

# **A preliminary investigation of the potential anticancer properties of 8-hydroxyquinoline derivatives**

by

Alet Pretorius

A dissertation submitted in fulfilment of the requirements for the  
degree

***Magister Scientiae***

in

**Pharmacology**

in the  
Faculty of Health Sciences,  
at the  
University of Pretoria

Supervisor: **Dr AD Cromarty**

Pretoria, June 2011

## Declaration

**University of Pretoria**

**Faculty of Health Sciences**

**Departement of Pharmacology**

**I, (full names):** Alet Pretorius

**Student number:** 25008553

**Subject of the work:** A preliminary investigation of the potential anticancer properties of 8-hydroxyquinoline derivatives.

## Declaration

1. I understand what plagiarism entails and am aware of the University's policy in this regard.
2. I declare that this project (e.g. essay, report, project, assignment, dissertation, thesis etc) is my own, original work. Where someone else's work was used (whether from a printed source, the internet or any other source) due acknowledgement was given and reference was made according to departmental requirements.
3. I did not make use of another student's previous work and submitted it as my own.
4. I did not allow and will not allow anyone to copy my work with the intention of presenting it as his or her own work.

Signature \_\_\_\_\_

## Acknowledgements

I would like to acknowledge the following people for their assistance and support throughout this study:

1. My supervisor, Dr A.D. Cromarty, for his guidance, patience and motivation throughout my postgraduate studies. It is greatly appreciated.
2. Prof C.E. Medlen for initiating the study.
3. Dr Inus Jansen van Rensburg for the synthesis of the experimental compounds.
4. The personnel at the UPBRC for their help in conducting the *in vivo* study.
5. Mr A Stander for his help with the methods for the flow cytometer.
6. Numerous personnel members at the Department of Pharmacology (University of Pretoria) for their cooperation and support, especially Dr G Joone and Ms Maryna Steinmann.
7. My parents for the opportunity to continue my studies and their support during the completion of the project.
8. My fiancé, Mr J.J. van Tonder, for his support and encouragement.
9. My Heavenly Father for the opportunity and grace to complete this project.

Finally, it should be recognized that the research presented here represents effort and cooperation of many more people than mentioned here but I would like to acknowledge their part and assistance in this work.

## Abstract

Derivatives of the quinoline moiety have been shown to exert a range of biological activities, including anti-neoplastic activity. The clinical application of quinoline derivatives in the treatment of malignancies has been limited due to non-selectivity. Four novel hydroxyquinoline derivatives were synthesised as potential anticancer agents. Each of these compounds contains the characteristic quinoline nucleus: a heterocyclic moiety containing a nitrogen atom. A hydroxyl group is present on C-8 and an azo bond links the heterocyclic quinoline core to a monocyclic benzene ring with various substituents. The aim of this study was to investigate the potential anticancer properties of the four hydroxyquinolines as pertains to their *in vitro* cytotoxicity, ability to circumvent multidrug resistance and possible mechanism of action. The acute *in vivo* toxicity profile of the two most promising compounds was also investigated.

The tumour specificity of a compound is an indicator of the selective cytotoxicity of a compound towards cancer cells, while maintaining minimal toxicity towards normal cells. To this end the four hydroxyquinolines were screened on a range of commercially available cancer cell lines and primary (normal) cultures. The cancer cell lines used included human cervical adenocarcinoma (HeLa), human estrogen receptor positive breast cancer (MCF-7) and several resistant cancer cell lines. Chicken embryo fibroblasts and human lymphocytes were included as primary cell cultures. From the results the cancer cell lines most sensitive to each compound were identified: breast (MCF-7) and leukaemia (Jurkat) cells were most sensitive to HQ5, a resistant colon cancer cell line (COLO 320DM) was most susceptible to HQ6 and HQ7, and HQ10 was most effective against cervical cancer (HeLa). Data indicated that HQ5 and HQ10 displayed the highest tumour specificities and these two promising hydroxyquinoline derivatives were selected for further investigation.

As quinoline derivatives have been reported to modulate multidrug resistance through the inhibition of the P-glycoprotein (P-gp) efflux pump, the effect of HQ5 and HQ10 on P-gp was evaluated in two experimental models. Firstly the experimental hydroxyquinolines were used in combination with the P-gp substrates doxorubicin, vinblastine and paclitaxel on three P-gp expressing cell lines to ascertain whether a synergistic combination could be observed. Secondly the direct effect of HQ5 and HQ10 on the function of P-gp was determined through the rhodamine 123 retention assay.

According to the results obtained from the combination therapy, the combinations of the experimental compounds and the known chemotherapeutic agents tested were at most additive. Data obtained from the rhodamine 123 accumulation assay revealed that HQ5 and HQ10 did not inhibit P-gp in the three P-gp expressing cell lines tested but appeared to enhance P-gp activity.

The mechanism of action of the two selected hydroxyquinolines was further investigated through flow cytometric analysis. The effect of HQ5 and HQ10 on the induction of apoptosis or necrosis in MCF-7 cells was determined. Results indicated that the two experimental compounds induced apoptosis in a dose and time dependent manner. After investigating the effect of the hydroxyquinolines on the cell cycle progression of MCF-7 cells, it was observed that HQ5 and HQ10 arrested the cell cycle at the G<sub>1</sub> checkpoint. Results suggest that at higher concentrations of HQ5 inhibition resembled a G<sub>2</sub> inhibitor.

In an acute *in vivo* cytotoxicity study the tolerability and safety profile of HQ5 and HQ10 were investigated. After daily intraperitoneal administration of either of the two compounds at two concentrations, no obvious histological signs of toxicity were reported. However a dose of 2 mg/kg per day of HQ10 caused a significant reduction in body weight. Haematological analysis revealed that administration of 0.1 mg/kg of HQ5 resulted in a significant decrease in white cell count. No other haematological parameters studied showed any difference between the animals in the control and experimental groups. It was thus concluded that daily dosing of HQ5 and HQ10 was well-tolerated and caused no severe toxicity. Chronic *in vivo* toxicity profiles were not determined in this study.

The *in vitro* studies suggested that HQ5 and HQ10 displayed promising anticancer properties. However, further investigation revealed several unfavourable characteristics, with regards to the solubility, purity and stability of these experimental hydroxyquinolines. In addition *in vivo* studies added further doubt on the success of these compounds in a clinical setting and it was concluded that these compounds were unsuitable for further development.



## Glossary of abbreviations

$\mu\text{l}$	Microlitre
$\mu\text{m}$	Micrometre
$\mu\text{M}$	MicroMolar
$\mu\text{mol/l}$	Micromoles per litre
$^{\circ}\text{C}$	Degree Celsius
%	Percentage
ABC proteins	ATP-binding cassette
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotranferase
ATCC	American Tissue Culture Collection
ATP	Adenosine-5'- triphosphate
$\text{cm}^2$	Centimetre squared
$\text{Ca}^{2+}$	Calcium ion
$\text{CaCl}_2$	Calcium chloride
CAM	Chorioallantoic membrane
CDK	Cyclin dependant kinase
$\text{CO}_2$	Carbon dioxide
CQ	Chloroquine
CYP450	Cytochrome P450
dL	Decilitre
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's Minimum Essential Medium

FCS	Foetal calf serum
FITC	Fuorescein isothiocyanate
G <sub>1</sub> phase	First gap/growth phase
G <sub>2</sub> phase	Second gap/growth phase
g	Gram
g/l	Gram per litre
g/mol	Gram per mole
<i>g</i>	Gravity
GGT	Gamma glutamyltransferase
Ham's F-12	Nutrient Mixture F-12 Ham's, Kaighn's Modification
HEPES	(4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid)
HR-NMR	High Resolution Nuclear Magnetic Resonance
HIV	Human Immunodeficiency Virus
Hb	Haemoglobin
IC <sub>20</sub>	Inhibitory concentration that results in 20% cell death
IC <sub>50</sub>	Inhibitory concentration that results in 50% cell death
ICH	International Conference on Harmonisation
i.p.	Intraperitoneal
JCRB	Japanese Collection of Research Bioresources
KCl	Potassium chloride
kDa	KiloDalton
l	Litre
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LD <sub>50</sub>	Median lethal dose
Mg <sup>2+</sup>	Magnesium ion
MgCl <sub>2</sub>	Magnesium chloride

Mg	Milligram
mg/kg	Milligram per kilogram
mg/ml	Milligram per millilitre
ml	Millilitre
mM	Millimolar
mmol/l	Millimole per litre
MCV	Mean corpuscular/ cell volume
MCHC	Mean corpuscular haemoglobin concentration
MDR	Multidrug resistance
MDR-TB	Multidrug resistant tuberculosis
M phase	Mitosis phase
MRP-1	Multidrug resistance-associated protein 1
MRP-2	Multidrug resistance-associated protein 2
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MQ	Mefloquine
NaHCO <sub>3</sub>	Sodium bicarbonate
NaCl	Sodium chloride
nm	Nanometre
nM	Nanomolar
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Cooperation and Development
p53	tumour protein 53
PBS	Phosphate buffered saline
PCV	Packed cell volume/ Hematocrit
pH	Percentage hydrogen
PHA	Phytohemagglutinin
Pgh-1	P-glycoprotein homologue 1
P-gp	P-glycoprotein
PI	Propidium iodide
pM	Picomolar
PPQ	Piperaquine

QN	Quinine
Rb	Product of the retinoblastoma susceptibility gene
RCC	Red blood cell count
RPMI 1640	Rosswell Park Memorial Institute medium
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen species
RSA	Republic of South Africa
S-phase	Synthesis phase
SPF	Specific pathogen free
SEM	Standard error of the mean
TB	Tuberculosis
U	Unit
U/l	Unit per litre
UK	United Kingdom
UPBRC	University of Pretoria Biomedical Research Centre
USA	United States of America
w/v	Weight per volume
WBC	White blood cell count
WHO	World Health Organisation
XDR-TB	Extensively multidrug resistant tuberculosis



## Table of contents

Declaration .....	i
Acknowledgements .....	ii
Abstract .....	iii
Glossary of abbreviations .....	v
Table of contents.....	ix
List of figures.....	xiii
List of tables .....	xvi
<b>Chapter 1: Introduction.....</b>	<b>1</b>
1.1. Literature review .....	1
1.1.1. Quinolines.....	1
1.1.2. Quinolines as antimicrobial agents .....	2
1.1.3. Quinolines in the treatment of malaria.....	4
1.1.4. Quinolines in the treatment of tuberculosis .....	6
1.1.5. Quinolines in the treatment of cancer.....	8
1.1.6. Multidrug resistance and combination therapy .....	10
1.1.7. Evaluation of a novel lead compound .....	11
1.2. General outline of the study.....	13
1.2.1. Experimental compounds .....	13
1.2.2. Research question.....	15
1.2.3. Aim of the study .....	15
1.2.4. Objectives of the study.....	15
<b>Chapter 2: <i>In vitro</i> cytotoxicity evaluation .....</b>	<b>17</b>
2.1. Introduction.....	17
2.2. Aim .....	18
2.3. Materials.....	19
2.3.1. Experimental compounds .....	19
2.3.2. Reagents .....	20



2.3.3. Cell culture.....	23
2.4. Methods.....	24
2.4.1. Isolation of chicken embryo fibroblasts .....	24
2.4.2. Lymphocyte preparation .....	25
2.4.3. Maintenance of primary cell culture and cancer cell lines.....	26
2.4.4. Preparation of cells for proliferation assays.....	26
2.4.5. Cell counting .....	27
2.4.6. Cell proliferation assay.....	27
2.4.7. MTT cell viability assay .....	27
2.4.8. Interpretation of results .....	28
2.5. Results .....	29
2.5.1. IC <sub>50</sub> concentrations on cancer cell lines .....	29
2.5.2. IC <sub>50</sub> concentrations on normal cell lines .....	37
2.5.3. Tumour specificity.....	41
2.6. Discussion .....	41
<b>Chapter 3: Combination therapy .....</b>	<b>44</b>
3.1. Introduction.....	44
3.2. Aim .....	46
3.3. Materials.....	46
3.3.1. Reagents .....	46
3.4. Methods.....	48
3.4.1. Maintenance and counting of cell culture .....	48
3.4.2. Determining the IC <sub>20</sub> of the standard chemotherapeutics with the MTT stain.....	48
3.4.3. Interpretation of results .....	49
3.4.4. Combination therapy.....	50
3.4.5. Interpretation of results .....	51
3.5. Results .....	51
3.5.1. IC <sub>20</sub> concentrations for doxorubicin, paclitaxel, verapamil and vinblastine....	51
3.5.2. Combination of HQ5, HQ10 and verapamil with doxorubicin, paclitaxel and vinblastine .....	52
3.6. Discussion .....	68



<b>Chapter 4: Rhodamine 123 retention assay .....</b>	<b>71</b>
4.1. Introduction.....	71
4.2. Aim .....	72
4.3. Materials.....	72
4.3.1. Reagents .....	72
4.4. Methods.....	73
4.4.1. Maintenance and counting of cell culture .....	73
4.4.2. Experimental procedure.....	74
4.4.3. Interpretation of results .....	74
4.5. Results .....	75
4.6. Discussion .....	78
<b>Chapter 5: Apoptosis/necrosis.....</b>	<b>81</b>
5.1. Introduction.....	81
5.2. Aim .....	82
5.3. Materials.....	82
5.3.1. Reagents .....	82
5.4. Methods.....	84
5.4.1. Maintenance and counting of cell culture .....	84
5.4.2. Experimental procedure.....	84
5.4.3. Interpretation of results .....	85
5.5. Results .....	85
5.6. Discussion .....	93
<b>Chapter 6: Cell cycle .....</b>	<b>96</b>
6.1. Introduction.....	96
6.2. Aim .....	98
6.3. Materials.....	98
6.3.1. Reagents .....	98
6.4. Methods.....	100
6.4.1. Maintenance and counting of cell culture .....	100



6.4.2. Experimental procedure.....	100
6.4.3. Interpretation of results .....	101
6.5. Results .....	101
6.6. Discussion .....	108
<b>Chapter 7: <i>In vivo</i> investigation of the acute toxicity profile of HQ5 and HQ10 in BALB/c mice .....</b>	<b>112</b>
7.1. Introduction.....	112
7.2. Aim .....	113
7.3. Materials.....	113
7.3.1. Animals.....	113
7.4. Methods.....	114
7.4.1. Dosing, route of administration and sample size .....	114
7.4.2. Evaluation of toxicity .....	115
7.4.3. Sample collection and termination of animals .....	115
7.4.4. Interpretation of results .....	115
7.5. Results .....	116
7.5.1. Observed adverse effects .....	116
7.5.2. Organ weights .....	117
7.5.3. Haematology .....	118
7.5.4. Serum markers of liver toxicity .....	120
7.5.5. Serum markers of kidney toxicity .....	122
7.6. Discussion .....	123
<b>Chapter 8: Final conclusions and recommendations.....</b>	<b>126</b>
8.1 Discussion .....	126
8.2 Conclusions and recommendations .....	131
References .....	133
<b>Appendix: Ethics protocol approvals .....</b>	<b>151</b>

## List of figures

### Chapter 1

<b>Figure 1.1:</b> The structures of (A) quinoline and (B) 8-hydroxyquinoline.....	1
<b>Figure 1.2:</b> Structures of quinoline derivatives used as antimicrobial agents where (A) nalidixic acid and (B) ciprofloxacin.....	2
<b>Figure 1.3:</b> Structures of quinoline derivatives used as antimalarial agents, where (A) quinine, (B) chloroquine, (C) mefloquine and (D) piperazine. ....	5
<b>Figure 1.4:</b> Structures of quinoline derivatives used as antitubercular agents, where (A) ofloxacin, (B) levofloxacin and (C) moxifloxacin.....	7
<b>Figure 1.5:</b> The hallmarks differentiating a cancer cell from a normal cell (Hanahan et al., 2000). ....	8
<b>Figure 1.6:</b> Structures of quinoline derivatives used as anticancer agents, where (A) streptonigrin, (B) lavendamycin, (C) linomide and (D) vesnarinone. ....	9
<b>Figure 1.7:</b> Structures of the experimental hydroxyquinolines where (A) HQ5 (5-phenylazo-8-hydroxyquinoline), (B) HQ6 (5-(2,4-dimethylphenyl)-azo-8-hydroxyquinoline), (C) HQ7 (5-(2,4,6-trimethylphenyl)-azo-8-hydroxyquinoline) and (D) HQ10 (5-(2,6-dibromo-4-fluoro)-azo-8-hydroxyquinoline).....	14

### Chapter 2

<b>Figure 2.1:</b> The mean growth inhibition of ASH-3 cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay.....	30
<b>Figure 2.2:</b> The mean growth inhibition of COLO 320DM cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay.....	31
<b>Figure 2.3:</b> The mean growth inhibition of HeLa cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay.....	32
<b>Figure 2.4:</b> The mean growth inhibition of HepG2 cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay.....	33
<b>Figure 2.5:</b> The mean growth inhibition of Jurkat cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay.....	34
<b>Figure 2.6:</b> The mean growth inhibition of MCF-7 cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay.....	35

**Figure 2.7:** The mean growth inhibition of SH-SY5Y cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay.....36

**Figure 2.8:** The mean growth inhibition of chicken embryo fibroblasts when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay .....38

**Figure 2.9:** The mean growth inhibition of resting lymphocytes when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay .....39

**Figure 2.10:** The mean growth inhibition of PHA-stimulated lymphocytes when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay ..... 40

### Chapter 3

**Figure 3.1:** The effect of exposure to HQ5 or HQ10 and doxorubicin, paclitaxel or vinblastine on COLO 320DM cells.....54

**Figure 3.2:** The effect of exposure to HQ5 or HQ10 and doxorubicin, paclitaxel or vinblastine on HepG2 cells.....59

**Figure 3.3:** The effect of exposure to HQ5 or HQ10 and doxorubicin, paclitaxel or vinblastine on SH-SY5Y cells. ....64

### Chapter 4

**Figure 4.1:** A graphic representation of the two experimental hydroxyquinolines on P-gp function of (A) COLO 320DM cells, (B) HepG2 cells and (C) SH-SY5Y cells..... 77

### Chapter 5

**Figure 5.1:** The effect of HQ5 on the induction of apoptosis or necrosis in MCF-7 cells after 5 days incubation .....89

**Figure 5.2:** The effect of HQ5 on the induction of apoptosis or necrosis in MCF-7 cells after 7 days incubation.. .....90

**Figure 5.3:** The effect of HQ10 on the induction of apoptosis or necrosis in MCF-7 cells after 5 days incubation .....91

**Figure 5.4:** The effect of HQ10 on the induction of apoptosis or necrosis in MCF-7 cells after 7 days incubation .....92

## Chapter 6

**Figure 6.1:** A simplified version of the cell cycle of a non-cancerous cell (adapted from [http://en.wikipedia.org/wiki/Cell\\_cycle](http://en.wikipedia.org/wiki/Cell_cycle)) .....96

**Figure 6.2:** A graphic representation of the controls of each stage of the cell cycle of MCF-7 cells after 48 hours ..... 106

**Figure 6.3:** A graphic representation of the effect of the experimental compounds on the stages of the cell cycle of MCF-7 cells after 48 hours..... 107

## Chapter 7

**Figure 7.1:** The percentage change in body weight of the female BALB/c mice receiving 5% DMSO vehicle alone or the two experimental compounds each at two different concentrations for six consecutive days. .... 117

**Figure 7.2:** The mean white blood cell count ( $\times 10^9$  cells/l) of the female BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each. .... 119

**Figure 7.3:** The mean AST values (U/l) of the female BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each ..... 121

**Figure 7.4:** The mean ALT values (U/l) of the female BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each..... 121

**Figure 7.5:** The mean GGT values (U/l) of the female BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each..... 122

## List of tables

### Chapter 2

<b>Table 2.1:</b> The various cells used in the study .....	26
<b>Table 2.2:</b> Mean drug concentration ( $\mu\text{M}$ ) of the four novel hydroxyquinolines causing 50% inhibition of cell growth of six cancer cell lines.....	29
<b>Table 2.3:</b> Mean drug concentration ( $\mu\text{M}$ ) of the four novel hydroxyquinolines causing 50% inhibition of cell growth of four non-cancerous normal cell culture .....	37
<b>Table 2.4:</b> The tumour specificities of the four experimental hydroxyquinolines .....	41

### Chapter 3

<b>Table 3.1:</b> The concentrations of doxorubicin, paclitaxel, verapamil and vinblastine used in the combination therapy experiments on COLO 320DM, HepG2 and SH-SY5Y cells. ....	49
<b>Table 3.2:</b> The concentrations of HQ5 and HQ10 that COLO 320DM, HepG2 and SH-SY5Y cells were exposed to during combination therapy experiments .....	50
<b>Table 3.3:</b> $\text{IC}_{20}$ concentrations (nM) of doxorubicin, paclitaxel, verapamil and vinblastine for COLO 320DM, SH-SY5Y and HepG2 cells. ....	52
<b>Table 3.4:</b> Results of combination therapy with HQ5 on COLO 320DM cells .....	55
<b>Table 3.5:</b> Results of combination therapy with HQ10 on COLO 320DM cells .....	56
<b>Table 3.6:</b> Results of combination therapy with verapamil on COLO 320DM cells .....	57
<b>Table 3.7:</b> Results of combination therapy with HQ5 on HepG2 cells .....	60
<b>Table 3.8:</b> Results of combination therapy with HQ10 on HepG2 cells.....	61
<b>Table 3.9:</b> Results of combination therapy with verapamil on HepG2 cells .....	62
<b>Table 3.10:</b> Results of combination therapy with HQ5 on SH-SY5Y cells.....	65
<b>Table 3.11:</b> Results of combination therapy with HQ10 on SH-SY5Y cells.....	66
<b>Table 3.12:</b> Results of combination therapy with verapamil on SH-SY5Y cells.....	67

## Chapter 4

<b>Table 4.1:</b> Rhodamine 123 uptake results on COLO 320DM, HepG2 and SH-SY5Y cells .....	76
---	----

## Chapter 5

<b>Table 5.1:</b> Apoptosis/necrosis analysis results of MCF-7 cells exposed to HQ5 at three different concentrations after 5 and 7 days exposure .....	87
---	----

<b>Table 5.2:</b> Apoptosis/necrosis analysis results of MCF-7 cells exposed to HQ10 at three different concentrations after 5 and 7 days exposure .....	88
--	----

## Chapter 6

<b>Table 6.1:</b> Cell cycle analysis results of MCF-7 cells exposed to HQ5 .....	104
---	-----

<b>Table 6.2:</b> Cell cycle analysis results of MCF-7 cells exposed to HQ10 .....	105
--	-----

## Chapter 7

<b>Table 7.1:</b> Summary of dosages used in the acute toxicity study carried out on BALB/c mice .....	114
--	-----

<b>Table 7.2:</b> The organ weight/body weight percentage of the BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each .....	118
---	-----

<b>Table 7.3:</b> Haematology results of the 50 BALB/c mice in the acute toxicity study indicated per treatment group.....	119
--	-----

<b>Table 7.4:</b> The results of liver serum markers performed on the 50 BALB/c mice in the acute toxicity study indicated per treatment group.....	120
---	-----

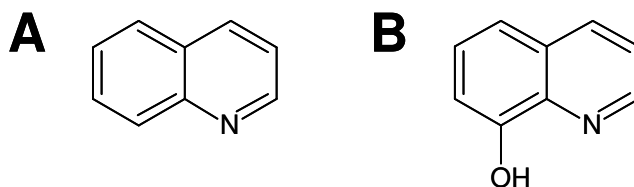
<b>Table 7.5:</b> The results of kidney serum markers performed on the 50 BALB/c mice in the acute toxicity study, indicated per treatment group.....	122
---	-----

## Chapter 1: Introduction

### 1.1. Literature review

#### 1.1.1. Quinolines

Heterocyclic structures containing a nitrogen atom have become of interest to medicinal chemists (Sankaran *et al.*, 2010). The indole and pyridine fragments of such molecules are of particular interest as both pyrrole and quinoline containing molecules are known pharmacophoric agents (Ferlin *et al.*, 2005). As the quinoline molecule is stable and easily synthesised, it is an ideal structure for use in the pharmacological setting. Due to the broad biological activity of quinolines, compounds containing this nucleus have been used in antimalarial, antibacterial, anti-tubercular and anti-inflammatory agents among others (Sankaran *et al.*, 2010). Quinolines are also widely recognized for their anti-neoplastic abilities.



**Figure 1.1:** The structures of (A) quinoline and (B) 8-hydroxyquinoline.

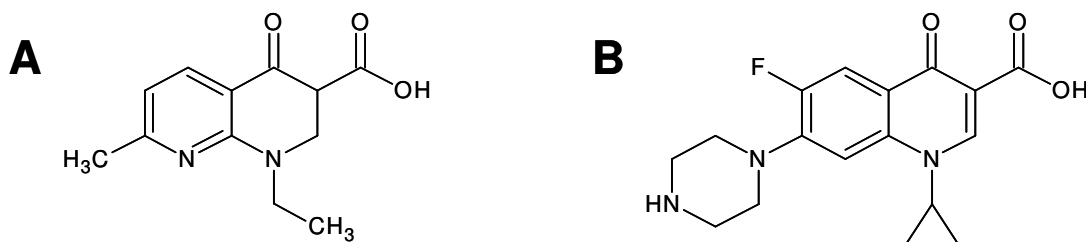
The quinoline moiety is widely found in nature, especially in plants abundant in alkaloids. A number of well-known quinolines were first isolated from natural products before synthesis in the laboratory setting was achieved: the antimalarial quinine was isolated in 1820 from the bark of the cinchona tree, and chimanine alkaloids, used in the treatment of leishmaniasis, were isolated from the bark of *Galipea longiflora* (Kouznetsov *et al.*, 2005).

The synthesis of quinoline derivatives can be performed through a variety of reactions, oftentimes starting with mono-substituted aniline, or *ortho*-substituted aniline (Kouznetsov *et al.*, 2005). One of the simplest syntheses for poly-substituted quinolines starting with *ortho*-substituted aniline is the Friedländer condensation method. This method is an acid or base catalysed condensation followed by cyclodehydration (De *et al.*, 2005).

The 8-hydroxyquinolines have many industrial and pharmaceutical applications. Arguably the most useful characteristic of 8-hydroxyquinolines in the industrial setting is its ability to form stable complexes with divalent and trivalent metal cations (Kim *et al.*, 2005) and as precursors to synthetic dyes and preservatives in cosmetics and tobacco products (Karpińska *et al.*, 2010). Metal quinolate complexes have been studied as light emitting devices (Khaorapapong *et al.*, 2010). In the agricultural setting 8-hydroxyquinoline derivatives are used as fungicides and insecticides. Clinically, 8-hydroxyquinoline derivatives have been used to treat malaria, cutaneous fungicidal infections and amoebic dysentery (Karpińska *et al.*, 2010).

### 1.1.2. Quinolines as antimicrobial agents

At the start of the twentieth century the focus of medical research moved in the direction of synthetic compounds with biological activity. To this end, Carl Browning investigated the potential antimicrobial activity of a series of synthetic dyes which resulted in the discovery of flavines as antimicrobials (Browning *et al.*, 1917). Further investigation of the biological activity of chromophores led Browning to identify the moieties within the active molecules, and in the case of acridine, the recognition of the quinoline fraction (Wainwright *et al.*, 2003). The structures of the quinoline derivatives discussed are shown in Figure 1.2.



**Figure 1.2:** Structures of quinoline derivatives used as antimicrobial agents where (A) nalidixic acid and (B) ciprofloxacin.

Since Browning's research, quinoline derivatives have been studied extensively for possible antimicrobial activity. It is noteworthy that all the quinoline derived antimicrobials have a carboxylic acid group at C-3. The evolution of quinoline derivatives into commercially available antimicrobials started with quinolone derivatives. Nalidixic acid was the first commercially available quinolone, and as it rapidly accumulated in the urinary tract (Heeb *et al.*, 2011), it was used for the treatment of uncomplicated urinary tract infections in the 1960's (O'Donnell *et al.*, 2010).

Manipulation of the structure of nalidixic acid yielded several 4-aminoquinolones and the fluoroquinolones with potent antimicrobial activity, (Heeb *et al.*, 2011). Fluoroquinolones were developed in the 1980's by adding fluorine in position 6 and piperazinyl derivative in position 7 of the structure of nalidixic acid (O'Donnell *et al.*, 2010). The 6-fluoro group improved on the pharmacokinetic limitations of nalidixic acid (Heeb *et al.*, 2011). Drugs from the quinolone class exert their effect by binding to topoisomerase IV and gyrase in the presence of DNA, causing a change in the DNA conformation and ultimately abrogating bacterial replication (Sanders *et al.*, 2011). As fluoroquinolone compounds were effective against a wider range of microbes, their use extended to the treatment of renal, respiratory, abdominal and sexually transmitted infections. The fluoroquinolone derivatives include drugs such as ciprofloxacin, norfloxacin and the newer moxifloxacin. Ciprofloxacin has been recognized as the most consumed antibiotic worldwide (Heeb *et al.*, 2011).

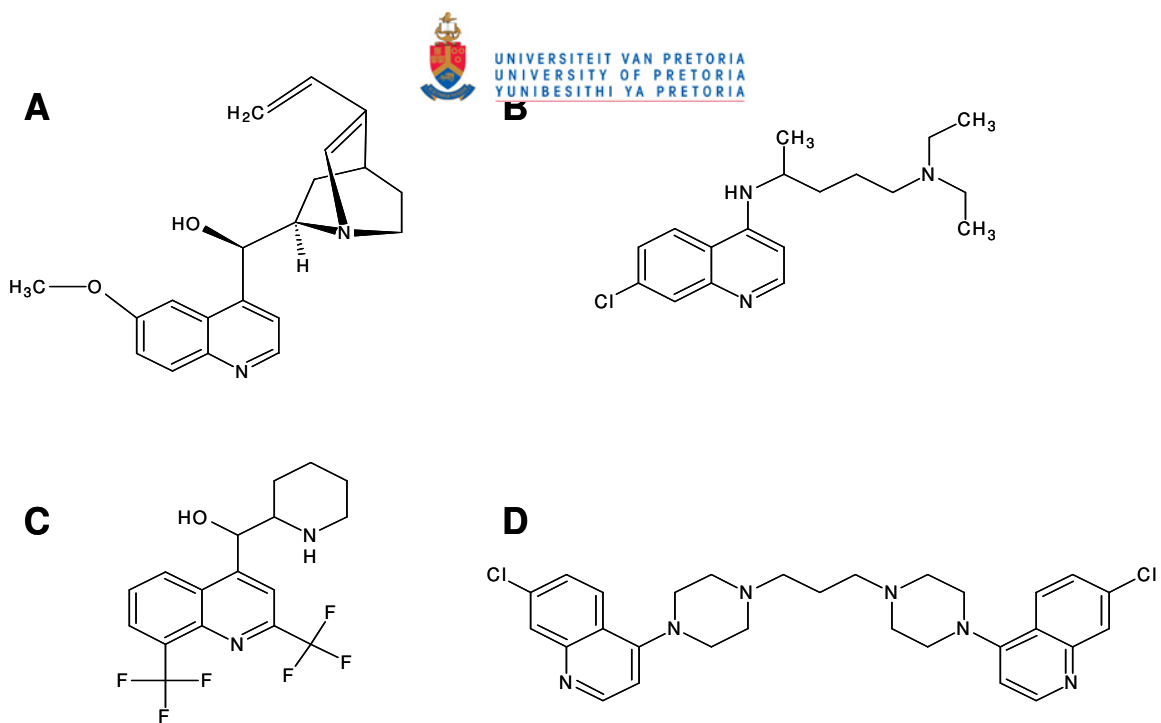
Unfortunately, resistance to quinoline derived antimicrobials developed. As quinolones are synthetic compounds, the development of resistance was considered improbable because no environmentally resistance genes are available from producer microbes. The mechanisms of resistance include mutations in the type IIA topoisomerases, the expression of a DNA topoisomerase protection protein and efflux pumps (Xiong *et al.*, 2011). However, several novel quinolone derivatives, such as delafloxacin and nemonoxacin believed to overcome resistance are currently in development (Stubbings *et al.*, 2009).

### 1.1.3. Quinolines in the treatment of malaria

According to the World Health Organisation's World Malaria Report, close to 1 million deaths result from malaria infection annually (WHO, 2010). Malarial infection is spread through the bite of the anopheline mosquito carrying sporozoites. The sporozoites travel to the liver and after a few asymptomatic weeks, merozoites are released into the blood supply. The merozoites invade the circulating erythrocytes, resulting in the clinical symptoms of malaria (Rosenthal *et al.*, 2001).

The parasite survives on the host's cytosolic haemoglobin which is ingested through endocytosis. Quinoline derivatives inhibit the haemozoin accumulation in infected erythrocytes: it has been speculated that drug-haem complexes are formed through interaction with the haem in the parasitic digestive vacuole, which results in the accumulation of the toxic haem and ultimately parasitic death (Muregi, 2010). The structures of anti-malarial quinoline derivatives discussed here are shown in Figure 1.3.

One of the first quinoline derivatives used in the treatment of malaria was quinine (QN), isolated from the cinchona tree indigenous to South America (Martinelli *et al.*, 2008). It is speculated that QN exerts its parasitocidal effect by inhibiting the parasite's ingestion of the host cell's cytosolic haemoglobin. Resistance to QN treatment is correlated to the amount of phospholipids and cholesterol in the red blood cell membrane and can be reversed by the co-administration of calcium channel blockers, natural alkaloids and calmodulin inhibitors (Henry *et al.*, 2008)



**Figure 1.3:** Structures of quinoline derivatives used as antimalarial agents, where (A) quinine, (B) chloroquine, (C) mefloquine and (D) piperaquine.

Chloroquine, a 4-aminoquinoline derivative of quinine, was developed during World War II by German scientists. Even though the mechanism of action has not been conclusively determined, it has been proposed that chloroquine (CQ) is trapped in the parasitic digestive vacuole, and accumulation thereof abrogates detoxification of the haem which results in parasite death. CQ was widely used due to its favourable safety profile, potency, affordability and convenient dosing. However, *P. falciparum* became resistant to CQ. The resistance is thought to be due to CQ efflux or a lack of accumulation in the digestive vacuole (Muregi, 2010). Several classes of drugs have been used to modulate CQ resistance *in vitro* including tricyclic antidepressants, antibiotics, histamine receptor antagonists and calcium channel blockers such as verapamil (Henry *et al.*, 2008). The reversal of CQ resistance involves the restoration of the pH in the digestive vacuoles to that of CQ susceptible parasites which increases chloroquine accumulation.

During World War II, American scientists developed several anti-malarial compounds, including the model for mefloquine (Muregi, 2010). The parasiticidal activity of mefloquine (MQ) is due to inhibition of endocytosis of the haem. Resistance to MQ is thought to be inferred through the expression of P-glycoprotein homologue 1 (Pgh1), a member of the ATP-binding cassette family of efflux transporters (Henry *et al.*, 2008). A

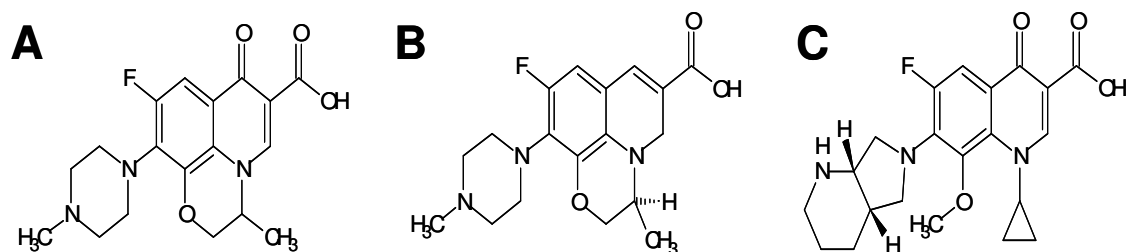
number of drugs have been identified which reverses MQ resistance *in vitro* through interaction with Pgh1. These include calmodulin inhibitors, synthetic surfactants and calcium channel blockers, although verapamil was found to be ineffective. The antifungal, ketoconazole, also modulates MQ resistance by inhibition of hepatic CYP450, which delays MQ degradation and thus increases the plasma drug level (Henry *et al.*, 2008).

The next generation of quinoline derived anti-malarial agents were the bisquinolines: dimeric 4-aminoquinolines in which two quinoline rings are connected by different linker regions (Muregi, 2010). The first of the bisquinolines, piperazine (PPQ), was first synthesised in the 1960's and, apart from proving extremely effective, it displayed a more favourable side effects profile than chloroquine. However, PPQ treatment can cause haemolysis in patients with a glucose-6-phosphate deficiency. In the Chinese National Malaria Control Programme PPQ replaced CQ and because of its effectiveness, a decrease in the number malaria cases was observed (Valecha *et al.*, 2010).

#### **1.1.4. Quinolines in the treatment of tuberculosis**

The World Health Organisation estimates that one third of the global population is infected with *Mycobacterium tuberculosis*, the bacteria causing tuberculosis (TB). As the immune system of healthy individuals can suppress the growth of TB bacilli, only 5-10% of the infected population will experience symptoms during their lifetime. Unfortunately, the combination of TB and HIV infections is deadly and TB remains the leading cause of death in HIV positive patients (WHO 2010).

The drugs used in the treatment of TB are classified in accordance with efficacy, toxicity, tolerability and affordability. First line drugs are used in combination in the initial six month regime and include rifampicin, isoniazid and ethambutol. A number of fluoroquinolones are used as second line drugs in the treatment of TB, and these include ciprofloxacin, ofloxacin, and the newer levofloxacin and moxifloxacin. The structures of these compounds are displayed in Figure 1.4.



**Figure 1.4:** Structures of quinoline derivatives used as antitubercular agents, where (A) ofloxacin, (B) levofloxacin and (C) moxifloxacin.

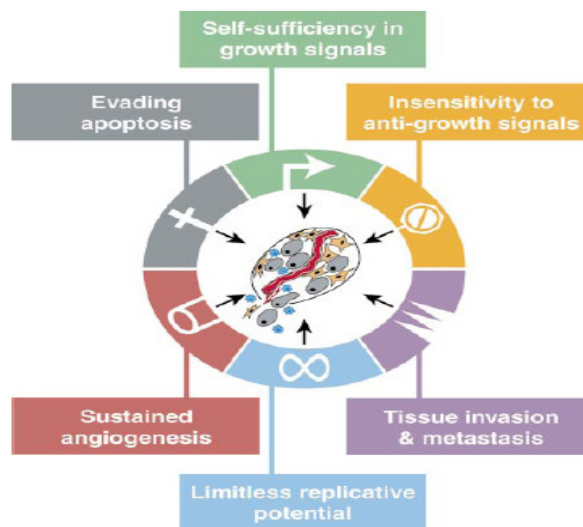
Fluoroquinolones are effective against extracellular, proliferating bacilli and intracellular, non-multiplying bacilli. Of the fluoroquinolones, moxifloxacin and gatifloxacin are the most effective and as such are prime candidates for inclusion in first line combination therapy (Laloo *et al.*, 2010). Some reports indicate that substituting ethambutol with ofloxacin in the first line treatment may reduce the treatment period from six to four months (Ziganshina *et al.*, 2009). However, as the efficacy of fluoroquinolones as first line combination therapy agents have not been proven conclusively, further investigation is still required.

The successful treatment of TB is hampered by the emergence of multidrug resistant TB (MDR-TB), which is defined as any strain of TB resistant to the first line drugs isoniazid and rifampicin, and extensively multidrug resistant TB (XDR-TB), which is defined as strains resistant to both first and second line drugs. The emergence of these strains of *M. tuberculosis* has been attributed to poor patient compliance, the use of monotherapy, inadequate dosing and poor drug quality (Chiang *et al.*, 2010). The use of fluoroquinolones in lower respiratory tract infections may be a contributing factor to fluoroquinolone resistant TB (Ziganshina *et al.*, 2009). Combination therapy with fluoroquinolones has been reported to cure MDR-TB (Chiang *et al.*, 2010). A diarylquinoline derivative, TMC207, is being investigated in Phase II clinical trials for the treatment of MDR-TB (Laloo *et al.*, 2010).

### 1.1.5. Quinolines in the treatment of cancer

Cancer remains a global health issue of the twenty first century. The World Health Organisation approximate that 7.6 million deaths in 2008 were cancer-related. Lung, stomach and liver cancer caused the most fatalities in 2008. The predicted death toll from cancer is predicted to be 11 million deaths in 2030 (WHO 2011).

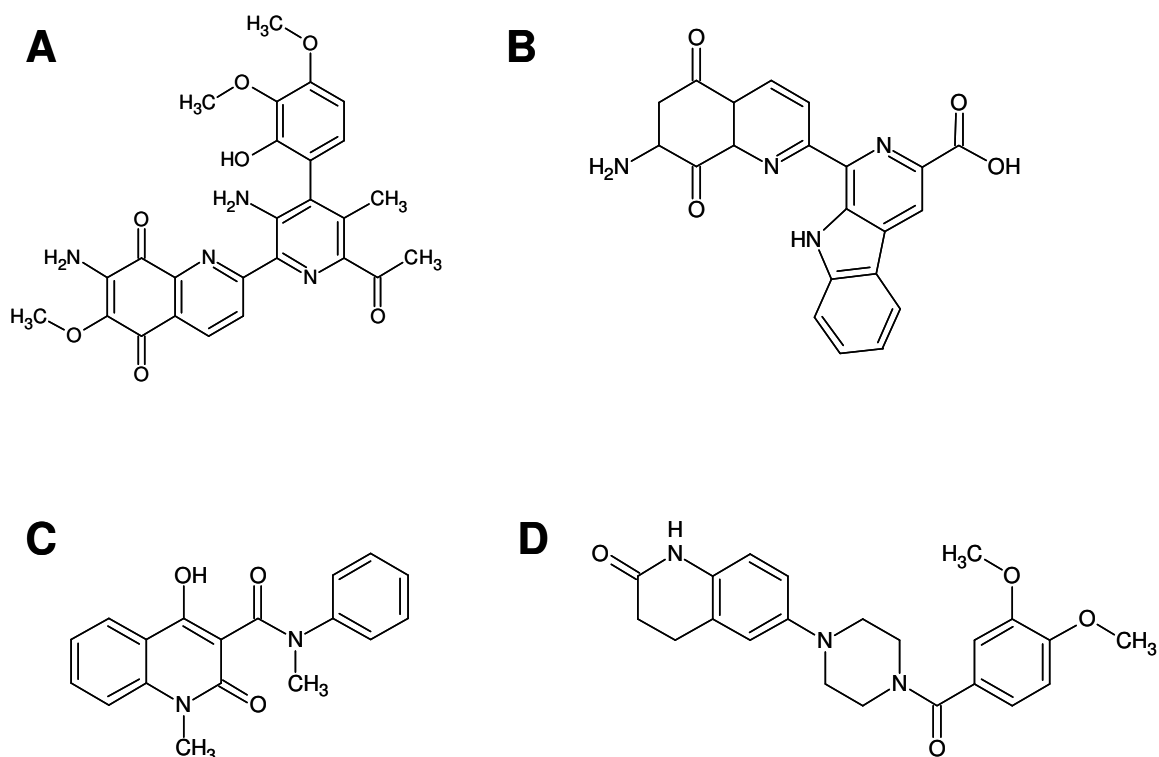
The transformation of a normal cell to cancerous tissue is a multi-factorial process leading to cells which proliferate uncontrollably, dedifferentiates and invades the body. The transformation involves a gain of function and production of oncogenes with a loss of function of tumour suppressor genes. Of the tumour suppressor genes, the *p53* is the most well-known and more than 50% of human cancers display a loss of the p53 protein function. This protein is involved in the induction of apoptosis, and loss of this proteins function is considered central to what has become known as the hallmarks of cancer (Figure 1.5). Due to the mutations in the oncogenes and tumour suppressor genes, cancerous cells become self-sufficient in growth signals and insensitive to growth inhibiting signals, resulting in uncontrolled cell proliferation. As homeostasis is disturbed, cancer cells proliferate limitlessly and, eventually, metastasis (Hanahan *et al.*, 2000).



**Figure 1.5:** The hallmarks differentiating a cancer cell from a normal cell (Hanahan *et al.*, 2000).

Conventional clinical treatment of cancer comprises of surgery, radiotherapy and systemic chemotherapy. Even though this regime is successful in the treatment of localized malignancies, death of the patient usually results from invasive and metastatic tumours. The dose-limiting side effects of many anti-neoplastic agents further influence the disease outcome. The development of more targeted anti-neoplastic agents with a reduced toxicity profile is thus long overdue.

Compounds that contain the planar polycyclic quinoline core are widely used in the treatment of cancer (Bolognese *et al.*, 2004). Most of these compounds exert their cytostatic effect through DNA intercalation, with further interactions with topoisomerase II. Cationic molecules have been shown to be the most effective DNA intercalators, and as such the quaternary or active nitrogen in the ring structure of quinoline containing compounds provides that characteristic (Bolognese *et al.*, 2004). The structures of the compounds which will be discussed are shown in Figure 1.6.



**Figure 1.6:** Structures of quinoline derivatives used as anticancer agents, where (A) streptonigrin, (B) lavendamycin, (C) linomide and (D) vesnarinone.

The aminoquinones, derived from the quinoline core, are used frequently in the clinical setting in the treatment of malignancies. This class of compounds includes streptonigrin and lavendamycin (Suh *et al.*, 2000). The active moiety in these compounds is reported to be the 7-aminoquinoline-5,8-dione nucleus. However, both streptonigrin and lavendamycin displayed severe cytotoxicity towards primary (normal) cell cultures, and were thus deemed unsuitable for further development. Derivatives of these molecules are being investigated as anti-neoplastic agents (Cai *et al.*, 2010).

Linomide, a quinoline-3-carboxamide, was known for its immunomodulatory effects, which was deemed promising in the treatment of autoimmune diseases. *In vivo* anti-neoplastic properties were also observed in a rat prostate cancer model. However, when cell culture was exposed to the same therapeutic concentration as that used in *in vivo* studies, no cytotoxicity was observed. Further investigation revealed that linomide is a potent anti-angiogenic agent, causing a decrease in tumour blood flow of 40% at a dose of 100 mg/kg/day in an *in vivo* model (Vukanovic *et al.*, 1993). During phase III trials of linomide in a population with multiple sclerosis, it was found that linomide caused serious cardiovascular events and the study was discontinued (Tan *et al.*, 2000).

The anticancer effects of vesnarinone, a positive inotropic quinoline derivative, were shown *in vivo* in various nude mouse models (Sato *et al.*, 1995; Honma *et al.*, 1999). The ability of vesnarinone to increase apoptosis in cancerous tissue and reduce the levels of p53 expression was also observed (Sato *et al.*, 1995). Unfortunately, a dose dependent increase in mortality rate due to vesnarinone treatment was observed in a 1995 trial (Cohn *et al.*, 1998).

#### **1.1.6. Multidrug resistance and combination therapy**

Multidrug resistance (MDR) has emerged as a major obstacle to the successful treatment of cancer. Multidrug resistance is classified as either initial resistance or acquired resistance after relapse. The mechanisms of resistance include drug efflux pumps, enzyme systems and limited vascular accessibility (Naito *et al.*, 1998). The ATP-binding cassette proteins (ABC) is a family of proteins which have been reported to contribute largely to transport-based multidrug resistance in cancer cells (Ullah, 2008). Therapeutic drug concentrations are thus not attained in cells expressing these ATP-dependant drug efflux pumps.

The most studied of the membrane transporters involved in MDR, P-glycoprotein (P-gp), is a 170 kDa membrane protein which is not only expressed in cancerous tissue, but also in the kidney, gastrointestinal tract and liver (Ullah, 2008) where it is thought to play a role in the efflux of toxic substances (Naito *et al.*, 1998). The expression of P-gp is higher in cancer cells derived from tissue that normally expresses P-gp, leading to decreased intracellular drug concentrations with a linked decrease in the efficacy of anthracyclins, vinca alkaloids, podophyllotoxins and taxanes. It has been reported in patient samples that the drug efflux pump, P-gp, is upregulated immediately after exposure to chemotherapeutic agents *in vivo* (Di Nicolantonio *et al.*, 2005).

The ability of the quinoline ring and its derivatives to modulate multidrug resistance were discovered when quinine, a well-known anti-malarial drug, was found to modulate drug-resistance in cancerous tissue to chemotherapy (Ullah, 2008). Since the coincidental finding, many quinolines have been investigated for potential resistance-reversal characteristics. It has been speculated that quinolines exert their chemo-sensitising effect via the inhibition of P-gp.

### **1.1.7. Evaluation of a novel lead compound**

Preclinical drug evaluation can be divided into two stages: discovery and development (Ruiz-Garcia *et al.*, 2008). The role of medicinal chemists in the discovery of therapeutic agents is undisputed. However, the role of the chemist should also extend to the determination of the physicochemical properties of a novel drug, including solubility, lipophilicity and metabolic stability (Rudnev *et al.*, 2006), and ensuring the purity of the compound through the use of in-depth analysis techniques such as LC-MS/MS and HR-NMR.

The use of cell culture techniques in preclinical development has become commonplace as it allows for the screening of a large number of novel compounds in order to ascertain the effect (whether cytotoxic or not) of the novel compounds. Cultured cells for screening can be selected to represent the disease state, or the biochemical anomalies characteristics of the disease of interest (Allen *et al.*, 2005). Generally novel anti-neoplastic compounds are screened on a variety of primary (normal) and cancer cell lines at physiologically achievable concentrations. Normal cell culture is included in the

screening process as an indicator of potential target organ toxicity (Ruiz-Garcia *et al.*, 2008), and is essential for determining the cancer specificity of a compound.

Screening on cell cultures also holds the possibility of determining the mechanism of action of a novel compound through various *in vitro* assays (Allen *et al.*, 2005). Mechanistic studies generally undertaken in the development of novel anti-neoplastic agents include ascertaining whether the compound induces apoptosis or necrosis, the effect of the compound on the cell cycle and, where interaction with specific transporters or receptors are suspected, assays specifically aimed at illuminating the interaction of the compound with that target.

*In vivo* toxicity testing must be undertaken on promising candidates before entry into the clinical arena. These studies are focused on assessing the safety, tolerability and pharmacokinetic parameters of a compound as pertains to organ accumulation, cardiotoxicity and geno-toxicity (Ruiz-Garcia *et al.*, 2008) and have been reported to be reliable indicators of human toxicity (Newell, 2004). Initially an acute toxicity study in rodents is undertaken to determine the highest concentration of an experimental compound that does not induce life-threatening toxicity. Thereafter the determined concentration of the drug is administered to a non-rodent species to ascertain that the concentration is non-lethal (DeGeorge *et al.*, 1998).

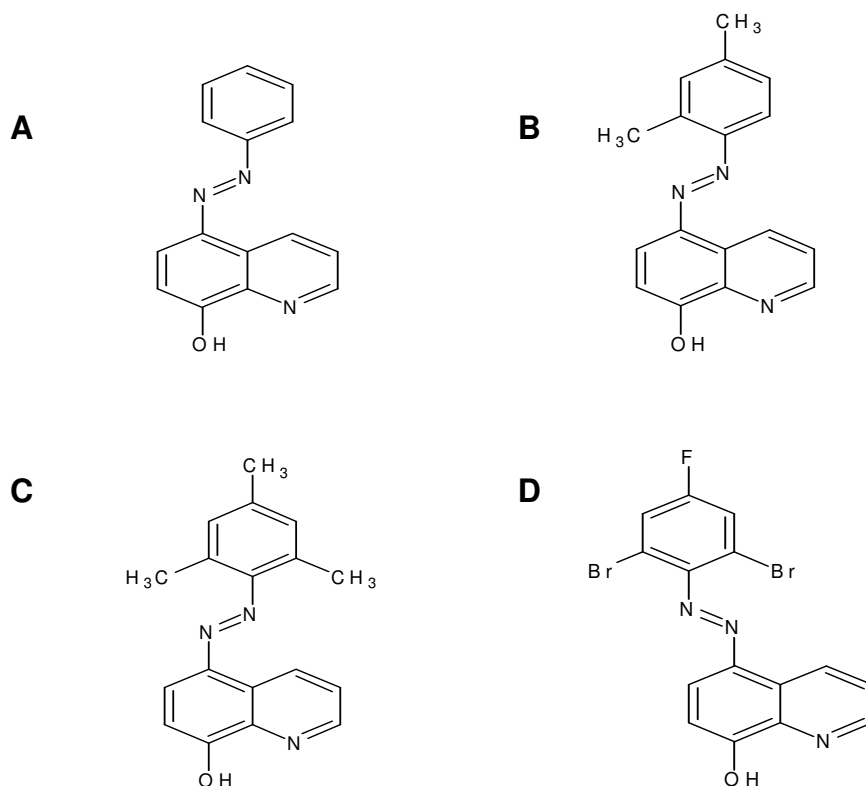
Many other aspects must be investigated during the discovery phase before first-in-man studies can commence. Pharmacokinetic parameters for a novel drug must be determined in order to illustrate the kinetic behaviour of a drug in an intact organism (Ruiz-Garcia *et al.*, 2008). The potential toxic effects of the compound's metabolites must be assessed. Studies on the potential interactions of the experimental compound with other drugs must be investigated (Baillie, 2008). These parameters can contribute to the early identification of potential failure or promising lead compounds as a potential drug.

## 1.2. General outline of the study

### 1.2.1. Experimental compounds

The four experimental hydroxyquinoline compounds were synthesised as part of a batch of compounds by Dr Inus Jansen van Rensburg under the supervision of Dr André Roodt at the Department of Chemistry, University of Bloemfontein. The initial samples were received in 2008 and proved to be specific to cancer cells during preliminary screening using HeLa cells (human cervical adenocarcinoma) and human lymphocytes (results not shown). These potential lead compounds were thus considered suitable candidates for further investigation.

The compounds used in the study were synthesised by Dr Inus Jansen van Rensburg in 2009. Infrared spectroscopy and thin layer chromatography were used to confirm the identity and purity of the compounds. The experimental compounds were (5-phenylazo-8-hydroxyquinoline), (5-(2,4-dimethylphenyl)-azo-8-hydroxy-quinoline), (5-(2,4,6-trimethylphenyl)-azo-8-hydroxyquinoline) and (5-(2,6-dibromo-4-fluoro)-azo-8-hydroxyquinoline) and coded as HQ5, HQ6, HQ7 and HQ10 respectively. The structures of the four experimental compounds are illustrated in Figure 1.7.



**Figure 1.7:** Structures of the experimental hydroxyquinolines where (A) HQ5 (5-phenylazo-8-hydroxyquinoline), (B) HQ6 (5-(2,4-dimethylphenyl)-azo-8-hydroxyquinoline), (C) HQ7 (5-(2,4,6-trimethylphenyl)-azo-8-hydroxyquinoline) and (D) HQ10 (5-(2,6-dibromo-4-fluoro)-azo-8-hydroxyquinoline).

The compounds each contain the characteristic quinoline ring – a heterocyclic moiety containing a nitrogen atom with a hydroxyl group on C-8. An azo-bond on C-5 links the heterocyclic quinoline core to a monocyclic substituted benzene ring. The experimental compounds are distinguished based on these substituents of the benzene ring, which ranges from hydrogen to methyl groups to halogens.

Due to the poor solubility profile of the compounds in water, solutions in DMSO were used for all experiments. The experimental compounds were soluble in DMSO at a concentration of 20 mM. However, HQ6 and HQ7 had a less favourable solubility profile than HQ5 and HQ10.

### 1.2.2. Research question

The following research questions were postulated.

- i. Will the four experimental hydroxyquinoline compounds induce a cytotoxic response in cancer cell lines *in vitro* at physiologically achievable concentrations while remaining non-toxic to primary (normal) cell cultures?
- ii. Which two of the four compounds are the most likely lead compounds?
- iii. Can the two selected hydroxyquinolines be shown to have favourable anticancer mechanisms of action in further *in vitro* studies?
- iv. Will the two selected hydroxyquinolines administered at estimated therapeutic concentrations be well-tolerated in an acute toxicity study in a BALB/c mouse model?
- v. Based on the *in vivo* tolerability and mechanism of action, are either of the two hydroxyquinolines an ideal candidate for further development as anti-neoplastic agent?

### 1.2.3. Aim of the study

The aim of this project is to investigate four synthetic hydroxyquinolines *in vitro* to determine their cytotoxicity, ability to circumvent multidrug resistance and possible mechanism of action.

A second aim is to determine the acute *in vivo* toxicity profile using the mouse model.

### 1.2.4. Objectives of the study

- i. To determine the cytotoxicity of the experimental compounds on a range of cancer cell lines and on non-cancerous cell cultures in order to ascertain the specificity of the compounds to cancer cells
- ii. To assess whether synergism between the experimental compounds and three known chemotherapeutics exists
- iii. To investigate the potential resistance circumventing properties of the experimental compounds as pertains to their ability to inhibit P-glycoprotein function



- iv. To determine whether the experimental compounds induce cell death through apoptosis or necrosis in a cancer cell line
- v. To investigate the effect of the experimental compounds on the cell cycle of a susceptible cancer cell line
- vi. To ascertain the tolerability of the experimental compounds *in vivo* in an acute toxicity model using female BALB/c mice

## Chapter 2: *In vitro* cytotoxicity evaluation

### 2.1. Introduction

The first demonstration of cells surviving outside of the intact organism was reported by Sir William Harvey who, in the sixteenth century, observed that a piece of myocardium covered in saliva remained contractile for extended periods of time. This remarkable discovery revealed the potential that cells could be cultured and maintained for further study, making *in vitro* screening of novel compounds with therapeutic potential a possibility. The use of cell culture has been valuable in identifying compounds that display selective toxicity towards cancer cells (Allen *et al.*, 2005).

The screening of compounds *in vitro* holds advantages over *in vivo* work such as the ability to control the experimental environment as well as being cost and time efficient (Kelloff *et al.*, 1995). A correlation has been reported between levels of cytotoxicity and *in vivo* activity (Hirschberg 1958; McKeage *et al.*, 2002). The accuracy of *in vitro* assays to predict *in vivo* toxicity was further studied by a collaboration of scientists in the 1990's where the available *in vivo* toxicity data was compared to a series of *in vitro* assays (National Toxicology Program Interagency Centre for the Evaluation of Alternative Toxicological Methods, 2000). The study found that for 43 of the 50 selected reference compounds *in vitro* assays accurately predicted *in vivo* toxicity. Thus *in vitro* activity can be a reliable predictor of *in vivo* activity.

A variety of commercially available cancer cell lines were used in the screening of the four novel synthetic hydroxyquinolines. These cell lines were chosen because of their unique characteristics. The Jurkat T lymphocytes (ATCC no TIB-152) are non-adherent cells well-known to be vulnerable to a range of stimuli (Wang *et al.*, 2006) and have been used widely for the evaluation of novel lead anticancer compounds (Tiekink, 2002). The HeLa cell line (ATCC no CCL-2), adenocarcinoma cells of the cervix, is an adherent line used worldwide as the 'laboratory model of a cancer cell' (Masters, 2002). The MCF-7 estrogen-receptor positive breast cancer cell line (ATCC no HTB-22) is the most widely studied immortal breast cancer cell line (Levenson *et al.*, 1997; Sánchez *et al.*, 2008). The COLO 320DM cell line (ATCC no CCL-220), a relatively chemotherapeutic resistant

human colon carcinoma cell line, has been reported to be an accurate indicator of drug cytotoxic potency (Tiekink, 2002). HepG2 cells (ATCC no HB-8065) a hepatoblastoma cell line, were included in the study as predictor of potential excessive hepatotoxicity, as liver toxicity has been one of the most frequent reasons for the withdrawal of drugs from the market (European Medicines Agency, 2008). The ASH-3 cell line (JCRB no 1073), derived from anaplastic thyroid carcinoma cells, is unresponsive to most therapies and was thus included in this study to indicate the cytotoxicity of the four hydroxyquinolines against such a resistant cancer (Sekiguchi *et al.*, 2001). The SH-SY5Y cell line (ATCC no CRL-2266), derived from human neuroblastoma cells, is known to highly express P-glycoprotein (P-gp), the membrane associated protein which confers multi-drug resistance to many cancers (Sieczkowski *et al.*, 2009) and which several quinoline derivatives have been shown to inhibit (Ullah, 2008).

Primary (normal) cells were also included in the study to predict the toxicity of the experimental compounds towards non-cancerous cells. The cells that were investigated in this study were resting human lymphocytes, PHA stimulated human lymphocytes and chicken embryo fibroblasts. It has been documented that phytohemagglutinin (PHA) cause an increase in the mitochondrial membrane potential of human lymphocytes (Chen, 1988).

## 2.2. Aim

The aims of this study were:

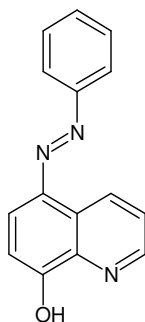
- i. To screen four hydroxyquinolines on 3 primary cell cultures and 7 cancer cell lines.
- ii. To determine the tumour specificity of the four experimental compounds.
- iii. To identify the two most promising compounds based on the results of the cytotoxicity studies for further investigation.

## 2.3. Materials

### 2.3.1. Experimental compounds

The four experimental compounds were dissolved in DMSO at a concentration of 20 mM and frozen in 50  $\mu$ l aliquots at  $-70^{\circ}\text{C}$ . The compounds were further diluted in cell culture medium just before use to ensure that the cells were exposed to a maximum of 5% DMSO. An initial concentration range of the hydroxyquinolines of 0.38  $\mu\text{M}$  to 50  $\mu\text{M}$  was used.

#### HQ5

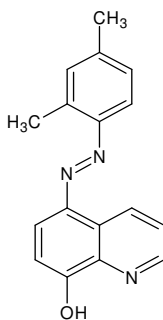


5-phenylazo-8-hydroxyquinoline

Molecular weight: 249.8 g/mol

A 20 mM stock solution was prepared by dissolving 3.75 mg of the compound in 750  $\mu$ l of DMSO.

#### HQ6

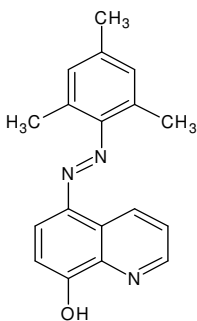


5-(2,4-dimethylphenyl)-azo-8-hydroxyquinoline

Molecular weight: 277.9 g/mol

A 20 mM stock solution was prepared by dissolving 4.16 mg of the compound in 750  $\mu$ l of DMSO. The solution was left on a shaker for 10 minutes before aliquots were made.

### HQ7

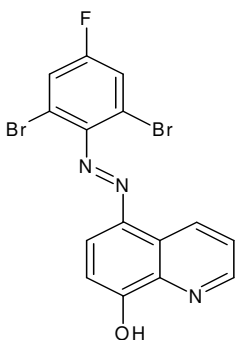


5-(2,4,6-trimethylphenyl)-azo-8-hydroxyquinoline

Molecular weight: 291.9 g/mol

A 20 mM stock solution was prepared by dissolving 4.36 mg of the compound in 750  $\mu$ l of DMSO. The solution was left on a shaker for 10 minutes before aliquots were made.

### HQ10



5-(2,6-dibromo-4-fluoro)-azo-8-hydroxyquinoline

Molecular weight: 424.1 g/mol

A 20 mM stock solution was prepared by dissolving 4.26 mg of the compound in 750  $\mu$ l of DMSO.

## 2.3.2. Reagents

### i. Ammonium chloride

An ammonium chloride solution was prepared by dissolving the following reagents in sequence in 1 l de-ionised water:

8.3 g  $\text{NH}_4\text{Cl}$  (Radchem, Johannesburg, RSA)

1 g  $\text{NaHCO}_3$  (Merck Chemicals, Wadeville, RSA)

74 mg EDTA (Labchem, Johannesburg, RSA)

Prior to use the solution was filter-sterilised with a 0.22  $\mu$ m filter.

### ii. Bovine foetal calf serum (FCS)

Bovine foetal calf serum was procured from PAA (Pasching, Austria). The serum was heat-inactivated prior to use by heating it at 56°C for 45 minutes. This was added to cell culture media to a concentration of 10%.

### **iii. Cell counting fluid**

The solution was prepared by adding 1 ml of 0.1% crystal violet solution and 2 ml acetic acid to 97 ml of de-ionised water.

### **iv. Dimethyl sulfoxide (DMSO)**

DMSO was procured from Sigma-Aldrich (St Louis, USA) and used undiluted.

### **v. Heparin**

Heparin, obtained from Sigma-Aldrich (St Louis, USA), was dissolved in distilled water at a concentration of 30 mg/ml. It was filter sterilized and stored at 4 °C. The ratio of heparin to blood used was 100 µl of heparin to 100 ml blood.

### **vi. Histopaque**

Histopaque was stored sterile at 4 °C and used undiluted at ambient temperature. The reagent was obtained from Sigma-Aldrich (St Louis, USA).

### **vii. MTT**

A mass of 250 mg of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), obtained from Sigma-Aldrich (St Louis, USA), was dissolved in 50 ml phosphate buffered saline (5 mg/ml). The solution was filter-sterilised with a 0.22 µm pore size filter and stored in foiled covered tubes at 4 °C.

### **viii. Penicillin/streptomycin**

A solution containing 10 000 U of penicillin and 10 000 µg streptomycin per millilitre was supplied by BioWhittaker (Walkersville, USA). 1% was added to cell culture media.

### **ix. Phosphate buffered saline (PBS)**

FTA Hemagglutination buffer was obtained from BD Biosciences (Sparks, USA). A solution containing 9.23 g/l in de-ionised water was made up and stored at 4 °C until use. The solution was filter-sterilised with a 0.22 µm pore size filter and stored at 4 °C is sterile PBS was required.

### **x. Phytohemagglutinin (PHA)**

Phytohemagglutinin (Remel, Kent, UK) was made up by adding 5 ml of distilled water to the freeze-dried bottle content. The stock solution was dispensed into 0.4 ml aliquots and stored at -20 °C. Prior to use, 1.6 ml of RPMI was added to a thawed aliquot.

### **xi. Trypsin/Versene**

A trypsin/Versene solution containing 0.25% trypsin and 0.1% EDTA in Ca<sup>++</sup> and Mg<sup>++</sup> free phosphate buffered saline was obtained from Highveld Biological (Johannesburg, RSA). Trypsin/Versene was stored at 4 °C.

### **xii. Cell culture media**

#### **Dulbecco's Modified Eagle's Medium**

DMEM powdered medium was procured from Sigma-Aldrich (St Louis, USA). A mass of 67.35 g of medium powder was dissolved in 5 litres sterile, deionised water. To this solution 18.5 g of NaHCO<sub>3</sub> was added to adjust the pH. The solution was filter sterilized twice with 0.22 µm cellulose acetate filters, dispensed into sterile 500 ml bottles, supplemented with 1% penicillin/streptomycin and stored at 4°C. Sterile heat inactivated FCS was added to a concentration of 10% prior to use.

#### **Eagle's Minimum Essential Medium**

EMEM powdered medium was supplied by Sigma-Aldrich (St Louis, USA). A mass of 48 g of medium powder was dissolved in 5 litres sterile, deionised water. To this solution 11 g of NaHCO<sub>3</sub> was added to adjust the pH. The solution was filter sterilized twice with 0.22 µm cellulose acetate filters, dispensed into sterile 500 ml bottles, supplemented with 1% penicillin/streptomycin and stored at 4°C. Sterile heat inactivated FCS was added to a concentration of 10% prior to use.

#### **Nutrient Mixture F-12 Ham, Kaighn's Modification**

Ham's F-12 powdered medium was obtained from Sigma-Aldrich (St Louis, USA). A mass of 55.5 g of medium powder was dissolved in 5 litres sterile, deionised water. To this solution 12.5 g of NaHCO<sub>3</sub> was added to adjust the pH. The solution was filter sterilized twice with 0.22 µm cellulose acetate filters, dispensed into sterile 500 ml bottles, supplemented with 1% penicillin/streptomycin and stored at 4°C. Sterile heat inactivated FCS was added to a concentration of 10% prior to use.

#### **Roswell Park Memorial Institute Medium 1640**

RPMI 1640 powdered medium was obtained from Sigma-Aldrich (St Louis, USA). A mass of 52 g of medium powder was dissolved in 5 litres sterile, deionised water. To this solution 10 g of NaHCO<sub>3</sub> was added to adjust the pH. The solution was filter sterilized twice with 0.22 µm cellulose acetate filters, dispensed into sterile 500 ml bottles,

supplemented with 1% penicillin/streptomycin and stored at 4°C. Sterile heat inactivated FCS was added to a concentration of 10% prior to use.

### **2.3.3. Cell culture**

#### **2.3.3.1. Carcinoma cell lines**

##### **i. ASH-3 (JCRB no 1073)**

Human anaplastic thyroid carcinoma cells were maintained in 1:1 DMEM:RPMI 1640 supplemented with 10% FCS. This cell line was included in the study as it is an extremely drug-resistant cell line expressing the MRP-1 transporter which is not related to P-glycoprotein multi-drug resistance (Sekiguchi *et al.*, 2001).

##### **ii. COLO 320DM (ATCC no CCL-220)**

Human colon cancer cells were maintained in RPMI 1640 supplemented with 10% FCS. This cell line was included in the study as it is known to express P-glycoprotein (Jansen *et al.*, 1995).

##### **iii. HeLa (ATCC no CCL-2)**

Human cervical adenocarcinoma cells were maintained in EMEM supplemented with 10% FCS. This cell line was included in the study as it has been reported to be an accurate indicator of cytotoxic potency against cancer cells in general (Tiekink, 2002).

##### **iv. HepG2 (ATCC no HB-8065)**

Human hepatoblastoma cells were maintained in EMEM supplemented with 10% FCS. This cell line was included in the study as it expresses both P-glycoprotein and MRP-2 transporters (Su *et al.*, 2003).

##### **v. Jurkat (ATCC no TIB -152)**

Human T-lymphocyte leukaemia cells were maintained in RPMI 1640 medium supplemented with 10% FCS. This cell line was included in the study as it is a non adherent cell line widely used in the screening of novel anticancer compounds (Tiekink, 2002).

**vi. MCF-7 (ATCC no HTB-22)**

Human breast carcinoma cells were maintained in DMEM supplemented with 10% FCS. This cell line was included in the study as it is a hormone dependent breast cancer cell line (Sánchez *et al.*, 2008).

**vii. SH-SY5Y (ATCC no CRL-2266)**

Human neuroblastoma cells were maintained in Ham's Nutrient F-12 mixture supplemented with 10% FCS. This cell line was included in the study as it is known to highly express P-glycoprotein (Sieczkowski *et al.*, 2009).

### **2.3.3.2. Normal cell lines**

**i. Chicken embryo fibroblasts**

Cells were isolated from fertilized chicken egg embryos. Ethical approval was obtained from the Animal Use and Care Committee at the University of Pretoria (H22/06).

**ii. Human lymphocytes**

Lymphocytes were isolated from heparinised peripheral human blood from drug naïve healthy volunteers. Ethical approval was obtained from the Faculty of Health Sciences Research Ethical Committee in August 2009.

## **2.4. Methods**

### **2.4.1. Isolation of chicken embryo fibroblasts**

Fertilised SPF chicken eggs were incubated at 38.5°C for 6-10 days. All further procedures were carried out under sterile conditions. The egg shells were cleaned with 70% ethanol and placed with the blunt side to the top in a small beaker. A section of the top of the shell was carefully cracked and the shell covering the air sac removed with sterile forceps. The forceps was sprayed with 70% ethanol and used to remove the shell membrane to reveal the chorioallantoic membrane (CAM). The CAM was broken and the embryo removed by grasping it carefully under the head. Once the embryo was in a Petri dish, it was decapitated. Fat and necrotic tissue was removed and the cleaned embryo moved to a new sterile Petri dish. The embryo was cut into small pieces, and the pieces transferred to a 15 ml centrifuge tube with a sterile plastic Pasteur pipette. The tube was

filled with RPMI+ which has been warmed to 37°C. Any fatty tissue floated, and was discarded.

The medium was aspirated and the cells washed with RPMI<sup>+</sup>. The tube was filled with 1% trypsin/versene in sterile PBS which had been warmed to 37°C, and left on a shaker for 20 minutes. The pellets of tissue were allowed to settle and the suspended cells transferred to a second 15 ml tube. The tube containing the pellet was again filled with 1% trypsin/versene solution and left on the shaker for 20 minutes. The tube containing the suspended cells was filled with RPMI<sup>+</sup> medium and centrifuged for 5 minutes at 300 *g*. The supernatant of the centrifuged tube was discarded, the cell pellet re-suspended in 5 ml medium and transferred to a 25 cm<sup>2</sup> cell culture flask. The cell culture flasks were kept in an incubator at 37°C and an atmosphere of 5% CO<sub>2</sub>. This process was repeated until sufficient quantities of cells were harvested. The cells were maintained by changing the medium every 3-4 days.

#### **2.4.2. Lymphocyte preparation**

A volume of 30 ml heparinised blood from three healthy adult human volunteers was loaded carefully onto 15 ml Histopaque 1077. The blood was centrifuged for 25 minutes at 650 *g* at room temperature. The top plasma layer was removed with a plastic Pasteur pipette and the lymphocyte/monocyte layer, approximately 12 ml, transferred to sterile 50 ml tubes. The tubes were filled with sterile RPMI-1640 medium. The mixture was centrifuged for 15 minutes at 200 *g* to rid the mixture of contaminating platelets. The supernatant was discarded and the tube filled with sterile RPMI-1640 before centrifuging again for 10 minutes at 200 *g*. The supernatant was again discarded and the tubes filled with sterile, cold ammonium chloride lysing solution. The tubes were left for 10 minutes on ice to lyse any contaminating erythrocytes. Thereafter the mixture was centrifuged for 10 minutes at 200 *g*. The supernatant was discarded and the cells re-suspended in 1 ml RPMI supplemented with 10% foetal bovine serum. The cells were then further diluted in RPMI<sup>+</sup> (RPMI supplemented with 10% foetal calf serum) to obtain the required cell concentrations for the experiments.

### 2.4.3. Maintenance of primary cell culture and cancer cell lines

The primary cell cultures and cancer cell lines to be used in this study together with their origin and growth characteristics are indicated in Table 2.1 below. All cells were kept in an incubator at 37°C and an atmosphere of 5% CO<sub>2</sub>. The cells were maintained by changing the medium every 3-4 days.

**Table 2.1:** The various cells used in the study

Name	Tissue type of origin	Medium required*	Growth characteristic	Seeding density (cells/ml)
ASH-3	Human anaplastic thyroid carcinoma	10% 1:1 RPMI:DMEM	Adherent	1 x 10 <sup>5</sup>
Chicken Embryo Fibroblasts	Chicken Embryo**	10% EMEM	Adherent	5 x 10 <sup>5</sup>
COLO 320DM	Human colon cancer	10% RPMI	Non-adherent	2 x 10 <sup>4</sup>
HeLa	Human cervical adenocarcinoma	10% EMEM	Adherent	2.5 x 10 <sup>4</sup>
HepG2	Human hepatoblastoma	10% EMEM	Adherent	2.5 x 10 <sup>4</sup>
Jurkat	Human T-lymphocyte leukaemia	10% RPMI	Non-adherent	3 x 10 <sup>4</sup>
Lymphocytes	Human blood***	10% RPMI	Non-adherent	2 x 10 <sup>6</sup>
MCF-7	Human breast carcinoma	10% DMEM	Adherent	2 x 10 <sup>4</sup>
SH-SY5Y	Human neuroblastoma	10% Ham's F12	Adherent	2.5 x 10 <sup>4</sup>

\* The culture medium will be supplemented with heat inactivated foetal bovine serum as indicated by the percentages

\*\* Obtained from 5-7 days old fertilized chicken eggs

\*\*\* Obtained from healthy, adult volunteers

### 2.4.4. Preparation of cells for proliferation assays

For adherent cells the medium was discarded and the cell culture flask rinsed with 0.25% trypsin/Versene (w/v). Approximately 3 ml of trypsin/Versene was then added to the flask and the flask re-incubated for 10-15 minutes. The cell suspension was transferred to a sterile 15 ml tube, filled with medium containing 10% foetal bovine serum to inactivate the trypsin/Versene. Loosely adherent cells were not trypsinised, but instead the medium was transferred directly from the cell culture flask to the 15 ml

centrifuge tube. The tube was centrifuged at 200 *g* for 6 minutes, the supernatant discarded and the cell pellet resuspended in 1 ml cell culture medium supplemented with 10% foetal bovine serum. The suspension was mixed thoroughly with an autopipette to obtain a homogenous cell suspension.

#### **2.4.5. Cell counting**

A volume of 50  $\mu$ l of the cell suspension was added to 450  $\mu$ l of cell counting fluid. The mixture was loaded onto a haemocytometer, and the cells counted using a Reichert Jung MicroStar 110 microscope at 400x magnification. Dilutions of the cell suspension in the appropriate medium supplemented with 10% foetal bovine serum were then made to achieve the correct cell concentration required for the proliferation assay.

#### **2.4.6. Cell proliferation assay**

A volume of 80  $\mu$ l of the appropriate medium supplemented with 10% foetal bovine serum was added to each well of a sterile, 96-well plate. A well containing 200  $\mu$ l of medium served as medium control. Three wells were also designated as vehicle controls and received 0.5% DMSO, cells and medium. A volume of 100  $\mu$ l of the cell suspension was then added to every well with an autopipette, and the plate incubated for 1 hour at 37°C in an atmosphere of 5% CO<sub>2</sub>. The experimental compounds were diluted with cell culture medium and 20  $\mu$ l of each concentration added to three wells. For human lymphocytes, stimulated wells also received 20  $\mu$ l of phytohemagglutinine. The primary cell culture, lymphocytes and chicken embryo fibroblasts, were incubated for 3 days. The cancer cell cultures were incubated for 7 days. After the incubation period, 20  $\mu$ l of MTT solution was added to perform the MTT viability assay as described below.

#### **2.4.7. MTT cell viability assay**

The MTT staining method, as described by Mossmann (1983) is based on the mitochondrial conversion of the tetrazolium salt (MTT) to formazan crystals during incubation. The pale yellow tetrazolium salt is cleaved by the mitochondrial dehydrogenase enzymes to form dark purple insoluble crystals. The conversion only occurs in live cells, thus the amount of purple formazan crystals present is directly proportional to the number of viable, metabolically active cells. To perform a MTT assay

20 µl of a MTT solution was added to each well and the plates re-incubated for 3.5-4 hours at 37°C in a 5% CO<sub>2</sub> incubator. Thereafter the plates were centrifuged for 10 minutes at 800 *g*. The supernatant was carefully removed without disturbing the pellet and the pellets washed with 150 µl PBS which was also removed after centrifugation. A volume of 100 µl of DMSO was added to each well and the plates shaken gently for approximately 1 hour on a shaker. The absorbance of the wells were measured using an EL<sub>x</sub>800 UV universal microplate reader (Bio-Tek Instruments Inc., Vermont, USA) at 570 nm with a reference wavelength of 630 nm.

#### **2.4.8. Interpretation of results**

A minimum of three independent experiments was performed. Each experiment included triplicate wells for each compound at each concentration. The results were expressed as percentage of cell viability compared to the untreated control, calculated as follows:

$$\% \text{ cell viability} = 100 \times \text{Mean absorbance of sample} / \text{Mean absorbance of control}$$

The standard error of the mean was also calculated. GraphPad Prism version 4.0 software was used to determine the compound concentration at which cell viability was inhibited by 50%, i.e. the IC<sub>50</sub>, using the nonlinear LOWESS spline fit method. To determine whether results obtained for the experimental compounds differ significantly from those obtained for the controls, the one-way ANOVA and Dunnett's *post-hoc* test were performed. A p-value of less than 0.05 was used as indicator of significance. GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) was used for all statistical calculations.

The tumour specificity was calculated using the following formula:

$$\text{Tumour Specificity} = \Sigma [\text{IC}_{50} (\text{normal cells})] / \Sigma [\text{IC}_{50} (\text{HeLa cells})]$$

From these results the two most promising candidates were selected for further investigation.

## 2.5. Results

### 2.5.1. IC<sub>50</sub> concentrations on cancer cell lines

The MTT viability assay was performed to determine the concentration of each of the four hydroxyquinolines where cell growth was inhibited by 50% compared to the untreated controls. This was done using cancer cell lines to indicate cytotoxic efficacy. Results are displayed in Table 2.2.

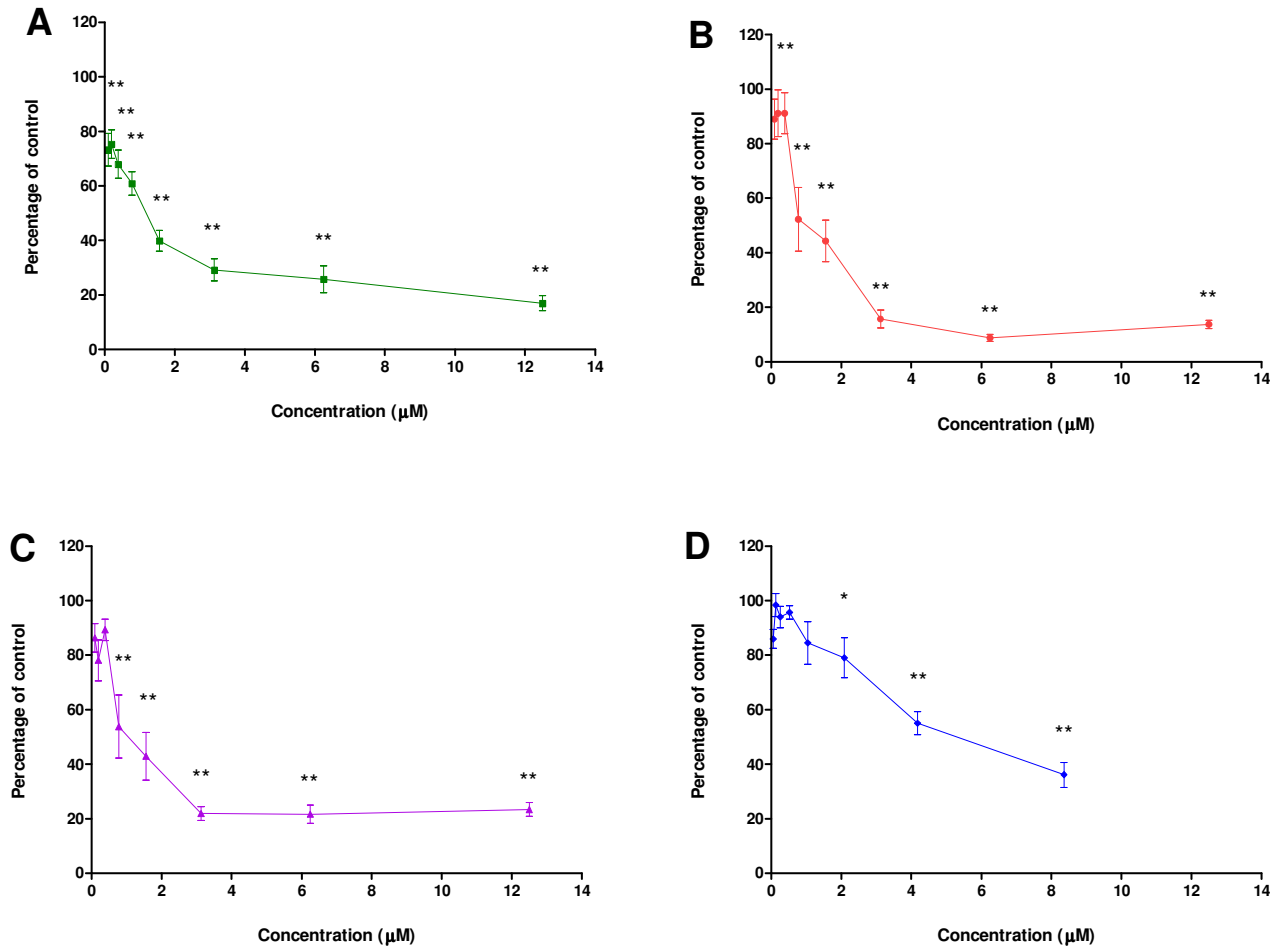
**Table 2.2:** Mean drug concentration ( $\mu\text{M}$ ) of the four novel hydroxyquinolines causing 50% inhibition of cell growth of six cancer cell lines, reported as mean concentration  $\pm$  SEM.

Drug	ASH-3	COLO 320DM	HeLa	HepG2	Jurkat	MCF-7	SH-SY5Y
HQ5	1.74 $\pm$ 0.30	0.65 $\pm$ 0.13	4.33 $\pm$ 0.48	1.21 $\pm$ 0.09	0.34 $\pm$ 0.07	0.35 $\pm$ 0.13	4.88 $\pm$ 1.18
HQ6	2.43 $\pm$ 0.17	0.48 $\pm$ 0.14	2.25 $\pm$ 0.39	12.34 $\pm$ 1.51	1.26 $\pm$ 0.06	1.21 $\pm$ 0.05	1.15 $\pm$ 0.05
HQ7	2.30 $\pm$ 0.18	0.54 $\pm$ 0.20	2.66 $\pm$ 0.57	13.65 $\pm$ 1.54	1.55 $\pm$ 0.14	1.16 $\pm$ 0.03	1.04 $\pm$ 0.05
HQ10	4.74 $\pm$ 0.37	4.98 $\pm$ 0.46	1.41 $\pm$ 0.19	7.23 $\pm$ 0.17	3.61 $\pm$ 0.06	5.85 $\pm$ 2.83	8.93 $\pm$ 1.94

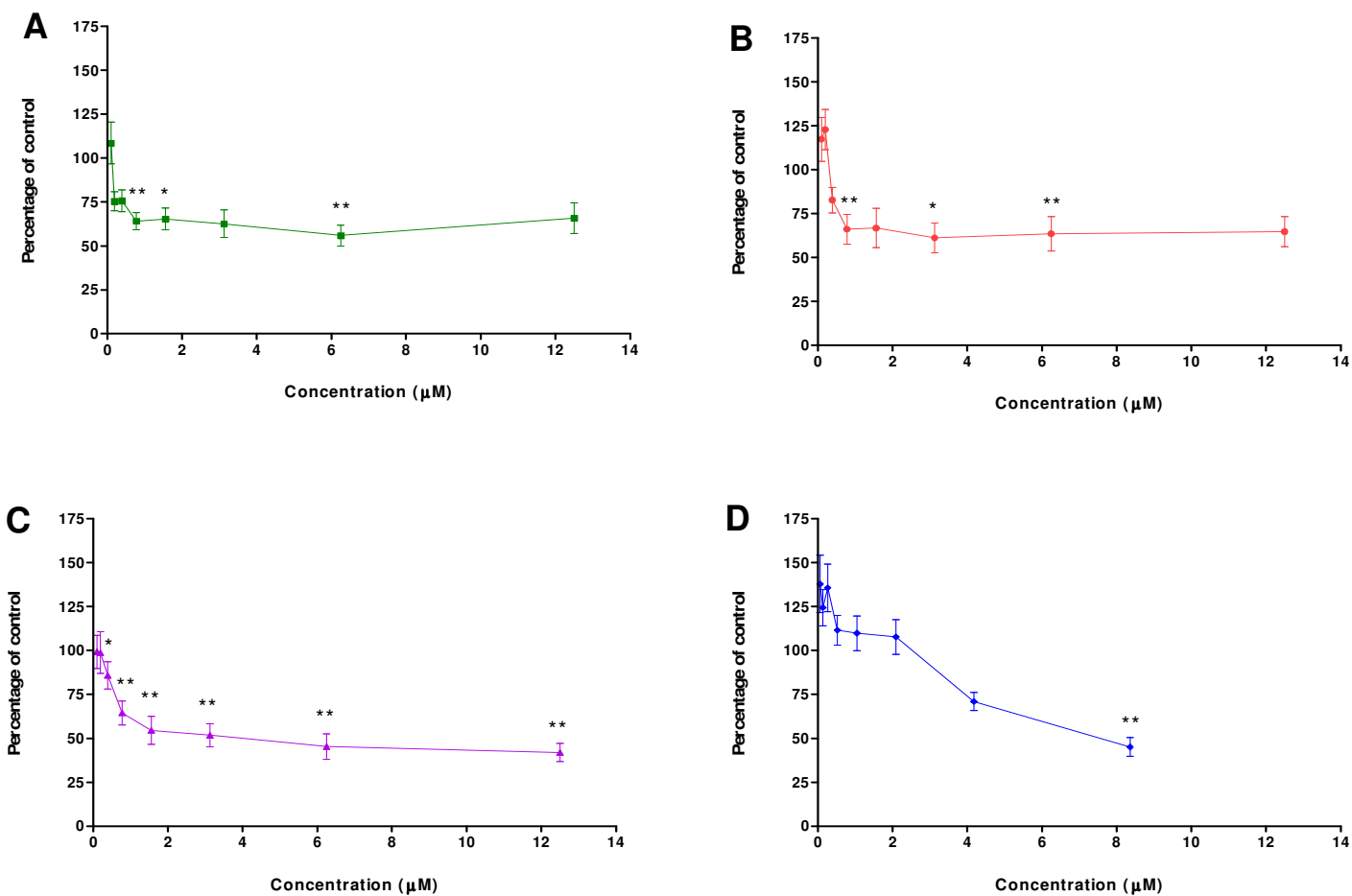
∅ Results are for a minimum of three repeats

§ Incubation period 7 days

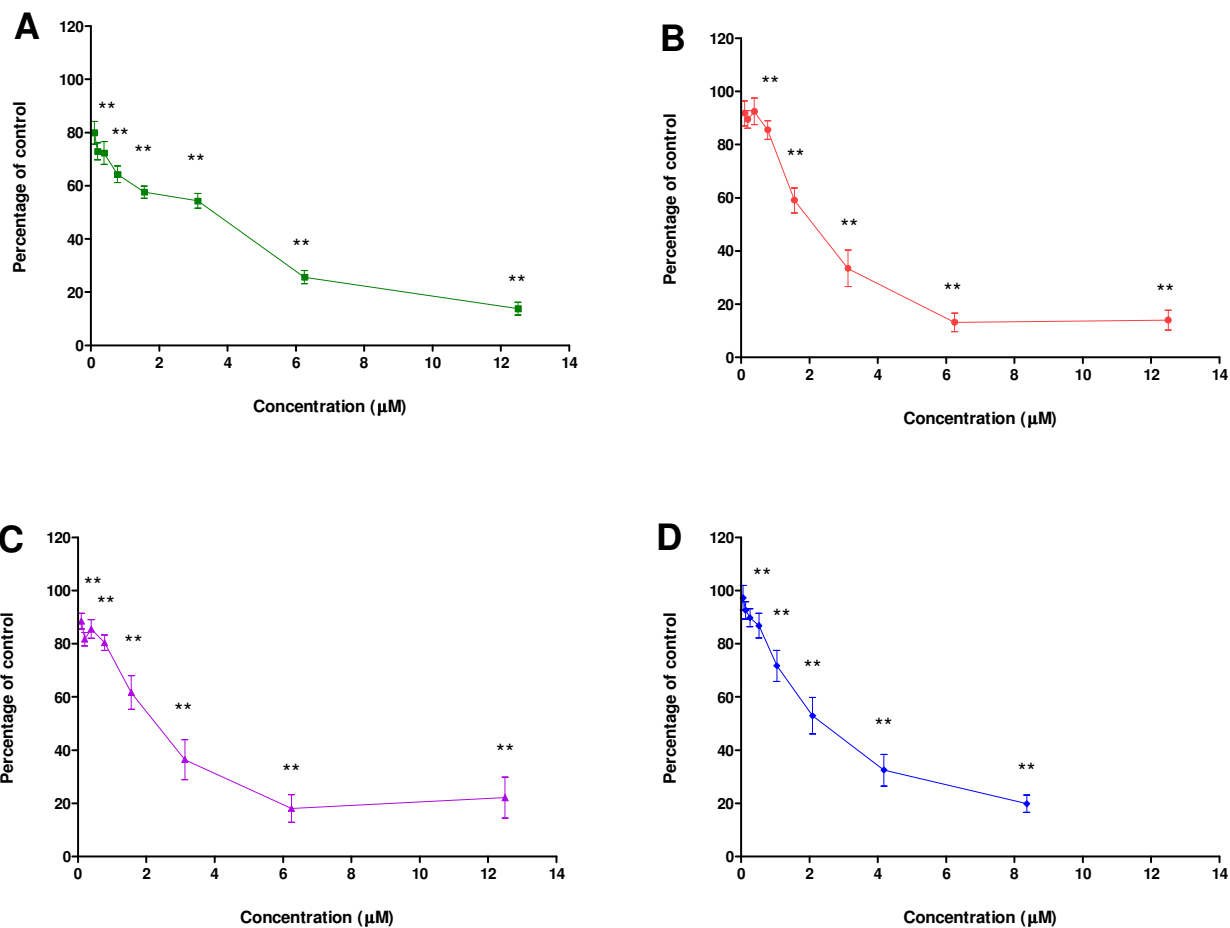
According to the results HQ5 had an IC<sub>50</sub> of 0.34  $\mu\text{M}$  towards Jurkat cells. Interestingly, HQ5 does not seem to be effective against HeLa cells (4.33  $\mu\text{M}$ ). HQ6 and HQ7 proved to be most toxic to COLO 320DM cells, with IC<sub>50</sub>'s of 0.48  $\mu\text{M}$  and 0.54  $\mu\text{M}$ , respectively. However, both of these compounds showed poor efficacy against HepG2 cells. HQ10 showed an IC<sub>50</sub> of 1.41  $\mu\text{M}$  against Jurkat cells. A graphic representation of the dose response of the cancer cell lines tested is shown in Figures 2.1 - 2.7.



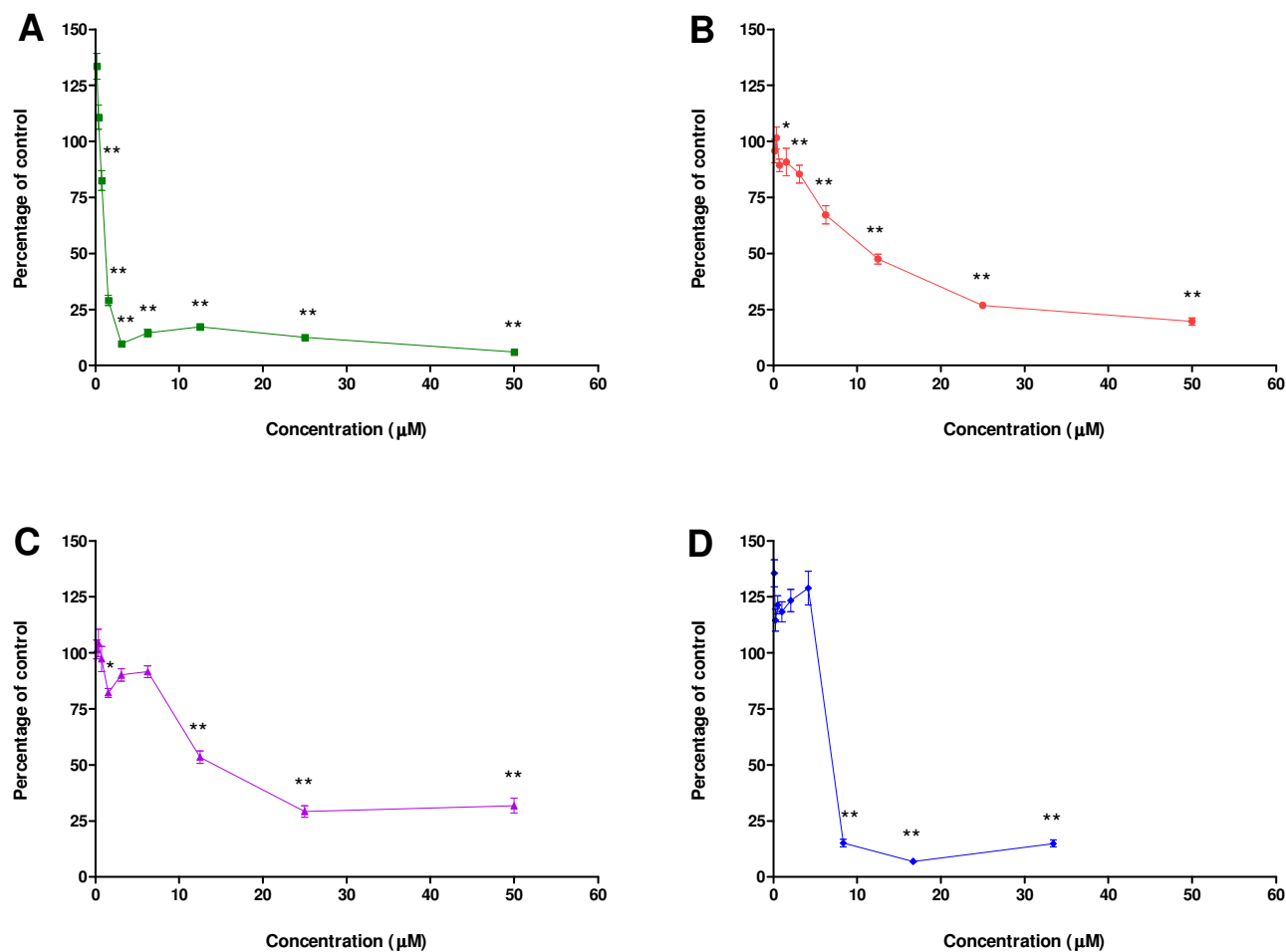
**Figure 2.1:** The mean growth inhibition of ASH-3 cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of four independent experiments was performed. All graphs were standardized to 120% of control, and show up to 14 µM. \* p < 0.05 \*\* p < 0.01



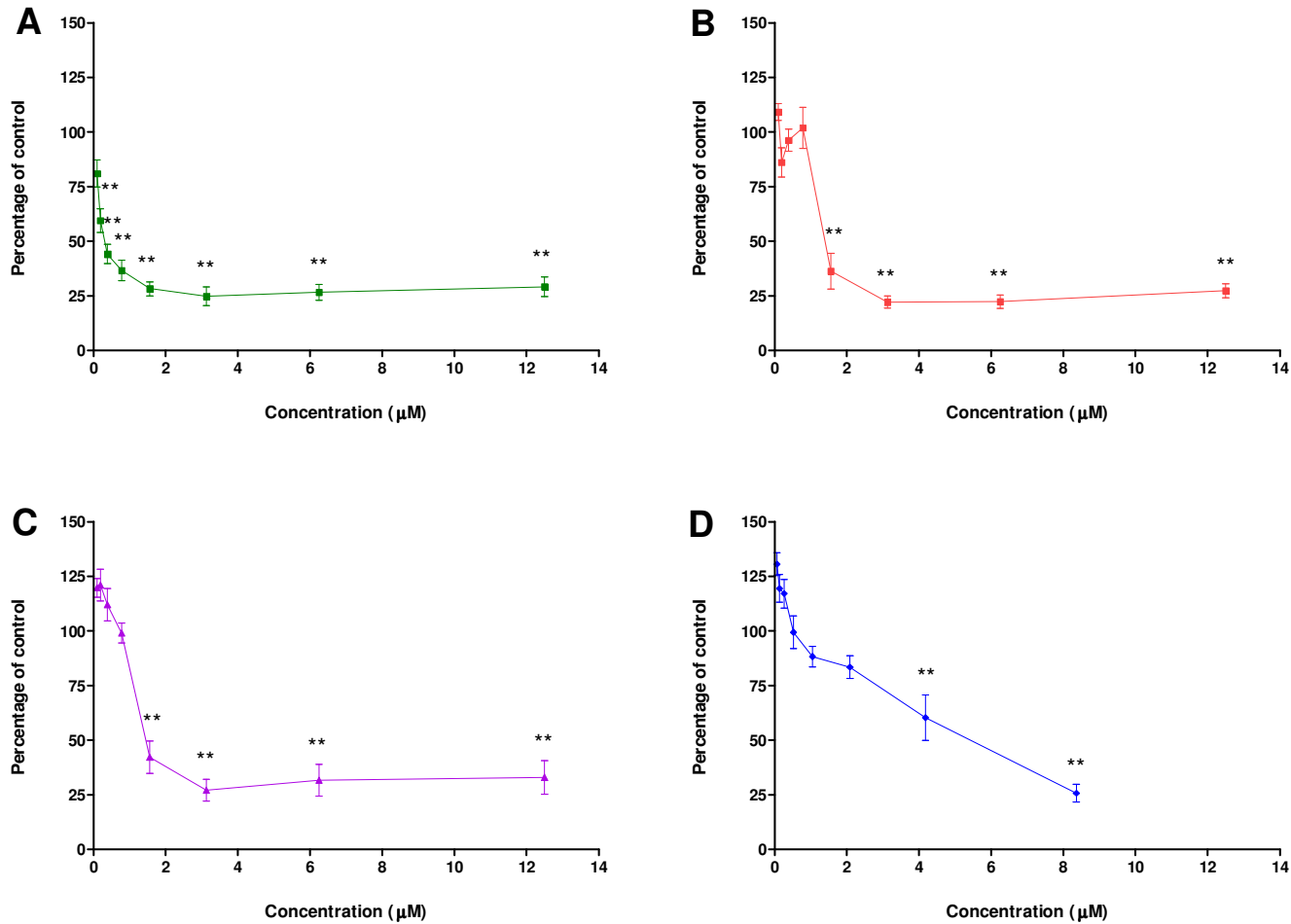
**Figure 2.2:** The mean growth inhibition of COLO 320DM cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of four independent experiments was performed. All graphs were standardized to 175% of control and show up to 14 µM. \*  $p < 0.05$  \*\*  $p < 0.01$



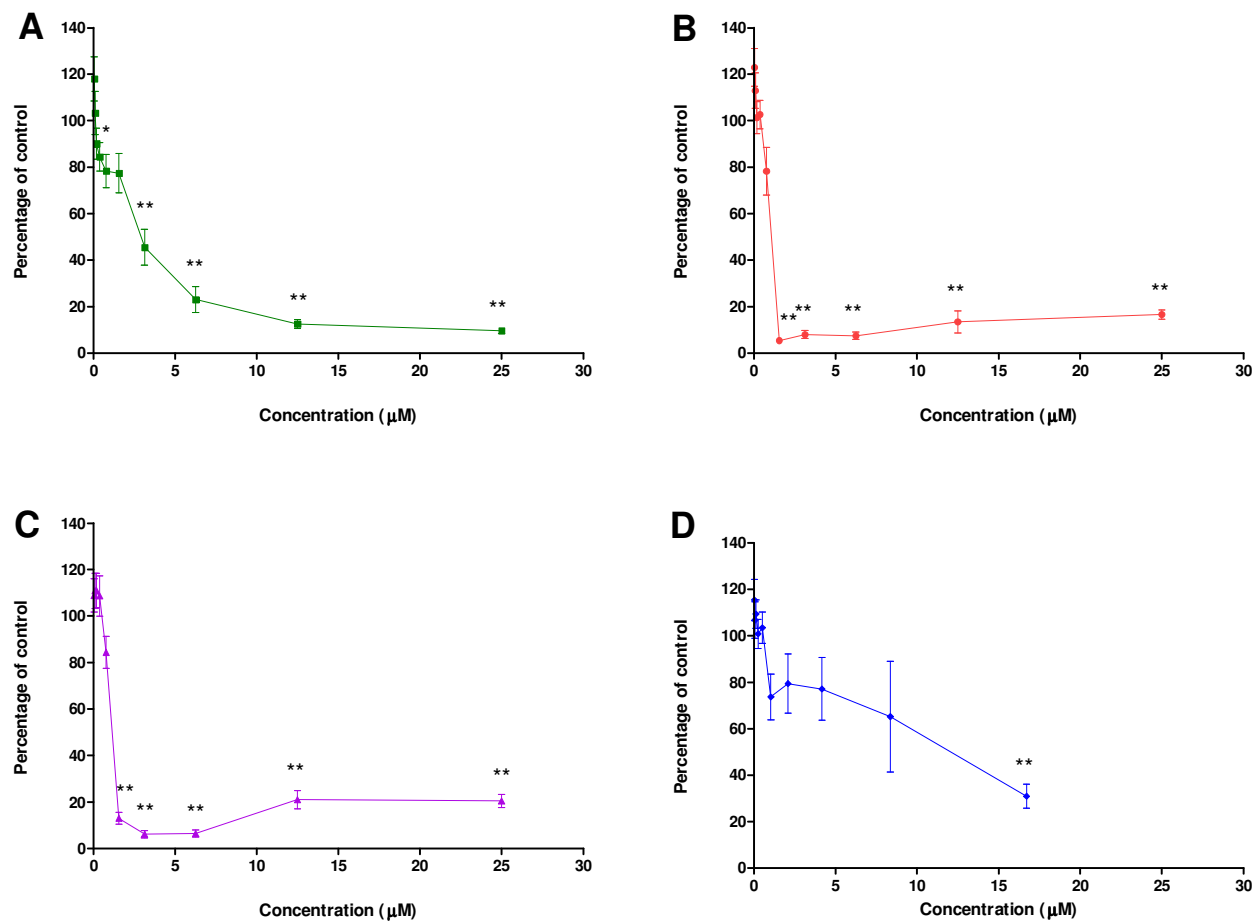
**Figure 2.3:** The mean growth inhibition of HeLa cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of five independent experiments was performed. All graphs were standardized to 120% of control and show up to 14 µM. \*  $p < 0.05$  \*\*  $p < 0.01$



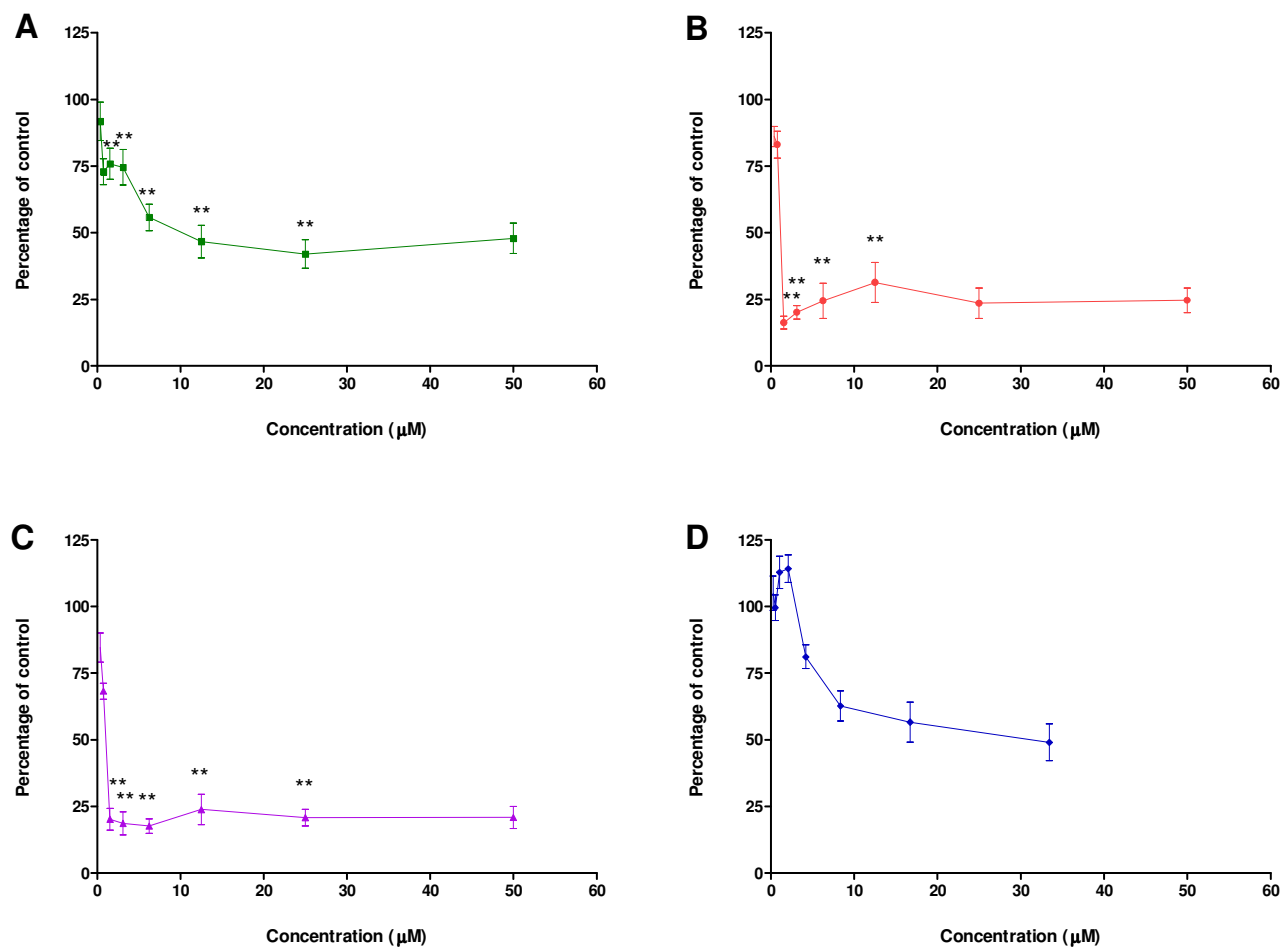
**Figure 2.4:** The mean growth inhibition of HepG2 cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of six independent experiments was performed. All graphs were standardized to 150% of control and show up to 60 µM. \* p < 0.05 \*\* p < 0.01



**Figure 2.5:** The mean growth inhibition of Jurkat cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of six independent experiments was performed. All graphs were standardized to 150% of control and show up to 14 µM. \*  $p < 0.05$  \*\*  $p < 0.01$



**Figure 2.6:** The mean growth inhibition of MCF-7 cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of three independent experiments was performed. All graphs were standardized to 140% of control and show up to 30 µM. \*  $p < 0.05$  \*\*  $p < 0.01$



**Figure 2.7:** The mean growth inhibition of SH-SY5Y cells when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of three independent experiments was performed. All graphs were standardized to 125% of control and show up to 60 µM. \*\* p < 0.01

### 2.5.2. IC<sub>50</sub> concentrations on normal cell lines

The MTT cell enumeration assay was performed to determine the concentration of each of the four hydroxyquinolines where cell growth was inhibited by 50% compared to the untreated controls. This was done on normal cell cultures to predict cytotoxicity, and results are summarised in Table 2.3.

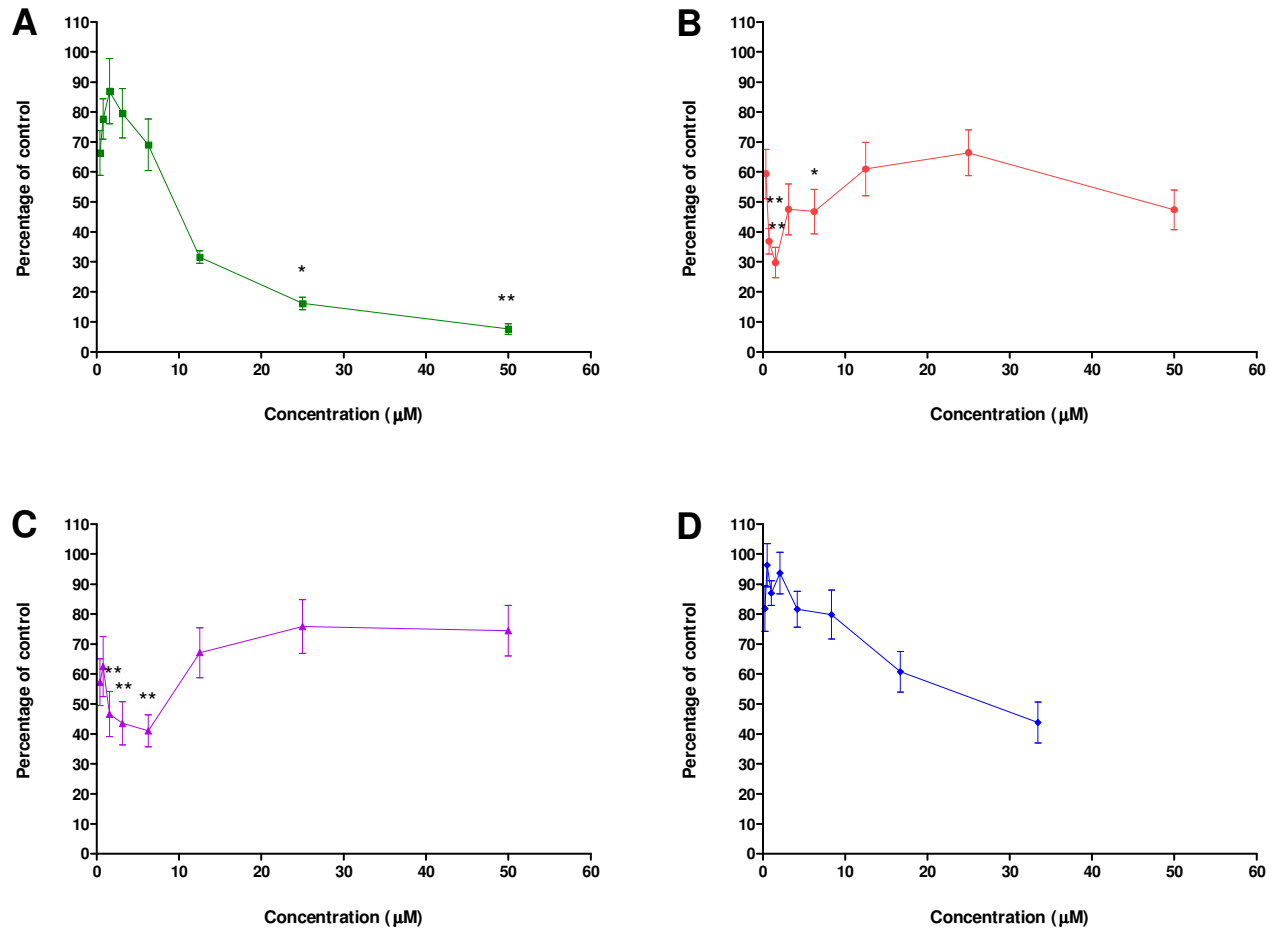
**Table 2.3:** Mean drug concentration ( $\mu\text{M}$ ) of the four novel hydroxyquinolines causing 50% inhibition of cell growth of four non-cancerous normal cell cultures, reported as mean concentration  $\pm$  SEM.

Drug	Resting Lymphocytes	PHA stimulated Lymphocytes	Chicken Embryo Fibroblasts
HQ5	14.73 $\pm$ 1.35	19.80 $\pm$ 1.01	1.08 $\pm$ 0.54
HQ6	3.19 $\pm$ 0.34	2.40 $\pm$ 0.16	1.62 $\pm$ 0.52
HQ7	2.10 $\pm$ 0.23	2.61 $\pm$ 0.13	1.03 $\pm$ 0.14
HQ10	19.08 $\pm$ 0.34	5.34 $\pm$ 0.26	7.54 $\pm$ 2.87

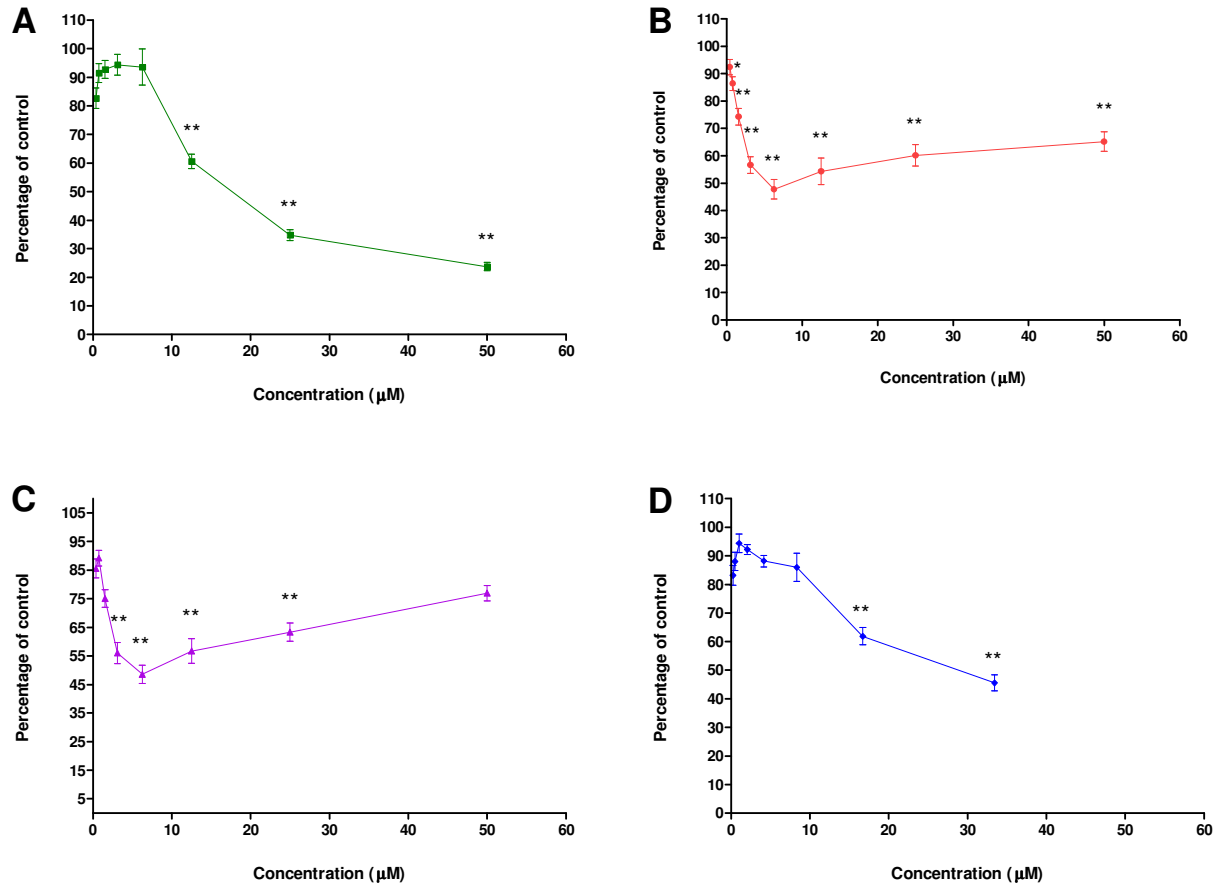
$\alpha$  Results for a minimum of five repeats

§ Incubation period 3 days

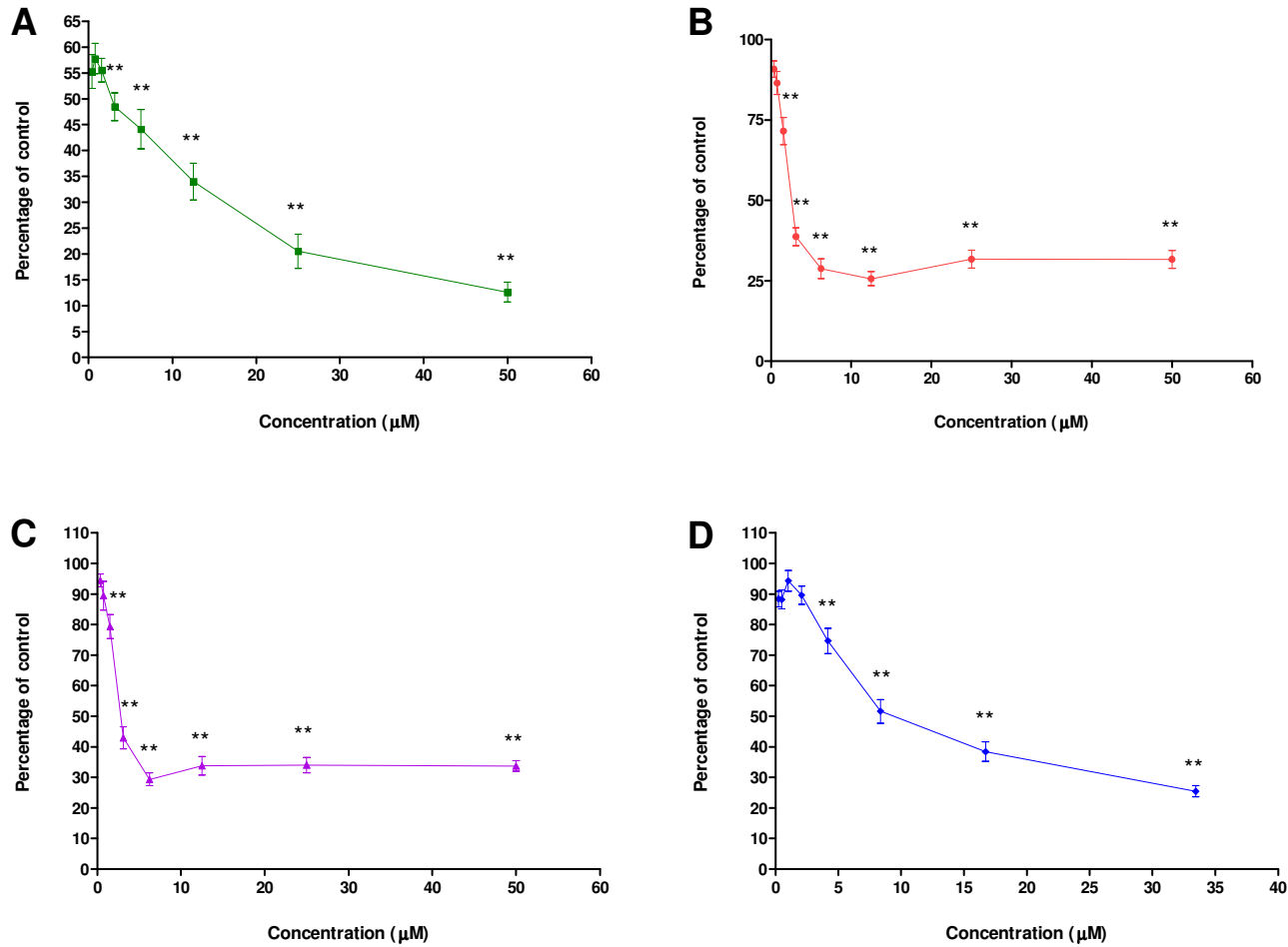
The results for HQ5 were considered promising as the compound only showed cytotoxicity against resting human lymphocytes at a relatively high concentration ( $>10 \mu\text{M}$ ). The compound does however appear cytotoxic to chicken embryo fibroblasts at a rather low concentration as is evident from the IC<sub>50</sub> value obtained ( $1.08 \mu\text{M}$ ). Low concentrations of HQ6 (from  $1.25 \mu\text{M}$ ) and HQ7 (from  $3.25 \mu\text{M}$ ) were cytotoxic to all the normal cell cultures tested. HQ10 did not cause toxicity in resting human lymphocytes ( $<16 \mu\text{M}$ ) or chicken embryo fibroblasts ( $<4 \mu\text{M}$ ). A graphic representation of the dose response of the chicken embryo fibroblasts and the two human lymphocyte cell culture tested are shown in Figures 2.8 - 2.10.



**Figure 2.8:** The mean growth inhibition of chicken embryo fibroblasts when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of five independent experiments was performed. All graphs are standardized to 110% of control and show up to 60 µM. \*  $p < 0.05$  \*\*  $p < 0.01$



**Figure 2.9:** The mean growth inhibition of resting lymphocytes when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of ten independent experiments was performed. All graphs were standardized to 110% of control and show up to 60 µM. \*  $p < 0.05$  \*\*  $p < 0.01$



**Figure 2.10:** The mean growth inhibition of PHA-stimulated lymphocytes when exposed to (A) HQ5, (B) HQ6, (C) HQ7 and (D) HQ10 as indicated by the MTT assay. A minimum of ten independent experiments was performed. All graphs were standardized to 110% of control and show up to 60 µM. \*  $p < 0.05$  \*\*  $p < 0.01$

### 2.5.3. Tumour specificity

The tumour specificity of novel chemotherapeutic agents are instrumental in identifying lead compounds which will show efficacy towards cancer cells, but with limited toxicity towards the body's normal cells. When calculated using the results for HeLa cells, the tumour specificity indicated that HQ10 were most specifically cytotoxic towards cancer cells (at 22.67), followed by HQ5 with a tumour specificity of 8.69. HQ6 and HQ7 both had tumour specificities below five (3.20 and 2.66, respectively). These results are indicated in Table 2.4.

**Table 2.4:** The tumour specificities of the four experimental hydroxyquinolines

<b>Drug</b>	<b>Tumour Specificity</b>
<b>HQ5</b>	8.69
<b>HQ6</b>	3.20
<b>HQ7</b>	2.66
<b>HQ10</b>	22.67

## 2.6. Discussion

During this study the four experimental hydroxyquinolines were screened against a range of cancer and normal cell lines. Results on the cancer cell lines showed that all four of the compounds displayed cytotoxicity towards cancer cells and are thus potential anti-neoplastic agents. The lowest  $IC_{50}$  concentration for HQ5 was achieved against the Jurkat cell line, HQ6 and HQ7 against the COLO 320DM cell line and HQ10 against the HeLa cell line. This indicates the cancer type each of the compounds will be most effective against in a treatment setting.

From the differences in the dose response curves it becomes clear, however, that the difference in structure and substituents of the four experimental hydroxyquinolines influences their cytotoxicity. The dose response curves obtained for HQ5, HQ6 and HQ7

follow mostly the same trend, whereas the dose response curves for HQ10 differ. The structure of HQ10 differs from the rest of the experimental compounds due to the presence of two halogens, bromine and fluorine. Even though the halogens are not typical substituents in medicinal compounds, previous studies of halogen-substituted quinolines have yielded promising results. A range of fluorinated quinoline derivatives screened against a panel of 60 cancer cell lines showed remarkable activity against leukaemia, melanoma and ovarian cancer cell lines (Lipunova *et al.*, 2000). Chandrika *et al.* (2008) showed that an iodine-substituted quinazoline derivative displayed remarkable toxicity towards the U937 leukaemia cell line. In this study it was found that HQ10 is selectively more toxic towards the HeLa and Jurkat leukaemia cell lines.

Other quinoline derivatives have also been reported to display anticancer properties. Quinoline derivatives containing the trace mineral selenium have been shown to induce cytotoxic effects in human leukaemia cells (Shahabuddin *et al.*, 2010). Studies on the T47D breast cancer cell lines indicated that quinolines substituted with, amongst others, amines, inhibited cell growth strongly (Gakhar *et al.*, 2008). Copper-containing quinoline-carboxamide derivatives were also reported to display anticancer properties against leukaemia, and a range of solid tumour cell lines (Zhang *et al.*, 2003).

The experimental compounds achieved promising IC<sub>50</sub> values against Jurkat leukaemia cells and MCF-7 breast cancer cells, with HQ5 scoring the lowest IC<sub>50</sub> in both cell lines. The results obtained using the COLO 320DM and HepG2 cell lines were not as consistent between the experimental compounds. By studying cell lines known to express high levels of P-gp such as COLO 320DM, HepG2 and SH-SY5Y, the effect of P-gp on the measured therapeutic efficacy of a compound can be assessed. A study comparing the effect of doxorubicin alone or in combination with a P-glycoprotein inhibitor on a cell line over-expressing P-glycoprotein clearly indicated that P-glycoprotein made a remarkable difference in the IC<sub>50</sub> value obtained (Dong *et al.*, 2009). The same trend was observed for paclitaxel (Dong *et al.*, 2009). Both HQ6 and HQ7 achieved fairly low IC<sub>50</sub> values against COLO 320DM and SH-SY5Y cells. HepG2 cells, however, proved to be fairly resistant to these two compounds with IC<sub>50</sub> values above 12 µM. HepG2 cells are known to express not only P-gp, but also the MRP2-transporter (Su *et al.*, 2003), another well described efflux transporter for xenobiotic compounds. HQ6 and HQ7 might thus be high affinity substrates for the MRP-2 efflux transporter. The

cytotoxicity results for HQ5 and HQ10 on the three P-gp expressing cell lines are remarkably similar as both compounds were found to be most toxic towards COLO 320DM cells of the three P-gp expressing cell lines.  $IC_{50}$  values for these compounds on SH-SY5Y cells were above 5  $\mu$ M, which exhibited the highest  $IC_{50}$  values observed for the compounds in the P-glycoprotein expressing cell lines.

During this study, human lymphocytes and chicken embryo fibroblasts were used as predictors of toxicity. The results obtained on the primary cell culture, human lymphocytes and chicken embryo fibroblasts used in this study showed that HQ10 exposure resulted in the least toxicity, as the highest  $IC_{50}$  concentrations were recorded for this compound. The toxicity to normal cells which results from chemotherapeutic agent exposure is related to the cytotoxic specificity of that compound and translates in an *in vivo* setting to the adverse events experienced by the patient during treatment (Huang *et al.*, 2009). As predicted by the results obtained on the normal cell culture, HQ10 scored the highest tumour specificity, followed by HQ5. These two compounds are thus predicted to have the most favourable *in vivo* toxicity profile.

As the experimental hydroxyquinolines were investigated for potential combination therapy agents, and not as monotherapy agents, the lack of tumour specificity does not deem these compounds unsuitable candidates. In a combination therapy set-up it is likely that each component will be administered in lower doses than those tested in this study. From the results obtained, HQ5 and HQ10 were selected for further investigation. Based on the toxicity against primary (normal) cell culture, the definitive lack of tumour specificity, unfavourable solubility profile and unusual dose response curves, HQ6 and HQ7 were deemed unsuitable candidates for further development. The remainder of the study was thus completed on HQ5 and HQ10. To assess whether these compounds were in fact suitable as therapeutic agents, a battery of mechanistic studies, including influence on apoptosis/necrosis, induction and P-gp inhibition, and combination with well-known chemotherapeutics were completed.

## Chapter 3: Combination therapy

### 3.1. Introduction

Multidrug resistance (MDR) has emerged as a major obstacle to the successful treatment of cancer. This phenomenon is characterized by the development of resistance to chemotherapeutic drugs unrelated in mechanistic and structural aspects (Koo *et al.*, 2008). In an effort to circumvent resistance, traditional cytotoxic chemotherapy regimens have been replaced with combination therapy (Via *et al.*, 2008).

The first successful combination therapy treatment in cancer was developed in the 1960's by Frei *et al.* (1961) for the treatment of acute lymphocytic leukaemia in children. Principles for the successful and appropriate combination of chemotherapeutic agents were developed thereafter. Many factors influence the suitability of compounds for use in combination regimens and the efficacy of the combination treatment, including the toxicity profiles and mechanisms of action of each drug in the combination. Compounds with overlapping toxicity are not used in a combination regimen as this would increase the likelihood of necessitated dose reduction that lowers the maximum tolerated dose of each compound. Furthermore, compounds with differing mechanisms of action are generally combined in order to avoid further resistance (Frei III, 1972).

Resistance can be classified as either cellular or non-cellular (Ullah, 2008). Non-cellular mechanisms include compromised angiogenesis which limits the accessibility of cytotoxic agents to cancerous tissue, or an environment unfavourable to agents dependant on pH gradient for cellular uptake. Cellular mechanisms include complex changes in enzyme systems and drug efflux based resistance (Naito *et al.*, 1998). Drug efflux based resistance refers to ATP-dependent transporters which transport hydrophobic xenobiotics out of a cell (Zloh *et al.*, 2004). In the context of cancer treatment this translates to ineffective therapeutic concentrations and unfavourable adverse effects profile.

The ATP-binding cassette (ABC) is a family of 13 proteins which have been reported to contribute largely to efflux based multidrug resistance in cancer cells (Meijerman *et al.*, 2008; Ullah, 2008). Therapeutic drug concentration is thus not reached inside cells

expressing these ATP-dependant drug efflux pumps. The focus of this study will be on a protein belonging to this family, P-glycoprotein (P-gp) as many quinolines have been reported to influence the functionality of this protein (Ullah, 2008; Zamora *et al.*, 1988). P-glycoprotein, a 170 kDa membrane protein, is not only expressed in cancerous tissue, but also in the kidney, gastrointestinal tract and liver (Ullah, 2008) where it is thought to play a role in the secretion of toxic substances (Naito *et al.*, 1998). The expression of P-gp is higher in cancer cells derived from tissue that normally expresses P-gp, leading to decreased intracellular drug concentrations and a decrease in the efficacy of anthracyclins, vinca alkaloids, podophyllotoxins and taxanes. It has been found that P-glycoprotein, the drug efflux pump, is rapidly up-regulated after *in vivo* exposure to chemotherapeutic agents in patient samples (Di Nicolantonio *et al.*, 2005) and in relapsed cancer (Koo *et al.*, 2008).

Modulators of MDR have evolved from the incidental discovery of the desired properties of medication used for other ailments to the development of compounds with a high affinity to transmembrane transport proteins with low pharmacokinetic interaction (Ullah, 2008). However, the use of many MDR reversal agents are limited due to unfavourable toxicity profiles (Koo *et al.*, 2008). The ability of the quinoline ring and its derivatives to modulate MDR were discovered when quinine, a well-known anti-malarial drug, was found to sensitize drug-resistant tumours to chemotherapy (Ullah, 2008). Compounds belonging to the quinoline family have since been investigated for potential resistance reversal characteristics. Among the most promising of these compounds is MS-209 which is a quinoline derivative with potent MDR modulating abilities that has been found to reach efficacious concentrations (3  $\mu$ M) after oral administration (Naito *et al.*, 2002). MS-209 has been reported to interact with both P-gp and MRP-1 (Narasaki *et al.*, 1997). It has been speculated that quinolines exert their chemosensitising effect via the inhibition of P-gp.

To investigate the potential effect of HQ5 and HQ10 on P-gp, cell lines known to express these transport proteins were exposed to a combination of the experimental compounds and anticancer drugs known to be substrates of each transporter. Reports indicate that COLO 320DM (Jansen *et al.*, 1995) and SH-SY5Y cells (Sieczkowski *et al.*, 2009) express P-glycoprotein and were thus included as P-gp model. HepG2 cells were included in this study as they express both P-glycoprotein and MRP-2 (Su *et al.*, 2003).

Doxorubicin (Le Bot *et al.*, 1994), paclitaxel (Chibale *et al.*, 2001) and vinblastine (Dey *et al.*, 1997) were included as they have all been reported to be P-gp substrates. Verapamil, reported as a P-gp inhibitor (Le Bot *et al.*, 1994) was included as positive inhibition control.

## 3.2. Aim

- i. To determine the concentrations of doxorubicin, paclitaxel and vinblastine which inhibit cell growth by 20% for COLO 320DM, SH-SY5Y and HepG2 cells respectively.
- ii. To investigate the effect of exposing COLO 320DM, SH-SY5Y and HepG2 cells to a combination of the test hydroxyquinolines and doxorubicin, paclitaxel or vinblastine at their respective  $IC_{20}$ .

## 3.3. Materials

### 3.3.1. Reagents

#### i. Doxorubicin

A stock solution of 1 mg/ml doxorubicin (Sigma-Aldrich St Louis, USA) was prepared in DMSO. Aliquots of the stock solution were made and stored at  $-80^{\circ}\text{C}$ . Dilutions were made in the appropriate cell culture medium prior to use.

#### ii. Eagle's Minimum Essential Medium

EMEM powdered medium was supplied by Sigma-Aldrich (St Louis, USA). A mass of 48 g of medium powder was dissolved in 5 litres sterile, deionised water. To this solution 11 g of  $\text{NaHCO}_3$  was added to adjust the pH. The solution was filter sterilized twice with  $0.22\ \mu\text{m}$  cellulose acetate filters, dispensed into sterile 500 ml bottles, supplemented with 1% penicillin/streptomycin and stored at  $4^{\circ}\text{C}$ . Sterile heat inactivated FCS was added to a concentration of 10% prior to use.

#### iii. Nutrient Mixture F-12 Ham, Kaighns's Modification

Ham's F-12 powdered medium was obtained from Sigma-Aldrich (St Louis, USA). A mass of 55.5 g of medium powder was dissolved in 5 litres sterile, deionised water. To this solution 12.5 g of  $\text{NaHCO}_3$  was added to adjust the pH. The solution was filter

sterilized twice with 0.22  $\mu\text{m}$  cellulose acetate filters, dispensed into sterile 500 ml bottles, supplemented with 1% penicillin/streptomycin and stored at 4°C. Sterile heat inactivated FCS was added to a concentration of 10% prior to use.

#### **iv. MTT**

A mass of 250 mg of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), obtained from Sigma-Aldrich (St Louis, USA), was dissolved in 50 ml phosphate buffered saline (5 mg/ml). The solution was filter-sterilised using a 0.22  $\mu\text{m}$  pore size filter and stored in foil covered tubes at 4°C.

#### **v. Paclitaxel**

Paclitaxel was procured from Sigma-Aldrich (St Louis, USA). A stock solution of 100  $\mu\text{M}$  was made in DMSO and stored in aliquots of 5  $\mu\text{l}$  at -80°C. Prior to use dilutions were made in the appropriate cell culture medium.

#### **vi. Phosphate buffered saline (PBS)**

FTA Hemagglutination buffer was obtained from BD Biosciences (San Jose, USA). A solution containing 9.23 g/l in de-ionised water was made and stored at 4°C until use.

#### **vii. Roswell Park Memorial Institute Medium 1640**

RPMI 1640 powdered medium was obtained from Sigma-Aldrich (St Louis, USA). A mass of 52 g of medium powder was dissolved in 5 litres sterile, deionised water. To this solution 10 g of  $\text{NaHCO}_3$  was added to adjust the pH. The solution was filter sterilized twice with 0.22  $\mu\text{m}$  cellulose acetate filters, dispensed into sterile 500 ml bottles, supplemented with 1% penicillin/streptomycin and stored at 4°C. Sterile heat inactivated FCS was added to a concentration of 10% prior to use.

#### **viii. Trypsin/Versene**

A trypsin/Versene solution containing 0.25% trypsin and 0.1% EDTA in  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  free phosphate buffered saline was obtained from Highveld Biological (Johannesburg, RSA). Trypsin/Versene solution was stored at 4°C.

#### **ix. Verapamil**

A 1mg/ml stock solution of verapamil (Sigma-Aldrich St. Louis, USA) was made in DMSO. Aliquots of 20  $\mu\text{l}$  were made and stored at -80°C. Aliquots were diluted to the required concentrations in the appropriate cell culture medium just prior to use.

## **x. Vinblastine**

Vinblastine was obtained from Sigma-Aldrich (St. Louis, USA). A stock solution of 1 mM was prepared in DMSO and stored at -80°C in aliquots of 5 µl. On the day of the experiment, dilutions were made in the appropriate cell culture medium.

## **3.4. Methods**

### **3.4.1. Maintenance and counting of cell culture**

Briefly, the cells were grown to approximately 80% confluence in the appropriate cell culture supplemented with 10% FCS. COLO 320DM cells were grown in RPMI, HepG2 cells in EMEM and SH-SY5Y cells in Ham's F12. As COLO 320DM cells are non-adherent cells, trypsin/vesene solution was not used to harvest the cells. HepG2 and SH-SY5Y cells were harvested with a trypsin/versene solution, counted with a haemocytometer and diluted to the cell concentration required. COLO 320DM cells were used at a concentration of  $2 \times 10^4$  cells/ml whilst HepG2 and SH-SY5Y cells were used at  $2.5 \times 10^4$  cells/ml.

### **3.4.2. Determining the IC<sub>20</sub> of the standard chemotherapeutics with the MTT stain**

In order to determine the concentration of the standard chemotherapeutics to be used in combination with the test hydroxyquinolines, the concentration at which doxorubicin, paclitaxel and vinblastine induced 20% cell death in COLO 320DM, HepG2 and SH-SY5Y cells were initially determined. COLO 320DM, HepG2 and SH-SY5Y cells were exposed to varying concentrations of doxorubicin, vinblastine and paclitaxel. The cell lines were also exposed to verapamil used as a P-gp positive inhibition control.

All experiments were performed in sterile, round-bottomed 96-well plates. Each plate contained a negative control and a vehicle control. A volume of 80 µl of cell culture medium was added to each well. To this, 100 µl of cell suspension was added. An hour after seeding, cells in the experimental wells were exposed to 20 µl of the different drugs at concentrations indicated in Table 3.1. The negative control wells received 20 µl of cell culture medium, and the vehicle control wells received 20 µl of the vehicle at the highest concentration used in the experiment.

**Table 3.1:** The concentrations of doxorubicin, paclitaxel, verapamil and vinblastine used in the combination therapy experiments on COLO 320DM, HepG2 and SH-SY5Y cells.

	COLO 320DM cells	HepG2 cells	SH-SY5Y cells
<b>Doxorubicin (nM)</b>	0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50	0.5, 1, 5, 10, 50, 100, 500, 1000	0.1, 0.5, 1, 5, 10, 50, 100, 500
<b>Paclitaxel (nM)</b>	0.05, 0.1, 0.5, 1, 5, 10, 50, 100	1, 5, 10, 50, 100, 500, 1000, 5000	0.05, 0.1, 0.5, 1, 5, 10, 50, 100
<b>Verapamil (nM)</b>	0.05, 0.1, 0.5, 1, 5, 10, 50, 100	0.1, 0.5, 1, 5, 10, 50, 100, 500 *	0.1, 0.5, 1, 5, 10, 50, 100, 500 *
<b>Vinblastine (nM)</b>	0.05, 0.1, 0.5, 1, 5, 10, 50, 100	0.5, 1, 5, 10, 50, 100, 500, 1000	0.05, 0.1, 0.5, 1, 5, 10, 50, 100 **

\* Concentration in  $\mu\text{M}$

\*\* Concentration in  $\text{pM}$

After adding the drugs, plates were incubated for 7 days at 37° C in an atmosphere of 5% CO<sub>2</sub>.

To perform a MTT cell enumeration assay, 20  $\mu\text{l}$  of MTT stock solution was added to each well and the plates re-incubated for 3.5-4 hours at 37°C in a 5% CO<sub>2</sub> incubator. Thereafter the plates were centrifuged for 10 minutes at 800 *g*. The supernatant was carefully removed without disturbing the pellet and the pellets washed with 150  $\mu\text{l}$  PBS which was removed after centrifugation. One hundred microlitres of DMSO was added to each well and the plates shaken gently for approximately 1 hour on a shaker. The absorbance of the wells were measured using an ELx800 UV universal microplate reader (Bio-Tek Instruments Inc., Vermont, USA) at 570 nm with a reference wavelength of 630 nm.

### 3.4.3. Interpretation of results

A minimum of three independent experiments were performed. Each experiment included triplicate wells for each compound at each concentration. The results were

expressed as percentage of cell viability compared to the untreated control, calculated as follows:

$$\% \text{ cell viability} = 100 \times \text{Mean absorbance of sample} / \text{Mean absorbance of control}$$

The standard error of the mean was also calculated. GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used to determine the concentration at which cell viability is inhibited by 20%, i.e. the IC<sub>20</sub>, using the LOWESS spline fit method.

### 3.4.4. Combination therapy

All experiments were performed in sterile, round-bottomed 96-well plates. Each plate contained a negative control, positive control and a vehicle control. A volume of 60 µl of cell culture medium was added to each well. To this, 100 µl of cell suspension was added. An hour after seeding, cells in the experimental wells were exposed to 20 µl of the hydroxyquinolines or verapamil at the concentrations indicated in Table 3.2. Plates were re-incubated for an hour before 20 µl of doxorubicin, paclitaxel or vinblastine was added to reach the IC<sub>20</sub> concentration. The negative control wells received 20 µl of cell culture medium, and the vehicle control wells received 20 µl of the vehicle at the highest concentration used in the experiment.

**Table 3.2:** The concentrations of HQ5 and HQ10 that COLO 320DM, HepG2 and Sh-SY5Y cells were exposed to during combination therapy experiments

Experimental Compounds	COLO 320DM cells	HepG2 cells	SH-SY5Y cells
HQ5 (µM)	0.12, 0.6, 1.2, 6, 12, 60, 120	0.5, 1, 5, 10, 50, 100, 500	1, 5, 10, 50, 100, 500, 1000
HQ10 (µM)	1, 5, 10, 50, 100, 500, 1000	3.5, 7, 35, 70, 350, 700, 3500	1.8, 9, 18, 90, 180, 900, 1800
Verapamil (nM)	9, 18, 90, 180, 900, 1800, 9000	12, 60, 120, 600, 1200, 6000, 12000 *	10, 50, 100, 500, 1000, 5000, 10000 *

\* Concentration in µM

After exposure, plates were incubated for 7 days at 37° C in an atmosphere of 5% CO<sub>2</sub>. To perform a MTT cell enumeration assay, 20 µl of a MTT stock solution was added to each well and the plates re-incubated for 3.5-4 hours at 37°C in a 5% CO<sub>2</sub> incubator. Thereafter the plates were centrifuged for 10 minutes at 800 *g*. The supernatant was carefully removed without disturbing the pellet and the pellets washed with 150 µl PBS which was removed after centrifugation. A volume of 100 µl of DMSO was added to each well and the plates shaken gently for approximately 1 hour on a shaker. The absorbance of the wells were measured using an EL<sub>x</sub>800 UV universal microplate reader (Bio-Tek Instruments Inc., Vermont, USA) at 570 nm with a reference wavelength of 630 nm.

### 3.4.5. Interpretation of results

A minimum of three independent experiments was performed. Each experiment included triplicate wells for each compound at each concentration. The results were expressed as percentage of cell viability compared to the untreated control, calculated as follows:

$$\% \text{ cell viability} = 100 \times \text{Mean absorbance of sample} / \text{Mean absorbance of control}$$

The standard error of the mean was also calculated. The results of the chemotherapy treated controls were compared to the results of the combination therapy with an unpaired Student's t-test. The difference of the mean was included to indicate the degree of synergism. Significance was set at a p-value < 0.05. GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) was used for all statistical calculations.

## 3.5. Results

### 3.5.1. IC<sub>20</sub> concentrations for doxorubicin, paclitaxel, verapamil and vinblastine

The MTT viability assay was performed to determine the concentration of doxorubicin, paclitaxel, verapamil and vinblastine which inhibited cell growth by 20% using COLO 320DM, HepG2 and SH-SY5Y cells respectively. Results are displayed in Table 3.3.

For COLO 320DM cells doxorubicin had the lowest IC<sub>20</sub> at 0.26 nM. The IC<sub>20</sub> values for verapamil and vinblastine on COLO 320DM cells were very comparable at 0.49 nM and

0.46 nM, respectively. The IC<sub>20</sub> concentration for paclitaxel was surprisingly high in comparison to the other standard chemotherapeutics at 14.34 nM. The lowest IC<sub>20</sub> obtained on HepG2 cells was for vinblastine at 4.78 nM. The results for doxorubicin, paclitaxel and verapamil on HepG2 cells were comparable and ranged from 21.15 nM to 26.00 nM. A surprisingly low IC<sub>20</sub> was obtained for vinblastine on SH-SY5Y cells (0.30 pM). The IC<sub>20</sub> values of doxorubicin, paclitaxel and verapamil were all below 10 nM.

**Table 3.3:** IC<sub>20</sub> concentrations of doxorubicin, paclitaxel, verapamil and vinblastine for COLO 320DM, SH-SY5Y and HepG2 cells. Results reported as mean ± SEM.

	COLO 320DM cells	HepG2 cells	SH-SY5Y cells
<b>Doxorubicin (nM)</b>	0.26 ± 0.16	26.00 ± 8.91	6.32 ± 1.80
<b>Paclitaxel (nM)</b>	14.34 ± 8.78	21.15 ± 8.35	2.28 ± 0.98
<b>Verapamil (nM)</b>	0.49 ± 0.21	25.3 ± 10.48	1.43 ± 0.85
<b>Vinblastine (nM)</b>	0.46 ± 0.24	4.78 ± 1.38	0.30 ± 0.15 *

\* Concentration in pM

▫ Results for a minimum of five repeats

### 3.5.2. Combination of HQ5, HQ10 and verapamil with doxorubicin, paclitaxel and vinblastine

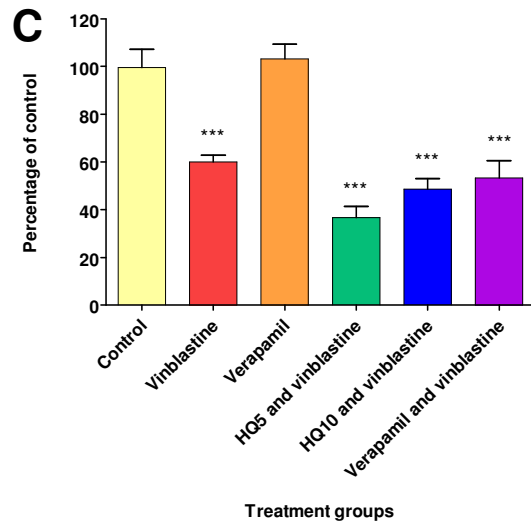
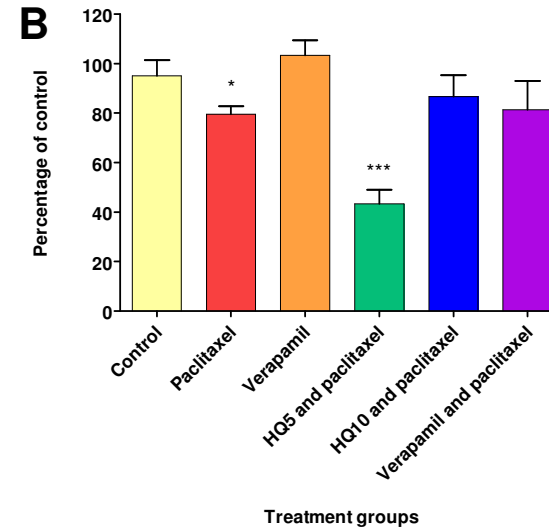
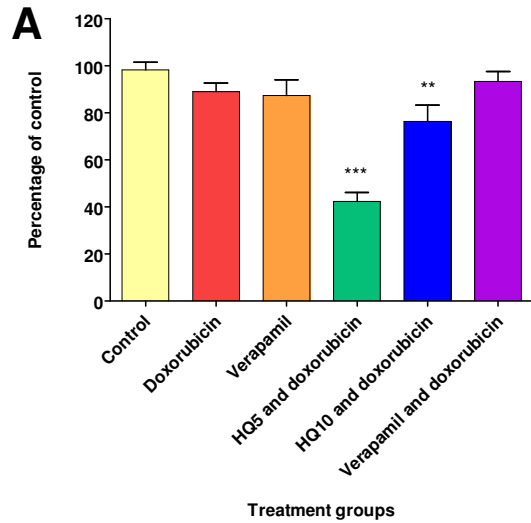
The MTT viability assay was performed to determine the effect of treatment with the two experimental hydroxyquinolines in combination with doxorubicin, paclitaxel and vinblastine, used at IC<sub>20</sub>. Three independent experiments were performed. The results which differ significantly from the untreated control are indicated.

Results for combination therapy on COLO 320DM cells are shown in Figure 3.1 and Tables 3.4 - 3.6. At a concentration of 0.12 μM of HQ5, the growth of COLO 320DM cells is inhibited significantly (p < 0.01) when used in combination with doxorubicin. A concentration of 1.2 μM of HQ5 in combination with doxorubicin showed the largest difference between the means. A concentration of 0.6 μM of HQ5 in combination with paclitaxel abrogated cell survival significantly (p < 0.0001). The paclitaxel combination with the largest difference between the means was found to be at 6 μM of HQ5. Cell growth was inhibited significantly where 0.012 μM HQ5 was used in combination with

vinblastine ( $p < 0.01$ ). The greatest difference between the means was observed where cells were treated with 6  $\mu\text{M}$  of HQ5 and vinblastine.

When HQ10 was used in combination with doxorubicin on COLO 320DM cells, a concentration of 0.1  $\mu\text{M}$  of HQ10 was sufficient to inhibit cell growth significantly ( $p < 0.01$ ). A combination with 10  $\mu\text{M}$  of HQ10 and doxorubicin showed the largest difference between the means. When HQ10 was used in combination with paclitaxel a significant decrease in cell survival was seen at 0.1  $\mu\text{M}$  and again from 10  $\mu\text{M}$  of HQ10. The most effective combination was with 50  $\mu\text{M}$  of HQ10. All combinations of vinblastine and HQ10 were found to be significantly different from the control. A combination of 100  $\mu\text{M}$  of HQ10 and vinblastine had the largest difference between the means.

Concentrations of 9  $\mu\text{M}$ , 90  $\mu\text{M}$  and 900  $\mu\text{M}$  of verapamil in combination with doxorubicin decreased growth of COLO 320DM cells very significantly ( $p < 0.01$ ). A combination of doxorubicin and 90  $\mu\text{M}$  of verapamil had the largest difference between the means. The results of the combination of verapamil and paclitaxel did not follow a dose response. Combination treatment with verapamil and vinblastine significantly decreased cell survival at concentrations tested. The difference of the mean was largest where verapamil at a concentration of 900  $\mu\text{M}$  was used with either paclitaxel or vinblastine.



**Figure 3.1:** The effect of exposure to HQ5 or HQ10 and (A) doxorubicin, (B) paclitaxel or (C) vinblastine on COLO 320DM cells. The experimental hydroxyquinolines and verapamil were used at the IC<sub>50</sub> concentration. Doxorubicin, paclitaxel and vinblastine were used at the IC<sub>20</sub> concentration. The graphs show up to 120% cell growth.

\* p < 0.05

\*\* p < 0.01

\*\*\* p < 0.001

**Table 3.4:** Results of combination therapy with HQ5 on COLO 320DM cells, reported as percentage untreated control  $\pm$  SEM.

HQ5 Concentration ( $\mu$ M)	Chemotherapeutics					
	Doxorubicin		Paclitaxel		Vinblastine	
	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means
<b>0.012</b>	113.20 $\pm$ 14.96	-12.35 $\pm$ 11.85	88.84 $\pm$ 6.17	3.20 $\pm$ 10.40	65.20 $\pm$ 5.31 **	34.38 $\pm$ 12.26
<b>0.06</b>	96.50 $\pm$ 14.53	4.318 $\pm$ 11.60	78.08 $\pm$ 6.20	13.96 $\pm$ 9.91	64.72 $\pm$ 4.54 **	34.86 $\pm$ 12.23
<b>0.12</b>	76.58 $\pm$ 7.31 **	24.25 $\pm$ 7.74	85.55 $\pm$ 11.04	6.49 $\pm$ 11.47	65.83 $\pm$ 8.44 *	33.76 $\pm$ 12.53
<b>0.6</b>	42.38 $\pm$ 3.76 ***	58.44 $\pm$ 6.58	43.23 $\pm$ 5.70 ***	48.81 $\pm$ 9.79	36.72 $\pm$ 4.59 ***	62.87 $\pm$ 12.14
<b>1.2</b>	32.69 $\pm$ 4.23 ***	68.13 $\pm$ 6.70	51.63 $\pm$ 10.88 **	40.41 $\pm$ 11.41	25.39 $\pm$ 3.10 ***	74.19 $\pm$ 11.29
<b>6</b>	41.96 $\pm$ 7.52 ***	58.86 $\pm$ 7.96	30.34 $\pm$ 6.11 ***	61.70 $\pm$ 9.51	19.01 $\pm$ 8.36 ***	80.57 $\pm$ 12.96
<b>12</b>	38.87 $\pm$ 8.15 ***	61.95 $\pm$ 8.08	33.87 $\pm$ 5.38 ***	58.17 $\pm$ 9.31	19.15 $\pm$ 4.84 *	80.44 $\pm$ 12.94

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

$\alpha$  MTT staining method used, 7 day incubation period, minimum  $n = 8$

§ All chemotherapeutics used at  $IC_{20}$  concentration (doxorubicin 0.26 nM, paclitaxel 14.35 nM, vinblastine 0.46 nM)

**Table 3.5:** Results of combination therapy with HQ10 on COLO 320DM cells, reported as percentage untreated control  $\pm$  SEM.

HQ10 Concentration ( $\mu$ M)	Chemotherapeutics					
	Doxorubicin		Paclitaxel		Vinblastine	
	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means
<b>0.1</b>	79.56 $\pm$ 5.04 **	21.26 $\pm$ 6.93	64.83 $\pm$ 5.38 **	27.21 $\pm$ 9.72	61.12 $\pm$ 4.83 **	38.46 $\pm$ 12.18
<b>0.5</b>	80.88 $\pm$ 8.82 *	19.94 $\pm$ 8.38	103.90 $\pm$ 7.47	-11.84 $\pm$ 10.69	49.54 $\pm$ 4.02 ***	50.04 $\pm$ 12.06
<b>1</b>	80.76 $\pm$ 8.01 *	20.06 $\pm$ 8.13	87.54 $\pm$ 4.17	4.50 $\pm$ 9.02	60.41 $\pm$ 3.83 **	39.17 $\pm$ 12.03
<b>5</b>	76.29 $\pm$ 6.94 **	24.54 $\pm$ 7.59	86.73 $\pm$ 8.59	5.31 $\pm$ 10.34	48.48 $\pm$ 4.62 ***	51.10 $\pm$ 12.14
<b>10</b>	34.89 $\pm$ 4.50 ***	65.94 $\pm$ 6.55	28.54 $\pm$ 3.72 ***	63.50 $\pm$ 8.94	26.64 $\pm$ 5.28 ***	72.95 $\pm$ 11.67
<b>50</b>	35.63 $\pm$ 7.15 ***	65.19 $\pm$ 7.68	26.75 $\pm$ 4.95 ***	65.29 $\pm$ 9.63	25.92 $\pm$ 5.53 ***	73.66 $\pm$ 11.72
<b>100</b>	51.68 $\pm$ 8.89 ***	49.15 $\pm$ 8.61	30.17 $\pm$ 8.00 ***	61.87 $\pm$ 10.82	24.24 $\pm$ 6.40 ***	75.34 $\pm$ 12.48

\* p < 0.05

\*\* p < 0.01

\*\*\* p < 0.001

$\alpha$  MTT staining method used, 7 day incubation period, minimum n = 8

§ All chemotherapeutics used at IC<sub>20</sub> concentration (doxorubicin 0.26 nM, paclitaxel 14.35 nM, vinblastine 0.46 nM)

**Table 3.6:** Results of combination therapy with verapamil on COLO 320DM cells, reported as percentage untreated control  $\pm$  SEM.

Verapamil Concentration ( $\mu$ M)	Chemotherapeutics					
	Doxorubicin		Paclitaxel		Vinblastine	
	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means
<b>0.9</b>	94.37 $\pm$ 11.44	6.45 $\pm$ 9.80	69.76 $\pm$ 9.99 *	28.74 $\pm$ 12.81	59.67 $\pm$ 6.95 ***	47.89 $\pm$ 7.983
<b>1.8</b>	100.60 $\pm$ 11.65	0.22 $\pm$ 9.91	70.53 $\pm$ 7.52 *	27.96 $\pm$ 12.19	58.49 $\pm$ 8.70 ***	49.07 $\pm$ 9.042
<b>9</b>	79.08 $\pm$ 7.22 **	21.74 $\pm$ 7.70	87.11 $\pm$ 4.48	11.39 $\pm$ 11.65	69.57 $\pm$ 5.26 ***	37.99 $\pm$ 6.86
<b>18</b>	92.16 $\pm$ 4.50	8.66 $\pm$ 7.08	71.22 $\pm$ 6.70	27.28 $\pm$ 12.69	53.29 $\pm$ 7.33 ***	54.27 $\pm$ 8.13
<b>90</b>	73.71 $\pm$ 6.53 **	27.11 $\pm$ 7.44	75.92 $\pm$ 6.61	22.57 $\pm$ 12.68	50.64 $\pm$ 5.32 ***	56.92 $\pm$ 6.89
<b>180</b>	93.80 $\pm$ 7.28	7.02 $\pm$ 8.56	67.21 $\pm$ 5.39 *	31.28 $\pm$ 11.78	52.69 $\pm$ 7.63 ***	54.87 $\pm$ 8.32
<b>900</b>	77.84 $\pm$ 6.57 **	22.98 $\pm$ 7.95	49.09 $\pm$ 4.49 ***	49.41 $\pm$ 11.65	39.98 $\pm$ 5.71 ***	67.58 $\pm$ 7.15

\* p < 0.05

\*\* p < 0.01

\*\*\* p < 0.001

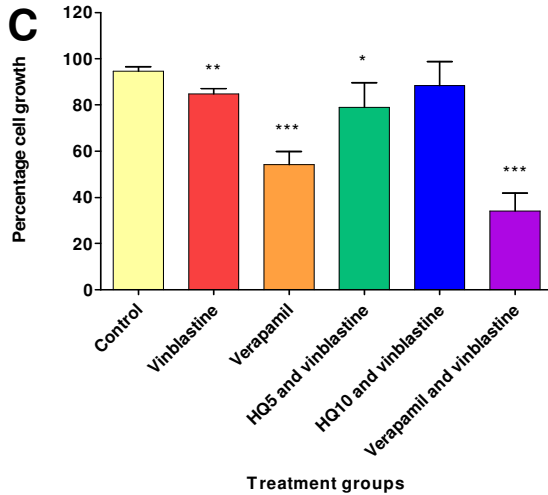
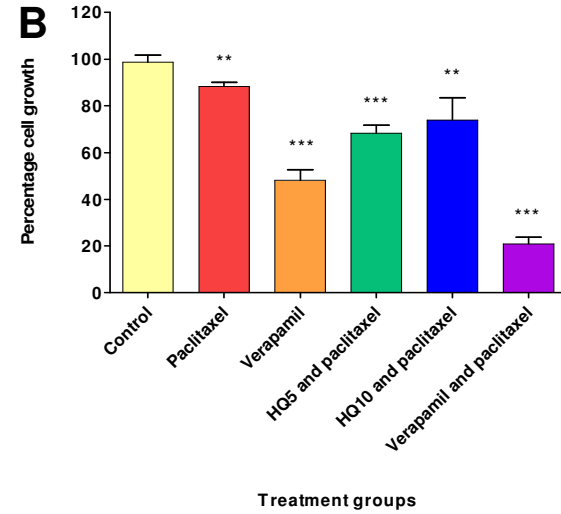
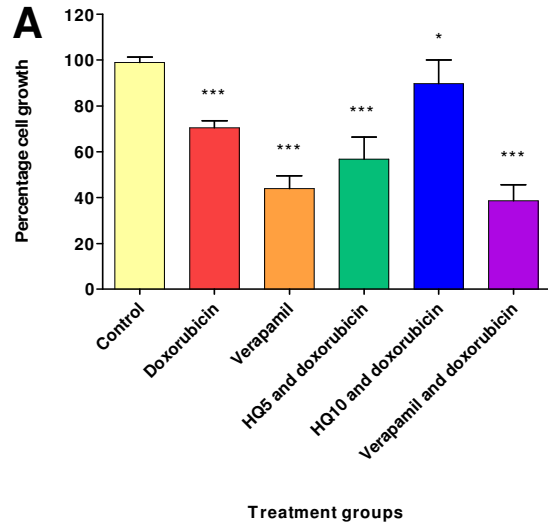
$\alpha$  MTT staining method used, 7 day incubation period, minimum n = 8

§ All chemotherapeutics used at IC<sub>20</sub> concentration (doxorubicin 0.26 nM, paclitaxel 14.35 nM, vinblastine 0.46 nM)

Results for combination therapy on HepG2 cells are shown in Figure 3.2 and Tables 3.7 - 3.9. All of the concentrations of HQ5 tested on HepG2 cells in combination with doxorubicin proved to inhibit cell growth significantly ( $p < 0.001$ ) except at 0.1  $\mu\text{M}$  HQ5. The greatest difference between the means was observed at 10  $\mu\text{M}$  of HQ5. The combination therapy with HQ5 and paclitaxel abrogated the growth of HepG2 cells significantly ( $p < 0.001$ ) at all concentrations studied. The largest difference between the means was observed at 50  $\mu\text{M}$  of HQ5. A significant decrease ( $p < 0.001$ ) in cell growth was only observed at in combination with vinblastine where concentrations exceeding 5  $\mu\text{M}$  of HQ5 were used. The greatest difference between the means was found to be at 10  $\mu\text{M}$  of HQ5 in combination with vinblastine.

Exposure to a combination of HQ10 and doxorubicin decreased the cell growth of HepG2 cells to less than 52% at concentrations of HQ10 exceeding 35  $\mu\text{M}$ . The largest difference between the means was observed for 70  $\mu\text{M}$  of HQ10. A significant decrease in cell growth was seen at all concentrations of the combination of paclitaxel and HQ10. The greatest difference between the means was found to be at 350  $\mu\text{M}$  of HQ10. A combination of vinblastine and 35  $\mu\text{M}$  of HQ10 abrogated 50% of cell growth. The greatest difference between the means was observed at 70  $\mu\text{M}$  of HQ10 in combination with vinblastine.

All concentrations of verapamil in combination with doxorubicin or paclitaxel on HepG2 cells inhibited cell growth significantly, except where 6  $\mu\text{M}$  of paclitaxel was used. The difference between the means was greatest where 1200  $\mu\text{M}$  of verapamil was used in combination with doxorubicin or paclitaxel. A significant reduction in cell growth was seen at concentrations exceeding 12  $\mu\text{M}$  of verapamil in combination with vinblastine. The greatest difference between the means was observed where 600  $\mu\text{M}$  of verapamil was use in combination with vinblastine.



**Figure 3.2:** The effect of exposure to HQ5 or HQ10 and (A) doxorubicin, (B) paclitaxel or (C) vinblastine on HepG2 cells. The experimental hydroxyquinolines and verapamil were used at the IC<sub>50</sub> concentration. Doxorubicin, paclitaxel and vinblastine were used at the IC<sub>20</sub> concentration. The graphs show up to 120% cell growth.

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

**Table 3.7:** Results of combination therapy with HQ5 on HepG2 cells, reported as percentage untreated control  $\pm$  SEM.

HQ5 Concentration ( $\mu$ M)	Chemotherapeutics					
	Doxorubicin		Paclitaxel		Vinblastine	
	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means
<b>0.05</b>	72.41 $\pm$ 2.47 ***	27.64 $\pm$ 4.21	74.63 $\pm$ 6.54 ***	24.11 $\pm$ 5.42	87.98 $\pm$ 7.56 *	9.75 $\pm$ 6.40
<b>0.1</b>	75.19 $\pm$ 10.38 ***	24.86 $\pm$ 6.64	72.93 $\pm$ 4.48 ***	25.81 $\pm$ 4.47	105.30 $\pm$ 15.74	-7.57 $\pm$ 9.47
<b>0.5</b>	55.57 $\pm$ 8.22 ***	44.49 $\pm$ 6.09	75.47 $\pm$ 7.73 ***	23.27 $\pm$ 6.06	105.70 $\pm$ 18.79	-7.97 $\pm$ 11.73
<b>1</b>	56.69 $\pm$ 9.70 ***	43.37 $\pm$ 6.79	68.28 $\pm$ 3.40 ***	30.46 $\pm$ 4.10	88.88 $\pm$ 13.69 *	8.85 $\pm$ 9.14
<b>5</b>	62.39 $\pm$ 6.66 ***	37.67 $\pm$ 5.40	40.17 $\pm$ 3.14 ***	58.57 $\pm$ 4.02	44.02 $\pm$ 7.73 ***	53.71 $\pm$ 6.50
<b>10</b>	48.00 $\pm$ 6.49 ***	52.06 $\pm$ 5.33	44.30 $\pm$ 3.98 ***	54.44 $\pm$ 4.22	30.13 $\pm$ 2.52 ***	67.60 $\pm$ 4.94
<b>50</b>	59.96 $\pm$ 5.30 ***	40.10 $\pm$ 5.04	34.22 $\pm$ 2.71 ***	64.53 $\pm$ 3.90	35.45 $\pm$ 6.81 ***	62.28 $\pm$ 6.20

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

$\alpha$  MTT staining method used, 7 day incubation period, minimum  $n = 8$

$\S$  All chemotherapeutics used at  $IC_{20}$  concentration (doxorubicin 26 nM, paclitaxel 21 nM, vinblastine 5 nM)

**Table 3.8:** Results of combination therapy with HQ10 on HepG2 cells, reported as untreated negative control  $\pm$  SEM.

HQ10 Concentration ( $\mu$ M)	Chemotherapeutics					
	Doxorubicin		Paclitaxel		Vinblastine	
	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means
<b>0.35</b>	84.04 $\pm$ 5.69 **	16.01 $\pm$ 5.01	80.00 $\pm$ 2.82 ***	18.74 $\pm$ 3.93	99.37 $\pm$ 14.81	-4.67 $\pm$ 9.37
<b>0.7</b>	94.34 $\pm$ 12.03	5.72 $\pm$ 7.96	84.18 $\pm$ 0.65 **	14.56 $\pm$ 4.50	104.10 $\pm$ 7.74	-9.42 $\pm$ 5.60
<b>3.5</b>	90.48 $\pm$ 9.82	9.58 $\pm$ 6.85	72.22 $\pm$ 9.03 ***	26.52 $\pm$ 6.79	110.70 $\pm$ 7.75 **	-16.03 $\pm$ 5.60
<b>7</b>	86.45 $\pm$ 7.64 *	13.60 $\pm$ 5.77	73.97 $\pm$ 9.46 **	24.77 $\pm$ 7.04	88.48 $\pm$ 10.36	6.21 $\pm$ 6.69
<b>35</b>	51.28 $\pm$ 8.88 ***	48.77 $\pm$ 6.28	54.39 $\pm$ 5.96 ***	44.35 $\pm$ 5.13	25.37 $\pm$ 3.46 ***	69.32 $\pm$ 3.88
<b>70</b>	40.66 $\pm$ 5.23 ***	59.39 $\pm$ 4.91	48.74 $\pm$ 3.48 ***	50.00 $\pm$ 4.03	14.45 $\pm$ 2.92 ***	80.24 $\pm$ 3.74
<b>350</b>	41.68 $\pm$ 7.28 ***	58.37 $\pm$ 5.67	6.50 $\pm$ 0.57 ***	92.25 $\pm$ 3.345	15.97 $\pm$ 1.98 ***	78.72 $\pm$ 3.39

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

$\alpha$  MTT staining method used, 7 day incubation period, minimum  $n = 8$

§ All chemotherapeutics used at  $IC_{20}$  concentration (doxorubicin 26 nM, paclitaxel 21 nM, vinblastine 5 nM)

**Table 3.9:** Results of combination therapy with verapamil on HepG2 cells, reported as percentage untreated control  $\pm$  SEM.

Verapamil Concentration ( $\mu$ M)	Chemotherapeutics					
	Doxorubicin		Paclitaxel		Vinblastine	
	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means
<b>1.2</b>	83.73 $\pm$ 8.46 *	15.20 $\pm$ 6.37	79.53 $\pm$ 7.94 **	19.22 $\pm$ 6.18	94.60 $\pm$ 11.71	0.09 $\pm$ 7.67
<b>6</b>	76.29 $\pm$ 6.53 ***	22.64 $\pm$ 5.58	84.78 $\pm$ 5.55 **	13.97 $\pm$ 4.93	83.11 $\pm$ 9.57	11.59 $\pm$ 6.53
<b>12</b>	79.97 $\pm$ 6.83 **	18.97 $\pm$ 5.72	84.18 $\pm$ 5.55 **	13.97 $\pm$ 4.93	75.18 $\pm$ 10.52 **	19.51 $\pm$ 6.77
<b>60</b>	38.64 $\pm$ 7.02 ***	60.29 $\pm$ 5.79	20.86 $\pm$ 2.89 ***	77.88 $\pm$ 3.95	33.99 $\pm$ 7.88 ***	60.70 $\pm$ 5.67
<b>120</b>	24.62 $\pm$ 3.58 ***	74.31 $\pm$ 4.81	24.23 $\pm$ 3.46 ***	74.52 $\pm$ 4.12	23.39 $\pm$ 7.17 ***	71.30 $\pm$ 5.23
<b>600</b>	26.84 $\pm$ 7.48 ***	72.09 $\pm$ 5.96	14.67 $\pm$ 2.50 ***	84.08 $\pm$ 3.71	12.01 $\pm$ 2.47 ***	82.68 $\pm$ 3.64
<b>1200</b>	21.43 $\pm$ 7.34 ***	77.57 $\pm$ 5.90	9.79 $\pm$ 2.90 ***	88.95 $\pm$ 3.83	15.99 $\pm$ 3.21 ***	78.70 $\pm$ 3.82

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

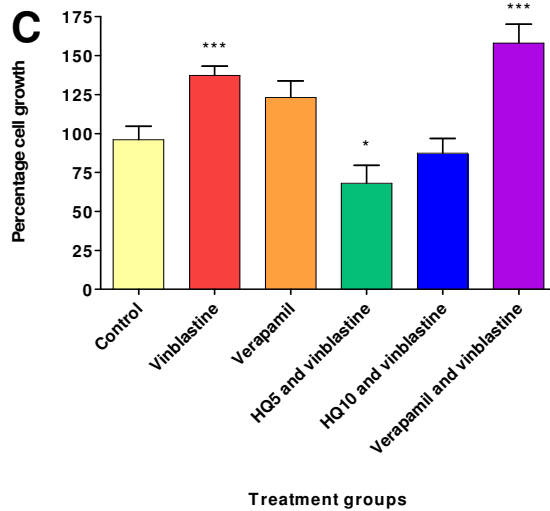
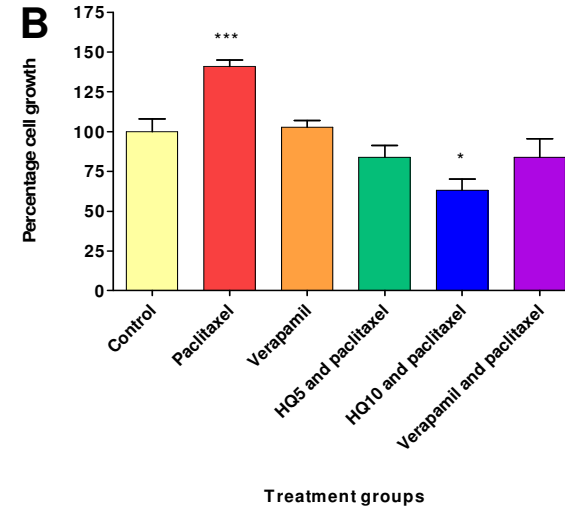
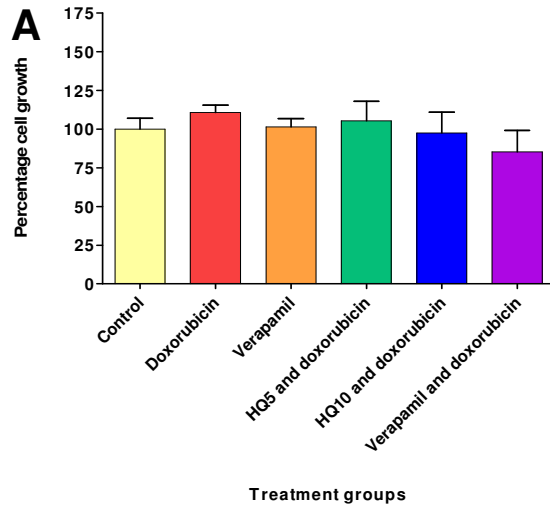
$\alpha$  MTT staining method used, 7 day incubation period, minimum  $n = 8$

$\S$  All chemotherapeutics used at  $IC_{20}$  concentration (doxorubicin 26 nM, paclitaxel 21 nM, vinblastine 5 nM)

Results for combination therapy on SH-SY5Y cells are shown in Figure 3.3 and Tables 3.10 - 3.12. A significant decrease in cell growth was observed where SH-SY5Y cells were exposed to concentrations of HQ5 exceeding 10  $\mu\text{M}$ . An increase in cell growth was observed where concentrations below 5  $\mu\text{M}$  of HQ5 were used in combination with paclitaxel. Cell growth was inhibited by 50% or less at concentrations exceeding 10  $\mu\text{M}$  of HQ5. Where vinblastine was used in combination with HQ5 a significant decrease in cell growth was observed from 5  $\mu\text{M}$  of HQ5. The greatest difference between the means was observed where 100  $\mu\text{M}$  of HQ5 was used in combination with doxorubicin, paclitaxel or vinblastine.

A significant increase in cell growth of SH-SY5Y cells was found where HQ10 was used at concentrations below 9  $\mu\text{M}$  in combination with doxorubicin, paclitaxel or vinblastine. At concentrations exceeding 18  $\mu\text{M}$  of HQ10 in combination with doxorubicin, cell growth was inhibited significantly ( $p < 0.001$ ). The largest difference between the means was observed where 90  $\mu\text{M}$  of HQ10 was used. Where SH-SY5Y cells were treated with HQ10 and paclitaxel, a significant decrease in cell growth was observed from 9  $\mu\text{M}$  of HQ10 and the greatest difference between the means was found to be where 180  $\mu\text{M}$  of HQ10 was used. Cell growth was abrogated by 50% or more only at 180  $\mu\text{M}$  of HQ10 in combination with vinblastine, which is also the combination with the greatest difference between the means.

The combinations of verapamil with doxorubicin, paclitaxel or vinblastine achieved abrogation of cell growth exceeding 50% at concentrations above the  $\text{IC}_{50}$  concentration for verapamil. The largest difference between the means was observed at 500  $\mu\text{M}$  verapamil in combination with doxorubicin, and 100  $\mu\text{M}$  verapamil in combination with paclitaxel. A significant increase in cell growth was observed where concentrations below and equal to the  $\text{IC}_{50}$  of verapamil were used in combination with vinblastine ( $p < 0.001$ ). The greatest difference between the means was found to be where 100  $\mu\text{M}$  of verapamil was used in combination with vinblastine.



**Figure 3.3:** The effect of exposure to HQ5 or HQ10 and (A) doxorubicin, (B) paclitaxel or (C) vinblastine on SH-SY5Y cells. The experimental hydroxyquinolines and verapamil were used at the IC<sub>50</sub> concentration. Doxorubicin, paclitaxel and vinblastine were used at the IC<sub>20</sub> concentration. The graphs show up to 175% cell growth.

\* p < 0.05  
\*\* p < 0.01  
\*\*\* p < 0.001

**Table 3.10:** Results of combination therapy with HQ5 on SH-SY5Y cells, reported as percentage untreated control  $\pm$  SEM.

HQ5 Concentration ( $\mu$ M)	Chemotherapeutics					
	Doxorubicin		Paclitaxel		Vinblastine	
	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means
<b>0.1</b>	109.50 $\pm$ 6.95	-9.525 $\pm$ 16.46	134.90 $\pm$ 9.85 *	-34.90 $\pm$ 15.73	109.60 $\pm$ 3.54	-13.53 $\pm$ 15.08
<b>0.5</b>	109.20 $\pm$ 16.23	-9.16 $\pm$ 16.23	131.30 $\pm$ 17.67	-31.27 $\pm$ 17.14	101.1 $\pm$ 7.90	-5.07 $\pm$ 16.41
<b>1</b>	109.10 $\pm$ 10.25	-9.10 $\pm$ 14.92	111.90 $\pm$ 7.60	-11.92 $\pm$ 15.38	100.90 $\pm$ 8.70	-4.88 $\pm$ 15.75
<b>5</b>	105.30 $\pm$ 12.83	-5.35 $\pm$ 14.32	101.2 $\pm$ 18.47	-1.18 $\pm$ 17.74	53.50 $\pm$ 9.15 *	42.56 $\pm$ 18.81
<b>10</b>	52.89 $\pm$ 10.71 **	47.11 $\pm$ 13.76	38.94 $\pm$ 10.02 ***	61.06 $\pm$ 15.76	47.12 $\pm$ 5.83 **	48.93 $\pm$ 15.31
<b>50</b>	47.57 $\pm$ 8.012 ***	52.43 $\pm$ 13.17	35.61 $\pm$ 5.79 ***	64.39 $\pm$ 15.16	56.33 $\pm$ 9.67 *	39.73 $\pm$ 15.93
<b>100</b>	34.32 $\pm$ 5.77 ***	65.68 $\pm$ 12.80	24.81 $\pm$ 2.68 ***	75.19 $\pm$ 14.92	43.73 $\pm$ 7.95 **	52.33 $\pm$ 15.62

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

$\alpha$  MTT staining method used, 7 day incubation period, minimum  $n = 8$

§ All chemotherapeutics used at  $IC_{20}$  concentration (doxorubicin 6 nM, paclitaxel 2 nM, vinblastine 0.3  $\mu$ M)

**Table 3.11:** Results of combination therapy with HQ10 on SH-SY5Y cells, reported as percentage untreated control  $\pm$  SEM.

HQ10 Concentration ( $\mu$ M)	Chemotherapeutics					
	Doxorubicin		Paclitaxel		Vinblastine	
	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means
<b>0.18</b>	126.10 $\pm$ 4.40	-26.12 $\pm$ 13.34	146.7 $\pm$ 11.08 **	-46.67 $\pm$ 15.31	163.70 $\pm$ 8.62 ***	-67.68 $\pm$ 15.73
<b>0.9</b>	140.30 $\pm$ 6.92 **	-40.31 $\pm$ 13.64	168.70 $\pm$ 14.26 ***	-68.65 $\pm$ 16.12	190.20 $\pm$ 12.03 ***	-94.15 $\pm$ 16.45
<b>1.8</b>	154.30 $\pm$ 12.24 ***	-54.28 $\pm$ 14.15	161.00 $\pm$ 14.20 ***	-61.00 $\pm$ 16.10	193.80 $\pm$ 16.71 ***	-97.74 $\pm$ 17.73
<b>9</b>	97.41 $\pm$ 13.48	2.59 $\pm$ 14.92	70.73 $\pm$ 9.76 *	29.27 $\pm$ 15.03	95.48 $\pm$ 11.67	0.58 $\pm$ 16.36
<b>18</b>	43.03 $\pm$ 9.54 ***	56.97 $\pm$ 13.48	49.28 $\pm$ 6.43 **	50.72 $\pm$ 14.45	75.86 $\pm$ 13.65	20.19 $\pm$ 16.85
<b>90</b>	42.76 $\pm$ 7.48 ***	57.24 $\pm$ 13.07	43.85 $\pm$ 5.69 ***	56.15 $\pm$ 14.35	51.29 $\pm$ 7.85 **	44.77 $\pm$ 15.60
<b>180</b>	64.25 $\pm$ 9.15 *	35.75 $\pm$ 13.40	41.27 $\pm$ 4.11 ***	58.73 $\pm$ 14.18	39.93 $\pm$ 4.76 ***	56.13 $\pm$ 15.19

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

$\alpha$  MTT staining method used, 7 day incubation period, minimum  $n = 8$

§ All chemotherapeutics used at  $IC_{20}$  concentration (doxorubicin 6 nM, paclitaxel 2 nM, vinblastine 0.3 pM)

**Table 3.12:** Results of combination therapy with verapamil on SH-SY5Y cells, reported as percentage untreated control  $\pm$  SEM.

Verapamil Concentration ( $\mu$ M)	Chemotherapeutics					
	Doxorubicin		Paclitaxel		Vinblastine	
	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means	Percentage of negative control	Difference between means
<b>1</b>	77.86 $\pm$ 8.07	22.14 $\pm$ 13.18	64.56 $\pm$ 10.83 *	35.44 $\pm$ 15.91	163.80 $\pm$ 12.66 ***	-67.72 $\pm$ 16.60
<b>5</b>	80.01 $\pm$ 14.15	19.99 $\pm$ 14.70	70.16 $\pm$ 18.21	29.84 $\pm$ 17.32	168.30 $\pm$ 15.75 ***	-72.19 $\pm$ 17.44
<b>10</b>	97.10 $\pm$ 16.93	2.89 $\pm$ 15.59	69.84 $\pm$ 11.16	30.16 $\pm$ 15.97	168.30 $\pm$ 11.09 ***	-72.25 $\pm$ 16.23
<b>50</b>	77.14 $\pm$ 12.85	22.86 $\pm$ 14.77	68.83 $\pm$ 8.35	31.17 $\pm$ 16.41	158.00 $\pm$ 12.27 ***	-61.94 $\pm$ 16.50
<b>100</b>	29.59 $\pm$ 6.22 ***	70.41 $\pm$ 13.54	35.36 $\pm$ 4.07 ***	64.64 $\pm$ 15.01	38.08 $\pm$ 4.07 **	57.97 $\pm$ 16.01
<b>500</b>	18.45 $\pm$ 2.57 ***	81.55 $\pm$ 13.21	42.62 $\pm$ 15.11 **	57.38 $\pm$ 16.36	49.76 $\pm$ 8.81 **	46.30 $\pm$ 16.55
<b>1000</b>	49.97 $\pm$ 4.71 **	50.03 $\pm$ 15.35	49.95 $\pm$ 7.00 **	50.05 $\pm$ 16.26	48.28 $\pm$ 10.78 *	47.77 $\pm$ 17.79

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

$\alpha$  MTT staining method used, 7 day incubation period, minimum  $n = 8$

$\S$  All chemotherapeutics used at  $IC_{20}$  concentration (doxorubicin 6 nM, paclitaxel 2 nM, vinblastine 0.3 pM)

### 3.6. Discussion

This study investigated the potential of the two experimental hydroxyquinolines, HQ5 and HQ10, as cytotoxic agents in combination with doxorubicin, paclitaxel and vinblastine on three P-glycoprotein (P-gp) expressing cell lines. Data indicate that both HQ5 and HQ10 abrogated the growth of P-gp expressing cell lines. The difference between the means of the untreated control and the different combinations of HQ5, HQ10 and verapamil with doxorubicin, paclitaxel and vinblastine was used as indicator of the degree of synergism achieved.

In the study of drug combinations it is necessary to define the different interactions of compounds. Enhancement or potentiation occurs when the combination of compounds results in an effect greater than that of the separate compounds. However, synergism is defined as an effect greater than potentiation. In contrast, when the combination of compounds displays less of an effect than the two compounds separately, the combination is antagonistic (Chou, 2006).

From the  $IC_{50}$  concentrations determined for verapamil on HepG2 and SH-SY5Y cells, it is apparent that these cell lines are not sensitive to verapamil. Verapamil is not an anti-neoplastic agent, but a calcium channel blocker that is also a potent P-gp inhibitor (Le Bot *et al.*, 1994). The concentrations of verapamil used in the combination experiments with these cell lines were relatively high. The doses of verapamil required to inhibit P-gp in a clinical setting limits the use of this compound, and gradually research into potential MDR modulators was focused on compounds designed specifically for this purpose.

Even though the experimental hydroxyquinolines were not designed specifically as inhibitors of P-gp, the effect of these compounds in combination with doxorubicin, paclitaxel and vinblastine was determined to ascertain if the test compounds could act in the same manner as quinoline derivatives that have been found to modulate drug resistance. Inhibition of P-gp by chloroquine has been reported (Savarino *et al.*, 2006). Sulphonamide derivatives of chloroquine and primaquine were reported to modulate MDR in breast cancer cells (Chibale *et al.*, 2001). Montelukast, a quinoline derivative used as anti-inflammatory agent, has been shown to modulate MDR through the

inhibition of MRP-2 (Roy *et al.*, 2009). From the structures of chemosensitising quinolines it has been postulated that a quinoline ring, a linker region, a piperazine ring and a hydrophobic group are necessary for MDR modulation (Kawase *et al.*, 2003).

Data indicate that on COLO 320DM cells the combinations of HQ5, HQ10 or verapamil the combinations with vinblastine resulted in the greatest decrease in cell growth. With HQ5, a dose dependent inhibition of cell growth was observed with doxorubicin, paclitaxel and vinblastine, although it was the combination of HQ5 with vinblastine that a minimum percentage of 65% cell growth was recorded at HQ5 concentrations below the  $IC_{50}$  concentration.

A dose response was observed with the combination of HQ10 and doxorubicin or vinblastine. For the combination of HQ10 and paclitaxel an increase in cell growth was observed which cannot be explained. No dose dependent inhibition of cell growth is evident in combinations of HQ10 with verapamil. The percentage cell growth recorded for the combination of verapamil and doxorubicin was extremely erratic and should be investigated further.

Vinblastine belongs to the vinca alkaloid family of anti-neoplastic agents. Compounds belonging to this class are known to destabilize microtubules resulting in the depolarization of the microtubules, and ultimately apoptosis (Perez, 2009). In terms of the cell cycle, vinblastine is thus expected to cause  $G_2/M$  arrest. As HQ5 and HQ10 in combination with vinblastine result in the enhancement of the cytotoxicity of vinblastine, cells are still allowed to enter the  $G_2/M$  phase where vinblastine exerts its effects. As the cells were exposed to the experimental compounds before the vinblastine was added, it is unlikely that the experimental compounds caused arrest at the S-phase as this would decrease the amount of cells in the  $G_2/M$  phase, and thus the efficacy of vinblastine.

The combination of HQ5 or HQ10 with vinblastine on HepG2 cells did not yield promising results: an increase in cell growth was reported where vinblastine was used in combination with HQ5 and no dose response was seen when used in combination with HQ10. The most effective combinations for HQ5, HQ10 and verapamil do not correlate: the combination of HQ5 and doxorubicin was most effective, no HQ10 combinations

showing inhibition of cell growth were found and the most effective combination with verapamil was with paclitaxel.

None of the drug combinations with HQ5 and HQ10 tested on SH-SY5Y cells displayed synergistic behaviour. An increase in cell growth was reported where HQ5 was used in combination with paclitaxel and for all of the combinations using HQ10 below its  $IC_{50}$  concentration. The combination of verapamil and vinblastine also showed an increase in cell growth at concentrations below the  $IC_{50}$  of verapamil. An inexplicable but repeatable increase in cell growth was reported for the highest concentration of verapamil used in combination with doxorubicin. The most effective combination for verapamil was with paclitaxel.

From the definition of synergism posed at the start of this section, no indication of any synergistic effects can be seen in the results obtained during this study. In general, potentiation rather than synergism, was observed. The only instance of antagonism observed was with the combination of HQ10 and doxorubicin on the SH-SY5Y cell line. The large differences in the concentrations tested did not allow for an exact determination of the concentration at which the potentiation or antagonism started. A logarithmic concentration scale was used in this study for all the compounds as it allows for the study of a wide range of concentrations.

The results obtained for the combination of HQ5, HQ10 and verapamil with doxorubicin, paclitaxel or vinblastine on three P-gp expressing cell lines were not always favourable and only a few combinations for each of the experimental hydroxyquinolines were deemed effective: HQ5 or HQ10 in combination with vinblastine on COLO 320DM cells, and HQ5 and doxorubicin on HepG2 cells. Whether the additive effect observed is caused by the inhibition of P-gp or a favourable combination of mechanisms of action cannot be speculated upon without further study.

## Chapter 4: Rhodamine 123 retention assay

### 4.1. Introduction

The plasma membrane surrounding cells consists of lipids and proteins arranged in a phospholipid bilayer (Cooper, 2000). The Singer-Nicolson fluid mosaic membrane model describes the cell membrane as a structure which allows lateral and rotational movement of proteins randomly distributed along the membrane (Vereb *et al.*, 2003). The proteins embedded in the plasma membrane perform the functions required such as transport of various molecules (Cooper, 2000). Some of these transporters are involved in drug resistance.

The ATP-binding cassette (ABC) is a large family of efflux proteins known to export a number of structurally unrelated compounds from the cell (Bachmeier *et al.*, 2005). As many chemotherapeutics count among the transported substrates of the ABC family, these transporters contribute to multidrug resistance in cancer cells (Meijerman *et al.*, 2008). The members of the ABC family comprise transmembrane domains and nucleotide binding domains (Wind *et al.*, 2011). Substrates are recognized by the transmembrane domain, which must undergo a conformational change in order to transport the substrates out of the cell. The nucleotide binding domain is located in the cytoplasm, and is the site for ATP-binding.

One member of the ABC family is of particular interest where multidrug resistance is concerned: P-glycoprotein (P-gp) that consists of twelve transmembrane domains, and two intracellular ATP-binding sites (Wind *et al.*, 2011). This exporter is responsible for the efflux of neutral and cationic hydrophobic compounds including certain classes of chemotherapeutic agents and partially positive dyes (Yeheeskely-Hayon *et al.*, 2009; Wind *et al.*, 2011). It has been reported that dye efflux studies are the most sensitive indicator of P-gp activity (Steiner *et al.*, 1998).

Rhodamine dyes are a family of cationic and relatively hydrophobic fluorophores (Yeheeskely-Hayon *et al.*, 2009). Rhodamine 123 has been demonstrated to be an efficient probe substrate used to demonstrate P-gp induction or inhibition. P-gp function

has been determined successfully using rhodamine 123 in a range of rodent and tissue culture models (Perloff *et al.*, 2003). Rhodamine 123 was therefore used as an indicator of changes in P-gp function of several cell lines, known to express P-gp, in the presence of the two hydroxyquinoline compounds selected for further investigation, namely HQ5 and HQ10.

The cell lines included in this study were chosen as they constitutively express P-gp. COLO 320DM cells (Jansen *et al.*, 1995) and SH-SY5Y cells (Sieczkowski *et al.*, 2009) are well-known to express relatively high amounts of P-gp. The HepG2 cell line is reported to express both P-gp and a second ABC transporter implicated in rapid xenobiotic efflux named MRP-2 (Su *et al.*, 2003).

## 4.2. Aim

To investigate the effect of both HQ5 and HQ10 on the P-gp activity of the COLO 320DM, SH-SY5Y and HepG2 cell lines.

## 4.3. Materials

### 4.3.1. Reagents

#### i. Bovine foetal calf serum (FCS)

Bovine foetal calf serum was procured from PAA (Pasching, Austria). The serum was heat-inactivated prior to use by heating it at 56°C for 45 minutes. A 1% solution of this inactivated serum was prepared in PBS before use.

#### ii. Dimethyl sulfoxide (DMSO)

DMSO was procured from Sigma-Aldrich (St Louis, USA) and used undiluted.

#### iii. Phosphate buffered saline (PBS)

FTA Hemagglutination buffer was obtained from BD Biosciences (Sparks, USA). A solution containing 9.23 g/l in de-ionised water was made and stored at 4°C until use. The solution was filter-sterilised through a 0.22 µm pore size filter and stored at 4°C if

sterile PBS was required. On the day of the experiment, 1% foetal calf serum was added, referred to as 1% PBS in the text.

#### **iv. Propidium iodide (PI)**

Propidium iodide was obtained from Sigma-Aldrich (St Louis, USA). A 3 mM stock solution was prepared in de-ionised water and stored in a glass vial covered in foil and stored at 4°C. The solution was used undiluted.

#### **v. Rhodamine 123**

Rhodamine 123 was procured from Sigma-Aldrich (St Louis, USA). A stock solution of 1mg/ml was prepared in DMSO and aliquots of 10 µl were stored at -70°C. Dilutions were made in cell culture medium prior to use.

#### **vi. Trypsin/Versene**

A trypsin/Versene solution containing 0.25% trypsin and 0.1% EDTA in Ca<sup>++</sup> and Mg<sup>++</sup> free phosphate buffered saline was obtained from Highveld Biological (Johannesburg, RSA). Trypsin/Versene was stored at 4°C.

#### **vii. Verapamil**

A 1 mg/ml stock solution of verapamil (Sigma-Aldrich St Louis, USA) was made in DMSO. Aliquots of 20 µl were made and stored at -70°C. Dilutions in 1% PBS were made to obtain the required concentrations immediately prior to use.

## **4.4. Methods**

### **4.4.1. Maintenance and counting of cell culture**

Briefly, cells were grown to approximately 80% confluence in cell culture medium containing 10% FCS. COLO 320DM cells were grown in RPMI, HepG2 cells in EMEM and SH-SY5Y cells in Ham's F12. As COLO 320DM cells are non-adherent cells, trypsin/vesene solution was not used to harvest these cells. The other cells were harvested using a trypsin/versene solution, washed with medium supplemented with FCS, counted with a haemocytometer and diluted to a concentration of 5 x 10<sup>4</sup> cells/ml.

#### 4.4.2. Experimental procedure

The method is based on the Chemicon<sup>®</sup> International Multidrug Resistance Direct Dye Efflux Assay instructions. HQ5 and HQ10 were used at concentrations of  $IC_{50}$ , two times  $IC_{50}$  and five times  $IC_{50}$ . Verapamil was used as positive control at a concentration of 250  $\mu$ M as it is a known P-gp inhibitor (Le Bot *et al.*, 1994).

After being harvested, cells were centrifuged at 200 *g* for 5 minutes and the pellet resuspended in 990  $\mu$ l 1% PBS. A volume of 50  $\mu$ l was removed from this solution and used for counting the cells. Thereafter 10  $\mu$ l of Rhodamine 123 solution was added to the cell suspension, gently mixed and the suspension incubated for 60 minutes at 4°C in the dark to load the cells with the rhodamine 123. After the incubation period the cell suspension was centrifuged at 200 *g* for 5 minutes and the pellet re-suspended in 1 ml 1% PBS. From this dye loaded cell suspension appropriate dilutions were made using 1% PBS into flow cytometry tubes so that each tube contained  $2.5 \times 10^4$  cells/500  $\mu$ l. Each tube was then treated with 500  $\mu$ l of one of the experimental compounds or verapamil diluted to the appropriate concentration in 1% PBS. An untreated control tube received only 500  $\mu$ l of cell suspension and 500  $\mu$ l 1% PBS. The tubes were incubated at 37°C in the dark for 1 hour. After the incubation period the cells were stained with PI in order to ensure that the rhodamine 123 content of only viable cells was recorded. The samples were analysed with a Beckman Coulter FC 500 Series flow cytometer. The channels used were FL1 (525 nm) and FL3 (620 nm). A total of 10 000 events were counted and gated to include only the PI (FL3) negative cells.

#### 4.4.3. Interpretation of results

Four independent experiments were performed for each cell line. The results obtained were in the form of histograms, displaying counts (number of cells) against fluorescent intensity. The decrease in the intensity of the rhodamine 123 fluorescence of the cells indicated active P-gp efflux of the tracer dye from the cells. The results were standardised against the untreated control. The standard error of the mean was also calculated. Data was analysed to determine whether results obtained for the experimental compounds differed significantly from those obtained for the controls using the Student's t-test. A p-value of less than 0.05 was used as indicator of significance. GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) was used for all statistical calculations.

## 4.5. Results

The Rhodamine 123 retention assay was used to investigate the influence of HQ5 and HQ10 on the function of the xenobiotic efflux transporter P-gp. For comparative purposes, a known inhibitor of P-gp was included as the positive control. Calculated results are summarised in Table 4.1 and represented graphically in Figure 4.1. The values in Table 4.1 indicate the percentage fluorescent intensity recorded where the results for the experimental groups were standardised against the untreated control.

For COLO 320DM cells, the positive P-gp inhibited control, treated with verapamil ( $855.2 \pm 12.3\%$ ), differed significantly from the untreated control ( $100.0 \pm 25.8\%$ ). The fluorescent intensity appeared to be decreased after exposure to either HQ5 or HQ10 in a dose dependent manner.

For HepG2 cells the fluorescence due to verapamil exposure was drastically higher than that of the untreated control ( $396.0 \pm 160.9\%$  and  $100.0 \pm 29.5\%$ , respectively). All three concentrations of HQ5 tested caused only a slight decrease in fluorescence with a significant decrease recorded for HQ5 at  $5 \times IC_{50}$  ( $22.1 \pm 1.9\%$ ). The highest concentration of HQ10 used significantly reduced the fluorescent intensity to  $6.0 \pm 2.3\%$ .

As with the other cell lines, the fluorescence recorded for the verapamil treated positive control in SH-SY5Y cells was significantly higher than that of the untreated control ( $1151.1 \pm 25.8\%$  and  $100.0 \pm 5.3\%$ , respectively). The fluorescent intensities obtained after treatment with the three concentrations of HQ5 studied were very comparable, with values ranging from 46% to 57%.

The fluorescent intensities obtained for HQ10 were much higher. Exposure to  $5 \times IC_{50}$  of HQ10 was resulted in a 38% increase in fluorescent intensity in comparison to the untreated control.

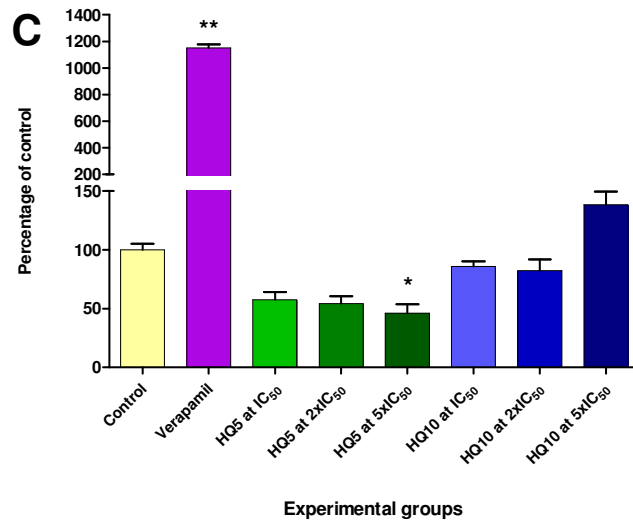
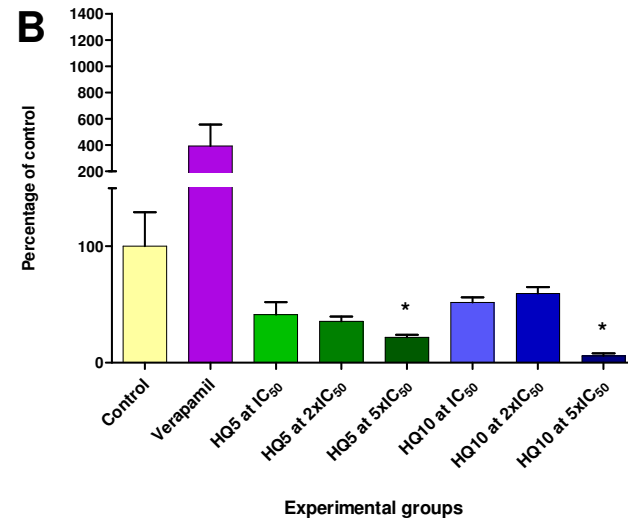
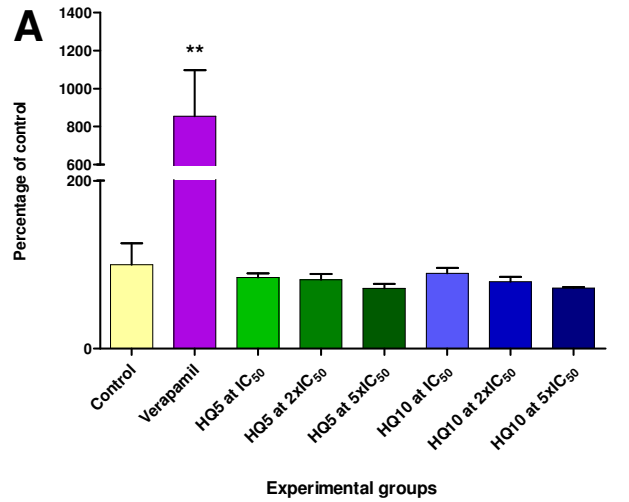
**Table 4.1:** Rhodamine 123 uptake results on COLO 320DM, HepG2 and SH-SY5Y cells. Results are reported as mean percentage of fluorescent intensity of untreated control  $\pm$  SEM.

	<b>COLO 320DM</b>	<b>HepG2</b>	<b>SH-SY5Y</b>
<b>Untreated Control</b>	100.0 $\pm$ 25.8	100.0 $\pm$ 29.5	100.0 $\pm$ 5.3
<b>Verapamil</b>	855.2 $\pm$ 12.3 **	396.0 $\pm$ 160.9 **	1151.1 $\pm$ 25.8 **
<b>HQ5 at IC<sub>50</sub></b>	84.75 $\pm$ 5.20	41.4 $\pm$ 10.9	57.6 $\pm$ 6.3
<b>HQ5 at 2 x IC<sub>50</sub></b>	82.2 $\pm$ 6.8	35.5 $\pm$ 4.3	54.2 $\pm$ 6.3
<b>HQ5 at 5 x IC<sub>50</sub></b>	72.0 $\pm$ 4.9	22.1 $\pm$ 1.9	46.1 $\pm$ 7.7 *
<b>HQ10 at IC<sub>50</sub></b>	89.8 $\pm$ 6.6	51.9 $\pm$ 4.2	85.9 $\pm$ 4.4
<b>HQ10 at 2 x IC<sub>50</sub></b>	80.1 $\pm$ 5.5	59.3 $\pm$ 5.7	82.6 $\pm$ 9.5
<b>HQ10 at 5 x IC<sub>50</sub></b>	72.2 $\pm$ 1.3	6.0 $\pm$ 2.3	138.4 $\pm$ 11.2

$\alpha$  Results for a minimum of four repeats

\* p < 0.05

\*\* p < 0.01



**Figure 4.1:** A graphic representation of the two experimental hydroxyquinolines on P-gp function of (A) COLO 320DM cells, (B) HepG2 cells and (C) SH-SY5Y cells. The graphs show up to 1400% of control.

\*  $p < 0.05$   
\*\*  $p < 0.01$

## 4.6. Discussion

The ability of HQ5 and HQ10 to inhibit the function of P-gp was investigated in COLO 320DM, HepG2 and SH-SY5Y cells as these cells are all known to express elevated levels of P-gp. The well-known P-gp inhibitor, verapamil, was used as a positive control for each cell line as the extent of P-gp expression is different for each of these cell lines. Verapamil caused a very significant increase in the fluorescence due to rhodamine 123 in all three cell lines confirming that rhodamine 123 does accumulate in the cells during the loading phase as well as the presence of the P-gp transporter and gave some scale to the extent of efflux activity for each cell line. From the significant difference in rhodamine 123 fluorescence caused by exposure to verapamil, it can be concluded that the assay was successful in measuring P-gp function.

Data collected for the cell lines after treatment with the experimental hydroxyquinolines showed no increase in intracellular fluorescence implying that there was little rhodamine 123 remaining in the cells, and thus the compounds had no inhibitory effect on P-gp function. No dose-dependent increase in rhodamine 123 fluorescence was observed for the three different concentrations of each compound tested. There was however a trend showing increased efflux of the rhodamine 123 which would imply an enhanced activity for P-gp.

Data indicate that a dose dependent decrease in rhodamine 123 concentration was observed for most of the cell lines for both of the experimental compounds. This is the opposite of what is expected to happen when a P-gp inhibitor is used: a P-gp inhibitor will cause a decrease in P-gp function and thus a higher concentration of rhodamine 123 should be found in the cell. The effect of HQ5 and HQ10 on the function of P-gp should be studied in greater depth before this anomaly can be explained. However, when SH-SY5Y cells were treated with  $5 \times IC_{50}$  of HQ10 an increase in the rhodamine 123 retention was observed. It may be that the function of P-gp was only influenced at the highest concentration of HQ10 used and that a concentration of HQ10 exceeding  $5 \times IC_{50}$  will have an even greater effect on P-gp function. At such a high concentration of HQ10 the likelihood of adverse effects including toxicity should be considered.

Reports in literature indicate that some quinoline derivatives have been proven to inhibit P-gp. It has been shown that a propylquinoline derivative inhibits P-gp in Caco-2 cells and rat everted gut sacs (Belliard *et al.*, 2003). Reports indicate that a novel quinoline derivative, MS-209, inhibited P-gp activity in K562/ADM leukaemia cells (Naito *et al.*, 2002). Vezmar *et al.* (2000) reported that a range of quinoline derivatives inhibited P-gp in HL60/AR and H69/AR cells.

Inhibition of P-gp is not the only parameter influencing rhodamine 123 retention. A number of anti-neoplastic agents have been shown to decrease the expulsion of rhodamine 123 from P-gp expressing primary bovine brain micro-vessel endothelial cells (Bachmeier *et al.*, 2005). Exposure to paclitaxel caused an increase in the intracellular concentration of rhodamine 123 comparable to that of the positive control, cyclosporine A. Doxorubicin was also observed to increase the intracellular concentration of rhodamine 123. These two anticancer agents are reported to be P-gp substrates (Le Bot *et al.*, 1994; Chibale *et al.*, 2001), so it can be speculated that these compounds compete with rhodamine 123 for the P-gp binding site and therefore slow the efflux through competition rather than inhibition. As no increase in rhodamine 123 retention was observed in the cell lines exposed to the two hydroxyquinoline experimental compounds, competitive inhibition would not explain the results observed in this study.

For compounds to have an effect on P-gp, they must be absorbed into the cell. The absorption of a compound into a cell is influenced, amongst other characteristics, by lipophilicity (Bachmeier *et al.*, 2005). Generally P-gp substrates tend to be lipophilic compounds as such compounds gain entry into the cell easily. A lack of effect on the intracellular rhodamine 123 concentrations may be explained by inadequate cell penetration by both the test compounds. The experimental compounds are however relatively hydrophobic, and thus can be assumed to have been absorbed into the cell. Other assays have shown selective cytotoxicity and effects on the cell cycle that imply that the compounds are in fact entering into the cell cytosol. However, whether penetration into the cell was sufficient to have an effect on P-gp is debatable. Also, as compounds which interact with the ABC cassette transporters are mostly hydrophobic (Bachmeier *et al.*, 2005), it may be that the experimental compounds are better substrates for the P-gp transporter than the chemotherapeutic drugs. Substrates may induce an up-regulation in the expression of P-gp, which would result in added

rhodamine 123 efflux once the hydroxyquinoline has been depleted in the cytosol. This is speculation as to the reason for the lack of the inhibitory response observed.

An up-regulation in P-gp expression has been reported due to a variety of stresses on a cancer cell: an increase in reactive oxygen species (ROS), glucose deprivation and hypoxia among others. Treatment with a number of chemotherapeutic drugs resulted in an increase in ROS, which in turn elevates the expression of P-gp (Callaghan *et al.*, 2008). Harmsen *et al.* (2010) reported that several anticancer drugs induced P-gp expression in the LS180 cell line.

Unlike COLO 320DM and SH-SY5Y cells, HepG2 cells express another ATP-dependent efflux pump, MRP-2, in addition to P-gp, (Gazzaniga *et al.*, 2008). Substrates of MRP-2 include cisplatin, methotrexate and the vinca alkaloids. Studies have shown a poor correlation between MRP activity and rhodamine 123 efflux (Steiner *et al.*, 1998; Perloff *et al.*, 2003) and thus MRP-2 should not have had a major influence on the rhodamine 123 efflux results, despite the fact that the HepG2 cells showed the most efficient efflux of the rhodamine 123.

The inability of the experimental hydroxyquinolines to inhibit P-gp function sufficiently to increase intracellular rhodamine 123 retention is unexpected as many other quinoline derivatives have been shown to effectively inhibit P-gp. The only exception was with the SH-SY5Y cells exposed to 5 x IC<sub>50</sub> of HQ10 and this should be investigated further but it should be noted that the increase is marginal compared to the result seen with verapamil which is a proven P-gp inhibitor. The additive effects observed in the combination therapy of HQ5 or HQ10 and vinblastine on COLO 320DM cells and HQ5 doxorubicin on HepG2 cells were thus not due to P-gp inhibition.

## Chapter 5: Apoptosis/necrosis

### 5.1. Introduction

Homeostasis in living tissue is maintained by careful regulation of cell proliferation and cell death (Raff, 1992). Cell death, whilst it can be induced by harmful external stimuli, also occurs at the end of a differentiated cell's lifespan (Darzynkiewicz *et al.*, 1992).

Apoptosis has been aptly nicknamed 'cell suicide' (Majno *et al.*, 1995). During this process the cell undergoes morphological and molecular changes which are closely regulated by caspases (Cohen, 1997) or the release of cytochrome c from the mitochondria (Sundquist *et al.*, 2006). The first stages of apoptosis are characterized by the shrinking of the cytoplasm and condensation of the DNA (Kerr *et al.*, 1994). Phosphatidyl serine, a phospholipid which is expressed on the cell surface only during apoptosis (Fadok *et al.*, 1992) acts as a signal to macrophages to eliminate the apoptotic cell (Aubry *et al.*, 1999) before inflammation and tissue damage occur. As the chromatin condensation progresses, the nucleus disintegrates, membrane blebbing occurs and apoptotic bodies enclosed by the cell membrane form.

In stark contrast to the organized process of apoptosis is the non-specific process of necrosis. During the first stage of necrosis, the integrity of the cell membrane is lost and the structure of the membrane compromised. Mitochondrial swelling also occurs. No change in the DNA of a cell undergoing necrosis has been reported (Darzynkiewicz *et al.*, 1992). Whereas apoptosis affects specific single cells, necrosis is mostly seen in adjoining cells. This may be due to the fact that necrosis mostly occurs in response to extreme damage to the cell (Kerr *et al.*, 1994).

It has been speculated that spontaneous decrease in tumour mass which has been reported in kinetic studies are due to apoptosis. Although the cause of this phenomena is complex, tumour necrosis factor  $\alpha$  released by macrophages in the vicinity of the tumour may play a role (Kerr *et al.*, 1994). Cytotoxic T-lymphocytes may also be responsible as an immunological reaction might be initiated. Tumour cell deletion can be

further encouraged through chemotherapeutic treatment. Apoptosis is the preferred cell elimination pathway as the inflammation which normally accompanies necrosis is avoided. The correlation between a chemotherapeutic agent's efficacy and its ability to induce apoptosis has been well documented (Darzynkiewicz *et al.*, 1992).

Annexin V-FITC binds specifically to phosphatidyl serine (Willingham, 1999; Aubry *et al.*, 1999) and was thus used to stain apoptotic cells.

Due to the large molecular weight of propidium iodide, this fluorochrome cannot cross an intact cell membrane (Willingham, 1999), and will thus initially only stain the DNA of cells undergoing necrosis. However, as apoptotic cells disintegrate into apoptotic bodies, the dye will indicate the later stage of this process. Propidium iodide was thus used to indicate necrotic and late apoptotic cells.

From the *in vitro* data obtained with the MTT assay, it is clear that both HQ5 and HQ10 are efficacious against MCF-7 cells. As the efficacy of most anti-neoplastic agents is linked to their ability to induce apoptosis, the ability of the two experimental compounds to cause apoptosis was investigated.

## **5.2. Aim**

To determine the whether HQ5 and HQ10 at various concentrations induces cell death in MCF-7 cells through apoptosis or necrosis.

## **5.3. Materials**

### **5.3.1. Reagents**

#### **i. Annexin V-FITC**

Annexin V-FITC was procured from BD Biosciences (Sparks, USA) and used undiluted. The solution was stored at 4 °C.

## **ii. Binding buffer**

Binding buffer was prepared by combining the following reagents at the specified concentrations:

10 mM HEPES buffer at pH 7.4

150 mM NaCl

5 mM KCl

1 mM MgCl<sub>2</sub>

1.8 mM CaCl<sub>2</sub>

The solution was prepared in de-ionised water. All reagents were obtained from Merck (Darmstadt, Germany) except the HEPES buffer which was procured from Sigma-Aldrich (St Louis, USA). The buffer was stored at 4 °C.

## **iii. Dulbecco's Modified Eagle's Medium**

DMEM was procured from Sigma-Aldrich (St Louis, USA). A mass of 67.35 g of medium powder was dissolved in 5 litres sterile, deionised water. To this solution 18.5 g of NaHCO<sub>3</sub> was added to adjust the pH. The solution was filter sterilized twice with 0.22 µm cellulose acetate filters, dispensed into sterile 500 ml bottles, supplemented with 1% penicillin/streptomycin and stored at 4 °C. Sterile heat inactivated FCS was added to a concentration of 10% prior to use.

## **iv. 99.9% Ethanol**

Ethanol was obtained from Illovo Sugar Limited (Durban, RSA).

## **v. Phosphate buffered saline (PBS)**

FTA Hemagglutination buffer was obtained from BD Biosciences (Sparks, USA). A solution containing 9.23 g/l in de-ionised water was made up and stored at 4 °C until use. On the day of the experiment, 1% foetal calf serum was added. As non-sterile PBS was required, no filter sterilization was necessary.

## **vi. Propidium iodide (PI)**

Propidium iodide was obtained from Sigma-Aldrich (St Louis, USA). A 3 mM stock solution was prepared in de-ionised water, covered in foil and stored at 4 °C. The solution was used undiluted.

## vii. Staurosporine

Staurosporine was procured from Sigma-Aldrich (St Louis, USA). A stock solution of 1 mg/ml or 2140  $\mu$ M was prepared in DMSO. Aliquots of 50  $\mu$ l was stored at -70°C and diluted in cell culture medium prior to use.

## 5.4. Methods

### 5.4.1. Maintenance and counting of cell culture

Briefly, MCF-7 cells were grown to approximately 80% confluence in DMEM with 10% FCS. The cells were harvested with a trypsin/Versene solution, counted with a haemocytometer and diluted to a concentration of  $2.5 \times 10^4$  cells/ml.

### 5.4.2. Experimental procedure

The experiments were set up in 25 cm<sup>2</sup> cell culture flasks with 4.5 ml of cell suspension and 0.5 ml of drug diluted in cell culture medium. Each flask received a single drug at a single concentration only. A control flask which received 4.5 ml of cell suspension and 0.5 ml of cell culture medium was used for comparative purposes. Staurosporine, a compound known to induce apoptosis in breast cancer cells (Mooney *et al.*, 2002), was used at a final concentration of 5  $\mu$ M as apoptosis control. Absolute ethanol (99.9%) was used as necrosis control. The two experimental compounds were investigated at concentrations of equal to the IC<sub>50</sub> value, double the IC<sub>50</sub> value and five times the IC<sub>50</sub> value. The compounds were diluted to the required concentrations in cell culture medium.

The cell suspension was decanted into the cell culture flasks and allowed to attach for 24 hours. Thereafter the experimental compounds were added to the similarly labelled flasks. The cells were exposed to 5  $\mu$ M staurosporine for 18 hours before analysis. The ethanol used as necrosis control was added after trypsinisation. After 120 and 168 hours exposure to the experimental compounds, the medium in the flasks were transferred to 15 ml centrifuge tubes and the flasks trypsinised. The trypsinised cells were added to the centrifuge tubes, and centrifuged for 5 minutes at 200 *g*.

The supernatant of the tube containing the cells for the necrosis control was discarded, and the pellet re-suspended in 200  $\mu$ l PBS. A volume of 800  $\mu$ l 99.9% ethanol was

added in a drop wise manner while the contents were gently mixed on a vortex mixer and the tube incubated at 4°C for 10 minutes. Thereafter the cells were washed with 5 ml PBS supplemented with 1% FCS and stained with annexin-V FITC and PI as described below.

For the remainder of the samples, the supernatant was discarded, 5 ml of PBS supplemented with 1% FCS added to each tube and the tubes re-centrifuged for 5 minutes at 200 *g*. The supernatant was again discarded and the pellet re-suspended in 950 µl binding buffer. A volume of 5 µl annexin V-FITC was added to each tube. The tubes were incubated for 15 minutes in the dark. Thereafter 2 µl PI was added to each tube and the contents of the tubes transferred to counting tubes.

The samples were analysed with a Beckman Coulter FC 500 Series flow cytometer. The channels used were FL1 (525 nm) and FL3 (620 nm). A total of 10 000 events were counted and quadrant analysis was performed using CXP software to obtain the percentage of cells in each quadrant depicting different extents of either apoptosis or necrosis.

### **5.4.3. Interpretation of results**

Three independent experiments were performed. The results obtained were in the form of scatter plots, displaying the percentage of cells in apoptosis or necrosis, or unaffected cells. The results are reported as mean percentage of cells in each quadrant  $\pm$  SEM. Statistical analysis was performed to determine whether results obtained for the experimental compounds differ significantly from those obtained for the controls. To this end, the one-way ANOVA and Dunnett's *post-hoc* test were performed. A p-value of less than 0.05 was used as indicator of significance. GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) was used for all statistical calculations.

## **5.5. Results**

This experiment investigated whether the two experimental compounds HQ5 and HQ10 induce apoptosis or necrosis in MCF-7 cells. For comparative purposes an inducer of

apoptosis and an inducer of necrosis were included in the experiment. Results are shown in Tables 5.1 and 5.2. A graphic representation of each tested time point is also shown (Figures 5.1 to 5.4).

For both time points the untreated control used in the experiments with HQ5 showed the distribution of cells expected in a growing population: the largest percentage of cells were alive ( $96.6 \pm 0.5\%$  after 5 days;  $91.2 \pm 1.9\%$  after 7 days). Significant differences were seen in the apoptosis control sample after 5 days where a smaller percentage of cells were viable ( $52 \pm 12.5\%$ ) and a fairly high number of cells were in the early stages of apoptosis ( $25.9 \pm 12\%$ ). After 7 days the percentage of cells in late apoptosis/late necrosis differed significantly ( $17.3 \pm 2\%$ ) from the untreated control. The percentage of cells in early necrosis and late necrosis differed significantly from the untreated control at both time points. A dose response was observed in the data obtained for HQ5 as the percentage of cells in early apoptosis increased from  $4.4 \pm 0.7\%$  after 7 days exposure to  $IC_{50}$  to  $13.9 \pm 1.7\%$  after 7 days exposure to  $5 \times IC_{50}$ . The same trend was seen in the percentage of cells in late apoptosis:  $1.0 \pm 0.1\%$  after 7 days exposure to  $IC_{50}$  and  $5.9 \pm 1.7\%$  after 7 days incubation with  $5 \times IC_{50}$ .

The untreated control used in the experiments with HQ10 showed a distribution of cells very similar to that seen in the experiments for HQ5: for both time points the largest percentage of cells were viable ( $91.4 \pm 0.6\%$  after 5 days;  $87.7 \pm 0.6\%$  after 7 days). A decrease in the percentage of viable cells was seen in the apoptosis control for both time points ( $69.8 \pm 1.6\%$  after 5 days;  $68.0 \pm 1.2\%$  after 7 days). The percentage of cells in late apoptosis differed significantly from the untreated control for both time points ( $15.5 \pm 2.1\%$  after 5 days;  $14.9 \pm 0.6\%$  after 7 days). The necrosis control showed a significant decrease in the percentage of viable cells and a significant increase of cells in early necrosis for both time points ( $85.7 \pm 3.8\%$  after 5 days;  $91.1 \pm 0.6\%$  after 7 days). A dose response was observed for the cells exposed to HQ10. After 7 days exposure the percentage of viable cells exposed to HQ10 at  $IC_{50}$  was  $92.6 \pm 0.06\%$ , whereas exposure to  $5 \times IC_{50}$  for 7 days decreased the percentage of viable cells significantly to  $12.9 \pm 0.6\%$ . The percentage of cells in early and late apoptosis also increased significantly from the sample exposed to HQ10 at  $IC_{50}$  ( $3.4 \pm 0.4\%$ ;  $0.6 \pm 0.2\%$ ) and HQ10 at  $5 \times IC_{50}$  ( $67.1 \pm 1.7\%$ ;  $19.4 \pm 1.7\%$ ).

**Table 5.1:** Apoptosis/necrosis analysis results of MCF-7 cells exposed to HQ5 at three different concentrations after 5 and 7 days exposure. Results are shown as mean percentage of cells  $\pm$  SEM.

		Exposure time:	Exposure time:
		5 days	7 days
<b>Control</b>	<b>Viable cells</b>	96.6 $\pm$ 0.5	91.2 $\pm$ 1.9
	<b>Early apoptosis</b>	1.8 $\pm$ 0.5	5.9 $\pm$ 1.3
	<b>Late apoptosis</b>	0.9 $\pm$ 0.1	2.5 $\pm$ 0.7
	<b>Early necrosis</b>	0.7 $\pm$ 1.3	0.4 $\pm$ 0.09
<b>Apoptosis control</b>	<b>Viable cells</b>	52.0 $\pm$ 12.5 **	70.0 $\pm$ 3.4 **
	<b>Early apoptosis</b>	25.9 $\pm$ 12 *	10.7 $\pm$ 2.5
	<b>Late apoptosis</b>	20.0 $\pm$ 1	17.3 $\pm$ 2 **
	<b>Early necrosis</b>	2.0 $\pm$ 0.8	2.0 $\pm$ 0.5
<b>Necrosis control</b>	<b>Viable cells</b>	1.4 $\pm$ 1 **	1.1 $\pm$ 0.2 **
	<b>Early apoptosis</b>	0.03 $\pm$ 0.03	0.00 $\pm$ 0.00
	<b>Late apoptosis</b>	47.8 $\pm$ 20.7 **	54.5 $\pm$ 5.6 **
	<b>Early necrosis</b>	50.8 $\pm$ 20.5 **	44.4 $\pm$ 5.5 **
<b>HQ5 at IC<sub>50</sub></b>	<b>Viable cells</b>	93.2 $\pm$ 2	94.5 $\pm$ 0.8
	<b>Early apoptosis</b>	2.7 $\pm$ 0.8	4.4 $\pm$ 0.7
	<b>Late apoptosis</b>	2.3 $\pm$ 1	1.0 $\pm$ 0.1
	<b>Early necrosis</b>	1.6 $\pm$ 0.3	0.2 $\pm$ 0.09
<b>HQ5 at 2 x IC<sub>50</sub></b>	<b>Viable cells</b>	85.4 $\pm$ 3.5	89.1 $\pm$ 2
	<b>Early apoptosis</b>	6.0 $\pm$ 2.2	5.0 $\pm$ 2.2
	<b>Late apoptosis</b>	5.8 $\pm$ 1.5	3.8 $\pm$ 0.8
	<b>Early necrosis</b>	2.8 $\pm$ 0.7	2.0 $\pm$ 1.3
<b>HQ5 at 5 x IC<sub>50</sub></b>	<b>Viable cells</b>	79.4 $\pm$ 2.4	74.7 $\pm$ 4.2 **
	<b>Early apoptosis</b>	8.7 $\pm$ 1.3	13.9 $\pm$ 1.7 *
	<b>Late apoptosis</b>	8.3 $\pm$ 2.4	5.9 $\pm$ 1.7
	<b>Early necrosis</b>	3.6 $\pm$ 1.0	5.5 $\pm$ 3.5

\* p < 0.05

\*\* p < 0.01

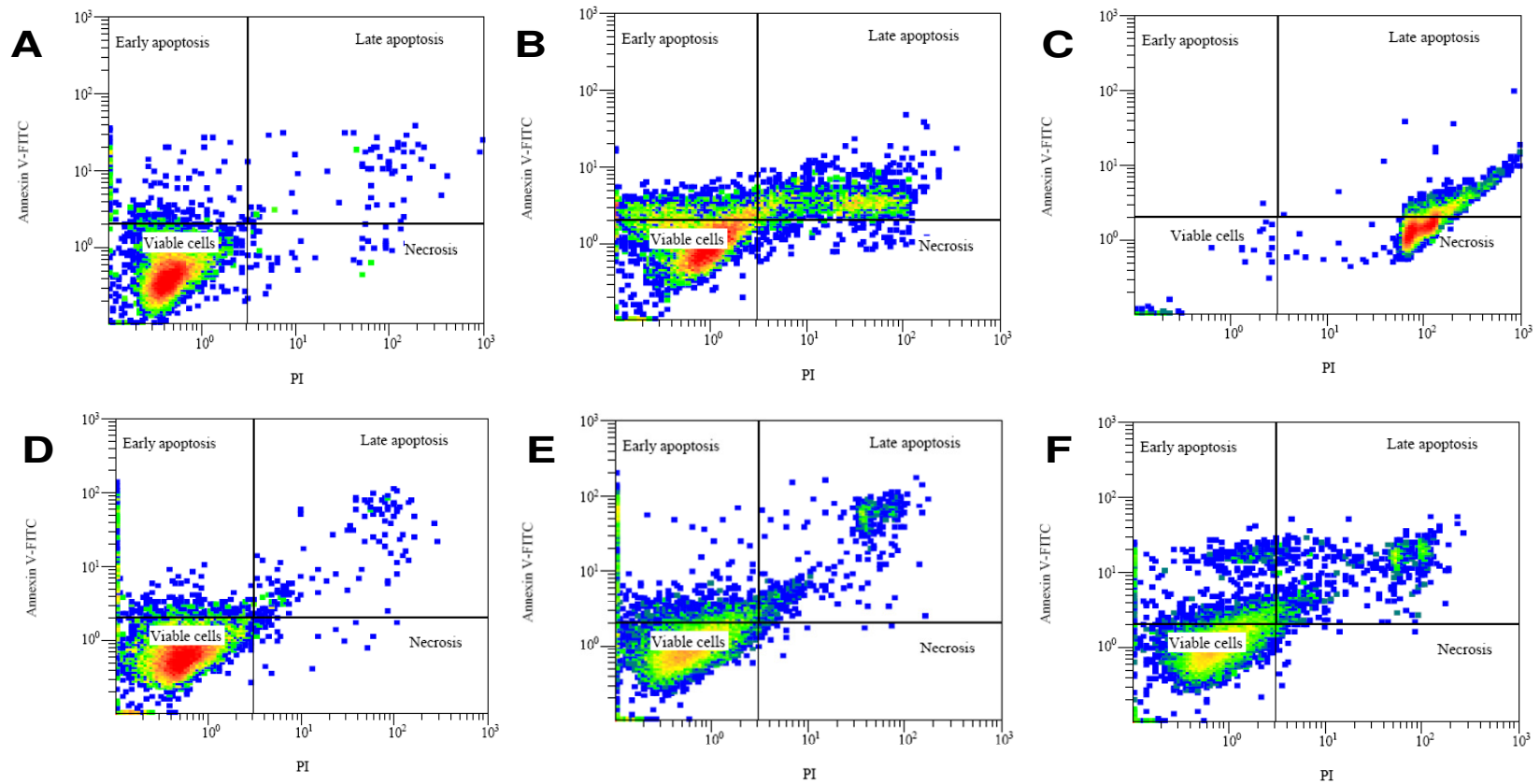
$\alpha$  A minimum of three independent experiments was performed.

**Table 5.2:** Apoptosis/necrosis analysis results of MCF-7 cells exposed to HQ10 at three different concentrations after 5 and 7 days exposure. Results are shown as mean percentage of cells  $\pm$  SEM.

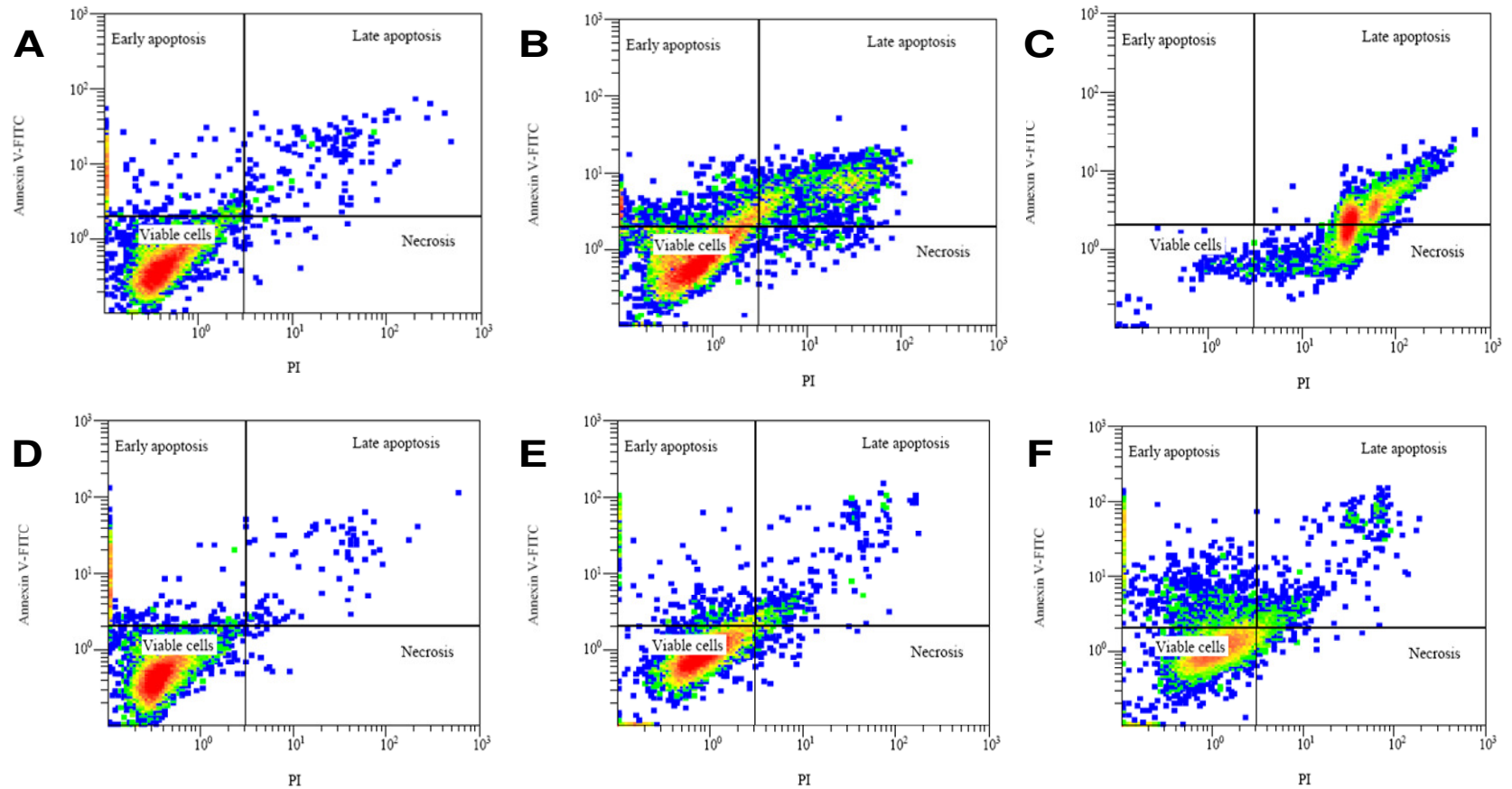
		Exposure time: 5 days	Exposure time: 7 days
<b>Control</b>	<b>Viable cells</b>	91.4 $\pm$ 0.6	87.7 $\pm$ 0.6
	<b>Early apoptosis</b>	5.2 $\pm$ 0.3	7.3 $\pm$ 0.3
	<b>Late apoptosis</b>	3.1 $\pm$ 0.2	4.5 $\pm$ 0.3
	<b>Early necrosis</b>	1.0 $\pm$ 0.4	0.45 $\pm$ 0.09
<b>Apoptosis control</b>	<b>Viable cells</b>	69.8 $\pm$ 1.6	68.0 $\pm$ 1.2
	<b>Early apoptosis</b>	7.8 $\pm$ 3.2	9.7 $\pm$ 0.7
	<b>Late apoptosis</b>	15.5 $\pm$ 2.1 **	14.9 $\pm$ 0.6 **
	<b>Early necrosis</b>	6.9 $\pm$ 3.6	7.4 $\pm$ 0.3 **
<b>Necrosis control</b>	<b>Viable cells</b>	7.1 $\pm$ 0.8 **	6.2 $\pm$ 0.4 **
	<b>Early apoptosis</b>	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	<b>Late apoptosis</b>	7.2 $\pm$ 3.4	2.7 $\pm$ 0.2
	<b>Early necrosis</b>	85.7 $\pm$ 3.8 **	91.1 $\pm$ 0.6 **
<b>HQ10 at IC<sub>50</sub></b>	<b>Viable cells</b>	70.3 $\pm$ 12.8	92.6 $\pm$ 0.06
	<b>Early apoptosis</b>	22.8 $\pm$ 11.7	3.4 $\pm$ 0.4
	<b>Late apoptosis</b>	5.8 $\pm$ 2.1	3.4 $\pm$ 0.8
	<b>Early necrosis</b>	1.2 $\pm$ 0.8	0.6 $\pm$ 0.2
<b>HQ10 at 2 x IC<sub>50</sub></b>	<b>Viable cells</b>	70.0 $\pm$ 2.2	48.0 $\pm$ 15.1 **
	<b>Early apoptosis</b>	24.6 $\pm$ 0.3	44.6 $\pm$ 14.0 **
	<b>Late apoptosis</b>	5.3 $\pm$ 1.8	7.2 $\pm$ 1.1
	<b>Early necrosis</b>	0.3 $\pm$ 0.2	0.2 $\pm$ 0.07
<b>HQ10 at 5 x IC<sub>50</sub></b>	<b>Viable cells</b>	26.7 $\pm$ 6.7 **	12.9 $\pm$ 0.6 **
	<b>Early apoptosis</b>	50.0 $\pm$ 9.0 **	67.1 $\pm$ 1.7 **
	<b>Late apoptosis</b>	21.3 $\pm$ 1.0 **	19.4 $\pm$ 1.7 **
	<b>Early necrosis</b>	2.3 $\pm$ 1.2	0.6 $\pm$ 0.1

\*\* p < 0.01

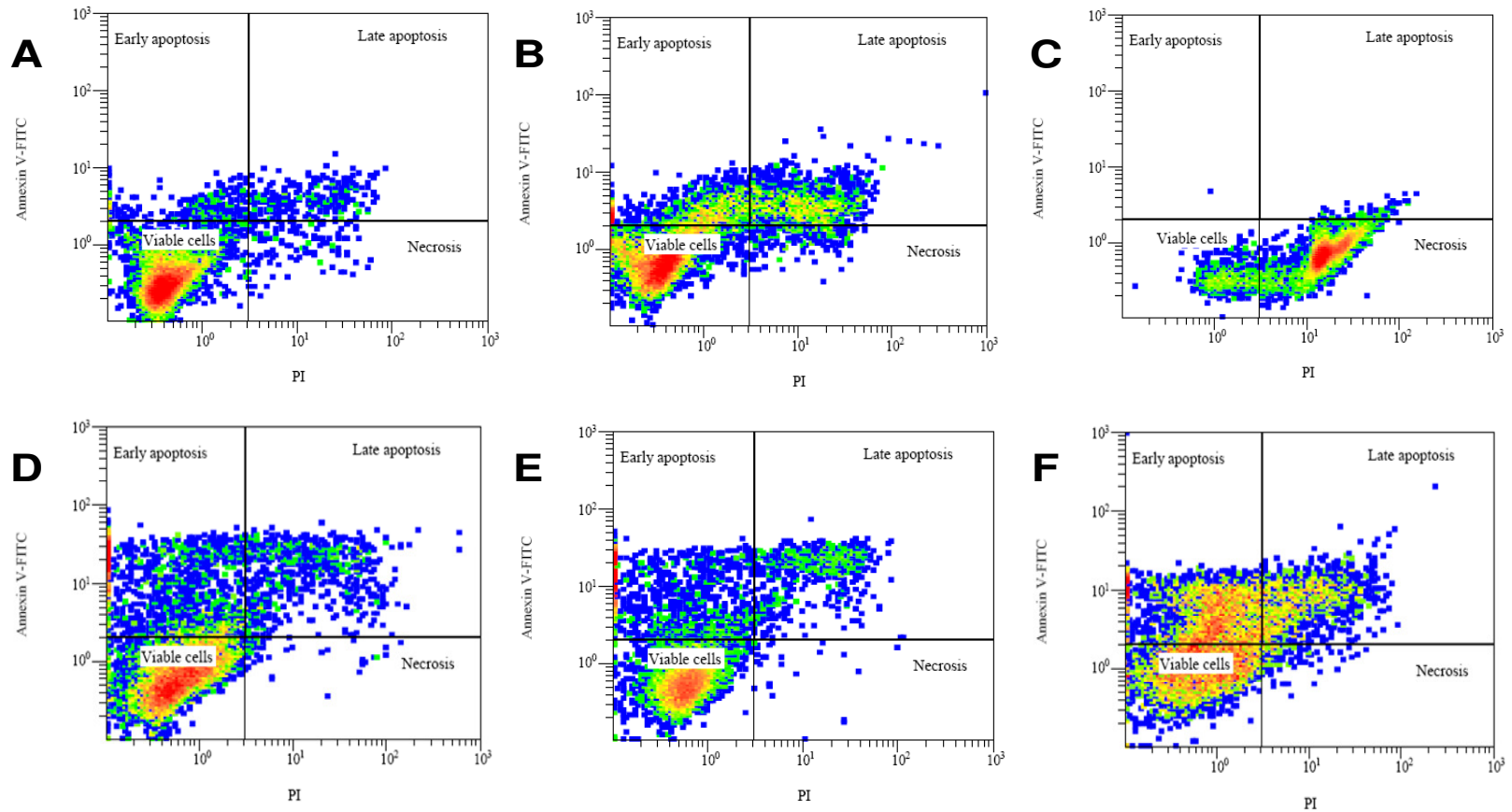
α A minimum of three independent experiments was performed.



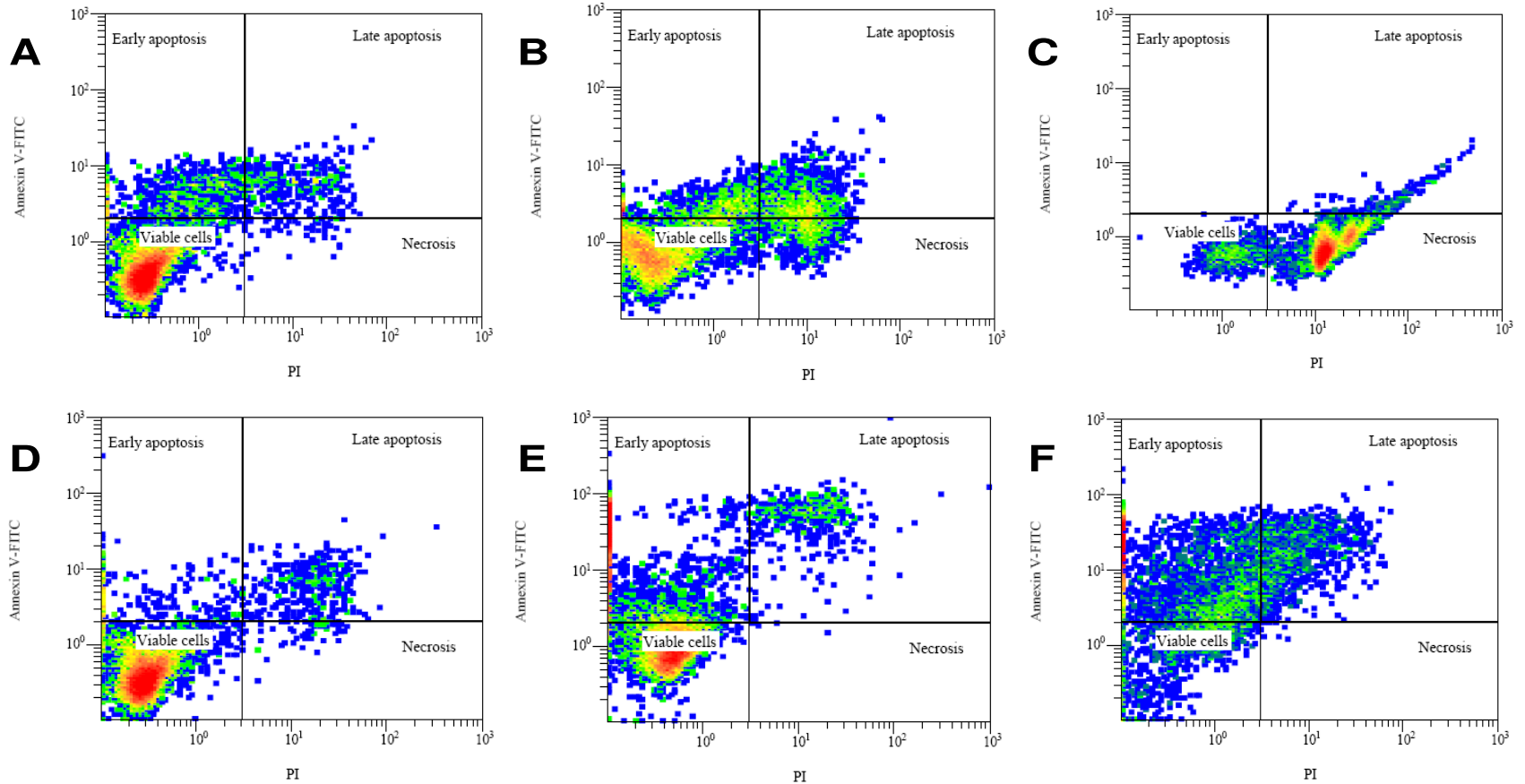
**Figure 5.1:** The effect of HQ5 on the induction of apoptosis or necrosis in MCF-7 cells after 5 days incubation. (A) untreated control, (B) apoptosis control, (C) necrosis control, (D) HQ5 at  $IC_{50}$ , (E) HQ5 at  $2 \times IC_{50}$ , (F) HQ5 at  $5 \times IC_{50}$ .



**Figure 5.2:** The effect of HQ5 on the induction of apoptosis or necrosis in MCF-7 cells after 7 days incubation. (A) untreated control, (B) apoptosis control, (C) necrosis control, (D) HQ5 at  $IC_{50}$ , (E) HQ5 at  $2 \times IC_{50}$ , (F) HQ5 at  $5 \times IC_{50}$ .



**Figure 5.3:** The effect of HQ10 on the induction of apoptosis or necrosis in MCF-7 cells after 5 days incubation. (A) untreated control, (B) apoptosis control, (C) necrosis control, (D) HQ10 at  $IC_{50}$ , (E) HQ10 at  $2 \times IC_{50}$ , (F) HQ10 at  $5 \times IC_{50}$ .



**Figure 5.4:** The effect of HQ10 on the induction of apoptosis or necrosis in MCF-7 cells after 7 days incubation. (A) untreated control, (B) apoptosis control, (C) necrosis control, (D) HQ10 at IC<sub>50</sub>, (E) HQ10 at 2 x IC<sub>50</sub>, (F) HQ10 at 5 x IC<sub>50</sub>.

## 5.6. Discussion

During this study it was investigated whether the two experimental compounds, HQ5 and HQ10, induce apoptosis or necrosis in MCF-7 breast cancer cells. The results obtained suggest that both HQ5 and HQ10 induced apoptosis in MCF-7 cells, and that the effects of each compound were most pronounced after 7 days of exposure. Data indicate that the experimental compounds exerted their effect in a dose and time dependent manner. It is remarkable that HQ5 and HQ10 seem to induce apoptosis at all three concentrations tested and at both time points studied. The percentage of cells in early apoptosis differs significantly from the untreated control for both HQ5 and HQ10 at  $5 \times IC_{50}$  after 7 days exposure. The effect of exposure to HQ10 at  $5 \times IC_{50}$  should not be misinterpreted as indicative as necrosis: rather the DNA of cells in late apoptosis can also stain positive for PI as the DNA may be exposed after membrane lysis (De Bruin *et al.*, 2008).

Mooney *et al.* (2002) showed that the increase in apoptosis induction (4.5 fold) plateaus after 16 hours treatment with staurosporine in MCF-7 cells. However, it should be noted that the percentage cells in early apoptosis ( $10.7 \pm 2.5\%$ ) in the apoptosis control for HQ5 after 7 days were reduced when compared to the percentage cells in early apoptosis ( $25.9 \pm 12.0\%$ ) after 5 days. The same trend was seen for the percentage cells in late apoptosis ( $20.0 \pm 1.0\%$  after 5 days,  $17.3 \pm 2.0\%$  after 7 days). The apoptosis control was treated with staurosporine 18 hours before sample preparation began at 5 and 7 days. Several apoptosis inducers, including tamoxifen, were tested during method development, but it was found that staurosporine was the most reliable and reproducible. Unfortunately, it appeared as though staurosporine-induced apoptosis occurs in a time dependent manner: the percentage of cells undergoing late and early apoptosis varied with a difference in exposure time of only 2 hours. Another explanation for this phenomenon may be that the cells reached close to 100% confluency at day 7. The growth rate of the cells would be drastically decreased due to contact inhibition, causing cells to senesce in the  $G_1$  phase of the cell cycle. The decreased growth rate might be correlated to the decreased apoptosis induction observed after 7 days incubation.

The pro-apoptotic activity of quinoline derivatives has been well documented. Studies on 23 substituted antiproliferative quinolines showed that only one compound did not cause apoptosis in MCF-7 breast cancer cells (Martirosyan *et al.*, 2004). Reports indicate that a quinoline derivative substituted with selenium induced apoptosis in two leukaemia cell lines (Shahabuddin *et al.*, 2010). An indolo-quinoline derivative was found to induce apoptosis in K562 leukaemia cells (Yang *et al.*, 2007).

In response to cancer treatment, cells have been observed to undergo apoptosis (Willingham, 1999). One of the advantages of apoptosis is that inflammation due to cell death is circumvented through the 'eat me' signal displayed by apoptotic cells (De Bruin *et al.*, 2008). The inflammation caused by necrosis leads to tissue repair and scarring, leading to permanent alterations in the tissue structure (Willingham, 1999). Apoptosis can be induced through various mechanisms categorised as intrinsic or extrinsic. Intracellular stress signals such as oxidative stress or severe DNA damage prompt mitochondrial leakage of cytochrome *c* which in turn activates caspase-9 (De Bruin *et al.*, 2008). Extrinsic signals can include interaction between pro-apoptotic ligands such as TNF $\alpha$  or the Fas ligand and their cell surface receptors (De Bruin *et al.*, 2008). Such interactions lead to the formation of a death-inducing signalling complex which will in turn activate caspase-8. Both caspase-9 and -8 recruit the executioner caspases responsible for degradation of the cell (De Bruin *et al.*, 2008).

Quinoline derivatives with a wide range of substituents have been shown to induce apoptosis in cancer cell lines through a variety of mechanisms. A 4-amino-3-acetylquinoline derivative induced apoptosis in L1210 leukaemia cells through activation of the p38 MAPK pathway (Jantova *et al.*, 2008). Jasinski *et al.* (2008) described the pro-apoptotic activity of a thiopyrano-quinoline derivative against H226 squamous cell lung carcinoma through the caspase cascade. Gakhar *et al.* (2008) observed that a quinoline derivative induced apoptosis in T47D breast cancer cells through the increased activation of caspase 3. A number of cyanopyrrolo quinoline derivatives have been reported to induce apoptosis in T47D breast cancer and HCT116 colon cancer cells through the inhibition of tubulin polymerization (Kemnitzer *et al.*, 2008).

The concentrations of the two experimental compounds tested in this study were higher than the doses which would be used in a clinical setting. However, the ability of these

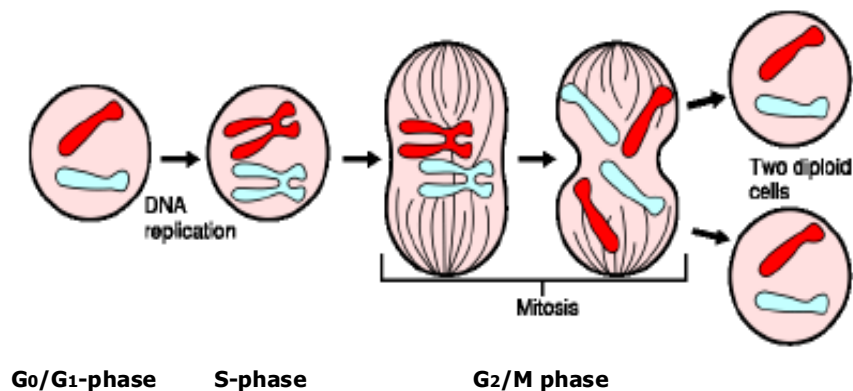
compounds to induce apoptosis is an attractive feature often seen in lead anticancer agents. Even though the mechanisms activated by HQ5 and HQ10 is unclear and cannot be speculated upon due to the wide variety of pathways activated by quinoline derivatives, data indicate that these two experimental compounds predominantly induce apoptosis in MCF-7 breast cancer cells.

## Chapter 6: Cell cycle

### 6.1. Introduction

The reproduction of cells occurs through the replication of DNA, the partition of the nucleus and separation of the cytoplasm into two daughter cells (Massagué, 2004). The process of cell reproduction has been termed the cell cycle. Although the cell cycle is an immensely complex process, it has been simplified to four distinct phases.

The cell cycle is initiated with the  $G_0$  to  $G_1$  gap phase change, referring to the first gap before the duplication of the DNA occurs (Hartwell *et al.*, 1989). The cell then enters the S phase during which the DNA content of the cell is replicated to ensure that both mother and daughter cell will contain identical DNA copies. The second gap phase, or  $G_2$  phase, occurs after DNA replication. Both of the gap phases,  $G_1$  and  $G_2$ , allow for the correction of replication errors (Massagué, 2004). Finally, the organelles, cytoplasm and nuclei of the daughter cells are separated during the M phase making use of the microtubules.



**Figure 6.1:** A simplified version of the cell cycle of a non-cancerous cell (adapted from [http://en.wikipedia.org/wiki/Cell\\_cycle](http://en.wikipedia.org/wiki/Cell_cycle); 2011)

The DNA content and ploidy level of each phase of the cell cycle is distinctive, and is indicative of the phase of the cell cycle a cell is in. A cell in the  $G_1$  phase contains only

one copy of DNA ( $N$ ). During the S-phase, DNA synthesis occurs and cells in this phase of the cell cycle contains between one and two copies of DNA. Once a cell reaches the  $G_2$  phase, it contains two complete copies of DNA ( $2N$ ) and undergoes mitosis (M phase) to form two daughter cells. It is possible to quantify the amount of DNA in a cell using flow cytometry and fluorescent dyes that bind DNA stoichiometrically, and thus to determine the phase of the cell cycle the cell is in (Ochatt, 2006).

During the four stages of the cell cycle, specific steps are taken to ensure the accurate replication of the cell. Progression between the phases of the cell cycle is regulated by cyclin dependent kinases (Shapiro *et al.*, 1999; Evan *et al.*, 2001) and mechanisms referred to as checkpoint controls (Collins *et al.*, 1997). Cyclin dependant kinases (CDK) are proteins which interact with cyclins to form an active unit which allows the cell cycle to proceed. CDK inhibitors will inhibit the formation of such a unit and thus result in cessation of the cell cycle. Checkpoint controls play a more indirect role by ensuring that an earlier stage of the cell cycle is properly completed before entering into the next stage (Hartwell *et al.*, 1989). For example, checkpoint controls detect damage to the DNA and to prevent potential mutations, halts the cell cycle until the DNA has been repaired otherwise apoptosis is initiated (Collins *et al.*, 1997; Kastan *et al.*, 2004; Massagué, 2004).

The transformation of a cell to a cancerous cell often results in the alteration of the cell cycle and its controls. The rate at which proliferation occurs is increased in cancer cells (Evan *et al.*, 2001) through an increase in the availability of cyclins and a decrease in CDK inhibitors (Shapiro *et al.*, 1999). The checkpoint controls are also affected in cancer cells: where irreparable damage would normally cause cell cycle blockade at the  $G_1/S$  or  $G_2/M$  boundaries, cancer cells continue through the cell cycle due to ineffective checkpoint controls (Shapiro *et al.*, 1999).

The abovementioned alterations in the cell cycle of cancerous tissue represent potential targets for clinical intervention (Evan *et al.*, 2001). Although the abrogation of  $G_1$  phase will decrease the number of cancer cells, this type of intervention is not always predictable (Shapiro *et al.*, 1999) as some cancer cells are incapable of  $G_1$  arrest, even though an increase in apoptosis is generally seen after treatment with a  $G_1$  blocker. S-phase inhibitors will mostly induce a cytostatic response especially when used as

monotherapy (Shapiro *et al.*, 1999). However, enhanced cytotoxicity has been reported when S-phase inhibitors are used in conjunction with other chemotherapeutic agents. The inhibition of the G<sub>2</sub> phase appears to be the most advantageous and clinically predictable: once DNA replication has occurred, a cell must divide to form two daughter cells. Otherwise a cell will undergo immediate apoptosis or re-enter the S-phase where apoptosis will be induced (Shapiro *et al.*, 1999). Thus inhibition in the G<sub>2</sub> phase, as in mitosis inhibition, is a very effective mechanism to induce apoptosis in cancer cells.

The two experimental hydroxyquinolines showing promising cytotoxic effects against MCF-7 cells during the initial phases of this study were assessed using a flow cytometric method to investigate their effects on the cell cycle using the MCF-7 cell line.

Flow cytometric procedures require the use of fluorescent dyes (Fried *et al.*, 1976). For the investigation of the experimental compounds on the cell cycle, propidium iodide (PI) was used to measure the relative DNA content of each cell. The DNA content of individual cells was used as an indicator of the ratio of cells in each cell cycle phase.

In order to exclude false results which can be obtained if PI binds to double stranded RNA, ribonuclease was added to all samples (Fried *et al.*, 1976). This enzyme ensured digestion of the RNA into lengths to which PI will not bind.

## **6.2. Aim**

To investigate the *in vitro* effect of HQ5 and HQ10 on the cell cycle of MCF-7 cells.

## **6.3. Materials**

### **6.3.1. Reagents**

#### **i. Curcumin**

Curcumin was obtained from Merck (Hohenbrunn, Germany). A stock solution of 1 mM was prepared in DMSO. The stock solution was dispensed into 100 µl aliquots and

stored at  $-70^{\circ}\text{C}$ . Appropriate dilutions into cell culture medium were made just prior to use.

### **ii. Dulbecco's Modified Eagle's Medium**

DMEM was procured from Sigma-Aldrich (St Louis, USA). A mass of 67.35 g of medium powder was dissolved in 5 litres sterile, deionised water. To this solution 18.5 g of  $\text{NaHCO}_3$  was added to adjust the pH. The solution was filter sterilized twice with  $0.2\ \mu\text{M}$  cellulose acetate filters, dispensed into sterile 500 ml bottles, supplemented with 1% penicillin/streptomycin and stored at  $4^{\circ}\text{C}$ . Sterile heat inactivated FCS was added to a concentration of 10% prior to use.

### **iii. 99.9% Ethanol**

Ethanol was obtained from Illovo Sugar Limited (Durban, RSA). A 70% ethanol solution was prepared by adding distilled water to 3.5 litres of 99.9% ethanol to make 5 L solution. The stock solution was dispensed into aliquots of smaller volumes and stored at  $-20^{\circ}\text{C}$ .

### **iv. Methotrexate**

Methotrexate was procured from Sigma-Aldrich (St Louis, USA). A stock solution of 0.1 M was prepared in DMSO and stored at  $-70^{\circ}\text{C}$  in aliquots of  $20\ \mu\text{l}$  until used. Dilutions were made in cell culture medium immediately prior to use.

### **v. Phosphate buffered saline (PBS)**

FTA Hemagglutination buffer was obtained from BD Biosciences (Sparks, USA). A solution containing 9.23 g/l of de-ionised water was made up and stored at  $4^{\circ}\text{C}$  until use. On the day of the experiment, 1% foetal calf serum was added.

### **vi. Staining solution**

The staining solution was prepared by dissolving the following reagents in PBS:

40  $\mu\text{g/ml}$  Propidium iodide

100  $\mu\text{g/ml}$  Ribonuclease A (RNase) from bovine pancreas

0.1% Triton-X

All of the reagents were obtained from Sigma-Aldrich (St Louis, USA).

As RNase is not stable in solution, it was added to the staining solution just before use.

The solution was stored in the dark at  $4^{\circ}\text{C}$  until used.

### **vii. Trypsin/Versene**

A trypsin/versene solution containing 0.25% trypsin and 0.1% EDTA in Ca<sup>++</sup> and Mg<sup>++</sup> free phosphate buffered saline was obtained from Highveld Biological (Johannesburg, RSA). Trypsin/versene was stored at 4°C.

## **6.4. Methods**

### **6.4.1. Maintenance and counting of cell culture**

Briefly, MCF-7 cells were grown to approximately 80% confluence in DMEM containing 10% FCS. The cells were harvested with a trypsin/versene solution, counted with a haemocytometer and diluted to a concentration of  $2.5 \times 10^4$  cells/ml.

### **6.4.2. Experimental procedure**

In order to synchronise the cells at G<sub>0</sub> before the start of the experiment, the cells were grown in DMEM without foetal bovine serum for 96 hours. After the “starvation period”, the cells were trypsinised and diluted to a concentration of  $2 \times 10^4$  cells/ml. Cell culture medium supplemented with 5% FCS was used in the experiment. The cells were allowed to attach for 24 hours before the experimental or control compounds were added. The experiments were set up in 25 cm<sup>2</sup> cell culture flasks with 4.5 ml of cell suspension and 0.5 ml of test compound diluted in cell culture medium. Each flask was dosed with a single drug at a single concentration. An untreated control flask, which received 4.5 ml of cell suspension and 0.5 ml of cell culture medium, was used for comparative purposes.

G<sub>1</sub> arrest was induced by continuing to grow the cells in cell culture medium deprived of foetal bovine serum. Methotrexate, which is regarded as a S-phase inhibitor (Zhu *et al.*, 1996) was used at a concentration of 10 µM as positive control. Curcumin, a known G<sub>2</sub> inhibitor (Simon *et al.*, 1998), was used at a concentration of 20 µM as a G<sub>2</sub> positive control. The concentrations of the two experimental compounds tested were equal to, double and five times the calculated IC<sub>50</sub> values. Stock solutions of the experimental compounds were diluted to the required concentrations in cell culture medium.

The incubation times used were 24, 48 and 72 hours of exposure to the experimental and control compounds. At the given time point the medium in each flask was transferred to a labelled 15 ml centrifuge tube. The flasks were rinsed with PBS and the PBS collected into the same tubes. This was to harvest the detached cells. Trypsin was then added to each flask, and the adherent cells allowed to detach at 37°C. The trypsinised cells were transferred to the original centrifuge tubes, then centrifuged for 5 minutes at 200 *g*. The cell free supernatant was discarded, 1 ml ice cold PBS added to each cell pellet and re-centrifuged for 5 minutes at 200 *g*. The supernatant was again discarded and the pellet re-suspended in 200 µl ice cold PBS supplemented with 1% FCS. A volume of 4 ml ice cold 70% ethanol was added drop wise to each tube while mixing on a vortex mixer to avoid clumping during the fixing step. The tubes were stored at 4°C for at least 24 hours. After the fixation step, the cells were centrifuged for 5 minutes at 200 *g* and the supernatant discarded. The pellet was re-suspended in 1 ml staining solution, and incubated at room temperature for 40 minutes. The samples were transferred to flow cytometer tubes and analysed with a Beckman Coulter FC 500 Series flow cytometer. The channel used was FL3 (620 nm). A maximum of 10 000 events was recorded per sample.

#### **6.4.3. Interpretation of results**

Three independent experiments were performed. The results obtained were in the form of DNA histograms, displaying the percentage of cells in each stage of the cell cycle, or the percentage of cells in apoptosis. The results are reported as mean percentage of cells in each phase  $\pm$  SEM. Statistical analysis was performed to determine whether the percentage cells in each stage of the cell cycle obtained for the experimental compounds differ significantly from those obtained for the controls using the Student's T-test. A p-value of less than 0.05 was used as indicator of significance. GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) was used for all statistical calculations.

### **6.5. Results**

In this experiment the effect of the two experimental hydroxyquinolines on the cell cycle of MCF-7 cells was investigated. For comparative purposes, an inhibitor of each phase

of the cell cycle was added. Results are shown in Tables 6.1 and 6.2. As the best results were obtained after exposure of 48 hours, those results are also shown graphically in Figure 6.2 and 6.3.

The untreated control serves as the model of the cell cycle of MCF-7 cells under standard conditions. A fluctuation in the percentage of cells in the G<sub>1</sub> phase over the three time points ranging from 47.9 ± 2.8% after 24 hours to 62.6 ± 4.4% after 72 hours could be seen. The percentage of cells in the S-phase and the percentage of cells undergoing apoptosis, are very comparable over the three time points. However, a 10% decrease of cells in G<sub>2</sub>/M phase is seen at 72 hours (11.9 ± 5.4%) as compared to the observations after 24 and 48 hours (23.5 ± 2.5% and 20.6 ± 3.8%, respectively). It would appear as though the G<sub>1</sub> phase inhibitor control reaches its maximum inhibitory potential after 72 hours as the percentage of cells in the G<sub>1</sub> phase increases from 57.9 ± 2.4% after 24 hours to approximately 70% after 72 hours. Even though the percentage of cells in the S-phase remains between 20 and 30% in the S-phase control, the effect of the inhibitor is notable when the percentage cells in the G<sub>2</sub>/M phase is taken into account: 7.8 ± 2.3% after 24 hours, 7.3 ± 2.6% after 48 hours and 7.9 ± 2.7% after 72 hours. These data follows the trend of a typical S-phase inhibitor as the amount of cells in the G<sub>2</sub>/M phase is substantially decreased when compared to the untreated control. When exposed to a G<sub>2</sub>/M inhibitor, it is expected that the percentage of cells in the G<sub>1</sub> phase will be very similar to that in the G<sub>2</sub>/M phase. The percentage of cells in the G<sub>1</sub> phase ranged from 23 to 29%, whilst the percentage of cells in the G<sub>2</sub>/M cells varied from 18 to 36%. Arrest in the G<sub>2</sub>/M phase causes a greater percentage of cell death, or apoptosis, than any of the other inhibitors. The maximum percentage of apoptotic cells (34.5 ± 15.0% after 72 hours of exposure) was indeed observed in the case of the G<sub>2</sub>/M inhibitor.

Each of the experimental compounds was tested at three different concentrations, using three selected time points. After 24 hours exposure to HQ5 the percentage of cells in the G<sub>1</sub> phase (approximately 43 to 48%) are higher than that of the percentage of cells in the G<sub>2</sub>/M phase (approximately 27 to 40%) for all the time points tested. Only 15 to 18% of cells are in the S-phase. The percentage of cells in the G<sub>1</sub> phase increases after 48 hours of exposure to 57.6 ± 3.5% at IC<sub>50</sub>, and 51.0 ± 6.5% at 5 × IC<sub>50</sub>. The percentage of cells in the G<sub>2</sub>/M phase varies from 15 to 28%. Again a smaller percentage of cells were

observed in the S-phase. However, after 72 hours of exposure the percentage of cells in G<sub>1</sub> phase decrease to  $56.2 \pm 7.2\%$  at IC<sub>50</sub>, and  $50.2 \pm 4.0\%$  at 5 x IC<sub>50</sub>, whereas the percentage of cells in the G<sub>2</sub>/M phase decreased to between 7 and 22%. The percentage of cells in the S-phase remains below 30%. At a concentration of 5 x IC<sub>50</sub> of HQ5, approximately 9% of the cells are apoptotic after an exposure time of 72 hours.

The percentage of cells in the G<sub>1</sub> phase after 24 hours of exposure to HQ10 ranges from 39 to 47%, in the S-phase from 20 to 25% and in the G<sub>2</sub>/M phase from 18 to 27%. After 48 hours of exposure the percentage of cells in the G<sub>1</sub> phase remains comparable to 24 hours of exposure:  $44.5 \pm 4.7\%$  at IC<sub>50</sub> and  $50.3 \pm 1.9\%$  at 5 x IC<sub>50</sub>. The percentage of cells in the S-phase after 48 hours (varying from 15 to 19%) of exposure is decreased in comparison to that of 24 hours of exposure. However, the percentage of cells in the G<sub>2</sub>/M phase remains almost constant (15 and 24%). After 72 hours of exposure the percentage of cells in the G<sub>1</sub> phase increases to between 50 and 69%. The percentage of cells in the S-phase decrease to below 15%, and the percentage of cells in the G<sub>2</sub>/M phase decreases to  $9.6 \pm 3.6\%$  at IC<sub>50</sub>. The percentage of apoptotic cells exposed to 5 x IC<sub>50</sub> concentration of HQ10 after 72 hours ( $19.4 \pm 10.7\%$ ) is notably higher than that observed for HQ5 at an equivalent concentration.

**Table 6.1:** Cell cycle analysis results of MCF-7 cells exposed to HQ5 at three different concentrations after 24, 48 and 72 hours. Results are shown as mean percentage of cells  $\pm$  SEM.

		Exposure time: 24 hours	Exposure time: 48 hours	Exposure time: 72 hours
<b>Untreated Control</b>	<b>Apoptosis</b>	5.0 $\pm$ 1.5	2.5 $\pm$ 0.8	1.9 $\pm$ 0.7
	<b>G<sub>1</sub> phase</b>	47.9 $\pm$ 2.8	47.4 $\pm$ 2.1	62.6 $\pm$ 4.4
	<b>S phase</b>	24.0 $\pm$ 1.4	20.9 $\pm$ 1.4	21.3 $\pm$ 2.9
	<b>G<sub>2</sub>/M phase</b>	23.5 $\pm$ 2.5	20.6 $\pm$ 3.8	11.9 $\pm$ 5.4
<b>G<sub>1</sub> control</b>	<b>Apoptosis</b>	3.9 $\pm$ 2.2	2.5 $\pm$ 0.7	4.3 $\pm$ 1.0
	<b>G<sub>1</sub> phase</b>	57.9 $\pm$ 2.4	61.2 $\pm$ 6.6	70.2 $\pm$ 9.0
	<b>S phase</b>	16.6 $\pm$ 2.7	18.3 $\pm$ 1.5	16.2 $\pm$ 7.3
	<b>G<sub>2</sub>/M phase</b>	18.3 $\pm$ 2.8	15.2 $\pm$ 4.5	7.5 $\pm$ 3.1
<b>S-phase control</b>	<b>Apoptosis</b>	12.0 $\pm$ 2.9	14.2 $\pm$ 2.3 **	24.7 $\pm$ 3.8 **
	<b>G<sub>1</sub> phase</b>	48.6 $\pm$ 4.7	39.0 $\pm$ 6.3	36.9 $\pm$ 4.7 *
	<b>S phase</b>	27.9 $\pm$ 7.9	24.0 $\pm$ 5.0	26.4 $\pm$ 3.2
	<b>G<sub>2</sub>/M phase</b>	7.8 $\pm$ 2.3 *	7.3 $\pm$ 2.6 *	7.9 $\pm$ 2.7
<b>G<sub>2</sub>/M control</b>	<b>Apoptosis</b>	15.2 $\pm$ 6.7	20.8 $\pm$ 4.4 *	34.5 $\pm$ 15.0
	<b>G<sub>1</sub> phase</b>	28.3 $\pm$ 4.6 *	28.2 $\pm$ 2.3 **	22.9 $\pm$ 5.2 **
	<b>S phase</b>	12.0 $\pm$ 3.5 *	17.1 $\pm$ 5.2	13.4 $\pm$ 4.6
	<b>G<sub>2</sub>/M phase</b>	35.4 $\pm$ 6.7	21.0 $\pm$ 6.1	17.6 $\pm$ 7.2
<b>HQ5 at IC<sub>50</sub></b>	<b>Apoptosis</b>	5.1 $\pm$ 1.0	4.1 $\pm$ 1.2	5.5 $\pm$ 2.6
	<b>G<sub>1</sub> phase</b>	43.4 $\pm$ 3.1	57.6 $\pm$ 3.5	56.2 $\pm$ 7.2
	<b>S phase</b>	17.6 $\pm$ 1.8 *	19.4 $\pm$ 7.5	23.1 $\pm$ 5.5
	<b>G<sub>2</sub>/M phase</b>	40.0 $\pm$ 9.5	15.3 $\pm$ 4.8	13.9 $\pm$ 8.4
<b>HQ5 at 2 x IC<sub>50</sub></b>	<b>Apoptosis</b>	6.6 $\pm$ 0.2	5.0 $\pm$ 1.7	7.7 $\pm$ 3.6
	<b>G<sub>1</sub> phase</b>	48.0 $\pm$ 5.6	54.1 $\pm$ 2.9	47.0 $\pm$ 13.3
	<b>S phase</b>	15.7 $\pm$ 0.6 **	14.7 $\pm$ 8.6	30.3 $\pm$ 13.0
	<b>G<sub>2</sub>/M phase</b>	27.4 $\pm$ 5.9	21.8 $\pm$ 5.1	6.5 $\pm$ 3.0
<b>HQ5 at 5 x IC<sub>50</sub></b>	<b>Apoptosis</b>	5.0 $\pm$ 0.5	6.8 $\pm$ 2.7	8.5 $\pm$ 2.5
	<b>G<sub>1</sub> phase</b>	43.1 $\pm$ 3.4	51.0 $\pm$ 6.5	50.2 $\pm$ 4.0
	<b>S phase</b>	15.0 $\pm$ 0.9 **	12.9 $\pm$ 4.5	12.4 $\pm$ 5.8
	<b>G<sub>2</sub>/M phase</b>	25.9 $\pm$ 0.8	27.2 $\pm$ 8.5	22.1 $\pm$ 6.4

\* p < 0.05

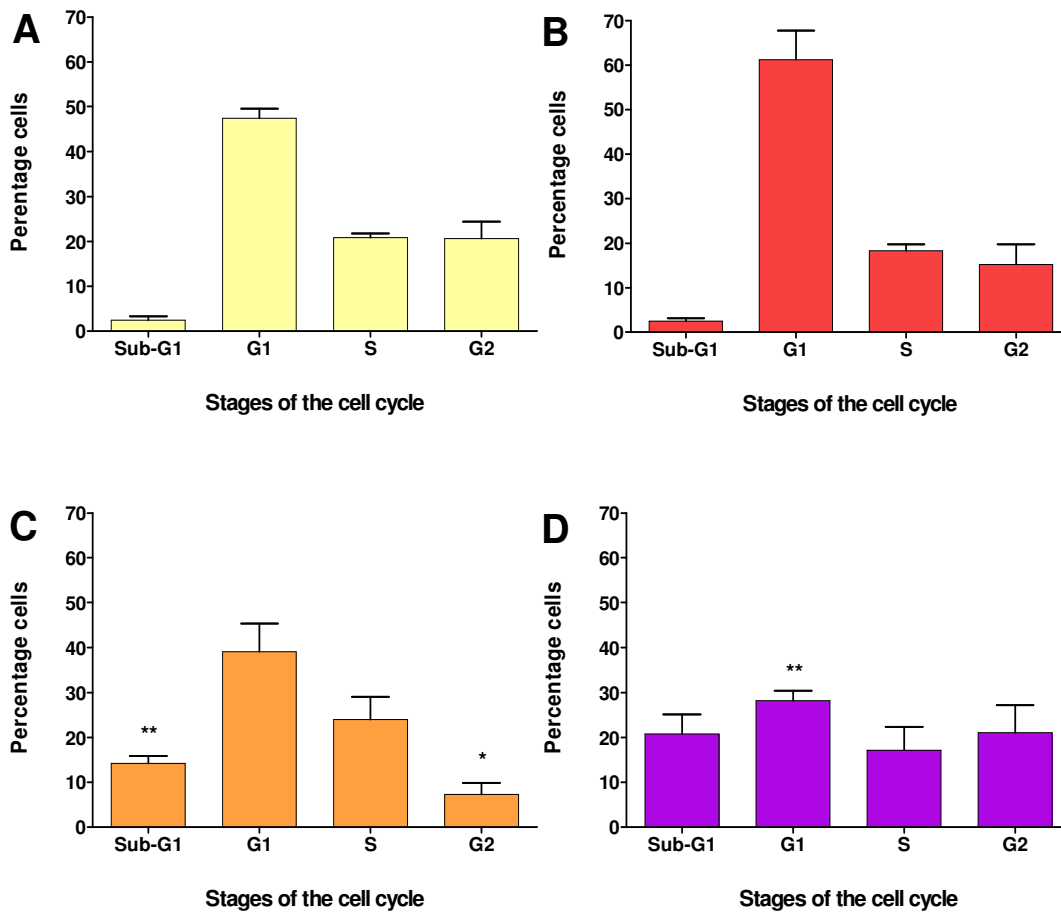
\*\* p < 0.01

**Table 6.2:** Cell cycle analysis results of MCF-7 cells exposed to HQ10 at three different concentrations after 24, 48 and 72 hours. Results are shown as mean percentage of cell  $\pm$  SEM.

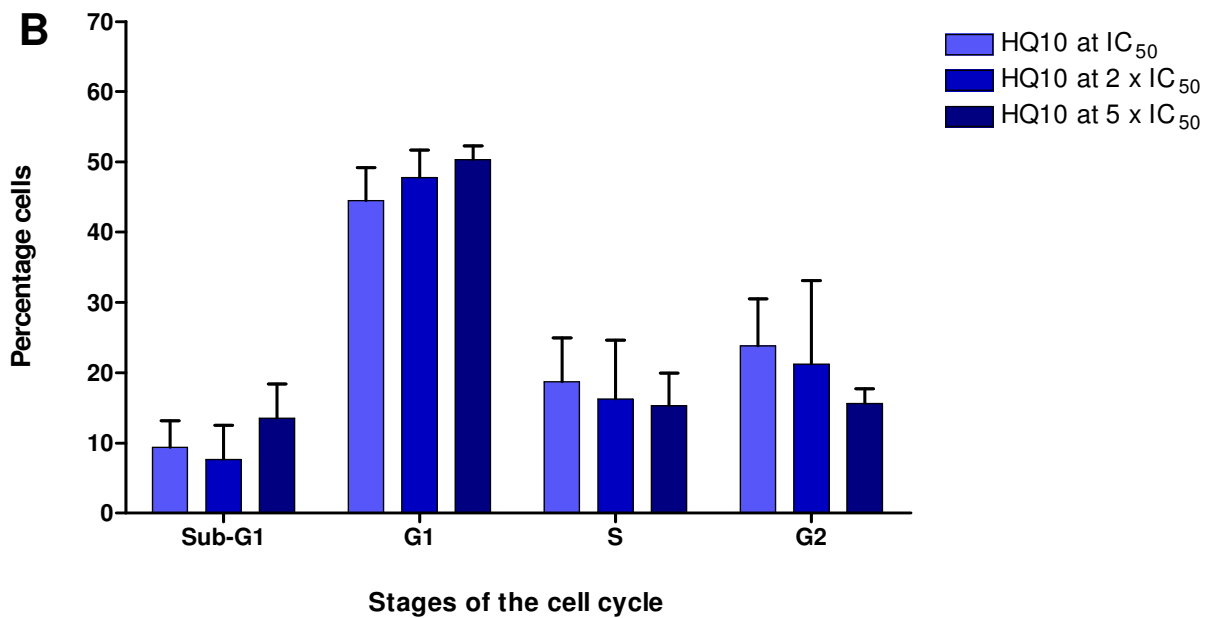
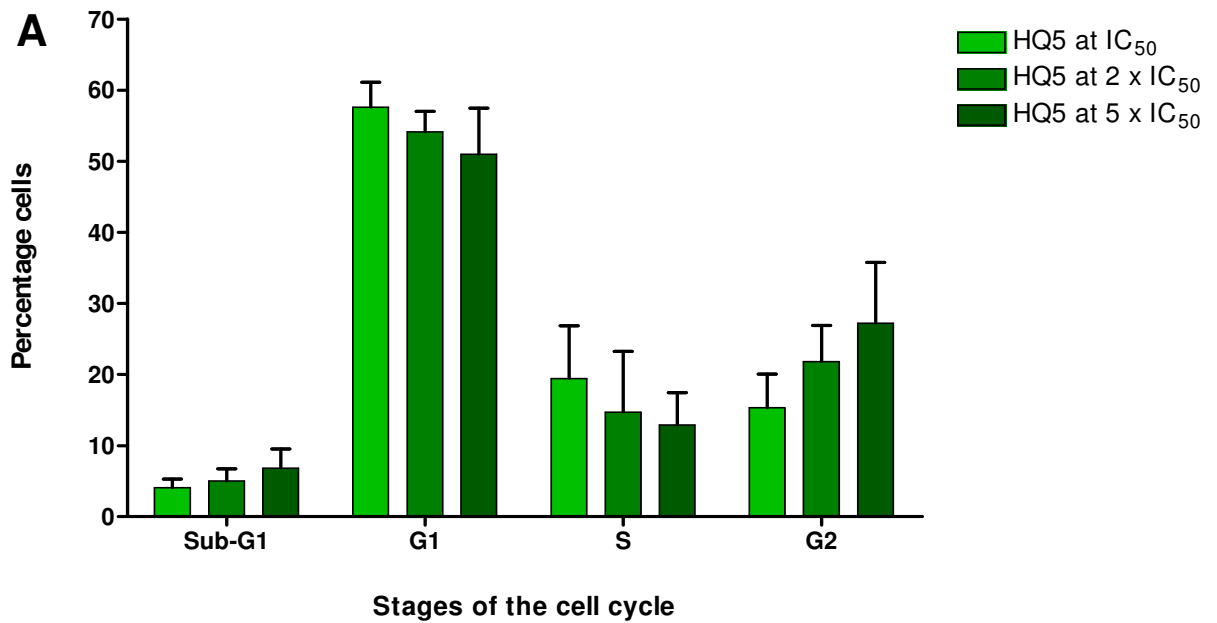
		Exposure time: 24 hours	Exposure time: 48 hours	Exposure time: 72 hours
<b>Untreated Control</b>	<b>Apoptosis</b>	5.0 $\pm$ 1.5	2.5 $\pm$ 0.8	1.9 $\pm$ 0.7
	<b>G<sub>1</sub> phase</b>	47.9 $\pm$ 2.8	47.4 $\pm$ 2.1	62.6 $\pm$ 4.4
	<b>S phase</b>	24.0 $\pm$ 1.4	20.9 $\pm$ 1.4	21.3 $\pm$ 2.9
	<b>G<sub>2</sub>/M phase</b>	23.5 $\pm$ 2.5	20.6 $\pm$ 3.8	11.9 $\pm$ 5.4
<b>G1 control</b>	<b>Apoptosis</b>	3.9 $\pm$ 2.2	2.5 $\pm$ 0.7	4.3 $\pm$ 1.0
	<b>G<sub>1</sub> phase</b>	57.9 $\pm$ 2.4	61.2 $\pm$ 6.6	70.2 $\pm$ 9.0
	<b>S phase</b>	16.6 $\pm$ 2.7	18.3 $\pm$ 1.5	16.2 $\pm$ 7.3
	<b>G<sub>2</sub>/M phase</b>	18.3 $\pm$ 2.8	15.2 $\pm$ 4.5	7.5 $\pm$ 3.1
<b>S-phase control</b>	<b>Apoptosis</b>	12.0 $\pm$ 2.9	14.2 $\pm$ 2.3 **	24.7 $\pm$ 3.8 **
	<b>G<sub>1</sub> phase</b>	48.6 $\pm$ 4.7	39.0 $\pm$ 6.3	36.9 $\pm$ 4.7 *
	<b>S phase</b>	27.9 $\pm$ 7.9	24.0 $\pm$ 5.0	26.4 $\pm$ 3.2
	<b>G<sub>2</sub>/M phase</b>	7.8 $\pm$ 2.3 *	7.3 $\pm$ 2.6 *	7.9 $\pm$ 2.7
<b>G<sub>2</sub>/M control</b>	<b>Apoptosis</b>	15.2 $\pm$ 6.7	20.8 $\pm$ 4.4 *	34.5 $\pm$ 15.0
	<b>G<sub>1</sub> phase</b>	28.3 $\pm$ 4.6 *	28.2 $\pm$ 2.3 **	22.9 $\pm$ 5.2 **
	<b>S phase</b>	12.0 $\pm$ 3.5 *	17.1 $\pm$ 5.2	13.4 $\pm$ 4.6
	<b>G<sub>2</sub>/M phase</b>	35.4 $\pm$ 6.7	21.0 $\pm$ 6.1	17.6 $\pm$ 7.2
<b>HQ10 at IC<sub>50</sub></b>	<b>Apoptosis</b>	5.7 $\pm$ 0.6	9.4 $\pm$ 3.7	4.7 $\pm$ 1.9
	<b>G<sub>1</sub> phase</b>	41.3 $\pm$ 0.8	44.5 $\pm$ 4.7	68.8 $\pm$ 1.6
	<b>S phase</b>	24.8 $\pm$ 0.1	18.7 $\pm$ 6.2	14.2 $\pm$ 5.0
	<b>G<sub>2</sub>/M phase</b>	26.5 $\pm$ 1.6	23.8 $\pm$ 6.7	9.6 $\pm$ 3.6
<b>HQ10 at 2 x IC<sub>50</sub></b>	<b>Apoptosis</b>	5.5 $\pm$ 1.1	7.6 $\pm$ 4.9	9.1 $\pm$ 4.5
	<b>G<sub>1</sub> phase</b>	39.0 $\pm$ 1.6	46.3 $\pm$ 4.8	50.8 $\pm$ 11.6
	<b>S phase</b>	25.5 $\pm$ 1.8	16.2 $\pm$ 8.4	9.3 $\pm$ 5.7
	<b>G<sub>2</sub>/M phase</b>	26.5 $\pm$ 2.2	21.2 $\pm$ 11.9	22.7 $\pm$ 10.3
<b>HQ10 at 5 x IC<sub>50</sub></b>	<b>Apoptosis</b>	14.8 $\pm$ 3.4 *	13.5 $\pm$ 4.9	19.4 $\pm$ 10.7
	<b>G<sub>1</sub> phase</b>	46.6 $\pm$ 2.7	50.3 $\pm$ 1.9	51.9 $\pm$ 9.8
	<b>S phase</b>	20.7 $\pm$ 5.5	15.3 $\pm$ 4.7	11.2 $\pm$ 2.1 *
	<b>G<sub>2</sub>/M phase</b>	18.2 $\pm$ 3.6	15.6 $\pm$ 2.1	14.0 $\pm$ 3.5

\* p < 0.05

\*\* p < 0.01



**Figure 6.2:** A graphic representation of the controls of each stage of the cell cycle of MCF-7 cells after 48 hours where (A) untreated control, (B) G<sub>1</sub> control and (C) S-phase control and (D) G<sub>2</sub> control. The graphs show up to 70%.



**Figure 6.3:** A graphic representation of the effect of the experimental compounds on the stages of the cell cycle of MCF-7 cells after 48 hours where (A) HQ5 and (B) HQ10. The graphs show up to 70%.

## 6.6. Discussion

The effect of the two experimental compounds, HQ5 and HQ10, on the cell cycle progression of MCF-7 cells was investigated using flow a cytometric relative DNA quantitation method. By comparing the results to those of known cell cycle phase transit inhibitors it can be deduced whether and where the inhibition occurs with the test compounds.

The untreated control showed a distinctive distribution of cells: the highest percentage of cells was found in the G<sub>1</sub> phase, with approximately equal percentages of cells in the S- and G<sub>2</sub>/M phases. However, it was expected that the percentage of cells in the G<sub>2</sub>/M phase would exceed the percentage of cells in the S-phase. Exposure to a G<sub>1</sub> inhibitor increased the percentage of cells in the G<sub>1</sub> phase with approximately 10% for each time point in comparison to the untreated control. The results obtained for the S-phase inhibitor show a significant decrease in the percentage of cells in the G<sub>2</sub>/M phase, indicating that the control was successful as an increase in the percentage of cells in the S-phase will lead to a reduction of the percentage of cells in the G<sub>2</sub>/M phase. Data obtained for the G<sub>2</sub>/M phase control showed a significant decrease of the percentage of cells in the G<sub>1</sub> phase which is suggestive of an accumulation of cells in the G<sub>2</sub>/M phase.

Data suggests that HQ5 behaves like a G<sub>1</sub> inhibitor at low concentrations, and tend to G<sub>2</sub>-like inhibition at the highest concentrations tested. HQ10, in contrast, appears to be a G<sub>1</sub> inhibitor. The effects of each compound are most pronounced after 48 hours of exposure: it may be that after 24 hours the compounds have not exerted their full effect, and from the percentage of cells in apoptosis, it is obvious that the compounds show cytotoxic effects towards the cells after 72 hours of exposure.

However, the significant difference in the percentage of cells exposed to HQ5 for 24 hours in the S-phase is suggestive of G<sub>1</sub>-like inhibition. Similarly, the significant decrease in the percentage of cells in the S-phase of cells treated with 5 x IC<sub>50</sub> of HQ10 after 72 hours indicates a G<sub>1</sub>-inhibitor.

The progression of cells from the G<sub>1</sub> phase to the S-phase is regulated by the product of the retinoblastoma susceptibility gene (Rb) and various members of the INK4 family. In a non-phosphorylated state, Rb forms a complex with members of the E2F transcription factor family which inhibits transcription (Massagué, 2004). Therefore phosphorylation of Rb by cyclin D-dependent kinases allows the cell to proceed to the S-phase. The members of the INK4 family act on CDK4 and CDK6 in particular, and lead to the inhibition of phosphorylation of Rb.

G<sub>1</sub> inhibitors are however not always successful in abrogating the cell cycle: some cell types treated with known G<sub>1</sub> inhibitors have been reported to progress to the S-phase through an unknown alternative mechanism (Shapiro *et al.*, 1999).

A number of quinoline derivatives have been shown to cause G<sub>1</sub> arrest. Pyrido-quinoline derivatives have been reported to induce G<sub>1</sub> inhibition in a prostate cancer cell line (Li *et al.*, 2006). Loehberg *et al.* (2007) observed that chloroquine arrested breast epithelial cell cycle at the G<sub>1</sub> phase through the upregulation of the p53 pathway. Another quinoline derivative, quinidine, has also been reported to cause G<sub>1</sub> inhibition in MCF-7 breast cancer cells through elevation of p53 and hypophosphorylated Rb (Zhou *et al.*, 2000). The mechanism through which the two experimental compounds, HQ5 and HQ10, induce G<sub>1</sub> arrest may be through the elevation of p53 levels, or inhibition of the phosphorylation of Rb. However, the precise mechanism would need to be confirmed through further studies.

The anomaly of the change from G<sub>1</sub> to G<sub>2</sub> inhibitor as observed in the results for HQ5 has been noticed in paclitaxel-treated cells. Paclitaxel, a drug which is often used in the clinical setting in the treatment of breast cancer (Wang *et al.*, 2000), is a known inhibitor of the G<sub>2</sub>/M phase, in particular for M-phase inhibition (Shah *et al.*, 2001). Interestingly, it has been reported that the concentration used and duration of exposure to paclitaxel influences the effect it has on the cell cycle (Wang *et al.*, 2000; Shah *et al.*, 2001). Paclitaxel appears to cause G<sub>1</sub> arrest at low concentrations (6 nM), and G<sub>2</sub>/M arrest at higher concentrations (above 100 nM) in A 549 cells, a non-small cell lung carcinoma cell line (Giannakakou *et al.*, 2001). Induction of p53 expression in the A 549 cells may account for the observed G<sub>1</sub> arrest. As paclitaxel is known to induce p53 expression in MCF-7 cells, it can be suggested that the same mechanism may be applicable in MCF-7

cells exposed to the experimental hydroxyquinolines. Thus a possible explanation for the results seen in this study can be that the experimental hydroxyquinolines have a similar response to paclitaxel in that they induce p53 expression at low concentrations.

The tumour suppressor protein, p53, is a mediator of the cell cycle and inducer of apoptosis. Hsu *et al.* (2008) did not observe an increase in the protein expression of p53 in A 549 cells treated with quinoline-dione derivatives. However, an interesting study on a benzimidazo-quinoline derivative showed that p53 was induced in a wild type p53 colon cancer cell line (HCT 116), but not in a colon cancer cell line with a mutated p53 gene (SW620) (Sedic *et al.*, 2008). The MCF-7 cell line used in this study with HQ5 and HQ10, is a wild type p53 breast cancer cell line (Fan *et al.*, 1995). As quinoline derivatives do not follow a predictable trend for the effects on p53 expression, it is difficult to speculate on the effect of HQ5 and HQ10 on p53 expression without further investigation.

Published studies show that several quinolines derivatives cause cell cycle arrest at the G<sub>2</sub>/M stage of the cell cycle. Reported studies of novel indoloquinoline derivatives indicate that these compounds induced cell cycle arrest at the G<sub>2</sub>/M stage when using the K562 leukaemia cell line (Yang *et al.*, 2007; Lamazzi *et al.*, 2000). An anilino-pyrroloquinoline derivative was shown to inhibit the G<sub>2</sub>/M stage of the OVCAR-3 ovarian cancer cell line (Via *et al.*, 2008). A range of cyano-pyrroloquinoline derivatives were shown to inhibit the cell cycle of HCT 116 colon cancer cells at the G<sub>2</sub>/M phase and it was suggested that tubulin polymerization may be affected (Kemnitzer *et al.*, 2008).

Paclitaxel affects both p53 induction and stabilization of microtubules (Shah *et al.*, 2001). Colchicine and other vinca alkaloids, on the other hand, inhibit tubulin polymerization, also resulting in cell cycle arrest at the G<sub>2</sub> phase. Similarly to the vinca alkaloids, aroylquinoline derivatives have been shown to inhibit tubulin polymerization through binding to the colchicine binding site of the tubulin complex (Nien *et al.*, 2010). However, 2-arylquinolin-4-one analogues were shown to inhibit tubulin polymerisation without interaction at the colchicine binding site (Chang *et al.*, 2009). Without further investigation it would be reckless to speculate on the effects of HQ5 pertaining to its interaction with tubulin or microtubules and the colchicine binding site.

The results of exposure to the two experimental hydroxyquinolines, HQ5 and HQ10, on the cell cycle of MCF-7 cells corresponds to the effects seen using certain other quinoline derivatives where cell cycle arrest is observed at the G<sub>1</sub> phase. At the highest concentration of HQ5 tested, it appears as though this compound behaves like a G<sub>2</sub>/M inhibitor.

Due to the complex nature and many potential causes of the arrest at each point of progression through the cell cycle, further investigation is needed to confirm the findings and to explain the exact mechanism by which these hydroxyquinolines cause cell cycle arrest.

## Chapter 7: *In vivo* investigation of the acute toxicity profile of HQ5 and HQ10 in BALB/c mice

### 7.1. Introduction

The *status quo* in anti-tumour drug development is the utilization of *in vitro* assays to prove the potential of a drug, followed by the *in vivo* assessment of toxicity and subsequently efficacy (Kelloff *et al.*, 1995). Even though *in vitro* assays offer many advantages, including management of cost, time and experimental conditions, *in vivo* testing remains essential as certain physiological influences, such as bioavailability, metabolism and excretion, can only be observed in an intact organism.

Approximately 80% of the animals used for research purposes are rodents (Lachmann, 1992). The use of mouse models in the assessment of the safety and toxicity of new drugs has become commonplace due to the reliability of the model to predict quantitative human toxicity (Newell *et al.*, 2004). Therefore, all novel pharmaceuticals are required to undergo *in vivo* acute toxicity testing before testing in humans commences (Robinson *et al.*, 2008).

Historically, the main objective of an acute toxicity study was to identify a single concentration (LD<sub>50</sub>) of a novel compound which resulted in fatality in half the animals in an experimental group (Botham, 2002). Up to 100 animals were required per group and oftentimes severe adverse events occurred before completion of the study. Recent trends in *in vivo* toxicity studies have moved away from the LD<sub>50</sub> method and towards more humane methods. Both the Organisation for the Economic Co-operation and Development (OECD) and International Conference on Harmonisation of the Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) recommend sequential dose escalating studies to determine a “no observed adverse effect level” (NOAEL), resulting in the use of fewer animals and limiting the number of animals experiencing serious adverse events.

A number of anti-neoplastic quinoline derivatives have been studied *in vivo*, the most well-known of which is linomide. Linomide, a quinoline-3-carboxamide, has been

successfully used as anti-angiogenic agent in the treatment of prostate, bladder and kidney cancers (Izawa *et al.*, 2001). The anticancer effects of vesnarinone, another quinoline derivative, were reported in various nude mouse models (Sato *et al.*, 1995; Honma *et al.*, 1999). Reports of adverse events suffered during cancer treatment with quinoline derivatives include thrombocytopenia, joint pain (Coutant *et al.*, 1998), leucopenia (Cohn *et al.*, 1998) and severe cardiotoxicity (Cohn *et al.*, 1998; Tan *et al.*, 2000).

Of the four hydroxyquinolines screened on various cancer cell lines and primary cell culture, two were selected for further investigation: HQ5 and HQ10. An *in vivo* acute toxicity study was carried out in a mouse model to determine the toxicity of these two hydroxyquinoline derivatives. To assess the toxicity of these compounds *in vivo*, several parameters were studied. The plasma levels of AST (aspartate aminotransferase), ALT (alanine aminotransferase) and GGT (gamma-glutamyltransferase) are known indicators of hepatotoxicity and cholestasis (Johnston, 1999). Similarly, elevated serum urea and creatinine levels generally suggest nephrotoxicity (Stevens *et al.*, 2005). Daily bodyweight measurements, end point organ weight and haematological studies on whole blood samples were also incorporated as markers for toxicity. A number of organs were collected after completion of the study for organ accumulation studies of the two hydroxyquinolines.

## 7.2. Aim

To investigate potential acute toxic effects of the hydroxyquinolines, HQ5 and HQ10, in the female BALB/c mouse model.

## 7.3. Materials

### 7.3.1. Animals

A total of 50 female BALB/c mice ( $\pm$  20 grams) of 6-8 weeks were used for this study. The animals were housed in standard mouse cages, in randomly selected groups of five to reduce the stress of solitary confinement. The environmental conditions were strictly regulated: 12-hour light/dark cycles, humidity of 40 – 60%, temperature 20 - 24°C, free access to both water and a normal pelleted mouse chow diet. Additional, sterilised

nesting materials were placed in the cages for enrichment. The animals were acclimatised for a minimum of one week with handling before the study commenced.

## 7.4. Methods

### 7.4.1. Dosing, route of administration and sample size

A volume of 150  $\mu$ l of a solution of drug was administered daily at approximately the same time to each animal intraperitoneally, from day 1 to day 5. The higher dosages administered were limited by the maximum concentration obtainable for each compound due to the very low solubility profile of the compounds. Other quinoline derivatives have been administered to BALB/c mice at higher dosages without reported adverse events (Ichikawa *et al.*, 1992; Fujita *et al.*, 2000).

As the compounds are not soluble in water, 5% DMSO was used as diluent. This vehicle has been reported to be tolerable to BALB/c mice *in vivo* (Yoon *et al.*, 2006; Clem *et al.*, 2008; Kayani *et al.*, 2008) and is below the published toxic doses of DMSO (Montaguti *et al.*, 1994; Gaylord Chemical Corporation, 1998). Animals in the control group received 150  $\mu$ l of 5% DMSO solution daily from day one to five.

The solution administered to the animals was prepared freshly prior to administration. The animals were weighed prior to administration, and the volume of dosages was adjusted accordingly. A description of the administration of the experimental compounds is given in Table 1.

**Table 7.1:** Summary of dosages used in the acute toxicity study carried out on BALB/c mice

	Control group	Dose 1	Dose 2
<b>HQ5</b>	DMSO-water solution, 150 $\mu$ l daily from day 1 to day 5 administered i.p. to 10 mice (5% DMSO)	150 $\mu$ l of a 0.05 mg/kg daily from day 1 to day 5 administered i.p. to 10 mice (5% DMSO)	150 $\mu$ l of a 0.1 mg/kg daily from day 1 to day 5 administered i.p. to 10 mice (5% DMSO)
<b>HQ10</b>	10 mice (5% DMSO)	150 $\mu$ l of a 1 mg/kg daily from day 1 to day 5 administered i.p. to 10 mice (5% DMSO)	150 $\mu$ l of a 2 mg/kg daily from day 1 to day 5 administered i.p. to 10 mice (5% DMSO)

#### **7.4.2. Evaluation of toxicity**

The animals were monitored daily for signs of adverse effects or toxicity, including abnormal behaviour or movement, weight loss, restricted mobility, rough hair coat and so forth. Any animal showing signs of pain and distress or whose ability to obtain food and water was severely compromised, was immediately euthanized via Isoflurane overdose and the animal assessed for the same toxicity parameters as those completing the study.

If any sign of toxicity had been observed in two or more animals of the same group, the entire group would have been euthanized and further testing at that specific dosage would have been stopped.

#### **7.4.3. Sample collection and termination of animals**

On day six, the mice were anaesthetised via Isoflurane inhalation, and while the mice were at the surgical plane of anaesthesia, blood samples were collected by cardiac puncture. Uncoagulated blood samples (800 µl/mouse - Li-heparin anticoagulant) were drawn by cardiac puncture. The animals were then terminated via Isoflurane overdose. All procedures were conducted by trained personnel at the University of Pretoria Biomedical Research Centre (UPBRC). Necropsies were performed and organ weights were recorded by VetPath, Onderstepoort. The blood samples were sent for haematological and clinical chemistry analysis at Idexx Laboratories.

#### **7.4.4. Interpretation of results**

To assist in comparison between the groups, the bodyweight measurements for each mouse over six days were normalized to the starting weight and reported as percentage change. Organ weights are reported as percentage body weight according to the following formula:

$$\text{Organ weight} = (\text{organ weight/body weight on day 6}) \times 100$$

The results of the haematological and chemical analysis of the blood are reported as mean  $\pm$  standard error of the mean (SEM). All results were analysed with a one-way ANOVA and Bonferroni post-hoc test. A p-value of  $<0.05$  were used as indicator of

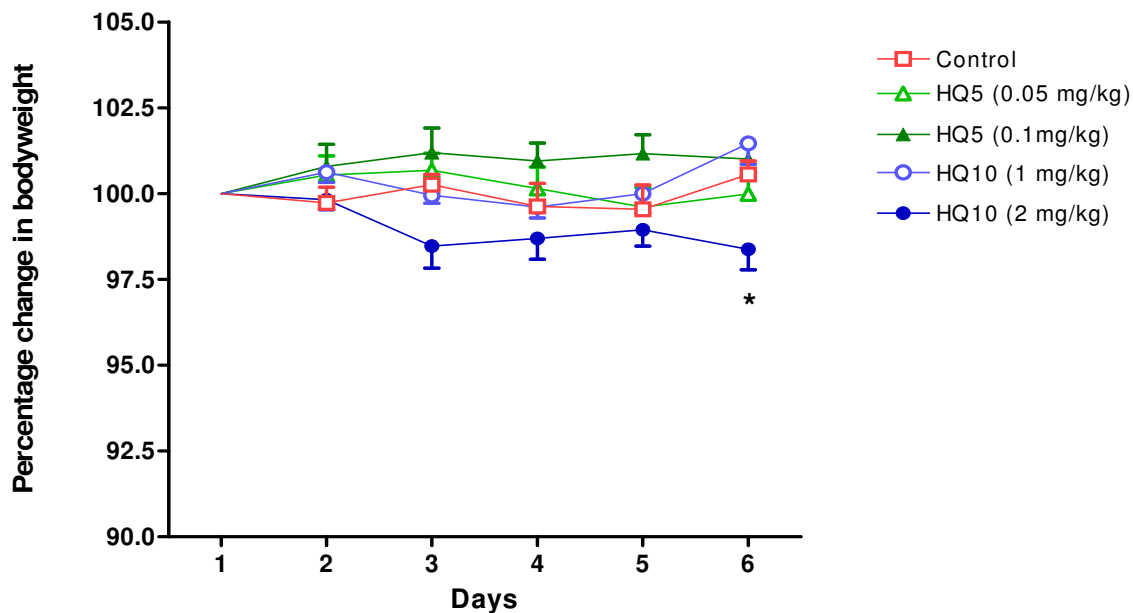
significance. GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) was used for all statistical calculations.

## 7.5. Results

### 7.5.1. Observed adverse effects

The mice were observed daily to detect any outward signs of toxicity due to the administration of the test compounds. No adverse effects were observed in any of the animals during the study period.

A dramatic decrease (10% or more) in body weight within a week can be considered an indicator of toxicity. All animals were weighed daily, and the percentage weight changes are illustrated in Figure 1. It is clear that all groups except the group receiving 2 mg/kg of HQ10 gained weight from day one to six. In the group receiving 0.1 mg/kg of HQ5, a total weight gain of 1% was observed over the study period, while the average weight of the mice dosed with 0.05 mg/kg of HQ5 were exactly the same on day 1 and day 6. The average weight loss of the mice administered 2 mg/kg of HQ10 was 2% on the final day of the study, which differed significantly in comparison to the control group. A weight increase of 1% was observed on day six in the animals in both the 1 mg/kg of HQ10 and control groups.



**Figure 7.1:** The percentage change in body weight of the female BALB/c mice receiving 5% DMSO vehicle alone or the two experimental compounds each at two different concentrations for six consecutive days.

\*  $p < 0.05$

### 7.5.2. Organ weights

The percentage organ weight/body weight was calculated to indicate whether an increase in weight of a specific organ resulted from the administration of the experimental compounds or vehicle. In comparison to the organ weights of the animals in the control group, none of the organ weights of the animals in any of the experimental groups differed significantly. Results are displayed in Table 7.2.

**Table 7.2:** The organ weight/body weight percentage of the BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each. Data is expressed as mean  $\pm$  SEM.

	<b>Spleen</b>	<b>Heart</b>	<b>Kidney</b>	<b>Liver</b>
<b>Control</b>	0.45 $\pm$ 0.08	0.55 $\pm$ 0.02	1.39 $\pm$ 0.03	5.56 $\pm$ 0.08
<b>HQ5 (0.05 mg/kg)</b>	0.49 $\pm$ 0.01	0.51 $\pm$ 0.02	1.35 $\pm$ 0.05	5.16 $\pm$ 0.20
<b>HQ5 (0.1 mg/kg)</b>	0.44 $\pm$ 0.01	0.50 $\pm$ 0.02	1.27 $\pm$ 0.04	4.70 $\pm$ 0.47
<b>HQ10 (1 mg/kg)</b>	0.45 $\pm$ 0.02	0.56 $\pm$ 0.03	1.30 $\pm$ 0.04	5.05 $\pm$ 0.12
<b>HQ10 (2 mg/kg)</b>	0.51 $\pm$ 0.02	0.56 $\pm$ 0.02	1.37 $\pm$ 0.04	5.20 $\pm$ 0.11

A slight increase in the spleen weight could be seen in the animals receiving 2 mg/kg of HQ10 when compared to the control group (0.51% body weight vs. 0.45% body weight). For both the groups receiving HQ5 the weight of the heart was slightly lower (0.50% body weight and 0.51% body weight) than that of the control group (0.55%). As far as the kidney weights of the animals were concerned, those of the animals receiving 0.05 mg/kg of HQ5 (1.35% body weight) and 2 mg/kg of HQ10 (1.37% body weight) were comparable to that of the animals in the control group (1.39% body weight). The kidney weights of the animals in the 0.1 mg/kg HQ5 (1.27% body weight) and 1 mg/kg HQ10 (1.30% body weight) were lower than that of the control animals. The percentage liver weights of the animals in the control group were the highest.

### 7.5.3. Haematology

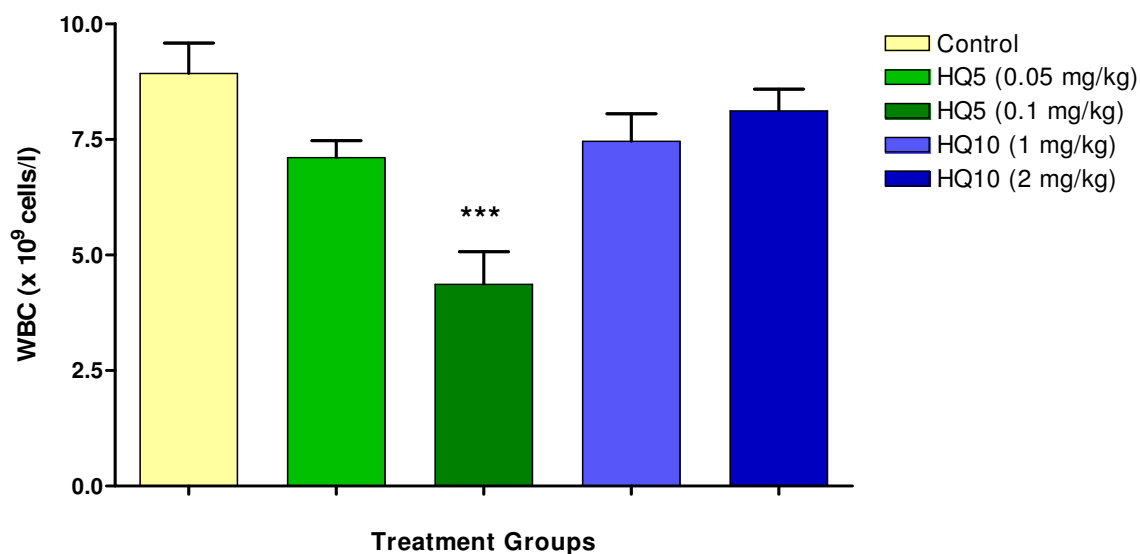
The results of the haematology tests are summarized in Table 7.3. Although the results recorded for each parameter differ between the treatment groups, only the white blood cell count for the animals receiving 0.1 mg/kg of HQ5 ( $4.37 \pm 0.71 \times 10^9$  cells/l) were significantly decreased in comparison to the control group ( $8.92 \pm 0.66 \times 10^9$  cells/l). However, the WCC for HQ5 at 0.1 mg/kg still fall within the normal range (Everds, 2007). Data indicate that all of the parameters investigated, except the mean cell volume (MCV), are lowered in all experimental groups in comparison to the control group. The MCV appear to be elevated by the ingestion of 2 mg/kg of HQ10. Haemoglobin levels

and white blood cell count (WBC) were found to be decreased slightly in the group receiving 0.1 mg/kg of HQ5, as illustrated in Figure 7.2.

**Table 7.3:** Haematology results of the 50 BALB/c mice in the acute toxicity study indicated per treatment group. Data is expressed as mean  $\pm$  SEM.

	RCC x 10 <sup>9</sup> /L	Hb g/dL	PCV %	MCV fL	MCHC g/dL rc	WBC x 10 <sup>9</sup> /L
<b>Control</b>	9.27 $\pm$ 0.30	14.88 $\pm$ 0.20	76.18 $\pm$ 1.83	82.51 $\pm$ 1.18	19.63 $\pm$ 0.36	8.92 $\pm$ 0.66
<b>HQ5 (0.1 mg/kg)</b>	8.82 $\pm$ 0.17	13.99 $\pm$ 0.22	72.65 $\pm$ 1.35	82.46 $\pm$ 0.27	19.26 $\pm$ 0.07	4.37 $\pm$ 0.71 ***
<b>HQ5 (0.05 mg/kg)</b>	9.00 $\pm$ 0.13	14.24 $\pm$ 0.18	73.73 $\pm$ 1.07	81.95 $\pm$ 0.25	19.33 $\pm$ 0.08	7.10 $\pm$ 0.37
<b>HQ10 (2 mg/kg)</b>	8.87 $\pm$ 0.16	14.22 $\pm$ 0.23	73.73 $\pm$ 1.37	83.15 $\pm$ 0.21	19.28 $\pm$ 0.05	8.11 $\pm$ 0.47
<b>HQ10 (1 mg/kg)</b>	9.04 $\pm$ 0.18	14.30 $\pm$ 0.27	74.51 $\pm$ 1.20	82.51 $\pm$ 0.43	19.19 $\pm$ 0.09	7.45 $\pm$ 0.60

\*\*\* p < 0.001 when compared to the untreated control



**Figure 7.2:** The mean white blood cell count (x 10<sup>9</sup> cells/l) of the female BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each. n = 10.

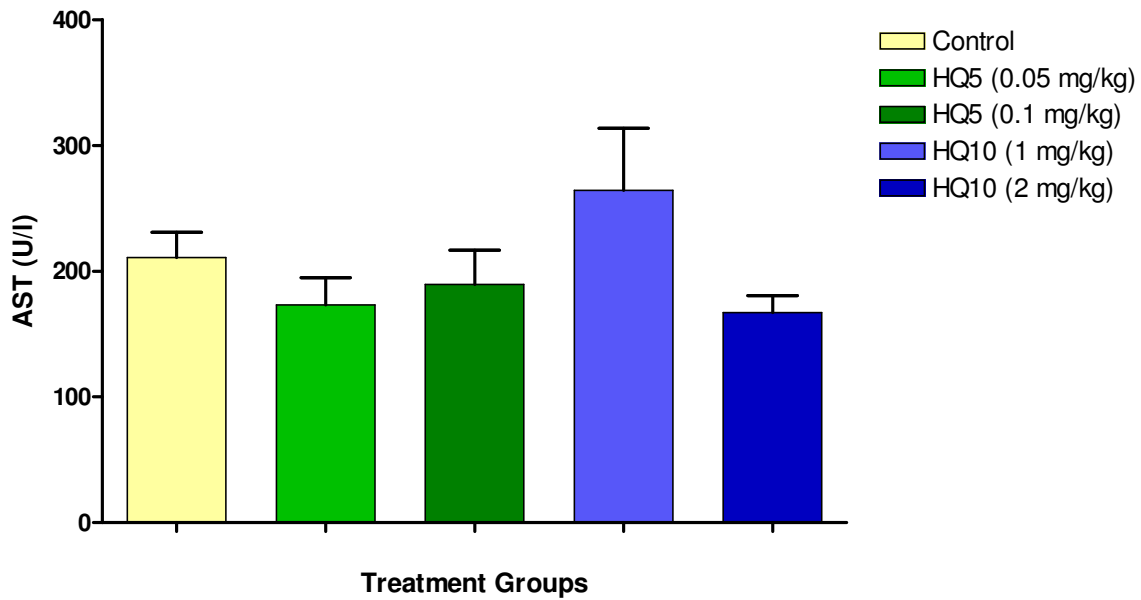
\*\*\* p < 0.001

#### 7.5.4. Serum markers of liver toxicity

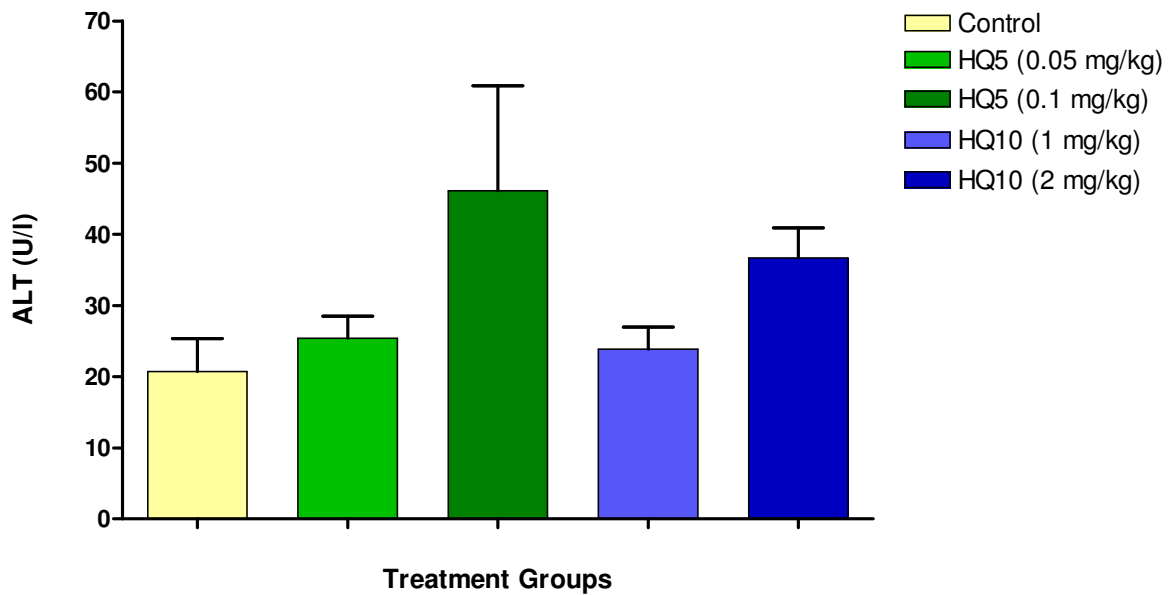
Concentrations of serum markers of liver toxicity, including ALT, AST and GGT, were determined for all the treatment groups in this study and are summarised in Table 7.4. Data indicated that even though the ALT value recorded for the animals receiving 0.1 mg/kg of HQ5 and 2 mg/kg of HQ10 were almost twice that of the animals in the control group, it was not increased significantly. The AST values recorded for the animals administered 1 mg/kg of HQ10 were slightly elevated, but the AST values for all the other groups were decreased in comparison to the control group. However, none of the differences were statistically significant. A slight elevation in the GGT values was seen in the animals that received 0.1 mg/kg of HQ5, although it was again not statistically significant. The results are represented graphically in Figures 7.3 – 7.5.

**Table 7.4:** The results of liver serum markers performed on the 50 BALB/c mice in the acute toxicity study indicated per treatment group. Data is expressed as mean  $\pm$  SEM.

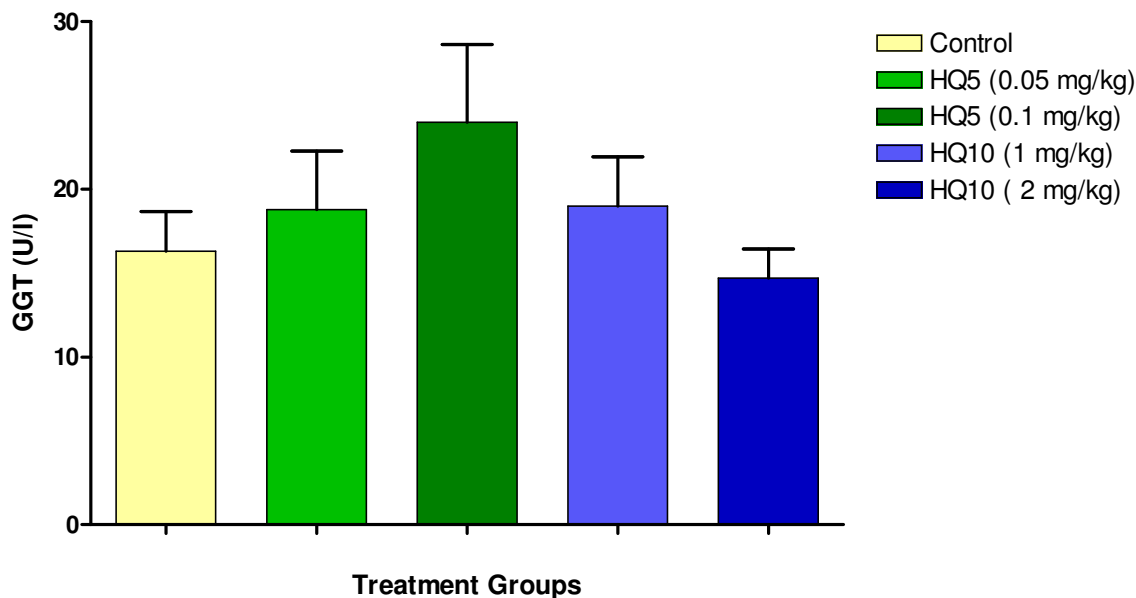
	AST U/l	ALT U/l	GGT U/l
<b>Control</b>	210.8 $\pm$ 20.1	20.7 $\pm$ 4.7	16.3 $\pm$ 2.4
<b>HQ5 (0.05 mg/kg)</b>	173.2 $\pm$ 21.5	25.4 $\pm$ 3.1	18.8 $\pm$ 3.5
<b>HQ5 (0.1 mg/kg)</b>	189.4 $\pm$ 27.2	46.1 $\pm$ 14.8	24.0 $\pm$ 4.6
<b>HQ10 (1 mg/kg)</b>	264.2 $\pm$ 49.5	23.9 $\pm$ 3.0	19.0 $\pm$ 2.9
<b>HQ10 (2 mg/kg)</b>	167.0 $\pm$ 13.4	36.7 $\pm$ 4.2	14.7 $\pm$ 1.7



**Figure 7.3:** The mean AST plasma concentrations (U/l) of the female BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each. The values were standardized to 400 U/l.



**Figure 7.4:** The mean ALT plasma concentrations (U/l) of the female BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each. The values were standardized to 70 U/l.



**Figure 7.5:** The mean GGT plasma concentrations (U/l) of the female BALB/c mice that received the 5% DMSO vehicle alone or the two experimental compounds at two different concentrations each. The values were standardized to 30 U/l.

### 7.5.5. Serum markers of kidney toxicity

The concentrations of serum urea and creatinine were determined in order to establish whether ingestion of the experimental compounds can induce nephrotoxicity. Results are indicated in Table 7.5.

**Table 7.5:** The results of kidney serum markers performed on the 50 BALB/c mice in the acute toxicity study, indicated per treatment group. Data is expressed as mean  $\pm$  SEM.

	Urea mmol/l	Creatinine $\mu$ mol/l
<b>Control</b>	6.03 $\pm$ 0.36	21.10 $\pm$ 1.08
<b>HQ5 (0.05 mg/kg)</b>	5.64 $\pm$ 0.21	19.00 $\pm$ 0.84
<b>HQ5 (0.1 mg/kg)</b>	6.77 $\pm$ 0.25	19.44 $\pm$ 1.17
<b>HQ10 (1 mg/kg)</b>	6.36 $\pm$ 0.45	21.40 $\pm$ 1.50
<b>HQ10 (2 mg/kg)</b>	5.47 $\pm$ 0.28	21.60 $\pm$ 0.79

Administration of 0.05 mg/kg of HQ5 and 2 mg/kg of HQ10 appeared to decrease the serum urea levels slightly. Diminished creatinine levels were reported in the groups receiving HQ5 (0.1 mg/kg and 0.05 mg/kg), although this was not significant. The administration of HQ10 did not seem to influence the concentration of creatinine significantly.

## 7.6. Discussion

An acute toxicity study was performed on 50 female BALB/c mice. During the study period no animals had to be terminated prior to the end of the study. All carcasses were sent for necropsy and haematological and clinical chemistry analysis was performed on blood samples.

The abnormalities observed during the necropsy was for the most part incidental. Atrophy of the fat deposits may have resulted from the daily intra peritoneal injections, although the position of the atrophied adipose tissue was not noted by the pathologist. Haemopericardium and haemothorax noted in four mice from different treatment groups can be attributed to the cardiac puncture.

As it is well-known that weight loss is suggestive of drug-induced toxicity (Clarke *et al.*, 2000; Gorbacheva *et al.*, 2005), weight measurements were taken daily to ascertain whether the experimental compounds had an effect on the appetite of the animals. Weight loss of 20% of original body weight was considered a sign of severe toxicity. The highest percentage of weight loss observed in any animal during this study was 4.6%. It must be mentioned that the weight gained during the study did not surpass 2.5% for any of the groups.

The organ weights revealed that the mean liver weight of the all the experimental groups was diminished in comparison to that of the control group. The liver is not only responsible for the metabolism of carbohydrates, proteins and lipids, but also for regulation of the glucose concentration in blood (Quimby *et al.*, 2007). This is achieved through the removal of glucose from the blood and storing it in the form of glycogen in the liver tissue. The decrease in liver mass might be an indicator that the animals relied on their glycogen supplies for the maintenance of blood sugar levels.

The results obtained from the haematology are, unfortunately, influenced by the sample collection, storage and analysis technique (Everds, 2007). Furthermore, inter-individual differences may cause large variations in the results. It is thus necessary to interpret haematology results by comparison to a published range for each parameter.

Erythrocyte parameters, including red blood cell count, mean cell haemoglobin and mean red cell volume, are used to determine whether the red blood cells have been compromised (Everds, 2007). Haematocrit (PCV) values were all considerably higher than the published normal ranges of 35 – 52%. An increase in PCV is observed in a state of dehydration, which is also common in sick animals (Everds, 2007). The mean red cell volume (MCV) values were elevated in comparison to the reference range. In contrast, the mean cell haemoglobin concentration (MCHC) for all mice was much lower than the 30 to 38 g/dL considered as normal for mice. The elevated MCV and MCHC observed in all of the treatment groups suggest an increase in the average red cell size and may be indicative of a diseased state. However, as elevated PCV, MCV and MCHC levels were reported for all the treatment groups, including the control group, it could be assumed these were not due to exposure to the experimental compounds.

As the liver is responsible for the metabolism of xenobiotics, liver function tests were performed. ALT and AST, two cytoplasmic enzymes responsible for the metabolism of alanine and aspartate respectively, are frequently used as indicators of hepatocellular damage (Masaki *et al.*, 2005). An increase in AST levels may also be an indicator of cardiac muscle damage (Quimby *et al.*, 2007) whereas a correlation exists between ALT concentrations and muscle necrosis (Ozer *et al.*, 2008). When compared to published values, both the average AST and ALT values for each treatment group fall within the normal range. However, it would appear as though the AST and ALT values for an individual mouse in this group, to which 0.1 mg/kg of HQ5 was administered, were highly elevated (370 and 163 U/l, respectively). It can thus be assumed that hepatocellular degeneration occurred. Although this is worrisome, the incident appeared to be isolated as no other animal in the same treatment group had elevated AST or ALT values.

Elevated serum GGT activity values were observed in the group receiving 0.1 mg/kg of HQ5 when compared to the control group. The concentration of serum GGT is typically indicative of hepatobiliary damage, and in particular of cholestasis (Ozer *et al.*, 2008).

This would tie in with the abnormally high AST and ALT values observed in an animal of the same group. The increased concentration of serum markers of liver function suggest that 0.1 mg/kg of HQ5 caused mild hepatotoxicity.

The serum markers for kidney toxicity did not indicate any significant difference between the control group and the experimental groups for any of the markers tested. However, three animals in the 2 mg/kg of HQ10 group did display slightly elevated urea levels (7.4, 7.8 and 9.2 mmol/L). Although there are several factors influencing serum urea levels, the most common are inflammation, a high protein diet and starvation (Quimby *et al.*, 2003). As the animals received a normal pelleted mouse chow diet, and an increase in white blood cell count was not observed in the experimental group receiving 2 mg/kg of HQ10, it would seem as though the ingestion of the experimental compound caused a decreased appetite.

In conclusion, it would appear as though the doses of the experimental hydroxyquinolines administered to the animals were overall well-tolerated. However, the data will have to be confirmed in a larger population study or chronic toxicity study.

## Chapter 8: Final conclusions and recommendations

### 8.1 Discussion

The purpose of this study was to investigate the anti-neoplastic activity of four novel hydroxyquinolines and to assess which of these compounds had the best chance of success as a new lead if developed further. The assessment was initiated in an *in vitro* setting through cytotoxicity screening and mechanistic studies. Two of the compounds with the best selectivity for HeLa cancer cells were subjected to a battery of tests using the COLO 320DM cell line as these cells were still sensitive to the compounds despite having drug resistant characteristics. In the later stages the assays were taken a step closer to clinical testing by performing the first *in vivo* tests of the selected compounds. Although organ accumulation studies of the compounds were also planned neither compound could be detected in any of the collected organ tissue.

The *in vitro* screening of the four experimental compounds was completed to ascertain whether exposure to these quinoline derivatives result in cytotoxicity in cancer cell lines, whilst remaining non-toxic towards normal cells at the same concentrations. To this end seven different commercially available cancer cell lines and three primary (normal) cell cultures were treated with a range of concentrations of the four hydroxyquinolines. A number of resistant cell lines (COLO 320DM, HepG2 and SH-SY5Y cells) were included in the study, as quinolines have been reported to modulate MDR through the inhibition of P-gp (Ullah 2008; Zamora *et al.*, 1988). The cytotoxic assays followed by MTT cell proliferation assay revealed that all four experimental compounds displayed anticancer properties. Data from these cell lines indicate that of these resistant cell lines, only COLO 320DM cells were sensitive to exposure to the experimental compounds. As HepG2 cells also express the MRP-2 xenobiotic transporter (Su *et al.*, 2003), it was speculated whether the experimental compounds are substrates for MRP-2. However, this did not fall within the scope of the project.

As pertains to the results on the primary cell culture, HQ5 and HQ10 were least toxic to normal cells (human lymphocytes and chicken embryo fibroblasts). HQ6 and HQ7

displayed significant cytotoxicity toward the primary cell cultures investigated. Data obtained from the cytotoxicity assays were used to determine which two of the four compounds displayed the most favourable tumour specificities. Based on the tumour specificities using HeLa and lymphocytes as cancer and normal cell models, HQ5 and HQ10 were identified as the most promising compounds and used in all the further investigations.

Based on literature reports of the probable different mechanisms of action by quinoline based drugs the potential mechanisms of action of HQ5 and HQ10 were investigated through a variety of *in vitro* assays aimed at assessing whether these compounds possess the biological activities generally associated with quinoline derivatives and exhibit anti-neoplastic properties.

Many quinoline derivatives have been reported to modulate MDR through the inhibition of P-glycoprotein (Ullah 2008; Zamora *et al.*, 1988), a transmembrane xenobiotics transporter contributing to multidrug resistance in cancer (Meijerman *et al.*, 2008; Ullah 2008). Due to the evidence of P-gp inhibition by quinoline derivatives in literature, the two selected experimental compounds were evaluated for their ability to abrogate P-gp function using two different approaches with separate assays. These were the drug combination therapy on resistant cell lines, and rhodamine 123 retention assay. If favourable results were achieved, these compounds would be potential treatment for resistant cancer.

Combination therapy aims at combining agents with different mechanisms of actions (Frei III 1972). The potential of the hydroxyquinolines, HQ5 and HQ10, as combination therapy agents was investigated by treating resistant cell lines with the combination of HQ5 or HQ10 and the known P-gp substrates doxorubicin, paclitaxel and vinblastine. These substrates were chosen in particular in order to ascertain whether HQ5 and HQ10 abrogate the function of P-gp and increase the cytotoxicity of these compounds. The concentrations of the two experimental compounds used were much higher than would be administered in a clinical setting. A distinct additive effect was obtained when HQ5 and HQ10 were combined with these anticancer drugs known to be P-gp substrates. However, a greater understanding of the mechanism of action of these compounds

could point to better combinations of drugs to achieve a synergistic effect with HQ5 and HQ10.

From the results obtained in the combination therapy assays, it was speculated that the experimental compounds cause cell cycle arrest in the G<sub>1</sub> phase. However, arrest of the cell cycle at the G<sub>1</sub> checkpoint is unpredictable as some variants of Rb are able to circumvent this block (Shapiro *et al.*, 1999). The effect of the two test compounds on the cell cycle of MCF-7 breast cancer cells was determined as this was a sensitive cell not having high P-gp activity which could possibly have affected the outcome of the results. Data indicated that both HQ5 and HQ10 arrested replication at the G<sub>1</sub> checkpoint of the cell cycle. Because cell cycle analysis was only completed on a single clone of the MCF-7 cell line, it remains unclear whether these compounds induce cell cycle arrest in a general manner that can be circumvented. Studies using different cell lines, which express different variants of Rb, will have to be performed to draw a conclusion.

If not circumvented, cell cycle inhibition at the G<sub>1</sub> checkpoint effectively induces apoptosis (Shapiro *et al.*, 1999). MCF-7 breast cancer cells were again used as model to investigate whether exposure to HQ5 and HQ10 induced apoptosis or necrosis. Data indicate that both HQ5 and HQ10 induced apoptosis in MCF-7 breast cancer cells in a time and dose dependent manner. HQ10 was, however, a more potent inducer of apoptosis than HQ5. As the substituents on the benzene ring are the only difference in the structure of HQ5 and HQ10, these data suggest that the halogens present in the structure of HQ10 result in more pro-apoptotic responses.

The lack of synergism observed in the combination therapy made it unlikely that HQ5 and HQ10 inhibited the function of P-gp. However, as it is a known characteristic of quinoline derivatives the effect of the compounds on P-gp was assessed directly through the rhodamine 123 retention assay. Rhodamine 123 is a fluorescent dye that is a P-gp substrate commonly used in studies of P-gp function (Perloff *et al.*, 2003). The assay is based on the principle that P-gp inhibitors will prevent the efflux of the dye from the cytosol, which would result in cells with a high fluorescent intensity as measured flow cytometrically. Data showed that the fluorescent intensity of cells treated with either of the two hydroxyquinolines had a reduced fluorescent intensity in comparison to the

untreated control. This assay indicates that HQ5 and HQ10 did not inhibit P-gp in the three cell lines tested but rather appeared to enhance the efflux activity.

Enhancement of P-gp activity can be explained through the upregulation of P-gp expression. The upregulation of P-gp has been reported in response to stress to the cell, including exposure to some anticancer agents, which are also P-gp substrates (Harmsen *et al.*, 2010). As no inhibition of P-gp was observed, it may be speculated that HQ5 and HQ10 are P-gp substrates and that the elevated concentration of P-gp substrates in the cytosol lead to an upregulation of P-gp. The lack of cytotoxicity towards “resistant” cell lines, as seen from the screening of HQ5 and HQ10 on P-gp expressing cell lines, also suggest that inhibition of P-gp function by these compounds are unlikely. These compounds will therefore not be useful in the treatment of resistant cancer, the effect that was hoped for with these compounds.

Data obtained from the rhodamine 123 retention assay indicate another possibility as to why no synergistic effects could be seen in the combination therapy of HQ5 or HQ10 with known P-gp substrates. The conclusions drawn after the completion of the rhodamine 123 retention assay are supported by the lack of synergism observed in the combination therapy. If the experimental hydroxyquinolines had inhibited P-gp, it is possible that the combination therapy might have yielded synergistic results. As HQ5 and HQ10 were shown not to abrogate the function of P-gp, the lack of synergy observed in the combination therapy is not surprising.

As all novel pharmaceutical compounds must undergo *in vivo* toxicity testing before first-in-man studies can be considered (Robinson *et al.*, 2008), an *in vivo* acute toxicity study was performed using the BALB/c mouse model. The rodent model is generally used as it has been recognised as a reliable model to predict human toxicity (Newell 2004). During the five days’ study period, no obvious signs of toxicity were observed. Even though slight differences in the haematological parameters tested were found between the experimental and untreated control group, it was concluded that the two experimental hydroxyquinolines at the concentrations tested were well tolerated and did not cause severe toxicity. However, these findings were for a short term acute toxicity study and would need to be confirmed using a lower dose chronic toxicity study.

Initially, *ex vivo* organ accumulation analysis was to be performed using LC-MS/MS analysis. The major organs were collected from the animals in the acute toxicity study after necropsy and worked up to extract the accumulated test compounds. A method was successfully developed which separated and quantitated standards of the compounds and an internal standard. When it was attempted to analyse the concentrations of HQ5 and HQ10 in a freshly spiked organ matrix, however, the compounds could not be detected. Further investigation led to the conclusion that HQ5 and HQ10 were unstable in aqueous solutions (data not shown). These findings were confirmed using two different methodologies on an LC-MS/MS system. The organ accumulation studies were not completed due to the failure to detect these compounds in aqueous protic solutions after as little as four hours after spiking. This did raise some further questions as to what was caused the inhibition of cell growth in the *in vitro* studies and what was being taken up by the mice after intraperitoneal administration.

The results of the *in vitro* studies proved that these experimental compounds possess some of the characteristics required of anticancer agents. HQ5 and HQ10 showed selective cytotoxicity against cancer cell lines which translates in a clinical setting to more targeted therapy with an improved side effects profile. Furthermore, the experimental hydroxyquinolines were observed to induce apoptosis in a time and dose dependent manner in a breast cancer cell model. It is generally considered advantageous for anti-neoplastic agents to induce cell death through apoptosis, rather than necrosis, as this avoids surrounding tissue damage due to inflammation (Darzynkiewicz *et al.*, 1992). Preliminary *in vivo* studies indicated that daily intraperitoneal administration of HQ5 and HQ10 did not result in severe toxicity.

However, several unfavourable characteristics were also identified. HQ5 and HQ10 did not inhibit P-gp as expected, but rather enhanced the function of P-gp. This property limits the therapeutic efficacy of these hydroxyquinoline derivatives in the treatment of resistant cancer. Furthermore cell cycle analysis indicated that the experimental compounds induced cell cycle arrest at the G<sub>1</sub> checkpoint. Inhibition of the cell cycle at this checkpoint has been shown to be unpredictable. The physico-chemical properties of these hydroxyquinoline derivatives further complicate matters as this would necessitate precautions to ensure the stability of the compounds before and during administration to humans.

In conclusion, of the four hydroxyquinoline derivatives initially tested, the two most promising compounds were selected for a battery of *in vitro* mechanistic studies and *in vivo* toxicity studies. While these compounds did display anticancer properties *in vitro* and appeared to be well-tolerated in a mouse model, a few serious obstacles were encountered during the study.

## 8.2 Conclusions and recommendations

The data obtained during this study clearly indicate the potential anticancer properties of the four hydroxyquinolines. Of the four experimental compounds, HQ5 and HQ10 were selected as the most promising candidates as the results from the initial *in vitro* cytotoxicity studies indicated that these compounds were the most selectively cytotoxic towards cancer cells with minimal cytotoxicity towards primary (normal) cell culture.

However, a number of major obstacles were encountered during the study. Sufficient testing on the purity and stability of the four hydroxyquinoline derivatives were not completed before the initiation of the study. A full description of the physico-chemical properties of these compounds was not provided. Information on the solubility of experimental compounds can lead to more strategic approaches to preclinical and *in vivo* studies. It was only during the last phase of the study that the impurity and instability of these compounds were recognised. Preclinical studies of novel anticancer compounds should in future not be attempted before sufficient information on the purity, stability and physico-chemical properties of the experimental compounds can be provided. It is also recognized that more in depth analysis techniques, such as LC-MS/MS, should be employed to ascertain the purity and stability of such compounds.

As it was recognised that some physico-chemical characteristics of 8-hydroxyquinolines could have affected the experimental results such as the adsorption of 8-hydroxyquinolines to glass, plastic containers and consumables were used where possible during this project. Derivatives of 8-hydroxyquinolines are also well-known for their metal-complexing abilities (Khaorapong *et al.*, 2010). These complexes remain lipophilic which may allow intracellular competitive metal complexation to be a possible

mechanism of action. During the *in vivo* study this metal complexation may have had an effect on metal dependent enzymes and thus the *in vivo* toxicity and tolerability profile obtained. However, this could also have been one of the reasons that the free compounds were not found in tissue extracts. The design of future studies on this class of compounds should involve greater precautions to ensure that these properties do not bring into question the reliability of the assays performed.

Moreover, it was established that the compounds exhibited some of the key characteristics of lead anticancer compounds. HQ5 and HQ10 were proven to have cytotoxic effects against cancer cell lines. Flow cytometric studies showed that the two experimental compounds induce apoptosis, rather than necrosis, in an *in vitro* setting using a breast cancer cell model. An *in vivo* acute toxicity study showed that these compounds were well-tolerated and caused no obvious signs of toxicity after short term exposure.

However there are also negative aspects which include the following: results from reliable assays proved that these compounds did not display synergism when used in combination with known anticancer drugs, did not inhibit the P-gp efflux pump known to expel many anticancer drugs and caused arrest in the cell cycle at the unreliable G<sub>1</sub> checkpoint. A further major concern was also the lack of chemical stability in protic aqueous solutions. Due to the lack of stability of these compounds (apparent or otherwise), the reliability of the results of the *in vitro* assays using extended times are unfortunately questionable, despite using metal free plastic labware as far as possible.

In addition the *in vivo* studies added further doubt on the success of these two hydroxyquinoline compounds in a clinical setting.

From all the results that were obtained, both positive and negative characteristics were identified for these two compounds but it was concluded that these compounds were unsuitable for further clinical development.

## References

- Allen DD, Caviedes R, Càrdenas AM, Shimahara T, Segura-Aguilar J, Caviedes PA. 2005. Cell lines as in vitro models for drug screening and toxicity studies. *Drug Development and Industrial Pharmacy* **31**: 757-768.
- Aubry JP, Blaecke A, Lecoanet-Henchoz S, Jeannin P, Herbault N, Caron G, Moine V, Bonnefoy JY. 1999. Annexin V used for measuring apoptosis in the early events of cellular cytotoxicity. *Cytometry* **37**: 197-204.
- Bachmeier CJ, Miller DW. 2005. A fluorometric screening assay for drug efflux transporter activity in the blood-brain barrier. *Pharmaceutical Research* **22**: 113-121.
- Baillie TA. 2008. Metabolism and toxicity of drugs. Two decades of progress in industrial drug metabolism. *Chemical Research in Toxicology* **21**: 129-137.
- Belliard AM, Leroy C, Banide H, Farinotti R, Lacour B. 2003. Decrease of intestinal P-glycoprotein activity by 2*n*-propylquinoline, a new oral treatment for visceral leishmaniasis. *Experimental Parasitology* **103**: 51-56.
- Bolognese A, Carreale G, Manfra M, Lavecchia A, Mazzoni O, Novellino E, La Colla P, Sanna G, Loddo R. 2004. Antitumor agents. 3. Design, synthesis and biological evaluation of pyridoisoquinolindione and dihydrothienoquinolindione derivatives with potent cytotoxic activity. *Journal of Medicinal Chemistry* **47**: 849-858.
- Botham PA. 2002. Acute systemic toxicity. *Institute for Laboratory Animal Research Journal* **43**: S27-S30.
- Browning CH, Gulbransen R, Kennaway EL, Thornton LHD. 1917. Flavine and brilliant green, powerful antiseptics with low toxicity to the tissues: their use in the treatment of infected wounds. *The British Medical Journal* **i**: 73-78.

Cai W, Hassani M, Karki R, Walter ED, Koelsch KH, Seradj H, Lineswala JP, Mirzaei H, York JS, Olang F, Sedighi M, Lucas JS, Eads TJ, Rose AS, Charkhzarrin S, Hermann NG, Beall HD, Behforouz M. 2010. Synthesis, metabolism and in vitro cytotoxicity studies on novel lavendamycin antitumor agents. *Bioorganic and Medicinal Chemistry* **18**: 1899-1909.

Callaghan R, Crowley E, Potter S, Kerr ID. 2008. P-glycoprotein: So many ways to turn it on. *Journal of Clinical Pharmacology* **48**: 365-378.

Chandrika PM, Yakaiah T, Rao ARR, Narsaiah B, Reddy NC, Sridhar V, Rao JV. 2008. Synthesis of novel 4,6-disubstituted quinazoline derivatives, their anti-inflammatory and anticancer activity (cytotoxic) against U937 leukemia cell lines. *European Journal of Medicinal Chemistry* **43**: 846-852.

Chang YH, Hsu MH, Wang SH, Huang LJ, Qian K, Morris-Natschke SL, Hamel E, Kuo SC, Lee KH. 2009. Design and synthesis of 2-(3-Benzo[*b*]thienyl)-6,7-methylenedioxyquinolin-4-one analogues as potent antitumor agents that inhibit tubulin assembly. *Journal of Medicinal Chemistry* **52**: 4883-4891.

Chemicon<sup>®</sup> International. 2003. *Multidrug resistance direct dye efflux assay instructions*. Chemicon<sup>®</sup> International.

Chiang C-Y, Centis R, Migliori GB, Lange C. 2010. Drug-resistant tuberculosis: past, present, future. *Respirology* **15**: 413-432.

Chibale K, Ojima I, Haupt H, Geng X, Pera P, Bernacki RJ. 2001. Modulation of human mammary cell sensitivity to paclitaxel by new quinoline sulfonamides. *Bioorganic and Medicinal Chemistry Letters* **11**: 2457-2460.

Chou TC. 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacological Reviews* **58**: 621-681.

Clarke SJ, Farrugia DC, Aherne GW, Pritchard DM, Benstead J, Jackman AL. 2000. Balb/c mice as a preclinical model for Raltitrexed-induced gastrointestinal toxicity. *Clinical Cancer Research* **6**: 285-296.

Clem B, Telang S, Clem A, Yalcin A, Meier J, Simmons A, Rasku MA, Arumugam S, Dean W, Eaton J, Lane A, Trent J, Chesney J. 2008. Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. *Molecular Cancer Therapy* **7**: 110-120.

Cohen GM. 1997. Caspases: the executioners of apoptosis. *Biochemical Journal* **326**: 1-16.

Cohn JN, Goldstein SO, Greenberg BH, Lorell BH, Bourge RC, Jaski BE, Gottlieb SO, McGrew F, DeMets DL, White BG. 1998. A dose-dependent increase in mortality with vesnarinone among patients with severe heart failure. *The New England Journal of Medicine* **339**: 1810-1816.

Collins K, Jacks T, Pavletich NP. 1997. The cell cycle and cancer. *Proceedings of the National Academy of Sciences* **94**: 2776-2778.

Cooper GM. 2000. The cell: a molecular approach. Second edition. Boston: Sinauer Associates.

Coutant R, Landais P, Rosilio M, Johnsen C, Lahlou N, Chatclain P, Carel JC, Ludvigsson J, Boitard C, Bougnères PF. 1998. Low dose liomide in Type I juvenile diabetes of recent onset: a randomized placebo-controlled double blind trial. *Diabetologia* **41**: 1040-1046.

Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P, Traganos F. 1992. Features of apoptotic cells measured by flow cytometry. *Cytometry* **13**: 795-808.

De SK, Gibbs RA. 2005. A mild and efficient one-step synthesis of quinolines. *Tetrahedron Letters* **46**: 1647-1649.

De Bruin EC, Medema JP. 2008. Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treatment Reviews* **34**: 737-749.

DeGeorge JJ, Ahn CH, Andrews PA, Brower ME, Giorgio DW, Goheer MA, Lee-Ham DY, McGuinn WD, Schmidt W, Sun CJ, Tripathi SC. 1998. Regulatory considerations for preclinical development of anticancer drugs. *Cancer Chemotherapy and Pharmacology* **41**: 173-185.

Dey S, Ramachandra M, Pastan I, Gottesman MM, Ambudkar SV. 1997. Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. *Proceedings of the National Academy of Sciences* **94**: 10594-10599.

Di Nicolantonio F, Mercer SJ, Knight LA, Gabriel FG, Whitehouse PA, Sharma S, Fernando A, Glaysher S, Di Palma S, Johnson P, Somers SS, Toh S, Higgins B, Lamont A, Gulliford T, Hurren J, Yiangou C, Cree IA. 2005. Cancer cell adaptation to chemotherapy. *BMC Cancer* **5**: 78-89.

Dong X, Mattingly CA, Tseng MT, Cho MT, Liu Y, Adams VR, Mumper RJ. 2009. Doxorubicin and paclitaxel-loaded lipid-based nanoparticles overcome multidrug resistance by inhibiting P-glycoprotein and depleting ATP. *Cancer Research* **69**: 3918-3926.

European Medicines Agency. 2008. Committee for Medicinal Products for Human Use Draft: Non-clinical Guideline on Drug-induced Hepatotoxicity.

Evan GI, Vousden KH. 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature* **411**: 342-348.

Everds NE. 2007. Hematology of the laboratory mouse. In *The mouse in biomedical research: normative biology, husbandry and models*, JG Fox, SW Barthold, MT Davisson, CE Newcomer, FW Quimby, AL Smith, Eds. San Diego: Academic Press.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *The Journal of Immunology* **148**: 2207-2216.

Fan S, Smith L, Rivert DJ, Duba Diane, Zhan Q, Kohn KW, Fornace AJ, O'Connor PM. 1995. Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. *Cancer Research* **55**: 1649-1654.

Ferlin MG, Marzano C, Via LD, Chilin A, Zagotto G, Guiotto A, Moro S. 2005. New water soluble pyrroloquinoline derivatives as new potential anticancer agents. *Bioorganic and Medicinal Chemistry* **13**: 4733-4739.

Frei III E, Freireich EJ, Gehan E, Pinkel D, Holland JF, Selawry O, Haurani F, Spurr CL, Hayes DM, James GW, Rothberg H, Sodee DB, Rundles RW, Schroeder LR, Hoogtraten B, Wolman IJ, Traggis DG, Cooper T, Ebaugh F, Taylor R. 1961. Studies of sequential and combination antimetabolite therapy in acute leukemia: 6-mercaptopurine and methotrexate. *Blood* **18**: 431-454.

Frei III E. 1972. Combination cancer therapy: presidential address. *Cancer Research* **32**: 2593-2607.

Fried J, Perez AG, Clarkson BD. 1976. Flow cytofluorometric analysis of cell cycle distributions using propidium iodide. *The Journal of Cell Biology* **71**: 172-181.

Fujita M, Fujita T, Higashino K. 2000. Antitumor effect of a quinolinone derivative, vesnarinone, on mouse hepatoma. *Hepatology Research* **17**: 65-71.

Gakhar G, Ohira T, Shi A, Hua DH, Nguyen TA. 2008. Antitumour effect of substituted quinolines in breast cancer cells. *Drug Development Research* **69**: 526-534.

Gaylord Chemical Corporation. 1998. *Dimethyl sulfoxide (DMSO) health effects information*. Slidell: Gaylord Chemical Corporation.

Gazzaniga P, Silvestri I, Gradilone A, Aglianò AM. 2008. The increasing use of intravesical therapies for stage T1 bladder cancer coincides with decreasing survival after cystectomy. *BJU International* **101**: 127-129.

Giannakakou O, Robey R, Fojo T, Blagosklonny MV. 2001. Low concentrations of paclitaxel induced cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. *Oncogene* **20**: 3806-3813.

Gorbacheva VY, Kondratov RV, Zhang R, Cherukuri S, Gudkov AV, Takahashi JS, Antoch MP. 2005. Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends on the functional status of the CLOCK/BMAL1 transactivation complex. *Proceedings of the National Academy of Sciences* **102**: 3407-3412.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* **100**:57-70.

Harmsen S, Meijerman I, Febus CL, Maas-Bakker RF, Beijnen JH, Schellens JHM. 2010. PXR-mediated induction of P-glycoprotein by anticancer drugs in a human colon adenocarcinoma-derived cell line. *Cancer Chemotherapy and Pharmacology* **66**: 765-771.

Hartwell LH, Weinert TA. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science* **246**: 629-634.

Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, Cámara M. 2011. Quinolones: from antibiotics to autoinducers. *FEMS Microbiology Reviews* **35**: 247-274.

Henry M, Alibert S, Rogier C, Barbe J, Pradines B. 2008. Inhibition of efflux of quinolones as new therapeutic strategy in malaria. *Current Topics in Medicinal Chemistry* **8**: 563-578.

Hirschberg E. 1958. Tissue culture in cancer chemotherapy screening. *Cancer Research* **18**: 869-878.

Honma Y, Yamamoto-Yamaguchi Y, Kanatani Y. 1999. Vesnarinone and glucocorticoids cooperatively induce G1 arrest and have an anti-tumour effect on human non-small cell lung carcinoma cells grown in nude mice. *British Journal of Cancer* **80**: 96-103.

Hsu ZS, Chen C, Lee PT, Chiu SJ, Liu HF, Tsai CC, Chao JI. 2008. 7-Chloro-6-piperidin-1-yl-quinoline-5,8-dione (PT-262), a novel synthetic compound induces lung carcinoma cell death associated with inhibiting ERK and CDC2 phosphorylation via a p53-independent pathway. *Cancer Chemotherapy and Pharmacology* **62**: 799-808.

Huang YF, Shangguan D, Liu H, Phillips JA, Zhang X, Chen Y, Tan W. 2009. Molecular assembly of an aptamer-drug conjugate for targeted drug delivery to tumor cells. *ChemBioChem* **10**: 862-868.

Ichikawa T, Lamb J, Christensson P, Hartley-Asp B, Isaacs J. 1992. The antitumor effects of the quinoine-3-carboxamide linomide on Dunning R-3327 rat prostatic cancers. *Cancer Research* **52**: 3022-3028.

Izawa JI, Dinney CPN. 2001. The role of angiogenesis in prostate and other urologic cancers: a review. *Canadian Medical Association Journal* **164**: 662-670.

Jansen WJM, Pinebo HM, Van der Wilt CL, Feller N, Bamberger U, Boven E. 1995. The influence of BIBW22BS, a dipyridamole derivative, on the antiproliferative effects of 5-fluorouracil, methotrexate and gemcitabine *in vitro* and in human tumour xenografts. *European Journal of Cancer* **31A**: 2313-2319.

Jantova S, Repický A, Letašiová S, Čipák L. 2008. 4-Amino-3-acetylquinoline-induced apoptosis of murine L1210 leukemia cells involves ROS-mitochondrial-mediated death signaling and activation of p38 MAPK. *Cell Biochemistry and Function* **26**: 609-619.

Jasinski P, Welsh B, Galvez J, Land D, Zwolak P, Ghandi L, Terai K, Dudek AZ. 2008. A novel quinoline, MT477: suppresses cell signaling through Ras molecular pathway, inhibits PKC activity and demonstrates *in vivo* anti-tumour activity against human carcinoma cell lines. *Invest New Drugs* **26**: 223-232.

Johnston DE. 1999. Special considerations in interpreting liver function tests. *American Family Physician*.

Karpińska G, Mazurek AP, Dobrowolski JC. 2010. On tautomerism and substituent effect in 8-hydroxyquinoline-derived medicine molecules. *Journal of Molecular Structure: THEOCHEM* **961**: 101-106.

Kastan MB, Bartek J. 2004. Cell-cycle checkpoints and cancer. *Nature* **432**: 316-323.

Kawase M, Motohashi N. 2003. New multidrug resistance reversal agents. *Current Drug Targets* **4**: 31-43.

Kayani AC, Close GL, Broome CS, Jackson MJ, McArdle A. 2008. Enhanced recovery from contraction-induced damage in skeletal muscles of old mice following treatment with the heat shock protein inducer 17-(allylamino)-17-demethoxygeldanamycin. *Rejuvenation Research* **11**: 1021-1030.

Kelloff GJ, Johnson JR, Crowell JA, Boone CW, DeGeorge JJ, Steele VE, Mehta MU, Temeck, JW, Schmidt WJ, Burke G, Greenwald P, Temple RJ. 1995. Approaches to the development and marketing approval of drugs that prevent cancer. *Cancer Epidemiology, Biomarkers and Prevention* **4**: 1-10.

Kemnitzer W, Kuemmerle J, Jiang S, Zhang HZ, Sirisoma N, Kasibhatla S, Crogan-Grundy C, Tseng B, Drewe J, Cai SX. 2008. Discovery of 1-benzoyl-3-cyanopyrrolo [1,2-a]quinolines as a new series of apoptosis inducers using a cell- and caspase-based high-throughput screening assay. Part 1: Structure-activity relationships of the 1- and 3-positions. *Bioorganic and Medicinal Chemistry Letters* **18**: 6259-6264.

Kerr JFR, Winterford CM, Harmon BV. 1994. Apoptosis: Its significance in cancer and cancer therapy. *Cancer* **73** (8): 2013-2026.

Khaorapapong N, Ogawa M. 2010. Formation of mono(8-hydroxyquinoine) lithium (I) complex in smectites by solid-solid reactions. *Journal of Physics and Chemistry of Solids* **71**: 1644-1650.

Kim S, Joo SW. 2005. Adsorption of 2'-biquinoline and 8-hydroxyquinoline on gold and silver nanoparticle surfaces. *Vibrational Spectroscopy* **39**: 74-80.

Koo JS, Choi WC, Rhee YH, Lee HJ, Lee EO, Ahn KS, Bae HS, Ahn KS, Kang JM, Choi SU, Kim MO, Lu J, Kim SH. 2008. Quinoline derivative KB3-1 potentiates paclitaxel induced cytotoxicity and cell cycle arrest via multidrug resistance reversal in MES-SA/DX5 cancer cells. *Life Science* **83**: 700-708.

Kouznetsov VV, Méndez LYV, Gómez CMM. 2005. Recent progress in the synthesis of quinolines. *Current Organic Chemistry* **9**: 141-161.

Lachmann P. 1992. The use of animals in research: medical progress depends on it. *British Medical Journal* **305**:6844.

Laloo UG, Ambaram A. 2010. New antituberculous drugs in development. *Current HIV/AIDS Reports* **7**: 143-151.

Lamazzi C, Léonce S, Pfeiffer B, Renard P, Guillaumet G, Rees CW, Besson T. 2000. Expeditious synthesis and cytotoxic activity of new cyanoindolo[3,2-*c*]quinolines and benzimidazo[1,2-*c*]quinazolines. *Bioorganic and Medicinal Chemistry Letters* **19**: 2183-2185.

Le Bot MA, Kernaleguen D, Robert J, Berlion M, Riché C. 1994. Modulation of anthracycline accumulation and metabolism in rat hepatocytes in culture by three revertants of multidrug resistance. *Cancer Chemotherapy and Pharmacology* **35**: 53-58.

Levenson AS, Jordan VC. 1997. MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Research* **57**: 3071-3078.

Li SY, Chen YL, Wang C, Tzeng CC. 2006. Synthesis and antiproliferative evaluation of certain pyrido[3,2-*g*]quinoline derivatives. *Bioorganic and Medicinal Chemistry* **14**: 7370-7376.

Lipunova GN, Nosova EV, Mokrushina GA, Sidorova LP, Charushin VN. 2000. Antitumour activity of the fluorinated derivatives of condensed quinolines and quinazolines. *Pharmaceutical Chemistry Journal* **34**: 20-23.

Loehberg CR, Thompson T, Kastam MB, Maclean KH, Edwards DG, Kittrell FS, Medina D, Conneely OM, O'Malley BW. 2007. Ataxia telangiectasia-mutated and p53 are potential mediators of chloroquine-induced resistance to mammary carcinogenesis. *Cancer Research* **67**: 12026-12033.

Majno G, Joris I. 1995. Apoptosis, oncosis and necrosis: An overview of cell death. *American Journal of Pathology* **146** (1): 3-15.

Martinelli A, Moreira R, Cravo PVL. 2008. Malaria combination therapies: advantages and shortcomings. *Mini-Reviews in Medicinal Chemistry* **8**: 201-212.

Martirosyan AR, Rahim-Bata R, Freeman AB, Clarke CD, Howard RL, Strobl JS. 2004. Differentiation-inducing quinolines as experimental breast cancer agents in the MCF-7 breast cancer cell model. *Biochemical Pharmacology* **68**: 1729-1738.

Masaki T, Chiba S, Tatsukawa H, Noguchi H, Kakuma T, Endo M, Seike M, Watanabe T, Yoshimatsu H. 2005. The role of histamine H<sub>1</sub> receptor and H<sub>2</sub> receptor in LPS-induced liver injury. *The FASEB Journal* **19**: 1245-1252.

Massagué J. 2004. G1 cell-cycle control and cancer. *Nature* **432**: 298-306.

Masters J. 2002. HeLa cells 50 years on: the good, the bad and the ugly. *Nature Reviews: Cancer* **2**: 315-319.

McKeage MJ, Maharaj L, Berners-Price SJ. 2002. Mechanisms of cytotoxicity and antitumour activity of Gold(I) phosphine complexes: the possible role of mitochondria. *Coordination Chemistry Reviews* **232**: 127-135.

Meijerman I, Beijnen JH, Schellens JHM. 2008. Combined action and regulation of phase II enzymes and multidrug resistance proteins in multidrug resistance in cancer. *Cancer Treatment Reviews* **34**: 505-520.

Mooney LM, Al-Sakkaf KA, Brown BL, Dobson PRM. 2002. Apoptotic mechanisms in T47D and MCF-7 human breast cancer cells. *British Journal of Cancer* **87**: 909 – 917.

Montaguti P, Molloni E, Cavallotti E. 1994. Acute intravenous toxicity of dimethyl sulfoxide, polyethylene glycol 400, dimethylformamide, absolute ethanol, and benzyl alcohol in inbred mouse strains. *Arzneimittelforschung* **44**: 566-570.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**: 55-63.

Muregi FW. 2010. Antimalarial drugs and their useful therapeutic lives: rational drug design lessons from pleiotropic action of quinolines and artemisinins. *Current Drug Technologies* **7**: 280-316.

Naito M, Tsuruo T. 1998. Therapeutic approach to drug resistant tumours. *Therapeutic Drug Monitoring* **20**: 557-580.

Naito M, Matsuba Y, Sato S, Hirata H, Tsuruo T. 2002. MS-209, a quinoline-type reversal agents, potentiates antitumor efficacy of docetaxel in multidrug-resistant solid tumor xenograft models. *Clinical Cancer Research* **5**: 582-588.

Narasaki F, Oka, M, Fukuda M, Nkano R, Ikeda K, Takatani H, Terachi K, Soda H, Yano O, Nakamura T, Doyle LA, Tsuruo T, Kohno S. 1997. A novel quinoline derivative, MS-209, overcomes drug resistance of human lung cancer cells expressing the multidrug resistance-associated protein (MRP) gene. *Cancer Chemotherapy and Pharmacology* **40**: 425-432.

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods. 2000. The Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) Summary.

Newell DR, Sylvester J, McDowell C, Vurtless SS. 2004. The cancer research UK experience of pre-clinical toxicology studies to support early clinical trials with novel cancer therapies. *European Journal of Cancer* **40**: 899-906.

Nien CY, Chen YC, Kuo CC, Hsieh HP, Chang CY, Wu JS, Wu SY, Liou JP, Chang JY. 2010. 5-Amino-2-arylquinolines as highly potent tubulin polymerization inhibitors. *Journal of Medicinal Chemistry* **53**: 2309-2313.

Ochatt SJ. 2006. Flow cytometry: ploidy determination, cell cycle analysis, DNA content per nucleus. In *Medicago truncatula* handbook. The Samuel Roberts Nobel Foundation.

O'Donnell F, Smyth TJP, Ramachandran VN, Smyth WF. 2010. A study of the antimicrobial activity of selected synthetic and naturally occurring quinolines. *International Journal of Antimicrobial Activity* **35**: 30-38.

Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. 2008. The current state of serum biomarkers of hepatotoxicity. *Toxicology* **245**: 194-205.

Perez EA. 2009. Microtubule inhibitors: Differentiating tubulin-inhibiting agents based on mechanisms of action, clinical activity, and resistance. *Molecular Cancer Therapeutics* **8**: 2086-2095.

Perloff MD, Störmer E, Van Moltke LL, Greenblatt DJ. 2003. Rapid assessment of p-glycoprotein and induction *in vitro*. *Pharmaceutical Research* **20**: 1177-1183.

Quimby FW, Luong RH. 2007. Clinical chemistry of the laboratory mouse. In *The mouse in biomedical research: normative biology, husbandry and models*, JG Fox, SW Barthold, MT Davisson, CE Newomer, FW Quimby, AL Smith, Eds. San Diego: Academic Press.

Quinn LA, Moore GE, Morgan RT, Woods LK. 1979. Cell lines from human colon carcinoma with unusual cell products, double minutes, and homogeneously staining regions. *Cancer Research* **39**: 4914-4924.

Raff MC. 1992. Social controls on cell survival and cell death. *Nature* **356**: 397-400.

Robinson S, Delogeas JL, Donald E, Dreher D, Festag M, Kervyn S, Lampo A, Nahas K, Noguez V, Ockert D, Quinn K, Old S, Pickersgill N, Somers K, Stark C, Stei P, Waterson L, Chapman K. 2008. A European pharmaceutical company initiative challenging the

regulatory requirement for acute toxicity studies in pharmaceutical drug development. *Regulatory Toxicology and Pharmacology* **50**: 345 – 352.

Rosenthal PJ, Miller LH. 2001. The need for new approaches to antimalarial chemotherapy. In *Antimalarial chemotherapy: mechanisms of action, resistance and new directions in drug discovery*, Rosenthal PJ, Eds. Totowa: Humana Press.

Roy U, Chahravarty G, Bentrup KHZ, Mondal D. 2009. Montelukast is a potent and durable inhibitor of multidrug resistance protein 2-mediated efflux of taxol and saquinavir. *Biological and Pharmaceutical Bulletin* **32**: 2002-2009.

Rudnev AV, Foteeva LS, Kowol C, Berger R, Jakupec MA, Arion VB, Timerbaev AR, Keppler BK. 2006. Preclinical characterization of anticancer gallium(III) complexes: solubility, stability, lipophilicity and binding to serum proteins. *Journal of Inorganic Biochemistry* **100**: 1819-1826.

Ruiz-Garcia A, Bermejo M, Moss A, Casabo VG. 2008. Pharmacokinetics in drug discovery. *Journal of Pharmaceutical Sciences* **97**: 654-690.

Sánchez CA, Rodriguez E, Varela E, Zapata E, Páez A, Massó FA, Montano LF, López-Marure R. 2008. Statin-induced inhibition of MCF-7 breast cancer cell proliferation is related to cell cycle arrest and apoptotic and necrotic cell death mediated by an enhanced oxidative stress. *Cancer Investigation* **26**: 698-707.

Sanders ME, Moore QC, Norcross EW, Sanfilippo CM, Hesje CK, Shafiee A, Marquart ME. 2011. Comparison of besifloxacin, gatifloxacin, and moxifloxacin against strains of *Pseudomonas aeruginosa* with different quinolone susceptibility patterns in a rabbit model of keratitis. *Cornea* **30**: 83-90.

Sankaran M, Kumarasamy C, Chokkalingam U, Mohan PS. 2010. Synthesis, antioxidant and toxicological study of novel pyrimido quinoline derivatives from 4-hydroxyl-3-acyl quinolin-2-one. *Bioorganic and Medicinal Chemistry Letters* **20**: 7147-7151.

Sato M, Harada K, Bando T, Shirakami T, Nakashiro K, Yoshida H, Nakai S, Kawai K, Adachi M. 1995. Characteristics of antitumour activity of 3,4-dihydro-6-[4-(3,4-dimethoxybenzoyl)-1-piperazinyl]-2(1H)-quinolinone (Vesnarinone) against a human adenoid squamous carcinoma forming cell line grown in athymic nude mice. *Cancer Letters* **91**: 1-9.

Savarino A, Lucia MB, Giordano F, Cauda R. 2006. Risks and benefits of chloroquine use in anticancer strategies. *The Lancet* **7**: 792-793.

Sedic M, Poznic M, Gehrig P, Scott M, Schlapbach R, Hranjec M, Karmiski-Zamola G, Pavelic K, Pavelic SK. 2008. Differential antiproliferative mechanisms of novel derivative of benzimidazo[1,2-a]quinoline in colon cancer cells depending on their p53 status. *Molecular Cancer Therapeutics* **7**: 2121-2132.

Sekiguchi M, Shiroko Y, Arai T, Kishino T, Sugawara I, Kusakabe T, Suzuki T, Yamashita T, Obara T, Ito K, Hasumi K. 2001. Biological characteristics and chemosensitivity profile of four human anaplastic thyroid carcinoma cell lines. *Biomedicine and Pharmacotherapy* **55**: 466-474.

Shah MA, Schwartz G. 2001. Cell cycle-mediated drug-resistance: An emerging concept in cancer therapy. *Clinical Cancer Research* **7**: 2168-2181.

Shahabuddin MS, Nambiar M, Choudhary B, Advirao GM, Raghavan SC. 2010. A novel DNA intercalator, butylamino-pyrimido[4',5':4,5]selenolo(2,3-b)quinoline, induces cell cycle arrest and apoptosis in leukemic cells. *Invest New Drugs* **28**: 35-48.

Shapiro GI, Harper JW. 1999. Anticancer drug targets: cell cycle and checkpoint control. *The Journal of Clinical Investigation* **104**: 1645-1653.

Sieczkowski E, Lehner C, Ambros PF, Hohenegger M. 2009. Double impact on p-glycoprotein by statins enhances doxorubicin cytotoxicity in human neuroblastoma cells. *International Journal of Cancer* **126**: 2025-2035.

Simon A, Allias DP, Duroux JL, Basly JP, Durand-Fontanier S, Delage S. 1998. Inhibitory effect of curcuminoids on MCF-7 cell proliferation and structure-activity relationships. *Cancer Letters* **129**: 111-116.

Steiner H, Polliack A, Kimchi-Sarfaty C, Libster D, Fibach E, Rund D. 1998. Differences in rhodamine-123 efflux in B-type chronic lymphocytic leukemia suggest possible gender and stage variations in drug-resistance gene activity. *Annals hematology* **76**: 189-194.

Stevens LA, Levey AS. 2005. Measurement of kidney function. *The Medical Clinics of North America* **89**: 457-473.

Stubbings W, Labischinski H. 2009. New antibiotics for antibiotic-resistant bacteria. *Biology Reports* **1**: 40.

Su Y, Zhang X, Sinko PJ. 2003. Human organic anion-transporting polypeptide OATP-A (SLC21A3) acts in concert with P-glycoprotein and multidrug resistance protein 2 in the vectorial transport of saquinavir in Hep G2 cells. *Molecular Pharmaceutics* **1**: 49-56.

Suh ME, Kang MJ, Yoo HW, Park SY, Lee CO. 2000. Synthesis and cytotoxicity of 2-methyl-1-substituted-imidazo [4,5-*g*]quinoline-4,9-dione and 7,8-dihydro-10*H*-[1,4]oxazino[3',4': 2,3]imidazo[4,5-*g*]quinoline-5,12-dione derivatives. *Bioorganic and Medicinal Chemistry* **8**: 2079-2083.

Sundquist T, Moravec R, Niles A, O'Brien M, Riss T. 2006. Timing your apoptosis assays. *Cell Notes* **16**: 18-21.

Tan IL, Lycklama à Nijeholt GJ, Polman CH, Adèr HJ, Barkhof F. 2000. Linomide in the treatment of multiple sclerosis: MRI results from prematurely terminated phase-III trials. *Multiple Sclerosis* **6**: 99-104.

Tiekink, E.R.T. 2002. Gold derivatives for the treatment of cancer. *Critical Reviews in Oncology/Hematology* **42**: 225-248.

Ullah MF. 2008. Cancer multidrug resistance (MDR): a major impediment to effective chemotherapy. *Asian Pacific Journal of Cancer Prevention* **9**: 1-6.

Valecha N, Phyo AP, Mayxay M, Newton PN, Krudsood S, Keomany S, Khanthavong M, Pongvongsa T, Ruangveerayuth R, Uthaisil C, Ubben D, Duparc S, Bacchieri A, Corsi M, Rao BHK, Bhataacharya PC, Dubhashi N, Ghosh SK, Dev V, Kumar A, Pukittayakamee S. 2010. An open-label, randomized study of dihydroartemisinin-piperaquine versus artesunate-mefloquine for falciparum malaria in Asia. *PLoS ONE* **5**: 1-13.

Vereb G, Szöllösi J, Matko J, Nagy P, Farkas T, Vigh L, Mátyus L, Waldmann TA, Damjanovich. 2003. Dynamic, yet structured: the cell membrane three decades after the Singer-Nicolson model. *Proceedings of the National Academy of Science* **100**: 8053-8058.

Vezmar M, Georges E. 2000. Reversal of MRP-mediated doxorubicin resistance with quinoline-based drugs. *Biochemical Pharmacology* **59**: 1245-1252.

Via LD, Gia O, Gasparotto V, Ferlin MG. 2008. Discovery of a new aniline-3H-pyrrolo[3,2-f]quinoline derivative as potential anticancer agent. *European Journal of Medicinal Chemistry* **43**: 429-434.

Vukanovic J, Passaniti A, Hirata T, Traystman RJ, Hartley-Asp B, Isaacs JT. 1993. Antiangiogenic effects of the quinoline-3-carboxamide linomide. *Cancer Research* **53**: 1833-1837.

Wainwright M, Kristiansen JE. 2003. Quinoline and cyanine dyes – putative antiMRSA drugs. *International Journal of Antimicrobial Agents* **22**: 479-486.

Wang P, Song JH, Song DK, Zhang J, Hao C. 2006. Role of death receptor and mitochondrial pathways in conventional chemotherapy drug induction of apoptosis. *Cell Signaling* **18**:1528-1535.

Wang TH, Wang HS, Soong YK. 2000. Paclitaxel-induced cell death: where the cell cycle and apoptosis comes together. *Cancer* **88**: 2619-2628.

Willingham MC. 1999. Cytochemical methods for the detection of apoptosis. *The Journal of Histochemistry and Cytochemistry* **47** (9): 1101-1109.

Wind NS, Holen I. 2011. Multidrug resistance in breast cancer: from *in vitro* models to clinical studies. *International Journal of Breast Cancer* **2011**: 1-12.

World Health Organisation. 2011. Cancer Fact Sheet 2011. [www.who.int](http://www.who.int)

World Health Organisation. 2011. Tuberculosis Fact Sheet 2010. [www.who.int](http://www.who.int)

World Health Organisation. 2011. World Malaria Report 2010. [www.who.int](http://www.who.int)

Xiong X, Bromley EHC, Oelschlaeger P, Woolfson DN, Spencer J. 2011. Structural insights into quinolone antibiotic resistance mediated by pentapeptide repeat proteins: conserved surface loops direct the activity of a Qnr protein from a Gram-negative bacterium. *Nucleic Acids Research* **39**: 3917-3927.

Yang SH, Chien CM, Lu CM, Chen YL, Chang LS, Lin SR. 2007. Involvement of c-Jun N-terminal kinase in G2/M arrest and FasL-mediated apoptosis by a novel indoloquinoline derivative, IQDMA, in K562 cells. *Leukemia Research* **31**: 1413-1420.

Yeheeskely-Hayon D, Regev R, Katzir H, Eytan GD. 2009. Competition between innate multidrug resistance and intracellular binding of rhodamine dyes. *FEBS Journal* **276**: 637-648.

Yoon MY, Kim SJ, Lee BH, Chung JH, Kim YC. 2006. Effects of dimethylsulfoxide on metabolism and toxicity of acetaminophen in mice. *Biological and Pharmaceutical Bulletin* **29**: 1618-1624.

Zamora JM, Pearce HL, Beck WT. 1988. Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Molecular Pharmacology* **33**: 454-463.

Zhang J, Ke X, Tu C, Lin J, Ding J, Lin L, Fun HK, You X, Guo Z. 2003. Novel Cu(II)-quinoline carboxamide complexes: Structural characterization, cytotoxicity and reactivity towards 5'-GMP. *BioMetals* **16**: 485-496.

Zhou Q, Melkounian ZK, Lucktong A, Moniwas M, Davies JR, Strobl JS. 2000. Rapid induction of histone hyperacetylation and cellular differentiation in human breast tumor cell lines following degradation of histone deacetylase-1. *The Journal of Biological Chemistry* **275**: 35256-35263.

Zhu X, Kumar R, Mandal M, Sharma N, Sharma HW, Dhingra U, Sokoloski JA, Hsiao R, Narayanan R. 1996. Cell cycle-dependent modulation of telomerase activity in tumor cells. *Proceedings of the National Academy of Sciences USA* **93**: 6091-6095.

Ziganshina LE, Squire SB. 2009. Fluoroquinolones for treating tuberculosis (review). John Wiley and Sons: The Cochrane Collaboration.

Zloh M, Kaatz GW, Gibbons S. 2004. Inhibitors of multidrug resistance (MDR) have affinity for MDR substrates. *Bioorganic and Medicinal Chemistry Letters* **14**: 881-885.