterized by distinct morphological and biochemical changes (Bortner and Cidlowski, 2007; Kunapuli *et al*, 2006; Patel *et al*, 1996; Zimmermann and Green, 2001). The apoptotic process starts without signs and symptoms of inflammation, and generally starts from the inside of the cell, involving the use of energy and active synthesis of specific proteins (Zlender, 2003). The intracellular concentration of ATP may be critical in the selection of the pathway to cell death. A high concentration of ATP favours apoptosis while a low concentration of ATP shifts cell death toward necrosis (Blaise *et al*, 2005; Kiechle and Zhang, 2002). The morphological changes that occur during apoptosis occurs in a desirable manner to the surroundings environment that it

decreases the risk of leakage of the intracellular content of the cell/s undergoing apoptosis where it could have harmful effects (Afford and Randhawa, 2000).

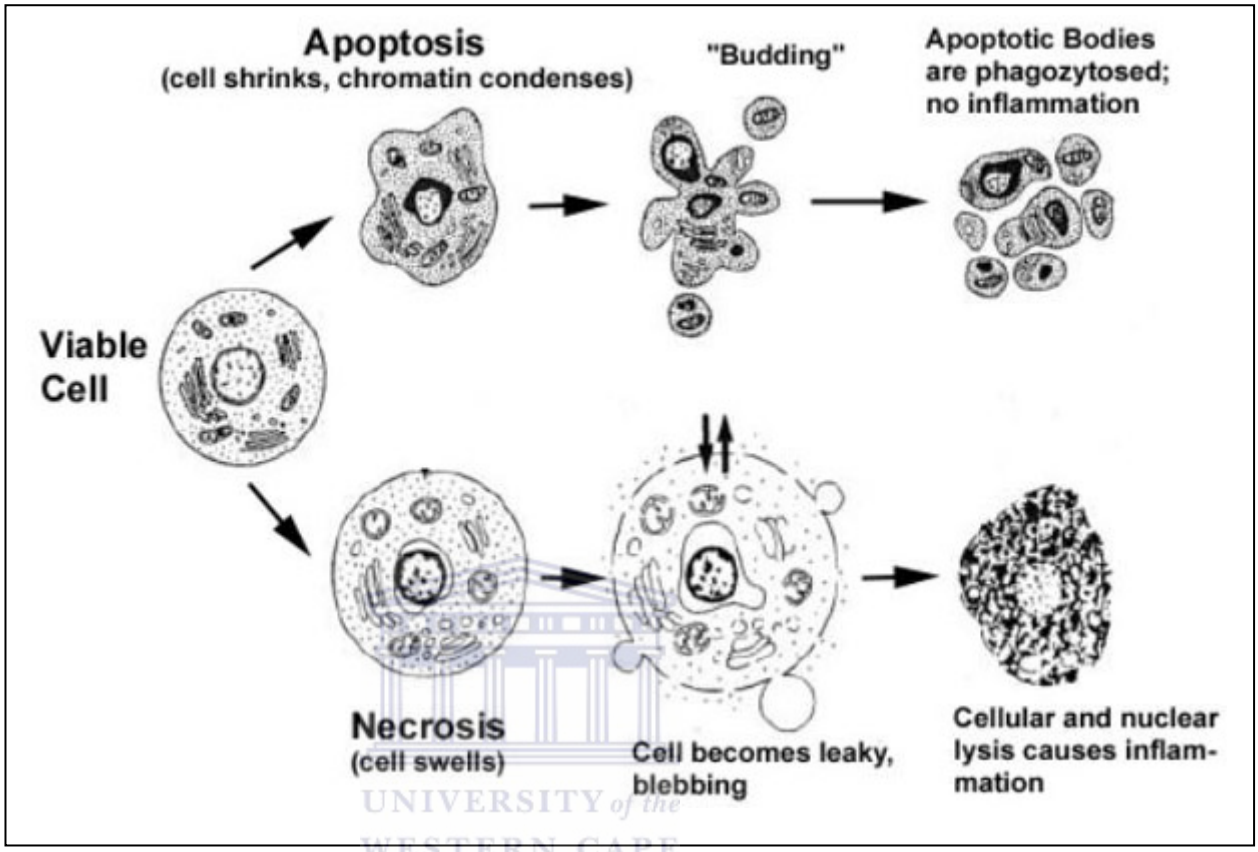

Figure 1.16: The morphology of apoptosis and necrosis as adapted from Van Cruchten and Van Den Broeck (2002).

Cells that are identified by means of microscopy have already loss adhesion and separated from their neighbouring cells (Kerr *et al*, 1972; Zimmermann *et al*, 2001). The initial morphological change is that of the nucleus. Chromatin condensation and segregation into sharply delineated masses that touch on the nuclear envelope are typically observed at the onset of apoptosis (Häcker, 2000). Chromatin masses are very electron dense and often show a characteristic crescent-like appearance. High magnification electron micrographs reveal that these masses are made up of closely packed, fine granular material. This initial condensation eventually leads to true nuclear pyknosis (Kerr, 2002; Lossi and Merighi, 2003; Takahashi *et al*, 2004).

In parallel with nuclear changes, cytoplasm condensation also occurs, and the cell membrane becomes convoluted with the onset of protuberances of various sizes that may give the cell a star like appearance (Kerr, 2002; Lossi and Merighi, 2003). As

the cytoplasm density increases, some vacuoles may develop (Lossi and Merighi, 2003). The cell organelles remain unaffected (Kinloch *et al*, 1999), although they become abnormally closely packed, likely as a consequence of the loss of cytosol (Lossi and Merighi, 2003). The condensation of the chromatin and cytosol lead to the change in cell shape and size of the cell (Monteiro *et al*, 2004). The cytoskeleton becomes disruptive (Liang and Fesik, 1997) and ribosomes detach from the rough endoplasmic reticulum and from polysomes, and eventually disappear (Lossi and Merighi, 2003).

As the process continues, the cell and its nucleus assume a more irregular shape (Lossi and Merighi, 2003) and undergo intense blebbing sometimes referred to as "boiling" of the plasma membrane (Singh *et al*, 1997). These blebs form numerous protusions at the cell surface and eventually break away from the plasma membrane forming packages known as apoptotic bodies (Saraste and Pulkki, 2000; Singh *et al*, 1997). These discrete fragments, still surrounded by an intact nuclear envelope display a sharp segregation of condensed chromatin in nuclear fragments and well preserved organelles (Lossi and Merighi, 2003) including intact lysosomes (Kinloch *et al*, 1999). The content of the apoptotic body depends on the cellular composition that is present in the cytoplasmic protuberance that gives rise to apoptotic bodies. Some might consist entirely of condensed nuclear chromatin whereas others are composed of cytoplasmic element (Kerr *et al*, 1972). Apoptotic bodies are rapidly cleared by means of phagocytosis by macrophages or neighbouring cells thereby avoiding inflammation in the surrounding tissues (Lossi and Merighi, 2003; Monteiro *et al*, 2004; Ricci and Zong, 2006; Zhang *et al*, 2003). *In vivo*, the fragments are engulfed by phagocytosis but in most *in vitro* systems this can not occur and consequently swelling and lysis are observed, which are also referred to as "secondary necrosis" (Kinloch *et al*, 1999; Singh *et al*, 1997). The maintenance of membrane integrity in apoptosis prevents the release of harmful cytoplasmic substances and the activation of inflammatory responses (Kunapuli *et al*, 2006; Maslinska, 2003). In contrast, necrotic cells increase cell permeability which leads to swelling of the cell and organelles resulting in rupture. The release of the cytoplasmic and nuclear content into the interstitial space results in inflammation (Erickson, 1997). Table 1.4 summarise the contrast of apoptosis and necrosis.

Table 1.4: A summary of the contrast of apoptosis and necrosis as adapted from (Allen *et al*, 1997; Kunapuli *et al*, 2006).

	Apoptosis	**Necrosis**
Occurrence	Scattered, single	Masive, tissue injury
Energy used	ATP (active process)	No ATP (passive process)
Cytoplasm	Shrinkage: condensed and dehydrated; normal organelles; later fragments	Swelling; ER and mitochondria swell
Nucleus	Chromatin condenses into crescentic masses adjacent to nuclear envelope; later fragments	Ill defined, randomly dispersed, smaller chromatin masses; later lysed
Plasma and nuclear membrane	Intense convolutions (blebbing but no loss of integrity); apoptotic bodies containing: normal cytoplasmic organelles; nuclear chromatin	Membrane injury/lysis; leakage of intracellular contents
DNA breakdown	Internucleosomal; gene activation; endonuclease	Random degradation including histones; diffuse
Tissue response	No inflammation; phagocytosis of apoptotic bodies by macrophages and adjacent cells	Inflammation; phagocytosis by macrophages

1.5.2 Biochemical features associated with apoptosis

Biochemical features associated with apoptosis include degradation of DNA by endogenous DNases, which cut the internucleosomal regions into double-stranded DNA fragments of 180-200 base pairs (Kunapuli *et al*, 2006; Saraste and Pulkki, 2000) leading to an oligonucleosomal "ladder" (Zimmermann *et al*, 2001). A variety of caspase substrates are involved in the regulation of DNA structure, repair and replication (Saraste and Pulkki, 2000). Other hallmarks are the externalization of phosphatidylserine (PS) on the plasma membrane (Hail *et al*, 2006) and proteolytic cleavage of a number of intracellular substrates (Zimmermann *et al*, 2001). Once the program for apoptosis has been initiated the biochemical events occur in a sequential order (figure 1.17).

It is these biochemical changes that lead to the morphological changes in appearance of an apoptotic cell (Allen *et al*, 1997). Three phases exist, the initial or induction phase, is the pro-apoptotic stimuli that trigger activation of the central molecular mechanism of apoptosis (Saraste and Pulkki, 2000; Wilson 1998). These signals are either extracellular or intracellular (Saikumar *et al*, 1999). The initiation phase depends on the cell type and stimulus which may influence the effector and or degradation phase (Hail *et al*, 2006). The second, committed or effector (execution) phase, the molecular executioner mechanisms become fully activated to induce apoptotic changes in nuclei (Saraste and Pulkki, 2000; Wilson 1998). This phase constitutes the activation of proteases, nucleases and other intermediaries that

participate in the final phase (Hail *et al*, 2006). The final or degradation phase is associated with evidence of hallmarks of apoptosis. These include morphologic changes, DNA fragmentation and the disposal of the cell (Saraste and Pulkki, 2000; Wilson 1998).

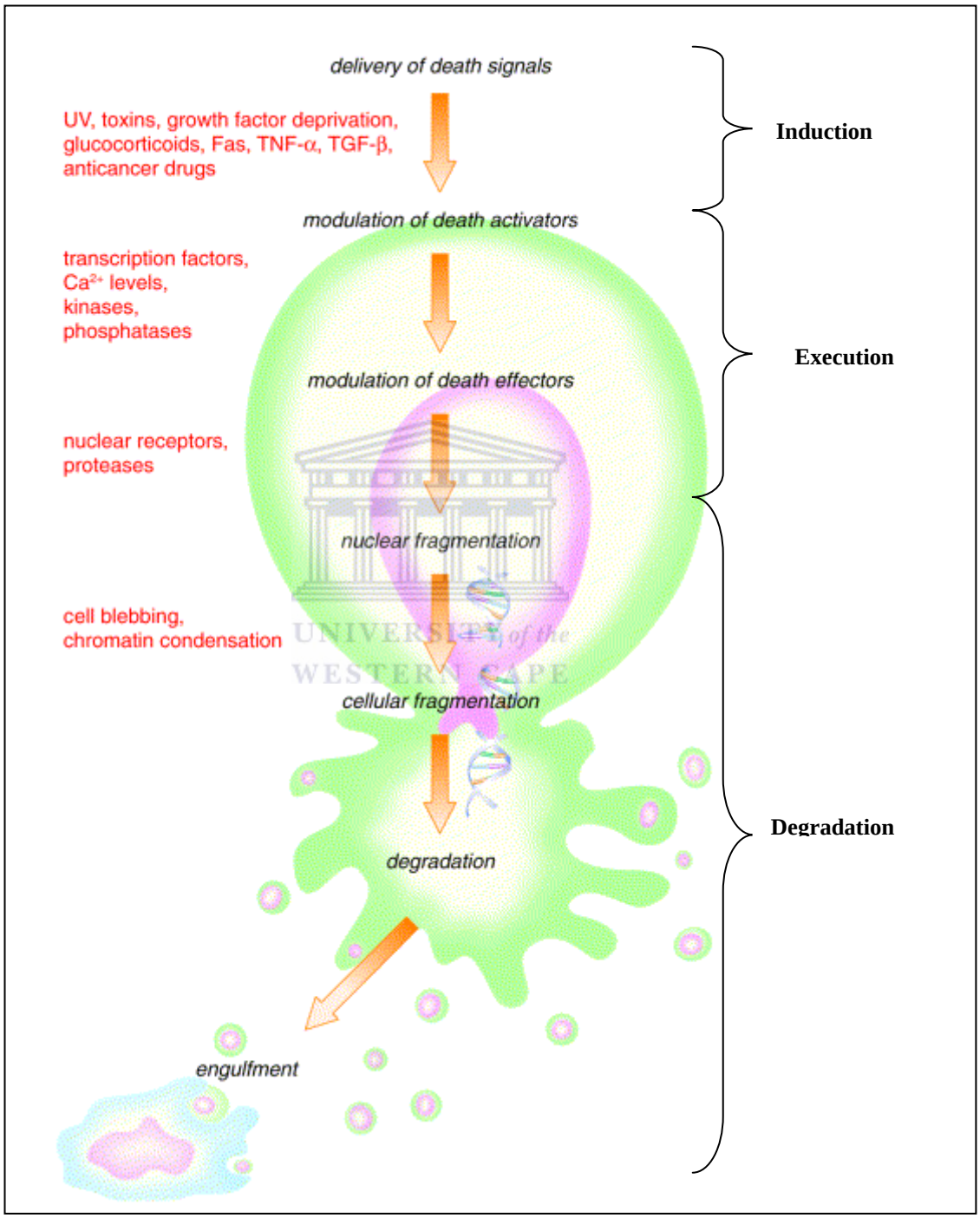

Figure 1.17: The phases of apoptosis, adapted from Kinloch and colleagues (1999).

Receiving extracellular or intracellular signals that are potentially apoptotic, cells activate caspases that are constitutively present in most mammalian cells (Hail *et al*,

2006; Monteiro *et al*, 2004; Saikumar *et al*, 1999). Caspase is a term derived from **c**ysteine dependent **asp**artate specific prote**ases** (Cohen, 1997; Dupont-Versteegden, 2006; Zhang *et al*, 2003) and originated from the discovery that the *C. elegans ced-3* gene encodes a homologue of the interleukin-1β processing enzyme (Jin and El-Deiry, 2005; Zhang *et al*, 2003). The activation of caspases cleave a variety of substrates including other caspases which eventually lead to the morphological changes indicated in apoptosis (Algeciras-Schimnich *et al*, 2002; Pützer, 2007).

Caspases are synthesised as inactive (proenzymes) zymogens and upon activation they are processed at a caspase cleavage site to generate their active form (Hajra and Liu, 2004; Jin and El-Deiry, 2005; Kunapuli *et al*, 2006; Zimmermann *et al*, 2001). All caspases have the same basic structure (Jin and El-Deiry, 2005; Monteiro *et al*, 2004; Zimmermann *et al*, 2001). However, they may differ in the primary sequence and in substrate specificity (Oliver and Vallette, 2005). Their common features are the pro-domain located at the amino-terminal portion, a large subunit (approximately 20 kDa) located in the middle of the molecule, and a carboxy terminal small subunit (approximately 10 kDa) (Jin and El-Deiry, 2005; Monteiro *et al*, 2004; Zimmermann *et al*, 2001). Activation involves proteolytic processing between domains, followed by association of the large and small subunits to form a heterodimer (Monteiro *et al*, 2004). Cleavages occur at aspartic acid (Asp-Xxx) sites strongly suggesting the possibility of autocatalytic activation (Monteiro *et al*, 2004; Zimmermann *et al*, 2001). Finally the two identical large subunits and the two identical smaller subunits will combine to form an active mature heterotetramer (Oliver and Vallette, 2005; Rupinder *et al*, 2007).

There are 14 members of the mammalian family of caspases (table 1.5) (Gopisetty *et al*, 2006; Monteiro *et al*, 2004; Rupinder *et al*, 2007; Zhang *et al*, 2003; Zimmermann *et al*, 2001). Twelve human caspases have been identified, with caspase-11 and -13 as the exception. Caspase-11 was identified as the mouse homolog of the human caspase-4, while caspase-13 has been shown to be the bovine homolog of human caspase-4 (Oliver and Vallette, 2005). Based on their functions Jin and colleague (2005) and Zhang and colleagues (2003) has classified caspases into three groups.

Table 1.5: The classification of mammalian caspases as adapted from Saikumar and colleagues (1999) and Jin and El-Deiry (2005).

Classification	Name of caspase	Other names	Size of prodomain
Apoptotic initiator caspases	Caspase 2	ICH-1, Nedd-2	Long
	Caspase 8	FLICE, Mach, Mch5	Long
	Caspase 9	ICE-LAP6, Mch6	Long
	Caspase 10	Mch4	Long
Apoptotic effector caspase	Caspase 3	Cpp-32, Yama, apopain	Short
	Caspase 6	Mch2	Short
	Caspase 7	Mch3, ICE-LAP3, CMH-1	Short
Inflammatory/Other caspases	Caspase 1	ICE	Long
	Caspase 4	TX, ICH-2, ICErel-II	Long
	Caspase 5	TY, ICErel-III	Long
	Caspase 11	mICH-3, mCASP-11	Long
	Caspase 12	mICH-4, mCASP-12	Long
	Caspase 13	ERICE	Long
	Caspase 14	MICE	Short

These groups are: (I) the inflammatory or other caspases, since caspase-1, -4, -5, and -11, -12, -13 and -14 regulate inflammation, while caspase-14 is associated with the keratinocyte differentiation (Jin and El-Deiry, 2005; Zhang *et al*, 2003). (II) The initiator (upstream) caspases (Fleischer *et al*, 2006) are caspases-2, -8, -9 and -10 (Jin and El-Deiry, 2005; Kunapuli *et al*, 2006; Wang *et al*, 2005; Zhang *et al*, 2003). The inactive form of the initiator caspases have a long N-terminal prodomain that functions as a protein interaction module by recognizable proteins that associate with proteins that trigger caspase activation (Fleischer *et al*, 2006; Wang *et al*, 2005). These long prodomains contain either a DED (death effector domain) caspase-8 and -10 or a CARD (caspase activation and recruitment domain) caspase-2 and -9 (Jin and El-Deiry, 2005) and are activated by autoproteolysis in response to death signals and initiate apoptosis (Lu *et al*, 2005; Shi, 2004). Caspase-8 is associated with apoptosis involving death receptors, and loss of cell adhesion, while caspase-9 is involved in the mitochondrial stress-induced apoptosis (Monteiro *et al*, 2004). (III) The effector (downstream) caspases (Fleischer *et al*, 2006) are the executioner caspases (-3, -6 and -7) which are characterized by the presence of a short prodomain (Fleischer *et al*, 2006; Jin and El-Deiry, 2005; Kunapuli *et al*, 2006; Monteiro *et al*, 2004). These caspases are processed and activated by the upstream caspases and perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates (Fleischer *et al*, 2006; Jin and El-Deiry, 2005; Monteiro et al, 2004).

The most prevalent caspase in the cell is caspase-3 (Jin and El-Deiry, 2005). In a review by Saikumar and colleagues (1999) they indicated that functional caspase-3 is required for the typical hallmarks of apoptosis and is crucial for the formation of apoptotic bodies, chromatin condensation and DNA fragmentation in all cell types. It was established that caspase-3 deficient animals showed impaired brain development and premature death (Saikumar *et al*, 1999). Caspase-3 is ultimately responsible for the majority of the apoptotic effects, although it is supported by caspase-6 and -7, the latter two playing a redundant role in most apoptotic pathways (Jin and El-Deiry, 2005). During apoptosis, caspase-3 cleaves an inhibitor, allowing the nuclease to cut the chromatin (Zimmermann *et al*, 2001). More than one hundred caspase substrates have been identified, some of them include inhibitor of caspase-activated DNase (ICAD), poly ADP-ribose polymerase (PARP), Bcl-2, lamin and several cytoskeleton binding proteins (Lu *et al*, 2005). According to Wang and colleagues (2005), the functions of caspases could be summarized as follow (I) the arrest of the cell cycle and the inactivation of DNA repair; (II) activation of the XIAP (inhibitor of apoptosis) and (III) the dismantling of the cytoskeleton. However, Lu and colleagues (2005) also indicated that the cleavage of the protein substrates caused DNA fragmentation, nuclear membrane disruption and chromatin condensation.

1.5.2.1 Pathways that activate caspase

Wang and colleagues (2005) indicated four pathways that activate caspase during apoptosis. They are the I) mitochondria mediated pathway, II) death receptor mediated pathway, III) granzyme B mediated pathway and IV) the endoplasmic reticulum (ER) mediated pathway (Wang *et al*, 2005). The two major pathways however, are the intrinsic (mitochondria mediated or type II) pathway and the extrinsic (death receptor mediated or type I) pathway (figure 1.18) (Bishopric *et al*, 2001; Fleischer *et al*, 2006; Hail *et al*, 2006; Lu *et al*, 2005). These two pathways merge and share mechanisms utilizing the caspase cascades (Lu *et al*, 2005). However, they can stimulate apoptosis independent of one another; yet, cross talk between the two pathways can also occur (Takahashi *et al*, 2004).

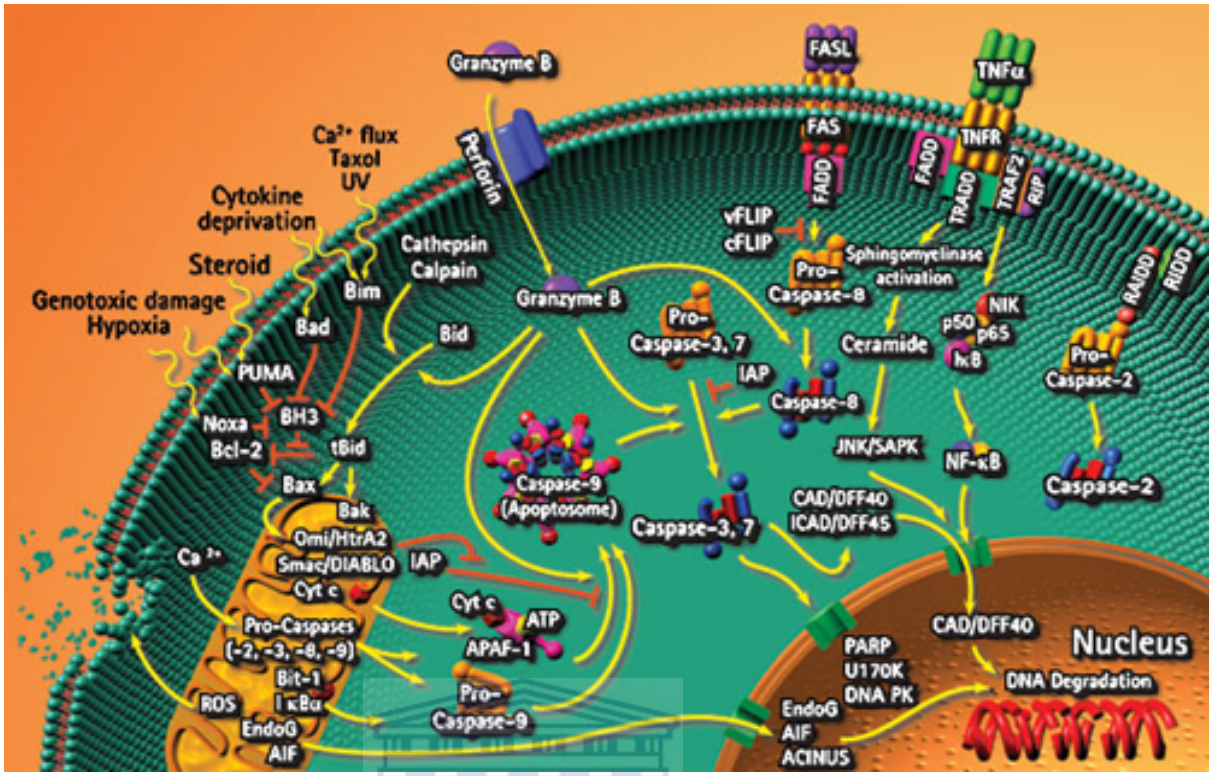

Figure1.18: A number of apoptotic signaling pathways (http://calbiochem.com/apoptosis, 2008).

1.5.2.2 The extrinsic or receptor mediated pathways

The extrinsic pathway is activated by binding of a ligand to a death receptor on the cell surface (Blaise *et al*, 2005; Hajra and Liu, 2004; Lu *et al*, 2005). The death receptors belong to the tumour necrosis factor receptor (TNFR) superfamily (Hajra and Liu, 2004; Wang *et al*, 2005) of which tumour necrosis factor-α receptor-1 (TNFR1) and Fas or APO-1 or CD95 are prominent examples (Bishopric *et al*, 2001; Gopisette *et al*, 2006; Wang *et al*, 2005). Another set of death receptors (DR4/5) known as tumour necrosis factor related apoptosis inducing ligand receptor (TRAILR) whom engage with the death ligand Apo2L, TRAIL (Danial and Korsmeyer, 2004; Gopisette *et al*, 2006). These death receptors are characterized by a cysteine rich repeat in their extracellular domain and a distinct domain within their cytoplasmic tails which is termed the death domain (DD) (Hajra and Liu, 2004; Van Cruchten and Van den Broeck, 2002; Wang *et al*, 2005). The Fas ligand (FasL) will bind to the Fas receptor and the TNFα binds to the TNFR1 (Van Cruchten and Van den Broeck, 2002). Once activated, these receptors recruit an adaptor molecule

which is the TNFR 1-associated death domain (TNFADD/TRADD) and Fas-associated death domain (FADD) (Blaise *et al*, 2005; Kiechle and Zhang, 2002; Wang *et al*, 2005). Binding of FADD or TNFADD leads to recruitment of procaspase-8, which binds to the adaptor molecule, and along with the receptor, form the death inducing signaling complex (DISC). At the DISC procaspase-8 is processed and caspase-8 is activated ensuring the activation of the down stream caspase-3 (Kunapuli *et al*, 2006; Rupinder *et al*, 2007; Wang *et al*, 2005; Zhang *et al*, 2003). However, in addition the initiator caspase-2 could also lead to the activation of caspase-3. The TNFR1 also mediates apoptosis through the recruitment of another adapter molecule known as receptor interactive protein associated ICH/CED-3 homologous protein death domain (RAIDD). RAIDD further binds with receptor interactive protein (RIP) through interactions between death domains and can activate caspase-2 (Rupinder *et al*, 2007). The activation of caspase-2 required for apoptosis in certain cell types is however, unclear. Researchers has reported the following findings; that caspase-2 activity induce cytochrome *c* release from mitochondria directly or indirectly via nuclear signaling, or by cleaving Bid, which in turn, translocates to the mitochondria and releases cytochrome *c* (Park *et al*, 2007). These findings suggest that caspase-2 could also activate caspase-3 (Huang *et al*, 2007; Park *et al*, 2007). Caspase-3 can then cleave a series of proteins such as PARP which plays a role in DNA repair. The cleavage of PARP accelerates DNA fragmentation by adversely affecting the DNA repair machinery (Porter *et al*, 1998). The cleavages may also lead to the prevention of depletion of NAD and ATP which would usually have been used for PARP activity (Cohen, 1997; Leist and Nicotera, 1997). Thus the ATP could therefore be utilized for the execution of apoptosis and cleaving of others such as nuclear lamins which occurs after PARP cleavages (Cohen, 1997).

1.5.2.3 The intrinsic or mitochondria mediated pathway

The intrinsic pathway is triggered by internal apoptotic signals and involves mitochondrial outer membrane permeability (Lu *et al*, 2005). Various apoptotic inducing signals (such as chemotherapeutic drugs, cytotoxic drugs, oxidants, radiation and growth factor deprivation) might directly or indirectly change mitochondrial membrane permeability and cause the release of the mitochondrial intermembrane proteins (Lu *et al*, 2005; Rupinder *et al*, 2007; Zhang *et al*, 2003).

The mitochondria contain a number of highly lethal substances that can initiate apoptosis when released into the cytosol (Bishopric *et al*, 2001). In response to these stimuli, the mitochondria release pro-apoptotic factors, such as apoptotic-inducing factor (AIF), Smac/Diablo and a serine protease, Omi. These factors are pro-apoptotic by inhibiting inhibitor of apoptosis protein (IAP), endo G (endonuclease G) and contributes to DNA fragmentation and cytochrome *c* release (Blaise *et al*, 2005; Lu *et al*, 2005). The electron transporter cytochrome *c*, is a cornerstone of apoptosis (Blaise *et al*, 2005).

A sequence of events in the typical intrinsic pathway usually follows a series of steps (Kiechle and Zhang, 2002; Wang *et al*, 2005). The mitochondrial outer membranes become permeable to proteins, resulting in the release of proteins normally found in the space between the inner and outer membranes as indicated above (Wang *et al*, 2005). The permeability of the membrane is due to the permeability transition pore (PTP). This is a multiprotein complex composed of voltage dependent anion channel (VDAC), adenine nucleotide translocator (ANT) and cyclophilin D (Susin *et al*, 1998). VDAC, located in the outer membrane, is responsible for most of the metabolites released from the mitochondria, including cytochrome *c* (Gogvadze *et al*, 2006; Shoshan-Barmatz *et al*, 2006). All mitochondria release cytochrome *c* within 5 min. Once the process is initiated, other mitochondrial contents are also released (Kiechle and Zhang, 2002). The release of cytochrome *c* leads to the formation of a heptameric wheel-like caspase-activating complex, which has been termed as an apoptosome (Rupinder *et al*, 2007; Wang *et al*, 2005). This is a high molecular weight complex composed of cytochrome *c*, apoptotic protease-activating factor-1 (Apaf-1), deoxyadenosine triphosphate (dATP), and procaspase-9, which forms a platform for the efficient processing and activation of caspase-9 (Hail *et al*, 2006; Hajra and Liu, 2004; Zhang *et al*, 2003). Caspase-9 has a CARD domain in the N-terminus, and this is a key site with which it associates with Apaf-1 and cytochrome *c* (Wang *et al*, 2005). Activated caspase-9 activates the effector caspases such as caspase-3, -6, and -7 (Blaise *et al*, 2005; Kuida, 2000; Ricci and Zong, 2006; Wang *et al*, 2005). Caspase-3 activation is detected following the formation of the apoptosome (Kiechle and Zhang, 2002). The effector caspase degrade vital cellular proteins, leading to cell death (Hajra and Liu, 2004; Zhang *et al*, 2003).

Activation of the executioner caspase is a point where the intrinsic and extrinsic apoptotic pathways converge (Zhang *et al*, 2003). Caspase-3 cleaves the signal components that therefore effect the morphological change that brings about cell death (Jin and El-Deiry, 2005; Zhang *et al*, 2003). Activation of caspase-3 leads to the translocation of phosphatidylserine located in the inner leaflet of the plasma membrane to the outer leaflet. This phenomenon could be confirmed by using the flow cytometry technique and detecting for annexin V which binds to externalized phosphatidylserine (Kiechle and Zhang, 2002). This translocation facilitates the recognition of apoptotic cells by macrophages which favour early phagocytosis of apoptotic cells (Kiechle and Zhang, 2002; Leist and Nicotera, 1997). As a final point, DNA fragment into 180-200 base pair units and multiples of these units is initiated by caspase-3 activation of inactive caspase-activated deoxyribonuclease by removing its inhibitor. However, exceptions to this generalized sequence of biochemical events do exits, because in some specific situations, the intrinsic apoptotic pathway may not require decreased mitochondrial transmembrane potential and cytochrome *c* release (Kiechle and Zhang, 2002). It is however, understandable that in this pathway of apoptosis, caspase-3 and -9 may be the most important as their activities influence the process of apoptosis as well as the type of cell death (Wang *et al*, 2005).

1.5.2.4 The role of Bcl-2 within the intrinsic or mitochondria mediated pathway

The Bcl-2 family plays a vital role in the promotion or inhibition of apoptosis (Allen *et al*, 1998; Cummings *et al*, 2004; Gil-Gómez *et al*, 1998; Schmitt *et al*, 2007). In humans more than 20 members of this family exist (Cummings *et al*, 2004; Hu and Kavanagh, 2003). The members of the Bcl-2 family are classified based on four conserved regions called Bcl-2 homology (BH) domains and are classified into three groups (Borner, 2003; Gopisetty *et al*, 2006; Willis and Adams, 2005). The anti-apoptotic (pro-survival) Bcl-2 family (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1) are characterised by the presence of all four BH1-4 domains. The pro-apoptotic (pro-death) Bax group (Bax, Bak, and Bok) has the BH1-3 domains and interact with Bcl-x_L and Bcl-2. The pro-apoptotic BH3-only group (Bim, Bad, Bid, Bik, Bmf, Puma, Noxa and Hrk proteins) comprises only a BH3 domain and therefore refered to as "BH3 only". The third class family members are thought to act by binding and

inactivating the anti-apoptotic members (Borner, 2003; Gopisetty *et al*, 2006; Wang *et al*, 2007; Willis and Adams, 2005).

The Bcl-2 family proteins regulate the permeabilization of the outer mitochondrial membrane (Fleischer *et al*, 2006) but it could also play a role in the mitochondrial independent apoptosis pathways (Kirkin *et al*. 2004). The Bcl-2 proteins that are anti-apoptotic can form heterodimers with the Bcl-2 proteins that are pro-apoptotic and the overexpression of one antagonizes the other effect (Gil-Gómez *et al*, 1998; Hu and Kavanagh, 2003). These proteins control the release of the apoptogens from the mitochondria to the cytosol and thus play a central role in the mitochondrial mediated pathway (Cummings *et al*, 2004; Hu and Kavanagh, 2003; Wang *et al*, 2007). Mitochondrial activation can be achieved by modulation of the molecules that act on mitochondria pores, altering the balance between pro- and anti-apoptotic members of the Bcl-2 family, either by downregulation of anti-apoptotic molecules, or by upregulation of pro-apoptotic partners (Fleischer *et al*, 2006). However, there remains uncertainty of how the Bcl-2 family functions biochemically (Kirkin *et al*, 2004). Some theories have been presented (Tamm *et al*, 2001), such as the following two: the binding to other proteins and ion-channel activity (Tamm *et al*, 2001).

The pro-apoptotic Bcl-2 proteins are important for the regulation of the release of mitochondrial cytochrome *c* (Hail *et al*, 2006). Caspase-8, when activated, will activate caspase-3 directly, or could trigger cytochrome *c* release from the mitochondria by specific processing of the pro-apoptotic Bcl-2 family member Bid (Kunapuli *et al*, 2006; Sinai *et al*, 2004). The BH3 only protein Bid connects the extrinsic to the intrinsic pathway (Kuribayashi *et al*, 2006; Ricci and Zong, 2006). Caspase-8 cleaves Bid to generate truncated Bid (tBid) which translocated to the mitochondria and activates pro-apoptotic Bax and Bak (Gogvadze *et al*, 2006; Gopisetty *et al*, 2006). The pro-apoptotic Bax family are able to alter the mitochondrial membrane allowing cytochrome *c* to be released into the cytoplasm (Dragovich *et al*, 1998; Hajra and Liu, 2004). The pro-apoptotic Bcl-2 family, Bax and Bak, induce cytochrome *c* release by forming pores in the mitochondrial membrane or by binding to VDAC and ANT components of the permeability transition pore (PTP) of the mitochondria (Fleischer *et al*, 2006; Kirkin *et al*, 2004; Schmitt *et al*, 2007). The PTP, too small to allow cytochrome *c* release and the

association with Bax/Bak result in the formation of channels which are specific for cytochrome *c* (Fleischer *et al*, 2006; Hu and Kavanagh, 2003).

The release of cytochrome *c* from the mitochondria promote the oligomerisation of the apoptosome which leads to the induction of apoptosis (Dragovich *et al*, 1998; Hajra and Liu, 2004; Hu and Kavanagh, 2003; Sinai *et al*, 2004). Cytochrome *c*, which, together with dATP or ATP, binds to the adaptor protein Apaf-1. This molecule then oligomerizes and forms the "apoptosome" complex (Kirkin *et al*, 2004; Kunapuli *et al*, 2006). Caspase-9 is recruited by the apoptosome and by dimerization or oligomerization becomes proteolytically activated. Active caspase-9 then triggers the activation of the effector caspases which will lead to the induction of apoptosis (Kirkin *et al*, 2004; Kunapuli *et al*, 2006; Sprick and Walczak, 2004).

The overproduction of Bcl-2 anti-apoptotic proteins prevents cell death (Hu and Kavanagh, 2003).The most important Bcl-2 proteins that promote suppression of apoptosis are the Bcl-2 and Bcl-x_L (Cummings *et al*, 2004). The anti-apoptotic Bcl-2 family maintains mitochondrial integrity by associating with VDAC, by being in competition with other proteins (Fleischer *et al*, 2006; Gogvadze *et al*, 2006; Kirkin *et al*, 2004; Sprick and Walczak, 2004). It has also been suggested that the anti-apoptotic family members, specifically Bcl-2 and Bcl-x_L forms heterodimers with Bax and Bak and thereby keeping them in check, not allowing the pro-apoptotic proteins to induce pores within the mitochondrial membrane (Kirkin *et al*, 2004). This would lead to the prevention of cytochrome *c* release from mitochondria (Dragovich *et al*, 1998; Hajra and Liu, 2004). However, Bcl-2 and Bcl-x_L had been reported to bind to Apaf-1 preventing Apaf-1 interactions with procaspase-9 (Tamm *et al*, 2001). This could lead to the suppression of apoptosis. However, researchers had dismissed these finding (Kirkin *et al*, 2004; Tamm *et al*, 2001).

The Bcl-2 family is regulated by p53, an important component of the intrinsic pathway (Blaise *et al*, 2005). Wild type of p53 protein is involved in cell cycle arrest at the G_1 and G_2 checkpoints and interacts with DNA repair or with Bcl-2 proteins that play a role in inhibition of apoptosis (Gregorc *et al*, 2003). In turn, p53 could increase the expression of the pro-apoptotic Bax, and decrease the expression of the anti-apoptotic Bcl-2 proteins (Blaise *et al*, 2005). However, during cancer p53

mutation and overexpression of anti-apoptotic Bcl-2 could be linked to many types of human cancers (Kirkin *et al*, 2004; Lin *et al*, 2005). The success of a tumour forming leads to cancer cells evading the self generated pro-apoptotic signals. It has been suggested that overexpression of Bcl-2 and Bcl-x_L may contribute to this process. Since both are survival factors and are drug resistant proteins (Cummings *et al*, 2004; Kirkin *et al*, 2004).

1.5.3 Apoptosis and treatment of cancer

Apoptosis is impaired in many human tumours, signifying that disruption of apoptotic function contributes significantly to the transformation of a normal cell into a tumour cell (Hu and Kavanagh, 2003). In treatment or prevention of cancer, understanding the mechanism of carcinogenesis, growth (proliferation) control and apoptosis is important, because we need to know what pathway the anti-cancer drug will influence to regain the cells ability to perform their vital cellular function (Trosko and Ruch, 2002).

Apoptosis is one of many cellular pathways currently under investigation as a therapeutic target for lung cancer treatment (Villaflor and Bonomi, 2005) also in general inducing tumour cell killing (Hu and Kavanagh, 2003). The switching on and off of apoptosis is determined by the ratio of pro-apoptotic and anti-apoptotic proteins (Hu and Kavanagh, 2003). Apoptotic strategies to remove cancer cells could involve direct induction of pro-apoptotic molecules, modulation of anti-apoptotic proteins or the restoration of tumour suppressor gene functions (Fleischer *et al*, 2006). Various cancer chemotherapeutic drugs are known to activate apoptotic mechanisms of tumour cell death. Apoptosis has been projected as a novel target for cancer chemoprevention where the rationale is to remove cells undergoing neoplastic transformation, in situations where other defence mechanisms fail to block the carcinogenesis process upstream (D'Agostini *et al*, 2005).

Cancer biology is based on an imbalance of proliferation and apoptosis caused by an increase in growth factor secretion, oncogene expression, loss of tumour suppressor genes and neovascularisation. According to Huber and Stratakis (2004) in their review, p53 and Bcl-2 are the two important genes taking the main position in the

activation and regulation of apoptosis and the p53 gene alterations are the most frequently found in human cancer (Huber and Stratakis, 2004).

Inducers of apoptosis have been used in cancer therapy (Hu and Kavanagh, 2003). These drugs seem to activate the intrinsic and extrinsic pathways separately, however, both apoptotic signals seem to be integrated at the mitochondrial level and are typically accompanied by the activation of the caspases which are central to most apoptotic pathways (Hu and Kavanagh, 2003; Soto-Cerrato *et al*, 2004). The defects in mitochondrial function could contribute therefore to the development and progression of cancer (Carew and Huang, 2002). The molecular mechanisms by which anti-cancer drugs induce apoptosis are mediated by mitochondrial dysfunction, which is regulated by the balance of pro-apoptotic and anti-apoptotic proteins in the Bcl-2 family (Kim *et al*, 2006). However, anti-cancer treatment also induce morphological features via both the apoptotic and nonapoptotic (autophagy) cell death. Apoptosis and autophagic cell death in some cases coincide *in vivo* in certain tissues. Thus both changes in cell morphology might coincide within the same cells (Kim *et al*, 2006).

However, apoptosis is probably the most essential defence mechanism against cancer because it regulates tissue homoeostasis by removal of redundant or potentially deleterious cells (Abu *et al*, 2005). According to Abu and colleagues (2005) more and more evidence suggests that some chemopreventive agents (examples include retinoids, vanilloids, butyroids, polyphenols, antioestrogens, and some non-steroidal anti-inflammatory drugs) trigger apoptosis in already transformed cells *in vivo* and *in vitro*, modulating the carcinogenic process.

1.6 An overview on lung cancer

Over 90% of all cancers of the lung develop in the bronchial epithelium (Jung, 1983). Human lung cancer consists of two major categories from a histological point of view (Danesi *et al*, 2003; Molina *et al*, 2008). Small cell lung carcinomas (SCLC) or oat cell and non-small cell lung carcinomas (Non-SCLC) (Forsberg *et al*, 1993; Sherwin *et al*, 1981). Non-SCLC accounts for approximately 80% of the cases that represents a heterogeneous group of cancers. The remaining 20% of the cases are related to SCLC (Lau *et al*, 2006; Nikliński *et al*, 2001). In 1981, the WHO

classified the Non-SCLC into a further three categories, squamous cell carcinoma (SQC) or epidermoid carcinoma, adenocarcinoma (ADO) and large cell carcinoma (LCC) (Banks-Schlegel *et al*, 1985; Forsberg *et al*, 1993; Herbst *et al*, 2008). These four types of cancer have been classified by various pathological criteria (Sherwin *et al*, 1981). A fifth but very rare lung cancer known as bronchio-alveolar carcinoma, also exists (Gazdar *et al*, 1983; Jung, 1983).

The ultrastructure, clinical presentation, course and response to therapy of SCLC distinct it from other forms of lung cancer. SCLC presumably arises from or is related to pulmonary endocrine cells expressing amine precursor uptake and decarboxylation (APUD) (Gazdar *et al*, 1983; Gazdar *et al*, 1981). Therefore, it is defined by a heterogeneous pattern of different neuroendocrine markers (Forsberg *et al*, 1993).

The Non-SCLC represents a mixed bag of histological appearances and biologic behaviour (Gazdar *et al*, 1983). However, common to the sub-groups is the expression of different epithelial markers while the neuroendocrine markers are low or absent (Forsberg *et al*, 1993). SQC development is preceded by many years of sequentially occurring pre-malignant changes, including mucus cell hyperplasia, squamous cell metaplasia, progressive atypia and *in situ* carcinoma (Carter, 1978; Jung, 1983). *In situ* carcinoma begins in the surface epithelium, usually in a segmental bronchus. From this focus it may spread in various directions and into mucus glands (Gazdar *et al*, 1983). Interestingly, the premalignant changes of squamous cell carcinoma and SCLC are identical, suggesting similar causative agents for these two different tumour types (Gazdar *et al*, 1983; Jung, 1983).

Most central ADO is thought to arise from surface epithelial cells. Whether they arise directly from multipotential basal cells or from more differentiated cells is controversial. Cellular distribution of glycoproteins after *in vitro* labeling suggests that ADO arise from a cell type committed to differentiate into ciliated epithelium (Dermer, 1981).

Studies of LCC indicated that many of them have ultrastructural features of SQC, ADO, or even endocrine tumours. Thus, LCC is a convenient waste bag, and consists

of very poorly differentiated ('undifferentiated') carcinomas whose various origins cannot readily be determined by light microscopy (Gazdar *et al*, 1983).

Bronchiolo-alveolar carcinoma is a sub-group of ADO. However, as bronchioles have no glandular elements, they are classified separately. Following nonspecific pulmonary injury, such as severe viral or repeated bacterial infections, small areas of multifocal epithelial proliferation arise in the respiratory bronchioles and alveolar structures (Jung, 1983). Thus, some authorities believe that this tumour arises from the type II alveolar epithelial cell (Jung, 1983) or their precursors, the Clara cells (Gazdar *et al*, 1983).

The pathogenesis of lung cancer involves the accumulation of multiple molecular abnormalities over a long period of time (Massion and Carbone, 2003). SCLC and Non-SCLC pathogenesis differ (Camps *et al*, 2006). The pathogenesis of Non-SCLC is discussed here, because our study focuses on a Non-SCLC cell line. The early genetic alterations in Non-SCLC involve loss of genomic regions of chromosomes 3p and 9p, deletions of chromosomal arm on 5p and mutations of p53 and K-ras. Loss of chromosomal regions on chromosomes 3p and 9p have been recognized as premature events and identified in pre-invasive lesions and in the normal appearing epithelium of smokers (Massion and Carbone, 2003). In contrast, p53 and K-ras mutations have been seen primarily in later stages of pre-neoplasia or frank invasive lesions. Enlargement of expanded regions on the q arm of chromosome 3 has been characterised in invasive carcinomas and only recently in pre-invasive lesions. K-ras mutations are most commonly seen in 30% of ADO of the lung but much less frequently in other subtypes. Mutated p53 is found in more than two thirds of lung cancers (Massion and Carbone, 2003). p53 mutations has been shown to play a key role in tumour development by dysregulation of cell cycle control and apoptosis induction. A tumour suppressor gene, p16, has been found to be inactive in over 40% of Non-SCLC. It is also important to note that cancer cells are not only characterised by mutations but also by a series of chromosomal aberrations including deletions and amplifications (Massion and Carbone, 2003).

Three quarters of clinical cases of lung cancer are Non-SCLC. Occasionally, patients with Non-SCLC can be cured by surgery or radiotherapy when the disease is

localized, but Non-SCLC is much less responsive to chemotherapy than SCLC. A better understanding of the basic biology of Non-SCLC might help improve treatment of this usually fatal disorder (Brower *et al*, 1986). It has been proven by Fox and colleagues (2004) that patients with a smoking history undergoing surgical resection for Non-SCLC had an increased morbidity and poorer long term survival. Establishing cell lines of Non-SCLC on a routine basis could provide an important tool of studying the disease (Brower *et al*, 1986). Since the knowledge of the mechanism of chemopreventative substances on human Non-SCLC is relatively limited, a Non-SCLC human squamous cell lung carcinoma cell line, NCI-H157 was used in this study.

1.6.1 The etiology of lung cancer: With reference to tobacco smoke and nicotine

Population studies have led to the suggestion that 80% of human cancers are due to environmental carcinogens. This would include physical and viral agents as well as naturally occurring or man-made chemicals (Doll and Peto, 1981). Carcinogens may be chemical (e.g tobacco smoke), physical (e.g metals such as nickel compounds, fibres such as asbestos), natural or man-made radiation in the air, or viruses. In addition, other agents (termed co-carcinogens or promoters) may add to the cancer inducing effect of common carcinogens, either by shortening the delay before the cancer appears or by increasing the number of tumours developing (Kawaguchi *et al*, 2006; Stoll, 1989).

The majority of human lung cancers are probably attributable to non-occupational factors, including smoking, air pollution, genetic susceptibility and atmospheric radioactivity (Gazdar *et al*, 1983). The first reports suggesting that tobacco might be related to lung cancer emerged perhaps as early as 1898, when it was observed that some tobacco workers exposed to tobacco dust developed lung cancer. Additional implications were drawn thereafter from cigar makers and cigar sellers with lung cancer autopsied at the Leipzig Pathological institute in 1900 through 1912. Generally, however, lung cancer was considered an uncommon entity for most of the first half of the previous century (Huber, 1989b). The first reports of tobacco smoking histories in men with lung cancer were published in 1939, just before the outbreak of World War II. These reports were generally ignored, as were case-control studies in 1943 in Germany and in 1948 in the Netherlands. In 1950, several

additional retrospective, case controlled studies on lung cancer were reported, and the interest in the role of tobacco smoking in the development of lung cancer soon thereafter intensified (Huber, 1989b).

An international working group positively associated tobacco smoke with cancer of the lung, oral cavity, pharynx, larynx, oesophagus, pancreas, urinary bladder and renal pelvis in 1985. However, seventeen years later a relationship between tobacco smoke and cancer incidence in other organs such as the nasal cavity, paranasal sinuses, nasopharynx, liver, stomach, kidney and uterine cervix was shown (Sasco *et al*, 2004).

Initial scientific reports, linking cigarette smoking with lung cancer, were published and reported in the lay press, giving the tobacco companies the break to claim that their products were safe. They promised to cooperate fully with tobacco-related research efforts, pledged to protect the public's health and they introduced filtered and low tar cigarettes (Giovino, 2002). However, low tar cigarettes have not been shown to have any health benefits, in part because people compensate for lower nicotine yields by smoking more cigarettes, taking more puffs per cigarette, increasing puff volume, depth, and duration and/or blocking ventilation holes (Giovino, 2002; Shields, 2002). In June 1997, representatives of 40 states and of five of the six American tobacco companies jointly announced an historic agreement. In this agreement, for the first time, after decades of investigation and the publishing of literally thousands of scientific studies, the tobacco industry acknowledged a link between smoking and lung cancer (Johnson, 1998a). The association between smoking and lung cancer has been studied in depth and in most countries the epidemiology of lung cancer reflects the epidemiology of smoking (Gazdar *et al*, 1983; Zhou *et al*, 2006).

1.7 Nicotine

1.7.1 Nicotine as a tobacco product

Nicotine is important in human biology for two reasons, (I) it appears to be the primary reason why people consume tobacco products, and (II) it may contribute to causation of some tobacco related diseases (Benowitz, 1986). Tobacco addiction had lead to extensive research (Benowitz, 1886; Editorials, 1991; Wonnacott *et al*, 1990)

of nicotine as a therapeutic agent for smoking cessation and cancer prevention (Vainio *et al*, 2001). However, the action of nicotine, have been extensively investigated in human, animal and a diversity of the cell systems (Yildiz, 2004).

Nicotine (1-methyl-2-[3-pyridyl-pyrrolidine], $C_{10}H_{14}N_2$) is an alkaloid (Brewer *et al*, 2004; Yildiz, 2004) and may exist as one of two stereoisomers (Wonnacott *et al*, 1990). Tobacco contains only (S)-nicotine (also called (-) or levo (*l*)-nicotine), which is the most pharmacologically active form (Huber, 1989b; Wonnacott *et al*, 1990). During the smoking process, some of the (S)-nicotine is converted to (R)-nicotine (also called (+) or dexto (*d*)-nicotine), which is much less pharmacologically active than the (S) isomer (Huber, 1989b; Wonnacott *et al*, 1990). About 90% of the nicotine in smoke is (S)-nicotine (Wonnacott *et al*, 1990). On average, a cigarette contains 6-17 mg of nicotine (Etter *et al*, 2003). Low yield cigarettes do not contain less nicotine; however, they are low yield because they remove tar and nicotine by filtration and or dilute the smoke with air (Benowitz, 1986). In most commercial cigarettes, only about 15% of the total nicotine appears in the mainstream smoke, where 25% to 40% is released to side stream smoke and 15% to 25% is deposited within the butt (and filter tip). The remainder is pyrolysed to nicotine decomposition products (Huber, 1989b).

1.7.2 Pharmacokinetics of nicotine

1.7.2.1 Absorption of nicotine

Nicotine is highly lipid soluble and therefore has a significant influence on biological systems (Meyer *et al*, 1971). Absorption of nicotine can occur through the oral cavity, skin, lung, urinary bladder and gastrointestinal tract (Yildiz, 2004). When tobacco smoke reaches the lung, nicotine is rapidly absorbed because of the large surface area, the large blood perfusion of the pulmonary capillary beds (Brewer *et al*, 2004), small diffusion distance and dissolution of nicotine into a fluid of a normal physiological range which facilitates transfer across cell membranes (Wonnacott *et al*, 1990). The rapid transfer across the lung and into the blood has been suggested to account for the higher dependence potential of inhaled nicotine compared to the transdermal, oral, nasal or buccal routes. Further, the rapid rate of transfer through the lungs is thought to cause the appearance of a transient large peak in blood nicotine levels a few seconds after an inhaled puff (Brewer *et al*, 2004).

However, it is also accepted that the transit time of nicotine from the lung to the brain is approximately 10 seconds after a puff. Nicotine binds to nicotinic receptors located in the brain and increase the number of nicotine receptor sites by two to three fold. This nicotine receptor proliferation may not be reversible in humans and this fact may contribute to the enhancement of nicotine addictions properties (Da Costa e Silva and Fishburn, 2004).

The absorption of nicotine across biological membranes depends on its pH, or its state of acid-base dissociation (Huber, 1989b). Nicotine is a weak base with a index of ionic dissociation (pK_a) of 8.0 (Sohn *et al*, 2003). The presence of both a pyrrolidine and a pyridine nitrogen means that nicotine is dibasic (Yildiz, 2004), where pyrrolidine has a pK_a 7.92 and pyridine has a pK_a 3.22 at physiological temperature and ionic strength (Brewer *et al*, 2004). This means at pH 8.0, 50% of nicotine is ionized and 50% is non-ionized (Sohn *et al*, 2003). Uncharged organic bases are lipophilic whereas charged organic bases are hydrophilic. As the pH of the aqueous solution increases the rate of nicotine absorption increases. Nicotine absorption decreases as the pH decreases (Yildiz, 2004). The smoke of tobacco found in most cigarettes is acidic (pH 5.5). At this range of pH the nicotine is primarily ionized. As a consequence, there is little buccal absorption of nicotine from cigarette smoke, even when it is held in the mouth (Benowitz, 1986).

When a cigarette is smoked, micro droplets reach the alveolar spaces and are deposited on the vast internal surface of the lung where they are immediately buffered to a physiological pH of near 7.4. This is close enough to the index of ionic dissociation so that a significant portion (more than 30% of the total amount) of the nicotine is in a nonprotonated state and as such, is rapidly and efficiently absorbed across the air blood barriers at the alveolar surface (Huber, 1989b). Absorption through the alveoli is also dependent on the nicotine concentration in the smoke (Yildiz, 2004). According to Yildiz (2004) observations demonstrated that absorption of nicotine through the buccal mucosa is poor and that through the lung is rapid. Studies has also indicated that nicotine plasma levels in non-inhaling smokers is around 2.5-8.0 ng/ml, whereas inhaling smoker's nicotine plasma levels reach 30-40 ng/ml (Yildiz, 2004). However, in contrast to cigarettes, other tobacco products such as pipe tobacco, cigars and European cigarettes are manufactured with air-cured

tobacco. Thus the smoke of these tobacco products is alkaline (the pH might be up to 8.5) and the nicotine of these products is absorbed well through the mouth (Sohn *et al*, 2003).

During tobacco harvesting and nicotine replacement therapy, absorption of nicotine occurs through the skin. Reabsorption of the extracted nicotine occurs through the urinary bladder (Yildiz, 2004). However, it is dependent on the pH of the urine and if the urine pH is below 6.0, nicotine cannot be absorbed. Absorption of nicotine within the gastrointestinal tract is poor because of the acidity of the stomach juices (Yildiz, 2004). An interesting phenomenon is that the smoker regulates the intake of nicotine. By the way they puff, regulating the frequency and intensity of the inhalation based on the available number of cigarettes and their chosen brand, to provide a certain amount of nicotine (Sohn *et al*, 2003). Evidence suggests that many smokers compensate when they switch to a light cigarette by inhaling more tar and nicotine, or increasing the number of cigarettes smoked per day. The risk of lung cancer is similar when smoking low tar cigarettes compared with high tar cigarettes (Sohn *et al*, 2003).

1.7.2.2 Distribution of nicotine

Smoking is being seen as "a unique form of systemic drug administration" because nicotine is delivered to the pulmonary system rather than to the portal or systemic venous circulations (Sohn *et al*, 2003). Nicotine found in the body only after tobacco smoke exposure, has a half-life of two hours (Shaham *et al*, 1993), whilst that of the products of nicotine metabolism, such as cotinine a major metabolite of nicotine, varies from one to two days (Shen *et al*, 1977). Due to its long half-life, cotinine is commonly used as a marker of nicotine intake (Benowitz, 1986). Pulmonary absorption of cigarette smoke distributes nicotine much more rapidly than any other manner of distribution in the human body (Sohn *et al*, 2003). In fact when a given dose of nicotine is ingested, for instance by smoking, about one half is removed from the blood stream within 15 to 30 minutes (Henningfield, 1985). The liver, lung and the brain have a high affinity for nicotine whilst the adipose tissue has a relative low affinity for nicotine. Immediately after *intra* pulmonary injection with nicotine, concentrations in arterial blood, lungs, and the brain are high, while concentrations in tissues such as muscle and adipose tissues are low (Duelli *et al*, 1998). The

consequence of this distribution pattern is that uptake of nicotine into the brain is rapid, occurring within approximately 10 seconds after a puff (Da Costa e Silva and Fishburn, 2004), and blood levels of nicotine fall due to peripheral tissue uptake for 20 or 30 minutes after administration (Duelli *et al*, 1998). Thereafter blood nicotine concentrations decline more slowly, the rate determined by rates of elimination and rates of distribution out of storage tissues (Wonnacott *et al*, 1990). However, according to a study done by Brewer and colleagues (2004) the lung may also act as a short term reservoir for nicotine for a 40 second period, delaying and depressing its appearance in the systemic arterial circulation.

Nicotine freely crosses the placenta of pregnant women and has been found in amniotic fluid and in umbilical cord blood of neonates. The amniotic fluid provides a reservoir for continued delivery of nicotine to the fetus, even when maternal levels are low (Wonnacott *et al*, 1990). Ahlsten and colleagues (1990) measured nicotine and cotinine in cord blood sera, and found that high concentrations of nicotine are transferred to the fetuses of smoking mothers. Babies of nursing, smoking mothers had been found to have a high cotinine concentration in their blood and their urine (Charlton, 1994). In children who are passive smokers, the level of urine cotinine has been shown to correlate with the number of cigarettes smoked by parents, especially by mothers (Halken *et al*, 1995). Cotinine and nicotine can also be measured in breast milk (Svensson, 1987) and concentrations of nicotine increased 10 fold when the mother smoked just before nursing (Halken *et al*, 1995). It has also been found by Schulte-Hobein and colleagues (1992) that cotinine excretion in urine from non breast fed infants of smoking mothers was even higher than that of adult passive smokers. However, the urinary cotinine excretion in breast fed infants of smoking mothers was at the same level as that of the mothers themselves (Schulte-Hobein *et al*, 1992).

1.7.2.3 Metabolism and elimination of nicotine

Nicotine is metabolized by several pathways (Price *et al*, 2004). Cotinine is the major metabolite (Dhar, 2004) of the primary C-oxidation pathway of nicotine biotransformation (Price *et al*, 2004). The liver is considered to be the major site of nicotine biotransformation, however to a small extend metabolism also occurs in the lung and kidney (Benowitz, 1986; Wonnacott *et al*, 1990). Various cytochrome P450

enzymes have been identified which mediate *in vitro* mammalian metabolism of nicotine to cotinine (Tricker, 2003). Nicotine is metabolized to *S*-nicotine $\Delta^{1'-5'}$-iminium ion by the genetically variable hepatic enzymes and then to the pharmacologically less active derivative, *S*-cotinine, by a cytosolic aldehyde oxidase enzyme (Denton *et al*, 2004; Price *et al*, 2004). In the rat liver nicotine is metabolized by CYP1A2, CYP2B1, CYP2C11 and other CYP forms: CYP2B1 is also constitutively expressed in the rat lung (Price *et al*, 2004). Variants of CYP2A6 have been implicated in the ability of people to metabolize nicotine at different rates (Denton *et al*, 2004).

There are more than 20 different derivates that are derived from nicotine (Dhar, 2004). In humans 70% of nicotine is oxidized to cotinine, 4% is oxidized to nicotine-N-oxide (Sohn *et al*, 2003), 9% is excreted unchanged in the urine and the metabolic outcome of the remaining 17% is still unknown (Dhar, 2004). Tobacco smoking also produce, metabolites other than those derived from nicotine such as trans, trans muconic acid and 1-hydroxy pyrene. These metabolites are produced from benzene and polycyclic aromatic hydrocarbons respectively, which are also present in environmental tobacco smoke (Dhar, 2004). Figure 1.19 represents the structure of the metabolic route to nicotine and figure 1.20 represents the quantitative scheme of nicotine metabolism based on the average excretion of metabolites as percentage of total urinary nicotine.

According to Yildiz (2004) nicotine could be excreted through urine, feaces, bile, saliva, gastric juice, sweat and breast milk. 2% to 35% of total elimination is conducted through renal excretion, which depends on urinary pH and urine flow (Sohn *et al*, 2003). However, if the pH of the urine is made alkaline the proportion of uncharged nicotine increases and reabsorption of nicotine occurs and as a result less nicotine is excreted (Yildiz, 2004). Nicotine levels in the blood fluctuate and the duration of urinary nicotine excretion is brief due to the short half life of 2.6 hours (Dhar, 2004; Tricker, 2003). It is therefore poorly suited as a marker for monitoring chronic exposure (Dhar, 2004). Cotinine, the principal metabolite of nicotine, with a half life of 16 hours is widely used as a biochemical marker of nicotine uptake to evaluate smoking status in surveys and treatment studies (Sohn *et al*, 2003; Tricker, 2004).

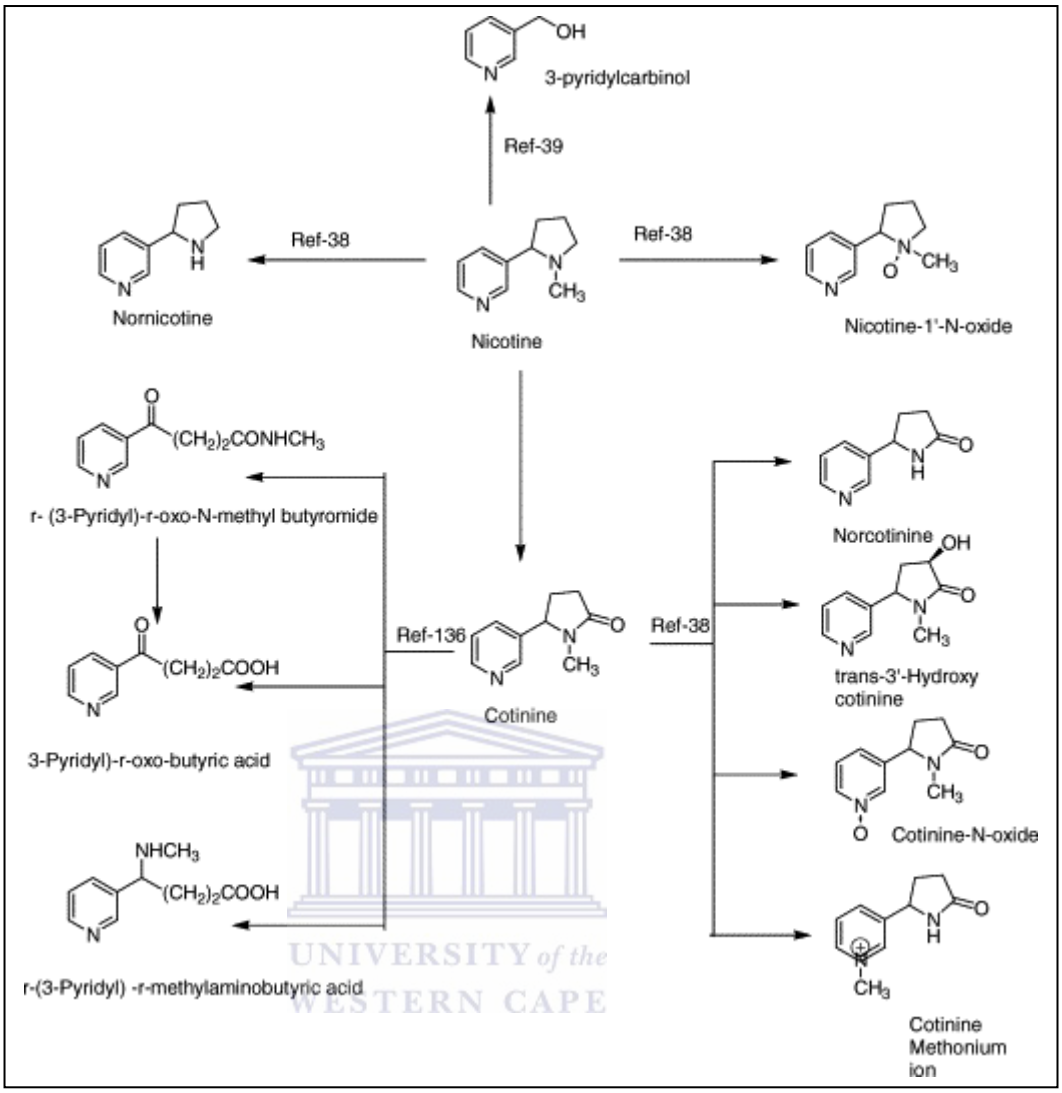

Figure 1.19: The structures of the nicotine metabolites (Dhar, 2004).

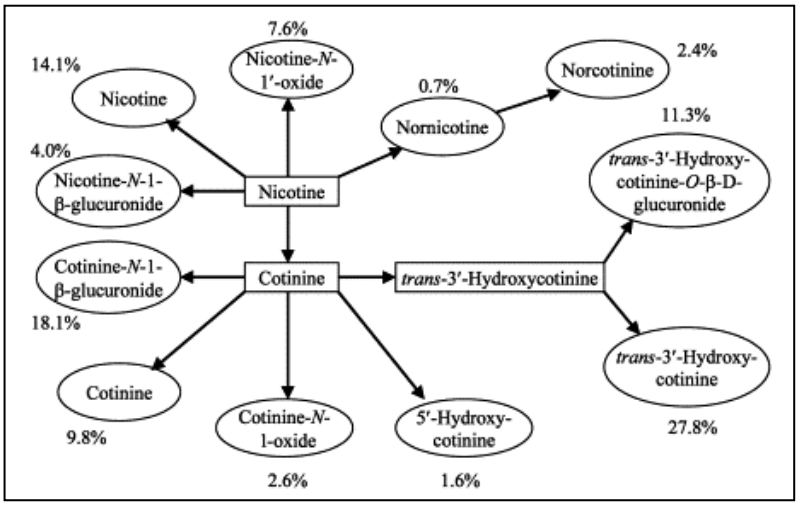

Figure 1.20: A quantitative outline of nicotine metabolism. The compounds in the square boxes detected in blood, circled compounds indicate major metabolites excreted in urine (Tricker, 2003).

Cotinine can be detected in body fluids such as blood, urine and saliva and the levels are on average 15 fold higher than levels of nicotine during regular smoking or nicotine replacement therapy (Sohn *et al*, 2003). Cotinine is transformed into secondary metabolites and the total cotinine plasma concentration is therefore determined by the summation of all four metabolites (Dhar, 2004). However, the clearance rate of nicotine in non-smokers is faster compared to nicotine clearance in habitual smokers (Tricker, 2003). This phenomenon could be because of a variant of CYP2A6 forms that have been implicated in the ability of people to metabolize nicotine at different rates (Denton *et al*, 2004).

1.7.3 Biological effects of nicotine

According to the literature, nicotine affects the organs of the body and it has been shown to either stimulate or inhibit cell proliferation. This depends on the concentration of nicotine and the tissue type.

Acetylcholine (ACh) is known primarily as a neurotransmitter and it is present at many sites outside the nervous system. The expression of so called “neuronal” nicotinic acetylcholine receptors (nAChRs) in several types of non-neuronal cells including airway epithelial cells occur in the lung (Wuenschell *et al*, 2004). Neurotransmitter receptors may, (I) promote neural cell replication, (II) initiate the switch from replication to differentiation, (III) enhance or retard axonogenesis or synaptogenesis, (IV) evoke or prevent apoptosis or (V) enable the appropriate migration and localization of specific cell populations within each brain region (Slotkin, 2004). These multiple developmental roles of neurotransmitters at the same time also render the developing brain vulnerable to neuroactive chemicals that elicit or block neurotransmitter responses (Slotkin, 2004).

The presence of a nAChRs suggest that extra-neuronal nicotinic receptor based signaling systems could be involved in autocrine or paracrine signaling in various tissues, including lung (Wuenschell *et al*, 2004). Heeschen and colleagues (2003) also suggested that nAChRs are not only expressed in neural, but also in non-neuronal cells such as endothelial cells and vascular smooth muscle cells. These receptors may be involved in the regulation of mitosis, differentiation, organization of the cytoskeleton, cell to cell contact, locomotion and migration in non-neuronal

cells (Heeschen *et al*, 2003). Nicotine enhanced endothelial cell numbers, reduced apoptosis and increased capillary network formation *in vitro*. It also enhanced angiogenesis/neovascularization in murine models of inflammation, tumour, atherosclerotic plague and ischemia (Heeschen *et al*, 2003).

The binding of nicotine to the nicotinic receptors for ACh, activates the receptors. These are a family of proteins formed by five homologous or identical subunits that are arranged symmetrically around a central ion channel. In the muscle as well as the neuron, different AChR isotypes exists. Muscle AChRs are composed of four different subunits (α, β, γ or ε and δ) (Maus *et al*, 1998). The neuronal AChRs may include only two types of subunits (α and β) or five copies of the same α subunit. At least eight different α subunits (α2-9) and three β subunits (β2-4) are expressed by the neurons. A diversity of neuronal AChRs results from the combination of different α and β subunits (Maus *et al*, 1998). Due to reports that ACh is synthesized and released by the bronchial epithelium cells (BECs) Maus and colleagues (1998) decided to investigate whether the epithelial cells that line the surface of the bronchial tree express functional AChRs sensitive to nicotine. They demonstrated previously that human skin keratinocytes express AChRs sensitive to acetylcholine and nicotine which regulate cell adhesion and motility.

Maus and colleagues (1998) also found in their study that human and rodent BECs express AChRs similar to those expressed by keratinocytes and by some neurons. The presence of AChRs sensitive to nicotine on the lining of the airways raises the possibility that the high concentrations of nicotine resulting from tobacco smoking will cause the receptors of the bronchial epithelium to be activated abnormally or desensitized or both. This may mediate or facilitate some of the toxic effects of cigarette smoking in the respiratory system. Their study therefore, provides several lines of evidence suggesting that human and rodent BECs express functional AChRs similar to those expressed by some neurons, which can be activated by nicotine. The presence of AChRs in BECs was demonstrated by the results of both structural and functional studies (Maus *et al*, 1998).

Nicotine delivered by cigarette smoke, activates the sympathetic nervous system. Activation of the sympathetic neural path results in the acceleration of heart rate,

increased myocardial contractility and constriction of some vascular beds (Arcavi *et al*, 1994). According to Lambers and Clark (1996) this is also true for the fetus. Pregnant mothers who consume nicotine via nicotine gum or cigarette smoke cause an increase in the fetal heart rate (Lambers and Clark, 1996) and fetal arterial blood pressure (Clark and Irion, 1992).

Nicotine absorbed during smoking increases the discharge of catecholamines from the adrenal medulla and from extra adrenal chromaffin tissue, which causes heart rate and blood pressure to rise (Cryer *et al*, 1976). Even rats treated with nicotine lost weight without significant reduction in food intake (Schecter and Cook, 1976). Activation of the sympathetic neural path by nicotine results in lipolysis with the release of free fatty acids which may contribute to the increase energy expenditure (Arcavi *et al*, 1994). The induction of lipolysis by nicotine could therefore help explain why the fat stores of nicotine treated animals, decreases (Winders and Grunberg, 1990). The increase in energy expenditure is thus believed to decrease the body weight of a smoker (Arcavi *et al*, 1994) and is responsible for smoking mothers to delivering low birth weight infants (Lambers and Clark, 1996).

Maternal smoking during pregnancy is also associated with alterations in pulmonary function at birth and greater incidence of respiratory illnesses after birth. Nicotine interacting with nAChRs during fetal monkey lung development caused lung hypoplasia and reduced surface complexity of developing alveoli. It also significantly increased numbers of type II cells and neuroendocrine cells in neuroepithelial bodies. These findings demonstrate that fetal monkey lung development could be altered by nicotine (Sekhon *et al*, 1999). It has also been found that the placentas of women who smoke show dramatic differences in morphology from gestation matched control placentas obtained from women who do not smoke. Such as an increase calcification in term placentas of women who smoke (Genbacev *et al*, 2000).

Maternal smoking has the potential to elicit fetal brain damage (Roy *et al*, 1998). Nicotine effectively reaches the amniotic fluid and fetal blood achieving concentrations similar to that in the maternal blood, if not more (Berger *et al*, 1998). Prenatal nicotine exposure produces neurobehavioral teratogenesis (Slotkin *et al*,

1997). This is associated with deficiencies in brain cell numbers that reflect, in part, effects on cell replication. It also involves delayed cell loss (Slotkin *et al*, 1997), synaptic abnormalities and behavioural deficits (Roy *et al*, 1998). According to Roy and colleagues (1998), a cloned cell line PC12, that developmentally resembles sympathetic neurons, indicates that nicotine of very high concentrations can inhibit DNA synthesis and neurite outgrowth. Cultured rat embryos similarly show physiochemical membrane disruption and oxidative damage by nicotine exposures at concentrations of 1 mM or more (Roy *et al*, 1998). A study conducted by Slotkin and colleagues (1997) showed that the fetal brain could be damaged due to nicotine exposure via an implanted mini-pump that infuse pregnant rats with nicotine from gestational days 4-12 or 4-21. The fetal and neonatal brain regions were examined for expression of the mRNA encoding *c-fos*, a nuclear transcription factor that becomes chronically elevated when cell injury or apoptosis are occurring (Slotkin *et al*, 1997). Their results indicated that prenatal nicotine exposure causes chronic elevations of *c-fos* expression in fetal and neonatal brain that are distinguishable from the later onset of the ability of acute nicotine to cause short-term stimulation of *c-fos* (Slotkin *et al*, 1997). Their data suggest that prenatal nicotine exposure evokes delayed neurotoxicity by altering the program of neural cell differentiation and that elevated *c-fos* expression provides an early marker of the eventual deficits (Slotkin *et al*, 1997). Studies by Trauth and colleagues (2000) proved that the adolescent brain were not as susceptible to nicotine induced neurotoxicity as the fetal brain.

This phenomenon can be due to the fact that the developing brain elicits inappropriate expression of genes leading to mitotic arrest, cell death and eventual shortfalls in neural cell numbers after nicotine exposure (Trauth *et al*, 2000). Nicotine, after binding to nicotine cholinergic receptors, alters the function of several CNS neurotransmitters, including dopamine (DA), noradrenaline (NA), 5-hydroxytryptamine (5-HT), glutamate, gamma aminobutyric acid (GABA) and endogenous opiod peptides (EOPs). Nicotine acts via nAChRs in the brain, which are diverse members of the neurotransmitter gated ion channel super family and have crucial neuromodulatory roles in the CNS (George and O'Malley, 2004). Nicotine in tobacco smoke reaches the human brain in about 10 seconds after a smoker inhales a puff and binds to nicotinic receptors located in the brain (Da Costa e Silva and Fishburn, 2004). Stimulation of the presynaptic nACh receptors increases neuron

transmitter release. Unlike most agonists, which down regulate receptor numbers with chronic exposure, chronic administration of nicotine leads to desensitization and inactivation of nACh receptors (George and O'Malley, 2004). An increase in the number of nicotine receptor sites in the brain occur and could thus contribute to enhance the addictive properties of nicotine (Da Costa e Silva and Fishburn, 2004) and long term behavioural defects thus eliciting nicotine as a neuroteratogen (Trauth *et al*, 2000).

However, nicotine is known to be a neuroprotective agent as well (Garrido *et al*, 2000; Slotkin *et al*, 2004). Garrido and colleagues (2000 and 2001) demonstrated that pre-treatment of normal cultured spinal cord neurons with 10 μM nicotine resulted in marked attenuation of all the apoptotic effects induced by arachidonic acid (Garrido *et al*, 2000 and 2001).

Lahmouzi and colleagues (2000) exposed rat gingival fibroblasts cultures to nicotine at various concentrations. They found that nicotine at concentrations of 0.05 μM to 1 mM had no affect on the DNA content or protein and collagen synthesis. However, at concentrations between 3 mM and 5 mM, growth was significantly diminished and the survival rate reduced (Lahmouzi *et al*, 2000). Hanes and colleagues (1991) showed that exposure of human gingival fibroblast to nicotine could lead to changes in cellular functions such as disruption of collagen synthesis and protein secretion.

Research done in 1979 by Ved Brat and colleagues investigated the effect of nicotine on cell division. They found that low concentrations of nicotine accelerated cytokinesis and high dosages prolonged the duration of metaphase in HeLa cells. They also suggested that the cytokinesis phenomenon might have been due to an increase in intracellular calcium pools (Ved Brat *et al*, 1979). This was because nicotine stimulates muscle contraction by a mechanism which involves its binding to membrane receptors and either the stimulation of uptake of calcium into the cell or its release from intracellular pools (Ved Brat *et al*, 1979). Studies in 1998 (Yamamura *et al*, 1998; Yoshida *et al*, 1998) proposed that an accumulation of intracellular calcium above a certain threshold level could be the initial step for cell death induction. Nuclear accumulation of calcium causes induction of DNA fragmentation that is induced by nicotine. Yoshida and colleagues (1998) showed

that nicotine did not stimulate the TNF but rather inhibited the production in human peripheral blood mononuclear cells. Nicotine also induced internucleosomal DNA fragmentaion in the human myelogenous leukemic cell lines, however, not in human peripheral blood lymphocytes and polymorphonuclear cells. This was because nicotine increased the intracellular levels of calcium to higher levels in the leukemic cells than in the normal leukocytes (Yoshida *et al*, 1998). Yamamura and colleagues (1998) found that nicotine could not induce internucleosomal DNA cleavage in human glioma and glioblastoma cell lines, even though nicotine induced increased levels of intracellular calcium. They suggested that these cell lines might have a chromatin structure resistant to endonuclease digestion (Yamamura *et al*, 1998).

A study done by Konno and colleagues (1986) on human promyelocytic HL-60 leukemia cells treated with 2 mM to 6 mM nicotine showed a dose and time dependent inhibition of cell growth. The ability of nicotine to suppress cell proliferation became more pronounced with treatment time. Nicotine induced a partial block in G_1 phase of the cell cycle, with a concomitant inhibition of S phase. They suggested that nicotine might have affected the synthesis of proteins which are involved in the progression of the cells from G_1 to S phase of the cell cycle (Konno *et al*, 1986).

Cigarette smoke has been reported to suppress proliferation, attenuate attachment and augment detachment of alveolar epithelial cells. It can also cause DNA single strand breaks in the cells and suppresses surfactant secretion and collagen production (Hoshino *et al*, 2001). It has also been found to inhibit the proliferation and migration of cultured human fetal lung fibroblast, suggesting that cigarette smoke may impair lung repair (Nobukuni *et al*, 2002) and contribute to the development of lung diseases (Hoshino *et al*, 2001). However, molecular alterations in bronchial epithelium, is also known to antedate the development of lung cancer (Barsky *et al*, 1998).

Cell death through apoptosis may be an important mechanism to prevent tumour development (Wright *et al*, 1993). Agents that inhibit apoptosis may thus function as tumour promoters. Nicotine could be seen as a promoter since it promotes tumour growth (Heeschen *et al*, 2001; Lawrence, 2003) and therefore nicotine addiction

could be linked to lung cancer (Hecht, 2002; Pfeifer *et al*, 2002). Wright and colleagues (1993) therefore investigated the effect of nicotine on the process of apoptosis. Their data, demonstrate that nicotine inhibits apoptosis induced by diverse stimuli including TNF, UV light, chemotherapeutic drugs and calcium ionophore. This phenomenon was observed in normal and transformed cells derived from a variety of species and tissues including tumour cell types related to tobacco use (Wright *et al*, 1993). According to the two stage model of carcinogenesis, tumours are initiated by exposure of cells to agents that cause mutations which tend to release cells from growth controls. Subsequent exposure to tumour promoting agents accelerates cancer development though by poorly understood mechanisms (Wright *et al*, 1993).

Lung cancer of diverse histological types express multiple, biologically active, high affinity membrane receptors for opioids and for nicotine (Maneckjee and Minna, 1994). Maneckjee and Minna (1994) showed that opioids acting via specific receptors inhibit the growth of human lung cancer cells while nicotine, acting through nAChRs, reverses this inhibition. The data indicated that engagement of opioid receptors in human lung cancer cells induces apoptosis, while engagement of nicotine receptors suppress apoptosis, which in some cases appear to be working through a PKC pathway. Maneckjee and Minna (1994) also suggest complexities in the system where blockade of C6 or C10 nicotinic receptors can lead to facilitation of apoptosis. These findings suggest new strategies for treatment and prevention of cancer using opioids or nicotine receptors antagonists and are consistent with the idea that nicotine functions as a tumour promoter (Maneckjee and Minna, 1994).

Several reports suggest that apoptosis may be an important mechanism in tumour cell death following treatment with chemotherapy and that alterations in apoptosis, such as those caused by the Bcl-2 oncogene, can lead to tumourigenesis (Maneckjee and Minna, 1994). A study done by Jenson and colleagues (1999) showed that nicotine activates the mitogen activated protein (MAP) kinase signaling pathway resulting in increased Bcl-2 protein with inhibition of apoptosis.

It has been suggested that nicotine induced inhibition of apoptosis involving Bcl-2, which might be a regulatory effect on Bcl-2 because nicotine induces extensive Bcl-

2 phosphorylation (Yildiz, 2004). Studies also showed, that nicotine acting through nicotinic acetylcholine receptors, suppresses apoptosis. This demonstrates that nicotine blocks the induction of apoptosis induced by opioids such as morphine or methadone (Yildiz, 2004). This evidence could suggest that nicotine contributes directly to lung carcinogenesis through stimulation of nAChRs in non-neuronal cells (Minna, 2003).

According to the study of Heusch and Maneckjee (1998) nicotine activates the MAP kinase signaling pathway in lung cancer cells, specifically extracellular signal regulated kinase (ERK2). This results in increased expression of the Bcl-2 protein and inhibition of apoptosis and blocks the inhibition of PKC and ERK2 activity in lung cancer cells by anti-cancer agents, such as therapeutic opioid drugs and this can adversely affect cancer therapy (Heusch and Maneckjee, 1998). Nicotine appears to have no effect on the activities of *c-jun* NH_2-terminal protein kinase (JNK) and p38 MAP kinase, which have also been shown to be involved in apoptosis. While exposure to nicotine can result in the activation of the two major signaling pathways (MAP kinase and PKC) that are known to inhibit apoptosis, nicotine regulation of MAP (ERK2) kinase activity is not dependent on PKC (Heusch and Maneckjee, 1998). These effects of nicotine occur at concentrations of 1 μM or less, that is generally found in the blood of smokers and could lead to disruption of the critical balance between cell death and proliferation, resulting in the unregulated growth of cells (Heusch and Maneckjee, 1998).

Minna (2003), showed that many lung cancers expressed nAChRs and that low concentrations of nicotine blocked the induction of apoptosis in these cells. Nicotine in pharmacologically relevant concentrations (in the 100 nM range which is attained in the plasma of smokers) induced phosphorylation of Akt on physiologically relevant serine and threonine residues within minutes (Minna, 2003). Akt is located at 14q32.3 in humans and sometimes called protein kinase B (PKB). Thus Akt activation plays a key role in inhibiting apoptosis in multiple tissues in response to growth factor deprivation or oncogene stimulation. It follows that Akt and its pathway are major targets for new cancer drug discovery. Nicotine also caused a loss of contact inhibition at high cell densities in culture and when lung epithelial cells were challenged with a variety of apoptotic inducing, stimuli nicotine inhibited the

induction of apoptosis (Minna, 2003). Nicotine could act as tumour promoter to facilitate the outgrowth of cells with genetic damage. It appears that nicotine stimulation of α7 nAChR transduces signals in a cascade to PI3K and Akt via Janus kinase 2 (JAK2). Other studies provide evidence for a novel signaling route coupling the stimulation of α7 nAChR to the activation of mitogen activated protein kinases ERK1/2 in a Ca^{2+} and protein kinase A-dependent (Minna, 2003). Activation of the serine/threonine kinase Akt in nonimortalized human airway epithelial cells *in vitro* by two components of cigarette smoke, nicotine and the tobacco specific carcinogen 4-methylnitrosamino-1-(3 pyridyl)-1-butanone (NNK) was demonstrated by West and colleagues (2003). Activation of Akt by nicotine or NNK occurred within minutes at concentrations achievable by smokers and depended upon α3-/α4 containing or α7 containing nicotinic acetylcholine receptors respectively. Redundant Akt activation by nicotine and NNK could contribute to tobacco related carcinogenesis by regulating two processes critical for tumourigenesis, cell growth and apoptosis (West *et al*, 2003). The serine/threonine kinase Akt (or protein B), controls key cellular processes such as glucose metabolism, cell cycle progression and apoptosis and activation can contribute to tumourigenesis (Lawrence, 2003; West *et al*, 2003).

The airway and alveolar epithelia contain pulmonary neuroendocrine cells (PNEC) whose structure indicates an endocrine function. They are also in contact with sensory nerve fibers. These cells often aggregate into distinct neuroepithelial bodies (NEB) (Van Lommel, 2001). PNEC and NEB seem to be most important in the fetal and neonatal lung as regulators of airway development and hypoxia-sensitive chemoreceptors. There is a link between these cells and specific types of lung cancer and their involvement in lung and paediatric pathology may be profound. An unusual property of PNEC is that they often aggregate into distinct corpuscles, the NEB, and sometimes remain as solitary neuroendocrine cells (Van Lommel, 2001). NEB and PNEC have been suspected to be sensitive to the chemical substances in tobacco smoke. Nicotine results in NEB proliferation and at times in the release or synthesis of active substances. In the fetal rhesus monkey, transplacental nicotine administration markedly increases the expression of the α7 subunit of the nicotinic cholinergic receptor in airway cells, including PNEC. Foetal hamster PNEC and human SCLC interaction of nicotine and α7 nicotinic acetylcholine receptor results

in Ca^{2+} influx, release of serotonin and over expression and activation of protein kinases (Van Lommel, 2001).

Proliferation of PNEC through exposure to nicotine may increase the release of bioactive substances. This may influence pulmonary vaso- and bronchomotor tone and induce mitogenic effects, leading to pulmonary pathologies and defective lung development. Since PNEC are stimulated by nicotine and carcinogens, and since they are cytologically similar to SCLC, they may be the progenitor cells of this type of lung cancer (Van Lommel, 2001). However, Waldum and colleagues (1996) showed that rats treated long term (two years) with nicotine with a concentration giving twice the plasma concentration found in heavy smokers, could not find any frequency of tumours in these rats compared with controls. Particularly there were no microscopic or macroscopic lung tumours or any increase in PNEC (Waldum *et al*, 1996). However, the body weight of the nicotine treated rats was reduced as compared to the controls. This study did not indicate any harmful effect of nicotine when given in its pure form of inhalation (Waldum *et al*, 1996).

Nicotine also affects the proliferation and the expression of the bombesin-like peptide autocrine system in human SCLC SHP77 cells compared with nonmalignant human bronchial epithelial BEAS 2B cells as non-neuroendocrine controls. Increase proliferation in the presence of nicotine may be due in part to increased levels of bombesin-like peptides in SHP77 cultured in nicotine (Novak *et al*, 2000). Nicotine effects on nonmalignant pulmonary neuroendocrine cells may provide additional insight into how nicotine itself may promote lung carcinogenesis. Nicotine has been implicated as playing a role in lung carcinogenesis. Nicotine stimulated the growth of human SCLC cells, human and hamster pulmonary neuroendocrine cells and human arterial smooth muscle cells. In bovine pulmonary artery endothelial cells, nicotine stimulated DNA synthesis and proliferation at concentrations as low as 10 nM, but toxicity was observed at concentrations higher than 1 μM (Novak *et al*, 2000). Nicotine stimulates secretion of a variety of growth factors and other small molecules which may act as secondary mitogens in autocrine or paracrine fashion. The involvement of other ligand/receptor/signal transduction pathways in the stimulatory actions of nicotine is also suggested by studies of signal transduction pathways. Such as where nicotine stimulates catecholamine release from bovine

adrenomedullary chromaffin cells PKC activation was also important. Mitogen activated kinases were also important as demonstrated in studies where the influence of nicotine on proliferation was inhibited (Novak *et al*, 2000).

With the growing amount of evidence it could thus be concluded that nicotine is a tumour promoter, neuroteratogen and a neuroprotector. Knowing this, how would nicotine replacement products influence a smoker's life?

1.7.4 The role of nicotine replacement therapy

Smoking is a public health problem, particularly for respiratory diseases, chronic obstructive pulmonary disease (COPD), asthma and lung cancer (Raherison *et al*, 2005). About one third of cancer patients who smoked prior to their diagnosis continued to smoke even though their survival and quality of life are adversely affected by smoking (Schnoll *et al*, 2004). Lung cancer patients who smoke should be strongly persuaded to stop smoking (Raherison *et al*, 2005). Significant risk reduction for cancers after cessation has been found to increase the life span from 5 to 15 years (Henningfield *et al*, 2005).

Clinicians are encouraged to motivate tobacco users to cease the use of tobacco. These clinicians should then abide by the Clinician Practice Guideline on Treating Tobacco Use and Dependence and guidelines set by the Food and Drug Administration (FDA) in the USA (Henningfield *et al*, 2005). Nicotine replacement therapies (NRTs) (George and O'Malley, 2004; Mahrer-Imhof *et al*, 2002), also known as alternative nicotine delivery systems (ANDS) (Kunze *et al*, 1999) have been approved as first line agents to treat nicotine dependence in the USA and other countries, including the slow acting transdermal nicotine patch (TNP) (George and O'Malley, 2004).

The two major medical disorders lead to the treatment of NRT is nicotine dependence, which is the disorder of maladaptive and chronic tobacco use and, nicotine withdrawal (Henningfield *et al*, 2005). The majority of smokers experience withdrawal symptoms after abstaining from nicotine. They are dysphoria, anxiety, irritability, decreased heart rate, insomnia, increased appetite and craving for cigarettes (George and O'Malley, 2004).

Most NRT forms deliver nicotine more slowly than smoking and the increase in nicotine blood levels is more gradual (Le Houezec, 2003). The following products are known as "acute" dosing forms (Henningfield *et al*, 2005), namely nicotine gum, nicotine nasal spray, nicotine vapour inhaler (George and O'Malley, 2004) and, since December 1999, nicotine lozenges and tablets for under the tongue have been available (Van den Berkmortel *et al*, 2000). The user determines the amount and time of the "acute" dosing products. The "passive" product is the TNP which is applied to the skin and delivered through the skin at a relatively steady rate (Henningfield *et al*, 2005). When smokers use these products they must cease all tobacco use before starting the NRT because of concerns about nicotine toxicity with concurrent NRT and tobacco use. The faster acting NRTs appear to be helpful in satiating the positive effects of nicotine administration through smoking, e.g smoking satisfaction, desire to smoke and anticipation of positive effects, and reducing acute craving, whereas the slow acting TNP formulation supplies constant low levels of nicotine which when adequately dosed, can relieve nicotine withdrawal symptoms (George and O'Malley, 2004).

At least three major mechanisms of action by which NRT medications support smoking cessation exits, namely I) the medication may reduce either general withdrawal symptoms or at least prominent ones, as a result enabling people to function while they learn to live without cigarettes. II) It may reduce the reinforcing effects of tobacco delivered nicotine. III) Nicotine, medication may provide some effects for which the patient previously relied on cigarettes, such as sustaining desirable mood and attention states, making it easier to handle stressful or boring situations, and managing hunger and body weight gain. However, the evidence for the operation of the mechanisms is not conclusive (Henningfield *et al*, 2005).

Information obtained through the literature of tobacco products indicates that it is responsible for the vast majority of health problems (Henningfield *et al*, 2005; Raherison *et al*, 2005; Sweanor, 2000). It is assumed that if a tobacco user can only refrain from smoking through the use of a therapeutic dose of 'clean' nicotine, this should be an option (Sweanor, 2000). Kunze and colleagues (1999) suggested that long term application of ANDS could be a means of preventing lung cancer and

other tobacco related diseases. ANDS are designed to provide nicotine without the harmful substances contained in cigarettes smoke (Kunze *et al*, 1999).

Although the evidence given in this review show that nicotine is a tumour promoter, neuroteratogen and a neuroprotector, the FDA has approved all the NRT medications, and they have determined it to be safe and effective in the aid of smoking cessation (Henningfield *et al*, 2005).

1.8 Essential fatty acids

'Essential fatty acids (EFAs) (figures 1.21 a and b) are essential nutrients that cannot be synthesized by the body and hence, have to be obtained exogenously' (Das, 1999a). EFAs may play an important role in the prevention and treatment of coronary artery disease, hypertension, arthritis, autoimmune disorders, cancer and other inflammatory states (Teitelbaum and Walker, 2001). However, dietary requirement generally varies with species, gender, age and the presence of physiological and pathological challenges for example, pregnancy, infancy, aging, infection, disease and other (Cunnane, 2003).

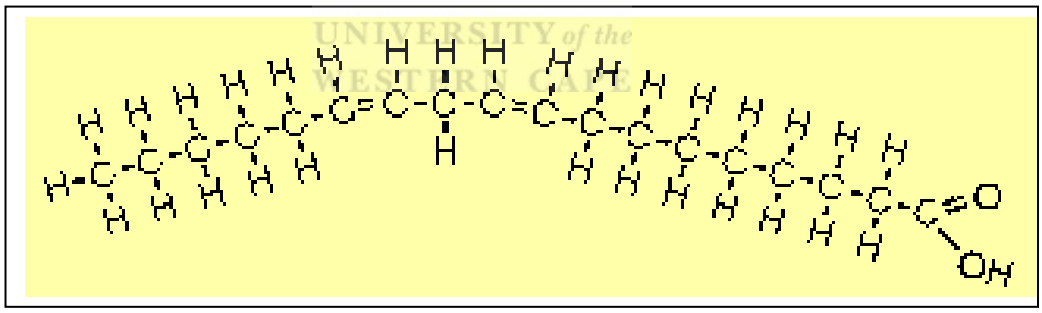

Figure 1.21 (a): The chemical structure of n-6 (http://waltonfeed.com/omega/ess_fat.html, 2006).

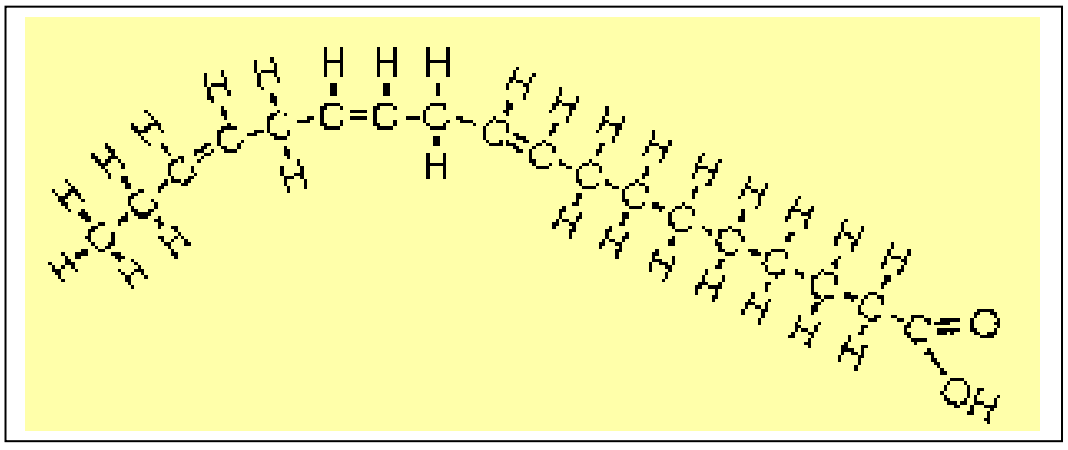

Figure 1.21 (b): The chemical structure of n-3 (http://waltonfeed.com/omega/ess_fat.html, 2006).

1.8.1 Metabolism of essential fatty acids

For both linoleic acid [LA (18:2n-6)] and α-linolenic acid [ALA (18:3n-3)] to function optimally they need to be metabolised in the tissues since they do not have any biological activity except to provide energy and to prevent fluid loss through the skin (Das, 1999a; Horrobin, 1982). The equilibrium between n-3 and n-6 fatty acids in the diet is important because of their competitive nature and their different biological roles (Smit *et al*, 2004). However, they are not interchangeable in animal and human tissues (Das, 1999a).

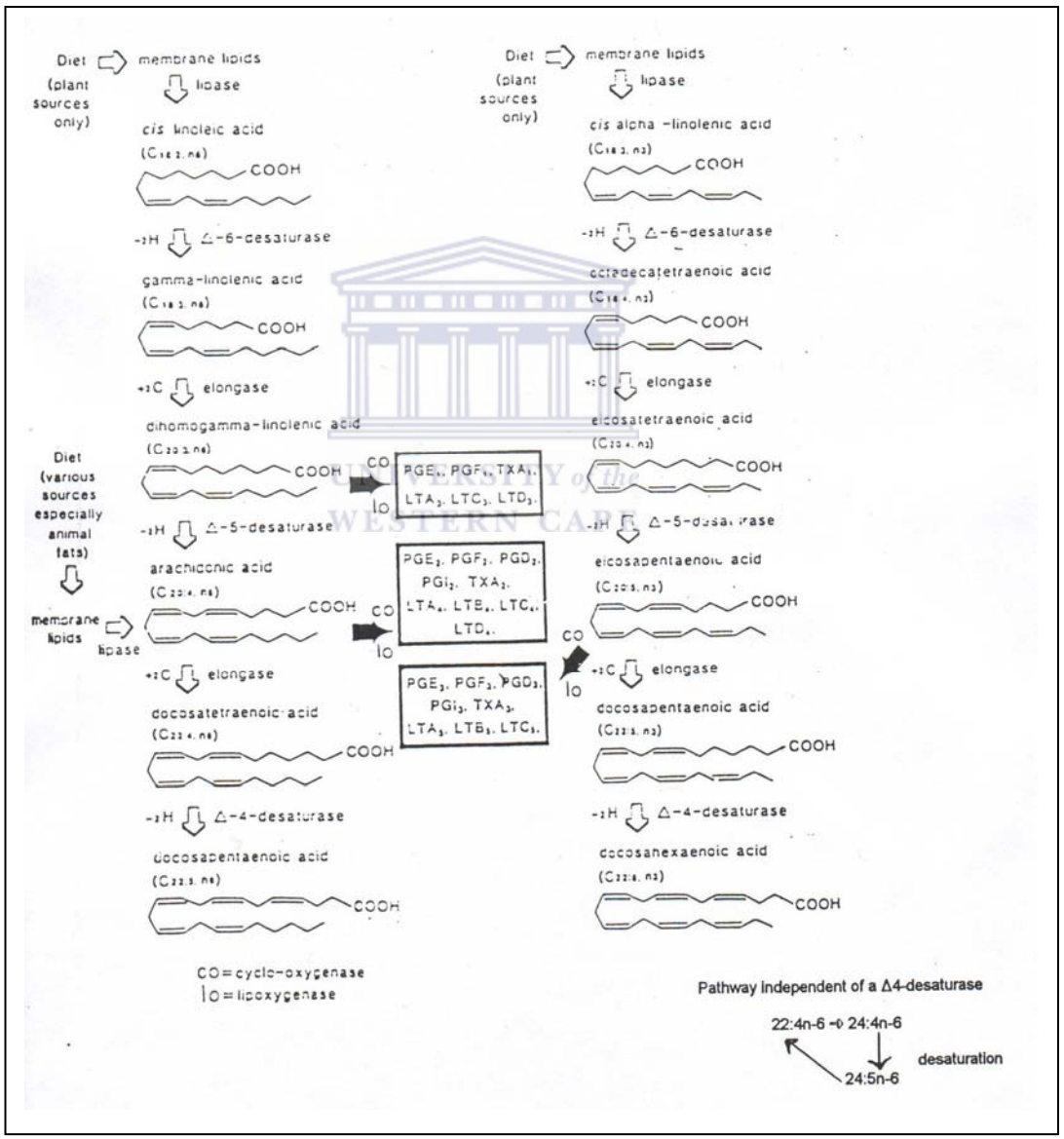

Figure 1.22: The pathway of synthesis of EFAs (Sprecher *et al*, 1994).

Figure 1.22 represents the metabolism of EFAs. Desaturation and elongation of LA (18:2n-6) to arachidonic acid [AA (20:4n-6)] and docosapentaenoic acid [DPA (22:5n-6)] and ALA (18:3n-3) to docosahexaenoic acid [DHA (22:6n-3)] (SanGiovanni and Chew, 2005), depends on the dietary contents of LA (18:2n-6) and ALA (18:3n-3) and the ratio of one to the other (Innis, 2000). In addition AA (20:4n-6), eicosapentaenoic acid [EPA (20:5n-3)] and DHA (22:6n-3) appears to inhibit desaturation of the 18 carbon precursors and is actively incorporated into lipids of the membranes (Innis, 2000).

1.8.2 The source of essential fatty acids in food

Since the n-6 and n-3 compete for enzymes responsible for their conversion and due to the fact that they are not interchangeable, humans must consume both 'parent' fatty acids (Davis and Kris-Etherton, 2003). LA (18:2n-6) is available in many vegetables, vegetable oils with the exception of palm, cocoa and coconut oils (Teitelbaum and Walker, 2001). Other rich sources include sunflower, safflower, corn, evening primrose seed, peanut and olive oils (Das, 1999a), whereas ALA (18:3n-3) is widely distributed in leafy vegetables, soya oil and linseed oil (Das, 1999a). The derived essential fatty acids such as GLA (18:3n-6) are present in human breast milk, evening primrose oil, and borage black current oils. Oats and barley contain small amounts of GLA (18:3n-6) which is believed to be responsible for their cholesterol lowering effects (Das, 1999a). DGLA (20:3n-6) is present in human breast milk and in trace amounts in organ meats such as adrenals, spleen, liver and kidney. AA (20:4n-6) is available in moderate amounts in meats, eggs; some see weeds, shrimps and prawns, whereas EPA (20:5n-3) and DHA (22:6n-3) are present mainly in marine oils especially migratory fish of the high seas such as salmon and herring oils (Das, 1999a; Horrobin, 1982).

The National Academies released a Dietary Reference Intakes Report for Energy and Macronutrients and Adequate Intakes (AI) have been set for LA (18:2n-6) and ALA (18:3n-3). The AI for LA (18:2n-6) should be 17 g/d for men and 12 g/d for women for both genders the age range is 19 to 50 years. The AI for ALA (18:3n-3) is 1.6 g/d for men and 1.1 g/d for women and for both in the aged range of 19 to greater then 70 years (Davis and Kris-Etherton, 2003). Insufficient dietary n-6 and n-3 could result in deficiency syndromes (Eritsland, 2000).

1.8.3 Functions of essential fatty acids

EFAs have at least four major roles in the human body. One: they are required for the structure of all membranes in the body (Horrobin and Manku, 1990). Particularly those in the brain, in the nervous and vascular systems (Van den Ham *et al*, 2001). They also determine the biological properties of the membranes and processing of the message received by the cell (Das, 1999a). This is due to their unsaturation that they confer on membrane properties of fluidity, flexibility and permeability (Horrobin and Manku, 1990). Two: they are the precursors for the so called eicosanoids, prostaglandins, leukotrienes and other oxygenated derivatives, derived in particular from DGLA (20:3n-6) (1 series prostaglandins), AA (20:4n-6) (2 series prostaglandins), EPA (20:5n-3) (3 series prostaglandins) and adrenic acid (homo-2-series prostaglandins) (Horrobin and Manku, 1990; Van den Ham *et al*, 2001). In almost all tissues of the body these very short lived substances are required for the second by second control of homeostasis (Horrobin and Manku, 1990). Three: they are required for the impermeability of the skin and possibly of other tissues. The skin is impermeable to the passage of water and the transport of many other substances. The impermeable barrier is entirely due to the presence of the n-6 EFAs (Horrobin and Manku, 1990). Fourth: the involvement in cholesterol transport and metabolism (Horrobin and Manku, 1990). The n-3 fatty acid plays especially a role in the prevention of coronary heart disease (Connor, 2000).

In addition they are also known to have substantial anti-bacterial, anti-fungal and anti-viral action (Das, 1999a). According to Das (1999a and b) the EFAs and their metabolites selectively kill tumour cells without harming the normal cells. This action of the fatty acids depends on their ability to augment free radical generation and lipid peroxidation process in the tumour cells (Das, 1999a and b).

1.9 Prostaglandins

In the early 1930's Von Euler, at the Karolinska Institute in Stockholm, first identified prostaglandins (PGs) in human semen (Baskett, 2003). Assuming that those compounds originated in the prostate, he then named it prostaglandins (Voet and Voet, 1995). Bergstrom and colleagues identified several members of the prostaglandin family in the early 1960's (Baker, 1990; Baskett, 2003). PGA_2 was the initial cyclopentenone prostaglandin to be discovered (Milne *et al*, 2005).

Prostanoids (prostaglandins and thromboxanes) (Helliwell *et al*, 2004b), prostacyclins (PGI_2), leukotrienes and lipoxins are all 20 carbon polyunsaturated fatty acids (Olson, 2003; Voet and Voet, 1995). A term given collectively to this remarkably diverse group of biologically active compounds is eicosanoids (Baker, 1990; Voet and Voet, 1995). With the exception of red blood cells most cells in the body produce PGs, which are released by almost any chemical or mechanical stimulus (Botting, 2006). PGs are not normally stored in the tissues but are biosynthesized from EFA as required (Baskett, 2003). PGs are short lived highly active hormone-like chemicals and minute quantities are released into the blood as required (Polgar *et al*, 1980).

1.9.1 The functions of prostaglandins

Prostaglandins have diverse biological effects, because they are involved in many physiological and pathological processes (Kimura *et al*, 2000; Pustisek and Lipozencic, 2001). It is playing a fundamental role in health, disease and therapeutic agents. Examples of these broad roles comprise gastric protection, peptic ulcer formation, intestinal fluid secretion, liver protection and damage, pregnancy, labour, abortion, glaucoma, blood pressure control, blood clotting, airway resistance and asthma, sleep, fever and modulation of inflammatory cells (Helliwell *et al*, 2004a; Schuster, 1998).

Although PGs interact directly with the cell membrane and modify the characteristics of the cells physiology (Olson, 2003), they may also act as intracellular messengers (Bos *et al*, 2004). On the other hand, the diverse activity profiles and generation of second messengers, principally cyclic AMP (cAMP), phosphatidylinositol turnover and Ca^{2+} , suggested that they interact with discrete receptors and that different receptors exist for each PG (Olson, 2003).

Bos and colleagues (2004) indicated that the five basic prostanoids could be divided into groups. The first group, $PGF_{2\alpha}$ and TXA_2, activates MAP kinase via the activation of the heterotrimeric G protein G_q, leading to the activation of the PKC pathway. The second group PGI_2 and PGD_2 cause MAP kinase inhibition through cAMP dependent pathways. The third group PGE_2 could either activate or inhibit the MAP kinase pathway depending on the subtype of receptor they bind to. The

receptors are classified on the basis of their response to a particular class of PGs (Zha *et al*, 2004). The agonists bind to their related receptors; thromboxane to TP, prostacyclin to IP, $PGF_{2\alpha}$ to FP, and PGE_2 to EP. The receptors are classified into subtypes depending on the second messenger produced. For example, four EP receptor subtypes have been described EP_1, EP_2, EP_3, and EP_4. PGE_2 thus has a wide spectrum of physiologic actions depending on the tissue distribution and subtypes of its receptors (Slater *et al*, 2002).

A strong link between prostanoid production and MAP kinase activation is found in the stimulation of mitosis. The endothelial growth factor (EGF) dependent stimulation of COX metabolites seems necessary for EGF dependent mitogenesis in Balb/c 3T3 cells, (figure 1.23). This role of prostanoid production has also been suggested for EGF induced pepsinogen release in Gastric chief cells (Bos *et al*, 2004). The type of G-protein that will be activated is dependend on any one of the five basic prostanoids and their receptors (Bos *et al*, 2004). Prostaglandin receptors combined to the guanine nucleotide binding (G) proteins will indirectly result in the stimulation or inhibition of second messengers. For example, the G_s and G_i proteins have stimulatory and inhibitory effects, respectively, on the enzyme adenylate cyclase, which catalyzes cAMP formation (Slater *et al*, 2002). The production of PGs differs from tissue to tissue (Botting, 2006) because of the variety of actions through the specific receptors of each PG (Nagata and Hirai, 2003).

PG as a signalling molecule thus plays a regulatory role in a broad variety of physiopathological processes, such as the immune response inflammation, cytoprotection and potent anti-viral activity (Elia *et al*, 1999) and are important regulators of cell proliferation, differentiation and apoptosis (Helliwell *et al*, 2004a). Prostanoids could therefore play a role in cell fate (Bos *et al*, 2004). Based on the functions of PGs, Horrobin (1980) suggested that cancer could be treated by the normalization of cancer cells using techniques harmless to normal tissues. He proposed that the majority of malignant cells have six common features, which do not appear in normal cells although each malignant cell type has its own individual features (Horrobin, 1980). The common features are aerobic glycolysis, the ability to switch off excess lactate production in the presence of excess oxygen, loss of feedback regulation of cholesterol biosynthesis, loss of the activity of delta-6-

desaturase the enzyme which converts LA to GLA, excess production of 2 series prostaglandins, loss of the ability to maintain intracellular calcium homeostasis, and a decrease in cAMP synthesis. He reasoned that the local deficiency PGE_1 or TXA_2 might give rise to the alterations in metabolism present in malignant cells. He therefore suggested that by providing GLA and DGLA and restoring the normal PGE_1 synthesis will enable the cell to bypass the biochemical block. Horrobin (1980) suggested that this procedure would normalize malignant cells and reverse cancer cell growth.

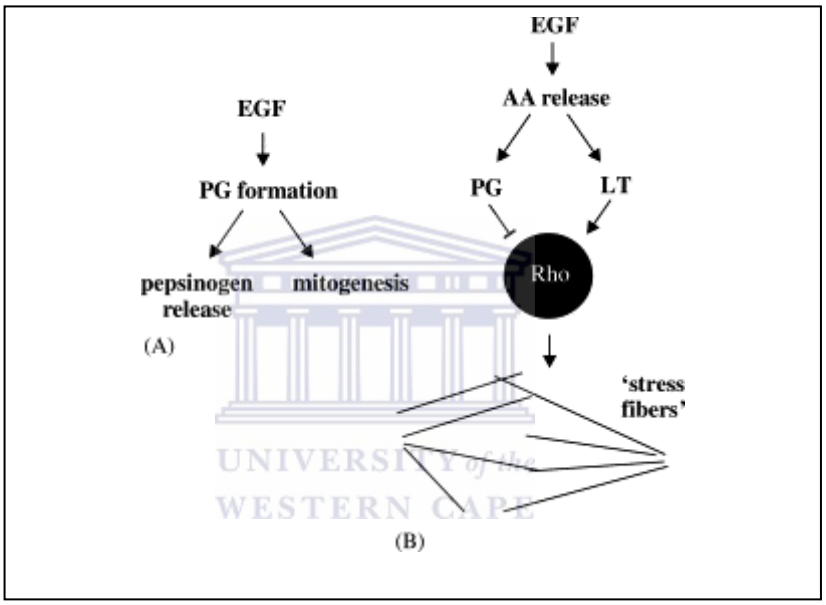

Figure 1.23: (A) EGF induced prostanoid production; (B) The release of arachidonic acid because of the cells exposure to EGF, leading to the successive production of stress fiber inhibiting prostanoids, and leukotrienes that, antagonistically, induce Rho-dependent stress fibers (Bos *et al*, 2004).

1.9.2 Prostaglandin and cancer: With reference to PGA_2

Substantiated evidence has indicated that certain PGs inhibit growth of tumour cells, therefore indicating an anti-proliferating effect (Choi *et al*, 1992). These effects of PGs on tumours have been described since the 1970s (Ishioka *et al*, 1988).

PGA compounds are considered as potential circulatory hormones (Rana and Gupta, 1984). They have been shown to be the most potent inhibitors of cell growth (Choi *et al*, 1992) in a variety of cultured cells (ElAttar and Virji, 1997; Holbrook *et al*, 1992; Parker, 1995). PGA_2 is the metabolite that has been enzymatically derived from PGE_2. The active moiety, in suppressing growth, are believed to be attributed to the

alpha and beta unsaturated carbonyl moiety in the cyclopentenone ring (Choi *et al*, 1992; Holbrook *et al*, 1992).

PGA_2 induce growth inhibition, independent of cAMP as evidence suggests (ElAttar and Virji, 1997; Ishioka *et al*, 1988). Conventional PGs such as PGE_2 act on cell surface receptors to exert their actions. In contrast, PGA_2 have no cell surface receptors but are actively transported into cells (Choi *et al*, 1992; ElAttar and Virji, 1997). This transportation occurs by means of a specific carrier on the cell membrane to eventually be transported into the nucleus where they bind to nuclear proteins (Choi *et al*, 1992; ElAttar and Virji, 1997). The uptake and accumulation in the nuclei are closely correlated with the extent of cell growth inhibition. Evidence suggests that the accumulation of PGA_2 into the cell nuclei is the first key step in the elicitation of their biological activities (Choi *et al*, 1992; Ohno and Hirata, 1993).

PGA_2 inhibition in growth of carcinoma cells relate to various mechanisms. The initial being to accumulate in the cell nuclei (Choi *et al*, 1992; Ohno and Hirata, 1993). Studies in the eighties by Ishioka and colleagues (1988) revealed that PGA_2 at 0.5 or 5.0 μg/ml arrested HL-60 (human promyelocytic leukaemia) cells in the G_0-G_1 phase of the cell cycle. In later years it has been proven to cause an arrest of the cell cycle in G_1 phase in a wide variety of cell types that are known to be insensitive to inhibition (Parker, 1995). PGA_2 was also found to inhibit the proliferation of B*l*6 melanoma (mouse melanoma cell line) and an erythroleukemia virus infected cells *in vivo* as well as *in vitro*, by influencing the cell cycle with a decline in the rate of progression, by distinctively arresting cells in the G_1 phase. This arrest relates to the decline in both *c-myc* and N-*myc* expression (Holbrook *et al*, 1992). Exposure of HeLa cells to PGA_2 resulted in a marked inhibition of cell proliferation which was associated with a significant induction of *gad 153* mRNA, a member of a novel class of genes linked with growth arrest and DNA damage (Choi *et al* 1992). Research done by Lin and colleagues (2000) using the human NSCLC H1299 cells demonstrated that PGA_2 decreased cyclin D1 expression by decreasing cyclin D1 mRNA stability and implicates a 390 base element in the 3'UTR in this regulation.

Glutathione (GSH) plays a key function in protecting cells against the toxic effects of reactive oxygen species and free radicals. A decrease in cellular GSH causes the

cell to become vulnerable to toxicants (Maher, 2005). Ohno and colleagues (1991) have demonstrated that PGA_2 stimulates the biosynthesis of γ-glutamylcysteine synthetase and elevated cellular GSH contents in various cultured mammalian cells. They suggested that pre-treatment of cells with PGA_2 could be effective to prevent the deleterious actions of reactive nucleophiles or radicals.

In 2001, Joubert and colleagues found that PGA_2 increased tyrosine kinase (TK) activity in both HeLa (human cervical carcinoma) and MCF-7 (human breast carcinoma) cells. The MCF-7 cells only indicated one protein of 55 kDa while the HeLa cells showed a range of proteins of about 21.5-97 kDa of tyrosine phosphorylation (Joubert *et al*, 2001). This was also a confirmation to an earlier study (Joubert *et al*, 1999) with two squamous oesophageal carcinoma cell lines (WHCO1 and WHCO3) and normal monkey kidney (NMK) cells exposed to PGA_2. PGA_2 caused an increase in the TK activity with a decrease in tyrosine phosphorylation of 55 kDa (Joubert *et al*, 1999). In 2003 Joubert and colleagues established an anti-mitogenic effect of PGA_2 on the HeLa and MCF-7 cell lines. PGA_2 also induced morphological changes in these cells such as chromatin aggregation, cell membrane blebbing and an uneven distribution of chromosomes (Joubert *et al*, 2003). Joubert and colleagues (2005) furthermore found that PGA_2 stimulated the increase of the expression levels of the pro-apoptotic protein Bax in HeLa cells.

Comparative studies of PGA_2 to chemotherapeutic agents (adriamycin, cyclophosphamide and hydroxyurea) have also indicated that PGA_2 had a more outspoken effect on DNA synthesis and cell proliferation in murine melanoma cells than the chemotherapeutic substances (ElAttar and Virji, 1997). PGA_2 seems to have a universal action of anti-tumour activity (ElAttar and Virji, 1997). However, in many studies the mechanism of action of this prostaglandin is not well understood and remains an enigma (Parker, 1995).

1.10 Chemoprevention

Chemoprevention is a fairly recent approach to cancer (Van Zandwijk and Hirsch, 2003), where the objective is the use of specific natural or synthetic substances to delay, reverse, suppress or prevent carcinogenic progression to invasive cancer

(Cohen and Khuri, 2002; Hecht, 2002; Vignot *et al*, 2005). This concept is based on epidemiological and experimental studies showing that certain compounds may influence carcinogenesis (Van Zandwijk and Hirsch, 2003). This is an approach that could be applied in populations at high risk of cancer (Hecht, 2002). The development in molecular biology and pharmacology has increased the probability that cancer prevention will rely on interventions collectively termed `chemoprevention' (Tamimi *et al*, 2002).

According to Saba and colleagues (2004) the term chemoprevention was used in the mid 1970s with the intention of reversing premalignant cells and preventing progression to invasive cancer. However, according to Gescher and Steward (2005) it was only in the early 1990s that this concept delivered results.

How do we distinguish between chemotherapy and chemoprevention? They have common elements however, there are also distinct differences. Chemoprevention focuses on reduction of incidence and is related to classical epidemiology, whereas chemotherapy focuses on prognosis and is related to clinical epidemiology. Chemoprevention is almost always systemic whereas, chemotherapy can be either systemic or, in certain cases, localized. Chemoprevention is usually of low frequency whereas; chemotherapy is generally a high frequency event (like death or metastasis). Finally, chemoprevention generally is administered to healthy people for whom serious side effects are unacceptable, whereas, chemotherapy applied to seriously ill patients, for whom side effects, even serious ones, may be tolerable (Tamimi *et al*, 2002).

Since carcinogenesis is a multistage process and frequently has a latency of many years or decades, there is significant opportunity for intervention (Tamimi *et al*, 2002). Chemoprevention is the intervention within the multistep carcinogenic development (Saba *et al*, 2004) by means of pharmacological or natural compounds (Cohen and Khuri, 2002). Chemoprevention is meant to interrupt the clonal propagation of aberrant cells by blocking DNA damage, retarding or reversing malignant phenotype or inducing apoptosis in the damaged cells of premalignant lesions (Cohen and Khuri, 2002).

Numerous models that assist the identification of intermediate biomarkers have been developed to outline the pathways through which carcinogenesis may occur (figure 1.24).

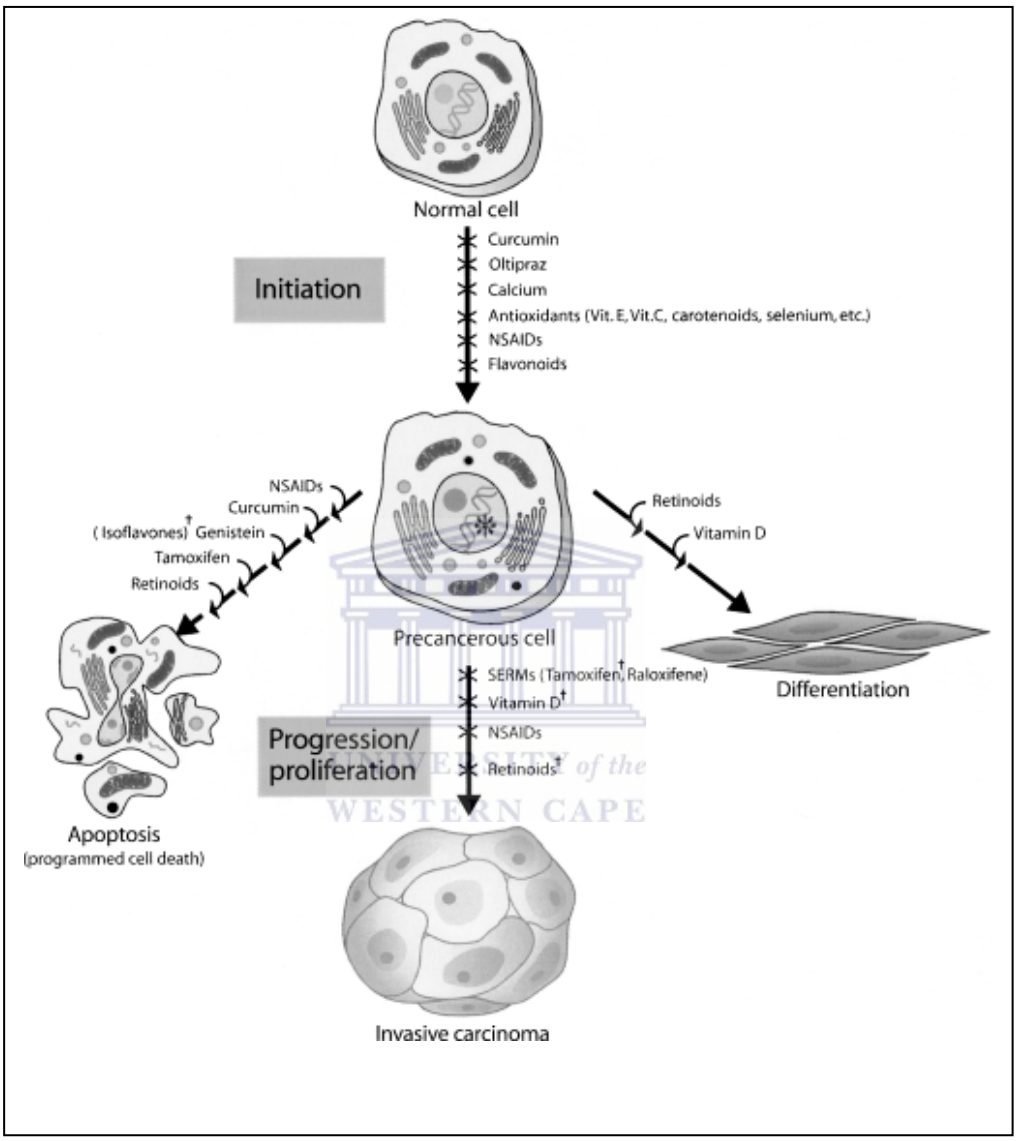

Figure 1.24: A postulated mechanism through which proposed chemopreventive agents may act. The Xs are indicative of the hypothesis to inhibit carcinogenesis and the arrows indicate the agents believed to divert the cell toward pathways away from carcinogenesis (Tamimi *et al*, 2002).

By serving as surrogate endpoints or intermediate endpoints the markers are pivotal in identifying chemopreventive agents (Tamimi *et al*, 2002; Veronesi and Bonanni, 2005). The intermediate endpoints can include specific molecular pathways, levels of circulating proteins, expression of histological markers, the presence of atypia in cytological samples, detection of genomic DNA from malignant cells in the peripheral blood, or other events that may be assessed in the pre-clinical phase and

are associated with cancer development (Veronesi and Bonanni, 2005). The use of these markers could allow chemopreventive studies to focus on stage arrest or reversion following treatment (Tamimi *et al*, 2002).

The strategies of chemoprevention could be considered at three different major levels, primary, secondary and tertiary. Primary prevention is defined as an intervention intended to prevent the development of cancer or hinder its progression. Primary prevention is directed at the normal healthy individuals of a population. Examples of this strategy would be the approach of smoking prevention and cessation treatments or the use of chemoprevention drugs in a group of asymptomatic smokers. Secondary chemoprevention is intended for persons with evidence of early disease, therefore to reverse the progression of premalignant lesions. Whereas, tertiary prevention involves reducing the morbidity of the established disease. An example of tertiary prevention is when a second primary tumour in patients are treated for or cured of an initial malignancy (Cohen and Khuri, 2002; Saba *et al*, 2004).

Numerous types of agents have shown promise as chemopreventive agents. These include anti-oestrogens, anti-inflammatories and anti-oxidants, and other diet derived agents (Tamimi *et al*, 2002).

1.10.1 Chemoprevention: With reference to lung cancer

The conventional treatment for lung cancer is surgery, radiation and chemotherapy. These interventions have produced a slight decrease in mortality rates. However, it appears unlikely that marked improvements will occur in the near future (Cohen and Khuri, 2002; Van Zandwijk, 2005). The key cause for this lack of progress is related to the fact that lung cancer is a conglomerate of diseases, where the symptoms elicit at a relatively late stage (Van Zandwij, 2005). The most effective way to reduce lung cancer mortality is prevention (Hecht, 2002). The poor lung cancer survival statistics encourages the development of new methods to control this most deadly form of cancer (Selvendiran *et al*, 2004; Van Zandwijk and Hirsch, 2003).

Cancer of the lung was an early target for chemoprevention trials (Tamimi *et al*, 2002). Epidemiological studies consistently suggested that people consuming diets

rich in fruits and vegetables are at a reduced risk of developing many types of cancers, including lung cancer (Hecht, 2002; Tamimi *et al*, 2002). Because of these studies, β-carotene was the original driving force as a chemopreventive agent. Even though there was no direct evidence to support the idea that β-carotene was going to be the `magic bullet' against cancer (Tamimi *et al*, 2002).

The relationship between diet and lung cancer has been extensively explored. In various studies interest has been focused on carotenoids, in particular β-carotene because of its antioxidant properties and the importance of this pro-vitamin for epithelial growth and differentiation (Van Zandwijk and Hirsch, 2003). However, β-carotene and other agents (include N-acetylcysteine, 13-cis-retinoic acid, retinyl palmitate) have failed in clinical trials (Hecht, 2002).

However, prostaglandin series 2 synthesized from AA by the action of COX-1 and COX-2 (also called PGH synthase or PG endoperoxide synthase) (Helliwell *et al*, 2004b; Reiter *et al*, 2004; Rowley *et al*, 2005; Schuster, 1998) has proven to become an experimental chemotherapeutic agent (Lin *et al*, 2000). Research done by Lin and colleagues (2000) using human Non-SCLC H1299 cells has demonstrated that PGA_2 down regulates cyclin D1 expression by decreasing cyclin D1 mRNA stability and implicates a 390 base element in the 3'UTR in this regulation. On the other hand, other targets for chemoprevention in lung cancer have also incorporated COX-2 inhibitors (Saba *et al*, 2004).

Treatment of lung cancer in the form of chemoprevention could be envisioned by considering the steps in retardation or blockage of the steps leading to genetic damage, or stimulation of protective processes. This would delay or stop the appearance of lung cancer (Hecht, 2002). Chemoprevention agents must possess a relevant mechanism of action, optimal pharmacokinetics, preclinical efficiency in *in vitro* and animal models, and have a toxicity profile. A potential agent for chemoprevention should be well tolerated and without major side effects. Given that this will be used for a long period of time in a large sector of the population, the majority of whom are well and asymptomatic and might never develop cancer. If however, the side effects are significantly severe it might counteract or undo any potential gain from such agents (Cohen and Khuri, 2002).

Cigarette smoking is not just going to vanish in the near future (Hecht, 2002). High risk cancers such as Non-SCLC, have apart from smoking cessation, no options to decrease their steadily increasing risk (Van Zandwijk and Hirsch, 2003). Those smokers, who fail in smoking cessation programmes, would also be candidates for chemoprevention, as would those who successfully stop, because the risk of lung cancer remains high for many years after cessation (Hecht, 2002). At present there are also no known chemopreventive agents that are effective against lung cancer in humans (Hecht, 2002). However, chemoprevention could logically be coupled with smoking cessation (Hecht, 2002) by the use of nicotine replacement therapy as discussed previously in the text.

1.11 Motivation

Lung cancer is the most important cause of cancer related mortality worldwide (Gregorc *et al*, 2003; Guessous *et al*, 2007). Eighty percent of lung cancer cases are Non-SCLC (Lau *et al*, 2006). Despite all the novel treatments available to lung cancer patients, the five year survival rate remains less than 15% (Gregorc *et al*, 2003; Huber and Stratakis, 2004). However, cancer prevention has become part of cancer control and the National Cancer Institute in the USA has made provision for support for the research and development of chemopreventive agents (Crowell, 2005).

Since the knowledge of the mechanism of chemoprevention substances on human Non-SCLC is relatively limited, finding a therapeutic outcome would prolong the patient's life. The supplement PGA_2, an inducer of apoptosis and controller of cellular differentiation of human lung cancer tissue, could act as a chemoprevention supplement.

The literature has clearly shown nicotine as a tumour promoter that stimulates cell growth while PGA_2 inhibits cell growth in a variety of cultured cancer cell lines. Nicotine, however, is being prescribed as a chemoprevention drug to non cancer and cancer patients. This could also be a factor contributing to the poorer long term survival of cancer patients. Using these two supplements in combination, would enable us to investigate which of the supplements would be more dominant in regulating the cell growth in stimulation or inhibition of a cultured cancer cell line.

This study investigated the effects of nicotine and PGA_2 as a stimulant or inhibitor on cellular proliferation and whether nicotine and PGA_2 had cytotoxic effects by indusing apoptosis in the lung cancer cell line NCI-H157. In this study the following were used:

1) nicotine, 1 mM, 1 µM and 1 nM and
2) PGA_2, 5 µg/ml, 10 µg/ml and 20 µg/ml, as well as
3) combination of nicotine and PGA_2; the supplement 5 µg/ml PGA_2 was added to 1 mM, 1 µM and 1 nM nicotine respectively. The same applied for 10 µg/ml and 20 µg/ml PGA_2.
4) these supplements were used at three different exposure times namely 24, 48 and 72 hours.

This study was carried out on the transformed human lung carcinoma NCI-H157 cell line and the objectives were:

To determine the effects of nicotine, PGA_2 and combinations thereof on:

1) the cell proliferation of the NCI-H157 cells
2) the morphology of interphase and dividing cells, as well as on the morphology of the dying cells were compared and quantified
3) the nucleolar organizer region (NOR) of these cells
4) the cytoskeleton (α-tubulin) of the cancer cells with aid of indirect immunofluorescence and to identify apoptotic cells using Hoechst 33342
5) the cell cycle progression and apoptosis induction in the transformed cells using flow cytometry (DNA propidium iodide stain, Annexin V and caspase-3) to identify possible apoptotic cells
6) the protein synthesis of mature and procaspase-3 by using SDS-PAGE and immunoblotting

CHAPTER II

Materials and Methods

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2.1 Chemicals and products

2.1.1 Amersham Pharmacia Biotech, UK supplied:

- ECL ™ Western blotting and analysis system

2.1.2 Biorad, UK supplied:

- Acrylamide/Bis 40%
- Filter paper
- Polyvinyl difluoride membrane
- Prestained standard Kaleidoscope
- Temed
- Tris buffer
- Tris/glycine/SDS buffer for SDS PAGE

2.1.3 Fluka, Germany supplied:

- Propidium iodide (PI)

2.1.4 Gibco BRL, Scotland supplied:

- Fetal bovine serum
- Gentamicin
- Penicillin/streptomycin
- Phosphate buffer saline (PBS)
- RPMI 1640
- Trypsin

2.1.5 Greiner, Germany supplied:

- Consumables (example, culture flasks)

2.1.6 Merck, Germany supplied:

- Anti-β-actin (Calbiochem)
- Anti-caspase-3 for Western blots (Calbiochem)

- Nicotine
- Other analytical grade chemicals

2.1.7 PharMingen, USA supplied:

- Annexin V-FITC
- Fluorescein isothiocyanate (FITC) Conjugated rabbit anti-active caspase 3 monoclonal antibody (flow cytometry)

2.1.8 Roche, Germany supplied:

- Staurosporine

2.1.9 Sigma, Germany supplied:

- Ammonium persulphate (APS)
- β-mercaptoethanol
- Crystal violet stain
- Goat anti-mouse IgG (whole molecule) FITC conjugate, affinity isolated antigen specific antibody
- Hepes
- Hoechst 33342
- Hydroxyurea
- Maleic acid
- Monoclonal anti-α-tubulin, Clone DM 1A
- Monoclonal anti-FITC biotin conjugate Clone FL-D6
- Poncheau S
- Prostaglandin A_2 (PGA_2)
- Sodium dodecyl sulphate (SDS)
- Triton X-100
- Tween® 20

2.2 Cell line

A squamous non-small cell lung cancer (Non-SCLC), NCI-H157 (human lung cancer) was used in this study. The NCI-H157 cells express methadone opioid receptors and C6 and C10 nicotinic acetylcholine receptors (Maneckjee and Minna,

1994). The cell line was purchased through Sterilab Services from the American Type Culture Collection (ATCC), Maryland, United States of America.

2.3 Culture medium

RPMI 1640 with glutamax was used and supplemented with 5% fetal bovine serum (FBS) (heat inactivated), 0.2% penicillin/streptomycin (5 000 units/ml penicillin and 5 000 µg/ml streptomycin utilizing penicillin G [sodium salt] and streptomycin sulphate) and 0.2% gentamicin (10 mg/ml gentamicin sulphate, prepared in distilled water). This medium was routinely used for continuous cultures.

2.4 Cell maintenance

The cells were grown and maintained as monolayer cultures. The stock cell cultures were maintained in a humidified 5% CO_2 atmosphere at 37^{o}C. The cells were re-supplemented twice a week and subcultured at weekly intervals. Early passage cell stocks, were stored at -150^{o}C to ensure a continuous supply.

2.5 Supplements

2.5.1 Nicotine

The purity of nicotine was 98%. The drug was directly made up in the cell culture medium (2.3), in the following three concentrations 1 mM, 1 µM and 1 nM and stored in dark vials at 4^{o}C until required.

2.5.2 Prostaglandin A_2 (PGA_2)

PGA_2, with a purity of 95%, after the removal of methyl acetate by degassing. The fatty acid was dissolved in ethanol to give a concentration of 10 mg/ml. This stock solution was stored in a dark vial, at -20^{o}C. When required the solution was diluted in the culture medium (2.3). The following three concentrations 5 µg/ml, 10 µg/ml and 20 µg/ml were used in this study.

The final ethanol concentrations in the growth medium did not exceed 0.1%. Ethanol was not used as a supplement in this study, but since it was used as a vehicle for PGA_2 it should be notified that research done by De Kock (1989) and Joubert and colleagues (1999) showed that up to 0.3% ethanol and up to 1% ethanol respectively, could be used as a vehicle and have no significant effect on cell growth.

2.5.3 Supplement combinations and exposure times

Throughout the study nicotine and the endogenous metabolite PGA_2 were used at the above mentioned concentrations and in combinations. Where 5 µg/ml PGA_2 was added to 1 mM, 1 µM and 1 nM nicotine respectively. This was also done for 10 µg/ml and 20 µg/ml PGA_2. Thus these supplements were used individually, but also in combination. Untreated cells were used as controls in all experiments. Three different exposure times namely 24, 48 and 72 hours were used in the study.

2.6 Staurosporine

During the study staurosporine (purity of 98%) was used as a positive inducer of apoptosis. Thus staurosporine acted as the positive control for apoptosis induction. Staurosporine is a broad spectrum kinase inhibitor known to induce apoptosis in many cell types. Kinases are enzymes that are critically involved in cell signaling and staurosporine prevents ATP binding to the kinase because of its stronger 'affinity' for the kinase (Martin *et al*, 1995). Studies done in our laboratories showed that a concentration of 1 µM (using DMSO as a vehicle) at an exposure time of 24 hours induced apoptosis (unpublished). Thus, a concentration of 1 µM was used at an exposure time of 24 hours. When required, the solution was diluted in the culture medium (2.3).

2.7 Proliferation studies

Cell proliferation studies were performed to assess how cell proliferation was affected by the different supplements.

2.7.1 Cell synchronization

A synchronized culture is one where cells pass through the division cycle as a relatively uniform cohort and represent, at different time points, cells of different cell cycle ages (Cooper, 2002). The length of the cell cycle of a cell line and the different time lengths for the different cell cycle phases could thus be determined in synchronized cells. Cell synchronization is thus a valuable tool to block a cell in a particular phase and therefore to analyse genes or proteins (Cooper, 2002; Bootsma, 1964).

The duration of the S phase, G_2/M transition period and mitosis were determined with the aid of mitotic cell count after removal of a block, hydroxyurea. Hydroxyurea block cells in the G_1 phase by inactivating ribonucleotide reductase. Ribonucleotide reductase is composed of two non-identical subunits: M1 and M2. M1, contains the allosteric binding sites for effector molecules, and M2 contains the tyrosyl radical, necessary for the reduction reaction and to which hydroxyurea binds (Thelander and Reichard, 1979). Hydroxyurea, by destroying the tyrosyl radical on the M2 subunit of ribonucleotide reductase, is a specific inhibitor of DNA synthesis (Albert and Nodzenski, 1989).

Cells were seeded at 300 000 cells per well, onto coverslips in a 6 well culture plate. After a 24 hour growth period (to allow the cells to become preconfluent) the cells were exposed to 1.5 mM hydroxyurea prepared in the culture medium (2.3) for 24 hours, which effectively blocked the cells at the G_1/S boundary. After the synchronization the mitotic cell count was less than 1%. The block was removed by washing the cells 3 times for 10 minute incubation periods with RPMI 1640 medium. This medium was without hydroxyurea. Duplicate coverslips were removed hourly for 24 hours after the removal of the G_1/S block. The experiment was terminated by fixing the cells on the coverslips in Bouin's fixative for a period of 20 minutes. The cells were then stained using a standard staining procedure for haematoxylin and eosin (H & E) (Kiernan, 1990). The coverslips were then mounted on microscopic slides, where after the mitotic cell count were determined by counting 1 000 stained cells per coverslip (De Kock, 1996). No statistical analysis was done, however data obtained where expressed in a graph as the number of cells counted.

2.7.2 Mitotic cell count

Cells were seeded onto sterilized coverslips (22 x 22 mm) in a 6 well culture plate. Cells were seeded at 300 000 cells per well in 2 ml culture medium. After a 24 hour growth period, to allow the cells to become preconfluent the cells were exposed to the supplements as explained in section 2.5.3. The experiment was terminated by fixing the cells on the coverslips in Bouin's fixative for a period of 20 minutes. The cells were then stained using a standard staining procedure for H & E (Kiernan,

1990). The coverslips were then mounted on microscopic slides, analyzed and the mitotic cell counts were calculated, using a Zeiss light microscope.

The total mitotic cell count (expressed as the number of cells counted) included all the normal phases of dividing cells as well as the cells in abnormal metaphases (including metaphase cells with lagging chromosomes and or metaphases with highly condensed chromosomes). This experiment was repeated six times. Data was then statistically analyzed.

2.7.3 Crystal violet staining (CV)

CV assay is a reliable and easy method for the monitoring of cell proliferation in cultures. This technique is a colorimetric assay, based on the estimation of cell numbers using crystal violet staining of cultures in 96 well culture plates *in situ* (Bonnekoh *et al*, 1989; Gillies *et al*, 1986). However, this assay does not distinguish between cell proliferation inhibition, decreased cell adherence, or cell death via apoptosis or lysis (Panzer, 1999).

Cells were seeded in a 96 well microtiter plate at 10 000 cells per well in a 100 µ*l* of culture medium. After a 24 hour growth period (to allow the cells to become preconfluent) the cells were exposed to 200 µ*l* of the supplements (after discarding of medium) as explained in section 2.5.3. After discarding of medium the experiment was terminated by the addition of 100 µ*l* of 1% gluteraldehyde in phosphate buffered saline (PBS) and incubated for 15 minutes. The fixative was discarded and the DNA of the cells was then stained for 30 minutes with a 100 µ*l* of 0.1% crystal violet (CV) in PBS per well. The culture plate was then immersed in running tap water for 15 minutes and air dried thoroughly. After the plate had dried, the metachromophore was extracted with 200 µ*l* of 0.2% Triton X-100 (in PBS) per well. The plate was incubated for 90 minutes and 100 µ*l* of the liquid content was transferred to a clean 96 well microtiter plate. All incubation was done at room temperature. The absorbance of the specimens was analyzed using an El_x800 Universal Microplate Reader. The absorbance was read at 570 nm, thus the color intensity was an indication of the DNA content, therefore an indication of cell progression (Joubert *et al*, 1999). The experiments were repeated six times.

2.7.4 Apoptotic count

H & E staining, which were performed as described previously in 2.7.2 was used to evaluate the apoptotic count. The apoptotic counts were obtained by counting 1 000 cells per coverslip. The total apoptosis (expressed as the number of cells counted) included all the cells with a morphological description of cell shrinkage, chromatin condensation and cytoplasmic blebbing, the apoptotic bodies. This experiment was repeated six times.

2.8 Morphological studies

A morphological assessment was done by means of observation, thus assessing for changes in the morphological development of the cells, which included apoptotic body formation, the observation of fragmentation and segregation of the nucleoli, the deformity of the cytoskeleton, with reference to α-tubulin and the viability of the cell.

2.8.1 Haematoxylin and eosin stain

Gross morphology was evaluated by H & E staining, which was performed as described previously in 2.7.2. Photographs were taken with a Zeiss light microscope and analyzed with the Matrox computer program. The magnification was x400, except where stated differently.

2.8.2 Silver staining for NOR activity

The following silver staining method is known as the Nucleolus Organizer Region silver (AgNOR) staining of cytological preparations (Ochs, 1998). The Nucleolar organizer regions (NORs) are chromosomal segments in which ribosomal RNA is encoded and the AgNOR technique reveals these regions (Hucumenoglu *et al*, 2002). This is a simple one step technique with the use of a protective colloidal developer in combination with aqueous silver nitrate (Busch and Smetana, 1970). With this technique the cultured cells were being impregnated with silver salts, which bound selectively to the region of mitotic chromosomes containing tandem repeats of the ribosomal RNA genes, the so called NOR located at the secondary constrictions of the NORs bearing chromosomes, and the nucleolus of the interphase (Ochs, 1998). The NORs are stained black to observe nuclear activity, while the chromosome arms stained yellow (Busch and Smetana, 1970). NOR related proteins are acidic non-

histone proteins stained by silver and although their real biological functions are contradictory, AgNOR result for each cell is accepted as a marker of cellular activity (Hucumenoglu *et al*, 2002). This technique was used to monitor the fragmentation and segregation of the interphase nucleolus and the chromosomal NORs, and therefore an indication of the cellular activation.

The following silver staining method for the NOR was modified. 300 000 Cells were seeded onto sterilized coverslips (22 x 22 mm) in 2 ml culture medium per petri dish (35 mm x 10 mm). Near confluent layers of cells were exposed to the supplements as explained in section 2.5.3. The experiment was terminated, by fixing the cells on the coverslips in 2% formaldehyde in PBS for 30 minutes. The cells were then washed thrice for a period of 5 minutes each time in PBS. The cells were then permeated with a solution of 95% ice cold ethanol in PBS at -20^{o}C for 5 minutes. Then washed again thrice for 5 minutes in PBS and thrice for 5 minutes in distilled water. For the selective staining of AgNORs, 2 drops of colloidal developer (2 gr powdered gelatin dissolved in a 100 ml dH_2O and heated until dissolved; then added 1 ml pure formic acid; solution stable for two weeks) and 4 drops of the aqueous silver nitrate (4 gr silver nitrate was added to 8 ml dH_2O) were pipetted onto the surface of a microscope slide and mixed. The coverslip was placed onto the mixture and the slide was placed onto the surface of a slide warmer that had been stabilized at 70^{o}C. Within 30 seconds, the silver staining mixture turned yellow and within less than 2 minutes became golden brown. The slide was then removed and the coverslip was rinsed under running de-ionized water. The following dehydration process was then followed: rinsing of the coverslips in 25%, 50%, 75%, 95% and a 100% ethanol for 5 minutes each, and twice for 5 minutes with xylene. The coverslips were then mounted with DPX mounting media on a microscope slide and left to dry (Howell and Black, 1980; Ochs, 1998). The slides were then analyzed using a Zeiss light microscope. Photographs were taken and analysis using the Matrox computer program. The magnification was x1 000, except where stated differently.

2.8.3 Indirect immunofluorescence

Fluorescence microscopy represents a simple, efficient approach for examining the distribution of specific molecules in cells. Such information is particularly important for the study of the cytoskeleton, since the molecular components involved often

undergo dramatic reorganizations in response to internal and external stimuli. Most applications of fluorescence microscopy to date involve fixing cells and staining with either specific antibodies (immunofluorescence) or specific fluorescent ligands, these ligands are known as fluorochromes (Carraway and Carraway, 1992).

2.8.3.1 α-Tubulin

Microtubules are dynamic components of the cell cytoskeleton (Waterman-Storer, 1998). During the M phase the mitotic spindle is required for segregation of chromosomes and is therefore essential for cell division. Anti-microtubule drugs or microtubule disrupting agents block the cell cycle at the M phase of proliferating cells by interfering with the function and integrity of the mitotic spindle (Gotoh *et al*, 1991). Alterations, of the cytoskeletal network changes the mechanical properties of the cell that are essential for functions such as locomotion and cytokinesis (Janmey, 1998). α-Tubulin was used to investigate the morphology of the cytoskeleton of the NCI-H157 cells.

The cells were seeded onto sterilized coverslips (22 x 22 mm) in a 6 well culture plate. Cells were seeded at 300 000 cells per well in 2ml culture medium. After a 24 hour growth period to allow the cells to become preconfluent, the cells were exposed to the drugs as explained in section 2.5.3. The experiment was terminated by fixing the cells with 2% formaldehyde in PBS for 10 minutes at room temperature. The coverslips were then rinsed three times for 5 minutes each time with PBS and then permeated with a solution of 97% methanol in PBS at -20^{o}C for 4 minutes. It was rinsed again three times for 5 minutes each time with PBS. Each coverslip was then drained thoroughly before adding 25 µ*l* of the primary antibody (usual range of dilution of the primary antibody is 1:500 to 1:1 000 in PBS) onto the coverslip. The coverslips were then placed cell-side down onto a microscope slide and incubated at 37^{o}C for 1 hour in a humid environment. The coverslips were then rinsed thrice for 5 minutes each time in PBS (at room temperature), 25 µ*l* of the Fluorescein Isothiocyanate (FITC) secondary antibody (anti-Mouse IgG [whole molecule]-FITC from goat) (dilution range of 1:32 to 1:100 in PBS) was placed onto the coverslip and incubated at 37^{o}C for 30 minutes in a humid environment. After incubation the coverslips were rinsed again three times for 5 minutes each time with PBS. 25 µ*l* (usual range of dilution 1:400 to 1:1 000 in PBS) of the monoclonal anti-FITC biotin

conjugate from mouse was placed onto the coverslip and incubated at 37^{o}C for 30 minutes in a humid environment. After incubation the coverslips were rinsed once again thrice for 5 minutes each time with PBS. This antibody (monoclonal anti-FITC biotin conjugate from mouse) was used as an amplification of the signal in the immunofluorescent detection of FITC.

The coverslips were then mounted in PBS/glycerol, and an anti-fading medium n-propylgalate. The slides were viewed for fluorescence with an Axiovert Zeiss microscope equipped with fluorescence attachments and excitation (488 nm)-emission (515-540 nm) filters for FITC. The camera was an Axiocam and photographs were analyzed using an Axiovision program. The magnification was x1 000, except where stated differently.

2.8.3.2 Viability staining

This method relies on the differences in the permeability of the cell membranes of live, dead and apoptotic cells to one DNA dye (fluorochrome), Hoechst 33342 (HO342) (Ormerod *et al*, 1992; Schmid *et al*, 1994). HO342 is a UV light excitable nucleic acid binding dye that is cell permeant. It stains the highly condensed chromatin of apoptotic cells uniformly and dimly stains the looser chromatin structure of viable cells. Apoptotic cells can take the form of crescents around the periphery of the nucleus or the entire nucleus can appear to be one or a group featureless, bright spherical beads (Samodien, 2003). HO342, unlike propidium iodide (PI) is not excluded by live or apoptotic cells (Ormerod *et al*, 1992; Schmid *et al*, 1994). Short time period exposure of cells to low concentrations of HO342 leads to strong labeling of apoptotic cells. Live cells, on the other hand, required much longer incubation with HO342 to obtain a comparable intensity of fluorescence. Therefore apoptotic cells are able to exclude PI but not HO342 (Ormerod *et al*, 1992; Schmid *et al*, 1994).

This technique was time and concentration dependent, since changes were only observed in the first few minutes after staining. The cells were seeded onto sterile petri dishes (35 mm x 10 mm). Cells were seeded at a concentration of 300 000 cells per dish in 2 ml culture medium. After a 24 hour growth period (to allow the cells to become preconfluent) the cells were exposed to the drugs as explained in section

2.5.3. The experiment was terminated by discarding the growth medium. The cells were not fixed, however, 2 ml of 1 μg/ml HO342 in cell culture medium (RPMI 1640) was added directly to each dish. After 30 minutes incubation at 37^{o}C the petri dishes were placed under an Axiovert Zeiss microscope and viewed with a UV light and a blue filter (excitation 358 nm and emission 461nm). The magnification was x200, except where stated differently.

2.9 Flow cytometry

Flow cytometry is designed for quantitative multiparametric analysis of isolated cells (Poirier, 1997). It is a methodology of choice to study various aspects of cell death including detection and quantitation of apoptotic or necrotic cells and offers the advantage of rapid analysis of the biological processes associated with cell death. The cytometer passes cells through a tightly focused beam of laser light. The interaction of the particle with the laser beam produces a light scatter in the forward direction (FSC-forward scatter, correlates with cell size) and a lateral direction (SCC-side scatter, correlating with granularity and/ or cell density). A reduction of both SCC and FSC (probably due to a rapture of plasma membrane and leakage of the cell content) characterize necrotic death. An initial increase in SCC (probably due to a chromatin condensation) and a reduction in FSC (due to cell shrinkage) characterize apoptosis (Samodien, 2003).

In this study flow cytometry was used to analyze the cell cycle (DNA profile) and apoptotic induction. This technique was used to separate cells on the basis of their light scattering properties, and the particular surface molecule, which they expressed, by the use of specific ligands (example antibodies) labeled with a fluorochrome. Cells were seeded at 500 000 cells per 25 cm^2 flasks and left for 24 hours to allow the cells to grow near confluent, before being exposed as explained in section 2.5.3. The experiment was terminated by trypsinizing the cells. Permeabilization of the cells were accomplished by fixing the cells in 3 ml 99.5% cold methanol and stored at -20^{o}C overnight, except where stated differently.

2.9.1 Determination of the DNA content for cell cycle progression

Analysis of cellular DNA by means of flow cytometry is a rapid and reliable approach for obtaining cell cycle frequency distributions of the cell populations

(Studzinski, 1995). The DNA content was determined by staining a single cell suspention with propidium iodide (PI). Sample preparation for PI labeling were seeded and fixed as explained in 2.9. Methanol was then removed by centrifugation at 2 500rpm for 5 minutes and the cells were then washed twice with PBS for 5 minute intervals. The sediments were resuspended in a 1 ml PBS containing 50 µg/ml PI and incubated for 20 minutes while shaking gently. The cells were then washed again twice with PBS for 5 minute period intervals. The pellets were resuspended in 1 ml PBS and the specimens were examined within 1 hour. Each analysis was based on at least 10 000 events. Analysis was done with a FACSCaliber flow cytometer and data were analyzed with a Cell Quest 3.3 program.

2.9.2 Annexin V

Annexin V-FITC is used to quantitatively determine the percentage of cells undergoing apoptosis (Allen *et al*, 1997). It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment (Vermes *et al*, 1995). Annexin V is a Ca^{2+} dependent phospholipid binding protein that has a high affinity for PS, and thus useful for identifying apoptotic cells with exposed PS. Cells that stain positive for Annexin V-FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both are either in the end stage of apoptosis, undergoing necrosis or are already dead. Cells that stain negative for both AnnexinV-FITC and PI are alive and not undergoing measurable apoptosis (Allen *et al*, 1997).

Cells were seeded at 500 000 cells per 25 cm^2 flasks and left for 24 hours to allow the cells to grow near confluent, before being exposed as explained in section 2.5.3. The experiment was terminated by trypsinization of the cells. The cells were not permeated because we wanted to distinguish dead from live cells. Cells were then resuspended in a 100 µl binding buffer (10 mM Hepes pH 7.4, pH with NaOH, 140 mM $NaCl_2$, stored at 4^oC). Adding to the cell suspension containing the binding buffer, 5µl Annexin V-FITC and 10 µl PI (prepared a 50 µg/ml solution in PBS) was added. The cell suspension was gently vortexed and the cells were incubated for 15 minutes in the dark at room temperature. After the incubation period, 400 µl binding

buffer was added to each tube and analysis was done within 1 hour. Each analysis was based on at least 10 000 events. Analysis was done with a FACSCaliber flow cytometer and data were analyzed with a Cell Quest 3.3 program.

2.9.3 Determination of caspase-3 active form

Active caspase-3 (32kDa) is a marker for cells undergoing early stages of apoptosis. Sample preparation for caspase-3 labeling were seeded and fixed as explained in 2.9. Methanol was removed by centrifugation at 2 500rpm for 5 minutes and the cells were then washed twice each time for 5 minutes with PBS. The cells were then resuspended in 2 ml of 20% fetal bovine serum in 3% bovine serum albumin in PBS for 30 minutes at room temperature. Samples were again centrifuged, and cells were incubated with 10 μl/ml/10^6 cells FITC-conjugated rabbit anti-active caspase-3 polyclonal antibody in 10% fetal bovine serum in 3% bovine serum albumin in PBS for 1 hour at room temperature. This antibody recognized human active caspase-3. Isotypic controls were exposed to 2% of irrelevant rabbit-IgG2b-kappa-FITC for 1 hour at room temperature. Subsequently, cell pellets were washed three times with PBS. Each analysis was based on at least 10 000 events. Analysis was done with a FACSCaliber flow cytometer and data were analyzed with a Cell Quest 3.3 program.

2.10 Western blotting (immunoblots)

This technique was performed to identify the pre-activated heterodimer caspase-3 at 30 kDa and the mature active heterotetramer caspase-3 at 60 kDa. This procedure can be devided into six steps: protein determination, sample preparation, electrophoretical separation of proteins by gel electrophoresis, transfer of the proteins to a suitable membrane, immunoreaction and detection of the transferred proteins.

2.10.1. Protein determination

Cells were seeded at 500 000 cells per 25 cm^2 flask and grown until preconfluent. Cells were exposed to the drugs at the various exposure time periods as explained in section 2.5.3. At termination, the flasks were placed on ice (4^oC) and cells were rinsed twice in 1 ml ice cold PBS pH 7.4, and harvested from the flasks and centrifuged at 2 000rpm for 3 minutes. The supernatant was discarded and the cells

were resuspended in 200 µ*l* PBS. The cells were stored at -70°C until used for protein determination.

A cell lysate was prepared for protein determination, by three freeze (-20°C) thaw (room temperature) cycles. An aliquot ± 90 µ*l* was then removed, and the protein content of the lysate was determined (in triplicate) using the method described by Lowry *et al*. (1951). The proteins of the cell lysates were determined using a Beckman DU640 Spectrophotometer at a wavelength of 650 nm. The remaining 110 µ*l* of the samples were diluted so that equal amounts of protein could be compared in experiments.

2.10.2 Sample preparation

A cell lysate was prepared as described in section 2.10.1, an aliquot removed and used for protein determination of the lysate. The remaining sample suspension was diluted 1:2 with an electrophoresis Laemmli sample buffer consisting of the following:

dH_2O	3.8 ml
0.5 M Tris-HCL (pH 6.8)	1.0 ml
Glycerol	0.8 ml
10% SDS	1.6 ml
β-mercaptoethanol	0.4 ml
0.5% Bromophenol blue	0.4 ml
Total volume	8.0 ml

For best results the β- mercaptoethanol was added to the sample buffer immediately before use. After the sample was diluted with the above mentioned sample buffer the solution was heated for 4 minutes at 90°C and then allowed to cool to room temperature. Equal amounts of 100 µg of protein per well were loaded onto the electrophoresis gel.

2.10.3 Separation of proteins by gel electrophoresis

A discontinuous gel electrophoresis was used since the two different gel layers cause the formation of highly concentrated bands of the sample in the staking gel and

greater resolution of the sample components in the lower (resolving) gel (Boyer, 1993).

The following protocol was followed:

	(4%) Stacking gel	(7.5%) Resolving gel
40% Acrylamide/Bis	2.5 ml	18.8 ml
0.5 M Tris-HCl (pH 6.8)	6.3 ml	------
1.5 M Tris-HCl (pH 8.8)	------	25 ml
10% SDS	250 µ*l*	1.0 ml
dH_2O	15.9 ml	54.7 ml
TEMED	25 µ*l*	50 µ*l*
10% APS	125 µ*l*	500 µ*l*
Total volume	25 ml	100 ml

A 7.5% resolving gel was cast then degassed and left to set at room temperature for 45 minutes. After the resolving gel had set, a 4% stacking gel was cast and left to set. Electrophoresis was carried out at a constant voltage of 200 V for 1 hour. A 5 times stock electrophoresis running buffer was made and consisted of the following: 15 gr Tris, 72 gr glycine, 5 gr SDS in 1 000 ml dH_2O (pH 8.3). Therefore, a working solution of 1 times running buffer was used during an electrophoresis run. A prestained Kaleidoscope standard of a broad range was used in this study, and a volume of 5 µ*l* of the standard, and 20 µ*l* per sample were loaded per well. After completion of electrophoresis the proteins were transferred to a polyvinyl difluoride membrane.

2.10.4 Transfer of proteins from SDS-polyacrylamide gel to a polyvinyl difluoride membrane

The proteins were directly transferred to a polyvinyl difluoride membrane in a wet electrophoretic blotting system. The gel was then washed for 5 minutes in transfer buffer (Tris-glycine buffer), (2.9 gr glycine, 5.8 gr Tris, 0.38 gr SDS and 200 ml methanol, diluted to a 1 000 ml by adding approximately 800 ml dH_2O; pH 8.3). This was done to remove the salts and detergents of the running buffer.

The polyvinyl difluoride membrane was however, presoaked in methanol and rinsed in dH_2O. Thereafter it was washed in transfer buffer. The filter paper as well as the porous pads was presoaked in transfer buffer. An electroblotting sandwiched was then assembled as followed:

- Porous pad
- Three sheets of Whatman 3 MM filter paper
- Polyvinyl difluoride membrane
- SDS-polyacrylamide gel
- Three sheets of Whatman 3 MM filter paper
- Porous pad

The sandwich was then place between two plastic supports and the entire construction was then immersed in an electrophoresis tank, containing the transfer buffer. An electrical current of 200 V was applied for approximately 2 hours. During this time, the proteins migrate from the gel towards the anode and became attached to the polyvinyl difluoride membrane. Transfer of the proteins, were carried out at room temperature.

The polyvinyl difluoride membrane was removed after the specified time period and placed in a solution of Poncheau S to verify the transfer of proteins onto the polyvinyl difluoride membrane. The stain is transient and is washed away during the processing of the Western blotting and is therefore completely compatible with all methods of immunological probing (Sambrook *et al*, 1989).

2.10.5 Immunoblotting and ECL detection

An enhanced chemiluminescence (ECL) Western blotting detection system was used in this study. The ECL Western blotting detection system is a light emitting non-radioactive method for detection of immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase labeled antibodies. All steps in the immunoreaction were performed at room temperature, unless stated otherwise.

After completion of the protein transfer, the polyvinyl difluoride membrane was briefly washed twice for a 5 minute period in Tris buffered saline (TBS) [6.05 gr Tris base and 8.76 gr sodium chloride was added to 800 ml dH_2O and adjusted to pH

7.5 then diluted to 1 000 ml with dH_2O]. The polyvinyl difluoride membrane was then incubated overnight in a 1% blocking solution [blocking stock reagent of 10% (w/v) was prepared by dissolving 10 gr of perfect block (supplied with kit) in a 100 ml maleic acid (2.32 gr maleic acid and 1.75 gr sodium chloride was dissolved in 100 ml dH_2O and the pH was adjusted to pH 7.5 and diluted to 200 ml with dH_2O); a working block solution of 1% was used for the experiment], at 4^oC without shaking. This was done to block non-specific binding of antibodies.

The polyvinyl difluoride membrane was then exposed to a washing procedure with washing buffer known as TBS-Tween® 20 (TBS-T) [Tween® 20 was diluted to 0.1% (v/v) concentration in TBS]. The membrane was briefly washed twice for a 5 minute period with washing buffer, then once for 15 minutes and twice for 5 minutes with fresh changes of washing buffer. The washing procedure was done on an orbital shaker.

Subsequent to the washing procedure the polyvinyl difluoride membrane was incubated with the primary antibody for an hour at room temperature. The primary antibody was diluted (1:500) in 1% blocking solution. Following the incubation period the polyvinyl difluoride membrane was washed in washing buffer (twice for a 5 minute period with washing buffer, then once for 15 minutes and twice for 5 minutes with fresh changes of washing buffer). The polyvinyl difluoride membrane was then incubated for an hour with a secondary antibody. The secondary antibody was diluted (1:1 000) in 1% block solution. The incubation with the secondary antibody was followed by the washing procedure (twice for 5 minute periods each time with washing buffer, then once for 15 minutes and twice for 5 minute periods each time with fresh changes of washing buffer).

Detection and developing step were executed according to the ECL Western blotting kit obtained from Amersham Pharmacia Biotech (United Kingdom). The detection solutions (supplied with the kit) were mixed in equal volumes sufficient to cover the membranes. The final volume was 0.125 ml/cm^2 membrane. The excess washing buffer was removed from the membrane. The detection reagent was then added to the membrane and then wrapped in cling wrap and incubated for 1 minute at room temperature without agitation. The detection fluid was then drained and the

membrane was wrapped in new cling wrap. Where the air pockets were gently smoothed out and the membrane was placed in a film cassette. A piece of hyperfilm was placed on the protein side of the membrane. The film was exposed for 15 seconds, developed for 4 minutes and then fixed.

2.11 Statistical analysis

Data were statistically analyzed with the statistical package Med Calc® software, Mariakerke, Belgium (Schoonjans *et al*, 1995). Data obtained from independent experiments are shown as the mean ± standard error of the mean (SEM). One way analysis of variance (ANOVA) analysis was carried out to compare the control and the treatment groups. A Student-Newman-Keuls test for all pair-wise comparisons was subsequently used and results were accepted as statistically significant when $P < 0.05$.

Flow cytometry data were represented as histograms. The values were tabulated and expressed as a percentage of a 100% gated events of 10 000 cells counted. A Becton Dickinson Immunocytometer was used and the data were analyzed with a Cell Quest 3.3 program.