Approaches for the Exploitation of Data Derived from Pharmacogenomics Screens

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Abstract

Since the discovery of the first immortal cell line HeLa in 1951, cancer cell lines (CCLs) have been a mainstay of cancer research. But it was not until the establishment of the National Cancer Institute of 60 cancer cell lines (NCI-60) that they became invaluable to cancer drug development. Over 30 years on from the beginning of large-scale cancer drug screening, many large-scale CCL panels now exist, including the Cancer Cell Line Encyclopedia (CCLE) and Genomics of Drug Sensitivity in Cancer (GDSC). These studies have had the reproducibility of their results questioned. With the increased attention of the reproducibility crisis and previous issues with cell line misidentification, scepticism is perhaps fair.

It is determined in analyses here that platinum drugs do not all have the same drug sensitivity profiles and confirmed a novel approach to determine cancer cell line sensitivity using clinically relevant thresholds. Data from two large-scale pharmacogenomics screens was also used to validate experimental work carried out with the compound YM155 with relation to *ABCB1* and *SLC35F2* expression levels. Reproducibility and replication of results is also examined using NCI-60 data and it was determined that high variability in drug sensitivity data existed despite standardisation of protocols.

Results here prove that if used correctly, despite flaws in the panels, the data can prove insightful in many ways. Particularly when used as a validation method for research carried out in a wet lab. It was also shown that reproducibility of data from these CCL panels should be considered when using for analysis as drug sensitivity data was shown to be highly variable, and that perhaps, despite high levels of standardisation there exists an intrinsic variability in biological systems.

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Abbreviations

ATCC American Type Culture Collection

AUC Area under curve

BR Breast cancer

CCC Compound/cell line combination

CCL Cancer cell line

CCLE Cancer Cell Line Encyclopedia

CGP Cancer Genome Project

CNS Central nervous system

CO Colon

CTRP Cancer Therapeutics Response Panel

DSMZ German collection of microorganisms and cell lines

EXN Number of experiments

FDA Food and drug administration

GDSC Genomics of Drug Sensitivity to Cancer

GIΔ GI50 fold change

GI50 Concentration for 50% of maximal inhibition of cell proliferation

IC50 Half maximal inhibitory concentration

LC Lung cancer

LE Leukaemia

ME Melanoma

NCI-60 National Cancer Institute of 60 cell lines

OV Ovary

PR Prostate

RCCL Resistant Cancer Cell Line Collection

RE Renal

WES Whole exome sequencing

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Chapter 1

Introduction

1 Cancer Cell Lines

1.1 HeLa – The Immortal Cell Line

In 1951 the human cell line HeLa was derived from cervical cancer cells from patient Henrietta Lacks (Gey *et al.*, 1952). This cell line is naturally "immortal" due to expression of telomerase during cell division which prevents shortening of telomeres and therefore cellular senescence, and even for a cancer cell, grows unusually fast (Ivankovic *et al.*, 2007). This made it the perfect candidate for medical and biological research, as it had until then been difficult to culture and sustain stocks of living cells for research. Although a cancer cell line, HeLa has been used in research for a myriad of diseases and many other scientific endeavours; for example, in 1953 the successful propagation of poliomyelitis virus in HeLa cells enabled the development of a vaccine for polio (Scherer *et al.*, 1953).

Before the discovery and commercialization of the HeLa cell line, it had not been possible for anybody to grow human cells in the laboratory for more than a few weeks, which limited human cell biological research. Most *in vitro* cancer research was carried on animal cells (Earle, 1943), tissue culture with such cells had been successful since early 20th century (Burrows, 1910). The stable growth of HeLa,

and ability to clone and mass produce the cell line with relative ease at low expense led to ground breaking scientific discovery.

The greatest implication of the HeLa cancer cell line was not only the potential new understanding of cancer, but the ability to now study many biological processes, diseases and drug mechanisms using human cells *in vitro*. Diseases including diphtheria (Strauss and Hendee, 1959) and adenoviruses (Boyer *et al.*, 1957) were researched using HeLa cells early on, and HeLa cells were used to understand the effects of x-rays on human cells (Yamada and Puck, 1961) and even the effect of zero gravity on human cells as HeLa cells were sent into space. The National Institutes of Health (NIH) determined that over 110,000 biomedical research articles cited the use of HeLa cell lines between 1953 and 2018. The HeLa cell line was only the first of what is now thousands of cell lines used in every day scientific research. Its advent and subsequent efforts to establish cancer cell lines from many different tissues and tumour types has allowed cancer researchers to study cancer in more dynamic ways. Before, research may have been reliant on use of patient biopsies, but these only represent a particular time in the life of that tumour, cancer cell lines have allowed researchers to investigate cancer traits that were previously difficult to do so including cancer initiation, progression and metastasis. However, as these new cell lines have been established over time, problems have occurred and the accuracy of scientific research with them has been questioned.

1.1.1 Misidentification and Cross-Contamination

Years after the discovery of the HeLa cell line, as more and more cell lines from different tissues were produced and established, a group of scientists began to question experimentally the relevance of cancer cell line models. Cell line species had previously been misidentified, and this had led to the formation of a cell line banks at the American Type Culture Collection (ATCC) (Clark and Geary, 1974), that would authenticate and store cell lines. But in 1966 Stanley Gartler introduced a method to determine misidentification within species was possible and reported that 18 cell lines claimed to be of different origins were actually all HeLa cell lines (Gartler, 1967). HeLa cell lines were easily available and so could be found in laboratories worldwide, and the combination of a lack of cross contamination awareness and the speed and ease at which HeLa cells can grow, other cells were overgrown by them. This was not a popular finding among much of the scientific community, but a group of scientists led by Walter Nelson-Rees took heed and their cytogenetic studies (Nelson-Rees and Flandermeyer, 1974) revealed that cell lines used to model various types of cancer were actually of HeLa origin, and the alarming frequency of cross contamination by HeLa cell lines meant that for years all cell lines were under suspicion of being HeLa cell lines.

By 1977, Nelson-Rees determined that the incidence of cell line misidentification submitted to international cell banks was as high as 16%, with two cases particularly disturbing (Nelson-Rees and Flandermeyer, 1977): Breast cancer cell line HBC was in fact of rat origin, and breast cancer cell line BrCa5 was of HeLa

origin, highlighting inter- and intraspecies contamination of cell cultures. This revelation, and Nelson-Rees, was perhaps unsurprisingly not popular amongst some scientists, and work continued to be published containing false information (Nelson-Rees, 2001). This eventually led to Nelson-Rees losing his position at the NIH in 1981, as he highlighted papers produced by peers that used cross-contaminated cell lines. Despite the concerns raised by Nelson-Rees, cell banks continued to distribute misidentified cell lines for the next two decades.

Cancer cell line research however, continued to thrive. In the late 1980s the National Cancer Institute (NCI) established a panel of 60 human cancer cell lines derived from nine different tissue sites, the NCI-60 human tumour cell lines screen was born. Their aim was to develop cancer drug discovery, which had been until then mainly carried out on leukaemia modelled in transplantable murine neoplasms, to solid tumours where the clinical activity of compounds previously identified had been relatively low or absent (Shoemaker *et al*., 1988). With the recent cross-contamination in mind, the NCI had to be careful in selection of cell lines for their panel. The advances in cytogenetic techniques used by Nelson-Rees and colleagues had already determined the KB cell line that was used by the NCI for early *in vitro* anticancer drug screening research was in reality derived from HeLa cells (Nelson-Rees and Flandermyer, 1976). Another concern the NCI had to deal with at conception of the NCI-60, was again linked with work by Nelson-Rees and colleagues. They had determined that the number of passages a cell line underwent, that is the number of times the cell culture had been subcultured, could lead to vast changes in characteristics that meant it no longer represented the primary tumour it was derived from (Nelson-Rees *et al.*, 1976). The NCI

attempted to avoid concerns of cross contamination of cell lines, authenticity of tissue and tumour type, and potential difference in cell characteristics caused by high passage number by using cell lines from trusted sources, carrying out cytogenetic techniques used by Nelson-Rees and colleagues, and using low-passage cell lines such as lung cancer cell lines from an NCI Navy Medical Oncology Branch (Gazdar *et al.*, 1980).

Despite the due diligence carried out at the inception of the NCI-60, the problem of misidentification and misclassification of cancer cell lines was further highlighted nearly two decades later. Advances in techniques used to highlight gene expression allowed scientists to more fully characterise tumour cell lines, and it was determined that the breast cancer cell line MDA-MB-435 more closely resembled a melanoma cell line (Ross *et al.*, 2000; Ellison *et al.*, 2002) and was in fact derived from the same patient as melanoma cell line M14 (Garraway *et al.*, 2005). Further DNA profiling of their cell lines determined that breast cancer cell line MDA-N was derived from MDA-MB-435 and so was also to be considered a melanoma cell line. Furthermore, central nervous system (CNS) cell lines SNB-19 and U251 were determined to originate from the same individual (Garraway *et al.*, 2005) and a cell line at first thought to be derived from the breast cancer cell line MCF-7 was determined to be of ovarian tumour origin. In 1998 DNA fingerprinting of MCF-7 and NCI-60 panel cell line MCF-7/ADR-RES suggested that the two cell lines were not derived from the same individual (Scudiero *et al.*, 1998), and the name was therefore changed to NCI/ADR-RES. Further analysis of the cell line through comparison with the other 59 cell lines in the panel (Roschke *et al.*, 2003) and more recently with single nucleotide polymorphism (SNP) array analysis

(Garraway *et al.*, 2005), it has been determined that the it is in fact derived from the same individual as ovarian cancer cell line OVCAR-8. It is thought that for the NCI/ADR-RES cell line alone, by the time it was confirmed as ovarian in origin, around 300 research papers had been published with the incorrect identification of the cell line (Liscovitch and Ravid, 2007).

So even though concerns of cancer cell line misidentification and cross-contamination brought to light by Gartler and Nelson-Rees were not met with resistance by all, and quality control steps were implemented and adhered to, the number of cell lines misidentified or misclassified had not been reduced by the start of the 21st century, but actually increased. The American type culture collection standards development organization workgroup ASN-0002 (Alston-Roberts *et al.*, 2010) reports that by 1999 there were reports that the incidence of cell line misidentification could be as high as 18% (MacLeod *et al.*, 1999) and a study of 500 leukaemia cell lines in 2003 determined that 15% of said cell lines were misidentified due to cross contamination, both from primary (originators of cell lines) and secondary sources (Drexler *et al.*, 2003). This finding was particularly interesting as there was no significant difference between the incidence of misidentified cell lines from primary sources (14.9%) and secondary sources (14.8%), that there was as much chance that cross contaminations occurred during attempted establishment of a new cell line as all being cross-contaminated by secondary investigators. A year later a survey was carried out, and out of 483 replies to the survey for active cell culture workers "32% used HeLa cells, 9% were unwittingly using HeLa contaminants, only 33% of the investigators tested their cell lines for authenticity and 35% obtained their cell

lines from other laboratories rather than from a major repository" (Alston-Roberts *et al.*, 2010; Buehring *et al.*, 2004). Continued reports of misidentification of cell lines, the complacency and denial from some scientists and the efforts of Nelson-Rees being ignored after his termination up to this time, led to a second misidentification revolution led by Roland Nardone. With the support of many scientists, organisations such as the NIH and the Howard Hughes Medical Institute, and editors of science journals, Nardone created a white paper that presented straight forward solutions to the misidentification problem (Nadone, 2007). The idea was simple, that funding agencies and journals would require cell line authentication before funding or publication was possible.

Researchers reaction to this was both of acceptance for the need to standardize cell line authentication and anger towards funding agencies and journals that had not addressed this problem sooner, but despite this acceptance misidentification has not been eliminated. In 2017 a study into 278 widely used cancer cell lines in China determined, by comparing DNA profiles with ATCC and DSMZ cell bank databases, that 46% of these 278 cell lines from 22 institutes were misidentified or cross-contaminated (Huang *et al.*, 2017). Huang *et al.* proceeded to report that they had found that 73.2% of cell lines established in China were misidentified cell lines and that the simplest form of misidentification was mislabelling of a cell line and their detection sensitivity for cross-contamination of another cell line was 5% meaning that with less complacency in laboratories, and regular cell line authentication, misidentification numbers could be drastically reduced. They also implied that methods of cell line authentication should be robust enough to determine cell misidentification. When authenticating the bile duct cancer cell line

HCCC-9810, the degenerative lung cancer cell line Calu-6 was an 88.9% match in the ATCC database (9-loci), yet when 21-loci were used to compare the match was only 48.2% and SNP profiles of the two cell lines also demonstrated that they were two different cell lines. So although still a problem, continued awareness of such, bides well for future prevention of cell line misidentification.

1.1.2 Tumour Heterogeneity and Genetic Variation

Misidentification and cross-contamination are not the only factor to consider when regarding cell line suitability in research. Tumours are extremely heterogeneous. This means that in a given tumour, we see cell subpopulations with distinct geno- and phenotypes that may display different biological behaviours, and may be more likely to proliferate in different cellular environments. It is a major factor in acquired drug resistance. Figure 1.1 shows cancer clonal theory (Nowell, 1976), cancer growth and subsequent mutations in individual cells leads to formation of sub-clonal populations that possess unique mutations compared to other sub-populations. Cancer cell lines are selected from tissues/tumours of interest to research cancers of a particular type, but due to the high levels of tumour heterogeneity, how well does a cancer cell line represent the tumour it originated from? One of the greatest advantages of work using cancer cell lines is that research can be carried out on them that would otherwise be impossible to *in vivo.* It is therefore important that cancer cell line models represent the heterogeneity of the origin tumour so that any research carried out on them is clinically relevant. Much research has been carried out to determine just how similar or different cancer cell lines in use are to their tumour of origin.

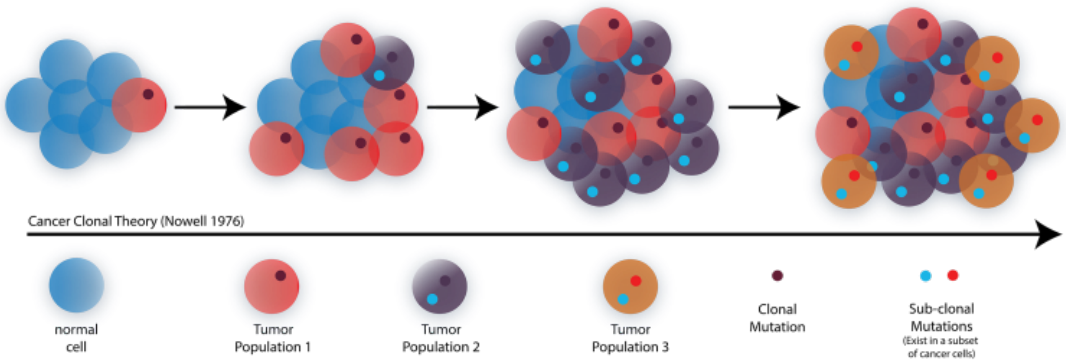

Figure 1.1 Cancer Clonal Theory. Genetic instability of cancer cells leads to production of variants and those with mutations that promote additional selective advantage thrive and create subpopulations within the tumour. Different environmental pressures are responsible for selection of cells that become dominant subpopulations. (Image downloaded from http://www.bioskyb.com/technology accessed 28/02/2020)

In 2006 a study of 51 breast cancer cell lines and 145 primary breast tumours compared genomic properties between cell line and primary tumour (Neve *et al.*, 2006). Hierarchical cluster analysis was carried out with cell line and gene expression patterns compared between cell lines and primary tumour, and the same method was used for genome aberrations. The frequency of significant increases or decreases in genome copy number were also compared between cell line and primary tumour. They concluded that the panel of 51 cell lines as a system, not individually, mirrors the genomic heterogeneity and the recurrent genome copy number abnormalities found in primary tumours, but that they also carry more aberrations, and increased amplification compared to primary

tumours. Furthermore, they believed that differences between the cell lines and primary tumours could be explained by the fact that many of the cell lines were obtained advanced-stage tumours so may represent the most malignant variants.

A study on colorectal cell lines compared genome-wide gene expression data from 22 colorectal cell lines and 276 primary colorectal tumours (Auman and McLeod, 2010) and determined that their comparison suggested that colorectal cell lines did not reflect the heterogeneity of colorectal primary tumours. They found that in eight different gene sets, the colorectal cell lines grouped together and posited that presence of nonparenchymal cells (that is cells that are not colorectal in origin) in primary tumour and gene expression alterations caused when creating the *in vitro* cell cultures were the reason for the differences observed between cell lines and primary tumour at the genome-wide gene expression level. This is a common theme among similar cancer cell line studies.

Ross *et al.* (2000) used the NCI-60 cell lines to determine whether gene expression in the cell lines corresponded to tissue of origin. Unlike previous studies into cancer/cancer cell heterogeneity mentioned earlier, no primary tumour samples were used, only the cell lines. They found that gene expression patterns related to the historical origins of the cell lines, that is the cell lines of the same tissues of origin mostly clustered together and that these cell line clusters were often distinguished by the expression of groups of genes responsible for characteristics of their particular tissue type. This is how melanoma cell lines MDA-MB-435 and

MDA-N were found to be misidentified as breast cancer cell lines, they clustered with melanoma cell lines and further investigation was carried out.

Gillet *et al.* (2012) iterate that genomic and transcriptomic changes should be clearly distinguished when comparing cell line and primary tumour as it has been repeatedly shown that driver mutations are almost always retained. This has been previously discussed (Neve *et al.*, 2006). Gillet *et al.* proceeded to compare expression profiles, for a selection of genes, of 112 ovarian tumour samples (80 untreated, 32 unpaired samples obtained at diagnosis or at recurrence after chemotherapy) with a panel of ovarian cancer cell lines either grown in three-dimensional culture or as xenografts, or from the NCI-60 panel. They discovered that cell lines, grown *in vitro* or *in vivo*, and clinical samples clustered in two distinct groups. Addition of the remainder of the NCI-60 cell lines (from 8 other tissues of origin) into this analysis, clustering was also in two groups: clinical samples and cell lines. The cell lines, regardless of their tissue of origin, were bore more resemblance to each other than the primary tumour samples they were supposed to model. Using 90 cell lines of six different tissue types they also determined, using fold change of gene expression for each tissue type (cell line and primary tumour), that cell lines and primary tumour of the same type are more similar to each other than cell line/primary tumours pairs from different tissues. So overall it was found that although cell lines have very different genomic signatures to the primary tumour of tissue type they originate from, they are closer to that primary tumour then any other tumour, but also that they are closer to cell lines of any tissue then they are to primary tumour of the same tissue.

Ben-David *et al.* (2018) took a slightly different approach to research of tumour heterogeneity and instead focused on heterogeneity in the cell lines themselves. They compared genetic profiles of 106 cell lines that were shared by two separate pharmacogenomics studies (the Broad Institute's Cancer Cell Line Encyclopedia (CCLE), and Sanger Institute's Genomics of Drug Sensitivity in Cancer (GDSC)), and also compared 27 strains of the breast cancer cell line MCF7 (19 of which had not undergone drug treatment). They also verified findings on multiple strains of 12 other cancer cell lines. It was determined that these "clonal" cancer cell lines were actually highly genetically heterogeneous. They found that when comparing two strains of a cancer cell line approximately 20% of mutations were observed in just one of the cell lines, when cloning cell lines variations in gene expression occurred *de novo*, genetic manipulations such as fluorescent reporters that are considered neutral can introduce genetic variation and that drug response can be affected by this observed variation between cancer cell lines.

The consensus of research on how the characteristics, and therefore heterogeneity, of the primary tumour is represented is that there are genomic similarities between cell line and primary tumour but maybe not as much transcriptomic similarity, that is gene expression profiles are different. However, Neve *et al.* (2006) suggests that perhaps the heterogeneity of the primary tumours is seen in cancer cells but between a panel of cell lines of the same tissue, and Ben-David *et al.* (2018) go one step further to suggest that cell lines of the same origin with heterogeneous variability may even be advantageous to cancer cell line research in a few ways:

1. It could enable the study of cooperative and competitive interactions between cell populations – that is it could help us understand which mutations may drive drug resistance or contribute to cancer population survival after treatment.
2. Comparisons between genetics between original cells and cells that are genetic variants (but originate from those cells) may give insight into associations between molecular features and phenotypes such as drug response.

1.1.3 Advantages of Using Cancer Cell Lines

As well as the limitations discussed, there are of course advantages to using cancer cell lines as an experimental model. Reviews by Namekawa *et al.* (2018) and Goodspeed *et al.* (2016) outline three main advantages for the use of cancer cell lines as molecular models of cancer. These advantages will be briefly discussed here.

1. Easily grown -

   Cancer cell lines, once derived from a patient sample, retain proliferative traits of the primary tumour. They also inherit "immortality" from their parent cells most often due to their expression of telomerase (Counter *et al.*, 1992; Chiu and Harley, 1997), so unlike normal cells there is no limit to the amount of times a cancer cell can divide. The combination of the

robustness, proliferative capabilities and immortality of cancer cell lines means that as long as grown on suitable growth media, they can easily be thawed from a frozen supply and be easily grown and maintained.

2. Relatively inexpensive –

Cancer cell lines, if not established from primary tumour in lab, would come at one initial cost. As cell lines can be frozen, defrosted, grown and frozen again, it is not necessary to purchase. But perhaps "inexpensive" should be thought of in terms of time, that is time to grow and maintain the cell lines. The process of growing the cancer cell lines is relatively easy when compared to other models, for example patient-derived xenografts require laborious and time consuming work (Namekawa *et al.*, 2018).

3. Amenable to high-throughput screening –

Due to the 2D nature of cancer cell line culture, that is they grow as monolayers, makes them perfect for use in high-throughput screening of drugs. The automated nature of this method allows many drugs to be screened on a large number of cell lines in a relatively short time compared to the process if it involved manually carrying out the drug screen on the cell lines individually.

As well as these advantages, I think it is important to consider some others. The high variety of cancer cell lines available, means that researchers already have

access to many different possible cancer models. Many types of cancer and tissue type are available, for example over 1000 cancer cell lines from 16 tissues are available from the ATCC. Another advantage is their genomic similarity with their primary tumour. As stated earlier, although cancer cell lines don't mirror primary tumour heterogeneity or gene expression profile to any great extent, there is a great deal of similarity between genomic alterations. Driver mutations in the primary tumour are present in the cancer cell line.

It has been suggested that potentially, rather than determining whether or not a cancer cell line model is suitable for research, the question "Do all cancer cell lines have equal value as tumour models?" (Gillet *et al.*, 2013) should be asked as perhaps some cell lines have greater clinical relevance. For example, low passage cancer cell lines are likely more genetically representative of the primary tumour than a cell line that has accumulated selective traits as it has acquired over many cell divisions. However they are or have been used and despite their limitations, they have increased in number and type highlighting the demand for cancer cell line models. This has led to the formation of cancer cell line panels, collections of large numbers of cancer cell lines with various implications for cancer research.

1.2 Pharmacogenomic Cell Line Studies

1.2.1 Cancer cell line panels

The number of cell line panels has increased in recent years. The first such panel was the American Type Culture Collection (ATCC), an organisation which was

established to collect, authenticate, store and distribute reference microorganisms, and in 1962 they began to do the same with cell lines. Other cancer cell line panels would not only store cell lines however, and in the 1980s as the demand for identification of novel anticancer compounds increased, a panel of cell lines was developed to test the efficacy and variability of chemotherapy agents over a range of tumour types. And so the National Cancer Institute began testing compounds on 60 cancer cell lines over 9 tissue types and the NCI-60 was born (Alley *et al.*, 1988; Grever *et al.*, 1992; Shoemaker, 2006). Around the same time the Japanese Foundation for Cancer Research established a panel of 39 cell lines with the same goals in mind (Yamori *et al.*, 1999; Yamori, 2003). Although 30 of the cell lines were also in the NCI-60, they also included six gastric cancer cell lines that represented the prevalence of stomach cancer in the Japanese population. Since the conception of these two panels, many more have spawned with different goals, for individual tumour types or for testing of certain drug types, or for investigation of mechanisms of drug resistance. The LL-100 is a cell line panel that collects 100 cell lines covering 22 entities of human leukaemia and lymphoma (Quentmeier *et al.*, 2019). They believed that all leukaemia and lymphoma cell lines were not represented by previous cell line panels and so developed this specialised cell panel. The Resistant cancer cell line collection (RCCL) is a cell line panel that collects cell lines that have acquired resistance compared to parental cell lines (Michaelis *et al.*, 2019). The RCCL collects 1300 cancer cell lines representing acquired resistance in 15 cancer entities and allows investigation into mechanisms of drug resistance, and biomarkers for alternative drug therapy on these resistant cell lines. The RCCL is another example of how cancer cell line panels can be used for different approaches to cancer research.

Perhaps the greatest advantage of these cell line panels though, it's to screen many compounds using high through-put methods. Four such pharmacogenomics cell line panels are particularly popular among the scientific community today: the NCI60; the Cancer Cell Line Encyclopedia (CCLE); the Genomics of Drug Sensitivity in Cancer (GDSC); and the Cancer Therapeutics Response Portal (CTRP). Between them they collect 1300 cancer cell lines of over 20 cancer types (Table 1.1). Here, each of these four pharmacogenomics cancer cell line panels will be discussed in further detail.

Table 1.1 Tissue representation of cell lines in large *in vitro* pharmacogenomics databases.

Malignancy	CCLE	GDSC	CTRP	NCI-60	Unique
Bladder	28	18	5	0	29
Bone	29	31	1	0	53
Breast	60	43	1	5	64
Cervix	0	12	0	0	12
Colon	63	35	37	7	70
Endometrium	28	10	11	0	29
Head and neck	35	23	2	0	44
Hematopoietic and lymphoid	181	113	24	6	227
Kidney	36	22	2	8	48
Liver	36	14	4	0	41
Lung	187	141	91	9	226
Nervous system	86	79	3	6	126
Osephagus	27	23	3	0	29
Ovary	52	20	26	7	57
Pancreas	46	17	10	0	47
Prostate	8	5	1	2	9
Skin	62	45	9	10	79
Soft tissue	21	17	3	0	28
Stomach	38	18	6	0	44
Testis	0	2	0	0	2
Thyroid	12	12	0	0	16
Other	11	7	3	0	20
Total # of cell lines	1,046	707	242	60	1300
Total # of agents tested	24	139	185	~50,000	

Adapted from table 1, Goodspeed *et al.* (2016).

1.2.2 NCI-60

As discussed previously, the NCI-60 was established in its fully operational form in 1990. The concept of this *in vitro* drug discovery cell line panel was devised in response to the limited success of identifying new anticancer compounds for human solid tumours using transplanted murine neoplasm models (Boyd, 1997). A total of 60 cell lines from nine different cancer types were established in the panel, a feat that was not easy at the time due to the concerns over cell line cross-contamination and misidentification that had been brought to light less than a decade earlier. Painstaking work was carried out to obtain low passage cell lines that represented the nine cancer types, ensuring patterns of expression were consistent with their primary tumours (Stinson *et al.*, 1992). Although it is now known that there were misidentification issues with a small number of the cell lines, they have now been reclassified and the NCI-60 still carries out research on the same cell lines over 30 years later.

The number of compounds screened by the NCI-60 is ~50,000. This number is unmatched in any other cell line panel. Early findings by the NCI-60 included the implication of two methods, the Mean Graph method and COMPARE algorithm. For the Mean Graph method the concentration for 50% of maximal inhibition of cell proliferation (GI50), the concentration that is lethal for 50% cells (LC50) and the total growth inhibition (TGI) are calculated for a compound for each cell line. The deviation of each cell line in relation to the overall mean for all the cell lines is plot and the Mean Graph is created. Figure 1.2 shows the Mean Graphs for the

two compounds carboplatin (A) and cisplatin (B). Cell lines with bars to the left of z-score of 0 (the mean) are classed as resistant to the compound, while bars to the right are classed as sensitive. The COMPARE algorithm uses the GI50, LC50 and TGI to essentially compare the Mean Graph patterns. Initial NCI findings using COMPARE observed that compounds that had similar Mean Graphs were often of similar structure, and further more whether similar in structure or not, would share the same or related mechanism of action (Paull *et al.*, 1995). This is highlighted in figure 1.2, the Mean Graphs are very similar for carboplatin and cisplatin. Carboplatin and cisplatin are both platinum drugs with similar structures, a doubly charged platinum ion surrounded by four ligands, and their mechanism of action is the same.

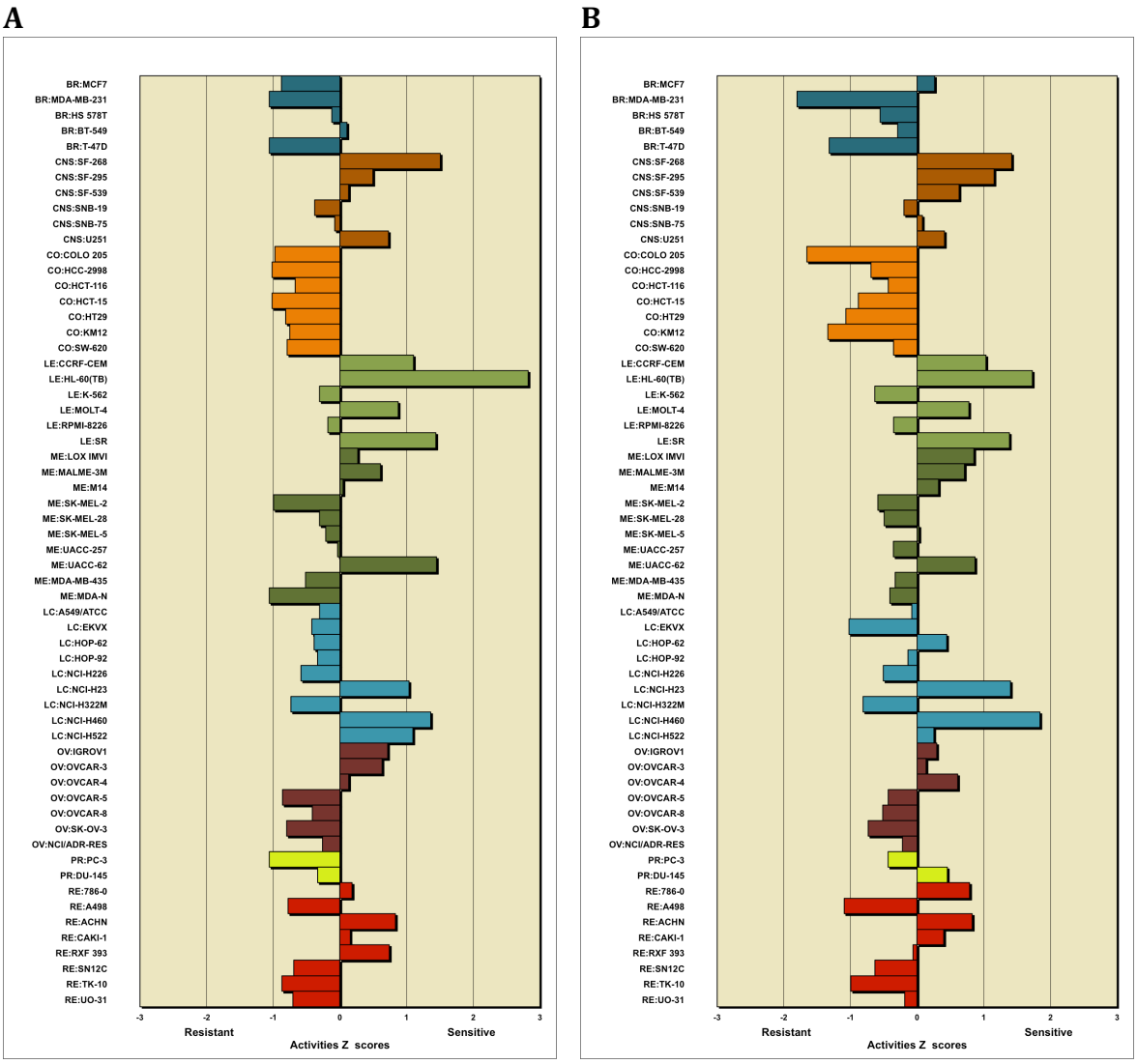

Figure 1.2 Mean Graphs using NCI-60 cancer cell line data. The relative activity of **A.** Carboplatin and **B.** Cisplatin on NCI-60 cell lines. Activity z score of 0 is the mean activity (GI50) of all cell lines. (Generated using NCI resource CellMiner https://discover.nci.nih.gov/cellminer/)

It is worth noting that the GI50 measurement is the most commonly used measurement from the NCI-60 and although it is often thought of as essentially the same as the more commonly known IC50 (Brooks *et al.* 2019), this is not the case if the potency of a compound is low. This is illustrated in figure 1.3. While IC50 is the drug concentration which reduces cell population to half of the control value, the GI50 is the drug concentration which reduces total cell growth by 50%. Essentially, the GI50 is a correction of the IC50 as the initial cell count is taken into

consideration. This is perhaps why the GI50 is preferred by the NCI60 over the IC50, the GI50 accounts for different growth rates. This is important when testing multiple cell lines or experimental conditions (Brooks *et al.*, 2019).

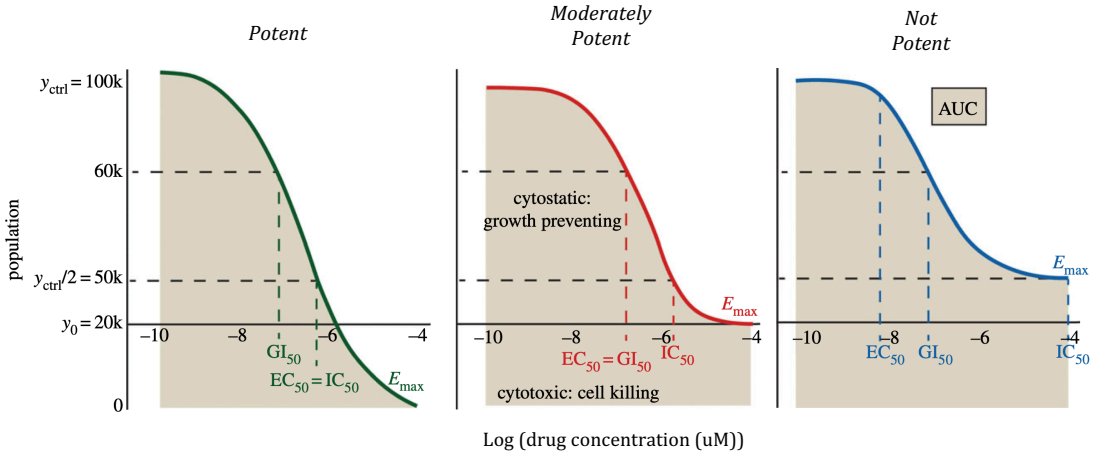

Figure 1.3 Difference in drug response metrics for compounds with different potency. Illustrated is the difference in concentration measurement of GI50, EC50 and IC50 in compounds that are A. potent, B. moderately potent and C. not potent.
Adapted from Figure 1, Brooks *et al.*, (2019).

Through this early work carried out by the NCI-60, many compounds were identified for *in vivo* testing and as a result the NCI developed novel ways to speed up the process by bypassing traditional human tumour xenografts (Hollingshead *et al.*, 1995), and when prescreening and high-throughput methods were implemented, the entire drug screening and testing process was a formidable tool in the battle against cancer. This has suited their method of the screening of many compounds on a relatively small number of cancer cell lines. Incidently, even reduced numbers of cell lines compared to the 60 in this panel support the COMPARE method developed by the NCI-60 (Yamori, 2003) making this a powerful tool for cell line panels. COMPARE analysis of a compound submitted for testing could arguably be the NCI-60s greatest contribution to cancer research.

Proscript Pharmaceuticals submitted the compound bortezomib for screening and COMPARE analysis and the result was COMPARE-negative. This meant that the bortezomib Mean Graph did not compare with any other compounds and indicated an unknown mechanism of action, one that was distinct from known anticancer drugs. This warranted further investigation and after eventual clinical trials, the FDA approved bortezomib, in the relatively short time of eight years from initial testing (Holbeck and Sausville, 2004).

In 2000 the NCI-60 began to move away from an NCI drug-discovery pipeline to a research tool for the scientific research community. As a result resources for the NCI-60 were reduced and compared to when the screen was implemented the workforce is now 20% what it was and the screening throughput has greatly decreased (Shoemaker, 2006). Despite this, the NCI-60 still remains a powerful tool, they continue to screen compounds sent to them by the researchers in the cancer research community and all data produced by them, including genomic and drug data, is easily available through a resource developed at the NCI called CellMiner (Shankavarum *et al.*, 2009).

1.2.3 Cancer Cell Line Encyclopedia

The Cancer Cell Line Encyclopedia (CCLE) project is a collaboration between the Broad Institute and Novartis Institutes for Biomedical Research (Barretina *et al.*, 2012). They undertook a different approach to the NCI-60. Rather than screen many compounds against few cell lines, they screened few compounds against many cell lines. This would give them best chance of achieving their goal, to

identify genomic predictors of drug response. The cell line panel consisted of ~1000 cancer cell lines, however , the drug screening was carried out with 24 compounds across only 504 of the cell lines, representing 21 different cancer types. A large-scale genomic dataset was generated using 947 of the cell lines, the data included mutational status of 1651 genes, DNA copy number, and mRNA expression levels. Measurements of cell line sensitivity were determined using 8-point dose response curves, resulting in values for maximal effect level (A_{max}), the concentration at half-maximal activity of the compound (EC50) and the concentration at which the drug response reached 50% inhibition (IC50). If the IC50 was not reached for a cell line, the maximum concentration of 8uM was given as the IC50 for that particular compound. Unlike the NCI-60, there was no additional screening of compounds, and this study was used to illustrate methods optimise cancer cell line research. Using the data they generated, they highlighted methods that they believed identified drug-gene and drug-lineage relationships.

They observed strong positive correlation for genomic features between cell lines and primary tumours of the same tissue type. That is for point mutation frequency, copy number and gene expression profile. This proved that CCLE cell lines likely represented the genomic features of primary tumours that shared the same tissue type. They found drug-gene relationships between RAF inhibitor PLX4720 and *BRAF* mutations, HER2 inhibitor lapatanib and *ERBB2* amplification, and Nutlin-3 and MDM2 overexpression to name a few. These were among the relationships they observed that were already known, but this acted as proof of concept that their approach using elastic net regression methods worked. So using the same methods they identified unknown relationships including topoisomerase I

inhibitor irinotecan sensitivity and SLFN11 expression, PD0325901 and AHR expression in *NRAS* mutant cancer, and IGF1 receptor inhibitor AEW541 and multiple myeloma.

The results from the CCLE analysis were significant themselves but perhaps the greatest strength of the cell line panel, is the number of cell lines from many different cancers, and the subsequent genomic analysis carried out upon them. As discussed previously, primary tumour heterogeneity is unlikely to be represented in a single cell line (Auman and McLeod, 2010), but with a large enough number of cell lines from a cancer type, perhaps tumour heterogeneity is represented over all of the cell lines (Neve *et al.*, 2006; Ben-David *et al.*, 2018). Additionally, a cell line panel with as many cell lines from as many tumour types with genomic analysis already carried out on them is obviously a great resource for scientific researchers. Domke *et al.* (2013) evaluated the ovarian cancer cell lines to determine whether they could be used as tumour models. Using the genomic data available and comparing to tumour sample genomic analysis, they determined that the 12 most suited candidate ovarian cell lines (from the 47 available in the CCLE) only accounted for 1% of PubMed citations out of the 47 analysed ovarian cell lines. Furthermore, they discovered that the two most frequently used ovarian cell lines that account for 60% PubMed citations, were poorly suited as models. They suggested though that cell line choice for a model is highly context specific, but cell line panels like CCLE can help to optimize the choice of cell lines for a particular tumour model.

Additional work by the CCLE team has recently been carried out (Ghandi *et al.*, 2019). As well as gene expression data for the whole genome, they have identified new characteristics for the cancer cell lines in their panel. Data available from their data portal now also includes genetic, RNA splicing, DNA methylation, histone H3 modification, microRNA expression and protein array data for various cell lines in the CCLE panel (for example, their whole-exome sequencing (WES) is only carried out on 329 cell lines while RNA sequencing (RNA-seq) is carried out for 1019 cell lines). In parallel work they have also measured the abundance of 225 metabolites in 928 of the cell lines in the panel (Li *et al.*, 2019). This additional CCLE cancer cell line analysis has strengthened an already valuable cancer cell line resource.

1.2.4 Genomics of Drug Sensitivity in Cancer

The Genomics of Drug Sensitivity in Cancer (GDSC) project is a collaboration between the Cancer Genome Project (CGP) at the Wellcome Sanger Institute and the Center for Molecular Therapeutics, Massachusetts General Hospital Cancer Center (Garnett *et al.*, 2012; Yang *et al.*, 2013). This study has received many comparisons with the CCLE as both are large-scale pharmacogenomics studies and both were published in the same year. As with the CCLE, the GDSC collects a large number of cell lines, 639 in the original study. However, 130 drugs were selected for screens, more than five times the amount used by the CCLE, though they didn't screen every cell line for every drug, a range of 205-507 cell lines were screened per drug. Another difference was the expression profiling between the two studies. Expression profiling of 14,500 genes was carried out in the GDSC

study, compared to just the 1651 for the original CCLE study (Barretina *et al.*, 2012).

Measurement of compound activity for cell lines in this study varied from the previous studies discussed. IC50 values were calculated using similar methods but calculations for cell lines that didn't reach an IC50 for a compound was not "capped" at the highest compound dose concentration as in the CCLE and NCI-60. An algorithm was used to extrapolate IC50 values, resulting in IC50s sometimes much higher than the maximum compound dose. Like the CCLE, all pharmacological data was carried out before analysis of the cell lines and further compound analysis (as the NCI-60 carries out) was not implemented.

Using multivariate analyses of variance (MANOVA) and elastic net regression methods revealed many significant gene-drug associations. As with the CCLE, known associations were observed in the data confirming proof of concept: *BRAF* mutations were associated with BRAF and MEK1/2 inhibitors and *HER2* mutation and amplification was associated with sensitivity to EGFR-family inhibitors. They also found that many transcriptional features can be associated with sensitivity of a cell line for a compound. For example, sensitivity to RAF or MEK1/2 inhibitors was associated with 67 genomic or transcriptional features other than *BRAF* mutation. The take away here, is that the GDSC analysis highlighted many gene-drug associations marking it as a potentially powerful resource in cancer cell line research, perhaps highlighted by their observation of PARP inhibitor sensitivity by *EWS-FLI1* positive Ewing's sarcoma cell lines.

A highly significant association was identified between the *EWS-FLI1* rearrangement indicative of Ewing's sarcoma tumours and PARP inhibitor olaparib (AZD2281). PARP inhibitors have activity in *BRCA1/2* mutant cancers and a comparison between PARP inhibitor sensitivity (olaparib and structurally distinct AG-0146099) in a 6-day viability assay confirmed a sensitivity to Ewing's sarcoma that was comparable to cancer cells with *BRCA1/2* mutations. The cancer cell lines tested on were not BRCA-deficient cell lines so the sensitivity observed was not due to *BRCA1/2* mutations, and *EWS*-FLI1 rearrangement was touted as a possible biomarker for PARP treatment of Ewing's sarcoma.

As with CCLE methods of analysis, the potential of the GDSC and other large-scale pharmacogenomics cell line studies is clear to see. Since the GDSCs initial analysis, there data repository, like the CCLEs, has grown. On a very user friendly online resource available at https://www.cancerrxgene.org all the GDSC data is easily accessed. Over recent years they have increased the amount of drugs and cell lines analysed and therefore more genomic information has been made available (Iorio *et al.*, 2016) and data and analysis is now available for two different sets of data: GDSC1 and GDSC2. The GDSC1 updates previous screening results and consists of 987 cell lines and 367 compounds. The GDSC2 uses the latest improved screening technology and consists of 809 cell lines and 198 compounds. Mutation, copy number, methylation and expression genomic data is available for the best part of all cell lines in the panel. This GDSC resource is perhaps the most user friendly and informative of the three pharmacogenomics cell line panels discussed so far.

1.2.5 Cancer Therapeutic Response Portal

The Cancer Therapeutic Response Portal (CTRP) was developed by researchers at the Center for the Science of Therapeutics at the Broad Institute (Basu *et al.*, 2013). Initially they gathered data from the CCLE cell panel, they used a subset of 242 of the CCLE cell lines and all genomic data for these cell lines. They determined their own sensitivity profile for the cell lines using an Informer Set 354 small molecules (35 FDA-approved drugs, 54 clinical candidates, 265 probes) and unlike the previous studies discussed, they chose the area under percent-viabilty curves (AUC) rather than IC50 as there measure of drug sensitivity. They stated the reason for this was that they believed the AUC measurement reflected both relative potency and total level of inhibition observed for a compound. Cell lines were determined sensitive to a compound if the AUC < 3.5 and resistant to a compound if AUC > 5.5 and used enrichment and regression analysis that used rank-based and parametric tests to determine whether genetic alterations and cell features were significantly enriched in these two groups (sensitive and resistant).

As in the CCLE and GDSC analyses, proof of concept was provided as known gene-drug associations were identified in the data. P-0850, a compound that is an analog to BRAF-V600E inhibitor vemurafenib was associated with sensitivity to *BRAF* mutated cell lines and that *EGFR*-Mutant lung cancer cell lines were highly sensitive to HER2/EGFR inhibitor neratinib. New found associations included findings that *MYC* mutated cell lines were associated with sensitivity to (-)-gallocatechin-3-monogallate (GCG) and ovarian cell lines were the most sensitive cell lines to two probes ML210 and RSL3. Perhaps the finding in this study with

the highest impact was that *CTNNB1* mutations in cell lines were associated with sensitivity to BCL inhibitor navitoclax.

Like the CCLE and GDSC, the CTRP has carried out further research and developed an online resource (Seashore-Ludlow *et al.*, 2015; Rees *et al.*, 2016). The additional work carried out by Seashore-Ludlow *et al.* (2015) involved looking at interactions between small molecule treatments using their own high-throughput method that allowed them to propose synergistic compound combinations. This method identified synergistic combinations that had been previously identified, such as IGF1R and MEK inhibitors and identified a snergy between crizotinib and BMS-754807 in neuroblastoma cell line NB1 but not in *KRAS*-mutant cell line LS513 showing that combination therapies are also targeted as targeted single therapies are. Much of the work from this study was establishing the data for the CTRP version 2 (V2). Rees *et al.* (2016) used this data to correlate the small molecule data with gene expression. One of the standout results from this study was the correlation of SLC35F2 and ABCB1 expression with cell line YM-155 sensitivity. They determined that high SLC35F2 and low ABCB1 expression facilitated cell line sensitivity to YM-155.

The CTRP online resource stores annotations for all data analysis carried out. In the CTRP v1 185 compounds and 242 cancer cell lines are studied, while these numbers increase for the CTRP v2 to 481 compounds and 860 cancer cell lines. Of the four cell line panels discussed here, the CTRP is probably the most underrated, but now has one of the most varied cancer cell line panels available.

1.2.6 Reproducibility between the studies

The CCLE and GDSC published their initial research within months of each other so it is of no surprise that comparison has been drawn between the two large-scale pharmacogenomics studies. Benjamin Haibe-Kains was at the forefront of this comparison. Haibe-Kains *et al.*, (2013) measured the correlation between the CCLE and GDSC analyses. 15 drugs and 471 cancer cell lines, with mutation data for 64 genes and expression of 12,153 genes were available in both studies to compare. Correlation between expression profiles of cell lines that were in both studies was generally good (median spearman correlation 0.85 between identical cell lines) and found agreement between mutations in the same cell lines that are in both studies (two-sided Wilcoxon rank-sum test p value <1x10e-16) but this was really where the agreement between the two studies ended according to Haibe-Kains.

Drug sensitivity comparison between yielded little correlation, only drugs 17-AAG and PD0325901 showed above fair correlation (0.61 and 0.53). After removal of extrapolated IC50 values calculated by the GDSC, correlation was still no better than fair. Repeating the correlation but using AUC faired little better, median spearman correlation for the 15 shared rugs 0.28 when using IC50 as sensitivity measurement and 0.35 when using AUC. Using linear regression models on the overlapping data to estimate gene-drug associations, results between the two studies were compared. Again highest correlations were poor, below 0.5, using IC50 or AUC as measure of drug sensitivity. Even using another

pharmacogenomics study as another comparison, the GlaxoSmithKline cell line collection (GSK) (Greshock *et al.*, 2010), poor correlation was observed between datasets, and this led to Kaibe-Hains concluding that inconsistency between studies despite correlation between genomic features between overlapping cell lines, was likely due to differences in pharmacological assays. He even went as far as to say perhaps differences were due to a lack of standardisation in experimental assays and that it was not unreasonable to assume that all drugs and cell lines should be questioned, not just the shared cell lines and drugs.

There was a response to this comparison study between CCLE and GDSC. From the authors of the original papers (Cancer Cell Line Encyclopedia Consortium and Genomics of Drug Sensitivity in Cancer Consortium, 2015). They began by repeating correlation analysis on overlapping drugs (15) and cell lines (471). Importantly though, they did not remove data that was extrapolated in the GDSC data, but instead capped the cell line IC50 at the maximum tested drug concentration. Correlation was better than reported by Haibe-Kains *et al.* and the authors defend the correlation between data by explaining that in these large pharmacogenomics data sets, there will exist a drug-insensitive cell line dominated distribution. Most of the compounds target specific oncogenic dependencies so it is perhaps unsurprising that there are relatively few "sensitive" cell lines to correlate IC50 values for. This was highlighted in a waterfall plot-based assessment. On average 94% of cell lines would be classed as insensitive to the relevant compounds. The same waterfall analyses demonstrated consistency between the two datasets, as cell lines were often classed as sensitive or resistant in both studies. The authors believe that the large-scale cell line pharmalogical

screening they undertake best suits research into discovery of small numbers of drug-sensitive cell lines among many other, often drug-insensitive cell lines.

The authors actually go on to discuss analysis that they carry out on their shared data. They used analysis of variance (ANOVA) and elastic net regression methods on the shared data and observed known biomarkers that correlated in both studies (*NRAS* mutation biomarker for MEK inhibitor PD0325901 sensitivity and *BRAF* mutations to BRAF inhibitor PLX4720 sensitivity to name a few). Overall the authors determined that the two screens exhibit broad convergence and also provide examples of consilience, that is they both independently and despite individual limitations, arrive at scientific agreement. A separate study (Haverty *et al.*, 2016) also agreed with the CCLE and GDSC authors that biologically grounded methods should be used rather than purely statistical methods. They went further and employed their own methods to compare the datasets between the two studies. They did observe common sensitive and resistant cell lines and the same biomarkers in both datasets and they highlighted the likelihood that experimental methodology was likely responsible for differences between the two studies. They highlighted three key differences, the assay type, cell seeding density and growth media, and acknowledged that if these are overcome any systemic error that remains are small enough to permit the generation of biomarker results that are reproducible in other laboratories.

This was not the end of the matter as another study was published from the Haibe-Kains group (Safikhani *et al.*, 2016). They argue that the latest study with authors from both CCLE and GDSC should not have just reanalysed the old data when they

had both added data to their respective datasets. Safikhani *et al.* also question what constitutes agreement, and suggest that perhaps some of the correlation coefficients that were said to be good, were not. They come to the same conclusion as in their first paper questioning the agreement between the CCLE and GDSC, that new standards need to be put in place to realize the full potential of large pharmacogenomics screens.

The discussion continued throughout 2016, and a thesis could probably be written on the comparison of the CCLE and GDSC alone. Further discussion is available (Geeleher *et al.*, 2016; Safikhani *et al.*, 2016; Mpindi *et al.*, 2016). It may be possible that the CCLE and GDSC strive to defend their work as does Haibe-Kains, so perhaps the study by Haverty *et al.* (2016) should be the leading authority on this subject. The general consensus though, amongst all the drama, is that these large-scale pharmacogenomics studies contain vast potential into future cancer drug biomarkers, but steps must be taken to standardise procedures where possible and ensure reproducibility, or at least agreement, of results between labs.

1.3 Platinum Drugs

1.3.1 Relevance

Platinum drugs are an important class of compounds that have been tested in the aforementioned pharmacogenomic cell line studies. Despite potential negative side effects of these chemotherapeutic agents (Ruggiero *et al.*, 2013), approximately 50% of all chemotherapy schedules include a platinum drug

(Wheate *et al.*, 2010). Although successful in treatment of a wide variety of cancer types, the side effects are potentially lethal and so can be dose limiting. This not only highlights a limitation of the drugs themselves but also the pharmacogenomics screens that they are tested in: how can we know if sensitivity of a drug in a cancer cell line reflects the sensitivity in a patient when dosage is unknown? Despite limitations the good far outweighs the bad and they have had success as an anti-cancer therapy and in models derived from pharmacogenomic cancer cell line studies (Mucaki *et al.*, 2019; Li *et al*, 2020). The three most widely used platinum drugs are cisplatin, carboplatin and oxaliplatin.

1.3.2 Structure of compounds

Platinum drugs are characterised by a platinum with 4 ligands attached. Three widely used platinum drugs are cisplatin, carboplatin and oxaliplatin and their similarities in structure confer to their mechanisms of action. Figure 1.4 illustrates the similar structure of the three compounds.

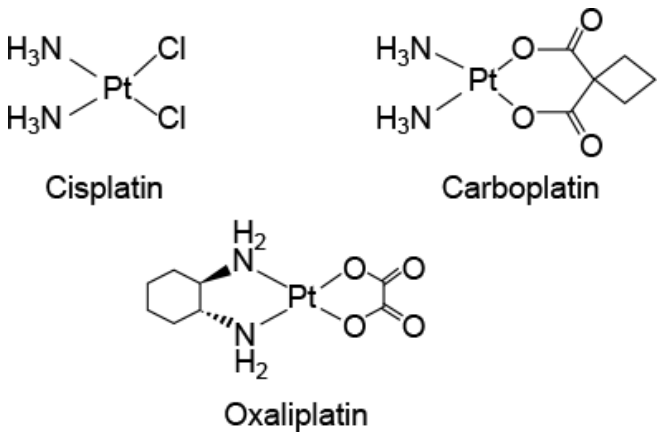

Figure 1.4 Molecular structure of three widely used platinum drugs.
Adapted from Figure 1, Barnard(2017).

1.3.3 Cisplatin

Platinum compound have become a vital weapon in the fight against cancer. Cisplatin was the first to be approved by the FDA in 1978 just nine years after its discovery. It has been used over the years to treat many cancer types and is often used in combination with other drugs. The target for cisplatin is nuclear DNA, guanine bases to be precise. It binds to both strands forming a crosslinking of the DNA, which attracts DNA repair machinery that binds irreversibly to the complex, thus preventing actual repair and replication (Gomez-Ruiz,2012). Despite the successes of cisplatin, including the treatment and eventual cure of testicular cancer (Einhorn, 1990), limitations have arisen with the drug. Firstly is the toxicity of the compound, specifically the main dose limiting nephrotoxicity. Secondly the resistance of cancers to cisplatin treatment, be it intrinsic or acquired resistance (Gottesman *et al.*, 2002).

One method of alleviating these limitations is by using cisplatin in a combined therapy. This lowers the dose of cisplatin, reducing toxicity, and if used with the correct drug can overcome resistance mechanisms in the patient (Achkar, 2018). There are, however, platinum drugs that are less toxic, and possess a mechanism of action that is different enough to cisplatin to avoid issues with resistance.

1.3.4 Carboplatin

Carboplatin was approved for use in 1986, and its mechanism of action is the same as cisplatins. Although activity of carboplatin is not equivalent to cisplatin, there

is thought to be little difference in efficacy (Go and Adjei, 1999; Santana-Davila *et al.*, 2014). But the reduced side effects of carboplatin, including the elimination of nephrotoxicity, makes it a better candidate for treatment for patients who are unable to tolerate cisplatin because of renal impairment, hearing impairment or neuropathy. Side effects do include low blood count which can lead to infection by opportunistic organisms (Shaw *et al.*, 1996). Mechanisms of resistance are thought to be the same as cisplatin (Stewart, 2007), so cannot be used as an alternative therapy to cisplatin in cases of resistance due to cross-resistance.

1.3.5 Oxaliplatin

Oxaliplatin was approved for use in 1996. Side effects due to toxicity are neither predominantly nephrotoxic like cisplatin or myelosuppressive like carboplatin, but rather neurotoxicity, its dose limiting side effect (Pasetto *et al.*, 2006). Oxaliplatin mechanism of action is similar to cisplatin and carboplatin, it forms cross-linking of DNA strands, however there are some differences in the pharmacology of the formation of these cross-links compared to cisplatin (Mishima *et al.*, 2002). This makes it a candidate as an alternative treatment to cisplatin or carboplatin in cases of platinum drug resistance. Oxaliplatin has also been successful in treatment of colon cancers which have always displayed intrinsic resistance to cisplatin and carboplatin (Comella *et al.*, 2009).

1.4 Data Reproducibility in the scientific community

1.4.1 Reproducibility crisis

There is a growing fear in the scientific community that much of the research carried out in the various scientific fields may be difficult, if not impossible to reproduce. The idea of this "reproducibility crisis" arose from the field of psychology in 2011 when a psychology research paper claiming it was possible to tell the future was published (Bem, 2011). This did not sit well with many scientists and they began questioning results of all research. Although talk of this crisis continued over the years and in other fields of study other than psychology such as neuroscience (Button *et al.*, 2013) and economics (Chang and Li, 2015), the possibility of this being a large-scale problem was really highlighted in 2015 when 270 scientists tried to replicate 100 experiments published in top journals with only 40% success (Open Science Collaboration, 2015). Figure 1.4 illustrates the results from this study, that the majority of attempts to reproduce the original results failed to do so. The replications that did work showed weaker effects than the original results and the results that were reproducible were generally the ones that had higher significance compared to ones that barely reached significance threshold. A number of reproduced results actually opposed original findings (below dotted line) but there were a number that actually exceeded the original findings (above diagonal line). Significance of reproduced results, however, was much lower, and nonsignificant results increased.

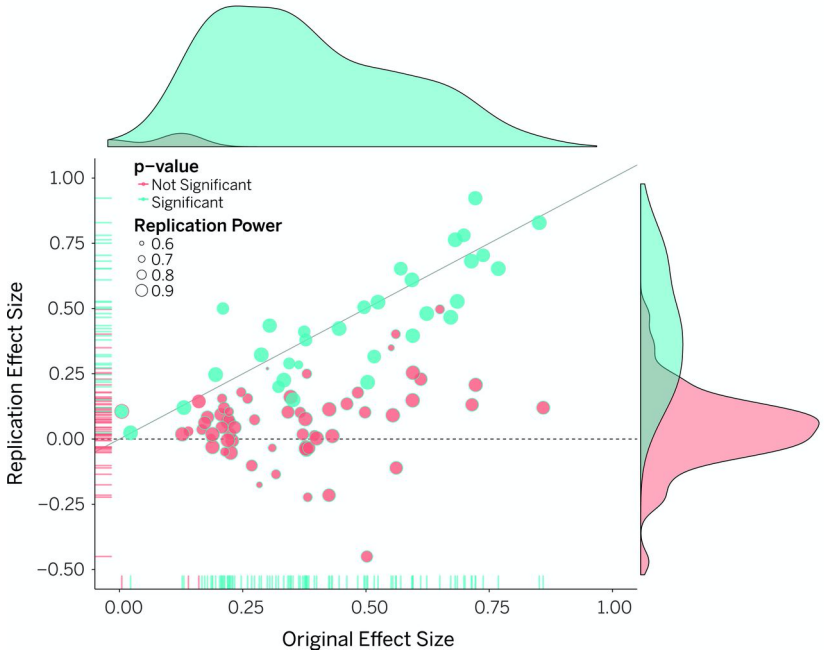

Figure 1.5 Original study effect size versus replication effect size (correlation coefficient). Diagonal line represents replication effect size equal to original effect size. Dotted line represents replication effect size of 0. Points below the dotted line were effects in the opposite direction of the original. Density plots are separated by significant (green) and nonsignificant (red) effects. Adapted from Figure 3, Open Science Collaboration (2015).

In the field of cancer biology, the Reproducibility Project: Cancer Biology is underway. A collaboration between Science Exchange and the Center of Open Science, they are attempting to replicate a subset of experimental results from a selection of cancer biology papers published between 2010 and 2012 . To date, 15 of the replication studies have been completed: Important parts of the original paper was reproduced in five replication studies (Kandela *et al.*, (2017); Aird *et al.*, 2017; Shan *et al.*, 2017; Showalter *et al.*, 2017; Essex *et al.*, 2019); another five replication studies reproduced some parts but failed to reproduce some parts (Lewis *et al.*, 2018; Vanden Heuvel *et al.*, 2018; Eaton *et al.*, 2018; Kim *et al.*, 2018; Sheen *et al.*, 2019); the results in two replication studies could not be interpreted (Horrigan *et al.*, 2017; Horrigan, 2017); and three replication studies failed to reproduce the results from the original paper (Mantis *et al.*, 2017; Repass *et al.*,

2018; Yan *et al.*, 2019). Early results as to whether these inconsistencies between original and replicated studies are indicative of a reproducibility crisis are open to interpretation and however results of the reproduced work are interpreted, questions about reproducibility and reproducibility or replication were, and are, a hot topic in the scientific community.

The terms "reproducibility" and "replication" seem to be used interchangeably throughout discussion, and perhaps they should not and the two terms should be defined. Reproducibility would describe the ability to reproduce results from a study by an independent research group using equipment and specimens (i.e. cell lines) from different sources to the original. An example of this has already been previously discussed. The CCLE and GDSC both independently required expression profiles for a collection of cancer cell lines using different platforms. Expression levels between the cell lines shared by both studies correlated, suggesting that measuring expression of cancer cell lines is reproducible, despite this not being the case with their pharmacological data. Replication would describe the ability to replicate the original research exactly, experimentally and with the same equipment and specimens. So perhaps the two terms should not be quite so interchangeable, as some may argue that failure to replicate results is worse than not being able to reproduce results. However the crisis is defined a recent increase in articles relating to "replication crisis" or "reproduction crisis" was highlighted by Wass *et al.* (2019). A PubMed search (on 12th January 2018) of the two terms yielded ~40 papers for each search compared to ~10 papers for each search in 2010 (Figure 1.5) showing the increased popularity of the notion that the scientific community is taking notice of a very real problem that faces

scientific research. Incidentally, as of March 2020, “replication crisis” yields 395 result in PubMed search and “reproducibility crisis” 465.

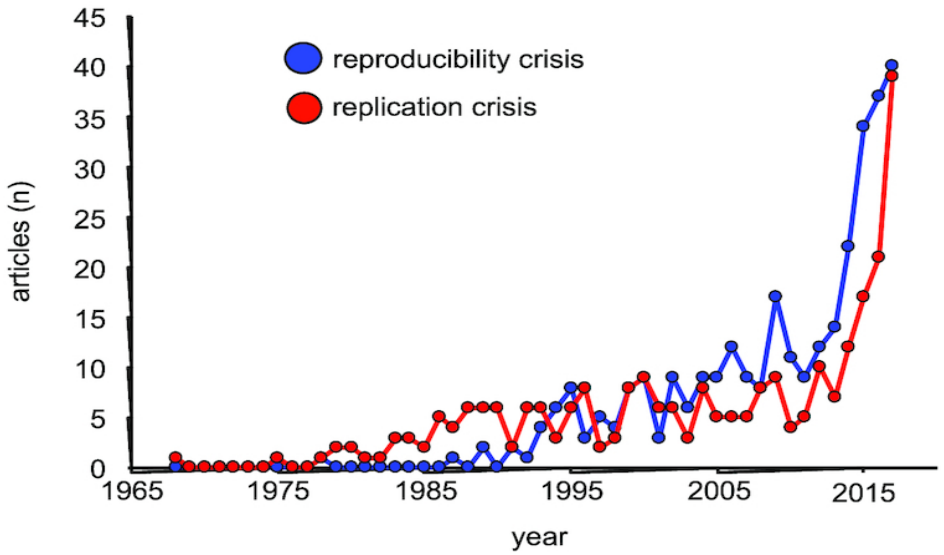

Figure 1.6 Number of articles that are identified by the search terms “replication crisis” and “reproducibility crisis” per year in PubMed. Adapted from Figure 1, Wass *et al.*, 2018.

1.4.2 Replication and reproducibility using cancer cell lines

As discussed previously, there has been a history of cancer cell lines being linked to scientific reproducibility. Some of the main issues with cancer cell lines and experimental reproducibility will briefly be revisited here in relation to their impact on the reproducibility crisis.

Cancer cell line misidentification and cross-contamination was common place in the early days of cell line research. Protocols that weren’t as stringent as recent times led to many publications towards the end of the 20th century that didn’t necessarily describe the cell lines that were actually used in the experiment, whether knowingly or not (Nelson-Rees, 2001). It is therefore fair to assume that

many results from research with cell lines from this time period may be difficult to reproduce or replicate as the cell lines may have been a completely different one to that described. Accepting that this was a problem, introduction of new methods of authentication such as short tandem repeat analysis and authenticated, standardised cell line collections such as that of the ATCC made available have all but abolished this problem, but there is still evidence of research being carried out recently with unauthenticated, misidentified cancer cell lines (Huang *et al.*, 2017).

Another issue with cancer cell lines is the heterogeneity within individual cell lines. It is quite possible that cell lines that go through many passages develop mutations other than those from parental cell lines (Nelson-Rees *et al.*, 1976). This could result in different strains of the same cell line, which could lead to reproducibility issues between two studies that unknowingly use different strains of the same cell line in their research (Ben-David *et al.*, 2018).

1.4.3 Methodological standards

Perhaps the area with the greatest focus with regards to the reproducibility crisis concerns methodological issues. There has been a reluctance to implement quality management procedures in the world of academia as work is often sensitive or secretive, and such procedures may end up with research divulged to a competitor lab. One could argue though, that pharmaceutical companies work with data as sensitive in other research labs yet they have managed to develop quality

management procedures, such as improved electronic record keeping and auditing of labs (Riedl and Dunn, 2013).

Two studies offer interesting insight to possible problems with methodological procedure in labs. Smaldino and McElreath (2016) suggest that scientific methodology evolves, that is researchers in a lab will inherit work practices from that lab and whoever is in charge of that lab, and then begin their own labs with these inherited work practices. If researchers were rewarded primarily for publishing, then practices to promote research publication will have been "naturally selected" and will drive their own lab. This is no bad thing of course, as these methods may be associated with greater academic success, but it is entirely possible that poor work practice can be "inherited" as well. Smaldino and McElreath tested their hypothesis, that there was more likely to be natural selection of bad science, by developing a model where they could simulate running laboratories. The labs had different areas of research, they ran experiments and published papers, but the amount of effort they spend testing their ideas (in the model). They found that spending more effort resulted in fewer reliable papers in the model and positive findings were easier to publish then negative findings. They concluded that the virtual labs shifted towards low-quality methods, widespread unreliable results and little effort.

Barnett *et al.* (2018) added to this model. They believe a "publish or peril" incentive drives quantity of research papers rather than quality and that this "natural selection" that was described by Smaldino and McElreath meant that the incentive was passed on to other labs an created a "competitive spiral". They found

that if they implemented auditing 1.35% of papers published in the model, the "competitive spiral" was avoided in 71% of simulations, auditing 1.94% avoided it in 95% of simulations. To give this context, by not adding random auditing to the model, the "competitive spiral" was only avoided in 0.2% of simulations. Barnett *et al.* believe that the fear of being audited maintains research quality and removes labs that report high levels of false positive findings.

As well as the random auditing of labs, other steps can be taken to help standardise aspects of the research environment. Assessing researchers properly has been discussed as an area that could be improved to alleviate reproducibility issues. Moher *et al.* (2018) suggests that current assessments of researchers do not focus on what makes a researcher a reliable member of the scientific community. They add that few assessments of scientists focus on the use of good or bad practices in the research environment, and suggest that more appropriate incentives for researchers may improve the research standard. They concluded that many things could help with better research assessment including implementing at the national level standardized assessment criteria that institutions could use to change promotion and tenure criteria and faculty assessment. They also admitted that current systems reward scientific innovation and maybe rewarding scientists who excel with reproducible research (Flier, 2017; Mogil and Macleod, 2017; Topol, 2016).

Journals and grant providers should also share the responsibility of increasing research reproducibility. Journals have not always been on board with this kid of rigour when faced with the possibility of research that is not reproducible. With

regards to the cell line misidentification, when asked if they would consider introducing cell line authentication as a requirement for publication, editors be financial suicide and reduce the amount of researchers submitting manuscripts to their journal (Alston-Roberts *et al.*, 2010). However, that was a relatively long time ago, and Nature Publications has produced a formatting guide (https://www.nature.com/nature/for-authors/formatting-guide) for submissions to their articles and has adapted it to make research and results as clear as possible. They have also compiled a code of ethics for researchers (https://www.nature.com/articles/d41586-018-02516-x).

Although implementing many of these methodological standards is no easy task, with cooperation between all parties involved in scientific research, we should expect change. Regardless, at least these once unknown or ignored issues are being discussed.

1.4.4 Publication pressure and bias

Already mentioned previously, publication pressure is possibly one of the biggest drivers of the reproducibility crisis. Publication number for a researcher or lab has often been deemed a measure of that researcher or labs success. The amount of publications can give a researcher an edge when applying for funding in an increasingly competitive funding environment. For the same reasons, positive findings are more likely to get published, especially in highly competitive journals that only accept few papers that will want novel discoveries to occupy their pages. In a random sample of 1316 papers it was determined that states in the USA with

researchers publishing more papers were significantly more likely to report positive results (Fanelli, 2010), and Between 1990 and 2007, the proportion of positive results in scientific literature increased from 70.2% to 85.9% (Mlinaric, 2017). The trend to publish just positive results has grown and is contributing to the reproducibility crisis.

To not publish "negative" results can have consequences. For example the Stroke Unit Trialists' Collaborative demonstrated that although 14 of 16 trials comparing a stroke unit with general medical care were "negative" for the effect on death, but taking all trials together it is estimated that stroke unit care reduces death by 17% (Stroke Unit Trialists' Collaboration, 2007). Whether this is contributing to the reproducibility crisis or not, this is a problem that should be addressed, but maybe it will take the journals being more accepting of so called "negative" results, because it is hard to blame a young researcher who's career depends on publication for striving for those "positive" results.

1.4.5 No crisis?

Despite the increased discussion on the matter and vast amount of data to support it, a minority would say that there either isn't a reproducibility crisis or it has been exaggerated, and if there is one, maybe it is good for science (Fanelli, 2018). In Fanelli's paper, it is argued, amongst other things, that researcher misconduct is so small it is unlikely to have a major impact on the literature and that "*P*-hacking" was unlikely to distort the literature. *P*-hacking is the art in which a researcher may continue to collect or select data until previously nonsignificant results

become significant (Head *et al.*, 2015; Wilcherts *et al.*, 2016). Fanelli believes that the "reproducibility crisis" narrative is not actually new and complaints about decline of research quality has been ever present (Babbage, 1830), and that rather than use the word "crisis", perhaps "revolution" or "challenge" should be used to compel and inspire more accurate, reproducible research.

One of the problems with declaring without doubt there is or isn't a reproducibility crisis, is that there is relatively little quantitative data to measure the level of reproducibility. As discussed earlier the Reproducibility Project: Cancer Biology has begun to attempt reproduction of papers, and the Open Science Collaboration published their findings on the matter. But there needs to be more reproduction of work to really understand the extent of the reproducibility crisis. Surveys are informative, but reliance is on the individual completing the survey to answer honestly. Of course one of the biggest questions is how does one measure reproducibility? What is deemed a successful replication of a result? As discussed earlier, the dispute between CCLE and GDSC groups and the Haibe-Kains research group highlights this. Some researchers just have a different definition of scientific reproducibility.

1.5 Overall Aims

The main aims of my thesis revolve around two main areas:

1. Investigating the methods used by large pharmacogenomic screens and look to develop novel methods to use this large amount of data made

available by the multiple screens. A large amount of data is available, in amounts that are often not possible to collect in a single lab or group. Therefore, I believe it is important to utilise this data to accompany, guide and reinforce work in the laboratory setting. While integration of computational analysis of cancer cell line drug screens and wet laboratory work is now common, it has often been difficult to translate results with relevance to the clinical setting. So novel approaches I consider will bear this in mind.

2. As an extension of looking to develop novel methods to use the data, I plan to investigate data variability or reproducibility in the screens. I will use the NCI-60 platform to do this as it has large amounts of data collected over three decades. It also used the same assay and experimental procedure over that time period so any variability is likely to be due to human error or the intrinsic variability that is present in biological systems. The questions surrounding the reproducibility crisis, as described in this introduction, have vast consequences in the scientific community. Hopefully analysis of this large data set collected over a long time period with standardised methods in a world leading research environment will go some way to a better understanding of variability in the sciences.

Chapter 2

2 Platinum drug resistance

2.1 Aims

The main aim here is to develop a better understanding of how similar or different platinum-based drugs cisplatin, carboplatin and oxaliplatin are in their pharmacological behaviour using drug sensitivity data for 60 cell lines in the NCI-60 database. A secondary aim will be to consider how well suited the NCI-60 cancer cell lines and methods are to the task by comparing what is known about the three platinum drugs and what is observed in the data, and determining how clinically relevant the data and analyses are by using a new method to determine drug sensitivity.

2.2 Introduction

Cisplatin, carboplatin and oxaliplatin are platinum drugs that consist of a central platinum atom with 4 attached ligands. They are used either individually or as part of a combination therapy to treat many types of cancer including bladder, cervical, ovarian, lung, gastric, breast, and head and neck, but have been particularly successful at treating testicular cancer (Einhorn, 1990). They cross-link DNA

strands caused resulting in irreparable damage and induction of cell death (Gomez-Ruiz,2012).

As with most drug treatments, side effects are common and sometimes severe. Nephrotoxicity can be a lethal side effect of cisplatin whereas myelosuppression is the dose limiting side effect for carboplatin and neurotoxicity for oxaliplatin (Pasetto *et al.*, 2006). The side effects of carboplatin are milder than those of cisplatin so is often used in patients that are not considered fit for cisplatin treatment.

There is an overlap in the resistance mechanisms against platinum-based drugs, but resistance to one compound is not always associated with cross-resistance to the other agents.

The NCI-60 is a 60 large cancer cell line panel with pharmacological data for 1000s of different compounds including the platinum group of compounds. Analysis of this data will determine whether cancer cell lines in the NCI-60 represent the cancer types that are treated by the platinum compounds and hopefully give some new insight into the similarities and differences of these three compounds, and whether or not they could hold any relevance to the clinical setting.

2.3 Methods

2.3.1 Platinum drug sensitivity data and Mean Graphs

Annotated cell line data and all pharmacogenomic data (negative log10 GI50 values) in this study were obtained from the online resource CellMiner (Shankavaram UT et al, 2009. Reinhold *et al.*, 2012) established by the National Cancer Institute (NCI) cell line panel, NCI-60.

The data consists of 60 cell lines from nine tissue types with pharmacogenomic data from three platinum drugs; cisplatin, carboplatin and oxaliplatin (only pharmacogenomic data for 50 cell lines was available for oxaliplatin after NCI-60 quality control). Negative log10 GI50 values for 139 individual compound/cell line combination experiments for cisplatin, 59 individual experiments for carboplatin and 3 individual experiments for oxaliplatin were available. The negative log10 GI50 values were transformed to micromolar (uM) concentration values and the mean GI50 concentration was calculated for each cell line for each drug.

Mean Graphs for each drug were obtained from the CellMiner resource. GI50 values undergo a quality control process to remove any that they deem as statistical outliers. In this instance a GI50 value may not be available to plot on the Mean Graph.

2.3.2 Clinically relevant threshold determination

Data processing was carried out using perl version 5.18.2, Microsoft Excel and R statistical packages version 3.4.4. Cell lines were determined to be either sensitive or resistant to a drug using perl and Microsoft Excel, using the Cmax for each drug as a threshold. Clinically relevant Cmax values used were obtained from available literature (Liston and Davis, 2017) and are displayed in table 2.2.

2.3.3 Platinum drug sensitivity correlation

Spearman correlations were determined for each drug-drug comparison (cisplatin-carboplatin, cisplatin-oxaliplatin, carboplatin-oxaliplatin) and displayed in a correlation matrix generated using R package corrplot (Wei and Simko, 2017). Correlation plots between drugs were generated using R. Correlation classifications are as defined in Table 2.1

Table 2.1 Classification of Correlation Coefficient Grouping

Correlation	Classification
0-0.1	None
0.1-0.2	Low
0.2-0.4	Moderate
0.4-0.6	Relatively Strong
0.6-0.8	Strong
0.8+	Very Strong

Table 2.2 Clinical Thresholds for Platinum Drugs

Compound	Threshold (Cmax, uM)
Cisplatin	14.4
Carboplatin	135
Oxaliplatin	4.96

2.3.4 Platinum drug NCI-60 cell line resistance

Using the cell line sensitivity status as determined using clinically relevant threshold, a heatmap was produced using R package ggplot2 (Wickham, 2016) to display cell line sensitivity for each drug. If the GI50 for a cell line was below or equal to the threshold for a compound it was designated -1 for sensitive and if above the threshold it was designated 1 for resistant. Venn diagrams to visualise cell lines sharing sensitivity or resistance between the drugs were produced using the R package VennDiagram (Chen and Boutros, 2011).

2.4 Results

2.4.1 Comparison of platinum compound sensitivity in the NCI60

The mean GI50 value over all experiments for a cell line was calculated for each of the three platinum compounds. The maximum experiment number for cisplatin was 138, for carboplatin 58 and oxaliplatin three. To determine if there was any similarity or difference in sensitivity profile over the NCI-60 panel, the GI50 data was compared using spearman's rank-order correlation. A strong positive correlation was observed between the sensitivity profile of cisplatin and carboplatin (0.84, $p<2.2E-16$, Figure 2.1A), and was the only significant relationship between the compounds regarding sensitivity.

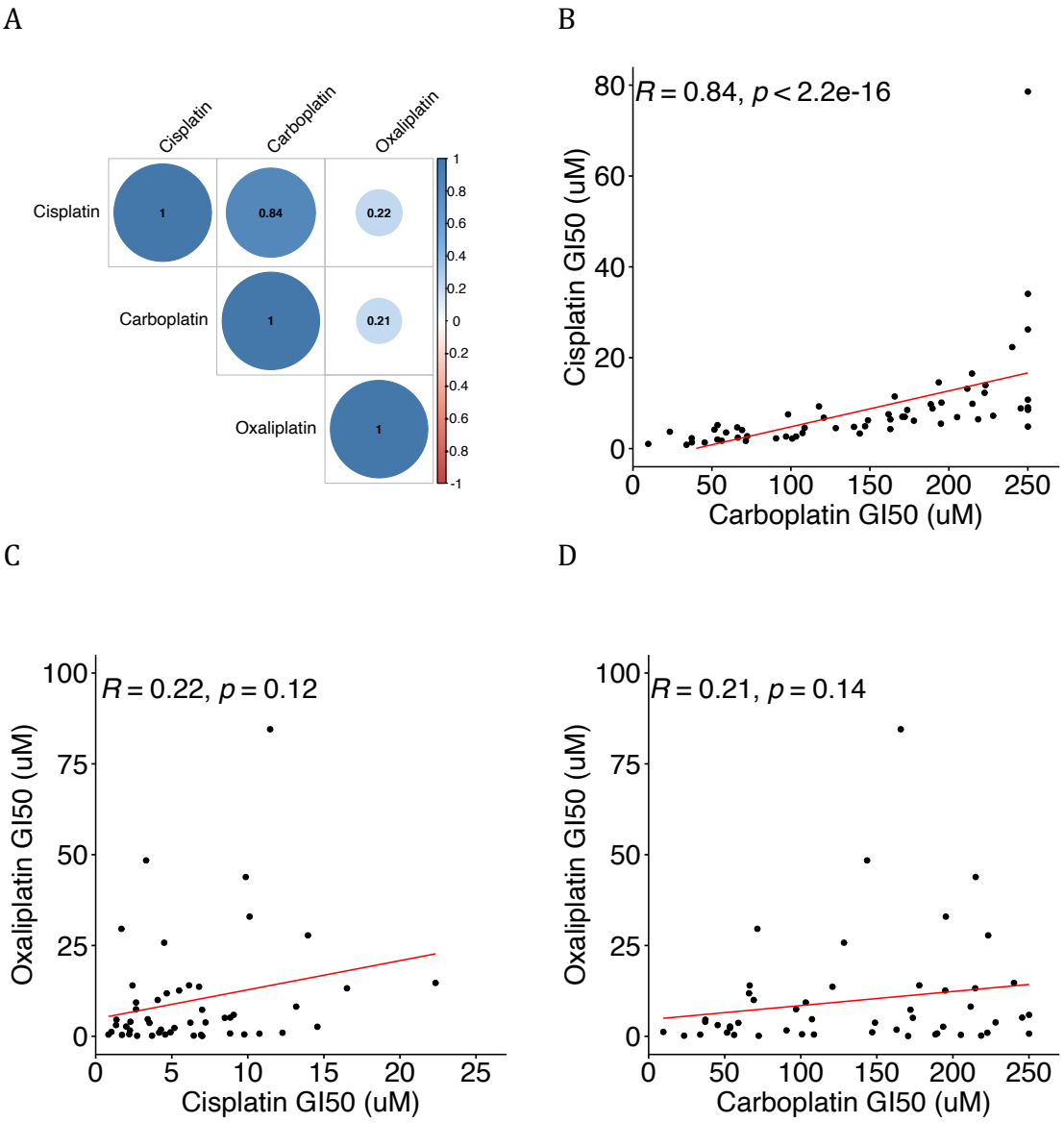

Figure 2.1 Platinum drug GI50 correlation in NCI-60 cell line panel. A. Correlation plot showing positive correlation (blue). The larger the circle, the higher the correlation. Scatter plots show correlation of **B.** cisplatin and carboplatin, **C.** cisplatin and oxaliplatin and **D.** carboplatin and oxaliplatin.

There is no significant correlation between oxaliplatin sensitivity and sensitivity of cisplatin or carboplatin (Spearman correlation: 0.22 and 0.21, p value: 0.12 and 0.14 respectively) over the NCI-60 cell line panel. Taken together this suggests that the mechanism of action between cisplatin and carboplatin is very similar,

while oxaliplatin, although also a platinum drug, has a different mechanism of action.

2.4.2 Comparison of compounds using Mean Graphs

Using the NCI-60 resource CellMiner, Mean Graphs for the three compounds (Figure 2.2) were obtained to further compare the three platinum compounds. They were created by calculating a z-score for all cell lines in the panel for each compound and plotting a bar graph either side of the mean (z-score = 0) so that bars left of the mean (-z-score) are classed resistant cell lines to the compound and bars right of the mean (+z-score) are classed sensitive. This really sets an arbitrary threshold of sensitivity using the mean, a cut-off such as the mean is unlikely to actually yield all sensitive and resistant cell lines but it is a good indicator of cell line sensitivity and works as a good comparison tool between compounds.

The Mean Graphs for cisplatin and carboplatin (Figure 2.2A and 2.2B) bear many similarities. For virtually every tissue type the same pattern of sensitivity or resistance is observed. Most notably for colon cell lines where all seven cell lines are classed as resistant to the compounds and for leukaemia and ovarian cell lines where identical cell lines are classed either sensitive or resistant for both compounds (three sensitive and four resistant). For the other tissues, pattern of sensitivity is close to identical barring one or two differences, for example breast cancer cell lines MCF7 and BT-549 are classed sensitive and resistant respectively

for cisplatin but the opposite for carboplatin, while the remaining three breast cancer cell lines are classed as resistant in both.

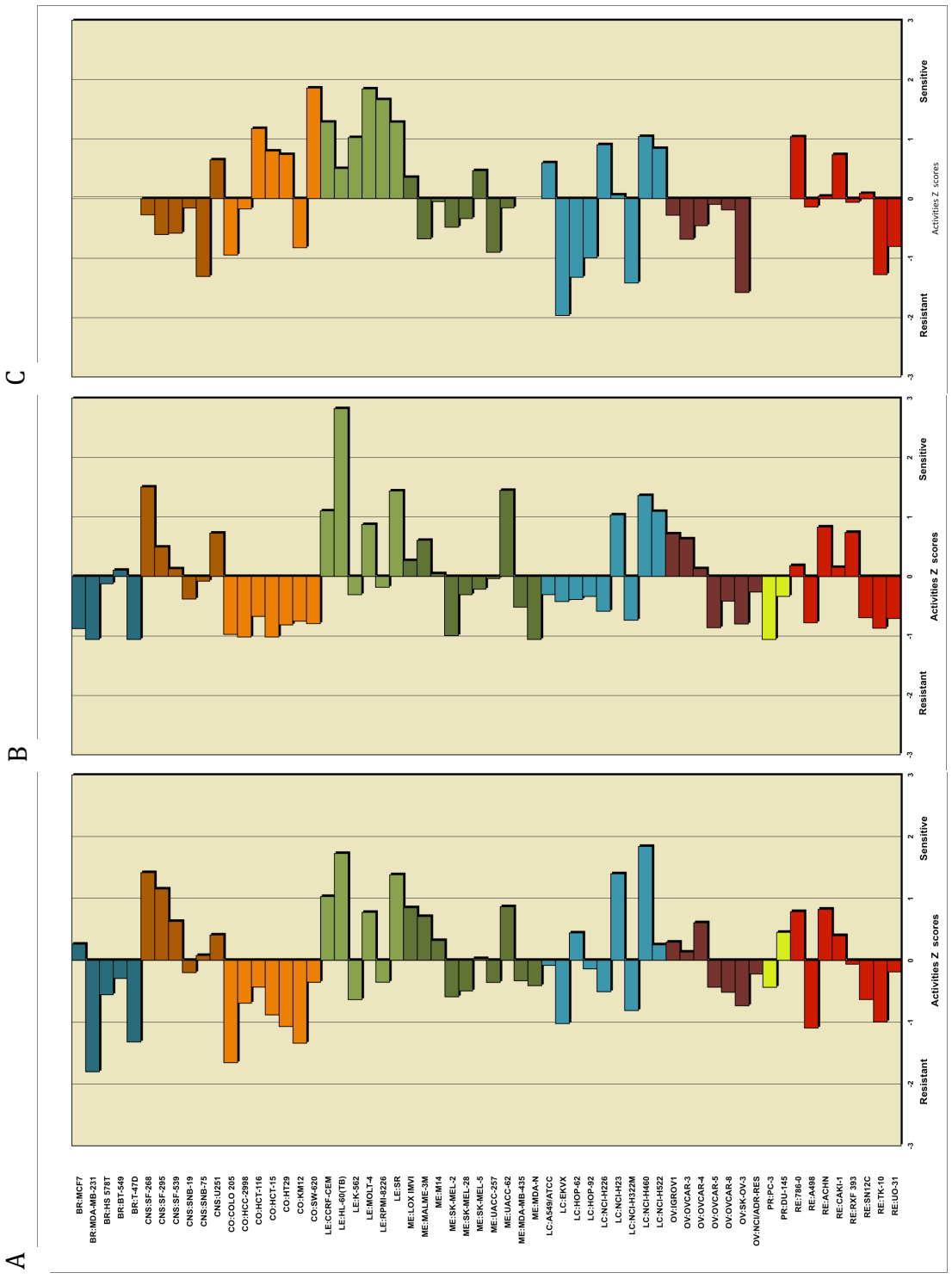

Figure 2.2 Mean Graphs displaying relative sensitivity of NCI-60 cell lines. For A. cisplatin, B. carboplatin and C. oxaliplatin.Cell lines with bars to the left of the mean (z-score = 0) are considered sensitive to compound, bars to the right of the mean resistant to the compound. Graphs obtained from NCI-60 resource CellMiner.

The oxaliplatin Mean Graph (Figure 2.2C) differs to those of cisplatin and carboplatin. The most striking differences are for CNS, colon and ovarian tissues. The general pattern of sensitivity for oxaliplatin is the opposite to that of the other two compounds. There is a general pattern of resistance for the CNS cell lines, five of the six are classed as resistant to oxaliplatin compared to two for carboplatin and one for cisplatin. For colon cell lines four of the seven are classed as sensitive compared to none in both cisplatin and carboplatin, and for ovarian cell lines six are classed as resistant to oxaliplatin while three of those six are classed as sensitive to the other two compounds. It is worth noting there that there was no data for one of the ovarian cell lines for oxaliplatin, as well as no data for the five breast cancer cell lines, two melanoma cell lines or the two prostate cell lines. For the remaining tissues (other than breast cancer and prostate due to missing data) there is similarity in sensitivity patterns between all drugs, although none of the leukaemia cell lines are classed resistant to oxaliplatin, while two are to cisplatin and carboplatin. This data is visualized in a simpler format that makes comparison more straightforward (Figure 2.3A).

2.4.3 Clinical threshold of sensitivity

Using a z score of 0 as a threshold for sensitivity, cisplatin and carboplatin sensitivity and therefore mechanism of action were similar, oxaliplatin was different. But how does a GI50 value for a compound and a cell line translate to a cancer patient? A GI50 for a cell line that is classed as sensitive (most likely relative to other cell lines in the panel) may represent a level that would be toxic to an actual human. So perhaps if there was a threshold of compound sensitivity that

accounted for a patient's tolerability of said compound, it may be possible to determine whether sensitivity observed in a cell line of certain cancer type would translate to a clinical setting.

With this in mind thresholds of sensitivity/resistance were set using the Cmax of each compound. The Cmax describes the maximum plasma concentration in a patient after dosing of a drug, and it is considered the concentration where side effects of the drug begin. So, the thought is that toxicity of a drug becomes more of a risk when side effects are higher, which would be at Cmax plasma concentration or above (Gatti *et al.*, 1999). If the treatment does not work at Cmax plasma concentration, the cancer should be considered resistant to the drug as higher concentrations may be lethal to the patient. Successful treatment at concentrations below the Cmax would mean cancer sensitivity to the drug. Figure 2.3B shows sensitivity profiles for the three platinum compounds.

Using the Cmax calculated thresholds (14.4uM for cisplatin, 135uM carboplatin, 4.96uM oxaliplatin) 54 cell lines were classed as sensitive to cisplatin and six resistant, 26 cell lines were sensitive to carboplatin and 34 resistant, and 28 cell lines were sensitive to oxaliplatin and 22 resistant (no data for 10 cell lines)(Figure 2.3B). The sensitivity profile was very different to that using the mean graph data. When considering the sensitivity profile using NCI-60 mean graph data, 53 cell lines had the same sensitivity allocation (sensitive or resistant) for both cisplatin and carboplatin, only 28 of those also shared with oxaliplatin (Figure 2.3C). When considering the sensitivity profile using the clinically relevant threshold this changed. Only 32 cell lines shared the same sensitivity allocation

between cisplatin and carboplatin while 18 of those had the same sensitivity profile as oxaliplatin (Figure 2.3D). Perhaps more strikingly, 17 cell lines had a sensitivity allocation unique to cisplatin and 16 had a cell line had a sensitivity allocation unique to carboplatin (Figure 2.3D), both compared to only 5 when considering the mean graph data (Figure 2.3C). The sensitivity profiles for cisplatin and carboplatin were no longer similar for cisplatin and carboplatin, while the sensitivity profile for oxaliplatin was more similar to both cisplatin (30 cell lines, 11 uniquely compared to 2 with mean graph data) and carboplatin (30 cell lines, 12 uniquely compared to 2 with mean graph data) (Figure 2.3C,D).

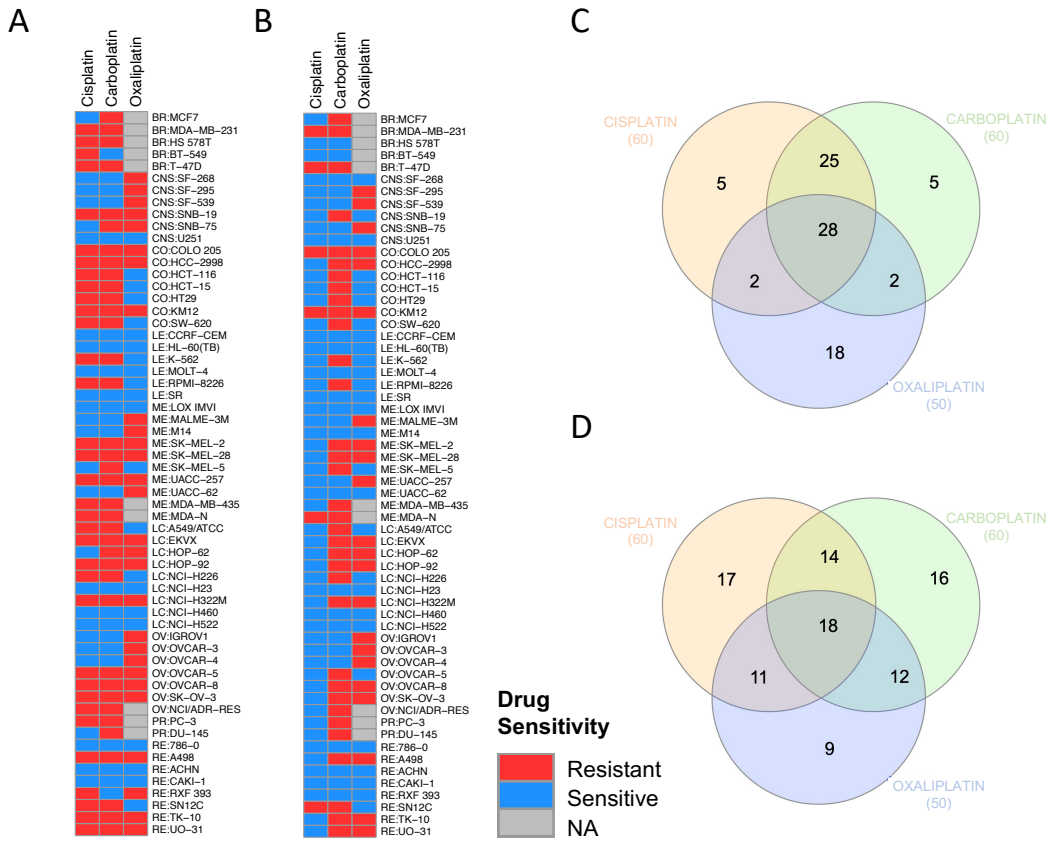

Figure 2.3 NCI-60 cell lines clinical sensitivity to platinum compounds. Cell lines sensitive (blue) and resistant (red) to platinum drugs using **A.** z-score of 0 as threshold of sensitivity (NCI-60 mean graph method) and **B.** clinically relevant threshold to determine drug sensitivity. Number of cell lines with shared drug sensitivity allocation (sensitive or resistant) between the platinum drugs when using **C.** z-score of 0 as threshold of sensitivity and **D.** clinically relevant threshold of sensitivity.

2.5 Discussion

2.5.1 Oxaliplatin mechanism of action/resistance is not the same as cisplatin and carboplatin

The mechanism of action (MOA) of oxaliplatin is similar to the MOAs of cisplatin and carboplatin MOA, but there are also differences. As well as causing DNA damage through the formation of DNA crosslinks, characteristic of platinum drugs, oxaliplatin also has immunologic mechanisms (Tesniere *et al.*, 2010) and exhibited synergism with other cytotoxic drugs (Fischel *et al.*, 2002). Results here using drug sensitivity data from the NCI-60 confirm that carboplatin and cisplatin behave similarly in vitro and both behave differently to oxaliplatin.

Similarity between the cisplatin and carboplatin Mean Graphs, yet difference to that of oxaliplatin is further confirmation that oxaliplatin MOA differs from the other two platinum drugs. The Mean Graphs can be treated like "compound fingerprints" as, although no two are identical, compounds with the same or similar "fingerprint" possess the same or similar MOA. The COMPARE algorithm available as a resource from the NCI-60 actually uses these Mean Graphs to identify compounds with similar MOA to input compounds, and although not analysed here, oxaliplatin has been used in the COMPARE algorithm previously. It was determined, using COMPARE, that cisplatin activity was similar to diamine-platinum compounds, alkylating agents and camptothecin analogs whereas

oxaliplatin activity was closer to other diaminocyclohexane platinum compounds and of acridine derivatives (Rixe *et al.*, 1996). Although not include in the analysis, one would presume that carboplatin activity would receive the same results as cisplatin due to the similarity in their Mean Graph and being a diamine-platinum compound.

The Mean Graphs also suggest a difference in mechanism of resistance (MOR) between cisplatin/carboplatin and oxaliplatin. Colon cancer is resistant or quickly becomes resistant to treatment from cisplatin, and therefore carboplatin, but the use of oxaliplatin with 5-fluorouracil has been effective in treating colon cancer (Fischel *et al.*, 2002; Alcindor and Beauger, 2011). In the Mean Graph all colon cell lines show resistance to cisplatin and carboplatin, yet five of the seven are sensitive to oxaliplatin. This may suggest a different MOR for cisplatin and carboplatin compared to oxaliplatin. It is possible that oxaliplatin can avoid cross-resistance from the other two compounds because it works so well in combination therapies, there are even reports that oxaliplatin resistance can be reversed when used in combination with another drug (Yin *et al.*, 2019, Wang *et al.*, 2018). It is likely that although oxaliplatin shares aspects of MOA and MOR of other platinum drugs but it is just as likely that it has additional MOA and MOR.

2.5.2 Clinically relevant thresholds

Determining which cell lines are “sensitive” or “resistant” in pharmacogenomics cell line panels is difficult, and potentially misleading. Cell lines are often designated one or the other in relation to the sensitivity of all the other cell lines

tested by the compound. For example, in this study, for the Mean Graph the NCI-60 use the mean as a threshold to designate a cell line resistant or sensitive. There is absolutely nothing wrong with this, it is still an informative tool with regards to compound activity in different cancer types and the direct comparison of drugs.

The idea was to use clinically relevant thresholds (Cmax) as indicators of drug sensitivity. Interestingly, there was less concordance between cisplatin and carboplatin in this analysis. 30/60 cell lines were either sensitive or resistant to both cisplatin and carboplatin. This did not differ substantially from the overlaps between cisplatin and oxaliplatin (29/50 cell lines) as well as cisplatin and oxaliplatin (30/50 cell lines). Hence, the efficacy profiles using clinical concentrations provide different results than the correlation of dose potencies. This is most probably the case because even closely related drugs differ in their pharmacokinetics, which contribute to the clinically achievable plasma levels (Passweg and Tichelli, 2009; Levi *et al.*, 2000; O'Dwyer *et al.,* 2000; Burger *et al.*, 2011). Thus, the use of clinical concentration as indicators of drug sensitivity adds complementary information to the direct correlation of drug sensitivity profiles.

2.6 Conclusions

The correlation of cisplatin, carboplatin, and oxaliplatin response data from the NCI-60 confirmed the anticipated higher similarity between cisplatin and carboplatin relative to oxaliplatin (Stewart, 2007). This finding is in agreement with the assumption that cisplatin and carboplatin largely share a mechanism of

action, whereas the mechanism of oxaliplatin seems to differ (Pasetto *et al.*, 2006). This is also observed using the mean graph analysis by the NCI-60. Sensitivity profiles were similar for cisplatin and carboplatin, while the oxaliplatin sensitivity profile was different to both. Furthermore, colorectal cancer cell lines displayed resistance to cisplatin and carboplatin, while they were sensitive to oxaliplatin. Colorectal cancer is intrinsically resistant to cisplatin with cross resistance due to the same mechanism of action to carboplatin, while it is sensitive to oxaliplatin therapy (Raymond *et al.*, 2002; Moltgen *et al.*, 2020).

However, the use of clinically achievable plasma levels as sensitivity thresholds to determine resistance profiles resulted in similar overlaps between all three drugs. This probably reflects that the clinical activity of a drug is not only determined by its mechanism of action but also by its pharmacokinetics (Wagstaff *et al.*, 1989; Levi *et al.*, 2000; O'Dwyer *et al.*, 2000; Burger *et al.*, 2011). This additional role of pharmacokinetics needs to be taken into account during the translation of preclinical findings.

Chapter 3

Publication:

YM155-adapted cancer cell lines reveal drug-induced heterogeneity and enable the identification of biomarker candidates for the acquired resistance setting

Martin Michaelis, Mark.N. Wass, Ian Reddin, Yvonne Voges, Florian Rothweiler, S tephanie Hehlgans, Jaroslav Cinatl, Marco Mernberger, Andrea Nist, Thorsten Sti ewe, Franz Rödel, Jindrich Cinatl jr.

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(Published at Cancers journal)

3.1 My contribution to the paper

All computational validation analysis using Genomics of Drug Sensitivity in Cancer (GDSC), Cancer Therapeutics Portal (CTRP), TARGET and The Cancer Genome Atlas (TCGA) and generation of accompanying figures.

cancers

MDPI

Article

YM155-Adapted Cancer Cell Lines Reveal Drug-Induced Heterogeneity and Enable the Identification of Biomarker Candidates for the Acquired Resistance Setting

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Abstract: Survivin is a drug target and its suppressant YM155 a drug candidate mainly investigated for high-risk neuroblastoma. Findings from one YM155-adapted subline of the neuroblastoma cell line UKF-NB-3 had suggested that increased ABCB1 (mediates YM155 efflux) levels, decreased SLC35F2 (mediates YM155 uptake) levels, decreased survivin levels, and *TP53* mutations indicate YM155 resistance. Here, the investigation of 10 additional YM155-adapted UKF-NB-3 sublines only confirmed the roles of ABCB1 and SLC35F2. However, cellular ABCB1 and SLC35F2 levels did not indicate YM155 sensitivity in YM155-naïve cells, as indicated by drug response data derived from the Cancer Therapeutics Response Portal (CTRP) and the Genomics of Drug Sensitivity in Cancer (GDSC) databases. Moreover, the resistant sublines were characterized by a remarkable heterogeneity. Only seven sublines developed on-target resistance as indicated by resistance to RNAi-mediated survivin depletion. The sublines also varied in their response to other anti-cancer drugs. In conclusion, cancer cell populations of limited intrinsic heterogeneity can develop various resistance phenotypes in response to treatment. Therefore, individualized therapies will require monitoring of cancer cell evolution in response to treatment. Moreover, biomarkers can indicate resistance formation in the acquired resistance setting, even when they are not predictive in the intrinsic resistance setting.

Keywords: acquired drug resistance; biomarkers; therapy monitoring; neuroblastoma; BIRC5; survivin; intrinsic drug resistance

1. Introduction

Sepantronium bromide (YM155) was introduced as an anti-cancer drug candidate that inhibits expression of the *BIRC5* gene, which encodes the protein survivin [1]. In the meantime, YM155 has been suggested to exert additional and/or alternative mechanisms of anticancer actions, including induction

of DNA damage, inhibition of NFκB signaling, induction of death receptor 5 expression, and/or suppression of MCL-1, XIAP, cIAP-1/2, BCL-2, BCL-XL, FLIP, EGFR, and/or mTORC [2–13].

A number of studies have investigated the potential of YM155 against neuroblastoma cells [14–17]. Neuroblastoma is the most common extracranial solid childhood tumor. Treatment outcomes in high-risk neuroblastoma patients remain unsatisfactory. About 50% of these patients relapse and have a 5-year-survial rate below 10% [18–21]. We have recently shown that suppression of survivin expression is the main mechanism through which YM155 exerts its anti-neuroblastoma effects [16]. Notably, the New Drug Development Strategy (NDDS, a project of Innovative Therapies for Children with Cancer, the European Network for Cancer Research in Children and Adolescents, and the International Society of Paediatric Oncology Europe Neuroblastoma) has categorized survivin as a high priority drug target in neuroblastoma and YM155 as a high priority drug [22].

The formation of acquired resistance is a central problem in (metastasized) cancer diseases that need to be treated by systemic drug therapy. Although many cancers initially respond well to therapy, resistance formation is common, and cures are rare [23]. Hence, biomarkers that indicate early therapy failure are needed to adapt therapies if resistance emerges. Liquid biopsies (e.g., circulating tumor cells) enable the monitoring of cancer cell evolution in patients with ever more detail [24]. However, the translation of the resulting information into improved therapies is hampered by a lack of understanding of the processes underlying acquired resistance formation and, in turn, a lack of biomarkers.

Most studies focus on biomarkers that indicate whether a certain cancer cell (population) is likely to respond to a certain treatment but not on biomarkers that indicate early that a current therapy has stopped working. This also applies to the previous studies that investigated the efficacy of YM155 in neuroblastoma [14,15,17]. However, it is known that intrinsic and acquired resistance mechanisms may substantially differ [25–27]. Using a single YM155-adapted neuroblastoma cell line, we identified increased ABCB1 (also known as P-glycoprotein or MDR1) expression, decreased SLC35F2 (solute carrier family 35 member F2) expression, decreased survivin expression, and loss-of-p53 function as potential markers of resistance formation to YM155 [16]. Given the tremendous (intra-tumor) heterogeneity in cancer [28], it is likely that the processes, which result in acquired resistance formation, are equally complex. If so, then a larger number of models of acquired resistance to a certain drug will be needed to adequately address the complexity of the resistance formation process.

To test this hypothesis, we here established and characterized 10 further YM155-adapted UKF-NB-3 neuroblastoma cell lines. Moreover, acquired resistance models may provide information that cannot be gained from the comparison of non-adapted cell lines with a varying resistance status. To investigate whether this is the case, the findings from the YM155-adapted UKF-NB-3 sublines were compared to data from the two large pharmacogenomics screens Genomics of Drug Sensitivity in Cancer (GDSC) and Cancer Therapeutic Response Portal (CTRP), which use non-adapted cancer cell lines [29,30], whether we can obtain information from our acquired resistance models that cannot be identified from traditional approaches using non-adapted cell lines. We also analyzed YM155 response data from the two large pharmacogenomics screens Genomics of Drug Sensitivity in Cancer (GDSC) and Cancer Therapeutic Response Portal (CTRP) [29,30]. We found a remarkable heterogeneity between the individual sublines, although they all were derived from the same parental cell line. An increase in cellular ABCB1 levels and/or a decrease in SLC35F2 levels indicate resistance formation to YM155, although the ABCB1 and/or SLC35F2 levels cannot be used to infer YM155 sensitivity in YM155-naïve cell lines. The use of the panel of YM155-adapted cell lines further enabled us to show that the cellular survivin levels and the *TP53* status do not reliably indicate resistance formation.

2. Results

2.1. YM155-Adapted UKF-NB-3 Sublines Display Pronounced YM155 Resistance

All YM155-adapted UKF-NB-3 sublines displayed pronounced YM155 resistance (Figure 1, Table S1). The YM155-adapted UKF-NB-3 sublines displayed between 38- and 76-fold increased YM155

IC50 values and between 30- and 135-fold increased IC90 values relative to UKF-NB-3 (Table S1). Representative photos of the morphology of the project cell lines are presented in Figure S1 and the doubling times in Table S1.

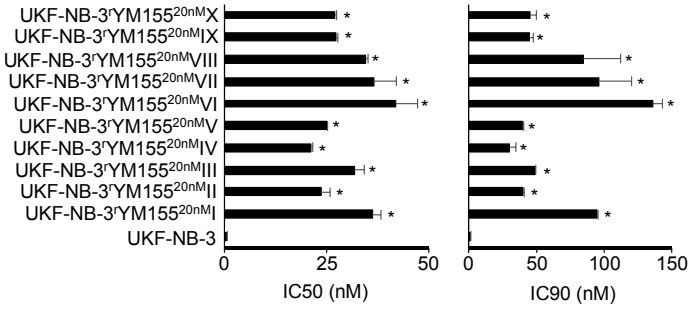

Figure 1. YM155 concentrations that reduce the viability of UKF-NB-3 and its YM155-adapted sublines by 50% (IC50) or 90% (IC90) as determined by MTT assay after 120 h of incubation. The sublines display significantly increased YM155 resistance. Numerical values are presented in Table S1. * $p < 0.05$ relative to UKF-NB-3.

2.2. The Cellular TP53 Status is Not a Reliable Indicator of YM155 Sensitivity

Originally, the cellular *TP53* status was described to not directly influence the anticancer action of YM155 [31]. In agreement, the analysis of the Genomics of Drug Sensitivity in Cancer (GDSC) and Cancer Therapeutics Response Portal (CTRP) databases did not indicate differences in the YM155 sensitivity between cell lines in dependence on their *TP53* status (wild-type or mutant) (Figure 2).

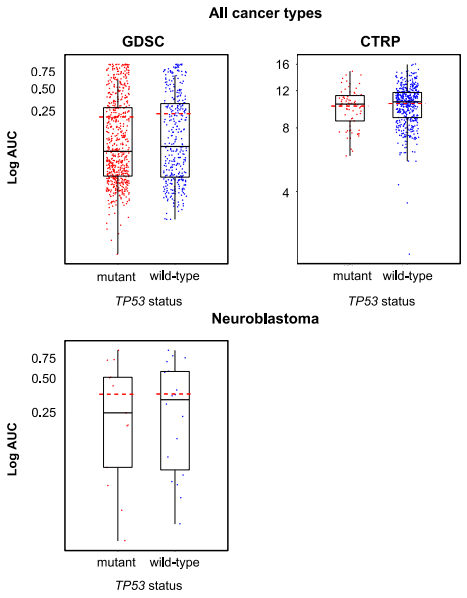

Figure 2. YM155 sensitivity in *TP53* wild-type and *TP53* mutant cancer cell lines based on the analysis of GDSC and CTRP data, both determined across all investigated cancer types/cell lines (GDSC, $p = 0.458$; CTRP, $p = 0.216$) and in a neuroblastoma-specific analysis (GDSC, $p = 0.922$; all 12 neuroblastoma cell lines in the CTRP harbor wild-type *TP53*). The comparisons did not reveal significant differences.

However, the activation of p53 signaling seems to be involved in the anticancer mechanism of action of YM155 at least in some cancer cells. We have previously shown in neuroblastoma cells that YM155 activates p53 signaling, that p53 activation using MDM2 inhibitors enhances the YM155 effects, and that p53 depletion reduces cancer cell sensitivity to YM155 [15]. In addition, a YM155-adapted UKF-NB-3 subline harbored a *TP53* mutation [16]. However, all 10 YM155-adapted UKF-NB-3 sublines that we investigated here displayed wild-type *TP53* as indicated by *TP53* next-generation sequencing. The cellular p53 levels also did not differ consistently between UKF-NB-3 and its YM155-resistant sublines (Figure S2). The YM155-resistant UKF-NB-3 sublines remained similarly sensitive to the MDM2 inhibitor and p53 activator nutlin-3 as UKF-NB-3 (Table S2). Hence, our findings do not suggest that YM155 adaption is generally associated with a loss of p53 function in neuroblastoma cells. The cellular *TP53* status is not a reliable indicator of YM155 sensitivity, neither in the intrinsic nor in the acquired resistance setting.

2.3. Cellular Survivin Levels Do Not Reliably Indicate YM155 Response

Some studies suggested cancer cells with high survivin levels to be particularly sensitive to YM155 [31–33]. However, other studies failed to detect an association between the cellular survivin status and YM155 activity [16,34]. When we compared the YM155 sensitivity between cancer cell lines with high and low survivin expression, we found statistically significant differences across all cell lines in the GDSC and CTRP datasets but not across the neuroblastoma cell lines (Figure 3). It was not possible to predict whether a certain cell line was sensitive to YM155 based on the cellular survivin level (Figure 3).

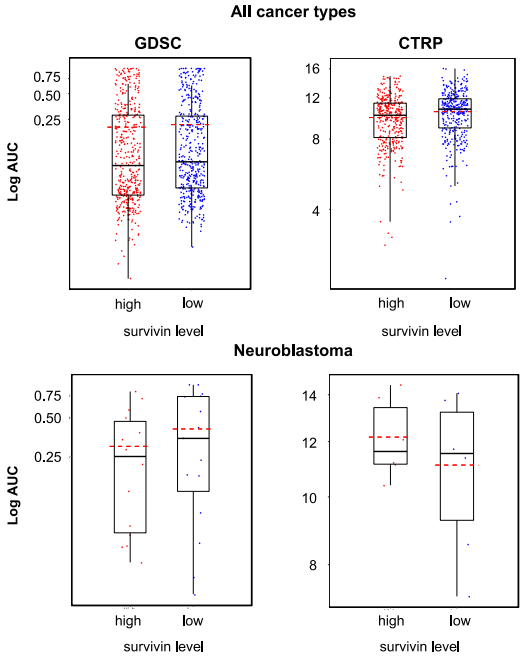

Figure 3. YM155 sensitivity in cell lines characterized by high or low survivin expression based on the analysis of GDSC and CTRP data, both determined across all investigated cancer types/cell lines (GDSC, $p = 0.048$; CTRP, $p < 0.001$) and in a neuroblastoma-specific analysis (GDSC, $p = 0.425$; CTRP, $p = 0.699$). Across all cell lines, high survivin expression was associated with increased YM155 sensitivity, but the YM155 sensitivity of individual cell lines could not be predicted based on the survivin levels.

Notably, a YM155-adapted UKF-NB-3 subline had previously displayed reduced survivin levels relative to the parental cell line [16]. However, the analysis of the 10 additional YM155-adapted UKF-NB-3 sublines in this study revealed that resistance acquisition to YM155 was not associated with a consistent change in the survivin expression patterns (Figure S3).

2.4. Acquired YM155 Resistance is Associated with Decreased Sensitivity to Survivin Depletion

Our previous findings suggested that YM155 predominantly exerts its antineuroblastoma effects via suppression of survivin expression [16]. Seven of the 10 YM155-adapted UKF-NB-3 sublines (I, III, V, VII, VIII, IX, X) displayed decreased sensitivity to siRNA-mediated survivin depletion, indicating that they developed on-target resistance. However, two sublines were similarly sensitive as parental UKF-NB-3 cells (II, VI), and one subline (IV) was more sensitive to survivin depletion (Figure 4, Figure S4). This shows that the YM155 resistance mechanisms differ between the individual UKF-NB-3 sublines. Notably, the viability of all sublines is still affected by survivin depletion, which shows that they have retained some level of survivin dependence.

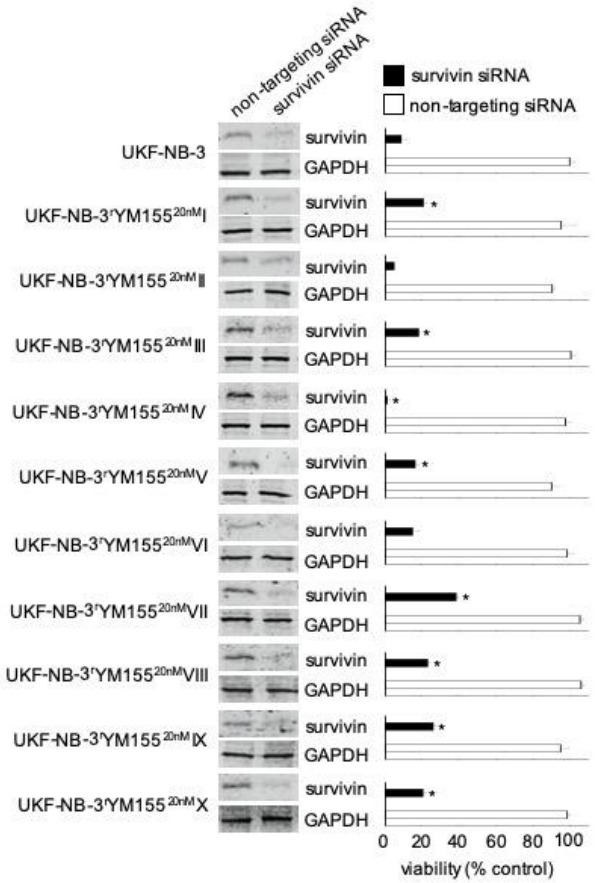

Figure 4. Effects of siRNA-mediated BIRC5/survivin depletion on the viability of UKF-NB-3 and its YM155-adapted sublines. Western blots confirm reduced survivin levels 48 h post-transfection. Viability of cells transduced with siRNA directed against BIRC5/survivin or non-targeting siRNA was determined relative to untreated control cells 168 h post transfection by MTT assay. The cell lines displayed varying levels of sensitivity to survivin depletion. * $p < 0.05$ relative to untreated cells. Uncropped blots are shown in Figure S4.

2.5. Relevance of Cellular ABCB1 and SLC35F2 Levels in the Context of YM155 Resistance

Increased cellular ABCB1 (mediates YM155 efflux) levels and decreased SLC35F2 (mediates cellular YM155 uptake) levels have previously been identified as important YM155 resistance mechanisms [14,16,17,35]. To further investigate the relationship between ABCB1 and SLC35F2 levels and YM155 sensitivity, we compared the YM155 sensitivity in cell lines that displayed low or high expression of the respective genes using GDSC and CTRP data. In agreement with previous data, high *ABCB1* expression (Figure 5) and low *SLC35F2* expression (Figure 6) were associated with reduced YM155 sensitivity. When we used transcriptomics data from the GDSC and CTRP to correlate the expression of all genes with YM155 sensitivity, *ABCB1* ranked as the gene whose expression was most strongly correlated to the YM155 AUC (area under the curve, unit used to quantify the drug response) (Table 1) in the GDSC and CTRP. *SLC35F2* expression was most strongly inversely correlated to the YM155 AUC (Table 2) in both data sets. There were no further overlaps among the top 10 genes between the two databases (Tables 1 and 2). However, the YM155 sensitivity of a certain cell line could not be reliably predicted based on the cellular ABCB1 and/or SLC35F2 levels (Figures 5 and 6).

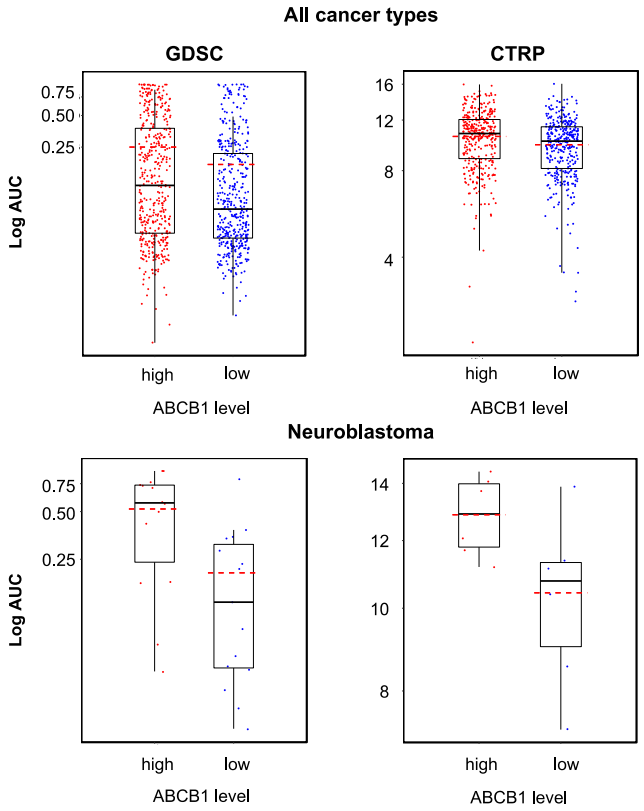

Figure 5. YM155 sensitivity in cancer cell lines characterized by high or low ABCB1 expression based on the analysis of GDSC and CTRP data, both determined across all investigated cancer types/cell lines (GDSC, $p < 0.001$; CTRP, $p < 0.001$) and in a neuroblastoma-specific analysis (GDSC, $p = 0.006$; CTRP, $p = 0.04$). High ABCB1 expression was associated with decreased YM155 sensitivity, but the YM155 sensitivity of individual cell lines could not be predicted based on the ABCB1 levels.

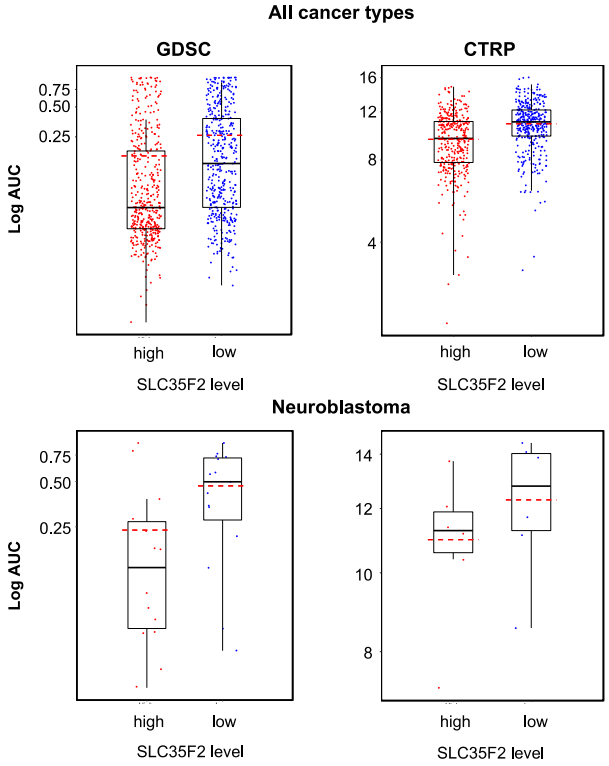

Figure 6. YM155 sensitivity in cancer cell lines characterized by high or low SLC35F2 expression based on the analysis of GDSC and CTRP data, both determined across all investigated cancer types/cell lines (GDSC, $p < 0.001$; CTRP, $p < 0.001$) and in a neuroblastoma-specific analysis (GDSC, $p = 0.033$; CTRP, $p = 0.310$). High SLC35F2 expression was associated with increased YM155 sensitivity, but the YM155 sensitivity of individual cell lines could not be predicted based on their SLC35F2 levels.

Table 1. Top 10 genes whose expression is most strongly correlated with the YM155 AUC in the Genomics of Drug Sensitivity in Cancer (GDSC) database and the Cancer Therapeutics Response Portal (CTRP) as indicated by the Pearson correlation coefficient.

GDSC			CTRP		
Gene	**Correlation Coefficient**	**FDR** [1]	**Gene**	**Correlation Coefficient**	**FDR**
ABCB1	0.3792069	2.82×10^{-6}	ABCB1	0.3624858	2.70×10^{-6}
FABP1	0.2972293	5.64×10^{-6}	CST3	0.3603422	5.39×10^{-6}
CDX2	0.2927057	8.46×10^{-6}	AKR1C3	0.3503176	8.09×10^{-6}
DDC	0.2922995	1.13×10^{-5}	EPS8	0.3453892	1.08×10^{-5}
CDH17	0.2652645	1.41×10^{-5}	ABHD2	0.3309384	1.35×10^{-5}
ANKS4B	0.2637047	1.97×10^{-5}	S100A6	0.3229691	1.62×10^{-5}
MYO1A	0.2626518	2.26×10^{-5}	ATP1B1	0.3111308	1.89×10^{-5}
PHGR1	0.2609317	2.54×10^{-5}	CD63	0.310712	2.16×10^{-5}
A1CF	0.252912	2.82×10^{-5}	AKR1C1	0.3073306	2.43×10^{-5}
GUCY2C	0.2513048	3.10×10^{-5}	ACVR1	0.30124	9.44×10^{-5}

[1] false discovery rate.

Table 2. Top 10 genes whose expression is most strongly inversely correlated with the YM155 AUC in the Genomics of Drug Sensitivity in Cancer (GDSC) database and the Cancer Therapeutics Response Portal (CTRP) as indicated by the Pearson correlation coefficient.

GDSC			CTRP		
Gene	**Correlation Coefficient**	**FDR [1]**	**Gene**	**Correlation Coefficient**	**FDR**
SLC35F2	−0.2643809	1.69×10^{-5}	SLC35F2	−0.3868041	9.17×10^{-5}
ALKBH8	−0.2042557	0.000121	CD19	−0.349156	8.90×10^{-5}
CWF19L2	−0.1926243	0.000180	CD79B	−0.346035	8.63×10^{-5}
RCSD1	−0.1855956	0.000226	SPIB	−0.341887	8.36×10^{-5}
P2RY8	−0.1834937	0.000257	SNX22	−0.338893	8.09×10^{-5}
RGS19	−0.1831031	0.000259	TCL1A	−0.338393	7.82×10^{-5}
FLI1	−0.1825892	0.000268	LOC100130458	−0.337940	7.55×10^{-5}
VAV1	−0.1819569	0.000273	BLK	−0.330471	7.28×10^{-5}
ATM	−0.1816322	0.000276	CD79A	−0.324465	7.01×10^{-5}
ARHGAP19	−0.1813872	0.000282	VPREB3	−0.322878	6.74×10^{-5}

[1] false discovery rate.

All YM155-adapted UKF-NB-3 sublines displayed increased ABCB1 levels relative to UKF-NB-3 (Figure 7, Figure S5). Acquired YM155 resistance was also generally associated with decreased SLC35F2 levels, in particular in the sublines I, IV, VI, and X (Figure 7, Figure S5). This indicates that increased ABCB1 levels and decreased SLC35F2 levels have potential as biomarkers, indicating YM155 resistance formation in response to YM155-based therapies, although cellular ABCB1 and SLC35F2 levels do not enable the prediction of YM155 sensitivity in YM155-naïve cells.

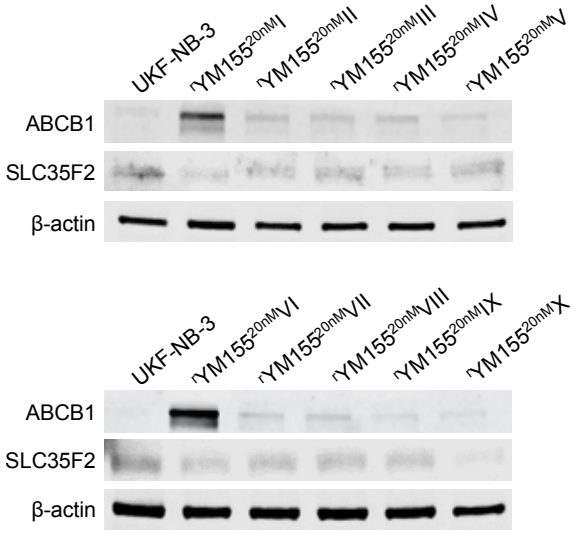

Figure 7. Representative Western blots indicating cellular levels of ABCB1 and SLC35F2 in UKF-NB-3 and YM155-adapted UKF-NB-3 sublines. YM155-adapted sublines are typically characterized by increased ABCB1 levels and decreased SLC35F2 levels. Uncropped blots are shown in Figure S5.

2.6. YM155-Adapted UKF-NB-3 Cells Remain Sensitive to DNA Damage Caused by Irradiation and Cytotoxic Drugs

YM155 has been proposed to exert its anticancer effects via the induction of DNA damage in some experimental systems [3,5,35,36]. To study whether the acquisition of YM155 resistance was associated with a generally increased resistance to DNA damage, UKF-NB-3 and its YM155-adapted UKF-NB-3 sublines were irradiated at a dose range of one to five Gy. None of the YM155-adapted UKF-NB-3 sublines displayed substantially reduced sensitivity to irradiation relative to UKF-NB-3

(Figure 8). Moreover, none of the YM155-resistant UKF-NB-3 sublines displayed reduced sensitivity to cisplatin (causes DNA crosslinks) or topotecan (topoisomerase I inhibitor), which cause DNA damage by different mechanisms (Figure 8, Table S2). There was also no coherent increase in resistance to the nucleoside analogue gemcitabine (Figure 8, Table S2). These data do not suggest that the activity of YM155 against UKF-NB-3 cells would predominantly depend on DNA damage induction.

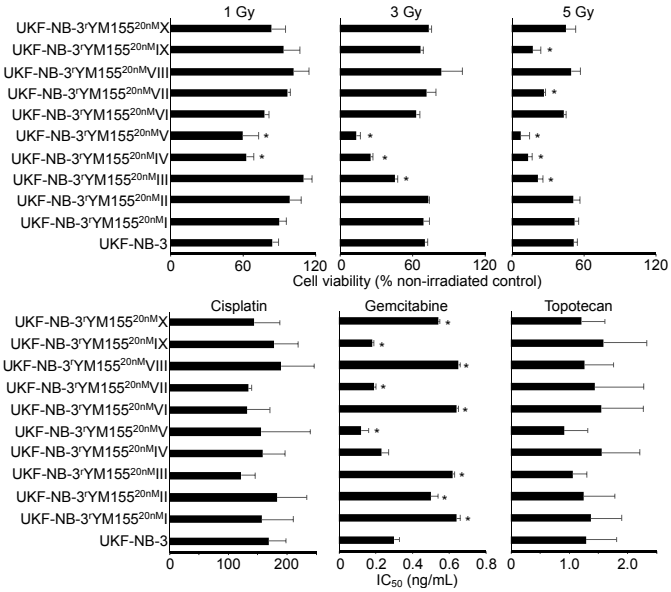

Figure 8. Sensitivity of UKF-NB-3 and its YM155-adapted sublines to irradiation and DNA-damaging drugs. The radiation response was determined 72 h after irradiation with 1, 3, or 5Gy by MTT assay. Drug concentrations that reduce cell viability by 50% (IC_{50}) were determined by MTT assay after 120 h of incubation. The YM155-adapted sublines did not display increased resistance to DNA damage induced by radiation or the drugs cisplatin, gemcitabine, or topotecan. * $p < 0.05$ relative to UKF-NB-3.

2.7. Heterogeneity among YM155-Adapted UKF-NB-3 Sublines

While the YM155-adapted UKF-NB-3 sublines displayed limited heterogeneity in response to treatment with cisplatin and topotecan, remarkable differences in the gemcitabine IC_{50}s were detected (Figure 8, Table S2). The fold difference between the YM155-adapted subline with the lowest gemcitabine IC_{50} (V, 0.12 ng/mL) and the subline with the highest IC_{50} (VIII, 0.65 ng/mL) was 5.4-fold. This heterogeneity is in agreement with the up to 29-fold difference observed in the cell viability in response to BIRC5/survivin depletion between the most sensitive (IV) and the most resistant (VII) subline (Figure 4). Resistance profiles to the destabilizing tubulin-binding agent vincristine also revealed a substantial heterogeneity between the YM155-resistant UKF-NB-3 sublines (Figure 9, Table S2), resulting in a fold difference of 127 between subline VI (vincristine IC_{50}: 714 ng/mL) and subline IX (vincristine IC_{50}: 5.6 ng/mL) (Table S2).

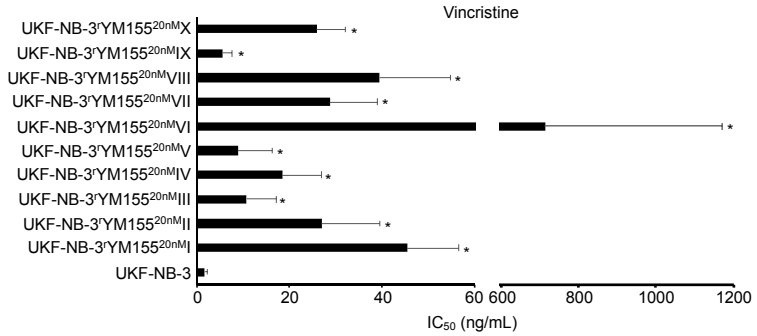

Figure 9. Vincristine concentrations that reduce cell viability by 50% (IC_{50}) were determined by MTT assay after 120 h of incubation. YM155-adapted sublines consistently displayed decreased vincristine sensitivity relative to UKF-NB-3. The vincristine IC_{50} values varied considerably. * $p < 0.05$ relative to UKF-NB-3.

3. Discussion

In a previous study, a YM155-adapted subline of the neuroblastoma cell line UKF-NB-3 was characterized by increased cellular ABCB1 levels, decreased SLC35F2 and survivin levels, and a *TP53* mutation [16]. Here, we systematically investigated the relevance of cellular ABCB1, SLC35F2, and survivin levels as well as the *TP53* status as potential biomarkers of YM155 resistance formation in the intrinsic resistance setting, using data derived from the GDSC and CTRP databases [29,30], and in the acquired resistance setting, using an additional set of 10 YM155-adapted UKF-NB-3 sublines, which were established in independent experiments.

Increased ABCB1 expression (mediates YM155 efflux) and decreased SLC35F2 expression (mediates cellular YM155 uptake) were identified as YM155 resistance mechanisms in panels of YM155-naïve cell lines that displayed varying levels of these proteins and in functional studies [14,16,17,37], which was further supported by our analysis of GDSC and CTRP data [29,30]. Despite their roles in determining YM155 resistance, however, cellular ABCB1 or SLC35F2 levels did not enable the prediction of whether an individual cell line would be sensitive to YM155 or not. The YM155-adapted UKF-NB-3 cell lines generally displayed elevated cellular ABCB1 levels and reduced SLC35F2 levels relative to UKF-NB-3. Hence, an increase in the cellular ABCB1 levels and/or a decrease in the SLC35F2 levels have potential as biomarkers that indicate resistance formation, even though the respective cellular levels do not reliably predict the YM155 response in YM155-naïve cells.

Initially, the *TP53* status was reported not to influence the anticancer effects of YM155 [31], which was further supported by our analysis of GDSC and CTRP data. In neuroblastoma cells, however, YM155 induced p53 signaling, p53 depletion reduced YM155 sensitivity, and a YM155-adapted UKF-NB-3 subline harbored a *TP53* mutation [16]. Here, all 10 YM155-adapted UKF-NB-3 sublines retained wild-type *TP53*. Thus, the role of p53 seems to depend on the individual cellular context. Neither the cellular *TP53* status nor the formation of *TP53* mutations can currently be considered as valid biomarkers for YM155 therapies.

The relevance of cellular survivin levels for cancer cell sensitivity to YM155 is not clear [16,31–35]. Our analysis of GDSC and CTRP data indicated that high survivin (BIRC5) expression was associated with increased YM155 sensitivity. However, it was not possible to infer the YM155 sensitivity of a particular cell line based on its survivin status. Reasons for this may include that survivin is not in all cell lines the major therapeutic target of YM155 as it is in neuroblastoma cells [1–12,16,36] and/or that off-target resistance mechanisms, such as ABCB1 and SLC35F2 expression, may affect YM155 efficacy independently of the survivin status [14,16,17,35].

The YM155-adapted UKF-NB-3 sublines displayed various survivin levels, demonstrating that resistance formation to YM155 is also not associated with a consistent change in cellular survivin levels.

Seven of the YM155-adapted cell lines displayed on-target resistance as indicated by reduced sensitivity to RNAi-mediated BIRC5/survivin depletion relative to parental UKF-NB-3 cells, further confirming that survivin is a target of YM155 in neuroblastoma cells. However, cellular survivin levels do not represent a reliable biomarker of resistance formation to YM155.

While YM155 was described to act via the induction of DNA damage in some cancer types [3,5,35,36], our previous results did not indicate a causative role of DNA damage induction in the anticancer effects of YM155 against neuroblastoma cells [16]. YM155 resistance formation in the YM155-adapted neuroblastoma cell lines was also not associated with generally decreased sensitivity to radiation or DNA damage caused by cisplatin (causes DNA crosslinks), gemcitabine (nucleoside analogue), or topotecan (topoisomerase I inhibitor). This indicates that YM155 resistance formation in neuroblastoma cells is not generally associated with an increased resistance to DNA damage induction.

In this study, the use of multiple models of acquired resistance enabled insights that could not be gained from just one drug-adapted subline. The previous investigation of one YM155-resistant UKF-NB-3 subline had suggested that changes in the cellular *TP53* status and survivin levels indicate resistance formation [16], which was not confirmed in our current panel of 10 YM155-adapted UKF-NB-3 sublines. Moreover, the use of multiple sublines provided a novel glimpse into the significant heterogeneity of the resistance formation process, even though all resistant sublines were derived from the same parental cell line. Only 7 of the 10 sublines developed on-target resistance mechanisms as indicated by reduced sensitivity to survivin depletion. The sublines also showed substantial variation in their sensitivity to irradiation (up to 7-fold difference at 5Gy), gemcitabine (up to 5-fold), and vincristine (up to 127-fold). Notably, a much higher heterogeneity would be expected in the clinical situation, in which tumors are already characterized by much higher heterogeneity than cancer cell lines and in which combination therapies are common.

4. Materials and Methods

4.1. Cells

The MYCN-amplified neuroblastoma cell line UKF-NB-3 was established from a bone marrow metastasis of a stage IV neuroblastoma patient [38]. Ten YM155-resistant UKF-NB-3 sublines were derived from the resistant cancer cell line (RCCL) collection (https://research.kent.ac.uk/industrial-biotechnology-centre/the-resistant-cancer-cell-line-rccl-collection/). They were established by adaptation of UKF-NB-3 cells (passage 87) to growth in the presence of YM155 20nM by previously described methods [39] and designated as UKF-NB-3^{r}YM155^{20nM}I to UKF-NB-3^{r}YM155^{20nM}X. All cells were propagated in IMDM supplemented with 10 % FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C. Cells were routinely tested for mycoplasma contamination (PlasmoTest™, Mycoplasma Detection kit, InvivoGen, Toulouse, France) and authenticated by short tandem repeat profiling.

To determine doubling times, 2×10^4 cells per well were plated into 6-well plates, incubated at 37 °C and 5% CO_2, and counted after 1, 2, 3, 5, and 7 days using a Neubauer chamber. Doubling times were then calculated using http://www.doubling-time.com/compute.php, which uses the equation:

Doubling time = duration × log(2)/log(final cell number) − log(initial cell number).

4.2. Viability Assay

Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay after 120 h of incubation modified as described previously [39]. Cells (2×10^4/100 µL per well in 96-well plates) were incubated in the presence or absence of drug for 120 h. Then, 25 µL of MTT solution (2 mg/mL (*w/v*) in PBS) were added per well, and the plates were incubated at 37 °C for an additional 4 h. After this, the cells were lysed using 100 µL of a buffer containing 20% (*w/v*) sodium dodecylsulfate and 50% (*v/v*) *N*,*N*-dimethylformamide with the pH adjusted to 4.7 at 37 °C for 4 h. Absorbance was determined at 570 nm for each well using a 96-well multiscanner. After subtraction of the background absorption, the results were expressed as the

percentage viability relative to control cultures that received no drug. Drug concentrations that inhibited cell viability by 50% (IC50) or 90% (IC90) were determined using CalcuSyn (Biosoft, Cambridge, UK).

4.3. TP53 Next-Generation Sequencing

TP53 next-generation sequencing was performed as previously described [16]. All coding exonic and flanking intronic regions of the human *TP53* gene were amplified from genomic DNA with Platinum™ Taq DNA polymerase (Life Technologies) by multiplex PCR using two primer pools with 12 non-overlapping primer pairs each, yielding approximately 180 bp amplicons. Each sample was tagged with a unique 8-nucleotide barcode combination using 12 differently barcoded forward and eight differently barcoded reverse primer pools. Barcoded PCR products from up to 96 samples were pooled, purified, and an indexed sequencing library was prepared using the NEBNext® ChIP-Seq Library Prep Master Mix Set for Illumina in combination with NEBNext® Multiplex Oligos for Illumina (New England Biolabs). The quality of sequencing libraries was verified on a Bioanalyzer DNA High Sensitivity chip (Agilent) and quantified by digital PCR. 2 × 250 bp paired-end sequencing was carried out on an Illumina MiSeq (Illumina) according to the manufacturer's recommendations at a mean coverage of 300×.

Read pairs were demultiplexed according to the forward and reverse primers and subsequently aligned using the Burrows-Wheeler Aligner against the Homo sapiens Ensembl reference (rev. 79). Overlapping mate pairs where combined and trimmed to the amplified region. Coverage for each amplicon was calculated via SAMtools (v1.1) [40]. To identify putative mutations, variant calling was performed using SAMtools in combination with VarScan2 (v2.3.9) [41]. Initially, SAMtools was used to create pileups with a base quality filter of 15. Duplicates, orphan reads, unmapped, and secondary reads were excluded. Subsequently, Varscan2 was applied to screen for SNVs and InDels separately, using a low-stringency setting with minimal variant frequency of 0.1, a minimum coverage of 20, and a minimum of 10 supporting reads per variant to account for cellular and clonal heterogeneity. Minimum average quality was set to 20 and a strand filter was applied to minimize miscalls due to poor sequencing quality or amplification bias. The resulting list of putative variants was compared against the IARC *TP53* (R17) database to check for known p53 cancer mutations.

4.4. Western Blot

Cells were lysed using Triton-X-100 sample buffer, and proteins were separated by SDS-PAGE. Detection occurred by using specific antibodies against β-actin (1:5000 dilution, Biovision through BioCat GmbH, Heidelberg Germany; secondary antibody: IRDye® 800CW Goat anti-Mouse IgG, dilution 1:25,000, Li-Cor Biosciences, Lincoln, NE, USA), SLC35F2 (1:200, Santa Cruz Biotechnology, Dallas, TX, USA; secondary antibody: IRDye® 800CW Goat anti-Mouse IgG, dilution 1:25,000, Li-Cor Biosciences, Lincoln, NE, USA), GAPDH (1:4000, Trevigen via Bio-Techne GmbH, Wiesbaden, Germany; secondary antibody: IRDye® 800CW Goat anti-Rabbit IgG, dilution 1:25,000, Li-Cor Biosciences), ABCB1 (1:1,000, Cell Signaling via New England Biolabs, Frankfurt, Germany; secondary antibody: IRDye® 800CW Goat anti-Rabbit IgG, dilution 1:25,000, Li-Cor Biosciences), p53 (1:1000, Enzo Life Sciences, Lörrach, Germany; secondary antibody: IRDye® 800CW Goat anti-Mouse IgG, dilution 1:25,000, Li-Cor Biosciences), and survivin (1:500, R&D Systems, Minneapolis, MN, USA; secondary antibody: IRDye® 800CW Goat anti-Rabbit IgG, dilution 1:25,000, Li-Cor Biosciences). Protein bands were visualized by laser-induced fluorescence using infrared scanner for protein quantification (Odyssey, Li-Cor Biosciences) and Image Studio Ver. 5.2 software (Li-Cor Biosciences) for densitometric analyses.

4.5. RNA Interference Experiments

Transient depletion of BIRC5/survivin was achieved using synthetic siRNA oligonucleotides (ON-TARGETplus SMARTpool) from Dharmacon (Lafayette, CO, USA). Non-targeting siRNA (ON-TARGETplus SMARTpool) was used as negative control. Cells were transfected by electroporation

using the NEON Transfection System (Invitrogen, Darmstadt; Germany) according to the manufacturer protocol. Cells were grown to 60–80% confluence, trypsinised, and 1.2×10^6 cells were re-suspended in 200 µL of resuspension buffer R including 2.5 µM siRNA. The electroporation was performed using two 20 ms pulses of 1400 V. Subsequently, the cells were transferred into cell culture plates or flasks, containing pre-warmed cell culture medium. During the set-up of the experiments, the SMARTpool was compared to two individual siRNAs (target sequences: GCAAAGGAAACCAACAAUA, GGAAAGGAGAUCAACAUUU) (Figure S6).

4.6. Irradiation Procedure

In 96-well cell culture plates, 10^4 cells per well were irradiated at room temperature (Greiner, Bio-ONE GmbH, Frickenhausen, Germany) with single doses of X-rays ranging from 1 to 5 Gy using a linear accelerator (SL 75/5, Elekta, Crawley, UK) with 6 MeV photons/100 cm focus–surface distance with a dose rate of 4.0 Gy/min. Sham-irradiated cultures were kept at room temperature in the X-ray control room while the other samples were irradiated.

4.7. Analysis of Data Derived from Large Pharmacogenomic Studies

All data (including drug response area under curve (AUC) data for YM-155-treated cancer cell lines, basal gene-expression for ABCB1, BIRC5 (the gene that encodes survivin), and SLC35F2, and genomic alterations of p53) in this study were obtained from two online resources: Version 2 of the Cancer Therapeutics Response Portal (CTRP v2) data [29,42] were obtained from the Cancer Target Discovery and Development (CTD^2) data portal (ocg.cancer.gov/programs/ctd2/data-portal). The Genomics of Drug Sensitivity in Cancer (GDSC) data were obtained from www.cancerrxgene.org [43,44].

The CTRP contains ABCB1, BIRC5, and SLC35F2 expression data for 823 cell lines and YM-155 AUC data for 715 cell lines. For 703 cell lines (including 12 neuroblastoma cell lines), gene expression data and YM155 AUC values were available. Whole exome sequencing (WES) data was available for 546 of the cell lines for which YM-155 sensitivity data was also available (including 11 neuroblastoma cell lines).

The GDSC contains ABCB1, BIRC5, and SLC35F2 expression data for 1019 cell lines and YM155 AUC data for 945 cell lines. Expression data and WES data were available for all 945 cell lines with YM-155 sensitivity data (including 30 neuroblastoma cell lines).

Data processing was performed using Perl version 5.26.0, and R statistical packages version 3.3.2. Cell lines were determined to display either high or low expression for each gene using the median gene expression as a threshold (i.e., low expression < = median expression, high expression > median expression). Box plots indicating the YM-155 sensitivity in cell lines that display low or high expression of a certain gene or wild-type or mutant *TP53* were produced using the ggplot2 package [45] in R.

Statistical tests were carried out in R and included Wilcoxon rank-sum test [46] and Pearson's correlation [47]. Correction for multiple comparisons was performed using the Benjamini–Hochberg procedure [37].

4.8. Statistics

Results are expressed as mean ± S.D. of at least three experiments. Comparisons between the means of two sample groups were performed using Student's *t*-test. The means of three or more sample groups were compared by one-way ANOVA followed by the Student–Newman–Keuls test. *P* values lower than 0.05 were considered to be significant.

5. Conclusions

Our data revealed a high phenotypic heterogeneity among a panel of 10 YM155-resistant sublines of the neuroblastoma cell line UKF-NB-3. This heterogeneity is of conceptual importance, because it shows that even a defined cancer cell population of limited intrinsic heterogeneity can develop various resistance mechanisms and phenotypes in response to treatment. From a clinical perspective,

this means that the close monitoring of cancer cell evolution in response to therapy will have to become an essential part of the design of individualized therapies. Notably, such insights can only be gained from preclinical model systems, such as drug-adapted cancer cell lines, which enable the repeated adaptation of a given cancer cell population to the same treatment but not from clinical material as every patient can only be treated once.

Our findings also demonstrate that biomarkers can indicate resistance formation, even when they do not enable the prediction of drug sensitivity in therapy-naïve cancer cells. Hence, the use of biomarkers differs between the intrinsic and the acquired resistance setting, and pre-clinical models of acquired drug resistance are needed for the identification of such biomarkers that herald resistance development.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/5/1080/s1, Figure S1: Representative photos of the project cell lines, Figure S2: Representative Western blots indicating cellular levels of p53 in UKF-NB-3 and YM155-adapted UKF-NB-3 sub-lines, Figure S3: Representative Western blots indicating cellular levels of survivin in UKF-NB-3 and YM155-adapted UKF-NB-3 sub-lines, Figure S4: Representative Western blots indicating cellular levels of survivin in UKF-NB-3 and its YM155-adapted UKF-NB-3 sub-lines 48h after transfection with non-targeting siRNA or siRNA directed against BIRC5/survivin, Figure S5: Representative Western blots indicating cellular levels of ABCB1 and SLC35F2 in UKF-NB-3 and YM155-adapted UKF-NB-3 sub-lines, Figure S6: Effects of different siRNAs in UKF-NB-3 cells, Table S1: YM155 concentrations (nM) that reduce the viability of UKF-NB-3 or YM155-adapted UKF-NB-3 sub-lines by 50% (IC_{50}) or 90% (IC_{90}), Table S2: Drug concentrations that reduce the viability of UKF-NB-3 or YM155-adapted UKF-NB-3 sub-lines by 50% (IC_{50}).

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Chapter 4

4 Variability of drug sensitivity in cancer cell lines

4.1 Aims

The aim is to investigate the variation in the concentration that causes 50% growth inhibition (GI50) in the National Cancer Institute 60 cancer cell line panel (NCI-60) for any compounds that the cell lines have been screened for multiple times. This will give insight into the perceived notion that standardisation of experimental procedure leads to replicability and reproducibility of results in the laboratory setting. The NCI-60 cell line screen is particularly suited for this task as it has been running for three decades using highly standardised procedures in a world leading research setting and thus have produced a large amount of data under the same conditions. Furthermore, many of the tested drugs are FDA approved so afford the chance to look at variability in drugs used in the clinical setting. After investigating variability of GI50 readings using all available data, a secondary aim will be to investigate whether any observed variability is caused by changes in sensitivity measurements over the long period of time that the NCI-60 has operated. This is made possible by large amount of testing over long time periods performed on a subset of 18 compounds.

4.2 Introduction

The NCI-60 was established in its full capacity in 1990 (Grever *et al.*, 1992) for the screening of many compounds (synthetic and natural) on their 60 cancer cell line panel. By 2007 the NCI-60 had screened close to 150,000 compounds and despite cuts to funding and personnel the screening throughput is still at around 3000 samples a year (Shoemaker, 2006).

The NCI-60s inception was at a period of time in cancer research where the validity and reliability of work was being questioned due to an inherent problem with misidentification and cross-contamination of cell lines (Gillet *et al.*, 2013). The NCI-60 ensured that rigorous authentication steps were taken and low passage cell lines used in their panel to truly represent the cancer types they sought to (Shoemaker, 2006). Despite this, four of the cell lines in the panel were found to be misidentified when short tandem repeat analysis became available (Masters *et al.*, 2001).

With the recent escalation of a supposed "reproducibility crisis" (Wass *et al.*, 2019) there is perhaps no better time to investigate the reliability of the NCI-60 cell lines with regards to compound sensitivity and whether the compound sensitivity to the cell lines in the panel have changed since the arrival of the NCI-60.

4.3 Methods

4.3.1 Data Source

All data were obtained from CellMiner [82] Version 2.2. Dose concentration range data (June 2018 release) were obtained from the National Cancer Institute DTP NCI bulk data for download pages (https://wiki.nci.nih.gov/display/NCIDTPdata/NCI-60+Growth+Inhibition+Data). Of the 52,585 NCI codes given to compounds tested on the NCI-60 cell line panel, 42,794 were given to unnamed compounds and 9,791 were given to 9,027 named compounds. 262 named compounds received two or more individual codes. Some GI50 values represent minimum or maximum drug concentrations where the actual GI50 was not reached. Since such values understate the actual data variation, we did not remove these data.

4.3.2 Data processing

Data was carried out using perl version 5.26.0, Microsoft Excel (2011) and R statistical packages version 3.4.4. Perl modules Statistics:Descriptive and Statistics::R were used. Packages used in R were robustbase, dplyr, webr, moonBook, tidyverse, reshape2, scales, gplots, ggpubr, ggExtra, RColorBrewer, corrplot, ggplot2, and tidyr.

The drug sensitivity data was converted from –log10 GI50, to the GI50 (uM) for all compound, all cell lines and all experiments. Using dose concentrations, any GI50 that was equal to or less than the minimum dose concentration or equal to or

greater than the maximum dose concentration was removed to minimise GI50 "estimate" values. Any compounds with only one experiment were removed as analysis would require calculating fold change between experiments. This left sensitivity data for 4030 compounds.

4.3.3 Number of experiments and experimental groups

The number of experiments for each individual compound/cell line combination was calculated by counting all experiments performed on the same experimental date as well as experiments on different dates. The relationship between number of experiments and maximum fold change was investigated by using Spearman's correlation coefficient as the distribution of maximum GI50 fold change was not normal.

The compound/cell line combinations were then assigned experimental groupings based on the number of experiments performed: all data, 5 or more experiments, 10 or more experiments, 20 or more experiments, and 100 or more experiments. This allowed comparison of "high" maximum fold changes (>2, >5, >10, >100, and >1000) for combinations with varied number of experiments. Additionally, compound/cell line combinations were assigned to experimental groups: 2 experiments, 3 to 5 experiments, 6 to 20 experiments and over 20 experiments. These experimental groupings enabled comparison of GI50 fold change statistics (mean, median, minimum, maximum, variance) for compound/cell line combinations with number of experiments ranging from lower to higher.

4.3.4 Concentration range and experimental groups

Maximum dose concentration range for a compound/cell line combination was determined by using the minimum and maximum dose concentration used in an experiment for an individual compound on an individual cell line. The minimum concentration range was $1.0 \times 10^{1.2}$ and the maximum concentration was $1.0 \times 10^{12.1}$. The relationship between dose concentration range and maximum fold change was investigated by using Spearman's correlation coefficient as the distribution of maximum GI50 fold change was not normal.

Compound/cell line combinations were assigned to groups based on the dose concentration range for that combinations: maximum concentration range less than 1.0×10^5, maximum concentration range 1.0×10^5 to 1.0×10^9 exclusive and maximum concentration range 1.0×10^9 and above. These experimental groupings enabled comparison of GI50 fold change statistics (mean, median, minimum, maximum, variance) for compound/cell line combinations between lower and higher concentration ranges.

4.3.5 FDA-approved compound analysis

All compounds that were classed as FDA-approved drugs by the NCI-60 in CellMiner Database Version 2.2 and where two or more experiments had been performed were extracted from the complete dataset. This created an FDA-approved dataset of 181 drugs for which 399,686 experiments for 9,970

individual drug/cell line combinations were performed. Analysis of relationship between the number of experiments/concentration ranges and maximum GI50 fold change for drug/cell line combinations was performed as for the complete dataset, described above.

4.3.6 Drift in drug sensitivity

The mean GI50 fold change was calculated for each experimental date (month) for the 18 compounds with 100 or more experiments for at least one cell line. The GI50 fold change between the first experimental date and the last experimental date was calculated using the mean GI50 on those dates. The first/last GI50 fold change was then compared to the maximum GI50 fold change for each compound/cell line combination and considered a candidate for a drift in sensitivity if it was 50% or more of the maximum fold change.

4.3.7 Cell line fold changes and correlation coefficients

The drug sensitivity data was converted from –log10 GI50, to the GI50 (μM) for all compound, all cell lines and all experiments. Maximum fold changes were calculated for each compound/cell line combination with more than one experiment (594,450) by dividing the maximum GI50 for a cell line by the minimum GI50.

Each compound and cell line combination had a fold change value and number of experiments for that combination. The fold change and number of experiments

was correlated using Spearman's correlation coefficient as the dataset was highly positively correlated.

4.3.8 Fold change and GI50 outliers

The adjusted boxplot method (Hubert and Vandervieren, 2008) was used to identify outlier thresholds. This method was chosen as the data set was highly skewed. It is also more robust than the alternative method considered, the tukey outlier method (Tukey, 1977). To use this method the medcouple (MC), a robust measure of skewness, had to be calculated (where $X_n = \{ x_1, x_2, \dots , x_n \}$ represents data for every compound/cell line combination):

$$MC(x_1, \dots , x_n) = med \frac{(x_j - med_k) - (med_k - x_i)}{x_j - x_i}$$

Where med_k is the median of X_n, and i and j have to satisfy $x_i \leq med_k \leq x_j$, and $x_i \neq x_j$.

Using the MC the upper (U) and lower (L) thresholds could be determined. If $MC \geq 0$:

$$L = Q_1 - 1.5 \times \exp(-3.5MC) \times IQR$$

$$U = Q_3 + 1.5 \times \exp(4MC) \times IQR$$

If $MC \leq 0$:

$$L = Q_1 - 1.5 \times \exp(-4MC)\, IQR$$

$$U = Q_3 + 1.5 \times \exp(3.5MC) \times IQR$$

If $MC = 0$ the adjusted boxplot method was not used but instead the Tukey method was used:

$$L = Q_1 - 1.5IQR$$

$$U = Q_3 + 1.5IQR$$

Where Q_1 is the lower quartile, Q_3 is the upper quartile and IQR is the interquartile range.

For each compound/cell line combination any GI50 value below L or above U were removed from the dataset. Analyses were performed on this dataset as previously described for the complete dataset.

4.3.9 Clustering method

Clustering analyses were performed using Euclidean clustering method. The optimum number of clusters was calculated using affinity propagation method. The R package apcluster was used for this analysis and pheatmap was used to visualise the clustering.

4.3.10 Experimental timeline

Experiment codes were transformed into dates and GI50 values for each cell line experiment was plot against those dates for each of 18 drugs with a maximum of 100 experiments or more for at least one cell line. The mean GI50 for an experimental day (of all the cell line GI50s) was calculated and also plotted against time on the experimental timeline plot.

For each date with multiple experiments on, the fold change between the lowest and highest GI50 was calculated. This was plot in heatmaps to determine how experimental variability changed over time. To measure fold change between consecutive experiments, on dates where multiple experiments occurred for a cell line, the mean was calculated for these experiments so one GI50 value was available to compare to the next GI50 in the timeline.

4.4 Results

4.4.1 Cell line sensitivity is highly variable

All drug sensitivity data derived from NCI60 testing are made available via Cellminer (https://wiki.nci.nih.gov/display/NCIDTPdata/NCI-60+Growth+Inhibition+Data). In total, 52,585 compounds were tested in the available NCI60 data resulting in 2.8 x 10^6 compound/cell line combinations (hereby referred to as CCCs). Two or more (up to 2,286) experiments were carried out for 11,841 compounds and 594,450 CCCs. Most compounds were tested on a high majority of the NCI-60 CCLs (Figure 4.1A).

Variability of the maximum GI50 fold change (hereby referred to as GIΔ) was high in the NCI-60 data. The highest GIΔ observed was for Cyanomorpholinodoxorubicin when used on colon cancer cell line (hereby referred to as CCL) COLO 205, a GIΔ of 3.16 x 10^{10} over 34 experiments (Figure 4.1B). Considering all data with two experiments or more, the mean GIΔ was 3.18

x 10^5 (SD = 5.71 x 10^7) and the median GIΔ was 1.57 (IQR = 1.12-3.36). The high mean illustrated the variation in GIΔ however, the low median was perhaps misleading. The data was highly positively skewed for both the number of experiments and the GIΔ. 484,908 (81.6%) of all CCCs were of two experiments, for 79,697 (13.4) of all CCCs there was no fold change between experiments and for 362,135 (60.9%) of all CCCs there was a GIΔ of less than 2. For this reason experimental groups were derived with two things in mind:

1. To attempt to have as close to equal number of CCCs in groups with ascending number of experiments.
2. To reduce the skewness of data by considering different minimum number of experiments in an experimental group.

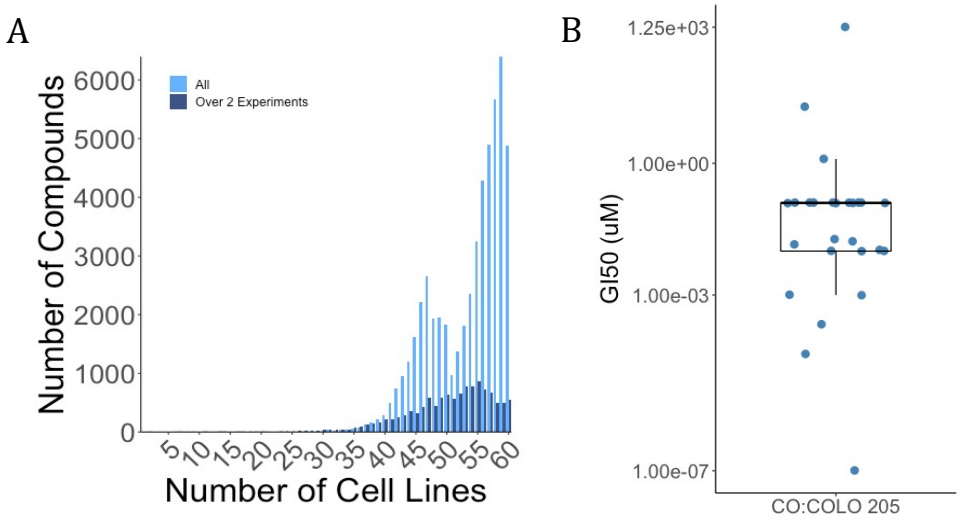

Figure 4.1 High level of variability in the large NCI-60 pharmacogenomic screen. A. Number of compounds tested with number of cell lines. B. Individual GI50 measurements recorded for the CCC with the highest GIΔ: compound cyanomorpholinodoxorubicin and colorectal CCL COLO 205.

With these experimental groups considered, variability in GIΔ was further illustrated when considering the proportion of all CCCs that had GIΔs over 2, 5, 10, 100 and 1000 experimental groups. 232,315 (39.1%) CCCs displayed GIΔs greater than 2, 108,247 (18.2%) CCCs displayed GIΔs greater than 5, 59,638 (10%) CCCs displayed GIΔs greater than 10, 19,089 (3.2%) CCCs displayed GIΔs greater than 100, and 8,320 (1.4%) CCCs displayed GIΔs greater than 1,000 (Figure 4.2A, Table 4.1). The mean and median maximum GIΔ for all CCCs was 318,410 (standard deviation (SD) = 5.71 x 10^7) and 1.6 (interquartile range (IQR) = 1.1-3.4), respectively. The low median fold change reflects the large number of experiments that were only performed twice. Only two experiments were performed for 99.9% (361,872) experiments of the 362,135 CCCs (60.9% of the total 594,450 of CCCs) that displayed maximum GI50 fold changes of less than two.

Table 4.1 – Experimental group data and frequency of "high" GIΔs

			Maximum GI50 Fold Change									
			Frequency					Percent of Total CCCs				
Group	Number of Compounds	Number of CCCs	>2	>5	>10	>100	>1000	>2	>5	>10	>100	>1000
All	11841	594450	232315	108247	59638	19089	8320	39.08	18.21	10.03	3.21	1.40
5+	738	30212	25496	19448	14660	7205	3832	84.39	64.37	48.52	23.85	12.68
10+	220	8517	8196	7461	6427	3948	2501	96.23	87.60	75.46	46.35	29.36
20+	62	2950	2853	2764	2651	2075	1594	96.71	93.69	89.86	70.34	54.03
100+	18	794	794	794	792	693	560	100.00	100.00	99.75	87.28	70.53
FDA	181	9970	5752	4213	3548	2065	1361	57.69	42.26	35.59	20.71	13.65

When we only considered experiments that were repeated more often, the median GI50 values increased considerably, as indicated below.

4.4.2 GI50 variability increases with the number of experiments

Each CCL in the NCI-60 panel was screened with a large number of compounds, some multiple times, increasing the number of experiments that have been carried out on a CCL for a compound. The number of times a CCL was screened with a compound was termed experiment number (hereby referred to as EXN). As EXN for a CCC increased, an increase in GIΔ was observed. To test this different experimental groups were considered: two experiments, three to five experiments, five to 20 experiments and greater than 20 experiments.

Using these experimental groups an increase in mean and median GIΔ was observed as EXN increased in that group. The mean and median GIΔs were 41,292 (SD = 1.09 x 10^7) and 1.4 (IQR = 1.1-2.5) for CCCs with two experiments, 97,002 (SD = 1.5 x 10^7) and 3.3 (IQR = 1.7-9.6) for CCCs with three to five experiments, 547,054 (SD = 4.5 x 10^7) and 11.2 (IQR = 3.9-98) for CCCs with five to 20 experiments and 53,343,445 (SD = 8.01 x 10^8) and 1,841 (IQR = 61.7-12,618) for CCCs with over 20 experiments (Figure 4.2B).

When only CCCs with a minimum of five experiments considered, the mean and median GIΔ for all combinations increased to 5.32 x 10^6 (SD = 2.48 x 10^8) and 10 (IQR = 3.1-98.7) when compared to all data. 25,496 (84.4%) of 30,212 CCCs displayed GIΔs greater than 2 and 3,832 (12.7%) GIΔs were greater than 1,000. For CCCs with at least 100 experiments, all 794 CCCs displayed a maximum fold

change greater than 5 and 70.5% (560 out of 794) displayed a GIΔ greater than 1,000 (Figure 4.2A).

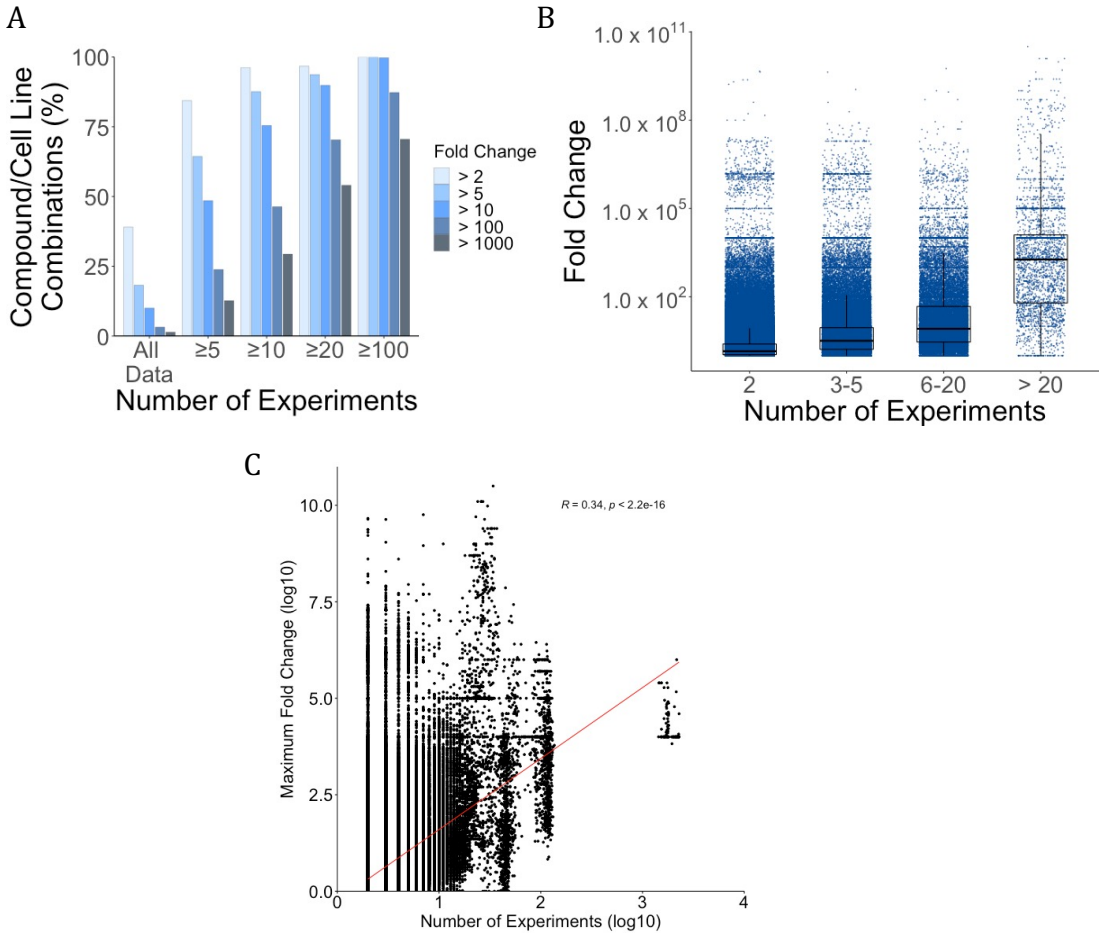

Figure 4.2 Variability increases with the number of experiments tested on a compound. A. Percentage of CCCs with GIΔs above the indicated thresholds in dependence of the number of experiments. **B.** GIΔ in dependence on the number of experiments per CCC. **C.** Scatterplot showing relationship between GIΔ and the number of experiments with statistics using Spearman's correlation.

Taken together, maximum GI50 fold changes increase with the number of experiments. In agreement, a significant correlation was detected between GIΔ and the number of experiments per CCC(Spearman correlation coefficient = 0.34, $p < 2.2 \times 10^{-16}$) (Figure 4.2C).

4.4.3 GI50 variability increases with the concentration range covered

Another factor considered was the concentration range covered. Each CCC was tested on at multiple dose concentrations over the time course of the NCI-60 screening, some over more than others. As with EXN, the concentration range covered was highly positively skewed. 547,256 (92%) of CCCs were tested over the standard five-dose screen (albeit with different maximum concentrations). Concentration ranges varied from $10^{1.2}$ to $10^{12.1}$ and 612 compounds were screened with multiple concentration ranges. To take the skewness into account, as with the EXN, multiple experimental groups were considered. As for EXN, when dose concentration range was increased, an increase in GIΔ was observed. To test this the three different experimental groups that were considered were dose ranges of: 1.0×10^5 or less, 1.0×10^5 up to 1.0×10^9 non-inclusive and 1.0×10^9 and above.

Using these experimental groups an increase in mean and median GIΔ was observed as dose concentration increased in that group. The mean and median GIΔs were 146.8 (SD = 4.76×10^4) and 1.5 (IQR = 1.1-2.9) for CCCs with a dose concentration range of 1.0×10^5 or less, 264,903 (SD = 3.35×10^7) and 10 (IQR = 3.2-87.1) for CCCs a dose concentration range of 1.0×10^5 up to 1.0×10^9, and 6.8×10^7 (SD = 8.44×10^8) and 289,734 (IQR = 2,004-1.5×10^6) for CCCs with a dose concentration range of 1.0×10^9 and above (Figure 4.3A). In agreement, a significant correlation was detected between maximum GIΔ and the dose

concentration range used for a CCC (Spearman correlation coefficient = 0.31, $p < 2.2 \times 10^{-16}$) (Figure 4.3B).

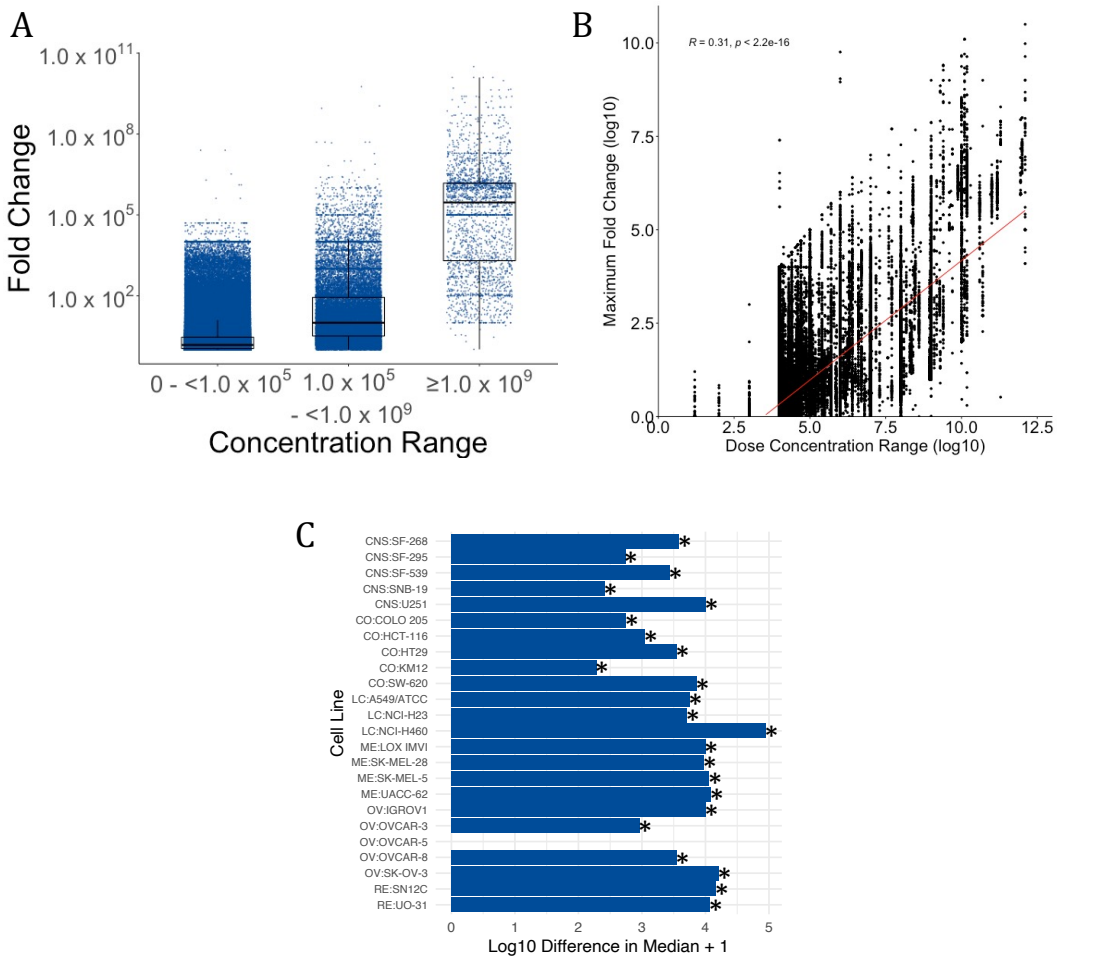

Figure 4.3 Variability increases as the dose concentration range tested on a compound increases. A. Distribution of GI50 fold changes in dependence of the concentration ranges in which compounds were tested. **B.** Scatterplot showing relationship between GIΔ and the dose concentration used with statistics using Spearman's correlation. **C.** Difference in median maximum GI50 fold change between individual dose concentration range and all dose concentration ranges. Blue bars represent positive difference in median GI50 fold change. * = significant, p value < 0.05, Wilcoxon Rank Sum test.

To further investigate whether a larger concentration range results in larger GIΔ, four compounds (doxorubicin, fluorouracil, cisplatin, vinblastine) with at least

100 experiments performed on more than 20 CCLs were considered. However, the vast majority of experiments were performed using the same concentration range for fluorouracil (99.5% of experiments), doxorubicin (99.3%), and vinblastine (91.2%). Only cisplatin was tested more frequently (21% of experiments) with different concentration ranges. Cisplatin was therefore used for further analysis.

Cisplatin had been tested more than 100 times in CCLs. 100 experiments were performed, in which 100 GI50 values for each cisplatin/cell line concentration in the most commonly used concentration range (0.05-500μM) were randomly selected and the GIΔ was calculated. A further random 100 selections were performed, but this time including all available concentration ranges. This was repeated 1,000 times and the median GIΔ for each cisplatin/cell line combination was calculated. In 23 of the 24 cisplatin/cell line combinations, the median GI50 fold change was significantly higher in the 100 random samples across all tested concentration ranges, than across 100 random samples from just one fixed concentration range (Figure 4.3C). This adds further evidence that the data variability increases when the covered concentration range increases.

4.4.4 Similar variability in GIΔ was present in FDA Approved Drugs

Since reliable clinical therapy outcomes depend on reproducible drug effects, it may be speculated that FDA-approved drugs are more robust in their drug response data than experimental agents. The same analyses that had been

performed on all data were repeated on NCI-60 data only including FDA approved drugs. The NCI60 database contained data on 181 FDA-approved drugs, which had been tested at least twice, resulting in 399,686 experiments investigating 9,970 individual CCCs. The number of experiments for CCCs ranged from 2 to 2,286.

The maximum GI50 fold change was 1.25×10^{10} observed for mithramycin in four cell lines, the colorectal CCL COLO-205 (26 experiments) (Figure 4.4A), the CNS CCLs SF-295 (24 experiments) and U251 (27 experiments), and the ovarian CCL IGROV1 (26 experiments). However, mithramycin was only used temporarily due to toxic side effects and is no longer used. The highest GIΔ for an FDA approved drug still in use was still high at 7.28×10^{7} for paclitaxel in CCL MDA-MB-435 (Figure 4.4B). Although FDA approved drugs are widely used as therapies, there was actually more GIΔ variability observed than for the complete dataset or for the non-FDA compounds with a mean GIΔ of 6.20×10^{6} (SD = 2.57×10^{8}) and a median GIΔ of 2.9 (IQR = 1.27-50) (Figure 4.4A). The mean and median GIΔ for non-FDA compounds were 218,154 (SD = 4.68×10^{7}) and 1.6 (IQR = 1.1-3.3) (Figure 4.4A).

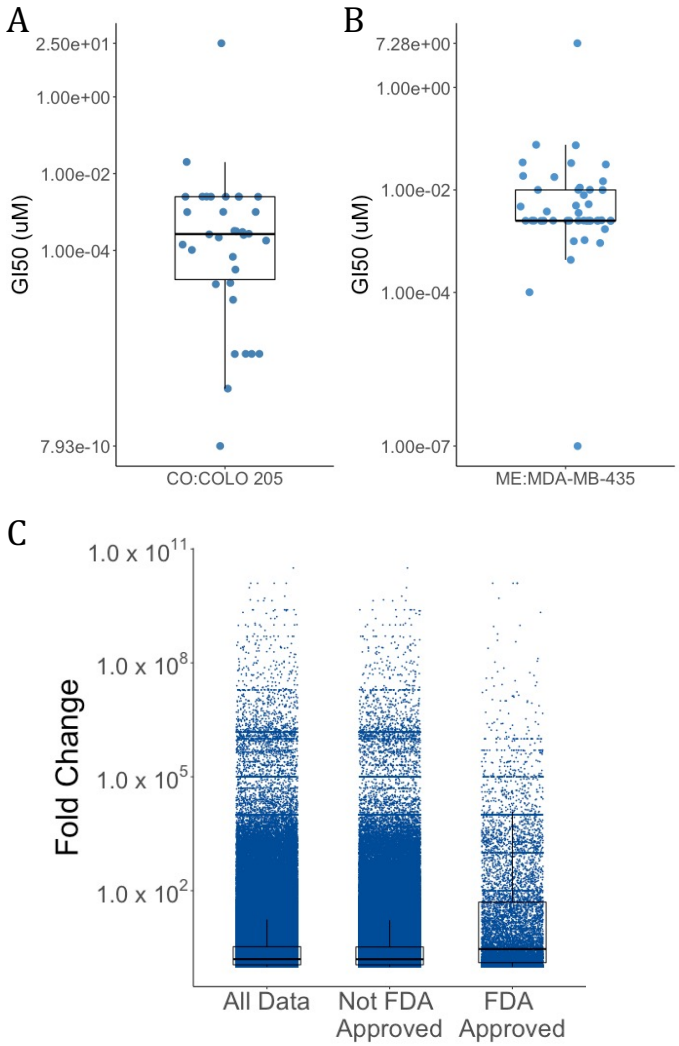

Figure 4.4 High level of GIΔ variability present in FDA approved drugs. Individual GI50 readings for **A.** mitramycin on colorectal CCL COLO 205 and **B.** paclitaxel on CCL MDA-MB-435**. C.** GIΔs for individual CCCs.

When considering all FDA approved CCCs, 5,752 (57.7%) had a GIΔ of greater than 2, 4,213 (42.3%) had a GIΔ of greater than 5, 3,547 (35.6%) had a GIΔ of greater than 10, 2,065 (20.7%) had a GIΔ of greater than 100 and 1,361 (13.6%) had a GIΔ of greater than 1,000 (Figure 4.5A, Table 4.1). For FDA approved CCCs with a minimum of five experiments considered, 3,533 (94.3%) CCCs displayed GIΔs greater than 2 and 1,295 (34.6%) GIΔs were greater than 1,000. For FDA approved CCCs with at least 100 experiments, 750 (99.9%) CCCs displayed a maximum fold

change greater than 5 and 544 (72.4%) displayed a GIΔ greater than 1,000 (Figure 4.5A, Table 4.2). As when considering all compounds, the higher the minimum number of experiments in experimental group, the higher the proportion of CCCs with higher GIΔ.

Table 4.2 – Number of CCCs with "high" fold changes for FDA approved drugs

Experimental Group	Number of CCCs	>2	>5	>10	>100	>1000
All Data	9971	5752	4213	3547	2065	1361
5 and Over	3745	3533	3206	2868	1849	1295
10 and Over	2718	2594	2470	2312	1633	1225
20 and Over	2187	2089	2008	1911	1461	1168
100 and Over	751	750	750	749	663	544

In agreement with the findings across all CCCs, the GIΔ also increased with EXN when the FDA approved CCCs were grouped into combinations with two experiments, 3 to 5 experiments, 6 to 20 experiments, and greater than 20 experiments. For CCCs with two experiments the mean and median GIΔ were 433.4 (SD = 16,619) and 1.4 (IQR = 1.1-2.3), for CCCs with 3 to 5 experiments 319.8 (SD = 2,337) and 4.2 (IQR = 1.7-23.7), for CCCs with 6 to 20 experiments 99,189 (SD = 1.14×10^6) and 17.86 (IQR = 5.2-93), and for CCCs with greater than 20 experiments 2.84×10^7 (SD = 5.5×10^8) and 1,578 (IQR = 43.4-10,000) (Figure 4.5B). The GIΔ was also correlated with the number of experiments performed (Spearman's correlation coefficient = 0.72, $p < 2.2 \times 10^{-16}$) (Figure 4.5C).

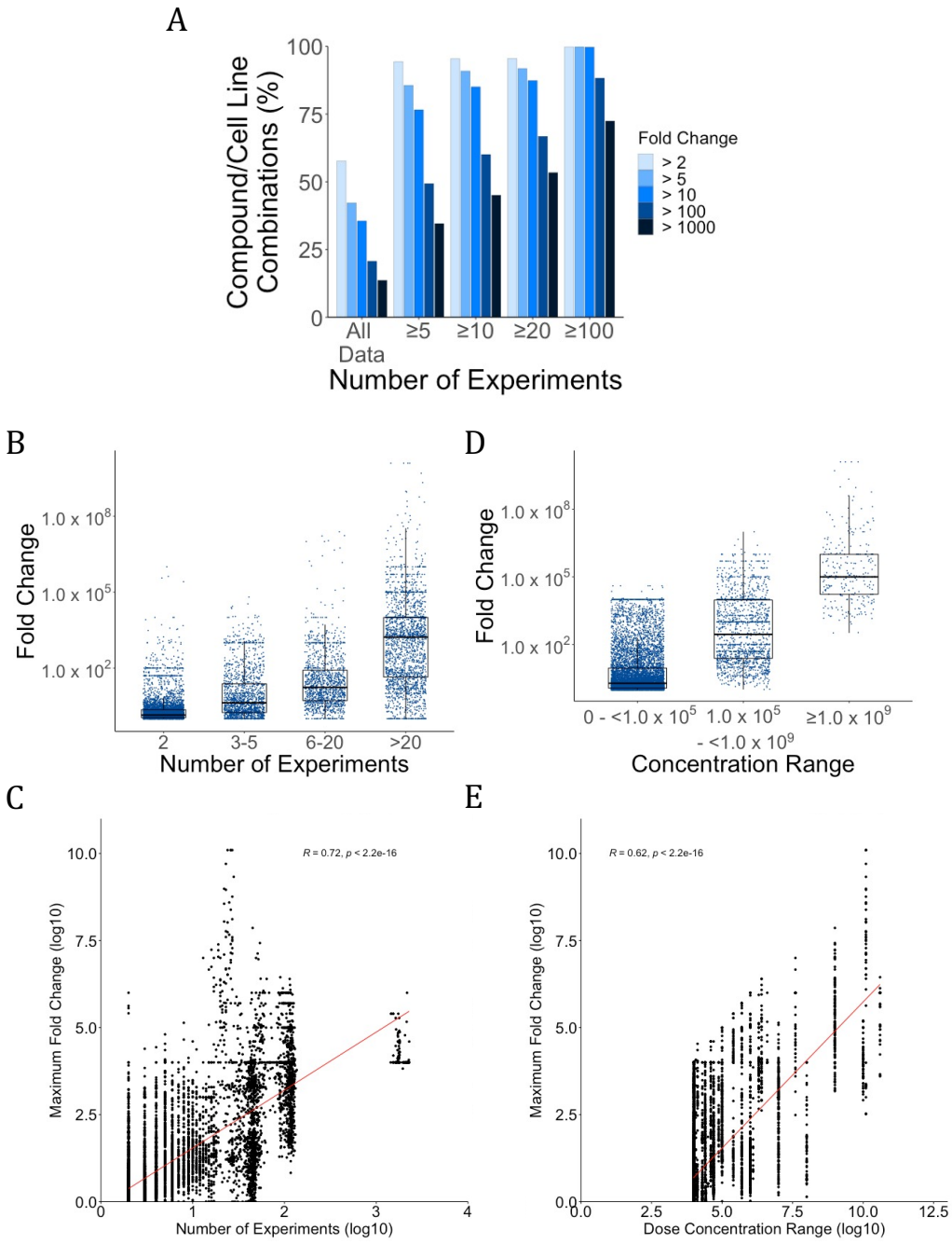

Figure 4.5 GIΔ increases as EXN and dose concentration range increase for FDA approved CCCs. A. Percentage of FDA approved CCCs with GIΔs above the indicated thresholds in dependence of the number of experiments. **B.** GIΔ in dependence on the number of experiments per FDA approved CCC. **C.** Scatterplot showing relationship between GIΔ and the number of experiments for FDA approved CCCs with statistics using Spearman's correlation. **D.** Distribution of GI50 fold changes in dependence of the concentration ranges in which FDA approved CCCs were tested. **E.** Scatterplot showing relationship between GIΔ and the dose concentration used for FDA approved CCCs with statistics using Spearman's correlation.

Moreover, the GIΔ increased with the concentration range covered (Figure 4.5D). The mean and median GIΔs were 378.6 (SD = 1,922) and 1.9 (IQR = 1.2-9.1) for CCCs with a dose concentration range of 1.0×10^5 or less, 44,413 (SD = 321,105) and 284.5 (IQR = 24.5-9,419) for CCCs a dose concentration range of 1.0×10^5 up to 1.0×10^9, and 2.19×10^8 (SD = 1.51×10^9) and 100,000 (IQR = 17,002-1.0×10^6) for CCCs with a dose concentration range of 1.0×10^9 and above (Figure 4.5D). In agreement, there was a significant correlation between the concentration range and the GIΔ (Spearman's correlation coefficient = 0.62, $p < 2.2 \times 10^{-16}$) (Figure 4.5E). Taken together, there is no indication that FDA-approved drugs would display less variability than experimental compounds.

4.4.5 Fold change outliers

For analysis of outliers there were two main aims. The first was to investigate whether there was any variability caused by specific CCLs or compounds. To determine whether certain individual CCLs or compounds were likely to be responsible for different levels of variation, the proportion of GIΔ outliers were calculated for both CCLs and compounds. To do this, only named compounds were used which reduced the dataset to 4030 total compounds. The second aim was to consider all individual GI50 values from the NCI-60 data and calculate the outlier GI50s and repeat previous analyses to see if the level of variability was still present, or was caused by individual outliers. Using these two methods made it possible to investigate whether individual compounds or CCLs were more likely to have high or low GIΔs that would be classed as outliers, suggesting more

variability in those CCLs and compounds, and also determine whether variability in the data set as a whole could be attributed to compound measurement outliers.

4.4.5.1 Compound outliers for cell lines

Outlier percentage was considered another possible measure of compound and CCL variability, and an indicator of experiment reproducibility. Each compound tested on a CCL was given a value, the GIΔ value between the lowest and the highest experimental GI50 for an individual CCL. Figure 4.6 shows the variation of GIΔ for each cell line and illustrates the likelihood of there being outliers in this data set. This gave a range of GIΔ values for each CCL that allowed us to determine how many, if any, compounds GIΔs could be considered outliers. For all data, outlier percentage (percentage of total compound GIΔs for a CCL considered an outlier) ranged from 4.45% (115 outliers from 2587 compounds, breast cancer CCL HS 578T) to 6.86% (189 outliers from 2754 compounds, lung cancer CCL HOP-92) (Figure 4.7). Of the total 4030 compounds, the GIΔ of 1273 (31.6%) compounds was classed as an outlier for at least one CCL. 12 compounds were GIΔ outliers for all 60 CCLs, seven of these compounds had a maximum EXN of over 100, while none of the compounds had an EXN of less than 19. 453 compounds were fold outliers for one CCL, EXN ranging from two to 15, although only nine of the compounds EXN was 10 or greater (Table 4.3).

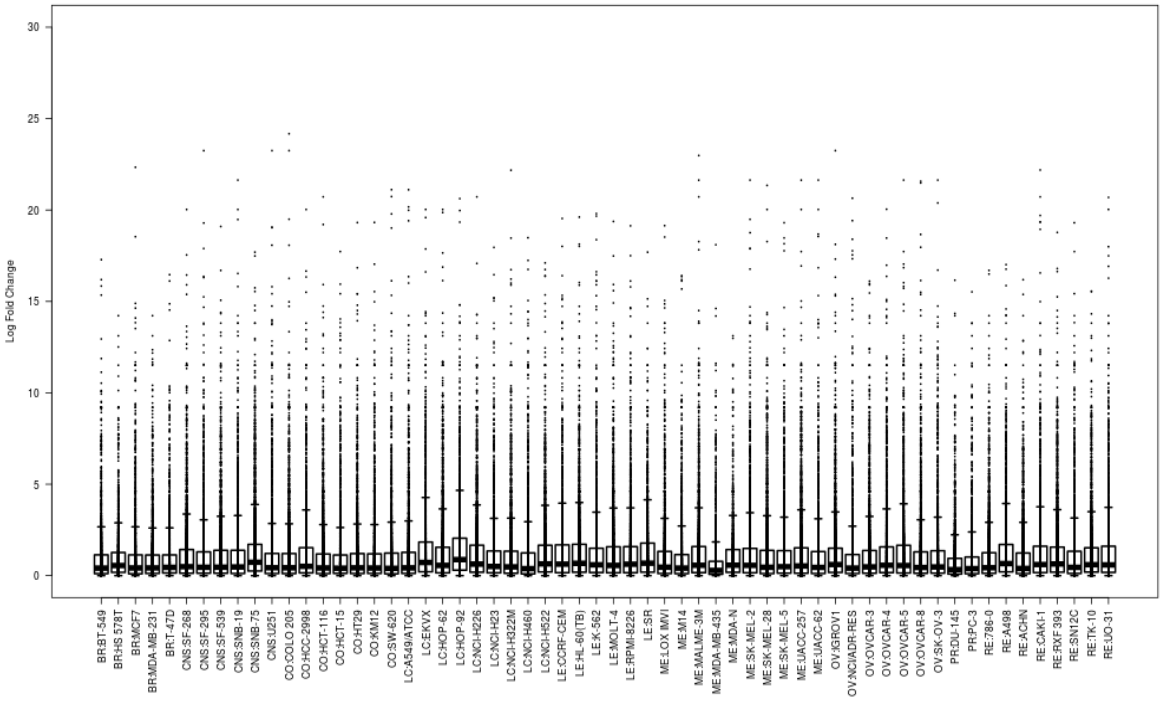

Figure 4.6 Variation of GIΔ in NCI-60 cancer cell lines using all experimental data. Each point is a fold change value for a compound. Cell line cancer type abbreviation and name on x axis.

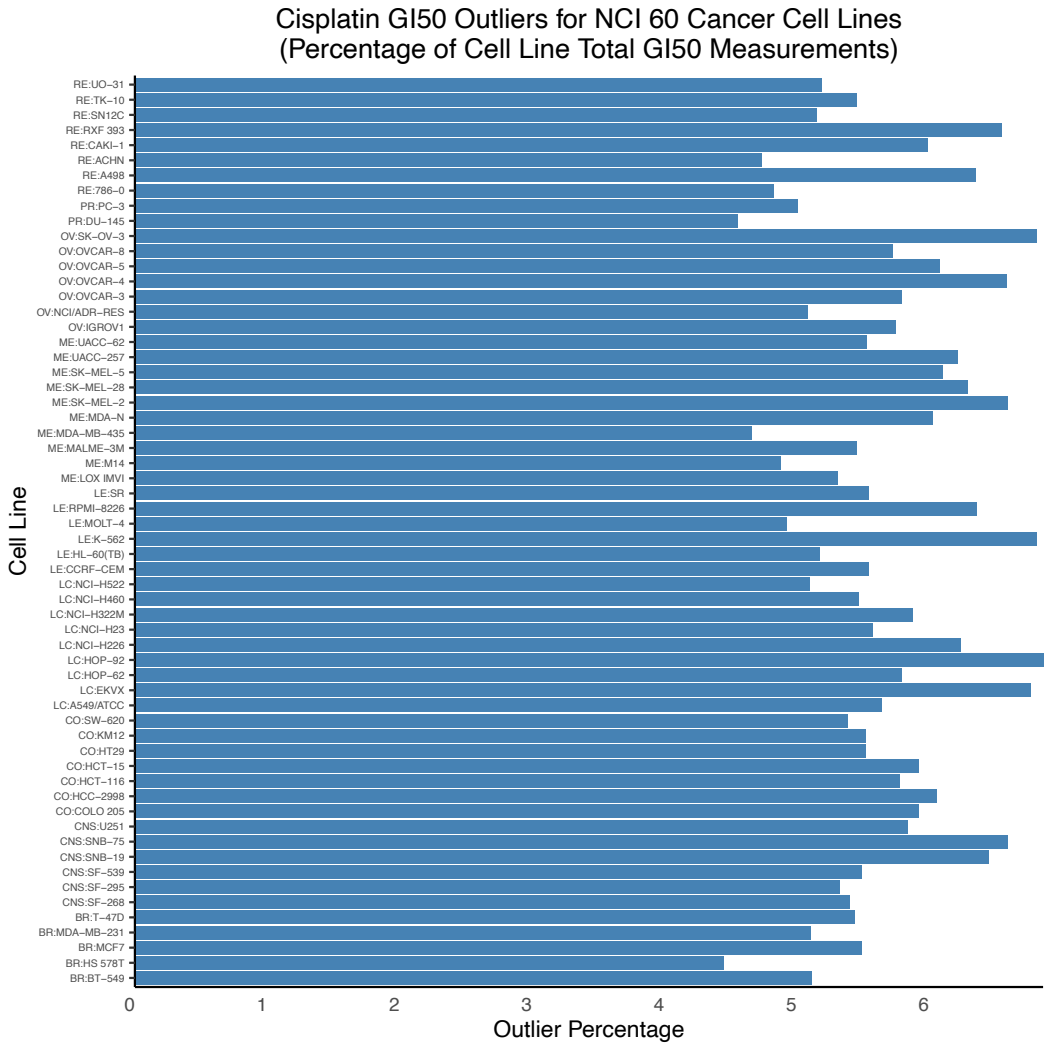

Figure 4.7 Percentage of GIΔ outliers for each NCI-60 cell line

Table 4.3 – Compound Outlier Frequency

	Compound			Number of Cell Lines a Compound is Outlier For			
Group	**Total**	**Outlier**	**Percentage Outlier**	**Minimum**	**Min Compound**	**Maximum**	**Max Compound**
All	4030	1273	31.59	1	453 Compounds	60	12 Compounds
5_+	562	149	26.51	1	44 Compounds	58	Vinblastine
10_+	205	37	18.05	1	9 Compounds	49	Maytansine
20_+	61	20	32.79	1	5 Compounds	41	2 Compounds
100_+	18	14	77.78	1	Bleomycin	11	Vinblastine
FDA Approved	178	52	29.21	1	11 Compounds	60	Vinblastine
Clinical Trial	69	29	42.03	1	11 Compounds	47	Dolastatin10

As with previous analyses, the alternative experimental groups (all data, 5+ experiments, 10+, 20+, 100+) were tested to determine the impact of large numbers of CCCs with low EXNs and no change in GI50. For all other testing groups, the minimum percentage of outliers was lower than for all data and the maximum percentage of outliers was higher than for all data. Percentage outlier numbers change very little, although the CCL with the lowest or highest outlier percentage differed among the experimental groups. Another notable difference occurred in the 100+ group, where the number of CCLs with no outliers increased from 37 to 45.

The number of compounds classed as outliers for a CCL decreased as a percentage of total compounds in the 5+ and 10+ groups (26.5% and 18% respectively), and was similar to the 20+ and FDA approved groups (32.8% and 29.2%). In the remaining two groups, 100+ (77.8%) and clinical trial (42.03%), the number of compounds as a percentage increased. Vinblastine and Maytansine were repeatedly among the outlier compounds most frequently present if not the most frequent for all groups they were present in, and particularly for the three groups with the most compounds (and therefore largest ranges of EXNs) there was a positive correlation between EXN and number of CCLs a compound was classed as an outlier for when considering all data(Spearman's correlation = 0.77, $p<2.2 \times 10^{16}$). This was not the case in all the other experimental groups however. For the 5+ (0.39,$p=1.61 \times 10^{-9}$), FDA approved (0.66,$p<2.2 \times 10^{16}$) and clinical trial (0.62,p=0.008) experimental groups, positive correlations were observed, but for 10+ (-0.6,$p<2.2 \times 10^{16}$), 20+ (-0.6,$p<2.2 \times 10^{16}$) and 100+ (-0.59,$p=1.04 \times 10^{-14}$) experimental groups a negative correlation was observed. This change from high

positive correlation to high negative correlation was likely due to the compounds with more than 20 experiments for one individual CCL, reducing the chance of the compound being an outlier.

4.4.5.2 Cell line outliers for compounds

A compounds maximum GIΔ over experiments for an individual CCL was used to determine CCL outliers for that compound. No CCL outliers were present for 1515 of the 4030 compounds. Of the remaining compounds with outliers present, the lowest outlier percentage was 1.67% (one out of 60 CCLs) for 32 compounds, while the highest was 40% (two out of five CCLs for compound 1h-pyrazolo[3,4-d]pyrimidine, 4-amino-1-(p-chlorobenzyl), NSC# 63629). Every CCL was an outlier for at least 73 compounds (CCL MDA-N) and for as many as 249 compounds (SNB-75). Again the analysis was carried out on the other experimental groups. Unsurprisingly, as the compounds per experimental group decreased, the number of times an individual CCL was an outlier also decreased. All groups bar the 5+ experimental group (and all data group) contained CCLs that were only outlier for one compound, and the CCL that appeared as an outlier in the most compounds was different for every group. For the complete data set there was a negative correlation between EXN and CCL outlier for individual compounds (correlation = -0.68, $p<2.2 \times 10^{-16}$). Again this is likely due to not many GIΔ from high EXNs being classed as outliers. No GIΔ from an EXN above 127 generated a CCL outlier for a compound, which is maybe not surprising as 95 of the 113 instances where EXN is above 127 were only observed once. Similar analysis was carried out on the alternative experimental groups to remove some

of the low EXN but results remained similar across the board, although correlation coefficients of -0.64 and -0.57 for 10+ and 20+ experimental groups were above significance threshold (p=0.57 and p=0.13). Little correlation was observed in the clinical trial experimental group, and again the correlation coefficient of -0.08 was not significant (p=0.75).

4.4.5.3 Variability in data after removal of outliers

GI50 outlier number is high in the NCI60 data. Of the 598,243 individual GI50 values considered for outlier analysis, 5.7% (34,216) were outliers. For CCLs with at least 5 experiments, 43.7% (13,208/30,212) CCCs had at least one GI50 outlier. The highest percentage of outliers was for 50% (7/14) of experiments for compound maytansine on prostate CCL DU-145 (Figure 4.8A), and the greatest number of outliers was 291 (of 1731 experiments, 16.8%) for fluorouracil on lung CCL HOP-62 (Figure 4.8B). The lowest percentage of outliers of those combinations with outliers present was 0.79% (1/128) of all experiments for cisplatin on CNS CCL U251. Outlier number increased with the number of experiments for a CCL with a Spearman's correlation coefficient of 0.25 ($p < 2.2 \times 10^{-16}$) however, when considering only CCCs with outliers, this increased to 0.52 ($p < 2.2 \times 10^{-16}$).

674 (91%) compounds had at least 1 outlier, and the compound with the greatest number of outliers was doxorubicin with 5504 (4.2% of total 129,815 experiments) over 59 of the 60 cell lines, which was also the highest among the compounds with outliers. The compound with the highest number of outliers

relative to the total experiments carried out was 15-desacetylsergeolide with 20.6% (53/257 experiments). A positive correlation was observed between maximum experiment number for a compound and the number of outliers (Spearman correlation coefficient = 0.52, $p < 2.2 \times 10^{-16}$).

All 60 cell lines had at least 84 outliers (breast CCL BT-549) and the greatest number of outliers was 1062 for renal CCL SN12C. Both also had the least and most number of outliers compared to total experiments, 0.44% (84/18,808) for BT-549 and 3.1% for SN12C (1062/34,408). The CCL with outliers for the most compounds was leukaemia CCL CCRF-CEM (303/735, 41.2% of all compounds) and the CCL with outliers for the least compounds was breast cancer CCL BT-549 (40/735, 5.4%).

Despite the large number observed, outliers were not responsible for the variability we see in the data. Removal of outlier GI50 values from the dataset does not change the overall pattern of variability. For data CCCs with at least 5 experiments and outliers removed the GIΔ after outlier removal was lower than when considering all data with 5 experiments or more with an observed mean GIΔ of 5.33×10^6 (SD = 2.48×10^8) for all data and 195,907 (SD = 1.63×10^7) for data after outlier removal. The median was 10 (IQR = 3.1-95.6) when considering all data with 5 experiments or more and 5.2 (IQR = 2.1-21.9) for data minus outlier GI50s (Figure 4.8C). Although variability, in the form of high GIΔs, decreased after outlier removal it was still very high.

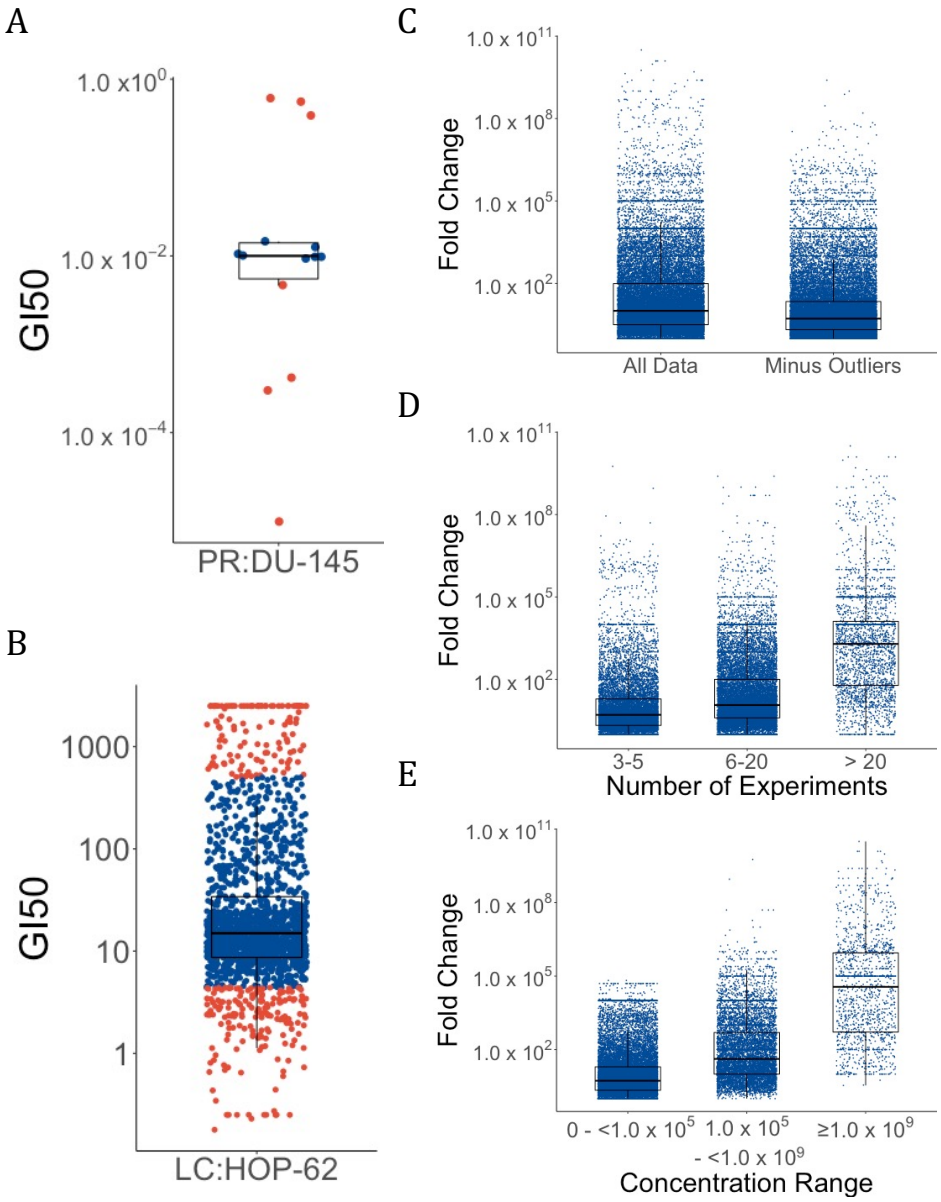

Figure 4.8 Analysis of NCI-60 GI50 data after removal of outliers. As many as 50% of all GI50 measurements for a compound/cell line combination were outliers (**A.** maytansine and prostate cell line DU-145) and as many as 291 GI50 measurements for a compound/cell line combination were outliers (**B.** fluorouracil and lung cancer cell line HOP-62). After removal of outlier GI50 measurements a high level of variability was still present (**C**) and GI50 variability increased with the number of experiments (**D**) and the dose concentration range covered (**E**). Each data point in A and B represent individual GI50 values, and for **C, D**, and **E** represents the maximum GI50 fold change for individual compound/cell line combinations.

A maximum GIΔ of 2.5 x 10^9 was observed for compound maytansine and ovarian CCL OVCAR-5 over 35 experiments. The number of high maximum fold changes was maintained after removal of outliers. 80% (20,826/26,108) of CCCs with 5 or more experiments, and 95% (2658/2806) with more than 20 experiments had maximum fold change of greater than 2. Maximum fold changes of over 100 were observed in 16.5% (4309/26,108) of CCCs with 5 or more experiments and 47.3% (1326/2806) of CCCs with 20 or more experiments. Even with outliers removed, 8.3% (232/2806) of GIΔ were over 100,000 for compound/cell line combinations with 20 or more experiments.

In agreement with the findings across all CCCs, the GIΔ increased with EXN when after removal of outliers CCCs were grouped into combinations with 3 to 5 experiments, 6 to 20 experiments, and greater than 20 experiments. For CCCs with 3 to 5 experiments 15,601 (SD = 5.35 x 10^5) and 2.8 (IQR = 1.8-7.5), for CCCs with 6 to 20 experiments 80,079 (SD = 8.01 x 10^6) and 7 (IQR = 2.8-27.2), and for CCCs with greater than 20 experiments 1.66 x 10^6 (SD = 5.08 x 10^7) and 69.3 (IQR = 10-2,231) (Figure 4.8D). A positive correlation between experiment number and fold change was also observed, the spearman correlation was 0.38 ($p < 2.2 \times 10^{-16}$) after outlier removal. The GIΔ also increased as dose concentration range increased after removal of outliers. Three different experimental groups that were considered were dose ranges of: 1.0 x 10^5 or less, 1.0 x 10^5 up to 1.0 x 10^9 non-inclusive and 1.0 x 10^9 and above. Using these experimental groups an increase in mean and median GIΔ was observed as dose concentration increased in that group. The mean and median GIΔs were 281 (SD = 2,478) and 3.4 (IQR = 1.6-9.4)

for CCCs with a dose concentration range of 1.0×10^5 or less, 22,255 (SD = 9.39×10^5) and 2.1 (IQR = 4.5-91) for CCCs a dose concentration range of 1.0×10^5 up to 1.0×10^9, and 3.93×10^6 (SD = 7.4×10^7) and 1,803 (IQR = $81.7\text{-}1.0 \times 10^5$) for CCCs with a dose concentration range of 1.0×10^9 and above (Figure 4.8E).

Although GI50 outliers were not responsible for the high levels of variability that was seen, the maximum fold change for CCCs with outlier data removed was drastically altered in many cases. A decrease in GIΔ was seen in 13,208 (43.7%) CCCs ranging from 0.005 (compound 6,7-diacetoxy-4'-methylisoflavan and melanoma CCL UACC-257) after removal of 1 outlier to 3.15×10^{10} (compound cyanomorpholinodoxorubicin and colorectal CCL COLO 205) after removal of 1 outlier. The mean change in maximum fold change for the 13,208 CCCs was 1.17×10^7.

4.4.6 GI50 Variability in cell lines with multiple experiments for a compound over time

4.4.6.1 Maximum GI50 fold change varies between cell lines and between compounds

Experimental timelines of the GI50 values for the 18 compounds, which had been tested at least 100 times in one or more cell lines were established (Table 4.4). This allowed us to investigate the variability of the GI50 during an experimental time frame spanning 324 months (July 1989 to July 2016), with individual compound experimental time frames ranging from 95 months (5 compounds) to 275 months (doxorubicin).

Table 4.4 – Compound Experiment Information – Compounds with 100 or More Maximum Experiments

Compound	Start Date	End Date	Time Frame (Months)	Maximum Experiment Number
Actinomycin D	1989-07	2009-12	245	132
Bleomycin	1989-07	2008-08	229	131
Carmustine	1989-07	2008-08	109	128
Chlorambucil	1989-07	2006-11	208	130
Cisplatin	1989-07	2010-04	249	139
Cytarabine	1989-07	1997-06	95	130
Daunorubicin	1989-07	1997-06	95	126
Doxorubicin	1993-08	2016-07	275	2348
Floxuridine	1989-07	1997-06	95	124
Fluorouracil	1993-08	2007-02	162	1886
M-AMSA	1989-07	2006-11	208	132
6-Mercaptopurine(1)	1989-07	2004-06	179	130
6-Mercaptopurine(2)	1989-07	1997-06	95	127
Methotrexate	1989-07	2006-11	208	134
Mitomycin	1989-07	2004-06	179	129
Thiotepa	1989-07	2008-08	229	136
Triethylenemelamine	1989-07	1997-06	95	128
Vinblastine	1989-07	1997-06	95	129

For 13 of the 18 compounds no change in GI50 was observed between consecutive experiment dates in multiple CCLs, in as few as two CCLs (for carmustine) and in as many as 52 CCLs (for methotrexate), while the lowest fold change between consecutive experiments for the remaining five compounds was no higher than 2.79 (mercaptopurine(2), SNB-19). The maximum fold change for a CCL between consecutive experiments varied from 27.67 (triethylenemelamine, MDA-MB-231) to 2.56E+10 (vinblastine, SNB-75) while a maximum fold change between consecutive experiments of at least 1.42E+03 was seen in 14 of the remaining 16 compounds. Maximum fold change was calculated for each CCL for each compound using the highest and lowest GI50 value at any point in the CCLs experimental time line, resulting in maximum fold change for a compound ranging from 8.09E+03 (thiotepa, BT-549) to 8.20E+10 (actinomycin d, HCT-116). The maximum fold values are not, however, a good indicator of GI50 variability for a

CCL or compound over time, this could just represent an outlier at a particular time. So the percentage of times a GIΔ between two consecutive experiments was ±2 or above and ±5 or above for each CCL for each compound was calculated. This allowed us to determine which compounds and CCLs were most variable over their experimental time frame. Figure 4.9 displays the results from this analysis and it is clear that some compounds possess far more GI50 variability from experiment to experiment than others.

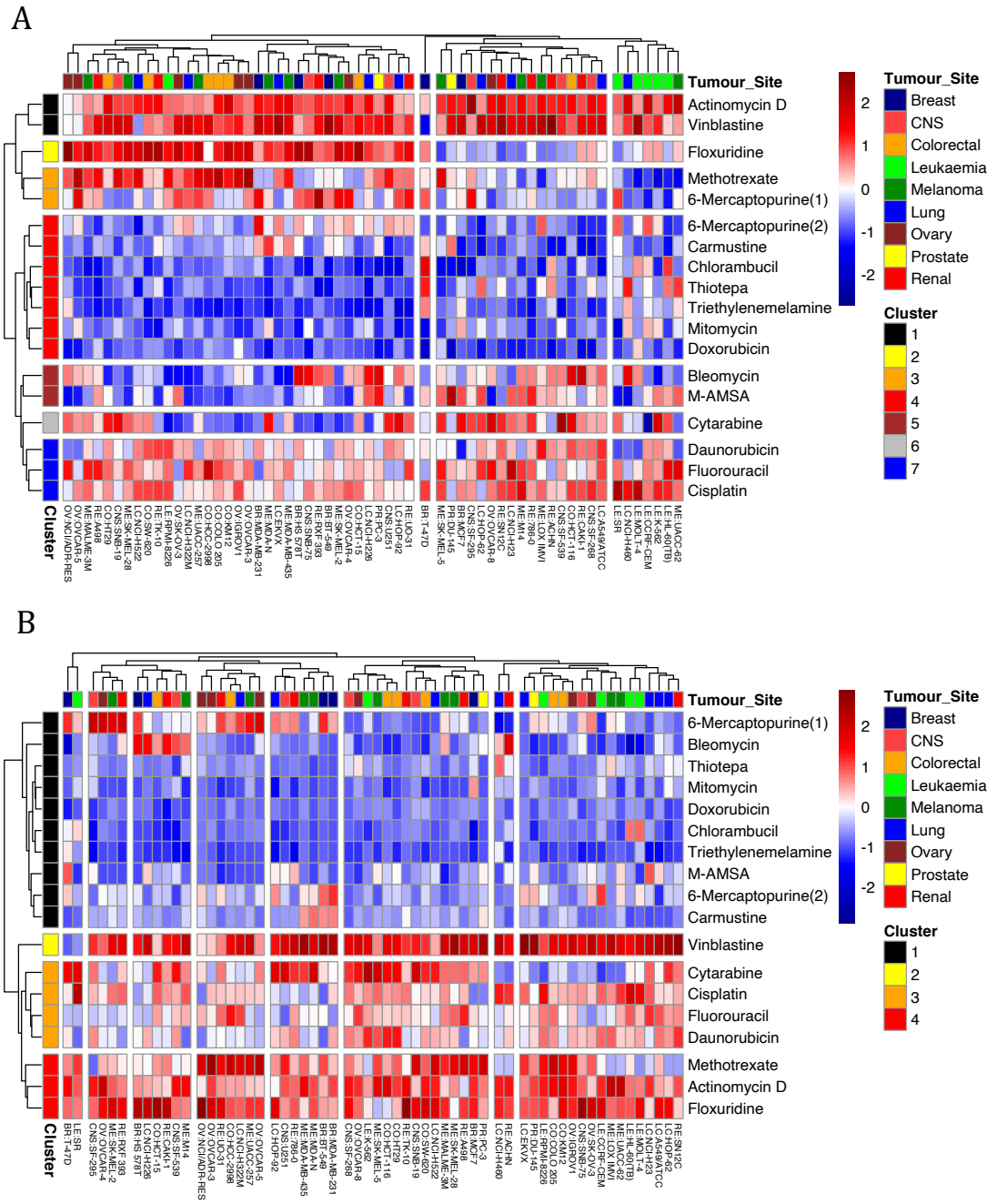

Figure 4.9 Percentage of GIΔ that are above A. ±2 and above B. ±5 between consecutive experiments for individual CCLs. Lower percentages represented as blue and higher percentages are represented as red, missing data for a cell line represented as grey. Data is scaled by column and so low values are represented by a negative score and high values represented by a positive score. Optimum number of clusters was determined using affinity propagation.

From the cluster analysis it is apparent that there were drugs that had a lower level of variability between consecutive experiments. This was represented by cluster 3 (7 drugs) when considering fold changes over 2 (Figure 4.9A) and cluster 1 (10 drugs) when considering fold changes over 5 (Figure 4.9B). Clusters 1 (2 drugs) and 7 (3 drugs) displayed high levels of variability when considering fold changes over 2 (Figure 4.9A), while clusters 2 (1 drug) and 4 (3 drugs) displayed high levels of variability for fold changes over 5 (Figure 4.9B). The remaining drugs displayed mixed levels of variability.

When considering the data for fold changes of greater than 2 between consecutive experiments (Figure 4.9A), there was some clustering of leukaemia CCLs. Five of the six leukaemia cell lines clustered in a cluster of seven. However, this was the only example of CCL clustering as CCLs from the same cancer site did not tend to cluster together. Considering the data for fold changes of greater than 5 between consecutive experiments (Figure 4.9B), again CCLs from the same cancer site did not tend to cluster together. Drugs that clustered together were not necessarily similar. When considering the data for fold changes of greater than 2 between consecutive experiments (Figure 4.9A), Actinomycin d and vinblastine clustered together in one cluster and were clearly the two drugs with the highest number of fold changes greater than 2 between experiments. However, they both have different mechanisms of action. Actinomycin d inhibits transcription by binding to DNA, while vinblastine is microtubule disrupting. Furthermore, drugs with similar mechanisms of action did not cluster together. Topoisomerase II inhibitors daunorubicin and doxorubicin, and thymidylate synthase inhibitor fluorouracil and its inactive form floxuridine did not cluster closely to each other. This was also

the case when considering fold changes of greater than 5. Although from Figures 4.9A and B it is clear that a drug appears to have similar numbers of high GI50 changes between consecutive experiments for all cell lines, whether there be many or few. This meant that there was little difference between profiles for each cell line. And drug mechanism of action or cancer site of CCLs did not contribute to variability in consecutive experiments.

Taking maximum fold change and the number of 'high' fold changes between consecutive experiments into consideration, vinblastine, actinomycin d and floxuridine appear to contain the most GI50 variation, whereas doxorubicin, chlorambucil and triethylenemelamine the least. The data certainly shows that maximum fold change can not be considered the only indicator of GI50 variability as Doxorubicin, with less variation when considering only 'high' fold changes (±2 and ±5) between experiments, giving the third highest maximum fold change (1.00E+08, IGROV1) and third highest fold change between two consecutive experiments (2.18E+06). This can be seen in the timeline plot for doxorubicin (Figure 4.10A), a large spike in the data is likely an outlier responsible for these high fold change values. Similarly, a large number of instances where there was no change in GI50 between consecutive experiments cannot be considered a reliable indicator of GI50 variability when used alone. In multiple CCLs for vinblastine for example (Figure 4.10B), there was no change in GI50 between multiple consecutive experiments. In one CCL, no change in GI50 was observed for 24.24% consecutive experiments, while 42% fold changes were of at least ±5. One very apparent observation that can be made is that all CCLs for all compounds

displayed GI50 variability over their experimental timeline, but to varying degrees.

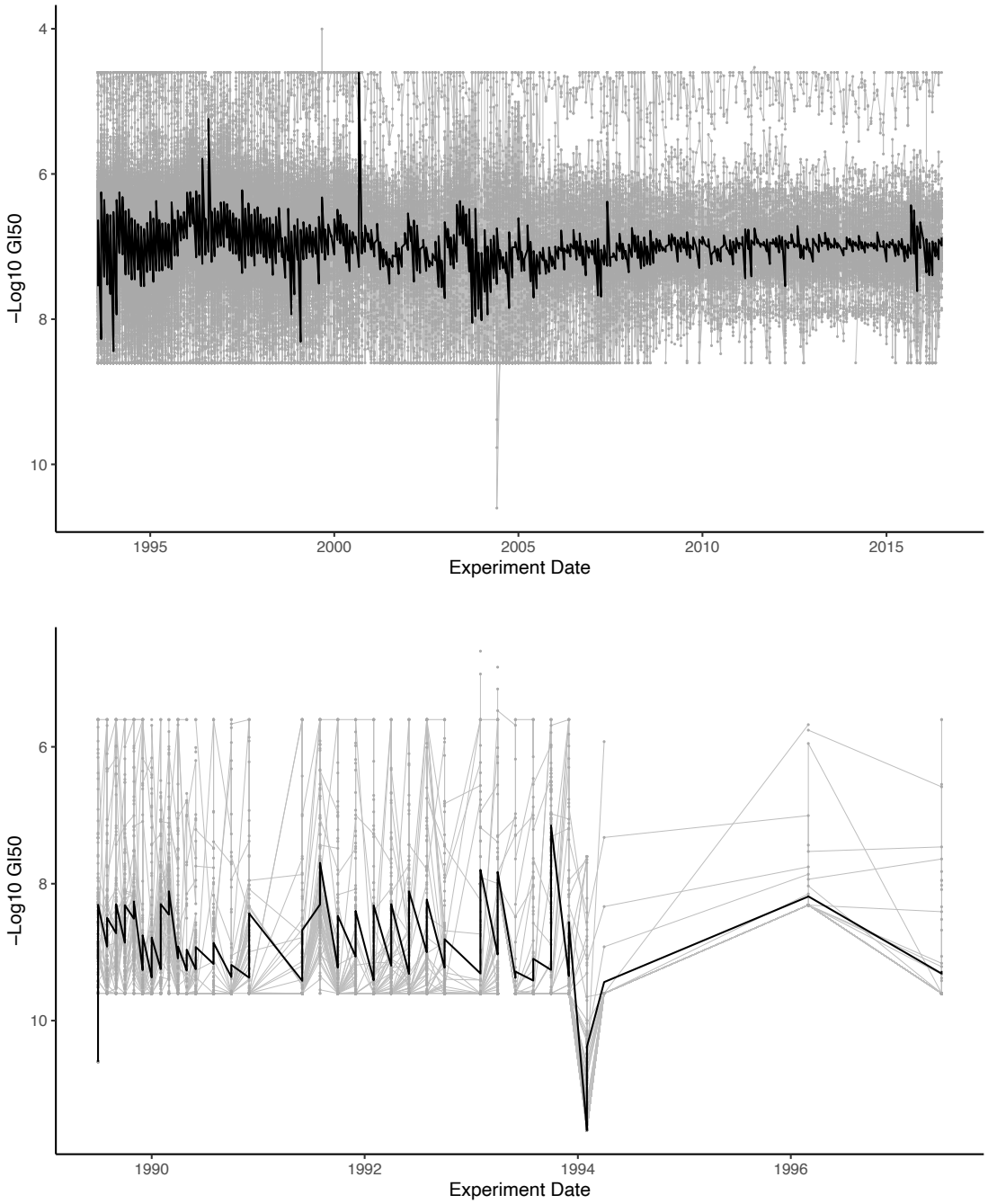

Figure 4.10 Experimental timeline for NCI-60 cell lines. Grey points represent GI50 for individual cell lines and the grey lines the change between GI50 over consecutive experiments in the timeline. The black line is the mean GI50 plotted line and represents the trend of GI50 change over the experimental timeline for A. doxorubicin and B. vinblastine. Changes in GI50 at October 1993 are highlighted.

4.4.6.2 GI50 Difference between first and last time point

As well as investigating the degree of variability in CCLs and compounds during the experimental timeline, GI50 at the beginning and end date of available data were compared for each CCL for each compound. Overall, fold changes between the start point and endpoint were variable between CCLs. For every single compound at least one CCL that had identical or close to identical start and end points (minimum fold varied from no change in GI50 (31 CCLs over 8 compounds) to a fold change of ±1.1 (fluorouracil, SK-OV-3)), while maximum fold changes ranged from 8.95 (chlorambucil, RXF 393) to as high as 5.00×10^8 (vinblastine, SNB-75). The direction of fold change between start and end point differs between compounds and between the individual CCLs for each compound. Vinblastine final GI50 was lower than start GI50 in 90% (54/60) of CCLs, while chlorambucil final GI50 was lower than start GI50 in 48.3% (29/60) of CCLs and higher than start GI50 in 51.7% (31/60) of CCLs. The final GI50 was higher than the start GI50 in as many as 59 of 60 CCLs for a compound (98.3%, cisplatin), and final GI50 was lower than start GI50 in as many as 58 of 60 CCLs (96.7%, actinomycin d) (Table 26). The analysis suggests that there is variability of compound GI50 between CCLs and over time, and in varying degrees. High variability was seen in vinblastine and actinomycin d for instance, in the form of high maximum fold changes between lowest and highest GI50 values for a CCL, high maximum fold changes (and large number of high fold changes) between experiment dates, and large start/end fold changes predominantly in one direction for the majority of CCLs. Chlorambucil GI50 fold changes were much lower and start/end fold changes were low and

shared between positive and negative fold changes, compared with other compounds this is relatively low variation.

CCLs may display substantial changes in genotype and phenotype over time (Ben-David *et al.*, 2018). Hence, part of the variability observed in drug sensitivity may be the consequence of a shift in drug response over time. CCCs, in which the fold change between the mean GI50 on the first experimental date and the mean GI50 on the last experimental date was 50% or greater than the maximum GI50 fold change for the data points in between were considered as candidates for a drift in drug sensitivity. Only six (0.56%) out of CCCs fulfilled these criteria (Figure 4.11). The distribution of the individual GI50 values for three of the CCCs (floxuridine/SK-OV-3, methotrexate/BT-549, 6-mercaptopurine/BT-549) did not indicate a GI50 shift over time (Figure 4.11A-C). For the other three CCCs (bleomycin/K-562, vinblastine/T-47D, M-AMSA/MDA-MB-435) a drift in sensitivity appears unlikely but cannot be excluded based on the data (Figure 4.11D-F). However, such observations are very rare. Moreover, a phenotypic drift in a cell line would be expected to result in changes in sensitivity to more than one drug over time.

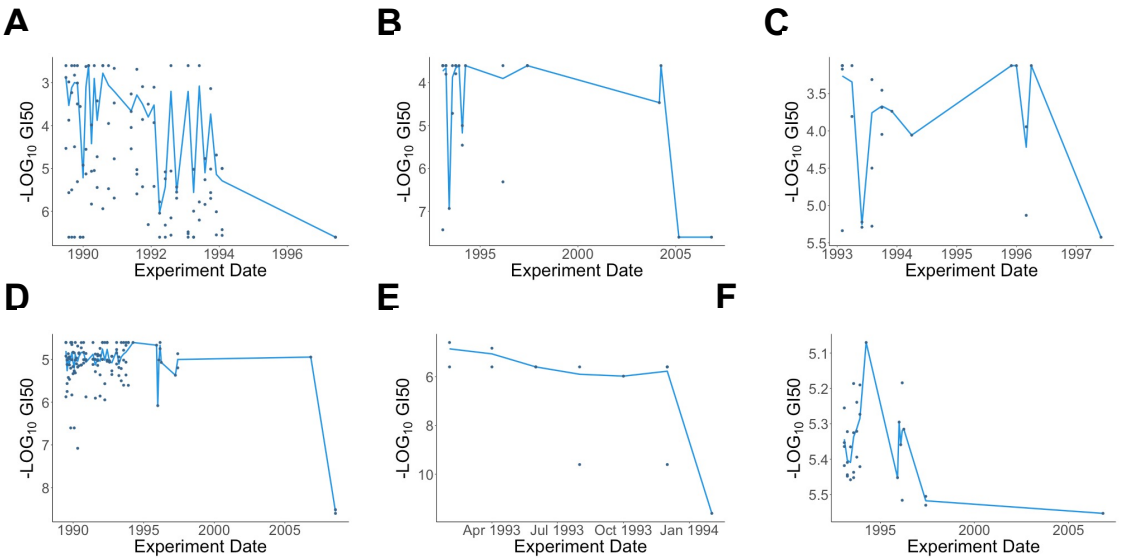

Figure 4.11 Experimental time lines for individual compound/cell line combinations. The 6 experimental timelines for compound/cell line combinations (amongst compounds for which at least one cell line has 100 experiments performed on) with a fold change between the mean GI50 at the first experimental date and final experimental date that is greater than 50% the value of the maximum GI50 for that combination. Compound/cell line combinations are **A.** floxuridine and ovarian cancer cell line SK-OV-3, **B.** methotrexate and breast cancer cell line BT-549, **C.** 6-mercaptopurine and breast cancer cell line BT-549, **D.** bleomycin and leukaemia cell line K-562, **E.** vinblastine and breast cancer cell line T-47D and F. M-AMSA and melanoma cell line MDA-MB-435. Plot line represents mean GI50 while data points represent individual experiments at experimental date.

4.4.6.3 Highly Tested Compound GI50 outlier numbers

Outlier analysis had been performed earlier for all NCI-60 data, however further outlier analysis was performed on the subset of drugs that had been experimented on the most in greater detail to determine the role outliers may have in drug sensitivity variability. The Outlier GI50 values ranged from zero for a CCL for a compound (multiple occasions) to as many as 32.26% of a CCLs GI50 values for a compound (Carmustine, MDA-MB-435) (Figure 4.12). CCL MDA-MB-435 displays more GI50 outliers across all compounds than any other CCL, with greater than 10% of GI50 values classified as outliers in nine of the 18 compounds, greater than

15% in seven, 20% in five and 25% in three. Similar numbers of outliers are also present in CCL MDA-N (seven, four, three and two respectively). In contrast, there were six CCLs with more than 10% (and no higher than 14.04%) of GI50 values classed as outliers in just one compound. No outliers are observed for CCL T-47D for eight of the 18 compounds, the most of all the CCLs, whereas no outliers were observed in only one compound for ten CCLs.

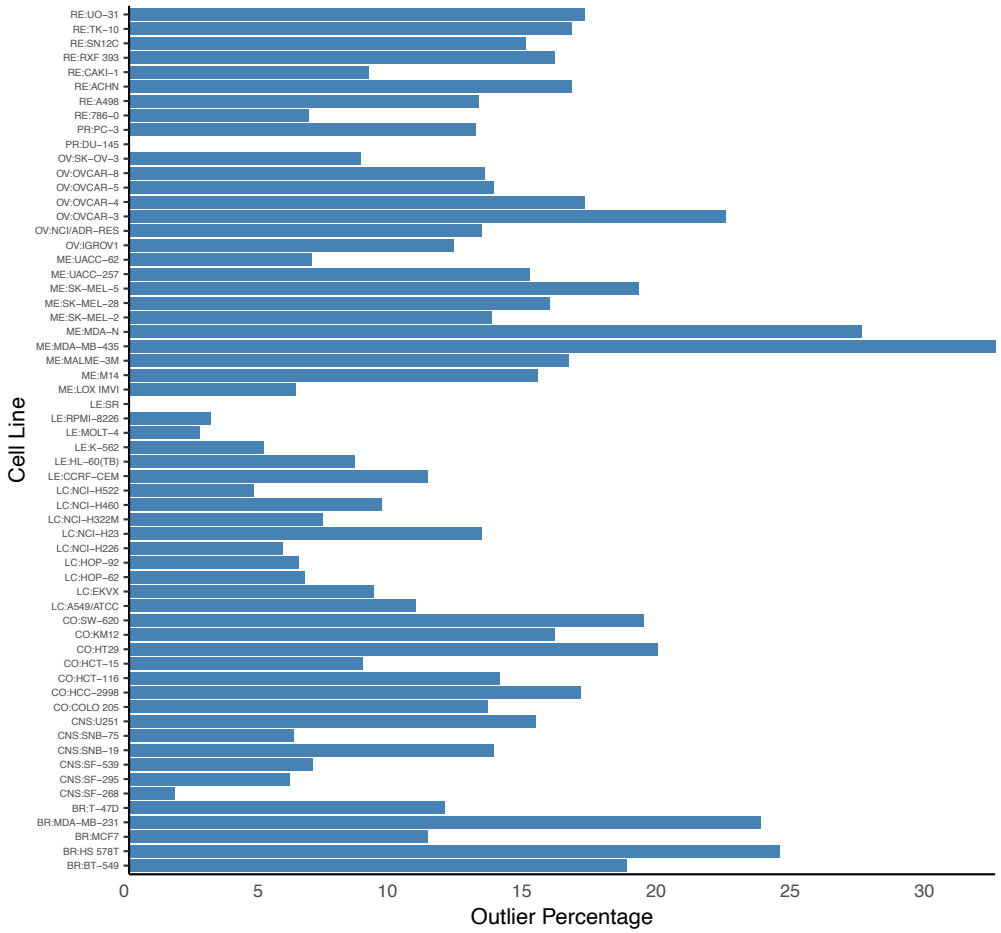

Figure 4.12 Percentage of carmustine GI50 values that are outliers for all cell lines

For three compounds, no outliers were present in more than 50% of CCLs: methotrexate (42/60), cytarabine (39/60) and floxuridine (31/60). The remaining CCLs did contain high levels of outliers for methotrexate, nine of the CCLs with over 10% of GI50 values outliers, and one with over 25% outliers.

cytarabine and floxuridine were two of the compounds with the greatest GI50 variation in fold change and maximum fold change analysis, so this suggested that due to the greater GI50 variability, there would no outliers from the varying range of GI50 values. However, for compounds previously determined to be two of the most variable, actinomycin d and vinblastine, there were only two CCLs with no GI50 outliers. For actinomycin d there were 26 CCLs with more than 10% GI50 outliers, 13 with more than 15%, ten with more than 20% and four CCLs with more than 25% GI50 outliers. CCLs did not have as many outliers for vinblastine but 11 CCLs had over 10% GI50 outliers, four with more than 15% and two with more than 20% GI50 outliers. The outlier percentages for each cell line for each compound are displayed in figure 4.13. It is clear that the compounds with the largest amount of outliers for CCLs are carmustine, actinomycin d and daunorubicin. The lowest amount of outliers are present for cytarabine, bleomycin and doxorubicin, and melanoma cell line MDA-MB-435 is the cell line with the most outliers.

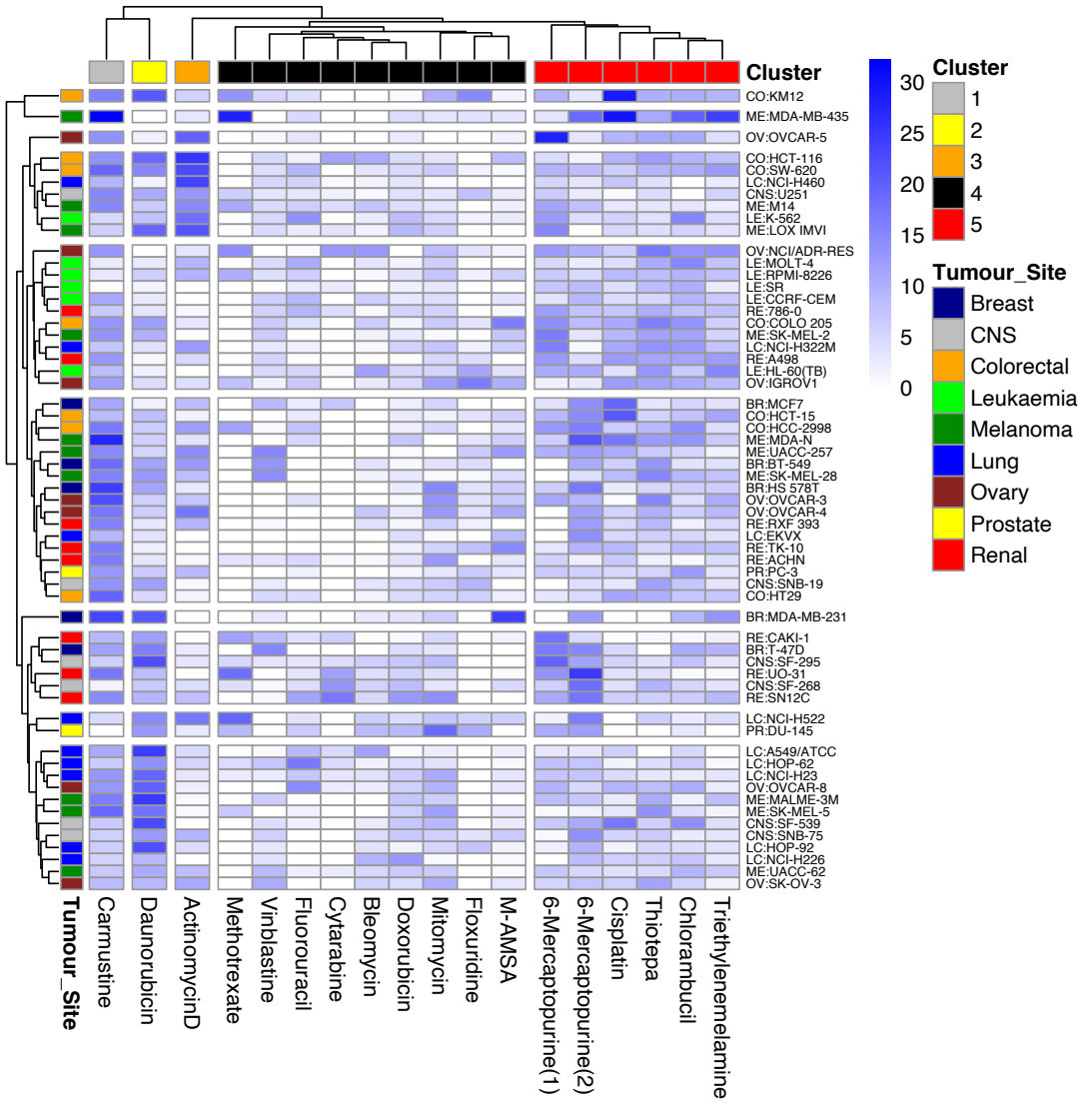

Figure 4.13 Percentage of GI50 values that are classed as outliers for individual cell lines. No outliers is represented as white, the more outliers present, the darker the blue.

The drugs clustered into 5 clusters. Actinomycin d (cluster 3) and daunorubicin (cluster 2) clustered individually with a large number of outliers in few CCLs. Carmustine displayed a large number of outliers for the majority of CCLs and also clustered individually (cluster 1). Cluster 4 (9 drugs) displayed relatively low amounts of GI50 outliers in in all CCLs and the remaining 6 drugs (cluster 5) displayed a

medium number of GI50 outliers across all CCLs (Figure 4.13). Interestingly, 3 of the 5 DNA synthesis inhibitors clustered in cluster 4 while 4 of the 5 alkylating agents clustered together in cluster 5. With carmustine displaying the highest number of GI50 outliers, it could perhaps be hypothesised that alkylating cancer treatments are more unstable than DNA synthesis inhibitors and DNA binding drugs.

CCLs all displayed similar profiles with regards to GI50 outliers. There were some examples of clustering by cancer site, leukaemia CCLs clustered closely together and displayed less outliers in carmustine, a drug that many GI50 outliers were recorded for other CCLs. There was evidence of clustering by other cancer sites. Colorectal, melanoma, renal, ovary and lung CCLs all displayed trends of clustering in the vicinity of other CCLs that were of the same cancer site. This suggests that maybe cell lines of particular cancer sites are prone to more or less variability than others.

Although outlier number is no doubt an indicator of some sort of variability with regards to GI50 measurements, it is not possible to determine whether or not a CCL or drug displays more or less

variability than another. Other factors that have been described throughout must also be taken into consideration.

4.4.6.4 Variability of GI50 for experiments with the same date

On dates where more than one experiment was undertaken for a CCL, the fold change between the lowest and highest GI50 for each relevant CCL was calculated and compared over experimental time frames. For 16 of the 18 compounds, the maximum EXN was seven and minimum two. For doxorubicin and fluorouracil though, the maximum EXN is much higher at 35 and 32 respectively (minimum EXN of two) (Table 4.5). The number of CCCs greatly increases for these two compounds as well. The number of combinations was 15,998 for doxorubicin and 9525 for fluorouracil while for the other compounds CCC number varies from 1639 to 2036. The number of dates experiments were carried out on also varied; 276 for doxorubicin, 163 for fluorouracil, and between 34 and 44 for the remaining 16 compounds.

For all compounds, but particularly doxorubicin and fluorouracil, fewer multiple experiments per date were carried out later in the experimental time frame. For doxorubicin and fluorouracil a moderately high positive correlation was observed between EXN on an experiment date and fold change between minimum and maximum GI50 (correlation - doxorubicin: 0.56, $p<2.2 \times 10^{-16}$; fluorouracil: 0.47, $p<2.2 \times 10^{-16}$). This was not the case for the remaining 16 compounds (Table 30), most likely due to lower EXNs and lower number of experimental dates.

Table 4.5 – Same date experiment number for compounds

	Experiments on Same Date				
Compound	Minimum	Maximum	Mean	Number of CCCs	Number Dates
Methotrexate	2	6	3.22	1814	40
6-Mercaptopurine(2)	2	5	3.3	1821	39
6-Mercaptopurine(1)	2	5	3.29	1738	37
Actinomycin D	2	5	3.27	1875	40
Chlorambucil	2	5	3.34	1798	38
Thiotepa	2	5	3.17	1996	45
Trethylenemelamine	2	5	3.31	1790	38
Fluorouracil	2	32	10.69	9525	163
Mitomycin	2	5	3.29	1822	39
Floxuridine	2	5	3.33	1700	34
Vinblastine	2	6	3.38	1639	34
Cytarabine	2	5	3.26	1711	37
Daunorubicin	2	5	3.32	1757	37
Cisplatin	2	5	3.16	2036	44
Doxorubicin	2	35	8.11	15998	276
Bleomycin	2	5	3.24	1866	40
MAMSA	2	7	3.41	1791	38
Carmustine	2	5	3.26	1821	38

For eight (check) of the 18 compounds an increased fold change was observed across most or all CCLs at a particular set of dates in the experimental time frame. For dates ranging from August 1993 to December 1993, fold changes were higher than for earlier and later dates (Figure 4.14). In figure 4.14 it can clearly be seen that in October 1993 GIΔ increases across all cell lines for both floxuridine (Figure 4.14A) and triethylenemelamine (Figure 4.14B). The large changes in GI50 as highlighted in figure 4.15 are undoubtedly responsible for the large GIΔ, but the change in GI50 is in opposite directions for floxuridine (4.15A) and triethylenemelamine (4.15B).

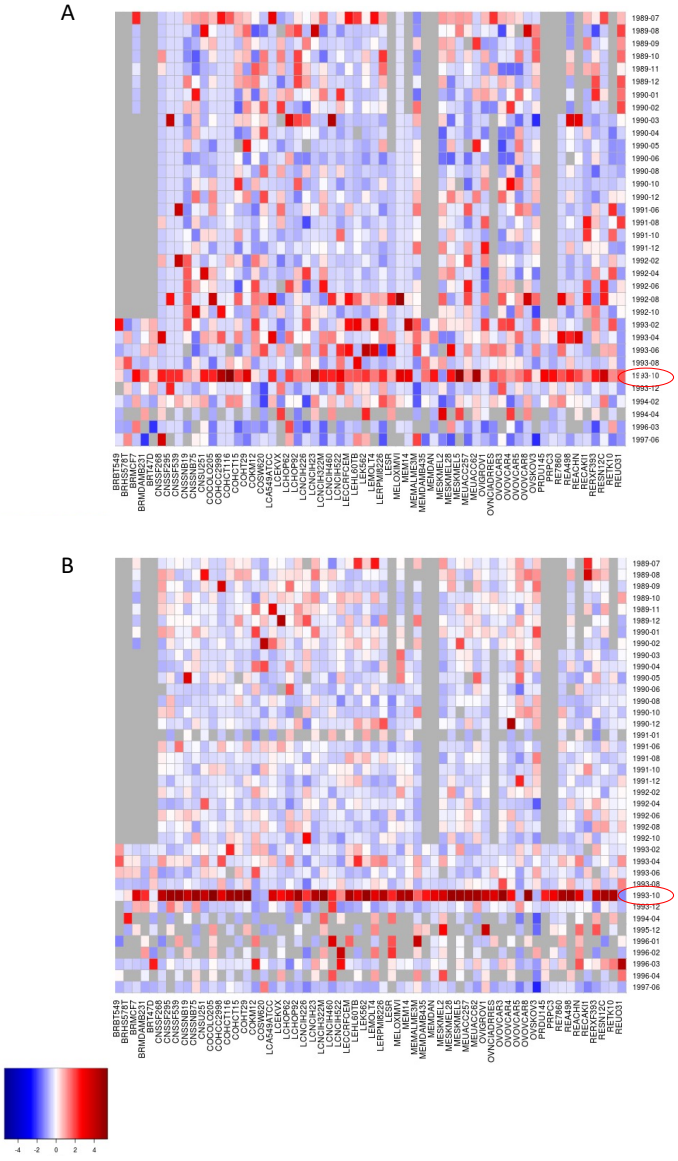

Figure 4.14 GIΔ between individual experiments at date of experiments. For A. floxuridine and B. triethylenemelamine. October 1993 highlighted. Data is scaled by column and so low values are represented by a negative score and high values represented by a positive score

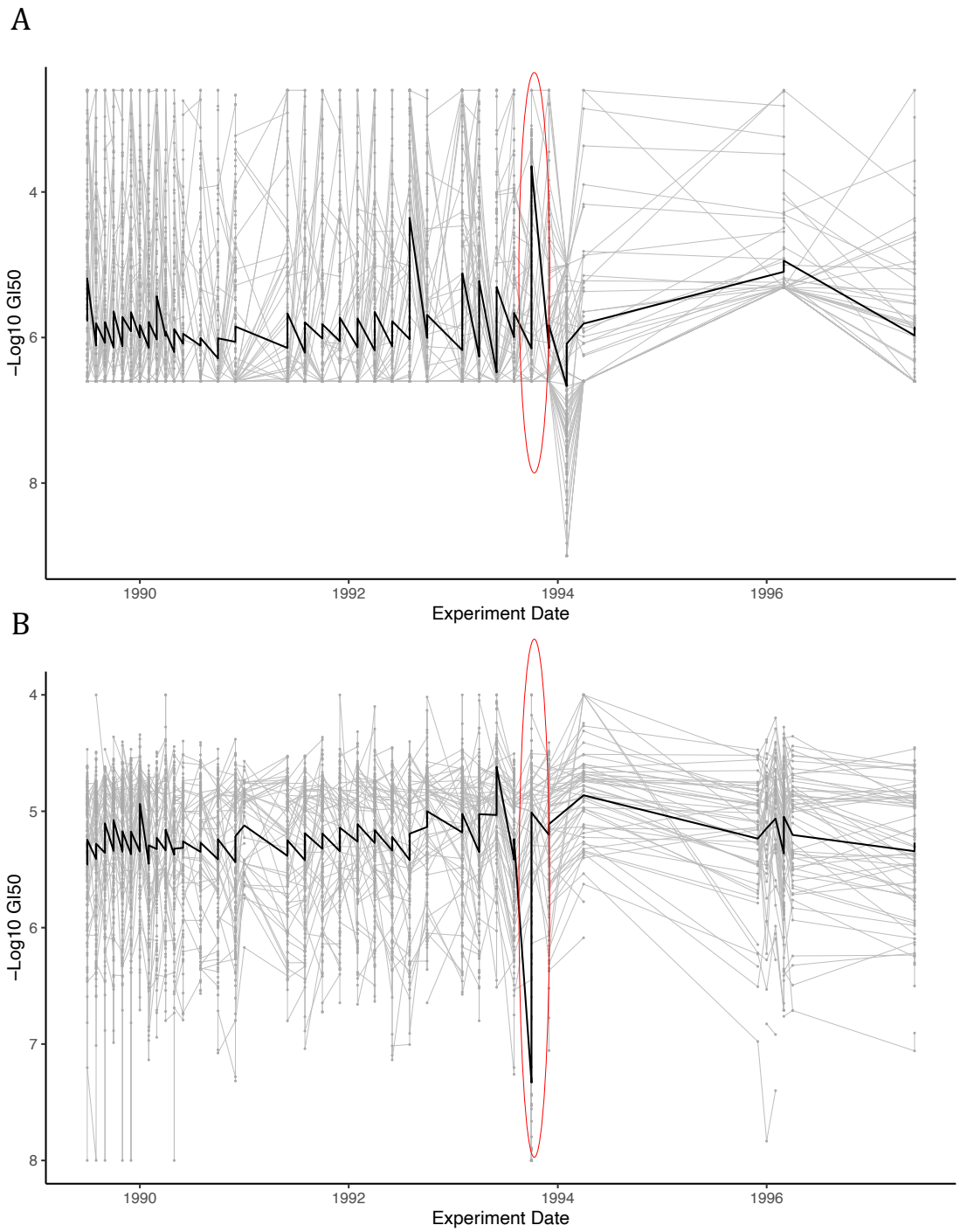

Figure 4.15 Experimental timeline for NCI-60 cell lines. Grey points represent GI50 for individual cell lines and the grey lines the change between GI50 over consecutive experiments in the timeline. The black line is the mean GI50 plotted line and represents the trend of GI50 change over the experimental timeline for A. floxuridine and B.Triethylenemelamine. Changes in GI50 at October 1993 are highlighted.

4.5 Discussion

4.5.1 GI50 variability is high in the NCI-60 drug sensitivity data

The most striking result from this analysis is the variability of GI50 for a compound. The highest GI50 observed was 3.16 x 10^{10} for cyanomorpholinodoxorubicin on colon CCL COLO 205. This is a huge amount of variation for one CCL. It is also not the only example of a high GIΔ, a GIΔ of over 2500 observed for 3064 individual CCC in the whole dataset, and a GIΔ of over 1.00 x 10^6 for 387 CCC. To put these figures in to context, generation of resistant CCLs *in vitro* requires an increase in IC50 compared to parental CCL of at least 2 fold for a resistant CCL to be established (McDermott *et al.*, 2014). Barr *et al.* (2013) described four non-small cell lung cancer (NSCLC) CCLs as cisplatin resistant after continuous exposure to cisplatin generated IC50 fold changes of between 4 and 15 compared to parental CCL. Even a fold increase in IC50 of 2500 described in actinomycin D resistant cell lines compared to parental cell lines (Biedler and Riehm, 1970) is dwarfed by the GIΔs observed in this analysis. By comparing these GIΔ to fold changes between parental and drug adapted CCLs, it is not being suggested that the CCLs are becoming resistant and sensitive as the GI50 changes, but this illustrates just how high a GIΔ this is.

These GIΔ are extraordinarily high, but it could be argued that they could be the result of calculating the fold change between two extreme outliers either direction from the median. But there are examples of multiple high GIΔ for individual cell

lines in the data. GIΔs were calculated for consecutive experiments (from now on these GIΔ will be GIΔC) in a compounds experimental timeline (experiments in order of date performed). Mcdermott *et al.* (2014) defined a CCL to be clinically relevant resistant if 2 to 5 fold different from parental cell. Although not investigating acquired resistance, these numbers were adopted in this investigation. A conservative (2) and stricter (5) threshold for what is determined to be a large GIΔ. For vinblastine over 75% of GIΔCs were over ±2 in 15 CCLs and over ±5 in 6 of those CCLs. 63.3% of all of the GIΔCs were over ±2 and 45.52% were over ±5. This is a large amount of variability between many experiments. Even for a compound with less variability in GI50, triethylenemelamine, 20% of GIΔCs were at least ±2 and 2.5% at least ±5. Large fold changes are seen between multiple individual experiments for CCLs, not just between the lowest and highest of a number of experiments. Cluster analysis suggested that individual drugs or CCLs were not prone to higher levels of variability between consecutive experiments. Drugs with the same mechanism of action did not display similar levels of variability between consecutive experiments and did not cluster together.

The GIΔ increased with both the number of experiments and the dose concentration range covered. This would perhaps be expected as the multiple testing gives rise to a higher probability of outliers, and the similarly with the dose concentration range, the higher the range the higher the probability that two GI50s will be further apart. Although FDA-approved drugs might have been expected to result in more robust data, this was not the case and they displayed a similar data variability as that determined across all compounds. In comparison,

the mean and median GIΔ was actually higher for FDA approved compounds when compared with all compounds, but this may have been because more experiments have been performed with the FDA drugs over more dose concentration ranges.

Reproducibility and replication are two words that have possibly never meant so much to the research community than now (Wass *et al.* 2019), and these results are certainly not conducive to either. The NCI-60 provides data that, if used correctly, grants insight into many aspects of cancer biology and drug development, in chapter 2 it was illustrated how the data could be used to successfully investigate and compare the mechanism of action in multiple compounds. When the average GI50 is used it is a great tool but the high GIΔ for CCLs illustrated throughout this chapter highlight the difficulty of working with CCLs. The agreement between two pharmacogenomics screens on CCL panels has been questioned in recent years. Haibe-Kaines *et al.* (2013) described poor correlation of drug response between the Cancer Cell Line Encyclopedia (CCLE) and the Genomics of Drug Sensitivity in Cancer (GDSC) pharmacogenomics studies. They suggested that differences in pharmacological assays and experimental approach may be the because of this disagreement in data. Haverty *et al.* (2016) also concluded that different experimental methodology could be the cause of discrepancies between the two studies and put forward the thought that perhaps there should be standardisation of experimental procedures in these large-scale cell line studies. This may indeed explain the difference in compound sensitivity between two studies with different lab protocols, but the NCI-60 uses the same protocol these huge GIΔ still occur. So can these large changes in GI50 be explained?

4.5.2 Genetic changes in cancer cell lines over time

The NCI-60 CCL panel has been operational for 30 years. It is entirely possible that the CCLs used in screening now have a large passage number. It is well documented that passage number can affect a CCLs characteristics over time (Esquenet *et al.*, 1997; Wenger *et al.*, 2004). Ben-David *et al.* (2018) reported that they found that strains of the same CCL from different sources were highly genetically heterogeneous, and that if two were compared, approximately 20% of mutations would be observed in just one. So the CCLs being screened now may have a different genetic profile to the same CCLs that were screened 30 years ago. It is possible that the sensitivity of CCLs to compounds may continuously change as the CCL undergoes division over the years. The analysis carried out here using an experimental timeline for the GI50 data of compounds that the highest number of screens have been carried out with is useful here to determine whether the possibility that CCLs have sensitized or become resistant to compounds can be entertained.

Cisplatin and actinomycin D are two compounds that the experimental timeline analysis was performed on. Figure 4.11 shows the timeline for each compound and the activity pattern of CCL GI50. For 58/60 CCLs actinomycin D GI50 is lower in the latest experiment compared to the GI50 at the beginning of the timeline. GIΔ for these CCLs range from 1.1 to 53. For 59/60 CCLs cisplatin GI50 is higher in the latest experiment compared to the GI50 at the beginning of the timeline. GIΔ ranges from 1.1 to 84 for these CCLs. Does this mass rise or fall in GI50 over this

time period suggest increased sensitivity towards actinomycin D or decreased sensitivity towards cisplatin for the NCI-60 CCLs? Looking at the two experimental timeline plots, it would seem as though this is not likely. GI50 is visibly variable on these plots, to a high degree. If the GI50 was gradually decreasing or increasing over the timeline, perhaps a case could be made for changes in CCL sensitivity over time. In both of these timelines a sharp up- or downtick is seen over the last few experimental dates, and for most of the cell lines which is strange in itself and may suggest that the actual screening procedure may have something to do with the large GIΔ.

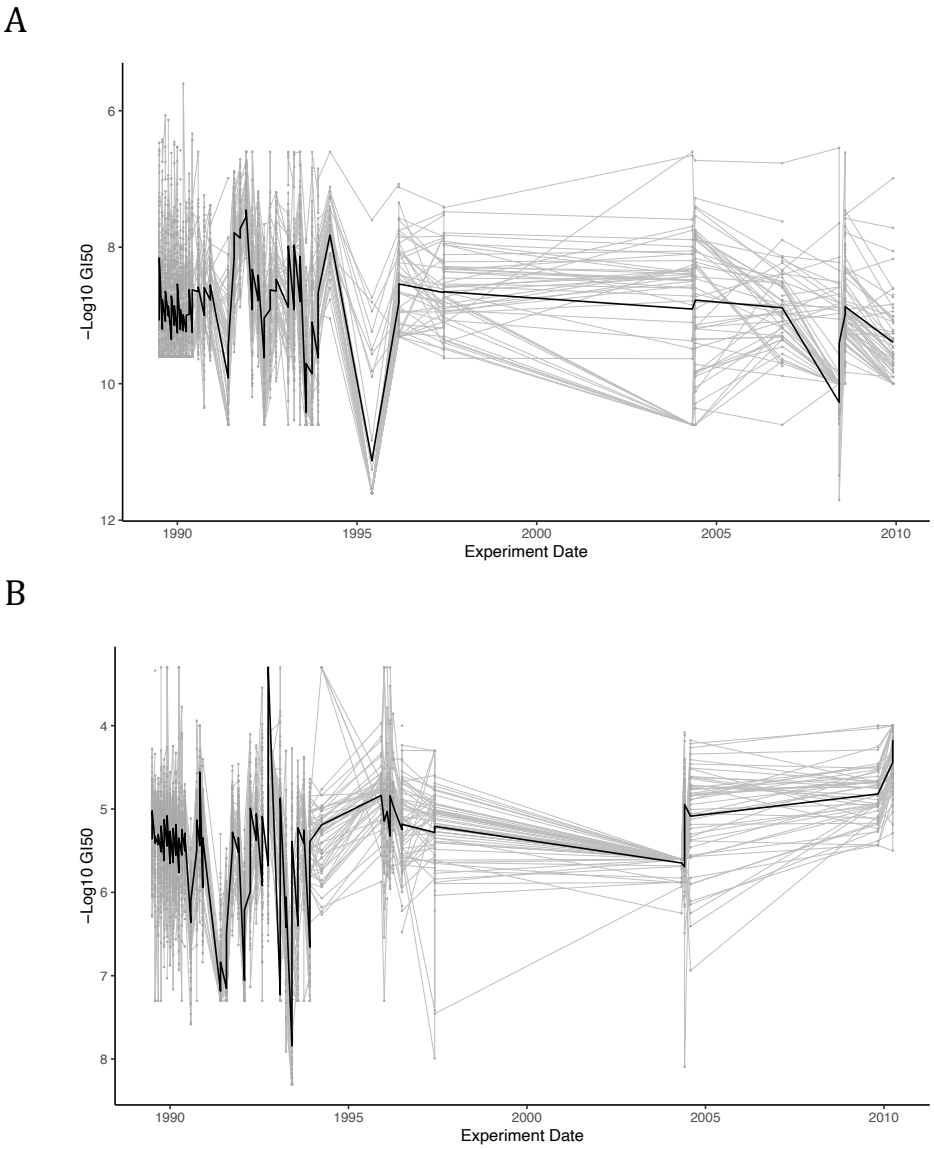

Figure 4.16 Experimental timeline for NCI-60 cell lines. Grey points represent GI50 for individual cell lines and the grey lines the change between GI50 over consecutive experiments in the timeline. The black line is the mean GI50 plotted line and represents the trend of GI50 change over the experimental timeline for A. actinomycin D and B. cisplatin.

4.5.3 Researcher or equipment error and August to December 1993

Lack of proper training or skills to carry out experimental protocols has been suggested as one of the possible contributors to the reproducibility crisis (Garijo *et al.*, 2013). Even though the actual screening of CCLs is high-throughput and performed by machines, there will always be a human element to the process. It is entirely possible that researcher or lab technician error causes the high GIΔs between experiments. Multiple experiments were carried out on the same CCC on the same experimental day at various times in the experimental timeline of these compounds, particularly early on in the timeline. This meant that for dates with multiple experiments, the GIΔ could be calculated between the highest and lowest GI50 for an individual CCL. Generally the number of experiments was low as was GIΔ, but a strange pattern emerged when viewing data in heatmaps. In months towards the end of 1993, large GIΔ suddenly occurred across all cell lines. Figure 4.9B shows this phenomenon for triethylenemelamine. For August 1993 GIΔ between experiments for a CCL ranged from 1.02 to 19.14 (median = 2.2, mean =3.2), then for October GIΔ suddenly increased and ranged from 1.1 to 2269.9 (median = 471.3, mean =630.9), and then in December GIΔ decreased again ranging from 1.07 to 260.6 (median = 1.6, mean = 7.7). If this was a lone case I think it could be ignored, but this happened for 5 of 18 compounds, and the same happened for 3 compounds but for a different month at the end of 1993. This is unlikely to be coincidental outliers for all compounds, so must be error in the procedure whether it be machine or technician, which then brings other GI50 measurements into question.

4.5.4 Variability of compound activity and compound stability

The experimental timeline analysis highlights how GI50 variability may be caused by compound activity. Vinblastine activity was amongst the most varied between CCLs, high maximum fold changes, relatively high fold changes between first and last experiments on the timeline, and produced many high GIΔs between experiments in all CCLs. Chlorambucil on the other hand produced relatively low maximum fold changes, relatively low fold changes between first and last experiment and produced relatively few high GIΔs between experiments for all CCL. It is difficult to determine the stability of the compounds with the data in this analysis, but if the compounds were to be ranked for stability, chlorambucil would be near the top and vinblastine would be near the bottom of those rankings. It is fair to question the effect that compound stability has on GI50 and it is known that compound stability, and therefore efficacy, can be affected by storage, light and how old they are (Bosanquet, 1986; Chan *et al.*, 2017) so perhaps further investigation into compound stability could lend insight into this high variability in GI50.

4.6 Conclusions

Reproducibility and replicability are easy to measure. If a result is repeated using the same method we have both. Variability in data is often easy to identify as well. However, measuring the level of variability for comparison is not so easy. For example it is not easy to determine whether individual CCLs, compounds or CCCs exhibit more or less variation in GI50 measurement than another. The GIΔ,

although high in many cases, cannot be used as a sole indicator of variability as this may be caused by a few outlier readings. The number of "high" fold changes between consecutive experiments cannot be used alone to determine variability. Firstly what is considered a "high" fold change? This is largely subjective. And using thresholds for "high" does not describe to what extent the fold change between experiments is actually high. For some CCCs the fold change may be very close to the fold change threshold, while for others it could be much higher. Looking at the difference between GI50 at the start of a CCC timeline and at the end is meaningful, but again large fold changes here could be due to outliers and it doesn't describe the level of variability observed between the two data points. And the number of outliers present for a CCC, although a possible indicator of GI50 variability, cannot be used alone. A CCC may have many outliers but only because the rest of the data is very similar to each other without much variation. Conversely, a CCC with no outliers may have none because the data is so variable, GI50 readings occur at so many different levels that none are considered outliers. To attempt to make some comparison I took all factors into account but the main observation is that high levels of GI50 variability is present in the GI50 drug screen, across many compounds and all cell lines.

Since the NCI60 screen was set up to account for and avoid known sources of data variability (Alley *et al.*, 1988; Shoemaker *et al.*, 1988), a significant part of the observed data variation is likely due to the inherent complexity of biological systems. It is not entirely clear what these findings mean and to which extent the further understanding of the parameters influencing experimental results in biological systems can be used in the future to increase data replicability.

Experiment heterogenisation, the testing of a hypothesis in many different (experimental) systems and datasets and different laboratories (Karp *et al.*, 2014; Ding *et al.*, 2017; Kafkafi *et al.*, 2017), has been suggested to provide more robust and meaningful data. This interpretation is also in line with the NCI60 strategy that emphasises that follow-up testing, including animal testing, is needed to identify drug leads (Shoemaker, 2006). However, further research will need to show whether and, if yes, to which extent such strategies will actually improve data replicability.

Chapter 5

Discussion

5.1 Utilisation of Pharmacogenomic Screens

The NCI-60, since its concept over 30 years ago, and its importance in the field of cancer research cannot be understated, perhaps not in terms of revolutionizing the world of cancer drug discovery in terms of new drugs discovered, but definitely in terms of large changes in the philosophy and practice of cancer drug research. That is not to say that it hasn't had success with drug development (Holbeck *et al.*, 2004; Bai *et al.*, 1991; Beck *et al.*, 2015), but its greatest contribution has been to bring forward the concept of representing the diversity of cancers in one panel in a cell line screen. This once novel idea has spawned further large-scale pharmacogenomics cell line screens the Cancer Cell Line Encyclopedia (CCLE) and the Genomics of Drug Sensitivity in Cancer (GDSC) which have much larger cell line panels that increase the ability to identify genetic alterations in a large number of tissues of origin and disease subtypes. Analysis carried out with drug sensitivity and genomic data from these cell line panels in earlier chapters has illustrated the potential that they possess.

It has never been easier to access the data provided, with multiple locations such as CellminerCDB (Augustin *et al.*, 2021) where data from multiple screens is available. Many computational methods have been used to exploit this data, and I have demonstrated some ways in which this is possible which I will now discuss further.

5.2 Translation to the Clinical Setting

Pharmacogenomic analysis performed with cancer cell lines is often preclinical with the aim to translate findings to the clinical setting to treat patients (Voskoglou-Nomikos, Pater and Seymour, 2003; Mirabelli, Coppola and Salvatore, 2019; Michaelis, Wass and Cinatl, 2019). With this in mind I aimed to investigate the possibility of using a different method to determine cancer cell line sensitivity that would be more relevant to the clinical setting. This was made possible using the GI50 values for three platinum drugs over the NCI-60 panel of cancer cell lines. I set threshold of sensitivity for the three platinum drugs cisplatin, carboplatin and oxaliplatin using the Cmax which is the therapeutically achievable dose in a patient (the dose before potentially lethal side effects)(Endrenyi *et al.*, 1991).

The sensitivity profiles for the three drugs using the NCI-60 mean graph data displayed many similarities between cisplatin and carboplatin, but both different to oxaliplatin. This would be expected as the mechanism of action (MOA) is the same for cisplatin and carboplatin, but slightly different for oxaliplatin (Comella *et al.*, 2009). Oxaliplatin is often used as an alternate therapy for cancers that possess intrinsic resistance or acquired resistance to cisplatin or carboplatin or

cross resistance due to adapted resistance to one or the other (Comella *et al.*, 2009). However, using the therapeutically achievable threshold the sensitivity profile was much different. Cisplatin and carboplatin no longer displayed similar profiles and the oxaliplatin profile was more similar to both. This is an important observation. While drug sensitivity analyses performed on large pharmacokinetic screens are of use as indicators of sensitivity in individual cancer types, more factors must be considered. The results here provide complementary information to the direct correlation of drug sensitivity profiles.

A consideration regarding the determination of therapeutically achievable threshold of resistance would be whether or not the Cmax is suitable for this. Firstly the Cmax varies greatly between patients (Marchant, 1981). So if Cmax is used as a threshold for sensitivity, perhaps the lowest Cmax found should be used, or the mean from a number of patients. The Cmax for the drugs in my analysis were found from the same source (Liston and Davis, 2017) so perhaps utilisation of other sources and the literature would allow more accurate thresholds to be determined. Secondly, the therapeutically achievable dose for a patient is often calculated differently for different drugs. For example, for some drugs the area under the curve (AUC) is used to determine the dose for a patient. This is the case for one of the drugs in this analysis, carboplatin (van Warmerdam *et al.*, 1995). This must be considered and in further work a therapeutically achievable dose should be calculated using the correct pharmacokinetic parameter where possible. Using pharmacokinetic parameters to determine cell line sensitivity has shown that there is a difference in sensitivity profiles in three platinum drugs compared to when determining drug sensitivity for cell lines with regards to each

other. This probably reflects that the clinical activity of a drug is not only determined by its mechanism of action but also by its pharmacokinetics (Wagstaff *et al.*, 1989; Levi *et al.*, 2000; O'Dwyer *et al.*, 2000; Burger *et al.*, 2011). This additional role of pharmacokinetics needs to be taken into account during the translation of preclinical findings.

5.3 Complementary Computational Analysis

A problem with cancer treatment today is that many patients that initially respond well to therapies, will ultimately stop responding to treatment, that is they develop acquired resistance to treatment. Biomarkers for acquired resistance are required to predict treatment failure and alternative therapies (Bao *et al.*, 2020), but this is difficult. Biomarkers are easier to identify for intrinsic resistance as initial response to treatment is easy to measure – it either works or it doesn't. But the heterogeneity of cancer means that it is likely that the processes resulting in acquired resistance are just as complex as the cancer itself. Preclinical model systems are required to determine biomarkers of acquired resistance, models that enable the repeated adaptation of a cancer cell population to the same treatment, something that can't be done with clinical material as patients can only be treated once. In this analysis I investigated the use of drug adapted cell lines as a preclinical model system, by complementing work carried out in the wet lab with a similar analysis using IC50 data from the CTRP and GDSC.

In a panel of 10 neuroblastoma cell lines adapted to the drug YM-155, wet lab collaborators identified a number of resistance mechanisms present that were not

in the parental cell line. The levels of two genes were of particular interest, multidrug resistance protein ABCB1 and solute carrier SLC35F2. ABCB1 levels were increased and SLC35F2 levels were decreased in the YM155 adapted cell lines. This was the case for all cell lines, so levels in these genes could be used to predict acquired resistance to YM-155 therapy if they were indeed responsible for the resistance to the drug. This was an example where computational analysis can be used to complement the work performed in the wet lab. I was able to use CTRP and GDSC data to perform confirmatory analyses.

Positive correlation between ABCB1 and YM-155 sensitivity, and negative correlation between SLC35F2 and YM-155 was observed in both CTRP and GDSC data. Furthermore, they both were the highest correlated genes in both datasets. This indicated that there was a relationship between the ABCB1 and SLC35F2 levels that we observed in the YM-155 adapted cell lines and YM-155 sensitivity. Further computational analysis indicated that this trend was apparent in the YM-155 naïve cell lines, cell lines that were classified as high ABCB1 or low SLC35F2 were generally less sensitive to YM-155 in both studies. This was also true when considering only neuroblastoma cell lines. However, importantly although the association between YM155 sensitivity and ABCB1/SLC35F2 levels in the GDSC and CTRP data is clear, it is not possible to predict YM155 sensitivity in an individual cell line based on ABCB1/SLC35F2 level alone. This finding confirmed that this was not intrinsic resistance as many cell lines that were sensitive to YM-155 were often high ABCB1 or low SLC35F2.

We demonstrated that it is essential to find biomarkers of acquired resistance to treatment that may not necessarily predict therapy outcome in therapy naive cancer cells. A realistic way to do this is by using pre clinical models of acquired drug resistance, a method of which we have shown in this study. The emergence of ABCB1 and SLC35F2 as biomarker candidates for YM155 resistance in our research highlights the potential of drug adapted cancer cell line models to identify biomarkers that can be used in the design of individualized therapies. These conclusions could not have been made if it wasn't for the computational analysis highlighting the importance of not only the use of pharmacogenomic data from cancer cell line screens but also how work performed in the wet lab can be enhanced by combining with computational analysis.

5.4 Variability of Data Within a Highly Standardised World Leading Laboratory

The NCI-60 has had to deal with data reproducibility questioned for many years. It was established at a time when cell line misidentification and cross-contamination were rife, and contained misidentified cell lines in its panel, and even though this was corrected in 2005, MDA-MB-435 was still used as a breast cancer cell line (it is actually derived from melanoma) as late as 2014 (Prasad and Gopalan, 2015). This has all been discussed in detail previously. Issues with the reproducibility of cancer cell line drug sensitivity in large screens data have also been discussed (Haibe-kains et al., 2013).

The NCI-60 data set made available ~2.8 million GI50 values, a huge amount of data, which allowed me to gain real-life insights into the extent of data variability in a standardised research environment. Variability was high throughout. Vinblastine was identified as a drug with high levels of variability in drug activity and chlorambucil showed lower levels of variability, although variability was still high. One of my aims was to investigate whether variability was due to changes in sensitivity over time, but this was not the case. Variability was high throughout. Moreover, variability increased when the number of experiments or dose concentration range tested on a cell line/compound increased, and high variability was not limited to experimental compounds, it was also observed at similar levels for widely used FDA approved drugs.

The variability observed for FDA approved anti-cancer drugs is of potential relevance, given that cytotoxic anti-cancer drugs are typically used at maximum tolerated doses that cannot be further increased without unacceptable toxicity (Eisenhauer *et al.*, 2000; Sachs *et al.*, 2000; Mansino *et al.*, 2019). Moreover, the maximum effects of targeted drugs, e.g. antibodies or kinase inhibitors, that interfere with cancer-specific structures or entities, do not further increase beyond the 'optimal biological dose', i.e. the dose at which the biological target is completely inhibited (Eisenhauer *et al.*, 2000; Sachs *et al.*, 2000; Mansino *et al.*, 2019). Hence, even a two-fold difference in the GI50 (which occurred in 25,496 (84.4%) of 30,212 compound/cell line combinations with at least five experiments) is of potential relevance, as a two-fold increase of the clinical dose of an anti-cancer drug is rarely feasible.

The NCI-60 uses highly standardised experimental procedures, for example the same assay has been used for the entirety of time the cell line panel has been tested. Reasons why there is therefore such a high level of variability present were discussed in chapter 4. Genetic changes to cell lines over time, drug stability and researcher error are but a few possible causes for the observed level of variability, however it may just be that the observed data variability probably reflects at least in part the underlying complex biology. This work raises awareness of the inherent variability of experimental results, and will hopefully allow us to develop a realistic understanding of the meaning of experimental data and inform and inspire further research that will eventually improve the robustness, reliability, and meaningfulness of research data.

5.5 Variables That May Affect Cancer Cell Line Screen Reliability

Other variables that may not be as obvious could also affect drug sensitivity in the pharmacogenomic screens. I explore some of them here with particular reference to the NCI-60 data and the variability analysis in chapter 4.

Intratumour clonal heterogeneity should be considered when variability as high as in the NCI-60 panel is observed. High levels of clonal heterogeneity is present in cancers of all types (Osuka *et al.* 2017; Kim *et al.*, 2018; Piotrowska *et al.*, 2018; Saeed *et al.*, 2018). It exists when different mutations arise in individual tumour cells in a tumour. The level of intratumour heterogeneity can vary greatly. Johnson *et al* (2014) showed that in 43% glioma patients at least 50% of mutations present

in the primary tumour were absent in metastatic or recurrent tumour sites. This may occur after drug therapy, a clonal population that is resistant to the therapy (due to genomic differences to other tumour cells that are sensitive to treatment) becomes a seed from which a new resistant tumour can grow (Kreso *et al.*, 2013). The drug treatment kills the sensitive tumour cell population but the resistant cells remain.

While the NCI-60 took many precautions while putting together their CCL panel (Alley *et al.*, 1988; Shoemaker *et al.*, 1988), it is likely that there was and still is a significant level of intratumoural heterogeneity present in the NCI-60 CCL collection. This may explain some of the GI50 variability observed in this analysis. Cell populations may exist in the CCLs that either have intrinsic resistance to treatments. If a large number of these cells are (unknowingly) picked to grow new populations for testing, a sample that exhibits a high level of resistance is created (Kreso *et al.*, 2013; Davis *et al.*, 2019). This may be what is happening at the NCI-60. At each experiment across the decades that tests have been performed, a CCL sample has been used that does not consist the same proportion of clonal populations that are present in other samples. Different numbers of cells that are resistant to the drug being tested. This would affect the GI50 reading and may be a cause of the variability we see between experiments.

Drug solubility may be an important factor in drug sensitivity measurement variability. Poor water solubility is an inherent property of many anti-cancer drugs. Hydrophobicity and lipophilicity are required for anti-cancer drugs to cross

cell membranes (Lipinski, 2000) and a low polar surface area increases permeability of the drug, but as the polar surface area decreases so too does the water solubility (Veber *et al.*, 2002). Balance between the two is difficult. If an anti-cancer drug possesses poor water solubility then this inhibits the anti-cancer action of the drug (Hoelder *et al.*, 2012; Tran *et al.*, 2019; Di *et al.*, 2012). Not all compounds in the NCI-60 screen are anti-cancer drugs, many are experimental and so water solubility may be unknown. The assay used in the NCI-60 screen is standardised but if storage of the drug changed or small errors were made when preparing the drug and assay, this could lead to variability in drug sensitivity measurement. Water solubility of drugs could be important when comparing the sensitivity profiles of drugs that have the same mechanism of action. The water solubility of cisplatin and carboplatin is different. Cisplatin is less soluble in water than carboplatin (Hamelers *et al.*, 2006) so this should be considered when testing and comparing.

Cell lines can maintain an intact intrinsic circadian clock that impacts on the drug sensitivity of cell lines (Cederroth *et al.*, 2019; Lee *et al.*, 2021). For example an HSP90 inhibitor reduced growth rate of a mouse melanoma in a time-of-day–specific manner, but efficacy was impaired in clock-deficient tumors (Lee *et al.*, 2021). Another example involves one of the platinum drugs used in chapter 2, oxaliplatin. Oxaliplatin development was halted after excessive toxicities in a phase I clinical trial. Later though it was found that if the drug was delivered at certain times in the day, the toxicities were avoided and eventually passed phase II (Levi *et al.*, 1992) and III (Levi *et al.*, 1997) trials using chronomodulated delivery. It is unknown whether drug screens in the pharmacogenomic cell line

studies are performed at the same time or if the circadian clock and cell cycle phase of the cell lines are considered or even known, but this could certainly be another variable that causes variability in drug sensitivity readings.

Chapter 6

Conclusions and Future Work

Pharmacogenomics screening of cancer cell line panels produces valuable drug sensitivity and genomic data. This data can be used to validate experimental work as was shown in chapter 3 when computational analysis of data from two of the panels (CTRP and GDSC) supported results observed in the wet lab. The analysis not only showed that ABCB1 and SLC35F2 levels were correlated with YM-155 sensitivity, but that levels in the cell lines in the panels could not be used to determine sensitivity to YM-155. This meant that ABCB1 and SLC35F2 were likely biomarkers of acquired resistance rather than intrinsic resistance. Data from the NCI-60 was used to develop a novel method to determine cell line sensitivity that could perhaps be of use when translating preclinical findings to the clinical setting. Using clinically relevant thresholds for sensitivity of platinum compounds, differences were seen between compounds that weren't apparent when only considering cell line sensitivity with relation to other cell lines. This additional role of pharmacokinetics, not just the mechanism of action, needs to be taken into account during the translation of preclinical findings. Finally, data variability was investigated using screening data from the NCI-60. The vast quantity of data and high levels of standardization at this world leading institute gave me the unique opportunity to test levels of variability in drug sensitivity data over three decades. Variability of GI50 values was surprisingly high, and not only in experimental compounds. Even widely used FDA approved drugs displayed

high levels of variability. Perhaps variability is an intrinsic trait of biological systems and rather than trying to replicate and reproduce work we should be attempting to satisfy the same hypotheses using different experimental methods.

Overall, I hope this work has highlighted some of the possibilities using data from pharmacogenomic cell line screens. Future work using this data and following on from some of the work here could include further investigation into some of the variables mentioned in the discussion. The clonal heterogeneity of cancer cell lines, drug solubility and cell line circadian rhythm. Another variable not mentioned is the doubling time of cell lines. Comparing the sensitivity of DNA damaging anti-cancer drugs in cell lines with different doubling times may be enlightening as assays are usually only incubated for 72 hours. Does a cell divide enough in that time for DNA damaging drugs to prevent growth in the cell line? Regarding variability of data, it would be interesting to investigate this in the other cell line panels. For example the GDSC has two datasets, GDSC1 and GDSC2, that has a large overlap of drugs. It would be interesting to see if there are differences between IC50 values for these drugs. Some of the drugs were also tested more than once at different locations (Massachusetts Hospital and Sanger Institute). It would also be interesting to compare IC50s for the cell lines in those drugs.

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Tran, P., Pyo, Y., Kim, D., Lee, S., Kim, J., and Park, J. (2019) Overview of the manufacturing methods of solid dispersion technology for improving the solubility of poorly water-soluble drugs and application to anticancer drugs **11:** 132.

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Appendix

Supplementary Data

Table S1. YM155 concentrations (nM) that reduce the viability of UKF-NB-3 or YM155-adapted UKF-NB-3 sub-lines by 50% (IC_{50}) or 90% (IC_{90}) as indicated by MTT assay after 120h of incubation and doubling times of the cells.

	IC_{50}	IC_{90}	Doubling times (h)
UKF-NB-3	0.55 ± 0.06	1.01 ± 0.24	30.8 ± 0.8
UKF-NB-3rYM155^{20nM}I	36.2 ± 2.0 (66)[1]	94.8 ± 0.7 (94)[2]	41.5 ± 5.2
UKF-NB-3rYM155^{20nM}II	23.6 ± 2.2 (43)	39.9 ± 0.9 (40)	39.2 ± 4.8
UKF-NB-3rYM155^{20nM}III	31.8 ± 2.3 (58)	49.1 ± 0.2 (49)	32.3 ± 1.1
UKF-NB-3rYM155^{20nM}IV	21.0 ± 0.6 (38)	29.8 ± 4.8 (30)	32.2 ± 2.0
UKF-NB-3rYM155^{20nM}V	25.1 ± 0.1 (45)	39.5 ± 0.7 (39)	35.3 ± 2.8
UKF-NB-3rYM155^{20nM}VI	41.9 ± 5.3 (76)	136 ± 7 (135)	36.3 ± 2.2
UKF-NB-3rYM155^{20nM}VII	36.5 ± 5.5 (66)	96.2 ± 23.9 (95)	46.1 ± 2.1
UKF-NB-3rYM155^{20nM}VIII	34.5 ± 0.6 (63)	84.7 ± 27.3 (84)	47.0 ± 2.5
UKF-NB-3rYM155^{20nM}IX	27.2 ± 0.5 (49)	44.8 ± 2.9 (44)	33.3 ± 0.2
UKF-NB-3rYM155^{20nM}X	26.9 ± 0.5 (49)	45.2 ± 4.7 (45)	41.9 ± 2.2

[1] fold resistance (IC_{50} YM155-adapted sub-line/ IC_{50} UKF-NB-3)
[2] fold resistance (IC_{90} YM155-adapted sub-line/ IC_{90} UKF-NB-3)

Table S2. Drug concentrations that reduce the viability of UKF-NB-3 or YM155-adapted UKF-NB-3 sub-lines by 50% (IC_{50}) as indicated by MTT assay after 120h of incubation.

	Nutlin-3	Vincristine	Cisplatin	Gemcitabine	Topotecan
	IC_{50} (µM)	IC_{50} (ng/mL)	IC_{50} (ng/mL)	IC_{50} (ng/mL)	IC_{50} (ng/mL)
UKF-NB-3	1.05 ± 0.25	1.75 ± 0.55	169 ± 29	0.30 ± 0.03	1.29 ± 0.52
UKF-NB-3rYM155^{20nM}I	0.57 ± 0.07 (0.5)[1]	45.5 ± 11.1 (26)	157 ± 54 (0.9)	0.64 ± 0.02 (2.1)	1.37 ± 0.53 (1.1)
UKF-NB-3rYM155^{20nM}II	1.31 ± 0.03 (1.2)	27.0 ± 12.6 (15)	183 ± 51 (1.1)	0.50 ± 0.04 (1.7)	1.25 ± 0.53 (1.0)
UKF-NB-3rYM155^{20nM}III	1.27 ± 0.01 (1.2)	10.8 ± 6.4 (6.2)	122 ± 24 (0.7)	0.62 ± 0.01 (2.1)	1.06 ± 0.24 (0.8)
UKF-NB-3rYM155^{20nM}IV	0.47 ± 0.03 (0.4)	18.5 ± 8.4 (11)	159 ± 38 (0.9)	0.23 ± 0.04 (0.8)	1.56 ± 0.65 (1.2)
UKF-NB-3rYM155^{20nM}V	0.99 ± 0.13 (0.9)	8.90 ± 7.39 (5.1)	156 ± 84 (0.9)	0.12 ± 0.04 (0.4)	0.91 ± 0.41 (0.7)
UKF-NB-3rYM155^{20nM}VI	0.64 ± 0.01 (0.6)	714 ± 456 (408)	132 ± 39 (0.8)	0.64 ± 0.01 (2.1)	1.55 ± 0.72 (1.2)
UKF-NB-3rYM155^{20nM}VII	1.27 ± 0.04 (1.2)	28.8 ± 10.2 (16)	134 ± 6 (0.8)	0.19 ± 0.01 (0.6)	1.44 ± 0.84 (1.1)
UKF-NB-3rYM155^{20nM}VIII	0.70 ± 0.01 (0.7)	39.5 ± 15.4 (23)	190 ± 56 (1.1)	0.65 ± 0.01 (2.2)	1.26 ± 0.50 (1.0)
UKF-NB-3rYM155^{20nM}IX	0.33 ± 0.01 (0.3)	5.63 ± 1.94 (3.2)	178 ± 41 (1.1)	0.18 ± 0.01 (0.6)	1.59 ± 0.74 (1.2)
UKF-NB-3rYM155^{20nM}X	0.64 ± 0.15 (0.6)	26.0 ± 6.2 (15)	144 ± 44 (0.9)	0.54 ± 0.01 (1.8)	1.21 ± 0.40 (0.9)

[1] fold resistance (IC_{50} YM155-adapted sub-line/ IC_{50} UKF-NB-3)

Figure S1

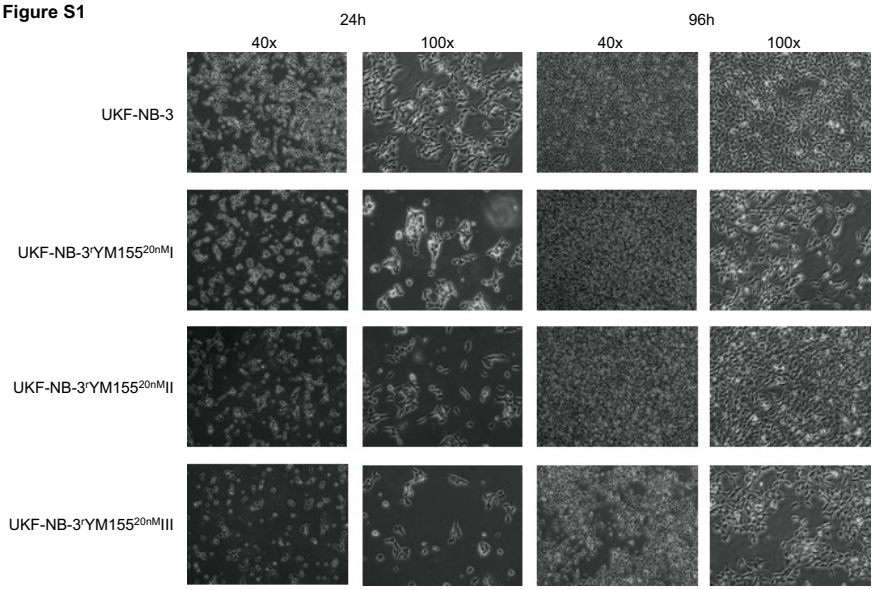


Figure S1

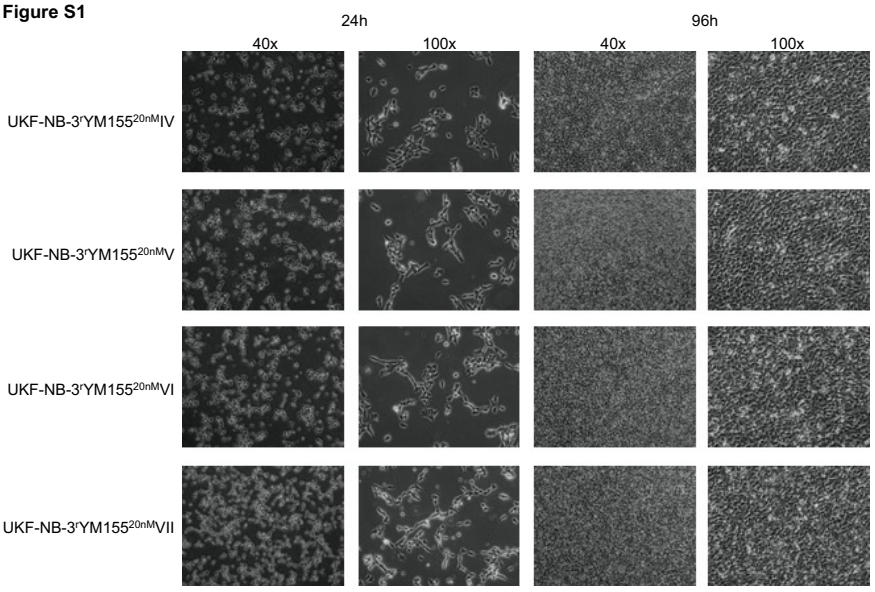


Figure S1

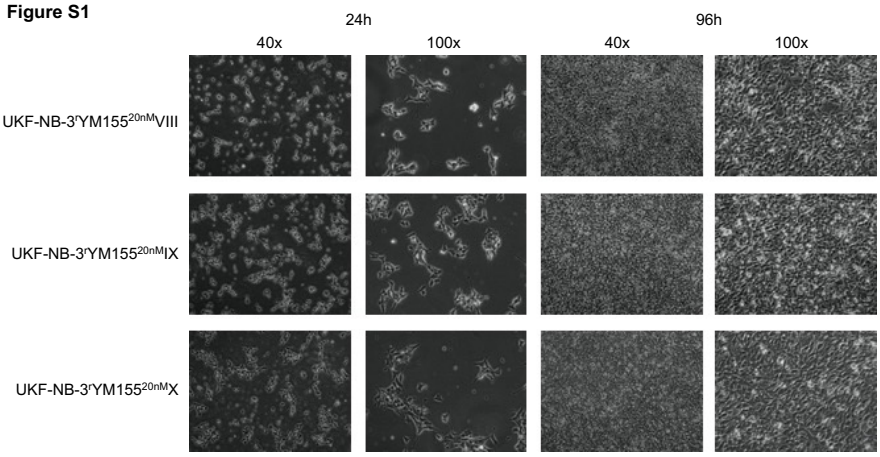


Figure S1. Representative photos of the project cell lines indicating cell morphology after different periods of cultivation and at different maginifications.

Figure S2

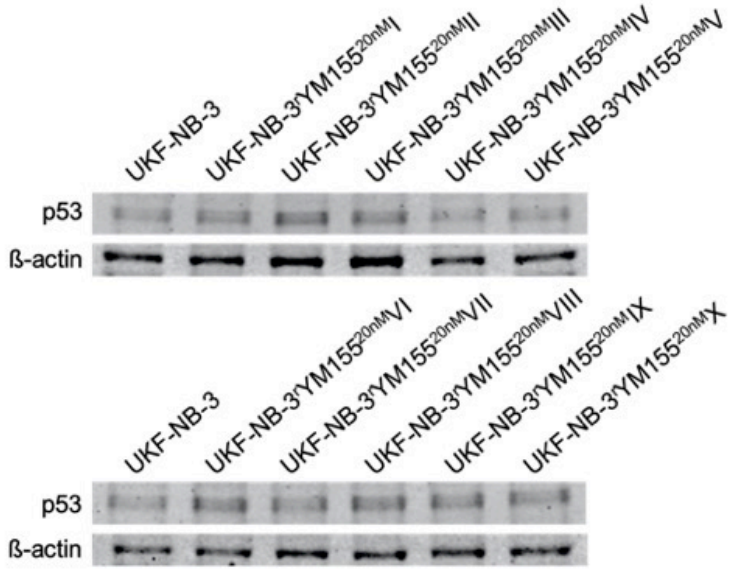


Figure S2. p53 levels in UKF-NB-3 and its YM155-adapted sublines.

Figure S2. Representative Western blots indicating cellular levels of p53 in UKF-NB-3 and YM155-adapted UKF-NB-3 sub-lines. Densitometric analysis was performed with QuantiOne (BioRad). p53 levels were normalised to β-actin expression and values relative to control cells are displayed.

Figure S2

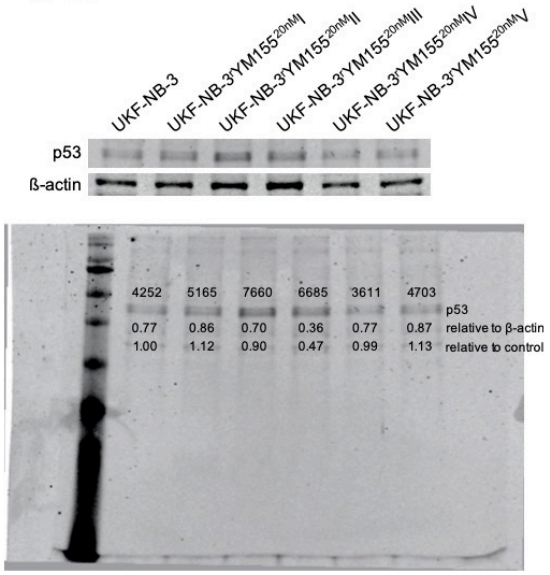
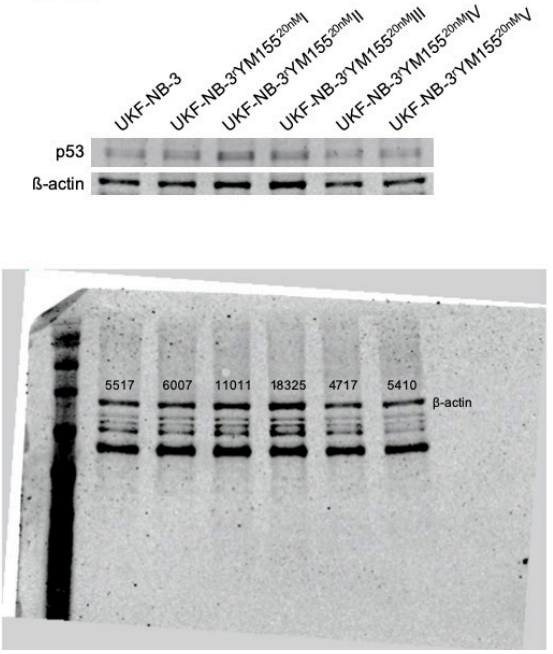


Figure S2



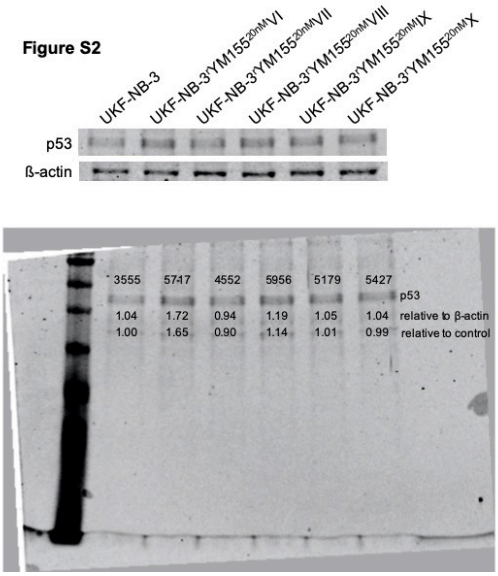


Figure S2
UKF-NB-3
UKF-NB-3ʳYM155 20nM VI
UKF-NB-3ʳYM155 20nM VII
UKF-NB-3ʳYM155 20nM VIII
UKF-NB-3ʳYM155 20nM IX
UKF-NB-3ʳYM155 20nM X
p53
ß-actin
3555 5747 4552 5956 5179 5427
p53
1.04 1.72 0.94 1.19 1.05 1.04 relative to β-actin
1.00 1.65 0.90 1.14 1.01 0.99 relative to control

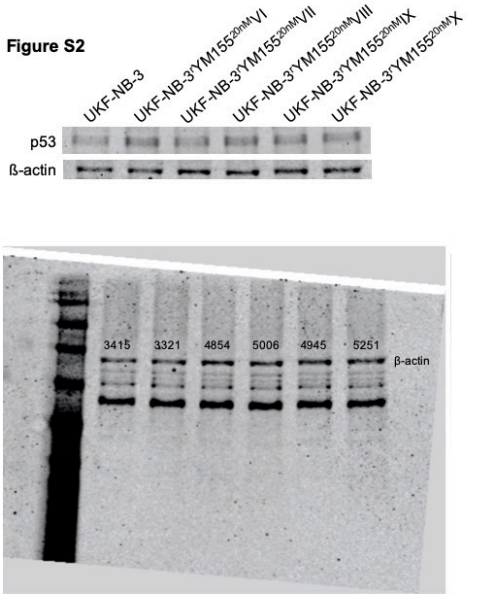


Figure S2
UKF-NB-3
UKF-NB-3ʳYM155 20nM VI
UKF-NB-3ʳYM155 20nM VII
UKF-NB-3ʳYM155 20nM VIII
UKF-NB-3ʳYM155 20nM IX
UKF-NB-3ʳYM155 20nM X
p53
ß-actin
3415 3321 4854 5006 4945 5251
β-actin

Figure S3

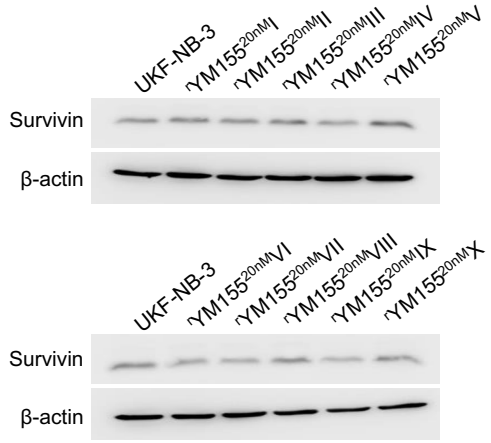


Figure S3. Representative Western blots indicating cellular levels of survivin in UKF-NB-3 and YM155-adapted UKF-NB-3 sub-lines. Densitometric analysis was performed with Image Studio Ver. 5.2 software (LICOR). Survivin levels were normalised to β-actin expression and values relative to control cells are displayed.

Figure S3

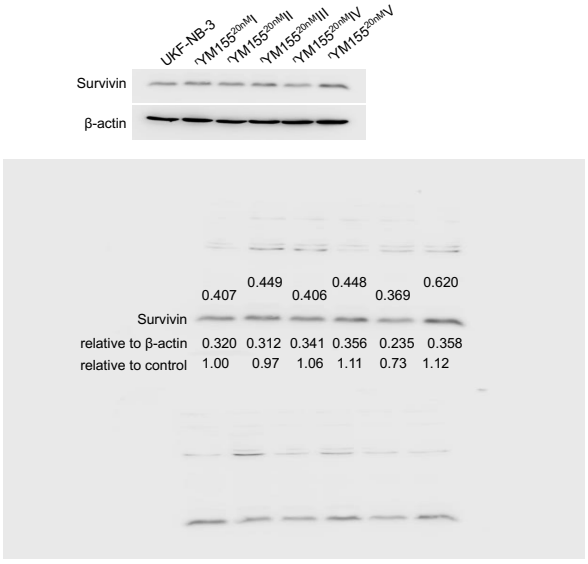


Figure S3

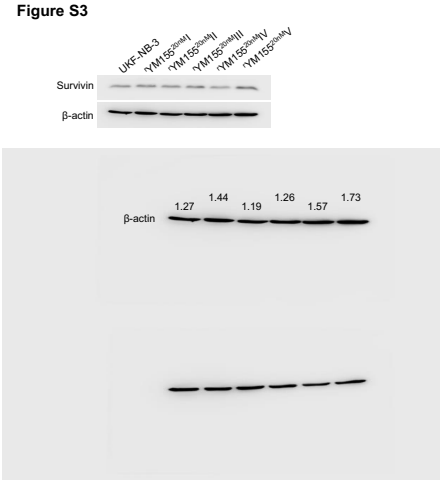


Figure S3

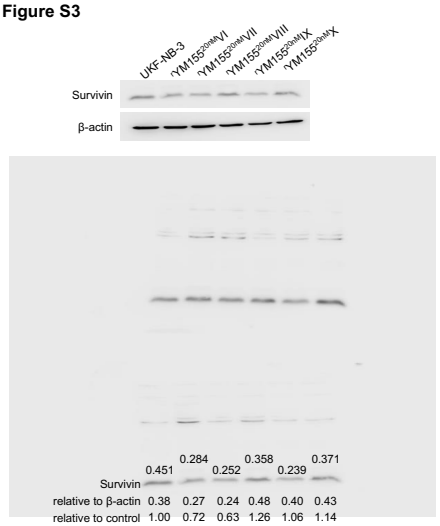


Figure S3

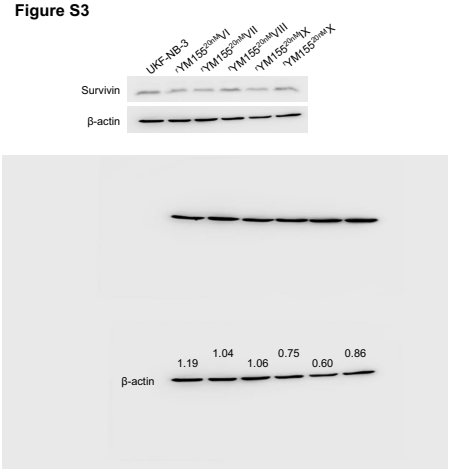


Figure S4
survivin

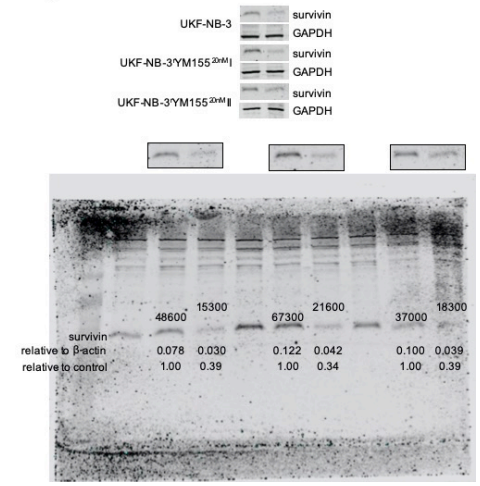


Figure S4
GAPDH

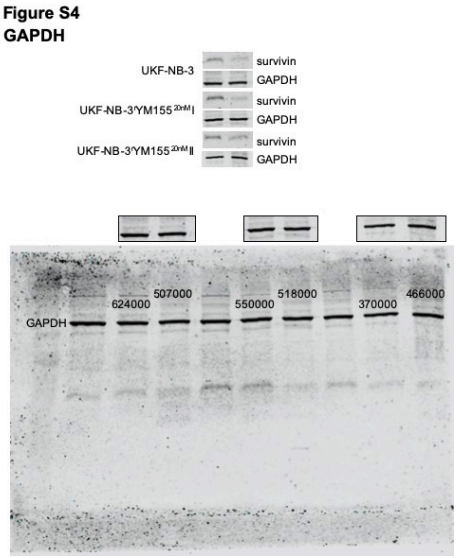


Figure S4
survivin

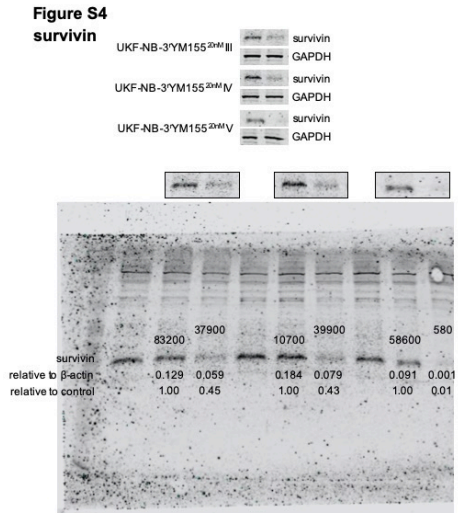
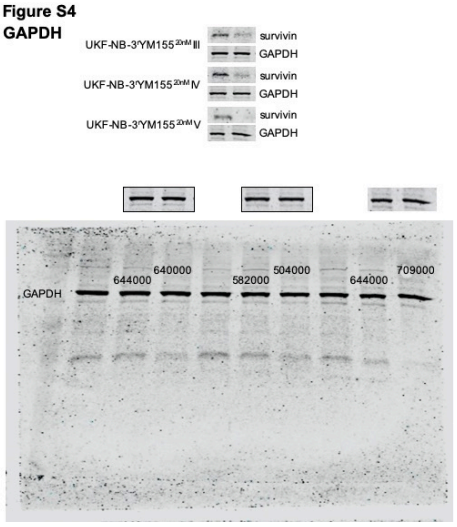
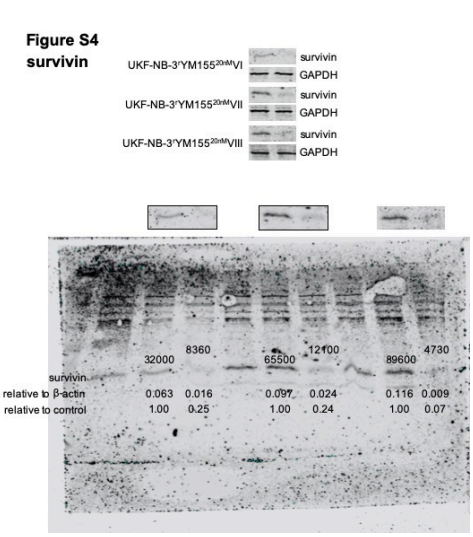
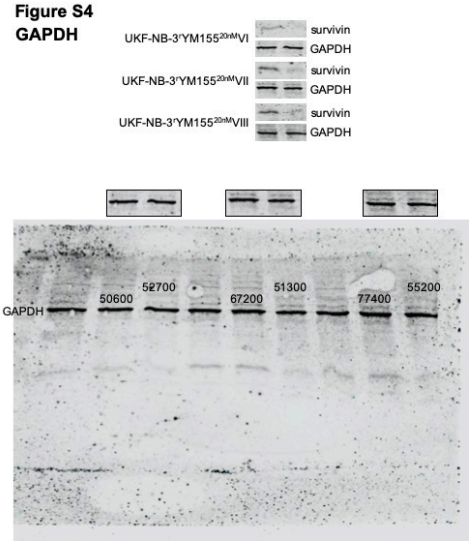
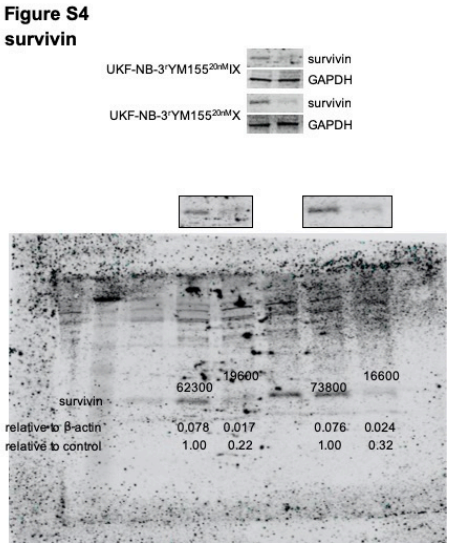


Figure S4
GAPDH









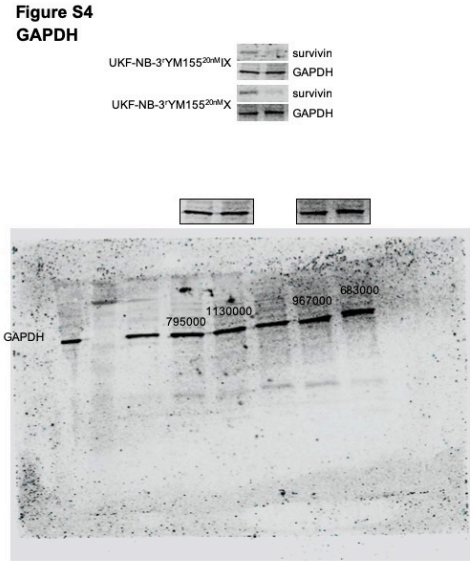


Figure S4. Representative Western blots indicating cellular levels of survivin in UKF-NB-3 and its YM155-adapted UKF-NB-3 sub-lines 24 h after transfection with non-targeting siRNA or siRNA directed against BIRC5/ survivin. Densitometric analysis was performed with QuantiOne (BioRad). Survivin levels were normalised to β-actin expression and values relative to control cells are displayed.

Figure S5
ABCB1

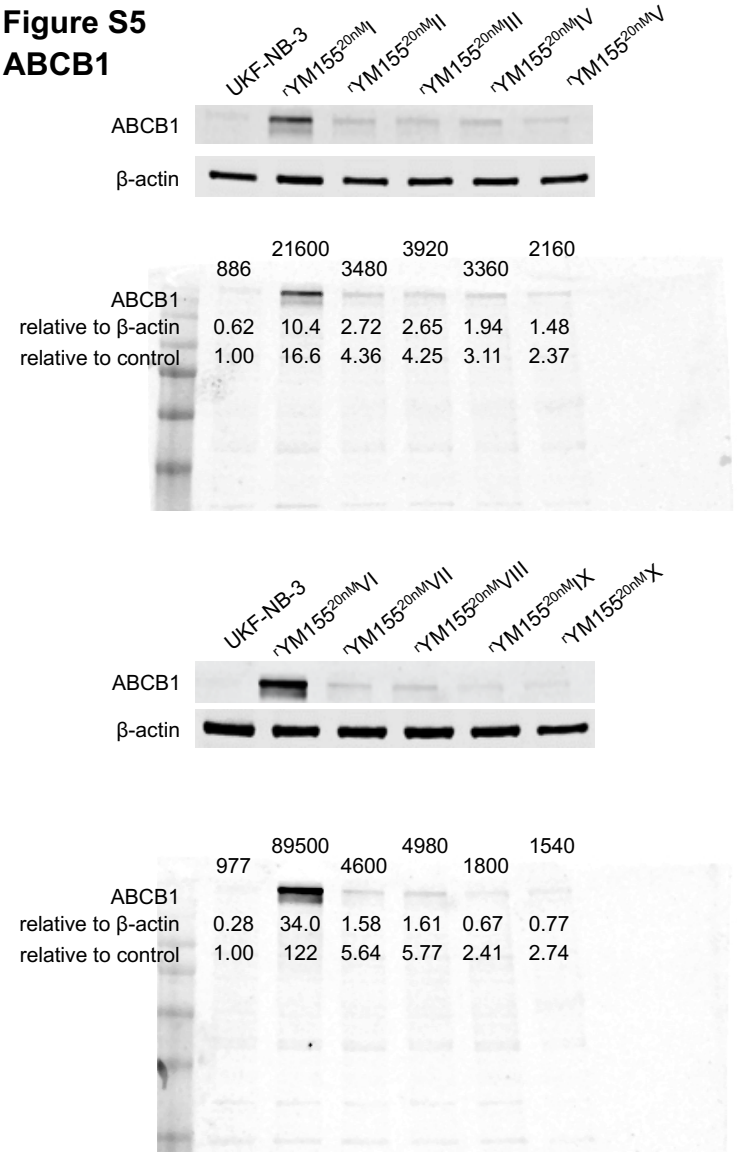


Figure S5
SLC35F2

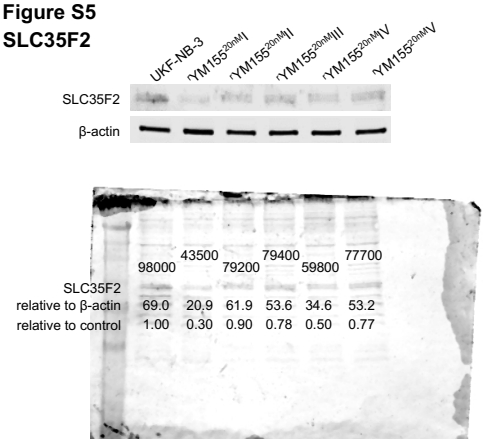


Figure S5
SLC35F2

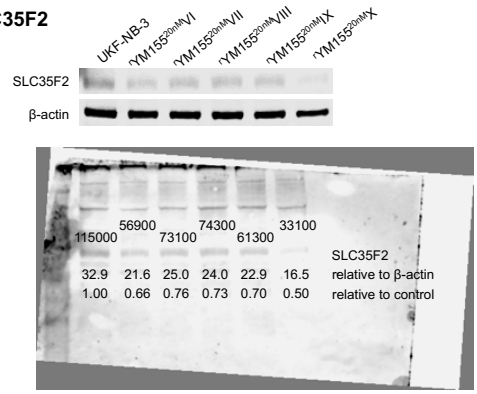


Figure S5
β-actin

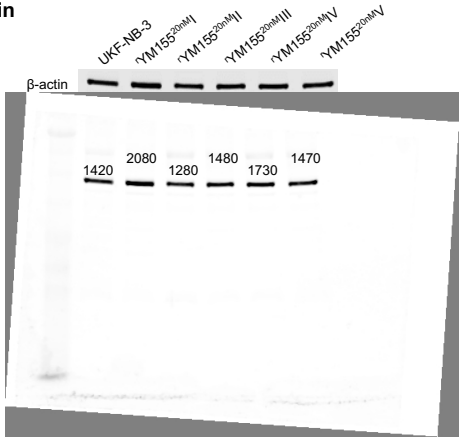


Figure S5
β-actin

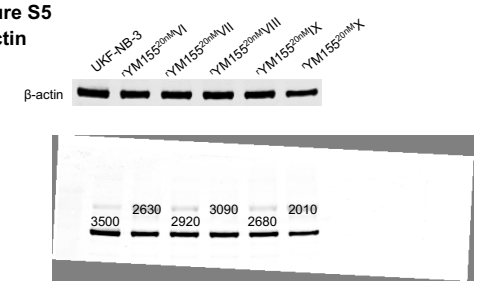


Figure S5. Representative Western blots indicating cellular levels of ABCB1 and SLC35F2 in UKF-NB-3 and YM155-adapted UKF-NB-3 sub-lines. Densitometric analysis was performed with QuantiOne (BioRad). ABCB1 and SLC35F2 levels were normalised to β-actin expression and values relative to control cells are displayed.

Figure S6

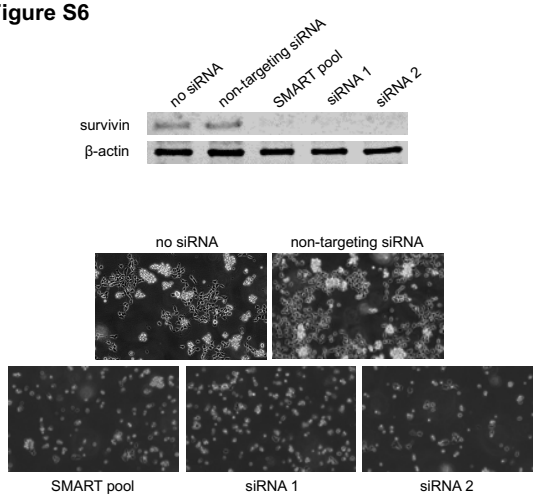


Figure S6. Western blots indicating cellular levels of survivin levels after transfection with the Dharmacon SMART pool (consisting of four siRNAs) and two individual siRNAs (siRNA 1, target sequence: GCAAAGGAAACCAACAAUA; siRNA 2, target sequence: GGAAAGGAGAUCAACAUUU) in UKF-NB-3 48h and representative images showing effects on cell viability..

Figure S6

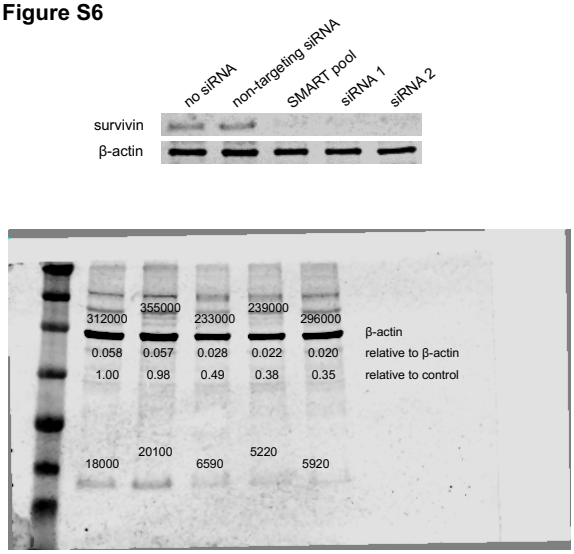


Figure S6. Original blots, densitometric analysis was performed with QuantiOne (BioRad). Survivin levels were normalised to β-actin expression and values relative to control cells are displayed.