The Effect of Doxorubicin on ABC Transporter Gene Expression in Triple Negative Breast Cancer

by

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Abstract

Breast cancer is the single foremost killer of women in the world today, and of this triple negative breast cancer (TNBC) is the most clinically aggressive kind. TNBC impacts roughly 15% of the worlds breast cancer cases and in New Zealand, having the highest rate of breast cancer incidence in the world. Since there is currently no TNBC specific treatment, the majority of people diagnosed with this subtype are often faced with treatment such as chemotherapy and radiation, this being the gold standard of treatment for these tumours. However, in many cases surgical excision of the main tumour mass in conjunction with said therapy is necessary. Surgical removal, as can be common and in fact one of the best therapeutic options for TNBC, poses a few issues. Among these is the likelihood of this subtype of cancer to metastasise. It is for this reason that TNBC tumours are characteristic of a high mortality rate. A considerably higher one than other breast cancers. Most vitally, however, a diagnosis can often be poor for TNBC patients and low survival rates are likely, due to a high incidence of chemotherapeutic resistance in TNBC tumours.

Typically, the cells within the tumours of all types develop chemoresistance through several different gene expression changes to regulate and defend themselves from cytotoxic agents. TNBC is no exception to this and by altering the expression of genes responsible for transport proteins (which are embedded in the cell membrane the cells) can increase the efflux of chemicals out of the cell. Functionally this serves to remove the toxic substrate before it has chance to affect the cells. The possibility that ATP-binding cassette (ABC) transporters could be targeted by drugs to make chemotherapy more efficacious in resistant cells could change the mortality rate for cancer patients. This is particularly useful whilst more direct therapeutic options not reliant on non-specific cell death and with fewer dangerous side-effects are researched and trialled.

To explore this, expression analysis of a host of genes related to ABC transporter proteins was conducted. The outcome of which was previously observed, in part, in broader spectrum studies to show some varied gene regulation in response to treatment over time with chemotherapy. The

experiment required TNBC cells to be induced into a state of resistance which from previous research had been shown to require at least 3 months of treatment of the cells at a dosage in accordance with the IC_{50} of the drug for the specific cell line in use. The cells used were MDA-MB-231 cells and the corresponding drug, doxorubicin. The cells, after three treatment cycles with the drug doxorubicin at a concentration of 1.24μM, had total RNA extracted before said RNA was converted to cDNA and expression quantities recorded using quantitative real-time polymerase chain reaction (RT-qPCR).

The data from this revealed a significant decrease in the expression of ABCG2, a xenobiotic transporter known to be responsible for removing foreign chemicals from the cells. A decrease in expression of significance was also observed in the gene ABCC2. This gene is commonly referred to as multidrug resistance protein 2 (MRP2) and as with ABCG2, it is expressed by cells to promote the clearance of drugs from the cytoplasm. Other genes showed a relative fold increase in expression when compared with reference genes, but no obvious effect was observed over treatment.

Studies in this field typically report cellular tolerance and development of multi-drug resistance (MDR) in MDA-MB-231 cells over a period of a maximum of 4 months of treatment. In this study therefore, it appears there may have been an underlying effect, likely the cause of cell death in the final stage of this experiment, that affected the development of chemoresistance. The slight increase in expression seen after the initial treatment of ABCC2 and ABCG2 could indicate that the process simply was not conducted over a long enough time period and that a significant effect may have been more likely if more allotment of time was given to this experiment.

Conclusively, there is a definite and significant effect on the expression levels of genes relating to the expression of certain elements of transport witnessed. The link between treatment and chemotherapeutic resistance development is a developing study that needs further study. Experimentation observing the effects observed in relation to the increased expression of ABCC2 and ABCG2 as well as confirmatory studies to solidify findings through use of a MTT assay should be considered in future studies.

Abbreviations

ABC: ATP-Binding Cassette

ABCC1: ATP Binding Cassette Subfamily C Member 1

ABCC2: ATP Binding Cassette Subfamily C Member 2

ACT- b: Beta-Actin

ADK: Adenosine Kinase

ASDR: Age-Standardized Death Rates

ASIR: Age-Standardized Incidence Rates

ATP: Adenosine Tri-Phosphate

ATTC: American Type Culture Collection

BCRP: Breast Cancer Resistance Protein

BLIA: Basal-Like Immune Activated

BLIS: Basal-Like Immunosuppressed

BRAF: B-Raf Proto-Oncogene

cAMP: Cyclic Adenosine Monophosphate

CAR-T Cell Therapy: Chimeric Antigen Receptor T-cell Therapy

CDK's: Cyclin-Dependent Kinases

CDKN2A: Cyclin Dependent Kinase Inhibitor 2A

cMOAT: cMOAT

DCIS: Ductal Carcinoma in Situ

DNA: Deoxyribonucleic acid

DOX: Doxorubicin

DOXOL: Doxorubiconol

DUB3: Ubiquitin Carboxyl-Terminal Hydrolase 17

EMT: Epithelial–Mesenchymal Transition

ER: Estrogen Receptor

FOXC1: Forkhead Box C1

GBD: Global Burden of Disease

GLOBOCAN: Global Cancer Incidence, Mortality and Prevalence

GSH: Glutathione

HER2: Human Epidermal Growth Factor Receptor 2

HPLC: High Performance Liquid Chromatography

IC_{50}: Inhibitory Concentration (50%, or half maximal)

IDC: Invasive Ductal Carcinoma

IDO1: Indoleamine 2, 3-dioxygenase 1

ILC: Invasive Luminal Carcinoma

iNOS: Nitric Oxide Synthase

ISG15: Interferon-Stimulated Gene 15

KRAS: Kirsten Rat Sarcoma virus

LAR: Luminal androgen receptor

LCIS: Luminal Carcinoma in Situ

LC-MS/MS: Liquid Chromatography with Tandem Mass Spectrometry

MDR: Multi-drug resistance

MES:	Mesenchymal
miRNA:	Micro-ribonucleic acid
MoH:	Ministry of Health
mRNA:	Messenger Ribonucleic Acid
MRP1:	Multi-Resistance Protein 1
MRP2:	Multidrug Resistance-Associated Protein 2
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH:	Nicotinamide Adenine Dinucleotide
NBD:	Non-Binding Domain
NF2:	Neurofibromatosis 2
NGS:	Next Generation Sequencing
NO:	Nitric Oxide
NOTCH-1:	Notch Homolog 1, Translocation-Associated
Pgp:	P-glycoprotein 1
PI3K:	Phosphoinositide 3-kinase
PCR:	Polymerase Chain Reaction
PR:	Progesterone Receptor
RNA:	Ribonucleic acid
RNAi:	Ribonucleic acid Interference
ROS:	Reactive Oxygen Species
RT-qPCR:	Reverse Transcriptase Quantitative Polymerase Chain Reaction
SAGE:	Serial Analysis of Gene Expression

SCL: Stem Cell Leukaemia

SEM: Standard Error of Means

STAT-3: Signal Transducer and Activator of Transcription 3

TMD: Transmembrane Domains

TNBC: Triple negative breast cancer

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree of diploma of a university or other institution of higher learning.

Signed: Robyn Heald

Date:23/9/2020

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Chapter 1

1.1 Introduction

1.1.1 Cancer, A Broad Overview

As the human population increases and medical aid becomes more readily accessible and affordable to a moderate proportion of the world, we are seeing the continuation of a trend that has been present since before the 1990s. Prior to the widespread availability of modern medicine, and in many developing countries today, diseases that are now known to be largely preventable now, such as influenza (or pneumonia), diphtheria, tuberculosis and polio were and are premature causes of death responsible for a large mortality globally. The development of vaccines and better treatments for these diseases have resulted in them becoming less of an issue and more and more people survive 50+ years. Converse to this, cancer has become more of an epidemic in the last 40 years and this is in part due to the disappearance of more preventable diseases and people living longer, as well as increased capability and precision we have testing for cancer, and changes in the environment.

Cancer, in medical terms, is defined as a group of diseases wherein cells of the body grow in an autonomous and abnormal manner (The, I.C.G.C., of Whole, T.P.C.A., & Genomes Consortium., 2020). The cells acting abnormally proffer the ability to continue through the cell cycle, unregulated and as such cells replicated in this manner are somatic clones of the original cell characterised as cancerous (Gerstung, M., et al., 2020). In order to propagate these somatic adaptations required to form a tumour, they must express genes differently and create exceptions that in normal healthy cells would be corrected in the maintenance of cell homeostasis and through DNA repair mechanisms. Typically, these adaptations are caused by the accumulation of mutations over time or by DNA damage from external sources (which can also be accumulative).

The largest known collection of contributors to the development of cancer are environmental factors. It is a vital part of cancer epidemiology to understand what the leading causes of cancer are in terms of the environmental effectors at work. As we have observed in the last five years (Bray, F., et al., 2018) from GLOBOCAN and World Health Organization (WHO) studies, the primary environmental contributors of carcinogenesis are UV radiation, smoking, the consumption of highly processed meats and large quantities of red meat, alcohol consumption and *Helicobacter pylori* infections (Clinton, S. K., Giovannucci, E. L., & Hursting, S. D., 2020; Bray, F., et al., 2018;Hu, Yi, et al., 2019). It is apparent from ongoing research into carcinogens and their identification that there is still much we are not aware of; or cases that do not yet have enough significant data concerning what in our environment could affect our bodies in such a way to promote tumour growth (Guyton, K. Z., et al., 2018). Even in the case of child cancer (incidence before the age of 20) where the majority of cancer originates in spontaneous genetic mutations that develop as tissues form in utero; the mothers environment can largely effect the developing foetus via the placental barrier (Steliarova-Foucher, E., et al., 2017).

Currently in development are methods of attempting to predict some of the potential chemical carcinogens through the use of neural networking, and more efficacious methods of discerning carcinogenic potential through the undertaking of in vitro tests in model organisms (Guyton, K. Z., et al., 2018; Wang, Y. W., et al., 2020). With a greater volume of tests and investigations being conducted we are becoming better equipped to help individuals identify and avoid carcinogens in their environments. This will hopefully lead to a decrease in the number of deaths we see globally should people be educated around the potential carcinogens or various carcinogenic factors in the world, however, it will likely take time for this to occur given how much we still have to learn about our environments and how they affect us as well as way our environments are continually changing.

According to the WHO, in 2020, we are estimated to see over 10 million new deaths from cancer globally, a statistic that holds up with predictions dating back as far as 2003, (Eaton, L., 2003), and that is likely to continue trending upwards unless we find solutions and can enact them in the near future. Cancer incidence has increased from a reported 10 million in 2000 as recorded by

Parkin (2001) to 18 million as recorded in 2018. Despite this, the rate of death has decreased. In studies by GLOBOCAN and WHO and the Global Burden of Disease (GBD) study, which viewed the age-standardized incidence rates (ASIR) and age-standardized death rates (ASDR) as observed in 29 groups of cancers between 1990 and 2017 the trend of deaths appears to be steadily decreasing across all demographics despite the increase in incidence (Bray, F., et al., 2018). The aforementioned GBD study by Lin et.al., (2019) quantified the burden of cancer across 195 countries and showed that in the 27 years under observation the world has seen a 2-fold increase in the number of fatalities caused by cancer, the highest burden being in those over the age of 50, with the highest incidence occurring on the Asian continent, likely in part due to population density.

Of the cancers represented in global data, lung cancer is the largest contributor of cancer-related deaths and, as of data recorded in 2018, comprises 45% of the cases present in most populations. Tumours related to the prostate and female breasts are also amongst the highest both in terms of incidence and mortality worldwide accounting for a predominant quantity of all cases (Lin et.al., 2019; Bray, F., et al., 2018). Males are 1.5 times more present in the dataset than women, reflecting both a higher prevalence of incidence and death in male cancer patients across all cancer types (Bray, F., et al., 2018). Greater survival rates globally have nevertheless been recorded and are predominantly due to several key factors. More sophisticated and reliable methods of identification of cancer in patients, that when combined with the development of more effective therapies and resulting earlier diagnosis has been key in providing better prognoses and outcomes in a greater proportion of cases.

This is not the case the world over, and in fact cancer has been deemed a group of diseases that disproportionately effect the developed world (Lundqvist, A., et al., 2016). As previously mentioned, this is largely likely due to improved survival rates past a certain age thanks to modern medicinal developments such as vaccines against influenza. However, the increased likelihood of survival comes with the increased probability of reproduction and in some cases, this leads to the propagation of genetic mutations across generations (Sharpe, K. H., et al., 2014). This increases the likelihood of cancer incidence, as a disease that by large is more likely with the more mutations

that cells have. In addition to this, access to many known carcinogens or substances with carcinogenic potential such as alcohol, tobacco and processed meats are far more prominent and available to those inhabiting developed countries than places less developed and as a result we see an increase in incidence seemingly favouring this proportion of the global population (Lundqvist, A., et al., 2016). The survival of these individuals is also largely based on their status socioeconomically as well as the country they live in and the healthcare standards they have access to (Lundqvist, A., et al., 2016; Sharpe, K. H., et al., 2014; Riba, L. A., et al., 2019). This explains the higher incidence, but lower mortality trends reported for many years now, and whilst any mortality is not ideal, there is at least more investment from richer countries into attempting to develop better treatments for cancer than if it was solely a developing world problem.

1.1.2 The Burden of Cancer Upon New Zealand

In New Zealand, data collected and curated by the New Zealand Ministry of Health shows that over a period of 9 years (2008-2017) reported cases of cancer incidence have increased from 20,476 to 24,453, with Māori incidence accounting for roughly 8.8% of total registered cancer cases in the country in 2008, increasing to 11% of cases in 2017 (New Zealand Ministry of Health., 2019). This is quite the far cry from increasing rates globally and shows that whilst the burden of cancer in NZ is not increasing at the same pace that global cases are, the impact on the indigenous population is becoming more pronounced within the dataset. Of this, and as visualised in Figure 1, 24,000, the largest contributors were cancers of the prostate, breast, colon/rectum skin and lungs. Prostate and breast cancers made up the largest percentage of cases registered in 2017 with around 12%-14% each, together totalling 25% of the registered cases that year. Each of these cancers impacts primarily one gender and therefore only half of their respective demographics, it is therefore logical that the effect of these cancers is roughly twice what the data shows when taken into that context.

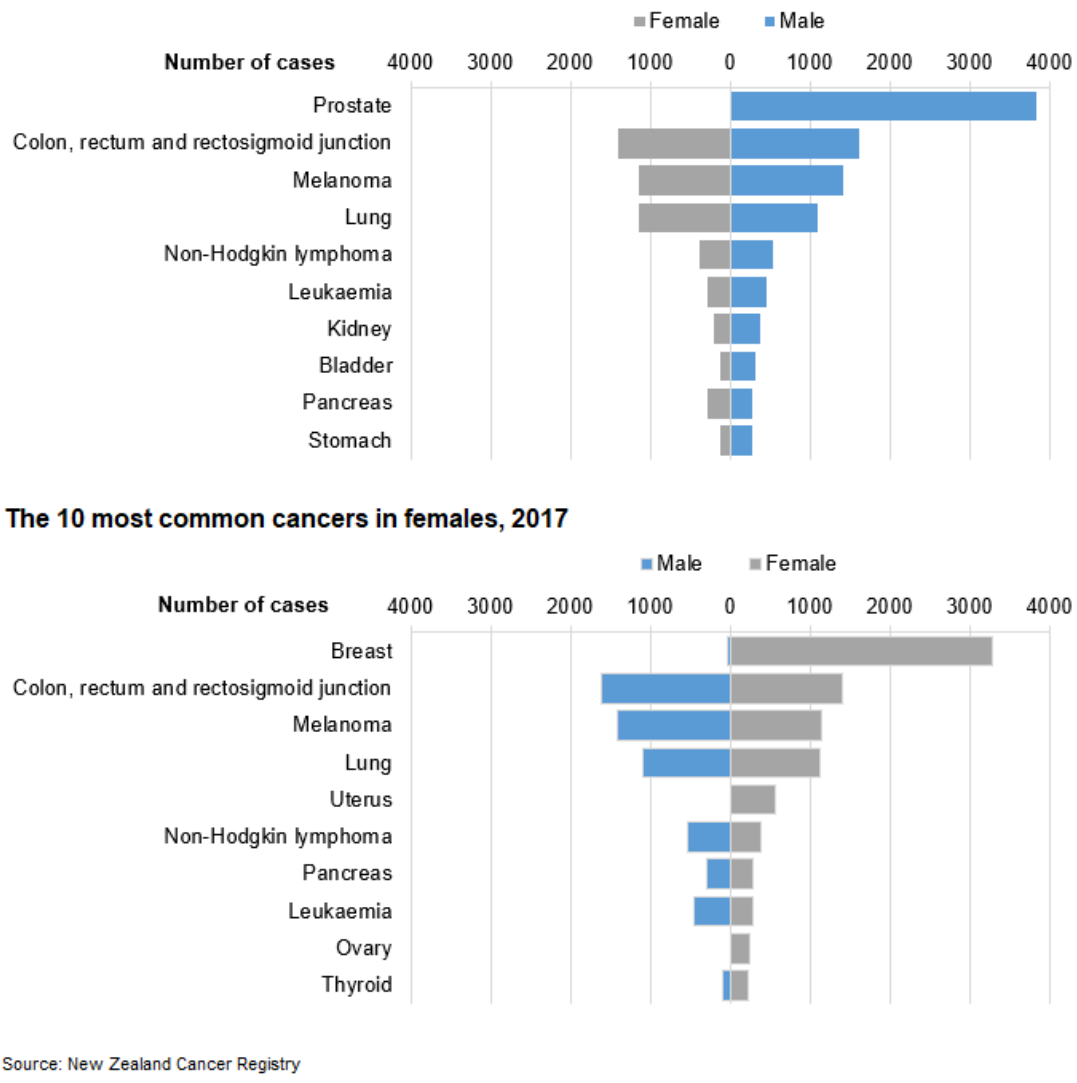

Figure 1: A summary of the ten cancers with the highest incidence in New Zealand as of 2017. (New Zealand Ministry of Health, 2019).

With regards to the national data as compared to data collected globally, Oceania made up 1.2% of the 8.6 million cases in 2018 (Bray, F., et al., 2018). And of these, the highest incidences were of lung, breast and colorectal cancers, showing that New Zealand largely follows trends of global case incidence and mortality with only slightly higher precedence of skin cancer within the dataset that is likely due to environmental factors such as a thinner layer of ozone in the atmosphere over some of Oceania that is not as evidently present in the global data (Mackenzie, R., 2017).

Data concerning indigenous populations are often scarce and in New Zealand there is still a disparity in the quality and amount of data collected on non-white populations and in particular

in Māori populations. Largely this has to do with record keeping and the willingness of indigenous populations to cooperate with research groups due to trust as well as a lack of mutual-respect and understanding for majority-led registration. Oftentimes a lack of consideration that is vital to be properly surveying these groups, as observed by Melkonian and constituents in a five year study concerning Alaskan and broadly American indigenous populations, can impact the quality and accuracy of data collected on these peoples (Melkonian et. al., 2020). This is particularly relevant in nations such as the United States of America and Australia, where there is an inherent lack of respect for indigenous populations that has had an effect on the way research is conducted on these populations and therefore has made it harder to assess the burden of cancer on indigenous populations globally. Information like this can be vital to understanding risk factors and the demographics and groups at risk of developing various types of cancers and linking environmental factors to incidence and mortality rates.

Environmental factors play a major role in the development and survivability of certain cancers and a large factor that dictates the environment that individuals inhabit is the socioeconomic circumstances that patients are in (Tweed et. al., 2018; Riba et. al., 2019). This contributes to the pattern we see regarding breast and prostate cancer in particular. Income and education are the major players in delineation of mortality as seen across multiple, large cohort, studies (Newman, A., 2017; Lunqvist, A., et al., 2016; Coughlin, S., 2019). And this holds particularly true in countries such as America where income directly correlates to the ability of individuals to afford healthcare and medical insurance which as seen in a paper by Ji, et al., 2020 is the largest contributing factor to survival of all cancers in that country (Ji, P., et al., 2020). There is a large disparity between American data to that of Europe, Asia and Oceania where healthcare is largely subsidised by the governments of those countries.

Beyond the socioeconomics of the country, there are other factors that effect the New Zealand population disproportionately to other countries, the aforementioned increased amount of UV penetration in the ozone layer around the Antarctic region. This has been extensively looked into as New Zealand has become the so-called “Melanoma Capital of the world” and has largely been put down to the willingness of the New Zealand population to spend large amounts of time

outdoors (Mackenzie, R., 2017). Smoking is another large factor of the impact of cancer in New Zealand and is considered to be the principal catalyst in incidence of mouth and lung cancer with almost 80% of global cases being linked to the smoking of tobacco (Tindle, H. A., et al., 2018; Lortet-Tieulent, J., et al., 2015).

Outside of the environmental causes of cancers in New Zealand there are also the cases that result from mutation and genetic predisposition, and this is more often than not the case with breast, prostate and colon cancer, all three of which we have seen have high incidence per capita in New Zealand.

1.1.3 Breast Cancer

As has been previously stated, breast cancer is one of the leading causes of mortality in women in New Zealand, currently rated as the third most common cause for premature death in the country according to the New Zealand Ministry of Health (MoH) as of 2019. These numbers (especially those concerning New Zealand) are predicted to increase with time as following the trends in incidence seen in GLOBOCAN research and private statistical analysis conducted by a host of researchers (Bray, F., et al., 2018; Lawrenson, R., et al., 2016; Heer, E., et al., 2020; Carioli, G., et al., 2018). Incidence and mortality in New Zealand has been reported to be higher than much of the rest of the world and this is specifically true for the Māori population. Māori women are more likely to be diagnosed with breast cancer than women of European descent in New Zealand for largely unknown reasons, although speculation points to a number of risk factors such as higher rates of obesity and alcohol intake as being somewhat responsible for some of the variance we see between ethnic groups (Lawrenson, R., et al., 2016). The most currently made available data supports this, as the New Zealand MoH report that the rate per 100,000 population was 130.8 in female Māori populations as compared to 94 in female Non-Māori populations. Of these incidences, Māori women are more likely to die than non-Māori women as a result of a worse prognosis and later detection of tumours. There have been initiatives to improve the detection in all populations as led by breast cancer screening initiatives established by the MoH

and there is a possibility that the increase in registration of Māori cases is in fact due to better detection, however mortality remains disproportionately higher in Non-European cases and this has been reflected in data.

Of those effected by breast cancer there are classifications within the group for sub-types of cancer typically determined by the position and status of the tumour as well as by the genetic factors affecting the patient and that result in the development of the tumour. The main groups of breast cancer are Luminal Carcinoma in situ (LCIS), ductal carcinoma in situ (DCIS) and then invasive variants of these two types, i.e. Invasive Ductal Carcinoma (IDC) and Invasive Luminal Carcinoma (ILC) (Zhao, H., 2020). The observed location and amount of penetration achieved by the tumour at the time of diagnosis dictates the type, and also largely the prognosis, as invasive tumours are more of a threat due to their proximity and ability to spread and metastasize via the circulatory and lymphatic systems (Zhao, H., 2020).

These groups typically present with specific receptor markers, namely; human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER) and progesterone receptor (PR) (Loibl, S., & Gianni, L., 2017).

As breast cells typically express the genes for these receptors on the surface of breast tissue cells, it can be a good indicator as to how to best treat the cancer, and the over or under expression of the genes relating to these receptor proteins are a guide to suggest whether hormone therapy can be used, perhaps not in treatment but in prevention of occurrence of the cancer.

HER2 positive breast cancers account for roughly 18% of breast cancer cases globally but have been found to account for a large proportion of cancer and mortality rates in New Zealand Māori and Pacifica populations. Māori women have been reported to show a higher rate of HER2+ diagnosis and also a higher rate of mortality if presenting with HER2+ tumours than other groups (Pernas, S., & Tolaney, S. M., 2019; Loibl, S., & Gianni, L., 2017; Lawrenson, R., et al., 2016). HER2+ treatment has been jettisoned forward with the recent discovery and application of pertuzumab, trastuzumab and alike derivatives in the treatment of tumours with HER2 markers. This has improved outcomes for those without metastatic tumours, and slightly improved

outcomes for those with metastatic tumours, however, around 50% of this latter category develop secondary tumours in the brain and this remains an issue for this diagnostic group in terms of treatment and planning for better outcomes and survival rates (Pondé, N., et al., 2018; Loibl, S., & Gianni, L., 2017).

ER and PR positive immunohistochemical cancer types are characterised by an increased concentration of oestrogen and progesterone receptors respectively in the tumour cells. Often these receptors appear in tandem and an immunohistochemical phenotype of ER positive/ PR positive/ HER2 negative (Wu, H., et al., 2017). This combination of factors is the most common receptor-typing for breast cancer that is in an invasive state and can be easier to treat than HER2 positive tumours due to increased response to hormone therapy (Xu, J., et al., 2018). As well as this typing, there are other combinations of positive and negative receptors that exist and can be treated with varying efficacy that depends more on the metastases of the tumour than the typing of the receptors on its surface. A combination of hormone therapies, chemotherapy and radiation as well as surgical excision of tumours are all utilised, however, it is the hormone therapy that has significantly improved the prognosis and survival of this group of cell types.

This is the case for all except the 10-20% tumours that have no markers at all (Al-Mahmood, Sumayah, et al., 2018). These tumours are known as triple negative breast cancers (TNBC) and as they have little to no markers for oestrogen, progesterone or HER2 overexpression on or in the cell and therefore cannot be treated with hormone therapy. Therapeutic approaches instead rely on the efficacy of chemotherapy, radiation and surgical excision in order to treat the cancer. Cancer subtypes with no histochemical markers typically have a longer, more difficult treatment plan as there is no hormone or immunotherapy or other effective therapeutic option approved for widespread use (Aydiner, A,. et al., 2015). It is for this reason that TNBC treatment relies on chemotherapy, to which it often becomes highly resistant, radiation and surgery and why this type of breast cancer was chosen as the subject of study (Wang, P., et al., 2015; Bertheau, P., et al., 2013).

1.1.4 Triple Negative Breast Cancer (TNBC)

Ordinarily considered to be the most aggressive type of cancer, TNBC has one of the higher mortality rates of all breast cancers and can be harder to diagnose and treat than the other 80% of cases (Urru, S., et al., 2018). The three biomarkers we standardise for the sake of diagnosis are considered largely defunct in terms of what we can identify of TNBC. Primarily this cluster of alike carcinomas are less like PR+/ER+ cancers of the same tissue type, and there is as much variation between TNBC tumours as there is between it and other types of breast cancer (Coussy, Florence, et al., 2019). Molecular typing and the identification of characteristics was vital to improve the treatments of other types of breast cancer and as such much research has been conducted to attempt the same in TNBC (Bianchini, G., et al., 2016). As such there has been little progress in identification of new targeted treatments but confirmation by consensus that there are four key subtypes to TNBC, each with its own unique phenotype and distinct mRNA profile (Burstein, M. D., et al., 2014). These subtypes have been identified as luminal androgen receptor (LAR), mesenchymal (MES), basal-like immunosuppressed (BLIS), and basal-like immune activated (BLIA). Genomic profiling of these types has largely been successful in terms of research, however, in many cases this is not practical for diagnostic purposes due to the cost and tissue sample requisites for this method. It is therefore down to the method of histochemical identification in many cases to confirm subtyping and through those means, attempt to find a more efficacious course of treatment for patients with TNBC.

LAR types of TNBC have been shown to account for around 30% to up to as much as 50% of all TNBC cases. This subtype is characterised by the presence of nuclear receptors that have multiple functions, but which largely are related to the activation of transcription in response to signal cascades from intra- and extra-cellular sources. (Gerratana, L., et al., 2018). More recent research such as that undertaken by Coussy et al. has resulted in an improved understanding of potential targets for more direct therapy such as anti-androgenics that could provide a better alternative to those with more chemo-unresponsive tumours (Coussy. F., et al., 2020; Coussy. F., et al., 2019).

This subtype accounts for the highest survivability within the breast cancer spectrum, and more easily identifiable than BLIS, BLIA and MES forms (Ding, Y. C., et al., 2019).

Immune activated and immuno-suppressed cases of basal-like TNBC are known to have less distinct molecular signatures and are harder to identify than the LAR subtype through immuno-histochemical means (Kim, S., et al., 2018). This is due to the difficulty in identification between subtypes as there are many cross-over genes and cellular effects that the four groups share. In gross terms, it has been documented that IDO1 and FOXC1 are key characteristic molecules for both BLIA and BLIS type TNBC. FOXC1 has long been associated with the proliferation of cells, and expression levels in basal-like breast cancer cells in particular (Kim, S., et al., 2018; Han, B., et al., 2017). IDO1 is a part of a Tryptophan (Trp) conversion pathway. Trp has, for several years now, been understood to play an important role in the mobilisation of T-cells and other immune response cells derived from myeloid tissue (Liu, M., et al., 2018). For BLIS tumours this is a key factor that appears to be present in 70% of tissue samples, however, this does not make it any less of a potential target for therapeutic response, should a more definite impact of this gene on TNBC or other cancers be found (Ding, Y., et al., 2019; Liu, M., et al., 2018).

MES TNBC cells are characterised primarily by the increased presence of epithelial-mesenchymal transition markers. These markers are responsible for cell differentiation between epithelial and mesenchymal cell states and have been shown in multiple cases to be associated with the development and invasiveness of tumours both within breast tissue and without (Elzamly, S., et al., 2018). The mechanisms for this cellular process are still under investigation but research to the effect has shown that there is some response of these tumours to novel drugs targeting the EMT pathways and the interaction thereof with integrin β3 (Liu, S., et al., 2020; Bianchini, G., et al., 2016). Treatments relating to this subtype of TNBC are particularly accessible to research given that one of the most common breast cancer cell lines, MDA-MB-231, are of this variety. This is vitally important as this type of breast cancer, which accounts for roughly 30% of TNBC, has one of the highest rates of developing chemo-resistance which has become one of the principal causes of the high mortality rate within TNBC (Hill, B S., et al., 2019).

TNBC tumours have a large amount of heterogeneity, which leads to further challenges in treating them. There are so many different types of cells that targeting each type based on markers or commonalities has been unsuccessful. As a result of this and is why for so long, chemotherapy and radiation therapy were the gold-standard of treatment. Instead of targeting specific cells with different markers etc, these therapies target masses of cells based on location or origin tissue, with all cells being targeted, whether they are healthy or cancerous. Cytotoxic therapies such as chemotherapy are being replaced in many cases by hormone and immune therapies, but to find a solution for TNBC more research into identification and underlying mutations and root causes cellular dysfunction must be undertaken.

1.1.5 MDA-MB-231 Cell Cultures

Cell cultures have long been used in vitro biological experimentation, especially in terms of the early development of drug-based therapies, as it provides a simple and easy to visualize, quantifiable model for assessing the way cells in the body may respond to a multitude of stimuli. In the case of breast cancer, there are manifold different cell culture options each with relatively different characteristics and from the different subtypes, as the development of a cell line is relatively straightforward once there has been consent given to harvest and maintain cells from an initial sample (Dove, A., 2014). The cell lines themselves come from patients who are confirmed to have, in this case, breast cancer, but a range of diseases and conditions that can be sampled direct from the tissue and immortalised before being frozen down. Cancer cells often requiring little aid in terms of immortalization as most tumours already replicate in an uncontrolled or regulated manner.

This was first done with a sample of cervical adenocarcinoma in 1951, with the isolation of what would become the immortalised HeLa cell line from Henrietta Lacks, a woman who would soon thereafter die from her tumour but whose cells continue to this day to be of use to researchers in this field wishing to model the tumour environment for research purposes (Lucey, B. P., Nelson-

Rees, W. A., & Hutchins, G. M., 2009). The topic of consent was brought up above as, famously, in the case of Henrietta Lacks, no consent was given by the patient, or her next of kin after her death and as such there was a large ethical debate that could have and should have been avoided through consent (Beskow, L. M., 2016). The ethical considerations concerning cell culture, is of importance and is acknowledged as a part of this experiment, however, an extensive review of this is beyond the scope of this research.

MDA-MB-231 cells are immortalised epithelial cells isolated from a breast adenocarcinoma tissue sample. The cells, as classified by ATCC, are of a basal-like morphology which is consistent with being a TP53 mutation carrier, as well as this mutation they are characteristically possessing of BRAF, CDKN2A, KRAS and NF2 (Komatsu, M., et al., 2012; Bamford, S., et al., 2004). MDA-MB-231 cells are also known to be in a metastatic phase which as a TNBC model is a beneficial feature given the characteristic aggression of this type of breast cancer (Kapoore, R. V., 2017). This increases the accuracy available to research utilizing these cells as it is common for TNBC cells to progress to a metastatic state faster or be identified as TNBC later due to the lack of hormone receptors seen in the cells phenotype (Tan, T., & Dent, R., 2018).

These cells are often used as a test model for experimentation such as the development of chemoresistance or the success of new drug combinations in treating the aggressive disease. The response of these cells developing chemoresistance has been largely investigated, and despite the hole still present in the knowledge we possess today, much is known about the genetic variation in broad terms that MDA-MB-231 cells undertake in order to survive chemotherapy. Previous work has highlighted the significant difference in cellular expression via the silencing of Notch-1, STAT3 and β-catenin increase, which in terms of function, is an increase in the features of the cell relating to stem-cell-likeness and known metastatic agents (Alkaraki, A., et al., 2020; Park, S. Y., Choi, J. H., & Nam, J. S., 2019). Another route of inquiry into this cell line and the mediation of DOX resistance was into the expression of Snail, a family of transcriptional factors that is linked to the adhesion of cells to nearby cellular contacts (Mariano, G., et al., 2015; Kajita, M., et al., 2004). This is not due to some feature of the cell line however, more that there are so many methods through which cell expression can be effected to revert or prevent the changes in

TNBC relating to MDR (Pindiprolu, S. K. S., et al., 2019). These genes have come under scrutiny due to the more prevalent sources of knowledge surrounding them and a long history of characterisation as important factors in cellular homeostasis and the cell cycle (Alshaer, W., et al., 2019). That there are so many genes likely linked to the effect of MDR is tantamount to the understanding and development of better treatments, and through the use of cells like MDA-MB-231s, we can further out knowledge to this end just as is intended with this study through the investigation of the expression of ABC transporter genes.

1.1.6 Cellular Mechanics

Cellular dysfunction with respect to cancer is typically dependent on several factors. These factors are primarily effectors of the cell cycle, but as evidenced with IDO1 as described above, they can work in conjunction with other cellular mechanisms in order to effect the cell biome as a whole and provide a niche better suited to the development and proliferation of a tumour (Liu, M., et al., 2018). Typically, the cell cycle is impeded or derailed in such a way that the mitotic replication of cells become uncontrolled and form an abnormal mass of cells.

Mitotic cellular replication is the process through which the majority of cells replicate in the body, and as such is a vital component to life through the growth and development it facilitates. This cycle, one typically defined by the stages of gap0 (G0), gap1 (G1), synthesis (S), gap2 (G2) and mitosis (M) is responsible for the maintenance of tissue homeostasis on a gross level and can only be undertaken due to the precise replication of genetic material within the cells undergoing mitosis (Hustedt, N., & Durocher, D., 2017). Cells that are undergoing replication, and therefore not in the quiescent phase (G0), must go through these steps for the cells to proliferate and for tissues to persist (Hustedt, N., & Durocher, D., 2017). Each of these stages is characterised by the activity of the cells through the phase in question, with gap phases serving largely as periods of preparation for cells to pass into either S or M phases where the DNA replication occurs and where the cells separate into two daughter cells respectively (Umeda, M., Aki, S. S., & Takahashi, N., 2019). The G phases are, however, invaluable in the process, in that they contain various cell

roadblocks or checkpoints, wherein cells may be arrested in their cycle should they be unfit to continue due to DNA damage. Subsequent of these roadblocks, cells fit for replication will progress on into the phase following or will be designated by cell signalling for apoptosis. The main signalling molecules that are responsible for the arrest and signalling for arrest of cells with significant DNA damage are tumour suppressor proteins and cyclin-dependent kinases, or CDKs (Hustedt, N., & Durocher, D., 2017). In healthy cells, CDKs use signalling pathways to arrest cells that have damaged DNA that requires repair; or to send that cell on into the M phase where the cell will replicate and split into two daughter cells with their own copies of intact and healthy DNA. However, this has been proven to not always be the case, as in many cancers CDKs have been identified as the cause of unconstrained mitotic replication, in regards to TNBC, CDK4/6 is a key component of the EMT pathway through the phosphorylation of deubiquitinase DUB3, which is required for the stabilization of adhesion regulating genes and therefore a key factor in the role of tumour development (Vijayaraghavan, S., et al., 2018). Overexpression of these regulators has opened a new route for prospective treatments as they are abundant and targetable using novel drugs.

Tumour proteins are also key regulators of the cell cycle, and as alluded to by the name, typically aid in the suppression of cellular development particularly in response to signals from the cell that indicate DNA damage, oxidative stress or other forms of cellular stress from extrinsic or intrinsic factors. Typically, tumour suppressor proteins are capable of interacting with cellular components via vital protein - RNA and protein - protein interactions that leads to either the activation or cessation of a cascade controlling the fate of cells across all replication phases but particularly during G phases. By and large these genes can either be mutants or wild type genes (lacking mutation) and the difference in activity is often stark with mutations at this locus (Karakostis, K., & Fåhraeus, R., 2019). Mutant genes responsible for the proteins policing cell turnover and replication such as these are often members of families and pathways that relate to tissue homeostasis, or the maintenance of the tissue itself, and as such a mutation causing the proteins to be upregulated or downregulated in the cells can lead to tissue creation and sustainment that is not considered normal.

Tumour protein 53 or TP53, is a key member of this family that in healthy cells receives a hormone or other chemical signal and as a result is expressed as the start of a pathway directly responsible for the repair of DNA. Mutations that occur in the genes responsible for proteins such as TP53 are some of the most common found in cancers of all types but specifically in breast cancers are accountable in 30% of all cases (Bertheau, P., et al., 2013). The highest proportion of these mutations are found to affect cells in basal-like carcinomas rather than their luminal counterparts. This gene is said to have many external effectors, with research into UV, alcohol and tobacco exposure showing direct links between these carcinogenic agents and the rate at which tumours develop (Zhao, M., et al., 2017). Alcohol in particular has been shown to activate the TP53 pathway as a direct result of consumption-related DNA damage, and as such creates an event at which a damaged copy of TP53 could allow for an uncontrolled cellular replication and cause a disruption to the continuation of normal cellular homeostasis (Zhao, M., et al., 2017).

There are many genes like TP53 that are vital in the maintenance of a functional cell cycle and the regulation of cell turnover, DNA repair and cell senescence/apoptosis and many are well known by researchers in this field. The most notable, and most recognisable aside from TP53 are the BRCA1 and BRCA2 genes (Yoshida, K., & Miki, Y., 2004). Like TP53, these genes are tumour suppressor genes that, upon activation, control whether cells are repaired or ready to pass through into mitosis (M Phase). The BRCA genes are particularly important to breast cancer because they are cellular regulators of cellular homeostasis that are found primarily in breast tissue (Yoshida, K., & Miki, Y., 2004). And BRCA1 in particular is often associated with and found in tumours without hormone or HER2 receptors, namely those that we previously defined as TNBC in nature. These other genes are more well-known than TP53 mutants due to media awareness, and are commonly regarded to be primarily heritable by nature (Song, Y., 2019). This is not always the case though and spontaneous non-germline mutations do occur with some regularity across all subtypes of breast cancer, and whilst they are not always the leading cause of the cancer, they can be used to determine vital diagnostic information such as likelihood of metastasizing, possibility of recurrence and risk for future generations (Lee, A., Moon, B. I., & Kim, T. H., 2020).

The reason that mutations at loci relating to proteins that control the cell cycle are so incredibly vital to our understanding of tumours is that the microbiome as a whole can be vastly effected by the up or downregulation of one gene, particularly in the case as something as important as a DNA repair initiator. This informs the way that we try to treat so many cases of cancer, in all tissues, because it impacts the way the cell regulates itself and all the processes therein. It is the knock-on or secondary effects that are often so important in therapy development as it is not currently possible for us to target genes directly with treatments.

1.1.7 Cancer Cell Dynamics

Complex in nature, the methods by which cells self-regulate, whilst broadly studied, still holds much to be discovered or uncovered through further research. Despite seemingly monumental discoveries surrounding cellular mechanics, there are things about growth, development, and maintenance that we still do not fully comprehend. It is through the discovery and furtherance of our knowledge in this field that we will better understand cancer and tumours as heterogeneous masses that do not follow all of the "rules" that we have established are true in healthy cells. Signalling, be it from hormones, growth factors, nearby cells or the extracellular matrix, is key to the way the cell continues to function and is the primary method cells have for activation or suppression of a whole host of cellular activity. However, as cells are largely impermeable to large chemicals such as oestrogen, vasopressin and most chemotherapy drugs. This means that they rely on the transport proteins residing in the cell membrane and the receptors both internal and external to the cell for the plethora of chemical effectors.

Cell membranes function around a model known as the mosaic model. The model shows a bi-layer of phospholipids interlaced with cholesterol for mobility, proteins making up transporters, and other macromolecular structures comprised of carbohydrates and various lipid structures to aid mobility and facilitate movement of the cell, of chemicals from within and without the cell membrane and most importantly to keep the cell stable (Hossain, K. R., & Clarke, R. J., 2019; Dunn, P. J., Salm, E. J., & Tomita, S. 2020). These transporters are required as the membrane

must remain a closed system so as to maintain the ionic charge within the cell membrane. This charge activates and chiefly aids in the movement of smaller particles through channel transporters or via diffusion. These are known as passive transporters and are almost all a part of the solute carrier (SLC) family of transport proteins (Xiao, Q., Zhou, Y., & Lauschke, V. M. 2020). Active transporters require an energy source and rely typically on ATP rather than the charge of the inside of the cell membrane to facilitate the transportation of small inorganic chemicals as well as larger, more complex molecules such as chemotherapeutic agents, hormones, lipids, vitamins, antioxidants and vital signalling chemicals.

ATP Binding Cassette (ABC) Transporters are a superfamily of transporters critical in the maintenance of the cell efflux via the lipid bilayer through the use of 'high energy' molecule hydrolysis. When functioning in their normal capacity, these proteins allow for said maintenance, a lack of which can result in conditions such as cystic fibrosis, anaemia and a host of neurological conditions that rely on the transport of hormones and other chemicals across membrane barriers throughout the nervous system. This family is composed of a multitude of membrane proteins that are coded for by 48 genes (Gillet, J. P., et al., 2020). Classified transporters of this family are segregated into two categories based on their architecture and function and these categories, referred to commonly as Class I and Class II are common across almost all living organisms however adapted for different functionality they may be.

In humans there are key domains that we have defined through the course of research that when working together make up the bulk of the ABC transporters we possess. Importantly eukaryotes, with few outliers, primarily utilise these transporters as exporters and only in very few cases are they used for the influx of molecules. Structurally however, this functionality plays little role in the composition of the proteins themselves and in fact there is only one protein component found in import ABC transporters and this is to capture the substrate required for transport. In general, there are four key domains to proteins of this family, these can be broken up into nucleotide-binding domains (NBDs) and transmembrane domains (TMDs). TMDs are the structural part of the protein that interfaces with the membrane of the cell. The proteins themselves are embedded within the lipid bilayer and are fused polypeptides commonly possessing all four functional units

that can have identical NBDs and TMDs or possess structurally different polypeptides that increase the diversity in substrates that can be transported.

As seen in Figure 2, the movement of drugs is mediated via the structural composites of the ABC

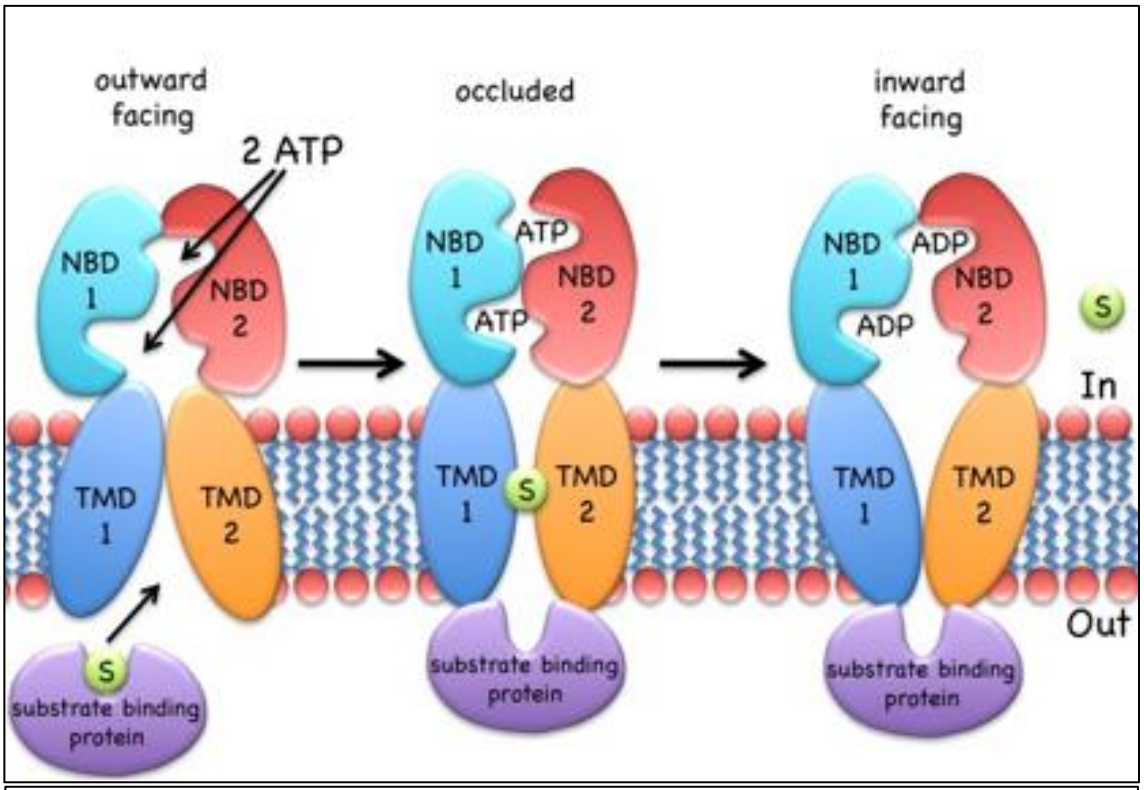

Figure 2: An outward facing ABC transporter changes structural confirmation to move Substrate "S" via the binding of ATP (Wilkins, S., 2015).

transporter. The use of a substrate binding protein as seen in the figure is not always used by the cell though this again can increase the diversity of substrates able to be transported out of eukaryotic cells. The movement of DOX from the inside of cells to the outside follow a mechanism such as this and ultimately in resistant cells occurs prior to the drug reaching the nucleus.

1.1.8 Chemotherapy and Doxorubicin

Chemotherapy is the process of treatment considered the gold standard for the treatment of TNBC and many other cancers without alternative treatment. The principle of chemotherapy is to utilize

cytotoxic drugs to target rapidly replicating and developing cells in the patient. As can be surmised by this functionality, it is destructive to cancer tumour cells but also to other cells in the body that have similar functions such as hair follicles, cells in the circulatory system and cells in the gut and greater digestive system. It is therefore less than ideal to still be using what is considered a poorly targeted method of treatment, particularly when so many TNBC tumours in particular are known to rapidly develop resistance to the drugs in question, however this cannot be avoided as for many types of cancer there exist few other therapeutic options.

Chemotherapy is often used in conjunction, referred to as adjuvant therapy, with surgery, to remove masses from the body and reduce the chances of tumour recurrence. It can also be used as a neoadjuvant therapy in order to shrink a tumour prior to surgery, or in cases of high-proliferation/metastatic patients to deal with the cancer as it moves to other parts of the body or in cases of relapse (Kashiwagi, S., et al., 2011). Often, TNBC patients fall into the latter category due to the clinical aggression of their tumours. This does not make the chemotherapy any less beneficial, rather the opposite is true, as the chemotherapy is at that point the most targeted therapy available. However, from studies we have seen that neoadjuvant chemotherapy can play a role in the development of resistance to the drugs in use and therefore only lead to further complication in the overall treatment process (Nedeljković, M., & Damjanović, A. 2019).

Cytotoxicity is affected upon the body via these drugs usually by means of the drugs disrupting the proliferative cycles and disrupting mitotic replication and in doing so disrupt the tumourigenesis at work (Silver, A. J., & Jaiswal, S. 2019). There are several ways through which the drugs can achieve this, and each method corresponds to a subgroup of cancer drugs. The main ones used in modern treatment are alkylating agents, antitumour antibiotics, antimetabolites, topoisomerase inhibitors, antineoplastics and plant alkaloids. In the treatment of breast cancer, the typically used drug regimens consist of combinations of alkylating agents, antitumour antibiotics in the form of anthracyclines, antimetabolites and in cases of metastases some treatments include plant-based vinca alkaloids. While each of these drugs serve the same purpose in causing cell death, there are various mechanisms through which this is achieved.

Alkylating agents functionally disrupt cell replication cycle by forming covalent bonds with nucleophilic structures in the DNA replication stages and as such disrupt the helical structure of the DNA as it is manipulated by helicases in the nucleus (Siddik, Z., 2002). These drugs have been shown to bond with guanine alkyl groups in particular during the first and second G phases in mitosis and as the cell moves into active replication the helical structure is disrupted leading the cell to recognise a critical error in the DNA structure and signal for the initiation of cell cycle arrest and apoptosis (Siddik, Z., 2002; Zanotto-Filho, A., et al., 2018; Hombach-Klonisch, S., et al., 2018).

The function of antimetabolites is linked directly to their structure. This class of drug is utilized for their structural or chemical mimicry of actual metabolites the cells require in order to be able to grow and proliferate as tumour cells do. By mimicking the structure of metabolites the antimetabolites can hamper the enzymatic resources of the cell by entering into enzyme led reactions in the place of metabolites and thereby decreasing the output of necessary synthesis essential to the continuity of nucleic acid production and protein turnover in the cells (Wu, Y., et al., 2017).

Vinca alkaloids are a subset of plant alkaloids derived from the Catharanthus roseus plant and are used across a variety of cancers for their ability to disrupt the process of spindle formation during mitosis and causing cell arrest before it can move into daughter cell cleavage. This activity is dependent on the high affinity of vinca alkaloids for the active binding sites of the tubulin molecules that are in an increased concentration within the cell prior as they are about to form the mitotic spindle. As the spindle does not form, replicated DNA remains in the cytoplasm and the cell goes into arrest (Anitha Sri, S., 2016). Anthracyclines are a class of antitumour antibiotics that have long been used in breast cancer treatment in particular (Jasra, S., & Anampa, J., 2018). The functionality of these unique antibiotics stems from the multifaceted interruption they can exhibit upon the tumour cells through disruption of DNA replication during each stage of the cell cycle (Cai, F., et al., 2019). This occurs via interaction between anthracyclines and DNA topoisomerases, enzymes that control and oversee the uncoiling of DNA as well as the cutting and stabilization of the DNA thereafter. The antibiotics work by binding with these enzymes and

thereby allowing the DNA to remain destabilized and unable to undergo the translation/transcription process. This effectively stops all mitotic cycling through damage to the DNA and causes the cell to undergo apoptosis (Marinello, J., 2018). Anthracyclines are of particular interest as they are the most common drug utilised in breast cancer chemotherapeutics and come with a host of issues, with cardiotoxicity and increasing incidence of metabolite-driven chemo resistance leading research into combinatorial therapies to increase the effect of these drugs without having to utilize higher and more toxic concentrations of the drug. While the use of several antibodies, such as erlotinib and through targeting methods such as glutamine-conjugates, is beginning to become more probable for future therapeutics, there are still many issues in particular with this form of breast cancer and its seemingly easily acquired resistance to many subtypes of chemotherapy drugs (Zhou, Z., et al., 2017; Zhou, P., et al., 2019).

1.1.9 The Pharmacokinetics of Doxorubicin

Doxorubicin (DOX), an anthracycline antibiotic used since the 1950s, is today one of the most commonly used cytotoxic drugs used in the treatment of TNBC (Speth, P. A. J., Van Hoesel, Q. G. C. M., & Haanen, C., 1988). Pharmacokinetics, the study of how drugs work within the body, has long been of relevance to describing how drugs are transported around the body, are metabolised, how they act upon the tissues that absorb them and the routes through which they are excreted. This drug and the family it comes from is characterized by possession of a tetracyclic ring possessing quinone-hydroquinone side chains, with a glycosidic bond to a Daunosamine group. DOX and its family of Anthracyclines are utilised therapeutically for a range of cancer treatments, but most frequently, as part of treatment regimens for ovarian cancer (Zou, Y., et al., 2018), lung sarcoma (Lv, L., et al., 2016), lymphoma (Xu, P., et al., 2017) and as indicated previously in breast cancer (Zhou, P., et al., 2019). Comprehension of drug pathways through pharmacokinetics has led to better treatment development and therefore plays a pivotal role in the research undertaken to improve efficacy across cancer types and different treatment regimes.

As per the previous description of the sub group it belongs to, DOX intercalates with DNA inside the nucleus of the cell due to the high affinity DNA has for the drug and prevents the ability of the cell to conduct DNA replication, causing apoptosis (Jawad, B., et al., 2019). However this is only one of the ways in which it can cause cell death to occur and it is why build-up of the drug in cardiac tissue has led to so many toxic outcomes for patients over the course of the last 60 years (Mordente, A., et al., 2009). As the cytotoxic drug builds up in bodily tissues, DOX interferes with the cell cycle through interaction with topoisomerases, intercalation of DNA, production of reactive oxidative species or through the stimulation of ceramide, all of which can lead to cells becoming compromised and leading to cell death. Despite these risks however, there has been a significant and documented effect of the use of anthracyclines such as DOX in the treatment of breast cancers in long-term studies; with as much as a 38% increase in survival between groups who were treated with DOX versus those who were not between 1985 and 2000 (Early Breast Cancer Trialists' Collaborative Group, 2005). Improved DOX treatments in particular have been formulated over time, and primarily due to concentration dependent toxicity, to be bound within a liposome capsule for better treatment outcomes (Tomankova, K., et al., 2015).

The drug itself is typically delivered intravenously in solution and is up-taken by tissues rapidly as the distributive half-life of the drug has been estimated around 5 minutes, during which time the drug binds to plasma proteins in the blood in order to reach tissues for uptake. At no point in this process does the drug or any of its metabolites pass the blood brain barrier. Elimination of the drug occurs primarily via the hepatobiliary system with up to 40% of the dosage used appearing in the bile and an additional 15% appearing in the urine within the course of 5 days (DrugBank, 2005). This data is widely standardised for the sake of convenience and it has been noted that anthracyclines as a family show great variation between individuals with different tumours and even those with tumours affecting the same tissues (Mordente, A., et al., 2009). Analysis of DOX clearance over the course of its use and study in relation to the pharmacokinetics of the drug has revealed that DOX is no exception to this variability in clearance and the extent

of that could provide information to the variation we see in patient response and incidence of cardiotoxicity (Pippa, L. F., et al., 2020; Lal, S. et al., 2017). Liquid chromatography coupled with mass spectrometry (LC–MS/MS) of urine, plasma and plasma ultrafiltrate, revealed that the variability was not only reserved to the clearance of DOX but also of it's most significant metabolite doxorubicinol (DOXOL). The data collected by Pippa, L. F., et al., from 12 patients, revealed an average value of 30.70 L·h−1 for total clearance, 0.66 L·h−1 for renal clearance, 29.97 L·h−1 for hepatic clearance and 0.39 L·h−1 for the formation clearance of the metabolite DOXOL. The geometric coefficients of variance for the clearance in these 12 patients revealed that 15 % and 17 % for unbound fractions of DOX and DOXOL were both particularly low in comparison to other pharmacokinetic parameters (Pippa, L. F., et al., 2020). This translated to confirmation of a large variation in the clearance of the drug by the 12 patients under study as was consistent with other relevant literature (Lal, S., et al., 2010; Lal, S. et al., 2017). The study also confirmed that whilst this data added insight to the understanding of DOX pharmacokinetics, there is still much that we do not know or cannot completely confirm using current methods as to the precise ways that DOX functions, is excreted, or is accumulated in the body.

This variability of the drugs elimination by the body means that anywhere between 50%-75% of the drug is cleared, the rest is broken down into metabolites through three major pathways, two of which are electron-driven reduction interactions which take place typically within the mitochondria of cells and account for the majority of drug metabolism in the cell (Pippa, L. F., et al., 2020). The third metabolic pathway is minor in comparison with the other two and relies on the deglycosilation of doxorubicin into its metabolites via a hydrolytic or reductive reaction (Renu, K., et al., 2018). The electron driven metabolism relies on a two-electron reaction from which only and alcohol metabolite is produced by the cell and a one-electron reaction from which ROS are produced. The latter is of significance as ROS creation is one of the principal ways in which toxicity to this drug can eventuate, particularly in the heart (Baxter-Holland, M., & Dass, C. R., 2018). These electron reactions are mediated by nicotinamide adenine dinucleotide hydrogenase (NADH) dehydrogenase and Aldo-Keto (ADK) reductase proteins in the cell and transported through cell membranes by a host of anion and ATP dependent transporters such as

P-glycoprotein 1, multidrug resistance-associated protein 1, ATP-binding cassette sub-family G member 2. These drugs are responsible for the influx and efflux of DOX and its metabolites and are key players in the development of drug resistance that tumour cells have to DOX as well as the cytotoxicity due to drug build up in cells particularly after treatment and in older patients (Lin, S. R., et al., 2020).

1.1.10 Multi-Drug Resistance

The ability of cellular masses such as cancer to form in the primary stages of disease is driven by the alteration and dysfunction of normal cellular pathways. This is principally driven by gene expression and the differential expression of genes becomes necessary for the propagation of the tumour in terms of cellular replication as well as the vascularization, and the increased survival mechanisms it must develop in order to maintain tumorigenic properties (Rejinold, N. S., et al., 2018). One such survival mechanism for tumours in patients that have been diagnosed and are undergoing treatment with cytotoxic drugs is to increase the number of transporters for the purpose of drug efflux present in the cell membrane (Yang, M., et al., 2018). This is believed to be the chief manner in which the development of MDR occurs and as such the genes coding for the exporters are of critical importance to the development of more effective cancer treatment. As with most biological phenomena, there are a host of factors which when combined are the cause and in part this can be divided down into two branches, one wherein the drug is not absorbed as efficiently and two where the drug is being exported from the cell to a greater extent.

As previously explored, there are no importers in the ABC transport family in eukaryotes and the majority of drug import is done via diffusion through passive/channel transport proteins (Cocucci, E., et al., 2016). These channels are relatively simple in structure and so far no evidence has expressly supported the notion that they could be decreased in number in cases of MDR development, rather that tumour cells appear to specialise export and increase the number of proteins that efflux drugs from the cell. This makes sense in terms of cell homeostasis and the

fact that the channel/diffusion importers are passive by nature and are often utilized in the transport of a wider range of substrates that the cell requires to grow than the ABC family of transporters that have a relatively narrow band of substrates. In particular, are the proteins P-170 glycoprotein (P-gp), multi-drug resistance protein 2 (MRP2) and breast cancer resistant protein (BCRP) which are most commonly seen to be associated and in some cases named for the effect they potentiate (Sinha, B. K., Perera, L., & Cannon, R. E., 2019).

The efficacy of many drugs, not only chemotherapeutic, is reliant on the presence and function of P-gp. This protein is well documented as being a principal driver in the development of MDR as one of the largest exporters of chemical toxins in body cells (Aller, S. G., et al., 2009). Evidence shows that this protein, coded for by the ABCB1 gene, can efflux as much as 99.6% of DOX delivered into the cell before the drug can penetrate the nuclear membrane (Mizutani, H., et al., 2005). This alone shows that there is significant reason to look into the ways that the treatment progress initiates this action by the cell and research carried out into alternative trafficking methods for the drugs as well as inhibitors of P-gp are being looked into. One such study by Xin Li et al., in 2019 showed that loading the drug (in this case DOX) into liposomes through the use of an aptamer conjugate complex of DOX enhanced the uptake of the drug by bypassing the efflux pumps through high affinity to the nuclear membrane in vitro (Li, X., et al., 2019). Cao, et al., (2019) also found that the association of DOX with a mixed micellar system, helped the drugs to circumnavigate the intracellular space and avoid immediate efflux by P-gp by being largely incompatible with the transporter (Cao, A., et al., 2019). Other studies have continued this vein of research, with various nanomicellar delivery systems being tested in breast cancer models and showing promise in treatment of resistant tumours through efflux avoidance strategies that have also appeared to reduce the chemotoxicity exhibited with free DOX in vivo (Cheng, X., et al., 2020; Cagel, M., et al., 2020; Zeng, X., et al., 2020).

ABCG2 is the gene known to code for the expression of BCRP, a protein named so due to the first isolation of the protein being in breast cancer tumour cells that were characteristically resistant to chemotherapy. BCRP is one of the ABC transporter family proteins and has proven to be key in the efflux of anthracyclines and mitoxantrone (novantrone), the latter being another

cytotoxic agent used commonly in the treatment of breast cancer (NCBI, 2020). These transporters play key roles in a multitude of cell membranes, with expression observed in the hematologic stem cells, endothelium of veins and capillaries, placental syncytiotrophoblasts, intestinal and colon epithelium, breast ducts and lobules, the bile canalicular membrane of hepatocytes, and to a lesser degree in renal cortical tubules (Eclov, R. J., 2018; Nayak, D., et al., 2020). Molecular analysis done on 52,000 tumours by the Caris Institute revealed that this protein is the second highest in expression levels in the cell of transporters from the ABC family, with 66% of the test group having positive markers for ABCG2 expression and showing signs of increased resistance to cytotoxic drugs, and in particular to anthracyclines (Feldman, R., et al., 2015). In the last year, the use of BCRP inhibitors in combination with chemo drugs such as DOX is a logical step in the direction of better treatment for patients with resistant tumours and chemical compounds such as Quinacrine, curcumin, triazole bridged flavonoid dimers and Wedelolactone have shown promise in in vitro testing to provide a reduction in the resistance of the tumour cells to the drug in use, but also in the case of Wedelolactone to downregulate the expression of ABCG2 without toxicity (Zhu, X., et al., 2019; Nayak, D., et al., 2020; Das, S., et al., 2019).

As ABCG2 plays a key role in the determination of drug absorption, distribution, metabolism and elimination, the inhibition or deregulation of this protein is crucial to the forward momentum of breast cancer treatment (Köhler, S. C., et al., 2018; Zhou, Q., et al., 2015).

According to NCBI, ATP Binding Cassette Subfamily C Member 1 (ABCC1), the gene which corresponds to and codes for the protein Multidrug Resistance-Associated Protein 1 (MRP1), is one of the major contributors to MDR in tumours. The protein itself is a multispecific organic anion transporter, with oxidized glutathione, cysteinyl leukotrienes, and activated aflatoxin B1 as substrates (NCBI, 2020). In the aforementioned study by Feldman, (Feldman, R., et al.) in 2015 there was only one protein in the study that had a higher incidence of markers across the 52,000 cohort study and that was MRP1. This protein was found in 88% of the tumours that were immunohistochemically profiled and the combination of this and the two prior proteins, has been shown to create a resistant phenotype present in roughly 29% of the cases studied with a higher

mortality rate than each individually could present. MRP1 is of interest to researchers as this protein, being part of ABC subfamily C, possesses a non-typical membrane-spanning domain additional to the two commonly found in all other ABC family sub-groups (Sampson, A., et al., 2019). The reason for this domain is still largely undetermined and though there is at this time no evidence it could play a role in MDR it is still a likely direction for future research as a potential mechanism through which this protein could be inhibited or otherwise effected to reduce MDR occurrence in tumours (Sampson, A., et al., 2019). ABCC1 is recorded as having far less substrate specificity than other proteins explored in this study and includes intercalators, topoisomerase II inhibitors, mitosis inhibitors, antifolates, and antiandrogens (Silbermann, K., et al., 2019). This is likely also a contributing factor as to why this protein was present in so many of the tumours in the Feldman study as cells require less specialisation to acquire or require the protein. And as with BCRP these proteins are expressed throughout the body in discreetly differentiated tissues as exporters of such a wide range of substrates. There are, as with the other proteins contributing to MDR, a variety of possible avenues down which a solution could lie, with specific attention being put into looking into the role that the imaging we use has effected the solutions we attempt to develop for MDR as a result of this protein in combination with others (Sampson, A., et al., 2019).

The less prevalent protein of the same family as MRP1, ABCC2, is a protein with like influence on the success of treatment for tumours with a variety of drugs. The protein itself is also known as canalicular multi-specific organic transporter (cMOAT) and whilst primarily is found in the canalicular domain of hepatocytes it is also present in the majority of excretory organs including the kidneys, colon, lungs and small intestine, it is also expressed in malignant cells where it is a key exporter of chemotherapeutics, in particular vinca alkaloids and anthracyclines (Alamolhodaei, N. S., et al., 2020). These protein transporters play a critical role in the elimination and bioavailability of a broad spectrum of drugs including endogenous glucuronides, sulphates and GSH conjugates from the cells (Gupta, S. K. et al., 2020). Inhibition through the utilization of microRNAs (miRNAs) has shown some promising results in terms of the ability of miRNAs to interact with the DNA coding for the resistance proteins and thereby potentially having the ability to reverse the effects of MDR (Bao, L., et al., 2012). As with the other genes in this family

and with DOX in particular, nanoparticle and liposome treatment conjugates are also being explored in order to combat the resistance via avoidance of transporters. A novel option for this gene as presented by Wang et al., supposed that a ubiquitin-like protein interferon-stimulated gene 15 (ISG15) could be utilized in order to treat cisplatin-resistant tumours in the ovaries through the suppression of ABCC2 translation in the nucleus, thereby providing some relief to the multiplicative effectors of MDR in tumours (Wang, J., et al., 2020). This has potential to be used in TNBC tumours as cisplatin has been used as a treatment for metastatic tumours in particular (Hill, D. P., et al., 2019).

These four genes are proven to be vital in the cumulative effect that is multiple drug resistance and whilst there is some data in terms of the effect that treatment has on the expression of MDR related proteins in particular those that are of such a vital role in the potential to alleviate the burden created by the increased mortality rate of chemoresistant tumours. The contribution this data may provide is the ability to better characterize the changes to the cell in a time-dependent manner in order to better inform future studies that focus on drug-based solutions or the development of medicinal means through which resistance can either be treated or prevented.

1.1.11 RT-qPCR

For some time now, thanks to the continued development into the study of genetics and of cell biology, there has been available to researchers a multitude of ways through which gene expression can be studied, quantified and analysed. Methods such as SAGE, and parallel sequencing technologies (next generation sequencing, NGS) are used today in the discovery of novel gene transcripts and the identification of editing events within the transcriptome without the prior need for knowledge of the sequences and mRNA under study (Teo, Z. L., Savas, P., & Loi, S., 2017). The two technologies that up until now have been widely utilised for the analysis of cancer cell expression profiles related changes in response to genotoxic compounds or other environmental stimuli are microarrays and quantitative reverse transcriptase polymerase chain

reactions (RT-qPCR). These methods are used in favour of SAGE as the cost and efficiency of SAGE is high compared to the relatively low cost of RT-qPCR, microarrays and NGS methods. And much of the later consideration when deciding on the most optimal method to use for a study comes down to the scale of the study and the purpose of the data once procured.

Generally, the use of microarray chips is reserved for broader studies that wish to observe the expression of up to thousands of known transcripts in one assay. This method has been extremely useful in improving the accuracy of cancer diagnostics, and in the realm of research has proven to be invaluable for a snapshot of whole-cell transcription as is the case in the study by Smith et al., in 2006 that utilised this technique to observe 224 genes throughout various cell pathways (Smith, L., et al., 2006). This study and those like it are useful in terms of identifying targets for future studies that have greater focus on, for example one family of proteins. Typically it would be unnecessary to use a microarray based approach when looking into the expression profiles of 5-50 genes and in these cases PCR-based methods such as RT-qPCR as these studies are typically for the sake of validation in terms of a potential or categorising of a target identified in a previous study concerning the same topic (VanGuilder, H. D., Vrana, K. E., & Freeman, W. M., 2008).

RT-qPCR itself was conceived around the year 1993, whereupon its developers won a Nobel Prize (VanGuilder, H. D., Vrana, K. E., & Freeman, W. M., 2008). The method built on the already extant notion of PCR, by utilizing reverse transcriptase, an important enzyme that can be used to synthesize a complementary strand of DNA (cDNA) from RNA, rather than through the use of DNA from samples. This allowed great advance to be made as previously research was limited to the research of that which was already DNA, and thereby changing the question being asked from what was being expressed to how it was expressed and how that expression could change.

Further expansion on this method brought about the ability to see the results of these experiments in real-time, which has allowed for a faster turn-around from the point of experimentation to data analysis which in a diagnostic setting has been crucial not only in cancer treatment and diagnostics, but across the board with a variety of diseases (Bustin, S. A., & Nolan, T., 2020).

These features make the use of RT-qPCR on ABC transporter family member genes ABCC1, ABCB1, ABCC2 and, ABCG2, the most efficient and fast approach to analyse and obtain data on the way in which these genes are effected by the treatment of cells by DOX.

1.2 Objective of the Study

It is still an unfortunate reality that we are unable to treat many kinds of cancer with real effectivity. Certainly, over the last 20 years, we have observed and developed methods that have increased the precision of radiation and chemotherapy as technological advance has allowed, but it is a pervasive issue in this field that our treatments are in many cases largely ineffective whilst also being destructive to the recipients. As a scientific community, we are working towards goals of more definitive treatment, as interrogation of the genome and cellular homeostatic response has led the way for research into more precise methods of treatment, for example, CAR-T cell therapy (Wang et. al., 2017). However, in the mean-time, we have a responsibility to attempt to increase the efficacy of the long-established treatments we are still using. In following with this logic, the intent of this study was to explore avenues through which the efficacy of chemotherapy could be increased with specific scrutiny being placed on the methods by which tumour cells defend themselves against harsh chemicals in an attempt to survive.

The processes of cellular efflux have been confirmed by numerous studies, such as Ughachukwu et al., (2012), Fletcher et.al., (2016) and Dréan et.al., (2018), to have a significant effect on the ability of a tumour to survive chemotherapy by increasing the rate at which the drugs in question are effluxed, i.e. removed, from the cells. Drug efflux is mainly controlled by proteins in the cellular membranes, known as ATP binding cassette (ABC) transporters and as such the expression of these proteins is critical to our understanding of drug resistance. Furthermore, while this has been studied in a general sense across many cancers, it was the goal of this study to make the experiment more relevant to New Zealand, particularly as a country that sits within the top ten countries in terms of breast cancer incidence per capita (GLOBOCAN, 2018).

Taking stock of chemotherapies commonly used in the treatment of a range of breast cancers, and the cells lines available, doxorubicin (DOX) was chosen as the drug for the treatment of MDA-MB-231 cells. These cells are characterized as triple-negative breast cancer (TNBC) cells, and as such lack expression of receptors for estrogen, progesterone and HER2 and are proven to show high heterogeneity compared to other breast cancer tissues (Foulkes, et. al., 2010). This subtype of breast cancer is often identified as the "most clinically aggressive" with a high cellular proliferation rate, leading to larger tumours more likely to metastasize, invade auxiliary lymph nodes and, therefore result in a far worse clinical prognosis (Anders & Carey., 2009; Chavez, et. al., 2012; Tan & Dent, 2018). The aggressive proliferation of these cells results in small windows during which treatment can be received with genuine effectivity and stresses the importance of developing better treatments with higher precision and a lower rate of failure (failure in this sense being the inability to control or eliminate the tumour growth). It is with this in mind that the study entailed herein was conducted to better comprehend how these cells combat the treatments and to allow future research to widen the window of effectivity for the drugs and methods in use today by taking advantage of that information.

For the sake of brevity, as this study was to be conducted over the course of one year, specificity was necessary and so the study concerns four target genes of interest, each related to the main components and regulators of large molecule efflux, ABC transporters. The target genes were selected from a host of genes from several studies that broadly inspected the interactions between ABC transporters and multidrug resistance (MDR) such as those by Amawi et.al (2019) and Fletcher et.al. (2016).

By studying genes linked with MDR we have the ability to develop methods to take action against the cellular processes that would decrease the effectivity of drugs in treating cancers of all kinds. It reduces the opportunity that tumours have of metastasizing and becoming more deadly to act more decisively and efficaciously particularly in those cancers, like TNBC, that have shown themselves to be more clinically aggressive. Analysis of the way that ABCC1, ABCB1, ABCG1 and, ABCC2 are affected in the cellular response to a constant dose of DOX over time will provide important data for the timeline and clinical approaches that have the most benefit and efficacy so

that treatment plans for patients may improve outcomes and prognosis while the scientific community develop treatments less destructive to the patients.

In order to observe the changes in expression as cellular drug resistance develops treatment was repeated several times and RT-PCR conducted on samples to quantitatively analyse how over the course of repeated treatment expression changed. The main aim of this consequently being to analyse the expression changes over a number of treatments to understand how certain vital target genes known to play roles in MDR are differentially expressed during chemotherapy in order to work towards the development of co-treatments or better delivery systems to prevent MDR in the future and improve the outcomes of patients diagnosed, not only with TNBC but with other tumour types known to rapidly develop MDR.

Chapter 2

2.1 Methods and Materials

2.1.1 Introduction

Doxorubicin (Adriamycin, DOX) is an anthracycline used commonly in the treatment of breast cancers of all sub-types, principally in conjunction with other drugs as the majority of breast cancer is treatable through the use of hormone-based therapies and cytotoxic drugs for maximum effect. However, in triple-negative breast cancer, a sub-type of breast cancer that does not possess the hormone receptors we see in HER2+, ER+ and PR+ breast cancers, we have yet to be able to find a hormone therapy that works due to the lack of common receptors. This makes chemotherapeutics the first viable choice in numerous TNBC cases, much to the detriment of patients diagnosed with these tumours as they are well known to swiftly develop chemoresistance, resulting in what is considered a very aggressive tumour type becoming unresponsive to treatment. This poses a greater risk for patients of metastasis and reduces the likelihood of survival and increases the chance of recurrence should the tumour be removed or treated initially.

To decrease future risk and decrease the burden on society that cancer places, we must look to develop treatments based on this new problem, multi-drug resistance (MDR). And first and foremost, in order to do this, we need to have the best understanding possible of the way that cells respond to drugs like DOX. One such way that we can achieve a better knowledge-base around this topic in these tumours is through analysis of the means by which the cells alter the expression of genes in direct response to a treatment regimen is with the use of real-time gene product analysis. The method to do so has been developed with reference to studies done prior that concerned a more extensive range of genes, not just those known to be primary initiators of MDR in breast cancer tumours. In these cases, changes in the expression profile of genes like BCRP1 were noted but not observed to the same specificity as intended with this methodology.

2.2 Materials

The reagents and materials utilised for the purpose of analysing and determining the gene expression of ABC-Transport related genes in MDA-MB-231 (ATCC® HTB-26™) cells are displayed in Table 1 including the suppliers and catalogue numbers. The cells themselves are described and a supplier detailed in Table 2.

Table 1:Reagents and Chemicals used in experimental processes.

Chemicals/ Reagents	**Supplier**
Phosphate-Buffered Saline	Sigma-Aldrich (MDL No. MFCD00131855)
TrypLE™ express enzyme	ThermoFisher (Catalogue:12604021)
Penicillin-Streptomycin Solution	ThermoFisher (Catalogue:15140122)
Fetal Bovine Serum (FBS)	ThermoFisher (Catalogue:10091155)
Doxorubicin Hydrochloride	Sigma-Aldrich (CAS Number: 25316-40-9)
Roswell Park Memorial Institute (RPMI) 1640 Medium	ThermoFisher (Catalogue:11875119)
L-Glutamine	ThermoFisher (Catalogue:25030081)
MagNA Pure Compact RNA Isolation Kit	Roche LifeSciences
LightCycler® 480 Master Reagents	Roche LifeSciences
Ethanol	N/A

Table 2:The breast cancer cells used in the experiment and their supplier and identifiers.

Cells/Cell Features	**Supplier**
MDA-MB-231 (ATCC® HTB-26™)/ Human adenocarcinoma cells from female mammary tissue known to express the WNT7B oncogene.	ATCC®

For the identification and analysis of the genes in question, a series of Oligo primers were developed and ordered from IDT and are represented in Table 3 and Table 4.

2.2.1 Primers

Table 3: Target genes and relevant primers and details pertaining to their use and the RNA to be extracted.

Target Gene	**Amplicon Size**	**Tm**	**Length**	**Sequence (5'- 3')**	**Primer Bank ID**
ABCB1 (F)	75bp	60.8 °C	21bp	TTGGCTGATGTTTGTGGGAAG	21536377c1
ABCB1 (R)		60.9°C	21bp	CCAAAAATGAGTAGCACGCCT	
ABCC1 (F)	184bp	60.2°C	21bp	GTGAATCGTGGCATCGACATA	86787725c1
ABCC1 (R)		62.6°C	20bp	GCTTGGGACGGAAGGGAATC	

ABCC2 (F)	131bp	60.5°C	23bp	CCCTGCTGTTCGATATACCAATC	188595701c1
ABCC2 (R)		60.4°C	23bp	TCGAGAGAATCCAGAATAGGGAC	
ABCG2 (F)	247bp	62.4°C	21bp	CAGGTGGAGGCAAATCTTCGT	62526032c1
ABCG2 (R)		60.2°C	22bp	ACCCTGTTAATCCGTTCGTTTT	

Table 4:Reference genes and relevant primers to be utilised in comparative measurement of the target gene expression.

Reference Gene	Amplicon Size	Tm	Length	Primer Sequence (5'- 3')	PrimerBank ID
RPS13 (F)	187bp	60.1°C	21bp	AAGTACGTTTTGTGACAGGCA	14591910c2
RPS13 (R)		61.9°C	23bp	CGGTGAATCCGGCTCTCTATTAG	
Act-β (F)	250bp	60.8°C	21bp	CATGTACGTTGCTATCCAGGC	4501885a1
Act-β (R)		60.2°C	21bp	CTCCTTAATGTCACGCACGAT	

2.2 Methods

2.2.1 Cell Lines and Cell Culture

For this experiment, MDA-MB-231 were purchased from American Type Culture Collection (Manassas, VA, USA) (ATCC® HTB-26™) cells were grown in Roswell Park Memorial Institute (RPMI) Medium that was supplemented with 100units/mL penicillin G sodium, 100µg/mL streptomycin, 4mM L-glutamine, and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2. Cells were passaged upon reaching 80% confluency to no more than passage 30 and samples were taken for RNA isolation and extraction as the cells were between 70% and 90% confluency. Two cultures of cells from the same line were kept in tandem, cycling and feeding for these cells was commenced on separate days from one another with two isolated containers of the same medium to prevent any potential cross-contamination from effecting all cells required for experimentation.

2.2.2 Selection of Target and Reference Genes

Target genes were selected from consideration of relevant literature concerning multidrug resistance in MDA-MB-231 cells and across all model TNBC cells in general (Wilkins, 2015; AL-Eitan, L. N., et al., 2019; Xiao, Q., Zhou, Y., & Lauschke, V. M. 2020). These genes were as listed in Table 3 and each corresponded to a significant gene within the ABC transporter family. In contrast to these genes and for the express purpose of comparison and verification of expression results and their significance, two reference genes were selected, RPS13 a ribosomal shock protein and beta (β)-actin, a gene that codes for cytoskeletal factors key in all cells.

2.2.3 Development of a Doxorubicin Resistant Cell Line

2.2.3.1 Cell Seeding

Cells were observed to determine confluence and health prior to the seeding process. Cells of 70-80% confluency were taken for passaging and firstly washed with 5mL pre-warmed sterile PBS. The determination of this level of confluency was done by consultation with figure 3 (National Cancer Institute, 2010). After carefully aspirating the PBS from the cells, so as to not disturb the cell layer, 2mL of TrypLE^{TM} express enzyme solution was introduced to the cell culture and incubated for 2 minutes at 37°C to increase the rate at which the adhesion between cells and the growth flask was broken. Cells in this mixture were then gently pipetted to disrupt any cells that may still have been attached to the growth surface and each other and 3mL complete RPMI warmed to 37°C added to solution in order to halt the trypsinization of the cells in culture. The cell solution was then taken in full and placed in a 15mL centrifuge tube and spun down at 200*g* for 5 minutes before removing the supernatant and resuspending in 1mL of non-supplemented RPMI. 10µL of this media and cell solution was then taken and mixed with the same volume of Trypan Blue solution and mixed briefly before pipetting 10µL into a hemocytometer under a cover slip and counting the living cells in each cell region and calculating the average.

The total number of cells in the whole solution was then calculated using the following equation:

$$No.\ of\ cells/mL = (average\ number\ of\ cells\ per\ square \times 2) \times 10^4$$

The cells were then seeded at a concentration of 2×10^5 cells/L, as a higher concentration of cells was beneficial to the treatment process wherein roughly 50% of the cells would be killed through exposure to doxorubicin.

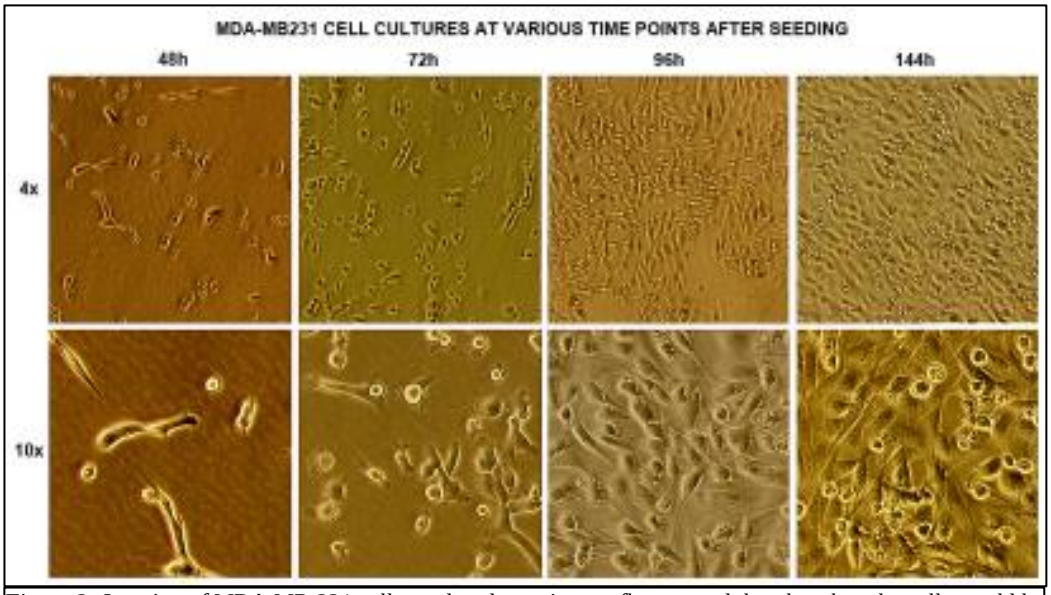

Figure 3: Imaging of MDA-MB-231 cells used to determine confluency and thereby when the cells would be split and seeded onto new plates. The 96-hour point was considered the 80% confluency point. (National Cancer Institute, 2010).

2.2.3.2 Treatment

In accordance with other, similar research, the design of the experiment took into account that at an approximate concentration of 1.24μM half of the cells in a culture of MDA-MB-231 half of the cells would die after treatment and that repetition of treatment would lead to a linear increase in resistance to the drug of choice across a recorded average of 2-5 "pulse" treatments at that concentration (Smith, L., et al., 2006; McDermott, M., et al., 2014; Tsou, S. H., et al., 2015; Carlisi, D., et al. 2017). With respect to this and in order to develop a line of cells resistant to Doxorubicin (DOX), cells at 80%-90% were washed with 5mL of PBS pre-warmed to 37°C prior to the treatment solution being pipetted into the growth flask. The treatment concentration was determined from the IC50 value of Doxorubicin specific to MDA-MB-231 cells (1.24μM), and

5mL of this was added to the cells for a 24 hour period at 37°C in a humidified atmosphere with 5% CO_2 (McDermott, M., et al., 2014). The treatment solution was then aspirated and washed once more with 37°C PBS before being recovered for 5-7 days in complete medium (RPMI). This process was repeated for one of the samples twice and thrice for the final sample, each done in duplicate.

2.2.3.3 Preparation of Tetrazolium Solution

An MTT stock solution of 12mM was prepared ahead of experimentation by adding 1mL of sterile PBS to a falcon tube containing 5mg of MTT powder. This mixture was vortexed/ sonicated until dissolved before being wrapped in aluminium foil to protect from UV exposure. The solution was stored in a refrigerator at 4°C for 2 days before use and could be stored up to 4 weeks should consequent use be required.

2.2.4 Cell Viability Assay

An MTT tetrazolium cell viability assay was necessary in the quantification and verification of the activity of the doxorubicin in treatment. The cells were first seeded into a 96-well plate at 1 x 10^4 cells/well (Note each well was seeded with a volume of 90µL of cell culture). The test included treatment of 4 test cell groups, those that had received DOX treatment, once, twice and thrice, and then a positive control containing cells that had not been exposed to DOX. Another test was conducted in the same plate in a well containing only media (without cells). These cells were then incubated at 37°C for 24 hours prior to the addition of 10µL of the yellow tetrazolium MTT reagent. Following the addition of the MTT reagent, the cultures were incubated at 37°C for 4 hours, till the presence of a purple precipitate was detectable. Once the precipitate was formed, 100µL of DMSO detergent solution was added and the cells

were left to sit at room temperature in a dark room for 2 hours. The plate was agitated for a brief period then absorbance was read using a spectrophotometer set to 570nm.

2.2.5 RNA-Extraction and RT-qPCR

Total RNA was extracted from the MDA-MB-231 treatment samples with the Roche MagNA Pure LC instrument using a MagNA Pure LC RNA isolation kit—High Performance (Roche Life Science, New Zealand). The total elution volume was 50 μL, which was stored at − 80 °C prior to analysis of gene expression. Absolute quantification of gene expression by one-step quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) was performed on the LightCycler 480 instrument II (Roche Diagnostics, Auckland, New Zealand) using the LightCycler® EvoScript RNA SYBR® Green I Master (Roche Diagnostics, Auckland, New Zealand), primers (Integrated DNA Technologies, Inc., Singapore) at a working concentration of 0.9 μmol L−1 and RNA in a final volume of 20 μL. There was no need for adjustment of magnesium as the master mix was optimised with a fixed concentration of Mg(OAc)2. The reactions were performed using the following thermocycling conditions: 60 °C for 15 min (reverse transcription/cDNA synthesis), 95 °C for 10 min (transcriptase inactivation and initial denaturation step) and 40 cycles of amplification (95 °C for 15 s for denaturation and 60 °C for 1 min for annealing and extension). All primers were sourced from PrimerBank (Spandidos, A., et.al. 2012) and were ordered from Integrated DNA Technologies (IDT, 2020 Integrated DNA Technologies, Inc.) as listed in Tables 3 and 4.

2.2.6 Data analysis

Expression of genes from these cells were detected through use of RT-qPCR to find the absolute quantified amount of cDNA in each sample of the primed sequences under observation in this

experiment. The quantified amount of cDNA was expressed as an absolute number of copies made of the gene, which was then manipulated utilizing the $2^{-\Delta\Delta Ct}$ formula, where $\Delta Ct = Ct_{GOI} - Ct_{Ref}$, and $\Delta\Delta Ct = \Delta Ct_{Treated} - \Delta Ct_{Normal}$ thereby giving the logarithmic fold change in expression, which, whilst having the drawbacks of the assumption of the PCR efficiency being equal to 2 it was accepted as a limitation of the study (Rao, X., et al., 2013). Only one of the two reference genes were used for the analysis of results, this being the readings obtained for ACT-b as RPS13 readings were not constant and therefore the Ct_{Ref} value was calculated as the mean of solely the ACT-b Ct values.

2.2.7 Statistical Analysis

Inquiry into the data was done through a one-way analysis of variance (ANOVA) with further validation conducted through a post-hoc (Dunnett's) multiple comparisons test in order to compare means between samples across each treatment to find significance to a p-value of $P < 0.05$ to inform statistical significance.

Chapter 3

3.1 Results

3.1.1 Gene Expression analysis utilising RT-qPCR

In order to determine the expression of the four target genes in conjunction with the reference genes, the samples were tested using RT-qPCR which produced the data shown in Figure 4. The data represented is the mean value of the crossing points (Cp) determined from two replications per sample per treatment. The crossing point itself is the value pertaining to the number of completed cycles at which the sample crosses the detection threshold and therefore is inversely correlated to the overall expression of the genes in these cells.

Figure 5 reveals that there was a significant decrease in expression between ABCC2 (P= 0.00167), ABCG2 (P= 0.01021) and RPSI3 (P= 7.84E-06) over the course of treatments. For the ABCC2 readings, the cells that were treated once with DOX had a fold increase of 19.18. This showed a significant effect (P-value < 0.05) over the course of just a single treatment in the expression of this gene and indicating that there was a significant decrease in the expression in response to treatment with DOX. The changes after this were not significant in terms of the difference to the expression of reference gene ACT-b, but there was a definite decrease in effect with regards to the initial response and therefore an increase in expression noted after this first sudden decrease as shown by a decrease in $2^{-(\Delta\Delta Ct)}$ from 19.18 to 0.41 and then 0.13.

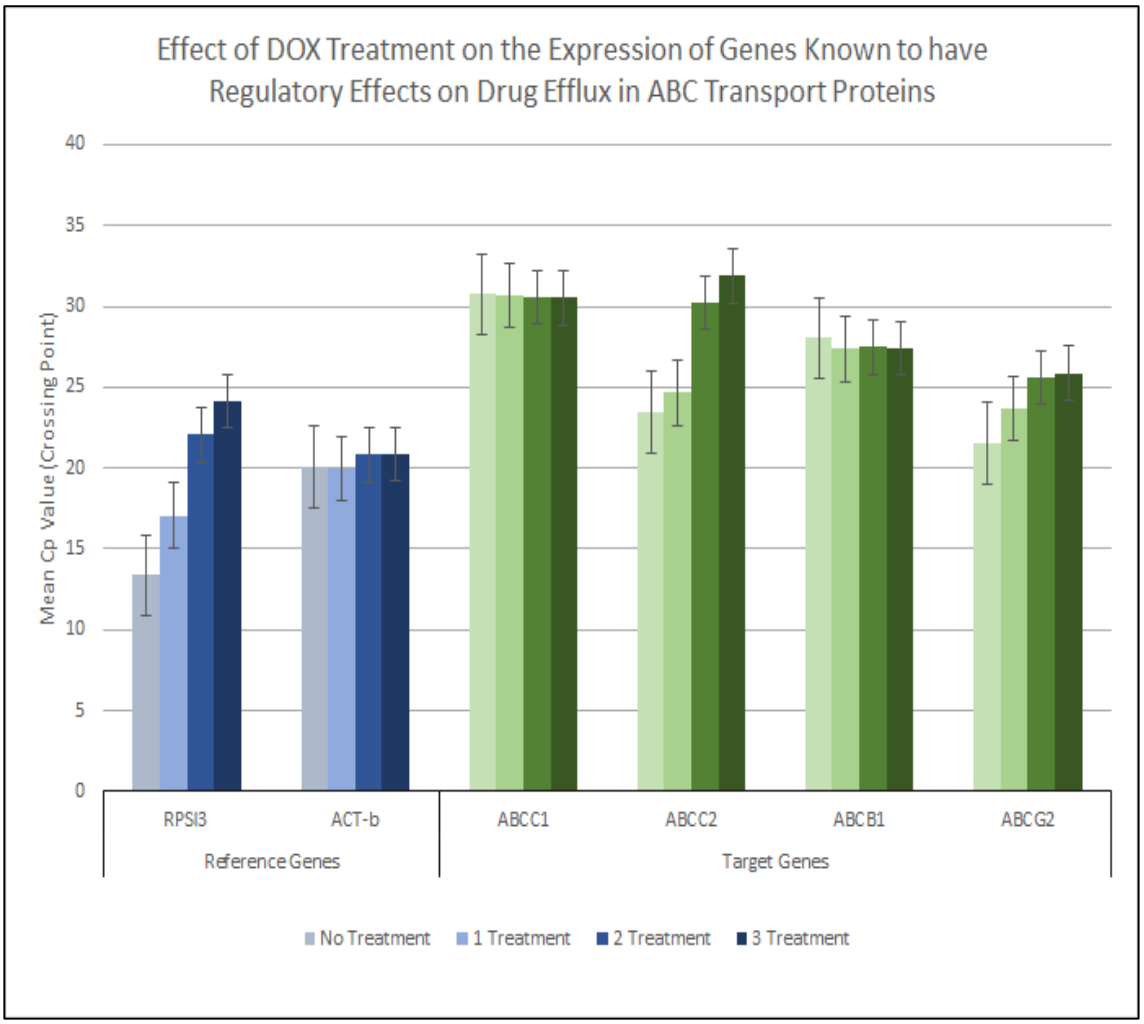

Figure 4: The Cp or crossing point of 6 genes was reported as an absolute quantity, the number of copies of gene targets is presented in this graph across 4 points with an increase of the number of treatments. Data is as a product of Cp means ± SEM.

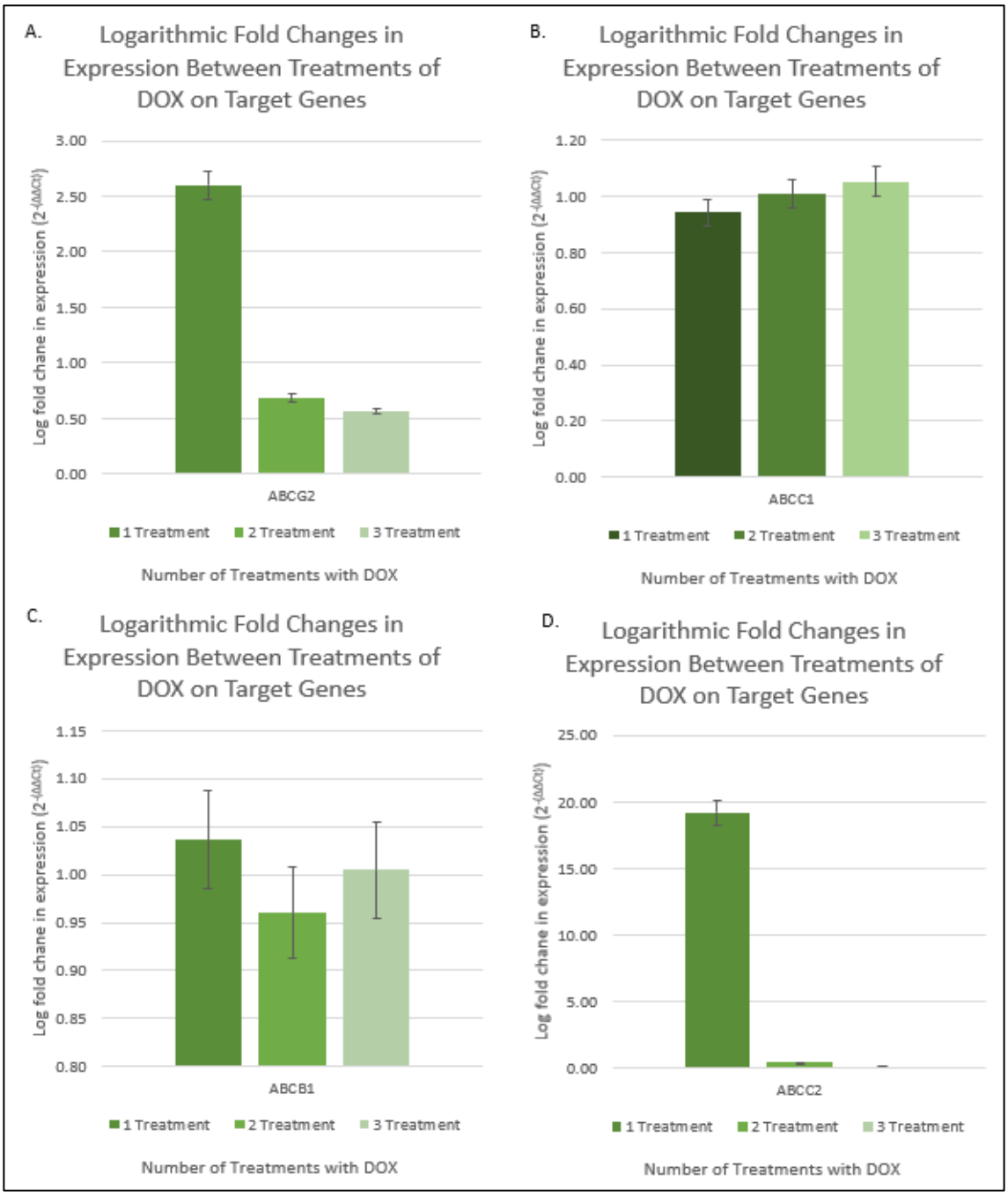

Figure 5: Graphs showing the log fold change in expression as determined via 2-(ΔΔCt) analysis of the Cp values, upon which ANOVA analysis revealed that the largest effect in terms of a change in expression was indeed as indicated from Cp data and the changes in ABCC2 and ABCG2 expression were significantly higher after one treatment before then falling to lower levels of expression post initial DOX treatment. $P<0.05$ for these assumptions as backed up by the Dunnett's multiple comparison test and reported here ± SEM. A, shows the fold expression of gene ABCG2; B, the fold expression of gene ABCC1; C., the fold expression of ABCB1 and finally D., shows the log expression change of ABCC1. Each 2-(ΔΔCt) was determined via difference between the expression of each treatment sample in contrast to the sample that received no treatment.

A noted decrease in the expression of ABCG2 was signalled by a 2.6 fold change in expression, the initial effect was the most in terms of fold change as with ABCC2. Fold change in expression

ranged from 2.6 after the first treatment of DOX to This indicated a significant exposure-dependent effect on the expression of ABCG2 as was the case with ABCC2 and as visualised in comparison to RPSI3 in figure 4. ANOVA analysis was conducted on this to identify the significance between gene expression with each treatment and found that whilst significant variation occurred in expression with samples tested for ABCG2 and ABCC2 there was no significant effect on expression for ABCC1 and ABCB1 between treatments, though the overall expression of these genes was roughly 2 and 3 fold difference from ACT-b.

3.1.2 Doxorubicin treatments

Insofar as the two genes that showed a significant effect of treatment, there was the most response in expression for both genes ABCC2 and ABCG2 between no treatment and the first, with the greatest fold change occurring in this period. This was typically followed by an increase in expression levels gradually over the course of the next two treatment cycles.

3.1.3 MTT Assay for Cytotoxicity

Cells used in this assay were seeded at a density of 4,700 cells/well and treated according to the method supplied, however, upon treatment with the tetrazolium solution cells were found to have detached in all treated cell samples and no purple solute had formed within the wells. As cell death had also occurred in remaining cultures there were no samples remaining to repeat the assay and therefore no results to report in terms of cytotoxicity. As all cells died it can be concluded that the cells were no longer viable and the reasons for this were explored thoroughly so as to determine the best course of action for potential consequent research.

Chapter 4

4.1 Discussion

4.1.1 Introduction

Gene expression is a vital part of the maintenance of cellular homeostasis and cell viability as without the expression of genes, the proteins so critical to cellular function, even in malignant tumour cells, are not made. Therefore, there is so much importance surrounding the understanding of situations where expression differs from what is considered a typical level in a cellular environment. It is normal to see changes in gene expression in the response of a cell to some extracellular influence and chemotherapy has been proven to be no exception to this (Starobova, H., et al., 2020; Kewitz, S., et al., 2016; Martínez-Campa, C., et al., 2017). Each of the genes selected for this experiment was chosen from a vast pool of candidates from research in this field that observed these cells through either DNA microarrays or other PCR-based methods (Fletcher, J. I., et al., 2016; Carlisi, D., et al., 2017; Tsou, S. H., et al., 2015). Prior research on this topic, viewed the expression experiments through the lens of resistance solutions or broad expression effects on a multitude of genes across TNBC cells, rather than narrowing down the topic to the cumulative changes leading to the development of MDR via ABC transporter protein expression. Of specific interest, was the way in which major genes considered to contribute to the effect were differentially expressed through the course of MDR development. To this end, a distinct effect on the expression of two genes was observed, in genes considered central to cell function specifically in the function of drug efflux and the protection granted by this activity to the DNA that would otherwise as a result of treatment become irreparably damaged. Each of the genes selected for analysis, in non-resistant cells are expected and have been documented as being expressed in a manner that would not prevent a range of xenobiotics, including anthracyclines like DOX from penetrating the nuclear membrane and being unable to act upon the DNA to kill the cell.

4.1.2 DOX Effect on Expression

The results of expression analysis were not as anticipated based off of the information gathered prior to undertaking the experiment. Each of these genes has some critical role in the development of MDR and so for there to be no upregulation of any of the target genes in cells as seen in the results, translates to signify that the cells were likely not yet resistant to DOX despite their improved survival during, and recovery after, each treatment. It is in this case where confirmation of the viability of the cells via MTT assay would have been useful in providing more quantitative insight into the effect the DOX treatments were having on the cells outside of the qualitative feedback collected throughout the course of the treatment and greater experimental process. However, in regards to the lack of resistance, there was evidence in the drop in $2^{-(\Delta\Delta Ct)}$ after the initial treatment that suggests that the cells may have been returning to a slightly more normal level of expression post the first treatment and as the cells moved into the final treatment the levels of expression in these two samples, despite the first reaction to DOX was normalizing. There was though, an overall lack of up-regulation of genes seen throughout the RT-qPCR data, the down-regulation of genes ABCC2 and ABCG2, coding for MRP2 and BCRP consecutively, further supported a secondary hypothesis that there was some underlying activity in the cell culture causing the cells to down-regulate xenobiotic transporters and that likely led to the cells dying as was observed immediately following the isolation of samples and prior to MTT analysis.

In similar expression-based inquiry performed on ABCG2, in colorectal and cervical cancer samples, incidences of decreased expression were linked to an increase in the cellular production of nitric oxide (NO) (Gupta, N., et al., 2006). Low or down-regulation of ABCG2 has been shown to increase the number of protoporphyrins that accumulate in the cell and through which the cell may utilize this and its properties as a heme precursor to produce nitric oxide via inducible nitric oxide synthase (iNOS) (Granados-Principal, S., et al., 2015). The effects of iNOS as an enzymatic producer of NO and that of NO overproduction itself has been supported via knockout studies and shown to increase the rate of tumorigenesis in cells (Gupta, N., et al., 2006). Potentially this could

have been the MDA-MB-231 cells attempting to up-regulate proliferation pathways after their chemotherapy recovery cycles.

Conversely to this point, there is a body of study that also acknowledges the role that ABCG2 has in other pathways as a promoter of cell proliferation and how down-regulation of the gene can concurrently cause the halting of the cell cycle (Zhao, Y., et al., 2020; Chen, Z., et al., 2010; Al-Momany, B. Z., et al., 2020). RNA interference (RNAi) methods connoted that the specific down-regulation of ABCG2 resulted in a reduction of cells in S phase and as a consequence of that were more likely to remain in G0 or G1 phases of the cell cycle where the cells arrested (Chen, Z., et al., 2010). Specifically in regards to DOX treatment, this validates the knowledge we have towards the mechanisms of DOX and how it has been seen to epigenetically effect the cell via down-regulation of ABCG2 in order to promote cell death (Zhao, Y., et al., 2020; Batrakova, E. V., et al., 2006). This effect begets the notion that prior to the development of resistance to DOX is developed in MDA-MB-231 cells there is a period during which the cells have decreased resistance and are more susceptible to treatment and potentially this could extend to being a period where effects on the cellular environment are not only easier to achieve but provide a window of opportunity for more precise treatment based around this gene. At the very least this could be a factor of future studies as the potential to utilize the lowered expression of ABCG2 in which time the cell is more susceptible to a range of xenobiotics and chemical substrates could lead to an important revelation for chemotherapeutics.

ABCC2 was the other gene in this experiment for which a change in expression was observed and the change therein, while not being consistent with a majority of the literature on how it was regulated by the cell after treatment, did show variation in expression and is therefore of importance to the characterisation of these genes in the scope of chemotherapeutics. This gene, as with ABCC2, was down-regulated over the course of the experiment and much like with this gene there is little in the way of literature describing a theory on why a cell undergoing a treatment of a concentration known to not be lethal to all cells in the culture would begin down-regulating the genes required to generate proteins that could combat the effect of the DOX.

One study carried out in 2016 by Litviakov, N. V., et al., conducted an *in vivo* study that discerned the effect of neoadjuvant chemotherapy on the generation of mutations at loci within major ABC transporter genes, two of which were ABCC2 and ABCG2. These genes were revealed by RT-qPCR and affymetrix microarray to have deletions within the MDR locus 83% of cases in ABCC2 and 79% of ABCG2 cases. All of these patients were recorded as having down regulated levels of these ABC transport genes and in these cases they were also observed as having less of a response to treatment initially before an increased response was recorded post-treatment. This provided insight into the fact that the allelic deletions in the cells likely resulted in lesser protein expression in the tumours, a factor consistent across studies. A potential reason for this could be the effect of the chemotherapy, not on the transport protein expression itself but in one of the regulatory pathways that contribute to the overall efflux of cytotoxins. Kinase-regulated pathways such as cAMP and PI3K, which are known to be principal players in cascade signalling within the cells, are likely the mode through which the activation of transcription for ABC transporters are effected and could be another route of inquiry to follow in future research (Crawford, R. R., et al., 2018; Litviakov, N. V., et al., 2016).

The lack of significant response from ABCC1 and ABCB1 is almost certainly a result of the lack of resistance the cells had developed to the DOX treatment. Due to the specificity of the primers used in the experiment, despite the gene itself having some conserved regions across some organisms such as chimpanzees, *C.elegans* and mice, it is unlikely that the exact region was present in case of a contaminant and therefore unlikely that this had a significant effect on the results of the PCR analysis (NCBI, 2020). This seems an unlikely route of future investigation without knowing the specific contaminant, if a contaminant was involved and not some other effect. Far more probable than the response being masked by another organism's expression is the fact that there was no induced resistance and these genes did not respond to treatment in the way that the other two targets did. It is possible that there are more underlying reasons as to why this is the case but as was the case with ABCG2 and ABCC2 down-regulation, the lack of effect is not something widely publicised in current literature.

There is not much research documenting the process of down-regulation of these genes in recent literature and as a consequence there is much speculation that can be done in the face of these results (Cherdyntseva, N. V., et al., 2016). Despite this obvious limitation, from the wealth of studies preceding this showing that up-regulation of these four genes is a sign of MDR it is unlikely that this was induced in the MDA-MB-231 cells over the course of this experiment. What was uncovered was the potential that early during the process of treatment or perhaps treatment at a specific concentration dependent on the cell mass could provide insight into a stage in treatment not particularly well characterized. Obviously this opportunity would be significantly easier to pinpoint in vitro, rather than in vivo studies, but it could drive a more precise chemotherapy treatment schedule that could provide various benefits if able to help prevent MDR from developing or reduce the speed at which it does.

That there was differential expression observed in the targets ABCG2 and ABCC2, is further evidence that despite thorough research into the development of resistant MDA-MB-231 cell lines during assay development and optimisation, there perhaps were not enough treatments conducted on the cells or perhaps there should have been a gradient in the treatment concentration in order to see results that better answered the initial question of how ABC transport protein expression would change in response to DOX treatment in TNBC cells. Despite this the fact that there was still insight to be gained as the cells passed through what appears to be an intermediary stage in the development of MDR has allowed for better insight into the development of MDR rather than only seeing the results of protein up-regulation (Pilco-Ferreto, N., & Calaf, G. M., 2016).

4.1.3 Reference Gene Selection

The use of reference genes for RT-qPCR is crucial for the analysis of data and can in many ways be the deciding factor of the experiment as the expression of reference genes is utilized in the normalization of the majority of samples. For the sake of good method development it is therefore important that multiple reference genes are used in the event that an unexpected result is obtained

and there is some effect on the expression of one of the genes selected for reference. Such was the case in this experiment, where ACT-b expression, as was expected, showed a standard and regular level of expression in the cells across each treatment and the only variation in expression was determined to be non-significant and likely due to PCR efficiency than a genuine change in expression. This gene and the other reference gene RPS13 were selected through the reading of literature using the same cell line and the same drug for treatment (Han, J., et al., 2019; Fu, Y., et al., 2015), and were considered strong candidates as being noted to have no known interaction with DOX or via the pathways DOX may effect.

The fact that there was some effect, and a statistically significant one, in the reference gene RPS13 further supports the theory that there was an underlying effect within the cell culture that had some impact on the expression of various genes in the cell and likely was the result of the cell death experienced during the MTT assay process. In normal MDA-MB-231 cell culture RPS13, which codes for the ribosomal protein s13, is a key structural element in the small ribosomal subunit that is highly conserved in eukaryotes and archaea (Ivanov, A. V., Malygin, A. A., & Karpova, G. G., 2011). There have been no previous indications that suggest they would be effected aside from a standardised increase in expression in cancer models (Buoso, E., et al., 2020). Despite this there is little research into the effectors of expression variance in this gene and this should be looked into further in the future.

4.1.4 Treatment Modification and Method Development

Initially the experimental design entailed an increase in the concentration over the course of 4 treatments in order to slowly build tolerance in the cells, beginning with a concentration of DOX just below the IC50 concentration as reported for MDA-MB-231 cells in numerous other studies and and building up to a more clinically relevant concentration of DOX such as would be used in treatments of actual patients (Smith, L., et al., 2006). This would have resulted in a final concentration of 32μM DOX in cell culture however, methods outlined as to achieving this were

not overtly clear and the reproducibility therefore deemed unlikely successful. Despite this, an attempt to achieve this method of resistance was carried out for 2 months, with cells poorly recovering from treatments and taking on average 3 weeks to recover from 30-50% survival of cells to a point where the doubling time of cells was back to an approximate 38 hours. During this time there were multiple cases of contamination and cells not recovering from treatment with characteristic signs of cytotoxic response such as non-uniform cell membranes, spontaneous detachment and a slower doubling time.

To this end, the protocol for treating cells was modified with much reference and examination done of literature concerning or relating to the development of DOX resistant cell lines utilising a method more in line with pulse therapy in order to treat the cells whilst attempting to decrease the amount of time required for recovery and still develop resistance (McDermott, M., et al., 2014).

Recovery of the cells was another major hurdle in the development of the assay as even when following guidelines set by previous successful studies, there was a period of time where the cells, even at half the IC50 concentration, were not recovering for nearly a month from the treatment. This was to be further investigated and changes to the method enacted when the cells at use at that time became contaminated

To remedy this or to at least attempt to provide more reproducible and reliable data, in the future the IC50 would be fund independently prior to commencement of research as this is one possible avenue through which the treatment cycle could be improved as it is likely that if not the potential contamination that perhaps the strain of MDA-MB-231 cells had increased sensitivity to DOX than has been seen in other research. Recovery, and the improvement of the process is key in the saving of time and increasing the efficient of the entire treatment cycle and also for the sake of obtaining results faster with more accuracy it could be argued that studies into the optimisation of cell recovery for the purpose of chemoresistance studies may need to be undertaken.

4.1.5 Cytotoxicity Assays

Assays used to explore and quantify the effect of cytotoxic substrates or drugs on the growth and survival of cells is an integral part of developing an entire cohesive picture of chemoresistance. For the present study this was applied in the form of an MTT assay, utilizing the conversion of yellow tetrazolium MTT to the soluble substrate Formazan that can be visualised as a purple precipitate suspended in solution that absorbs light characteristically at 540nm. The use of this assay is well documented and for an experiment utilizing a treatment strategy of multiple treatments of the same concentration over time, the expected outcome would be a curve showing gradual increase in the cell viability over time. Unfortunately, this was not the case. Cells were plated onto the 96-well plates from the experimental 6-well plates after a feeding cycle for the purpose of running this assay concurrently with the freezing down of the cultures that were to be used for the extraction of RNA for the RT-qPCR. During this there was some concern about the health of the samples but due to time constraints on the experiment, the decision was made to progress to the assay and expression experiments. After 24 hours the cells in the 96-well plate that had been seeded around 10,000 cells per well were looking irregular in shape and despite relatively low confluence the cells appeared to have been detaching. The cells remaining from the two experimental procedures that were in the incubators at that time were also looking unhealthy and were mostly detached in turbid media. The cells in the 96-well plate had not reached this point and as such the tetrazolium was added to the culture and left to incubate for 4 hours. At the end of this period there should have been the appearance of a purple precipitate however there was no visible colour change to the solution and under observation under microscope, the cells were more detached. At this point the cells proceeded to the next step of the assay but it was unsurprising to find them dead after the following steps.

Significant consideration was taken as to the most optimal viability assay for these cells in this experiment and with regards to the DOX treatment the cells would be receiving. With awareness of the fact that some chemical compounds can interact with assay substrates literature consulted

on this topic revealed no possible issues with the tetrazolium assay in combination with DOX treated MDA-MB-231 cells (Abel, S. D., & Baird, S. K., 2018; Marinello, P. C., et al., 2019; Alkaraki, A., et al., 2020).

4.1.6 Contamination in Research

It is probable, given the manifold contamination events that occurred in the development stages of the experiment and during the treatment phase, that the death of these cells was due to contaminants rather than any phenom of tumour biology. Infection by yeast or bacterial organisms is one of the most common impacts on research concerning and utilizing cell cultures for any kind of experimentation as the impact can cause setbacks of months (Lincoln, C. K., & Gabridge, M. G., 1998). This experimental procedure was designed with these effects in mind and changes to the initial protocol were required to account for an increased rate of infection that appeared to be a lab-wide issue. Initial tests were conducted through the use of only one culture of cells in order to attempt to reduce the materials used during the course of the experiment, however, after several attempts to successfully treat cells and have them recover only to observe infection by yeast or fungal organism as diagnosed through observation of rapid-onset turbidity and pH change in the growth medium (Niehues, H., et al., 2020).

All cell cultures being experimented on were cycled on different days with media split into two separate containers to prevent any contamination affecting both sets of cells. This was done in response to issues early in the experimental process wherein the cell culture became contaminated and the progress of two treatments and the recovery period required for both was lost in primary and duplicate cultures. One potential solution for this would have been to sample the infection and develop an assay to identify the contaminant potentially using sequencing and determine the identity of the contaminant and be able to go forward targeting that contaminant through a change of protocol or to attempt to act prophylactically to avoid cases if the addition of PenStrep or other antibiotics is not sufficient (Lin, J., et al., 2019; Niehues, H., et al., 2020; JR, R., & Mundayoor,

S., 2020). With regards to this though it should be noted that in terms of the ability of cell culture to be used as accurate models for the purposes of studying expression *in vitro* there is some argument that the use of antibiotics in culture experiments can lead to less accurate results or results that do not translate directly into real-life cancer cases as living patients can not be treated with antibiotics as continuously as they are used in in *vitro studies* (McDermott, M., et al., 2014).

Chapter 5

IV.I Conclusion

In conclusion, there were several key findings that were uncovered through this experiment. The first of which is that there could be a window of down-regulation in ABCG2 and ABCC2 genes prior to the development of the resistance to DOX and during this time it could be concluded that the cell would be more susceptible to treatment, not only with DOX but with other drugs such as Paclitaxel and Cisplatin, both of which TNBC tumours are known to develop MDR for. This discovery should be further studied as it could create a potential avenue for better treatment of this type of tumour that has long been considered aggressive and often results in poor survival rates. As well as this, a hole has been identified in the literature surrounding ABC transporter genes and the effects that could be observed during the development of MDR to drugs in *in vitro* studies and the lack of comprehension the current body of study has for effects that may cause down-regulation of these genes.

The latter point, that down-regulation was achieved in this experiment of two crucial genes to the expression of an MDR phenotype, should receive further inquiry, as even if this was achieved through a contamination event, there may be some effect causing this that could be used to the advantage of future therapeutic approach. However, and in regard to potential contaminants, there are several limitations that were discovered and addressed upon reflection of this experiment that may impact the reproducibility of this experiment and that is an issue going forward that will not be easy to account for. And whilst the failure of the MTT assay to quantify the viability of cells may have reduced the ability of this body of work to be trustworthy or of the reliability of the final results, there were visual cues and expression results that are still valid even without the confirmation of the cells ability to be viable after DOX treatment that are worth inquiring into in the future. Finally, time and the lack thereof is the bane of anyone with a deadline and this case was no different; At the very least the experiment and methodology utilized in this work should provide a solid starting point for studies wishing to use MDA-MB-231 cells or doxorubicin for

the investigation of gene expression in ABC transporters in order to provide better chemotherapy treatment in the future.

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