Development and characterization of pro-apoptotic drug candidates for anticancer drug discovery

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ABSTRACT:

Cancer is one of the leading causes of death worldwide. According to the WHO, cancer accounted for 7.4 million deaths world wide in 2004. The metallo-compound cisplatin has been used for years as an effective antitumor agent for treating solid tumours such as breast, bladder, lung, oesophageal, and head and neck carcinomas. However, the use of cisplatin as an antitumor agent has been limited because of its association with problems such as lack of selectivity for cancer cells over normal cells, development of resistance to cisplatin treatment, and side effects such as nephrotoxicity. Recent studies on anticancer drugs have focussed on alternative anticancer agents such as gold compounds in both Au(I) and (III) oxidation states, which have shown to be potential anticancer drug agents because of their ability to induce apoptosis in several human cancer cells. Some gold complexes have shown to be able to selectively kill cancer cells over normal cells.

METHODS: In view of this, fifteen bidentate amino--and iminophosphine ligands coded (TTL) and their corresonding mono- and dinuclear Au(I) complexes coded (TTC) were synthesised and screened for their pro-apoptotic effects on a panel of cell lines. This panel consisted of ten human cancer cell lines (A549J, Caski, HepG2, HeLa, Hek293, H157, H157, Jurkat, MCF-7, and MG-63), one non-cancerous human cell line (KMST-6), and two rodent cell lines (CHO and 3T3). To further elucidate the mechanism of apoptosis, other markers of apoptosis such as mitochondrial membrane depolarisation, caspase-3 activation and DNA fragmentation were investigated. The mode of action through which these gold complexes and ligands induce apoptosis were also studied by investigating the generation of ROS, lipid peroxidation, the uptake of Au and thioredoxin redox system.

RESULTS: This demonstrated that some of the phosphine ligands and gold(I) complexes selectively inhibited the growth of cultured human cancer cells. These compounds induced morphological changes in cultured cells that were indicative of apoptosis. An apoptosis assay which detects phosphatidyl serine exposure confirmed that some of the phosphine ligands and gold(I) complexes selectively induced apoptosis in cancer cells. TTC18 and TTL5 demonstrated the highest pro-apoptotic activity and were further investigated. Cells treated with TTC18 and TTL5 were also positive for other markers of apoptosis, which included the activation of caspase-3, the fragmentation of DNA and the depolarisation of mitochondria. Cell cycle analysis shows that TTL5 induced cell cycle block in the G1 phase. This was not observed for TTC18 though it induced apoptosis in H157 cells. Both TTC18 and TTL5 induced the production of ROS. However, the oxidative damage induced by TTL5 was significantly higher in comparison to TTC18. The cytoprotective effects of several antioxidants were investigated. L-glutathione was shown to protect H157 cells against the effects of TTC18. ICP-MS data also show that the intracellular levels of Au are reduced in the presence of L-glutathione.

CONCLUSION: This study demonstrated that some of the phosphine ligands and gold(I) complexes tested here show promise as anticancer agents. On the whole, the cytotoxicity of the gold(I) complexes were much higher compared to the phosphine ligands, but it is particularly TTC18 and TTL5 that can potentially be developed further as anticancer agents.

KEYWORDS: Anti-cancer drug, Apoptosis, Cytotoxicity, Cytoprotection, Gold(I) complexes, Thioredoxin, Lipid peroxidation, Oxidative damage, Phosphine ligands, Reactive Oxygen Species.

STATEMENT OF ORIGINALITY

This work has not been previously submitted for any degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material that was previously published or written by another person except where due reference is made in the thesis itself.

S.S.E. Kanyanda

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At the heart of it all is the **Almighty God** who makes everything possible.

DEDICATION:

This research project is dedicated to my FAMILY

ABBREVIATIONS:

7-AAD	7-amino actinomycin D
ANT	Adenine nucleotide translocator
AO	Acridine orange
ATP	Adenosine triphosphate
AIDS	Acquired immune deficiency syndrome
Anexin-V PE	Annexin-V phycoerythrin
Apaf-1	Apoptosis activating factor -1
AIF	Apoptosis-inducing factor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia related
Bax	Bcl-2 associated x protein
Bcl-2	B cell leukaemia-2
BH	Bcl-2 Homology domains
Bid and tBid	(Truncated) BH3 interacting domain
CAD	Caspase-activated deoxyribonuclease
CARD	Caspase recruitment domain
Caspase	Cysteine aspartic acid-specific proteases
CDKs	Cyclin dependent kinase
CDKIs	Cyclin dependent kinase inhibitors
CD-95	Cluster of differentiation
CED-3	Cell-death abnormality-3
Cisplatin	Cis-dichlorodiammineplatinum(II) complex
CLM	Confocal laser microscopy
D4-GD1	Inhibitor of small G protein
DD	Death domain
DED	Death effector domain

DIABLO	Direct IAP binding protein with low pI
DISC	Death-inducing signalling complex
DMEM	Dulbeco's modified medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
$DiOC_6$ (3)	3,3′-diehexyloxocarbocyanine iodide
DcR	Decoy receptor
DR	Death receptor
DR-3	Death receptor-3
EDTA	Ethylene diamine tetra acetic acid
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain
Fas	Fibroblast-associated
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FLICE	FADD-Like IL-1β-converting enzyme
IAPs	Inhibitors of apoptosis;
ICAD	Inhibitor of caspase-activated DNase.
ICE	Interleukin-1-β-convertin caspase enzyme
IM	Inner membrane
ICE	Interleukin-1-beta converting enzyme (now known as caspase-1)
LMW	Low molecular weight
MAPK	Mitogen-activated protein kinase
Mdm2	Murine double-minute 2
Myc	Mylocytoma
NFκB	Nuclear factor κB
NRU_{50}	Concentration at which 50% of cells take up Neutral Red dye
OM	Outer membrane

OPG	Osteoprotegerin
p53	Phosphoprotein 53 (tumour protein/TP53)
E2F	Family of transcription factors involved in cell cycle
PARP	Poly (ADP) ribose polymerase
PT Pore	Mitochondrial permeability transition pore
PBS	Phosphate buffered saline
PCD	Programmed cell death
PI3K/Akt	Phosphatidylinositol 3-kinase (serine/threonine-protein kinase)
PKC	Protein kinase c
PS	Phosphotidylserine
Rb	Retinoblastoma
ROCKI	Rho-associated coiled-coil forming kinase
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
SARs	Structural activity relationships
SMs	Secondary metabolites
Smac	Second mitochondria-derived activator of caspase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TMRE	Tetramethylrhodamineethylester
TNF	Tumour necrosis factor
TRADD	Tumour necrosis factor receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TEM	Transmission electron microscopy
TE	Tris-EDTA [Ethylenediamine tetracetic acid]
VDAC	Voltage -dependent anion channel
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1

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CHAPTER ONE

1.1 Cancer defined

Cancer is not a single disease and different authors have defined it in different ways. Foster (2008), defined cancer as a class of diseases that result from the deregulation of the cell cycle whereby damaged or mutated cells which under normal circumstances are destroyed, are allowed to progress through the cell cycle thereby accumulating mutations that result in the uncontrolled growth of the cells, and this results in the formation of tumours or increased production of abnormal cells that have lost proper function. Foster (2008), further stated that these mutations are mainly found in proto-oncogenes as well as in tumour suppressor genes. Hanahan and Weinberg (2000); Croce (2009), described cancer as a disease that involves mutations in the genome and these mutations produce oncogenes with dominant gain of function and tumour suppressor genes as well as microRNA genes that have recessive loss of function. While Huang *et al*., (1997), reported that cancer is caused by compound mutations in a single cell and its progeny and that germ-line mutations may predispose a person to heritable /familial cancers. Another description of cancer by Coleman and Tsongalis (2001) is that cancer is a genetic disease or a disease of abnormal gene expression.

1.2 Cancer incidence

Cancer affects people and animals of different ages including foetuses however, for most cancers the risk increases with age (Irigaray *et al*., 2007). Parkin *et al*., (2000), gave an

estimated figure of over ten million new cancer cases every year world wide however, this figure excluded non-melanoma skin cancers. Out of this figure over six million deaths were estimated. In the USA cancer is the second leading cause of death the first being accidental deaths (Gurib-Fakim, 2006; Jemal *et al.,* 2008). Jemal *et al*., (2008) reported an overall estimate of 1.44 million new cancer cases in the USA alone without the inclusion of carcinoma in situ of any site except urinary bladder, basal cell and squamous cell cancers of the skin. Kushi *et al*., (2006), indicated that about 500,000 people die of cancer each year worldwide indicating that cancer is a world wide problem.

1.3 Causes of cancer

Most of the research work on the causes, development and effects of cancer focus mainly on three main research areas: (i) those that focus on agents as well as events that cause or aid in genetic changes in cells that eventually become cancerous (Su *et al*., 2002) (ii) studies that show the damage in affected genes (Angèle and Hall, 2000) (iii) biological studies that show genetic changes in both normal and cancerous cells (Mathews *et al.,* 2009). There are various agents or events that have been linked to causing or initiating the development of cancer. Some of such agents or events may include: *dysfunction of the immune system, hereditary hormonal imbalances, mutations due to chemical carcinogens or ionizing radiation, bacterial, viral or parasitic infections and* occupational *exposures* (Irigaray *et al*., 2007).

Table 1.2: Causes of cancer

Cause	Example	References
Dysfunction of the immune system	The human immunodeficiency virus (HIV) and associated malignancies (ARL)	Wood and Harrington, 2005 Bower *et al.*, 2006
Hereditary	About 10% of ovarian cancers (OC)	Lynch and Chapelle, 2003 Russo *et al*., 2009
Hormonal imbalances	Benign and premenopausal breast diseases.	Berstein *et al*., 2006; Linkov *et al*., 2008.
Mutations due to chemical carcinogens	Nasal cavities and paranasal sinuses, stomach, liver, kidney, uterine cervix, myeloid leukaemia, lung, oral cavity and pancreas	Toyomura *et al*., 2004, Sasco *et al*., 2004; Pöschl and seitz, 2004.
Ionizing radiation	Childhood leukemia, basal cell carcinoma (BCC), skin melanoma, nonmelanocytic skin cancers	Feychting *et al*., 2005; English, 1997; Rukin *et al*., 2007.
Bacterial, viral or parasitic infections	Adenocarcinoma of the distal stomach	Irigaray *et al*., 2007; Wen and Moss, 2008
Transmission	Anogenital carcinomas	Teksam *et al.*, 2004; Edgren *et al* 2007.
Pollution	Skin cancer, keratosis and hyperpigmentation, kidney, lung and liver cancers	Hopenhayn-Rich *et al*., 1996.
Occupational Exposures	Lung cancers; hepatocellular carcinoma	Siemiacki. *et al*., 2004; Alberg *et al*., 2007.
Hereditary	Ovarian cancers (OC)	Lynch and Chapelle, 2003 Russo *et al*., 2009.

1.4 Classification of cancer

Tumours are complex and as such it is necessary to classify them so that it can be easy to identify them. Members of a class inherit the properties of their ancestors as such prediction of prognosis can be made and drugs that can target a particular class of cancer can be developed and compared between homogeneous groups of patients (Louis *et al*., 2001). Louis *et al*., (2001), further reported that cancer classification is the basis on which clinicians make critical therapeutic recommendations to their individual patients. Louis *et al*., (2001), gave an example of neuro-oncologits that they apply therapies in a relatively uniform way for all patients with a given tumour type. Tumours have been grouped and classified based on features that are common to the members of the group/groups (Berman, 2004). The tumours are classified based on the type of cell that resembles the tumour and the tissue that is thought to be the origin of the tumour. Accordingly, the tumours are named using words with Latin or Greek root (Louis *et al*., 2001). Cancers are generally categorised/classified as follows:

Table 1.2: Classification and description of some cancers

Cause	Example	References
Dysfunction of the immune system	The human immunodeficiency virus (HIV) and associated malignancies (ARL)	Wood and Harrington, 2005 Bower *et al.*, 2006
Hereditary	About 10% of ovarian cancers (OC)	Lynch and Chapelle, 2003 Russo *et al*., 2009
Hormonal imbalances	Benign and premenopausal breast diseases.	Berstein *et al*., 2006; Linkov *et al*., 2008.
Mutations due to chemical carcinogens	Nasal cavities and paranasal sinuses, stomach, liver, kidney, uterine cervix, myeloid leukaemia, lung, oral cavity and pancreas	Toyomura *et al*., 2004, Sasco *et al*., 2004; Pöschl and seitz, 2004.
Ionizing radiation	Childhood leukemia, basal cell carcinoma (BCC), skin melanoma, nonmelanocytic skin cancers	Feychting *et al*., 2005; English, 1997; Rukin *et al*., 2007.
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Occupational Exposures	Lung cancers; hepatocellular carcinoma	Siemiacki. *et al*., 2004; Alberg *et al*., 2007.
Hereditary	Ovarian cancers (OC)	Lynch and Chapelle, 2003 Russo *et al*., 2009.

1.5 Cancer pathophysiology

Six fundamental elements that underlie the pathology of human cancers were described by Hanahan and Weinberg (2000), to be self sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, sustained angiogenesis, limitless replicative potential, tissue invasion and metastasis. However, Kroemer and Pouyssegur(2008), added another "avoidance of immuno-surveillance" to the list of fundamental elements that underlie the pathology of human cancer. Further to this, another emerging fundamental element is the alterations in cellular bioenergetics (Pathania *et al*., 2009). The main contributors to cancer pathophysiology being mutations in oncogenes and also epigenetics come into play.

1.5.1 Oncogenes

The major cause of cancer is the alterations in oncogenes, microRNA genes and tumour-suppressor genes (Croce, 2008). Most of these alterations are somatic events, however, germ-line mutations have been reported to predispose a person to heritable or familial cancers (Emery *et al*., 2001). It is believed that a single genetic alteration is uncommon for the development of cancer but it is evident that a multistep process of sequential alterations in many oncogenes, tumour-suppressor genes, or microRNA genes is required for cancer development (Doucas and Berry, 2006).

1.5.2 Proto-oncogenes

Proto-oncogenes are genes that are responsible for coding proteins that regulate cell differentiation, growth, signal transduction and mitosis in normal cells (Salgia and Abidoye, 2006). Some examples of proto-oncogenes include MYC, ERK, BCL-2 and RAS (Salgia and Abidoye, 2006; Doucas and Berry, 2006). Proto-oncogenes however, when mutated, or over expressed or when their original function has been modified with small modifications, they become oncogenes. Oncogenes are genes that have the potential to change normal cell machinery function into a cancerous tumour cell. According to Croce CM, (2008); Hanahan and Weinberg, (2000); oncogenes were discovered in retroviruses as the initiators of cancers in animals and avians. These genes modify the normal functions of cells. For instance, they are responsible for deregulation of the cell cycle machinery and have been implicated as important contributors to human carcinogenesis (Hanahan and Weinberg, 2000). There are three main ways by which proto-oncogenes have been known to transform into oncogenes. Such ways are: point mutation whereby a single nucleotide base repair is inherently altered or translocation in which a segment of the chromosome breaks off and attaches to another chromosome or by amplification whereby there is an increase in the number of copies of the proto-oncogene (Hanahan and Weinberg, 2000; Doucas and Berry, 2006).

1.5.3 Epigenetics

Epigenetics is the study of heritable changes in gene expression that are not due to any alteration in the DNA sequence for instance DNA methylation (Esteller, 2008) and several histone modifications that are involved in chromatin remodelling (Kristensen *et al*., 2009). DNA methylation is known to take place mainly at the carbon-5 position of cytocine residues within CpG dinucleotides in mammalian cells. This process is done by DNA methyltransferases (DMNT's) and S-adenosyl-methionine (SAM). DNA methylation is a normal process in mammalian embryogenesis. However, a wide variety of cancers have shown to either have a decrease or an increase in DNA methylation (Feinberg and Vogelstein, 1983). To a large extent DNA hypomethylation in most cancers is a frequent occurrence (Yegnasubramanian *et al*., 2008; Lana *et al.,* 2009). In some cancers hypermethylation of DNA has also been reported (Herman, 1999; Sasaki *et al*., 2003; Yegnasubramanian *et al*., 2008; Zou *et al*., 2009). Interestingly, DNA methylation and histone modifications are reversible processes and Esteller(2008), indicated that dormant hypermethylated tumour suppressor genes can be awakened with drugs which can re-express the DNA methylated genes in cancer cells by using demethylating agents and rescue their functionality. Esteller (2008), pointed out that demethylating agents have not shown activity against solid tumours. Conversely, Donepudi *et al.,* (2007), indicated that hypomethylating agents have shown effectiveness at low doses in the treatment of hematologic malignancies.

1.5.4 Self-sufficiency in growth signals

Growth factors (GFs) are the main growth–regulatory polypeptides that stimulate cell proliferation in culture and most likely also *in vivo* (Goustin *et al*., 1986). Insulin and adrenocorticotropic hormones are known polypeptides however, GFs differ in the mode in which they are elicited and delivered to the responding cell (Goustin *et al*., 1986). According to Kroemer and Pouyssegur (2008), GFs activate receptor tyrosine kinases (RTKs), and these in turn stimulate two other key signal-transducing kinase pathways: the Ras→Raf→MAP kinase (ERK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway. Kroemer and Pouyssegur, (2008), further reported that ERK and PI3K converge to activate mammalian target of rapamycin (mTOR) which is responsible for cell growth stimulation. Almost all cancers are known to have mutations in genes such as Neuroblastoma (N-Ras), Kirsten (K-Ras), B-Raf, Harvey (H-Ras), the p110a PI3K subunit, and RTKs, which regulate cell signalling and growth (Rajkumar, 2001). Alternatively mutations may occur in the downstream effector genes (such as the kinases Akt and PDK1) (Kroemer and Pouyssegur, 2008). Growth factor receptors (GFRs) have also been implicated to play a major physiological role in the normal process of growth and differentiation (Rajkumar, 2001). Accordingly, Rajkumar (2001), further mentioned that this process is achieved through the binding of the growth factor to its receptor subsequently leading to receptor dimerization and phosphorylates a number of cytoplasmic proteins, which also sets off a cascade of events that lead to the activation of transcription factors in the nucleus. The transcription factors lead to increased mRNA synthesis, translation of the mRNA, increased protein synthesis, which eventually leads to

either cell growth or differentiation (Rajkumar, 2001; Harrington *et al*., 2007). Hanahan and Weinberg, (2000), reported that mitogen growth signals are needed for normal cells to move from a quiescent condition into an active proliferative condition. According to Hanahan and Weinberg, (2000), in cell culture, normal cells require more than one growth factor for proliferation. When grown under normal laboratory conditions, normal cells usually deplete growth factors more frequently than other components in the media and accordingly become rate limiting for proliferation. On the other hand, for cancer cells, the loss or decrease in specific growth factors is a common incidence and often may lead to growth advantage, a basic feature of cancer cells (Goustin *et al* .,1986). Similarly, Pathania *et al*., (2009), mentioned that a basic characteristic feature of cancer cells is self-sufficiency in the absence of GFs.

1.5.5 Limitless replicative potential

Almost all normal vertebrate cells undergo a finite number of cell divisions known as Hayflick limit and this cell division is determined by an intrinsic mitotic clock that involves the maintenance of telomeres (Effros and Walford , 1984). Following the finite number of divisions, the cell eventually enters into senescence, a state in which cells stop replication (Bodnar *et al*., 1998). Normal cells for instance human fibroblasts can divide 60 to 80 times before they can halt their replication process. Contrary, cancer cells may replicate indefinitely if they have a continued supply of nutrients (Granger *et al*., 2002). According to Bellantuono, (2004), the maintenance of telomeric DNA is what underlies

the ability of tumours to possess unlimited replicative potential. Current evidence indicates that the capability to sustain tumour growth and metastasis is contained in a subpopulation of cells called stem cells (SCs) or tumour-initiating cells (Klonisch *et al*., 2008; Todaro *et al*., 2010). It is suggested that stem cells have the capabilities of self-renewal in which case they grow indefinitely and differentiate or develop into different types of cells or tissues, following mitogen stimulation (Granger *et al* 2002; Moore, 2007). It is believed that impairment of stem cell differentiation leads to unlimited replication in cancer cells (Klonisch *et al.,* 2008; Kitamura *et al*., 2009). Cancer stem cells (CSCs) have been found to express stem cell-like surface markers such as CD34 in leukemia cells and CD44 in breast cancers (Kitamura *et al*., 2009; Pfeiffer and Schalken, 2010), which can be used to identify cancerous cells from a mixture of cells.

1.5.6 Telomeres

Telomeres are hexameric DNA sequences found at the ends of chromosomes characterized in all vertebrates by tandems of *TTAGGG/CCCTAA* repeats. Telomeres prevent chromosomal end-to-end fusions and maintain genomic stability (Herbert, 2008). Telomeric DNA ends in a single-strand G-rich overhang presumably between 50 and 300 nucleotides at the 3' end (Herbert, 2008). It has been proposed that the telomere bends back on itself forming a "T-loop" structure and telomeric DNA together with its associated capping protein complexes have been given the name telosome or shelterin (Granger *et al*., 2002; Weinert, 2005). In most normal human cells telomeres shorten with

each cellular division because of "end-replication problem" (Granger *et al*., 2002). During replication, DNA polymerase adds a DNA primer only in the 5′to 3′ direction in order to initiates synthesis and is done in segments known as Okazaki fragments (Timson *et al*., 2000). However, the 3′ terminal end of the lagging strand of linear DNA cannot be fully synthesized since there is no DNA at end of the chromosome to serve as a template for the next Okazaki fragment to fill in the gap between the last Okazaki fragment and the end of the chromosome as a result, the ends of the chromosomes are not replicated hence the telomere continuously shorten with each round of replication (Granger *et al*., 2002; Herbert, 2008). In a normal situation cells undergo replicative senescence the moment telomeres have shortened to a certain length. However, cells that have lost critical checkpoint controls for example p53/Rb can bypass senescence and their telomeres continue to shorten and they usually die (Dikmen, 2009). In rare instances however, the presence of critically short or dysfunctional telomeres can result in end fusions and genomic instability, (Dikmen, 2009**),** as a result a cell acquires mutations that can lead to the cell progress towards cancer (Dikmen, 2009). Some telomere-associated proteins known to play a role in telomerase's accessibility include the telomeric repeat binding factors 1 and 2 (TRF1 and TRF2), TRF-1 interacting protein 2 (TIN2), POT1 (protection of telomeres 1), *TPP1, Rap1* and tankyrase 1, which is a telomeric member of the Poly adenosine diphosphate (ADP)-ribose polymerase (PARP) family of proteins (Herbert, 2008).

Telomerase is a specialized reverse transcriptase enzyme produced by cells that adds hexameric repeats of *TTAGGG* DNA sequences to the ends of chromosomes to prevent

the shortening of telomeres that occur during cell division (Testorelli, 2003). In effect, the adding of TTAGGG repeats to the depleted ends of chromosomes extend the 3' end of chromosomes and this results in the continued multiplication of the cell (Artandi, 2010). Most healthy cells are reported not to make telomerase (Artandi, 2010). One exception however, is the stem cells (SC). Stem cells are involved in the continued renewal of blood and skin cells, and also those cells that line the intestinal tract. The main human enzyme consists of a reverse transcriptase protein (TERT) (Kirwan and Dokal, 2009). Premature shortening of telomere and mutation of telomerase components are associated with syndromes such as dyskeratosis congenita and idiopathic pulmonary fibrosis (Kirwan and Dokal, 2009). Telomerase and the maintenance of telomeres are key players in the ability of stem and cancer cells to bypass senescence and be immortal (Ju and Rudolph, 2006). For this reason telomerase are attractive targets for the development of novel anti-cancer therapeutics (Aubert and Lansdorp, 2008).

Most types of cancer cells are known to have telomerase but most normal somatic cells do not express telomerase or that telomerase is limited in normal cells (Testorelli, 2003), which means that the enzyme might be used as a target for anticancer drugs (Rezler *et al.,* 2002). The increased presence of telomerase in cancer cells makes them retain their telomerase homeostasis and potentially survive indefinitely (Cian *et al.,* 2008). On the other hand the limited presence of telomerase in non-cancer cells made them to divide with minimal cell divisions and eventually die or go into senescence. Using this scenario, agents can be developed to specifically target telomerase to allow them to shrink and

disappear without disrupting the functioning of most normal cells. Current anticancer drugs kill both cancerous and non-cancerous cells, and development of agents that would target telomerase would be less toxic as they would specifically kill the cancer cells over the non-cancerous cells thereby reducing side effects (Klonisch *et al*., 2008). An example of a telomerase inhibitor is GRN163L which is in phase I/II clinical trials and appears to be promising anticancer agent (Kelland, 2007).

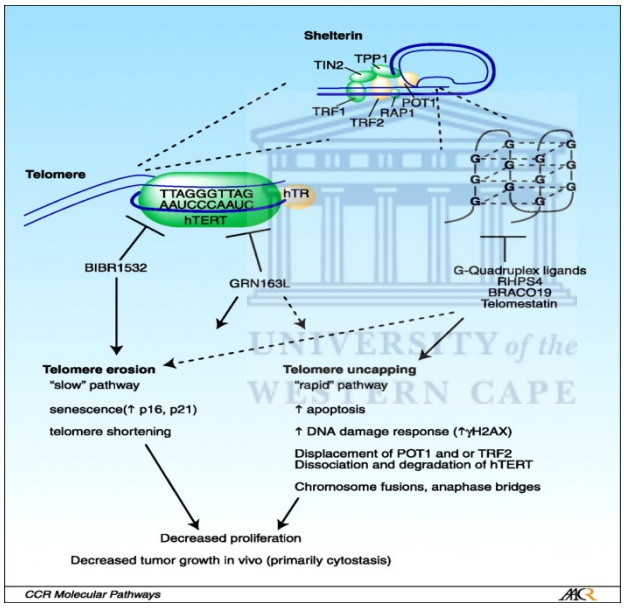

Figure 1.1: The telomerase/telomere pathway and main points of possible therapeutic intervention. Pathway modulators may result in either mainly telomere erosion (e.g., BIBR1532) resulting in relatively slow phenotypic anticancer effects or predominantly telomere uncapping (e.g., RHPS4) producing more rapid anticancer effects. Agents such as GRN163L probably mainly affect telomere erosion but also seem to induce telomere uncapping (Adapted from Kelland, 2007).

1.5.7 Sustained Angiogenesis

Vasculogenesis is the term used to describe the initial steps involved in the formation of the vascular system during embryogenesis (Alessi *et al*., 2004). From the pre-existing vessels angiogenesis sets (sprouts) in to form new blood and lymphatic vessels

(Sköldenberg *et al*., 2000; Djonov *et al*., 2000). Further, Sköldenberg *et al*., (2000), mentioned that angiogenesis is one of many mechanisms required for the building and maintenance of blood supply of the body's tissues, disposition of metabolic waste products and also allowing metastatic spread. This however, (Cox*, et al.,* 2000), occurs in response to the metabolic demands of tissues and tumours. It is well known that all solid tumours are angiogenesis-dependant (and most probably many leukaemia's as well) (Keshet and Ben-Sasson, 1999; Bjerkvig, 2009)**.** In cancer development, angiogenesis is crucial as the supply of oxygen and nutrients are required to support the growth of the solid tumour or leukemia thereby overcoming hypoxia and starvation (Bjerkvig, 2009. Tumour growth is also dependent on the balance between proliferating cells and cells dying through apoptosis and necrosis (Alessi *et al*., 2004). Cells resist apoptotic cell death because of the presence of mutations in genes that eventually produce phenotype cells which resist apoptosis and therefore have a survival advantage (Alessi *et al*., 2004).

There are four major steps implicated in the development of angiogenesis and these are: (i) breaking through the basal lamina that envelopes existing blood vessels by proteases (ii) migration of endothelial cells towards a source signal and sprouting (iii) proliferation of endothelial cells and (iv) formation of tubes/new basement membrane with final blood flow (Gambino, 2002; Gupta and Qin, 2003). Several other factors have been implicated in angiogenesis however, one of the major potent and predominant factor implicated is the vascular endothelial growth factor (VEGF), a member of six structurally related families of proteins (Clark and Jones**,** 1999; Li and Eriksson**,** 2001). In order for VEGF to be

produced, it has to be stimulated by upstream activators, such as growth factors, oncogenes, cytokines, hormones and other environmental factors (Djonov *et al*., 2003). Once VEGF is produced, it promotes cell proliferation and growth, and also cell survival as observed with experiments on endothelial cells (Gerhardt *et al.,* 2003). The VEGF family interacts with vascular endothelial growth factor receptors (VEGFRs) to exert their angiogenic effects (Demir *et al*., 2006; 2007). The binding of VEGF to its receptors on the surface of endothelial cells activates intracellular tyrosine kinases, triggering multiple downstream signals that promote angiogenesis. Although there are many variants of VEGF and their receptors, the angiogenic effects of VEGF pathway are primarily mediated through the interaction of VEGF-A (the most common variant, often referred to as VEGF) with receptor-2 (VEGFR-2) (Li and Eriksson**,** 2001; Gambino *et al*., 2002). The role of other non-VEGF factors is not fully known however; it is thought that they play secondary roles in angiogenesis, most likely additional non-angiogenic pathways (Risau *et al*., 1988; Djonov *et al*., 2003; Ferrara, 2004).

Matter (2001) proposed that drugs on tumour angiogenesis be classified into two main classes namely (i) drugs that interfere with the process of forming new blood vessels, and (2) vasculotoxins that destroy newly formed blood vessels thereby producing antitumour effects. Keshet and Ben-Sasson, (1999), mentioned that the supply of both nutrients and oxygen in angiogenesis are critical in maintaining the growth of tumours. As such Matter, (2001), Ryan *et al*., (2005), suggested that inhibiting the supply of both nutrients and oxygen to the tumour could render the tumour incapable of growth. Therefore

development of anticancer drugs that can target the tumour vasculature rather than the tumour itself could be of fundamental importance. Recently several research works in anticancer drug has focused into the development of drugs that inhibit angiogenesis growth factors or block their receptors (Bjerkvig *et al*., 2009). Of particular importance is the targeting of the VEGF-signalling pathway, which has an important role in stimulating neovascular growth and survival (Wedge *et al*., 2002; Ryan *et al*., 2005). Some of such drugs include ZD6474 an orally bio available inhibitor of VEGF receptor-2 tyrosine kinase activity developed by AstraZeneca. Wedge *et al* (2002) showed that ZD6474 could selectively inhibit tyrosine kinase activity of VEGFR-2 of VEGF-stimulated endothelial cells. . ZD6474 blocks VEGF receptor-2 pathways another drug Avastin is reported to bind to VEGF (Herbst *et al*., 2007). Another anticancer agent that targets the VEGF is Bevacizumab developed by Avastin, Genentech a recombinant humanized monoclonal antibody, which was approved for the treatment of colorectal cancer and non-small cell lung cancer by the Food and Drug Agency (FDA) (Heinzman *et al*., 2008). According to Heinzman *et a*l., (2008), the mechanism through which Bevacizumab acts is that it binds to VEGF with high specificity subsequently neutralizing the growth factor and preventing its interaction with its receptors. Endothelial cells are inhibited from proliferation resulting in the hindrance of tumour progression (Bamias and Dimopoulos, 2003; Heinzman *et al*., 2008).

1.5.8 Tissue invasion and metastasis

Metastasis is the ability of cancerous cells to break off from their original sites and form tumours at a different part of the body (Campbell and Der, 2004; Stafford *et al*., 2008; Geiger and Peeper, 2009). Cancer invasion is the processes whereby cancer cells break away from a tumour, circulate through the circulatory or lymph system and eventually enters adjacent tissue (Campbell and Der, 2004). Sierra (2005); Geiger and Peeper, (2009), reported that tissue invasion and metastasis occur in a sequence of distinct steps, which have been termed as a "metastatic cascade. Cancer invasion and metastasis are said to be two most lethal of cancer phenomena responsible for 90% of cancer-related deaths (Ruiter, 2001; Neal and Berry, 2006; Geiger and Peeper, 2009), and metastasis is said to be the hallmark of malignancy (Iiizumi *et al*., 2008). Iiizumi *et al*., (2008) hinted that at the moment there are no efficient anticancer drugs available for treating patients whose cancers have metastasized. Campbell and Der (2004) indicated that the signalling and the mechanisms that promote tissue invasion and metastasis are extremely intricate.

1.5.8.1 Promoters and suppressors of tissue invasion and metastasis.

Cells have genes that can promote tissue invasion and metastasis and also genes that can suppress issue invasion and metastasis. Most cancer cells however, have the ability to overcome the effects of most of the tissue suppressor genes (Hwang-Verslues *et al*., 2008). Some of the membrane bound metastasis suppressor genes include KAI1, cadherins, ovarian cancer G-protein coupled receptor, and CD44 (Yoshida *et al*., 2000; Stafford *et al*., 2008**)**. KISS1, Nm23, RhoGDI2, BRMS1, SSeCKs, MAP kinase kinase 4 (MKK4/7/p380), TIMPs, Drg1, RKIP, DCC1, gelsolin, caspase-8, RRM1, and DCC1 are

some of the cytoplasmic metastasis suppressors genes reported (Shevde *et al.,* 2002; Robinson *et al*., 2003; Mitchell *et al*., 2006; Woolworth *et al*., 2009). Metastasis suppressors that are said to reside predominantly in the nucleus include CRSP3/TXNIP, BRMS1, Nm23 and Drg1 (Yoshida *et al*., 2000**)**. However, it is reported that genes BRMS1, Nm23 and Drg1 can also be localized to both the cytosol and the nucleus which means that they may function as metastasis suppressors in both compartments but Yoshida *et al*., (2000), argued that further studies are required to iron out the precise role of each gene in each of the cellular compartments.

Under normal conditions tissue cells adhere to one another with cell-to-cell adhesion molecules and to a mesh of protein known as extracellular matrix (ECM) composed of molecules such as glycosaminoglycans, proteoglycans, collagens and non-collagenous glycoproteins and these fill the space between the cells (Rozario and DeSimone, 2009). The extracellular matrix allows interactions of proteins on the cell surface of different cells thereby allowing the cells to survive and also proliferate as they exchange different important molecules. One of such molecules is a nuclear protein called E type cyclin dependent kinase 2 (E-CDK2) which regulates cell growth, division as well as apoptosis (Guo and Hay 1999; O'Cnnor *et al*., 2000). According to Chin *et al*., (2005), cells begin their reproductive cycle only after they attach to a surface. Chin *et al*., (2005), further reported that inhibitory effects of E-CDK2 in the nuclei of cells stops functioning or shuts down if the cells have not attached to anything and the cells stop growing and eventually die by apoptosis. Cancer cells however, are able to exist without being anchored and their E-CDK2 protein remains active and allows the cancer cells to grow and reproduce

(Okegawa *et al*., 2002). The reasons E-CDK2 remains active are unknown, but researchers think that oncogenes may be responsible. Other abnormalities of cancer cells include missing of adhesion molecules such as cadherins and intergrins (Okegawa *et al*., 2002), and also the increased involvement of matrix metalloproteases (MMPs) enzymes which are known to be involved in cell proliferation, differentiation, remodelling of the extracellular matrix (ECM), vascularisation and cell migration (Chang and Werb, 2001). Cancer cells are reported to use proteases such as urokinase plasminogen activator (uPA), cathepsin B (CB) cathepsin D and the enzymes MMPs to dissolve basement membranes and other extracellular matrices, which allows the cancer cells to penetrate the basement membranes of blood vessels and have access to other parts of the body (Chin *et al*., 2005). Other studies have shown that certain tumours have an affinity to specific organs of the body and they only anchor to those specific organs even if the cancer cells reached all other organs in the body and it is only when the cancer cells reach those specific organs do they metastasis and reproduce (Terranova *et al*., 1986; Brooks *et al*., 2010).

1.5.8.2 Targeting tissue invasion and metastasis

Metastatic cancers originate from a primary site and despite the spread they are still named by their primary site for instance, if breast cancer spreads to the lungs, it remains breast cancer (Labianca *et al*., 2004; Bodner *et al.,* 2006). Consequently, treatment for metastatic cancers depends on where the cancer originated from and not by its secondary site (Iiizumi *et al*., 2008). Further, Iiizumi *et al*., (2008), reported that one major problem with cancer treatment is that a small percentage of metastatic cancers are discovered but

their primary tumour is not identified and in such cases treatment is based on the location of the cancer rather than the origin. Both primary and metatastic tumours can be treated concurrently by blocking the promoters or the suppressors required for tissue invasion, metastasis or tumour vascularisation (Sarkar *et al.,* 2005). Some of such drugs developed to that effect include different kinds of agents that block the ligand-recepter interaction of metastasis-promoters such as hepatocyte growth factor (HGF) (figure 1.2) and its receptor c-*Met* (Comoglio and Vigna, 1995; Christensen *et al*., 2005). Other agents include those that antagonize and inhibit the transcriptional activity of metastasis promoter (β-Catenin) such as autocrine motility factor (AMF), urokinase plasminogen activator (uPA) and matrix metalloproteinase (MMP) (Iiizumi *et al*., 2008). Additionally Yoshida *et al*., (2000) and Iiizumi *et al*., (2008), found that small molecules such as NM23, Kiss-1, MKK4 and NDRG1 have been developed in order to restore the expression or mimic the function of metastasis-suppressor genes (MSG).

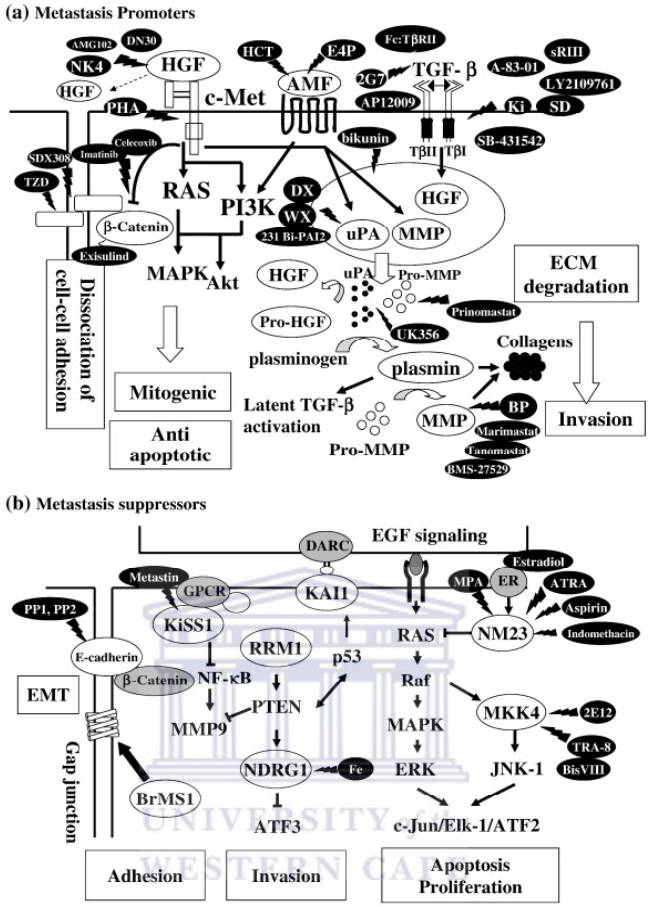

Figure 1.2: Signal pathway of tumour metastasis. Tumour metastasis is a result of complex interplay of both positive (a) and negative (b) factors. These pathways and their factors are potential targets for anti-metastatic therapy. The drugs currently under development are shown as black oval shapes (Adapted from Iiizumi *et al*., (2008).

1.5.9 Evasion of apoptosis (a universal characteristic of all cancer cells)

Apoptosis is defined as cell death properly programmed to eliminate unwanted cells from an organism and is essential for the maintenance of the organism's homeostasis (McCabe and Dlamini 2005; Prevarskaya *et al*., 2010). The term apoptosis was introduced into modern scientific writing by Kerr, Willey and Currie (1972), in which they described it as a cell death (programmed cell death/cell suicide) different from necrosis. The term was

chosen for it's meaning for it represented the falling of leaves as used in ancient Greek (Kerr *et al*., 1972; Cruchten, 2002). Since then this term has been in accepted use in biomedical sciences. While cell proliferation and tissue growth are important, apoptosis is equally important for the normal development of the tissue including morphogenesis and removal of harmful cells (Tsujimoto and Shimizu, 2000). On the other hand, (McCabe and Dlamini, 2005; Prevarskaya *et al*., 2010), stated that increased levels of apoptosis as well as low levels of apoptosis have profound effects on the organism. Increased apoptosis can lead to several pathological conditions, for instance motor neuron diseases such as amyotrophic lateral sclerosis (ALS) cerebral dementia such as Alzheimer's and inherited ataxias (Honig and Rosenberg 2000), type 1 diabetes which involves destruction of immune-mediated islets of Langerhans and AIDS (Badley and Dockrell, 1997). On the other hand, decreased apoptosis can lead to diseases such as inflammation, cancer, restenosis, autoimmune diseases and persistent infections (Reed, 2000). Players in the apoptotic pathways have been identified and used as targets for anticancer drugs. The targeting of such players in the apoptotic pathways can either have a direct pro-apoptotic effect or can cause cancer cells to become susceptible to cytotoxics (Melet *et al.,* 2008). It has been reported (Marzano *et al*, 2006; Melet *et al.,* 2008), that most cancers develop drug resistance because of lack of successful induction of apoptosis and also because of defects in the apoptotic pathway. According to Hanahan and Weinberg (2000), acquired resistance toward apoptosis is a hallmark of most if not all types of cancer and Fulda and Debatin 2004, 2006; Abdollahi and Folkman, 2009), reported that almost all cancer cells contain mutations in their genes that enable them to evade apoptosis. This is achieved

through several means one example is the one which involves the active interaction between oncogenes and/or mutated *tumour suppressor genes*. For instance over 50% of cancers are reported to have mutations in their tumour suppressor gene p53 an important pro-apoptotic regulator gene (Ashkenazi, 2002). The mutation of p53 has been implicated in the facilitation of tumourigenesis (Ashkenazi, 2002; 2008a, 2008b). Another means by which cancer cells evade apoptosis is through continued elevated signalling of oncogenes thereby driving increased cellular proliferation genes such as Myc, and Ras (Fadeel and Orrenius, 2005). Other studies however, have reported that Myc under other circumstances can induce apoptosis for instance under cellular stress, DNA damage, or when levels of survival factors are low (Ziegler and Groscurth, 2004). Another means by which cancer cells overcome apoptosis as mentioned by Fulda and Debatin (2004), is through up regulation of the anti-apoptotic PI3 kinase (PI3K)-Akt/PKB survival pathway which can result due to loss of the tumour suppressor gene PTEN, a phospholipid phosphatase that normally down regulates the Akt survival signal or other signals such as IGF-1/2 that can trigger the oncogene Ras (Frisch and Screaton, 2001; Fulda and Debatin 2004). Yet another means by which cancer cells evade apoptosis is through the up regulation of the expression of the non-signalling decoy receptor for the FAS ligand, which may prevent activation of the FAS receptor (Ashkenazi, 2002).

1.5.9.1 Characteristics of apoptotic and necrotic cell death.

Apoptosis is characterised with distinct set of biochemical and physiological changes involving the endoplasmic reticulum (Duvall and Wyllie, 1986), cytoplasm, mitochondria, nucleus and plasma membrane (Bortner *et al*., 1995; Frédérich *et al.,* 2003; Edinger and Thompson, 2004; Krysko *et al*., 2008). The final feature of apoptosis is the budding off of the nucleus into numerous fragments of 50-300 kbp and eventually into approximately 180-bp fragments that can be viewed upon agarose gel electrophoresis as characteristic DNA ladders (Zamzani and Kroemer, 1999; Lawen, 2003). These fragments are encapsulated within apoptotic bodies (Bortner *et al*., 1995; Frédérich *et al.,* 2003), and since they are surrounded by an intact plasma membrane, they are easily engulfed by macrophages (Lawen, 2003), and apoptosis usually occurs without leakage of cell contents and usually without inflammation (Edinger and Thompson, 2004). However, Otsuki *et al*., (2003), further reported that should phagocytosis not occur, the apoptotic bodies may lyse and this may result in apoptotic necrosis. While apoptosis is a normal cell death, it can as well be initiated. Lawen, (2003), reported that anti-cancer agents can induce apoptosis. Accidental cell death on the other hand, referred to as or cellular necrosis, is the consequence of injury or toxicity and results in cell lysis (Frohlich and Madeo, 2000; LaCasse *et al*, 2005). In this form of cell death, the cell swells and disintegrates in an unordered manner, eventually leading to the destruction of the cellular organelles and finally rupture of the plasma membrane and leakage of the cell contents into the surrounding environment with the consequent of inflammation (Lawen, 2003). According to Otsuki *et al.*, 2003), necrosis is terminal-stage cell death, which has no morphological definition.

1.5.9.2 Mechanisms of Apoptosis and the mammalian plasma membrane

The mammalian cell membrane is a semi-fluid mosaic structure made of phospholipids, proteins and some cholesterol. Phospholipids are the major components of the membrane and are arranged in the form of a "bi-layer", each arranged in an amphipathic structure (Avers, 1982; Fadeel, 2004). The major feature associated with apoptosis is the loss of the phospholipid asymmetry and the subsequent exposure of negatively charged phosphatidylserine (PS) from the inner to the outer surface of the cell membrane. This charge is necessary for recognition and engulfment of the apoptotic cell by macrophages (Fadok *et al*., 2001). Furthermore, Fadok *et al*., (2001), reported that the plasma membrane asymmetry in viable cells is maintained by the activity of an aminophospholipid translocase, which is believed to be a 120-kDa Mg^{2+}-dependent adenosine triphosphatase (ATPase) This adenosine triphosphatase (ATPase) transfers any phosphatidylserine (and, to some extent, phosphatidylethanolamine) that may have reached the outer leaflet back to the inner leaflet of the plasma membrane (Wyllie *et al*., 1984). Fadok *et al.,* (2001), demonstrated that the rapid appearance of phosphatidylserine (PS) on the cell surface during cellular activation and during apoptosis, is mainly due to the activation of a lipid-nonspecific membrane phospholipid scrambling. This activity results in the movement of phospholipids bi-directionally across the plasma membrane, and in so doing increases the surface expression of phosphatidylserine. For this reason, (Barrett *et al*., 2001; Fadok *et al.,* 2001; Fadeel, 2004), reported that phosphatidylserine is commonly used as a marker for apoptosis since macrophages attach and engulf cells exposing phosphatidylserine on their surface.

1. 5.9.3 Pathways of Apoptosis

1.5.9.3.1 The Intrinsic Pathway

The intrinsic pathway of apoptosis is activated by mitochondrial disruption following cytochrome *c* release (Reed and Pellecchia; 2005). Cytochrome *c* is associated with permeabilisation of mitochondrial outer membrane and the subsequent formation of the apoptosome, which in turn activates executioner caspases such as caspase-3 (Regula, *et al*., 2003). There is evidence that the "apoptosome" oligomeric structure of approximately 1 MDa is formed by the interaction of cytochrome *c*, Apaf-1, ATP and procaspase-9 (Zou, 2003; Nagata, 2005). This pathway is reported to be initiated by growth factor withdrawal, UV irradiation, ischemia, oxidative stress and cytotoxic drugs (Ashe and Berry, 2003). Members of the Bcl-2 family are reported to control this pathway of apoptosis (Lawen, 2003).

1.5.9.3.2 The Extrinsic Pathway

This involves the binding of cell death signal to cell surface receptors that transduce extracellular signals across the plasma membrane and through the activation of intracellular signalling pathways, bring about the appropriate functional response (Farrow, 2000). Some cell surface receptors are needed to transmit apoptotic signals initiated by ligands (e.g., specific antibodies) or by natural ligands called death receptors (DRs) to the inside of the cell and this plays a critical role in instructive apoptosis (Schlegel and Williamson, 2001; Ashkenazi, 2008b). Death receptors are part of the tumour necrosis factor (TNF) gene super-family and provide a rapid and also efficient route to apoptosis

(Kumar *et al.,* 2005). These receptors can activate caspases within seconds of ligand binding, causing an apoptotic cell death within hours (Nagata, 1997, 2000). Both extrinsic and intrinsic pathways of apoptosis however, converge to induce effector caspases which are the final executioners of cell death (Lawen, 2003), although, other studies have reported caspase-independent forms of apoptosis (Richard et al., 2002; Ajiro *et al*., 2008; Liang *et al*., 2008).

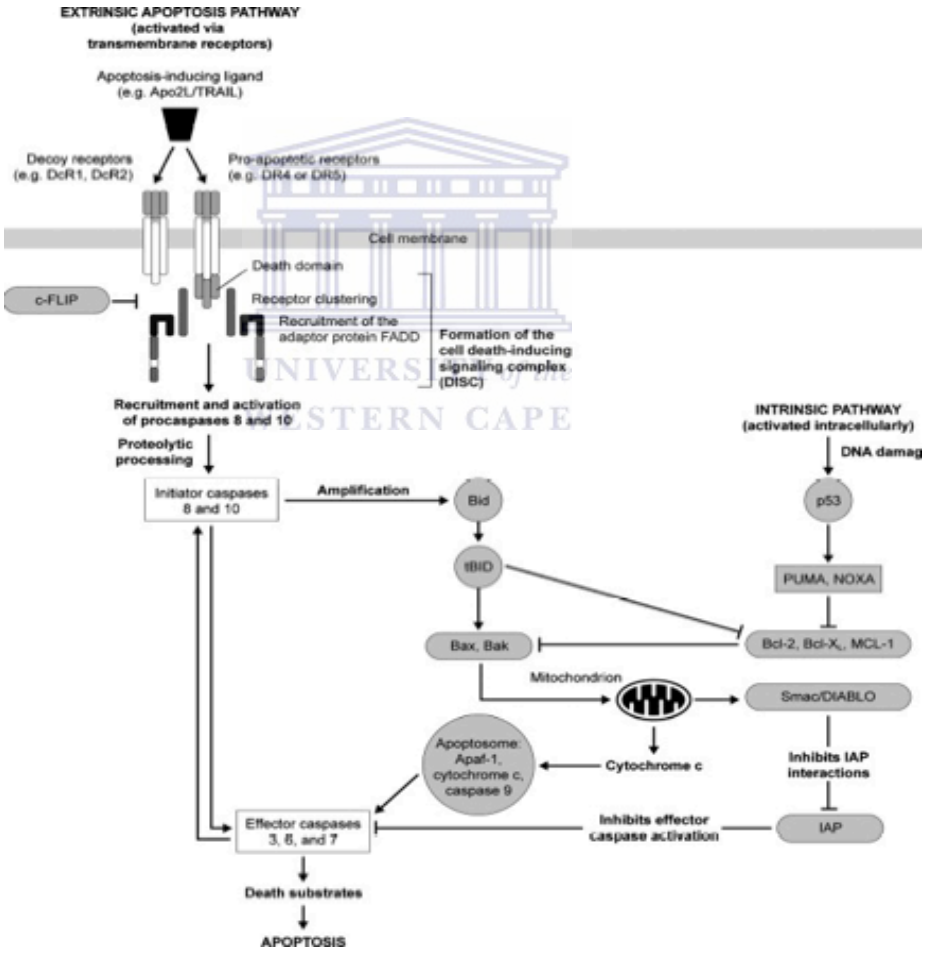

Figure 1.3. The two major apoptosis pathways. Unlike the intrinsic pathway, the extrinsic pathway operates independently of *p53*. Agents that target the extrinsic pathway therefore provide a novel approach to activating apoptosis in cancer cells regardless of *p53* status (Adapted from Ashkenazi, 2008b).

Table 1.3: Some of the features measured in apoptosis cell research

Feature	**Some Detection Techniques**	**References**
Structural evaluation	Light microscopy/TEM/Phase contrast microscopy Fluorescence microscopy	Gorman *et al*., (1996). Otsuki *et al*., 2003).
Exposure of Phosphatidylserine	Annexin V/ APOP*ercentage*™ Apoptosis assay	Meyer *et al*., 2007 Krysko *et al*., (2008 AB). Vermes *et al*., (1995)
Caspase activation/ Cleavage	Flow cytometry / colorimetry /Western blot	Krysko *et al*., (2008). Sabraham and Shaham, (2004).
DNA fragmentation	Flow cytometry / Fluorescence microscopy/agarose gel	Otsuki *et al*., (2003) Sgonc and Gruber (1998). Nagase *et al*., (2002)
Mitochondrial depolarization	Flow cytometry (TMRE/JC-1/$DiOC_6$ /Mito Flow)	Otsuki *et al*., 2003). Jayaraman, (2005).
Bad/Bax/Bak/Bcl-2/BxL/-p53/Cytochrome-c/PARP-1 cleavage	Flow cytometry / western blot	Prasad *et al*., (2006) Rigobello, (2004)
DNA perturbations	Propidium Iodide/Acridine orange/ 5-bromo-2t deoxyuridine (BrdU) (Cell cycle analysis)	Darzynkiewicz Z *et al*., (2001); Ho *et al*., (2009) Bertino *et al*., (2003)
Cellular viability assay	Neutral Red/Annexin V /MTT assay	Repetto *et al*.,(2008); Mosmann, (1983). Babich and Borenfreund, (1991).
ROS production	Dihydroethidine (DHE) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA)	Prasad *et al*., (2006) Wei *et al*., (2000) Arrigo, (1999)
Analysis of lactate dehydrogenase release (LDH)	CytoTox 96 Assay	Krysko *et al*., (2008).
Analysis of cytokeratin 18 release.	ELISA	Krysko *et al*., (2008).
Cytokines	ELISA	Krysko *et al*., (2008).
Single cell layer	Hematoxylin and Eosin staining	Otsuki *et al*., (2003).
MDA product.	Lipid Peroxidation	Catala, (2009)

1.5.9.4 Role of Caspases in Apoptosis

Caspases are a group of cysteine proteases which are members of the interleukin-1beta-converting enzyme family and are responsible for cleaving target proteins at specific aspartate residues and are essential for carrying out apoptosis in eukaryotic cells (Wang and Lenardo, 2000; Mita *et al.,* 2006; Chowdhury *et al*., 2008). All caspases exist within the cell as pro-enzymes but become active upon activation resulting in a cascade of events that ultimately cause apoptosis (Wang and Lenardo, 2000). Earlier studies put the number of members of caspases in mammals to 14 (Wang and Lenardo, 2000), however, recent reports indicate that there are 15, although caspase-15 has not been identified in the human and mouse genomes (Eckhart, 2005; Chowdhury *et al*., 2008). The caspases have further been grouped into two major sub-families, namely inflammatory and apoptotic caspases (Logue and Martin, 2008). The apoptotic caspases have further been subdivided into two sub-groups, initiator caspases and executioner caspases (Chowdhury *et al*., 2008). The substrates of apoptotic caspases are responsible for cellular disassembling, while inflammatory caspases mediate the proteolytic activation of inflammatory cytokines. Thornberry and Lazebnik, (1998), reported that specificity of individual caspase is due to its ability to recognise three amino acid residues lying upstream of the aspartate residue in the substrate.

The apoptotic caspases have been classified into two groups: the initiator (the upstream) and the effector/executioner (downstream) caspases (Sabraham and Shaham, 2004; Rupinder *et al.,* 2007). The initiators are categorized by their long prodomains (i.e. having

>90 amino acids) and they contain either death effector domain (DED) (for instance caspase-2, -8, -9a -10 and-12) in mammals (Chowdhury *et al*., 2008**),** or Dronc and Dredd in fruit flies (Kilicc, 2002), while the executioner (downstream) caspases contain short prodomains (20-30 amino acids), (such as caspase-3, caspase-6 and caspase-7) in mammals (Ashkenazi, 2002; Chowdhury *et al*., 2008) and Drice, Decoy, Damm, Dcp1 and Strica in fruit flies (Kilicc, 2002). The only apoptotic caspase found in the nematode worm *Ceanorhabditis elegans* is the cell-death abnormality-3 (CED-3) which works as both initiator as well as effector caspase **(**Kilicc, 2002). The other caspases such as -1, -4, and -5 are reported to be mainly involved in cytokine maturation but have not been reported to be involved in apoptosis (Stennicke and Salvesen, 1998). Following activation, caspases prodomains cleave into large and small subunits (Lawen, 2003), with eventual formation of active caspases as illustrated in figure 1.5. Once the initiator caspases are formed, they cleave further to trigger effector caspases (Sabraham and Shaham, 2004). It is the effector caspases that cleave cellular substrates with eventual cell death. Effector caspase -3, is reported to cleave a lot of critical cellular substrates, for instance inhibitor of caspase-activated DNase (ICAD), Rho-associated coiled-coil forming kinase (ROCKI), poly (ADP-ribose) polymerase (PARP) a DNA repair enzyme, actin (Lawen, 2003; Sabraham and Shaham, 2004).

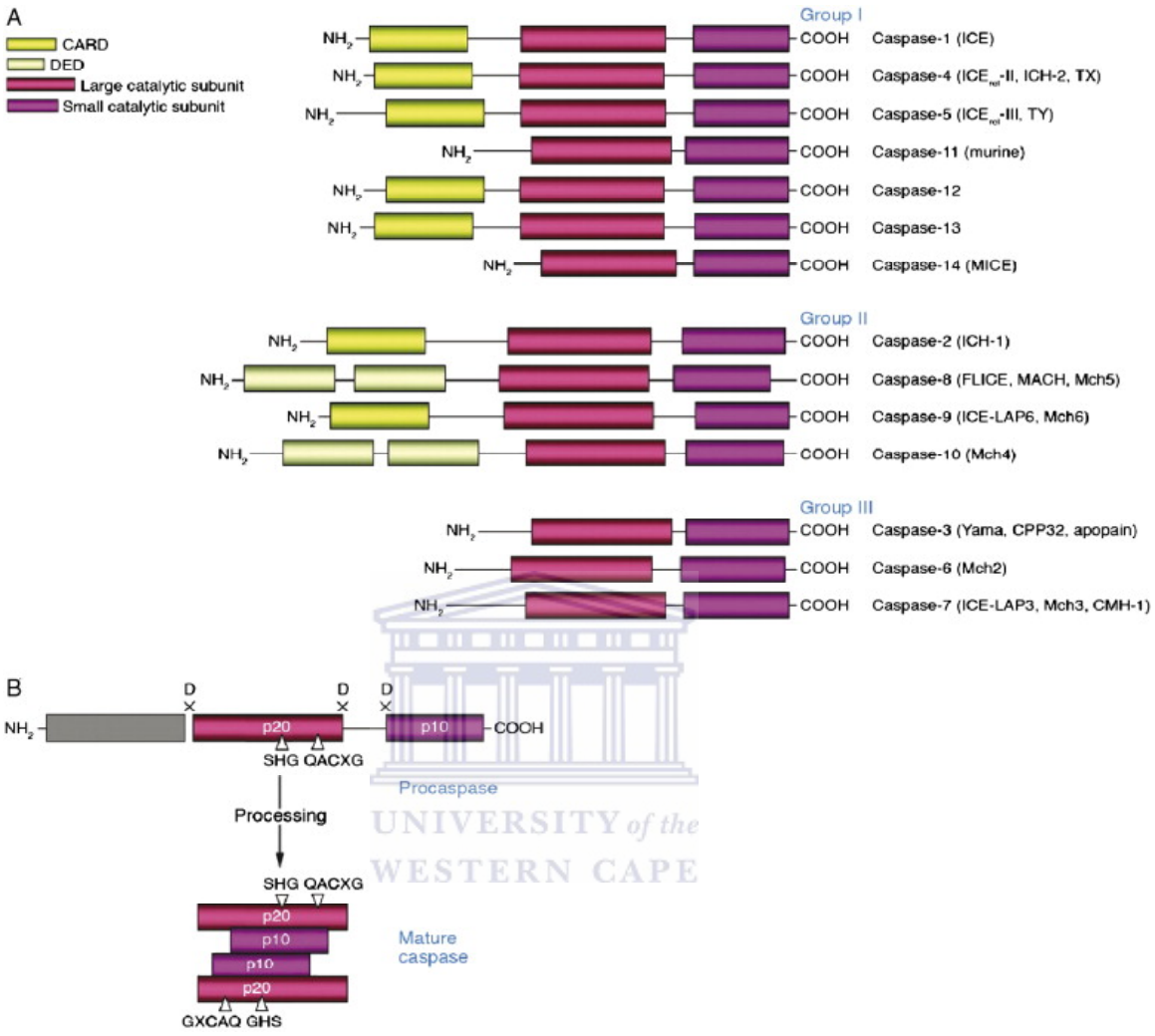

Figure 1.4. Caspase structure. (A) The caspase family. Three major groups of caspases are presented. Group I: inflammatory caspases; group II: apoptosis initiator caspases; group III: apoptosis effector caspases. The CARD, the DED, and the large (p20) and small (p10) catalytic subunits are indicated. (B) Scheme of procaspase activation. Cleavage of the procaspase at the specific Asp-X bonds leads to the formation of the mature caspase, which comprises the heterotetramer $p20_2$–$p10_2$, and the release of the prodomain. The residues involved in the formation of the active centre are shown (Adapted from Rupinder, 2007).

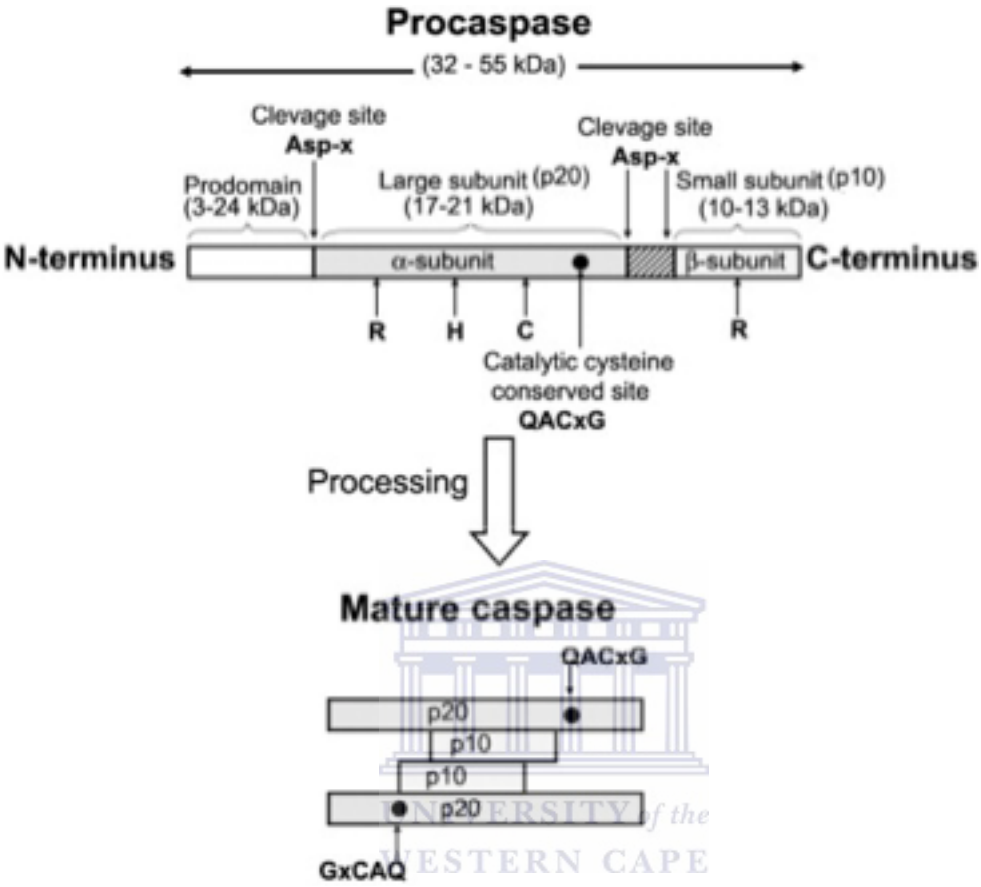

Figure 1.5: A schematic representation of structural features of mammalian caspases. C, H and R represent the active site residues. (adapted from Chowdhury *et al.,* 2008).

1.5.9.5 The role of Bcl-2 family members in apoptosis

The Bcl-2 family, which consist of 25 anti-apoptotic and pro-apoptotic members in mammals, control the mitochondrial pathway of apoptosis (Tsujimoto**,** 1998; Gross *et al*., 1999). The Bcl-2 family members interact to maintain a balance between proliferating and dying cells (Hu and Kavanagh**,** 2003). Anti-apoptotic members hinder the release of mitochondrial apoptogenic factors such as cytochrome *c* and AIF (apoptosis-inducing factor) into the cytoplasm (Tsujimoto**,** 1998), while the pro-apoptotic members induce the

release of caspases from death antagonists via heterodimerization and also by inducing the release of mitochondrial apoptogenic factors into the cytoplasm to activate a cascade of caspase activation (Galteland *et al*., 2005). In this case, the Bcl-2 family members are like critical life-death decision makers within the common pathway of apoptosis (Tsujimoto**,** 1998). Over expression of anti-apoptotic Bcl-2 family members disturb the ratio of the pro- and anti-apoptotic Bcl-2 proteins and hence prevent cell death (Kang and Reynolds, 2009). However, when pro-apoptotic Bcl-2 family members are over expressed, apoptosis proceeds (Hu and Kavanagh 2003). Changes in the mitochondrial function determines whether apoptosis will take place or not (Friend *et al*., 1986). The release of cytochrome *c* from the mitochondria has been implicated as a switch to turn apoptosis on or off and this is regulated by the pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family (Hu and Kavanagh, 2003). The pro-apoptotic proteins are found in the cytosol under normal circumstances where they act as scavengers of cellular damage or stress (Er *et al*., 2006). Following cellular stress or damage however, these proteins relocate to the mitochondria surface where the anti-apoptotic proteins are normally located (Hu and Kavanagh**,** 2003). This results in the interaction between pro- and anti-apoptotic proteins and causes disruption of the normal function of the anti-apoptotic proteins leading to the formation of pores in the mitochondria and hence the release of cytochrome *c* and other pro-apoptotic molecules from the intermembrane space (Gross *et al*., 1999). The release of cytochrome *c* aids in the formation of the apoptosome, which activates the caspase cascade (Gross *et al*., 1999). One other important molecule which is activated upon DNA damage is the p53 tumour suppressor gene (Mak and Yeh, 2002; Ashkenanzi, 2008), which when over

expressed has been implicated to arrest cells in the G1 and G2/M phases of the cell cycle (Ashe and Berry, 2003), and in the case of severe DNA damage and depending on cell type as well as oncogene composition of the cell, p53 is reported to initiate apoptosis by inducing the transcription of the pro-apoptotic proteins such as Bad or genes that generate induction of ROS (Ashe and Berry, 2003; Paradies *et al*., 2010). Most cancers are reported to have loss of p53 function which if properly functioning prevents the development of cancer (Maximov and Maximov, 2008).

The BCL-2 family of proteins are known to posses up to four conserved BCL-2 homology (BH) domains termed BH1, BH2, BH3, and BH4, in correspondence to α-helical segments (Gross *et al*., 1999), (figure 1.6) and these have been subdivided into three classes namely, anti-apoptotic, pro-apoptotic, and the 'BH3-only' pro-apoptotic members (Breckenridge and Xue, 2004). The first subdivision of anti-apoptotic Bcl-2 members, comprise of Bcl-X_L, Bcl-2, Bcl-w, A1/Bfl-1, Mcl-1 and Boo/Diva/Bcl-B (figure 1.7) and these have been implicated in the inhibition of cytochrome *c* release by blocking the activation of the multi-domain pro-apoptotic proteins Bax and Bak (Breckenridge and Xue, 2004; Tahir *et al*., 2007). Most of anti-apoptotic members are reported to show sequence conservation in all four domains (Cory and Adams 2002). The second subdivision includes the multi-domain pro-apoptotic protein members Bax and Bak (Cory and Adams 2002). The third group comprises pro-apoptotic members, such as Bad, Bik, Bid, Bim, Hrk, Bmf, Noxa, and Puma, which act as molecular sensors of cellular stress or damage (Regula *et al.,* 2003; Paradies, 2010). They are discriminated from Bax and Bak

in that they have only the BH3 domain and they are mobilized and activated to induce apoptosis by modulating the activity of the other Bcl-2 family members (Tahir *et al*., 2007). It has been shown that most pro-apoptotic members show less sequence conservation of the first α-helical segment, BH4 (Gross *et al*., 1999) (figure 1.7). All the proteins in these three classes are able to form either homo-oligomer or hetero-dimers with one another and seem to have well-defined functions in the regulation of mitochondrial membrane permeabilization (MMP) (Gross *et al*., 1999; Kirkin and Zörnig, 2004).

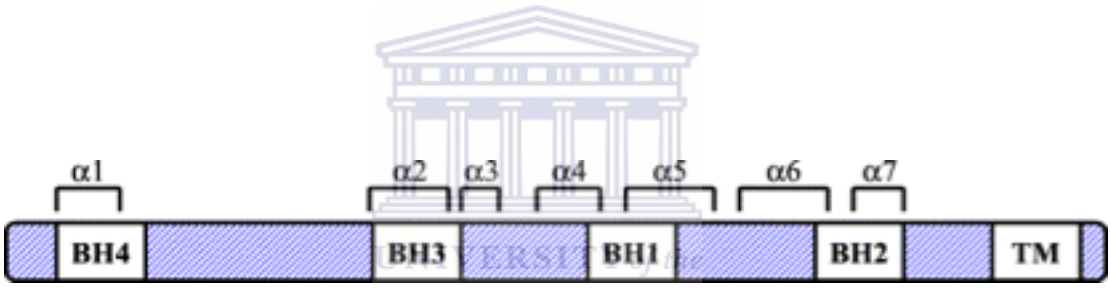

Figure 1.6: Bcl-2 protein domain organisation. Abbreviations: BH1-4, Bcl-2 homology domains; TM, transmembrane domain; α1–7, known alpha-helical regions (adapted from Cory and Adams, 2002).

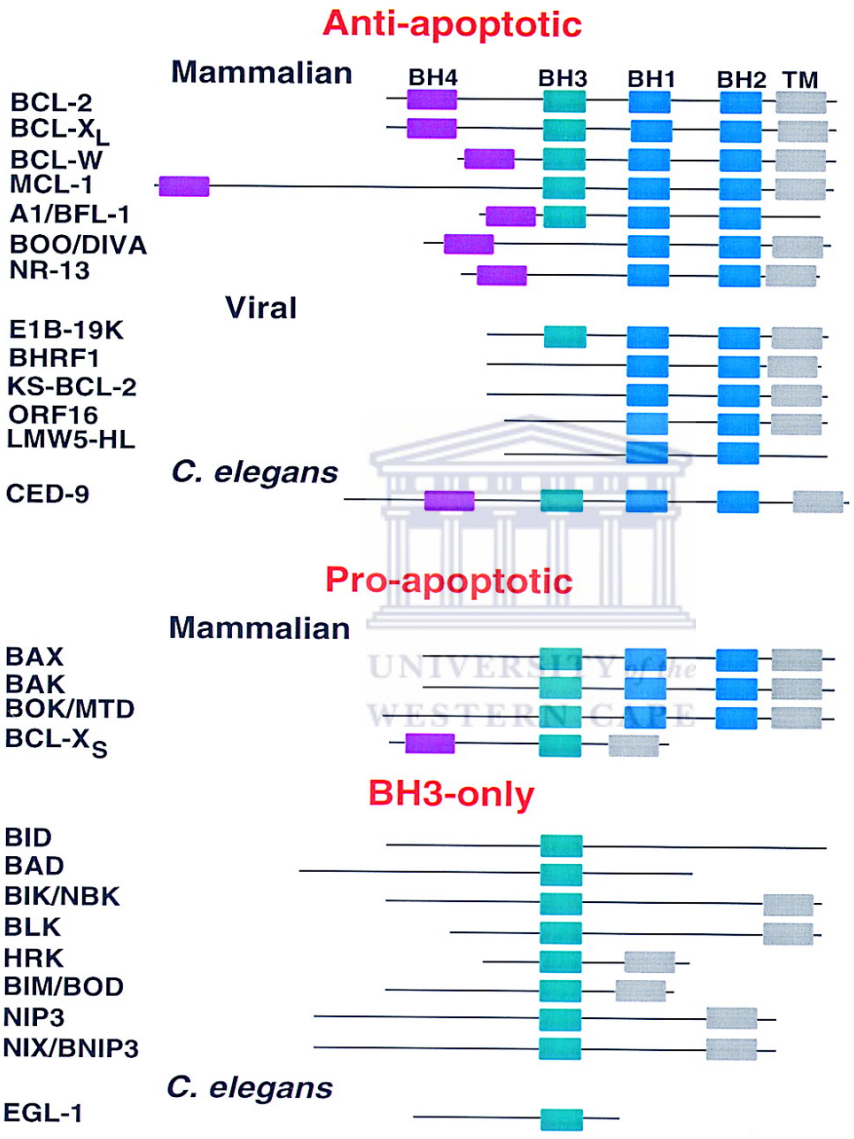

Figure 1.7. Summary of anti-apoptotic and pro-apoptotic BCL-2 members (Adapted from Gross *et al*., 1999).

1.5.10 The cell cycle and cancer

1.5.10.1 Defining the cell cycle

The cell cycle is defined by DeWolf and Gaston, (2005), as the manner by which cells grow, replicate their genome, then separate the 2 copies of the genome into two genetically identical daughter cells (Bertino et al., 2003). In simple term it is a process of cell division. The process however, is regulated by multifaceted molecular connections. The process mimics a clock with orderly movement as the cell moves through the phases of the cycle with each phase executed with a precise predetermined timetable. The phases of the cycle are divided into 4 phases, which are G_1—a phase whereby the cell prepares to synthesize DNA, S-phase is a period of DNA synthesis, G_2-phase is the period whereby the cell prepares itself for cell division and M-phase is the phase of cell division (mitosis) (Weinberg and Lundberg, 1999).

1.5.10.2 Cell cycle, Insensitivity to anti-growth signals and Disease

Deregulation of the cell cycle has a consequence on disease development. The cell cycle is involved in the control of cellular proliferation, growth, and sensitizing cells to apoptosis so as to keep a balance between proliferating cells and dying cells (Shah and Schwartz, 2001). As previously reported in section 1.5.10, increased cell proliferation can lead to diseases such as cancer while decreased cell proliferation can lead to diseases such as neurodegenerative disorders, autoimmune diseases, viral infections, stroke, anaemia and AIDS (Pucci *et al* 2000). In the case of cancer, there are basically three major factors that have been implicated in cancer development. Since apoptosis and cell proliferation are

interlinked, the events that are implicated in the evasion of apoptosis section 1.5.10 are the same that are implicated in cells to have insensitivity to antigrowth signals. One of the factors is that cells avoid the apoptotic program by down-regulating the apoptotic inducers such as p53 (Bennett, 1999**).** The second factor is that survival signals such as phosphatidylinostol-3-Kinase (P13K) are inappropriately or excessively induced (Dong *et al.,* 1999). The third factor involves mutations in proto-oncogenes or tumour suppressor genes. Mutations in proto-oncogenes or tumour suppressor genes may allow cancerous cells to grow and divide thereby passing on mutations to daughter cells that can resist apoptosis (Dong *et al.,* 1999). According to Pucci *et al.,* (2000); Bertino *et al*., (2003), understanding the basic knowledge of the stages of the cell cycle is of ultimate importance because novel therapies that can target genes which are involved in circumventing apoptosis or inhibiting the proper functioning of the cell cycle at each phase of the cell cycle for instance the cyclin dependent kinase inhibitors (CDKIs) can be developed.

1.5.10.3 Stages of the cell cycle

There are four stages of the cell cycle however, the process has been divided into two main events: the S-phase (DNA doubling phase) during which chromosomes replicate and the M-phase (halving of DNA phase) during which the replicated chromosomes segregate into two daughter nuclei (Arellano and Moreno, 1997). In between S and M phases there are two gaps: Gap-1 (G_1) is the phase also known as growth phase. It is a phase in which various enzymes that are required in S phase for DNA replication are synthesized at an increased rate and the cell also prepares itself for replication of the chromosomes (Resende

et al., 2010). The duration of time in which cells spend in G_1 is reported to be extremely variable, even among different cells of the same species (Shackelford *et al*., 2000). Following the G_1 is the S phase in which DNA is now starting to be synthesized (doubling phase of DNA) and at the end of DNA synthesis duplication of the centrosome takes place (Singh *et al*., 2009). The G_2 phase is the phase prior to M phase. In G_2 phase microtubules that are necessary during the mitosis stage are produced (Dekoj *et al*., 2007). M phase is the mitosis phase in which the cell eventually divides into two and it does not begin until the previous S phase has completed (Lewis, 1990). The G_0 phase also known as the "post-mitotic" is the phase in which the cells go into quiescent (Ishidate *et al*., 2000). Non-proliferating cells of multi-cellular organisms are usually found in G_0 stage (Yoshikawa, 2000), and these are mostly adult cells that usually enter the G0 from G-1 and neurons may remain quiescent for long periods of time, possibly indefinitely (Yoshikawa, 2000; Wang *et al*., 2010). Most cells however, remain in interphase, i.e. the period between cell divisions (comprising G_1, S and G_2 phases), for at least ninety percent of the cell cycle. DNA repair genes are said to be active throughout the cell cycle, mostly during G_2 after DNA replication and before the chromosomes prepare for mitosis (Pucci *et al*., 2000).

1.5.10.4 Cell cycle and check control points

In general there are two ways by which genes control the cell cycle (Bertino *et al*., 2003). Some genes control and promote the production of proteins that are required for the cell cycle to take place as well as control the initiation of each phase of the cell cycle. On the other hand, there are genes that negatively regulate the cell cycle by inhibiting the cell from

moving from one phase of the cell cycle to the next step/phase. As cell proliferation proceeds however, there are check points that guarantee that all the genetic material are kept integral, not damaged, not incomplete or miscopied. Should there be any problem(s) to the genetic material, the checkpoints halt the cell from proceeding to the next phase of the cell cycle until the problem has been rectified. In the case that the damage cannot be rectified, the cell is removed by apoptosis (Pucci *et al*., 2000). The stages of the cell cycle have three classified DNA damage checkpoints. These check points are G1/S (G1) checkpoint, intra-S phase checkpoint, and G2/M checkpoint. Within these check points there are many more checkpoints such as spindle checkpoint and morphogenesis checkpoint (Hartwell and Weinert, 1989). The spindle checkpoint arrests cell cycle at M phase until all chromosomes are aligned on spindle. This checkpoint is very important for equal distribution of chromosomes (Hartwell and Weinert, 1989). Morphogenesis checkpoint detects abnormalities in cytoskeleton and arrests cell cycle at G2/M transition (McMillan *et al.,* 1999). Schwartz and Rotter (1998), reported four key check points in the cell cycle. At the end of the G_1 phase, G_2 phase and after DNA has been replicated in the S phase to check for damages. Again at the end of the M phase a checkpoint is available to stop cytokinesis in case the chromosomes are not properly aligned on the mitotic spindle. Checkpoint failure often causes mutations and genomic arrangements resulting in genetic instability (Bertino *et al.*, 2000). Genetic instability is a major factor of birth defects and in the development of many diseases, most notably cancer. Therefore, checkpoint studies are very important for understanding mechanisms of genome maintenance as they have direct

impact on the ontogeny of birth defects and the cancer biology (Schwartz and Rotter, 1998).

1.5.10.5 Cell cycle checkpoints and anticancer drugs

Most anticancer drugs are designed to target different stages of the cell cycle so that the cell should not proceed through to the next stage (Taylor, 2009). Damage to DNA or the spindle apparatus normally triggers cell cycle arrest or apoptosis, depending on the degree of damage and the cellular context. Cell cycle arrest most frequently occurs at the G1/S or G2/M boundaries (Shapiro and Harper, 1999). Some anticancer agents for instance alkylating, anti-tumour antibiotics, platinum compounds and other miscellaneous agents, work in such away that they bind to DNA in the S phase of the cell cycle in order to stop DNA synthesis (Gonzalez *et al.*, 2001; Pasettoa *et al.*, 2006). Some plant-derived anticancer agents such as vinca alkaloids (vinblastine, vincristine, vinorelbine), docetaxol and paclitaxel target the microtubules to stop the cells from synthesizing components required for mitosis or hinder chromosome separation (Das *et al*., 2001; Jordan and Wilson, 2004; Shah and Schwartz, 2006). Taxanes are known to stabilize the microtubules causing a G2/M arrest, followed by apoptosis (Darwiche *et al*., 2007). Anti-metabolites stop production of nucleotides, which are used for DNA synthesis, and some anticancer drugs target cells in G_1 to inhibit nucleotides production for DNA synthesis. Some of such drugs include 6- mercaptopurine (6-MP), 6-thioguanine (6-TG), azathioprine, 5-fluorouracil (5-FU) and methotrexate (Bertino *et al*., 2003). Some anticancer drugs destruct the metabolic processes of the cell to stop the cell from its metabolic processes

(antimetabolites) (Fuksa *et al*., 2010). One such drug is methotrexate (MTX) which when used in combination with other drugs such as 5-fluorouracil and cyclophosphamide can be used in the adjuvant treatment of cancer of the breast and it targets the enzyme dihydrofolate reductase (DHFR) of rapidly growing and reproducing cells by binding and deactivating the enzyme which is key for DNA synthesis (Bast *et al*., 2000; Su and Ciftci, 2002).

1.5.10.6 Role of cyclins and cyclin-dependent kinases (Cdks) in cell cycle regulation.

Cyclins are activating partners of the conserved family of protein kinases that regulate the passage of cells through the cell cycle by forming complexes with other protein kinases known as cyclin dependent kinases (Pines, 1995). Cyclin-dependent kinase (Cdks), are proteins, which belong to a well-conserved family of serine/threonine protein kinases and act as control switches in the regulation of the cell when activated as the cell goes through several stages of the cell cycle (Pucci *et al.*, 2000; Sledge *et al*., 2003). While Cdks are positively regulated by cyclins to move the cell from one phase to the other, cyclin dependent kinase inhibitors (Cdks) negatively regulate movement of the cell from one phase to the other (Golsteyn, 2005; Shah and Schwartz, 2006; Laine *et al*., 2010). To date according to (Yata and Esashi, 2009), there are eleven human CDKs identified however, CDKs 1, 2, 4 and 6 have well characterized roles in cell cycle regulation, while the role of the others is not clear (Makin and Dive, 2001). On the other hand, a considerable number of cyclins has also been identified to date viz: cyclins A-T (Golsteyn, 2005). The most notable cyclins however, are cyclins D, A, E, and B1 since they have shown to subdivide the cell

cycle into different clear stages (Golsteyn, 2005; Laine *et al*., , 2010). These cyclins have been found in abundance during specific phase of the cell cycle in which they are required and subsequently decrease during phases in which they are not required (Shah and Schwartz, 2001). It was shown by Weigel and Moore, (2007); Laine *et al*., (2010); that cyclin D1 is expressed and can be detected in early G1 of the cell cycle while cyclin E is expressed and can be detected in late G1 specifically G1/S peak (Lee *et al*., 2010; Song *et al.,* 2010). Expressions of cyclin A can be detected in early G2/M phase while B1 can be detected in late G2/M phase (Golsteyn, 2005; Nakayama and Nakayama, 2005; Visudtiphole *et al*., 2009). Cyclin H has been shown to be expressed in all phases of the cell cycle (Vermeulen *et al*., 2003 a, b).

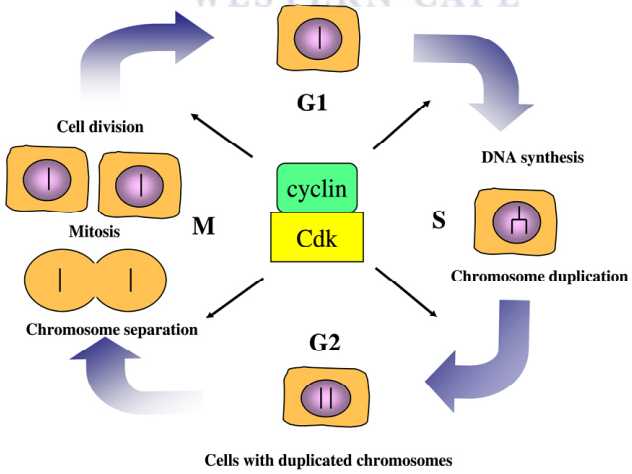

Figure 1.8: The eukaryotic cell cycle and its control mechanisms. The cell cycle consists of four phases: G_1, S, G_2 and M. Regulation of the cell cycle is done by the Cyclin-dependent kinases.

1.5.10.7 Role of p53 in cell cycle regulation

The p53 protein is a transcription factor that has been mapped to human chromosome 17p13 and consists of 393 amino acids that bind to DNA (Atalay and Ozturk, 2000; Buganim and Rotter, 2009). Under normal conditions the p53 protein is present at low basal levels in the cells and is very unstable (el-Deiry *et al*., 1992). However, following nutrient starvation, DNA damage (by chemotherapeutic drugs, UV light and protein kinase inhibitors that involve kinases called ataxia telangiectasia related (ATR) and casein kinase II), **(**Lowe and Lin, 2000), hypoxia and activation by oncogenes, the p53 protein is induced to greater levels (Moll and Petrenko, 2003). Its activation may involve different post-translational modifications including phosphorylation methylation and acetylation (Selivanova, 2010). Phosphorylation is the key event of its activation and may involve some 15 different kinases (Moll and Petrenko 2003; Najjar and Fagard, 2010), and its ubiquitination by the ubiquitin-ligase mouse double minute (Mdm2), negatively regulates p53 activity (Najjar and Fagard, 2010).

The p53 protein has several functions, however, the major function is to sense damage to the DNA and halt the progression of the cell through the cycle in G_1 (Agarwal, 1995), by activating the transcription of another protein called p21 $^{Waf1/Cip1}$ which blocks the activity of Cdk2 which is required for the progression of the cell through G_1 (Minnella *et al*., 2002; Lozano and Zambetti, 2005). In case of DNA damage, cell cycle blocking enables the cell to repair the DNA damage before it is replicated. In the event that the DNA damage is so severe that repair is not possible, p53 induces the cell to undergo apoptosis (Lozano and

Zambetti, 2005). The p53 protein has been coined the “guardian of the genome” and also known as “a tumour suppressor gene” because it allows only good cells to progress through the cell cycle while "bad" cells are forced to commit suicide (el-Deiry *et al*., 1992). If the p53 protein is mutated, cells with damaged DNA can progress through the cell cycle and might most likely result into the development of tumours/cancers (Vousden, 2000). In the case that a person inherits only one functional copy of the p53 gene from his/her parents, the person is predisposed to cancer and usually develops numerous independent tumours in different tissues of the body during early adulthood, a condition known as Li-Fraumeni syndrome although this condition is rare (Senzer *et al*., 2007). Mutations in both copies of the p53 gene and non-functioning of p53 protein has however been implicated in more than half of all human cancers (Bennett, 1999). Apart from activating the transcription factor p21, the p53 protein is also known to induce the 14-3-3σ (sigma) protein a unique member of 14-3-3 family of proteins, which negatively regulates the cell cycle but has a positive feedback effect on p53 activity in response to DNA damage to initiate cell cycle checkpoint control (Lee and Lozano, 2006).

Yet other studies have reported of another tumour suppressor protein known as STAT-1, a transcription factor, which has been implicated, to inhibit cell growth and promote apoptosis (Townsend *et al*., 2004). Following DNA damage, STAT-1 has been shown to interact directly with p53 to initiate cell cycle block and induce apoptosis if necessary (Townsend *et al*., 2004). Hence, although STAT-1 negatively regulates Mdm2, it also acts as a co-activator for p53 (Vermeulen *et al*., 2003b). Therefore STAT-1 is another member

of a growing family of proteins implicated as a partner in the modulation of the p53 activated apoptosis pathway **(**Najjar and Fagard, 2010). The p53 protein is also known for its ability to relocalise death receptors like for instance Fas from the Golgi to the cell surface (el-Deiry *et al*., 1992; Bennett*,* 1999; Vousden, 2000), and also for its direct involvement in the intrinsic pathway of apoptosis (Marchenko *et al*., 2000).

1.5.10.8 The Retinoblastoma (Rb) gene and its role in cell cycle control

The Rb gene was the first tumour suppressor to be identified through human genetic studies in 1986 and it encodes a nuclear protein of 928 amino acids (Wiman, 1993; Feakins *et al*., 2003). Further studies on Rb gene led to the identification of p107 and p130, two related Rb proteins which are more closely related to each other than either one is to Rb (Wiman, 1993; Yoshikawa**,** 2000; Zhu, 2005). These three proteins (Rb, p107 and p130) are commonly known as "pocket proteins" because they share onco-protein binding sequences (Cobrinik, 2005). While Rb has several functions such as maintenance of chromosome stability (Zheng and Lee, 2002), regulation of replication machinery (Zheng and Lee, 2002), regulation of differentiation (male/female) (Berckmans and De Veylder**,** 2009), the major role of Rb however, is to regulate the cell cycle machinery specifically at the G1 phase of the cell cycle (Seeley, 2007), in accordance with promoter-specific transcription factors (Zieske *et al.,* 2004). The transcription factors associated with Rb are the E2Fs and DPs (Zheng *et al*., 1999). Currently there are eight known E2Fs and two known DPs (Wiman, 1993). E2F1, 2, and 3a are commonly believed to be "activator E2Fs" regulated by Rb while E2F3b, E2F4, E2F5, E2F6 and E2F7 are "repressor E2Fs," (Figure 1.9). E2F4

and E2F5 are regulated by p107/p130, although this is not conclusive (Wiman, 1993; Cobrinik**,** 2005). However, Rb is best known as a repressor of the E2F/DP family of transcription factors, which control expression of genes involved in cell proliferation and survival (Zheng *et al*., 1999; Zhu, 2005). The Rb protein is mostly present in a hypophosphorylated form in quiescent cells (Zieske, 2004). Following appropriate stimulation however, Rb protein is phosphorylated by activated cyclin-dependent kinases (Cobrinik**,** 2005). This phosphorylation dislocates the interaction of Rb protein with the transcription factor E2F. Once free, E2F activates the transcription of genes necessary for DNA synthesis (Zheng *et al*.,1999). The active hypophosphorylated form of the retinoblastoma protein (pRb) binds and blocks the action of the transcription factor E2F, inhibiting transition from the G1 phase to the S phase of the cell cycle (Boer and Murray, 2000; Hanahan and Weinberg, 2000; Harashima and Schnittger**,** 2010). The increased concentration of Rb protein at promoter site coupled with E2F, binds adjacent transcription factors, preventing their contact with the basal transcription complex, in so doing blocking transcription (Day *et al*., 1997). It has also been reported by Vidal and Koff, (2000); Bertino *et al.,* (2003); Feakins *et al*., (2003) that cyclin D1 stimulates phosphorylation of Rb by associating with cyclin-dependent kinases (CDKs) and p16 binds to CDKs 4 and 6, blocking their association with D-type cyclins and in so doing preventing the phosphorylation of Rb and blocking the activity of E2F. Hanahan and Weinberg, (2000); Zhu, (2005), noted that the repression of E2F is crucial to Rb tumour suppression activity therefore mutations to the Rb gene have profound consequences since there is a disturbance in the ability of Rb to interact and repress E2F. Wiman, (1993); Mittnacht, (2005);

Mastrangelo *et al* (2007), reported that the deletion of both Rb alleles have shown to play a rate-limiting role in retinoblastoma and also in the sarcomas that mainly arise in families that carry the mutated Rb gene as well as non-retinal, sporadic cancers; for instance small cell carcinoma of the lung, breast, bladder, prostate and glioblastomas. Maximum phosphorylation of Rb proteins is related with S phase of the cell cycle (Mancini 1994; Zhu 2005). Some studies have focused on the restoration of Rb gene functionality using novel small-molecule inhibitors of CDKs and to some extent this has clinically been successful (Sridhar *et al*., 2006).

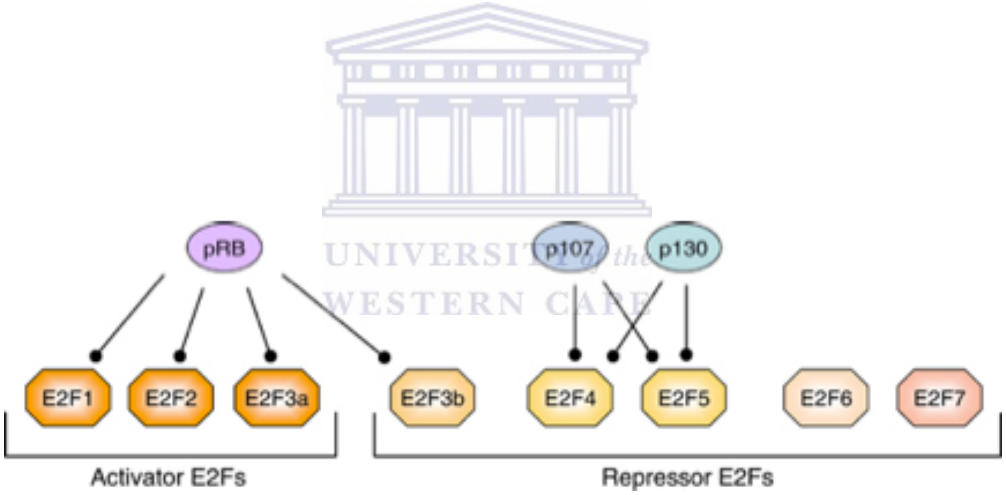

Figure 1.9: Interactions among pocket proteins and E2F transcription factors. Pocket proteins can be subdivided into the pRB and p107/p130 groups. pRB prefers to bind to the activator E2Fs, E2F1, E2F2, and E2F3a; as well as to E2F3b, which function mainly as a repressor. p107 and p130 prefers to bind the repressor E2Fs, E2F4 and E2F5. E2F6 and E2F7 forming transcriptional repressor complexes but do not bind pocket proteins. The DP1 and DP2 proteins form heterodimers with E2F1−6 to allow binding to DNA (not shown), whereas E2F7 binds as a homodimer (Adapted from Cobrinik, 2005).

1.5.11 Role of PARP-1 in DNA repair

Poly(ADP-ribose) polymerase (PARP-1) is a 113-kDa protein composed of 1014 amino acids and the gene coding for this protein has been mapped at the q41–q42 position of

chromosome 1 (Bouchard *et al*., 2003). PARP-1 has been reported to be involved in several functions such as DNA replication, transcription, DNA repair, apoptosis, chromatin structure, cell cycle arrest, initiation of cell death induced by different stimuli following DNA damage and stabilization of the genome (Carrozza *et al*., 2009; Sabisz *et al*., 2010). Although PARP-1 has several functions, the major function of this enzyme however, is to help in base excision repair (BER) and recombination of single-stranded DNA strand breaks through the activation of DNA repair and check point control enzymes (Bouchard *et al*., 2003; Süsse *et al*., 2004**)**. Following DNA damage, (Caldecott *et al*., 1996; Malanga *et al* 1998), reported that the enzyme binds to only one strand of a broken DNA and then recruits XRCC1, DNA ligase IIIα, DNA polymerase β and polynucleotide kinase to the broken end. Additionally, Bouchard *et al*., (2003), reported that PARP-1 binds to DNA strand breaks with high affinity and subsequently poly(ADP-ribosyl)ates itself and other nuclear proteins involved in chromatin structure, DNA base excision repair and recombination and this complex then repairs the broken part. Wesierska-Gadek *et al*., (2003), suggested that the amino-terminal and central fragments of PARP-1 were required for complexion of PARP-1 with p53 protein subsequent modification for poly(ADP-ribosyl)ation. According to Bouchard *et al*., (2003), Poly(ADP-ribosyl)ation occurs in all cells with nucleus but not in yeast.

1. 6 Metallo-organic compounds

Metal compounds are synthesized drugs whose centre contains a metal with other substances known as ligands bound to it (Huang *et al*., 2005). The metal and the ligand together form a complex known as organo-metallic complex (Huang *et al*., 2005). When metals bind to elements N, O or S they form a chelate ring that binds to metals more tightly as compared to non-chelate form (Huang *et al*., 2005). Biologically, the ligand is responsible to change the biological activity of the metal in which case the ligand is used as a carrier of the metal to its active site (Shaw, 1999). According to Berners-Price and Sadler, 1996; Shaw, 1999, in some cases, an inactive metal drug might be administered but in the processes becomes active after undergoing ligand exchange or other reactions and become what is termed as a pro-drug. These reactions may be involved in metal substitutions or redox reactions (Berners-Price and Sadler, 1996).

1.6.1 Metal compounds as medicinal agents

Historically the use of metal compounds as medicinal agents dates back to as early as the 16th century with reports documenting the therapeutic use of metals or metal containing compounds as anticancer treating agents (Huang *et al.,* 2005). The earliest metal containing compounds to be studied in medicinal chemistry are the iron complexes as they were and are still being used for the treatment of hypochromic anaemia caused by iron deficiency (Schwietert and McCue, 1999)**.** To date several metal containing compounds have been synthesized and used as medicinal agents in the treatment of various ailments for instance antimony (antiprotozoal) (Maltezou, 2009), platinum

(anticancer) (Timerbaev *et al*., 2006; Michalke, 2010**)**, silver (antimicrobial) (Ahmad, 2006), gold (antiarthritic) (Alama *et al.*, 2009), iron (antimalarial) (Alama *et al*., 2009**)**, vanadium (antidiabetic) (Rehder *et al*., 2003; Ahmad, 2006) and bismuth (antiulcer) (Ahmad, 2006) among others. Metals can be used in medicine because they have the ability to bind and interact with important biological molecules such as proteins and DNA (Gras, 2010). This comes about because these biological molecules are sufficient in electrons while metal ions are deficient in electrons as a result, the metal ions tend to bind to the biological molecules (Huang *et al.,* 2005). In addition, metal ions also tend to bind to small molecules that are crucial to life such as O_2 (Schindler *et al*., 1998). More also in some cases the presence of metals results in the generation of reactive oxygen species (ROS), whose presence is believed to play crucial though poorly understood roles that can alter drug-induced cytotoxic responses and affect cancer pathogenesis (Sasabe *et al.,* 2010). Scientists take advantage of the benefits which metals offer and use them to modulate biological systems (Sanchez-Ruiz**,** 2010). For instance, metals can bridge substrate to enzymes or metals can participate in the correct tertiary folding of proteins (Lange SJ and Que Jr, 1998). While metals can be used in biological systems however, care should be noted that transition metals can generate potentially harmful effects, hence their levels in normal homeostasis or therapeutic intervention should be strictly controlled because most are toxic especially when used in excess (Fairbrother *et al.,* 2007). All other drugs require the understanding of their mechanism of action. Similarly, the use of metals in drug development also depends mainly on the understanding of their mechanism of action (MOA) upon which their selective toxicities can be controlled (Reedijk, 2008).

1.6.2 Challenges in metal drug discovery

The biggest challenge faced with metal drug discovery is that most metal compounds have poor physico-chemical properties such as inadequate solubility, hydrolytic instability and that most of the compounds tend to readily decompose when exposed to solvents, humidity, light or air (Schwietert and McCue, 1999). Additionally, most metal drugs are cytotoxic and also lack selectivity to act only on the affected cells but rather they act on both affected as well as non-affected cells. As if this is not adequate, there has been limited knowledge about the mode of action (MOA) by which metal drugs brings about their biological activities, how much of the drug has been assimilated and how much has been inactivated **(**Timerbaev *et al*., 2006). Because of these problems there has been a general reluctance in the development of metal-based drugs (Timerbaev *et al.,* 2006; Hindo *et al*., 2009).

1.6.3 Cisplatin as an anticancer agent

Cisplatin is a metallo-compound and has been used as an anticancer agent for over 25 years (Gonzalez *et al*., 2001; Alderdena, 2006) in the treatment of solid tumours such as testicular, ovarian, head and neck, and bladder cancers **(**Lebwohl and Canetta, 1998; Timerbaev *et al.*, 2006**),** with a cure rate of as high as 90% (Reedijk, 2003). It is of particular importance to note that cisplatin exhibits anti-tumour activity while its trans isomer does not show any activity (Reedijk and Lohman, 1985). This shows that minor variations in the structure of a metal can bring about powerful effects on its biological

toxicities (Ciccarrelli *et al*., 1985; Brabec and Marc, 1993; Brabec and Kaspaarkova, 2005). Although cisplatin has been widely used successfully as an anti-cancer agent for several years, however, its use has had several problems. Some of such problems include lack of selectivity in killing of tumour tissue, toxicity and acquired drug resistance **(**Hindo *et al*., 2009). For these reasons over the years, several thousands of platinum analogues have been synthesized and tested with the major urge of developing novel anticancer drugs that have modes of action (MOA) distinct from those of the parent cisplatin (Pasetto *et al*., 2006; Hindo *et al*., 2009). Only a small number of compounds (between 28 and 40) were taken into clinical trials because they showed to have enhanced therapeutic index as compared to that of cisplatin (Lebwohl and Canetta, 1998; Michalke, 2010). Out of the compounds that went into clinical trials apart from cisplatin however, only three Pt drugs were approved as anti-cancer drugs (carboplatin, world wide; oxaliplatin in a few countries only; nedaplatin in Japan only) (Lebwohl and Canetta, 1998). Of these, carboplatin has shown to provide significant advantage over cisplatin in reducing some of cisplatin's toxicities. Notwithstanding carboplatin receiving worldwide approval in reducing some of cisplatin's toxicities, its use however, has not enlarged the spectrum of platinum-sensitive cancers, and neither has it proved active in cisplatin-resistant cancers (Weiss and Christian, 1993; Pasetto *et al.,* 2006). Another obstacle faced with carboplatin is its myelosuppression (Dygai *et al*., 2007).

1.6.4 Gold compounds

Gold (Au) is a metallic element that has been used in a variety of forms for many centuries and it exists in many different oxidative states: –I, 0, II, III, IV, and V (Fricker, 1996; Alama *et al.*, 2009). According to Fricker, (1996), only AU(0), I and III, are stable in aqueous solutions however, AU(I) and AU(III) are unstable in respect to AU(0). Fricker, (1996), further reported that AU(I) is more stable than AU(III) but AU(III) complexes are strong oxidizing agents as they get reduced to AU(I) and are therefore more toxic than AU(I). Tiekink, (2002) and Rackham *et al*., (2007), reported that "Soft" gold(I), (meaning easily polarisable) (Tiekink, 2002), binds only weakly to "hard" oxygen and nitrogen ligands; however, it exhibits a very high affinity for "soft" donor atoms (sulphur and/or phosphorus) (Tiekink, 2002), and thus forms numerous complexes with sulphur, selenium, and phosphorus containing ligands, while AU(III) complexes have preferences for hard atoms donors such as nitrogen, oxygen and carbon. Biologically, the range of sulphur-containing molecules with biological activity is diverse, as a result many investigations have been evaluated on the anti-tumour activity of phosphinegold(I) complexes directed at cysteine residues within critical *in vivo* putative targets (Huang *et al.,* 2005).

1.6.4.1 Gold (Au) compounds for treatment of Rheumatoid arthritis

Rheumatoid arthritis is a painful and disabling chronic autoimmune disease, which causes inflammation and progressive erosion of the joints (Gonzalez-Gay *et al*., 2005). So far the cause of the disease is not known. Current treatment mainly focuses on alleviating the

symptoms and preventing the progressive destructive processes of the disease (Gandin *et al*., 2010). The treatment for this disease uses anti-inflammatory agents, analgetics and so-called disease modifying antirheumatic drugs (DMARDs) which use methotrexate (MTX) or combined therapy of intermediate to high doses of glucocorticoids and combinations of MTX with TNF blockers (Smolen and Weinblatt, 2008) or the disease can be treated with gold complexes such as aurothioglucose (solganol), aurothiomalate (myocrisin) aurothiosulfate (sanocrysin), aurothiopropanol sulfonate (allocrysin) and triethylphosphinegold(I)tetraacetylthioglucose (auranofin) to arrest or slow down the disease progression and lower bone and cartilage damage (Fricker, 1996; Kean 1997; Gandin *et al*., 2010). The use of DMARDs is critical since once damage to the joints is done, the damage is usually irreparable and anti-inflammatory agents and analgesics cannot help alleviate the problem. Of the above-mentioned gold salts auranofin triethylphosphine (2,3,4,6-tetra-*O*-acetyl-β-1-d-thiopyranosato-S) gold(I), is of particular interest because it can be administered orally as opposed to the other gold salts, which are normally given by injection and moreover, auranofin is the first metal phosphine complex which was introduced into clinical practice for cryotherapy, after successful studies conducted on gold(I) thiolate compounds (Gandin *et al*., 2010**)**. Cryotherapy has been practiced for more than 60 years (Song *et al*., 1999; Tiekink, 2002; Ott, 2009). Another important feature of auranofin is that it is labile and its liability has been linked to be due to the metal–thioglucose bond, which suggests that slight modification of this interaction could produce changes in its biological profile (Gandin *et al*., 2010).

1.6.4.2 Gold(I) complexes with multiple phosphine ligands

Phosphine is a compound with a chemical formula PH_3 (Bond *et al*., 1969). Phosphines are also said to be a group of organophoshorus compounds with the formula R3P (where R=organic derivative/condensed aromatic group) (Tefteller *et al.,* 1965). In most cases phosphine ligands PR_3 carry alkyl and aryl substituent at the phosphorus atom. In a more general sense however, R may also be a halogen atom (phosphorus halides, PX_3) or, for example, an OR group (phosphites, $P(OR)_3$) (Tefteller *et al.,* 1965). Generally, organic phosphines (phosphanes) PR3 play an important role as ligands in coordination chemistry and also in organometallic chemistry (Vogler and Kunkely, 2002). Organophosphines are easy to synthesize, are excellent ligands for transition metals and are also important in catalysts where they complex to various metal ions/complexes derived from chiral phosphines that can catalyze reactions to give chiral products (Pascariu *et al.,* 2009). As a result, the steric attributes of phosphine ligands are easily controlled and one can be able to fine-tune the reactivity of the metal complex (Pascariu *et al.,* 2009). Additionally, phosphines are also known to stabilize transition metals in low oxidation states but their versatility as ligands is also documented by their ability to coordinate transition metals in higher oxidation states including metals with d^0 electronic configuration owing to their electron donating and accepting abilities (Vogler and Kunkely, 2002). Phosphine is used extensively as a fumigant and it is highly toxic as it can easily kill in fairly low concentrations (Bond *et al*., 1969). Ott, (2009), reported that agents with multiple phosphine ligands attached to the gold(I) central atom are mostly active implicating $[Au(dppe)_2]$ as being the lead compound for their activities (Ott, 2009) (figure 1.10).

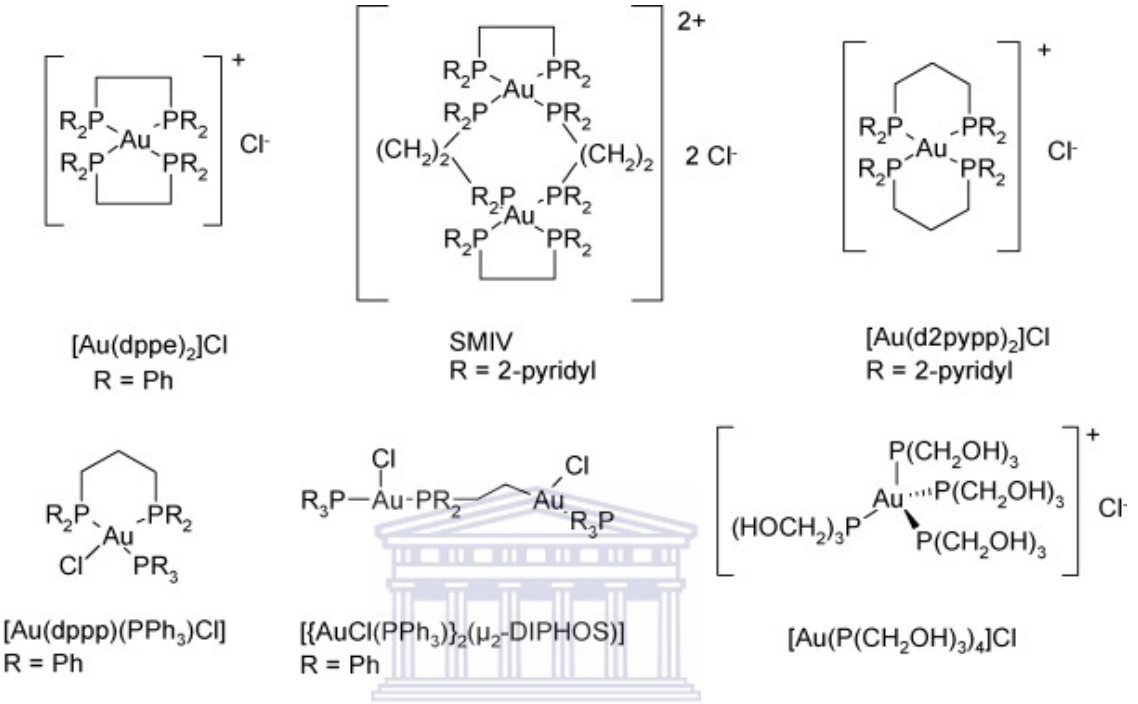

Figure 1.10: Examples of Gold(I) complexes with multiple phosphine ligands (Adapted from Ott, 2009).

1.6.4.3 Gold (Au) compounds for the treatment of cancer

The use of gold in medicine dates back to antiquity when Arabic and Chinese physicians are reported to have used gold preparations for the treatment of various ailments (Tiekink, 2002), and also gold alloys in restorative dentistry (Möller, 2002). Following the discovery of metal platinum complexes as anticancer agents, researchers have since resorted to investigating other novel inorganic anti-tumour agents that can specifically kill cancer cells, but have less toxic side effects than their platinum counterparts (Irena, 2006). One of such metal compound is gold. Gold complexes have been reported to have

anticancer properties and are currently attracting considerable attention due to their wide and diverse structural types and varied ligand binding modes which offers considerable potential in fine-tuning their biological properties (Tiekink 2002; Bradley *et al*., 2007). That is why apart from being used in cryotherapy, auranofin has also been studied for its antitumor activities and so far results have shown that auranofin possesses *in vivo* antitumor activity against P388 murine leukemia and also *in vitro* cytotoxic potency against both B16 melanoma and P388 leukemia cells (Simon *et al*., 1981; Mirabelli *et al*., 1985). Of late *in vitro* studies on auranofin have shown that auranofin is effective against in cisplatin-resistant human ovarian cancer cells. Cells treated with auranofin have reportedly exhibited increased levels of TrxR activity indicating that cytotoxic activity that underlie phosphine Au(I) drugs exhibits a mechanism of action (MOA) different from that caused by cisplatin (Huang *et al.,* 2005; Marzano, 2008**;** Ott, 2009). In particular, auranofin has been implicated as a potent inhibitor of thioredoxin reductase, whereby it alters the redox state of the cell leading to an increased production of hydrogen peroxide and oxidation of the components of the thioredoxin (Trx) system, with resultant creation of conditions that enhanced apoptosis (Marzano, 2008). Gold complexes have been reported to induce apoptosis in several cancer cells as well as cells that have acquired resistance to specific drugs. Their mode of action has been attributed to inhibition of mitochondrial and cytosolic proteins (figure1.11) mainly glutathione and thioredoxin systems (Arnér and Holmgren, 2006; Che and Siu, 2010).

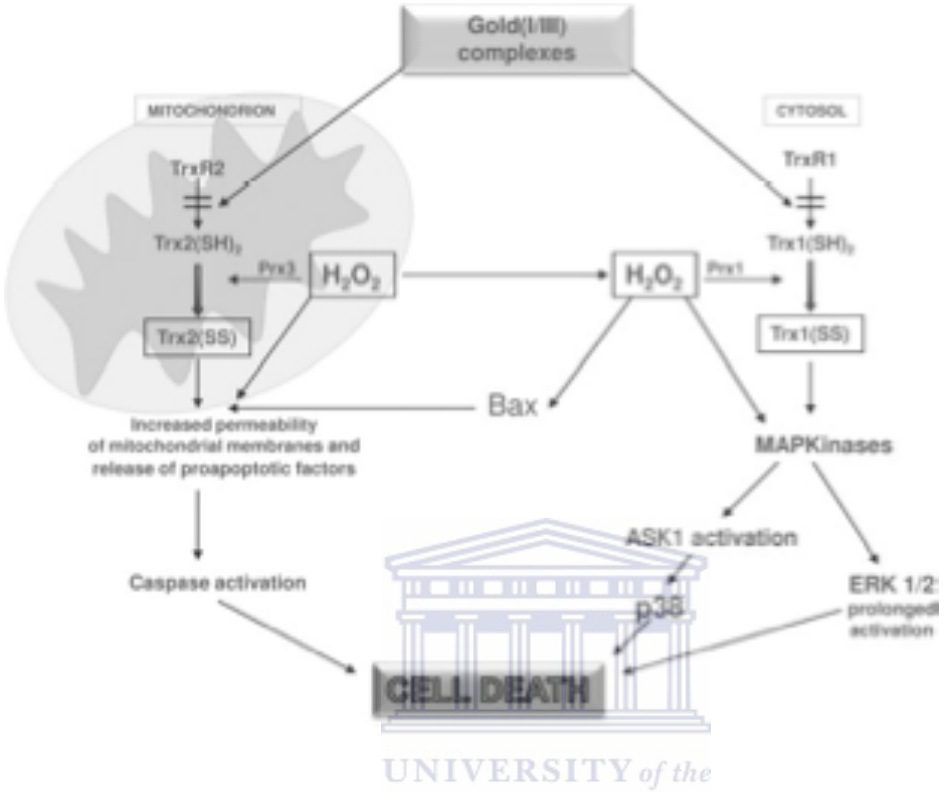

Figure 1.11: Model depicting the mechanism of action of cell death induction by gold(I/III) compounds. The mitochondrial respiratory chain produces superoxide anion that dismutes to hydrogen peroxide and oxidizes thioredoxin in a reaction mediated by peroxiredoxin. Thioredoxin reductase, inhibited by gold(I/III) complexes, is unable to reduce back oxidized thioredoxin that accumulates together with hydrogen peroxide and both act on several different intramitochondrial targets leading to the opening of the mitochondrial permeability transition pore and/or to an increase of the permeability of the outer membrane. Hydrogen peroxide is then released to the cytosol where causes oxidation of Trx1, that, similarly, to mitochondrial thioredoxin (Trx2), cannot be reduced back by the gold(I/III)-inhibited thioredoxin reductase. Oxidized thioredoxin stimulates the MAP kinases pathways leading to cell death **(**Adapted from Bindoli *et al*., 2009).

Recent studies have shown that gold nanoparticles can be used to target drug delivery to particular cancers and in such way increase their efficiency such as has been used in pancreatic cancer treatment (Patra *et al*., 2010). Yet another study by Lum *et al*., (2010), showed that some gold compounds induced apoptosis as well as prolonged the survival of hepatocellular carcinoma (HCC)-bearing rats and also inhibited the tumour growth of

mice bearing nasopharyngeal carcinoma (NPC), neuroblastoma and colon carcinoma. Lum *et al*., (2010), showed that gold-1a prolonged the survival of NPC metastasis-bearing mice and also inhibited intra-hepatic and lung metastasis. Histological studies, showed that gold-1a markedly reduced tumour microvessel formation. Consistently, their *in vitro* studies, showed that gold-1a inhibited migration and invasion of C666-1 human NPC cells. Conclusively, data from Lum *et al*., (2010), strongly supported the use of gold(III) compounds for the treatment of cancer metastasis.

1.7 Problem statement

Despite cisplatin's success story in combating most of the solid tumours (Zhang and Lippard, 2003; Elwell, 2006; Keter *et al*., 2008), most cancers however, frequently develop resistance to cisplatin, lack sensitivity, increased DNA repair capacity, poor pharmacokinetics profile, and failure to selectively kill cancer cells over normal cells (Alderdena *et al*. 2006). Cisplatin treatment also increases production of intracellular thiols (e.g. glutathione and metallothionein), which leads to harmful side effects such as: nausea, emetogenesis, hair loss, neurotoxicity, ototoxicity, nephrotoxicity and myelosuppression among others (Kumar and Clark, 1990; Brabec and Kasparkova, 2005; Wang and Lipard, 2005; Bravo *et al*., 2010)**.** These limitations have prompted a search for more effective and less toxic metal-based anti-tumour agents (Brabec and Kasparkova, 2005; Elwell, 2006).

1.8 Hypothesis

In this study novel water soluble classes of phosphine ligands which will have minor variations in their structure and their respective Au (1) complexes will be synthesized and assessed for possible anticancer activities. This is in respect to recent studies which have shown that different classes of gold-based compounds, in both Au(I) and Au(III) oxidation states, induce apoptosis in several cancer cells *via* mitochondrial cell death pathways (Barnard and Price, 2007; Rackham, 2007; Ott, 2009). Bis-chelated Au(I) phosphine complexes have to some extent demonstrated to selectively induce apoptosis in cancer cells over normal cells (Rackham, 2007) though with a narrow spectrum. We hypothesize that the variations made to the newly synthesized structures of these compounds will bring the much-needed change in the biological activity of these compounds.

1.9 The aims of the project

The aims of this project are two fold: Firstly, to screen the compounds for pro-apoptotic activity and evaluate their potential anticancer agents. Secondly, to evaluate the mechanism of action of the promising compounds.

1.10 The specific objectives

1. To screen the fifteen synthesized bidentate amino-and iminophosphine ligands for pro-apoptotic activity on a panel of human cell lines and to determine their potential as anticancer agents.

2. To screen the fifteen synthesized bidentate mono and dinuclear Au(I) complexes for pro-apoptotic effects on a panel of human cell lines and to determine their potential as anticancer agents.

3. To investigate the underlying mechanisms through which the bidentate amino-and iminophosphine ligands or their cognate mono and dinuclear Au(I) complexes induce biological activities.

CHAPTER TWO OUTLINE

2.1 General stocks

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- **2.1.2 Commercial kits/antibodies/molecular probes**
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2.2.16.3 Calculations

2.2.17 RNA isolation

2.2.17.1 Isolation of RNA from cultured mammalian cells

2.2.17.2 RNA gel electrophoresis

2.2.18 Preparation of cDNA using ImProm-II™ Reverse Transcriptase system.

2.2.19 Polymerase Chain Reaction (PCR)

2.2.20 The pcDNA™ 3.1 D/V5-His-TOPO cloning vector

2.2.21 Genotype of Bacterial strains

2.2.22 Selection of transformed bacteria

2.2.23 Storage of bacterial strains and clones

2.2.24 TOPO® cloning procedure

2.2.24.1 Design of PCR primers

2.2.24.2 Amplification of gene of interest

2.2.24.3 TOPO® Ligation reaction

2.2.24.4 Transformation of *E.coli* cells

2.2.24.5 Screening for transformed colonies by PCR

2.2.25 Agarose gel electrophoresis of DNA

2.2.26 Purification of DNA fragments from agarose gels

2.2.27 Preparation of Plasmid DNA

2.2.28 Sequencing of cloned DNA product

2.2.29 DNA transfection

2.2.30 Extraction of proteins from cell lines

2.2.31 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

2 .2.31.1 Preparation of samples

2.2.31.2 Preparation of gels

2.2.31.3 Loading and running of samples

2.2.31.4 Staining and Destaining of SDS-PAGE gels

2.2.32 The Western blot assay

2.2.32.1 Blot Stripping

CHAPTER TWO

2.1 General Stocks

2.1.1 Chemicals	Supplier
Acrylamide:bis-acrylamide 40% (37.5:1)……....	Promega
Agarose…….	Promega
Ammonium acetate……..	Merck
Ammonium persulphate (APS)……	Merck
Ampicillin……..	Roche
Ascobic acid…….	Sigma
Boric acid…….	Sigma
Bovin Serum Albumin (BSA)…….	Roche
Bromophenol blue……	Roche
Cesium chloride (CsCl)……	Roche
Calcium chloride……..	Sigma
Catalase……	Sigma
Cisplatin……	Sigma
Coomassie Brilliant Blue R250…….	Sigma
Diethyl pyrocarbonate (DEPC)……	Roche
Diethyldithiocarbamate (DDTC)……..	Alexis

Dimethyl sulphoxide (DMSO)…….	Sigma
Dithiothreitol (DTT)…….	Roche
Dulbecco's Modified Eagle Medium (DMEM)……..	GIBCO
DNA molecular weight marker……	Fermentas
Dream *Taq*……..	Fermentas
E.coli One Shot® TOP10F' Chemically competent cells…….	Invitrogen
Ethanol…….	Merck
Ethidium bromide…….	Merck
Ethylene diamine tetra-acetic acid (EDTA)…….	Merck
Foetal calf serum (FCS)……...	Roche
G418……..	Roche
Glucose…….	Sigma
Glycerol……	Saarchem UniVAR
Glycine…….	Merck
Ham's F12……	Invitrogen
Hydrochloric acid…….	Saarchem UniVAR
Hydrogen peroxide (H_2O_2)……..	Sigma
L-Broth…….	Merck
L-glutathione (reduced)……	Sigma
Magnesium Chloride……	Merck
Metafectene® Pro Transfection reagent ……..	Biontex

Methanol……..	Merck
4-Morpholine propanesulphonic acid (MOPS)…….	Roche
n-butanol……..	Merck
Nutrient Agar……	Merck
Oligonucleotides……..	Inqaba
Paraformaldehyde……	Sigma
Penicillin-Streptomycin……	Invitrogen
Pfu *Taq* Polymerase…..……	Fermentas
Phosphate Buffered Saline (PBS) without $CaCl^{2+}$ and $MgCl^{2+}$….	GIBCO
Potassium acetate…….	Merck
Potassium chloride……...	Merck
Propan-2-ol……...	Merck
Propidium Iodide……..	Sigma
Proteinase K…….	Roche
Pyrrolidine dithiocarbamate (PDTC)……	Alexis
RNase A……	Roche
RiboRuler ™ High-range RNA ladder…….	Fermentas
Roswell Park Memorial Institute (RPMI) medium……..	Invitrogen
Sodium acetate…….	Merck
Sodium chloride……	Merck
Sodium dodecyl sulphate (SDS)……...	Roche

N,N,N´,N´-Tetramethylethylene-diamine (TEMED)……..	Promega
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)……..	Sigma
Thiobarbituric acid……...	Sigma
Trichloroacetic acid……..	Sigma
Tris(hydroxymethyl) aminomethane……	BDH
Triton X-100…….	Sigma
Trypsin……..	Invitrogen
Tryptone……	Merck
Tween 20……..	Merck

2.1.2 Commercial kits/antibodies/molecular probes	**Supplier**
Anti-Actin (C-2) HPR antibody……	Santa Cruz
Active caspase-3-FITC assay…….....	BD Biosciences
Annexin V PE assay…….....	BD Biosciences
APO-Direct™ (TUNEL) assay…….....	BD Biosciences
APOP*ercentage*™ apoptosis assay…….....	Biocolor
CM-H_2DCFDA molecular probe……..……...	Invitrogen
CytoBuster™ protein extraction reagent……..	Novagen
Goat anti-mouse IgG –HPR…….....	Santa Cruz
Glutathione Assay…….....	Sigma
ImProm-ll™ Reverse Transcriptase System …….....	Promega

NucleoSpin TriPrep RNA extraction kit…….	Fermentas
pcDNA™ 3.1 Directional TOPO® expression kit …….....	Promega
Tetramethyl Rhodamine (TMRE) …….....	Promega
Anti-Trx-2 antibody…….....	Santa Cruz
Thioredoxin reductase assay…….....	Sigma
Wizard® plus DNA purification system …….....	Promega
Wizard® SV gel and PCR clean up system …….....	Promega

2.1.3 General stock solutions and buffers

Ampicillin

100mg/ml stock was prepared in sterile deionised water, filter sterilised using a 0.22µM filter and stored at -20°C.

Ammonium Persulfate (APS) (10%)

APS 10% solution was prepared fresh daily in a microcentrifuge tube and the appropriate amount of water (e.g. 40mg would require 400µL water) was added and the tube was maintained on ice for the day.

Digestion Buffer

100mM NaCl, 10mM Tris-Cl (pH 8), 25mM EDTA (pH 8), and 0.5% SDS. Proteinase K (to a final concentration of 0.1mg/ml) was added just before use.

Glycerol Bromophenol Blue (GBB)

30% glycerol (v/v), 15mM EDTA (pH 8.0) and 0.5% bromophenol blue (w/v).

L-Broth

1% tryptone, 0.5% yeast extract, 0.5% NaCl and 0.2% glucose.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

A stock solution was prepared by making a 5mg/ml solution in PBS

Neutralisation Solution

3M potassium acetate (pH 5.0).

Paraformaldehyde Fixative

Paraformaldehyde (16g) was dissolved in 80 ml of deionised water by stirring at 70^{o}C in a fume cupboard. One drop of 2M NaOH was added. The solution was cooled down slowly to room temperature and the volume was adjusted to 100ml with deionised water. The solution was filtered through a 0.45-micron filter and a 100ml of 2x PBS added. The paraformaldehyde solution was stored at 4^{o}C wrapped in foil.

Phenylmethanesulfonylfluoride/ phenylmethylsulfonylfluoride (PMSF)

A 10mM stock solution was prepared in isopropanol.

Propidium iodide (PI)

A 1mg/ml stock solution of PI was prepared in 3.8mM sodium citrate pH 7.0 and a working stock was prepared by combining 950µL PBS, 400µL PI and 100µL RNase A.

Protein Lysis buffer

150mM NaCl, 10mM Tris, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1% (w/v) Triton X-100, 10mM DTT, 0.5mM PMSF pH 7.4.

RNase (DNase free)

A 20mg/ml stock solution was prepared in a buffer containing 0.1M sodium acetate and 0.3mM EDTA (pH was adjusted to 4.8 with acetic acid). This solution was boiled for 15 minutes and cooled quickly by placing it in ice water and stored at -20^{o}C.

Tris EDTA (TE) Buffer

1M Tris-Cl (pH 7.5) and 500mM EDTA (pH 8.0).

All solutions were made in deionised water unless otherwise stated.

2.2. Methods

2.2.1 Synthesis of Compounds

The methods for the synthesis of phosphine ligands and gold complexes were previously described (Williams *et al*., 2007). In short 15 phosphine ligands were synthesized as shown in **Figure 2.1-A** The ligands were named **TTL**2, 4, 5, 6, 9, 11, 14, 15, 18, 19, 20,

22, 23, 25 and 28 **(table 2.1)**. The phosphine ligands were complexed with ClAu(tht) in a 1: 1 ratio to produce cognate mononuclear (**Figure 2.1-B**) or in a 1:2 ratio dinuclear Au complexes (**Figure 2.1-C**). The complexes were named **TTC**2, 4, 5, 6, 9,11,14,15,18,19, 20, 22, 23, 25 and 28 (**table 2.2)**.

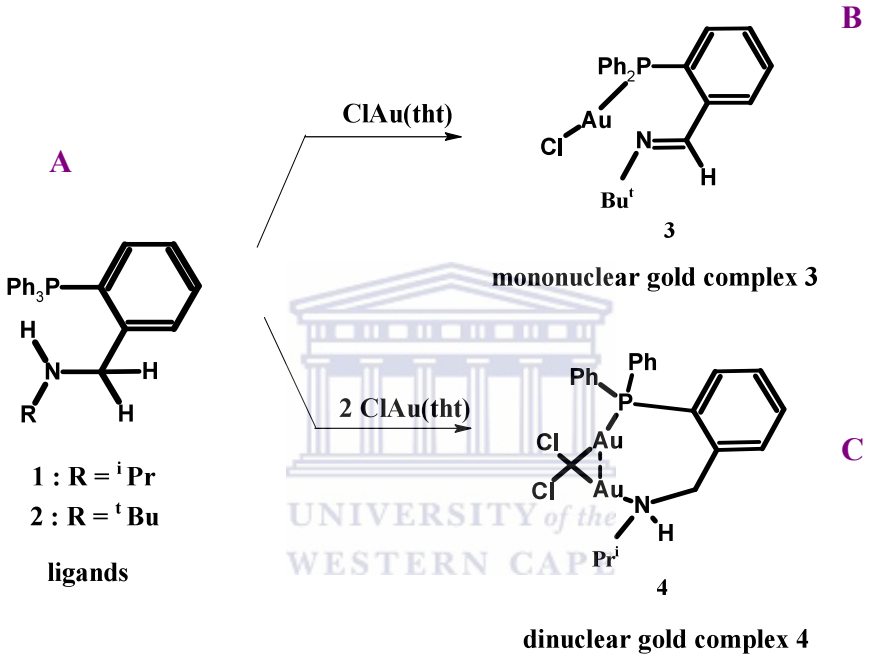


Figure 2.1 Synthesis of phosphine ligands (A), which were coded (TTL) and corresponding gold complexes (B or C), which were coded (TTC).

Table 2.1: Phosphine ligands

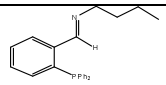
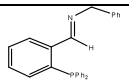
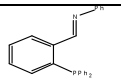
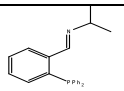
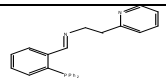
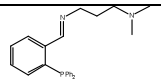
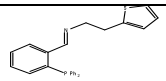
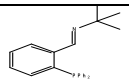
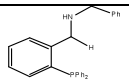
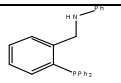
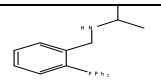
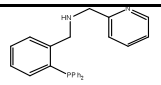
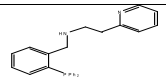
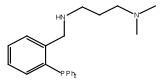
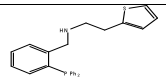
Sample Code	Structure	(M.W.)
TT L**2**		$C_{23}H_{24}NP$ Exact Mass: 345.165 Mol. Wt.: 345.417
TT L**4**		$C_{26}H_{22}NP$ Exact Mass: 379.149 Mol. Wt.: 379.433
TT L**5**		$C_{25}H_{20}NP$ Exact Mass: 365.133 Mol. Wt.: 365.407
TT L**6**		$C_{22}H_{22}NP$ Exact Mass: 331.149 Mol. Wt.: 331.391
TT L**9**		$C_{26}H_{23}N_2P$ Exact Mass: 394.160 Mol. Wt.: 394.448
TT L**11**		$C_{24}H_{27}N_2P$ Exact Mass: 374.191 Mol. Wt.: 374.458
TT L**14**		$C_{25}H_{22}NPS$ Exact Mass: 399.121 Mol. Wt.: 399.488
TT L**15**		$C_{23}H_{24}NP$ Exact Mass: 345.165 Mol. Wt.: 345.417
TT L**18**		$C_{26}H_{24}NP$ Exact Mass: 381.165 Mol. Wt.: 381.449
TT L**19**		$C_{25}H_{22}NP$ Exact Mass: 367.149 Mol. Wt.: 367.423
TT L**20**		$C_{22}H_{24}NP$ Exact Mass: 333.165 Mol. Wt.: 333.406
TT L**22**		$C_{25}H_{23}N_2P$ Exact Mass: 382.160 Mol. Wt.: 382.437
TT L**23**		$C_{26}H_{25}N_2P$ Exact Mass: 396.176 Mol. Wt.: 396.464
TT L**25**		$C_{24}H_{29}N_2P$ Exact Mass: 376.207 Mol. Wt.: 376.474
TT L**28**		$C_{25}H_{24}NPS$ Exact Mass: 401.137 Mol. Wt.: 401.504

Table 2.2: Gold complexes

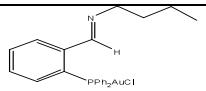
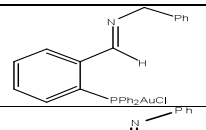
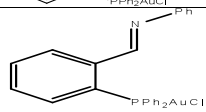
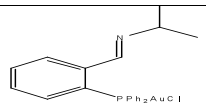
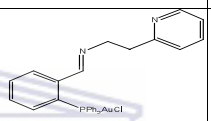
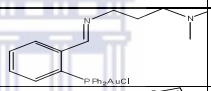
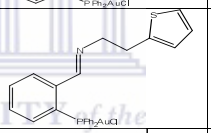
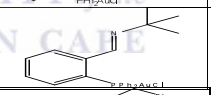
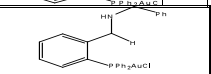
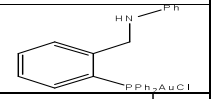
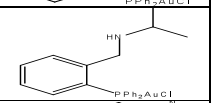
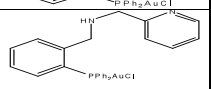
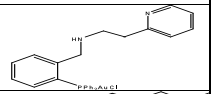
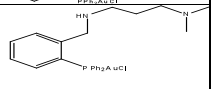
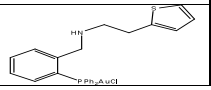
Sample code	Structure	Molecular weight and formula.
TT C**2**		$C_{23}H_{24}AuClNP$ Exact Mass: 577.100 Mol. Wt.: 577.837
TT C**4**		$C_{26}H_{22}AuClNP$ Exact Mass: 611.084 Mol. Wt.: 611.853
TT C**5**		$C_{25}H_{20}AuClNP$ Exact Mass: 597.069 Mol. Wt.: 597.826
TT C**6**		$C_{22}H_{22}AuClNP$ Exact Mass: 563.084 Mol. Wt.: 563.810
TT C**9**		$C_{26}H_{23}AuClN_2P$ Exact Mass: 626.095 Mol. Wt.: 626.868
TT C**11**		$C_{24}H_{27}AuClN_2P$ Exact Mass: 606.127 Mol. Wt.: 606.878
TT C**14**		$C_{25}H_{23}AuClNPS$ Exact Mass: 631.056 Mol. Wt.: 631.907
TT C**15**		$C_{23}H_{24}AuClNP$ Exact Mass: 577.100 Mol. Wt.: 577.837
TT C **18**		$C_{26}H_{24}AuClNP$ Exact Mass: 613.100 Mol. Wt.: 613.869
TT C **19**		$C_{25}H_{22}AuClNP$ Exact Mass: 599.084 Mol. Wt.: 599.842
TT C **20**		$C_{22}H_{24}AuClNP$ Exact Mass: 565.100 Mol. Wt.: 565.826
TT C **22**		$C_{25}H_{23}AuClN_2P$ Exact Mass: 614.095 Mol. Wt.: 614.857
TT C **23**		$C_{26}H_{25}AuClN_2P$ Exact Mass: 628.111 Mol. Wt.: 628.883
TT C **25**		$C_{24}H_{29}AuClN_2P$ Exact Mass: 608.142 Mol. Wt.: 608.894
TT C **28**		$C_{25}H_{24}AuClNPS$ Exact Mass: 633.072 Mol. Wt.: 633.923

Table 2.3: Cell lines used in this study

CELL LINE	SPECIES	MEDIA	SUPPLEMENTS
A549 (Human lung adenocarcinoma epithelial)	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
Caski (Cervical Cancer)	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
CHO (Chinese Hamster Ovary)	Chinese Hamster	F-12 Hams	Foetal Calf Serum, Penicillin/Streptomycin
HepG2 (Human hepatocellular liver carcinoma)	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
HeLa (Cervical Adenocarcinoma)	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
Hek 293-T (Human Embryonic Kidney	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
HT-29 (Colon adenocarcinoma grade II)	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
H157 (Non Small Cell Lung Carcinoma)	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
Jurkat (Leukemia Cells)	Human	RPMI	Foetal Calf Serum, 2 mM glutamine, Penicillin/Streptomycin
KMST-6 (Non Cancer Human Fibroblast)	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
MCF-7 (Breast adenocarcinoma)	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
MG-63 (Osteogenic Sarcoma)	Human	DMEM	Foetal Calf Serum, Penicillin/Streptomycin
3T3 (Embryonic Fibroblast)	Mouse	DMEM	Foetal Calf Serum, Penicillin/Streptomycin

2.2.2 Preparing metallo-compounds

Stock solutions of the metallo-compounds were prepared in DMSO and working concentrations were prepared in cell culture media. The final DMSO concentration was less than 0.1%.

2.2.3 Culturing of cells

A vial of frozen cultured cells was taken from the -150°C-freezer. The cells were thawed in a water-bath set at 37°C by submerging the vial in water. The cells were transferred into cell culture flasks containing the appropriate pre-warmed complete culture medium and incubated at 37°C in a humidified incubator containing 5% CO_2.

2.2.4 Cell count

Cell counts were performed as per the manufacturer's instructions using the Countess™ automated cell counter (Invitrogen).

2.2.5 Morphological evaluation treated and untreated cells

Different cell lines (listed in **Table 2.3)** were cultured in 6 well culture plates to 90% confluency. The cells were treated with various concentrations of the compounds ranging from 5µM to 50µM, while the negative control cells were left untreated. The cells were incubated for 24 hours at 37°C in a humidified CO_2 incubator. Following incubation, the

cells were studied using an inverted Nikon light microscope. Photographs were taken at 20X magnification using a Leica EC3 digital camera.

2.2.6 The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

Cell proliferation was determined using the MTT assay following the methods described by Mosmann (1983); Eguchi *et al.*, (1997) and Freimoser *et al.,* (1999). Adherent and suspension cell cultures were processed with minor differences.

Adherent cells were trypsinized and a concentration of 2.4×10^4 cells/ml was prepared in 50ml tubes. The cells were plated (100µL/well) in 96 well cell culture plates. The cells were cultured at 37°C in a humidified CO_2 incubator until 90% confluency was reached. The culture medium was removed and replaced with 100µL of fresh medium, which contained various concentrations of the test compounds. The concentrations of the test compounds ranged from 5 to 100µM. Triplicate wells were done for each concentration. The concentrations of positive control (cisplatin) ranged from 100 to 2000 µM. The cells were incubated at 37°C in a humidified CO_2 incubator for 20 hours. After 20 hours, 10µL of 5mg/ml MTT solution was added to each well and the plates were incubated for a further 4 hours. At the end of the 24hour incubation period, the media was removed and the plate was blotted on a paper towel. DMSO (100µL) was added to each well. The plates were placed on a rotating shaker for 10 minutes. Thereafter the optical density was read at

560nm using a LabSystems Multiscan Plus microplate reader. Cellular viability was calculated using the following formulae:

$$\% \text{ Cell Viability} = \frac{\text{sample absorbance} - \text{cell free sample blank}}{\text{mean media control absorbance}} \times 100$$

The results were expressed as a percentage of the control. Results of cellular viability were tabulated as mean absorbance of each compound expressed as a percentage of the untreated control. IC_{50} values were tabulated from the graphs as compound concentrations that reduced the absorbance at 560 nm by 50% of the untreated control wells. Triplicate experiments were conducted and the results were expressed as means ± SD.

2.2.7 Measurement of cell surface modifications

2.2.7.1 The APOP*ercentage*™ apoptosis assay

The assay was done as described by Meyer *et al.,* (2007). In brief, the cells were plated in 24 well cell culture plates at a cell density of 2.5×10^4 cells/ml and incubated for 24 hours at 37°C in a humidified CO_2 incubator. The cells were treated for 24 hours with various concentrations of the test compounds, ranging from 5 to 50µM. As a positive control, cells were also treated with 0.5mM cisplatin. Following the 24hour treatment, the medium containing the floating cells was removed and transferred to 15ml centrifuge tubes. The adherent cells were trypsinized and combined with the floating cells. The cells were recovered by centrifugation. The cells were washed twice with PBS and stained for 30 minutes with APOP*ercentage*™ dye. The cells were analyzed on a FACScan™ (Becton

Dickson) instrument equipped with a 488nm Argon laser. APOP*ercentage*™ fluorescence was measured using the FL3 channel.

2.2.7.2 The Annexin V-PE labelling assay by Flow Cytometry.

Cells were seeded at 1.5×10^6 cells/ml in 6 well cell culture plates and were incubated at 37°C in a humidified CO_2 incubator until 90% confluency. The cells were treated with various concentrations of the test compounds ranging from 15 to 50µM. After a 24hour treatment, both the floating and adherent cells were transferred to a 15ml tube. The cells were harvested by centrifugation and washed twice with PBS. The cell pellet was resuspended in 1x binding buffer at a concentration of ~$1.0x10^6$ cells/ml. The cells were incubated for 15 minutes in the dark at room temperature after 5µL Annexin V-PE of was added. 7-Amino-actinomycin D (5µL) was added to the cells before the cells were analyzed on a FACScan™ (Becton Dickson) instrument equipped with a 488 nm Argon laser. Forward scatter (FSC) and side scatter (SSC) settings were used to differentiate cells from cell debris. Cell fluorescence was measured by using both the FL3 channel (7-AAD) and FL2 channels (Annexin-V-PE). A minimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST Pro software (BD Biosciences).

2.2.8 Measurement of Mitochondrial membrane potential (ΔΨ) using TMRE.

Cells were cultured in 6 well cell culture plates at 1.0×10^6 cells/ml at 37°C in humidified CO_2 incubator. When the cells reached 90% confluency, the culture media was removed and replaced with media containing 30µM of the test compounds. The cells were incubated

at 37°C in humidified CO_2 incubator for various time points ranging from 0 to 12hours. At each time point, floating and adherent cells were transferred to a 15ml tube. The cells were recovered by centrifugation. The cells were incubated for 30 minutes in medium containing 1μM TMRE. The cells were washed with PBS and analyzed by flow cytometry using a FACScan™ (Becton Dickson) instrument equipped with a 488 nm Argon laser. Forward scatter (FSC) and side scatter (SSC) was used to differentiate population of cells and cell debris. TMRE fluorescence was measured using the FL-1 channel. A minimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST Pro software (BD Biosciences).

2.2.9 Evaluation of caspase-3 activation

Cells were cultured as indicated in **section 2.2.7.2** and were treated with 30μM of the test compounds or 0.5mM of cisplatin (positive control) and incubated at 37°C for 24 hours in a humidified CO_2 incubator. Following incubation, floating and adherent cells were transferred to a 15ml tube and washed twice with cold PBS by centrifugation. The cells were resuspended in 0.5ml Cytofix/Cytoperm™ at a concentration of ~$1.0x10^6$ cells/ml then incubated on ice for 20 minutes. The cells were centrifuged for two minutes at 2000-x *g* and washed twice with 0.5 ml Perm/Wash™ buffer at room temperature. Total number of samples was determined and the amount of Perm/Wash buffer and antibody calculated so that each sample received 100μL Perm/Wash™ buffer and 20μL antibody. Thereafter, the cells were incubated for 30 minutes at room temperature in the dark. At the end of the incubation period, the cells were washed twice in 1.0ml Perm/Wash™ buffer. The cells

were resuspended in 0.5ml Perm/Wash™ buffer and analyzed on a FACScan™ (Becton Dickson) instrument equipped with a 488 nm Argon laser as a light source. Cell fluorescence was measured by setting the Forward (FSC) and Side Scatter (SSC) to differentiate cell populations and cell debris. On a log histogram dot plot, FL1 channel (Active Caspase-3 FITC) was measured against relative cell numbers. A minimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST PRO Software (BD Biosciences).

2.2.10 Evaluation of time and dose response by detecting caspase-3 activation

To evaluate dose response, H157 cells were treated with various concentrations of TTC18 that ranged from 5μM to 50μM for 24 hours at 37°C for 24 hours in a humidified CO_2 incubator. For time response, the cells were treated with 50μM and incubated at 37°C for various time points that ranged fro 0 to 24 hours. Thereafter the caspase-3 assay described in **section 2.2.9** was followed. The cells were analyzed on a FACScan™ (Becton Dickson) instrument equipped with a 488nm Argon laser as a light source. The experiments were done in triplicate.

2.2.11 Measurement of DNA perturbations

2.2.11.1 Evaluation of DNA Fragmentation by APO-DIRECT™

Cells were cultured in 6 well cell culture plates at a cell density of $2.0x10^6$ cells per ml at 37°C for 24 hours in a humidified CO_2 incubator. The cells were allowed to reach 90% confluency before they were treated with various concentrations of the test compounds that

ranged from 30μM to 50μM. As positive control cells were treated with 0.5mM cisplatin. Upon addition of the test compounds, the cells were incubated at 37°C for 24 hours in a humidified CO_2 incubator. Following treatment, floating and adherent cells were transferred to a 15ml tube and centrifuged. The cells were fixed in 5ml 1% (W/V) paraformaldehyde in PBS and placed on ice for 15 minutes and centrifuged for 5 minutes at 300 x *g*. The supernatant was discarded and the pellet was washed twice with PBS and resuspended in 0.5ml PBS. Ice-cold 70% (v/v) ethanol (5ml) was added to the cells in order to permeabilize the cells. The cells were stored at –20°C for 48 hours prior to staining. After permeabilization, the cells were harvested by centrifuging the tube for 15 minutes at 300 x *g* and the alcohol was removed by aspiration being careful not to disturb the cell pellet. The pellet was washed twice with wash buffer. Thereafter, 50μL of staining solution (provided in the kit) was added to the tube and incubated at 37°C for 4 hours and 1.0 ml of rinse buffer was added to the tube and centrifuged for 300 x *g* for five minutes. This procedure was repeated twice, before 1ml PI/Rnase A (which was provided in kit) solution was added to the cell pellet and incubated for 30 minutes at room temperature. The cells were analyzed on a FACScan™ (Becton Dickson) instrument equipped with a 488 nm Argon laser as a light source. Forward (FSC) and Side Scatter (SSC) setting were used to differentiate cell populations and cell debris. Green fluorescence (FITC-dUTP) was measured using the FL1 channel. A minimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST PRO Software (BD Biosciences).

2.2.11.2 Cell cycle analysis using the propidium iodide assay

In this assay H157 cells were cultured in 6 well cell culture plates at $2.0x10^6$ cells per ml and were cultured to 90% confluency. After which, the cells were treated with 15µM of complexes TTC18 and TTL5 respectively for various time points that ranged from 3 to 36 hours incubated at 37°C in 5% humidified incubator. At the end of each time point, floating cells were transferred to 15ml tubes and centrifuged for 5 minutes at 10,000 x *g* and the supernatant was discarded. The cells were washed twice with cold PBS and the pellet resuspended in 200µL PBS before fixing with 2ml cold 70% ethanol drop wise to avoid cell aggregation. The cells were left at -20°C for 48 hours. After that, the samples were centrifuged at 250 x *g* for 10 min at 4°C and washed twice with cold PBS. Then 250µL PBS, PI and Rnase A mixture was added to the cell pellet, mixed gently and incubated at 37°C for 30 minutes at room temperature in the dark. Cells were analyzed on a FACScan™ (Becton Dickson) instrument equipped with a 488 nm Argon laser as a light source. Forward (FSC) and Side Scatter (SSC) to discriminate cell populations and cell debris. Two dual parameter and two single parameter displays were created. A gated standard dual parameter display was done by displaying DNA peak signal on the Y-axis and the DNA width displayed on X-axis dot plot while DNA content was displayed on linear histogram dot plot (FL2). A minimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST PRO Software (BD Biosciences).

2.2.12 Determination of ROS

To evaluate intracellular ROS, the molecular probe 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was used as described by Wei *et al*., (2000) with minor modifications. In brief H157 and Jurkat cells were cultured in 24 well cell culture plates and were treated with 30µM of the most active gold complex, TTC18 or the most active phosphine ligand TTL5. Additionally H_2O_2 at 400 µM was used as a positive control. The cells were treated for 24 hours at 37°C in 5% humidified incubator. Following treatment, floating and adherent cells were transferred to 15 ml tubes and washed twice with PBS with each wash subjected to 300 x *g* of centrifugation for 5 minutes. Cells were stained with 7.5 µM (CM-H_2DCFDA) prepared in PBS and incubated for 30 minutes at 37°C in a 5% humidified CO_2 incubator following which they were analyzed on a FACScan™ (Becton Dickson) instrument equipped with a 488 nm argon laser. Cell fluorescence (DCF) was measured using (FL-1 channel) against relative cell numbers.

2.2.13 Determination of Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) assay (used to monitor lipid peroxidation) was done as described by Wei *et al*., (2000) with minor modifications. Jurkat, H157, KMST-6 cells were cultured in 6 well culture plates at 1.0 x 10^6 cells/ml and were incubated at 37°C in a 5% humidified CO_2 incubator until a 90% confluency was reached. The cells were treated with 50µM of TTC18 or TTL5 or as a positive control 400µM of H_2O_2 was used. The cells were incubated for 24 hours at 37°C in a humidified CO_2

incubator. Subsequently, floating and adherent cells were transferred to 15ml tubes and washed twice with PBS subjecting each washing centrifugation at 10,000-x *g* for 5 minutes. The cells were resuspended in the residue PBS and transferred to 2 ml centrifuge tubes to which 2.8% of trichloroacetic acid (400µL) and 0.67% of thiobarbituric acid (600µL) were added and mixed and incubated at 95°C for 1 hour. The cells were cooled and n-butanol (300µL) was added to each tube and mixed vigorously. The cells were centrifuged at 10,000-x *g* for 10 minutes in a micro centrifuge and 100µL of the supernatant was transferred into triplicate wells of a 96 well culture plate. Absorbance of the samples were read at 532nm using a LabSystems Multiscan Plus plate reader. Results were expressed as percentage of untreated cells.

2.2.14 Investigating the cyto-protective potential of antioxidants against oxidative effects of gold complex and phosphine ligand induced cell death.

2.2.14.1 The cyto-protective potential of vitamin C

Various cell lines (Jurkat, H157 and KMST6) were seeded at 2.0 x10^4 in 24 well cell culture plates and were allowed to reach a 90% confluency in an incubator set at 37°C with 5% CO_2 humidified atmosphere. The cells were treated with various concentrations of vitamin C that ranged from 200µM to 2000µM for 24 hours. Following the treatment, the cells were evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay to access if vitamin C on its own induced apoptosis. Having established the basal apoptosis level of vitamin C a single dose of vitamin C that did not have apoptotic effects

on the cells was used to treat the cells for 24 hours before the cells were treated with 50µM of TTC18 or TTL5. Alternatively, the cells were concurrently treated with vitamin C and TTC18 or TTL5. The cells were incubated for 24 hours at 37°C 5% humidified CO_2 incubator and the cells were evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay as described.

2.2.14.2 The cytoprotective potential of PDTC and DDTC

Various cell lines (Jurkat, H157 and KMST6) were seeded at 2.0 $x10^4$ in 24 well tissue culture plates and were allowed to reach 90% confluency in 37°C incubator with 5% CO_2 humidity. The cells were treated for 24 hours with various concentrations of PDTC or DDTC that ranged from 100µM to 200µM. Following the treatment, the cells were evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay to assess the basal level at which PDTC or DDTC induced apoptosis. Having established the basal apoptosis level of PDTC or DDTC a single dose of PDTC or DDTC that did not have apoptotic effects on the cells was used to treat the cells for 24 hours. Following treatment the media containing PDTC or DDTC was removed and replaced with media that contained 50µM of TTC18 or TTL5. Alternatively, the cells were concurrently treated with 200µM PDTC or DDTC and 50µM TTC18 or TTL5. The cells were treated for 24 hours at 37°C in 5% humidified CO_2 incubator. Following treatment, the cells were evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay.

2.2.14.3 Investigating the cytoprotective potential of catalase

H157 were seeded at 2.0 x10^5 in 12 well tissue culture plates and were allowed to reach 90% confluency in an incubator set at 37°C with 5% CO_2 humidity. There after, the cells treated with various concentrations of catalase that ranged from 150µM to 200µM for 24 hours. Following the treatment, the cells were evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay to access the basal level at which catalase induces apoptosis. Having established the basal apoptosis level of catalase, the cells were treated with a single dose of 200µM catalase for 24 hours at 37°C with 5% CO_2 humidity incubator after which the media containing catalase was removed and replaced with media that contained 50µM of TTC18 or TTL5 and cells were put back in the incubator and treated for 24 hours. Following treatment, the cells were evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay as described.

2.2.14.4 The cytoprotective potential of L-glutathione (reduced)

Various cell lines (Jurkat, H157 and KMST6) were seeded at 2.0 x10^4 in 24 well tissue culture plates and were allowed to reach 90% confluency at 37°C incubator with 5% CO_2 humidity. Thereafter the cells were treated for 24 hours with various concentrations of L-glutathione that ranged from 2mM to 5mM. Following treatment, the cells were evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay to assess the basal level at which L-glutathione induced apoptosis. The media containing the antioxidant L-glutathione was removed and replaced with media that contained 50µM of TTC18 or TTL5 and the cells were treated for various time points. Alternatively the cells were

concurrently treated with 2.5mM of L-glutathione and 50µM of TTC18 or TTL5 also for various time points and cells were evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay.

2.2.15 Investigating Au uptake using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis

H157 cells were cultured at 37°C with 5% CO_2 humidity in 12 well culture plates seeded at 2.0x 10^5 cells /ml and were allowed to reach 90% confluency before they were concurrently treated with 2.5mM L-glutathione and 50µM TTC18 or TTL5 or only treated with 50µM TTC18 or TTL5 for different time points that ranged from 30 minutes to 8 hours. Cell treatment was started with the highest time point. Following treatment, floating and adherent cells were transferred to 15ml tubes and cells were washed twice with 1ml cold PBS centrifuging at 10,000 x *g* for 5 minutes for each wash. After last wash, the supernatant was completely removed and the pellet was resuspended in 300µL of ice cold Milli-Q and 500µL 70% HNO_3. Following which, the cells were digested at 70°C for 2 hours. At the expiry of the digestion period, samples were diluted 1:10 with Milli-Q water in preparation for Inductively coupled plasma mass spectrometry (ICP-MS) analysis. Gold analysis was done as described by (Rackham *et al*., 2007). In short, analysis was done using an Agilent 7700 ICP-MS with a micromist concentric nebulizer, and a double-pass spray chamber maintained at 2°C. The instrument was calibrated in the range 1– 1000 ppb using a NIST-traceable reference standard. A quality control standard

was analysed to verify the accuracy of the analysis, which was better than 98% confidence.

2.2.16 Determination of Thioredoxin

In order to determine the DNTB reduction due only to the thioredoxin reductase activity present in the sample, two assays were performed: the first measurement was of the total DNTB reduction by the sample and the second one was the DNTB reduction by the sample in the presence of the thioredoxin reductase inhibitor solution. The difference between the two results was the DTNB reduction due to thioredoxin reductase activity.

The assay was performed at room temperature (25°C) in 96 well plates in order to assay for more samples, however the results were tabulated as for 1ml samples. The assay was carried out using the Sigma assay kit. All reagents were prepared according to the manufacture's instructions with no modifications.

2.2.16.1 Preparation of cells extracts

H157 cells were cultured to 90 % confluency in 6 well tissue culture plates at 37°C in a humidified CO_2 incubator and were treated with various concentrations of TTC18 or TTL5 that ranged from 30 to 50µM for 24hours. Following treatment, adherent and floating cells were transferred to 15ml tubes and washed twice with PBS centrifuging for 5 minutes at 300-x *g* for each wash. After the last wash, supernatant was completely discarded and a 1 ml volume of CelLytic containing protease inhibitor at 1:100 was added

to the cell pellet. The cells were vigorously mixed and centrifuged for 10 minutes at 10,000-x *g* and the supernatant was used as the enzyme sample.

2.2.16.2 The thioredoxin assay

The assay procedure is as shown in **table 2.4.** A working buffer of 180µL was placed in each well of a 96 well culture plate and the other components were added according to the reaction scheme in **table 2.4.** For the total activity of the unknown sample x µL of the sample and 70-x µL of 1x assay buffer were added and for the inhibition of thioredoxin reductase reaction, x µL of the sample, 10-x µL of 1x assay buffer, and 4µL of diluted inhibitor solution were added and the solutions were mixed by gently tapping the plate on the side or gently shaking using a plate shaker. The reaction scheme for 96 well plate is shown in **table 2.4.**

Table 2.4: Thioredoxin reaction scheme for 96 well Plate

Sample	Enzyme	1x assay Buffer	Diluted inhibitor Solution	Working Buffer	DNTB
Blank	0	14µL	0	180µL	6µL
Positive control	2 µL	12µL	0	180µL	6µL
Unknown sample	x µL	14-x µL	0	180µL	6µL
Unknown sample with Inhibitor	x µL	10-x µL	4µL	180µL	6µL

The reaction was started by the addition of 6µL of the DTNB solution to each well and mixed by gently tapping the plate on the side or gently shaking using a plate shaker. The rate of formation of the yellow colour was determined by measuring the increase in absorption (ΔA_{412}/min) for each reaction using a POLARstar Omega plate reader and the amount of enzyme activity present was calculated.

The spectrophotometer was set at 412 nm using an enzymatic kinetic program as follows:

Delay=120 seconds

Interval=10 seconds

Number of readings=6

2.2.16.3 Calculations for a 96 well plate

$$\text{Unit/ml} = \frac{\Delta A_{412}/\text{min (thioredoxin reductase)} \times \text{dilution} \times \text{vol}}{\text{Enzvol}}$$

ΔA_{412}/min (thioredoxin reductase) = [ΔA_{412}/min (sample)- [ΔA_{412}/min (sample)+ inhibitor)]

dil: sample dilution factor

vol: volume of reaction in ml

enzol: volume of enzyme in ml

The calculation of the enzymatic activity was adjusted for difference in path length between 1 ml curette (1cm) and the plate, by dividing the calculated activity (unit/ml) obtained by 0.55.

Unit definition: One unit of mammalian thioredoxin reductase will cause an increase in ΔA_{412} of 1.0 per minute per ml (when measured in a non-coupled assay containing DNTB alone) at pH 7.0 at 25^{o}C.

2.2.17 RNA isolation

2.2.17.1 Isolation of RNA from cultured mammalian cells

RNA isolation and purification was done using the SV Total RNA Isolation System. When preparing RNA care was taken not to contaminate the samples with ribonucleases by working on ribonucleases-free environment and wearing clean disposable gloves that were changed regularly. All solutions were prepared according to the kit manufacture's instructions. RNA was isolated from KMST-6 cells cultured in 25 cm^2 cell culture flasks and isolation was done when the cells were 80% confluent. To isolate RNA, the media was removed and cells were washed twice with ice-cold sterile PBS and trypsinized. After which, the cells were transferred to 15ml centrifuge tubes and centrifuged at 300-x *g* for 5 minutes at room temperature. The supernatant was discarded and the pellet resuspended in residual PBS and washed twice at room temperature with ice-cold sterile PBS by centrifugation at 300-x *g* for 5 minutes each wash. After the last wash, the cells were resuspended in 500µL PBS from which a cell count of 1.0 x 6^{10} cells/ml was prepared and transferred to 2ml centrifuge tubes and centrifuged at 300-x *g* for 5 minutes. The supernatant was discarded and 175µL Lysis Buffer was added to the tubes. The cells were mixed and 35µL of RNA dilution buffer was added to the 175µL and mixed by inverting the tubes 3-4 times. The tubes were placed in a heating block set at

70ºC and heated for 3 minutes and centrifuged for 10 minutes at 12,000-x *g* at room temperature. Following centrifugation the cleared lysine solution was transferred to a new microcentrifuge tube and 200µL of 95% ethanol was added to the cleared lysine.

Collection tubes and spin columns for each sample were assembled by placing the spin columns in the collection tubes. The sample mixture to which ethanol was added was transferred to the spin columns/collection tubes assembly and centrifuged at 12,000-x *g* for 1 minute at room temperature. The solution collected in the collection tubes was discarded and the spin columns put back into the collection tubes. Then 600µL of RNA wash solution was added to the spin columns and again centrifuged for 1 minute at 12,000-x *g*. There after, 50µL of freshly prepared DNase incubation mixture 40 µL Core Buffer, 50µL 0.09M $MnCl_2$ and 5 µL DNase 1 enzyme prepared in this order and mixed by pipetting) was added to each of the membrane spin column tubes and incubated for 15 minutes at 20-25ºC. After incubation, 200µL DNase stop solution was added to the spin columns and centrifuged for 1 minute at 12,000-x *g* at room temperature. After which 600µL RNA wash solution was added to the spin columns and centrifuged for 1 minute at 12,000-x *g*. The collection tubes were emptied and 250µL RNA wash solution was added to the spin columns and centrifuged for 2 minutes at high speed. The spin columns were transferred into new collection tubes. RNA was eluted by addition of 100µL nuclease-free water to the spin column membranes making sure to completely cover the surface of the membranes and then centrifuged for 1 minute at 12,000-x *g*. The spin columns were discarded and the eluted purified RNA was quantified using a

Namedrop. The samples were aliquot into 20µL aliquots, and the concentration was indicated on the tubes and stored at -80ºC.

2.2.17.2 RNA gel electrophoresis

To assess the total RNA quality, the RNA was size fractionated by electrophoresis. RNA was electrophoresed on 1% agarose gel prepared in 1xTAE buffer then 0.5µg/ml ethidium bromide was added. The samples were prepared by adding 5 µL RNA, 1 µL of 6x DNA loading dye, 6µL formamide (for denaturation of RNA) and heated at 65ºC for 5 minutes. RNA high-range (0.2-6 kb) molecular weight marker to estimate the size of RNA fragments were loaded onto a pre-cast gel alongside the samples and electrophoresed at 70 V for 30 minutes. RNA was visualized with short wave UV light on a transilluminator and photographed using the Sony UVP image Store 5000 photographic system.

2.2.18 Preparation of cDNA using ImProm-II™ Reverse Transcriptase system.

For first strand cDNA synthesis, the ImProm-II™ Reverse Transcriptase system kit for reverse transcription (Invitrogen) was used. A tube containing the RNA of interest with known RNA concentration was removed from the -80ºC freezer and placed on ice to thaw. A required volume according to concentration was removed from the tube and any unused portion of the sample was returned to the freezer. The RNA volume was determined with consideration to the final volume of the reaction mixture. The reaction mixture for

annealing the oligo dT primer to the target RNA consisted of the components listed in **table 2.5**

Table 2.5: Reaction mixture for annealing the dT oligo

Reaction Component	**Volume**
RNA up to 1µg/reaction	XµL
Primer - oligo (dT), (0.5µg/reaction)	XµL
Nuclease-Free Water	XµL
Final Volume	**5.0µL**

The tube was closed tightly and placed into a polymerase chain reaction (PCR) machine and was heated to 70ºC for 5 minutes and immediately chilled on ice-water for at least 5 minutes. After which, the tube was short spun for 10 seconds in a microcentrifuge to collect any condensed parts and maintain the original volume. The tube was kept closed tight on ice until the reverse transcription reaction was added.

Reverse transcription reaction components were added to the tube while on ice and the reaction components are shown in **table 2.6.**

Table 2.6: Reaction mixture for the first strand cDNA synthesis reaction

Reaction Component	Volume
ImpProm-ll^{TM} 5x reaction buffer	XµL
$MaCl_2$ (final concentration 1.5-8.0mM)	4.0µL
dNTP Mix (final concentration 0.5mM each dNTP)	1.0µL
Recombinant Ribonuclease Inhibitor	20u
ImProm-ll^{TM} Reverse Transcriptase	1.0µL
Nuclease-Free Water to 15µL final volume	XµL
Final Volume	**15.0µL**

The 5µL of the target RNA was transferred to the 15µL of the reverse transcription reaction mixture on ice to have a final volume of 20µL. The tube was placed in a PCR machine and incubated for 1 hour at 42ºC followed by incubation at 70ºC for 15 minutes to inactivate reverse transcriptase activity, and cooled to 4ºC for 5 minutes. The synthesised cDNA was used for amplification using the standard PCR cycling protocol. The resulting cDNA was quantified using a Nanodrop and the resultant first strand cDNA preparation was aliquoted into 5µL aliquots and kept at -20ºC. To assess the successfulness of the reverse transcription procedure, 1µL of the cDNA product was PCR amplified using specific primers (Reaction mixture shown in **table 2.7**) and to view the results of the PCR reaction the samples were run on a 1% agarose gel.

Table 2.7: Reaction mixtures for PCR amplification

Reaction component	**1x**
Pfu DNA polymerase 10x buffer with $MgSO_4$	2.5µL
dNTPs mix 10mM each	1.0µL
FP (1µM)	1.5µL
RP (1µM)	1.5µL
Pfu polymerase Taq (1.25 u)	0.25µL
Nuclease free water	14.25µL
Template (1µg)	1.0µL
$MgCl_2$	3.0µL
Total reaction volume	**25µL**

2.2.19 Colony PCR

The compositions of the colony PCRs were the same as described for the amplification of *Trx-2* in **table 2.7**, except that the amplifications were performed with Dream *Taq* polymerase (Fermentas). The PCR conditions are tabled in **table 2.8**.

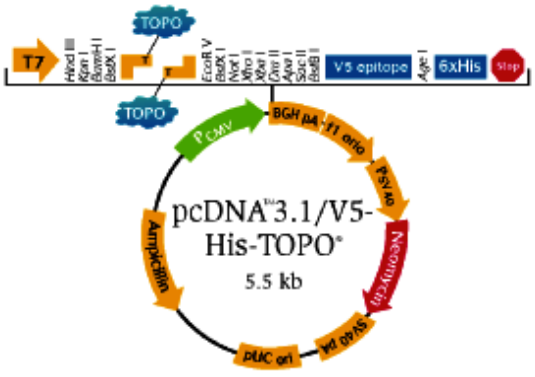
Table 2.8: PCR reaction conditions

Condition	Temperature (°C)	Time	
Denaturation	95	2 minutes	
Denaturation	92	30 seconds	*35 cycles*
Annealing	61	30 seconds	
Amplification	72	30 seconds	
Extension	72	10 minutes	
Hold	4		

2.2.20 The pcDNA™ 3.1 D/V5-His-TOPO cloning vector

The pcDNA™ 3.1 D/V5-His-TOPO cloning vector (**Figure 2.2)** 5.5kb vector designed for high-level stable and transient expression in mammalian hosts. Accordingly, high-level stable and non-replicative transient expression can be carried out in most mammalian cells. Stably transfected cells can be generated by selecting the cells in the Neomycin. The vector contain human cytomegalovirus immediate-early (CMV) promoter for high level expression and a topoisomerase-I activated TOPO TA box which allow for the directional insertion of PCR products by ligation.

Figure 2.2: pcDNA™ 3.1 D/V5-His-TOPO cloning vector mammalian expression vector.



2.2.21 Genotype of Bacterial strains

One Shot® TOP10 Chemically competent cells: Genotype of TOP10 Cells. F⁻ mcr A Δ (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ*lac*X74*rec*A1 *ara*D139 Δ (*ara-leu*) 7697 *gal*U *gal*K rpsL (Str^R) *end* A1 *nup*G López *et al*., (2009), was used in this experiment.

2.2.22 Selection of transformed bacteria

For experiments with *E. coli* containing ampicillin resistant plasmids, transformed cells were plated on Nutrient-agar with ampicillin at 100 µg/ml. Selection was maintained during growth in liquid culture by the inclusion of ampicillin at 100 µg/ml.

2.2.23 Storage of bacterial strains and clones

Overnight cultures were diluted by the addition of an equal volume of 80% sterile glycerol. Stocks were stored at -80ºC.

2.2.24 TOPO® cloning procedure

2.2.24.1 Design of PCR primers for the amplification of *Trx-2*

PCR primers were designed in such away that 4 base pair sequences (CACC) that are necessary for directional cloning on the 5' end of the forward primer were included and were fused in frame for optimal expression. The designed PCR primers were: FORWARD PRIMER (>>>): 5'-**CACCACC**ATGGCTCAGCGACTTCTTC-3' and REVERSE PRIMER (<<<): 5'-TCAGCCAATCAGCTTCTTCA-3'. The underlined sequence will allow for the directional cloning of the PCR product. The sequence in red corresponds to the human Trx-2 sequence (NM_012473.3).

2.2.24.2 PCR amplification of Trx-2

A thermostable proofreading DNA polymerase (Pfu *Taq* Polymerase) and the designed PCR primers were used to produce the PCR product.

2.2.24.3 TOPO® Ligation reaction

The reaction was carried out at room temperature (~25°C). The reaction mixture is described in **table 2.9**.

Table 2.9: TOPO® Ligation reaction

Reagent	**Volumes**
Fresh PCR product	3µL
Salt Solution	1µL
Sterile Water	1µL
TOPO® Vector	1µL
Final volume	**6µL**

The reaction was gently mixed and incubated for 5 minutes at room temperature and then placed on ice.

2.2.24.3 Transformation of *E.coli* cells

For DNA transformations One Shot® TOP10 Chemically competent cells were thawed on ice and an aliquot (2µL) of the cells were added to the 6uL TOPO ligation reaction. This reaction was gently mixed then incubated for 20 minutes on ice. The cells were heat shocked for 30 seconds at 42ºC without shaking and immediately transferred the tube on ice. Following which, SOC medium (250µL) was added. The tube was placed in a shaking incubator (200rpm, at 37ºC) for 1 hour. An aliquot (50uL) of the culture was plated on a

nutrient agar plate containing 100µg/ml ampicillin and incubated at 37ºC overnight. Plates were checked for growth and if present, the clones were individually picked for analysis for the presence of positive clones.

2.2.24.4 Analyzing for transformed colonies by PCR

Colonies from the ligation plates were picked and each dissolved in 10µL of sterile distilled H_2O. The colony mixture was used as a template for PCR reaction. PCR reactions were carried out as described in **section 2.2.19.1** for standard PCR using specific oligonucleotides at a concentration of 10pmole. A negative control, substituting 1µL of deionised water for the bacterial suspension was also performed. The products of the PCR were analysed by agarose gel electrophoresis. Glycerol stocks and large scale plasmid preparations were prepared for positive clones.

2.2.25 Agarose gel electrophoresis of DNA

DNA was size fractionated by agarose gel electrophoresis on 1% gel containing 0.5µg/ml ethidium bromide and electrophoresed in 1 x TBE buffer. The DNA sample was mixed with glycerol Bromophenol Blue (GBPB) before loading onto the gel. DNA size markers (Fermentas LabAid™ GeneRuler™ DNA ladders-10bp-48502) were loaded alongside the samples to estimate the size of DNA fragments. After electrophoresis the DNA was visualized with short wave UV light on a transilluminator and photographed using the Sony UVP Image Store 5000 photographic system. When DNA was to be recovered from the gel, a hand held long wavelength lamp was used to avoid damage to the DNA and a sterile

blade used to excise the desired band from the gel. DNA was purified from gels as described in **section 2.2.27**.

2.2.26 Purification of DNA fragments from agarose gels

PCR fragments from agarose gels were purified with Wizard SV gel and PCR clean up system (Promega). The excised agarose embedded fragment was weighed. Capture buffer (100µL) was added for every 10mg of agarose. The agarose was melted in the buffer by placing the tube in a heating block set at 60ºC. After incubation the sample was transferred to a GFX column within a collection tube and centrifuged for 30 seconds at 13,000-x *g*. The flow through was discarded and the column placed back in the collection tube. Wash buffer (500µL) was added to the column and the tube was centrifuged for 1 minute at 13,000-x *g*. The collection tube was removed and placed in a 1.5ml centrifuge tube. Elution buffer (20µL) was placed directly on the matrix of the column and incubated at room temperature for 1 minute. The tube was centrifuged for 1 minute at 13, 000-x *g* to collect the DNA. The DNA was aliquoted and stored at -20ºC.

2.2.27 Preparation of Plasmid DNA

Glycerol stocks of the appropriate plasmids were thawed on ice. LB (50ml) containing the appropriate antibiotic was inoculated with the glycerol stock. The culture was incubated (with shaking) overnight at 37°C. The culture was centrifuged at 6,000x *g* at 4°C in a Beckman centrifuge. The plasmid DNA was isolated as per the manufacturer's

instructions using the Wizard® plus DNA purification system. Plasmid DNA was stored at -20°C.

2.2.28 Sequencing of cloned DNA product

Clones to be sequenced were plated on Nutrient agar containing the appropriate antibiotic and submitted to Inqaba Biotech core sequencing facility for sequencing.

2.2.29 DNA transfection of cultured cells

Transient and stable cells were propagated with the aid of Mectafectene® Pro. Cells were seeded in a 6-well cell culture plate at $2.0x10^5$ cells/ml and incubated at 37ºC in a humidified CO_2 incubator until growing area was 90% covered. The complete medium was removed from the wells and cells were washed 2x with serum free medium. After that 1 ml of serum free medium was added to the cells. Stock solution and the genetic material used for the transfection were removed from the fridge and kept at room temperature to warm. Before use the stock solutions were gently mixed. The following solutions were prepared in two separate wells of a 96 well tissue culture plate.

Solution A: 0.5-1.5 µg of DNA in a 50µL serum-and antibiotic-free cell culture medium.

Solution B 1.0-7.0 µL of Metafectene® in 50µL serum-and antibiotic-free cell culture medium.

The DNA lipid ratio was kept at 1:2 (µg DNA: µL Metafectene®). The solutions were mixed gently by pipetting once. Then the DNA solution was added to the transfection reagent i.e. Solution A was added to Solution B and not the other way round. The combined mixture was pipetted once gently up and down and incubated at Room temperature for 20 minutes. The DNA/Lipid complex was added drop wise to the cells and swirled with care. The plates were incubated at 37ºC in a 5% humidified CO_2 incubator for 2 hours and then 1ml of complete medium was added to the cells and incubated for 24 hours before start of selections. G418 was used for selections at 800 µg/ml for 3 to 4 weeks until resistant colonies were visible, after which cells were maintained in 500 µg/ml of the antibiotic.

2.2.30 Extraction of proteins from cell lines

Transfected and un-transfected KMST-6 cells were grown to 90% confluency in T25 cell culture flasks. Cells were washed twice with 1x PBS following which 500µL of CytoBuster was added to the flask and cells were incubated for 5 minutes at room temperature. Cells were scrapped with a cell scrapper and transferred to a microcentrifuge tube and centrifuged for 5 minutes at 16,000xg at 4ºC. The supernatant was transferred to a new microcentrifuge tube and stored at -20ºC.

2.2.31 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

2 .2.31.1 Preparation of samples

Protein concentrations of samples were determined using the Bradford assay. Protein samples were loaded onto gels at 1 µg/µL following heating at 95ºC for 10 minutes.

2.2.31.2 Preparation of gels

Proteins were separated by denaturing SDS-PAGE under reducing conditions according to the method of Laemmli (1970). Briefly, gels were made from 40% stock of premixed 37.5:1 acrylamide: bisacrylamide and consisted of separating and stacking gels which were poured between two assembled glass plates separated by a 1.5mm thick comb spacer. The gels were prepared in 1.5 mm Hoeffer dual gel casters and about 10 ml were enough for one gel. The separating gel was poured to about 1 cm below the wells of the comb and a bout 1ml water-saturated 1-butanol was overlayed on top of the separating gel and was let to set. Once set, the butanol was poured off and gel rinsed with deionised water. Stacking gel (~5 ml) was poured over the running gel and to make wells the comb was inserted into the stacking gel immediately and was let to set. When the stacking gel was set, the comb was removed and the gel was transferred to the Mighty Small apparatus (Hoeffer) containing running buffer.

2.2.31.3 Loading and electrophoresis of samples

Two equal volumes of the samples were loaded into two different gels and electrophoresis at 100V/cm until the loading dye barely leaked out of the gel into the running buffer. One of the gels was blotted onto a PVDF membrane while the other was stained with Coomassie stain.

2.2.31.4 Staining and Destaining of SDS-PAGE gels

The Coomassie blue stain gel was stained with coomassie solution for 30 minutes. Destaining was achieved by multiple washes with SDS-PAGE destaining solution while shaking.

2.2.32 The Western blot assay

A Bio-Rad MiniProtein Trans Blot system was used to transfer proteins separated by SDS-PAGE onto PVDF membrane. Gels, sponges, Whatman paper were equilibrated in pre-chilled transfer buffer for 30 minutes. PVDF membrane was immersed in 100% methanol for 15 to 20 seconds and was left immersed in pre-chilled transfer buffer for about 30 minutes to equilibrate before blotting took place. To assemble the transfer apparatus, the sponge was placed on the black side of the transfer cassette, followed by the Whitman paper, the gel, the PVDF membrane, Whatman paper and lastly the sponge once more. Then bubbles were squeezed out by simply rolling a tube over the sponge. The cassette was closed and placed in the Bio-Rad MiniProtein tank making sure that the black side is also on the same black side of the tank. Pre-chilled transfer buffer was added to cover the tank

and protein was electro-blotted onto the PVDF membrane at 100V constant for 1 hour 20 minutes. Following blotting, the PVDF membrane was blocked with 5% BSA in TBST at room temperature for 20 minutes. The PVDF membrane was immediately incubated in 1:500 dilution of the primary antibody prepared in TBST to which 0.25M NaCl was added and incubated overnight at 4ºC while shaking. The membrane was rinsed at room temperature three times with 10 ml TBST, each rinse lasting 5 minutes while shaking. Thereafter the membrane was incubated in secondary antibody diluted at 1:2,000 in TBST and incubated at room temperature for 45 minutes while shaking. Finally the membrane was rinsed twice with TBST for 5 minutes each rinse and once with TBS without tween-20 also for 5 minutes while shaking. The membrane was placed on Saran Wrap making sure that the side with the blotted protein is upright. Super Signal West Pico Chemiluminescent substrate was prepared by mixing 1ml of solution A and 1 ml of solution B as per manufacture's instructions (Thermo Scientific) and the substrate mixture was pipetted over the membrane making sure that the substrate covered the entire membrane. The substrate was left on the membrane for 5 to 10 minutes, and then the membrane was placed between two clear plastic sheets placed inside an x-ray cassette. CL-XPosure film (Thermo Scientific) was placed over the membrane and exposed for 2 to 5 minutes. The film was removed and developed using the automated Curix 60 AGFA-Gevaert N.V (F-Nr.1419) film processing system.

2.2.32.1 Blot Stripping

The blot was stripped for another primary antibody. The stripping was performed by pre-wetting the membrane in methanol for a few seconds, 5 minutes wash in deionised water, 5 minutes incubation in 0.2M NaOH, another 5 minutes wash in deionised water and finally 5 minutes wash in TBST. The membrane was then blocked with blocking buffer as described in **section 2.2.33**. Following stripping and blocking, the membrane was stained with another antibody (actin antibody).

CHAPTER THREE OUTLINE

3.1 Introduction

3.2 Evaluating the effects of TTL and TTC on cell morphological

3.3 Measurement of cellular viability using the MTT assay

3.4 Evaluating the activation of apoptosis using the APOP*ercentage*™ assay

3.5 Evaluating the cleavage of caspase-3

3.6 Evaluating dose and time dependent activation of caspase-3

3.7 Evaluation of DNA Fragmentation using the APO-DIRECT™ assay

3.8 Investigating the effects of TTC18 and TTL5 on cell cycle

3.9 Evaluating mitochondrial depolarisation using the TMRE assay

3.10 Summary

CHAPTER THREE: Investigating the toxicity of phosphine ligands and gold(I) complexes.

3.1 Introduction

When a cell has undergone apoptosis, it is systematically disposed of without causing damage or stress to neighbouring cells (as reviewed in chapter 1, **section 1.5.9.1**). The dying cell has characteristic morphological changes, which include cell shrinkage, disappearance of microvilli on plasma membrane, the chromosomal DNA is fragmented into nucleosome size units of about 200 bp (as reviewed in chapter 1, **section 1.5.9.1**), as well as the nucleus and the cell itself is fragmented. It is possible to identify apoptotic cells using *in vitro* assays. Since the apoptotic cell undergoes cell condensation and therefore reduction in cell volume, the morphological changes can be observed by light microscopy, and its condensed nuclei can be stained with fluorescent dyes such as Hoechst or DAPI (Whiteside *et al*., 1998; Boya *et al*., 2005). The apoptotic cell also exposes phosphatidyl-serine (PS) on the cell surface (reviewed in chapter 1, section **1**.**1.5.9.2**). PS externalisation can be observed with dyes such as APOP*ercentage*™ or fluorescent-labelled Annexin V.

Mitochondria also play a key role in apoptosis (reviewed in chapter 1, section **1.5.9.1**) and Lum and Nagley, 2003). Dissipation of the mitochondrial membrane potential (⊗¬m) is a key event in the intrinsic apoptosis pathway. Mitochondrial membrane potential can be measured by employing fluorescent dyes such as rhodamine 123 (R123), and tetramethylrhodamine ethyl ester (TMRE), which are

fluorescent probes that can be used to monitor the membrane potential of mitochondria (Scaduto Jr. and Grotyohann, 1999). The opening of pores in the mitochondria results in the release of signalling proteins, which includes cytochrome *c* (Reviewed in chapter 1, section **1.5.9.3.1**). Cytochrome *c* is found in mitochondrial intermembrane space and upon its release to the cytosol through the outer membrane forms the apoptosome (a complex with Apaf-1, pro-caspase-9 and ATP or dATP). The apoptosome triggers activation of downstream post mitochondrial caspases like caspase-3 and caspase-7 (Lum and Nagley, 2003; Patrushev *et al*., 2004). This activation of downstream caspases by proteolytic cleavage consequently results in the biochemical and morphological changes that are characteristic of apoptosis, consequently resulting in DNA fragmentation (Lum and Nagley, 2003). The fragmented DNA can be detected by assays such as Terminal deoxynucleotide transferase dUTP Nick End Labelling (TUNEL) or by electrophoresis of the isolated DNA on agarose gel and changes in DNA content of the cell can also be measured by cell cycle analysis (Frédérich *et al*., 2003). The neutral red assay or the MTT assay can be used to assess cell viability and the determination of IC_{50}. It is imperative that in order to assess apoptosis several assays be employed in order to affirm the results.

The aims of this chapter are:

- To screen 15 bidentate amino and iminophosphine ligands for apoptosis induction in a panel of cell lines shown in chapter 2 (**table 2.3**).

- To screen 15 mono and dinuclear Au(I) gold complexes for apoptosis induction in a panel of cell lines shown in chapter 2 (**table 2.3**).

3.2 Evaluating the effects of TTL and TTC on cell morphological

Darzynkiewicz (1997) indicated the importance of confirming the mode of cell death by the use of light and electron microscopy. To assess morphological changes caused due to apoptosis induction, various cell lines shown in **table 2.3, morphological features of cells treated with TTL and TTC were evaluated according to method described in section 2.2.5** Morphological changes that can be associated with cell death and potentially apoptosis could be observed for some of the phosphine ligands and the complexes (**tables 3.1** and **3.2**). The morphological changes that were observed bioactive compounds included cell shrinkage, inhibition of cell growth and cell detachment. Some of the compounds failed to induce any morphological changes in the cells. Jurkat cells were highly sensitive to the effects of both TTL and TTC compounds. Thirteen cells lines were screened in this study and 50μM of TTC18 induced morphological changes in all of them (table 3.2). TTL5 was the most toxic phosphine ligand, inducing morphological changes in eight of the cell lines tested in this study (table 3.1).

3.3 Measurement of cellular viability using the MTT assay

The tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was developed by Mosmann in 1983 to measure cellular growth and survival by detecting living cells. In this assay, metabolically active and therefore live cells reduce MTT to yield non-water-soluble violet formazan crystals.

Metabolically inactive and therefore dead cells fail to reduce MTT. The MTT assay was used to determine the IC_{50} value for both TTL (**table 3.3**) and TTC (**table 3.4**) samples on a panel of 13 cell lines. The procedure is described in chapter 2, section **2.2.6**. A comparison between the phosphine ligands (TTL) and gold complexes (TTC) shows that the IC_{50} values for the most of the phosphine ligands is above 100µM, while the IC_{50} values for most of the gold complexes were below 50µM. The IC_{50} values for the positive control, cisplatin was above 100µM for all the cell lines used in this study.

Table 3.1: Summary of morphological changes observed in cultured cells in response to treatment with phosphide ligands (TTL). The cells were treated for 24hours with 50µM of the compounds and then studied under the light microscope.

Cell line	Phosphine Ligands														
	2	4	5	6	9	11	14	15	18	19	20	22	23	25	28
A549J	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
Caski	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CHO	*	*	•	*	*	*	*	*	•	*	*	*	*	*	*
HeLa	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
HepG2	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
HT29	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
H157	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Jurkat	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
KMST6	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
MCF7	*	*	*	*	*	•	*	*	*	*	*	*	*	*	*
MG-63	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
Hek293T	*	*	•	*	•	•	*	*	*	*	*	*	*	*	*
3T3	*	*	*	*	*	*	•	*	*	*	*	*	*	*	*

* No discernible morphological changes observed.

• Morphological changes (cell shrinkage and cell detachment) indicative of apoptosis were observed.

Table 3.2: Summary of morphological changes observed in cultured cells in response to treatment with phosphide ligands (TTC). The cells were treated for 24hours with 50μM of the compounds and then studied under the light microscope.

Cell line	Gold Complex														
	2	4	5	6	9	11	14	15	18	19	20	22	23	25	28
A549J	*	*	*	*	*	*	*	*	●	*	*	*	*	●	*
Caski	*	●	*	*	*	*	*	*	●	*	●	*	*	*	*
CHO	●	●	●	●	●	●	●	●	●	●	●	*	●	*	●
HeLa	*	●	●	*	*	●	*	*	●	●	●	●	*	●	*
HepG2	*	*	*	*	*	*	*	*	●	*	*	●	*	*	*
HT29	*	●	*	*	*	*	*	*	●	*	●	*	*	●	*
H157	●	●	*	●	*	*	*	*	●	●	●	●	*	*	*
Jurkat	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
KMST6	*	*	*	*	*	*	*	*	●	*	*	*	*	*	*
MCF7	●	●	*	*	*	*	*	*	●	*	*	●	*	●	*
MG-63	●	●	*	*	*	*	*	*	●	*	*	●	*	●	*
Hek293T	●	●	*	*	*	*	*	*	●	●	●	●	*	●	*
3T3	●	●	*	*	*	*	*	*	●	●	●	●	*	*	*

* No discernible morphological changes observed.

● Morphological changes (cell shrinkage and cell detachment) indicative of apoptosis were observed.

Table 3.3: Cytotoxicity of phosphine ligands (TTL). A panel of cells were treated for 24hrs with 50µM of the compounds and cell viability was assessed using the MTT assay. Treatments that caused apoptosis in more than 50% of the cells are in red.

Phosphine Ligand	A549J	Caski	CHO	HepG2	HeLa	HT29	H157	Jurkat	KMST6	MCF7	MG-63	Hek	3T3
TTL2	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
TTL4	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	90±0.14	>100
TTL5	>100	>100	>100	>100	35±0.16	>100	28±0.01	<10	75±0.6	>100	50±0.3	<10	>100
TTL6	>100	>100	>100	>100	>100	>100	>100	100	100	>100	>100	20±0.8	>100
TTL9	>100	>100	>100	>100	>100	>100	>100	25±0.05	100	>100	>100	90±0.58	>100
TTL11	>100	>100	>100	>100	>100	>100	>100	80±0.00	>100	>100	>100	50±0.48	50±0.3
TTL14	>100	>100	>100	98±0.02	>100	>100	>100	80±0.01	>100	>100	>100	20±0.34	20±26
TTL15	>100	>100	>100	>100	>100	>100	>100	>100	100	>100	>100	20±0.45	20±0.76
TTL18	>100	>100	>100	95±0.04	95±0.56	>100	>100	80±0.01	>100	>100	>100	10±0.28	>100
TTL19	35±00	55±06	35±06	>100	>100	48±0±06	>100	10±0.00	35±0.00	35±0.06	>100	30±0.00	>100
TTL20	>100	>100	>100	100	>100	>100	>100	>100	70±0.00	50±0.3	>100	50±0.00	>100
TTL22	>100	>100	>100	50±0.5	100	>100	>100	>100	>100	>100	>100	100	>100
TTL23	>100	>100	85±0.1	>100	100	>100	>100	>100	95±0.5	100	15±0.05	10±0.36	18±0.00
TTL25	>100	>100	>100	>100	98±0.04	>100	>100	20±0.2	>100	>100	>100	>100	>100
TTL28	>100	>100	80±0.1	>100	95±0.02	>100	100	35±0.13	95±0.4	100	100	>100	>100

Table 3.4: Cytotoxicity of gold complexes (TTC). A panel of cells were treated for 24hrs with 50µM of the compounds and cell viability was assessed using the MTT assay. Treatments that caused apoptosis in more than 50% of the cells are in red.

Gold Complex	A549J	Caski	CHO	HepG2	HeLa	HT29	H157	Jurkat	KMST6	MCF7	MG-63	Hek	3T3
TTC2	>100	90±0.01	>100	>100	>100	90±0.00	60±0.05	>100	>100	50±0.7	>100	25±0.00	50±0.28
TTC4	50±0.01	80±0.2	30±0.02	30±0.02	90±0.04	15±0.00	30±0.9	20±0.00	38±0.00	20±0.1	25±0.1	<10	45±0.13
TTC5	>100	>100	37±0.01	>100	85±0.00	60±0.00	98±0.05	18±0.00	>100	48±0.05	<10	50±0.00	15±0.4
TTC6	60±0.04	>100	35±0.01	20±0.00	15±0.00	80±0.06	28±0.03	50±0.00	90±0.05	20±0.05	95±0.05	18±0.00	80±0.05
TTC9	>100	>100	85±0.05	>100	15±0.00	>100	>100	80±0.5	>100	>100	100	60±0.02	>100
TTC11	60.0.01	>100	25±0.00	20±0.00	100	10±0.00	25±0.09	<10	48±0.01	15±0.05	27±0.05	<10	15±0.2
TTC14	100	>100	65±0.04	48±0.02	12±0.00	<10	98±0.03	25±0.01	>100	98±0.06	20±0.06	<10	<10
TTC15	>100	98±0.02	74±0.03	48±0.01	34±0.07	35±0.01	96±0.14	25±0.00	>100	30±0.08	40±0.08	26±0.00	25±0.6
TTC18	63+0.0	50±0.01	18±0.00	10±0.01	35±0.04	20±0.02	28±0.01	<10	37±0.00	10±0.06	12±0.06	<10	<10
TTC19	20+0.00	75±0.22	60±0.00	10±0.01	<10	10±0.00	45±0.22	45±0.01	>100	10±0.00	20±0.00	10±0.3	<10
TTC20	>100	>100	70±0.01	48±0.01	100	50±0.00	>100	<100	>100	48±0.06	25±0.06	10±0.56	18±0.00
TTC22	>100	80±0.02	>100	100	35±0.029	90±0.02	50±0.02	20±0.3	>100	90±0.1	30±0.1	50±0.08	50±0.3
TTC23	30±0.01	68±0.00	25±0.01	10±0.00	100	12±0.01	28±0.00	50±0.01	30±0.00	10±0.07	80±0.07	<10	<10
TTC25	>100	70±0.01	90±0.01	25.0.00	12±0.06	50±0.01	30±0.01	<10	>100	>100	<10	10±0.02	018±0.1
TTC28	30±0.00	85±0.01	27±0.00	<10	22±0.00	45±0.05	25±0.01	28±0.02	30±0.00	<10	<10	28±0.02	45±0.32
Cisplatin	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

3.4 Evaluating the induction n of apoptosis using the APOP*ercentage*™ assay

The APOP*ercentage*™ apoptosis assay was used to assess the induction of apoptosis in cultured cells. As previously described the principle of the assay is based on a dye that the dye enters the cells upon phosphatidylserine transmembrane movement ('flip-flop' mechanism).

A panel of cell lines were cultured to 90% confluence in 24 well cell culture plates. Thereafter, the cells were either left untreated or treated with a single dose of 50μM of the phosphine ligands (TTL) or gold complexes (TTC) for 24 hours. Following treatment, the cells were stained with APOP*ercentage*™ apoptosis dye as described in section **2.2.7.1** and analysed by flow cytometry. Results for TTL and TTC compounds are tabulated in **table 3.5** and **table 3.6**, respectively. The data is also summarised in **table 3.7** and **table 3.8**, showing which compounds induced more than 50% cell death. The induction of apoptosis under these conditions was selective. In general, the TTC compounds were more bioactive than the TTL compounds. TTL5 demonstrated higher pro-apoptotic activity than the other phosphine ligands, inducing more than 50% apoptosis in five cell lines (A549J, Caski, CHO, HepG2, Jurkat and KMST6). The most bioactive gold complexes were TTC2, TTC4, TTC18, TTC20, TTC22 and TTC25, with TTC18 demonstrating the highest pro-apoptotic activity. TTC18 induced more than 50% apoptosis in all thirteen cell lines used in this study. Jurkat cells were highly sensitive to the effects of both TTL and TTC compounds.

Table 3.5: Apoptotic activity of phosphine ligands (TTL). A panel of cells were treated for 24hrs with 50µM of the compounds and apoptosis was assessed using the APOP*ercentage*™ assay. Treatments that caused apoptosis in more than 50% of the cells are in red.

Phosphine Ligand	A549J	Caski	CHO	HepG2	HeLa	HT29	H157	Jurkat	KMST6	MCF7	MG-63	Hek	3T3
TTL2	44±1	44±2	12±3	24±2	43±2	22±1	48±3	68±8	22±2	42±1	22±3	44±3	43±3
TTL4	32±1	45±1	13±2	32±4	27±2	24±2	45±1	99±0	30±4	48±2	15±1	38±4	15±4
TTL5	57±3	36±4	88±1	50±1	30±2	27±4	48±4	98±0	50±5	10±2	20±2	22±2	22±2
TTL6	20±1	44±3	18±1	25±3	22±3	18±3	22±2	98±1	23±2	12±3	28±2	48±3	35±3
TTL9	17±2	40±5	28±1	36±2	40±3	29±1	37±2	98±1	21±1	22±2	11±3	66±1	28±2
TTL11	41±3	32±2	16±2	28±1	38±2	28±3	44±2	98±1	20±3	63±6	19±1	50±2	32±1
TTL14	22±1	38±2	18±1	20±4	49±4	33±4	32±1	98±1	19±1	10±3	11±1	44±3	27±2
TTL15	27±3	46±4	13±1	18±2	40±3	36±2	38±3	84±1	26±2	60±10	41±2	23±1	29±3
TTL18	31±1	18±3	15±3	22±3	40±2	41±2	36±1	98±1	28±3	49±1	11±3	25±3	35±1
TTL19	39±1	48±2	19±1	38±5	32±4	43±2	30±3	98±1	43±1	8±3	22±2	19±2	41±2
TTL20	22±2	26±4	32±1	24±1	28±2	44±2	22±5	98±1	44±1	5±2	20±1	30±3	33±1
TTL22	34±3	20±2	18±3	15±4	12±2	24±1	14±3	40±1	40±3	25±1	20±3	18±2	28±2
TTL23	38±2	28±2	26±1	36±2	16±1	18±1	24±3	38±1	38±2	32±2	31±4	16±1	32±1
TTL25	22±1	37±3	10±2	28±2	26±3	24±3	72±1	28±1	48±2	17±4	22±2	15±2	36±2
TTL28	20±2	39±3	28±1	33±1	21±1	27±1	40±3	41±1	33±1	40±2	25±3	11±3	34±4

Table 3.6: Apoptotic activity of gold complexes (TTC). A panel of cells were treated for 24hrs with 50µM of the compounds and apoptosis was assessed using the APOP*ercentage*™ assay. Treatments that caused apoptosis in more than 50% of the cells are in red.

Gold Complex	A549J	Caski	CHO	HepG2	HeLa	HT29	H157	Jurkat	KMST6	MCF7	MG-63	Hek	3T3
TTC2	50±3	54±2	100±0	46±5	43±2	48±2	98±1	99±0	22±2	88±5	92±4	72±4	50±1
TTC4	66±4	45±1	53±2	45±2	77±2	66±3	97±2	98±0	30±4	98±1	75±4	60±6	54±2
TTC5	30±2	36±4	46±1	32±1	77±2	34±1	44±4	98±0	15±3	10±2	20±2	42±2	17±4
TTC6	40±2	44±3	98±1	30±2	62±3	30±1	42±2	98±1	23±2	12±3	28±2	48±3	24±2
TTC9	37±4	40±5	98±1	40±3	40±3	22±3	40±2	98±0	21±1	22±2	11±3	46±1	36±1
TTC11	26±4	32±2	96±2	34±1	58±2	35±2	44±2	98±0	20±3	8±2	19±1	40±2	28±2
TTC14	45±2	38±2	98±1	28±4	49±4	27±2	32±1	98±0	19±1	10±3	11±1	44±3	40±3
TTC15	46±2	46±4	98±1	20±3	40±3	25±4	38±3	88±4	26±2	9±2	41±2	43±1	42±1
TTC18	80±4	88±3	66±3	85±2	80±2	76±5	99±0	99±0	70±5	99±1	91±5	85±5	77±2
TTC19	40±3	48±2	98±1	48±2	72±4	28±4	30±3	98±0	43±1	8±3	22±2	46±2	66±2
TTC20	48±3	66±4	98±1	50±5	68±2	59±2	99±0	98±0	44±1	5±2	20±1	40±3	56±1
TTC22	49±2	60±2	18±3	64±1	62±2	40±2	78±3	80±5	40±3	98±1	60±6	50±4	62±1
TTC23	48±2	48±2	44±1	44±3	36±1	40±1	82±2	98±1	38±2	32±2	31±4	30±1	34±2
TTC25	65±2	40±3	10±2	45±1	56±3	63±3	42±1	98±0	48±2	97±1	88±8	61±4	28±3
TTC28	46±2	48±4	32±6	38±4	42±1	41±2	40±3	98±0	33±1	40±2	25±3	41±3	46±1

Table 3.7: Summary of pro-apoptotic activities of phosphine ligands (TTL) in cultured cells.

Cell line	Phosphine Ligands														
	2	4	5	6	9	11	14	15	18	19	20	22	23	25	28
A549J	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
Caski	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CHO	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
HeLa	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
HepG2	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
HT29	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
H157	*	*	*	*	*	*	*	*	*	*	*	*	*	*	•
Jurkat	•	•	•	•	•	•	•	•	•	•	•	*	*	*	*
KMST6	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
MCF7	*	*	*	*	*	•	*	•	*	*	*	*	*	*	*
MG-63	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Hek293T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
3T3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

* Induced less than 50% apoptosis.

• Induced more than 50% apoptosis.

Table 3.8: Summary of pro-apoptotic activities of gold complexes (TTC) in cultured cells.

Cell line	Gold Complexes														
	2	4	5	6	9	11	14	15	18	19	20	22	23	25	28
A549J	●	●	*	*	*	*	*	*	●	*	*	*	*	●	*
Caski	●	*	*	*	*	*	*	*	●	*	●	●	*	*	*
CHO	●	●	*	●	●	●	●	●	●	●	●	*	*	*	*
HeLa	*	●	●	●	*	●	*	*	●	●	●	●	*	●	*
HepG2	*	*	*	*	*	*	*	*	●	*	●	●	*	*	*
HT29	*	●	*	*	*	*	*	*	●	*	●	*	*	●	*
H157	●	●	*	*	*	*	*	*	●	*	●	●	●	*	*
Jurkat	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
KMST6	*	*	*	*	*	*	*	*	●	*	*	*	*	*	*
MCF7	●	●	*	*	*	*	*	*	●	*	*	●	*	●	*
MG-63	●	●	*	*	*	*	*	*	●	*	*	●	*	●	*
Hek293T	●	●	*	*	*	*	*	*	●	*	*	●	*	●	*
3T3	●	●	*	*	*	*	*	*	●	*	●	●	*	*	*

* Induced less than 50% apoptosis.

● Induced more than 50% apoptosis.

3.5 Evaluating the cleavage of caspase-3

Based on the results obtained for the APOP*ercentage*™ asay, the gold complex TTC18 and the phosphine ligand TTL5 stood out as the most bioactive compounds. Consequently these two compounds were selected for further testing. Two cell lines, Jurkat and H157 were selected to evaluate whether apoptosis induced by TTC18 and TTL5 is also associated with the activation of caspase-3.

The activation of downstream caspases by proteolytic cleavage results in biochemical and morphological changes that are characteristic of apoptosis. It should however be noted that caspase-independent forms of apoptosis have been reported (Liang *et al*., 2008). Evaluation of the involvement of caspases especially the effector caspases provides an insight into whether the mode of apoptosis is caspase-independent or not. Jurkat and H157 cells were treated with TTC18 and TTL5. The anti-active caspase-3-PE antibody was used to assess whether treatment with these compounds result in the cleavage of caspase-3 (**Figure 3.1**). The presence of the anti-active caspase-3-PE antibody was confirmed by flow cytometry. Both TTL5 and TTC18 induced caspase-3 cleavage in H157 and Jurkat cells as shown in figure 3.1 compared to untreated cells. However, the percentage of cells showing the presence of cleaved caspase-3 was higher for TTC18. In addition, caspase-3 cleavage was higher number in Jurkat cells compared to H157 cells.

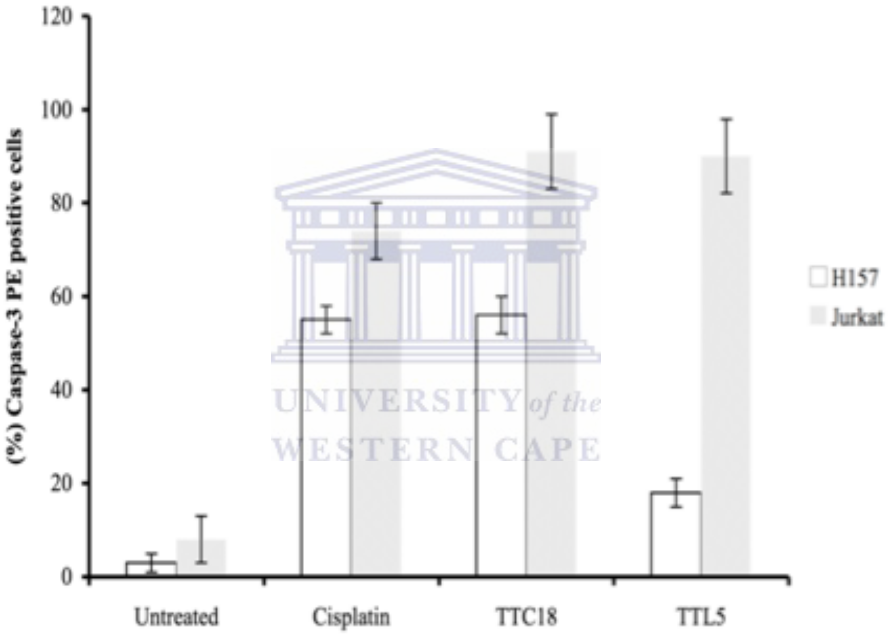


Figure 3.1: The activation of caspase-3 in Jurkat and H157 cells. Jurkat and H157 cells were treated for 24 hours with 30μM of the gold complex TTC18 and the phoshine ligand TTL5. Cisplatin was used a positive control. The cleavage of caspase-3 was assessed using an anti-active caspase-3-PE antibody. Cells were analyzed by flow cytometry on a FACScan™(Becton Dickson) instrument using the FL-3 channel. The experiment was done in triplicate and data is represented in a bar graph as the mean percentage anti-active caspase-3 positive cells.

3.6 Evaluating dose and time dependent activation of caspase-3

A dose is defined as the amount of chemical or physical agent that comes into contact with a living organism or part of a living organism or an amount absorbed into the organism (Rozman and Doull**,** 1998). Rozman and Doull defined toxicity as the accumulation of injury over short or long periods of time, which renders an organism incapable of functioning within the limits of adaptation, meaning that toxicity is a function of time in addition to the dose. As regards to dose dependence, Levy (1982), Ishaque and Aighewi (2008), defined dose dependence as the change in effects that treatment like for instance radiation, chemotherapy or antibiotics may have on an organism and is key to scientific study of poisons. In essence, this effect can be assessed over a period of time. If the effects change in response to change in the dosage or treatment, then the effects are referred to as dose-dependence (Rozman and Doull, 2001; Ishaque and Aighewi, 2008). Again if the increase in time also results in effect change, then it can categorically be said that effect has dependence on time. Rozman and Doull (2001) indicated that in toxicological studies analysis of time as a variable of toxicity showed the existence of at least three independent time scales (toxicokinetic, toxicodynamic and exposure frequency/duration), which interact with the dose and consequently result in the huge toxicological effects known as toxicity.

The present study was aimed at investigating the relationship of time and dose to gold compound and ligand-induced apoptosis in cell death. It is important to know

whether induction of apoptosis with these compounds responds in accordance with drug concentration as well as duration of treatment. Knowing dose and time responses helps to quantify a therapeutic agent such as a drug to be taken at one time or stated intervals. Paracelsus (1493-1541) in Tan and Yeow (2003), publication, indicated that "all substances are poisons: there is none which is not a poison, however the dose makes the poison. It depends only upon the dose whether a poison is poison or not. At extremely low doses, a given substance may be non-toxic and even beneficial (hormesis concept), while at intermediate doses, it may be toxic. At high doses, it may be lethal. Meaning that varying the amount of the poison affected the severity of the effects. This therefore underscores the importance of understanding dose response relationships".

Since apoptosis requires active cell participation and is therefore primarily caused by physiological stimuli, a variety of varied doses may lead the cell to die by apoptosis or necrosis (Lennon *et al*., 1991). One other important aspect of dose-response relationship is the concept of threshold. Eaton and Klaassen, (1996), reported that most types of toxicities do not occur below the level at which there are no effects from the exposure to the chemical. Further, Gibaldi *et al*., (1982); Eaton and Klaassen, (1996), reported that the human body has defences against many toxic agents. For instance, cells in human organs, especially in the liver and kidneys, break down chemicals into nontoxic substances that can be eliminated from the body in urine and faeces. In this way, the human body can take some toxic insult (at a

dose that is below the threshold) and still remain healthy (Levy, 1982; Eaton and Klaassen, 1996). To assess the effect of dosage, H157 were treated with various concentrations (5, 15, 30, 50µM) of the most active gold complex TTC18 (**Figure 3.2 A**) and to assess the effect of exposure time the cells were treated for various time points (**Figure 3.2 B**). **Figure 3.2 A** shows a dose-dependent increase in caspase-3 cleavage. No caspase-3 cleavage was observed at 5µM. **Figure 3.2 B** shows that caspase-3 cleavage started to increase between 6 and 12 hours.

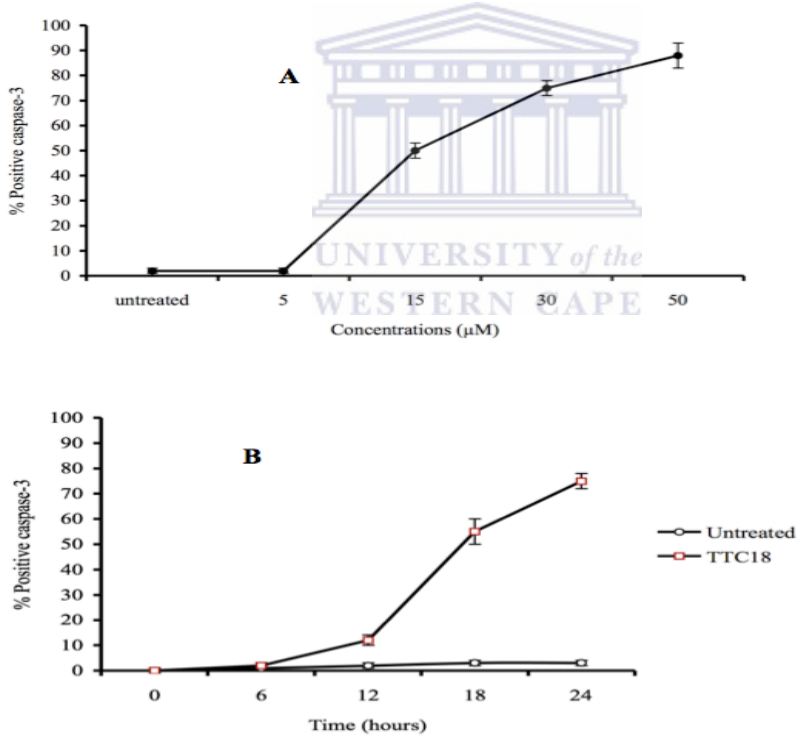


Figure 3.2: Dose and time dependent activation of caspase-3. H157 cells were treated for 24 hours with increasing concentrations (5 to 50µM) of the gold complex TTC18 (panel **A**). To assess time response the cells were treated with a single dose of 50µM for 6, 12, 18 and 24 hours (panel **B**). The cells were stained with anti-active caspase-3-PE antibody and subsequently analyzed by flow cytometry. The experiment was done in triplicate and data is represented in line graphs as the mean percentage anti-active caspase-3 positive cells.

3.7 Evaluation of DNA Fragmentation using the APO-DIRECT™ assay

The final step in the apoptotic pathway is DNA fragmentation with resultant cell death following the activation of endonucleases in the apoptotic pathway and is also one of the hallmarks of apoptotic cell death. As reviewed in chapter 1, **section 1.5.9.4.1**, it is well documented that endonucleases degrade chromatin higher order structures into fragments of ~300 kb and 50 kb lengths and subsequently into 200 bp ladders (Nagata, 2000). In order to detect DNA fragmentation, several assays are available such as the agarose gel electrophoresis (Pablo *et al*., 1998), fiberglass filters (Chow *et al*., 1989) or staining with bis-benzimidazole, Hoechst 33342 and propidium iodide (Ormerod *et al*., 1993). Another method frequently used to detect DNA fragmentation is the TUNEL assay. Apoptotic cells are identified by using the enzyme TdT that catalyzes the addition of dUTPs that are labelled with a marker such as FITC. DNA strand break labelling appears to be most specific as DNA strand break sites are identified by the conjugate FITC-labelled dUTP's (Darzynkiewicz *et al.,* 1997; Huerta *et al*., 2007). The assay however, can also label cells that have undergone severe DNA damage. It is possible to detect the degraded DNA using several assays such as electron microscopy (EM), DNA laddering using agarose gel electrophoresis. H157 cells were treated for 24 hrs with 30μM TTC18 and TTL5. DNA damage was assessed using the APO-DIRECT™ assay (**Figure 3.3**). Both TTL5 and TTC18 induced DNA fragmentation in H157 and Jurkat cells. However, the percentage of cells showing the presence of fragmented DNA was higher for TTC18.

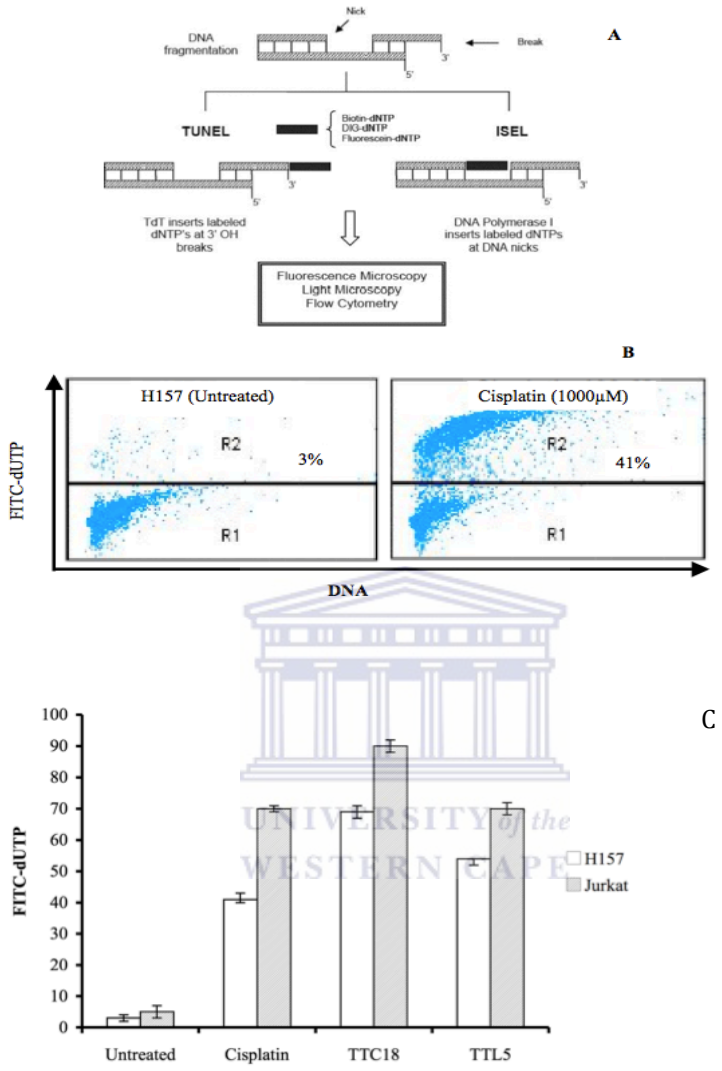


Figure 3.3: DNA fragmentation in H157 cells. Panel **A** is a demonstration of the TUNEL and *In situ* end-labelling (ISEL) techniques (Huerta *et al*., 2007). Following treatment DNA fragmentation was assessed using the APO-DIRECT™ assay and the cells were analysed by flow cytometry using a FACScan™ (Becton Dickson) instrument. FITC-dUTP labelling was measured using the FL-1 channel. Panel **B** shows two dot plots of untreated and cisplatin treated cells. The DNA content is on the X-axis and FITC-dUTP is on the Y-axis. The region labelled, R1 represents cells that are negative for FITC-dUTP, while R2 represents cells that are positive for FITC-dUTP. The numbers in R2 is the percentage of cells in R2 region. Panel **C** is a summary of the results for H157 cells treated for 24 hours with 30µM of gold complex TTC18 and phosphine ligand TTL5. The experiment was done in triplicate and data is represented in a bar graph as the mean percentage FITC-dUTP positive cells.

3.8 Investigating the effects of TTC18 and TTL5 on cell cycle

As the cell proliferation proceed from one stage of the cell cycle to the other, all mechanisms necessary for apoptosis are present throughout the cell cycle (Alenzi, 2004). DNA synthesis can be halted at any stage of the cell cycle when cells are exposed to harmful environmental agents such as mutagenic chemicals or radiation (Papamichos-Chronakis *et al*., 2006). In essence, DNA synthesis is halted in order for the cell to repair damaged DNA. If the DNA damage is repairable, the damage is repaired and cell proliferation continues, however if the damage is severe, the cell commits suicide (Wyllie *et al.,* 1984). In the case of anticancer drug development, Pucci *et al.,* (2000) and Bertino *et al*., (2003) pointed out that the basic understanding of the stages of the cell cycle is imperative as novel therapies may be developed to target the genes that are involved in circumventing apoptosis or inhibiting the proper functioning of the cell cycle for example the cyclin dependent kinase inhibitors.

One of the methods used to perform cell cycle analysis is the propidium iodide (PI) method. PI is a nucleic dye that intercalates single or double stranded DNA/RNA and fluoresces red. Since PI also stains RNA, this should first be removed with ribonuclease. PI can be used to identify the percentage of cells that are in one of the three phases of the cell cycle or to demonstrate the presence of apoptotic bodies (fragmented cells with low DNA content), which appear in the Sub-G1 phase. The intensity of the PI signal is directly proportional to the DNA content. Following

staining with the PI, cells that have lost DNA will take up less of the dye and will appear to the left of the G1 peak (the so- called “sub-G1 peaks”, i.e. cells with lower fluorescence level than G0+G1 cells) and are hence considered apoptotic. The major disadvantage is that apoptotic G2-Phase cells that exhibit a reduced DNA content could represent the DNA content of a G1-cell. Therefore it may not be detected as apoptotic and this can result in an underestimation of the apoptotic population.

To assess whether TTC18 and TTL5 affect cell cycle progression, H157 cells were treated for 6, 12, 18 and 24 hours with 15μM of TTC18 and TTL5 (**Figure 3.4**). The sub-G1 population for the untreated (**Figure 3.4 C**) and TTL5 treated (**Figure 3.4 D**) cells were below 2%, while the sub-G1 population for TTC18 treated cells incrementally increased from 2% to 12% over the 24hr period. This increase in the sub-G1 population was accompanied by a decrease in the G1 population, which later lead to the blockage of cell cycle progression at G1. Cells treated with TTL5 showed a time dependent increase in the G1 population, which appeared to be associated with a decrease in S and G2/M phases.

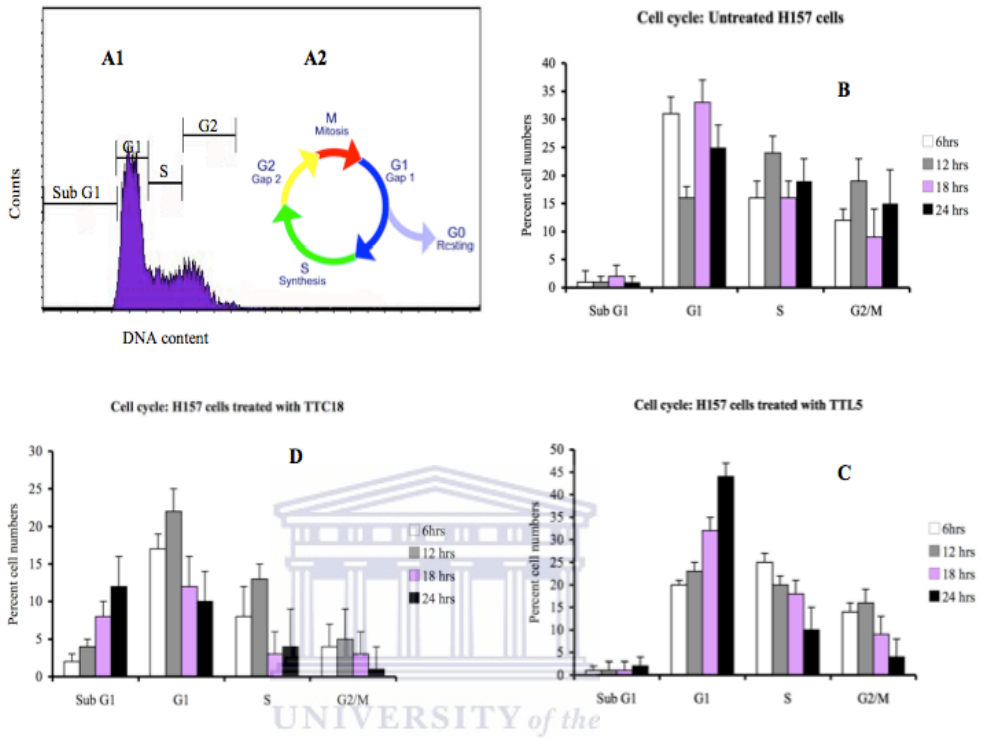


Figure 3.4: Cell cycle analysis of H157 cells. H157 cells were treated for 6, 12, 18 and 24 hours with 15µM of gold complex TTC18 and phosphine ligand TTL5 and stained with PI as described in chapter 2, section 2.2.10.2. Cells were analysed using a FACScan™ (Becton Dickson) instrument equipped with a 488 nm argon laser as a light source. Panel A shows how the analysis was done. Panel **A1** shows histogram plot of the DNA content on the X-axis and counts on the Y-axis. Indicated on the histogram are the three main stages (G1, G2 and S-phase) of the cell cycle and the Sub-G1 phase. Panel **A2** shows the stages of the cell cycle. Panels **B**, **C** and **D** show the cell cycle profile of untreated, TTL5 treated and TTC18 treated H157 cells, respectively. The experiment was done in triplicate and data is represented in a bar graph as the mean percentage cells.

3.9 Evaluating mitochondrial depolarisation using the TMRE assay

There are several functions associated with mitochondria. Mitochondria are involved in bioenergetics, apoptosis and cell signalling (Lum and Nagley, 2003). Mitochondria have been implicated in influencing life and death decisions by initiating or inhibiting cell death (Krohn *et al*. 1999). The depolarisation of the mitochondrial membrane potential was shown to be required for the subsequent release of pro-apoptotic factors from the mitochondria. However, Krohn *et al*., (1999), showed that apoptosis can also occur in the absence of mitochondrial depolarisation.

The molecular probe tetramethylrhodamine ethyl ester (TMRE) can be used to assess mitochondrial membrane depolarisation or loss of the electrochemical gradient across the mitochondrial membrane (Jayaraman, 2005). TMRE is a cationic, lipohilic dye that accumulates inside the membrane regions of healthy functioning mitochondria according to the Nernst equation potential in a voltage dependent manner (Kronhn *et al*., 1999). TMRE fluoresces bright orange/red in viable cells, which dissipates when the cells become apoptotic. The loss of fluorescence can be measured by flow cytometry. To evaluate mitochondrial membrane depolarisation, H157 cells were treated for 6, 12, 18 and 24 hours with 30μM of the gold complex TTC18, the phoshine ligand TTL5. The mitochondrial membrane potential (⊗¬m) was evaluated as described in chapter 2, **section 2.2.8** (**Figure 3.5**). Both TTC18 and TTL5 induced mitochondrial depolarisation in H157 cells in a time dependent manner. However, the number of cells with depolarised mitochondria was higher for TTC18 than TTL5. There was a significant increase in

depolarization with cells treated with TTC18 compared to cells treated with TTL5 at 18 and 24hrs.

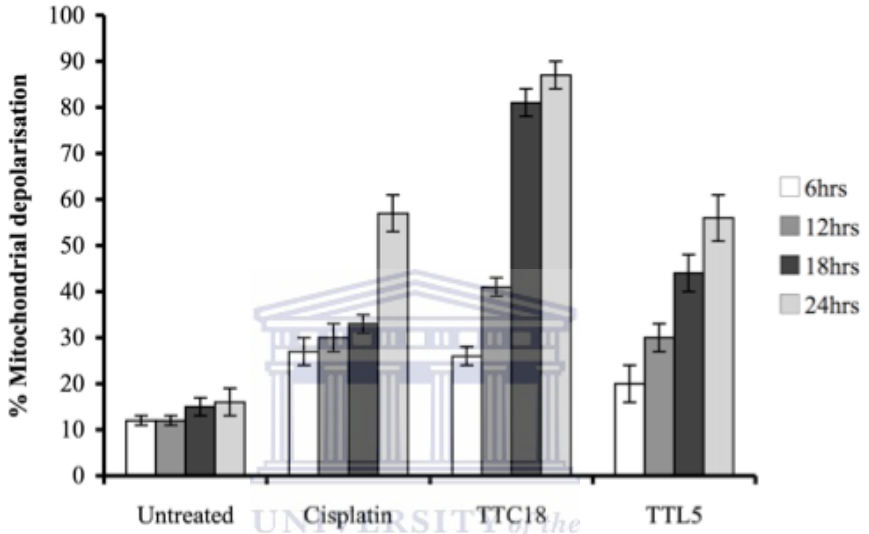


Figure 3.5: Assessing mitochondrial depolarisation using TMRE. H157 cells were cultured in 6 well plates as described in chapter 2, **section 2.2.8**. The cells were either left untreated or treated with 30µM of TTC18, or TTL5. The cells were stained with TMRE at 6, 12, 18 and 24 hours. TMRE fluorescence was measured on a FACScan™(Becton Dickson) instrument using the FL-3 channel.

3.10 Summary

The toxicity of fifteen phosphine ligands and fifteen gold(I) complexes were evaluated on a panel of 13 cell lines. Light microscopy was used to assess whether 50μM of these compounds can induce any morphological changes in these cells. The IC_{50} values for all 30 compounds were determined for each cell line using the MTT assay. The pro-apoptotic activity of all 30 compounds was evaluated on the panel of cell lines. The cells were treated for 24 hours with 50μM of the compounds and apoptosis was assessed using the APOP*ercentage*™ assay. Based on the results obtained for the MTT assay and the APOP*ercentage*™ assay, the gold(I) complexes were more bioactive than the phosphine ligands. The gold complexes TTC2, TTC4, TTC18, TTC20, TTC22, TTC25 and the ligand TTL5 were noticeably the most active compounds. The sensitivity of the cell lines varied, with Jurkat cells being very susceptible to the compounds.

The two most active compounds TTC18 and TTL5 were further tested on two cell lines (H157 and Jurkat). Three additional apoptosis assays, the caspase-3 cleavage assay, the DNA fragmentation assay and the mitochondrial depolarisation assay were used to confirm the activation of apoptosis. TTC18 was more bioactive than TTL5 and Jurkat cells were more sensitive to the effects of the two compounds. The effects of TTC18 and TTL5 on the mitochondrial potential of H157 cells were assessed using TMRE probe.

The effects of TTC18 and TTL5 on the cell cycle regulation of H157 cells were investigated over a 24 hour period. TTC18 induced apoptosis in H157 cells, while TTL5

induced cell cycle block in the G1 phase.

CHAPTER THREE OUTLINE

3.1 Introduction

3.2 Evaluating the effects of TTL and TTC on cell morphological

3.3 Measurement of cellular viability using the MTT assay

3.4 Evaluating the activation of apoptosis using the APOP*ercentage*™ assay

3.5 Evaluating the cleavage of caspase-3

3.6 Evaluating dose and time dependent activation of caspase-3

3.7 Evaluation of DNA Fragmentation using the APO-DIRECT™ assay

3.8 Investigating the effects of TTC18 and TTL5 on cell cycle

3.9 Evaluating mitochondrial depolarisation using the TMRE assay

3.10 Summary

CHAPTER THREE: Investigating the toxicity of phosphine ligands and gold(I) complexes.

3.1 Introduction

When a cell has undergone apoptosis, it is systematically disposed of without causing damage or stress to neighbouring cells (as reviewed in chapter 1, **section 1.5.9.1**). The dying cell has characteristic morphological changes, which include cell shrinkage, disappearance of microvilli on plasma membrane, the chromosomal DNA is fragmented into nucleosome size units of about 200 bp (as reviewed in chapter 1, **section 1.5.9.1**), as well as the nucleus and the cell itself is fragmented. It is possible to identify apoptotic cells using *in vitro* assays. Since the apoptotic cell undergoes cell condensation and therefore reduction in cell volume, the morphological changes can be observed by light microscopy, and its condensed nuclei can be stained with fluorescent dyes such as Hoechst or DAPI (Whiteside *et al*., 1998; Boya *et al*., 2005). The apoptotic cell also exposes phosphatidyl-serine (PS) on the cell surface (reviewed in chapter 1, section **1**.**1.5.9.2**). PS externalisation can be observed with dyes such as APOP*ercentage*™ or fluorescent-labelled Annexin V.

Mitochondria also play a key role in apoptosis (reviewed in chapter 1, section **1.5.9.1**) and Lum and Nagley, 2003). Dissipation of the mitochondrial membrane potential ($\Delta\Psi m$) is a key event in the intrinsic apoptosis pathway. Mitochondrial membrane potential can be measured by employing fluorescent dyes such as rhodamine 123 (R123), and tetramethylrhodamine ethyl ester (TMRE), which are

fluorescent probes that can be used to monitor the membrane potential of mitochondria (Scaduto Jr. and Grotyohann, 1999). The opening of pores in the mitochondria results in the release of signalling proteins, which includes cytochrome *c* (Reviewed in chapter 1, section **1.5.9.3.1**). Cytochrome *c* is found in mitochondrial intermembrane space and upon its release to the cytosol through the outer membrane forms the apoptosome (a complex with Apaf-1, pro-caspase-9 and ATP or dATP). The apoptosome triggers activation of downstream post mitochondrial caspases like caspase-3 and caspase-7 (Lum and Nagley, 2003; Patrushev *et al*., 2004). This activation of downstream caspases by proteolytic cleavage consequently results in the biochemical and morphological changes that are characteristic of apoptosis, consequently resulting in DNA fragmentation (Lum and Nagley, 2003). The fragmented DNA can be detected by assays such as Terminal deoxynucleotide transferase dUTP Nick End Labelling (TUNEL) or by electrophoresis of the isolated DNA on agarose gel and changes in DNA content of the cell can also be measured by cell cycle analysis (Frédérich *et al*., 2003). The neutral red assay or the MTT assay can be used to assess cell viability and the determination of IC_{50}. It is imperative that in order to assess apoptosis several assays be employed in order to affirm the results.

The aims of this chapter are:

- To screen 15 bidentate amino and iminophosphine ligands for apoptosis induction in a panel of cell lines shown in chapter 2 (**table 2.3**).

- To screen 15 mono and dinuclear Au(I) gold complexes for apoptosis induction in a panel of cell lines shown in chapter 2 (**table 2.3**).

3.2 Evaluating the effects of TTL and TTC on cell morphological

Darzynkiewicz (1997) indicated the importance of confirming the mode of cell death by the use of light and electron microscopy. To assess for morphological changes caused due to apoptosis induction, various cell lines shown in **table 2.3** were cultured in 6 well culture plates at a density of 2.5 x 10^5 cells per ml and were allowed to grow to 90% confluency. The cells were either left untreated or treated with 50µM of the gold complexes or the phosphine ligands and incubated at 37°C for 24 hours in a humidified CO_2 incubator. The morphology of the treated cells was studied by light microscopy. Morphological changes that can be associated with cell death and potentially apoptosis could be observed for some of the phosphine ligands and the complexes (**tables 3.1** and **3.2**). The morphological changes that wee observed bioactive compounds included cell shrinkage, inhibition of cell growth and cell detachment. Some of the compounds failed to induce any morphological changes in the cells. Jurkat cells were highly sensitive to the effects of both TTL and TTC compounds. Thirteen cells lines were screened in this study and 50µM of TTC18 induced morphological changes in all of them. TTL5 was the most toxic phosphine ligand, inducing morphological changes in eight of the cell lines tested in this study.

3.3 Measurement of cellular viability using the MTT assay

The tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

Table 3.1: Summary of morphological changes observed in cultured cells in response to treatment with phosphine ligands (TTL). The cells were treated for 24 hours with 50μM of the compounds and then studied under the light microscope.

Cell line	Phosphine Ligands														
	2	4	5	6	9	11	14	15	18	19	20	22	23	25	28
A549J	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
Caski	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CHO	*	*	•	*	*	*	*	*	•	*	*	*	*	*	*
HeLa	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
HepG2	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
HT29	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
H157	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Jurkat	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
KMST6	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
MCF7	*	*	*	*	*	•	*	*	*	*	*	*	*	*	*
MG-63	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
Hek293T	*	*	•	*	•	•	*	*	*	*	*	*	*	*	*
3T3	*	*	*	*	*	*	•	*	*	*	*	*	*	*	*

* No discernible morphological changes observed.

• Morphological changes (cell shrinkage and cell detachment) indicative of apoptosis were observed.

Table 3.2: Summary of morphological changes observed in cultured cells in response to treatment with gold complexes (TTC). The cells were treated for 24 hours with 50μM of the compounds and then studied under the light microscope.

Cell line	Gold Complex														
	2	4	5	6	9	11	14	15	18	19	20	22	23	25	28
A549J	*	*	*	*	*	*	*	*	•	*	*	*	*	•	*
Caski	*	•	*	*	*	*	*	*	•	*	•	*	*	*	*
CHO	•	•	•	•	•	•	•	•	•	•	•	*	•	*	•
HeLa	*	•	•	*	*	•	*	*	•	•	•	•	*	•	*
HepG2	*	*	*	*	*	*	*	*	•	*	*	•	*	*	*
HT29	*	•	*	*	*	*	*	*	•	*	•	*	*	•	*
H157	•	•	*	•	*	*	*	*	•	•	•	•	*	*	*
Jurkat	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
KMST6	*	*	*	*	*	*	*	*	•	*	*	*	*	*	*
MCF7	•	•	*	*	*	*	*	*	•	*	*	•	*	•	*
MG-63	•	•	*	*	*	*	*	*	•	*	*	•	*	•	*
Hek293T	•	•	*	*	*	*	*	*	•	•	•	•	*	•	*
3T3	•	•	*	*	*	*	*	*	•	•	•	•	*	*	*

* No discernible morphological changes observed.

• Morphological changes (cell shrinkage and cell detachment) indicative of apoptosis were observed.

(MTT) assay was developed by Mosmann in 1983 to measure cellular growth and survival by detecting living cells. In this assay, metabolically active and therefore live cells reduce MTT to yield non-water-soluble violet formazan crystals. Metabolically inactive and therefore dead cells fail to reduce MTT. The insoluble purple formazan product formed can serve to estimate the number of mitochondria and hence the number of living cells in the sample. The amount of formazan crystals formed within the living cells can be solubilised with a solution of dimethyl sulfoxide (DMSO) or an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate (SDS) in diluted hydrochloric acid to form a coloured solution. According to Mosmann (1983), the absorbance of this coloured solution can be measured and quantified using a spectrophotometer set at a wavelength between 500 and 600 nm. The absorption maximum wavelength is dependent on the solvent employed. This method can therefore be used to measure cellular cytotoxicity, proliferation or activation (Freimoser *et al*., 1999). The MTT assay was used to determine the IC_{50} value for both TTL (**table 3.3**) and TTC (**table 3.4**) samples on a panel of 13 cell lines. The procedure is described in chapter 2, section **2.2.6**. A comparison between the phosphine ligands (TTL) and gold complexes (TCC) shows that the IC_{50} values for the most of the phosphine ligands is above 100µM, while the IC_{50} values for most of the gold complexes were below 50µM. The IC_{50} values for the positive control, cisplatin was above 100µM for all the cell lines used in this study.

Table 3.3: IC_{50} values (in μM) for phosphine ligands (TTL) as determined by the MTT assay. IC_{50} values of 50μM and below are in red.

Phosphine Ligand	A549J	Caski	CHO	HepG2	HeLa	HT29	H157	Jurkat	KMST6	MCF7	MG-63	Hek	3T3
TTL2	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
TTL4	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	90±0.14	>100
TTL5	>100	>100	>100	>100	35±0.16	>100	28±0.01	<10	75±0.6	>100	50±0.3	<10	>100
TTL6	>100	>100	>100	>100	>100	>100	>100	100	100	>100	>100	20±0.8	>100
TTL9	>100	>100	>100	>100	>100	>100	>100	25±0.05	100	>100	>100	90±0.58	>100
TTL11	>100	>100	>100	>100	>100	>100	>100	80±0.00	>100	>100	>100	50±0.48	50±0.3
TTL14	>100	>100	>100	98±0.02	>100	>100	>100	80±0.01	>100	>100	>100	20±0.34	20±26
TTL15	>100	>100	>100	>100	>100	>100	>100	>100	100	>100	>100	20±0.45	20±0.76
TTL18	>100	>100	>100	95±0.04	95±0.56	>100	>100	80±0.01	>100	>100	>100	10±0.28	>100
TTL19	35±00	55±06	35±06	>100	>100	48±0±06	>100	10±0.00	35±0.00	35±0.06	>100	30±0.00	>100
TTL20	>100	>100	>100	100	>100	>100	>100	>100	70±0.00	50±0.3	>100	50±0.00	>100
TTL22	>100	>100	>100	50±0.5	100	>100	>100	>100	>100	>100	>100	100	>100
TTL23	>100	>100	85±0.1	>100	100	>100	>100	>100	95±0.5	100	15±0.05	10±0.36	18±0.00
TTL25	>100	>100	>100	>100	98±0.04	>100	>100	20±0.2	>100	>100	>100	>100	>100
TTL28	>100	>100	80±0.1	>100	95±0.02	>100	100	35±0.13	95±0.4	100	100	>100	>100

Table 3.4: IC_{50} values (in µM) for gold complexes (TTC) as determined by the MTT assay. IC_{50} values of 50µM and below are in red.

Gold Complex	A549J	Caski	CHO	HepG2	HeLa	HT29	H157	Jurkat	KMST6	MCF7	MG-63	Hek	3T3
TTC2	>100	90±0.01	>100	>100	>100	90±0.00	60±0.05	>100	>100	50±0.7	>100	25±0.00	50±0.28
TTC4	50±0.01	80±0.2	30±0.02	30±0.02	90±0.04	15±0.00	30±0.9	20±0.00	38±0.00	20±0.1	25±0.1	<10	45±0.13
TTC5	>100	>100	37±0.01	>100	85±0.00	60±0.00	98±0.05	18±0.00	>100	48±0.05	<10	50±0.00	15±0.4
TTC6	60±0.04	>100	35±0.01	20±0.00	15±0.00	80±0.06	28±0.03	50±0.00	90±0.05	20±0.05	95±0.05	18±0.00	80±0.05
TTC9	>100	>100	85±0.05	>100	15±0.00	>100	>100	80±0.5	>100	>100	100	60±0.02	>100
TTC11	60.0.01	>100	25±0.00	20±0.00	100	10±0.00	25±0.09	<10	48±0.01	15±0.05	27±0.05	<10	15±0.2
TTC14	100	>100	65±0.04	48±0.02	12±0.00	<10	98±0.03	25±0.01	>100	98±0.06	20±0.06	<10	<10
TTC15	>100	98±0.02	74±0.03	48±0.01	34±0.07	35±0.01	96±0.14	25±0.00	>100	30±0.08	40±0.08	26±0.00	25±0.6
TTC18	63+0.0	50±0.01	18±0.00	10±0.01	35±0.04	20±0.02	28±0.01	<10	37±0.00	10±0.06	12±0.06	<10	<10
TTC19	20+0.00	75±0.22	60±0.00	10±0.01	<10	10±0.00	45±0.22	45±0.01	>100	10±0.00	20±0.00	10±0.3	<10
TTC20	>100	>100	70±0.01	48±0.01	100	50±0.00	>100	<100	>100	48±0.06	25±0.06	10±0.56	18±0.00
TTC22	>100	80±0.02	>100	100	35±0.029	90±0.02	50±0.02	20±0.3	>100	90±0.1	30±0.1	50±0.08	50±0.3
TTC23	30±0.01	68±0.00	25±0.01	10±0.00	100	12±0.01	28±0.00	50±0.01	30±0.00	10±0.07	80±0.07	<10	<10
TTC25	>100	70±0.01	90±0.01	25.0.00	12±0.06	50±0.01	30±0.01	<10	>100	>100	<10	10±0.02	018±0.1
TTC28	30±0.00	85±0.01	27±0.00	<10	22±0.00	45±0.05	25±0.01	28±0.02	30±0.00	<10	<10	28±0.02	45±0.32
Cisplatin	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

3.4 Evaluating the activation of apoptosis using the APOP*ercentage*™ assay

The APOP*ercentage*™ apoptosis assay was used to assess the induction of apoptosis in cultured cells. As previously described the principle of the assay is based on a dye that the dye enters the cells upon phosphatidylserine transmembrane movement ('flip-flop' mechanism).

A panel of cell lines were cultured to 90% confluence in 24 well cell culture plates. There after, the cells were either left untreated or treated with a single dose of 50μM of the phosphine ligands (TTL) or gold complexes (TTC) for 24 hours. Following treatment, the cells were stained with APOP*ercentage*™ apoptosis dye as described in section **2.2.7.1** and analysed by flow cytometry. Results for TTL and TTC compounds are tabulated in **table 3.5** and **table 3.6**, respectively. The data is also summarised in **table 3.7** and **table 3.8**, showing which compounds induced more than 50% cell death. The induction of apoptosis under these conditions was selective. In general, the TTC compounds were more bioactive than the TTL compounds. TTL5 demonstrated higher pro-apoptotic activity than the other phosphine ligands, inducing more than 50% apoptosis in five cell lines (A549J, Caski, CHO, HepG2, Jurkat and KMST6). The most bioactive gold complexes were TTC2, TTC4, TTC18, TTC20, TTC22 and TTC25, with TTC18 demonstrating the highest pro-apoptotic activity. TTC18 induced more than 50% apoptosis in all thirteen cell lines used in this study. Jurkat cells were highly sensitive to the effects of both TTL and TTC compounds.

Table 3.5: Determining the percentage apoptosis induced by 50μM of the phosphine ligands (TTL) in a panel of cell lines. The cells were treated for 24 hours and apoptosis was quantified using the APOP*ercentage*™ apoptosis. Treatments that resulted in more than 50% cell death are indicated in red.

Phosphine Ligand	A549J	Caski	CHO	HepG2	HeLa	HT29	H157	Jurkat	KMST6	MCF7	MG-63	Hek	3T3
TTL2	44±1	44±2	12±3	24±2	43±2	22±1	48±3	68±8	22±2	42±1	22±3	44±3	43±3
TTL4	32±1	45±1	13±2	32±4	27±2	24±2	45±1	99±0	30±4	48±2	15±1	38±4	15±4
TTL5	57±3	36±4	88±1	50±1	30±2	27±4	48±4	98±0	50±5	10±2	20±2	22±2	22±2
TTL6	20±1	44±3	18±1	25±3	22±3	18±3	22±2	98±1	23±2	12±3	28±2	48±3	35±3
TTL9	17±2	40±5	28±1	36±2	40±3	29±1	37±2	98±1	21±1	22±2	11±3	66±1	28±2
TTL11	41±3	32±2	16±2	28±1	38±2	28±3	44±2	98±1	20±3	63±6	19±1	50±2	32±1
TTL14	22±1	38±2	18±1	20±4	49±4	33±4	32±1	98±1	19±1	10±3	11±1	44±3	27±2
TTL15	27±3	46±4	13±1	18±2	40±3	36±2	38±3	84±1	26±2	60±10	41±2	23±1	29±3
TTL18	31±1	18±3	15±3	22±3	40±2	41±2	36±1	98±1	28±3	49±1	11±3	25±3	35±1
TTL19	39±1	48±2	19±1	38±5	32±4	43±2	30±3	98±1	43±1	8±3	22±2	19±2	41±2
TTL20	22±2	26±4	32±1	24±1	28±2	44±2	22±5	98±1	44±1	5±2	20±1	30±3	33±1
TTL22	34±3	20±2	18±3	15±4	12±2	24±1	14±3	40±1	40±3	25±1	20±3	18±2	28±2
TTL23	38±2	28±2	26±1	36±2	16±1	18±1	24±3	38±1	38±2	32±2	31±4	16±1	32±1
TTL25	22±1	37±3	10±2	28±2	26±3	24±3	72±1	28±1	48±2	17±4	22±2	15±2	36±2
TTL28	20±2	39±3	28±1	33±1	21±1	27±1	40±3	41±1	33±1	40±2	25±3	11±3	34±4

Table 3.6: Determining the percentage apoptosis induced by 50μM of the gold complexes (TTC) in a panel of cell lines. The cells were treated for 24 hours and apoptosis was quantified using the APOP*ercentage*™ apoptosis. Treatments that resulted in more than 50% cell death are indicated in red.

Gold Complex	A549J	Caski	CHO	HepG2	HeLa	HT29	H157	Jurkat	KMST6	MCF7	MG-63	Hek	3T3
TTC2	50±3	54±2	100±0	46±5	43±2	48±2	98±1	99±0	22±2	88±5	92±4	72±4	50±1
TTC4	66±4	45±1	53±2	45±2	77±2	66±3	97±2	98±0	30±4	98±1	75±4	60±6	54±2
TTC5	30±2	36±4	46±1	32±1	77±2	34±1	44±4	98±0	15±3	10±2	20±2	42±2	17±4
TTC6	40±2	44±3	98±1	30±2	62±3	30±1	42±2	98±1	23±2	12±3	28±2	48±3	24±2
TTC9	37±4	40±5	98±1	40±3	40±3	22±3	40±2	98±0	21±1	22±2	11±3	46±1	36±1
TTC11	26±4	32±2	96±2	34±1	58±2	35±2	44±2	98±0	20±3	8±2	19±1	40±2	28±2
TTC14	45±2	38±2	98±1	28±4	49±4	27±2	32±1	98±0	19±1	10±3	11±1	44±3	40±3
TTC15	46±2	46±4	98±1	20±3	40±3	25±4	38±3	88±4	26±2	9±2	41±2	43±1	42±1
TTC18	80±4	88±3	66±3	85±2	80±2	76±5	99±0	99±0	70±5	99±1	91±5	85±5	77±2
TTC19	40±3	48±2	98±1	48±2	72±4	28±4	30±3	98±0	43±1	8±3	22±2	46±2	66±2
TTC20	48±3	66±4	98±1	50±5	68±2	59±2	99±0	98±0	44±1	5±2	20±1	40±3	56±1
TTC22	49±2	60±2	18±3	64±1	62±2	40±2	78±3	80±5	40±3	98±1	60±6	50±4	62±1
TTC23	48±2	48±2	44±1	44±3	36±1	40±1	82±2	98±1	38±2	32±2	31±4	30±1	34±2
TTC25	65±2	40±3	10±2	45±1	56±3	63±3	42±1	98±0	48±2	97±1	88±8	61±4	28±3
TTC28	46±2	48±4	32±6	38±4	42±1	41±2	40±3	98±0	33±1	40±2	25±3	41±3	46±1

Table 3.7: Summary of pro-apoptotic activities of phosphine ligands (TTL) in cultured cells.

Cell line	Phosphine Ligands														
	2	4	5	6	9	11	14	15	18	19	20	22	23	25	28
A549J	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
Caski	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CHO	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
HeLa	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
HepG2	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
HT29	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
H157	*	*	*	*	*	*	*	*	*	*	*	*	*	*	•
Jurkat	•	•	•	•	•	•	•	•	•	•	•	*	*	*	*
KMST6	*	*	•	*	*	*	*	*	*	*	*	*	*	*	*
MCF7	*	*	*	*	*	•	*	•	*	*	*	*	*	*	*
MG-63	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Hek293T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
3T3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

* Induced less than 50% apoptosis.

• Induced more than 50% apoptosis.

Table 3.8: Summary of pro-apoptotic activities of gold complexes (TTC) in cultured cells.

Cell line	Gold Complexes														
	2	4	5	6	9	11	14	15	18	19	20	22	23	25	28
A549J	●	●	*	*	*	*	*	*	●	*	*	*	*	●	*
Caski	●	*	*	*	*	*	*	*	●	*	●	●	*	*	*
CHO	●	●	*	●	●	●	●	●	●	●	●	*	*	*	*
HeLa	*	●	●	●	*	●	*	*	●	●	●	●	*	●	*
HepG2	*	*	*	*	*	*	*	*	●	*	●	●	*	*	*
HT29	*	●	*	*	*	*	*	*	●	*	●	*	*	●	*
H157	●	●	*	*	*	*	*	*	●	*	●	●	●	*	*
Jurkat	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
KMST6	*	*	*	*	*	*	*	*	●	*	*	*	*	*	*
MCF7	●	●	*	*	*	*	*	*	●	*	*	●	*	●	*
MG-63	●	●	*	*	*	*	*	*	●	*	*	●	*	●	*
Hek293T	●	●	*	*	*	*	*	*	●	*	*	●	*	●	*
3T3	●	●	*	*	*	*	*	*	●	*	●	●	*	*	*

* Induced less than 50% apoptosis.

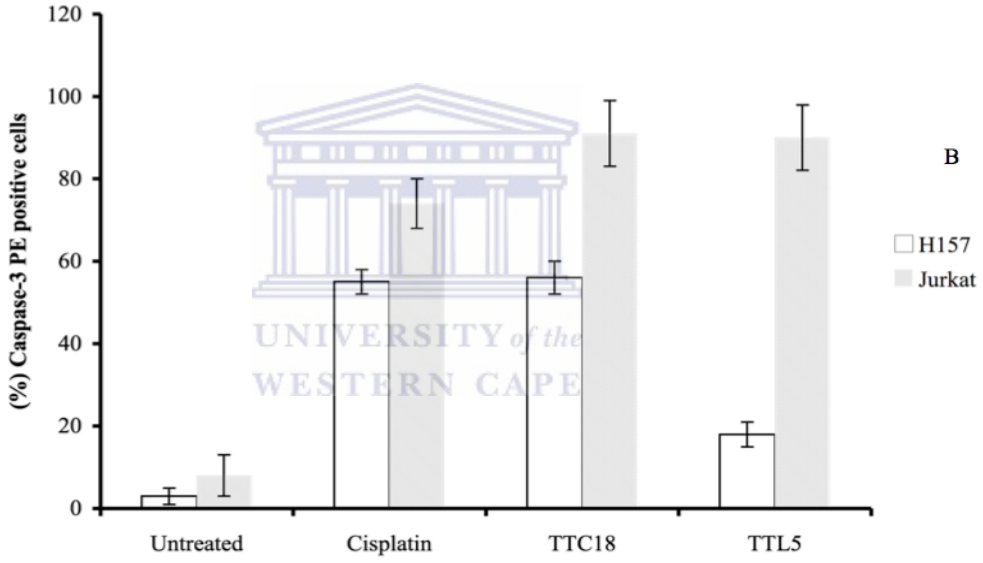
● Induced more than 50% apoptosis.

3.5 Evaluating the cleavage of caspase-3

Based on the results obtained for the APOP*ercentage*™ asay, the gold complex TTC18 and the phosphine ligand TTL5 stood out as the most bioactive compounds. Consequently these two compounds were selected for further testing. Two cell lines, Jurkat and H157 were selected to evaluate whether apoptosis induced by TTC18 and TTL5 is also associated with the activation of caspase-3.

The activation of downstream caspases by proteolytic cleavage results in biochemical and morphological changes that are characteristic of apoptosis. It should however be noted that caspase-independent forms of apoptosis have been reported (Liang *et al*., 2008). Evaluation of the involvement of caspases especially the effector caspases provides an insight into whether the mode of apoptosis is caspase-independent or not. Jurkat and H157 cells were treated with TTC18 and TTL5. The anti-active caspase-3-PE antibody was used to assess whether treatment with these compounds result in the cleavage of caspase-3 (**Figure 3.1**). The presence of the anti-active caspase-3-PE antibody was confirmed by flow cytometry. Both TTL5 and TTC18 induced caspase-3 cleavage in H157 and Jurkat cells. However, the percentage of cells showing the presence of cleaved caspase-3 was higher for TTC18. In addition, caspase-3 cleavage was higher number in Jurkat cells.

Figure 3.1: The activation of caspase-3 in Jurkat and H157 cells. Jurkat and H157 cells were treated for 24 hours with 30μM of the gold complex TTC18 and the phoshine ligand TTL5. Cisplatin was used a positive control. The cleavage of caspase-3 was assessed using an anti-active caspase-3-PE antibody. Cells were analyzed by flow cytometry on a FACScan™(Becton Dickson) instrument using the FL-3 channel. The experiment was done in triplicate and data is represented in a bar graph as the mean percentage anti-active caspase-3 positive cells.



3.6 Evaluating dose and time dependent activation of caspase-3

A dose is defined as the amount of chemical or physical agent that comes into contact with a living organism or part of a living organism or an amount absorbed into the organism (Rozman and Doull**,** 1998). Rozman and Doull defined toxicity as the accumulation of injury over short or long periods of time, which renders an organism incapable of functioning within the limits of adaptation, meaning that toxicity is a function of time in addition to the dose. As regards to dose dependence, Levy (1982), Ishaque and Aighewi (2008), defined dose dependence as the change in effects that treatment like for instance radiation, chemotherapy or antibiotics may have on an organism and is key to scientific study of poisons. In essence, this effect can be assessed over a period of time. If the effects change in response to change in the dosage or treatment, then the effects are referred to as dose-dependence (Rozman and Doull, 2001; Ishaque and Aighewi, 2008). Again if the increase in time also results in effect change, then it can categorically be said that effect has dependence on time. Rozman and Doull (2001) indicated that in toxicological studies analysis of time as a variable of toxicity showed the existence of at least three independent time scales (toxicokinetic, toxicodynamic and exposure frequency/duration), which interact with the dose and consequently result in the huge toxicological effects known as toxicity.

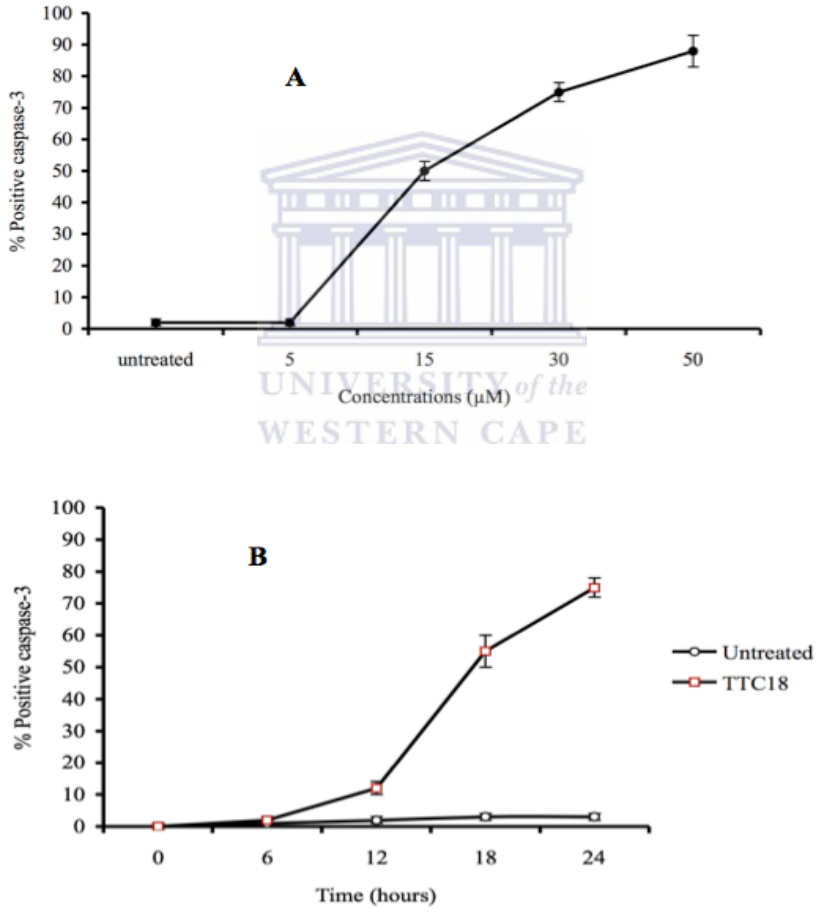
The present study was aimed at investigating the relationship of time and dose to gold compound and ligand-induced apoptosis in cell death. It is important to know

whether induction of apoptosis with these compounds responds in accordance with drug concentration as well as duration of treatment. Knowing dose and time responses helps to quantify a therapeutic agent such as a drug to be taken at one time or stated intervals. Paracelsus (1493-1541) in Tan and Yeow (2003), publication, indicated that "all substances are poisons: there is none which is not a poison, however the dose makes the poison. It depends only upon the dose whether a poison is poison or not. At extremely low doses, a given substance may be non-toxic and even beneficial (hormesis concept), while at intermediate doses, it may be toxic. At high doses, it may be lethal. Meaning that varying the amount of the poison affected the severity of the effects. This therefore underscores the importance of understanding dose response relationships".

Since apoptosis requires active cell participation and is therefore primarily caused by physiological stimuli, a variety of varied doses may lead the cell to die by apoptosis or necrosis (Lennon *et al*., 1991). One other important aspect of dose-response relationship is the concept of threshold. Eaton and Klaassen, (1996), reported that most types of toxicities do not occur below the level at which there are no effects from the exposure to the chemical. Further, Gibaldi *et al*., (1982); Eaton and Klaassen, (1996), reported that the human body has defences against many toxic agents. For instance, cells in human organs, especially in the liver and kidneys, break down chemicals into nontoxic substances that can be eliminated from the body in urine and faeces. In this way, the human body can take some toxic insult

(at a dose that is below the threshold) and still remain healthy (Levy, 1982; Eaton and Klaassen, 1996). To assess the effect of dosage, H157 were treated with various concentrations (5, 15, 30, 50μM) of the most active gold complex TTC18 (**Figure 3.2 A**) and to assess the effect of exposure time the cells were treated for various time points (**Figure 3.2 B**). **Figure 3.2 A** shows a dose-dependent increase in caspase-3 cleavage. No caspase-3 cleavage was observed at 5μM. **Figure 3.2 B** shows that caspase-3 cleavage started to increase between 6 and 12 hours.

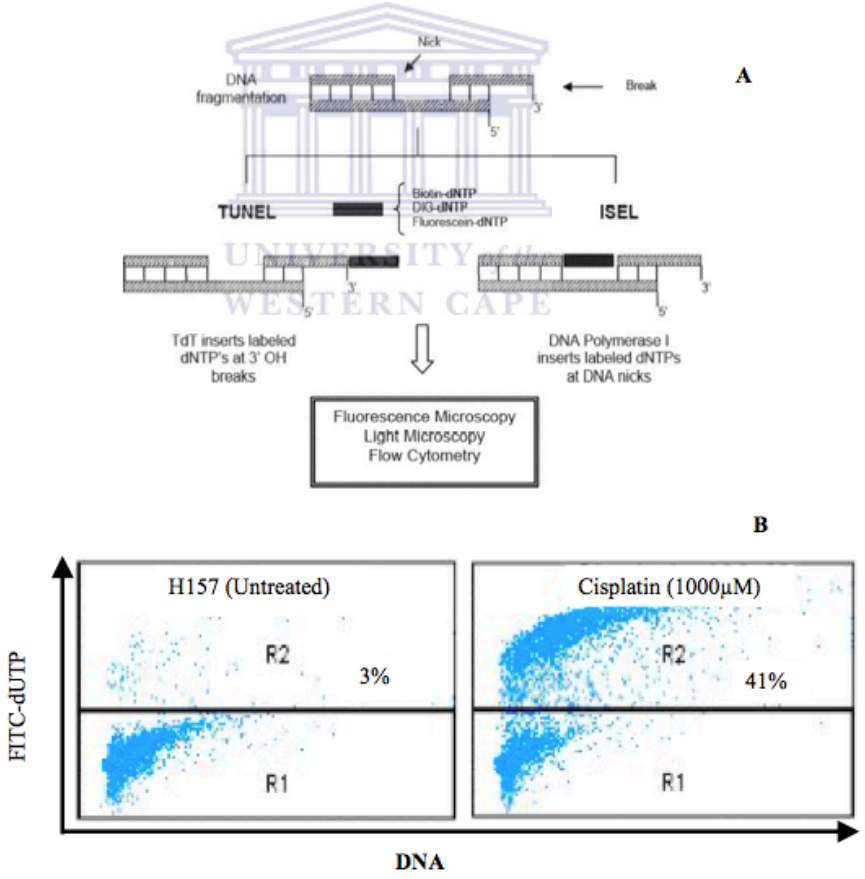
Figure 3.2: Dose and time dependent activation of caspase-3. H157 cells were treated for 24 hours with increasing concentrations (5 to 50µM) of the gold complex TTC18 (panel **A**). To assess time response the cells were treated with a single dose of 50µM for 6, 12, 18 and 24 hours (panel **B**). The cells were stained with anti-active caspase-3-PE antibody and subsequently analyzed by flow cytometry. The experiment was done in triplicate and data is represented in line graphs as the mean percentage anti-active caspase-3 positive cells.



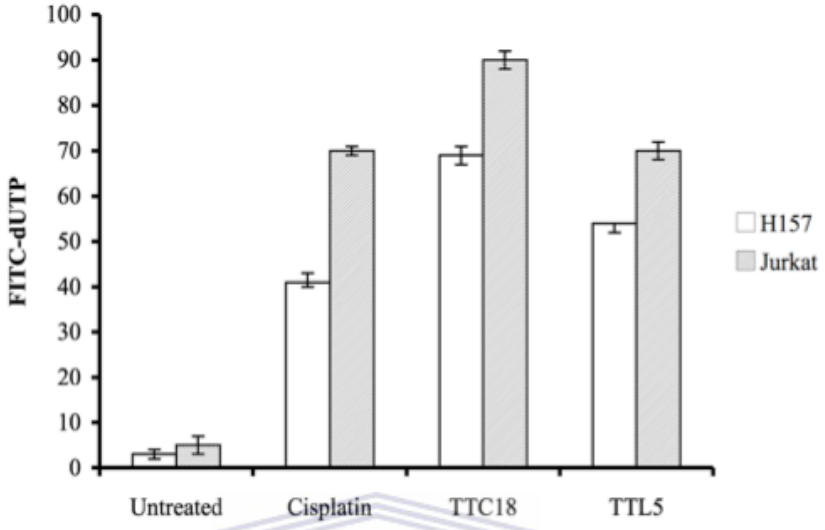
3.7 Evaluation of DNA Fragmentation using the APO-DIRECT™ assay

The final step in the apoptotic pathway is DNA fragmentation with resultant cell death following the activation of endonucleases in the apoptotic pathway and is also one of the hallmarks of apoptotic cell death. As reviewed in chapter 1, **section 1.5.9.4.1**, it is well documented that endonucleases degrade chromatin higher order structures into fragments of ~300 kb and 50 kb lengths and subsequently into 200 bp ladders (Nagata, 2000). In order to detect DNA fragmentation, several assays are available such as the agarose gel electrophoresis (Pablo *et al*., 1998), fiberglass filters (Chow *et al*., 1989) or staining with bis-benzimidazole, Hoechst 33342 and propidium iodide (Ormerod *et al*., 1993). Another method frequently used to detect DNA fragmentation is the TUNEL assay. Apoptotic cells are identified by using the enzyme TdT that catalyzes the addition of dUTPs that are labelled with a marker such as FITC. DNA strand break labelling appears to be most specific as DNA strand break sites are identified by the conjugate FITC-labelled dUTP's (Darzynkiewicz *et al.,* 1997; Huerta *et al*., 2007). The assay however, can also label cells that have undergone severe DNA damage. It is possible to detect the degraded DNA using several assays such as electron microscopy (EM), DNA laddering using agarose gel electrophoresis. H157 cells were treated for 24 hrs with 30μM TTC18 and TTL5. DNA damage was assessed using the APO-DIRECT™ assay (**Figure 3.3**). Both TTL5 and TTC18 induced DNA fragmentation in H157 and Jurkat cells. However, the percentage of cells showing the presence of fragmented DNA was higher for TTC18.

Figure 3.3: DNA fragmentation in H157 cells. Panel **A** is a demonstration of the TUNEL and *In situ* end-labelling (ISEL) techniques (Huerta *et al*., 2007). Following treatment DNA fragmentation was assessed using the APO-DIRECT™ assay and the cells were analysed by flow cytometry using a FACScan™ (Becton Dickson) instrument. FITC-dUTP labelling was measured using the FL-1 channel. Panel **B** shows two dot plots of untreated and cisplatin treated cells. The DNA content is on the X-axis and FITC-dUTP is on the Y-axis. The region labelled, R1 represents cells that are negative for FITC-dUTP, while R2 represents cells that are positive for FITC-dUTP. The numbers in R2 is the percentage of cells in R2 region. Panel **C** is a summary of the results for H157 cells treated for 24 hours with 30µM of gold complex TTC18 and phosphine ligand TTL5. The experiment was done in triplicate and data is represented in a bar graph as the mean percentage FITC-dUTP positive cells.



C



3.8 Investigating the effects of TTC18 and TTL5 on cell cycle

As the cell proliferation proceed from one stage of the cell cycle to the other, all mechanisms necessary for apoptosis are present throughout the cell cycle (Alenzi, 2004). DNA synthesis can be halted at any stage of the cell cycle when cells are exposed to harmful environmental agents such as mutagenic chemicals or radiation (Papamichos-Chronakis *et al*., 2006). In essence, DNA synthesis is halted in order for the cell to repair damaged DNA. If the DNA damage is repairable, the damage is repaired and cell proliferation continues, however if the damage is severe, the cell commits suicide (Wyllie *et al.,* 1984). In the case of anticancer drug development, Pucci *et al.,* (2000) and Bertino *et al*., (2003) pointed out that the basic understanding of the stages of the cell cycle is imperative as novel therapies may be developed to target the genes that are involved in circumventing

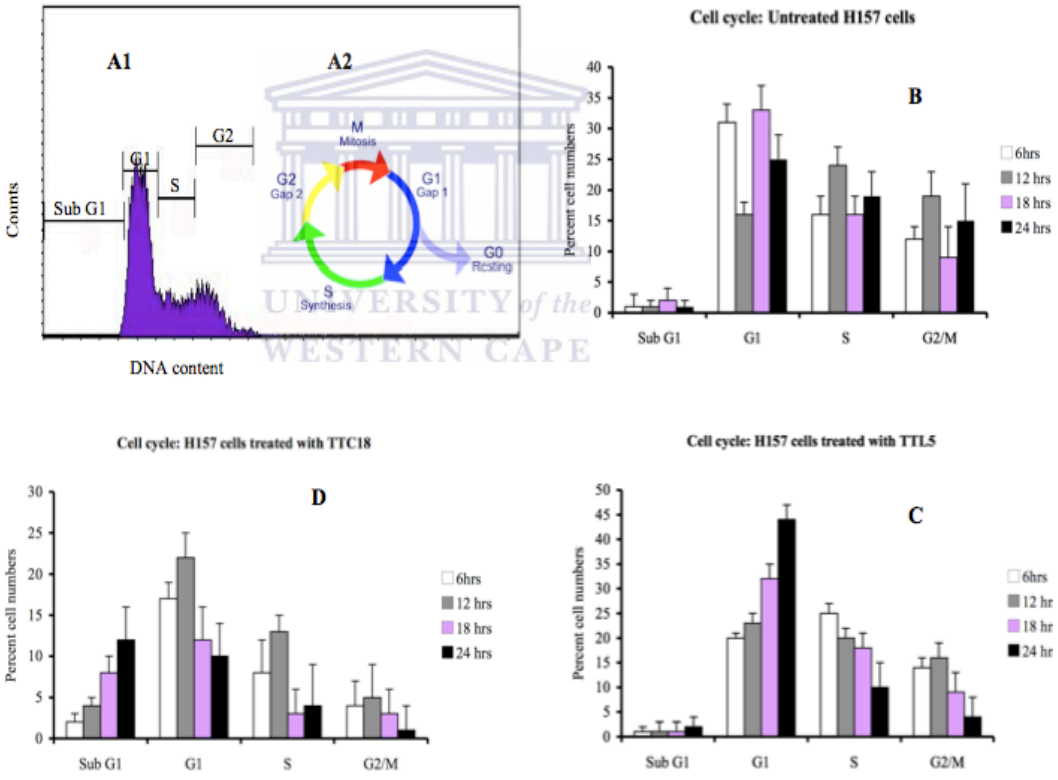
apoptosis or inhibiting the proper functioning of the cell cycle for example the cyclin dependent kinase inhibitors.

One of the methods used to perform cell cycle analysis is the propidium iodide (PI) method. PI is a nucleic dye that intercalates single or double stranded DNA/RNA and fluoresces red. Since PI also stains RNA, this should first be removed with ribonuclease. PI can be used to identify the percentage of cells that are in one of the three phases of the cell cycle or to demonstrate the presence of apoptotic bodies (fragmented cells with low DNA content), which appear in the Sub-G1 phase. The intensity of the PI signal is directly proportional to the DNA content. Following staining with the PI, cells that have lost DNA will take up less of the dye and will appear to the left of the G1 peak (the so- called "sub-G1 peaks", i.e. cells with lower fluorescence level than G0+G1 cells) and are hence considered apoptotic. The major disadvantage is that apoptotic G2-Phase cells that exhibit a reduced DNA content could represent the DNA content of a G1-cell. Therefore it may not be detected as apoptotic and this can result in an underestimation of the apoptotic population.

To assess whether TTC18 and TTL5 affect cell cycle progression, H157 cells were treated for 6, 12, 18 and 24 hours with 15μM of TTC18 and TTL5 (**Figure 3.4**). The sub-G1 population for the untreated (**Figure 3.4 C**) and TTL5 treated (**Figure 3.4 D**) cells were below 2%, while the sub-G1 population for TTC18 treated cells incrementally increased from 2% to 12% over the 24hr period. This increase in the

sub-G1 population was accompanied with a decrease in the G1 population. Cells treated TTL5 showed a time dependent increase in the G1 population, which appeared to be associated with a decrease in S and G2/M phases.

Figure 3.4: Cell cycle analysis of H157 cells. H157 cells were treated for 6, 12, 18 and 24 hours with 15µM of gold complex TTC18 and phosphine ligand TTL5 and stained with PI as described in chapter 2, section 2.2.10.2. Cells were analysed using a FACScan™ (Becton Dickson) instrument equipped with a 488 nm argon laser as a light source. Panel A shows how the analysis was done. Panel **A1** shows histogram plot of the DNA content on the X-axis and counts on the Y-axis. Indicated on the histogram are the three main stages (G1, G2 and S-phase) of the cell cycle and the Sub-G1 phase. Panel **A2** shows the stages of the cell cycle. Panels **B**, **C** and **D** show the cell cycle profile of untreated, TTL5 treated and TTC18 treated H157 cells, respectively. The experiment was done in triplicate and data is represented in a bar graph as the mean percentage cells.

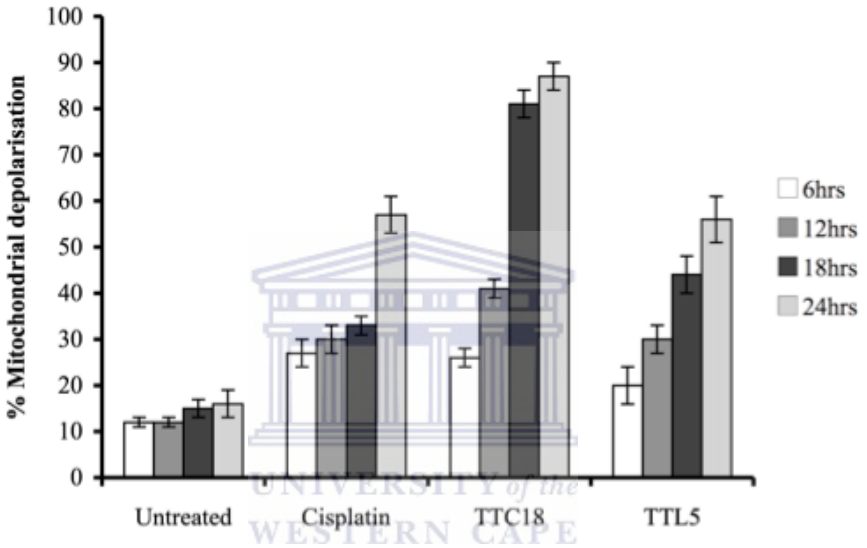


3.9 Evaluating mitochondrial depolarisation using the TMRE assay

There are several functions associated with mitochondria. Mitochondria are involved in bioenergetics, apoptosis and cell signalling (Lum and Nagley, 2003). Mitochondria have been implicated in influencing life and death decisions by initiating or inhibiting cell death (Krohn *et al*. 1999). The depolarisation of the mitochondrial membrane potential was shown to be required for the subsequent release of pro-apoptotic factors from the mitochondria. However, Krohn *et al*., (1999), showed that apoptosis can also occur in the absence of mitochondrial depolarisation.

The molecular probe tetramethylrhodamine ethyl ester (TMRE) can be used to assess mitochondrial membrane depolarisation or loss of the electrochemical gradient across the mitochondrial membrane (Jayaraman, 2005). TMRE is a cationic, lipohilic dye that accumulates inside the membrane regions of healthy functioning mitochondria according to the Nernst equation potential in a voltage dependent manner (Kronhn *et al*., 1999). TMRE fluoresces bright orange/red in viable cells, which dissipates when the cells become apoptotic. The loss of fluorescence can be measured by flow cytometry. To evaluate mitochondrial membrane depolarisation, H157 cells were treated for 6, 12, 18 and 24 hours with 30μM of the gold complex TTC18, the phoshine ligand TTL5. The mitochondrial membrane potential ($\Delta\Psi m$) was evaluated as described in chapter 2, **section 2.2.8** (**Figure 3.5**). Both TTC18 and TTL5 induced mitochondrial depolarisation in H157 cells in a time dependent manner. However, the number of cells with depolarised mitochondria was higher for TTC18 than TTL5.

Figure 3.5: Assessing mitochondrial depolarisation using TMRE. H157 cells were cultured in 6 well plates as described in chapter 2, **section 2.2.8**. The cells were either left untreated or treated with 30µM of TTC18, or TTL5. The cells were stained with TMRE at 6, 12, 18 and 24 hours. TMRE fluorescence was measured on a FACScan™(Becton Dickson) instrument using the FL-3 channel.



3.10 Summary

The toxicity of fifteen phosphine ligands and fifteen gold(I) complexes were evaluated on a panel of 13 cell lines. Light microscopy was used to assess whether 50μM of these compounds can induce any morphological changes in these cells. The IC_{50} values for all 30 compounds were determined for each cell line using the MTT assay. The pro-apoptotic activity of all 30 compounds was evaluated on the panel of cell lines. The cells were treated for 24 hours with 50μM of the compounds and apoptosis was assessed using the APOP*ercentage*™ assay. Based on the results obtained for the MTT assay and the APOP*ercentage*™ assay, the gold(I) complexes were more bioactive than the phosphine ligands. The gold complexes TTC2, TTC4, TTC18, TTC20, TTC22, TTC25 and the ligand TTL5 were noticeably the most active compounds. The sensitivity of the cell lines varied, with Jurkat cells being very susceptible to the compounds.

The two most active compounds TTC18 and TTL5 were further tested on two cell lines (H157 and Jurkat). Three additional apoptosis assays, the caspase-3 cleavage assay, the DNA fragmentation assay and the mitochondrial depolarisation assay were used to confirm the activation of apoptosis. TTC18 was more bioactive than TTL5 and Jurkat cells were more sensitive to the effects of the two compounds. The effects of TTC18 and TTL5 on the mitochondrial potential of H157 cells were assessed using TMRE probe.

The effects of TTC18 and TTL5 on the cell cycle regulation of H157 cells were investigated over a 24 hour period. TTC18 induced apoptosis in H157 cells, while TTL5

induced cell cycle block in the G2 phase.

CHAPTER FOUR OUTLINE

4.1 Introduction

4.2 Assessing ROS generation

4.3 Assessing lipid peroxidation

4.4 Evaluating the cytoprotective effects of antioxidants

4.5 Evaluating the uptake of TTC18 using ICP Mass Spectrometry analysis

4.6 Summary

CHAPTER FOUR: Assessing TTC18 and TTL5 as inducers of oxidative stress.

4.1 Introduction

Living cells are in a tightly regulated intracellular redox state, which is regulated by thiol containing molecules such as glutathione and thioredoxin. Redox homeostasis is important in order for biological processes such as DNA synthesis, cell cycle regulation, the transcriptional activation of genes, enzyme activation and programmed cell death (Arrigo, 1999). Redox homeostasis controls the level of intracellular reactive oxygen species (ROS), which are formed as side products of biological reactions that use the electron transfers, for example oxidative phosphorylation. It is also noted that small amounts of ROS are important in modulating cell metabolism, gene expression and also in post-translational modification of proteins (Sies, 1991). When the intracellular levels ROS produced by oxidizing agents such as peroxides, radiations, toxins, high doses of inflammatory cytokines or glutathione depriving drugs exceed the levels of antioxidants, ROS can become toxic causing oxidative injuries or oxidative stress (Arrigo, 1999). Oxidative stress has been implicated in many diseases including cancer atherosclerosis, acquired immunodeficiency syndrome (AIDS), rheumatoid arthritis (RA), Alzheimer's, aging and Parkinson's (Townsend *et al*., 2003). Several studies demonstrated that gold compounds to induce apoptosis by increasing intracellular levels of ROS (Townsend *et al*., 2003; Leonard *et al*., 2004; Wang *et al*., 2005; Omata *et al*., 2006),

The aim of this chapter is:

To further investigate the mechanisms through which the gold complex TTC18 and the phosphine ligand TTL5 induce apoptosis. This chapter will specifically evaluate the production of ROS in cultured cells treated with these compounds.

4.2 Assessing ROS generation

Several assays are available to assess intracellular ROS production. One such assay use 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), a non-fluorescent probe that freely permeates into cells. CM-H2DCFDA is not fluorescent and is hydrolysed to DCFH by intracellular esterases when inside the cell. DCFH is oxidized to the highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF) in the presence of ROS. DCF can be detected by flow cytometry and can therefore be used to detect intracellular ROS.

H157 and Jurkat cells were treated for 24 hours with 50μM TTC18 and TTL5. ROS production was evaluated using CM-H2DCFDA (**Figure 4.1**). Both TTC18 and TTL5 induced the production of ROS in H157 and Jurkat cells. However, ROS production in Jurkat cells was higher. Jurkat cells also produced higher levels of ROS in response to H_2O_2-treatment. In addition, TTC18 produced higher levels of ROS in comparison to TTL5 in H157 only.

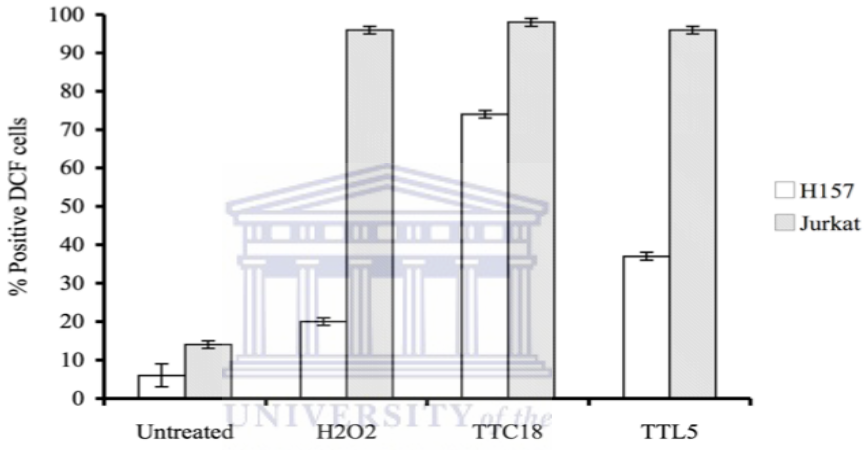


Figure 4.1: ROS production in Jurkat and H157 cells. Jurkat and H157 cells were treated for 24 hours with 50μM of TTC18 and TTL5. As a positive control, cells were also treated with 400μM of H_2O_2. The production of ROS was evaluated using the CM-H2DCFDA molecular probe. Cell fluorescence was measured by flow cytometry as described in chapter 2, **section 2.2.12**. The experiment was done in triplicate and data is represented in a bar graph as the mean percentage DCF positive cells.

4.3 Assessing lipid peroxidation

Lipid peroxidation is defined as the oxidative degradation of the cell membrane phospholipids and polyunsaturated fatty acids (PUFA) (Kelly *et al*., 1998) and the process can be catalyzed by free radicals (non enzymatic lipid peroxidation) or enzymes (enzymatic lipid peroxidation) (Halliwell and Chirico, 1993; Gutteridge, 1995; Türkdogan *et al*., 1998). During lipid peroxidation, free radicals remove electrons from lipids in the cell membranes resulting in cell damage and increased production of free radicals. It is the free radicals that cause oxidative damage to nuclear DNA and proteins (Türkdogan *et al*., 1998). Lipid hydroperoxides are the initial products that are formed when lipids are damaged by oxidative stress and are used as indicators of oxidative stress (Kelly *et al*., 1998; Halliwell and Chirico, 1993; Halliwell and Chirico, 1993; Kell *et al*., 1998; Shao *et al*., 2009; Catala, 2009). Lipid hydroperoxides have been reported by Niki *et al*., (2005) as unstable products of lipid peroxidation and are substrates for several enzymes including glutathione peroxidases and phospholipases. Some of the hydroperoxides generated as a result of advanced lipid peroxidation include 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxyl-2- hexenal (4-HHE) (Catala, 2009) and malondialdehyde (MDA) (Halliwell and Chirico, 1993; Niki *et el*., 2005).

Catala (2009) reported that hydroperoxides could be reduced to their corresponding alcohols by glutathione peroxides. However, Catala, (2009) reported that a decrease in glutathione peroxides could result in the degradation of the hydroperoxides that can lead to oxidative stress. Niki *et al*., (2005) reported that oxidative damage occurs when oxidative stress exceeds the antioxidant capacity of the cell. However, exposure to low

levels of stress may enhance the defence capacity of the cell.

Recent studies have shown that gold based compounds in both (I) and (III) oxidation states have pro-apoptotic and anti-proliferative properties against selected human cell lines and have been reported to induce apoptosis through the inhibition of thioredoxin reductase and subsequent increase in hydrogen peroxide and deregulation of mitochondrial functions which consequently leads to cell death (Kerimova *et al*., 2000; Rigobello *et al*., 2007; Casini *et al*., 2007; Rackham *et al*., 2007; Bindoli, 2009).

One of the ways to assess oxidative damage is the measurement of the end products of lipid peroxidation as they are generally accepted markers of oxidative stress (Williamson *et al*., 2008). A well-established assay for screening and monitoring lipid peroxidation is the thiobarbituric acid reactive substances (TBARS) assay (Botsoglou *et al*., 1994; Sheu *et al.,* 2003). Most researchers use the TBARS assay to evaluate presence of MDA in drugs, food, as well as human and animal tissue samples. MDA is a secondary lipid oxidation product. However, because thiobarbituric acid reacts with a number of other oxidation products including 4-HNE, other unsaturated aldehydes and endoperoxides from enzymatic routes, the TBARS assay is not very specific. The principle of the TBARS assay is based on the production of a coloured adduct from the reaction of lipid peroxidation products. One of the lipid peroxidation products is MDA. The MDA forms a 1:2 adduct with thiobarbituric acid (TBA). This adduct can be measured calorimetric method at 532nm or fluorometric method measured at 553nm (Wei *et al*., 2000) or by HPLC (Halliwell and Chirico, 1993; Botsoglou *et al.,* 1994).

Oxidative damage in H157 cells treated for 24 hours with TTC18 and TTL5 was assessed using the TBARS assay to evaluate the production of MDA (**Figure 4.2**) using the calorimetric method. H157 cells produced MDA in response to TTC18 and TTL5 treatment. However, MDA production was significantly higher for TTL5 in comparison to TTC18.

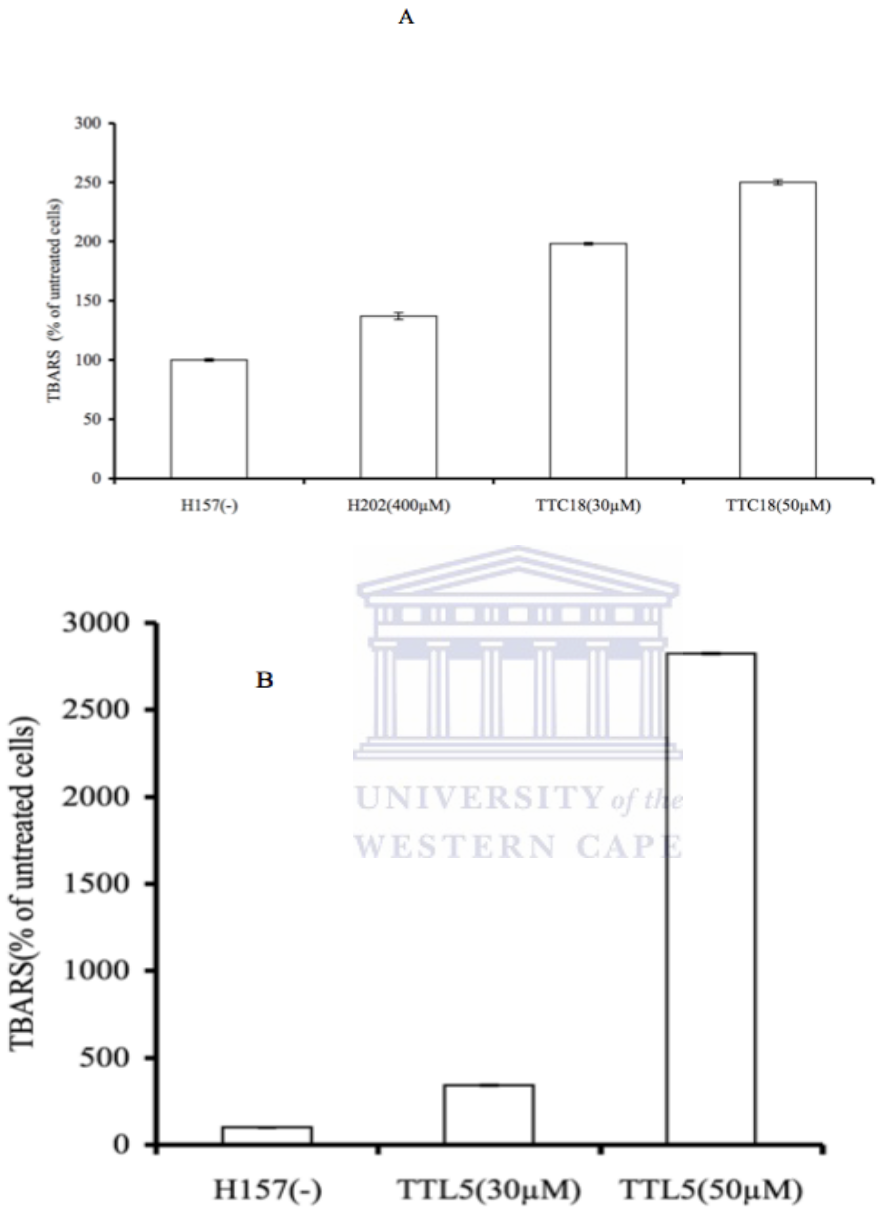


Figure 4.2: Assessing oxidative stress using the TBARS assay. H157 cells were either left untreated or treated for 24 hours with two concentrations (30µM or 50µM) of the gold complex TTC18 (**A**) and the phosphine ligand TTL5 (**B**). As a positive control, the cells were also treated with 400µM H_2O_2. TBARS was determined using the procedure described in chapter 2, **section 2.2.13**.

4.4 Evaluating the cytoprotective effects of antioxidants

Several antioxidants have been reported to protect cells from the effects of free radicals (Mittler, 2002), and xenobiotics (Sandermann Jr., 1992; Nordberg and Arnér, 2001). Glutathione (GSH) is the main antioxidant regulatory system in the cell (Arrigo, 1999; Leonard *et al*., 2004). Glutathione is one of the systems that cells use to maintain redox homeostasis and to cope with high levels of ROS produced during oxidative stress (Arrigo, 1999). The cells protect themselves from excess ROS by blocking the production of ROS, or by sequestering transition metals that have free electrons, or by scavenging or detoxifying the ROS with enzymes, or antioxidants such as vitamin C or E or thiol-containing molecules (Arrigo, 1999). A number of anticancer drugs have been implicated to exert their cytotoxic effects by altering GSH levels in the cells (Sies, 1991) and as noted by Tew (1994) and also by Stordal and Davey (2007), increased levels of cellular GSH could play a major role in desensitising cells to apoptosis. Wang *et al.*, (2005) further stated that cells treated with anticancer agents may inhibit GSH and this may facilitate ROS accumulation in the cells and increase cytotoxicity. Leonard *et al*, (2004) further noted that cells attempt to defend themselves from the cytotoxic effects of ROS by neutralizing the effects of ROS with antioxidants and that the balance between oxidants and antioxidant defences are critical in maintaining redox homeostasis.

Thiol-containing molecules such as reduced glutathione and ascorbic acid (vitamin C) are known to be strong antioxidants (Stordal and Davey, 2007; Cotgreave and Gerdes, 1998), and Diethyldithiocarbamate (DDTC)

and Pyrrolidine dithiocarbamate (PDTC) are widely used dithiocarbamates in clinical use (Zhu *et al*., 2002). Many of the biological effects of dithiocarbamates are a result of their metal-chelating properties (Schreck *et al*., 1992; Zhu *et al*., 2002). Dithiocarbamates potentially inhibit/block the damaging effects of oxidizing compounds (Schreck *et al*., 1992). Enzymes such as superoxide dismutase and catalase also play important roles in converting superoxide to H_2O.

In **sections 4.2** and **4.3** it was demonstrated that that the gold complex TTC18 and phosphine ligand TTL5 induced the production of intracellular ROS, which is also associated with oxidative stress. In order to determine if antioxidants could protect the Jurkat, H157 and KMST-6 cells from the oxidative effects of the gold complex (TTC18) and the phosphine ligand (TTL5), the cells were pre-treated with several antioxidants, which included vitamin C (**Figure 4.3**), PDTC (**Figure 4.4**), DDTC (**Figure 4.5**), catalase (**Figure 4.6**) and L-glutathione (**Figure 4.7**).

Treatments with the increase in concentrations of antioxidants only were not toxic to the cells (**Figure 4.3-A**, **Figure 4.4-A**, **Figure 4.5-A**, **Figure 4.6-A** and **Figure 4.7-A**). Pre-treatments with vitamin C (**Figure 4.3-B**), PDTC (**Figure 4.4-B**) DDTC, (**Figure 4.5-B**), catalase (**Figure 4.6-B**), and L-glutathione (**Figure 4.7-B**) failed to protect Jurkat, H157 and KMST-6 cells from the effects of TTC18 and TTL5. Since pre-treatment with the antioxidants failed to protect the cells against the effects of TTC18 and TTL5, the cells were also concurrently treated with the antioxidants and TTC18 or TTL5. Concurrent treatment with vitamin C, PDTC, DDTC also failed to protect the cells (data not shown). However, the concurrent treatment with L-glutathione and TTC18 protected H157 cells

against the effects of TTC18 (**Figure 4.7-C**). The cell viability increased from 2% (TTC18 only) to 80% (concurrent treatment with L-glutathione and TTC18). However, Jurkat and KMST-6 cells were not protected by L-glutathione.

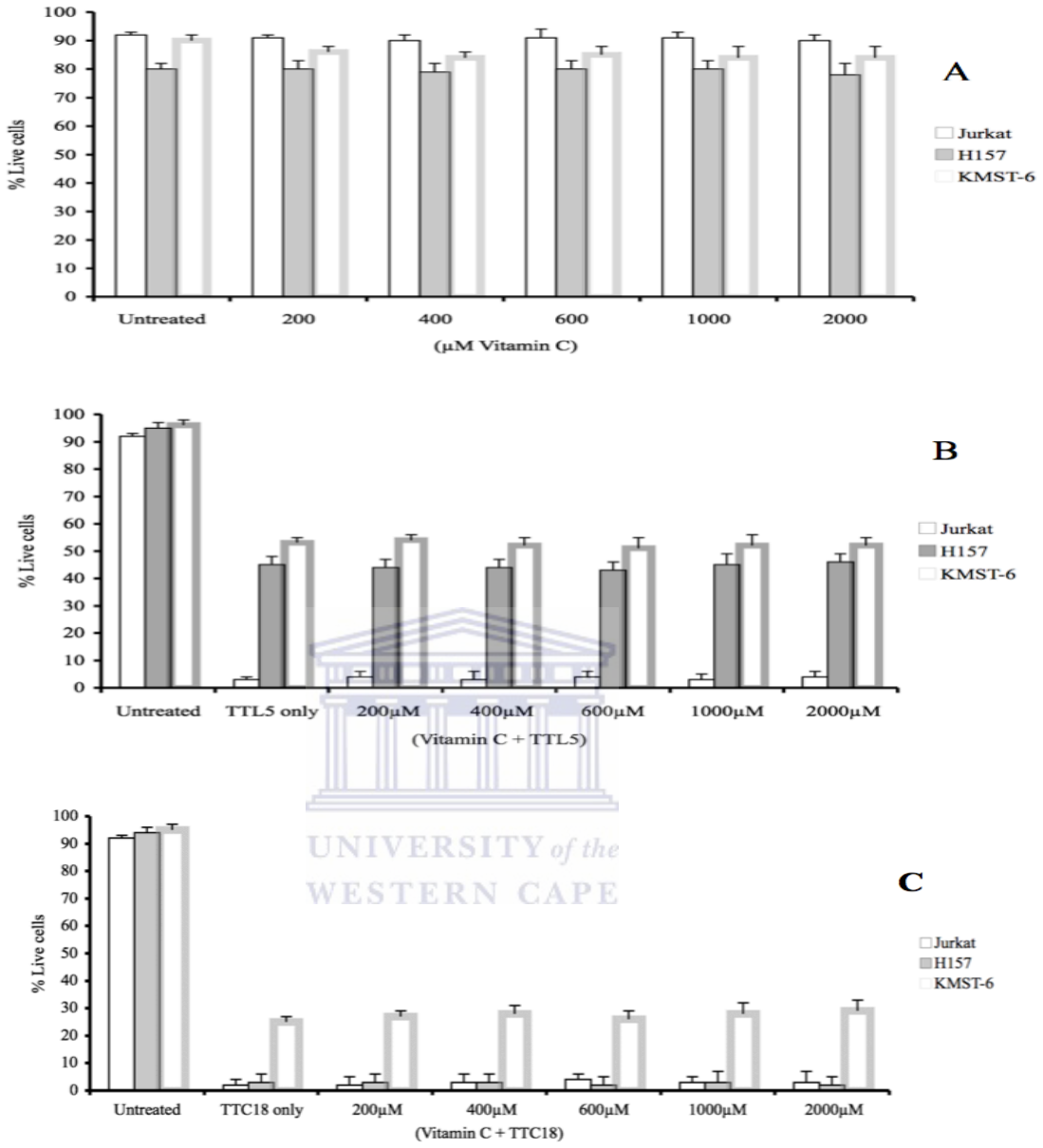


Figure 4.3: Investigating the cytoprotective potential of vitamin C. Jurkat, H157 and KMST6 cell were either left untreated or treated with increasing concentrations of vitamin C (200 to 2 000µM) for 24 hours and evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay (panel **A**). To investigating the cytoprotective potential of the antioxidant vitamin C, the cells were pre-treated with 2000µM of vitamin C for 24 hours then treated with 50µM of phosphine ligand TTL5 (panel **B**) or 50µM of gold complex TTC18 (panel **C**) and evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay. The experiment was done in triplicate and data is represented in bar graphs as the mean percentage apoptotic cells.

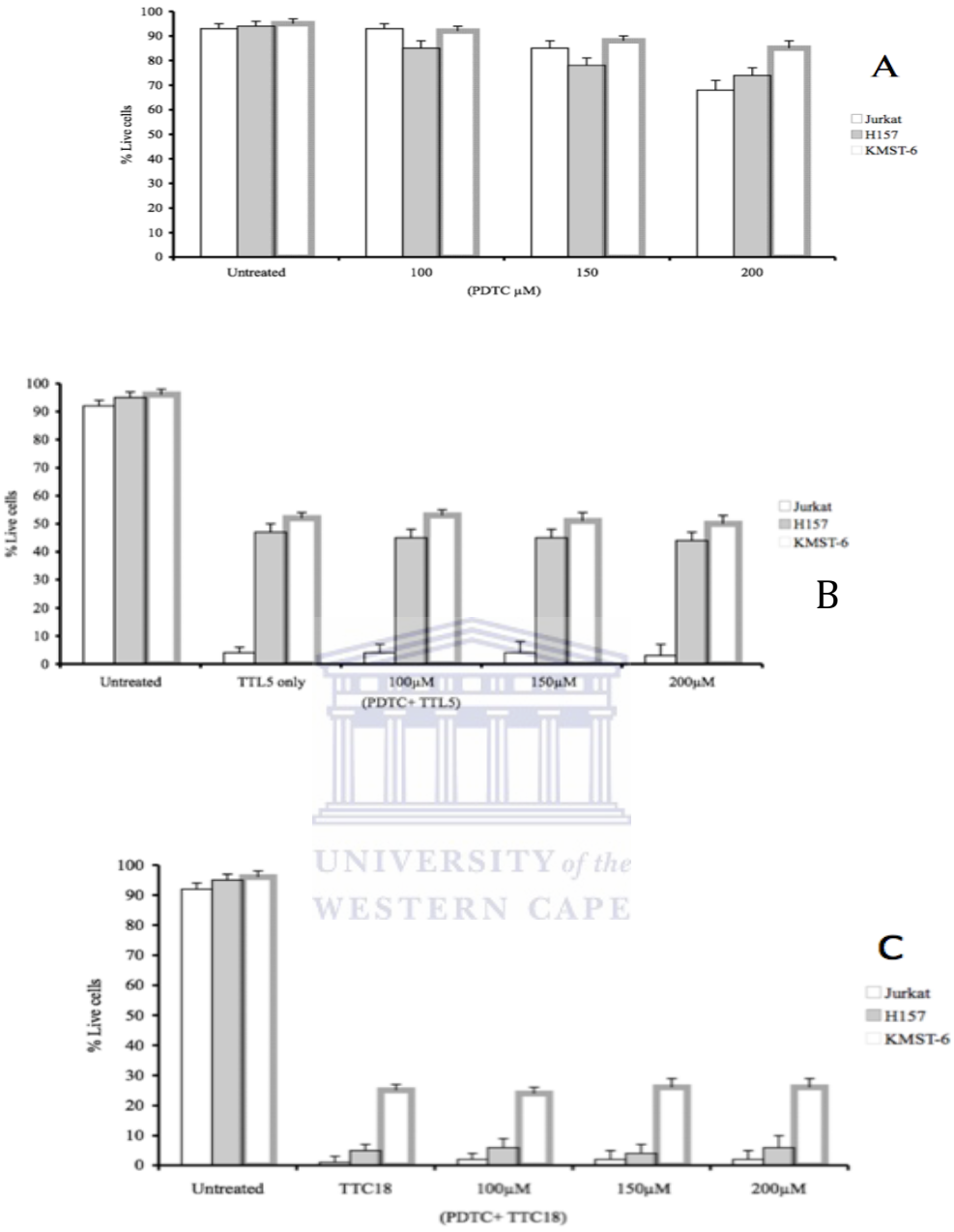


Figure 4.4: Investigating the cytoprotective potential of PDTC. Jurkat, H157 and KMST6 cell were either left untreated or treated with increasing concentrations of PDTC (100 to 200µM) for 24 hours and evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay (panel **A**). To investigating the cytoprotective potential of the antioxidant PDTC, the cells were pre-treated with 100µM of PDTC for 24 hours then treated with 50µM of phosphine ligand TTL5 (panel **B**) or 50µM of gold complex TTC18 (panel **C**) and evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay. The experiment was done in triplicate and data is represented in bar graphs as the mean percentage apoptotic cells.

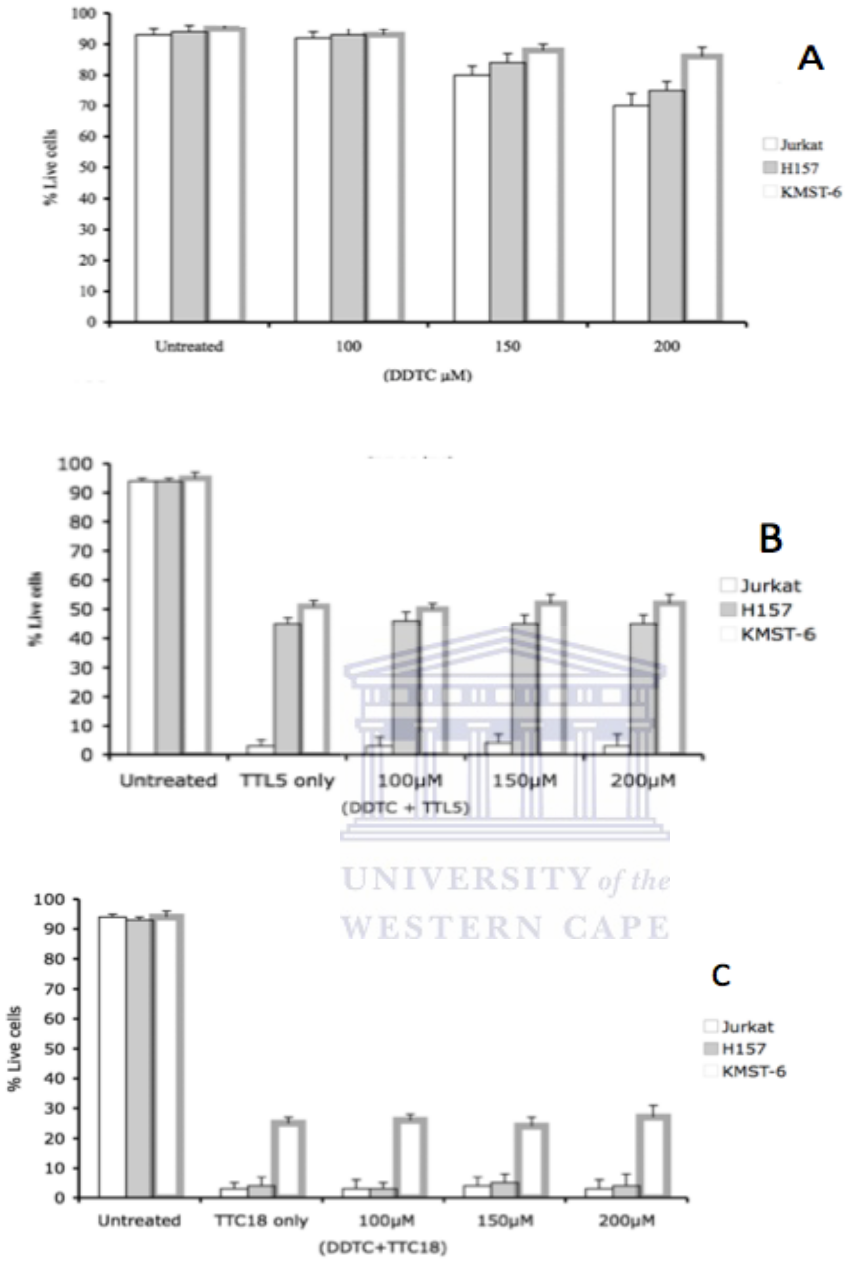


Figure 4.5: Investigating the cytoprotective potential of DDTC. Jurkat, H157 and KMST6 cell were either left untreated or treated with increasing concentrations of DDTC (100 to 200µM) for 24 hours and evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay (panel **A**). To investigating the cytoprotective potential of the antioxidant DDTC, the cells were pre-treated with 100µM of DDTC for 24 hours then treated with 50µM of phosphine ligand TTL5 (panel **B**) or 50µM of gold complex TTC18 (panel **C**) and evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay. The experiment was done in triplicate and data is represented in bar graphs as the mean percentage apoptotic cells.

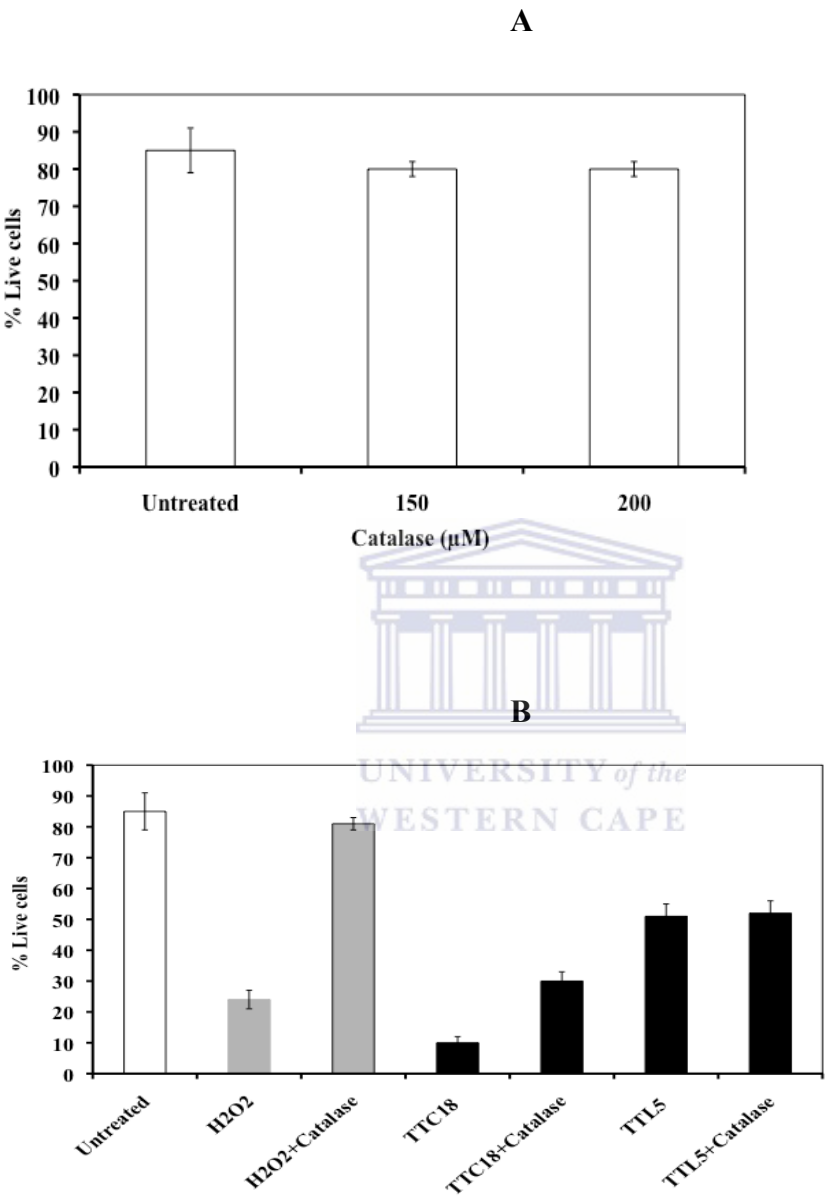


Figure 4.6: Investigating the cytoprotective potential of catalase. H157 cells were either left untreated or treated with two concentrations of catalase (150 and 200µM) for 24 hours and evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay (panel **A**). To investigating the cytoprotective potential of the catalase, the cells were pre-treated with 150µM catalase for 24 hours and then treated with 50µM of phosphine ligand TTL5 or 50µM of gold complex TTC18 (panel **B**) and evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay. The experiment was done in triplicate and data is represented in bar graphs as the mean percentage apoptotic cells.

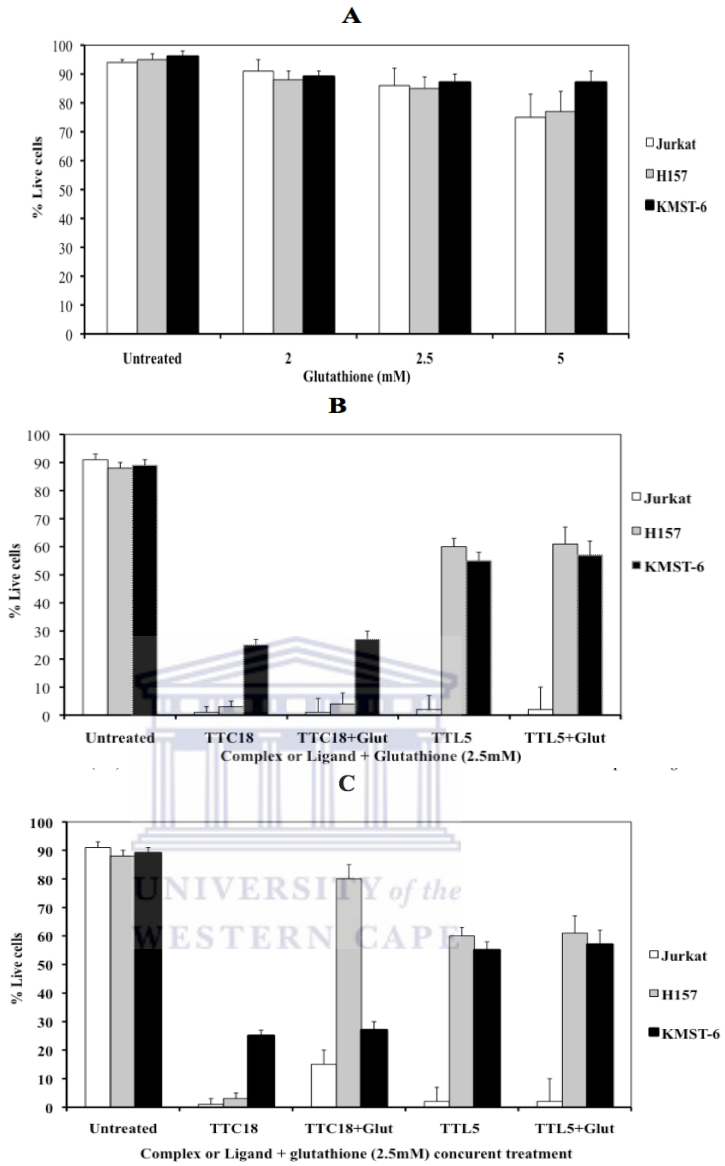


Figure 4.7: Investigating the cytoprotective potential of L-glutathione. Jurkat, H157 and KMST6 cell were either left untreated or treated with increasing concentrations of L-glutathione (2 to 5mM) for 24 hours and evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay (panel **A**). To investigating the cytoprotective potential of the antioxidant L-glutathione, the cells were pre-treated for 24 hours with 2.5mM L-glutathione then treated for 24 hours with 50µM of phosphine ligand TTL5 or 50µM of gold complex TTC18 (panel **B**). Alternatively, the cells were co-treated for 24 hours with 2.5mM L-glutathione and 50µM TTL5 or 50µM TTC18 (panel **C**). The cells were analysed for the induction of apoptosis using the APOP*ercentage*™ apoptosis assay. The experiment was done in triplicate and data is represented in bar graphs as the mean percentage apoptotic cells.

4.5 Evaluating the uptake of TTC18 using ICP Mass Spectrometry analysis

In section 4.4 it was shown that concurrent treatment with TTC18 and L-glutathione protects H157 cells against apoptosis. In order to elucidate whether L-glutathione affects cellular uptake of TTC18, the uptake assay described in chapter two, **section 2.2.15** was performed. The role of speciation in correlation with cellular uptake and cytotoxicity can aid in determining the amount of drug to treat the cells with (Dopp *et al*., 2010). In many cases for bioactivity to occur, the host cells must be able to take up the drug (Ghezzi *et al*., 2004), although compound uptake does not necessarily constitute to bioactivity. Some compounds maybe taken up by cells but remain biologically inactive (Okada *et al*., 1993; McKeage *et al*., 2000). In this section the uptake of the most active gold complex TTC18 over a 8-hour period was evaluated in H157 cells in the absence and presence of the antioxidant, L-glutathione. **Figure 4.8** shows that the amount of gold present in H157 cells were much lower when cells are concurrently treated with TTC18 and L-glutathione.

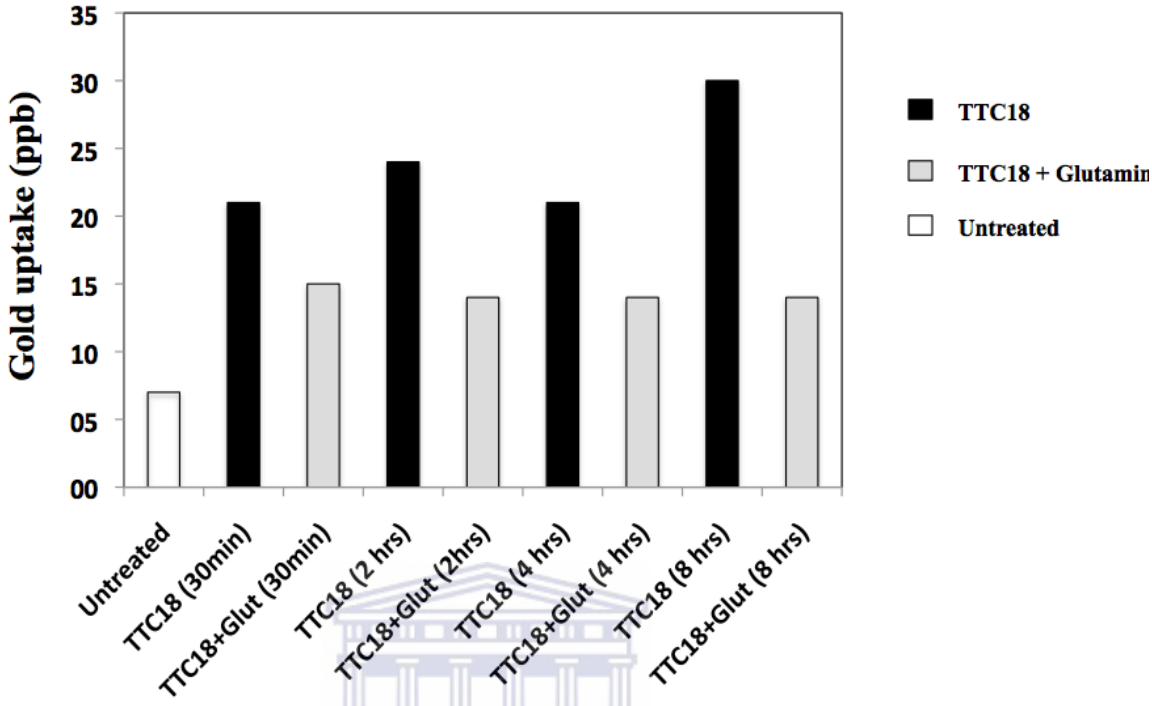


Figure 4.8: Assessing the cellular uptake of TTC18. H157 cells were either left untreated or treated with 30µM of the gold complex (TTC18) for 30min, 2hrs, 4hrs and 8hrs in the presence and absence of 2.5mM L-glutathione. Following treatments, the Au uptake assay described in chapter 2, **section 2.2.15.**

4.6 Summary

TTL18 and TTL5 induced ROS in both Jurkat and H157 cells. However, Jurkat and H157 responded very differently to the treatments. Close to 100% of the Jurkat cells treated with TTL18, TTL5 and the positive control (H_2O_2) were positive for ROS production, while the response of H157 cells varied depending on the treatment (figure 4.1). TTC18 induced ROS in a higher number of H157 cells. Both TTL5 and TTC18 induced oxidative stress in H157 cells. However, treatment with 50μM TTL5 produces 10× more MDA in these cells (figure 4.2). The lipid peroxidation caused by TTL5 in H157 cells is therefore significantly higher. Pre-treatment of H157, Jurkat and KMST-6 cells with antioxidants (vitamin C, PDTC, DDTC catalase and L-glutathione) failed to protect these cells against the effects of TTL5 and TTC18 (figures 4.3-4.6). Concurrent treatment with the gluthatione and TTC18 protected H157 cells but not Jurkat and KMST-6 cells against the effects of TTC18 (figure 4.7). The ICP-MS results show that H157 cells take up TTC18 within 30 min of the treatment and that the concurrent treatment with L-glutathione reduces the uptake of TTC18 into H157 cells (figure 4.8).

CHAPTER FIVE OUTLINE

5.1 Introduction

5.2 Gold complexes as inhibitors of thioredoxin and glutathione

5.3 Cloning of *Trx-2*

5.4 Over expression of Trx-2 in H157 cells

5.5 Evaluating the cytoprotective potential of Trx-2 over expression

5.6 Summary

CHAPTER FIVE: Evaluation of cytoprotective potential of thioredoxin.

5.1 Introduction

As discussed previously in chapter four (**section 4.4**), there are several antioxidants known to protect cells from xenobiotics. Two other major intracellular redox systems that have been reported to protect cells from xenobiotics are the thioredoxin (Trx) and glutathione systems, which also play other roles such as controlling cell proliferation and cancer development (Marks, 2006). The Trx system is composed of thioredoxin (Trx), thioredoxin reductase (TrxR1) and nicotinamide adenine dinucleotide phosphate (NADPH) (Arnér and Holmgren, 2006). Peter Reichard and co-workers discovered the thioredoxin system in 1964. They reported that the system involves hydrogen donation for the enzymatic synthesis of cytidine deoxyribonucleoside diphosphate by ribonucleotide reductase, which was observed in *Escherichia coli* (Holmgren and Lu, 2010). Since the system was reported, many studies have shown that thioredoxin reductase (TrxR) is a selenoenzyme, which has three isoforms namely: TrxR1 found in the cytosol, TrxR2 found in mitochondria and TrxR3 or TGR (thioredoxin glutathione reductase) found mainly in testis (Madeja *et al*., 2005; Holmgren and Lu, 2010). The Trx enzyme contains a conserved –Cys–Gly–Pro–Cys– active site, which is essential for the redox regulatory function of the Trx (Madeja *et al*., 2005; Holmgren and Lu, 2010). TrxR performs various biological functions that are essential in life of higher organisms (Bindoli *et al*., 2009). The studies done by Bindoli *et al*., (2009), showed that the disruption of either *TrxR1* or *TrxR2* genes is embryonic lethal phenotype. It was shown that TrxR1 null embryos were affected mainly by compromised cell proliferation whereas TrxR2 null embryos suffered from severe anaemia and improper heart

development (Bindoli *et al*., 2009). On the other hand, it was shown that TrxR is over expressed in many tumour cells and the increased presence of TrxR is thought to contribute to drug resistance **(**Madeja *et al*., 2005; Marks, 2006; Casini, 2008; Che and Siu, 2010). Trx is therefore emerging as a new target for anti-cancer drug discovery (Arnér and Holmgren, 2006; Liu *et al*., 2009).

5.2 Gold compounds as inhibitors of thioredoxin and glutathione

Several studies have reported the use of Auranofin and other gold (I) complexes as anti-arthritic drugs, and also showed that gold complexes inhibit the growth of cultured tumour cells *in vitro* and many have shown to have anti-mitochondrial activity (McKeage, 2002). Additionally, other studies have shown that gold complexes induced apoptosis in a variety of cancer cells as well as cancer cells that had acquired resistance to specific anti-cancer drugs (Marks, 2006; Powis and Kirkpatrick, 2007). Their mode of action has been attributed to inhibition of mitochondrial and cytosolic proteins mainly glutathione and thioredoxin systems (Arnér and Holmgren, 2006; Che and Siu, 2010). It has been shown that inhibition of glutathione and thioredoxin reductase (Tiekink, 2002; Gandin, 2010), results in the alteration in the balance of hydrogen peroxide production and its removal results in the disturbance of the normal electron flow along the respiratory chain (Arnér and Holmgren, 2006; Cox, 2008; Ott, 2009). The mitochondrial respiratory chain produces superoxide anion that dismutes to hydrogen peroxide and oxidizes thioredoxin in a reaction mediated by peroxiredoxin (Bindoli *et al*., 2009). According to Bindoli *et al*., (2009), gold(I/III) complexes inhibit thioredoxin reductase, and inhibition of TrxR leads to its own accumulation as well as accumulation of hydrogen peroxide inside the cell. Bindoli *et al*., (2009), further reported that increased accumulation of H_2O_2 results in

mitochondrial perturbations leading to the opening of the mitochondrial permeability transition pore and/or increase in the permeability of the mitochondrial outer membrane consequently releasing cytochrome *c* as well as H_2O_2 to the cytosol where H_2O_2 causes oxidation of Trx1, and oxidation of mitochondrial thioredoxin (*Trx-2*), which cannot be reduced back to thioredoxin reductase. Alteration of the redox state results in the creation of conditions that enhance apoptosis (Marzano *et al*., 2007), for instance stimulation of the MAP kinases pathways that leads to cell death (Bindoli *et al*., 2009) and release of cytochrome *c* that binds with apaf-1, ATP and procaspase-9 to form apoptosome complex that activate caspase-9 and in turn activate down stream executioner caspases leading to cell death (Rigobello *et al*., (2004). Both cancer cells and non cancerous cells are known to be resistant to permeability transition (Rigobello *et al*., 2004), therefore, compounds or drugs that disrupt permeability transition, are considered to be of therapeutical value (Bindoli *et al*., 2009; Liu *et al*., 2009; Che and Siu, 2010). The over expression of mitochondrial thioredoxin (*Trx-2*) has been reported to protect cells from the effects of oxidizing agents and to assess this cyto-protective effect against the gold complex or phosphine ligand oxidative cell death, *Trx-2* was over expressed and cells were subjected to apoptosis induction.

The aim of this chapter:

To assess whether the over expression of Trx-2 protects H157 cells against the effects of the gold complex (TTC18) and the phosphine ligand (TTL5).

5.3 Cloning of *Trx-2*

Forward and reverse primers were designed for the amplification of *Trx-2* and cloning of the PCR product into the pcDNA™ 3.1 D/V5-His-TOPO cloning vector. Total RNA was

isolated from KMST-6 cells. The total RNA was used as a template to synthesize a cDNA library of KMST-6 cells using the ImProm-II™ Reverse Transcriptase system as described in chapter 2, **section 2.2.18**. The cDNA library was used as template to amplify *Trx-2* (**Figure 5.1**). The expected size for the human *Trx-2* gene product is 496 bp. The PCR produced a 500bp product, which is in agreement with the expected result.

The *Trx-2* PCR product was cut from the agarose gel and the DNA was isolated from the gel as described in chapter 2, **section 2.2.26**. The isolated DNA was used as an insert for the ligation reaction into the pcDNA™ 3.1 D/V5-His-TOPO cloning vector as described in chapter 2, **section 2.2.24.3**. The ligation products were transformed into One Shot® TOP10 Chemically competent *E.coli* cells (described in chapter 2, **section 2.2.24.4**). The transformed colonies were screened by colony PCR for the presence of the *Trx-2* PCR product (described in chapter 2, **section 2.2.24.4**). Six colonies were screened for the presence of *Trx-2* (**Figure 5.2**). Four of the colonies produced a ~500bp PCR product. Glycerol stocks were prepared for all four colonies as described in chapter 2, **section 2.2.23**. Colony number 4 was selected for sequence analysis. The colony was plated on Nutrient agar and submitted to Inqaba Biotech core sequencing facility for sequencing. The DNA sequence data was analyzed using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/). The DNA sequence was searched against the human RefSeq database (blastn). The BLAST output is shown in **Figure 5.3**. The human Trx-2 DNA sequence (accession number, NM_012473.3) was the most significant match. The alignment between clone 4 and NM_012473.3 shows an identity or match of 100% for the 496 base pairs.

The DNA sequence for colony number 4 was translated into protein sequence using the ExPASy Bioinformatics Resource Tool (http://web.expasy.org/translate). The translated sequence aligned with the protein sequence for human Trx-2 (accession number, NP_036605.2). The two protein sequences were aligned using the Align Two (or more) sequences BLAST tool (bl2seq) (**Figure 5.4**). The alignment between clone 4 and NP_036605.2 shows an identity or match of 100% for the 165 residues.

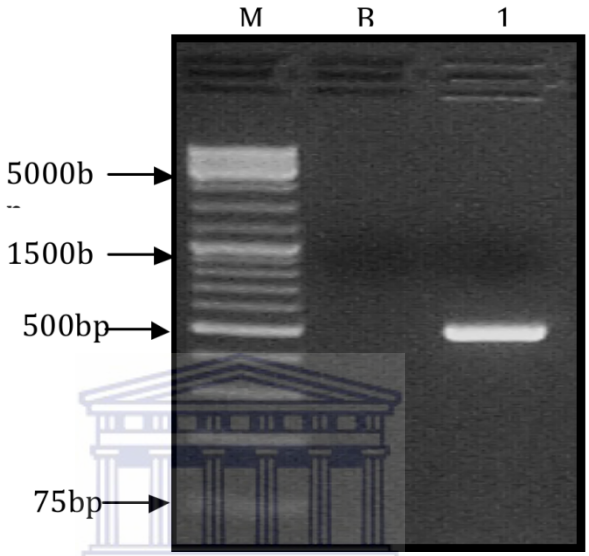


Figure 5.1: PCR amplification of *Trx-2*. Trx-2 was PCR amplified from a cDNA library prepared from KMST-6 cells and electrophoresed on a 1% agarose gel. Lane M is the molecular weight marker, lane B is the negative control (water control) and lane 1 is the PCR product produced from KMST-6 cells.

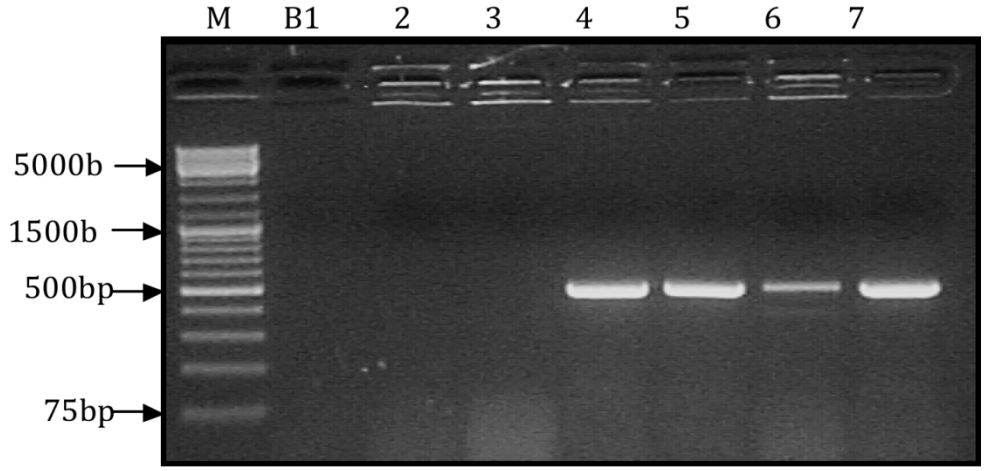
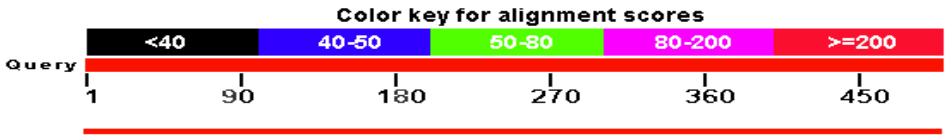


Figure 5.2: Screening transformed colonies for the presence of *Trx-2* by colony PCR. Six colonies were selected for screening. The PCR products were electrophoresed on a 1% agarose gel. Lane M is the molecular weight marker while lanes B1 is the water blank, while lanes 2, 3, 4, 5, 6 and 7 is the screen for the six different clones.

A



B

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
NM_012473.3	Homo sapiens thioredoxin 2 (TXN2), nuclear gene encoding	917	917	100%	0.0	100%	UEGM

C

>ref|NM_012473.3| UEGM Homo sapiens thioredoxin 2 (TXN2), nuclear gene encoding mitochondrial protein, mRNA
Length=1342

GENE ID: 25828 TXN2 | thioredoxin 2 [Homo sapiens] (Over 10 PubMed links)

Score = 917 bits (496), Expect = 0.0
Identities = 496/496 (100%), Gaps = 0/496 (0%)
Strand=Plus/Plus

```
Query  1    TGGCTCAGCGACTTCTTCTGAGGAGGTTCCTGGCCTCTGTCATCTCCAGGAAGCCCTCTC  60
Sbjct  79   TGGCTCAGCGACTTCTTCTGAGGAGGTTCCTGGCCTCTGTCATCTCCAGGAAGCCCTCTC  138
Query  61   AGGGTCAGTGGCCACCCCTCACTTCCAGAGCCCTGCAGACCCCACAATGCAGTCCTGGTG  120
Sbjct  139  AGGGTCAGTGGCCACCCCTCACTTCCAGAGCCCTGCAGACCCCACAATGCAGTCCTGGTG  198
Query  121  GCCTGACTGTAACACCCAACCCAGCCCGGACAATATACACCACGAGGATCTCCTTGACAA  180
Sbjct  199  GCCTGACTGTAACACCCAACCCAGCCCGGACAATATACACCACGAGGATCTCCTTGACAA  258
Query  181  CCTTTAATATCCAGGATGGACCTGACTTTCAAGACCGAGTGGTCAACAGTGAGACACCAG  240
Sbjct  259  CCTTTAATATCCAGGATGGACCTGACTTTCAAGACCGAGTGGTCAACAGTGAGACACCAG  318
Query  241  TGGTTGTGGATTTCCACGCACAGTGGTGTGGACCCTGCAAGATCCTGGGGCCCGAGGTTAG  300
Sbjct  319  TGGTTGTGGATTTCCACGCACAGTGGTGTGGACCCTGCAAGATCCTGGGGCCCGAGGTTAG  378
Query  301  AGAAGATGGTGGCCAAGCAGCACGGGAAGGTGGTGATGGCCAAGGTGGATATTGATGACC  360
Sbjct  379  AGAAGATGGTGGCCAAGCAGCACGGGAAGGTGGTGATGGCCAAGGTGGATATTGATGACC  438
Query  361  ACACAGACCTCGCCATTGAGTATGAGGTGTCAGCGGTGCCCACTGTGCTGGCCATGAAGA  420
Sbjct  439  ACACAGACCTCGCCATTGAGTATGAGGTGTCAGCGGTGCCCACTGTGCTGGCCATGAAGA  498
Query  421  ATGGGGACGTGGTGGACAAGTTTGTGGGCATCAAGGATGAGGATCAGTTGGAGGCCTTCC  480
Sbjct  499  ATGGGGACGTGGTGGACAAGTTTGTGGGCATCAAGGATGAGGATCAGTTGGAGGCCTTCC  558
Query  481  TGAAGAAGCTGATTGG  496
Sbjct  559  TGAAGAAGCTGATTGG  574
```


Figure 5.3: Analysis of sequence data for colony number 4. BLAST output for DNA sequence obtained for colony 4. **A** shows a graphic summary of the BLAST hits on the query sequence (colony number 4), **B** shows a description of the most significant hit and **C** shows the alignment to the most significant hit.

```
>lcl|25285 gi|21361403|ref|NP_036605.2| thioredoxin, mitochondrial precursor
[Homo sapiens]
Length=166

 Score =  342 bits (876),  Expect = 1e-125, Method: Compositional matrix adjust.
 Identities = 165/165 (100%), Positives = 165/165 (100%), Gaps = 0/165 (0%)

Query  1    MAQRLLLRRFLASVISRKPSQGQWPPLTSRALQTPQCSPGGLTVTPNPARTIYTTRISLT  60
            MAQRLLLRRFLASVISRKPSQGQWPPLTSRALQTPQCSPGGLTVTPNPARTIYTTRISLT
Sbjct  1    MAQRLLLRRFLASVISRKPSQGQWPPLTSRALQTPQCSPGGLTVTPNPARTIYTTRISLT  60

Query  61   TFNIQDGPDFQDRVVNSETPVVVDFHAQWCGPCKILGPRLEKMVAKQHGKVVMAKVDIDD  120
            TFNIQDGPDFQDRVVNSETPVVVDFHAQWCGPCKILGPRLEKMVAKQHGKVVMAKVDIDD
Sbjct  61   TFNIQDGPDFQDRVVNSETPVVVDFHAQWCGPCKILGPRLEKMVAKQHGKVVMAKVDIDD  120

Query  121  HTDLAIEYEVSAVPTVLAMKNGDVVDKFVGIKDEDQLEAFLKKLI  165
            HTDLAIEYEVSAVPTVLAMKNGDVVDKFVGIKDEDQLEAFLKKLI
Sbjct  121  HTDLAIEYEVSAVPTVLAMKNGDVVDKFVGIKDEDQLEAFLKKLI  165
```

Figure 5.4: Sequence alignment of clone 4 and Trx-2. The DNA sequence of clone 4 was converted into protein sequence and aligned to the protein sequences of Trx-2 (NP_036605.2).

5.4 Over expression of Trx-2 in H157 cells

The Trx-2 PCR product was successfully cloned into the pcDNA™ 3.1 D/V5-His-TOPO cloning vector to produce the pcDNA™ 3.1 D/V5-His-TOPO/Trx-2 construct. Plasmid DNA was prepared for colony 4 as described in chapter two, **section 2.2.27**. This DNA was used to transfect H157 cells as described in chapter two, **section 2.2.29**. Stably transfected cells were generated by selecting the transfected cells in G418. The over expression of the Trx-2 in the transfected cells was investigated using Western blot analysis (**Figure 5.5**). Total protein was isolated from H157 cells and mutant H157 cells that was transfected with the pcDNA™ 3.1 D/V5-His-TOPO/Trx-2 vector as described in chapter two, **section 2.2.31**. The protein samples were electrophoresed on a polyacrylamide gel as described in chapter two, **section 2.2.31** and Western blot analysis was performed as described in chapter two, **section 2.2.32** using an anti-Trx-2 antibody. **Figure 5.5** shows that cells transfected with the pcDNA™ 3.1 D/V5-His-TOPO/Trx-2 vector express higher levels of Trx-2 compared to H157 control cells.

To confirm that the increased levels of Trx-2 also increase the thioredoxin reductase activity in H157 cells, the thioredoxin reductase activity was measured in H157 cells and the Trx-2 transfected H157 cells as described in chapter 2, **section 2.2.16**. **Figure 5.6** shows that the thioredoxin reductase activity) is higher in the Trx-2 transfected H157 cells (i.e. cells over expressing Trx-2 compared to untreated controls. Treatment with the gold complex TTC18 completely suppressed the thioredoxin reductase activity in H157 cells, while treatment with the phosphine ligand TTL5 had very little or no significant effect on the thioredoxin reductase activity.

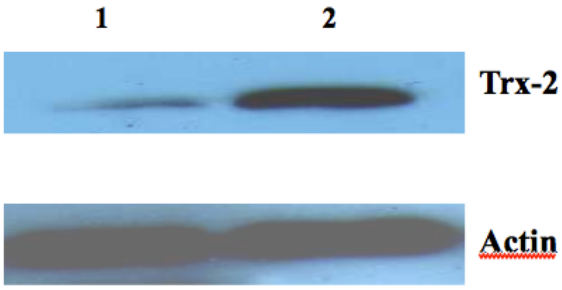


Figure 5.5: Evaluating the expression of Trx-2 by Western blot analysis. The expression of Trx-2 in H157 (lane 1) and H157 cells transfected with pcDNA™ 3.1 D/V5-His-TOPO/Trx-2 vector (lane 2) were investigated using the anti-Trx-2 antibody. The anti-actin antibody was used to confirm equal total protein loading in lanes 1 and 2.

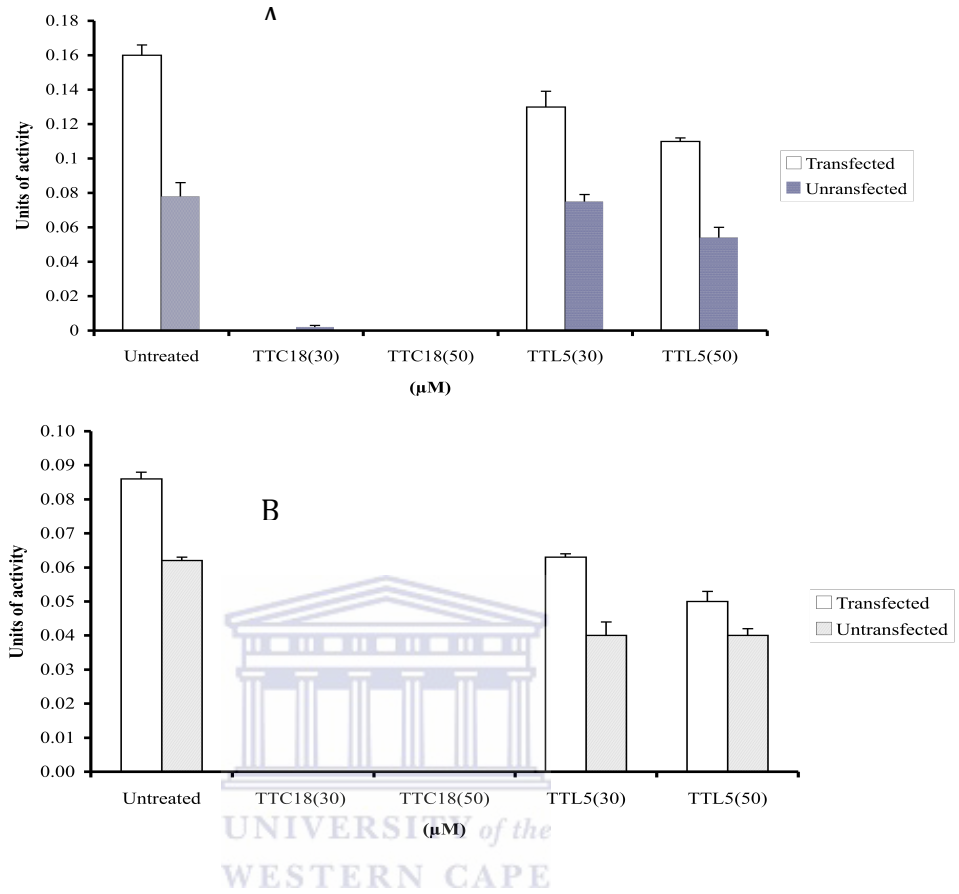


Figure 5.6: Thioredoxin reductase activity in H157 cells that over express Trx-2. The thioredoxin reductase activity was determined in H157 cells (untransfected) and mutant H157 cells that over express Trx-2 (transfected). The cells were also treated with TTC18 and TTL5 for 24 hours. **A** shows thioredoxin reductase activity in the absence of the inhibitor, while **B** shows thioredoxin reductase activity in the presence of the inhibitor.

5.5 Evaluating the cytoprotective potential of Trx-2 over expression

Sections 5.3 and **5.4** showed that H157 cells transfected with the pcDNA™ 3.1 D/V5-His-TOPO/Trx-2 vector over express Trx-2 and that the over expression of Trx-2 is also associated with increased thioredoxin reductase activity the mutant H157 cells. Moreover, TTC18 completely inhibited this activity

To evaluate whether the over expression of Trx-2 can protect cells against the effects of TTC18 and TTL5, the mutant H157 cells were treated with these compounds and the induction of apoptosis was assessed using the APOP*ercentage*™ assay. **Figure 5.7** shows that the percentage apoptosis observed in H157 cells and H157 cells that over expression Trx-2 is the same. TTL5 at 50μM and TTC18 at 30 and 50μM induced apoptosis in untransfected and transfected cells compared to untreated controls. Overexpression of Trx-2 did not have a significant effect on apoptosis induced by these agents compared to untransfected cells.

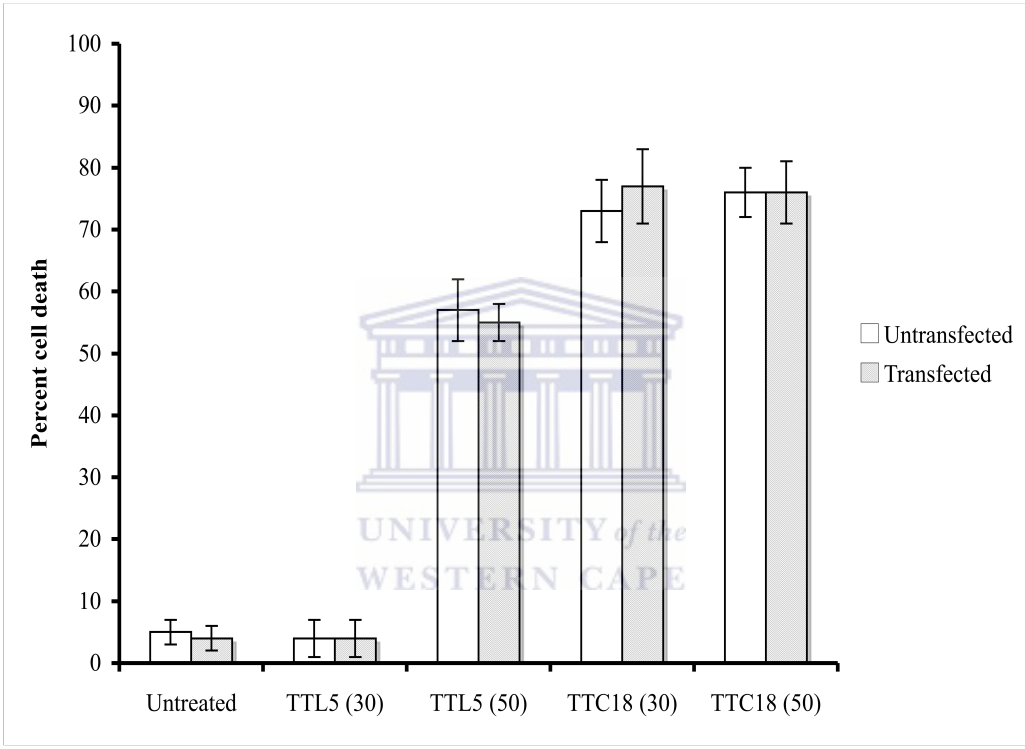


Figure 5.7: Assessing the cytoprotective effects of Trx-2 over expression. H157 cells (untransfected) and H157 cells that over express Trx-2 (transfected) were treated for 24 hours with TTC18 and TTL5. The induction of apoptosis was determined by flow cytometry as described in chapter 2, **section 2.2.7.1**.

5.6: Summary

The human gene encoding Trx-2 was successfully amplified from the cDNA library of KMST-6 cells. This PCR product was cloned into the pcDNA™ 3.1 D/V5-His-TOPO vector. Sequence analysis confirmed that the DNA sequence and the translated protein sequence match the human Trx-2 gene with a 100% identity. No mutations were found. H157 cells were transfected with the pcDNA™ 3.1 D/V5-His-TOPO/Trx-2 plasmid DNA and cells that stably over express Trx-2 were generated by selecting the transfected cells in G418. The over expression of Trx-2 was confirmed by Western blot analysis (figure 5.5), which shows that the transfected cells express more Trx-2 in comparison to untransfected H157 cells. H157 cells that were tranfected with pcDNA™ 3.1 D/V5-His-TOPO/Trx-2 also showed higher thioredoxin reductase activity than untransfected H157 cells. Treatment with TTC18 completely suppressed thioredoxin reductase activity in both untransfected and transfected H157 cells (figure 5.6). There was a moderate reduction in thioredoxin reductase activity in response to TTL5 treatment in both untransfected and transfected H157 cells (figure 5.6). The over expression of Trx-2 failed to protect H157 cells against the effects of TTC18 and TTL5.

CHAPTER SIX OUTLINE

6.1. Phosphine ligands and gold(I) complexes induce morphological changes in cultured cells

6.2 Phosphine ligands and gold(I) complexes selectively inhibit cell growth of cancer cells

6.3 Phosphine ligands and gold(I) complexes selectively induce apoptosis in cancer cells

6.4 TTC18 and TTL5 activate caspase-3

6.5 TTC18 and TTL5 induce DNA fragmentation

6.6 TTL5 block cell cycle progression in G1 phase

6.7 TTC18 and TTL5 induce mitochondrial depolarisation

6.8 TTC18 and TTL5 induce ROS production

6.9 TTC18 and TTL5 cause oxidative damage

6.10 L-glutathione protect H157 cells against TTC18

6.11 Intracellular levels Au is reduced in the presence of L-glutathione

6.12 Over expression of Trx-2 do not protect cells against the effects of TTC18 and TTL5

6.13 Conclusion

6.14 Future perspectives

CHAPTER 6: DISCUSSIONS, CONCLUSION AND FUTURE PERSPECTIVES

6.1. Phosphine ligands and gold(I) complexes induce morphological changes in cultured cells

Based on morphological features cell death can be classified into different types (Häcker, 2000; Kroemer *et al*., 2005; Roos and Kaina, 2006). Yet other studies have also reported cell deaths with no observable morphological changes (Jänicke *et al*., 1998). Morphological observations can be combined with biochemical assays in order to evaluate the underlying mechanisms of apoptosis (Häcker, 2000).

In this study the pro-apoptotic activity of 15 phosphine ligands (TTL) and the 15 gold(I) complexes (TTC) were evaluated. The gold(I) complexes and the phosphine ligands selectively induced morphological changes in the panel of thirteen cell lines that were screened in this study. This panel consisted of 10 human cancer cell lines, 1 non-cancerous human cell line and two rodent cell lines (**Table 2.3**). The morphological changes that were observed were studied by light microscopy and included cell shrinkage and cell detachment. Based on the morphological changes that were observed, the effects of some of the compounds were more severe than others. Some of the compounds were able to induce morphological changes in particular cell lines, but failed to have the same effect on other cell lines. In general, the gold(I) complexes were more bioactive than the phosphine ligands under the conditions that this study was performed.

The gold(I) complexes induced morphological changes in a wide range of cell types (summarized in **Table 3.2**). All the gold complexes induced noticeable morphological changes in Jurkat cells and in CHO cells, except TTC20 and 25, which failed to induce noticeable morphological changes in CHO cells. TTC2, TTC4, TTC5, TTC6, TTC11, TTC18, TTC19, TTC20, TTC22 and TTC25 induced morphological changes in more than two cell lines. However, TTC18 induced noticeable morphological changes in all the cell lines tested.

Compared to the gold complexes, the phosphine ligands failed to induce noticeable morphological changes in most of the cell lines (summarised in **Table 3.1**). Jurkat cells appeared to be very sensitive to the phosphine ligands, with all 15 phosphine ligands inducing noticeable morphological changes in Jurkat cells. TTL5 also induced noticeable morphological changes in large number of different cell lines, which include CHO, HeLa, HepG2, A549J, H157, KMST-6, MG-63 and Hek 293-T cells. TTL5 stood out as the most active compound amongst the phosphine ligands.

6.2. Phosphine ligands and gold(I) complexes selectively inhibit cell growth of cancer cells

The IC_{50} values for all 30 compounds (15 phosphine ligands and the 15 gold(I) complexes) were determined on the panel of thirteen cell lines. The IC_{50} values obtained for the phosphine ligands and gold complexes are shown in **Tables 3.3** and **3.4**, respectively. For the purpose of this study, compounds with IC_{50} values of 50μM or lower were considered to be significantly bioactive. The MTT assay showed that the IC_{50} values

for most of the phosphine ligands were above 50μM. Only two of the phosphine ligands (TTL5 and TTL19) had IC_{50} values below 50μM in more than 3 cell lines. The IC_{50} values for TTL19 and TTL5 were below 50μM in seven cell lines (A549J, CHO, HT29, Jurkat, KMST-6, MCF-7 and Hek293-T) and five cell lines (HeLa, H157, Jurkat, MG-63 and Hek293-T), respectively. Jurkat, 3T3 and Hek293-T cells were more sensitive to phosphine ligands, while Caski cells were the most resistant cell line.

In comparison the IC_{50} values for most the gold(I) complexes were below 50μM. Except for TTC2 and TTC9, all the other complexes had IC_{50} values below 50μM in more than 3 cell lines. Based on the MTT assay 3T3, HepG2 and Jurkat cells were more sensitive to the effects of the gold(I) complexes.

6.3. Phosphine ligands and gold(I) complexes selectively induce apoptosis in cancer cells

For the purpose of this study, compounds that induced apoptosis in more than 50% of the cells treated for 24 hours with 50μM of the compound were considered to have significant pro-apoptotic activity. The APOP*ercentage*™ assay showed that most of the TTL compounds failed to induce apoptosis in more than 50% of the cells treated with 50μM of the ligands (**Table 3.5** and the summary in **Table 3.7**) and were therefore not considered to have significant pro-apoptotic activity. Jurkat cells were more sensitive to the phosphine ligands. Eleven of the phosphine ligands (TTL2, TTL4, TTL5, TTL6, TTL9, TTL11, TTL14, TTL15, TTL18, TTL19 and TTL20) induced more than 50% apoptosis in

Jurkat cells. TTL5 was the most active phosphine ligand, inducing significant pro-apoptotic activity in five of the cell lines (A549J, CHO, HepG2, Jurkat and KMST-6).

The APOP*ercentage*™ assay showed that most of the gold complexes induced significant levels of apoptosis in the panel of cell lines tested in this study (**Table 3.6** and the summary in **Table 3.8**). Six of the gold(I) complexes (TTC2, TTC4, TTC18, TTC20, TTC22 and TTC25) induced significant levels of apoptosis in more than three cell lines. However, the most active gold(I) complex was TTC18 since it induced significant levels of apoptosis in all thirteen cell lines tested in this study, while the least active gold(I) complex was TTC28. The non-cancerous KMST-6 cells were highly resistant to the effects of the gold(I) complexes. TTC18 is the only gold(I) complex that was able to induce significant levels of apoptosis in these cells.

The APOP*ercentage*™ assay demonstrated that the phosphine ligands and gold(I) complexes selectively induced apoptosis in a number of human cancer cell lines. The most active phoshine ligand (TTL5) and gold(I) complex (TTC18) were selected for further study. The objectives were to investigate whether these compounds also activate other markers of apoptosis and to elucidate the mechanism of action. Two cell lines; H157 and Jurkat were selected for this study. Three additional markers of apoptosis (caspase-3 cleavage, mitochondrial depolarisation and DNA fragmentation) were used to study the activation of apoptosis.

6.4. TTC18 and TTL5 activate caspase-3

The activation of caspase-3 is a universal marker for the induction of apoptosis. The cleavage of caspase-3 was assessed in H157 and Jurkat cells following treatment with TTC18, TTL5 and cisplatin (used as a positive control). The results (**Figure 3.1**) showed that all three treatments induced caspase-3 cleavage in both cell lines. However, the percentage cells that were positive for caspase-3 cleavage was higher for Jurkat cells than H157 cells, suggesting that Jurkat cells were more sensitive to the effects of TTC18, TTL5 and cisplatin. H157 cells responded very differently to TTC18 and TTL5. TTC18 induced caspase-3 cleavage in about ~60% H157 cells, compared to ~20% for TTL5 (**Figure 3.1**), suggesting that TTC18 is more cytotoxic than TTL5.

In order to evaluate whether the cleavage of caspase-3 in H157 is a dose and time dependent event, the cells were either treated with increasing doses (5, 15, 30 and 50μM) of TTC18 for 24 hours or the cells were treated with 50μM TTC18 for different time periods (6, 12, 18 and 24 hours). **Figure 3.2-A** shows a concentration dependent increase in the number of cells staining positive for cleaved caspase-3. **Figure 3.2-B** shows time dependent increase in caspase-3 activation over 24 hours.

6.5. TTC18 and TTL5 induce DNA fragmentation

DNA fragmentation is one of the hallmarks of apoptosis. The APO-DIRECT™ assay was used to evaluated DNA fragmentation in H157 and Jurkat cells following the treatment of the cells with 30μM TTC18 and 30μM TTL5. As a positive control, the cells were also

treated with 1mM cisplatin. The dot plots in **Figure 3.3-B** show the results generated for untreated H157 cells and H157 cells treated for 24 hours with 1mM cisplatin. The dot plots compare DNA content (X-axis) and FITC-dUTP labelling (Y-axis). R1 and R2 represent the viable and apoptotic (i.e. FITC-DUTP positive cells), respectively. **Figure 3.3-C** shows a summary of the data for the DNA fragmentation assay. Cisplatin, TTL5 and TTC18 induced DNA fragmentation in both H157 and Jurkat cells. Compared to H157 cells, Jurkat cells were more sensitive to the effects of cisplatin, TTL5 and TTC18. The results for cisplatin showed that ~68% of Jurkat cells and ~40% of H157 cells were positive for DNA fragmentation. A similar trend was observed for TTL5 and TTC18 where the number of Jurkat cells that were positive for DNA fragmentation was significantly higher in comparison to H157 cells.

6.6. TTL5 block cell cycle progression in G1 phase

Cell cycle analysis can also be used to study the activation of apoptosis. In addition, this assay can also be used to assess if the test compound has any effect on cell cycle progression. The mechanisms of several anticancer drugs are based on blocking cell cycle progression in fast growing cells, which include cancer cells. This assay can therefore also provide information on the potential of TTC18 and TTL5 as anticancer agents and give some insight into the possible mechanism of these compounds. H157 cells were treated for 24 hours with low doses (15µM) of TTC18 and TTL5. The cell cycle progression of the cells was evaluated at 6, 12, 18 and 24 hours. The rational was to investigate the effects of a sub-lethal dose of the gold(I) complex on the cell cycle. The results (**Figure 3.4-D**) showed that TTC18 treatment resulted in an incremental increase in the cells in

sub-G1 population over 24 hours. This implies that TTC18 induced apoptosis H157 cells. In general, the G1 population for TTC18 treated cells declined over the 24hr treatment. However, it is not clear if TTC18 induced cell cycle block in the H157 cells.

On the contrary, TTL5 treatment did not cause an increase in the sub-G1 population (**Figure 3.4-C**). The G1 cell population increased over the 24hr treatment. This was accompanied with a decrease in the S and G2/M cell populations. This suggests that TTL5 treatment results in a cell cycle block in the G1 phase. It is likely that the cells trapped in the G1 phase will eventually activate apoptotic pathways if the cells are treated for a longer period.

6.7. TTC18 and TTL5 induce mitochondrial depolarisation

Mitochondrial depolarization is an event that is associated with the activation of the intrinsic apoptosis pathway. In this study the TMRE probe was used to assess whether TTC18- and TTL5-induced apoptosis involve mitochondrial depolarization. H157 cells were treated with 30μM TTC18 or 30μM TTL5, and the mitochondrial potential of the cells were assessed at 6, 12, 18 and 24 hours (**Figure 3.5**). Both TTC18 and TTL5 resulted in a time dependent increase in the number of cells with depolarised mitochondria. However, it was clear that the number of H157 cells with depolarized mitochondria was higher for TTC18 treated cells than for TTL5 treated cells (**Figure 3.5**).

Taken together these results confirm that both TTC18 and TTL5 induce apoptosis in cultured cells. The induction of apoptosis is associated with the cleavage of caspase-3, DNA fragmentation and mitochondrial depolarization. However, the mechanism of TLL5 and TTC18 induced cell death appears to be very different. At low concentrations (15μM) TTL5 induced cell cycle block in the G1 phase. Under the same conditions TTC18 induced apoptosis as indicated by the increase in sub-G1 population. Furthermore, the data also confirm that TTC18 is a more effective pro-apoptotic agent compared to TTL5.

6.8. TTC18 and TTL5 induce ROS production

The CM-H_2DCFDA probe was used to investigate the production of ROS in H157 and Jurkat cells following treatment with TTC18 and TTL5 (**Figure 4.1**). As a positive control, the cells were also treated with H_2O_2. TTC18, TTL5 and H_2O_2 induced ROS production in both H157 and Jurkat cells. However, the number of cells that were positive for ROS production was significantly higher for Jurkat cells than H157 cells. Interestingly, H_2O_2 failed to generate ROS production H157 cells.

6.9. TTC18 and TTL5 cause oxidative damage

ROS production does not necessarily result in oxidative damage. To evaluate whether treatment with TCC18 and TTL5 cause oxidative damage the TBARS assay was performed. This assay indirectly monitors lipid peroxidation by evaluating the presence of MDA, which is a secondary lipid oxidation product. Lipid peroxidation is thus an indication of oxidative damage. The TBARS assay was performed on H157 cells treated

with 30µM and 50µM TTC18 and TTL5 (**Figure 4.2**). Both TTC18 and TTL5 induced TBARS in H157 cells, suggesting that the ROS produced by TTC18 and TTL5 result in oxidative damage. However, H157 cells produced significantly higher levels of TBARS in response to TTL5 treatment. TBARS production in H157 cells treated with 50µM TTL5 was 30× higher compared to TBARS production in untreated H157 cells. In comparison, TBARS production in H157 cells treated with 50µM TTC18 was only 2.5× higher than the untreated control. These results appear to contradict the results obtained for the ROS assay, since ROS production was higher for TTC18 than TTL5. However, the CM-H_2DCFDA probe does not detect of all types of ROS. In addition, it should be noted that the production of ROS does not necessarily result in membrane damage (Millie *et al*., 2006).

6.10. L-glutathione protect H157 cells against TTC18

This study showed that the mechanism of TTC18 and TTL5 induced cytotoxicity may involve the production of ROS, which cause oxidative damage in H157 cells. To investigate this further, cells were pre-treated for 24 hours with antioxidants (vitamin C, catalase, L-glutathione, PDTC and DDTC) before treatment with TTC18 and TTL5. Antioxidants can potentially protect cells against the oxidative effects of oxidizing agents such as TTC18 and TTL5. To evaluate the potential toxicity of the antioxidants, KMST-6, H157 and Jurkat cells were treated with increasing concentrations of the antioxidants (Vitamin C, catalase, PDTC, DDTC and L-glutathione) and apoptosis was assessed using the APOP*ercentage*™ assay (**Figure 4.3-A**, **Figure 4.4-A**, **Figure 4.5-A**, **Figure 4.6-A** and **Figure 4.7-A**). None of the antioxidants tested in this study induced cell death in

KMST-6, H157 or Jurkat cells. To evaluate whether the antioxidants can protect the cells against the effects of TTC18 and TTL5, the cells (KMST-6, H157 and Jurkat) were concurrently treated with TTC18 or TTL5 and the antioxidants (**Figure 4.3-B** and **-C**, **Figure 4.4-B** and **-C**, **Figure 4.5-B** and **-C**, **Figure 4.6-B** and **Figure 4.7-B**). The antioxidants failed to protect the cells. Since it was possible that a 24hr pre-treatment with the antioxidants resulted in a transient increase in the intracellular levels of the antioxidants, the cells were also concurrently treated with the antioxidants and TTC18 or TTL5. Vitamin C, catalase, PDTC and DDTC failed to protect the cells against the effects of TTC18 and TTL5 (data not shown). L-glutathione also failed to protect the cells against the effects of TTL5, however it was able to protect H157 cells against the effects of TTC18 (**Figure 4.7-C**). The viability of H157 cells increased from ~2% in the absence of L-glutathione to ~80% in the presence of L-glutathione. Interestingly, the other two cell lines Jurkat and KMST-6 were not protected by the concurrent treatment with L-glutathione.

6.11. Intracellular levels Au is reduced in the presence of L-glutathione

To evaluate the uptake of TTC18 into cells, ICP-MS was used to assess the intracellular levels of Au in H157 cells following treatment with TTC18. H157 cells were treated with 30μM TTC18 and the presence of Au was assessed at 30min, 2hrs, 4hrs and 8hrs (**Figure 4.8** shows that Au accumulated in the cells within 30min after treatment with TTC18. It was previously shown that concurrent treatment with L-glutathione protect H157 cells against the effects of TTC18. To investigate whether L-glutathione affected intracellular

levels of Au in H157 cells during treatment with TTC18, this experiment was also performed in the presence of L-glutathione. **Figure 4.8** show that the intracellular levels of Au were lower when H157 cells were concurrently treated with L-glutathione and TTC18. This confirms the cytprotective effects of L-glutathione.

6.12. Over expression of Trx-2 do not protect cells against the effects of TTC18 and TTL5

This study showed that TTC18 and TTL5 induce apoptosis in human cancer cells and that the mechanism of cell death most probably involves the generation of ROS. However, the treatment of cells with antioxidants (vitamin C, catalase, PDTC and DDTC) failed to protect the cells against the effects of TTC18 and TTL5. L-glutathione was the only antioxidant that protected H157 cells against the effects of TTC18.

Two intracellular redox systems (the thioredoxin and glutathione systems) have been described (Arnér and Holmgren, 2006; Bindoli *et al*., 2009; Che and Siu, 2010). Previous studies investigated the involvement of redox systems in the mechanism of cytoprotection (Patenaude *et al*., 2004). The thioredoxin reductase enzymes (Trx-1, Trx-2 and Trx-3) are often targeted in these studies (Eriksson, *et al*, 2009; Zeng and Wang, 2010). To further investigate the mechanism of TTC18 and TTL5 induced apoptosis, the human *Trx-2* gene was cloned and the protein encoded by this gene was over expressed in H157 cells.

The human *Trx-2* gene was PCR amplified from a cDNA library prepared from total RNA isolated from KMST-6 cells (**Figure 5.1**). The PCR product was cloned into the

mammalian expression vector, pcDNA™ 3.1 D/V5-His-TOPO. The successful cloning of *Trx-2* was confirmed by sequence analysis. BLAST analysis shows a 100% match to the human *Trx-2* gene sequence with the sequence ID, NM_012473.3 (**Figure 5.3**). A protein sequence alignment between the human *Trx-2* protein sequence (NP_036605.2) and the cloned sequence show a 100% match (**Figure 5.4**). This data confirms the cloning of human *Trx-2* sequence into the pcDNA™ 3.1 D/V5-His-TOPO vector. Furthermore that the DNA sequence was cloned in the right frame and that no mutations, substitutions or deletions were present in the sequence.

H157 cells were transfected with plasmid DNA of the pcDNA™ 3.1 D/V5-His-TOPO vector into which *Trx-2* was cloned. Cells that are stably transfected with the pcDNA™ 3.1 D/V5-His-TOPO-Trx-2 vector was generated by selecting the cells in G418 antibiotic. To confirm that these cells over express Trx-2, total protein was extracted from cells that were stably transfected and Western blot analysis was performed using an anti-Trx-2 antibody (**Figure 5.5**). The Western blot analysis show that transfected cells express more Trx-2 protein compared to untransfected control cells. The anti-actin antibody was used as a loading control to show that the same amount of total protein was loaded for the two protein samples.

The over expression of Trx-2 in H157 cells should result in increased thioredoxin reductase activity in the transfected cells as shown in **Figure 5.6**. The thioredoxin reductase activity in the untreated control cells is significantly higher in the transfected

cells compared to the untransfected cells. Treatment with TTC18 completely suppressed thioredoxin reductase activity in both transfected and untransfected H157 cells, while TTL5 had very little effect on the thioredoxin reductase activity in these cells.

It is clear that the thioredoxin redox system is involved in the mechanism of TTC18 induce cell death. The suppression of thioredoxin reductase activity may possibly contribute to the effects of TTC18. To investigate whether the over expression of Trx-2 can protect H157 cells, the Trx-2 over expressing cells were treated with TTC18 and TTL5 and cell death was quantified using the APOP*ercentage*™ assay. **Figure 5.7** shows that the over expression of Trx-2 failed to protect H157 cells against the effects of TTL5 and TTC18. It is likely that the level of Trx-2 over expression is not sufficient to block the effects of TTC18. Alternatively, other Trx genes should be targeted.

6.13. Conclusion

The major objective of this study was to screen the toxicity of 15 novel phosphine ligands and 15 novel gold(I) complexes and to explore the potential application of these compounds as potential anticancer agents. The investigations into the cytotoxicity of the compounds involved analysing the pro-apoptotic activities of the compounds in a panel of cultured human cancer cell lines. This study demonstrated that some of phosphine ligands and gold(I) complexes tested show promise as anticancer agents. Particularly TTC18 and TTL5 can potentially be further developed as anticancer agents. These compounds selectively induced apoptosis in cultured human cancer cell lines. The mechanism of

cytotoxicity involves the generation of ROS and consequent oxidative stress, which activate apoptotic pathways.

6.14. Future perspectives

This study focussed very much on only two of the 30 compounds (TTC18 and TTL5). It would be advisable to investigate some of the other compounds that also showed good cytotoxic activity in the cancer cells. Therefore, TTC2, TTC4, TTC20 and TTC22 should be further investigated.

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