

Evaluation of the efficacy of selected anticancer compounds in multidrug resistant cell culture models

MJ Rossouw

 **orcid.org / 0000-0002-3401-8829**

Dissertation submitted in fulfilment of the requirements for the degree *Masters of Science in Pharmaceutics* at the North West University

Supervisor: Dr C Gouws
Co-supervisor: Prof JH Hamman
Co-supervisor: Dr C Willers

Graduation: May 2019
Student number: 24093580

The financial assistance of the National Research Foundation (NRF) and the South African Medical Research Council towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF or the MRC.



**National
Research
Foundation**



“Who you are is defined by what you’re willing to struggle for.”

— **Mark Manson**

ABSTRACT

Small cell lung cancer (SCLC) has an aggressive disseminating nature and the relapse of cancer patients after initial treatment is common. Acquired multidrug resistance (MDR) can often be attributed to over-expression of ATP-binding cassette efflux transporters on the cell surface of SCLC cells. In the current study, three SCLC cell lines were evaluated as potential anticancer research models, specifically for investigations to overcome efflux-based MDR in cancer. These cell lines included the drug-sensitive H69V cell line, the multidrug resistance-associated protein 1 (MRP1) hyperexpressing H69AR cell line and the multidrug resistant NCI-H69/LX4 cell line that over-expresses P-glycoprotein (P-gp). A non-cancerous porcine embryonic kidney cell line (LLC-PK1) was included to evaluate the cytotoxic effects of each anticancer drug and to serve as a chemotherapeutic selectivity control.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as a preliminary screening tool to determine the relative cell viability inhibitory concentration (IC) of selected chemotherapeutic drugs (i.e. paclitaxel, cisplatin and irinotecan) on the SCLC cell lines. Following the MTT assay, each cell line was treated with selected concentrations of the anticancer drugs based on the MTT results, which were adjusted daily relative to the wet biomass of the cells. This follow-up advanced cell viability assessment included quantification of intracellular adenosine triphosphate (ATP) and extracellular adenylate kinase (AK) of the different cell lines treated with the selected drugs.

The ATP and AK cell viability assessment accurately indicated chemosensitivity of the H69V cell line, as well as resistance to treatment with drugs known to be substrates for the hyperexpressed efflux transporters. The P-gp over-expressing NCI-H69/LX4 cell line exhibited resistance to treatment with the P-gp substrate, paclitaxel (resistance ratio of 215.90; IC₅₀ 613.05 nM), while the H69AR cell line (hyperexpressing MRP1) was resistant to irinotecan treatment (resistance ratio of 16.50; IC₅₀ 7 716.70 nM), which is known to be a substrate for MRP1-mediated efflux. Cisplatin (ATP, 0.90; AK, 1.21), irinotecan (ATP, 0.94; AK, 1.80) and paclitaxel (ATP, 0.88; AK, 1.94) were cytotoxic to varying degrees in the non-cancerous LLC-PK1 cell line at drug sensitive line (H69V) IC₅₀ concentrations (170.75 nM; 467.01 nM; 2.84 nM), but significantly higher concentrations than those shown to be effective in the SCLC cell lines were needed to determine LLC-PK1 IC₅₀ values (2 619.60 nM; 15 534.00 nM; 29.73 nM). The H69AR cell line also showed resistance to cisplatin (resistance ratio of 2.50; IC₅₀ 427.34 nM), an MRP2 substrate, indicating cross-resistance potential between MRP1 and MRP2 transporters (transporter familial resistance).

To confirm reports of transporter hyperexpression, a quantitative reverse transcription polymerase chain reaction was completed on all of the cell lines. When normalised to the NCI-H69/LX4 cell

line (presented as 0), MRP1 expression was found to be present in the H69V (0.106) and LLC-PK1 (-0.076) cell lines in varying degrees, but as literature reported in the H69AR cell line MRP1 was hyperexpressed (2.037). P-gp expression quantification, normalised to the H69AR cell line (presented as 0), confirmed published reports of hyperexpression in the NCI-H69/LX4 cell line (5.95). No detectable P-gp levels were found to be present in the H69V and LLC-PK1 cell lines.

The anticancer results of the selected chemotherapeutic agents were comparable to published *in vivo* and clinical trial conclusions, confirming the predictive potential of the selected cell lines for cancer treatment screening, including the effectiveness of compounds against MDR in lung cancer. Therefore, the presented SCLC cell model platform can be used as an effective *in vitro* tool to screen the efficacy of new compounds or to study efflux-based MDR in lung cancer.

Keywords: *Cell viability, chemotherapy, in vitro cancer model, multidrug resistance, small cell lung cancer, drug screening.*

ACKNOWLEDGEMENTS

I would like to extend my most sincere gratitude towards the major role players that supported me during the completion of my MSc dissertation. The work contained herein would not have been achieved if it wasn't for the following influences:

- First and foremost to my Heavenly Father without whom I would have never even made it through my undergraduate degree, nevertheless my masters. Thank you for the excessive blessings I have received throughout my entire life and that You were always a place of solace in times of need.
- My parents, Marinda and Andrew Rossouw, who supported me in every possible way they could. I cannot begin to state my appreciation for the continuous financial assistance, your willingness to listen to my endless rants and general support of all aspect of my life. I could not have asked for better parents!
- To my significant other, Clara Olivier. These past two years have been tough, but here we are at the end of it. Your relentless love, understanding and devotion to my aspirations meant the world to me. Your unwavering support made all of the difference and I look forward to the next chapter in our lives.
- To my study leader, Prof Chrisna Gouws, I would like to thank you for introducing me to the marvels of science. I would like to express my utmost gratitude for all of the countless hours you spent editing my chapters, assisting me with grant applications, generally pointing me in the right direction and assisting me with my presentation. I will always appreciate your calming demeanour during our most stressful of times and the opportunities you granted me. Thank you for believing in me and playing such a large role in the successful completion of my project.
- Dr Clarissa Willers, I cannot begin to stress enough how large of a role you played in our lives the past two years. Thank you for all of the weekends you gave up to assist us in the laboratory to ensure the success of our study. Your continued positivity, support and willingness to help meant the world to me. I have great respect for your selfless sacrifices you made to ensure the successful outcome of our projects.
- Dr Carlemi Calitz, when I first met you I didn't quite know what to expect from this bossy PhD student ordering us around and calling us ducks. Thank you for exceeding my initial expectations and showing me what an absolute passion for science looks like. I will always appreciate your words of support and introducing us to the world of cell culturing. Your

energy and kindness is contagious. I wish you the best of luck with your future endeavours in Sweden.

- To my co-supervisor, Prof Sias Hamman, I would like to thank you for always making time to attend our presentations and adding valuable insights during all aspects of my study. My journey to this research topic started with you and I greatly appreciate your belief in me from day one.
- Prof Krzysztof Wrzesinski, the time you spent advising and helping us with the study layout is greatly appreciated.
- To my laboratory partner and co-masters candidate, Roan Swanepoel. Thank you for all of your support during the completion of our projects. I wish you the best upon your return as a PhD student.
- To Prof Suria Ellis for her assistance with all of the statistical aspects of this study.
- To the North-West University for the masters, institutional and additional research bursaries.

TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGEMENTS

TABLE OF CONTENTS	i
-------------------------	---

LIST OF TABLES	vii
----------------------	-----

LIST OF FIGURES	x
-----------------------	---

LIST OF ABBREVIATIONS	xiii
-----------------------------	------

LIST OF DEFINITIONS	xix
---------------------------	-----

CHAPTER 1: INTRODUCTION	1
-------------------------------	---

1.1 Background and Justification.....	2
---------------------------------------	---

1.2 Problem statement	3
-----------------------------	---

1.3 General aim.....	4
----------------------	---

1.4 Specific objectives.....	4
------------------------------	---

1.5 Presentation of results.....	4
----------------------------------	---

1.6 Chapter layout of this dissertation	5
---	---

1.7 References.....	8
---------------------	---

CHAPTER 2: LITERATURE BACKGROUND	11
--	----

2.1 Introduction	12
------------------------	----

2.2 Multidrug resistance in cancer	12
--	----

2.2.1 What is multidrug resistance?	12
---	----

2.2.2 How is multidrug resistance achieved in cancer cell lines.....	13
--	----

2.2.2.1 Macroscopic pharmacokinetic resistance.....	13
---	----

2.2.2.2 Tumour microenvironment multidrug resistance mechanisms.....	16
--	----

2.2.2.3 Efflux pumps of the ABC-transporter superfamily	19
---	----

2.2.2.3.1 P-glycoprotein:	21
---------------------------------	----

2.2.2.3.2 Multidrug resistance-associated protein (ABCC1)	22
---	----

2.2.2.3.3	Other clinically significant transporters	26
2.3	Small cell lung carcinoma	27
2.4	Chemotherapeutic model compounds	28
2.4.1	Cisplatin	28
2.4.2	Irinotecan	31
2.4.3	Paclitaxel.....	35
2.5	<i>In vitro</i> models to study multidrug resistance in cancer	38
2.5.1	SCLC models	39
2.6	Summary.....	41
2.7	References.....	41
CHAPTER 3: ARTICLE SUBMITTED TO CURRENT CANCER DRUG TARGETS FOR PUBLICATION		55
AUTHOR CONTRIBUTION.....		56
1. INTRODUCTION.....		58
2. MULTIDRUG RESISTANT CANCER TREATMENT APPROACHES		59
3. <i>IN VITRO</i> MODELS FOR MULTIDRUG RESISTANT CANCER TREATMENT SCREENING.....		59
3.1	Conventional cell-based models.....	60
3.2	Complex cell-based models.....	61
3.2.1	Integrated discrete multiple organ culture (IdMOC) system:	61
3.2.2	Microfluidic channel-based systems:	62
3.2.2.1	Multi-organ co-culture in tumour-on-a-chip system:	62
3.2.2.2	Lung-on-a-chip microfluidic device:.....	63
3.2.2.3	3D lung cancer microfluidic constructs:.....	63
3.2.3	3D spheroid cell cultures:	63
3.2.3.1	3D multicellular models mediating MDR:	64
3.2.3.2	Hollow fibre-based spheroid cultures:	64
3.2.3.3	Cell printing:	65

4. <i>IN VIVO</i> MODELS FOR MULTIDRUG RESISTANT CANCER TREATMENT SCREENING	65
4.1 <i>In vivo</i> hollow fibre assay models	65
4.2 Xenograft cancer models.....	65
4.2.1 Cell-derived xenografts:.....	66
4.2.2 Patient-derived xenografts:.....	67
4.3 Genetically engineered mouse models.....	67
5. IMPROVEMENTS FOR PRECLINICAL MDR TREATMENT SCREENING	68
6. CONCLUSIONS	69
7. CONFLICT OF INTEREST	69
8. ACKNOWLEDGEMENTS.....	70
9. REFERENCES	80

CHAPTER 4: PRELIMINARY *IN VITRO* CYTOTOXICITY SCREENING USING THE METHYL THIAZOLYL TETRAZOLIUM ASSAY

4.1 Introduction	92
4.2 The methyl thiazolyl tetrazolium assay	92
4.3 Materials and Methods	93
4.3.1 Study design	93
4.3.2 General materials and reagents	94
4.3.3 Culturing of small cell lung carcinoma cell lines.....	95
4.3.3.1 NCI-H69V chemosensitive cell line	95
4.3.3.2 H69AR MRP-1 hyperexpressive cell line	95
4.3.3.3 NCI-H69/LX4 P-gp hyperexpressive cell line	96
4.3.3.4 LLC-PK1 (Porcine kidney) cell line.....	96
4.3.4 Seeding of cells in 96-well plates.....	96
4.3.4.1 Seeding of the adherent cell lines: NCI-H69V, H69AR and LLC-PK1	96
4.3.4.2 Seeding of the NCI-H69/LX4 suspension cell line.....	97
4.3.5 Preparation of model chemotherapeutic drugs	97

4.3.5.1	Cisplatin	98
4.3.5.2	Irinotecan.....	98
4.3.5.3	Paclitaxel.....	99
4.3.6	MTT cytotoxicity assay	99
4.3.6.1	Adherent cell line assay procedure.....	99
4.3.6.2	Suspension cell line assay procedure.....	100
4.3.7	Data analyses	100
4.3.8	Statistical analysis	102
4.4	Results and Discussion	103
4.4.1	Cisplatin	103
4.4.1.1	Cell viability inhibition of the LLC-PK1 cell line following treatment with cisplatin	103
4.4.1.2	Cell viability inhibition of the NCI-H69V cell line following treatment with cisplatin	105
4.4.1.3	Cell viability inhibition of the H69AR cell line following treatment with cisplatin	107
4.4.1.4	Cell viability inhibition of the NCI-H69/LX4 cell line following treatment with cisplatin	109
4.4.1.5	Brief summary for cisplatin.....	113
4.4.2	Irinotecan	114
4.4.2.1	Cell viability inhibition of the LLC-PK1 cell line following treatment with irinotecan.....	114
4.4.2.2	Cell viability inhibition of the NCI-H69V cell line following treatment with irinotecan.....	116
4.4.2.3	Cell viability inhibition of the H69AR cell line following treatment with irinotecan.....	117
4.4.2.4	Cell viability inhibition of the NCI-H69/LX4 cell line following treatment with irinotecan	119
4.4.2.5	Brief summary for irinotecan	123

4.4.3	Paclitaxel.....	124
4.4.3.1	Cell viability inhibition of the LLC-PK1 cell line following treatment with paclitaxel.....	124
4.4.3.2	Cell viability inhibition of the NCI-H69V cell line following treatment with paclitaxel.....	126
4.4.3.3	Cell viability inhibition of the H69AR cell line following treatment with paclitaxel.....	128
4.4.3.4	Cell viability inhibition of the NCI-H69/LX4 cell line following treatment with paclitaxel	130
4.4.3.5	Brief summary for paclitaxel.....	133
4.5	Conclusion	134
4.6	References.....	136

CHAPTER 5: ARTICLE SUBMITTED FOR PUBLICATION IN EXPERIMENTAL CELL

RESEARCH.....	144
AUTHOR CONTRIBUTION:	145
ABSTRACT:	147
1. Introduction.....	148
2. Materials and methods	149
2.1 Experimental design.....	149
2.2 Chemicals and reagents used.....	151
2.3 Cell culture maintenance.....	152
2.4 Preparation of chemotherapeutic drugs.....	152
2.5 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay ..	153
2.6 Soluble protein quantification	154
2.7 Intracellular adenine triphosphate cell viability assay	154
2.8 Extracellular adenylate kinase cell death assay	155
2.9 Quantitative reverse transcription polymerase chain reaction.....	155
2.10 Calculations and statistical analysis	156
3. Results and discussion.....	157

3.1 Pre-screening of relative cell viability inhibition potential	157
3.2 Advanced anticancer efficacy assessment	157
3.2.1 Irinotecan anticancer efficacy	159
3.2.2 Cell responses following treatment with paclitaxel	162
3.2.3 Cell responses following treatment with cisplatin	165
3.3 ABC-transporter expression in the cell models.....	168
4. Conclusion.....	169
5. Acknowledgements	169
6. Funding	170
7. Conflict of interest.....	170
8. References.....	170
CHAPTER 6: FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS	176
6.1 Introduction	177
6.2 Efflux transporter hyperexpression validation	177
6.3 Preliminary efficacy screening.....	178
6.4 Advanced efficacy screening.....	179
6.5 Final conclusion	180
6.6 Future recommendations	181
6.7 References	182
APPENDIX A: AUTHOR GUIDELINES FOR CURRENT CANCER DRUG TARGETS	183
APPENDIX B: AUTHOR GUIDELINES FOR EXPERIMENTAL CELL RESEARCH	202
APPENDIX C: PROOF OF ATTENDANCE AT THE FIRST CONFERENCE OF BIOMEDICAL AND NATURAL SCIENCES AND THERAPEUTICS 2018.....	212
APPENDIX D: SEEDING DENSITIES FOR CONFLUENCY DETERMINATION OF CELL LINES	214
APPENDIX E: ETHICS APPROVAL AND REGISTRATION OF COMMERCIAL CELL LINE USE	219
APPENDIX F: ISOLATED ENLARGED GRAPHS INCLUDED IN CHAPTER 5.....	221

LIST OF TABLES

Chapter 2

Table 2.1:	Examples of how pharmacokinetic factors can influence drug delivery to tumour sites	14
Table 2.2:	Tumour microenvironment factors influencing MDR	16
Table 2.3:	Chemotherapeutic drugs that are susceptible to P-gp transporter efflux	22
Table 2.4:	Common chemotherapeutic drugs that are susceptible to MRP1 based transporter efflux	24
Table 2.5:	Common chemotherapeutic drugs that are susceptible to MRP2 based transporter efflux	26
Table 2.6:	Cisplatin IC ₅₀ values indicating the relative potency of the drug across several cancer cell lines	30
Table 2.7:	Irinotecan IC ₅₀ values indicating the relative potency of the drug across several cancer cell lines	34
Table 2.8:	Paclitaxel IC ₅₀ values indicating the relative potency of the drug across several cancer cell lines	37

Chapter 3

* Table numbering format adapted according to submitted journal requirements

Table 1:	<i>In vitro</i> & <i>In vivo</i> efflux-based models used for anticancer drug screening	71
-----------------	---	----

Chapter 4

Table 4.1:	Model chemotherapeutic drugs used in this study	95
-------------------	---	----

Table 4.2:	Cell viability inhibition concentrations (IC) of cisplatin on the LLC-PK1 cell line (nM), as determined with Probit analysis	103
Table 4.3:	Cell viability inhibition concentrations (IC) of cisplatin on the NCI-H69V cell line (nM), as determined with Probit analysis	105
Table 4.4:	Cell viability inhibition concentrations (IC) of cisplatin on the H69AR cell line (nM), as determined with Probit analysis	108
Table 4.5:	Cell viability inhibition concentrations (IC) of cisplatin on the NCI-H69/LX4 cell line (nM), as determined with Probit analysis	111
Table 4.6:	Cell viability inhibition concentrations (IC) of irinotecan on the LLC-PK1 cell line (nM), as determined with Probit analysis	114
Table 4.7:	Cell viability inhibition concentrations (IC) of irinotecan on the NCI-H69V cell line (nM), as determined with Probit analysis	116
Table 4.8:	Cell viability inhibition concentrations (IC) of irinotecan on the H69AR cell line (nM), as determined with Probit analysis	119
Table 4.9:	Cell viability inhibition concentrations (IC) of irinotecan on the NCI-H69/LX4 cell line (nM), as determined with Probit analysis	121
Table 4.10:	Cell viability inhibition concentrations (IC) of paclitaxel on the LLC-PK1 cell line (nM), as determined with Probit analysis	124
Table 4.11:	Cell viability inhibition concentrations (IC) of paclitaxel on the NCI-H69V cell line (nM), as determined with Probit analysis	126
Table 4.12:	Cell viability inhibition concentrations (IC) of paclitaxel on the H69AR cell line (nM), as determined with Probit analysis	128
Table 4.13:	Cell viability inhibition concentrations (IC) of paclitaxel on the H69/LX4 cell line (nM), as determined with Probit analysis	131
Table 4.14:	Summary of 50% inhibition concentrations and their respective resistance factors for the model drugs on the various SCLC cell lines	135

Chapter 5

* Table numbering format adapted according to submitted journal requirements

Table 1:	Relative cell viability inhibitory concentrations for cisplatin, irinotecan and paclitaxel on each cell line, as determined following MTT analysis	158
-----------------	--	-----

LIST OF FIGURES

Chapter 1

- Figure 1.1:** A diagram depicting the layout of all experimental aspects of this study. 7

Chapter 2

- Figure 2.1:** Typical full ABC-transporter (e.g. P-gp) embedded in a lipid bilayer 21
- Figure 2.2:** Large conventional MRP-transporter structure in a lipid bilayer 23
- Figure 2.3:** Replacement of chloride molecules with water molecules for activation of the cisplatin drug in cancer cells 28
- Figure 2.4:** Cisplatin-induced DNA damage leading to cell cycle arrest processes to either repair or initiate cellular death in cancerous cells 29
- Figure 2.5** Metabolic pathways of irinotecan with the Carboxylesterases transformed metabolite (SN-38) being the primary active form of the drug and SN-38G being the inactive metabolite 32
- Figure 2.6** Typical path followed in cancerous cells after the introduction and activation of irinotecan. 33
- Figure 2.7** Typical path followed in cancerous cells after the introduction of paclitaxel. 36
- Figure 2.8** The three SCLC cell lines derived from the H69 parental cell line that will be incorporated into this study 39
- Figure 2.9** The porcine kidney cell line (LLC-PK1) included as a healthy tissue standard. 40

Chapter 4

Figure 4.1:	Percentage cell viability inhibition following treatment of the LLC-PK1 cell line with cisplatin, relative to the untreated control	104
Figure 4.2:	Percentage cell viability inhibition following treatment of the NCI-H69V cell line with cisplatin, relative to the untreated control	106
Figure 4.3:	Percentage cell viability inhibition following treatment of the H69AR cell line with cisplatin, relative to the untreated control	110
Figure 4.4:	Percentage cell viability inhibition following treatment of the NCI-H69/LX4 cell line with cisplatin, relative to the untreated control	112
Figure 4.5:	Percentage cell viability inhibition following treatment of the LLC-PK1 cell line with irinotecan, relative to the untreated control	115
Figure 4.6:	Percentage cell viability inhibition following treatment of the NCI-H69V cell line with irinotecan, relative to the untreated control	118
Figure 4.7:	Percentage cell viability inhibition following treatment of the H69AR cell line with irinotecan, relative to the untreated control	120
Figure 4.8:	Percentage cell viability inhibition following treatment of the NCI-H69/LX4 cell line with irinotecan, relative to the untreated control	122
Figure 4.9:	Percentage cell viability inhibition following treatment of the LLC-PK1 cell line with paclitaxel, relative to the untreated control	125

Figure 4.10:	Percentage cell viability inhibition following treatment of the NCI-H69V cell line with paclitaxel, relative to the untreated control	127
Figure 4.11:	Percentage cell viability inhibition following treatment of the H69AR cell line with paclitaxel, relative to the untreated control	129
Figure 4.12:	Percentage cell viability inhibition following treatment of the NCI-H69/LX4 cell line with paclitaxel, relative to the untreated control	132

Chapter 5

*** Figure numbering format adapted according to submitted journal requirements**

Figure 1:	A diagram depicting the experimental design for this study.	150
Figure 2:	Cell responses following daily treatment with irinotecan (nM) for 96 h.	161
Figure 3:	Cell responses following daily treatment with paclitaxel (nM) for 96 h.	164
Figure 4:	Cell responses following daily treatment with Cisplatin (nM) for 96 h.	167
Figure 5:	Relative quantification of MRP1 (A) and P-gp (B) efflux transporter gene expression in the LLC-PK1, H69V, H69AR and NCI-H69/LX4 cell lines, respectively.	168

LIST OF ABBREVIATIONS

μ CCA	micro-scale cell culture analogue
2D	Two-dimensional
3D	Three-dimensional
3Rs	Replacement, Reduction and Refinement
A	
ABC	Adenosine triphosphate binding cassette
ADME	Absorption, Distribution, Metabolism and Elimination
ADP	Adenosine diphosphate
ADR	Adriamycin
AK	Adenylate kinase
AKDR	Adenylate kinase detection reagent
ANOVA	Analysis of variances
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B	
BBB	Blood-brain-barrier
BCRP	Breast cancer-resistance protein
BSA	Bovine serum albumin
C	

cDNA	Complementary Deoxyribonucleic acid
CDXs	Cell-derived xenografts
CES	Carboxylesterases
CO ₂	Carbon dioxide
CRC	Human colorectal carcinoma
CSCs	Cancer stem cells
Ct	Threshold cycle
CyCAV	Cyclophosphamide, cisplatin, doxorubicin and etoposide
CYP P450	Cytochrome P450
CYP3A4	Cytochrome P3A4

D

DMBA	Dimethylbenz(a)anthracene
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin

E

E ₂ 17βG	Estradiol glucuronide
ECACC	European Collection of Authenticated Cell Cultures
ECM	Extracellular matrix

ED	Extensive-stage disease
EDTA	Ethylenediaminetetraacetic acid
EP	Etoposide/Cisplatin
EP	European Pharmacopoeia
F	
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FGF-2	Fibroblast growth factor-2
fLUC	Firefly luciferase
G	
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEMMs	Genetically engineered mouse models
GSH/GST	Glutathione S-transferase
H	
H ₂ O	Water
HEK	Human embryonic kidney
HFA	Hollow fibre assay
HIF-HRE	Hypoxia-inducible factors responsive elements
HPLC	High-performance liquid chromatography
I	
IC	Inhibition Concentration
IdMOC	Integrated discrete multiple organ culture

IHC	Immunohistochemical
IP	Cisplatin/Irinotecan
K	
kDa	kilodaltons
L	
LD	Limited-stage disease
M	
MCC	Merkel cell carcinoma
MDCK	Madin-Darby canine kidney
MDR	Multidrug-resistant
mdr-1	Multi-Drug Resistance Gene
microPET	Micropositron emission tomography
MNU	N-methyl-N-nitrosourea
mRNA	Messenger Ribonucleic acid
MRP	Multidrug resistance-associated protein
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
N	
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
NBD	Nucleotide binding sites
NCI	National Cancer Institute
NEAA	Non-essential amino acids

NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NSCLC	Non-Small Cell Lung Cancer
NWU	North-West University
P	
PARP	Poly-(adenosine diphosphate-ribose) polymerase
PBPK	Physiologically-based pharmacokinetic model
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PDXs	Patient-derived xenografts
PEI	Polyethyleneimine
P-gp	P-glycoprotein
pKa	Acidic dissociation value
PLGA	Poly(D,L-lactide-co-glycolide)
PVDF	Polyvinylidene fluoride
Q	
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
R	
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species

RPMI	Roswell Park Memorial Institute
RR _{drug}	Resistance ratio calculated for each drug
RT-PCR	Real-time polymerase chain reaction
S	
SAS	Statistical Analysis System
SCID	Severely compromised immunodeficient
SCLC	Small cell lung carcinoma
SDF1	Stromal cell-derived factor-1
si-RNA	Small interfering RNA
SN-38	7-Ethyl-10-hydroxycamptothecin
SOP	Standard Operating Procedure
T	
TALEN	Transcription activator-like effector nucleases
TBP	TATA-binding protein
TMD	Transmembrane hydrophobic domains
U	
USA	United States of America
V	
VEGF	Vascular endothelial growth factor

LIST OF DEFINITIONS

Chapter 1

Anti-neoplastic	Agents that inhibits, prevents or halts the growth of a tumour.
Apoptotic	Energy driven programmed cellular death.
Cell cycle arrest	Cellular checkpoint control mechanism activated when cells stop replication to ensure acceptable division and either initiate damage repair mechanisms or apoptosis/necrosis.
Depolymerisation	The process of converting a polymer into a monomer.
Efflux	The flowing out of a compound from the intracellular compartment.
Hyperexpression	The increased presence of a certain molecule on the cellular membrane surface due to increased genetic encoding.
Inhibition concentration	The degree of growth inhibition present in a cellular population after exposure to a predetermined amount of cytotoxic compound.
<i>in vitro</i>	Performing a given procedure on a biological molecule outside of the living organism in a controlled environment.
Malignant cells	Cancerous cells that are able to invade and kill healthy cells.
Metastasis	Ability to develop malignant growths at secondary sites in the human body.
Mono-therapy	Treatment of a disease with one single drug.
Non-conformative	Not responding to predefined external interventions as projected.
Palliative	Relieving symptoms of the disease without removing the cause.
Pleural	Fluid-filled space in the lung.

Polymer	Polymers are materials made of long, repeating chains of molecules.
Semisynthetic	Synthesised from a naturally occurring material.
S-phase-specific cell killing	Mechanism of inducing cellular death during the S phase of cellular replication.
Substrate specificity	The binding of certain compounds to binding sites only conformable to their structure, thus causing specificity of interaction.
Differentiate	The structure of characteristics changed during growth and development of the cells.

Chapter 2

Air-liquid interface	A method of <i>in vitro</i> cell culturing where the basal side of cells are grown in contact with cell culture medium while the top of the cells is exposed to air.
Allele	A variant form of a gene, wherein humans (diploid organism) one is inherited from each parent.
Arylmethoxy-derivatives	P-Glycoprotein-mediated drug efflux inhibitors
Athymic	Mouse model lacking a thyroid gland
ATPase activity	Enzymatic ATP activation through hydrolysis
Bidirectional transport	The movement of objects over a membrane in opposite directions
Bio-accumulation	The absorption and accumulation of a material by an organism, faster than the rate of catabolism and excretion
Biomimetic	Artificial or synthetic methods that mimic biochemical processes

Bioreactors	A synthetic manufactured device that supports a biologically active environment
Cadherin	Cell adhesion molecule that binds cells together
Cellular “plasticity”	Stem cells ability to switch to new identities
Clinical studies	The introduction of newly developed medical strategies, drugs or devices in human subjects to test for efficacy.
Clinical trial randomisation	To eliminate bias, subjects are randomly allocated to either receiving treatment under investigation or receiving treatment as the control
Clonogenic	Testing cell survival and proliferation cellular biology technique after exposure of specific agents
Competitive inhibition	Competing for a binding site by two substrate molecules thus interrupting a chemical pathway and response
Cross-contamination	The process in which bacteria or a substance that causes an undesirable effect (like other cells or metabolites) is transferred from the one object to another
Daughter cell	The cell formed by the division of a predecessor (parental cell)
Epigenetic	Genetic influences on gene expression causing phenotype changes
ex vivo	Takes place outside of an organism
Excised	Removal of a tissue or material
Exosome	Eukaryotic fluid based vesicles
Exponential growth	Increase in size and numbers at a constantly growing rate
Firefly luciferase	Light emitting enzyme

Haematological malignancies	Cancer that begins in blood-forming tissues
High-throughput assays	Large-scale automated experiments for screening purposes
Histopathological	Microscopic examination of materials to study manifestations of the disease
Human tumour explants	The removal of tumours from human hosts for <i>ex vivo</i> experimentation
Hydrostatic pressure	The pressure exerted by a fluid at equilibrium due to the force of gravity
Immunocompromised	Having an impaired immune system
Immunodeficient	Absent immune system that causes an inability to fight infectious disease by the host (also see Immunocompromised)
Immunohistochemical analysis	Protein expression analysis in tissue samples
<i>in silico</i>	Performed using computer simulation
Intraperitoneal	Within the peritoneum (membranes that line the walls of the abdominal cavity)
Mechanistic studies	Uncover the mechanisms of disease
Metronomic chemotherapy	Administering comparatively lower dosages of chemotherapy at higher frequencies to decrease the drug-free periods
Micelle	Spherical formation in aqueous solution
Microlitre	1×10^{-6} Litres
Micro-metastatic	The small collection of cancer cells that underwent metastasis from their original population

Microparticles	Particles with dimensions between 1×10^{-6} and 1×10^{-3} m
Microphysiological system	A system incorporating multiple physiological tissues or cells <i>ex vivo</i>
Microphysiometers	Analytical instrument used <i>in vitro</i> to analyse biochemical and biological systems
Micro-positron emission tomography	An imaging test that uses radioactive tracers in a special dye to scan diseases
Microvascular	Small vascular network
Morphology	Study the form of things
mRNA	RNA molecule that conveys information from the DNA to the ribosome
Murine	Mouse models
Nanoparticle	A particle that is in the nanometer range (1×10^{-9} m)
Necrotic	Death of cells and tissue
Neuroendocrine cells	Cells that receive neuronal input and respond by releasing hormones
Non-competitive inhibition	Enzymatic inhibition where an inhibitor reduces the activity of an enzyme and bind equally well to the enzyme regardless if it is already bound to a substrate
Oncogenes	A gene that can result in the formation of cancer
Oncolytic virus	Specifically attacks cancer cells, causing cellular death
Oocytes	A cell in an ovary
Orthoallobanks	The transplantation of cells or tissue from their equivalent organ in humans into murine models and creating a database where disease progression is mapped

Phenotype	The composition of observable traits and characteristics
Physiologically-based pharmacokinetic model	A mathematical modelling technique for predicting pharmacokinetic factors (ADME) of chemical substances in humans and other animal species.
Phytochemical	Active compounds in the treatment of diseases found in plants
Picolitre	1 x10 ⁻¹² Litre
Active plant constituents	See Phytochemical
Proliferation	The rapid increase of cellular population
Quiescent cells	Cells that do not divide, but retains the ability to re-enter cellular proliferation
Radiolabelled tracers	Radioactive decay of a chemical compound leaves traces as it moves, thus allowing the mapping of the compound through organisms
Reactive oxygen species	Chemically reactive species containing oxygen mostly responsible for the killing reaction in cells activated by the immune system
Replication-competent viruses	Viruses that contain all of the necessary components to replicate themselves
Sacrificed mice	Mice that are killed during <i>in vivo</i> investigations
Scaffold-dependant	<i>In vitro</i> introduction of a scaffold-like substance to help cells grow into a certain formation, usually utilised during 3D cell culture models
Site-specific recombinases	Targeted replacement of specific sites in DNA, using recombinase enzymes
Sub-atmospheric pressure	Pressure smaller than the normal atmospheric pressure (101,325 pascals)

Subcellular	Structures/occurrences within the cell and/or looking at below cellular level/scope.
Subcutaneous	Situated under the skin
Surrogate	Substituting a person/object in a specific role with someone/something else
Symbiotic growth	Mutually beneficial growth where both sides benefit from the relationship
Thrombocytopenic	Low blood platelet count
Transcription activator-like effector nucleases	Restriction enzymes used to cut certain sequences of DNA
Transgenes	Transferral of a gene from one organism to another
Transgenic	An organism containing genic material from another organism
Tumorigenesis	Formation of tumours
Tumour regression rates	The rate at which tumours shrink after the introduction of chemotherapy
Tyrosine kinase inhibitors	Drugs that stop the enzyme tyrosine kinase from transferring a phosphate group from ATP to a protein
Universal medium	Cell culture growth medium that accommodates the metabolic needs of a large range of cells
Vascularisations	The formation of a vascular network
Vectors	Vehicle that artificially carries foreign genetic material into another cell
Vesicular transport assays	Using inside-out vesicles from membranes, interactions with surface transporters can be viewed

Western blot assay	Assay to detect certain proteins in a sample
Zinc finger nuclease-mediated gene knockout	Engineered DNA-binding proteins that cause DNA double-strand breaks at user-defined areas, thus allowing genome editing

Chapter 3

3D-culturing	The artificial environment created for cells to grow and interact outside of the living organism in all three dimensions.
Acidic dissociation	The quantified strength of an acid in solution.
Adherent	Sticking or growing to a surface.
Amphipathic	Having both hydrophilic and hydrophobic characteristics.
Angiogenesis	Formation of blood vessels in tumours.
Apical	Growing tip/pinnacle of the cells.
Ascites	Accumulation of fluids in the abdominal area.
Basolateral	Outward facing part of the cellular membrane.
Bio-activated	Dormant compound pharmacologically activated through biological precursors in the body.
Canaliculi	Small passageways.
Carcinogen-induced carcinomas	The use of agitating agents typically responsible for causing cancer to induce the abnormal growth of cells into cancerous tumours.
Chemotherapeutic	One or more chemical compounds used to treat cancers usually by killing the cancerous cells.
Chemotherapy-naive patients	Patients who never before received any chemotherapeutic treatments.

Cleavage complex	Covalently linked enzymes on a strand of broken DNA, typically induced through chemotherapeutic interventions.
Complete response	No detectable cancers present in the patient after treatment.
Continuous cell line	Chemically, virally or naturally induced transformation that allows cells to divide indefinitely through evasion of cellular senescence
Convection	Increased fluid flow pattern.
Core consensus motifs	Nucleotide sequence of DNA, RNA, or an amino acid sequence of proteins.
Cytokine	Cell signalling agent having an effect on other cells.
Denaturation	The loss of structure in proteins or nucleic acids due to external stress.
Electrophile	Positively charged or neutral species attracted to electrons.
Endoplasmic reticulum	Intracellular organelle primarily responsible for protein and lipid synthesis.
Enterocytes	Intestinal absorptive cells.
Eukaryotic	Cells that have a nucleus enclosed within membranes.
Exogenous	A foreign compound having an external cause or origin.
Extracellular matrix	Serves as the structural component in which more specialised structures are embedded.
Filamentous	Long thread-like shape
Flippase processes	Transmembrane lipase transporter processes for aiding the movement of phospholipid molecules.
Genetic alterations	Modification of the genetic make-up to induce certain required characteristics.
Genomic sequences	DNA nucleotide sequence order.

Glutathione conjugated-and-unconjugated	Metabolic processes mediated through the enzyme glutathione which primarily converts xenobiotics into metabolites for excretion. Some metabolites formed presented with cytotoxic, genotoxic, or mutagenic capabilities.
Guanosine triphosphate	A nucleotide that channels chemical energy for biosynthetic pathways through carrying phosphates and pyrophosphates.
Helices	Plural form of the word helix that describes a curve through three-dimensional spaces (DNA helix).
Hematopoietic	Immature blood cells that can convert into platelets, red blood cells and white blood cells.
Hepatocytes	A cell that originates from the liver.
Histocompatibility antigens	Facilitates cell surface inspection by binding to intracellular or extracellular peptides and presenting them to the T cells.
Histocultures	Tissue cultures containing multiple cell types extracted from the <i>in vivo</i> environment and are maintained in growth medium either in collagen gel or as free-floating tissues.
Homeostatic	State of physiological normality and stability within an organism where all the processes function at optimum levels.
Hypoxic	Deprivation of oxygen
Immortalised cell line	Chemically, virally or naturally induced transformation that allows cells to divide indefinitely through evasion of cellular senescence (see continuous cell line)
Immune-regulation	The manipulation of immune components (e.g. lymphocytes) to maintain a predetermined function pertaining to immunology.
<i>in vivo</i>	Testing of tissues or cells within living organisms.
Incidences	The probability that a certain medical condition will occur in a population at a given time.

Interstitial	Space between structures or organs.
Isoform	Proteins with similar but not identical amino acid sequences.
kDa	Kilodalton is a unit for molecular mass.
Lipid bilayer	Boundaries of a cell, containing two layers of fat cells.
Lumen	Central open space
Lysosomes	Present in the cytoplasm of eukaryotes that presents as an enclosed membrane containing degradative enzymes.
Macromolecules	A large molecule created through polymerisation of monomers
Microsomes	Present in dead or dying cells and is vesicles made up of pieces from the endoplasmic reticulum. Microsomes are present when breaking up eukaryotic cells in the laboratory.
Mitosis	Cellular division in which two daughter cells are created, each containing identical type and amount of chromosomes as the original parent cell.
Morphology	The particular shape of the described object.
Mortality	Describes the number of deaths caused by the described disease.
Murine	Mice or relating rodents
Nucleophile	Electron pair donator species
Nucleophilic attack	The selective bonding of a nucleophile to a positively charged atom or groups of atoms.
Nucleotide binding domains	Domains that bind and hydrolyse ATP as well as playing a role in channel opening and closing (dependant on the specific domain).
Overall response	A reported predefined tumour size reduction in a certain amount of time after treatment
Oxygenation	Addition of oxygen to the system.

Pharmacodynamics	How the body reacts to drugs.
Pharmacokinetics	The study of drug absorption, distribution, metabolism, and excretion in the host.
Pleural effusion	Fluid build-up between the tissues that line the lungs and the chest.
Polymorphs	Various crystalline structures of a material.
Pre-clinical	The stage of testing before human trials.
Primary cell lines	Dissociation of cells from a parental animal or plant tissue.
Prodrugs	Compound that is pharmacologically activated after being metabolised within the host.
Prokaryotic	Organism without any membrane-bound organelles.
Prophylactic cranial irradiation	Radiating the brain to stop metastasis of cancer cells to the cranial region and thus prolonging survivability of patients.
Proteoglycans	Glycosylated proteins attached to anionic glycosaminoglycan and are especially present in connective tissues.
Proximal	Situated closer to the centre of the body or attachment area.
Purine residues	DNA purine base pairs
Remission	Patients present with no sign of cancer after treatment.
Resistance spectrum	Ability to propagate resistance to a number of structurally different drugs from separate classes.
Rheology	The study of the flow of matter.
Sequester	Isolation or removal of a material.
Stroma (tumour)	All of the elements supporting the viability of a tumour including basement membrane, fibroblasts, extracellular matrix, immune cells, and vasculature.

Syncytiotrophoblasts	Endothelial cells covering the placental villi.
Syngeneic transplantation	The transplantation of blood-forming stem cells.
Traditional flat culturing	The culture of cells as a monolayer on cellular life-sustaining surfaces.
Transmembrane domains	Facilitates a two-way transportation of certain molecules across the cellular surface.
Tyrosine kinase activity	Transfers a phosphate group from ATP to a protein in a cell.
Vascularisation	The development of capillaries in organic tissue.
Xenobiotic	A material foreign to the body and can include synthetic compounds (chemotherapy)
Xenografts	The transplantation of cells or tissues into an organism that originates from a different species.

Chapter 4

Aliquots	Divided into smaller volumes from a larger solution.
Anaphylaxis	Severe life-threatening allergic reaction to stimuli.
Antitumorigenic	Counteract the formation of tumours.
Biological variation	The variation of response between the similar class of cells or tissues due to individual unique reactions to stimuli.
Cell suspension	Cells that have the unique characteristics of growing as non-adherent colonies in growth medium or adherent cells that were subjected to trypsination and are loosely floating in cell medium before reseeding.
Centrifuge	The application of centrifugal force to separate particles.

Colorimetric assay	Determining certain parameters by quantifying colour compounds in a solution.
Confidence limits	The limits in which the true value will definitely be present.
Confluence	Percentage coverage of the culture dish surface that is covered by growing cells.
Cultured	The growing of cells under controlled conditions outside of the host.
Dose-response	The rate of cellular death in a population is directly determined by the administered dose.
Familial resistance	Resistance propagated the same transporters family, but in different sub-classes (e.g. MRP1 propagating resistance towards an MRP2 substrate).
High throughput	Scientific experimentation on a large scale.
Histological	Microscopic investigation and study of tissue subjected to disease.
L-glutamine	Biosynthesis of proteins is mediated by using this α -amino acid.
Myelosuppression	Inhibition of bone marrow activity.
Nephrotoxicity	Damage due to toxicity in kidney cells.
Neutropenia	An abnormally low blood count of white blood cells.
Nicotinamide adenine dinucleotide	A coenzyme that facilitates redox reactions by acting as a carrier of electrons from one reaction to another.
Normalised response	The data is manipulated to be comparable to a single reference parameter.
Optical density	This value is measured as absorbance and is quantified as the ratio of the intensity of light that fell on a material and the intensity of the retransmitted light.

Parental line	The original cell line from which the derivatives were made.
Passages	The number of times that the cells were subcultured.
Peripheral neuropathy	Damage to peripheral nerves causing numbness and pain.
Platinum-based therapy	The use of cell-damaging compounds containing platinum.
Porcine	Originates from a pig.
Pyruvate	Intermediate in several metabolic pathways made from glucose through glycolysis.
Second-line therapy	After the failure of the initial first line regimes, this class of treatments follows.
Serial dilutions	The stepwise dilution of a substance in solution.
Spectrophotometry	Quantification of the amount of light that is able to transmit through or reflect off a certain compound. These values are determined through wavelengths.
Standard deviations	Variability of repeats in the same sample from the mean value.
Stock solutions	The concentrated solution that will be diluted to weaker working solutions before use.
Sub-culturing	Transferring some of the cells from a previous culture to the new separate growth medium.
Succinate	Metabolic intermediated anion from succinate acid which is involved in numerous processes including producing ATP.
Trypsinisation	The dissociation of adherent cells from the surface by using the proteolytic enzyme, trypsin.

Viability inhibition The percentage inhibition of cellular populations treated with cytostatic or cytotoxic compounds compared to their untreated counterpart.

Working solutions Actual concentrations exposed to cellular populations that were created through serial dilutions of stock solutions.

Chapter 5

Acquired multidrug resistant cells Cells that underwent genetic changes to become multidrug resistant due to continuous exposure to ineffective concentrations of cytotoxic drugs

Biochemical markers Biological components present in blood, urine, tissue or other bodily fluids that serve as a sign of disease or abnormality.

Cell cycle progression delays Delaying the replication processes due to an abnormality or specific signalling instructions from the host (see cell cycle arrest).

Cross-resistant effects The ability of ABC transporters to propagate resistance to another entity of compounds that are structurally unrelated to their specific substrate compounds.

Dissemination The ability to spread cancer through the host (metastasis).

Extensive-stage disease (SCLC) The spreading of cancer to more than one radiotherapeutic treatment site (e.g. another part of the body).

Extrinsic pro-apoptotic processes Activation of cellular death through extracellular ligands (signal producing substance connected to a biomolecule) binding to cell surface death receptors. Cytotoxic cells from the immune system usually activate this process.

Fluorescence Emission of light by a substance after absorbance

Gene expression Information from a gene is used to synthesise a gene product

Hematologic Disease affecting the blood

Intrinsic pro-apoptotic processes	Intracellular cues such as DNA damage activates cell death precursors (e.g. caspase)
Localised limited-stage disease	The containment of cancer to a single area (e.g. one lung or nearby lymph nodes)
Luminescence	The emission of light from a substance not subjected to heating (as in fluorescence)
Oxidative phosphorylation	The oxidation of nutrients by enzymes to release the energy needed to produce ATP
Photosensitivity	Overreaction causing destabilisation of compounds after receiving photons from light
Seeded	The process of spreading a defined amount of cells in suspension onto a cell culture surface for culturing
Supernatant	Liquid above a solid residue
Surgical resected tumour biopsies	The removal of tissue from tumours inside of the host through operative measures
Unilateral	Affecting only one side of the organ, body or structure.

Chapter 6

Soluble protein	Hydrophilic amino acids that are most commonly situated in the cytoplasm (e.g. mitochondria, nucleus etc.)
Combinatory drug	A drug that can facilitate better therapeutic results when combined with another compound
Probit analysis	Statistical method to quantify the relationship between a stimulus (dose) and the response.
Embryonic	Relating to an embryo
Metabolic shift	Cells redirect intracellular energy towards secondary processes to counteract disruptive external stimuli

CHAPTER 1

This chapter presents the background, justification, problem statement, general aim, specific objectives, results presentation and chapter layout for this study.

INTRODUCTION

1.1 Background and justification

Small cell lung carcinoma (SCLC) is categorised as a malignant, undifferentiated lung carcinoma subgroup originating in the pulmonary neuroendocrine cells, and is thus described as an adenocarcinoma (Kalemkerian *et al.*, 2013). SCLC is the most prevalent of the adenocarcinomas and represents 15-20% of all invasive lung cancers (Öberg *et al.*, 2012; Kalemkerian *et al.*, 2013). Patients suffering from SCLC have a dire prognosis due to this cancer's rapid doubling time, combined with aggressive metastasis. Typically, less than 5 % of diagnosed patients live longer than 5 years (Elias, 1997; Kaur *et al.*, 2016).

Chemotherapy is seen as the cornerstone for the treatment of both limited and extensive SCLC (Paumier & Le Péchoux, 2010). The standard first-line treatment for SCLC includes variations of DNA targeting drugs (e.g. carboplatin, cisplatin) and topoisomerase 2 targeting drugs (i.e. doxorubicin, etoposide) (Sundstrøm *et al.*, 2002; Pass *et al.*, 2012; Früh *et al.*, 2013). Thoracic and cranial irradiations are mostly used for palliative and prophylactic (against metastasis) care (Warde & Payne, 1992; Paumier & Le Péchoux, 2010; Früh *et al.*, 2013). Even though initial reports show tumour drug sensitivity in chemo-naive patients, almost all patients present with an inevitable relapse of multidrug resistance (MDR) cancer (Oronsky *et al.*, 2017). The development of MDR in tumour cells is commonly defined as an overall resistance towards different types and combinations of chemotherapeutic drugs, because of cellular and non-cellular mutations (Krishna & Mayer, 2000). MDR can either be acquired after exposure to chemotherapy, or it can present as a pre-existing resistance within a tumour (Baguley, 2010). One of the mechanisms through which cancer cells can develop MDR is the over-expression of efflux transporters. The adenosine triphosphate (ATP)-binding cassette (ABC)-transporters are cellular transmembrane proteins, responsible for the efflux of solutes across the cell membrane. These transporters play a predominant role in cell susceptibility towards exogenous substances with apoptotic potential (Gottesman *et al.*, 2002). These solutes can range from biological material to chemotherapeutic drugs (xenobiotics), and the efflux of these materials ensures overall cell health and survival (Fletcher *et al.*, 2010). Tumour-specific studies have established that the ABC-transporters frequently over-expressed in SCLC, which act as the main drivers of MDR in this cancer type, are P-glycoprotein (P-gp or ABCB1) and multidrug resistance-associated protein 1 (MRP1 or ABCC1) (Triller *et al.*, 2006; Alvarez *et al.*, 2010). By manipulating the expression of these transporters in human cancer cell lines, several potential MDR models have been

established. For example, the H69 cell line was established from the pleural fluid of a human subject with SCLC, and manipulated to generate sub-clones (Little *et al.*, 1983) such as H69V (an adherent epithelial SCLC cell line, H69AR and NCI-H69/LX4 (both of which are drug resistant) (Twentyman *et al.*, 1986).

There are chemotherapeutic drugs used in SCLC therapeutic regimens, known to show an affinity for some of the abovementioned transporters. Irinotecan is used as a second line monotherapy for SCLC (Sevinc *et al.*, 2011). Although both P-gp and MRP1 ABC-transporters have an effect on irinotecan efflux, further studies have highlighted the dominant substrate affinity of the MRP1 sub-family for this drug and its active metabolite (SN-38) (Chu *et al.*, 1999). Paclitaxel is usually given once weekly as a second-line monotherapy in relapsed or refractory SCLC (Smit *et al.*, 1998; Yamamoto *et al.*, 2006), and pharmacokinetic studies have indicated that the ABC-transporter, P-gp, has a significant substrate affinity towards this drug (Ambudkar *et al.*, 1999). Cisplatin is a widely used anti-neoplastic drug and is specifically used in combination therapy for the first line treatment of SCLC. Combination therapies of cisplatin/irinotecan and cisplatin/paclitaxel have also yielded promising results (Sevinc *et al.*, 2011; van Meerbeeck *et al.*, 2011). ABC-transporter efflux studies indicated drug resistance to cisplatin is predominately caused by MRP2 transporters, with minimum effects exerted by P-gp and MRP1 (Stordal & Davey, 2007).

1.2 Problem statement

SCLC is an aggressive lung cancer that has a poor prognosis and results in the highest mortality rates among lung cancer types. Chemotherapeutic treatment gradually loses its apoptotic potential in these tumours, due to genetic mutations leading to the over-expression of efflux transporter proteins (i.e. P-gp, MRP1, etc.). These transporters synergistically lower intracellular concentrations of the anticancer drugs, and thereby cause MDR. Researchers have trouble studying the potential effects of efflux transporters on chemotherapeutic compound efficacy over a wide dosage range, and across several periods of drug exposure. The mechanisms of drug failure are therefore not always clear or understood, because of the limited information preclinical assays and clinical studies present. Also, a lot of valuable research is frequently lost during subsequent discontinuation of drug development.

The use of mammalian cell lines does have their limitations, as highlighted in **Chapter 3**, including numerous inter-laboratory differences, morphological changes of cells between passages and the lack of a physiological carrier system limits the complete reliability of cellular reactions during experiments.

1.3 General aim

The aim of this study is to determine the suitability of selected mammalian multidrug resistant cell lines as models for *in vitro* anticancer drug screening when exposed to established chemotherapeutic compounds. Furthermore, the predictive capacity of these models to investigate ABC-transporter induced MDR was also assessed. The general cytotoxicity of each anticancer drug was confirmed in non-cancerous porcine kidney cell line.

1.4 Specific objectives

The specific objectives for this study entailed the following.

- Culturing three human small cell lung carcinoma cell lines (H69V, H69AR and NCI-H69/LX4), and a non-cancerous porcine embryonic kidney cell line (LLC-PK1), as two-dimensional *in vitro* models.
- Determining the anticancer efficacy of a selection of chemotherapeutic compounds (irinotecan, paclitaxel and cisplatin) on the various cell lines following exposure for 96 h, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as a preliminary screening tool.
- Calculating the concentration of the model compounds needed to cause a 50% and 75% inhibition in cell population compared to an untreated value (IC_{50} and IC_{75}) in the selected cell lines through statistical evaluation of the MTT data.
- Treating the cell lines with the calculated IC_{50} and IC_{75} concentrations of each model compound in terms of wet biomass for 96 h, followed by evaluation of cellular responses using more sensitive assays measuring soluble protein content, intracellular adenosine triphosphate (ATP) and extracellular adenylate kinase (AK) levels.
- Confirming the increased expression levels of the P-gp and MRP1 efflux transporters in the MDR cell lines through gene expression assays.

1.5 Presentation of results

The results contained herein was presented at the first Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST) hosted by the University of Stellenbosch. The conference proceedings were held from the 7th until 10th of October 2018 at the Spier wine farm, Western Cape. The acceptance of my submitted abstract allowed me to partake

in the young scientist competition, hosted by the Academic Pharmaceutical Society of South Africa (APSSA). (See **Appendix C**)

1.6 Chapter layout of this dissertation

This dissertation is a compilation of chapters, with some chapters consisting of manuscripts prepared for publication in scientific journals.

Chapter 1 provides the background and justification for the study. It also includes the problem statement, aim, specific objectives and the outline of the chapters.

Chapter 2 consists of a literature review on the overall impact of cancer on mankind, the occurrence of MDR in cancer, efflux transporters as mechanisms used in MDR with a special focus on P-gp and MRP1, as well as SCLC as a devastating disease and the chemotherapeutic drugs used for its treatment.

Chapter 3 includes a review manuscript, discussing the literature regarding the various models available for efflux-based multidrug resistant cancer treatment screening. These can be classified as either *in vitro* or *in vivo* models. This manuscript will be submitted for publication to the journal *Current Cancer Drug Targets*. The author of this dissertation contributed significantly to this manuscript and wrote the complex cell-based models section.

Chapter 4 provides the materials, methods, results and discussion of the preliminary screening of each model drug in the various cell lines as determined with the MTT assay. The relative cell viability inhibitory concentrations determined are indicative of the drug sensitivity of each cell line, and confirmed literature reports of drug resistance towards certain chemotherapeutic compound substrates.

Chapter 5 is presented as an original research manuscript, describing the responses of each cell line to treatment with the different model compounds. IC_{50} and IC_{75} values determined with the preliminary MTT screening was used, and soluble protein content, intracellular ATP levels and AK release were assessed following exposure for 96 h. This chapter will be submitted to the journal *Experimental Cell research*.

Chapter 6 contains concluding remarks on the use of these cell lines as preclinical drug development models, and future recommendations are also presented.

References are provided at the end of each individual chapter.

Appendix A includes the “Instructions to Authors” of the journal *Current Cancer Drug Targets*.

Appendix B includes the “Instructions to Authors” of the journal *Experimental Cell Research*.

Appendix C includes the proof of attendance at the CoBNeST conference.

Appendix D includes confluency determination for each cell line.

Appendix E includes the ethics approval and registration of commercial cell line use.

Appendix F includes the isolated graphs as present in chapter 5 as single larger images.

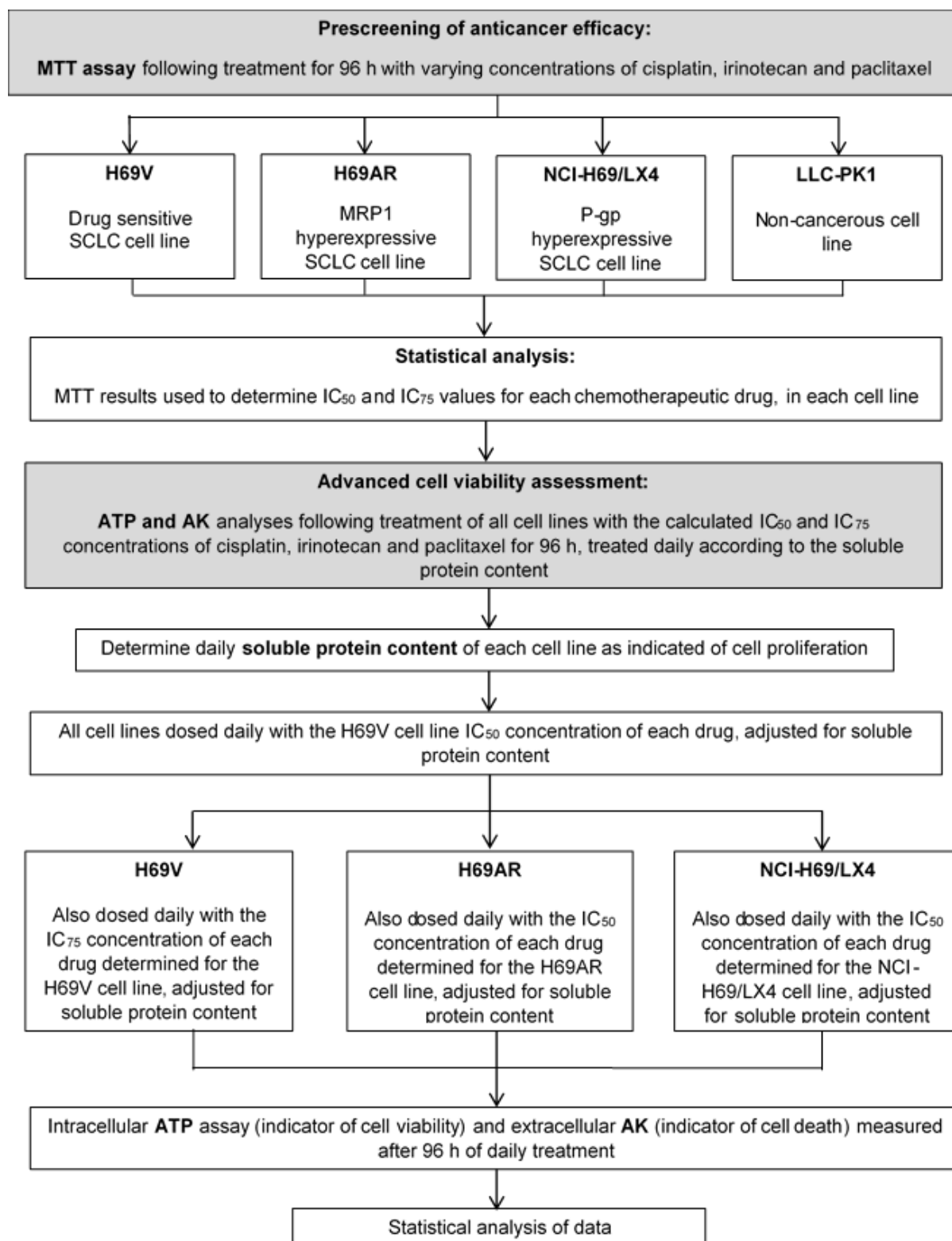


Figure 1.1: A diagram depicting the layout of all experimental aspects of this study.

1.7 References

- Alvarez, A.I., Real, R., Pérez, M., Mendoza, G., Prieto, J.G. & Merino, G. 2010. Modulation of the activity of ABC transporters (P-glycoprotein, MRP2, BCRP) by flavonoids and drug response. *Journal of Pharmaceutical Sciences*, 99(2): 598-617.
- Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I. & Gottesman, M.M. 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter 1. *Annual Review of Pharmacology and Toxicology*, 39(1): 361-398.
- Baguley, B.C. 2010. Multiple drug resistance mechanisms in cancer. *Molecular Biotechnology*, 46(3): 308-316.
- Chu, X.-Y., Suzuki, H., Ueda, K., Kato, Y., Akiyama, S.-I. & Sugiyama, Y. 1999. Active efflux of CPT-11 and its metabolites in human KB-derived cell lines. *Journal of Pharmacology and Experimental Therapeutics*, 288(2): 735-741.
- Elias, A.D. 1997. Small cell lung cancer: state-of-the-art therapy in 1996. *Chest*, 112(4): 251S-258S.
- Fletcher, J.I., Haber, M., Henderson, M.J. & Norris, M.D. 2010. ABC transporters in cancer: more than just drug efflux pumps. *Nature Reviews Cancer*, 10(2): 147-156.
- Früh, M., De Ruyscher, D., Popat, S., Crino, L., Peters, S., Felip, E. & Group, E.G.W. 2013. Small-cell lung cancer (SCLC): ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, 24(suppl 6): vi99-vi105.
- Gottesman, M.M., Fojo, T. & Bates, S.E. 2002. Multidrug resistance in cancer: the role of ATP-dependent transporters. *Nature Reviews Cancer*, 2(1): 48-58.
- Kalemkerian, G.P., Akerley, W., Bogner, P., Borghaei, H., Chow, L.Q., Downey, R.J., Gandhi, L., Ganti, A.K.P., Govindan, R. & Grecula, J.C. 2013. Small cell lung cancer. *Journal of the National Comprehensive Cancer Network*, 11(1): 78-98.
- Kaur, G., Reinhart, R.A., Monks, A., Evans, D., Morris, J., Polley, E. & Teicher, B.A. 2016. Bromodomain and hedgehog pathway targets in small cell lung cancer. *Cancer Letters*, 371(2): 225-239.
- Krishna, R. & Mayer, L.D. 2000. Multidrug resistance (MDR) in cancer: mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *European Journal of Pharmaceutical Sciences*, 11(4): 265-283.

- Little, C.D., Nau, M.M., Carney, D.N., Gazdar, A.F. & Minna, J.D. 1983. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. *Nature*, 306(5939): 194-196.
- Öberg, K., Hellman, P., Ferolla, P., Papotti, M. & Group, E.G.W. 2012. Neuroendocrine bronchial and thymic tumors: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, 23(suppl 7): vii120-vii123.
- Oronsky, B., Reid, T.R., Oronsky, A. & Carter, C.A. 2017. What's new in SCLC? A review. *Neoplasia*, 19(10): 842-847.
- Pass, H.I., Carbone, D.P., Johnson, D.H., Minna, J.D., Scagliotti, G.V. & Turrisi, A.T. 2012. Principles and practice of lung cancer: the official reference text of the international association for the study of lung cancer (IASLC): Lippincott Williams & Wilkins.
- Paumier, A. & Le Péchoux, C. 2010. Radiotherapy in small-cell lung cancer: Where should it go? *Lung Cancer*, 69(2): 133-140.
- Sevinc, A., Kalender, M.E., Altinbas, M., Ozkan, M., Dikilitas, M., Camci, C. & Oncology, A.S.o.M. 2011. Irinotecan as a second-line monotherapy for small cell lung cancer. *Asian Pacific Journal of Cancer Prevention*, 12(4): 1055-1059.
- Smit, E., Fokkema, E., Biesma, B., Groen, H., Snoek, W. & Postmus, P. 1998. A phase II study of paclitaxel in heavily pretreated patients with small-cell lung cancer. *British Journal of Cancer*, 77(2): 347.
- Stordal, B. & Davey, M. 2007. Understanding cisplatin resistance using cellular models. *IUBMB Life*, 59(11): 696-699.
- Sundstrøm, S., Bremnes, R.M., Kaasa, S., Aasebø, U., Hatlevoll, R., Dahle, R., Boye, N., Wang, M., Vigander, T., Vilsvik, J., Skovlund, E., Hannisdal, E. & Aamdal, S. 2002. Cisplatin and etoposide regimen is superior to cyclophosphamide, epirubicin, and vincristine regimen in small-cell lung cancer: Results from a randomized phase III trial with 5 years' follow-up. *Journal of Clinical Oncology*, 20(24): 4665-4672.
- Triller, N., Korošec, P., Kern, I., Košnik, M. & Debeljak, A. 2006. Multidrug resistance in small cell lung cancer: Expression of P-glycoprotein, multidrug resistance protein 1 and lung resistance protein in chemo-naive patients and in relapsed disease. *Lung Cancer*, 54(2): 235-240.

Twentyman, P., Fox, N., Wright, K. & Bleehen, N. 1986. Derivation and preliminary characterisation of adriamycin resistant lines of human lung cancer cells. *British Journal of Cancer*, 53(4): 529.

van Meerbeeck, J.P., Fennell, D.A. & De Ruyscher, D.K. 2011. Small-cell lung cancer. *The Lancet*, 378(9804): 1741-1755.

Warde, P. & Payne, D. 1992. Does thoracic irradiation improve survival and local control in limited-stage small-cell carcinoma of the lung? A meta-analysis. *Journal of Clinical Oncology*, 10(6): 890-895.

Yamamoto, N., Tsurutani, J., Yoshimura, N., Asai, G., Moriyama, A., Nakagawa, K., Kudoh, S., Takada, M., Minato, Y. & Fukuoka, M. 2006. Phase II study of weekly paclitaxel for relapsed and refractory small cell lung cancer. *Anticancer Research*, 26(1B): 777-781.

CHAPTER 2

This chapter contains relevant literature background regarding small cell lung carcinoma, multidrug resistance in cancer and the chemotherapeutic compound and cell line models used in this study.

LITERATURE BACKGROUND

2.1 Introduction

Cancer is defined as the uncontrolled and abnormal division of cells resulting in the formation of malignant tumour growth. These uncontrolled cells can metastasise to different regions of the body through the bloodstream and lymphatic system and cause over 200 different kinds of cancer. The disease progression rate statistical analysis in populations projected that by 2025 cancer will result in 11.4 million deaths, along with 19.3 million new cancer diagnoses (Ferlay *et al.* 2010). The majority of diagnoses worldwide in men are lung cancer and in women, it is primarily breast cancer, but incidences differ in various populations and are dependent on lifestyle factors (e.g. smoking and hormone treatments). Of significant concern is when a 59% increase in cancer diagnoses will be seen in less-developed countries, which are already struggling to provide primary health care to cancer patients, let alone sophisticated cancer treatment regimens (Bray *et al.*, 2012). The ineffective treatment of cancers with out-dated regimens due to the inability of individuals to afford these complex treatments leads to the prevalence of multidrug resistant cancers with severe mortality rates. The premature death of individuals can have a severe impact on both the economy and the overall wellbeing of these developing countries.

2.2 Multidrug resistance in cancer

2.2.1 What is multidrug resistance?

Multidrug resistance (MDR) can be defined as the resistance capabilities cancer cells develop, due to cellular and non-cellular mutations, to withstand the apoptotic induction of different classes and combinations of chemotherapeutic regimens (Krishna & Mayer, 2000). This resistance is inclusive for a wide range of structurally unrelated chemotherapeutic compounds and not necessarily limited to a single class of drugs to which the patient was previously exposed to (Baguley, 2010). Multidrug resistant cancers can be categorised into two main groups, namely those who have intrinsic resistant capabilities and those who present with acquired resistance (Gottesman, 2002; Baguley, 2010).

During most chemotherapeutic treatment regimens, a chemo-naive cancer patient initially responds remarkably well to the treatments and can even be in remission for a certain period of time only to have the cancer return after a few months/years, sometimes with complete non-responsiveness to chemotherapy. In this case cancer has undergone adaptive

mutations and will not respond to the previous cancer regimens used in this patient and potentially also not to other structurally unrelated compounds. This phenomenon is known as acquired MDR (Goldstein *et al.*, 1989; Binkhathlan & Lavasanifar, 2013). In some cases a chemo-naive patient does not respond to any of the first line treatments and cancer cell characteristics are similar to that of acquired MDR. This intrinsic resistance is just as fatal and is most likely due to aggressive mutations within the original cancer cell line (Ozben, 2006; Lage, 2008; Baguley, 2010).

2.2.2 How is multidrug resistance achieved in cancer cell lines

2.2.2.1 Macroscopic pharmacokinetic resistance

Pharmacokinetic parameters can be seen as a multi-factorial study of the bodily action on drugs (Undevia *et al.*, 2005). An initial approach to characterise and explain chemo-drug failure in cancer treatment by using systematic macroscopic pharmacokinetics failed to encapsulate and explain every aspect of the MDR phenomenon, but it gave an investigational starting point for researchers. By compartmentalising the classic ADME-factors (absorption, distribution, metabolism and elimination) researchers were able to track the reason for drug failures and to what extent each factor played a role in this drug efficacy deviation in the host. Pharmacokinetic ADME properties of drugs can be manipulated to minimize insufficiencies caused by compound structure properties, but as seen clinically this will only marginally increase the effectiveness of drug therapy. Drug delivery was found not to be hindered exclusively by shortcomings of the drug but rather by multiple factors ranging from tumour microenvironments to cellular adaptations.

Even when considering all of the pharmacokinetic factors mentioned in **Table 2.1**, drug failure was still imminent and manipulation of drug properties only marginally increased cancer cell death. A major obstacle for drug effectiveness was found to be the permeation capabilities of the chemotherapeutic drug compounds into the solid tumours (Alfarouk *et al.*, 2015).

Table 2.1: Examples of how pharmacokinetic factors can influence drug delivery to tumour sites (adapted from Finlay & Baguley, 2000; Undevia *et al.*, 2005; Garattini, 2007; Alfarouk *et al.*, 2015)

Absorption	<ul style="list-style-type: none"> • Nausea and vomiting (a common side effect in most chemotherapeutic regimens) • Inadequate dosage form/infusion and release into the bloodstream • Physiological factors progressing drug breakdown or inhibiting absorption • Intestinal absorption mechanisms and metabolic systems with genetic variations
Distribution	<ul style="list-style-type: none"> • The extravascular fluid build-up in cancer patients (pleural effusion and ascites) causing redistribution • Binding of anticancer drugs to plasma protein (Plasma albumin) and subsequent deactivation • Sequester of anticancer drugs by binding to alpha-acid glycoprotein (commonly elevated protein in cancer patients) • Percentage body fat lowering distribution of lipophilic compound concentration to tumour sites

Metabolism

(P=Properties relating specifically to prodrugs)

- Hepatic damage can influence the concentration of active ingredient present in the bloodstream (P)
- Chronic inflammation (which is the case in most cancers) lowers metabolic capabilities (P)
- Phase 1 metabolism by microsomal CYP P450 enzymes and epoxide hydrolases followed by Phase 2 metabolism and excretion by ABC-transporters

Excretion (in malignancies influencing metabolic and excretion organs)

- Altered hepatic biliary blood flow causing host toxicity
- Renal insufficiency
- Urinary pH influencing precipitation and excretion

Factors influencing pharmacokinetic parameters

- Age
 - Gender
 - Race/ethnic background
 - Body fat percentage
 - Diseases/illnesses
 - Pharmacodynamics (drug-drug interactions)
 - Genetic polymorphs (metabolic enzymes)
-

2.2.2.2 Tumour microenvironment multidrug resistance mechanisms

Solid tumours are complex heterogeneous organ-like structures embedded in an extracellular communications matrix, equipped with their own vascular system (Trédan *et al.*, 2007). By taking a molecular approach to MDR, we can witness a complex cascade of synergistic mechanisms in the tumour microenvironment working against xenobiotic/foreign substances to limit any adverse effects on the cancer cells in the host. These conjunctive mechanisms were found to be the backbone of MDR and can be held responsible for most of the chemo-drug failures. **Table 2.2** provides a summary of several of these microenvironmental mechanisms used by cancer cells to achieve MDR.

Table 2.2: Tumour microenvironment factors influencing MDR

Mechanisms	
Increased inflammation in the tumour environment	<ul style="list-style-type: none"> • Increased angiogenesis and vascularisation • The release of fibroblasts and growth factors
Interstitial space	<ul style="list-style-type: none"> • Increased pressure due to enhanced vascularisation and permeability • Further pressure due to lack of lymphatic drainage • Blockage of convective flow
Vascularisation	<ul style="list-style-type: none"> • Heterogeneous vascularisation causes uneven distribution of chemotherapeutic drugs • Uneven distribution of oxygen and nutrients causes hypoxic resistance • Rheology and geometric resistance
Extracellular matrix	<ul style="list-style-type: none"> • Tyrosine kinase activity of structural proteins • Cell density limits drug penetration

pH	<ul style="list-style-type: none"> Alkaline and acidic properties affect drugs with a certain pKa parameter (ion-trapping mechanism) Immune-regulation
Hypoxia	<ul style="list-style-type: none"> Gene destabilisation (aggressive mutations) Increased ABC-transporter expression P53 mutations The absence of free radicals Failure of oxygen-dependent chemotherapy Deregulation of the immune system due to oxygen starvation
ABC-transporters	<ul style="list-style-type: none"> Active efflux of xenobiotic and chemotherapy drugs out of the cell and lysosomes Modulation of certain enzymes (CYP3A4) to increase metabolism
Other	<ul style="list-style-type: none"> Drug sequestration into lysosomes Mutations in target molecules (e.g. topoisomerase 2)

The extracellular matrix (ECM), a structural framework consisting of proteoglycans and fibrous proteins (Frantz *et al.*, 2010), of a solid tumour has important behavioural roles in the tumour's metastatic, transformation potential and drug sensitivity properties (Trédan *et al.*, 2007). The wound-like characteristics of the tumour stroma have been found to potentiate numerous inflammatory processes resulting in stiffened tissue and the release of fibroblasts and growth factors e.g. vascular endothelial growth factor (VEGF). These growth factors and fibroblasts ultimately initiate angiogenesis and tissue growth similar to wound healing (Chang *et al.*, 2004; Ellis & Hicklin, 2008). This inflammatory driven angiogenesis causes vascularisation of the tumour with remarkably increased permeation capabilities resulting in high interstitial pressure (Arroyo & Iruela-Arispe, 2010). Solid tumours also present with the lack of a lymphatic drainage system. This lymphatic system usually carries macromolecules (e.g. proteins and chemotherapeutic drugs) from the interstitial space to the blood and the

lack of this drainage mechanism further increases the pressure that is already present in this space (Narang & Varia, 2011). The pressure caused by this interstitial fluid inhibits the effective transport of anticancer molecules dependent on the convection transport mechanism (induced inward flow) to enter the tumour environment, thus lowering cytotoxic effects (Heldin *et al.*, 2004). Solid tumours also present with heterogeneous vascularisation with better circulation in the outer proliferating tissue than in the necrotic centres. This means systematic chemotherapeutic drugs are not delivered to large parts of the tumour and because of weak vessel spacing, substances cannot properly diffuse to all of the tissue compartments (Ullah, 2008).

Small cell lung cancer (SCLC) cells exist in a particularly rich extracellular matrix environment and studies investigating matrix components (fibronectin, collagen and laminin), found drug failure can be directly linked to the protein tyrosine kinase activity of these proteins. The ECM proteins are bound to SCLC-cells through connective cell surface glycoproteins called integrins (β 1 and α -subunits) that ultimately control this mechanism of MDR. These matrix proteins inhibit chemotherapeutic driven apoptotic processes (caspase-3) by means of β 1-integrin-stimulating antibodies and tyrphostin-25 (Rintoul & Sethi, 2002; Hodgkinson *et al.*, 2007). The ECM also consists of proteins with a fibrous nature (e.g. glycosaminoglycan) that increase the cell density and resists convective flow into the cell, hindering drug delivery. This resistance mechanism has been observed in xenografts and histocultures during the administration of paclitaxel, doxorubicin and vinblastine. The permeation only increased during apoptotic driven processes causing a decrease in cell density, substantiating the claim of density-dependent MDR (Grantab *et al.*, 2006; Kim *et al.*, 2011).

Analysis of the tumour microenvironment has found a pH difference in the extracellular (acidic) and intracellular (basic) compartments, creating a pH gradient. This pH gradient has numerous effects on increasing tumour malignancy and survivability in the host (e.g. immune-regulation and cellular replication dominance), but also introduces another drug barrier for chemotherapeutic drugs, namely the “ion-trapping mechanism” (Neri & Supuran, 2011; Parks *et al.*, 2011). The differences in pH cause drug acidic dissociation value (pKa) dependent ionisation and sequestration into lysosomes, resulting in lowered active drug concentrations in the host tumour cells. Weak basic drugs are affected the most by this phenomenon due to ionisation deactivation processes already taking place in the interstitial compartment (Mahoney *et al.*, 2003; Webb *et al.*, 2011).

Due to heterogeneous vascularisation, resistant blood flow (rheology/geometric resistance) and overall perfusion limiting factors in the tumour environment, cancer cells can either be exposed to intermittent supplies of nutrients and oxygen or reside in a state of permanent starvation (Fukumura & Jain, 2007; Müller *et al.*, 2014). This allows for cell behavioural predictive categorisation into the permanent isolation of vascularisation (chronic hypoxia) and intermittent supply of oxygen (perfusion-limited hypoxia) (Rofstad *et al.*, 2007). Initially, cancer starvation was thought to be a viable mechanism to initiate cancer cell death in tumours, but upon observation of hypoxic cell behaviour a different conclusion was made. These intermittent (diffusion limited) hypoxic cells are the drivers of the malignant nature found in cancers due to destabilisation of the normal gene structure, causing aggressive mutations that can be linked to some of the most prominent MDR mechanisms in tumours. Gene destabilisation was found to cause deactivating mutations in apoptotic precursors (p53), increased expression of adenosine triphosphate (ATP) binding cassette (ABC)-transporters and gene expression increases (HIF-HRE) influencing supplementation pathways of crucial resources (e.g. vasodilatation, angiogenesis and glycolysis) (Harris, 2002; Rohwer & Cramer, 2011; Keith *et al.*, 2012; Rebutti & Michiels, 2013). The hypoxic environment in the cells also causes oxygen dependent drug treatment failures (radiation/chemotherapy) that either relies on the formation of free radicals or metabolism of drugs to active substrates (e.g. paclitaxel to 6- α -hydroxypaclitaxel) (Semenza, 2004; Denko, 2008; Huang *et al.*, 2010). The host's immune system is also oxygen dependent in order to facilitate the natural apoptotic inducing mechanism of malignant cancer cells and the absence of proper oxygenation de-escalates this process and increases tumour survivability (Antonioli *et al.*, 2013).

Another molecular mechanism attributing to MDR is an intra-molecular resistance mechanism known as drug sequestration. Intracellular organelles like lysosomes, Golgi apparatus and secretory compartments act as masking carrier vessels, sequestering drug molecules such as cisplatin, followed by ABC-transporter efflux (Gillet & Gottesman, 2010).

2.2.2.3 Efflux pumps of the ABC-transporter superfamily

The ABC-transporters are a family of 48 cellular transmembrane proteins responsible for the efflux of solutes across the cell membrane or sequestration into lysosomes and plays a predominant role in cell susceptibility to exogenous pathogenic substances with apoptotic potential (Gottesman *et al.*, 2002). Emphasis is particularly placed on these transporters, as the resistance of cell lines to chemotherapeutic drugs can be directly proportional to transporter expression (Wu *et al.*, 2014). Solute can range from biological endogenous

substances needed for life-sustaining processes (including lipids and metabolic products) to exogenous cytotoxic xenobiotics (chemotherapeutics) and the expulsion or transport of these materials ensures host health and survival (Fletcher *et al.*, 2010a). These transporters are present in a wide spectrum of anatomic membranes (e.g. intestinal membrane, blood-brain-barrier and hematopoietic membranes), both defending and maintaining homeostasis in the host by processes such as immune-regulation (transport of histocompatibility antigens into endoplasmic reticulum), intercellular transport of lipid-and-peptide molecules and inductive interactions with metabolic enzymes (increases CYP3A4 expression) (Wacher *et al.*, 1998; Sharom, 2008). Emphasis of their importance in self-preservation is demonstrated by their presence in both prokaryotic (cytoplasmic membrane) and eukaryotic (plasma membrane and organelle membranes) organisms, but unfortunately some of the 48 different ABC-transporters (subclasses grouped from A to G) are also paradoxically linked to some life-threatening malignancies (e.g. cancer and cystic fibrosis) (Dean *et al.*, 2001; Wilkens, 2015).

Genomic analysis found that all of the ABC-transporters share 30% of their genomic sequences with each other, each possessing core consensus motifs (Walker A, Walker B and signature C) needed for ATP-binding (Walker *et al.*, 1982; Toyoda *et al.*, 2008). Full ABC-transporter molecules are equipped with two intracellular nucleotide binding domains (NBDs) for hydrolyses of ATP molecules to generate energy for transport of molecules against the concentration gradient (active transport) across the plasma and intracellular membrane to the outside of the cell (Binkhathlan & Lavasanifar, 2013). These transporters are also equipped with a range of transmembrane domains (6 helical membrane spanning α -helices per domain) that encapsulates a drug binding site chamber, providing a pathway for substrates and this ultimately determines transporter specificity (Szakacs *et al.*, 2008). The energy generated by the hydrolysis of the ATP at the NBD-sites initiates conformational changes in the transmembrane domain to alternate between inward and outward facing helices, exposing substrates to binding sites and causing active transport across the cell membrane (Dean, 2009). **Figure 2.1** provides an example of how a full ABC-transporter is positioned in the phospholipid bilayer.

Tumour-specific studies have established that the most pharmacologically relevant and over-expressed ABC-transporters in cancers (especially SCLC) are P-glycoprotein (P-gp or ABCB1) and multidrug resistance-associated protein 1 (MRP1 or ABCC1). These transporters are also the main drivers of MDR in the majority of cancer cells and they have a broad overlapping spectrum of substrate specificity aiding their MDR-inducing capabilities

(Triller *et al.*, 2006; Alvarez *et al.*, 2010). Breast cancer resistance protein (BCRP or ABCG2) is the third most prevalent ABC-transporter and will only be discussed briefly.

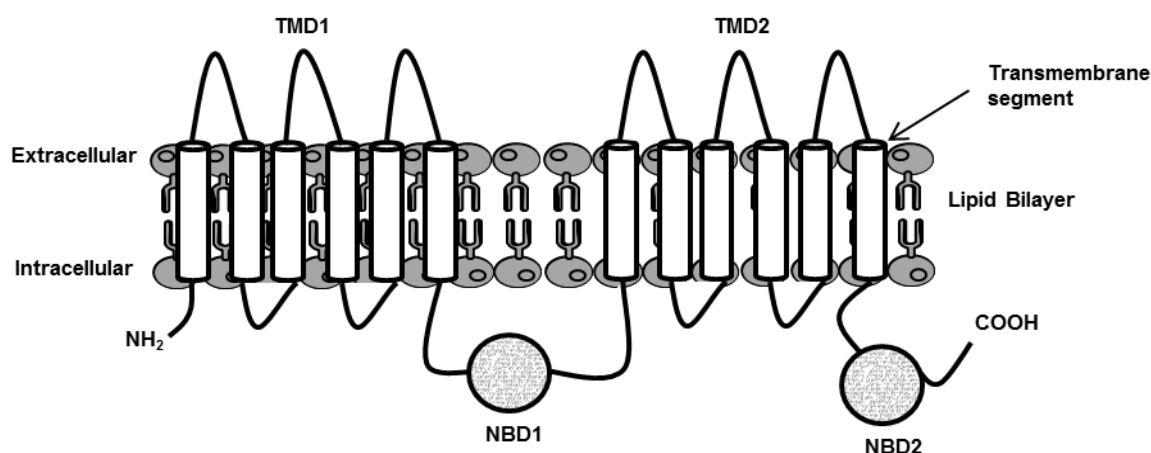


Figure 2.1: Typical full ABC-transporter (e.g. P-gp) embedded in a lipid bilayer (adapted from Choudhuri & Klaassen, 2006). *TMD*; *Transmembrane domain*, *NBD*; *Nucleotide binding domain*.

2.2.2.3.1 P-glycoprotein

P-glycoprotein, also known as multidrug resistant protein 1 (MDR1) or ABCB1-transporter, was the first ABC-transporter to be categorised in Chinese hamster ovary multidrug-resistant cells conferring resistance to colchicine (Ling & Thompson, 1974; Higgins, 2007). It has later been found to also cause the majority of chemotherapeutic drug failures during cancer treatment and quickly became an important target to overcome MDR in cancer. This 170-kDa protein is specifically localised in the Golgi apparatus and rough endoplasmic reticulum, thus ensuring intracellular homeostasis and defence by means of drug sequestration and efflux (Bendayan *et al.*, 2006; Eckford & Sharom, 2009).

P-gp has been found to have extremely broad substrate specificity, to be active in both positively and negatively charged hydrophobic substrates while partitioning into the lipid bilayer and to serve as the rate-limiting step in efflux (lipophilicity) (Seelig & Landwojtowicz, 2000). Hydrogen bonds are directly associated with the lipophilic characteristics ($\log-P \geq 2.92$), and substrate specificity to the P-gp protein is measured by means of hydrogen bond strength and the number of bonds (Zhou, 2008). These amphipathic substrates have molecular weights ranging between 300 Mm and 1000 Mm, as well as having a positive charge in the host's pH (Sharom, 2008). This specificity included the lion's share of

chemotherapeutic drugs (listed in **Table 2.3**) and expression of this protein in cancer cells was directly proportional to the degree of MDR in general (Fletcher *et al.*, 2010a).

Administering treatments to block the P-gp efflux action is not always necessarily a feasible pathway due to the presence of this protein on the apical membranes of excretion and secretion organs including the intestines, canaliculi of the bile ducts, kidney tubular cells, pancreatic membranes, placenta and blood-brain-barrier (BBB). These organs are susceptible to pathogenic substances and removal of their primary defence mechanism can have catastrophic toxicological consequences (Giacomini *et al.*, 2010). P-gp transporters also have important physiological roles in the host including transporting numerous endogenous metabolites, facilitating flippase processes (cytoplasmic leaflet to extracellular leaflet), and cytokine/hormone transport. Selectivity of treatments are not always adequate and systematic toxicity is a given during P-gp modulating/blocking treatments.

Table 2.3: Chemotherapeutic drugs that are susceptible to P-gp transporter efflux (adapted from Sharom, 2008; Zhou, 2008)

P-gp substrate chemotherapeutic agents:	<ul style="list-style-type: none"> • Anthracyclines (daunorubicin, doxorubicin) • Vinca-alkaloids (Vincristine, Vinblastine) • Taxanes (Paclitaxel, docetaxel) • Epipodophyllotoxins (Etoposide, Teniposide) • Imatinib • Camptothecins (Topotecan, Irinotecan (SN-38)) • Anthracenes (Bisantrene, mitoxantrone) • Colchicine
--	---

2.2.2.3.2 Multidrug resistance-associated protein (ABCC1)

The multidrug resistance-associated proteins are a family of nine full 190-kDa transporters, also known as MRP (protein) or ABCC (transcribing gene), that specifically transports

glutathione conjugated-and-unconjugated substrates out of the cell. The MRP transporters play a significant role in host homeostasis by means of transport and modulation of physiological substances including immune-and-steroid hormone components such as leukotrienes, prostaglandins and oestrogens (Leslie *et al.*, 2005b; Cole, 2014a).

These transporters differ from the conventional P-gp protein in both its substrate specificity and its structure. The larger MRP transporters are equipped with three transmembrane hydrophobic domains (TMD), along with the conventional 2 NBDs, and are responsible for the main efflux potential in the ABCC family (Choudhuri & Klaassen, 2006). **Figure 2.2** illustrates a conventional MRP-transporter located in a phospholipid bilayer.

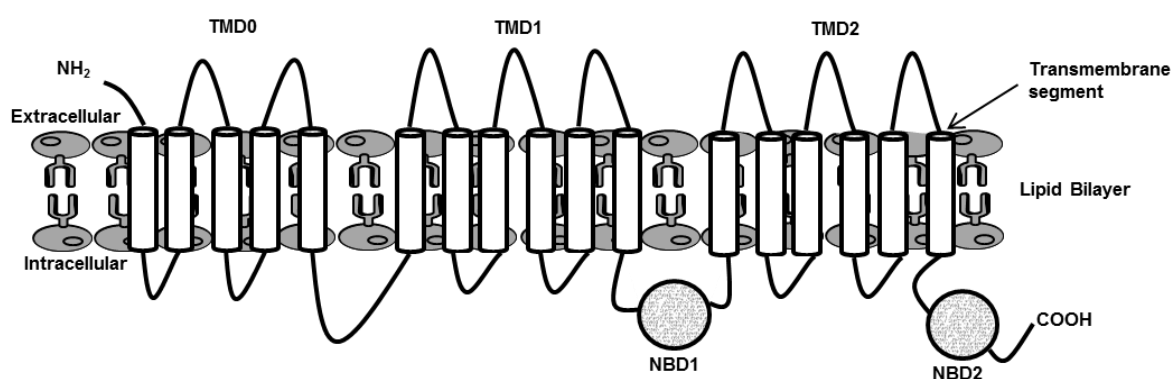


Figure 2.2: Large conventional MRP-transporter structure in a lipid bilayer (adapted from Choudhuri & Klaassen, 2006). *TMD*; *Transmembrane domain*, *NBD*; *Nucleotide binding domain*

Second only to P-gp, the MRP1 transporter of the MRP-family fulfils a substantial role in cancer MDR. The MRP-transporters were discovered when an adriamycin exposed cell line (HL60/ADR) exerted drug resistance via an energy-dependent mechanism without the presence of the known P-gp efflux proteins. Further investigation led to the isolation of the MRP-transporter DNA and provided the first evidence of multiple cofactors in efflux based drug resistance (Kruh & Belinsky, 2003). This transporter was later also cloned from a human SCLC cell line (H69AR) that was continuously exposed to increasing doses of the anticancer compound, adriamycin, to hyperexpress the MRP1 transporter (Mirski *et al.*, 1987; Cole *et al.*, 1992). The characterisation of this protein led to the discovery of eight more transporters (ABCC2-6 and ABCC-10-12), all descendants from the ABCC-genes and contributors to MDR (Dean & Allikmets, 2001; Kruh & Belinsky, 2003).

MRP1 increases its substrate affinity spectrum by using the glutathione present at millimolar concentrations in cell membranes and is thus aptly known as a glutathione, as well as an

ATP-dependent transporter (Cole & Deeley, 2006). Human genome studies found MRP1 to be predominantly present in the basolateral endothelial polarised membranes of the lung, prostate and breast cancer cells, explaining particular resistance in these cancers (Cole, 2014a).

In cytotoxicity studies, relatively low resistance capabilities have been found for MRP1 transporters with the use of P-gp substrates such as paclitaxel and vinblastine. This implies that transporters like MRP1 has a synergistic auxiliary function to strengthen resistance in cells towards chemotherapeutics, but needs a network of several transporters to effectively maintain a status of total MDR. In other words, where P-gp lacks certain aspects of cell defence (e.g. metabolites substrate specificity), transporters like MRP1 compensates for these shortcomings to maintain the MDR-status and vice versa (Cole, 2014b).

Possible clinical significance can be achieved by means of receptor modulation in a disease specifically plagued by this transporter such as acute myeloid-and-lymphoblastic leukaemia as well as lung cancers to control and diminish MDR in these cells. However, considering the role this transporter has in ensuring cell viability and overall health of the host more refined and tumour specific transporter modulating agents are needed to lower overall systematic toxicity.

In **Table 2.4** one can see the vast range of drugs as well as natural substrates that are affected by this transporter, thus indicating the problematic consequences that can follow when causing a complete blockage of this MRP-1 transporter as a method to overturn MDR.

Table 2.4: Common chemotherapeutic drugs that are susceptible to MRP1 based transporter efflux (adapted from Borst *et al.*, 2000; Eckford & Sharom, 2009; Fletcher *et al.*, 2010b)

Classes of drugs	MRP1 substrates
Chemotherapeutic drugs	<ul style="list-style-type: none"> • Anthracyclines (Doxorubicin, Daunorubicin, epirubicin) • Camptothecins (Topotecan, Irinotecan) • Vinca Alkaloids (Vinblastine, Vincristine) • Methotrexate • Mitoxantrone

	<ul style="list-style-type: none"> • Imatinib • Etoposide • Colchicine • Saquinavir • Flutamide • Geldanamycin
Conjugated metabolites (GSH)	<ul style="list-style-type: none"> • Leukotriene C₄ • Prostaglandin A₂ • Prostaglandin J₂
Conjugated metabolites (Glucuronide)	<ul style="list-style-type: none"> • E₂17βG
Conjugated Metabolites (Sulphate)	<ul style="list-style-type: none"> • Estrone-3-sulfate
Unconjugated	<ul style="list-style-type: none"> • Folic acid • Bilirubin

Although the MRP-2 transporters have similar structural components to that of the MRP-1 family (**Figure 2.2**), it has distinctive substrate specificity to some endogenous and xenobiotic compounds (**Table 2.5**) (Hipfner *et al.*, 1997; Leslie *et al.*, 2005b). What makes this transporter function significantly different from P-gp and other MRP transporters is their specific expression on the apical membranes of polarised cells of proximal renal tubular cells, enterocytes, syncytiotrophoblasts (placenta) and hepatocytes (Jedlitschky *et al.*, 2006). The expression of the MRP-2 transporter on the latter mentioned areas plays a large homeostatic role in the pharmacokinetic regulation of xenobiotic and endogenous compounds. Although there are numerous overlapping substrates between P-gp and MRP1 with the MRP2 substrate, chemotherapeutic drug cisplatin seems to be primarily subjected to MRP2 efflux (Sprowl *et al.*, 2012).

Table 2.5: Common chemotherapeutic drugs that are susceptible to MRP2 based transporter efflux (adapted from König *et al.*, 2003; Jedlitschky *et al.*, 2006; Zhou *et al.*, 2008)

Classes of drugs	MRP2 substrates
Chemotherapeutic drugs	<ul style="list-style-type: none"> • Cisplatin • Doxorubicin • Etoposide • Methotrexate • Vincristine • Vinblastine • Camptothecin • Mitoxantrone
Conjugated metabolites (GSH)	<ul style="list-style-type: none"> • Leukotriene C4 • Leukotriene D4 • Leukotriene E4
Conjugated metabolites (Glucuronide)	<ul style="list-style-type: none"> • Bilirubin • 17β-glucuronosyl estradiol
Conjugated Metabolites (Sulphate)	<ul style="list-style-type: none"> • Bile salts

2.2.2.3.3 Other clinically significant transporters

The original identification of transporter related drug failure was narrowed down mainly to three primary mammalian transporters namely P-glycoprotein (ABCB1/P-gp), breast cancer-resistance protein (ABCG2) and multidrug resistance-associated proteins (ABCC/MRP) (Leslie *et al.*, 2005a; Mao & Unadkat, 2015). Although other transporters from the ABC-family have their role in drug resistance, the abovementioned transporters were responsible for most of the reported MDR.

The BCRP is a relatively small 70 kDa (half the size of P-gp) efflux protein that was originally cloned from a multidrug resistant breast cancer (hence aptly named). The BCRP transporter is structurally different to its MRP and P-gp transporter counterparts but has a broad resistance spectrum overlapping significantly with the latter named transporters (Mao & Unadkat, 2015). The BCRP transporter contributes greatly to the resistance population found in many recurring and fatal lung cancers, thus warranting the inclusion of this transporter in many broad studies (Usuda *et al.*, 2011), but due to the scope of this study, BCRP will be excluded.

2.3 Small cell lung carcinoma

Statistical analysis of population cancer incidence by the World Health Organization found that lung cancer accounts for 10-15% of newly diagnosed cancer cases and has a mortality rate of 1 in 4 cancer fatalities (Ferlay *et al.*, 2015). Although SCLC only attributes to about 13% of the newly diagnosed cases, it was found to be the most aggressive and fatal among the lung cancers since 90% of the patients are older immuno-compromised smokers or previous smokers. Survival rates longer than 5 years in SCLC patients ranges between 15-21% and overall curative potential is slanted to a negative outlook (van Meerbeeck *et al.*, 2011b; Ferlay *et al.*, 2012; Bray *et al.*, 2013). A more recent analysis has determined that only 1 in 4 patients survived a stage IV small cell lung carcinoma diagnosis for longer than one year (Dayen *et al.*, 2017). Statistical analysis in South Africa by the National Cancer Registry in 2013 indicated that lung cancer makes out 4.91% of cancers in men and 2.52% of all cancers in women with 20% of all lung cancer diagnosis being attributed to SCLC (Singh *et al.*, 2014).

Initially, high response rates to treatments are evident, with over 90% of chemotherapy-naive patients experiencing tumour regression with the commencement of treatment (Stordal & Davey, 2007). Malignancy of this specific cancer is propagated by the fact that the relapse of this disease is almost always evident as multidrug resistant cancers, with a tendency to metastasise to other organs resulting in fatal consequences (van Meerbeeck *et al.*, 2011b).

The severity of this disease justifies intense research on possible curative regimes and the design of *in vitro* models to screen anticancer drugs, before advancing to *in vivo* and clinical studies. This has led to the design and characterisation of the SCLC H69 continuous cell line, originally retrieved from primary tumour sites or pleural fluids in humans, and subsequent cell strains that were manipulated to present with certain characteristics (Little *et al.*, 1983; Carney *et al.*, 1985). Current standard first-line treatments include platinum-based

chemotherapy with radiation. Prophylactic cranial irradiation to limit metastasis to the brain is recommended in both responsive treatment strategies as a palliative measure to prolong survival (Slotman *et al.*, 2009).

Treatment approaches for MDR cancer, in general, is discussed briefly in the review manuscript presented in **Chapter 3**.

2.4 Chemotherapeutic model compounds

2.4.1 Cisplatin

The platinum compound, cisplatin, was first identified as a possible antibiotic due to the changes induced on the bacterial morphology of *Escherichia coli*. These changes (filament elongation) ultimately resulted in the inhibition of cell division of these bacteria and antineoplastic research followed shortly afterwards in murine tumour models. The immense antineoplastic results found in *in vivo* murine cancer models motivated further intensive clinical trials and the Food and Drug Administration (FDA) approved cisplatin as the first chemotherapeutic drug to be used in anticancer therapy (Rosenberg *et al.*, 1965; Rosenberg *et al.*, 1969)

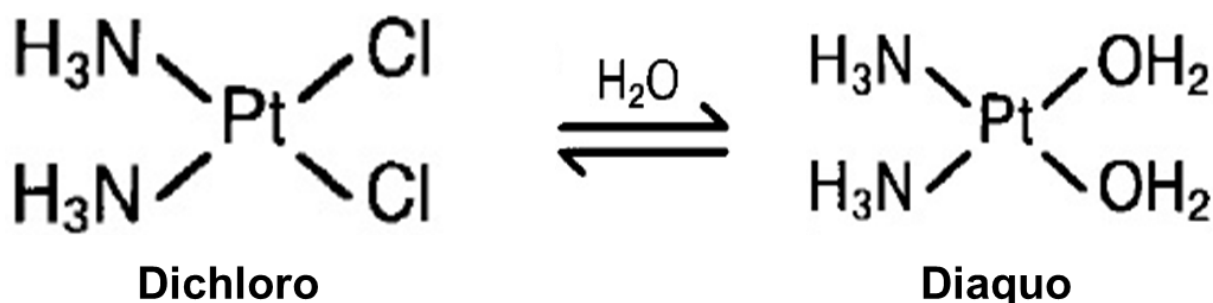


Figure 2.3: Replacement of chloride molecules with water molecules for activation of the cisplatin drug in cancer cells (adapted from Go & Adjei, 1999).

Cisplatin is activated in cancer cells by replacing the chloride groups with water molecules (**Figure 2.3**), driven by the presence of high water concentrations in these cells, and results in a hydrolysed electrophile product. This aqueous complex has the ability to react with numerous macromolecules, thus resulting in the desired cancer cell death (Go & Adjei, 1999). Cisplatin's antineoplastic properties can be attributed to its damaging effects on cell DNA such as denaturation of DNA strands, covalent binding to genetic bases and intra-

strand cross-links that result in cell apoptosis and death (Dasari & Tchounwou, 2014). This mechanism disrupts RNA-transcription and DNA-replication, leading to cell cycle arrest and apoptosis as illustrated in **Figure 2.4** (Stordal & Davey, 2007). The N7 reactive centre on purine residues acts as another binding location for this drug and the nucleophile DNA bases most subjected to cisplatin binding have been found to be adenine and guanine (Pinto & Lippard, 1985; Dasari & Tchounwou, 2014). The targeting of these binding sites substantiates the hypothesis that the main mechanism of induced cell toxicity by cisplatin is by exerting DNA damage, resulting in the cell activating apoptotic pathways (Siddik, 2003).

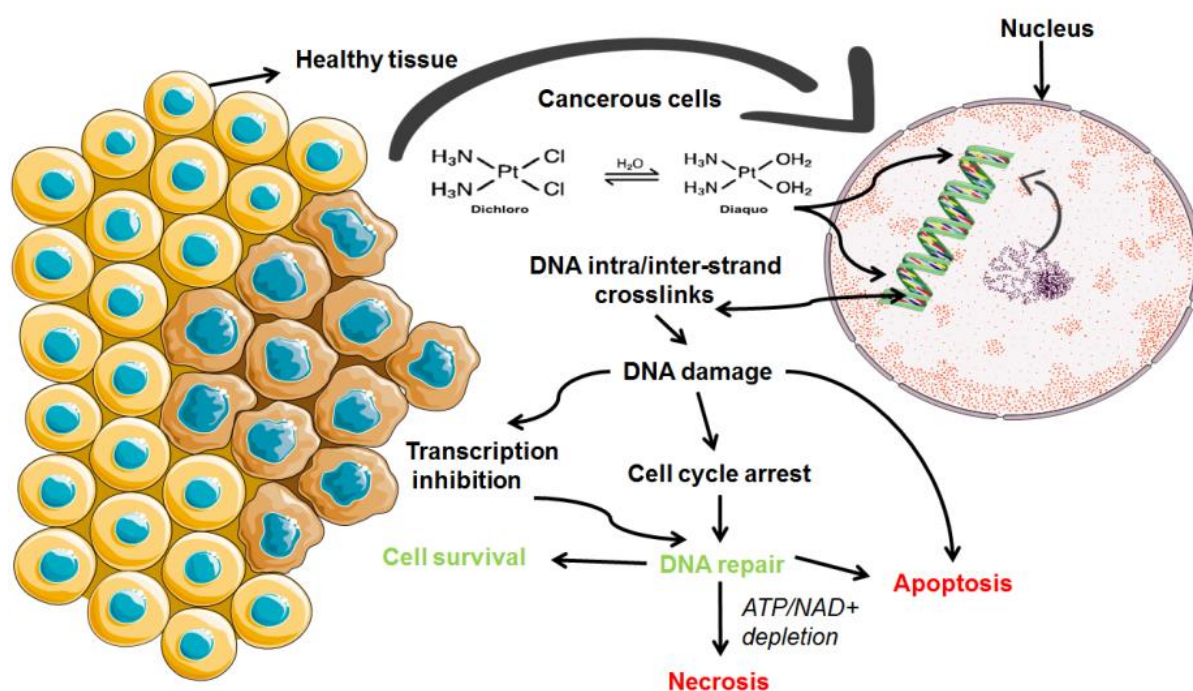


Figure 2.4: Cisplatin-induced DNA damage leading to cell cycle arrest processes to either repair or initiate cellular death in cancerous cells (adapted from Ma, P.A. *et al*, 2015).

Cisplatin is a widely used antineoplastic drug and is specifically used in combination therapy (etoposide/cisplatin) for first-line treatment of SCLC (Früh *et al.*, 2013). Platinum-based drugs (like cisplatin) are described as key players in optimal chemotherapeutic treatment regimens and they are often combined with other chemotherapeutic drugs to widen their scope of action (Go & Adjei, 1999). The combination of other chemotherapeutic drugs with cisplatin is justified by their differing mechanisms of action, thus allowing for a more aggressive treatment with a smaller chance of a recurring MDR-cancer. An example of this is the cisplatin/irinotecan, cisplatin/paclitaxel and cisplatin/etoposide (current first-line therapy) treatments. During clinical trials the difference in cancer death mechanisms of each

drug allows for a synergistic effect, thus increasing the effectiveness of the treatments. The effectiveness was witnessed in the response rate (complete and overall response) of patients with limited and extensive SCLC disease after exposure to the treatments, as well as a decrease in recurring MDR-cancer (Sevinc *et al.*, 2011; van Meerbeeck *et al.*, 2011a).

Inhibition concentration (**IC₅₀**) is a term used during *in vitro* drug development studies to describe the dosage needed to inhibit half of the population cells in your sample. The inclusion of this value indicates the potency of your drug compounds in different cell lines and will be extensively explored in **Chapter 4**. In **Table 2.6** the relative potency of Cisplatin *in vitro* is examined by including published IC₅₀ dosages.

Table 2.6: Cisplatin IC₅₀ values indicating the relative potency of the drug across several cancer cell lines (adapted from Soriano *et al.*, 1999; Su *et al.*, 2000; Jin *et al.*, 2010; Yin *et al.*, 2012)

<i>In Vitro</i> Models		IC₅₀
<i>Leukaemia cell lines</i>		
HL60	Promyelocytic leukaemia	1300 nM
CEM	T-lymphoblastic leukemia	900 nM
K562	Chronic myelogenous leukemia	23000 µM
<i>Cervical carcinoma</i>		
HeLa cells	Epitheloid cervix carcinoma	± 20 000-30 000 nM
<i>Squamous cell carcinomas (SCC)</i>		
HX155cisR	Cervical squamous cell carcinoma (Cisplatin resistant)	70 000 nM
<i>Breast carcinoma</i>		
MCF-7	Breast cancer	19 900 nM

Lung cancer cell lines

NCI-H69	Small Cell lung cancer	\pm 300 000 - 400 000 nM
NCI-H345	Small Cell lung cancer	\pm 500 000 nM
SHP77	Small Cell lung cancer	\pm 400 000 nM
A549	Non-Small Cell lung cancer	7 900 nM
NCI-H460	Non-Small Cell lung cancer	\pm 500 000 nM
NCI-H157	Non-Small Cell lung cancer	\pm 500 000 nM

Gastric carcinoma

SGC-7901	Cellosaurus Gastric carcinoma	2 400 nM
----------	-------------------------------	----------

2.4.2 Irinotecan

Carboxylesterases (CES) are enzymes from the esterase family that are responsible for the metabolism of ester-bond containing molecules, by means of hydrolysis, in the body. This enzyme has immense importance in the functioning of several drug delivery mechanisms (like prodrugs) and the majority of the esterase family belongs to the CES-1 and CES-2 isoforms (Sato *et al.*, 2012). The presence of esterase was initially thought to be primarily localised in the lumen of the endoplasmic reticulum and the cytosol of liver cells, but upon peripheral cellular investigation, the presence of esterase was also found to be expressed in many other tissues (including solid tumour cells) (Xu *et al.*, 2002).

Irinotecan is a semisynthetic plant-derived prodrug that is bio-activated by carboxylesterases to an active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38) (**Figure 2.5**). Comparative investigation of the carboxylesterases isoforms revealed CES-2 had a 12.5-fold higher affinity towards irinotecan molecules with a 5-fold increase in hydrolysis velocity compared to its counterpart, concluding that this isoform was the main metabolic driver behind drug activation (Charasson *et al.*, 2004). Even though primary bio-activation of irinotecan was believed to be located in the liver, due to the increased CES-2 activity present in these cells,

only 2-5% of systematic SN-38 was actually produced due to the weak substrate specificity irinotecan actually has for CES-2 (Senter *et al.*, 2001).

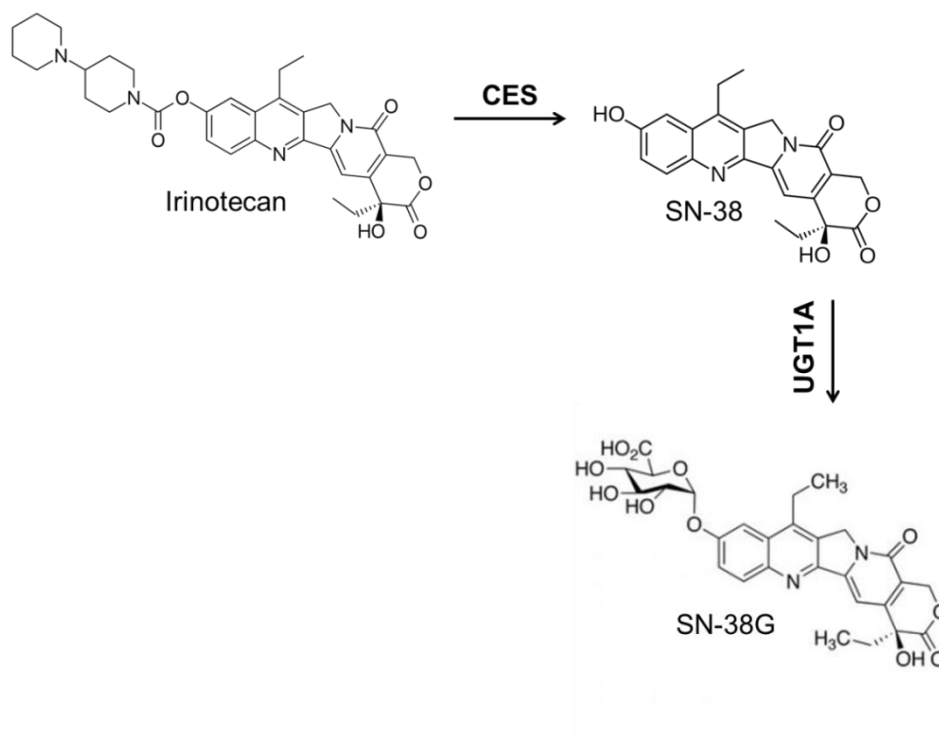


Figure 2.5: Metabolic pathways of irinotecan with the Carboxylesterases transformed metabolite (SN-38) being the primary active form of the drug and SN-38G being the inactive metabolite (adapted from Smith *et al.*, 2006). *UGT1A*; uridine diphosphate glucuronosyltransferase isoform 1A1, *CES*; Carboxylesterase.

The fact that irinotecan had significant anti-tumour activity in some cancers has led researchers to deviate from the belief that the activation site for irinotecan is solely isolated to the liver CES. It has later been confirmed that different tumours express their own degree of CES-2 levels, ultimately explaining the degree of chemosensitivity each tumour had towards irinotecan monotherapy (Xu *et al.*, 2002). A higher CES-2 expression in these tumours, together with SN-38 metabolites produced by the liver, has a synergistic effect thus resulting in a better chemotherapeutic outcome. Small Cell Lung Cancer has been found to be the cancer with one of the highest CES-activity levels, explaining the superior cytotoxic effects irinotecan has on SCLC (van Ark-Otte *et al.*, 1998). Cytotoxicity of this drug *in vitro* is highlighted with the inclusion of **Table 2.7** that indicates the IC₅₀ values needed for each cancer cell line.

The metabolite SN-38 is a topoisomerase-1 inhibitor and blocks the cleavage complex, resulting in double-strand breaks in cell DNA that induces S-phase-specific cell death (**Figure 2.6**). The entrapment of the cleavage complex results in DNA damage when replication and translation follow (Kuhn, 1998). Topoisomerase-1 has immense importance in mitotic processes by exerting its effects primarily on the DNA structure. It does this in the absence of ATP-processes by inducing controlled rotation (swivel) of the 5'-hydroxyl end around the intact chain, resulting in supercoiled strand relaxation. Before coil relaxation can be achieved, a single strand breakage is needed and this is achieved by means of a catalytic process. Topoisomerase-1 supplies a tyrosyl residue that mediates a nucleophilic attack on the DNA chain's phosphate bases, resulting in a covalent bond (topoisomerase-1 cleavage complex) at the broken DNA's 3'-end. The 5'-hydroxyl end is generated at the other side (thus coining the term nicking and closing) during this attack, allowing negatively and positively supercoiled relaxation (Pommier, 2009). This enzyme mediates DNA synthesis (initiation and elongation), separation of chromatids during mitotic replication and transcription of RNA by coil relaxation (Teicher, 2008). Primary metabolic breakdown by the glucuronidated process is located in the hepatic microsomes and the final inactive product for excretion is described as SN-38G (**Figure 2.5**) (Iyer *et al.*, 1998).

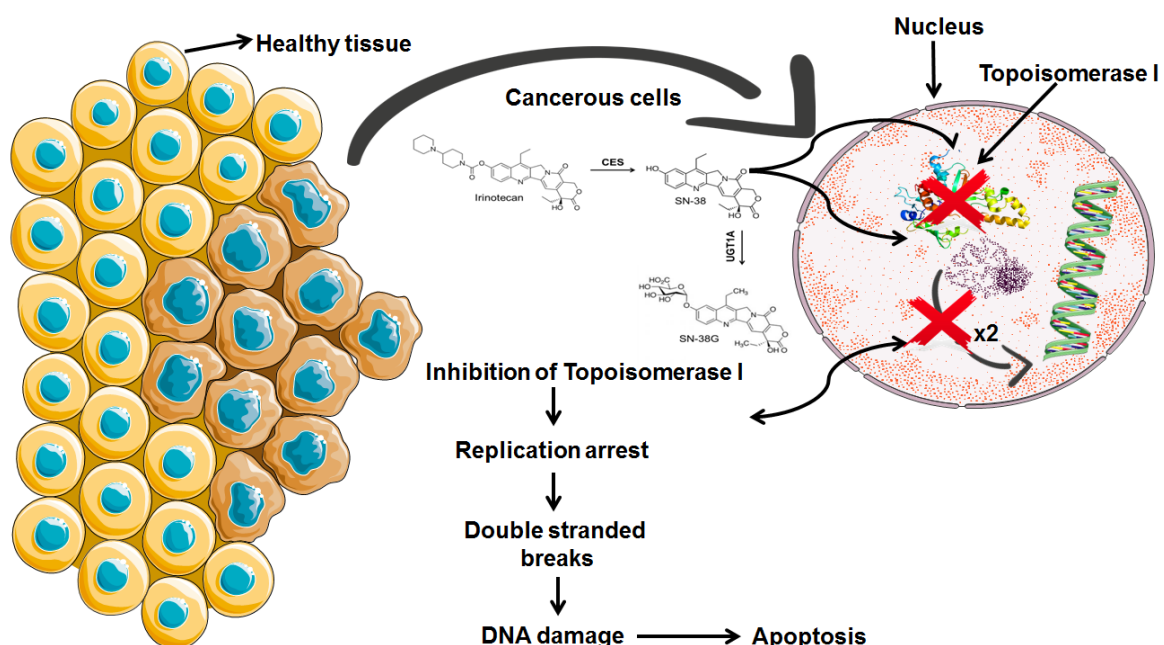


Figure 2.6: Typical path followed in cancerous cells after the introduction and activation of irinotecan (Kuhn, 1998; Basic cellular components were downloaded from <https://smart.servier.com>).

Table 2.7: Irinotecan IC₅₀ values indicating the relative potency of the drug across several cancer cell lines (adapted from Shrivastava *et al.*, 1998; van Ark-Otte *et al.*, 1998; Takag & Saotome, 2001)

<i>In vitro</i> models		IC ₅₀
<i>Human colorectal tumour cell lines</i>		
LoVo	Colorectal adenocarcinoma	15.8 μ M
HT-29	Colon adenocarcinoma	5.17 μ M
DLD-1	Human colorectal adenocarcinoma	2.889 μ M
<i>Pancreatic cancer cell lines</i>		
KP-3	Pancreatic adenosquamous carcinoma	5.94 μ M (3700 ng/ml)
H48N	Pancreatic ductal adenocarcinoma	674 nM (420 ng/ml)
PANC-1	Pancreatic carcinoma of ductal origin	144.43 nM (90 ng/ml)
<i>Lung cancer cell lines</i>		
NCI-H69	Lung small cell carcinoma	259-850 nM
NCI-H322	Bronchi alveolar carcinoma	2.1 μ M
GLC4/ADR	Small Cell Lung Carcinoma	1.5 μ M
SBC-3	Small Cell Lung Carcinoma	552.52 nM
SBC-3/ADM	Small Cell Lung Carcinoma, Adriamycin resistant	860.48 nM
SBC-3/CDP	Small Cell Lung Carcinoma, Cisplatin resistant	516.10 nM

The MRP1 sub-family was proven to be the predominant transporter responsible for the efflux of irinotecan and its active metabolite (SN-38) (Chu *et al.*, 1999). The other dominant transporter responsible for Irinotecan drug failure was found to be BCRP, but due to its absence in SCLC, it was excluded from this study (Pommier, 2006).

The general first line treatment for extensive SCLC is a combination of etoposide and cisplatin (EP-combination). During phase 2 clinical trials of irinotecan, a 50% response rate was witnessed during monotherapy with this drug in chemo-naive patients, compared to the 35% response rate seen in patients using etoposide as a single agent therapy. Irinotecan is thus used as a second line monotherapy for SCLC, due to its efficient single agent activity (Sevinc *et al.*, 2011). This single agent activity led to the introduction of a cisplatin/irinotecan (IP) regimen that yielded fluctuating results between significantly better response rates with progression-free survival and results that matched those of the EP-regimen. It was later confirmed that these unsatisfactory results were due to the antagonistic effects of the drug combinations, whereas synergistic effects were only achieved during optimal drug-ratio formulations (Tardi *et al.*, 2009).

2.4.3 Paclitaxel

Paclitaxel is classified as a natural taxane that inhibits DNA, RNA and protein synthesis by stimulating polymerisation and causing microtubule destabilisation. In short, this suppresses spindle-microtubule dynamics, slowing mitosis and ultimately resulting in the activation of cell apoptotic precursors as illustrated in **Figure 2.7** (Mooberry, 2011). Cancer cell susceptibility in microtubule destabilisation processes and other mitotic poisons (such as vinca alkaloids) is caused mainly by constant mitosis, which occurs faster in cancer cells than in their healthy cell counterparts, thus limiting toxicity of small doses in the host (Jordan & Wilson, 2004).

Microtubules are filamentous components in the cytoskeleton of cells, which is cardinal for maintenance and development of the eukaryotic cell shape. It also has a crucial role in the transport of cell components (e.g. vesicles and mitochondria), regulating mitotic processes (like cell division) and enabling communicative (signalling) capabilities in cells, ultimately regulating a large part of cell homeostasis and differentiation (Jordan & Wilson, 2004). Microtubule targeting is deemed as one of the most effective targets in cancer therapy and led to the design of the taxanes, of which paclitaxel was the flagship drug. Justification of targeting microtubules is primarily sought in the monumental role of cytoskeletons in mitotic

processes. Microtubule-based motor proteins have been found to be the primary differentiation drivers, by forming mitotic spindles that physically segregates chromosomes into daughter cells by orchestrating equatorial chromosome positioning followed by segregation to opposite poles (Sharp *et al.*, 2000). These microtubule subsets are constantly transitioning between rapidly growing (plus-end) and shrinking (minus-end) at the different ends to accommodate cellular changes or to adapt to external stimuli. This guanosine triphosphate-energy dependent process is known as dynamic instability and is needed for the proper polymerisation of microtubules and effective mitosis (Burbank & Mitchison, 2006).

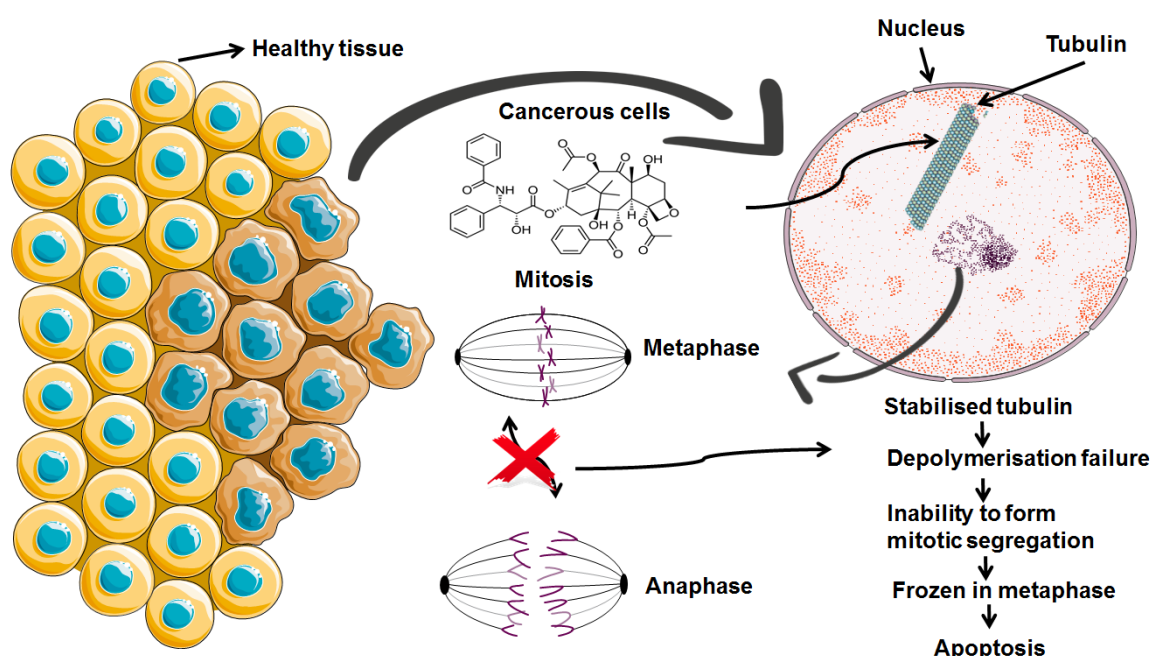


Figure 2.7: Typical path followed in cancerous cells after the introduction of paclitaxel (Basic cellular components were downloaded from <https://smart.servier.com>).

Paclitaxel was proven to be effective in a broad range of solid tumours including lung and breast cancers, head, neck and ovarian carcinoma, acute leukaemia and Kaposi's sarcoma (Fonseca *et al.*, 2002). Paclitaxel administered at a high dose (250 mg/m² as a 24 h infusion every 21 days) in chemo-naïve SCLC-patients, had a response rate between 30-40% and a 29% response rate with a survival median of 100 days in refractory disease (at 175 mg/m² as a 3 h infusion every 21 days) (Ettinger *et al.*, 1995; Smit *et al.*, 1998; Kirschling *et al.*, 1999). This proves that this drug has single-agent activity comparable to the first line agents, but dose adaptations are needed to compensate for severe toxicity in a host (such as

neutropenia, haematological toxicity and hypersensitivity reactions). Due to a limited response timeframe (3 months), another drug is usually added to compensate for shortened effects (Yamamoto *et al.*, 2006).

Pharmacokinetic studies indicated that the ABC-transporter, P-gp, has significant substrate specificity towards this drug (Ambudkar *et al.*, 1999). The increased presence of this specific transporter in relapsed and intrinsic resistant cancers can be directly correlated with paclitaxel treatment failure (Goncalves *et al.*, 2001). Initial good response to paclitaxel-based chemotherapy can be explained by the general absence of P-gp transporters in natural occurring *in vivo* SCLC (Campling *et al.*, 1997). The P-gp mediated resistance can thus be seen as an inductive mechanism and this phenomenon explains the limited response timeframe seen with this paclitaxel single agent regime.

Table 2.8: Paclitaxel IC₅₀ values indicating the relative potency of the drug across several cancer cell lines (adapted from Liebmann *et al.*, 1993; Tolba *et al.*, 2013; Vydra *et al.*, 2013; Frink *et al.*, 2016)

<i>In vitro</i> models		IC ₅₀
<i>Human colorectal tumour cell lines</i>		
HT-29	Colon adenocarcinoma	2.80 nM
<i>Pancreatic tumour cell lines</i>		
PC-sh	Pancreatic adenocarcinoma	7.50 nM
PC-zr	Pancreatic adenocarcinoma	4.00 nM
<i>Squamous cell carcinomas (SCC)</i>		
H157	SCC of the buccal mucosa	0.41 nM
<i>Skin cancer cell lines</i>		
WM793B	Melanoma cell line	30.20 nM

1205Lu	Melanoma cell line	25.00 nM
<hr/>		
<i>Lung cancer cell lines</i>		
NCI-H1648	Lung adenocarcinoma	4.69-5.25 nM
H1819	Lung adenocarcinoma	1.95 nM
NCI-H2087	Non-small cell lung adenocarcinoma	0.37 nM
NCI-H358	Primary bronchi alveolar carcinoma	1.20 nM
A549	lung adenocarcinoma	4.10 nM
<i>Prostate cancer cell lines</i>		
PC-3	Prostate adenocarcinoma	67.00 nM
DU-145	Prostate carcinoma derived from metastatic site (brain)	8.00 nM
LNCaP	Prostate carcinoma derived from metastatic site: (Left Supraclavicular Lymph Node)	6.30 nM
<hr/>		

2.5 *In vitro* models to study multidrug resistance in cancer

As mentioned earlier in this chapter (2.2.2), MDR can present itself in numerous forms in cancer and drug failure is dependent on a wide range of factors. Pre-clinical *in vitro* models are an essential part of drug development since they have the remarkable predictive potential for both intrinsic and acquired MDR gathered from either immortalised or primary cell lines. The latter mentioned cells are extensively researched and drug sensitivity profiles are documented in a few databases for possible active drug agent screening (Forbes *et al.*, 2010; Barretina *et al.*, 2012; Xavier *et al.*, 2016; Yang *et al.*, 2016). These characterised cell lines can be manipulated to express certain mechanism conferring known MDR in the clinical setting, thus allowing a targeted predictive determination of clinical drug failure (Rosa *et al.*, 2014).

These cell lines merely represent the building blocks of pre-clinical drug screening models and the incorporation of these cells into novel culturing methods underlines their true potential. As comprehensively explored earlier in this chapter (**section 2.2.2.2**), tumours have a complex multifactorial microenvironment (extracellular matrix) that can mediate resistance in conjunction with several intracellular methods (e.g. ABC-transporters) (Housman *et al.*, 2014). Although immense strides were accomplished in drug development using traditional flat culturing of these cells, novel strategies to imitate actual *in vivo* environments (e.g. by 3D-culturing) presents with an endless capacity for drug failure detection (Fang & Eglén, 2017). **Chapter 3** highlights advances made in incorporating models using efflux based cell lines for multidrug resistant cancer drug screening.

2.5.1 SCLC models

Currently, SCLC cell lines are primarily investigated through multiple mechanisms including classic immortalised cell line *in vitro* culturing (e.g. H69-cell line), primary *in vitro* cultures (patient personalised model) and *in vivo* models (syngeneic transplantation, genetic alterations and carcinogen-induced carcinomas) (Kellar *et al.*, 2015).

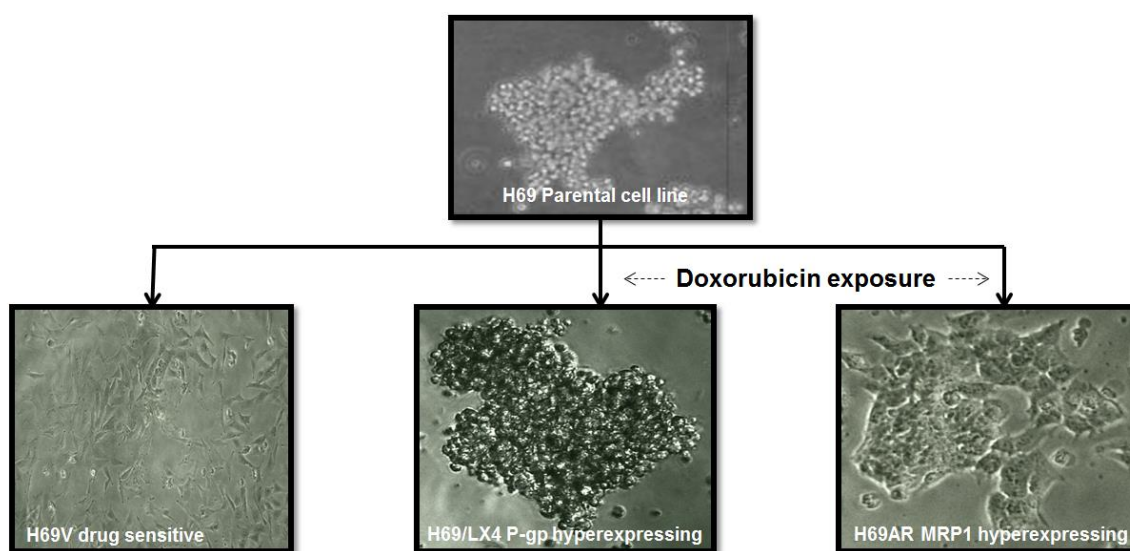


Figure 2.8: The three SCLC cell lines derived from the H69 parental cell line that will be incorporated into this study (H69 parental cell line image was adapted from Kraus *et al.*, 2002; **x10** Magnification under a light microscope).

Established immortalised cell lines will be incorporated into this study with one sensitive cell line (H69V) and two others (H69AR; H69/LX4) presenting with overexpression of MDR-inducing ABC-efflux transporters due to previous systematic drug exposure. The adherent H69V (ECACC 91091803) cell line was derived from the parental H69 cell line isolated from the epithelial tissue of a patient diagnosed with SCLC and will serve as the drug-sensitive control group (Carney *et al.*, 1985). The H69AR cell line was established from the parental cell line by means of a 14 month gradually increased exposure to doxorubicin and MRP-1 induction was reported (Mirski *et al.*, 1987; Mao *et al.*, 1999). Finally, the NCI-H69/LX4 (ECACC 96042329) cell line was also exposed to and maintained in doxorubicin and amplification of a separate MDR inducing gene to that in H69AR was witnessed termed *mdr-1*, resulting in P-gp overexpression (Twentyman *et al.*, 1987; Reeve *et al.*, 1989). The above-mentioned cell lines are illustrated in **Figure 2.8**.

These three cell lines were selected due to their shared parental heritage and their irrespective method of inducing MDR through separate efflux transporters. The introduction of ABC-transporter chemotherapeutic substrates to their designated cell line will help to characterise each cell line with comprehensive assays.

In **Chapter 4**, traditional flat cultures were separately cultivated as chemosensitive SCLC (H69V), two MDR SCLC cell lines each primarily expressing either P-gp (NCI-LX4) or MRP-1 (NCI-H69AR) transporters and a porcine kidney cell line (LLC-PK1) included as a healthy tissue standard (**Figure 2.9**). These cell lines grown as traditional flat cultures will be exposed to different chemotherapy drugs and the MTT assay will be used to determine the relative toxicity of each drug on the separate cell lines.

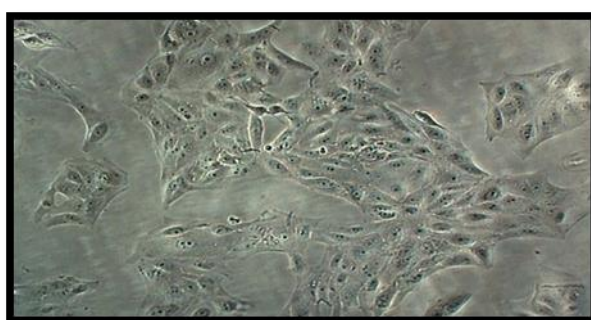


Figure 2.9: The porcine kidney cell line (LLC-PK1) included as a healthy tissue standard (x10 Magnification under a light microscope).

2.6 Summary

It is clearly evident that cancer and the treatment thereof can not be narrowed down to a single simple treatment approach. The inability of chemotherapy to isolate cell damage only to cancerous cells shows the complexity of both the disease as well as its treatment. This is complicated further by the occurrence of multidrug resistance. The prioritisation of developing thoroughly characterised cancer cell models are increasingly required by the pharmaceutical industry to minimise the toxicological impact of failed treatments during drug trials. By determining the mechanisms of drug reactions on cancer cell lines before going to expensive human and animal trials, a more cost-effective approach can be followed, thus allowing more refined research with smaller chances of development setbacks due to unforeseen pharmacological reactions to the physiological tumour microenvironment. Furthermore, adequate *in vitro* testing will allow better reduction and refinement of animal use during *in vivo* trials as required by the ethically responsible three-R approach. The three-R approach insists that researchers must always try to replace, reduce or refine the use of animals in their studies (Russel *et al.*, 1959).

2.7 References

- Alfarouk, K.O., Stock, C.M., Taylor, S., Walsh, M., Muddathir, A.K., Verduzco, D., Bashir, A.H., Mohammed, O.Y., Elhassan, G.O. & Harguindey, S. 2015. Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp. *Cancer Cell International*, 15(1): 71.
- Alvarez, A.I., Real, R., Pérez, M., Mendoza, G., Prieto, J.G. & Merino, G. 2010. Modulation of the activity of ABC transporters (P-glycoprotein, MRP2, BCRP) by flavonoids and drug response. *Journal of Pharmaceutical Sciences*, 99(2): 598-617.
- Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I. & Gottesman, M.M. 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter 1. *Annual Review of Pharmacology and Toxicology*, 39(1): 361-398.
- Antonioli, L., Blandizzi, C., Pacher, P. & Haskó, G. 2013. Immunity, inflammation and cancer: a leading role for adenosine. *Nature Reviews Cancer*, 13(12): 842-857.
- Arroyo, A.G. & Iruela-Arispe, M.L. 2010. Extracellular matrix, inflammation, and the angiogenic response. *Cardiovascular Research*, 86(2): 226-235.

- Baguley, B.C. 2010. Multiple drug resistance mechanisms in cancer. *Molecular Biotechnology*, 46(3): 308-316.
- Barretina, J., Caponigro, G., Stransky, N., Venkatesan, K., Margolin, A.A., Kim, S., Wilson, C.J., Lehár, J., Kryukov, G.V. & Sonkin, D. 2012. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*, 483(7391): 603.
- Bendayan, R., Ronaldson, P.T., Gingras, D. & Bendayan, M. 2006. In situ localization of P-glycoprotein (ABCB1) in human and rat brain. *Journal of Histochemistry & Cytochemistry*, 54(10): 1159-1167.
- Binkhathlan, Z. & Lavasanifar, A. 2013. P-glycoprotein inhibition as a therapeutic approach for overcoming multidrug resistance in cancer: current status and future perspectives. *Current Cancer Drug Targets*, 13(3): 326-346.
- Borst, P., Evers, R., Kool, M. & Wijnholds, J. 2000. A family of drug transporters: the multidrug resistance-associated proteins. *Journal of the National Cancer Institute*, 92(16): 1295-1302.
- Bray, F., Jemal, A., Grey, N., Ferlay, J. & Forman, D. 2012. Global cancer transitions according to the Human development index (2008–2030): a population-based study. *The Lancet Oncology*, 13(8): 790-801.
- Bray, F., Ren, J.S., Masuyer, E. & Ferlay, J. 2013. Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *International Journal of Cancer*, 132(5): 1133-1145.
- Burbank, K.S. & Mitchison, T.J. 2006. Microtubule dynamic instability. *Current Biology*, 16(14): R516-R517.
- Campling, B.G., Young, L.C., Baer, K.A., Lam, Y.M., Deeley, R.G., Cole, S. & Gerlach, J.H. 1997. Expression of the MRP and MDR1 multidrug resistance genes in small cell lung cancer. *Clinical Cancer Research*, 3(1): 115-122.
- Carney, D.N., Gazdar, A.F., Bepler, G., Guccion, J.G., Marangos, P.J., Moody, T.W., Zweig, M.H. & Minna, J.D. 1985. Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Research*, 45(6): 2913-2923.
- Chang, H.Y., Sneddon, J.B., Alizadeh, A.A., Sood, R., West, R.B., Montgomery, K., Chi, J.T., Van De Rijn, M., Botstein, D. & Brown, P.O. 2004. Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. *PLoS Biology*, 2(2): e7.

- Charasson, V., Bellott, R., Meynard, D., Longy, M., Gorry, P. & Robert, J. 2004. Pharmacogenetics of human carboxylesterase 2, an enzyme involved in the activation of irinotecan into SN-38. *Clinical Pharmacology & Therapeutics*, 76(6): 528-535.
- Choudhuri, S. & Klaassen, C.D. 2006. Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters. *International Journal of Toxicology*, 25(4): 231-259.
- Chu, X.Y., Suzuki, H., Ueda, K., Kato, Y., Akiyama, S.I. & Sugiyama, Y. 1999. Active efflux of CPT-11 and its metabolites in human KB-derived cell lines. *Journal of Pharmacology and Experimental Therapeutics*, 288(2): 735-741.
- Cole, S., Bhardwaj, G., Gerlach, J., Mackie, J., Grant, C., Almquist, K., Stewart, A., Kurz, E., Duncan, A. & Deeley, R. 1992. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science*, 258(5088): 1650-1654.
- Cole, S.P.C. 2014a. Multidrug resistance protein 1 (MRP1, ABCC1), a “multitasking” ATP-binding cassette (ABC) transporter. *Journal of Biological Chemistry*, 289(45): 30880-30888.
- Cole, S.P.C. 2014b. Targeting multidrug resistance protein 1 (MRP1, ABCC1): Past, present, and future. *Annual Review of Pharmacology and Toxicology*, 54(1): 95-117.
- Cole, S.P.C. & Deeley, R.G. 2006. Transport of glutathione and glutathione conjugates by MRP1. *Trends in Pharmacological Sciences*, 27(8): 438-446.
- Dasari, S. & Tchounwou, P.B. 2014. Cisplatin in cancer therapy: molecular mechanisms of action. *European Journal of Pharmacology*, 0: 364-378.
- Dayen, C., Debieuvre, D., Molinier, O., Raffy, O., Paganin, F., Virally, J., Larive, S., Desurmont-Salasc, B., Perrichon, M. & Martin, F. 2017. New insights into stage and prognosis in small cell lung cancer: an analysis of 968 cases. *Journal of Thoracic Disease*, 9(12): 5101.
- Dean, M. 2009. ABC transporters, drug resistance, and cancer stem cells. *Journal of Mammary Gland Biology and Neoplasia*, 14(1): 3-9.
- Dean, M. & Allikmets, R. 2001. Complete characterization of the human ABC gene family. *Journal of Bioenergetics and Biomembranes*, 33(6): 475-479.
- Dean, M., Hamon, Y. & Chimini, G. 2001. The human ATP-binding cassette (ABC) transporter superfamily. *Journal of Lipid Research*, 42(7): 1007-1017.

- Denko, N.C. 2008. Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nature Reviews Cancer*, 8(9): 705-713.
- Eckford, P.D. & Sharom, F.J. 2009. ABC efflux pump-based resistance to chemotherapy drugs. *Chemical Reviews*, 109(7): 2989-3011.
- Ellis, L.M. & Hicklin, D.J. 2008. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nature Reviews Cancer*, 8(8): 579-591.
- Ettinger, D.S., Finkelstein, D.M., Sarma, R.P. & Johnson, D.H. 1995. Phase II study of paclitaxel in patients with extensive-disease small-cell lung cancer: an eastern cooperative oncology group study. *Journal of Clinical Oncology*, 13(6): 1430-1435.
- Fang, Y. & Eglén, R.M. 2017. Three-dimensional cell cultures in drug discovery and development. *Stem Discovery*, 22(5): 456-472.
- Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C. & Parkin, D.M. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer*, 127(12): 2893-2917.
- Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D., Forman, D. & Bray, F. 2012. GLOBOCAN, v1.0. *Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11*.
- Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D., Forman, D. & Bray, F. 2015. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11.
- Finlay, G.J. & Baguley, B.C. 2000. Effects of protein binding on the *in vitro* activity of antitumour acridine derivatives and related anticancer drugs. *Cancer Chemotherapy and Pharmacology*, 45(5): 417-422.
- Fletcher, J.I., Haber, M., Henderson, M.J. & Norris, M.D. 2010a. ABC transporters in cancer: more than just drug efflux pumps. *Nature Reviews Cancer*, 10(2): 147-156.
- Fletcher, J.I., Haber, M., Henderson, M.J. & Norris, M.D. 2010b. ABC transporters in cancer: more than just drug efflux pumps. *Nature Reviews Cancer*, 10(2): 147.
- Fonseca, C., Simões, S. & Gaspar, R. 2002. Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and *in vitro* anti-tumoral activity. *Journal of Controlled Release*, 83(2): 273-286.

- Forbes, S.A., Bindal, N., Bamford, S., Cole, C., Kok, C.Y., Beare, D., Jia, M., Shepherd, R., Leung, K. & Menzies, A. 2010. COSMIC: mining complete cancer genomes in the catalogue of somatic mutations in cancer. *Nucleic Acids Research*, 39(suppl_1): D945-D950.
- Frantz, C., Stewart, K.M. & Weaver, V.M. 2010. The extracellular matrix at a glance. *Journal of Cell Science*, 123(24): 4195-4200.
- Frink, R.E., Peyton, M., Schiller, J.H., Gazdar, A.F., Shay, J.W. & Minna, J.D. 2016. Telomerase inhibitor imetelstat has preclinical activity across the spectrum of non-small cell lung cancer oncogenotypes in a telomere length dependent manner. *Oncotarget*, 7(22): 31639-31651.
- Früh, M., De Ruyscher, D., Popat, S., Crino, L., Peters, S., Felip, E. & Group, E.G.W. 2013. Small-cell lung cancer (SCLC): ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, 24(suppl_6): vi99-vi105.
- Fukumura, D. & Jain, R.K. 2007. Tumor microenvironment abnormalities: causes, consequences, and strategies to normalize. *Journal of Cellular Biochemistry*, 101(4): 937-949.
- Garattini, S. 2007. Pharmacokinetics in cancer chemotherapy. *European Journal of Cancer*, 43(2): 271-282.
- Giacomini, K.M., Huang, S.-M., Tweedie, D.J., Benet, L.Z., Brouwer, K.L., Chu, X., Dahlin, A., Evers, R., Fischer, V. & Hillgren, K.M. 2010. Membrane transporters in drug development. *Nature Reviews Drug Discovery*, 9(3): 215-236.
- Gillet, J.P. & Gottesman, M.M. 2010. Mechanisms of multidrug resistance in cancer. *Multi-drug resistance in cancer*. Humana Press: 47-76.
- Go, R.S. & Adjei, A.A. 1999. Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin. *Journal of Clinical Oncology*, 17(1): 409-409.
- Goldstein, L.J., Galski, H., Fojo, A., Willingham, M., Lai, S.L., Gazdar, A., Pirker, R., Green, A., Crist, W. & Brodeur, G.M. 1989. Expression of multidrug resistance gene in human cancers. *Journal of the National Cancer Institute*, 81(2): 116-124.
- Goncalves, A., Braguer, D., Kamath, K., Martello, L., Briand, C., Horwitz, S., Wilson, L. & Jordan, M. 2001. Resistance to Taxol in lung cancer cells associated with increased microtubule dynamics. *Proceedings of the National Academy of Sciences*, 98(20): 11737-11742.

- Gottesman, M.M. 2002. Mechanisms of cancer drug resistance. *Annual Review of Medicine*, 53(1): 615-627.
- Gottesman, M.M., Fojo, T. & Bates, S.E. 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nature Reviews Cancer*, 2(1): 48-58.
- Grantab, R., Sivanathan, S. & Tannock, I.F. 2006. The penetration of anticancer drugs through tumor tissue as a function of cellular adhesion and packing density of tumor cells. *Cancer Research*, 66(2): 1033-1039.
- Harris, A.L. 2002. Hypoxia—a key regulatory factor in tumour growth. *Nature Reviews Cancer*, 2(1): 38-47.
- Heldin, C.-H., Rubin, K., Pietras, K. & Östman, A. 2004. High interstitial fluid pressure—an obstacle in cancer therapy. *Nature Reviews Cancer*, 4(10): 806-813.
- Higgins, C.F. 2007. Multiple molecular mechanisms for multidrug resistance transporters. *Nature*, 446(7137): 749-757.
- Hipfner, D.R., Almquist, K.C., Leslie, E.M., Gerlach, J.H., Grant, C.E., Deeley, R.G. & Cole, S.P. 1997. Membrane topology of the multidrug resistance protein (MRP): A study of glycosylation-site mutants reveals an extracytosolic NH₂ terminus. *Journal of Biological Chemistry*, 272(38): 23623-23630.
- Hodkinson, P., Mackinnon, A. & Sethi, T. 2007. Extracellular matrix regulation of drug resistance in small-cell lung cancer. *International Journal of Radiation Biology*, 83(11-12): 733-741.
- Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N. & Sarkar, S. 2014. Drug resistance in cancer: an overview. *Cancers*, 6(3): 1769-1792.
- Huang, L., Ao, Q., Zhang, Q., Yang, X., Xing, H., Li, F., Chen, G., Zhou, J., Wang, S. & Xu, G. 2010. Hypoxia induced paclitaxel resistance in human ovarian cancers via hypoxia-inducible factor 1 α . *Journal of Cancer Research and Clinical Oncology*, 136(3): 447-456.
- Iyer, L., King, C.D., Whittington, P.F., Green, M.D., Roy, S.K., Tephly, T.R., Coffman, B.L. & Ratain, M.J. 1998. Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. *The Journal of Clinical Investigation*, 101(4): 847-854.

- Jedlitschky, G., Hoffmann, U. & Kroemer, H.K. 2006. Structure and function of the MRP2 (ABCC2) protein and its role in drug disposition. *Expert Opinion on Drug Metabolism & Toxicology*, 2(3): 351-366.
- Jin, K.L., Park, J.-Y., Noh, E.J., Hoe, K.L., Lee, J.H., Kim, J.-H. & Nam, J.-H. 2010. The effect of combined treatment with cisplatin and histone deacetylase inhibitors on HeLa cells. *Journal of Gynecologic Oncology*, 21(4): 262-268.
- Jordan, M.A. & Wilson, L. 2004. Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, 4(4): 253-265.
- Keith, B., Johnson, R.S. & Simon, M.C. 2012. HIF1 α and HIF2 α : sibling rivalry in hypoxic tumour growth and progression. *Nature Reviews Cancer*, 12(1): 9-22.
- Kellar, A., Egan, C. & Morris, D. 2015. Preclinical murine models for lung cancer: clinical trial applications. *BioMed Research International*, 2015: 621324.
- Kim, S.-H., Turnbull, J. & Guimond, S. 2011. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *Journal of Endocrinology*, 209(2): 139-151.
- Kirschling, R.J., Grill, J.P., Marks, R.S., Kugler, J.W., Gerstner, J.B., Kuross, S.A., Michalak, J.C., Windschitl, H.E., Krewer, K.D. & Jett, J.R. 1999. Paclitaxel and G-CSF in previously untreated patients with extensive stage small-cell lung cancer: a phase II study of the North Central cancer treatment group. *American Journal of Clinical Oncology*, 22(5): 517.
- König, J., Nies, A.T., Cui, Y., Keppler, D., Holland, I. & Cole, S. 2003. MRP2, the apical export pump for anionic conjugates. *ABC proteins: from bacteria to man*.
- Kraus, A.C., Ferber, I., Bachmann, S.O., Specht, H., Wimmel, A., Gross, M.W., Schlegel, J., Suske, G. and Schuermann, M., 2002. *In vitro* chemo- and radio-resistance in small cell lung cancer correlates with cell adhesion and constitutive activation of AKT and MAP kinase pathways. *Oncogene*, 21(57): 8683–8695.
- Krishna, R. & Mayer, L.D. 2000. Multidrug resistance (MDR) in cancer: mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *European Journal of Pharmaceutical Sciences*, 11(4): 265-283.
- Kruh, G.D. & Belinsky, M.G. 2003. The MRP family of drug efflux pumps. *Oncogene*, 22(47): 7537–7552.

- Kuhn, J.G. 1998. Pharmacology of irinotecan. *Oncology (Williston Park)*, 12(8 Suppl 6): 39-42.
- Lage, H. 2008. An overview of cancer multidrug resistance: a still unsolved problem. *Cellular and Molecular Life Sciences*, 65(20): 3145-3167.
- Leslie, E.M., Deeley, R.G. & Cole, S.P. 2005a/b. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defence. *Toxicology and Applied Pharmacology*, 204(3): 216-237.
- Liebmann, J., Cook, J., Lipschultz, C., Teague, D., Fisher, J. & Mitchell, J. 1993. Cytotoxic studies of paclitaxel (Taxol[®]) in human tumour cell lines. *British Journal of Cancer*, 68(6): 1104-1109.
- Ling, V. & Thompson, L.H. 1974. Reduced permeability in CHO cells as a mechanism of resistance to colchicine. *Journal of Cellular Physiology*, 83(1): 103-116.
- Little, C.D., Nau, M.M., Carney, D.N., Gazdar, A.F. & Minna, J.D. 1983. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. *Nature*, 306(5939): 194-196.
- Ma, P.A., Xiao, H., Li, C., Dai, Y., Cheng, Z., Hou, Z. and Lin, J., 2015. Inorganic nanocarriers for platinum drug delivery. *Materials Today*, 18(10): 554-564.
- Mahoney, B.P., Raghunand, N., Baggett, B. & Gillies, R.J. 2003. Tumor acidity, ion trapping and chemotherapeutics: I. Acid pH affects the distribution of chemotherapeutic agents *in vitro*. *Biochemical Pharmacology*, 66(7): 1207-1218.
- Mao, Q., Leslie, E.M., Deeley, R.G. & Cole, S.P. 1999. ATPase activity of purified and reconstituted multidrug resistance protein MRP1 from drug-selected H69AR cells. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1461(1): 69-82.
- Mao, Q. & Unadkat, J.D. 2015. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport—an update. *The AAPS journal*, 17(1): 65-82.
- Mirski, S.E., Gerlach, J.H. & Cole, S.P. 1987. Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. *Cancer Research*, 47(10): 2594-2598.
- Mooberry, S.L. 2011. Microtubules as a target for anticancer drugs. *New Frontiers and Treatment Paradigms for Metastatic Breast Cancer*, 28: 7.
- Müller, K., Fedosov, D.A. & Gompper, G. 2014. Margination of micro-and nano-particles in blood flow and its effect on drug delivery. *Scientific Reports*, 4: 4871.

- Narang, A.S. & Varia, S. 2011. Role of tumor vascular architecture in drug delivery. *Advanced Drug Delivery Reviews*, 63(8): 640-658.
- Neri, D. & Supuran, C.T. 2011. Interfering with pH regulation in tumours as a therapeutic strategy. *Nature Reviews Drug Discovery*, 10(10): 767-777.
- Ozben, T. 2006. Mechanisms and strategies to overcome multiple drug resistance in cancer. *FEBS letters*, 580(12): 2903-2909.
- P.R. Xavier, C., Pesic, M. & Helena Vasconcelos, M. 2016. Understanding cancer drug resistance by developing and studying resistant cell line models. *Current Cancer Drug Targets*, 16(3): 226-237.
- Parks, S.K., Chiche, J. & Pouyssegur, J. 2011. pH control mechanisms of tumor survival and growth. *Journal of Cellular Physiology*, 226(2): 299-308.
- Pinto, A.L. & Lippard, S.J. 1985. Binding of the antitumor drug cis-diamminedichloroplatinum (II)(cisplatin) to DNA. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, 780(3): 167-180.
- Pommier, Y. 2006. Topoisomerase I inhibitors: camptothecins and beyond. *Nature Reviews Cancer*, 6(10): 789-802.
- Pommier, Y. 2009. DNA topoisomerase I inhibitors: chemistry, biology, and interfacial inhibition. *Chemical Reviews*, 109(7): 2894-2902.
- Rebucci, M. & Michiels, C. 2013. Molecular aspects of cancer cell resistance to chemotherapy. *Biochemical Pharmacology*, 85(9): 1219-1226.
- Reeve, J.G., Rabbitts, P.H. & Twentyman, P.R. 1989. Amplification and expression of mdr1 gene in a multidrug resistant variant of small cell lung cancer cell line NCI-H69. *British Journal of Cancer*, 60: 339.
- Rintoul, R.C. & Sethi, T. 2002. Extracellular matrix regulation of drug resistance in small-cell lung cancer. *Clinical Science*, 102(4): 417-424.
- Rofstad, E.K., Galappathi, K., Mathiesen, B. & Ruud, E.-B.M. 2007. Fluctuating and diffusion-limited hypoxia in hypoxia-induced metastasis. *Clinical Cancer Research*, 13(7): 1971-1978.
- Rohwer, N. & Cramer, T. 2011. Hypoxia-mediated drug resistance: Novel insights on the functional interaction of HIFs and cell death pathways. *Drug Resistance Updates*, 14(3): 191-201.

- Rosa, R., Monteleone, F., Zambrano, N. & Bianco, R. 2014. *In vitro* and *in vivo* models for analysis of resistance to anticancer molecular therapies. *Current Medicinal Chemistry*, 21(14): 1595-1606.
- Rosenberg, B., Van Camp, L. & Krigas, T. 1965. Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. *Nature*, 205: 698.
- Rosenberg, B., Vancamp, L., Trosko, J.E. & Mansour, V.H. 1969. Platinum compounds: a new class of potent antitumour agents. *Nature*, 222: 385.
- Russell, W.M.S., Burch, R.L. and Hume, C.W., 1959. The principles of humane experimental technique (Vol. 238). London: Methuen.
- Sato, Y., Miyashita, A., Iwatsubo, T. & Usui, T. 2012. Simultaneous absolute protein quantification of carboxylesterases 1 and 2 in human liver tissue fractions using liquid chromatography tandem mass spectrometry. *Drug Metabolism and Disposition*: 112.
- Seelig, A. & Landwojtowicz, E. 2000. Structure–activity relationship of P-glycoprotein substrates and modifiers. *European Journal of Pharmaceutical Sciences*, 12(1): 31-40.
- Semenza, G.L. 2004. Intratumoral hypoxia, radiation resistance, and HIF-1. *Cancer Cell*, 5(5): 405-406.
- Senter, P.D., Beam, K.S., Mixan, B. & Wahl, A.F. 2001. Identification and activities of human carboxylesterases for the activation of CPT-11, a clinically approved anticancer drug. *Bioconjugate Chemistry*, 12(6): 1074-1080.
- Sevinc, A., Kalender, M.E., Altinbas, M., Ozkan, M., Dikilitas, M., Camci, C. & Oncology, A.S.o.M. 2011. Irinotecan as a second-line monotherapy for small cell lung cancer. *Asian Pacific Journal of Cancer Prevention*, 12(4): 1055-1059.
- Sharom, F.J. 2008. ABC multidrug transporters: structure, function and role in chemoresistance: *Pharmacogenomics*, 9 (1): 105-127.
- Sharp, D.J., Rogers, G.C. & Scholey, J.M. 2000. Microtubule motors in mitosis. *Nature*, 407(6800): 41-47.
- Shrivastava, P., Hanibuchi, M., Yano, S., Parajuli, P., Tsuruo, T. & Sone, S. 1998. Circumvention of multidrug resistance by a quinoline derivative, MS-209, in multidrug-resistant human small-cell lung cancer cells and its synergistic interaction with cyclosporin A or verapamil. *Cancer Chemotherapy and Pharmacology*, 42(6): 483-490.

- Siddik, Z.H. 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene*, 22(47): 7265.
- Singh, E., Sengayi, M., Urban, M., Babb, C., Kellett, P. and Ruff, P., 2013. The South African National Cancer Registry: an update. *The Lancet*.
- Slotman, B.J., Mauer, M.E., Bottomley, A., Faivre-Finn, C., Kramer, G.W.P.M., Rankin, E.M., Snee, M., Hatton, M., Postmus, P.E., Collette, L. & Senan, S. 2009. Prophylactic cranial irradiation in extensive disease small-cell lung cancer: short-term health-related quality of life and patient reported symptoms—results of an international phase iii randomized controlled trial by the eortc radiation oncology and lung cancer groups. *Journal of Clinical Oncology*, 27(1): 78-84.
- Smit, E., Fokkema, E., Biesma, B., Groen, H., Snoek, W. & Postmus, P. 1998. A phase II study of paclitaxel in heavily pretreated patients with small-cell lung cancer. *British Journal of Cancer*, 77(2): 347.
- Smith, N.F., Figg, W.D. & Sparreboom, A. 2006. Pharmacogenetics of irinotecan metabolism and transport: An update. *Toxicology in Vitro*, 20(2): 163-175.
- Soriano, A.F., Helfrich, B., Chan, D.C., Heasley, L.E., Bunn, P.A. & Chou, T.-C. 1999. Synergistic effects of new chemopreventive agents and conventional cytotoxic agents against human lung cancer cell lines. *Cancer Research*, 59(24): 6178.
- Sprowl, J.A., Gregorc, V., Lazzari, C., Mathijssen, R.H., Loos, W.J. & Sparreboom, A. 2012. Association of ABCC2 polymorphisms with cisplatin disposition and efficacy. *Clinical Pharmacology and Therapeutics*, 91(6): 1022-1026.
- Stordal, B. & Davey, M. 2007. Understanding cisplatin resistance using cellular models. *IUBMB life*, 59(11): 696-699.
- Su, W.-C., Chang, S.-L., Chen, T.-Y., Chen, J.-S. & Tsao, C.-J. 2000. Comparison of *in vitro* growth-inhibitory activity of carboplatin and cisplatin on leukemic cells and hematopoietic progenitors: the myelosuppressive activity of carboplatin may be greater than its antileukemic effect. *Japanese Journal of Clinical Oncology*, 30(12): 562-567.
- Szakacs, G., Varadi, A., Özvegy-Laczka, C. & Sarkadi, B. 2008. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME–Tox). *Drug Discovery Today*, 13(9): 379-393.

- Takag, T. & Saotome, T. 2001. Chemotherapy with irinotecan (CPT-11), a topoisomerase-I inhibitor, for refractory and relapsed non-Hodgkin's lymphoma. *Leukemia & Lymphoma*, 42(4): 577-586.
- Tardi, P.G., Dos Santos, N., Harasym, T.O., Johnstone, S.A., Zisman, N., Tsang, A.W., Bermudes, D.G. & Mayer, L.D. 2009. Drug ratio-dependent antitumor activity of irinotecan and cisplatin combinations *in vitro* and *in vivo*. *Molecular Cancer Therapeutics*, 8(8): 2266-2275.
- Teicher, B.A. 2008. Next generation topoisomerase I inhibitors: Rationale and biomarker strategies. *Biochemical Pharmacology*, 75(6): 1262-1271.
- Tolba, M.F., Esmat, A., Al-Abd, A.M., Azab, S.S., Khalifa, A.E., Mosli, H.A., Abdel-Rahman, S.Z. & Abdel-Naim, A.B. 2013. Caffeic acid phenethyl ester synergistically enhances docetaxel and paclitaxel cytotoxicity in prostate cancer cells. *IUBMB life*, 65(8): 716-729.
- Toyoda, Y., Hagiya, Y., Adachi, T., Hoshijima, K., Kuo, M. & Ishikawa, T. 2008. MRP class of human ATP binding cassette (ABC) transporters: historical background and new research directions. *Xenobiotica*, 38(7-8): 833-862.
- Trédan, O., Galmarini, C.M., Patel, K. & Tannock, I.F. 2007. Drug resistance and the solid tumor microenvironment. *JNCI: Journal of the National Cancer Institute*, 99(19): 1441-1454.
- Triller, N., Korošec, P., Kern, I., Košnik, M. & Debeljak, A. 2006. Multidrug resistance in small cell lung cancer: Expression of P-glycoprotein, multidrug resistance protein 1 and lung resistance protein in chemo-naive patients and in relapsed disease. *Lung Cancer*, 54(2): 235-240.
- Twentyman, P., Fox, N. & White, D. 1987. Cyclosporin A and its analogues as modifiers of adriamycin and vincristine resistance in a multi-drug resistant human lung cancer cell line. *British Journal of Cancer*, 56(1): 55.
- Ullah, M.F. 2008. Cancer multidrug resistance (MDR): a major impediment to effective chemotherapy. *Asian Pacific Journal of Cancer Prevention*, 9(1): 1-6.
- Undevia, S.D., Gomez-Abuin, G. & Ratain, M.J. 2005. Pharmacokinetic variability of anticancer agents. *Nature Reviews Cancer*, 5(6): 447-458.
- Usuda, J., Ichinose, S., Ishizumi, T., Ohtani, K., Inoue, T., Saji, H., Kakihana, M., Kajiwara, N., Uchida, O., Nomura, M., Ohira, T. & Ikeda, N. 2011. Klotho predicts good clinical outcome in patients with limited-disease small cell lung cancer who received surgery. *Lung Cancer*, 74(2): 332-337.

- van Ark-Otte, J., Kedde, M., Van Der Vijgh, W., Dingemans, A., Jansen, W., Pinedo, H., Boven, E. & Giaccone, G. 1998. Determinants of CPT-11 and SN-38 activities in human lung cancer cells. *British Journal of Cancer*, 77(12): 2171.
- van Meerbeeck, J.P., Fennell, D.A. & De Ruyscher, D.K. 2011. Small-cell lung cancer. *The Lancet*, 378(9804): 1741-1755.
- Vydra, N., Toma, A., Glowala-Kosinska, M., Gogler-Piglowska, A. & Widlak, W. 2013. Overexpression of heat shock transcription factor 1 enhances the resistance of melanoma cells to doxorubicin and paclitaxel. *BMC Cancer*, 13: 504-504.
- Wacher, V.J., Silverman, J.A., Zhang, Y. & Benet, L.Z. 1998. Role of p-glycoprotein and cytochrome p450 3a in limiting oral absorption of peptides and peptidomimetics. *Journal of Pharmaceutical Sciences*, 87(11): 1322-1330.
- Walker, J.E., Saraste, M., Runswick, M.J. & Gay, N.J. 1982. Distantly related sequences in the alpha-and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *The EMBO Journal*, 1(8): 945.
- Webb, B.A., Chimenti, M., Jacobson, M.P. & Barber, D.L. 2011. Dysregulated pH: a perfect storm for cancer progression. *Nature Reviews Cancer*, 11(9): 671-677.
- Wilkins, S. 2015. Structure and mechanism of ABC transporters. *F1000prime reports*: 7.
- Wu, Q., Yang, Z., Nie, Y., Shi, Y. & Fan, D. 2014. Multi-drug resistance in cancer chemotherapeutics: mechanisms and lab approaches. *Cancer Letters*, 347(2): 159-166.
- Xu, G., Zhang, W., Ma, M.K. & McLeod, H.L. 2002. Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. *Clinical Cancer Research*, 8(8): 2605-2611.
- Yamamoto, N., Tsurutani, J., Yoshimura, N., Asai, G., Moriyama, A., Nakagawa, K., Kudoh, S., Takada, M., Minato, Y. & Fukuoka, M. 2006. Phase II study of weekly paclitaxel for relapsed and refractory small cell lung cancer. *Anticancer Research*, 26(1B): 777-781.
- Yang, W., Lightfoot, H., Bignell, G., Behan, F., Coker, T., Haber, D., Engelman, J., Stratton, M., Benes, C. & McDermott, U. 2016. Genomics of drug sensitivity in cancer (GDSC): A resource for biomarker discovery in cancer cells. *European Journal of Cancer*, 69: S82.
- Yin, R., Gou, S., Sun, Y. & Liu, X. 2012. *In vitro* biological evaluation of platinum(II) complexes with 1-(methoxy substituted benzyl) azetidine-3,3-dicarboxylato ligands. *Bioorganic & Medicinal Chemistry*, 20(4): 1461-1467.

Zhou, S.-F. 2008. Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica*, 38(7-8): 802-832.

Zhou, S.-F., Wang, L.-L., Di, Y.M., Xue, C.C., Duan, W., Li, C.G. & Li, Y. 2008. Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development. *Current Medicinal Chemistry*, 15(20): 1981-2039

CHAPTER 3

ARTICLE SUBMITTED TO *CURRENT CANCER DRUG TARGETS* FOR PUBLICATION

This chapter is presented as a review article to be submitted for publication in *Current Cancer Drug Targets*. It is structured and prepared as prescribed by the journal's instructions to authors, which are included in Appendix A.

AUTHOR CONTRIBUTION

In this review article, I was responsible for the compilation of all research pertaining to complex cell-based models, including the following headings in the article:

- 3.2 Complex cell-based models
- 3.2.1. Integrated discrete multiple organ culture (IdMOC) system
- 3.2.2. Microfluidic channel-based systems
- 3.2.2.1. Multi-organ co-culture in tumour-on-a-chip system
- 3.2.2.2. Lung-on-a-chip microfluidic device:
- 3.2.2.3. 3D lung cancer microfluidic constructs
- 3.2.3. 3D spheroid cell cultures
- 3.2.3.1. 3D multicellular models mediating MDR
- 3.2.3.2. Hollow fibre-based spheroid cultures
- 3.2.3.3. Cell printing
- Table 1: advanced complex cell-cultures

Other Author contributions:

Dr Clarissa Willers acted as the primary contributor to the review article and was responsible for compilation of all the researcher's contributions as well as overall editing. Furthermore, she contributed to the following parts:

- 4. IN VIVO MODELS FOR MULTIDRUG RESISTANT CANCER TREATMENT SCREENING
- 4.1. In vivo hollow fibre assay models
- 4.2. Xenograft cancer models
- 4.2.1. Cell-derived xenografts
- 4.2.2. Patient-derived xenografts
- 4.3. Genetically engineered mouse models
- 5. IMPROVEMENTS FOR PRECLINICAL MDR TREATMENT SCREENING
- 6. CONCLUSIONS
- Table 1: Cell-derived xenografts
- Table 1: Patient-derived xenografts
- Table 1: Genetically engineered mouse models

Roan Swanepoel acted as Co-author to the review article and was responsible for compilation of the following headings:

- 3.1. Conventional cell-based models
- Table 1: 2D flat cultures

Dr Hanna Svitina acted as Co-author to the review article and was responsible for compilation of the following headings:

- Introduction

Prof Josias H. Hamman acted as Co-author to the review article and was responsible for compilation of the following headings:

- 2. MULTIDRUG RESISTANT CANCER TREATMENT APPROACHES

Prof C Gouws acted as final editor to the review article

Efflux-Based Models for Multidrug Resistant Cancer Treatment Screening

Clarissa Willers*^a, Michael J. Rossouw^a, Roan A. Swanepoel^a, Hanna Svitina^a, Josias H. Hamman^a and Chrisna Gouws^a

^aPharmacem™, Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa.

Abstract: Efflux transporters of the adenosine triphosphate (ATP)-binding cassette (ABC)-superfamily play an important role in the development of multidrug resistance (MDR) in cancer. The overexpression of these transporters can directly contribute to the failure of chemotherapeutic drugs. Several *in vitro* and *in vivo* models exist to screen for the efficacy of chemotherapeutic drugs against multidrug resistant cancer, facilitated by efflux transporters. This article reviews a range of efflux-based models used to test the efficacy of compounds to overcome MDR in cancer. These models are classified as either *in vitro* or *in vivo* and are categorised from the most basic, conventional models to more complex and advanced systems. Each model's origin, advantages and limitations, as well as specific efflux-based MDR applications are discussed. Accordingly, future modifications to existing models or new research approaches are suggested to develop prototypes that closely resemble the true nature of multidrug resistant cancer in the human body. It is evident from this review that a combination of both *in vitro* and *in vivo* preclinical models can provide a better understanding of cancer itself, than using a single model only.

Keywords: Cancer, efflux transporters, genetically engineered mouse models, multidrug resistance, preclinical screening model, two dimensional and three dimensional cell culture, xenograft.

1. INTRODUCTION

According to the International Agency for Research on Cancer, 18.1 million new cancer cases were registered around the world in 2018. The estimated number of deaths due to cancer in 2018 were approximately 9.6 million and this number is expected to increase to 29.5 million by 2040 (<https://gco.iarc.fr/>), mainly because of phenomena such as multidrug resistance (MDR). Multidrug resistance is defined as the ability of a living cell to display resistance to multiple unrelated anticancer drugs that are structurally and/or functionally different [1]. To date, MDR has been shown to develop via cell death inhibition, drug inactivation, deoxyribonucleic acid (DNA) damage repair, drug target alteration, the epithelial-mesenchymal transition and epigenetic modifications, but the most widely studied MDR mechanism is that of drug efflux involving adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporters [2-3]. At least 49 structurally related transporters, known collectively as the ABC-superfamily, have been identified and categorised into 7 subfamilies. Of these, 16 are primarily involved in human diseases [4], of which P-glycoprotein (P-gp; MDR1/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP1/ABCG2) are the major drug transporters widely implicated in drug resistant cancers [5-6]. P-gp, MRP1 and BCRP1 are present in the epithelial lining of the lungs, testes, skin, kidneys, small intestines and in heart and muscle tissues, acting as a barrier against many

epithelium, whereas MRP1 has a basolateral cell localisation [9]. All three transporters have significantly overlapping resistance profiles [9], but MRP1 does not confer resistance to taxanes and some classes of tyrosine kinase inhibitors [10-11], while BCRP1 is not inhibited by arylmethoxy-derivatives [12].

The more ABC transporters are expressed in the cancer tissue, the lower the chance will be to have effective anticancer results with chemotherapy. For example, a recent study on children with acute myeloid leukaemia found a strong association between the relapse of patients and the number of overexpressed ABC transporters such as ABCA3, ABCB1, ABCC3 and ABCG2 in the cancer cells [13]. Furthermore, a compensatory effect between different ABC transporters has been proposed in the sense that the down-regulation of one transporter is interchanged by the up-regulation of another [14-15]. This highlights the challenge with the development of new anticancer drugs that should be able to deal with complex interactions between different efflux transporters.

This review presents and discusses the different *in vitro* and *in vivo* models currently available to evaluate the treatment of efflux-based MDR in cancer. The *in vitro* models that are discussed include both conventional cell culture-based models cultured as basic two-dimensional (2D) flat cultures and more complex cell-based models that make use of specialised matrices or objects to culture non-conventional or three-dimensional (3D) models. The *in vivo* models that are discussed include cell- and patient-derived xenografts as well as genetically engineered mouse models (GEMMs). In addition to the discussions of these selected models, a comprehensive summary of both *in vitro* (Table 1) and *in vivo* models (Table 1) are given which have been used in previous studies to investigate the anticancer efficacy of

*Address correspondence to Clarissa Willers at Pharmacem™, Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa; Tel: +27-18-285-2486; Fax: +27-87-231-5432; E-mail: 20672322@nwu.ac.za

xenobiotics [7-8]. Through staining, P-gp and BCRP1 have been observed predominantly in the apical side of certain

compounds by influencing efflux transporter activity and/or expression. Lastly, a few suggestions are provided on future modifications that can be made to existing models, as well as newly developed approaches, that can accurately imitate the true drug response of tumours occurring in the human body.

2. MULTIDRUG RESISTANT CANCER TREATMENT APPROACHES

Common treatments of cancer include surgery, radiation therapy and chemotherapy, or combinations thereof [16]. Chemotherapy specifically is important in the treatment of metastatic and haematological malignancies [17]. The phenomenon of MDR is, however, a major challenge causing failures in chemotherapy, but the mechanisms responsible for both intrinsic and acquired MDR also provide potential targets for new anticancer drugs to overcome MDR. Therefore, many innovative therapeutic strategies focus specifically on reversing, suppressing or evading MDR mechanisms in tumours [1, 3, 18].

Inhibition of efflux transporters, specifically P-gp, by co-administration of transporter inhibitors has been explored as a treatment strategy to overcome MDR. Different P-gp inhibitors have been investigated (including first, second and third generation inhibitors) by using *in silico*, *in vitro* and *in vivo* models [2-3,19]. However, many clinical trials that tested the efficacy of co-administration of P-gp inhibitors with anticancer drugs could not provide sufficient evidence that this strategy could overcome MDR in most cancers. Several reasons were given for this outcome such as associated toxicity, poor planning or lack of randomisation in clinical trial design and the fact that little effort was made to determine if the tumours in the subjects did actually express P-gp [20]. Besides competitive and non-competitive inhibition of P-gp, modulation of P-gp expression has also been investigated as a strategy to overcome chemoresistance. Several signalling pathways that modulate P-gp expression in cancer cells have been targeted to regulate P-gp function and expression. However, P-gp modulators appeared to have selective efficacy depending on the specific anticancer drug that it was combined with [21]. Despite the failure to produce significant improvements in the treatment of multidrug resistant cancer with co-administering efflux transporter inhibitors, research relevant to this approach remains ongoing. In a recent study, it was shown that combining an approved anticancer drug that suppresses efflux activity (i.e. regorafenib) with another anticancer drug (i.e. paclitaxel), resulted in increased concentrations of paclitaxel in the tumours and thereby provided a synergistic anti-tumour effect [2].

Some of the other strategies that have been attempted to overcome MDR in chemotherapy, besides efflux inhibition, are briefly outlined here. Collateral sensitivity is the use of compounds that selectively kill multidrug resistant cells without killing the non-resistant parental cells from which they were derived. The genetic alterations accrued by cancer cells to obtain resistance against one compound may provide

hypersensitivity towards another compound, which can be exploited. An important aspect of this strategy is that its efficacy is independent of other mechanisms of MDR in cancer cells [17]. Another strategy that was proposed to overcome MDR is ribonucleic acid (RNA) interference (RNAi) therapy. In brief, this approach aims to silence or down-regulate the expression of specific genes by means of destructing mRNA molecules as triggered by other RNA molecules [3]. Metronomic chemotherapy is the long-term administration of low, but active, concentrations of chemotherapeutic drugs on a frequent basis that target endothelial cells and thereby provide an anti-angiogenic effect [22]. The combination of metronomic chemotherapy and immunotherapy exhibited promising results in both preclinical and clinical studies, but more research is necessary to be conclusive [23]. Microparticles have been identified as vectors to spread MDR to drug sensitive cancer cells by means of a non-genetic mechanism [24]. Compounds that are capable of inhibiting formation of microparticles are under investigation to potentially overcome MDR in cancer [25]. Replication competent viruses have been used to kill cancer cells without harming normal cells. In order to improve the anticancer activities of oncolytic viruses, they can be engineered to express exogenous transgenes for tumour proteins (e.g. tumour protein p53) [26]. Several models are available to study the anti-tumour effect of potential compounds in an attempt to overcome MDR caused by efflux transporter up-regulation.

3. *IN VITRO* MODELS FOR MULTIDRUG RESISTANT CANCER TREATMENT SCREENING

For the screening of the effectiveness of anticancer drugs, it is essential to use a viable test system that truly represents the disease as it appears in human subjects. Systems used as preclinical screening models include, among others, *in vitro* approaches such as cultured stem cells or other unicellular or multicellular cell lines [27-29]. Cultured human cancer cells form a strong pillar in anticancer drug screening and an exhaustive number of high-throughput assays and types of cellular material are available for measuring anticancer effects [30-31]. Furthermore, it is relatively easy to perform genetic manipulation of these cells that can provide information on the genetic mutations occurring in cancer cells that correlates with tumour tissues [31-33]. Essentially, *in vitro* approaches refine preclinical results before advancing to animal and human trials, thus reducing ethical concerns, time and cost. *In vitro* cell culture-based models also have certain drawbacks as outlined in the next sections.

Cultured cell models can range from very simple systems to very intricate and complex models consisting of multiple cell types (i.e. co-cultures), additional matrices or mechanical support. For the purpose of this review, the *in vitro* cell-based models used to screen for the anticancer efficacy of efflux-targeting MDR treatments are divided into conventional (basic mammalian cell lines cultured as 2D or flat cultures) and complex (mammalian cell lines cultured

with additional matrices, foreign objects or as 3D cultures) cell-based models.

3.1. Conventional cell-based models

For the purpose of this review, “conventional models” include all adherent cell cultures which are grown in an artificial environment in a 2D state as a monolayer or flat culture, although some suspension cells can also be classified as conventional cell-based models. Conventional mammalian cell culture models have served as the primary pillar for the evaluation of tumour biology, drug screening and mechanistic studies [30].

The introduction of cell cultures in 1950 provided an exciting new method for cancer drug screening in an *in vitro* environment [34]. Retrospectively, during the mid-1970’s the Chinese hamster ovary cell line was the first cell model to indicate a correlation between the drug-resistant phenotype and the drug efflux P-gp membrane transporter, which was indicated by a change in the drug permeation rate [35]. Drug resistance was induced in the cultured cells through exposure to actinomycin D, which resulted in a 2500-fold increase in resistance compared to the parental cell line [36]. In the 1980’s, the United States National Cancer Institute (NCI) listed 60 human tumour cell lines which can be used for *in vitro* drug screening and discovery [37], which is available online on the NCI’s website. These cell lines encompass nine different tumour organs/systems including blood, prostate, colon, central nervous system, ovaries, kidney, skin, breast and lung. This NCI-60 cell panel is considered an important platform for high-throughput models for characterisation and a biologically based approach to investigate treatment of malignancies [38].

The initial technique used in developing the drug-resistant Chinese hamster cell model in the mid-1970’s is still used in modern cell culture laboratories, which entails continuous exposure of a parental cell line to an anticancer drug that can result in a drug-resistant daughter cell line. Furthermore, conventional efflux cell culture models and modified cell culture models are used together with suitable assays during drug screening to investigate MDR. Determination of P-gp protein levels can be done through a Western blot assay [39]. Several other cell viability, proliferation and cytotoxicity assays (e.g. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)), bidirectional transport studies and clonogenic assays are commonly used to compare the sensitivity or resistance of both the parental and daughter cell lines towards anticancer drugs [40-43]. Furthermore, the inhibition of ABC transporters and the extent of substrate efflux can be determined through fluorescent dye accumulation and vesicular transport assays [44-45].

The effect of the ABC transporter family on chemotherapeutics, and potential inhibitors of these transporters, has been studied using the conventional cell lines as listed by the NCI (see Table 1). Human leukaemia

K562 and K562/Dox cells were used to evaluate the influence of metformin on the P-gp efflux of doxorubicin and the cytotoxicity of doxorubicin was shown to increase in the presence of metformin [43]. A human embryonic kidney (HEK293) cell line was used to modify an inverted membrane vesicle and estradiol-17- β -glucuronide was subsequently identified as a substrate for MRP2, P-gp and BCRP. This shared substrate was then used to determine the transport inhibition ability of nine inhibitors, showing different inhibitory properties towards the studied ABC transporters [46]. The ability of cordycepin, a phytochemical in dietary mushrooms, to modulate the functionality and expression of MRP1, P-gp and BCRP transporter proteins was assessed in the following cell lines: MDR human non-small cell lung carcinoma (NSCLC) cell line NCI-H460/MX20, MDR human hepatocellular carcinoma cell line HepG2-vin and MDR human cervical cancer cell line KB-vin. The findings purported cordycepin to influence P-gp ATPase activity, resulting in the inhibition of the P-gp transporter and thereby illustrated cordycepin as a promising substance to be used in combination with chemotherapy [47].

In a study conducted by Kim *et al.* [48], a doxorubicin resistant breast cancer cell line (MCF-7/ADR) was obtained by exposing the parental MCF-7 cell line to 0.3 μ M doxorubicin (Adriamycin[®]) over an extended period of time. The combined effect of doxorubicin and resveratrol subsequently decreased the gene expression of MDR1 and MRP1 in this breast cancer model, which simultaneously increased the intracellular doxorubicin concentration. The same MDR MCF-7/ADR model was also used to establish a novel micelle-based drug delivery system to increase the uptake of paclitaxel into the tumour tissue [49]. The MDR MCF-7/ADR cell line is one of the most widely used conventional models in cancer research, although its true origin is still questionable. Therefore, it should rather be used for hormone-responsive breast cancer studies due to its expression of oestrogen and progesterone receptors [50-51].

The cytotoxic ability of faspaplysin (a natural product with anticancer activity) was screened between 134 and 1740 nM against a panel of cell lines including NSCLC, small cell lung cancer (SCLC), unrelated cell lines and the HEK293 cell line. The concluding remarks suggested the alteration of reactive oxygen species (ROS), topoisomerase I and poly-(adenosine diphosphate (ADP)-ribose) polymerase (PARP) activity through the influence of several cellular mechanisms [42]. Other conventional cell models, including a cell clone of human epidermoid carcinoma (KB-3-1), namely KB-CV60, were used to evaluate the ability of sulindac sulfide to inhibit MRP1 efflux [44]. More recently, it was shown that the efflux of doxorubicin and rhodamine 123, through ABCB1 and ABCG2 transporters, was inhibited by abemaciclib in hyper-expressing cell lines such as KB, MCF-7, the human colon carcinoma cell line S1 and HEK293 cells [52].

Contributing further to the development of drug screening models is the transfection of a parent cell line with microRNA or small interfering RNA (siRNA). These models enable targeted alteration of the transporter expression of the parental line in the hope of modifying the extent of drug efflux, resulting in anticancer drug accumulation in the tumour and consequently increased therapeutic effects [53-54]. However, the shortcomings of using nucleic acid (RNA and DNA) interference therapies include ineffective tumour targeting, reduced RNA stability and short circulation time, which limit its clinical use [55]. In a study conducted by Yang *et al.* [56], a co-delivery system containing both cells [58].

Resistance in a paclitaxel resistant human colon cell line (HCT 116) was reduced through incorporation of paclitaxel into a nanoparticle delivery system [59]. Furthermore, an exosome-encapsulated paclitaxel delivery system was tested in P-gp expressing Madin-Darby canine kidney (MDCK) and RAW 264.7 macrophage cells, indicating a potential strategy for future chemotherapeutic treatment [48]. The screening of a multifunctional nanoparticle containing paclitaxel and curcumin was recently performed on a breast cancer cell line expressing P-gp (MCF-7/ADR). This delivery system was able to inhibit the P-gp mediated efflux of paclitaxel, thus increasing its intracellular accumulation [60].

In a sub-clone of human colorectal adenocarcinoma cells (Caco-2; sub-clone C2BBel), the zinc finger nuclease-mediated gene knockout approach was used to reduce the extent of transport facilitated by several ABC transporters. This subsequently produced a successful tool for explaining transport interactions [61]. Human cervix carcinoma (HeLa) cells have also been used for transcription activator-like effector nucleases (TALEN)-mediated Nanog gene knockout, resulting in weakened malignancy [62].

Although continuous 2D cell lines are easily maintained and the same cell line can be used for long-term experimental evaluations, these models do not fully correlate with *in vivo* conditions [63]. The monolayer cellular growth pattern of conventional cells is a problem, since few primary tumours can grow as a monolayer *ex vivo* [64]. Other drawbacks of conventional cell models are the absence of 3D architecture, limited cell-cell and cell-extracellular matrix interactions and uncontrolled exponential growth [65-67]. All these drawbacks add to the need for development of more advanced cell culture systems in an attempt to overcome these limitations.

3.2. Complex cell-based models

The lack of models that consider and include the organisational physiological structure of human tissues is deemed the major precursor of clinical drug failure during *in vivo* trials [68-69], while large scale screening of anticancer drug compounds in *in vivo* models (e.g. rodent models) is for the most part unpractical and subject to ethical scrutiny [70-

siRNA (to silence MDR1 mRNA) and paclitaxel, showed increased cytotoxicity of paclitaxel in MCF-7 and MCF7/taxol cells. The ovarian carcinoma cell line (SKOV-3) was used to evaluate the effectiveness of a nanoscale metal-organic framework co-delivery system of siRNA and cisplatin, to decrease the expression of MDR genes, and an increased chemotherapeutic efficacy was observed [57]. More recently, a hollow carbon nanosphere particle was used to treat MCF-7 and MCF-7/ADR cells by delivering either cisplatin or doxorubicin, in combination with siRNAs targeting MDR1. The findings included a down regulation of up to 96% of MDR1 protein expression in the MCF-7/ADR

71]. Intrinsic limitations of conventional models cause isolation of certain characteristics, thus ignoring major pharmacokinetic parameters. This inability to accurately extrapolate to humans caused a major void in clinical trials and eventually stimulated the design of more complex systems resulting in advanced *in vitro* models [72]. These models are designed to better reproduce the primary elements of MDR, allowing better screening of drug compounds before they advance to the later stages of clinical trials. For the purpose of this review, advanced cell culture models are defined as mammalian cells incorporated into advanced systems with more realistic intercellular communications and other features that better resemble those of the *in vivo* microenvironment. Table 1 provides some examples of complex cell-based models.

3.2.1. Integrated discrete multiple organ culture (IdMOC) system:

The integrated discrete multiple organ culture (IdMOC) system was designed to consist of individual wells, each containing their own cell culture and growth medium, embedded in a larger outer chamber [73]. Once the cells in each well reached experimental viability, a universal medium containing the test compound is added to such an extent that it overflows into all the wells and fills up the outer chamber, thereby creating an interconnected system. This universal medium serves as a blood surrogate between the different cells in the system, containing metabolites and waste products produced by each organ-representative well, and thereby mimic the *in vivo* state by exposing the blood surrogate to every part of the system. This overcomes the problem of cell type isolation that is present in traditional 2D cell culture studies. The universal medium can be analysed to determine accumulative system metabolism, whereas each individual cell culture can be individually analysed for organ toxicity, bio-accumulation and cytotoxicity [74-77]. The IdMOC system was designed to incorporate primary hepatocytes, cell cultures representing several organs and a cancer cell line to allow more effective testing of potential anticancer drugs on multiple organ systems [78]. This model currently permits for the screening of organ selective toxicity, cytotoxicity, drug-drug interactions and metabolic activation and also enables analyses of pharmacokinetic parameters (e.g. MDR1 and MRP1 influence) [73].

The major advantages of the IdMOC system include the ability to do high-throughput screening assays, retaining the homogeneity of the individual culture conditions of each cell line and the possibility of inter-cell metabolite interactions between each organ-representative. The model also functions as a low cost alternative for screening with an adaptable microenvironment to optimise growth conditions for each individual cell type. A major problem with this system, however, is the elevated risk for cross contamination. The model is also not designed to homogeneously spread universal medium through the system like a classic blood circulatory system, which makes this model vulnerable to criticism. Another restriction lies in the disparity of the metabolic needs of the various cultures, potentially causing saturation or starvation of some of the cells receiving universal medium [77, 79]. Time-dependent interactions between the organs are also not simulated, reducing this model's capability of giving exact quantitative results [80].

The application of the IdMOC system in MDR studies was suggested by Uzgare and Li [79] in a review of the IdMOC model. Targeted disruption of specific transporters responsible for MDR (e.g. MDR1, MRP1, etc.), and the pharmacokinetic consequences thereof can be investigated in this model due to the incorporation of liver cells [79]. A cytotoxicity study on tamoxifen was completed using the IdMOC system incorporating primary cells of different origin namely hepatocytes (liver), cortical cells (kidney), small airway epithelial cells (lung), astrocytes (central nervous system), aortic endothelial cells (blood vessels) and the MCF-7 human breast adenocarcinoma line. This allowed for the effective quantification of cytotoxicity in normal cells compared to cancer cells. Each individual organ system provided results comparable to those seen in literature during human trial cytotoxicity studies, thus validating this model [79, 81].

3.2.2. Microfluidic channel-based systems:

The idea of microfluidic systems was conceptualised around the design of a system encapsulating complicated, time intensive and expensive laboratory protocols into a relatively inexpensive, portable cell culture model that involves the manipulation of relatively small amounts of fluid (microlitre or picolitre) in a channel-based system on a chip [82-83]. Microfluidic systems were designed to overcome the absence of pharmacokinetic features in *in vitro* cell-based models as well as to refine, automate and reduce the associated costs of current laboratory practises [84]. The incorporation of multiple barriers commonly affecting drug delivery into organ/tissue compartments, as well as an interconnected microfluidic channel arrangement acting as a vascular circulating system, allowed for testing the compounds' permeability capabilities in the *in vivo* setting. Culture medium acts as a blood surrogate in the microfluidic channels, delivering important nutrients into the cells, while carrying metabolites away from the cells. The microfluidic channel model is scaled according to human physiological

parameters, which provides a realistic degree of drug exposure to each specific physiological barrier. This also allows a better understanding of pharmacokinetic interactions at each of the barriers, thus increasing predictive capabilities during extrapolation to the *in vivo* situation [85]. Microfluidic chips containing cell cultures are equipped with microphysiometers capable of measuring numerous metabolic parameters (e.g. oxygen consumption, glucose uptake, lactate production) by means of microsensors [86]. The incorporation of multiple-well plates (e.g. 96-well plates) also makes this model a viable option for high-throughput screening [87]. Its dynamic nature ensures the real-time monitoring of cell culture parameters needed for drug development, while minimising standard deviations of results due to the concentrated amounts of reagent used in these studies. Human cell lines can be used in these systems, each chosen to have the desired characteristics of the designated organ portrayed in the model (e.g. multidrug resistant cancer combined with a normal epithelial cell line). A physiologically-based pharmacokinetic model (PBPK) is usually used adjacently in combination with the microfluidic channel systems in order to mathematically translate absorption, distribution, metabolism and excretion (ADME) data obtained from the micro-scale cell culture analogue (μ CCA) model into actual *in vivo*-reliable results [88]. The model can also be manipulated to test permeability potential through different barriers by sequential organisation of the tissue chambers (e.g. intestinal lumen before the cancer cells) [89-90]. Microfluidic based systems also have the capability to present drug synergistic effects that cannot be seen in traditional *in vitro* studies and the replicated vascular system allows for different dose exposure parameters, giving results similar to those *in vivo*. Currently, there are vast selections of these chips, each containing unique mechanisms and added protocols. Organ systems included in this design is lung, liver, intestinal, urinary, breast, brain and bone marrow tumours [91-93].

The microfluidic channel system incorporates conventional cell models, which presents its own range of drawbacks including cell changes and differentiations through passages and physiological structure differences compared to cells within organs *in vivo*. Adapting this model for high-throughput screening studies is difficult and expensive because of the expertise needed to maintain viability and sterility for all the different cell cultures in play [89]. Different types of microfluidic channel systems have been developed and used in studies as briefly described in the sections below.

3.2.2.1. Multi-organ co-culture in tumour-on-a-chip system:

The next step in developing advanced organ-on-a-chip systems was to incorporate multiple organ cell cultures in the same system. This creates an *in vivo*-like microphysiological system that represents all of the major tissues responsible for

metabolic processes, as well as influencing drug pharmacokinetic and pharmacodynamics [94].

One example of a multi-organ co-culture as part of a tumour-on-a-chip design included HepG2/C3A (hepatocellular carcinoma / liver cells), MEG-01 (megakaryoblast / bone marrow), MES-SA (drug sensitive uterine sarcoma) and MES-SA/DX-5 (MDR uterine sarcoma) cell lines. The HepG2/C3A cell line was included to view the effect of the metabolism of doxorubicin towards the less active doxorubicinol. The MEG-01 cell line represented the thrombocytopenic effect of this drug due to bone marrow suppression, while the cancer cell line MES-SA/DX-5 was transfected to over-express P-gp, thereby inducing MDR. This P-gp over-expression allowed the researchers to determine if the required concentration of drug needed to have cytotoxic effects on the resistant cancer cells, was in an acceptable range to avoid systemic toxicity [95].

3.2.2.2. Lung-on-a-chip microfluidic device:

This novel biomimetic device was developed to improve the model by incorporating key characteristics of tissues that were absent from the traditional microfluidic channel system. The lung-on-a-chip microfluidic device was designed to accommodate an extracellular matrix coating (consisting of collagen and fibronectan), whilst human alveolar epithelial cells and human pulmonary microvascular endothelial cells were cultured to confluent monolayers on opposing membrane sides, which allowed airflow between the barriers. This airflow allowed for an air-liquid interface similar to the alveolar airspace found in human lungs. The compartmentalised design enabled controlled delivery of nutrients to the different cell cultures. Two vacuum inducing micro-chambers were installed to mimic sub-atmospheric pressure driven stretching of the membranes to replicate normal breathing. In this model, the researchers attempted to replicate the major factors of pulmonary physiology in order to evaluate how drug compounds may act in these systems and to compare the outcomes to actual *in vivo* results [96].

3.2.2.3. 3D lung cancer microfluidic constructs:

Polydimethylsiloxane (PDMS) round bottom micro-wells were constructed as microchip bases and hydrostatic pressure was used to guide the introduced cancer cell suspension to the membranes. Gravitational forces kept the cells in place and forced the formation of 3D cell culture constructs. The mesothelioma cell line (H2052) and primary cell lines from patients (NSCLC, squamous carcinoma and pericytes) were introduced into the model as mono- and co-cultures to test the effect of stromal cells on MDR, using cisplatin as chemotherapeutic compound. This system allowed viable assays, but a concentration gradient generator was proposed for future, more realistic studies [97].

3.2.3. 3D spheroid cell cultures:

Complex 3D spheroid cell cultures are being incorporated into modern *in vitro* screening models, which

attempt to bridge the gap between traditional flat culture shortcomings and the limitations experienced with *in vivo* studies [98-99]. This design more realistically mimics the physiological characteristics of *in vivo* tumours by allowing cell-cell interaction as well as better communication within the microenvironment (e.g. extracellular matrix). Intercellular communication includes the activation of signalling pathways, which mediates a cascade of effects including molecular, subcellular and cellular changes. These changes can include alterations in protein/gene expression, molecular exchanges, enhanced cellular transport effects (endocytosis/exocytosis) and cellular behavioural changes (e.g. apoptosis, proliferation, cell shape, motility) [100-101].

3D spheroid cell culture is seen as the first and oldest 3D cell culture model construct and dates back to the 1970's. This model depends on the tumour cells' capability to differentiate into heterogeneous multi-cellular spheroids by either introducing them into an extracellular matrix (ECM) as a scaffold-dependant (i.e. scaffold-based) construct or by introducing external factors to enforce spheroid growth (non-scaffold based). Scaffold-based cell culture models include matrix-on-top, matrix-embedded, matrix-encapsulation, spinner flasks and the micro-patterned plate technique. Non-scaffold-based cell culture models employ ultra-low attachment plates, hanging drop, microgravity (rotating wall-vessels/bioreactors or clinostat), spontaneous aggregation, magnetic levitation and 3D bioprinting [102]. After the cells start piling up on each other in 3D spheroid cultures, they develop surface receptors known as integrins, which are used to anchor themselves onto the ECM and thereby sustaining tumour growth and enhancing cell-cell communication [103-104].

The connection of these cells to the microenvironment initiates increased protein and gene expression levels of MDR precursors, which are generally limited in conventional models. Changes that were found to occur in these cell structures included metabolic changes, increased responses to cellular stress, cellular structural changes, increased signal transduction, slowing down proliferation cycles (quiescent cells), limiting drug surface contact, inhibiting apoptotic precursors and elevated expression of cellular transporter proteins, all playing a role in the increased resistance of these cell models to cancer treatment [102]. The capability of these cells to use cadherin molecules and integrin surface receptors to mediate resistance against drug treatment, allowed for the design of effective MDR cell culture models [105-107].

The combination of acquired MDR characteristics with surface ABC transporters created a cellular model that more accurately predicted the *in vivo* occurrences of drug interactions. The major advantage of this 3D spheroid cell culture model is the formation of the *in vivo*-like necrotic centres (although recent publications showed some spheroid systems to be devoid of necrotic centres) and an overall heterogeneity [108]. Modern 3D tumour cell model concepts

incorporated angiogenesis to recreate the *in vivo* microenvironment even more closely. Vascularisations of breast and prostate 3D spheroid cellular models (MCF-7, MDA-MB-231, LNCaP and PC3) were initiated by incorporating glycosaminoglycan-based hydrogel culture systems containing vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and stromal cell-derived factor-1 (SDF1), thus creating a multi-parametric bioengineered tumour angiogenesis microenvironment. This vascularisation ultimately resulted in increased chemoresistance as well as tumour regression rates comparable to that observed clinically [109].

Further investigation found that the introduction of an exogenous ECM into the 3D cell culture model design can increase the *in vivo* imitation of cancer cells, ultimately resulting in more predictable screening. This model has the capacity not only for screening of anticancer compounds, but also to investigate protein changes (mRNA inducing protein expression and complex interactions), cellular communication and cellular metabolic and functional processes (ATP production and cell migration/invasion). In most larger 3D cell spheroids (i.e. exceeding 150 μm in diameter), the nutritional starvation and hypoxic conditions of the tumour centre causes necrosis accompanied by micro-metastatic behaviour, thus allowing investigation of anticancer compound evasion mechanisms, as well as the overall influence that these physiological changes may have on tumour biology [110-112].

The superiority of this model lies in its ability to shy away from the exponential growth phase priority that 2D cell cultures exhibit, by re-directing energy to develop advanced tumour-like structures [65]. This mechanism of sophisticated self-assembly occurs because the cells reach a “dynamic equilibrium”, and this phenomenon essentially bridges the gap between *in vivo* and *in vitro* screening [65]. The development of 3D multicellular tumour spheroids is also purported to be more time and cost effective in the identification of novel anticancer drugs and produce data of superior quality compared to that of conventional models [113].

Three-dimensional cell models have been shown to have greater chemo- and radiotherapy resistance compared to their 2D counterparts and they show similar characteristics to those expressed by native cancers, including lactate accumulation, oxygen/hypoxia and proliferation. It has also been suggested that 3D cell culture models lead to more aggressive cancer phenotypes, including sustained proliferative signalling, inducing angiogenesis, resisting cell death, genome instability and mutations, and deregulating cellular energetics [114]. The 3D spheroids can also be used to determine lethal doses closely related to *in vivo* values [115]. It has also been claimed that in 3D cell culture models, the non-hologynic exposure of the cells within the spheroid to the drug being tested and the non-constant proliferation rate of the cells inside the spheroid, will

contribute to the deeper understanding of mechanistic properties of cancer treatments [116].

Unfortunately, 3D cell culture operating techniques can be time consuming and the reproducibility of some techniques are questionable [117]. Furthermore, if the size of the spheroid is greater than 150 μm , necrosis of the core can occur due to inadequate nutrient and oxygen diffusion [118], although this is greatly dependent on the type of spheroid system being used [108]. Drug penetration can also be restricted by the size of the spheroid, diffusion through the ECM and cell-to-cell and cell-matrix adhesion, influencing the effect of chemotherapy [114].

3.2.3.1. 3D multicellular models mediating MDR:

One practical application of a 3D multicellular model consisted of two NSCLC cell lines, isolated from primary lung cancer pleural effusions in patients who have not received any chemotherapy. The two cell lines INER-37 (well-differentiated adenocarcinoma) and INER-51 (weakly-differentiated adenocarcinoma) each had a differing histological appearance, with the former cell line being a smaller aggregate ($550 \pm 25 \mu\text{m}$), whilst the latter appeared to be a much larger aggregate ($1314 \pm 30 \mu\text{m}$). These two cell lines were cultured separately as multicellular spheroid aggregates as well as monolayer models, while differentiation and changes in drug sensitivity were monitored. The ultimate goal was to disprove that drug resistance in these multicellular models was primarily caused by lower surface exposure of drugs to cells, due to lower diffusion of drugs into the larger spheroids, as had been previously stated [119]. Upon gene analysis using real-time polymerase chain reaction (RT-PCR) it was found that INER-37 induced gene expression of both *mdr1* and *mpr1* in multicellular spheroids, while this was not the case in the 2D cell culture model. The *mdr1* gene is ultimately responsible for the expression of the major MDR surface efflux transporter, P-gp [120]. INER-51 showed no changes to *mdr1* expression between culture conditions, and *mpr1* expression was absent in both models. Other changes were also induced, such as larger cells going into a quiescent state thus allowing less DNA damage and faster DNA repair capabilities. Several anticancer compounds (including etoposide, doxorubicin and camptothecin) were used in this study to compare the chemo sensitivity of cells grown in multicellular tumour models (3D models) and those in conventional 2D models. Ultimately, it was found that the 3D models conferred MDR to cells through numerous gene expression changes, most notably the induction of MDR transporters e.g. P-gp [107].

3.2.3.2. Hollow fibre-based spheroid cultures:

Biocompatible polyvinylidene fluoride (PVDF) hollow fibres were introduced into this model as an inducing agent of tumour cells, to accumulate into solid masses resembling spheroids [121]. Large concentrations of tumour cells are injected into the hollow lumen of the fibres, and thereafter

they are periodically sealed and left in Petri dishes to proliferate into solid spheroid masses. The fibres, which acted as bioreactors, are then separated and transferred into cell culture plate wells, containing the test drug. This model produces versatile drug resistant 3D cancer spheroids, but the growth of these cells are severely limited by the fibre walls. This approach needs consideration of maximum cell growth and the time that is paired with it, to prevent a decline in cell numbers. Migration studies are also not permitted in these models, since the hollow fibres serve as a barrier to large molecules, limiting these studies to only toxicity screening [122-124]. Nevertheless, this model allows the quantification of multiple metabolic precursors and has immense potential in MDR studies, but to our knowledge, this model has not featured immensely in efflux-based resistance studies.

3.2.3.3. Cell printing:

Cell printing is based on bio-additive technology with the ability to build 3D spheroid constructs in a carrier medium [125]. This technology entails implanting the cells layer by layer, and assembles a heterogeneous tumour model with a relative high density capacity. Biological 3D printing can be modified to produce high-throughput *in vivo*-like tumour models with ECM-cell interactions on a large scale. It allows controlled cell distribution by the researchers, thus creating a biomimetic design that resembles the *in vivo* situation, as well as allowing alterations to the tumour morphology. These alterations can be used to test specific tumour properties, accommodate certain assays and to investigate tumour behaviour [125, 126]. The ability to adjust cell morphology gives researchers the opportunity to create models with specific MDR properties, e.g. increased MDR1 gene expression.

4. IN VIVO MODELS FOR MULTIDRUG RESISTANT CANCER TREATMENT SCREENING

Although *in vitro* cell culture-based models are fundamental building-blocks in preclinical cancer research, *in vivo* animal models are still used as the decisive factor in the preclinical anticancer drug screening process before further clinical trials in humans may be conducted [127-128]. *In vivo* animal models resemble in part the *in vivo* responses and physiological conditions in humans [129], providing predictive means to study patient responses to anticancer drugs. Mice are the most commonly used animal model in cancer treatment research as their genome is well-characterised, making the manipulation of their genes much easier with the established genetic engineering techniques already available. Furthermore, their small size and short development period alleviates excessive breeding and maintenance costs in comparison to larger animals [130-132]. The most frequently used mouse cancer models involve xenografts, where human cell-derived tumours or human tumour explants (patient-derived) are implanted into immunocompromised mice, and genetically engineered mouse models (GEMMs) that are genetically manipulated to

form spontaneous tumours. As both these models have been applied in cancer research to study efflux transporter-related MDR, they will be discussed in the following sections. However, it is important to mention that other rodent models also exist, but have not necessarily been commonly utilised for efflux-related MDR cancer studies.

Mouse allografts involve the injection of immunocompetent mice with murine cancer cells or tumours. This model eliminates the bias of xenografts, which use only immunocompromised mice [133-134]. However, the interspecies differences between human and mouse cells result in poor translation between the drug efficacy in allograft models compared to humans, as comprehensively reviewed by Rangarajan and Weinberg [131]. Despite such discrepancies, mouse allografts have been used to generate orthoallobanks of NSCLC for preclinical drug screening [135], as well as basal cell carcinoma models for carcinogenesis and drug pathway studies [136].

By exposing rodents to carcinogens such as DMBA (dimethylbenz(a)anthracene), MNU (N-methyl-N-nitrosourea) and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), spontaneous carcinogen-induced tumours can develop [137-138]. However, the locality of tumour formation is not always constant or controllable. In addition, there is the unpredictable tumour formation rate and the adverse effects (cancer-unrelated) of such chemical substances on overall tissues [139].

4.1. *In vivo* hollow fibre assay models

In 1995, the NCI of the USA developed the hollow fibre assay (HFA) for the preliminary *in vivo* screening of anticancer drugs as a means to bridge the gap between *in vitro* cell-based assays and *in vivo* xenograft models [140]. This assay entails the culturing of 12 human cancer cell lines (of 6 different histologies) in highly permeable biocompatible PVDF hollow fibres, followed by implanting them into nude mice at both subcutaneous and intraperitoneal locations. After anticancer drug exposure for 4-6 days, the fibres are removed and cytotoxicity effects detected with colorimetric tetrazolium-based dye (i.e. MTT) [140-141]. The majority of HFA-based data have been used in prioritising anticancer drugs with a high probability of antitumour activity in follow-up *in vivo* xenograft models. By implementing a few modifications to the original NCI protocol such as extending the drug exposure period, using immunocompetent mice or conducting other end-point measurements, several studies have proven the potential of HFA to study antitumour vasculature drugs and tumour angiogenesis [142-143], DNA damaging agents [144], proteasome-inhibiting drugs [145] and the antitumour effects of active plant constituents [146-147]. The antitumour activity determined with the HFA has been found to positively correlate with that of xenograft models [148-149].

4.2. Xenograft cancer models

Xenograft cancer models are based on the implantation of a tumour(s) into immunocompromised mice, which ensures that no rejection of the induced tumour occurs [150]. Such immunocompromised mice can either be athymic nude mice, with a mutation in the *Foxn1* gene [151], or severely compromised immunodeficient (SCID) mice, with a nucleotide polymorphism in the *Prkdc* gene causing abnormalities to or complete absence of T and B lymphocytes [152-153]. The tumour implantations can either be done orthotopically or heterotopically [154-155]. Orthotopic models are formed when the tumour is directly implanted into its organ of origin, whereas heterotopic models are placed directly underneath the skin (subcutaneous). As subcutaneous heterotopic xenografts can be observed with the naked eye in hairless (nude) mice and the procedure is much easier and less invasive than orthotopic surgeries, this model is frequently used for rapid cytotoxic or cytostatic anticancer drug screening [156-157]. In contrast, orthotopic tumours provide a more appropriate measure to study the tumour at its primary site of origin and to evaluate possible metastasis and invasion [156]. However, orthotopic models have to be excised from sacrificed mice to measure the tumour volume, and therefore only end-point measurements can be taken. Xenograft cancer models can be classified as either cell-derived or patient-derived, as discussed in the following sections, with the focus on efflux transporter-based models used for anticancer drug screening applications.

4.2.1. Cell-derived xenografts:

The transplantation of *in vitro* cultured human cancer cell lines into mice with compromised immune systems is the traditional way to generate a xenograft. The specific type of induced cancer, the exact amount of cells required to form a solid tumour and the time it takes for tumour formation, all depend on the specific cancer cell line used [151, 154, 158]. Given that a considerable number of established cell lines are available for transplantation, a wide range of human cancer types can be studied. Nonetheless, the continuous passaging of the *in vitro* cell lines can lead to drifted cells with different genetic characteristics than their primary cancer tissue of origin [159-160]. In addition, the homogeneity of an injected cell mass does not recapitulate the complexity of a tumour with evolved cells in symbiotic growth [161]. The implantation of tumour cells in different sites than where they normally occur as a disease in humans can also contribute to discrepancies in translation [162]. As previously mentioned, the use of immunocompromised mice can also provide misleading results as not all cancers arise in immunodeficient patients [133, 163]. Even though cell-derived xenografts (CDXs) have several limitations, this *in vivo* model is still frequently used for preclinical anticancer treatment screening.

Overall, for the screening of anticancer drugs against MDR tumours associated with efflux transporter up-regulation, human cancer cells showing efflux transporter

over-expression are injected either subcutaneously (used predominantly) or orthotopically into immunocompromised mice generating a CDX. After the allocated drug treatment period, the cell-based tumour growth is evaluated for subsequent changes to the tumour size and volume. This is followed by immunohistochemical (IHC) analysis or Western Blot and quantitative reverse transcription polymerase chain reaction (RT-qPCR) experiments to identify the transporter protein expression levels. By using these end-point measurements, CDXs can provide valuable information regarding the efflux transporter proteins responsible for drug resistance in specific cancers [164-166], the modulation effect of drug combinations on efflux-related MDR in cancer [167-169] and the potential of herbal medicine to re-sensitise tumours towards anticancer drugs [170].

By using *in vivo* CDXs in conjunction with *in vitro* cytotoxicity assays, several studies have proposed the combinational therapy of drugs as an effective regiment to overcome cancer MDR. Table 1 provides a broad overview of a range of CDXs used for efflux transporter-based anticancer drug screening. Argov *et al.* [169] studied the modulation effect of fluoxetine (Prozac) on the moderate drug resistance of doxorubicin in subcutaneous human colon cancer (HCT-15) CDXs expressing only P-gp. Fluoxetine was found to reverse moderate MDR *in vitro* by inhibiting the P-gp efflux pump and improving doxorubicin intracellular accumulation. Tumour progression was drastically delayed when fluoxetine was combined with doxorubicin *in vivo*, which proved the combination therapy to be as efficient as bevacizumab treatment. Chakravarty *et al.* [168] used the aggressive MDR breast cancer cell line, MCF-7/Dox, in orthotopic xenografts to investigate the anticancer efficacy of the clinically approved antiretroviral drug, nelfinavir. Multiple exposures to nelfinavir *in vitro* enhanced intracellular doxorubicin levels and inhibited P-gp expression and efflux. The co-exposure to nelfinavir and doxorubicin resulted in a 65-80% reduction of *in vivo* tumour growth, in comparison to each drug alone. As this breast cancer model over-expressed P-gp, nelfinavir was shown to counteract MDR by inhibiting P-gp activity. Another study utilised human embryonic kidney cells (HEK293) transfected with the ABCG2 transporter, also known as BCRP, to generate ABCG2-expressing xenografts [170]. These MDR xenografts were implemented to assess the potential of MBLI-87, a non-toxic ABCG2-specific inhibitor, to increase irinotecan accumulation by modulating ABCG2-related drug resistance. The MBLI-87 inhibitor was able to re-sensitise the resistant tumours to irinotecan therapy, although it had no significant effect when treatments were discontinued for 14 days and reconstituted thereafter. This incident was similar to clinical findings also stating premature discontinuation to be a cause of survival failure [171]. Cell-derived xenografts have also been used to evaluate the anticancer efficacy of drug-loaded nanoparticles against efflux-related cancer MDR. Patil *et al.* [172] used

poly(D,L-lactide-co-glycolide) (PLGA) and polyethyleneimine (PEI) to induce gene silencing in P-gp over-expressing xenograft models (JC cells used). By using nanoparticles encapsulating PLGA-PEI and paclitaxel and with the surface functionalised with biotin for specific tumour targeting, the efficacy of paclitaxel to inhibit *in vivo* tumour growth was significantly higher compared to treatments without gene silencing. Therefore, the use of dual-agent nanoparticles focusing on gene silencing and cytotoxic drug encapsulation shows potential to defeat cancer MDR, especially when the particle surface is coated with specific ligands commonly recognised by cancer cell receptors, such as biotin [172].

4.2.2. Patient-derived xenografts:

In an attempt to improve on the shortcomings of CDXs, the direct transplantation of a patient's primary tumour material as fresh explants into immunocompromised mice has been developed [153, 173-174]. These models, known as patient-derived xenografts (PDXs) or tumour grafts, more closely recapitulate the parental tumour behaviour, histology, genetic diversity and metastatic patterns [175-177]. Bearing in mind that the tumour is directly transplanted into the murine host, the *in vitro* manipulation of the tumour is completely eliminated, ensuring the preservation of the original tumour heterogeneity. With the genomic analysis of 1075 established PDXs and corresponding cell lines, Gao *et al.* [178] demonstrated that several signalling pathways were over- or under-represented in cell lines, whereas matching PDXs showed accurate representation thereof. Moreover, only minimum genetic drift occurred between the passages of a particular PDX, in contrast to the loss of certain developmental pathways in the cell lines upon continuous *in vitro* culturing. Such similarities between PDXs and the original tumours were also observed for human breast cancer models [175]. This explains the deferral of the *in vitro* NCI-60 cell panels, used for more than 25 years as the golden standard for anticancer drug screening, in favour of PDX collections that closely mimic the human tumour drug response [179]. The concept of using PDXs as mouse surrogates (newly referred to as "avatars") in chemotherapeutic research has evolved into a promising practice to improve patient treatment regimens. A patient's tumour is used to generate a number of PDXs in various murine hosts and these xenografts are then studied concurrently or ahead of the actual patient treatment program [180]. This "avatar" approach can help guide the therapeutic management of the clinical trial and introduce key features necessary for the development of personalised medicine [181]. Patient-derived xenografts have also been shown to accurately portray the biodistribution of anticancer drugs when coupled to radiolabelled tracers and examined with micropositron emission tomography (microPET) imaging [182].

Patient-derived xenografts are more commonly implanted subcutaneously, but orthotopic and mammary fat pad

implantations have also been done before [174-175]. The development of validated PDX models is a long-term process with high costs. Just the average tumour latency, from tumour implantation to graft appearance, takes up to 12 months. Sadly, patients with rapidly growing tumours will most probably not benefit from their "avatar" models, established for their personalised therapy, as these model development programs are too time-consuming. Furthermore, the engraftment into an immunocompromised host has the potential to select for a more aggressive phenotype than originally present in the human patient [175]. The lack of an intact immune system in the immunocompromised murine host hinders the ability to study cancer-related immune responses in PDXs [183-184]. However, this limitation has been addressed with bioengineering approaches using GEMMs (discussed in the following section), immune cells and fibroblasts to establish an immunocompetent mouse model [185].

Irrespective of the abovementioned disadvantages of PDXs, these models can provide predictive preclinical means to evaluate patient drug responses, especially those related to efflux transporters involved in cancer MDR (Table 1). Earlier research conducted by Arvelo *et al.* [186] investigated the potential of the P-gp inhibitor, verapamil, to counter MDR in SCLC PDXs. By combining verapamil with CyCAV, a multifaceted chemotherapeutic dosing consisting of cyclophosphamide, cisplatin, doxorubicin (adriamycin) and etoposide, the drug efficacy was improved against the resistant tumours. Therefore, verapamil inhibited the P-gp efflux transporter responsible for MDR in SCLCs. A recent study investigated the role of the transporter ABCB5 in the drug resistance of neuroendocrine skin cancer or Merkel cell carcinoma (MCC) [187]. After the treatment of intraperitoneal MCC PDXs with carboplatin or etoposide, the expression level of ABCB5 was 8-fold higher than the control PDXs. But, when these anticancer drugs were combined with an ABCB5 blocking antibody, tumour growth was significantly reduced and tumour apoptosis drastically enhanced. These results clearly showed the potential of ABCB5-targeted drug resistance reversal therapies to enhance the success of MCC treatment in the clinic.

4.3. Genetically engineered mouse models

Genetically engineered mouse models (GEMMs) are the most advanced *in vivo* models used for human cancer research. These models involve the genetic manipulation of cancer-related genes for drug efficacy screening, studying the interactions between chemotherapeutic agents as well as research on the role of these target genes in tumour biology and tumorigenesis [133]. For the purpose of this review, GEMMs can be categorised as either transgenic or endogenous [163, 188]. In essence, transgenic GEMMs are generated through the microinjection of cDNA with specific promoters into the nuclei of fertilised oocytes, which induces the expression of targeted oncogenes [189-190]. However,

the expression level of such oncogenes may not always be predictable or accurately controlled, as apoptosis or senescence may also result from such manipulations [191]. Endogenous GEMMs, on the other hand, can either lose the ability to express oncogenes through the disruption or inactivation of the target allele (through knockout techniques) or express modified regions of the target allele by the insertion of the desired mutation into the target allele (knock-in approaches) [189]. Yet, several of these allele disruption approaches can cause embryonic lethality or developmental defects as these mutations are present in the whole mouse [192]. Through the use of conditional alleles requiring site-specific recombinases to induce mutations, the locality of mutations can be restricted to specific tissues [133].

The use of GEMMs in cancer research harbours a number of improvements on other model pitfalls. As GEMMs are generated in the presence of an intact immune system [193], the inadequacy of xenografts to study cancer-related immune responses is overcome [134]. Furthermore, the histopathological and molecular analyses of GEMMs have shown the accurate recapitulation of essential characteristics of human tumours, which is not always the case for CDXs. Liu *et al.* [194] developed a mouse model for human BRCA1-associated breast cancer through the simultaneous deletion of the *Brca1* and *p53* alleles. These tumours were found to closely resemble human basal-like breast cancers based on expansive growth, the expression of basal epithelial biomarkers and genomic instability. A year later, Rottenberg and colleagues used the same breast cancer GEMMs (*K14cre;Brca1^{F/F};p53^{F/F}*) to investigate the mechanism involved in the acquired resistance of the PARP inhibitor, AZD2281 [195]. The over-expression of the P-gp efflux transporter was the observed mechanism of acquired resistance as the P-gp inhibitor, tariquidar, successfully reversed the resistance. By breeding mice carrying *Abcg2*-deleted alleles with the *K14cre;Brca1^{F/F};p53^{F/F}* GEMM, Zander *et al.* [196] confirmed that the ABCG2 transporter confers resistance to the topoisomerase I inhibitor, topotecan, *in vivo*. Therefore, this specific model proves to be a valuable predictor of human *BRCA1*-mutated breast cancers in preclinical trials.

Knockout GEMMs have been used to investigate the role of efflux transporters in anticancer drug failure and cancer MDR. Table 1 provides evidence of several knockout models. An *Mrp4* knockout mouse model showed that MRP4 restricts topotecan drug distribution and its over-expression caused resistance to topotecan [197]. Marchetti *et al.* [198] used *Mdr1a/1b^{-/-}* (P-gp) knockout mice to study the pharmacokinetics of the C-4 methyl carbonate paclitaxel analogue, BMS-275,183, in the presence of the proton pump inhibitor, pantoprazole. After an exposure period of 6 h, the apparent oral bioavailability of *Mdr1a/1b^{-/-}* mice in comparison to wild-type mice was 97% and 63%, respectively. Significant increases were also observed in the absolute brain concentrations of BMS-275,183 in knockout

mice, compared to control groups. The *in vivo* double-knockout GEMMs confirmed the affinity of BMS-275,183 for the P-gp efflux transporter. Not only knockout approaches are used; knock-in models can also provide information on efflux transporters. Gu *et al.* [199] created a mouse model by inserting a firefly luciferase (*fLUC*) gene into the murine *mdr1a* genetic locus. This model proved to faithfully show basal *mdr1a* expression, as well as induced expression after xenobiotic treatment. Changes in luminescence intensities were observed after treatment with paclitaxel, docetaxel and pregnenolone-16 α -carbonitrile. These correlated to the statistically significant induction of *mdr1a.fLUC* expression in drug-exposed mice compared to controls, demonstrating the feasibility of linking the conditional knock-in system with a luminescent reporter gene for potential *in vivo* tumour bio-imaging.

5. IMPROVEMENTS FOR PRECLINICAL MDR TREATMENT SCREENING

For the past 30 to 40 years, the NCI made significant contributions to the list of standard drugs available for chemotherapy. The majority of these drugs were tested on the NCI-60 cell panel, even though today researchers are sceptic about using conventional 2D flat cultures for anticancer efficacy screening. Such scepticism can be substantiated by the proven discrepancies in conventional 2D cell culture growth patterns, when compared to human tumour tissues. Calitz *et al.* [200] and Wrzesinski *et al.* [65] clearly note the main differences occurring between cells cultured in 2D and 3D. Conventional 2D cultures require synthetic growth surfaces with artificial nutrient sources, while regular sub-culturing (e.g. trypsinisation) of the cell population is necessary to ensure the availability of sufficient growth space and nutrients. Therefore, 2D cell cultures are in a constant state of proliferation and active cell growth to recover from sub-culturing. In contrast, dynamic 3D spheroids have reached a state of “dynamic equilibrium” after about 18 days’ post-trypsinisation [201]. During this stage, these spheroids start to physiologically mature and form functional structures and characteristics of the original tumour tissue. Given that the dynamic spheroids are allowed to completely recover from laboratory practices (e.g. trypsinisation), the formation of an ECM is an important feature of such 3D cultures. An ECM ensures proper cell-to-cell communication, which is important in true human tumours. This explains why the NCI recently shifted its preclinical screening focus to the use of other models, such as PDXs [179].

Considering that each *in vitro* and *in vivo* model discussed in this paper has its own advantages and limitations, no single model can comprehensively elucidate on the entire efflux transporter-facilitated drug resistance mechanism of cancer as yet. In fact, a combination of both *in vitro* and *in vivo* models would enhance the preclinical decision-making of a chosen drug for further human clinical trials, where the advantages of a particular model

overshadow the shortcomings of the other model. For example, preliminary screening of various drug targets at a wide range of concentrations for anticancer efficacy using conventional 2D flat cell cultures, followed by further in depth screening of the most potent compounds (as determined by 2D screening) using advanced 3D cancer spheroids. From this, only the most promising drug targets with the least detrimental side-effects are tested in PDXs, after which one drug is selected for human trials. This approach can greatly decrease the number of animals needed for research purposes, potentially decreasing drug development costs and addressing the ethical issues involved with animal-based research. It may also increase the success rate of clinical trials, since more accurate pre-screening was possible. It is also suggested that clinicians and academic researchers collaborate continuously, to ensure early communication and advice on relevant findings.

Keeping in mind that human tumours form complex structures, interacting with other normal tissues and no tumour exists as the single cell types found in monoculture models. Therefore, several future directions to overcome cancer MDR should be aimed at developing techniques to produce co-cultures containing different cancer cell types. High-throughput hanging drop technology has been used to successfully produce a 3D co-culture of DLD1 colon cancer cells with mouse NIH3T3 fibroblasts, for the validation of RNAi therapy [202]. Another study co-cultured NSCLC cells with T-lymphocytes, using scaffolds, to develop a 3D model for the exploration of tumour-immune responses [203]. Therefore, the use of *in vitro* 3D co-cultures in general can provide a more realistic portrayal of the tumour-related responses associated with anticancer drug treatments.

Adding to the heterogeneity and complexity of a human tumour is the fact that tumours can contain their own sub-population of stem cells responsible for cellular “plasticity”. These cancer stem cells (CSCs) can shift as a response to external/internal stimuli from a quiescent state to actively proliferating; from chemosensitive to chemoresistant (by overexpressing ABC-efflux transporters) and from an epithelial to a mesenchymal phenotype (see Fiori *et al.* [204] and Zakaria *et al.* [205] for a comprehensive discussion on CSC biology). The hypothesis is that these CSCs hold the key to cancer recurrence (after remission has been medically confirmed) or the emergence of the same type of cancer in various organs. Therefore, by targeting CSCs specifically, improved anticancer treatment strategies can evolve for clinical applications.

3D cell culture models have proven to be suitable for use in CSC research. Stankevicius *et al.* [206] used scaffold-attached laminin rich ECM and scaffold-free multicellular spheroid models (liquid-overlay technique) to investigate CSC marker expression, associated with self-renewal and epithelial to mesenchymal transition, of human colorectal carcinoma (CRC) cells (DLD1 and HT29). In comparison to 2D culture conditions, the CRC cells showed higher

expression of the CSC-associated genes when cultured in 3D. This is in accordance with the findings of Reynolds *et al.* [207], who found that CSC content was the highest in 3D embedded spheroid models compared to 2D cultures.

To this end, complex 3D cell culture-based models continuously demonstrate truthful resemblances to the *in vivo* or human-like environment in which tumours can arise. Seeing as tumour complexity and stem cell characteristics can add to the long list of tumour properties portrayed in 3D models, this approach can contribute to this approach becoming the gold standard in efflux-based MDR screening of anticancer drugs.

6. CONCLUSIONS

The causative effect of drug efflux transporters, especially those of the ABC-superfamily, to the occurrence of MDR in cancer is widely recognised. A collection of therapeutic strategies exist to combat efflux-based MDR mechanisms in cancer including the co-administration of efflux transporter inhibitors/modulators, RNAi therapy, metronomic chemotherapy combined with immunotherapy, to name but a few. The preclinical screening of such therapeutic strategies form a fundamental part of the drug development process and both *in vitro* and *in vivo* models are used for this purpose; each with their own strengths and limitations. *In vitro* models applied can range from easily maintained conventional 2D flat cultures (having poor translatability towards *in vivo* tissues) to more intricate 3D culture systems that closely imitate the true *in vivo* microenvironment. *In vivo* animal models, predominantly used for efflux-based MDR treatment screening, involve the grafting of either cell-derived or patient-derived tumours into immunodeficient rodents to generate xenografts. Although predictive patient drug responses can be obtained from xenografting, the model development is extremely time-consuming and the absence of a fully functioning immune system in the murine host restricts studies on cancer immune responses. By genetically manipulating cancer-associated genes in mouse models, accurate representations of human tumour properties can be achieved while using models with intact immune systems.

Given that cancer research to overcoming efflux transporter-driven MDR is a multifaceted approach, no particular model should be used solitary. In fact, the utilisation of a complete “package” of preclinical screening models would provide a better understanding and refinement of which therapeutic strategies to explore in further effective clinical trials.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest, financial or otherwise. This work was carried out with the financial support of the National Research Foundation (NRF) of South Africa and the South African Medical Research Council (MRC). Any opinions, findings and

conclusions or recommendations expressed in this material are those of the authors only, and are not necessarily to be attributed to the NRF or the MRC.

ACKNOWLEDGEMENTS

All the authors contributed substantially to this paper.

Table 1. *In vitro* & *In vivo* efflux-based models used for anticancer drug screening

Model	Drug target	Transporter studied	Outcome	Reference
2D flat cultures				
MCF-7 (MCF-7/ADR) cells (Doxorubicin-resistant human breast cancer cells)	<ul style="list-style-type: none"> • Resveratrol • Doxorubicin 	MDR1 (P-gp) MRP1 / MRP2 BCRP	<ul style="list-style-type: none"> • Doxorubicin-induced cell death is potentiated by resveratrol. • Enhanced intracellular doxorubicin accumulation is facilitated by resveratrol. • MDR1 and MRP1 mRNA-expression is down regulated. • Resveratrol acts as a P-gp inhibitor. • No effect was witnessed on MRP2 and BCRP. 	[208]
LoVo and LoVoDX cells (colon cancer cells resistant to doxorubicin)	<ul style="list-style-type: none"> • Melatonin • Doxorubicin 	P-gp	<ul style="list-style-type: none"> • In some concentrations melatonin intensified the cytotoxicity effect of doxorubicin in the LoVoDX cells. 	[209]
H460/MX20; HEK293/pcDNA3.1; HEK/R482T cells (Large cell lung cancer cells; human embryonic kidney cells)	<ul style="list-style-type: none"> • CCTA-1523 (ABCG2 inhibitor) • Mitoxantrone • Doxorubicin • SN-38 • Cisplatin 	ABCG2	<ul style="list-style-type: none"> • CCTA-1523 is a potent, selective & reversible modulator of ABCG2. • CCTA-1523 enhances the cytotoxicity of mitoxantrone & SN-38. 	[210]
SW620/Ad300 cells (human colon cancer cells)	<ul style="list-style-type: none"> • Regorafenib (multikinase inhibitor) • Paclitaxel • Doxorubicin • Vincristine • Cisplatin 	ABCB1	<ul style="list-style-type: none"> • Regorafenib reversed the ABCB1-mediated MDR & increased the accumulation of [3H]-paclitaxel in ABCB1-overexpressing cells, by suppressing efflux activity of ABCB1, but not altering expression level & localisation of ABCB1. • Regorafenib & paclitaxel synergistically shrink resistant colorectal tumours. • Regorafenib inhibits the efflux activity of ABCB1 transporter. 	[211]

K562 and K562/ADM cells (human chronic myeloid leukaemia (CML) cells)	<ul style="list-style-type: none"> • Imatinib • Etoposide 	MRP1 MDR1 / P-gp	<ul style="list-style-type: none"> • Use of imatinib may be preferable over the use of etoposide in the treatment of CML. 	[212]
BxPC3, Cfpac-1, and HPAC cells (human pancreatic cancer cells)	<ul style="list-style-type: none"> • CG200745 (HDAC inhibitor) • Erlotinib • Gemcitabine 	MRP3 MRP4	<ul style="list-style-type: none"> • A synergistic inhibitory & apoptotic effect with CG200745 was witnessed when combined with gemcitabine/erlotinib in pancreatic cancer cells. • Decreased expression of MRP mRNA. 	[213]
KB _{v200} (drug resistant) and HEK293/ABCB1 stable transfected cells (human embryonic kidney cells)	<ul style="list-style-type: none"> • Pristimerin 	P-gp / ABCB1 / MDR1	<ul style="list-style-type: none"> • Pristimerin inhibited cell proliferation & induced apoptosis in both cell lines. • Decreased P-gp activity in a dose-dependent manner was independent of mRNA levels but primarily owing to its protein stability. • Disturbed the subcellular distribution of P-gp with decreased location in the plasma membrane. 	[214]
Micelles developed				
MCF-7 / ADR cells (human drug resistant breast cancer cells)	<ul style="list-style-type: none"> • Doxorubicin • Hyaluronic acid-g-poly(L-histidine) (HA-PHis) and d-α-tocopheryl polyethylene glycol 2000 (TPGS2k) copolymers 	P-gp	<ul style="list-style-type: none"> • Incorporation of doxorubicin into a nanoparticle limited distribution (thus reducing cardio toxicity). • The doxorubicin-loaded mixed micelles (HPHM/TPGS2k) caused increased MDR sensitisation due to reversal of transporter efflux. • Blank HPHM/TPGS2k inhibited P-gp activity by reducing mitochondrial membrane potential & depletion of ATP but without inhibition of P-gp expression. 	[215]
MCF-7 and MCF-7/ADR cells (human breast cancer cells and their multidrug resistant phenotype)	<ul style="list-style-type: none"> • Paclitaxel • d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) and the mPEG-SS-PTX conjugate 	P-gp	<ul style="list-style-type: none"> • TPGS is a P-gp inhibitor that can block the cancer cell action of pumping drugs out of cells. • Mixed micelles can effectively improve the accumulation of paclitaxel in multidrug resistant MCF-7 cells. 	[216]

SKOV-3TR and A2780-Adr cells (Ovarian carcinoma cells)	<ul style="list-style-type: none"> • Paclitaxel • Tariquidar (P-gp inhibitor) • Transferrin (Tf)-modified polyethylene glycol-phosphatidyl ethanolamine-based micellar delivery system 	P-gp	<ul style="list-style-type: none"> • Tariquidar increased the intracellular paclitaxel levels & its cytotoxicity. • Tf-targeting of these micellar nanoformulations can further enhance their efficacy by targeting deeper layers of tumours. 	[217]
Nanoparticles developed				
MCF-7/T cells (Taxol-resistant breast cancer cells)	<ul style="list-style-type: none"> • Selenium / Ruthenium (nanoparticles) 	P-gp	<ul style="list-style-type: none"> • The delivery of siRNA by means of Se/Ru- Metal-Organic Framework nanoparticles, decreased MDR genes in MCF-7/T. 	[218]
HCT 116 cells (human colon cancer cells)	<ul style="list-style-type: none"> • Paclitaxel combined with rod-shaped gadolinium arsenite nanoparticles 	P-gp	<ul style="list-style-type: none"> • The hybrid paclitaxel-loaded gadolinium arsenite nanoparticle is used to decrease MDR of paclitaxel. 	[219]
MCF-7 and MCF-7/ADR cells (Doxorubicin resistant breast cancer cells)	<ul style="list-style-type: none"> • Doxorubicin in combination with siMDR1 	P-gp	<ul style="list-style-type: none"> • The liposome decreased doxorubicin resistance. 	[220]
T24 and TCC-SUP cells (Bladder cancer cells & acquired resistant cells towards gemcitabine and vinblastine)	<ul style="list-style-type: none"> • Nanoparticle albumin-bound (nab) paclitaxel 	ABCB1 (P-gp)	<ul style="list-style-type: none"> • Hyperexpressive ABCB1 cell lines showed a comparable resistance pattern towards both paclitaxel & nab paclitaxel. 	[221]
MCF-7/ADR cells (Doxorubicin resistant breast cancer cells)	<ul style="list-style-type: none"> • Doxorubicin combined with microRNAs 	P-gp	<ul style="list-style-type: none"> • The higher miR-129-5p expression led to P-gp inhibition & decreased cellular efflux of doxorubicin. 	[55]

Advanced complex cell-cultures

<p>HEK cells cultured as scaffold-free, 3D spheroids formed in nonadhesive micro-molds</p> <p>(human embryonic kidney cells)</p>	<ul style="list-style-type: none"> • Ko143 (inhibitor) • Gefitinib • Elacridar 	<p>ABCG2</p>	<ul style="list-style-type: none"> • This model gave a better idea of pharmacokinetic characteristics of transporter inhibitors. • Drug uptake and penetration was modelled more effectively. • Overall effectiveness of inhibitors was lower than in 2D models due to diffusion barriers in spheroids. • Elacridar showed effectiveness 5 hours after removal, thus showing differentiation abilities of the model between static and dynamic inhibitors. 	<p>[222]</p>
<p>NHLF, SAEC and HMVEC-L co-cultured cells dispensed on poly-HEMA-coated plates and centrifuged to form 3D aggregates.</p>	<ul style="list-style-type: none"> • Recombinant human Wnt5a • Cisplatin • LiCl (chemical activator of Wnt5a) • IWR-1 (pathway inhibitor) 	<p>ABCB1 ABCG2</p>	<ul style="list-style-type: none"> • Transporter expression was induced in primary healthy lung tissue 3D aggregates, by adding precursors responsible for transporter expression. • Chemical manipulation of Wnt5a pathway induced or reduced transporter expression. 	<p>[223]</p>
<p>MCF-7 and DOX resistant MCF-7/ADR cells entrapped in alginate-oligochitosan microcapsules to form 3D aggregates.</p>	<ul style="list-style-type: none"> • Doxorubicin derivatives (Palm-N2H-DOX, N-Palm-DOX, DOX-5FU, DOX-TPP and DOX-AMG) 	<p>BCRP P-gp</p>	<ul style="list-style-type: none"> • Determining the most effective derivative (Palm-N2H-DOX) in the MDR 3D cancer model. • This model had increased resistance towards drugs in comparison to flat culture models. 	<p>[224]</p>
<p>Adriamycin-resistant cells, MCF-7R, and parental control cell line, MCF 7, seeded in silk-collagen scaffolds to form 3D aggregate models.</p>	<ul style="list-style-type: none"> • Doxorubicin • Carboplatin • Paclitaxel 	<p>P-gp MRP2</p>	<ul style="list-style-type: none"> • 3D aggregates had distinct cell proliferation, cellular aggregate formation and expression of drug resistance-related genes/proteins compared to that of 2D models. • The distinctive characteristics made the model more comparable to the <i>in vivo</i> environment. • Drug resistance of the 3D model increased due to alterations in the cell cycle distribution and increased presence of breast cancer stem cells. 	<p>[225]</p>

HER2-positive breast cancer cell lines cultured as 3D models using the poly-HEMA method.	<ul style="list-style-type: none"> • Neratinib (HER2 targeted therapy) • Docetaxel (chemotherapy) 	HER2	<ul style="list-style-type: none"> • This 3D model indicated a higher innate resistance due to alterations in cell survival precursors such as the receptor proteins, drug transporters and metabolic enzymes when compared to the 2D cells. • An overall increased expression of drug targets, cell survival proteins and drug transporters was also reported when compared to the 2D counterpart. • An overall increased cellular viability was reported when 3D cells were dosed with toxic concentrations in 2D. 	[226]
Cell-derived xenografts				
Subcutaneous implantations				
ABCG2-HEK293 cells (human embryonic kidney cells)	<ul style="list-style-type: none"> • MBLI-87 (ABCG2 inhibitor) • Irinotecan 	ABCG2 / BCRP	<ul style="list-style-type: none"> • Significant sensitisation to irinotecan. • Increased irinotecan's effect. • MBLI-87 prevents drug efflux by ABCG2 inhibition. 	[170]
ABCB1-KB-C2; H460/MX-20 & ABCC10-HEK/MRP7 cells (human non-small cell lung cancer cells, human embryonic kidney cells)	<ul style="list-style-type: none"> • Nilotinib 	ABCB1; ABCG2; ABCC10	<ul style="list-style-type: none"> • Nilotinib potentiates the anticancer effect of paclitaxel & doxorubicin in MDR xenograft models. 	[227]
SKOV-3 cells (human ovarian cancer cell line)	<ul style="list-style-type: none"> • NSC23925 (P-gp inhibitor) • Paclitaxel 	P-gp / MDR1	<ul style="list-style-type: none"> • Combination of paclitaxel & NSC23925 showed inhibition of tumour growth. • NSC23925 prevented development of paclitaxel resistance <i>in vivo</i>. 	[164]
MCF-7 / ADR cells (human breast cancer cells resistant to adriamycin)	<ul style="list-style-type: none"> • Psi-Pgp-tGC nanoparticles • Doxorubicin 	P-gp	<ul style="list-style-type: none"> • Psi-Pgp-tGC nanoparticles down-regulated P-gp expression. • Nanoparticles potentiated doxorubicin-mediated inhibition of tumour growth & showed lower tumour volume. 	[228]

KB _{V200} cells (human keratin-forming HeLa-derivative, ABCB1-overexpressing cells)	<ul style="list-style-type: none"> • Trametinib • Vincristine • Doxorubicin 	ABCB1	<ul style="list-style-type: none"> • Trametinib in combination with vincristine inhibited the tumour growth of ABCB1-overexpressing xenografts. • Trametinib used in combination therapy has the potential to combat efflux pump related MDR. 	[229]
MDA-MB-231 and BT-474 cells transfected with shRNA targeting ABCC1 or ABCC3 (human breast cancer cells)	<ul style="list-style-type: none"> • Doxorubicin • Mitoxantrone • 5-fluorouracil 	ABCC1 & ABCC3	<ul style="list-style-type: none"> • Like ABCC1, ABCC3 is also overexpressed in primary breast cancers. • ABCC3 was responsible for drug resistance, whereas ABCC3 knockdown reversed resistance. 	[230]
C4-2B, TaxR and DU145-DTXR cells (Docetaxel resistant human prostate cancer cells)	<ul style="list-style-type: none"> • Bicalutamide • Enzalutamide • Docetaxel 	ABCB1	<ul style="list-style-type: none"> • Combination of bicalutamide & docetaxel overcomes resistance. • Bicalutamide and enzalutamide inhibit ABCB1 transporter activity. 	[231]
HCT-15 cells (human colorectal adenocarcinoma)	<ul style="list-style-type: none"> • Fluoxetine (Prozac) • Doxorubicin • Bevacizumab (avastin) 	P-gp; MRP & BCRP – tested, but not detected	<ul style="list-style-type: none"> • Combination of doxorubicin & fluoxetine showed significant slow-down of tumour progression, comparable to aggressive treatment with bevacizumab. • Fluoxetine improved doxorubicin intracellular accumulation & uptake. • Ability of fluoxetine to modulate resistance <i>in vivo</i>. 	[169]
JFCR013-2 cells (human non-small cell lung cancer cells resistant to ceritinib)	<ul style="list-style-type: none"> • Ceritinib • Crizotinib • Alectinib 	P-gp	<ul style="list-style-type: none"> • P-gp overexpression is the major cause of ceritinib & crizotinib resistance in NSCLC cells. • Ceritinib & crizotinib are P-gp substrates. 	[166]
T24 cells (human bladder cancer cells)	<ul style="list-style-type: none"> • Emodin • Cisplatin 	MRP1	<ul style="list-style-type: none"> • By downregulating the MRP1 expression, emodin improves the chemosensitivity to cisplatin. 	[167]
H460 cells (human large cell lung carcinoma cells)	<ul style="list-style-type: none"> • Sulindac • Doxorubicin 	MRP1	<ul style="list-style-type: none"> • Sulindac reduced doxorubicin resistance by inhibiting MRP1 activity. 	[232]

JC cells (mouse primary mammary gland adenocarcinoma cells)	<ul style="list-style-type: none"> • Paclitaxel • Poly(D,L-lactide-co-glycolide) nanoparticles 	P-gp	<ul style="list-style-type: none"> • Nanoparticles, containing both paclitaxel and P-gp targeted siRNA, showed effective MDR1 gene silencing & the improved accumulation of paclitaxel in drug-resistant cells. 	[172]
Orthotopic implantations				
MCF-7/DOX cells (human breast cancer cells)	<ul style="list-style-type: none"> • Nelfinavir (inhibitor) • Doxorubicin 	P-gp	<ul style="list-style-type: none"> • Co-exposure to the anti-HIV-drug nelfinavir enhanced the antitumor efficacy of doxorubicin. 	[168]
MDA-MB-231 cells (human breast cancer cells)	<ul style="list-style-type: none"> • Curcumin • Paclitaxel • Cisplatin • Doxorubicin 	ABCG2 ABCC1	<ul style="list-style-type: none"> • Curcumin downregulated ABCG2 and ABCC1 expression. • Curcumin enhanced the sensitivity of the cancer to paclitaxel, cisplatin, doxorubicin & mitomycin C. 	[233]
Patient-derived xenografts				
Subcutaneous implantations				
Small cell lung cancer	<ul style="list-style-type: none"> • Cyclophosphamide • Cisplatin • Doxorubicin • Etoposide • Verapamil (inhibitor) 	P-gp / MDR1	<ul style="list-style-type: none"> • The co-administration of verapamil enhanced the antitumor efficacy of CyCav treatment regimen. 	[186]
Human colorectal tumours	<ul style="list-style-type: none"> • PF-309 (inhibitor) 	P-gp / ABCB1 gene	<ul style="list-style-type: none"> • PF-309 efficacy is affected by the expression of P-gp in tumours. 	[234]
Merkel cell carcinoma (MCC)	<ul style="list-style-type: none"> • Carboplatin • Etoposide 	ABCB5	<ul style="list-style-type: none"> • MCC cancers showing carboplatin and etoposide resistance, had high ABCB5 expression levels. • Tumour growth was reduced by the reversal of drug resistance, through the administration of ABCB5 blockade. 	[187]

Non-small cell lung cancer	<ul style="list-style-type: none"> • Etoposide • Carboplatin • Gemcitabine • Paclitaxel • Erlotinib 	BCRP MDR1 MRP1	<ul style="list-style-type: none"> • A low correlation between BCRP expression & PDX response to etoposide was found. • No other efflux transporter expression levels were correlated to any drug responses <i>in vivo</i>. 	[235]
Retinoblastoma (eye cancer)	<ul style="list-style-type: none"> • Topotecan • Melphalan 	ABCB1 ABCC1 ABCG2	<ul style="list-style-type: none"> • The single exposure to melphalan or topotecan did not influence the expression levels of the efflux transporters in the PDXs. 	[236]

Genetically engineered mouse models

Knock-in mice

<i>mdr1a.fLUC</i> knock-in mice	<ul style="list-style-type: none"> • Paclitaxel • Docetaxel • Pregnenolone-16α-carbonitrile (PCN) 	MDR1	<ul style="list-style-type: none"> • By inserting a firefly luciferase (<i>fLUC</i>) gene into the <i>mdr1a</i> locus of the murine host, luminescence intensities could be accurately detected. • <i>mdr1a.fLUC</i> expression was shown to change with paclitaxel, docetaxel or PCN treatments. 	[199]
---------------------------------	--	------	---	-------

Knockout mice

<i>Mdr1a/1b</i> ^{-/-} knockout mice	double	<ul style="list-style-type: none"> • BMS-275,183 (analogue of paclitaxel) • Pantoprazole (proton pump inhibitor) 	P-gp / ABCB1 MRP2 / ABCC2 BCRP / ABCG2	<ul style="list-style-type: none"> • By genetically deleting P-gp, it was shown that P-gp plays a role in the pharmacokinetics & brain distribution of BMS-275,183. 	[198]
<i>Mrp4</i> ^{-/-} knockout mice		<ul style="list-style-type: none"> • Topotecan 	MRP4	<ul style="list-style-type: none"> • The distribution of the anticancer drug, topotecan, is restricted by the <i>Mrp4</i> transporter. • The overexpression of <i>Mrp4</i> causes topotecan resistance. 	[197]
<i>Mdr1a/b/Mrp2</i> ^{-/-} knockout mice	triple	<ul style="list-style-type: none"> • Irinotecan • Methotrexate • Doxorubicin 	P-gp / MDR1 MRP2	<ul style="list-style-type: none"> • Methotrexate is a good substrate for <i>Mrp2</i>, whereas irinotecan is poorly effluxed. 	[237]

Doxorubicin-sensitive & doxorubicin-resistant <i>Brca1^{-/-};p53^{-/-}</i> tumours in <i>K14cre;Brca1^{F/F};p53^{F/F}</i> mice	<ul style="list-style-type: none"> • Doxorubicin • Tariquidar 	MDR1	<ul style="list-style-type: none"> • Doxorubicin resistance is caused by increased expression of the <i>Mdr1</i> gene encoding P-gp. • The third-generation P-gp inhibitor, tariquidar, can reverse this resistance. 	[238]
<i>Brca1^{-/-};p53^{-/-}</i> tumours in <i>K14cre;Brca1^{F/F};p53^{F/F}</i> mice	<ul style="list-style-type: none"> • Doxorubicin • AZD2281 (inhibitor) • Tariquidar (inhibitor) 	P-gp / <i>Abcb1a</i> and <i>Abcb1b</i>	<ul style="list-style-type: none"> • The up-regulation of P-gp induced acquired resistance to AZD2281. • The addition of the P-gp inhibitor, tariquidar, reversed this resistance. 	[195]
<i>Abcg2</i> -deleted alleles bred with <i>K14cre;Brca1^{F/F};p53^{F/F}</i> mice	<ul style="list-style-type: none"> • Topotecan 	BCRP / ABCG2	<ul style="list-style-type: none"> • ABCG2 contributes to topotecan resistance <i>in vivo</i>. 	[196]
<i>Slco1a/1b^{-/-};1A2^{tg}</i> ; <i>Slco1a/1b^{-/-};1B1^{tg}</i> ; <i>Slco1a/1b^{-/-};1B3^{tg}</i> mice	<ul style="list-style-type: none"> • Paclitaxel • Methotrexate 	OATP1B1 OATP1B3 OATP1A2	<ul style="list-style-type: none"> • Methotrexate is a substrate of all three human OATP1A/B transporters. • Paclitaxel is transported by OATP1B3 and OATP1A2, but not OATP1B1. 	[239]

REFERENCES

- [1] Krishna, R.; Mayer, L.D. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *Eur. J. Pharm. Sci.*, **2000**, *11*, 265-283.
- [2] Wang, J.; Seebacher, N.; Shi, H.; Kan, Q.; Zhenfeng, D. Novel strategies to prevent the development of multidrug resistance (MDR) in cancer. *Oncotarget*, **2017**, *8*(48), 84559-84571.
- [3] Saraswathy, M.; Gong, S. Different strategies to overcome multidrug resistance in cancer. *Biotechnol. Adv.*, **2013**, *31*(8), 1397-1407.
- [4] Tarling, E.J.; de Aguiar Vallim, T.Q.; Edwards, P.A. Role of ABC transporters in lipid transport and human disease. *Trends Endocrinol. Metab.*, **2013**, *24*(7), 342-350.
- [5] Luker, G.D.; Flagg, T.P.; Sha, Q.; Luker, K.E.; Pica, C.M.; Nichols, C.G.; Piwnica-Worms, D. MDR1 P-glycoprotein reduces influx of substrates without affecting membrane potential. *J. Biol. Chem.*, **2001**, *276*(52), 49053-49060.
- [6] Goldstein, L.J.; Galski, H.; Fojo, A.; Willingham, M.; Lai, S.; Gazdar, A.; Pirker, R.; Green, A.; Crist, W.; Brodeur, G.M.; Lieber, M.; Cossman, J.; Gottesman, M.M.; Pastan, I. Expression of a multidrug resistance gene in human tumors. *J. Natl. Canc. Inst.*, **1989**, *81*, 116-124.
- [7] Qian, X.; Cheng, Y.H.; Mruk, D.D.; Cheng, C.Y. Breast cancer resistance protein (Bcrp) and the testis-an unexpected turn of events. *Asian. J. Androl.*, **2013**, *15*(4), 455-460.
- [8] Flens, M.J.; Zaman, G.J.R.; Van der Valk, P.; Izquierdo, M.A.; Schroeijers, A.B.; Scheffer, G.L.; Van der Groep, P.; De Haas, M.; Meijer, C.J.L.M.; Scheper, R.J. Tissue distribution of the multidrug resistance protein. *Am. J. Pathol.*, **1996**, *148*(4), 1237-1247.
- [9] Szakacs, G.; Varadi, A.; Ozvegy-Laczka, C.; Sarkadi, B. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). *Drug Discov. Today*, **2008**, *13*(9-10), 379-393.
- [10] Sodani, K.; Patel, A.; Kathawala, R.J.; Chen, Z.-S. Multidrug resistance associated proteins in multidrug resistance. *Chin. J. Canc.*, **2012**, *31*(2), 58-73.
- [11] Brooks, T.A.; Kennedy, D.R.; Gruol, D.J.; Ojima, I.; Baer, M.R.; Bernacki, R.J. Structure-activity analysis of taxane-based broad-spectrum multidrug resistance modulators. *Anticancer Res.*, **2004**, *24*, 409-416.
- [12] Colabufo, N.A.; Berardi, F.; Perrone, R.; Rapposelli, S.; Digiaco, M.; Vanni, M.; Balsamo, A. Synthesis and biological evaluation of (hetero)arylmethoxy- and arylmethylamine-phenyl derivatives as potent P-glycoprotein modulating agents. *J. Med. Chem.*, **2008**, *51*, 1415-1422.
- [13] Bartholomae, S.; Gruhn, B.; Debatin, K.M.; Zimmermann, M.; Creutzig, U.; Reinhardt, D.; Steinbach, D. Coexpression of Multiple ABC-Transporters is Strongly Associated with Treatment Response in Childhood Acute Myeloid Leukemia. *Pediatr. Blood Cancer*, **2016**, *63*(2), 242-247.
- [14] Aberuyi, N.; Rahgozar, S.; Khosravi Dehaghi, Z.; Moafi, A.; Masotti, A.; Paolini, A. The translational expression of ABCA2 and ABCA3 is a strong prognostic biomarker for multidrug resistance in pediatric acute lymphoblastic leukemia. *OncoTargets Ther.*, **2017**, *10*, 3373-3380.
- [15] Efferth, T.; Gillet, J.P.; Sauerbrey, A.; Zintl, F.; Bertholet, V.; de Longueville, F.; Remacle, J.; Steinbach, D. Expression profiling of ATP-binding cassette transporters in childhood T-cell acute lymphoblastic leukemia. *Mol. Cancer Ther.*, **2006**, *5*(8), 1986-1994.
- [16] Yura, Y. Presage of oncolytic virotherapy for oral cancer with herpes simplex virus. *Jpn. Dent. Sci. Rev.*, **2017**, *53*(2), 53-60.
- [17] Pluchino, K.M.; Hall, M.D.; Goldsborough, A.S.; Callaghan, R.; Gottesman, M.M. Collateral sensitivity as a strategy against cancer multidrug resistance. *Drug Resist. Updat.*, **2012**, *15*(1-2), 98-105.
- [18] Ozben, T. Mechanisms and strategies to overcome multiple drug resistance in cancer. *FEBS Lett.*, **2006**, *580*(12), 2903-2909.
- [19] Srivalli, K.M.R.; Lakshmi, P.K. Overview of P-glycoprotein inhibitors: a rational outlook. *Braz. J. Pharm. Sci.*, **2012**, *48*(3), 353-367.
- [20] Gottesman, M.M.; Pastan, I.H. The Role of Multidrug Resistance Efflux Pumps in Cancer: Revisiting a JNCI Publication Exploring Expression of the MDR1 (P-glycoprotein) Gene. *J. Natl. Cancer Inst.*, **2015**, *107*(9).
- [21] Callaghan, R.; Luk, F.; Bebawy, M. Inhibition of the multidrug resistance P-glycoprotein: time for a change of strategy? *Drug. Metab. Dispos.*, **2014**, *42*(4), 623-631.
- [22] Hida, K.; Kikuchi, H.; Maishi, N.; Hida, Y. ATP-binding cassette transporters in tumor endothelial cells and resistance to metronomic chemotherapy. *Cancer Lett.*, **2017**, *400*, 305-310.
- [23] Chen, Y.L.; Chang, M.C.; Cheng, W.F. Metronomic chemotherapy and immunotherapy in cancer treatment. *Cancer Lett.*, **2017**, *400*, 282-292.

- [24] Bebawy, M.; Combes, V.; Lee, E.; Jaiswal, R.; Gong, J.; Bonhoure, A.; Grau, G.E. Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells. *Leukemia*, **2009**, 23(9), 1643-1649.
- [25] Vysotski, S.; Winzelberg, R.; Babayeva, M. Overcoming cancer multidrug resistance through inhibition of microparticles. *GSTF J. Adv. Med.Res.*, **2016**, 1(4).
- [26] Bressy, C.; Hastie, E.; Grdzlishvili, V.Z. Combining oncolytic virotherapy with p53 tumor suppressor gene therapy. *Mol. Ther. Oncolytics*, **2017**, 5, 20-40.
- [27] Du, G.; Fang, Q.; den Toonder, J.M. Microfluidics for cell-based high throughput screening platforms - A review. *Anal. Chim. Acta*, **2016**, 903, 36-50.
- [28] Shaheen, S.; Ahmed, M.; Lorenzi, F.; Nateri, A.S. Spheroid-Formation (Colonsphere) Assay for *in Vitro* Assessment and Expansion of Stem Cells in Colon Cancer. *Stem Cell Rev.*, **2016**, 12(4), 492-499.
- [29] Vandenhoute, E.; Drolez, A.; Sevin, E.; Gosselet, F.; Mysiorek, C.; Dehouck, M.P. Adapting coculture *in vitro* models of the blood-brain barrier for use in cancer research: maintaining an appropriate endothelial monolayer for the assessment of transendothelial migration. *Lab. Invest.*, **2016**, 96(5), 588-598.
- [30] Barretina, J.; Caponigro, G.; Stransky, N.; Venkatesan, K.; Margolin, A.A.; Kim, S.; Wilson, C.J.; Lehar, J.; Kryukov, G.V.; Sonkin, D.; Reddy, A.; Liu, M.; Murray, L.; Berger, M.F.; Monahan, J.E.; Morais, P.; Meltzer, J.; Korejwa, A.; Jane-Valbuena, J.; Mapa, F.A.; Thibault, J.; Bric-Furlong, E.; Raman, P.; Shipway, A.; Engels, I.H.; Cheng, J.; Yu, G.K.; Yu, J.; Aspesi, P., Jr.; de Silva, M.; Jagtap, K.; Jones, M.D.; Wang, L.; Hatton, C.; Palesscandolo, E.; Gupta, S.; Mahan, S.; Sougnez, C.; Onofrio, R.C.; Liefeld, T.; MacConaill, L.; Winckler, W.; Reich, M.; Li, N.; Mesirov, J.P.; Gabriel, S.B.; Getz, G.; Ardlie, K.; Chan, V.; Myer, V.E.; Weber, B.L.; Porter, J.; Warmuth, M.; Finan, P.; Harris, J.L.; Meyerson, M.; Golub, T.R.; Morrissey, M.P.; Sellers, W.R.; Schlegel, R.; Garraway, L.A. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*, **2012**, 483(7391), 603-607.
- [31] Clarke, C.J.; Holyoake, T.L. Preclinical approaches in chronic myeloid leukemia: from cells to systems. *Exp. Hematol.*, **2017**, 47, 13-23.
- [32] Wilding, J.L.; McGowan, S.; Liu, Y.; Bodmer, W.F. Replication error deficient and proficient colorectal cancer gene expression differences caused by 3'UTR polyT sequence deletions. *PNAS*, **2010**, 107(49), 21058-21063.
- [33] Ashraf, S.Q.; Nicholls, A.M.; Wilding, J.L.; Ntouroupi, T.G.; Mortensen, N.J.; Bodmer, W.F. Direct and immune mediated antibody targeting of *ERBB* receptors in a colorectal cancer cell-line panel. *PNAS*, **2012**, 109(51), 21046-21051.
- [34] Hickman, J.A.; Graeser, R.; De Hoogt, R.; Vidic, S.; Brito, C.; Gutekunst, M.; Van der Kuip, H. Three-dimensional models of cancer for pharmacology and cancer cell biology: Capturing tumor complexity *in vitro/ex vivo*. *Biotechnol. J.*, **2014**, 9(9), 1115-1128.
- [35] Juliano, R.L.; Ling, V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta*, **1976**, 455(1), 152-162.
- [36] Biedler, J.L.; Riehm, H. Cellular resistance to actinomycin D in Chinese hamster cells *in vitro*: cross-resistance, radioautographic, and cytogenetic studies. *Cancer Res.*, **1970**, 30, 1174-1184.
- [37] Shoemaker, R.H. The NCI60 human tumour cell line anticancer drug screen. *Nat. Rev. Cancer*, **2006**, 6(10), 813-823.
- [38] Zampella, J.G.; Rodic, N.; Yang, W.R.; Huang, C.R.; Welch, J.; Gnanakkan, V.P.; Cornish, T.C.; Boeke, J.D.; Burns, K.H. A map of mobile DNA insertions in the NCI-60 human cancer cell panel. *Mob. DNA*, **2016**, 7, 20.
- [39] Kim, M.S.; Haney, M.J.; Zhao, Y.; Mahajan, V.; Deygen, I.; Klyachko, N.L.; Inskoe, E.; Piroyan, A.; Sokolsky, M.; Okolie, O.; Hingtgen, S.D.; Kabanov, A.V.; Batrakova, E.V. Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine*, **2016**, 12(3), 655-664.
- [40] Wong, H.L.; Rauth, A.M.; Bendayan, R.; Manias, J.L.; Ramaswamy, M.; Liu, Z.; Erhan, S.Z.; Wu, X.Y. A new polymer-lipid hybrid nanoparticle system increases cytotoxicity of doxorubicin against multidrug-resistant human breast cancer cells. *Pharm. Res.*, **2006**, 23(7), 1574-1585.
- [41] Corcoran, C.; Rani, S.; O'Brien, K.; O'Neill, A.; Prencipe, M.; Sheikh, R.; Webb, G.; McDermott, R.; Watson, W.; Crown, J.; O'Driscoll, L. Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes. *PLoS One*, **2012**, 7(12), e50999.
- [42] Hamilton, G. Cytotoxic effects of fascaplysin against small cell lung cancer cell lines. *Mar. Drugs*, **2014**, 12(3), 1377-1389.
- [43] Xue, C.; Wang, C.; Liu, Q.; Meng, Q.; Sun, H.; Huo, X.; Ma, X.; Liu, Z.; Ma, X.; Peng, J.; Liu, K. Targeting P-glycoprotein expression and cancer cell energy metabolism: combination of metformin and 2-deoxyglucose reverses the multidrug resistance of K562/Dox cells to doxorubicin. *Tumour Biol.*, **2016**, 37(7), 8587-8597.

- [44] Whitt, J.D.; Keeton, A.B.; Gary, B.D.; Sklar, L.A.; Sodani, K.; Chen, Z.-S.; Piazza, G.A. Sulindac sulfide selectively increases sensitivity of ABCC1 expressing tumor cells to doxorubicin and glutathione depletion. *J. Biomed. Res.*, **2016**, *30*(2), 120-133.
- [45] Ghavami, G.; R Kazemali, M.; Sardari, S. Informatics of drug synergism in naturally occurring anticancer agents. *Recent Pat. Anti-Cancer Drug Discov.*, **2011**, *6*(1), 26-44.
- [46] Pedersen, J.M.; Khan, E.K.; Bergström, C.A.S.; Palm, J.; Hoogstraate, J.; Artursson, P. Substrate and method dependent inhibition of three ABC-transporters (MDR1, BCRP, and MRP2). *Eur. J. Pharmaceut. Sci.*, **2017**, *103*, 70-76.
- [47] Teng, Y.-N.; Chang, C.-S.; Lee, T.-E.; Hung, C.-C. Cordycepin re-sensitizes multidrug resistance cancer cells to chemotherapeutic agents through modulating P-glycoprotein expression and ATPase function. *J. Funct. Foods*, **2016**, *26*, 681-690.
- [48] Kim, T.H.; Shin, Y.J.; Won, A.J.; Lee, B.M.; Choi, W.S.; Jung, J.H.; Chung, H.Y.; Kim, H.S. Resveratrol enhances chemosensitivity of doxorubicin in multidrug-resistant human breast cancer cells via increased cellular influx of doxorubicin. *Biochim. Biophys. Acta*, **2014**, *1840*(1), 615-625.
- [49] Zhao, D.; Zhang, H.; Yang, S.; He, W.; Luan, Y. Redox-sensitive mPEG-SS-PTX/TPGS mixed micelles: An efficient drug delivery system for overcoming multidrug resistance. *Int. J. Pharm.*, **2016**, *515*(1-2), 281-292.
- [50] Vickers, P.J.; Dickson, R.B.; Shoemaker, R.H.; Cowan, K.H. A multidrug-resistant MCF-7 human breast cancer cell line which exhibits cross-resistance to antiestrogens and hormone-independent tumor growth *in vivo*. *Mol. Endocrin.*, **1988**, *2*(10), 886-892.
- [51] Ke, W.; Yu, P.; Wang, J.; Wang, R.; Guo, C.; Zhou, L.; Li, C.; Li, K. MCF-7/ADR cells (re-designated NCI/ADR-RES) are not derived from MCF-7 breast cancer cells: a loss for breast cancer multidrug-resistant research. *Med. Oncol.*, **2011**, *28 Suppl 1*, S135-S141.
- [52] Wu, T.; Chen, Z.S.; To, K.K.W.; Fang, X.; Wang, F.; Cheng, B.; Fu, L. Effect of abemaciclib (LY2835219) on enhancement of chemotherapeutic agents in ABCB1 and ABCG2 overexpressing cells *in vitro* and *in vivo*. *Biochem. Pharmacol.*, **2017**, *124*, 29-42.
- [53] Yhee, J.Y.; Song, S.; Lee, S.J.; Park, S.-G.; Kim, K.-S.; Kim, M.G.; Son, S.; Koo, H.; Kwon, I.C.; Jeong, J.H.; Jeong, S.Y.; Kim, H.K.; Kim, K. Cancer-targeted MDR-1 siRNA delivery using self-cross-linked glycol chitosan nanoparticles to overcome drug resistance. *J. Control. Release*, **2015**, *198*, 1-9.
- [54] Chen, S.; Jiao, J.-W.; Sun, K.-X.; Zong, Z.-H.; Zhao, Y. MicroRNA-133b targets glutathione S-transferase π expression to increase ovarian cancer cell sensitivity to chemotherapy drugs. *Drug Des. Dev. Ther.*, **2015**, *9*, 5225-5235.
- [55] Yi, H.; Liu, L.; Sheng, N.; Li, P.; Pan, H.; Cai, L.; Ma, Y. Synergistic Therapy of Doxorubicin and miR-129-5p with Self-Cross-Linked Bioreducible Polypeptide Nanoparticles Reverses Multidrug Resistance in Cancer Cells. *Biomacromolecules*, **2016**, *17*(5), 1737-1747.
- [56] Yang, S.D.; Zhu, W.J.; Zhu, Q.L.; Chen, W.L.; Ren, Z.X.; Li, F.; Yuan, Z.Q.; Li, J.Z.; Liu, Y.; Zhou, X.F.; Liu, C.; Zhang, X.N. Binary-copolymer system base on low-density lipoprotein-coupled N-succinyl chitosan lipic acid micelles for co-delivery MDR1 siRNA and paclitaxel, enhances antitumor effects via reducing drug. *J. Biomed. Mater. Res. B Appl. Biomater.*, **2017**, *105*(5), 1114-1125.
- [57] He, C.; Lu, K.; Liu, D.; Lin, W. Nanoscale metal-organic frameworks for the co-delivery of cisplatin and pooled siRNAs to enhance therapeutic efficacy in drug-resistant ovarian cancer cells. *J. Am. Chem. Soc.*, **2014**, *136*(14), 5181-5184.
- [58] Zhang, L.; Yang, X.; Li, Y.; Zheng, W.; Jiang, X. Hollow carbon nanospheres as a versatile platform for co-delivery of siRNA and chemotherapeutics. *Carbon*, **2017**, *121*, 79-89.
- [59] Chen, F.-y.; Zhang, Y.; Chen, X.-y.; Li, J.-q.; Xiao, X.-p.; Yu, L.-l.; Tang, Q. Development of a hybrid paclitaxel-loaded arsenite nanoparticle (HPAN) delivery system for synergistic combined therapy of paclitaxel-resistant cancer. *J. Nanopart. Res.*, **2017**, *19*(4), 155.
- [60] Baek, J.-S.; Cho, C.-W. A multifunctional lipid nanoparticle for co-delivery of paclitaxel and curcumin for targeted delivery and enhanced cytotoxicity in multidrug resistant breast cancer cells. *Oncotarget*, **2017**, *8*(18), 30369-30382.
- [61] Sampson, K.E.; Brinker, A.; Pratt, J.; Venkatraman, N.; Xiao, Y.; Blasberg, J.; Steiner, T.; Bourner, M.; Thompson, D.C. Zinc finger nuclease-mediated gene knockout results in loss of transport activity for P-glycoprotein, BCRP, and MRP2 in Caco-2 cells. *Drug Metab. Dispos.*, **2015**, *43*(2), 199-207.
- [62] Ding, Y.; Yu, A.Q.; Li, C.L.; Fang, J.; Zeng, Y.; Li, D.S. TALEN-mediated *Nanog* disruption results in less invasiveness, more chemosensitivity and reversal of EMT in Hela cells. *Oncotarget*, **2014**, *5*(18), 8393-8401.
- [63] Phelan, K.; May, K.M. Mammalian cell tissue culture techniques. *Curr. Protoc. Pharmacol.*, **2016**, *73*, 12.1.1-12.1.23.
- [64] Naipal, K.A.T.; Verkaik, N.S.; Sánchez, H.; Van Deurzen, C.H.M.; Den Bakker, M.A.; Hoeijmakers, J.H.J.; Kanaar, R.; Vreeswijk, M.P.G.; Jager, A.; van Gent, D.C. Tumor slice culture system to assess drug

- response of primary breast cancer. *BMC Cancer*, **2016**, 16(1), 78.
- [65] Wrzesinski, K.; Rogowska-Wrzesinska, A.; Kanlaya, R.; Borkowski, K.; Schwämmle, V.; Dai, J.; Joensen, K.E.; Wojdyla, K.; Carvalho, V.B.; Fey, S.J. The cultural divide: exponential growth in classical 2D and metabolic equilibrium in 3D environments. *PLoS one*, **2014**, 9(9), e106973.
- [66] Sarisozen, C.; Abouzeid, A.H.; Torchilin, V.P. The effect of co-delivery of paclitaxel and curcumin by transferrin-targeted PEG-PE-based mixed micelles on resistant ovarian cancer in 3-D spheroids and *in vivo* tumors. *Eur. J. Pharm. Biopharm.*, **2014**, 88(2), 539-550.
- [67] Breslin, S.; O'Driscoll, L. Three-dimensional cell culture: the missing link in drug discovery. *Drug Discov. Today*, **2013**, 18(5-6), 240-249.
- [68] Mak, I.W.Y.; Evaniew, N.; Ghert, M. Lost in translation: animal models and clinical trials in cancer treatment. *Am. J. Transl. Res.*, **2014**, 6(2), 114-118.
- [69] Katt, M.E.; Placone, A.L.; Wong, A.D.; Xu, Z.S.; Searson, P.C. *In vitro* tumor models: advantages, disadvantages, variables, and selecting the right platform. *Front. Bioeng. Biotechnol.*, **2016**, 4, 12.
- [70] Tannenbaum, J.; Bennett, B.T. Russell and Burch's 3Rs then and now: the need for clarity in definition and purpose. *J. Am. Assoc. Lab. Anim. Sci.*, **2015**, 54(2), 120-132.
- [71] Linzey, A. *Why animal suffering matters: Philosophy, theology, and practical ethics*. Oxford University Press: Northants, **2013**.
- [72] Griffith, L.G.; Swartz, M.A. Capturing complex 3D tissue physiology *in vitro*. *Nat. Rev. Mol. Cell Biol.*, **2006**, 7(3), 211.
- [73] Li, A.P. The use of the integrated discrete multiple organ co-culture (IdMOC (R)) system for the evaluation of multiple organ toxicity. *Altern. Lab. Anim.*, **2009**, 37(4), 377-385.
- [74] Li, A.P.; Bode, C.; Sakai, Y. A novel *in vitro* system, the integrated discrete multiple organ cell culture (IdMOC) system, for the evaluation of human drug toxicity: comparative cytotoxicity of tamoxifen towards normal human cells from five major organs and MCF-7 adenocarcinoma breast cancer cells. *Chem.-Biol. Interact.*, **2004**, 150(1), 129-136.
- [75] Li, A.P. Preclinical *in vitro* screening assays for drug-like properties. *Drug Discov. Today*, **2005**, 2(2), 179-185.
- [76] Gayathri, L.; Karthikeyan, B.S.; Rajalakshmi, M.; Dhanasekaran, D.; Li, A.P.; Akbarsha, M.A. Metabolism-dependent cytotoxicity of citrinin and ochratoxin A alone and in combination as assessed adopting integrated discrete multiple organ co-culture (IdMOC). *Toxicol. In Vitro*, **2018**, 46, 166-177.
- [77] Li, A.P.; Uzgare, A.; LaForge, Y.S. Definition of metabolism-dependent xenobiotic toxicity with co-cultures of human hepatocytes and mouse 3T3 fibroblasts in the novel integrated discrete multiple organ co-culture (IdMOC) experimental system: Results with model toxicants aflatoxin B1, cyclophosphamide and tamoxifen. *Chem.-Biol. Interact.*, **2012**, 199(1), 1-8.
- [78] Li, A.P. *In vitro* evaluation of human xenobiotic toxicity: scientific concepts and the novel integrated discrete multiple cell co-culture (IdMOC) technology. *ALTEX*, **2008**, 25(1), 43-49.
- [79] Uzgare, A.R.; Li, A.P. New paradigm in toxicity testing: Integrated discrete Multiple Organ Co-cultures (IdMOC) for the evaluation of xenobiotic toxicity. *ALTEX Proc.*, **2013**, 2, 39-46.
- [80] Sung, J.H.; Esch, M.B.; Prot, J.-M.; Long, C.J.; Smith, A.; Hickman, J.J.; Shuler, M.L. Microfabricated mammalian organ systems and their integration into models of whole animals and humans. *Lab on a Chip*, **2013**, 13(7), 1201-1212.
- [81] Li, A.P.; Uzgare, A.; Saminathan, H.; Doshi, U. In *Integrated Discrete Multiple Organ Co-culture (IdMOC) System for the Evaluation of Multiple Organ Drug Distribution, Drug Metabolism, and Organ Specific Drug Toxicity*, Drug Metabolism Reviews, Informa healthcare: London, England, **2011**; pp 141-142.
- [82] Whitesides, G.M. The origins and the future of microfluidics. *Nature*, **2006**, 442(7101), 368-373.
- [83] El-Ali, J.; Sorger, P.K.; Jensen, K.F. Cells on chips. *Nature*, **2006**, 442(7101), 403-411.
- [84] Guo, M.T.; Rotem, A.; Heyman, J.A.; Weitz, D.A. Droplet microfluidics for high-throughput biological assays. *Lab on a Chip*, **2012**, 12(12), 2146-2155.
- [85] Dittrich, P.S.; Manz, A. Lab-on-a-chip: microfluidics in drug discovery. *Nat. Rev. Drug Discov.*, **2006**, 5(3), 210-218.
- [86] Weltin, A.; Slotwinski, K.; Kieninger, J.; Moser, I.; Jobst, G.; Wego, M.; Ehret, R.; Urban, G.A. Cell culture monitoring for drug screening and cancer research: a transparent, microfluidic, multi-sensor microsystem. *Lab on a Chip*, **2014**, 14(1), 138-146.
- [87] Weibel, D.B.; Whitesides, G.M. Applications of microfluidics in chemical biology. *Curr. Opin. Chem. Biol.*, **2006**, 10(6), 584-591.
- [88] Sung, J.H.; Kam, C.; Shuler, M.L. A microfluidic device for a pharmacokinetic-pharmacodynamic (PK-PD) model on a chip. *Lab on a Chip*, **2010**, 10(4), 446-455.
- [89] Kashaninejad, N.; Nikmaneshi, M.R.; Moghadas, H.; Kiyomarsi Oskouei, A.; Rismanian, M.; Barisam, M.; Saidi, M.S.; Firoozabadi, B. Organ-tumor-on-a-chip for

- chemosensitivity assay: a critical review. *Micromachines*, **2016**, 7(8), 130.
- [90] Ghaemmaghami, A.M.; Hancock, M.J.; Harrington, H.; Kaji, H.; Khademhosseini, A. Biomimetic tissues on a chip for drug discovery. *Drug Discov. Today*, **2012**, 17(3-4), 173-181.
- [91] Bhise, N.S.; Ribas, J.; Manoharan, V.; Zhang, Y.S.; Polini, A.; Massa, S.; Dokmeci, M.R.; Khademhosseini, A. Organ-on-a-chip platforms for studying drug delivery systems. *J. Control. Release*, **2014**, 190, 82-93.
- [92] Caplin, J.D.; Granados, N.G.; James, M.R.; Montazami, R.; Hashemi, N. Microfluidic organ-on-a-chip technology for advancement of drug development and toxicology. *Adv. Healthc. Mater.*, **2015**, 4(10), 1426-1450.
- [93] van der Helm, M.W.; van der Meer, A.D.; Eijkel, J.C.; van den Berg, A.; Segerink, L.I. Microfluidic organ-on-chip technology for blood-brain barrier research. *Tissue barriers*, **2016**, 4(1), e1142493.
- [94] Maschmeyer, I.; Lorenz, A.K.; Schimek, K.; Hasenberg, T.; Ramme, A.P.; Hübner, J.; Lindner, M.; Drewell, C.; Bauer, S.; Thomas, A. A four-organ-chip for interconnected long-term co-culture of human intestine, liver, skin and kidney equivalents. *Lab on a Chip*, **2015**, 15(12), 2688-2699.
- [95] Tatosian, D.A.; Shuler, M.L. A novel system for evaluation of drug mixtures for potential efficacy in treating multidrug resistant cancers. *Biotechnol. Bioeng.*, **2009**, 103(1), 187-198.
- [96] Huh, D.; Matthews, B.D.; Mammoto, A.; Montoya-Zavala, M.; Hsin, H.Y.; Ingber, D.E. Reconstituting organ-level lung functions on a chip. *Science*, **2010**, 328(5986), 1662-1668.
- [97] Ruppen, J.; Wildhaber, F.D.; Strub, C.; Hall, S.R.; Schmid, R.A.; Geiser, T.; Guenat, O.T. Towards personalized medicine: chemosensitivity assays of patient lung cancer cell spheroids in a perfused microfluidic platform. *Lab on a Chip*, **2015**, 15(14), 3076-3085.
- [98] Imamura, Y.; Mukohara, T.; Shimono, Y.; Funakoshi, Y.; Chayahara, N.; Toyoda, M.; Kiyota, N.; Takao, S.; Kono, S.; Nakatsura, T. Comparison of 2D-and 3D-culture models as drug-testing platforms in breast cancer. *Oncol. Rep.*, **2015**, 33(4), 1837-1843.
- [99] Lee, J.; Lilly, G.D.; Doty, R.C.; Podsiadlo, P.; Kotov, N.A. *In vitro* toxicity testing of nanoparticles in 3D cell culture. *Small*, **2009**, 5(10), 1213-1221.
- [100] Kriston-Vizi, J.; Flotow, H. Getting the whole picture: High content screening using three-dimensional cellular model systems and whole animal assays. *Cytometry Part A*, **2017**, 91(2), 152-159.
- [101] Elliott, N.T.; Yuan, F. A review of three-dimensional *in vitro* tissue models for drug discovery and transport studies. *J. Pharm. Sci.*, **2011**, 100(1), 59-74.
- [102] Nath, S.; Devi, G.R. Three-Dimensional Culture Systems in Cancer Research: Focus on Tumor Spheroid Model. *Pharmacol. Ther.*, **2016**, 163, 94-108.
- [103] Antoni, D.; Burckel, H.; Josset, E.; Noel, G. Three-dimensional cell culture: a breakthrough *in vivo*. *Int. J. Mol. Sci.*, **2015**, 16(3), 5517-5527.
- [104] Breslin, S.; O'Driscoll, L. Three-dimensional cell culture: the missing link in drug discovery. *Drug Discov. Today*, **2013**, 18(5-6), 240-249.
- [105] Desoize, B.; Jardillier, J.-C. Multicellular resistance: a paradigm for clinical resistance? *Crit. Rev. Oncol. Hematol.*, **2000**, 36(2), 193-207.
- [106] Shield, K.; Ackland, M.L.; Ahmed, N.; Rice, G.E. Multicellular spheroids in ovarian cancer metastases: Biology and pathology. *Gynecol. Oncol.*, **2009**, 113(1), 143-148.
- [107] Barrera-Rodríguez, R.; Fuentes, J.M. Multidrug resistance characterization in multicellular tumour spheroids from two human lung cancer cell lines. *Cancer Cell Int.*, **2015**, 15(1), 47.
- [108] Wrzesinski, K.; Fey, S.J. Metabolic Reprogramming and the Recovery of Physiological Functionality in 3D Cultures in Micro-Bioreactors. *Bioengineering (Basel)*, **2018**, 5(1).
- [109] Bray, L.J.; Binner, M.; Holzheu, A.; Friedrichs, J.; Freudenberg, U.; Hutmacher, D.W.; Werner, C. Multiparametric hydrogels support 3D *in vitro* bioengineered microenvironment models of tumour angiogenesis. *Biomaterials*, **2015**, 53, 609-620.
- [110] LaBarbera, D.V.; Reid, B.G.; Yoo, B.H. The multicellular tumor spheroid model for high-throughput cancer drug discovery. *Expert Opin. Drug Discov.*, **2012**, 7(9), 819-830.
- [111] Hirschhaeuser, F.; Menne, H.; Dittfeld, C.; West, J.; Mueller-Klieser, W.; Kunz-Schughart, L.A. Multicellular tumor spheroids: an underestimated tool is catching up again. *J. Biotechnol.*, **2010**, 148(1), 3-15.
- [112] Ma, H.-I.; Jiang, Q.; Han, S.; Wu, Y.; Tomshine, J.C.; Wang, D.; Gan, Y.; Zou, G.; Liang, X.-J. Multicellular tumor spheroids as an *in vivo*-like tumor model for three-dimensional imaging of chemotherapeutic and nano material cellular penetration. *Mol. Imaging*, **2012**, 11(6), 487-498.
- [113] Wang, J.-Z.; Zhu, Y.-X.; Ma, H.-C.; Chen, S.-N.; Chao, J.-Y.; Ruan, W.-D.; Wang, D.; Du, F.-g.; Meng, Y.-Z. Developing multi-cellular tumor spheroid model (MCTS) in the chitosan/collagen/alginate (CCA) fibrous scaffold for anticancer drug screening. *Mater. Sci. Eng. C*, **2016**, 62, 215-225.

- [114] Longati, P.; Jia, X.; Eimer, J.; Wagman, A.; Witt, M.-R.; Rehnmark, S.; Verbeke, C.; Toftgård, R.; Löhr, M.; Heuchel, R.L. 3D pancreatic carcinoma spheroids induce a matrix-rich, chemoresistant phenotype offering a better model for drug testing. *BMC Cancer*, **2013**, *13*(1), 95.
- [115] Fey, S.J.; Wrzesinski, K. Determination of drug toxicity using 3D spheroids constructed from an immortal human hepatocyte cell line. *Toxicol. Sci.*, **2012**, *127*(2), 403-411.
- [116] Lovitt, C.J.; Shelper, T.B.; Avery, V.M. Advanced cell culture techniques for cancer drug discovery. *Biology*, **2014**, *3*(2), 345-367.
- [117] Vinci, M.; Gowan, S.; Boxall, F.; Patterson, L.; Zimmermann, M.; Lomas, C.; Mendiola, M.; Hardisson, D.; Eccles, S.A. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biology*, **2012**, *10*(1), 29.
- [118] Chatzinikolaïdou, M. Cell spheroids: the new frontiers in *in vitro* models for cancer drug validation. *Drug Discov. Today*, **2016**, *21*(9), 1553-1560.
- [119] Minchinton, A.I.; Tannock, I.F. Drug penetration in solid tumours. *Nat. Rev. Cancer*, **2006**, *6*(8), 583-592.
- [120] Greiner, B.; Eichelbaum, M.; Fritz, P.; Kreichgauer, H.-P.; Von Richter, O.; Zundler, J.; Kroemer, H.K. The role of intestinal P-glycoprotein in the interaction of digoxin and rifampin. *J. Clin. Invest.*, **1999**, *104*(2), 147-153.
- [121] Eghbali, H.; Nava, M.M.; Mohebbi-Kalhari, D.; Raimondi, M.T. Hollow fiber bioreactor technology for tissue engineering applications. *Int. J. Artif. Organs*, **2016**, *39*(1), 1-15.
- [122] Casciari, J.J.; Hollingshead, M.G.; Alley, M.C.; Mayo, J.G.; Malspeis, L.; Miyauchi, S.; Grever, M.R.; Weinstein, J.N. Growth and chemotherapeutic response of cells in a hollow-fiber *in vitro* solid tumor model. *J. Natl. Cancer Inst.*, **1994**, *86*(24), 1846-1852.
- [123] Hassan, S.B.; de la Torre, M.; Nygren, P.; Karlsson, M.O.; Larsson, R.; Jonsson, E. A hollow fiber model for *in vitro* studies of cytotoxic compounds: activity of the cyanoguanidine CHS 828. *Anti-cancer drugs*, **2001**, *12*(1), 33-42.
- [124] Eghbali, H.; Nava, M.; Mohebbi-Kalhari, D.; Raimondi, M. Hollow fiber bioreactor technology for tissue engineering applications. *Int. J. Artif. Organs*, **2016**, *39*(1), 1-15.
- [125] Moon, S.; Hasan, S.K.; Song, Y.S.; Xu, F.; Keles, H.O.; Manzur, F.; Mikkilineni, S.; Hong, J.W.; Nagatomi, J.; Haeggstrom, E. Layer by layer three-dimensional tissue epitaxy by cell-laden hydrogel droplets. *Tissue Eng. Part C Methods*, **2009**, *16*(1), 157-166.
- [126] Boland, T.; Xu, T.; Damon, B.; Cui, X. Application of inkjet printing to tissue engineering. *Biotechnol. J.*, **2006**, *1*(9), 910-917.
- [127] ICH, General considerations for clinical trials ICH Topic E8. In *ICH Guidance for Industry: Health Canada*, **1998**.
- [128] Junod, S.W. FDA and Clinical Drug Trials: A Short History. In *U.S. Food and Drug Administration*, **2013**.
- [129] Stakleff, K.D.S.; Von Gruenigen, V.E. Rodent models for ovarian cancer research. *Int. J. Gynecol. Cancer*, **2003**, *13*, 405-412.
- [130] Roper, J.; Hung, K.E. Priceless GEMMs: genetically engineered mouse models for colorectal cancer drug development. *Trends Pharmacol. Sci.*, **2012**, *33*(8), 449-455.
- [131] Rangarajan, A.; Weinberg, R.A. Comparative biology of mouse versus human cells: modelling human cancer in mice. *Nat. Rev. Cancer*, **2003**, *3*, 952-959.
- [132] Cheon, D.J.; Orsulic, S. Mouse models of cancer. *Annu. Rev. Pathol.*, **2011**, *6*, 95-119.
- [133] Sharpless, N.E.; DePinho, R.A. The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat. Rev. Drug Discov.*, **2006**, *5*, 741-754.
- [134] Dranoff, G. Experimental mouse tumour models: what can be learnt about human cancer immunology? *Nat. Rev. Immunol.*, **2012**, *12*, 61-66.
- [135] Ambrogio, C.; Carmona, F.J.; Vidal, A.; Falcone, M.; Nieto, P.; Romero, O.A.; Puertas, S.; Vizoso, M.; Nadal, E.; Poggio, T.; Sanchez-Céspedes, M.; Esteller, M.; Mulero, F.; Voena, C.; Chiarle, R.; Barbacid, M.; Santamaria, D.; Villanueva, A. Modeling lung cancer evolution and preclinical response by orthotopic mouse allografts. *Cancer Res.*, **2014**, *74*(21), 5978-5988.
- [136] Wang, G.Y.; So, P.-L.; Wang, L.; Libove, E.; Wang, J.; Epstein, E.H.J. Establishment of murine basal cell carcinoma allografts - a potential model for preclinical drug testing and for molecular analysis. *J. Invest. Dermatol.*, **2011**, *131*(11), 2298-2305.
- [137] Schuller, H.M.; Jorquera, R.; Reichert, A.; Castonguay, A. Transplacental induction of pancreas tumors in hamsters by ethanol and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Res.*, **1993**, *53*, 2498-2501.
- [138] Rao, M.S.; Reddy, J.K. Histogenesis of pseudoductular changes induced in the pancreas of guinea pigs treated with N-methyl-N-nitrosourea. *Carcinogenesis*, **1980**, *1*(12), 1027-1037.
- [139] Qiu, W.; Su, G.H. Challenges and advances in mouse modeling for human pancreatic tumorigenesis and

- metastasis. *Cancer Metastasis Rev.*, **2013**, 32(1-2), 83-107.
- [140] Hollingshead, M.G.; Alley, M.C.; Camalier, R.F.; Abbott, B.J.; Mayo, J.G.; Malspeis, L.; Grever, M.R. *In vivo* cultivation of tumor cells in hollow fibers. *Life Sci.*, **1995**, 57(2), 131-141.
- [141] Decker, S.; Hollingshead, M.; Bonomi, C.A.; Carter, J.P.; Sausville, E.A. The hollow fibre model in cancer drug screening: the NCI experience. *Eur. J. Cancer*, **2004**, 40, 821-826.
- [142] Shnyder, S.D.; Hasan, J.; Cooper, P.A.; Pilarinou, E.; Jubb, E.; Jayson, G.C.; Bibby, M.C. Development of a modified hollow fibre assay for studying agents targeting the tumour neovasculature. *Anticancer Res.*, **2005**, 25, 1889-1894.
- [143] Phillips, R.M.; Pearce, J.; Loadman, P.M.; Bibby, M.C.; Cooper, P.A.; Swaine, D.J.; Double, J.A. Angiogenesis in the hollow fibre tumour model influences drug delivery to tumour cells: implications for anticancer drug screening programs. *Cancer Res.*, **1998**, 58, 5263-5266.
- [144] Veiga, J.P.; Cooper, P.A.; Pors, K.; Patterson, L.H.; Bibby, M.C.; Shnyder, S.D. Use of the hollow fiber assay for the evaluation of DNA damaging agents. *J. Pharmacol. Toxicol. Meth.*, **2011**, 64, 226-232.
- [145] Bachmann, A.S.; Opoku-Ansah, J.; Ibarra-Rivera, T.R.; Yco, L.P.; Ambadi, S.; Roberts, C.C.; Chang, C.E.; Pirrung, M.C. Syrbactin Structural Analog TIR-199 Blocks Proteasome Activity and Induces Tumor Cell Death. *J. Biol. Chem.*, **2016**, 291(16), 8350-8362.
- [146] Mi, Q.; Pezzuto, J.M.; Farnsworth, N.R.; Wani, M.C.; Kinghorn, A.D.; Swanson, S.M. Use of the *in vivo* hollow fiber assay in natural products anticancer drug discovery. *J. Nat. Prod.*, **2009**, 72(3), 573-580.
- [147] Liu, X.; Yang, Q.; Zhang, G.; Li, Y.; Chen, Y.; Weng, X.; Wang, Y.; Wang, Y.; Zhu, X. Anti-tumor pharmacological evaluation of extracts from *Stellera chamaejasme* L based on hollow fiber assay. *BMC Complement. Altern. Med.*, **2014**, 14, 116.
- [148] Lee, K.H.; Rhee, K.H. Correlative effect between *in vivo* hollow fiber assay and xenografts assay in drug screening. *Cancer Res. Treat.*, **2005**, 37(3), 196-200.
- [149] Johnson, J.I.; Decker, S.; Zaharevitz, D.; Rubinstein, L.V.; Venditti, J.M.; Schepartz, S.; Kalyandrug, S.; Christian, M.; Arbuck, S.; Hollingshead, M.; Sausville, E.A. Relationships between drug activity in NCI preclinical *in vitro* and *in vivo* models and early clinical trials. *British J. Cancer*, **2001**, 84(10), 1424-1431.
- [150] Gould, S.E.; Junttila, M.R.; de Sauvage, F.J. Translational value of mouse models in oncology drug development. *Nat. Med.*, **2015**, 21(5), 431-439.
- [151] Fogh, J.; Fogh, J.M.; Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.*, **1977**, 59, 221-226.
- [152] Cekanova, M.; Rathore, K. Animal models and therapeutic molecular targets of cancer: utility and limitations. *Drug Des. Devel. Ther.*, **2014**, 8, 1911-1921.
- [153] Morton, C.L.; Houghton, P.J. Establishment of human tumor xenografts in immunodeficient mice. *Nat. Protoc.*, **2007**, 2(2), 247-250.
- [154] Schuh, J.C. Trials, tribulations, and trends in tumor modeling in mice. *Toxicol. Pathol.*, **2004**, 32 Suppl 1, 53-66.
- [155] Shaw, T.J.; Senterman, M.K.; Dawson, K.; Crane, C.A.; Vanderhyden, B.C. Characterization of intraperitoneal, orthotopic, and metastatic xenograft models of human ovarian cancer. *Mol. Ther.*, **2004**, 10, 1032-1042.
- [156] Kumar, S.; Bajaj, S.; Bodla, R.B. Preclinical screening methods in cancer. *Indian J. Pharmacol.*, **2016**, 48(5), 481-486.
- [157] Killion, J.J.; Radinsky, R.; Fidler, I.J. Orthotopic models are necessary to predict therapy of transplantable tumors in mice. *Cancer Metastasis Rev.*, **1998**, 17(3), 279-284.
- [158] Dipersio, L.P. Regional growth differences of human tumour xenografts in nude mice. *Lab. Anim.*, **1981**, 15, 179-180.
- [159] Haddad, T.C.; Yee, D. Of mice and (wo)men: is this any way to test a new drug? *J. Clin. Oncol.*, **2008**, 26, 830-832.
- [160] Hughes, P.; Marshall, D.; Reid, Y.; Parkes, H.; Gelber, C. The cost of using unauthenticated, over-passaged cell lines: how much more data do we need? *BioTechniques*, **2007**, 43(5), 575-584.
- [161] Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell*, **2011**, 144(5), 646-674.
- [162] Junttila, M.R.; De Sauvage, F.J. Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature*, **2013**, 501, 346-354.
- [163] Frese, K.K.; Tuveson, D.A. Maximizing mouse cancer models. *Nat. Rev. Cancer*, **2007**, 7(9), 645-658.
- [164] Yang, X.; Feng, Y.; Gao, Y.; Shen, J.; Choy, E.; Cote, G.; Harmon, D.; Zhang, Z.; Mankin, H.; Hornicek, F.J.; Duan, Z. NSC23925 prevents the emergence of multidrug resistance in ovarian cancer *in vitro* and *in vivo*. *Gynecol. Oncol.*, **2015**, 137(1), 134-142.
- [165] Redaelli, S.; Perini, P.; Ceccon, M.; Piazza, R.; Rigolio, R.; Mauri, M.; Boschelli, F.; Giannoudis, A.; Gambacorti-Passerini, C. *In vitro* and *in vivo* identification of ABCB1 as an efflux transporter of bosutinib. *J. Hematol. Oncol.*, **2015**, 8, 81.

- [166] Katayama, R.; Sakashita, T.; Yanagitani, N.; Ninomiya, H.; Horiike, A.; Friboulet, L.; Gainor, J.F.; Motoi, N.; Dobashi, A.; Sakata, S.; Tambo, Y.; Kitazono, S.; Sato, S.; Koike, S.; John Iafrate, A.; Mino-Kenudson, M.; Ishikawa, Y.; Shaw, A.T.; Engelman, J.A.; Takeuchi, K.; Nishio, M.; Fujita, N. P-glycoprotein Mediates Ceritinib Resistance in Anaplastic Lymphoma Kinase-rearranged Non-small Cell Lung Cancer. *EBioMedicine*, **2016**, *3*, 54-66.
- [167] Li, X.; Wang, H.; Wang, J.; Chen, Y.; Yin, X.; Shi, G.; Li, H.; Hu, Z.; Liang, X. Emodin enhances cisplatin-induced cytotoxicity in human bladder cancer cells through ROS elevation and MRP1 downregulation. *BMC Cancer*, **2016**, *16*, 578.
- [168] Chakravarty, G.; Mathur, A.; Mallade, P.; Gerlach, S.; Willis, J.; Datta, A.; Srivastav, S.; Abdel-Mageed, A.B.; Mondal, D. Nelfinavir targets multiple drug resistance mechanisms to increase the efficacy of doxorubicin in MCF-7/Dox breast cancer cells. *Biochimie*, **2016**, *124*, 53-64.
- [169] Argov, M.; Kashi, R.; Peer, D.; Margalit, R. Treatment of resistant human colon cancer xenografts by a fluoxetine-doxorubicin combination enhances therapeutic responses comparable to an aggressive bevacizumab regimen. *Cancer Lett.*, **2009**, *274*(1), 118-25.
- [170] Arnaud, O.; Boumendjel, A.; Geze, A.; Honorat, M.; Matera, E.L.; Guitton, J.; Stein, W.D.; Bates, S.E.; Falson, P.; Dumontet, C.; Di Pietro, A.; Payen, L. The acridone derivative MBLI-87 sensitizes breast cancer resistance protein-expressing xenografts to irinotecan. *Eur. J. Cancer*, **2011**, *47*(4), 640-648.
- [171] Stein, W.D.; Yang, J.; Bates, S.E.; Fojo, T. Bevacizumab reduces the growth rate constants of renal carcinomas: a novel algorithm suggests early discontinuation of bevacizumab resulted in a lack of survival advantage. *Oncologist*, **2008**, *13*(10), 1055-1062.
- [172] Patil, Y.B.; Swaminathan, S.K.; Sadhukha, T.; Ma, L.; Panyam, J. The use of nanoparticle-mediated targeted gene silencing and drug delivery to overcome tumor drug resistance. *Biomaterials*, **2010**, *31*(2), 358-365.
- [173] Fiebig, H.-H.; Burger, A.M. *Human tumor xenografts and explants*. Humana Press Inc.: Totowa, **2002**.
- [174] Williams, S.A.; Anderson, W.C.; Santaguida, M.T.; Dylla, S.J. Patient-derived xenografts, the cancer stem cell paradigm, and cancer pathobiology in the 21st century. *Lab. Invest.*, **2013**, *93*(9), 970-982.
- [175] Marangoni, E.; Vincent-Salomon, A.; Auger, N.; Degeorges, A.; Assayag, F.; de Cremoux, P.; de Plater, L.; Guyader, C.; De Pinieux, G.; Judde, J.G.; Rebutti, M.; Tran-Perennou, C.; Sastre-Garau, X.; Sigal-Zafrani, B.; Delattre, O.; Dieras, V.; Poupon, M.F. A new model of patient tumor-derived breast cancer xenografts for preclinical assays. *Clin. Cancer Res.*, **2007**, *13*(13), 3989-3998.
- [176] DeRose, Y.S.; Wang, G.; Lin, Y.C.; Bernard, P.S.; Buys, S.S.; Ebbert, M.T.; Factor, R.; Matsen, C.; Milash, B.A.; Nelson, E.; Neumayer, L.; Randall, R.L.; Stijleman, I.J.; Welm, B.E.; Welm, A.L. Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes. *Nat. Med.*, **2011**, *17*(11), 1514-1520.
- [177] Tentler, J.J.; Tan, A.C.; Weekes, C.D.; Jimeno, A.; Leong, S.; Pitts, T.M.; Arcaroli, J.J.; Messersmith, W.A.; Eckhardt, S.G. Patient-derived tumour xenografts as models for oncology drug development. *Nat. Rev. Clin. Oncol.*, **2012**, *9*, 338-350.
- [178] Gao, H.; Korn, J.M.; Ferretti, S.; Monahan, J.E.; Wang, Y.; Singh, M.; Zhang, C.; Schnell, C.; Yang, G.; Zhang, Y.; Balbin, O.A.; Barbe, S.; Cai, H.; Casey, F.; Chatterjee, S.; Chiang, D.Y.; Chuai, S.; Cogan, S.M.; Collins, S.D.; Dammassa, E.; Ebel, N.; Embry, M.; Green, J.; Kauffmann, A.; Kowal, C.; Leary, R.J.; Lehar, J.; Liang, Y.; Loo, A.; Lorenzana, E.; Robert McDonald, E., 3rd; McLaughlin, M.E.; Merkin, J.; Meyer, R.; Naylor, T.L.; Patawaran, M.; Reddy, A.; Roelli, C.; Ruddy, D.A.; Salangsang, F.; Santacroce, F.; Singh, A.P.; Tang, Y.; Tinetto, W.; Tobler, S.; Velazquez, R.; Venkatesan, K.; Von Arx, F.; Wang, H.Q.; Wang, Z.; Wiesmann, M.; Wyss, D.; Xu, F.; Bitter, H.; Atadja, P.; Lees, E.; Hofmann, F.; Li, E.; Keen, N.; Cozens, R.; Jensen, M.R.; Pryer, N.K.; Williams, J.A.; Sellers, W.R. High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. *Nat. Med.*, **2015**, *21*(11), 1318-1325.
- [179] Ledford, H. US cancer institute overhauls cell lines. *Nature*, **2016**, *530*, 391.
- [180] White, R.d.V. Can mouse avatars guide cancer treatment? *OncLive*, **2013**.
- [181] Richmond, A.; Su, Y. Mouse xenograft models vs GEM models for human cancer therapeutics. *Dis. Model. Mech.*, **2008**, *1*, 78-82.
- [182] Gangloff, A.; Hsueh, W.-A.; Kesner, A.L.; Kiesewetter, D.O.; Pio, B.S.; Pegram, M.D.; Beryt, M.; Townsend, A.; Czernin, J.; Phelps, M.E.; Silverman, D.H.S. Estimation of paclitaxel biodistribution and uptake in human-derived xenografts *in vivo* with 18F-Fluoropaclitaxel. *J. Nucl. Med.*, **2005**, *46*, 1866-1871.
- [183] Malaney, P.; Nicosia, S.V.; Dave, V. One mouse, one patient paradigm: New avatars of personalized cancer therapy. *Cancer Lett.*, **2014**, *344*(1), 1-12.
- [184] Dey, N.; Sun, Y.; Leyland-Jones, B.; De, P. Evolution of Tumor Model: From Animal Model of Tumor to Tumor Model in Animal. *J. Cancer Ther.*, **2013**, *04*(09), 1411-1425.

- [185] Shultz, L.D.; Brehm, M.A.; Garcia-Martinez, J.V.; Greiner, D.L. Humanized mice for immune system investigation: progress, promise and challenges. *Nat. Rev. Immunol.*, **2012**, *12*, 786-798.
- [186] Arvelo, F.; Poupon, M.F.; Bichat, F.; Grossin, F.; Bourgeois, Y.; Jacrot, M.; Bastian, G.; Le Chevalier, T. Adding a reverser (Verapamil) to combined chemotherapy overrides resistance in small cell lung cancer xenografts. *Eur. J. Cancer*, **1995**, *31A*(11), 1862-1868.
- [187] Kleffel, S.; Lee, N.; Lezcano, C.; Wilson, B.J.; Sobolewski, K.; Saab, K.R.; Mueller, H.; Zhan, Q.; Posch, C.; Elco, C.P.; DoRosario, A.; Garcia, S.S.; Thakuria, M.; Wang, Y.E.; Wang, L.C.; Murphy, G.F.; Frank, M.H.; Schatton, T. ABCB5-Targeted Chemoresistance Reversal Inhibits Merkel Cell Carcinoma Growth. *J. Invest. Dermatol.*, **2016**, *136*(4), 838-846.
- [188] van Miltenburg, M.H.; Jonkers, J. Using genetically engineered mouse models to validate candidate cancer genes and test new therapeutic approaches. *Curr. Opin. Genet. Dev.*, **2012**, *22*(1), 21-27.
- [189] Lee, H. Genetically engineered mouse models for drug development and preclinical trials. *Biomol. Ther.*, **2014**, *22*(4), 267-274.
- [190] Tuveson, D.A.; Jacks, T. Technologically advanced cancer modeling in mice. *Curr. Opin. Genet. Dev.*, **2002**, *12*, 105-110.
- [191] Sarkisian, C.J.; Keister, B.A.; Stairs, D.B.; Boxer, R.B.; Moody, S.E.; Chodosh, L.A. Dose-dependent oncogene-induced senescence *in vivo* and its evasion during mammary tumorigenesis. *Nat. Cell Biol.*, **2007**, *9*, 493-505.
- [192] Lee, E.Y.; Chang, C.Y.; Hu, N.; Wang, Y.C.; Lai, C.C.; Herrup, K.; Lee, W.H.; Bradley, A. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature*, **1992**, *359*, 288-294.
- [193] Legrand, N.; Ploss, A.; Balling, R.; Becker, P.D.; Borsotti, C.; Brezillon, N.; DeBarry, J.; De Jong, Y.; Deng, H.; Di Santo, J.P.; Eisenbarth, S.; Eynon, E.; Flavell, R.A.; Guzman, C.A.; Huntington, N.D.; Kremsdorf, D.; Manns, M.P.; Manz, M.G.; Mention, J.-J.; Ott, M.; Rathinam, C.; Rice, C.M.; Rongvaux, A.; Stevens, S.; Spits, H.; Strick-Marchand, H.; Takizawa, H.; Van Lent, A.U.; Wang, C.; Weijer, K.; Willinger, T.; Ziegler, P. Humanized mice for modeling human infectious disease: challenges, progress, and outlook. *Cell Host Microbe*, **2009**, *6*, 5-9.
- [194] Liu, X.; Holstege, H.; van der Gulden, H.; Treur-Mulder, M.; Zevenhoven, J.; Velds, A.; Kerkhoven, R.M.; van Vliet, M.H.; Wessels, L.F.; Peterse, J.L.; Berns, A.; Jonkers, J. Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. *Proc. Natl. Acad. Sci. U.S.A.*, **2007**, *104*(29), 12111-12116.
- [195] Rottenberg, S.; Jaspers, J.E.; Kersbergen, A.; van der Burg, E.; Nygren, A.O.; Zander, S.A.; Derksen, P.W.; de Bruin, M.; Zevenhoven, J.; Lau, A.; Boulter, R.; Cranston, A.; O'Connor, M.J.; Martin, N.M.; Borst, P.; Jonkers, J. High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc. Natl. Acad. Sci. U S A*, **2008**, *105*(44), 17079-17084.
- [196] Zander, S.A.; Kersbergen, A.; van der Burg, E.; de Water, N.; van Tellingen, O.; Gunnarsdottir, S.; Jaspers, J.E.; Pajic, M.; Nygren, A.O.; Jonkers, J.; Borst, P.; Rottenberg, S. Sensitivity and acquired resistance of BRCA1;p53-deficient mouse mammary tumors to the topoisomerase I inhibitor topotecan. *Cancer Res.*, **2010**, *70*(4), 1700-1710.
- [197] Leggas, M.; Adachi, M.; Scheffer, G.L.; Sun, D.; Wielinga, P.; Du, G.; Mercer, K.E.; Zhuang, Y.; Panetta, J.C.; Johnston, B.; Scheper, R.J.; Stewart, C.F.; Schuetz, J.D. MRP4 confers resistance to topotecan and protects the brain from chemotherapy. *Mol. Cell Biol.*, **2004**, *24*(17), 7612-7621.
- [198] Marchetti, S.; Pluim, D.; Beijnen, J.H.; Mazzanti, R.; van Tellingen, O.; Schellens, J.H.M. Effect of the drug transporters ABCB1, ABCC2, and ABCG2 on the disposition and brain accumulation of the taxane analog BMS-275,183. *Invest. New Drugs*, **2014**, *32*, 1083-1095.
- [199] Gu, L.; Tsark, W.M.; Brown, D.A.; Blanchard, S.; Synold, T.W.; Kane, S.E. A new model for studying tissue-specific mdr1a gene expression *in vivo* by live imaging. *Proc. Natl. Acad. Sci. U S A*, **2009**, *106*(13), 5394-5399.
- [200] Calitz, C.; Hamman, J.H.; Fey, S.J.; Wrzesinski, K.; Gouws, C. Recent advances in three-dimensional cell culturing to assess liver function and dysfunction: from a drug biotransformation and toxicity perspective. *Toxicol. Mech. Methods*, **2018**, *28*(5), 369-385.
- [201] Wrzesinski, K.; Fey, S.J. After trypsinisation, 3D spheroids of C3A hepatocytes need 18 days to re-establish similar levels of key physiological functions to those seen in the liver. *Toxicol. Res.*, **2013**, *2*(2), 123-135.
- [202] Thoma, C.R.; Stroebel, S.; Rosch, N.; Calpe, B.; Krek, W.; Kelm, J.M. A high-throughput-compatible 3D microtissue co-culture system for phenotypic RNAi screening applications. *J. Biomol. Screen.*, **2013**, *18*(10), 1330-1337.
- [203] Alonso-Nocelo, M.; Abuin, C.; Lopez-Lopez, R.; de la Fuente, M. Development and characterization of a three-dimensional co-culture model of tumor T cell infiltration. *Biofabrication*, **2016**, *8*(2), 025002.
- [204] Fiori, M.E.; Villanova, L.; De Maria, R. Cancer stem cells: at the forefront of personalized medicine and

- immunotherapy. *Curr. Opin. Pharmacol.*, **2017**, *35*, 1-11.
- [205] Zakaria, N.; Satar, N.A.; Abu Halim, N.H.; Ngalm, S.H.; Yusoff, N.M.; Lin, J.; Yahaya, B.H. Targeting Lung Cancer Stem Cells: Research and Clinical Impacts. *Front. Oncol.*, **2017**, *7*, 80.
- [206] Stankevicius, V.; Kunigenas, L.; Stankunas, E.; Kuodyte, K.; Strainiene, E.; Cicenias, J.; Samalavicius, N.E.; Suziedelis, K. The expression of cancer stem cell markers in human colorectal carcinoma cells in a microenvironment dependent manner. *Biochem. Biophys. Res. Commun.*, **2017**, *484*(4), 726-733.
- [207] Reynolds, D.S.; Tevis, K.M.; Blessing, W.A.; Colson, Y.L.; Zaman, M.H.; Grinstaff, M.W. breast cancer spheroids reveal a differential cancer stem cell response to chemotherapeutic treatment. *Sci. Rep.*, **2017**, *7*(1), 10382.
- [208] Kim, T.H.; Shin, Y.J.; Won, A.J.; Lee, B.M.; Choi, W.S.; Jung, J.H.; Chung, H.Y.; Kim, H.S. Resveratrol enhances chemosensitivity of doxorubicin in multidrug-resistant human breast cancer cells via increased cellular influx of doxorubicin. *Biochim. Biophys. Acta*, **2014**, *1840*(1), 615-625.
- [209] Fic, M.; Gomulkiewicz, A.; Grzegorzolka, J.; Podhorska-Okolow, M.; Zabel, M.; Dziegiel, P.; Jablonska, K. The impact of melatonin on colon cancer cells' resistance to doxorubicin in an *in vitro* study. *Int. J. Mol. Sci.*, **2017**, *18*(7), 1396.
- [210] Patel, A.; Li, T.-W.; Anreddy, N.; Wang, D.-S.; Sodani, K.; Gadhia, S.; Kathawala, R.; Yang, D.-H.; Cheng, C.; Chen, Z.-S. Suppression of ABCG2 mediated MDR *in vitro* and *in vivo* by a novel inhibitor of ABCG2 drug transport. *Pharmacol. Res.*, **2017**, *121*, 184-193.
- [211] Wang, Y.-J.; Zhang, Y.-K.; Zhang, G.-N.; Al Rihani, S.B.; Wei, M.-N.; Gupta, P.; Zhang, X.-Y.; Shukla, S.; Ambudkar, S.V.; Kaddoumi, A.; Shi, Z.; Chen, Z.-S. Regorafenib overcomes chemotherapeutic multidrug resistance mediated by ABCB1 transporter in colorectal cancer: *In vitro* and *in vivo* study. *Cancer Lett.*, **2017**, *396*, 145-154.
- [212] Husaini, R.; Ahmad, M.; Zakaria, Z. Effectiveness of imatinib mesylate over etoposide in the treatment of sensitive and resistant chronic myeloid leukaemia cells *in vitro*. *Exp. Ther. Med.*, **2017**, *13*(6), 3209-3216.
- [213] Lee, H.S.; Park, S.B.; Kim, S.A.; Kwon, S.K.; Cha, H.; Lee, D.Y.; Ro, S.; Cho, J.M.; Song, S.Y. A novel HDAC inhibitor, CG200745, inhibits pancreatic cancer cell growth and overcomes gemcitabine resistance. *Sci. Rep.*, **2017**, *7*, 41615.
- [214] Yan, Y.-Y.; Wang, F.; Zhao, X.-Q.; Wang, X.-K.; Chen, Y.-F.; Liu, H.; Xie, Y.; Fu, L.-W. Degradation of P-glycoprotein by pristimerin contributes to overcoming ABCB1-mediated chemotherapeutic drug resistance *in vitro*. *Oncol. Rep.*, **2017**, *37*(1), 31-40.
- [215] Qiu, L.; Qiao, M.; Chen, Q.; Tian, C.; Long, M.; Wang, M.; Li, Z.; Hu, W.; Li, G.; Cheng, L.; Cheng, L.; Hu, H.; Zhao, X.; Chen, D. Enhanced effect of pH-sensitive mixed copolymer micelles for overcoming multidrug resistance of doxorubicin. *Biomaterials*, **2014**, *35*(37), 9877-9887.
- [216] Zhao, D.; Zhang, H.; Yang, S.; He, W.; Luan, Y. Redox-sensitive mPEG-SS-PTX/TPGS mixed micelles: An efficient drug delivery system for overcoming multidrug resistance. *Int. J. Pharm.*, **2016**, *515*(1), 281-292.
- [217] Zou, W.; Sarisozen, C.; Torchilin, V.P. The reversal of multidrug resistance in ovarian carcinoma cells by co-application of tariquidar and paclitaxel in transferrin-targeted polymeric micelles. *J. Drug Target.*, **2017**, *25*(3), 225-234.
- [218] Chen, Q.; Xu, M.; Zheng, W.; Xu, T.; Deng, H.; Liu, J. Se/Ru-Decorated Porous Metal-Organic framework nanoparticles for the delivery of pooled siRNAs to reversing multidrug resistance in taxol-resistant breast cancer cells. *ACS Appl. Mater. Interfaces*, **2017**, *9*(8), 6712-6724.
- [219] Chen, F.-y.; Zhang, Y.; Chen, X.-y.; Li, J.-q.; Xiao, X.-p.; Yu, L.-l.; Tang, Q. Development of a hybrid paclitaxel-loaded arsenite nanoparticle (HPAN) delivery system for synergistic combined therapy of paclitaxel-resistant cancer. *J. Nanopart. Res.*, **2017**, *19*(4), 155.
- [220] Saw, P.E.; Park, J.-K.; Jon, S.; Farokhzad, O.C. A drug-delivery strategy for overcoming drug resistance in breast cancer through targeting of oncofetal fibronectin. *Nanomedicine*, **2017**, *13*(2), 713-722.
- [221] Vallo, S.; Köpp, R.; Michaelis, M.; Rothweiler, F.; Bartsch, G.; Brandt, M.P.; Gust, K.M.; Wezel, F.; Blaheta, R.A.; Haferkamp, A. Resistance to nanoparticle albumin-bound paclitaxel is mediated by ABCB1 in urothelial cancer cells. *Oncol. Lett.*, **2017**, *13*(6), 4085-4092.
- [222] Curran, S.; Achilli, T.-M.; Leary, E.; Wilks, B.T.; Vantangoli, M.M.; Boekelheide, K.; Morgan, J.R. A 3D spheroid system to evaluate inhibitors of the ABCG2 transporter in drug uptake and penetration. *Technology*, **2015**, *03*(01), 54-63.
- [223] Vesel, M.; Rapp, J.; Feller, D.; Kiss, E.; Jaromi, L.; Meggyes, M.; Miskei, G.; Duga, B.; Smuk, G.; Laszlo, T.; Karner, I.; Pongracz, J.E. ABCB1 and ABCG2 drug transporters are differentially expressed in non-small cell lung cancers (NSCLC) and expression is modified by cisplatin treatment via altered Wnt signaling. *Respir. Res.*, **2017**, *18*(1), 52.
- [224] Akasov, R.; Drozdova, M.; Zaytseva-Zotova, D.; Leko, M.; Chelushkin, P.; Marc, A.; Chevalot, I.; Burov, S.; Klyachko, N.; Vandamme, T. Novel doxorubicin derivatives: synthesis and cytotoxicity study in 2D and

- 3D *in Vitro* Models. *Adv. Pharm. Bull.*, **2017**, 7(4), 593-601.
- [225] Ding, Y.; Liu, W.; Yu, W.; Lu, S.; Liu, M.; Kaplan, D.L.; Wang, X. Three-dimensional tissue culture model of human breast cancer for the evaluation of multidrug resistance. *J. Tissue Eng. Regen. Med.*, **2018**, 12(9), 1959-1971.
- [226] Breslin, S.; O'Driscoll, L. The relevance of using 3D cell cultures, in addition to 2D monolayer cultures, when evaluating breast cancer drug sensitivity and resistance. *Oncotarget*, **2016**, 7(29), 45745.
- [227] Tiwari, A.K.; Sodani, K.; Dai, C.L.; Abuznait, A.H.; Singh, S.; Xiao, Z.J.; Patel, A.; Talele, T.T.; Fu, L.; Kaddoumi, A.; Gallo, J.M.; Chen, Z.S. Nilotinib potentiates anticancer drug sensitivity in murine ABCB1-, ABCG2-, and ABCC10-multidrug resistance xenograft models. *Cancer Lett.*, **2013**, 328(2), 307-317.
- [228] Yhee, J.Y.; Song, S.; Lee, S.J.; Park, S.G.; Kim, K.S.; Kim, M.G.; Son, S.; Koo, H.; Kwon, I.C.; Jeong, J.H.; Jeong, S.Y.; Kim, S.H.; Kim, K. Cancer-targeted MDR-1 siRNA delivery using self-cross-linked glycol chitosan nanoparticles to overcome drug resistance. *J. Control. Release*, **2015**, 198, 1-9.
- [229] Qiu, J.-G.; Zhang, Y.-J.; Li, Y.; Zhao, J.-M.; Zhang, W.-J.; Jiang, Q.-W.; Mei, X.-L.; Xue, Y.-Q.; Qin, W.-M.; Yang, Y.; Zheng, D.-W.; Chen, Y.; Wei, M.-N.; Shi, Z. Trametinib modulates cancer multidrug resistance by targeting ABCB1 transporter. *Oncotarget*, **2015**, 6(17), 15494-15509.
- [230] Balaji, S.A.; Udupa, N.; Chamallamudi, M.R.; Gupta, V.; Rangarajan, A. Role of the drug transporter abcc3 in breast cancer chemoresistance. *PLoS One*, **2016**, 11(5), e0155013.
- [231] Zhu, Y.; Liu, C.; Armstrong, C.; Lou, W.; Sandher, A.; Gao, A.C. Antiandrogens Inhibit ABCB1 efflux and atpase activity and reverse docetaxel resistance in advanced prostate cancer. *Clin. Cancer Res.*, **2015**, 21(18), 4133-4142.
- [232] O'Connor, R.; Heenan, M.; Connolly, L.; Larkin, A.; Clynes, M. Increased anti-tumour efficacy of doxorubicin when combined with sulindac in a xenograft model of an MRP-1-positive human lung cancer. *Anticancer Res.*, **2004**, 24, 457-464.
- [233] Zhou, Q.; Ye, M.; Lu, Y.; Zhang, H.; Chen, Q.; Huang, S.; Su, S. Curcumin improves the tumoricidal effect of mitomycin c by suppressing abcg2 expression in stem cell-like breast cancer cells. *PLoS One*, **2015**, 10(8), e0136694.
- [234] Bradshaw-Pierce, E.L.; Pitts, T.M.; Tan, A.C.; McPhillips, K.; West, M.; Gustafson, D.L.; Halsey, C.; Nguyen, L.; Lee, N.V.; Kan, J.L.; Murray, B.W.; Eckhardt, S.G. Tumor P-glycoprotein correlates with efficacy of pf-3758309 in *in vitro* and *in vivo* models of colorectal cancer. *Front. Pharmacol.*, **2013**, 4, 22.
- [235] Rolff, J.; Dorn, C.; Merk, J.; Fichtner, I. Response of Patient-Derived Non-small cell lung cancer xenografts to classical and targeted therapies is not related to multidrug resistance markers. *J Oncol*, **2009**, 2009, 814140.
- [236] Winter, U.; Mena, H.A.; Negrotto, S.; Arana, E.; Pascual-Pasto, G.; Laurent, V.; Sunol, M.; Chantada, G.L.; Carcaboso, A.M.; Schaiquevich, P. Schedule-dependent antiangiogenic and cytotoxic effects of chemotherapy on vascular endothelial and retinoblastoma cells. *PLoS One*, **2016**, 11(7), e0160094.
- [237] Vlaming, M.L.; Mohrmann, K.; Wagenaar, E.; de Waart, D.R.; Elferink, R.P.; Lagas, J.S.; van Tellingen, O.; Vainchtein, L.D.; Rosing, H.; Beijnen, J.H.; Schellens, J.H.; Schinkel, A.H. Carcinogen and anticancer drug transport by Mrp2 *in vivo*: studies using Mrp2 (Abcc2) knockout mice. *J. Pharmacol. Exp. Ther.*, **2006**, 318(1), 319-327.
- [238] Pajic, M.; Iyer, J.K.; Kersbergen, A.; van der Burg, E.; Nygren, A.O.; Jonkers, J.; Borst, P.; Rottenberg, S. Moderate increase in Mdr1a/1b expression causes *in vivo* resistance to doxorubicin in a mouse model for hereditary breast cancer. *Cancer Res.*, **2009**, 69(16), 6396-6404.
- [239] van de Steeg, E.; van Esch, A.; Wagenaar, E.; Kenworthy, K.E.; Schinkel, A.H. Influence of human OATP1B1, OATP1B3, and OATP1A2 on the pharmacokinetics of methotrexate and paclitaxel in humanized transgenic mice. *Clin. Cancer Res.*, **2013**, 19(4), 821-832.

Received:

Revised:

Accepted:

CHAPTER 4

Chapter 4 presents the first experimental phase where the preliminary degree of inferred cytotoxicity was tested through the metabolic methyl thiazolyl tetrazolium assay.

PRELIMINARY *IN VITRO* CYTOTOXICITY SCREENING USING THE METHYL THIAZOLYL TETRAZOLIUM ASSAY

4.1 Introduction

Half-maximal inhibitory concentration (IC_{50}) is defined as the concentration of a specific compound needed to have an inhibitory effect on the biological activity of cells (*in vitro*) of 50% compared to an untreated control, under standardised conditions (Cortés *et al.*, 2001). It is described as one of the most commonly used pharmacological parameters to determine the effectiveness of drug compounds *in vitro* (Aykul & Martinez-Hackert, 2016). IC_{50} values are generally calculated through cytotoxicity assays such as the MTT colorimetric assay. The MTT assay allows cells exposed to serial dilutions of predetermined concentrations of a compound to be evaluated relative to cells that are left untreated (normalised response) throughout the experiment. This allows researchers to determine the relative effectiveness, potency and dose-response of the tested compounds on selected cancer cell lines, providing a guideline when advancing to *in vivo* studies (Nikzad *et al.*, 2014). The determination of IC_{50} values can be achieved in a high throughput manner with an acceptable degree of incurred cost, reproducibility and predictability of actual toxicity, thus making it an ideal parameter to determine the relative toxicity of selected chemotherapeutics (Shimoyama *et al.*, 1989).

4.2 The methyl thiazolyl tetrazolium assay

The MTT assay was incorporated into the study design as a preliminary screening tool, providing a general measure of the cytotoxicity potential of each of the selected drugs on the three SCLC cell lines. The MTT assay is able to determine a cell's ability to survive after exposure to cytotoxic compounds by measuring the metabolic capabilities of the cell population (Mosmann, 1983). Tetrazolium, initially a yellow water-soluble salt, is metabolised in the mitochondria of living cells through the cleavage of the tetrazole ring by cytochrome b and c sites (Berridge & Tan, 1993). The unique ability of this formazan salt to transfer across both the cell and mitochondrial membranes are attributed to the positively charged tetrazole ring that facilitates this movement through the negative membrane potential (Berridge *et al.*, 2005). The cleaved product of the metabolised tetrazolium is a

water-insoluble purple crystalline formazan, that can only be dissolved by organic solvents such as dimethyl sulfoxide (DMSO). By measuring the absorbance of the dissolved crystals through spectrophotometry, a positive correlation can be drawn between the number of crystals produced and the number of living cells present in a sample (Fotakis & Timbrell, 2006; Li *et al.*, 2015).

It is important to note that, although reduction of the salt is primarily based in the cell mitochondria, it has been reported that MTT-salt reduction is also facilitated intracellularly through other factors such as nicotinamide adenine dinucleotide (NADH), succinate and pyruvate. Furthermore, the crystalline formation may damage some cellular membranes and quiescent cells may appear to be metabolically inactive. These characteristics may potentially contribute to a possible false positive result (Berridge *et al.*, 2005). In spite of these challenges, the MTT assay is a high throughput, cost-effective assay with highly reproducible results. And although it has been criticised for excluding some important aspects in viability testing, it is still regularly used as the primary source of quantification of living cells in chemotherapeutic studies (Alves *et al.*, 2016; Tabatabaei Mirakabad *et al.*, 2016).

For the purpose of this study, the MTT assay was employed as a preliminary screening method to determine the relative IC₅₀ ranges of the reference chemotherapeutic compounds chosen for this study, in each individual cell line. These ranges could then be applied, using more comprehensive and accurate viability assays, to characterise the cell models (as presented in **Chapter 4**).

4.3 Materials and Methods

4.3.1 Study design

The aim of this study was to validate the use of three derivatives of an SCLC cell line in parallel as *in vitro* SCLC cancer models, and in combination as an efflux based drug resistant cancer research platform. Each model cell line has specific drug resistance characteristics for this purpose. The NCI-H69V cell line was derived from the chemosensitive H69-parental cell line, and thus chosen as the control cell line (Khan *et al.*, 1991; Krohn *et al.*, 2014b). The H69AR line was chosen for the reported overexpression of the multidrug resistance-associated protein 1 (MRP-1) surface transporters, while NCI-H69/LX4 has hyperexpressed levels of the well-known multidrug resistance (MDR) inducing efflux transporter, P-glycoprotein (P-gp). The LLC-PK1 cell line (pig embryonic kidney line typical of normal kidney tubular epithelium) was included to view the general cytotoxic

effects of these drugs on noncancerous cells, thus providing a relative systemic toxicity prediction model.

To evaluate and validate these three SCLC cell lines as models for chemotherapeutic drug screening and MDR research, three well-known and characterised chemotherapeutic drugs were chosen as model compounds. Each has a different substrate-specificity towards MDR-inducing surface ABC-transporters. The first drug, cisplatin, has particular specificity towards the multidrug resistance-associated protein 2 (MRP-2) transporter sub-group and should be excluded from excessive primary transporter expulsion from any of the cell lines used in this study (Korita *et al.*, 2010). Irinotecan has been reported to be primarily effluxed by MRP-1 transporters and was therefore incorporated to confirm resistance of the H69AR cell line (Abdallah *et al.*, 2016b). Paclitaxel is a well-known P-gp substrate, indicating primary MDR effects of the NCI-H69/LX4 cell line (El-Araby *et al.*, 2017).

Although the cell lines were chosen for this study because of the abovementioned surface transporter characteristics, it is important to note that they do not exclusively express only one surface transporter. The model drugs were used in this study to view the different degrees of resistance of each cell line and to study the prevalent cellular interactions.

4.3.2 General materials and reagents

The specific details of the model compounds used in this study are presented in **Table 4.1**.

Other reagents and materials included 0.1 µm sterile filtered phosphate buffered saline (PBS) (Hyclone; Thermo Fisher Scientific, Johannesburg, South Africa); trypsin EDTA (Lonza; Whitehead Scientific (Pty) Ltd, Cape Town, South Africa), L-glutamine (Whitehead Scientific (Pty) Ltd), 1% non-essential amino acids (NEAA) (Lonza; Whitehead Scientific (Pty) Ltd), sodium chloride (Merck, Johannesburg, South Africa) and 1% penicillin/streptomycin (Separations, Johannesburg, South-Africa). Acrodisc 25mm PF syringe filters 0.8/0.2 µm supor membrane (Pall Corporation); Triton X-100, thiazolyl blue tetrazolium bromide, dimethyl sulfoxide (DMSO) and trypan blue solution were purchased from Sigma-Aldrich (Johannesburg, South Africa). Foetal bovine serum (FBS) 10%, cell culture flasks (75cm²) (Corning Inc.); Costar[®] flat bottom 96-well plates (Corning Inc.) and Costar[®] v-bottom 96-well plates (Corning Inc.) were purchased from The Scientific group (Johannesburg, South Africa).

Table 4.1: Model chemotherapeutic drugs used in this study

Drugs	Reference number	Lot number	Company
Cisplatin European Pharmacopoeia (EP) Reference Standard	C2210000	6.0	Sigma-Aldrich, Johannesburg, South Africa
Paclitaxel from <i>Taxus brevifolia</i> , ≥95% (HPLC), powder	T7402	MKBZ4464V	Sigma-Aldrich, Johannesburg, South Africa
Irinotecan hydrochloride	I1406	WXBC3715V	Sigma-Aldrich, Johannesburg, South Africa

4.3.3 Culturing of small cell lung carcinoma cell lines

Derivatives of the pleural fluid isolated parental SCLC cell line (H69) were cultured in 75 cm² cell culture flasks at 37°C with 5% CO₂ and 95% humidified air in an ESCO CelCulture CO₂ incubator (ESCO Micro Pte Ltd; Changi, Singapore). Sub-culturing was initiated when the cultures reached 80% confluence as assessed visually through light microscopy (**See Appendix D**).

4.3.3.1 NCI-H69V chemosensitive cell line

The NCI-H69V (ECACC #91091803) cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco; Thermo Fisher Scientific) supplemented with 2 mM L-glutamine, 10% FBS, 1% NEAA and 1% penicillin/streptomycin. The NCI-H69V cells were sub-cultured through trypsinisation, and the growth medium was replaced every third day.

4.3.3.2 H69AR MRP-1 hyperexpressive cell line

The H69AR (ATCC® #CRL11351™) cell line was cultured in RPMI 1640 medium (ATCC modification) (Gibco; Thermo Fisher Scientific) supplemented with 20% FBS, 2 mM

L-glutamine, 1% NEAA and 1% penicillin/streptomycin. The H69AR cells were sub-cultured through trypsinisation and growth medium was replaced every second day.

4.3.3.3 NCI-H69/LX4 P-gp hyperexpressive cell line

The NCI-H69/LX4 (ECACC #96042329) cell line was cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 0.4 µg/ml doxorubicin hydrochloride (Sigma-Aldrich), 10% FBS, 1% NEAA and 1% penicillin/streptomycin. No trypsinisation was necessary as the cells are cultured as aggregates in suspension. As soon as this cell line reached 80% confluence ($\pm 4 \times 10^6$ cells/ml), it was sub-cultured to avoid overgrowth.

4.3.3.4 LLC-PK1 (Porcine kidney) cell line

The LLC-PK1 (ATCC® CL-101™) cell line was cultured in high glucose (4.5 mg/L) Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine (4mM), sodium pyruvate and phenol red (Hyclone; Thermo Fisher Scientific), further supplemented with 2 mM L-glutamine, 10% FBS, 1% NEAA and 1% penicillin/streptomycin. The LLC-PK1 cells were sub-cultured through trypsinisation and growth medium was replaced every second day.

4.3.4 Seeding of cells in 96-well plates

4.3.4.1 Seeding of the adherent cell lines: NCI-H69V, H69AR and LLC-PK1

After the cells reached the desired confluence of $\pm 80\%$, they were subjected to trypsinisation. Cells were washed twice with 10 ml PBS, and 3 ml trypsin was added to detach the cells. The flasks were incubated at 37°C for 4 min (9-10 min for LLC-PK1), and after detachment 1 ml FBS was added to speed up the recovery of the cells. The trypsin was deactivated through the addition of 5 ml of growth medium, and the cells were washed from the flask surface with a serological pipette. The entire cell suspension was transferred to a 15 ml tube and centrifuged in an EBA21 bench top centrifuge (Hettich, Tuttlingen, Germany) at $140 \times g$ for 5 min. The supernatant was removed and the cell pellet re-suspended in 5 ml growth medium. The cell suspension was counted with the trypan blue live staining method (0.4%) with a haemocytometer, using an inverted light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan), as previously described (Stoddart, 2011).

The cell suspensions were diluted to 35 000 cells/ml (7 000 cells/well) for the NCI-H69V and LLC-PK1 cell lines, while the H69AR line had to be seeded at 80 000 cells/ml (16 000

cells/well). These concentrations were predetermined to ensure the cells reached optimal confluence (80%) in the 96-well plates after 96 h incubation without overgrowth. The cells were seeded in a final volume of 200 μ l per well in flat-bottomed 96-well plates and incubated at 37°C with 5% CO₂ and 95% humidified air for 24 h. To ensure that the edge effect had no impact on the experimental procedures, the outer wells were filled with PBS.

4.3.4.2 Seeding of the NCI-H69/LX4 suspension cell line

The NCI-H69/LX4 cells grow as a cell suspension with no significant adherent characteristics, although individual cells tend to lose their ability to stay in suspension after a few passages and can lightly adhere to the flask surface. These cells were scraped with a cell scraper from the surface to detach them. The cell suspension was transferred to a 15 ml tube and centrifuged at 200 x *g* for 5 min. The supernatant was removed and the cell pellet was re-suspended in 5 ml growth medium.

Due to these cells' sensitivity to trypan blue, the Scepter 2.0 automated cell counter, (Millipore, Massachusetts, USA) with 60 μ m sensors was used to determine the cell concentration in the suspension. The cell suspension was counted in duplicate by adding 100 μ l cell suspension to 900 μ l PBS and correcting the final count with a dilution factor of 10. The primary cell suspension was diluted in growth medium to 150 000 cells per/ml (30 000 cells/well) and 200 μ l/well was pipetted into V-bottom 96-well plates. The seeded plates were incubated at 37°C with 5% CO₂ and 95% humidified air for 24 h. In order to prevent an edge effect, the outer wells were filled with PBS.

4.3.5 Preparation of model chemotherapeutic drugs

A fresh stock solution of each model compound was prepared on a weekly basis, and aliquots were stored at the respective optimal storage conditions. Each specific stock solution was subjected to serial dilution in complete medium with additives (except NCI-H69/LX4), to create the concentration series used to determine the approximate IC₅₀ values of each model compound in each cell line. The stock solutions for the NCI-H69/LX4 line were prepared in growth medium with no added doxorubicin hydrochloride, to prevent possible drug-drug interactions.

The concentration series were prepared daily in microcentrifuge tubes and stored in a block heater (Stuart scientific, Staffordshire, UK) at 37°C until dosage commenced. All microcentrifuge tubes were briefly mixed on a vortex mixer (Benchmark Scientific Inc., New

Jersey, USA). For compounds with very poor solubility in aqueous solutions DMSO was used as a solvent, never exceeding final exposure of the cells after dilution of more than 0.1% (Chen & Thibeault, 2013). A vehicle control was included where necessary.

Adherent cells were treated daily by removing the spent medium and replacing it with 200 μ l growth medium containing the specified drug concentrations. Each concentration was evaluated in triplicate. The plates containing the suspension NCI-H69/LX4 line were first centrifuged at 250 x g for 5 min in a 3-16 KL benchtop centrifuge (Sigma, Osterode am Harz, Germany) before exchanging the growth medium containing the model compounds. Each pellet was carefully agitated to re-suspend the cells.

4.3.5.1 Cisplatin

According to the European Pharmacopoeia, cisplatin is characterised as a water-soluble (0.25 g/100 ml) powder that needs to be protected from light due to photosensitivity of the compound. To limit premature activation and possible destabilisation of the drug, a 2 mM stock solution was prepared in marginally acidic 0.9% saline (distilled, autoclaved water with sodium chloride powder (Merck, Johannesburg, South Africa; pH 5.5) (Williams & Lokich, 1992). The saline solution was subjected to filter sterilisation (0.2 μ m) before dissolving the cisplatin powder. The sterilised saline was added to the cisplatin powder and the solution was placed in a block heater at 37°C to assist with solubilisation. Aluminium foil was used to cover the stock solution and it was vortexed until total dissolution was evident. The stock was divided into 4 aliquots and stored between 2 – 8 °C for a maximum of 1 week. In this formulation state cisplatin was characterised as being stable for up to 30 days, but to ensure the credibility of the compound a fresh solution was prepared every week during the cytotoxicity tests to avoid slow transformation to the trans-conformation in solution (Karbownik *et al.*, 2012).

4.3.5.2 Irinotecan

Irinotecan is described as a pale yellow powder that is soluble in DMSO at approximately 20 mg/ml and should be stored at -20°C. The preparation of the stock solution was based on a recent publication (Wissing *et al.*, 2013), and the concentrations ranged between 1.4×10^{-2} M and 1.53×10^{-4} M, depending on the working solutions of the experiment. A stock solution was prepared using DMSO as an organic solvent and stored at -20°C in 4 separate aliquots. The stock solution was thawed daily in a block heater at 37°C and diluted with growth medium.

4.3.5.3 Paclitaxel

Paclitaxel is described as a water-insoluble white powder that is soluble in organic solvents such as DMSO at approximately 171 mg/ml. The stock solution concentrations ranged between 1.054×10^{-3} M and 1.054×10^{-4} M, depending on the working solutions of the experiment. A stock solution was prepared using DMSO as an organic solvent and stored at -20°C in 4 separate aliquots. As a precautionary measure, all of the stock samples were stored covered in aluminium foil to avoid any exposure to light (Duggett *et al.*, 2017). The stock solution was thawed daily in the block heater at 37°C and diluted with growth medium. These dosages were kept in the block heater at 37°C until treatment.

4.3.6 MTT cytotoxicity assay

The various cell lines were seeded at predetermined densities, to reach approximately 80% confluence at the time of the MTT assay. Treatment was initiated 24 h after seeding (T0), and the MTT cytotoxicity assay was initiated after 96 h of drug exposure (T96). The tetrazolium powder was freshly prepared at 5 mg/ml in PBS and administered to the cells at a final concentration of 0.5 mg/ml. Due to the light-sensitive nature of MTT, all experiments were performed with the overhead laminar flow hood lights switched off. Triton X-100 was prepared at a concentration of 0.2% in PBS to initiate the dead cell control. All solutions were preheated to 37°C in a water bath before use.

Each experimental plate contained a DMSO background control, untreated cells to indicate 100% cell viability (or no viability inhibition) and a dead cell control to represent 97-100% relative inhibition of cell viability.

4.3.6.1 Adherent cell line assay procedure

After exposure of the cells (H69V, H69AR and LLC-PK1) to the individual drugs in their respective concentration ranges for 96 h, the cells were washed with PBS after removing the growth medium by gently adding 100 μl of preheated PBS, immediately removing the PBS and then repeating this step. Following removal of the PBS, 180 μl of non-additive RPMI 1640 medium was added to act as the MTT carrier. A volume of 20 μl MTT was then added to bring the final volume of each well to 200 μl (0.5 mg/ml MTT exposure). Triton X-100 (200 μl) was added directly to the dead cell control wells with an exposure time of 15 min. The DMSO control wells were left throughout this procedure with their original medium. The 96-well plate was wrapped in aluminium foil and left to incubate at 37°C with 5% CO_2 and 95%

humidified air for 4 h. After incubation, the 200 μ l medium was replaced with 200 μ l DMSO in all of the wells (including the DMSO, untreated and dead cell control wells). The plate was placed on a compact rocker (Bio-Rad Laboratories Ltd., Johannesburg, South Africa) for 1 h to ensure all of the crystals were dissolved. The optical density was subsequently measured at a wavelength of 560 nm, with a background reading at 630 nm, using a SpectraMax Paradigm plate reader (Molecular devices, Separations, Johannesburg, South Africa).

4.3.6.2 Suspension cell line assay procedure

The V-bottom 96-well plates containing the H69-LX4 cell line were centrifuged at 250 x g for 5 min before removing the growth medium. Triton X-100 (0.2% in PBS) was administered to the dead cell control wells for 15 min. The washing procedure was facilitated by adding 100 μ l of non-additive medium to all of the wells except the dead cell controls and centrifuging the plate again. The medium was removed from the wells and 90 μ l of non-additive RPMI 1640 medium was added together with 10 μ l of MTT solution. With each addition of medium following centrifugation, the cells were properly agitated to ensure homogenous exposure.

After the addition of MTT, the plates were covered with aluminium foil and left to incubate at 37°C with 5% CO₂ and 95% humidified air for 4 h. The 96-well plate was centrifuged at 1 218 x g for 5 min to pellet floating crystals after the incubation period. After removing 50 μ l MTT-containing medium and replacing it with 150 μ l DMSO, the crystals were left to dissolve while the plate was on a compact rocker for 1 h. The reading of the optical density followed the same procedures as that of the adherent cell lines.

4.3.7 Data analyses

The amount of formazan crystals produced is directly proportional to the number of viable cells present (Stepanenko & Dmitrenko, 2015). The solution of these purple coloured crystals dissolved in DMSO has a maximum absorbance at \pm 560 nm and can be analysed to determine cell viability. As an added measure, a standard wavelength was added of 630 nm to act as the background reference to eliminate the effect of light scattering, bubbles and cell debris (Riss *et al.*, 2004). The absorbance values for each of the groups (untreated control, dead cell control and dosage ranges) were expressed as a percentage cell viability, relative to the untreated control.

The following equation was used to determine the viability of the cells:

$$\% \text{ Cell viability} = (\Delta \text{ sample} - \Delta \text{ blank}) / (\Delta \text{ untreated control} - \Delta \text{ blank}) \times 100 \quad \text{Eq. 1.}$$

Δ Sample is the difference between the true wavelength value (560 nm) and that of the background wavelength (630 nm), measured for all of the groups on the plate (e.g. DMSO control, dead cell control, drug treatments). The equation can thus be defined as absorbance at 560 nm – absorbance at 630 nm.

Δ Blank is the difference between the true wavelength value (560 nm) and that of the background wavelength (630 nm), measured for the DMSO control. The equation can thus be defined as DMSO blank at 560 nm – DMSO blank at 630 nm.

Δ Untreated control is the difference between the true wavelength value (560 nm) and that of the background wavelength (630 nm), measured for the untreated control. The equation can thus be defined as untreated control at 560 nm – untreated control at 630 nm.

The following equation was used to determine the relative percentage inhibition of cell viability (IC):

$$\% \text{ IC} = 100\% - \% \text{ cell viability} \quad \text{Eq. 2.}$$

Where cell viability was calculated with Equation 1.

The IC_{50} values of each model compound in each cell line were defined as the drug concentration inhibiting cell viability to 50% of the untreated control, as determined following the MTT assay.

The following equation was used to determine the relative resistance ratios inferred by the MDR cell lines towards each drug.

$$\text{RR}_{\text{drug}}: \text{IC}_{50} (\text{MDR}) / \text{IC}_{50} (\text{chemosensitive}) \quad \text{Eq. 3.}$$

Where RR_{drug} refers to the resistance ratio of a multidrug resistant cell line for a specific chemotherapeutic drug compared to the chemosensitive cell line. IC_{50} (MDR) is the dosage needed to cause a viability reduction of 50% of the cell population in a multidrug resistant cell line (H69AR or NCI-H69/LX4 cell line), and IC_{50} (sensitive) is the dosage needed to cause a viability reduction of 50% of the cell population in a drug sensitive cell line (NCI-H69V cell line).

4.3.8 Statistical analysis

All the treatments were performed in triplicate. The MTT data was analysed with SPSS statistical analysis software (IBM Analytics, Version 25). The IC₂₅, IC₅₀ and IC₇₅ values for each treatment on each cell line were calculated by using the Probit Analysis Method, which included both the lower and upper 95% confidence limits. The best fit model was used to determine the regression analysis.

4.4 Results and Discussion

4.4.1 Cisplatin

4.4.1.1 Cell viability inhibition of the LLC-PK1 cell line following treatment with cisplatin.

The LLC-PK1 cell line was treated with cisplatin between 25 nM and 10 000 nM, and the % relative cell viability inhibition (compared to the untreated cell control) as measured with the MTT assay, is presented in **Figure 4.1**. Although the relative viability inhibition was for the most part below 20%, it was still a marked response. This reaffirms the inherent toxicity of many chemotherapeutic drugs (Bahl *et al.*, 2006). The relative cell viability inhibition for the higher dosages exceeded 90%, which almost matched the dead cell control. This highlights the toxic nature of cisplatin and highlights the irrelevance of dosing cisplatin in such extreme concentrations due to unacceptable levels of cytotoxicity in healthy cells (Pabla & Dong, 2008). This is also the reason why cisplatin is mainly administered in lower concentrations in combination with other drugs in the treatment of SCLC (Eckardt *et al.*, 2006; Natale *et al.*, 2008; Zatloukal *et al.*, 2010a).

The Cisplatin IC₂₅, IC₅₀ and IC₇₅ values in the LLC-PK1 cell line, determined by means of Probit analysis of the MTT data, and their 95% limit ranges are presented in **Table 4.2**. These IC values can be compared to the extrapolated observable values seen in **Figure 4.1**, and the high level of correlation substantiates the statistical values calculated and supports the conclusions made in this chapter.

Table 4.2: Cell viability inhibition concentrations (IC) of cisplatin on the LLC-PK1 cell line (nM), as determined with Probit analysis

LLC-PK1			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC ₂₅	603.60	332.10	870.50
IC ₅₀	2619.60	2314.90	2960
IC ₇₅	4635.60	4207.10	5140.10

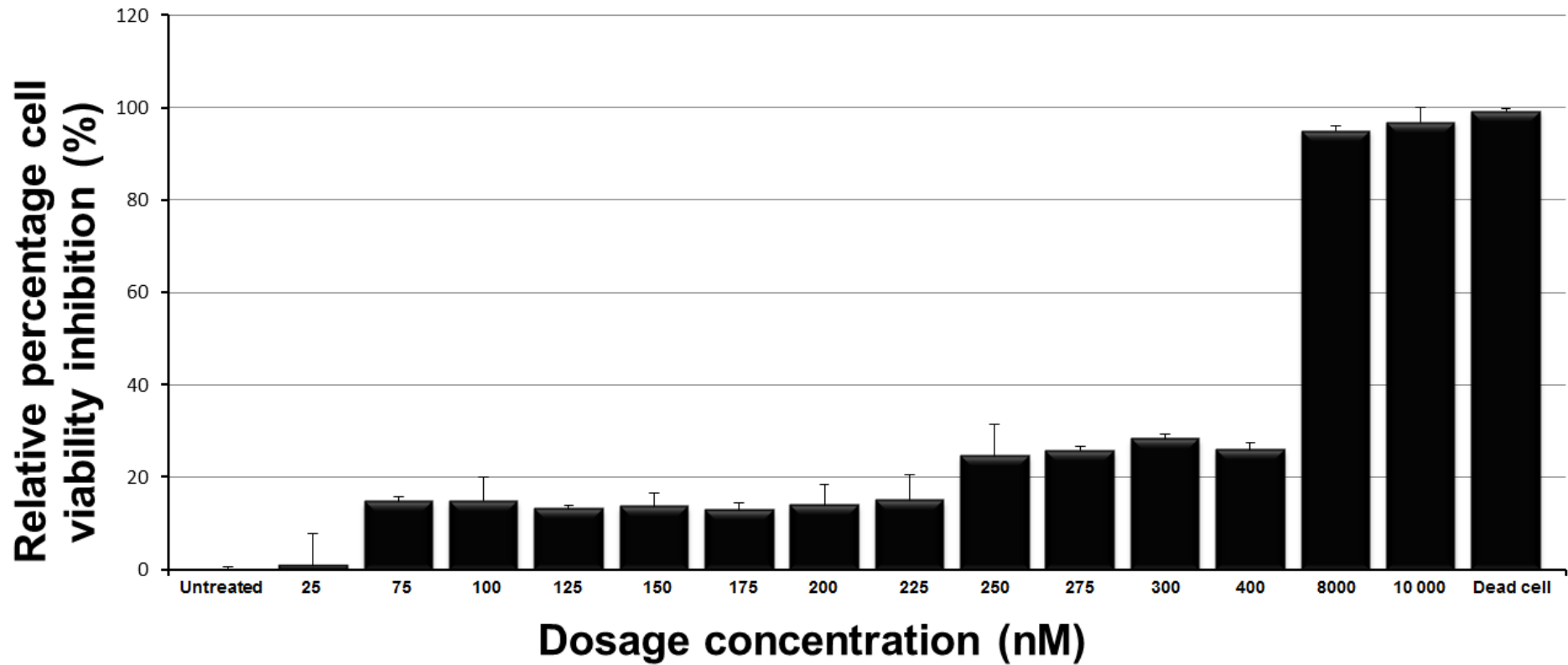


Figure 4.1: Percentage cell viability inhibition following treatment of the LLC-PK1 cell line with cisplatin, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

4.4.1.2 Cell viability inhibition of the NCI-H69V cell line following treatment with cisplatin

The NCI-H69V cell line was treated with cisplatin between 25 nM and 400 nM for 96 h, and the relative % cell viability inhibition (compared to the untreated control) as determined with the MTT assay is presented in **Figure 4.2**. Cisplatin platinum-based therapy is described as an essential part of first-line therapy when designing a chemotherapeutic regimen for SCLC (Abrams *et al.*, 2003; Dasari & Tchounwou, 2014). Following exposure of the chemosensitive NCI-H69V cell line to multiple concentrations of the compound, the data ranged from 11.26% relative inhibition potential (25 nM) to 90.66% (400 nM). The extensive inhibitory concentration range presented in **Figure 4.2** indicated that relatively low concentrations of Cisplatin were needed to have cytotoxic effects on the NCI-H69V SCLC line, and a dose-dependent effect was clearly evident. Standard deviations between the triplicate concentration range values were relatively small and major deviations, such as seen for 25 nM (11.7%), can be attributed to biological variation and lower repeatability when cells are exposed to minimally responsive concentrations of the compound. Large standard deviations are commonly seen with ineffective drug concentrations due to some cells being able to fix broken DNA and initiating repair pathways to avoid cellular death, while others are unable to withstand the chemotherapeutic damage (Clewell & Andersen, 2016).

The IC₂₅, IC₅₀ and IC₇₅ values for cisplatin in the chemosensitive NCI-H69V cell line are presented in **Table 4.3**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.2**, thus confirming the accuracy of the method used to determine the IC values.

Table 4.3: Cell viability inhibition concentrations (IC) of cisplatin on the NCI-H69V cell line (nM), as determined with Probit analysis

NCI-H69V			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC ₂₅	102.86	90.45	114.16
IC ₅₀	170.75	158.86	181.47
IC ₇₅	283.47	271.52	296.40

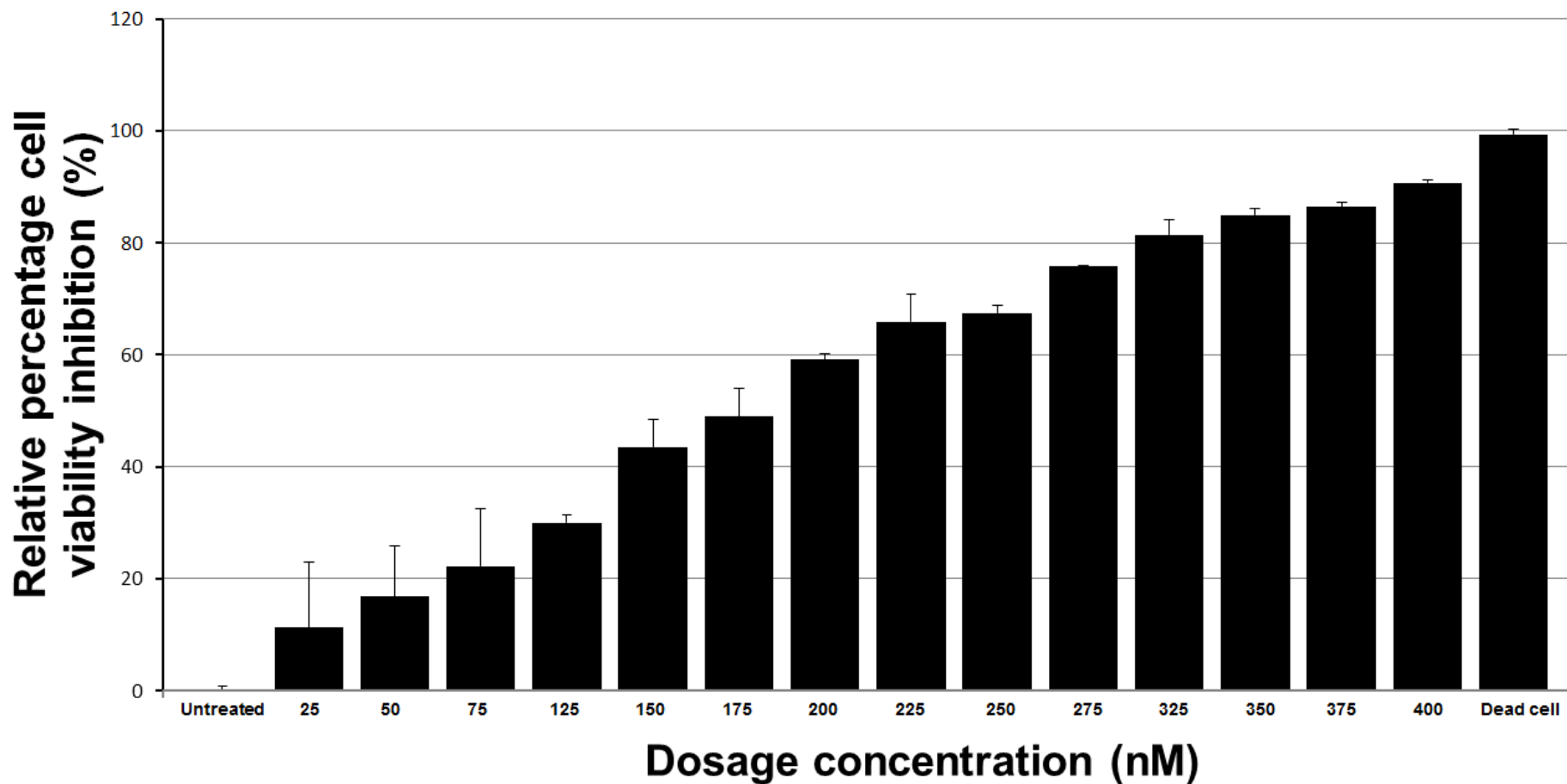


Figure 4.2: Percentage cell viability inhibition following treatment of the NCI-H69V cell line with cisplatin, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

No publications presenting IC_{50} values for cisplatin in the NCI-H69V cell line could be found. Although such values are available for the parental line (NCI-H69), the parental cell line does not provide an accurate depiction of the response of the NCI-H69V variant (Kasahara *et al.*, 1991; Stordal & Davey, 2007). A previous study comparing the NCI-H69V and parental cell line, NCI-H69, found major discrepancies between the two cell lines. This included major histological differences that notably increased resistance to chemotherapy in the NCI-H69V line (Krohn *et al.*, 2014a). When comparing the calculated IC_{50} value for cisplatin on the chemosensitive NCI-H69V line (170.75 nM) with that obtained from the noncancerous LLC-PK1 cell line (2 619.60 nM), it is evident that cisplatin has a much higher antitumorigenic effect than overall cytotoxicity. This supports its use as a chemotherapeutic drug for the treatment of SCLC.

4.4.1.3 Cell viability inhibition of the H69AR cell line following treatment with cisplatin

The chemoresistant (MRP-1 hyperexpressive) H69AR cell line was treated with cisplatin (25 nM and 500 nM), and the relative % cell viability inhibition (relative to the untreated control) as determined with the MTT assay is presented in **Figure 4.3**. The inhibition capacity of Cisplatin ranged between 16.67% (25 nM) and 61.65% (500 nM), thus allowing estimation of IC_{50} values.

The IC_{25} , IC_{50} and IC_{75} values for cisplatin in the MRP1 hyperexpressing chemo-resistant H69AR cell line are presented in **Table 4.4**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.3**, thus confirming the accuracy of the method used to determine the IC values.

The dosage cisplatin needed to induce a reduction of relative cell viability to 50% (compared to the untreated control) in the H69AR cells was 2.5 times higher than for the drug sensitive, NCI-H69V cell line. The highest dosage concentration of 400 nM, a concentration resulting in cytotoxic effects of more than 90% in NCI-H69V cells, was only marginally higher than the IC_{50} value in this experiment on the H69AR cell line; thus highlighting the resistance of this cell line to cisplatin (an MRP-2 substrate).

Table 4.4: Cell viability inhibition concentrations (IC) of cisplatin on the H69AR cell line (nM), as determined with Probit analysis

NCI-H69AR			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC₂₅	115.03	86.15	141.57
IC₅₀	427.34	382.35	482.83
IC₇₅	1588	1236	2261

This resistance can potentially be attributed to various factors, of which a degree of familial resistance between the multidrug resistance-associated proteins (MRP) may be one. It seems that even though primary expulsion is mediated through MRP-2 transporters, some resistance is mediated by the MRP-1 transporter group as seen in ovary cell lines (Itamochi *et al.*, 2002; Ueno *et al.*, 2018). Furthermore, resistance may be attributed to cell histology differences specifically influencing the cellular uptake of cisplatin (down-regulation of plasma membrane copper transporter), cellular efflux (up-regulation and presence of ABC-transporters/copper-extruding enzymes) and intracellular inactivation (e.g. Metallothioneins, GSH/GST) (Galluzzi *et al.*, 2011).

Despite the drastically increased resistance factor (2.5 times that of the chemosensitive dosage) of this cell line, cisplatin still exhibited therapeutically viable concentrations restricted to the nanomolar range. Although a previous study placed the IC₅₀ values of cisplatin on the H69AR cell line at about 120.1 - 131.7 nM, this differs from the estimation of this study (Heuser *et al.*, 2005). However, inter-laboratory variations, cell line passages, as well as culturing periods during the MTT assay could easily have affected the final outcome. Major inter-laboratory variations included the shorter exposure time (24-48h) of previous studies as well as differing cell line passages influenced the final drug concentrations throughout.

4.4.1.4 Cell viability inhibition of the NCI-H69/LX4 cell line following treatment with cisplatin

The drug resistant NCI-H69/LX4 (P-gp hyperexpressive) cell line was treated with cisplatin between 500 nM and 10 000 nM, and the relative % cell inhibition (compared to the untreated and dead cell controls) is presented in **Figure 4.4**. The inhibition capabilities of cisplatin were determined to be between 34.90 % (500 nM) and 99.20% (10 000 nM).

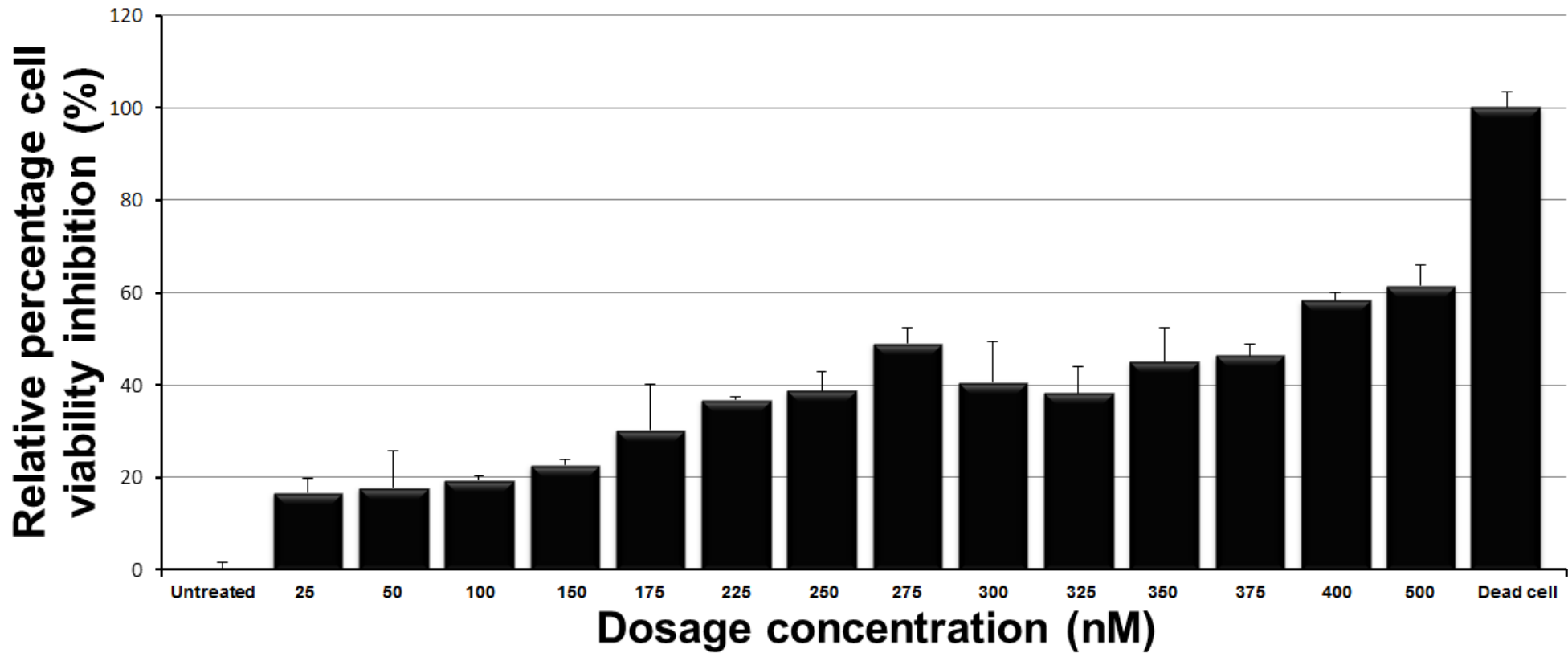


Figure 4.3: Percentage cell viability inhibition following treatment of the H69AR cell line with cisplatin, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

The IC₂₅, IC₅₀ and IC₇₅ values for cisplatin in the P-gp hyperexpressing chemo-resistant NCI-H69/LX4 cell line are presented in **Table 4.5**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.4**, thus confirming the accuracy of the method used to determine the IC values.

Table 4.5: Cell viability inhibition concentrations (IC) of cisplatin on the NCI-H69/LX4 cell line (nM), as determined with Probit analysis

NCI-H69/LX4			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC₂₅	620.41	345.17	943.21
IC₅₀	2155.45	1533.22	2769.73
IC₇₅	7488.59	6420.92	8626.68

From these results, it was clear that the NCI-H69/LX4 cell line required considerably higher concentrations of cisplatin to induce a tumoricidal effect, than in the other cell lines. A major resistance factor of 12.6 fold was calculated for this cell line compared to the sensitive NCI-H69V cell line. This drastic increase in resistance may possibly be attributed to the ability of the overexpressed P-gp transporter to efflux a broad spectrum of drugs, potentially including cisplatin, out of the cells (Ma *et al.*, 2017). It is, however, important to reiterate that although the LX cell line hyperexpress P-gp efflux transporters, MRP-2 transporters are not necessarily absent and may still play a significant role in resistance of the cell line to treatment. Even though lower concentrations of cisplatin were ineffective, a point of transporter saturation may have been reached at very high concentrations. The therapeutically questionable concentration of 10 000 nM did give a cell inhibition percentage of 99.20 %.

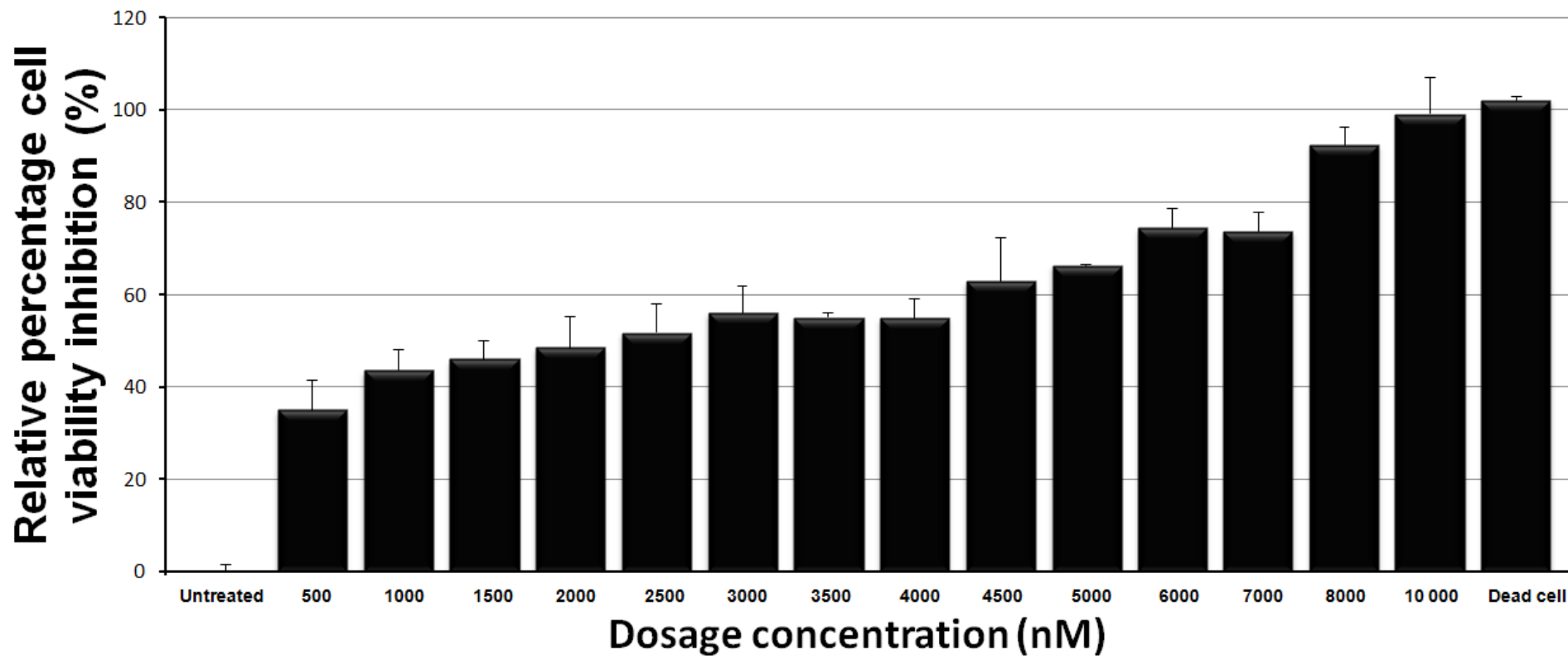


Figure 4.4: Percentage cell viability inhibition following treatment of the NCI-H69/LX4 cell line with cisplatin, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

4.4.1.5 Brief summary for cisplatin

The DNA-damaging chemotherapeutic drug cisplatin has a relatively low therapeutic dosing range when treating chemo-naive cancer. This can be seen when reviewing the effects of the nanomolar concentrations of cisplatin in the chemosensitive NCI-H69V cell line, with a relative viability reduction of 90.66% (400 nM) as seen in **Figure 4.2**. However, the effectiveness of this drug during initial treatment is overshadowed by its susceptibility to MDR inducing factors, such as being exported by the most prominent transporter families (MRP/P-gp). Due to extreme life-threatening adverse effects (e.g. nephrotoxicity, myelosuppression and anaphylaxis) reported in early clinical trials with high therapeutic dosages, simply increasing the drug concentration to combat MDR is clearly not a viable option (Von Hoff *et al.*, 1979). These nephrotoxic effects are illustrated in the porcine kidney 1-LLC cells, where approximately 20% of the healthy cell population were metabolically inhibited upon dosing with cisplatin at therapeutically effective levels (**Figure 4.1**). Larger standard deviations seen for low dosages throughout these experiments are discussed in **Section 4.4.1.2.2**.

Clinically, the susceptibility of cisplatin to being effluxed by the MDR inducing transporters is the reason why this drug is never administered as a monotherapeutic agent. Modern regimes use combination therapies (e.g. cisplatin/etoposide, cisplatin/irinotecan), with or without radiation, to treat both limited and extensive disease states (Turrisi *et al.*, 1999; Noda *et al.*, 2002; Hanna *et al.*, 2006; Saito *et al.*, 2006). The efficacy of these treatments can be attributed to the combinatory drugs having different mechanisms of action, as well as being substrates to different transporters. This multiple approach treatment plan lowers the chance of acquired MDR, but even these regimes are overshadowed by the inevitable relapse most SCLC patients experience (Jackman & Johnson, 2005; Rossi, 2016).

4.4.2 Irinotecan

4.4.2.1 Cell viability inhibition of the LLC-PK1 cell line following treatment with irinotecan

The noncancerous LLC-PK1 cell line was treated with irinotecan concentrations between 150 nM and 10 000 nM, and the % relative cell viability inhibition (compared to the untreated cell controls) is presented in **Figure 4.5**. The irinotecan concentrations showed minimal activity in the LLC-PK1 cell line as seen in **Figure 4.5**. Even when including concentrations as high as 10 000 nM, minimal inhibitory effects were witnessed (25.62 %). Therefore, irinotecan does not seem to have such high cytotoxicity compared to cisplatin, which is well-known for its detrimental effects on cancer patient health (Tsang *et al.*, 2009). The fluctuating inhibition seen throughout the lower concentration ranges (150 nM – 950 nM) can be attributed to biological variation and activated repair mechanisms (as mentioned in **Section 4.4.1.2.2**).

The IC_{25} , IC_{50} and IC_{75} values for irinotecan in the LLC-PK1 cell line are presented in **Table 4.6**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.5**, thus confirming the accuracy of the method used to determine the IC values.

Table 4.6: Cell viability inhibition concentrations (IC) of irinotecan on the LLC-PK1 cell line (nM), as determined with Probit analysis

LLC-PK1			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC_{25}	9055.70	7801.70	10665.60
IC_{50}	15534	13494.20	18398.20
IC_{75}	22012.30	19036.80	26280.70

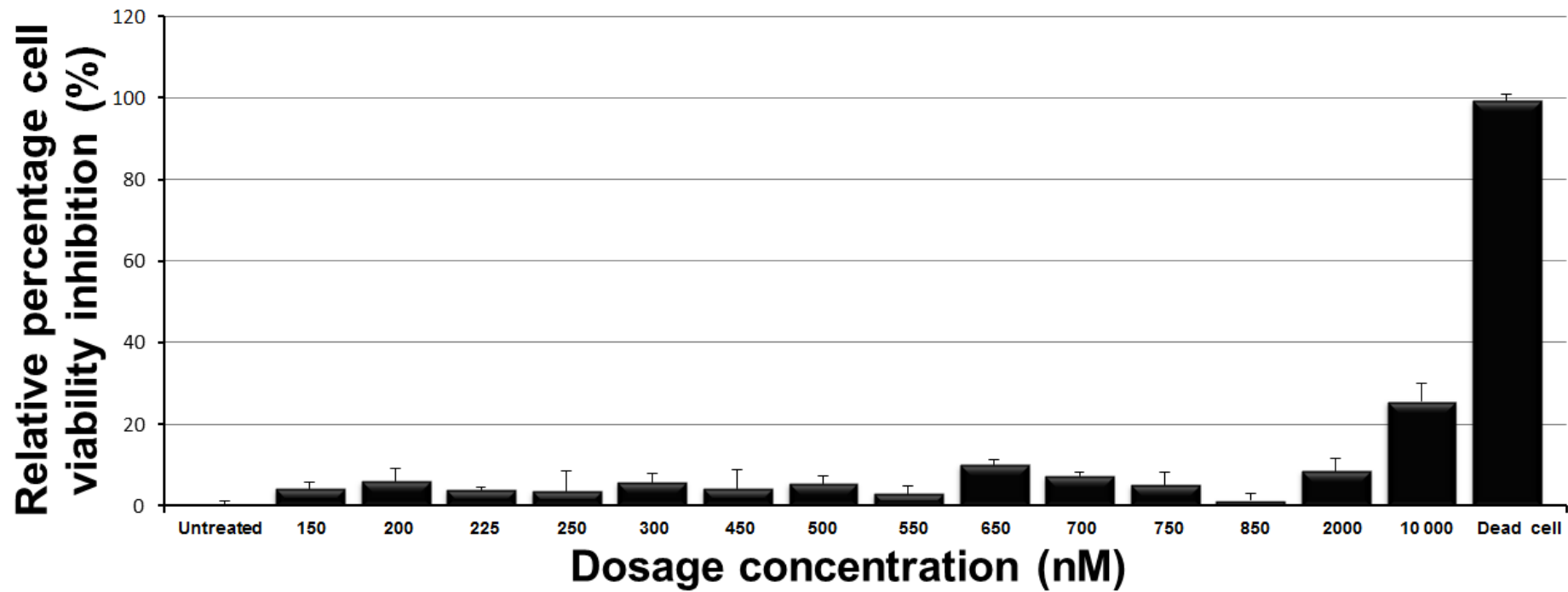


Figure 4.5: Percentage cell viability inhibition following treatment of the LLC-PK1 cell line with irinotecan, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

4.4.2.2 Cell viability inhibition of the NCI-H69V cell line following treatment with irinotecan

The chemosensitive H69V cell line was treated with irinotecan between 150 nM and 950 nM for 96 h, and the relative cell viability inhibition (compared to the untreated control) as determined with the MTT assay is presented in **Figure 4.6**. The potency of irinotecan was found to be lower than that of cisplatin and paclitaxel using this *in vitro* screening assay. This may be ascribed to the fact that irinotecan (CPT-11), a DNA topoisomerase inhibitor, is first activated through intracellular esterase (as mentioned in **Chapter 3**) before becoming potent. This relatively lower potency doesn't affect its clinical efficacy (as seen in the LLC-PK1 cell line) since dose-limiting cytotoxicity usually only occurs at much higher dosages, thus rendering this drug as a viable treatment option with slightly higher dosages (Ando *et al.*, 2004). Following exposure of the chemosensitive cell line to this cytotoxic pro-drug, an inhibition percentage ranging from 8.23% (150 nM) to 90.76 % (950 nM) was achieved, thus proving the efficacy of this drug in the treatment of SCLC (**Figure 4.6**).

The IC_{25} , IC_{50} and IC_{75} values for irinotecan in the chemosensitive NCI-H69V cell line are presented in **Table 4.7**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.6**, thus confirming the accuracy of the method used to determine the IC values.

Table 4.7: Cell viability inhibition concentrations (IC) of irinotecan on the NCI-H69V cell line (nM), as determined with Probit analysis

NCI-H69V			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC_{25}	160.35	115.38	200.78
IC_{50}	467.01	435.04	498.18
IC_{75}	773.68	738.81	811.46

The IC_{50} range obtained showed a close correspondence with *in vitro* cytotoxicity assays previously conducted on another drug sensitive SCLC cell line, H69, where they found the IC_{50} to be 259-850 nM (van Ark-Otte *et al.*, 1998). Van Ark-Otte also showed that there was a high prevalence of CES in general present in lung cancer cell lines. Therefore, it can be assumed that the NCI-H69V cells possess high levels of intracellular carboxylesterase (CES) necessary for the conversion of CPT-11 to the active substrate, SN38. To our knowledge, no studies currently exist in which this compound has been tested on the NCI-H69V cell line. A clear dose-dependent response was observed during the treatments, with the therapeutic concentrations remaining relatively low (in the nanomolar range). This drug can be deemed highly active in the chemosensitive SCLC cells. Standard deviations remain minimal and as mentioned before, the lower concentrations having the highest deviations can once again be attributed to biological variation.

4.4.2.3 Cell viability inhibition of the H69AR cell line following treatment with irinotecan

The resistant H69AR (MRP-1 hyperexpressive) cell line was treated with irinotecan between 20 nM and 12 000 nM for 96 h, and the % relative cell viability inhibition (compared to the untreated cell controls) as determined by the MTT assay is presented in **Figure 4.7**. Following exposure of the H69AR cell line to multiple concentrations of irinotecan, the data ranged between 4.30 % (20 nM) and 50.08 % (7 000 nM). Interestingly, a decline in inhibition to 48.51 % was noted at 12 000 nM. Irinotecan is described as a major substrate for MRP-1 efflux transporters, and resistance was expected in this series of experiments since the latter mentioned transporter is abundantly present in the H69AR cell membranes (Abdallah *et al.*, 2016a).

The IC_{25} , IC_{50} and IC_{75} values for irinotecan in the MRP1 hyperexpressive chemo-resistant H69AR cell line are presented in **Table 4.8**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.7**, thus confirming the accuracy of the method used to determine the IC values.

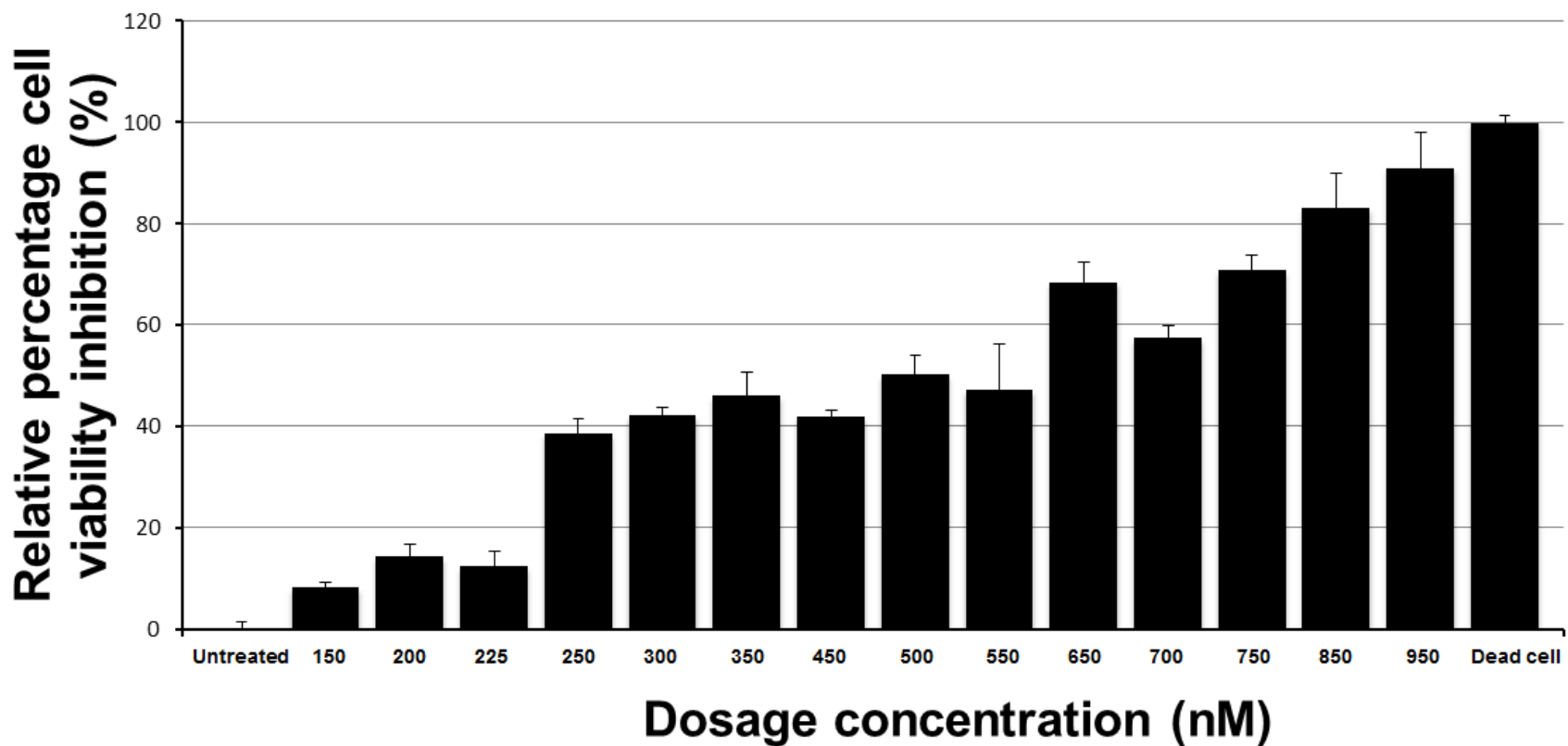


Figure 4.6: Percentage cell viability inhibition following treatment of the NCI-H69V cell line with irinotecan, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

Table 4.8: Cell viability inhibition concentrations (IC) of irinotecan on the H69AR cell line (nM), as determined with Probit analysis

H69AR			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC₂₅	-1145.20	-2278.70	-169.60
IC₅₀	7716.70	6925.50	8568.30
IC₇₅	16578.60	15236.10	18199.70

This cell line was proven to be 16.5 times more resistant towards irinotecan than its chemosensitive counterpart, NCI-H69V. An early plateau was already reached at 2 500 nM (42.87 %) and maintained (with minor standard deviations) up to a therapeutically non-viable concentration of 12 000 nM (48.51 %). At the highest concentration, the cells seemed to become more resistant and cell inhibition dropped slightly; thus indicating that they might have undergone possible cellular adaptations toward resistance. This trend proved that the inclusion of higher concentration ranges probably would not provide informative results since the cells could just continue to adapt. Standard deviations between the triplicate concentrations were relatively small throughout and major deviations, such as that seen at 200 nM, can be attributed to biological variation, especially seen in lower ineffective dosages where some cells in the population are affected and others are able to resist the effect of the chemotherapy. These reported deviations decrease drastically when exposing cells to more definitive concentrations, thus inducing cell death of vulnerable parts of the population early on and leaving a drug resistant homogenous cell line after 96 hours.

4.4.2.4 Cell viability inhibition of the NCI-H69/LX4 cell line following treatment with irinotecan

The P-gp hyperexpressive NCI-H69/LX4 cell line was treated with irinotecan between 30 000 nM and 200 000 nM, and the % relative cell viability inhibition (compared to the untreated control) as determined with the MTT assay is presented in **Figure 4.8**. Following exposure of the NCI-H69/LX4 cell line to multiple concentrations of irinotecan, inhibition ranges were determined to be between 46.40 % (30 000 nM) and 75.26 % (200 000 nM).

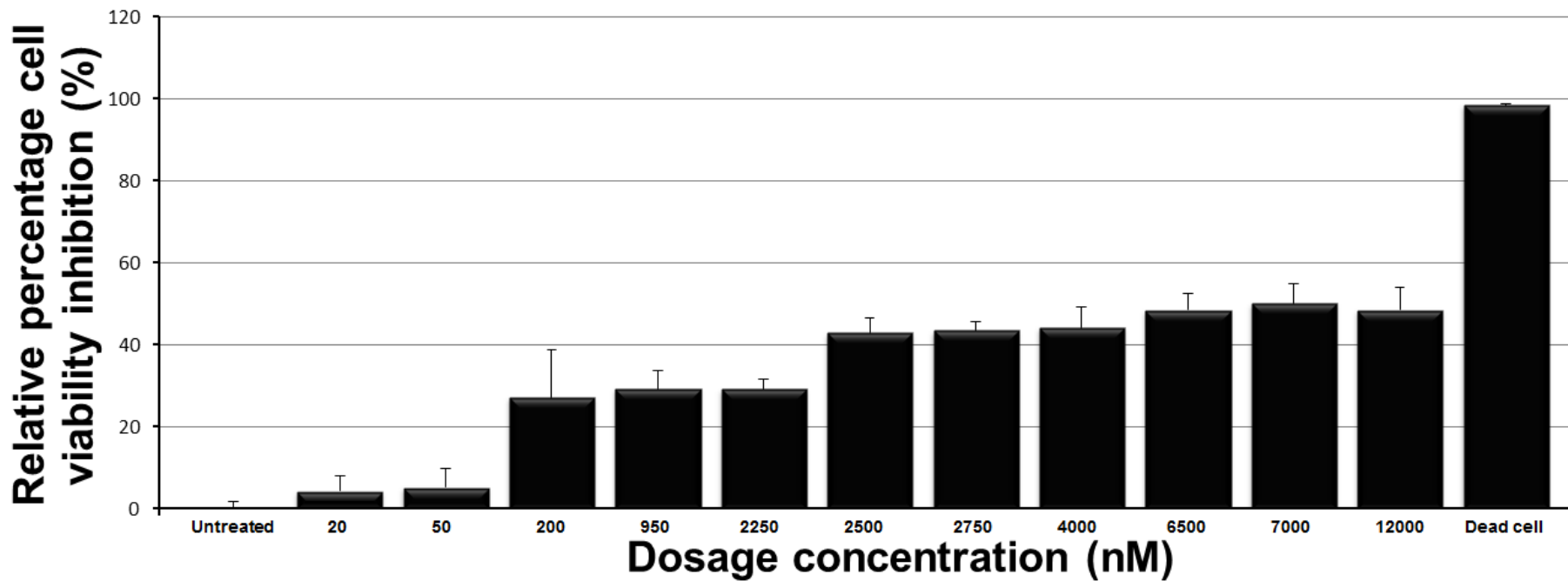


Figure 4.7: Percentage cell viability inhibition following treatment of the H69AR cell line with irinotecan, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

The IC₂₅, IC₅₀ and IC₇₅ values for irinotecan in the P-gp hyperexpressive chemo-resistant NCI-H69/LX4 cell line are presented in **Table 4.9**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.8**, thus confirming the accuracy of the method used to determine the IC values.

Table 4.9: Cell viability inhibition concentrations (IC) of irinotecan on the NCI-H69/LX4 cell line (nM), as determined with Probit analysis

NCI-H69/LX4			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC ₂₅	13 380.80	17 474.70	93 668.50
IC ₅₀	57 491.60	50 623.24	63 830.80
IC ₇₅	247 016	212 579.55	300 080

The resistance factor measured up to 123.1 folds that of the NCI-H69V sensitive line. The drug resistance seen in the NCI-H69/LX4 cell line can be attributed to irinotecan being largely subjected to efflux by the P-gp transporter (Yamamoto *et al.*, 2001; Bansal *et al.*, 2009; Tariq *et al.*, 2015). As mentioned before, irinotecan is a primary substrate for the MRP-1 efflux transporter, but the broad spectrum resistance conferred by P-gp towards various substances could have propagated a degree of resistance to irinotecan (Callaghan *et al.*, 2014). Furthermore, although the cell line is known to hyperexpress P-gp transporters, it does not imply that MRP-1 transporters are not abundant or absent. Even though excessive concentrations were needed to see a dose-dependent effect of irinotecan in this cell line, it was still clearly evident and an IC₅₀ range could be determined. Minimal standard deviations between triplicate concentration ranges were reported and substantiate the homogenous nature of this cell line, attributable to its maintenance in a cytotoxic agent (doxorubicin) that kills vulnerable parts of the cellular population.

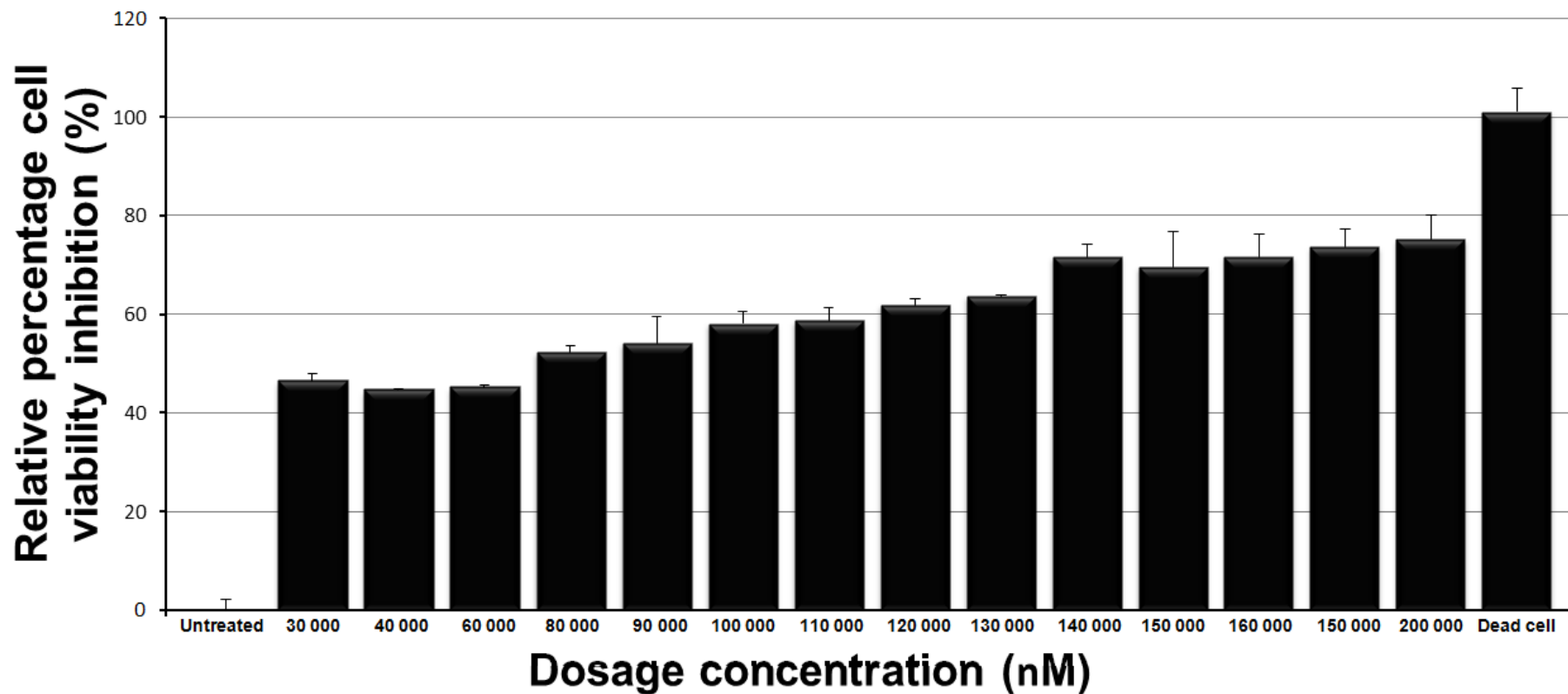


Figure 4.8: Percentage cell viability inhibition following treatment of the NCI-H69/LX4 cell line with irinotecan, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

4.4.2.5 Brief summary for irinotecan

Irinotecan as a prodrug provides its therapeutic profile with both advantages and disadvantages. Its prodrug form, CPT-11, ensures that prolonged therapeutic value can be achieved with high dosages while having a relatively low systemic toxicity potential. Major adverse effects that can restrict dosings, such as neutropenia and diarrhoea, are manageable due to a relatively slow conversion of the prodrug to the active cytotoxic metabolite (SN-38) by the liver (< 3%); thus decreasing systemic circulation (Lévesque *et al.*, 2013; Liu *et al.*, 2013). The disadvantage of this drug is that it is dependent on this conversion to become therapeutically active, which limits the exposure of cancer cells. As mentioned in **Chapter 2.4.2**, SCLC is reported to have high levels of the CES-enzyme needed to convert this drug, thus increasing its clinical efficacy in this specific cancer type. This selective toxicity was witnessed when the porcine kidney cells (LLC-PK1) were treated with the same dosages as those administered to the NCI-H69V drug sensitive cell line. Inhibition percentages as low as 1.36 % were observed at high concentrations (850 nM). This theory was further investigated by exposing the cells to 10 000 nM Irinotecan. Still, a relatively low inhibition of 25.62 % was found with this therapeutically unrealistic dosage. The considerably low cytotoxicity of this drug at therapeutic concentrations similar to that of cisplatin, emphasizes its selectivity towards SCLC cells.

Irinotecan induced a relatively effective drug response within the nanomolar range, thus reiterating the fact that this drug is an acceptable choice when treating SCLC. However, the major problem with this chemotherapeutic compound is its extreme susceptibility to the MRP-1 efflux transporters. While dosing the H69AR cell line, we required unrealistically high concentrations to overcome the MRP-1 efflux linked resistance in order to see at least 50% cell inhibition (7 716.70 nM). This concentration can be deemed as the turnover point where the cells no longer reacted to the irinotecan treatment. The inhibitory potential might have decreased with increased dosages, due to the overexpression of MRP-1 transporters on the cellular surfaces which propagates further resistance.

Although serious resistance was also witnessed in the NCI-H69/LX4 cell line (IC_{50} = 57 491.60 nM), adaptive mechanisms such as that seen in the H69AR cell line was not as prominent and 75% of the population was calculated to be inhibited at 247 016 nM. The generated results substantiate the use of irinotecan in present-day programs focused on combination therapy, especially when cancer MDR is an issue.

4.4.3 Paclitaxel

4.4.3.1 Cell viability inhibition of the LLC-PK1 cell line following treatment with paclitaxel

The noncancerous LLC-PK1 cell line was treated with paclitaxel between 0.25 nM and 20 nM and the % relative cell inhibition (compared to the untreated cell controls) as measured with the MTT assay is presented in **Figure 4.9**. As mentioned previously (**Section 4.4.1.1**) chemosensitive concentration ranges were incorporated into the LLC-PK1 cell dosing range. Negligible activity was witnessed throughout the dosages proven to be potent in previous studies dosing similar drug sensitive small cell lung cancer concentrations (Liebmann *et al.*, 1993a). This proves this cell line's ability to resist life-threatening toxicity during therapeutic dosages, but exposure to higher concentrations most likely present in chemo-resistant cells can have detrimental effects on the host in terms of nephrotoxicity. As mentioned earlier (**section 4.4.1.2.2**), relatively large standard deviations seen throughout the administered dosages are attributable to lower repeatability when cells are exposed to ineffective concentrations. This is due to cellular evasive and repair mechanisms being more effective with manageable dosages.

The IC₂₅, IC₅₀ and IC₇₅ values for Paclitaxel in the LLC-PK1 cell line are presented in **Table 4.10**. The IC values determined by means of Probit analysis and their 95% limit ranges can be directly correlated with the observable results seen in **Figure 4.9**, thus confirming the accuracy of the method used to determine the IC values.

Table 4.10: Cell viability inhibition concentrations (IC) of paclitaxel on the LLC-PK1 cell line (nM), as determined with Probit analysis

LLC-PK1			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC ₂₅	23.21	20.98	3.00x10 ¹¹
IC ₅₀	29.73	24.13	1.06x10 ²⁸
IC ₇₅	38.08	27.42	3.80x10 ⁴⁴

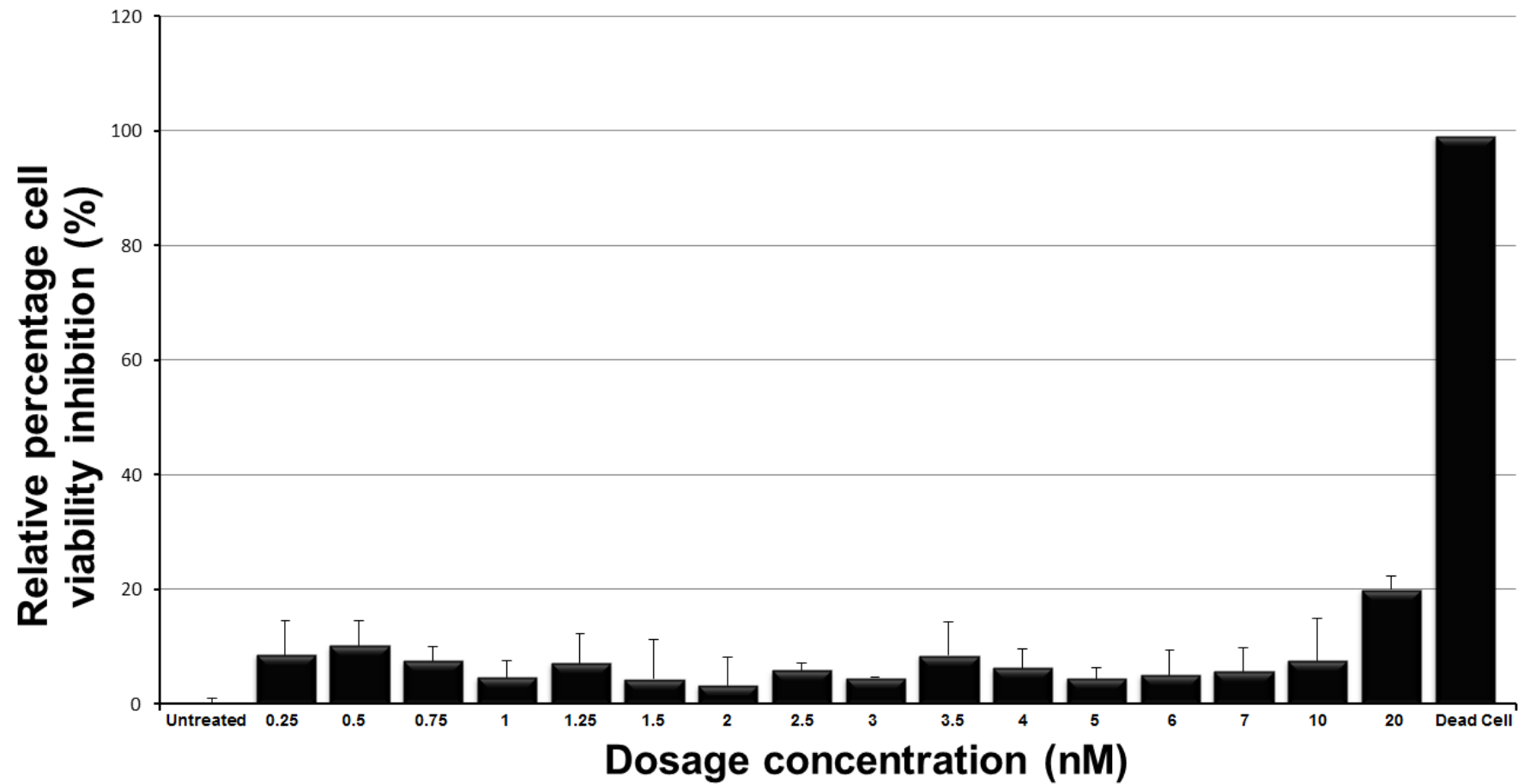


Figure 4.9: Percentage cell viability inhibition following treatment of the LLC-PK1 cell line with paclitaxel, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

4.4.3.2 Cell viability inhibition of the NCI-H69V cell line following treatment with paclitaxel

The chemosensitive NCI-H69V cell line was treated with paclitaxel between 0.25 nM and 3 nM for 96 h, and the relative % cell viability inhibition (compared to the untreated control) as determined with the MTT assay is presented in **Figure 4.10**. Paclitaxel, a microtubule targeting cytotoxic drug, proved to be immensely potent on the NCI-H69V cell line. Following exposure of the NCI-H69V cell line to multiple concentrations of the compound, cell inhibition was witnessed in dosage ranges as low as 0.25 nM (5.10 %) to 3 nM (57.84%); thus confirming the therapeutic value of this drug. These values are consistent with other human carcinoma cell lines which generally range between 2 nM - 20 nM ranges, thus reinforcing the validity of the paclitaxel results (Liebmann *et al.*, 1993b). A clear dose-dependent effect was witnessed. Reportable interdosage standard deviations, although minor, is present due to the small increase (0.5 nM) between dosages and the total therapeutic spectrum (0.25 nM to 3 nM) being immensely refined, thus possibly causing some cells to behave differently between triplicate concentrations.

The IC_{25} , IC_{50} and IC_{75} values for paclitaxel in the chemosensitive NCI-H69V cell line are presented in **Table 4.11**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.10**, thus confirming the accuracy of the method used to determine the IC values.

Table 4.11: Cell viability inhibition concentrations (IC) of paclitaxel on the NCI-H69V cell line (nM), as determined with Probit analysis

NCI-H69V			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC_{25}	1.78	1.64	1.91
IC_{50}	2.84	2.65	3.08
IC_{75}	4.53	4.05	5.26

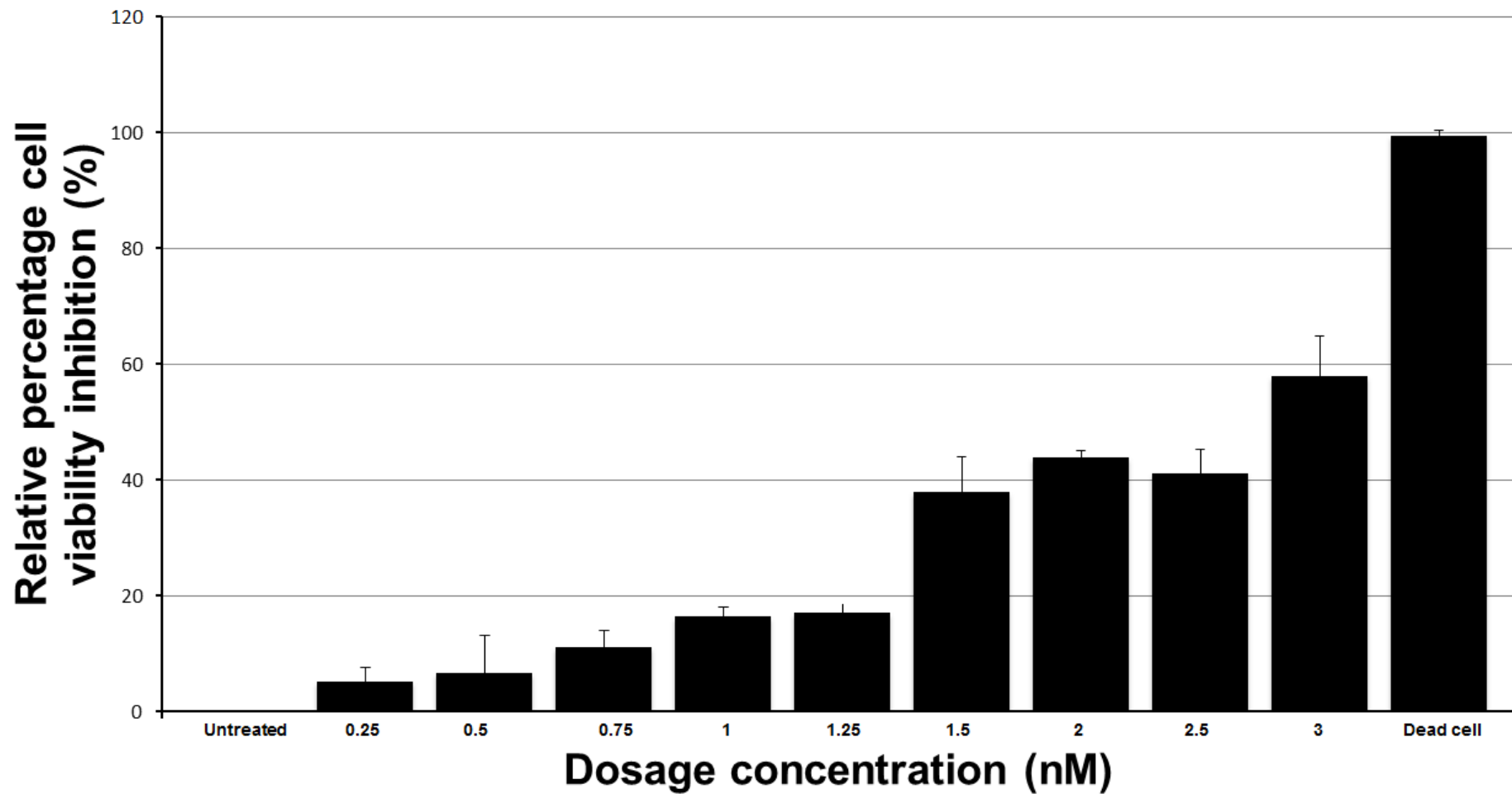


Figure 4.10: Percentage cell viability inhibition following treatment of the NCI-H69V cell line with paclitaxel, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

When comparing the IC values obtained from the NCI-H69V cell line with those from the non-cancerous LLC-PK1 line, it is clear that Paclitaxel has a higher anticancerous effect than overall cytotoxicity.

4.4.3.3 Cell viability inhibition of the H69AR cell line following treatment with Paclitaxel

The H69AR cell line was treated with paclitaxel between 0.25 nM and 6 nM for 96 h, and the relative % cell inhibition (compared to the untreated control) as determined with the MTT assay is presented in **Figure 4.11**. Due to the MRP-1 hyperexpressive nature of the H69AR cells and the minimal effect MRP-1 has on paclitaxel molecules, minor resistance capabilities compared to the sensitive cell line (NCI-H69V) was expected. Following exposure of the H69AR cell line to multiple concentrations of Paclitaxel, the inhibition data ranged from 14.10% (0.25 nM) to 70.32% (6 nM), thus providing a broad range for IC₅₀ value estimations.

The IC₂₅, IC₅₀ and IC₇₅ values for Paclitaxel in the MRP1 hyperexpressive chemo-resistant H69AR cell line are presented in **Table 4.12**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.11**, thus confirming the accuracy of the method used to determine the IC values.

Table 4.12: Cell viability inhibition concentrations (IC) of paclitaxel on the H69AR cell line (nM), as determined with Probit analysis

H69AR			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC₂₅	3.45	3.18	3.67
IC₅₀	4.75	4.54	4.97
IC₇₅	6.54	6.09	7.20

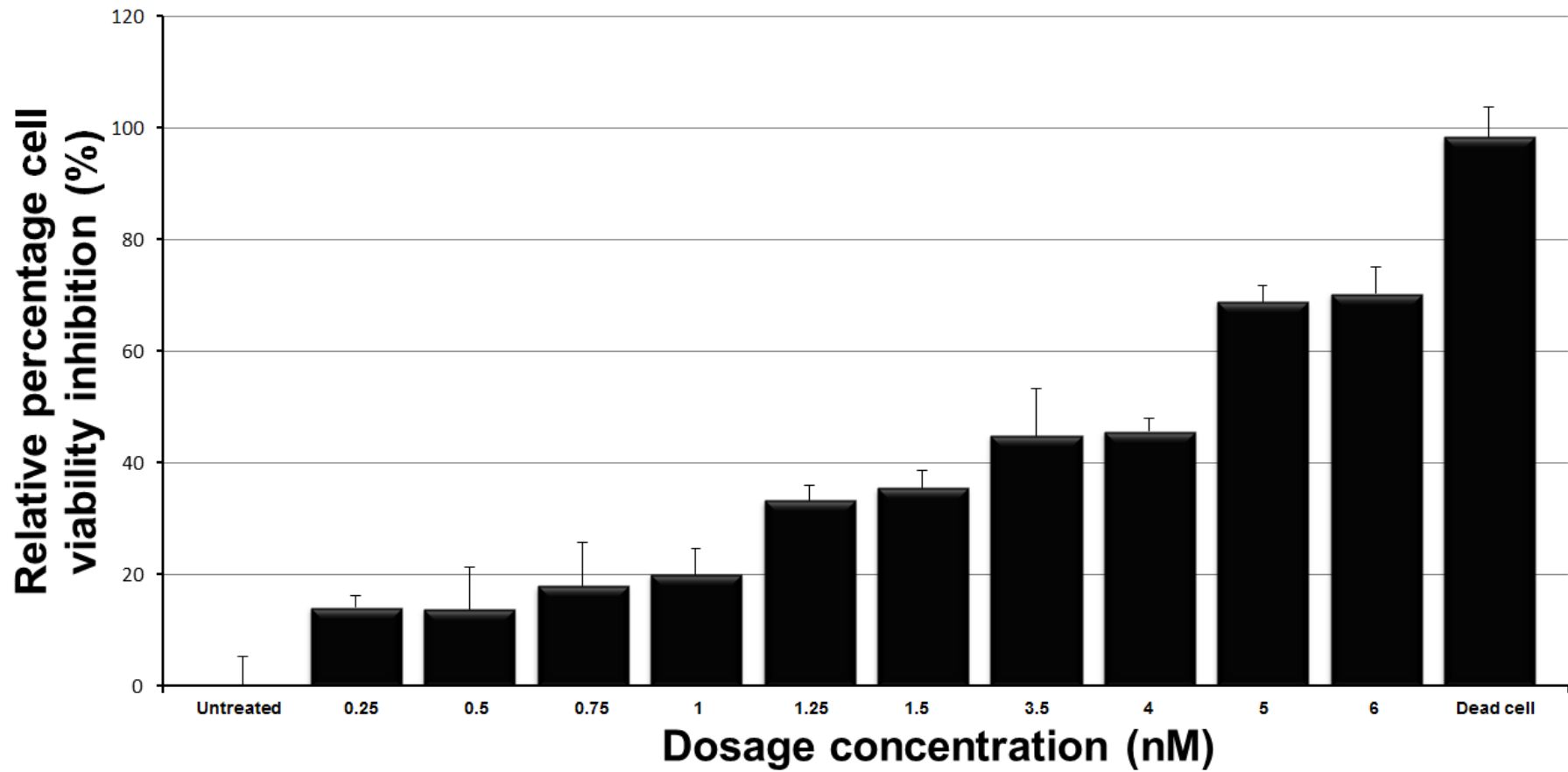


Figure 4.11: Percentage cell viability inhibition following treatment of the H69AR cell line with paclitaxel, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

Paclitaxel had a resistance factor 1.7 times that of the sensitive cell line. The slight increase in resistance against Paclitaxel, primarily a P-gp substrate, in an MRP-1 hyperexpressive cell line can possibly be attributed to numerous other molecular mechanisms, including adaptive changes in β -tubulin target molecules, changes in apoptotic proteins, changes in the composition of cellular lipids and the overexpression of cytokine (Interleukin 6) that further up-regulates proteins associated with anti-apoptotic and angiogenesis of tumours (Salgado *et al.*, 2003; Yusuf *et al.*, 2003). However, previous studies found a negligible association between paclitaxel resistance and the hyperexpression of MRP-1, which implies that the resistance mediated in this cell line is due to other factors not related to the MRP-genes (Arts *et al.*, 1999; Brinkmann *et al.*, 2015). The most probable explanation can be that of transporter heterogeneity on the cellular surface of the H69AR cells, consequently showing the secondary presence of P-gp transporters. The IC_{50} ranges are similar to those reported for numerous other human lung carcinoma cell lines, ranging between 2.5 - 7.5 nM (Liebmann *et al.*, 1993a; Frink *et al.*, 2016). A clear dose-dependent effect was witnessed for this cell line.

4.4.3.4 Cell viability inhibition of the NCI-H69/LX4 cell line following treatment with paclitaxel

The NCI-H69/LX4 cell line was treated with Paclitaxel between 300 nM and 1 500 nM for 96 h and the % cell inhibition (relative to the untreated control) as determined with the MTT assay is presented in **Figure 4.12**. Upon exposure to multiple concentrations of the compound, the P-gp hyperexpressive NCI-H69/LX4 cell line showed inhibition ranging between 16.42% (300 nM) and 89.96% (1 500 nM). Based on the fact that very high dosages were required to obtain an IC_{50} value in the NCI-H69/LX4 cell line, as well as a dose-dependent response, Paclitaxel was suspected to undergo major efflux by the P-gp transporter as it is a known substrate for P-gp (Sharom, 2014; Bender *et al.*, 2017). Reported standard deviations throughout the dosed concentrations can be directly attributable to the large population of cells present as floating aggregates, thus limiting homogenous exposure of a fraction of the population. Furthermore, the reliance of this cell line on their ABC transporter, P-gp, to export its substrate Paclitaxel may cause transporter exhaustion on outer cells, thus causing cellular death earlier in some cells and a delayed response in others.

The IC₂₅, IC₅₀ and IC₇₅ values for Paclitaxel in the P-gp hyperexpressive chemo-resistant NCI-H69/LX4 cell line are presented in **Table 4.13**. The IC values determined by means of Probit analysis, and their 95% limit ranges, can be directly correlated with the observable results seen in **Figure 4.12**, thus confirming the accuracy of the method used to determine the IC values.

Table 4.13: Cell viability inhibition concentrations (IC) of paclitaxel on the H69/LX4 cell line (nM), as determined with Probit analysis

NCI-H69/LX4			
Inhibitory concentration percentage	Calculated concentration (nM)	95% confidence limits (nM)	
IC ₂₅	412.59	385.05	437.79
IC ₅₀	613.05	588.41	636.79
IC ₇₅	910.92	875.83	950.93

A resistance factor increase of 215.9 was determined for the P-gp hyperexpressive cell line NCI-H69/LX4, in comparison to the drug-sensitive NCI-H69V cell line. In some cases, the exposure of cancer cells to extremely high concentrations of paclitaxel (10 000 nM) resulted in the cells increasing their resistance compared to lower doses (Liebmann *et al.*, 1993a). However, this phenomenon did not come forth in the tested ranges and was also not investigated further for the purpose of this study.

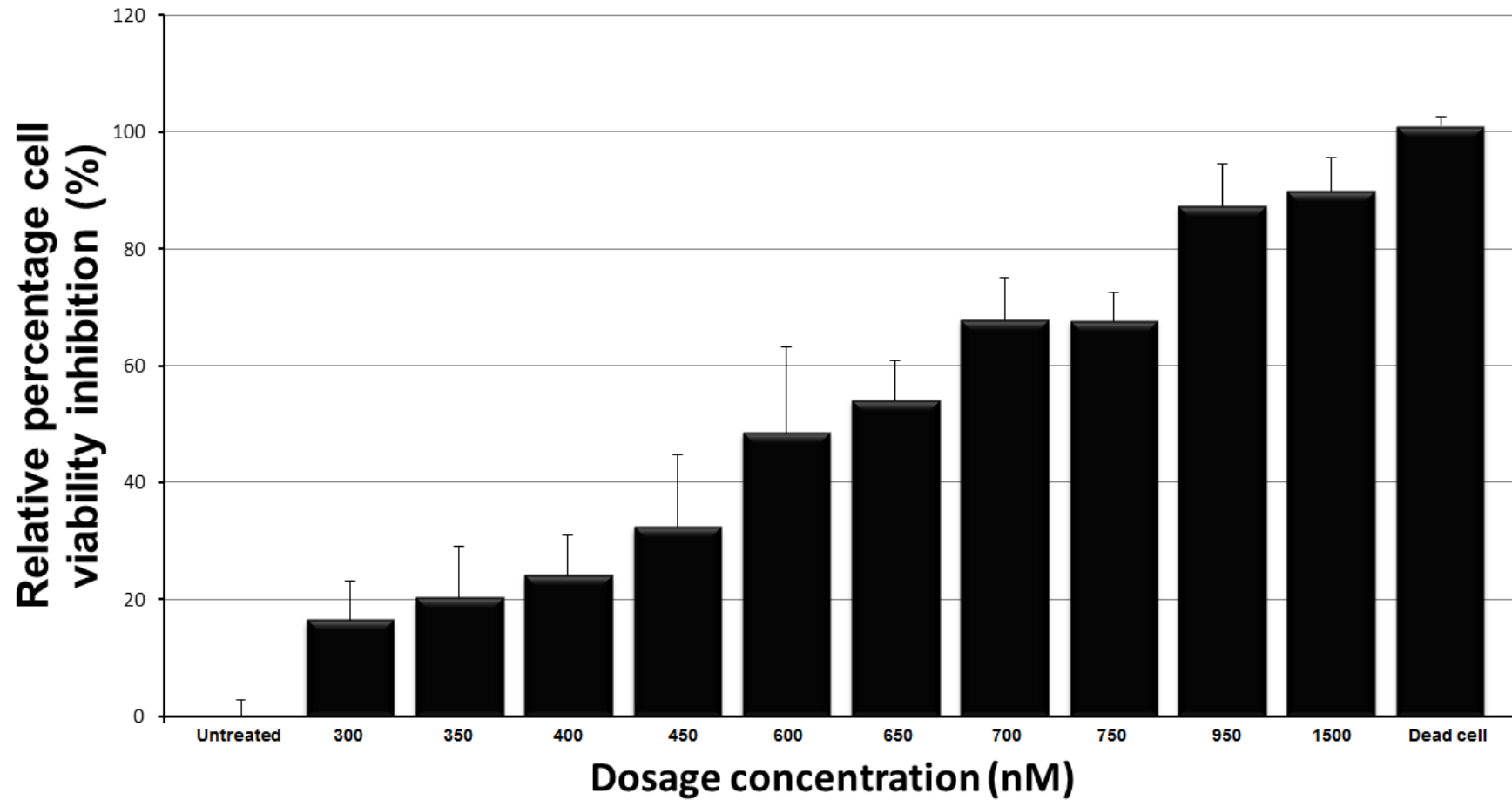


Figure 4.12: Percentage cell viability inhibition following treatment of the NCI-H69/LX4 cell line with paclitaxel, relative to the untreated control ($n = 3$; error bars indicate standard deviation).

4.4.3.5 Brief summary for paclitaxel

Paclitaxel exerts its cytotoxic effects by disrupting cellular differentiation and homeostasis through targeting the cellular structure proteins, microtubules. This aggressive mechanism of inducing cellular toxicity limits the dosing of this drug to small quantities at a time, due to serious systemic toxicity effects (e.g. myelosuppression and peripheral neuropathy) (Marupudi *et al.*, 2007). Unfortunately, the continuous exposure of cells to these lower concentrations allow for the development of adaptive mechanisms ranging from mutations in the targeted microtubules, all the way to the overexpression of P-gp transporters of which Paclitaxel is a specific substrate. Paclitaxel is so effective because it exerts relatively minimal cross-resistance among major efflux transporters such as MRP-1, and it is effective at very low concentrations. Therefore, this drug is ideal for the treatment of relapsed SCLC patients previously treated with substrates commonly associated with the MRP family (e.g. Irinotecan). For this reason, paclitaxel is recommended as a second-line chemotherapy for relapsed or refractory cancer in stage 4 disease states (Yamamoto *et al.*, 2006).

As mentioned earlier (**Section 4.4.3.4**), paclitaxel infers a forced adaption of MDR on cells exposed to extremely high concentrations of the drug. This characteristic serves as a reminder of the limitations of this drug in chemotherapy and the ability of SCLC cells to undergo adaptive mechanisms as a coping mechanism after exposure to paclitaxel. The cellular models will eventually reach a threshold of cytotoxicity and a rebound of multidrug resistant cells will emerge.

4.5 Conclusion

As expected, resistance occurred when each efflux transporter hyperexpressive cell line was exposed to the chemotherapeutic compound known to be a substrate for the particular efflux pump overexpressed. An interesting observation was the increased resistance to the drugs not known to be substrates, in the H69AR and NCI-H69/LX4 cell lines. This is most probably attributable to cross-resistance between transporters and the presence of the substrate transporters in a lower degree on the cell surfaces, which will be established later on through real-time polymerase chain reaction assays. This phenomenon of cross-resistance was most noticeable in the NCI-H69/LX4 cells, where P-gp conferred immense resistance to all of the tested drug compounds (**Figure 4.12; 4.8 and 4.4**). As expected, irinotecan reached a plateau of efficacy at extremely high doses on the H69AR cell line (**Figure 4.7**) and paclitaxel dosages had to overcome a massive resistance factor of 215.9 to produce an IC_{50} value (**Table 4.12**). Familial MRP resistance was also observed when the MRP-2 substrate, cisplatin, was introduced in the H69AR cell line with a resistance factor of 2.5 needed to be overcome to drug resistance (**Table 4.4**).

The NCI-H69V drug sensitive cell line had comparable results with all of the tested drug compounds to that of previously published experiments using drug sensitive lung cancer cells. This substantiates the calculation of resistance factors in the H69AR and NCI-H69/LX4 cell lines, thus giving an estimate of the resistance capabilities of each cell line.

The LLC-PK1 noncancerous cell line responded as expected to the chemotherapeutic drugs throughout the exposure. When treatment was limited to dosages given in the NCI-H69V and H69AR cell lines, negligible toxicity was witnessed in the LLC-PK1 cell line. This explains the inclusion of these drugs in both restricted and extensive-stage disease of untreated SCLC, given their relatively low life-threatening systemic toxicity in most patients (Ettinger *et al.*, 1995; Lara Jr *et al.*, 2009).

Clinically, irinotecan and platinum-based therapy (e.g. cisplatin) is currently utilised as a first-line therapy in the treatment of SCLC, due to their synergistic effects when administered as a combination (Zatloukal *et al.*, 2010b). The standard treatment of cisplatin and etoposide was proven to be less effective than that of the formerly mentioned combination in the treatment of Japanese patients, thus prompting the shift towards topoisomerase I inhibitors (e.g. irinotecan) (Noda *et al.*, 2002). Paclitaxel mono-therapy is specifically introduced to

relapsed patients who were exposed to prior chemotherapy, due to the fact that limited cross-resistance was found with cisplatin (Smit *et al.*, 1998). This multifaceted approach

Table 4.14: Cell viability inhibitory concentrations (IC) for cisplatin, irinotecan and paclitaxel on each cell line, as determined following MTT analysis

Cell line	Drug	IC ₅₀ (nM) [§]	95% limits (nM)	Resistance ratio	IC ₇₅ (nM) [§]	95% limits (nM)
H69V	Cisplatin	170.75	158.86 - 181.47	N/A	283.47	271.52 - 296.40
	Irinotecan	467.01	435.04 - 498.18	N/A	773.68	738.81 - 811.46
	Paclitaxel	2.84	2.65 - 3.08	N/A	4.53	4.05 - 5.26
H69AR	Cisplatin	427.34	382.35 - 482.83	2.50	1 588.00	1 236.00 – 2 261.00
	Irinotecan	7 716.70	6 925.50 – 8 568.30	16.50	16 578.60	15 236.10 – 18 199.70
	Paclitaxel	4.75	4.54 - 4.97	1.70	6.54	6.09 - 7.20
NCI-H69/LX4	Cisplatin	2 155.45	1 533.22 – 2 769.73	12.60	7 488.59	6 420.92 – 8 626.68
	Irinotecan	57 491.60	50 623.24 – 63 830.80	123.10	247 016.00	212 579.55 – 300 080.00
	Paclitaxel	613.05	588.41 - 636.79	215.90	910.92	875.83 - 950.93
LLC-PK1	Cisplatin	2 619.60	2 314.90 – 2 960.00	15.30	4 635.60	4 207.10 – 5 140.10
	Irinotecan	15 534.00	13 494.20 – 18 398.20	33.30	22 012.30	19 036.80 – 26 280.70
	Paclitaxel	29.73	24.13 – 1.06 x 10 ²⁸	10.50	38.08	27.42 – 3.80 x 10 ⁴⁴

[§]Resistance factor was calculated by dividing the absolute IC₅₀ values of the resistant cell line (H69AR or H69/LX4) with that of the sensitive line (NCI-H69V).

towards treatment is used because each chemotherapeutic drug has a unique apoptosis inducing target and ABC-transporter substrate specificity; accordingly lowering the cancer cells' ability to develop multidrug cross-resistance (e.g. microtubule mutations causing resistance towards paclitaxel). This ultimately ensures that secondary treatment regimens are available when relapse occurs, especially in the SCLC cells which in most patients show this recurrence as inevitable (Morabito, 2016).

The response of the cells used in this study, on each of the selected drug compounds, is comparable to the theoretical expectations mentioned when cellular characteristics are kept in mind. The IC₅₀ concentration ranges generated in these experiments can be incorporated into more extensive metabolic and cytotoxic assays, which provide better accuracy and more mechanistic clarity. Due to the MTT assay merely being an indicator of the cells' ability to be metabolically active, a more thorough approach is necessary to differentiate between cells that are quiescent, apoptotic or necrotic. This will help map the cells' general responses to the chemotherapeutic compounds more accurately, and will finally verify the models' ability to reflect realistic reactions as seen *in vivo*.

4.6 References

- Abdallah, E.A., Fanelli, M.F., Souza, e.S., Virgílio, Machado Netto, M.C., Gasparini Junior, J.L., Araújo, D.V., Ocea, L.M.M., Buim, M.E.C., Tariki, M.S. & Alves, V.d.S. 2016a/b. MRP1 expression in CTCs confers resistance to irinotecan-based chemotherapy in metastatic colorectal cancer. *International Journal of Cancer*, 139(4): 890-898.
- Abrams, T.J., Lee, L.B., Murray, L.J., Pryer, N.K. & Cherrington, J.M. 2003. SU11248 inhibits KIT and platelet-derived growth factor receptor β in preclinical models of human small cell lung cancer. *Molecular Cancer Therapeutics*, 2(5): 471-478.
- Alves, C., Pinteus, S., Horta, A. & Pedrosa, R. 2016. High cytotoxicity and anti-proliferative activity of algae extracts on an *in vitro* model of human hepatocellular carcinoma. *SpringerPlus*, 5(1): 1339.
- Ando, M., Kobayashi, K., Yoshimura, A., Kurimoto, F., Seike, M., Nara, M., Moriyama, G., Mizutani, H., Hibino, S. & Gemma, A. 2004. Weekly administration of irinotecan (CPT-11) plus cisplatin for refractory or relapsed small cell lung cancer. *Lung Cancer*, 44(1): 121-127.
- Arts, H.J., Katsaros, D., de Vries, E.G., Massobrio, M., Genta, F., Danese, S., Arisio, R., Scheper, R.J., Kool, M. & Scheffer, G.L. 1999. Drug resistance-associated markers P-

glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 2, and lung resistance protein as prognostic factors in ovarian carcinoma. *Clinical Cancer Research*, 5(10): 2798-2805.

Aykul, S. & Martinez-Hackert, E. 2016. Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis. *Analytical Biochemistry*, 508: 97-103.

Bahl, A., Sharma, D., Julka, P. & Rath, G. 2006. Chemotherapy related toxicity in locally advanced non-small cell lung cancer. *Journal of Cancer Research and Therapeutics*, 2(1): 14-16.

Bansal, T., Mishra, G., Jaggi, M., Khar, R.K. & Talegaonkar, S. 2009. Effect of P-glycoprotein inhibitor, verapamil, on oral bioavailability and pharmacokinetics of irinotecan in rats. *European Journal of Pharmaceutical Sciences*, 36(4): 580-590.

Bender, J., Fang, J. & Simon, R. 2017. A computational study of the inhibition mechanisms of P-glycoprotein mediated paclitaxel efflux by kinase inhibitors. *BMC Systems Biology*, 11(1): 108.

Berridge, M.V., Herst, P.M. & Tan, A.S. 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology Annual Review*, 11: 127-152.

Berridge, M.V. & Tan, A.S. 1993. Characterization of the cellular reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Archives of Biochemistry and Biophysics*, 303(2): 474-482.

Brinkmann, U., Hoffmeyer, S. & Mornhinweg, E. 2015. Polymorphisms in the human gene for the multidrug resistance-associated protein 1 (mrp-1) and their use in diagnostic and therapeutic applications: U.S. Patent Application 14/276,577.

Callaghan, R., Luk, F. & Bebawy, M. 2014. Inhibition of the multidrug resistance P-Glycoprotein: Time for a change of strategy? *Drug Metabolism and Disposition*, 42(4): 623-631.

Chen, X. & Thibeault, S. 2013. Effect of DMSO Concentration, cell density and needle gauge on the viability of cryopreserved cells in three dimensional hyaluronan hydrogel. Engineering in medicine and biology society (EMBC), 35th Annual International Conference of the IEEE: 6228-6231.

- Clewell, R.A. & Andersen, M.E. 2016. Approaches for characterizing threshold dose–response relationships for DNA-damage pathways involved in carcinogenicity *in vivo* and micronuclei formation *in vitro*. *Mutagenesis*, 31(3): 333-340.
- Cortés, A., Cascante, M., Cárdenas, M.L. & Cornish-Bowden, A. 2001. Relationships between inhibition constants, inhibitor concentrations for 50% inhibition and types of inhibition: new ways of analysing data. *Biochemical Journal*, 357(Pt 1): 263-268.
- Dasari, S. & Tchounwou, P.B. 2014. Cisplatin in cancer therapy: molecular mechanisms of action. *European Journal of Pharmacology*, 740: 364-378.
- Duggett, N.A., Griffiths, L.A. & Flatters, S.J.L. 2017. Paclitaxel-induced painful neuropathy is associated with changes in mitochondrial bioenergetics, glycolysis, and an energy deficit in dorsal root ganglia neurons. *Pain*, 158(8): 1499-1508.
- Eckardt, J.R., von Pawel, J., Papai, Z., Tomova, A., Tzekova, V., Crofts, T.E., Brannon, S., Wissel, P. & Ross, G. 2006. Open-label, multicenter, randomized, phase III study comparing oral topotecan/cisplatin versus etoposide/cisplatin as treatment for chemotherapy-naive patients with extensive-disease small-cell lung cancer. *Journal of Clinical Oncology*, 24(13): 2044-2051.
- El-Araby, M.E., Omar, A.M., Khayat, M.T., Assiri, H.A. & Al-Abd, A.M. 2017. Molecular mimics of classic p-glycoprotein inhibitors as multidrug resistance suppressors and their synergistic effect on paclitaxel. *PloS one*, 12(1): e0168938.
- Ettinger, D.S., Finkelstein, D.M., Sarma, R.P. & Johnson, D.H. 1995. Phase II study of paclitaxel in patients with extensive-disease small-cell lung cancer: an Eastern Cooperative Oncology Group study. *Journal of Clinical Oncology*, 13(6): 1430-1435.
- Fotakis, G. & Timbrell, J.A. 2006. *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, 160(2): 171-177.
- Frink, R.E., Peyton, M., Schiller, J.H., Gazdar, A.F., Shay, J.W. & Minna, J.D. 2016. Telomerase inhibitor imetelstat has preclinical activity across the spectrum of non-small cell lung cancer oncogenotypes in a telomere length dependent manner. *Oncotarget*, 7(22): 31639-31651.
- Galluzzi, L., Senovilla, L., Vitale, I., Michels, J., Martins, I., Kepp, O., Castedo, M. & Kroemer, G. 2012. Molecular mechanisms of cisplatin resistance. *Oncogene*, 31(15): 1869-1883.

- Hanna, N., Bunn Jr, P.A., Langer, C., Einhorn, L., Guthrie Jr, T., Beck, T., Ansari, R., Ellis, P., Byrne, M. & Morrison, M. 2006. Randomized phase III trial comparing irinotecan/cisplatin with etoposide/cisplatin in patients with previously untreated extensive-stage disease small-cell lung cancer. *Journal of Clinical Oncology*, 24(13): 2038-2043.
- Heuser, M., Kopun, M., Rittgen, W. & Granzow, C. 2005. Cytotoxicity determination without photochemical artifacts. *Cancer Letters*, 223(1): 57-66.
- Itamochi, H., Kigawa, J., Sultana, H., Iba, T., Akeshima, R., Kamazawa, S., Kanamori, Y. & Terakawa, N. 2002. Sensitivity to anticancer agents and resistance mechanisms in clear cell carcinoma of the ovary. *Japanese Journal of Cancer Research*, 93(6): 723-728.
- Jackman, D.M. & Johnson, B.E. 2005. Small-cell lung cancer. *The Lancet*, 366(9494): 1385-1396.
- Karbownik, A., Szalek, E., Urjasz, H., Głęboka, A., Mierzwa, E. & Grześkowiak, E. 2012. The physical and chemical stability of cisplatin (Teva) in concentrate and diluted in sodium chloride 0.9%. *Contemporary Oncology*, 16(5): 435-439.
- Kasahara, K., Fujiwara, Y., Nishio, K., Ohmori, T., Sugimoto, Y., Komiya, K., Matsuda, T. & Saijo, N. 1991. Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. *Cancer Research*, 51(12): 3237-3242.
- Khan, M., Freshney, R., Murray, A., Merry, S., Plumb, J. & McNicol, A. 1991. Identification and characterisation *in vitro* of cells with a non-SCLC cell-like phenotype derived from a continuous SCLC cell line. *Anticancer Research*, 11(5): 1687-1695.
- Korita, P.V., Wakai, T., Shirai, Y., Matsuda, Y., Sakata, J., Takamura, M., Yano, M., Sanpei, A., Aoyagi, Y. & Hatakeyama, K. 2010. Multidrug resistance-associated protein 2 determines the efficacy of cisplatin in patients with hepatocellular carcinoma. *Oncology Reports*, 23(4): 965-972.
- Krohn, A., Ahrens, T., Yalcin, A., Plönes, T., Wehrle, J., Taromi, S., Wollner, S., Follo, M., Brabletz, T. & Mani, S.A. 2014. Tumor cell heterogeneity in small cell lung cancer (SCLC): phenotypical and functional differences associated with epithelial-mesenchymal transition (EMT) and DNA methylation changes. *PloS one*, 9(6): e100249.
- Krohn, A., Ahrens, T., Yalcin, A., Plönes, T., Wehrle, J., Taromi, S., Wollner, S., Follo, M., Brabletz, T., Mani, S.A., Claus, R., Hackanson, B. & Burger, M. 2014b. Tumor Cell Heterogeneity in Small Cell Lung Cancer (SCLC): Phenotypical and Functional Differences

Associated with Epithelial-Mesenchymal Transition (EMT) and DNA Methylation Changes. *PloS one*, 9(6): e100249.

Lara Jr, P.N., Natale, R., Crowley, J., Lenz, H.J., Redman, M.W., Carleton, J.E., Jett, J., Langer, C.J., Kuebler, J.P. & Dakhil, S.R. 2009. Phase III trial of irinotecan/cisplatin compared with etoposide/cisplatin in extensive-stage small-cell lung cancer: clinical and pharmacogenomic results from SWOG S0124. *Journal of Clinical Oncology*, 27(15): 2530-2535.

Lévesque, É., Bélanger, A.-S., Harvey, M., Couture, F., Jonker, D., Innocenti, F., Cecchin, E., Toffoli, G. & Guillemette, C. 2013. Refining the UGT1A haplotype associated with irinotecan-induced hematological toxicity in metastatic colorectal cancer patients treated with 5-fluorouracil/irinotecan-based regimens. *Journal of Pharmacology and Experimental Therapeutics*, 345(1): 95-101.

Li, W., Zhou, J. & Xu, Y. 2015. Study of the *in vitro* cytotoxicity testing of medical devices. *Biomedical Reports*, 3(5): 617-620.

Liebmann, J., Cook, J., Lipschultz, C., Teague, D., Fisher, J. & Mitchell, J. 1993. Cytotoxic studies of paclitaxel (Taxol®) in human tumour cell lines. *British Journal of Cancer*, 68(6): 1104-1109.

Liu, X., Cheng, D., Kuang, Q., Liu, G. & Xu, W. 2013. Association between UGT1A1* 28 polymorphisms and clinical outcomes of irinotecan-based chemotherapies in colorectal cancer: a meta-analysis in Caucasians. *PloS one*, 8(3): e58489.

Ma, J., Hu, X., Li, J., Wu, D., Lan, Q., Wang, Q., Tian, S. & Dong, W. 2017. Enhancing conventional chemotherapy drug cisplatin-induced anti-tumor effects on human gastric cancer cells both *in vitro* and *in vivo* by Thymoquinone targeting PTEN gene. *Oncotarget*, 8(49): 85926-85939.

Marupudi, N.I., Han, J.E., Li, K.W., Renard, V.M., Tyler, B.M. & Brem, H. 2007. Paclitaxel: a review of adverse toxicities and novel delivery strategies. *Expert Opinion on Drug Safety*, 6(5): 609-621.

Morabito, A. 2016. Is combined chemotherapy with cisplatin, etoposide and irinotecan the new standard treatment for patients with sensitive relapsed small cell lung cancer? *Translational Cancer Research*: S1369-S1371.

Mosmann, T., 1983. Use of MTT colorimetric assay to measure cell activation. *J Immunol Methods*, 65, pp.55-63.

Natale, R., Lara, P., Chansky, K., Crowley, J., Jett, J., Carleton, J., Kuebler, J., Lenz, H., Mack, P. & Gandara, D. 2008. S0124: a randomized phase III trial comparing irinotecan/cisplatin (IP) with etoposide/cisplatin (EP) in patients (pts) with previously untreated extensive stage small cell lung cancer (E-SCLC). *Journal of Clinical Oncology*, 26(15_suppl): 7512-7512.

Nikzad, S., Baradaran-Ghahfarokhi, M. & Nasri, P. 2014. Dose-response modeling using MTT assay: a short review. *Life Science Journal*, 11(9s): 432-437.

Noda, K., Nishiwaki, Y., Kawahara, M., Negoro, S., Sugiura, T., Yokoyama, A., Fukuoka, M., Mori, K., Watanabe, K. & Tamura, T. 2002. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *New England Journal of Medicine*, 346(2): 85-91.

Pabla, N. & Dong, Z. 2008. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney International*, 73(9): 994-1007.

Riss, T.L. and Moravec, R.A., 2004. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay and Drug Development Technologies*, 2(1): 51-62.

Rossi, A. 2016. Relapsed small-cell lung cancer: platinum re-challenge or not. *Journal of Thoracic Disease*, 8(9): 2360-2364.

Saito, H., Takada, Y., Ichinose, Y., Eguchi, K., Kudoh, S., Matsui, K., Nakagawa, K., Takada, M., Negoro, S. & Tamura, K. 2006. Phase II study of etoposide and cisplatin with concurrent twice-daily thoracic radiotherapy followed by irinotecan and cisplatin in patients with limited-disease small-cell lung cancer: West Japan Thoracic Oncology Group 9902. *Journal of Clinical Oncology*, 24(33): 5247-5252.

Salgado, R., Junius, S., Benoy, I., Van Dam, P., Vermeulen, P., Van Marck, E., Huget, P. & Dirix, L.Y. 2003. Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *International Journal of Cancer*, 103(5): 642-646.

Sharom, F.J. 2014. Complex interplay between the P-glycoprotein multidrug efflux pump and the membrane: its role in modulating protein function. *Frontiers in Oncology*, 4: 41.

Shimoyama, Y., Kubota, T., Watanabe, M., Ishibiki, K. & Abe, O. 1989. Predictability of *in vivo* chemosensitivity by *in vitro* MTT assay with reference to the clonogenic assay. *Journal of Surgical Oncology*, 41(1): 12-18.

- Smit, E.F., Fokkema, E., Biesma, B., Groen, H.J.M., Snoek, W. & Postmus, P.E. 1998. A phase II study of paclitaxel in heavily pretreated patients with small-cell lung cancer. *British Journal of Cancer*, 77: 347-351.
- SPSS Inc. (2017). IBM SPSS Statistics Version 25, Release 23.0.0, Copyright© IBM Corporation and its licensors. <http://www-01.ibm.com/software/analytics/spss/>
- Stepanenko, A. & Dmitrenko, V. 2015. Pitfalls of the MTT assay: Direct and off-target effects of inhibitors can result in over/underestimation of cell viability. *Gene*, 574(2): 193-203.
- Stoddart, M.J. 2011. Cell Viability Assays: Introduction. Stoddart, M.J., ed. *Mammalian Cell Viability: Methods and Protocols*. Totowa, NJ: Humana Press: 1-6.
- Stordal, B. & Davey, M. 2007. Understanding cisplatin resistance using cellular models. *IUBMB life*, 59(11): 696-699.
- Tabatabaei Mirakabad, F.S., Akbarzadeh, A., Milani, M., Zarghami, N., Taheri-Anganeh, M., Zeighamian, V., Badrzadeh, F. & Rahmati-Yamchi, M. 2016. A Comparison between the cytotoxic effects of pure curcumin and curcumin-loaded PLGA-PEG nanoparticles on the MCF-7 human breast cancer cell line. *Artificial Cells, Nanomedicine, and Biotechnology*, 44(1): 423-430.
- Tariq, M., Negi, L.M., Talegaonkar, S., Ahmad, F.J., Iqbal, Z. & Khan, A.M. 2015. Liquid Chromatographic Method for Irinotecan Estimation: Screening of P-gp Modulators. *Indian Journal of Pharmaceutical Sciences*, 77(1): 14-23.
- Tsang, R.Y., Al-Fayea, T. & Au, H.-J. 2009. Cisplatin overdose. *Drug safety*, 32(12): 1109-1122.
- Turrisi, A.T., Kim, K., Blum, R., Sause, W.T., Livingston, R.B., Komaki, R., Wagner, H., Aisner, S. & Johnson, D.H. 1999. Twice-daily compared with once-daily thoracic radiotherapy in limited small-cell lung cancer treated concurrently with cisplatin and etoposide. *New England Journal of Medicine*, 340(4): 265-271.
- Ueno, M., Shiomi, T., Mochizuki, S., Chijiwa, M., Shimoda, M., Kanai, Y., Kataoka, F., Hirasawa, A., Susumu, N. & Aoki, D. 2018. ADAM9 is over-expressed in human ovarian clear cell carcinomas and suppresses cisplatin-induced cell death. *Cancer Science*, 109(2): 471-482.

- van Ark-Otte, J., Kedde, M.A., van der Vijgh, W.J.F., Dingemans, A.M., Jansen, W.J.M., Pinedo, H.M., Boven, E. & Giaccone, G. 1998. Determinants of CPT-11 and SN-38 activities in human lung cancer cells. *British Journal of Cancer*, 77: 2171-2176.
- Von Hoff, D., Schilsky, R., Reichert, C., Reddick, R., Rozenzweig, M., Young, R. & Muggia, F. 1979. Toxic effects of cis-dichlorodiammineplatinum (II) in man. *Cancer Treatment Reports*, 63(9-10): 1527-1531.
- Williams, D.A. & Lokich, J. 1992. A review of the stability and compatibility of antineoplastic drugs for multiple-drug infusions. *Cancer Chemotherapy and Pharmacology*, 31(3): 171-181.
- Wissing, M.D., Mendonca, J., Kim, E., Kim, E., Shim, J.S., Kaelber, N.S., Kant, H., Hammers, H., Commes, T., Van Diest, P.J., Liu, J.O. & Kachhap, S.K. 2013. Identification of cetrimonium bromide and irinotecan as compounds with synthetic lethality against NDRG1 deficient prostate cancer cells. *Cancer Biology & Therapy*, 14(5): 401-410.
- Yamamoto, N., Tsurutani, J., Yoshimura, N., Asai, G., Moriyama, A., Nakagawa, K., Kudoh, S., Takada, M., Minato, Y. & Fukuoka, M. 2006. Phase II study of weekly paclitaxel for relapsed and refractory small cell lung cancer. *Anticancer Research*, 26(1B): 777-781.
- Yamamoto, W., Verweij, J., de Bruijn, P., de Jonge, M.J., Takano, H., Nishiyama, M., Kurihara, M. & Sparreboom, A. 2001. Active transepithelial transport of irinotecan (CPT-11) and its metabolites by human intestinal Caco-2 cells. *Anti-Cancer Drugs*, 12(5): 419-432.
- Yusuf, R., Duan, Z., Lamendola, D., Penson, R. & Seiden, M. 2003. Paclitaxel resistance: molecular mechanisms and pharmacologic manipulation. *Current Cancer Drug Targets*, 3(1): 1-19.
- Zatloukal, P., Cardenal, F., Szczesna, A., Gorbunova, V., Moiseyenko, V., Zhang, X., Cisar, L., Soria, J.-C., Domine, M. & Thomas, M. 2010. A multicenter international randomized phase III study comparing cisplatin in combination with irinotecan or etoposide in previously untreated small-cell lung cancer patients with extensive disease. *Annals of Oncology*, 21(9): 1810-1816.

CHAPTER 5

ARTICLE SUBMITTED FOR PUBLICATION IN *EXPERIMENTAL CELL RESEARCH*

This chapter is presented as a research article for publication in *Experimental Cell Research*. It is formatted according to the journal's instructions to authors, which are included in **Appendix B**.

AUTHOR CONTRIBUTION

This original research article was fully compiled and executed by the primary author, MJ Rossouw. All the authors mentioned in the article participated in the study design where Dr C Willers, Dr C Calitz and Dr H Svitina provided guidance and support with the experimental phase. Prof C Gouws and Prof JH Hamman supervised the experimental phase as well as the compilation of the research article. All the mentioned authors reviewed the final document compiled by MJ Rossouw and provided comments and improvement

EVALUATION OF THREE SMALL CELL LUNG CARCINOMA CELL LINES AS MODELS FOR *IN VITRO* EFFLUX-BASED MULTIDRUG RESISTANT CANCER TREATMENT SCREENING

Rossouw, Michael Jacques¹, Willers, Clarissa¹, Calitz, Carlemi¹, Svitina, Hanna.¹, Hamman, Josias Hendrik¹ & Gouws, Chrisna.¹

¹Pharmacem™, Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa.

Abbreviations:

ABC, ATP-binding cassette; ANOVA, Analysis of variance; ATCC, American Type Culture Collection; ATP, adenosine triphosphate; AK, adenylate kinase; AKDR, adenylate kinase detection reagent; BSA, bovine serum albumin; cDNA, complementary deoxyribonucleic acid; CES-2, carboxylesterase-2; Ct, Threshold cycle; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; ECACC, European Collection of Authenticated Cell Cultures; FBS, foetal bovine serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IC, inhibitory concentration; MDR, multidrug resistance; MRP1, multidrug resistance-associated protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaCl, sodium chloride; NEAA, non-essential amino acids; nM, nanomolar; PBS, phosphate buffered saline; PCR, polymerase chain reaction; P-gp, P-glycoprotein; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RNA, ribonucleic acid; RPMI, Roswell Park Memorial Institute; SCLC, small cell lung cancer; TBP, TATA-binding protein; USA, United States of America.

ABSTRACT:

Small cell lung cancer (SCLC) has an aggressive disseminating nature and the relapse of cancer patients after initial treatment is common. Acquired multidrug resistance (MDR) can often be attributed to the over-expression of ABC-efflux transporters on the surfaces of SCLC cells. The two most prevalent hyperexpressed ABC-transporters reported in SCLC are P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP). In the current study, three SCLC cell lines were evaluated as potential in vitro models for anticancer research, specifically investigations to overcome efflux-based MDR in cancer. These SCLC cell lines included the drug-sensitive H69V cell line, the MDR H69AR cell line which hyperexpresses MRP1 efflux transporters, and the resistant P-gp hyperexpressing NCI-H69/LX4 cell line. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as a preliminary screening tool to determine the relative cell viability inhibitory potential (IC) of selected chemotherapeutic drugs (i.e. paclitaxel, cisplatin and irinotecan) on the selected SCLC cell lines. Based on the MTT pre-screening results, concentrations for the anticancer drugs were chosen and each cell line was treated with these selected concentrations of the drugs for 96 hours. Cell viability was subsequently assessed with adenosine triphosphate (ATP) and adenylate kinase (AK) assays. Non-cancerous porcine kidney cells (LLC-PK1) were included to evaluate the cytotoxic potential of each selected anticancer drug on normal cells. The results confirmed drug resistance of both the H69AR and NCI-H69/LX4 cell lines, relative to the drug-sensitive H69V cell line. Furthermore, the H69AR cell line was also resistant to cisplatin, a known MRP2 substrate, indicating possible cross-resistance between the transporter families. The NCI-H69/LX4 cell line exhibited increased resistance to paclitaxel, which was expected since this drug is a known substrate for the P-gp efflux transporter. The selected anticancer drugs exhibited slight cytotoxic effects on the non-cancerous cell line, although it was at significantly higher concentrations than the effective concentrations in the SCLC cell lines. The results indicated that the three selected SCLC cell lines can be used as predictive in vitro models for efflux-based MDR cancer treatment studies.

Keywords:

Cell viability, chemotherapy, *in vitro* cancer model, multidrug resistance, small cell lung cancer, drug screening.

1. Introduction

Lung cancer is the second most prevalent cancer in both men and women, and 13% thereof is attributed to small cell lung cancer (SCLC) [1]. High proliferation rates and rapid systematic dissemination of SCLC make it the type of lung cancer with the highest mortality rate [2]. In most cases, patients are diagnosed at a stage of high dissemination, termed as an extensive-stage disease, and are then left with a mere 5-year survival rate of just 1-2% compared to 10-13% for localised limited-stage disease [3]. In most instances, the inevitable occurrence of relapse due to acquired multidrug resistance (MDR) of cells after initial treatment further contributes to the dire prognosis of this disease [4].

One of the major obstacles in SCLC research is the lack of reproducible and clinically applicable results from *in vitro* models during the development of treatment strategies. For this reason, researchers suggest pushing towards human-derived surgical resected tumour biopsies as a replacement for preclinical models [5]. Although this technique encapsulates the unique heterogeneity of tumour cells, this system has been shown to have limitations in terms of its use as an *in vitro* model [6], such as the inability to perform large-scale (high-throughput) experiments due to patient privacy rights and restrictions on the distribution of human tissues [7]. In addition, reproducibility between experiments could not be obtained with this model, and several biological shortcomings have been reported for these primary cells when used as *in vitro* models for cancer research [8].

The most notable mechanism of MDR is the over-expression of adenosine triphosphate (ATP)-binding cassette (ABC) transporters in cancer cells, specifically the P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) efflux transporters [9]. ABC-transporters are membrane proteins responsible for the unilateral ATP-driven active efflux of a range of organic and inorganic substrates from within the cells that can include but are not limited to, xenobiotics, endogenous toxins, amino acids and cholesterol [10, 11]. The efflux of chemotherapeutic drugs from cancer cells by means of these transporters is described as one of the major targets for researchers in the design of drugs to overcome MDR, and the thorough characterisation of test models that mimic this MDR mechanism is aptly seen as important [12, 13].

In the current study, the use of three commercial SCLC cell lines was evaluated as potential preclinical models for the screening of MDR cancer treatments. To that extent, well-characterised first line SCLC chemotherapeutic drugs, that are known substrates for the transporters hyperexpressed in the selected MDR cell lines, were included as model

compounds. Cisplatin, which is known to be a substrate for MRP2-mediated efflux, was included to evaluate familial and cross-resistance effects in the MRP1 hyperexpressing cell line (H69AR). The non-cancerous LLC-PK1 cell line was also included as a control.

2. Materials and methods

2.1 Experimental design

This study evaluated cell viability in different *in vitro* cell culture models, including three SCLC cell lines with different chemotherapy sensitivities and a non-cancerous pig embryonic kidney cell line (LLC-PK1) (depicted in **Figure 1**). Firstly, the concentration ranges needed for each selected chemotherapeutic drug in each selected cell line to inhibit cell viability (inhibitory concentration, IC) were determined by using the MTT assay. The data obtained from this prescreening were analysed using the Probit Analysis method, to calculate IC values with 95% confidence intervals for each drug in each cell line. Then, the efficacy of the chemotherapeutic drugs was evaluated in terms of cell viability using more sensitive assays, namely by measuring soluble protein content, adenylate kinase (AK) and ATP levels.

Paclitaxel and irinotecan were specifically included in this study due to their affinity for the transporter proteins of interest, the former drug being a substrate for P-gp (hyperexpressed in the NCI-H69/LX4 cell line) and the latter being an MRP1 substrate (hyperexpressed in the H69AR cell line) [14, 15]. Cisplatin, a known MRP2 substrate [16], was included to view familial susceptibility to efflux by the MRP1 hyperexpressing cell line (H69AR) since both transporters belong to the MRP family. Cross-resistance susceptibility of P-gp transporters hyperexpressed by the NCI-H69/LX4 cell line was also explored with the inclusion of cisplatin.

Control groups included in the study design consisted of the LLC-PK1 cell line as a non-cancerous reference to determine chemotherapeutic selectivity, while also being useful to view clinical reproducibility when reviewing nephrotoxicity. All cell lines receiving no drugs acted as their own untreated controls, and the H69V cell line also served as a drug-sensitive control for the resistant cell lines, enabling calculation of a resistance ratio.

The analytical controls included a DMSO-only background control for the MTT assay since DMSO solvent was used for crystal solubilisation, as well as cells exposed to 0.2% Triton X-100 as a dead cell control. Bovine serum albumin standard was used as a reference compound for the soluble protein quantification, an ATP standard as a reference compound

for the intracellular ATP quantification and a dead cell control consisting of known concentrations dead cells for each cell line to quantify extracellular AK.

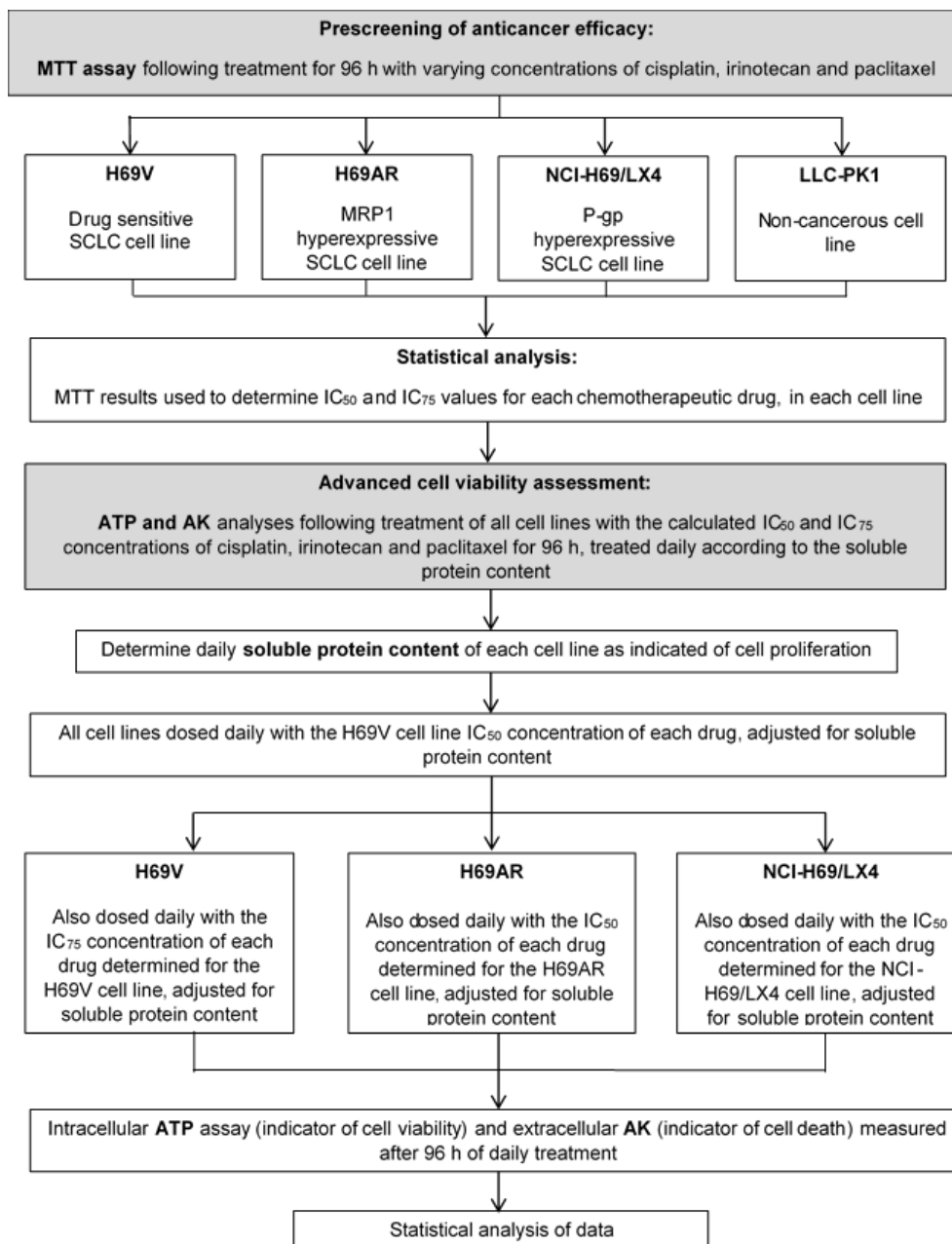


Figure 1: A diagram depicting the experimental design for this study.

2.2 Chemicals and reagents used

The thiazolyl blue tetrazolium bromide (#M5655), Triton X-100 (#T8787), dimethyl sulfoxide (#D5879), ATP disodium salt hydrate standard (#A26209), Roswell Park Memorial Institute (RPMI) 1640 medium (#11835-063), Dimethyl sulfoxide (#D5879-1L, DMSO) cisplatin (#C2210000), paclitaxel (#T7402) and irinotecan (#I1406) were purchased from Sigma-Aldrich (Johannesburg, South Africa). The Quick Start™ Bradford Protein Assay containing dye reagent concentrate and 2 mg/ml bovine serum albumin standards were purchased from Bio-Rad (Johannesburg, South Africa). The lysis buffer used in the protein analysis was donated by Professor Krzysztof Wrzesinski from Celvivo® [17]. CellTiter-Glo® Luminescent Cell viability assay and CytoTox-Glo® digitonin lysis buffer were both purchased from Promega (Anatech Instruments (Pty) Ltd., Gauteng, South Africa). The ToxiLight® BioAssay kit (#LT07-117) containing the AK assay buffer and the AK detection reagent was purchased from Lonza (Whitehead Scientific (Pty) Ltd). Sodium Chloride (NaCl) was purchased from Merck (Gauteng, South Africa).

Trypsin EDTA (#BE-17-161F), L-glutamine (#BE17-605E) and non-essential amino acids (NEAA; #BE13-114E) were purchased from Lonza (Whitehead Scientific (Pty) Ltd, Cape Town, South Africa), while penicillin/streptomycin (#DE17-602E) was purchased from Separations (Johannesburg, South-Africa). RPMI 1640 medium was supplemented with Doxorubicin hydrochloride purchased from Sigma-Aldrich (ATCC adaptation; #D1515-10mg; Johannesburg, South Africa). Costar® cell culture flasks (75cm²/flat bottom 96-well/v-bottom 96-well /black 96-well) and foetal bovine serum (FBS) were purchased from The Scientific group (#430641U; #3596; #3894; #3912; Johannesburg, South Africa) were purchased from Thermo Fisher scientific (Johannesburg; South-Africa)

PureLink™ RNA Mini Kit (#12183018A) was purchased from Invitrogen (Carlsbad, USA) and RNA later (#76104) was purchased from Qiagen (Hilden, Germany). The High-Capacity cDNA Reverse Transcription Kit (#4368813), TaqMan™ Fast Advanced Master Mix (#4444556), FAM-labelled TaqMan™ Gene Expression Assays (#4351372), P-gp (#4331182; ABCB1-Hs00184500_m1), MDR1 (#4331182; ABCC1-Hs00219905_m1), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #4331182; Hs99999905_m1), TATA-binding protein (TBP; #4331182; Hs00427620_m1), Dulbecco's Modified Eagle's Medium (DMEM; #SH30243.01) supplemented with L-glutamine (4mM), sodium Pyruvate and Phenol Red as well as Phosphate buffered saline (#SH30256.01; PBS) were purchased from Thermo Fisher scientific (Johannesburg; South-Africa).

2.3 Cell culture maintenance

The H69V (#91091803) and NCI-H69/LX4 (#96042329) cell lines were purchased from the European Collection of Authenticated Cell Cultures (ECACC). Both were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 % FBS, 1 % NEAA, 1 % penicillin/streptomycin. This will be referred to as a supplemented medium. Doxorubicin hydrochloride (0.4 µg/ml) was added for the NCI-H69/LX4 cell line to maintain drug resistance. The H69AR (ATCC® #CRL11351™) cell line was purchased from American Type Culture Collection (ATCC) and maintained in RPMI 1640 (ATCC® adaptation) medium supplemented with 20% FBS, 2 mM L-glutamine, 1 % NEAA and 1% penicillin/streptomycin. The LLC-PK1 (ATCC® CL-101™) cell line, also purchased from ATCC®, was maintained in high glucose (4.5 mg/L) Dulbecco's Modified Eagle's Medium (DMEM). This was supplemented with 2 mM L-glutamine, 10 % FBS, 1 % NEAA and 1 % penicillin/streptomycin.

Adherent cell lines cultured in flat bottom 96-well plates were sub-cultured through trypsinisation. The supplemented medium was replaced every second day for the H69AR and LLC-PK1 lines, while for H69V supplemented medium was replaced every third day during normal cell maintenance. The NCI-H69/LX4 cells were cultured in 96-well V-bottom plates and were not subjected to trypsinisation due to the cells growing as aggregates in suspension. Sub-culturing was only initiated when the flask reached 80% confluence ($\pm 4 \times 10^6$ cells/ml).

All cells were cultured at 37°C, with 5% CO₂ and 95% humidified air in an ESCO CelCulture CO₂ incubator (ESCO Micro Pte Ltd; Changi, Singapore). Throughout the experiments, cells were plated while in their exponential growth phase. Cell densities for seeding were pre-determined to reach approximately 80% confluence after 96 h treatment and ranged from 7 000 cells/well ($\pm 21\,875$ cells/cm²) for the H69V and LLC-PK1 cell lines, to 16 000 cells/well ($\pm 50\,000$ cells/cm²) for the H69AR and 30 000 cells/well ($\pm 93\,750$ cells/cm²) for the NCI-H69/LX4 cell lines.

2.4 Preparation of chemotherapeutic drugs

Cisplatin solution was prepared by diluting the compound to 2 mM stock solutions in saline (0.9% *m/v*), which was sterilised by means of 0.2 µm syringe filtration. Stock solutions were stored between 2 – 8 °C for one week, with minimum exposure to light [18, 19]. Irinotecan solution was stored at -20 °C in aliquots, dissolved in DMSO and prepared at concentrations

between 14 mM and 15,3 mM as previously published [20]. Paclitaxel was also dissolved in DMSO and stored at -20°C as aliquots with concentrations ranging from 1.054 mM to 105,4 µM. In all instances, exposure to light was kept to a minimum by dosing and preparing paclitaxel in dimly lighted flow cabinets [21].

The final DMSO concentration to which the cells were exposed never exceeded 0.5% v/v, due to the cytotoxic nature of this solvent [22]. Each frozen drug aliquot solution was allowed to thaw at 37°C in a block heater prior to initiation of experiments. Further dilutions were prepared with supplemented medium, excluding doxorubicin hydrochloride to prevent potential drug-drug interactions.

2.5 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was included as a preliminary screening tool to determine the relative drug sensitivity of the different cancer cell lines to the three selected compounds. The cells, seeded in 96 well plates as set out in section 2.4, were incubated for 24 h in supplemented medium to recover prior to initiation of drug treatments. Each selected drug was prepared in the specific supplemented medium and was replaced at 24 h intervals during treatment. The MTT assay was performed after 96 h exposure of the cells to each drug, as previously published [23]. No drug compounds were added to the untreated cell control, DMSO background control or the dead cell control. These wells did receive fresh supplemented medium daily.

After 96 h, the medium supernatant (supplemented with drugs in the treated samples) was removed from each well, and the cells washed twice with 100 µL PBS. It was then replaced with non-additive growth medium containing MTT (0.5 mg/ml). The DMSO background control wells only received 200 µL of non-additive growth medium during incubation, while the dead cell control was first exposed to 0.2 % Triton X-100 for 15 min before the addition of MTT. The plates were incubated at 37 °C, with 5 % CO₂ and 95 % humidified air for 4 h. The plates were then centrifuged at 1000 x g for 5 min and the supernatant was discarded. Subsequently, all the wells (including the DMSO control) received 200 µL DMSO to dissolve the purple formazan crystals formed as metabolic products, and the absorbance of these metabolic products in solution was measured on a SpectraMax Paradigm plate reader (Molecular devices, Separations, Johannesburg, South Africa) at 560 nm, with a background reading at 630 nm. The extra background reading was included to eliminate background interference, including bubbles, cell debris and light scattering [24]. The absorbance of the

solutions of the untreated controls was established as 100% cell viability after the absorbance value of the DMSO background control was subtracted from all the samples. Three samples were measured for each experimental or control group, and the relative percentage cell viability inhibition was calculated by subtracting the measured viability from the 100%.

Throughout the experiments, the NCI-H69/LX4 suspension cell line, seeded in 96 well v-bottom plates, was first centrifuged at 250 x *g* for 5 min in a 3-16 KL benchtop centrifuge (Sigma, Osterode am Harz, Germany) to minimise cell loss during medium withdrawal, before initiating dosing or assays.

2.6 Soluble protein quantification

The soluble protein content in each well of the 96-well plates, seeded as described in section 2.4, was determined in 24 h intervals using the Bradford protein assay as previously published [25]. This enabled calculation of the approximate wet biomass of each experimental group, allowing treatment to be adjusted accordingly. This adjustment ensured that the cells were constantly exposed to the same amount of drug throughout the experiment per wet biomass. This corresponds better to *in vivo* trials where dosing is calculated in terms of body mass [17].

Briefly, the Bradford protein assay entailed removal of the spent cell culture medium and replacing it with 158 μL water. Lysis buffer (2 μL) was then added to all wells. The protein assay dye reagent (40 μL) was added to all wells, to a final volume of 200 μL /well. Absorbance was measured on a SpectraMax Paradigm plate reader at 595 nm. The protein content was quantified relative to a bovine serum albumin (BSA) standard.

2.7 Intracellular adenine triphosphate cell viability assay

Highly sensitive intracellular ATP detection assays, such as the CellTiter-Glo[®] Luminescent Cell viability assay, can more accurately indicate the viability of cells after treatment with chemotherapeutic compounds [26]. Cells were seeded as described in Section 2.4, in black 96-well plates. After 24 h recovery, all cell lines were treated daily with the calculated concentrations of the chemotherapeutic compounds, adjusted for wet biomass. After 96 h treatment, intracellular ATP levels were measured as previously published (Calitz *et al.*, 2018). The spent medium was collected from each sample well for AK quantification (see section 2.9), and replaced with 100 μL PBS. An ATP standard concentration series was also plated in triplicate (3 x 100 μL), and 100 μL CellTiter-Glo[®] luminescent lysis buffer was

added to all wells. The luminescence values were then measured with a Spectramax® paradigm plate reader with one kinetic window, 10 measurement cycles with 0.3 s measurement interval time, 2 s delay per measurement and an additional 0.5 s delay per position change. All samples were quantified relative to the known ATP standard, and all data were normalized to the untreated control.

2.8 Extracellular adenylate kinase cell death assay

Cytotoxicity can be indicated by the presence of extracellular cytosolic adenylate kinase (AK) due to cytoplasmic membrane rupture, which is an important marker of cell death [27]. Spent medium (200 μ L) was collected from each sample in the ATP assay plate following daily treatment for 96 h, to determine AK content. The collected medium was centrifuged at 140 x *g* for 5 min, and 160 μ L of the supernatant was then transferred to a clean microcentrifuge tube. The transferred sample was centrifuged again at 15 000 x *g* for 15 min. Finally, 140 μ L of the supernatant was transferred to a new tube for the assay.

A known dead cell standard for each cell line was prepared by treating a known concentration of cells with CytoTox-Glo® digitonin lysis buffer (Promega). The cell concentrations used for the preparation of the standard were 378 000 cells/ml for the H69V cell line; 839 000 cells/ml for the H69AR cell line; 1 310 000 cells/ml for the NCI-H69/LX4 cell line and 124 000 cells/ml for the LLC-PK1 cell line. The dead cell standard was diluted with heat-treated medium and exposed to the same conditions as the samples.

Samples were loaded in triplicates of 20 μ L each in a black 96-well plate, and 100 μ L AK detection reagent was added to each well. The plate was covered and placed on a compact rocker for 20 min. Luminescence was measured using a SpectraMax Paradigm plate reader at one kinetic window, 10 measurement cycles with 0.3 s measurement interval time, 2 s delay per measurement, additional 0.5 s delay per position changed. All samples were quantified relative to the dead cell standards, and results were normalized relative to the untreated controls.

2.9 Quantitative reverse transcription polymerase chain reaction

To confirm maintained hyperexpression of the P-gp and MRP1 efflux transporters by the H69AR and NCI-H69/LX4 cell lines, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed. The cells were seeded as described in section 2.4 and incubated for 96 h. The cells were then trypsinised, centrifuged at 200 x *g*, re-suspended in 200 μ L RNAlater, and stored at +4°C for 24 h or -80°C for long-term storage. Total RNA

was extracted with the PureLink™ RNA Mini Kit according to the manufacturer's guidelines. Extracted RNA was quantified using a NanoDrop™ One/OneC UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

cDNA was synthesized using 2 µg total RNA and the High-Capacity cDNA reverse transcription kit. Subsequently, real-time PCR was carried out using TaqMan™ Fast Advanced master mix according to the manufacturer's instructions, on the QuantStudio™ 3 real-time PCR systems cycler (Applied Biosystems, Singapore). FAM-labelled TaqMan™ gene expression assays were used for the following genes: P-gp (ABCB1-Hs00184500_m1), MDR1 (ABCC1-Hs00219905_m1), GAPDH (Hs99999905_m1) and TBP (Hs00427620_m1). GAPDH and TBP were used to normalize the data, and to guarantee comparability of mRNA expression in all samples analysed. All PCR analyses were performed in biological triplicates. Threshold cycle (Ct) values were determined using QuantStudio™ design and analysis software v1.4.3 (Applied Biosystems), and data were further analysed with ExpressionSuite software v1.1 (Applied Biosystems). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [28].

2.10 Calculations and statistical analysis

SPSS statistical analysis software, version 25 (IBM Analytics, New York, USA) was used to analyse the MTT data from the preliminary efficacy screening, using the Probit analysis method to calculate IC values for each chemotherapeutic drug in each cell model, with 95% confidence intervals. The best fit model was used to determine the regression analysis.

The IC_{50} and IC_{75} values calculated from the MTT results were also used to calculate resistance ratios by dividing IC_{50} concentration of each drug into the drug resistant cell lines by the IC_{50} values determined for each drug in the drug sensitive cell line (H69V). The resistance ratio provides an indication of the resistance of a cell line to a specific treatment.

The statistical analysis programme, STATISTICA 13.3 (Statsoft, TIBCO Software Inc., CA, USA) was used for the statistical analyses of the advanced anticancer efficacy screening findings, to determine if statistically significant differences ($p < 0.05$) occurred as a result of each drug treatment compared to its untreated control, as well as between the different concentrations of a drug. Two-way analysis of variance (ANOVA) was performed, followed by post-hoc tests: Bonferroni's multiple comparison test (soluble protein content/gene expression), Tukey's HSD (ATP & AK) and Kruskal-Wallis (ATP & AK). A nonparametric Mann-Whitney U test was also applied ($n = 6$).

3. Results and discussion

3.1 Pre-screening of relative cell viability inhibition potential

The results from the MTT assays were statistically analysed to determine the IC_{50} and IC_{75} concentrations for each compound on each cell line, with a 95% confidence range, as presented in **Table 1**. Data generated by these studies were used as a preliminary indicator of drug sensitivity and resistance of the selected cell culture models investigated in this study.

The results obtained from the initial MTT assays, although very simplistic, already corresponded to the predicted outcomes. Severe drug resistance to the respective substrate compounds was observed for both efflux transporter hyperexpressing cell lines, as indicated by the resistance ratios in **Table 1**. In the MRP1 hyperexpressing H69AR cell line, the MRP1 substrate irinotecan (a known MRP1 substrate) had a resistance ratio of 16.50, compared to ratios below 3.00 for the other compounds in this cell line. Paclitaxel (a known P-gp substrate) had a similarly increased resistance ratio for the P-gp hyperexpressing NCI-H69/LX4 cell model, indicating efficient efflux of this drug by the cells and subsequent evasion of cell death. As expected, lower levels of cytotoxicity were seen for irinotecan (IC_{50} of 15 534.00 nM) in the LLC-PK1 cell line than for the other chemotherapeutic drugs (IC_{50} of 2 619.60 nM and 29,73 nM), having shown no dose-dependent kidney damage clinically [29]. The other drugs that are known to cause severe clinical nephrotoxicity [30, 31], also had higher levels of cytotoxicity in the embryonic kidney cells.

3.2 Advanced anticancer efficacy assessment

Chemotherapeutic compounds can induce programmed cell death (apoptosis) through various pharmacological mechanisms of action. Apoptosis is an ATP-dependent process to activate biochemical markers that induce cell death [32]. ATP levels in tumour tissue microenvironments are naturally high to combat host immune responses [33, 34], drive angiogenesis [35], fulfil tumour energy needs through activation of mitochondrial oxidative phosphorylation for intracellular ATP production [36] and to activate glucose transporters [37]. When reviewing the dependence of cell survival on these factors, the viability of cells can accurately be determined by quantifying intracellular ATP content.

Table 1: Relative cell viability inhibitory concentrations for cisplatin, irinotecan and paclitaxel on each cell line, as determined following MTT analysis

Cell line	Drug	IC ₅₀ (nM) [§]	95% limits (nM)	Resistance ratio	IC ₇₅ (nM) [§]	95% limits (nM)
H69V	Cisplatin	170.75	158.86 - 181.47	N/A	283.47	271.52 - 296.40
	Irinotecan	467.01	435.04 - 498.18	N/A	773.68	738.81 - 811.46
	Paclitaxel	2.84	2.65 - 3.08	N/A	4.53	4.05 - 5.26
H69AR	Cisplatin	427.34	382.35 - 482.83	2.50	1 588.00	1 236.00 – 2 261.00
	Irinotecan	7 716.70	6 925.50 – 8 568.30	16.50	16 578.60	15 236.10 – 18 199.70
	Paclitaxel	4.75	4.54 - 4.97	1.70	6.54	6.09 - 7.20
NCI-H69/LX4	Cisplatin	2 155.45	1 533.22 – 2 769.73	12.60	7 488.59	6 420.92 – 8 626.68
	Irinotecan	57 491.60	50 623.24 – 63 830.80	123.10	247 016.00	212 579.55 – 300 080.00
	Paclitaxel	613.05	588.41 - 636.79	215.90	910.92	875.83 - 950.93
LLC-PK1	Cisplatin	2 619.60	2 314.90 – 2 960.00	15.30	4 635.60	4 207.10 – 5 140.10
	Irinotecan	15 534.00	13 494.20 – 18 398.20	33.30	22 012.30	19 036.80 – 26 280.70
	Paclitaxel	29.73	24.13 – 1.06 x 10 ²⁸	10.50	38.08	27.42 – 3.80 x 10 ⁴⁴

[§]n = 3

The release of AK into the extracellular environment is also a known indicator of cytoplasmic membrane rupture, which is an important marker of cell death [27]. By including assays such as the soluble protein content during the 96 h treatment, the cytostatic behaviour could

also potentially be identified. Expressing the ATP and AK results in terms of this protein content allowed mapping of the chemo-evasive behaviour of the cell population [38].

3.2.1 Irinotecan anticancer efficacy

Irinotecan (CPT-11) is a prodrug that is metabolised to a more cytotoxic metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), through carboxylesterase-2 (CES-2) enzyme activation in the liver and blood [39]. The targeted enzyme for cytotoxicity induction is the Topoisomerase 1 enzyme, which is generally responsible for DNA structural changes and relegating strands of DNA during transcription and replication [40]. Inhibition of this enzyme results in double-stranded DNA breaks and replication arrest, which eventually causes cell death [41]. Fast dividing healthy cells are subject to toxicity through systemic activation of the drug, and common dose-limiting adverse effects include hematologic and gastrointestinal problems, with neutropenia and diarrhoea having primary debilitating effects on patients [42].

Cell responses following treatment with 467.01 nM; 773.68 nM; 7716.70 or 57 491.60 nM irinotecan for 96 h, including soluble protein content over time, intracellular ATP levels per soluble protein content (normalised relative to the untreated controls) and extracellular AK levels per soluble protein content (normalised relative to the untreated controls) are shown in **Figure 2**.

No significant protein content changes were induced in the noncancerous LLC PK1 cell line (**Figure 2.1A**), but some of the cells succumbed to the treatment with a significant reduction in intracellular ATP (0.94) and a significant increase in AK (1.80) levels (**Figure 2.1B and C**). This was indicative of cytotoxicity, even though the population growth was not significantly affected, yet.

The drug sensitive cell line, H69V, showed a statistically significant ($p < 0.01$; Kruskal-Wallis; Tukey's HSD tests & Mann-Whitney U test) concentration-dependent reduction in cell viability, relative to the untreated control for all of the measured responses, as seen in **Figure 2.2**. Severe depletion of intracellular ATP with cells actively releasing AK indicated the sensitivity of the cell line to irinotecan treatment.

Following treatment of the H69AR cell line (MRP1 hyperexpressive) with irinotecan, a known MRP1 substrate, adaptive MDR appeared to be induced further in the cell line at extremely high concentrations, as seen in **Figure 2.3**. Although growth appeared to be stunted in the H69AR cell line following treatment with 7 716.70 nM irinotecan (IC_{75} concentration) for 96 h

(**Figure 2.3A**), no significant AK release indicative of cell death was seen for either concentration. This could indicate that due to enhanced efflux, tolerable intracellular concentrations of irinotecan active metabolites (SN-38) allowed DNA repair during the cell cycle arrest. The notable decrease in ATP between dosages (0.95 for 467.01 nM; 0.84 for 7 716.70 nM) ($p < 0.05$; Kruskal-Wallis; Tukey's HSD tests & Mann-Whitney U test) shown in **Figure 2.3.B**, with no increased AK release, confirmed that the cells are extremely resistant against irinotecan and its metabolites due to the most likely use of ATP for cellular repair. The concentration irinotecan needed to inhibit growth in the H69AR cell line (7 716.70 nM) was also 16.5 fold higher than the active dosage in the drug-sensitive H69V cell line (467.01 nM), further showing the resistance of the cell line to treatment with an MRP1 substrate.

When exposing the P-gp hyperexpressive NCI-H69/LX4 cell line to irinotecan (**Figure 2.4**), some drug resistance was apparent at the lower concentration (H69V IC_{50} of 467.01 nM), as the dosage had no significant cytotoxic effect. The drastically increased NCI-H69/LX4 IC_{50} value of 57 491.6 nM did result in significant changes ($p < 0.01$; Kruskal-Wallis; Tukey's HSD tests & Mann-Whitney U test), with ATP lowered to 0.60 and AK increasing to 6.63. When administering such a high concentration, the soluble protein content showed a decline after 72 h (786.08 $\mu\text{g}/\text{well}$) compared to the lower concentration (944.21 $\mu\text{g}/\text{well}$) and the untreated control (954.67 $\mu\text{g}/\text{well}$), with high levels of cell death after 96 h most probably due to ABC-transporter saturation. The resistance inferred by the hyperexpressed P-gp transporter in the NCI-H69/LX4 cell line could indicate the potential of the cell model to be resistant towards multiple drugs, not necessarily only primary P-gp substrates. This suggests the potential for cross-resistance. This observed irinotecan expulsion by P-gp has been explored previously, and dual therapy with P-gp inhibitors has been suggested in response [43].

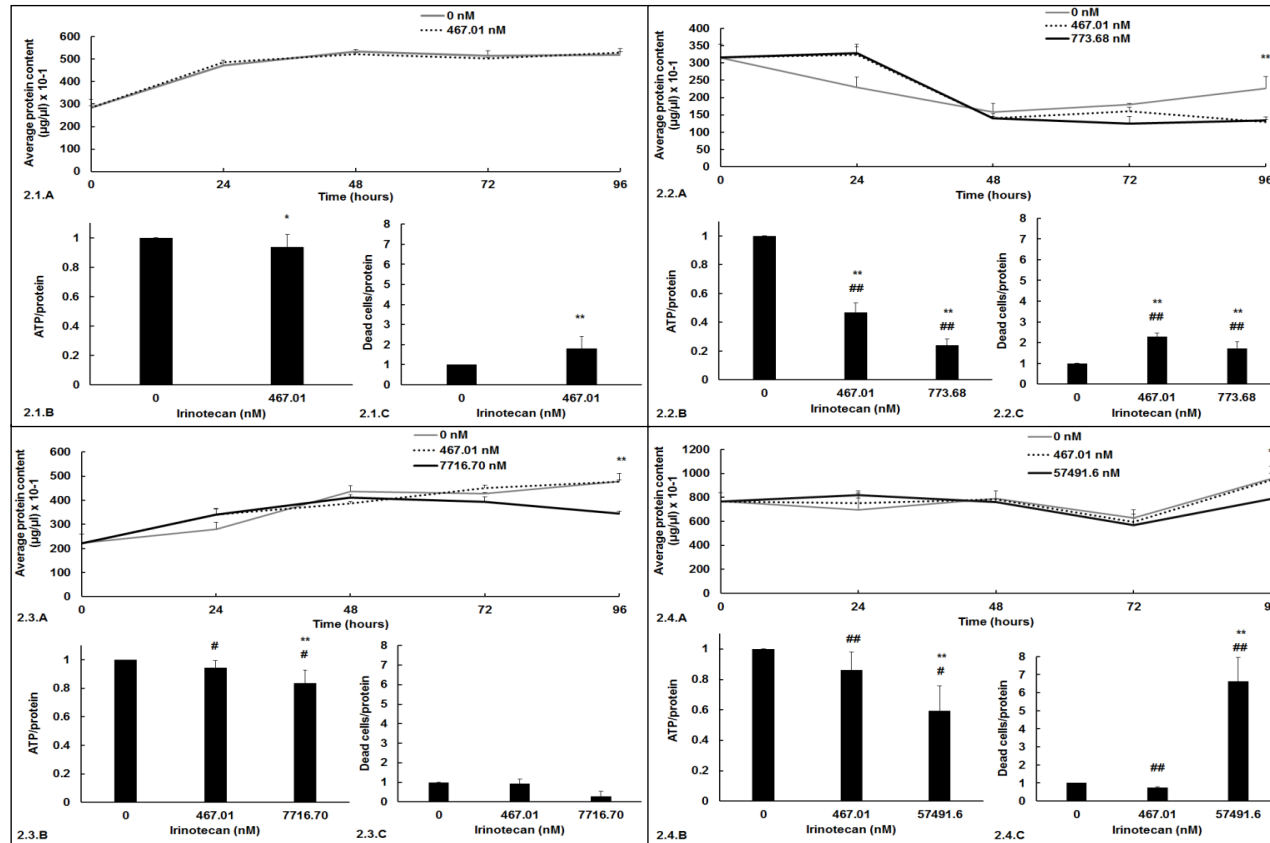


Figure 2: Cell responses following daily treatment with irinotecan (nM) for 96 h. Soluble protein content over time (A), intracellular ATP levels per soluble protein content (normalised relative to the untreated control) (B) and extracellular AK levels per soluble protein content (normalised relative to the untreated control) (C) are shown for the LLC-PK1 (1), H69V (2), H69AR (3) and NCI-H69/LX4 (4) cell lines, respectively. Error bars denote the standard deviation ($n = 6$), # indicate statistically significant inter-dosage differences ($p < 0.05$); ## indicate statistical significant inter-dosage differences ($p < 0.01$); * indicate statistically significant differences relative to the untreated control ($p < 0.05$); ** indicate statistically significant differences relative to the untreated control ($p < 0.01$).

3.2.2 Cell responses following treatment with paclitaxel

Paclitaxel is known to induce mitotic arrest after microtubule destabilisation due to polymerisation, thus inhibiting DNA and RNA synthesis and eventually forcing the activation of apoptotic precursors [44, 45]. Paclitaxel has also been shown to induce significant toxicity in healthy tissues, with major dose-limiting side effects including neutropenia and neuropathy. This prompted the regime of 24 h infusion every 3 weeks, maximum [46].

Cell responses following treatment with 2.84 nM; 4.53 nM; 4.75 nM or 613.05 nM paclitaxel for 96 h, including soluble protein content over time, intracellular ATP levels per soluble protein content (normalised relative to the untreated controls) and extracellular AK levels per soluble protein content (normalised relative to the untreated controls) are shown in **Figure 3**.

Severe toxicity, as reported clinically, was also observed in the porcine kidney cells (LLC-PK1) as shown in **Figure 3.1**, with significant ($p < 0.01$; Kruskal-Wallis; Tukey's HSD tests & Mann-Whitney U test) decline in ATP (0.88; **Figure 3.1B**) and increase in overall cell death indicated by AK release (1.94; **Figure 3.1.C**) at a low drug concentration of 2.84 nM. The lack of adaptation of the cells to the toxicity was also apparent, with a notable decline ($p < 0.05$; Bonferroni test) in protein content (482.34 $\mu\text{g}/\text{well}$) when compared to 519.87 $\mu\text{g}/\text{well}$ in the untreated control (**Figure 3.1A**), confirming restriction of dosing.

Patients not previously exposed to chemotherapeutic treatment presenting with SCLC, similar to the drug-sensitive H69V cell line, responded adequately to paclitaxel in a clinical setting with a response rate of up to 53% during monotherapy [47]. This initial response was mimicked in the H69V cells, with a remarkable reduction in the cellular protein content after 96 h when comparing the untreated samples (226.97 $\mu\text{g}/\text{well}$) to the protein content after exposure to 2.84 nM (148.24 $\mu\text{g}/\text{well}$) and 4.53 nM (152.40 $\mu\text{g}/\text{well}$), as seen in **Figure 3.2A**. The efficacy was further substantiated by severely decreased ATP levels (**Figure 3.2B**) in the 2.84 nM (0.45) and 4.53 nM (0.38) treated chemosensitive cells. Furthermore, there was a significant amount ($p < 0.05$; Kruskal-Wallis; Tukey's HSD tests & Mann-Whitney U test) of cell death as indicated by the AK release (1.63 at 2.84 nM; 1.51 at 4.53 nM) after 96 h when compared to the untreated control (1.00) (**Figure 3.2C**).

When paclitaxel was administered to the MRP1 hyperexpressing H69AR cell line, relatively small changes in drug concentrations were needed to see the same effect as observed in the chemosensitive cell line (**Figure 3.3**). This drug sensitivity in a reported MDR cell line is due to the lack of resistance of MRP1 transporters towards taxanes (such as paclitaxel), thus prompting their use as potent combinatory drugs during chemotherapy [48]. The

inclusion of a higher dose (4.75 nM) resulted in a soluble protein content decline (304.89 µg/well) after 48 h, while the same decline was only witnessed in the lower dose (2.84 nM) after 72 h (315.90 µg/well), as shown in **Figure 3.3A**. Severely depleted intracellular ATP (0.39) was also seen for the higher treatment concentration of 4.75 nM, but with no associated AK increase (1.28) (**Figure 3.3**). ATP driven repair could be the reason for the higher ATP content. Alternatively, ATP was increased to drive P-gp related efflux of the drug, since P-gp is expressed in H69AR cells even though it is not hyperexpressed (see **Figure 5.A**). Although the same trend was seen for the ATP and soluble protein content at the lower concentration of 2.84 nM, AK release was increased significantly to (1.57), potentially indicating that active cell death or apoptosis occurred prior to the assessment at 96 h.

Paclitaxel is a well-known substrate of the ABC-transporter, P-gp [49], and severe resistance against this drug was expected in the NCI-H69/LX4 cell line hyperexpressing this transporter. When reviewing the protein content of the treated (1 282.70 µg/well at 2.84 nM and 1 369.23 µg/well at 613.05 nM) and untreated samples (954.67 µg/well) relatively small differences were present over the first 72 h, but significantly increased growth following both treatments at 96 h. This suggested that paclitaxel had no inhibitory effect on the proliferation abilities of the cells, seen in **Figure 3.4A**. Although a dose-dependent protein content reduction was not evident, a significant difference ($p < 0.01$; Kruskal-Wallis; Tukey's HSD tests & Mann-Whitney U test) was present when reviewing the ATP content (**Figure 3.4B**) after exposure to 2.84 nM (0.55) and 613.05 nM (0.45). However, the inverse was true for the amount of dead cells after exposure to 2.84 nM (0.70) and 613.05 nM (0.42) as indicated by the AK release (see **Figure 3.4B and 3.4C**). On closer inspection, it could be proposed that active cell death or apoptosis occurred around 48 h as indicated by a slight decrease in protein content, but that a sub-population of cells survived due to possible adaptation to the treatment. This would result in increased use of intracellular ATP to produce more cells while inhibiting overall metabolic capabilities. This energy shift towards replication, and likely also to powering of the P-gp efflux transporters, possibly minimised cell death.

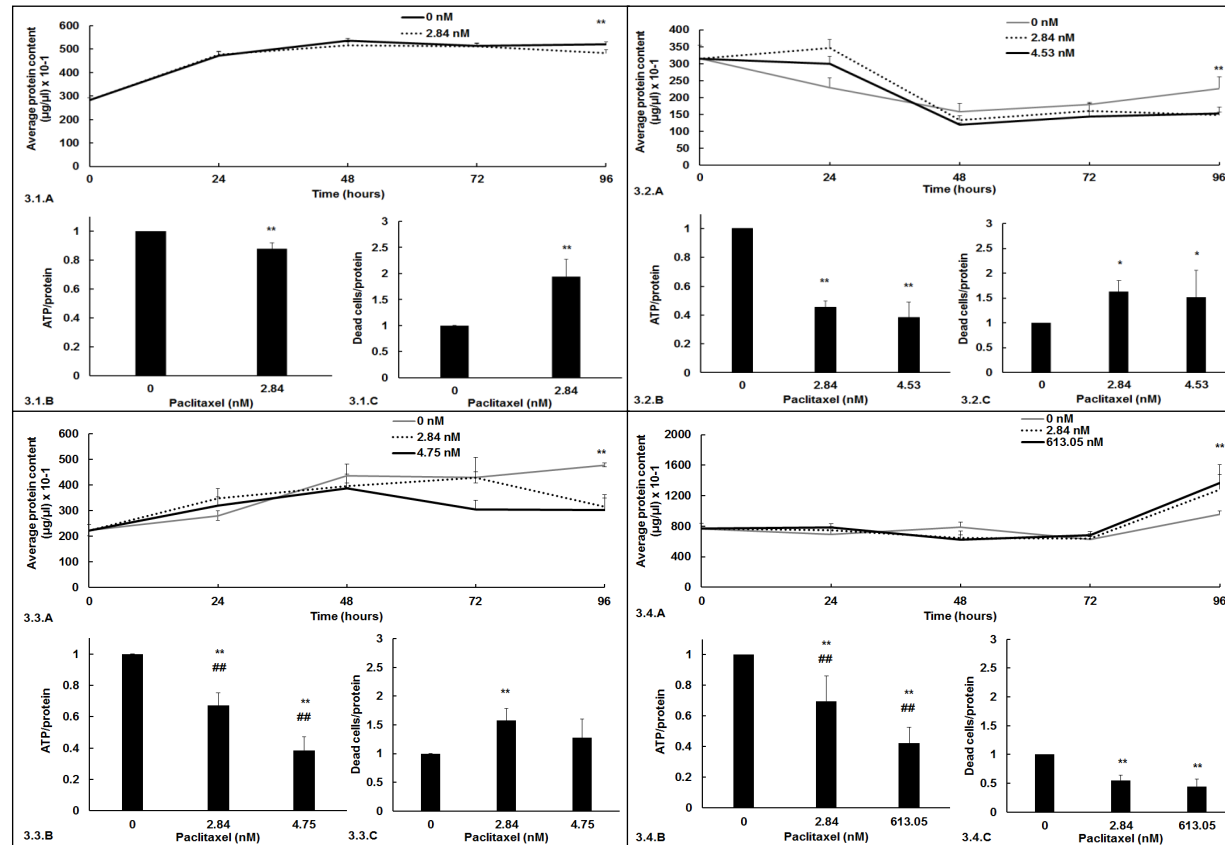


Figure 3: Cell responses following daily treatment with paclitaxel (nM) for 96 h. Soluble protein content over time (A), intracellular ATP levels per soluble protein content (normalised relative to the untreated control) (B) and extracellular AK levels per soluble protein content (normalised relative to the untreated control) (C) are shown for the LLC-PK1 (1), H69V (2), H69AR (3) and NCI-H69/LX4 (4) cell lines, respectively. Error bars denote the standard deviation ($n = 6$), # indicate statistically significant inter-dosage differences ($p < 0.05$); ## indicate statistical significant inter-dosage differences ($p < 0.01$); * indicate statistically significant differences relative to the untreated control ($p < 0.05$); ** indicate statistically significant differences relative to the untreated control ($p < 0.01$).

3.2.3 Cell responses following treatment with cisplatin

Cisplatin induces cytotoxicity through targeting nucleus DNA and formation of DNA-adducts, thus inhibiting replication and transcription [50]. If the cells are not capable of restoring adduct damage inflicted by cisplatin through repair proteins (e.g. nucleotide excision repair proteins) during cell cycle progression delays, cell death is initiated through either extrinsic (e.g. tumor necrosis factor- α) or intrinsic (cytochrome-c release) pro-apoptotic processes [51]. Cisplatin has the notable effect of starving cells from intracellular ATP through blocking of oxidative phosphorylation [52], possibly explaining the low metabolic activity determined with the MTT assay (see **Table 1**).

Cell responses following treatment with 170.75 nM; 283.47 nM; 427.34 nM or 2 155.45 nM cisplatin for 96 h, including soluble protein content over time, intracellular ATP levels per soluble protein content (normalised relative to the untreated controls) and extracellular AK levels per soluble protein content (normalised relative to the untreated controls) are shown in **Figure 4**.

As shown in **Figure 4.1A and 4.1C**, a very significant decline ($p < 0.01$; Bonferroni's post hoc test) in the protein content (480.25 $\mu\text{g}/\text{well}$ for 170.75 nM vs. 519.87 $\mu\text{g}/\text{well}$ for untreated cells) and increase in AK release (1.21; $p < 0.05$; Kruskal-Wallis; Tukey's HSD tests & Mann-Whitney U test) were reported in the non-cancerous porcine kidney cell line (LLC-PK1) following treatment with cisplatin. This could indicate cell death taking place in a controlled manner, after 96 h treatment. The significant decline in intracellular ATP levels following treatment with 170.75 nM (0.90; $p < 0.01$; Kruskal-Wallis; Tukey's HSD tests & Mann-Whitney U test) as seen in **Figure 4.1B**, in combination with the lower protein content and increased AK, indicated apoptosis of the cells. Nephrotoxicity is commonly seen as a side effect in clinical cisplatin treatment [30, 53], and notable toxicity is documented in patients after 10 days [54]. This requires treatment to be limited to 5 days at a time. The toxicity seen in **Figure 4.1** predicted this response and, therefore, showed clinically representative results.

The drug sensitive H69V cell line had no significant inter-dosage differences in soluble protein content (150.64 $\mu\text{g}/\text{well}$ for 170.75 nM and 140.38 $\mu\text{g}/\text{well}$ for 283.47nM), ATP (0.41 and 0.53, respectively) or the amount of dead cells (1.76 and 2.11, respectively), as seen in **Figure 4.2**. However, significance ($p < 0.05$) was observed between the treatments and the untreated control after 96 h for all cell responses evaluated. This highlighted the susceptibility of the chemo-naive SCLC cells to treatment with cisplatin.

Cisplatin resistance is primarily affected by the multidrug resistance-associated protein 2 (MRP2) ABC-transporter [55], but some level of cross-resistance have been reported for other transporters (e.g. P-gp and MRP1) [56]. As shown in **Figure 4.3**, compelling significance ($p < 0.01$; Bonferroni's post hoc test; Kruskal-Wallis; Tukey's HSD tests & Mann-Whitney U test) was found for all parameters measured following treatment with the H69AR IC₅₀ dosage of 427.34 nM. The elevated ATP concentration (0.85 compared to 0.80 at the lower H69V IC₅₀ concentration of 170.75 nM), together with the high level of dead cells as indicated by the AK release (1.34), showed that the MRP1 hyperexpressive cell line (H69AR) was probably undergoing apoptosis, while some cells were potentially already in late-stage necrosis (**Figure 4.3B & C**). The soluble protein content plateau at 430.58 µg/well seen for the lower dosage of 170.75 nM (**Figure 4.3A**), can possibly be attributed to cell cycle arrest initiated by the cells to repair DNA damage caused by manageable levels of intracellular cisplatin [57].

Although no significant protein changes were measured in the NCI-H69/LX4 cells (954.66 µg/well at 96 h) following treatment with 170.75 nM (969.62 µg/well) or 2155.45 nM (885.86 µg/well) (**Figure 4.4A**), the P-gp hyperexpressive cell line had decreased ATP (0.87) and increased AK release (3.92; $p < 0.01$) following treatment with the NCI-H69/LX4 IC₅₀ concentration of 170.75 nM cisplatin, suggesting apoptosis taking place (**Figure 4.4B & C**). The higher dosage of 2 155.45 nM cisplatin most likely initiated cell death much earlier, since the intracellular ATP was reduced significantly ($p < 0.01$), with no associated increase of extracellular AK levels.

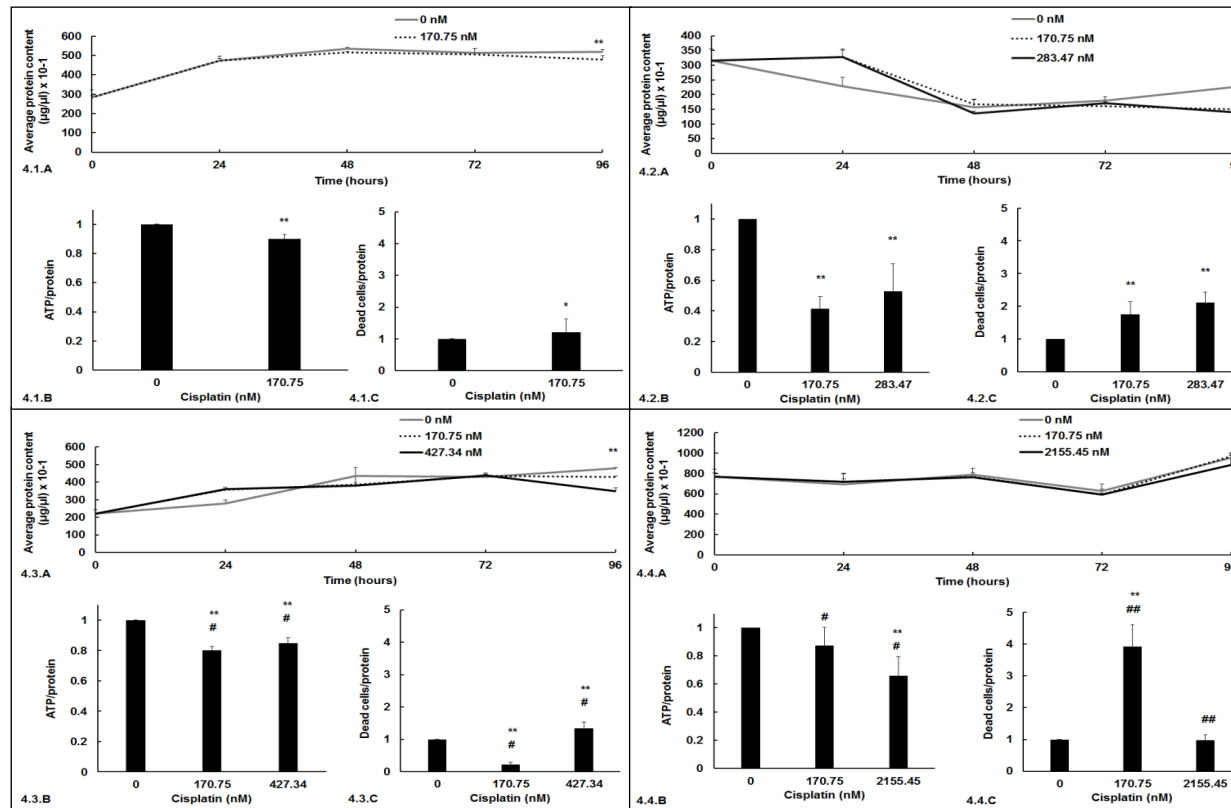


Figure 4: Cell responses following daily treatment with Cisplatin (nM) for 96 h. Soluble protein content over time (A), intracellular ATP levels per soluble protein content (normalised relative to the untreated control) (B) and extracellular AK levels per soluble protein content (normalised relative to the untreated control) (C) are shown for the LLC-PK1 (1), H69V (2), H69AR (3) and NCI-H69/LX4 (4) cell lines, respectively. Error bars denote the standard deviation ($n = 6$), # indicate statistically significant inter-dosage differences ($p < 0.05$); ## indicate statistical significant inter-dosage differences ($p < 0.01$); * indicate statistically significant differences relative to the untreated control ($p < 0.05$); ** indicate statistically significant differences relative to the untreated control ($p < 0.01$).

3.3 ABC-transporter expression in the cell models

The quantitative reverse transcription polymerase chain reaction assay (qRT-PCR) was used to quantify P-gp and MRP1 efflux transporter expression in the MDR cell lines, relative to the chemosensitive H69V cell line, and is presented as relative ratios (**Figure 5**). MRP1 transporter expression could be detected in all of the cell lines, but as illustrated in **Figure 5A**, statistically significant increased MRP1 expression was measured in the H69AR cell line compared to the NCI-H69/LX4 cell line ($p < 0.01$; Bonferroni's post-hoc test). Analysis of expression of the P-gp transporter (**Figure 5B**), indicated that only the two MDR cell lines expressed detectable levels of the transporter. Although there was some degree of P-gp expression by the H69AR cell line, statistically significant hyperexpression of P-gp was present in the NCI-H69/LX4 cell line compared to the H69AR cell line ($p < 0.01$; Bonferroni's post-hoc test).

These results substantiated the claims of efflux-based MDR in the two SCLC cell lines used in this study (e.g. MRP1 hyperexpression in the H69AR cell line and P-gp hyperexpression in the NCI-H69/LX4 cell line).

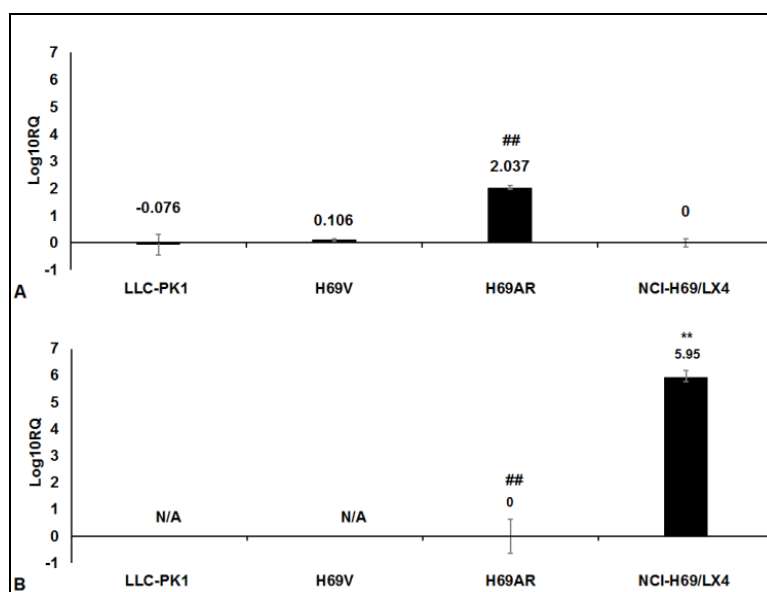


Figure 5: Relative quantification of MRP1 (A) and P-gp (B) efflux transporter gene expression in the LLC-PK1, H69V, H69AR and NCI-H69/LX4 cell lines, respectively. H69AR was used as the reference sample for P-gp expression, and the NCI-H69/LX4 cell line was used as the reference sample for MRP1 expression. Standard deviation is indicated with error bars, $n = 3$. ## indicate statistically significant differences between H69AR and the other cell lines ($p < 0.01$); ** indicate statistically significant differences between NCI-H69/LX4 and the other cell lines ($p < 0.01$); N/A indicates that no expression could be detected in the sample.

4. Conclusion

Based on cell viability assays such as MTT conversion and intracellular ATP quantification, this study provided a comprehensive evaluation of cell responses of three chemosensitive and efflux-based drug resistant SCLC cell lines following treatment with well characterised chemotherapeutic agents. The selected SCLC cell lines and the non-cancerous LLC-PK1 cell line included in this study were shown to respond to the selected treatments as anticipated, and the results corresponded well with previous studies or clinical trials. The cell models indicated substrate vulnerability to ABC transporters, possible cross-resistant effects as well as ABC-familial resistance conveyed by MRP1 transporters to an MRP2 substrate. Furthermore, the non-cancerous LLC-PK1 cell line showed increases in cytotoxicity following treatment with drugs known to be nephrotoxic in the clinical setting. The results suggested the potential use of combinatory drugs since the MDR H69AR cell model indicated sensitivity towards paclitaxel treatment. These cell models also indicated possible adaptation to treatment causing cell growth, propagated by MDR cell lines when confronted with chemotherapeutic drug substrates for their specific hyperexpressed transporters (e.g. paclitaxel in the NCI-H69/LX4 cell line). This could explain why certain cancers emerge stronger and more aggressive after initial treatment.

The ability of the cell models in parallel as a screening platform to predict such widespread resistance, may allow researchers to better identify effective drug combinations to counter ABC-transport efflux when introducing novel drugs. The use of the presented MDR SCLC cell lines as preclinical models, to comprehensively study and understand the reaction of resistant cancer cells towards specific drug treatments is important in the development of novel compounds and may contribute to a clinical trial design that will reduce unnecessary *in vivo* studies and maximise outputs.

Although many shortcomings have previously been reported for *in vitro* cell models in terms of cancer research, their importance in development and refinement of drugs prior to clinical trials cannot be ignored and continued use is warranted.

5. Acknowledgements

We would like to thank Prof. Suria Ellis at the North West University Statistical Consultation Services for her assistance with the statistical analyses. We would also like to thank Prof. Krzysztof Wrzesinski from CelVivo IVS, Denmark, for his contributions regarding the study design.

6. Funding

The work contained herein was financially supported by National Research Foundation (NRF) of South Africa under grant number [91460; 112971], as well as the Medical Research Council of South Africa (SAMRC) under Self-Initiated Research grant, and hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to these institutions.

7. Conflict of interest

The authors declare no conflict of interest.

8. References

- [1] J.P. van Meerbeeck, D.A. Fennell, D.K. De Ruyscher, Small-cell lung cancer, *Lancet* 378 (2011) 1741-1755.
- [2] C.L. Hodgkinson, C.J. Morrow, Y. Li, R.L. Metcalf, D.G. Rothwell, F. Trapani, R. Polanski, D.J. Burt, K.L. Simpson, K. Morris, Tumorigenicity and genetic profiling of circulating tumor cells in small-cell lung cancer, *Nature Medicine* 20 (2014) 897.
- [3] B.E. Lally, J.J. Urbanic, A.W. Blackstock, A.A. Miller, M.C. Perry, Small cell lung cancer: have we made any progress over the last 25 years?, *The Oncologist* 12 (2007) 1096-1104.
- [4] B.G. Campling, L.C. Young, K.A. Baer, Y.M. Lam, R.G. Deeley, S.P. Cole, J.H. Gerlach, Expression of the MRP and MDR1 multidrug resistance genes in small cell lung cancer, *Clin Cancer Res* 3 (1997) 115-122.
- [5] Y.T. Chen, B. Feng, L.B. Chen, Update of research on drug resistance in small cell lung cancer chemotherapy, *Asian Pac J Cancer Prev* 13 (2012) 3577-3581.
- [6] M.E. Katt, A.L. Placone, A.D. Wong, Z.S. Xu, P.C. Searson, *In Vitro* tumor models: advantages, disadvantages, variables, and selecting the right platform, *Front Bioeng Biotechnol* 4 (2016) 12.
- [7] A.F. Gazdar, L. Girard, W.W. Lockwood, W.L. Lam, J.D. Minna, Lung cancer cell lines as tools for biomedical discovery and research, *Jnci-J Natl Cancer I* 102 (2010) 1310-1321.

- [8] V.C. Daniel, L. Marchionni, J.S. Hierman, J.T. Rhodes, W.L. Devereux, C.M. Rudin, R. Yung, G. Parmigiani, M. Dorsch, C.D. Peacock, D.N. Watkins, A primary xenograft model of small-cell lung cancer reveals irreversible changes in gene expression imposed by culture *in vitro*, *Cancer Res* 69 (2009) 3364-3373.
- [9] J.J. Yeh, N.Y. Hsu, W.H. Hsu, C.H. Tsai, C.C. Lin, J.A. Liang, Comparison of chemotherapy response with P-glycoprotein, multidrug resistance-related protein-1, and lung resistance-related protein expression in untreated small cell lung cancer, *Lung* 183 (2005) 177-183.
- [10] S. Wilkens, Structure and mechanism of ABC transporters, *F1000Prime Rep* 7 (2015) 14.
- [11] I.B. Holland, ABC transporters, mechanisms and biology: an overview, *Essays Biochem* 50 (2011) 1-17.
- [12] Q. Wu, Z. Yang, Y. Nie, Y. Shi, D. Fan, Multi-drug resistance in cancer chemotherapeutics: mechanisms and lab approaches, *Cancer Lett* 347 (2014) 159-166.
- [13] G. Housman, S. Byler, S. Heerboth, K. Lapinska, M. Longacre, N. Snyder, S. Sarkar, Drug resistance in cancer: an overview, *Cancers (Basel)* 6 (2014) 1769-1792.
- [14] E.A. Abdallah, M.F. Fanelli, E.S.V. Souza, M.C. Machado Netto, J.L. Gasparini Junior, D.V. Araujo, L.M. Ocea, M.E. Buim, M.S. Tariki, S. Alves Vda, V. Piana de Andrade, A.L. Dettino, C. Abdon Lopes de Mello, L.T. Chinen, MRP1 expression in CTCs confers resistance to irinotecan-based chemotherapy in metastatic colorectal cancer, *Int J Cancer* 139 (2016) 890-898.
- [15] M.E. El-Araby, A.M. Omar, M.T. Khayat, H.A. Assiri, A.M. Al-Abd, Molecular mimics of classic p-glycoprotein inhibitors as multidrug resistance suppressors and their synergistic effect on paclitaxel, *Plos One* 12 (2017) e0168938.
- [16] P.V. Korita, T. Wakai, Y. Shirai, Y. Matsuda, J. Sakata, M. Takamura, M. Yano, A. Sanpei, Y. Aoyagi, K. Hatakeyama, Y. Ajioka, Multidrug resistance-associated protein 2 determines the efficacy of cisplatin in patients with hepatocellular carcinoma, *Oncol Rep* 23 (2010) 965-972.
- [17] K. Wrzesinski, S.J. Fey, After trypsinisation, 3D spheroids of C3A hepatocytes need 18 days to re-establish similar levels of key physiological functions to those seen in the liver, *Toxicology Research* 2 (2013) 123-135.

- [18] D.A. Williams, J. Lokich, A review of the stability and compatibility of antineoplastic drugs for multiple-drug infusions, *Cancer Chemother Pharmacol* 31 (1992) 171-181.
- [19] A. Karbownik, E. Szalek, H. Urjasz, A. Gleboka, E. Mierzwa, E. Grzeskowiak, The physical and chemical stability of cisplatin (Teva) in concentrate and diluted in sodium chloride 0.9%, *Contemp Oncol (Pozn)* 16 (2012) 435-439.
- [20] M.D. Wissing, J. Mendonca, E. Kim, E. Kim, J.S. Shim, N.S. Kaelber, H. Kant, H. Hammers, T. Commes, P.J. Van Diest, J.O. Liu, S.K. Kachhap, Identification of cetrimonium bromide and irinotecan as compounds with synthetic lethality against NDRG1 deficient prostate cancer cells, *Cancer Biology & Therapy* 14 (2013) 401-410.
- [21] N.A. Duggett, L.A. Griffiths, O.E. McKenna, V. de Santis, N. Yongsanguanchai, E.B. Mokori, S.J. Flatters, Oxidative stress in the development, maintenance and resolution of paclitaxel-induced painful neuropathy, *Neuroscience* 333 (2016) 13-26.
- [22] M. Singh, K. McKenzie, X. Ma, Effect of dimethyl sulfoxide on *in vitro* proliferation of skin fibroblast cells, *Journal of Biotech Research* 8 (2017) 78.
- [23] S. Léonce, V. Perez, M. R Casabianca-Pignede, M. Anstett, E. Bisagni, A. Pierré, G. Atassi, *In vitro* cytotoxicity of S16020-2, a new olivacine derivative, *Investigational new drugs* 14 (1996) 169-180
- [24] T.L. Riss, R.A. Moravec, Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays, *Assay and Drug Development Technologies* 2 (2004) 51-62.
- [25] C. Calitz, J.H. Hamman, A.M. Viljoen, S.J. Fey, K. Wrzesinski, C. Gouws, Toxicity and anti-proliferic properties of *Xysmalobium undulatum* water extract during short-term exposure to two-dimensional and three-dimensional spheroid cell cultures, *Toxicol Mech Methods* 28 (2018) 641-652.
- [26] P.E. Andreotti, I.A. Cree, C.M. Kurbacher, D.M. Hartmann, D. Linder, G. Harel, I. Gleiberman, P.A. Caruso, S.H. Ricks, M. Untch, C. Sartori, H.W. Bruckner, Chemosensitivity testing of human tumors using a microplate adenosine-triphosphate luminescence assay - clinical correlation for cisplatin resistance of ovarian-carcinoma, *Cancer Research* 55 (1995) 5276-5282.

- [27] D. Squirrell, J. Murphy, Rapid detection of very low numbers of micro-organisms using adenylate kinase as a cell marker, *A practical guide to industrial uses of ATP luminescence in rapid microbiology* (1997) 107-113.
- [28] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, *25* (2001) 402-408.
- [29] R.R. Barakat, M. Markman, M. Randall, *Principles and practice of gynecologic oncology*, Lippincott Williams & Wilkins, 2009.
- [30] N. Pabla, Z. Dong, Cisplatin nephrotoxicity: mechanisms and renoprotective strategies, *Kidney Int* 73 (2008) 994-1007.
- [31] S.O. Rabah, Acute Taxol nephrotoxicity: Histological and ultrastructural studies of mice kidney parenchyma, *Saudi J Biol Sci* 17 (2010) 105-114.
- [32] Y. Fuchs, H. Steller, Live to die another way: modes of programmed cell death and the signals emanating from dying cells, *Nature Reviews Molecular Cell Biology* 16 (2015) 329-344.
- [33] B. Allard, M.S. Longhi, S.C. Robson, J. Stagg, The ectonucleotidases CD 39 and CD 73: Novel checkpoint inhibitor targets, *Immunological reviews* 276 (2017) 121-144.
- [34] U. Schenk, M. Frascoli, M. Proietti, R. Geffers, E. Traggiai, J. Buer, C. Ricordi, A.M. Westendorf, F. Grassi, ATP Inhibits the generation and function of regulatory t cells through the activation of purinergic p2x receptors, *Sci Signal* 4 (2011) ra12-ra12.
- [35] P. Pellegatti, L. Raffaghello, G. Bianchi, F. Piccardi, V. Pistoia, F. Di Virgilio, Increased level of extracellular atp at tumor sites: *in vivo* imaging with plasma membrane luciferase, *Plos One* 3 (2008) e2599.
- [36] A.I. Tarasov, E.J. Griffiths, G.A. Rutter, Regulation of ATP production by mitochondrial Ca^{2+} , *Cell Calcium* 52 (2012) 28-35.
- [37] Y. Liu, Y. Cao, W. Zhang, S. Bergmeier, Y. Qian, H. Akbar, R. Colvin, J. Ding, L. Tong, S. Wu, J. Hines, X. Chen, A small-molecule inhibitor of glucose transporter 1 downregulates glycolysis, induces cell-cycle arrest, and inhibits cancer cell growth *in vitro* and *in vivo*, *Mol Cancer Ther* 11 (2012) 1672-1682.
- [38] M. Leist, B. Single, H. Naumann, E. Fava, B. Simon, S. Kuhnle, P. Nicotera, Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis to necrosis, *Exp Cell Res* 249 (1999) 396-403.

- [39] R. Khanna, C.L. Morton, M.K. Danks, P.M. Potter, Proficient metabolism of irinotecan by a human intestinal carboxylesterase, *Cancer Res* 60 (2000) 4725-4728.
- [40] J.G. Kuhn, Pharmacology of irinotecan, *Oncology (Williston Park, N.Y.)* 12 (1998) 39-42.
- [41] Y. Pommier, M. Cushman, The indenoisoquinoline noncamptothecin topoisomerase I inhibitors: update and perspectives, *Mol Cancer Ther* 8 (2009) 1008-1014.
- [42] X. Bao, J. Wu, S. Kim, P. LoRusso, J. Li, Pharmacometabolomics reveals irinotecan mechanism of action in cancer patients, *J Clin Pharmacol* (2018) 1-15.
- [43] T. Bansal, G. Mishra, M. Jaggi, R.K. Khar, S. Talegaonkar, Effect of P-glycoprotein inhibitor, verapamil, on oral bioavailability and pharmacokinetics of irinotecan in rats, *Eur J Pharm Sci* 36 (2009) 580-590.
- [44] S.L. Mooberry, Microtubules as a target for anticancer drugs, *New frontiers and Treatment paradigms for metastatic breast cancer, Clinical Advances for Hematology & Oncology* 28 (2011) 7-9.
- [45] B.A. Weaver, How Taxol/paclitaxel kills cancer cells, *Molecular biology of the cell* 25 (2014) 2677-2681.
- [46] T.K. Bergmann, H. Green, C. Brasch-Andersen, M.R. Mirza, J. Herrstedt, B. Holund, A. du Bois, P. Damkier, W. Vach, K. Brosen, C. Peterson, Retrospective study of the impact of pharmacogenetic variants on paclitaxel toxicity and survival in patients with ovarian cancer, *Eur J Clin Pharmacol* 67 (2011) 693-700.
- [47] R.J. Kirschling, J.P. Grill, R.S. Marks, J.W. Kugler, J.B. Gerstner, S.A. Kuross, J.C. Michalak, H.E. Windschitl, K.D. Krewer, J.R. Jett, Paclitaxel and G-CSF in previously untreated patients with extensive stage small-cell lung cancer: a phase II study of the North central cancer treatment group, *Am J Clin Oncol* 22 (1999) 517-522.
- [48] R.J. Kathawala, P. Gupta, C.R. Ashby, Jr., Z.S. Chen, The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade, *Drug Resist Updat* 18 (2015) 1-17.
- [49] S.V. Ambudkar, S. Dey, C.A. Hrycyna, M. Ramachandra, I. Pastan, M.M. Gottesman, Biochemical, cellular, and pharmacological aspects of the multidrug transporter, *Annu Rev Pharmacol Toxicol* 39 (1999) 361-398.
- [50] L. Amable, Cisplatin resistance and opportunities for precision medicine, *Pharmacol Res* 106 (2016) 27-36.

- [51] S. Dasari, P.B. Tchounwou, Cisplatin in cancer therapy: molecular mechanisms of action, *Eur J Pharmacol* 740 (2014) 364-378.
- [52] S.K. Aggarwal, A histochemical approach to the mechanism of action of cisplatin and its analogues, *J Histochem Cytochem* 41 (1993) 1053-1073.
- [53] A.A. Saad, M.I. Youssef, L.K. El-Shennawy, Cisplatin induced damage in kidney genomic DNA and nephrotoxicity in male rats: the protective effect of grape seed proanthocyanidin extract, *Food Chem Toxicol* 47 (2009) 1499-1506.
- [54] I. Arany, R.L. Safirstein, Cisplatin nephrotoxicity, *Seminars in nephrology*, Elsevier, 2003, Vol. 23, pp. 460-464.
- [55] S.Z. Ke, X.Y. Ni, Y.H. Zhang, Y.N. Wang, B. Wu, F.G. Gao, Camptothecin and cisplatin upregulate ABCG2 and MRP2 expression by activating the ATM/NF- κ B pathway in lung cancer cells, *International Journal of Oncology* 42 (2013) 1289-1296.
- [56] L. Galluzzi, L. Senovilla, I. Vitale, J. Michels, I. Martins, O. Kepp, M. Castedo, G. Kroemer, Molecular mechanisms of cisplatin resistance, *Oncogene* 31 (2012) 1869-1883.
- [57] V. Velma, S.R. Dasari, P.B. Tchounwou, Low Doses of Cisplatin induce gene alterations, cell cycle arrest, and apoptosis in human promyelocytic leukemia Cells, *Biomark Insights* 11 (2016) 113-121.

CHAPTER 6

This chapter presents the final conclusions of this study, and discusses some future recommendations.

FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1 Introduction

Acquired MDR was first induced in HeLa cells through actinomycin D exposure more than 50 years ago, that led to the characterisation and exploration of 48 human adenine triphosphate (ATP)-binding cassette (ABC) efflux transporters (Robey *et al.*, 2018). From a family of 48 ABC transporters, those predominantly responsible for cancer tissue defence has been found to be P-glycoprotein (P-gp) and multidrug resistance protein 1 (MRP1) transporters (Fletcher *et al.*, 2016). Although the science behind efflux induced MDR in cancer was broadly investigated, most patients with stage IV small cell lung carcinoma (SCLC) will eventually succumb to resistance within 1 year (25.7 % survival rate) due to the inability of chemotherapeutic drugs to surpass these transporters during treatment (Dayen *et al.*, 2017). This low survival rate hinders research through the introduction of a wider range of dosages with varying periods of drug exposure, thus limiting the study of evasion strategies in ABC transporter-induced MDR SCLC. The inability to fully explore MDR in these cancers allows only an elementary understanding of how cells react to chemotherapeutic drugs during treatment in clinical trials, and ultimately scientists are still unable to comprehensively explain how drug failure can be overcome.

The particular importance of *in vitro* models containing acquired ABC induced MDR through pre-exposure to chemotherapeutic drugs is highlighted by the fact that SCLC has an almost inevitable relapse after initial treatment (van Meerbeeck *et al.*, 2011). Since these cells were exposed to chemotherapeutic drugs, they change to resistant cells with similar drug resistance profiles as MDR cancer *in vivo*.

In this study, the primary goal was to evaluate the suitability of three SCLC cell lines with varying treatment sensitivities for preclinical screening of MDR cancer treatment. This was established through exposure of the three SCLC *in vitro* models to varying concentrations of established chemotherapeutic drugs, each known to be a substrate for a specific efflux transporter. The inclusion of non-cancerous pig embryonic kidney cells (LLC-PK1) also enabled testing of dose-dependent cytotoxicity.

6.2 Efflux transporter hyperexpression validation

To confirm the drug resistance of the selected SCLC cell lines reported in literature, quantification of the efflux transporters' gene expression was performed. Based on the results from the gene expression assays, all of the cell lines expressed MRP1 transporter mRNA (messenger ribonucleic acid) to some

extent, with the exception of the H69AR cell line which hyperexpressed this transporter (see **Figure 5.5A**) as reported in literature (Hipfner *et al.*, 1999). P-gp expression was below the detection limits in the LLC-PK1 and the chemosensitive H69V cell lines (see **Figure 5.5B**). P-gp hyperexpression was confirmed in the NCI-H69/LX4 line as reported in literature, while the H69AR cell line expressed P-gp to a limited extent (Sandvold *et al.*, 2010). Although minor, the presence of this transporter clearly played a part in inferring some P-gp-related resistance (as discussed in **Section 4.3**).

6.3 Preliminary efficacy screening

To screen the efficacy of the selected anticancer drugs in the four model cell lines, the cells were subjected to daily treatment with a range of concentrations of the known transporter substrates. After 96 h treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to estimate the concentration of each chemotherapeutic drug needed to inhibit cell viability 50% (IC₅₀) relative to the untreated controls, for each cell line.

From the MTT preliminary screening results, it was apparent that the MDR cell lines were severely resistant to treatment with their specific transporter substrate model compounds, thus confirming previous findings of how MDR is inferred by ABC transporters (Robey *et al.*, 2018). Chemotherapeutic compounds reported to be substrates for the specific transporters hyperexpressed in the SCLC cell lines used in this study, accurately portrayed the resistance to treatment expected. Efficacy of the MRP1 substrate, irinotecan, was clearly reduced in the MRP1 hyperexpressive H69AR cell line (see **Figure 4.7**), while even greater resistance was seen in the P-gp hyperexpressive cell line, NCI-H69/LX4, when treated with the P-gp substrate, paclitaxel (see **Figure 4.12**). The cell models were even capable of predicting the cross-resistance commonly seen with P-gp hyperexpressive cells, as well as the familial resistance seen within the MRP family. This was demonstrated by the resistance of the H69AR cell line observed following treatment with an MRP2 substrate, cisplatin (see **Figure 4.3**).

Furthermore, the MTT assay illustrated the ability of the MDR H69AR cell line to desensitise itself to treatment with MRP1 substrates such as irinotecan, after excessive dosing (see **Figure 4.7**). This highlighted how meticulous the treatment of cancer should be selected, and why drug failure is so prominent in heavily pre-treated individuals. The similar drug sensitivity profiles for paclitaxel in the H69V and the MDR H69AR cell lines showed the lack of resistance to a strong P-gp substrate like paclitaxel in the presence of hyperexpressed MRP1 transporters. This suggested the potential use of drugs like cisplatin as a combinatory drug, as well as a second line treatment in heavily pre-treated individuals. The small dosage increase needed to see the same level of cytotoxicity in the H69AR line with paclitaxel as

for its drug-sensitive H69V counterpart, also made sense when considering the low levels of P-gp expression in the H69AR cell line (see **Figure 5.5B**).

6.4 Advanced efficacy screening

The data obtained with the MTT assay following the concentration series treatments were statistically evaluated with the Probit analyses method. The calculated IC_{50} and IC_{75} values (concentrations needed to reduce cell viability with 50% or 75%, relative to the untreated controls) were subsequently used to study the efficacy of the model drugs in the specific cell models, using more sensitive assays. These assays included evaluation of intracellular ATP, extracellular adenylate kinase (AK) and cell populations through soluble protein quantification.

Introduction of extensive assays following treatment with the dosages determined from the MTT data enabled formulation of a more complete picture of drug evasion mechanisms in the cancer cell lines. By measuring intracellular ATP in the cells after exposure to the various model drugs and quantifying cell death by measuring AK release, it was possible to differentiate between cell cycle arrest, apoptosis and necrosis. A metabolic shift occurred in most of the cell lines, where ATP was most probably utilised to power efflux transporters, activate cell cycle arrest to fix damaged deoxyribonucleic acid (DNA) and to initiate general chemo-evasive measures (Sholl-Franco *et al.*, 2010; Göddeke *et al.*, 2018). Using only the MTT assay, this shift of energy could easily have been reported as a false positive for sensitivity to a chemotherapeutic drug, thus showing the importance of the inclusion of the more sensitive assays. The quantification of the total soluble protein provided an estimate of cell population size since stunted or exponential growth could easily be observed from the protein graphs.

Although some cytotoxicity due to irinotecan was reported in the LLC-PK1 cell line when reviewing the AK and ATP levels in the cells (shown in **Figure 2.1**), soluble protein levels showed no notable decline in population growth. This was most probably due to the ability of the cell populations to initiate repair mechanisms. Treatment of the drug-sensitive H69V cell line induced severe depletion of ATP, reduced soluble protein content as well as increased AK release, substantiating severe dose-dependent chemosensitivity. The MDR cell lines, however, showed severe resistance to treatment with this MRP1 substrate. H69AR, the MRP1 hyperexpressing cell line, only indicated cytotoxic events after the first 48 hours at extremely high dosages. Thereafter, cell cycle arrest probably initiated cell repair mechanisms. This supports the claims of severe resistance inferred against MRP1 substrates. Cross-resistance was seen to some degree in the P-gp hyperexpressing NCI-H69/LX4 cell line, with cell death being activated

only after severely increasing concentration (indicated by depleted ATP and increased AK release in **Figure 2.4**).

Paclitaxel induced toxicity at low concentrations in the LLC-PK1 non-cancerous cell line, with a notable decline in soluble protein and ATP content, as well as an increase in AK (**Figure 3.1**). This correlates well with previous clinical reports of nephrotoxicity (Rabah, 2010). The drug sensitive H69V cell line, as well as the MRP1 hyperexpressing H69AR cell line, showed significant dose-dependent cell death (**Figure 3.2 & 3.3**). This is due to paclitaxel's inability to be effluxed by the hyperexpressed MRP1 transporters (Borst *et al.*, 2000). The NCI-H69/LX4 cell line hyperexpressing P-gp showed severe resistance to paclitaxel since this compound is a known P-gp substrate. The cells appeared to activate a metabolic shift, possibly using ATP to increase cell proliferation (increased soluble protein content in **Figure 3.4**) after exposure to the treatment. The low AK levels also showed how the cells remained unaffected by extremely high dosages.

Cisplatin had similar effects on the LLC-PK1 cell line as previously seen clinically, with severe toxicity induced in the kidney cells (discussed in **Section 3.2.3**). Furthermore, the drug sensitive cell line, H69V, was also very sensitive to cisplatin treatment, although the concentrations did not appear to affect the rate of death. Some familial resistance to this MRP2 substrate was seen in the MRP1 hyperexpressing cell line (H69AR), since the cells were able to efflux enough of the lower drug concentration (170.75 nM) to activate cell repair mechanisms (indicated by cytostatic cellular behaviour as indicative by protein content and low AK release in **Figure 4.3.A & C**). An increased dosage (427.34 nM) did circumvent this repair, suggesting only partial resistance or saturation of the transporters. In the P-gp hyperexpressing NCI-H69/LX4 cell line, only a few vulnerable populations in the suspension cell line succumb to this treatment. The surviving populations are extremely resistant and showed no response, thus indicating severe MDR.

6.5 Final conclusion

The use of mammalian cancer cell lines for the *in vitro* screening of cancer compounds have enabled immeasurable advances in the development of modern chemotherapy. In numerous cases drug development programs skim over the potential use of these cell lines, only to experience therapeutic failure during *in vivo* or human clinical trials. The failure of these drugs could possibly have been predicted in some instances through the use of comprehensively characterised cell models. Such an approach could potentially limit the associated costs of these failures and reduce the ethical implications seen with human and animal trials.

This study clearly demonstrated the potential of the three H69 cell variants as models to study SCLC drug resistance *in vitro*. The incorporation of these cell models combined as a preclinical screening platform has immense potential to predict possible resistance and clinical drug failure. These models may also provide valuable information and give a more comprehensive approach to understanding MDR in cancer.

6.6 Future recommendations

The models presented in this study have endless capabilities, one of which may be to incorporate combinatory compounds to view how MDR cancers respond to multiple DNA damaging agents. The combination of these standard chemotherapeutic drugs at different concentrations could provide valuable insight into how certain drug ratios can either induce MDR circumvention or induce further resistance.

Another promising application for these models is the inclusion of combinations containing standard chemotherapeutic drugs and phytochemicals from traditional plant remedies. This would not only enable identification of potentially detrimental herb-drug interactions but also allow preclinical screening of these interactions to circumvent MDR through competitive interaction with ABC transporters. This could be exploited to increase intracellular chemotherapeutic compound concentrations to increase efficacy.

Although results comparable to clinical information was obtained with the LLC-PK1 cell line, its isolation from a porcine hosts' kidney is a limitation. The inclusion of a non-cancerous pulmonary cell line from human origin should produce more valuable information in terms of drug selectivity to lung tissue. Another possibility is to include multiple human cell lines from metabolic organs (kidney/hepatic) to view possible dose-limiting toxicity in the clinical setting.

Although valuable information was gathered from the 2D models, an important future application for these characterised cell lines would be their incorporation into novel 3D *in vitro* cell models. These 3D cell models have the potential to refine translation towards the actual *in vivo* environment and may fill the gaps of physiological irrelevance commonly seen with *in vitro* models. 3D cell models are described as the future of drug discovery due to their physiological similarity to actual tumour microenvironments, thus making the incorporation of well-defined multidrug resistant cell lines into these models an opportunity to further refine drug development, and possibly decrease clinical drug failures (Elliott & Yuan, 2011; Breslin & O'Driscoll, 2013).

6.7 References

- Borst, P., Evers, R., Kool, M. & Wijnholds, J. 2000. A Family of Drug Transporters: the Multidrug Resistance-Associated Proteins. *JNCI: Journal of the National Cancer Institute*, 92(16): 1295-1302.
- Breslin, S. & O'Driscoll, L. 2013. Three-dimensional cell culture: the missing link in drug discovery. *Drug Discovery Today*, 18(5-6): 240-249.
- Dayen, C., Debieuvre, D., Molinier, O., Raffy, O., Paganin, F., Virally, J., Larive, S., Desurmont-Salasc, B., Perrichon, M. & Martin, F. 2017. New insights into stage and prognosis in small cell lung cancer: an analysis of 968 cases. *Journal of Thoracic Disease*, 9(12): 5101.
- Elliott, N.T. & Yuan, F. 2011. A review of three-dimensional *in vitro* tissue models for drug discovery and transport studies. *Journal of pharmaceutical sciences*, 100(1): 59-74.
- Fletcher, J.I., Williams, R.T., Henderson, M.J., Norris, M.D. & Haber, M. 2016. ABC transporters as mediators of drug resistance and contributors to cancer cell biology. *Drug Resistance Updates*, 26: 1-9.
- Göddeke, H., Prieß, M., Groenhof, G. & Schäfer, L. 2018. Is ATP Hydrolysis the Power Stroke in ABC Transporters? *Biophysical Journal*, 114(3): 148a.
- Hipfner, D.R., Deeley, R.G. & Cole, S.P. 1999. Structural, mechanistic and clinical aspects of MRP1. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1461(2): 359-376.
- Rabah, S.O. 2010. Acute Taxol nephrotoxicity: Histological and ultrastructural studies of mice kidney parenchyma. *Saudi Journal of Biological Sciences*, 17(2): 105-114.
- Robey, R.W., Pluchino, K.M., Hall, M.D., Fojo, A.T., Bates, S.E. & Gottesman, M.M. 2018. Revisiting the role of ABC transporters in multidrug-resistant cancer. *Nature Reviews Cancer*, 18 (7): 452–464
- Sandvold, M.L., Galmarini, C., Myhren, F. & Peters, G. 2010. The activity of the lipophilic nucleoside derivatives elacytarabine and CP-4126 in a panel of tumor cell lines resistant to nucleoside analogues. *Nucleosides, Nucleotides and Nucleic Acids*, 29(4-6): 386-393.
- Sholl-Franco, A., Fragel-Madeira, L., Macama, A.d.C.C., Linden, R. & Ventura, A.L.M. 2010. ATP controls cell cycle and induces proliferation in the mouse developing retina. *International Journal of Developmental Neuroscience*, 28(1): 63-73.
- van Meerbeeck, J.P., Fennell, D.A. & De Ruyscher, D.K. 2011. Small-cell lung cancer. *The Lancet*, 378(9804): 1741-1755.

APPENDIX A

AUTHOR GUIDELINES FOR CURRENT CANCER DRUG TARGETS

Guidelines for the publication of the content written in the review article for the journal, Cancer Drug Targets (Impact factor 2.622; ISSN: 1873-5576)

A.1 INTRODUCTION

A1.1 ONLINE MANUSCRIPT SUBMISSION

Manuscripts must be submitted by one of the authors of the manuscript, and should not be submitted by anyone on their behalf. The principal/corresponding author will be required to submit a Copyright Letter along with the manuscript, on behalf of all the co-authors (if any). The author(s) will confirm that the manuscript (or any part of it) has not been published previously or is not under consideration for publication elsewhere. Furthermore, any illustration, structure or table that has been published elsewhere must be reported, and copyright permission for reproduction must be obtained.

For all online submissions, please provide soft copies of all the materials (main text in MS Word or Tex/LaTeX), figures / illustrations in TIFF, PDF or JPEG, and chemical structures drawn in ChemDraw (CDX) / ISISDraw (TGF) as separate files, while a PDF version of the entire manuscript must also be included, embedded with all the figures/illustrations/tables/chemical structures etc. It is advisable that the document files related to a manuscript submission should always have the name of the corresponding author as part of the file name, i.e., "Cilli MS text.doc, Cilli MS Figure 1, etc.

It is imperative that before submission, authors should carefully proofread the files for special characters, mathematical symbols, Greek letters, equations, tables, references and images, to ensure that they appear in proper format.

References, figures, tables, chemical structures etc. should be referred to in the text at the appropriate place where they have been first discussed. Figure legends/captions should also be provided.

A successful electronic submission of a manuscript will be followed by a system-generated acknowledgement to the principal/corresponding author. Any queries therein should be addressed to info@benthamscience.org

A.2 EDITORIAL POLICIES

For journals and eBooks, the following publication policies are applied by Bentham Science.

A2.1 Peer Review

Bentham Science Publishers follows the single blind peer-review procedure for submissions of all manuscripts to its journals, except for a selected number of patent journals where double blind review is followed.

All submitted articles/eBook chapters are subjected to an extensive peer review in consultation with members of the journal s editorial board and independent external referees (usually three reviewers). All manuscripts/chapters are assessed rapidly and the decision based on all the peer reviewers' comments, taken by the journal s Editor-in-Chief/eBook Editor, is then conveyed to the author(s).

Submissions from the Editor-in-Chief will undergo independent peer-review and will be submitted to another Editor for his decision on acceptance.

A2.2 Copyediting and Proofs

Articles and eBook chapters must be written in good English in a clear and correct style in order to maintain uniformity throughout the text. Articles/chapters submitted are copyedited before they are published.

A.2.3 Reprints

High-quality, bound/unbound, print/e-prints can be purchased for all published articles and book chapters.

A.2.4 Editorial Policies

Articles/chapters must be submitted by one of the authors of the manuscript, and should not be submitted by anyone on their behalf. The principal/corresponding author will be required to submit a Copyright Letter along with the manuscript, on behalf of all the co-authors (if any). The author(s) will confirm that the manuscript (or any part of it) has not been published previously or is not under consideration for publication elsewhere. Furthermore, any illustration, structure or table that has been published elsewhere must be reported, and copyright permission for reproduction must be obtained.

A.2.5 Appeals and Complaints

Generally, the editorial decisions are not reverted. However, authors who think that their manuscript was rejected due to a misunderstanding or mistake may seek an explanation for the decision. Appeals must give sound reasoning and compelling evidence against the criticism raised in the rejection letter. A difference of opinion as to the interest, novelty, or suitability of the manuscript for the journal will not be considered as an appeal. The EIC and other relevant editors will consider the appeal and the decision thereafter taken by the journal will be deemed final. Acceptance of the manuscript is not guaranteed even if the journal agrees to reconsider the manuscript, and the reconsideration process may involve previous or new reviewers or editors and substantive revision.

Authors who wish to make a complaint should refer them to the Editor-in-Chief of the journal concerned. Complaints to the Publisher may be emailed to info@benthamscience.org

A.2.6 Conflict of Interest

Financial contributions to the work being reported should be clearly acknowledged, as should any potential conflict of interest.

A.2.7 Plagiarism Prevention

Bentham Science uses the iThenticate software to detect instances of overlapping and similar text in submitted manuscripts. iThenticate software checks content against a database of periodicals, the Internet, and a comprehensive article database. It generates a similarity report, highlighting the percentage of overlap between the uploaded article and the published material. Any instance of content overlap is further scrutinized for suspected plagiarism according to the publisher's Editorial Policies. Bentham Science allows an overall similarity of 20% for a manuscript to be considered for publication. The similarity percentage is further checked keeping the following important points in view

A.2.7.1 Low Text Similarity

The text of every submitted manuscript is checked using the Content Tracking mode in iThenticate. The Content Tracking mode ensures that manuscripts with an overall low percentage similarity (but may have a higher similarity from a single source) are not overlooked. The acceptable limit for similarity of text from a single source is 5%. If the similarity level is above 5%, the manuscript is returned to the author for paraphrasing the text and citing the original source of the copied material.

It is important to mention that the text taken from different sources with an overall low similarity percentage will be considered as a plagiarized content if the majority of the article is a combination of copied material.

A.2.7.2 High Text Similarity

There may be some manuscripts with an overall low similarity percentage, but a higher percentage from a single source. A manuscript may have less than 20% overall similarity but there may be 15% similar text taken from a single article. The similarity index in such cases is higher than the approved limit for a single source. Authors are advised to thoroughly rephrase the similar text and properly cite the original source to avoid plagiarism and copyright violation.

A.2.7.3 Types of Plagiarism

We all know that scholarly manuscripts are written after thorough review of previously published articles. It is therefore not easy to draw a clear boundary between legitimate representation and plagiarism. However, the following important features can assist in identifying different kinds of plagiarized content. These are:

- Reproduction of others words, sentences, ideas or findings as one's own without proper acknowledgement.
- Text recycling, also known as self-plagiarism. It is an author's use of a previous publication in another paper without proper citation and acknowledgement of the original source.
- Poor paraphrasing: Copying complete paragraphs and modifying a few words without changing the structure of original sentences or changing the sentence structure but not the words.
- Verbatim copying of text without putting quotation marks and not acknowledging the work of the original author.
- Properly citing a work but poorly paraphrasing the original text is considered as unintentional plagiarism. Similarly, manuscripts with language somewhere between paraphrasing and quoting are not acceptable. Authors should either paraphrase properly or quote and in both cases, cite the original source.
- Higher similarity in the abstract, introduction, materials and methods, and discussion and conclusion sections indicates that the manuscript may contain plagiarized text. Authors can easily explain these parts of the manuscript in many ways. However, technical terms and sometimes standard procedures cannot be rephrased; therefore Editors must review these sections carefully before making a decision.

A.2.7.4 Plagiarism in Published Manuscripts

Published manuscripts which are found to contain plagiarized text are retracted from the journal's website after careful investigation and approval by the Editor-in-Chief of the journal. A Retraction Note as well as a link to the original article is published on the electronic version of the plagiarized manuscript and an addendum with retraction notification in the particular journal.

A3. COPYRIGHT AND LICENSE

A3.1 Open Access Plus (Subscription Journals and eBooks)

Accepted articles can be published online for free open access. Open access publishing provides maximum dissemination of the article to the largest audience. All authors will be asked to indicate whether or not they wish to pay to have their paper made freely available on publication. If authors do not select the 'Open Access Plus' option, then their article will be published with standard subscription-based access.

A3.2 Copyright (Subscription Journals and eBooks)

Editors/Authors who contribute in a Bentham's Journal/eBook will transfer copyright to their work to Bentham Science Publishers. Submission of a manuscript to the respective journals implies that all editors/authors have read and agreed to the content of the copyright letter.

A3.3 Ethical Approval of Studies and Informed Consent

For human or animal experimental investigations, it is a prerequisite to provide a formal review and approval, or review and waiver, by an appropriate institutional review board or ethics committee, which should be documented in the paper. For investigations undertaken on human subjects, the manner in which the informed consent was obtained from the study participants (i.e., oral or written) should be stated in the Methods section.

Authors are encouraged to obtain patient consent when they use confidential case material. Consent is not necessary in the case of very brief case vignettes which do not contain identifying information or if the case material is disguised sufficiently to prevent identification of the patient.

In obtaining consent, the author(s) should discuss the purpose(s) of publication, the possible risks and benefits to the patient and the patient's right to withhold or withdraw consent. In the case of a minor patient, consent should be obtained from the parent(s) or guardian(s).

A.4 STANDARD PROTOCOL ON APPROVALS, REGISTRATIONS, PATIENTS CONSENTS & ANIMAL PROTECTION

All clinical investigations must be conducted according to the Declaration of Helsinki principles. Authors must comply with the guidelines of the International Committee of Medical Journal Editors (www.icmje.org) with regard to the patient's consent for research or participation in a study. Patients' names, initials, or hospital numbers must not be mentioned anywhere in the manuscript (including figures). Editors may request that authors provide documentation of the formal review and recommendation from the institutional review board or ethics committee responsible for oversight of the study.

A4.1 Errata and Corrections in Published Articles

Authors and readers are encouraged to notify the Editor-in-Chief if they find errors in published content, authors names and affiliations or if they have reasons for concern over the legitimacy of a publication. In such cases the journal will publish an ERRATUM in consultation with Editor-in-Chief and authors of the article, and/or replace or retract the article.

A4.2 Article Withdrawal

Articles in Press (articles that have been accepted for publication or published as E-pub Ahead of Schedule but which have not been formally published with volume/issue/page information) that include errors, or are determined to violate the publishing ethics guidelines such as multiple submission, fake claims of authorship, plagiarism, fraudulent use of data or the like, may be Withdrawn from the journal. Withdrawal means that the article files are removed and replaced with a PDF stating that the article has been withdrawn from the journal in accordance with BSP Editorial Policies.

A4.3 Article Retraction

Published articles (with volume/issue/page information) which may contain infringements of professional ethical codes, such as multiple submissions, bogus claims of authorship, plagiarism, fraudulent use of data or the like are retracted.

- A retraction note titled Retraction: [article title] signed by the authors and/or the Editor-in-Chief is published in the paginated part of a subsequent issue of the journal and listed in the contents list.
- In the electronic version, a link is made to the original article.
- The online article is preceded by a screen containing the retraction note. It is to this screen that the link resolves; the reader can then proceed to the article itself.
- The original article is retained unchanged with a watermark on the PDF indicating on each page that it is retracted.
- The HTML version of the document is removed.

A4.4 Redundant (multiple) publication/ Re-publication

Abstracts and posters of conferences, results presented at meetings (for example, to inform investigators or participants about findings), results databases (data without interpretation, discussion, context or conclusions in the form of tables and text to describe data/information where this is not easily presented in tabular form) are not considered prior publication.

Authors who wish to publish translations of the articles that have been published elsewhere should ensure that they have appropriate permission(s), indicate clearly that the material has been translated and re-published, and indicate clearly the original source of the material. The Editor-in-Chief may request copies of related publications if he/she is concerned about overlap and possible redundancy.

A.5 PERMISSION FOR REPRODUCTION

Bentham Science has collaborated with the Copyright Clearance Center to meet its customer s licensing, besides rights & permission needs.

The Copyright Clearance Center s RightsLink® service makes it faster and easier to secure permission from Bentham Science journal titles. Simply visit Journals by Title and locate the desired content. Then go to the article s abstract and click on Rights and Permissions to open the RightsLink s page. If you are unable to locate the content you wish to use or are unable to secure the rights you are seeking, please e-mail us at permissions@benthamscience.org

Published/reproduced material should not be included unless written permission has been obtained from the copyright holder, which should be forwarded to the Editorial Office in case of acceptance of the article for publication.

A.6 DISCLAIMER

Responsibility for the content published by Bentham Science Publishers in any of its journals, including any opinions expressed therein, rests exclusively with the author(s) of such content. To the maximum extent permitted by applicable law, BSP (on its own behalf, and on behalf of its staff and members of its editorial board) disclaims responsibility for any and all injury and/or damage (whether financial or otherwise) to persons or property, resulting directly or indirectly from any ideas, methods, instructions or products (including errors in the same) referred to in the content of any of BSP s journals. Any dispute arising, including any claim shall be governed exclusively by the laws of the United Arab Emirates, as applied in the Emirate of Sharjah.

A.7 COPYRIGHT / SELF-ARCHIVING POLICY

A.7.1 Copyright

Authors who publish in Bentham Science Publishers print & online journals will transfer copyright of their work to **Bentham Science Publishers**. Submission of a manuscript to the respective journals implies that all authors have read and agreed to the content of the Copyright Letter or the Terms and Conditions. It is a condition of publication that manuscripts submitted to this journal have not been published and will not be simultaneously submitted or published elsewhere. Plagiarism is strictly forbidden, and by submitting the article for publication the authors agree that the publishers have the legal right to take appropriate action against the authors, if plagiarism or fabricated information is discovered. By submitting a manuscript the authors agree that the copyright of their article is transferred to the publishers if and when the article is accepted for publication. Once submitted to the journal, the authors will not withdraw their manuscript at any stage prior to publication.

A.7.2 Copyright letter

It is mandatory that a signed copyright letter also be submitted along with the manuscript by the author to whom correspondence is to be addressed, delineating the scope of the submitted article, declaring the potential competing interests, acknowledging

contributions from authors and funding agencies, and certifying that the paper is prepared according to the '**Instructions for Authors**'. All inconsistencies in the text and in the reference section, and any typographical errors must be carefully checked and corrected before the submission of the manuscript. The article should not contain any such material or information that may be unlawful, defamatory, fabricated, plagiarized, or which would, if published, in any way whatsoever, violate the terms and conditions as laid down in the copyright agreement. The authors should acknowledge that the publishers have the legal right to take appropriate action against the authors for any such violation of the terms and conditions as laid down in the copyright agreement. Download the Copyright letter

A.8 PERMISSION FOR REPRODUCTION

Bentham Science has collaborated with the Copyright Clearance Center to meet our customer's licensing, besides rights & permission needs.

The Copyright Clearance Center's RightsLink® service makes it faster and easier to secure permission from Bentham Science's journal titles. Simply visit Journals by Title and locate the desired content. Then go to the article's abstract and click on "Rights and Permissions" to open the RightsLink's page. If you are unable to locate the content you wish to use or you are unable to secure the rights you are seeking, please e-mail us at permissions@benthamscience.org

Published/reproduced material should not be included unless written permission has been obtained from the copyright holder, which should be forwarded to the Editorial Office in case of acceptance of the article for publication.

A.9 SELF-ARCHIVING

By signing the Copyright Letter the authors retain the rights of self-archiving. Following are the important features of self-archiving policy of Bentham Science journals

1. Authors can deposit the first draft of a submitted article on their personal websites, their institution's repositories or any non-commercial repository for personal use, internal institutional use or for permitted scholarly posting.
2. Authors may deposit the ACCEPTED VERSION of the peer-reviewed article on their personal websites, their institution's repository or any non-commercial repository such as PMC, arXiv after **12 MONTHS of publication** on the journal website. In addition, an acknowledgement must be given to the original source of publication and a link should be inserted to the published article on the journal's/publisher's website.
3. If the research is funded by NIH, Wellcome Trust or any other Open Access Mandate, authors are allowed the archiving of published version of manuscripts in an institutional repository after the mandatory embargo period. Authors should first contact the Editorial Office of the journal for information about depositing a copy of the manuscript to a repository. Consistent with the copyright agreement, Bentham Science does not allow archiving of FINAL PUBLISHED VERSION of manuscripts.
4. The link to the original source of publication should be provided by inserting the DOI number of the article in the following sentence: "The published manuscript is available at EurekaSelect via [http://www.eurekaselect.com/openurl/content.php?genre=article&doi= \[insert DOI\]](http://www.eurekaselect.com/openurl/content.php?genre=article&doi=[insert DOI])
5. There is no embargo on the archiving of articles published under the OPEN ACCESS PLUS category. Authors are allowed deposition of such articles on institutional, non-commercial repositories and personal websites immediately after publication on the journal website.

A.10 MANUSCRIPTS PUBLISHED

The Journal publishes peer-reviewed mini- and full-length Review Articles, research papers, letters and drug clinical trial studies written in English. Single topic/thematic issues may also be considered for publication.

A.10.1 Single Topic Issues

These special issues are peer-reviewed and may contain invited or uninvited review/mini-review articles. A Single Topic Issue Editor will offer a short perspective and co-ordinate the solicitation of manuscripts between 3-5 (for a mini-thematic Issue) to 6-10 (for full-length thematic Issue) from leading scientists. Authors interested in editing a single topic issue in an emerging topic of cancer drug targets may submit their proposal to the Editor-in-Chief at thematicissue@benthamscience.org for consideration.

A.10.2 Conference Proceedings

For proposals to publish conference proceedings in this journal, please contact us at email: proceedings@benthamscience.org.

A.11 MANUSCRIPTS LENGTH

A.11.1 Review Articles

The length of a published comprehensive review article is from 35000 to 40000 words with 100 or more references excluding figures, structures, photographs, schemes, tables, etc.

A.11.2 Mini-Reviews

Mini-reviews should be 6000-10000 words with 75 or more references excluding figures, structures, photographs, schemes, tables etc.

A.11.3 Systematic Reviews

Systematic Reviews include systematic updates on review protocols, methods, research, and results from all relevant fields for any studies and updates on already published issues. The total number of words for a published systematic review is from 35000 to 40000 words with 100 or more references excluding figures, structures, photographs, schemes, tables etc.

A.11.4 Registration of Systematic Reviews

Bentham Science Publishers supports retrospective registration of systematic reviews, in a suitable registry (such as PROSPERO). The registered systematic review must include the registration number as the last line of the manuscript abstract.

A.11.5 Research Articles

Research articles should be of 4000-6000 words with 75 or more references excluding figures, structures, photographs, schemes, tables, etc.

A.11.6 Letters

Letters should be 3000-6000 words with 40 or more references excluding figures, structures, photographs, schemes, tables, etc.

A.11.7 Book Reviews

The journal publishes book reviews on recently published books (both print and electronic) relevant to the journal. Proposals for publishing book reviews should first contact the editor at ccdt@benthamscience.org stating the title of the book, publisher, year of publication and book summary. No books must be sent to Bentham Science prior to their confirmation to the publisher concerned.

A.11.8 Randomized Drug Clinical Trial Studies

Trial studies should be 1500 to 4000 words with 50 or more references excluding figures, structures, photographs, schemes, tables etc.

A.11.9 Case Reports

Case reports should describe new observations of findings or novel/unique outcomes relevant to the field. The total number of words for a published case report is 1500 to 2000 words with 40 or more references excluding figures, structures, photographs, schemes, tables etc.

There is no restriction on the number of figures, tables or additional files e.g. video clips, animation and datasets, that can be included with each article online. Authors should include all relevant supporting data with each article (Refer to Supplementary Material section).

A.12 MANUSCRIPT PREPARATION

The manuscript should be written in English in a clear, direct and active style. All pages must be numbered sequentially, facilitating in the reviewing and editing of the manuscript.

A.12.1 Microsoft word template

It is advisable that authors prepare their manuscript using the template available on the Web, which will assist in preparation of the manuscript according to Journal's Format.

A.12.2 Sections in manuscripts

Manuscripts submitted for research and review articles in the journal should be divided into the following sections

- Title
- Title page
- Structured Abstract
- Graphical Abstract
- Keywords
- Text Organization
- Conclusion
- List of Abbreviations
- Consent for Publication
- Conflict of Interest
- Acknowledgements
- References
- Appendices
- Figures/Illustrations (if any)
- Chemical Structures (if any)
- Tables (if any)
- Supportive/Supplementary Material (if any)

A.12.3 Title

The title of the article should be precise and brief and must not be more than 120 characters. Authors should avoid the use of non-standard abbreviations and question marks in titles. The first letter of each word should be in capital letters except for articles, conjunctions and prepositions.

Authors should also provide a short 'running title'. Title, running title, byline, correspondent footnote and keywords should be written as presented in original manuscripts.

A.12.4 Title Page

Title page should include paper title, author(s) full name and affiliation, corresponding author(s) names complete affiliation/address, along with phone, fax and email.

A.12.5 Structured Abstract

The abstract of an article should be its clear, concise and accurate summary, having no more than 250 words, and including the explicit sub-headings (as in-line or run-in headings in bold). Use of abbreviations should be avoided and the references should not be cited in the abstract. Ideally, each abstract should include the following sub-headings, but these may vary according to requirements of the article.

- Background
- Objective
- Method
- Results
- Conclusion

A.12.6 Graphical Abstract

A graphic must be included with each manuscript for use in the Table of Contents (TOC). This must be submitted separately as an electronic file (preferred file types are EPS, PDF, TIFF, Microsoft Word, PowerPoint and CDX etc.). A graphical abstract, not exceeding 30 words along with the illustration, helps to summarize the contents of the manuscript in a concise pictorial form. It is meant as an aid for the rapid viewing of the journals' contents and to help capture the readers' attention. The graphical abstract may feature a key structure, reaction, equation, etc. that the manuscript elucidates upon. It will be listed along with the manuscript title, authors' names and affiliations in the contents page, typeset within an area of 5 cm by 17 cm, but it will not appear in the article PDF file or in print.

Graphical Abstracts should be submitted as a separate file (must clearly mention graphical abstract within the file) online via Bentham's Content Management System by selecting the option "supplementary material".

A.12.7 Keywords

6 to 8 keywords must be provided. Choose important and relevant keywords that researchers in your field will be searching for so that your paper will appear in a database search. In biomedical fields, MeSH terms are a good 'common vocabulary' source to draw keywords from <https://www.nlm.nih.gov/mesh/meshhome.html>.

A.12.8 Text Organization

The main text should begin on a separate page and should be divided into Title page, Abstract and the main text. For Research Articles/Letters the manuscript should begin with the Title page and Abstract followed by the main text, which must be structured into separate sections as Introduction, Materials and Methods, Results, Discussion, and Conclusion, List of Abbreviations, Conflict of Interest, Acknowledgements and References. For Review, the manuscript should be divided into title page, abstract and the main text. The text may be subdivided further according to the areas to be discussed, which should be followed by the List of Abbreviations (if any), Conflict of Interest, Acknowledgements and Reference sections. The review article should mention any previous important reviews in the field and contain a comprehensive discussion starting with the general background of the field. It should then go on to discuss the salient features of recent developments. The authors should avoid presenting material which has already been published in a previous review.

The authors are advised to present and discuss their observations in brief. The manuscript style must be uniform throughout the text and 10 pt Times New Roman font should be used. The full term for an abbreviation should precede its first appearance in the text unless it is a standard unit of measurement. The reference numbers should be given in square brackets in the text. Italics should be used for Binomial names of organisms (Genus and Species) for emphasis and for unfamiliar words or phrases. Non-assimilated words from Latin or other languages should also be italicized e.g. *in vivo*, *in vitro*, *per se*, *et al.* etc.

A.13 SECTION HEADINGS

Section headings should be numbered sequentially, left aligned and have the first letter capitalized, starting with the introduction. Sub-section headings however, should be in lower-case and italicized with their initials capitalized. They should be numbered as 1.1, 1.2, etc.

A.13.1 INTRODUCTION

The Introduction section should include the background and aims of the research in a comprehensive manner.

A.13.2 MATERIALS AND METHODS

This section provides details of the methodology used along with information on any previous efforts with corresponding references. Any details for further modifications and research should be included.

A.13.3 EXPERIMENTAL

Repeated information should not be reported in the text of an article. A calculation section must include experimental data, facts and practical development from a theoretical perspective.

A.13.4 RESULTS

Results should be precise.

A.13.5 DISCUSSION

This should explore the significance of the results of the work, present a reproducible procedure and emphasise the importance of the article in the light of recent developments in the field. Extensive citations and discussion of published literature should be avoided.

The Results and Discussion may be presented together under one heading of "Results and Discussion". Alternatively, they may be presented under two separate sections ("Results" section and "Discussion" Sections). Short sub-headings may be added in each section if required.

A.13.6 CONCLUSION

A small paragraph summarizing the contents of the article, presenting the final outcome of the research or proposing further study on the subject, may be given at the end of the article under the Conclusion section.

A.13.7 GREEK SYMBOLS AND SPECIAL CHARACTERS

Greek symbols and special characters often undergo formatting changes and get corrupted or lost during preparation of manuscript for publication. To ensure that all special characters used are embedded in the text, these special characters should be inserted as a symbol but should not be a result of any format styling (Symbol font face) otherwise they will be lost during conversion to PDF/XML.

Authors are encouraged to consult reporting guidelines. These guidelines provide a set of recommendations comprising a list of items relevant to their specific research design. Chemical equations, chemical names, mathematical usage, unit of measurements, chemical and physical quantity & units must conform to SI and Chemical Abstracts or IUPAC.

All kinds of measurements should be reported only in International System of Units (SI).

A.13.8 APPENDICES

In case there is a need to present lengthy, but essential methodological details, use appendices, which can be part of the article. An appendix must not exceed three pages (Times New Roman, "10 point font", 900 max. words per page). The information should be provided in a condensed form, ruling out the need of full sentences. A single appendix should be titled APPENDIX, while more than one can be titled APPENDIX A, APPENDIX B, and so on.

A.13.9 SUPPORTIVE/SUPPLEMENTARY MATERIAL

We do encourage to append supportive material, for example a PowerPoint file containing a talk about the study, a PowerPoint file containing additional screenshots, a Word, RTF, or PDF document showing the original instrument(s) used, a video, or the original data (SAS/SPSS files, Excel files, Access Db files etc.) provided it is inevitable or endorsed by the journal's Editor.

Supportive/Supplementary material intended for publication must be numbered and referred to in the manuscript but should not be a part of the submitted paper. In-text citations as well as a section with the heading "Supportive/Supplementary Material" before the "References" section should be provided. Here, list all Supportive/Supplementary Material and include a brief caption line for each file describing its contents.

Any additional files will be linked into the final published article in the form supplied by the author, but will not be displayed within the paper. They will be made available in exactly the same form as originally provided only on our Web site. Please also make sure that each additional file is a single table, figure or movie (please do not upload linked worksheets or PDF files larger than one sheet). Supportive/Supplementary material must be provided in a single zipped file not larger than 4 MB.

Authors must clearly indicate if these files are not for publication but meant for the reviewers'/editors' perusal only.

A.13.10 LIST OF ABBREVIATIONS

If abbreviations are used in the text either they should be defined in the text where first used, or a list of abbreviations can be provided.

A.14 AVAILABILITY OF MATERIALS

The NIH acknowledges the misidentification and/or cross-contamination of cell cultures e.g. HeLa cells being used in a research study as a serious problem. In order to ensure the validation of the work and proper utilization of resources, it is a prerequisite that correct reagents be used in studies dealing with established human (tumor) cell lines that have been cultured for more than 4 years up to the date of submission of the manuscript. Cell lines such as short-term cultures of human tumors, murine cell lines (as a catalog of DNA profiles is not yet available) and tumor cell lines established in the course of the study that is being submitted, are presently exempt from this rule. To minimize the risk of working with misidentified and/or contaminated cell lines, tests such as isoenzyme analysis, karyotyping/cytogenetic analysis and, more recently, molecular techniques of DNA profiling may be carried out to authenticate cell cultures. These tests may help confirm or establish the identify profile for a cell line. Bentham Science recommends that all cell lines be authenticated prior to submitting a paper for review. Authors are therefore required to provide authentication of the origin and identity of the cells by performing cell profiling either in their own laboratory or by outsourcing an approved laboratory or cell bank. Authentication is required when a new line is established or acquired, before freezing a cell line, if the performance of the line is not consistent or results are unexpected, if using more than one cell line, and before publication of the study.

The cell lines profile should be cross-checked with the profile of the donor tissue of other continuous cell lines such as provided by the authentic data bank such as www.dsmz.de/fp/cgi-bin/str.html , ATCC® etc.

A.15 DECLARATION OF INTEREST/FUNDING SOURCES

A.15.1 Conflict of Interest

Financial contributions and any potential conflict of interest must be clearly acknowledged under the heading 'Conflict of Interest'. Authors must list the source(s) of funding for the study. This should be done for each author.

A.15.2 Acknowledgements

All individuals listed as authors must have contributed substantially to the design, performance, analysis, or reporting of the work and are required to indicate their specific contribution. Anyone (individual/company/institution) who has substantially contributed to the study for important intellectual content, or who was involved in the article's drafting the manuscript or revising must also be acknowledged.

Guest or honorary authorship based solely on position (e.g. research supervisor, departmental head) is discouraged.

The specific requirements for authorship have been defined by the International Committee of Medical Journal Editors (ICMJE; www.icmje.org). Examples of authors' contributions are: 'designed research/study', 'performed research/study', 'contributed important reagents', 'collected data', 'analyzed data', 'wrote paper' etc. This information must be included in the submitted manuscript as a separate paragraph under the heading 'Acknowledgements'. The corresponding author is responsible for obtaining permission from all co-authors for the submission of any version of the manuscript and for any changes in the authorship.

Standard Protocol on Approvals, Registrations, Patient Consents & Animal Protection

All clinical investigations must be conducted according to the Declaration of Helsinki principles. For all manuscripts reporting data from studies involving human participants, formal review and approval by an appropriate institutional review board or ethics committee is required. For research involving animals, the authors should indicate whether the procedures followed were in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals (grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals_prepub.pdf; published by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

A.15.3 Consent for Publication

If the manuscript has an individuals' data, such as personal detail, audio-video material etc., consent should be obtained from that individual. In case of children, consent should be obtained from the parent or the legal guardian.

A specific declaration of such approval and consent-to-disclose form must be made in the copyright letter and in a stand-alone paragraph at the end of the article especially in the case of human studies where inclusion of a statement regarding obtaining the written informed consent from each subject or subject's guardian is a must. The original should be retained by the guarantor or corresponding author. Editors may request to provide the original forms by fax or email.

All such case reports should be followed by a proper consent prior to publishing.

A.16 REFERENCES

References must be listed in the ACS Style only. All references should be numbered sequentially [in square brackets] in the text and listed in the same numerical order in the reference section. The reference numbers must be finalized and the bibliography must be fully formatted before submission.

Some important points to remember:

- All references must be complete and accurate.
- All authors must be cited and there should be no use of the phrase *et al.*
- Date of access should be provided for online citations.
- Journal names should be abbreviated according to the Index Medicus/MEDLINE.
- Punctuation should be properly applied as mentioned in the examples given above.
- Superscript in the in-text citations and reference section should be avoided.
- Abstracts, unpublished data and personal communications (which can only be included if prior permission has been obtained) should not be given in the references section. The details may however appear in the footnotes.
- The authors are encouraged to use a recent version of EndNote (version 5 and above) or Reference Manager (version 10) when formatting their reference list, as this allows references to be automatically extracted.

A.17 FIGURES / TABLES

A.17.1 Figures/Illustrations

All authors must strictly follow the guidelines below for preparing illustrations for publication in **Current Cancer Drug Targets**. If the figures are found to be sub-standard, then the manuscripts will be rejected.

The authors are expected to submit good quality figure(s) in PDF, PPT, MS Word, TIFF or JPEG versions, which, if required, should be improved yourself or by professional graphic designers of your organization/ country. You may even consider approaching our contracted service providers Eureka Science for Graphics Enhancement Services.

The Graphics Designing team at Eureka Science can assist in improving the quality of your images at affordable rates. Eureka Science has contracted special rates with us of US \$125 for the improvement of up to five figures, with any additional figures being charged at US \$20 each.

The quality of Graphic Enhancement Services offered by Eureka Science can be viewed at <http://www.eureka-science.com/images/Binder1.pdf>, along with valuable feedback on their services at <http://www.eureka-science.com/testimonials.php>. You may contact Eureka Science at info@eureka-science.com

Note: Availing Graphics Enhancement Services do not guarantee acceptance of the manuscript for publication. The final acceptance/decision on the manuscript is taken by the EIC.

A.17.2 Guideline for Figures/Illustrations

Illustrations must be provided according to the following guideline:

- Illustrations should be embedded in the text file, and must be numbered consecutively in the order of their appearance. Each figure should include only a single illustration which should be cropped to minimize the amount of space occupied by the illustration.
- If a figure is in separate parts, all parts of the figure must be provided in a single composite illustration file.
- Photographs should be provided with a scale bar if appropriate, as well as high-resolution component files.

A.17.3 Scaling/Resolution

Line Art image type is normally an image based on lines and text. It does not contain tonal or shaded areas. The preferred file format should be TIFF or EPS, with the color mode being Monochrome 1-bit or RGB, in a resolution of 900-1200 dpi.

Halftone image type is a continuous tone photograph containing no text. It should have the preferred file format TIFF, with color mode being RGB or Grayscale, in a resolution of 300 dpi.

Combination image type is an image containing halftone, text or line art elements. It should have the preferred file format TIFF, with color mode being RGB or Grayscale, in a resolution of 500-900 dpi.

A.17.4 Formats

Illustrations may be submitted in the following file formats

- **Illustrator**
- **EPS** (preferred format for diagrams)
- **PDF** (also especially suitable for diagrams)
- **PNG** (preferred format for photos or images)
- **Microsoft Word** (version 5 and above; figures must be a single page)
- **PowerPoint** (figures must be a single page)
- **TIFF**
- **JPEG** (conversion should be done using the original file)

- **BMP**
- **CDX** (ChemDraw)
- **TGF** (ISISDraw)

Bentham Science Publishers does not process figures submitted in GIF format.

For TIFF or EPS figures with considerably large file size restricting the file size in online submissions is advisable. Authors may therefore convert to JPEG format before submission as this results in significantly reduced file size and upload time, while retaining acceptable quality. JPEG is a 'lossy' format, however. In order to maintain acceptable image quality, it is recommended that JPEG files are saved at High or Maximum quality.

Zipit or Stuffit tools should not be used to compress files prior to submission as the resulting compression through these tools is always negligible.

Please refrain from supplying:

1. Graphics embedded in word processor (spreadsheet, presentation) document.
2. Optimized files optimized for screen use (like GIF, BMP, PICT, WPG) because of the low resolution.
3. Files with too low a resolution.
4. Graphics that are disproportionately large for the content.

Technical requirements for graphic/ figure submissions.

A.17.5 Requirement

Width = 8.5 inches (In-between the required size)

Height = 11 inches (In-between the required size)

Pixels/Inches = 300 (minimum dpi)

All figures should be in vector scale (except half tone, photograph.)

Note for authors: To maintain publication quality, figures submitted in colour will be published in colour only.

Chemical Structures

Chemical structures must be prepared in ChemDraw/CDX and provided as separate file.

A.17.6 Structure Drawing Preferences

[As according to the ACS style sheet]

Drawing Settings

Chain angle	120°
Bond spacing	18% of width
Fixed length	14.4 pt (0.500cm, 0.2in)
Bold width	2.0 pt (0.071cm, 0.0278in)
Line width	0.6 pt (0.021cm, 0.0084in)
Margin width	1.6 pt (0.096cm)
Hash spacing	2.5 pt (0.088cm, 0.0347in)

Text Settings

Font	Times New Roman / Helvetica
Size	10 pt

Under the Preference Choose

Units	points
Tolerances	3 pixels

Under Page Setup Use

Paper	US letter
Scale	100%

A.17.7 Tables

- Data Tables should be submitted in Microsoft Word table format.
- Each table should include a title/caption being explanatory in itself with respect to the details discussed in the table. Detailed legends may then follow.
- Table number in bold font i.e. Table **1**, should follow a title. The title should be in small case with the first letter in caps. A full stop should be placed at the end of the title.
- Tables should be embedded in the text exactly according to their appropriate placement in the submitted manuscript.
- Columns and rows of data should be made visibly distinct by ensuring that the borders of each cell are displayed as black lines.

- Tables should be numbered in Arabic numerals sequentially in order of their citation in the body of the text.
- If a reference is cited in both the table and text, please insert a lettered footnote in the table to refer to the numbered reference in the text.
- Tabular data provided as additional files can be submitted as an MS Excel spreadsheet.

A.18 AUTHORS AND INSTITUTIONAL AFFILIATIONS

The names of the authors should be provided according to the previous citations or as the authors would want them to be published along with the institutional affiliations, current address, telephone, cell & fax numbers and the email address. Email address must be provided with an asterisk in front of the name of the principal author. The corresponding author(s) should be designated and their complete address, business telephone and fax numbers and e-mail address must be stated to receive correspondence and galley proofs. Also it is suggested to regularly update the profile on SCOPUS and other databases.

A.18.1 CHANGES TO AUTHORSHIP

Authors must provide a final list of authors at the time of submission, ensuring the correct sequence of the names of authors, which will not be considered for any addition, deletion or rearrangement after final submission of the manuscript. If a change is essentially required, it can only be done on Editor's approval, for which the Editor must receive the following from the corresponding author

(a) the reason for change in the author list and the sequence

(b) written confirmation from all co-authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, confirmation from the author being added or removed is mandatory.

Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors after the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

A.19 PAGE CHARGES/QUICK TRACK PUBLICATION

A.19.1 PAGE CHARGES

No page charges will be levied to authors for the publication of their Review Articles. To publish research articles, however, the publication charges are **\$365 US**, and for a contribution of Short Communication/Letters the publication charges are **\$190 US**.

A.19.2 QUICK TRACK Publication

For this journal an optional fast publication fee-based service called QUICK TRACK is available to authors for their submitted manuscripts. Authors who opt for this fee-based service do not have to pay any additional page charges.

QUICK TRACK allows online publication within 2 weeks of receipt of the final approved galley proofs from the authors. Similarly the manuscript can be published in the next forthcoming PRINT issue of the journal. The total publication time, from date of first receipt of manuscript to its online publication is 10 weeks, subject to its acceptance by the referees and modification (if any) by the authors within one week.

Authors who have availed QUICK TRACK service in a BSP journal will be entitled for an exclusive 30% discount if they again wish to avail the same services in any Bentham journal.

For more information please contact the Editorial Office by e-mail at ccdt@benthamscience.org.

A.20 LANGUAGE AND EDITING

Manuscripts containing language inconsistencies will not be published. Authors should seek professional assistance for correction of grammatical, scientific and typographical errors before submission of the revised version of the article for publication. Professional editing services may also be sought by the team available at Bentham Science.

A.21 PROOF CORRECTIONS

Authors will receive page proofs of their accepted paper before publications. To avoid delays in publication, proofs should be checked immediately for typographical errors and returned within **48 hours**. Major changes are not acceptable at the proof stage. If unable to send corrections within **48 hours** due to some reason, the author(s) must at least send an acknowledgement on receiving the galley proofs or the article will be published exactly as received and the publishers will not be responsible for any error occurring in the published manuscript in this regard.

The corresponding author will be solely responsible for ensuring that the revised version of the manuscript incorporating all the submitted corrections receives the approval of all the co-authors of the manuscript.

APPENDIX B

AUTHOR GUIDELINES FOR *EXPERIMENTAL CELL RESEARCH*

Guidelines for the publication in the journal, *Experimental Cell Research* (Impact factor 3.309; ISSN: 0014-4827)

B.1. INTRODUCTION

Experimental Cell Research (ECR) is dedicated to publishing manuscripts that significantly advance our understanding of cell and molecular biology. Examples of areas of interest to ECR are the cell cycle; gene regulation; chromatin; sub cellular organization; signal transduction; nuclear import-export; intracellular trafficking; programmed cell death; the dynamics and function of the cytoskeleton, cell membrane, and extra cellular matrix; the biology of meiosis and mitosis; cell motility and migration; mechanisms of cellular growth control and differentiation; and cell-cell interaction between cells in tissues or in culture. Manuscripts in the area of cancer research, particularly those that address fundamental aspects of cell and molecular biology, are also welcomed. Reports describing new experimental techniques and methods or of theoretical work having a direct bearing on experimental approaches are also appropriate for inclusion in the journal. Experimental manuscripts will be given the highest priority and can range from the sub cellular to the organism level, including work on transgenic animals. Purely descriptive work, including descriptions of RNA or protein expression patterns, is given lower priority but can be accepted if it provides important new or unexpected information.

Acceptance of manuscripts will be based on scientific excellence and priority as judged by editors and reviewers, on relevance to the central themes of the journal, and on the interest of the results to a broad readership of cell and molecular biology. A manuscript of high intrinsic quality may be returned if it seems better suited to a more specialized journal. Also, manuscripts that present preliminary observations or an incremental increase in understanding are not encouraged.

Manuscripts are published in ECR under the condition that the author(s) agree to provide, in a timely manner, unique non-commercial reagents to other investigators upon request.

B.2 TYPES OF PAPERS

Manuscripts can be published in the format of **regular articles**. There is no strict size limitation on regular articles, although they usually do not exceed 15,000 words, preceded by an abstract not to exceed 200 words. ECR also publishes review articles, which should be between 3000 and 5000 words in length. ECR does not accept unsolicited **review articles** unless they are preapproved by the Editor-in-Chief. All review articles will be subject to review before publication.

B.3 SUBMISSION CHECKLIST

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

B.3.1 Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

- E-mail address
- Full postal address

B.3.1.1 All necessary files have been uploaded:

Manuscript:

- Include keywords
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided
- Indicate clearly if colour should be used for any figures in print

Graphical Abstracts / Highlights files (where applicable)

Supplemental files (where applicable)

B.3.1.2 Further considerations

- Manuscript has been 'spell checked' and 'grammar checked'
- All references mentioned in the Reference List are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Internet)
- A competing interests statement is provided, even if the authors have no competing interests to declare
- Journal policies detailed in this guide have been reviewed
- Referee suggestions and contact details provided, based on journal requirements

B.4 BEFORE YOU BEGIN

B.4.1 Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted. 2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches.

B.4.2 Submission declaration and verification

Submission of an article implies that the work described has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

B.4.3 Preprints

Please note that preprints can be shared anywhere at any time, in line with Elsevier's sharing policy. Sharing your preprints e.g. on a preprint server will not count as prior publication (see 'Multiple, redundant or concurrent publication' for more information).

B.4.4 Use of inclusive language

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

B.4.5 Changes to authorship

Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

B.4.6 Article transfer service

This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. Please note that your article will be reviewed again by the new journal.

B.4.7 Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement'. An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has pre-printed forms for use by authors in these cases.

For gold open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement'. Permitted third party reuse of gold open access articles is determined by the author's choice of user license.

B.4.8 Author rights

As an author you (or your employer or institutions) have certain rights to reuse your work.

B.4.9 Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

B.4.10 Funding body agreements and policies

Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some funding bodies will reimburse the author for the gold open access publication fee.

B.4.11 Open access

This journal offers authors a choice in publishing their research:

B.4.12 Subscription

- Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs.

- No open access publication fee payable by authors.
- The Author is entitled to post the accepted manuscript in their institution's repository and make this public after an embargo period (known as green Open Access). The published journal article cannot be shared publicly, for example on ResearchGate or Academia.edu, to ensure the sustainability of peer-reviewed research in journal publications. The embargo period for this journal can be found below.

B.4.13 Gold open access

- Articles are freely available to both subscribers and the wider public with permitted reuse.
- A gold open access publication fee is payable by authors or on their behalf, e.g. by their research funder or institution.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

B.4.14 Green open access

Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our green open access page for further information. Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form. This journal has an embargo period of 12 months.

B.5 PREPARATION

B.5.1 NEW SUBMISSIONS

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process. As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay-out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

B.5.2 References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

B.5.3 Formatting requirements

There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.

If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.

Divide the article into clearly defined sections.

Please note that the instructions related to Abbreviations, Abstract, Graphical abstract & Keywords still apply to all new submissions.

B.5.4 Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final.

B.6 REVISED SUBMISSIONS

B.6.1 Use of word processing software

Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

B.7 ARTICLE STRUCTURE

B.7.1 Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

B.7.2 Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

B.7.3 Results

Results should be clear and concise.

B.7.4 Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

B.7.5 Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

B.7.6 Essential title page information

- **Title:** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- **Author names and affiliations:** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
- **Corresponding author:** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
- **Present/permanent address:** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

B.7.7 Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

B.7.8 Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files.

B.7.9 Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

B.7.10 Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

B.7.11 Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

B.7.12 Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

B.7.13 Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

B.7.14

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

B.8 ARTWORK

B.8.1 Image manipulation

Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

B.8.2 Electronic artwork

General points

- Make sure you use uniform lettering and sizing of your original artwork.
- Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Indicate per figure if it is a single, 1.5 or 2-column fitting image.
- For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
- Please note that individual figure files larger than 10 MB must be provided in separate source files.

B.8.3 Figure captions

Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

B.9 REFERENCES

B.9.1 Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

B.9.2 Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

B.9.3 Data references

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

B.9.4 References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

B.9.5 References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following

B.10 DATA VISUALIZATION

Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research.

B.11 SUPPLEMENTARY MATERIAL

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

B.12 RESEARCH DATA

This journal encourages and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. If you are sharing data in one of these ways, you are encouraged to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation.

B.12.1 Data in Brief

You have the option of converting any or all parts of your supplementary or additional raw data into one or multiple data articles, a new kind of article that houses and describes your data. Data articles ensure that your data is actively reviewed, curated, formatted, indexed, given a DOI and publicly available to all upon publication. You are encouraged to submit your article for Data in Brief as an additional item directly alongside the revised version of your manuscript. If your research article is accepted, your data article will automatically be transferred over to Data in Brief where it will be editorially reviewed and published in the open access data journal, Data in Brief. Please note an open access fee of 500 USD is payable for publication in Data in Brief.

B.12.2 Data statement

To foster transparency, we encourage you to state the availability of your data in your submission. This may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you will have the opportunity to indicate why during the submission process, for example by stating that the research data is confidential. The statement will appear with your published article on ScienceDirect.

B.13 AFTER ACCEPTANCE

B.13.1 Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.

If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.

We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

B.13.2 Offprints

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Webshop. Corresponding authors who have published their article gold open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link

APPENDIX C

PROOF OF ATTENDANCE OF THE FIRST CONFERENCE OF BIOMEDICAL AND NATURAL SCIENCES AND THERAPEUTICS 2018

This Appendix includes a proof of attendance letter, as sent by Prof Helmuth Reuter (Chair of the organising committee).

This serves to confirm that

Michael Jacques Rossouw

attended the

First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST) 2018

at

Spier Conference Centre, Stellenbosch, and Cape Town, South Africa



Prof Helmuth Reuter
Chair: Organising committee

VISION 2040 | VISIE 2040 | UMBONO 2040 
Stellenbosch University has launched its Vision 2040 and Strategic Framework 2019-2024. Click here to find out more. 

APPENDIX D

SEEDING DENSITIES FOR CONFLUENCY DETERMINATION OF CELL LINES

This Appendix includes multiple seeding densities summarised in tables, to determine the correct concentration of cells at the beginning of the experiments for ideal confluency.

D.1 Seeding densities for confluency determination

Before the experiments could commence, the correct seeding density had to be determined to ensure optimal cellular populations were present upon exposure to chemotherapeutic drugs. If too many cells were present from the beginning, they would overgrow the wells and cellular death would be due to other factors not relating to cellular damage through compounds.

The following tables contain the ranges of cells seeded in 96 well plates and their relative confluence after 120 h in the H69V, H69AR and LLC-PK1 cell line.

The NCI-H69/LX4 cell line forms aggregates in suspension, and doesn't adhere to the tissue culture flask surface, the confluency determination used for adherent cell lines is not applicable. Therefore, this cell line is sub-cultured when the clusters start to become dense (darker colour) in the centre.

Usually, a T75 cm² flask of this cell line that can be cultured for 6-7 days has $\pm 8 \times 10^4$ cells/cm² before being sub-cultured. This calculates to approximately 25600 cells/well for a 96-well of 0.32 cm². Since an exposure period of only 96 h was used in this project, it means that at least 30000 cells/well were required for such a short culturing period.

Table D.1: Confluency (%) of the H69V cell line over a period of 120h when seeded at different concentrations

After seeding					
Cells/well (0.32cm ²)	24h	48h	72h	96h	120h
3000	25	25	30	30	40
4000	30	35	35	45	50
5000	30	40	45	50	60
6000	30	45	50	60	70
7000	35	50	55	70	80
8000	40	50	60	70	85
9000	50	60	65	80	90
10000	50	75	80	90	100

Table D.2: Confluency (%) of the H69AR cell line over a period of 120h when seeded at different concentrations

After seeding					
Cells/well (0.32cm ²)	24h	48h	72h	96h	120h
16000	15	30	45	60	80
18000	25	40	50	65	85
20000	30	50	60	80	90
22500	35	55	70	90	100
25000	50	65	80	100	ND
27500	55	75	85	100	ND
30000	60	80	90	100	ND
32500	70	85	90	100	ND

ND – not determined

Table D.3: Confluency (%) of the LLC-PK1 cell line over a period of 120h when seeded at different concentrations

After seeding					
Cells/well (0.32cm ²)	24h	48h	72h	96h	120h
2000	25	25	30	35	40
2500	30	30	40	40	45
3000	35	40	40	45	50
3500	40	45	45	50	60
4000	45	45	50	55	60
5000	50	55	60	65	70
6000	55	60	65	75	80
7000	60	65	70	80	90

APPENDIX E

ETHICS APPROVAL AND REGISTRATION OF COMMERCIAL CELL LINE USE

This Appendix includes the approval from the ethics committee for the use of commercial cell lines for experimentation.



Private Bag X6001, Potchefstroom
 South Africa 2520

Tel: 018 299-1111/2222
 Web: <http://www.nwu.ac.za>

Faculty of Health Sciences Ethics Office for
 Research, Training and Support

Health Research Ethics Committee (HREC)

Tel: 018-285 2291
 Email: Wayne.Towers@nwu.ac.za

15 June 2017

Prof Sias Hamman
 Pharmaceutics

Dear Prof Hamman

FEEDBACK ON AMENDMENTS TO APPLICATION FOR COMMERCIAL CELL LINE REGISTRATION

We would like to thank you for submitting the amendments to your application for the registration of the commercial cell lines that are in use or in storage within your facility. We hereby approve the addition of the extra personnel and students to the already approved project. If further amendments are to be made, please submit the registration form, indicating the updated information, with a cover letter stating clearly the amendment being made. We would, however, like to again emphasise that should you do research that involves the following procedures, a full application should be submitted to either the Health Research Ethics Committee (HREC) or the AnimCare committee, dependant on the source of the cells to be used:

- Establishing and/or use of primary cell cultures (except when purchased from a commercial source).
- Potential commercialization of a cell line developed from a primary cell culture.
- Any genetic manipulation of a cell line.
- Infection with micro-organisms which may be biohazardous to humans/others.

The following staff members are included under this approval:

Ms L Badenhorst	Dr W Pheiffer
Prof L du Plessis	Prof J Steenekamp
Dr C Gouws	Dr D Steyn
Prof S Hamman	Dr J Viljoen
Ms S Lowe	Dr J Wentzel
Mr H Netshimbupfe	Dr C Willers
Prof P Pretorius	

As agreed previously, we have also received registration forms for the following students that will be making use of cell lines in their research projects. These students are also included under this approval:

Simoné Eicker	Michelle Niemand
Morné Fouché	Michael Jacques Rossouw
Werner Gerber	Roan Albertus Swanepoel

Should you have any further queries, please feel free to contact Ms Hildah Kilani at your earliest convenience (E-mail: Ethics-AnimCare@nwu.ac.za; Tel: 018 299 1208). We wish you well in your future endeavours.

Yours sincerely

Prof Minnie Greeff
 Head of Health Sciences Ethics
 Office for Research, Training and Support

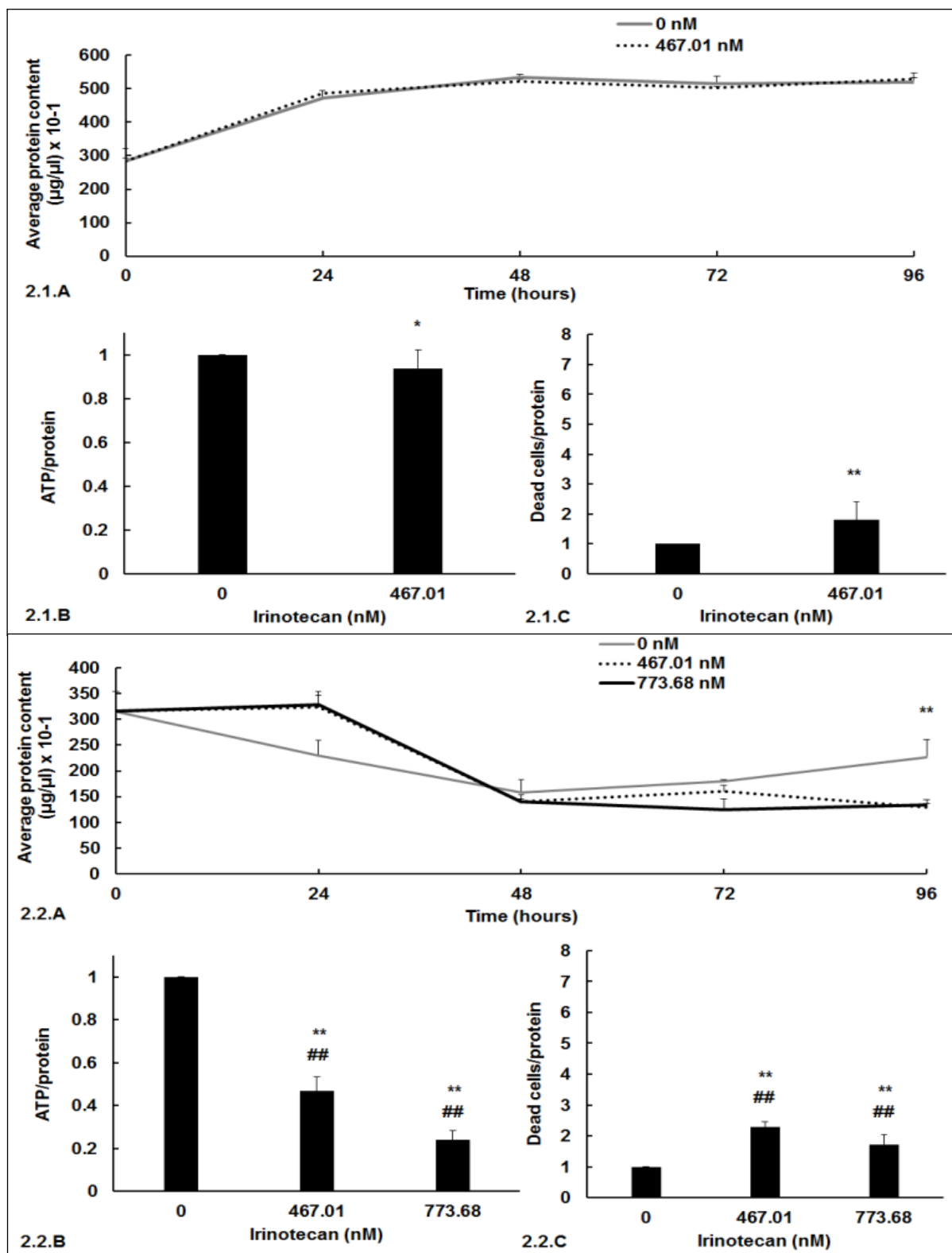
Dr Wayne Towers
 Chairperson: HREC

APPENDIX F

ISOLATED ENLARGED GRAPHS INCLUDED IN CHAPTER 5

This Appendix includes the isolated graphs as present in chapter 5 as single larger images.

Figure 2: Cell responses following daily treatment with irinotecan (nM) for 96 h. Soluble protein content over time (A), intracellular ATP levels per soluble protein content (normalised relative to the untreated control) (B) and extracellular AK levels per soluble protein content (normalised relative to the untreated control) (C) are shown for the LLC-PK1 (1), H69V (2), H69AR (3) and NCI-H69/LX4 (4) cell lines, respectively. Error bars denote the standard deviation ($n = 6$), # indicate statistically significant inter-dosage differences ($p < 0.05$); ## indicate statistical significant inter-dosage differences ($p < 0.01$); * indicate statistically significant differences relative to the untreated control ($p < 0.05$); ** indicate statistically significant differences relative to the untreated control



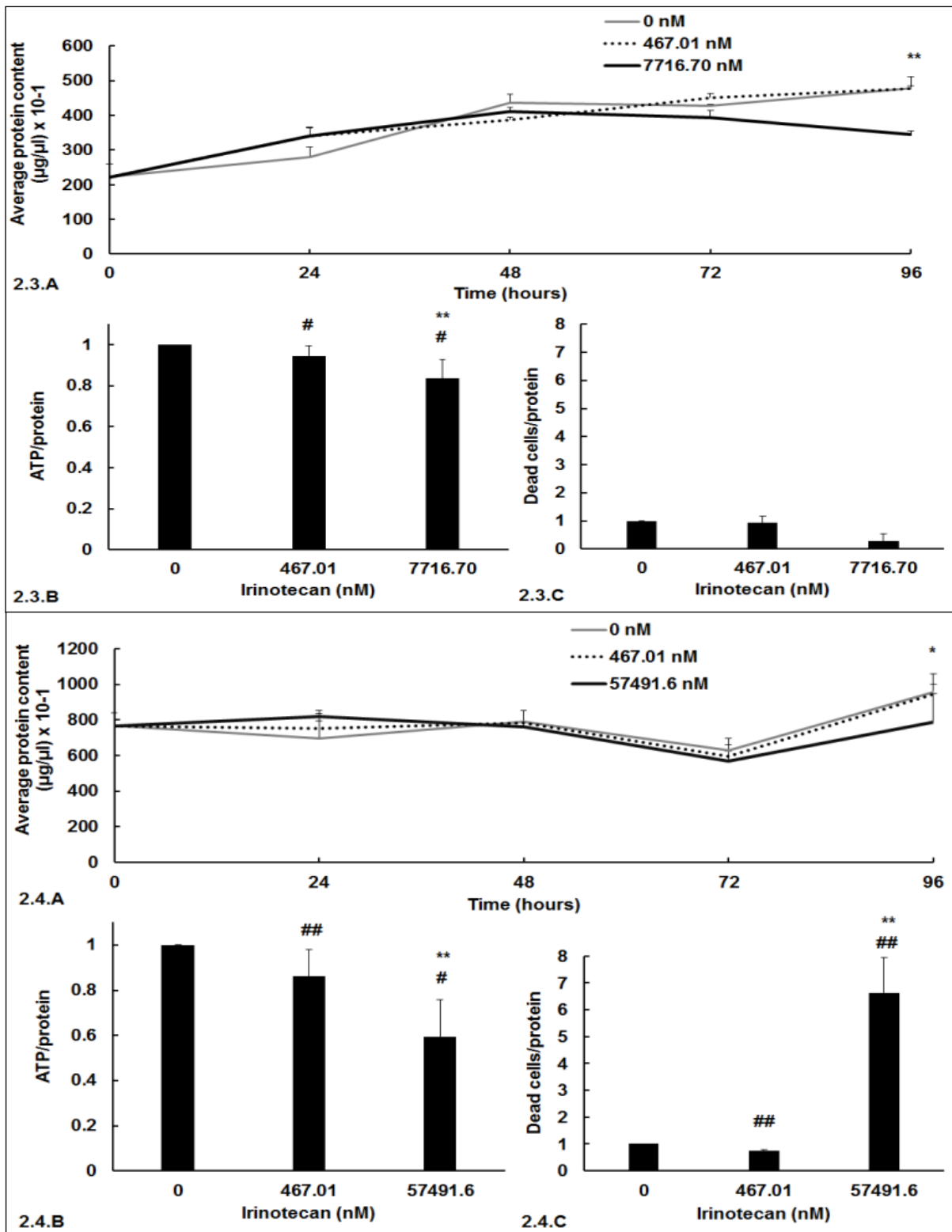
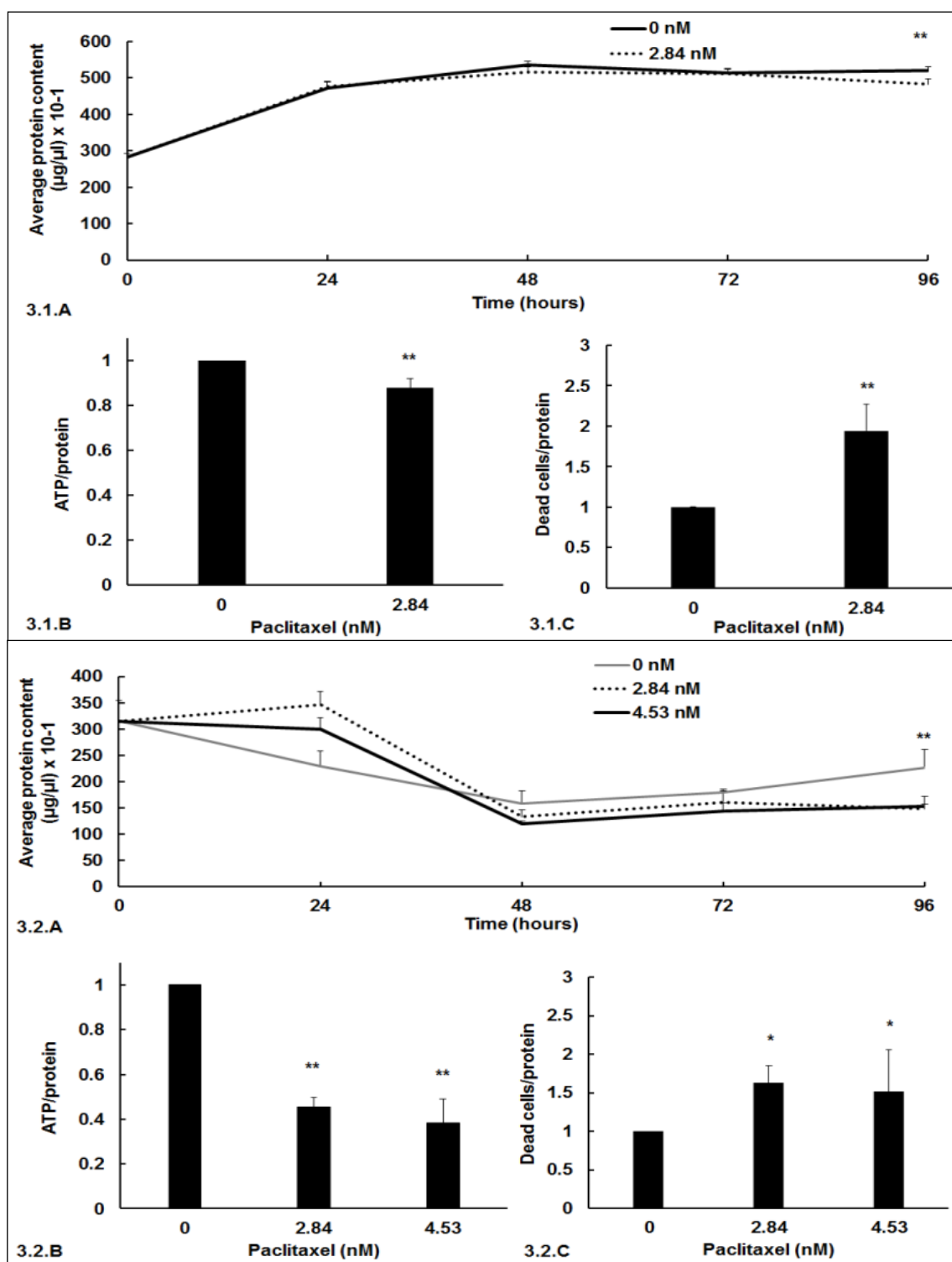


Figure 3: Cell responses following daily treatment with paclitaxel (nM) for 96 h. Soluble protein content over time (A), intracellular ATP levels per soluble protein content (normalised relative to the untreated control) (B) and extracellular AK levels per soluble protein content (normalised relative to the untreated control) (C) are shown for the LLC-PK1 (1), H69V (2), H69AR (3) and NCI-H69/LX4 (4) cell lines, respectively. Error bars denote the standard deviation ($n = 6$), # indicate statistically significant inter-dosage differences ($p < 0.05$); ## indicate statistical significant inter-dosage differences ($p < 0.01$); * indicate statistically significant differences relative to the untreated control ($p < 0.05$); ** indicate statistically significant differences relative to the untreated control



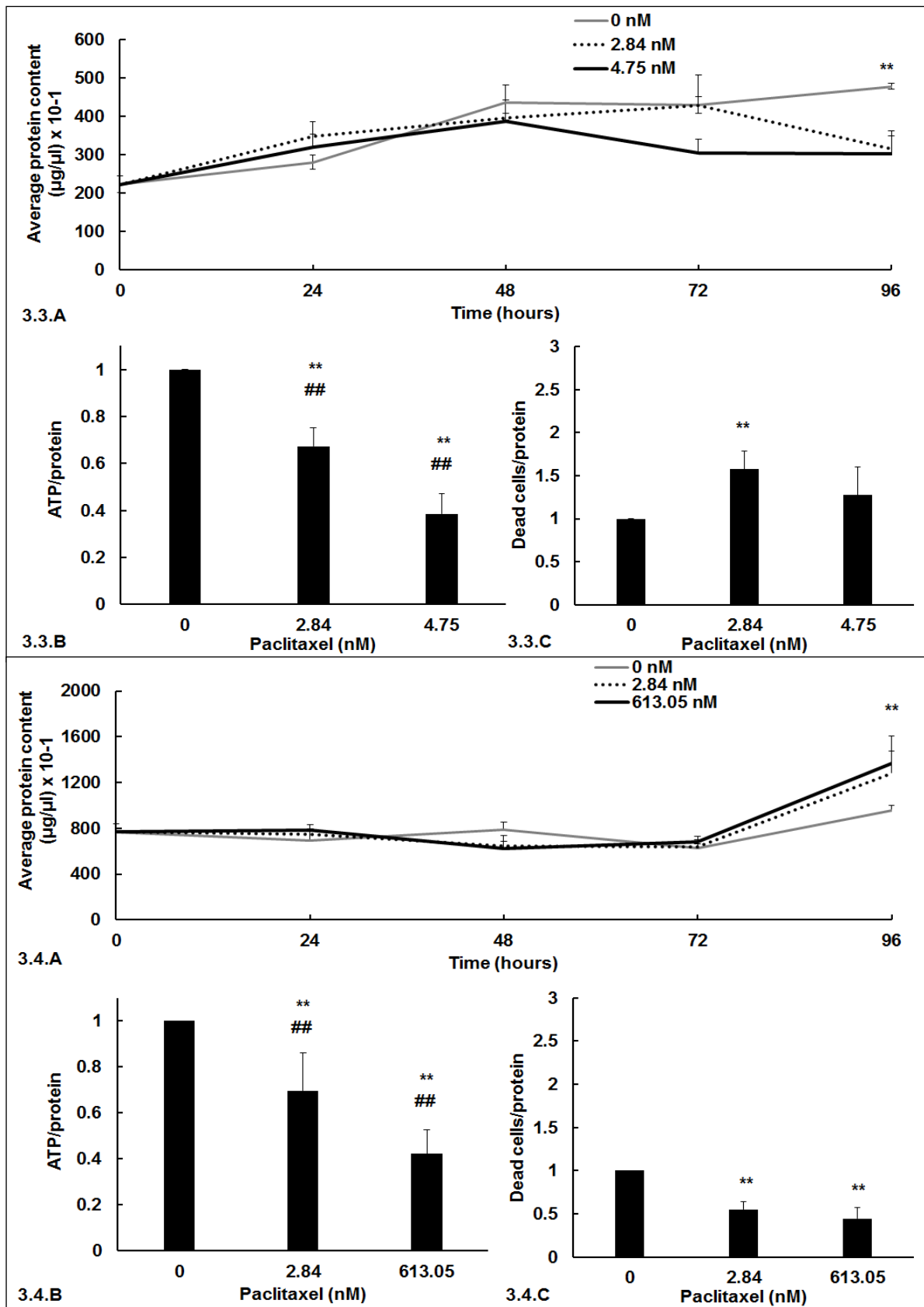


Figure 4: Cell responses following daily treatment with Cisplatin (nM) for 96 h. Soluble protein content over time (A), intracellular ATP levels per soluble protein content (normalised relative to the untreated control) (B) and extracellular AK levels per soluble protein content (normalised relative to the untreated control) (C) are shown for the LLC-PK1 (1), H69V (2), H69AR (3) and NCI-H69/LX4 (4) cell lines, respectively. Error bars denote the standard deviation ($n = 6$), # indicate statistically significant inter-dosage differences ($p < 0.05$); ## indicate statistical significant inter-dosage differences ($p < 0.01$); * indicate statistically significant differences relative to the untreated control ($p < 0.05$); ** indicate statistically significant differences relative to the untreated control ($p < 0.01$).

