Pharmacokinetics and pharmacogenetics of actinomycin D in children with cancer

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Abstract

Actinomycin D (Act D) has been used successfully to treat several cancers for over 50 years and continues to play a key role in the treatment of paediatric tumours. The aim of this thesis was to investigate Act D pharmacokinetic variability in children with cancer, alongside *in vitro* and *in vivo* drug transporter and pharmacogenetic studies.

The transport of Act D by ABC transporter proteins was investigated in chapter 2. Growth inhibition and intracellular and cellular efflux assays in MDCKII cells demonstrated that Act D was a substrate for ABCB1, ABCC2 and to a lesser extent ABCC1. The *in vivo* relevance of these findings was assessed using knockout mouse models in chapter 3.

Act D pharmacokinetics was investigated in Abcb1a/1b and Abcc2 knockout mice. Abcb1a/1b$^{-/-}$ mice had 1.6-fold higher $AUC_{0\text{-}6h}$ compared to wild-type (WT). Abcc2$^{-/-}$ mice exhibited an Act D $AUC_{0\text{-}6h}$ of 76% that seen in WT mice. Brain concentrations of Act D were also significantly higher in Abcb1a/1b$^{-/-}$ mice compared to WT, although no difference was observed in liver and kidney concentrations. These findings suggest that Abcb1a/1b and possibly Abcc2 play an important role in Act D pharmacokinetics but have only a minimal impact on tissue distribution.

Following this the pharmacokinetics of Act D in children with cancer was investigated. Analysis of pharmacokinetic data from 74 patients showed large inter-patient variability in Act D pharmacokinetics. Clearance ranged from 15 – 341 ml/min and was positively correlated to patient age and body size. A 10-fold range in clearance normalised to body surface area was observed, indicating that factors other than body size may be involved in determining Act D pharmacokinetics.

Finally, the relevance of ABCB1 and ABCC2 SNPs to Act D pharmacokinetics was investigated in 64 patients. Genotyping analysis of 3435C>T and 2677G>T/A, showed that patients with more than one variant allele in these two

SNPs had 1.7-fold higher Act D clearance compared to patients who were WT at both SNPs (200 versus 115 ml/min/m^2, respectively).

The work presented in this thesis has advanced our understanding of Act D pharmacokinetics and the potential factors that influence patient exposure. Act D has been confirmed as a substrate for both ABCB1 and ABCC2, and these transporters have been shown to influence Act D pharmacokinetics *in vivo*. Patient variability in Act D exposure has been confirmed in a large patient cohort, and it has been demonstrated that some of this variability can be attributed to ABCB1 genotype.

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Abbreviations

7-AD	7-aminoactinomycin D
+ve	Positive
4-HT	4-hydroxytamoxifen
6-MP	6-mercaptopurine
6-TG	6-thiopurine
ABC	ATP-binding cassette
ABCB1	ATP-binding cassette, sub-family B member 1
Abcb1a	ATP-binding cassette, sub-family B, member 1A in mice
Abcb1b	ATP-binding cassette, sub-family B, member 1B in mice
ABCC1	ATP-binding cassette, sub-family C member 1
ABCC2	ATP-binding cassette, sub-family C member 2
Abcc2	ATP-binding cassette, sub-family C, member 2 in mice
ABCC3	ATP-binding cassette, sub-family C member 3
ABCC4	ATP-binding cassette, sub-family C member 4
ABCG2	ATP-binding cassette, sub-family G member 2
Abcg2	ATP-binding cassette, sub-family G, member 2 in mice
Act D	Actinomycin D
ADME	Absorption, distribution, metabolism and excretion
ADP	Adenosine diphosphate
ALL	Acute lymphoblastic leukaemia
ALT	Alanine transaminase
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
A-RMS	Alveolar RMS
AST	Aspartate transaminase
ATP	Adenosine triphosphate
$AUC_{0\text{-}24h}$	Area under the plasma-concentration time curve from 0-24h
$AUC_{0\text{-}6h}$	Area under the plasma-concentration time curve from 0-6h
$AUC_{0\text{-}\infty}$	Area under the plasma-concentration time curve from 0-infinity
BAD	British Association of Dermatologists
B/P	Brain-to-Plasma concentration ratio
BCA	Bicinchoninic acid
BCRP	Breast cancer resistance protein

BSA	Bovine serum albumin
BWS	Beckwith-Wiedemann syndrome
C	Cyclophosphamide
CARBO	Carboplatin
cDNA	Complementary DNA
CIS	Cisplatin
Cl	Clearance
Cl-F	Clearance from full sample sets
Cl-FSA	Clearance from full sample sets normalised to body surface-area
Cl-L	Clearance from limited sample sets
C_{max}	Maximum plasma concentration
CML	Chronic myelogenous leukaemia
CNS	Central nervous system
CsA	Cyclosporin A
CTC	Common toxicity criteria
CVC	Central venous catheter
CYP2D6	CYP2D6 cytochrome P450, family 2, subfamily D, polypeptide 6
D'	Lewontin's D-prime
Da	Daltons
DD	Denys-Drash Syndrome
ddH_2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
E	Exon
ECL+	Enhanced chemiluminescence plus
EDTA	Ethylenediaminetetraacetic acid
ERG	Ets Related Gene
EPI	Epirubicin
E-RMS	Embryonal RMS
ES	Ewing's sarcoma
ET	Etoposide
ETV1	Ets translocation variant 1
ETV4	Ets translocation variant 4

EWS	EWSR1 gene
FEV	Ets oncogene family
FH	Favourable histology
FLI1	ETS transcription factor family
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase in mice
GFR	Glomerular filtration rate
GI	Growth inhibition
h	hour
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
HPLC	High pressure liquid chromatography
I	Intron
IRSG	The Intergroup Rhabdomyosarcoma Study Group
IS	Internal standard
K/P	Kidney-to-Plasma concentration ratio
kb	kilobase
kDa	Kilo-dalton
KO143	Specific ABCG2/BCRP transport inhibitor
L/P	Liver-to-Plasma concentration ratio
LC/MS	Liquid chromatography mass spectrometry
LD	Linkage disequilibrium
MAF	Minor allele frequency
MDCKII	Madin-Darby Canine Kidney Type II
MDR	Multidrug resistance
MDR1	Multidrug resistance protein 1
MeCN	Acetonitrile
min	Minutes
MK571	ABCC/MRP-family transport inhibitor
MP	Mercaptopurine
mRNA	Messenger ribonucleic acid
MRP1	Multidrug resistance-associated protein 1
MRP2	Multidrug resistance-associated protein 2
MTS	Tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MTX	Methotrexate
NCA	Non-compartmental analysis
NSCLC	Non-small cell lung cancer

NWTS	The National Wilms Tumour Study
OAT1	Organic anion transporter 1
OAT2	Organic anion transporter 2
pa	peak area
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
P-gp	P-glycoprotein
PVDF	Polyvinylidene fluoride
QC	Quality control
RMS	Rhabdomyosarcoma
RNA	Ribonucleic acid
RT	Radiotherapy
SA	Body surface-area
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SIOP	Society of Paediatric Oncology
SMN	Secondary Malignant Neoplasms
SNP	Single nucleotide polymorphisms
SRB	Sulfarhodamine B
SPE	Solid phase extraction
$T_{1/2}$	Terminal phase half-life
TagSNPs	SNPs which capture other alleles due to linkage disequilibrium
TBS/T	Tris-Buffered Saline and Tween 20
TCA	Trichloroacetic acid
TGN	Thioguanine nucleotides
tIMP	Thioinosine monophosphate
T_{max}	Time of maximum plasma concentration
TPMT	Thiopurine methyltransferase
UH	Unfavourable histology
UV	Ultraviolet light
VA	Vincristine and actinomycin D
VAC	Vincristine, actinomycin D and cyclophosphamide
VAI	Vincristine, actinomycin D and ifosfamide
VCR	Vincristine
-ve	Negative

VIDE	Vincristine, ifosfamide, doxorubicin and etoposide
VIE	Vincristine, ifosfamide and etoposide
VOD	Veno-occlusive disease
Vss	Volume of distribution at steady-state plasma concentrations
WAGR	Wilms-Aniridia-Genitourinary anomalies-mental Retardation syndrome
WT	Wild-type

Chapter 1. Introduction

1.1 Cancer

1.1.1 Background

Cancer is a broad term for a wide range of diseases all characterised by uncontrolled cell proliferation. Cell proliferation in the body is tightly controlled, resulting in cell number homeostasis and maintenance of normal tissue structure and function. Cancer occurs when regulation of this vital process is lost, as cancer cells have acquired functional capabilities which allow them to survive, proliferate and spread outside the control of normal cellular processes.

The six hallmarks of cancer dictate that cancer cells must be able to:

- sustain chronic proliferation
- circumvent growth suppressors
- activate invasion and metastasis
- replicate
- induce angiogenesis
- avoid apoptosis.

These acquired hallmarks can be the result of genetic instability, where random mutations or chromosome translocations pave the way for tumourigenesis (Hanahan and Weinberg, 2000). Recently, this has been expanded to include new emerging hallmarks such as deregulating cellular energetics and avoiding immune destruction, in addition to enabling characteristics which include

genome instability and tumour-promoting inflammation (Hanahan and Weinberg, 2011).

1.1.2 Cancer in adults

Cancer is predominately a disease of the elderly, with 1/3 of the UK population developing some form of cancer during their lifetime. In the UK, 60% of cancer cases are in patients older than 65 years, and 1/3 are in patients older than 75 years. Since the 1970s, the incidence of cancer has steadily increased in males by 20% and females by 40%. This increase in cancer incidence has been strongly linked to lifestyle choices. For instance in the case of malignant melanoma, due to increased sun and UV exposure, or liver cancer, due to poor diet and alcohol abuse. According to the most recent CancerStats data from Cancer Research UK (Figure 1.1), breast cancer is the most common malignancy in females, whilst prostate cancer is the most common in males (http://info.cancerresearchuk.org/cancerstats).

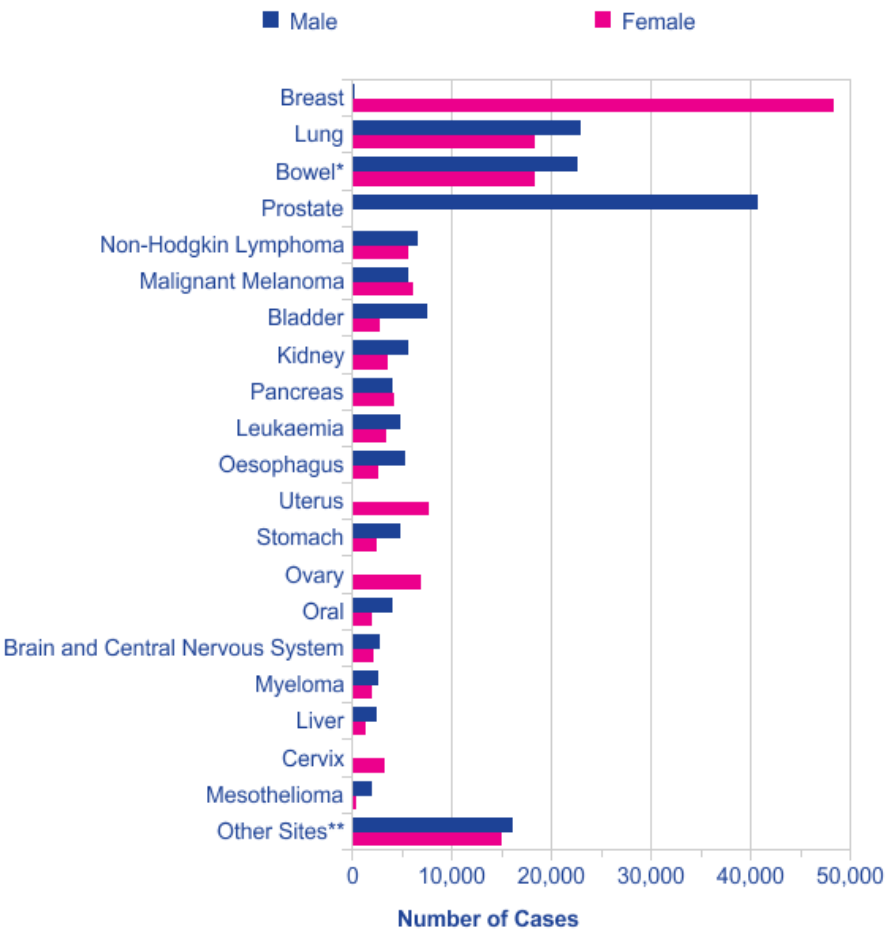

Figure 1.1. The twenty most common adult cancers in the UK in 2009.

*Bowel including anus. **4% of all female cancer cases and 3% of all male cancer cases are registered without specification of the primary site.

Modern multimodal approaches to cancer treatment, in addition to increased public awareness and earlier detection, have helped to dramatically increase the ten-year survival rates of many adult cancers since the 1970s (Figure 1.2). Survival to ten years in common cancers such as breast cancer in women has increased from 39% to 77%, while prostate cancer survival rates have increased from 20% to 69% since 1971. However, the same has not happened in some aggressive tumour types such as lung and pancreatic cancer, where ten year survival rates remain below 10% (http://info.cancerresearchuk.org/cancerstats).

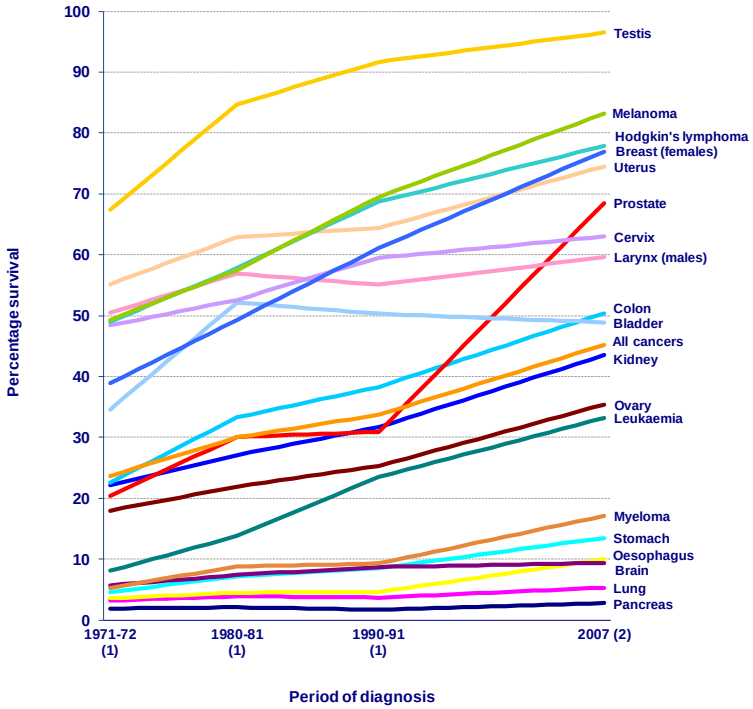

Figure 1.2. Ten year survival (%) in adults (15-99 years) in England and Wales 1971-2007.

1.1.3 Paediatric cancer

Childhood cancer is relatively rare, with only 0.5% of cancer sufferers younger than 16 years in western populations. However, while the risk of developing some form of cancer during the first 15 years of life is around 1 in 500, paediatric cancer remains the leading cause of non-accidental death in children in the UK. Despite a rising incidence of paediatric cancers over the last 50 years mortality rates have been declining (Figure 1.3), with average 5 year survival rates increasing from 30% to 78% (http://info.cancerresearchuk.org/cancerstats).

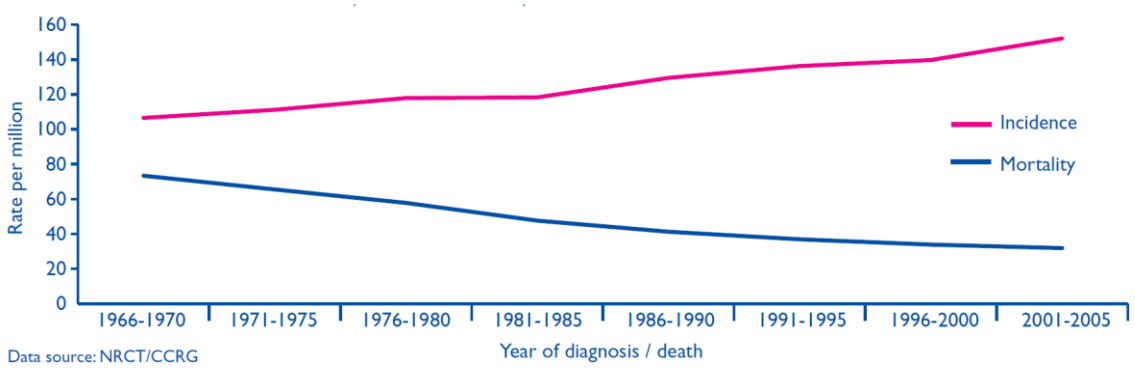

Figure 1.3. Average incidence and mortality rates for all paediatric cancers between 1966-2005.

Cancers seen in the paediatric population are very different to those seen in adults, with some of the major adult cancers, such as lung cancers, being extremely rare in children. Paediatric cancer classification and incidence are shown in Figure 1.4. Leukaemia is by far the most common malignancy in childhood (Figure 1.4), accounting for approximately one third of all paediatric cancers. Acute Lymphoblastic Leukaemia (ALL) accounts for 79% of childhood leukaemia (Pui and Evans, 2006; CancerStats, 2010), and 25% of all paediatric cancers (Pui *et al.*, 2004). Acute Myeloid Leukaemia (AML) is the second most common type of leukaemia in children, accounting for 15% of leukaemia cases in the UK (http://info.cancerresearchuk.org/cancerstats).

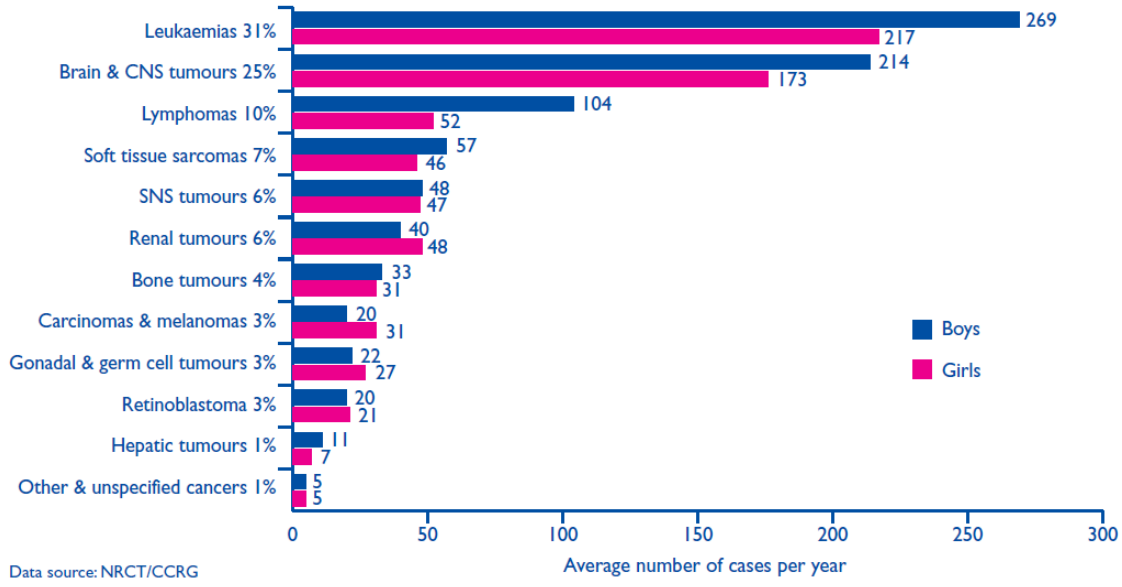

Figure 1.4. Average number of cases and percentage of new cases of childhood cancer. Results are separated by sex and diagnostic group.

1.2 Cancer Therapy

1.2.1 Introduction

Treatment of both adult and paediatric cancers often requires a multidisciplinary approach, depending on disease stage and progression. Several types of cancer therapy exist, and are usually used in combination as multimodal treatment to rid the body of cancerous cells.

1.2.2 Surgery

Surgical removal is widely used and is a treatment option for many patients with solid tumours. Surgery can be carried out before or after chemotherapy depending on the staging of the tumour. In conjunction with chemotherapy, surgery is an excellent way of large scale tumour eradication (Stiller and Draper, 2005).

1.2.3 Radiotherapy

Radiotherapy uses concentrated ionizing radiation, focused on a particular area, to control the development and proliferation of malignant cells. It is commonly used in combination with surgery and chemotherapy. Radiotherapy can be used pre-surgery to shrink tumour bulk or post-surgery to kill any cells not removed by the surgical procedure (Stiller and Draper, 2005).

1.2.4 Chemotherapy

In 1942, Louis Goodman and Alfred Gilman investigated the potential for using chemical warfare agents as potential cancer therapeutics. Based on observations using mice, it was found that nitrogen mustards, similar to those used in chemical weapons, caused tumour regression in a non-Hodgkin's lymphoma patient. Towards the end of the 1940s, Sydney Farber investigated the effect of folic acid on paediatric patients with ALL, after previous work showed that folate deficiency was linked to megaloblastic anaemia (Wills *et al.*, 1937). Farber discovered that administration of folic acid actually increased the rate of leukaemic cell proliferation, leading to the development of the folate analogues, aminopterin and amethopterin (Methotrexate), which act to block the function of enzymes requiring folate. Remission was induced in children with ALL following treatment with antifolates, and normal bone marrow function was restored (Farber and Diamond, 1948). From these observations, nitrogen mustards and antifolates were discovered as chemotherapeutic agents and modern chemotherapy was born (Gilman, 1963).

Modern day chemotherapy involves the use of a plethora of cytotoxic drugs, either on their own, or more frequently in combination, with the aim of achieving

complete disease remission and eradication of minimal residual disease, or as palliation of symptoms (Chabner and Roberts Jr, 2005; Stiller and Draper, 2005). Chemotherapy is adapted for each patient depending on their disease state and was the first form of cancer therapy to provide systemic treatment and to target unknown distant metastases. Most paediatric cancers respond very well to treatment with cytotoxic drugs as they have a very high proliferation rate, a property targeted by many cytotoxic agents (Stiller and Draper, 2005).

1.2.5 Toxicity

Most conventional cytotoxic agents are usually administered intravenously and as such have the potential to reach many cells in the body. As a result of this, the use of cytotoxic drugs often causes some toxicity to normal healthy cells. Toxicity tends to occur in the normal cells, such as the bone marrow, gastrointestinal mucosa and liver cells, that are actively replicating (Stiller and Draper, 2005).

1.2.6 Molecular Targeted Approach

Until recently chemotherapy was based on a largely non-specific approach to treatment, with the use of cytotoxic drugs merely targeting actively dividing cells. By far the biggest advance in chemotherapy over the past decade has been the shift in focus to cancer specific, targeted therapies. This requires a greater understanding of the cancer aetiology at a genetic level, which then allows the development of specific drugs.

A great success story in this area is the identification and use of imatinib, in the treatment of Chronic Myeloid Leukaemia (CML). Approximately 95% of patients with CML have the Philadelphia chromosome, which is formed by a

translocation between chromosome 9 and 22, creating a fused BCR-ABL gene. This fused gene causes the expression of a constitutively-active tyrosine kinase which results in uncontrolled cell proliferation. A specific inhibitor of the BCR-ABL fusion protein was developed, targeting the ATP binding site on the ABL kinase (Druker *et al.*, 1996). Imatinib and related drugs have transformed the treatment of CML, with 5 year survival rates of 89% (Druker *et al.*, 2006). Targeted therapy is therefore a way of specifically targeting markers only found in tumour cells, allowing significantly increased survival with much less toxicity. However, specific molecular targets have not been identified for the majority of tumours.

1.3 Actinomycin D

1.3.1 The Actinomycins

In 1940, the Actinomycins became one of the first group of antibiotics isolated from *Streptomyces* by Waksman and Woodruff (Waksman and Woodruff, 1940). *Streptomyces* bacteria produce many different actinomycins including, A, B, C, D, I, J and X (Vining and Waksman, 1954), all containing an identical phenoxazine ring linked to different peptides (Figure 1.5) (Brockmann, 1954; Brockmann and Muxfeldt, 1955). Different actinomycins are produced depending on the availability of peptide components within the bacteria (Roussos and Vining, 1956).

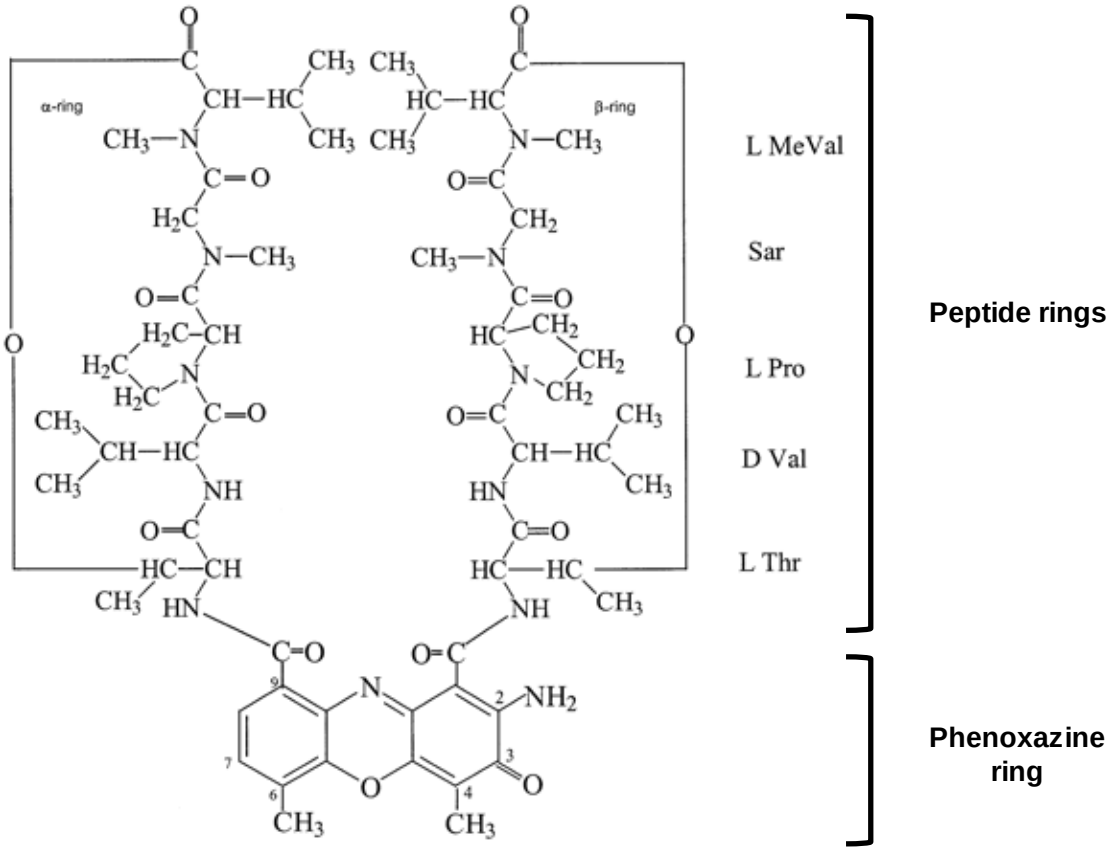

Figure 1.5. The chemical structure of Actinomycin D

1.3.2 Actinomycin D in cancer therapy

Actinomycins were discovered during the search for novel anti-microbial agents, but research was stopped when they were found to be too toxic for use as antibiotics (Farber, 1966). It took a further 12 years for the actinomycins to be identified as potential anti-tumour agents, when in 1952 Hackmann demonstrated the activity of actinomycin C in animal models (Hackmann, 1952; Hackmann, 1953) and Ravina et al., observed clinical activity of actinomycin C (Ravina *et al.*, 1954). Between 1946 and 1954, Sydney Farber and colleagues at Harvard Medical School were working to improve the survival rate of children with Wilms tumour. The treatment regimen of choice at that time involved surgery to remove the tumour and subsequent radiotherapy to prevent reoccurrence, leading to a survival rate of 40%. It was hypothesised that poor prognosis patients who presented with metastases, particularly involving the

lung, would benefit from the administration of adjuvant chemotherapy. Over this eight year period a wide range of chemotherapeutic agents were tested in children with Wilms tumour, but survival rates did not improve.

Following Hackmann's demonstration of the activity of actinomycin C, Farber et al. found that actinomycin D (Act D) was the most potent anti-cancer agent by weight, when treating transplanted mouse tumours (Farber, 1955; Farber *et al.*, 1956). Act D was first administered to a child with advanced terminal Wilms tumour with lung metastases in 1954. Following the death of the child three weeks post-Act D treatment, the post-mortem showed that the metastases, previously observed in the lungs by X-ray, had either disappeared or become collagenous. Act D was also administered to a second child with a ruptured Wilms tumour and a large lung metastasis. No evidence of tumour was present three months after treatment with Act D and the child was alive without tumour after eleven years (Farber, 1966). Early studies also showed Act D could potentiate the effects of radiation when given in combination for Wilms tumour therapy (D'Angio *et al.*, 1959).

In 1960, the long-term treatment program for all patients with Wilms tumour was modified to include Act D immediately following surgery and radiotherapy. Benefits of this change were observed when early patient studies showed 43% survival in patients receiving surgery and radiotherapy, compared to 92% survival in patients receiving the surgery and radiotherapy plus Act D (Fernbach and Martyn, 1966). In addition, Act D improved treatment outcome in patients with or without metastases; 89% of patients presenting with no metastases were tumour free after 2 years, compared to 40% previously, and 53% of

patients presenting with metastases were tumour free after 2 years, compared to no patients previously (Farber, 1966).

Act D is now an integral part of multimodal therapy for many paediatric tumours, particularly Wilms tumour, rhabdomyosarcoma (RMS) and Ewing's sarcoma (ES), where it is used in combination with radiotherapy and other anti-cancer agents such as vincristine and doxorubicin (Metzger and Dome, 2005). Although rarely used, Act D is also effective in some adult cancers, such as Gestational trophoblastic disease (Turan *et al.*, 2006) and Ewing's sarcoma arising in adult patients (Ferrari *et al.*, 2010).

1.3.3 Mechanism of action

Act D binds to DNA mainly at guanine (Reich *et al.*, 1961; Reich *et al.*, 1962), preventing the synthesis of RNA (Goldberg *et al.*, 1962a). It has also been shown to inhibit the incorporation of nucleotide triphosphates into DNA, again resulting in prevention of RNA synthesis, possibly due to stalling of DNA polymerase replication machinery (Goldberg *et al.*, 1962b). Structures of Act D bound to DNA reveal that the phenoxazine ring intercalates into DNA between the dinucleotide sequence d(pGpX), whilst the peptide subunits lie in the minor groove of the DNA helix, forming hydrogen bonds with the opposing guanine residues (Sobell, 1985). Another study indicated that Act D binds tightly to double-stranded DNA, but poorly to double-stranded RNA, RNA-DNA double-strands or single–stranded DNA or RNA (Sobell and Jain, 1972). Act D has also been shown to induce DNA strand breaks when incubated with purified DNA topoisomerase I and II, perhaps revealing another mechanism of action (Wassermann *et al.*, 1990).

1.3.4 Toxicity

Act D is a relatively well-tolerated drug with minimal side effects associated with its use. However, liver toxicities including veno-occlusive disease (VOD) have been reported in 1.7 to 13.5% of cases (Green *et al.*, 1988; Raine *et al.*, 1991; Bisogno *et al.*, 1997), with reported incidence rates increasing due to a greater awareness of VOD by paediatric oncologists. Occurring suddenly after the 2nd or 3rd injection, liver toxicity usually causes fever, anaemia and thrombocytopaenia (Davidson and Pritchard, 1998). Liver toxicity and the associated development of VOD, whilst undesirable, is usually a reversible process. However, VOD associated with multi-organ failure has been reported, leading to high death rates (D'Antiga *et al.*, 2001).

A study carried out in the US has shown age to be a risk factor for VOD. RMS patients under 3 years were 11% more likely to develop liver toxicity than patients aged 3 and over (Arndt *et al.*, 2004). A more recent study of over 3000 patients confirmed the higher incidence of Act D related toxicity in patients <1 year old, and observed that toxicity was more likely to occur early in treatment, although the incidence was lower in patients who initially tolerated the treatment (Langholz *et al.*, 2011). However, the major contributing risk factor appears to be the presence of Wilms tumour. A review in 1995 by Kanwar et al. found that, out of 49 patients with chemotherapy-associated VOD related to Act D treatment, 41 patients had Wilms tumour (Kanwar *et al.*, 1995). Although disease stage is not thought to be a direct risk factor of developing VOD, patients with stage III Wilms tumour who require radiotherapy are possibly at a greater risk due to the exposure to radiation and the positioning of the liver (Bisogno *et al.*, 1997).

The National Wilms Tumour Study (NWTS) group in the United States and International Society of Paediatric Oncology (SIOP) group in Europe advise differing dosing regimens for Act D; the former recommend dosing based on body weight, and the latter suggests dosing based on body surface area, thus complicating the discussion of dosing of Act D as a risk factor. However studies by both clinical research groups have repeatedly changed their advice to clinicians, indicating that further information on the pharmacology of Act D is required. Recent pharmacokinetic studies have suggested that dosing via body surface area is not optimal, with clearance more closely correlated to body weight (Veal *et al.*, 2005; Mondick *et al.*, 2008).

Studies into the late effects of Wilms tumour therapy reveal that secondary malignant neoplasms (SMN) and cardiotoxicity are common. SMNs occur in between 4.8-7.0% of patients 30 years following treatment and this has been linked to radiotherapy and anthracyclines administration (Levitt, 2012). Anthracyclines have also been linked with cardiotoxicity, with 25% patients in one study having abnormalities during echocardiograms (Sorensen *et al.*, 1995). However, Act D has not been linked with secondary effects due to chemotherapy, with study suggesting that nephrectomy followed by vincristine and Act D treatment has helped to minimize late effects of Wilms tumour treatment (Bailey *et al.*, 2002).

1.4 Wilms tumour

1.4.1 Epidemiology

Wilms tumour or nephroblastoma is the most common renal malignancy in childhood, accounting for 90% of renal tumours in people under the age of 15,

and 6% of all paediatric cancers in the UK (CancerStats, 2010). Wilms tumour was first described in 1814 by Rance, but received its name after a surgeon, Max Wilms, identified nephroblastoma as a mixture of three tissues; blastema, stroma and epithelium (Rance, 1814; Wilms, 1899). The term “Wilms tumour” is now used loosely to describe a solid tumour arising in the kidney during childhood; however in practice these tumours can vary significantly at a pathological, clinical and genetic level.

Wilms tumour usually arises in a single kidney, and a slight predominance is seen in girls. Globally, a higher incidence of Wilms tumour is seen in the black population, 10 per million, compared to 6-9 per million in the Caucasian population, and only 3 per million in East Asian children (Stiller and Parkin, 1990; Stiller and Draper, 2005; Varan, 2008). Peak incidence occurs between 1-3 years old in the UK (http://info.cancerresearchuk.org/cancerstats).

1.4.2 Aetiology

Wilms tumour arises through three different mechanisms; sporadic formation, inherited genetic mutations or in conjunction with congenital abnormalities. Around 10-15% of patients with Wilms tumour have a positive family history (Ruteshouser *et al.*, 2008).

Some Wilms tumours arise as the result of hereditary factors. WT1, present in around 20% of sporadic tumours (Huff, 1998; Ruteshouser *et al.*, 2008), was the first gene thought to be involved in tumourigenesis, identified through patients with Wilms-Aniridia-Genitourinary anomalies-mental Retardation (WAGR) syndrome who presented with Wilms tumour. WT1 is expressed in the kidney, gonads, spleen and mesothelium, where it encodes four zinc finger

transcription factors. WT1 plays an important role in normal kidney development. It is detected early during embryonic development (Pritchard-Jones *et al.*, 1990), and $Wt1^{-/-}$ mice fail to develop kidneys (Kreidberg *et al.*, 1993). WT1 acts as a tumour suppressor gene in Wilms tumour, as patients who are heterozygous for germline mutations in WT1 have inactivated WT1 and are therefore predisposed to Wilms tumour. However, this is not universally the case, as upregulation has been described in some leukaemias, suggesting an alternative role as an oncogene (Huff, 2011).

CTNNB1, the gene encoding β-catenin, responsible for control of the cell adhesion and the WNT pathway, is also independently found to be mutated in around 15% of Wilms tumours (Koesters *et al.*, 1999). CTNNB1 and WT1 mutations are found in 79% of Wilms tumours, suggesting the possibility that a single mutation in WT1 or CTNNB1 may not be sufficient to initiate tumourigenesis (Huff, 2011). This hypothesis was strengthened by the generation of mice where Wt1 ablation was induced by tamoxifen treatment, following kidney development. In these mice tumourigenesis occurred only in combination with upregulation of insulin-like growth factor 2 (Hu *et al.*, 2011), a mutation found in patients with Beckwith-Wiedemann syndrome (BWS) who are predisposed to Wilms tumour.

More recently a third gene, WTX, has been shown to be mutated in around 20% of Wilms tumours (Rivera *et al.*, 2007; Huff, 2011). WTX function is at present unknown, but it is thought to be involved in the WNT pathway, similar to CTNNB1, promoting ubiquitination and degradation of β-catenin (Major *et al.*, 2007). A recent study has shown WTX mutations to be present in 18.4% of 125 sampled patients, and that the WTX mutation is independent of WT1 status.

Defects in WT1, CTNNB1 and WTX account for around 1/3 of the mutations found in Wilms tumour patients (Ruteshouser *et al.*, 2008; Huff, 2011).

Around 10% of Wilms tumour cases occur in conjunction with congenital abnormalities (Narod *et al.*, 1997). Wilms tumour occurs in around 4-5% of patients with Beckwith-Wiedemann Syndrome (Porteus *et al.*, 2000) with a chromosomal defect occurring at 11p15.5. A second abnormality, WAGR syndrome is associated with a chromosomal deletion at 11p13, and patients suffering from WAGR have a 30% risk of Wilms tumour (Fischbach *et al.*, 2005). Denys-Drash Syndrome is associated with a defect on the WT1 gene (Büyükpamukçu *et al.*, 1992).

1.4.3 Staging

Two major staging systems are in place for Wilms tumour, NWTS in North America and SIOP in Europe (Figure 1.1). NWTS favours surgery and staging before chemotherapy, whereas SIOP advise initial chemotherapy followed by surgery.

Stage	SIOP (after chemotherapy)	NWTS (before chemotherapy)
I	- Tumour contained in the kidney - Protrusion or bulging allowed into pelvic/ureter systems but infiltrations not allowed - Vessels of renal sinus not involved, but intrarenal vessels may be.	- Tumour contained in the kidney - Total removal - Vessels of renal sinus not involved beyond 2mm - Rupture does not occur during removal
II	- Tumour extends outside of kidney - Total removal - Capsule, adjacent tissues, renal sinus and renal vessels can be involved	- Tumour extends outside of kidney - Total removal - Local spillage and intrarenal vessel involvement allowed
III	- intra-abdominal tumour - incomplete surgical removal - renal hilus, lymph, ureteral and peritoneal involvement - peritoneal metastases	- intra-abdominal tumour - incomplete surgical removal - renal hilus, abdominal lymph nodes and peritoneal involvement
IV	- distant metastases i.e. lung, liver, bones & brain	- distant metastases i.e. lung, liver, bones & brain
V	- Bilateral tumours at diagnosis	- Bilateral tumours at diagnosis

Table 1.1. Staging systems in use in North America (NWTS) and Europe (SIOP).

Adapted from (Kaste *et al.*, 2007; Bhatnagar, 2009).

1.4.4 Treatment

Early treatment of Wilms tumour involved complete surgical resection on presentation and carried high death rates. The first planned surgery to remove Wilms tumour in 1871 was performed on an adult patient and the first nephrectomy on a child with Wilms tumour was in 1877. Radiation was added to standard therapy following evidence that pre-surgery radiotherapy helped to reduce surgery-associated deaths, and post-operatively increase cure rates (Friedlander, 1916; Prather and Friedman, 1936; Farber, 1966). As previously mentioned, in the mid-1950s Farber et al. recognised the importance of including a chemotherapeutic agent in the Wilms tumour treatment regimen,

and tested many agents until Act D was shown to greatly increase the survival of Wilms tumour patients (Farber, 1966).

Modern treatment of Wilms tumour involves a multidisciplinary approach, incorporating chemotherapy, surgery and radiotherapy, with cure rates of almost 90% (Metzger and Dome, 2005). Both SIOP and the NWTS have carried out numerous clinical trials to determine the optimal treatment regimen for Wilms tumour, however, their recommendations differ substantially. SIOP have carried out six clinical trials between 1971 and 2001 (Table 1.2). The SIOP treatment regimen is based entirely on pre-operative chemotherapy to reduce tumour mass with the aim of reducing tumour rupture during surgery. In North America, NWTS have performed five clinical trials (Table 1.3), but have not considered pre-operative chemotherapy.

SIOP trail No.	Date	No. of patients	Aims	Conclusions	Ref.
SIOP 1	1971-1974	398	- benefits of pre-operative radiotherapy - duration of post-operative chemotherapy	- pre-operative chemotherapy reduces tumour ruptures during surgery - no benefit to prolonged post-operative surgery	(Lemerle *et al.*, 1976)
SIOP 2	1974-1976	138	- confirm the findings of SIOP 1	- pre-operative chemotherapy benefits confirmed - post-operative chemotherapy should be no longer than 6 months	(Oncology, 2001)
SIOP 5	1977-1979	433	- compare pre-operative radiotherapy vs. chemotherapy	- chemotherapy is as good as radiotherapy at avoiding tumour rupture during surgery	(Lemerle *et al.*, 1983)
SIOP 6	1980-1987	1095	- duration of stage I treatment - need for anthracycline in post-operative chemotherapy - need for radiotherapy in stage II –ve nodes	- post-operative chemotherapy -18 weeks as effective as 38 weeks (92% vs. 88% 2hr DFS) - stage II +ve nodes/III – anthracycline treatment increased DFS (74% vs. 49%) - stage II –ve nodes - relapses seen in non-irradiated group	(Tournade *et al.*, 1993)
SIOP 9	1987-1991	852	- optimal pre-operative chemotherapy duration in localised tumours	- 4 weeks as good as 8 weeks - 1% vs. 3% rupture rate at surgery - 84% vs. 83% 2yr EFS - 92% vs. 87% 5yr survival rate	(Tournade *et al.*, 2001)
SIOP 93-01	1993-1999	1104	- optimal post-operative chemotherapy length	- 4 weeks as good as 18 weeks in stage I tumours	(Reinhard *et al.*, 2004)

Abbreviations- DFS; disease free survival, EFS; event free survival, +ve; positive, -ve; negative,

Table 1.2. A summary of the aims and conclusions of all SIOP WT clinical trials published to date.

NWTS trial No.	Date	No. of patients	Aims	Conclusions	Ref.
NWTS-1	1969-1975	741	- elimination of radiotherapy in low risk patients - is the combination of Act D/VCR required? - examining tumour histology	- Radiotherapy not given to low risk patients - combination therapy better than single agent - FH and UH histologic classifications	(Sutow *et al.*, 1982)
NWTS-2	1975-1979	950	- reduce treatment in low risk patients - will an anthracycline improve outcome - 50% dose for children <12 months	- 6 months as good as15 months - anthracycline improved outcome for stage II and III patients - 50% dosage in children <12 months is safe - prognosis for FH is good, UH is bad - new staging system introducing lymph nodes	(Grundy *et al.*, 1989)
NWTS-3	1979-1986	2496	- study histology vs. outcome - refine treatment to staging, prevent over-treatment - late survivor studies	- patients must be divided into FH and UH groups - Stage I FH – no radiotherapy - Stage III FH – Act D/VCR/DOX + RT - Stage II-IV UH - Act D/VCR/DOX/C	(Grundy *et al.*, 1989)
NWTS-4	1986-1995	3335	- reduce cost by decreasing clinical visits and drug treatments	- Pulse intensive method created - cost decreased	http://www.nwtsg.org/about/clinical_trials.html
NWTS-5	1995-2002	3031	- increase survival rate in children with FH - link histology to prognosis and outcome	- histology can be a factor in patients with FH - surgery alone may be adequate in children <2 years - Anthracycline could benefit children with lung metastases.	(Dome *et al.*, 2006)

Abbreviations – VCR; Vincristine, DOX; Doxorubicin, C; Cyclophosphamide, RT; radiotherapy, UH; unfavourable histology, FH; favourable histology

Table 1.3. A summary of the aims and conclusions of all NWTS clinical trials published to date.

A major benefit to post-operative chemotherapy is the ability to stage a tumour before administering chemotherapy, preventing patients with benign tumours from being over-treated and ensuring that patients with high-risk Wilms tumour are treated adequately. Patients treated on SIOP protocols receive chemotherapy before histological examination of their tumours, therefore some patients may receive chemotherapy unnecessarily. However, the potential reduction in tumour size following pre-operative chemotherapy allows for simpler surgery with a lower risk of tumour rupture and the possibility of sparing kidney tissue. Current treatment protocols for both SIOP and NWTS are summarised in Table 1.4. Although protocols by SIOP and NWTS are different, the two treatment plans report equivalent overall survival rates (Bhatnagar, 2009).

Stage	SIOP		NWTS
	Pre-operative	**Post-operative (after staging)**	
Localised	4 weeks Act D/VCR		
Metastatic	6 weeks Act D/VCR/EPI		
I		Low grade – None Int grade – 18 weeks Act D/VCR	FH/UH – 18 weeks Act D/VCR
II		-ve lymph nodes – 28 weeks Act D/VCR/EPI +ve lymph nodes - 28 weeks Act D/VCR/EPI + RT tumour bed	FH - 18 weeks Act D/VCR UH – 24 weeks Act D/VCR/DOX/C/ET + RT tumour bed and involved sites
III		+ve lymph nodes - 28 weeks Act D/VCR/EPI + RT tumour bed High grade – 34 weeks EPI/IF/ET/CARBO + RT	FH - 24 weeks Act D/VCR/DOX/ + RT tumour bed and involved sites UH – 24 weeks Act D/VCR/DOX/C/ET + RT tumour bed and involved sites
IV		Treat as per local stage + RT and excision of metastases	FH - 24 weeks Act D/VCR/DOX/ + RT tumour bed and involved sites UH – 24 weeks Act D/VCR/DOX/C/ET + RT tumour bed and involved sites

Abbreviations - VCR; Vincristine, EPI; Epirubicin, ET; Etoposide, RT; radiotherapy, CARBO; Carboplatin, DOX; Doxorubicin, C; Cyclophosphamide; IF, Ifosfamide; Int, Intermediate; +ve, positive; -ve, negative;

Table 1.4. Treatment regimens advised by SIOP and NWTS (Bhatnagar, 2009).

1.5 Rhabdomyosarcoma (RMS)

1.5.1 Epidemiology

RMS is the most common soft tissue sarcoma of childhood; classed as a small, round, blue-cell tumour (Dagher and Helman, 1999). In the UK, RMS has an incidence rate of around 16 per million, with approximately 80% of those occurring from birth to 9 years old. This makes RMS, the third most common

extra-cranial paediatric solid tumour after neuroblastoma and Wilms tumour (Stiller and Draper, 2005).

RMS tumours are derived from mesenchymal cells, which mature into numerous cells throughout the body including muscle, fibrous structures and fat (Helman and Meltzer, 2003; Garcia-Castro *et al.*, 2008). As such possible primary tumour sites for RMS are numerous, with head and neck (40%), genitourinary tract (20%), and the extremities (20%) being the most common (Dagher and Helman, 1999).

1.5.2 Aetiology

RMS can occur sporadically, or as a result of a germline familial mutation such as Li-Fraumeni syndrome associated with a p53 mutation, or with BWS (Malkin *et al.*, 1990; Li *et al.*, 1997).

RMS has two main subtypes; Alveolar RMS (A-RMS) accounting for 20%, having an alveolar like appearance, and Embryonal RMS (E-RMS) accounting for 80%, consisting of spindle cells (Stiller and Draper, 2005). A-RMS cells have been found to carry chromosomal translocations creating fusion oncogenes. The most frequently seen translocation t(2;13)(q35;q14) creates a PAX3-FOXO1 fusion gene (Galili *et al.*, 1993; Shapiro *et al.*, 1993) or the less common variant t(1;13)(p36;q14) creates a PAX7-FOXO1 fusion gene (Davis *et al.*, 1994). Both fuse a DNA binding domain (PAX) with a transactivating domain (FOXO1), thought to contribute to tumourigenesis (Linardic, 2008). In contrast E-RMS has been shown to occur through loss of heterozygosity (LOH) at 11p15.5, resulting in a loss of maternal DNA, but a duplication of paternal DNA (Visser *et al.*, 1997). However, translocations in patients with E-RMS have more

recently been reported, with the majority again involving chromosome 2 (Pressey *et al.*, 2008).

1.5.3 Staging and grouping

The Intergroup Rhabdomyosarcoma Study Group (IRSG) employs separate grouping and staging systems. Patients are separated into groups following an initial surgical procedure, depending on the extent of remaining disease before chemotherapy and radiotherapy (Table 1.5).

Group	Definition
I	- Localised - completely removed - clear margins - no lymph node involvement
II	- Localised - Mostly removed - microscopically involved margins - microscopically involved lymph nodes, mostly removed
III	- Localised - incomplete removal
IV	- Distant metastases at diagnosis

Table 1.5. The IRSG pre-chemotherapy grouping system for RMS patients

The staging system is then used to separate patients based on primary tumour site, lymph node involvement and presence of metastases (Table 1.6).

Stage	Primary tumour site	Tumour size (cm)	Lymph nodes	Distant metastases
1	- Orbit - head/neck (non-PM) - GU (non-bladder/prostate) - biliary tract	Any size	Yes/No	No
2	Any other site	≤ 5	No	No
3	Any other site	≤ 5 > 5	Yes Yes/No	No
4	All sites	Any size	Yes/No	Yes

Abbreviations- PM; Parameningeal, GU; genito-urinary,

Table 1.6. The IRSG staging system for RMS patients

1.5.4 Treatment

Before chemotherapy became a standard part of the multidisciplinary approach to treat RMS, surgery represented the sole treatment option. Current protocols are the result of four randomised clinical trials (IRSI-IV) performed by the IRSG. Following surgery and staging, the optimal combination of chemotherapeutic agents and radiotherapy was determined. A summary of these clinical trials and their findings can be found in Table 1.7.

Chemotherapy is usually given as a combination of vincristine, Act D and cyclophosphamide (VAC), but for low-risk sub-types vincristine and Act D (VA) alone are used. Chemotherapy can be given to shrink the tumour size before surgery and post-operatively. Radiation is used to treat areas where surgical removal is difficult, such as the head and neck, and to control the proliferation of any remaining cancerous cells post-surgery. Patients with RMS will undergo surgical removal of the tumour if appropriate, as determined by stage, with the aim of minimising mutilation (Dagher and Helman, 1999). Cure rates for RMS

following IRS-IV are high, with an average of 70% of patients surviving to 5 years (Crist *et al.*, 2001), compared to 55% at the start of IRS-I (Maurer *et al.*, 1988; Hayes-Jordan and Andrassy, 2009).

IRS trial No.	Date	No. of Patients	Aims	Conclusions	Ref.
I	1972-1978	686	- RT clinical benefit in Group I - Clinical benefit of C in Group II - Clinical benefit of DOX for Group III/IV patients	- RT does not benefit Group I patients - C does not benefit Group II patients - DOX does not benefit Group III patients - 55% overall 5 year survival - survival after relapse 12% after 2 years	(Maurer *et al.*, 1988)
II	1978-1984	999	- C clinical benefit in Group I/II - DOX clinical benefit in Group III/IV	- C gives no clinical benefit in Group I and II patients - DOX gives no clinical benefit - 63% overall 5 year survival (up 8% from IRS-I)	(Maurer *et al.*, 1993)
III	1984-1991	1062	- Risk-based treatment - Is 1 year therapy safe for group I/II - DOX clinical benefit in Group II - CIS and ET benefit in Group III/IV	- patients should be treated by risk stratification - 1 year therapy safe for low risk patients - DOX gives no benefit to low risk patients - intensive regimens better than pulsed regimens of IRS-II	(Crist *et al.*, 1995)
IV	1987-1997	883	- combinations of three drug therapies tested - VAC, VAI or VIE	- All combinations equally effective in patients with local/regional disease - VA and RT gives cure in Group II patients	(Crist *et al.*, 2001)
V	1997-ongoing		- to test the benefit of T or I in therapy for low risk patients	- not completed	(Raney *et al.*, 2001)

Abbreviations – VCR; Vincristine, DOX; Doxorubicin, C; Cyclophosphamide, VAC; vincristine/Act D/cyclophosphamide, VAI; vincristine/Act D/ifosfamide, VIE; vincristine/ifosfamide/etoposide, VA; vincristine/Act D, RT; radiotherapy, CIS; Cisplatin, T;topotecan, I; ifosfamide

Table 1.7. A summary of the aims and conclusions of IRS-I-IV clinical trials.

1.6 Ewing's sarcoma

1.6.1 Epidemiology

ES is an aggressive sarcoma, predominately of the bone and to some extent soft tissue. ES has an incidence of 1-3 per million in western populations, making it the second most common primary bone tumour in childhood and adolescence (Janknecht, 2005; Potratz *et al.*, 2012). The median age for diagnosis of ES is 14-15 years, however 20% of patients are over 20 years of age, and diagnosis is not uncommon in patients over 30 years (Cotterill *et al.*, 2000; Paulussen *et al.*, 2001). ES has a slight predominance in males and is found in Caucasian patients more often than in patients of Asian or African descent (Worch *et al.*, 2010). In children and adolescents the primary site of ES is commonly in the pelvis, chest wall and limbs, whereas in adults the presence of extraskeletal soft tissue ES is common (Applebaum *et al.*, 2011).

1.6.2 Aetiology

ES is a small round blue cell malignancy with >90% of ES cells positive for the CD99 surface antigen. ES cancer cells have been found to commonly harbour chromosome translocations, which are responsible for uncontrolled cell proliferation and cell division (Potratz *et al.*, 2012). The EWS-FLI1 translocation is found in 85% of ES patients (Delattre *et al.*, 1994). This involves the transactivation domain of EWSR1 gene on chromosome 22 (EWS), and a DNA-binding domain from the ETS transcription factor family on chromosome 11 (FLI1), to create the EWS-FLI1 aberrantly active transcription factor, promoting transcription and cell division. In the 15% of patients that do not contain the EWS-FLI1 translocation, 10% carry translocations involving EWS and Ets Related Gene (ERG), and translocations involving Ets translocation variant 1

(ETV1), Ets translocation variant 4 (ETV4) or Ets oncogene family (FEV) occur rarely, all creating aberrant transcription factors (Janknecht, 2005).

1.6.3 Treatment

VAC chemotherapy was added to the treatment protocol for ES in the 1970s, before which surgery and radiotherapy represented the standard approach (Jaffe *et al.*, 1976). The introduction of multiple chemotherapeutic agents to the treatment protocol has resulted in an increase in 5-year survival rates from 50% to 70% (Potratz *et al.*, 2012). The on-going clinical trials Euro-EWING 99 (Figure 1.6) and EWING 2008 dictate the treatment regimen for patients with ES. Patients are grouped by risk, determined by metastatic state and successful tumour removal following surgery. EWING 2008 addresses the issue of whether high dose chemotherapy in high risk patients affects outcome (ClinicalTrials.gov, 2009).

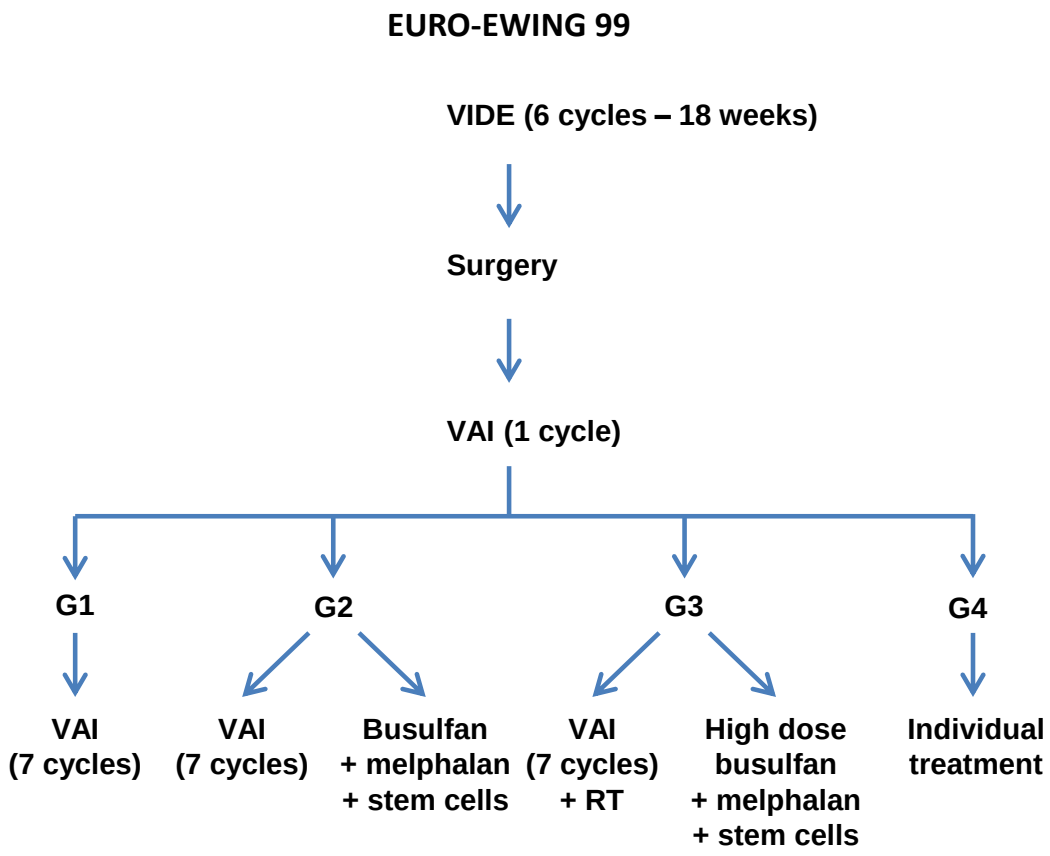

Figure 1.6. The protocol for ES patients enrolled on the current EURO-EWING 99 clinical trial.

All patients receive 6 cycles of VIDE (vincristine, ifosfamide, doxorubicin and etoposide), followed by surgery and one further cycle of VAI (vincristine, actinomycin D, and ifosfamide). Patients are then grouped (see below) and randomised depending on their grouping. Abbreviations - G1; Group 1 – successful treatment, not spread, G2; Group 2 – treatment not fully successful, total tumour removal not possible, G3; Group 3 – unsuccessful treatment and lung metastases, G4; unsuccessful treatment and metastases in lungs and other places, RT; radiotherapy (http://cancerhelp.cancerresearchuk.org/trials).

1.7 Pharmacokinetics and drug disposition

1.7.1 Introduction

Pharmacokinetics can be described in simple terms as the study of what the body does to the drug. Four processes govern the fate of a drug within the body; Absorption, Distribution, Metabolism and Excretion (ADME) (Figure 1.7).

Understanding these four processes can be very important in designing dosing regimens for patients.

Chemotherapy is an extremely important and well used tool in modern cancer care. However, not all patients fully benefit from chemotherapy, with both under and over-treatment, a common observation in cancer therapy. Pharmacokinetic studies provide an important insight into how patients are responding to treatment, and whether current treatment is appropriate. Pharmacokinetic models which take into account these processes are used to interpret pharmacokinetic data more easily, providing a useful way of characterising individual drug pharmacokinetics.

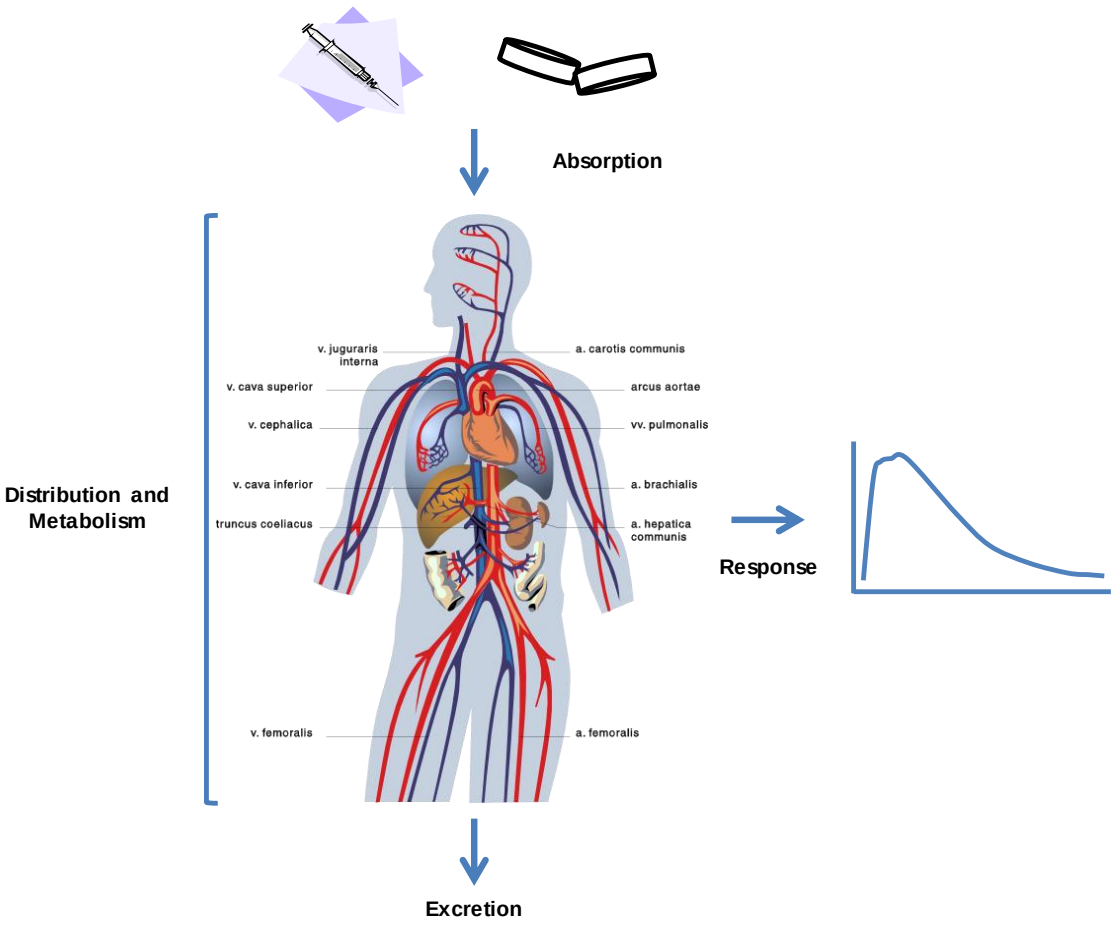

Figure 1.7. The relationship between the four processes influencing drug pharmacokinetics. Adapted from (http://www.diseasespictures.com/circulatory-system/).

Pharmacokinetics is the relationship between drug absorption, distribution around the body metabolism and subsequent drug excretion. All these processes govern the plasma concentration-time profile and thus drug response.

Clinical trials involving the collection of pharmacokinetic data are therefore important as through these potential variations between patients can be seen. Information relating to differences in pharmacokinetics between patients can be used to identify more appropriate dosing regimens for individual patients (Gibson and Skett, 2001; Katzung, 2007).

1.7.2 Pharmacokinetics of anti-cancer drugs

The standard dosage regimen for many drugs may not be adequate for all patients. Pharmacokinetic studies allow individual exposures to externally

administered compounds in patients to be investigated. Observed variations can be correlated to patient characteristics such as age and size and patient covariates such as glomerular filtration rate (GFR) and toxicity. Tailoring treatment based on pharmacokinetic studies has been shown to improve patient outcome for a number of important drugs.

Carboplatin and methotrexate are both good examples of anti-cancer drugs where treatment can be tailored based on pharmacokinetics, resulting in more patients treated within a specific therapeutic range. Individualised therapy for carboplatin has been extensively researched. Large inter-individual variation in carboplatin pharmacokinetics had been observed, suggesting that therapy could be improved by a more individualised approach to dosing (Newell *et al.*, 1987; Madden *et al.*, 1992; Murry *et al.*, 1993). The majority of carboplatin is cleared via the kidneys, and a direct correlation was seen between carboplatin clearance and renal function (Figure 1.8), resulting in therapy being adapted for the GFR of the patient (Harland *et al.*, 1984; Calvert *et al.*, 1985; Calvert *et al.*, 1989; Sorensen *et al.*, 1992; Newell *et al.*, 1993; Thomas *et al.*, 2000).

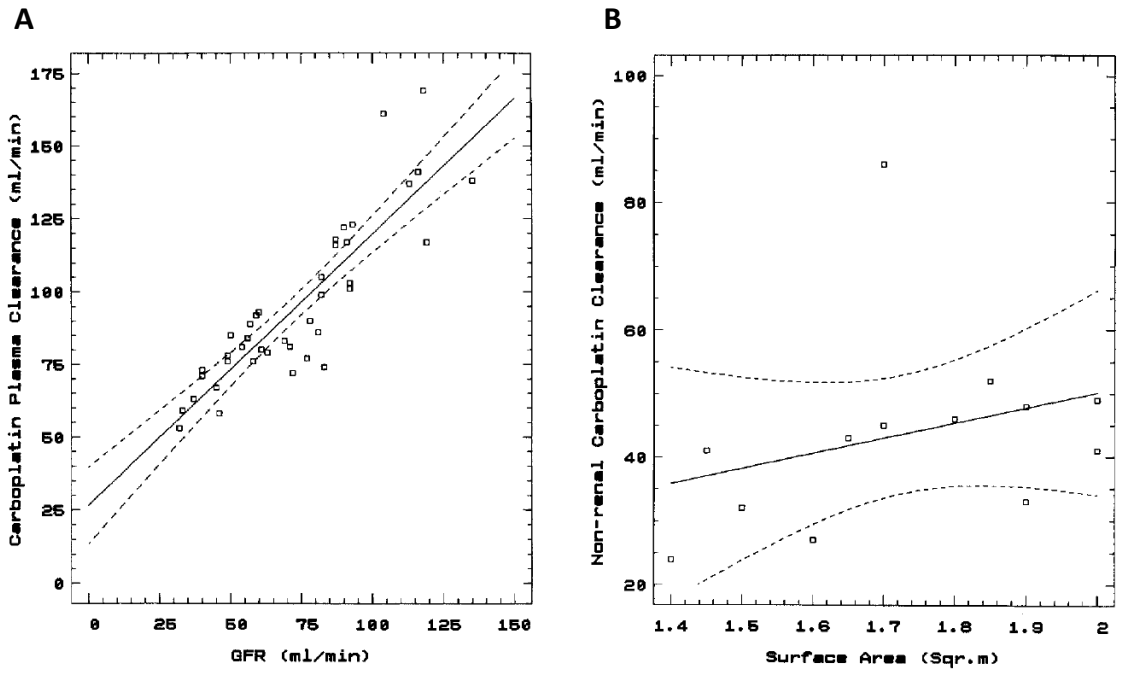

Figure 1.8. Carboplatin clearance in relation to (A) GFR and (B) body surface area (Calvert *et al.*, 1989).

Carboplatin clearance was shown to correlate better with pre-treatment GFR (r=0.875), compared to body surface area (r=0.312).

In children with ALL, a 3 to 10-fold difference in clearance of anti-cancer drugs has been reported (Rodman *et al.*, 1993). For example, in patients with ALL treated on the same conventional methotrexate (MTX) treatment regimen, low plasma concentrations and therefore a high rate of systemic clearance were associated with poor outcome (Evans *et al.*, 1986). To combat this varied outcome with conventional therapy, a clinical trial was conducted commencing in 1988, with the aim of dosing patients on the basis of their systemic clearance of the administered anti-cancer agents, rather than by body size. Previously, it had been demonstrated that steady-state plasma concentrations below 16μM during a continuous infusion of MTX were associated with an increased rate of relapse (Evans *et al.*, 1986), therefore 20μM (25% above 16μM) was set as the lowest acceptable plasma concentration. For those patients randomised to the individualised therapy arm, plasma concentrations were taken at 1 and 6h to

determine the individual rate of clearance and steady-state MTX concentration. If required, the drug infusion rate was altered to increase or decrease overall MTX exposure (target range 580-950µM.h), but the steady-state plasma concentration was not allowed to be less than 20µM. In the short-term, more patients treated with the individualised approach were within the target compared to those on conventional therapy ($p<0.001$), with only 7.7% of patients outside the target range. Overall, patients on individualised treatment required fewer courses of chemotherapy, had lower systemic toxicity and the 5 year survival in B-lineage patients increased from 66% to 76% (Figure 1.9)(Evans *et al.*, 1998).

Based on these and other examples, pharmacokinetic individualisation offers the potential to improve therapy with other drugs used in cancer treatment.

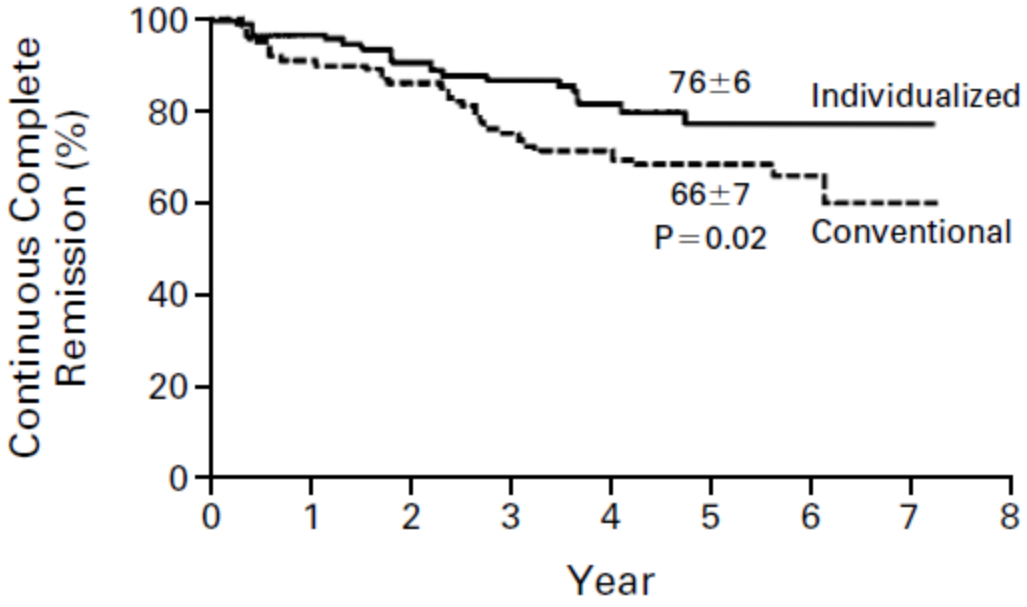

Figure 1.9. Continuous complete remission Kaplan-Meier curves comparing individualised vs. conventional administration of methotrexate.

Individualised dosing based on systemic clearance improved 5 year survival rates to 76 ± 6% compared to 66 ± 7% using conventional therapy (Evans *et al.*, 1998).

1.7.3 Pharmacokinetics of actinomycin D

Although Act D was discovered over 70 years ago, and has been used as an anti-cancer agent for over 50 years, very few studies investigating the pharmacokinetic of Act D have been published. In 1975, Tattersall et al. carried out the first pharmacokinetic analysis using ^{3}H-labelled Act D administered to 3 patients. This study showed substantial accumulation of Act D in the bone marrow, tumour samples and nucleated cells. No accumulation was seen in the brain and after 5 days 86% of the total Act D remained unmetabolised. In one patient it was demonstrated that only 34.6% of the administered Act D was found to be excreted after 5 days; 20.3% was due to renal clearance and 14.3% was due to hepatic clearance (Tattersall *et al.*, 1975). Tritiated-Act D was also administered intravenously to rat, monkey and dog animal models, with rapid depletion from the plasma and no significant metabolism observed. Total excretion was 42.6%, 31.0% and 16.9% in rat, monkey and dogs respectively, with on average 1.3-fold more elimination of the kidneys than liver (Galbraith and Mellett, 1975).

In 1977 a pharmacokinetic model for Act D in the beagle dog was published, again using ^{3}H-labelled Act D. A flow-limited model was presented, suggesting that Act D distribution was limited by blood flow rate rather than cell permeability, assuming that the concentration of Act D in tissues is in equilibrium with that in the blood (Lutz *et al.*, 1977).

However, using radioimmunoassay (RIA) and an anti-Act D antibody, Brothman et al., reported a large difference in plasma half-life between dogs (0.78min) and humans (1.78min). These data indicated the need for more investigations in patients, rather than relying on animal pharmacokinetic models (Brothman *et*

al., 1982). Further assay development led to an anti-Act D.BSA conjugate being developed for use with an ELISA (Fujiwara *et al.*, 1988).

However, a more specific assay was needed which was suitable for Act D quantification in clinical samples. All previous methods were unsuitable, either due to a lack of specificity and/or the use of a radiolabelled drug in patients. In 2003, Veal et al. published the first method suitable for quantification of Act D in human plasma, using liquid chromatography-mass spectrometry (LC/MS). The LC/MS approach provided a robust, specific and sensitive method for studying clinical samples.

Development of this LC/MS method was initiated due to concerns expressed by clinicians who desired more useful information regarding the pharmacology of Act D and its potential impact on toxicity in patients (Veal *et al.*, 2003b). Using this assay, a study of 31 patients under the age of 21 years, who received Act D as part of their standard treatment, was published in 2005. Initial plasma concentration data from these 31 patients indicated the extent of inter-patient variation in Act D concentrations in plasma (Figure 1.10). The pharmacokinetic variability observed between patients strongly indicated that current surface-area based dosing regimens are not optimal, with some patients having extremely high Act D exposures and others having markedly lower plasma levels.

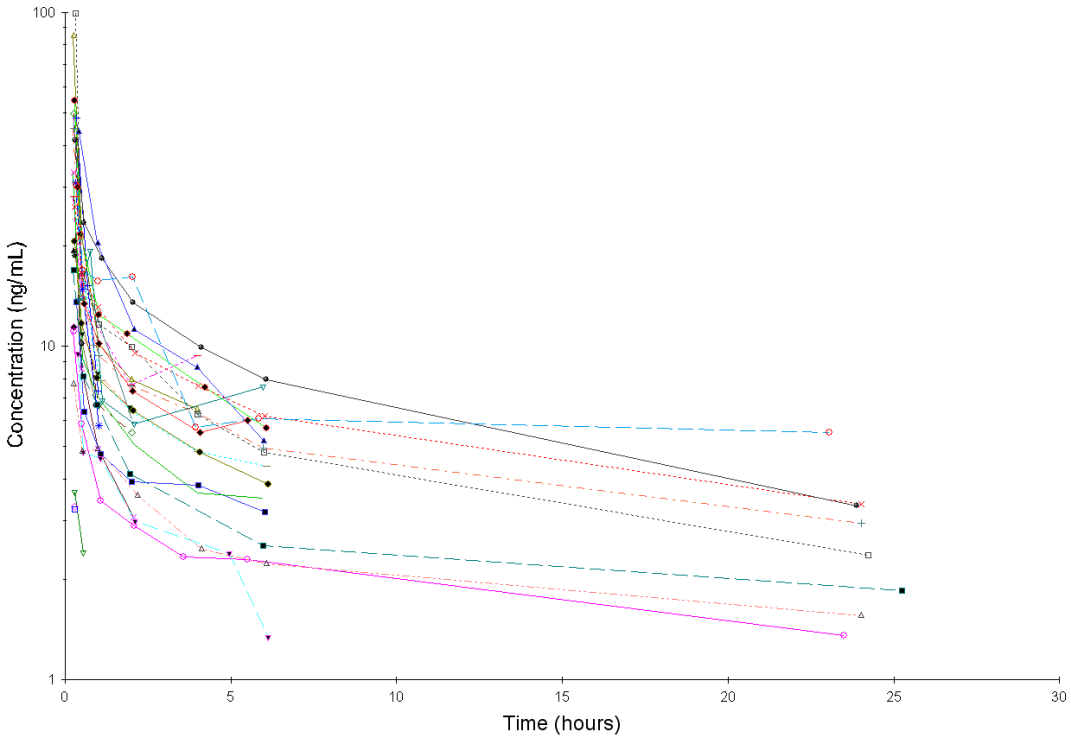

Figure 1.10. Act D plasma concentration-time profiles for 31 paediatric patients.

Blood samples were taken were between 15mins and 24h. Each individual line represents pharmacokinetic data from an individual patient (Veal *et al.*, 2005).

These published data suggested that Act D exposure is inversely related to weight, with lower exposures generally observed in larger children, a finding that may be connected with the practice of dose capping Act D at a maximum dose of 2mg. Despite the limited number of patients, these data also suggested that higher plasma concentrations may be associated with a higher risk of toxicity (Veal *et al.*, 2005). Using these data, a three-compartment pharmacokinetic model with first order elimination was proposed. Age and gender had no effect on Act D pharmacokinetics in patients, and body weight was the best descriptor of body size (Mondick *et al.*, 2008). This initial study and the proposed model were the starting point for further investigation of Act D

pharmacology; continuing recruitment in a larger population and to investigate other sources of variability.

1.8 ATP-binding cassette transporters

1.8.1 Introduction

ATP-binding cassette (ABC) transporters were discovered and extensively studied during the 1970s. Chinese hamster ovary cells that had been continuously treated with low, non-toxic concentrations of Act D conferred resistance to Act D and cross-resistance to multiple agents. It was noted that resistance was inversely proportional to the level of radiolabelled drug present in the cell nucleus. Therefore resistance appeared to be due to lower intracellular concentrations of Act D (Biedler and Riehm, 1970). Later, it was shown that accumulation of daunorubicin in cells was higher when co-incubated with vincristine and vinblastine, perhaps indicating resistance via the same saturable efflux mechanism (Dano, 1973). Subsequent work demonstrated that resistance in Chinese hamster ovary cells was due to a membrane protein alteration at the cell surface, which was not present in the wild-type (WT) cells; this 170kDa membrane component was termed “P-glycoprotein” (P-gp) (Juliano and Ling, 1976). Expression of P-gp was confirmed in mammalian cell lines resistant to colchicine, anthracyclines such as daunorubicin and cytotoxic antibiotics including Act D (Kartner *et al.*, 1983a).

The gene responsible for P-gp was first isolated from human cancer cell lines in 1986 (Roninson *et al.*, 1986), with the 4.5kb mRNA coding for P-gp found to be upregulated in human cancer the same year (Shen *et al.*, 1986). Since the discovery of P-gp, subsequently known as MDR1 or ABCB1, 48 further ABC

transport proteins have been identified in humans, and have been divided into seven subfamilies, designated A-G (Dean *et al.*, 2001; Dean, 2002), based on structure and sequence homology; of these transport proteins ABCB1, ABCC1 and ABCC2 (MRP1 and MRP2) and ABCG2 (BCRP) are the most extensively characterised. ABCB1 has twelve transmembrane regions, with two ATP binding domains, resulting in a transmembrane protein capable of exporting a wide range of hydrophobic substances against high concentration gradients (Chen *et al.*, 1986; Ambudkar *et al.*, 1999). Two mechanisms of action for ligand efflux have been proposed; these are detailed in Figure 1.11.

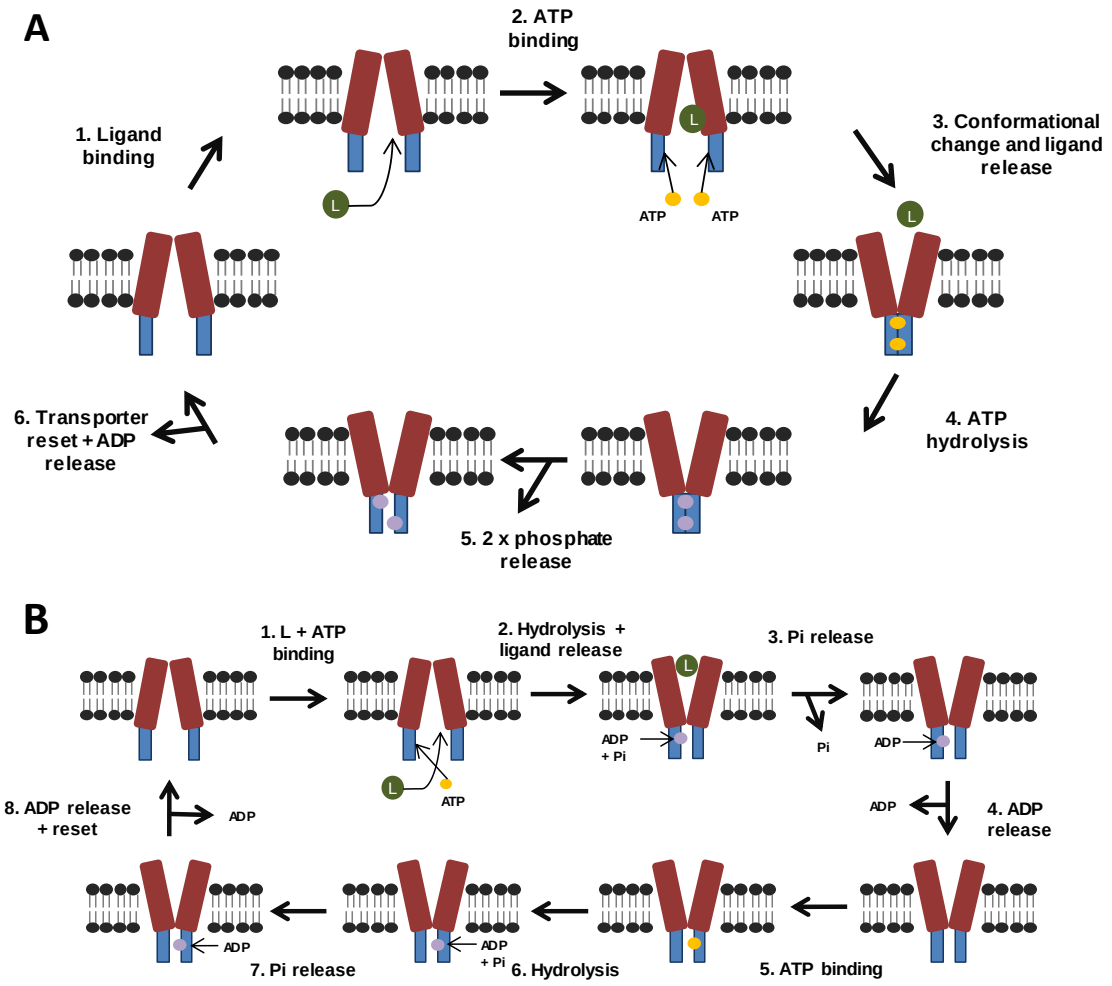

Figure 1.11. The two proposed transport cycles of ABCB1.

(A) Model proposing ATP binding causes a conformational change, causing ligand efflux. Subsequent ATP hydrolysis and phosphate release causes transporter reset. (B) Model proposing hydrolysis of one ATP molecule causes ligand efflux. Binding and hydrolysis of a second ATP molecule causes transporter resetting. Red rectangles; transmembrane substrate binding domain, Blue rectangles; ATP binding domains. Adapted from (Ambudkar *et al.*, 2006).

1.8.2 ABC transporters in normal tissues

Using a combination of mRNA and immunohistochemistry techniques, endogenous P-gp expression has been confirmed at many sites such as the liver, kidney and intestines and indeed throughout the body (Fojo *et al.*, 1987; Thiebaut *et al.*, 1987). It is now recognised that ABC transport proteins have a vital role to play in removing substances from the body. ABCB1, ABCC2 and

ABCG2 are all present at the apical membrane of hepatocytes and proximal tubule cells in the liver and kidneys, where they are thought to contribute to the elimination of substrates via the bile and urine (Figure 1.12) (Thiebaut *et al.*, 1987; Ho and Kim, 2005). These transporters are also present at the apical membrane of enterocytes, where ABCB1 in particular has been shown to prevent uptake of substrates via the intestines (Lown *et al.*, 1997). ABCB1, ABCC2 and ABCG2 are also found at many important “sanctuary sites” such as blood-brain, blood-testis, and blood-placenta barriers (Tatsuta *et al.*, 1992; Melaine *et al.*, 2002; Vahakangas and Myllynen, 2009). In contrast, ABCC1 is present at the basolateral membrane of hepatocytes and proximal tubular cells, facilitating the return of compounds to the blood (Borst and Elferink, 2002). Thus, ABC transporters have pivotal physiological roles, actively protecting the body from harmful exogenous compounds. Full expression details and notable transporter substrates can be seen in Table 1.8.

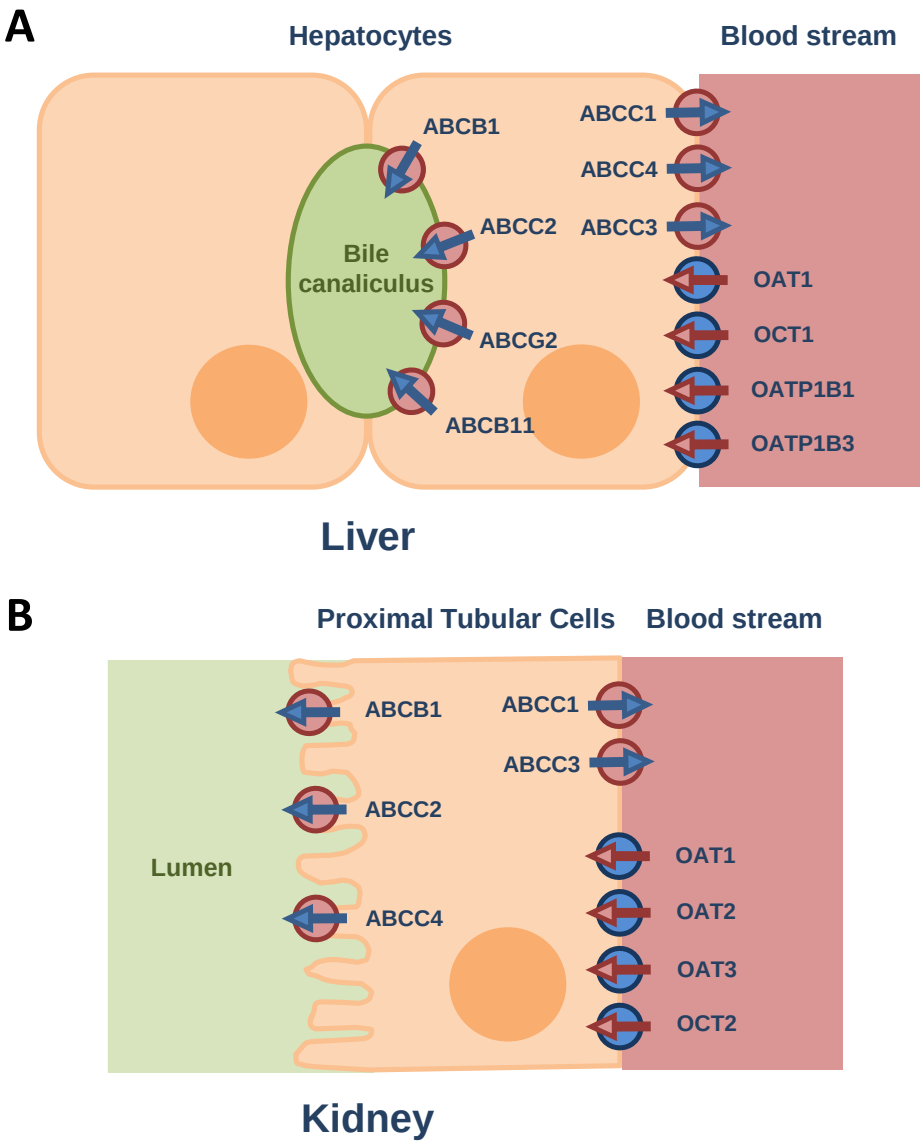

Figure 1.12. Location of important transport proteins in the liver (A) and kidneys (B).

In both the liver and kidney, ABCB1, ABCC2 and ABCG2 are present at the apical membrane actively removing substrates from the hepatocytes for biliary excretion and from the proximal tubular cells for urinary excretion. In addition, ABCC1 and ABCC3 transports substrates back into the blood stream, working against import transporters such as OAT1 and OAT2. ABCC4 is present at the basolateral membrane in hepatocytes, and the apical membrane in proximal tubular cells. Adapted from (Ho and Kim, 2005).

Transporter	Tissue distribution	Non-chemotherapy substrates	Cancer chemotherapy substrates	Ref.
ABCB1	Liver, kidney, intestine, blood-brain, blood-testis and blood-placenta barrier	Hydrophobic, neutral and cationic organic compounds. Common drugs.	Anthracyclines, actinomycin D, etoposide, vincristine, vinblastine, imatinib	(Biedler and Riehm, 1970; Dano, 1973; Fairchild *et al.*, 1987; Sehested *et al.*, 1992; Mahon *et al.*, 2003)
ABCC1	All tissues	Glutathione and other conjugates, leukotriene C4, bilirubin conjugates	Anthracyclines, vincristine, methotrexate	(Cole *et al.*, 1992; Leier *et al.*, 1994; Jedlitschky *et al.*, 1997; Zhang *et al.*, 2002; Rigato *et al.*, 2004)
ABCC2	Liver, kidney, intestine, pancreas, blood-brain barrier	As ABCC1 but preference for bilirubin conjugates. Non bile-salts organic anions.	Doxorubicin, vincristine, cisplatin, SN-38	(Taniguchi *et al.*, 1996; Chu *et al.*, 1997b; Jedlitschky *et al.*, 1997; Cui *et al.*, 1999; Faneyte *et al.*, 2004)
ABCG2	Placenta, intestine, breast, liver,		Anthracyclines mitoxantrone, topotecan,	(Schellens *et al.*, 2000; Robey *et al.*, 2003)

Table 1.8. Tissue distribution and selected transporter substrates for ABCB1, ABCC1, ABCC2 and ABCG2 specific to cancer.

1.8.3 Transport of chemotherapeutic agents by ABC transporters in animals

Since the generation of ABC transporter knockout mice, the endogenous role of ABC transporters in drug disposition and elimination has become clearer. One of the original cytotoxics used to characterise multidrug resistance (MDR) was doxorubicin. ABCB1-mediated cellular resistance to doxorubicin was first noted in MCF-7 breast cancer cells, which had been subjected to continuous low-level

exposure of doxorubicin (Fairchild *et al.*, 1987). This led to a pharmacokinetic study of doxorubicin administered intravenously in Abcb1a$^{-/-}$ mice revealing that absence of Abcb1a had a profound effect on doxorubicin disposition, with higher plasma exposure and higher tissue accumulation in the liver (van Asperen *et al.*, 1999). Altered plasma exposure to etoposide in Abcb1a/1b$^{-/-}$;Abcc2$^{-/-}$ double knockout mice has also been observed following oral and intravenous dosing, with higher plasma exposure as well as reduced urinary and biliary excretion, compared to WT mice (Lagas *et al.*, 2010).

ABCB1 has been shown to lower the bioavailability of orally administered cyclosporine by reducing absorption in the intestines in humans (Lown *et al.*, 1997). The importance of ABCB1 expression in the intestine was demonstrated in mice, where complete reversal of digoxin transport in the enterocytes was achieved by coadministration with the ABCB1 inhibitor, PSC833 (Mayer *et al.*, 1997). In the brain vinblastine accumulates 3-fold higher in Abcb1a deficient mice compared to the WT following intravenous administration, demonstrating that ABCB1 is critically important at the blood-brain barrier, preventing uptake of potential neurotoxins into the brain and central nervous system (CNS) (Schinkel *et al.*, 1994; Schinkel *et al.*, 1996).

Both *in vitro* and *in vivo* investigations are now performed routinely with old and new anti-cancer drugs, demonstrating that ABC transporters have the potential to directly impact the pharmacokinetics of anti-cancer agents. More recent investigations suggest that investigating the transport of novel agents by ABCB1 and other ABC transporters can lead to important findings, particularly as transport is often different depending on substrate. Reduced accumulation of the B-Raf inhibitor vemurafenib was seen *in vitro* in human ABCB1 and mouse

Abcg2 over-expressing cells, but an increase in plasma exposure was not observed in the subsequent *in vivo* study in Abcb1a/1b$^{-/-}$;Abcg2$^{-/-}$ mice. Instead, following intravenous dosing, vemurafenib accumulated in the brain in these mice due to the absence of Abcb1a, Abcb1b and Abcg2 at the blood-brain barrier (Mittapalli *et al.*, 2012). Similar results were seen with both the tyrosine kinase inhibitor sunitinib and its active metabolite N-desethyl sunitinib, both showing significantly higher brain exposure after intravenous dosing, with no change in plasma concentration in comparable mouse models (Tang *et al.*, 2012a; Tang *et al.*, 2012b).

1.8.4 Multidrug resistance

Modern cancer therapy, although potentially extremely effective, is limited by the development of drug resistance in tumours. Resistance can be acquired or intrinsic and is thought to occur via one or more of the following mechanisms:

- reduced drug uptake into cells
- changes to cellular drug targets rendering drugs ineffective
- increased drug efflux from cells

MDR is often caused by greater expression of ABC transporters in tumour cells, commonly ABCB1, ABCC2 and ABCG2. Increased drug efflux in resistant cancer cells results in lower drug accumulation, therefore reducing the therapeutic effect.

Determining the significance of MDR in solid malignancies has been impaired by heterogeneity of tumour tissue, the expression of ABC transporters in the

original non-cancerous tissues and different analysis techniques. However, ABCB1 has been linked to reduced response in breast cancer patients following treatment (Trock *et al.*, 1997), in addition to being present in 80% of soft tissue sarcomas following doxorubicin therapy (Abolhoda *et al.*, 1999). ABC transporters have also been found to be overexpressed in many solid malignancies, with ABCC1 and ABCC3 overexpressed in small cell lung cancer (SCLC) (Young *et al.*, 1999) and ABCG2 in paediatric hepatoblastoma (Vander Borght *et al.*, 2008).

However, perhaps the best studied example of MDR in patients comes from haematological malignancies, due to the ease of collection and examination of tumour material. Numerous studies have shown that MDR is common in patients with AML, with one reporting ABCB1 expression in 50% of AML leukaemic blasts of patients (Leith, 1998). Greater expression of the ABCB1 gene product has been shown in elderly patients (Marie and Legrand, 1999; Pallis *et al.*, 2002), and this has been linked to more inferior outcome. However, this data could be masked by the fact that younger patients with leukaemias are treated using more intensive therapy (Steinbach and Legrand, 2007). ABCB1 overexpression as a consequence of imatinib resistance has also been reported in a leukaemic cell line model (Mahon *et al.*, 2003) with this translating to an increase in ABCB1 transcripts following imatinib therapy (Galimberti *et al.*, 2005).

1.8.5 MDR reversing agents

MDR reversing agents were developed with the aim of improving the effectiveness of therapy and clinical outcome in patients who have tumours with MDR. The first generation of MDR inhibitors were known substances that were

found to be effective in reversing MDR in a cell line model, these were verapamil and cyclosporin A (CsA) (Tsuruo *et al.*, 1981). However, to achieve ABCB1 inhibition verapamil and CsA needed to be used at concentrations that were too toxic. This prompted the generation of the first compound specifically designed to inhibit ABCB1, the CsA analog PSC833 or valspodar (Twentyman and Bleehen, 1991). Valspodar is 10-fold more potent than CsA, however during a phase III clinical trial patients receiving valspodar in addition to vincristine and doxorubicin exhibited greater toxicity compared to the control arm, due to inhibition of hepatic ABCB1 and increased exposure to both doxorubicin and vincristine (Friedenberg *et al.*, 2006). Subsequent third generation inhibitors have also shown similar toxicity problems in phase III clinical trials (Shukla *et al.*, 2011).

ABCB1 reversing agents also have the potential to alter brain accumulation of substrates. This represents an attractive route for oncologists to improve penetration of anti-cancer agents across the blood-brain barrier, thereby improving the efficacy of some drugs used to treat brain tumours. The use of imatinib in the treatment of gliomas in the brain is severely limited by ABCB1 and ABCG2 removal at the blood-brain barrier. Tariquidar, a third-generation ABCB1 and ABCG2 inhibitor increased brain penetration of imatinib in mice (Gardner *et al.*, 2009). However due to the expression of both ABCB1 and ABCG2 throughout the body, this also caused a significant increase in liver and plasma concentrations increasing the treatment-associated toxicity. Additionally, brain accumulation of dasatinib, another BCR-ABL inhibitor was shown to be dramatically higher in mice treated with the third generation inhibitor elacridar (Tang *et al.*, 2011). However, in this study plasma concentration was not

effected by administration of the ABCB1 inhibitor, perhaps indicating substrate or inhibitor specific effects on pharmacokinetics.

1.8.6 Other roles for ABC transporters in cancer

ABC transporters are often present in tumours prior to chemotherapy, with expression often indicative of poor cell differentiation and prognosis. In colorectal carcinomas ABCB1 expression levels correlate with invasion and metastases, with the highest ABCB1 expression seen at the site of invasion (Weinstein *et al.*, 1991). In primary untreated neuroblastoma, high expression of ABCC1 and ABCC4 is independently associated with poor clinical outcome. Interestingly, whereas ABCC1 is known to transport drugs used in neuroblastoma therapy, ABCC4 does not (Norris *et al.*, 2005; Haber *et al.*, 2006). Another example of this phenomenon is in untreated non-small cell lung cancer (NSCLC) where, ABCG2 expression is predictive of poor prognosis, although no agent used to treat NSCLC is known to be an ABCG2 substrate (Oda *et al.*, 2005). These studies perhaps indicate a role independent of efflux, or the potential for substrates other than chemotherapeutic agents being removed from cells when ABC transporters are highly expressed, leading to tumour progression.

1.9 Pharmacogenetics

1.9.1 Introduction

Pharmacogenetics is the study of genetic variation, which influences response to drug treatment. Genetic variation has been found to occur in numerous protein classes including drug metabolising and transport proteins. The first evidence for inherited drug response was observed in 1950, when anti-malarial

drugs were found to cause haemolysis in patients deficient in glucose-6-phophate dehydrogenase (Alving *et al.*, 1956), the mechanism for which was described in 1988 (Hirono and Beutler, 1988). At around the same time, variations in renal clearance profiles of isoniazid, used to treat tuberculosis, was observed (Hughes, 1953). Patients exhibiting poor conversion of isoniazid to acetylisoniazid were more likely to suffer from common adverse reactions, and these have been termed poor acetylators (Hughes *et al.*, 1954). This was later found to be due to an ethnically diverse genetic polymorphism present in around 50% of Europeans and 10% of the East Asian population (Mitchell and Bell, 1957; Harris *et al.*, 1958; Evans *et al.*, 1960). Also in the 1950s, succinylcholine a muscle relaxant was found to be associated with a rare side effect causing prolonged paralysis following surgery (Lehmann and Ryan, 1956). This was shown to be due an autosomal recessive inherited mutation in cholinesterase, following which a biochemical test was developed to screen for the deficiency (Kalow, 1990).

Following this the first example of germline mutation in the cytochrome P450 metabolising enzyme family was identified in CYP2D6. This was found to prevent expression, the result of which was hypotension in 8% of patients administered with debrisoquine (Mahgoub *et al.*, 1977).

Since these early observations, the field of pharmacogenetics has expanded greatly, particularly with the advent of modern molecular biological techniques. Genetic variations are very common, with single nucleotide polymorphisms (SNPs) occurring every 1000-3000 bases. These variants can occur at any point along the gene, in the coding or non-coding region. SNPs in the coding region of a gene can be synonymous, where no amino acid change occurs, or

nonsynonymous, resulting in an altered amino acid sequence (Sachidanandam, 2001). It has been suggested that between 20-95% of differences in drug response between patients, may be caused by genetic variability (Scripture *et al.*, 2005). Genetic mutations can often change the expression or activity of the protein, having the potential to influence both pharmacokinetics and pharmacodynamics. A number of significant examples have been demonstrated for drugs used to treat cancer.

1.9.2 Pharmacogenetics and anti-cancer agents

Soon after polymorphisms in CYP2D6 were discovered, altered response to thiopurines in the treatment of ALL was observed. Thiopurine methyltransferase (TPMT) is an enzyme with unknown endogenous function that methylates thiopurines, preventing their conversion into toxic thioguanine nucleotides (TGNs). 6-mercaptopurine (6-MP) and its prodrug azathioprine are used in the treatment of ALL and as immune suppressant (Veal *et al.*, 2003a). 6-MP is metabolised by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) before conversion into TGNs, which are then incorporated into DNA, exerting a cytostatic effect (Bertino, 1991). TPMT competes with HGPRT to methylate thiopurines, preventing their conversion to TGNs (Figure 1.13). High concentrations of TGNs are extremely toxic to cells and can cause widespread toxicity.

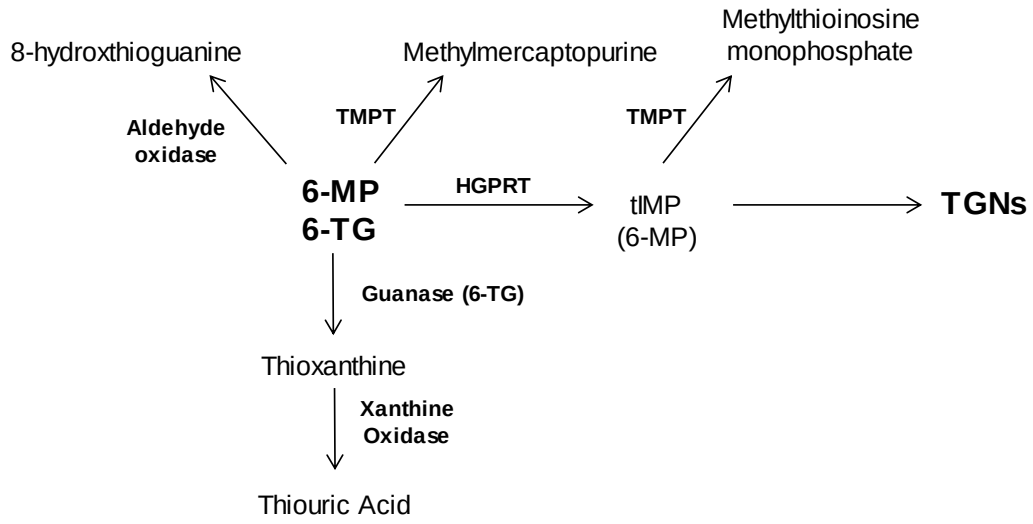

Figure 1.13. Metabolic pathway of the thiopurines 6-MP and 6-Thioguanine (6-TG).

Adapted from (Veal *et al.*, 2003a).

Caucasians can be separated into three groups according to TPMT activity: high activity (88.6%), intermediate activity (11%) and no activity (0.3%) (Figure 1.14) (Weinshilboum and Sladek, 1980). TPMT is subjected to several common SNPs which are grouped into haplotypes. The most common variant haplotype in Caucasians is TPMT*3A (Yates *et al.*, 1997), which results in amino acid changes causing a high rate of TPMT ubiquitination and proteolysis (Tai *et al.*, 1997). The lower level of TPMT in these individuals results in higher levels of TGNs and is found in around 1 in 300 people (Weinshilboum, 1984). As such, patients on 6-MP therapy with these SNPs suffer from life threatening toxicity, causing widespread myelosuppression (Ben Ari *et al.*, 1995) and hepatotoxicity (Lennard *et al.*, 1989) and therefore require individualised dose reductions, based on phenotype or genotype (Abraham *et al.*, 2006).

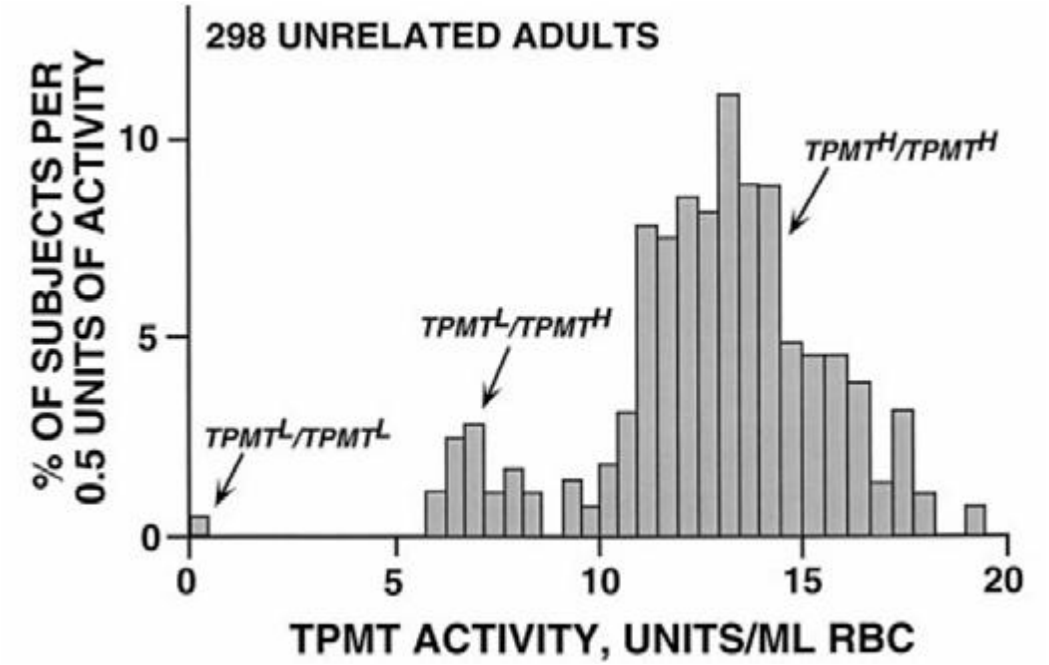

Figure 1.14. TPMT activity in red blood cells in 298 health volunteers.

The trimodal distribution of TPMT activity can clearly be seen in the Caucasian population, with high activity in homozygous wild type volunteers, intermediate activity seen in heterozygous volunteers and the lowest activity seen amongst those patients who were homozygous variant. $TPMT^{L}/TPMT^{L}$; no activity, $TPMT^{L}/TPMT^{H}$; intermediate activity, $TPMT^{H}/TPMT^{H}$; high activity (Weinshilboum and Sladek, 1980).

TPMT represents a very good example of a polymorphism that is relevant to cancer, and which has been implemented clinically. Due to the strength of the evidence, the British Association of Dermatologists (BAD) and the Clinical Pharmacogenetics Implementation Consortium have updated their guidelines for the safe and effective use of 6-MP and azathioprine (Relling *et al.*, 2011). BAD recommend that TPMT activity should be determined in each patient before administration, with patients exhibiting intermediate activity receiving a reduced dose, and patients with no TPMT activity being ineligible for treatment. Although these recommendations are in place, treatment-associated toxicities should be routinely monitored as evidence exists of variable toxicity amongst those patients with the same TPMT status (Meggitt *et al.*, 2011).

Another highly polymorphic gene that can influence drug exposure is CYP2D6, a member of the cytochrome P450 family. The cytochrome P450 family are responsible for metabolising up to 80% of all prescribed drugs (Eichelbaum *et al.*, 2006), 25% of which can be attributed to CYP2D6 (Zanger *et al.*, 2004). SNPs identified in CYP2D6 have a broad range of effects; increased or decreased expression, altered protein function, and some are still currently uncharacterised. Tamoxifen is an oestrogen receptor antagonist used in breast cancer treatment, and is metabolised to its active forms, 4-hydroxytamoxifen (4-HT) and endoxifen by CYP2D6 (Teh and Bertilsson, 2012). Tamoxifen and 4-HT exposure in patients is highly variable, with a major cause of this variation believed to be linked to CYP2D6 genotype. Impact of CYP2D6 genotype is highly dependent on race. In Caucasian post-menopausal oestrogen receptor positive breast cancer patients, those who were homozygous for the *4 genotype were shown to have shorter relapse free survival, compared to patients who were heterozygous or WT (Schroth *et al.*, 2007; Schroth *et al.*, 2009). However, in Asian breast cancer patients, the *10 genotype is associated with low 4-HT and endoxifen (Lim *et al.*, 2007; Lim *et al.*, 2011) and this is predictive of lower progression free survival and increased recurrence in the Japanese population (Kiyotani *et al.*, 2008).

1.9.3 ABC transporter genetic variation and clinical relevance

ABCB1 is a highly polymorphic gene with over 100 exonic and many more intronic SNPs. The three most common and extensively studied SNPs on the ABCB1 gene are 1236C>T, 2677G>T/A and 3435C>T. Clinical studies investigating the effect of these polymorphisms on pharmacokinetics and patient response and toxicity are numerous and are largely inconsistent,

appearing to be dependent on drug type and patient ethnicity (Tang *et al.*, 2002).

1236C>T is a synonymous SNP, however studies have demonstrated greater exposure to temozolomide, an orally administered anti-cancer agent, in patients with the CC genotype (Schaich *et al.*, 2009) and conversely patients with the TT genotype receiving irinotecan therapy experience higher irinotecan and SN-38 exposure (Mathijssen *et al.*, 2003).

Inconsistent reports are also associated with 2677G>T/A, a non-synonymous SNP causing an amino acid change from serine to threonine or alanine. At amino acid position 2677, the G allele is associated with greater efflux of ABCB1 substrate digoxin (Kim *et al.*, 2001), whilst higher exposure of cyclosporin A has been observed in patients homozygous for the variant T allele (Zhang *et al.*, 2008).

Hoffmeyer et al. first associated the TT homozygotes at allele 3435 with lower ABCB1 expression and consequently higher digoxin exposure in patients in 2000 (Hoffmeyer *et al.*, 2000). Although this is a synonymous SNP, expression of the T allele has been linked to altered mRNA and protein conformation (Kimchi-Sarfaty *et al.*, 2007), and higher mRNA turnover due to lower mRNA stability and altered protein folding structures (Wang and Sadee, 2006). As such the ABCB1 SNP 3435C>T has been the subject of extensive research. Although lower expression associated with the TT genotype was also shown in kidney epithelial tumours (Siegsmund *et al.*, 2002) and mammary and ovarian carcinomas (Sauer *et al.*, 2002), higher expression in homozygous TT patients has been reported in the intestine and placenta of Japanese healthy volunteers

(Dey, 2006). Liver expression of ABCB1 has also been shown to be unchanged with genotype (Owen *et al.*, 2005). Away from cancer, 3435C>T genotype also has variable impact on the exposure of antiretrovirals in patients. Homozygous TT patients have high exposure of nelfinavir (Zhu *et al.*, 2004), whilst in contrast patients with the homozygous CC genotype have high exposure of atazanavir (Rodriguez Novoa *et al.*, 2006).

ABCB1 SNPs have also been found to be predictive of outcome. The 3435T allele has been linked to greater overall survival in multiple myeloma patients treated with bortezomib and pegylated liposomal doxorubicin (Buda *et al.*, 2010), whilst it is suggested that the 2677A allele is predictive of lower overall survival and progression-free survival in breast cancer patients treated with doxorubicin (Bray *et al.*, 2010).

Due to the inconsistency of these reports, many studies now include haplotype analysis with 1236C>T, 2677G>T/A and 3435C>T. Studies in an Asian breast cancer patient population found that patients with one variant allele from all three common SNPs of ABCB1 (C1236T, G2677T/A and C3435T), had significantly higher exposure to doxorubicin (Figure 1.15) (Lal *et al.*, 2008). In the same study patients homozygous WT for all three SNPs had lower doxorubicin exposure (Lal *et al.*, 2008), however, single SNP analysis performed before haplotype analysis revealed limited impact on doxorubicin pharmacokinetics.

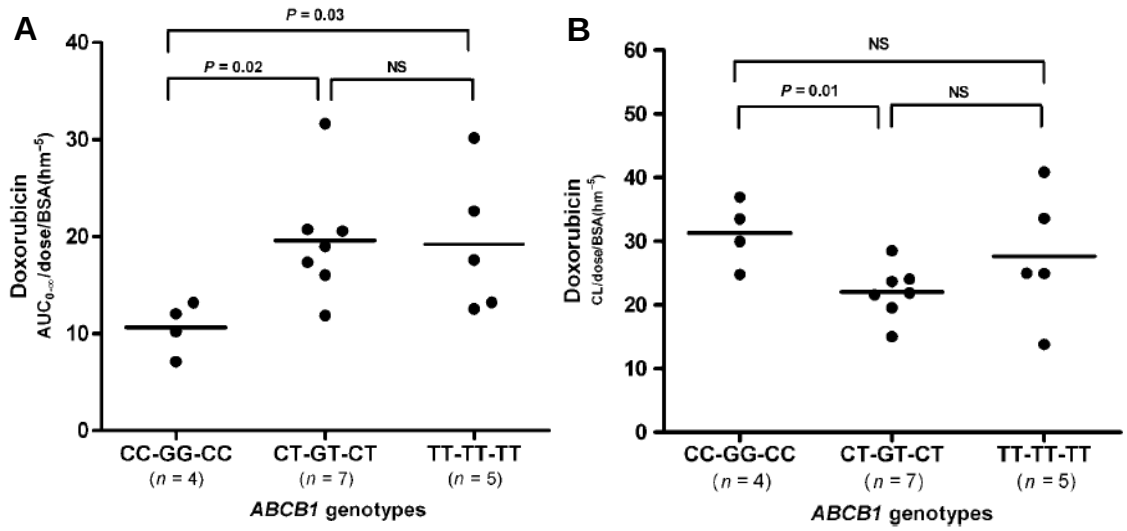

Figure 1.15. Influence of the three common ABCB1 SNPs on overall exposure and clearance of doxorubicin in Japanese patients with breast cancer.

Japanese breast cancer patients who were homozygous WT for all three common ABCB1 SNPs had (A) lower exposure than heterozygous or homozygous variant patients and (B) higher clearance than heterozygous patients (Lal *et al.*, 2008)

SNPs in other ABC transport proteins have been less well studied than ABCB1. To date, few studies regarding ABCC1 SNPs have been reported in humans. However, one study has identified 2012G>T (Gly621Val), present in around 10% of the Caucasian population, as a risk allele for doxorubicin-related cardiotoxicity in patients with non-Hodgkin's lymphoma (Wojnowski *et al.*, 2005).

A major endogenous function of ABCC2 is to facilitate the removal of bilirubin conjugates from the body. This was first elucidated in patients with Dubin-Johnson Syndrome, where loss of function of ABCC2 leads to hyperbilirubinemia. There are several SNPs in ABCC2 with potential clinical importance, however, by the far the most studied SNP is -24C>T. This is present in around 18% of the Caucasian population and is located in the 5' untranslated region (UTR), potentially influencing transcription due to its

location upstream of the ABCC2 gene. Patients with at least one copy of the -24T allele have been shown to have lower ABCC2 mRNA in the kidney (Haenisch *et al.*, 2007), however other studies have not confirmed this in either the intestine (Moriya *et al.*, 2002; Haenisch *et al.*, 2008) or placenta (Meyer zu Schwabedissen *et al.*, 2005).

Clinical studies investigating the effect of this SNP on drug disposition, have demonstrated higher AUC of the irinotecan metabolite SN-38 in patients homozygous for the TT allele (Zhou *et al.*, 2005; Fujita *et al.*, 2008). Other studies have demonstrated that an increase in the ratio SN-38:SN-38 glucuronide is indicative of lower elimination in homozygous TT patients (Han *et al.*, 2007), indicating reduced expression or function of ABCC2 associated with this SNP. In addition to the impact on the pharmacokinetics of irinotecan and its metabolites, presence of the variant allele T is associated with higher risk of diclofenac induced hepatotoxicity. This is potentially due to higher hepatic concentrations of diclofenac and its metabolites remaining in the liver, due to lower ABCC2-mediated elimination (Daly *et al.*, 2007).

1249G>A (Val417Ile), a second commonly studied ABCC2 SNP and is found in around 20% of Caucasians. It has been shown to cause lower mRNA and protein expression in some studies (Meyer zu Schwabedissen *et al.*, 2005), and changes to membrane localisation in neuroepithelial tumours have been seen (Vogelgesang *et al.*, 2004).

ABCG2 is a half transporter known to transport a wide range of endogenous and exogenous substrates throughout the body. Several SNPs have been identified within the ABCG2 gene with the potential to be clinically relevant. The

non-synonymous SNP 421C>A (Gln141Lys) is present in 12% of the Caucasian population, has been widely studied and is responsible for altered drug disposition and pharmacokinetics. The mechanism behind this is unclear, with studies reporting altered transport activity, protein stability, reduced protein expression and enhanced degradation as possible reasons for the impact of this SNP (Imai *et al.*, 2002; Kondo *et al.*, 2004; Mizuarai *et al.*, 2004; Morisaki *et al.*, 2005; Tamura *et al.*, 2007; Furukawa *et al.*, 2009).

Investigations into anti-cancer agents in children with ALL treated with ABCG2 and ABCB1 substrates have shown that those patients carrying at least one variant allele at the 421C>A position, as well as the 3435TT genotype in ABCB1, were more likely to suffer from toxic encephalopathy (Erdilyi *et al.*, 2008). Patients with the variant allele being treated with docetaxel in hormone-refractory prostate cancer, show better response and survival, perhaps due to an increase in intracellular docetaxel concentration in the tumour cells (Hahn *et al.*, 2006). Also in cancer patients administered with gefitinib, those with the variant allele had significantly higher steady-state plasma concentrations (Cusatis *et al.*, 2006; Li *et al.*, 2007), with similar results being seen with administration of rosuvastatin and sulfasalazine (Zhang *et al.*, 2006; Yamasaki *et al.*, 2008). However, as with ABCB1 genotype-phenotype relationships, the literature is highly inconsistent. Studies involving nitrofurantoin, lamivudine, indinavir and zidovudine have all failed to show any impact on drug disposition and pharmacokinetics (Anderson *et al.*, 2006; Adkison *et al.*, 2008). Heterozygotes at amino acid position 421 have higher oral bioavailability of diflomotecan and topotecan than homozygous WT patients. However, as the pharmacokinetic profile for irinotecan is unchanged between the genetic groups

(Sparreboom *et al.*, 2004; Sparreboom *et al.*, 2005; Han *et al.*, 2007), the effect of this SNP appears to be substrate specific.

Another SNP which is found frequently in the Asian population, 34G>A, was found to exhibit decreased expression of ABCG2 *in vitro* (Mizuarai *et al.*, 2004), however, this appears to have not translated into a related finding *in vivo* as studies have shown no link between 34G>A genotype and pharmacokinetic parameters (Kondo *et al.*, 2004).

1.10 Summary and Aims

While Act D plays a key role in the successful treatment of paediatric malignancies including Wilms tumour, RMS and ES, its use could be further improved. Although relatively rare, severe hepatotoxicity in the form of VOD, is a major concern for clinicians when treating patients with Act D. While it is still not clear why some patients develop VOD, links to patient age and disease have been suggested. Therefore current aims are to maintain high survival rates, whilst reducing treatment-related toxicities.

Previous pharmacokinetic studies have shown the extent of inter-individual variation in Act D exposure, indicating that current surface area-based dosing is not optimal. Continued pharmacokinetic evaluation is needed with a particular emphasis on a greater number of early (5min) and late (24-26h) samples to more fully characterise Act D pharmacokinetics and to identify key factors determining Act D exposure. Additional data are also required to further investigate the potential correlation between high plasma concentrations in patients and toxicity.

ABC transport proteins are present at vital cell membranes throughout the body, and as such have the potential to markedly influence drug disposition and elimination. Studies in mice have shown that systemic removal of ABC transport proteins significantly alters the pharmacokinetic profile and tissue distribution of many anti-cancer agents, however, this is highly substrate and transporter specific. ABC transporters are highly polymorphic, and various SNPs have been shown to potentially alter protein expression and function. Clinical studies involving patients receiving ABC transporter substrates as part of their standard chemotherapy regimen have shown a link between ABCB1 genotype and drug exposure or clinical outcome. Whilst the literature is sometimes contradictory regarding clinical association and ABC transporter genotype, this represents a potentially interesting prospect for studies with Act D.

The aims of this project are:

1. To determine Act D transport *in vitro* using MDCKII cell-lines over-expressing the ABC transporters, ABCB1, ABCC1, ABCC2 and ABCG2.
2. To determine the effect of ABC transporters on Act D pharmacokinetics and tissue accumulation *in vivo,* using relevant ABC transporter knockout mice.
3. To determine the pharmacokinetics of Act D in an expanded paediatric patient population, with a wider range of sampling time points, and to investigate potential correlations between plasma concentrations and clinical endpoints.
4. To investigate pharmacogenetic variation in ABC transport proteins relevant to Act D pharmacology, correlating genetic variations with pharmacokinetic data, clinical response and toxicity.

Chapter 2. *In vitro* transport in ABC-transporter over-expressing cell lines

2.1 Introduction

Since its introduction into the standard treatment regimen in 1960 (Fernbach and Martyn, 1966), very few detailed pharmacokinetic studies have been performed with Act D. In the only pharmacokinetic study involving significant patient numbers to date, 31 children receiving Act D demonstrated that whilst treatment is generally well tolerated, it is associated with a large variation in exposure between patients on similar dosing regimens.

ABC transporters are present throughout the body, removing a wide range of substrates from cells. ABCB1, ABCC2 and ABCG2 are all present on the apical membrane in liver, kidney and intestine, facilitating the elimination of substrates via the bile, urine and faeces (Ho and Kim, 2005). ABCB1 is also found at the blood-brain barrier, blood-testis barrier, and the blood-placenta barrier, protecting vital sites from harmful toxins (Schinkel *et al.*, 1996; Melaine *et al.*, 2002; Vahakangas and Myllynen, 2009). In contrast, ABCC1 is present on the basolateral membrane, transporting substrates into the blood (Borst and Elferink, 2002).

Many anti-cancer agents such as doxorubicin (Fairchild *et al.*, 1987; Ueda *et al.*, 1987) and etoposide (Guo *et al.*, 2002; Allen *et al.*, 2003) are substrates for ABC transporters. The impact of ABC transporters on drug exposure by influencing drug absorption and elimination could be clinically relevant. Since the initial work undertaken to characterise ABCB1 (Biedler and Riehm, 1970; Juliano and Ling, 1976), no detailed confirmatory studies have been carried out

to confirm Act D as a substrate for ABCB1 and it is unclear whether Act D is a substrate for any other major ABC transport proteins.

To predict the potential impact of drug transporters on Act D exposure in patients, Act D transport was investigated *in vitro* in MDCKII cell lines, stably over-expressing human ABCB1, ABCC1, ABCC2 or ABCG2. Using these cell lines, Act D growth inhibition (GI) studies, intracellular accumulation assays, cellular efflux assays and fluorescent competition assays were developed and utilised. In addition expression and function of the appropriate ABC transporters in MDCKII cell lines was tested using western blotting and known, well-studied ABC transporter substrates such as doxorubicin and mitoxantrone.

2.2 Materials and methods

2.2.1 Materials

Drug transporter inhibitors KO143 and MK571 were supplied by Tocris Bioscience (Bristol, UK), methanol and ethanol were supplied by Fisher Scientific (Loughborough, UK) and all other chemicals were supplied by Sigma-Aldrich (Dorset, UK) unless otherwise specified.

2.2.2 Cell lines

WT polarized Madin-Darby canine kidney (MDCKII) cell lines and those stably transfected with human ABCB1 (MDR1/P-gp) (Schinkel *et al.*, 1993; Schinkel *et al.*, 1995; Evers *et al.*, 1997), ABCC1 (MRP1) (Evers *et al.*, 1997), ABCC2 (MRP2) (Evers *et al.*, 1998) and ABCG2 (BCRP) cDNA (Pavek *et al.*, 2005), were kindly provided by Dr A.H. Schinkel (Amsterdam, the Netherlands). These cell lines were used for growth inhibition, intracellular accumulation, cellular efflux, and fluorescent competition assays.

2.2.3 Culture of cell lines

Cells were cultured, in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 2mM L-glutamate, 2mM penicillin, and 2mM of streptomycin, at 37^{o}C, 5% CO_2 in a humidified incubator, and were routinely screened for mycoplasma. Cells were grown as a monolayer and were passaged at approximately 80% confluency, using 2xTrypsin-EDTA.

2.2.4 Western Blotting

Cells were seeded at 25,000 cells per well in 6-well plates and were left for between 1-4 days to establish growth. Cell lysates were extracted and protein

concentration determined. Cells were lysed and scraped from 6-well plates in lysis buffer (62.5mM Tris-HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS)) containing 1x protease inhibitor (Thermo-Scientific, Rockford, USA) and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo-Scientific, Rockford, USA). Cell lysates were diluted 1:20 in lysis buffer, and 10µl of each sample was loaded in quadruplicate onto a 96-well plate. Once all samples had been loaded, 190µl of assay buffer (50:1 mixture of buffers A:B) was added to each well using a 12 lane multichannel pipette. Plates were then incubated for 30 min at 37^{o}C, 5% CO_2 in a humidified incubator. After 30 min, the plates were removed from the incubator, and absorbance was measured at 562nm using a BMG Labtech FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK). Protein concentrations were determined using a standard curve of bovine serum albumin at concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2mg/ml.

Depending on protein concentration, samples were diluted and 20µg of lysate was added to a 1.5ml capped vial with 1x NuPage LDS sample loading buffer and 1/10 β-mercaptoethanol. Samples were briefly mixed by vortex and pulse spun then incubated at 98^{o}C for 10 min to denature the protein, and kept at on ice if being used the same day, or stored at -20^{o}C until needed. Samples were loaded onto 4-20% Tris-Glycine gels (Life Technologies, Paisley, UK), with 1x running buffer (25mM Trizma base, 0.19M Glycine with 0.1 % SDS) using SeeBlue Plus2 Pre-Stained Standard as a molecular marker (Life Technologies, Paisley, UK). Gels were run in an Invitrogen XCell *SureLock*™ Novex Mini-Cell with a Bio-Rad Powerpac 300 (Hemel Hempstead, UK) at a constant 150v for approximately 1.5 h, until the dye had reached the bottom of the gel.

Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Before use, PVDF membranes were soaked in methanol for 15s, soaked in double-distilled water (ddH_2O) for 2min and then stored in transfer buffer (25mM Trizma base, 0.19M Glycine, 20% methanol) until required. For protein transfer, a Bio-Rad Mini-PROTEAN 2-D Electrophoresis Cell using a Bio-Rad Powerpac 300 was used at a constant 30v overnight. Membranes were then blocked in 1x TBS/T (20mM Trizma Base, 140mM NaCl, pH 7.6 with 0.1% Tween20) with 5% milk (5% w/v, Marvel, Lincolnshire, UK) for 2 h with gentle agitation. Following membrane blocking, the membrane was incubated with the appropriate antibody at the appropriate concentration in blocking buffer overnight at 4^{o}C, with gentle agitation (Table 2.1).

Protein	**Antibody**	**Concentration**	**Expected molecular weight (kDa)**
ABCB1/MDR1/P-gp	C219, Abcam, Cambridge, UK	1:200	170
ABCC1/MRP1	MRPm6, Enzo Life Sciences, Exeter, UK	1:1000	190
ABCC2/MRP2	M2-III-6, Kamiya Biosciences, Seattle, USA	1:1000	170-180
ABCG2/BCRP	BXP-21, Kamiya Biosciences, Seattle, USA	1:1000	70 (half transporter)
Actin	Ab3280, Abcam, Cambridge, UK	1:25000	42

Table 2.1. Antibodies for western blot analysis

Optimised concentrations for an overnight incubation at 4^{o}C in blocking buffer.

Following overnight incubation with primary antibody, the membrane was washed three times in 1xTBS/T to wash off any unbound primary antibody. After washing, membranes were incubated with the secondary antibody, goat anti-mouse conjugated to horse radish peroxidase (P0447, Dako, Cambridge) in 1x

TBS/T with 5% milk at a 1:1000 dilution, with gentle agitation for 2h. The membrane was then washed three times in 1x TBS/T for 10min each, removing unbound secondary antibody. To detect bound secondary antibody, enhanced chemiluminescence + (ECL+) (GE Healthcare, Buckinghamshire, UK) was used, and the membrane was covered for 5min. Excess ECL+ was then removed and the membrane was imaged with a Fujifilm LAS3000 using Fujifilm Image Reader version 1.1 (Billingham, UK). Genetools version 4.01 was used to perform densitometry on the migrated proteins (Syngene, Cambridge, UK). Transporter expression was compared to actin expression for each lane and the output termed “relative protein expression”.

2.2.5 Growth curve with MDCKII cell lines

Growth curves were carried out to determine an appropriate density to seed MDCKII-WT, MDCKII-ABCB1, MDCKII-ABCC2 and MDCKII-ABCG2 cells for a 96h growth inhibition investigation, to ensure that the cells would be in the exponential phase of growth. To compensate for the edge effect, 200µl ddH_2O was first added to the outer wells of the 96-well plates. Following this, the cell count of each cell line was determined using a Neubauer haemocytometer supplied by Hawlsey (Lancing, UK) and cells were seeded at varying densities (250 – 40,000 cells per well) in 96-well plates, with four identical plates being set up for each cell line on day 0. Subsequently, one plate per cell line was fixed at 24, 48, 72 and 96h by removing medium and replacing with 250µl 0.6mM TCA in phosphate buffered saline (PBS) and placing at 4^oC. After 1h, the TCA was removed and the plates were washed with water, and stored at 4^oC until all time points had been completed. The plates were stained by adding 100µl of 0.4% sulfarhodamine B (SRB) to each well for 1h, washed in 1% glacial acetic

acid and allowed to dry overnight. Once dry, the stain was solubilised in 100µl 10mM Tris (pH 10.5) (Skehan *et al.*, 1990) and absorbance readings were taken using a Spectramax 250 microplate reader at 570nm (Molecular Devices, USA).

2.2.6 Drug stocks and dilutions

Act D stocks were made up in methanol, verapamil stocks were made up in ddH_2O, and all other stocks (doxorubicin, mitoxantrone, SN-38, MK571 and KO143) were made up in dimethyl sulfoxide (DMSO). Dilutions were carried out to ensure the maximum concentration of methanol, ddH_2O or DMSO in media on the cells was 1:500 (0.2%) for all experiments and all experiments were run with a solvent control to ensure any solvent effect would be detected.

2.2.7 Growth Inhibition

Growth inhibition (GI) assays were carried out in 96-well plates. According to growth curves, all cells were seeded at a density of 4,000 cells/well on day 0, and allowed to establish growth over 24h, with 200µl ddH_2O added in all outer wells to compensate for the edge effect. Cells were treated with varying concentrations (0-50µM) of an appropriate drug (Act D, doxorubicin, mitoxantrone or SN-38) in triplicate on day 1 in the presence or absence of the appropriate transporter inhibitor; verapamil (1µM) for ABCB1, MK571 (25µM) for ABCC1/2 and KO143 (0.2µM) for ABCG2. Drug, verapamil and MK571 concentrations were chosen after initial GI experiments were carried out to determine half-maximal inhibitory concentration (IC_{50}) values for the various agents in these cell lines, and 0.2µM was chosen for KO143 from concentrations used in the literature (Allen *et al.*, 2002). Following a 72h drug

incubation, Promega tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) substrate (12µl) was added and plates were returned to the incubator for 2-4h. The plates were read on a Spectramax 250 (Molecular devices, USA) at 490nm and results reported as a percentage of growth inhibition compared to cells incubated with medium alone.

2.2.8 Intracellular accumulation of actinomycin D in MDCKII cell lines

Initial experiments of various seeding densities in 6-well plates determined that 25,000 cells per well ensured that the cells were approximately 80% confluent after 4 days growth and therefore still in exponential phase of growth. All cell lines were seeded at 25,000 cells per well in 6-well plates on day 0 and left to establish growth over 4 days. On day 4 individual plates of cells were incubated with 0.01-2µM Act D alone for up to 12h to determine a dose which did not cause cellular toxicity following a 12h incubation. Experiments were then carried out over 12h with 0.01µM Act D, and over 4h with 0.1µM Act D (to ensure detectability on the LC/MS) and increasing concentrations (0-25µM) of the appropriate inhibitor (2.2.7). After the defined time course, medium was removed and the cells were washed twice with 1ml ice-cold PBS to remove excess Act D and to prevent any further ABC-mediated drug transport. Methanol (200µl) was added to each well and cell scrapers used to collect cells. Intracellular Act D concentrations in cell lysates were then determined by LC/MS (0).

2.2.9 Cellular efflux of actinomycin D in MDCKII cell lines

For efflux experiments, cell growth was established as per 2.2.8 and cells were incubated with 0.1µM Act D for 4h at 37^{o}C, 5% CO_2 in a humidified incubator. The medium was then replaced with medium containing no drug and intracellular levels were determined at 0, 1 and 2h to determine rate of drug efflux. Following completion of incubations, intracellular Act D was extracted as per 2.2.8 and concentrations were determined (2.2.10.3).

2.2.10 Analysis of actinomycin D by liquid chromatography-mass spectrometry in cell extracts

2.2.10.1 Chemicals

HPLC-grade solvents were supplied by Fisher Scientific (Loughborough, UK), concentrated ammonia was supplied by BDH (Dorset, UK).

2.2.10.2 Preparation of working standards

Act D was weighed and dissolved in methanol to obtain a 1mg/ml solution. The 1mg/ml working standard stock solution was used to make a 1µg/ml solution in methanol, and from this the following working standards were prepared in methanol: 10, 5, 2, 1, 0.5 and 0.25ng/ml. Working standards were stored at -20^{o}C until needed.

2.2.10.3 Preparation of samples for analysis via liquid chromatography-mass spectrometry

Full LC/MS assay validation was performed with human plasma and can be seen in 4.2.3. Prior to performing intracellular accumulation and efflux experiments (as described in 2.2.8 and 2.2.9), extraction of Act D was attempted from cells in suspension in tissue culture medium. This caused ion

suppression with no Act D peak visible. Moving to a methanol based extraction method resulted in consistent and reliable Act D peaks and standard curves. Comparing chromatograms between Act D in human plasma, methanol or cell lysates in methanol all retention times are comparable (3.84-3.87min, Figure 2.1) and the method was deemed acceptable for use in the *in vitro* setting.

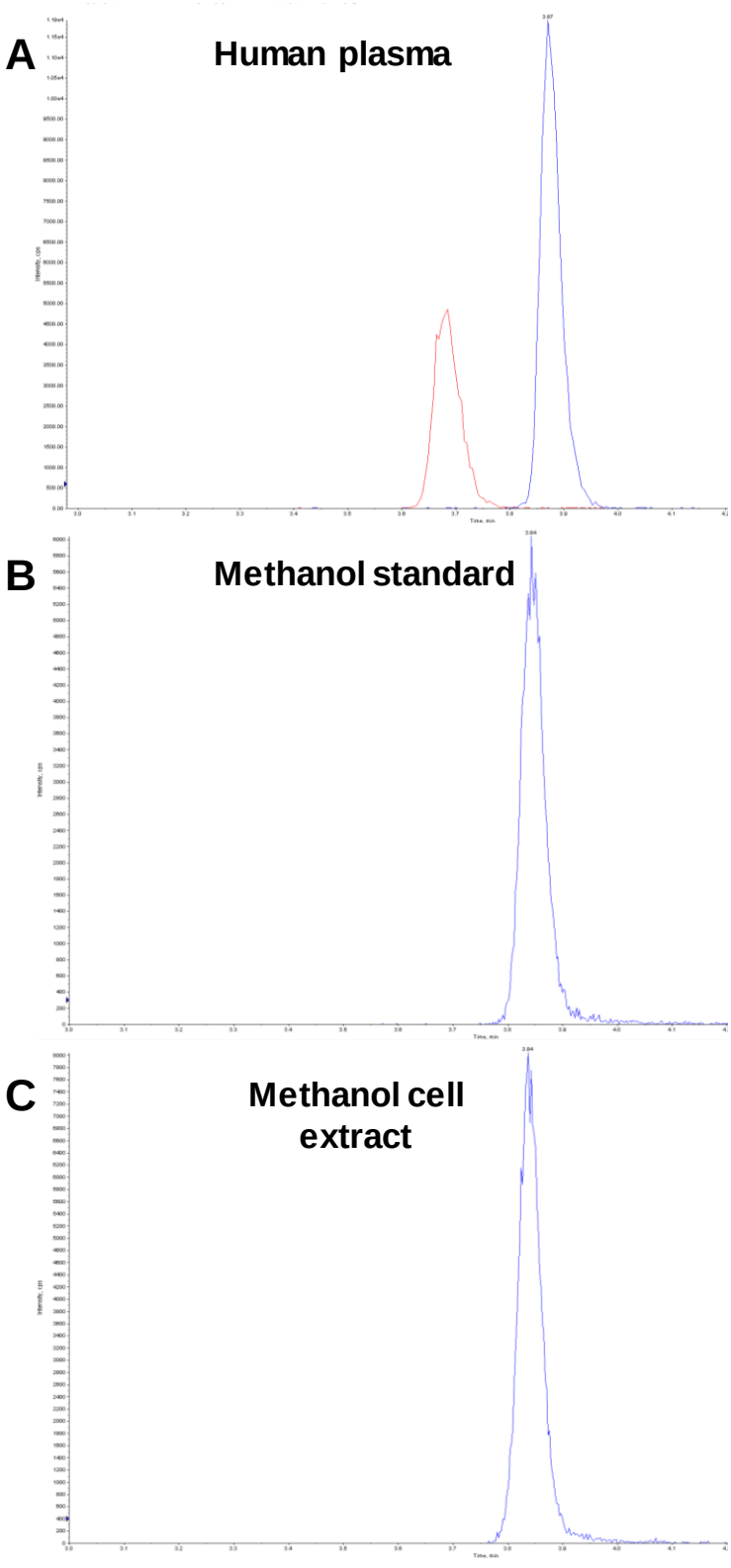

Figure 2.1. Comparisons between chromatograms of Act D (A) extracted from human plasma, (B) in methanol and (C) in methanol from cell lysates.

The retention time of Act D in (A) human plasma (3.87min), (B) methanol (3.84min) and (C) methanol and cell lysates (3.84min) are all comparable between matrices.

Therefore cell lysates in methanol were centrifuged at 15,300g to remove cell debris and appropriately diluted in methanol to give a 100µl sample. Before injection onto the LC/MS 100µl 1% acetic acid pH 4 was added to all samples. For each separate LC/MS run, a standard curve in methanol (2.2.10.2) was run in duplicate before the cell samples, in addition to a low, medium and high standard being placed at the end of each run to ensure consistency. Standards were back calculated from the equation of the standard curve, with an allowed error of ± 15%.

2.2.10.4 Liquid chromatography-mass spectrometry method

An API4000 LC/MS/MS (Applied Biosystems, California, USA) was used for analysis attached to a Series 200 micropump, autosampler and Peltier column oven (Perkin Elmer Ltd, Beckonsfield, UK). A Luna 3µ C_8 (2) Mercury column (20 x 4mm) fitted with a security guard cartridge C_8 (4 x 2mm) was used (Phenomenex, Cheshire, UK). The gradient was set as shown in Table 2.2, with a 0.5ml/min flow rate and an injection volume of 50µl.

Time (min)	A (%)	B (%)
0.0	40	60
2.0	40	60
3.0	0	100
6.0	0	100
10.0	40	60

Table 2.2. Liquid chromatography conditions for Act D and 7-aminoactinomycin D separation.

A: acetate buffer (1% acetic acid, adjusted to pH4 using strong ammonia), B: 100% methanol.

Turbo spray ionisation was performed in positive ion mode with nitrogen gas at the following optimal settings; collision gas, 3, curtain gas, 10, ion source gas 1, 85 and ion source gas 2, 60. The ion spray voltage was set at 5500 with an interface temperature of 450^{o}C. Optimal mass-spectrometry conditions for Act D and 7-aminoactinomycin D (7-AD) can be found in Table 2.3.

Analyte	Retention time (min)	Q1 mass (Da)	Q2 mass (Da)	Declustering Potential	Entrance Potential	Collision Energy	Collision Exit Potential
Act D	3.8	1255.79	857.60	136	10	47	26
7-AD	3.6	1270.77	872.70	136	10	49	26

Table 2.3. Mass spectrometer gas and temperature settings for Act D and 7-AD

2.2.11 Inhibition assay using fluorescent transporter substrates

Fluorescence inhibition assays were carried out in black, clear bottom 96-well plates (Greiner Bio-One, Stonehouse, UK). MDCKII-WT and MDCKII-ABCB1 cells were seeded at 1,000 cells per well, to ensure low confluency for easy imaging, and allowed to establish growth over 4 days. Cells were then pre-incubated with varying levels of Act D (0 – 100µM) for 1h. The fluorescent ABCB1 substrate doxorubicin (10µM) was added for a further 1h, to assess doxorubicin efflux in the presence of Act D. Hoechst 33342 was used as a nuclear stain once cells had been formalin-fixed and permeabilised with 0.1% Triton-X. Cells were then imaged for doxorubicin fluorescence on a BD HT Pathway (BD Biosciences, Oxford, UK). The excitation wavelength for doxorubicin was 488nm and a 515nm long-pass filter was used to monitor emitted fluorescence.

2.2.12 Statistical analysis

For the growth inhibition, intracellular accumulation and cellular efflux assays it was not possible to determine whether the data was either normally or non-normally distributed as there is only 3 data points per time point or drug concentration. Non-parametric analysis such as the Mann-Whitney U test or the Kruskal-Wallis one-way analysis of variance (ANOVA) cannot be performed on data sets that contain only 3 repeats each, and will result in an inaccurate significance being reported (Mundry and Fischer, 1998).

Therefore for GI assays, drug concentrations were log-transformed and non-linear regression was performed, with upper limit set to 100% and lower limit set to 0%. An F-test was then used to compare GI_{50} values. For intracellular accumulation and cellular efflux assays, the intracellular concentrations were log-transformed if variances were not equal, to achieve equal variances. Following this, two-way ANOVAs were performed and multiple comparisons were used to compare all cell lines against the MDCKII-WT parental cell line at each time point or concentration. Bonferroni's correction was used to correct for the number of these tests performed. Multiple comparisons were not used to compare differences between time points or inhibitor concentrations in the same cell lines. The significance reported is therefore under the assumption that if more data were generated, it would be normally distributed, which is highly likely when performing repeats of experiments under identical conditions. GraphPad Prism 6.00 was used for all statistical analysis, following Bonferroni's correction $p<0.05$ was accepted as statistically significant.

LC/MS data were quantitated using Analyst Software 1.4.2 (Applied Biosystems), with optimal settings as follows: Act D, peak splitting factor, 2

smoothing width, 3 and for 7-AD, peak splitting factor, 2 smoothing width, 11. The standard curve was set to linear, and was fitted automatically by the software, and from this percentage accuracies and unknown sample concentrations were calculated.

2.3 Results

2.3.1 Confirmation of transporter expression in MDCKII cell lines

ABC transporter expression was confirmed in each MDCKII cell line by western blot analysis. As subsequent assays would be carried out over 4 days of cell growth, the expression of ABCB1 was initially investigated over 4 days in MDCKII-WT and MDCKII-ABCB1 cell lines. ABCB1 was detected in all MDCKII-ABCB1 samples, and was uniform over 3 days (2.01-2.12 relative expression, Figure 2.2B). Minimal levels of ABCB1 were detected in MDCKII-WT, due to the endogenous expression of canine ABCB1 in these cell lines (0.01-0.03 relative expression, Figure 2.2A). Densitometry performed on the day 4 sample of MDCKII-ABCB1 suggested a lower expression after 3 days proliferation, however due to the corruption of the migrated protein band this result is not reliable. Western blotting did not improve following repeats.

As consistent expression of ABC transporters was observed in MDCKII over 4 days, subsequent transporter expression analysis was carried out after 4 days proliferation. Again, ABCB1 expression was confirmed in the MDCKII-ABCB1 cell line, with analysis in ABCC2 and ABCG2 over-expressing cell lines (Figure 2.3) revealing comparable ABCB1 expression to the parental in MDCKII-ABCC2 cell line (0.12 vs. 0.14 relative expression), but lower expression in the MDCKII-ABCG2 cell line (0.02 vs. 0.12 relative expression).

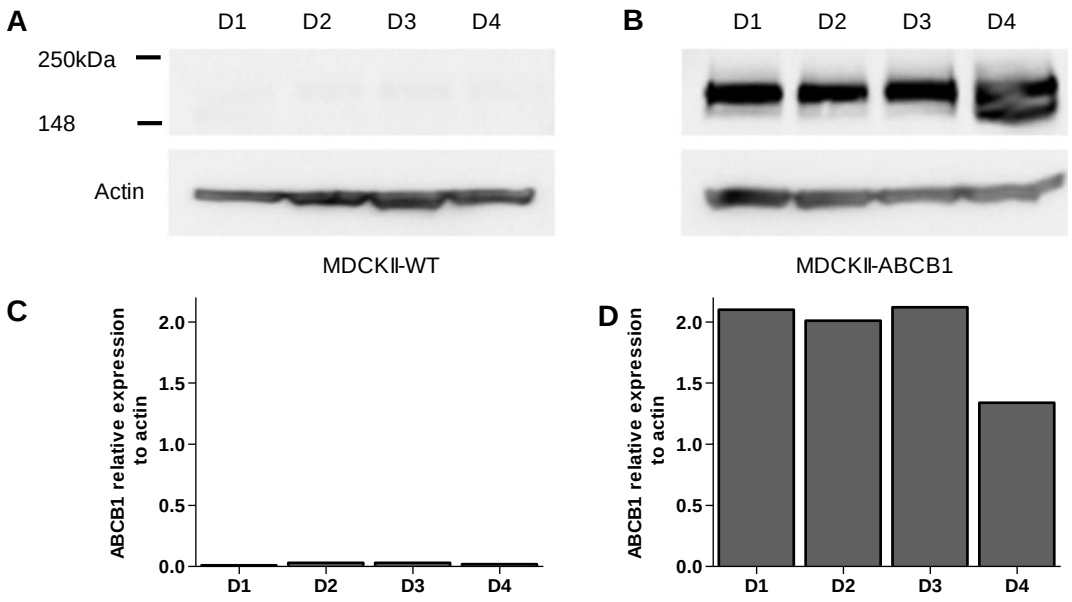

Figure 2.2. Expression of human ABCB1 in MDCKII-WT and MDCKII-ABCB1 cell lines.

Expression of ABCB1 was determined by western blot analysis. Cell lines were allowed to grow for 1-4 days and western blotting was performed on cell lysates. ABCB1 expression in (A) MDCKII-WT and (B) MDCKII-ABCB1 were normalised against actin as an endogenous control. Relative expression in (C) MDCKII-WT cells was 0.01-0.03 compared to actin and (D) MDCKII-ABCB1 cells was 2.01-2.12, cells over 3 days. Abbreviations- D, Day. ABCB1 molecular weight: 170kDa, Actin molecular weight: 42kDa.

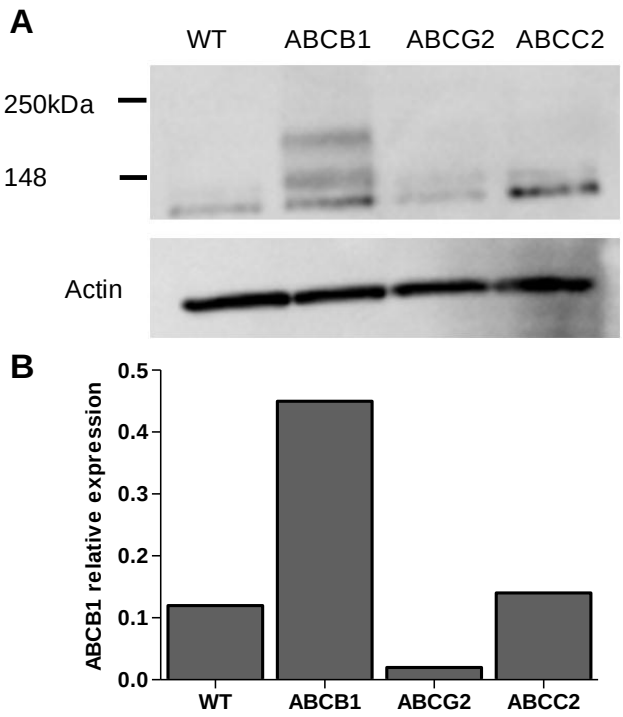

Figure 2.3. Expression of ABCB1 in MDCKII WT, -ABCB1, -ABCG2 and –ABCC2 cell lines.

Expression of ABCB1 in MDCKII WT, -ABCB1, -ABCG2 and –ABCC2 cell lines was determined by western blot analysis. Cell lines were grown for 4 days and western blotting was performed on cell lysates. ABCB1 expression in all cell lines (A) was normalised to actin as an endogenous control in each sample. (B) Relative expression: MDCKII-WT: 0.12, MDCKII-ABCB1: 0.45, MDCKII-ABCG2: 0.02, MDCKII-ABCC2: 0.14. ABCB1 molecular weight: 170kDa, Actin molecular weight: 42kDa.

Higher expression of ABCC1 was seen in the ABCC1 over-expressing cell line compared to parental (0.78 vs. 0.18 relative expression, Figure 2.4A/C) and higher ABCC2 expression was confirmed in the MDCKII-ABCC2 cell line compared to parental (1.60 vs. 0.08 relative expression, Figure 2.4B/D). Further analysis in other MDCKII cell lines was not carried out due to the time spent optimising antibody conditions for the ABCC1 and ABCC2 antibodies.

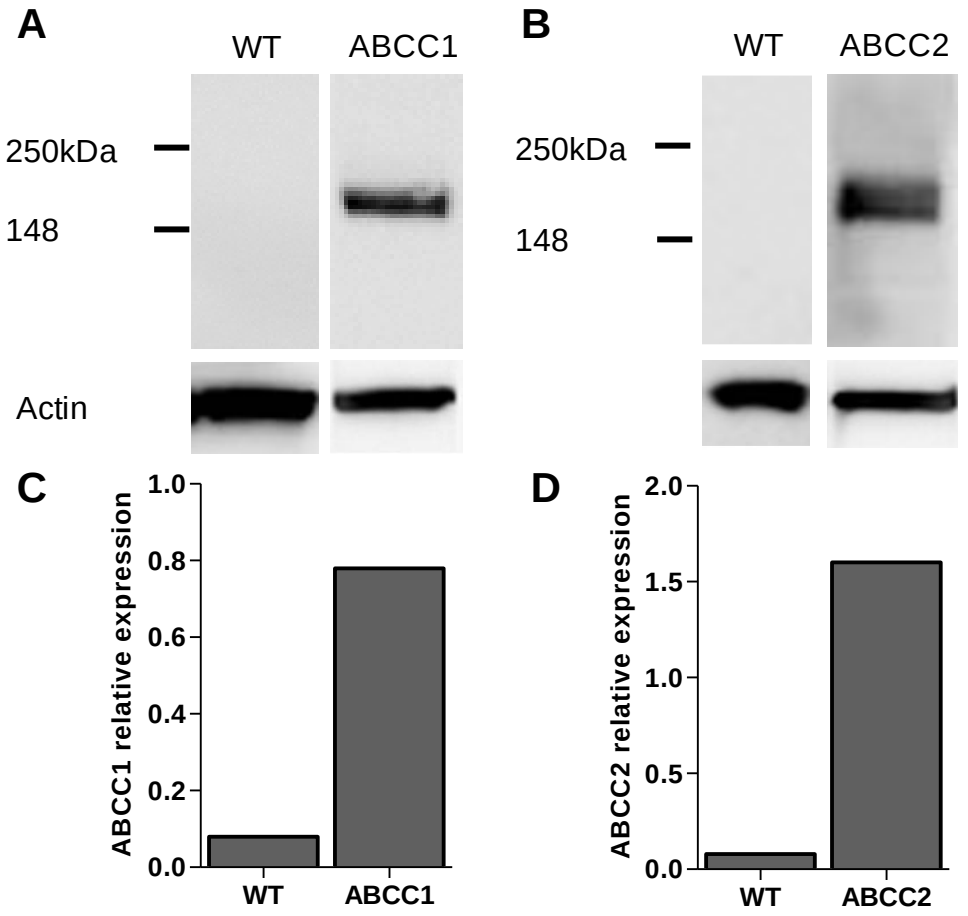

Figure 2.4. Expression of ABCC1 and ABCC2 in MDCKII-WT, MDCKII-ABCC1 and MDCKII-ABCC2 cell lines.

Expression of (A) ABCC1 in MDCKII WT and MDCKII-ABCC1 cell lines and (B) ABCC2 in MDCKII-WT and MDCKII-ABCC2 cell lines was determined by western blot analysis. Cell lines were grown for 4 days and western blotting was performed on cell lysates. (C) ABCC1 and (D) ABCC2 expression in relevant cell lines was normalised to actin as an endogenous control in each sample. Relative expression of ABCC1: MDCKII-WT: 0.08, MDCKII-ABCC1: 0.78 and ABCC2: MDCKII-WT: 0.08, MDCKII-ABCC2: 1.60.

High levels of ABCG2 expression were confirmed in MDCKII-ABCG2 cell lines (Figure 2.5) compared to MDCKII-WT and MDCKII-ABCB1 cell lines (0.85 vs. 0 relative expression). Densitometry did suggest ABCG2 expression in MDCKII-ABCC2 cell lines (0.85 vs. 0.16 relative expression), but this was not clearly visible in the gel image.

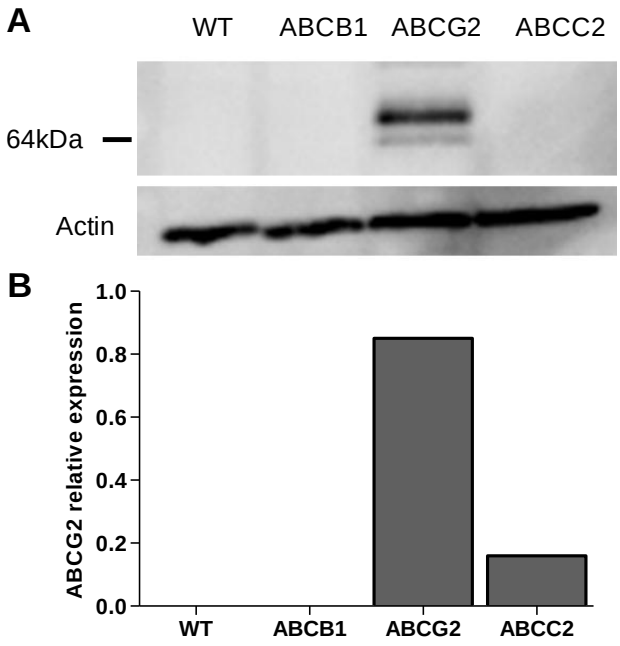

Figure 2.5. Expression of ABCG2 in MDCKII WT, -ABCB1, -ABCG2 and –ABCC2 cell lines.

(A) Expression of ABCG2 in MDCKII WT, -ABCB1, -ABCG2 and –ABCC2 cell lines was determined by western blot analysis. Cell lines were grown for 4 days and western blotting was performed on cell lysates. (B) ABCG2 expression in all cell lines was normalised to actin as an endogenous control in each sample. Relative expression: MDCKII-WT: 0, MDCKII-ABCB1: 0, MDCKII-ABCG2: 0.85, MDCKII-ABCC2: 0.16.

2.3.2 Growth curves using MDCKII-WT, ABCB1, ABCC2 and ABCG2

Following confirmation of ABC transporter expression, growth curves were constructed in MDCKII-WT (Figure 2.6A), ABCB1 (Figure 2.6B), ABCC2 (Figure 2.6C) and ABCG2 (Figure 2.6D) cell lines to determine the optimal seeding densities for a 4 day incubation in 96-well plates. In all four cell lines, 4,000 cells per well was sufficient to initiate and maintain exponential phase growth over

96h. As transporter expression did not have an effect on cell growth, it was deemed appropriate for all cell lines to be seeded at 4,000 cells per well, including MDCKII-ABCC1 cells for which growth curves were not constructed due to being sourced late in the study.

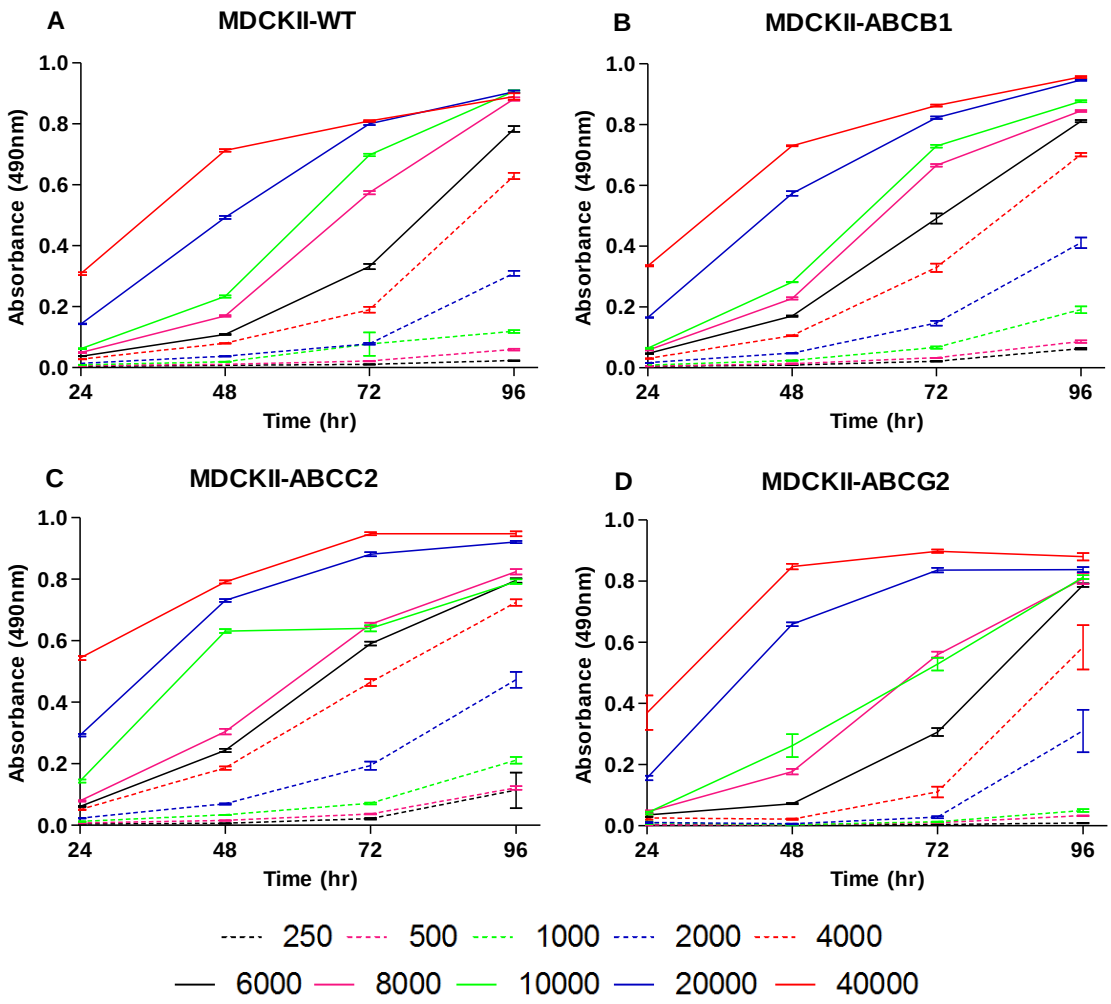

Figure 2.6. Growth curves in MDCKII cell lines.

MDCKII-WT (A), MDCKII-ABCB1 (B), MDCKII-ABCC2 (C) and MDCKII-ABCG2 (D) cells were seeded at the indicated densities in 96-well plates and allowed to grow for 96h. Cell growth was determined using SRB staining with results expressed as absorbance at 490nm. The optimal seeding density of 4,000 cells per well was chosen.

2.3.3 Growth inhibition of known ABC transporter substrates in MDCKII cell lines

Known ABC transporter substrates were used to assess ABC transporter function. Doxorubicin was used as a well-characterised ABCB1 substrate

(Fairchild *et al.*, 1987; Ueda *et al.*, 1987). When incubated with a concentration range of doxorubicin of 0-50μM, ABCB1 over-expressing cells were 16-fold less sensitive than the parental cell line (GI_{50} values 890nM vs. 55.7nM, p<0.0001, Table 2.4, Figure 2.7A). Inhibition of ABCB1 by verapamil (1μM) over a 72h period resulted in almost complete reversal of resistance in the MDCKII-ABCB1 cells (GI_{50} without verapamil, 890nM vs. with verapamil, 142nM, p<0.0001, Table 2.4, Figure 2.7C). Inhibition with verapamil also caused a 2.9-fold reduction (GI_{50} without verapamil, 55.7nM vs. with verapamil, 19.5nM, p<0.0001, Figure 2.7B, Table 2.4) in the parental cell, due to the endogenous expression of canine ABCB1. No difference was observed in sensitivity to doxorubicin compared to the parental cell line in MDCKII-ABCC2 and MDCKII-ABCG2 cell lines.

	MDCKII-WT		**MDCKII-ABCB1**		**MDCKII-ABCC2**		**MDCKII-ABCG2**	
	GI_{50} (nM)	**95% CI**	**GI_{50} (nM)**	**95% CI**	**GI_{50} (nM)**	**95% CI**	**GI_{50} (nM)**	**95% CI**
Doxorubicin	55.7	(34.3, 90.4)	890****	(529, 1501)	39.3	(33.0, 47.0)	47.3	(36.2, 61.7)
Doxorubicin + Verapamil	19.5†	(10.4, 36.7)	142†	(72.3, 280)	-	-	-	-
SN-38	335	(307, 365)	659****	(526, 826)	5668****	(4861, 6609)	445	(341, 580)
Mitoxantrone	475	(395, 584)	5545****	(4564, 6737)	300	(249, 361)	4793****	(3730, 6172)
Mitoxantrone + KO143	690	(571, 834)	-	-	-	-	523†	(362, 757)

Abbreviation: -, not applicable

GI_{50} assessed by MTS assay after 72h of drug exposure. Values are the mean of ≥ 3 experiments.

****p<0.0001 compared to MDCKII-WT under the same treatment conditions.

†p<0.0001 compared to the same cell line without inhibitor treatment.

Table 2.4. Growth inhibition of doxorubicin, SN-38 and mitoxantrone in MDCKII cell lines over-expressing specific drug transporters

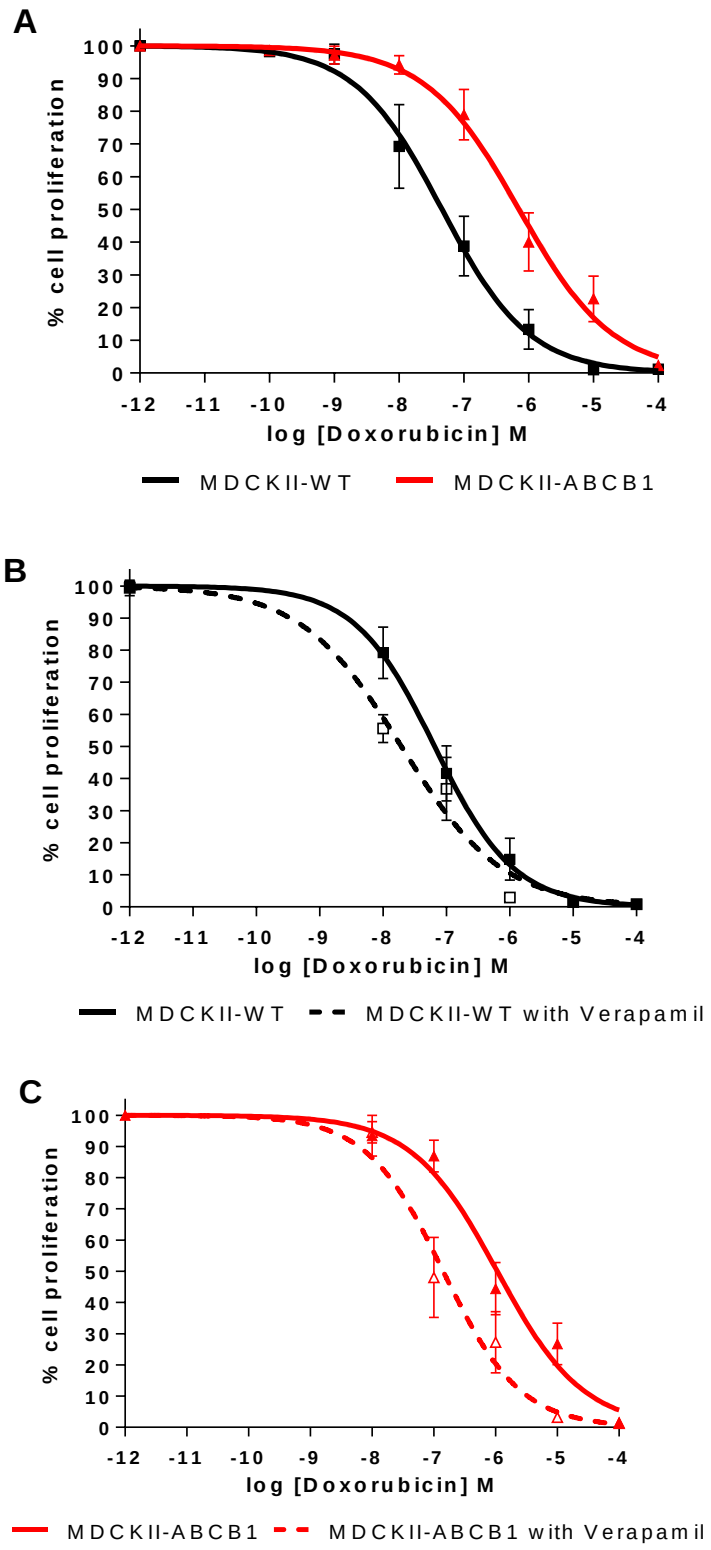

Figure 2.7. Doxorubicin growth inhibition in MDCKII-WT and MDCKII-ABCB1 cell lines in the presence or absence of verapamil.

Cells were seeded and allowed 24h to establish growth and were then incubated with 0-50µM doxorubicin either alone (A) or in the presence of 1µM verapamil (B and C) for 72h following which MTS substrate was added to determine cell sensitivity. Each point represents mean ± SEM from ≥ 3 experiments. GI_{50} values were compared by F-test.

SN-38, the active metabolite of irinotecan is a well-established ABCC2 substrate (Chu *et al.*, 1997a; Fujita *et al.*, 2008). When MDCKII cells were incubated with SN-38 (0-20µM), ABCC2 over-expressing cells were 17-fold less sensitive than the parental cell line (GI_{50} values 5.67µM vs. 0.34µM, $p<0.0001$, Figure 2.8, Table 2.4). A small yet statistically significant difference in sensitivity was also seen in MDCKII-ABCB1 (GI_{50} values 0.66µM vs. 0.34µM, $p<0.0001$, Table 2.4) and MDCKII-ABCG2 cell lines (GI_{50} values 0.45µM vs. 0.34µM, $p<0.0001$, Table 2.4). Adequate, selective inhibition of ABCC2 was not possible during this initial investigation.

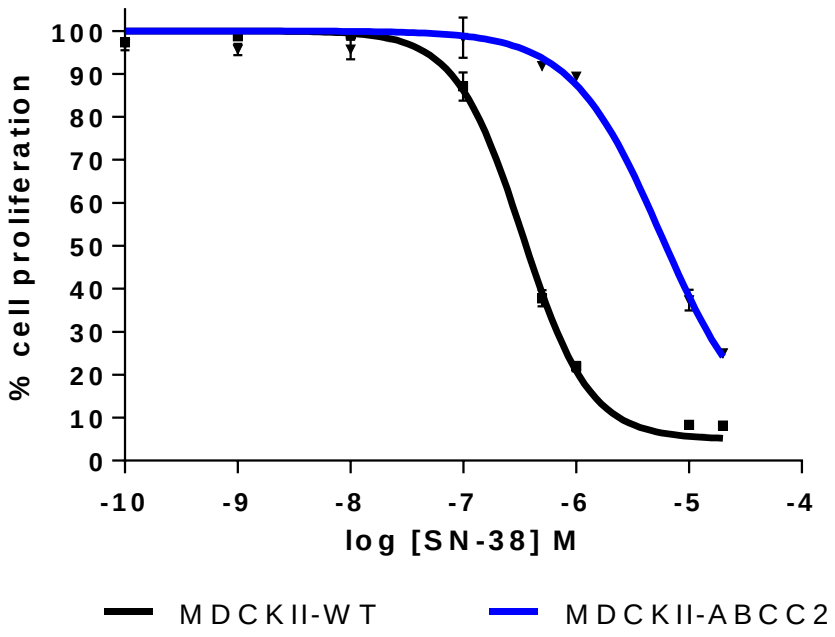

Figure 2.8. SN-38 growth inhibition in MDCKII-WT and MDCKII-ABCC2 cell lines.

Cells were seeded and allowed 24h to establish growth and were then incubated with 0-20µM SN-38 for 72h following which MTS substrate was added to determine cell sensitivity. Each point represents mean ± SEM from ≥ 3 experiments. GI_{50} values were compared by F-test.

Using mitoxantrone as a model substrate for ABCG2 (Doyle *et al.*, 1998), ABCG2 over-expressing cells were 10-fold less sensitive than MDCKII-WT (GI_{50} values 4.79µM vs. 0.48µM, $p<0.0001$, Figure 2.9A, Table 2.4). Use of the

specific ABCG2 inhibitor KO143 (0.2µM) further confirmed this by complete reversal of resistance in MDCKII-ABCG2 cell lines over a 72h period (GI_{50} in the absence of KO143, 4.79µM vs. in the presence of KO143, 0.52µM, $p<0.0001$, Figure 2.9C, Table 2.4). No effect was observed of incubation with KO143 in MDCKII-WT cells (Figure 2.9B, Table 2.4). MDCKII-ABCB1 cells were also less sensitive to mitoxantrone than the parental cell line (5.55nM vs. 0.48nM, $p<0.0001$, Table 2.4) due to mitoxantrone also being an ABCB1 substrate (Kamiyama *et al.*, 2006).

These experiments demonstrate that MDCKII-ABCB1, MDCKII-ABCC2, MDCKII-ABCG2 and the parental cell lines are functioning as models to assess the transport of Act D.

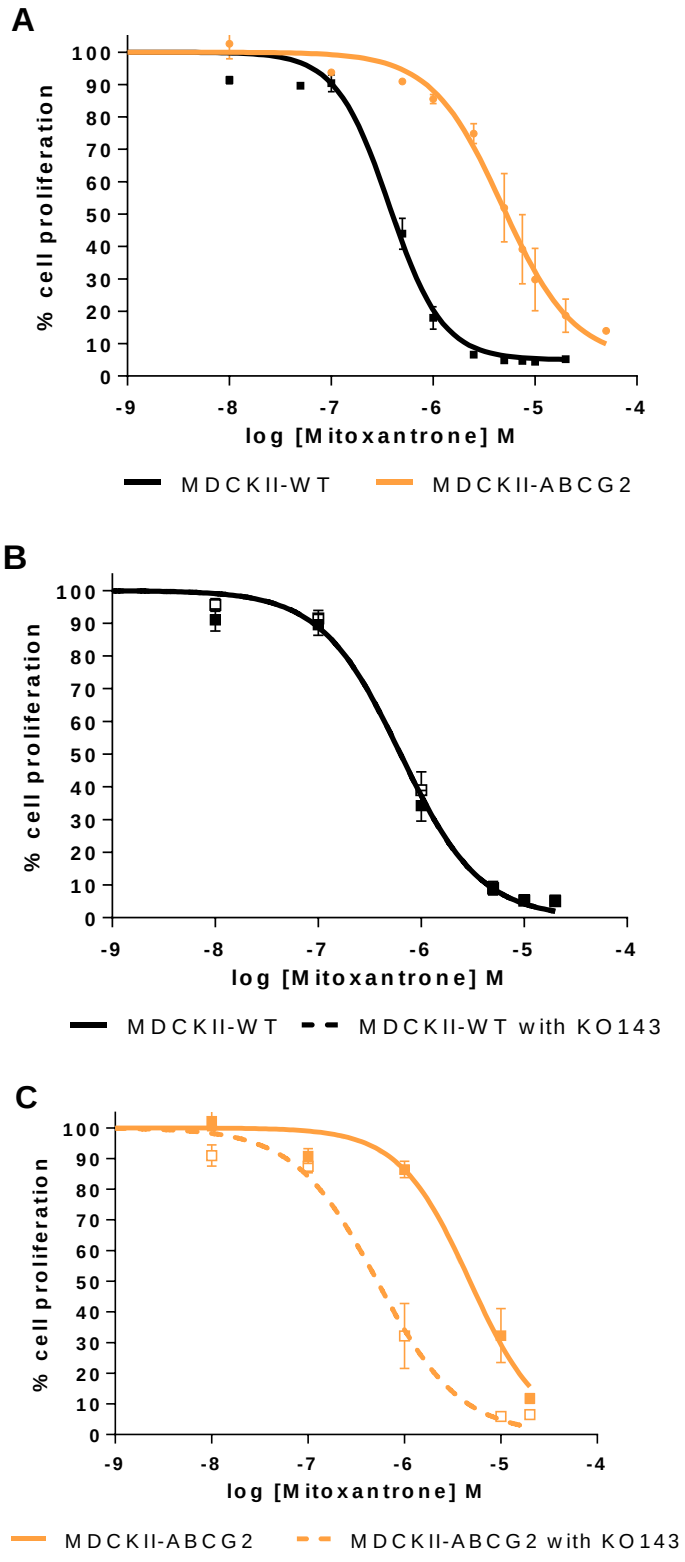

Figure 2.9. Mitoxantrone growth inhibition in MDCKII-WT and MDCKII-ABCG2 cell lines in the presence or absence of KO143.

Cells were seeded and allowed 24h to establish growth and were then incubated with 0-20μM mitoxantrone either alone (A) or in the presence of 0.2μM KO143 (B and C) for 72h following which MTS substrate was added to determine cell sensitivity. Each point represents mean ± SEM from ≥ 3 experiments. GI_{50} values were compared by F-test.

2.3.4 Growth inhibition due to actinomycin D in MDCKII cell lines

Growth inhibition by Act D was carried out as an initial step to investigate the possible transport of Act D by ABCB1, ABCC1, ABCC2 and ABCG2. When treated with 0-10μM of Act D over 72h, MDCKII-ABCB1 cells were 59-fold less sensitive than the parental cell line (GI_{50} values 745nM vs. 12.7nM, p<0.0001, Table 2.5, Figure 2.10) indicating that Act D undergoes ABCB1-mediated efflux, therefore reducing growth inhibition. Inhibition of ABCB1 with verapamil (1μM) over a 72h period resulted in partial reversal of resistance in the MDCKII-ABCB1 cell line, with a 6-fold lower GI_{50} (745nM vs. 129nM, p<0.0001, Figure 2.10, Table 2.5). Inhibition with verapamil also caused a 3-fold reduction (GI_{50} values 12.7nM vs. 4.4nM, p<0.0001, Table 2.5, Figure 2.10) in GI_{50} in the parental cell line, due to endogenous canine ABCB1 expression.

	MDCKII-WT		**MDCKII-ABCB1**		**MDCKII-ABCC1**		**MDCKII-ABCC2**		**MDCKII-ABCG2**	
	GI_{50} (nM)	**95% CI**	**GI_{50} (nM)**	**95% CI**	**GI_{50} (nM)**	**95% CI**	**GI_{50} (nM)**	**95% CI**	**GI_{50} (nM)**	**95% CI**
Act D	12.7	-	745****	(680.8, 905.3)	25.7****	(18.3, 36.1)	40.4****	(32.2, 50.7)	14.0	(11.7, 16.8)
Act D + Verapamil	4.40†	(3.31, 5.85)	129†	(109.8, 150.5)	-	-	-	-	-	-
Act D + MK571	4.10†	(3.02, 5.55)	-	-	5.90†	(3.35, 10.4)	32.3	23.3, 44.7)	-	-
Act D + KO143	13.1	(10.2, 16.9)	-	-	-	-	-	-	13.0	(10.3, 15.4)

Abbreviation: -, not applicable

GI_{50} assessed by MTS assay after 72h of drug exposure. Values are the mean of ≥ 3 experiments.

****p<0.0001 compared to MDCKII-WT under the same treatment conditions.

†p<0.0001 compared to the same cell line without inhibitor treatment.

Table 2.5. Growth inhibition of Act D in MDCKII cell lines over-expressing specific drug transporters

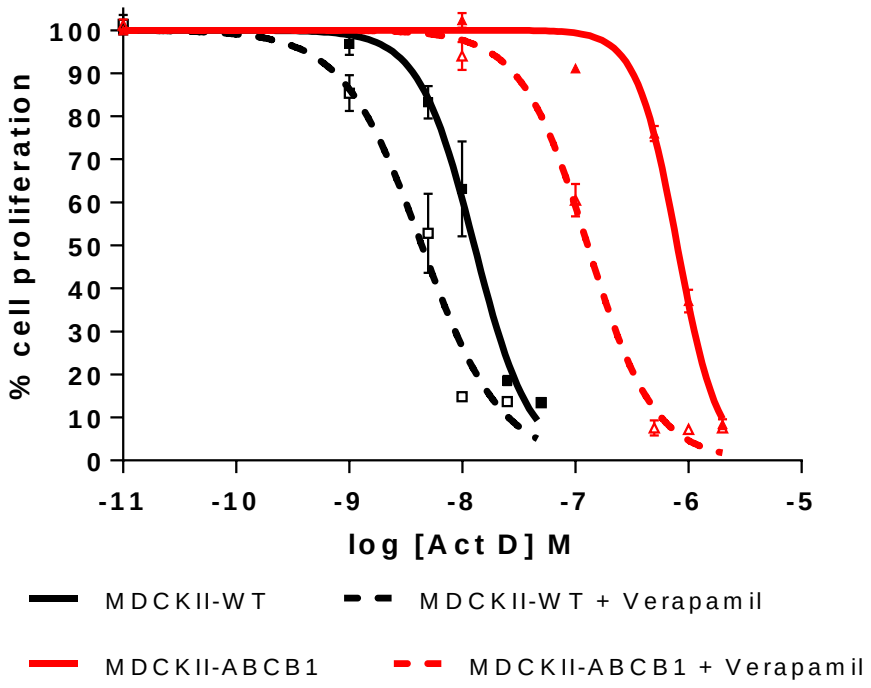

Figure 2.10. Act D growth inhibition in MDCKII-WT and MDCKII-ABCB1 cell lines in the presence or absence of verapamil.

Cells were seeded and allowed 24h to establish growth and were then incubated with 0-10µM Act D either alone or in the presence of verapamil (1µM) for 72h, following which MTS substrate was added to determine cell sensitivity. Each point represents mean ± SEM from ≥ 3 experiments. GI_{50} values were compared by F-test.

ABCC1 and ABCC2 over-expressing cell lines showed reduced sensitivity to Act D compared to the parental cell line. GI_{50} values were 25.7nM for ABCC1 and 40.4nM for ABCC2, compared to the parental GI_{50} of 12.7nM ($p<0.0001$, Figure 2.11, Table 2.5), indicating that Act D may be a substrate for ABCC1 and ABCC2. To further investigate this, experiments were carried out with the pan-ABCC family inhibitor MK571. MK571 (25µM) increased sensitivity 3-fold in the parental cell line ($p<0.0001$, Figure 2.12A, Table 2.5), whilst a greater than 4-fold increase was seen in MDCKII-ABCC1 ($p<0.0001$, Figure 2.12B, Table 2.5). There was no effect on the sensitivity of the MDCKII-ABCC2 cell line ($p=0.23$, Figure 2.12C, Table 2.5).

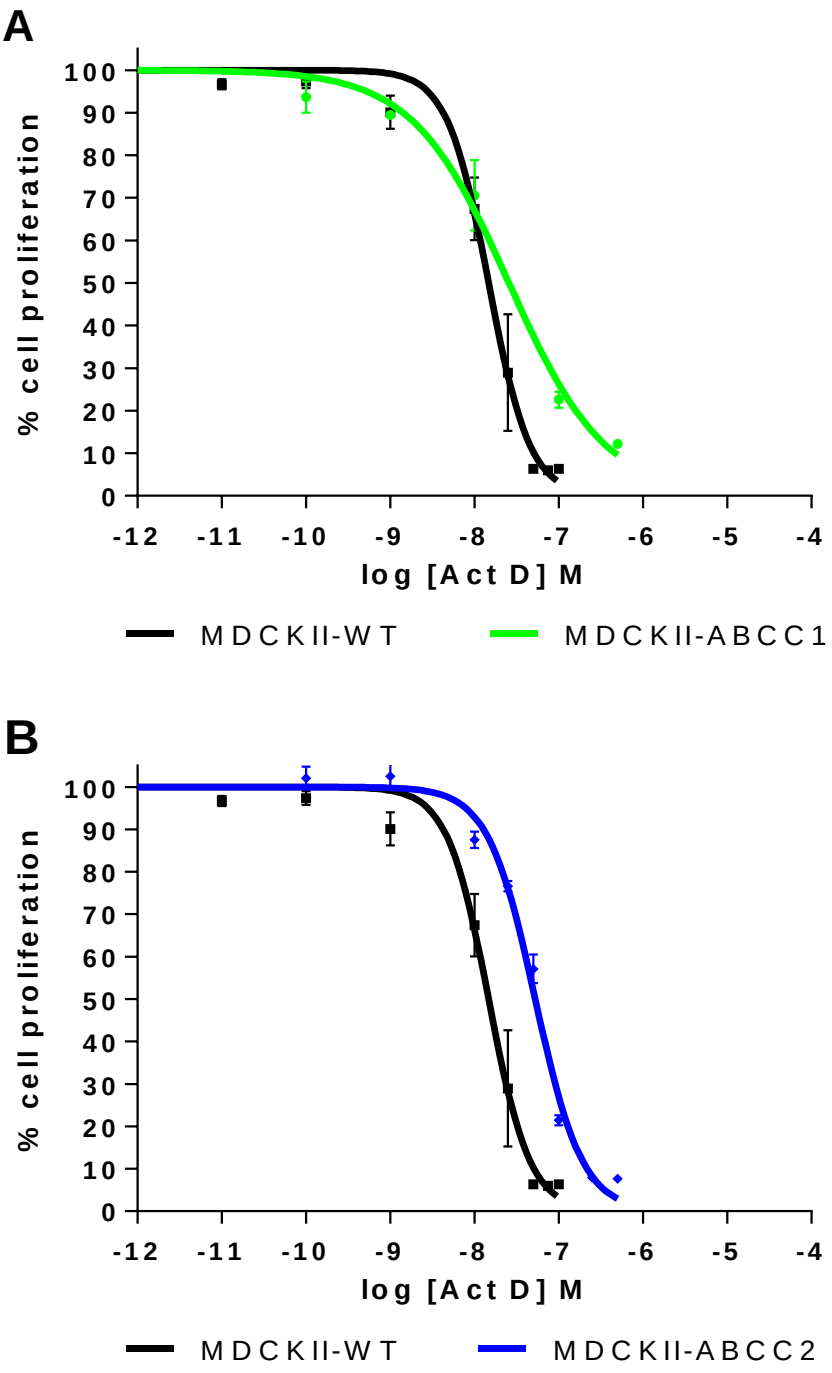

Figure 2.11. Act D growth inhibition in MDCKII-WT, MDCKII-ABCC1 and MDCKII-ABCC2 cell lines.

Cells were seeded and allowed 24h to establish growth and were then incubated with 0-10µM Act D for 72h following which MTS substrate was added to determine cell sensitivity. Each point represents mean ± SEM from ≥ 3 experiments. GI_{50} values were compared by F-test.

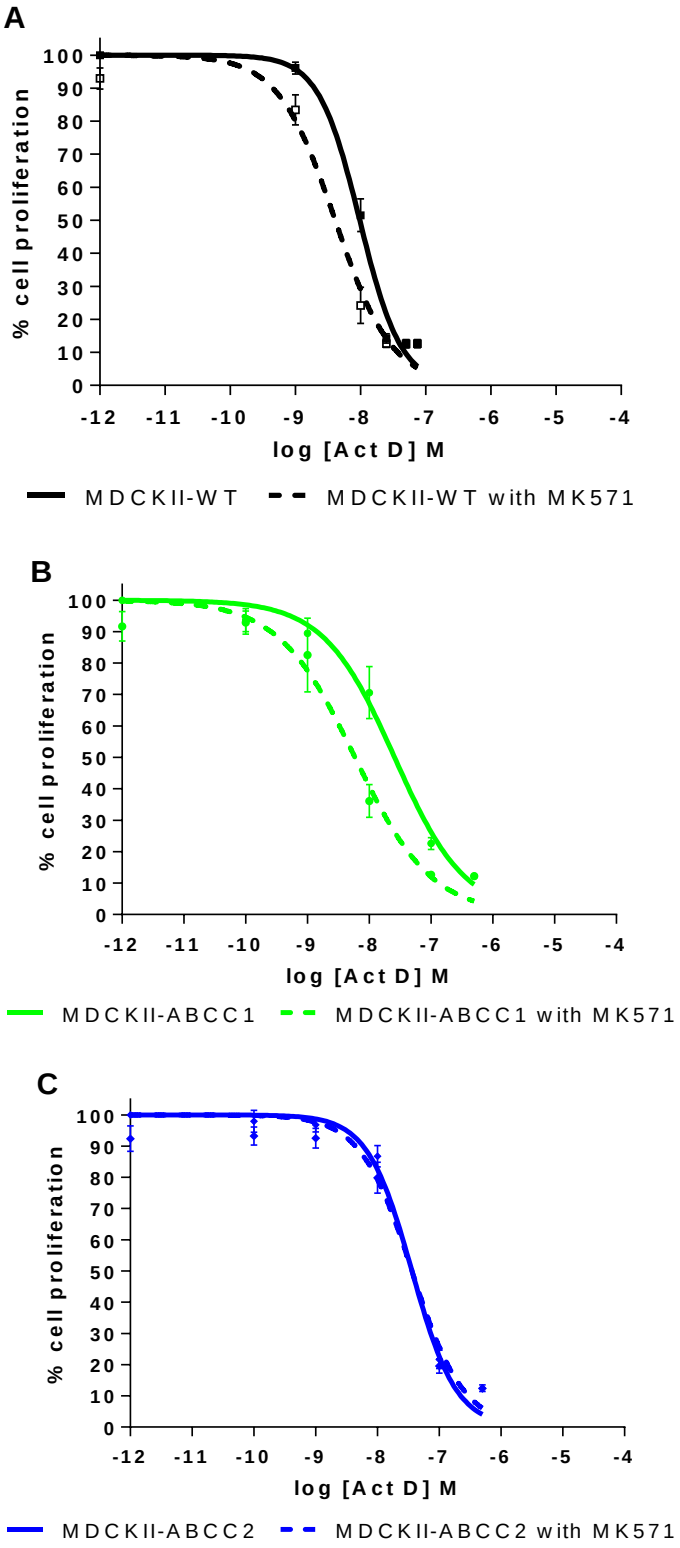

Figure 2.12. Act D growth inhibition in MDCKII-WT, MDCKII-ABCC1 and MDCKII-ABCC2 cell lines with MK571.

Cells were seeded and allowed 24h to establish growth and were then incubated with 0-10µM Act D in the presence of MK571 (25µM) for 72h following which MTS substrate was added to determine cell sensitivity. Each point represents mean ± SEM from ≥ 3 experiments. GI_{50} values were compared by F-test.

No difference in sensitivity was observed between the parental cell line and ABCG2 over-expressing cells, when incubated with Act D alone or in combination with the specific ABCG2 inhibitor KO143 (p=0.74, Figure 2.13, Table 2.5).

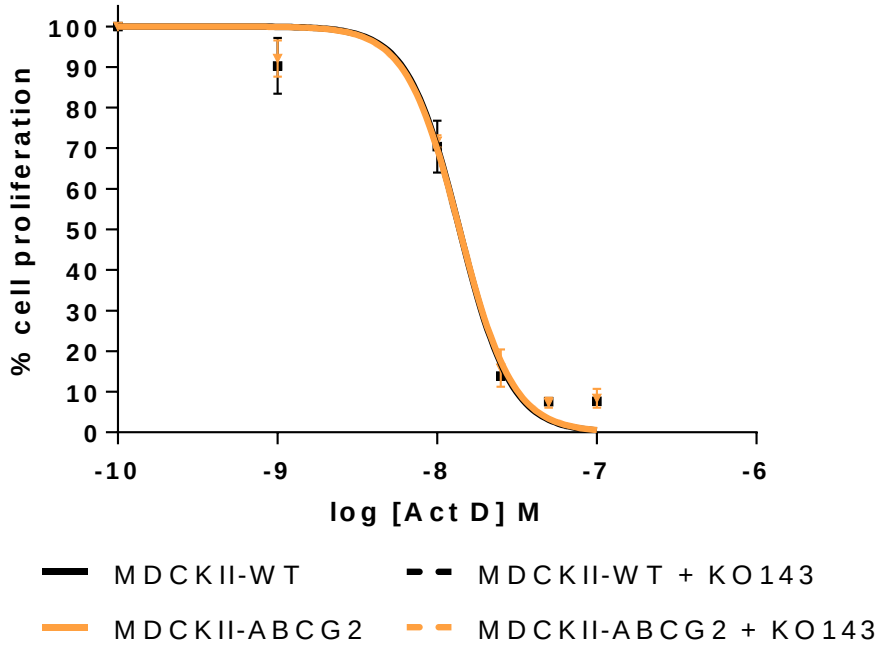

Figure 2.13. Act D growth inhibition in MDCKII-WT and MDCKII-ABCG2 cell lines in the presence or absence of KO143 (0.2µM).

Cells were seeded and allowed 24h to establish growth and were then incubated with 0-10µM Act D either alone or in the presence of KO143 (0.2µM) for 72h following which MTS substrate was added to determine cell sensitivity. Each point represents mean ± SEM from ≥ 3 experiments. GI_{50} values were compared by F-test.

2.3.5 Intracellular accumulation of actinomycin D in MDCKII cell lines

To confirm the results obtained from growth inhibition studies, intracellular concentrations of Act D were determined in all cell lines across a wide concentration range. Initially, to determine an acceptable dose which would not cause cell toxicity over 12h, all MDCKII cell lines were incubated with 0.01-2µM Act D for between 2 and 12h; 0.01µM was used over 12h and 0.1µM was used over 4h during inhibition studies (Table 2.6).

	Act D concentration (μM)																			
	0.01				0.1				0.5				1				2			
Time (h)	**2**	**4**	**6**	**12**	**2**	**4**	**6**	**12**	**2**	**4**	**6**	**12**	**2**	**4**	**6**	**12**	**2**	**4**	**6**	**12**
MDCKII-WT	-	-	-	-	-	-	T	T	-	-	T	T	-	-	T	T	-	T	T	T
MDCKII-ABCB1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-
MDCKII-ABCC1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	-	-	T	T
MDCKII-ABCC2	-	-	-	-	-	-	-	T	-	-	T	T	-	-	T	T	-	-	T	T
MDCKII-ABCG2	-	-	-	-	-	-	T	T	-	-	T	T	-	T	T	T	-	T	T	T

Abbreviations: -, No toxicity* observed T, Toxicity* observed

Table 2.6. Observed cellular toxicity following incubation with 0.01-2μM Act D for 2-12h.

All MDCKII cell lines were seeded in 6-well plates and allowed to establish growth over 4 days, cells were then incubated with a concentration range of Act D (0.01-2μM). *Cells were examined using a microscope and toxicity was defined as cells detaching from the base of the well at 2, 4, 6 and 12h. Each reading was from ≥ 3 experiments.

When incubated for between 2 and 12 hours with Act D (0.01μM) alone, a time-dependent accumulation of Act D was observed. After 12h, mean Act D accumulation in MDCKII-ABCB1 was 10.2% of that seen in the parental cell line (0.98nM vs. 0.1nM, $p<0.0001$, Figure 2.14A, Table 2.7). When co-incubated with verapamil, intracellular levels of Act D increased in a verapamil concentration-dependent manner, indicating that Act D efflux is reduced when ABCB1 is inhibited.

Cell line	Act D concentration (nM)			
	2h	4h	6h	12h
MDCKII-WT	0.24	0.43	0.66	0.98
MDCKII-ABCB1	0.06****	0.06****	0.07****	0.10****
MDCKII-ABCC1	0.17	0.22	0.31*	0.40**
MDCKII-ABCC2	0.16	0.19*	0.22***	0.28***
MDCKII-ABCG2	0.43	0.75	0.88	1.27

*p < 0.05, **p < 0.01, ***p<0.001, ****p < 0.0001 – compared with MDCKII-WT cell line at the same accumulation time point, using two-way ANOVA with Bonferroni's multiple comparisons test

Table 2.7. Intracellular accumulation of 0.01μM Act D in MDCKII- cell lines over 12h

Treating MDCKII-ABCB1 cells with 10μM verapamil caused a 7.9-fold increase in intracellular Act D (Figure 2.14B, Table 2.8). Complete reversal of intracellular concentration was achieved with verapamil concentrations of 10μM or greater. These results, alongside those seen during growth inhibition studies further suggest a role for ABCB1 in the transport of Act D.

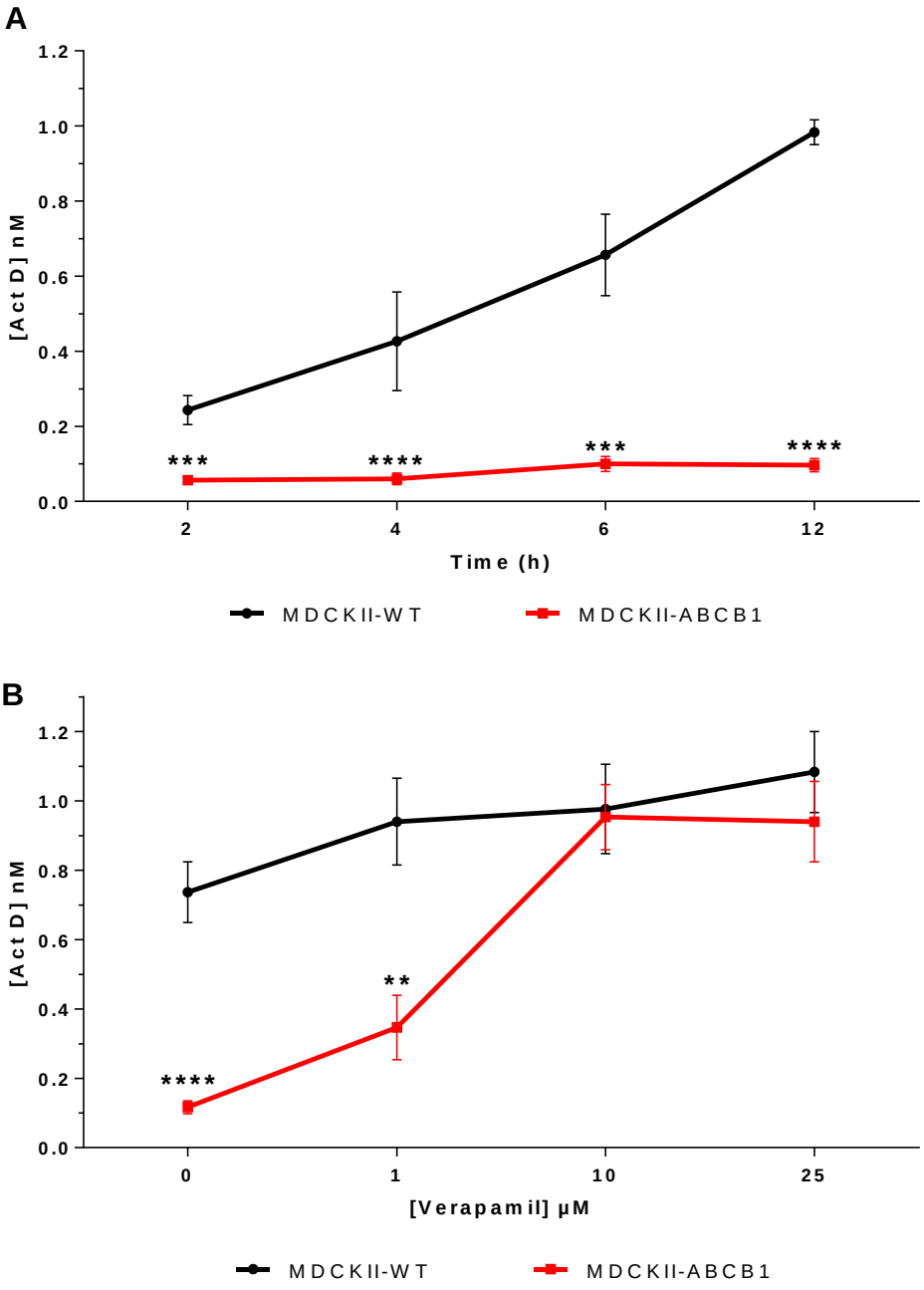

Figure 2.14. Intracellular accumulation of Act D in MDCKII-WT and MDCKII-ABCB1 in the presence or absence of verapamil.

Following 4 days growth, cells were incubated with (A) 0.01µM Act D for between 2 and 12h or (B) 0.1µM Act D for 4h in the presence or absence of verapamil (0-25µM). Cellular contents were extracted at the appropriate time point and intracellular Act D concentrations were determined by LC/MS analysis. Each point represents mean ± SEM from ≥ 3 experiments. **p<0.01, ***p<0.001 and ****p<0.0001 compared to MDCKII-WT at the same experimental conditions using the two-way ANOVA with Bonferroni's correction for multiple comparisons.

Cell line	Act D concentration (nM)								
	No inhibitor	[Verapamil] μM			[MK571] μM			[KO143] μM	
		1	10	25	1	10	25	1	10	25
MDCKII-WT	0.76	0.94	0.96	1.15	0.94	0.92	1.27	0.71	0.78	0.85
MDCKII-ABCB1	0.12***	0.35**	0.95	0.94	-	-	-	-	-	-
MDCKII-ABCC1	0.55	-	-	-	0.55	0.68	0.91	-	-	-
MDCKII-ABCC2	0.39**	-	-	-	0.41**	0.56	0.87	-	-	-
MDCKII-ABCG2	1.85*	-	-	-	-	-	-	1.43	1.04	1.1

Abbreviations: -, not applicable

*p < 0.05, **p < 0.01, ***p<0.001 – compared with MDCKII-WT cell line at the same inhibitor concentration, using the two-way ANOVA with Bonferroni's multiple comparisons test.

Table 2.8. Intracellular accumulation of Act D (0.1μM) over 4 h in MDCKII-cell lines in the presence or absence of specific drug transport inhibitors

Accumulation studies over 12h indicated that Act D is also transported by ABCC1 and ABCC2, with 2.5-fold ($p<0.01$) and 3.5-fold ($p<0.001$) lower accumulation respectively, compared to the parental cell line (Figure 2.15A, Table 2.7). A small trend towards increased Act D accumulation (1.24-fold in MDCKII-ABCC1 and 1.41-fold in MDCKII-ABCC2, Figure 2.15B, Table 2.8) was seen when co-incubating Act D with MK571, but this was not statistically significant.

Throughout this investigation, MDCKII-ABCG2 cell lines consistently had higher intracellular concentrations of Act D than any other cell line, including the parental. Between 2-6h, intracellular Act D concentrations in MDCKII-ABCG2 were 1.6-fold higher than the parental, and 1.3-fold higher than the parental at 12h, however these results were not statistically significant (Figure 2.16A, Table

2.7). Use of the specific ABCG2 inhibitor KO143 reduced Act D accumulation with increasing inhibitor concentration. When MDCKII-WT and MDCKII-ABCG2 cells were incubated for 4h in the absence of KO143, intracellular Act D concentrations were 1.92-fold higher in MDCKII-ABCG2 than the parental ($p<0.05$, Figure 2.16B, Table 2.8), but upon KO143 treatment the intracellular Act D concentration in MDCKII-ABCG2 was lower than without inhibition (1.14nM vs. 1.42nM, Figure 2.16B, Table 2.8). This trend continued with increasing KO143 concentrations.

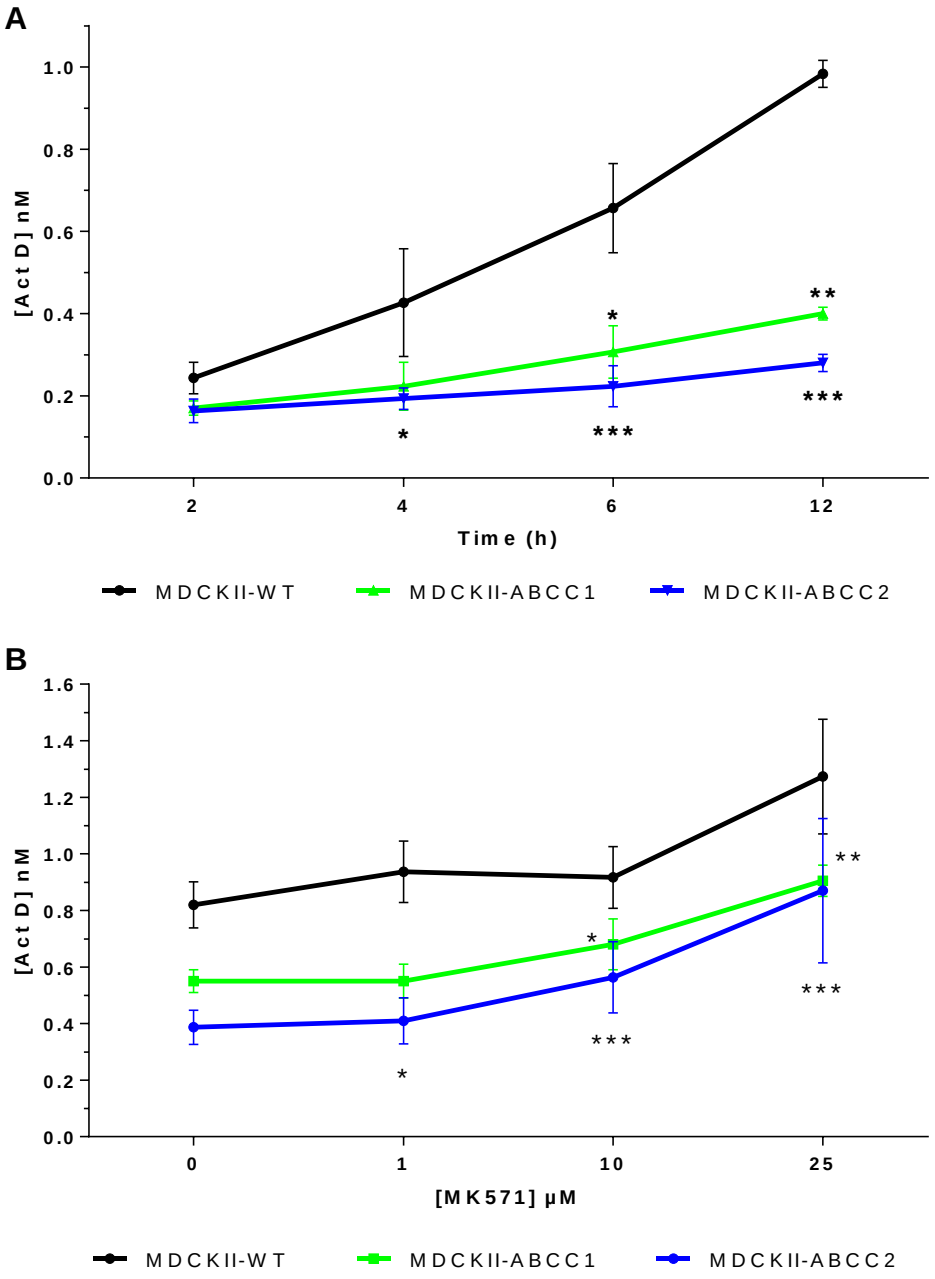

Figure 2.15. Intracellular accumulation of Act D in MDCKII-WT, MDCKII-ABCC1 and ABCC2 in the presence or absence of MK571.

Following 4 days growth, cells were incubated with (A) 0.01µM Act D for between 2 and12h or (B) 0.1µM for 4h in the presence or absence of MK571 (0-25µM) . Cellular contents were extracted at the appropriate time point and intracellular Act D concentrations were determined by LC/MS analysis. Each point represents mean ± SEM from ≥ 3 experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to MDCKII-WT at the same experimental conditions using the two-way ANOVA with Bonferroni's correction for multiple comparisons.

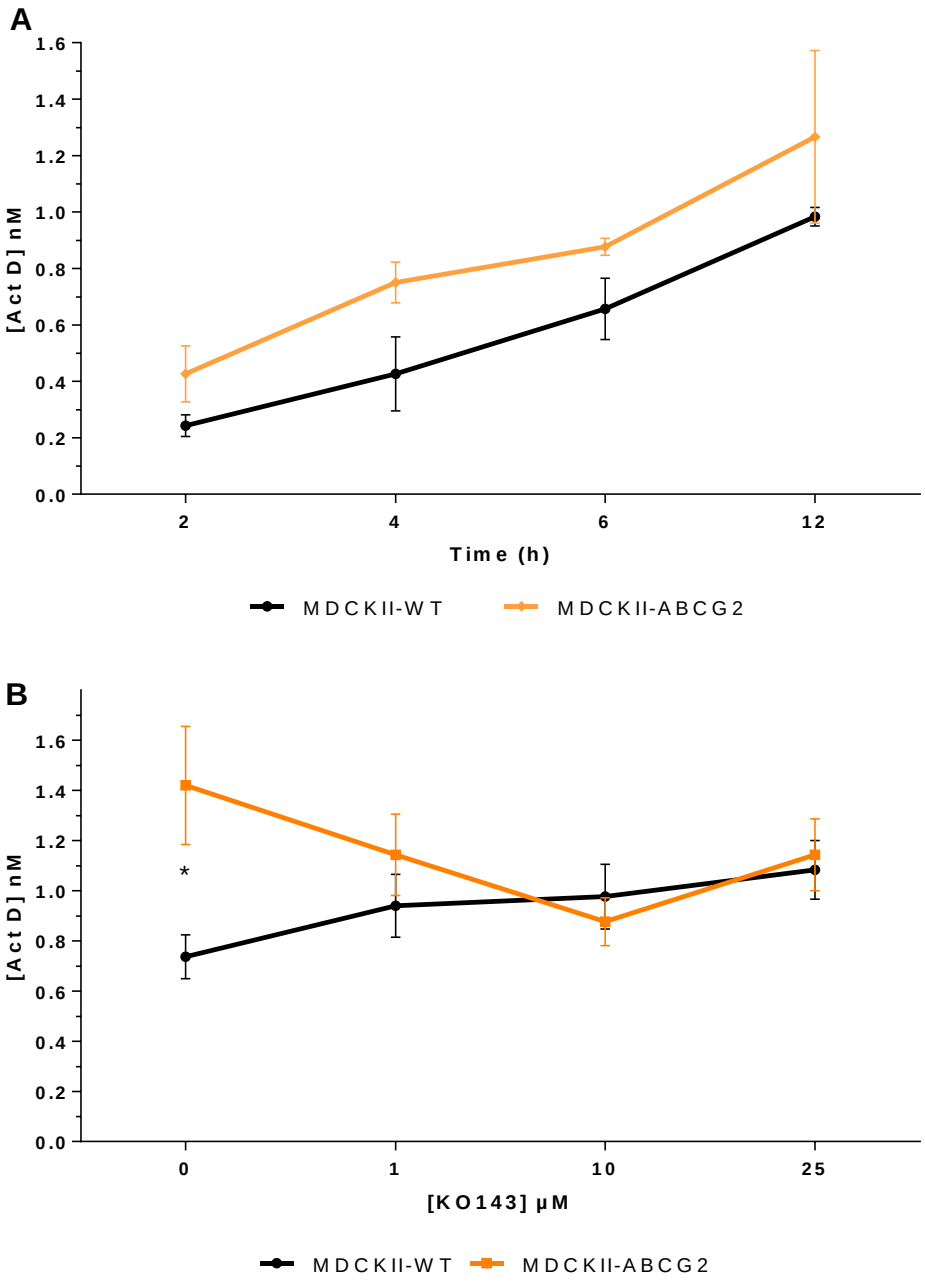

Figure 2.16. Intracellular accumulation of Act D in MDCKII-WT and MDCKII-ABCG2 in the presence or absence of KO143.

Following 4 days growth, cells were incubated with (A) 0.01µM Act D for between 2 and12h or (B) 0.1µM for 4h in the presence or absence of MK571 (0-25µM) . Cellular contents were extracted at the appropriate time point and intracellular Act D concentrations were determined by LC/MS analysis. Each point represents mean ± SEM from ≥ 3 experiments. *$p<0.05$ compared to MDCKII-WT at the same experimental conditions using the two-way ANOVA with Bonferroni's correction for multiple comparisons.

2.3.6 Cellular efflux of actinomycin D in MDCKII cell lines

To further confirm the results seen in both the GI and intracellular accumulation assays, Act D efflux was assessed following a 4h incubation. Over 2h efflux, a time-dependent reduction in intracellular Act D was seen in all cell lines. Consistent with previous data, MDCKII-ABCB1 cell lines initially had 12.7% of the Act D intracellular concentration compared to the parental cells (0.61 vs. 4.8nM, $p<0.0001$, Table 2.9, Figure 2.17). Over 2h the intracellular Act D concentration decreased 5.5-fold in ABCB1 over-expressing cells (0.61 to 0.11nM, $p<0.0001$, Figure 2.17, Table 2.9), whilst the Act D concentration in the parental cell line decreased by only 1.5-fold (4.8 to 3.2nM, Figure 2.17, Table 2.9).

	Act D concentration (nM)		
	0h	**1h**	**2h**
MDCKII-WT	4.80	4.04	3.19
MDCKII-ABCB1	0.61****	0.15****	0.11****
MDCKII-ABCC1	4.60	3.93	2.40
MDCKII-ABCC2	2.75*	1.55***	1.29***
MDCKII-ABCG2	8.20*	5.85	5.29*

*p < 0.05, ***p < 0.001, ****p < 0.0001

– compared with MDCKII-WT cell line at the same efflux time point, using two-way ANOVA with Bonferroni's multiple comparisons test.

Table 2.9. Cellular Efflux of Act D (0.1μM), following a 4h incubation in MDCKII cell lines

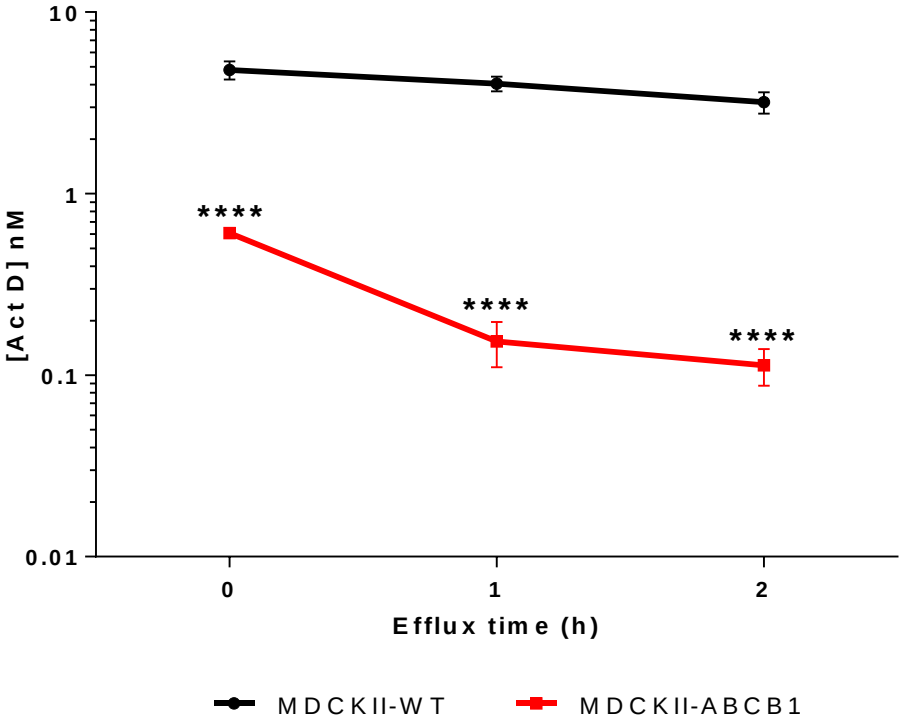

Figure 2.17. Cellular efflux of Act D in MDCKII-WT and MDCKII-ABCB1 cell lines following a 4h incubation.

After 4 days growth, cells were incubated with Act D (0.1µM) for 4h, following which the 0h time point was extracted and spiked media was removed and replaced with blank media for the 1 and 2h time points. Intracellular concentrations were determined by LC/MS analysis. Each point represents mean ± SEM from ≥ 3 experiments. ****p<0.0001 compared to MDCKII-WT at the same sample time, using the two-way ANOVA with Bonferroni's correction for multiple comparisons.

Supporting both GI and intracellular accumulation assays, initial intracellular Act D concentrations in MDCKII-ABCC1 and MDCKII-ABCC2 cells were lower than MDCKII-WT (4.6 and 2.75nM ($p<0.05$) vs. 4.8nM respectively, Figure 2.18, Table 2.9) followed by a 1.9- and 2.1-fold reduction in intracellular Act D respectively during efflux observed over 2h.

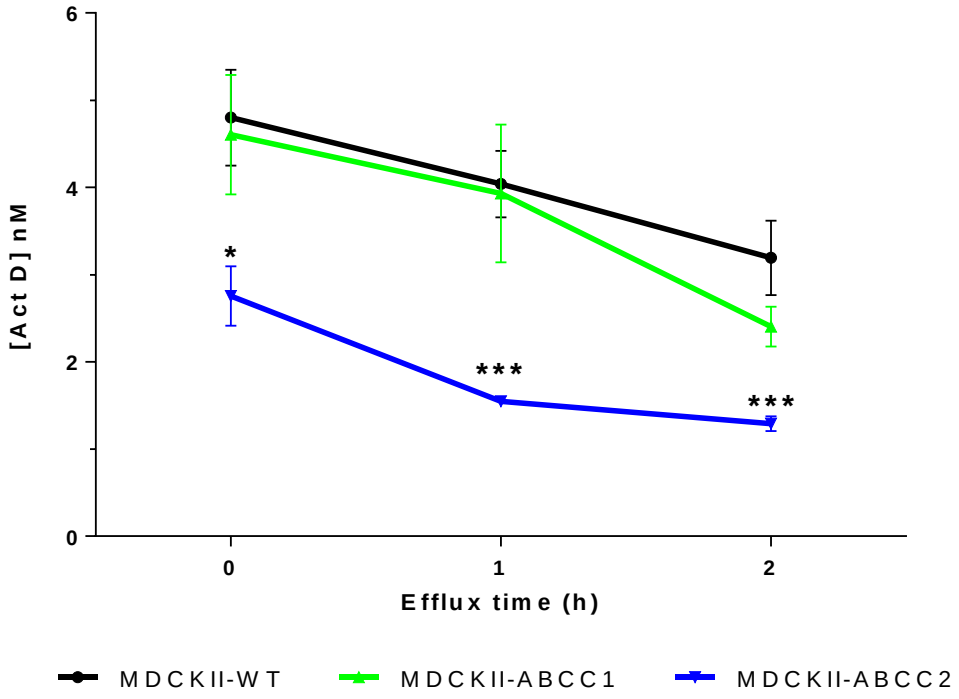

Figure 2.18. Cellular efflux of Act D in MDCKII-WT, MDCKII-ABCC1 and MDCKII-ABCC2 cell lines following a 4h incubation.

After 4 days growth, cells were incubated with Act D (0.1µM) for 4h, following which the 0h time point was extracted and spiked media was removed and replaced with blank media for the 1 and 2h time points. Intracellular concentrations were determined by LC/MS analysis. Each point represents mean ± SEM from ≥ 3 experiments. *p<0.05 and ***p<0.001 compared to MDCKII-WT at the same sample time, using the two-way ANOVA with Bonferroni's corrections for multiple comparisons.

MDCKII-ABCG2 cells again had higher accumulation of Act D after the initial 4h incubation (8.20 vs. 4.80, p<0.05, Figure 2.19, Table 2.9) confirming results seen during intracellular accumulation studies. Similarly to the parental cell line, a 1.6-fold reduction in Act D was seen during efflux over 2h.

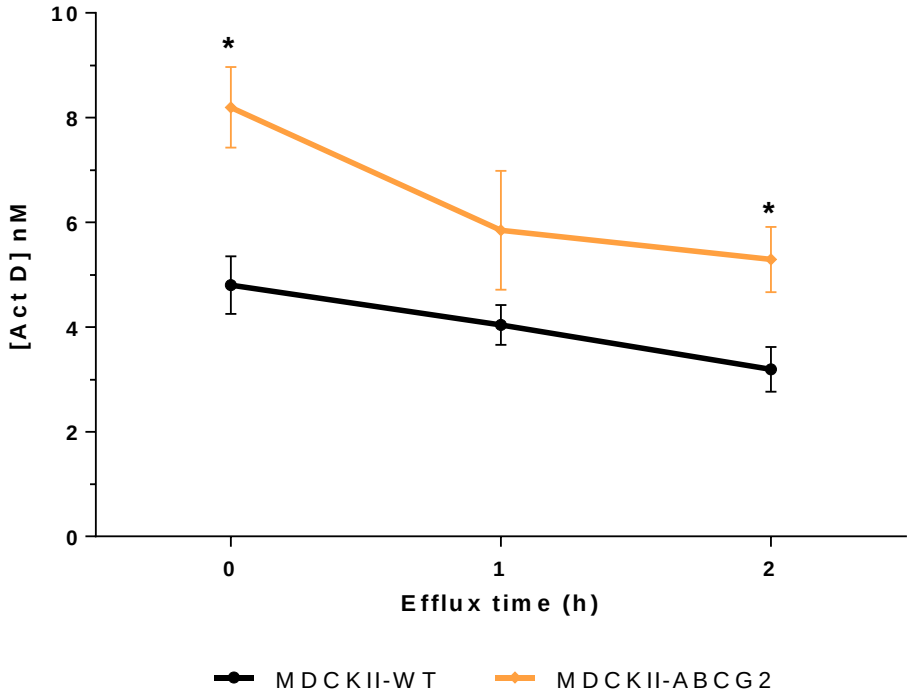

Figure 2.19. Cellular efflux of Act D in MDCKII-WT and MDCKII-ABCG2 cell lines following a 4h incubation.

After 4 days growth, cells were incubated with Act D (0.1µM) for 4h, following which the 0h time point was extracted and spiked media was removed and replaced with blank media for the 1 and 2h time points. Intracellular concentrations were determined by LC/MS analysis. Each point represents mean ± SEM from ≥ 3 experiments. *$p<0.05$ compared to MDCKII-WT at the same sample time.

2.3.7 Drug transport inhibition assay using doxorubicin and actinomycin D

Inhibition of ABCB1-mediated efflux was used to confirm the results obtained from Act D growth inhibition and intracellular drug accumulation experiments (Figure 2.20). Pre-incubation with Act D (0-100µM) resulted in an increase in intracellular levels of the fluorescent ABCB1 substrate doxorubicin. Intracellular doxorubicin levels in the parental cells did not appear to increase further following Act D concentrations of 0.1-1µM, whereas in the ABCB1 over-

expressing cell line concentrations of 1-25μM were required to inhibit doxorubicin efflux.

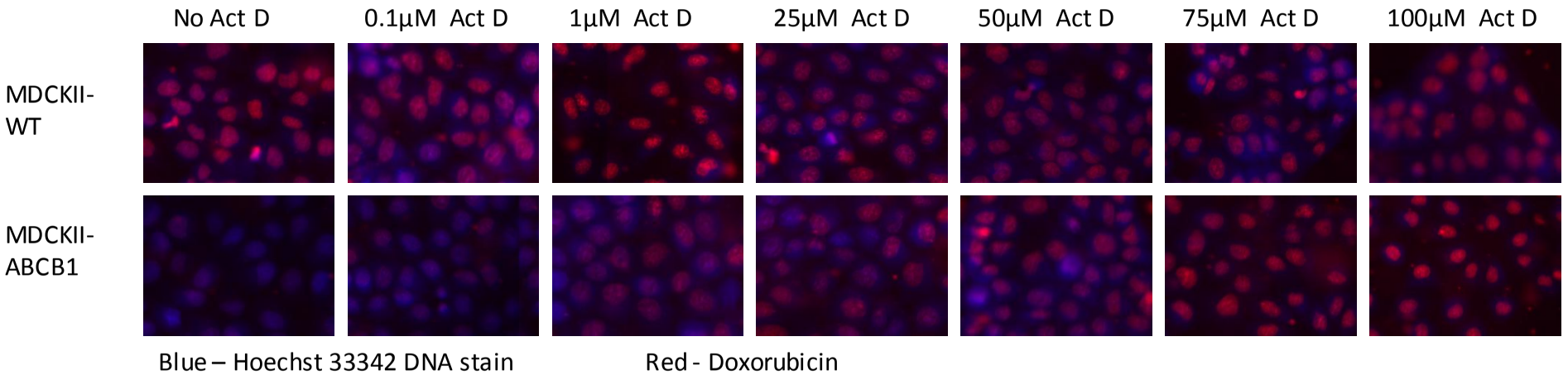

Figure 2.20. Act D inhibition assay using the fluorescent ABCB1 substrate doxorubicin.

MDCKII-WT and MDCKII-ABCB1 cell lines were pre-incubated with varying concentrations of Act D for 1h and 10µM of fluorescent ABCB1 substrate doxorubicin was added for a further 1h, to assess doxorubicin efflux in the presence of Act D.

2.4 Discussion

Studies were undertaken to determine the *in vitro* transport of Act D and whether Act D is a substrate for clinically-relevant ABC transporters. Data generated from these *in vitro* studies may provide useful information which has the potential to help us understand inter-patient variability in Act D pharmacokinetics (Veal *et al.*, 2005). Cellular resistance to Act D has previously been reported in a Chinese hamster ovary model (Biedler and Riehm, 1970), and confirmed to be caused by ABCB1 (Juliano and Ling, 1976). However, no detailed subsequent study has been conducted to confirm the transport of Act D by ABCB1 or other ABC transporters.

In the current study the transport of Act D by a number of ABC transporters was characterised. Using the well-studied MDCKII cell line model, transporter expression was initially confirmed by western blot analysis. Uniform high ABCB1 expression was demonstrated over 4 days in MDCKII-ABCB1 compared to all other cell lines. However, low-level ABCB1 expression was observed in all cell lines due to endogenous canine ABCB1 expression. High ABCG2 expression was observed in MDCKII-ABCG2 cell lines, but not for other cell lines. Expression analysis for ABCC1 and ABCC2 was only performed in MDCKII-WT and their over-expressing cell lines due to time constraints. MDCKII-ABCC1 cells had high expression levels of ABCC1 compared to the parental cell line and similarly MDCKII-ABCC2 cells had high expression of ABCC2 compared to the parental cells.

Following confirmation of appropriate ABC transporter expression and determination of an appropriate seeding density, cell function was assessed with known transporter substrates using GI assays. Doxorubicin, one of the

original anti-cancer agents shown to be a substrate for ABCB1, was first established as an ABCB1 substrate in doxorubicin resistant MCF-7 breast cancer cells. Doxorubicin resistant cells had GI_{50} values 200-fold higher than the WT cell line, with a 45-fold amplification of ABCB1 cDNA (Fairchild *et al.*, 1987). In this study, MDCKII-ABCB1 expressing cells were 16-fold less sensitive to doxorubicin than MDCKII-WT, indicating active ABCB1 efflux. This was further confirmed by inhibition of ABCB1 with the competitive substrate verapamil, causing almost complete restoration of sensitivity. MDCKII-ABCC2 cells were 17-fold less sensitive than the parental cell line to SN-38, a known ABCC2 substrate, with small differences in sensitivity also being seen in MDCKII-ABCB1 and MDCKII-ABCG2 cell lines (2- and 1.3-fold respectively), confirming active efflux of SN-38 by ABCC2. Specific reversal of resistance by inhibition was not possible due to the lack of an available specific inhibitor. Finally, MDCKII-ABCG2 cells were 10-fold less sensitive than MDCKII-WT to mitoxantrone, an ABCG2 substrate. Inhibition of ABCG2 by KO143 (0.2µM) caused complete reversal of resistance only in the MDCKII-ABCG2 cell line, therefore confirming active transport of mitoxantrone by ABCG2.

MDCKII-WT, -ABCB1, -ABCC2, and –ABCG2 cell lines were therefore functioning as a model to study the transport of ABCB1, ABCC2 and ABCG2 substrates. MDCKII-ABCC1 over-expressing cells were sourced late in the study, therefore it was not possible to fully test their function. However, transporter expression was confirmed by western blot analysis and MDCKII cell lines are a recognised model for studying ABC transporters and their substrates.

GI studies initially confirmed Act D as an ABCB1 substrate as a 59-fold difference in sensitivity was seen between the parental and ABCB1 over-expressing cells. This indicates that Act D is removed from MDCKII-ABCB1 cells, therefore decreasing its growth inhibitory effect. Verapamil was subsequently used to successfully reverse this resistance, confirming the role of ABCB1 in decreasing the growth inhibitory effect.

Transport was further investigated by directly looking at intracellular concentrations of Act D in both MDCKII-WT and MDCKII-ABCB1 cell lines. A 7.9-fold lower intracellular accumulation was observed in cells over-expressing ABCB1 at a fixed concentration of Act D (0.01µM) over a 12h period, confirming that Act D was being actively removed from the MDCKII-ABCB1 cells by ABCB1. Again, verapamil was used to inhibit ABCB1-mediated transport, resulting in a concentration-dependent increase in Act D accumulation in MDCKII-ABCB1 cells.

Cellular efflux experiments over 4h further confirmed lower Act D accumulation in ABCB1 expressing cells. Following removal of spiked media a 5.5-fold time-dependent reduction in intracellular levels in MDCKII-ABCB1 cells was observed compared to a 1.5-fold reduction in MDCKII-WT 2h after drug removal.

The inhibition assay was used to demonstrate that doxorubicin fluorescence was reduced in MDCKII-ABCB1 cells, as compared to MDCKII-WT cells. Incubating cells with increasing concentrations of Act D resulted in competition for ABCB1 efflux, inhibiting doxorubicin removal from cells. This caused an increase in doxorubicin fluorescence in both cell lines, with Act D demonstrating

a higher affinity for efflux than doxorubicin. Due to the over-expression of ABCB1, a greater concentration of Act D was required to saturate the transporter and prevent doxorubicin efflux in MDCKII-ABCB1 cells compared to MDCKII-WT. These four independent assessments strongly indicate that Act D is a substrate of the efflux protein ABCB1.

Growth inhibition assays showed lower sensitivity in both MDCKII-ABCC1 and MDCKII-ABCC2 cell lines compared to MDCKII-WT (2-fold and 3.2-fold respectively). Although initial investigations into ABCC2 function were not carried out in combination with an inhibitor due to the lack of appropriate specific inhibitors, the pan-ABCC family inhibitor MK571 was used to investigate Act D transport. Upon inhibition with MK571, a 4-fold increase in sensitivity was seen in MDCKII-ABCC1, whilst in the MDCKII-ABCC2 cell line treatment with MK571 had no effect on Act D sensitivity. However, MDCKII-WT were also 3-fold more sensitive when treated with MK571, possibly indicating either some low-level toxicity when incubating with 25μM MK571 or endogenous ABCC1/2 expression.

Again the role of these transporters was investigated with intracellular accumulation and cell efflux assays. Over 12h, accumulation was significantly lower in both MDCKII-ABCC1 (2.45-fold) and MDCKII-ABCC2 (3.5-fold) cell lines, as compared to MDCKII-WT, providing additional confirmation of the results of the GI studies, that Act D is undergoing active ABCC1 and ABCC2 mediated efflux. To further investigate this, the pan-ABCC family inhibitor MK571 was used. Over 4h, a concentration-dependent increase in Act D accumulation was observed in both the ABCC1 and ABCC2 over-expressing cell lines, but this was not significant. In cellular efflux experiments a significant

time-dependent reduction in intracellular Act D was observed over 1 h in ABCC2 over-expressing cells (1.6-fold), compared to the parental cell line, further supporting the GI and intracellular accumulation assay data suggesting ABCC2-mediated transport.

No evidence of Act D transport by ABCG2 was found during GI, intracellular accumulation or cellular efflux assays. Interestingly, intracellular Act D concentrations were consistently higher in MDCKII-ABCG2 cells than MDCKII-WT cells throughout both intracellular (1.6-fold between 2-6 hours) and cellular efflux assays (1.7-fold after 4 hours), however, this was not reflected in GI studies. Evidence from investigations in rats suggests that Oatp1 and Oatp2 are bidirectional import transporters, using endogenous glutathione as an electrochemical driving force for transporter function (Li *et al.*, 1998; Li *et al.*, 2000). The data here could suggest a role for ABCG2 as a bidirectional transporter, importing Act D into the cell causing an increase in intracellular Act D concentrations and a decrease in Act D efflux. However, as ABCG2 transport is ATP-dependent, co-transport would not be expected and as such there are no data available in the literature to support this. In MDCKII-ABCG2 cells, ABCG2 is present on the apical membrane, removing substrates from the cell (Pavek *et al.*, 2005), it is therefore unlikely that this effect is the result of ABCG2 over-expression. According to densitometry performed on MDCKII-ABCG2 cells after being probed for ABCB1, lower expression of endogenous ABCB1 was observed in the MDCKII-ABCG2 cell lines (23-fold), which could give rise to reduced Act D efflux in these cells, therefore increasing Act D intracellular accumulation.

The data presented here confirm, through a systematic investigation, that Act D is a substrate for ABCB1, ABCC2 and to lesser extent ABCC1. ABCB1 and ABCC2 are both present at the apical membrane in the liver and kidneys eliminating substances from the body via the bile and urine. It is therefore possible that these transporters have an important role in determining the pharmacokinetics of Act D. Data generated in ABC transporter knockout mouse models have highlighted the potential importance of ABC transporter expression, demonstrating altered pharmacokinetics when mice are administered ABC transporter substrates (Schinkel *et al.*, 1996; van Asperen *et al.*, 1999; Lagas *et al.*, 2010). The impact of ABCB1 and ABCC2 on the transport of Act D *in vivo* must therefore be investigated in an appropriate model.

Chapter 3. Pharmacokinetics and tissue distribution of actinomycin D in mice

3.1 Introduction

Pharmacokinetic studies in animals and patients investigate the fate of externally administered compounds through the processes of absorption, distribution, metabolism and excretion. These processes are governed by the expression of drug-metabolising enzymes and transporter proteins, as well as physiological functions such as glomerular filtration. The presence of ABC transporters at sites such as the intestinal lumen prevents absorption of orally administered drugs, whereas in the liver and kidney they facilitate the elimination of drugs via the bile and urine.

The availability of ABC transporter knockout mice has made it possible to study the impact of individual ABC transporters and to investigate the role of drug transporters in influencing the pharmacokinetics of drugs. It is often difficult to predict the effect of complete systemic knockout, and as such results vary substantially between different chemotherapeutic agents. Administration of etoposide, both orally and intravenously, in Abcb1a/1b$^{-/-}$;Abcc2$^{-/-}$ mice causes a significant increase in plasma $AUC_{0-\infty}$, due to reduction of urinary and biliary excretion (Lagas *et al.*, 2010). In other studies, reduced concentrations of vemurafenib were seen *in vitro* in MDCKII-ABCB1 and MDCKII-Abcg2 cells, but there was no increase in plasma exposure in the subsequent *in vivo* study in Abcb1a/1b$^{-/-}$;Abcg2$^{-/-}$ mice. Instead vemurafenib concentrations were higher in the brain in the knockout mice, due to the absence of Abcb1a, Abcb1b and Abcg2 at the blood-brain barrier (Mittapalli *et al.*, 2012).

To date, limited *in vivo* pre-clinical pharmacokinetic studies have been performed with Act D. Using tritiated Act D in rats, monkeys and dogs rapid depletion from plasma was been observed, coupled with increased linear accumulation in tissues except the brain with 12-31% of Act D excreted via the urine depending on species (Galbraith and Mellett, 1975; Lutz *et al.*, 1977). Preliminary Act D pharmacokinetic data in 31 patients suggest a marked variability in exposure in patients being treated with Act D (Veal *et al.*, 2005).

The evidence presented in Chapter 2 suggests transport of Act D by ABCB1, ABCC1 and ABCC2 *in vitro* which could, in part, be responsible for the observed inter-patient variation in Act D exposure. In this chapter, the relevance of this will be investigated in an appropriate pre-clinical model. Mice lacking Abcb1a/1b (Abcb1a/1b$^{-/-}$) and Abcc2 (Abcc2$^{-/-}$) as well as their WT counterparts will be used to assess the role of Abcb1a/1b and Abcc2 in the transport of Act D *in vivo*.

3.2 Materials and methods

3.2.1 Materials

Mice were treated with Act D (Merck Sharp & Dohme, UK) supplied by the Pharmacy, Royal Victoria Infirmary, Newcastle upon Tyne.

3.2.2 Preliminary pharmacokinetic study in CD-1 mouse and ABC-transporter knockout mice

All *in vivo* experiments were reviewed and approved by the institutional animal welfare committee, and performed according to National Cancer Research Institute Guidelines (Workman *et al.*, 2010) and the Animals (Scientific Procedures) Act 1986. CD-1 mice were chosen to carry out a preliminary pharmacokinetic study in non-knockout mice to confirm the current LC/MS method would be sufficiently sensitive and the chosen dosage regimen would provide plasma concentrations of Act D similar to those seen in human patients. For the initial study, 12 female CD-1 mice were used. ABC transporter knockout mice were chosen based on results obtained during *in vitro* investigations. Abcb1a/1b$^{-/-}$(Schinkel *et al.*, 1994; Schinkel *et al.*, 1997), Abcc2$^{-/-}$ (Vlaming *et al.*, 2006) constitutive knockout mice, along with the parental FVB strain were supplied by Taconic (Hudson, USA) with 12 mice used per strain.

3.2.3 Treatment regimen

Mice were dosed based on the equivalent dose in children of 1.5mg/m^2 (0.06mg/kg). Using the species conversion factor from human to mouse (dose (mg/kg) x 12) an initial dose of 0.72mg/kg was obtained (Freireich *et al.*, 1966). However previous Act D *in vivo* data demonstrated that using 0.5mg/kg in mice resulted in limited toxicity over 24h, but an increase in dose to 1mg/kg caused

toxicity and death in 9 out of 10 mice (Robinson and Waksman, 1942), therefore 0.5mg/kg was chosen. A 500µg vial of Act D suitable for *in vivo* administration was reconstituted in 1.1ml ddH_2O to obtain a 500µg/ml solution. This was further diluted 10-fold to 50µg/ml in saline and administered intravenously at 10ml/kg (0.5mg/kg) via the tail vein.

3.2.4 Mouse plasma collection

At the appropriate sample time, mice were anesthetised with 10 ml/kg of an anaesthetic cocktail (0.75mg/kg fentanyl citrate, 25mg/kg fluanisone, 12.5mg/kg midazolam), and euthanized by cervical dislocation. Blood was taken via cardiac puncture using a heparin-coated needle and syringe, and placed in 1.5ml capped vials. Plasma was separated by centrifugation (1,000g for 5min) and stored at -20^{o}C until needed.

3.2.5 Mouse tissue collection

To assess Act D concentrations in tissue, each mouse was dissected following cardiac puncture and the brain, liver and kidneys were removed, wrapped in aluminium foil and immediately snap-frozen in liquid nitrogen. Once all animals had been dissected, tissues were stored at -80^{o}C until needed.

3.2.6 Analysis of actinomycin D in mouse plasma by LC/MS

Details of the LC/MS method are provided in 2.2.10 and assay validation can be seen in 4.2.3. Act D chromatography following extraction from mouse plasma and tissue was identical to that from human plasma. The chromatograms and retention times for all matrices were identical (3.87-3.88min, Figure 3.1). Repeated analyses of both plasma and tissue samples were consistent,

therefore it was deemed appropriate to use the same assay to analyse Act D concentrations in mouse plasma and tissues.

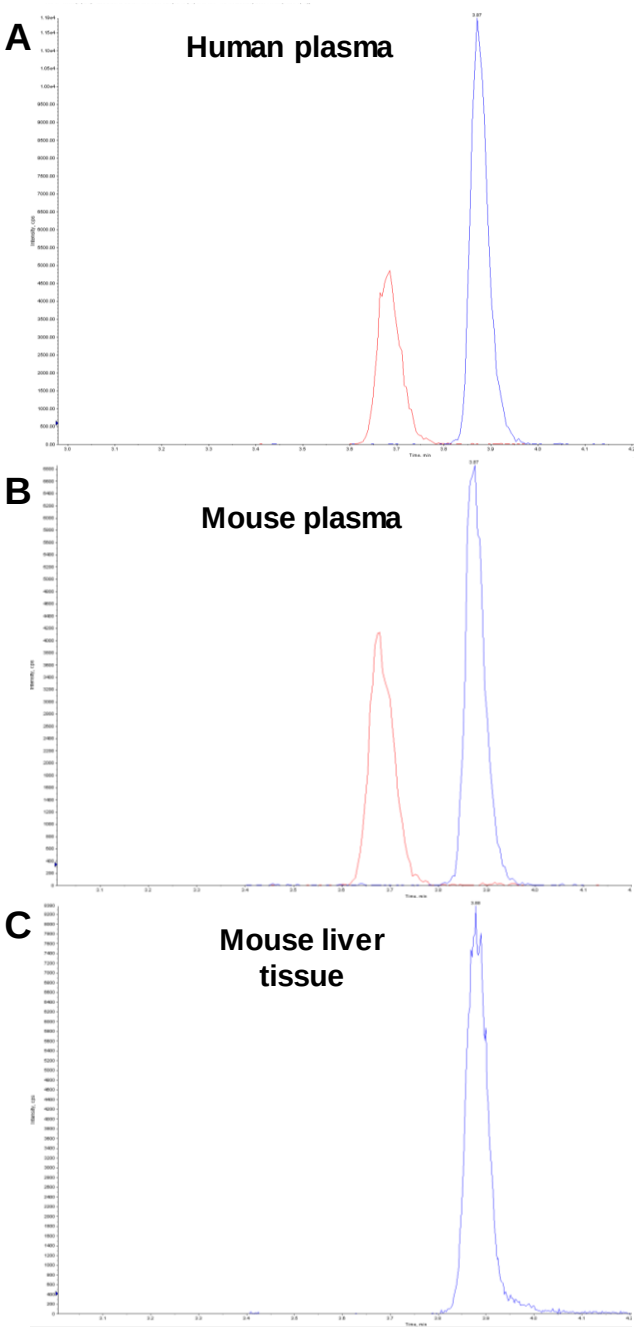

Figure 3.1. Comparisons between chromatograms of Act D (A) extracted from human plasma, (B) in extracted from mouse plasma and (C) extracted from mouse liver tissue.

The retention time of Act D in (A) human plasma (3.87min), (B) mouse plasma (3.87min) and (C) mouse liver tissue (3.88min) are all comparable between matrices.

3.2.6.1 Preparation of working standards, QCs and internal standard

Act D was weighed and dissolved in methanol to obtain a 1mg/ml solution. Separate Act D and stock solutions were prepared for working standards and QCs. The 1mg/ml working standard stock solution was used to make a 1µg/ml solution in human plasma, and from this the following working standards were prepared in human plasma: 10, 5, 2, 1, 0.5 and 0.25ng/ml. The 1mg/ml QC stock solution was used to prepare a 1µg/ml solution in human plasma and from this 10, 2 and 0.5ng/ml QC samples were prepared in human plasma. Working standards and QC samples were stored at -20^{o}C until needed. 7-aminoactinomycin D (7-AD) was used as an internal standard (IS), with a 1mg/ml solution prepared in methanol diluted 1:200 in methanol, to give a 5µg/ml working solution. Both 7-AD solutions were stored at 4^{o}C until needed.

3.2.6.2 Actinomycin D extraction from mouse plasma

Preliminary pharmacokinetic study samples were assayed at 1:3 and 1:10 dilutions (diluted in blank human plasma) as the concentration of Act D in the mouse plasma was unknown. Subsequently, analysis of knockout mice samples was carried out at 1:10 dilutions for the samples at 15 and 30min and 1:3 dilutions for the samples at 2 and 6h, ensuring all sample were within the range of the standard curve.

The 5µg/ml IS working solution was diluted 1:200 in ddH_2O to give a 25ng/ml solution. Patient samples were centrifuged at 15,300g for 5min, then 0.6ml was mixed with 0.6ml ddH_2O and 50µl 25ng/ml IS solution (final concentration 1ng/ml) in a 10mm borosilicate tube. Gilson Aspec XL4 (Anachem, UK) and Oasis solid phase extraction (SPE) cartridges (Part no. WAT058882) (Waters, UK) were used for extraction, using 100% acetonitrile (MeCN), 5% MeCN in

ddH_2O and ddH_2O as follows: (1) SPE columns were conditioned with 1ml 100% MeCN, (2) SPE columns were washed with 1ml ddH_2O, (3) 1ml sample was loaded onto the SPE column, (4) SPE was washed with 5% MeCN, and (5) Act D was eluted with 2ml 100% MeCN into a 12mm borosilicate tube. Samples were then evaporated to dryness under nitrogen using a Turbovap LV solvent evaporator (Biotage, Uppsala, Sweden).

3.2.7 Preparation of mouse liver and kidney samples for LC/MS analysis

The concentration of Act D in mouse liver and kidneys was determined using the method of addition. Tissue samples were washed in PBS, weighed and homogenised in 3 x PBS (i.e. 1g tissue: 3ml PBS) using a Pro scientific 200 Homogeniser (Oxford, CT, USA). Standards were prepared in MeCN by diluting the 1mg/ml working solution to 10µg/ml. From this working standards were then prepared in MeCN at concentrations of 150, 100 and 50ng/ml. After appropriate dilution in PBS, 50µl of the same tissue sample was pipetted into eight 1.5ml capped vials with 10µl of 0, 50, 100 or 150ng/ml standard, added to the duplicate samples. To each 1.5ml vial, 440µl MeCN was added to extract Act D and the tubes were mixed by vortex for 10s. These tubes were then centrifuged at 15,300g for 5min, transferred to 12mm borosilicate tubes and evaporated to dryness under nitrogen using a Turbovap LV solvent evaporator (Biotage, Uppsala, Sweden). Dried samples were then reconstituted in 200µl mobile phase (50:50 A:B), mixed by vortex for 20s and centrifuged at 15,300g for 5min. The supernatant was transferred to small volume insert tubes and placed in the Peltier tray for analysis. Results are expressed as µg/L, however, these have been normalised to tissue weight.

3.2.8 Preparation of mouse brain samples for LC/MS analysis

The mouse brain tissue was washed, weighed and homogenised as per 3.2.7. Due to low sample volumes of brain tissue following homogenisation, it was not possible to produce an adequate spiked standard curve for the analysis of Act D concentrations in the brain. It was therefore not possible to use the method of addition and 1ml MeCN was added to 100µl of undiluted brain homogenate to extract Act D within the tissue. Samples were then centrifuged, dried down under nitrogen and reconstituted as per 3.2.7. Results are expressed as peak area (pa), however, these have been normalised to tissue weight.

3.2.9 RNA extraction from mouse liver

RNA extraction was carried out using a Qiagen RNeasy® Mini Kit according to the manufacturer's instructions (Qiagen, Crawley, UK). Briefly, between 15-30mg of liver tissue per sample (one per mouse) was weighed and homogenised in Buffer RLT using a Pro Scientific 200 Homogeniser (Oxford, CT, USA). Following centrifugation, the supernatant was mixed with 70% ethanol and transferred to an RNeasy spin column, where cell debris was removed by further centrifugation and column washing with Buffer RW1 and twice with Buffer RPE. Total RNA was then eluted using RNase-free water. RNA concentration was determined against an RNase-free water blank using a NanoDrop ND-1000 (Thermo Scientific, Rockford, USA).

3.2.10 Reverse transcription of RNA to cDNA

Reverse transcription was carried out using the Reverse Transcription System supplied by Promega (Southampton, UK). All extracted RNA from mouse liver was reverse transcribed in the same assay to ensure uniform PCR conditions

and amplification for all samples. For each sample, a 20µl reaction mix (5mM $MgCl_2$, 1x Reverse transcription buffer, 1mM dNTP mixture, 1u/µl Recombinant RNasin® Ribonuclease Inhibitor, 15u/µg AMV Reverse Transcriptase, 0.5µg Random primers, Nuclease-Free water and 1µg sample total RNA) was set up on ice in 0.2ml PCR tubes. Samples were then incubated at room temperature for 10min, and transferred to a GeneAmp PCR System 9700 (Applied Biosystems, California, USA). The PCR conditions used are shown in Figure 3.2, cDNA was stored at 4°C for short-term storage or -20°C for long term storage.

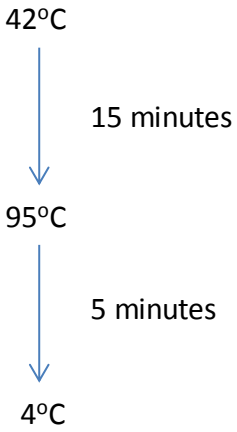

Figure 3.2. Reverse transcriptase PCR conditions.

3.2.11 PCR primers, design and validation for detection of mouse Abcb1a, Abcb1b and Abcc2

Validated QuantiTect Primer Assays for Abcb1a and Abcc2, along with mouse Gapdh as an endogenous control were supplied by Qiagen (Crawley, UK). Acceptable primer assay kits were not available for Abcb1b, therefore the Abcb1b cDNA sequence was used to design 4 primer sets (Table 3.1) which were supplied by Invitrogen (Paisley, UK).

Primer set	Forward	Reverse	Exon
Cyclophilin	5′-ATGGCACTGGTGGCAAGTCC-3′	5′-TTGCCATTCCTGGACCCAAA-3′	
1	5′–ACGTGAGGTGGTGATGGAGTTTGA-3′	5′-CCAGCCAATCTGCATAGCGAAACA-3′	1/2
2	5′–ACGTGAGGTGGTGATGGAGTTTGA-3′	5′-CCCAAATACGCCAACAGCAGGTTT-3′	3/4
3	5′-ATCAGCAACAGCAGTCTGGAGGAA-3′	5′-GGCACCAAAGTGAAACCTGGATGT-3′	5/6
4	5′-TGTGGATGAAGATGTGCCTCTGGT-3′	5′-CAGCGCAAAGTACGCCAACAAGTA-3′	16/17

Table 3.1. Primer sequences for 4 Abcb1b primer sets for Real-time PCR.

Real-time PCR was performed using a Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX kit supplied by Invitrogen (Paisley, UK). Validation of all primers was carried out using five serial 1 in 10 dilutions of WT mouse cDNA (3.2.10), adding 1µl of cDNA per well in triplicate to a MicroAmp® Optical 384-well plate (Applied Biosystems, California, USA). According to the manufacturer's instructions, 9µl of reaction master mix (1x SYBR green master mix, 0.2µM forward and reverse primers) was added to each well. Abcb1a and Abcc2 primers were validated against Gapdh as an endogenous control, whilst Abcb1b was validated against cyclophilin. No template controls in triplicate were also used for each primer set. Plates were sealed using MicroAmp® Optical Adhesive Film and Real-time PCR under standard conditions (Figure 5.3) was carried out using an ABI PRISM® 7900HT (Applied Biosystems, California, USA). Real-time PCR data was analysed using SDS version 2.3 and data was exported to Microsoft Excel 2010 for further analysis. Primers were accepted as valid if the Ct value (cycle threshold) data was linear over 3 concentrations of

cDNA and the slope of the ΔCt (the difference between endogenous control Ct and target primer Ct) against cDNA concentration was not greater than 0.1.

3.2.12 Detection of Abcb1a, Abcb1b and Abcc2 by Real-time PCR

All previously generated cDNA (3.2.10) was diluted 1 in 10 to produce a working stock for all samples. Each sample was run twice in triplicate on a 384-well plate, once using endogenous control primers (Gapdh or Cyclophilin), and once using the primers for the gene of interest (Abcb1a, Abcb1b or Abcc2). Expression of Abcb1a, Abcb1b and Abcc2 was analysed in all samples and compared to the endogenous control. Plate set up, master mix, Real-time PCR and software were all as previously described (3.2.11). The ΔC_T ($C_{T\ target} - C_{T\ reference}$) was calculated individually for each cDNA sample, following which outliers and contaminated samples were removed from the data set. The $\Delta\Delta C_T$ ($\Delta C_{T\ test\ sample} - \Delta C_{T\ calibrator\ sample}$) was also calculated, representing the difference in expression relative to one representative sample from the WT mice group.

3.2.13 Statistical analysis

Pharmacokinetic data from thirty-six mice were analysed by WinNonlin Professional version 5.3 (Pharsight Corp, Mountain View, CA, USA). Data from three mice per time point were combined and non-compartmental analysis (NCA) was performed once for each mouse strain. The area under the plasma concentration versus time curve from drug administration to 6h ($AUC_{0\text{-}6h}$) were estimated where possible using the logarithmic trapezoidal rule. AUC extrapolation to infinity ($AUC_{0\text{-}\infty}$) was performed using an estimate of $t_{1/2}$ from

slope of the concentration vs. time curve using the final two pharmacokinetic sample time data points. Clearance was estimated as dose divided by $AUC_{0-\infty}$.

It was not possible to statistically compare overall exposure data between mouse variants as samples were not paired and once all pharmacokinetic samples had been combined at the appropriate time points, only one curve per mouse variant existed. Therefore mean Act D concentrations in plasma, liver, kidney and brain at individual time points were compared as per the intracellular accumulation cellular efflux statistical analysis which can be found in 2.2.12. Mean tissue-to-plasma ratios and transporter expression data were compared using the one-way ANOVA. Multiple comparisons were used to analysis differences in Abcb1a/1b-/- mice and Abcc2-/- against WT mice and individual time points. Bonferroni's multiple comparisons were used to correct for this multiple testing. $p<0.05$ following Bonferroni's correction was taken as significant. LC/MS data were analysed as per 2.2.12.

3.3 Results

3.3.1 Actinomycin D pharmacokinetics in CD-1 mice

No previous pharmacokinetic study of Act D had been performed in mice, therefore an initial study in CD-1 mice was undertaken to determine an adequate dosage regimen and to ensure the current LC/MS method would be sufficiently sensitive. Following administration of 0.5mg/kg Act D to CD-1 mice, a time-dependent decrease in Act D plasma concentrations was observed (Figure 3.3). The overall exposure to Act D in CD-1 mice ($AUC_{0\text{-}6h}$ 131.7µg/L.h, Table 3.2) was higher but comparable to that seen previously in patients ($AUC_{0\text{-}6h,}$ range 18.7-81.7 µg/L.h) (Veal *et al.*, 2005). No visible signs of acute toxicity were seen with this single dose, therefore a dose of 0.5mg/kg was taken forward into the knockout mouse study.

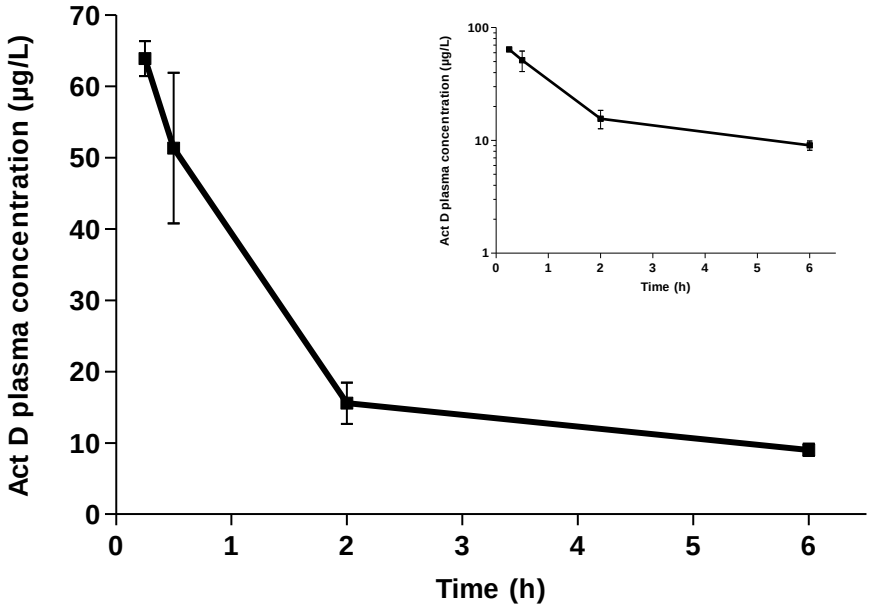

Figure 3.3. Act D plasma concentration-time profile for CD-1 mice.

CD-1 mice were treated with 0.5mg/kg Act D administered via the tail vein, following which blood samples were taken at 15min, 30min, 2h and 6h. Act D concentrations in mouse plasma samples were determined by LC/MS analysis. Inset – semi-logarithmic representation of data. Each point represents mean ± SEM from 3 mice.

Time (h)	Act D concentration (µg/L)
0.25	63.9 ± 3.5
0.5	51.3 ± 15.0
2	15.6 ± 4.1
6	9.0 ± 1.2
AUC_{0-6h}, µg/L.h	132†
$AUC_{0-\infty}$ µg/L.h	160†
Cl (ml/min)	3.13†
C_{max}, µg/L	63.9 ± 3.5
T_{max}, h	0.25
$t_{1/2}$, h	2.15†

Individual plasma concentration time points are mean ± SD from 3 mice.

†No SD reported as data is from combined pharmacokinetic sample non-compartmental analysis.

Table 3.2. Plasma Act D concentrations and pharmacokinetic parameters in CD-1 mice following intravenous administration of 0.5mg/kg Act D

3.3.2 Actinomycin D pharmacokinetics in wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice

To investigate the role of Abcb1a/1b and Abcc2 on pharmacokinetics, a dose of 0.5 mg/kg Act D was administered to WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice and blood samples were taken between 15min and 6h. Following administration, plasma concentrations of Act D decreased with time (Figure 3.4, Table 3.2). Act D plasma concentrations were consistently higher in Abcb1a/1b$^{-/-}$ mice than WT (Figure 3.4, Table 3.3) over the 6h period. The C_{max} at 15min was 1.4-fold higher in Abcb1a/1b$^{-/-}$ mice compared to WT (93.7µg/L vs. 68.6µg/L, Table 3.3, Figure 3.4). A significant 1.7-fold difference in plasma concentration was observed 30min post-administration between Abcb1a/1b$^{-/-}$ and WT (71.6µg/L

vs. 43.3µg/L, p<0.05, Table 3.3/Figure 3.4). Act D plasma concentrations in Abcb1a/1b$^{-/-}$ were 3.3-fold higher than the WT mice (20.1µg/L vs. 6.0µg/L, p<0.0001, Table 3.3, Figure 3.4), resulting in a 1.6-fold higher $AUC_{0\text{-}6h}$ ($AUC_{0\text{-}6h}$; 242µg/L.h vs. 151µg/L.h, Table 3.3) than the parental. In addition, the terminal phase half-life ($t_{1/2}$) was 1.5-fold longer in Abcb1a/1b$^{-/-}$ mice than WT, resulting in a 1.9-fold greater AUC when extrapolating to infinity ($AUC_{0\text{-}\infty}$ 322.6 µg/L.h vs. 168.1µg/L.h, Table 3.3). Clearance (Cl) in Abcb1a/1b$^{-/-}$ was 52% of that observed in WT mice, whilst Cl in Abcc2$^{-/-}$ mice was 1.2-fold higher compared to WT mice. This indicates that absence of systemic Abcb1a/1b is associated with a reduced rate of elimination of Act D, higher Act D plasma concentrations and a higher overall exposure over 6 h.

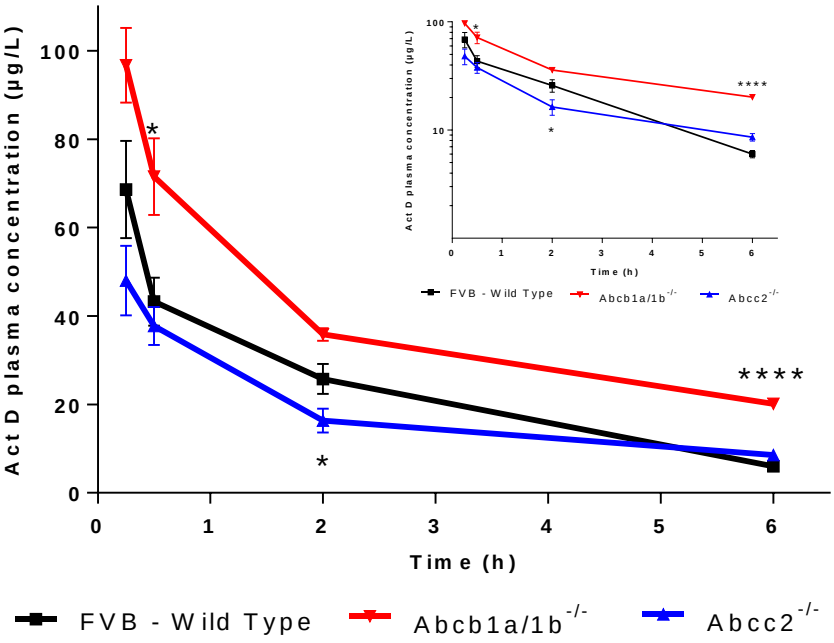

Figure 3.4. Act D plasma concentration-time profiles for wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice.

Act D (0.5mg/kg) was administered to WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice and blood samples were taken at 15min, 30min, 2h and 6h. Act D concentrations in mouse plasma were determined by LC/MS analysis. Inset – semi-logarithmic representation of data. Each point represents mean ± SEM from 3 mice. *p<0.05 and ****p<0.0001 compared to the WT, using the two-way ANOVA with Bonferroni's correction for multiple comparisons.

In contrast to Abcb1a/1b$^{-/-}$ mice, Abcc2$^{-/-}$ mice generally had lower plasma concentrations of Act D than the WT (Figure 3.4, Table 3.3), with significantly lower plasma concentrations being observed in Abcc2$^{-/-}$ mice after 2h (16.3µg/L vs. 25.8µg/L, $p<0.05$, Figure 3.4, Table 3.3). $AUC_{0\text{-}6h}$ in Abcc2$^{-/-}$ mice was 76% of that in WT mice (115µg/L.h vs. 152µg/L.h, Table 3.3) and similarly the C_{max} in Abcc2 deficient mice was 73% of that seen in WT mice (48.0µg/L vs. 68.6µg/L, Figure 3.4/Table 3.3). The $t_{1/2}$ in Abcc2$^{-/-}$ was longer than the parental (2.46h vs. 1.92h), and consequently a similar $AUC_{0\text{-}\infty}$ for parental and Abcc2$^{-/-}$ was observed (168.1µg/L.h vs. 145.2µg/L.h).

	Act D concentration (µg/L)		
	WT	Abcb1a/1b$^{-/-}$	Abcc2$^{-/-}$
0.25h	68.6 ± 15.6	96.7 ± 12.0	48.0 ± 11.1
0.5h	43.3 ± 7.7	71.6 ± 12.3*	37.7 ± 6.1
2h	25.8 ± 4.8	35.8 ± 2.0	16.3 ± 3.7*
6h	6.0 ± 0.7	20.1 ± 0.8****	8.6 ± 1.0
AUC_{0-6h}, µg/L.h	152†	242†	115†
$AUC_{0-\infty}$, µg/L.h	168†	323†	145†
Cl (ml/min)	2.97†	1.55†	3.44†
C_{max}, µg/L	68.6 ± 15.6	96.7 ± 12.0	48.0 ± 11.1
T_{max}, h	0.25	0.25	0.25
$t_{1/2}$, h	1.92†	2.78†	2.46†

Individual plasma concentration time points are mean ± SD from 3 mice.

*p<0.05 and ****p<0.0001 compared to the WT, using the two-way ANOVA with Bonferroni's correction for multiple comparisons.

†No SD reported as data is from combined pharmacokinetic sample non-compartmental analysis.

Table 3.3. Plasma Act D concentrations and pharmacokinetic parameters in WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice following intravenous administration of 0.5mg/kg Act D

3.3.3 Actinomycin D accumulation in wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mouse liver

To investigate the impact of Abcb1a/1b and Abcc2 knockout on Act D accumulation, mouse livers were taken at 15min, 30min, 2h and 6h following administration and Act D concentrations were determined. Act D liver concentrations decreased with time in WT and Abcb1a/1b$^{-/-}$ over 6 hours (Figure 3.5). The C_{max} in Abcb1a/1b$^{-/-}$ was 1.3-fold higher than in the parental (2,323µg/L vs. 1,810µg/L, Table 3.4) and this was seen at the earliest

observation of 15min post-administration. The C_{max} in Abcc2$^{-/-}$ mice was reached 30min post-administration and was 85% of that seen in WT (1,540µg/L vs. 1,810µg/L, Table 3.4). Liver Act D concentrations were not markedly different between knockout and WT mice. As such, no difference was seen in overall liver exposure ($AUC_{0\text{-}6h}$) between knockout and WT mice (Table 3.4). The $t_{1/2}$ in Abcb1a/1b$^{-/-}$ mice was 1.5-fold longer (3.37h vs. 2.21h, Table 3.4) resulting in a higher $AUC_{0\text{-}\infty}$ than the WT (6,594µg/L.h vs. 5,387µg/L.h), although due to the limited data and therefore the difficulty in estimating $t_{1/2}$, these results should be considered with caution.

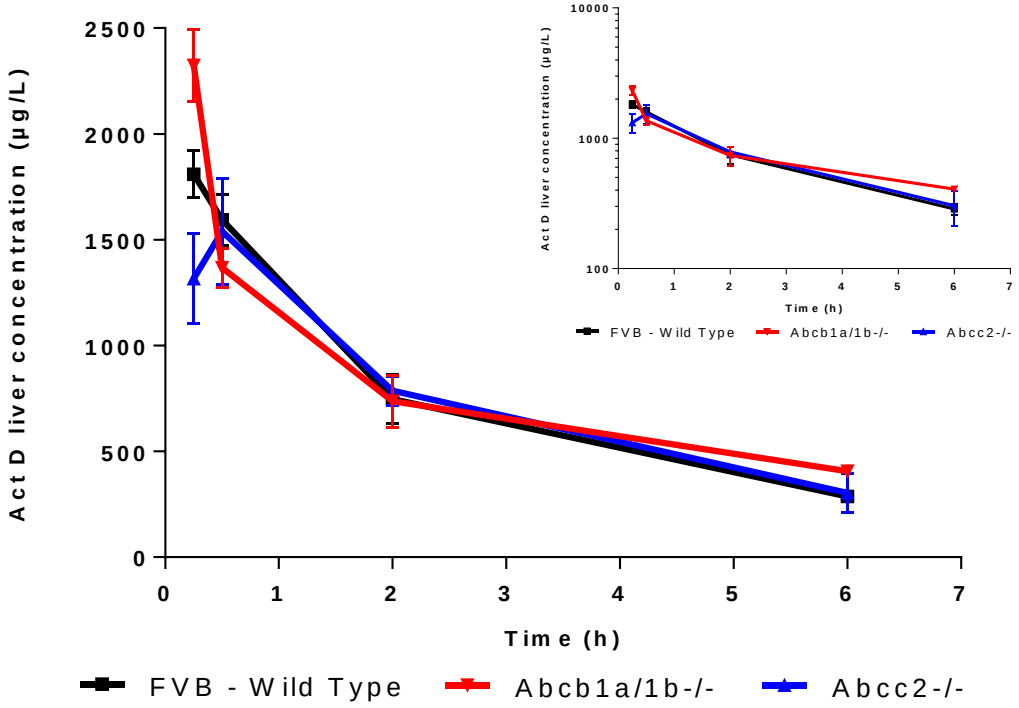

Figure 3.5. Act D liver concentration-time profiles for wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice.

Act D (0.5mg/kg) was administered to WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice and liver samples were taken at 15min, 30min, 2h and 6h. Act D concentrations in mouse livers were determined by LC/MS analysis. Inset – semi-logarithmic representation of data. Each point represents mean ± SEM from 3 mice.

	Act D concentration (µg/L)		
	WT	Abcb1a/1b$^{-/-}$	Abcc2$^{-/-}$
0.25h	1810 ± 155	2323 ± 239	1317 ± 302
0.5h	1593 ± 167	1367 ± 133	1540 ± 356
2h	747 ± 165	737 ± 172	787 ± 99
6h	287 ± 40	407 ± 9	303 ± 128
AUC_{0-6h}, µg/L.h	4473†	4616†	4445†
$AUC_{0-\infty}$, µg/L.h	5387†	6594†	5516†
C_{max}, µg/L	1810 ± 155	2323 ± 239	1540 ± 356
T_{max}, h	0.25	0.25	0.5
$t_{1/2}$, h	2.21†	3.37†	2.44†

Individual liver concentration time points are mean ± SD from 3 mice.

†No SD reported as data is from combined pharmacokinetic sample non-compartmental analysis.

Table 3.4. Liver Act D concentrations and pharmacokinetic parameters in WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice following intravenous administration of 0.5mg/kg Act D

The relationship between Act D accumulation in liver tissue and the plasma concentration (L/P ratio) was determined for all sample times. Over the 6h of exposure to Act D, there was a small trend towards increasing L/P ratio in WT mice (26.4g^{-1} to 47.8g^{-1}) and Abcc2$^{-/-}$ (27.4g^{-1} to 35.4g^{-1}). In contrast, this trend was not observed in Abcb1a/1b$^{-/-}$ mice, as the L/P ratio remained constant over 6h and lower L/P ratio was observed after 6h in Abcb1a/1b$^{-/-}$ mice as compared to WT (20.4g^{-1} vs. 47.8g^{-1}, $p<0.05$, Figure 3.6). This observation suggests that despite overall higher plasma concentrations over 6h in Abcb1a/1b$^{-/-}$ mice, this does not translate into higher accumulation in the liver.

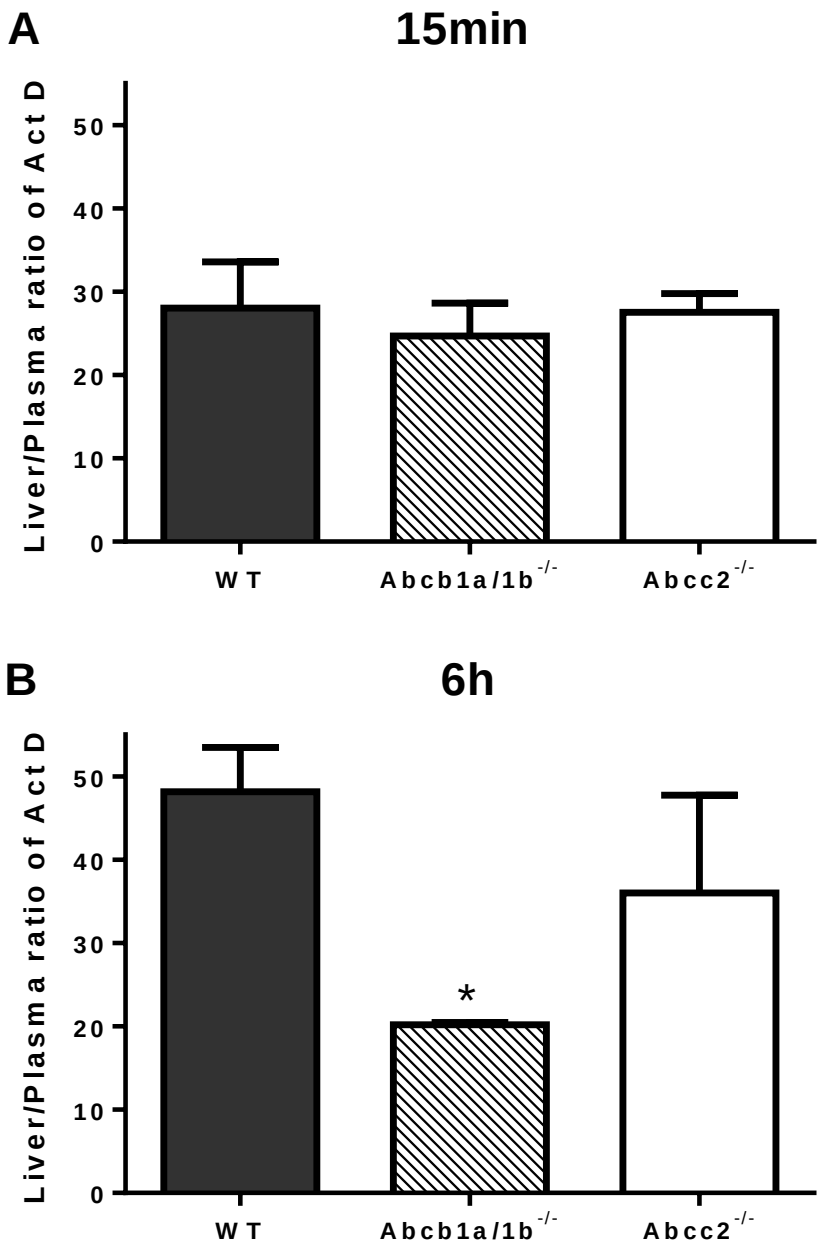

Figure 3.6. Act D liver-to-plasma ratios at 15min and 6h in wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice.

The relationship between Act D liver accumulation and plasma concentration was investigated at 15min (A) and 6h (B). Liver-to-plasma ratios were calculated by dividing the liver concentration by the corresponding plasma concentration at 15min and 6h. Each point represents mean ± SEM from 3 mice. *$p<0.05$ compared to the WT, by using the one-way ANOVA with Bonferroni's correction for multiple comparisons.

3.3.4 Actinomycin D accumulation in wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mouse kidney

To investigate the impact of systemic Abcb1a/1b and Abcc2 removal on Act D accumulation in kidney tissue, mouse kidneys were taken at the time points previously mentioned and Act D concentrations in whole kidney were determined. As with the liver, Act D concentrations in the kidney decreased in all three mouse variants over 6h (Figure 3.7/Table 3.5). Comparable $AUC_{0\text{-}6h}$ and C_{max} values were observed in knockout and WT mice (Table 3.5). Act D accumulation in kidneys from Abcb1a/1b$^{-/-}$ mice was 1.2-fold higher than the WT (13,163µg/L vs. 10,713µg/L) at 30min post-administration, however this difference was not statistically significant. $T_{1/2}$ in Abcb1a/1b and Abcc2 deficient mice were 1.2- and 1.5-fold higher than the WT animals, respectively. A longer $t_{1/2}$ resulted in higher $AUC_{0\text{-}\infty}$ in Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice compared to WT (48,799 and 46,701 vs. 35,754µg/L.h, Table 3.5).

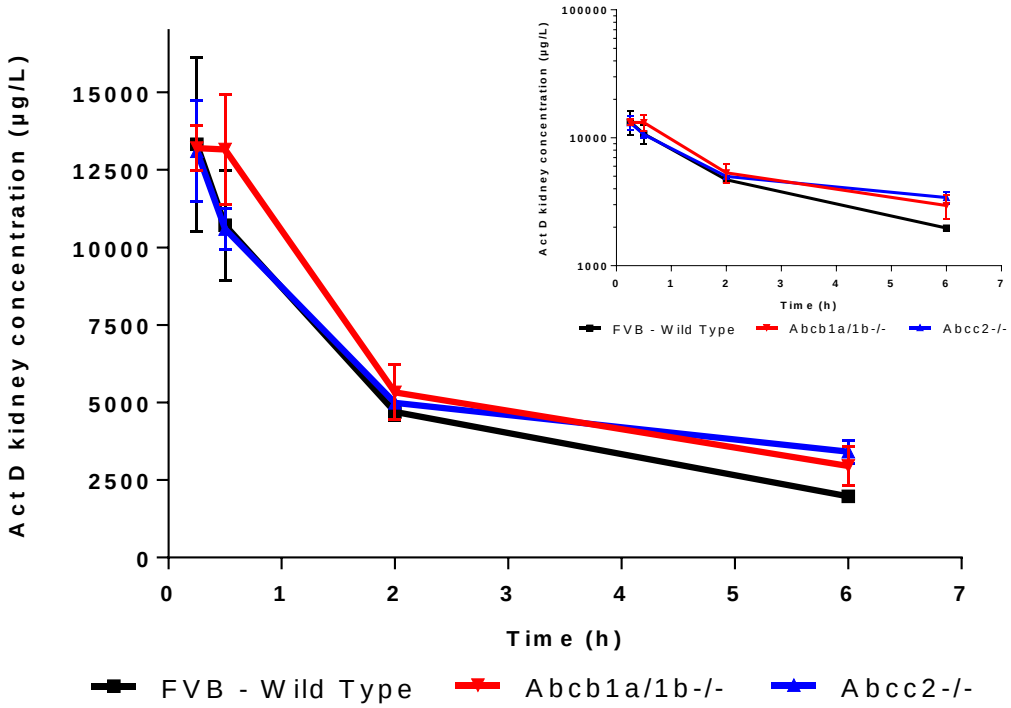

Figure 3.7. Act D kidney concentration-time profiles for wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice.

Act D (0.5mg/kg) was administered to WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice and kidney samples were taken at 15min, 30min, 2h and 6h. Act D concentrations in mouse kidneys were determined by LC/MS analysis. Inset – semi-logarithmic representation of data. Each point represents mean ± SEM from 3 mice.

	Act D concentration (µg/L)		
	WT	Abcb1a/1b$^{-/-}$	Abcc2$^{-/-}$
0.25	13323 ± 3960	13207 ± 1025	13107 ± 2318
0.5	10713 ± 2498	13163 ± 2521	10593 ± 944
2	4693 ± 415	5327 ± 1257	4987 ± 161
6	1970 ± 150	2957 ± 872	3410 ± 527
$AUC_{0\text{-}6h}$, µg/L.h	29552†	35381†	33079†
$AUC_{0\text{-}\infty}$, µg/L.h	35754†	46701†	48799v
C_{max}, µg/L	13323 ± 3960	13207 ± 1025	13107 ± 2318
T_{max}, h	0.25	0.25	0.25
$t_{1/2}$, h	2.18†	2.65v	3.2†

Individual kidney concentration time points are mean ± SD from 3 mice.

†No SD reported as data is from combined pharmacokinetic sample non-compartmental analysis.

Table 3.5. Kidney Act D concentrations and pharmacokinetic parameters in WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice following intravenous administration of 0.5mg/kg Act D

Comparisons between plasma and kidney Act D concentrations (K/P ratio) were also investigated between 15min and 6h. Increases in K/P ratio were observed with time in WT (194g^{-1} to 329g^{-1}, at 15min and 6h, respectively) and Abcc2$^{-/-}$ (273g^{-1} to 398g^{-1}). Again, this trend was not observed in Abcb1a/1b$^{-/-}$ mice. Over 6h, the K/P ratio in Abcb1a/1b$^{-/-}$ remained constant (137g^{-1} to 147g^{-1}). After 6h the K/P in Abcb1a/1b$^{-/-}$ mice was 2.2-fold lower than the WT (147g^{-1} vs. 329g^{-1}, $p<0.05$, Figure 3.8). In agreement with the liver data, this indicates that a high plasma $AUC_{0\text{-}6h}$ in Abcb1a/1b$^{-/-}$ mice does not translate into higher kidney accumulation.

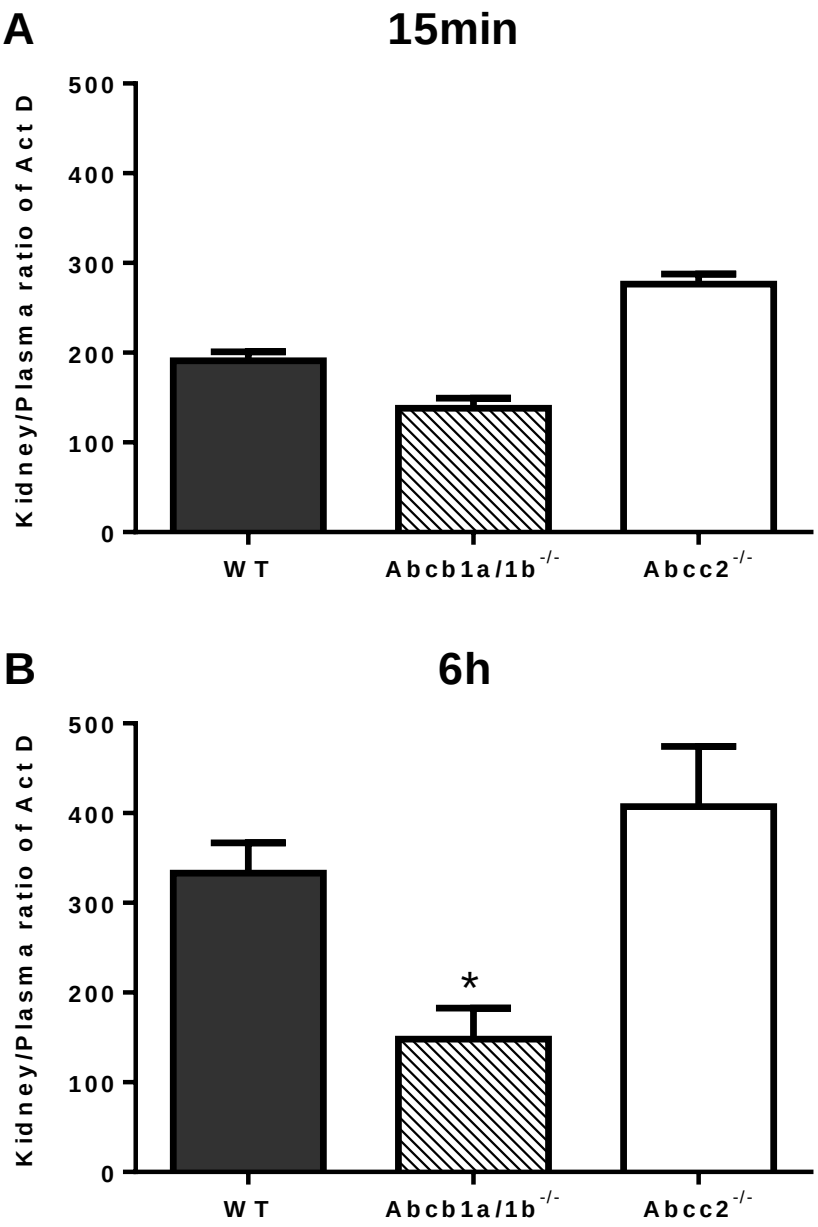

Figure 3.8. Act D kidney-to-plasma ratios at 15min and 6h in wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice.

The relationship between Act D kidney accumulation and plasma concentration was investigated at 15min (A) and 6h (B). Kidney-to-plasma ratios were calculated by dividing the kidney concentration by the corresponding plasma concentration at 15min and 6h. Each point represents mean ± SEM from 3 mice. *p<0.05 compared to the WT, using the one-way ANOVA with Bonferroni's correction for multiple comparisons.

3.3.5 Actinomycin D accumulation in wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mouse brain

Finally, Act D accumulation in the brain of all mouse variants was investigated over 6h; results are expressed as peak area (pa). Act D brain penetration was comparable between different groups at 15min (Figure 3.9, Table 3.6). This was followed by a time-dependent decrease in Act D brain concentrations in WT mice, whereas Act D concentration in the brains of Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice were maintained over 6 hours resulting in 2.3-fold and 1.3-fold higher $AUC_{0\text{-}6h}$ values respectively, compared to WT (6,802 and 3,785 vs. 2,943pa.h, Figure 3.9, Table 3.6). However, Act D concentrations in the brain were highly variable between both animals and time points, with Abcc2$^{-/-}$ mice having the lowest Act D concentration at 2h. $T_{1/2}$ and thus extrapolation to estimate $AUC_{0\text{-}\infty}$ could not be performed for Abcb1a/1b and Abcc2 deficient mice as there was no consistent decrease in Act D concentrations between 2 and 6h.

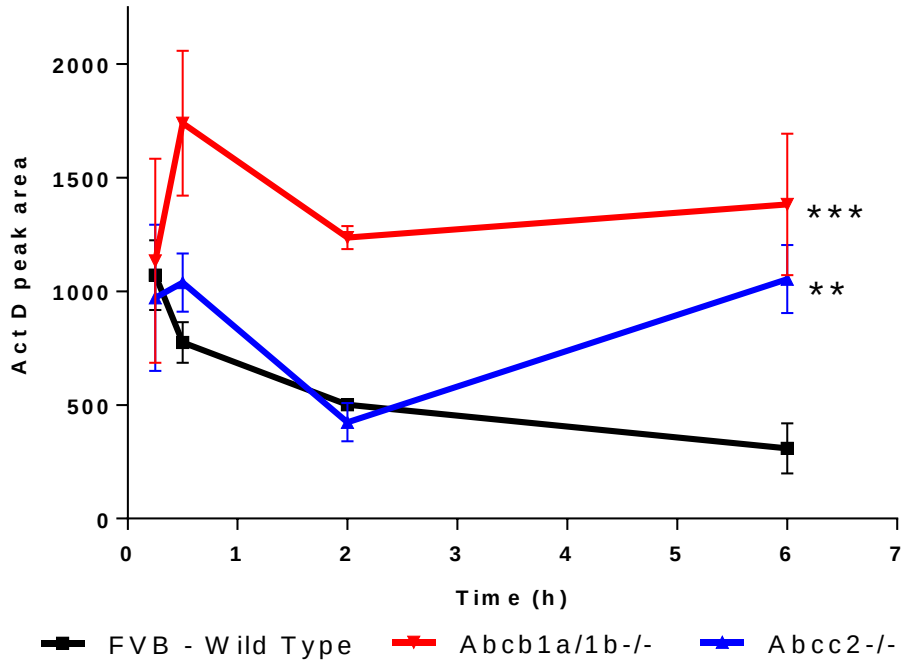

Figure 3.9. Act D brain peak area-time profiles for wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice.

Act D (0.5mg/kg) was administered to WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice and brain samples were taken at 15min, 30min, 2h and 6h. Act D concentrations in mouse brains were determined by LC/MS analysis. Inset – semi-logarithmic representation of data. Each point represents mean ± SEM from 3 mice. **p<0.01 and ***p<0.001 compared to WT at the same time point, using the two-way ANOVA with Bonferroni's correction for multiple comparisons.

	Act D peak area (pa)		
	WT	Abcb1a/1b$^{-/-}$	Abcc2$^{-/-}$
0.25h	1071 ± 217	1134 ± 635	972 ± 455
0.5h	775.3 ± 127	1420 ± 521	1039 ± 181
2h	501 ± 28.9	912.7 ± 461	424.3 ± 120
6h	309 ± 156	1383 ± 439***	733 ± 470**
$AUC_{0\text{-}6h}$, pa.h	2943†	6802†	3785†
C_{max}, pa	1071 ± 217	1420 ± 521	1039 ± 181
T_{max}, h	0.25	0.5	0.5

Individual plasma concentration time points are mean ± SD from 3 mice.

**p<0.01 and ***p<0.001 compared to WT at the same time point using the two-way ANOVA with Bonferroni's correction for multiple comparisons.

†No SD reported as data is from combined pharmacokinetic sample non-compartmental analysis.

Table 3.6. Brain Act D concentrations and pharmacokinetic parameters in WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice following intravenous administration of 0.5mg/kg Act D.

Brain-to-plasma (B/P) Act D concentration ratios were investigated up to 6h post administration. B/P ratios increased in all mouse variants over 6h. A 3.3-fold (15.6 to 51.6pa/µg/L) increase in B/P ratio was observed in WT mice, compared to a 5.9-fold (11.7 to 68.6 pa/µg/L) increase in Abcb1a/1b$^{-/-}$ mice and a 4.2-fold (20.2 to 85.5 pa/µg/L) increase in Abcc2$^{-/-}$ mice (Figure 3.10) over this time period. This indicates that despite a reduction in Act D plasma concentrations over time, Act D is accumulating in the brains of all mice, to the greatest extent in Abcb1a/1b deficient mice, confirming Act D transport at the blood-brain barrier.

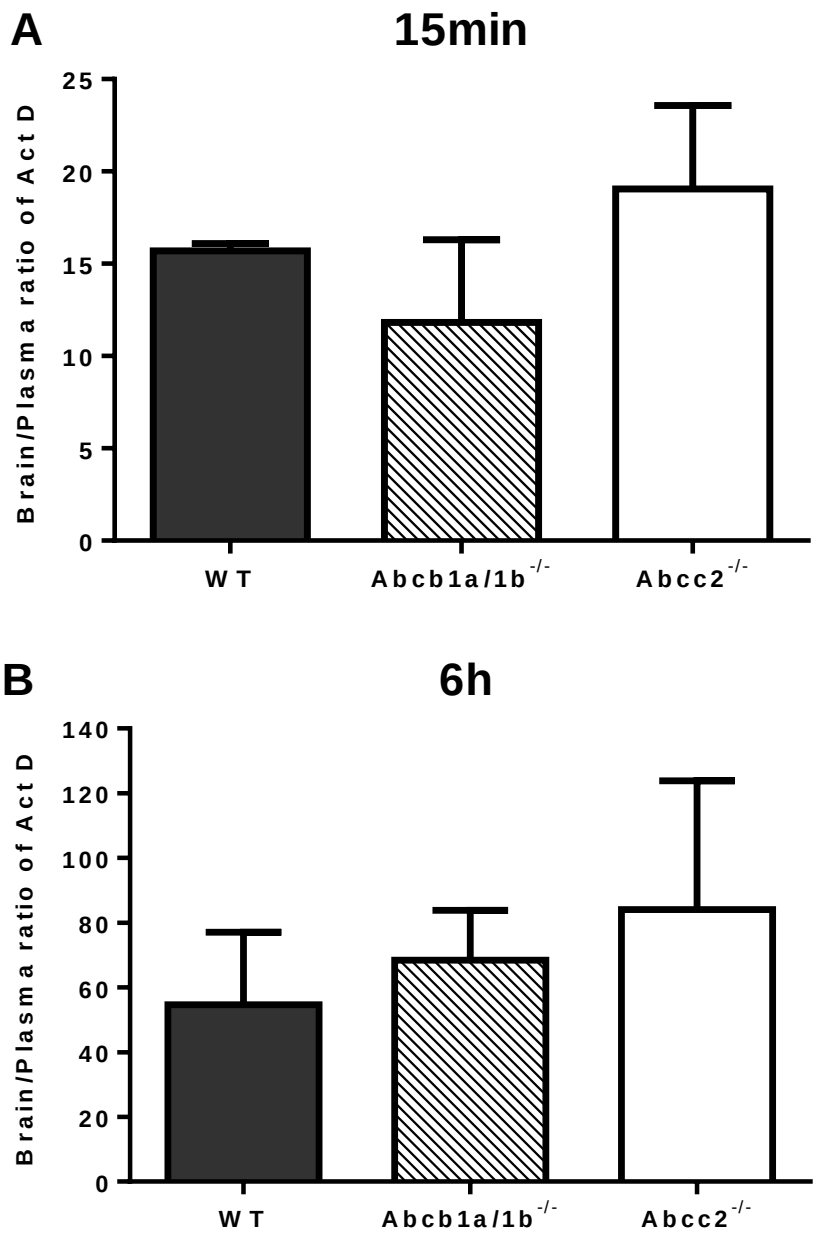

Figure 3.10. Act D brain-to-plasma ratios at 15min (A) and 6h (B) in wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice.

The relationship between Act D brain penetration and plasma concentration was investigated at 15min (A) and 6h (B). B/P ratios were calculated by dividing the brain concentration by the corresponding plasma concentration at 15min and 6h. Each point represents mean ± SEM from 3 mice.

3.3.6 Expression of Abcb1a, Abcb1b and Abcc2 in wild-type, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice.

Abcb1a, Abcb1b and Abcc2 expression was analysed in all mice to assess whether differential expression of these transporters was responsible for the lower plasma AUC_{0-6} seen in Abcc2$^{-/-}$. The expression of Abcb1a and Abcc2 in all mice was evaluated using commercially available primer kits from Qiagen (see 3.2.11), hepatic mRNA and real-time PCR. All four primer sets designed to assess the expression of Abcb1b did not amplify the target DNA sequence in a linear fashion over 3 log concentrations of template DNA, therefore they failed validation checks (see 3.2.11) and Abcb1b expression could not be determined.

Abcb1a expression in Abcb1a/1b$^{-/-}$ mice was 4% of the WT expression (0.04 vs. 1.00, $p<0.0001$, Figure 3.11A). Unexpectedly, Abcb1a expression was also significantly lower than WT in Abcc2$^{-/-}$ mice (0.65 vs. 1.00, $p<0.0001$, Figure 3.11A). Similar results were also observed when evaluating the expression of Abcc2 in all mice. Abcc2 expression in Abcc2$^{-/-}$ was 26-fold less than in the WT (0.04 vs. 1.02, $p<0.0001$, Figure 3.11B), and 2.0-fold less in Abcb1a/1b$^{-/-}$ mice (0.52 vs. 1.02, $p<0.0001$, Figure 3.11B).

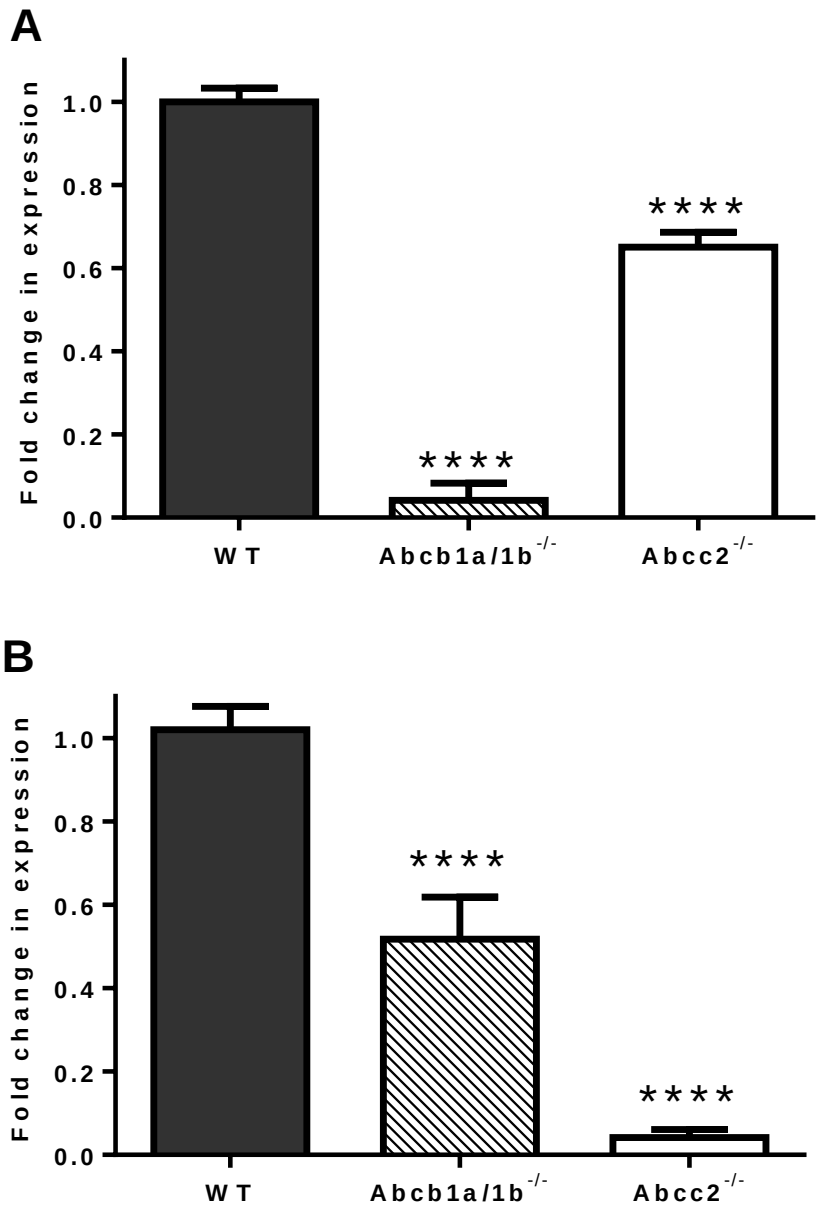

Figure 3.11. Expression of Abcb1a (A) and Abcc2 (B) in WT, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice livers.

Livers from all mice were dissected and used to assess Abcb1a (A) and Abcc2 (B) expression. mRNA was extracted from the liver tissue and reverse transcribed to cDNA. Real-time PCR was performed using specific Abcb1a and Abcc2 primers to initiate replication and SYBR green was used to detect amplified DNA. Results are normalised to Gapdh expression. Values represent the mean fold change ± SEM of 12 samples, compared with WT ΔCt values. ****p<0.0001 compared to WT, using the one-way ANOVA with Bonferroni's correction for multiple comparisons.

3.4 Discussion

Studies have been undertaken to investigate the relevance of Act D transport by ABCB1 and ABCC2 in an appropriate animal model. Abcb1a/1b$^{-/-}$, Abcc2$^{-/-}$ and WT mice were used to investigate the *in vivo* pharmacokinetics and distribution of Act D, along with the parental WT mice, allowing the systemic impact of transporter knockout to be fully investigated.

A preliminary study in CD-1 mice was undertaken to determine an appropriate dosage regimen, as well as confirming adequate sensitivity of the LC/MS method and the suitability of current extraction techniques. The pharmacokinetic profile observed at a dose of 0.5mg/kg in mice was similar to that previously seen in patients (Veal *et al.*, 2005). The C_{max} in mice was recorded at the first sampling time of 15min, followed by a decrease in Act D concentrations in plasma over the 6h studied. The $AUC_{0\text{-}6h}$ in CD-1 mice was higher than in previous studies in patients (131.7µg/L.h vs. 18.7-81.7 µg/L.h (Veal *et al.*, 2005)), but no acute toxicities were observed over 6h and plasma concentrations were detectable in all samples by LC/MS analysis.

Following confirmation that the dose and LC/MS method were appropriate, the transporter knockout mouse studies were performed. Investigations in Abcb1a$^{-/-}$ mice with the ABCB1 substrate doxorubicin have demonstrated higher plasma AUC and half-life ($t_{1/2}$) and lower clearance compared to WT mice following intravenous administration. High doxorubicin accumulation was also seen in the liver in the absence of Abcb1a (van Asperen *et al.*, 1999). In the current study, significant differences between Act D plasma concentrations at 15 and 30min post-administration were observed between WT and Abcb1a/1b$^{-/-}$ mice. At the 30min time point, plasma concentrations in Abcb1a/1b$^{-/-}$ mice were 1.7-fold

higher ($p<0.05$) in Abcb1a/1b$^{-/-}$ mice than the WT mice. This trend continued at 2 and 6h, with 1.4-fold and 3.3-fold ($p<0.0001$) higher plasma concentrations, respectively, in Abcb1a/1b$^{-/-}$ compared to WT. Higher plasma concentrations in Abcb1a/1b$^{-/-}$ resulted in a higher overall exposure and lower Act D Cl, with a 1.6-fold higher $AUC_{0\text{-}6h}$ and Cl being 52% of that observed in WT mice. Half-life was longer in Abcb1a/1b which resulted in a 1.9-fold higher $AUC_{0\text{-}\infty}$ over the WT. However, a low final plasma concentration caused difficulty in estimating $AUC_{0\text{-}\infty}$ in WT. These data show that Abcb1a/1b knockout had a significant impact on Act D exposure resulting in higher plasma concentrations and a slower elimination from the plasma compared to the WT.

Previously investigations using Abcc2$^{-/-}$ mice have demonstrated higher plasma exposure in Abcc2 knockout mice, when intravenously administered with anti-cancer ABCC2 substrates methotrexate (Vlaming *et al.*, 2006) and etoposide (Lagas *et al.*, 2010). In this study, plasma concentrations in Abcc2$^{-/-}$ mice were consistently lower than the WT. The C_{max} 15min post-administration, was 70% of that seen in the parental mice (48.0 vs. 68.6µg/L), and this continued at 30min and 2h, with plasma concentrations 87% (37.7 vs. 43.3µg/L) and 63% (16.3 vs. 25.8µg/L) of those seen in WT mice respectively. However, this data only reached significance at the 2h time point. These lower Act D plasma concentrations resulted in a 1.3-fold lower overall exposure to Act D over 6h compared to WT, and a 1.2-fold higher Cl. The sharp reduction in plasma concentrations in WT mice between 2 and 6h resulted in a shorter $t_{1/2}$ in WT than in Abcc2$^{-/-}$ mice (1.9h vs. 2.5h). $AUC_{0\text{-}\infty}$ estimation with limited pharmacokinetic sampling was highly dependent on the concentration of the final sample. This resulted in comparable $AUC_{0\text{-}\infty}$ values being determined for Abcc2$^{-/-}$ and WT mice (146µg/L.h vs. 168µg/L.h). Although plasma

concentrations in Abcc2$^{-/-}$ were generally lower, no significant difference in results between Abcc2$^{-/-}$ and WT were observed.

Interestingly, higher Act D accumulation was not seen in either the liver or kidney of Abcb1a/1b$^{-/-}$ mice. Although higher liver concentrations were initially observed in Abcb1a/1b$^{-/-}$ mice at 15min, similar time-dependent decreases in Act D liver concentrations were seen in all mice variants, and consequently no difference in $AUC_{0\text{-}6h}$ was observed. Similarly to Abcb1a/1b$^{-/-}$ mice, Act D plasma concentrations in Abcc2$^{-/-}$ mice did not appear to impact on accumulation in the liver. Although there was an initially lower plasma concentration in Abcc2$^{-/-}$ livers at 15min, there was no difference in Act D accumulation between Abcc2$^{-/-}$ and WT mice, resulting in unchanged C_{max}, $AUC_{0\text{-}6}$ and $AUC_{0\text{-}\infty}$ values.

There was also no difference between Abcb1a1b$^{-/-}$, Abcc2$^{-/-}$ and WT mice in Act D concentration in the kidneys. Act D concentration in kidney tissue decreased with time at a similar rate in all mice, with no difference observed in C_{max} or $AUC_{0\text{-}6h}$. The $t_{1/2}$ was greater in Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice compared to WT, resulting in a 1.3-fold and 1.4-fold higher $AUC_{0\text{-}\infty}$ than WT, respectively. However, due to the limited number of pharmacokinetic samples analysed, $AUC_{0\text{-}\infty}$ was highly dependent on the last pharmacokinetic sample at 6h. The final liver concentration in WT mice was lower than those seen in both Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice, resulting in the percentage extrapolation to infinity being lower in WT mice compared to both variant strains; $AUC_{0\text{-}\infty}$ was 17% extrapolated from $AUC_{0\text{-}6h}$ in WT mice, compared to 24% and 32% in Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice respectively.

It is possible that the lack of difference seen in both liver and kidney concentrations in Abcb1a/1b$^{-/-}$ mice is due to high Act D plasma protein binding, therefore preventing high plasma concentrations causing a reciprocal increase in liver and kidney concentrations compared to the wild-type. Studies carried out in rats have demonstrated 8% plasma protein binding (Wosilait and Eisenbrandt, 1971), whilst plasma protein binding in humans has been reported as 5% (Dart, 2004). Therefore this low level of protein binding is unlikely to affect the disposition of Act D in the mice.

Contrary to both liver and kidney data, increased concentrations of Act D were seen in brain tissue from Abcb1a/1b$^{-/-}$ compared to WT. Over the 6h studied, Act D brain concentration reduced in WT mice, whereas it remained constant in Abcb1a/1b$^{-/-}$ mice resulting in a 1.5-fold higher brain concentration at 6h (2957 vs.1970pa, $p<0.001$). Overall exposure to the brain over a 6h period was 2.3-fold higher in Abcb1a/1b$^{-/-}$ mice versus the WT. Brain accumulation of Act D in Abcc2$^{-/-}$ followed a similar pattern to that observed in Abcb1a/1b$^{-/-}$ mice. C_{max} was reached at 30min, therefore indicating an initial slow distribution phase not seen in the WT. This was followed by a decline in Abcc2$^{-/-}$ brain concentrations to 2h, and an increase in Act D brain concentration between 2-6h. However, data generated from brain tissue was highly variable and should be treated with caution. Additional experiments with an increased number of time points and animals would be needed to assess whether this later increase in brain concentration, seen in Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ is reproducible.

Tissue-to-plasma ratios can be used to correct tissue concentration for differences in plasma concentrations, allowing distribution to be examined independent of plasma concentration (Lagas *et al.*, 2009; Poller *et al.*, 2011). In

this study, L/P ratios between Abcb1a/1b$^{-/-}$, Abcc2$^{-/-}$ and WT were comparable at 15min post-administration, however, the L/P ratio was lower in Abcb1a/1b$^{-/-}$ as compared to Abcc2$^{-/-}$ and WT at 6h (47.8g^{-1} vs. 20.4g^{-1} and 26.4g^{-1}, respectively, $p<0.05$).

In agreement with the liver data, no differences in K/P ratios were observed between Abcb1a/1b$^{-/-}$, Abcc2$^{-/-}$ and WT 15min post administration (138, 277 and 191g^{-1}, respectively). The K/P ratio in Abcb1a/1b$^{-/-}$ mice remained constant over 6h and was 2.2-fold lower than the WT and 2.8-fold lower than Abcc2$^{-/-}$ at this time point (148 vs. 329g^{-1}, 148 vs. 408g^{-1} $p<0.05$). The K/P ratio increased over 6h in both Abcc2$^{-/-}$ (273g^{-1} to 398g^{-1}) and WT (194g^{-1} to 329g^{-1}) mice, indicating continued accumulation in the kidney over 6h, despite declining plasma concentrations. These data would indicate that high Act D plasma concentrations in Abcb1a/1b$^{-/-}$ were not influencing the concentration of Act D in the liver and kidney over 6h compared to both Abcc2$^{-/-}$ and WT mice. Finally, B/P ratio contrasted those data observed in the liver and kidneys. When plasma concentration was taken into account, no difference in B/P ratio was observed between all mice variants, indicating brain concentration was proportionate to the concentration of Act D in the plasma.

Contradictory reports exist as to whether collateral upregulation of various ABC transporters occurs in knockout mice. Abcc3 upregulation has been shown in Abcc2$^{-/-}$ mice liver with Abcc4 upregulation in the kidney (Vlaming *et al.*, 2006; Lagas *et al.*, 2010). Using a different strain of Abcc2$^{-/-}$, Chu et al. found no upregulation of either transporter (Chu *et al.*, 2006), however a separate report from a different laboratory using the same mice did find upregulation of Abcc3 (Nezasa *et al.*, 2006). Abcb1a1b$^{-/-}$ and Abcc2$^{-/-}$ mice used in this study were

investigated for Abcb1a and Abcc2 expression. Abcb1a expression was lower in Abcc2$^{-/-}$ than the WT and Abcc2 expression was lower in Abcb1a/1b$^{-/-}$ mice. Interestingly, this does not explain the low Act D concentrations observed in the plasma of Abcc2$^{-/-}$ mice. In light of the previously discussed publications it would have been beneficial to determine the expression of other ABC transporters for example Abcc3 and Abcc4 in Abcc2$^{-/-}$ mice to establish whether upregulation of either transporter was responsible for the Act D plasma concentrations observed.

The data presented in this chapter show that knockout of either Abcb1a/1b or Abcc2 may influence the *in vivo* pharmacokinetics of Act D. Abcb1a and Abcb1b knockout reduces the elimination of Act D from plasma, resulting in higher plasma concentrations and plasma exposure.

Due to the role of Abcb1a/1b and Abcc2 at the apical membrane of proximal tubular cells in kidney and hepatocytes in the liver eliminating substances from the body, knockout of these transporters would be expected to cause accumulation of substrates in both the liver and kidney. However, the data presented in this study demonstrate that despite high Act D plasma exposure in Abcb1a/1b deficient mice, this does not impact on Act D concentration in the liver or kidneys. However, ABC transporter knockout did appear to alter Act D penetration across the blood-brain barrier. Although not significant, Abcc2 knockout appeared to result in a lower Act D plasma $AUC_{0\text{-}6h}$, the reason for which is not understood. The absence of differences in Act D distribution to the liver and kidneys of all mice may be due to the reciprocal expression of Abcc2 in Abcb1a/1b$^{-/-}$ mice and vice versa. An investigation using a double knockout mouse model (Abcb1a/1b$^{-/-}$; Abcc2$^{-/-}$) would therefore be potentially informative.

Irrespective of transporter knockout genotype, Act D accumulation over 6h in the kidneys was 6.6 to 7.7-fold higher than in the liver, potentially demonstrating a major route for Act D elimination from the body. A limitation to the current study is the lack of Act D analysis in the urine and faeces. This would allow the full impact of Abcb1a, Abcb1b and Abcc2 on Act D elimination in the liver and kidneys to be investigated.

As Abcb1a/1b and to a degree Abcc2 have been shown to be involved in the *in vivo* disposition of Act D, it is important to investigate whether common SNPs in ABCB1 and ABCC2, which have clinical relevance, could be responsible for inter-patient variation in Act D exposure.

Chapter 4. Pharmacokinetics of actinomycin D in a paediatric patient population

4.1 Introduction

Although Act D has been used clinically for many years, very few pharmacokinetic studies have been carried out, and as such little is known about its drug disposition in humans. Early pharmacokinetic studies used tritiated Act D demonstrated accumulation in nucleated cells in addition to tumour accumulation (Tattersall *et al.*, 1975). Act D half-life in dogs (0.8min) was less than half that of humans (1.8min) when using a RIA (Brothman *et al.*, 1982). This inter-species variation and the use of non-specific analytical methods indicate that more human pharmacokinetic trials need to be performed.

The main side-effect associated with Act D therapy is treatment-related toxicity. Although rare, this can be life-threatening, and remains a major concern when treating potentially curable patients. Age at treatment is a major risk factor, with patients less than 3 years old almost 4-fold more likely to have some form of toxicity following Act D treatment (Arndt *et al.*, 2004). This observation was confirmed in a retrospective study, with patients aged <1 year at treatment showing an even greater incidence of toxicity (Langholz *et al.*, 2011).

The current study was originally driven by the concerns of clinicians over treatment-related toxicity and its potential association with inter-patient variability in Act D pharmacokinetics and exposure. This led to the development of a highly sensitive method for measuring Act D in patient plasma using LC/MS (Veal *et al.*, 2003b), and a clinical trial evaluating the pharmacokinetics of Act D

in patients was performed (Veal *et al.*, 2005). Initial reports in 31 patients highlighted the inter-patient variation in Act D exposure with a greater than 4-fold range in $AUC_{0\text{-}6h}$ being observed. Higher Act D exposure was observed in smaller children, and despite the limited number of patients this was linked to an increase in toxicity risk. Conversely, low Act D exposure was reported in older, larger patients where the practice of dose capping at 2mg may result in under dosing. However, the major limitations of this initial report were low patient numbers and limited pharmacokinetic sampling beyond 6h after drug administration, requiring much of the data to be extrapolated out to 24h to obtain pharmacokinetic parameter estimates.

In this chapter, Act D pharmacokinetic data from two large clinical trials conducted in the UK are presented. Eligible patients were less than 21 years old and were due to receive Act D as part of their standard treatment regimen. A major aim of these two clinical trials was to address the limitations of the initial study, increasing patient numbers and including pharmacokinetic sampling at earlier and later time points, thus allowing more accurate characterisation of Act D pharmacokinetics. Act D pharmacokinetic data will be assessed against patient characteristic and covariate data recorded at the time of treatment. The link between Act D patient exposure and treatment-related toxicity will be investigated.

4.2 Materials and methods

4.2.1 Clinical study

Study protocols were approved by UK Trent Multicentre Research Ethics Committee and written informed consent was obtained from all patients or parents where appropriate. Eligible patients were under 21 years old and were receiving Act D as part of their standard chemotherapy. The trials were registered through the appropriate clinical trials registries (PK 2003 08; REC: 03/04/074 CTA: 23198/0001/001, PK 2006 07; REC 05/MRE04/62, CTA: 2005-002996-34, ClinicalTrials.gov identifier: NCT00900354) prior to patient recruitment. Baseline toxicity data prior to Act D treatment was obtained from patients' notes and details of concomitant medications being administered prior to and/or in combination with Act D were recorded. Additional patient characteristics and clinical parameters were also recorded for each patient participating in the study for post-study analysis.

Patients were dosed at 1.5mg/m^2 (max 2mg) intravenously. However, the standard protocol dose could be given as a split dose (0.75mg/m^2 over 2 days) and is altered for infants that were <1 year or <10kg in body weight. For example, doses are frequently changed to 25µg/kg for patients between 0-6months, and adjusted to 1mg/m^2 for patients aged 6months to 1 year, but less than 10kg. In practice, patients were not dosed at 1.5mg/m^2 until they reach their third birthday (Arndt *et al.*, 2004).

Blood samples (2ml) were collected in heparinised tubes from patients with a central line, before administration, and at 5, 15 and 30min, and 2, 4, 8, 24 and 26h post administration. Actual sampling times were recorded; not all samples

were available for all patients. Blood samples were immediately centrifuged at 1,200g for 10min at 4^{o}C. Plasma was separated and stored at -20^{o}C prior to analysis by LC/MS.

4.2.2 Analysis of actinomycin D by LC/MS

Chemicals and LC/MS method details can be found in 2.2.10. Preparation of working standards and samples for LC/MS analysis can be found in 3.2.6.

4.2.3 Assay validation

A standard curve of between 0.25-10ng/ml was established to be linear in 10 assays (mean r^2=0.998 ± 0.001) and the LC/MS assay was validated using human plasma. The level of detection (LOD) and level of quantification (LOQ) for Act D were both determined to be 0.125ng/ml (Figure 4.1). Further work was not carried out to establish the true LOD (a peak 3 times the baseline) and LOQ (a peak 10 times the baseline) as 0.125ng/ml was below the concentration needed for the range of the standard curve in human plasma.

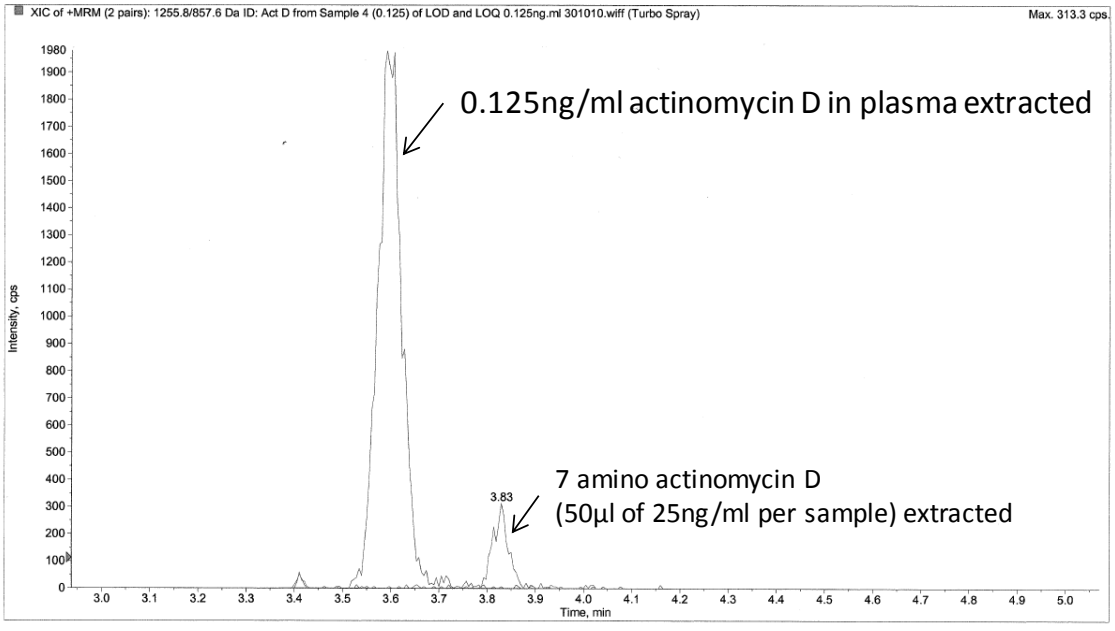

Figure 4.1. LOD and LOQ determination in human plasma.

Intra- and inter-assay precision and accuracy were determined in human plasma as per Table 4.1. The coefficient of variation was deemed acceptable if <10% and the concentration of samples had to be within 15% of the concentration injected to be deemed accurate.

Added (ng/ml)	Intra-assay precision (n=10)			Inter-assay precision (n=8)		
	Found (ng/ml)	CV (%)	Accuracy (%)	Found (ng/ml)	CV (%)	Accuracy (%)
0.5	0.47 ± 0.03	6.0	94	0.496 ± 0.02	3.1	99
2	2.1 ± 0.07	3.6	105	1.91 ± 0.19	9.3	96
10	9.9 ± 0.63	6.4	99	10.0 ± 0.25	2.5	100

Data expressed as mean ± SD for 'found' values.

Abbreviations – CV; coefficient of variation (SD/mean).

Table 4.1. Intra- and inter-assay precision and accuracy of the Act D LC/MS assay in human plasma.

In addition, Act D stability during a run was tested. Following sample extraction and reconstitution, Act D is determined to be stable for 24h at 4^{o}C or room temperature.

4.2.4 Non-compartmental pharmacokinetic data analysis

Pharmacokinetic data from 108 patients were analysed by WinNonlin Professional version 5.3 (Pharsight Corp, Mountain View, CA, USA). Non-compartmental analysis (NCA) was performed on all 108 sample sets. The area under the plasma concentration versus time curve from drug administration to 6h (AUC_{0-6}, n=93) and 24h (AUC_{0-24}, n=66) were estimated where possible using the logarithmic trapezoidal rule. AUC extrapolation to infinity ($AUC_{0-\infty}$, n=108) was performed using an estimate of $t_{1/2}$ from slope of the concentration vs. time curve using the final 3-4 pharmacokinetic sample time data points.

Clearance was estimated as dose divided by $AUC_{0-\infty}$ for 107 patients, as dose information was not available for one patient.

4.2.5 Statistical analysis

Linear regression was performed to assess possible associations between only clearance values obtained from full sample sets (those with early (5-30min) and late (20-26h) samples, and was termed Cl-F) and patient characteristics. An r^2 value is reported for each linear regression performed; a p-value is also given to represent the deviation of the slope from zero. Overall means between groups were compared using the unpaired t-test (two groups) and one-way ANOVA (three groups) with Bonferroni's multiple comparisons. If data were not normally distributed, the data were log-transformed to achieve a normal distribution, after which an unpaired t-test or one-way ANOVA with Bonferroni's correction for multiple comparisons was performed to compare means. Normality was checked by producing a histogram of the data. LC/MS data were quantitated as per 2.2.12.

4.3 Results

4.3.1 Patient characteristics

One hundred and forty two patients receiving Act D as part of their standard chemotherapy regimen were recruited on to the study between August 2004 and February 2012. The study population had a median age of 4.5 years (range 0.4-19.8 years) with identical numbers of males and females. Wilms tumour was the most common tumour type in patients recruited on to the study (52%), followed by RMS (27%) and ES (18%). Other tumour types included a paraspinal undifferentiated sarcoma, an embryonal sarcoma of the liver, a pleuropulmonary blastema and a non-RMS tumour. Full patient characteristics can be found in Table 4.2.

Characteristic		No of patients (%) (n=142)	No. of patients with valid PK results (%) (n=108)	No. of patients with valid PG results (%) (n=126)	Matched PK-PG results (%) (n=98)
Age (years)	<1	7 (5)	7 (6)	5 (4)	5 (5)
	1 – 3	39 (27)	28 (26)	34 (27)	24 (24)
	3 - 10	72 (51)	52 (48)	63 (50)	48 (49)
	10 - 21	24 (17)	21 (19)	24 (19)	21 (21)
Gender	Male	71 (50)	58 (54)	61 (48)	45 (46)
	Female	71 (50)	50 (46)	65 (52)	53 (54)
Weight (kg)	<10	13 (9)	12 (11)	10 (9)	9 (9)
	10 - 30	92 (65)	70 (65)	81 (64)	64 (65)
	30 - 60	25 (18)	22 (20)	23 (18)	21 (21)
	>60	4 (3)	4 (4)	4 (1)	4 (4)
	Unknown	8 (6)	0 (0)	8 (6)	0 (0)
SA (m^2)	<0.5	18 (13)	15 (14)	15 (12)	13 (13)
	0.5 – 1	87 (61)	66 (61)	75 (60)	59 (60)
	>1	28 (20)	26 (24)	27 (21)	25 (26)
	Unknown	9 (6)	1 (1)	9 (7)	1 (1)
Diagnosis	Wilms tumour	74 (52)	45 (42)	63 (50)	39 (40)
	Rhabdomyosarcoma	39 (27)	35 (32)	35 (28)	32 (33)
	Ewing's sarcoma	25 (18)	24 (22)	24 (19)	23 (23)
	Other	4 (3)	4 (4)	4 (3)	4 (4)
Ethnicity[†]	White British	80%	-	-	-
	White other	4	-	-	-
	Pakistani	3	-	-	-
	Asian other	5	-	-	-
	Black Caribbean	1	-	-	-
	Black African	2	-	-	-
	Black other	2	-	-	-
	Any other mixed background	2	-	-	-
	Other	1	-	-	-

Abbreviations: SA; body surface area, PK; pharmacokinetic, PG; pharmacogenetic

[†]Data not collected in this study, therefore a representative UK paediatric cancer population with a similar number of patients has been used to demonstrate the likely patient demographics (Veal *et al.*, 2012).

Table 4.2. Patient characteristics for all 142 patients recruited onto study

4.3.2 Actinomycin D pharmacokinetics

Out of the 142 patients recruited, blood samples for pharmacokinetic analysis were taken from 131 patients. Data from our laboratory suggest that Act D is stable in human plasma for no longer than six months and as such pharmacokinetic data from 23 patients was deemed invalid due to analysis taking place beyond this time period. A total of 601 plasma samples from 108 patients were valid, and have been used to generate pharmacokinetic data. Full pharmacokinetic data for the 108 patients can be found in Table A.1 in the appendix. Full sample sets with early (5-30min) and late (20-26h) time points were provided by 74 patients (69%).

A large degree of variability in plasma concentrations was observed between patients (Table 4.3, Figure 4.2). For those patients whose first sample was taken 5min post-administration, the median C_{max} was 129µg/L (range 64.7-193µg/L, n=14), compared to 22.7µg/L (range 7.5-122µg/L, n=81) for those patients whose first sample was taken at 15min. The median Act D concentration in plasma samples taken 24h after administration was 1.8µg/L (range 0.7-4.3µg/L, n=66).

Depending on treatment protocol, doses of Act D ranged from 0.19-2.5mg (0.43-1.62mg/m^2). Following non-compartmental pharmacokinetic analysis (see 4.2.4) the mean $t_{1/2}$ was estimated at 13.7 ± 10.3h. AUC_{0-6h} and AUC_{0-24h} were calculated for 93 and 66 patients respectively (Table 4.3). The median AUC_{0-6h} was 2.8mg/L.min (range 1.1-10mg/L.min), indicating a 9-fold variation in Act D exposure up to 6h in patients. A large degree of variability was also seen in AUC_{0-24h}, with a 6-fold range observed from 2.3-13.7mg/L.min.

Sample time (min)	No. of samples	Median	Minimum	Maximum
5	14	129.0	64.7	193
15	91	24.4	7.5	122
30	104	9.7	3.3	91.9
60	16	6.3	3.3	38.1
120	102	4.7	1.4	44.8
240	97	3.6	1.4	19.5
360	39	3.2	1.2	9.1
480	29	2.8	1.2	6.6
1320	12	1.7	1.1	4
1440 (24h)	67	1.8	0.7	4.8
1560 (26h)	30	2.0	0.7	4.3
$AUC_{0\text{-}6h}$ (mg/L.min)	93	2.8	1.1	10
$AUC_{0\text{-}24h}$ (mg/L.min)	66	5.6	2.3	13.7
$AUC_{0\text{-}\infty}$ (mg/L.min)	108	6.3	1.4	19.8
Cl-F (ml/min)	74	116	14.8	341
Vss (L)	80	135	2.5	638
GFR (ml/min/1.73m^2)	52	116	64.0	278
Creatinine (µmol/L)	104	38	18	90

Abbreviations- $AUC_{0\text{-}6h}$; area under the concentration time curve between 0 and 6h, $AUC_{0\text{-}24h}$; area under the concentration time curve between 0 and 24h, $AUC_{0\text{-}\infty}$; area under the concentration time curve extrapolated to infinity, Cl-F; Clearance from patients with both early (5-30min) and late (20-26h) samples, Vss; volume of distribution at steady-state conditions calculated from patients with C_{max} at 15min, GFR; glomerular filtration rate.

Table 4.3. Pharmacokinetic, GFR and creatinine data from 108 patients receiving Act D

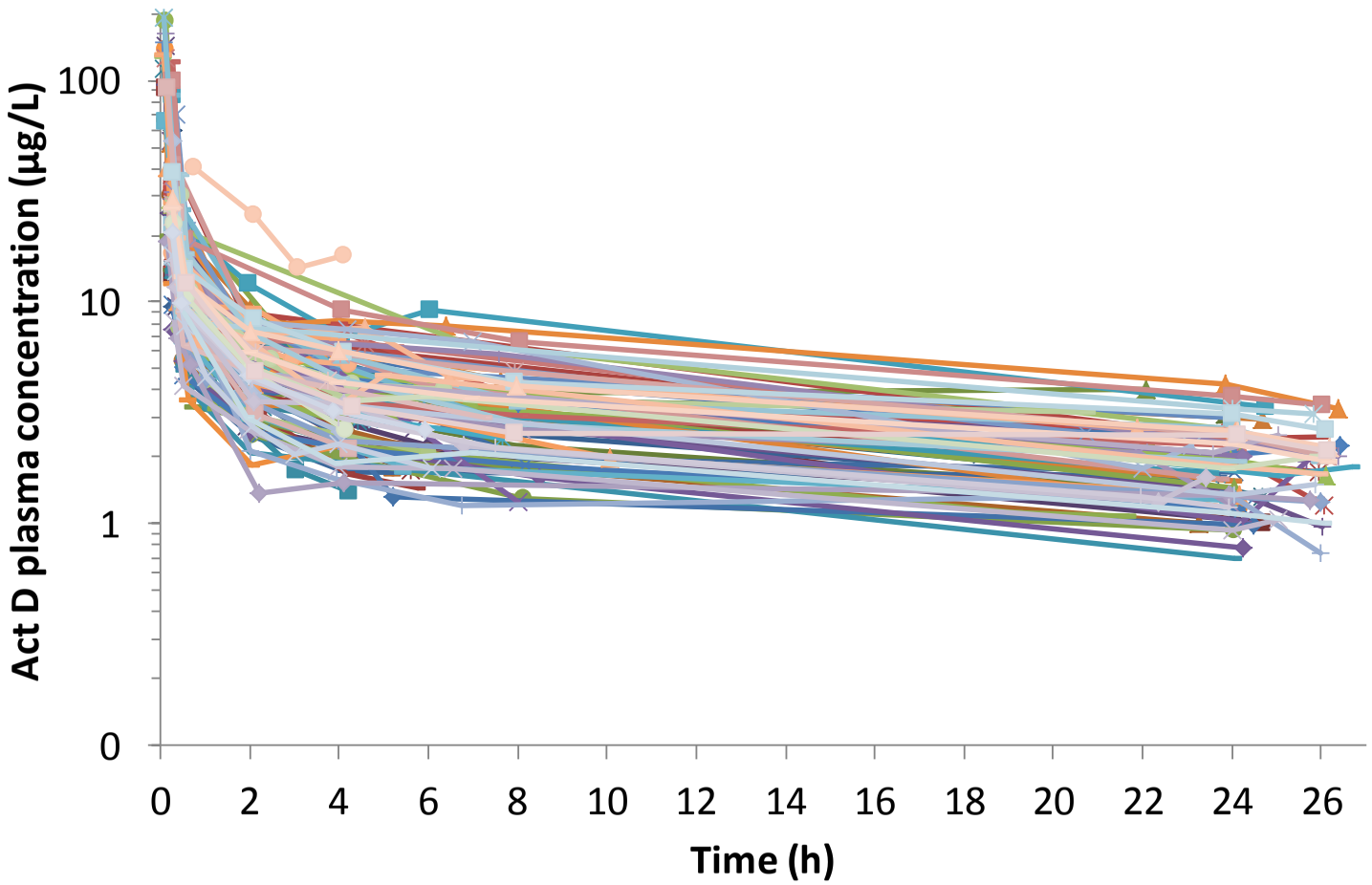

Figure 4.2. Act D plasma concentration-time profiles for 108 patients.

Blood samples were collected between 5min and 26h from patients following intravenous bolus administration of Act D. Plasma was separated from whole blood, and Act D concentrations were determined using LC/MS. Each line represents Act D concentrations in plasma samples from one patient.

4.3.3 Patient covariate analysis

The primary pharmacokinetic end points used were estimated clearance, based on dose and $AUC_{0-\infty}$. For those patients without full sample sets (no pharmacokinetic sampling after 8h), $AUC_{0-\infty}$ was estimated using the latest samples available (72 to 480min) and the estimated clearance was termed Cl-L. The median $AUC_{0-\infty}$ estimated from 108 patients was 6.3mg/L.min (range 1.4-19.8mg/L.min) and subsequently, mean clearance was estimated as 154 ± 103ml/min, indicating a 42-fold range in clearance among patients. Clearance in patients with limited samples (Cl-L, n=33) was 1.7-fold higher, compared to those with full sample sets (Cl-F, n=74) (211 vs. 127ml/min, p = 0.003, Figure 4.3), indicating that AUC is underestimated when data were limited to 6h after administration. Given that the clearance for Cl-L is likely to be an overestimate, covariate analysis was therefore carried out using Cl-F only.

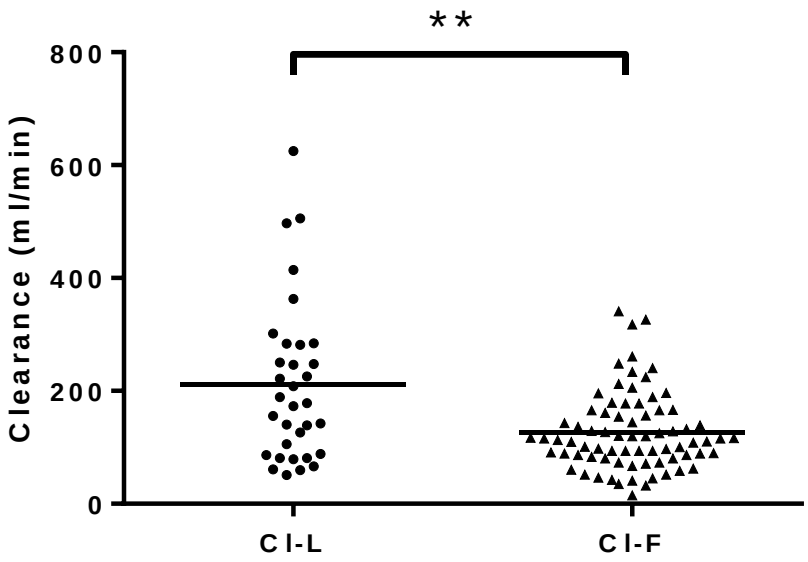

Figure 4.3. Comparisons between Cl-L and Cl-F.

Estimated clearance from 108 patients were separated into two groups based on the last pharmacokinetic sample time; <8h = Cl-L, n=33, >20h = Cl-F, n=74. **p=0.003. Mean represented by horizontal bar.

4.3.4 Actinomycin D clearance and patient characteristics

Positive linear relationships were observed between Cl-F and patient age (Figure 4.4A), SA (Figure 4.5A) and weight (Figure 4.6A). To investigate the relationship between Cl-F and these patient characteristics further, patient age, weight and SA were grouped according to age and size. Patients who were above 3 years old had 1.8-fold higher mean Cl-F than patients less than 3 years old (144 vs. 78.2ml/min, p=0.0002, Figure 4.4B). Similarly, patients above 0.5m^2 had Cl-F 2.3-fold higher than those below 0.5m^2 (138 vs. 59ml/min, p=0.0005, Figure 4.5B) and those above 10kg had 3-fold greater Cl-F compared to smaller patients (136 vs. 46.8ml/min, p=0.0008, Figure 4.6B). Associations between Cl-F normalised to surface area (Cl-FSA) and patient age, SA and weight were also investigated. After normalising for body size, variation was lower, with a 10-fold range in Cl-FSA observed (33.7-339ml/min/m^2), compared to 23-fold variation in Cl-F.

Creatinine and GFR data before Act D treatment were provided for 104 and 52 patients respectively (Table 4.3). The median GFR recorded in patients was 116ml/min/1.73m^2 (range 64-278 ml/min/1.73m^2) and the median creatinine was 38µmol/l (range 18-90 µmol/l). When comparing these two clinical parameters to Cl-F, positive correlations were observed with creatinine (r^2 = 0.3, Figure 4.7A), however no association was found between Cl-F and GFR (Figure 4.7B).

The type of catheter used to administer Act D appeared to have a minor influence on Cl-F (Figure 4.8A). Those patients fitted with a double-lumen Hickman line had 1.5-fold higher mean CL-F than those fitted with a port-a-cath (142 vs. 94.3ml/min, p<0.05, Figure 4.8A) and 1.4-fold higher mean CL-F than those patients with a single-lumen Hickman line (142 vs. 103ml/min, Figure

4.8A). However, these differences were also confounded by the interaction between patient age and line type. The mean age of patients with a double-lumen Hickman line was 2.4-fold higher than those with a single-lumen Hickman line (7.3 vs. 3.3yr, $p<0.01$, Figure 4.8B) and 1.4-fold higher than those with a port-a-cath (7.3 vs. 5.3yr, Figure 4.8B). Line type had no influence on C_{max} at 5 or 15min. Gender (Figure 4.9A) and Act D infusion time (Figure 4.9B) had no impact on Cl-F.

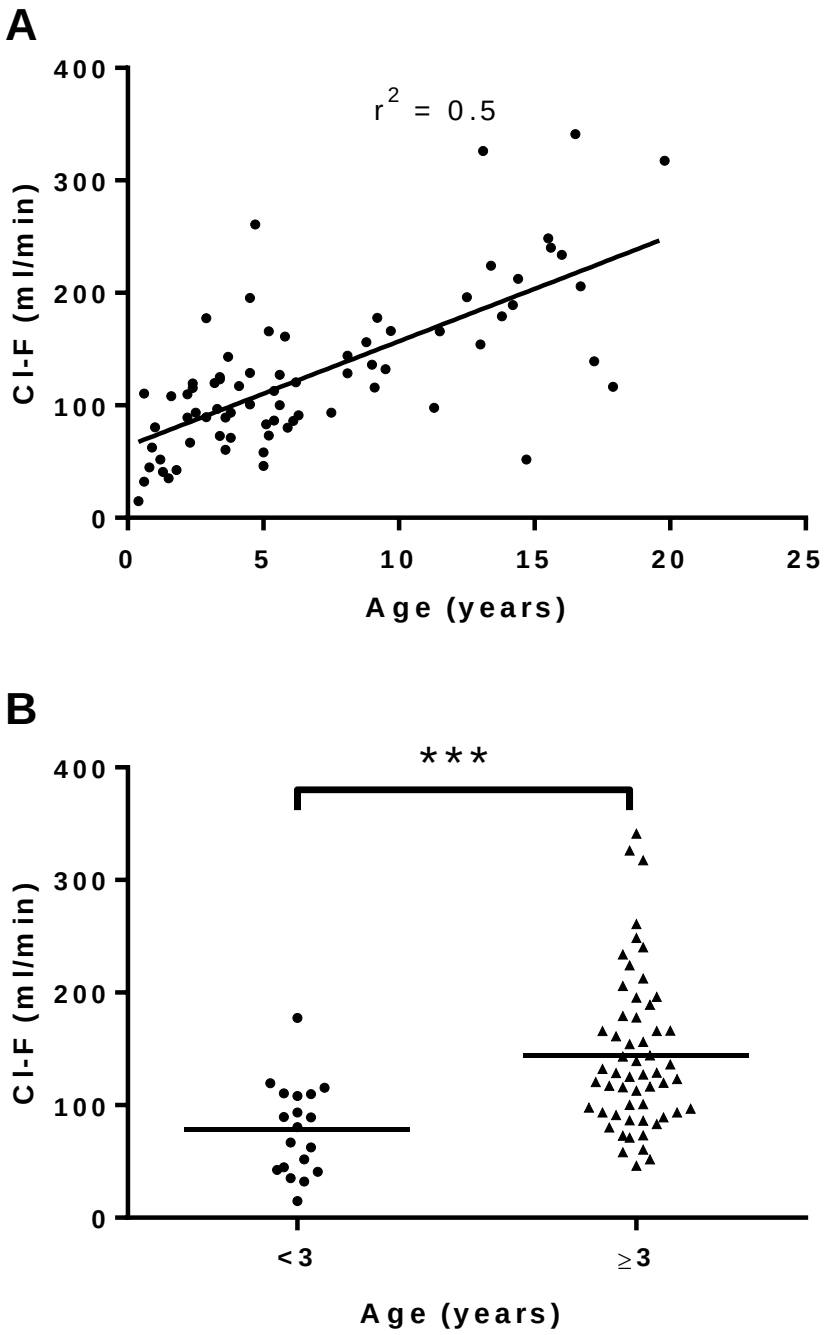

Figure 4.4. Relationship between Cl-F and patient age following intravenous administration of Act D.

Patient age (A) was plotted against Cl-F. Linear regression was used to assess correlations between Cl-F and age ($p<0.0001$), r^2 values are presented on graphs where applicable. Patients were also grouped according to age (B). Means were tested using the unpaired t-test. ***$p<0.001$.

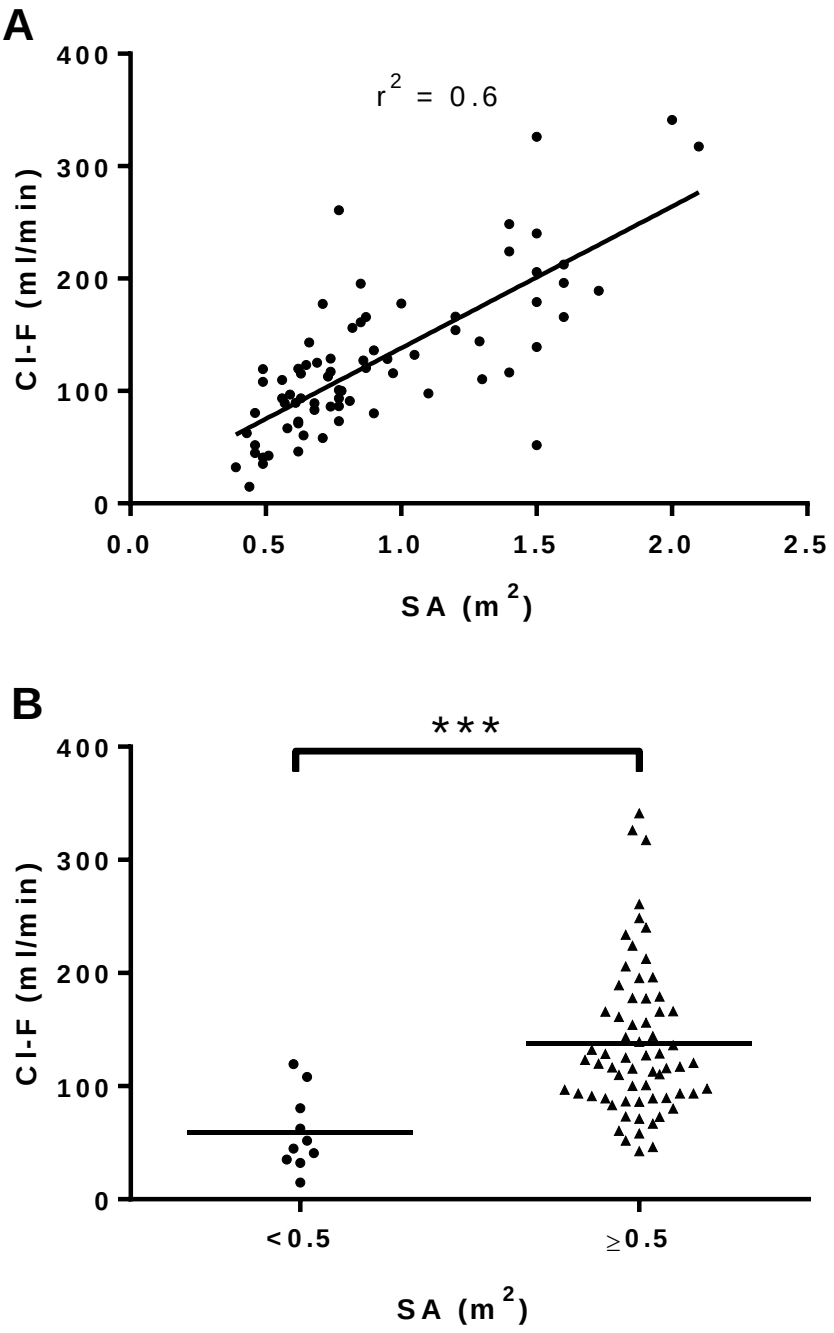

Figure 4.5. Relationship between Cl-F and SA following intravenous administration of Act D.

SA (A) was plotted against Cl-F. Linear regression was used to assess correlations between Cl-F and SA ($p<0.0001$), r^2 values are presented on graphs where applicable. Patients were also grouped according to SA (B). Means were tested using the unpaired t-test. ***$p<0.001$.

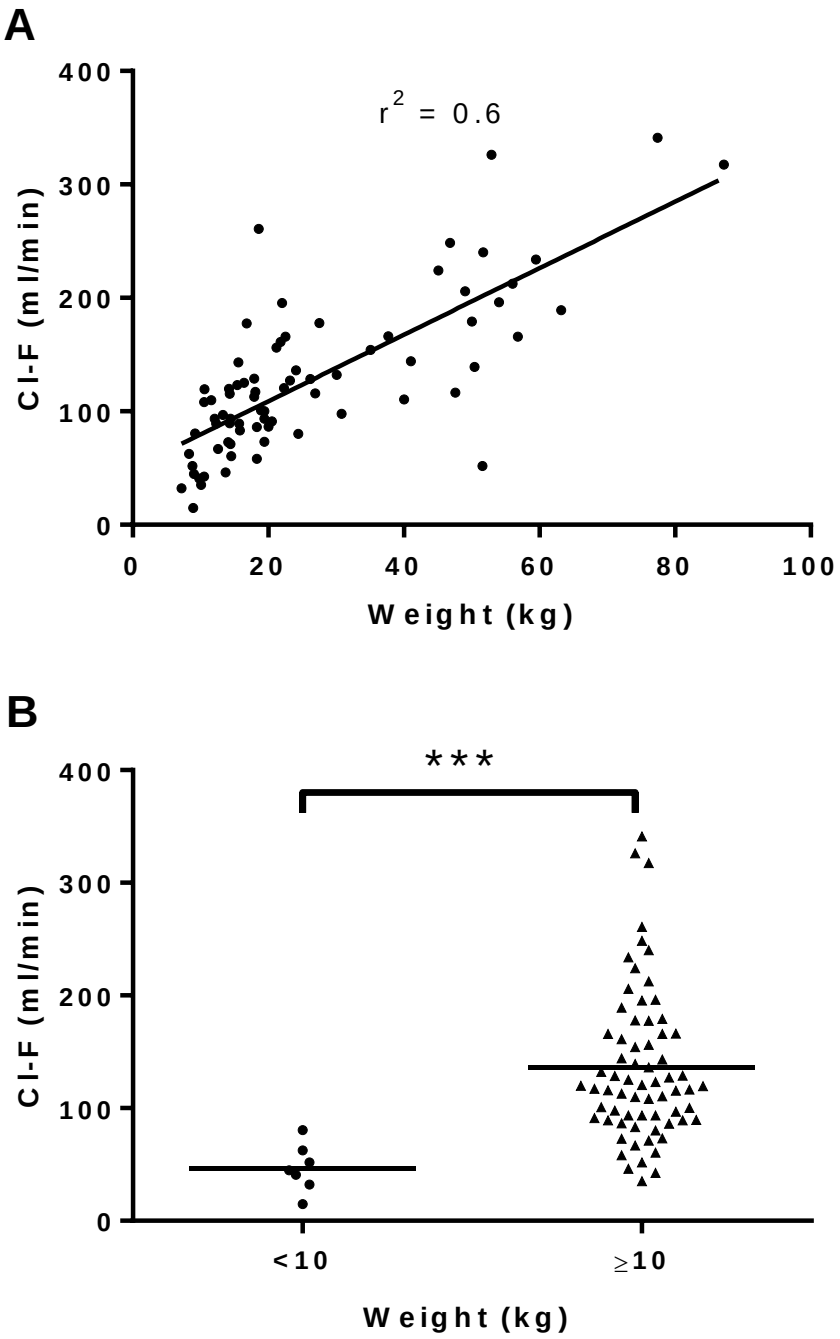

Figure 4.6. Relationship between Cl-F and weight following intravenous administration of Act D.

Weight (A) was plotted against Cl-F. Linear regression was used to assess correlations between Cl-F and weight ($p<0.0001$), r^2 values are presented on graphs where applicable. Patients were also grouped according to weight (B). Means were tested using the unpaired t-test. ***$p<0.001$.

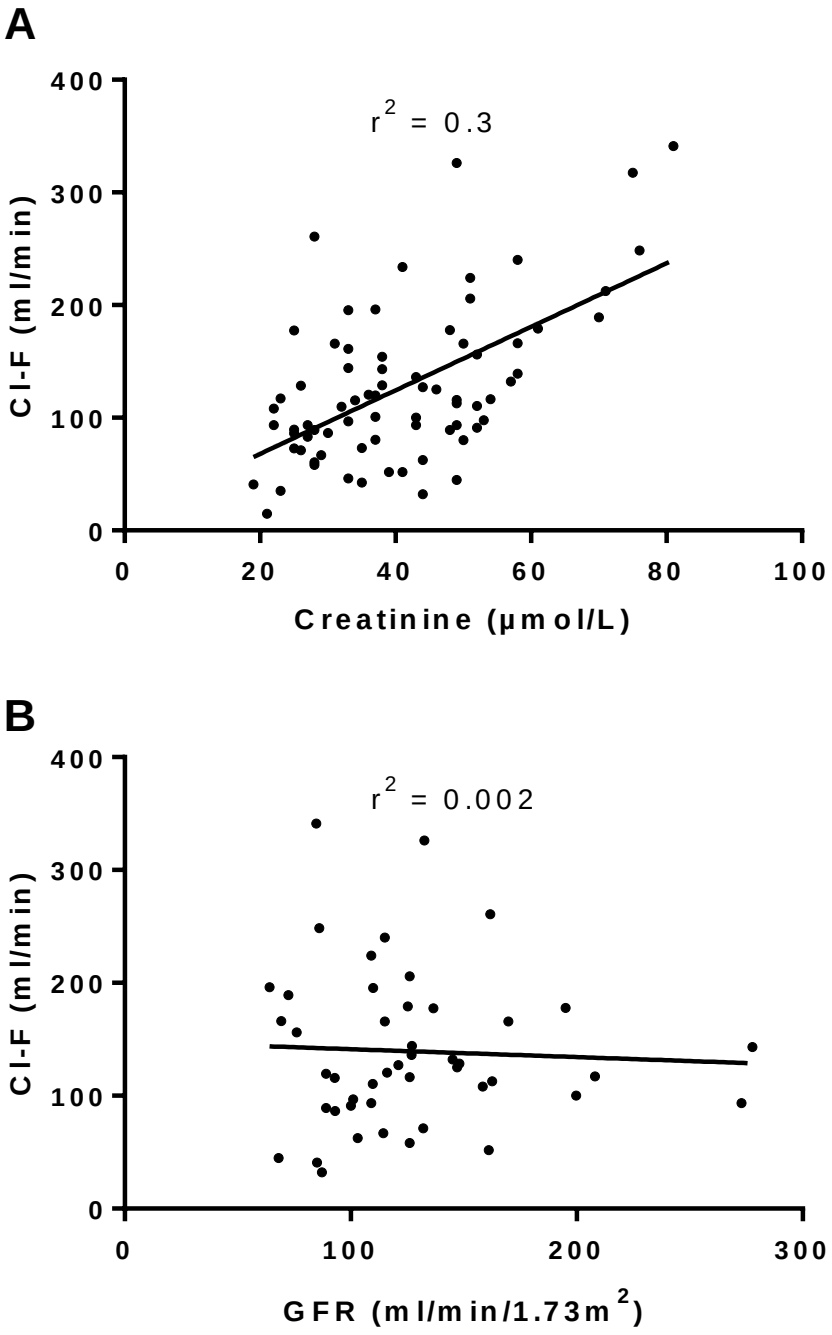

Figure 4.7. Relationship between creatinine and GFR and Cl-F following intravenous administration of Act D.

Creatinine (A) and corrected GFR (B) were plotted against Cl-F. Linear regression was used to assess correlations between creatinine ($p<0.0001$) and GFR and Cl-F, r^2 values are presented on graphs where applicable.

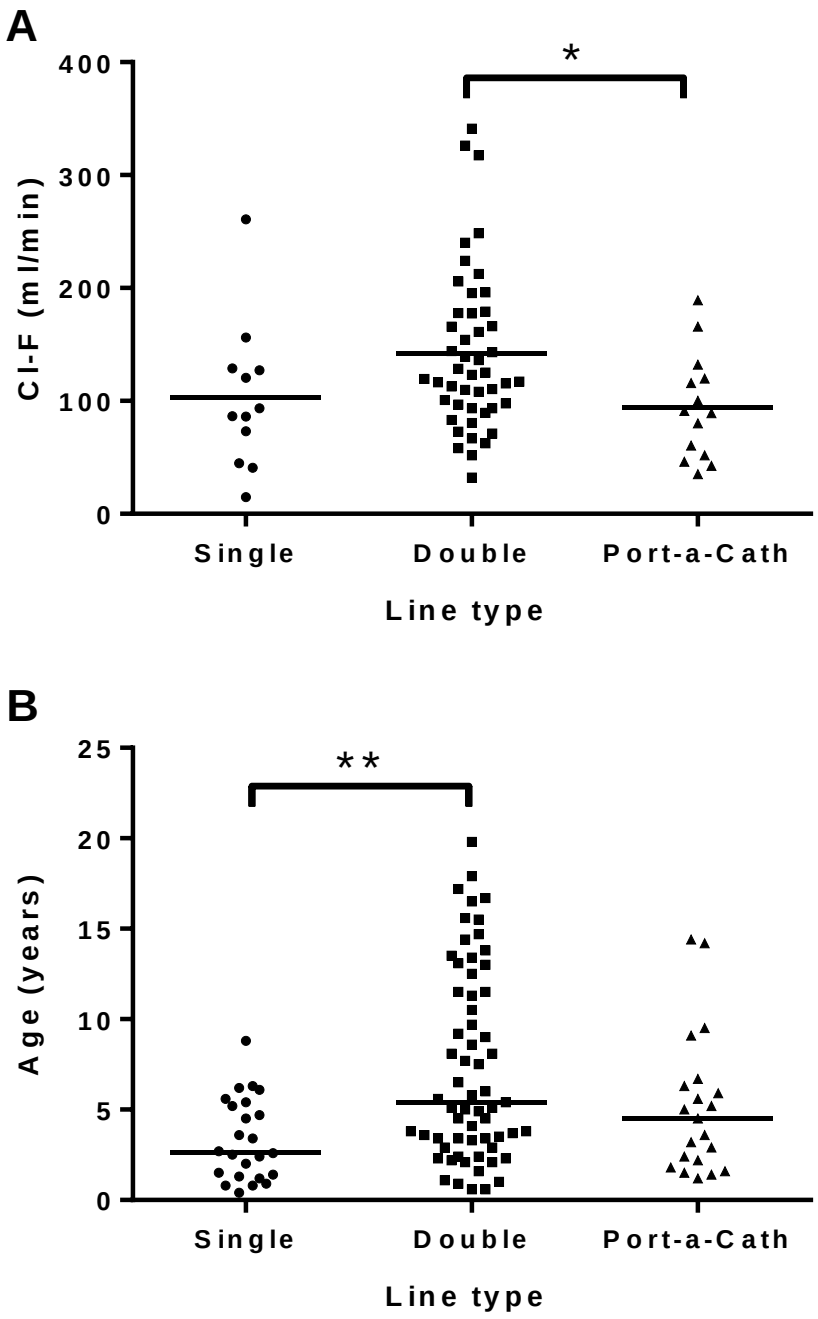

Figure 4.8. Relationship between catheter type and Cl-F and age.

Catheter type was investigated against (A) Act D CL-F and (B) age. Means were tested using the one-way ANOVA with Bonferroni's multiple comparison tests. *p<0.05 and **p<0.01. (B) Data was log transformed to achieve a normal distribution before using the one-ANOVA with Bonferroni's correction for multiple comparisons. Horizontal bar represents; (A) Mean, and (B) median.

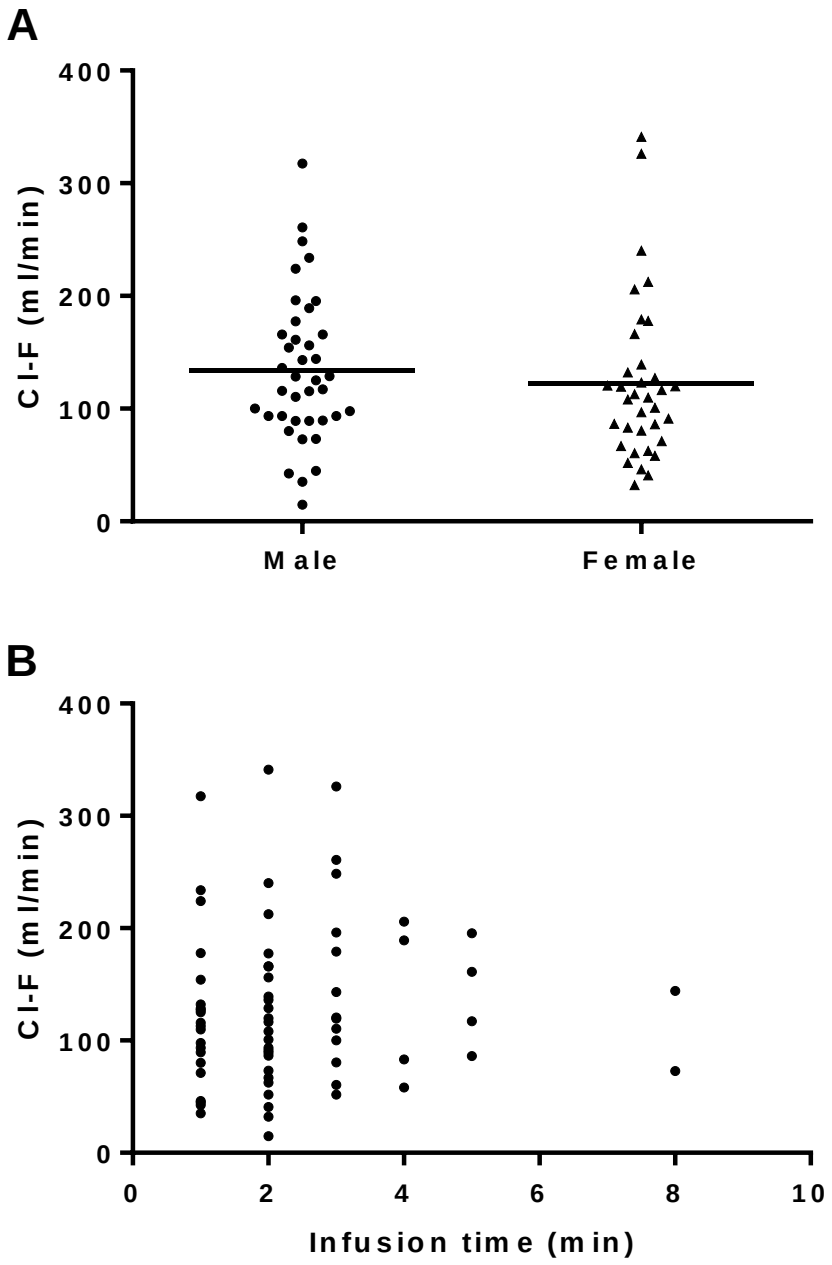

Figure 4.9. Relationship between Cl-F and gender and infusion time.

Cl-F was investigated against (A) gender and (B) Act D infusion time. (A) Means were tested using the unpaired t-test. (B) The association between Cl-F and infusion time was assessed using linear regression, $r^2 = 0.01$.

4.3.5 Tumour type

The impact of tumour type on Act D pharmacokinetic parameters was also investigated. CI-F in ES patients was 1.8-fold higher than patients with Wilms tumour (170 vs. 94ml/min, $p<0.01$, Figure 4.10A). However, this association was strongly influenced by confounding factors such as patient age and consequently body size. Patients with ES were significantly older than those with Wilms tumour (median age 9.2 vs. 3.0 years, Figure 4.10B, $p<0.0001$) and those with RMS (9.2 vs.5.0, Figure 4.10B, $p<0.01$).

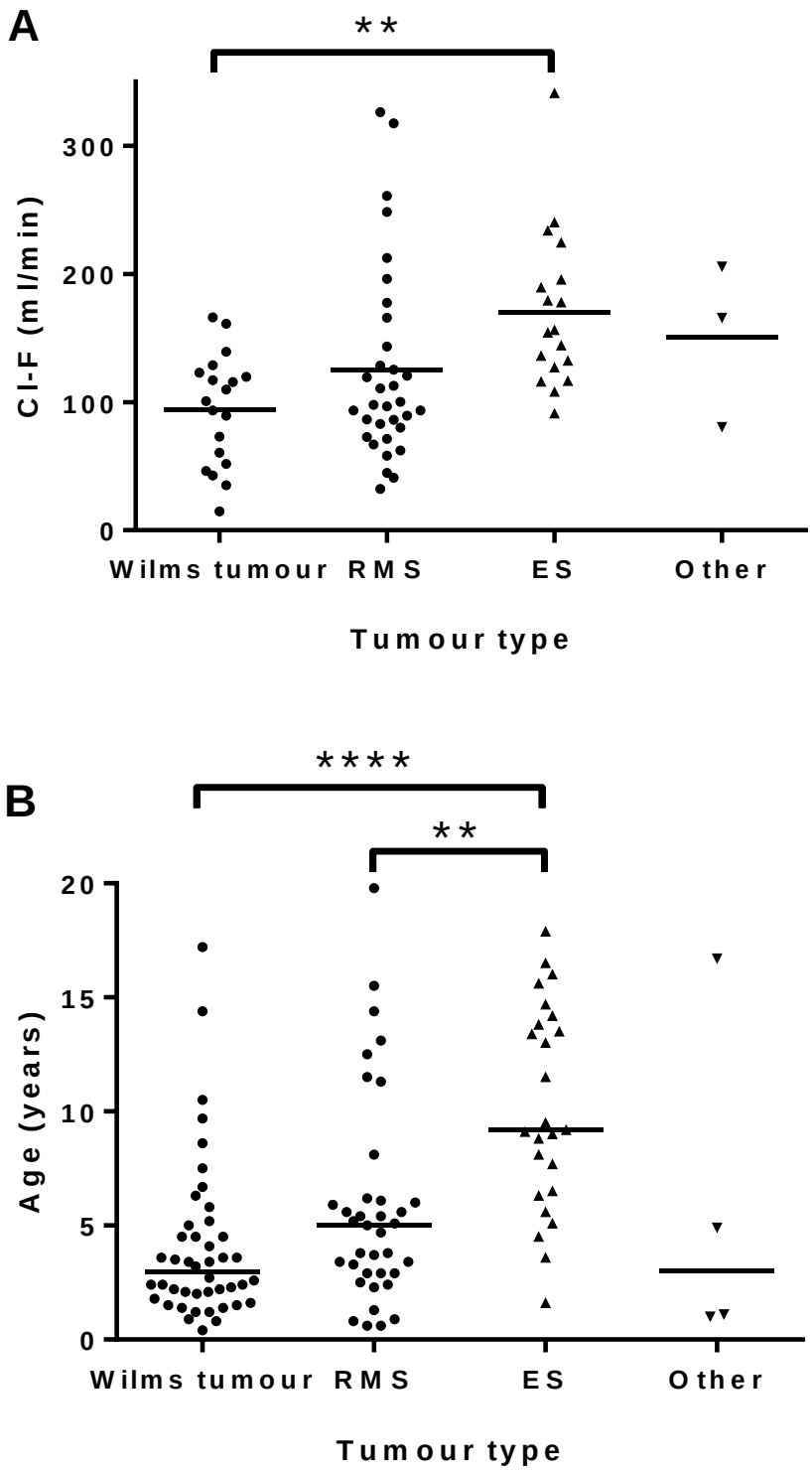

Figure 4.10. Relationship between tumour type, Act D clearance and age.

Tumour type was plotted against Cl-F (A) and patient age at diagnosis (B). **p<0.01, ***p<0.001, ****p<0.0001 compared to ES. (B) Data was log transformed to achieve a normal distribution before using the one-ANOVA with Bonferroni's correction for multiple comparisons. Horizontal bar represents; (A) mean, and (B) median.

4.3.6 Actinomycin D toxicity

Act D treatment was relatively well tolerated; the most common side-effects observed were haematological toxicities. Reduced granulocytes occurred in 45% of patients, 35% of which were considered CTC grade 3 or 4 and grade 3 or 4 leucocyte and haemoglobin toxicity were seen in 26% and 15% of patients respectively. Grade 3 or 4 infection (14%), reduced platelets (9%) and fever (10%) were also observed. Elevated alanine transaminase (ALT) and aspartate transaminase (AST) concentrations were observed in 8% and 2% of patients respectively, however only two cases of grade 3/4 toxicity associated with liver function was recorded. Overall, following administration of Act D, 36 patients (28%) had no adverse events, 63 patients (49%) had one or more grade 1/2 toxicities, and 59 patients (46%) had one or more grade 3/4 toxicities. A summary of all toxicity data is shown in Table 4.4.

No statistically significant associations were found between patient age, weight and SA and grade 3 or 4 treatment related toxicities (Figure 4.11). Analysis of Act D pharmacokinetic parameters also revealed no relationship between peak plasma concentration, clearance or dose and incidence of grade 3 or 4 toxicity (Figure 4.12 and Figure 4.13). It should be noted that any analysis of the influence of Act D pharmacokinetics on toxicity could be confounded by the co-administration of other chemotherapy.

Toxicity	No. of patients (%)	
	All CTC grades	Grades (3/4)
Granulocytes	57 (45)	44 (35)
Leucocytes	56 (44)	33 (26)
Haemoglobin	53 (42)	19 (15)
Infection	21 (17)	18 (14)
Platelets	20 (16)	12 (9)
Fever	20 (16)	12 (9)
ALT	10 (8)	1 (1)
Neuroconstipation	6 (5)	0
Neuromotor	5 (4)	0
AST	3 (2)	1 (1)
Neurosensory	3 (2)	1 (1)
Bilirubin	3 (2)	0
Hepatic enlargement	0	0
Ascites	0	0
Haemorrhage	0	0
Neurocorticol	0	0

Note: Toxicity grades based on National Cancer Institute Common Toxicity Criteria (version 3.0)

Table 4.4. Act D treatment-related toxicity in 127 patients, 102 of which had corresponding pharmacokinetic samples.

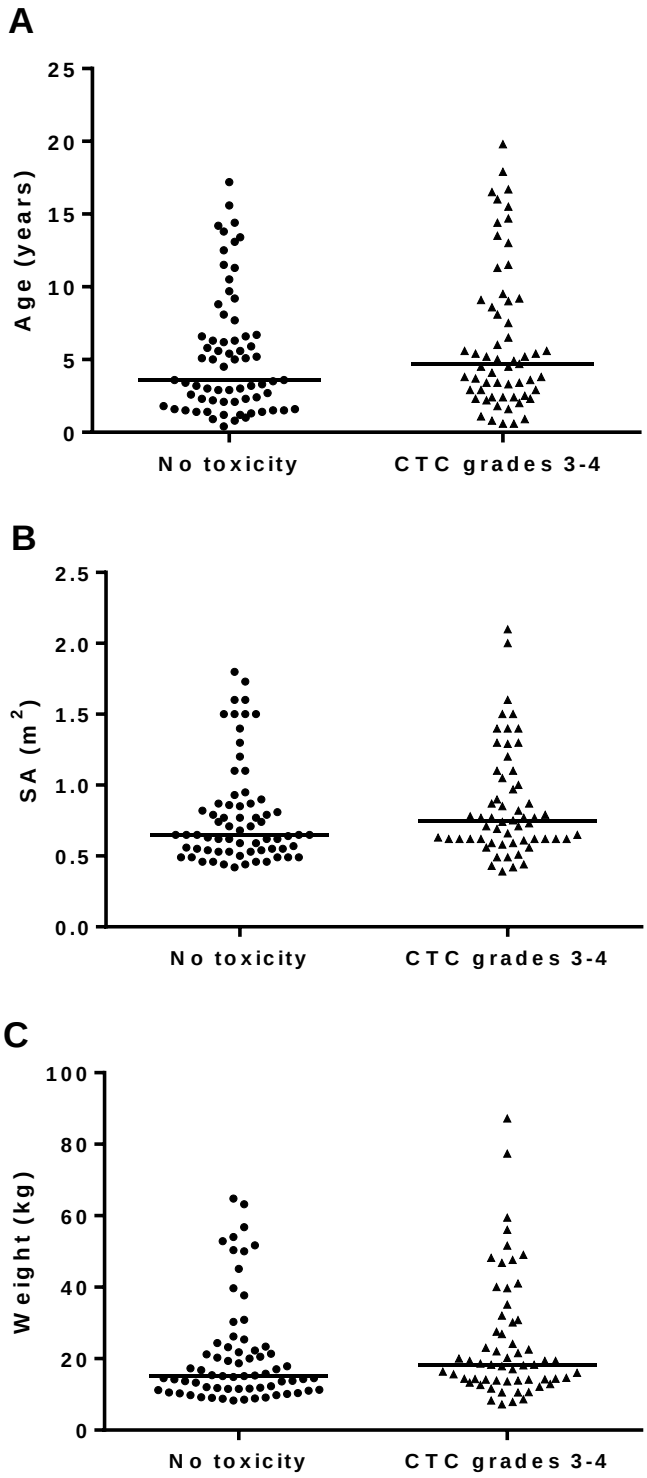

Figure 4.11. Relationship between patient age, weight and body surface area and incidence of CTC grade 3-4 toxicity following Act D administration.

Data for age, SA and weight was log transformed to achieve a normal distribution prior to using the unpaired t-test to compare means.(A) Patient age, p=0.2, (B) weight, p=0.2 and (C) body surface area, p=0.3. N=127. Median is identified by a horizontal bar. No toxicity = includes patients with CTC grade 1-2 toxicity.

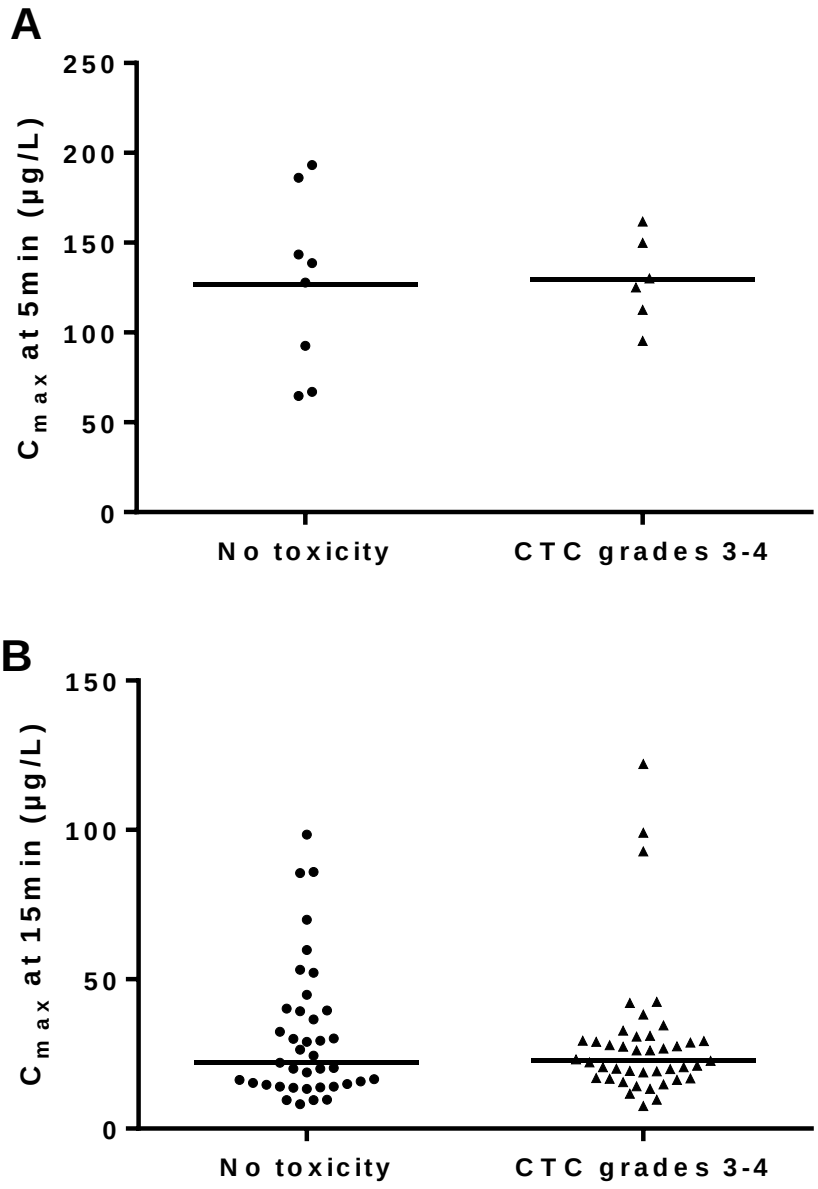

Figure 4.12. Relationship between incidence of CTC grade 3-4 toxicity and C_{max} at 5min and C_{max} at 15min following Act D administration.

(A) C_{max} at 5min, p=0.9, (B) C_{max} at 15min, p=0.9. (B) Data was log transformed to achieve a normal distribution before using the unpaired t-test to compare means. Horizontal bar represents; (A) mean, and (B) median. No toxicity = includes patients with CTC grade 1-2 toxicity.

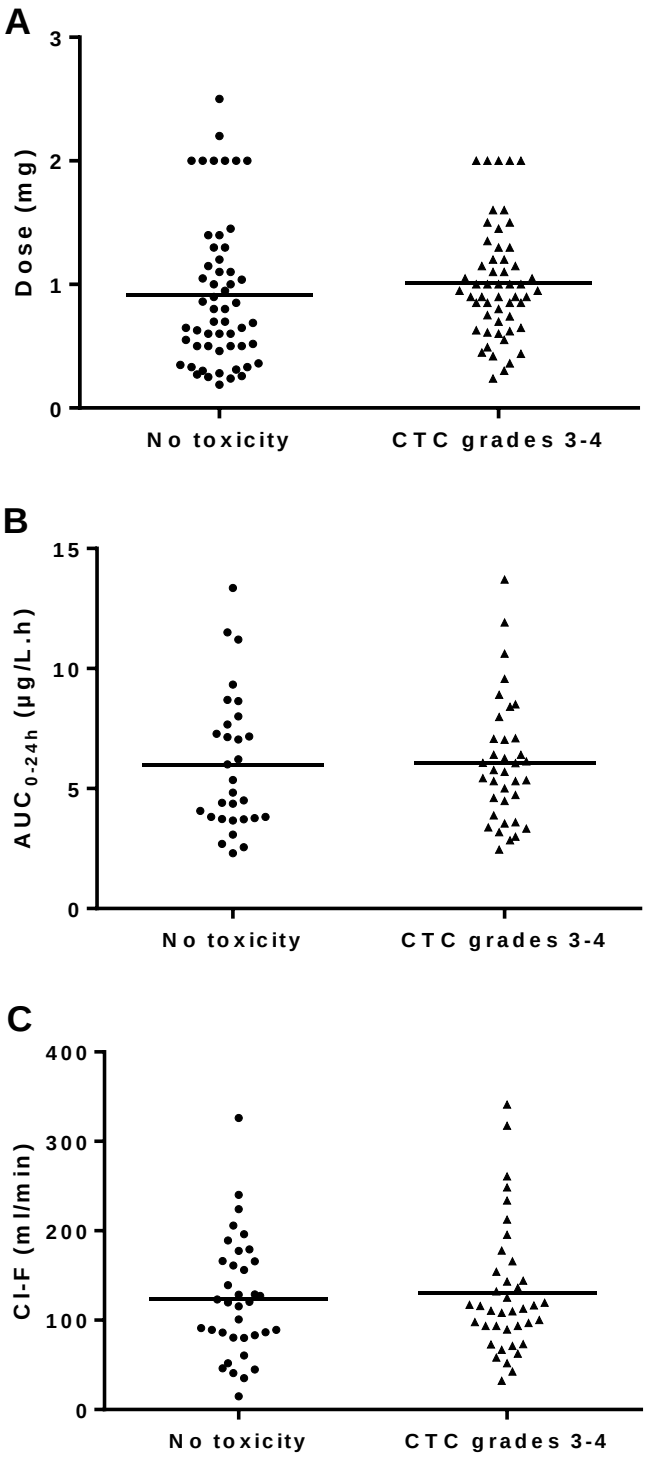

Figure 4.13. Relationship between incidence of CTC grade 3-4 toxicity and dose, $AUC_{0\text{-}24h}$ and Cl-F following Act D administration.

(A) Dose (mg), p=0.3, (B) $AUC_{0\text{-}24h}$, p=0.9 and (C) Cl-F, p=0.7. Mean is identified by a horizontal bar. No toxicity = includes patients with CTC grade 1-2 toxicity

4.4 Discussion

Administration of Act D to paediatric patients over the last 50 years has helped to greatly improve 5 year survival rates in WT, RMS and ES, now standing at 85%, 63% and 64% respectively. Despite this, toxicity remains the major concern with Act D treatment. Inter-patient variation in Act D exposure has previously been reported (Veal *et al.*, 2005), however, low sample numbers and limited early and late sample time points have hindered definitive characterisation of Act D pharmacokinetics.

Over a 7.5 year period, 142 patients were recruited on to this study, 131 of which provided pharmacokinetic samples. Valid pharmacokinetic data were available from 108 patients, compared to 31 recruited in the previously published study (Veal *et al.*, 2005). Early time points were available from 14 (13%) patients and full sample sets were available from 74 (69%) patients, allowing a more comprehensive analysis of Act D pharmacokinetic parameters.

The data presented in this study confirm the large degree of inter-individual variability in the pharmacokinetics of Act D in children. A 3-fold range (64.7-193µg/L) in C_{max} at 5min and 16-fold range (range 7.5-122µg/L) in C_{max} at 15min were observed, and this variation continued to 24h, where the Act D concentrations varied 7-fold (0.7-4.8µg/L). Non-compartmental pharmacokinetic analysis was carried out on data from all 108 valid sample sets. Act D $AUC_{0\text{-}6h}$ varied 9-fold (range 1.1-10mg/L.min) and $AUC_{0\text{-}24h}$ varied 6-fold (range 2.3-13.7mg/L.min). This compares to the previous study, where in 31 patients a 4.4-fold variation in $AUC_{0\text{-}6h}$ (1.12-4.90mg/L.min) and a 31-fold range in C_{max} (3.2-99.2µg/L) were observed. The data presented here are therefore comparable to

those reported by Veal et al in 2005, with a greater degree of variation expected due to a larger sample size.

Due to a 13-fold range in Act D dose (0.19-2.5mg), the primary pharmacokinetic end point used was estimated clearance, calculated from dose in milligrams and $AUC_{0-\infty}$ in mg/ml.min (Cl = Dose / $AUC_{0-\infty}$). $AUC_{0-\infty}$ was estimated for all patients using the log-trapezoidal rule; mean $AUC_{0-\infty}$ was 6.3mg/L.min (range 1.4-19.8mg/L.min). $AUC_{0-\infty}$ estimates from limited sample sets were significantly under-estimated compared to $AUC_{0-\infty}$ from patients with full sample sets (4.1 vs. 9.0mg/L.min, $p<0.0001$). After dose was taken into account by estimating clearance, the difference between groups was still significant (211 vs. 127 ml/min, $p=0.003$), therefore only Cl-F were used for covariate and toxicity analysis. A large degree of variability in Cl-F was observed, with a 23-fold range (14.8 to 341ml/min) compared to 7-fold range (48-345ml/min) previously reported (Veal *et al.*, 2005).

The relationship between age, weight and SA was investigated in this paediatric population. A large interrelationship between body size indicators age, weight and SA was observed. Cl-F was positively correlated to age, weight and SA (r^2 = 0.6 for all). When separating these data into age groups, the mean Cl-F in patients under 3 years was 54% of that in patients 3 years old and above (78.2 vs. 144ml/min, $p=0.0002$). Predictably, this was also observed with patient weight and SA; Cl-F was 2.9-fold higher in patients of 10kg or above (46.8 vs. 136ml/min, $p = 0.0008$) and 2.3-fold higher in patients who were above 0.5m^2 (59 vs. 138ml/min, $p=0.0005$). However, these measures of body size were not predictive of Cl-F. When selecting patients that were between 2-3 years old at the time of treatment, a 2.7-fold range in Cl-F was seen (66.9-178ml/min). A

similar result was obtained for patient SA and weight; patients that were between 10-15kg had a 3-fold range in Cl-F (35.1-120ml/min) and patients whose SA was between 0.6-0.7m^2 also had a 3-fold range in Cl-F (46.3-143ml/min). Variability in CI-F was still apparent when adjusting for patient age, weight or SA, confirming that other factors in addition to body size need to be considered in order to achieve consistent Act D exposure in children.

There was no evidence to suggest that gender or Act D infusion time had an influence on Cl-F. Although tumour type was found to be associated with Cl-F, this was a result of confounding factors such as patient age and body size. Patients with ES had a median age of 9.2 years compared to 3.0 and 5.0 years in WT and RMS, respectively. Unfortunately, perhaps due to complexity of the treatment regimens used to treat paediatric tumours, concomitant therapy data was unavailable therefore it was not possible to assess any drug-drug interactions which may be occurring in this patient population.

Paediatric oncology patients almost invariably have a semi-permanent fixed central venous catheter (CVC), to facilitate the administration of drugs and for routine biochemical sampling. While this CVC provides a convenient access point for obtaining blood samples for pharmacokinetic analysis, a number of concerns need to be addressed. Firstly, any dead-space in the line must be removed before the pharmacokinetic sample is obtained and secondly, the risk of line contamination must be minimised by an appropriate flushing procedure between drug administration, and pharmacokinetic sampling. The paediatric oncology centres contributing to this pharmacokinetic study are staffed by experienced research nurses who receive specific training for pharmacokinetic

studies and adhere to appropriate flushing and sampling procedures, standardised across all of the centres.

It has recently been reported that binding of administered Act D to the catheter may result in contamination of pharmacokinetic samples, with an initial contribution to the measured plasma concentration of 19%. This contribution was modelled to decrease exponentially with time, and to be removed by an appropriate catheter clearing procedure (Edwards *et al.*, 2012). An initial comparison of Cl-F between different catheter types indicated a difference, which might be interpreted as suggesting a problem with sample contamination with single-lumen lines or port-a-caths. However, line type was closely linked to age, which also influences pharmacokinetics. It was not possible to separate these confounded potential influences on pharmacokinetics in the current study. Based on previous experience with pharmacokinetic sampling using a range of CVCs, it is unlikely that line contamination has a significant effect on this dataset.

Several studies have demonstrated that treatment with Act D can cause life-threatening toxicity (Green *et al.*, 1988; Raine *et al.*, 1991; Bisogno *et al.*, 1997), with a reported incidence of 1.7-13.5%. However, in the current study, Act D treatment appeared to be well tolerated. Haematological toxicities were by far the most common adverse effect suffered by patients, with CTC grade 1-4 haematological toxicities occurring in 45% of the population. Increases in ALT or AST, signifying hepatotoxicity, were rare, occurring in 8% and 2% of patients respectively. Only two patients had CTC grade 3 or 4 elevated AST or ALT, therefore covariate comparisons were not possible.

No significant correlations were observed between pharmacokinetic parameters and treatment related toxicity in terms of individual toxicities. After grouping patients that experienced CTC grade 3 or 4 toxicity and those that did not, no influence of age, weight, SA, C_{max}, Cl-F or dose was found.

Consistent with previous studies, the data presented here demonstrate inter-patient variability in Act D exposure in patients treated on the same dosage regimen. Due to the low number of young and smaller patients, meaningful comparisons with patient age or size and pharmacokinetic parameters were not previously possible. In the current study, full sample sets were available from 19 patients (25%) under the age of 3 years, and 7 patients (9%) under 10kg.

Clearance of Act D has been shown to be positively associated with patient age, weight and body size. Current dosing guidelines suggest a dose cap of Act D of 2mg regardless of patient age, weight or SA. The data from this study suggest that this dose cap could result in some larger patients being under-dosed, resulting in potentially sub-therapeutic Act D exposures. In addition to this, dose administered had no impact on treatment-related toxicities, further questioning the relevance of a 2mg dose cap. No evidence exists in the literature to justify the rationale for this dose capping. Vincristine is another anti-cancer drug which is subjected to dose capping. An investigation in children with leukaemia demonstrated no pharmacokinetic rationale for the dose cap at 2mg, and similar to our data, suggested that older, larger patients receive less intensive chemotherapy due to under-dosing (Frost *et al.*, 2003).

Patient age and weight have both been identified as possible risk factors for the development of toxicity following Act D treatment (Arndt *et al.*, 2004; Veal *et al.*,

2005; Langholz *et al.*, 2011). In our study patients below 3 years old were shown to have 1.8-fold lower systemic Act D clearance, compared to patients older than 3 years (Cl-F 78.2 vs. 144ml/min, p=0.0002). Similar findings were observed with patient weight, where patients below 10kg had 3-fold lower Cl-F compared to those above 10kg. These data suggest that smaller, younger patients have relatively higher Act D exposures. This could highlight the possibility of greater toxicity risk, although this cannot be confirmed without any patients with hepatotoxicity being reported in this study.

Estimating $AUC_{0-\infty}$ for those patients without late pharmacokinetic sampling was challenging using non-compartmental analysis. The use of population pharmacokinetic approaches would allow an appropriate pharmacokinetic model to be generated, and subsequently, more accurate $AUC_{0-\infty}$ estimation especially for those patients lacking later pharmacokinetic samples. Act D pharmacokinetics in patients has previously been fitted to a three-compartment model with first order elimination (Mondick *et al.*, 2008; Edwards *et al.*, 2012). Confirmation of this model, or generation of a new model, would allow more accurate extrapolation for $AUC_{0-\infty}$ for those patients with limited sample sets.

Although many of the agents used to combat paediatric cancer today have been used for many years, for drugs such as Act D, there remain considerable gaps in our knowledge. The success seen over the last four decades in improving survival rates of children with cancer should not deter efforts to further improve therapy, especially given the potential to reduce life-threatening toxicities. The main aim now for pharmacokinetic studies is to provide supportive data to maintain current survival rates and reduce treated-associated toxicities.

This study has demonstrated a large degree in variability in Act D exposure between patients, which appears to be partially attributed to patient age and size. Act D has been shown both in Chapter 2 and Chapter 3 be a good substrate for ABCB1, and to a lesser extent ABCC2. It is possible that SNPs in these proteins, that have been previously shown to influence drug disposition of important anti-cancer agents, could be responsible for some of this variation.

Chapter 5. Pharmacogenetic analysis of patients being treated with actinomycin D

5.1 Introduction

Inter-patient variation in drug exposure is a very common observation for many anti-cancer agents, in both adult and paediatric patient populations. With the advent of modern biological techniques, it has been suggested that much of the inter-patient variation observed could be due to genetic differences among individuals. SNPs occur every 1000-3000 bases (Sachidanandam, 2001) and are implicated in 20-95% of differences in drug response (Evans and McLeod, 2003).

Act D exposure in paediatric patients has been shown previously to be highly variable, with the pharmacokinetic variability observed between patients strongly indicating that current surface-area based dosing regimens are not optimal (Veal *et al.*, 2005). Consistent with these previous data, marked inter-patient variability in Act D exposure was confirmed in the current study (Chapter 4). Patient age and size have previously been associated with Act D treatment-related toxicity, with younger, smaller patients more likely to experience some form of toxicity during treatment (Arndt *et al.*, 2004; Langholz *et al.*, 2011).

The data presented in the current study suggest that younger patients with small body surface area and body weight appear to have low clearance, indicating high Act D exposure and therefore the possibility of an increased risk of toxicity. Conversely, older patients with a large surface area and body weight exhibit relatively high clearance values, resulting in low Act D exposure and the possibility of sub-therapeutic dosing. Therefore identification of factors that

could be predictive of Act D pharmacokinetics could be important in the optimisation of Act D dosage regimens.

ABC transporters are present throughout the body, and are well recognised to have a key role in removing both endogenous and exogenous substances from the body (Ho and Kim, 2005). Many commonly used drugs, including anti-cancer agents have been shown to be substrates for various ABC transporters, and as such, ABC transporters have the potential to impact drug disposition and elimination from the body. SNPs in these transporters that influence the function or expression of ABC transporters therefore have the potential to alter drug transport and pharmacokinetics. For example, SNPs in ABCB1 have previously been shown to alter exposure of the commonly used anti-cancer agent doxorubicin in Asian breast cancer patients (Lal *et al.*, 2008) and be predictive of clinical outcome in breast cancer and multiple myeloma patients (Bray *et al.*, 2010; Buda *et al.*, 2010). However, clinical studies investigating the impact of ABCB1 SNPs are highly inconsistent.

Act D has been confirmed as a good substrate for ABCB1 and ABCC2 through growth inhibition and intracellular accumulation assays in Chapter 2. Following this in Chapter 3 ABC transporter function was confirmed to affect the pharmacokinetics of Act D in an ABC transporter knockout mouse model. Based on these findings the potential effects of clinically-relevant SNPs and their associated diplotypes for both ABCB1 and ABCC2, on the pharmacokinetics of Act D are investigated in the current chapter.

5.2 Materials and methods

5.2.1 Patient eligibility, treatment and blood sampling

Patients were eligible and treated as per (4.2.1). Whole blood samples (5-10ml) were collected in tubes containing EDTA as an anti-coagulant, and stored at -20^{o}C for pharmacogenetic analysis.

5.2.2 DNA extraction from whole blood

QIAamp DNA blood Maxi kits were used to extract DNA from whole blood according to the manufacturer's instructions. Briefly, 5-10ml of whole blood was mixed with 500µl QIAGEN Protease , and all tubes were made up to 10ml with PBS. Buffer AL (12ml) was used to lyse the cells during a 10min incubation at 70^{o}C and following this, 100% ethanol (10ml) was added to each tube to bind the DNA. This solution was added to a QIAamp Maxi column. Subsequent centrifugation steps (2,000g for 5min) ensured that all waste was removed, leaving the DNA bound to the QIAamp Maxi column. The column was washed twice, once with Buffer AW1 (5ml) and once with Buffer AW2 (5ml), and the DNA was eluted with 1ml Buffer AE. DNA purity and concentration was measured against a Buffer AE blank using a NanoDrop ND-1000 (Thermo Scientific, Rockford, USA).

5.2.3 Genotype analysis

Genetic analysis was performed on genomic DNA obtained and extracted as described (5.2.2). To assess whether ABCB1 and ABCC2 variants are responsible for inter-patient variability in Act D exposure, tagSNPs were selected using the international HapMap database and Haploview with the integrated tagger tool to capture alleles and regions of interest. The tagger

software was set to output tagSNPs based on the CEU (U.S. residents of northern and western European ancestry) and TSI (Tuscans in Italy) databases, with a minor allele frequency (MAF) of 0.1 and a minimum r^2 value of 0.8 for both genes. In addition to this, SNPs were also chosen if clinical relevance had previously been shown. TagSNPs were used to minimise the amount of genotyping that was required, preventing SNPs in linkage disequilibrium (LD) being individually genotyped. This resulted in 18 tagSNPs in ABCB1 encompassing 67 polymorphic sites (Figure 5.1, Table 5.1) and 11 tagSNPs in ABCC2 encompassing 27 polymorphic sites (Figure 5.2, Table 5.2), spanning the majority of both genes.

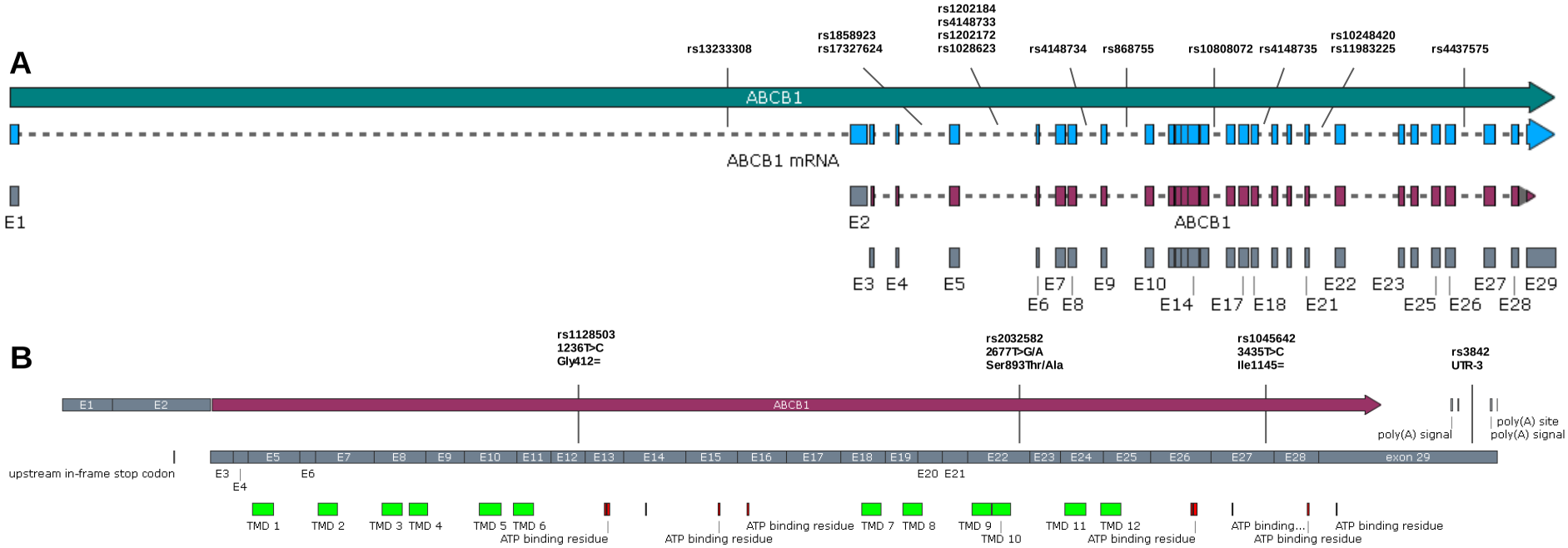

Figure 5.1. Locations of genotyped tagSNPs in the human ABCB1 gene and mRNA chosen based on minor allele frequency and clinical association

The locations of the 14 intronic tagSNPs are displayed on the ABCB1 gene (A) and the 4 exonic tagSNPs SNPs are displayed on the ABCB1 mRNA (B).

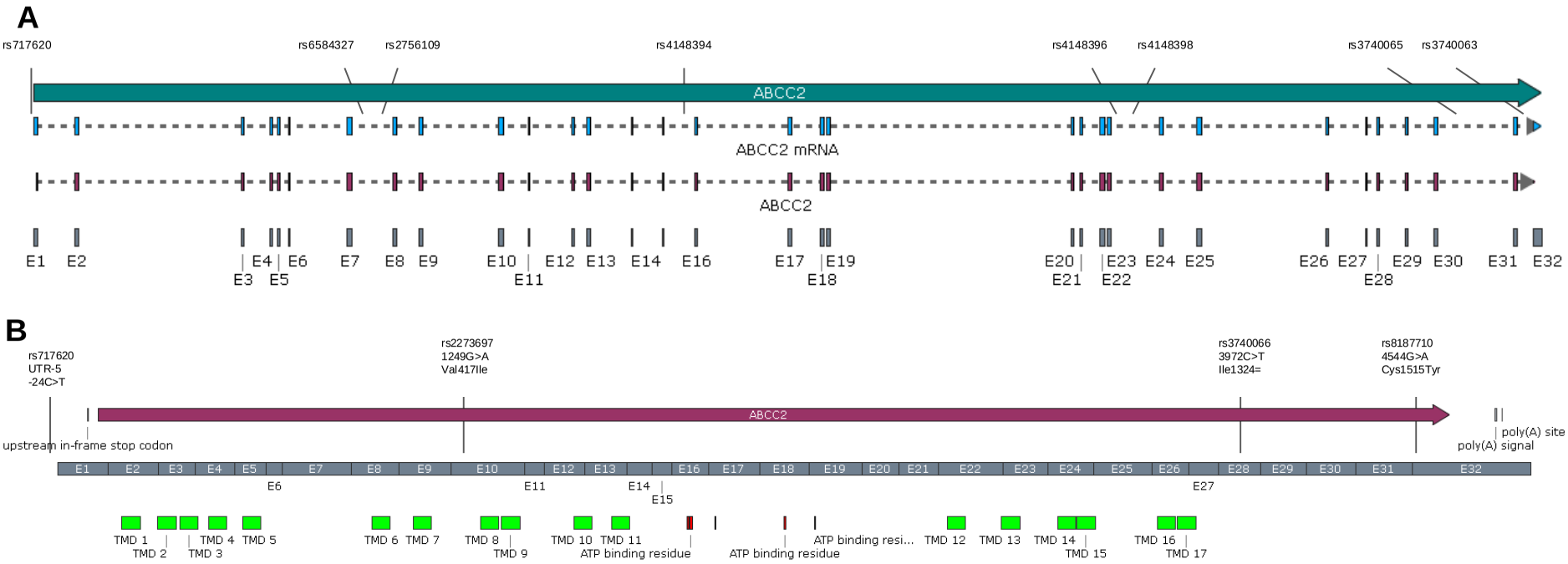

Figure 5.2. Locations of genotyped tagSNPs in the human ABCC2 gene and mRNA chosen based on minor allele frequency and clinical association.

The locations of the 7 intronic tagSNPs are displayed on the ABCC2 gene (A) and the 4 exonic tagSNPs are displayed on the ABCC2 mRNA (B).

ABCB1 tagSNPs	Captured alleles	MAF	Exon/Intron	Gene location NG_011513	Clinical association
rs13233308	-	0.43	Intron 1-2	102605G>A	No (Xing *et al.*, 2006)
rs1858923		0.42	Intron 4-5	126349T>C	-
	rs12535512		Intron 4-5	127231A>G	-
	rs3789243		Intron 4-5	126679T>C	Possible (Haerian *et al.*, 2011; He *et al.*, 2011)
rs17327624	-	0.13	Intron 4-5	130748C>A	(Bochud *et al.*, 2008)
rs1202184		0.5	Intron 5-6	133664G>A	(Krupoves *et al.*, 2009; Lin *et al.*, 2011)
	rs11763872		Intron 4-5	130350A>G	
rs4148733		0.1	Intron 5-6	134333T>C	(Bochud *et al.*, 2008)
	rs17327442		Intron 4-5	130350A>G	
rs1202172		0.45	Intron 5-6	136591G>T	(Wong *et al.*, 2008; Krupoves *et al.*, 2009)
	rs1989830		Intron 5-6	141902T>C	
	rs1202175		Intron 5-6	138415C>T	
	rs1202185		Intron 5-6	134181G>A	
	rs1202179		Intron 5-6	143286G>A	
	rs1202182		Intron 4-5	132261C>T	
	rs1202171		Intron 5-6	136520A>T	
	rs1202186		Intron 5-6	134307G>A	
	rs1202181		Intron 4-5	131415C>T	
	rs1202180		Intron 5-6	143725G>A	
rs10280623		0.25	Intron 5-6	145021A>G	(Uhr *et al.*, 2008)
	rs10260862		Intron 5-6	146083C>G	
	rs12334183		Intron 5-6	146185A>G	
	rs10808071		Intron 26-27	206757T>C	
	rs2235015		Intron 5-6	148001G>T	
rs4148734		0.29	Intron 8-9	153968C>T	(Potocnik *et al.*, 2004; Potocnik *et al.*, 2008)

ABCB1 tagSNPs	Captured alleles	MAF	Exon/Intron	Gene location NG_011513	Clinical association
	rs2235035		Intron 14-15	168479C>T	
	rs10259849		Intron 5-6	146723G>A	
	rs1922240		Intron 9-10	164211A>G	
	rs1922241		Intron 9-10	161671C>	
rs868755		0.38	Intron 9-10	157635A>C	(Huebner *et al.*, 2009; Krupoves *et al.*, 2009)
	rs6949448		Intron 26-27	205751A>G	
	rs2235046		Intron 17-18	173499A>G	
	rs1202169		Intron 7-8	151715A>G	
	rs2520464		Intron 4-5	146479G>A	
	rs10276036		Intron 10-11	167367G>A	
	rs4148738		Intron 21-22	184516G>A	
	rs1202167		Intron 6-7	150506G>A	
	rs1202168		Intron 7-8	151603C>T	
	rs11975994		Intron 8-9	154834C>T	
rs1128503	-	0.45	Exon 12	167964C>T 1236C>T Gly412=	
rs10808072		0.47	Intron 15-16	171102T>C	
	rs2235033		Intron 14-15	168422T>C	
	rs1202170		Intron 8-9	152459G>A	
	rs6961665		Intron 10-11	166147G>T	
	rs2235013		Intron 15-16	168939G>A	
rs4148735		0.43	Intron 18-19	174684G>A	(Krupoves *et al.*, 2009)
	rs6961419		Intron 18-19	175429A>G	
	rs4148737		Intron 18-19	176413A>G	
	rs2091766		Intron 16-17	173061G>A	
rs10248420	-	0.18	Intron 21-22	182579T>C	(Krupoves *et al.*, 2009)

ABCB1 tagSNPs	Captured alleles	MAF	Exon/Intron	Gene location NG_011513	Clinical association
rs11983225		0.16	Intron 21-22	186045A>G	(Krupoves *et al.*, 2009)
	rs11760837		Intron 21-22	184549A>G	
	rs2235040		Intron 21-22	181815G>A	
	rs2235067		Intron 23-24	197643G>A	
	rs10268314		Intron 19-20	177896A>G	
	rs4148739		Intron 21-22	186516A>G	
	rs10244266		Intron 9-10	159098A>C	
	rs10280101		Intron 22-23	193980T>G	
	rs10276603		Intron 18-19	176038A>G	
	rs12720067		Intron 19-20	178209G>A	
	rs10274587		Intron 21-22	183082C>T	
	rs10225473		Intron 22-23	192919T>C	
	rs2032583		Intron 22-23	187004T>C	
rs2032582	-	0.47	Exon 22	186947G>T/A 2677G>T/A Ser893Thr/Ala	
rs4437575		0.47	Intron 26-27	208249T>C	(Lin *et al.*, 2011)
	rs2235048		Intron 27-28	209054C>T	
rs1045642	-	0.43	Exon 27	208920T>A 3435C>T Ile1145=	
rs3842	-	0.16	Exon 29	214199A>G UTR-3	

All 18 tagSNPs genotyped in ABCB1 are listed along with the 67 polymorphic sites that are “captured” by the chosen tagSNPs due to their linkage disequilibrium. Their location and clinical significance (if appropriate) are also listed.

Abbreviations: MAF, minor allele frequency

Table 5.1. TagSNPs in the ABCB1gene genotyped in this study.

ABCC2 tagSNP	Captured alleles	MAF	Exon/Intron	Gene location NG_011798	Clinical association
rs717620	-	0.23	Exon1	5116C>G -24C>T UTR-5	(Zhou *et al.*, 2005; Daly *et al.*, 2007; Haenisch *et al.*, 2007; Han *et al.*, 2007; Fujita *et al.*, 2008; Sun *et al.*, 2010; Franke *et al.*, 2011)
rs6584327		0.48	Intron 7-8	19867A>C	(Sookoian *et al.*, 2008a; Sookoian *et al.*, 2009)
	rs2756103		Intron 1-2	5585A>C	
	rs4148397		Intron 23-24	54858A>G	
	rs4148388		Intron 2-3	12303G>A	
	rs4148386		Intron 2-3	11006G>A	
	rs7393105		Intron 2-3	9559C>A	
	rs2804400		Intron 3-4	15797C>T	
	rs4148385		Intron 2-3	10715A>C	
	rs4919395		Intron 1-2	5501A>G	
	rs4148389		Intron 2-3	12449G>A	
	rs2756105		Intron 2-3	9580C>T	
	rs2756104		Intron 1-2	6564C>T	
rs2756109	-	0.45	Intron 7-8	21284G>T	(Sloan *et al.*, 2012)
rs2273697		0.23	Exon 10	26353G>A 1249G>A Val417Ile	(Vogelgesang *et al.*, 2004; Meyer zu Schwabedissen *et al.*, 2005; Haenisch *et al.*, 2008; Kim *et al.*, 2010)
	rs11190291		Intron 11-12	28548C>T	
rs4148394		0.3	Intron 15-16	34881A>C	(Sookoian *et al.*, 2008a)
	rs2002042		Intron 19-20	50469C>T	
rs4148396			Intron 23-24	54482T>C	(Cecchin *et al.*, 2012)
	rs2804398		Intron 7-8	21172A>T	
	rs3740074		Intron 15-16	34066C>T	

ABCC2 tagSNP	**Captured alleles** rs2073337 rs3740073	**MAF**	**Exon/Intron** Intron 12-13 Intron 16-17	**Gene location NG_011798** 29964A>G 39498T>C	**Clinical association**
rs4148398	-	0.33	Intron 23-24	55160A>G	-
rs3740066	-	0.34	Exon 28	66745C>T 3972C>T Ile1324=	(Sookoian *et al.*, 2008a; Cecchin *et al.*, 2012; Qu *et al.*, 2012)
rs3740065	-	0.1	Intron 29-30	68231A>G	(Kiyotani *et al.*, 2010)
rs3740063	-	0.41	Intron 31-32	73261A>G	-
rs8187710	-	0.05	Exon 32	73832G>A 4544G>A Cys1515Tyr	(Wojnowski *et al.*, 2005; Elens *et al.*, 2009; Sookoian *et al.*, 2009; Ni *et al.*, 2010; Elens *et al.*, 2011; Simon *et al.*, 2012)

All 11 tagSNPs genotyped in ABCC2 are listed along with the 27 polymorphic sites that are "captured" by the chosen tagSNPs due to their linkage disequilibrium. Their location and clinical significance (if appropriate) are also listed.

Abbreviations: MAF, minor allele frequency

Table 5.2. TagSNPs in the ABCC2 gene genotyped in this study.

5.2.4 Patient genotyping by Real-time Polymerase Chain Reaction using the TaqMan® method

According to DNA concentrations determined in 5.2.2, patient DNA was diluted in ddH_2O to 10ng/µl to produce a working stock of DNA for each patient. TaqMan® SNP primer sets and probes for all 29 SNPs (5.2.3) were obtained from Applied Biosystems.

Prior to adding DNA samples, a reaction master mix was made up (Table 5.3) in a sterile 5ml tube and 24µl was added to each required well of a 0.1 ml MicroAmp Fast Optical 96-well plate (Applied Biosystems).

Reagent	Initial Concentration	Final Concentration	Volume per sample	Volume for full plate
Master Mix	2x	1x	12.5µl	1250µl
SNP primers	20x	1x	1.25µl	125µl
ddH_2O			10.25µl	1025µl

Table 5.3. Volume required for each component of the TaqMan PCR reaction mixture.

Full plate volume = 100 samples (96 wells + 4 for error).

Following this, 1µl of DNA (10ng) was added to each well. No-template controls (reaction master mix only) were placed at the beginning and end of the samples, and where possible, known genotype controls were used to validate the primers. Plates were sealed using MicroAmp® Optical Adhesive Film and Real-Time PCR was carried out (Figure 5.3) using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, California, USA). Upon completion, endpoint allelic discrimination was performed at 60^oC, allowing quantitative analysis of the fluorescence of both the wild type and variant reporters in each well of the 96-well plate. Applied Biosystems Sequence Detection Software (SDS) version 1.4 was used to run the Real-time PCR system, analyse data and assign

genotypes. Example allelic discrimination plots can be found in Figure A.1 and Figure A.2 in the appendix.

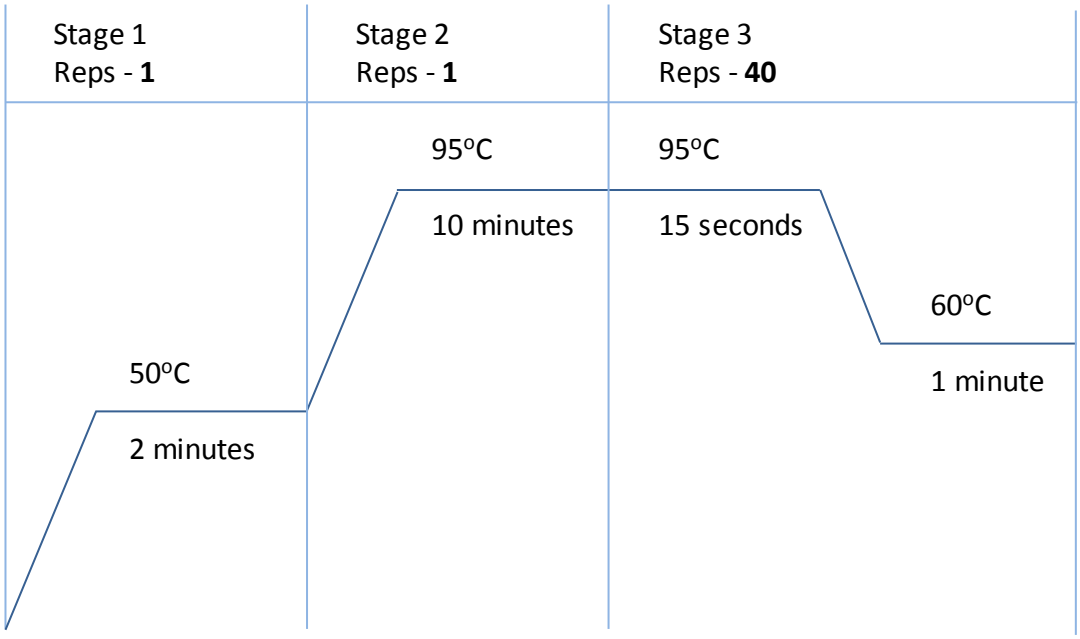

Figure 5.3. Real-time PCR conditions used for the TaqMan® and SYBR Green methods.

5.2.5 Statistical analysis

Hardy-Weinberg equilibrium was assessed using the Chi-squared test, a p-value of >0.05 was taken as data being consistent with Hardy-Weinberg equilibrium. D' and R-squared between all SNPs within ABCB1 and ABCC2 were calculated using the program pwld (David Clayton, Cambridge Institute for Medical Research, Cambridge University, UK) in Stata/SE 11.2 (StataCorp, College Station, Texas). These values were imported into Microsoft Excel 2010 and were plotted against each other using the conditional formatting feature to create the plot in Figure 5.4.

Genotypes were analysed separately and the ABCB1 SNPs 1236C>T, 2677G>T/A and 3435C>T were grouped into diplotypes according to Table 5.4

for analysis against pharmacokinetic parameters. Overall means between groups were compared using the unpaired t-test (two groups) and one-way ANOVA (three groups) with Bonferroni's correction for multiple comparisons. If data were not normally distributed, the data were log-transformed to achieve a normal distribution, after which an unpaired t-test or one-way ANOVA with Bonferroni's correction for multiple comparisons was performed to compare means. Normality was checked by producing a histogram of the data. If normality could not be achieved by log-transforming the data, a Kruskal-Wallis one-way ANOVA was performed.

Due to the large number of statistical tests performed, the p-value accepted as significant will be changed accordingly. As 29 statistical tests were performed on Cl-F, a p-value of 0.002 (0.05/29) will be accepted as significant.

Diplotype	Allele 1			Allele 2		
	1236C>T	2677G>T/A	3435C>T	1236C>T	2677G>T/A	3435C>T
1	C	G	C	C	G	C
2	C	G	C	*	*	*
3	C	G	C	T	T/A	T
4	T	T/A	T	*	*	*
5	T	T/A	T	T	T/A	T
6	C	G	-	C	G	-
7	C	G	-	♦	♦	-
8	C	G	-	T	T/A	-
9	T	T/A	-	♦	♦	-
10	T	T/A	-	T	T/A	-
11	-	G	C	-	G	C
12	-	G	C	-	♦	♦
13	-	G	C	-	T/A	T
14	-	T/A	T	-	♦	♦
15	-	T/A	T	-	T/A	T

* Any combination of alleles that is not mutually exclusive with another diplotype consisting of all three SNPs.
♦ Any combination of alleles that is not mutually exclusive with another diplotype consisting of only the 2677 and 3435 SNPs.

Table 5.4. Diplotype groupings for ABCB1 SNPs 1236C>T, 2677G>T/A and 3435C>T. Diplotypes 1-5 are based on all 3 SNPs. Diplotypes 6-10 include only diplotypes in SNPs 1236C>T and 2677G>T/A, whilst diplotypes 11-15 include only diplotypes in SNPs 2677G>T/A and 3435C>T. Diplotypes based on (Sissung *et al.*, 2008).

Post-hoc power calculations were carried out using the 3435C>T SNP data and the one-way ANOVA power calculator in Minitab 16. The population standard deviation was 50.5 and the maximum difference between the three groups was set at 30.9 with same size mean of 21 patients per genotype used.

5.3 Results

5.3.1 DNA extraction and quantification

DNA was extracted from whole blood from 126 patients. Following extraction DNA was quantified; mean DNA concentration was 310 ± 178µg.

5.3.2 Genotyping

One hundred and twenty six patients provided samples for genetic analysis Twenty nine tagSNPs, encompassing 94 polymorphic sites across ABCB1 and ABCC2 were chosen to be genotyped based on clinical relevance and a minor allele frequency of ≥ 0.1 (10%). At least one copy of the variant allele in the three common exonic ABCB1 SNPs, 1236C>T, 2677G>T/A and 3435C>T were present in 43%, 43% and 48% of patients respectively. The rare ABCB1 SNP 2677A allele, causing a change in amino acid from serine to alanine, was present in 9 patients (allele frequency – 0.04). The commonly-studied ABCC2 exonic SNPs rs2273697 and rs3740066 were present in 22% and 35% of individuals on study. Although rs8187710 in ABCC2 had a minor allele frequency of only 0.05 in a Caucasian population according to HapMap data, it was selected to be genotyped as it is a non-synonymous SNP with clinical relevance (Table 5.2). Full genotype and allelic frequencies can be found for ABCB1 in Table 5.5 and ABCC2 in Table 5.6. All genotype frequencies were comparable to those previously observed in Caucasian populations (Table 5.1 and Table 5.2) and all were in Hardy-Weinberg equilibrium.

ABCB1 Polymorphism	No of patients with genotype (frequencies)			Allelic frequencies‡	
	Allele 1†	Heterozygous	Allele 2†	p	q
1236C>T	44 (0.35)	55 (0.44)	27 (0.21)	0.567	0.433
2677G>T/A	35 (0.28)	GT - 58 (0.46) GA – 7 (0.06) TA – 2(0.02)	24 (0.19)	0.536	0.429 (A - 0.036)
3435C>T	28 (0.22)	64 (0.51)	34 (0.27)	0.476	0.524
rs11983225	1 (0.01)	30 (0.24)	95 (0.75)	0.127	0.873
rs868755	47 (0.37)	58 (0.46)	21 (0.17)	0.603	0.397
rs1202172	10 (0.08)	53 (0.42)	63 (0.50)	0.290	0.710*
rs10280623	2 (0.02)	43 (0.34)	81 (0.64)	0.187	0.813
rs4148734	68 (0.54)	47 (0.37)	11 (0.09)	0.726	0.274
rs10808072	33 (0.26)	62 (0.49)	31 (0.25)	0.508	0.492
rs4148735	27 (0.21)	52 (0.41)	47 (0.37)	0.421	0.579
rs1858923	30 (0.24)	63 (0.50)	33 (0.26)	0.488	0.512
rs4148733	1 (0.01)	36 (0.29)	89 (0.71)	0.151	0.849
rs3842	96 (0.76)	30 (0.24)	0 (0)	0.881	0.119
rs4437575	38 (0.30)	65 (0.52)	23 (0.18)	0.560	0.440
rs10248420	82 (0.65)	42 (0.33)	2 (0.02)	0.817	0.183
rs13233308	31 (0.25)	65 (0.52)	30 (0.24)	0.504	0.496
rs1202184	40 (0.32)	55 (0.44)	31 (0.25)	0.536	0.464
rs17327624	78 (0.62)	41 (0.33)	7 (0.06)	0.782	0.218

†Allele 1- homozygous for allele 1, Allele 2 – homozygous for allele 2

‡Hardy-Weinberg notation for allele frequencies (p, frequency for allele 1; q, frequency for allele 2)

Table 5.5. Genotype and allelic frequencies for the studied ABCB1 SNPs

ABCC2 Polymorphism	No of patients with genotype (frequencies)			Allelic frequencies‡	
	Allele 1†	Heterozygous	Allele 2†	p	q
rs6584327	22 (0.18)	56 (0.44)	48 (0.38)	0.397	0.603
rs4148396	53 (0.42)	55 (0.44)	18 (0.14)	0.639	0.361
rs4148394	68 (0.54)	49 (0.39)	9 (0.07)	0.734	0.266
rs2273697	5 (0.04)	46 (0.37)	75 (0.60)	0.222	0.778
rs2756109	26 (0.21)	61 (0.48)	39 (0.31)	0.448	0.552
rs3740065	107 (0.85)	17 (0.14)	2 (0.02)	0.917	0.083
rs717620	5 (0.04)	36 (0.29)	85 (0.68)	0.183	0.817
rs4148398	16 (0.13)	53 (0.42)	57 (0.45)	0.337	0.663
rs3740063	46 (0.37)	56 (0.44)	24 (0.19)	0.587	0.413
rs3740066	16 (0.13)	55 (0.44)	55 (0.44)	0.345	0.655
rs8187710	1 (0.01)	10 (0.08)	115 (0.91)	0.048	0.952

†Allele 1- homozygous for allele 1, Allele 2 – homozygous for allele 2

‡Hardy-Weinberg notation for allele frequencies (p, frequency for allele 1; q, frequency for allele 2)

Table 5.6. Genotype and allelic frequencies for the studied ABCC2 SNPs

5.3.3 Linkage disequilibrium

In this population LD was observed between several genotyped SNPs within the ABCB1 gene and within the ABCC2 gene (Figure 5.4), based on Lewontin's D'. Lewontin's D' calculations take into account LD irrespective of allele frequencies. When applying the more rigorous parameter, r^2, the number of SNPs in LD ($r^2 \geq 0.8$) was lower. Out of the three ABCB1 SNPs that are commonly found in LD (1236C>T, 2677G>T/A and 3435C>T), only 1236C>T and 2677G>T/A were in LD in this population according to r^2 correlations (0.82). Full results of LD analysis for all ABCB1 and ABCC2 SNPs are shown in Figure 5.4A and Figure 5.4B respectively.

Following the analysis of genotype-phenotype correlations, the LD data were used if similar results were seen, to determine whether they were due to SNPs in LD.

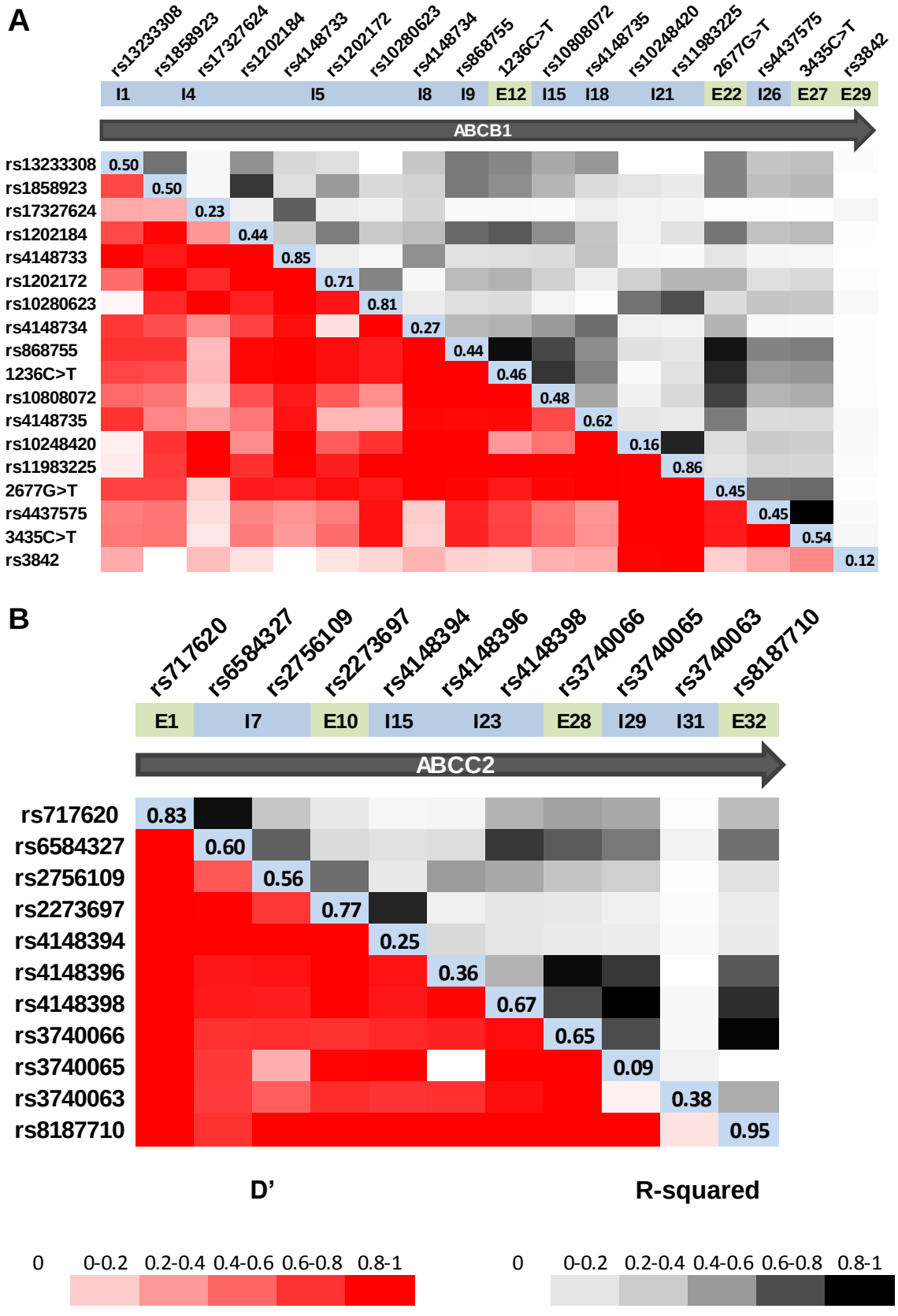

Figure 5.4. Linkage disequilibrium for ABCB1 and ABCC2 SNPs.

Visible values (blue) are minor allele frequencies observed in this population. Lewontin's D' (red) and r^2 value (black) were used to assess LD between SNPs in (A) ABCB1 and (B) ABCC2. SNPs are ordered according to location on the gene. Abbreviations: I=intron, E=exon

5.3.4 ABCB1 genotype-phenotype association

Relationships between transporter genotype and pharmacokinetic parameters were investigated. Since a strong correlation was observed between Cl-F and patient age, SA and weight, Cl-F was normalised to SA (Cl-FSA) and the impact of transporter genotype investigated. Estimates of Cl-FSA were available from 74 patients, 64 of whom had accompanying genetic data, allowing the impact of transporter genotype to be assessed.

There was no apparent influence of the ABCB1 SNP 1236C>T on Cl-FSA when considering each genotype separately (Figure 5.5A, Table 5.7). However, when combining all patients with at least one copy of the variant T allele, these patients had a 1.2-fold higher Cl-FSA than WT patients (153 vs. 125ml/min/m^2, p=0.05, Figure 5.5B). Similarly the 3435C>T polymorphism, had no effect on Cl-FSA (Table 5.7, Figure 5.6A) until variant allele carriers were considered as a group, where those patients had 1.3-fold higher Cl-FSA than patients with two WT alleles (150 vs. 116ml/min/m^2, p=0.05, Figure 5.6B). Patients carrying at least one copy of the variant T allele at position 2677 also exhibited a 1.3-fold higher Cl-FSA than other individuals (154 vs. 121ml/min/m^2, p=0.02, Figure 5.7B, Table 5.7). However, these data cannot be considered significant due to the number of statistic tests performed; p<0.002 identifies significance. Meaningful analysis to determine the effect of the 2677A SNP on Cl-FSA could not be performed, as only 3 patients with the A allele had accompanying pharmacokinetic data.

No other ABCB1 SNP had a statistically significant effect on Cl-FSA (Table 5.7) and ABCB1 genotype did not have an impact on the volume of distribution at steady-state (Vss) concentrations of Act D (Table 5.8).

ABCB1 Polymorphism	Mean Cl-FSA (ml/min/m^2)			Significance‡
	Allele 1†	Heterozygous	Allele 2†	
1236C>T	125	157	144	0.10
2677G>T/A	122	156 (GA – 117)	145	0.10
3435C>T	115	153	144	0.12
rs11983225	-	144	144	0.96♦
rs868755	125	155	153	0.1
rs1202172	144	137	151	0.75
rs10280623	-	145	144	0.93
rs4148734	149	145	113	0.34
rs10808072	145	152	127	0.33
rs4148735	111	158	146	0.04
rs1858923	152	144	136	0.66
rs4148733	-	130	149	0.21♦
rs3842	142	152	-	0.48♦
rs4437575	142	154	104	0.04
rs10248420	148	138	-	0.39♦
rs13233308	129	152	142	0.38
rs1202184	155	145	125	0.24
rs17327624	145	137	144	0.74

†Allele 1- homozygous for allele 1, Allele 2 – homozygous for allele 2

‡Statistical analysis performed was the one-way ANOVA. One test was performed per genotype, assessing global significance between genotype and Cl-FSA. **p<0.002 accepted as significant**. ♦Unpaired t-test as testing was performed on less than three groups.

Table 5.7. Effect of genotyped ABCB1 SNPs on Cl-FSA

ABCB1 Polymorphism	Median Vss (L)			Significance‡
	Allele 1†	Heterozygous	Allele 2†	
1236C>T	178	150	211	0.61
2677G>T/A	197	148	246	0.35
3435C>T	247	144	231	0.04
rs11983225	-	156	171	0.57
rs868755	193	144	246	0.21
rs1202172	201	165	151	0.83
rs10280623	-	158	163	0.58
rs4148734	173	155	217	0.55
rs10808072	173	155	155	0.75
rs4148735	140	156	174	0.77
rs1858923	161	154	178	0.90
rs4148733	-	139	172	0.16
rs3842	164	153	-	0.80
rs4437575	215	151	178	0.18
rs10248420	173	151	365	0.37
rs13233308	158	164	161	0.89
rs1202184	171	155	158	0.95
rs17327624	155	158	263	0.32

†Allele 1- homozygous for allele 1, Allele 2 – homozygous for allele 2

‡Statistical analysis performed using the Kruskal-Wallis one-way ANOVA. One test was performed per genotype, assessing global significance between genotype and Vss. **p<0.002 accepted as significant.**

Table 5.8. Effect of genotyped ABCB1 SNPs on Vss.

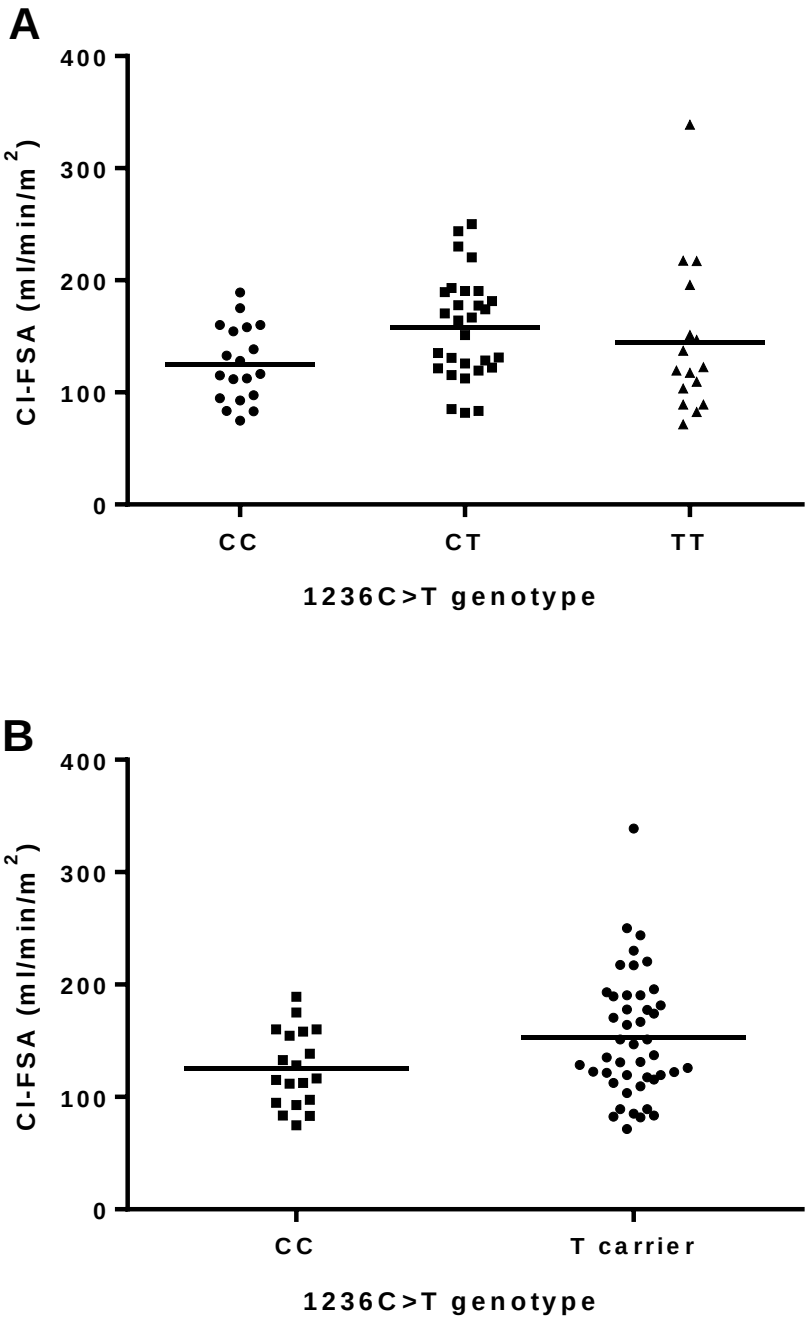

Figure 5.5. Effect of ABCB1 SNP 1236C>T on Cl-FSA.

Mean values represented with a horizontal bar. CL-FSA was assessed against (A) all genotypes and (B) those patients that carried the variant allele T compared to CC patients. Number of patients: CC=19, CT=29, TT=16, CC=19, T carrier=45.

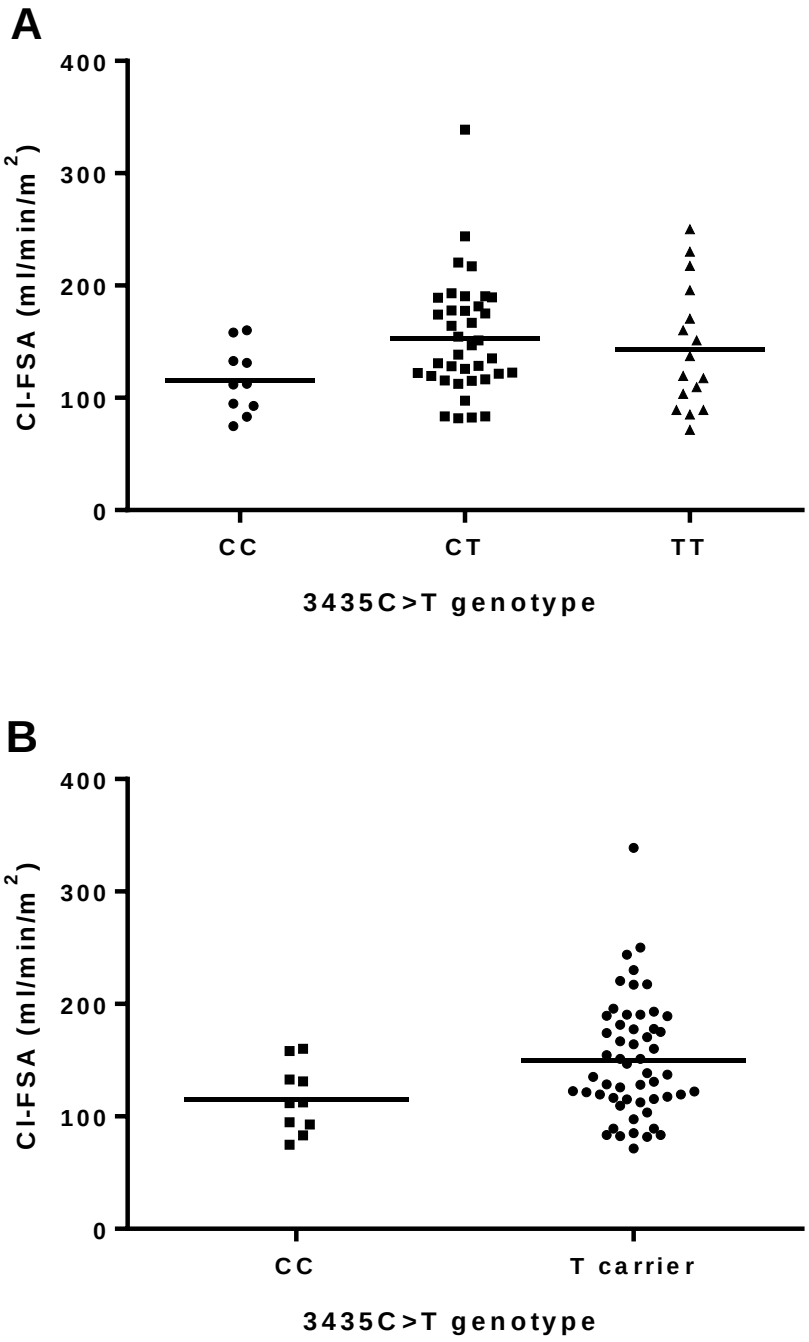

Figure 5.6. Effect of ABCB1 SNP 3435C>T on Cl-FSA.

Mean values represented with a horizontal bar. CL-FSA was assessed against (A) all genotypes and (B) those patients that carried the variant allele T compared to CC patients. Number of patients: CC=10, CT=38, TT=16, CC=10, T carrier=54.

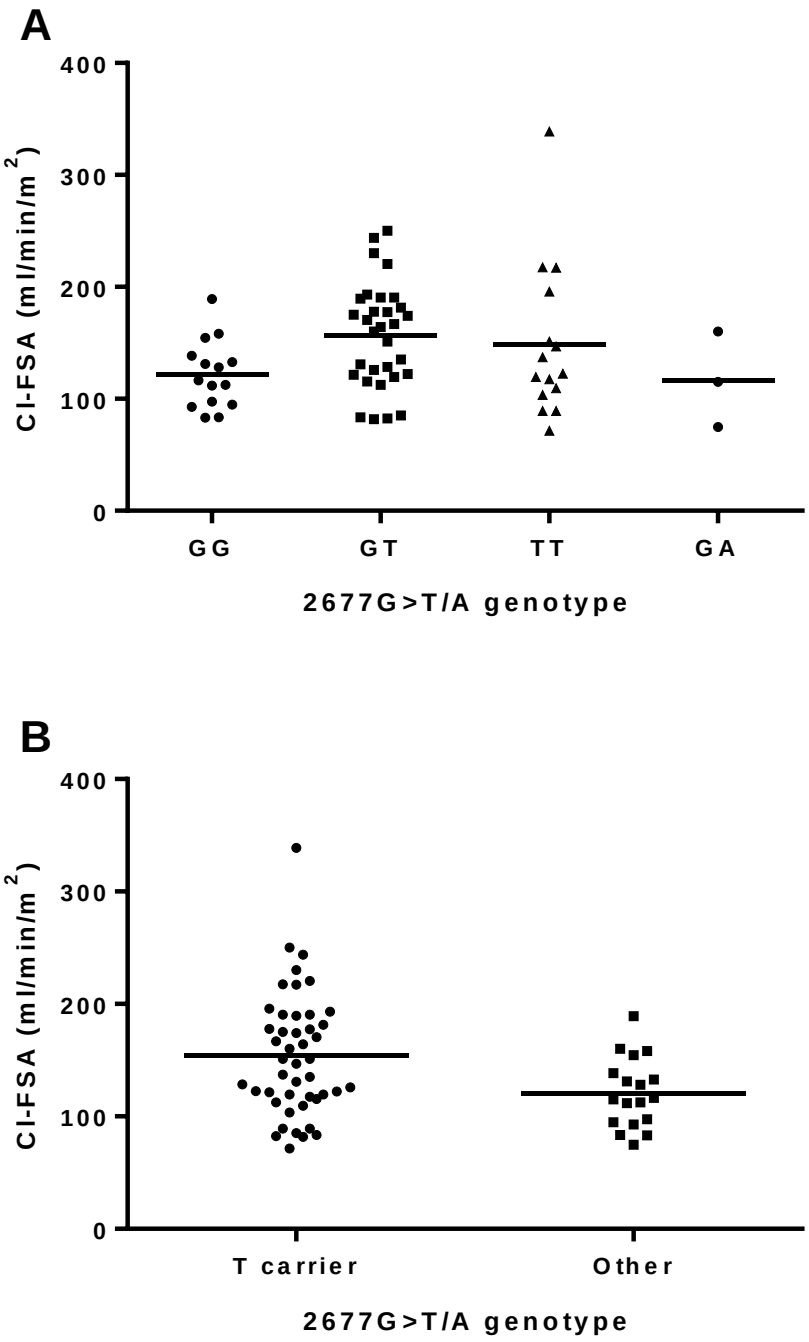

Figure 5.7. Effect of ABCB1 SNP 2677G>T/A on Cl-FSA.

Mean values represented with a horizontal bar. CL-FSA was assessed against (A) all genotypes and (B) those patients that carried the variant allele T compared to all other patients. Number of patients: GG=15, GT=31, TT=15, GA=3, T carrier=46, Other=18

5.3.5 ABCB1 diplotype analysis

The three widely studied ABCB1 SNPs, 1236C>T, 3435C>T and 2677G>T/A are in LD and diplotype analysis has previously shown an additive effect on drug transport when considering all three SNPs (Salama *et al.*, 2006; Sissung *et al.*, 2011). Diplotype analysis was performed according to Table 5.4.

Association between diplotypes 1-5 and Cl-FSA are shown in Figure 5.8A and Table 5.9. Patients with diplotype 4 had 1.6-fold higher Cl-FSA than those with diplotype 1, but this did not withstand correction for multiple testing (183 vs. 115ml/min/m^2, p=0.06). When considering only the polymorphisms at base pairs 3435 and 2677 (diplotypes 11-15), a trend of higher Cl-FSA with increasing numbers of variant alleles was observed (Figure 5.8C, Table 5.9). Patients with diplotype 14 had 1.7-fold higher Cl-FSA than WT patients (diplotype 11) (200 vs. 115ml/min/m^2, p<0.01, Table 5.9). Differences between Cl-FSA and diplotype were also observed between diplotype 14 and 12 (mean 200 vs. 127ml/min/m^2, p<0.05) and diplotype 14 and 15 (mean 200 vs. 127ml/min/m^2, p<0.05). No statistically significant associations were found between diplotypes 6-10 (1236C>T and 2677G>T/A) and Cl-FSA. Although trends between Cl-FSA and ABCB1 diplotype did appear to be seen, these data cannot considered significant due to the number of statistical tests performed.

Diplotype [Δ]	No. of patients (%)	Cl-FSA (ml/min/m^2)			Significance[†]
		Mean	Minimum	Maximum	
1	8 (13)	115	83.3	158	
2	10 (16)	132	74.7	189	
3	25 (40)	152	82.0	244	
4	9 (14)	183	82.7	339	
5	11 (17)	127	71.7	218	0.03
6	15 (23)	122	83.3	189	
7	4 (6)	143	74.7	175	
8	29 (45)	157	82.0	250	
9	1 (2)	82.7[‡]			
10	15 (23)	148	71.7	339	0.38
11	8 (13)	115	83.3	158	
12	9 (14)	127	74.7	189	
13	27 (42)	151	82.0	244	
14	9 (14)	191	85.1	339	
15	11 (17)	127	71.7	218	0.003

[†]Statistical analysis performed was a one-way ANOVA. One test was performed per genotype, assessing global significance between diplotype and Cl-FSA. **p<0.002 accepted as significant.**

[‡]Only one patient was diplotype 9 had associated pharmacokinetic samples. Diplotype 6-10 analysis should therefore be treated with caution

[Δ]Note – one patient did not fit into any diplotype between 1-5 involving all three SNPs.

Table 5.9. Effect of ABCB1 diplotypes on Cl-FSA.

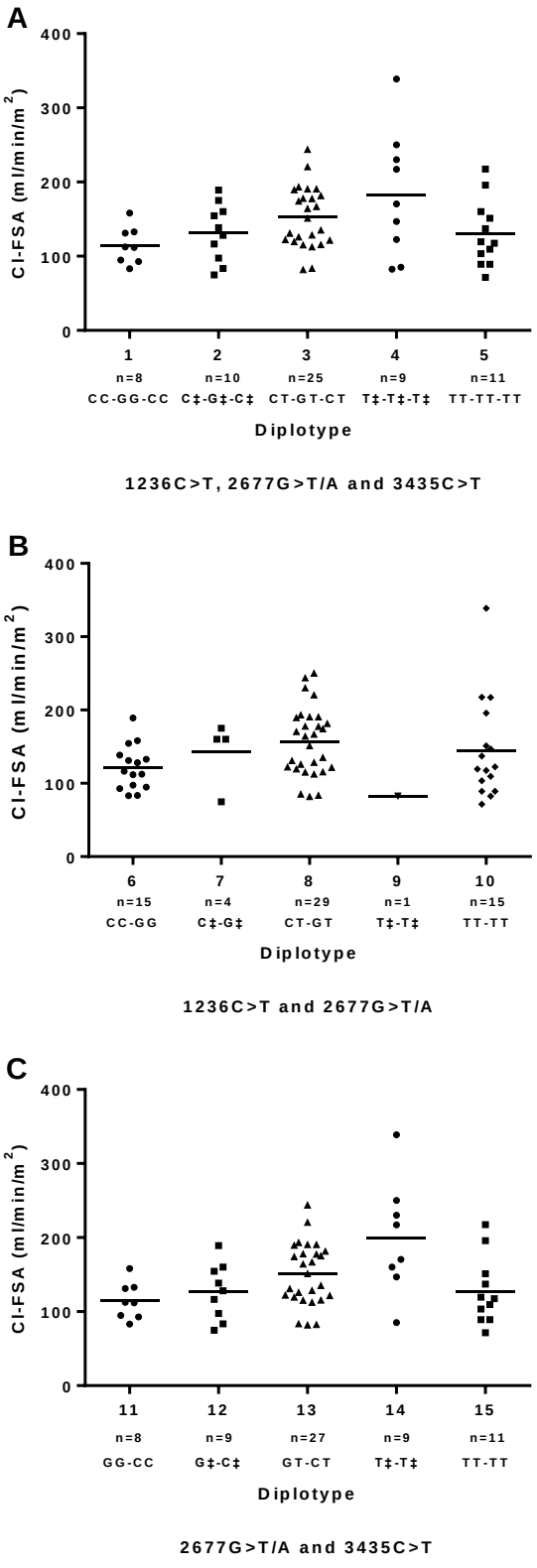

Figure 5.8. Effect of ABCB1 diplotypes 1-15 on Cl-FSA in 63 paediatric patients with diplotype 1-5, 64 patients with diplotypes 6-15 following Act D administration.

Mean values represented with a horizontal bar. The impact of diplotypes (A) 1-5, (B) 6-10 and (C) 11-15 on Cl-FSA was investigated. ‡ Any combination of alleles that is not mutually exclusive with another diplotype within the diplotype group.

5.3.6 ABCC2 genotype-phenotype association

The impact of ABCC2 genotype on Cl-FSA was also investigated. Associations between genotyped exonic ABCC2 SNPs and Cl-FSA are shown in Figure 5.9 and Figure 5.10. No statistically significant relationships were seen between exonic or intronic ABCC2 SNPs and Cl-FSA (Table 5.10) and ABCC2 genotype had no impact on Vss (Table 5.11).

ABCC2 Polymorphism	Mean Cl-FSA (ml/min/m^2)			Significance‡
	Allele 1†	Heterozygous	Allele 2†	
rs6584327	157	135	149	0.46
rs4148396	148	140	144	0.83
rs4148394	150	138	133	0.60
rs2273697	151	149	140	0.75
rs2756109	146	140	149	0.84
rs3740065	144	157	98	0.48
rs717620	-	139	146	0.64♦
rs4148398	139	137	150	0.62
rs3740063	149	131	166	0.16
rs3740066	167	130	151	0.17
rs8187710	-	170	142	0.24♦

†Allele 1- homozygous for allele 1, Allele 2 – homozygous for allele 2

‡Statistical analysis performed was a one-way ANOVA. One test was performed per genotype, assessing global significance between genotype and Cl-FSA.
♦Unpaired t-test as testing was performed on less than three groups. **p<0.002 accepted as significant.**

Table 5.10. Effect of genotyped ABCC2 SNPs on Cl-FSA

ABCC2 Polymorphism	Median Vss (L)			Significance‡
	Allele 1†	Heterozygous	Allele 2†	
rs6584327	139	177	171	0.46
rs4148396	164	213	126	0.09
rs4148394	174	140	177	0.55
rs2273697	212	158	153	0.59
rs2756109	158	175	152	0.81
rs3740065	171	158	76	0.36
rs717620	-	148	171	0.93
rs4148398	127	210	172	0.16
rs3740063	156	223	146	0.37
rs3740066	140	217	171	0.42
rs8187710	-	199	155	0.29

†Allele 1- homozygous for allele 1, Allele 2 – homozygous for allele 2

‡Statistical analysis performed using the Kruskal-Wallis one-way ANOVA. One test was performed per genotype, assessing global significance between genotype and Vss. **p<0.002 accepted as significant.**

Table 5.11. Effect of genotyped ABCC2 SNPs on Vss.

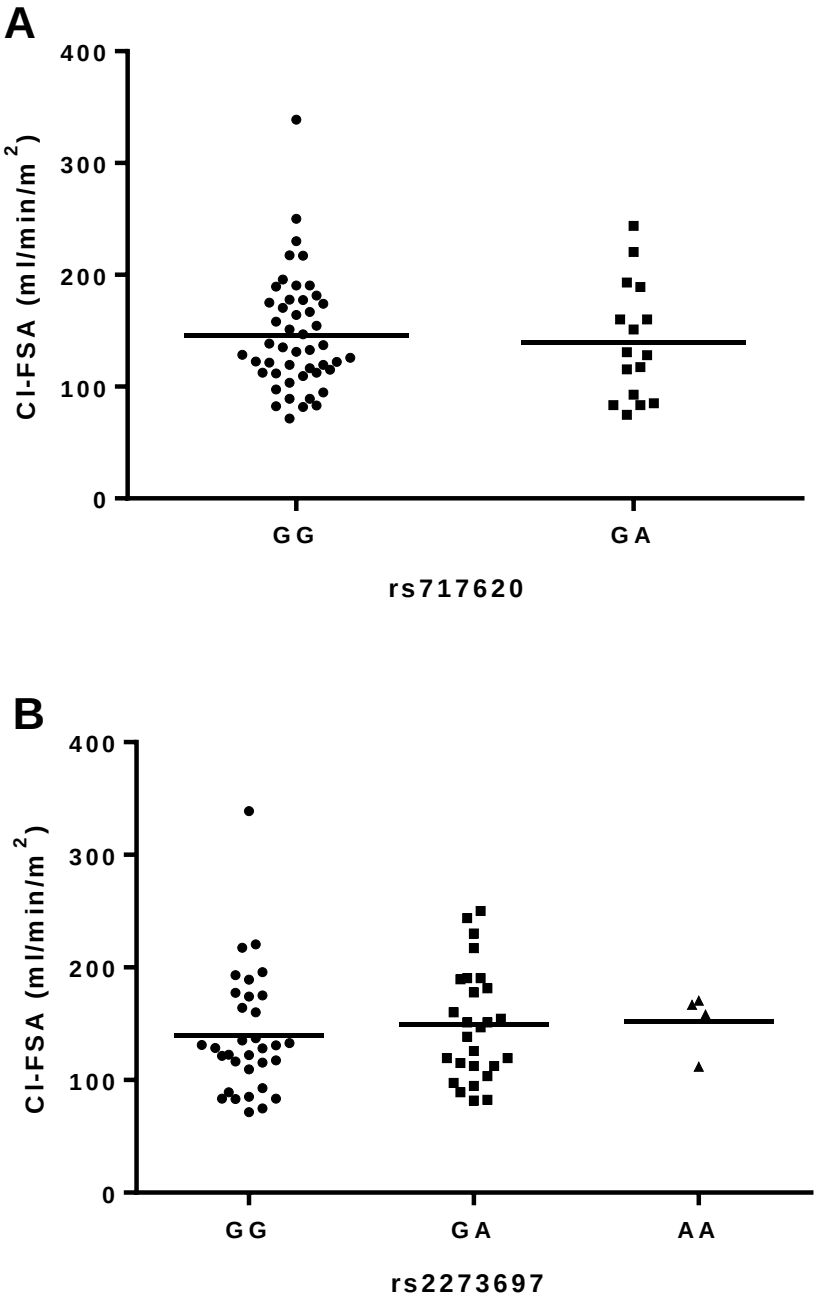

Figure 5.9. Effect of exonic ABCC2 SNPs rs717620 and rs2756109 on Cl-FSA.

Mean values represented with a horizontal bar. Cl-F was assessed against (A) the exon 1 5' UTR SNP rs717620 and (B) exon 10 non-synonymous SNP rs2273697.

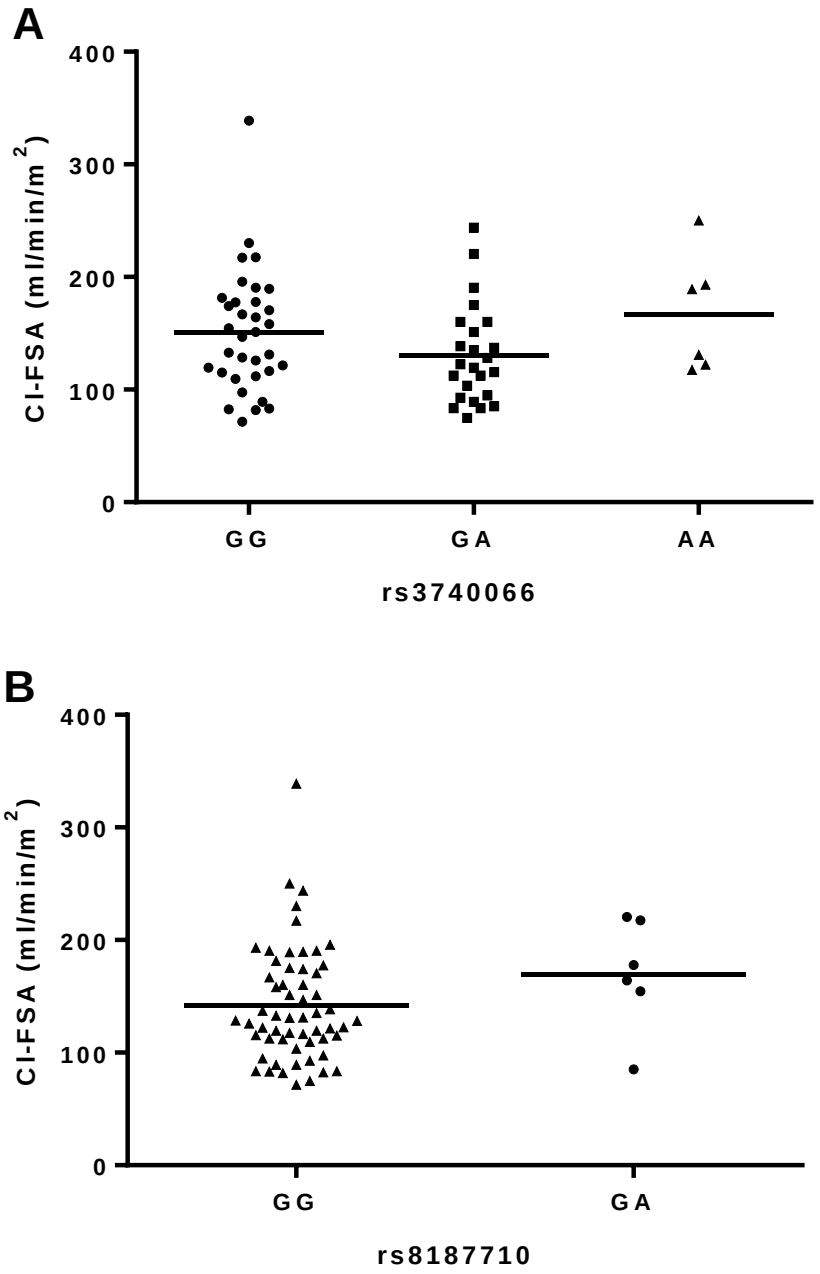

Figure 5.10. Effect of exonic ABCC2 SNPs rs3740066 and rs8187710 on Cl-FSA.

Mean values represented with a horizontal bar. Cl-F was assessed against (A) the exon 28 synonymous SNP rs3740066 and (B) exon 32 non-synonymous SNP rs8187710.

5.3.7 ABCB1 diplotype and ABCC2 SNP association with actinomycin D treatment-related toxicity

ABCB1 diplotype data were also assessed against toxicity as a result of Act D therapy. ABCB1 diplotype had no effect on treatment-associated CTC grade 3-4 toxicity (Figure 5.11). The incidence of toxicity did appear higher in heterozygous patients with diplotypes 3, 8 and 13, however this result did not reach significance, perhaps due to low patient numbers. No associations were observed between ABCC2 SNPs and incidence of toxicity however this could be due concomitant chemotherapy administered alongside Act D.

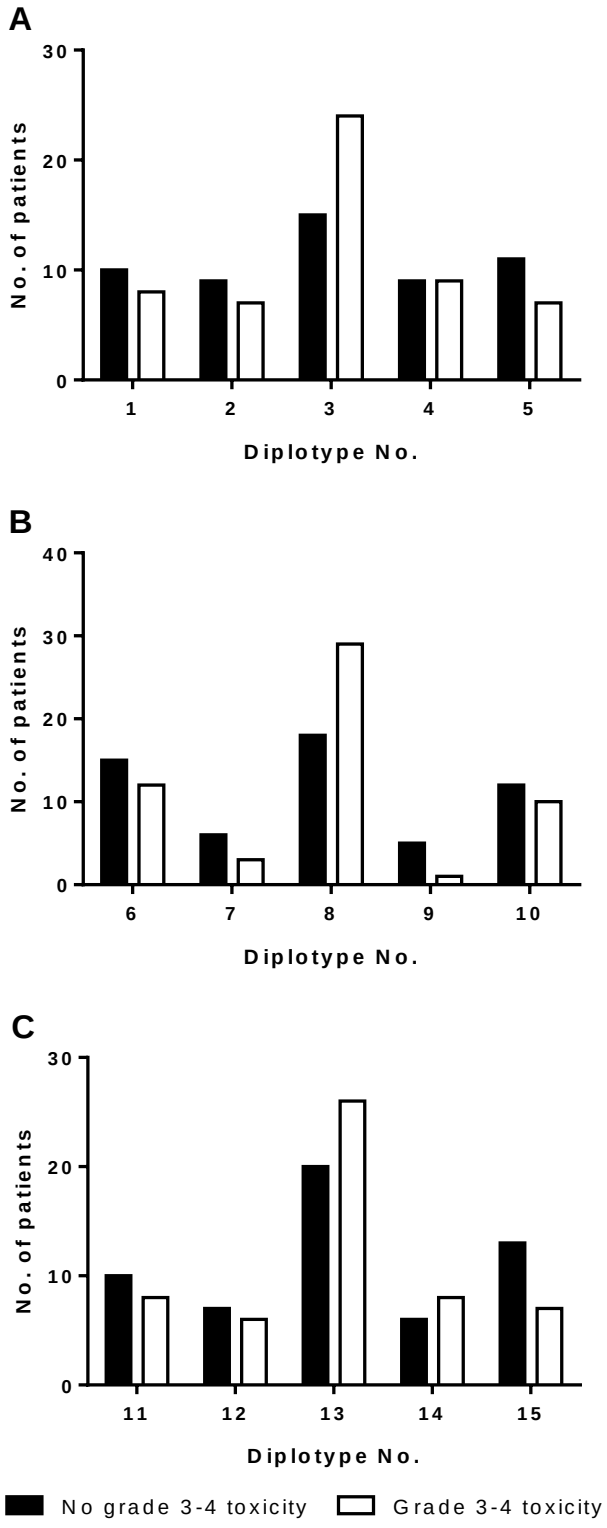

Figure 5.11. Effect of ABCB1 diplotype on Act D treatment related toxicity.

The number of patients with (A) diplotypes 1-5, (B) diplotypes 6-10 and (C) diplotypes 11-15 were separated as to whether they experienced Act D treatment related CTC grade 3/4 toxicity. (A) p=0.49, and (C) p=0.53 by Chi-squared test and (B) p=0.16 by Fisher's exact test, **p<0.002 accepted as significant.**

5.3.8 Post-hoc study power calculation

To assess the power of the current study a post-hoc power calculation was performed using the genotype data and Cl-F values from the common ABCB1 SNP 3435C>T. Setting the same size at 16 patients (i.e. the number of patients that were 3435TT) , this current study in this genotype had a power of 30%. With identical genotypic and pharmacokinetic variation, to achieve a power of 80%, this study would require an estimated total of 212 patients to potentially achieve significance. However, if more patients were recruited more pharmacokinetic variability is likely to occur.

5.4 Discussion

Inter-individual variation in drug exposure between patients is a major issue facing clinicians in modern cancer therapy. This may be particularly relevant for drugs used to treat tumours with better survival rates, where the majority of patients respond well, but a small percentage may experience unacceptable toxicity. As such, it is important to consider genetic variation in drug metabolising or transport proteins, as polymorphisms in these genes have the potential to alter pharmacokinetics and pharmacological phenotype.

In the current study of 108 patients, inter-patient variation in Act D exposure previously observed in 31 patients (Veal *et al.*, 2005) was confirmed, with a 23-fold range in Act D Cl-F observed in patients across all age groups (14.8-341ml/min, n=74). Due to the strong correlation between Act D Cl-F and patient age and body size, Cl-F was normalised to SA. A 10-fold range in Cl-FSA (33.7-339ml/min/m^2) was still observed indicating that variation in patient exposure could be attributed to factors other than patient age and body size.

Data presented in this chapter represents the first investigation of the potential impact of common and clinically-relevant SNPs of ABCB1 and ABCC2 on the pharmacokinetics of Act D in patients. Using the International HapMap project data, 29 candidate SNPs in ABCB1 and ABCC2 were chosen based on previous clinical relevance, and/or a minor allele frequencies of 0.1 (10%) or above. It was also important that the SNPs chosen covered as much of each gene as possible; LD data allowed tagSNPs to be chosen that would capture other SNPs in the same region. The SNPs chosen covered 96 polymorphic sites across both genes.

LD analysis of both ABCB1 and ABCC2 SNPs revealed interesting data. Within the ABCB1 and ABCC2 genes, despite variations in allelic frequencies, many SNPs appeared to be in close LD according to the D' value, however when r^2, which takes into consideration allele frequency as well as linkage, this was not the case. The ABCB1 SNPs 1236C>T, 2677G>T/A and 3435C>T have been extensively studied with many anti-cancer agents. The 2677G>T/A SNP is a non-synonymous SNP, where the variant allele T results in a serine to threonine conversion or the variant allele A results in a serine to alanine conversion. Both 1236C>T and 3435C>T are synonymous SNPs, with inconsistent data concerning their effect on drug exposure. Reduced digoxin exposure in patients is associated with TT genotype at position 3435 (Hoffmeyer *et al.*, 2000), whilst conflicting studies have reported higher exposure of temozolomide in CC patients (Schaich *et al.*, 2009) and irinotecan in patients with a TT genotype at position 1236 (Mathijssen *et al.*, 2003).

In clinical studies, the 3435C>T SNP has been associated with both up- and down-regulation (Hoffmeyer *et al.*, 2000; Dey, 2006), in addition to being predictive of longer overall survival in multiple myeloma patients (Buda *et al.*, 2010). In the study presented here, no influence was observed of individual genotypes on Cl-FSA when studied as separate genotypes. When combining all patients who carry one copy of the variant T allele for 1236C>T, 3435C>T and 2677G>T/A, Cl-FSA was 1.2-, 1.3- and 1.3-fold higher respectively than homozygous WT patients. However, this borderline significance cannot be accepted as significant due to the high number of statistical tests performed.

Clinical studies investigating the impact of 1236C>T, 3435C>T and 2677G>T/A on pharmacokinetics are inconsistent, and as such they are commonly studied

as haplotypes (Salama *et al.*, 2006; Kimchi-Sarfaty *et al.*, 2007). Single SNP analysis in Asian breast cancer patients revealed only a minor impact on pharmacokinetic parameters. However, in the same study, haplotype analysis suggested higher doxorubicin exposure in patients who were heterozygous for each SNP, and higher clearance in those patients who were homozygous WT for all three SNPs (Lal *et al.*, 2008).

In this patient population, no significant association between patient diplotype and Act D pharmacokinetics was observed. A trend did appear to be seen between Cl-FSA and diplotypes 11 to 15, which include only those polymorphisms at base pairs 2677 and 3435. However, as no difference was observed when analysing these SNPs individually, it was unlikely that diplotype analysis would reveal further information. Analysis of diplotypes 11-15 was close to acceptable significance ($p=0.003$ vs. $p<0.002$), however, this may have been driven by the apparent large difference between diplotype 11 and 14, which collectively only represented 17 patients.

Although not significant, these observations are in contrast to those for doxorubicin published by Lal et al. as the data presented here may suggest that patients with variant alleles have higher clearance compared to homozygous WT patients. Interestingly, patients with diplotype 15, who are homozygous variant at 2677 and 3435, had similar Cl-FSA to homozygous WT patients (127 vs. 115ml/min/m^2), and lower Cl-FSA than patients with diplotype 14 (127 vs. 200ml/min/m^2), however this may be due to low patient numbers. This phenomenon of homozygous WT and variant patients with similar clearance was also noted following doxorubicin therapy in a breast cancer setting (Lal *et al.*, 2008).

Diplotype analysis using 1236C>T, 3435C>T and 2677G>T/A also revealed a trend between diplotype and Cl-FSA, but significance was not reached. Diplotypes 6-10 examined the combination of alleles at base pairs 1236 and 2677. Statistically significant associations between diplotypes 6-10 and Cl-FSA were not observed. However, similar to other diplotype groupings, Cl-FSA appeared to be higher in heterozygous patients, but due to only one patient with diplotype 9, meaningful conclusions could not be drawn.

Although data regarding the transport of Act D by ABCB1 throughout this work have been consistent from cell line models, to knockout mice and finally the impact of genotype on pharmacokinetic parameters, this is not the case for ABCC2. Cell lines over-expressing ABCC2 were less sensitive to Act D and had lower intracellular accumulation and higher cellular efflux of Act D than their WT counterparts. However, data from Abcc2$^{-/-}$ mice unexpectedly indicated consistently lower Act D plasma concentrations compared to the WT mice, but this was not globally significant.

In addition to investigating correlations between ABCB1 genotype and Cl-FSA, clinically relevant and common SNPs in ABCC2 were also chosen, and these genotypes were assessed against Cl-FSA. Rs2273697 has been shown to cause lower mRNA and protein expression in the placenta (Meyer zu Schwabedissen *et al.*, 2005), and one study reported a neurological adverse drug reaction following carbamazepine therapy associated with the variant genotype (Kim *et al.*, 2010). Lower expression and subsequent lower protein levels of ABCC2 could therefore alter drug elimination and pharmacokinetics. In this study 37% of patients were heterozygous for this SNP, however the data presented here do not suggest any link between patient genotype at position

1249 and Cl-FSA. Rs717620, rs8187710 and rs3740066 are all SNPs located in the exonic regions of ABCC2. Rs717620 has been associated with increased platinum response in patients (Sun *et al.*, 2010), whereas rs8187710 and rs3740066 have been reported to be related to development of certain liver diseases (Sookoian *et al.*, 2008a; Sookoian *et al.*, 2008b; Sookoian *et al.*, 2009). However, patient genotype at these three positions did not have an impact on Act D Cl-FSA.

Examples of studies that have shown transport of anti-cancer agents to be relevant both *in vitro* and *in vivo*, that fail to impact of the pharmacokinetics in patients are available throughout the literature. A recent study involving docetaxel has shown *in vitro* in CHO cells transfected with OATP1B1 and OATP1B3 demonstrated transport of docetaxel by these transporters. Docetaxel clearance was then shown to be 18-fold lower in Oat1b2$^{-/-}$ mice, a rodent transporter with 60% sequence homology to human OATP1B1 and OATP1B3. However, similarly to this study, the large difference in both *in vitro* and *in vivo* disposition of docetaxel was not found to be clinically relevant patients when examining comparing common SNPs in human OATP1B1 and OATP1B3 against docetaxel clearance in 141 patients (de Graan *et al.*, 2012). In addition to this, post-hoc power calculations performed using data from the 3435C>T SNP, estimate that at least 212 patients would be required on this study for potential significance to occur. However, post-hoc power calculations should be considered with caution as their use is often controversial (Hoenig and Heisey, 2001).

Despite relatively high 5 year survival rates in Wilms tumour, ES and RMS (85%, 64% and 63% respectively), a major drawback of the clinical use of Act D

is the treatment-associated toxicities observed. Previous studies have shown toxicity risk to be correlated to patient age, weight and body size (Arndt *et al.*, 2004; Veal *et al.*, 2005; Langholz *et al.*, 2011). Act D treatment-related toxicity has been assessed against relevant ABCB1 diplotype. Those patients with or without CTC grade 3 or 4 toxicities were assessed against ABCB1 diplotypes. Toxicity incidence did not appear to be influenced by diplotype, except in those patients who had diplotype 3, 8 and 13. Across diplotypes 3, 8 and 13, where patients are heterozygous for all SNPs, Act D treatment-related toxicity occurred in 62, 69 and 57% of patients respectively, however this was not significant and only limited patient data were available to correlate toxicity with other diplotypes.

In this genotype-phenotype study, the relevance of genetic variation in ABCB1 is inconclusive. Associations between patient genotype and pharmacokinetic parameters have demonstrated that the inter-patient variability in Act D exposure in paediatric patients could be influenced by ABCB1, but not ABCC2 genotype. Pharmacogenetic and corresponding pharmacokinetic samples were available from only 64 patients in this current study. Continued recruitment to increase the number of patients who provide both pharmacokinetic and pharmacogenetic samples would be vital for a more conclusive outcome to be potentially achieved. In addition to this, the effect of ABCB1 and ABCC2 pharmacogenetics should be validated in a larger more ethnically diverse cohort of patients.

Chapter 6. Conclusion

Act D is one of seven naturally occurring actinomycins isolated from *Streptomyces* in 1940 by Waksman and Woodruff (Waksman and Woodruff, 1940). Identified during the search for novel anti-microbial agents (Farber, 1966), it was a further 12 years until their potential as chemotherapeutic agents was recognised (Hackmann, 1952; Hackmann, 1953; Ravina *et al.*, 1954). When Act D was discovered, the average cure rate of Wilms tumour at 20 years post-diagnosis was 40%, and patients who presented with metastases faired far worse than those that did not (Farber, 1966). Sydney Farber and colleagues hypothesised that addition of a systemic anti-cancer agent to the treatment regimen to those patients with metastases would be of great benefit. This led to a wide range of chemotherapeutic agents being tested for efficacy against Wilms tumour, resulting in Act D being added to the long-term treatment program in 1960. Addition of Act D to treatment protocol significantly increased the number of patients that were tumour free 2 years after diagnosis; 89% vs. 40% previously in patients with no metastases, and 53% vs. 0% in patients who presented with metastases.

Despite being used as a chemotherapeutic agent for over 50 years, limited information is known about the pharmacokinetics of Act D in animals or humans. Early pharmacokinetic studies were mainly performed in animals, with only two studies to date involving humans.

Concerns over treatment-associated toxicity and its possible link with inter-patient variability in Act D exposure in patients led to the development of a highly sensitive LC/MS method suitable for detection of Act D in human plasma

(Veal *et al.*, 2003b). Subsequently, Act D pharmacokinetic data in 31 patients indicated large variations in Act D exposure in patients ($AUC_{0\text{-}6h}$ 1.12-4.90mg/L.min) (Veal *et al.*, 2005). This study also demonstrated that those with high Act D exposure tended to be smaller children, indicating a potential increased toxicity risk, a finding confirmed in a recent large-scale retrospective study in the United States (Langholz *et al.*, 2011). Perhaps due in part to the practice of Act D dose capping at 2mg, older children were shown to have lower Act D exposure. Due to the lack of meaningful Act D pharmacokinetic data in the literature, it was unclear at the start of the first study which pharmacokinetic time points would be required for accurate pharmacokinetic parameter estimates. A major limitation of this initial study was the sparsity of pharmacokinetic samples collected beyond 6h, with extensive extrapolation to 24h therefore required for many patients in order to estimate pharmacokinetic parameters.

To address the limitations of the initial study, two large clinical trials were set up, increasing patient numbers and including pharmacokinetic samples at earlier and later time points, to allow more accurate pharmacokinetic parameter estimation; the results of these studies are presented in Chapter 4. Consistent with the findings from Veal et al. 2005, a large inter-patient variability was observed with Act D clearance in patients on the same dosage regimen (14.8 to 341ml/min). Again, in agreement with the previous study, pharmacokinetic parameters correlated well with age and patient size; clearance in patients under 3 years old was estimated to be 54% of that seen in patients older than 3 years. No link was seen between Act D treatment-related toxicities and any pharmacokinetic parameters, however toxicity was mainly haematological, with only two cases of hepatotoxicity reaching grade 3 or 4. It was also not possible

to remove confounding factors such as concomitant administration of other anti-cancer drugs during analysis of treatment-associated toxicities.

Variability in clearance could not be attributed to factors such as dose, line-type or tumour-type and variability between patients was still apparent when adjusting for patient age, weight or SA. Therefore it is possible that variation in genes affecting the disposition of Act D, such as ABC transporters, could underlie the inter-individual differences in the pharmacokinetics of Act D.

MDCKII cell lines are an excellent and well exploited resource in determining whether drugs are substrates for ABC transporters. Act D has long been thought of as a substrate for ABCB1. Indeed, the early investigations into MDR were carried out in Chinese hamster ovary cells resistant to Act D (Juliano and Ling, 1976). Later the resistance was found to be caused by upregulation of a cell membrane P-glycoprotein, now known as MDR1 or ABCB1 (Kartner *et al.*, 1983a; Kartner *et al.*, 1983b; Ling *et al.*, 1983; Ueda *et al.*, 1986). Perhaps due to its limited clinical use, determining the clinical relevance of transport of Act D by ABCB1 or other ABC transport proteins has seen little attention since the early the 1980s.

MDCKII cell lines which individually over-express the human forms of ABCB1, ABCC1, ABCC2 and ABCG2 were used to investigate the transport of Act D. Through growth inhibition assays, cell lines over-expressing either ABCB1, ABCC1 or ABCC2 were significantly less sensitive to Act D compared to MDCKII-WT. This observation led to the development of intracellular accumulation and cellular efflux assays which, using LC/MS, allowed the quantification of intracellular Act D following defined treatments. Intracellular Act

D was lower in ABCB1, ABCC1 and ABCC2 over-expressing cells compared to MDCKII-WT and significant Act D efflux was shown to be greater in MDCKII-ABCB1 than any other cell line. These three techniques demonstrated that Act D was a good substrate for ABCB1 and ABCC2.

Based on these findings, Abcb1a/1b$^{-/-}$ and Abcc2$^{-/-}$ mice were used to determine the relevance of ABCB1 and ABCC2-mediated Act D transport in an animal model. Previous data using knockout mice suggested that transport by ABC transporters *in vitro* is not always indicative of *in vivo* transport and potential clinical relevance, with results varying substantially between chemotherapeutic agents. For example, etoposide exposure is significantly higher in Abcb1a/1b$^{-/-}$;Abcc2$^{-/-}$ mice due to reduced renal and hepatic excretion (Lagas *et al.*, 2010). However, no alteration in plasma pharmacokinetics was observed when administering vemurafenib to Abcb1a/1b$^{-/-}$;Abcg2$^{-/-}$ mice despite indications in MDCKII cell lines that vemurafenib was transported by ABCB1 and mouse Abcg2 (Mittapalli *et al.*, 2012).

In this study altered pharmacokinetics were observed in both strains of mice compared to the WT. Lack of Abcb1a/1b caused a consistent significant increase in plasma concentrations over 6h, resulting in higher Act D exposure compared to WT mice. Interestingly, absence of Abcc2 caused a decrease in plasma concentrations over 6h but this was not significant. Although altered plasma pharmacokinetics were observed in both knockout mouse models, removal of these transporters had no impact on the Act D concentrations in the liver or kidneys. However, a significantly higher Act D brain concentration in Abcb1a/1b$^{-/-}$ indicated that the presence of ABCB1 at the blood-brain barrier does limit penetration of Act D into the brain. Through a systematic approach

Act D was confirmed as an ABCB1 and ABCC2 substrate *in vitro* and knockout of Abcb1a/1b significantly influences its pharmacokinetics.

Clinical studies investigating the role of ABC transport SNPs to date are largely inconsistent, and substrate specific, and have not previously been performed with Act D. The common ABCB1 SNP 3435C>T has been found to cause both lower expression in the Caucasian population (Hoffmeyer *et al.*, 2000), whilst it has been associated with higher expression in certain tissues of the Japanese population (Dey, 2006). Results are also variable when considering another ABCB1 SNP 1236C>T, with higher exposure to temozolomide in CC patients (Schaich *et al.*, 2009) and higher exposure to irinotecan in TT patients (Mathijssen *et al.*, 2003).

Finally, in Chapter 6, the hypothesis was investigated that the variability seen in Act D pharmacokinetics in children is influenced by ABCB1 and/or ABCC2 SNPs. When considering single SNPs, the variant allele T in ABCB1 SNPs 1236C>T, 2677G>T/A and 3435C>T had higher Act D clearance normalised to patient SA but this was not significant. Similarly, diplotype analysis of these three common ABCB1 SNPs revealed a trend of increasing clearance with the variant alleles at position 2677 and 3435, however this cannot be considered significant and required the removal of 1236C>T from diplotype analysis.

Paired pharmacokinetic and pharmacogenetic data were only available from 64 patients in this current study. Another limitation of this study is the lack of data concerning patient ethnicity. A larger more ethnically diverse patient population would therefore be required to appropriately determine the effect of common ABCB1 and ABCC2 polymorphisms on the exposure of Act D.

Early studies have demonstrated that Act D is minimally metabolised (Tattersall *et al.*, 1975), and further work in human liver microsomes in this laboratory, has as yet, failed to identify metabolites. ABCB1 and other efflux protein expression is regulated by the pregnane X receptor (PXR) (Synold *et al.*, 2001) which can be activated by large structurally diverse anti-cancer agents such as vincristine and paclitaxel (Harmsen *et al.*, 2010). It would therefore be interesting to investigate the potential induction of ABCB1 via PXR with Act D. Future investigations into the metabolism and excretion of Act D, in addition to ABCB1 induction, would be helpful to fully determine the factors influencing Act D pharmacokinetics, to ensure all patients receive a non-toxic, therapeutic dose.

References

Abolhoda, A., Wilson, A.E., Ross, H., Danenberg, P.V., Burt, M. and Scotto, K.W. (1999) 'Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin', *Clin Cancer Res*, 5(11), pp. 3352-6.

Abraham, J., Earl, H.M., Pharoah, P.D. and Caldas, C. (2006) 'Pharmacogenetics of cancer chemotherapy', *Biochim Biophys Acta*, 1766(2), pp. 168-83.

Adkison, K.K., Vaidya, S.S., Lee, D.Y., Koo, S.H., Li, L., Mehta, A.A., Gross, A.S., Polli, J.W., Lou, Y. and Lee, E.J. (2008) 'The ABCG2 C421A polymorphism does not affect oral nitrofurantoin pharmacokinetics in healthy Chinese male subjects', *Br J Clin Pharmacol*, 66(2), pp. 233-9.

Allen, J.D., Van Dort, S.C., Buitelaar, M., van Tellingen, O. and Schinkel, A.H. (2003) 'Mouse breast cancer resistance protein (Bcrp1/Abcg2) mediates etoposide resistance and transport, but etoposide oral availability is limited primarily by P-glycoprotein', *Cancer Res*, 63(6), pp. 1339-44.

Allen, J.D., van Loevezijn, A., Lakhai, J.M., van der Valk, M., van Tellingen, O., Reid, G., Schellens, J.H., Koomen, G.J. and Schinkel, A.H. (2002) 'Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C', *Mol Cancer Ther*, 1(6), pp. 417-25.

Alving, A.S., Carson, P.E., Flanagan, C.L. and Ickes, C.E. (1956) 'Enzymatic deficiency in primaquine-sensitive erythrocytes', *Science*, 124(3220), pp. 484-5.

Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I. and Gottesman, M.M. (1999) 'Biochemical, cellular, and pharmacological aspects of the multidrug transporter', *Annu Rev Pharmacol Toxicol*, 39, pp. 361-98.

Ambudkar, S.V., Kim, I.W. and Sauna, Z.E. (2006) 'The power of the pump: mechanisms of action of P-glycoprotein (ABCB1)', *Eur J Pharm Sci*, 27(5), pp. 392-400.

Anderson, P.L., Lamba, J., Aquilante, C.L., Schuetz, E. and Fletcher, C.V. (2006) 'Pharmacogenetic characteristics of indinavir, zidovudine, and lamivudine therapy in HIV-infected adults: a pilot study', *J Acquir Immune Defic Syndr*, 42(4), pp. 441-9.

Applebaum, M.A., Worch, J., Matthay, K.K., Goldsby, R., Neuhaus, J., West, D.C. and Dubois, S.G. (2011) 'Clinical features and outcomes in patients with extraskeletal Ewing sarcoma', *Cancer*, 117(13), pp. 3027-32.

Arndt, C., Hawkins, D., Anderson, J.R., Breitfeld, P., Womer, R. and Meyer, W. (2004) 'Age is a risk factor for chemotherapy-induced hepatopathy with vincristine, dactinomycin, and cyclophosphamide', *J Clin Oncol*, 22(10), pp. 1894-901.

Bailey, S., Roberts, A., Brock, C., Price, L., Craft, A.W., Kilkarni, R., Lee, R.E., Skillen, A.W. and Skinner, R. (2002) 'Nephrotoxicity in survivors of Wilms' tumours in the North of England', *Br J Cancer*, 87(10), pp. 1092-8.

Ben Ari, Z., Mehta, A., Lennard, L. and Burroughs, A.K. (1995) 'Azathioprine-induced myelosuppression due to thiopurine methyltransferase deficiency in a patient with autoimmune hepatitis', *J Hepatol*, 23(3), pp. 351-4.

Bertino, J.R. (1991) 'Improving the curability of acute leukemia: pharmacologic approaches', *Semin Hematol*, 28(3 Suppl 4), pp. 9-11.

Bhatnagar, S. (2009) 'Management of Wilms' tumor: NWTS vs SIOP', *J Indian Assoc Pediatr Surg*, 14(1), pp. 6-14.

Biedler, J.L. and Riehm, H. (1970) 'Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies', *Cancer Res*, 30(4), pp. 1174-84.

Bisogno, G., de Kraker, J., Weirich, A., Masiero, L., Ludwig, R., Tournade, M.F. and Carli, M. (1997) 'Veno-occlusive disease of the liver in children treated for Wilms tumor', *Med Pediatr Oncol*, 29(4), pp. 245-51.

Bochud, M., Eap, C.B., Maillard, M., Johnson, T., Vollenweider, P., Bovet, P., Elston, R.C., Bergmann, S., Beckmann, J.S., Waterworth, D.M., Mooser, V., Gabriel, A. and Burnier, M. (2008) 'Association of ABCB1 genetic variants with renal function in Africans and in Caucasians', *BMC Med Genomics*, 1, p. 21.

Borst, P. and Elferink, R.O. (2002) 'Mammalian ABC transporters in health and disease', *Annu Rev Biochem*, 71, pp. 537-92.

Bray, J., Sludden, J., Griffin, M.J., Cole, M., Verrill, M., Jamieson, D. and Boddy, A.V. (2010) 'Influence of pharmacogenetics on response and toxicity in breast cancer patients treated with doxorubicin and cyclophosphamide', *Br J Cancer*, 102(6), pp. 1003-9.

Brockmann, H. (1954) 'Chemie und Biologie des Actinomycins.', *Angew. Chem.*, (66), pp. 1-10.

Brockmann, H. and Muxfeldt, H. (1955) 'Die Konstitution des Despeptido-Actinomycins.', *Angew. Chem.*, (67), pp. 617-618.

Brothman, A., Davis, T., Duffy, J. and Lindell, T. (1982) 'Development of an Antibody to Actinomycin D and its application for the detection of Serum levels by Radioimmunoassay', *Cancer Research*, 42, pp. 1184-1187.

Buda, G., Ricci, D., Huang, C.C., Favis, R., Cohen, N., Zhuang, S.H., Harousseau, J.L., Sonneveld, P., Blade, J. and Orlowski, R.Z. (2010) 'Polymorphisms in the multiple drug resistance protein 1 and in P-glycoprotein 1 are associated with time to event outcomes in patients with advanced multiple myeloma treated with bortezomib and pegylated liposomal doxorubicin', *Ann Hematol*, 89(11), pp. 1133-40.

Büyükpamukçu, M., Kutluk, T., Büyükpamukçu, N., Sarıalioğlu, F. and Akyüz, C. (1992) 'Renal tumors with pseudohermaphroditism and glomerular disease', *Acta Oncol*, 31, pp. 745-748.

Calvert, A.H., Harland, S.J., Newell, D.R., Siddik, Z.H. and Harrap, K.R. (1985) 'Phase I studies with carboplatin at the Royal Marsden Hospital', *Cancer Treat Rev*, 12 Suppl A, pp. 51-7.

Calvert, A.H., Newell, D.R., Gumbrell, L.A., O'Reilly, S., Burnell, M., Boxall, F.E., Siddik, Z.H., Judson, I.R., Gore, M.E. and Wiltshaw, E. (1989) 'Carboplatin dosage: prospective evaluation of a simple formula based on renal function', *J Clin Oncol*, 7(11), pp. 1748-56.

CancerStats (2010) *Childhood Cancer - Great Britain & UK*. Cancer Research UK website: UK, C.R.

Cecchin, E., D'Andrea, M., Lonardi, S., Zanusso, C., Pella, N., Errante, D., De Mattia, E., Polesel, J., Innocenti, F. and Toffoli, G. (2012) 'A prospective validation pharmacogenomic study in the adjuvant setting of colorectal cancer patients treated with the 5-fluorouracil/leucovorin/oxaliplatin (FOLFOX4) regimen', *Pharmacogenomics J*.

Chabner, B. and Roberts Jr, T. (2005) 'Chemotherapy and the war on cancer', *Nature Reviews: Cancer*, 5, pp. 65-72.

Chen, C.J., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M. and Roninson, I.B. (1986) 'Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells', *Cell*, 47(3), pp. 381-9.

Chu, X.Y., Kato, Y., Niinuma, K., Sudo, K.I., Hakusui, H. and Sugiyama, Y. (1997a) 'Multispecific organic anion transporter is responsible for the biliary excretion of the camptothecin derivative irinotecan and its metabolites in rats', *J Pharmacol Exp Ther*, 281(1), pp. 304-14.

Chu, X.Y., Kato, Y. and Sugiyama, Y. (1997b) 'Multiplicity of biliary excretion mechanisms for irinotecan, CPT-11, and its metabolites in rats', *Cancer Res*, 57(10), pp. 1934-8.

Chu, X.Y., Strauss, J.R., Mariano, M.A., Li, J., Newton, D.J., Cai, X., Wang, R.W., Yabut, J., Hartley, D.P., Evans, D.C. and Evers, R. (2006) 'Characterization of mice lacking the multidrug resistance protein MRP2 (ABCC2)', *J Pharmacol Exp Ther*, 317(2), pp. 579-89.

ClinicalTrials.gov (2009) *Study in Localized and Disseminated Ewing Sarcoma (EWING 2008)*. Available at: http://clinicaltrials.gov/ct2/show/NCT00987636 (Accessed: 16/07/12).

Cole, S.P., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M. and Deeley, R.G. (1992) 'Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line', *Science*, 258(5088), pp. 1650-4.

Cotterill, S.J., Ahrens, S., Paulussen, M., Jurgens, H.F., Voute, P.A., Gadner, H. and Craft, A.W. (2000) 'Prognostic factors in Ewing's tumor of bone: analysis of 975 patients from the European Intergroup Cooperative Ewing's Sarcoma Study Group', *J Clin Oncol*, 18(17), pp. 3108-14.

Crist, W., Gehan, E.A., Ragab, A.H., Dickman, P.S., Donaldson, S.S., Fryer, C., Hammond, D., Hays, D.M., Herrmann, J., Heyn, R. and et al. (1995) 'The Third Intergroup Rhabdomyosarcoma Study', *J Clin Oncol*, 13(3), pp. 610-30.

Crist, W.M., Anderson, J.R., Meza, J.L., Fryer, C., Raney, R.B., Ruymann, F.B., Breneman, J., Qualman, S.J., Wiener, E., Wharam, M., Lobe, T., Webber, B., Maurer, H.M. and Donaldson, S.S. (2001) 'Intergroup rhabdomyosarcoma study-IV: results for patients with nonmetastatic disease', *J Clin Oncol*, 19(12), pp. 3091-102.

Cui, Y., Konig, J., Buchholz, J.K., Spring, H., Leier, I. and Keppler, D. (1999) 'Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells', *Mol Pharmacol*, 55(5), pp. 929-37.

Cusatis, G., Gregorc, V., Li, J., Spreafico, A., Ingersoll, R.G., Verweij, J., Ludovini, V., Villa, E., Hidalgo, M., Sparreboom, A. and Baker, S.D. (2006) 'Pharmacogenetics of ABCG2 and adverse reactions to gefitinib', *J Natl Cancer Inst*, 98(23), pp. 1739-42.

D'Angio, G.J., Farber, S. and Maddock, C.L. (1959) 'Potentiation of x-ray effects by actinomycin D', *Radiology*, 73, pp. 175-7.

D'Antiga, L., Baker, A., Pritchard, J., Pryor, D. and Mieli-Vergani, G. (2001) 'Veno-occlusive disease with multi-organ involvement following actinomycin-D', *Eur J Cancer*, 37(9), pp. 1141-8.

Dagher, R. and Helman, L. (1999) 'Rhabdomyosarcoma: An overview', *The Oncologist*, 4, pp. 34-44.

Daly, A.K., Aithal, G.P., Leathart, J.B., Swainsbury, R.A., Dang, T.S. and Day, C.P. (2007) 'Genetic susceptibility to diclofenac-induced hepatotoxicity: contribution of UGT2B7, CYP2C8, and ABCC2 genotypes', *Gastroenterology*, 132(1), pp. 272-81.

Dano, K. (1973) 'Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells', *Biochim Biophys Acta*, 323(3), pp. 466-83.

Dart, R.C. (2004) *Medical Toxicology*. 3rd edn. Philadelphia: Lippincott, Williams and Wilkins.

Davidson, A. and Pritchard, J. (1998) 'Actinomycin D, Hepatic Toxicity and Wilms' Tumour - a Mystery Explained?', *European Journal of Cancer*, 34(8), pp. 1145-1147.

Davis, R., D'Cruz, C. and Lovell, M. (1994) 'Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma.', *Cancer Research*, 54, pp. 2869-2872.

de Graan, A.J., Lancaster, C.S., Obaidat, A., Hagenbuch, B., Elens, L., Friberg, L.E., de Bruijn, P., Hu, S., Gibson, A.A., Bruun, G.H., Corydon, T.J., Mikkelsen, T.S., Walker, A.L., Du, G., Loos, W.J., van Schaik, R.H., Baker, S.D., Mathijssen, R.H. and Sparreboom, A. (2012) 'Influence of polymorphic OATP1B-type carriers on the disposition of docetaxel', *Clin Cancer Res*, 18(16), pp. 4433-40.

Dean, M. (2002) 'The Human ATP-Binding Cassette (ABC) Transporter Superfamily', in *The Human ATP-Binding Cassette (ABC) Transporter Superfamily*

Dean, M., Rzhetsky, A. and Allikmets, R. (2001) 'The human ATP-binding cassette (ABC) transporter superfamily.', *Genome Res.*, 11, pp. 1156-1166.

Delattre, O., Zucman, J., Melot, T., Garau, X.S., Zucker, J.M., Lenoir, G.M., Ambros, P.F., Sheer, D., Turc-Carel, C., Triche, T.J. and et al. (1994) 'The Ewing family of tumors--a subgroup of small-round-cell tumors defined by specific chimeric transcripts', *N Engl J Med*, 331(5), pp. 294-9.

Dey, S. (2006) 'Single nucleotide polymorphisms in human P-glycoprotein: its impact on drug delivery and disposition', *Expert Opin Drug Deliv*, 3(1), pp. 23-35.

Dome, J.S., Cotton, C.A., Perlman, E.J., Breslow, N.E., Kalapurakal, J.A., Ritchey, M.L., Grundy, P.E., Malogolowkin, M., Beckwith, J.B., Shamberger, R.C., Haase, G.M., Coppes, M.J., Coccia, P., Kletzel, M., Weetman, R.M., Donaldson, M., Macklis, R.M. and Green, D.M. (2006) 'Treatment of anaplastic

histology Wilms' tumor: results from the fifth National Wilms' Tumor Study', *J Clin Oncol*, 24(15), pp. 2352-8.

Doyle, L.A., Yang, W., Abruzzo, L.V., Krogmann, T., Gao, Y., Rishi, A.K. and Ross, D.D. (1998) 'A multidrug resistance transporter from human MCF-7 breast cancer cells', *Proc Natl Acad Sci U S A*, 95(26), pp. 15665-70.

Druker, B.J., Guilhot, F., O'Brien, S.G., Gathmann, I., Kantarjian, H., Gattermann, N., Deininger, M.W., Silver, R.T., Goldman, J.M., Stone, R.M., Cervantes, F., Hochhaus, A., Powell, B.L., Gabrilove, J.L., Rousselot, P., Reiffers, J., Cornelissen, J.J., Hughes, T., Agis, H., Fischer, T., Verhoef, G., Shepherd, J., Saglio, G., Gratwohl, A., Nielsen, J.L., Radich, J.P., Simonsson, B., Taylor, K., Baccarani, M., So, C., Letvak, L. and Larson, R.A. (2006) 'Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia', *N Engl J Med*, 355(23), pp. 2408-17.

Druker, B.J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G.M., Fanning, S., Zimmermann, J. and Lydon, N.B. (1996) 'Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells', *Nat Med*, 2(5), pp. 561-6.

Edwards, A.Y., Skolnik, J.M., Dombrowsky, E., Patel, D. and Barrett, J.S. (2012) 'Modeling and simulation approaches to evaluate pharmacokinetic sampling contamination from central venous catheters in pediatric pharmacokinetic studies of actinomycin-D: a report from the children's oncology group', *Cancer Chemother Pharmacol*, 70(1), pp. 83-94.

Eichelbaum, M., Ingelman-Sundberg, M. and Evans, W.E. (2006) 'Pharmacogenomics and individualized drug therapy', *Annu Rev Med*, 57, pp. 119-37.

Elens, L., Tyteca, D., Panin, N., Courtoy, P., Lison, D., Demoulin, J.B. and Haufroid, V. (2011) 'Functional defect caused by the 4544G>A SNP in ABCC2: potential impact for drug cellular disposition', *Pharmacogenet Genomics*, 21(12), pp. 884-93.

Elens, L., Yombi, J.C., Lison, D., Wallemacq, P., Vandercam, B. and Haufroid, V. (2009) 'Association between ABCC2 polymorphism and lopinavir accumulation in peripheral blood mononuclear cells of HIV-infected patients', *Pharmacogenomics*, 10(10), pp. 1589-97.

Erdilyi, D.J., Kamory, E., Csokay, B., Andrikovics, H., Tordai, A., Kiss, C., Filni-Semsei, A., Janszky, I., Zalka, A., Fekete, G., Falus, A., Kovacs, G.T. and Szalai, C. (2008) 'Synergistic interaction of ABCB1 and ABCG2 polymorphisms predicts the prevalence of toxic encephalopathy during anticancer chemotherapy', *Pharmacogenomics J*, 8(5), pp. 321-7.

Evans, D.A., Manley, K.A. and Mc, K.V. (1960) 'Genetic control of isoniazid metabolism in man', *Br Med J*, 2(5197), pp. 485-91.

Evans, W.E., Crom, W.R., Abromowitch, M., Dodge, R., Look, A.T., Bowman, W.P., George, S.L. and Pui, C.H. (1986) 'Clinical pharmacodynamics of high-dose methotrexate in acute lymphocytic leukemia. Identification of a relation between concentration and effect', *N Engl J Med*, 314(8), pp. 471-7.

Evans, W.E. and McLeod, H.L. (2003) 'Pharmacogenomics--drug disposition, drug targets, and side effects', *N Engl J Med*, 348(6), pp. 538-49.

Evans, W.E., Relling, M.V., Rodman, J.H., Crom, W.R., Boyett, J.M. and Pui, C.H. (1998) 'Conventional compared with individualized chemotherapy for childhood acute lymphoblastic leukemia', *N Engl J Med*, 338(8), pp. 499-505.

Evers, R., Cnubben, N.H., Wijnholds, J., van Deemter, L., van Bladeren, P.J. and Borst, P. (1997) 'Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1', *FEBS Lett*, 419(1), pp. 112-6.

Evers, R., Kool, M., van Deemter, L., Janssen, H., Calafat, J., Oomen, L.C., Paulusma, C.C., Oude Elferink, R.P., Baas, F., Schinkel, A.H. and Borst, P. (1998) 'Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA', *J Clin Invest*, 101(7), pp. 1310-9.

Fairchild, C.R., Ivy, S.P., Kao-Shan, C.S., Whang-Peng, J., Rosen, N., Israel, M.A., Melera, P.W., Cowan, K.H. and Goldsmith, M.E. (1987) 'Isolation of amplified and overexpressed DNA sequences from adriamycin-resistant human breast cancer cells', *Cancer Res*, 47(19), pp. 5141-8.

Faneyte, I.F., Kristel, P.M. and van de Vijver, M.J. (2004) 'Multidrug resistance associated genes MRP1, MRP2 and MRP3 in primary and anthracycline exposed breast cancer', *Anticancer Res*, 24(5A), pp. 2931-9.

Farber, S. (1955) 'Carcinolytic Action of Antibiotics: Puromycin and Actinomycin D', *Amer J Path*, (31).

Farber, S. (1966) 'Chemotherapy in the treatment of leukemia and Wilms' tumor', *JAMA*, 198(8), pp. 826-36.

Farber, S. and Diamond, L.K. (1948) 'Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid', *N Engl J Med*, 238(23), pp. 787-93.

Farber, S., Maddock, C.L. and Swaffield, M.N. (1956) 'Studies on the Carcinolytic and Other Biological Activity of Actinomycin D', *Proc Amer Assoc Cancer Res*, (2).

Fernbach, D.J. and Martyn, D.T. (1966) 'Role of dactinomycin in the improved survival of children with Wilms' tumor', *JAMA*, 195(12), pp. 1005-9.

Ferrari, S., Palmerini, E., Alberghini, M., Staals, E., Mercuri, M., Barbieri, E., Longhi, A., Cantero, L., Cesari, M., Abate, M., Balladelli, A., Picci, P. and Bacci, G. (2010) 'Vincristine, doxorubicin, cyclophosfamide, actinomycin D, ifosfamide, and etoposide in adult and pediatric patients with nonmetastatic Ewing sarcoma. Final results of a monoinstitutional study', *Tumori*, 96(2), pp. 213-8.

Fischbach, B., Trout, K., Lewis, J., Luis, C. and Sika, M. (2005) 'WAGR syndrome: a clinical review of 54 cases.', *Pediatrics*, 116, pp. 984-988.

Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M. and Pastan, I. (1987) 'Expression of a multidrug-resistance gene in human tumors and tissues', *Proc Natl Acad Sci U S A*, 84(1), pp. 265-9.

Franke, R.M., Lancaster, C.S., Peer, C.J., Gibson, A.A., Kosloske, A.M., Orwick, S.J., Mathijssen, R.H., Figg, W.D., Baker, S.D. and Sparreboom, A. (2011) 'Effect of ABCC2 (MRP2) transport function on erythromycin metabolism', *Clin Pharmacol Ther*, 89(5), pp. 693-701.

Freireich, E.J., Gehan, E.A., Rall, D.P., Schmidt, L.H. and Skipper, H.E. (1966) 'Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man', *Cancer Chemother Rep*, 50(4), pp. 219-44.

Friedenberg, W.R., Rue, M., Blood, E.A., Dalton, W.S., Shustik, C., Larson, R.A., Sonneveld, P. and Greipp, P.R. (2006) 'Phase III study of PSC-833 (valspodar) in combination with vincristine, doxorubicin, and dexamethasone (valspodar/VAD) versus VAD alone in patients with recurring or refractory multiple myeloma (E1A95): a trial of the Eastern Cooperative Oncology Group', *Cancer*, 106(4), pp. 830-8.

Friedlander, A. (1916) 'Sarcoma of the kidney treated by the roentgen ray', *Am J Dis Child*, (12), pp. 328-330.

Frost, B.M., Lonnerholm, G., Koopmans, P., Abrahamsson, J., Behrendtz, M., Castor, A., Forestier, E., Uges, D.R. and de Graaf, S.S. (2003) 'Vincristine in childhood leukaemia: no pharmacokinetic rationale for dose reduction in adolescents', *Acta Paediatr*, 92(5), pp. 551-7.

Fujita, K., Nagashima, F., Yamamoto, W., Endo, H., Sunakawa, Y., Yamashita, K., Ishida, H., Mizuno, K., Matsunaga, M., Araki, K., Tanaka, R., Ichikawa, W., Miya, T., Narabayashi, M., Akiyama, Y., Kawara, K., Ando, Y. and Sasaki, Y. (2008) 'Association of ATP-binding cassette, sub-family C, number 2 (ABCC2) genotype with pharmacokinetics of irinotecan in Japanese patients with metastatic colorectal cancer treated with irinotecan plus infusional 5-fluorouracil/leucovorin (FOLFIRI)', *Biol Pharm Bull*, 31(11), pp. 2137-42.

Fujiwara, K., Saita, T., Tekenawa, N., Matsumoto, N. and Kitagawa, T. (1988) 'Enzyme-linked Immunosorbent assay for the quantification of Actinomycin D using β-D-Galactosidase as a Label', *Cancer Research*, 48, pp. 4843-4847.

Furukawa, T., Wakabayashi, K., Tamura, A., Nakagawa, H., Morishima, Y., Osawa, Y. and Ishikawa, T. (2009) 'Major SNP (Q141K) variant of human ABC transporter ABCG2 undergoes lysosomal and proteasomal degradations', *Pharm Res*, 26(2), pp. 469-79.

Galbraith, W. and Mellett, L. (1975) 'Tissue disposition of 3H-actinomycin D (NSC-3053) in the rat, monkey, and dog', *Cancer Chemother Rep*, 59(6), pp. 1601-1609.

Galili, N., Davis, R., Fredericks, W., Mukhopadhyay, S., Rauscher III, F., Emanuel, B., Rovera, G. and Barr, F. (1993) 'Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma', *Nature Genetics*, 5, pp. 230-235.

Galimberti, S., Cervetti, G., Guerrini, F., Testi, R., Pacini, S., Fazzi, R., Simi, P. and Petrini, M. (2005) 'Quantitative molecular monitoring of BCR-ABL and MDR1 transcripts in patients with chronic myeloid leukemia during Imatinib treatment', *Cancer Genet Cytogenet*, 162(1), pp. 57-62.

Garcia-Castro, J., Trigueros, C., Madrenas, J., Perez-Simon, J.A., Rodriguez, R. and Menendez, P. (2008) 'Mesenchymal stem cells and their use as cell replacement therapy and disease modelling tool', *J Cell Mol Med*, 12(6B), pp. 2552-65.

Gardner, E.R., Smith, N.F., Figg, W.D. and Sparreboom, A. (2009) 'Influence of the dual ABCB1 and ABCG2 inhibitor tariquidar on the disposition of oral imatinib in mice', *J Exp Clin Cancer Res*, 28, p. 99.

Gibson, G.G. and Skett, P. (2001) *Introduction to Drug Metabolism*. 3 edn.

Gilman, A. (1963) 'The initial clinical trial of nitrogen mustard.', *Am. J. Surg*, 105, pp. 574-578.

Goldberg, B., Rabinowitz, M. and Reich, E. (1962a) 'Basis of actinomycin action I. DNA binding and inhibition of RNA-polymerase synthetic reactions by actinomycin', *Biochemistry*, 48, pp. 2094-2101.

Goldberg, I.H., Rabinowitz, M. and Reich, E. (1962b) 'Basis of Actinomycin action II. Effect of actinomycin on the nucleotide triphosphate-inorganic pyrophosphate exchange', *Proc Natl Acad Sci U S A*, 49, pp. 226-229.

Green, D.M., Finklestein, J.Z., Norkool, P. and D'Angio, G.J. (1988) 'Severe hepatic toxicity after treatment with single-dose dactinomycin and vincristine. A report of the National Wilms' Tumor Study', *Cancer*, 62(2), pp. 270-3.

Grundy, P., Breslow, N., Green, D.M., Sharples, K., Evans, A. and D'Angio, G.J. (1989) 'Prognostic factors for children with recurrent Wilms' tumor: results from

the Second and Third National Wilms' Tumor Study', *J Clin Oncol*, 7(5), pp. 638-47.

Guo, A., Marinaro, W., Hu, P. and Sinko, P.J. (2002) 'Delineating the contribution of secretory transporters in the efflux of etoposide using Madin-Darby canine kidney (MDCK) cells overexpressing P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP1), and canalicular multispecific organic anion transporter (cMOAT)', *Drug Metab Dispos*, 30(4), pp. 457-63.

Haber, M., Smith, J., Bordow, S.B., Flemming, C., Cohn, S.L., London, W.B., Marshall, G.M. and Norris, M.D. (2006) 'Association of high-level MRP1 expression with poor clinical outcome in a large prospective study of primary neuroblastoma', *J Clin Oncol*, 24(10), pp. 1546-53.

Hackmann, C. (1952) '[Experimental investigations on the effects of actinomycin C (HBF 386) in malignancies]', *Z Krebsforsch*, 58(4-5), pp. 607-13.

Hackmann, C. (1953) '[HBF 386 (actinomycin C), a natural substance of cytostatic effect]', *Strahlentherapie*, 90(2), pp. 296-300.

Haenisch, S., May, K., Wegner, D., Caliebe, A., Cascorbi, I. and Siegmund, W. (2008) 'Influence of genetic polymorphisms on intestinal expression and rifampicin-type induction of ABCC2 and on bioavailability of talinolol', *Pharmacogenet Genomics*, 18(4), pp. 357-65.

Haenisch, S., Zimmermann, U., Dazert, E., Wruck, C.J., Dazert, P., Siegmund, W., Kroemer, H.K., Warzok, R.W. and Cascorbi, I. (2007) 'Influence of polymorphisms of ABCB1 and ABCC2 on mRNA and protein expression in normal and cancerous kidney cortex', *Pharmacogenomics J*, 7(1), pp. 56-65.

Haerian, B.S., Lim, K.S., Mohamed, E.H., Tan, H.J., Tan, C.T., Raymond, A.A., Wong, C.P., Wong, S.W. and Mohamed, Z. (2011) 'Lack of association of ABCB1 haplotypes on five loci with response to treatment in epilepsy', *Seizure*, 20(7), pp. 546-53.

Hahn, N.M., Marsh, S., Fisher, W., Langdon, R., Zon, R., Browning, M., Johnson, C.S., Scott-Horton, T.J., Li, L., McLeod, H.L. and Sweeney, C.J. (2006) 'Hoosier Oncology Group randomized phase II study of docetaxel, vinorelbine, and estramustine in combination in hormone-refractory prostate cancer with pharmacogenetic survival analysis', *Clin Cancer Res*, 12(20 Pt 1), pp. 6094-9.

Han, J.Y., Lim, H.S., Yoo, Y.K., Shin, E.S., Park, Y.H., Lee, S.Y., Lee, J.E., Lee, D.H., Kim, H.T. and Lee, J.S. (2007) 'Associations of ABCB1, ABCC2, and ABCG2 polymorphisms with irinotecan-pharmacokinetics and clinical outcome in patients with advanced non-small cell lung cancer', *Cancer*, 110(1), pp. 138-47.

Hanahan, D. and Weinberg, R.A. (2000) 'The hallmarks of cancer', *Cell*, 100(1), pp. 57-70.

Hanahan, D. and Weinberg, R.A. (2011) 'Hallmarks of cancer: the next generation', *Cell*, 144(5), pp. 646-74.

Harland, S.J., Newell, D.R., Siddik, Z.H., Chadwick, R., Calvert, A.H. and Harrap, K.R. (1984) 'Pharmacokinetics of cis-diammine-1,1-cyclobutane dicarboxylate platinum(II) in patients with normal and impaired renal function', *Cancer Res*, 44(4), pp. 1693-7.

Harmsen, S., Meijerman, I., Febus, C.L., Maas-Bakker, R.F., Beijnen, J.H. and Schellens, J.H. (2010) 'PXR-mediated induction of P-glycoprotein by anticancer drugs in a human colon adenocarcinoma-derived cell line', *Cancer Chemother Pharmacol*, 66(4), pp. 765-71.

Harris, H.W., Knight, R.A. and Selin, M.J. (1958) 'Comparison of isoniazid concentrations in the blood of people of Japanese and European descent; therapeutic and genetic implications', *Am Rev Tuberc*, 78(6), pp. 944-8.

Hayes-Jordan, A. and Andrassy, R. (2009) 'Rhabdomyosarcoma in children', *Curr Opin Pediatr*.

He, T., Mo, A., Zhang, K. and Liu, L. (2011) 'ABCB1/MDR1 polymorphism and colorectal cancer risk: a meta-analysis of case-control studies', *Colorectal Dis*.

Helman, L.J. and Meltzer, P. (2003) 'Mechanisms of sarcoma development', *Nat Rev Cancer*, 3(9), pp. 685-94.

Hirono, A. and Beutler, E. (1988) 'Molecular cloning and nucleotide sequence of cDNA for human glucose-6-phosphate dehydrogenase variant A(-)', *Proc Natl Acad Sci U S A*, 85(11), pp. 3951-4.

Ho, R.H. and Kim, R.B. (2005) 'Transporters and drug therapy: implications for drug disposition and disease', *Clin Pharmacol Ther*, 78(3), pp. 260-77.

Hoenig, J.M. and Heisey, D.M. (2001) 'The Abuse of Power: The Pervasive Fallacy of Power Calculations for Data Analysis', *The American Statistician*, 55(1), pp. 1-6.

Hoffmeyer, S., Burk, O., von Richter, O., Arnold, H.P., Brockmoller, J., Johne, A., Cascorbi, I., Gerloff, T., Roots, I., Eichelbaum, M. and Brinkmann, U. (2000) 'Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo', *Proc Natl Acad Sci U S A*, 97(7), pp. 3473-8.

Hu, Q., Gao, F., Tian, W., Ruteshouser, E.C., Wang, Y., Lazar, A., Stewart, J., Strong, L.C., Behringer, R.R. and Huff, V. (2011) 'Wt1 ablation and Igf2

upregulation in mice result in Wilms tumors with elevated ERK1/2 phosphorylation', *J Clin Invest*, 121(1), pp. 174-83.

Huebner, C., Browning, B.L., Petermann, I., Han, D.Y., Philpott, M., Barclay, M., Gearry, R., McCulloch, A., Demmers, P. and Ferguson, L.R. (2009) 'Genetic analysis of MDR1 and inflammatory bowel disease reveals protective effect of heterozygous variants for ulcerative colitis', *Inflamm Bowel Dis*, 15(12), pp. 1784-93.

Huff, V. (1998) 'Wilms Tumour Genetics', *Am J Med Genet*, (79), pp. 260-267.

Huff, V. (2011) 'Wilms' tumours: about tumour suppressor genes, an oncogene and a chameleon gene', *Nat Rev Cancer*, 11(2), pp. 111-21.

Hughes, H.B. (1953) 'On the metabolic fate of isoniazid', *J Pharmacol Exp Ther*, 109(4), pp. 444-52.

Hughes, H.B., Biehl, J.P., Jones, A.P. and Schmidt, L.H. (1954) 'Metabolism of isoniazid in man as related to the occurrence of peripheral neuritis', *Am Rev Tuberc*, 70(2), pp. 266-73.

Imai, Y., Nakane, M., Kage, K., Tsukahara, S., Ishikawa, E., Tsuruo, T., Miki, Y. and Sugimoto, Y. (2002) 'C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance', *Mol Cancer Ther*, 1(8), pp. 611-6.

Jaffe, N., Paed, D., Traggis, D., Salian, S. and Cassady, J.R. (1976) 'Improved outlook for Ewing's sarcoma with combination chemotherapy (vincristine, actinomycin D and cyclophosphamide) and radiation therapy', *Cancer*, 38(5), pp. 1925-30.

Janknecht, R. (2005) 'EWS-ETS oncoproteins: the linchpins of Ewing tumors', *Gene*, 363, pp. 1-14.

Jedlitschky, G., Leier, I., Buchholz, U., Hummel-Eisenbeiss, J., Burchell, B. and Keppler, D. (1997) 'ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2', *Biochem J*, 327 (Pt 1), pp. 305-10.

Juliano, R.L. and Ling, V. (1976) 'A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants', *Biochim Biophys Acta*, 455(1), pp. 152-62.

Kalow, W. (1990) 'The Pennsylvania State University College of Medicine 1990 Bernard B. Brodie Lecture. Pharmacogenetics: past and future', *Life Sci*, 47(16), pp. 1385-97.

Kamiyama, N., Takagi, S., Yamamoto, C., Kudo, T., Nakagawa, T., Takahashi, M., Nakanishi, K., Takahashi, H., Todo, S. and Iseki, K. (2006) 'Expression of ABC transporters in human hepatocyte carcinoma cells with cross-resistance to epirubicin and mitoxantrone', *Anticancer Res*, 26(2A), pp. 885-8.

Kanwar, V., Albuquerque, M., Ribeiro, R., Kauffman, W. and Furman, W. (1995) 'Veno-occlusive disease of liver after chemotherapy for rhabdomyosarcoma: case report with review of the literature', *Med Ped Oncol*, 24, pp. 334-340.

Kartner, N., Riordan, J.R. and Ling, V. (1983a) 'Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines', *Science*, 221(4617), pp. 1285-8.

Kartner, N., Shales, M., Riordan, J.R. and Ling, V. (1983b) 'Daunorubicin-resistant Chinese hamster ovary cells expressing multidrug resistance and a cell-surface P-glycoprotein', *Cancer Res*, 43(9), pp. 4413-9.

Kaste, S., Dome, J., Babyn, P., Graf, N., Grundy, P., Godzinski, J., Levitt, G. and Jenkinson, H. (2007) 'Wilms tumour: prognostic factors, staging, therapy and late effects', *Pediatr Radiol*, 38, pp. 2-17.

Katzung, B.G. (2007) *Basic and Clinical Pharmacology*. Tenth Edition edn.

Kim, R.B., Leake, B.F., Choo, E.F., Dresser, G.K., Kubba, S.V., Schwarz, U.I., Taylor, A., Xie, H.G., McKinsey, J., Zhou, S., Lan, L.B., Schuetz, J.D., Schuetz, E.G. and Wilkinson, G.R. (2001) 'Identification of functionally variant MDR1 alleles among European Americans and African Americans', *Clin Pharmacol Ther*, 70(2), pp. 189-99.

Kim, W.J., Lee, J.H., Yi, J., Cho, Y.J., Heo, K., Lee, S.H., Kim, S.W., Kim, M.K., Kim, K.H., In Lee, B. and Lee, M.G. (2010) 'A nonsynonymous variation in MRP2/ABCC2 is associated with neurological adverse drug reactions of carbamazepine in patients with epilepsy', *Pharmacogenet Genomics*, 20(4), pp. 249-56.

Kimchi-Sarfaty, C., Oh, J.M., Kim, I.W., Sauna, Z.E., Calcagno, A.M., Ambudkar, S.V. and Gottesman, M.M. (2007) 'A "silent" polymorphism in the MDR1 gene changes substrate specificity', *Science*, 315(5811), pp. 525-8.

Kiyotani, K., Mushiroda, T., Imamura, C.K., Hosono, N., Tsunoda, T., Kubo, M., Tanigawara, Y., Flockhart, D.A., Desta, Z., Skaar, T.C., Aki, F., Hirata, K., Takatsuka, Y., Okazaki, M., Ohsumi, S., Yamakawa, T., Sasa, M., Nakamura, Y. and Zembutsu, H. (2010) 'Significant effect of polymorphisms in CYP2D6 and ABCC2 on clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients', *J Clin Oncol*, 28(8), pp. 1287-93.

Kiyotani, K., Mushiroda, T., Sasa, M., Bando, Y., Sumitomo, I., Hosono, N., Kubo, M., Nakamura, Y. and Zembutsu, H. (2008) 'Impact of CYP2D6*10 on

recurrence-free survival in breast cancer patients receiving adjuvant tamoxifen therapy', *Cancer Sci*, 99(5), pp. 995-9.

Koesters, R., Ridder, R., Kopp-Scheider, A., Betts, D., Adams, V., Niggli, F., Briner, J. and von Knebel Doeberitz, M. (1999) 'Mutational activation of the beta-catenin proto-oncogene is a common event in the development of Wilms' tumors', *Cancer Research*, 59, pp. 3880-3882.

Kondo, C., Suzuki, H., Itoda, M., Ozawa, S., Sawada, J., Kobayashi, D., Ieiri, I., Mine, K., Ohtsubo, K. and Sugiyama, Y. (2004) 'Functional analysis of SNPs variants of BCRP/ABCG2', *Pharm Res*, 21(10), pp. 1895-903.

Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D. and Jaenisch, R. (1993) 'WT-1 is required for early kidney development', *Cell*, 74(4), pp. 679-91.

Krupoves, A., Seidman, E.G., Mack, D., Israel, D., Morgan, K., Lambrette, P., Costea, I., Deslandres, C., Grimard, G., Law, L., Levy, E. and Amre, D.K. (2009) 'Associations between ABCB1/MDR1 gene polymorphisms and Crohn's disease: a gene-wide study in a pediatric population', *Inflamm Bowel Dis*, 15(6), pp. 900-8.

Lagas, J.S., Fan, L., Wagenaar, E., Vlaming, M.L., van Tellingen, O., Beijnen, J.H. and Schinkel, A.H. (2010) 'P-glycoprotein (P-gp/Abcb1), Abcc2, and Abcc3 determine the pharmacokinetics of etoposide', *Clin Cancer Res*, 16(1), pp. 130-40.

Lagas, J.S., van Waterschoot, R.A., van Tilburg, V.A., Hillebrand, M.J., Lankheet, N., Rosing, H., Beijnen, J.H. and Schinkel, A.H. (2009) 'Brain accumulation of dasatinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by elacridar treatment', *Clin Cancer Res*, 15(7), pp. 2344-51.

Lal, S., Wong, Z.W., Sandanaraj, E., Xiang, X., Ang, P.C., Lee, E.J. and Chowbay, B. (2008) 'Influence of ABCB1 and ABCG2 polymorphisms on doxorubicin disposition in Asian breast cancer patients', *Cancer Sci*, 99(4), pp. 816-23.

Langholz, B., Skolnik, J.M., Barrett, J.S., Renbarger, J., Seibel, N.L., Zajicek, A. and Arndt, C.A. (2011) 'Dactinomycin and vincristine toxicity in the treatment of childhood cancer: A retrospective study from the Children's Oncology Group', *Pediatr Blood Cancer*, 57(2), pp. 252-7.

Lehmann, H. and Ryan, E. (1956) 'The familial incidence of low pseudocholinesterase level', *Lancet*, 271(6934), p. 124.

Leier, I., Jedlitschky, G., Buchholz, U., Cole, S.P., Deeley, R.G. and Keppler, D. (1994) 'The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates', *J Biol Chem*, 269(45), pp. 27807-10.

Leith, C. (1998) 'Multidrug resistance in leukemia', *Curr Opin Hematol*, 5(4), pp. 287-91.

Lemerle, J., Voute, P.A., Tournade, M.F., Delemarre, J.F., Jereb, B., Ahstrom, L., Flamant, R. and Gerard-Marchant, R. (1976) 'Preoperative versus postoperative radiotherapy, single versus multiple courses of actinomycin D, in the treatment of Wilms' tumor. Preliminary results of a controlled clinical trial conducted by the International Society of Paediatric Oncology (S.I.O.P.)', *Cancer*, 38(2), pp. 647-54.

Lemerle, J., Voute, P.A., Tournade, M.F., Rodary, C., Delemarre, J.F., Sarrazin, D., Burgers, J.M., Sandstedt, B., Mildenberger, H., Carli, M. and et al. (1983) 'Effectiveness of preoperative chemotherapy in Wilms' tumor: results of an International Society of Paediatric Oncology (SIOP) clinical trial', *J Clin Oncol*, 1(10), pp. 604-9.

Lennard, L., Van Loon, J.A. and Weinshilboum, R.M. (1989) 'Pharmacogenetics of acute azathioprine toxicity: relationship to thiopurine methyltransferase genetic polymorphism', *Clin Pharmacol Ther*, 46(2), pp. 149-54.

Levitt, G. (2012) 'Renal tumours: long-term outcome', *Pediatr Nephrol*, 27(6), pp. 911-6.

Li, J., Cusatis, G., Brahmer, J., Sparreboom, A., Robey, R.W., Bates, S.E., Hidalgo, M. and Baker, S.D. (2007) 'Association of variant ABCG2 and the pharmacokinetics of epidermal growth factor receptor tyrosine kinase inhibitors in cancer patients', *Cancer Biol Ther*, 6(3), pp. 432-8.

Li, L., Lee, T.K., Meier, P.J. and Ballatori, N. (1998) 'Identification of glutathione as a driving force and leukotriene C4 as a substrate for oatp1, the hepatic sinusoidal organic solute transporter', *J Biol Chem*, 273(26), pp. 16184-91.

Li, L., Meier, P.J. and Ballatori, N. (2000) 'Oatp2 mediates bidirectional organic solute transport: a role for intracellular glutathione', *Mol Pharmacol*, 58(2), pp. 335-40.

Li, M., Squire, J. and Weksberg, R. (1997) 'Molecular genetics of Beckwith-Wiedmann syndrome', *Curr Opin Pediatr*, 9, pp. 623-629.

Lim, H.S., Ju Lee, H., Seok Lee, K., Sook Lee, E., Jang, I.J. and Ro, J. (2007) 'Clinical implications of CYP2D6 genotypes predictive of tamoxifen pharmacokinetics in metastatic breast cancer', *J Clin Oncol*, 25(25), pp. 3837-45.

Lim, J.S., Chen, X.A., Singh, O., Yap, Y.S., Ng, R.C., Wong, N.S., Wong, M., Lee, E.J. and Chowbay, B. (2011) 'Impact of CYP2D6, CYP3A5, CYP2C9 and CYP2C19 polymorphisms on tamoxifen pharmacokinetics in Asian breast cancer patients', *Br J Clin Pharmacol*, 71(5), pp. 737-50.

Lin, K.M., Chiu, Y.F., Tsai, I.J., Chen, C.H., Shen, W.W., Liu, S.C., Lu, S.C., Liu, C.Y., Hsiao, M.C., Tang, H.S., Liu, S.I., Chang, L.H., Wu, C.S., Tsou, H.H., Tsai, M.H., Chen, C.Y., Wang, S.M., Kuo, H.W., Hsu, Y.T. and Liu, Y.L. (2011) 'ABCB1 gene polymorphisms are associated with the severity of major depressive disorder and its response to escitalopram treatment', *Pharmacogenet Genomics*, 21(4), pp. 163-70.

Linardic, C. (2008) 'PAX3-FOXO1 fusion gene in rhabdomyosarcoma', *Cancer Letters*, 270, pp. 10-18.

Ling, V., Kartner, N., Sudo, T., Siminovitch, L. and Riordan, J.R. (1983) 'Multidrug-resistance phenotype in Chinese hamster ovary cells', *Cancer Treat Rep*, 67(10), pp. 869-74.

Lown, K.S., Mayo, R.R., Leichtman, A.B., Hsiao, H.L., Turgeon, D.K., Schmiedlin-Ren, P., Brown, M.B., Guo, W., Rossi, S.J., Benet, L.Z. and Watkins, P.B. (1997) 'Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine', *Clin Pharmacol Ther*, 62(3), pp. 248-60.

Lutz, R., Galbraith, W., Dedrick, R., Shrager, R. and Mellett, L. (1977) 'A model for the kinetics of distribution of actinomycin-d in the beagle dog', *The Journal of Pharmacology and Experimental Therapeutics*, 200(3), pp. 469-478.

Madden, T., Sunderland, M., Santana, V.M. and Rodman, J.H. (1992) 'The pharmacokinetics of high-dose carboplatin in pediatric patients with cancer', *Clin Pharmacol Ther*, 51(6), pp. 701-7.

Mahgoub, A., Idle, J.R., Dring, L.G., Lancaster, R. and Smith, R.L. (1977) 'Polymorphic hydroxylation of Debrisoquine in man', *Lancet*, 2(8038), pp. 584-6.

Mahon, F.X., Belloc, F., Lagarde, V., Chollet, C., Moreau-Gaudry, F., Reiffers, J., Goldman, J.M. and Melo, J.V. (2003) 'MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models', *Blood*, 101(6), pp. 2368-73.

Major, M., Camp, N., Berndt, J., Yi, X., Goldenberg, S., Hubbert, C., Biechele, T., Gingras, A., Zheng, N., Maccoss, M., Angers, S. and Moon, R. (2007) 'Wilms tumor suppressor WTX negatively regulates WNT/b-catenin signaling', *Science*, 316, pp. 1043-1046.

Malkin, D., Li, F. and Strong, C. (1990) 'Germline p53 mutations in a familial syndrome of breast cancers, sarcomas, and other neoplasms.', *Science*, 250, pp. 1233-1238.

Marie, J.P. and Legrand, O. (1999) 'MDR1/P-GP expression as a prognostic factor in acute leukemias', *Adv Exp Med Biol*, 457, pp. 1-9.

Mathijssen, R.H., Marsh, S., Karlsson, M.O., Xie, R., Baker, S.D., Verweij, J., Sparreboom, A. and McLeod, H.L. (2003) 'Irinotecan pathway genotype analysis to predict pharmacokinetics', *Clin Cancer Res*, 9(9), pp. 3246-53.

Maurer, H.M., Beltangady, M., Gehan, E.A., Crist, W., Hammond, D., Hays, D.M., Heyn, R., Lawrence, W., Newton, W., Ortega, J. and et al. (1988) 'The Intergroup Rhabdomyosarcoma Study-I. A final report', *Cancer*, 61(2), pp. 209-20.

Maurer, H.M., Gehan, E.A., Beltangady, M., Crist, W., Dickman, P.S., Donaldson, S.S., Fryer, C., Hammond, D., Hays, D.M., Herrmann, J. and et al. (1993) 'The Intergroup Rhabdomyosarcoma Study-II', *Cancer*, 71(5), pp. 1904-22.

Mayer, U., Wagenaar, E., Dorobek, B., Beijnen, J.H., Borst, P. and Schinkel, A.H. (1997) 'Full blockade of intestinal P-glycoprotein and extensive inhibition of blood-brain barrier P-glycoprotein by oral treatment of mice with PSC833', *J Clin Invest*, 100(10), pp. 2430-6.

Meggitt, S.J., Anstey, A.V., Mohd Mustapa, M.F., Reynolds, N.J. and Wakelin, S. (2011) 'British Association of Dermatologists' guidelines for the safe and effective prescribing of azathioprine 2011', *Br J Dermatol*, 165(4), pp. 711-34.

Melaine, N., Lienard, M.O., Dorval, I., Le Goascogne, C., Lejeune, H. and Jegou, B. (2002) 'Multidrug resistance genes and p-glycoprotein in the testis of the rat, mouse, Guinea pig, and human', *Biol Reprod*, 67(6), pp. 1699-707.

Metzger, M. and Dome, J. (2005) 'Current Therapy for Wilms' Tumor', *The Oncologist*, 10, pp. 815-826.

Meyer zu Schwabedissen, H.E., Jedlitschky, G., Gratz, M., Haenisch, S., Linnemann, K., Fusch, C., Cascorbi, I. and Kroemer, H.K. (2005) 'Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation', *Drug Metab Dispos*, 33(7), pp. 896-904.

Mitchell, R.S. and Bell, J.C. (1957) 'Clinical implications of isoniazid, PAS and streptomycin blood levels in pulmonary tuberculosis', *Trans Am Clin Climatol Assoc*, 69, pp. 98-102; discussion 103-5.

Mittapalli, R.K., Vaidhyanathan, S., Sane, R. and Elmquist, W.F. (2012) 'Impact of P-Glycoprotein (ABCB1) and Breast Cancer Resistance Protein (ABCG2) on the Brain Distribution of a Novel BRAF Inhibitor: Vemurafenib (PLX4032)', *J Pharmacol Exp Ther*, 342(1), pp. 33-40.

Mizuarai, S., Aozasa, N. and Kotani, H. (2004) 'Single nucleotide polymorphisms result in impaired membrane localization and reduced atpase activity in multidrug transporter ABCG2', *Int J Cancer*, 109(2), pp. 238-46.

Mondick, J., Gibiansky, L., Gastonguay, M., Skolnik, J., Cole, M., Veal, G., Boddy, A., Adamson, P. and Barrett, J. (2008) 'Population Pharmacokinetic Investigation of Actinomycin-D in Children and Young Adults', *J Clin Pharmacol*, 48, pp. 35-42.

Morisaki, K., Robey, R.W., Ozvegy-Laczka, C., Honjo, Y., Polgar, O., Steadman, K., Sarkadi, B. and Bates, S.E. (2005) 'Single nucleotide polymorphisms modify the transporter activity of ABCG2', *Cancer Chemother Pharmacol*, 56(2), pp. 161-72.

Moriya, Y., Nakamura, T., Horinouchi, M., Sakaeda, T., Tamura, T., Aoyama, N., Shirakawa, T., Gotoh, A., Fujimoto, S., Matsuo, M., Kasuga, M. and Okumura, K. (2002) 'Effects of polymorphisms of MDR1, MRP1, and MRP2 genes on their mRNA expression levels in duodenal enterocytes of healthy Japanese subjects', *Biol Pharm Bull*, 25(10), pp. 1356-9.

Mundry, R. and Fischer, J. (1998) 'Use of statistical programs for nonparametric tests of small samples often leads to incorrect P values: examples fromAnimal Behaviour', *Anim Behav*, 56(1), pp. 256-9.

Murry, D.J., Sandlund, J.T., Stricklin, L.M. and Rodman, J.H. (1993) 'Pharmacokinetics and acute renal effects of continuously infused carboplatin', *Clin Pharmacol Ther*, 54(4), pp. 374-80.

Narod, S.A., Hawkins, M.M., Robertson, C.M. and Stiller, C.A. (1997) 'Congenital anomalies and childhood cancer in Great Britain', *Am J Hum Genet*, 60(3), pp. 474-85.

Newell, D.R., Pearson, A.D., Balmanno, K., Price, L., Wyllie, R.A., Keir, M., Calvert, A.H., Lewis, I.J., Pinkerton, C.R. and Stevens, M.C. (1993) 'Carboplatin pharmacokinetics in children: the development of a pediatric dosing formula. The United Kingdom Children's Cancer Study Group', *J Clin Oncol*, 11(12), pp. 2314-23.

Newell, D.R., Siddik, Z.H., Gumbrell, L.A., Boxall, F.E., Gore, M.E., Smith, I.E. and Calvert, A.H. (1987) 'Plasma free platinum pharmacokinetics in patients treated with high dose carboplatin', *Eur J Cancer Clin Oncol*, 23(9), pp. 1399-405.

Nezasa, K., Tian, X., Zamek-Gliszczynski, M.J., Patel, N.J., Raub, T.J. and Brouwer, K.L. (2006) 'Altered hepatobiliary disposition of 5 (and 6)-carboxy-2',7'-dichlorofluorescein in Abcg2 (Bcrp1) and Abcc2 (Mrp2) knockout mice', *Drug Metab Dispos*, 34(4), pp. 718-23.

Ni, W., Ji, J., Dai, Z., Papp, A., Johnson, A.J., Ahn, S., Farley, K.L., Lin, T.S., Dalton, J.T., Li, X., Jarjoura, D., Byrd, J.C., Sadee, W., Grever, M.R. and Phelps, M.A. (2010) 'Flavopiridol pharmacogenetics: clinical and functional evidence for the role of SLCO1B1/OATP1B1 in flavopiridol disposition', *PLoS One*, 5(11), p. e13792.

Norris, M.D., Smith, J., Tanabe, K., Tobin, P., Flemming, C., Scheffer, G.L., Wielinga, P., Cohn, S.L., London, W.B., Marshall, G.M., Allen, J.D. and Haber, M. (2005) 'Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and confers resistance to irinotecan in vitro', *Mol Cancer Ther*, 4(4), pp. 547-53.

Oda, Y., Saito, T., Tateishi, N., Ohishi, Y., Tamiya, S., Yamamoto, H., Yokoyama, R., Uchiumi, T., Iwamoto, Y., Kuwano, M. and Tsuneyoshi, M. (2005) 'ATP-binding cassette superfamily transporter gene expression in human soft tissue sarcomas', *Int J Cancer*, 114(6), pp. 854-62.

Oncology, I.S.o.P. (2001) 'Information of SIOP Wilms Tumour studies', *Welcome to the page of the SIOP-WILMS studies!* Available at: http://www.siop.nl/pagina_182.html (Accessed: 12/07/12).

Owen, A., Goldring, C., Morgan, P., Chadwick, D., Park, B.K. and Pirmohamed, M. (2005) 'Relationship between the C3435T and G2677T(A) polymorphisms in the ABCB1 gene and P-glycoprotein expression in human liver', *Br J Clin Pharmacol*, 59(3), pp. 365-70.

Pallis, M., Turzanski, J., Higashi, Y. and Russell, N. (2002) 'P-glycoprotein in acute myeloid leukaemia: therapeutic implications of its association with both a multidrug-resistant and an apoptosis-resistant phenotype', *Leuk Lymphoma*, 43(6), pp. 1221-8.

Paulussen, M., Ahrens, S., Dunst, J., Winkelmann, W., Exner, G.U., Kotz, R., Amann, G., Dockhorn-Dworniczak, B., Harms, D., Muller-Weihrich, S., Welte, K., Kornhuber, B., Janka-Schaub, G., Gobel, U., Treuner, J., Voute, P.A., Zoubek, A., Gadner, H. and Jurgens, H. (2001) 'Localized Ewing tumor of bone: final results of the cooperative Ewing's Sarcoma Study CESS 86', *J Clin Oncol*, 19(6), pp. 1818-29.

Pavek, P., Merino, G., Wagenaar, E., Bolscher, E., Novotna, M., Jonker, J.W. and Schinkel, A.H. (2005) 'Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine', *J Pharmacol Exp Ther*, 312(1), pp. 144-52.

Poller, B., Iusuf, D., Sparidans, R.W., Wagenaar, E., Beijnen, J.H. and Schinkel, A.H. (2011) 'Differential impact of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) on axitinib brain accumulation and oral plasma pharmacokinetics', *Drug Metab Dispos*, 39(5), pp. 729-35.

Porteus, M., Narkool, P., Neuberg, D., Guthrie, K., Breslow, N., Green, D. and Diller, L. (2000) 'Characteristics and outcome of children with Beckwith-Wiedemann syndrome and Wilms' tumor:a report from the national Wilms' Tumor Study Group', *J Clin Oncol*, 18, pp. 2026-2031.

Potocnik, U., Ferkolj, I., Glavac, D. and Dean, M. (2004) 'Polymorphisms in multidrug resistance 1 (MDR1) gene are associated with refractory Crohn disease and ulcerative colitis', *Genes Immun*, 5(7), pp. 530-9.

Potocnik, U., Glavac, D. and Dean, M. (2008) 'Common germline MDR1/ABCB1 functional polymorphisms and haplotypes modify susceptibility to colorectal cancers with high microsatellite instability', *Cancer Genet Cytogenet*, 183(1), pp. 28-34.

Potratz, J., Dirksen, U., Jurgens, H. and Craft, A. (2012) 'Ewing sarcoma: clinical state-of-the-art', *Pediatr Hematol Oncol*, 29(1), pp. 1-11.

Prather, G.C. and Friedman, H.P. (1936) 'The Immediate Effect of Preoperative Radiation in Cortical Tumours of the Kidney', *N Engl J Med*, 215(15), pp. 655-663.

Pressey, J., Mroczek-Musulman, E., Murata-Collins, J., Saito, J. and Hammers, Y. (2008) 'Embryonal rhabdomyosarcoma with a novel t(2;6)(p23;p21.1)', *Cancer Genetics and Cytogenetics*, 187, pp. 39-42.

Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D. and et al. (1990) 'The candidate Wilms' tumour gene is involved in genitourinary development', *Nature*, 346(6280), pp. 194-7.

Pui, C.H. and Evans, W.E. (2006) 'Treatment of acute lymphoblastic leukemia', *N Engl J Med*, 354(2), pp. 166-78.

Pui, C.H., Relling, M.V. and Downing, J.R. (2004) 'Acute lymphoblastic leukemia', *N Engl J Med*, 350(15), pp. 1535-48.

Qu, J., Zhou, B.T., Yin, J.Y., Xu, X.J., Zhao, Y.C., Lei, G.H., Tang, Q., Zhou, H.H. and Liu, Z.Q. (2012) 'ABCC2 polymorphisms and haplotype are associated with drug resistance in Chinese epileptic patients', *CNS Neurosci Ther*, 18(8), pp. 647-51.

Raine, J., Bowman, A., Wallendszus, K. and Pritchard, J. (1991) 'Hepatopathy-thrombocytopenia syndrome--a complication of dactinomycin therapy for Wilms' tumor: a report from the United Kingdom Childrens Cancer Study Group', *J Clin Oncol*, 9(2), pp. 268-73.

Rance, T. (1814) 'Case of fungus haematodes of the kidnies', *Med Phys J*, (32), pp. 19-25.

Raney, R.B., Maurer, H.M., Anderson, J.R., Andrassy, R.J., Donaldson, S.S., Qualman, S.J., Wharam, M.D., Wiener, E.S. and Crist, W.M. (2001) 'The Intergroup Rhabdomyosarcoma Study Group (IRSG): Major Lessons From the

IRS-I Through IRS-IV Studies as Background for the Current IRS-V Treatment Protocols', *Sarcoma*, 5(1), pp. 9-15.

Ravina, A., Pestel, M. and Thielen, R. (1954) '[Clinical applications of the cytostatic and antitumoral properties of actinomycin C (sanamycin): importance of a new method of administration]', *Presse Med*, 62(56), pp. 1159-60.

Reich, E., Franklin, R.M., Shatkin, A.J. and Tatum, E.L. (1961) 'Effect of actinomycin D on cellular nucleic acid synthesis and virus production', *Science*, 134(3478), pp. 556-7.

Reich, E., Franklin, R.M., Shatkin, A.J. and Tatumel (1962) 'Action of actinomycin D on animal cells and viruses', *Proc Natl Acad Sci U S A*, 48, pp. 1238-45.

Reinhard, H., Semler, O., Burger, D., Bode, U., Flentje, M., Gobel, U., Gutjahr, P., Leuschner, I., Maass, E., Niggli, F., Scheel-Walter, H.G., Stockle, M., Thuroff, J.W., Troger, J., Weirich, A., von Schweinitz, D., Zoubek, A. and Graf, N. (2004) 'Results of the SIOP 93-01/GPOH trial and study for the treatment of patients with unilateral nonmetastatic Wilms Tumor', *Klin Padiatr*, 216(3), pp. 132-40.

Relling, M.V., Gardner, E.E., Sandborn, W.J., Schmiegelow, K., Pui, C.H., Yee, S.W., Stein, C.M., Carrillo, M., Evans, W.E. and Klein, T.E. (2011) 'Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing', *Clin Pharmacol Ther*, 89(3), pp. 387-91.

Rigato, I., Pascolo, L., Fernetti, C., Ostrow, J.D. and Tiribelli, C. (2004) 'The human multidrug-resistance-associated protein MRP1 mediates ATP-dependent transport of unconjugated bilirubin', *Biochem J*, 383(Pt 2), pp. 335-41.

Rivera, M., Kim, W., Wells, J., Driscoll, D., Brannigan, B., Han, M., Kim, J., Feinberg, A., Gerald, W., Vargas, S., Chin, L., Iafrate, A., Bell, D. and Haber, D. (2007) 'An X chromosome gene, WTX, is commonly inactivated in Wilms tumor', *Science*, 315, pp. 642-645.

Robey, R.W., Honjo, Y., Morisaki, K., Nadjem, T.A., Runge, S., Risbood, M., Poruchynsky, M.S. and Bates, S.E. (2003) 'Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity', *Br J Cancer*, 89(10), pp. 1971-8.

Robinson, H.J. and Waksman, S.A. (1942) 'Studies on the toxicity of actinomycin', *The Journal of Pharmacology and Experimental Therapeutics*, 74(1), pp. 25-32.

Rodman, J.H., Relling, M.V., Stewart, C.F., Synold, T.W., McLeod, H., Kearns, C., Stute, N., Crom, W.R. and Evans, W.E. (1993) 'Clinical pharmacokinetics

and pharmacodynamics of anticancer drugs in children', *Semin Oncol*, 20(1), pp. 18-29.

Rodriguez Novoa, S., Barreiro, P., Rendon, A., Barrios, A., Corral, A., Jimenez-Nacher, I., Gonzalez-Lahoz, J. and Soriano, V. (2006) 'Plasma levels of atazanavir and the risk of hyperbilirubinemia are predicted by the 3435C-->T polymorphism at the multidrug resistance gene 1', *Clin Infect Dis*, 42(2), pp. 291-5.

Roninson, I.B., Chin, J.E., Choi, K.G., Gros, P., Housman, D.E., Fojo, A., Shen, D.W., Gottesman, M.M. and Pastan, I. (1986) 'Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells', *Proc Natl Acad Sci U S A*, 83(12), pp. 4538-42.

Roussos, G.G. and Vining, L.C. (1956) 'Isolation and properties of pure actinomycins', *J. Chem. Soc.*, pp. 2469-2474.

Ruteshouser, E.C., Robinson, S.M. and Huff, V. (2008) 'Wilms tumor genetics: mutations in WT1, WTX, and CTNNB1 account for only about one-third of tumors', *Genes Chromosomes Cancer*, 47(6), pp. 461-70.

Sachidanandam, R. (2001) 'A map of the human genome sequence variation containing 1.42 million single nucleotide polymorphisms', *Nature*, 409, pp. 928-933.

Salama, N.N., Yang, Z., Bui, T. and Ho, R.J. (2006) 'MDR1 haplotypes significantly minimize intracellular uptake and transcellular P-gp substrate transport in recombinant LLC-PK1 cells', *J Pharm Sci*, 95(10), pp. 2293-308.

Sauer, G., Kafka, A., Grundmann, R., Kreienberg, R., Zeillinger, R. and Deissler, H. (2002) 'Basal expression of the multidrug resistance gene 1 (MDR-1) is associated with the TT genotype at the polymorphic site C3435T in mammary and ovarian carcinoma cell lines', *Cancer Lett*, 185(1), pp. 79-85.

Schaich, M., Kestel, L., Pfirrmann, M., Robel, K., Illmer, T., Kramer, M., Dill, C., Ehninger, G., Schackert, G. and Krex, D. (2009) 'A MDR1 (ABCB1) gene single nucleotide polymorphism predicts outcome of temozolomide treatment in glioblastoma patients', *Ann Oncol*, 20(1), pp. 175-81.

Schellens, J.H., Maliepaard, M., Scheper, R.J., Scheffer, G.L., Jonker, J.W., Smit, J.W., Beijnen, J.H. and Schinkel, A.H. (2000) 'Transport of topoisomerase I inhibitors by the breast cancer resistance protein. Potential clinical implications', *Ann N Y Acad Sci*, 922, pp. 188-94.

Schinkel, A.H., Kemp, S., Dolle, M., Rudenko, G. and Wagenaar, E. (1993) 'N-glycosylation and deletion mutants of the human MDR1 P-glycoprotein', *J Biol Chem*, 268(10), pp. 7474-81.

Schinkel, A.H., Mayer, U., Wagenaar, E., Mol, C.A., van Deemter, L., Smit, J.J., van der Valk, M.A., Voordouw, A.C., Spits, H., van Tellingen, O., Zijlmans, J.M., Fibbe, W.E. and Borst, P. (1997) 'Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins', *Proc Natl Acad Sci U S A*, 94(8), pp. 4028-33.

Schinkel, A.H., Smit, J.J., van Tellingen, O., Beijnen, J.H., Wagenaar, E., van Deemter, L., Mol, C.A., van der Valk, M.A., Robanus-Maandag, E.C., te Riele, H.P. and et al. (1994) 'Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs', *Cell*, 77(4), pp. 491-502.

Schinkel, A.H., Wagenaar, E., Mol, C.A. and van Deemter, L. (1996) 'P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs', *J Clin Invest*, 97(11), pp. 2517-24.

Schinkel, A.H., Wagenaar, E., van Deemter, L., Mol, C.A. and Borst, P. (1995) 'Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A', *J Clin Invest*, 96(4), pp. 1698-705.

Schroth, W., Antoniadou, L., Fritz, P., Schwab, M., Muerdter, T., Zanger, U.M., Simon, W., Eichelbaum, M. and Brauch, H. (2007) 'Breast cancer treatment outcome with adjuvant tamoxifen relative to patient CYP2D6 and CYP2C19 genotypes', *J Clin Oncol*, 25(33), pp. 5187-93.

Schroth, W., Goetz, M.P., Hamann, U., Fasching, P.A., Schmidt, M., Winter, S., Fritz, P., Simon, W., Suman, V.J., Ames, M.M., Safgren, S.L., Kuffel, M.J., Ulmer, H.U., Bolander, J., Strick, R., Beckmann, M.W., Koelbl, H., Weinshilboum, R.M., Ingle, J.N., Eichelbaum, M., Schwab, M. and Brauch, H. (2009) 'Association between CYP2D6 polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen', *JAMA*, 302(13), pp. 1429-36.

Scripture, C.D., Sparreboom, A. and Figg, W.D. (2005) 'Modulation of cytochrome P450 activity: implications for cancer therapy', *Lancet Oncol*, 6(10), pp. 780-9.

Sehested, M., Friche, E., Jensen, P.B. and Demant, E.J. (1992) 'Relationship of VP-16 to the classical multidrug resistance phenotype', *Cancer Res*, 52(10), pp. 2874-9.

Shapiro, D., Sublett, J., Li, B., Downing, J. and Naeve, C. (1993) 'Fusion of PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma', *Cancer Research*, 53, pp. 5108-5112.

Shen, D.W., Fojo, A., Roninson, I.B., Chin, J.E., Soffir, R., Pastan, I. and Gottesman, M.M. (1986) 'Multidrug resistance of DNA-mediated transformants is linked to transfer of the human mdr1 gene', *Mol Cell Biol*, 6(11), pp. 4039-45.

Shukla, S., Ohnuma, S. and Ambudkar, S.V. (2011) 'Improving cancer chemotherapy with modulators of ABC drug transporters', *Curr Drug Targets*, 12(5), pp. 621-30.

Siegsmund, M., Brinkmann, U., Schaffeler, E., Weirich, G., Schwab, M., Eichelbaum, M., Fritz, P., Burk, O., Decker, J., Alken, P., Rothenpieler, U., Kerb, R., Hoffmeyer, S. and Brauch, H. (2002) 'Association of the P-glycoprotein transporter MDR1(C3435T) polymorphism with the susceptibility to renal epithelial tumors', *J Am Soc Nephrol*, 13(7), pp. 1847-54.

Simon, N., Marsot, A., Villard, E., Choquet, S., Khe, H.X., Zahr, N., Lechat, P., Leblond, V. and Hulot, J.S. (2012) 'Impact of ABCC2 polymorphisms on high-dose methotrexate pharmacokinetics in patients with lymphoid malignancy', *Pharmacogenomics J*.

Sissung, T.M., Baum, C.E., Deeken, J., Price, D.K., Aragon-Ching, J., Steinberg, S.M., Dahut, W., Sparreboom, A. and Figg, W.D. (2008) 'ABCB1 genetic variation influences the toxicity and clinical outcome of patients with androgen-independent prostate cancer treated with docetaxel', *Clin Cancer Res*, 14(14), pp. 4543-9.

Sissung, T.M., Gardner, E.R., Piekarz, R.L., Howden, R., Chen, X., Woo, S., Franke, R., Clark, J.A., Miller-DeGraff, L., Steinberg, S.M., Venzon, D., Liewehr, D., Kleeberger, S.R., Bates, S.E., Price, D.K., Rosing, D.R., Cabell, C., Sparreboom, A. and Figg, W.D. (2011) 'Impact of ABCB1 allelic variants on QTc interval prolongation', *Clin Cancer Res*, 17(4), pp. 937-46.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S. and Boyd, M.R. (1990) 'New colorimetric cytotoxicity assay for anticancer-drug screening', *J Natl Cancer Inst*, 82(13), pp. 1107-12.

Sloan, J.A., de Andrade, M., Decker, P., Wampfler, J., Oswold, C., Clark, M. and Yang, P. (2012) 'Genetic variations and patient-reported quality of life among patients with lung cancer', *J Clin Oncol*, 30(14), pp. 1699-704.

Sobell, H.M. (1985) 'Actinomycin and DNA transcription', *Proc Natl Acad Sci U S A*, 82(16), pp. 5328-31.

Sobell, H.M. and Jain, S.C. (1972) 'Stereochemistry of actinomycin binding to DNA. II. Detailed molecular model of actinomycin-DNA complex and its implications', *J Mol Biol*, 68(1), pp. 21-34.

Sookoian, S., Castano, G., Burgueno, A., Gianotti, T.F. and Pirola, C.J. (2008a) 'Association of the multidrug-resistance-associated protein gene (ABCC2) variants with intrahepatic cholestasis of pregnancy', *J Hepatol*, 48(1), pp. 125-32.

Sookoian, S., Castano, G., Gianotti, T.F., Gemma, C. and Pirola, C.J. (2009) 'Polymorphisms of MRP2 (ABCC2) are associated with susceptibility to nonalcoholic fatty liver disease', *J Nutr Biochem*, 20(10), pp. 765-70.

Sookoian, S., Castano, G. and Pirola, C.J. (2008b) 'Role of ABCC2 common variants in intrahepatic cholestasis of pregnancy', *World J Gastroenterol*, 14(13), pp. 2126-7.

Sorensen, B.T., Stromgren, A., Jakobsen, P., Nielsen, J.T., Andersen, L.S. and Jakobsen, A. (1992) 'Renal handling of carboplatin', *Cancer Chemother Pharmacol*, 30(4), pp. 317-20.

Sorensen, K., Levitt, G., Sebag-Montefiore, D., Bull, C. and Sullivan, I. (1995) 'Cardiac function in Wilms' tumor survivors', *J Clin Oncol*, 13(7), pp. 1546-56.

Sparreboom, A., Gelderblom, H., Marsh, S., Ahluwalia, R., Obach, R., Principe, P., Twelves, C., Verweij, J. and McLeod, H.L. (2004) 'Diflomotecan pharmacokinetics in relation to ABCG2 421C>A genotype', *Clin Pharmacol Ther*, 76(1), pp. 38-44.

Sparreboom, A., Loos, W.J., Burger, H., Sissung, T.M., Verweij, J., Figg, W.D., Nooter, K. and Gelderblom, H. (2005) 'Effect of ABCG2 genotype on the oral bioavailability of topotecan', *Cancer Biol Ther*, 4(6), pp. 650-8.

Steinbach, D. and Legrand, O. (2007) 'ABC transporters and drug resistance in leukemia: was P-gp nothing but the first head of the Hydra?', *Leukemia*, 21(6), pp. 1172-6.

Stiller, C.A. and Draper, G.J. (2005) *Cancer in Children*. 5 edn. Oxford University Press.

Stiller, C.A. and Parkin, D.M. (1990) 'International variations in the incidence of childhood renal tumours', *Br J Cancer*, 62(6), pp. 1026-30.

Sun, N., Sun, X., Chen, B., Cheng, H., Feng, J., Cheng, L. and Lu, Z. (2010) 'MRP2 and GSTP1 polymorphisms and chemotherapy response in advanced non-small cell lung cancer', *Cancer Chemother Pharmacol*, 65(3), pp. 437-46.

Sutow, W.W., Breslow, N.E., Palmer, N.F., D'Angio, G.J. and Takashima, J. (1982) 'Prognosis in children with Wilms' tumor metastases prior to or following primary treatment: results from the first National Wilms' Tumor Study (NWTS-1)', *Am J Clin Oncol*, 5(4), pp. 339-47.

Synold, T.W., Dussault, I. and Forman, B.M. (2001) 'The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux', *Nat Med*, 7(5), pp. 584-90.

Tai, H.L., Krynetski, E.Y., Schuetz, E.G., Yanishevski, Y. and Evans, W.E. (1997) 'Enhanced proteolysis of thiopurine S-methyltransferase (TPMT) encoded by mutant alleles in humans (TPMT*3A, TPMT*2): mechanisms for the genetic polymorphism of TPMT activity', *Proc Natl Acad Sci U S A*, 94(12), pp. 6444-9.

Tamura, A., Wakabayashi, K., Onishi, Y., Takeda, M., Ikegami, Y., Sawada, S., Tsuji, M., Matsuda, Y. and Ishikawa, T. (2007) 'Re-evaluation and functional classification of non-synonymous single nucleotide polymorphisms of the human ATP-binding cassette transporter ABCG2', *Cancer Sci*, 98(2), pp. 231-9.

Tang, K., Ngoi, S.M., Gwee, P.C., Chua, J.M., Lee, E.J., Chong, S.S. and Lee, C.G. (2002) 'Distinct haplotype profiles and strong linkage disequilibrium at the MDR1 multidrug transporter gene locus in three ethnic Asian populations', *Pharmacogenetics*, 12(6), pp. 437-50.

Tang, S.C., Lagas, J.S., Lankheet, N.A., Poller, B., Hillebrand, M.J., Rosing, H., Beijnen, J.H. and Schinkel, A.H. (2011) 'Brain accumulation of sunitinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by oral elacridar and sunitinib coadministration', *Int J Cancer*.

Tang, S.C., Lagas, J.S., Lankheet, N.A., Poller, B., Hillebrand, M.J., Rosing, H., Beijnen, J.H. and Schinkel, A.H. (2012a) 'Brain accumulation of sunitinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by oral elacridar and sunitinib coadministration', *Int J Cancer*, 130(1), pp. 223-33.

Tang, S.C., Lankheet, N.A., Poller, B., Wagenaar, E., Beijnen, J.H. and Schinkel, A.H. (2012b) 'P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) restrict brain accumulation of the active sunitinib metabolite N-desethyl sunitinib', *J Pharmacol Exp Ther*, 341(1), pp. 164-73.

Taniguchi, K., Wada, M., Kohno, K., Nakamura, T., Kawabe, T., Kawakami, M., Kagotani, K., Okumura, K., Akiyama, S. and Kuwano, M. (1996) 'A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation', *Cancer Res*, 56(18), pp. 4124-9.

Tatsuta, T., Naito, M., Oh-hara, T., Sugawara, I. and Tsuruo, T. (1992) 'Functional involvement of P-glycoprotein in blood-brain barrier', *J Biol Chem*, 267(28), pp. 20383-91.

Tattersall, M.H., Sodergren, J.E., Dengupta, S.K., Trites, D.H., Modest, E.J. and Frei, E., 3rd (1975) 'Pharmacokinetics of actinoymcin D in patients with malignant melanoma', *Clin Pharmacol Ther*, 17(6), pp. 701-8.

Teh, L.K. and Bertilsson, L. (2012) 'Pharmacogenomics of CYP2D6: molecular genetics, interethnic differences and clinical importance', *Drug Metab Pharmacokinet*, 27(1), pp. 55-67.

Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M.M., Pastan, I. and Willingham, M.C. (1987) 'Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues', *Proc Natl Acad Sci U S A*, 84(21), pp. 7735-8.

Thomas, H., Boddy, A.V., English, M.W., Hobson, R., Imeson, J., Lewis, I., Morland, B., Pearson, A.D., Pinkerton, R., Price, L., Stevens, M. and Newell, D.R. (2000) 'Prospective validation of renal function-based carboplatin dosing in children with cancer: A United Kingdom Children's Cancer Study Group Trial', *J Clin Oncol*, 18(21), pp. 3614-21.

Tournade, M.F., Com-Nougue, C., de Kraker, J., Ludwig, R., Rey, A., Burgers, J.M., Sandstedt, B., Godzinski, J., Carli, M., Potter, R. and Zucker, J.M. (2001) 'Optimal duration of preoperative therapy in unilateral and nonmetastatic Wilms' tumor in children older than 6 months: results of the Ninth International Society of Pediatric Oncology Wilms' Tumor Trial and Study', *J Clin Oncol*, 19(2), pp. 488-500.

Tournade, M.F., Com-Nougue, C., Voute, P.A., Lemerle, J., de Kraker, J., Delemarre, J.F., Burgers, M., Habrand, J.L., Moorman, C.G., Burger, D. and et al. (1993) 'Results of the Sixth International Society of Pediatric Oncology Wilms' Tumor Trial and Study: a risk-adapted therapeutic approach in Wilms' tumor', *J Clin Oncol*, 11(6), pp. 1014-23.

Trock, B.J., Leonessa, F. and Clarke, R. (1997) 'Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance', *J Natl Cancer Inst*, 89(13), pp. 917-31.

Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. (1981) 'Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil', *Cancer Res*, 41(5), pp. 1967-72.

Turan, T., Karacay, O., Tulunay, G., Boran, N., Koc, S., Bozok, S. and Kose, M.F. (2006) 'Results with EMA/CO (etoposide, methotrexate, actinomycin D, cyclophosphamide, vincristine) chemotherapy in gestational trophoblastic neoplasia', *Int J Gynecol Cancer*, 16(3), pp. 1432-8.

Twentyman, P.R. and Bleehen, N.M. (1991) 'Resistance modification by PSC-833, a novel non-immunosuppressive cyclosporin [corrected]', *Eur J Cancer*, 27(12), pp. 1639-42.

Ueda, K., Cardarelli, C., Gottesman, M.M. and Pastan, I. (1987) 'Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine', *Proc Natl Acad Sci U S A*, 84(9), pp. 3004-8.

Ueda, K., Cornwell, M.M., Gottesman, M.M., Pastan, I., Roninson, I.B., Ling, V. and Riordan, J.R. (1986) 'The mdr1 gene, responsible for multidrug-resistance, codes for P-glycoprotein', *Biochem Biophys Res Commun*, 141(3), pp. 956-62.

Uhr, M., Tontsch, A., Namendorf, C., Ripke, S., Lucae, S., Ising, M., Dose, T., Ebinger, M., Rosenhagen, M., Kohli, M., Kloiber, S., Salyakina, D., Bettecken, T., Specht, M., Putz, B., Binder, E.B., Muller-Myhsok, B. and Holsboer, F. (2008) 'Polymorphisms in the drug transporter gene ABCB1 predict antidepressant treatment response in depression', *Neuron*, 57(2), pp. 203-9.

Vahakangas, K. and Myllynen, P. (2009) 'Drug transporters in the human blood-placental barrier', *Br J Pharmacol*, 158(3), pp. 665-78.

van Asperen, J., van Tellingen, O., Tijssen, F., Schinkel, A.H. and Beijnen, J.H. (1999) 'Increased accumulation of doxorubicin and doxorubicinol in cardiac tissue of mice lacking mdr1a P-glycoprotein', *Br J Cancer*, 79(1), pp. 108-13.

Vander Borght, S., Komuta, M., Libbrecht, L., Katoonizadeh, A., Aerts, R., Dymarkowski, S., Verslype, C., Nevens, F. and Roskams, T. (2008) 'Expression of multidrug resistance-associated protein 1 in hepatocellular carcinoma is associated with a more aggressive tumour phenotype and may reflect a progenitor cell origin', *Liver Int*, 28(10), pp. 1370-80.

Varan, A. (2008) 'Wilms' Tumor in Children: An Overview', *Nephron Clinical Practice*, 108, pp. 83-90.

Veal, G.J., Cole, M., Errington, J., Parry, A., Hale, J., Pearson, A.D., Howe, K., Chisholm, J.C., Beane, C., Brennan, B., Waters, F., Glaser, A., Hemsworth, S., McDowell, H., Wright, Y., Pritchard-Jones, K., Pinkerton, R., Jenner, G., Nicholson, J., Elsworth, A.M. and Boddy, A.V. (2005) 'Pharmacokinetics of dactinomycin in a pediatric patient population: a United Kingdom Children's Cancer Study Group Study', *Clin Cancer Res*, 11(16), pp. 5893-9.

Veal, G.J., Coulthard, S.A. and Boddy, A.V. (2003a) 'Chemotherapy individualization', *Invest New Drugs*, 21(2), pp. 149-56.

Veal, G.J., Errington, J., Rowbotham, S., Illingworth, N.A., Malik, G., Cole, M., Daly, A.K., Pearson, A.D. and Boddy, A. (2012) 'Adaptive dosing approaches to the individualization of 13-cis-retinoic acid (isotretinoin) treatment for children with high-risk neuroblastoma', *Clin Cancer Res*.

Veal, G.J., Errington, J., Sludden, J., Griffin, M.J., Price, L., Parry, A., Hale, J., Pearson, A.D. and Boddy, A.V. (2003b) 'Determination of anti-cancer drug actinomycin D in human plasma by liquid chromatography-mass spectrometry', *J Chromatogr B Analyt Technol Biomed Life Sci*, 795(2), pp. 237-43.

Vining, L.C. and Waksman, S.A. (1954) 'Paper chromatographic identification of the actinomycins', *Science*, 120(3114), pp. 389-90.

Visser, M., Sijmons, C., Bras, J., Arceci, R., Godfried, M., Valentijn, L., Voute, P. and Baas, F. (1997) 'Allelotype of pediatric rhabdomyosarcoma', *Oncogene*, 15, pp. 1309-1314.

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. and Schinkel, A.H. (2006) 'Carcinogen and anticancer drug transport by Mrp2 in vivo: studies using Mrp2 (Abcc2) knockout mice', *J Pharmacol Exp Ther*, 318(1), pp. 319-27.

Vogelgesang, S., Kunert-Keil, C., Cascorbi, I., Mosyagin, I., Schroder, E., Runge, U., Jedlitschky, G., Kroemer, H.K., Oertel, J., Gaab, M.R., Pahnke, J., Walker, L.C. and Warzok, R.W. (2004) 'Expression of multidrug transporters in dysembryoplastic neuroepithelial tumors causing intractable epilepsy', *Clin Neuropathol*, 23(5), pp. 223-31.

Waksman, S.A. and Woodruff, H.B. (1940) 'Bacteriostatic and bactericidal substances produced by a soil Actinomyces', *Proc Soc Exp Biol Med*, 45, pp. 609-214.

Wang, D. and Sadee, W. (2006) 'Searching for polymorphisms that affect gene expression and mRNA processing: example ABCB1 (MDR1)', *AAPS J*, 8(3), pp. E515-20.

Wassermann, K., Markovits, J., Jaxel, C., Capranico, G., Kohn, K. and Pommier, Y. (1990) 'Effects of Morpholinyl Doxorubicins, Doxorubicin, and Actinomycin D on Mammalian DNA Topoisomerases I and II', *Molecular Pharmacology*, 38, pp. 38-45.

Weinshilboum, R.M. (1984) 'Human pharmacogenetics: Introduction', *Fed Proc*, 43(8), pp. 2295-7.

Weinshilboum, R.M. and Sladek, S.L. (1980) 'Mercaptopurine pharmacogenetics: monogenic inheritance of erythrocyte thiopurine methyltransferase activity', *Am J Hum Genet*, 32(5), pp. 651-62.

Weinstein, R.S., Jakate, S.M., Dominguez, J.M., Lebovitz, M.D., Koukoulis, G.K., Kuszak, J.R., Klusens, L.F., Grogan, T.M., Saclarides, T.J., Roninson, I.B. and et al. (1991) 'Relationship of the expression of the multidrug resistance gene product (P-glycoprotein) in human colon carcinoma to local tumor aggressiveness and lymph node metastasis', *Cancer Res*, 51(10), pp. 2720-6.

Wills, L., Clutterbuck, P.W. and Evans, B.D. (1937) 'A new factor in the production and cure of macrocytic anaemias and its relation to other haemopoietic principles curative in pernicious anaemia', *Biochem J*, 31(11), pp. 2136-47.

Wilms, M. (1899) 'Die Mischgeschwülste der Niere', *Leipzig, A Georgi,*, pp. 1-99.

Wojnowski, L., Kulle, B., Schirmer, M., Schluter, G., Schmidt, A., Rosenberger, A., Vonhof, S., Bickeboller, H., Toliat, M.R., Suk, E.K., Tzvetkov, M., Kruger, A., Seifert, S., Kloess, M., Hahn, H., Loeffler, M., Nurnberg, P., Pfreundschuh, M., Trumper, L., Brockmoller, J. and Hasenfuss, G. (2005) 'NAD(P)H oxidase and multidrug resistance protein genetic polymorphisms are associated with doxorubicin-induced cardiotoxicity', *Circulation*, 112(24), pp. 3754-62.

Wong, M.L., Dong, C., Maestre-Mesa, J. and Licinio, J. (2008) 'Polymorphisms in inflammation-related genes are associated with susceptibility to major depression and antidepressant response', *Mol Psychiatry*, 13(8), pp. 800-12.

Worch, J., Matthay, K.K., Neuhaus, J., Goldsby, R. and DuBois, S.G. (2010) 'Ethnic and racial differences in patients with Ewing sarcoma', *Cancer*, 116(4), pp. 983-8.

Workman, P., Aboagye, E.O., Balkwill, F., Balmain, A., Bruder, G., Chaplin, D.J., Double, J.A., Everitt, J., Farningham, D.A., Glennie, M.J., Kelland, L.R., Robinson, V., Stratford, I.J., Tozer, G.M., Watson, S., Wedge, S.R. and Eccles, S.A. (2010) 'Guidelines for the welfare and use of animals in cancer research', *Br J Cancer*, 102(11), pp. 1555-77.

Wosilait, W.D. and Eisenbrandt, L.L. (1971) 'Biliary excretion of (3 H)-actinomycin D in the rat', *Life Sci I*, 10(18), pp. 1051-5.

Xing, Q., Gao, R., Li, H., Feng, G., Xu, M., Duan, S., Meng, J., Zhang, A., Qin, S. and He, L. (2006) 'Polymorphisms of the ABCB1 gene are associated with the therapeutic response to risperidone in Chinese schizophrenia patients', *Pharmacogenomics*, 7(7), pp. 987-93.

Yamasaki, Y., Ieiri, I., Kusuhara, H., Sasaki, T., Kimura, M., Tabuchi, H., Ando, Y., Irie, S., Ware, J., Nakai, Y., Higuchi, S. and Sugiyama, Y. (2008) 'Pharmacogenetic characterization of sulfasalazine disposition based on NAT2 and ABCG2 (BCRP) gene polymorphisms in humans', *Clin Pharmacol Ther*, 84(1), pp. 95-103.

Yates, C.R., Krynetski, E.Y., Loennechen, T., Fessing, M.Y., Tai, H.L., Pui, C.H., Relling, M.V. and Evans, W.E. (1997) 'Molecular diagnosis of thiopurine S-methyltransferase deficiency: genetic basis for azathioprine and mercaptopurine intolerance', *Ann Intern Med*, 126(8), pp. 608-14.

Young, L.C., Campling, B.G., Voskoglou-Nomikos, T., Cole, S.P., Deeley, R.G. and Gerlach, J.H. (1999) 'Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response', *Clin Cancer Res*, 5(3), pp. 673-80.

Zanger, U.M., Raimundo, S. and Eichelbaum, M. (2004) 'Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry', *Naunyn Schmiedebergs Arch Pharmacol*, 369(1), pp. 23-37.

Zhang, D.W., Cole, S.P. and Deeley, R.G. (2002) 'Determinants of the substrate specificity of multidrug resistance protein 1: role of amino acid residues with hydrogen bonding potential in predicted transmembrane helix 17', *J Biol Chem*, 277(23), pp. 20934-41.

Zhang, W., Yu, B.N., He, Y.J., Fan, L., Li, Q., Liu, Z.Q., Wang, A., Liu, Y.L., Tan, Z.R., Fen, J., Huang, Y.F. and Zhou, H.H. (2006) 'Role of BCRP 421C>A polymorphism on rosuvastatin pharmacokinetics in healthy Chinese males', *Clin Chim Acta*, 373(1-2), pp. 99-103.

Zhang, Y.T., Yang, L.P., Shao, H., Li, K.X., Sun, C.H. and Shi, L.W. (2008) 'ABCB1 polymorphisms may have a minor effect on ciclosporin blood concentrations in myasthenia gravis patients', *Br J Clin Pharmacol*, 66(2), pp. 240-6.

Zhou, Q., Sparreboom, A., Tan, E.H., Cheung, Y.B., Lee, A., Poon, D., Lee, E.J. and Chowbay, B. (2005) 'Pharmacogenetic profiling across the irinotecan pathway in Asian patients with cancer', *Br J Clin Pharmacol*, 59(4), pp. 415-24.

Zhu, D., Taguchi-Nakamura, H., Goto, M., Odawara, T., Nakamura, T., Yamada, H., Kotaki, H., Sugiura, W., Iwamoto, A. and Kitamura, Y. (2004) 'Influence of single-nucleotide polymorphisms in the multidrug resistance-1 gene on the cellular export of nelfinavir and its clinical implication for highly active antiretroviral therapy', *Antivir Ther*, 9(6), pp. 929-35.

Appendix

Actinomycin D pharmacokinetic data in 108 patients

Patient	SA (m^2)	Dose (mg)	Weight (kg)	AUC_{0-6h} (µg/L.h)	AUC_{0-24h} (µg/L.h)	$AUC_{0-\infty}$ (µg/L.h)	Cl-F (ml/min)	C_{max} (µg/L)	T_{max} (h)
11	0.77	0.80	18.7	73.0	-	96.0	14.8	59.8	15
12	0.62	0.60	14.2	27.5	67.8	83.6	-	13.3	17
13	0.77	0.85	19.4	42.8	117.2	194.1	-	29.1	17
15	0.63	0.63	14.3	16.9	51.3	90.9	-	9.6	20
16	0.57	0.55	12.3	24.8	72.8	102.7	51.8	14.0	17
17	0.44	0.25	8.3	44.2	-	51.6	44.9	30.2	14
18	1.10	1.35	32.0	-	-	36.0	-	18.8	19
22	0.77	0.90	20.2	30.1	-	60.5	42.6	42.4	15
24	0.85	0.95	21.8	26.3	63.7	98.3	-	14.1	15
25	0.46	0.26	8.8	28.8	63.7	83.7	35.1	20.3	17
26	0.62	0.61	13.6	26.8	-	96.1	-	25.9	23
29	0.44	0.19	8.9	22.6	191.7	213.3	-	33.4	25
33	0.74	0.80	17.9	22.9	-	103.5	-	13.2	23
34	0.42	0.24	8.5	21.5	-	28.1	-	5.8	35
35	0.77	0.85	19.4	29.3	88.6	151.4	32.2	26.2	16
38	0.55	0.36	11.9	94.9	-	115.5	62.4	98.4	13
41	0.53	0.33	11.0	-	-	83.3	66.9	36.5	13
42	0.62	-	14.1	24.9	-	34.0	-	14.2	20
43	0.65	0.69	15.4	23.5	-	93.5	156.1	9.5	17
44	1.80	2.00	64.8	54.5	-	65.9	-	92.6	7
45	0.65	0.70	15.3	0.0	-	23.5	80.6	14.9	19
46	0.53	0.33	11.2	81.0	-	91.8	-	143.4	7
47	0.62	0.63	14.2	-	-	83.0	40.9	112.6	5
49	0.62	0.49	14.6	18.3	-	45.8	89.2	19.9	16
50	0.74	0.80	18.1	15.2	55.4	113.9	-	16.8	18
51	0.87	1.04	23.4	36.8	-	41.8	89.3	22.1	20
53	1.20	0.90	37.7	18.7	-	90.3	109.7	20.0	11

Patient	SA (m^2)	Dose (mg)	Weight (kg)	AUC_{0-6h} (µg/L.h)	AUC_{0-24h} (µg/L.h)	$AUC_{0-\infty}$ (µg/L.h)	Cl-F (ml/min)	C_{max} (µg/L)	T_{max} (h)
55	0.42	0.24	7.9	-	-	48.3	119.7	95.4	6
56	0.54	0.52	11.5	-	-	23.9	-	13.8	18
57	0.62	0.65	13.7	57.6	144.0	234.1	-	52.2	13
60	0.51	0.30	10.5	27.0	83.6	117.4	-	27.9	18
61	1.40	2.00	46.8	24.1	68.9	134.2	-	16.9	17
62	2.00	1.50	77.4	16.6	49.9	73.3	-	16.7	20
63	0.71	1.00	16.8	26.8	64.1	93.9	115.5	20.1	17
64	0.82	0.46	21.2	13.6	38.5	49.1	-	9.7	18
65	0.46	0.50	9.2	22.9	62.0	103.3	46.3	3.4	140
66	1.50	1.10	51.7	60.2	61.2	76.3	195.6	30.1	16
69	0.39	0.36	7.2	60.2	141.8	186.0	60.6	42.0	18
70	0.87	1.30	22.5	51.5	102.2	130.7	123	27.6	16
71	0.87	1.30	22.3	35.6	-	179.7	-	39.3	16
72	0.49	0.75	10.5	32.0	78.8	115.8	136	16.3	17
73	0.43	0.42	8.3	43.7	94.8	112.2	-	32.8	17
74	0.73	1.10	17.9	38.9	106.9	162.7	119.4	13.3	29
75	1.50	2.20	50.0	53.0	130.4	204.5	108	39.5	18
77	0.77	1.20	18.6	17.8	-	76.7	-	19.3	18
78	0.85	0.65	22.0	16.3	40.9	55.4	128.8	7.5	15
79	0.90	1.40	24.4	71.7	222.7	291.3	117	85.9	15
80	1.10	1.60	30.8	52.0	158.7	272.5	73	30.8	19
81	0.90	0.70	24.1	16.3	47.5	85.8	93.6	9.7	15
82	0.66	1.00	15.6	31.0	83.5	116.3	93.4	15.6	15
83	1.60	2.00	56.8	46.7	127.8	201.1	100.7	186.2	7
84	0.49	0.74	10.6	24.3	58.8	103.3	-	13.3	18
85	0.69	1.05	16.4	36.4	87.8	139.7	-	21.0	17
86	0.54	0.35	11.5	-	-	30.9	-	67.0	6
87	0.63	0.95	14.4	38.5	89.6	169.5	166.1	34.5	16
88	0.86	1.30	23.2	49.8	119.5	170.4	89.5	32.4	15
89	1.00	1.50	27.5	36.8	90.1	140.5	72.8	29.3	14

Patient	SA (m^2)	Dose (mg)	Weight (kg)	AUC_{0-6h} (µg/L.h)	AUC_{0-24h} (µg/L.h)	$AUC_{0-\infty}$ (µg/L.h)	Cl-F (ml/min)	C_{max} (µg/L)	T_{max} (h)
90	0.65	1.00	16.0	39.0	-	59.1	96.8	28.8	18
91	0.65	0.50	14.5	26.9	-	53.6	154	15.8	18
92	1.73	2.50	63.2	53.2	144.8	220.1	161	138.6	7
93	0.97	1.45	26.9	52.4	140.1	208.5	93.4	149.8	5
94	1.30	2.00	40.0	40.2	198.7	301.4	71.3	122.0	15
95	0.81	1.20	20.5	-	186.8	219.5	177.5	127.8	5
96	1.60	2.00	56.0	37.6	100.8	156.9	143.3	29.4	15
97	0.46	0.27	9.0	26.9	62.8	100.2	-	64.7	6
98	1.50	2.00	52.9	29.9	75.0	102.2	-	40.2	10
99	0.74	1.10	18.3	52.8	-	213.0	144.2	69.9	20
100	0.78	1.15	19.4	58.8	129.1	191.0	83	125.2	6
101	0.82	0.62	21.5	21.2	-	36.5	-	7.6	25
102	1.05	1.60	30.1	62.0	133.4	201.7	-	161.8	6
103	0.93	0.70	25.4	57.1	-	135.7	125.2	85.6	17
104	1.30	2.00	39.7	-	-	117.2	116.6	130.2	5
105	2.10	2.00	87.2	19.1	50.5	105.0	224.3	14.8	20
106	0.71	1.15	18.3	78.4	173.1	329.1	240.2	99.0	15
108	1.29	1.00	41.0	35.2	90.5	115.6	112.7	31.1	18
109	0.77	0.86	19.1	-	-	82.7	86.1	193.2	5
110	1.10	1.40	30.3	-	-	77.4	51.9	16.5	17
111	0.56	0.55	11.6	19.8	42.7	83.6	100.3	26.8	15
112	0.49	0.31	10.1	60.7	121.3	144.7	58.2	44.8	15
113	1.50	2.00	50.4	33.5	-	239.5	86.6	29.4	18
114	0.49	0.50	9.8	14.3	40.0	203.5	260.8	18.8	10
115	0.56	0.85	12.1	40.1	97.9	151.6	91.1	22.2	15
116	0.68	1.00	15.8	47.2	119.1	200.8	233.9	19.3	22
117	1.40	1.05	47.6	46.2	117.9	150.1	165.7	19.2	16
118	0.61	0.90	14.3	40.5	107.6	167.6	120.6	27.4	15
119	0.87	1.30	23.0	80.9	-	104.1	127.1	30.2	27
120	1.60	2.00	54.0	30.6	83.6	170.0	-	14.7	18

Patient	SA (m^2)	Dose (mg)	Weight (kg)	AUC_{0-6h} (µg/L.h)	AUC_{0-24h} (µg/L.h)	$AUC_{0-\infty}$ (µg/L.h)	Cl-F (ml/min)	C_{max} (µg/L)	T_{max} (h)
121	0.62	0.90	14.1	39.6	-	206.1	-	37.4	27
122	0.77	0.85	18.9	31.9	84.8	140.7	80.1	19.6	22
123	0.49	0.30	10.5	-	-	63.5	-	53.2	15
124	0.71	0.85	17.0	-	-	64.0	115.9	92.8	10
126	1.40	1.05	45.1	14.0	37.8	78.0	128.4	8.2	19
127	0.77	1.15	20.0	43.0	128.2	221.4	341.1	13.7	20
128	0.59	0.60	13.3	-	-	164.3	177.9	40.7	45
129	0.58	0.44	12.6	26.9	64.9	109.6	97.9	20.5	17
130	1.40	1.00	48.2	28.8	-	66.6	132.2	23.2	10
131	0.59	0.90	13.3	30.7	77.9	155.0	-	26.2	15
132	1.20	0.90	35.1	24.3	59.0	97.4	248.4	11.7	20
133	0.62	0.95	14.4	48.9	117.6	221.9	165.7	38.2	15
134	0.95	1.45	26.2	44.1	106.9	188.2	110.6	29.0	15
135	0.62	0.60	13.6	24.7	-	44.4	212.4	16.3	16
136	0.49	0.28	9.8	-	-	52.8	326.3	24.4	17
137	0.59	0.60	12.9	-	-	40.6	-	22.7	18
138	0.68	0.5	15.7	25.8	62.2	93.4	317.6	15.3	16
139	-	1.2	59.45	20.0	48.3	85.5	139.2	20.0	15
140	1.5	2	49.05	36.7	90.6	162.0	196.1	26.4	15
141	0.44	0.45	8.6	35.1	-	53.5	205.7	20.6	15
142	0.64	0.65	14.5	26.5	72.3	178.8	179.3	12.1	34

Table A.1. Pharmacokinetic parameters for all 108 patients with valid sample sets.

Allelic discrimination plot examples 1

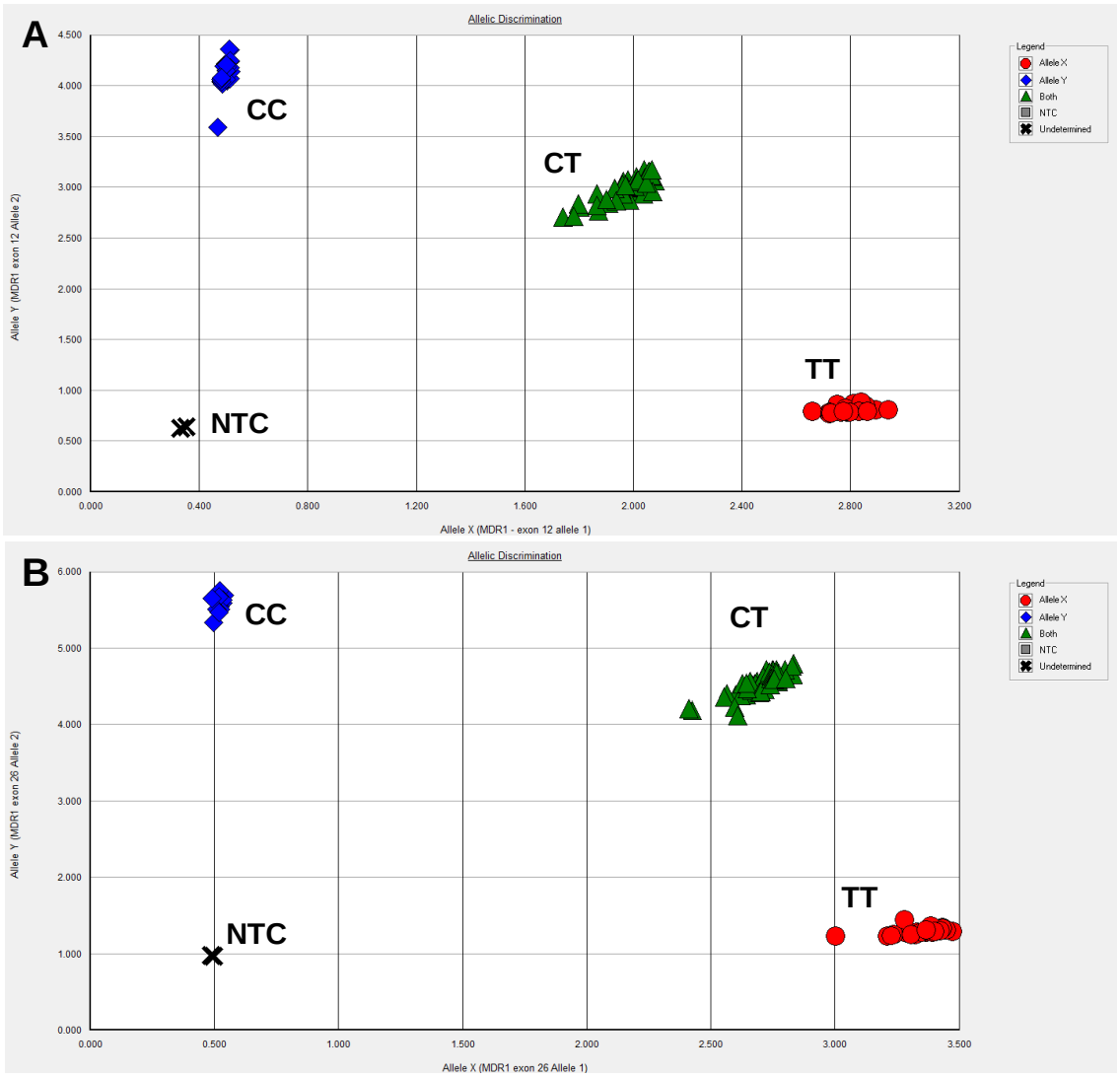

Figure A.1. Example allelic discrimination plots for ABCB1 SNPs (A) 1236C>T and (B) 3435C>T

Genotypes are labelled next to their corresponding allelic discrimination cluster.

Abbreviations- NTC; no template control.

Allelic discrimination plot examples 2

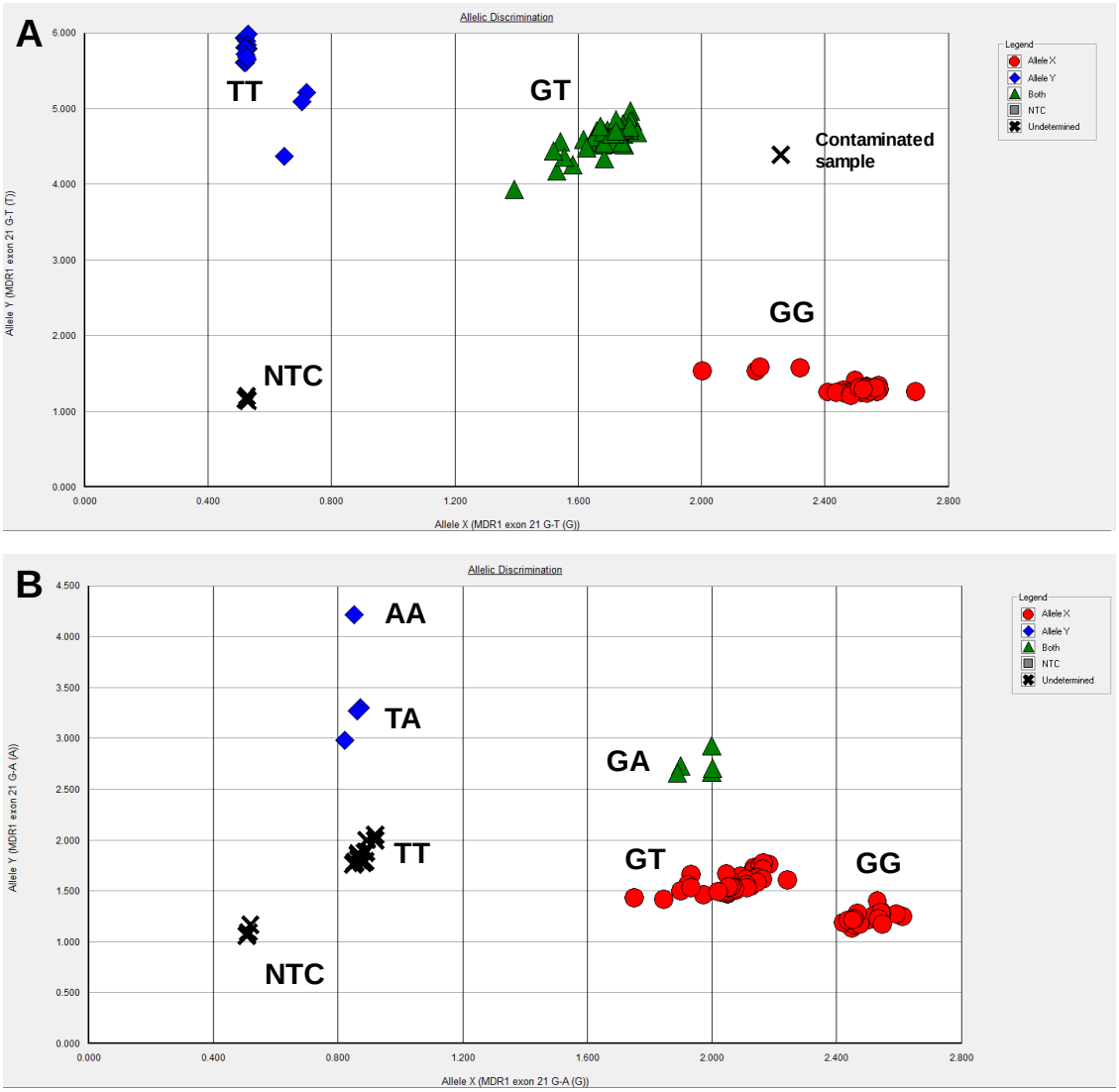

Figure A.2. Example allelic discrimination plots for ABCB1 SNP 2677G>T/A

Genotypes are labelled next to their corresponding allelic discrimination cluster. Both the (A) 2677G>T SNP assay and (B) 2677G>A SNP assay were used to determine patient genotype at position 2677 on ABCB1.

Abbreviations- NTC; no template control.

Conference abstracts

BACR annual meeting 2010, Edinburgh

Poster presentation

Investigating the clinical significance of pharmacogenetic factors affecting the transport of Actinomycin D in children with cancer

Christopher Hill, David Jamieson, Colin Brown, Alan V. Boddy and Gareth J. Veal

Actinomycin D (Act D) has been used routinely to treat certain types of cancer in both adults and children for over 30 years. It has been previously shown that treatment of patients with Act D is not optimal, with varied patient exposures being recorded. ABC transporters are expressed throughout the body. They have been shown to play a major role in the transport of anti-cancer agents such as doxorubicin, potentially affecting clinical response and toxicity.

Act D transport was investigated using MDCKII-WT, ABCB1, ABCG2 and ABCC2 over-expressing cell lines. Growth inhibition (GI) and intracellular accumulation of Act D was determined from a range of Act D concentrations (0-2µM). In addition, transport competition experiments were also carried out exploiting the fluorescent properties of known transporter substrates.

GI of Act D indicate a 40 –fold decrease in sensitivity in cells over-expressing ABCB1 (GI_{50}- 0.85µM) and a 3-fold decrease in cells over-expressing ABCC2 (GI_{50}- 0.06µM) compared to the control cell line (GI_{50}- 0.02µM). Higher intracellular levels of Act D were observed in the untransfected parental cell line than those over-expressing ABCB1, an effect that was abrogated by use of the ABCB1 inhibitor verapamil. In addition Act D has been shown to increase intracellular levels of the fluorescent ABCB1 substrate doxorubicin, by competition for ABCB1 efflux.

A pharmacokinetic study is also being carried out alongside the investigation into the transport of Act D to determine a possible link between SNPs in various transport proteins and clinical response and toxicity.

AACR annual meeting 2011, Orlando, Florida

Poster presentation

Investigating the relationship between pharmacogenetic variation in ABCB1 and ABCC2 tagSNPs and actinomycin D pharmacokinetics in children

Christopher Hill, Alan V. Boddy and Gareth J. Veal

Actinomycin D (Act D) has been used successfully to treat adult and childhood cancers for over 30 years. It continues to play a key role in the treatment of Wilms tumour patients, with 5-year survival rates >80% currently observed. While treatment is generally well tolerated, veno-occlusive disease (VOD) is a potentially life-threatening side effect observed in 1.7-13.5% of patients. Dose-intensity of Act D treatment has been highlighted as a possible risk-factor for VOD in paediatric patient populations.

ATP binding cassette (ABC) transporters are expressed throughout the body to protect against exogenous compounds. In the liver and kidney, ABC transporters facilitate the removal of compounds via biliary or renal excretion and have previously been shown to play a major role in the transport of anti-cancer agents such as doxorubicin and irinotecan. Common single nucleotide polymorphisms (SNPs) for these transporters have been shown to alter clinical exposure to various anti-cancer agents. We have therefore carried out translational and clinical studies focusing on Act D pharmacokinetics in Wilms tumour patients and pharmacogenetic variation in key ABC transporters.

Initial experiments were carried out to determine the potential importance of ABC transporter expression on the in vitro activity and transport of Act D, using MDCKII-WT alongside ABCB1, ABCG2 and ABCC2 over-expressing cell lines. Growth inhibition (GI) and intracellular accumulation experiments were carried out over a range of Act D concentrations (0-10μM). Pharmacokinetic analysis of samples obtained from patients receiving Act D was carried out using a validated LC/MS method and blood samples were obtained from all patients for pharmacogenetic analysis. Finally, the International HapMap database was used to determine tagSNPs for ABCB1 and ABCC2 with minor allele frequencies of greater than 0.1 in a Caucasian population, allowing the link

between ABCB1 and ABCC2 haplotypes and Act D exposure to be investigated.

GI experiments indicated a 40–fold decrease in Act D sensitivity in cells over-expressing ABCB1 (GI_{50}: 0.85µM) and a 3-fold decrease in cells over-expressing ABCC2 (GI_{50}: 0.06µM) compared to the control cell line (GI_{50}: 0.02µM). Higher intracellular levels of Act D (normalized per mg protein) were observed in the WT parental cell line (650nM) as compared to those over-expressing ABCB1 (215nM), following a 6h incubation with 2µM Act D. This effect was abrogated by use of the ABCB1 inhibitor verapamil. Pharmacokinetic analysis of samples obtained from 56 patients indicated a large inter-patient variability in Act D exposure ($AUC_{0\text{-}24h}$ range: 1.6 - 11.4 µg/ml.min). Ongoing investigations into pharmacogenetic variation in ABCB1 and ABCC2 tagSNPs in these patients provide novel preliminary data relating to links between ABCB1 and ABCC2 haplotypes and Act D exposure in children with cancer.

AACR annual meeting 2012, Chicago, Illinois

Poster presentation

Pharmacokinetics and pharmacogenetics of Actinomycin D in an animal model and paediatric patient population

Christopher Hill, Alan V. Boddy and Gareth J. Veal

ATP binding cassette (ABC) transporters, such as ABCB1 (MDR1/P-gp), are expressed throughout the body to protect against exogenous toxins. ABC transporters control the removal of many anti-cancer drugs from the body via biliary or renal excretion, such as doxorubicin and etoposide. Actinomycin D (Act D) has been used successfully to treat adult and childhood cancers for over 30 years. It continues to play a key role in the treatment of Wilms tumour patients, with 5-year survival rates >80% currently observed. Treatment is generally well tolerated, however, it has been previously demonstrated in a small paediatric population that Act D exposure in patients is associated with a large degree of inter-patient variability. Common single nucleotide polymorphisms (SNPs) in ABC transporters have been previously shown to alter clinical exposure to various anti-cancer agents. We have therefore carried out translational and clinical studies to investigate the transport of Act D *in vitro* and *in vivo* using well established cell line and animal models.

Growth inhibition (GI) and intracellular accumulation studies, over a range of Act D (0-10µM), were initially carried out *in vitro* to determine the role of ABC transporter expression in MDCKII-WT, ABCB1, ABCC1, ABCC2 and ABCG2 cell lines. To assess the importance of ABCB1 and ABCC2 transporter expression *in vivo*, Act D pharmacokinetics and exposure were determined in Abcb1a/1b and Abcc2 knockout mice over 6 hours. In addition, liver, kidney and brain tissue samples were also taken from mice treated with Act D to assess tissue accumulation. Further pharmacokinetic sampling has also been carried out from patients receiving Act D as part of their standard treatment, with analysis using a validated LC/MS method and blood samples were obtained from all patients for pharmacogenetic analysis.

When treated with a concentration range of Act D, a 59-fold decrease in Act D sensitivity in ABCB1 expressing cells (GI_{50}: 745nM), a 2-fold decrease in ABCC1 (GI_{50} : 25.7nM) and a 3-fold decrease (GI_{50}: 40.4nM) in ABCC2 compared to the parental cell line (GI_{50}: 12.7nM). Intracellular accumulation of Act D was lower in ABCB1 over-expressing cell lines compared to the parental, following incubation with Act D for 6 hours. This effect was abrogated by use of the ABCB1 inhibitor verapamil. In vivo pharmacokinetic analysis revealed a 1.64-fold increase in $AUC_{0\text{-}6hours}$ in mice with no Abcb1a/1b expression and a 0.78-fold reduction in $Abcc2^{-/-}$ mice compared to the wild type. Pharmacokinetic analysis of samples obtained from 58 patients indicated a large inter-patient variability in Act D exposure ($AUC_{0\text{-}24h}$ range: 1.6 - 11.4 µg/ml.min). Ongoing investigations into pharmacogenetic variation in ABCB1 and ABCC2 tagSNPs in these patients provide novel preliminary data relating to links between ABCB1 haplotypes and Act D exposure in children with cancer.

Publications

Hill, C. R., Jamieson, D., Thomas, H. D., Brown, C. D. A., Boddy, A. V., and Veal, G. J. 2012. Characterisation of the roles of ABCB1, ABCC1, ABCC2 and ABCG2 in the transport and pharmacokinetics of Actinomycin D in vitro and in vivo. *Biochemical Pharmacology*, E-pub, ahead of print. DOI: http://dx.doi.org/10.1016/j.bcp.2012.10.004