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Initial analysis of drug-induced heterogeneity in cancer cell lines

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in

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2. Abstract

Drug-induced heterogeneity of cancer cells is a known concept that is not well understood. Heterogeneity is a common characteristic of cancer and describes how cancer cells of the same tumour can show distinct morphologies and genetic phenotypes, due to an increased cell cycle and thus elevated occurrence of mutations. The exposure of cancer to drugs is thought to be a diver of heterogeneity leading to acquired-drug resistance. This is when a cancer mass that was once sensitive to a particular drug is no longer effected by it and results in treatment failure, leading to loss of life of the patient if no other treatments are available. The problem with heterogeneity and acquired-drug resistance is they can't be easily studied due to the need for multiple and extensive biopsies of the patient's cancer. Therefore the only way to study the process of the formation of drug-induced heterogeneity is through experimental investigations into model cell lines.

Here we attempt to standardise an adaptation protocol for the formation of resistant cell lines, with the aim of creating a protocol that allows comparison between different cell lines and different drugs. We also wish to better understand the formation of resistance in UKF-NB-3 cell lines with the hope of later identifying cross-resistance with other drugs. Moreover, we aim the better understand heterogeneity by establishing clonal cell lines of UKF-NB-3 and exposing them to a number of tubulin-binding drugs, with the aim of making comparisons between the clones and other established clones with acquired resistance to a number of drugs.

We found that repeated adaptation of cell lines to the same drug results in resistant heterogenic sub-lines. We also concluded that exposure of cells to drugs of a similar mechanism of action can lead to varying results.

3. Introduction

I) Neuroblastoma

Neuroblastoma is a childhood cancer that is often diagnosed in the first year of life and accounts for 15% of paediatric oncology deaths in children aged between 0 to 14 years old (Ries *et al.* 2007) . Patients with a high-risk phenotype have a long-term survival rate of less than 40% (Maris *et al*, 2007). Like with most cancers, a high risk phenotype is defined by the spread of the cancer to other parts of the body, and for neuroblastoma, around 60% of cases result in a metastatic disease state (Cheung & Dyer, 2013). Neuroblastoma is an embryonal tumour from cells of sympathoadrenal linage of the neural crest (Anderson *et al,* 1991). This is only present during embryogenesis, suggestive of why the cancer is mainly found amongst young children, resulting in tumour development within the sympathetic nervous system, primarily (65%) within the abdomen but also identified in the neck, chest and pelvis regions (Cheung & Dyer, 2013; Maris *et al*, 2007).

There are two types of Neuroblastoma; Familial or Sporadic. Familial neuroblastoma is rare and linked to a hereditary cause (Maris *et al* 2002). It is characterized by mutations in the paired-like homeobox 2b (*PHOX2B*) which encodes transcription factors that promote cell cycle exit and neural differentiation (Mosse *et al.* 2004). Other mutations also include those found in the anaplastic lymphoma receptor tyrosine kinase (*ALK*) gene which regulates proliferation and differentiation (Cheung & Dyer, 2013). Sporadic neuroblastoma is commonly associated with the amplification of the *MYCN* gene, occuring in 22% of tumours and often results in a negative outcome for the patient (Brodeur, 2003).

Metastises often develop in cortical bone, bone marrow, liver and lymph nodes (Quinn, 1979) resulting in a diagnosis of the cancer after an inicial mis-diagnosis of a blood-born

cancer. There is often a poor outcome for those whos cancer has reached stage 4 i.e. when the cancer begins to metastasise. However this is not the case for those whos cancer is in the rare and special 4S disease stage. This is a unique occurance, found in 5% of cases, where the matastesises that have disseminated to the afore mentioned organs, spontaneously regress (D'Angio *et al*, 1971). These patients then fall into a low-risk category with a survival rate between 85-90% unless *MYCN* amplifications are observed (Nickerson *et al* 2000).

Though there is a low survival rate for patients with a high risk phenotype, greater than 50% of these paitents would have originally responeded well to selective multi-modal therapies. This relapes is often attributed to the development acquired drug resistance or clonal evolution (the expantion of risistant cells within a heterogeneous tumor environment).

II) Microtubules

Microtubules make up part of cytoskeleton, a system of filaments involved in the organisation and stability of the cell. Microtubules play a specific role in cell division and make up cells mitotic spindles.

They are typically comprised of 13 protofilaments that form a hollow tube approximately 25nm in diameter. Protofilaments consist of a heterodimer of α- and β-tubulin molecules that when bound to GDP through an active site on the β-tubulin, causes a bent confirmation that prevents their incorporation into a microtubule (Howard *et al*, 2007). However, exchange of GDP for GTP allows the dimer to become incorporated into the microtubule and thus GTP-tubulin is often thought of as the fuel for polymerization. The hydrolysis of GTP, on the other hand, permits disassembly of the dimer (Abal, 2003).

Microtubules have two districted ends, one (referred to as the minus-end) is anchored to the centrosome while the plus-end is highly dynamic and fast growing and thus is the site of

polymerisation and depolymerisation (Etienne-Manneville, 2010). The regulation of microtubule growth is dictated by the interactions with a family of proteins known as microtubule associated proteins (MAPs). They also control the number of microtubules present during mitosis.

III) Tubulin binding agents

Tubulin binding agents (TBA) interfere with the dynamics of microtubules during mitosis. They aim to cause cell cycle arrest leading to the prevention of cellular proliferation and in some cases, trigger apoptosis of the cell. TBAs consist of a wide range of drugs, both natural and synthetic, that can be separated depending on their mode of action, as TBAs can both stabilise microtubules preventing their depolymerisation or destabilise them, encouraging depolymerisation and preventing further growth of the tubule (Kavallaris, 2010). Examples of different drugs can be seen below in **Table 1.**

Destabilising agents		Stabilising agents
Vina binding site	Colchicine binding site	Taxane binding site
Vincristine	Combretastatin A4	Docetaxel
Vinblastine	2-Methoxyestradiol	Epithilone B
		Paclitaxel

Table 1. A characterised selection of TBAs and their binding sites on the microtubule.

Stabilising agents bind to the taxane pocket of β-tubulin molecules within the microtubule (depicted in figure 1). It has been shown that the binding of stabilising agents causes a conformational change within the corresponding tubulin molecule (specifically causing a short-helix at the M-loop within the β-tubulin molecule) that allows for lateral tubulin interactions within the microtubule, thereby stabilizing the molecule and preventing its

depolymerisation (Prota *et al,* 2013). Paclitaxel, for example, mimics the nucleotide GTP and thus promotes stabilisation by preventing GTP hydrolysis that permits microtubule disassembly (Abal, 2003).

Destabilising agents, or polymerisation inhibitors, prevent further tubulin molecules from being added and thus have the ability to reduce the microtubule polymer mass at high concentrations. Colchicine analogues bind to soluble β-tubulin creating a complex that can be incorporated in to the microtubule during polymerisation (see figure 1). However, the complex brings about a conformational change blocking further tubulin dimers from binding and results in disablement of the microtubule due to structural instability (Chen *et al,* 2009).

In contrast, Vinca-alkaloids bind to β-tubulin at the Vinca-binding domain (see figure 1) of dimers that are already incorporated into the microtubule. They tend to bind with greater affinity to molecules at the ends of microtubules and this high affinity remains in low drug concentrations (Jordan, 2012).

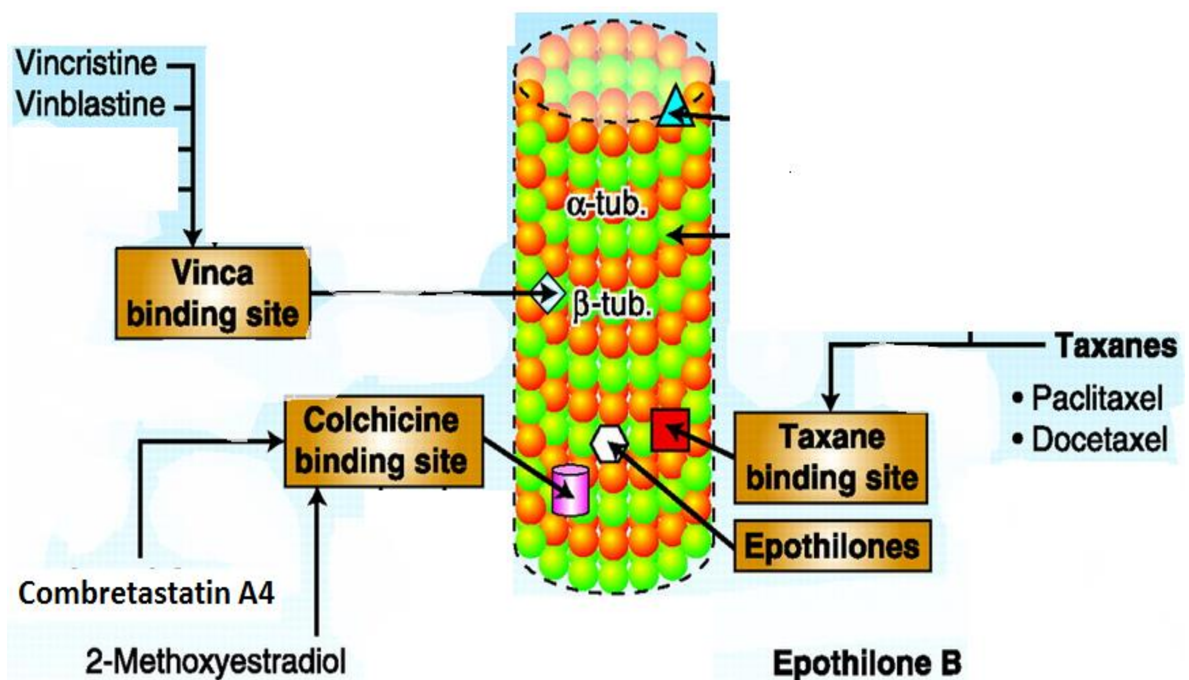

Figure 1. A microtubule and the binding sites of the Tubulin binding drugs.
(Originally extracted from Morris P.G *et al.* 2008)

IV) Drug resistance

Treatment failure can often be attributed to chemotherapeutic resistance of the Neuroblastoma cells, especially in metastatic cancers where 90% of treatment failure can be credited to resistance (Longley *et al* 2005). Drug resistance can be either intrinsic (previously untreated cells are unaffected by drug) or aquired (initially respond to therapy before developing resistance) and it is this aquired drug resistance that results in the low long-term suervival rates of high-risk paitents.

It is widely understood that there are multiple durg resistance mechanisms exploited by cancer cells that contribute to aquired resistance. A cancer cell may have one or more of

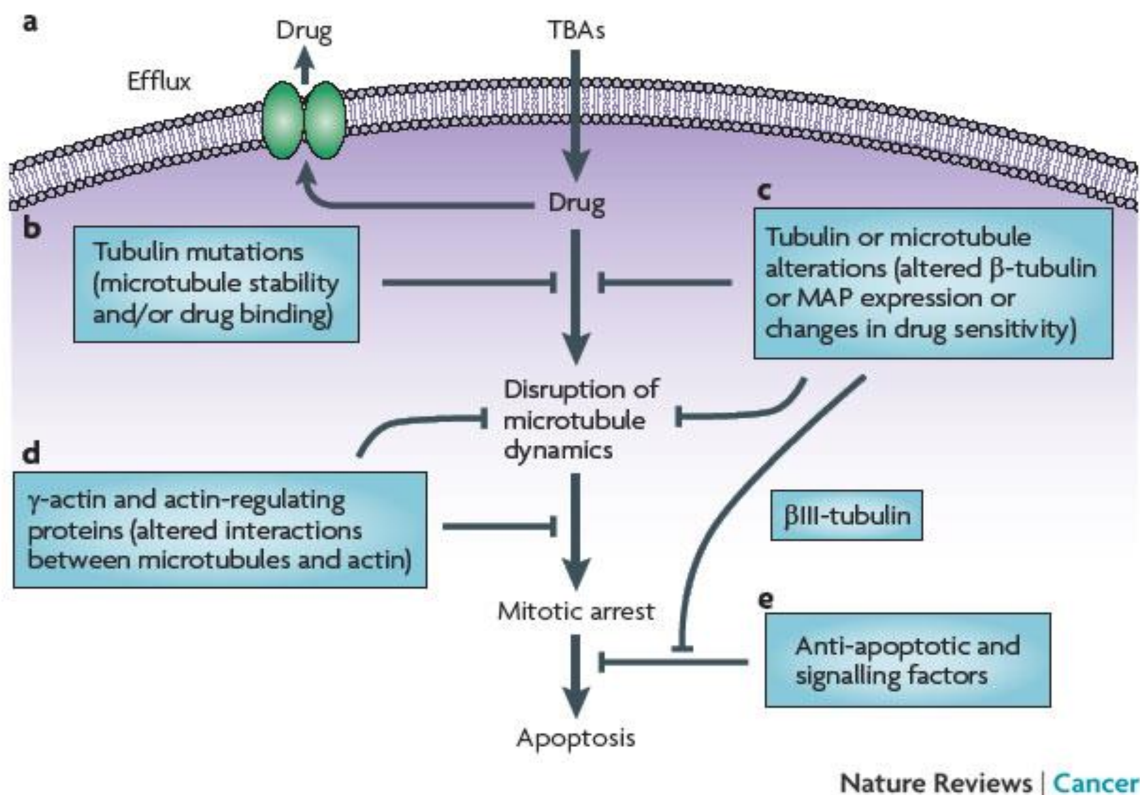

Figure 2. A diagram to show the mechanisms of cancer cells in resistance to tubulin-binding anti-cancer drugs. a) Efflux of drug through the ABC transporter preventing the drug from reaching its target site. b) Genetic mutations affecting microtubule stability preventing binding of the drug. c) Alterations to tubulin and microtubules preventing the drug from binding. d) Changes to the cytoskeleton reduces the effect of the drug once bound. e) Changes to anti-apoptotic factors prevent the cytotoxic effects of the drug. (Extracted from Kavallaris, 2010)

these mechanisms and due to the heterogenic nature of cancer, difference cells within the same tumour mass can have entierly different mechanisms of action. Furthermore Multidrug resistance can occur after treatment with a particular anti-cancer drug, resulting in cross-resistance to other drugs the cancer has yet to be exposed to (Ambudkar *et al.* 1999; Leslie *et al.* 2005). Figure 2 depics an overview of the known mechanisms involved in cancer cell resistance to tubulin-binding drugs.

The first mechanism involves the efflux of the drug through ATP binding cassette (ABC) transporters (figure 2 a). This transmembrane transporter is involved in multiple cellular transport processes, including the transference of anti-cancer drugs across the cytoplasmic membrane. Evidence for its use as a resistance mechanisms is supported by the finding that multiple ABC transporters were found to be highly expressed on cancer cells (Holohan *et al.* 2013).

Genetic mutations/post-translational modifications to tubulin have also been identified as a resistance mechanism as they lead to increased expression of drug targets (figure 2 b). Though this seems counter intuitive, Holohan (2013) argues that this increase reduces the effectiveness of the drugs as more must bind to have the same effect as in a cancer without these mutations. Further tubulin alterations, such as the expression of different β-tubulin isoforms, can result in reduced sensitivity to tubulin-binding agents. For example, the over expression of βIII-tubulin results in a lowered efficacy of a particular tubulin-binding agent due to it having less affinity than normal β-tubulin (Kavallaris, 2010), depicted in figure 2 c).

Additional mutations in γ-actin and its regulatory proteins results in changes to the cells cytoskeleton (Figure 2 d). This in turn has an influence on the ability of the drug to induce cell death. In addition, changes to the apoptotic signalling pathways have also been

identified in cells with resistance to tubulin binding agents, as seen in figure 2 e) (Kavallaris, 2010).

V) Tumour Heterogeneity

It is understood that cancer cells within a tumour can differ genetically from one another due to the increased rate of cell division and high level of mutations. It has also been argued that this heterogeneity can be further effected by the selection pressures from anti-cancer drugs that favour the less sensitive cells. In short, the continuous formation of mutations give rise to a heterogeneous cell population which when introduced to drug treatments, exerts selective pressures that favour more resistance cells resulting in a process that develops resistant called clonal evolution.

VI) Aims

The two main aims of the project are as follows. Firstly, we aim to better understand Intra-tumour heterogeneity by investigating the drug sensitivity profiles of non-resistant UKF-NB-3 clones. And secondly, we aim to understand acquired drug resistance through the establishment of resistant UKF-NB-3 cell lines through adaptation using a standardised protocol. Specifically, this Thesis will focus on establishing and standardising the protocol and assessing its validity.

4. Materials and Methods

I) Cells

The MYCN-amplified neuroblastoma cell line UKF-NB-3, was established from a bone marrow metastasis of a patient with stage 4 neuroblastoma (Kotchetkov et al., 2003). The drug-resistant sub-lines and single-cell derived clones of UKF-NB-3 were derived from the resistant cancer cell line (RCCL) collection (www.kent.ac.uk/stms/cmp/RCCL/RCCLabout.html). Drug-resistant UKF-NB-3 sub-lines have been established as previously described (Kotchetkov et al., 2003 Michaelis *et al.* 2011).

II) Reagents

500mL Iscove's Modified Dulbecco's Medium (IMDM), 100IU/mL penicillin and 100mg/mL streptomycin were supplied by Life technologies (Paisley, UK) and foetal bovine serum (FBS) was obtained from Sigma-Aldrich (Ayrshire, UK).

Phosphate Buffered Saline (PBS) was prepared by dissolving one PBS salt tablet (Oxoid Limited, Hampshire, UK) in 100mL of ddH_2O. The solution was then sterilised using an autoclave and aliquoted as needed before being stored at 2°C-4°C. Trypsin and EDTA, was purchased from Life Technologies (Paisley, UK)

Sodium Dodecyl Sulphate (SDS) was obtained from Fisher Scientific (Loughborough, UK). SDS solution was prepared by dissolving 200g SDS powder in a mixture of, 400mL of purified water (Barnstead NANOpure Diamond) and 400mL of dimethylformamide (DMF; Fisher Scientific, Loughborough, UK) and adjusting the pH to 4.7. SDS was stored at room temperature.

500mg of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT; Universal Biologicals, Cambridge, UK) was dissolved in 250mL of PBS prior to sterile filtration in a laminar flow hood using a 0.22µM bottle-top filter ( ) and a vacuum pump. The MTT solution was stored between 2°C-4°C and protected from sunlight.

Dimethyl Sulphoxide (DMSO) and 0.04% trypan blue solution were purchased from Sigma-Aldrich (Ayrshire, UK).

III) Drugs

Combretastatin A4, 2-methoxystradiol, vincristine, vinblastin, docetaxel and epothilone B were obtained from Cambridge Biosciences (Cayman Chemicals, USA). All compounds were stored as a stock solution at 1mg/mL in DMSO, except for paclitaxel which was diluted in ethanol (Fisher Scientific, UK). L181.1 and L181.2 were obtained from Dr Maxwell Casely-Hayford (Medway School of Pharmacy, Universities of Kent and Greenwich, Chatham, UK.

IV) Cell Passaging

The cells grew in T25 flasks at a ratio of 1 in 10 in an environment of 37°C and 5% CO_2. When the cells reached about 70% confluency, the cell culture medium (IMDM supplemented with 10% FBS, 1% penicillin and 1% streptomycin) was removed and the cells were washed using 2mL of PBS. Then, the cells were incubated for 1-2 minutes in 1mL of Trypsin (0.05%, w/v)/EDTA (0.02%, w/v) solution at 37°C and 5% CO_2 in order to detach the cells from the bottom of the flask. Next, the cells were then resuspended in 9mLs of pre-warmed cell culture medium. The cells were transferred into a new flask. The splitting rates ranged from 1:10 to 1:50 according to the experimental requirements.

V) Cell count

20µL cell suspension was mixed with 40µL of trypan blue solution and 20µl PBS and then counted in a haemocytometer (Hawksley; Lancing, UK). Viable (unstained) cells were then counted under a microscope at 40x magnification.

VI) Cell Viability Assay

MTT assays were performed in 96-well plates (Greiner Bio One Ltd; Stonehouse, UK) as previously described (Kotchetkov et al., 2003; Michaelis *et al.* 2011). 100µL of cell cultured medium was added to the outer wells and positive control wells (figure 3).

Wells	1	2 (-ve)	3	4	5	6	7	8	9	10	11 (+ve)	12
A	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL
B	100µL	50µL									100µL	100µL
C	100µL	50µL									100µL	100µL
D	100µL	50µL									100µL	100µL
E	100µL	50µL									100µL	100µL
F	100µL	50µL									100µL	100µL
G	100µL	50µL									100µL	100µL
H	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL	100µL

Figure 3 A schematic of where media is added in a 96 well plate. The wells 2B-2G (negative control wells) were used as cell only controls. The wells 11B-11G (positive control wells) were used to determine the minimum value indicating the level of background absorbance created by the presence of cell culture medium in the absence of cells. Different colours indicate a difference between the wells.

Then, 50µL of cell suspension were added to the wells serving as untreated cell control and to the wells in which the cells were grown in the presence of drug (figure 4). If not indicated otherwise, 5000 cells/well were used.

Wells	1	2 (-ve)	3	4	5	6	7	8	9	10	11 (+ve)	12
A												
B		50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL		
C		50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL		
D		50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL		
E		50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL		
F		50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL		
G		50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL	50µL		
H												

Figure 4 A schematic of where cells are added in a 96 well plate. Wells 2B-2G were used as cell only control wells to determine maximum value when incubated without drug. Different colours indicate a difference between the wells.

In a separate drug block (Corning Inc. New York, USA), 8 point serial drug dilutions were prepared. If not stated otherwise, 1 in 4 dilution steps were applied. 50µL of each drug dilution were added to the respective wells (figure 5). The arrangement of 96 well plates allowed for two drugs to be tested against one cell line at any one time. It also allowed for triplicates of each drug to be achieved.

Wells	1	2 (-ve)	3	4	5	6	7	8	9	10	11 (+ve)	12
A												
B		1st Drug	100	25	6.25	1.5625	0.390625	0.097656	0.024414	0.006104	50µL	
C			100	25	6.25	1.5625	0.390625	0.097656	0.024414	0.006104		
D			100	25	6.25	1.5625	0.390625	0.097656	0.024414	0.006104		
E		2nd Drug	50	12.5	3.125	0.78125	0.195313	0.048828	0.012207	0.003052	50µL	
F			50	12.5	3.125	0.78125	0.195313	0.048828	0.012207	0.003052		
G			50	12.5	3.125	0.78125	0.195313	0.048828	0.012207	0.003052		
H												

Figure 5 A schematic of where the drugs are added in a 96 well plate. The concentrations are in nM. The starting concentrations of 100nM and 50nM are examples of possible 8 point, 1 in 4 drug dilutions and the starting concentration depend on the drug being added. 50µL of drug were added to each well.

The plates were incubated for 120hrs at 37°C and 5% CO_2. Then 25µL of MTT reagent were added to all wells, prior to a further incubation period of four hours. Then 100µL SDS solution was added and the cells were incubated over night at 37°C and 5% CO_2 in order to dissolve the non-soluble Formazan salt that was formed through MTT metabolisation in the mitochondria. The absorbance was measured at 600nm using a BMG Labtech Fluostar Omega plate reader (Ortenberg, Germany).

VII) Data analysis

The cell viability of drug-treated cells was determined relative to untreated control according to the following formula:

$$(Absorbance\ of\ drug\ treated\ cells\ (nM) -\ Mean\ absorbance\ of\ cell\ free\ background\ (nM))/(Mean\ absorbance\ of\ untreated\ control\ cells\ (nM) -\ Mean\ ab$$

The concentrations that reduced cell viability by 50% IC50 or 90% IC90 were determined using Calcusyn (Biosoft; Cambridge, UK).

5. Results

I) Sensitivity of the clonal UKF-NB-3 sub-lines to tubulin-biding agents

The clonal UKF-NB-3 sub-lines were tested for sensitivity to the tubulin-binding agents Combretastatin A4 and 2-Methoxyestradiol (microtubule destabilising agents targeted to the colchicine domain), Vincristine and Vinblastine (microtubule destabilising agents targeted to the vinca-domain), and Docetaxel and Epothilone B (microtubule stabilising agents that target the taxoid domain). The drugs L181.1 and L181.2 (novel Combretastatin A4 derivatives) were also screened against the clonal UKF-NB-3 sublines.

In order to detect differences in the drug sensitivity of the individual clones, we determined the mean IC50 of a drug in all clones. Then we identified clones in which the IC50 was >2-fold higher or lower than the mean. In addition, we identified >2-fold differences between the individual clones.

The Combretastatin A4 IC50 values of the investigated clones range from 0.24nM to 0.55nM (figure **6a**). This is similar to the Combretastatin A4 IC50 in the UKF-NB-3 cell line (0.4nM ± 0.05nM). The mean IC50 value was 0.24nM. Only clones 4 and 7 displayed an IC50 value that was >2-fold different from the mean IC50 (see Appendix **I b)**).

The IC50 values for the Combretastatin A4 derivatives L181.1 and L181.2 means ranged from 55nM to 82nM (**Figure 6b**) and 175nM to 336nM (**Figure 6c**) respectively. Though all three sets of data appear to have the same slightly variable IC50 pattern amongst the clones, the amount of drug required to reach the IC50s for L181.1 and L181.2, when compared to that required for Combretastatin A4, are notably higher with up to a 200-fold and 1000-fold increase respectively (**Table 2)**. This shows that the derivatives have a lower molecular potency. Interestingly, all of the individual data points for both drugs lie within a 2-fold range. The IC50 for clone 7, however, was >2-fold lower than the mean IC50 but only very slightly (6nM relative to an IC50 of 266.27nM).

	Combretastatin A4	L181.1	Relative difference	L181.2	Relative difference
Clone 1	0.24nM±0.15nM	55.23nM±16.11nM	230.13	249.10nM±88.58nM	1037.92
Clone 2	0.37nM±0.2nM	66.19nM±25.22nM	178.89	306.03nM±65.75nM	827.11
Clone 3	0.37nM±0.05nM	57.61nM±23.77nM	155.86	175.44nM±44.91nM	474.16
Clone 4	0.55nM±0.55nM	82.44nM±22.96nM	149.89	336.37nM±77.46nM	611.58
Clone 7	0.53nM±0.14nM	72.78nM±29.15nM	137.32	264.42nM±90.99nM	498.91

Table 2. Comparison of IC50 values of Combretastatin A4 and its two derivatives, L181.1 and L181.2, of all clonal sub-lines and the relative differences between each derivative and Combretastatin A4. The data shows the relative potency of Combretastatin A4 to its two derivatives. Values given to 2 decimal places.

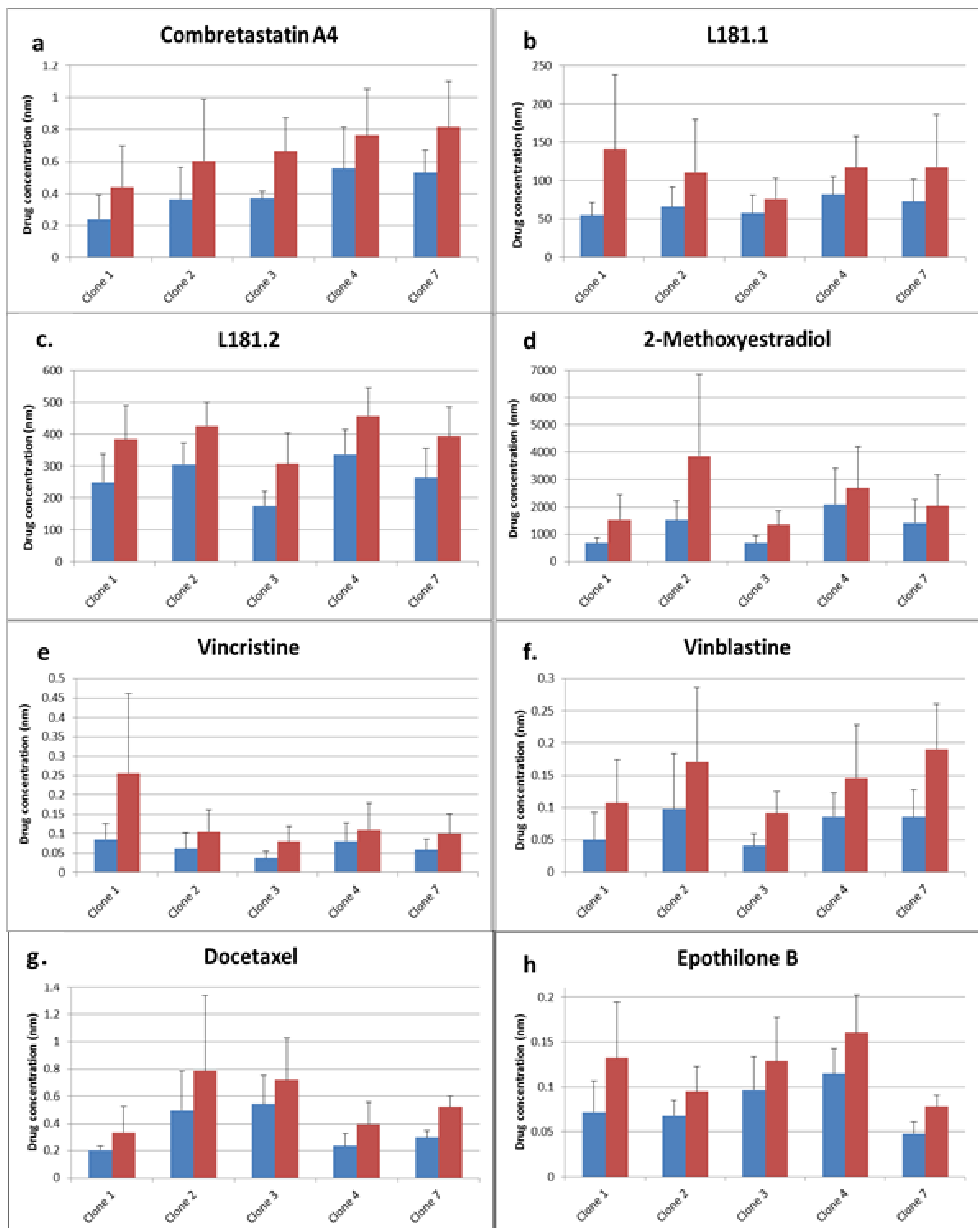

Figure 6 Drug concentrations (mean ± S.D.) at which cell viability is reduced by 50% (IC50) and 90% (IC90) for each of the UKF-NB-3 clones 1-4 and 7 as determined by MTT assay after a 120 h incubation period. The blues bars represent the IC50 values while the red bars depict the IC90 values. a, Cells incubated with Combretastatin A4. Highest start concentration used was 2000nM, with a modal concentration of 100nM. **b,** Cells incubated with L181.1. Highest concentration used was 50000nM. **c,** Cells incubated with L181.2. Highest concentration used was 80000nM. **d,** Cells incubated with 2-methoxyestradiol. Highest concentration used was 100000nM, with a modal start concentration of 30000nM. **e,** Cells incubated with Vincristine. Highest concentration used was 80nM with a modal start concentration of 10nM. **f,** Cells incubated with Vinblastine. Highest concentration used was 30nM, with a modal start concentration of 15nM. **g,** Cells incubated with Docetaxel. Highest concentration used was 100nM, with a modal start concentration of 10nM. **h,** Cells incubated with Epothilone B. Highest concentration used was 100nM. Other start concentrations include 50nM and 5nM.

The IC50s for the drug 2-methoxyestradiol can be seen in figure **6d**. Though no significant data was identified, the ranges between the individual data points for each clone differ dramatically, suggesting a high amount of variability between the clones. Clones 1 and 3 had the least amount of variability, with the data ranging from 366.69nM and 592.23nM respectively, while clones 2, 4 and 7, however, had large ranges of 1610.87nM, 3216.77nM and 1187.83nM.

Figures **6e and 6f,** depict the IC50s of the vinca-alkaloid drugs, vincristine and vinblastine. The IC50 values of the clones are almost identical when you compare the two drugs, suggesting similar properties. The data could also suggest that the clones retained the same anti-cancer drug resistance mechanisms. The cumulative data of all clones from both drugs showed no significant differences. Individually, when testing vincristine, Clones 2 and 3 showed some data points that did not fall in the two-fold ranges (2 and 1 respectively), while for Vinblastine clones 1 had 4 IC50s, 3 which were <2- and 1 which was >2-fold difference, and clone 2 had 2 less and 1 greater than a 2-fold difference. Again, as seen with most of the drugs, the differences aren't very large so do not reap a significant result.

The figure **6g,** showing the IC50 values for Docetaxel (range of 0.2nM to 0.55nM), again shows no significant differences between the clones. However the data points range significantly between clones, with clone 1 having a range of 0.05965nM while clone 2 has a range 10-fold greater at 0.5775nM

Again, figure 6**h,** depicting the mean IC50 range of 0.05nM-0.11nM, no significant differences between the clones when testing Epothilone B. However the data does support the finding that Epothilone B has increased efficacy compared to Docetaxel, as the mean IC50s of all the clones have a <4.5-fold increase from Epothilone B to Docetaxel, though the same binding site is used.

II) Adaptation of UKF-NB-3 to Paclitaxel by a standardised protocol

In order to investigate resistance acquisition in UKF-NB-3 cell lines a protocol was developed in order to standardise the way in which resistant cell lines are developed. The aim is to create comparable resistant cell lines to different drugs that will allow for the possible identification of cross-resistance. This is a long term study that began before I started my masters project and involves the adaptions of UKF-NB-3 to multiple tubulin-binding agents. As a result, I did not complete the first 17 weeks of work on the project and I only collected data from the cell lines adapted to the tubulin stabilising agent, Paclitaxel.

The protocol consists of exposing the 10 sub-lines of UKF-NB-3 cells to the previously defined IC50 concentration of paclitaxel (0.57nM), on alternate weeks. In these weeks, the number of cells required for incubation with the drug was 100,000 cells/ml, while in weeks were there was no drug, only 10,000 cells/ml was essential. At the end of each week the number of remaining cells was determined allowing the calculation of the fold-difference between the number of cell at the start and after a 168 hour incubation period.

Figures **7 and 8,** show the number of cells determined each week of the study, so far.

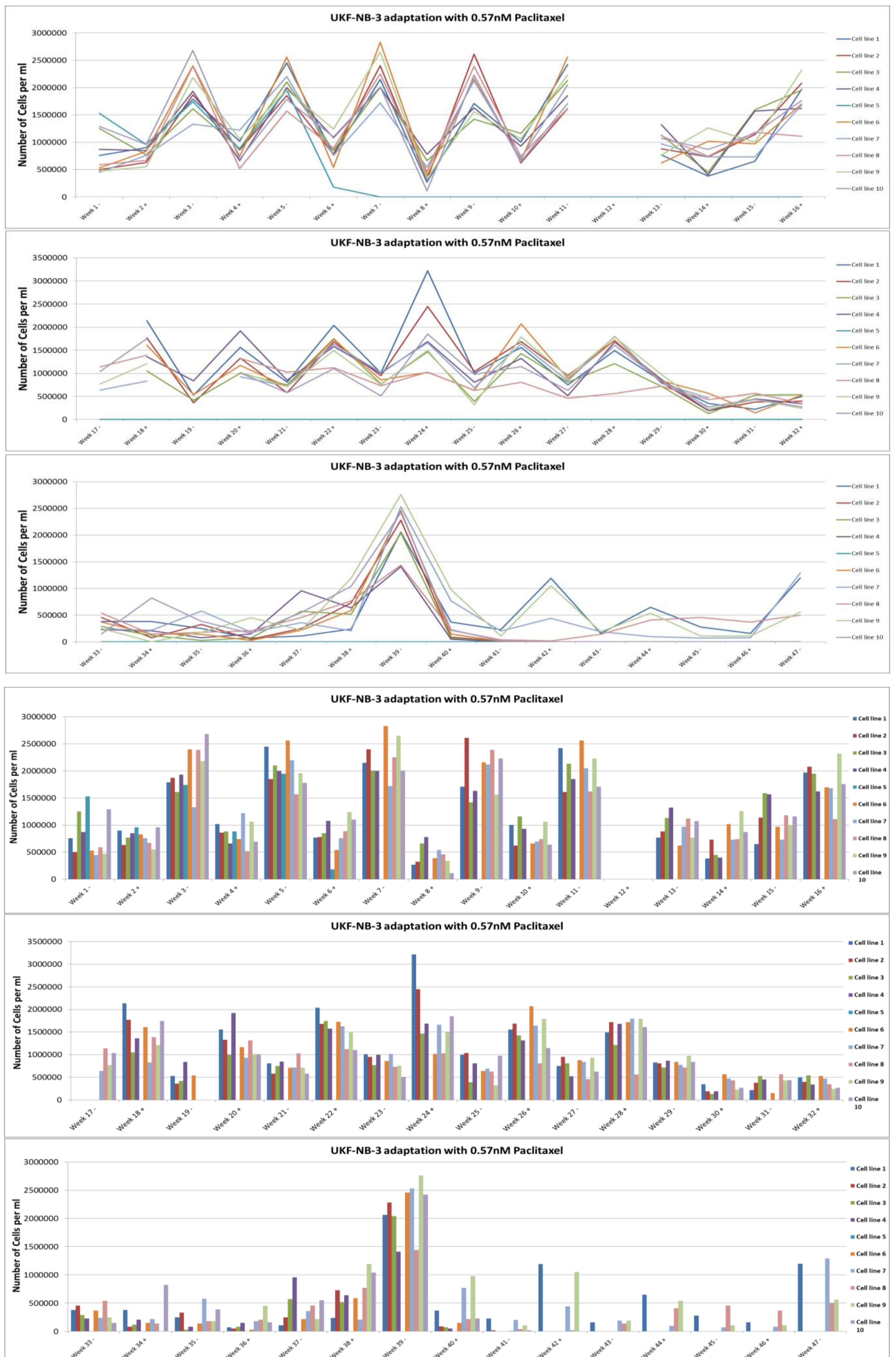

Figure 7. The number of cells per mL after each 168 hour incubation in either the presence (+) or absence (-) of 0.57nM Paclitaxel for cell lines 1-10. In week without drugs, the starting number of cells was 10,000/ml and for the weeks where drug was added, 100,000 cells/ml were required. From week 29 onwards, 10,000 cells/ml were added regardless. Cell line 5 was lost in week 7, followed by the loss of cell lines 3 and 4 in week 41 and cell lines 2,6 and 10 in week 42. No data was collected in week 12 and only for half the cell lines in weeks 17 and 19.

While both graphs in the figure show the same set of data, **7a,** allows the visualisation of the general change in cell number of the cell line UKF-NB-3 over the continuous weeks. The most observable trend (which can be seen in both figure **7a and 7b)** is the initial decline in the cell number in weeks where the cells were incubated with the drug. This is most likely down to the chemotherapeutic effects of the drug. However, from week 16 onwards, the number of cells counted when incubated with drug was higher than that counted in the drug free weeks. This suggests that the cells were becoming increasingly resistant to paclitaxel and the higher cell number reflects the 10-fold increase in the cell number incubated in those weeks.

Figure **7b,** on the other hand, allows a clearer look at the number of cell of each individual sub-line and how they differ from week to week. One interesting finding is that the number of cells counted for cell line 1 remains consistently higher than the other cell lines throughout the study, along with cell lines 9 and 10 (see raw data in Appendix). However, the higher IC50 values do not directly correlate with cell line survival since cell line 10 was lost in week 42. Moreover, cell line 8 generally had lower cell numbers relative to the over sub-lines, but when the cells were passaged, cell line 8 always out grew the other cell lines with it often achieving the highest confluence.

The data from both graphs shows a general decline in the number of cells in both the weeks with and without drug, as the duration of the study lengthens. The high cell numbers of the cell line in week 39 are explained by construction works during which the lab could not be accessed. Therefore, the cells were cultivated for 312 hours instead of 168 hours.

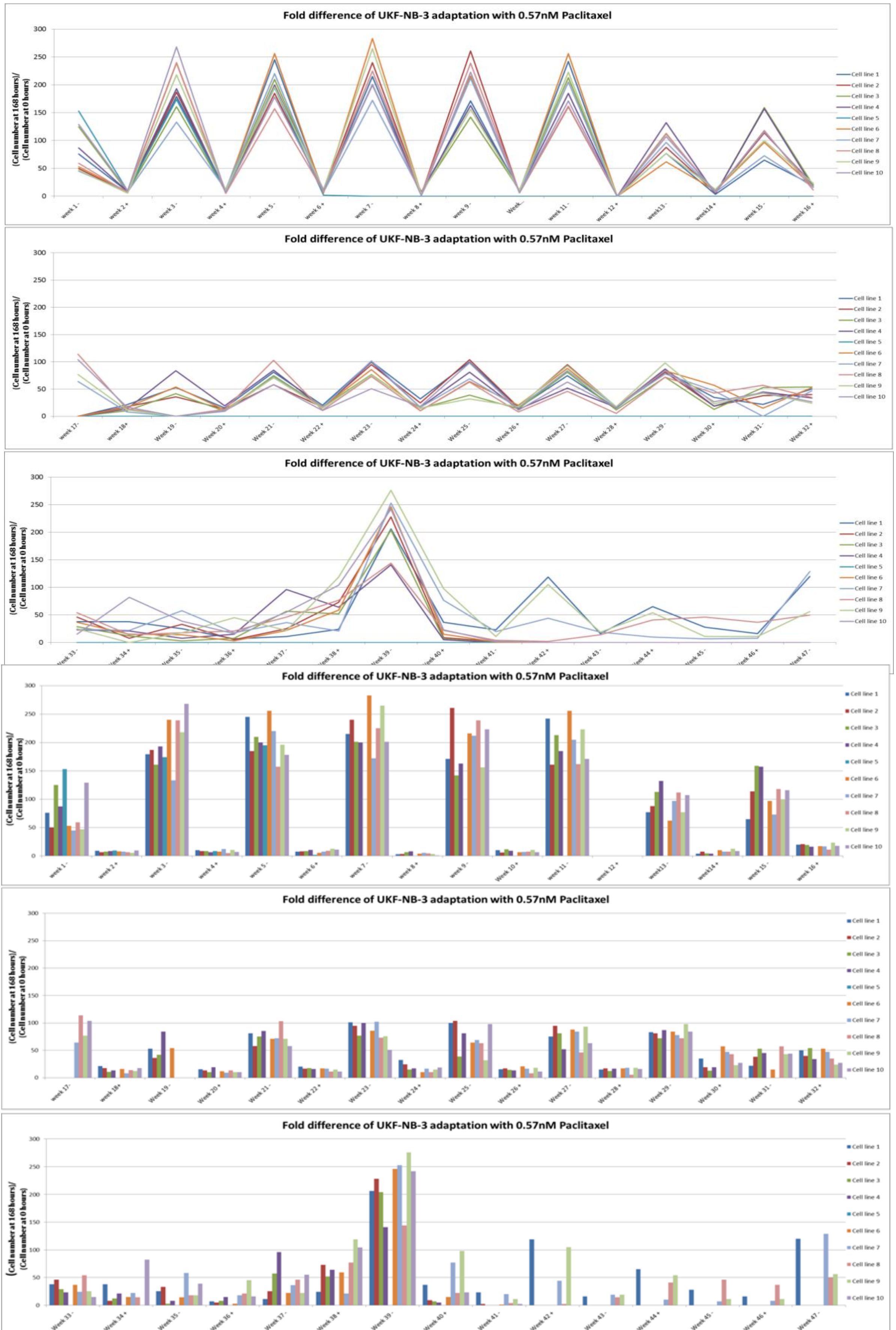

Figure 8 The fold difference between the cell number at 0 hours and the number of cells after 168 hour incubation for all 10 cell lines. In week without drugs, the starting number of cells was 10,000/ml and for the weeks where drug was added, 100,000 cells/ml were required. From week 29 onwards, 10,000 cells/ml were added regardless. Cell line 5 was lost in week 7, followed by the loss of cell lines 3 and 4 in week 41 and cell lines 2,6 and 10 in week 42. No data was collected in week 12 and only for half the cell lines in weeks 17 and 19.

Figure **8 a and b** (also see Appendix II b)-d))**,** depict the fold-difference in cell number between the number of cells incubated at the beginning of each week and the resulting cell number after the incubation period. Again figure 8**a,** shows the general trend of the change in cell number across the weeks, while figure **8b,** allows the comparison of individual data points. By calculating the fold difference, the change in cell number from the beginning of the incubation and the end, is observable and thus a clearer picture of the effect of paclitaxel on the resistance adaptation of each cell line can been seen. To better understand the effect the new protocol was having on the cells as a whole, the data was separated into weeks with drug and weeks without. The two new sets of data were then split in to 3 sections, each depicting a different phase in the study which can be visually identified in figure **8a**. The first groups were weeks 1-15 (no drug) and 2-14 (with drug), showing the initial phase, the second groups were weeks 17-29 (no drug) and 16-28 (with drug) where the growth began to change and the final section contained the remaining weeks 31-47 (no drug) and 30-46 (drug). The mean fold-difference was then identified for each week and thus the mean for each section was then determined (see Table 3 and Appendix II e)). By analysing the data in this way the general effect across all cell lines can be observed.

	Without Paclitaxel			0.57nM Paclitaxel		
Weeks	1-15	17-31	33-47	2-14	16-28	30-46
Mean	154.0625	18.05625	42.4375	6.08	13.94429	26.6111111
STDV	54.34077494	20.39655083	62.49760995	3.104835	1.689604	15.1541615

Table 3. The analysis of the individual cell lines as a whole, grouped depending on the growth patters identified from the fold-difference data.

This data shows that initially, there is a greater fold increase (154-fold ±54) when the cell lines were incubated without drug, while in the presence of the drug, there is only a small increase of 6-fold (±3), again supporting the idea that Paclitaxel is having anti-proliferative effect on the cells. However, from week 16 onwards, there is an increase in the fold difference of cells in the presence of the drug, where the average increase of all cell lines is

14-fold (±2). This further continues in to the remaining weeks where the mean fold differences average at 27 (±15). This data shows that, even when the cell number is reduced at the beginning of the incubation, as the weeks were the drug is present go by, the greater the difference in cell number, meaning their ability to grow in the presence of the drug increases.

Interestingly, when there is no drug, the general fold-difference dramatically decreases to 18-fold (±20) after week 17, suggesting a reduced ability to grow after incubation with paclitaxel. The growth picks up again, however, but remaining lower than at the beginning of the study, with the average increase in the cell number of 42-fold (±62). This indicates that the cells are becoming adapted to the growing conditions and thus becoming for resistant to the effects of paclitaxel.

Understanding the growth of the cell lines is important as it gives an indication to how well the cells have adapted to the drug due via increased growth. However, it does not give a quantifiable value that enables comparison to other cell lines etc. In order to gage the resistance profile of the cell lines, an MTT assay was performed every 4 weeks, resulting in the generation of IC50 values for each cell line, as depicted in figure 9 . Unfortunately, as the project changed hands, MTTs were unable to be conducted, resulting in a gap in the data between weeks 12 and 30.

In the initial screening in week 4, all cell lines showed an increase in their IC50 values when compared to the IC50 of a UKF-NB-3 cell line (0.57nM). Though, a slight lowering of the IC50s can be seen during week 8, in general, the IC50s stayed constant, with most cell line IC50s not reaching above 1nM. This indicates that the exposure to paclitaxel creates initial resistance but as no results had a >2-fold difference compared to UKF-NB-3, the findings do not count as noteworthy.

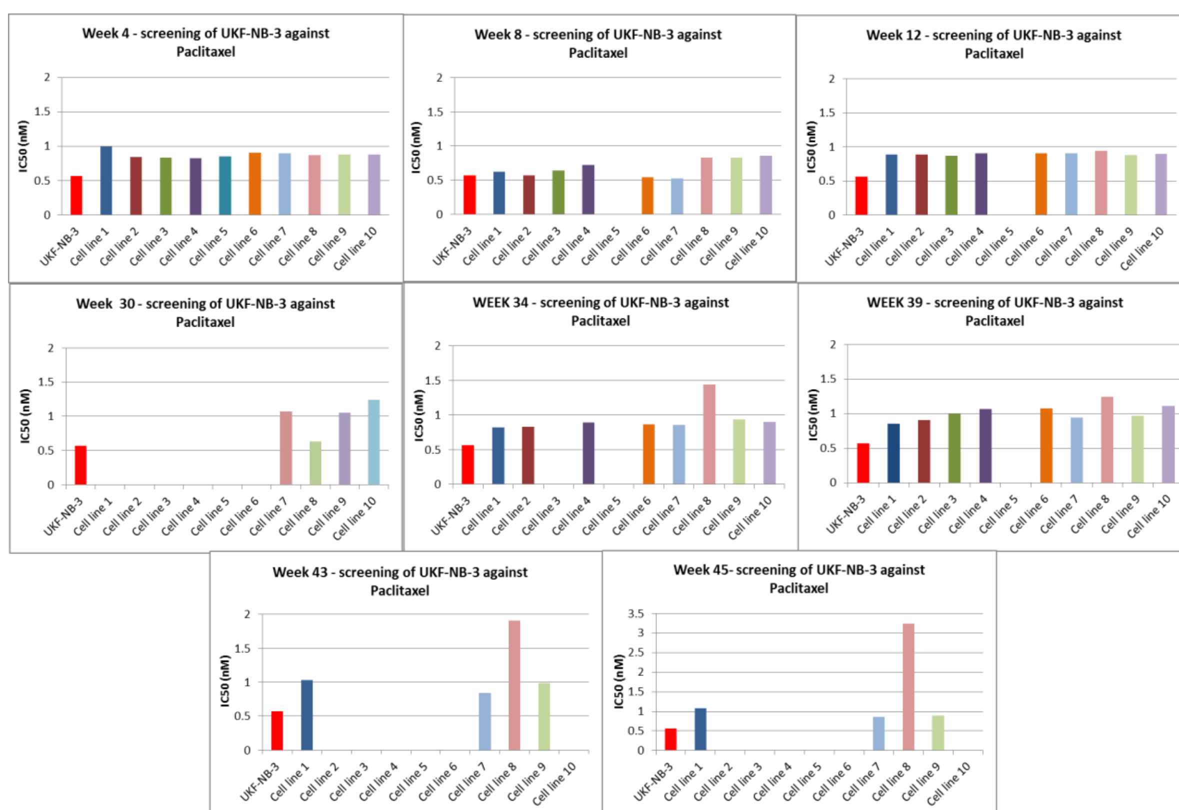

Figure 9 Drug concentrations at which cell viability is reduced by 50% (IC50) for each of the 10 UKF-NB-3 cell lines adapted to 0.57nM of Paclitaxel, as determined by MTT assay after a 120 hour incubation. Cell line 5 was lost in week 7, followed by the loss of cell lines 3 and 4 in week 41 and cell lines 2,6 and 10 in week 42. No data could be collected for cell lines 1-4 and 6 from the MTT assay run in week 30, as well as cell line 3 from the MTT assay run in week 34.

However, this is not the case for cell line 8 which shows an increase in its IC50 from week 30 onwards where it reaches its peak in week 45 in which it takes 3.25nM of paclitaxel to reduce cell viability by 50%. These results are greater than a 2-fold difference when compared to the original cell line. This trend in cell line 8 can be seen more clearly in figure 10 (also see appendix II h)). The figure shows that though the IC50s of all cell lines increase above that

of the sensitive UKF-NB-3 cell line, the IC90s remain the same. Again this is not the case for cell line 8, where its IC90 increases >2-fold higher than that of the parental cell line (0.99nM) from week 30. The comparison of just cell line 8 can be found in the Appendix **II i)**).

Though it appears that when UKF-NB-3 sub-lines are incubated with paclitaxel on alternate weeks, their IC50s increase and thus appear to be resistance, due to the lack of statistically significant data this cannot be concluded. However, striking data is generated in the final weeks for cell line 8, with a substantial increase in the IC90. Furthermore, the addition of fluctuating cell numbers depending on whether or not the drug is present, seems to allow time for the cell lines to recoup, thereby reducing the number of cell lines lost though out the adaptation process.

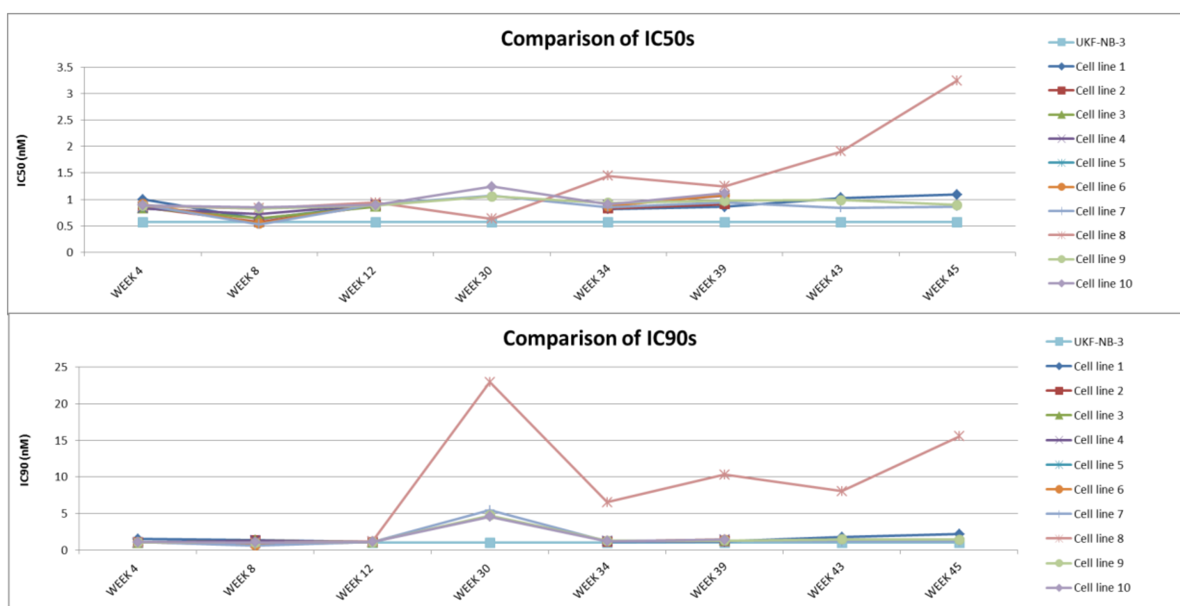

Figure 10. Comparison of drug concentrations at which cell viability is reduced by 50% (IC50) and 90% (IC90) of all 10 UKF-NB-3 Paclitaxel adapted cell lines over 8 weeks of MTT assays of 120 hour incubation period.

6. Discussion

I) Sensitivity of the clonal UKF-NB-3 sub-lines to tubulin-biding agent

UKF-NB-3 is a MYCN-amplified Neuroblastoma cell line. 5 single cell derived clones (1,2,3,4 and 7) were then produced to study intra-tumour heterogeneity, with the aim of determining the effects on the sub-lines after inoculation with a panel of tubulin-binding agents. These include the microtubule destabilising agents Combretastatin A4 and 2-methoxestradiol (bind to the colchicine domain) and Vincristine and vinblastine (that bind to the vinca-domain), as well as the microtubule stabilising agents docetaxel and Epothilone B (that bind to the taxoid domain). The drugs, L181.1 and L181.2, which are derivatives of Combretastatin A4 were also screened against the clonal UKF-NB-3 sub-lines, though their mechanism of action is at this point, still unknown.

We found that the data suggest some heterogeneity. Though there were no significant results, the difference between the cell lines can be clearly seen in the level of the spread of data and the high value ranges. It suggests that there was no uniformed repose to the drugs. It also highlights the heterogeneity within the sublines. For example, when looking at the raw data for the clones tested against 2-methoxyestradiol, the IC50s change dramatically from one week of testing to the next.

The data highlights just how difficult it is to experimentally determine the effects of anti-cancer drugs on the cells. Often when analysing resistance data, a standard value is used to determine the IC50 of the sensitive cell lines. However our data shows that even sensitive cells can produce a range of values and thus this use of a standard IC50 values may not be

valid. The data highlights the need for control (sensitive) cell lines to be tested alongside experimental cells in order for the results to be comparable.

It is important to note that this clonal work is only a small part of a larger project going on in the lab and that this data was collected in order to make comparisons between sensitive clones and clones from a resistance subline. This work was undertaken by others within the lab and will be analysed properly when all the data is collected. It is for this reason I cannot say much about the data other than the fact that there were on clearly defined observable differences.

II) Adaptation of UKF-NB-3 to Paclitaxel by a standardised protocol

To further understand the effects of drug induced heterogeneity, we adapted 10 sub-lines of UKF-NB-3 to the microtubule stabilising drug, Paclitaxel. The aim was to devise and test a new standardised protocol for the adaption of cell lines to anti-cancer drugs that would allow the further study and understanding of drug-induced resistance. The aim was to study the drug-induced heterogeneity in a given cancer cell population using a cancer cell line as a model. An additional aim was to develop standardised drug adaptation protocols that enable the comparison of drugs for their potential to induce resistance in a given cancer cell population.

Our protocol, focuses on alternating drug incubation with UKF-NB-3 cells, compared to most adaptation protocols where cells are continuously exposed to the drug in question. Here, we incubated 10,000 cells/ml for all ten cell lines, in the absence of Paclitaxel. Following a 168hour incubation, the cells were passaged, this time inoculating 100,000 cells/ml in the presence of 0.57nM paclitaxel, the drug concentration at which paclitaxel reduced cell

viability by 50% in the cell line UKF-NB-3. After a further 168 hour incubation, the original growing conditions were reinstated and this continued until the 29th week where it was decided that the remaining 9 cell lines (cell line 5 was lost in week 7) would be passaged with 10,000 cells/ml regardless of whether the drug was present or not. This decision was made because, when incubated with the drug and thus 100,000 cells/ml, the cells to reached the ideal passaging confluency of 70% before the 168 hour incubation was completed. This resulted in a lack of viable cells for the following incubation, due to the environmentally stressful conditions they were subjected to. Though I completed analysis on the cells after the 47th week, the study is still ongoing. Also, though I analysed all 47 weeks, I took over the passaging of the cells from the 18th week onwards.

We found that through using a standardised drug adaptation protocol, heterogenic cellular sub-lines were created with resistance to Paclitaxel.

The need for a protocol like this enables the study of resistance formation, where it was not previously possible. Currently, to achieve this, regular biopsies must be taken from a patient which is neither possible to practically achieve nor is it fair or ethical for the patient. Furthermore, biopsies don't allow for the overall view of the cancer as only a small number of cells are sampled from one section of the cancer. By having a protocol which is known to appropriately adapted cells to a particular drug, it will reduce the need for patient biopsies.

The protocol will also allow for the comparison of cross resistance with drugs of a similar and different mode of action. This has therapeutic implications as it will allow for quicker and more effect treatment strategies as clinicians will be able to suggest the appropriate drug for a patient with a known drug resistance. By creating a catalogue of resistance profiles and cross-resistance profiles the ideal treatment course can be suggested, reducing the need for a trial and error treatment course, as is common practise at the moment.

There are current protocols for drug resistance adaptation but these focus on continuous exposure to the drug in question instead of alternating incubations (Biedler J. L. *et. al. (*1970); Liang X. J. *et al* (2003)). This method of resistance formation also tends to result in the early loss of cell lines as the sensitive cells are killed by the drug. With the weekly changing cell number we implicate here, this early loss of cell lines are overcome.

Though assessing cells through a viability assay allows an insight as to the resistance profile of the sub-lines and though this can be compared to the other sub-lines and even other cell lines, it is not possible to understand what is going on at a molecular level. This means the true heterogenic nature of the sub-lines cannot be established. To achieve this, I would suggest in future experiments that samples are taken at regular intervals and subjected to genetic analysis to better understand what is going on within the cells. For example, testing for certain cell surface receptors etc. to define what resistance mechanisms are being utilised my each cell line. This will also be advantageous in a clinical setting, as once these are determined, the appropriate drugs can be suggested that are known to bypass the pathway in question.

Furthermore, I'd suggest undertaking other viability test other than just an MTT assay. This is because MTT assays assess metabolic activity of the cells, a process which can be slowed in living cells though because of the way the assay is completed, can often identify this cells ad dead when they are not. In short, the assay cannot differentiate between anti-proliferating cells and those that have under gone apoptosis. Also the viability assays could be performed more regularly than every four weeks to get a clearer understanding resistance acquisition. However, there are experimental constraints with this the process can take several hours to set up and be completed and this is only lengthened when the number of samples are increased. For future study, I'd suggest using and automated MTT-

assay (Pieters *et al* (1989). By using this method the chances of human error effecting the results is reduced.

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8. Appendix

I) Sensitivity of the clonal UKF-NB-3 sub-lines to tubulin-biding agent

a) Clonal UKF-NB-3 MTT assay raw data

Clone 1	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Combretastatin A4	0.14897	0.18749	L181.1	49.38247	68.55376	L181.2	318.6081	441.4491	2-Methoxyestridol	487.5684	672.3367
	0.11134	0.15021		88.34911	266.62		184.9381	308.799		854.2541	2796.847
	0.1375	0.25861		32.39066	310.6975		147.8318	380.4975		603.7796	1222.287
	0.1176	0.91184		48.09395	102.0231		134.7334	184.2196		814.9076	1430.274
	0.13822	0.18678		49.12855	68.28539		316.0976	490.5753			
	0.40166	0.55539		56.08728	75.53245		315.2895	437.9436			
	0.46501	0.62092		54.54444	73.94514		326.0883	449.3189			
	0.43979	0.5951		63.85189	165.3963						
	0.20127	0.47434									
Mean	0.240151	0.437853	Mean	55.22854	141.3817	Mean	249.0838	384.6861	Mean	690.1274	1530.436
STDV	0.149527	0.259479	STDV	16.10855	97.1075	STDV	88.58143	105.9965	STDV	174.1587	902.7955

Clone 1	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Vincristine	0.07542	0.13177	Vinblastine	0.02359	0.04342	Docetaxel	0.18092	0.11871	Epothilone B	0.1057	0.1782
	0.05431	0.55714		0.02078	0.07061		0.17392	0.23598		0.06392	0.08343
	0.06558	0.11858		0.03301	0.0766		0.22517	0.46833		0.05941	0.07891
	0.14495	0.21556		0.03973	0.07875		0.23357	0.52282		0.10765	0.14641
				0.08172	0.1797					0.10501	0.13621
				0.0203	0.07554					0.03052	0.23843
				0.13377	0.2243					0.02757	0.06441
Mean	0.085065	0.255763	Mean	0.050414	0.106989	Mean	0.203395	0.33646	Mean	0.071397	0.132286
STDV	0.040844	0.205457	STDV	0.042524	0.067235	STDV	0.030324	0.191164	STDV	0.035148	0.062493

Clone 2	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Combretastatin A4	0.63256	1.35713	L181.1	105.4617	155.002	L181.2	344.6278	468.6414	2-Methoxystridol	721.2361	1552.073
	0.21657	0.3554		56.11809	78.1146		349.44	473.6159		2332.106	3081.587
	0.13479	0.18951		53.27577	72.63186		295.1211	416.4452		1202.524	8239.646
	0.16027	0.29832		37.74916	50.22972		164.4761	266.4966		1837.484	2557.72
	0.43625	0.59146		87.88626	232.5356		351.1456	475.3751			
	0.54633	1.0243		56.66459	76.12372		323.7453	446.8586			
	0.12842	0.17661					313.6424	436.2004			
	0.55556	0.99592									
	0.57077	0.72577									
	0.42527	0.58008									
	0.22286	0.3734									
Mean	0.366332	0.606173	Mean	66.1926	110.7729	Mean	306.0283	426.2333	Mean	1523.337	3857.756
STDV	0.196196	0.384167	SD	25.22233	69.52655	SD	65.74942	73.70531	SD	706.8904	2989.407

Clone 2	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Vincristine	0.04766	0.06327	Vinblastine	0.07235	0.17084	Docetaxel	0.21441	0.21441	Epothilone B	0.05178	0.07108
	0.02448	0.05203		0.02258	0.07302		0.79191	1.31497		0.05348	0.07285
	0.03972	0.05507		0.22214	0.30921		0.48613	0.83612		0.06041	0.07993
	0.06121	0.10606		0.05944	0.08246					0.08867	0.13349
	0.03155	0.05234		0.18775	0.31409					0.08475	0.1157
	0.05247	0.12369		0.02599	0.0734						
	0.08896	0.14904									
	0.04897	0.12078									
	0.1605	0.22199									
Mean	0.061724	0.104919	**Mean**	0.098375	0.170503	**Mean**	0.497483	0.7885	**Mean**	0.067818	0.09461
SD	0.041423	0.057051	**SD**	0.085421	0.115361	**SD**	0.288917	0.551823	**SD**	0.017601	0.02828

Clone 3	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Combretastatin A4	0.36493	0.50797	L181.1	54.66705	74.07168	L181.2	244.2089	425.5668	2-Methoxyestridol	355.1614	628.0094
	0.40848	1.00643		113.9389	139.4645		181.4269	305.2839		947.3902	1616.651
	0.32327	0.53388		48.00662	67.84123		196.0916	407.9404		715.4084	1310.921
	0.35278	0.93666		33.60468	45.97296		128.0119	177.1792		742.3052	1836.539
	0.31456	0.52264		51.5068	70.78815		124.1326	228.4749			
	0.43878	0.59406		54.37091	73.76594		178.7628	298.1034			
	0.39547	0.54887		51.15159	70.41612						
				53.65113	73.02119						
Mean	0.371181	0.664359	**Average**	57.61221	76.91772	**Average**	175.4391	307.0915	**Average**	690.0663	1348.03
STDV	0.045515	0.212527	**SD**	23.77036	26.89961	**SD**	44.90971	97.31261	**SD**	246.1355	526.1829

Clone 3	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Vincristine	0.07605	0.13193	Vinblastine	0.02702	0.04719	Docetaxel	0.3125	0.36869	Epothilone B	0.05809	0.07757
	0.03524	0.04905		0.0623	0.12272		0.65749	0.87732		0.05538	0.07481
	0.03955	0.05489		0.02313	0.0709		0.67247	0.91985		0.09766	0.11522
	0.02371	0.09681		0.06084	0.10189					0.11232	0.18414
	0.02646	0.05128		0.02826	0.07501					0.09829	0.1366
	0.01983	0.05095		0.04457	0.13416					0.15625	0.18435
	0.05319	0.15602									
	0.04106	0.07346									
	0.01885	0.04589									
Average	0.037104	0.07892	**Average**	0.04102	0.091978	**Average**	0.547487	0.721953	**Average**	0.096332	0.128782
SD	0.018456	0.040638	**SD**	0.017534	0.033349	**SD**	0.203642	0.306673	**SD**	0.037392	0.048853

Clone 4	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Combretastatin A4	0.41846	0.573	L181.1	95.85316	162.7126	L181.2	385.1259	510.0137	2-Methoxyestridol	533.2332	719.7684
	0.24402	0.51063		97.65625	115.2165		361.1747	485.6784		3750	4424.314
	0.4662	0.62213		41.72769	54.21211		381.2216	506.072		2094.302	2839.282
	0.97084	1.28312		57.61294	77.09188		181.029	279.8634		2012.794	2754.746
	0.49055	0.64672		97.65625	115.2165		345.2281	469.2629			
	0.94172	1.2537		90.82931	148.5403		364.4276	489.0056			
	0.47764	0.63372		95.73871	149.3613						
	0.78125	0.92173									
	0.50735	0.66351									
	0.24505	0.50384									
Mean	0.554308	0.76121	**Average**	82.43919	117.4787	**Average**	336.3678	456.6493	**Average**	2097.582	2684.528
STDV	0.259353	0.291244	**SD**	22.96408	40.14851	**SD**	77.46045	87.85484	**SD**	1314.645	1518.334

Clone 4	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Vincristine	0.15876	0.22015	Vinblastine	0.05794	0.08142	Docetaxel	0.19286	0.33345	Epothilone B	0.13766	0.17627
	0.04315	0.05865		0.06606	0.08936		0.15603	0.28291		0.11791	0.19435
	0.04073	0.05613		0.11015	0.19434		0.19029	0.33475		0.11141	0.18396
	0.04421	0.05974		0.06045	0.08353		0.11584	0.15481		0.13188	0.17088
	0.08325	0.11413		0.15935	0.20231		0.31729	0.54261		0.06016	0.07967
	0.05059	0.06621		0.06496	0.08822		0.32751	0.56077		0.12746	0.15787
	0.07621	0.10608		0.08441	0.28525		0.33896	0.57302			
	0.04258	0.05806									
	0.1496	0.20824									
	0.04659	0.06219									
	0.14685	0.20746									
Average	0.080229	0.11064	**Average**	0.086189	0.146347	**Average**	0.234111	0.397474	**Average**	0.114413	0.1605
SD	0.048066	0.068027	**SD**	0.037072	0.081155	**SD**	0.091599	0.162578	**SD**	0.028215	0.041456

Clone 7	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Combretastatin A4	0.67773	1.19749	L181.1	58.67172	78.1683	L181.2	127.5841	370.402	2-Methoxyestridol	1029.804	1757.551
	0.8132	1.12117		113.4838	188.3121		314.1706	436.7597		2217.629	2965.719
	0.41263	0.56691		53.743	73.11638		205.7726	332.2356		1988.043	2728.914
	0.61224	1.04038		36.63241	49.09422		297.564	414.1997		1725.252	2576.114
	0.6675	1.1935		119.644	250.1562		147.1275	196.9866			
	0.49317	0.64934		62.61558	82.13798		363.4984	488.0559			
	0.45318	0.60885		61.9537	94.80197		336.6599	460.3682			
	0.10942	0.14823		75.48866	125.1174		322.9645	446.0377			
Mean	0.529884	0.815734	**Average**	72.77911	117.6131	**Average**	264.4177	393.1307	**Average**	1740.182	2507.074
STDV	0.14351	0.28895	**SD**	29.1523	68.2766	**SD**	90.99242	93.63723	**SD**	514.5388	524.7602

Clone 7	IC50	IC90		IC50	IC90		IC50	IC90		IC50	IC90
Vincristine	0.06478	0.13648	Vinblastine	0.09067	0.16667	Docetaxel	0.35212	0.61283	Epothilone B	0.05529	0.07472
	0.03131	0.05216		0.02998	0.12703		0.2666	0.45551		0.0234	0.06284
	0.03705	0.05222		0.09901	0.17573		0.27947	0.4968		0.0506	0.06984
	0.079	0.10968		0.06549	0.17609					0.0518	0.07109
	0.04199	0.05745		0.14444	0.30909					0.05691	0.07638
	0.03392	0.06108								0.05984	0.09617
	0.06417	0.11761								0.0374	0.09575
	0.11677	0.20623									
	0.05812	0.10767									
Average	0.058568	0.100064	**Average**	0.085918	0.190922	**Average**	0.299397	0.521713	**Average**	0.047891	0.078113
SD	0.027303	0.051204	**SD**	0.042324	0.069074	**SD**	0.046111	0.081565	**SD**	0.012983	0.012927

b) Table to assess Significance of the mean clone data

	Combretastatin A4		L181.1		L181.2		2-Methoxyestridiol	
Clone 1	0.24015111		55.2285		249.084		690.127	
Clone 2	0.36633182		66.1926		306.028		1523.34	
Clone 3	0.37118143		57.6122		175.439		690.066	
Clone 4	0.554308		82.4392		336.368		2097.58	
Clone 7	0.52988375		72.7791		264.418		1411.85	
Mean of all clones	0.240151111		66.85032943		266.2673626		1282.593024	
2 fold range	0.12	0.4803	33.53	133.7	133.134	532.534	641.295	2565.18

Vincristine		Vinblastine		Docetaxel		Epothilone B			
0.08507		0.05041		0.2034		0.0714			
0.06172		0.09838		0.49748		0.06782			
0.0371		0.04102		0.54749		0.09633		<2-fold	
0.08023		0.08619		0.23411		0.11441		2-fold	
0.05857		0.08592		0.2994		0.04789		>2-fold	
0.064538152		0.072383171		0.356374619		0.079570314			
0.03227	0.129076	0.03619	0.144766	0.17819	0.712749	0.03979	0.159141		

II) Adaptation of UKF-NB-3 to Paclitaxel by a standardised protocol

a) Weekly cell number raw data

	Week 1 -	Week 2 +	Week 3 -	Week 4 +	Week 5 -	Week 6 +	Week 7 -	Week 8 +	Week 9 -	Week 10 +
Cell line 1	760000	900000	1790000	1020000	2450000	770000	2150000	270000	1710000	1000000
Cell line 2	500000	630000	1870000	860000	1850000	780000	2400000	320000	2610000	620000
Cell line 3	1250000	770000	1610000	880000	2100000	850000	2010000	660000	1420000	1160000
Cell line 4	870000	850000	1930000	660000	2000000	1080000	2000000	780000	1630000	930000
Cell line 5	1530000	960000	1740000	880000	1950000	180000	-	-	-	-
Cell line 6	530000	830000	2400000	740000	2560000	540000	2830000	390000	2160000	660000
Cell line 7	450000	760000	1330000	1220000	2200000	760000	1720000	540000	2120000	700000
Cell line 8	590000	670000	2390000	520000	1570000	890000	2250000	460000	2390000	740000
Cell line 9	470000	550000	2180000	1060000	1960000	1240000	2650000	340000	1560000	1060000
Cell line 10	1290000	960000	2680000	690000	1780000	1100000	2010000	110000	2230000	640000

	Week 11 -	Week 12 +	Week 13 -	Week 14 +	Week 15 -	Week 16 +	Week 17 -	Week 18 +	Week 19 -	Week 20 +
Cell line 1	2420000	-	770000	380000	650000	1970000	-	2140000	530000	1560000
Cell line 2	1610000	-	880000	730000	1140000	2080000	-	1770000	360000	1330000
Cell line 3	2130000	-	1130000	450000	1590000	1950000	-	1050000	420000	1000000
Cell line 4	1850000	-	1320000	400000	1570000	1620000	-	1360000	840000	1920000
Cell line 5	-	-	-	-	-	-	-	-	-	-
Cell line 6	2560000	-	620000	1020000	970000	1700000	-	1610000	540000	1170000
Cell line 7	2050000	-	970000	730000	730000	1680000	640000	830000	-	930000
Cell line 8	1620000	-	1120000	740000	1180000	1110000	1140000	1390000	-	1320000
Cell line 9	2230000	-	770000	1260000	1000000	2320000	770000	1210000	-	1020000
Cell line 10	1710000	-	1070000	870000	1160000	1760000	1040000	1750000	-	1010000

	Week 21 -	Week 22 +	Week 23 -	Week 24 +	Week 25 -	Week 26 +	Week 27 -	Week 28 +	Week 29 -	Week 30 +
Cell line 1	810000	2040000	1010000	3220000	1000000	1560000	750000	1490000	830000	350000
Cell line 2	580000	1680000	950000	2450000	1040000	1690000	950000	1720000	810000	190000
Cell line 3	750000	1750000	770000	1470000	390000	1430000	810000	1210000	720000	130000
Cell line 4	850000	1580000	1000000	1690000	810000	1320000	520000	1680000	870000	190000
Cell line 5	-	-	-	-	-	-	-	-	-	-
Cell line 6	710000	1730000	860000	1020000	640000	2070000	880000	1720000	840000	570000
Cell line 7	720000	1630000	1020000	1660000	690000	1640000	840000	1800000	780000	470000
Cell line 8	1030000	1120000	730000	1030000	630000	810000	460000	560000	720000	430000
Cell line 9	710000	1490000	760000	1500000	320000	1790000	930000	1790000	980000	230000
Cell line 10	580000	1100000	510000	1850000	980000	1150000	630000	1610000	840000	270000

	Week 31 -	Week 32 +	Week 33 -	Week 34 +	Week 35 -	Week 36 +	Week 37 -	Week 38 +	Week 39 -	Week 40 +
Cell line 1	220000	500000	380000	380000	250000	70000	110000	240000	2060000	370000
Cell line 2	380000	400000	460000	80000	330000	50000	250000	730000	2280000	90000
Cell line 3	530000	540000	290000	120000	30000	80000	570000	520000	2040000	70000
Cell line 4	450000	340000	230000	210000	80000	150000	960000	640000	1410000	50000
Cell line 5	-	-	-	-	-	-	-	-	-	-
Cell line 6	150000	530000	370000	150000	140000	30000	220000	590000	2460000	150000
Cell line 7	-	470000	240000	220000	580000	180000	360000	210000	2530000	770000
Cell line 8	570000	350000	540000	140000	180000	210000	460000	770000	1440000	220000
Cell line 9	430000	240000	250000	-	180000	450000	220000	1190000	2760000	980000
Cell line 10	440000	270000	150000	820000	390000	160000	550000	1040000	2420000	230000

	Week 41 -	Week 42 +	Week 43 -	Week 44 +	Week 45 -	Week 46 +	Week 47 -
Cell line 1	230000	1190000	160000	650000	280000	160000	1200000
Cell line 2	20000	-	-	-	-	-	-
Cell line 3	-	-	-	-	-	-	-
Cell line 4	-	-	-	-	-	-	-
Cell line 5	-	-	-	-	-	-	-
Cell line 6	10000	-	-	-	-	-	-
Cell line 7	200000	440000	190000	100000	70000	80000	1290000
Cell line 8	40000	20000	140000	410000	460000	370000	500000
Cell line 9	110000	1050000	190000	540000	110000	110000	560000
Cell line 10	20000	-	-	-	-	-	-

c) Calculated fold difference raw data

	week 1 -	week 2 +	week 3 -	week 4 +	week 5 -	week 6 +	week 7 -	week 8 +	week 9 -	Week 10 +
Cell line 1	76	9	179	10.2	245	7.7	215	2.7	171	10
Cell line 2	50	6.3	187	8.6	185	7.8	240	3.2	261	6.2
Cell line 3	125	7.7	161	8.8	210	8.5	201	6.6	142	11.6
Cell line 4	87	8.5	193	6.6	200	10.8	200	7.8	163	9.3
Cell line 5	153	9.6	174	8.8	195	1.8	-	-	-	-
Cell line 6	53	8.3	240	7.4	256	5.4	283	3.9	216	6.6
Cell line 7	45	7.6	133	12.2	220	7.6	172	5.4	212	7
Cell line 8	59	6.7	239	5.2	157	8.9	225	4.6	239	7.4
Cell line 9	47	5.5	218	10.6	196	12.4	265	3.4	156	10.6
Cell line 10	129	9.6	268	6.9	178	11	201	1.1	223	6.4

	week 11 -	week 12 +	week13 -	week14 +	week 15 -	week 16 +	week 17-	week 18+	Week 19 -	Week 20 +
Cell line 1	242	-	77	3.8	65	19.7	-	21.4	53	15.6
Cell line 2	161	-	88	7.3	114	20.8	-	17.7	36	13.3
Cell line 3	213	-	113	4.5	159	19.5	-	10.5	42	10
Cell line 4	185	-	132	4	157	16.2	-	13.6	84	19.2
Cell line 5	-	-	-	-	-	-	-	-	-	-
Cell line 6	256	-	62	10.2	97	17	-	16.1	54	11.7
Cell line 7	205	-	97	7.3	73	16.8	64	8.3	-	9.3
Cell line 8	162	-	112	7.4	118	11.1	114	13.9	-	13.2
Cell line 9	223	-	77	12.6	100	23.2	77	12.1	-	10.2
Cell line 10	171	-	107	8.7	116	17.6	104	17.5	-	10.1

	Week 21 -	Week 22 +	Week 23 -	Week 24 +	Week 25 -	Week 26 +	Week 27 -	Week 28 +	Week 29 -	Week 30 +
Cell line 1	81	20.4	101	32.2	100	15.6	75	14.9	83	35
Cell line 2	58	16.8	95	24.5	104	16.9	95	17.2	81	19
Cell line 3	75	17.5	77	14.7	39	14.3	81	12.1	72	13
Cell line 4	85	15.8	100	16.9	81	13.2	52	16.8	87	19
Cell line 5	-	-	-	-	-	-	-	-	-	-
Cell line 6	71	17.3	86	10.2	64	20.7	88	17.2	84	57
Cell line 7	72	16.3	102	16.6	69	16.4	84	18	78	47
Cell line 8	103	11.2	73	10.3	63	8.1	46	5.6	72	43
Cell line 9	71	14.9	76	15	32	17.9	93	17.9	98	23
Cell line 10	58	11	51	18.5	98	11.5	63	16.1	84	27

	Week 31 -	Week 32 +	Week 33 -	Week 34 +	Week 35 -	Week 36 +	Week 37 -	Week 38 +	Week 39 -	Week 40 +
Cell line 1	22	50	38	38	25	7	11	24	206	37
Cell line 2	38	40	46	8	33	5	25	73	228	9
Cell line 3	53	54	29	12	3	8	57	52	204	7
Cell line 4	45	34	23	21	8	15	96	64	141	5
Cell line 5	-	-	-	-	-	-	-	-	-	-
Cell line 6	15	53	37	15	14	3	22	59	246	15
Cell line 7	0	47	24	22	58	18	36	21	253	77
Cell line 8	57	35	54	14	18	21	46	77	144	22
Cell line 9	43	24	25	0	18	45	22	119	276	98
Cell line 10	44	27	15	82	39	16	55	104	242	23

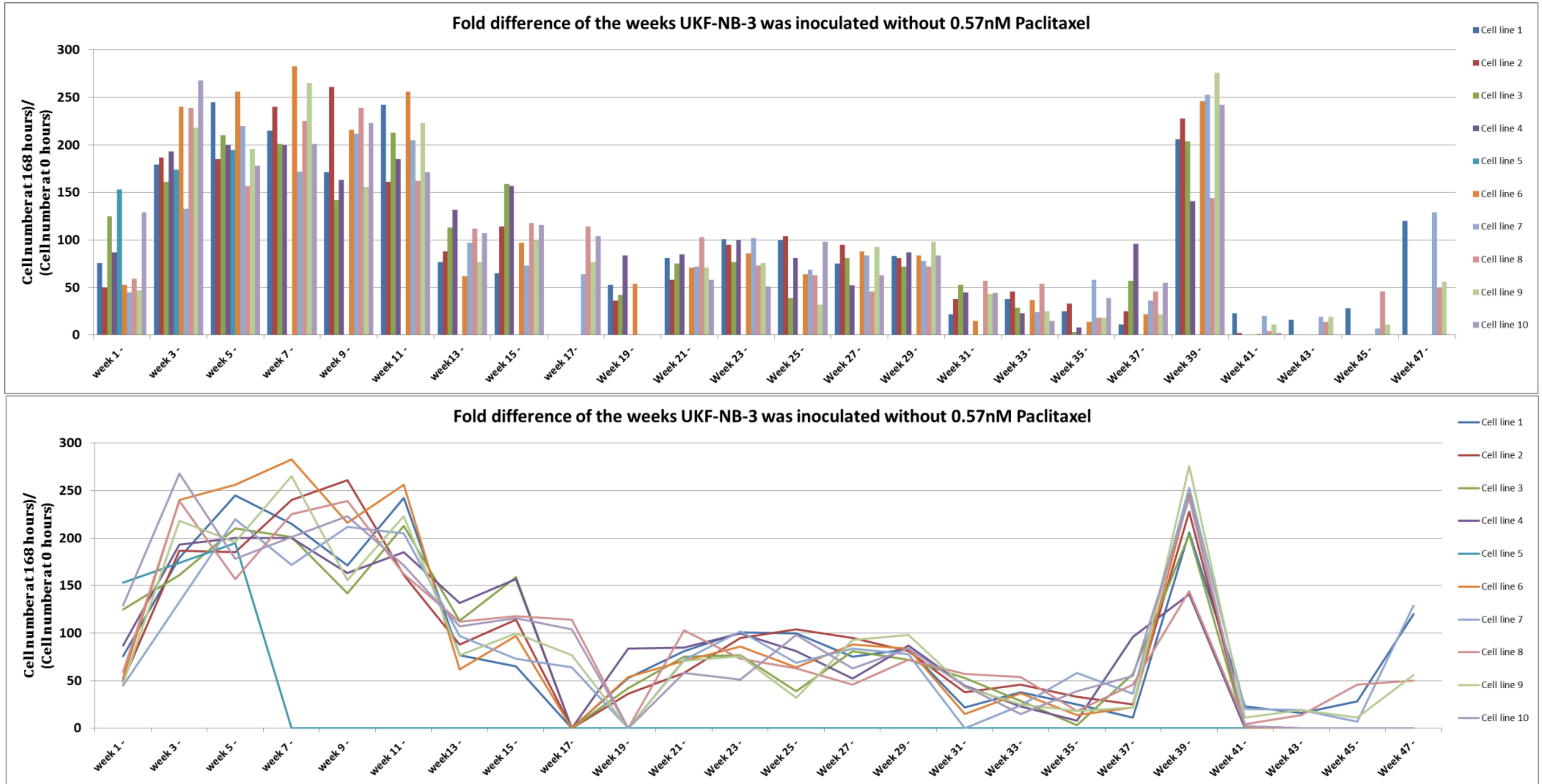
	Week 41 -	Week 42 +	Week 43 -	Week 44 +	Week 45 -	Week 46 +	Week 47 -
Cell line 1	23	119	16	65	28	16	120
Cell line 2	2	-	-	-	-	-	-
Cell line 3	-	-	-	-	-	-	-
Cell line 4	-	-	-	-	-	-	-
Cell line 5	-	-	-	-	-	-	-
Cell line 6	1	-	-	-	-	-	-
Cell line 7	20	44	19	10	7	8	129
Cell line 8	4	2	14	41	46	37	50
Cell line 9	11	105	19	54	11	11	56
Cell line 10	2	-	-	-	-	-	-

d) Graph to show the fold difference of the cell number of weeks with drug only

Fold difference of the weeks UKF-NB-3 was inoculated with 0.57nM Paclitaxel

Fold difference of the weeks UKF-NB-3 was inoculated with 0.57nM Paclitaxel

e) Graph to show the fold difference in only the weeks incubated without paclitaxel



f) Raw data table for the mean fold-differences of all cell lines for each week.

No drug	week 1 -	week 3 -	week 5 -	week 7 -	week 9 -	week 11 -	week13 -	week 15 -
Mean	82.4	199.2	204.2	200.2	178.3	181.8	86.5	99.9
STDV	39.6462132	41.37578465	29.96961424	77.71000222	73.76547	71.757	36.8457747	46.510333

	week 17-	Week 19 -	Week 21 -	Week 23 -	Week 25 -	Week 27 -	Week 29 -	Week 31 -
	35.9	26.9	67.4	76.1	65	67.7	73.9	31.7
	48.24578	30.920867	27.05221	31.17852	33.5029	29.09009	27.02859	21.0082

	Week 33 -	Week 35 -	Week 37 -	Week 39 -	Week 41 -	Week 43 -	Week 45 -	Week 47 -
	29.1	21.6	37	194	6.3	6.8	9.2	35.5
								651

Drug	week 2 +	week 4 +	week 6 +	week 8 +	Week 10 ·	week 12 +	week14 +
Mean	7.88	8.53	8.19	3.87	7.51	0	6.58
STDV	1.39028374	2.093402334	3.025612886	2.367863547	3.26818	0	3.61410816

	week 16 +	week 18+	Week 20 +	Week 22 +	Week 24 +	Week 26 +	Week 28 +
	16.19	13.11	11.26	14.12	15.89	13.46	13.58
	6.538	5.978749	4.9964877	5.715243	8.607548	5.885047	6.084187

	Week 30 +	Week 32 +	Week 34 +	Week 36 +	Week 38 +	Week 40 +	Week 42 +	Week 44 +	Week 46 +
	28.3	36.4	21.2	13.8	59.3	29.3	27	17	7.2
	17.23079	16.56771	24.10071	12.96834	37.0706533	32.84661	46.94678	25.88865	11.97961231

g) Raw data from paclitaxel MTT assay

WEEK 4		
Cell lines	IC50	IC90
UKF-NB-3	0.567648	0.987432
Cell line 1	0.995	1.47632
Cell line 2	0.84734	1.01233
Cell line 3	0.83691	1.00156
Cell line 4	0.83041	0.99484
Cell line 5	0.85872	1.02407
Cell line 6	0.9116	1.0783
Cell line 7	8.99E-01	1.06505
Cell line 8	0.87068	1.03637
Cell line 9	0.8789	1.04482
Cell line 1	0.88416	1.05021

WEEK 8		
Cell lines	IC50	IC90
UKF-NB-3	0.567648	0.987432
Cell line 1	0.62571	1.31984
Cell line 2	0.57333	1.2779
Cell line 3	0.64425	1.27503
Cell line 4	0.72267	1.28903
Cell line 5	-	-
Cell line 6	0.54234	0.64584
Cell line 7	5.25E-01	0.62825
Cell line 8	0.82973	0.99414
Cell line 9	0.83073	0.98091
Cell line 1	0.85262	1.01778

WEEK 12		
Cell lines	IC50	IC90
UKF-NB-3	0.567648	0.987432
Cell line 1	0.89017	1.08695
Cell line 2	0.88779	1.08451
Cell line 3	0.86619	1.06227
Cell line 4	0.90388	1.10101
Cell line 5	-	-
Cell line 6	0.9036	1.10073
Cell line 7	0.90416	1.1013
Cell line 8	0.93799	1.13585
Cell line 9	0.87907	1.07554
Cell line 1	0.89349	1.09036

WEEK 30		
Cell lines	IC50	IC90
UKF-NB-3	0.567648	0.987432
Cell line 1	-	-
Cell line 2	-	-
Cell line 3	-	-
Cell line 4	-	-
Cell line 5	-	-
Cell line 6	-	-
Cell line 7	1.06778	5.4323
Cell line 8	0.6332	22.95891
Cell line 9	1.05579	4.70775
Cell line 1	1.24188	4.55892

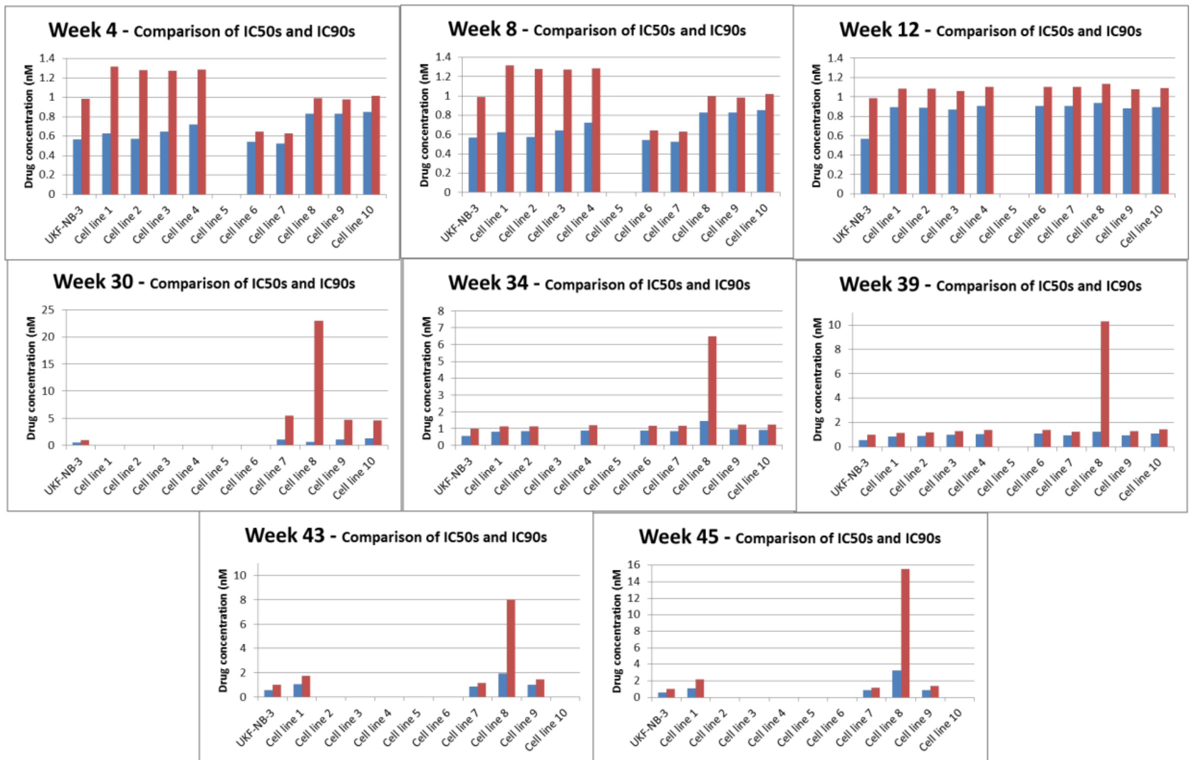
WEEK 34		
Cell lines	IC50	IC90
UKF-NB-3	0.567648	0.987432
Cell line 1	0.81803	1.12625
Cell line 2	0.82602	1.13461
Cell line 3	-	-
Cell line 4	0.89416	1.2052
Cell line 5	-	-
Cell line 6	0.86669	1.1769
Cell line 7	0.85361	1.16336
Cell line 8	1.44336	6.49989
Cell line 9	0.93209	1.24393
Cell line 1	0.90402	1.2153

WEEK 39		
Cell lines	IC50	IC90
UKF-NB-3	0.567648	0.987432
Cell line 1	0.8574	1.16728
Cell line 2	0.9079	1.21928
Cell line 3	0.99316	1.30552
Cell line 4	1.0653	1.37712
Cell line 5	-	-
Cell line 6	1.08094	1.39248
Cell line 7	0.94263	1.25463
Cell line 8	1.24552	10.28793
Cell line 9	0.97133	1.28361
Cell line 1	1.11173	1.42258

WEEK 43		
Cell lines	IC50	IC90
UKF-NB-3	0.567648	0.987432
Cell line 1	1.02859	1.75516
Cell line 2	-	-
Cell line 3	-	-
Cell line 4	-	-
Cell line 5	-	-
Cell line 6	-	-
Cell line 7	0.84259	1.1519
Cell line 8	1.9097	8.01299
Cell line 9	0.98778	1.45946
Cell line 1	-	-

WEEK 45		
Cell lines	IC50	IC90
UKF-NB-3	0.567648	0.987432
Cell line 1	1.08791	2.17353
Cell line 2	-	-
Cell line 3	-	-
Cell line 4	-	-
Cell line 5	-	-
Cell line 6	-	-
Cell line 7	0.86105	1.17107
Cell line 8	3.2476	15.53666
Cell line 9	0.89321	1.40653
Cell line 1	-	-

h) The comparative data of the MTT assay IC50 and IC90 results of all cell lines. The blue bars represent the IC50s while the red bars depict the IC90s.



i) Comparison of the IC50 and IC90 data for cell line 8 only.