

**Molecular Mechanisms of Drug
Resistance in K562 Multidrug Resistant
Leukaemic Cell Lines**

Jodie Stephenson

PhD

2005

CERTIFICATE OF AUTHORSHIP/ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Candidate

Production Note:

Signature removed prior to publication.

ACKNOWLEDGEMENTS

During my PhD I received invaluable assistance and support from my supervisor, Associate Professor Anita Piper. Anita was instrumental in providing guidance throughout the entire project for which I am forever grateful.

I also wish to thank Associate Professor Mary Davey, my co-supervisor, for her expert help with tissue culture and supplying the K562 cell lines.

Thank you to Michelle Byrne for making the luciferase constructs and for her friendship in the lab.

Thank you also to Dr Merlin Crossley, for donating the CMV- β -galactosidase plasmid, and all members of UTS department of Cell and Molecular Biology, past and present.

I am deeply grateful to my family, Dad and Mum, Melinda and Sean, Daniel and Sophie, Jeremy and Louise, Nanny and Puppa, Grandma, Scruffy and Fergus for all their continuous love and support.

A special mention is owed to Declan, for his love, friendship, patience and for taking wonderful care of me over the years. Without him life wouldn't be the same.

For their love, fun and laughter, I owe many thanks to all of my close friends especially Anna, Amy and Rob, Simon and Lindsay, Larina and Shaun, Rachel and Jo, Tanya, Bernie, Marky and Rosco, and in particular Roie, whose daily teatime antics kept me sane.

TABLE OF CONTENTS

Certificate of authentication	i
Acknowledgements	ii
List of figures and tables	vii
Abbreviations	x
Abstract	xiii
CHAPTER 1 Introduction	1
1.1 Cancer	1
1.2 The Problem of Multidrug Resistance in Cancer	2
1.3 P-glycoprotein Mediated MDR	5
1.3.1 P-glycoprotein expression in normal tissues	9
1.3.2 P-glycoprotein expression in cancer <i>in vivo</i> and <i>in vitro</i>	10
1.4 Mechanisms for the activation of P-gp	13
1.4.1 Methylation	13
1.4.2 Gene rearrangement	15
1.4.3 Gene amplification	16
1.4.4 Transcriptional activation	16
1.4.5 Post transcriptional mechanisms	25
1.5 The current study	26
CHAPTER 2 Characterisation of experimental cell lines	28
2.1 Introduction	28
2.2 Materials and Methods	29
2.2.1 Cell lines and culture	29
2.2.2 Cytotoxicity assays	29
2.2.3 Total RNA extraction	29

2.2.4	RNA transfer and Northern hybridisation	31
2.2.5	Isolation of genomic DNA	32
2.2.6	Slot Blot Transfer and Southern hybridisation	33
2.2.7	Drug induction	34
2.2.7.1	3 day treatments	34
2.2.7.2	4 and 16 hour drug induction treatments	34
2.2.8	Trichostatin A treatment of cells	35
2.3	Results	35
2.3.1	Drug Resistance levels of K562 MDR cell lines	35
2.3.2	<i>MDR1</i> mRNA levels in K562 and MDR cell lines	37
2.3.3	<i>MDR1</i> gene copy number	40
2.3.4	Stability of MDR in K562 MDR sublines	40
2.3.5	Effect of 3 day re-exposure to drug	44
2.3.6	<i>MDR1</i> mRNA Levels in re-stimulated K562 sublines	51
2.3.7	Induction by short term exposure to <i>MDR1</i> substrate drugs	51
2.3.8	Induction by short term exposure to cisplatin	54
2.3.9	Induction by short term exposure to rifampicin	61
2.3.10	Effect of short term exposure to the histone deacetylase inhibitor TSA	61
2.4	Summary of Results	63
 CHAPTER 3 Investigation of binding to transcription factor sites in the <i>MDR1</i> promoter		68
3.1	Introduction	68
3.2	Materials and Methods	70
3.2.1	EMSAs	70
3.2.1.1	Nuclear extract preparation	70
3.2.1.2	Preparation of labelled binding DNA	71
3.2.1.3	Binding reactions	73
3.2.2	Transient transfections	73

3.2.2.1	Reporter assays	74
3.2.2.2	Statistical analysis	75
3.3	Results	75
3.3.1	Preparation of nuclear extracts	75
3.3.2	Y-box binding factor levels in K562 and MDR sublines	76
3.3.3	Identification of Y-box binding factor	79
3.3.4	-55GC box binding factor levels in K562 and MDR sublines	81
3.3.5	Transient transfection of <i>MDR1</i> promoter	85
3.3.6	Repressor Binding Protein levels in K562 and K/EPR MDR cell lines	88
3.4	Summary of Results	91
 CHAPTER 4 <i>MDR1</i> Promoter Methylation Studies		93
4.1	Introduction	93
4.2	Materials and Methods	94
4.2.1	Genomic sequencing	94
4.2.1.1	Bisulphite conversion of genomic DNA	94
4.2.1.2	PCR of converted genomic DNA	95
4.2.1.3	Subcloning of PCR products	95
4.3	Results	98
4.3.1	Methylation status in K562 and K/EPR cells	98
4.3.2	Protein binding levels at CpG sites 43/44	98
4.4	Summary of Results	101
 CHAPTER 5 <i>MDR1</i> mRNA Stability Studies		105
5.1	Introduction	105
5.2	Materials and Methods	106
5.2.1	Determination of inhibitory actinomycin D concentration	106
5.2.2	<i>MDR1</i> mRNA stability determination	106
5.2.3	<i>MDR1</i> RT-PCR	107
5.3	Results	108

5.3.1 Optimal actinomycin D concentration	108
5.3.2 <i>MDR1</i> mRNA half life	108
CHAPTER 6 Discussion	114
6.1 MDR and its reversible induction in K562 MDR sublines	114
6.2 Transcriptional regulators of MDR1	117
6.3 GC box binding protein involvement in <i>MDR1</i> activation	121
6.4 NRE binding protein involvement in <i>MDR1</i> activation	122
6.5 <i>MDR1</i> transfection studies	123
6.5.1 <i>MDR1</i> promoter studies	123
6.5.2 Downstream region	123
6.6 Effect of TSA on MDR levels	124
6.7 Methylation status of the <i>MDR1</i> promoter	125
6.8 Stability of <i>MDR1</i> mRNA in K562 MDR sublines	126
6.9 Future directions	128
6.10 Conclusions	130
References	132

LIST OF FIGURES AND TABLES

Figure 1.1	Structure of P-gp	6
Table 1.1	Representative compounds that interact with P-gp	8
Figure 1.2	The <i>MDR1</i> promoter structure	17
Figure 2.1	Resistance levels of K/EC32 cells	36
Figure 2.2	Resistance levels of K/EPR cells	38
Figure 2.3	Resistance levels of K/DNR cells	39
Figure 2.4	Northern blot for the determination of <i>MDR1</i> mRNA levels in K562 MDR cell lines	41
Table 2.1	Normalised <i>MDR1</i> mRNA levels in K562 MDR cell lines	41
Figure 2.5	Comparison of the <i>MDR1</i> gene copy number in K562 MDR cell lines	42
Table 2.2	Normalised <i>MDR1</i> gene copy number in K562 MDR cell lines	42
Figure 2.6	Stability of MDR	43
Figure 2.7	The effect of 3 day drug exposure on the K/EC32 cell line	45
Figure 2.8	The effect of short-term drug exposure on the K/EC32 and K/EC32T cell lines	47
Figure 2.9	The effect of 3 day drug exposure on the K/EPR cell line	48
Figure 2.10	The effect of 3 day drug exposure on the K/DNR cell line	49
Figure 2.11	The effect of 3 day drug exposure on the K562 cell line	50
Figure 2.12	Northern blot for the determination of <i>MDR1</i> mRNA levels in K562 MDR cell lines	52
Table 2.3	Correlation of <i>MDR1</i> mRNA and relative resistance levels in K562 MDR sublines following 3 day drug treatment	53
Figure 2.13	The effect of short-term epirubicin exposure on the K/EPR cell line	55
Figure 2.14	The effect of short-term paclitaxel exposure on the K/EPR cell line	56
Table 2.4	Comparison of K/EPR cell paclitaxel IC ₅₀ 's before and after 4 or 16 hour epirubicin or paclitaxel treatment	57

Figure 2.15	The effect of short-term cisplatin exposure on the K/EPR cell line - paclitaxel cytotoxicity	59
Figure 2.16	The effect of short-term cisplatin exposure on the K/EPR cell line – vinblastine cytotoxicity	60
Figure 2.17	The effect of short-term rifampacin exposure on the K/EPR cell line	62
Figure 2.18	Effect of TSA on growth and epirubicin resistance of K562 cells	64
Figure 2.19	Effect of TSA on growth and epirubicin resistance of K/EPR cells	65
Figure 3.1	EMSA for Y-box binding probe	77
Figure 3.2	EMSA for (a) Y-box binding probe and (b) USF binding probe	78
Table 3.1	Normalised Y-box binding factor levels in K562 MDR sublines	82
Figure 3.3i	EMSA using (a) single stranded Y-box binding oligonucleotide and (b) Supershifting of Y-box binding factor with anti-NF-YA antibody	82
Figure 3.3ii	EMSA for supershifting of the Y-box factor with anti NF-YA	83
Figure 3.4	EMSA for (a) USF and (b) –55GC box binding factors	84
Table 3.2	Normalised –55GC box binding factor levels in K562, K/EPR and K/EPR drug treated cells	86
Figure 3.5	Relative <i>MDR1</i> promoter activity in K562 and K/EPR cells	87
Figure 3.6	<i>MDR1</i> promoter activity in K/EPR untreated and K/EPR treated cells	89
Figure 3.7	EMSA for (a) NRE binding factor and (b) USF	90
Table 3.3	Normalised NRE binding factor levels in K562, K/EPR and K/EPR drug treated cells	90
Figure 4.1	Sequence of the <i>MDR1</i> promoter	99
Table 4.1	Methylation status of CpG sites in the <i>MDR1</i> promoter region (- 462 to +782bp) in K562 and K/EPR cells	100
Figure 4.2	EMSA for (a) binding to CpG sites 43 & 44 and (b) USF binding sequence	102
Figure 4.3	Transcription factor analysis of sequence surrounding CpG sites 43 and 44	103

Table 4.2	Transcription factor binding sequences	103
Figure 5.1	Optimal actinomycin D concentration for K562 and K/EPR cells cell lines	109
Figure 5.2	The effect of short-term drug exposure on the K562 and K/EPR	110
Figure 5.3	<i>MDR1</i> mRNA half-life for K/EPR cells before and after Epirubicin treatment	112
Table 5.1	Comparison of <i>MDR1</i> cDNA levels and paclitaxel resistance before and after 3 days epirubicin treatment.	113

ABBREVIATIONS

A	adenine or adenosine
ABC	ATP-binding cassette
AMP	adenosine monophosphate
ATCC	American type culture collection
ATP	adenosine triphosphate
5'-azadC	5'-azadeoxycytidine
bp	base pairs
BSA	bovine serum albumin
C	cytosine or cytidine
CAT	chloramphenicol acetyl transferase
cAMP	cyclic AMP
cDNA	complementary DNA
Ci	curie
CO ₂	Carbon dioxide
CpG	CG dinucleotide
CRE	cAMP regulator elements
CREB	cAMP-responsive element binding protein
CRS	cAMP response sequences
CTP	cytosine triphosphate
DEPC	diethylpyrocarbonate
DH ₂ O	deionised water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	(unspecified) deoxyribonucleotide triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
Egr	early growth response
EGTA	ethylene glycol-bis-(2-aminoethyl ether) N, N, N', N'-tetracetic acid

EMSA	electrophoretic mobility shift assay
ET-743	Ecteinascidin-743
EtBr	ethidium bromide
5-FU	5-Fluorouracil
fmole	femtomole
g	grams
G	guanine or guanosine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
GST	glutathione S-transferase
GTP	guanine triphosphate
HEPES	N-2-hydroxyethyl-piperazine-N ¹ -2-ethanesulfonic acid
HSRE	heat shock response element
IC50	50% inhibitory concentration
kb	kilobase
kDa	kilodalton
L	litre
MDR	multidrug resistance
MDR1	multidrug resistance gene 1
MDR2	multidrug resistance gene 2
mM	millimolar
$\mu\text{J}/\text{cm}^2$	microjoules per centimetre squared
μM	micromolar
ml	millilitre
mRNA	messenger RNA
MRP	multidrug resistance associated protein
MRP1	multidrug resistance associated protein gene 1
MTT	3,4,5-dimethylthiazole-2,5 diphenyl tetra bromide
MW	molecular weight
nm	nanometer
nM	nanomolar
NRE	negative response element

P/CAF	CREB binding protein associated factor
PCR	polymerase chain reaction
P-gp	p-glycoprotein
PKA	type 1 cAMP-dependant kinase
PKC	protein kinase C
pmole	picomole
PABP	poly(A)-binding protein
poly(A)	polyadenylated
RB	retinoblastoma
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SRE	stress response elements
SSC	sodium chloride sodium citrate
SSPE	Sodium chloride sodium phosphate + Ethylenediaminetetraacetic acid
SSRE	serum starvation response element
ssYB-1	single stranded YB-1
T	thymine or thymidine
TBE	Tris-borate buffer
TBP	TATA binding protein
TE	Tris EDTA
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	tris(hydroxymethyl) aminomethane
TSA	trichostatin A
TSS	transcription start site
tRNA	transfer RNA
TTP	thymidine triphosphate
U	unit
UV	ultraviolet

ABSTRACT

A major problem in chemotherapy is that many cancers are intrinsically drug resistant or later become resistant. Resistance to one drug is often accompanied by cross-resistance to many unrelated drugs and this is known as multidrug resistance (MDR). MDR is commonly associated with a 170kDa glycoprotein called P-glycoprotein (P-gp), which is thought to act as an ATP-dependant drug efflux pump and which is encoded for by the *MDR1* gene in humans. *MDR1* transcription can be initiated by various mechanisms such as demethylation of *MDR1* promoter sequences or translocation of the *MDR1* gene. In cells already expressing P-gp this expression can be increased by mechanisms such as amplification of the *MDR1* gene copy number or an increase in the rate of mRNA translation or stability. However the focus of many studies is on the regulation of *MDR1* transcription through the binding of transcription factors to specific sequences in the *MDR1* promoter region, in particular an inverted CCAAT element known as the Y-box and the -55GC box.

The current study investigated the mechanisms for MDR in a series of K562 derived MDR cell lines demonstrating varying levels of low level MDR. Levels of resistance of each of the K562 MDR cell lines were all confirmed by performing vinblastine and paclitaxel cytotoxicity assays on the cell lines. Northern hybridisation of total RNA isolated from K562 MDR cells indicated a positive correlation existed between the level of *MDR1* mRNA expressed in the MDR cells and the level of MDR displayed by the cells. Southern blot hybridisation of DNA from the cells with an *MDR1* probe indicated that increase of *MDR1* mRNA in the K562 MDR cells was not due to amplification of the *MDR1* gene. Bisulphite genomic sequencing of K562 and MDR cell lines revealed the *MDR1* promoter in both cell lines to be almost completely unmethylated apart from two distinct sites of methylation in K562 cells at two CpG sites downstream of the transcription start at +421 and +423bp respectively.

Treatment of the K562 cells with the histone deacetylase inhibitor Trichostatin A (TSA) increased the cells resistance to epirubicin, however no effect was seen upon TSA treatment of the K562 MDR cells. This suggests there is a difference in the chromatin structure in the two cell lines.

It was further demonstrated that resistance levels of K562 derived MDR cell lines declined with increasing time in drug free culture, but could be restimulated by short term drug exposure to P-gp substrate drugs, in some cases in as little as after 4 hours exposure, suggesting a transcriptional mechanism of *MDR1* upregulation is likely to be responsible for the induction. Consistent with this, mRNA stability studies indicated that the drug induction of K562 MDR cells that resulted in increased MDR and *MDR1* mRNA levels was not mediated by an increase in the stability of *MDR1* mRNA, as the rate of mRNA decay was the same in treated and untreated control cells. However, electrophoretic mobility shift assays (EMSA) of the two major transcription factors involved in *MDR1* regulation, the Y-box binding protein (identified as NF-Y) and the –55GC box binding protein indicated no difference in nuclear levels of these two proteins in untreated and drug induced cells. Thus the mechanism for up-regulation of *MDR1* activity in K562 MDR cell lines is most likely due to activation via alteration of other factors or via changes in chromatin structure regulating accessibility of transcription factors.