Reversing ABCC2-mediated oxaliplatin resistance in human PANC-1 cells using the CRISPR-Cas9 system.

Camren Cullen

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Abstract:

Pancreatic ductal adenocarcinoma (PDAC) is considered to be a highly aggressive malignancy that often shows minimal symptoms, early signs of the disease are difficult and often impossible to identify until the later stages of the disease due to the location of the pancreas. Oxaliplatin is a critically important treatment for not only pancreatic cancer but also many other gastrointestinal cancers, it plays a key role in improving the disease and increasing the life expectancy of patients. However, due to acquired drug resistance and accumulated toxicities the efficiency of oxaliplatin in treating PDAC is limited. High doses of the drug can cause a variety of toxic effects in patients, while low doses of the drug are less effective at eradicating the tumor and can lead to resistance to the drug. Previous studies have indicated that the membrane transporter protein (MRP2) works as an efflux pump to move cytotoxic compounds out of the cell and plays a role in the cellular accumulation of cytotoxic platinum drugs. It is suggested that the MRP2 protein is over expressed in pancreatic cancer cells leading to the increased efflux of oxaliplatin out of the cell limiting its efficiency. From this we have hypothesized that resistance could be reduced by knocking out the ABCC2 gene (encoding MRP2). The CRISPR-Cas9 gene editing system can be used to knock out your gene of interest in an in vivo cell model. We hypothesized that the CRISPR-Cas9 system can be used to knock out the ABCC2 gene in Panc1 cells.

Panc1 cells were transfected with sgRNA and Cas9 protein using the CRISPRMAX transfection kit, the efficiency of the ABCC2 knockout was assessed using a T7 endonuclease cleavage assay. An accumulation study of the ABCC2 substrate 5-carboxy-2',7'-dichlorofluorescein (CDCF) was used to distinguish the difference in accumulation between the knockout cells and the wildtype. MTT assay was undertaken to compare the

differences of oxaliplatin sensitivity between MRP2 knockout and the wildtype PANC1 cells. The results show the feasibility of the CRISPR-Cas9 system in knocking out the ABCC2 gene. The genomic cleavage assay yields a cleavage efficiency of 42.43%. We were able to see an increase in CDCF accumulation and oxaliplatin cytotoxicity in the MRP2 knockout cells in comparison to the wildtype. The results show that the use of CRISPR-Cas9 to silence the ABCC2 gene does reduce the function of the MRP2 protein and improves the oxaliplatin sensitivity in PANC1 cells.

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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, it contains no material previously published or written by other people. Except for work that has been explicitly defined and acknowledged. To the best my knowledge the material has not come previous work that has been submitted for an award or any other degree/diploma of a university or other institute.

Signature:

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Abbreviations

°c	Degrees Celsius
µl	Microlitre
5FU	Fluorouracil
AAV	Adeno-associated Virus
ABC	ATP-binding cassette
ATP	Adenosine triphosphate
CDCF	(5)6-carboxy-2,'7′,dichlorofluorescein
CDCFDA	(5)6-carboxy-2,'7′,dichlorofluorescein diacetate
CRISPR	Clustered regularly interspaced short palindromic repeats
CT	Computed tomography
DACH	Diaminocyclohexane
DMY	Dihydromyricetin
DNA	Deoxyribose nucleic acid
DSB	Double strand break
EUS	Endoscopic ultrasonography
FOLFOX	Folinic acid, Fluorouracil and Oxaliplatin
FOLIFIRNOX	Fluorouracil, Irinotecan, Oxaliplatin and Leucovorin
g	Grams
gRNA	Guide RNA

HDR	Homologous directed repair
LDH-A	Lactate dehydrogenase
MDCT	Multi-detector row computed tomography
MDR	Multidrug resistance
ml	Millilitre
MMR	Mismatch repair
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRP	Multi-drug resistance protein
NHEJ	Non-homologous end joining
PAM	Photo spacer adjacent motif
PBS	Phosphate Buffered Saline
PC	Pancreatic Cancer
PCR	Polymerase Chain Reaction
PDAC	Pancreatic adenocarcinoma
pmol	Picomole
PSC	Pancreatic stellate cells
RNA	Ribonucleic acid
RNA	Ribonucleic acid
RNPs	Ribonucleoprotein

SCLC	Small cell lung cancer
SD	Standard deviation
siRNA	Small interfering RNA
TALENS	Transcription activator-like effector nucleases
ZFNs	Zinc finger nucleases

Chapter one

Introduction:

Cancer continues to create an enormous burden on society. It is a disease that effects both economically developed, and undeveloped countries. Over the last few decades, the occurrence of cancer has continued to increase significantly due to the growth and aging of the population, as well as an increased prevalence of risk factors in society associated with urbanization and economic development. This has prompted an increase in the study of this field to greater understand the complexity, heterogeneity of the disease, and high epidemiology among humans (Hazafa et al., 2020; Torre et al., 2015b). The development of resistance to multiple drugs with varying structures and targets has been grounds for concern and has created a major obstacle in producing effective cancer therapy (Szakács et al., 2006). The ability to understand the relationship between epigenetic alterations, their effects on gene expression has become important in cancer research. The knowledge that epigenetic alterations are reversible has opened up new therapeutic pathways for treating various diseases most importantly cancer (Ansari et al., 2021).

Failure of chemotherapy in cancer treatment and chemoresistance occurs due to efflux of the chemotherapeutic agents out of the cancer cells (Fletcher et al., 2010). A new technology that has been developed to fight the disease is gene editing, the technology provides a means of producing deletion, mutation, or substitution in genes of interest. The technology consists of zinc finger nucleases (ZNFs), transcription activator-like effector nucleases (TALENS), and clustered regularly interspaced palindromic repeats associated RNA guided Cas9 (CRISPR-Cas9). These technologies utilise nucleases to generate

double strand breaks (DSB) in the DNA and then the repair pathway specific to each system repairs the break (Suleiman et al., 2021).

GLOBOCAN 2018 has estimated cancer incidence and mortality produced by the International Agency for Research of cancer. They were able to estimate that there will be 18.1 million new cancer cases develop and 9.6 million cancer related deaths in 2018 (Bray et al., 2018).

1.1 Pancreatic cancer.

1.1.1 What is pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is an incurable, lethal disease with a growing incidence rate. The disease has a devastating prognosis mostly due to the failure of many chemotherapies (Cid-Arregui & Juarez, 2015; Neesse et al., 2011). Adenocarcinoma is considered the most common and most lethal type of pancreatic cancer (PC), however, there are other rare tumour types such as sarcoma, lymphoma, pseudopapillary and intraductal papillary mucinous (McCarter, 2018). Pancreatic adenocarcinoma is considered a highly aggressive malignancy and shows profound resistance to treatment regimens (Bardeesy & DePinho, 2002).

Pancreatic cancer originates in the tissues of the pancreas, the pancreas is an organ in the abdomen that lies behind the lower part of the stomach (Mayo Foundation for Medical Education and Research, 2021) (Figure 1.2). The pancreas can be found in the retroperitoneal and is relatively insensate, this means that early signs of the disease will be difficult if not impossible to identify until the symptoms of the disease obstruct the duodenum, pancreatic or binary duct (McCarter, 2018). In the development of PDAC, pancreatic stellate cells (PSC) are important as they are over-expressed in this particular form of PC. In a normal pancreas PSC accounts for about 4% of pancreatic cells. For the

most part these cells remain dormant however, after response to cytokines and oxidative stress PSCs will proliferate and produce large amounts of extra cellular matrix (ECM) components. Activated PSCs are major drivers of cytokine and ECM production and culminate the perfect microenvironment for PDAC (Schober et al., 2014). In the western world most gastric cancer patients are diagnosed when the tumour has become inoperable. For these patients' chemotherapy is the only treatment option (Wagner et al., 2006). In most cases, late diagnosis is due to the non-specific symptoms associated with the disease. Some of the symptoms of pancreatic cancer are painless jaundice, epigastric back pain and non-specific gastrointestinal symptoms, including; bloating, food intolerance, pancreatic insufficiency, and weight loss (McCarter, 2018). The symptoms associated with pancreatic cancer can differ at the time of presentation and may be dependent on the location of the tumour within the pancreas (Figure 1.1). The pancreas is typically divided into the head, neck, body and tail (Figure 1.1;1.2) (Mizrahi et al., 2020).

There are multiple risk factors that can increase the prevalence of PC, the risk factors for this disease include; family history, smoking, chronic pancreatitis, obesity, diabetes mellitus, heavy alcohol use and possible dietary factors (Cruz et al., 2014). Smoking is considered a significant risk factor for pancreatic cancer, with a 2012 study showed the current rate of smoking for that year correlated with a twofold increased risk of pancreatic cancer. This study also showed that the risk of developing the disease increased with the number of cigarettes smoked and the length of time spent smoking (Bosetti et al., 2012). Individuals that have a family history of pancreatic cancer have also shown a heightened risk of developing PC. If we can gain a better understanding of the genetic aspect of this disease, we can provide a basis for cancer risk counselling and early screening for pancreatic cancer (Klein et al., 2004).

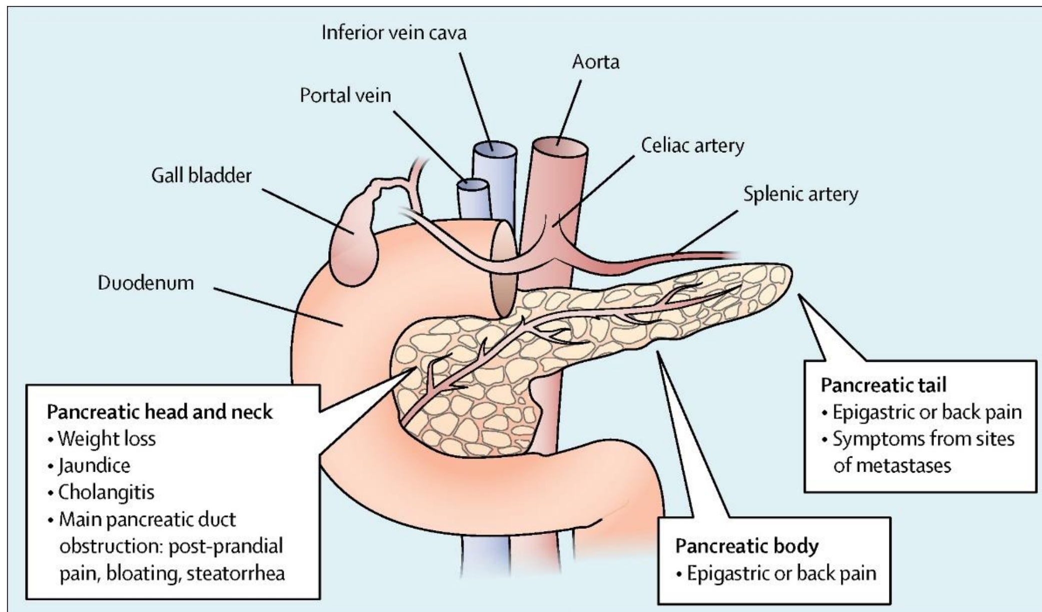

Figure 1.1 Common signs and symptoms at the site of tumour (Mizrahi et al., 2020)

Pancreatic adenocarcinomas form due to the progression of lesions occurring in the pancreatic ducts. There is a genetic aspect to these carcinomas consisting of mutations in KRAS, TP53 and SMAD4/DPC4 (Bardeesy & DePinho, 2002). The aggressive nature of this disease is related to the genetic mechanisms causing uncontrolled growth and metastases (Cid-Arregui & Juarez, 2015).

There are 5 different stages of pancreatic cancer, below are the following stages that are used for the diagnosis of pancreatic cancer.

Stage 0 is carcinoma in situ. These cells are found in the lining of the pancreas, these abnormal cells are able to become cancerous, once cancerous can spread into nearby normal tissues.

In stage I cancer has developed but is found only in the pancreas. At this stage tumours can be divided into two stages A and B. In stage A, the tumour is 2 centimetres or smaller

while in stage B, the tumour is larger than 2 centimetres but has not become larger than 4 centimetres.

Stage II can be divided into stages A and B depending on the size and the spread of the tumour. In stage A the tumour has become larger than 4 centimetres, while in stage B, the tumour can be any size and has spread to 1-3 nearby lymph nodes.

By stage III the tumour can be any size and tumour cells have spread to four or more lymph nodes and/or major blood vessels near the pancreas.

In stage IV the tumour has become metastatic, the tumour has become any size and has spread to one or more different parts of the body such as the liver, lungs and peritoneal cavity (PDQ Adult Treatment Editorial Board, 2002)

Over the last two decades gemcitabine has been considered the gold standard of treatment for pancreatic cancer as well many other gastric cancers, however recently a new method of chemotherapy has surpassed gemcitabine. Combining fluorouracil, irinotecan, oxaliplatin and leucovorin (FOLIFIRNOX) has shown great therapeutic advantage in treating the disease over gemcitabine, with a greater survival rate in patients and increased tumour response (Cid-Arregui & Juarez, 2015). A constant issue surrounding the treatment of PC is multi drug resistance (MDR). The expression of the ATP-binding cassette (ABC) transporters by cancer cells is considered one of the major mechanisms responsible for insufficient drug accumulation which can lead to resistance and decreased tumour response (Adamska & Falasca, 2018).

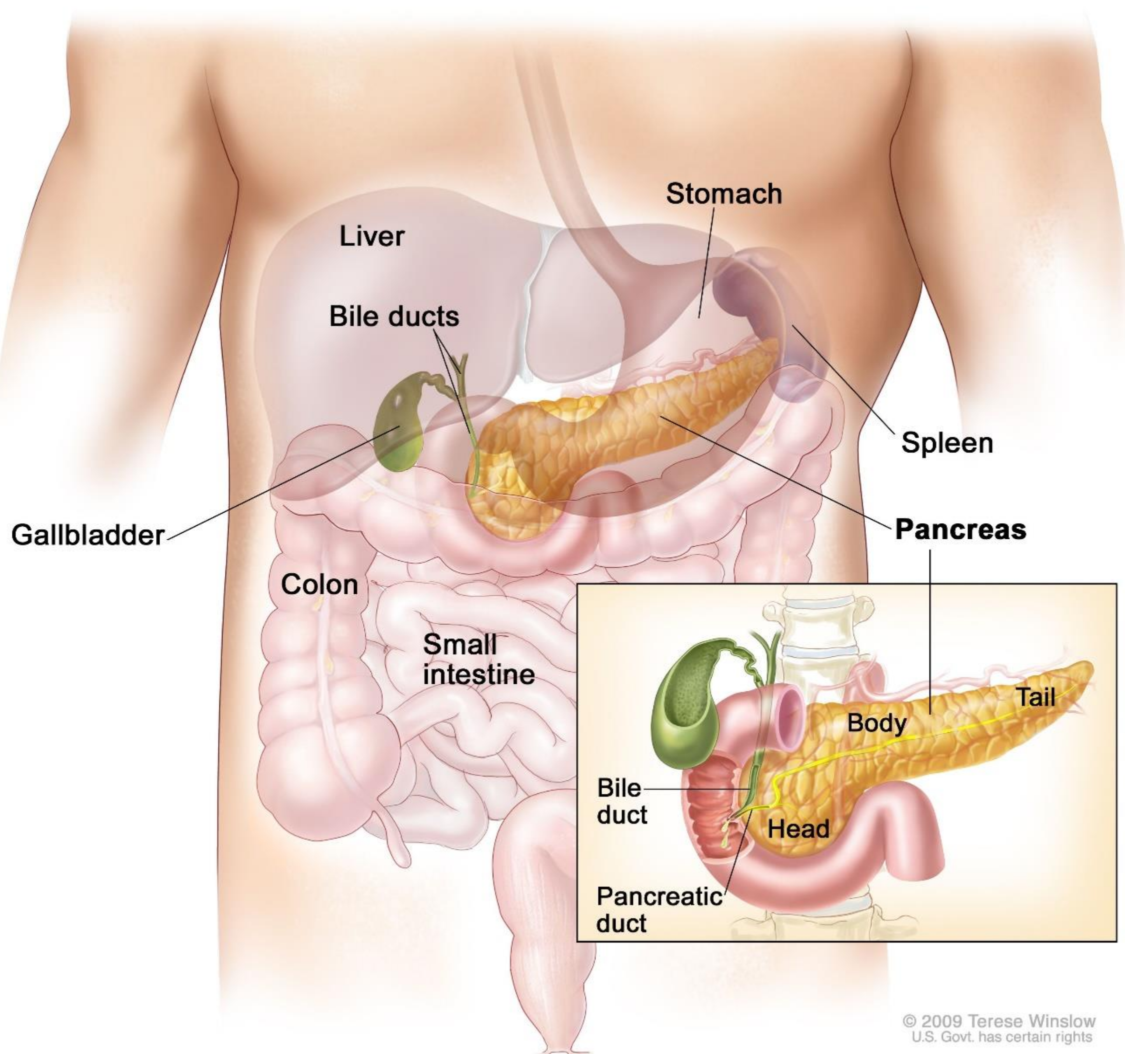

Figure 1.2 Anatomy of the pancreas within the body and enlarged. The pancreas has three major areas; head, body and tail. It is found in the abdomen near the stomach and intestines (PDQ Adult Treatment Editorial Board, 2002).

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1.1.2 How pancreatic cancer affects New Zealand and the rest of the world

Pancreatic cancer is the seventh leading cause of cancer-related deaths worldwide (Rawla et al., 2019a). New Zealand has high rates of PC with The New Zealand cancer registry showing that in 2018, 23,296 non-Maori and 2,862 Maori people had cancer, of those

26,158 kiwis 632 people had pancreatic cancer coming second to colorectal cancer at 3,189 people (Ministry of Health, 2020). Of the 632 people in New Zealand with pancreatic cancer 328 were male while 304 were female (Ministry of Health, 2020). If we compare that to the 2017 rates of 563 kiwis having pancreatic cancer, 248 being male and 279 being female (Ministry of Health, 2019), we can see that there is an increase in the rate of pancreatic cancer from 2017 to 2018.

PDAC is a common type of PC and is considered one of the leading causes of cancer related deaths all over the world. Patients with PC tend to have an overall survival rate of 5-years at less than 5% (Rajabpour et al., 2017). It is the fourth most common cause of cancer related death in United States and its incidence is increasing (Al-Hawary, 2016). Globally 432,242 new deaths due to PC occurred in 2018, with 458,918 new cases reported (Rawla et al., 2019b). With GLOBCAN estimating that by 2040 355,317 cases of PDAC are estimated to occur (Rawla et al., 2019b)

Despite the advancements in survival rates of different cancer types over the last 40 years, there has been minimal progression in treatment and survival for pancreatic cancer patients having seen no substantial changes. (Falasca et al., 2016).

Between 2009 and 2013, a span of 5 years, the annual change in incidence of PC increased by 1% in men and 1.1% in women, with the 5 year survival rate increasing from 2.5% to 8.5% between 2006 and 2012 (Rahib et al., 2014; Saung & Zheng, 2017). In 2010 the incidence of pancreatic cancer was 12.1 per 100,000, it is projected that by 2030 the incidence will grow to 15.1 and then is believed to climb to 18.6 by 2050 with an average growth 1.1% (Cho & Petrov, 2020).

1.1.3 Methods of imaging and diagnosing pancreatic cancer

There are multiple methods employed to identify and diagnose pancreatic cancer, some of the most prominent diagnostic procedures used in PC diagnosis are image-based technologies, these technologies include; computed tomography (CT), endoscopic ultrasound, and magnetic resonance imaging (MRI) (Chrystoja et al., 2013). The most popular forms of imaging that are used are ultrasonography, multi-detector row computed tomography (MDCT), endoscopic ultrasonography (EUS), and MRI (E. S. Lee & Lee, 2014). Ultrasonography has been used as a first line diagnostic tool. It is used in patients who are presenting with jaundice and abdominal pain. This technique can be both non-invasive and cost-effective (E. S. Lee & Lee, 2014). It provides a fast and efficient assessment of the pancreas in most patients and it has a high sensitivity for detecting biliary tract dilation and establishing the level of obstruction. Although, this method is less reliable in visualising the pancreatic parenchyma and duct due to body habitus and overlying bowel gas that can obstruct the view (Balci & Semelka, 2001; Brambs & Claussen, 1993). In most cases using ultrasound by itself does not always provide consistency in accurately diagnosing and staging, making it a valuable tool for initial assessment of a patient suspected to have the disease. However, it needs to be followed up by other imaging procedures (Michl et al., 2006). Tissue harmonic imaging is another useful technique for diagnosing PC, with reports demonstrating improved image quality (Shapiro et al., 1998). Though, the additional use of ultrasound can further improves the technique. The technique requires the administration of intravenous bolus micro-bubbles. The micro-bubbles resonate and produce a strong echo when an ultrasound beam strikes the circulating contrast (Kitano et al., 2004; Michl et al., 2006). There has been evidence to suggest that EUS as a diagnostic tool has produced a great impact on the clinical evaluation of PC. EUS produces a higher resolution image of the pancreas, far surpassing

that of the transabdominal ultrasound (US), CT, or MRI (Kitano et al., 2019). Endoscopic ultrasonography differentiation of malignant to benign tumours of the pancreas has been possible in tumours larger than 30mm in size but difficult in tumours less than 20mm in size (Yasuda et al., 1988). While MDCT is most commonly used as a pre-operative tool for examining patients that are suspected to have pancreatic cancer (E. S. Lee & Lee, 2014).

1.1.4 Treatments for pancreatic cancer:

Surgical resection has been considered the only potentially curative treatment for pancreatic ductal adenocarcinomas. Of the patients that are recommended for surgical resection fewer than 20% have a survival expectancy of five years after the surgery is complete (Cruz et al., 2014). Although surgical treatment is often not an option for most patients with fewer than 20% of patients have surgically resectable tumours (Kleeff et al., 2016). The disease has perineural and vascular local growth with early distant metastases that prevents surgical resection (Kleeff et al., 2016). Instead an effective treatment of metastatic cancer usually requires the use of toxic chemotherapy. For most patients multiple drugs are used, as resistance to single drugs is a common occurrence (Szakács et al., 2006)

Chemotherapy is an important part of pancreatic cancer treatment for both metastatic disease but also for after curative resection. It has been reported that adjuvant chemotherapy can significantly improve disease free survival (Springfeld et al., 2019). While there have been numerous research efforts and developments of various anticancer drugs and treatment strategies over the past decade, there has been minimal improvement in patient survival (Vetvicka et al., 2021).

Oxaliplatin is a therapeutic agent that is found in FOLFIRNOX treatment, there has been intrinsic or acquired resistance associated with oxaliplatin which is a major reason why treatments fail in gastric cancer limiting therapeutic success (H. Zhang et al., 2018). The current standard of treatment is six months chemotherapy of FOLFIRINOX for patients that are fit enough for the protocol, otherwise six months of gemcitabine (Springfeld et al., 2019). A combination of multiple chemotherapeutic agents has recently overtaken the long-standing gemcitabine as the first line response. FOLFIRINOX and gemcitabine plus nab-paclitaxel has also been found to be effective (Chin et al., 2018). Although there are many treatment options that clinicians can consider, treatment choice needs to be tailored to the individual person, taking into consideration their performance status and the side effects of the chemotherapy agents (Chin et al., 2018).

FOLFOX is another 5FU, oxaliplatin anti-cancer treatment that is often used as a second line treatment option (Zaanan et al., 2014). A FOLFOX regimen may consist of 85 mg/m^2 for a two-hour infusion, Folinic acid 400 mg/m^2 for a two hour infusion, followed by a 5FU bolus of 400 mg/m^2, then a further 2400 mg/m^2 5FU infusion over 46 hours. Such a regimen can be administered every two weeks until the patient's disease progression changes or toxicities limit the treatment (Zaanan et al., 2014)

Producing a knockdown affect via gene silencing has shown promise in effectiveness in tumour and stromal cells by inhibiting tumour promoting genes (Vetvicka et al., 2021). Biswas et al., (2019) showed promising data that using siRNA to silence the MRP2 protein can increase the accumulation of oxaliplatin in pancreatic and colorectal cancer cell lines. Pharmacogenetics is a method to select patients that benefit from a specific therapy that best matches the individual's tumour and the tumour's genetic profile (el Hassouni et al., 2019). This form of treatment aims to identify individuals predisposed to risk of toxicity from chemotherapeutic agents (W. Lee et al., 2005). A major challenge associated with

pharmacogenetics is identifying subgroups of patients that will benefit from the treatments (el Hassouni et al., 2019).

The overall goal of chemotherapy is to eradicate tumour cells without damaging normal host tissue (Kwok et al., 2017)

1.1.5 Chemotherapy resistance in Pancreatic cancer

There are some major challenges that impede the success of cancer chemotherapy the most important is multidrug resistance (MDR). MDR can be attributed to the overexpression of membrane transporter proteins (J. Zhou et al., 2006). Studies have indicated that there are multiple mechanisms that produce drug resistance in pancreatic cancer, these include changes in individual genes and signalling pathways, the influence of the microenvironment surrounding the tumour, and the presence of highly resistant stem cells (Long et al., 2011). Tumour microenvironment plays an important role in the uptake of chemotherapeutic agents. The microenvironment consists of the interstitial tissue that surrounds the cancer cells. These are pancreatic stellate cells, fibroblasts, endothelial cells, inflammatory cells, nerve cells and non-cellular components such proteoglycans and fibrosis proteins (Gnanamony & Gondi, 2017; JA et al., 2015). These components are known to interact with cancer cells and produce a desmoplastic reaction that induces migration and chemoresistance (Schober et al., 2014). There is also a fibrous stroma that surrounds the cancer core constituting to around 90% of tumour volume which can impede the proper delivery of anti-cancer drugs (Gnanamony & Gondi, 2017). The environment that a tumour is surrounded by can influence its resistance to chemotherapy. Hypoxia, in Pancreatic cancer cells, is a mechanism known to cause chemoresistance. Hypoxia is important for the growth of PDAC, metastasis and chemoresistance. Lactate dehydrogenase (LDH-A) is a major check point for the switch to anaerobic glycolysis, this ensures the supply of energy and anabolites to maintain a hypoxic environment. LDH-A is

significantly increased in a hypoxic environment which inhibits the cytotoxic effect of gemcitabine creating chemoresistance to gemcitabine in PDAC (M et al., 2014)

More than 165 genes have been identified as a protagonist in the growth of drug resistance of pancreatic tumours, some of these include mucins, NF-κB, RAS and CXCR4 (Quiñonero et al., 2019). MRP2 was also found to induce drug resistance in pancreatic cancer, MRP2 is a multidrug resistant protein from the ABC family (Noma et al., 2008). Multidrug resistance proteins are responsible for the catalysis and detoxification of xenobiotics and excretion of metabolites and can be controlled at the transcriptional level by interaction of exogenous compounds (Pułaski et al., 2005). In multiple pancreatic cancer tissues MRP2 over expression was observed in 77.5% (31/40) tissue types compared to normal pancreatic tissues (Noma et al., 2008).

If we are able to increase the efficiency of drug delivery and decrease drug efflux this will have a great impact in the success of treatment for pancreatic cancer (Long et al., 2011). There had been evidence to suggest that dihydromyricetin (DMY) has antitumor properties. By treating colorectal cancer with DMY, Wang et al (2016) found that chemosensitivity to oxaliplatin was enhanced and oxa-induced apoptosis was increased, suggesting that DMY treatment inhibited the MRP2 expression (Z. Wang et al., 2016). There is also plenty of evidence to suggest that silencing multi drug resistant proteins such as the MRP2 protein may be able to increase the cellular accumulation of anti-cancer drugs within cancer cells and thus improve therapeutic efficacy (Biswas et al., 2019; Myint et al., 2015, 2019a).

1.2 Oxaliplatin

Oxaliplatin is a third-generation platinum derivative that blocks DNA replication and transcription by causing DNA breaks and inhibiting DNA synthesis. The use of oxaliplatin

treatment has been approved for multiple cancer types, including pancreatic cancer. (Kwok et al., 2017). The drug undergoes a rapid nonenzymatic biotransformation where it forms multiple different reactive platinum intermediates. These intermediates bind rapidly and comprehensively to plasma proteins and erythrocytes (Culy et al., 2000a). This therapeutic agent has shown efficiency both in vitro and in vivo in tumour cell lines. The efficiency is believed to be a result of platinum-DNA abducts forming within the cell, these appear to be more efficient in blocking DNA replication and have shown a greater cytotoxic than adducts formed by cisplatin (Raymond et al., 1998). Oxaliplatin has proved to have a better safety profile than cisplatin as it continues to shows a lack of cross-resistance with cisplatin and carboplatin, while its cytotoxicity is harmonious with fluorouracil and folinic acid (di Francesco et al., 2002; Lévi et al., 2000). Although oxaliplatin has a better safety profile than cisplatin, oxaliplatin is very similar to cisplatin and both have similar mechanisms of inducing apoptosis (Mehmood, 2014)

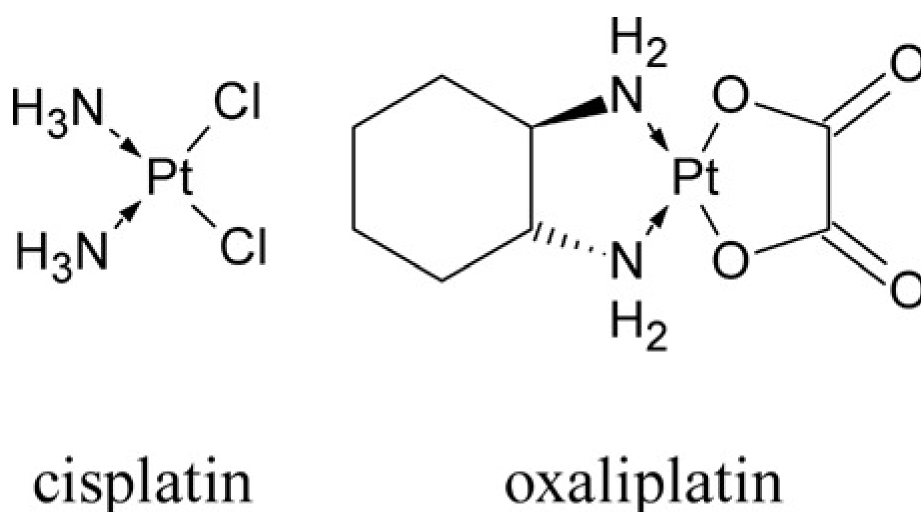


Figure 1.3 The molecular structure of cisplatin and oxaliplatin chemotherapeutic agents side by side (Jerremalm et al., 2006).

Oxaliplatin is often compared to cisplatin however the drug differs from cisplatin and carboplatin due to oxaliplatin's possession of a bulky diaminocyclohexane (DACH) moiety and the presence of an oxalate leaving group (Stein & Arnold, 2012). Oxaliplatin has become the preferred treatment as it has shown success in treating cisplatin resistant cancers. This could be due to the fact that oxaliplatin has a different activity profile compared to cisplatin (Fojo et al., 2005; Stordal et al., 2005). However, in the Stordal et al., (2005) study, they found that oxaliplatin may not be as effective as cisplatin in lung cancer treatment. The study tested resistance development in SCLC cell lines using oxaliplatin and cisplatin. Both developed drug resistance but in cisplatin a 2 fold higher dose was cytotoxic in low resistant cells whereas a 2 fold higher treatment of oxaliplatin still had viable cells suggesting that may not be as effective.

1.2.1 Clinical use

Oxaliplatin, known commercially as Eloxatin, is commonly used in conjunction with other chemotherapeutic compounds (Simpson et al., 2003). Oxaliplatin is often combined with fluorouracil 5FU/folinic acid (Simpson et al., 2003). There have been multiple clinical trials that have focused on the efficiency oxaliplatin treatment. These include; metastatic colorectal cancer, advanced ovarian cancer, non-Hodgkins lymphoma, non-small cell lung cancer and breast cancer (Culy et al., 2000a).

Oxaliplatin has demonstrated high activity in treating colorectal cancer, more so when combined with other therapeutic agents (M. L. Chen et al., 2010). A previous study has shown that when using oxaliplatin treatment for two hours over 6 months, the overall survival of patients increased by 4.3 months compared to patients treated with gemcitabine (11.1 months vs 6.8) (Conroy et al., 2011). In this trial the treatment regimen for oxaliplatin was 85mg per square meter over two hours, immediately followed by leucovorin at 4oomg per square meter over two hours, with an addition of 180 mg per

square meter of irinotecan after 30 minutes given over 90 minutes. The treatment was then followed fluorouracil at 400 mg per square meter. This was done over a 46 hour period every two weeks for six months, patients were then followed up with every three months until death (Conroy et al., 2011).

FOLFIRNOX is a common treatment protocol for pancreatic cancer. The treatment is made up of oxaliplatin, irinotecan, leucovorin and fluorouracil (C. T et al., 2011). The regimen is commonly used in colorectal cancer but has more recently been used as a treatment for pancreatic cancer (Deyme et al., 2018). It is currently the first line treatment for metastatic pancreatic cancer in patients under 75 years of age., with phase II and III trials showing an increase in survival of 10-11 months (Thibodeau & Voutsadakis, 2018). While FOLFOX is a combination of fluorouracil, leucovorin and oxaliplatin (Lieu et al., 2019), it has previously been a second line treatment in advanced pancreatic cancer once gemcitabine has failed (Yoo et al., 2009).

Cancer research UK indicates this will be the expected treatment procedure for oxaliplatin.

Day	Treatment
1	Oxaliplatin through a drip into the blood stream Folinic acid is injected into the bloodstream at the same time Followed by an injection of 5FU into a cannula or central line into the bloodstream Lastly an infusion of 5FU through a drip or pump into the blood stream for 22 hours
2	Folinic acid as an injection or through a drip into the bloodstream for 2 hours An injection into the bloodstream of 5FU A 5FU infusion through a drip or pump in to the bloodstream for 22 hours

3-14	No treatment for 12 days

Table 1.1 Cancer research UK 2 week cycle for FOLFOX treatment, treatment continues for 12 cycles (Cancer Research UK, 2021).

1.2.2 Mechanism of action

Oxaliplatin has a non-hydrolysed diaminocyclohexane (DACH) carrier ligand, which is maintained in the final cytotoxic metabolites of the drug (di Francesco et al., 2002). The DACH ligand is bulky which is thought to enhance the cytotoxicity of the drug as well as remove cross-resistance between oxaliplatin other platinum compounds (Wiseman et al., 2012). Like cisplatin, oxaliplatin targets DNA producing intrastrand cross links mainly 1,2-GG (di Francesco et al., 2002). Oxaliplatin works like an alkylating agent on DNA. It forms the cross links between adjacent guanine bases d(GpG) or it can form between adjacent guanine-adenine bases d(GpA), creating the cytotoxic lesions (Misset et al., 2000). There are three types of cross links oxaliplatin can induce; DNA intra-strand crosslinks, DNA inter-strand crosslinks and DNA-protein crosslinks (Alcindor & Beauger, 2011a; Faivre et al., 2003), these various cross links act to inhibit the synthesis of DNA (Wiseman et al., 2012). The drug is made up of heavy metal compounds, these compounds inhibit the synthesis of RNA, DNA and protein in cells (OnciLink, 2020). Apoptosis of cancer cells can be caused by the formation of DNA lesions, arrest of DNA synthesis, inhibition of RNA synthesis and triggering of immunologic reaction (Alcindor & Beauger, 2011a). In the body it undergoes rapid nonenzymatic transformation where it forms reactive intermediates (Culy et al., 2000a)

In vitro oxaliplatin, studies show antiproliferative activity against several tumour cell lines. The drug displays a much greater cytotoxicity against cell lines when compared with treatments using cisplatin and carboplatin (Culy et al., 2000b).

In comparison to cisplatin and carboplatin, the DACH-platinum DNA adducts that are formed by oxaliplatin are considered to be much bulkier and more hydrophobic. The DACH-platinum adducts that are formed by oxaliplatin are generally more effective at inhibiting DNA synthesis more cytotoxic than other platinum adducts. The DACH ring also provides steric hindrance, it prevents the mismatch repair (MMR) enzyme complex from binding to oxaliplatin adducts (Misset et al., 2000).

Oxaliplatin is believed to arrest DNA synthesis by preventing the incorporation of thymidine in nucleic acid synthesis. This is an antimetabolite-like effect that cause the arrest of the mitotic process. This may not be a process that occurs solely because of oxaliplatin however, because the drug is often combined with 5FU and 5FU is a thymidylate synthase inhibitor making it unclear if the action occurs because of oxaliplatin or if it's a joint effort between the two therapies (Alcindor & Beauger, 2011b)

The drug also inhibits messenger RNA synthesis through three methods, binding transcription factors, inhibition of RNA polymerase and DNA adducts. During the initial stages, platinum DNA adducts act as binding sites for transcription factors. Some transcription factors have a strong chemical affinity to platinum which prevents the natural binding of the transcription factors to their promotor site. RNA polymerase is prevented by the nucleosomal DNA adducts they have the potential to block the RNA polymerase access to the DNA template (Alcindor & Beauger, 2011b)

1.2.3 Pharmacokinetics

The pharmacokinetics of oxaliplatin has been evaluated in various clinical studies. Oxaliplatin is diluted in 5% glucose injection to achieve a final concentration between 0.2 and 2 mg/mL and administered by i.v. infusion over 2 to 6 hours. Oxaliplatin has a prolonged stability making it also suitable for continuous infusions over 4 to 5 days (Lévi

et al., 2000). Blood samples were taken before, during and after infusion to determine the total and ultrafiltrate of platinum. The maximum plasma concentration of platinum is reached at the end of infusion (Lévi et al., 2000) and is dependent on the time of peak delivery during a treatment regimen, with total and free platinum being significantly lower at 0100 hours compared to 0700 or 1600 hours (Culy et al., 2000a). Compared to the constant rate infusion, chronomodulated treatment has shown to reduce toxicity significantly and improve antitumour activity in treating GI cancer patients(Lévi et al., 2000).

Oxaliplatin metabolism is characterised as a rapid nonenzymatic reaction in the blood to form reactive platinum intermediates, which produce a rapid and extensive binding to plasma proteins and erythrocytes (Culy et al., 2000b). Accordingly in the blood, 3 species can be found: total platinum, ultrafilterable or 'free' platinum (representing a mixture of all unbound, active and inactive platinum species) and erythrocyte platinum (Lévi et al., 2000). There was a significant correlation between of oxaliplatin in patients and the degradation rate in whole blood (Ehrsson et al., 2002). Oxaliplatin is not degraded by CYP450-mediated metabolism and it is not an inhibitor of common CYP450 isoenzymes (Graham et al, 2000), indicating the minimum possibilities of CYP450-mediated drug-oxaliplatin interactions.

The pharmacokinetics of oxaliplatin has been well fit using a 3-compartment model, with a presumption of rapid cellular permeability of oxaliplatin possibly due to facilitated uptake. In the blood, platinum binds irreversibly to plasma proteins and erythrocytes. For example, approximately 40% of the blood platinum is associated with the erythrocytes at the end of a 2-hour infusion and the distribution half-life of free plasma platinum is estimated to be 10-25 minutes. (Lévi et al., 2000). Platinum in the blood is not considered clinically important and is rapidly cleared from the plasma by covalent binding to tissues and renal

elimination (Graham M. A, Lockwood G. F, Greenslade D, Brienza S, Bayssas M, 2000), However, the elimination half-life of free platinum is ranging from 252 to 273 hours (Graham et al, 2000; (Lévi et al., 2000), suggesting there is some redistribution between different tissue compartments. Five days post administration, renal platinum elimination accounts for 53.8% (± 9.1%) of the administered dose, while faecal excretion is only 2.1% (±1.9%). The elimination half-life of erythrocytic platinum is 29-50 days (Lévi et al., 2000), but this may not have significant impact on the systematic exposure of free platinum (the only biologically active form). When understanding the pharmacokinetics of the drug it is important to distinguish between free and bound platinum. Free or unbound platinum exerts anti-tumour activity and toxic effects while bound platinum is mostly inactive (Deyme et al., 2018)

Oxaliplatin is more prone to react with sulphur-containing compounds, complexes with sulphur containing compounds have previously been found in plasma from patients treated with oxaliplatin (Jerremalm et al., 2006). Oxaliplatin is often paired with other drugs such as 5-fluorouracil and folinic acid as the combination of these don't affect the pharmacokinetics of oxaliplatin (JM et al., 1998).

1.2.4 Oxaliplatin toxicity

Oxaliplatin has become an fundamental part of numerous chemotherapy protocols, while oxaliplatin has only mild hematologic and gastrointestinal side effects, its dose limiting toxicity causes a cumulative sensory neurotoxicity that is very similar to that of cisplatin. However, there is an important difference between the two oxaliplatin produces a more rapid and complete reversibility of toxic effects in patients (Grothey, 2003). Haematological toxicities is a common occurrence in patients receiving oxaliplatin treatment, the symptoms of this include mild anaemia (Grade1-2) and approximately 90%

of recipients develop measurable spleen enlargement. This is known as oxaliplatin induced splenomegaly (Lees et al., 2020).

Studies showed oxaliplatin treatment dependently induced toxicity and splenomegaly, liver injury induced by oxaliplatin treatment has become a limiting factor affecting the efficiency of oxaliplatin based chemotherapy in patients with colorectal cancer (Lees et al., 2020; Lu et al., 2020). The result from a 2020 study showed dose dependent decreases in white and red blood cell counts in mice. The study also showed that spleen weight significantly increased after treatment with oxaliplatin indicating that spleen weight and splenomegaly was affected by the treatment (Lees et al., 2020).

Despite similar co-morbidities, cancer stage, demographics and treatment schedule, oxaliplatin induced peripheral neurotoxicity develops differently within patients with variating levels of severity (Marmiroli et al., 2017). Oxaliplatin causes damage to the peripheral sensory fibres and dorsal root ganglia neurons (Renn et al., 2011). Previous studies in mice found that with the treatment of oxaliplatin nerve conduction velocities and action potential amplitude significantly decreased, along with neuronal atrophy and multinucleolated dorsal root ganglia neurons (Renn et al., 2011). The findings also showed that the drug induced significant mechanical allodynia and cold hyperalgesia starting from the first week of treatment (Renn et al., 2011). The prominent toxicity of oxaliplatin is a very unique acute sensory neuropathy that is triggered or aggravated by exposure to cold but is rapidly reversible without impairment of sensory function (Grothey, 2003).

Intravenous calcium and magnesium have been extensively used in an attempt to reduce the oxaliplatin induced cumulative neurotoxicity (Loprinzi et al., 2014). High doses of intravenous calcium and magnesium (CaMg) can be given before and after FOLFOX treatment to prevent neurotoxicity (Gamelin et al., 2004; Loprinzi et al., 2014), Gamelin et

al treated 91 patients being exposed to FOLFOX with infusions of Ca gluconate and Mg sulphate before and after oxaliplatin. The results showed that the infusions of Ca/Mg reduce the incidence and intensity of oxaliplatin induced symptoms. Although a few studies showed that there was no evidence to support that using calcium and magnesium protects patients from oxaliplatin induced neurotoxicity (Loprinzi et al., 2014). A 2001 study attempted to determine if the scheduling of oxaliplatin treatment would have an effect on the onset of peripheral neurotoxicity and ototoxicity using a rat model. There were 5 different trial groups used for this study. Group 1: oxaliplatin 4 mg/kg twice weekly, Group 2: oxaliplatin 2.4 mg/kg for 5 consecutive days every other day, Group 3: oxaliplatin 2.2 mg/kg every other day, Group 4 oxaliplatin 3 mg/kg every other day and Group 5 was an untreated control. The treatment period was for 5 weeks as well as a follow up period of 5 weeks. The findings show that general toxicity was observed in the rats treated with oxaliplatin and a decrease in weight was observed in all the rats when compared to the control group. Neuropathological examination showed dorsal root ganglia nucleolar, nuclear and somatic size reduction in the treated rats as well sensory nerve conduction velocity reduction. The overall conclusions of their study suggested that mice treated with shorter interval times and higher doses had severity in the toxicities documented but all mice treated with oxaliplatin displayed toxicities to varying extents and these differences could be related to the cumulative oxaliplatin dose. However, the neurotoxicity appeared to be reversable after the 5 week follow up period (Cavaletti et al., 2001). From this study we can assume that even through changing the treatment course and intervals between treatments patients will more than likely still suffer from some form of toxicity as discussed in the mice example and to varying intensities depending on the treatment schedule.

Neurotoxicity is one of the major implications of oxaliplatin treatment however there are other forms of toxicity that arise from it as well. Patients being treated with oxaliplatin also suffer from gastrointestinal toxicity and pulmonary toxicity. Gastrointestinal toxicity presents as nausea and vomiting, the main treatment method for these side effects are prophylactic antiemetic therapy and corticosteroids. Pulmonary toxicity is in 0.7% of patients, it displays as pulmonary fibrosis which can be fatal. Oxaliplatin has to be discontinued when pulmonary toxicity causes respiratory symptoms such as dyspnoea, crackles or radiological pulmonary infiltrates (Medsafe NZ, 2020).

1.2.5 Limitations

There are limitations associated with chemotherapy that affects the clinical applicability of oxaliplatin, these limitations pave the way for undesirable toxic effects to healthy cells which results in sub-standard drug accumulation at the tumour. If drug accumulation decreases it requires an increased doses of chemotherapy creating a cycle that will lead to increased toxic effects (Tummala et al., 2016). Resistance to oxaliplatin is another a major limiting factor of the drug, resistance occurs for a number of reasons these include uptake of the drug or enhanced efflux as well as intracellular sequestration, a decrease DNA abduct formation, increased DNA repair and decreased DNA adduct formation (Noordhuis et al., 2019). A 2005 study looked at the acquired resistance in response to repeated oxaliplatin and cisplatin treatment in H69 small cell lung cancer (SCLC). They did two treatment plans for four days and two days. The four day plan treated cells using 400 ng/ml of oxaliplatin this treatment plan produced viable cells that after eight treatments showed resistance. Treatments of two hours at 2000 ng/ml produced some viable cells but these cell lines weren't stably resistant. This could suggest that shorter time frames between treatments could be more effective at reducing resistance and treating the disease however some form of resistance still occurs regardless (Stordal et al., 2005)

Oxaliplatin is an important agent in the combinational regimen for colorectal cancer and other gastrointestinal malignancies, but tumour resistance develops rapidly limiting therapeutic efficacy (Myint et al., 2019b). Application of oxaliplatin for the treatment of pancreatic cancer is restricted due to its toxic side effects and drug resistance (X. Li et al., 2015). High doses of oxaliplatin eliminate PC effectively but cause severe side effects, while low doses of the treatment elicit a poor response and drug resistance (X. Li et al., 2015). Gemcitabine is another treatment option for pancreatic cancer, but it is no longer the gold standard of treatment, A 2012 study showed that only 6% of patients responded to the standard of gemcitabine treatment (Ying et al., 2012). Resistance to chemotherapy can occur when there is a decrease in drug accumulation or an increase in the efflux of the drug out of the cell. Both of these lead to a decrease in intracellular concentrations of the compound inside (Misset et al., 2000).

The expression of the ATP-binding cassette (ABC) transporters by cancer cells is considered one of the major mechanisms responsible for insufficient drug accumulation (Adamska & Falasca, 2018). MRP has been found to decrease the accumulation of drugs within a cell by increasing the efflux of drugs from the cell acting as a drug pump (Zaman et al., 1994). Overexpression of MRP2 in Panc1, Caco-2 and HepG2 cells has been associated with decreased accumulation of oxaliplatin. MRP2 was differentially expressed in tumours of colorectal cancer patients who did not respond to oxaliplatin chemotherapy (Myint et al., 2019a).

1.2.7 Cellular transport

Oxaliplatin's ability to cross cell membrane by passive diffusion is limited by its hydrophilicity. The drug is hydrophobic and in order for the drug to cause cytotoxicity in tumour cells it must enter the cell and react with DNA to form DNA abducts (Myint et al., 2019a; Virag et al., 2011; D. Wang & Lippard, 2005).

Platinum based derivatives such as oxaliplatin have potent antitumour activity, along with severe side effects such as nephron-, oto and neurotoxicity associated with their use. In order for sufficiently high concentrations of intracellular platinum drugs, the drugs need to cross the plasma membrane to reach their targets. Oxaliplatin is a charged compound at the physiological environment and thus its cellular uptake is typically mediated by specialised plasma membrane proteins called transporters. Transporter mediated uptake has been suggested to play a pivot role in the determination of cellular accumulation and thus efficacy and toxicity of platinum drugs (Harrach & Ciarimboli, 2015). Oxaliplatin is also proven to be a substrate of an efflux transporter multidrug resistance protein 2 (MRP2) which is involved in the development of drug resistance (Biswas et al., 2019) (Harrach & Ciarimboli, 2015; Renes et al., 2000). Multidrug resistance proteins pump out exogenous compounds /xenobiotics and their metabolites and are often controlled at the transcriptional level by various mechanisms (Pułaski et al., 2005). Previous studies have shown that in vitro interaction of oxaliplatin with MRP2 and the silencing of MRP2 in Caco2 and Panc1 cells by siRNA increased platinum accumulation and oxaliplatin sensitivity (Biswas et al., 2019). Myint et al., (2015) found direct evidence for the role MRP2 in mediating the transport of oxaliplatin-derived platinum. The study also noted that platinum accumulation in the membrane vesicles increased with increasing exposure times to oxaliplatin. Myint et al., (2015) investigated the active transport of platinum during invitro exposure to oxaliplatin. They tested this in the presence or absence of ATP, known substrates and inhibitors of MRP2 in comparison to control membrane vesicles that don't express MRP2. The experiments undertaken used inside-out membrane vesicles that express the MRP2 protein to investigate the transport of platinum during exposure to oxaliplatin (Myint et al., 2015).The overall results of their study showed higher intravesicular accumulation of platinum increased with membranes overexpressing MRP2

compared to the control membrane vesicles. MRP2 mediated transport of oxaliplatin was ATP-dependant and the kinetics data was best fit by a Michaelis-menten model (Km = 301 µM; Vmax = 2680 pmol Pt/mg protein/10 minutes) (Myint et al., 2015).

Biswas et al., (2019) and Myint et al., (2019) studies also provide further evidence that MRP2 transports oxaliplatin. Both studies used siRNA to knock down the MRP2 protein in HepG2, Panc1 and Caco2 cells. Biswas et al., (2019) found an over expression of MRP2 in Caco2 and Panc1 cells was associated with a decrease in accumulation of oxaliplatin, and silencing MRP2 by siRNA increased oxaliplatin induced apoptotic rate in both cell lines. Myint et al., (2019) results showed HepG2 cells endogenously overexpressing MRP2 had decreased levels of oxaliplatin accumulation and cytotoxicity. After, siRNA knock down of MRP2 increased the accumulation of oxaliplatin in both cell lines (Biswas et al., 2019; Myint et al., 2019a).

1.3 Membrane Transporters

Drug transporters are membrane proteins that are expressed in various different tissue types including heart, intestine, liver, kidney and the immune system and central nervous system (Girardin, 2006). Transporters play a pivotal role in pharmacokinetics generally by influencing drug absorption, distribution and excretion and facilitating efflux of drug metabolites. Targeting specific transporter proteins and thorough characterisation of transporter profiles may represent a novel strategy for drug delivery and design (Girardin, 2006; Mizuno et al., 2003). 48 drug transporters are members of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter super family. The multidrug resistance protein MDR (ABCB1), the multidrug resistance-associated protein MRP1 (ABCC1) and MRP2 (ABCC2), and the breast cancer resistance protein BCRP (ABCG2) are ATP-dependent efflux transporters expressed in the blood brain barrier (Girardin, 2006). ABC transporters are ubiquitous membrane proteins that couple the transport of

diverse substrates across cellular membranes using the energy generated from ATP hydrolysis (Hollenstein et al., 2007). There 48 ABC genes in the human genome divided into 7 subfamilies (Dean, 2005). These transporters mediate the active transport of drugs/solutes across the cell membrane by using the energy generated from ATP hydrolysis. Interestingly, these transporters have been also suggested to be important virulence factors in bacteria because they play roles in nutrient uptake and secretion of toxins (Davidson & Chen, 2004). Multiple mutations in ABC transporters cause or contribute to a number of different disorders (Dean, 2005). Several ABC transporters have been found to be overexpressed in cancer cell lines cultured under select pressures, it was found at least 12 ABC transporters from four ABC subfamilies showed drug resistance of cells maintained cultured (Szakács et al., 2006).

Walker A and B domains can be found in all ATP-binding proteins. ABC transporters also have a specific C motif located just upstream from the walker B site (Hyde et al., 1990). The C domain is specific to ABC transporters, distinguishing them from other ATP-binding domains (Hyde et al., 1990). It has been identified that ATP-binding cassette (ABC) multidrug transporters are associated with chemoresistance. The extrusion of the anticancer drugs by members of the ABC family has become one of the most important mechanisms of multidrug resistance (Fletcher et al., 2016; Mirakhorli et al., 2012a)

MRPs represent the largest subfamily of ABC transporter superfamily and MRP1 was the first cloned member (a glycoprotein of about 190kDa) from the multidrug-resistant lung cancer cells in humans (Büchler et al., 1996; Cole et al., 1992). In vitro, studies have found that various MRP transporters can collectively confer resistance to anticancer drugs and/or their conjugated metabolites including alkylators (e.g. platinum drugs), antimetabolites (e.g. anti-folate, nucleoside and nucleotide analogues), microtube stabilisers (e.g. vincristine), arsenical and antimonial oxyanions and peptide-based agents (Deeley et al.,

2006). In vivo, several MRPs are major contributors to the distribution and elimination of a wide range of both anticancer and non-anticancer drugs (Deeley et al., 2006)

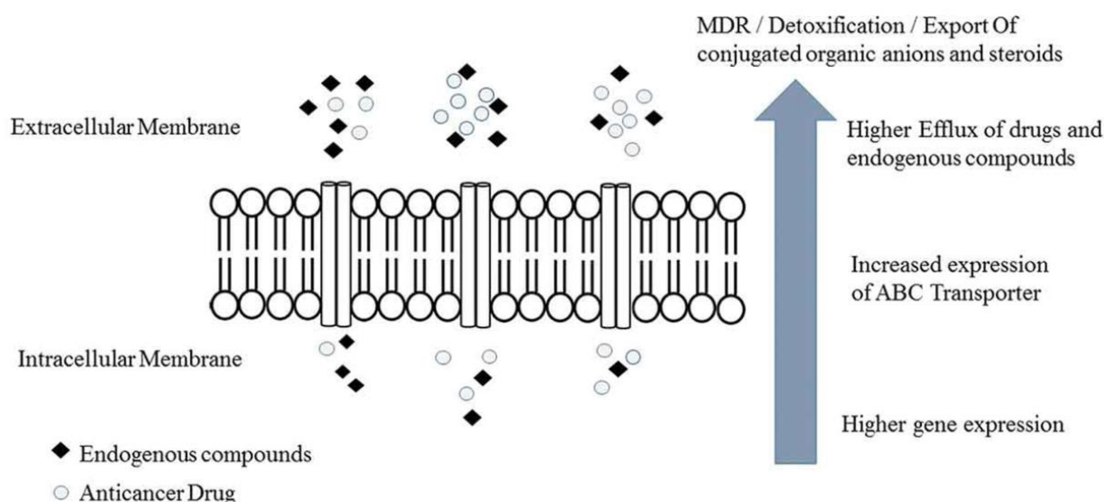


Figure 1.4: Diagram of anticancer and endogenous compounds being exported out of the cell via the ABC transporter (Bugde et al., 2017).

1.3.1 MRP2 Protein:

The multidrug resistance protein (ABBC2; MRP2) is an ATP-binding cassette transporter expressed in the liver, bile duct, kidney and intestine and its substrates include multiple and exogenous endogenous organic anions, such as bilirubin and drugs and toxic chemicals. MRP2 plays an important role in detoxification and chemotherapy by transporting a wide range of compounds (Jedlitschky et al., 2006; Payen et al., 2002). The MRP2 protein expression has been reported to be upregulated in some cancerous tissues and notably downregulated in liver cholestasis. The levels of the protein are also altered in hepatocytes in response to hormones such as glucocorticoids. Due to MRP2 playing a role in drug elimination, changes in the expression of the protein may lead to variations in drug

absorption, distribution, metabolism and elimination and thus their efficacy and toxicity (Payen et al., 2002).

1.3.2 Physiological role of MRP2

MRP2 is a protein that is encoded by the ABCC2 gene. The protein consists of two ATP binding domains and two transmembrane domains, with the addition of a third amino proximal transmembrane region (Jemnitz et al., 2010a; König et al., 1999). The hepatobiliary and renal elimination of many drugs and their metabolites is mediated by MRP2 (Keppler, 2011). The MRP2 protein is localized at major physiological barriers such as the apical membrane domain of polarised cells, the canalicular membrane of liver cells, hepatocytes, renal proximal tubular cells, enterocytes of the small and large intestines, and syncytiotrophoblasts of the placenta (Jedlitschky et al., 2006; Jemnitz et al., 2010a). The transporter is most highly expressed in the liver, where it facilitates the elimination of its drug substrates as well as some endogenous compounds (e.g. glutathione, bilirubin glucuronides and conjugates) into the bile leading to decreased bioavailability and thus bioactivity (Z. Chen et al., 2016). As stated before, the protein is expressed in the intestines. In the intestine this protein plays an important role as the transcellular barrier to its substrates. The expression and activity of MRP2 can vary in this area due to changes in physiological and pathophysiological conditions. This is expected to effect drug absorption and disposition and thus efficacy and toxicity (Arana et al., 2016).

Studies using rats lacking the apical MRP2 indicate that anionic conjugates of endogenous and exogenous substrates cannot exit cells, suggesting that MRP2 is essential for the transport of substrates across the cell membrane (König et al., 1999). A non-functioning MRP2 protein also has detrimental effects. The absence of a functioning MRP2 from the hepatocyte canalicular membrane causes Dubin-Johnson syndrome (DJS). Dubin-Johnson syndrome (DJS) is a rare recessive autosomal liver disorder, the disorder is caused by

conjugated hyperbilirubinemia caused by absence of the MRP2 protein (CC et al., 1997; Keitel et al., 2003). Patients with DJS have impaired hepatobiliary transport of non-bile salt organic anions (CC et al., 1997). This disorder indicates the importance of the MRP2 protein in the human body for the efflux of substrates out of cells as accumulation can cause detrimental effects. However, as discussed earlier, changes in expression can also lead to detrimental effects (Payen et al., 2002). Transcription of MRP2 is mediated by nuclear receptors, these are activated by endo- and xenobiotics. They bind to elements within the MRP2 promotor to induce a response that leads to an increase in MRP2 mRNA synthesis leading to the induction of MRP2 protein synthesis. Regulation of MRP2 can be controlled by xenobiotics predominately binding to membrane receptors or interacting with membrane proteins. Transcription of MRP2 can also be regulated by cAMP. For example, intracellular levels of cAMP may trigger many different cellular signals in enterocytes in turn leading to the upregulation of MRP2 protein in their apical membrane (Arana et al., 2016).

1.3.3 Role of MRP2 in cancer pharmacology

ABC transporters are known to mediate cellular transport of a number of substates including chemotherapy agents conferring resistance in some cancer cell lines (Fletcher et al., 2016; Mirakhorli et al., 2012a). Many different ABC transporters are found to be expressed at higher levels in tumour subtypes that are less differentiated (Fletcher et al., 2010). MRPs 1- 5 are associated with MDR often caused by an accelerated active transport and diminished intracellular accumulation of drug substrates (S.-F. Zhou et al., 2008). One of the main ABC transporters involved in resistance is MRP2, the protein has been previously identified to play a role in cisplatin resistance (Minamino et al., 1996). Overexpression of MRP2 protein has been found in multiple cancer types and associated with resistance to platinum-based drugs. Those cancer types include ovarian carcinomas,

hepatocellular carcinomas and colorectal cancer (Materna et al., 2005; Mirakhorli et al., 2012b; Nies et al., 2001). MRP2 is overexpressed in colorectal cancer throughout the course and development of the disease (Mirakhorli et al., 2012b). Studies have found that the expression of MRP2 is higher in cancerous colorectal tissues compared to non-cancerous tissues. These same studies found that the high expression of MRP2 in these cancerous regions lead to a reduced sensitivity to cisplatin (E et al., 2000). MRP2 also confers resistance to other cytotoxic drugs including etoposide, doxorubicin, and epirubicin (Cui et al., n.d.). MRP2 is known to share substrates with MRP1 as well as other ABC transporters. Some of the common human substrates for the MRP2 protein include bilirubin, leukotriene C4, chemotherapeutic agents. A universal feature of ABC transporters is their ability to incur MDR, although there have been multiple strategies developed to revert MDR targeting MRP2 alone may not be effective. This is due to other ABC transporters being able to transport many of MRP2s substrates (Arana et al., 2016; Kapse-Mistry et al., 2014; König et al., 1999)

Shen et al (2012) found by supressing MRP2 using nuclear translocation of Nrf2 the drug resistant phenotype was reversed. While other studies have shown that in vitro interaction of oxaliplatin with MRP2 and the siRNA mediated knock-down of MRP2 in Caco2 and Panc1 cells increased platinum accumulation and oxaliplatin sensitivity (Biswas et al., 2019). By using inside-out membrane vesicles that express the human MRP2 protein, Myint et al., 2015 found higher intravesicular accumulation of platinum in membranes overexpressing MRP2 compared to control membrane vesicles, providing direct evidence that MRP2 transports oxaliplatin-derived platinum (Myint et al., 2015). The study also noted that platinum accumulation in the membrane vesicles is ATP-dependant and is also increased with increasing exposure times to oxaliplatin (Myint et al., 2015), suggesting

MRP2 control the steady-state intracellular level of platinum drugs. In vivo mice, studies have shown that the transport of chemotherapeutic drug cisplatin is actively transported by the MRP2 protein. In null-MRP2 mice, concentrations of cisplatin were two-fold higher than mice with a functioning MRP2 (Wen et al., 2014).

1.3.4 Implications of MRP2 in oxaliplatin therapy

There have been many reports that state MRP2 plays a role in the transport of substrates across membranes using energy derived from ATP hydrolysis (Jemnitz et al., 2010b; Myint et al., 2019a). MRP2 has been found to decrease the accumulation of drugs within a cell by increasing the efflux of drugs from the cell acting as a drug pump (Zaman et al., 1994). The protein can also transport uncharged compounds in cotransport with glutathione and thus modulate the pharmacokinetics of many drugs (Jedlitschky et al., 2006). MRP2 has been suggested to play a major role in the pharmacokinetics and tumour distribution of oxaliplatin. A major limitation of oxaliplatin treatment is resistance to the treatment and the toxic side effects associated with it (X. Li et al., 2015; Tummala et al., 2016). Overexpression of MRP2 in Panc1, Caco-2 and HepG2 cells has caused a decreased accumulation of oxaliplatin. A 2019 oncogenomic dataset study showed that MRP2 was differentially expressed in tumours of colorectal cancer patients who did not respond to oxaliplatin chemotherapy (Myint et al., 2019a). Biswas et al., (2019) reported the effects of MRP2 on oxaliplatin therapy, the study aimed to silence MRP2 by using siRNA. They were able to determine that MRP2 was overexpressed in both Caco2 and Panc1. They achieved this by surface staining with an anti-MRP2 primary and control isotope IgG2a antibody. Their results showed Caco2 and Pac1 cells had a much higher fluorescent signal compared to the isotope control. With an understanding that MRP2 was significantly over expressed on the surface of the cell lines they were interested in, the next step was to transfect the cells with siRNA, targeting MRP2. The siRNA knocked down the MRP2

protein resulting in reduced transport function. The study reported that MRP2 knock down cells were more sensitive towards oxaliplatin, as the treatment inhibited the growth of transfected cells more efficiently than control cells (Biswas et al., 2019). These studies indicate that MRP2 has a role in limiting tumour response to oxaliplatin. The studies show that reducing MRP2 activity may restore cellular oxaliplatin accumulate in certain cell lines overexpressing MRP2 and consequently potentiate its cytotoxic effects, suggesting that in the above cancer cell lines MRP2 transports oxaliplatin out of cells limiting the tumour response to treatment (Biswas et al., 2019; Myint et al., 2019a). Screening patients for MRP2 level and specific MRP inhibitors will help overcome the multidrug resistance in cancer (L. Zhang et al., 2019). If patients can be screened for MRP2 levels this will identify patients that will benefit from oxaliplatin treatment alone and show those that may need an MRP2 inhibitor to aid treatment (Myint et al., 2019a). To translate these preclinical results into clinical practice, an alternative approach (e.g. CRISPR/cas9 system) may be required to verify our previous results since siRNA based studies could have some limitations such as off-target effects.

1.4 CRISPR-Cas9

1.4.1 Mechanism of CRISPR-Cas9 system

In recent years, the genome editing tool known as CRISPR (clustered regularly interspaced short palindromic repeats) -Cas9 has emerged. It has quickly become an efficient and user-friendly way to genetically modify genomes (Bhaya et al., 2011; Cong et al., 2013; Shi et al., 2020). The CRISPR-Cas9 system has greatly simplified genome editing and has been applied in a wide array of applications such as stem cell editing, gene therapy, tissue engineering and creation of disease models and transgenic plants (ThermoFisher, n.d.) The mechanism for CRISPR-Cas9 was developed from a system that naturally occurs in many bacteria and archaea. The system provides an acquired immune response against viruses

and plasmids that have entered the bacteria by using CRISPR RNAs to guide the silencing of nucleic acids (van der Oost et al., 2009). The genetic material from invasive elements is then inserted into these loci and inheritable DNA-encoded immunity is built up over time (van der Oost et al., 2009). The host then incorporates these short sequences from the invading genetic elements into a region of its own genome. This is distinguished by clustered regularly interspaced short palindromic repeats (CRISPRS)(Levy et al., 2015). The palindromic repeats are separated by short stretches of DNA called spacers. These are acquired from extrachromosomal elements (Nishimasu et al., 2018). When these sequences are transcribed and processed into small RNA's, the guides multifunctional protein complexes (Cas proteins) to recognise and cleave incoming foreign genetic material (Levy et al., 2015).

There are three distinct stages involved in the CRISPR system, adaptation, expression and interference (Garneau et al., 2010). Adaptation is the process in which short pieces of DNA called spaces are acquired from foreign elements and integrated into the CRISPR array (Cong et al., 2013), including the integration of short sequences of the invaders as spacers, expression of CRISPRs and subsequent processing to small guide RNAs, and interference of target DNA by crRNA guides (Deveau et al., 2010). In the second stage (expression), the short crRNAs can be synthesized base on the long primary transcript of a CRISPR locus (Makarova et al., 2011). Finally, the third step is interference directed by crRNA guides to target DNA (van der Oost et al., 2009)

This system has become a primary option for knocking out, as well as knocking in, specific genomic loci in various organisms. It requires a single-strand guide RNA (gRNA) to direct the Cas9 enzyme. This enzyme snips the target DNA at the specific spot through DNA pairing (Cong et al., 2013; Shi et al., 2020). It is a powerful genome editing tool, with a number of human clinical trials using CRISPR-Cas to combat lung, prostate and

renal cell cancers (Kang et al., 2017; Mahmood & Yang, 2012). In order to alter the genome cells are transfected with CRISPR-Cas9. Transfection is the process by which CRISPR-Cas9 DNA, mRNA or protein systems are delivered into target cells. There are multiple approaches including, lipid nanoparticle mediated transfection, viral delivery, and physical methods such as electroporation (ThermoFisher, n.d.). The system is composed of two major components including a single guide RNA and a Cas9 nuclease. The latter is usually isolated and purified from *Streptococcus pyogenes,* this is what cleaves the DNA (Saber et al., 2020). The guide RNA locates and targets a specific site for the Cas9 protein to cleave this specific site is next to a protospacer adjacent motif (PAM). PAMs are usually a few base pairs long and are located downstream from the cut site (Kurien & Scofield, 2006).

The system has more than one repair pathway that can be utilised to inactivate genes (Knockouts) or to incorporate new sequences (Knock-ins), which have been used to correct disease-associated genetic mutations (Acharya et al., 2020). The CRISPR-Cas9 system has two repair pathways that can repair the double stranded breaks (DBS) cleaved by the Cas9 protein. These are known as non-homologous end joining (NHEJ) and homology directed repair (HDR) (Figure 1.5) (Ghosh et al., 2019a). These two pathways vary in many aspects but both pathways use the same signalling propagation cascade to drive cellular responses to Cas9 induced DSB (Yang et al., 2020a).

NHEJ is an error-prone DNA DSB repair pathway, the pathway generates insertions and deletions. In most cases the pathway will cause a deletion but less frequently it will also produce insertions. This mechanism is mostly for gene disruption as the pathway is active throughout the cell cycle. It is suggested that the end process for this mechanism comprises of the removal of mismatched or damaged nucleotides by nucleases and phosphodiesterase's as well as subsequent strand extension by polymerases (Baliou et al.,

2018; Menon & Povirk, 2016). In the NHEJ repair system the DNA ends are chemically ligated back together. This is done using Ku heterodimers, Ku bind to DSB ends and act as a molecular scaffold for the repair proteins (Baliou et al., 2018; Hsu et al., 2014). When a DSB occurs, the cell's initial reaction is usually the NHEJ pathway. When you compare it to other pathways this one is robust and predominately faster making it a more ideal mechanism for producing CRISPR knockouts. The pathway has two strategies it can employ, c-NHEJ and alt-NEHJ. Each strategy is dependent on the DNA ends after DSB. There is a complex that drives the NEHJ pathway known as the Ku heterodimer (M. Liu et al., 2019). The Ku heterodimer is the main component of the NHEJ pathway, Ku heterodimers bind to broken DNA ends to facilitate the ligation of the broken end. It recruits other proteins to aid in the processing and ligation of broken ends (Fell & Schild-Poulter, 2012).

During the HDR process a donor DNA strand matches with the sequence flanking each side of the DSB, introducing new genetic information into the genome (Baliou et al., 2018; Drost & Clevers, 2016; Ran et al., 2013). Rad51 proteins can bind DSB during the initial phase of HDR. This recruits accessory factors that direct genomic recombination by using homology arms on exogenous repair template (Hsu et al., 2014). The HDR pathway requires a donor strand of DNA matching the flanking sequences each side of the DSB. After this, the donor sequence is then incorporated into the genome (Baliou et al., 2018; Drost & Clevers, 2016; Ran et al., 2013). The pathway uses homologous recombinant proteins that are expressed in the G2 phase of the cell cycle which has previously shown to cause difficulties editing postmitotic cells (Hsu et al., 2014). Strategies that manipulate the choice of repair and favour HDR have become popular and can assist in the utilization of CRISPR-Cas9 system, achieving a more precise genome editing (Yang et al., 2020b).

NHEJ is often the preferred choice in repair pathway in mammalian cells for multiple reasons. NHEJ is active throughout the whole cell cycle while HDR only occurs during the S/G2 phase. NHEJ occurs a lot faster than HDR and NHEJ can supress the HDR process (Yang et al., 2020a).

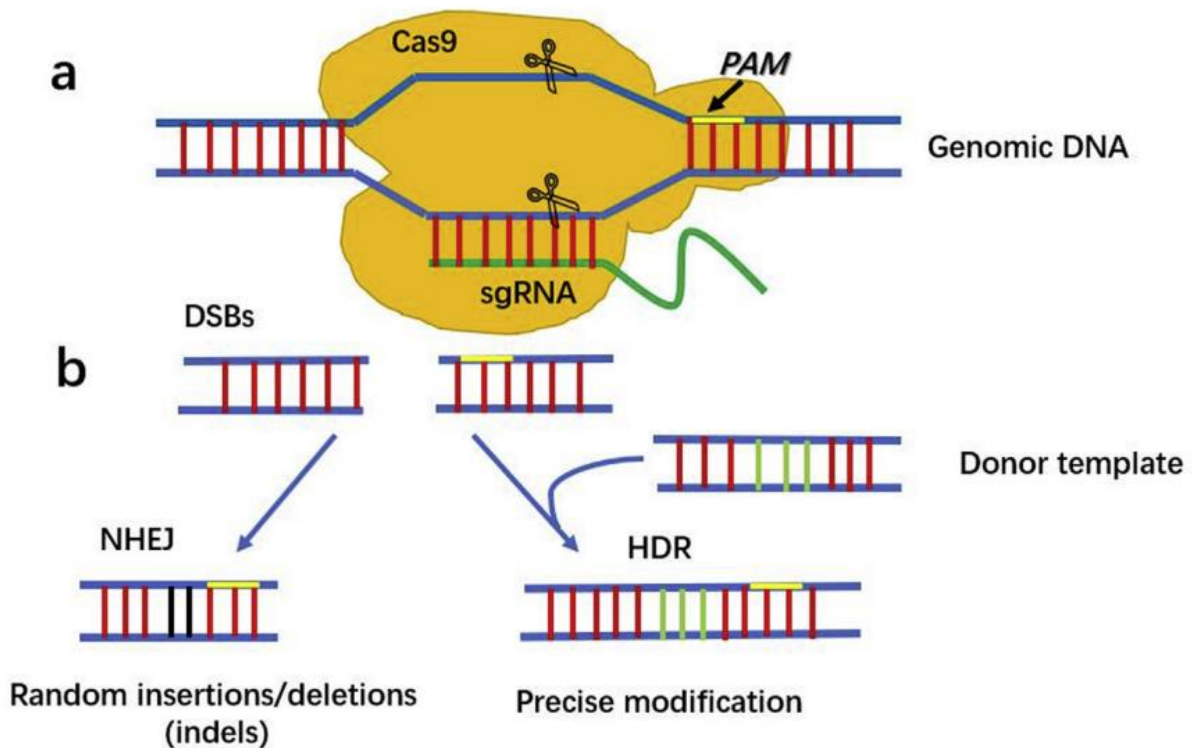


Figure 1.5: The mechanism of the CRISPR-Cas9 system (M. Chen et al., 2019a)

There are many delivery systems for CRISPR-Cas. One very popular type of delivery is viral delivery. There are four major classes of viral vectors applied as delivery tools retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses (Schmidt & Grimm, 2015). The appropriate choice of delivery system is important for the components to be efficiently and effectively delivered to targeted cells (L. Li et al., 2015). Viruses have evolved natural vectors for the transfer of foreign genetic information into cells (Kay et al., 2001). Adeno-associated viruses (AAV) are largely used for CRISPR genome editing (Xu et al., 2019). AAV are considered to be one of the more suitable viral vectors to package,

deliver and express CRISPR components for gene editing (Lau & Suh, 2017). Nonviral vectors are another method of transfection, these include lipid-based vectors (L. Li et al., 2015) A lipid-based mechanism for CRISPR transfection is a lipofectamine based method. Lipofectamine reagents are widely considered as the gold standard in the safe delivery of exogenous DNA or RNA into cells (Cardarelli et al., 2016). This method allows for transfection without having to synthesise the Cas9 protein or gRNA inside the cell. The Cas9 protein and gRNA are packed into liposomes, these liposomes can easily pass through the cell membrane (Biagioni et al., 2018; Yip, 2020).

1.4.2 Application of system in cancer

CRISPR-Cas9 has been widely used to identify potential therapeutic targets involved in cancer (B. Liu et al., 2019). It can be used for precisely manipulating the genes known to be associated with cancer growth and development in order to treat the disease (Manvati & Dhar, 2020). The use of CRISPR-Cas9 continues to demonstrate great promise for cancer treatment in cancer immunotherapy, by manipulating the cancer hallmarks and eliminating or inactivating carcinogenic viral infections. However, there are still a few hurdles that need to be overcome in order to increase efficacy, including genomic editing efficiency, delivery system, off-target effects, the fitness of transgenic cells and systemic difficulties such as immunogenic effects (Cheng et al., 2020). Accumulating evidence revealed that CRISPR-Cas9 can be used to silence the oncogenes, tumour suppressor genes and genes associated with drug resistance. significantly inhibiting the tumour survival/growth in breast, lung, liver, colorectal and prostate cancers (Hazafa et al., 2020). The silencing of genes involved in the proliferation and survival of cancer cells has shown to remarkably reduce cancer cell growth and promotes apoptosis, inhibiting tumour growth (M. Chen et al., 2019b). For example, a 2019 study employed CRISPR-Cas9 to knock out CD133 in colon cancer cells by using a lentiviral vector delivery, to investigate the roles of CD133 in

the colon cancer hallmark development. The results of this study suggest the CD133 knockouts showed significant inhibitory effects on the ability of cell migration and invasion despite their limited effects on colon tumour generation (W. Li et al., 2019).

1.4.2.1 Lipofectamine based delivery in cancer

A useful method in which the Cas9 protein and gRNA can be introduced into cancer cells is using a lipid-based delivery method. This method of transfection is beneficial as cells undergo transfection without having to synthesise the gRNA and the Cas9. The process uses combined ribonucleoproteins (RNPs) Cas9-gRNA presenting a very rapid and robust knock-out with the complete clearing of the Cas9 after 24 hours of transfection. The complete clearing after 24 hours is beneficial as it reduces the risk of off target effects (Biagioni et al., 2018; Kim et al., 2014; Zuris et al., 2015). RNPs also significantly reduced the rate of off-target mutagenesis and cell death when compared to plasmid delivery methods. RNPS avoid the risk of insertional mutagenesis by integration of the vector into the host genome (Biagioni et al., 2018). Nucleic acids are negatively charged and so is the cell membrane, this means that nucleic acids cannot pass through the cell membrane. Liposomes can be imposed with positive charges. By packing the Cas9 protein and gRNA into liposomes, the system is able to pass through the cell membrane and into the cell (Yip, 2020).

1.4.3 CRISPR Screening

CRISPR-Cas based genetic screens are an emerging powerful tool used for the large scale identification of new targets involved in cancer immunotherapy (D. Liu et al., 2020). CRISPR screening is an application of the CRISPR technology that can be used multiple different factors at once. There are two types of screens the first being a pooled screen, pooled screens deliver lentiviral guide RNAs to a large population of cells. While the other

form of a screening is an array, for an array screen a 96 well plate or 3184 plate is used to look systematically at individual genes or constructs (Goldmeyer, 2021).

Programable RNA-guided nucleases such as CRISPR/Cas9 system provide a mechanism of precision genome editing in diverse model systems and in human cells. Functional screens using large libraries of DNA guides can cross-examine a large area of space to identify specific genes and genetic elements involved in biological processes (Guo et al., 2017).

Pooled genome screening studies have uncovered genes that are responsible for survival, proliferation, drug resistance, viral susceptibility and many other important functions (So et al., 2019).

A basic yet important screen that is often used is a viability screen. The idea of these screens is to find genes that affect the fitness of the cell. By the end of the screen, modifications to the genome that reduce cell fitness will be depleted or absent. These screens are often called negative selection screens and have been used in cancer biology to determine particular mutations that have detrimental effects (Doench, 2017).

CRISPR screening can be used to knockout multiple genes with multiple different guide RNA in human cancer cell lines to identify cells that are essential for cell viability (Shalem et al., 2014). This study targeted 18,080 genes using 64,751 unique guide sequences to screen genes whose loss is involved in resistance to vemurafenib in melanoma cell lines (Shalem et al., 2014)

Previous studies have found that cancer cells can under express and overexpress some determinates such as PD-L1, MHC-1 and CD47. These determinates help to evade recognition and elimination by immune cells. Modifying the expression of these determinants by manipulating their regulators, can aid in immunotherapy (Burr et al.,

2017; D. Liu et al., 2020). By using CRISPR screening Burr et al were able to identify the uncharacterised protein CMTM6 as a critical regulator of PD-L1 in a broad range of cancer cells.

1.4.4 Challenges

Despite the therapeutic potential of CRISPR-Cas there are multiple technical issues surrounding the technology that stop the system from being used clinically (Kang et al., 2017), such as the fitness of edited cells, editing efficiency, delivery methods and potential off target effects (M. Chen et al., 2019a)

CRISPR-Cas9 is considered a highly effective tool for gene editing but produces considerable off target effects in cell culture using human cell lines as well as several other organisms (Xiao et al., 2014). When using a large genome there may be DNA sequences that are identical or closely resemble the sequence you are interested in. This can result in the non-specific cleavage of Cas nucleases of non-targeted gene areas giving rise to mutations. These mutations are what is known as off target effects (Kang et al., 2017). Off target mutations have been a major concern in the CRISPR-Cas system, as reports of off target effects where the Cas9 endonuclease is able to cut sites other than those targeted has limited the appeal of this technology (Fu et al., 2013; Hay et al., 2017). The targeting specificity is believed to be controlled by the 20-nt sgRNA and rely on the presence of a PAM sequence adjacent to the sequence of interest in the genome, however the potential for off targets can still occur with the potential of three to five base pair mismatches in the PAM distal part of the sgRNA guiding sequence (X. H. Zhang et al., 2015). Off targets are a cause for concern in CRISPR because off target mutations have the potential to cause genomic instability and disrupt the function of normal genes. The mutations it can cause are often large deletions and genomic rearrangements that have the potential to cause lethal mutations or loss of function in genes (Naeem et al., 2020; X. H. Zhang et al., 2015).

The delivery of Cas9 and the synthesis of guide RNA have been suggested to represent a major hurdle that limits the genomic editing efficiency and ease of use (Liang et al., 2015). In order for the system to be successful the delivery method needs to be effective at cell targeting, rapid clearance of the CRISPR components after transfection and minimal cytotoxicity, however, there are challenges associated with delivery methods (M. Chen et al., 2019b; Mout et al., 2017) Choosing an appropriate mechanism for delivery has been suggested to be critical to improve genome editing efficiency in targeted cells (L. Li et al., 2015). The enormous size of Cas9 protein is associated with packing problems (Ghosh et al., 2019b; Hazafa et al., 2020). Adeno-associated viral particles have shown great promise but have proven difficult to use efficiently as AAV's have a loading capacity of approximately 4.7 Kb (Kay, 2011; Swiech et al., 2015). Using viral vectors also gives long term exposure invo that may increase the potential for off target effects as well the risk for immunogenicity (F. Chen et al., 2020). To avoid the pitfalls of viral delivery methods, using non-viral delivery methods such as nanoparticle delivery were developed (F. Chen et al., 2020). A challenge associated with non-viral delivery methods is that non-viral vectors are unable to penetrate cells as efficiently (Jiang & Doudna, 2017; L. Li et al., 2015)

The heterogeneity of cancer is rather significant, genomic aberration profiles are different not only in tumours between patients but also in tumours during different stages or from different sites within one patient. This can create many difficulties in genome manipulation in cancer (M. Chen et al., 2019b). The fitness of edited cells is important, by editing cells you can change the overall fitness. If cells have an increased fitness in comparison to the unedited cells the edited cells will develop a selective advantage (Cox et al., 2015).

However, in some diseases edited cells do not exhibit a change in fitness, this means the number of cells modified required to achieve the desired therapeutic effect will be higher (Cox et al., 2015)

When you compare unedited cells to edited ones, edited cells often possess a fitness disadvantage, that results in less therapeutic benefits. Edited cells often show a weaker ability to proliferate and differentiate. If edited cells have a selective advantage over unedited cells the number of edited cells initially needed to cause a therapeutic advantage will be reduced decreasing the need for high efficiency (M. Chen et al., 2019b). However in cancer, cancer cells have the advantage of growth including rapid proliferation and longer survival. In terms of CRISPR-Cas, this means a higher gene editing efficiency is required. Meaning, to produce a therapeutic benefit, a large number of cells will need to be effectively transfected (M. Chen et al., 2019b).

The repair pathway used can cause challenges in the gene editing process, as each pathway plays a significant role in controlling editing rates. DSB breaks can be repaired by two different mechanisms. These mechanisms are known as NHEJ and HDR. Due to there being more than one repair mechanism, differences in efficiency between the two occur, not only between cell types, but also in different cell states. NHEJ is active throughout the whole cell cycle whereas HDR mostly operates during the S/G2 phase. This means that NHEJ can be considered more flexible and more efficient for creating indels to knockout genes. HDR is more efficient at precise gene modifications but is considered relatively sluggish depending on the DNA template being used (M. Chen et al., 2019b; Yin et al., 2016). The rate of HDR pathway is considered to be relatively low, due to homologous recombinant proteins being mainly expressed in the G2 phase of the cell cycle. Because HDR is expressed in the G2 phase it causes difficulties in gene editing of postmitotic cells such as neurons and cardiac myocytes (Hsu et al., 2014). The HDR repair pathway can be difficult to use in cancer cell lines as some cancer cells have a deactivated HDR pathway and can only repair DSB using the NEHJ pathway. This removes the ability to create knockins using the HDR pathway in these cell lines (S. Li et al., 2021)

1.5 Aims

For this study we hypothesise that silencing MRP2 may be achieved by a CRISPR/Cas9 system. This thesis aims to knock out MRP2 by using a liposome-mediated delivery of ABCC2 guide-RNA/CAS9 protein ribonucleoprotein complexes in Panc1 cell lines and to evaluate the effect of MRP2 silencing on oxaliplatin sensitivity in human PDAC Panc1cell lines.

Chapter 2:

Methods

2.1 Chemicals and reagents

Table 2.1 Chemicals and reagents used in this study and their sources.

Reagents	**Sources (Suppliers)**
Lipofectamine ™ CRISPRMAX™ Cas9 transfection reagent (Cas9 Plus™ lipofectamine and CRISPRMAX™ lipofectamine	ThermoFisher
TrueCut™ Cas9 protein	ThermoFisher
RPMI	ThermFisher
Opti-MEM medium	ThermoFIsher
Fetal Bovine Serum (FBS)	Mediray
TrypLE™ express enzyme	ThermoFisher
GeneArt® genomic cleavage detection kit	ThermoFisher

Table 2.2 Key reagents with catalogue numbers

Description	**Catalogue number**
Invitrogen TrueGuide™ sgRNA Modified (ABCC2)	A35511

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide	M2128-1G
Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent	CMAX00001
TrueCut™ Cas9 Protein v2 (100 µg)	A36498
GeneArt™ Genomic Cleavage Detection Kit	A24372

GeneArt™ Platinum™ Cas9 Nuclease

Added In Cart	Gene	CRISPR Sequence	Direction	PAM	Binding Sites	gRNA Format
	ABCC2	TGGTCCTGGATTTATACACG	(-)	TGG	3	GeneArt™ P...

GCD Primer	Forward Oligo	Reverse Oligo	GCD Band Sizes
Set1	AATGCAGTGGTGAGGTTTAC	AGAAATGCAGCAAGGGAC	628 [405 bp,223 bp]

Figure 2.1 image of the forward and reverse primer corresponding to the ABCC2 CRISPR-Cas9 sGRNA for genomic cleavage assay.

2.2 Cell lines and cell culture

The cell lines provided were from an already established wild type cell at the AUT university. The cells were defrosted from frozen stock by briefly immersing the vial in a 37°C water bath (1-2 min with constant agitation). 2 ml of RPMI (complete media) was added to a 15 ml centrifuge tube, followed by the contents of the defrosted vial. These were spun at 250 x g for 3 min. The supernatant was discarded and the pellet was resuspended with 2 ml of complete, next 4 ml of complete media was added to a T25 flask followed by the 2 ml of resuspended cells. The cells were the kept in a 37°C CO_2 incubator to allow the cells to grow over the surface of the flask.

2.3 Transfection

In order to establish a knockout cell line constituents from ThermoFisher were used. This was applied following the guide lines of the manufacturer.

2.3.1 Seeding cells

Once the cells had reached 70-80% confluency the culture medium was removed, and the cells were washed with PBS solution. After the cells were washed, an aliquot of 2 ml of trypsin was added and the flask was placed in the incubator at 37°C for 3-5 minutes. In order to stop trypsinisation an aliquot 4 ml of culture medium was added to the flask containing the detached cells. The contents were then transferred to a 15 ml centrifuge tube followed by centrifugation at 240 g for 5 minutes. The supernatant was removed, and 1 ml of warm culture medium was added to resuspend the cells. An aliquot of 10 µl of the cell suspension was removed using a pipette, placed on parafilm and mixed with 0.4% Trypan Blue (10µl). This mixture (10) was then added to one side of the haemocytometer until the chamber under the coverslip is filled. Each counting square on the haemocytometer was counted using a microscope with a 10X objective and the average number of cells per sqaure was calculated. The viable cell concentrations was then calculated using the following equation.

Viable cell concentration (number/mL) = Mean viable cell/sqaure × Dilution Factor × 10(to the power of 4)

PANC-1 cells (100,000 cells per/well) were then placed in a 12 well plate for transfection.

2.3.2 Transfection

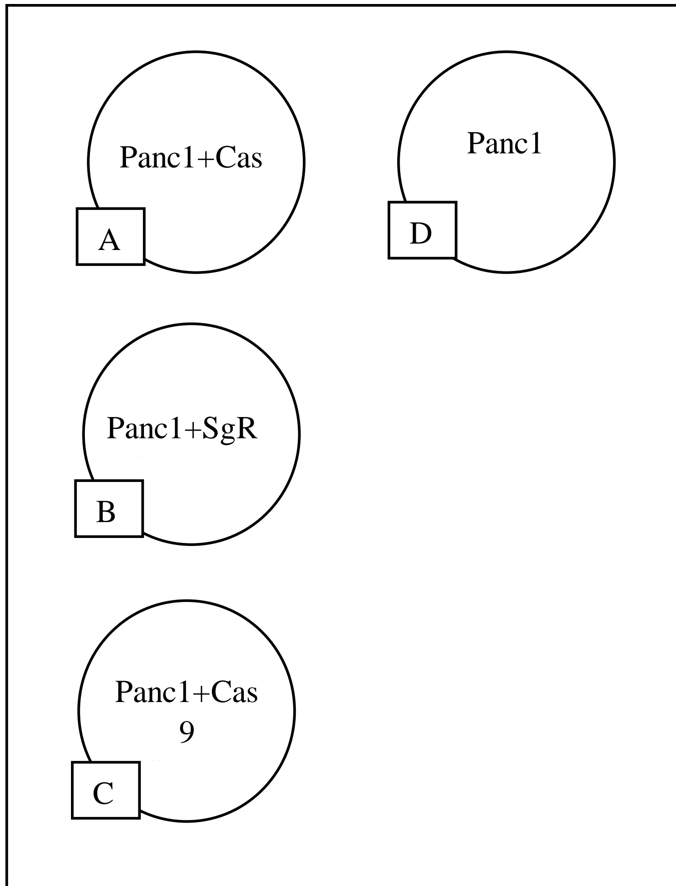


Figure 2 .2 The layout of a transfection experiment displayed on a 6 well plate

Four microcentrifuge tubes prepared as the following:

Two control groups A and B

Cas9 only (A): 50 µl of Opti-MEM medium was added, followed by 0.5 µl of TrueCut™ Cas9 protein V2 and 5 µl of Lipofectamine Cas9 plus. Lastly a mixture of 3 µl of Lipofectamine CRISPRMAX and 50 µl Opti-MEM was added to the solution. This mixture was incubated at room temperature for 15 minutes before being added to the cells.

SgRNA only (B): 50 µl of Opti-MEM medium was added, followed by 3 µl Invitrogen TrueGuide™ sgRNA and 5 µl of Lipofectamine Cas9 plus. Lastly a mixture of 3 µl Lipofectamine CRISPRMAX and 50 µL Opti-MEM was added to the solution. This mixture was incubated at room temperature for 15 minutes before being added to the cells.

ABCC2 KO (C): 5 µl of Opti-MEM was added, followed 0.5 µl of TrueCut™ Cas9 protein, 3 µl Invitrogen TrueGuide™ sgRNA and 5µl Lipofectamine Cas9 plus. Lastly a mixture of 3 µl Lipofectamine CRISPRMAX and 50 µl of Opti-MEM was added to the solution. This mixture is incubated at room temperature for 15 minutes before being added to the cells.

Wild type (D): 50 µl of Opti-MEM was added, followed by Lipofectamine Cas9 plus. Lastly a mixture of 3 µl of Lipofectamine CRISPRMAX and 50 µl of Opti-MEM to the solution. This mixture was incubated at room temperature for 15 minutes before being added to the cells.

The solutions were then mixed by gently swirling the plates, the plates were then incubated at 37° C for 48 hours in a CO_2 incubator. After the incubation period the culture medium was removed and the cells were washed with 500 µl of PBS solution and then the culture medium was replaced with fresh culture medium.

Solution 1:

Invitrogen TrueGuide™ sgRNA: 45 pmol

TrueCut™ Cas9 protein V2: 2500 ng

Lipofectamine Cas9 PLUS: 3 µl

Opti-MEM medium (no FBS): 50 µl for KO reagent

Solution 2:

Lipofectamine CRISPRMAX: 3 µl

Opti-MEM medium (no FBS) 50 µl

2.4 Genomic cleavage assay

In this study the technique used to verify the gene editing efficiency was the GeneArt® Genomic Detection Kit. This kit was applied following the manufacturer's instructions.

2.4.1 Harvest cells.

One the cells had reached confluency after transfection the cells were harvested; these cells were spun down at 200 x g for 5 minutes at 4°C. Then the supernatant is removed and the steps for cell lysis are followed.

2.4.2 Cell lysis and DNA extraction

Cell lysis occurs as followed, 10^6 were used. Fifty µl of cell lysis buffer was mixed with 2 µl of protein degrader in a microcentrifuge tube. This solution was then used to resuspend the cell pellet, the cell suspension was then transferred to a PCR tube and was run following the program in a thermal cycler.

Temp	Time
68^{oc}	15 min
95^{oc}	10 min
4^{oc}	Hold*

PCR amplification was followed directly after completion.

2.4.3 PCR amplification

The cell lysate was briefly vortexed, and the following was added to each PCR tube.

Component	Sample	Control
Cell lysate	2 µl	-
10 µl F/R primer mix	1 µl	-
Control template & primers	-	1 µl

ampliTaq Gold 360 master mix	25 µl	25 µl
Water	22 µl	24 µl
Total	50 µl	50 µl

After the following constituents were added a PCR reaction was run following the below conditions.

Stage	Temp	Time	Cycles
Enzyme activation	95°C	10 min	1x
Denature	95°C	30 sec	40x
Anneal	55°C	30 sec	40x
Extend	72°C	30 sec	40x
Final extension	72°C	7 min	1x
Hold	4°C	Hold	1x

2.4.4 Verifying the PCR product

10µl of PCR product was loaded in a 2% agarose gel along with a DNA ladder. The gel is then run for 10 minutes at 50V then 1 and ½ hours at 100V, after this the gel is imaged.

2.4.6 Cleavage assay

The purpose of this step is to randomly anneal the PCR fragments with and without the indels to form heterogenous DNA duplexes. 2 µl of PCR product was combined with 1 µl of 10X Detection Reaction Buffer in a PCR tube, the volume was then brought to 9 µl with water. Once combined the solution was briefly spun in a centrifuge tube to ensure there are no bubbles, the PCR tube was then placed in a thermal cycler and the following program was run.

Steps	**Heterogenous DNA duplexes condition**
1	95 °C, 10 min
2	95–85 °C, −2 °C s −1
3	85 °C, 1 min
4	85–75 °C, −0.3 °C s−1
5	75 °C, 1 min
6	75–65 °C, −0.3 °C s−1
7	65 °C, 1 min
8	65–55 °C, −0.3 °C s−1
9	55 °C, 1 min
10	55–45 °C, −0.3 °C s−1
11	45 °C, 1 min
12	45–35 °C, −0.3 °C s−1
13	35 °C, 1 min
14	35–25 °C, −0.3 °C s−1
15	25 °C, 1 min
16	25–4 °C, −0.3 °C s−1
17	4 °C, hold

Once the program was finished, enzyme digestion was initiated. This was done by adding 1 µl of detection enzyme to the test samples and 1 µl of water to all the negative samples and then incubate at 42°C for 30 minutes. After 30 minutes, the mixture was vortexed briefly and spun down before immediately loading to the 2% agarose gel.

2.4.5 Gel analysis

A 100 ml of agarose gel solution was prepared in a 250 ml flask, the gel concentration was 2%. The flask was heated in the microwave until the agar was fully dissolved. The solution was then poured into a gel gasket with a comb on the top, avoiding any bubbles.

Once set 10 µl of each of the samples mixed with loading dye were added to the gel as well as a DNA ladder.

The gel is then run for 10 minutes at 50 V then 1 and ½ hours at 100 V, after this the gel is imaged.

2.5 CDCF Accumulation

2.5.1 Clones

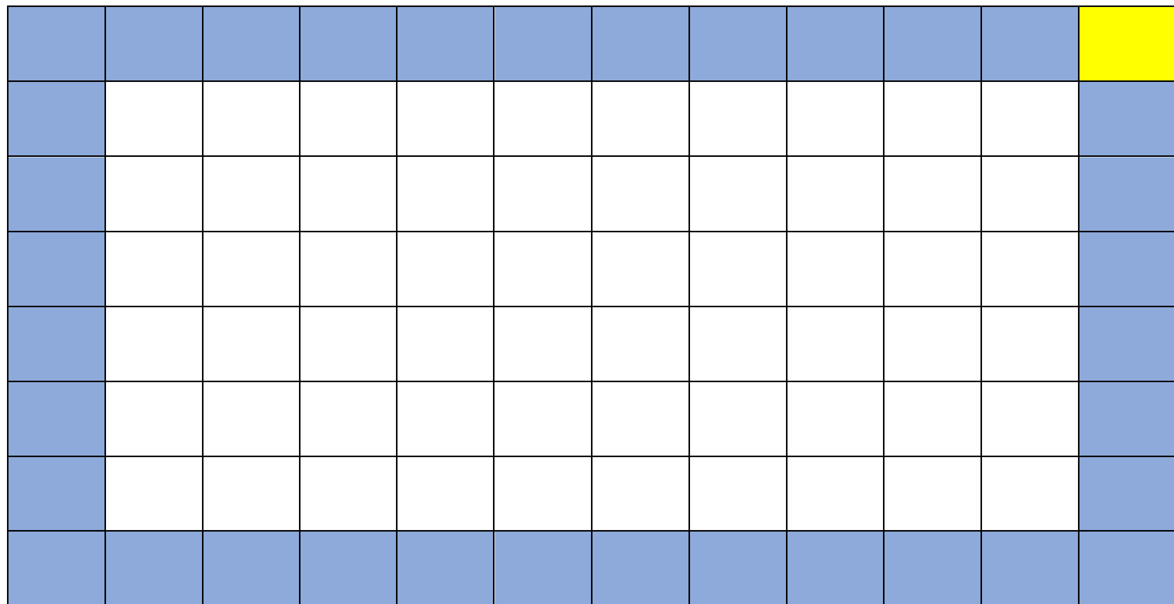
Once the cells had reached confluency the medium was removed from the cells and the cells were washed and detached from the surface of the flask as done previously. They were spun down in the centrifuge at 250 x *g* for 5 minutes, the supernatant was then removed and 1ml of culture medium was added to resuspend the pellet. 10 µl of the cell suspension was removed and placed on parafilm, it was the mixed with 10 µl of 0.4% Trypan Blue. This mixture (10) was added to one half of a haemocytometer until the chamber under the coverslip was full. Each counting square on the haemocytometer was counted using a microscope with a 10X objective and the average number of cells per square was calculated.

The average seeding density we wanted per well was 0.5 this was to ensure that the average number of cells per well was 0.5 to reduce the risk of there being more than 1 cell per well.

To achieve this, we had a cell density of 5 cells/ml, 100 µl of 5 cells/ml solution was aliquoted to each well of the 96 plate. One well in the corner of the plate was seeded with 1000 cells this was done to focus the microscope each time before looking for individual

clones.

Once the cells were seeded into the 96 well plate they were observed under a microscope and wells displaying singular cells were circled with a marker to come back to. The cells were kept in a 37°C CO_2 incubator.



Sterile PBS

1000 cells/well

0.5 cells/well

Figure 2.3: 96 well plate layout for the production of single cell clones

The cells were checked on every few days to ensure they were growing and to cross at any wells where the cells had died. After two to three weeks of growth in the 96 well plate the cells were moved to 6 well plates. Using a multichannel pipette, the culture medium was removed and discarded, the cells were washed with PBS and then detached from the surface of the well using 100 µl of trypsin. Once detached the cells were spun down to form a pellet and the cell density is too small for a viable pellet to be produced, instead

double the amount of culture medium was added to each well and then the cell suspension of each individual well was transferred to a new well in a 6 well plate, where another 2 ml of culture medium was added. A total of 10 clones were originally produced across two 6 well plates.

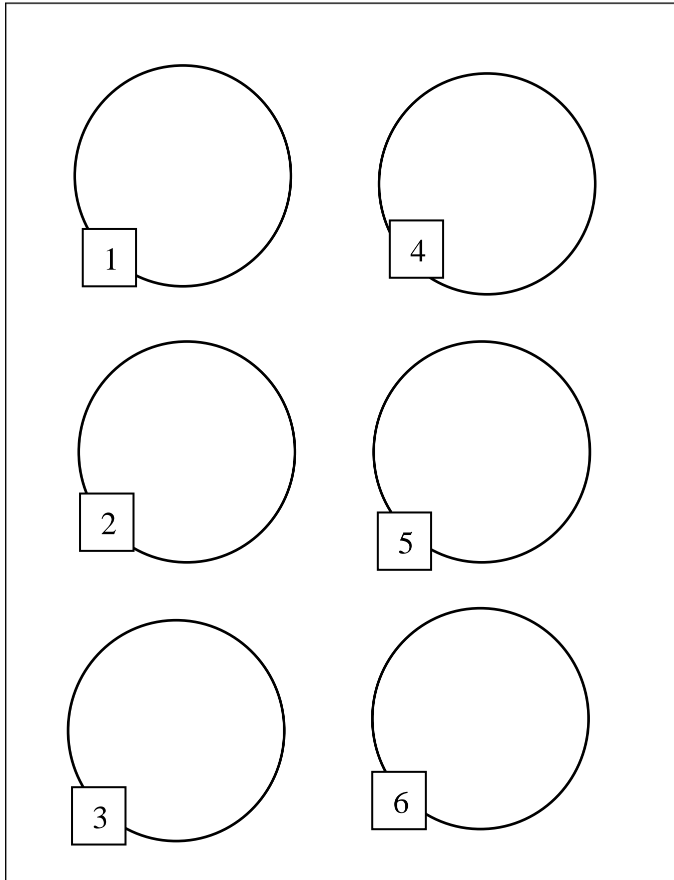


Figure 2.4: Layout of the clones in a 6 well plate, each number indicates the name of clone.

The clones in the 6 well plates were left to grow until they became confluent enough to be transferred to t25 flasks in a 37°C CO_2 incubator. Once they had reached confluency the clones were washed with PBS and detached from the surfacing using 0.5 ml of trypsin after trypsinisation 2ml of culture medium was added again the cells were not spun down in the centrifuge but instead were directly transferred to the T25 flasks. Each T25 flask had

5 ml of culture medium added to the flask before the cells suspension was added. The T25 flasks were then placed back in the 37°C CO_2 incubator to allow the cells to grow.

2.5.2 Accumulation study.

Once the cells are 70-80% confluent the cells were washed with PBS and detached with trypsin before being spun down in a centrifuge at 250 g for 5 minutes. The supernatant was carefully removed and 1ml HBBS was added to resuspend the cells. 10µl of cell suspension is mixed with 10 µl of Trypan blue, after being mixed thoroughly 10 µl of this mixture is placed on one side of a haemocytometer for cell counted. The required cell density $0.5x10^6$ cells/ml.

The accumulation of CDCF was performed by incubating 1ml of cells with 3µl of CDCFDA for 15 minutes at 37°C. After 15 minutes of incubation the accumulation is stopped by adding 3 ml of ice cold PBS and centrifuged for 5 minutes at 250 *g*, this step is carried out a second time. After two lots of washing the resuspended cells are lysed with 200 µl of 0.01% triton in PBS. The cell lysate was shaken for 10 minutes before being plated in a black 96 well plate and placed in the plate reader.

2.6 MTT Assay

2.6.1 Reagent preparation

12 mM MTT stock solution was prepared by adding 1ml of sterile PBS to one 5 mg vial of MTT. The solution was mixed by vortexing until dissolved. The solution was then filtered to remove the material that did not dissolve.

2.6.2 Culturing cells

The cells were seeded onto a 96 well plate, the PANC1 cells were seeded at 8,000 cells/100 µl/well (Figure 2.3). The cells were then incubated for 24 hours in complete medium in a CO_2 incubator. After attachment 100µl of oxaliplatin solutions was added to

each well (refer to figure 2.5 for different concentrations). The cells were then incubated for 2 hours in a CO_2 cell culture incubator, after 2 hours all of the drug solution was removed and 200 µl of complete medium was added and the cells were incubated in a CO_2 incubator for an additional 70 hours.

		0 µM	1.56 µM	3.125 µM	6.25 µM	12.5 µM	25 µM	50 µM	100 µM	200 µM	
		0 µM	1.56 µM	3.125 µM	6.25 µM	12.5 µM	25 µM	50 µM	100 µM	200 µM	
		0 µM	1.56 µM	3.125 µM	6.25 µM	12.5 µM	25 µM	50 µM	100 µM	200 µM	
		0 µM	1.56 µM	3.125 µM	6.25 µM	12.5 µM	25 µM	50 µM	100 µM	200 µM	
		0 µM	1.56 µM	3.125 µM	6.25 µM	12.5 µM	25 µM	50 µM	100 µM	200 µM	
		0 µM	1.56 µM	3.125 µM	6.25 µM	12.5 µM	25 µM	50 µM	100 µM	200 µM	

Panc1 cells (8000 cell/well)

Panc1 cells (8000 cell/well)

Figure 2.5 Layout of the MTT assay on a 96 well plate with drug concentrations

2.6.3 Labelling cells

Immediately after 70 hours all of the culture medium was removed from the cells and the control and replaced with 100 µl of fresh culture medium. 10 µl of the 12 mM MTT stock solution was added to each of the wells including the control wells and incubated at 37°C for 3 hours.

2.6.4 Detection

After the 3 hour incubation period all but 25 µl of the medium was removed from each well and 150 µl of DMSO was added, then mixed thoroughly for 30 minutes on a plate shaker. Once the plate had been shaken 30 minutes it was placed in a plate reader and the absorbance was read at 540 nm with a reference wave length of 680 nm.

Chapter 3

Results

To investigate the feasibility of knocking out the ABCC2 alleles in the Panc1 cell lines using CRISPR-Cas9 system multiple methods were employed. These cells lines were produced by using a lipid based CRISPR-Cas9 transfection method, which should allow for an efficient creation of gene knock outs. In this study we attempted to knock out the ABCC2 genes by transfecting one guide RNA with a Cas9 protein and then assessed the genomic cleavage efficiency. CDCF accumulation and an MTT assay was used to compare the phenotypic differences between the wild type and the transfected cell lines.

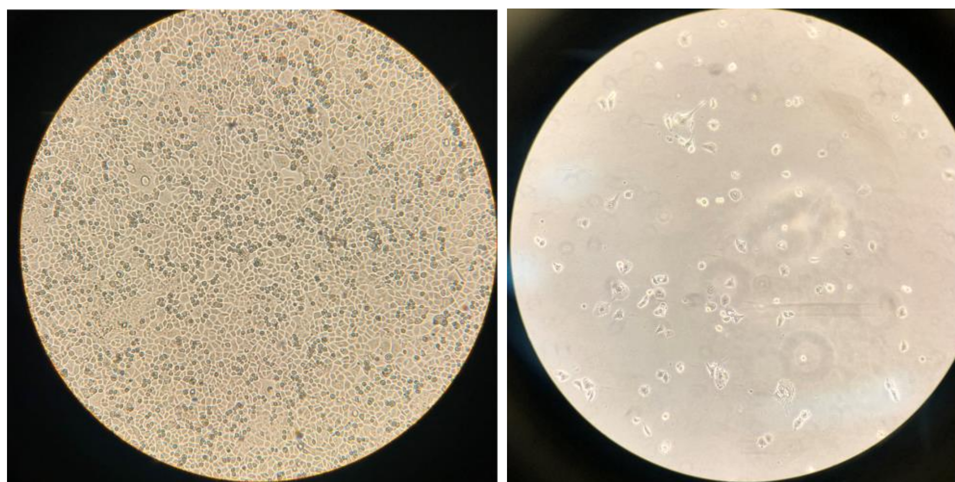


Figure 3.1 (A) An image of high density culture of Panc1 mixing population knockouts, (B) An image of low density culture of Panc1 knockout clones.

3.2 Analyses of gene editing efficiency

Analysis of gene editing efficiency was undertaken using the GeneArt genomic cleavage detection kit. It was a T7 endonuclease 1(T7E1) based method, this is used to quantify how

well the gene editing protocol created indels in the genome of the Panc1 cell line. This functional assay provides us with a means of quantifying the on target efficiency of our knockouts by giving us a visual representation of the indel produced.

Lane 1 2 3 4 5 6

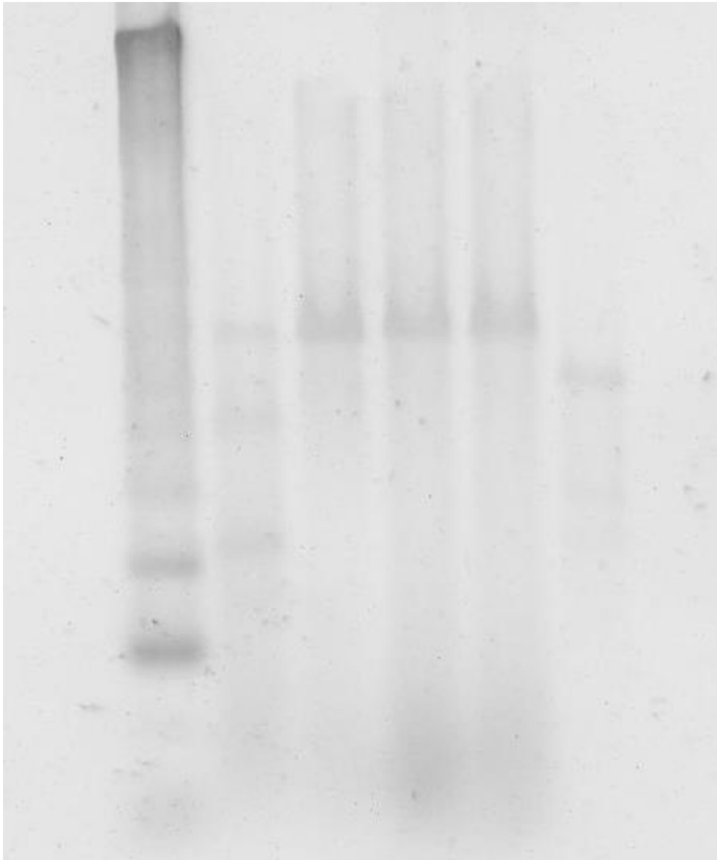


Figure 3.2 The first gel image of genomic detection cleavage assay using, PCR products derived from CRISPR/Cas9 knock out (lane 2), wild type (lane 3), sgRNA only treated (lane 4) and Cas9 only treated (lane 5) Panc1 cells. Lane 6 represents a positive control of the assay displaying one parental band of 516 bp and two cleavage products of 225 and 291 bp in size.

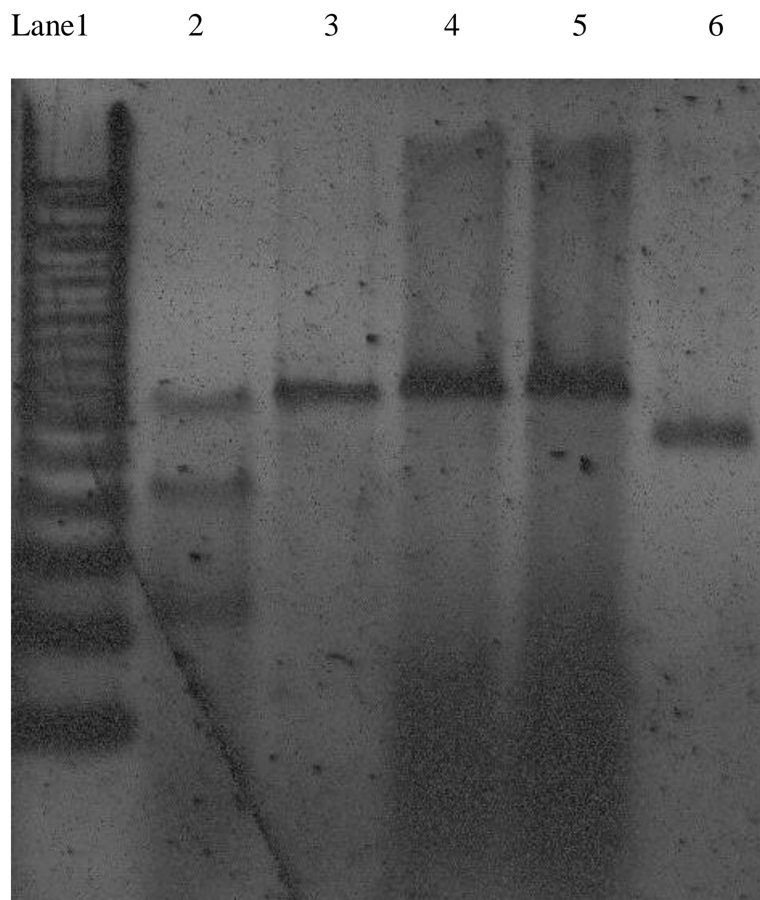


Figure 3.3 Second Gel image of Genomic Detection Cleavage Assay using using, PCR products derived from CRISPR/Cas9 knock out (lane 2), wild type (lane 3), sgRNA only treated (lane 4) and Cas9 only treated (lane 5) Panc1 cells. Lane 2 displays one parental band of 628 bp and two cleavage products of 405 and 223 bp in size.

The results show that the Knockout cell line has three bands present (lane 2) on the gel, one parental band and two distinctive cleavage bands. The cleavage bands are around 405 and 223 base pairs long while the parental band is around 628 base pairs long.

Two genomic cleavage assays were run, (Figure 3.2 and 3.3). The mean of the two results estimates a cleavage efficiency of 42.43 ± 4.3%.

This assay is beneficial as we can physically visualise the parental band and two cleaved bands to confirm if an insertion or deletion (Indel) was produced as well as estimate the efficiency of the transfection. This information provides us with an estimated percentage of the cells that were transfected. What this assay doesn't provide us with is what changes to the gene has occurred, we are unable to determine of the cells that have been transfected if any of them are homozygous for the knockout. For us to be able to determine this we would need to undertake DNA sequencing.

3.3 CDCF uptake study

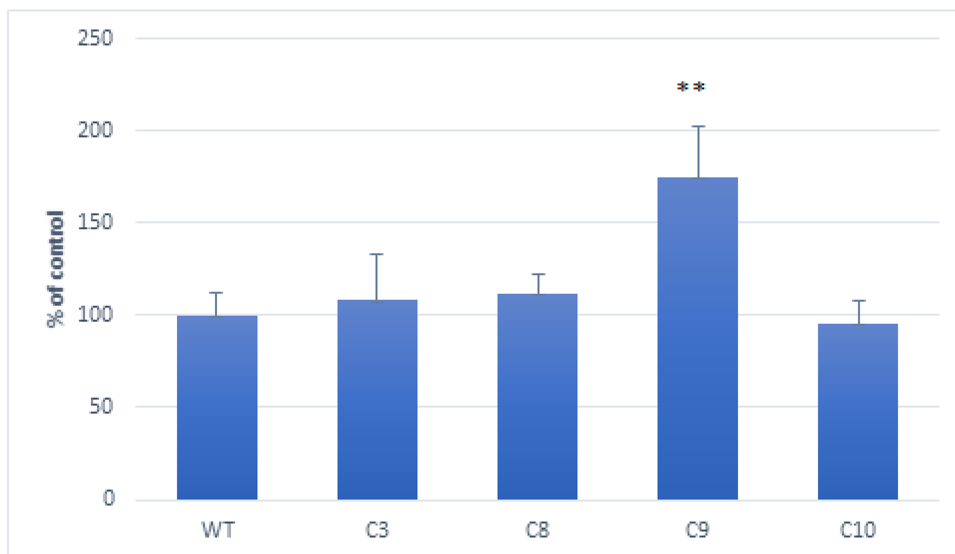


Figure 3.4 Accumulation of CDCF in wild type, and clones 1,8,9,10 knock out Panc1 cells. A one-way ANOVA analysis followed by a Dunnett multiple comparisons gave a $P< 0.01$ ** , Data shown are means ± SD., n=3.

From the results above three of the four clones show a similar fluorescence intensity to the wild type cells while cellular accumulation of CDCF in clone 9 increases by 75% compared to the wild type.

The CDFC uptake study is important for this study as it provided a means of quantifying the accumulation of products that are transported by the MRP2 protein. The non-fluorescent CDCFDA is converted to CDFC inside of the cell and then is transported out of the cell by MRP2. By undertaking this study, we are able to see if the accumulation of CDFC has increased in the cells with a suspected MRP2 knockout. However, the CDFC uptake study is extremely light sensitive it was important for us to ensure that we limited light exposure as much as we possibly can. In order to limit light exposure samples during wash stages were wrapped in tinfoil and when we had to transfer the cells into the 96 well plate it was done under low light conditions. This is because if the CDCFDA was exposed to light for prolonged periods of time it could alter the results that are produced.

3.4 Induced cytotoxicity and cell viability

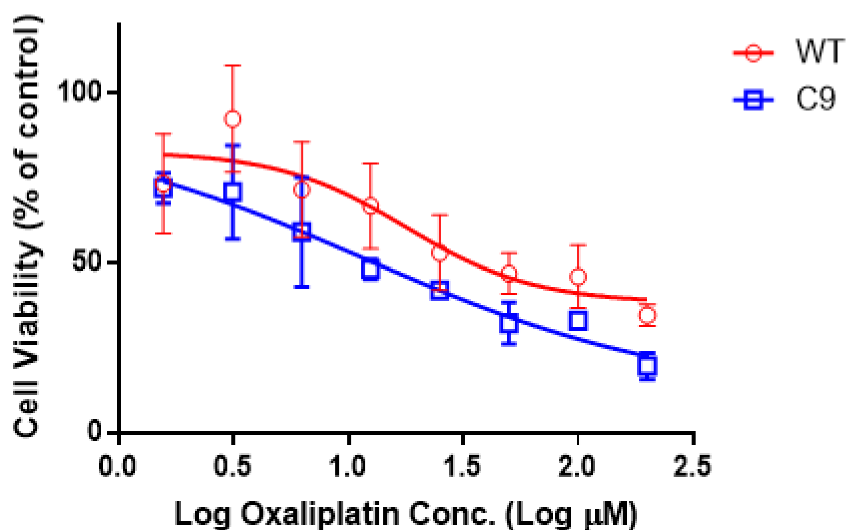


Figure 3.5 Log graph of Oxaliplatin induced cytotoxicity in wild type and clone 9 ABCC2 knock out Panc1 cells. Data shown are means ± SD., n=6.

Table 3.1 The IC_{50} values of the wild type and clone 9 Panc1 cells from induced oxaliplatin induced toxicity (n = 6).

	WT	C9
IC50 (µM)	20.88 (95% CI: 2.688 to 162.1)	12.89 (95% CI: 5.079 to 32.71)

We compared the chemosensitivity of a singular MRP2 knocked out Panc1 cell line with the wild type Panc1 cell line as shown in figure 3.5

The concentration of cell viability curves was well fit the negative sigmoidal model, both cell lines show a slightly decrease in cell viability at lower concentrations, although not steep there is a consistent decrease in cell viability as the concentration increases before the viability begins to plateau and straighten out.

C9 has an IC50 value of 12.89 while the WT cell line has an IC50 number of 20.88 but the 95% confidence interval is overlapped. While this is a preliminary result, further studies may be required to confirm the impact of MRP2 KO on oxaliplatin cytotoxicity.

It was important for us to understand that there are multiple factors that can inhibit the results of the MTT assay. Culture conditions can skew the results, these conditions include the age and the passage number of the cells. The method is designed to measure cytotoxicity, it actually measures the activity of mitochondrial enzymes (Gutiérrez et al.,

2017). The MTT is taken up through passive diffusion and is reduced by mitochondrial enzymes (Kuete et al., 2017).

Cell's health and viability is believed to decrease as passage number increases, some researchers will discard cells with a high passage number to ensure consistent results. There are no guidelines that specifically suggest the optimal passage range (Kwist et al., 2016). The age of and passage of ours cells needed to be taken into consideration, by using cells at a later passage the supposed decrease in cell health could limit the cells rate of mitochondrial enzymes potentially altering the results of the assay. Two cell lines were used in this assay, Wild type Panc1 cells and Clone 9 Knockout cells. Both of these cell lines were of a similar passage number this should reduce any inconsistencies that could have occurred between the cell lines.

Chapter 4

Discussion

4.1 Introduction

Cancer constitutes a significant burden on today's society, the prevalence of cancer is continuously increasing due to the growth of an aging population as well as the increasing prevalence of risk factors in society (Torre et al., 2015a). Pancreatic cancer (PC) is an incurable lethal cancer with a devastating prognosis (Cid-Arregui & Juarez, 2015; Neesse et al., 2011). It has quickly become the seventh leading cause of cancer related deaths worldwide (Rawla et al., 2019a). One of the main reasons for pancreatic cancer being is considered so deadly is that signs of the disease are often not found until the disease has progressed into the late stages of cancer development (McCarter, 2018), with symptoms of the disease not readily displayed in the earlier stages. But the main reason this is such a lethal disease is the failure of chemotherapies in actively treating PC (Cid-Arregui & Juarez, 2015; Neesse et al., 2011).

Previously the first line treatment method for pancreatic cancer was gemcitabine but recently the gold standard has shifted become mixture of more than one chemo-drug called FOLFIRNOX to limit resistance (Springfeld et al., 2019). FOLIFNROX is a combination of drugs, these include folinic acid, fluorouracil, irinotecan and oxaliplatin (Cancer Research UK, n.d.). Previous studies have indicated that by using treatments containing oxaliplatin they have been able increase the survival rate of patients by 4.3 months compared to the survival patients being treated with gemcitabine. However, oxaliplatin has shown some limitations surrounding its effectiveness in treating cancer (Conroy et al., 2011). Treatments with oxaliplatin are limited due oxaliplatin toxicity, high doses of

oxaliplatin have proved effective at eliminating the disease but can cause severe side effects while low doses of the treatment show a poor response and can induce drug resistance (X. Li et al., 2015). As discussed in section 3.1 membrane transporters proteins play an important role in drug transport (Girardin, 2006), the ABC transporter family have multidrug resistant proteins (MRP), the MRP family have been known to confer resistance to platinum-based compounds such as cisplatin (Deeley et al., 2006; Girardin, 2006). Previous lab's work points to the strong potential for ABC transporters to limit oxaliplatin accumulation and sensitivity in human GI cancer lines (e.g Caco-2, HepG2 and PANC1), in which endogenous overexpression of MRP2 was associated with decreased oxaliplatin accumulation and consequently diminished antitumour cytotoxicity (Biswas et al., 2019; Myint et al., 2019a). Also, in membrane vesicles prepared from cells overexpressing the human ABCC2 gene, which encodes MRP2, we showed that MRP2 transports oxaliplatin (Km=301μM; Vmax= 2680 pmol Pt/mg protein/ 10 minutes) (Myint et al., 2015), and that oxaliplatin stimulates MRP2 ATPase activity (EC50 = 8.3 ± 0.7 μM; Hill slope = 2.7) (Biswas et al., 2019). In mice bearing tumour xenografts of a human GI tumour line with endogenous MRP2 over-expression demonstrated that inhibiting MRP2 with myricetin caused increased in vivo sensitisation to oxaliplatin antitumour activity with little or no increase in host toxicity (Myint et al., 2019a).

A possible solution to drug resistance in cancer is gene editing, CRISPR-Cas9 is an efficient and user-friendly method of editing the human genome (Bhaya et al., 2011). The CRISPR system provides a means of knocking out specific genome loci in an organism of interest by using a single strand guide RNA to direct the Cas9 enzyme to snip the target DNA at a specific spot of interest (Cong et al., 2013; Shi et al., 2020). The system is composed of two major components a single guide RNA and a Cas9 nuclease, the Cas9 nuclease uses the guide RNA to locate the appropriate site to cleave the DNA (Saber et al.,

2020). Once the Cas9 protein cleaves the DNA causing a double strand break one of two pathways will take place NHEJ and HDR, to create knockouts the NHEJ pathway is undertaken (Acharya et al., 2020; Ghosh et al., 2019b). NHEJ is an error prone pathway in response to DNA DSB caused by Cas9 cleavage the system chemically ligates the DNA ends back together creating indels disrupting the gene (Baliou et al., 2018; Ghosh et al., 2019b; Menon & Povirk, 2016). The CRISPR-Cas9 system has been used to inhibit tumour cell growth in multiple different cancer types breast, lung, liver, colorectal and prostate cancer by targeting oncogenes with relative success (Hazafa et al., 2020).

The aim of this study was to increase the cytotoxicity of oxaliplatin in Panc1 cells reducing the drug efflux by knocking out the MRP2 protein using the CRISPR-Cas9 system, therefore increasing the efficiency of the drug. In order to determine whether or not we were successful our first objective was to confirm the on target effects of the CRISPR-Cas9 editing system, and estimate the gene efficiency.

4.2 Summary of findings

The results of this project showed that by transfecting Panc1 cells with CRISPR-Cas9 we were able to produce cell lines with an MRP2 knockout. We ran multiple assays in order to determine whether or not the transfection was successful. A genomic cleave assay was undertaken using the T7 endonuclease method, this yielded a cleavage efficiency of 42.5% in the knock out panc1 cell line. Clones of the of the potential knock out cells were then produced and compared against the wild type cell line using the CDCF uptake study (Fig 3.4). The results showed that three of the four viable clones showed a similar fluorescent signal to the wild type cells however one of the clones, clone 9 had a fluorescent signal 75% higher than the wild type. Clone 9 was then used for an MTT assay (Fig 4.5), Clone 9 and wild type cells were treated with varying concentrations of oxaliplatin. The IC50 number (Table 3.1) shows a decrease in cell viability at a much lower concentration for the

clone 9 compared to the wild type, with an IC50 value of 12.89 μM in clone 9 cells (MRP2 knockouts) and an IC50 value of 20.80 μM wild type PANC1 cells. The results suggest that by using gene editing techniques in cancer therapy to inhibit the effects of multi drug resistant proteins we have been able to make cancer cells more susceptible to chemotherapy treatment.

4.3 Gene editing system for the improvement of chemotherapy

Our results provide some evidence that gene editing in combination with chemotherapy acts to limit cell viability. From this we can assume that if cell viability is reduced tumour growth should become limited. Surgical resection has been considered the only curative treatment for pancreatic cancer, though fewer than 20% of patients actually have surgically resectable tumours (Cruz et al., 2014; Kleeff et al., 2016). The inability to operate is due to the disease not being identified early on because of the non-specific symptoms that mostly occur in the later stages of the disease (McCarter, 2018) For both resecteble and non-resectable tumours chemotherapy is used to improve the survival rates of patients, although resistance to single use drugs has been a commonly occurring problem associated with chemotherapy (Springfeld et al., 2019; Szakács et al., 2006). Previously Gemcitabine was considered to be the gold standard for treatment of the disease but recently a combination of multiple drugs has shown to be a more effective form of treatment (Cid-Arregui & Juarez, 2015). FOLFIRNOX a combination of drugs one of which being oxaliplatin has by far surpassed gemcitabine as the preferred treatment option (Chin et al., 2018).

Previous studies involving oxaliplatin have shown that the drug increased the survival rate of patients by 4.3 months when compared against patients treated with gemcitabine (Conroy et al., 2011). Oxaliplatin has shown great promise as a treatment method for pancreatic cancer though the therapy does incur resistance as well as many undesirable

toxic effects (X. Li et al., 2015). A major limitation with oxaliplatin treatment is the dose limiting toxicity, the therapy induces neurotoxicity which damages the peripheral sensory fibres and dorsal root ganglia (Grothey, 2003; Renn et al., 2011). High doses of the treatment are effective at eliminating PC effectively but cause severe side effects and toxicity, while low doses elicit a poor tumour response and create drug resistance (X. Li et al., 2015). By using gene editing we should be able to enhance the efficiency of the treatment by targeting specific genes that confer chemoresistance, which could lead to actively killing cells at a lower dosage of chemotherapy. If we can use smaller doses of chemotherapy we should be able to reduce the levels of toxicity and enhancing the outcome of the cancer treatment. It was important for the study to choose an appropriate gene to target, for this experiment we chose MRP2. MRP2 was chosen because of its association to confer resistance to oxaliplatin. MRP2 plays an important role facilitating detoxification and transport of chemotherapy by the efflux of the drug out of the cells (Z. Chen et al., 2016; Jedlitschky et al., 2006; Zaman et al., 1994). Previous studies had shown that Panc1 cancer cells have a decreased accumulation of oxaliplatin in cells that differentially expressed MRP2 (Myint et al., 2019a).

Our results showed that by knocking out the MRP2 protein we were successfully able to increase the cytotoxicity of oxaliplatin by increasing the accumulation of the drug and decreasing cell viability. However, the results of the of MTT assay in figure 3.5 was only undertaken using one of the clones produced from the original knock out cell line (Clone 9). We can be confident that clone 9 could potentially be a knock out cell line due to the 75% increase in fluorescence compared to the wild type with a p value less than 0.01 (Fig 3.4). While if we compare the results of the rest of the clones with the wild type we see no significant increase in fluorescence meaning the that the MRP2 protein is still successfully pumping the compound out of the cells. This coincides with the cleavage efficiency

percentage; the 42.43% efficiency means we can say we successfully transfected around 42.43% of the cells in our cell line of interest. Indicating that although we were able to successfully transfect cells using CRISPR-Cas9 not every cell was able to be transfected. If we had produced an MTT assay on all of the clones as opposed to just clone 9, we may have found that those cells did not show a decrease in cell viability with low levels of oxaliplatin.

4.4 CRISPR-Cas9 efficiency

CRISPR-Cas9 has shown great therapeutic potential, however there are still many technical issues that stop this technology from being used in a clinical aspect (Kang et al., 2017), these issues include the fitness of edited cells, editing efficiency, delivery of constituents into cells, and potential off target effects (M. Chen et al., 2019b). Genomes can consist of DNA sequences that are identical or closely resemble the sequence you are interest in, this can cause the non-specific cleavage of Cas nucleases at non-target gene areas giving rise to mutations known as off target effects (Kang et al., 2017).

The decision on what guide RNA to use was based on what was available on the Thermofisher website as well the site chop chop. The guide RNA we chose was not the top scoring option available on Thermofisher but was considered high scoring. It was important that we didn't base our decision on one source for our guide RNA. To ensure that the guide RNA of interest was suitable for this project we used chop chop to determine the ranking, efficiency and off target effects. The efficiency of the guide RNA on Chop Chop is 64.7%, the efficiency of our knockouts only showed to be 42.43%. This could be due to multiple factors one of those being off-target effects. By comparing the Thermofisher sequence with the sequences on chop chop we are able to estimate the off-target effects of the guide RNA of interest, Chop Chop indicated that the sequence we chose had 3 potential off targets that were commonly found when using this particular

guide RNA sequence.

Thermofisher had a Top scoring guide RNA that could be considered for more desirable for this project, when compared in chop chop it did rank higher than the one chosen and with an estimated one off target effect but it had a lower efficiency than the guide RNA we chose. It showed an efficiency of 50.52% on chop chop which could suggest it may not be as effective. In order to determine if one guide RNA would be more effective than another both could have been used for this project. By using more than one guide RNA in two sperate Panc1 cell lines the overall project would have had a greater impact as we would have not only been able to compare the efficiencies of multiple guide RNA but been able to see if the cell lines transfected with each guide RNA had varying cell viabilities.

A potential mechanism to test more than one guide RNA at once is CRISPR screening. CRISPR screening can be used to test multiple different guide sequences as well as multiple different genes at once (Goldmeyer, 2021; Shalem et al., 2014). The heterogeneity of cancer causes significant variation between tumours in different patients but also between tumours at different stages within one patient (M. Chen et al., 2019b). This could suggest that although the guide RNA chosen for this project yielded good results for previous studies, the diversity of the cancer genome means that the guide RNA may not be as compatible with this cell line. By using a genetic screen we would be able to test multiple guide RNA against one cell line and find the most effective guide RNA for that cell line in particular.

As discussed earlier the repair pathway chosen plays a significant role in the success of gene editing. For this study we chose to use the NHEJ repair method to produce our knockouts, this method is an error prone repair pathway that produces indels after double strand breaks (Baliou et al., 2018; Ghosh et al., 2019b; Menon & Povirk, 2016). This method of producing knockouts is active during all stages of the cell cycle making flexible

and efficient (M. Chen et al., 2019a; Yin et al., 2016). We chose this repair pathway due to its efficiency compared to the other repair pathway. The other major repair pathway is known as HDR, past studies have shown that efficiency of this pathway is rather low. The HDR pathway requires a donor strand of DNA matching the flanking sequences each side of the DSB, the donor sequence is then incorporated into the genome (Baliou et al., 2018; Drost & Clevers, 2016; Ran et al., 2013). The pathway uses homologous recombinant proteins that are expressed in the G2 phase of the cell cycle which has previously shown to cause difficulties in editing postmitotic cells (Hsu et al., 2014). Strategies that manipulate the choice of repair and favour HDR have been becoming more popular and can assist in the utilization of the CRISPR-Cas9 system achieving a more precise genome editing (Yang et al., 2020b). It has been reported that by inhibiting the NHEJ pathway HDR will be activated as a compensatory mechanism after the generation of a Cas9 mediated DSB (Devkota, 2018; M. T et al., 2015). A 2015 study looked at supressing key NHEJ molecules to disable the NHEJ pathway so that it can’t disrupt the HDR pathway. The key molecules they looked into supressing were the KU70, KU80, DNA ligase IV by silencing SCR7, they also looked into the coexpression of adenovirus 4 E1B55K and E4orf6p proteins. Supressing KU70 and DNA ligase IV showed a 5 fold increase in the efficiency of the HDR pathway, When coexpressed with the Cas9 system E1B55k and E4orf6 improved the efficiency of the pathway up to 8 fold higher. Another approach to increase HDR is to use siRNA or shRNA to knockdown NHEJ effectors (Devkota, 2018). Robert et al (2015) used siRNA to target DNA-PKcs and DNA ligase IV, the results showed that knockdown of the DNA ligase and DNA-PKcs stimulated HDR by approximately 3 fold while reducing NHEJ. With the ever improving rate of CRISPR-Cas9 for producing knockins and utilising the HDR pathway a possible next step for this study would be to use the HDR pathway to insert 1 or more stop codons into our gene of interest. We know that

the NHEJ pathway is error prone and produces indels and still has the ability to produce off target effects. The down fall of this pathway is that the changes made to the genome are random and so we cannot be certain of the changes we are making without going through the extensive process of gene sequencing. While the HDR pathway produces precise changes to the genome just with a lower transfection rate. If we have the ability to increase the rate of transfection using the HDR pathway, knocking in stop codons can produce a more precise knockout/knock down affect. If we were to introduce three stop codons into the MRP2 protein we could have a higher chance of producing a more effective knockout to the ABCC2 gene. The insertion of a stop codon in a protein coding region of a target gene can cause the production of a truncated protein as synthesis is stopped after the stop codon, a truncated protein doesn't function (Kato, 2019).

4.5 MRP2-mediated active transport of oxaliplatin

ABC transporters are ubiquitous membrane proteins found to transport various substrates across membranes via the hydrolysis of ATP (Hollenstein et al., 2007). Mutations in ABC transporters have contributed to a number different disorders (Davidson & Chen, 2004), with several ABC transporters having been found to be overexpressed in cancer cell lines (Szakács et al., 2006). Due to the overexpression of ABC transporters in cancer cells the extrusion of anticancer drugs by these transporters has become one of the of the most widely recognised mechanisms of multidrug resistance (Fletcher et al., 2016; Mirakhorli et al., 2012a). MRP2 is expressed on the apical membrane domain of polarised cells such as hepatocytes, renal proximal tubular cells and enterocytes (Jedlitschky et al., 2006). The MRP2 protein was chosen as our gene of interest due to previous studies relating to this protein. It has been previously identified that the MRP2 protein plays a role in the accumulation of platinum-based drugs. A 2019 study showed that the mechanisms underlying poor responses to oxaliplatin in gastrointestinal cancer involved MRP2 actively

transporting oxaliplatin out of the cells, resulting in a net reduction of oxaliplatin accumulating. The reduction in accumulation decreased the inhibitory effects oxaliplatin has on cell growth (Myint et al., 2019b). This study used the colorectal patient oncogenomic database and found that MRP2 was significantly over expressed in colorectal tumours of patients who did not respond to oxaliplatin based chemotherapy (Myint et al., 2019a). The application of oxaliplatin to treat pancreatic cancer is limited, this is due to the toxic side effects and drug resistance. The drug resistance that is associated with MRP2 is prevalent in Panc1 cells that express a differentiated MRP2 protein and has shown a decreased accumulation of oxaliplatin and an increase in the efflux of drugs out of cells (X. Li et al., 2015; Myint et al., 2015; Zaman et al., 1994). The results from this study can support the hypothesis that MRP2 plays a role in the efflux of oxaliplatin, in chapter 3 we show that by knocking out MRP2 we are able to decrease the uptake of the fluorescent compound CDFC in one of cell lines (Figure 3.4) reiterating that MRP2 is responsible for the transport of compounds out of cells. By treating that cell line with oxaliplatin and comparing it to the wild type we were able to see a decrease in cell viability (Figure 3.5) suggesting that the efflux of oxaliplatin out of cell became limited once the MRP2 function had been disrupted. This provides evidence that MRP2 limits the efficiency of oxaliplatin treatment by transporting the drug out of the Panc1 cells, although it is not indication that MRP2 is over expressed in these cells. In order to determine if MRP2 was over expressed the concentration of the MRP2 protein would need to be compared between panc1 cell lines and healthy pancreatic cells.

4.6 Limitations of this thesis and future directions

One of the major limitations of this study was the number of functional assays undertaken. For this study we only produced three functional assays. It could have been more beneficial to have also done a western blot and gene sequencing. The functional assays

produced were beneficial for the project, however showed some limitations. By doing a genomic cleavage assay we were able to estimate the efficiency at which we transfected the cells, or a percentage of cells that may have the knockout. As discussed in chapter 3 the cleavage assay does not indicate to what extent the knockout was produced and how the gene has been altered. We were also able to do a CDFC accumulation study this was done using clones and comparing them to wild type Panc1 cells. Of the 4 viable clones that were used in this assay only one produced promising results suggesting that clone 9 may have been a homozygous clone for the MRP2 knockout. This assay was beneficial in that we were able to see that knocking out the MRP2 protein increased the accumulation of a model MRP2 substrate CDCF, but from this assay we cannot confirm that clone 9 was homozygous due to the lack of direct genomic sequencing results. If gene sequencing had been carried out, we have been able to confirm whether or not clone 9 was homologous for the knockout and to what extend we had produced the knock out in the rest of the clone cell lines. Another assay we were unable to produce was a western blot, western blotting can be used to measure protein expression. Measuring protein expression would have been important to this study as it would have provided a means of measuring the expression of MRP2 in our cell lines. Currently this study shows the effects the transfection has on the cells and how that can improve oxaliplatin treatment in Panc1 cells but it doesn't give us a means of measuring the protein expression/abundance after the transfection has occurred to ensure that we had been successful in reducing or halting the expression of MRP2 at the protein level.

Another limitation of this study is that it is carried out in vitro, studies that are carried out in vitro are carried out in ideal environments. The cells live in an optimum environment with specific culture medium and at a somewhat maintained temperature. This doesn't accurately reflect how the study would take place during in patient studies. During

transfection in vitro the guide RNA and Cas9 protein are exposed directly to the cell and only have to pass through the cell membrane. In patients the transfection process would face a series of limitations, these include how the CRISPR mechanism would be effectively inserted into the body and then how the mechanism of transfection would withstand being exposed to different elements inside the body. In vitro studies only use a select sample of cells and therefore doesn't accurately represent entire populations of people. The heterogeneity of cancer is quite significant, there is a large number of variations not only between patients but also within tumours and at different stages of tumour growth (M. Chen et al., 2019b). In patients pancreatic cancer is often in later stages when discovered, this means that is very likely the patient has become metastatic. Due the nature of cancer being very genetically diverse even withing one singular patient, the results of CRISPR transfection may yield a very low efficiency in comparison to in vitro studies. This makes it difficult to predict if the results produced in an in vitro study would translate well into a clinical aspect.

For future research we should move towards animal trials to try and replicate how the study may impact humans, providing more clinical relevancy. A study used mice with a null-MRP2 knockout to understand the function of MRP2 in the accumulation and toxicity of cisplatin in the kidneys of mice. For this study, the mice were purchased already with a null-MRP2 mutation, therefore no gene editing was carried out during the study. After exposure to cisplatin for 24 hours the null-MRP2 mice showed a concentration of platinum in the kidneys two-fold higher than in the wild type mice (Wen et al., 2014). Another in vivo study used mice with HepG2 subcutaneous xenografts to understand the association myricetin had on oxaliplatin treatment. Mice treated with oxaliplatin showed sensitivity to the anticancer drug and it was believed that by concurrently treating the mice with myricetin and oxaliplatin the sensitivity and toxicity to oxaliplatin would decrease. The

mice were implanted with HepG2 cells to induce tumours in the mice, once a tumour was established mice were treated with a combination of oxaliplatin and myricetin. They found that the combined treatment slowed tumour growth and extended the survival of mice while reducing the toxic effects (Myint et al., 2019a). Both of these studies lay a good foundation to use mice as an animal model for our future research as well as it being feasible to target MRP2 in mice. Myint et al (2019) indicates that it is possible to produce mice with HepG2 tumours by implanting tumour cells into the mice, we could replicate this in our study using Panc1 tumour cells and implanting them subcutaneously into mice ideally inducing pancreatic cancer tumours. The mice would then need to be transfected using CRISPR-Cas9 as we did with the cells in this study, in attempt to knock out the MRP2 protein in the tumour cells within the mice. Once the transfection is done the mice would be treated with oxaliplatin and tested in various aspects to understand if tumour growth had decreased. By using an animal model we are able to form some understanding as to how this process may take place in humans with similar limitations being in place, this makes the research more clinically relevant.

Myint et al (2019) studied the associations between tumour expression of MRP2 and patient response to oxaliplatin, what they found was that MRP2 was differentially expressed in patients that showed a poor response to FOLFOX treatment, a treatment that uses oxaliplatin. The results from our study provide evidence that MRP2 limits the accumulation of oxaliplatin in Panc1 cells increasing cell viability. If patient response is poor in people who differentially express MRP2 and our data suggests that limiting MRP2 can decrease cell viability, then a potential step in the future is screening patients for MRP2 levels. If we screen patients based on their MRP2 levels we may be able to determine what patients may benefit more from oxaliplatin treatment than others. By treating patients with lower levels of MRP2 expression this ideally should reduce the

amount of oxaliplatin needed to treat the tumour. Using lower amounts of oxaliplatin should in turn reduce toxicity in patients and oxaliplatin resistance.

4.7 Overall conclusions

In summary, this thesis has demonstrated that by using CRISPR-Cas9 gene editing system we have been able to produce MRP2 knockouts in Panc1 cell lines. This has led to an increased accumulation of the model MRP2 substrate, therefore enhancing oxaliplatin cytotoxicity in Panc1 cells. The results reported in this thesis are consistent with what previous studies have shown, that due to MRP2 being a transporter protein it limits the accumulation of oxaliplatin in certain cancer cells making it less effective.

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