

A thesis submitted in fulfillment of the requirements for the degree of
MAGISTER SCIENTIAE in the Department of Pharmacology, Faculty
of Health Science, University of Pretoria

AN EVALUATION OF 6-THIOGUANINE DERIVATIVES AS
POTENTIAL ANTI-CANCER AGENTS

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FOREWORD

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I would also like to express my sincere gratitude to my mentors Prof CE Medlen and Prof JR Snyman who provided guidance and valuable contributions throughout my project. I would also like to convey my gratitude to the following personnel from the Department of Pharmacology for their expertise and assistance regarding each aspect of my research namely; Dr Gisela Joone and Margo Nel (cell culturing and flow cytometry), Dr Duncan Cromarty (Spectrophotometry and flow cytometry), Chrisna (flow cytometry) and Dr Piet Bekker who provided guidance for the statistical analysis of my results.

Lastly but not least, I would like to express my thanks to my family and friends for their continuous support, patience and understanding throughout the duration of my studies.

SUMMARY

During neoplastic development cellular requirements for the micronutrients and macronutrients increase dramatically for the primary metabolites, sugar and amino-acids. This concept of increased cellular needs lead to the development of antimetabolites as anticancer agents. Antimetabolites, are structurally similar to that of physiological metabolites, but have the ability to interfere with normal metabolic functioning of the cell that eventually leads to cell death. One such agent used in chemotherapy is 6-thioguanine. Another group of agents that also only recently showed potential for the used in cancer therapy are the Au containing anti-arthritis gold compounds, such as Auranofin. Both these groups, antimetabolites and Au containing compounds have different intracellular targets e.g. DNA(6-Thioguanine) and thioredoxin reductase [Au(I) and Au(III)], respectively.

No study thus far has attempted to combine gold to 6-thioguanine nor has it been tested on non-cancerous and cancer cell lines. In this study two novel Au(I) compounds were synthesized i.e. a Au (I) metal 6-thioguanine (EKJC56) compound and Au (I)-phosphine 6-thioguanine (EKJC80A) compound (Figure 1).

The cytotoxic effect and selectivity of these novel compounds were determined on several cancerous and normal cell cultures using the MTT assay. The cells were exposed to varying concentrations of these compounds. The novel compounds (EKJC56ST, EKJC56, and EKJC80A) showed higher toxicity towards all the cancerous cell lines tested, while EKJC56ST and EKJC56

showed only slight inhibition of the proliferation of normal cell cultures. These novel compounds proved to be more selective towards the hormone dependent cell line compared to the non-hormonal cell lines. The Au-phoshine compound, EKJC80A were non selective for both cancerous cells and normal cell cultures. The mechanism of action of these compounds were evaluated and showed a higher percentage of induction early apoptosis compared to an untreated control group. These results were supported when the effect of these derivatives were evaluated on the activation of caspase-3 activity, a known marker of early apoptosis. On evaluation of the effect of these compounds on the mitochondrial membrane it was observed that the mitochondrial membrane of the HeLa cell line became hyperpolarized, but did not prove to be statistical significant. No significant changes in cell cycle phases were observed when cell cycle progression was investigated.

Due to limited amounts of thioredoxin reductase enzyme an observational study was conducted to determine the possible involvement of the Au(I) side chain of the novel compounds on the functionality of this enzyme. It was observed that these novel Au(I) containing compounds were as effective as Auranofin to inhibit this enzyme at a concentration as low as 0.1 μ M. Further enzymatic studies are still required to support the hypothesis.

This study can assist in developing new multi-acting chemotherapeutic drugs that may have the potential to effectively and selectively treat cancer and minimize the development of resistance during treatment.

LIST OF ABBREVIATIONS :

A2780	: Cisplatin sensitive human ovarian cancer
A2780cis	: Cisplatin resistance human ovarian cancer
ANOVA	: Analysis of variance
ATCC	: American type cell culture
Au	: Gold
BSA	: Bovine serum albumin
CO ₂	: Carbon dioxide
°C	: Degrees Celsius
Ca ²⁺	: Calcium
CoLo320DM	: Human colon cancer cell line
DMEM	: Dulbecco's Minimal Essential Medium
DMSO	: Dimethyl sulfoxide
Du145	: Human prostate carcinoma cell line
EDTA	: Ethylene diamine tetra-acetate
EMEM	: Eagles Minimal essential Medium
HCT116	: Mismatch repair mechanism-deficient colorectal cell line.
HeLa	: Human cervical carcinoma cell line
HEPES	: N-2-Hydroxyethylpiperazine – N'2-ethane sulfonic acid
HIFCS	: Fetal calf serum
IC ₅₀	: Inhibitory concentration at which 50% of cell growth is inhibited
JC-1	: 5,5',6,6',-tetrachloro-1,1'3,3' tetraethyl benzimidazolyl carbocyanine iodide

Jurkat	: Human T-cell line
MCF12A	: Human non-malignant breast cell carcinoma
MCF7	: Human malignant breast cell carcinoma
MMP	: Mitochondrial membrane potential/permeabilization
MMR	: Mismatch Repair mechanism
MNL	: Mononuclear leucocytes
MTP	: Mitochondrial transition pore
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaCl	: Sodium Chloride
PBS	: Phosphate Buffer Saline
PCD	: Programmed cell death
PHA	: Phytohaemaglutamine
PI	: Propidium Iodide
RPMI	: Roswell Park Memorial Institute
SEM	: Standard error of mean
6-TG	: 6-Thioguanine

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CHAPTER 1

INTRODUCTION

1. LITERATURE

1. 1 INTRODUCTION

During neoplastic development, the cellular nutritional requirements for primary metabolites, sugars and amino acids increases (Colombo *et al.*, 2001). This concept of increased cellular nutritional needs, has led to the development of anti-metabolites, such as 6-thioguanine (Figure 1a), as anticancer agents. These chemotherapeutic agents were used since the 1940's in the treatment of acute leukaemias (Karran and Bignami, 1996; Krynetskaia *et al.*, 2000; Lennard *et al.*, 2002).

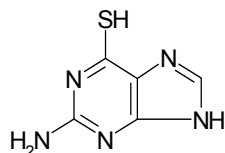
The anti-cancer potential of gold (I) compounds was recognized when arthritic patients treated with gold (I) salts showed lower incidences of tumour formation (Tiekink, 2002). Various cancer cell lines have shown sensitivity after treatment with these anti-arthritic gold compounds. Since both the gold (I) and 6-thioguanine containing compound represent succinct classes of antitumour classes with distinctly different modes of action the design of the drug candidates that contain both Au(I) and 6-thioguanine present an opportunity to broaden the scope of responsive tumours. These new chemotherapeutic compounds may reduce the possibility of resistance formation as the nucleotide and the gold metal targets a different mechanism within the intracellular structure. The presence of the nucleotide can increase cellular uptake of the drug due to the structural similarity of the 6-TG to that of physiological nucleotides. (Galmarini *et al.*, 2002)

Various studies have shown that linear gold compounds such as Au (I)-phosphine and Au(I)-sulphur complexes mechanistically act on mitochondrial function, rather than targeting DNA as the square planar gold(III) compounds as reviewed by Tiekink, 2002 and McKeage *et al.*, 2002.

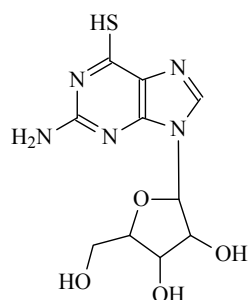
No study thus far has attempted to combine gold to 6-thioguanine nor has it been tested on non-cancerous and cancer cell lines. In this study two novel Au(I) compounds were synthesized i.e. a Au (I) metal 6-thioguanine (EKJC56) compound and Au (I)-phosphine 6-thioguanine (EKJC80A) compound (Figure 1.1). The anti-tumour activity of these compounds were compared to that of 6-thioguanine and a metabolite thereof, 6-thioguanine riboside. This metabolite contains a sugar side chain, thereby allowing it to bypass the enzyme, hypoxanthine guanine phosphoribosyl transferase which is the major cause of resistance in 6-thioguanine resistant cancers. This study can assist in developing new multi-acting chemotherapeutic drugs that may have the potential in effectively and selectively treat cancer and minimise the development of resistance during treatment.

The purine anti-metabolite, 6-thioguanine, was used to treat adults and children suffering from acute lymphoblastic leukaemia since the 1940's (Colombo *et al.*, 2001; Revankar *et al.*, 1990). This drug has, however, been associated with serious, but reversible side effects, such as neutropenia, anaemia, thrombocytopenia and prolonged clotting time (Boobis *et al.*, 1999). The inactive 6-thioguanine enters the purine salvage pathway and is converted to the active

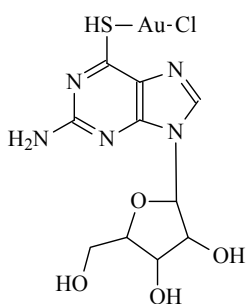
but, toxic metabolite, 6-thioguanilyc acid by the enzyme hypoxanthine guanine transferase (Figure 1.2)(Sciandrello *et al.*, 2003; Glaab *et al.*, 1998). The active metabolite is able to incorporate itself into the DNA. During the first cell cycle the sulphur group of the active metabolite of 6-TG is methylated to S⁶ – methylthioguanine, (Sciandrello *et al.*, 2003; Glaab *et al.*, 1998).



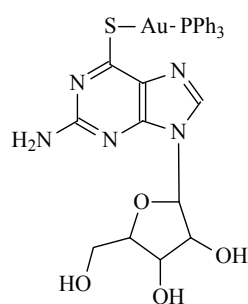
(a) 6-Thioguanine (6-TG)



(b) 6-Thioguanine riboside (EKJC56ST)



(c) EKJC 56



(d) EKJC 80A

Figure 1.1: Molecular structures of 6-TG, EKJC 56 ST (6-Thioguanine riboside), EKJC 56, and EKJC 80A

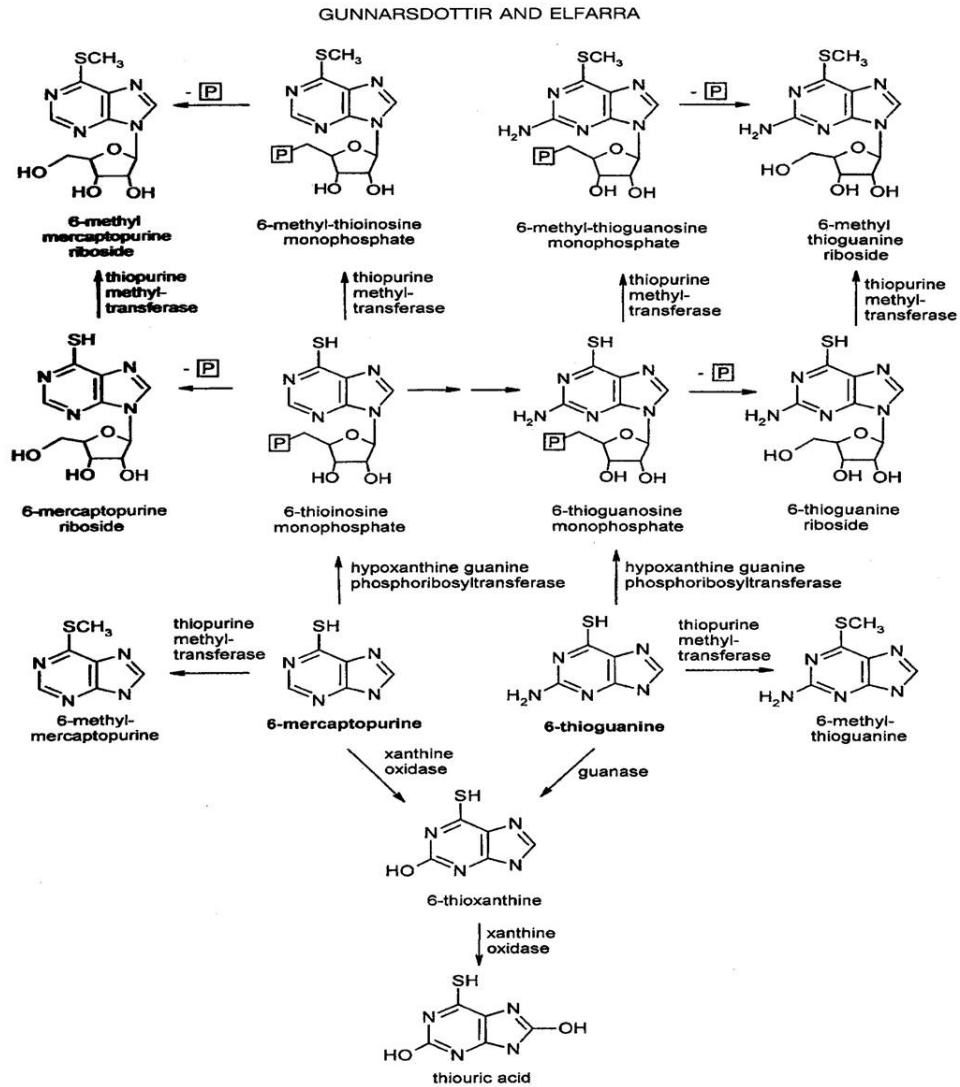


Figure 1.2 Schematic presentation of the metabolic pathway of 6-TG
(Gunnarsdottir and Elfarrá, 2002)

During the next cell cycle the mismatch repair mechanism (MMR) recognizes the 6 TG/cytidine and 6-TG/thymidine mismatches on the daughter strand and repairs the mismatch. However the MMR is unable to excise the faulty nucleic acid on the parent strand, inducing DNA strand breaks and the activation of various pro-apoptotic signalling pathways i.e. the ATR / Chkl 1 pathway, hypophosphorilation of Bad, release of cytochrome c and activation of the caspases (Stojic *et al.*, 2004; Bignami *et al.*, 2003; Bernstein *et al.*, 2002)(Figure 1.3).

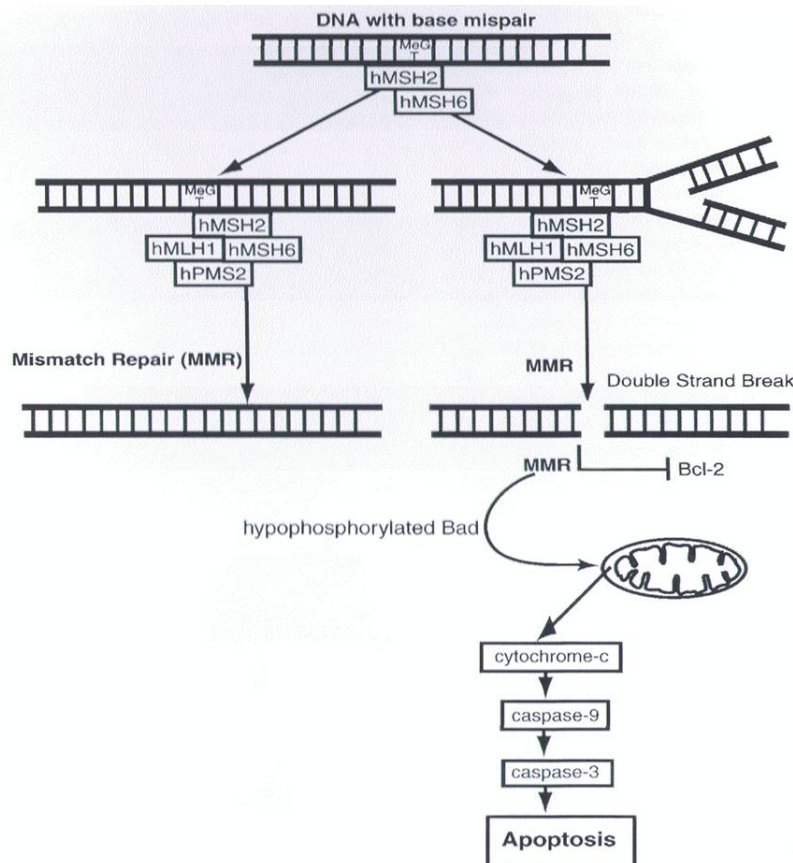


Figure 1.3 The mismatch repair mechanism (Bernstein *et al.*, 2002)

The fate of 6-thioguanine is also dependent on the activity of two other enzymes, i.e. thiopurine S-methyl transferase and xanthine oxidase. These enzymes convert 6-thioguanine to the inactive metabolites, 2-amino-6 methylpurine and 6-thiouric acid as shown in Figure 1.4. These inactive metabolites may be responsible for some level of hepatotoxicity associated with 6-TG treatment, but not to the extent as 6-mercaptopurine treatment (Boobis *et al.*, 1999).

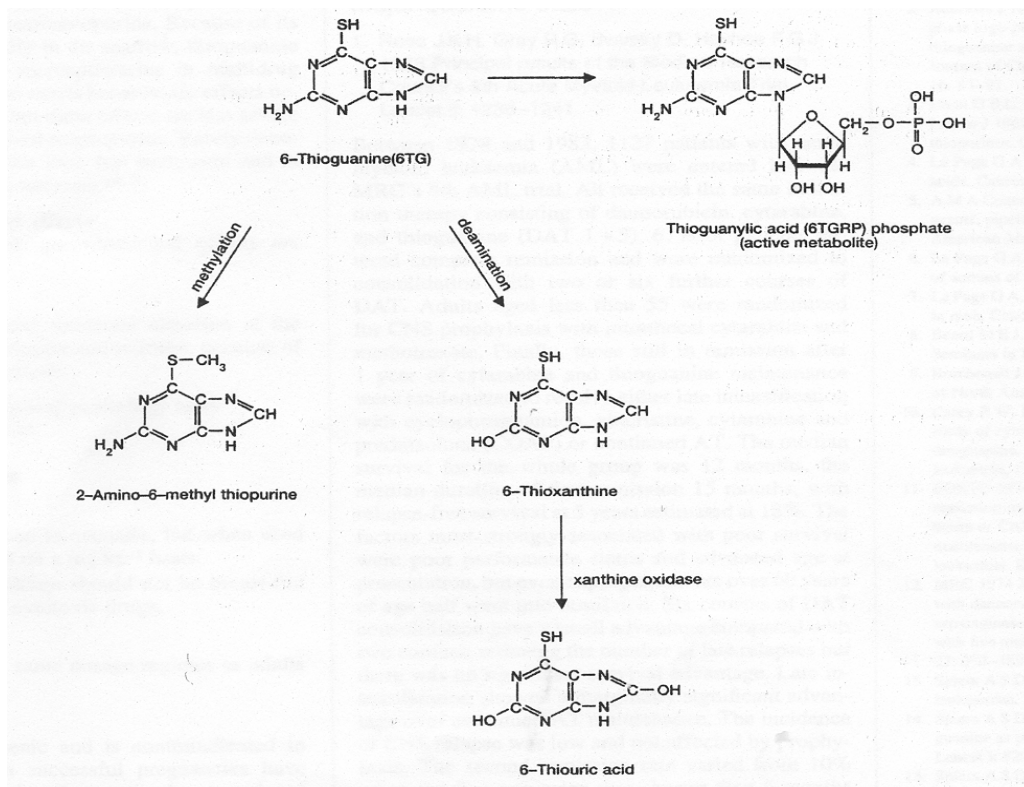


Figure 1.4 Breakdown of 6-TG to the active and inactive metabolites (Boobis *et al.*, 1999).

Another study suggested that thiopurines not only caused MMR induced apoptosis, but that the toxic active metabolite could inhibit various enzymes

necessary for *de novo* purine synthesis eg. S-phosphoribosyl-1 pyrophosphate amido transferase, IMP dehydrogenase and ribonucleotide reductase, causing purine starvation (Galmarini *et al.*, 2002; Bernstein *et al.*, 2002). Recently, it was shown that thiopurine incorporation was site dependent and could alter the cleaving effect of topoisomerase II activity by either contributing to the cytotoxic effects or enhancing secondary cancer formation (Krynetskaia *et al.*, 2000; Boulieu and Lenoir, 1995). Mutation at either of the above mentioned repair mechanisms could result in treatment failure and tumour re-growth (Kaina, 2003). Mutations at these loci are responsible for hereditary non-polyposis colorectal and pancreatic cancer (Stojic *et al.*, 2004).

Gold (I) compounds have been used since the 1970's as anti-arthritic agents, but the potential use in the treatment of AIDS, bronchial asthma, malaria and cancer has recently become more apparent (Tiekink, 2002; Baguley and Ferguson, 1998). The anti cancer abilities of the gold (I) compounds were recognized in the 1980's, when arthritic patients treated with gold salts indicated lower incidences of tumour formation. A study on P388 leukaemia inoculated mice also showed an increase in the lifespan of mice treated intra-peritoneally with gold-phoshine compounds as reviewed by Tiekink (2002) and McKeage *et al.*, (2002). Another study conducted on the inhibitory effect of the Au(I) thiolates also showed a reduction in tumour growth and tumour metastasis in mice with Lewis carcinoma (Giraldi T *et al.*; 1984). The most commonly used gold (I) compounds are auronofin, thioglucose and aurothiomalate. These compounds are potent immunosuppressive and anti-inflammatory agents and have a wide range of

effects on lymphocyte and macrophage functions (Karran *et al.*, 2003; Yamashita *et al.*, 1999; Sakuma *et al.*, 2004).

Early studies conducted by Berner Price *et al.*, 1986 showed that Au(I) phosphine compound [(AuCl)₂(DPPE)], produced DNA crosslinks which in turned caused DNA strand breaks this in turn leads to the subsequent inhibition of proteins required for DNA and RNA synthesis. However, it was the study conducted by Hoke (1989) that indicated the importance of the mitochondria as a possible mechanism of action for the Au(I) compounds, by facilitation the uncoupling of the oxidative phosphorylation chain. Since then further studies has provided supporting evidence that the Au(I) and Au(III) compounds have a prominent mitochondrial mode of action by decreasing various mitochondrial functions (Kaina, 2003; Rigobello *et al.*, 2004; Barnard *et al.*, 2004).

Au (I) has a high affinity for the thiolates (cysteine residues in proteins, free cysteine and glutathione) and phosphine containing enzymes (Roberts and Shaw, 1998). Several studies have indicated that Au (I) compounds act directly on the functions of mitochondrial enzymes, especially thioredoxin reductase, glutathione reductase and DNA polymerases (Kaina, 2003; Rigobello *et al.*, 2004; Barnard *et al.*, 2004). Thioredoxin and the glutathione reductase redox system plays an important physiological role in cellular metabolism by protecting cells against oxidative damage (Barnard *et al.*, 2004).

In vitro studies showed cells treated with auranofin (a gold(I)-phosphine compound) did not induce significant changes in cell cycle progression, when compared to 6 TG which in turn caused G2/M cell cycle arrest (Adams *et al*, 1995). Another study conducted, suggested that auranofin interacted with cell membrane thiol-groups causing displacement of the Au from the parent drug, thus allowing the cleaved Au to move freely in the intracellular space (McKeage *et al*, 2002).

Structural modification of conventional chemotherapeutic agents with Au(I) side chain can enhance antitumour activity of these agents. This study can assist in developing new multi-acting chemotherapeutic drugs that can target not one but numerous sites within the cancerous cell. These new chemotherapeutic agents have the potential to effectively and selectively treat cancer and potentially minimalising the development of cellular drug resistance during the treatment.

1.2. PROJECT DISCUSSION

1.2.1 HYPOTHESIS

Incorporation of various gold (I)- sugar derivatives into 6-thioguanine (6-TG) can increase anti-tumour activity and overcome resistance of tumour cells against 6-TG.

1.2.2 AIM

The aims of the study are:

- I. To compare the cytotoxic activity and selectivity of gold(I) sugar - 6TG derivatives with that of 6-TG against several sensitive, and resistant tumour cell lines and normal cell cultures.
- II. To determine the mechanism of action of these compounds.

1.2.3 OBJECTIVES

- To compare selectivity of experimental drugs with 6-TG using various cancerous and non-cancerous cell cultures using the MTT colorimetric assay. (Chapter 2)
- To compare the cytotoxicity of the experimental compounds with 6-TG on a 6-TG resistant cell line using the MTT colorimetric assays. (Chapter 2)
- To determine whether the mechanism of action of both Au and the 6-TG are operational in the Au(I)- 6TG and the Au(I) phosphine –6TG compounds. The following assays were done with a brief rationale given to determine the possible mechanism of action of the compounds.

Background and methodology of each of the assays are described in detail in their relevant chapters.

- **Apoptosis (Chapter 3)**

Both 6-TG and Au(I) compounds are known to induce apoptosis.

- **Caspase-3 activation (Chapter 4)**

Activation of the effector caspase is indication of the induction early apoptosis.

- **Mitochondrial membrane potential (Chapter 5)**

Changes in the mitochondrial membrane indicate disruption of the electron balance within the cell.

- **Mitochondrial thioredoxin reductase activity (Chapter 6)**

Au (I)-phosphine compounds such as Auranofin are known to inhibit various mitochondrial enzyme especially thioredoxin reductase.

- **Cell cycle analysis (Chapter 7)**

6-TG induces a G2/M cell cycle arrest whereas the Au(I) compounds do not induce a significant change in the cell cycle.

1.2.4 STATISTICAL ANALYSIS

Guidance for the statistical analysis of the results was provided by Dr Piet Bekker from the Department of Biostatistics, Medical Research Council.

1.2.5 ETHICAL APPROVAL

Ethical approval has been obtained from a previous *in vitro* study conducted in the Department of Pharmacology for the use of the following primary cell cultures: human lymphocytes, porcine hepatocytes and chick embryo fibroblast (Student protocol no.167).

Protocol was accepted by the Student Protocol Committee in November 2006.



1.2.6 STUDY DURATION

The experimental procedures were conducted over a period of two years.



CHAPTER 2

CYTOTOXICITY

2. CYTOTOXICITY

2.1 INTRODUCTION

Cytotoxicity assays were performed to evaluate the sensitivity and selectivity of the novel 6-TG Au(I) compounds on several cancer cell lines as well as normal cell cultures. Cells were exposed to varying concentrations of the experimental compounds for a period of time ranging from either a few hours to 7 days depending on the cell type. After the cells were exposed to the experimental compounds for a period of time the percentage of viable cells in each well of the cell culture plates was determined by adding MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide](Mosmann, 1983). Metabolically active cells have the ability to convert the tetrazolium salts to dark blue formazan crystals. The amount of formazan that forms correlates with cell viability and can be measured by means of spectrophotometry.

2.2 CELL LINES AND CELL CULTURES

The following cell lines were used to investigate *in vitro* cytotoxicity of the novel 6-TG compounds:

- **MCF 7** (mammary, adenocarcinoma, human)
- **MCF 12A** (mammary gland, human)
- **HeLa** (cervix, adenocarcinoma, human)
- **COLO 320** (Colon, adenocarcinoma, human)
- **A2780** (ovary, carcinoma, human)
- **A2780cis** (ovarian, carcinoma, human resistant to cisplatin)
- **HCT 116** (colorectal, carcinoma, human) (MMR deficient cell line)

- **Jurkat** (peripheral blood, human, leukaemia, T cell)
- **Du 145** (prostate cancer)

The following normal, primary cell cultures were used to investigate *in vitro* cytotoxicity of the novel 6-TG compounds:

- **Chick embryo fibroblast**
- **Human mononuclear leukocytes (MNL)**
- **Porcine hepatocytes**

2.3 AIM

To compare the cytotoxic activity and selectivity of gold(I) sugar - 6TG compounds with that of 6-TG against several sensitive, and resistant tumour cell lines and normal cell cultures. Additionally, since the compounds EKJC56 and EKJC80A differ structurally only by virtue of the Cl and PPh₃ coordination to gold respectively, this study will investigate differences in the cytotoxicity arising from halide and phosphine substitutions.

2.4 METHOD

2.4.1 INSTRUMENTS AND REAGENTS REQUIRED

- 0.25% Trypsin / EDTA solution (Highveld Biological, Jhb, SA)
- 15ml and 50ml centrifuge tubes(AEC-Hamersham, Jhb, SA)
- 20-200 micro pipet
- 3-[4,5 dimethylthiozol-2-yl]-2;5-diphenyl tetrazolium bromide (MTT solution)
(Sigma-Aldrich, Jhb, SA)

- Ammonium chloride (NH₄CL) (Merck, Jhb, SA)
- Crystal violet cell counting fluid (Merck, Jhb, SA)
- Dimethyl sulphoxide (DMSO)(Sigma-Aldrich, Jhb, SA)
- EDTA (Sigma-Aldrich, Jhb, SA)
- Fetal calf serum (FCS)(Adcock Ingram, Jhb, SA)
- FTA hemaglutimine (The Scientific group, Jhb, SA)
- Glacial acetic acid (Lab Chem., Jhb, SA)
- Heparin (Sigma-Aldrich, Jhb, SA)
- Histoplaque 1077 (Sigma-Aldrich, Jhb, SA)
- Inverted microscope 4x10 optical lens
- Penicillin / Streptomycin (Adcock-Ingram, Jhb, SA)
- Phytohemaglutamine (PHA) (Bioweb (Pty)Ltd., SA)
- Shaker (Model: VRN-200, Gemmy industrial corp. Taiwan)
- Sodium bicarbonate (NaHCO₃) (Merck, Jhb, SA)
- sterile 96-well round bottom microtiter plates (AEC-Hamersham, Jhb, SA)
- Laminar flow cabinet
- Water jacketed CO₂ incubator

See Appendix A for preparation of reagents and medium.

2.4.2 EXPERIMENTAL PROCEDURES

2.4.2.1 Cell lines and Primary cell cultures

The following general procedure was followed for both cancerous cell lines and normal cell cultures tested during this study. Tables 2.1a and Table 2.1b represent the medium, cell concentration and dilution factors used for each cell line and cell culture. The final incubation period for each assay is also recorded.

Procedure

A volume of 80µl of medium supplemented with 10% heat inactivated FCS and 1% penicillin-streptomycin was dispensed into each well of a 96-well microtiter plate. (See Table 2.1 and Table 2.2) A volume of 100µl of cell suspension was added to each well and incubated at 37°C in an atmosphere of 5% CO₂ for 1 hour. After incubation, 20µl of the experimental compounds were added in triplicate to wells at varying concentrations ranging from 100µM to 0.0125µM. The untreated control group received 20µl of growth medium. The cell culture plates were then incubated for a period of 7 days at a temperature of 37°C in an atmosphere of 5% CO₂.

After the incubation period, 20 µl MTT was added to each well and cell culture plates were re-incubated for a further 3-4 hours at 37°C in an atmosphere of 5% CO₂. This step was followed by centrifuging the cell culture plates for 10 min at 2000rpm's (800G's). The supernatant was discarded and cells pellets were washed twice by adding 150µl PBS and centrifuging for 10 min at 2000rpm's (800G's). The supernatant was carefully removed after each wash step and cells

were allowed to dry overnight before 100µl DMSO was added to each well. Cell culture plates containing the DMSO/ formazan solution were placed on a shaker for approximately 2 hours or until all the formazan crystals were solubilised. Viable cells were quantified, using an UV 900 Micro-ELISA spectrophotometer, at a test wavelength of 570 nm and a reference wavelength of 630 nm.

2.4.2.2 Lymphocyte proliferation assay

Venous blood was collected from healthy human volunteers using heparin containing vacutubes. The hepanarised blood was carefully layered onto the density separating solution, Histoplaque 1077 and centrifuged at 2000rpm's (800G's) for 25 minutes. After centrifugation the mononuclear leukocyte monolayer was transferred to sterile 50ml centrifuge tubes. The mononuclear leucocyte cell suspension was washed twice with freshly prepared PBS before diluting to the 2.0×10^6 cells / ml using RPMI medium supplemented with 10% heat inactivated FCS and 1% penicillin-streptomycin.

A volume of 60µl of RPMI growth medium, supplemented with 10% heat inactivated FCS and 1% penicillin-streptomycin, was dispensed into each well of a 96-well microtiter plate. 100µl of the mononuclear leukocyte cell suspension was added to each well and incubate at 37°C in an atmosphere of 5% CO₂ for 1 hour. After 1 hour of incubation, 20µl of the relevant drug was added in triplicate wells at varying concentrations ranging from 0.0125 to 100µM. The cell culture plates allocated for lymphocyte stimulation received 20µl PHA 15 minutes after the drugs had been added. The unstimulated cell culture plates, however, did not

receive the 20µl PHA but received 20µl of the growth medium. The untreated control groups received an additional 20µl growth medium. Cells cultures plates were incubated for 3 days at 37°C in an atmosphere of 5%CO₂.

After the incubation period, 20 µl MTT was added to each well and cell culture plates were re-incubated for a further 3-4 hours at 37°C in an atmosphere of 5% CO₂. This step was followed by centrifuging the cell culture plates for 10 min at 2000rpm's (800G's). The supernatant was discarded and cells pellets were washed twice by adding 150µl PBS and centrifuging for 10 min at 2000rpm's (800G's). A pipet was used to carefully remove supernatant from the wells without disturbing the cell pellet after each wash step. Cells were allowed to dry overnight before 100µl DMSO was added to each well. Cell culture plates containing the DMSO/ formazan solution were placed on a shaker for approximately 2 hours or until all the formazan crystals were solubilised. Viability was determined by measuring the absorbance of the formazan / DMSO solution, using an UV 900 Micro-ELISA spectrophotometer, at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Table 2.1a. Standard cell concentration, growth medium and incubation period required for cancerous cell lines used during the cytotoxic assay.

Cell line	Initial concentration (cells / ml)	Dilution	Growth medium	Incubation period
Hela	2.0×10^4	1/5	EMEM	7 days
CoLo320DM	2.5×10^4	1/5	RPMI	7days
HCT-116	2.0×10^4	1/5	McCoy 5A	7days
MCF-7	2.0×10^4	1/5	DMEM	7 days
MCF-12A	2.0×10^4	1/5	DMEM	7days
A2780	2.0×10^4	1/5	RPMI	7days
A2780cis	2.0×10^4	1/5	RPMI	7days
Jurkat	3.0×10^4	1/5	RPMI	7days

Table 2.1b Standard cell concentration, growth medium and incubation period required for the normal cell cultures (Hepatocytes, Fibroblast, Stimulated and Unstimulated lymphocytes) used during the cytotoxicity assay.

Cell line	Initial concentration (cells / ml)	Growth medium	Incubation period
Hepatocytes	2.0×10^6	DMEM+	7days
Fibroblast	2.0×10^6	RPMI+	7days
PHH stimulated lymphocytes	2.0×10^6	RPMI+	3days
Non-stimulated lymphocytes	2.0×10^6	RPMI+	3days

2.6 STATISTICAL ANALYSIS OF RESULTS

The results of the cytotoxic assay are expressed as the mean percentage (%) of \pm SEM. A statistical program GRAPHPAD was used to determine the IC_{50} concentration of the drug treated groups. Data from the treated groups were compared with the untreated control group using the Students paired t-test. A p value of < 0.05 was considered to be significant, a p-value of < 0.01 more significant and a p value of < 0.001 to be highly significant, indicated as *, **, and *** respectively. See Appendix B for statistical definitions.

2.7 RESULTS and DISCUSSION

2.7.1 Non-hormonal cell lines

Non hormonal cell lines (HCT-116, Jurkat and CoLo320DM) were treated with the experimental compounds (6-TG, EKJC56ST, EKJC56, and EKJC80A) at concentrations ranging from concentrations from as little as 0.006 to 100 μ M. During the prescreening phase of the study an estimated dose range for each of the experimental compounds were determined by incubating each cell line with high concentrations of the experimental compounds and down titrating the concentration of the drug until an expected IC₅₀ value was obtained. Assays repeats involving the experimental compounds incorporated this dose range throughout. 6-TG was used as a standard in all tests conducted. Cells were incubated and harvested as described in Section 2.4.2.1. The MMT assay was used to quantify the percentage of viable cells. The effect of the experimental compounds on the non-hormonal cell lines (CoLo320DM, Jurkat and HCT116) are illustrated in Figure 2.1, Figure 2.2 and Figure 2.3 respectively, while Table 2.2 expresses the mean IC₅₀ values obtained from the MTT assay.

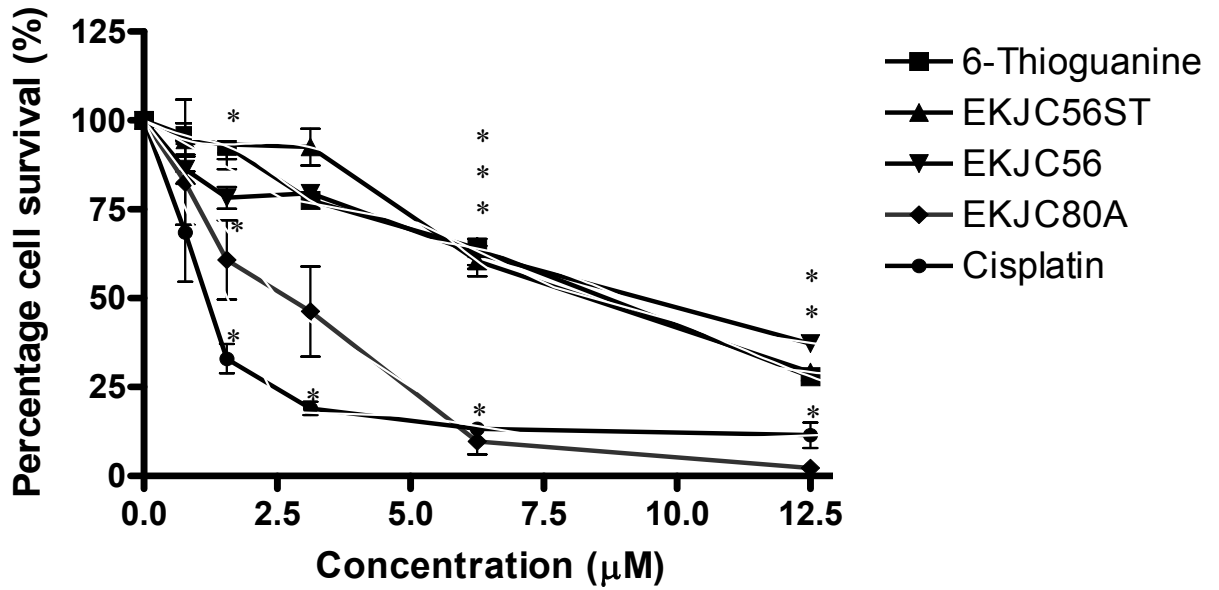


Figure 2.1 The inhibitory effect of 6-TG, EKJC56ST, EKJC56, EKJC80A and cisplatin at varying concentrations on the percentage of cell survival on the 6-TG resistant cell line, HCT-116.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

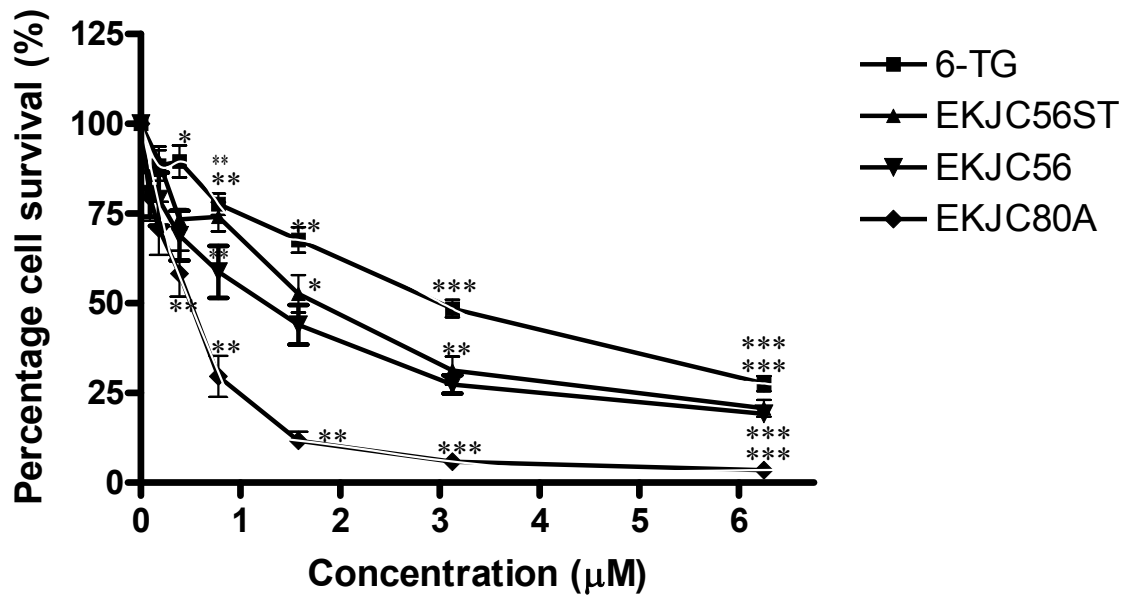


Figure 2.2 The inhibitory effect of 6-TG, EKJC56ST, EKJC56, and EKJC80A at varying concentrations on the percentage of cell survival of the CoLo 320DM cell line.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

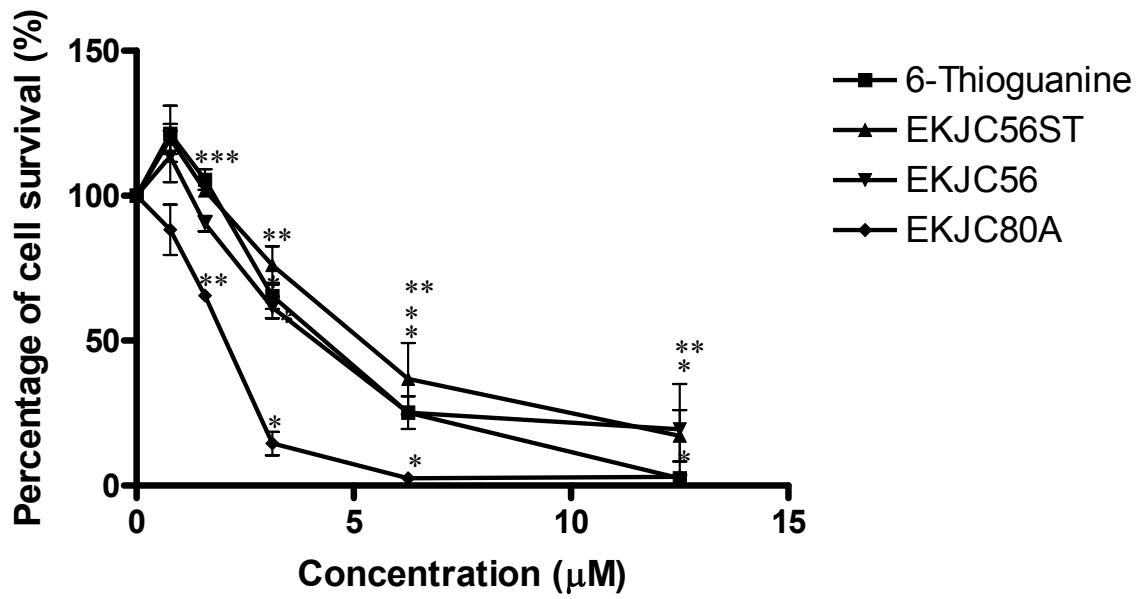


Figure 2.3 The inhibitory effect of 6-TG, EKJC56ST, EKJC56, and EKJC80A at varying concentrations on the percentage of cell survival of the Jurkat cell line.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

TABLE 2.2 ⁽¹⁾Mean *IC₅₀ of cell lines treated with 6-TG, EKJC56, EKJC56ST, EKJC80A and Cisplatin after a seven day incubation period

Mean IC ₅₀ * value (±SEM) of cell lines treated (µM)			
Treatment	CoLo320DM	HCT 116	Jurkat
6-TG	3.108±0.485	9.6 ±0.60	4.217 ±0.309
EKJC56ST	1.739±0.490	7.34 ±0.43	4.445 ±0.443
EKJC56	0.672 ±0.605	9.07 ±0.663	4.031 ±0.609
EKJC80A	0.441 ±0.190	3.83 ±0.67	1.845 ±0.115
Cisplatin	0.329 ±0.047	1.35 ±0.07	1.738 ±0.217

⁽¹⁾ **Mean of at least 4 different experimental repeats**
***IC₅₀ is the concentration at which 50% of cell growth inhibited**

On evaluation of the effect of these derivatives on cellular growth in non-hormone dependent cell lines (Jurkat, HCT-116 and CoLo320DM), treatment with the metabolite, EKJC56ST and novel Au(I) derivatives (EKJC56 and EKJC80A) proved that these compounds inhibit cell growth in a dose response manner (Figure 2.2, Figure 2.4 and Figure 2.5). The cytotoxic effect of EKJC56ST and EKJC56 on the MMR-deficient cell lines (HCT-116 and Jurkats) were similar to that of 6-TG. EKJC80A were the most cytotoxic of all the novel 6-TG compounds tested and was the only compound that elicited a higher activity towards the 6-TG resistant cell line, HCT-116. Cisplatin, a standard treatment, used to treat solid tumors resulted in the lowest IC₅₀ values between of all the compounds tested when tested on the non-hormonal cancers.

2.7.2 Hormone dependent cell lines

Several hormone-dependent cell lines (MCF-7, MCF12A, HeLa, A2780, A2780cis and DU145) were treated with varying concentrations of the novel compounds ranging from concentrations from as little as 0.006 to 100 μ M. During the prescreening phase of the study an estimated dose range for each of the experimental compounds on all cell lines were determined. These dose ranges were used during experimental repeats. The 6-TG was used as a standard in all tests conducted. Cells were incubated and harvested as described in Section 2.4.2.1. The MTT assay was used to quantify the percentage of viable cells. The inhibitory effect of the experimental compounds on hormonal cell lines (MCF-7 and HeLa) are illustrated in Figure 2.4a, Figure 2.4b, Figure 2.5a and Figure 2.5b respectively, while Table 2.3 expresses the mean IC₅₀ values obtained from the MTT assay on all the hormonal cell lines (MCF7, MCF12A, HeLa, Du145, A2780, and A2780A).

It was observed that all the novel 6-TG compounds were highly selective for the hormone dependent cell lines (MCF-7, MCF12A, HeLa, A2780, A2780cis and DU145) compared to the non-hormonal cell lines (Jurkat, HCT-116 and CoLo320DM). Figures 2.1-2.5b clearly indicated that these novel compounds inhibit cell growth in a dose-dependent manner in both the hormonal (MCF-7, MCF12A, HeLa, A2780, A2780cis and DU145) and non-hormonal cell lines (Jurkat, HCT-116 and CoLo320DM). Figures 2.4a-2.5b only represents the overall effect of the most selective (MCF-7) and the least selective group (HeLa). These novel derivatives were highly selective towards the hormone dependent

cell lines and resulted in lower IC₅₀ values compared to the non-hormonal cell lines as previous discussed in section 2.7.1 and are summarized in Table 2.2.

The results from this MTT assay are summarized in Table 2.2 and represent the mean IC₅₀ concentration±SEM. It was interesting to note that the Au(I)-compounds (EKJC56 and EKJC80A) showed higher selectivity towards the A2780, A2780cis, MCF12A and MCF7 cell lines. The novel compounds indicated similar activity to 6-TG towards both the prostate cancer, Du145 and the cervical cancer cell line, HeLa. Cisplatin, a compound that is used as standard therapy in many cancers, proved to be more effective in inhibiting cellular growth than the experimental compounds, resulting in the lowest IC₅₀ values the cell lines tested. The inhibitory effect of EKJC80A, EKJC56 and EKJC56ST showed to be significant (p value < 0.05) at almost all the concentrations used up to 0.039µM in all the cell lines. Treatment with 6-TG proved to be significant at concentrations of up to 0.07 µM (p value< 0.05).

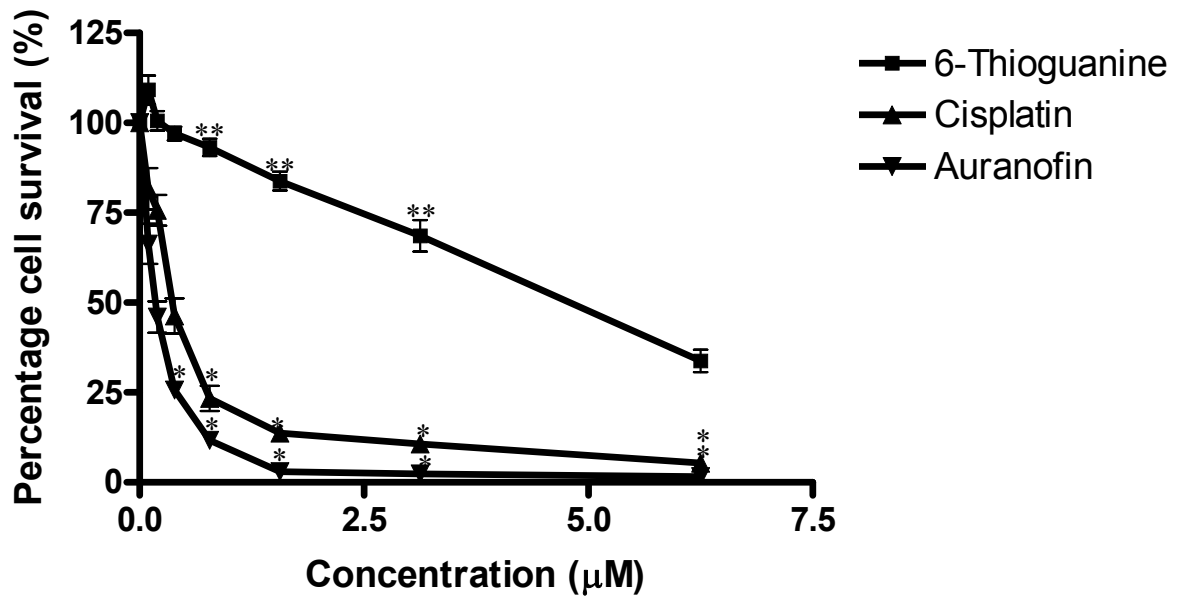


Figure 2.4a The inhibitory effect of 6-TG, Cisplatin and Auranofin on the percentage cellular survival of the MCF-7 cell line.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

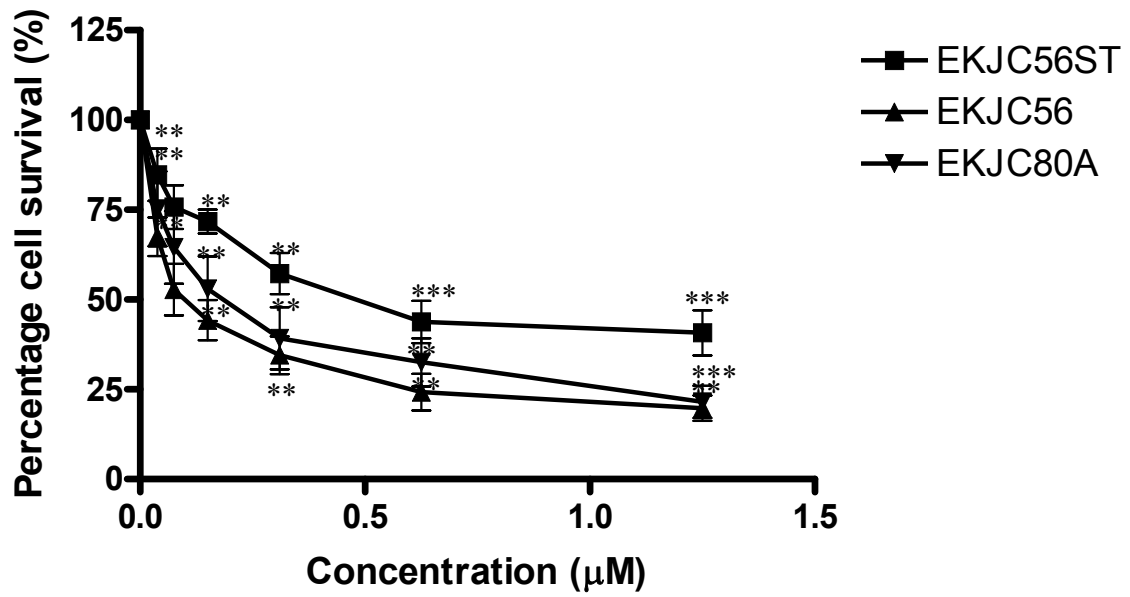


Figure 2.4b The inhibitory effect of EKJC56ST, EKJC56 and EKJC80A on the percentage cellular survival of the MCF-7 cell line.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

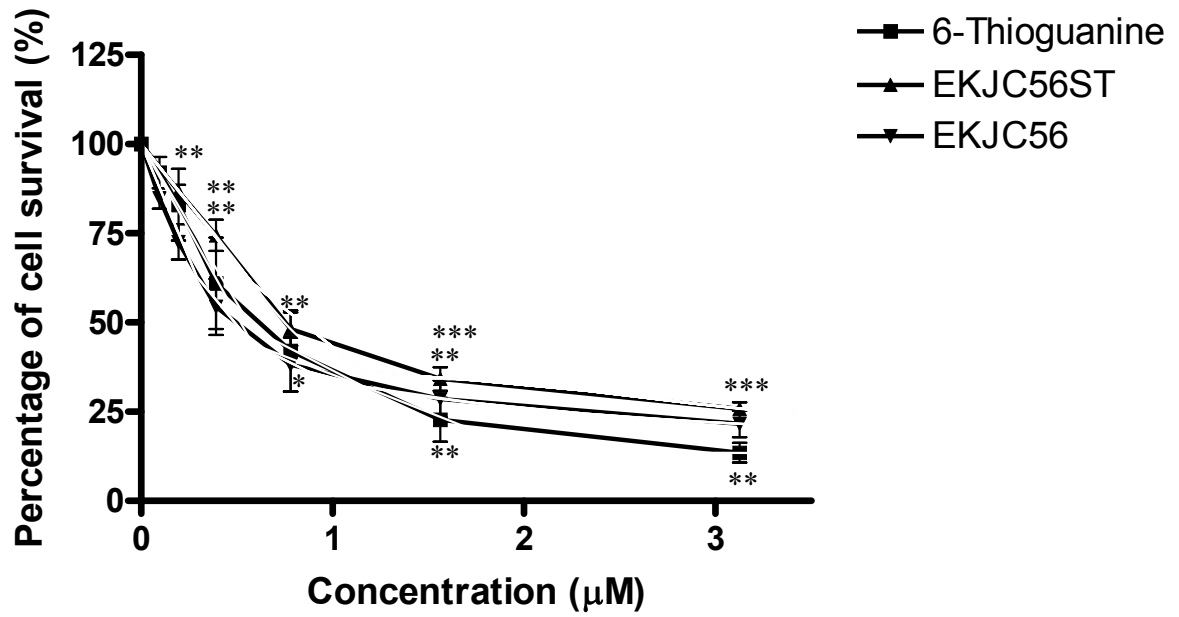


Figure 2.5a The inhibitory effect of EKJC80A, cisplatin and Auranofin on the percentage cellular survival of the HeLa cell line.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

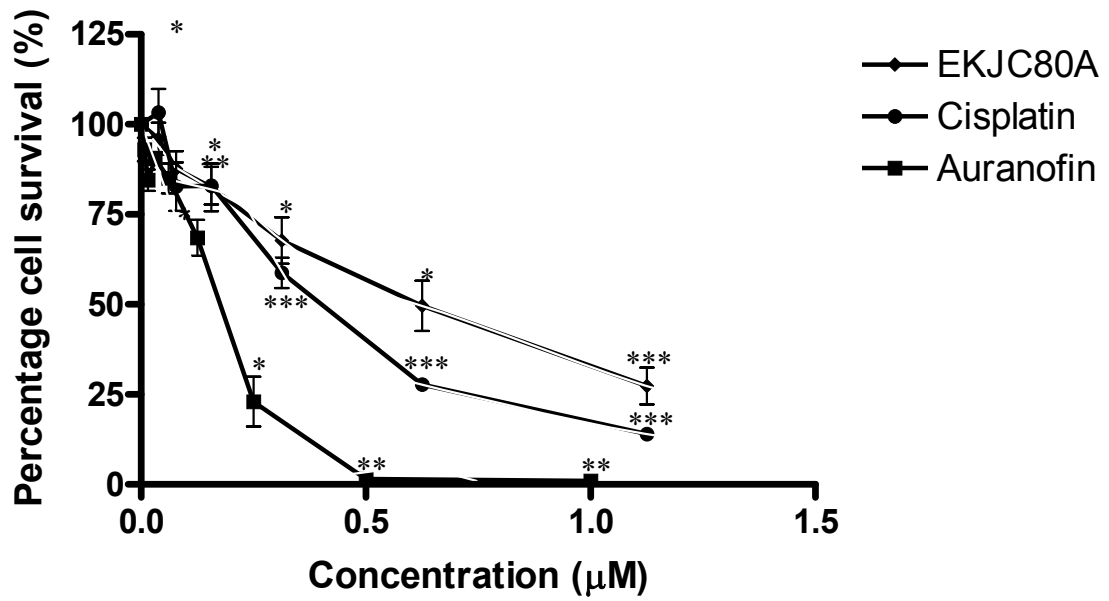


Figure 2.5b The inhibitory effect of EKJC80A, cisplatin and Auranofin on the percentage cellular survival of the HeLa cell line.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

TABLE 2.3 Mean⁽¹⁾ IC₅₀ (*) of cell lines treated with 6-TG, EKJC56, EKJC56ST, EKJC80A and Cisplatin after a seven day incubation period

Mean IC ₅₀ * value (±SEM) of cell lines treated (µM)						
Treatment	A2780	A2780cis	MCF-7	MCF12A	HeLa	Du145
6-TG	1.56 ±0.115	1.73 ±0.149	4.70 ±0.403	1.64±0.159	0.44 ±0.155	4.91 ±0.62
EKJC56ST	1.33 ±0.183	1.11 ±0.127	0.18 ±0.053	0.95 ±0.038	0.59 ±0.031	5.24 ±0.87
EKJC56	0.2 ±0.07	1.26 ±0.28	0.09 ±0.04	0.72 ±0.171	0.56 ±0.166	5.91 ±0.50
EKJC80A	0.31 ±0.04	0.96±0.64	0.17 ±0.051	1.18 ±0.37	0.54 ±0.165	0.6±0.053
Cisplatin	0.22 ±0.01	0.37 ±0.013	0.90 ±0.12	0.50 ±0.12	0.70 ±0.097	0.50 ±0.047

⁽¹⁾ Mean of at least 4 individual experimental repeats

*IC₅₀ is the concentration at which 50% of cell growth inhibited

2.4.3 Normal primary cell cultures

Porcine hepatocytes, chick embryo fibroblast, stimulated and unstimulated primary cells cultures were treated with varying concentrations of the experimental compounds ranging from concentrations from as little as 0.006 to 100 μ M. 6-TG was used as standard in all test conducted. Cells were incubated and harvested as described in Section 2.4.2.1 and Section 2.4.2.2. The MMT assay was used to quantify the percentage of viable cells.

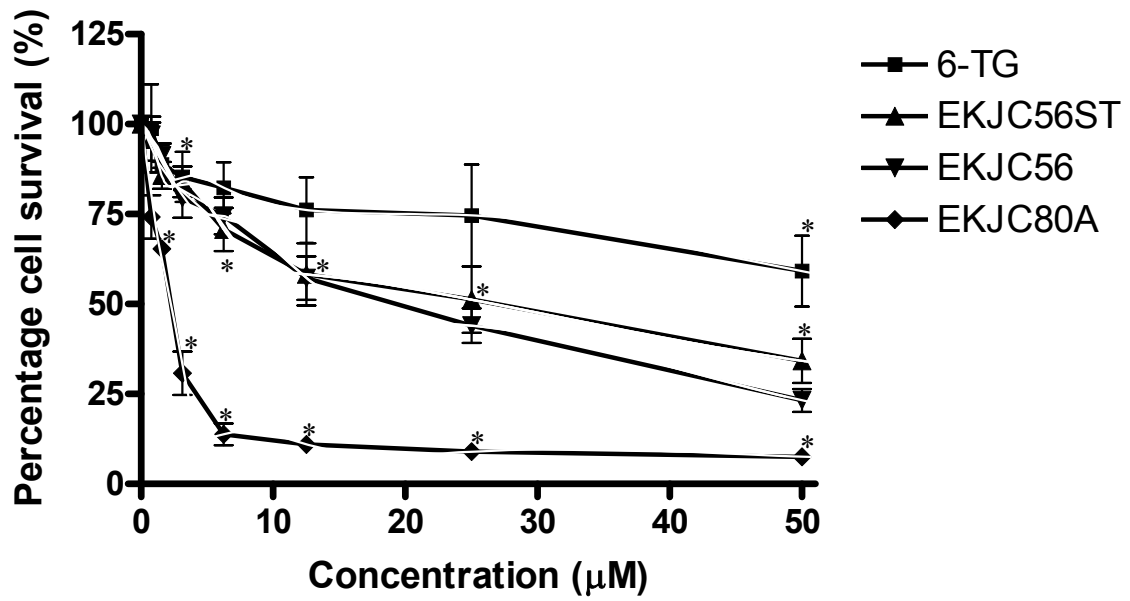


Figure 2.6 The inhibitory effect of 6-TG, EKJC56ST, EKJC56, and EKJC80A on the percentage cellular survival of the primary porcine hepatocytes.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

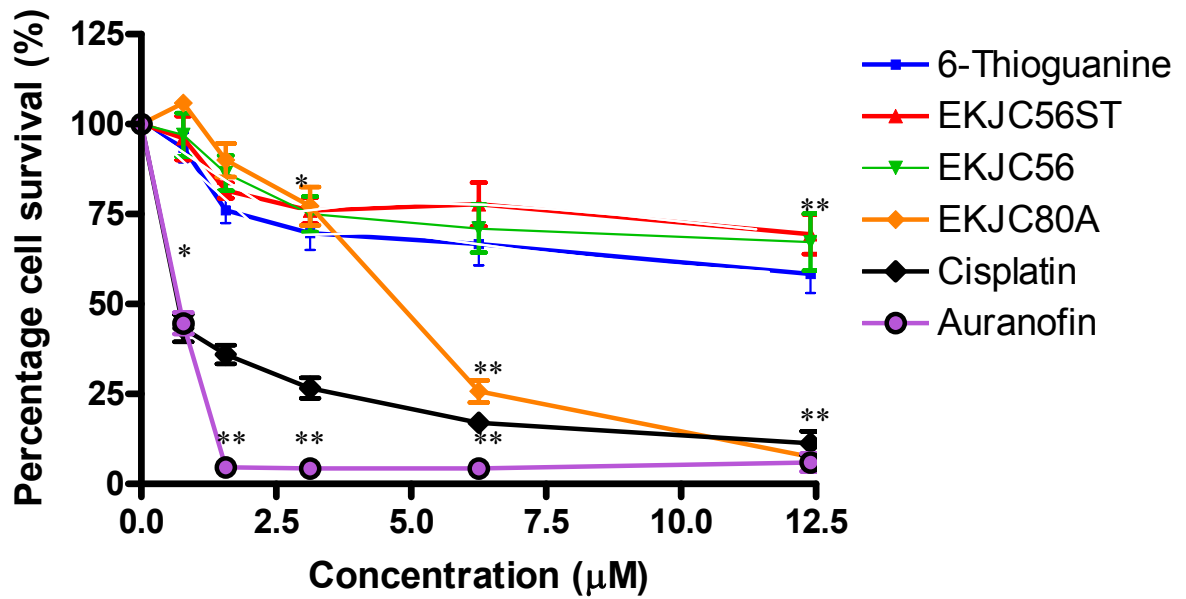


Figure 2.7. The inhibitory effect of 6-TG, EKJC56ST, EKJC56, EKJC80A, cisplatin and auranofin on the percentage cellular survival of the primary chick embryo fibroblast.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

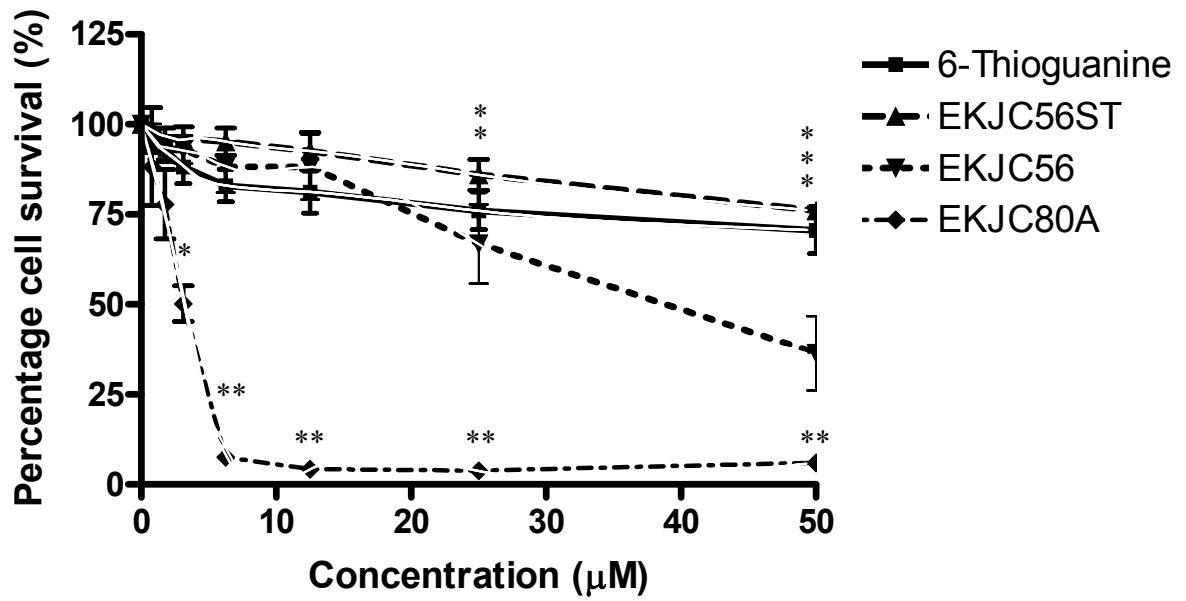


Figure 2.8. The inhibitory effect of 6-TG, EKJC56ST, EKJC56, and EKJC80A on the percentage of cellular survival of unstimulated human lymphocytes.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

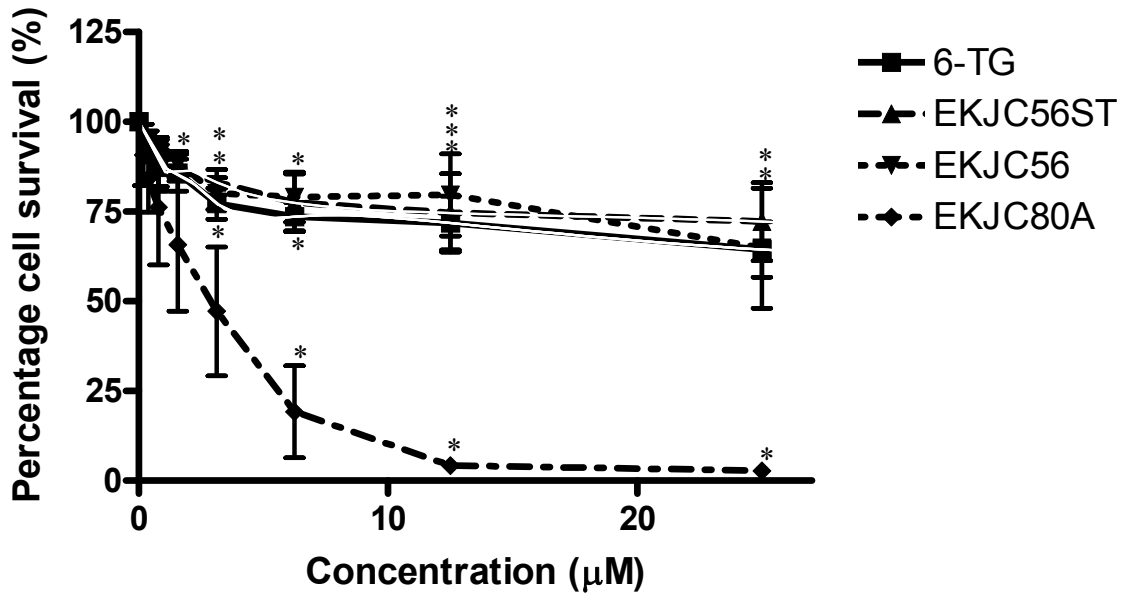


Figure 2.9. The inhibitory effect of 6-TG, EKJC56ST, EKJC56, and EKJC80A on the percentage of cellular survival of stimulated human lymphocytes after a 3 day incubation period.

* $p < 0.05$

** $p < 0.005$

*** $p < 0.0001$

TABLE 2.4 Mean IC50 of normal cell cultures treated with 6-TG, EKJC56, EKJC56ST, EKJC80A and Cisplatin after a seven day incubation period

Mean IC50* value (\pm SEM) of cell lines treated (μ M)				
Treatment	Fibroblast	Porcine Hepatocytes	Stimulated Lymphocytes	Unstimulated Lymphocytes
6-TG	>100	30.23 \pm 6.213	>100	>50
EKJC56ST	>100	16.50 \pm 3.246	>100	>50
EKJC56	57.69 \pm 3.954	13.13 \pm 0.543	>50	36.34 \pm 7.78
EKJC80A	5.02 \pm 0.09	2.31 \pm 0.36	3.17 \pm 0.22	3.13 \pm 1.244
Cisplatin	0.96 \pm 0.16	6.64 \pm 2.314	ND	ND

⁽¹⁾ Mean of at least 4 different experimental repeats

*IC50 is the concentration at which 50% of cell growth inhibited

ND – not done

On evaluation of the effect of the novel 6-TG derivatives on normal cell cultures including the primary porcine hepatocytes, chick embryo fibroblast, stimulate and non-stimulated lymphocytes indicated that 6-TG, EKJC56ST and EKJC56 showed very little toxicity towards these primary cell cultures. EKJC80A again proved to be the most toxic of the compounds tested resulting in the lowest IC50 concentrations in all the primary cell cultures (primary porcine hepatocytes, chick embryo fibroblast, stimulate and non-stimulated lymphocytes) tested.

The porcine hepatocytes seemed to be mostly affected by the Au(I) phosphine derivative, EKJC80A and inhibited cell growth significantly ($p < 0.05$) between 1.78-100 μ M whereas the other compounds (6-TG, EKJC56ST and EKJC56) did not inhibit cell growth significantly ($p \neq 0.05$) even at the concentrations as high as 12.5 μ M used. EKJC80A, inhibited cellular growth in a dose-dependent manner towards the stimulated-, unstimulated lymphocytes and chick embryo fibroblast and did not exhibited selectivity between normal and cancerous cell lines as previously discussed in Section 2.7.1 and Section 2.7.2. Therefore making this compound less favorable for potential *in vivo* studies. EKJC80A proved to be 6 times more toxic on the porcine hepatocytes and 30 times more toxic on the stimulated lymphocytes than 6-TG.

2.8 CONCLUSION

Results from the cytotoxic assay indicated that the novel compounds (EKJC56ST, EKJC56 and EKJC80A) showed promising antitumour activity towards the hormonal (MCF-7, MCF12A, HeLa, A2780, A2780cis and DU145) and non-hormonal cell lines (Jurkat, HCT-116 and CoLo320DM).

However, the Au(I)-phosphine derivative of 6-TG was the most cytotoxic of all the novel compounds tested and it did not differentiate between malignant and non-malignant cell lines and cell cultures. The results correlated with data from several articles that have shown that the Au(I)- and Au(III)-phosphine compounds have higher cytotoxic activity than gold-sulphur and gold – chloride containing compounds. **(See Chapter 1)**

It was further observed that the novel Au(I) compounds (EKJC56 and EKJC80A) had a slightly higher or even higher cytotoxic effect on several cell lines tested compared to 6-TG and EKJC56ST, thus supporting the hypothesis that the Au(I) side chain may be acting on a different mechanism within the cellular environment.

The results clearly showed that the addition of the sugar side-chain enhanced the activity of parent compound, 6-TG, in several of the cell lines tested. As mentioned in chapter 1, resistance towards 6-TG is also dependent on the status of hypoxanthine guanine transferase activity within the cell. This enzyme converts 6-TG to 6-thioguanosine monophosphate (a metabolite of 6-TG

containing a phospho sugar side chain). Addition of the phospho-sugar side chain to 6-TG can therefore enable EKJC56ST to bypass hypoxanthine guanine transferase in the metabolic pathway. Deficiency in this enzyme is the main cause of resistance towards 6-TG in 6-TG resistant cell lines; therefore limiting the amount of 6-TG converted to the active and toxic metabolite 6-thioguanilyc acid (**See Chapter 1**). This addition of the phospho-sugar side-chain can potentially increase the amount of 6-TG available for incorporation into the DNA while eliciting a more potent effect on tumour growth.

The results from the cytotoxicity assay strengthen the hypothesis that by altering pre-existing chemotherapeutic agents such as 6-TG with an Au side-chain the potency and selectivity of conventional chemotherapeutic agents can be increased. The Hela cell line was selected for further experimental procedures as this cell line was sensitive to all the compounds screened.



CHAPTER 3

APOPTOSIS AND NECROSIS: CELL DEATH PATHWAYS

3.APOPTOSIS AND NECROSIS : CELL DEATH PATHWAYS

3.1. INTRODUCTION:

Literature has described two major mechanisms of cell death with each being unique in their morphological changes and biological characteristics (Leach AP., 1998). Necrosis is a pathological passive death and occurs after severe tissue damage. This process of cell death is characterized, firstly, by morphological changes that include cellular swelling (i.e. loss of ion-influx control), cell membrane rupture, and finally spilling of intracellular contents to surrounding tissues evoking an inflammatory response. Other organelles such as the mitochondria are easily damaged whereby the nucleus still remains intact. (Leach AP.,1998; Kuan NK and Passaro E., 1998).

Apoptosis differs from necrosis in the sense that it is an active physiological process that requires ATP to maintain cellular integrity. This form of cell death is tightly regulated by intracellular genes at various stages of the cell cycle. Apoptosis is, however, characterized by several structural and biochemical changes which include the following: nuclear alterations, cell membrane alterations, mitochondrial alterations, DNA alterations and protein degradation (Woodle ES, and Kullcami., 1998)

Apoptosis forms part of a complex cascade of events within the eukaryotic cell such as the activation of various genes, transcription of pro-apoptotic protease, kinases, phosphates, and nucleases.

Cells can be forced to undergo apoptosis as a result of various intrinsic and extrinsic factors from several intracellular locations. Most chemotherapeutic agents have the ability to induce apoptosis by either causing intracellular stress, irreversible damage to DNA or the inhibition of vital intracellular proteins (Lowe SW *et al*, 2000). Cisplatin, a chemotherapeutic drug is known to induce apoptosis by intercalating with the DNA, causing irreversible DNA strand breaks. Unfortunately these compounds are non-selective and healthy cells are also targeted leading to the classical side-effects experienced when patients are treated with these agents.

Annexin V-FITC, a Ca²⁺ dependent phospholipid-binding protein has a high affinity for the phospholipid, phosphatidylserine (PS). PS is translocated from the inner to the outer plasma membrane during apoptosis. Binding of Annexin V-FITC to PS can measure the percentage of cells undergoing apoptosis.

Propidium iodide is used to detect non-viable cells by entering through damaged cell membranes and intercalates with the DNA of necrotic cells. Cells that stain positive for the Annexin V-FITC and negative for the propidium iodide are undergoing apoptosis, those cells that stain positive for both the Annexin V-FITC and propidium iodide are either at the end of apoptosis, undergoing necrosis or are non-viable. Cells that do not stain with either of the two stains are viable and not undergoing measurable apoptosis (Tuschl and Schwab, 2004; Michie *et al.*, 2003).

3.2 AIM

To determine if apoptosis can be induced in the HeLa cell line after treatment with the novel 6-TG compounds (EKJC56ST, EKJC56 and EKJC80A) compared to the parent compound 6-TG.

3.3 METHOD

3.3.1 Instruments and reagents required

- Sterile laminar flow cabinet,
- Water jacketed CO₂ incubator,
- Inverted microscope 4x10 optical lens,
- Shaker (Model: VRN-200, Gemmy industrial corp. Taiwan), 2
- 0-200 micro pipet,
- 15ml centrifuge tubes(AEC-Hamersham, Jhb, SA),
- 25cm³ cell culture flask (AEC-Amersham, P/L, Jhb, SA),
- Annexin V-FITC (BD-Bioscience, Jhb, SA)
- Propidium iodide (BD-Bioscience, Jhb, SA),
- Binding buffer (See Appendix A for preparation),
- 6-Thioguanine (Sigma-Aldrich, Johannesburg, South Africa),
- Auranofin (Sigma-Aldrich, Johannesburg, South Africa)

3.3.2 Experimental procedure

A volume of 9ml of HeLa cell suspension at a concentration of 1×10^5 cells/ ml was dispensed into 25cm³ cell culture flasks and incubated for 3 hours. After the incubation period 1ml of the experimental compounds (6-TG, EKJC56, EKJC56ST and EKJC80A) at a concentration of once and twice the concentration of the IC 50 concentration (See Table 2.2) were added to each of the cell culture flask. The cell culture flasks were incubated for a period of 24hours at temperature of 37°C at an atmosphere of 5%CO₂. The untreated control group received 1 ml EMEM growth medium, supplemented with 10% heat inactivated FCS and 1% penicillin-streptomycin.

After the required incubation period, cells were detached from cell culture flasks by carefully scraping the bottom of the flasks. The cell suspensions were then transferred to 15ml sterile centrifuge tubes and washed with freshly prepared PBS that were supplemented with 1% FCS. Tubes were centrifuged for 5 min at 1000 rpm's (200G's) and the supernatant discarded. Cells were then washed twice with freshly prepared PBS supplemented with 1% FCS and re-suspended in 1 ml binding buffer supplemented with 10% FCS. A volume of 100µl of HeLa cell suspension was transferred to 5ml flow cytometric tubes and 10µl of a 50µg/ml PI solution and 5µl Annexin V-FITC (Fluorescence conjugated Annexin-V) (*BD Bioscience, Johannesburg, South Africa*) was added. The PI/ Annexin FITC cell suspension was incubated at room temperature for 15 minutes and were kept in the dark area, because both the PI and Annexin V-FITC is light sensitive. A volume of 400µl binding buffer was added to each of the tubes and was vortexed

gently. Fluorescent intensity of both the PI and Annexin V-FITC on each of the experimental groups was measured using a Beckman Coulter FACS 500 Flow Cytometer.

3.4 STATISTICAL ANALYSIS OF RESULTS

The experiment was repeated 5 times on each of the experimental groups and the results obtained from this assay are expressed as the mean percentage (%)±SEM. A statistical program GRAPHPAD was used to determine the mean values and SEM of each concentration of the drug treated groups. Data was statistical analyzed using the Students' paired t-test and ANOVA. Results were considered significant at a p value < 0.05, a p-value of < 0.01 more significant and a p value of < 0.001 to be highly significant are indicated as *, ** and *** respectively. See Appendix B for definitions and explanations of statistical methods used for data analysis.

3.5 RESULTS AND DISCUSSION

The results of the effect of the experimental compounds on the percentage of cell entering the cell death pathways i.e. apoptosis, early- or late necrosis are illustrated in the Table 3.1, Table 3.2 and Table 3.3. An analysis of variance (ANOVA) statistical analysis was performed to determine whether there was any difference between the treatment groups, and concentrations used (IC50 value) on the percentage of viable cells after a 24 hour incubation period (Table 3.1). The results indicated that the differences between the concentrations were highly significant ($p < 0.0001$). Treatments were also significantly different ($p = 0.0152$) from each other, however no significant differences occurred between the interaction of the treatment groups and concentrations ($p = 0.1809$).

Table 3.1 Analysis of variance on the effects of treatments and concentrations on the percentage of viable cells after treatment with the experimental compounds.

Source	df	Sum of Squares	Mean of Square	F	p- value
Treatment	4	271.9	67.99	3.37	0.0152
Concentration	2	2244	1122	55.69	0<0001
Interaction	8	240.7	30.08	1.49	0.1809
Residual error	56	1128	20.15		
Total	70				

An analysis of variance (ANOVA) statistical analysis was performed to determine whether there was any difference between the treatment groups, and concentrations used (IC50 value) on the percentage of cells undergoing early apoptosis after a 24 hour incubation period (Table 3.2). The results from these experiments showed that the differences between concentrations were highly significant ($p < 0.0001$). Treatments also differed more significantly ($p = 0.0184$) from each other, however, no interaction occurred between the treatment groups and concentrations ($p = 0.1869$).

Table 3.2 Analysis of variance on the effects of treatments and concentrations on the percentage of HeLa cells entering early apoptosis after treatment with the experimental compounds.

Source	df	Sum of Squares	Mean of Square	F	p- value
Treatment	4	190.0	47.51	3.24	0.0184
Concentration	2	2295	1148	78.31	<0.0001
Interaction	8	173.1	21.63	1.48	0.1869
Residual error	56	820.8	14.66		
Total	70				

An analysis of variance (ANOVA) statistical analysis was performed to determine whether there was any difference between the treatment groups, and concentrations used (IC50 value) on the percentage of cells undergoing late apoptosis after a 24 hour incubation period (Table 3.3). The results from these experiments showed that the differences in all the groups were non significance ($p > 0.05$)

Table 3.3 Analysis of variance on the effects of treatments and concentrations on the percentage of HeLa cells entering late apoptosis after treatment with the experimental compounds.

Source	df	Sum of Squares	Mean of Square	F	p- value ^f
Treatment	4	26.58	6.646	0.47	0.7573
Concentration	2	50.55	25.28	1.79	0.1766
Interaction	8	36.03	4.504	0.32	0.9557
Residual error	56	791.3	14.13		
Total	70				

An analysis of variance (ANOVA) statistical analysis was performed to determine whether there was any difference between the treatment groups, and concentrations used (IC50 value) on the percentage of cells undergoing necrosis after a 24 hour incubation period (Table 3.4). The results from these experiments showed that the differences in all the groups were non significant.

Table 3.4 Analysis of variance on the effects of treatments and concentrations on the percentage of HeLa cells entering necrosis after treatment with the experimental compounds.

Source	df	Sum of Squares	Mean of Square	F	p- value
Treatment	4	5.511	1.378	0.53	0.7152
Concentration	2	25.35	12.68	4.86	0.0113
Interaction	8	12.64	1.581	0.61	0.7687
Residual error	56	146	2.607		
Total	70				

The percentage of viable cells (mean (%) of \pm SEM) and cells entering apoptosis, early – and late apoptosis treated with the low and high dose of the experimental compounds are shown in Table 3.4 and Figure 3.5 respectively.

Table 3.5 Mean percentage (\pm SEM) of HeLa cells entering early apoptosis, late apoptosis, necrosis and viable cells after a 24 hour exposure to 6-TG , EKJC56ST, EKJC56, EKJC80A and Auranofin at concentrations double the IC50 concentration. The Student's paired t-test was used to determine significance between the treatment groups and the untreated control group with * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

	Viable cells (%)	Early apoptosis (%)	Late apoptosis (%)	Necrosis (%)
Untreated Control	74.58 \pm 1.13	9.87 \pm 3.71	9.87 \pm 1.66	2.38 \pm 0.40
6-TG (0.44 μM)	67.11 \pm 1.87*	18.78 \pm 1.47*	6.38 \pm 0.51	3.182 \pm 0.50
EKJC56ST (0.59 μM)	64.03 \pm 3.37*	22.22 \pm 1.50*	7.61 \pm 0.50	3.73 \pm 0.78
EKJC56 (0.5 μM)	66.69 \pm 1.19**	18.39 \pm 2.02*	7.36 \pm 1.01	2.17 \pm 0.11
EKJC80A (0.7 μM)	55.54 \pm 3.04*	26.91 \pm 1.75*	8.23 \pm 0.55	3.52 \pm 0.57
Auranofin (0.1 μM)	56.78 \pm 1.98**	26.65 \pm 1.31**	10.15 \pm 0.83	3.77 \pm 1.01

Table 3.6 Mean percentage (\pm SEM) of HeLa cells entering early apoptosis, late apoptosis, necrosis and viable cells after a 24 hour exposure to 6-TG , EKJC56ST, EKJC56, EKJC80A and Auranofin at concentrations double the IC50 concentration. The Student's paired t-test was used to determine significance between the treatment groups and the untreated control group with * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

	Viable cells (%)	Early apoptosis (%)	Late apoptosis (%)	Necrosis (%)
Untreated Control	74.58 \pm 1.13	9.87 \pm 3.71	9.87 \pm 1.66	2.38 \pm 0.40
6-TG (0.9 μM)	68.27 \pm 1.06**	16.98 \pm 0.74*	7.77 \pm 1.02	3.13 \pm 0.46
EKJC56ST (1.18 μM)	66.69 \pm 1.19**	18.39 \pm 2.02	7.34 \pm 1.01	2.17 \pm 0.11
EKJC56 (1.0 μM)	61.43 \pm 3.05**	21.64 \pm 1.11**	7.36 \pm 1.32	3.132 \pm 0.41
EKJC80A (1.4 μM)	63.77 \pm 2.65*	21.89 \pm 1.65**	7.46 \pm 0.89	3.66 \pm 1.00
Auranofin (0.2 μM)	64.68 \pm 2.83*	20.53 \pm 3.37	8.14 \pm 0.99	2.70 \pm 0.521

Results obtained from the cell death induction are represented in the Table 3.5 and Table 3.6. HeLa cells treated with 6-TG significantly increase higher number of cells entering early apoptosis compared to the control group (18.78% vs. 9.87%). HeLa cells treated with EKJC56ST (22.22%), EKJC56 (18.39%) and EKJC80A (26.91%) also resulted in a higher percentage of cells entering early apoptosis to that of the untreated control group (9.87%). EKJC80A, the Au(I)-phosphine compounds induced a slightly higher number of cells to enter early apoptosis than 6-TG. The percentage of cells undergoing late apoptosis or necrosis phase was not significant in any of the experimental groups (6-TG, EKJC56ST, EKJC56, EKJC80A and Auranofin) at both the low and higher concentration groups.

The results from this study showed significant changes in cells undergoing early apoptosis after treatment with the novel compounds compared to the untreated control group.

Statistical analysis indicated that 6-TG, EKJC56ST, EKJC56 and Auranofin did not differ significantly from each other ($p > 0.05$) in respect to the percentage of cells entering early and late apoptosis. EKJC80A, however, differed significantly from 6-TG and EKJC56 in regards to percentage of cells entering early apoptosis.

3.6 CONCLUSION

Apoptosis is a more preferred method of cell death after treatment with chemotherapeutic agents, by clearing the physiological system of the dysfunctional or damaged cells. These results indicated that the novel compounds (EKJC56ST, EKJC56 and EKJC80A) are able to induce early apoptosis significantly compared to the untreated control group. Incubating HeLa cells at a higher concentration of the experimental compounds did not increase the number of cells entering apoptosis significantly after 24 hours. Further studies are required to observe the effect of the experimental compounds on the progression of cells into early / late apoptosis and necrosis if the incubation periods with the experimental compounds are prolonged.

Since HeLa cells are known to have a cell doubling time of approximately 19-28 hours a significant increase in cells entering apoptosis after 24-hour treatment with 6-TG was. As previously discussed in chapter 1; 6-TG requires the active metabolite to be incorporated into the DNA; therefore the effect of 6-TG will only be seen during the next cell cycle.

Addition of the gold side-chain increased the quantity (percentage) of cells entering apoptosis compared to the other non-gold containing compounds, but the potency of the compounds may be more dependent on the phosphine ligand. Results from this study suggest that treatment with the novel Au(I) compounds increases the percentage of cells entering early apoptosis but not proved to be more significant to that of the 6-TG treated group.

These results supports the hypothesis that addition of the sugar and Au(I) side-chains into 6-TG produces drug candidates that follows the same cell death pathway (i.e. early apoptosis) as that of 6-TG and Auranofin. Chapters 5, 6 and 7 are directed towards investigating the intracellular mechanism of action of these compounds.



Chapter 4

APOPTOSIS AND NECROSIS: CASPASE-3 ACTIVATION

4. APOPTOSIS AND NECROSIS: CASPASE-3 ACTIVATION

4.1 INTRODUCTION

The mitochondria play a crucial part in regulating cell death and cell survival. Apoptosis can be induced following intrinsic and extrinsic stimulation that results in the release of pro-apoptotic factors. This reaction elicits signals that can originate from various intracellular structures with the subsequent release of cytochrome c from the mitochondria. This substrate will in turn bind to dATP, Apaf-1 to form a multimeric complex known as an apoptasom. This apoptosom will then activate the inactive caspase 9. Active caspase 9 in turn will activate various other downstream effector caspases such as caspase 3, 6, 7. These effector caspases have the ability to act on several intracellular structures within the cell, including the nucleus, cytoskeleton and cytoplasm (Bernstein C, *et al.*, 2002; Philchenkov A, *et al.*, 2004).

Many cancers have down regulation of pro-apoptotic enzymes including the caspase family. These cysteine proteases that are activated from the inactive proenzyme are important indicators of early apoptosis (BD Pharmagen Technical Data Sheet).

4.2 AIM

To determine if caspase-3, a predictor of early apoptosis, can be activated in the HeLa cell line after treatment with the novel 6-TG compounds (EKJC56ST, EKJC56 and EKJC80A) compared to the parent compound 6-TG.

4.3 METHOD

4.3.1 Instruments Required

(See Appendix A for preparation of reagents)

- 15ml centrifuge tubes(AEC-Hamersham, Jhb, SA)
- 20-200 micro pipet
- 25cm³ cell culture flask (AEC-Amersham, P/L, Jhb, SA)
- Auranofin (Sigma-Aldrich, Johannesburg, South Africa)
- Camptothecin (Sigma-Aldrich, Johannesburg, South Africa)
- Microscope 4x10 optical lens
- PE-conjugated Monoclonal Rabbit Anti-active Caspase-3 Antibody Kit (BD Pharmagen, Johannesburg, South Africa)
- Shaker (Model: VRN-200, Gemmy industrial corp. Taiwan)
- Sterile laminar flow cabinet
- Water jacketed CO₂ incubator

4.3.2 Experimental Procedures

This method was done according to the package insert of the BD Pharmagen TM using PE-conjugated monoclonal active caspase-3 antibody apoptosis kit.

HeLa cells were detached from the cell culture flask (See previous preparation method) and washed twice with freshly prepared PBS by centrifuging the cell suspension at 1000rpm's for 10min. Supernatant was discarded and the pellet resuspend into 500µl Cytofix / CytopermTM solution. Cells were incubated on

ice for 20min and centrifuged at a speed of 1000rpm's for 10min. Cytofix / Cytoperm™ solution was carefully removed and discarded. The cells were washed a further two times using the 500µl Perm / Wash™ Buffer. 100µl Perm / Wash buffer and 20µl antibody was require per test sample. Cell / PermWash/ Antibody were incubated at room temperature for 30 min. After the incubation the 1ml Perm / Wash Buffer was added to the test sample, washed and the supernatant discarded. Cell pellets was resuspended into 500µl Perm / Wash™ Buffer and immediately analyzed on a Beckmann Coulter FACS 500 flow cytometer.

4.4 STATISTICAL ANALYSIS OF RESULTS

The results of the cytotoxic assay are expressed as the mean percentage (%) of \pm SEM. A statistical program GRAPHPAD was used to determine the mean values each of the treatment groups. Data was statistical analyzed with the Students' paired t-test and differences between concentrations in the treatment groups were determined using the analysis of variance (ANOVA). A p value of < 0.01 was considered to be significant, a p-value of < 0.001 more significant and a p value of < 0.0001 to be highly significant, indicated as *, **, and *** respectively. Also see Appendix B for explanation and definitions of statistical methods used for analysis.

4.5 RESULTS AND DISCUSSION

HeLa cells were treated with the experimental compounds (6-TG, EKJC56ST, EKJC56, EKJC80A and Auranofin) for a period of 24 hours at the concentration and double the concentration of the IC₅₀ value. Camphothecin (1 and 10 μ M), a standard agent used in the industry to activate caspase-3, was used as positive control in this assay. The effect of the experimental compounds is illustrated in Figure 4.1 and Figure 4.2.

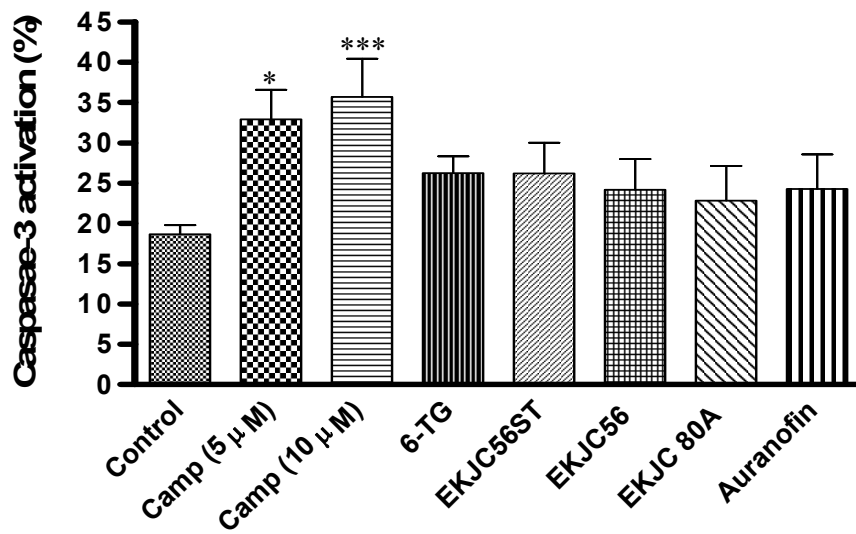


Figure 4.1 Histogram representing the percentage of caspase3 activation in the HeLa cell line after treatment with 6-TG, EKJC56ST, EKJC56, EKJC80A and Auranofin at the IC_{50} concentration for a period of 24hours. Camphothecin (5 and 10 μ M) is used as a positive control in this assay.

* $p < 0.01$

** $p < 0.001$

*** $p < 0.0001$

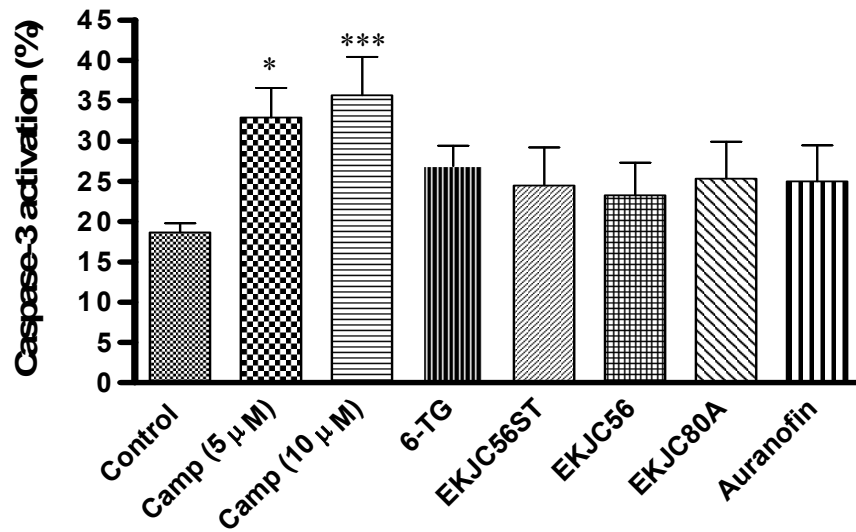


Figure 4.2 Histogram representing the percentage of caspase3 activated in the HeLa cell line after treatment with 6-TG, EKJC56ST, EKJC56, EKJC80A and Auranofin at double the IC₅₀ concentration for a period of 24hours. Camphothecin (5 and 10μM) is used as a positive control in this assay

* $p < 0.01$

** $p < 0.001$

*** $p < 0.0001$

The results show that HeLa cells treated with the experimental compounds markedly increases the activation of caspase-3 (24.16-26.85%) compared to the untreated control group (18.67%). Treatment with Camphothecin were highly significant ($p < 0.0001$) at a concentration of 10 μ M by almost doubling the percentage of activated caspase-3 compared the untreated control (35.68% vs 18.67%). This change was also seen in all the other experimental groups tested and ranged from (35.68-24.16%).

The increase in the activation of caspase-3 correlated with the results that were obtained in Chapter 3. The presence of the effector's caspases such as caspase 3, 6 and 7 are markers of the induction of early apoptosis. No significant changes between the different concentrations of the drugs were observed during this assay when data was analyzed using the Student paired t-test and the ANOVA test. Treatment groups were not significant different from each other using the analysis of variance. Figure 4.1 and 4.2 illustrates the activation of caspase-3 in the treated and untreated control groups.

The results obtained from the flow cytometric analysis of cell death induction indicated that all the experimental compounds (EKJC56ST, EKJC56, and EKJC80A) induced early apoptosis when compared to the untreated control groups. The data obtained from this assay supports the hypothesis that the novel derivatives (EKJC56ST, EKJC56 and EKJC80A) are able to follow the same cell death induction pathway as parent compound 6-TG.

These results further justified the previous findings in Chapter 3 that early apoptosis is indeed induced. It is known that caspase-3 is released explicitly during the early stages of apoptosis. The results showed an increase of caspase-3 activation due to the binding of the monoclonal anti-body to this activated enzyme, which could be detected using flow cytometry. Camptothecin, a known inducer of caspase-3 activity was used as positive control. However, the results did not show significant differences ($p>0.05$) between the different treatment groups or concentration used in the activation of caspase-3 activity.

4.6 CONCLUSION

These results supports the previous findings in Chapter 3, that like 6-TG and Auranofin, the novel compounds investigated here induce HeLa cells to enter early apoptosis.



CHAPTER 5 MITOCHONDRIAL MEMBRANE POTENTIAL

5. MITOCHONDRIAL MEMBRANE POTENTIAL

5.1 INTRODUCTION

The mitochondria received a considerable amount of attention as potential chemotherapeutic targets during the past 40 years. Mitochondria are not only responsible for most homeostatic processes within the intracellular environment but its role in regulating cell proliferation has become an essential part in drug development. Functions include the production of up to 80-90% of ATP through oxidative phosphorylation, Ca^{2+} flux, pro and anti-apoptotic signaling. Alterations in these unique organelles contribute to the pathophysiology of many diseases and resistance towards chemotherapeutic treatments including arthritis and cancer (Barnard et al, 2004).

It was found that neoplastic cells have higher mitochondrial and plasma membrane potentials than normal cells (Chen, 1988). This difference in membrane potential reduces the uptake of numerous compounds such as cisplatin and contributes to treatment failure. The recent developments of lipophilic cationic therapeutic compounds have the ability to overcome this barrier, allowing these compounds to enter the cell. This results in the accumulation of the lipophilic compounds within the highly polarized structure, inducing the required effect (Berners-Price *et al.*, 1999).

The mitochondrial transition pore, a voltage dependent, inner membrane channel, regulates the mitochondrial membrane potential and is considered to play an essential part in apoptosis (Barnard *et al.*, 2004). Opening of these pores

are characterized by several events resulting in depletion in ATP, dissipation of $\Delta\Psi_m$, (Mitochondrial membrane potential) reduction in oxidative phosphorylation, increase production of free radicals and reactive oxygen species, with the subsequent mitochondrial swelling (Patenaude *et al.*, 2004). Under physiological conditions the mitochondria produce high quantities of oxidative end products (H_2O_2 and O_2^-). Literature has described several detoxification systems whereby these potential damaging agents are adequately removed. The most research being done on the glutathione / glutathione reductase, glutathione / glutathione peroxidase, thioredoxin / thioredoxin reductase and peridoxin systems (Patenaude *et al.*, 2004; Omata *et al.*, 2006).

Mitochondria of cancer cells have higher mitochondrial membrane potential than normal healthy cells. The fluorescent dye, 5,5',6,6',-tetrachloro-1,1',3,3' tetraethyl benzimidazolyl carbocyanine iodide, or JC-1 have the ability to distinguish between low and highly polarized cells. JC-1 can form multiple aggregates (J-aggregates) that fluoresce red-orange (590nm) when excited at 488nm in cells with high mitochondrial membrane potential. At lower membrane potential JC-1 can only form monomers which emits green light (525-535nm) when excited at 488nm. This method makes the effect of these experimental compounds on the mitochondria visible. The advantage of using the JC-1 stain is that it can quantitatively and qualitatively make detection of the shift of fluorescence possible as the mitochondrion becomes depolarized (Smiley *et al.*, 1991; Cassarelli *et al.*, 1993).

Various studies have shown that Au (I) compounds can induce mitochondrial permeability transition which is characterized by mitochondrial swelling and reduction in mitochondrial membrane potential. The mechanism is not clearly understood but it is believed that there is a direct inhibition of oxidative phosphorylation and of thioredoxin reductase (Rigobello *et al.*, 2005; Barnard *et al.*, 2004).

5.2 AIM

To determine the effect of the novel 6-TG compounds (EKJC56ST, EKJC56 and EKJC80A) on mitochondrial membrane potential of the HeLa cell line.

5.3 METHOD

5.3.1 Instruments required (See Appendix A for preparation of reagents)

- Sterile laminar flow cabinet,
- Water jacketed CO₂ incubator,
- 20-200 µl pipet,
- 15ml centrifuge tubes(AEC-Hamersham, Jhb, SA),
- cell culture Petri dish (AEC-Amersham, P/L, Jhb, SA),
- 6-thioguanine (Sigma-Aldrich, Johannesburg, South Africa),
- auranofin (Sigma-Aldrich, Johannesburg, South Africa)
- valinomycin (Sigma-Aldrich, Johannesburg, South Africa)

5.3.2 Experimental procedure

This method was carried out according to the method described by Cossarizza and Salvioli, 1997.

A volume of 2.7ml of HeLa cell suspension at a concentration of 1×10^5 cells/ml was dispensed into sterile Petri dishes and incubated for 3 hours at 37°C in an atmosphere of 5% CO_2 . After the incubation period the experimental compounds (6-TG, EKJC56, EKJC56ST, EKJC80A and Auranofin) were added to the cell suspension at the concentration and double the concentration of the IC_{50} concentration. The cell and drug suspension was incubated for 24hours at 37°C in an atmosphere of 5% CO_2 . Cells were harvested and transferred to sterile 15ml centrifuge tubes. Cells were centrifuge for 5min at 800G's and the supernatant discarded. The cell pellet was resuspended into 1ml EMEM supplemented with 10% heat inactivated FCS. 900 μl of cell suspension was transferred to sterile 15ml centrifuge tubes and 100 μl of a 1.5 μg / ml JC-1 solution was added (see Appendix A). The cell / JC-1 suspension was incubated for 20min in the dark at an atmosphere of 5% CO_2 at 37°C . After the incubation period cells were centrifuge at 1700 rpm's (500G's) for 5min and the supernatant discarded. The pellet was washed twice with 2ml PBS and centrifuged at 1700 rpm's (500G's) for 5 min.

The pellet was resuspended in 1ml PBS supplemented with 10% fetal calf serum. The fluorescence of the formation J-aggregates (Green fluorescence-

hyperpolaration) and monomers formation (Orange fluorescence-depolarization) was measured using a Beckmann Coulter FACS 500 flow cytometer.

5.4 STATISTICAL ANALYSIS OF RESULTS

The results of the cytotoxic assay are expressed as the mean percentage (%) of \pm SEM. A statistical program GRAPHPAD was used to determine the mean values each of the treatment groups. Data was statistical analyzed with the Students' paired t-test and differences between concentrations in the treatment groups were determined using the analysis of variance (ANOVA). A p value of < 0.05 was considered to be significant, a p-value of < 0.001 more significant and a p value of < 0.001 to be highly significant, indicated as *, **, and *** respectively. Also see Appendix B for explanation and definitions of statistical methods used for analysis.

5.5 RESULTS AND DISCUSSION

The ratio of the fluorescence of the JC-1 monomers and JC-1 conjugates after treatment at both high and low concentrations with the experimental compounds are illustrated in Figure 5.1 and Figure 5.2 and the mean ratio (FL1/FL2) \pm SEM tabulated in Table 5.1 and Table 5.2.

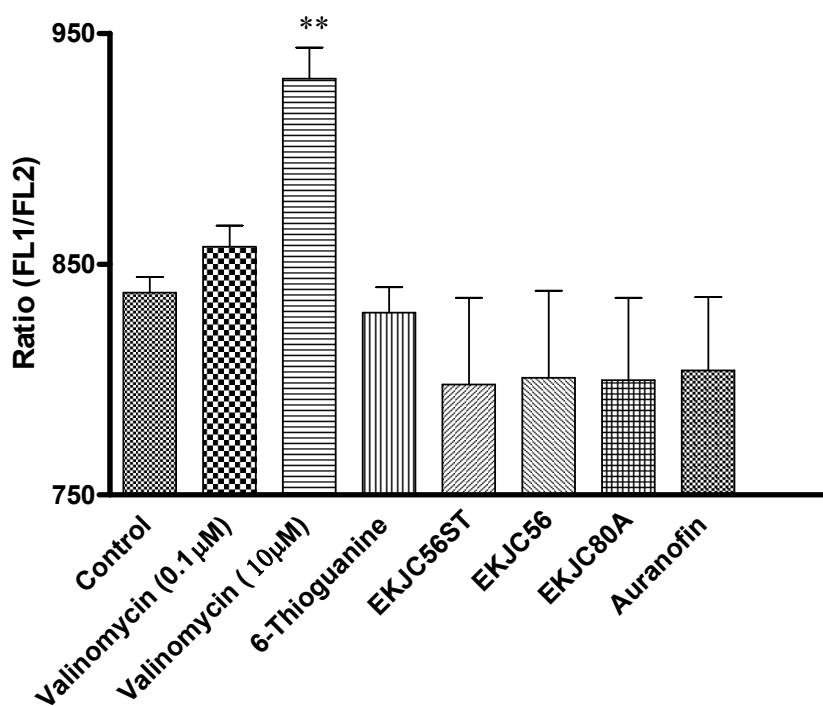


Figure 5.1 Histogram representing the changes in mitochondrial membrane potential (Ratio-FL1/FL2) after treatment with the novel compounds (EKJC56ST, EKJC56 and EKJC80A) at concentrations equal to the IC50 concentration.

* $p < 0.05$

** $p < 0.001$

*** $p < 0.0001$

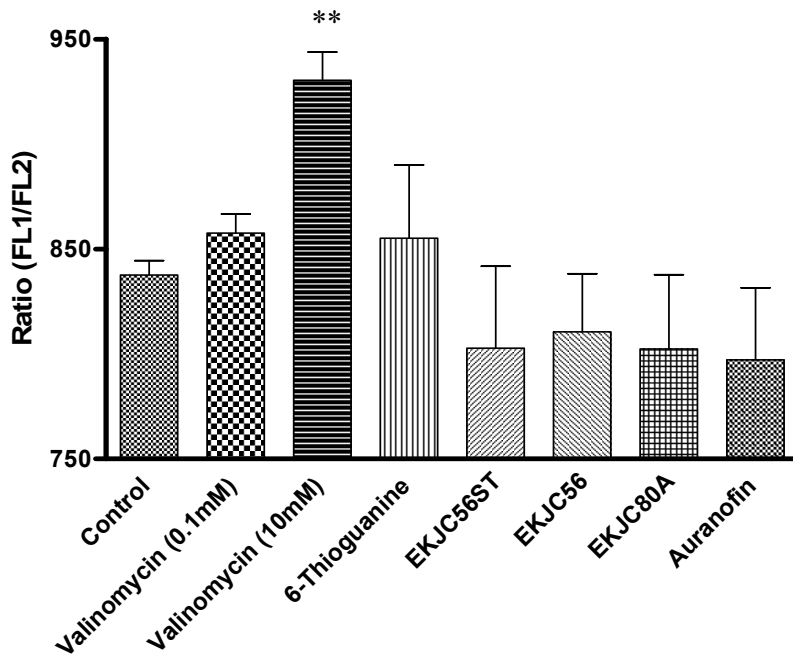


Figure 5.2 Histogram representing the changes in mitochondrial membrane potential (Ratio-FL1/FL2) after treatment with the novel compounds (EKJC56ST, EKJC56 and EKJC80A) at higher concentrations.

* $p < 0.05$

** $p < 0.001$

*** $p < 0.0001$

Treatment group	FL1/FL2	p-value
Untreated Control	837.6±6.88	
Valinomycin	857.60±9.10 (0.1µM)	0.087
6-TG	829.00±10.97 (0.44µM)	0.301
EKJC56ST	796.00±36.98 (0.59µM)	0.273
EKJC56	800.80±37.75 (0.5µM)	0.339
EKJC80A	799.80±35.60 (0.7µM)	0.292
Auranofin	804.00±31.76 (0.1µM)	0.293

Table 5.1 Changes in the mitochondrial membrane potential after HeLa cells were exposed to concentration equal to the IC50 concentrations for 6-TG, EKJC56ST, EKJC56 and EKJC80A following a 24hours exposure period. Results are expressed mean ratio±SEM and the p-value obtained using the Students paired t-test (Treatment vs control)

* $p < 0.05$

** $p < 0.001$

*** $p < 0.0001$

Treatment group		
	FL1/FL2	p-value
Untreated Co	837.6±6.88	
Valinomycin	928.60±12.74 (10µM)	0.0007**
6-TG	855.20±34.93 (0.9µM)	0.663
EKJC56ST	802.80±39.06 (1.18µM)	0.378
EKJC56	810.60±27.96 (1.0µM)	0.308
EKJC80A	802.04±35.28 (1.4µM)	0.351
Auranofin	797.20±34.27 (0.2µM)	0.254

Table 5.2 Changes in the mitochondrial membrane potential after HeLa cells were exposed to concentrations double the IC50 concentration for 6-TG, EKJC56ST, EKJC56 and EKJC80A following a 24hours exposure period. Results are expressed mean ratio±SEM and the p-value obtained using the Students paired t-test (Treatment vs control).

* $p < 0.05$

** $p < 0.001$

*** $p < 0.0001$

An analysis of variance (ANOVA) statistical analysis was performed to determine whether there was any difference between the treatment groups, and concentrations used (IC₅₀ value) on the polarity of the HeLa cells mitochondrial membrane after a 24 hour incubation period (Table 5.3) with the experimental compounds. The results from this experiment indicated that treatments were significant ($p < 0.0001$) different from each other. No significant differences were observed between the concentrations and interaction between groups / concentration.

Table 5.3 Analysis of variance on the effects of treatments and concentrations on the percentage of HeLa cells entering early apoptosis after treatment with the experimental compounds.

Source	df	Sum of Squares	Mean of Square	F	p- value
Treatment	5	47430	9485	2.93	0.0183
Concentration	1	8888	4444	1.37	0.2599
Interaction	10	33510	3351	1.04	0.4231
Residual error	72	23300	3236		
Total	89				

From the above results, no significant changes were observed in mitochondrial membrane potential after exposure to both low and high concentrations of the experimental compounds (6-TG, EKJC56, EKJC56ST, EKJC80A and Auranofin) after 24 hours. Treatment with 6-TG, EKJC56ST, EKJC56, EKJC80A and Auranofin, resulted in the mitochondrial membrane becoming slightly hyperpolarized at both the high and low concentrations used, but the hyperpolarization was not significant in these treatment groups. Treatment with valinomycin (0.1 and 10 μ M) however resulted in a significant loss ($p < 0.001$) in MMP of 13% compared to the untreated control group (941 vs 834). In contrast, Auranofin did not elicit the depolarization of the MMP as anticipated but instead hyperpolarized the HeLa cell mitochondrial membrane compared to the untreated control group.

5.6 Conclusion

Previous research conducted have shown that both Au(I) and Au(III) compounds are highly selective in targeting the mitochondria and that these compounds have the ability to induce the dissipation of the mitochondrial membrane potential. (Rigobello *et al.*, 2002; Rigobello *et al.*, 2005; Barnard *et al.*, 2004) The concentrations of these gold compounds used on normal isolated rat liver mitochondria ranged between 0.02-20 μ M. These research articles did not use the fluorescent probe, JC-1, to determine disruption of the MMP, but utilized other methods to detect the consequences of the Au(I) and Au(III) compounds on the integrity of the MMP, including mitochondrial swelling, production of NO and H₂O₂ (Rigobello *et al.* 2005; Barnard *et al.* 2004)

The results did not support loss in MMP after treatment with auranofin as published in these research articles. A possible justification for our results could be that these studies tested the effect Au(I) and Au(III) compounds only on healthy isolated rat liver mitochondria and not on cancer cells. Thus no results have been published to support the dissipation of the $\Delta\psi_m$ in cancer cell lines after treatment with Au(I) and Au (III) compounds. An article published by Wigdal *et al*, (2002) referred to a previous study conducted whereby Cytochrome c was released prior to the loss of MMP in HeLa cells. This indicated the possibility that the induction of apoptosis is not dependent on the dissipation of the MMP for apoptosis to be in progress. An article published by Giovannini *et al* 2002 suggested that cells such as the lymphocytes became hyperpolarized prior to the loss of the MMP even though apoptosis was in progress.

The results obtained during this assay suggested that the mitochondrial membrane of HeLa cells did not become hyperpolarized nor polarized after treatment with all the experimental compounds.



CHAPTER 6

THIOREDOXIN REDUCTASE

ACTIVITY

6. THIOREDOXIN REDUCTASE ACTIVITY

6.1 INTRODUCTION

During this stage of the study the mechanism of action of the gold (I) and gold (I)-phosphine compounds were investigated. Au (I) has a high affinity for the following soft ligands i.e. thiolates (cysteine residues in proteins, free cysteine and glutathione) and phosphine containing enzymes (Roberts and Shaw, 1998). Several studies have indicated that Au (I) compounds act directly on the mitochondria by inhibiting various mitochondrial enzymes especially thioredoxin reductase glutathione reductase and DNA polymerases (Kaina, 2002; Rigobello *et al.*, 2004; Barnard *et al.*, 2004).

Thioredoxin and the glutathione reductase redox system play an important physiological role in cellular metabolism by protecting cells against oxidative damage. Thioredoxin reductase is a member of a small group of selenium containing flavoenzymes. Thioredoxin reductase has various metabolic functions that involve synthesis of deoxyribonucleotides, reduction of thioredoxin, selenonite, lipoic acids, hydroperoxides and hydrogenperoxide. The thioredoxin reductase system is needed in all three stages of cancer prevention, early detection and effective treatment (Baguley *et al.*, 1998).

A thioredoxin assay kit was purchased from Sigma-Aldrich, Jhb, SA. This colometric assay is based on the reduction of 5, 5' dithiobis (2-nitrobenzoic) acid (DTNB) in the presence of NADPH to a bright yellow 5-thio-2-nitrobenzoic acid

(TNB) product which can then be measured at a wavelength of 412nm using a Beckmann Coulter FACS 500 flow cytometer.

6.2 AIM

To determine the effect of the novel 6-TG compounds (EKJC56 and EKJC80A) on thioredoxin activity in HeLa cells.

6.3 METHOD

6.3.1 Instruments required

- UV spectrophotometer
- Quartz cuvettes
- Pipettes
- Thioredoxin reductase assay kit (*Sigma-Aldrich, Johannesburg, South Africa*)
- Sterile water

6.3.2 Experimental procedures

This method was conducted according to the package insert of the Thioredoxin reductase assay kit. (Sigma Aldrich, JHB, SA)

The spectrophotometer was set to a wavelength of 412nm. The program was set to allow for a 5 second delay before the first reading was measured and a reading was taken every 30 seconds for a 10 minute period. A volume of 900µl of working buffer was added to each 1ml cuvette. The reaction was started by

adding 30 μl of the DNTB solution to each cuvette and mixing by means of inverting the cuvette. Rate of the formation of the yellow product (TNB) was determined by measuring the absorbance for each of the cuvettes over a period of 10 minutes ($\Delta A_{412}/\text{min}$)

The rate of formation can be described as the ability of one unit of mammalian thioredoxin reductase will cause an increase in ΔA_{412} of 1.0 unit per minute per when used with DTNB alone at a pH of 7.07 at a temperature of 25°C.

6.4 RESULTS AND DISCUSSION

Due to limited availability of thioredoxin reductase, direct inhibition of rat liver thioredoxin reductase was only performed during a single experimental run. During this assay only the novel Au(I) compounds EKJC56 and EKJC80A were selected to assess the possible inhibitory potential of these novel Au -6 TG compounds. Therefore further investigation of this assay to include 6-TG, and EKJC56 is required to established if these compounds ability to inhibit thioredoxin reductase to the extent of that of Auranofin was used to verify the inhibition of thioredoxin reductase. The inhibitory effect of the two novel Au(I) compounds (EKJC56 and EKJC80A) at varying concentrations (0.1, 1 and 10 μM) were investigated and the results are shown in Figure 6.1-6.3.

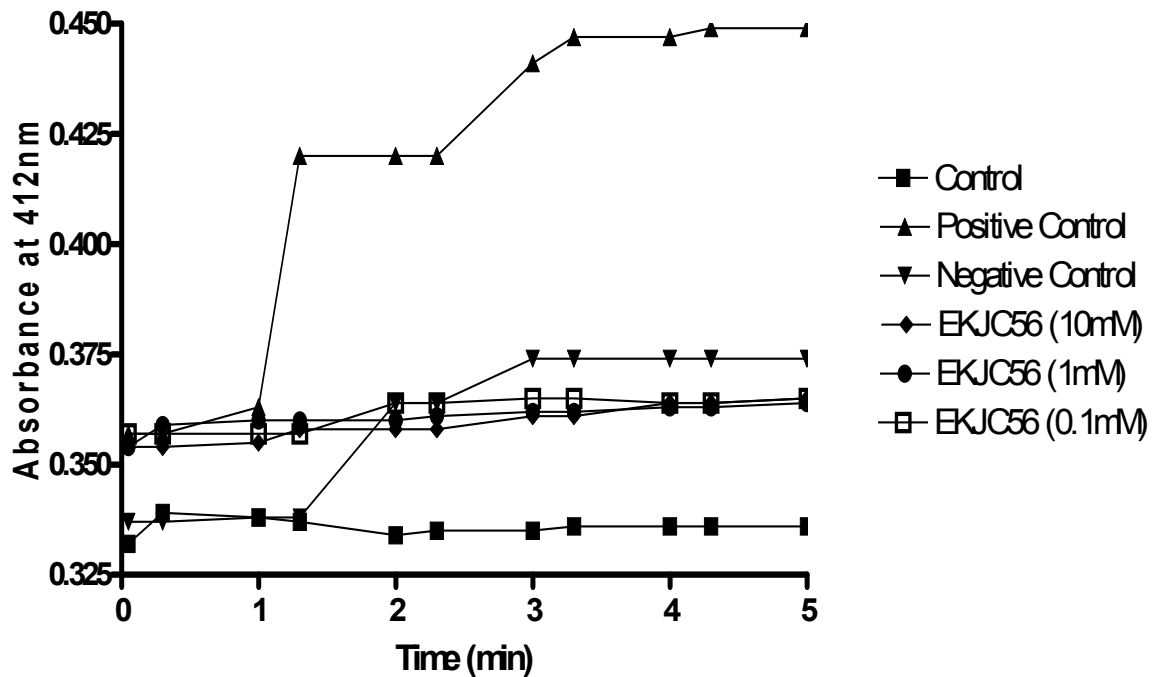


Figure 6.1 Graph representing the change in absorbance (A_{412nm}) of TNB following the addition of EKJC56 at varying concentrations measured over time at set time intervals.

Control: No enzyme + Substrate + Working buffer

Positive Control: Enzyme + Substrate + Working Buffer

Negative control: Enzyme + Substrate + Inhibitor + Working buffer

Drug treated: Enzyme + Substrate + Drug + Working buffer

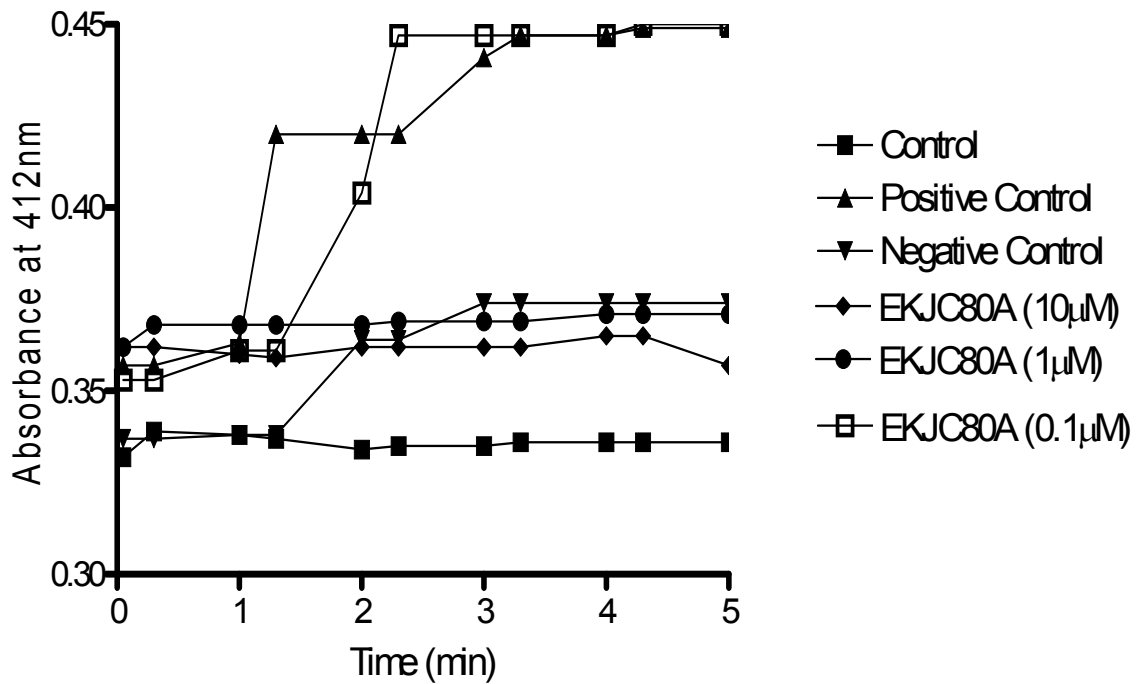


Figure 6.2 Graph representing the change in absorbance (A_{412nm}) of TNB following the addition of EKJC80A at varying concentrations measured over time at set time intervals.

Control: No enzyme + Substrate + Working buffer

Positive Control: Enzyme + Substrate + Working Buffer

Negative control: Enzyme + Substrate + Inhibitor + Working buffer

Drug treated: Enzyme + Substrate + Drug + Working buffer

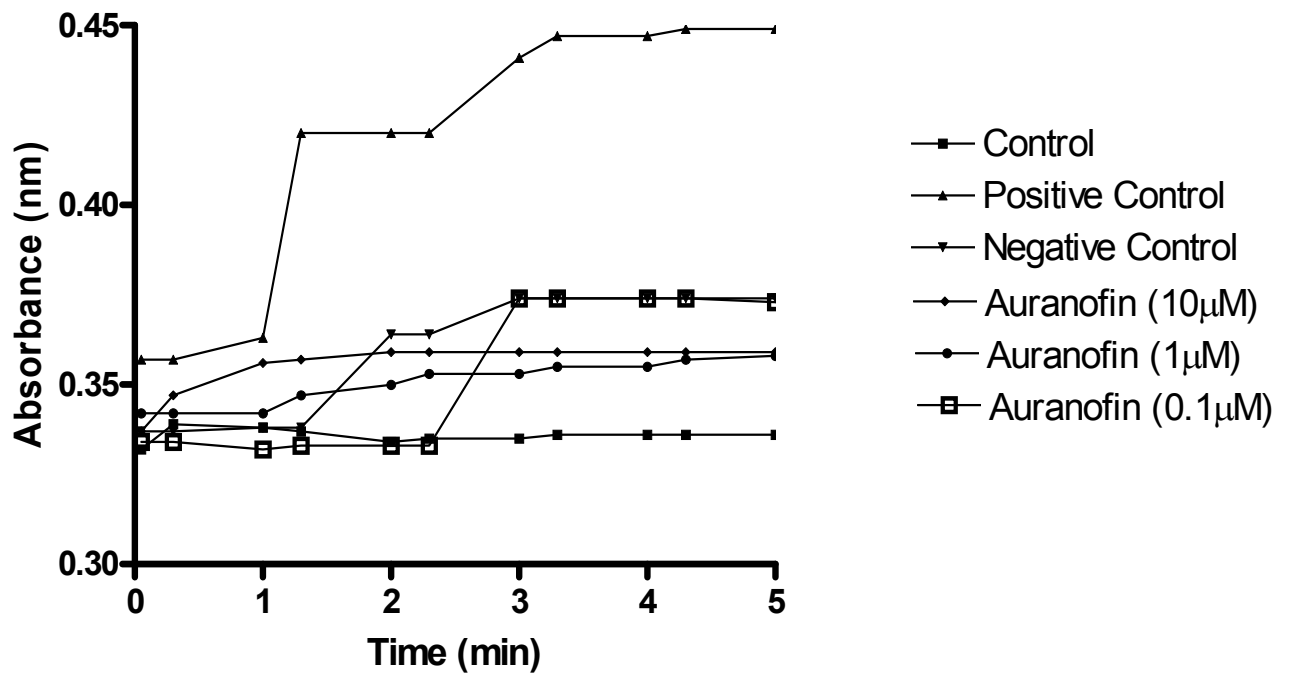


Figure 6.3 Graph representing the change in absorbance (A_{412nm}) of TNB following the addition of Auranofin at varying concentrations measured over time at set time intervals.

Positive Control: Enzyme + Substrate + Working Buffer

Negative control: Enzyme + Substrate + Inhibitor + Working buffer

Drug treated: Enzyme + Substrate + Drug + Working buffer

The preliminary results indicate that the novel Au(I)-sugar (EKJC56) and Au-phoshine (EKJC80A) were as effective as Auranofin in inhibiting the formation of the bright yellow product, TNB. During this assay it was observed that EKJC56 was the most effective in inhibiting the production of TNB when compared to both EKJC80A and Auranofin. EKJC80A inhibited the production of TNB at both 1 and 10 μ M, but resulted in the production of TNB after 1min. A similar results was observed for the untreated positive control.

These results support the hypothesis that these novel Au(I) compounds have the ability to inhibit the enzyme, thioredoxin reductase, contributing to the enhanced anticancer activity of 6-TG derivatives. Further enzymatic analysis is still required to prove that these Au(I) compounds do act on thioredoxin reductase.

6.5 CONCLUSION

Although only one experimental procedure could be performed the preliminary results indicated that the Au(I) derivatives were potent inhibitors of the enzyme thioredoxin reductase, and therefore inhibited the production of the product TNB,. however, further biochemical analysis of this possible mechanism of action should be explored.



CHAPTER 7

CELL CYCLE ANALYSIS

7. CELL CYCLE ANALYSIS

7.1 INTRODUCTION

Many articles have described cancer as a cell cycle disease. Cancer cells attain proliferative abilities through various mutations within the genome resulting in the inactivation or up regulation of various cell regulatory proteins. The cell cycle can be divided into four distinct phase G1 (growth), S (DNA synthesis), G2 (gap) and M (mitosis) with each phase unique according to it's' function (McLaughlin F *et al.*, 2003). In normal proliferative cells various mechanisms tightly regulate the progression of cells through the phases by the formation, activation and deactivation of phase specific cyclins and cyclin dependent kinases (Park et al., 2002). Within each phase various checkpoints within the cell cycle, allows the cell to delay transit into the next phase to repair any damage. Defects in these checkpoints and the proteins expressed during these phases are contributing factors in cancer. If the cell is severely damaged and un-repairable it will be subject to apoptosis (Schafer KA,. 1998).

Understanding cancer one needs to take inconsideration the various contributing factors in the etiology of the disease such as the biological, immunological, environmental and genetic background. The complexity of the disease by the constant discovery of novel tumour suppressor genes, mutations, molecular pathways and tumour markers (Jefford and Irminger-Finger., 2006) Upregulation of oncogenes and suppression of the tumour suppression genes are defined characteristic of this disease (McLaughlin et al., 2003).

McLaughlin et al. (2003) indicated that new research supported the role of Chk1 at various points in the cell cycle, especially the G2/M phase also known as the DNA-damage checkpoint in response to replication inhibition during the S phase. The research also indicated that Chk2 appeared to be mostly required for the G1/S phase checkpoint in response to ionizing radiation and radiomimetic drugs. Various DNA damaging agents such as cisplatin arrest the cell cycle in the G1 phase. Other agents that also target DNA such as thiopurines have the ability to arrest cell cycle in the G2/ M phase (Adams *et al*, 1995).

As discussed previously in Chapter 1 the structural similarity of 6-TG to physiological nucleotides allows for the antimetabolite to be incorporated into the DNA, resulting in irreversible DNA strand breaks, followed by the arrest of cell cycle in the G2/M phase (Adams *et al*, 1995). In contrast to 6-TG, Auranofin does not induce significant changes in cell cycle progression.

7.2 AIM

To determine the effect of the novel 6-TG compounds (EKJC56ST, EKJC56 and EKJC80A) on cell cycle progression on the HeLa cell line.

7.3 METHOD

7.3.1 Instruments and reagents required (See Appendix A for preparation of reagents)

- RNaseA (DNase-free) (BD Bioscience, Johannesburg, South Africa)
- Propidium Iodide

- BD FACS 500 Flow cytometer

7.3.2 Experimental procedure

The following assay was carried out according to the method described by Jayadev (1994)

A volume of 9ml of HeLa cell suspension at a concentration of 1×10^5 cells /ml was prepared and incubated for a period of 3 hours at 37°C in an atmosphere of 5% CO_2 . The experimental compounds were added at concentrations equal to and twice that of the IC_{50} value. The cell culture / drug suspension were incubated for a period of 24 hours and 48 hours at 37°C in an atmosphere of 5% CO_2 . After the incubation period, cells were detached from cell culture flasks by adding approximately 2ml of trypsin. The detached cells were transferred to 15ml centrifuge tubes. Tubes were filled with medium supplemented with 5%FCS and the cell suspension centrifuged at 1000rpm's (200G's) for 5min at 4°C . The supernatant was discarded and the cell pellet was gently vortexed. Cells were re-suspended in 1ml cold PBS, and 4ml absolute ethanol at -20°C was added.

The cell / alcohol suspension was kept on ice overnight allowing the cells to fixate. After the fixation period cells were centrifuged at 1000rpm (200G's) for 5min at a temperature of 4°C .

The supernatant was discarded and the cell pellet re-suspended in 1 ml cold PBS. A volume of 125 μl of a 200 μg / ml solution of DNase-free, RNase A was

added to the cells and incubated at 37°C for 15 minutes. This step was followed by the addition of 100µl of a 5 mg / ml propidium iodide solution.

The cell suspension was incubated for 30 minutes at ambient temperature. The cell / PI solution was kept in the dark to prevent the deterioration of the PI as this compound is highly light sensitive. The cell suspension was vortexed gently and 100µl of the cell suspension was transferred to clean flow cytometric tubes and read within 2 hours on a Beckmann Coulter FACS 500 flow cytometer

7.4 STATISTICAL ANALYSIS OF RESULTS

The results of the cell cycle analysis are expressed as the mean percentage (%) \pm SEM. A statistical program GRAPHPAD was used to determine the mean values each of the treatment groups. Data was statistical analyzed with the Students' paired t-test. A p-value less than 0.05 were considered significant. See Appendix B for definitions and statistical methods used.

7.5 RESULTS AND DISCUSSION

Figure 7.1 and 7.2 represents the effect of 6-TG, EKJC56ST, EKJC56, EKJC80A and Auranofin on cell cycle progression of the HeLa cell line after 24 and 48hours, respectively.

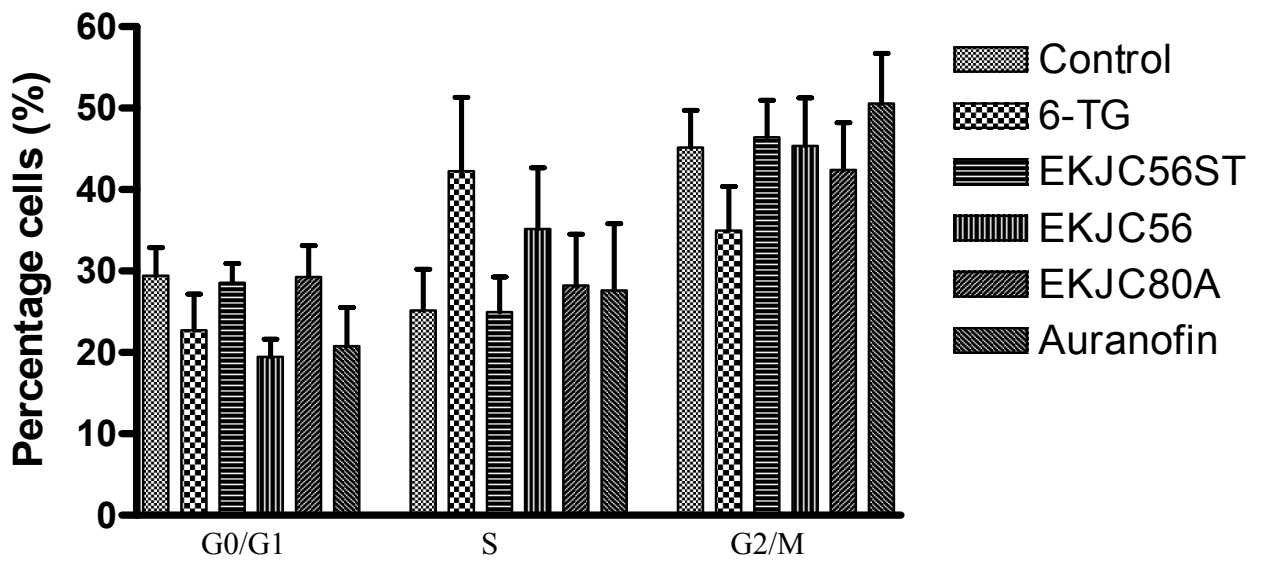


Figure 7. 1 Flow cytometric analysis of cell cycle progression (G0/G1, S and G2/m phase) after a 48hour exposure to 6-TG, EKJC56ST, EKJC56, EKJC80A and Auranofin, The results are expressed as the mean+standard error bars represents 6 individual experiments.

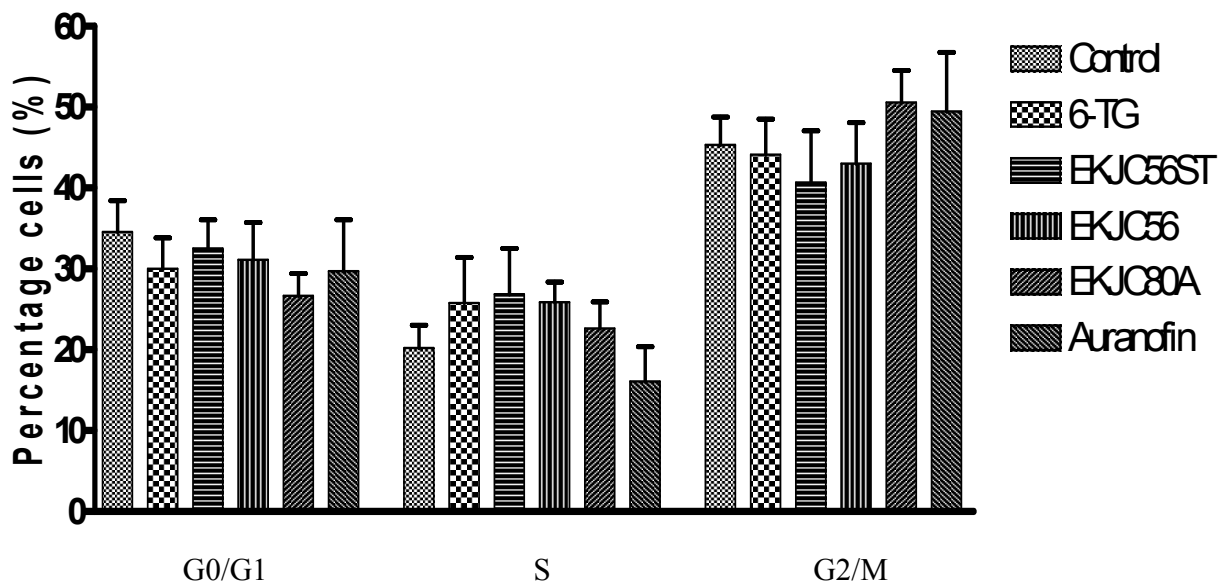


Figure 7.2 Flow cytometric analysis of cell cycle progression (G0/G1, S and G2/m phase) after a 48hour exposure to 6-TG, EKJC56ST, EKJC56, EKJC80A and Auranofin, The results are expressed as the mean+standard error bars represents 7 individual experiments.

Cells treated at both the low and higher concentration of 6-TG showed an increase in the S-phase block (43%) after 24h incubation. However, at 48hours the percentage of cells in the S-phase decreased from 43% to 24% with a subsequent increase in cells in the G2/M phase (24% to 44%) were observed but were not significant. These results correlated with the data from a previous study done by Adam *et al.* to determine the effect of 6-TG on cell cycle progression. EKJC56ST, EKJC56 and EKJC80A produced very similar results to that the untreated control group and clearly indicated that these compounds do not cause arrest in cell cycle progression after 24hours or 48hours. EKJC56 was the only compound that caused a slight increase in the percentage of cells in the S-phase at 24hours compared to the untreated control (35% vs 25%), but this change was not significant ($p>0.05$). Only EKJC80A seemed to increase the number of cells entering the G2/M phase, after 48hours compared to the untreated control group (50% vs 44%) with these results being attained independently of the concentrations used. We can speculate that EKJC56ST, EKJC56 and EKJC80A does not influence cell cycle progression to the extent as 6-TG and that these compounds are acting directly on another mechanism and does not effect DNA synthesis, as does the parent compound, 6-TG.

No significant changes were observed in the treatment groups following changes in concentration of the drug candidates after both 24 and 48hour incubation periods. 6-TG was the only compound that produced significant changes in cell cycle progression after both 24 and 48hours.

7.5 CONCLUSION

The results obtained from evaluating the progression of the HeLa cell into the different phases of the cell cycle, reveal that the novel derivatives do not act directly on the DNA as observed for the parent compound 6-TG. Therefore the experimental compound, EKJC56 and EKJC80A, may act in a similar way as the Au(I) containing compound (Auranofin) by targeting another target intracellular; such as the mitochondria (See Chapter 5) rather than disrupting the DNA structure itself.

CHAPTER 8 CONCLUSION

8. CONCLUSION

This study has shown that structural modification of pre-existing chemotherapeutic agents such as 6-TG can enhance the antitumour activity of these agents. Addition of both a sugar and Au(I) side chain increased cytotoxicity towards several cancerous cell lines with limited toxicity towards normal cell cultures. The addition of only the sugar side-chain into the structure of 6-TG, increased the toxicity of 6-TG and was shown to be highly selective for several of the hormone dependent cell lines. This derivative showed toxicity equal to that of the parent compound, 6-TG, in both the non-hormone cancers and normal cell cultures. By observing this trend in the hormone dependent cell lines, it can be speculated that these hormone-dependent cell lines are highly dependent on the activity of hypoxanthine guanine transferase, an enzyme required for the incorporation of the sugar into 6-TG. Deficiency of this enzyme is the primary cause of 6-TG treatment failure in many patients. It is indicated that designing compounds that have the capability to bypass various metabolic processes may potentially be used to treat hypoxanthine guanine transferase deficient cancers. Of all the derivatives tested, the Au(I)-Phosphine derivatives of 6-TG, EKJC80A, proved to be the most cytotoxic to both normal and cancerous cell lines, and is not suitable for *in vivo* testing due to its non selectivity.

On evaluation of the type of cell death induced using flow cytometry, it was observed that all these novel derivatives could induce early apoptosis, a more organized process of removing dead and damaged cells from the physiological system. To confirm these findings, a caspase-3 monoclonal antibody was used to

detect the activation of caspase-3, an proteolytic enzyme predominately present during the early stages of apoptosis. This assay confirmed the findings regarding the cell death pathway after treatment with these novel derivatives.

The depolarization effect of the both the Au(I) and Au(III) compounds on the mitochondrial membrane have been described in several research articles. During this assay depolarization of mitochondrial membrane was not observed as expected (Rigobello *et al.*, 2002; Rigobello *et al.*, 2005; Barnard *et al.*, 2004). Auranofin also did not induce depolarization of the mitochondrial membrane but rather unexpectedly hyperpolarized the mitochondrial membrane after 24 hours.

The data did not support loss in MMP after treatment with auranofin as published in several research articles (Rigobello *et al.*, 2002; Rigobello *et al.*, 2005; Barnard *et al.*, 2004). A possible justification for the results could be that these studies tested the effect Au(I) and Au(III) compounds on healthy isolated rat liver mitochondria only and not on cancer cells. Thus no results have been published to support the dissipation of the $\Delta\psi_m$ in cancer cell lines after treatment with Auranofin. An article published by Wigdal *et al.*, (2002) referred to a previous study conducted whereby Cytochrome c was released prior to the loss of MMP in HeLa cells. This indicates the possibility that apoptosis may be in progress before the dissipation of the MMP is observed.

The observational study indicated that both the Au(I) derivatives of 6-TG were as potent as Auranofin in directly inhibiting thioredoxin reductase at concentrations

as low as 0.1 μ M. However, further enzymatic biochemical analysis is still required to support these findings.

On assessment of cell cycle progression a slight increase in cells entering in the G2/M phase was observed when HeLa cells were treated with EKJC80A and Auranofin after 48hours. The novel derivatives did not seem to significantly affect one of the cell cycle phases to such an extent and results were very comparable to that of the untreated control group. Similar results were obtained as previously published for 6-TG which showed cell lag in the S-phase (24hours), before entering the G2/M phase (48hours) (Adams *et al*, 1995).

In conclusion, this study has shown that structural modification of pre-existing chemotherapeutic agents such as 6-TG can enhance the antitumour activity and selectivity of these conventional chemotherapeutic treatments. This study supports the hypothesis that derivatives of 6-TG can be used as potential anticancer agents. However, further studies will need to be conducted to provide clarity on the precise mechanism of action of these compounds. From the promising results obtained from this study, it may be suggested that these derivatives should be tested *in vivo* to determine safety and efficacy.

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APPENDIX A: PREPARATION OF REAGENTS

Ammonium chloride solution (NH₄Cl)

Dissolve 8.3g ammonium chloride, 1g NaHCO₃, 74g EDTA (all obtained from Sigma-Aldrich, Johannesburg, South Africa) into 2000ml of distilled water. Sterile filter solution through a 0.2 µm pore size filter and store under sterile conditions in the refrigerator until required.

Binding buffer

Dissolve 0.238g HEPES buffer powder (BD Bioscience, Johannesburg, South Africa) into 100ml distilled H₂O. Add 876g NaCl; 37,3mg KCl, 26,5mg CaCl₂, and 9,5mg MgCl₂ to the HEPES solution. Adjust pH of HEPES buffer to 7.4.

Cytofix/Cytoperm™ solution (1x)

This solution is used for the fixation and permeabilization of the cells before staining with the conjugated PE caspase-3 antibody. Keep refrigerated until use.

Dulbecco's Modified Eagle medium (DMEM)

Dissolve DMEM medium powder (obtained from Sigma-Aldrich, Johannesburg, South Africa) into 5L sterile water and stir at a speed of 100 rpm's. Divide into 500ml bottles. Remove 55ml from the 500 ml DMEM medium and supplement the solution with 5ml of a 1% penicillin / streptomycin (obtained from Adcock-Ingram, Johannesburg, South Africa) and 50ml heat inactivated fetal calf serum (HI-FCS) (obtained from Adcock-Ingram, Johannesburg, South Africa)

ESSENTIAL MODIFIED EAGLE'S MEDIUM (EMEM)

Dissolve EMEM medium powder (obtained from Sigma-Aldrich, Johannesburg, South Africa) into 5L sterile water and stir at a speed of 100 rpm's. Adjust the pH of the solution to 4 with 1N HCl to completely dissolve the remaining powder. This is followed by adding 2mg of NaHCO₃ (obtained from Sigma-Aldrich, Johannesburg, South Africa) or 26.7ml of a 7.5% w/v NaHCO₃ to each litre of medium. Re-adjust the pH to 7.1 with either 1N HCl or 1N NaOH. Sterilize medium through a 0.22µm filter. Divide into 500ml sterile bottles. Remove 55ml of the 500 ml medium and supplement the solution with 5ml of a 1% penicillin / streptomycin (obtained from Adcock-Ingram, Johannesburg, South Africa) and 50ml heat inactivated fetal calf serum (HI-FCS) (obtained from Adcock-Ingram, Johannesburg, South Africa). Keep medium refrigerated.

Heparin

Dissolve 30g of heparin (*Sigma- Aldrich, Johannesburg, South Africa*) into 90 ml of distilled water. Sterilize the solution by filtering through a 0.2 µm pore size filter and store in the refrigerator.

Use 0.1ml (5 units) of the heparin per ml of blood.

Leukocyte counting fluid

Mix 0.1ml of a 1% (100mg / 100 ml W/V) crystal violet solution (*Merck, Johannesburg, South Africa*), 2ml glacial acetic acid (*Lab Chem., Johannesburg, South Africa*) with 97 ml distilled water. Keep solution refrigerated.

McCoy 5A medium

Dissolve McCoy 5A medium powder (obtained from Sigma-Aldrich, Johannesburg, South Africa) into sterile water with a sterile stirrer at a speed of 100 rpm's. Adjust the pH of the solution to 4 with 1N HCl to completely dissolve the remaining powder. After the powder has completely dissolved add 2mg of NaHCO₃ (obtained from Sigma-Aldrich, Johannesburg, South Africa) or 26.7ml of a 7.5% w/v NaHCO₃ to each litre of medium. Re-adjust the pH to 7.1 with either 1N HCl or 1N NaOH. Sterilize medium through a 0.22µm filter. Divide into 500ml sterile bottles. Remove 55ml of the 500 ml RPMI 1640 medium and supplement the solution with 5ml of a 1% penicillin / streptomycin (obtained from Adcock-Ingram, Johannesburg, South Africa) and 50ml heat inactivated fetal calf serum (HI-FCS) (obtained from Adcock-Ingram, Johannesburg, South Africa). Keep medium refrigerated.

3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT solution)

Dissolve 200mg MTT stain powder (*Sigma- Aldrich, Johannesburg, South Africa*) into 40ml freshly prepared PBS (prepared as described previously). Sterile filter the solution using a 2µm pore size filter. Keep solution refrigerated and keep solution away from light as the solution is light sensitive.

Perm/Wash™ buffer (10x)

The buffer solution has multiple functions and is used for the following: permeabilization of cells before staining, antibody diluent and cell wash buffer. Keep buffer refrigerated until use. Upon use dilute 1:10 in sterile H₂O.

Phosphate buffered saline (PBS)

Dissolve 9.23g of FTA haemaglutamine buffer powder (*The Scientific Group, Johannesburg, South Africa*) into 1000ml sterile distilled water. Adjust pH to 7.2 using either HCL or NaOH.

Phytohaemaglutamine (PHA)

Add 5ml of sterile distilled water to the freeze dried PHA in its bottle (BIOWEB, Eden Glen, Johannesburg, South Africa) and mix gently. Dispense 0.2 ml PHA solution into sterile plastic tubes and store at –20⁰C until required.

Add 0.8 ml of a 10 % RPMI 1640 solution (See below) to the 0.2ml PHA, diluting the solution 1:4, just before use. Upon use add 20 µl of the PHA to each well of the 96-well plate.

Roswell Park memorial medium (RPMI) 1640

Dissolve RPMI 1640 medium powder (obtained from Sigma-Aldrich, Johannesburg, South Africa) into sterile water with a sterile stirrer at a speed of 100 rpm's. Adjust the pH of the solution to 4 with 1N HCl to completely dissolve the remaining powder. After the powder has completely dissolved add 2mg of NaHCO₃ (obtained from Sigma-Aldrich, Johannesburg, South Africa) or 26.7ml

of a 7.5% w/v NaHCO₃ to each litre of medium. Re-adjust the pH to 7.1 with either 1N HCl or 1N NaOH. Sterilize medium through a 0.22µm filter. Divide into 500ml sterile bottles. Remove 55ml from the 500 ml RPMI 1640 medium and supplement the solution with 5ml of a 1% penicillin / streptomycin (obtained from Adcock-Ingram, Johannesburg, South Africa) and 50ml heat inactivated fetal calf serum (HI-FCS) (obtained from Adcock-Ingram, Johannesburg, South Africa). Keep medium refrigerated.

Propidium iodide staining for apoptosis

Prepare a 50µg/ml solution by dissolving 2.5mg propidium iodide (PI) powder into 50ml PBS (See above). Keep solution in the dark as PI is light sensitive.

APPENDIX B: STATISTICAL TERMINOLOGY

Confidence Interval: A range of values that has a specific probability of containing the estimated rate of interest.

Analysis of Co-Variance (ANCOVA): A statistical method that is used to determine the effect of the inclusion of variables (i.e. concentration) or covariates into the model. Inclusion of these covariates can therefore increase statistical power as it will explain the variability between groups.

Chi-Squared distribution: One the most widely used in theoretical probability distributions in statistical significant test. It is considered a good test to consider when under reasonable assumption the calculated quantities can be proven to have distributions similar to the chi-square distributions if the null hypothesis is true.

Degrees of Freedom (df): A term that is used to describe number of values in the final calculation that is free to change. This value is calculated by the number of subject and number of groups.

F ratio: Ratio of the two square values. If the ratio is close to 1.0 one can assume the Null hypothesis to be true

IC₅₀: Inhibitory concentration at which 50% of cellular growth are inhibited.

Mean: Average of individual repeats.

Mean of Squares: Is calculated by dividing the sum of squares by the number of degrees of freedom

Probability (p-value): The chance that an event occurs and are expressed as a percentage. In statistics the most commonly used probability 95% ($p=0.05$) or if specified 99% ($p=0.01$).

Standard deviation (SD): A term used to define the quantitative variability or scatter if a sample follows the Gaussian distribution, this means that 68% of data points will be within one SD of the mean and 95% within two SD of the mean.

Standard Error of the Mean (SEM): A term used to describe the measurements on how far the sample mean differs from the true population mean. The larger the sample the smaller the SEM and therefore is closer to the mean population. The standard mean is used to calculate the confidence interval.

Statistically significant: Describes differences between groups whereas the differences are considered significant if the chance is greater than 95% of the time.

Students paired T-test: Data is derived from a sample from the same population that has been measured at 2 different time points (before and after

treatment). Confidence interval can then be determined between two sets of paired observations.

Sum of Squares: Associated with degrees of freedom and is commonly used in statistical models.