The study of acquired drug resistance in cancer cell lines

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Declaration

No part of this thesis has been submitted in support of an application for any other degree or other qualification of the University of Kent, or any other University or Institution of learning.

Thomas Jackson-Soutter
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Contents

Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine Triphosphate
CABA	Cabazitaxel
CDK	Cyclin Dependent Kinase
CIS	Cisplatin
DMSO	Dimethylsulphoxide
DOCE	Docetaxel
EPOB	Epothilone b
FBS	Foetal Bovine Serum
IC50	Inhibitory Concentration 50
IMDM	Iscove's Modified Dulbecco's Medium
kDa	Kilo Daltons
Mo Ab	Monoclonal antibody
MW	Molecular Weight
NB	Neuroblastoma
nM	Nano Molar
PBS	Phosphate Buffered Saline
PCL	Paclitaxel
P-gp	P-glycoprotein
PI3K	Phosphoinositide 3-kinase
SDS	Sodium Dodecyl Sulfate
TAE	Tris-acetic-Acid-EDTA
VCR	Vincristine
µl	µLitre
µM	µMolar

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Abstract

Cytotoxic drugs are frequently used in the treatment of cancer. However whilst most patients initially respond well to cytotoxic therapies, acquired resistance often develops. In this project we aim to improve understanding of the mechanisms of acquired drug resistance and contribute to the development of a novel assay for determining the potential of different cancer drugs to cause acquired resistance.

UKF-NB-3 cell lines, initially sensitive to docetaxel, paclitaxel, cabazitaxel and epothilone-b, were cultivated in the presence of each drug. Once resistance was conferred in the surviving sub-lines, the concentration of drug was increased to determine the extent of resistance. The sub-lines of UKF-NB-3 demonstrated resistance to Docetaxel and Paclitaxel. However, cabazitaxel and epothilone-b sub-lines were unable to adapt to the respective drugs. This demonstrates that resistance is more easily acquired to docetaxel and paclitaxel than cabazitaxel and epothilone b. To investigate the resistance mechanism responsible, cross resistance against a panel of selected cytotoxic drugs was determined. The surviving cell lines were resistant to additional drugs with high affinity to ABCB1 transporters. The expression of the drug efflux proteins (ABCB1) and tubulin was determined using confocal microscopy and found to be overexpressed in the resistant sub-lines.

This project will lead to a standard protocol for determining the potential of anti-cancer drugs to cause acquired resistance in cancer cells. In this study we have acquired evidence that cultivation of UKF-NB-3 cell line in higher concentrations of docetaxel can result in increased degrees of resistance, possibly depending on the heterogeneity of the cell population. We have additionally characterised the mechanism of resistance through investigation of ATP-binding cassette (ABC) transporters and tubulin expression. With

further work, this project will lead to improved understanding of resistance to tubulin binding agents, the cellular pathways responsible and ultimately improved therapeutic strategies and for drug adaptation of cell lines.

Chapter 1

Introduction

1.1. Acquired resistance to cancer therapy

357,000 cancer diagnoses were made during 2014. In the same year there were 163,000 cancer related deaths, accounting for 29% of all deaths in the UK (Cancer Research UK 2017) Treatment is chosen depending on the stage and type of cancer, however the majority of successful treatment results from surgery and tumour resection. This will often be in combination with radiotherapy, chemotherapy or targeted therapy; depending on the stage and type of tumour. The variation in the use of treatments between different stages of cancer has been described in a study by Miller et al. This showed that early stage tumours (stage 1 and 2) are most preferably treated with surgery alone. In some cases this is followed by radiotherapy. Late stage tumours (stage 3 and 4) are most frequently treated with surgery and a combination of radiotherapy and chemotherapy. The increased risk of metastasis in late stage tumours and the inaccessibility of many tumours mean surgery alone is not suitable. As a result systemic courses of chemotherapy and targeted therapies are used to treat inoperable and secondary metastatic tumours (Miller et al. 2016). Acquired resistance to pharmacological cancer therapies accounts for 90% of therapeutic failure in patients with metastatic disease and is the leading cause of therapeutic failure in cancer treatment (Longley & Johnston 2005). Metastatic disease is most frequently treated with systemic chemotherapy or targeted therapies, and it is here that drug resistance has the greatest impact upon patient outcome.

Resistance to anti-cancer drugs may be intrinsic or acquired. Intrinsic resistance occurs when the tumour does not respond to the drugs administered from the start of treatment. These tumours have the phenotype required to overcome the action of the drug before treatment begins. In cases of acquired resistance the tumour initially responds to treatment resulting in tumour regression however tumours often stop responding to the administered drug, relapse occurs and tumour progression continues.

1.2. Neuroblastoma

Neuroblastoma occurs almost exclusively in children under 5 years old. Primary tumourgenesis originates from undifferentiated precursor neural crest cells usually within the abdomen or adrenal glands but tumours can occur anywhere in the sympathetic nervous system (Maris 2010) . Metastatic sites include bone marrow, skin, liver and in rare cases the spinal cord. Around 100 children are diagnosed every year within the UK, accounting for 15% of paediatric oncology deaths (Mullassery et al. 2014)(Park et al. 2008). Success of treatment varies with age, with higher success rates in younger children. 83% of children diagnosed under the age of 1 survive beyond five years compared to just 43% of children diagnosed between the ages of 1 and 4 years old (Cancer Research UK, 2017)(ChildrenwithCancer.org).

The age of diagnosis and stage of disease are important predictors of treatment outcome. Diagnosis of neuroblastoma during stage 1 and 2 in patients under 1 year of age is often successfully treated with surgery with no further treatment required (Alvarado et al. 2000). Additionally patients under 1 year of age with stage 3 and metastatic stage 4 disease have also had long term treatment success with tumour resection and systemic

chemotherapy frequently providing positive outcomes (Bowman et al. 1997). By comparison, this success has not been replicated in patients over 1 year of age, with older patients showing substantially higher mortality rates (Matthay et al. 1999). This trend may partially be accounted for by the occurrence of unresectable tumours due to infiltration into local organs as a result of later diagnosis (Maris 2010). This trend is still stark in current outcomes and little progress has been made in reducing the mortality rate in children over 1 year of age.

The pathogenesis of neuroblastoma is relatively well established and can progress in multiple different ways. It is described by Mullassery *et al.* as enigmatic due to the broad spectrum of clinical progression (Mullassery et al. 2014). Neuroblastoma may demonstrate rapid progression in some cases, and in other cases spontaneous regression of late stage tumours.

1.3. MYCN amplification

Predictors of treatment outcome have been identified. It has been well established for some time that MYCN amplification is associated with poor prognosis and is seen frequently in stage 3 and 4 disease types (Brodeur et al. 1984)(Puissant et al. 2013). The encoded protein is around 50 kDa in size and upregulates transcription through binding with a basic helix-loop-helix (bHLH) domain within the nucleus. A study by Schwabb *et al.* first showed that MYCN induced cell proliferation and was an important regulator of the cell cycle, cellular differentiation and apoptosis (Schwab et al. 1985)(Gustafson & Weiss 2010). In normal cells the expression of N-MYC is well regulated. A Study by Sjostrom *et* al. found that phosphorylation by cyclin dependent kinase-1 (CDK1) allows its entry to the

nucleus where the bHLH is able to bind with MYCN protein within the nucleus. The bound proteins then bind to Max, a helix loop helix transcription factor, enabling transcription of target genes (Sjostrom et al. 2005).

In addition to MYCN amplification being associated with poor prognosis, it has also been identified as a therapeutic marker. A study conducted by Dolman *et al.* found neuroblastoma cell lines possessing MYCN amplification responded better to a cyclin-dependant kinase inhibitor AT7519. This study aimed to evaluate cyclin-dependent kinases as therapeutic targets against neuroblastoma. The efficacy of AT7519 was determined against a number of neuroblastoma cell lines with and without MYCN amplification. In the study, it was found that MYCN amplified cell lines were significantly more sensitive to AT7519 than cell lines without MYCN amplification, showing that inhibition of cyclin dependant kinases more often results in apoptosis in MYCN amplified neuroblastoma. This was confirmed in the same publication through investigation of PARP cleavage after treatment with AT7519. PARP cleavage was increased in MYCN amplified neuroblastoma cell lines treated with AT7519. PARP cleavage was increased further through treatment with increasing concentrations of AT7517. This was not seen in neuroblastoma cell lines without MYCN amplification strongly suggesting that CDK inhibition upregulates apoptosis in MYCN amplified neuroblastoma cell lines (Dolman et al. 2015). The association of CDK inhibition and apoptosis in MYCN amplified neuroblastoma was also described by Molenaar *et al.* Here mRNA expression was analysed across 88 neuroblastoma tumours, identifying CDK2 overexpression as a poor prognostic marker of neuroblastoma. Using transient siRNA transfection, CDK2 gene was silenced in amplified and non-amplified MYCN neuroblastoma cell lines. This resulted in strong induction of apoptosis in MYCN amplified neuroblastoma cell lines. This effect was reversed when MYCN was also silenced in addition to CDK2.

From these results it can be concluded that apoptosis through CDK inhibition is dependent on MYCN over expression. Therefore CDK inhibitors will not induce apoptosis in cells with normal MYCN expression, providing an attractive therapeutic target considering the poor therapeutic outcomes seen with MYCN amplification (Molenaar et al. 2009)(Brodeur et al. 1984).(Puissant et al. 2013).

MYCN proto-onco gene has been extensively studied across different cancers as a well-established transcription factor. MYCN has roles in cell proliferation and apoptosis however little is known about its activation in neuroblastoma (Gustafson & Weiss 2010). PI3K is partly responsible for its regulation. A study by Kumar *et al*. in 2006 found that PI3K stabilizes MYCN and enables its entry through the nuclear membrane, enabling transcription of downstream target genes (Kumar et al. 2006). MYCN also has a non-transcriptional roll in cell cycle control (Gustafson & Weiss 2010). A study by Staller *et al.* demonstrated that MYC-Max dimers are able to inhibit other transcription factors for example Miz-1. Miz-1 transcription factor is able to promote transcription of proteins associated with cell cycle arrest. This downregulation of cell cycle arrest associated proteins contributes to loss of proliferative control and oncogenesis (Staller et al. 2001). This is further evidenced by the epidemiological analysis of neuroblastoma tumours performed by Brodeur *et al.* (Brodeur et al. 1984).

MYCN amplification is an important factor to consider when using neuroblastoma as an in vitro model of disease as we have done in our own study. The transcriptional and non-transcriptional role of MYCN in cell cycle regulation is still not fully understood in neuroblastoma. As such the amplification of MYCN which is present in the UKF-NB-3 cell line used in our own study may have unanticipated effects on the cells.

This is an example of how multiple different mechanisms play a role in cell proliferation and the complex intrinsic mutations observed in cancer cell lines.

1.4. Tubulin binding agents

Tubulin is an attractive target for novel anti-cancer drugs as it plays a vital role in cellular proliferation. Tubulin consists of α and β subunits and is arranged into microtubules for intracellular trafficking of proteins and organelles through the cytoskeleton. Additionally it is arranged into mitotic spindles for mitosis (Parker et al. 2014). The dynamic nature of tubulin subunits is key in the role of microtubules in intracellular trafficking as they are frequently required to lengthen and shorten by binding and dissociating of soluble tubulin heterodimers.

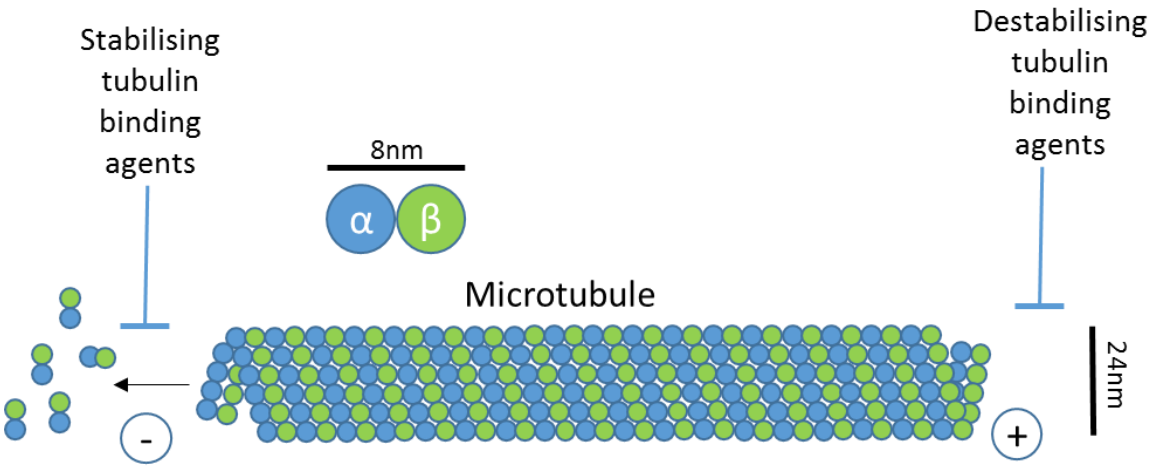


Figure 1.1, Schematic diagram of microtubule structure, assembled from polymerisation of α-β-tubulin heterodimers. Stabilising tubulin binding agent such as docetaxel, paclitaxel, cabazitaxel and epothilone b inhibit microtubule disassembly. Destabilising tubulin binding agents such as vincristine inhibit microtubule assembly.

Considering the importance of the processes made possible by the dynamic nature of microtubules, inhibiting this process in rapidly proliferating cells is an obvious target for anti-cancer drugs.

A study in 1967 by Perdue *et al.* screened extracts from 15,000 species of plants, aiming to discover novel anti-cancer therapies. (Perdue *et al.* 1969). Taxol (now known as paclitaxel) was identified from extracts from the bark of the pacific yew tree. Shortly after it was proposed by Schiff *et* al. that paclitaxel was able to promote microtubule formation. This study using HeLa cell models, also found that cell cycle arrest occurred during mitosis (SCHIFF et al. 1979). McGuire *et al*. conducted a study into its efficacy against advanced ovarian cancer, finding that twelve of the forty patients evaluated in the trial responded to paclitaxel with effects lasting between 3 and 18 months (McGuire et al. 1989).

A more recent study by Hornick *et al*. aimed to elucidate the exact mechanisms of paclitaxel action in *vivo*. Here the assembly of tubulin was examined using GFP expressing α-tubulin and live cell imaging. It was observed that treatment of human cancer cells with paclitaxel results in the release of mitotic spindles away from the centrosome during mitosis (Hornick et al. 2008). Another study by Chen *et al*. also found that treatment with low doses of paclitaxel resulted in multi-polar microtubules and microtubule dysregulation (Chen & Horwitz 2002). It is not however clear that these effects have clinical relevance to the same extent as microtubule stabilisation.

Microtubule stabilisation is the most well established mechanism of paclitaxel action with proven clinical relevance. A study by Nogales *et al*. first characterised the binding of paclitaxel. Using electron crystallography, they observed high density areas found at every two tubulin subunits.

It was therefore concluded that each tubulin heterodimer possessed a binding site for paclitaxel on the β-tubulin subunit, causing sections of the microtubule to become more ordered and stable, reducing the dynamics of the microtubule (Nogales et al. 1995). This increased polymerisation and stabilisation of microtubules leads to cell cycle arrest during mitosis, followed by apoptosis, with greater cytotoxic effect on rapidly proliferating cells. This mechanism of action has been supported almost universally with recent studies inducing tubulin destabilisation resulting in paclitaxel resistance. A study by Ahmed *et al*. found that paclitaxel induced microtubule stabilisation was dependent on extracellular matrix protein TGFBI (transforming growth factor β induced) protein. Underexpression of TGFBI was found to be sufficient to cause paclitaxel resistance through destabilisation of microtubules. It was also observed in the ovarian cancer cell line SKOV-3TR, with paclitaxel resistant phenotype, that TGFBI was underexpressed compared to a paclitaxel sensitive counterpart. Within the same study it was also found that paclitaxel resistant cell lines could be resensitised to paclitaxel using TGFBI protein, causing paclitaxel induced microtubule polymerisation (Ahmed et al. 2007)

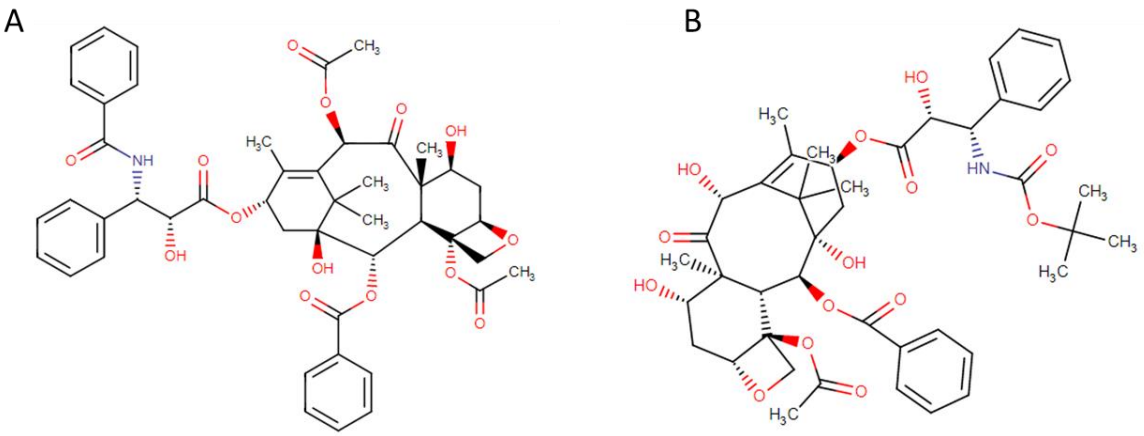


Figure 1.2, Structure of paclitaxel (**A**) and docetaxel (**B**). Structures sourced from (www.drugbank.ca)

Like paclitaxel, docetaxel (the second taxoid derivative) was derived from the European yew tree and also promotes microtubule stabilisation through the same mechanisms as paclitaxel (Lavelle et al. 1995). Docetaxel was found to be 2.5 fold more potent than paclitaxel in a study by Ringel *et al*. (Ringel & Horwitz 1991). Lavelle *et al*. proposed that docetaxel is more effectively retained intracellularly than paclitaxel. This may evidence the 2.5 fold increase in toxicity which was observed by Ringel *et al*. (Lavelle et al. 1995) (Ringel & Horwitz 1991). Additionally, Docetaxel was found to have higher affinity to the shared binding site with paclitaxel (Díaz et al. 1998).

Cabazitaxel is a relatively novel member of the taxane family of anti-cancer agents, possessing lower binding affinity to P-glycoprotein (ATP-binding cassette transporter B1, referred to hereafter as ABCB1) than docetaxel and paclitaxel. Like other taxanes, cabazitaxel binds to tubulin, stabilising and inhibiting the disassembly of the microtubules, leading to cell cycle arrest during mitosis and subsequent apoptosis (Jordan & Wilson 2004) (Tsao et al. 2011). Cabazitaxel was found to be more potent than docetaxel against a range of human cancer cell lines which have overexpression of ABCB1 (Mita et al. 2009).

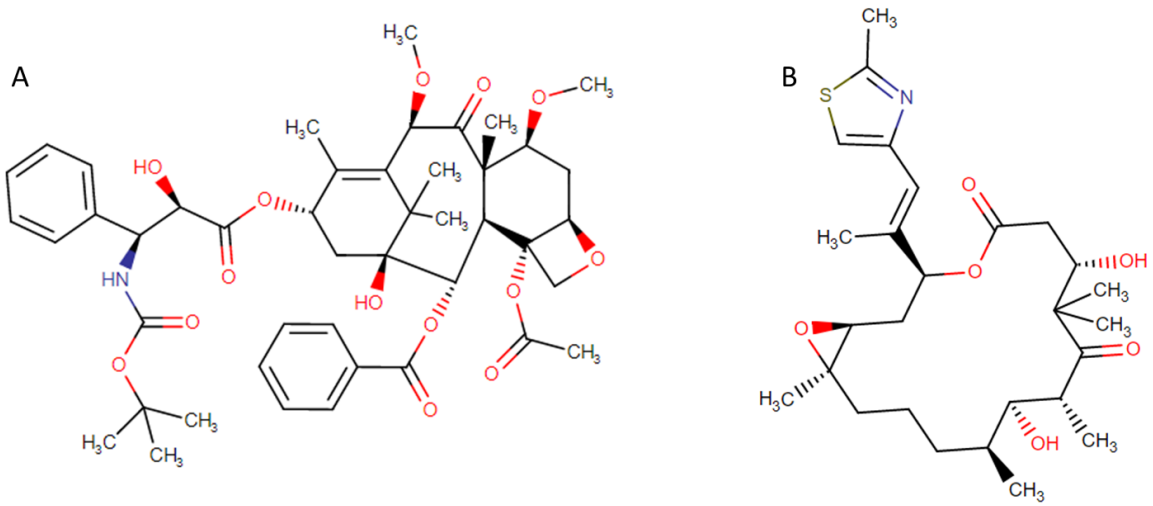


Figure 1.3, Structure of cabazitaxel (**A**) and epothilone b (**B**). Structures sourced from (www.drugbank.ca)

Further evidence of the low affinity to ABCB1 was found by Bart *et al*. Here it was observed that cabazitaxel was able to maintain higher concentrations beyond the blood-brain barrier. ABCB1 is a highly active transporter in the blood brain barrier. The low affinity of cabazitaxel for ABCB1 likely means it is not effluxed across the barrier as readily as other drugs and substrates of ABCB1, leading to higher concentrations of cabazitaxel in the brain. This provides further applications for its use in the treatment of brain metasteses (Bart et al. 2000). This was investigated further by Cisternino *et al*. through observing radiolabelled cabazitaxel in mouse models. It was found that cabazitaxel showed superior penetration of the blood brain barrier. However Cisternino *et al*. hypothesised that after penetration of the blood brain barrier ABCB1 transporters became saturated with cabazitaxel, therefore preventing any further efflux from the neural tissue. To conclude this, the concentrations of cabazitaxel were measured on in neural tissue and in the serum. They found that serum concentrations did not increase once the saturating dose had been reached. Suggesting that cabazitaxel may have very low affinity for ABCB1 efflux (Cisternino et al. 2003). This hypothesis has not however been supported by other published work, however the data does suggest that there is minimal binding affinity between cabazitaxel and ABCB1.

Despite binding tubulin and stabilising microtubules in the same manner as taxanes, Epothilone B is not a member of the taxane family of drugs and possess distinct mechanisms of action. In a study by Nettles *et al*. it was shown through electron crystallography that epothilones have a distinct binding site from that of taxanes (Nettles et al. 2004).

Epothilone b was also found to have a greater effect on tubulin polymerisation than taxanes (Kowalski et al. 1997). The same study also found that epothilone b (like cabazitaxel) had low binding affinity to ABCB1. This was supported by the findings of Altmann *et al*. who observed that epothilone b showed cytotoxicity in cell lines with ABCB1 mediated paclitaxel resistant phenotypes. It was also observed that paclitaxel resistant sublines with ABCB1 overexpression were not cross-resistant to epothilone b. The paclitaxel resistance within these cell lines was completely reversed in the presence of ABCB1 inhibitors. (Altmann et al. 2000)

These finding show that epothilone b and cabazitaxel resistance is not mediated by ABCB1, however resistance to docetaxel and paclitaxel is highly dependent on its overexpression.

1.5. ABC transporters

ATP binding cassette (ABC) transporters are a superfamily of around 49 membrane transport proteins with broad substrate specificity responsible for mediating membrane transport of numerous substrates (Vasiliou et al. 2009). Consequently overexpression of ABC transporters are a major mechanism of anti-cancer multi-drug resistance (MDR) through increased efflux of anti-cancer drugs, thus decreasing the intracellular concentration and reducing the toxicity of drugs with affinity to one or more ABC transporters. ABC transporters are present in many healthy eukaryotic and prokaryotic cells and function to export toxic metabolites. The first ABC transporter to be identified as a mechanism of drug resistance was ABCB1 or P-glycoprotein. This was established as a drug efflux protein in a study by Juliano *et al*. which used Chinese hamster ovary (CHO) cells as a model to investigate causes of drug resistance.

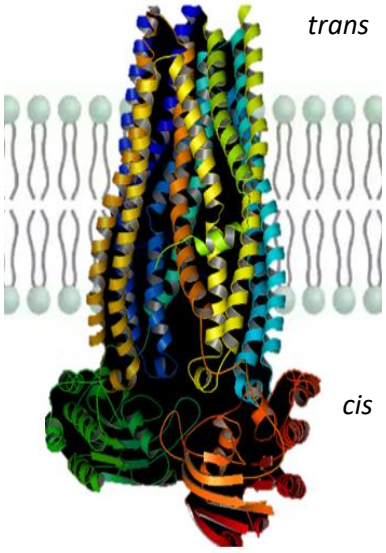


Figure 1.4, Model of ABCB1 structure, showing its position within the plasma membrane. Nucleotide binding domains are shown in green and orange on the *cis* side of the plasma membrane. Transmembrane domains are shown in blue and yellow. The helices within the transmembrane domains are substrate binding sites.

They found that cells possessing MDR phenotypes had additional membrane proteins when compared to the CHO cells which did not show MDR phenotypes. Expression of the additional membrane proteins correlated to the degree of drug resistance and ABCB1 (P-glycoprotein) was identified (Juliano & Ling 1976). The superfamily contains seven subgroups from ABCA through to ABCG, categorised through structural similarities. Whilst the ATP binding domains remain structurally similar across subgroups, there is a substantial degree of structural diversity seen in the transmembrane domains (Beek et al. 2014). Substrate transport is achieved through coupling the hydrolysis of the phosphate bond between the β and γ-phosphates of ATP, producing ADP. The energy released during this reaction is used to transport substrates across membranes (Poolman et al. 2005).

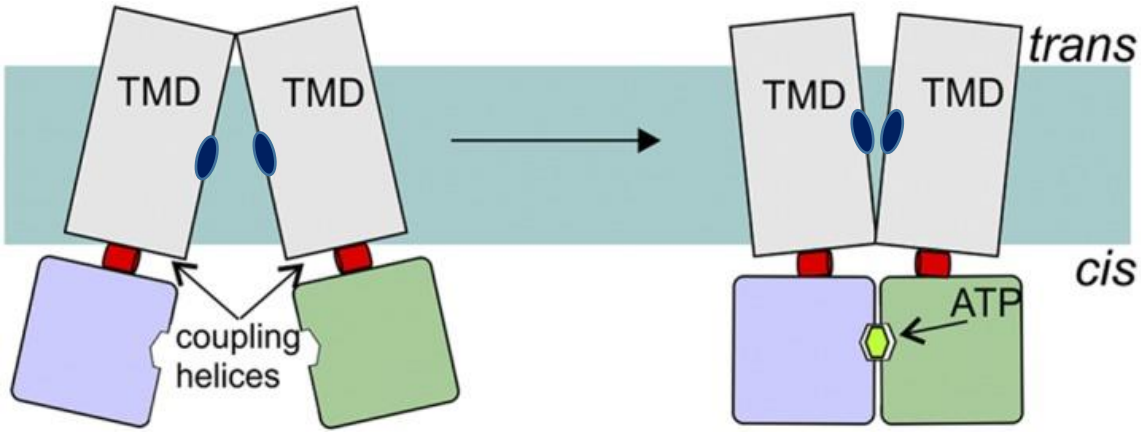


Figure 1.5, Schematic diagram of conformational change caused by ATP hydrolysis. Nucleotide binding domains are represented in pale blue and green, transmembrane domains are shown in grey. The conformational change in the nucleotide binding domains is transferred to the transmembrane domains through coupling helices shown in red. The substrate binding site is represented by dark blue ovals

Image adapted from (ter Beek et al. 2014)

ABCB1, encoded by MDR1 gene, possesses the broadest range of substrate specificity and hence is most associated with MDR.

Figure 1.4, shows the structure of ABCB1, possessing two intracellular nucleotide binding domains and six hydrophobic transmembrane domains, totalling 170kDa.

Substrate specificity is determined by the transmembrane domains. Substrates are able to bind to “substrate binding pockets” in the helices of the transmembrane domains. ATP hydrolysis at the nucleotide binding sites causes a conformational change opening the substrate binding site to the extracellular space and closing access to the Cis side of the cellular membrane. This process is demonstrated in **figure 1.5.** (ter Beek et al. 2014)

As substrate binding occurs within the helices of the transmembrane domains, and the sub-families of ABC transporters are categorised based on structural similarities, it follows that different sub-families are able to transport different substrates as a result of the structural differences seen in the transmembrane domains. Likewise, any structural similarities will result in specificity to the same substrate. Consequently multiple different drugs can be substrates of the same ABC transporter. Additionally, different ABC proteins possessing the same helical domains are capable of effluxing the same drug. It is this combination of structural similarities and differences across the superfamily of ABC transporters that contributes so substantially to MDR.

Table 1.6, shows the ABC transporters for which the drugs used in this project are substrates and the known mechanism of action for each drug.

Drug	Drug class and cellular targets	ABC transporter affinity
Docetaxel	Tubulin binding agent – Binds reversibly to beta-tubulin stabilising microtubules, inhibiting mitosis	ABCB1, ABCC10
Paclitaxel	Tubulin binding agent- Binds beta subunit of tubulin stabilising microtubules required for mitosis and cytoskeleton	ABCA3, ABCB1, ABCB4, ABCB11, ABCC1, ABCC2, ABCC10,
Cabazitaxel	Tubulin binding agent – stabilisation of microtubules	Non identified
Epothilone-B	Tubulin binding agent – stabilisation of microtubules	Non identified
Cisplatin	DNA damage – DNA alkylation	ABCA1, ABCA3, ABCC2, ABCC6
Vincristine	Tubulin binding agent – destabilisation of microtubules	ABCA3, ABCB1, ABCC1, ABCC2, ABCC3, ABCC10
Crizotinib	Tyrosine Kinase Receptor inhibitor	Non identified
Topotecan	DNA damage – Single strand breaks	ABCB1, ABCB5, ABCC2, ABCC4, ABCG2,

Table 1.1, showing the drugs used in this project, the main mechanism of action and the ABC transporters though which they are effluxed.

(Ween et al. 2015)(Wils et al. 1994)(Kathawala et al. 2015)

Chapter 2

Materials and methods

2.1. Materials and reagents

2.1.1. Drugs used for resistant cell culture and cross-resistance panel

Docetaxel, Paclitaxel and Epothilone-B were purchased from Cayman Chemical. Cabazitaxel was purchased from Selleckchem. All drugs were dissolved in Dimethyl Sulfoxide (DMSO) purchased from Sigama Aldrich and stored at -20^{o}C. Crizotinib was purchased from Sigma-Aldrich. Vincristine, Topotecan, Docetaxel, Paclitaxel and Epothilone-B were sourced from Cayman Chemical. These drugs were dissolved in Dimethyl Sulfoxide (DMSO) and stored at -20^{o}C. Cisplatin was purchased from Accord Healthcare, dissolved in 0.9% NaCl (saline) and stored at room temperature protected from light.

2.1.2. Materials and reagents for cell culture

All cell culture was undertaken in sterile conditions using a biosafety category 2 tissue culture hoods. Industrial methylated spirit (IMS) was used for sterilisation. All equipment was sterilised with IMS and autoclaved if suitable.

Cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM) purchased from Life technologies and supplemented with 10% Foetal Bovine Serum purchased from Sigma Aldrich and 1g/100ml Penicillin-Streptomycin purchased from Life Technologies.

PBS was made using 1 tablet per 100ml dH_2O. Tablets were purchased from Oxoid.

Trypsin solution was purchased from Sigma Aldrich and contained 0.12% trypsin dissolved in 0.05% EDTA solution.

Disposable equipment such as 1ml, 2ml, 5ml, 10ml, 25ml and 50ml stripettes and 15ml and 50ml falcon tubes were acquired from Sarstedt. 25cm^3 and 75cm^3 vented tissue culture flasks were also purchased from Sarstedt. 96 well and 24 well tissue culture plates were purchased from Greiner.

2.1.3. Materials and reagents used in cell viability assays

MTT (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) was purchased from Serva Electrophoresis and made up to 2% solution using dH_2O. MTT solution was sterile filtered, protected from light exposure and stored at 4^{o}C.

20% sodium dodecyl sulphate (SDS) solution was used to solubilise cells after incubation. SDS solution was made using 40% dH_2O, 40% dimethylformamide (DMF) and 20% SDS purchased from Fisher Scientific and the pH adjusted to pH 4. SDS was stored at room temperature and warmed to 37^{o}C in the water bath before use.

0.1% Trypan Blue was used for viable cell counts and sourced from Sigma Aldrich and stored at room temperature.

10% Trichloroacetic acid (TCA) and 0.4% Sulfurhodamine B in 1% acetic acid was purchased from Sigma Aldrich. 10mM Tris-buffer was sourced from Fisher Scientific.

2.1.4. Materials and reagents used for fluorescent microscopy

Anti-P Glycoprotein rabbit monoclonal antibody was purchased from Abcam. Anti-beta tubulin mouse monoclonal antibody was also purchased from Abcam. Poly-l-lysine, paraformaldehyde, tween buffer and Triton-X was all sourced from Sigma Aldrich.

The Zeiss LSM 880 confocal microscope with airyscan was used to capture fluorescent images. ALEXA – anti-mouse 647 and ALEXA – anti-rabbit 488 fluorophores were used.

2.2. Methods

2.2.1. Cell culture

The MYCN-amplified UKF-NB-3 cell line was established from a bone marrow metastasis of stage 4 neuroblastoma patient. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) purchased from Life technologies and supplemented with 10% Foetal Bovine Serum purchased from Sigma Aldrich and 1g/100ml Penicillin-Streptomycin purchased from Life Technologies. They were incubated at 37^{o}C in the presence of 5% CO_2.

Cells lines were passaged in sterile conditions using a biosafety category 2 tissue culture hoods. Industrial methylated spirit (IMS) was used for sterilisation. Old media was first removed from the tissue culture flask and disposed of. The cells, still adherent to the back of the flask were gently washed with 2mls PBS for T25 or 6ml for T75. The PBS was then aspirated and 1 ml 0.12% trypsin dissolved in 0.05% EDTA solution or 3ml for T75 and incubated at 37^{o}C and 5% CO_2 for five minutes or until the cells have visibly detached from the back of the tissue culture flask. The detached cells and trypsin is then resuspended with IMDM with 10% FBS Aldrich and 1g/100ml Penicillin-Streptomycin to the final volume of the tissue culture flask

2.2.2. Morphological analysis

Photographs were taken to observe the morphology of the cell lines using an Olympus CKX53 light microscope and camera. Confocal Microscopy was used for the capture of fluorescent images. All parameters used on the confocal microscope were kept the same for each sample.

2.2.3. Cell viability assays

2.2.3.1. MTT cell viability / drug sensitivity assay

Cell viability was investigated using MTT (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) reduction assay. Cells were trypsinised and seeded into a 96 well plate at a density of 5000 cells per well and incubated in the presence of serial dilutions of drug for 120 hours at 37^{o}C at 5% CO_2. Positive control wells containing only media and cells (to determine uninhibited growth) and negative control wells containing just media (to determine background absorbance from media) were also included. The cells were then incubated for four hours in the presence of 2% MTT reagent (added in the tissue culture hood), which is reduced in the mitochondria to formazan. This is then solubilised overnight with 20% acidified sodium dodecyl sulphate (SDS) (pH4) and the optical density read at 600nm. The process has been previously described (Michaelis et al. 2011).

2.2.3.2. SRB assay

SRB (Sulfurhodamine B) assays were used as an alternative assay for cell viability. Cells were trypsinised and seeded at 5000 cells per well in a 96 well plate with serial dilutions of drug and incubated at 37^{o}C and 5% CO_2 for 120 hours in serial drug dilutions. Positive control wells containing only media and cells (to determine uninhibited growth) and negative

control wells containing just media (to determine background absorbance from media) were also included. Cells were then fixed using 70µl per well of 10% TCA (purchased from Sigma Aldrich) for 30 minutes and washed with distilled water before staining with 70µl SRB dye for a further 30 minutes. Plates are then washed with 1% acetic acid to remove excess dye and any non-adherent cells still present. The plates are dried and resuspended in 100µL of 10mMol Tris buffer and the optical density read at 490nM. The protocol has previously been described (Walton et al. 2012)

2.2.4. Immunostaining for fluorescent microscopy

Anti-P Glycoprotein rabbit monoclonal antibody was purchased from Abcam. Anti-beta tubulin mouse monoclonal antibody was also purchased from Abcam.

Sterile cover slips were placed into 24 well plates and treated with 0.01% poly-l-lysine (purchased from Sigma-Aldrich). 0.5ml poly-l-lysine was added to each well and incubated at room temperature for 5 minutes. The poly-l-lysine was aspirated and the cover slips washed three times with 1 ml sterile milli-q water suitable for tissue culture. The cover slips were then left to dry for 2 hours in the tissue culture hood.

Once dry, the plates were seeded with $3x10^5$ cells/ml. Growth media was aspirated from the tissue culture flask, the cells were washed with PBS and detached by incubating at 37^oC in 5% CO_2 for 5 minutes in 1ml 0.05% trypsin 0.12% EDTA. Once detached from the tissue culture flask, cells were resuspended in 9ml IMDM supplemented with 10% FBS and 1g/100ml penicillin-streptomycin. Plates were incubated at 37^oC in 5% CO_2 for 24 hours.

The confluency of the coverslips within the plates was checked under the microscope before continuing when confluency reached 40-50%.

The media was aspirated from the wells and coverslips washed with 1ml PBS. The PBS is then aspirated and the cells fixed using 4% paraformaldehyde at pH8 (using NaOH acquired from Thermo Fisher) for 20 minutes before being washed three times with PBS and blocked using 1% BSA in PBS. The blocking solution was aspirated and the cells washed three times in PBS. Optimisation of the antibodies was performed and it was found that 1 in 250 dilution in 1% BSA in PBS was most suitable for both antibodies.

Antibodies used were Anti-P Glycoprotein rabbit monoclonal antibody (Abcam) and Anti-beta-tubulin mouse monoclonal antibody (Abcam). 25µl drops of antibody and 1% BSA in PBS were dropped onto Parafilm.

Coverslips were then removed from wells and placed cell side down using a sterile needle and forceps and incubated overnight at 4^{o}C. Cover slips were then washed three times in 0.1% tween PBS.

50µl secondary antibody was dropped onto Parafilm and the cover slips placed cell side down using sterile needle and forceps and incubated at room temperature for 2 hours in a damp and dark box. ALEXA – anti-mouse 647 and ALEXA – anti-rabbit 488 fluorophores were used. The coverslips were then rinsed with 0.1% Tween PBS and fixed to microscope slides using Prolong-Diamond with DAPI, dropped ono the slides.

2.2.5. Mycoplasma testing

2.2.5.1. Genomic DNA extraction

Genomic DNA was extracted from cell cultures using QIAGEN DNeasy Blood and tissue DNA extraction kit. Cell pellets, containing $5x10^6$ cells were resuspended in phosphate-buffered saline (PBS) and lysed in 5% proteinase K. Ethanol is then added to the mix to stop proteinase action. The solution is then centrifuged through a DNeasy Mini spin column, fixing the DNA within the column. Wash steps are then performed to elute cell debris, purifying the DNA held within the column. The purified DNA is then eluted and quantified using Thermo-Fischer NanoDrop spectrophotometer. Cell samples were taken from flasks at 90-100% confluency, as specified in the test kit protocols.

2.2.5.2. Conventional PCR amplification of 16S rRNA coding region

For PCR amplification of 16S rRNA coding region, two mycoplasma detection kits were used; Venor GeM Classic mycoplasma detection kit for conventional PCR, purchased from Minerva Biolabs. And LookOut Mycoplasma PCR detection Kit, purchased from Sigma-Aldrich. Hot-start DNA Taq polymerase was used for all reactions.

GeM Classic mycoplasma detection kit was performed in accordance with the provided protocol. The concentration of purified DNA within the samples obtained from the DNeasy purification kit was quantified using Thermo-Fischer NanoDrop spectrophotometer.

The nucleic acid concentration was then standardised across samples. Each sample reaction contained reaction buffer, internal control DNA, primers and nucleotides all provided by Minerva Biolabs within the GeM Classic Mycoplasma detection kit. Genomic DNA was also added at the same concentration across all samples. MB Taq polymerase was used with these reactions and the total reaction volume made up with PCR grade water.

The reaction mix was then amplified using Techne PCR thermal cycler. The program for the thermal cycles was performed in accordance with the provided protocol. Gel electrophoresis was performed to separate the PCR products. 12µl of PCR product and Promega 6x loading dye was loaded onto 1.5% agarose gel with TAE and ran at 90V until suitable separation of DNA markers. The agarose gels were imaged using Syngene G-box transilluminator.

Sigma-Aldrich LookOut Mycoplasma PCR Detection kit was used as an alternative to the Minerva Biolabs kit in an effort to increase sample throughput. DNA extraction was first performed using the DNeasy blood and tissue DNA extraction and purification kit as previously described.

Sample DNA, rehydration buffer and JumpStart Taq polymerase were added to the provided reaction tubes. The nucleotides and primers for 16S rRNA coding region and an unspecified internal control sequence were provided within the reaction tubes. The program for the thermal cycler was set up in accordance with the protocol provided with the LookOut Mycoplasma detection kit. 12µL of the PCR product and Promega 6x loading dye was loaded into 1.5% agarose gel with TAE running buffer. Gel electrophoresis was performed at 90V until sufficient separation of the DNA marker had occurred. The agarose gel was imaged using the Syngene G-box transilluminator.

Chapter 3

Results

3.1. Introduction

UKF-NB-3 cell lines were treated with 4 different tubulin binding agents using a standardised protocol. Sub-lines were derived from UKF-NB-3 initially sensitive to Docetaxel, Paclitaxel, Cabazitaxel and Epothilone-B were cultured long term in the initial IC50 of each drug. This project focussed on the investigation of resistance in UKF-NB-3rDOCE1 and UKF-NB-3rDOCE4 sub-lines, as part of an ongoing project aiming to develop a standard protocol to determine the potential of anti-cancer drugs to cause acquired resistance.

The IC50 values of Docetaxel, Paclitaxel, Cabazitaxel and Epothilone-B were determined in UKF-NB-3. Five sub-lines were then split for each drug, creating a total of 20 sub-lines. These sub-lines were cultivated one week in the presence of the predetermined IC50 concentration of each respective drug, and one week without. 100,000 cells were initially passaged for each week cultured in the presence of drug, and 10,000 cells in the weeks cultured without. For some cell lines it was necessary to reduce the cell number to 10,000 cells in the weeks cultivated with drug as the sub-lines began to adapt to drug treatment. This is described in the following chapter. Development of resistance was profiled using MTT cell viability assays performed every 4 weeks on each cell line.

The standard protocol defined at the start of the assay for cell culture is summarised as follows:

- Cell lines were pasaged after seven days of growth
- 10,000 cells/ml taken at each passage
- The IC50 concentration of each drug was added to enriched IMDM growth medium at every other passage.
- The sensitivity of each sub-line to each respective drug was recorded every four weeks using the MTT drug sensitivity assay. Untreated UKF-NB-3 cell lines were used to compare the development of resistance in each cell line.

Figure 3.1 and **Figure 3.2** show a schematic diagram of the experimental design. My work was exclusively focused on the docetaxel treated sub-lines. Data that was produced by other lab members working on the same project will be appropriately acknowledged.

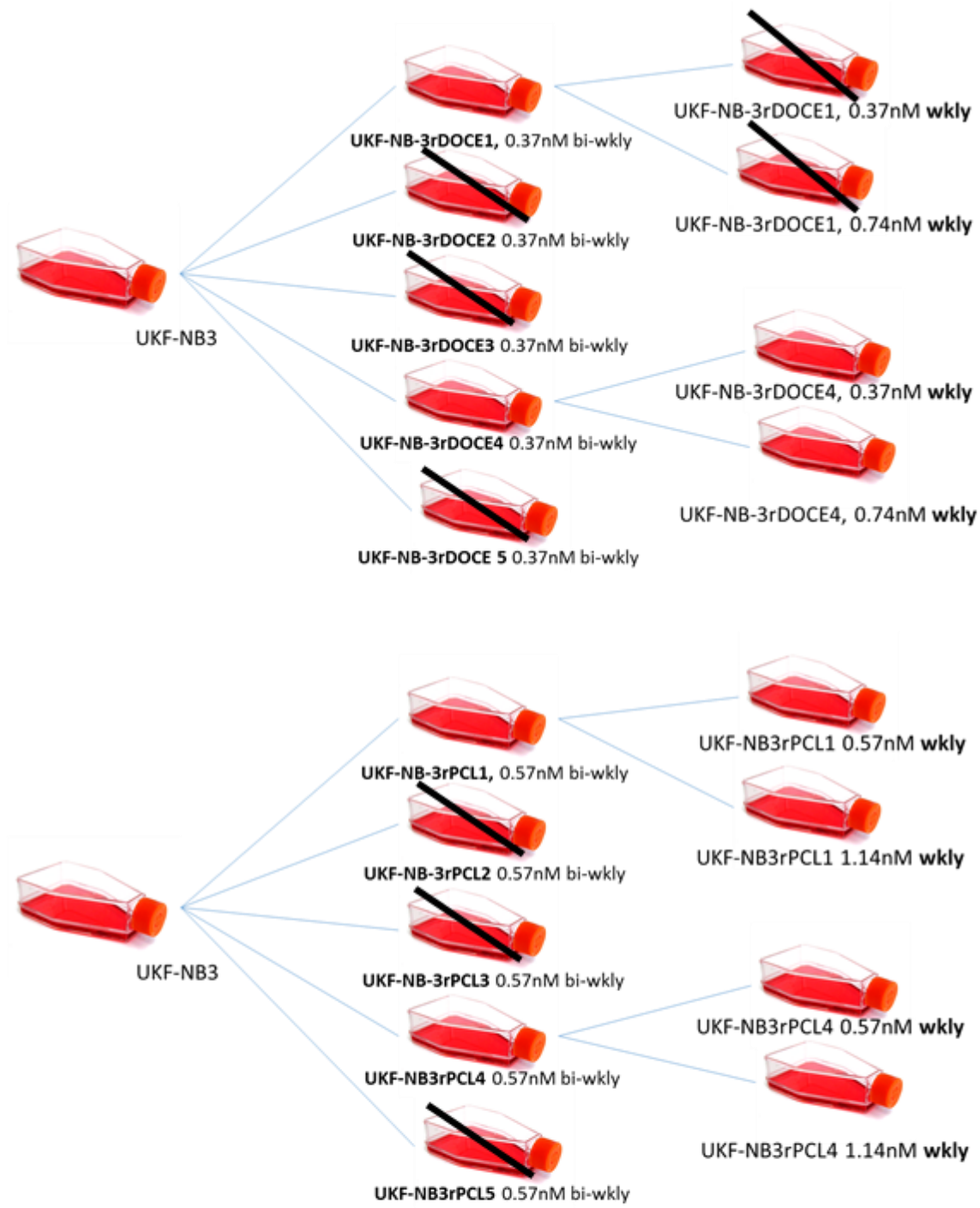


Figure 3.1. Diagram of experimental setup, showing Docetaxel and Paclitaxel resistant cell lines adapted from UKF-NB3 through exposure to the IC50 concentration of each drug. Also showing sublines with increased conditions of drug exposure, IC50 concentration added weekly and double IC50 concentration added weekly. 10,000 cells per ml were taken over in each split every week. Thick black lines indicate the cell line did not survive. During the course of this project Docetaxel cell lines were cultivated by Emilie Saintas and Tom Jackson-Soutter. Paclitaxel cell lines were cultured by Hannah Onafuye.

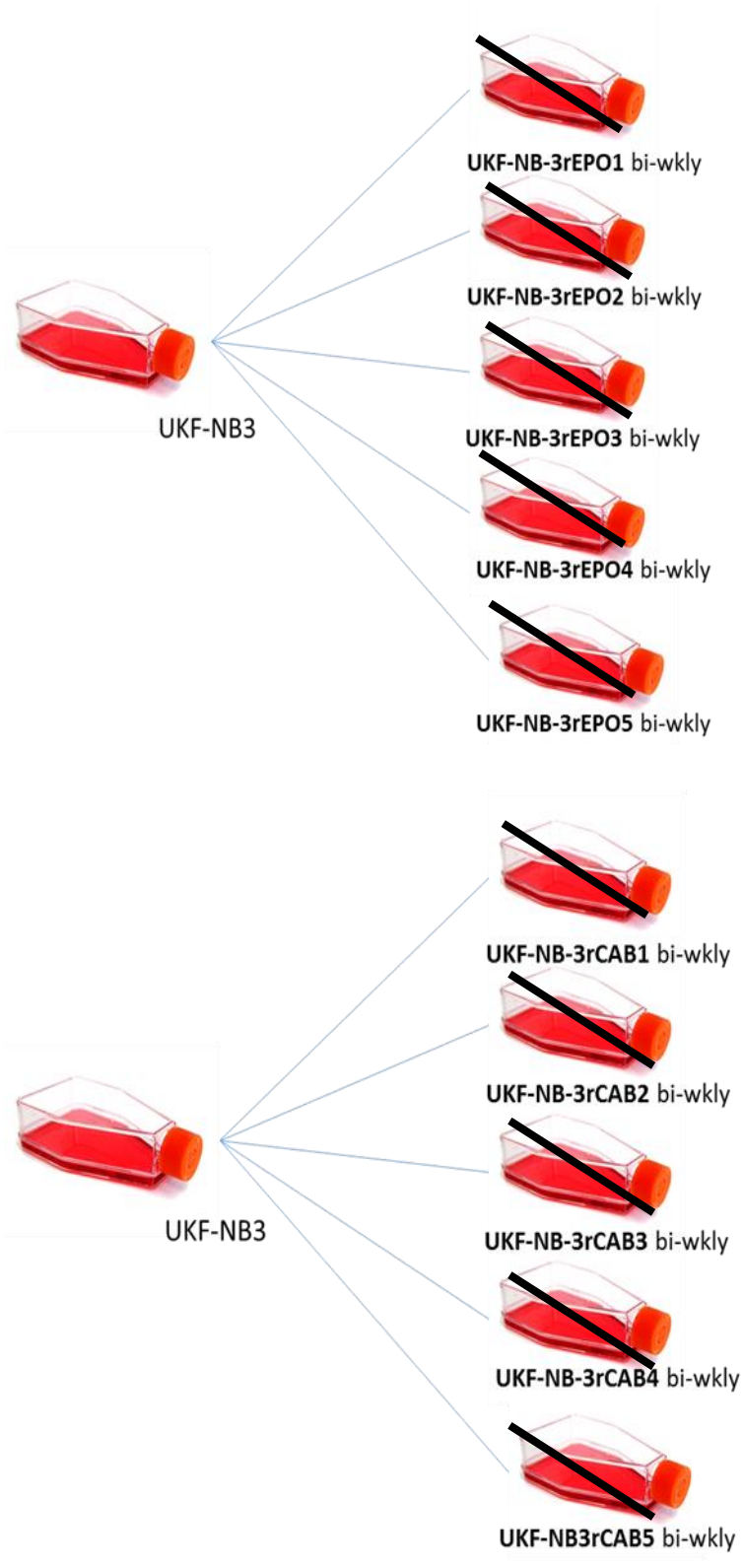


Figure 3.2. Diagram of experimental set up for Cabazitaxel and Epothilone B. Thick black lines indicate the cell line could not adapt to the standard protocol for drug exposure.

3.2. Adaptation of Cell lines to Docetaxel

This project aimed to develop a standard protocol for the adaptation of cancer cell lines to anti-cancer drugs to assess the potential of different drugs to cause acquired resistance. This resulted in the adaptation of two of the five UKF-NB-3 sub-lines treated with docetaxel. Similarly, two of the five UKF-NB-3 sub-lines treated with paclitaxel were also able to adapt to the presence of the drug. Conversely, UKF-NB-3 sub-lines treated with the IC50 concentration of epothilone-b were unable to adapt. Nor were the UKF-NB-3 sub-lines treated with cabazitaxel. Consequently, all 10 sublines (5 for each drug) treated with epothilone b and cabazitaxel respectively, died during the experiment as they were unable to adapt to the presence of each drug.

Of the five UKF-NB-3 sub-lines treated with docetaxel, only UKF-NB-3rDOCE1 and UKF-NB-3rDOCE4 were able to adapt. UKF-NB-3rDOCE2, UKF-NB-3rDOCE3 and UKF-NB-3rDOCE5 were unable to adapt to the standard protocol and the presence of docetaxel in the growth medium.

My work was focussed on investigating if it was possible to confer further resistance upon the surviving sublines treated with docetaxel. To accomplish this further sub-lines were split from UKF-NB-3rDOCE1 and UKF-NB-3rDOCE4 and the conditions of drug exposure increased. In these further sub-lines the IC50 concentration of Docetaxel was added at every passage and in a separate subline, double the IC50 concentration was added at every passage. The development of resistance in the surviving original sub-lines following the standard protocol has been included in **figure 3.3** The IC50 values of all 20 cell lines grown in the presence of drug were recorded at four week time points using MTT assays throughout the course of the experiment.

The sensitivity of UKF-NB-3 which had no previous exposure to the drugs was also recorded at the same time intervals as the resistant cell lines using MTT assays.

The initial IC50 values were lower than that of the sensitive UKF-NB-3 cell line, demonstrating that the addition of Docetaxel to the culture medium and the addition of docetaxel during the drug sensitivity assay has a cumulative effect on the cells. This is likely the result of increased cellular stress caused by the cumulative effect of docetaxel toxicity.

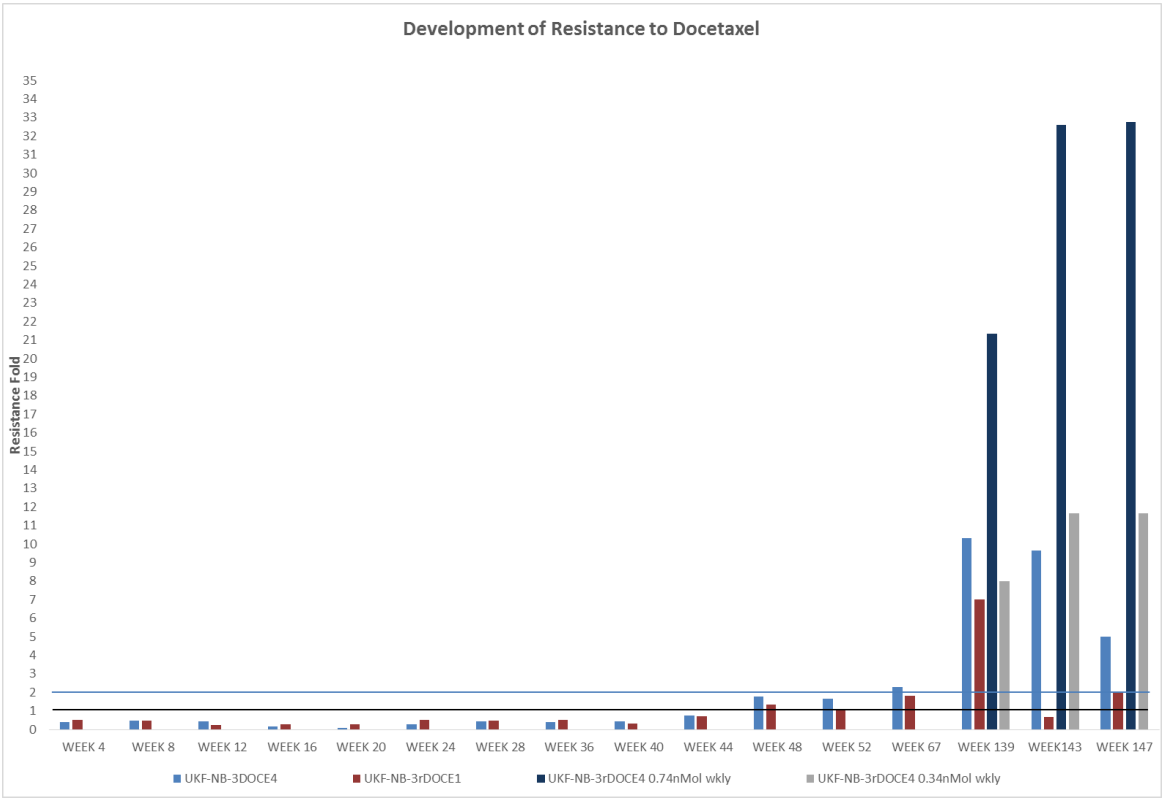


Figure 3.3. Graph of fold change in resistance across the course of the experiment from week 4 to week 147 in surviving cell lines UKF-NB-3rDOCE1 and UKF-NB-3rDOCE4. Data points have been omitted from this graph to demonstrate the change in sensitivity to Docetaxel of each sub-line over the entire course of the experiment. The black line represents the resistance of UKF-NB-3 to Docetaxel. The blue line represents a two fold increase in the resistance of untreated UKF-NB-3. Data was obtained through IC50 values taken at 4 week time points using MTT assays. Data was produced by Emilie Saintas, Lyto Yiangou and Tom Jackson-Soutter.

Figure 3.3 shows the sensitivity of the surviving sub-lines UKF-NB-3rDOCE1 and UKF-NB-3rDOCE4 at the start of the study and the most recent levels of resistance. Figure 3.3 clearly demonstrates that resistance has been conferred to UKF-NB-3rDOCE4 and UKF-NB-3rDOCE1 over the course of the study, suggesting that resistance to docetaxel increases with time when sublines follow the standard adaptation protocol.

Cell lines were defined as resistant once a two-fold increase in the IC50 concentration of docetaxel had been observed. **Figure 3.3** shows the development of resistance in the original UKF-NB-3 sub-lines treated with docetaxel. UKF-NB-3rDOCE4 proved to be up to 10 fold more resistant to docetaxel than UKF-NB-3. UKF-NB-3rDOCE1 demonstrated lower levels of resistance than UKF-NB-3rDOCE4 and on occasion showed lower resistance to docetaxel than UKF-NB-3.

To conclude the treatment of UKF-NB-3 sub-lines with different tubulin binding agents, sub-lines of UKF-NB-3 were not able to adapt to cabazitaxel and epothilone b. Resistance was not acquired and the cell lines died relatively early in the experiment. Of the five UKF-NB-3 sub-lines cultured in paclitaxel two were able to adapt and showed evidence of acquired resistance. Similarly, two of the five UKF-NB-3 sublines cultured in docetaxel were able to adapt to the presence of drug and again showed evidence of acquired resistance. This suggests that resistance is more easily acquired to docetaxel and paclitaxel than it is to epothilone b and cabazitaxel

Once resistance had been conferred, further sublines were derived from the surviving sub-lines treated with docetaxel and paclitaxel. The conditions of drug exposure were altered, increasing selective pressure on the sub-lines to investigate if the resistance of the sub-lines could be increased further by increasing drug exposure.

The further sub-lines were cultured in double the IC50 concentration of drug added at each passage, and the IC50 concentration of each drug added at each passage. This part of the project focussed entirely on docetaxel treated sub-lines and so the data for paclitaxel treated sub-lines has not been included. Resistance of the docetaxel treated sublines with increased exposure to docetaxel is shown in **Figure 3.3**.

3.3. Increased drug exposure in resistant sublines

After 130 weeks following the standardised adaptation protocol, only two paclitaxel treated sub-lines (UKF-NB-3rPCL1 and UKF-NB-3rPCL4) and two docetaxel treated sub-lines (UKF-NB-3rDOCE1 and UKF-NB-3rDOCE4) were still growing. The remaining docetaxel, paclitaxel, cabazitaxel, and epothilone B-treated cell lines did not survive the standardised adaptation protocol. Despite the long term treatment with docetaxel and paclitaxel, the surviving sub-lines did not consistently show a minimum of a two-fold increase in the IC50 value (which was defined as the criteria for confirming resistance formation) for each drug respectively. Consequently, we adapted the protocol to induce clear resistance phenotypes in the surviving cell lines through splitting further sub-lines from the original sub-lines. The original sublines continued to be treated according to the original protocol in parallel to the further sub-lines. One set of sub-lines (UKF-NB-3rDOCE4 0.37nMol wkly, UKF-NB-3rDOCE1 0.37nMol wkly) was cultured in the presence of the IC50 concentration of Docetaxel in UKF-NB-3 continuously.

Two further sub-lines (UKF-NB-3rDOCE4 0.74 nMol wkly, UKF-NB-3rDOCE 1 0.74 wkly) were continuously cultured in the presence of twice the IC50 concentration of Docetaxel in UKF-NB-3. 0.74 nMol of Docetaxel was added to the growth medium every week.

These sublines were taken from UKF-NB-3rDOCE1 and UKF-NB-3rDOCE4 at week 100. The origin of the resistant sublines can be seen in **figure 3.4.**

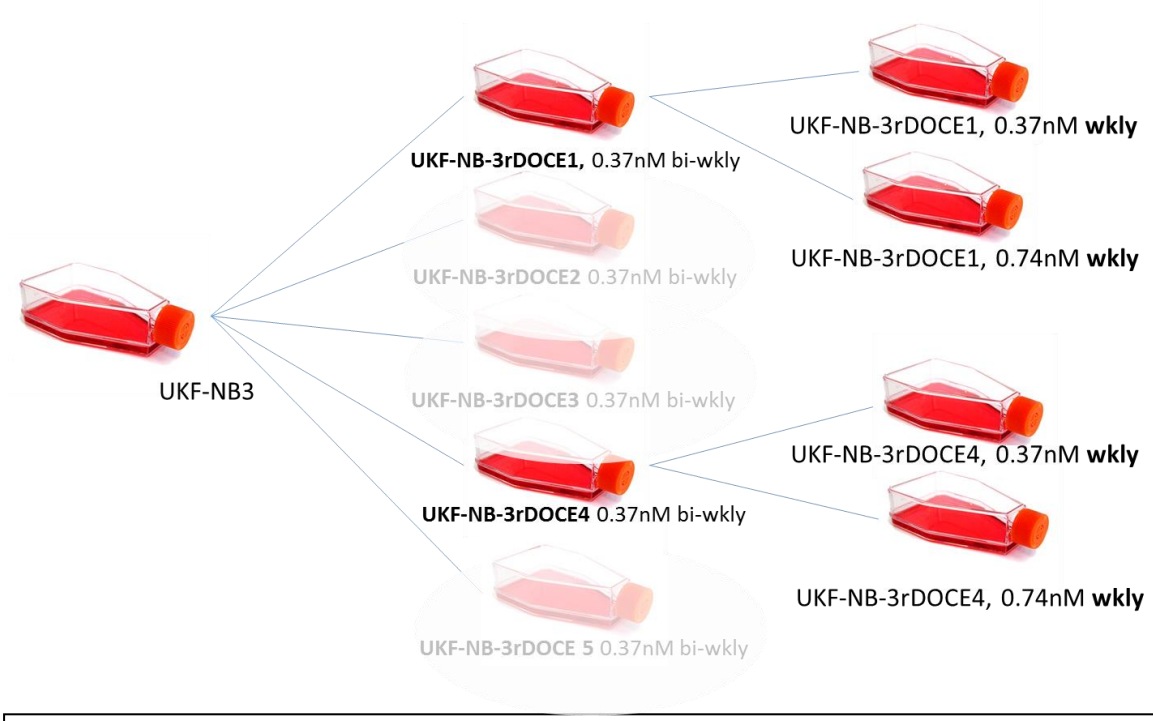


Figure 3.4. Diagram to demonstrate the origin of Docetaxel sublines.

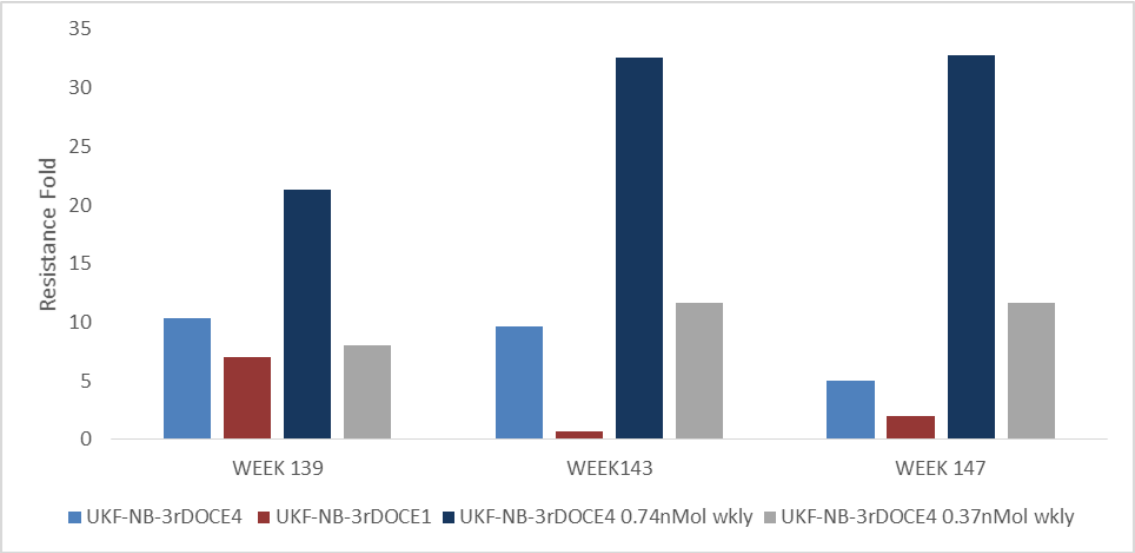


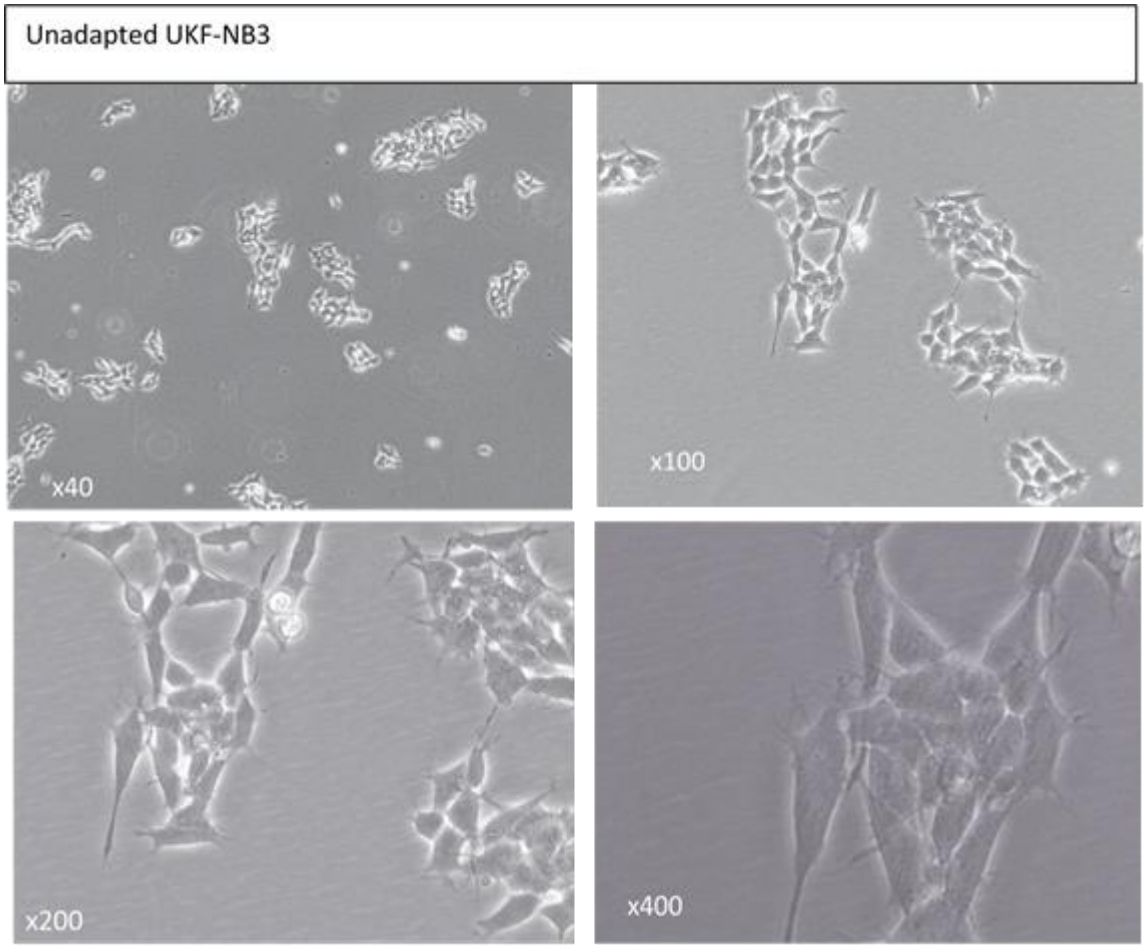
Figure 3.5. Fold increase in resistance of UKF-NB3r DOCE 4 and UKF-NB3 DOCE 1 sublines

Of the two sets of sublines with increased exposure to docetaxel, (UKF-NB-3rDOCE4 0.37nMol wkly, UKF-NB-3rDOCE1 0.37nMol wkly) and (UKF-NB-3rDOCE4 0.74 nMol wkly, UKF-NB-3rDOCE1 0.74 wkly), only those derived from UKF-NB-3rDOCE4 were able to adapt to the increased conditions of docetaxel exposure. The sub-lines derived from UKF-NB-3rDOCE1 did not survive.

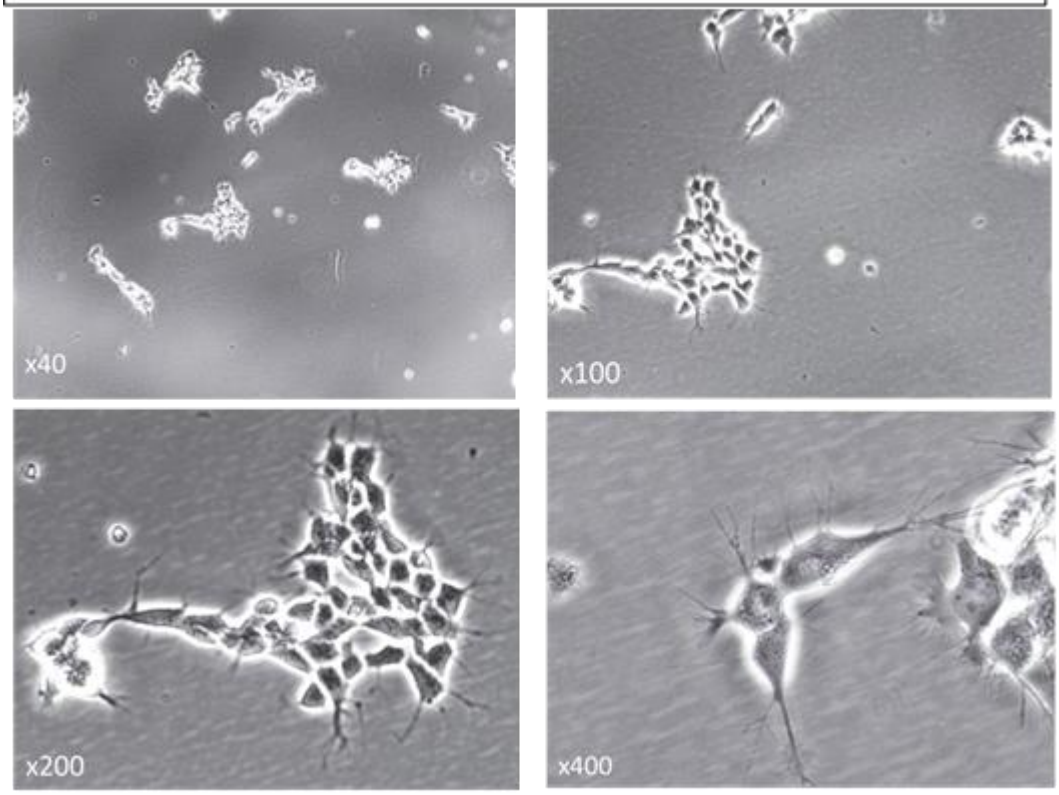
Figure 3.5 Shows the IC50 values of the two original sub-lines in parallel with the sublines derived from UKF-NB-3rDOCE4. This shows that UKF-NB-3rDOCE4 0.74 nMol wkly consistently showed higher levels of resistance than the two original sub-lines treated with docetaxel according to the standard protocol. It is also clear that UKF-NB-3rDOCE4, 0.37nMol wkly also demonstrated increased resistance compared to the original sub-lines, suggesting that increased exposure to docetaxel once resistance has been conferred increases the level of resistance observed.

3.4. Cellular Morphology

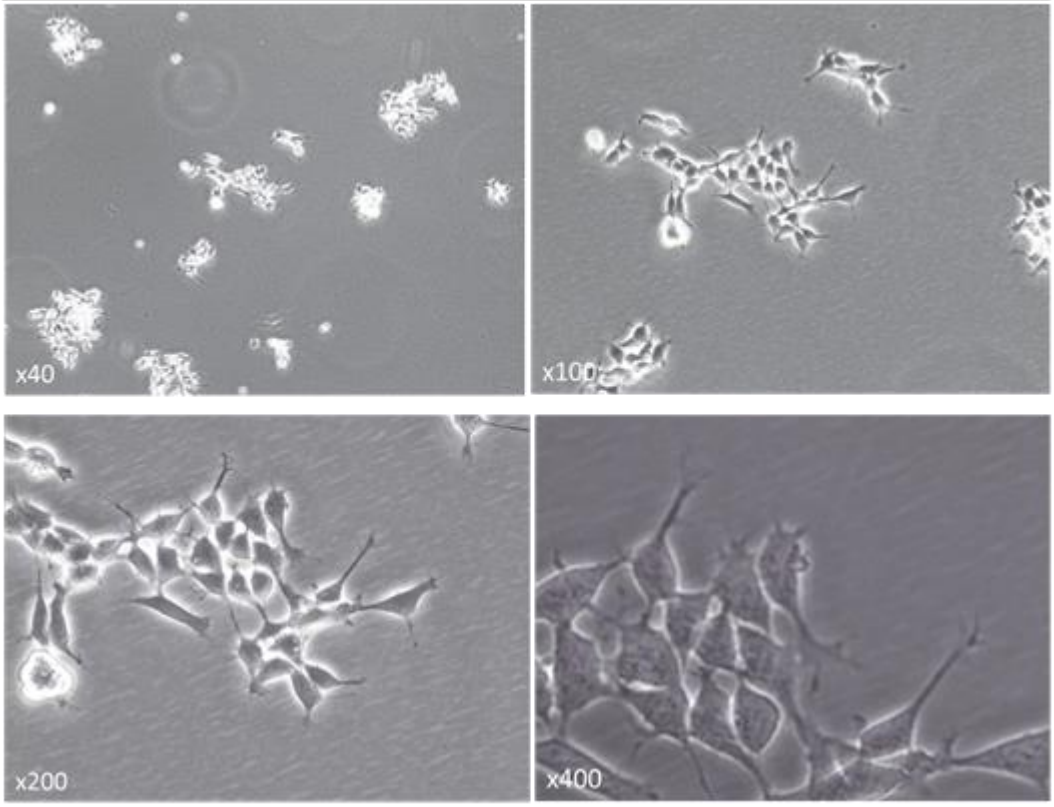
Images of the adapted cell lines were taken to investigate any potential differences in cellular morphology which may have resulted exposure to Docetaxel. Images were taken of unadapted UKF-NB-3 and the adapted cell lines cultured with the addition of Docetaxel to the growth medium. Images were taken 3 days post passage with 1000 cells per ml.



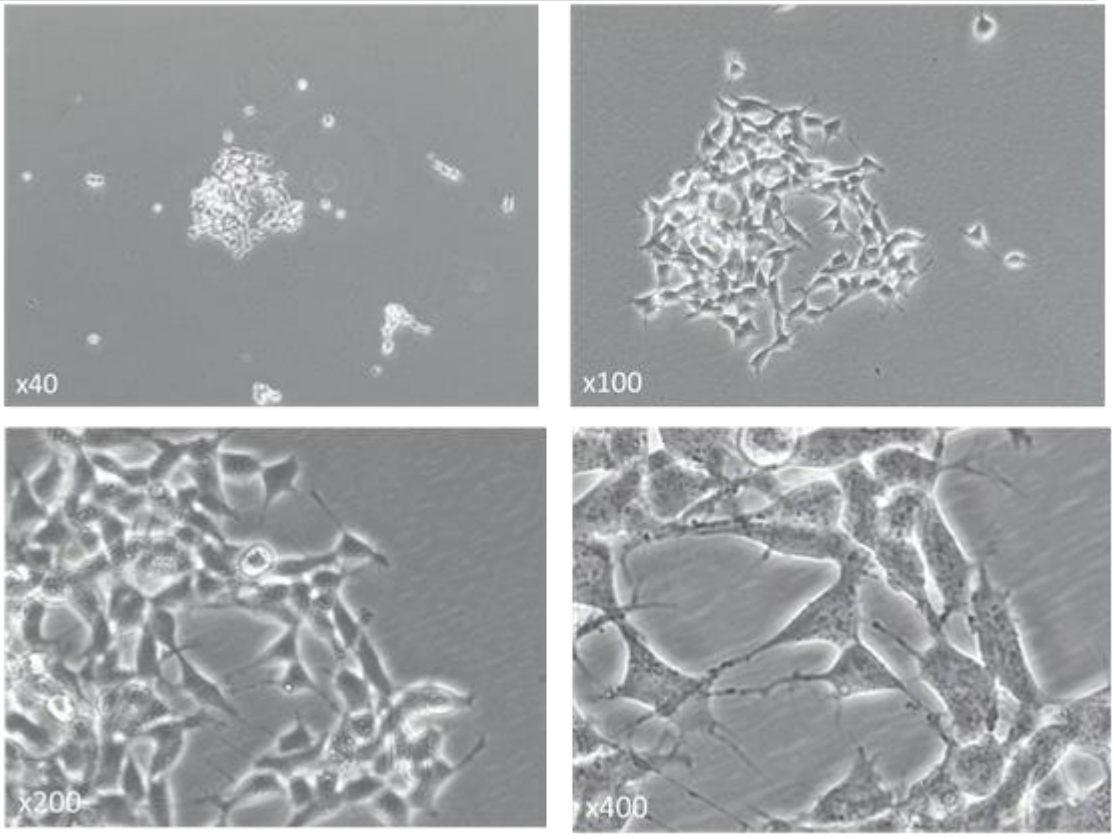
UKF-NB3r DOCE 1, 0.37nMol bi-wkly



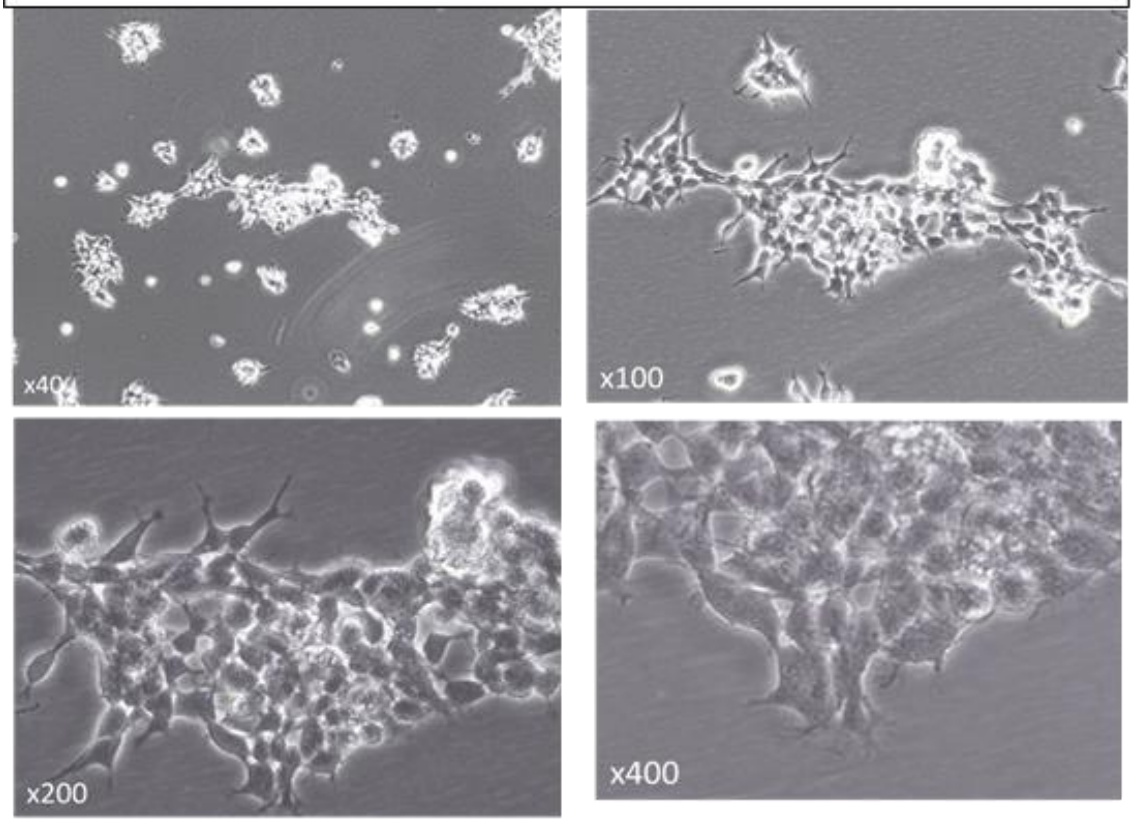
UKF-NB3r DOCE4, 0.37nMol bi-wkly



UKF-NB3r DOCE4 0.37nMol wkly



UKF-NB3r DOCE4, 0.74nMol wkly



The cell lines showed no obvious differences in morphology or appearance using light microscopy. The Docetaxel adapted cell lines all had similar appearances, growth rates and behaved the same with regards to the process of tissue culture. In the images taken it appears that UKF-NB-3rDOCE1 0.37nMol wkly was slightly more filamentous than the unadapted UKF-NB-3 cell line, however may be a result of the specific images taken and further investigation would be required to draw any further conclusions. All images were taken after 3 days of growth post passage with 10000 cells. The images show that all flasks were at similar confluency and hence the growth of all Drug adapted cell lines was relatively similar, regardless of the concentration of Docetaxel in the growth media. However growth curves would need to be produced to draw any further conclusions on patterns of growth.

3.5. Cross-resistance screen

In order to further investigate the mechanisms of resistance, we selected a panel of drugs with different mechanisms of action to establish the IC50 values of in each sub-line. Cross-resistance to multiple drugs with similar mechanisms of action or similar mechanisms of resistance would suggest a resistance mechanism in the pathways common to those drugs which resistance has been acquired. A brief description of the mechanisms of action for each drug can be found in **table 1.1**.

IC50 values for a panel of eight drugs were determined using MTT drug sensitivity assays. Cross-resistance was investigated for the following drugs:

- Docetaxel
- Paclitaxel
- Cabazitaxel
- Epothilone-B
- Cisplatin
- Vincristine
- Crizotinib
- Topotecan

A summary of the results can be found in **table 3.1**. The cross-resistance screen showed that resistance was conferred in all docetaxel adapted cell lines to paclitaxel and vincristine in addition to docetaxel.

Drug	ABC transporter affinity	IC50 value (nMol)				
		UKF-NB-3	UKF-NB-3rDOCE1	UKF-NB-3rDOCE4	UKF-NB-3DOCE4 0.37nMol wkly	UKF-NB-3rDOCE4 0.74nMol wkly
Docetaxel	ABCB1, ABCC10	0.01	0.15	0.28	0.31	0.87
Paclitaxel	ABCA3, ABCB1, ABCB4, ABCB11, ABCC1, ABCC2, ABCC10,	0.88	2.18	4.77	3.32	5.63
Cabazitaxel	Non identified	0.11	0.14	0.13	0.13	0.15
Epothilone-B	Non identified	0.04	0.06	0.04	0.06	0.06
Cisplatin	ABCA1, ABCA3, ABCC2, ABCC6	0.52	0.26	0.81	0.55	0.60
Vincristine	ABCA3, ABCB1, ABCC1, ABCC2, ABCC3, ABCC10	0.07	0.18	0.18	0.27	0.59
Crizotinib	Non identified	0.41	0.35	0.65	0.44	0.37
Topotecan	ABCB1, ABCB5, ABCC2, ABCC4, ABCG2,	6.91	4.44	8.70	7.70	6.44

Table 3.1. Summary of properties of drugs used in cross resistance screen. IC50s have been averaged from cell line with alternating weeks of Docetaxel exposure. Drugs to which cell lines show resistance have been highlighted in red text.

(Ween et al. 2015)(Wils et al. 1994)(Kathawala et al. 2015)

Sensitivity of sub-lines to docetaxel

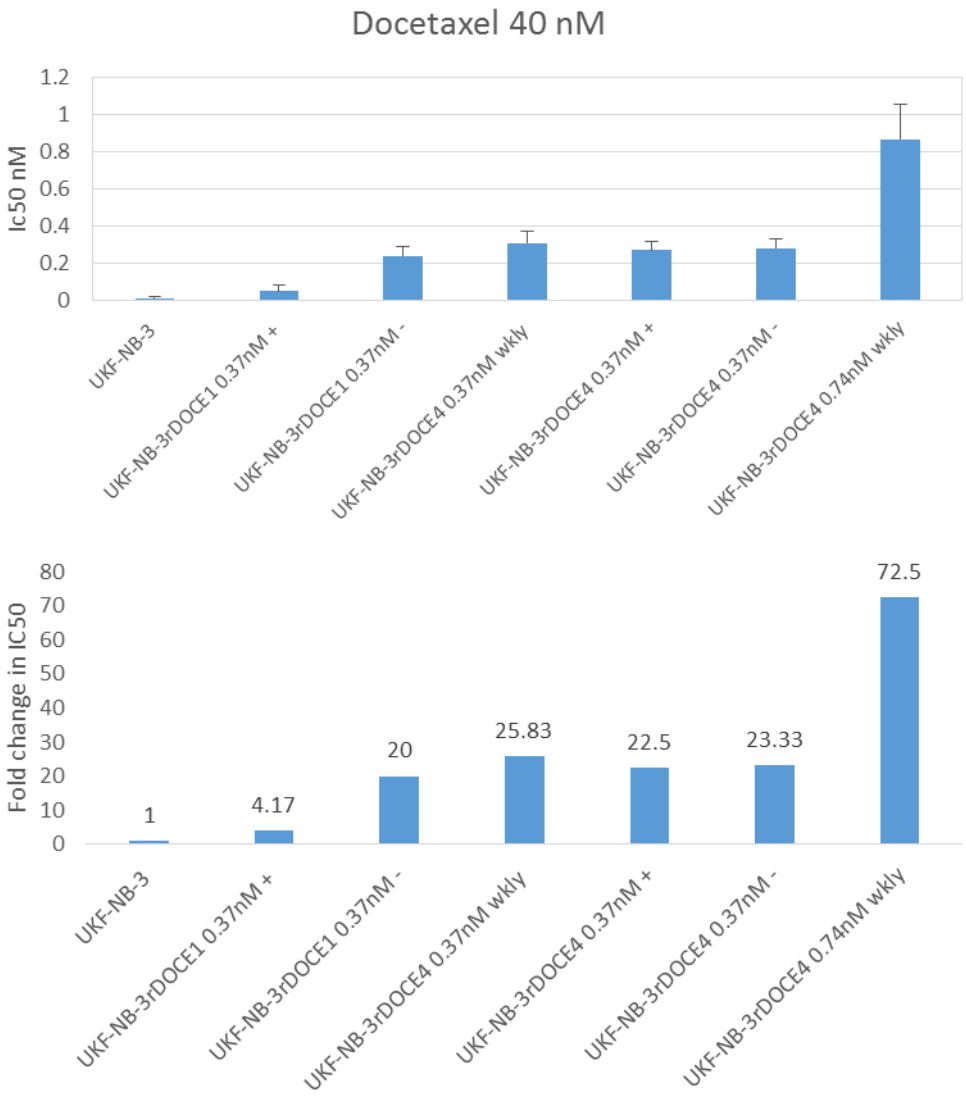


Figure 3.6 Resistance to cisplatin and fold change in resistance compared to unadapted UKF-NB3 cell line

UKF-NB-3DOCE4 demonstrated the highest degree of resistance of the two sub-lines following the standard protocol, supporting the data acquired from the monthly MTTs. The degree of resistance to docetaxel in UKF-NB-3rDOCE1 and UKF-NB-3DOCE4 both depended on drug exposure over the previous week of growth. Sensitivity to docetaxel was increased with the presence of docetaxel in the culture medium by 4.8 fold in UKF-NB-3rDOCE1.

This increase was substantially smaller in UKF-NB-3rDOCE4 and will be discussed further in chapter 4. UKF-NB-3rDOCE4 0.74nMol wkly showed the highest degree of resistance to docetaxel and demonstrated a 73 fold increase in the IC50 of docetaxel when compared against UKF-NB-3. The sub-line grown continuously in docetaxel (UKF-NB-3rDOCE4 0.37 wkly) also showed increased degrees of resistance compared to UKF-NB-3. The sub-lines with increased exposure (UKF-NB-3rDOCE4 0.37 wkly and UKF-NB-3rDOCE4 0.74nMol wkly) were more resistant to docetaxel than the sub-lines following the standard adaptation protocol (UKF-NB-3rDOCE1 and UKF-NB-3rDOCE4). The data described here can be found in **figure 3.6**.

Docetaxel shows binding affinity to ABCB1 and ABCC10. (Wils et al. 1994)(Kathawala et al. 2015) **Table 3.1** shows any affinity of each drug to ABC transporters.

Sensitivity of sub-lines to paclitaxel

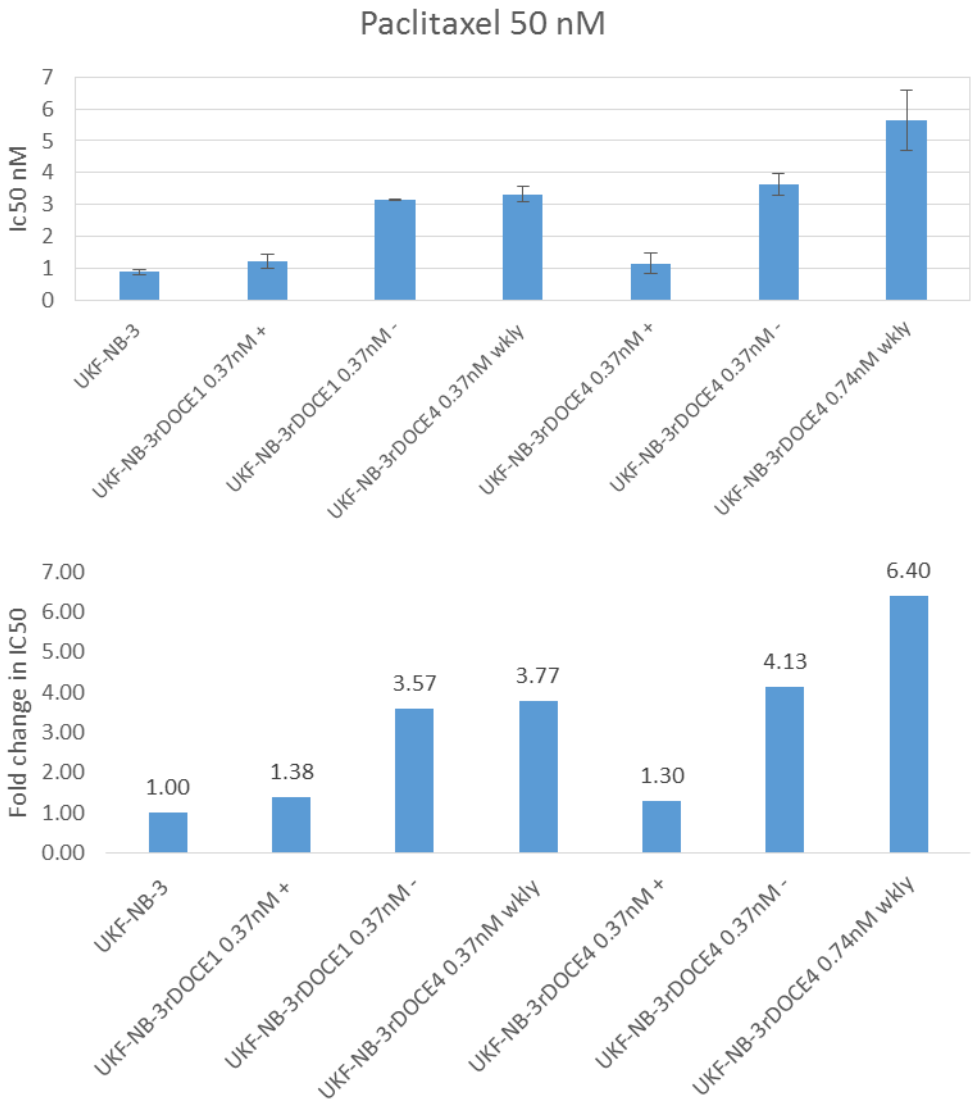


Figure 3.7. Resistance to cisplatin and fold change in resistance compared to unadapted UKF-NB3 cell line

Both sub-lines following the standard protocol displayed resistance (defined as a two-fold increase in the IC50 concentration) to paclitaxel with the absence of docetaxel from the culture medium. There were substantial differences in the degree of resistance between cultivation in the presence of docetaxel cultivation without.

The increased sensitivity in the presence of docetaxel was most pronounced in UKF-NB-3rDOCE4 with a 3.2 fold decrease in resistance with the addition of docetaxel.

As with the resistance seen against docetaxel, UKF-NB-3rDOCE4 0.74nMol wkly showed the highest degree of resistance against paclitaxel with 6.4 fold increase compared to UKF-NB-3 and 1.5 fold increase compared to UKF-NB-3rDOCE4. Both sub-lines cultured with additional exposure to docetaxel demonstrated higher resistance to paclitaxel than the two sub-lines following the standard protocol cultured in the presence of docetaxel. Paclitaxel, like docetaxel, has well established resistance ABCB1 mediated resistance mechanism.

The addition of 0.37nMol Docetaxel to the growth medium every week in UKF-NB-3r DOCE4 0.37nMol wkly showed increased resistance to Paclitaxel compared to the cell lines which followed the standard adaptation protocol. The increase in resistance however was not as pronounced as that of UKF-NB-3r DOCE4 0.74nMol wkly.

Sensitivity of cell lines to cabazitaxel

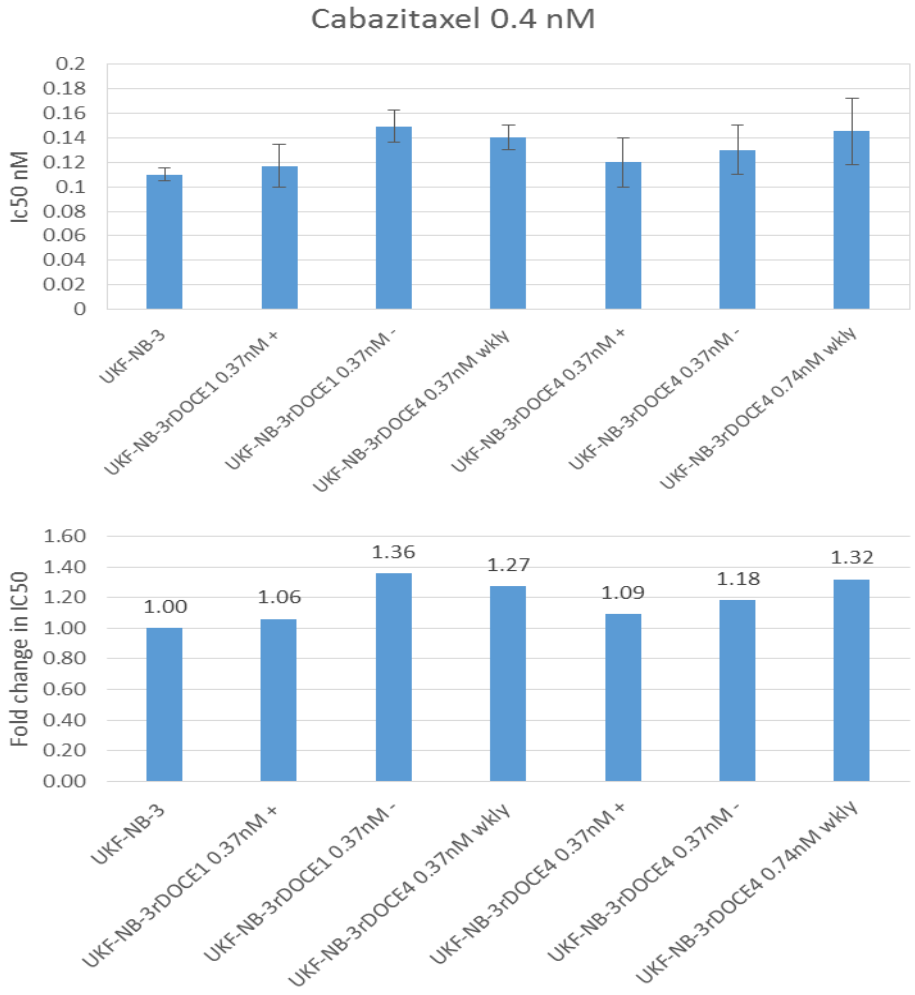


Figure 3.8. Resistance to cisplatin and fold change in resistance compared to unadapted UKF-NB3 cell line

All cell lines were showed very high sensitivity to Cabazitaxel and a two fold increase in the IC50 was not observed in any cell line. This supports the findings from the start of the drug adaptation assay which showed that unadapted UKF-NB-3 cell lines were unable to adapt to the presence of Cabazitaxel in the growth media. Whilst none of docetaxel treated sub-lines developed a two fold increase in resistance, the IC50 concentration for Cabazitaxel did increase slightly for the adapted cell lines. Furthermore the adapted cell lines which showed greatest increases in resistance compared to the unadapted UKF-NB-3 were also slightly more resistant than other cell lines to cabazitaxel. Additionally the pattern of increased resistance in the absence of Docetaxel was also observed.

Sensitivity of sub-lines to epothilone b

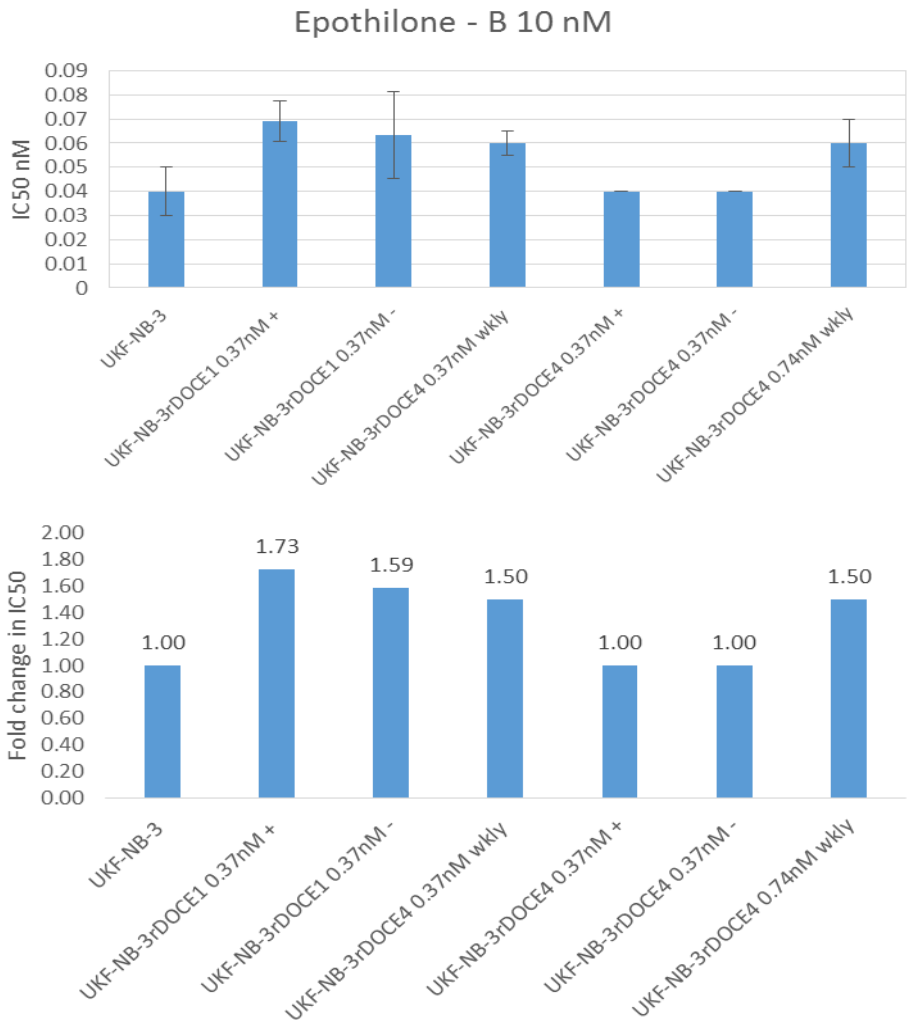


Figure 3.9. Resistance to epothilone b and fold change in resistance compared to unadapted UKF-NB3 cell line

Resistance to Epothilone-B was not observed with no substantial increase in IC50 values for any of the cell lines. The slight increases in IC50 of Epothilone-B were not in line with patterns of resistance previously described for other drugs. UKF-NB-3rDOCE 1 showed higher resistance when cultured in the presence of Docetaxel compared to when the same cell line was cultured in the absence of Docetaxel. This again supports the findings of the standard adaptation protocol, which also showed UKF-NB-3 was unable to adapt to treatment with cabazitaxel.

Sensitivity of sub-lines to cisplatin

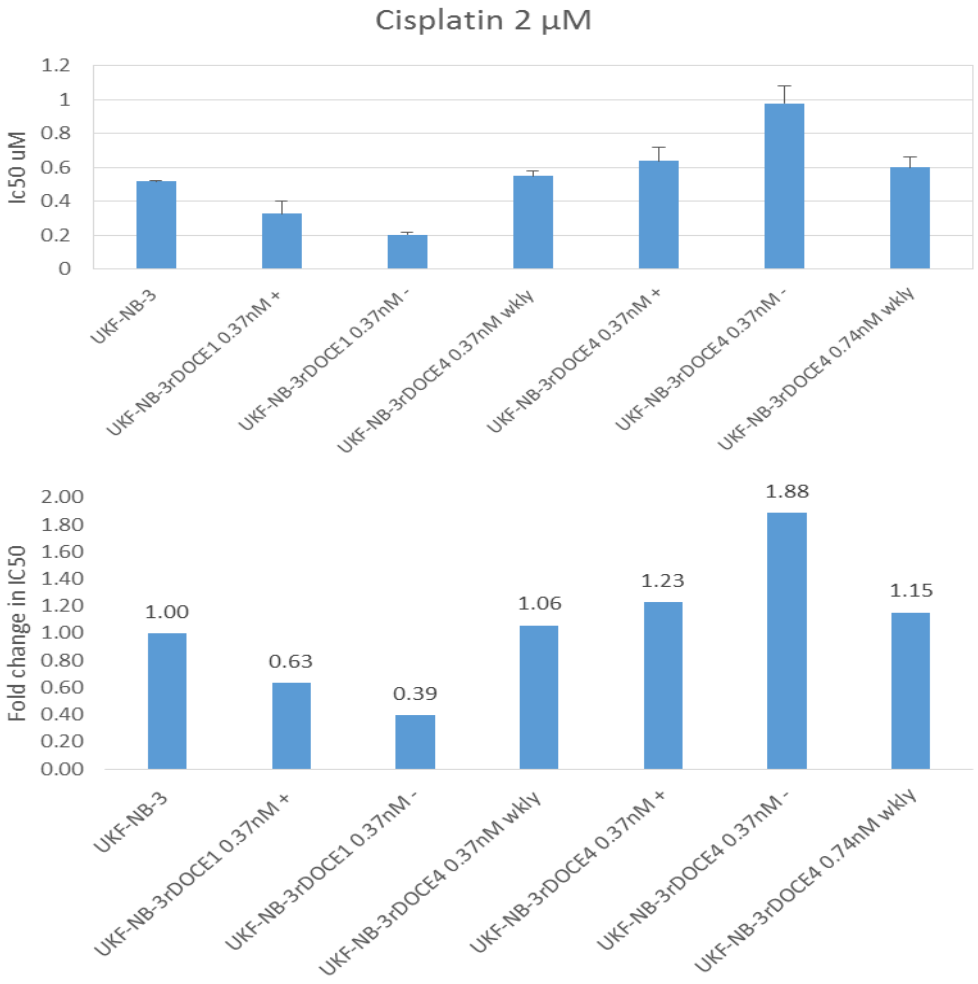


Figure 3.10. Resistance to cisplatin and fold change in resistance compared to unadapted UKF-NB3 cell line

UKF-NB-3rDOCE4 (cultured in the absence of Docetaxel) showed the greatest increase in IC50 across all cell lines adapted to Docetaxel at 0.98uMol compared to the unadapted UKF-NB-3 with an IC50 concentration of 0.52uMol. This was not a twofold increase and so the cell lines cannot be defined as resistant to Cisplatin. UKF-NB-3r DOCE1 became more sensitive to Cisplatin than the unadapted UKF-NB-3 cell line which had an IC50 of 0.52uMol compared to 0.32uMol for UKF-NB-3r DOCE1 in the presence of Docetaxel and 0.2uMol in the absence of Docetaxel. This increased sensitivity in UKF-NB-3rDOCE1 was also observed in weeks without the addition of Docetaxel to the growth medium against Crizotinib and Topotecan. (See **figure 3.12** and **figure 3.13**)

Sensitivity of sub-lines to vincristine

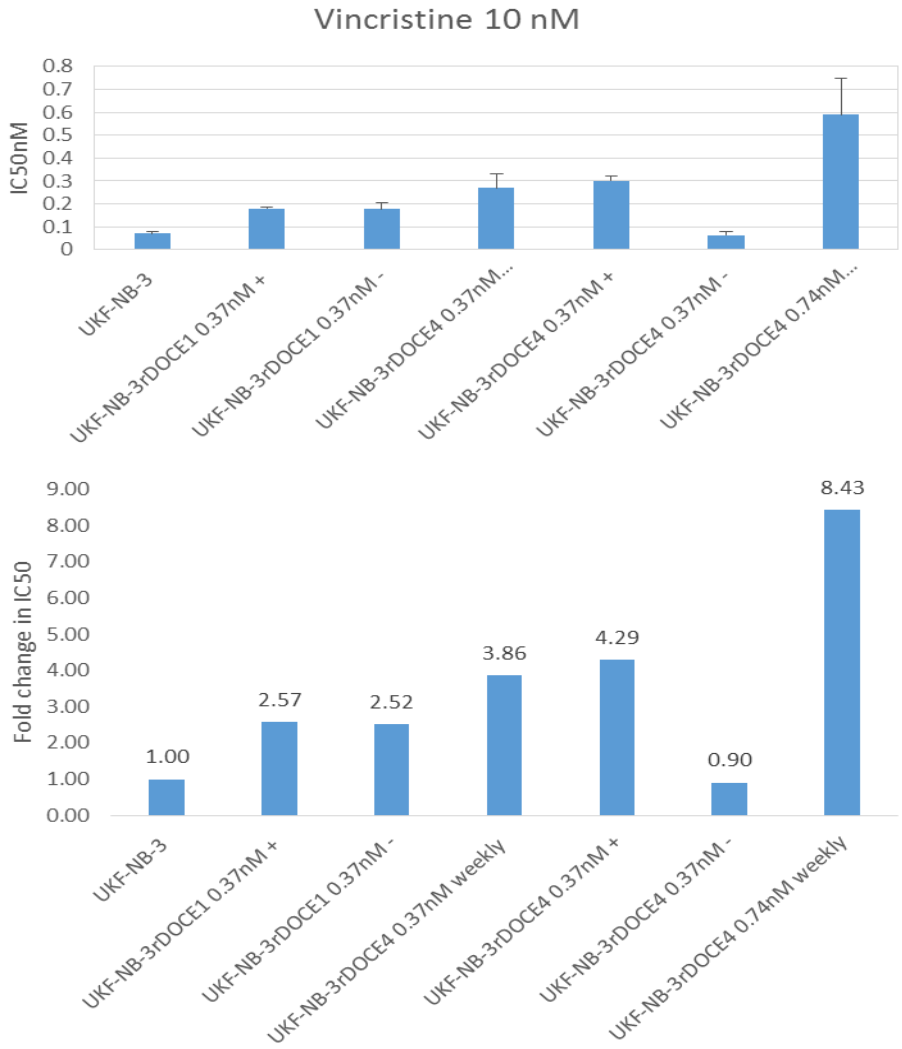


Figure 3.11. Resistance to vincristine and fold change in resistance compared to unadapted UKF-NB3 cell line

All Docetaxel adapted cell lines showed more than a twofold increase in IC50 to Vincristine, defining them as resistant cell lines. UKF-NB-3r DOCE4 cultured in the absence of Docetaxel in the growth media did not demonstrate any increase in resistance and became more sensitive to Vincristine than unadapted UKF-NB-3. The IC50 of this cell line under these conditions of drug exposure fell to 0.06nMol. The IC50 of the UKF-NB-3 was 0.07nMol giving a fold change of 0.9.

This increased sensitivity beyond that of the unadapted UKF-NB-3 control cell line was not seen against any other drug tested within the cross-resistance panel in UKF-NB-3rDOCE4. UKF-NB-3rDOCE4 also showed the greatest variation between IC50 for weeks cultured in and out of Docetaxel against Vincristine showing a 4.7 fold increase in the IC50 of Vincristine for weeks cultured in the presence of Docetaxel. The increased resistance in the Cell line cultured in the presence of Docetaxel was also observed to an extent for UKF-NB-3rDOCE1 which had a very slight increase in IC50 concentration of Vincristine from 0.17nMol (in the absence of Docetaxel), to 0.18nMol (with the addition of Docetaxel to the growth medium). Despite the change being negligible for UKF-NB-3 DOCE1, the trend for the two sub-lines following the standard adaptation protocol displaying increased resistance in the presence of docetaxel was not observed against the other drugs in docetaxel or paclitaxel. In both drugs the pattern of resistance was completely opposed. Resistance in Docetaxel and Paclitaxel was higher for both drugs in both sub- lines following the standard adaptation protocol in the weeks with the absence of Docetaxel from the growth medium, contrary to what was seen in vincristine.

UKF-NB-3rDOCE4 0.74nMol wkly was the least sensitive to vincristine with an IC50 of 0.59nMol compared to of 0.07nMol of UKF-NB-3 and 0.18nMol of UKF-NB-3rDOCE4. A fold increase of 3.3 on the cell line from which it was derived. The resistance of UKF-NB-3r DOCE4 0.37nMol wkly was higher than the resistance in UKF-NB-3rDOCE1, as was observed for Docetaxel and Paclitaxel. The degree of resistance was lower in UKF-NB-3rDOCE4 0.37nMol wkly than it was in UKF-NB-3rDOCE4 (cultured in the presence of Docetaxel.) This was not the case for Docetaxel and Paclitaxel, both of which showed higher levels of resistance to Docetaxel and Paclitaxel for UKF-NB-3rDOCE4 0.37nMol wkly than UKF-NB-3rDOCE 4.

Sensitivity of sublines to crizotinib

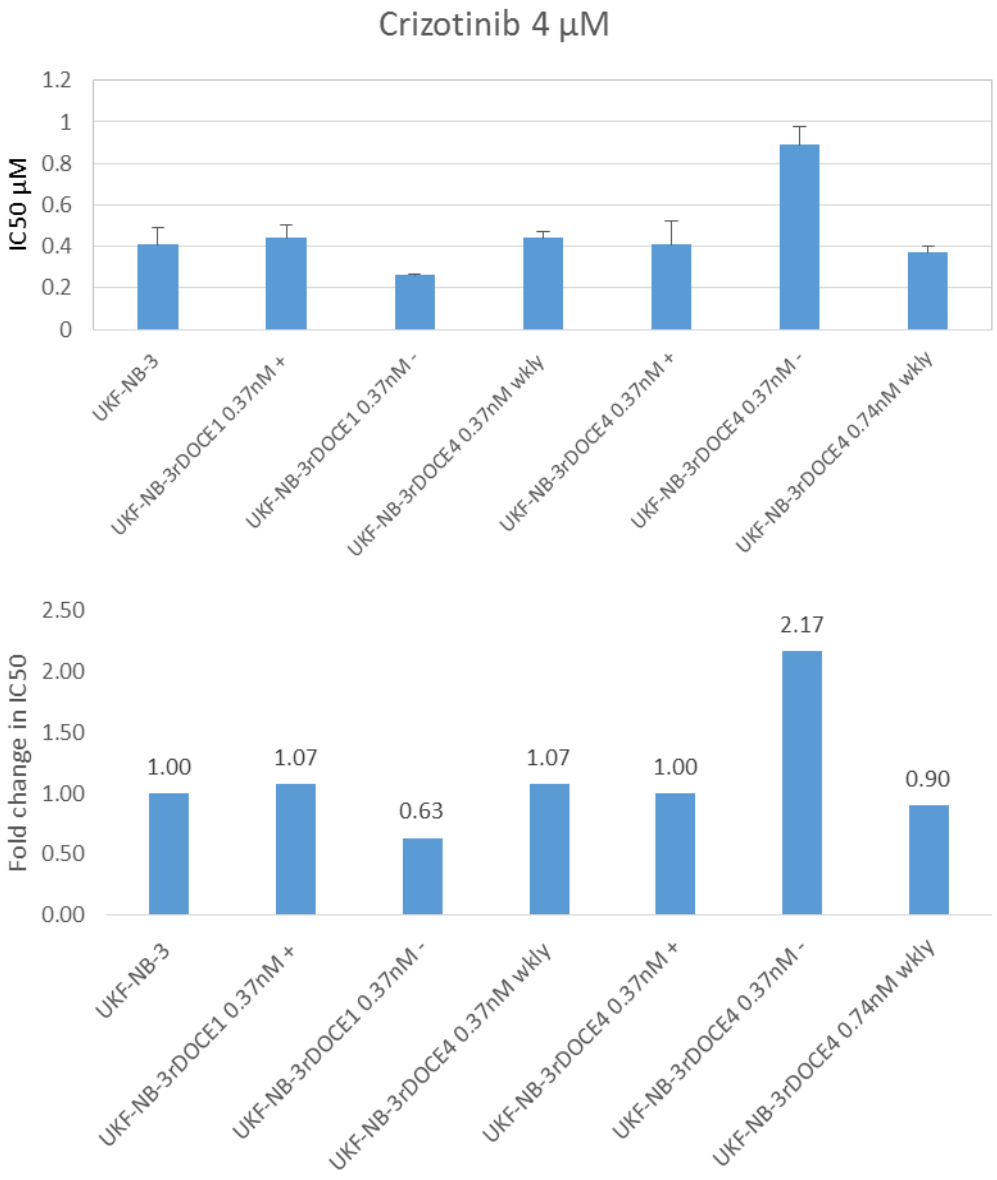


Figure 3.12. Resistance to crizotinib and fold change in resistance compared to unadapted UKF-NB3 cell line

Resistance was observed in UKF-NB-3rDOCE4 (cultured in the absence of Docetaxel) from the growth medium against Crizotinib, with an IC50 value of 0.89nMol compared to 0.41 in the unadapted UKF-NB-3 cell line. This was not replicated by any other sub-line. Nor was it replicated in the same sub-line in the presence of docetaxel.

Sensitivity of sub-lines to topotecan

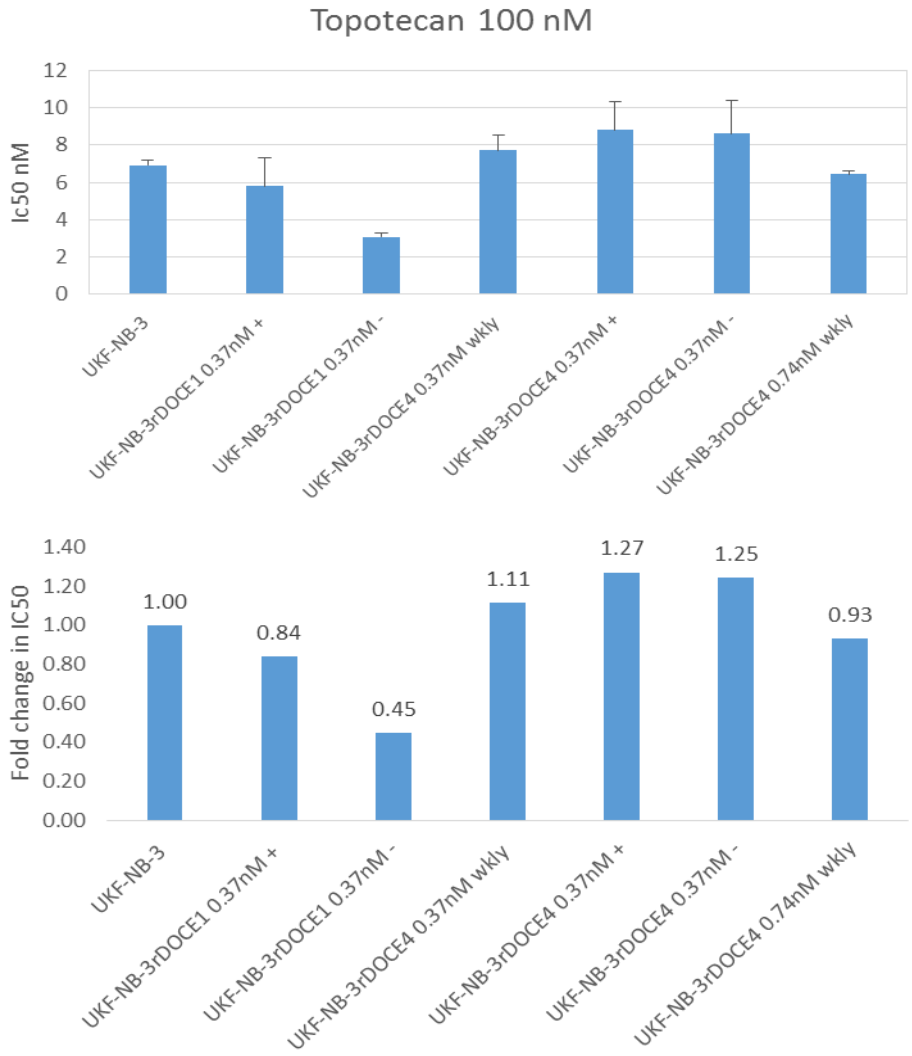


Figure 3.13. Resistance to topotecan and fold change in resistance compared to unadapted UKF-NB3 cell line

None of the sub-lines displayed any resistance to topotecan. UKF-NB-3rDOCE1 was more sensitive than UKF-NB-3, as was UKF-NB-3rDOCE4 0.74nMol wkly.

Conclusion of Cross-resistance screen

Both Docetaxel treated cell lines (UKF-NB-3r DOCE1 and UKF-NB-3rDOCE4) and the sublines with increased exposure to Docetaxel (UKF-NB-3rDOCE4 0.37nMol wkly and UKF-NB-3rDOCE4 0.74 wkly) demonstrated a twofold increase in IC50 against Docetaxel, Paclitaxel and Vincristine. This was not seen against the other drugs used in the panel. UKF-NB-3rDOCE4 0.74nMol showed the greatest increase in resistance and was the least sensitive to Docetaxel, Paclitaxel and Vincristine.

The sub-lines with increased exposure (UKF-NB-3rDOCE4 0.37nMol wkly and UKF-NB-3rDOCE4 0.74 wkly) demonstrated greater degrees resistance than those following the standard adaptation protocol (UKF-NB-3r DOCE1 and UKF-NB-3rDOCE4). UKF-NB-3rDOCE4 0.74 wkly showed the greatest degree of resistance.

Variation in the sensitivity to each drug was observed between weeks depending on exposure to Docetaxel in the culture medium. Cultivation in the presence of docetaxel had varying effects. Under these conditions sensitivity to Docetaxel and Paclitaxel increased, however the opposite was true against Vincristine where sensitivity increased in the absence of Docetaxel.

For the drugs for which resistance was not established, docetaxel resistance seemed to have little effect on sensitivity, suggesting the resistance pathway responsible for the observed docetaxel resistance is common to paclitaxel and vincristine resistance but not to topotecan, crizotinib, cisplatin or epothilone b. However, some small changes in sensitivity were seen against these drugs and resistance was established under certain conditions for example UKF-NB-3r DOCE4 without Docetaxel against Crizotinib.

Subsequently resistance pathways associated with these drugs should not be ruled out as more than one resistance mechanism may be active. Despite this, the major cause of docetaxel resistance here appears to be common to paclitaxel and vincristine resistance. As a result of the cross-resistance screen and the patterns of cross resistance observed, we decided to investigate further the expression of ABCB1 efflux proteins using fluorescent microscopy. To investigate further the changes in resistance to tubulin binding agents, we additionally stained for tubulin expression.

Future work will elucidate the mechanisms by which resistance has been acquired to Docetaxel and Epothilone B. Exome sequencing data will be analysed to asses any mutational changes which may have resulted in the expression of a resistant phenotype to Docetaxel and Paclitaxel. Alternatively the Exome analysis will confirm that the resistant phenotype has always been present in the population and the observed resistance was intrinsic to the specific population of UKF-NB-3 selected for each sub-line.

Expression of ABCB1 and β-tubulin

In order to further investigate the mechanisms of resistance to docetaxel we used confocal microscopy to investigate the expression of ABCB1 and β-tubulin. Here we clearly saw that ABCB1 was upregulated in the sublines with the most resistance to docetaxel, supporting the evidence of ABCB1 overexpression found in the cross-resistance panel. The degree of resistance seen in the cross resistance screen appeared to correlate with ABCB1 expression. UKF-NB-3rDOCE4 0.74nMol wkly demonstrated the highest degree of resistance to docetaxel, paclitaxel and vincristine in the cross resistance panel. Here, with confocal microscopy we have shown that ABCB1 is most highly expressed within this cell line.UKF-NB-3rDOCE4 0.74nMol wkly demonstrated the highest degree of resistance and the highest degree of ABCB1 expression. UKF-NB-3rDOCE4 0.37nMol wkly appeared to show the second highest degree of expression of ABCB1 compared to the other sub-lines.

We also observed increased tubulin expression in the resistant sub-lines compared to UKF-NB-3. The confocal images had quite high background signal for β-tubulin fluorophores. As a result, it is difficult to say exactly how much the expression of β-tubulin changes between each resistant subline. However there is a distinct increase in expression between UKF-NB-3 and UKF-NB-3rDOCE4 0.74nMol wkly

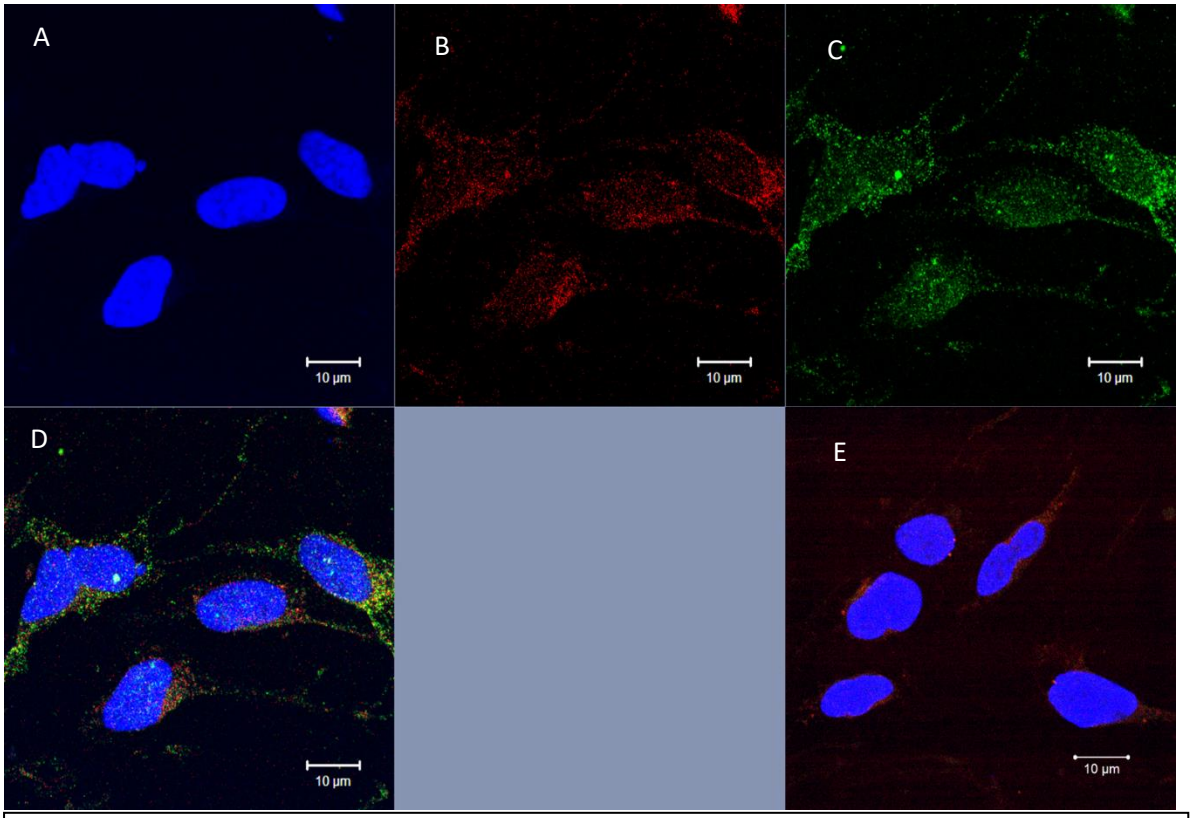


Figure 3.14, UKF-NB-3 (**A**) DAPI staining of UKF-NB-3 nuclei. (**B**) ALEXA 647 fluorophore bound to β-tubulin primary antibody. (**C**) ALEXA 488 fluorophore bound to anti-P-gp primary antibody. (**D**) Merge of A,B and C. (**E**) Merge of UKF-NB-3 negative control.

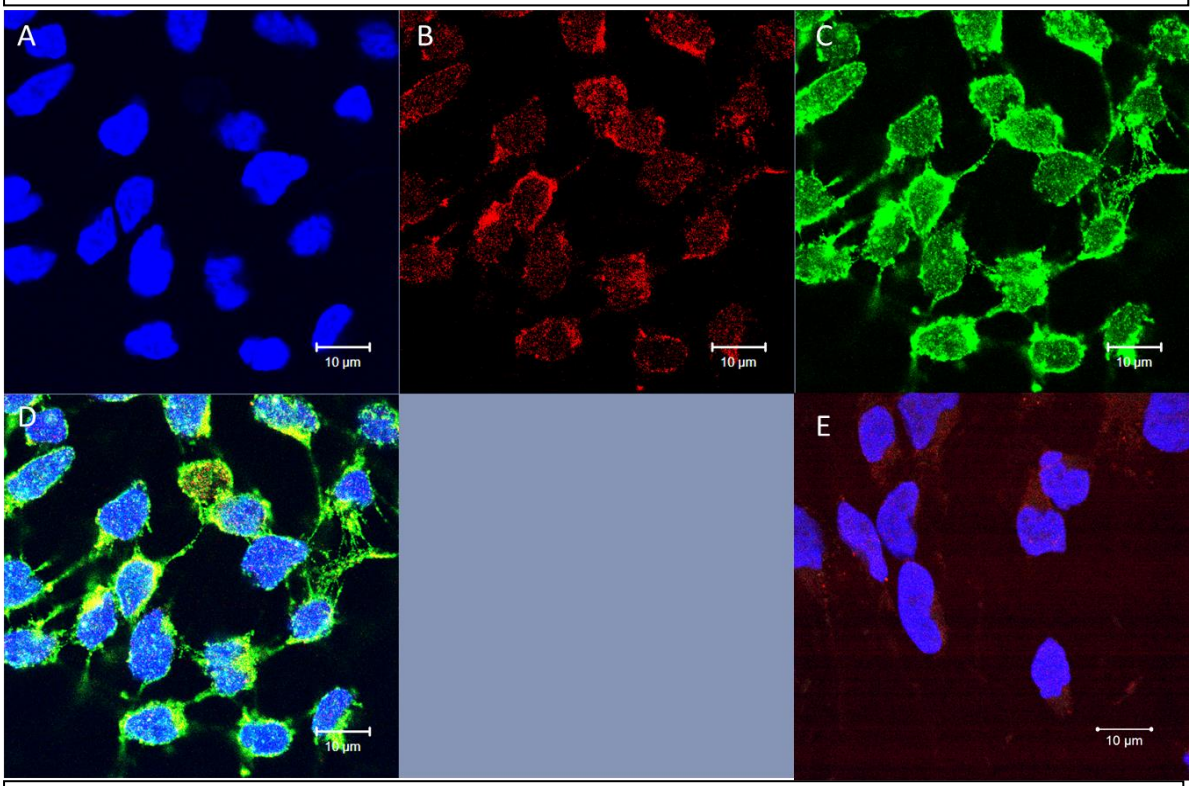


Figure 3.15, UKF-NB-3rDOCE4 0.74nMol wkly (**A**) DAPI staining of UKF-NB-3rDOCE4 0.74nMol wkly nuclei. (**B**) ALEXA 647 fluorophore bound to mouse anti-β-tubulin primary antibody. (**C**) ALEXA 488 fluorophore bound to anti-P-gp primary antibody. (**D**) Merge of A,B and C. (**E**) Merge of UKF-NB-3rDOCE4 0.74nMol negative control.

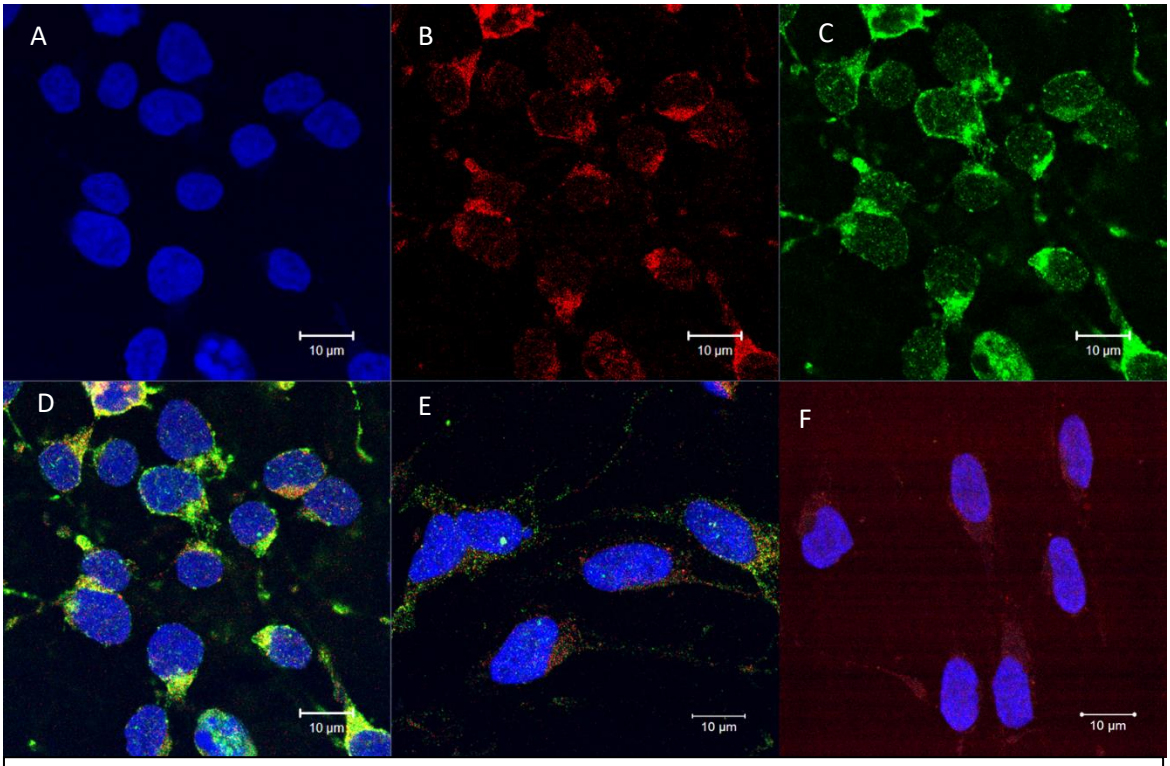


Figure 3.16UKF-NB-3rDOCE4, (A,B,C,D) UKF-NB-3 (E). (**A**) DAPI staining of UKF-NB-3rDOCE4 nuclei. (**B**) ALEXA anti-mouse 647 fluorophore bound to mouse anti-β-tubulin primary antibody. (**C**) ALEXA – anti-rabbit 488 fluorophore bound to anti-P-gp primary antibody. (**D**) Merge of A,B and C. (**E**) merge of UKF-NB-3. (**F**) Merge of UKF-NB-3rDOCE4 negative control.

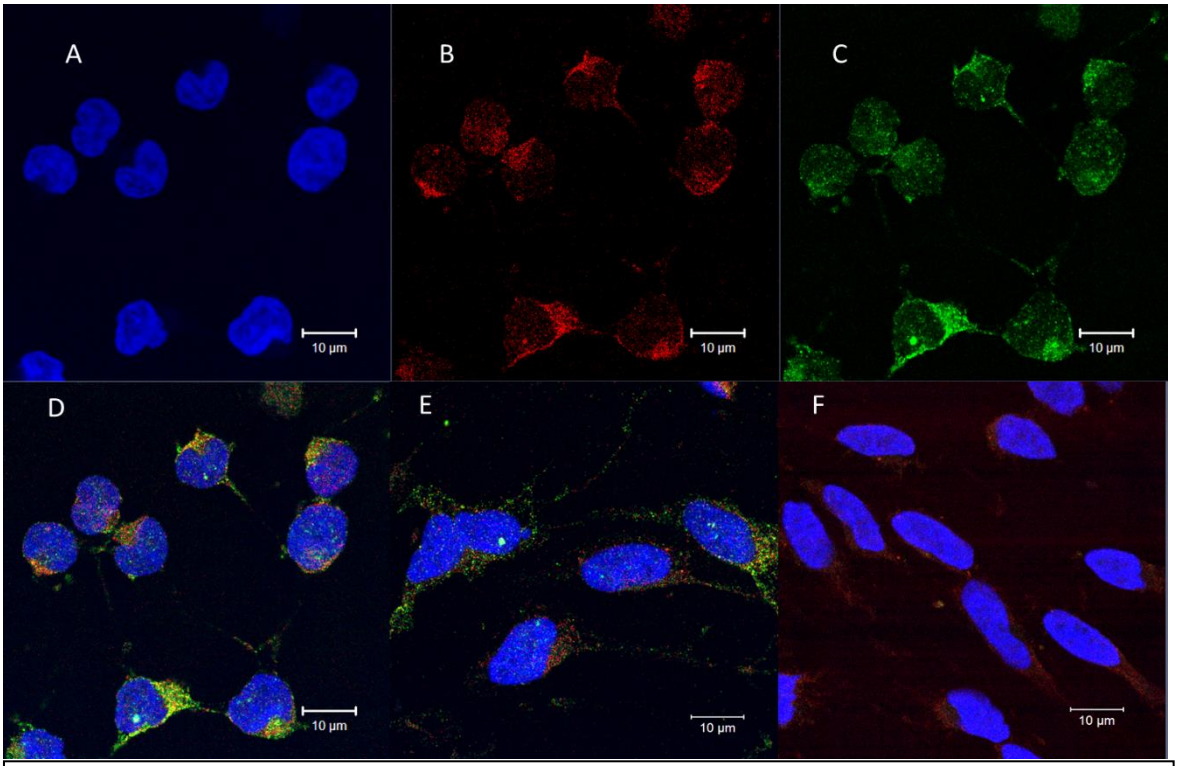


Figure 3.17, UKF-NB-3rDOCE1, (A,B,C,D) UKF-NB-3 (E). (**A**) DAPI staining of UKF-NB-3rDOCE1 nuclei. (**B**) ALEXA anti-mouse 647 fluorophore bound to mouse anti-β-tubulin primary antibody. (**C**) ALEXA – anti-rabbit 488 fluorophore bound to anti-P-gp primary antibody. (**D**) Merge of A,B and C. (**E**) merge of UKF-NB-3. (**F**) Merge of UKF-NB-3rDOCE1 negative control.

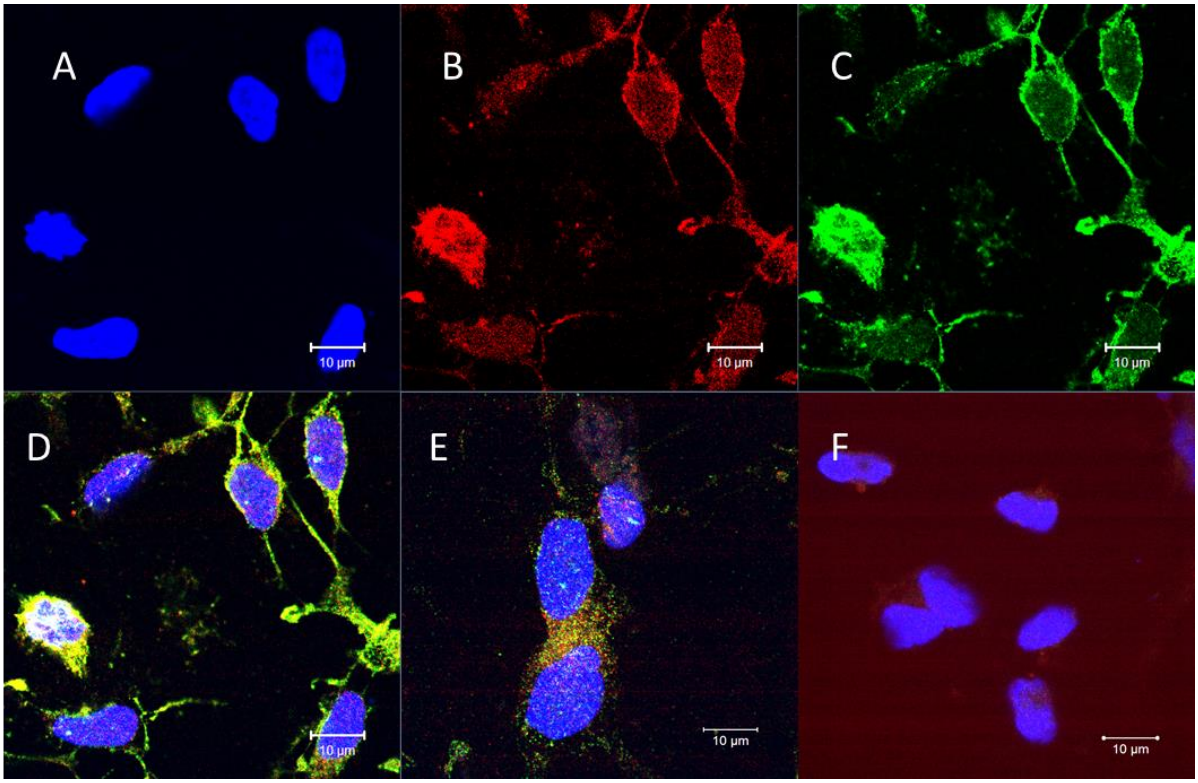


Figure 3.18, UKF-NB-3rDOCE4 0.37nMol wkly, (A,B,C,D) UKF-NB-3 (E). (**A**) DAPI staining of UKF-NB-3rDOCE4 0.37nMol wkly nuclei. (**B**) ALEXA 647 fluorophore bound to anti-β-tubulin primary antibody. (**C**) ALEXA 488 fluorophore bound to anti-P-gp primary antibody. (**D**) Merge of A,B and C. (**E**) merge of UKF-NB-3. (**F**) Merge of UKF-NB-3rDOCE4 0.37nMol wkly negative control.

Here we saw that ABCB1 expression correlated with the degree of resistance to drugs with known ABCB1 mediated resistance mechanisms, supporting the evidence acquired from the cross-resistance panel. The highest expression was seen in the sublines cultivated in additional exposure to docetaxel which also possessed the highest degrees of resistance. The sub-line with the lowest expression of ABCB1 was UKF-NB-3rDOCE1. This sub-line also showed the lowest degrees of resistance. The images also show β-tubulin to be most overexpressed in the sub-lines with the highest degrees of resistance (UKF-NB-3rDOCE4 0.37nMol wkly and UKF-NB-3rDOCE4 0.74nMol wkly).

Chapter 4

Discussion

The potential for different drugs to cause anti-cancer drug resistance is still poorly understood. In this project we have evaluated the development of a standardised protocol for assessing the potential of individual drugs to cause acquired resistance. To do this we have increased docetaxel exposure in UKF-NB-3 sub-lines which have been adapted to docetaxel. Here we found that cultivating cell lines adapted to docetaxel in twice the IC50 concentration results in a substantially increased degree of resistance to a range of drugs, each possessing high binding affinity to ABCB1. It therefore follows that long term exposure of UKF-NB-3 cell lines results in ABCB1 mediated multi-drug resistance. Additionally, once resistance has been acquired, increasing the concentration of docetaxel in the growth medium results in substantially higher degrees of resistance not only to docetaxel, but also to paclitaxel and vincristine suggesting that subpopulations of cells exist within the sublines which possess the phenotype required to tolerate higher concentrations of drug. Furthermore, the failure of UKF-NB-3rDOCE1 sublines to adapt to increased exposure to docetaxel support the hypothesis that individual subpopulations also exist within the main subline which rely on other cells for resistance.

The treatment of UKF-NB-3 with docetaxel, paclitaxel, cabazitaxel and epothilone b resulted in the adaptation and development of resistant sublines to docetaxel and paclitaxel.

This immediately demonstrates that acquired resistance is more favourably developed to docetaxel and paclitaxel, both of which have high binding affinity to ABCB1 with well-established ABCB1 mediated drug resistance pathways. UKF-NB-3 was not able to adapt to treatment with cabazitaxel and epothilone b. These sublines remained sensitive to both drugs, which are known not to be effluxed by ABCB1 due to their much lower binding affinity.

Adaptation of cell lines to docetaxel

Of the five UKF-NB-3 sublines lines treated with docetaxel, two (UKF-NB-3rDOCE1 and UKF-NB-3DOCE4) possessed the necessary phenotype for resistance. The other three sublines remained sensitive to docetaxel when cultivated in accordance to the standardised adaptation protocol defined at the beginning of the study. This was replicated in sub-lines cultivated in paclitaxel according to the standardised adaptation protocol, with two of the five sublines surviving.

This was not observed in the sublines treated with cabazitaxel and epothilone b, all of which died, remaining sensitive to the drugs. This occurred despite all four drugs targeting β-tubulin. Docetaxel and paclitaxel are known to cause stabilisation of microtubules leading to cell cycle arrest during mitosis and subsequent apoptosis. Cabazitaxel and epothilone b cause more tubulin polymerisation in microtubules through the same mechanism of action as docetaxel and paclitaxel (Mita et al. 2009), however both cabazitaxel and epothilone b have much lower binding affinity for ABCB1 efflux proteins (Tsao et al. 2011) (Parker et al. 2014). Suggesting a mechanism for resistance through over expression of ABCB1.

To further investigate the resistance induced by the standardised adaptation protocol to docetaxel and paclitaxel, we split two further sets of sublines from the original surviving docetaxel treated sub-lines. From each surviving sub-line two further flasks were split, one cultivated in 0.37nM added every week (UKF-NB-3rDOCE4 0.37nMol wkly, UKF-NB-3rDOCE1 0.37nMol wkly) and one cultivated in double the IC50 concentration of each drug added weekly (UKF-NB-3r DOCE 4 0.74 nMol wkly, UKF-NB-3r DOCE 1 0.74 nMol wkly). The origin of these sublines can be seen in **figure 3.1** and **figure 3.2.** The same was done with the two surviving paclitaxel treated sub-lines. Two splits were cultivated in the IC50 concentration of paclitaxel added every week (UKF-NB-3rPCL4 0.37nMol wkly, UKF-NB-3rPCL1 0.37nMol wkly) and two splits were cultivated in double the IC50 concentration, added every week (UKF-NB-3rPCL 4 0.74 nMol wkly, UKF-NB-3rPCL1 0.74 nMol wkly).

Both sets of sublines split from the original paclitaxel treated sublines were able to adapt to growth in the increased conditions of drug exposure. This project has focussed on docetaxel resistance, hence data on these sublines has not been included.

The sublines split from the original docetaxel treated subline were able to adapt to the increased conditions of docetaxel exposure in some sublines but not in others. UKF-NB-3rDOCE1 sublines lines were not able to adapt to the increased conditions of exposure to docetaxel, these sublines died. However UKF-NB-3rDOCE4 sublines were able to adapt to growth in docetaxel added every week and twice the IC50 concentration of docetaxel added every week.

The differences seen in the ability of each subline to adapt to increased drug exposure suggest the sublines are heterogeneous cell populations, possessing different phenotypes for resistance within the same subline (Goodspeed et al. 2016)(Meacham & Morrison 2013)

Through the process of splitting the sub-lines (taking 10,000 cells from the population into the next week) and the selective pressures exerted upon the cells by the addition of drug (making the resistant phenotypes more favourable) (Ovens & Naugler 2012)(Greaves 2013) it is likely that the cells possessing the resistant phenotype have been more common throughout the general population within each sub-line. Consequently it is more likely that these resistant cells will be part of the 10,000 cells taken into the next week and that these cells will be capable of growth in the presence of drug.

Conversely, if the 10,000 cells taken from the previous flask during the split possesses too few of these cells possessing a resistant phenotype for the flask to become properly confluent within a week, then these sublines are likely to die.

Furthermore, this suggests that cancer cells with docetaxel sensitive phenotypes may rely upon others within the population for their resistance to docetaxel. This could be a contributing factor to the death of some of the sub-lines. For example if within the 10,000 cells taken at each split, there were too few cells with resistant phenotypes compared to those without (and relied upon the resistance of other cells within the population) to support resistance in the new population, the sub-line is likely to die (Sun et al. 2016).

The resistant phenotype displayed by these cells may be present intrinsically. In this case the resistant subline are simply a result of the selective pressures exerted upon the subline by exposure to drug. Here the resistant cells would have always been present within the population and it is only through the gradual removal of the non-resistant cells that we see resistance in individual sub-lines as a whole.

In those subline which could not adapt and died, the process of taking only 10,000 cells at each passage would have removed the resistant cells through creating a genetic "bottleneck". This is one possible explanation for the survival of certain sublines over others.

Alternatively the resistant phenotypes observed could be a result of mutations caused by exposure to drug, or somatic mutations. The combined data from this project suggests that ABCB1 overexpression is responsible for the resistance to docetaxel (Ween et al. 2015).

Further work would be required to conclude exactly whether the resistant phenotypes were intrinsically present within the population from the beginning of the study, or if the exposure to tubulin binding agents has resulted in somatic mutations within the cell lines causing over expression of ABCB1 and the development of acquired resistance. The latter hypothesis may be supported by the observation that cell lines became more resistant over the course of the experiment, possibly due to increasing mutations as a result of prolonged drug exposure. Furthermore, the increased exposure to docetaxel (UKF-NB-3rDOCE4 0.74nMol) resulted in increased resistance levels to docetaxel, possibly as a result of the increased concentration of drug causing mutations resulting in ABCB1 overexpression. To fully understand this, we have extracted genomic DNA from the cell lines for exome sequencing and analysis. This data can be compared to UKF-NB-3 cell line and any mutations resulting from docetaxel treatment can be identified. The heterogeneity of the sub lines could be determined using fluorescent activated cell sorting (FACS) (Hölzenspies et al. 2015). The exact proportion of resistant and non-resistant phenotypes within sublines lines, and the proportion of resistant cells compared to other sublines could be investigated.

The hypothesis of acquired resistance of the sub-line as a whole through docetaxel induced mutations seems unlikely considering the mechanism of docetaxel action. Tubulin binding agents are less likely to result in somatic mutations than a DNA damaging agent such as cisplatin, which induces apoptosis through DNA alkylation (Basu & Krishnamurthy 2010). With this in mind, it is more likely that resistant phenotypes were already present within the cell populations.

The development of resistance was monitored through the determination of IC50 values at 4 week time points. This showed that whilst in general resistance increased to docetaxel increased over time, there were occasions where the IC50 value fell below that of UKF-NB-3, suggesting that the sublines were not resistant to docetaxel during these weeks. This however was obviously not the case as the sublines were still able to proliferate in the presence of docetaxel. These anomalies are likely to be a result of the selective nature of the MTT assay (van Tonder et al. 2015). As only 5000 cells were used per well of the 96 well plate for the MTT assay (seeding density was selected to maintain log phase growth throughout incubation period) it is possible that these cells selected did not possess the resistant phenotype resulting in data which showed increased sensitivity to docetaxel. Another explanation for the abnormally low degrees of resistance seen in some weeks could be that the cells selected for the MTT assay were already under stress after cultivation in docetaxel and were not able to cope as well with the further stresses of the MTT assay (trypinising and additional drug). The increased stress placed on the cells by the addition of docetaxel can be seen in **figure 3.6.** Here the degree of resistance to docetaxel was substantially higher in the weeks when subline UKF-NB-3rDOCE1 was cultivated without the addition of docetaxel to the growth medium.

This was not observed for UKF-NB-3rDOCE4 however this sub-line was able to produce further sublines (UKF-NB-3rDOCE4 0.37nMol wkly and UKF-NB-3rDOCE4 0.74 nMol wkly) which UKF-NB-3rDOCE1 was not (see **figure 3.1**). This demonstrates that UKF-NB-3rDOCE 4 was able to cope with higher concentrations of docetaxel than UKF-NB-3rDOCE1, therefore the accumulative effect of docetaxel during the viability assays had much less impact on cell viability. The reduced effect of accumulated docetaxel in UKF-NB-3rDOCE4 additionally suggests that this subline demonstrates higher expression of ABCB1. The reduced cell viability in UKF-NB-3rDOCE1 with the addition of docetaxel in the growth medium could be the result of saturation of ABCB1 transporters, causing increased intracellular accumulation of docetaxel during the MTT assay, resulting in increased stabilisation of tubulin and reduced cell viability (Lin & Yamazaki 2003). Conversely, higher expression of ABCB1 would allow increased efflux of docetaxel, despite the already increased intracellular concentration caused by the additional docetaxel in the growth medium. We saw this effect in UKF-NB-3rDOCE4 suggesting more expression of ABCB1 than in the other cell lines which became less viable in the presence of additional docetaxel.

Characterisation of docetaxel resistance

Once resistance to the surviving docetaxel treated cell lines had been established, we further characterised the resistance through selecting a panel of drugs to determine any patterns of cross resistance. Here we investigated the sensitivity of the docetaxel treated sub-lines of UKF-NB-3 to docetaxel, paclitaxel, cabazitaxel, epothilone b, vincristine, cisplatin, crizotinib and topotecan.

Here we found that long term treatment of UKF-NB-3 sub-lines with docetaxel conferred multi-drug resistance (MDR) to the sub-lines against docetaxel, paclitaxel and vincristine.

The sub-lines remained sensitive to the other drugs included in the panel. It has been very well established that paclitaxel and docetaxel have very similar mechanisms of action with the major difference being the higher potency of docetaxel over paclitaxel (Ringel & Horwitz 1991). As both drugs have similar binding sites on β-tubulin (Lavelle et al. 1995), and both induce apoptosis through the stabilisation and polymerisation of microtubules leading to cell cycle arrest during mitosis, it is not surprising that sub- lines with resistance to one additionally show resistance to the other. Furthermore, both drugs also have very well established ABCB1 mediated resistance pathways, leading to the development of cabazitaxel and other low ABCB1 affinity taxanes. Both docetaxel and paclitaxel are readily effluxed from the cell through their high affinity to ABCB1 (Parker et al. 2014). This consequently reduces the intracellular concentration of the drugs below the effective dose, allowing repair mechanisms to overcome the action of the drug which remains within the cell. Therefore its overexpression will reduce the efficacy of docetaxel, paclitaxel and any other drug with high enough binding affinity to ABCB1.

The docetaxel treated cell lines additionally showed resistance to vincristine, also a tubulin binding agent. Vincristine however differs from the action of paclitaxel and docetaxel. Whereas the taxanes inhibit microtubule disassembly, vincristine binds to free tubulin heterodimers, preventing the polymerisation of microtubules. Vincristine, like paclitaxel and docetaxel has high affinity for ABCB1 (Böhme et al. 1995) (Gidding 1999).

Potency of vincristine is dependent on its intracellular concentration and cells exposed to vincristine can continue through the cell cycle with low enough concentrations of vincristine (Takano et al. 1993). This demonstrates how ABCB1 overexpression is able to cause resistance.

Of all the drugs included in the panel to assess cross-resistance, all those which are known substrates of ABCB1 (with the exception of topotecan) had greatly reduced cytotoxicity on all surviving UKF-NB-3 cell lines treated with docetaxel. Furthermore, the drugs to which UKF-NB-3 cells treated with docetaxel did not show resistance do not have ABCB1 as a known mechanism of resistance (Ween et al. 2015). However, paclitaxel, docetaxel and vincristine are also known substrates of ABCC10 as shown in **table 1.6** and **table 3.1**. This data alone suggests that resistance could be mediated by ABCB1 and ABCC10 as none of the other drugs included in the cross-resistance panel are known to be substrates of ABCC10 (Hopper-Borge et al. 2004)

The data obtained from the cross-resistance panel and the patterns of resistance demonstrated strongly suggests that the resistance observed in the UKF-NB-3 sub-lines treated with docetaxel was mediated by ABCB1 overexpression.

Expression of ABCB1 and β-tubulin

We investigated the expression of ABCB1 further using confocal microscopy. As the sub-lines were treated with a tubulin binding agent, and demonstrated cross-resistance to tubulin binding agents, we additionally stained for β-tubulin (in addition to ABCB1) to determine any changes in tubulin expression or distribution across the different sub-lines.

Here, we saw higher degrees of ABCB1 expression in the docetaxel treated sub-lines with higher degrees of resistance to drugs with ABCB1 mediated resistance mechanisms. UKF-NB-3rDOCE4 0.74nMol wkly, showed the highest degree of resistance to docetaxel, paclitaxel and vincristine (all of which are known to have ABCB1 mediated resistance) also had the highest degree of ABCB1 expression. (See **figure 3.14 – figure 3.18**) From the overexpression of ABCB1 seen in the confocal microscopy, resistance to drugs known to have ABCB1 mediated resistance observed in the cross-resistance panel and with evidence from the existing literature (Lombard et al. 2017), we can strongly support the hypothesis that docetaxel resistance through long term exposure is heavily mediated by ABCB1 drug efflux. To conclude definitively if this is the case, functional assays would need to be performed to confirm that the expressed ABCB1 transporters are functionally active in effluxing drug. This is discussed further later in this chapter.

The confocal images used here also showed β-tubulin to be overexpressed. The negative control showed relatively high levels of non-specific binding of tubulin secondary antibody. Given more time, the immunostaining would be repeated with further wash steps and additional optimisation of antibody concentration. Despite this, there is evidence in the literature of tubulin overexpression associated with tubulin binding agent resistance. A study by Mozzetti *et al*. suggested that this was the most prominent mechanism of paclitaxel resistance in breast cancer (Mozzetti et al. 2005)(Ferrandina et al. 2006). Consequently, it is possible that resistance is mediated by both ABCB1 and tubulin over expression.

In the images used in this study, there is quite a high amount of background signal for ALEXA 647 fluorophore (bound to anti-β-tubulin primary antibody) (**figure 3.14-figure**

3.18). This could be reduced with further wash steps using tween buffer during the immunostaining protocol (as described in chapter 2). Additional optimisation of the primary and secondary antibodies used could also result in lower background signal. The protocol used here was optimised for use with UKF-NB-3 and the use of poly-l-lysine was found to be essential for cell adherence. This however could also have effects on the amount of background signal as a result of unbound secondary antibody getting caught within the poly-l-lysine coating.

In order to validate this work and the cell lines used, and considering the high number of passages and the long term nature of the study, testing for mycoplasma contamination was performed, with results negative for mycoplasma. Additional steps were also taken to ensure the sub-lines remained separate. By working with only one sub line at a time in the tissue culture hood and using different sets of reagents for each sub-line, the identity of the sublines was maintained and any risk of cross-contamination eliminated. Additionally, each data point represents an n number of at least three biological replicates. Likewise, the microscopy images (both brightfield and confocal) were representative of the tissue culture flask or slide from which the image was acquired and confocal setting were identical for acquisition of each image. We also produced some cell viability data using SRB assays to ensure docetaxel was having no effect on the efficiency of the assay (Riss et al. 2004). The IC50 data produced was similar and we did not continue with SRB assays.

Future work

Whilst we have strong evidence that resistance to docetaxel is ABCB1 dependent within our docetaxel treated sub-lines, further work could prove this beyond doubt. Conducting the cross resistance panel in the presence of chemical inhibitors of ABCB1 such as verapamil and zosuquidar could prove the resistance is dependent on ABCB1 overexpression (Raza et al. 2015). This would be required to confirm the ABCB1 transporters are functional and actively effluxing the drugs. To prove this hypothesis, sensitivity to the drugs would be restored with inhibition of ABCB1. Additional work to quantify expression could be done using flow cytometry. Here the cells would be labelled with fluorescent antibody for ABCB1 in a similar method to that used here for fluorescent microscopy the process is described by (Strouse et al. 2013) . The signal from the bound fluorophore could then be counted to give a quantitative figure of ABCB1 expression. By comparing this to the degrees of resistance observed across cell lines further evidence could be gained for an ABCB1 mediated resistance pathway. FACS could also be used to determine the degree of heterogeneity within each sub-lines, confirming if the survival of some sub-lines over others was likely to be a result of the selective process of splitting the sublines with only 10,000 cells each week (Hölzenspies et al. 2015). Here, high levels of heterogeneity within the sublines which followed the standardised adaptation protocol (UKF-NB-3rDOCE1 and UKF-NB-3rDOCE4) and a high degree of homogeneity within the further sub-lines with increased drug exposure (UKF-NB-3rDOCE4 0.37nMol wkly and UKF-NB-3rDOCE4 0.74 nMol wkly) would give weight to this hypothesis and determine if resistance to docetaxel was intrinsic or acquired. Furthermore, repeats of fluorescent microscopy would add validity to the claims of ABCB1 overexpression. With more time,

optimising the concentration of the antibodies used could reduce background signal, allowing further comparisons on β-tubulin expression and investigation into its overexpression as a resistance mechanism. Western blots could be a lower cost alternative to investigate further ABCB1 and β-tubulin expression.

Conclusion

This project is part of a larger project which will result in a specific protocol for defining drug adaptation in cancer cell lines. This protocol will define the ability of different anti-cancer drugs to cause acquired resistance in cancer cell lines. The work done here will contribute towards the protocol, allowing the degree of resistance caused to be assessed. With a standard protocol to determine individual drug adaptation and the extent of resistance caused, clinically relevant drugs can be tested for their potential to cause acquired drug resistance in cancer patients and the degree of resistance that the drug may be able to cause. Higher degrees of drug resistance may require clinically unachievable doses to overcome the resistance. This protocol will allow this to be quantified for individual drugs. Furthermore, we have shown that docetaxel treatment can result in substantial degrees of resistance, likely to be mediated by ABCB1 overexpression resulting in multidrug resistance.

This project has made three main conclusions. Long term exposure to docetaxel can result in ABCB1 overexpression within sub-lines of UKF-NB-3. Specific sub-populations of UKF-NB-3 show phenotypes for different degrees of docetaxel resistance. Finally, the standard adaptation protocol defined at the start of the study is a suitable for determining the potential of drugs to cause acquired resistance in cancer cells. This part of the project showed that by increasing exposure to drug during the standard protocol it would be possible to determine the degree of resistance caused by exposure to a specific drug being used clinically or a novel drug candidate for clinical trial.

References

Ahmed, A.A. et al., 2007. The extracellular matrix protein TGFBI induces microtubule stabilization and sensitizes ovarian cancers to paclitaxel. *Cancer cell*, 12(6), pp.514–27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18068629 [Accessed July 23, 2017].

Altmann, K.H., Wartmann, M. & O'Reilly, T., 2000. Epothilones and related structures--a new class of microtubule inhibitors with potent in vivo antitumor activity. *Biochimica et biophysica acta*, 1470(3), pp.M79-91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10799747 [Accessed July 23, 2017].

Alvarado, C.S. et al., 1997. Natural history and biology of stage A neuroblastoma: a Pediatric Oncology Group Study. *Journal of pediatric hematology/oncology*, 22(3), pp.197–205. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10864050 [Accessed July 1, 2017].

Anon, Cancer mortality statistics | Cancer Research UK. Available at: http://www.cancerresearchuk.org/health-professional/cancer-statistics/mortality#heading-Five [Accessed June 30, 2017a].

Anon, Data collection and quality implications | Cancer Research UK. Available at: http://www.cancerresearchuk.org/health-professional/cancer-statistics/cancer-stats-explained/data-collection-implications#heading-Nine [Accessed June 30, 2017b].

Anon, Neuroblastoma | Children with Cancer UK. Available at: https://www.childrenwithcancer.org.uk/childhood-cancer-info/cancer-types/neuroblastoma/ [Accessed July 1, 2017c].

Bart, J. et al., 2000. The blood-brain barrier and oncology: new insights into function and modulation. *Cancer Treatment Reviews*, 26(6), pp.449–462. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0305737200901944 [Accessed July 23, 2017].

Basu, A. & Krishnamurthy, S., 2010. Cellular responses to Cisplatin-induced DNA damage. *Journal of nucleic acids*, 2010. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20811617 [Accessed July 26, 2017].

ter Beek, J., Guskov, A. & Slotboom, D.J., 2014. Structural diversity of ABC transporters. *The Journal of general physiology*, 143(4), pp.419–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24638992 [Accessed July 22, 2017].

Böhme, A., Ganser, A. & Hoelzer, D., 1995. Aggravation of vincristine-induced neurotoxicity by itraconazole in the treatment of adult ALL. *Annals of hematology*, 71(6), pp.311–2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8534764 [Accessed July 24, 2017].

Bowman, L.C. et al., 1997. Genetic Staging of Unresectable or Metastatic Neuroblastomain Infants: a Pediatric Oncology Group Study. *JNCI Journal of the National Cancer Institute*, 89(5), pp.373–380. Available at: https://academic.oup.com/jnci/article-lookup/doi/10.1093/jnci/89.5.373 [Accessed July 1, 2017].

Brodeur, G. et al., 1984. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science*, 224(4653). Available at: http://science.sciencemag.org/content/224/4653/1121 [Accessed July 3, 2017].

Chen, J.-G. & Horwitz, S.B., 2002. Differential Mitotic Responses to Microtubule-stabilizing and -destabilizing Drugs. *Cancer Research*, 62(7). Available at: http://cancerres.aacrjournals.org/content/62/7/1935.long [Accessed July 22, 2017].

Cisternino, S. et al., 2003. Nonlinear accumulation in the brain of the new taxoid TXD258 following saturation of P-glycoprotein at the blood-brain barrier in mice and rats. *British journal of pharmacology*, 138(7), pp.1367–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12711638 [Accessed July 23, 2017].

Díaz, J.F. et al., 1998. Changes in microtubule protofilament number induced by Taxol binding to an easily accessible site. Internal microtubule dynamics. *The Journal of biological chemistry*, 273(50), pp.33803–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9837970 [Accessed July 23, 2017].

Dolman, M.E.M. et al., 2015. Cyclin-Dependent Kinase Inhibitor AT7519 as a Potential Drug for MYCN-Dependent Neuroblastoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 21(22), pp.5100–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26202950 [Accessed July 20, 2017].

Ferrandina, G. et al., 2006. Class III β-Tubulin Overexpression Is a Marker of Poor Clinical Outcome in Advanced Ovarian Cancer Patients. *Clinical Cancer Research*, 12(9). Available at: http://clincancerres.aacrjournals.org/content/12/9/2774.long [Accessed July 27, 2017].

Gidding, C., 1999. Vincristine revisited. *Critical Reviews in Oncology/Hematology*, 29(3), pp.267–287. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1040842898000237 [Accessed July 24, 2017].

Goodspeed, A. et al., 2016. Tumor-Derived Cell Lines as Molecular Models of Cancer Pharmacogenomics. *Molecular cancer research : MCR*, 14(1), pp.3–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26248648 [Accessed July 26, 2017].

Greaves, M., 2013. Cancer stem cells as “units of selection”. *Evolutionary applications*, 6(1), pp.102–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23396760 [Accessed July 26, 2017].

Gustafson, W.C. & Weiss, W.A., 2010. Myc proteins as therapeutic targets. *Oncogene*, 29(9), pp.1249–59. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20101214 [Accessed July 3, 2017].

Hölzenspies, J., Cruz, G. Dela & Brickman, J.M., 2015. Resolving Heterogeneity: Fluorescence-Activated Cell Sorting of Dynamic Cell Populations from Feeder-Free Mouse Embryonic Stem Cell Culture. In *Methods in molecular biology (Clifton, N.J.)*. pp. 25–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26026884 [Accessed July 26, 2017].

Hopper-Borge, E. et al., 2004. Analysis of the Drug Resistance Profile of Multidrug Resistance Protein 7 (ABCC10). *Cancer Research*, 64(14), pp.4927–4930. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15256465 [Accessed July 26, 2017].

Hornick, J.E. et al., 2008. Live-cell analysis of mitotic spindle formation in taxol-treated cells. *Cell motility and the cytoskeleton*, 65(8), pp.595–613. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18481305 [Accessed July 22, 2017].

Jordan, M.A. & Wilson, L., 2004. Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, 4(4), pp.253–265. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15057285 [Accessed July 22, 2017].

Juliano, R.L. & Ling, V., 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 455(1), pp.152–162. Available at: http://linkinghub.elsevier.com/retrieve/pii/0005273676901607 [Accessed July 22, 2017].

Kathawala, R.J. et al., 2015. ATP-binding cassette subfamily B member 1 (ABCB1) and subfamily C member 10 (ABCC10) are not primary resistance factors for cabazitaxel. *Chinese journal of cancer*, 34(3), pp.115–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25962593 [Accessed July 16, 2017].

Kowalski, R.J., Giannakakou, P. & Hamel, E., 1997. Activities of the microtubule-stabilizing agents epothilones A and B with purified tubulin and in cells resistant to paclitaxel (Taxol(R)). *The Journal of biological chemistry*, 272(4), pp.2534–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8999970 [Accessed July 23, 2017].

Kumar, A., Marqués, M. & Carrera, A.C., 2006. Phosphoinositide 3-kinase activation in late G1 is required for c-Myc stabilization and S phase entry. *Molecular and cellular biology*, 26(23), pp.9116–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17015466 [Accessed July 20, 2017].

Lavelle, F. et al., 1995. Preclinical evaluation of docetaxel (Taxotere). *Seminars in oncology*, 22(2 Suppl 4), pp.3–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7740328 [Accessed July 23, 2017].

Lin, J.H. & Yamazaki, M., 2003. Role of P-Glycoprotein in Pharmacokinetics. *Clinical Pharmacokinetics*, 42(1), pp.59–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12489979 [Accessed July 26, 2017].

Lombard, A.P. et al., 2017. ABCB1 mediates cabazitaxel-docetaxel cross-resistance in advanced prostate cancer. *Molecular Cancer Therapeutics*, p.molcanther.0179.2017. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28698198 [Accessed July 26, 2017].

Longley, D. & Johnston, P., 2005. Molecular mechanisms of drug resistance. *The Journal of Pathology*, 205(2), pp.275–292. Available at: http://doi.wiley.com/10.1002/path.1706 [Accessed June 30, 2017].

Maris, J.M., 2010. Recent advances in neuroblastoma. *The New England journal of medicine*, 362(23), pp.2202–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20558371 [Accessed July 3, 2017].

Matthay, K.K. et al., 1999. Treatment of High-Risk Neuroblastoma with Intensive Chemotherapy, Radiotherapy, Autologous Bone Marrow Transplantation, and 13- *cis* -Retinoic Acid. *New England Journal of Medicine*, 341(16), pp.1165–1173. Available at: http://www.nejm.org/doi/abs/10.1056/NEJM199910143411601 [Accessed July 1, 2017].

McGuire, W.P. et al., 1989. Taxol: A Unique Antineoplastic Agent with Significant Activity in Advanced Ovarian Epithelial Neoplasms. *Annals of Internal Medicine*, 111(4), p.273. Available at: http://annals.org/article.aspx?doi=10.7326/0003-4819-111-4-273 [Accessed July 22, 2017].

Meacham, C.E. & Morrison, S.J., 2013. Tumour heterogeneity and cancer cell plasticity. *Nature*, 501(7467), pp.328–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24048065 [Accessed July 26, 2017].

Michaelis, M. et al., 2011. Adaptation of cancer cells from different entities to the MDM2 inhibitor nutlin-3 results in the emergence of p53-mutated multi-drug-resistant cancer cells. *Cell death & disease*, 2(12), p.e243. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22170099 [Accessed June 22, 2017].

Miller, K.D. et al., 2016. Cancer treatment and survivorship statistics, 2016. *CA: A Cancer Journal for Clinicians*, 66(4), pp.271–289. Available at: http://doi.wiley.com/10.3322/caac.21349 [Accessed June 30, 2017].

Mita, A.C. et al., 2009. Phase I and Pharmacokinetic Study of XRP6258 (RPR 116258A), a Novel Taxane, Administered as a 1-Hour Infusion Every 3 Weeks in Patients with Advanced Solid Tumors. *Clinical Cancer Research*, 15(2), pp.723–730. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19147780 [Accessed July 23, 2017].

Molenaar, J.J. et al., 2009. Inactivation of CDK2 is synthetically lethal to MYCN over-expressing cancer cells. *Proceedings of the National Academy of Sciences of the United States of America*, 106(31), pp.12968–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19525400 [Accessed July 20, 2017].

Mozzetti, S. et al., 2005. Class III beta-tubulin overexpression is a prominent mechanism of paclitaxel resistance in ovarian cancer patients. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 11(1), pp.298–305. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15671559 [Accessed July 27, 2017].

Mullassery, D., Farrelly, P. & Losty, P.D., 2014. Does Aggressive Surgical Resection Improve Survival in Advanced Stage 3 and 4 Neuroblastoma? A Systematic Review and Meta-analysis. *Pediatric Hematology and Oncology*, 31(8), pp.703–716. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25247398 [Accessed July 3, 2017].

Nettles, J.H. et al., 2004. The Binding Mode of Epothilone A on , -Tubulin by Electron Crystallography. *Science*, 305(5685), pp.866–869. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15297674 [Accessed July 23, 2017].

Nogales, E. et al., 1995. Structure of tubulin at 6.5 Å and location of the taxol-binding site. *Nature*, 375(6530), pp.424–427. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7760939 [Accessed July 23, 2017].

Ovens, K. & Naugler, C., 2012. Preliminary evidence of different selection pressures on cancer cells as compared to normal tissues. *Theoretical biology & medical modelling*, 9, p.44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23146329 [Accessed July 26, 2017].

Park, J.R., Eggert, A. & Caron, H., 2008. Neuroblastoma: Biology, Prognosis, and Treatment. *Pediatric Clinics of North America*, 55(1), pp.97–120. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0031395507001575 [Accessed July 3, 2017].

Parker, A.L., Kavallaris, M. & McCarroll, J.A., 2014. Microtubules and their role in cellular stress in cancer. *Frontiers in oncology*, 4, p.153. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24995158 [Accessed July 22, 2017].

Puissant, A. et al., 2013. Targeting MYPuissant, A. et al., 2013. Targeting MYCN in neuroblastoma by BET bromodomain inhibition. Cancer discovery, 3(3), pp.308–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23430699 [Accessed July 3, 2017].CN in neuroblastoma by BET bromodoma. *Cancer discovery*, 3(3), pp.308–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23430699 [Accessed July 3, 2017].

Raza, A. et al., 2015. Effects of third generation P-glycoprotein inhibitors on the sensitivity of drug-resistant and -susceptible isolates of Haemonchus contortus to anthelmintics in vitro. *Veterinary Parasitology*, 211(1–2), pp.80–88. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25986327 [Accessed July 26, 2017].

Ringel, I. & Horwitz, S.B., 1991. Studies with RP 56976 (taxotere): a semisynthetic analogue of taxol. *Journal of the National Cancer Institute*, 83(4), pp.288–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1671606 [Accessed July 23, 2017].

Riss, T.L. et al., 2004. *Cell Viability Assays*, Eli Lilly & Company and the National Center for Advancing Translational Sciences. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23805433 [Accessed July 26, 2017].

SCHIFF, P.B., FANT, J. & HORWITZ, S.B., 1979. Promotion of microtubule assembly in vitro by taxol. *Nature*, 277(5698), pp.665–667. Available at: http://www.nature.com/articles/277665a0 [Accessed July 22, 2017].

Schwab, M., Varmus, H.E. & Bishop, J.M., 1985. Human N-myc gene contributes to neoplastic transformation of mammalian cells in culture. *Nature*, 316(6024), pp.160–2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4040214 [Accessed July 3, 2017].

Sjostrom, S.K. et al., 2005. The Cdk1 Complex Plays a Prime Role in Regulating N-Myc Phosphorylation and Turnover in Neural Precursors. *Developmental Cell*, 9(3), pp.327–338. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1534580705002947 [Accessed July 3, 2017].

Staller, P. et al., 2001. Repression of p15INK4b expression by Myc through association with Miz-1. *Nature cell biology*, 3(4), pp.392–9. Available at: http://www.nature.com/doifinder/10.1038/35070076 [Accessed July 20, 2017].

Strouse, J.J. et al., 2013. Fluorescent substrates for flow cytometric evaluation of efflux inhibition in ABCB1, ABCC1, and ABCG2 transporters. *Analytical Biochemistry*, 437(1), pp.77–87. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23470221 [Accessed July 26, 2017].

Sun, D. et al., 2016. Differential selective pressure alters rate of drug resistance acquisition in heterogeneous tumor populations. *Nature Publishing Group*. Available at: https://www.nature.com/articles/srep36198.pdf [Accessed July 26, 2017].

Takano, Y., Okudaira, M. & Harmon, B.V., 1993. Apoptosis Induced by Microtubule Disrupting Drugs in Cultured Human Lymphoma Cells. *Pathology - Research and Practice*, 189(2), pp.197–203. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0344033811800920 [Accessed July 24, 2017].

van Tonder, A., Joubert, A.M. & Cromarty, A.D., 2015. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC research notes*, 8, p.47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25884200 [Accessed July 26, 2017].

Tsao, C.-K. et al., 2011. Clinical development of cabazitaxel for the treatment of castration-resistant prostate cancer. *Clinical Medicine Insights. Oncology*, 5, pp.163–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21695098 [Accessed July 23, 2017].

Vasiliou, V., Vasiliou, K. & Nebert, D.W., 2009. Human ATP-binding cassette (ABC) transporter family. *Human genomics*, 3(3), pp.281–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19403462 [Accessed July 22, 2017].

Walton, M.I. et al., 2012. CCT244747 is a novel potent and selective CHK1 inhibitor with oral efficacy alone and in combination with genotoxic anticancer drugs. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 18(20), pp.5650–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22929806 [Accessed June 22, 2017].

Ween, M.P. et al., 2015. The role of ABC transporters in ovarian cancer progression and chemoresistance. *Critical Reviews in Oncology/Hematology*, 96(2), pp.220–256. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1040842815001018 [Accessed July 16, 2017].

Wils, P. et al., 1994. Polarized transport of docetaxel and vinblastine mediated by P-

glycoprotein in human intestinal epithelial cell monolayers. *Biochemical Pharmacology*, 48(7), pp.1528–1530. Available at: http://linkinghub.elsevier.com/retrieve/pii/0006295294905800 [Accessed July 16, 2017].