MRP2 as a Targetable Oxaliplatin Resistance Factor in Gastrointestinal Cancer

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Abstract

Despite the severe adverse effects, toxicity and limited efficacy due to the development of multidrug resistance (MDR), chemotherapy is still the treatment of choice for most of the cancer patients. One of the formidable MDR mechanisms is the up-regulation of various efflux pumps, known as the ATP binding cassette (ABC) transporters, which can efficiently remove drugs from the cell, thus causing the decreased efficacy of chemotherapeutic drugs. The platinum-based regimens are important in the clinical treatment of gastrointestinal cancer. While many membrane transporters are reported to play important roles in platinum-based chemoresistance, a recent study showed that multidrug resistance-associated protein 2 (MRP2) mediated the ATP-dependent active transport of oxaliplatin-derived platinum in membrane vesicle models. This study suggested that oxaliplatin is a substrate for MRP2. However, the mechanistic mechanisms of MRP2-oxaliplatin interactions and the contribution of MRP2 to oxaliplatin resistance remain unclear.

The purpose of this thesis is to investigate oxaliplatin interaction with MRP2 transporter using a colourimetric ATPase assay and assess whether MRP2 confers oxaliplatin resistance in MRP2 overexpressing gastrointestinal cancer cells, particularly colorectal cancer cells (Caco-2), hepatocellular cancer cells (HepG2) and pancreatic cancer cells (PANC-1).

Human MRP2-expressing membrane vesicles prepared from Sf9 insect cells were used for ATPase studies (Chapter 3). The amount of inorganic phosphate released from substrate-stimulated ATP hydrolysis was measured by a colourimetric assay. Oxaliplatin stimulated vanadate-sensitive Sf9/MRP2 ATPase activity appeared linear within 30 min. Concentration-dependent effects of oxaliplatin on Sf9/MRP2 ATPase activity were determined at 20 min and the data was best fit with a sigmoidal dose-response model to generate an EC_{50} value of 8.3 ± 0.7 μM and a Hill coefficient of 2. Oxaliplatin-stimulated Sf9/MRP2 ATPase activity was significantly inhibited by well-defined MRP2 inhibitors benzbromarone and myricetin. Oxaliplatin does not interact with wild-type ABC transporters in Sf9 cells. Taken together, our results suggest oxaliplatin is a human MRP2 substrate possibly with two binding sites on MRP2.

In above mentioned human gastrointestinal cell lines, silencing the *ABCC2* gene led to increased cellular accumulation of oxaliplatin-derived platinum and enhanced

anticancer activity of oxaliplatin (Chapter 4 and 5). In HepG2, Caco-2 and PANC-1 cells, after siRNA transient knockdown of *ABCC2* gene, the ABCC2 mRNA level and cell surface MRP2 expression were significantly decreased. The sensitivity to oxaliplatin-induced growth inhibition were enhanced in ABCC2-siRNAs transfected HepG2, Caco-2 and PANC-1 cells by up to two-fold compared with control-siRNA transfected cells.

To explore whether the marked suppression of tumour proliferation was attributed to the inhibition of MRP2-mediated platinum efflux, the mechanisms underlying the enhanced efficacy were explored. Silencing *ABCC2* gene resulted in about 2-fold increase in the oxaliplatin-derived platinum accumulation in HepG2 and Caco-2 cells.
The apoptosis assays revealed that modulating MRP2 transporter either by siRNA knockdown of *ABCC2* gene or by an MRP2 inhibitor myricetin, significantly enhanced the oxaliplatin-induced apoptosis rate in gastrointestinal cancer cells (Chapter 6).

In conclusion, oxaliplatin was confirmed as a substrate of MRP2 transporter. Furthermore, modulation of MRP2 in human gastrointestinal cancer cells using either siRNA-mediated transient gene knockdown or an MRP2 inhibitor myricetin increased the sensitivity of oxaliplatin and cellular accumulation of oxaliplatin-derived platinum. Screening tumour MRP2 expression levels to select patients for treatment with oxaliplatin alone or in combination with MRP2 modulation, could improve outcomes of gastrointestinal cancer treatment.

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Attestation of Authorship

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

Signed:

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1. Piyush Bugde, Riya Biswas, Fabrice Merien, Jun Lu, Dong-Xu Liu, Mingwei, Chen, Shufeng Zhou & Yan Li, The therapeutic potential of targeting ABC transporters to combat multi-drug resistance. *Expert Opinion on Therapeutic Targets*, 2017. 21(5): p. 511-530.

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Conference abstracts

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List of abbreviations

°C	Degree Celsius
5-FU	5-fluorouracil
ABC	ATP-binding cassette
ADME	Absorption, distribution, metabolism and elimination
ANOVA	Analysis-of-variance
ATP	Adenosine-5'-triphosphate
ATP7A	P-type ATPase 7A
ATP7B	P-type ATPase 7B
AUC	Area under the curve
BBB	Blood brain barrier
BCRP	Breast cancer resistance protein
bp	Base pair
BSA	Bovine serum albumin
CDCFDA	5(6)-carboxy-2,'7'-dichlorofluorescein diacetate
CDCF	5(6)-carboxy-2',7'-dichlorofluorescein
cDNA	Complementary deoxyribonucleic acid
Cmax	Maximum plasma concentration
Ct	Cycle threshold
CTR1	Copper transporter 1

DACH	Diaminocyclohexane
DDI	Drug-drug interaction
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
EC_{50}	Half maximal effective concentration
EGFR	Epidermal growth factor receptor
FBS	Foetal bovine serum
FDA	Food and drug administration
FOLFOX	Folinic acid, fluorouracil and oxaliplatin
FOLFIRI	Folinic acid, fluorouracil and irinotecan
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GI	Gastrointestinal
HCC	Hepatocellular carcinoma
hr	Hour
IC_{50}	Half maximal inhibitory concentration
ICP-MS	Inductively coupled plasma mass spectrometry
i.v.	Intravenous
kDa	Kilodaltons
KD	Knockdown
kg	Kilogram
Km	Concentration at half Vmax

KO	Knockout
LTC4	Leukotriene C4
M	Molar concentration
µM	Micromolar concentration
mM	Millimolar concentration
µg	Micro-gram
mg	Milli-gram
MgATP	Adenosine 5'-triphosphate magnesium salt
µl	Micro-litres
ml	Milli-litres
mol	Mole
MDR	Multidrug resistance Minutes
min	Minutes
MEM	Minimum essential media
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MMR	Mismatch repair
NER	Nucleotide excision repair
OCT	Organic cation transporter
ng	Nano-gram
nM	Nanomolar concentration
nmol	Nanomole
NBD	Nucleotide binding domain
pmol	Picomole
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PD	Pharmacodynamics
Pen-strep	Penicillin-streptomycin
PFA	Paraformaldehyde
PFS	Progression-free survival
P-gp	P-glycoprotein
Pi	Inorganic phosphate
PK	Pharmacokinetics
ppb	Parts-per-billion
Pt	Platinum
RNA	Ribonucleic acid
RNAi	RNA interference
R^2	Coefficient of determination
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
qPCR	Quantitative polymerase chain reaction
SLC	Solute carrier
siRNA	Small interfering RNA
TMD	Transmembrane domain
Vmax	Maximum velocity
XELOX	Oxaliplatin and capecitabine

Chapter 1 General Introduction

1.1. Overview of Gastrointestinal (GI) cancer

Cancer is an abnormal growth of cells that have lost control over death mechanisms and continuously expand and invade other adjacent normal tissues. There are more than 200 types of cancer that can spread from almost every cell type, including prostate cancer, colorectal cancer, breast cancer, lung cancer and lymphoma [1]. Each cell type gives rise to a distant type of cancer and depending on both location of the cells and genetic aberration, multiple forms of cancer can develop from each cell type. These cancerous cells mostly form a mass of cancerous tissue known as a tumour, which invades from the primary site and spreads throughout the body, forming a metastatic cancer. Numerous factors, such as the growth rate of a tumour, the extent of invasiveness, the degree of differentiation and metastatic potential determine the degree of tumour malignancy.

Cancers can be classified into five groups, including leukaemia (cancer that starts in blood-forming tissues such as bone marrow), lymphoma and myeloma (cancer that begins from lymphatic system and lymph nodes), carcinoma (cancer that begins in skin, lungs, pancreas or the tissues that cover internal organs), sarcoma (cancer of mesodermal cells, mainly blood vessels, bone, muscle and connective tissue) and central nervous system cancer (cancer that originates in the tissues of the brain and spinal cord) [2].

Gastrointestinal (GI) cancer refers to malignant conditions of the GI tract and organs of digestive system, including the oesophagus, biliary system, stomach, pancreas, liver, colon and rectum. GI cancers are responsible for more cancer deaths than any other types of cancer [3]. GI cancer is mainly divided into the upper digestive tract and lower digestive tract. The upper digestive tract includes oesophageal, stomach, pancreatic, liver and gallbladder cancer. The lower digestive tract includes colorectal and anal cancer. Oesophageal cancer is the sixth most common cancer in the world and characterised by a high incidence rate [4]. The two major types of oesophageal cancer are adenocarcinoma and squamous cell carcinoma [3]. Stomach or gastric cancer is the fourth most common type of cancer and the second leading cause of cancer death globally [5]. The most common type of gastric cancer is adenocarcinoma [6]. Australia and New Zealand are low-risk areas for stomach cancer [7]. Pancreatic cancer has a

poor prognosis and the major risk factors for pancreatic cancer are advanced age, chronic pancreatitis, diabetes and smoking [3]. The most common type of pancreatic cancer is ductal adenocarcinoma. Hepatocellular or liver cancer is usually caused by hepatitis B or C, cirrhosis or aflatoxins [8]. Colorectal cancer is the third most common cancer globally [5]. The majority of colorectal cancers are adenocarcinomas.

Cells become cancerous because of the acquired changes in deoxyribonucleic acid (DNA) sequences in the normal cells. While approximately 10% of cancers can be traced directly from inherited genetic defects [9], DNA damage may be caused by environmental factors that increase the risk of cancer, including smoking, dietary factors, chemical and radiation exposure, obesity, diabetes and other environmental pollutants [10]. In most cases, that cause of cancer is probably due to the combination of both genetic makeup and environmental factors. The causes of cancer are complex, diverse, highly variable and still uncertain. Cancer can affect people of any age group; however, but the risk of developing cancer increases with age. According to the American Cancer Society in 2018, the most common cancers in men are prostate, lung and colon and in women are breast, lung and GI cancer [11].

Cancers can be detected in many ways, including early signs and symptoms, screening tests, other medical imaging or biopsies. The most common types of cancer treatments are surgery, chemotherapy, immunotherapy, radiation therapy, targeted therapy, hormone therapy and stem cell transplant [12].

1.1.1. Impact of cancer worldwide

Cancer is one of the major public health problems throughout the world in both developed and developing countries and is the second leading cause of death in the United States. According to GLOBOCAN 2012 estimation, approximately 14.1 million new cancer cases, including 7.4 million (53%) in males and 6.7 million (47%) in females were reported [13, 14] and nearly 8.2 million cancer-related deaths have been reported [12]. In 2012, around 46% of all cancer deaths throughout the world were caused by lung, liver, stomach, bowel and pancreas cancer.

According to the National Cancer Institute [7], in 2018 it is estimated that 1,735,350 new cases of cancer will be diagnosed and around 609,640 people will die from cancer-related disease [7]. Based on the 2011-2015 SEER Cancer Statistic Review [15], the

number of new cancer incidences is 439.2 per 100,000 and the number of cancer deaths is 163.5 per 100,000 [16]. Based on the recent increase of cancer incidence, globally, it is predicted that by 2030 there will be 23.6 million new cancer cases [5, 17]. The alarming rate of cancer incidences and associated mortality calls for an immediate action plan. Hence, it is urgent to develop an effective and affordable mode of early detection, diagnosis and treatment of cancer. Despite the development of many new cancer treatments, including chemotherapy, immunotherapy and surgery, based on these reports, we still estimate that cancer is one of the most common health problems in the world.

According to worldwide estimated new cases, the most common cancers in 2018 are breast, lung, prostate, colorectal, melanoma, bladder, non-Hodgkin lymphoma, kidney and renal pelvis, endometrial, leukaemia, pancreatic, thyroid and liver cancer [7].

1.1.2. GI cancer status in New Zealand

According to the New Zealand Cancer Registry in 2011, cancer is the third most common cause of death in New Zealand. In 2015, the incidence of new cancers was estimated as 331.7 per 100,000 and according to the 2013 report, the death rate from cancer was estimated as 122.8 per 100,000 population [18, 19]. It has been observed that cancer is a common disease in the aged population and 57% of new cancers are registered for people above 65 years old [19].

In New Zealand, the most common type of cancer registered is prostate cancer (3129 cases) followed by colorectal cancer (3075), breast cancer (3046), melanoma (2366) and lung cancer (2037) [20]. About 27.3% of males and 28.7% of females suffer from prostate cancer and breast cancer respectively, which are considered the most common types of cancer. The next most commonly registered cancers are colorectal cancer and melanoma. Lung cancer causes the highest death rate. According to worldwide cancer mortality statistics, in New Zealand, lung cancer is the most common cause of death from cancer. Colorectal cancer is the second most common cause of death followed by breast cancer, prostate cancer and pancreatic cancer [19]. The rate of stomach cancer incidence and cancer death is higher in men than in women in New Zealand. Therefore, GI cancers including stomach, colorectal, liver and pancreatic cancer are the most commonly estimated cancers and cause of cancer deaths both worldwide and New

Zealand. In this thesis, we are mainly focusing on liver, pancreatic and colorectal cancer.

Hepatocellular carcinoma (HCC) or liver cancer is the most common type of cancer worldwide with 782,451 incidences per annum and 745,533 cases of cancer death per annum. The second most common cause of cancer death in the world is liver cancer [5, 21]. In New Zealand, liver cancer is not so common with an estimated incidence rate 6.6 per 100,000 population (296 cases per annum) and an estimated mortality rate 5.3 per 100,000 population (236 cases per annum).

Pancreatic cancer is considered a fatal cancer with the poorest survival rate, only 25-30% five-year survival rate after surgery [22]. Worldwide, pancreatic cancer is the twelfth most common cancer and the seventh leading cause of cancer mortality, with 330,391 deaths, according to GLOBOCON 2012 [5]. In New Zealand, pancreatic cancer is one of the most common cancer types with an incidence rate of 10.9 per 100,000 population (486 cases per annum) and is the fifth leading cause of cancer mortality with an estimated mortality rate 10.7 per 100,000 population (476 cases per annum).

According to GLOBOCON 2012, Colorectal or bowel cancer is considered to be the third most commonly diagnosed cancer with an estimated 1,360,602 new cases (19.3 per 100,000 population) and 693,933 cases of cancer death (9.8 per 100,000 population). The Australia/New Zealand region has the highest rate of colorectal cancer in the world. In New Zealand, colorectal cancer is the second most common type of cancer after prostate cancer in males and breast cancer in the females. In 2012, the estimated new cases reported were 3018 (67.6 per 100,000) and mortality cases were 1321 (29.6 per 100,000).

From the above statistics, we can conclude that GI cancer is one of the main cancers with the highest incidence rate and mortality rate in the world and New Zealand. Therefore, it is worthwhile to study, explore and modify current treatment approaches of GI cancer for effective outcomes in GI cancer patients.

1.1.3. Chemotherapy regimens for GI cancer

Chemotherapy is a method of treating cancer using anticancer drugs to kill cancer cells or inhibit their proliferation. Mostly, other types of therapy like surgery and radiation therapy remove or damage cancer cells in a certain area, but chemotherapy can work throughout the whole body. Chemotherapy is usually given orally or intravenously before surgery to shrink the tumour size and/or after surgery to destroy the remaining cancer cells or kill cancer cells that have returned or spread to the other parts of the body. For some advanced cancer cases where a tumour is hard to resect or remove by surgery, chemotherapy is given as a primary course of treatment to deter tumour size that is causing pain and other problems. Chemotherapy is usually given in cycles in which a dose of one or more drugs is given in regular intervals followed by several days of no treatment or a period of rest. The resting period gives normal cells time to recover from drug side effects [23].

1.1.3.1. Chemotherapy drugs used for liver cancer

Chemotherapeutic agents have been extensively studied in liver cancer, but have not demonstrated an improvement in overall survival benefits compared with other therapies that do not involve drug treatment. The most common combination of drugs used is the ECF regimen (epirubicin, cisplatin and 5-fluorouracil). This combination of drugs shrinks only a small portion of tumours and responses do not last long. Oxaliplatin-based combination chemotherapy has improved liver cancer patients' outcomes, especially GEMOX (oxaliplatin and gemcitabine), FOLFOX4 (oxaliplatin, folinic acid and 5-fluorouracil) and XELOX (oxaliplatin and capecitabine) [24, 25]. The combination of sorafenib (multi-kinase inhibitor) with different regimens has slightly improved outcomes in patients with advanced HCC [26]. Therefore, liver cancer is still a refractory disease due to tumour resistance and/or the toxicity of chemotherapeutic agents.

1.1.3.2. Chemotherapy drugs used for pancreatic cancer

A fluorinated analogue of deoxycytidine, gemcitabine, is the main chemotherapeutic drug used as the first-line agent for the treatment of pancreatic cancer. Gemcitabine-based combination treatments have been assessed for advanced pancreatic cancer and have yielded a better response and longer progression-free survival (PFS) than the

treatment with gemcitabine alone, such as a combination of gemcitabine and capecitabine [27]. Erlotinib, a tyrosine kinase inhibitor of EGFR is also used along with gemcitabine [28, 29]. In another clinical study, oxaliplatin-based combination chemotherapy, FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan and oxaliplatin), was reported with good performance status. Oxaliplatin-based, GEMOXEL (gemcitabine, oxaliplatin and capecitabine) regimen is commonly used to treat pancreatic cancer, which has improved the survival status of patients [30-33]. The overall survival of pancreatic cancer patients was better when gemcitabine was combined with the platinum-based drug [34, 35].

1.1.3.3. Chemotherapy drugs used for colorectal cancer

Oxaliplatin is used as a first-line treatment regimen for metastatic colorectal cancer. Drugs that are commonly used for colorectal cancer are 5-Fluorouracil, Capecitabine (Xeloda), Irinotecan (Camptosar), Oxaliplatin (Eloxatin), Trifluridine and tipiracil (Lonsurf). In most cases, a combination of two or more drugs is used for chemotherapy. The most common oxaliplatin-based combination treatment regimens used for colorectal cancer include FOLFOX (oxaliplatin, 5-fluorouracil, folinic acid), XELOX (oxaliplatin, capecitabine) and FOLFOXIRI (oxaliplatin, 5-Fluorouracil, folinic acid, irinotecan) [36-39]. Recently, several other drugs have been used for the treatment of colorectal cancer. These include panitumumab (Vectibix), cetuximab (Erbitux), bevacizumab (Avastin), ramucirumab (Cyramza) and aflibercept (Zaltrap). These novel drugs are used along with 5-fluorouracil, irinotecan and oxaliplatin for metastatic colorectal cancer [40].

From the above section, we can note that the platinum-based drug, oxaliplatin, is a common anticancer drug that is used in combination chemotherapy for the treatment of GI cancers including liver, pancreatic and colorectal cancer.

1.2. Platinum-based drugs

Nowadays, different types of drugs are used for treating human malignancies, such as cancer of the lung, colon, rectum, breast and ovary; however, platinum-based drugs are considered one of the most important chemotherapeutic drugs for cancer treatment [41]. Since the late 1970s, several different platinum-based drugs have been introduced as chemotherapeutic agents for the treatment of various cancer tumours worldwide, such

as cisplatin, carboplatin and oxaliplatin, and locally, such as nedaplatin, lobaplatin and heptaplatin [42, 43]. However, the cellular responses that confer the resistance to platinum (Pt) drugs are multifactorial and inexplicit [44]. Their clinical utility is generally limited by tumor resistance. The intracellular mechanism by which cells acquire resistance to Pt drugs include (a) increased detoxification of drugs by thiol groups; (b) improved tolerance to nuclear lesion, which ultimately leads to reduced apoptosis; and (c) diminished accumulation of cisplatin [45] , carboplatin [46] and oxaliplatin [47]. The structures of different types of clinically important platinum-based drugs are shown in figure 1.1.

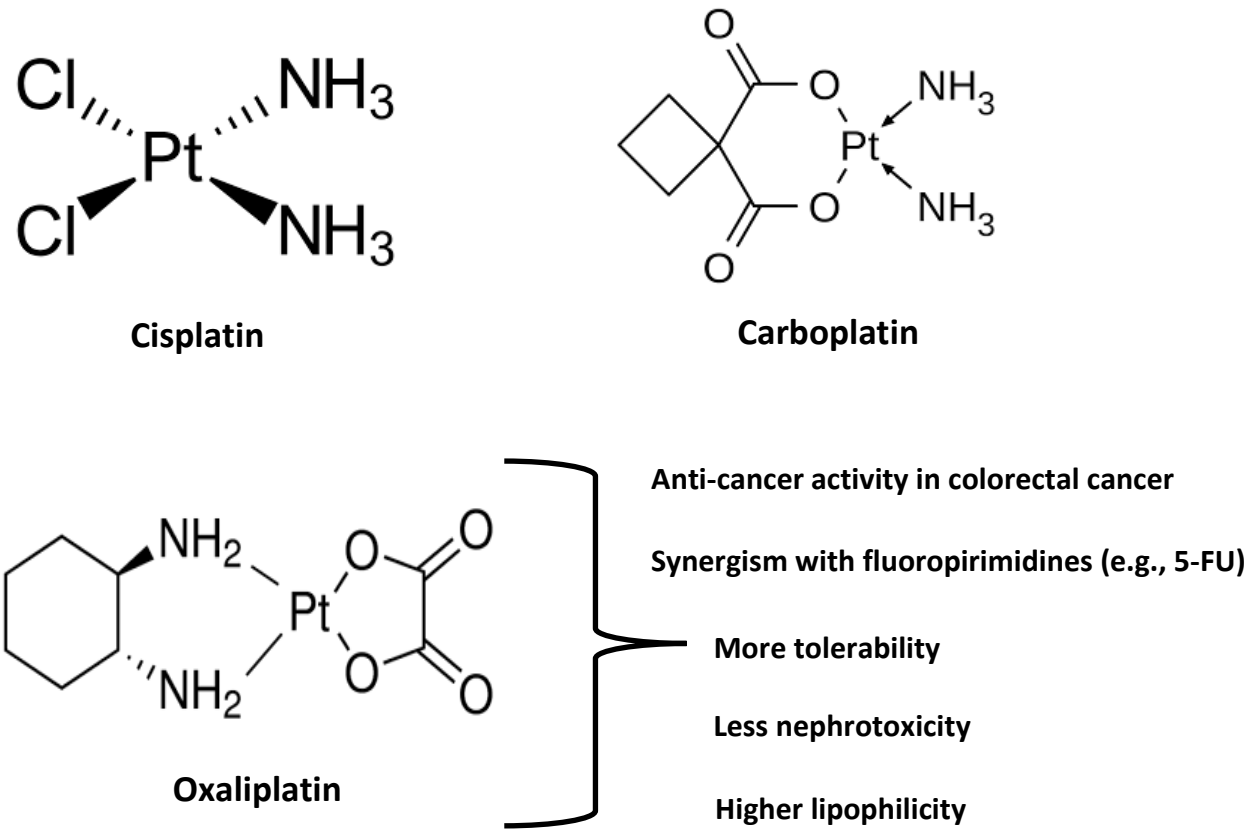


Figure 1.1 Chemical structures of the clinically important platinum drugs

Structures of some of the clinically used platinum drugs and schematic representation of features of oxaliplatin compared to other platinum-based drugs.

1.1.4. Oxaliplatin: Third generation Platinum-based drug

Oxaliplatin is a platinum (II) drug similar to carboplatin and cisplatin. This drug was invented by Yoshinori Kidani at Nagoya City University in 1976, and successively approved by FDA in 2002. Oxaliplatin was originally only used for metastatic colorectal cancer; later it was used in adjuvant therapy in combination with 5-FU and leucovorin for curing different carcinomas [48]. Oxaliplatin, or trans-L-dach (1R, 2R-diaminocyclohexane) oxalatoplatinum (L-OHP), varies from cisplatin and carboplatin by its possession of a massive diaminocyclohexane (DACH) moiety and the presence of an oxalate "leaving group". Oxaliplatin has completely different characteristics from cisplatin and carboplatin, which is summarised as: (i) Among the other platinum-based drugs, only oxaliplatin is considered to be a curative treatment for colorectal cancer [49]; (ii) Unlike cisplatin and carboplatin, oxaliplatin can be synergised with 5-FU and capecitabine [50]; (iii) oxaliplatin showed more tolerability and less nephrotoxicity than cisplatin, but unique progressive and cumulative sensitive neuropathy [51]; (iv) different DNA platination due to huge size of oxaliplatin-derived adducts compared to cisplatin and carboplatin adducts; (v) different mechanism of DNA adduct repair, which mainly occurs by nucleotide excision repair (NER) and DNA mismatch repair (MMR) [48].

In terms of mechanism of action, the primary target for all the platinum-based drugs is DNA and the formation of intrastrand and interstrand cross DNA links. However, there is a possibility that cisplatin and oxaliplatin have differences in drug transport and mechanisms of action as they conferred different activity in different types of cells [52]. For example, MMR complex plays an important role in determining whether cells enter to cell cycle for cell growth or proceed to apoptosis and death depending on the DNA damage. Oxaliplatin-DNA adducts are not recognised by MMR complex, but MMR detects the cytotoxic adducts induced by cisplatin [53-56]. Therefore, some cancer cells with loss of MMR complex, such as colorectal cancer cells, are not sensitive to cisplatin or carboplatin, but substantially sensitive to oxaliplatin. Therefore, oxaliplatin is considered a clinically important drug in the treatment of cisplatin- and carboplatin-resistant cancers [53-56].

1.1.5. Mechanism of action

Oxaliplatin-induced cytotoxicity affects the inside of cancer cells through various mechanisms, including deoxyribonucleic acid (DNA) lesions, DNA arrest and

inhibition of DNA during nucleic acid synthesis, as well as immunologic mechanisms of nucleic acid synthesis [57].

The primary mechanism of action of platinum complexes is the formation of DNA adducts. After entering a cell, the oxalate group in oxaliplatin is displaced by water molecules, converting into a charged active electrophile compound. The positively charged molecule then reacts with nucleophilic molecules inside the cells like DNA, RNA and protein. The platinum atom mainly binds covalently to DNA at the N^7 position of guanine and form platinum-based monoadducts, intrastrand and interstrand crosslinks. Most of the crosslinks are intrastrand and the majority of links formed are 1,2-d(GpG) crosslinks. Oxaliplatin mainly induces different types of intra and interstrand DNA crosslinks with DNA intrastrand crosslinks, which leads to DNA lesions [58, 59]. At the same concentration, oxaliplatin forms fewer crosslinks than cisplatin, but is able to induce similar or higher cytotoxic effects with fewer DNA lesions [58, 60]. These crosslinks result in alteration of the structure of DNA with bending towards the major groove and exposition of the minor groove [45].

Oxaliplatin directly inhibits thymidylate synthesis, which ultimately blocks DNA synthesis [59]. Platinum-DNA adducts further block the synthesis of mRNA by binding to transcription factors or inhibiting RNA polymerase [61].

Moreover, before apoptosis, oxaliplatin-induced immunogenic signals on the surface of cancer cells trigger the production of interferon gamma and this interacts with dendritic cells, which leads to the immunogenic death of cancer cells [62].

1.1.6. Metabolism of oxaliplatin

Cellular uptake of Pt drugs is mainly prevented due to diminished drug accumulation, which is caused by the neutral intact drug. A number of transporters which regulates Pt drugs accumulation, do not necessarily transport intact drugs. Furthermore, outside the cells, the neutral drug is prevented from hydrolysis due to the high chloride level present in plasma [63]. Therefore, one or more biotransformation products of Pt drugs are required, which will contribute to the pool of drugs that enter the cells. Oxaliplatin is administered to cancer patients intravenously; hence, the bioavailability is 100% [64]. After administration, oxaliplatin and its intermediates are distributed in high

concentrations to the kidneys, spleen, intestine, liver and red blood cells within two hours [51, 65].

Oxaliplatin consists of a non-leaving group, 1,2-diaminocyclohexane (DACH) and a leaving group, oxalate, which accounts for the water solubility of oxaliplatin [64, 66]. In plasma, oxaliplatin rapidly undergoes a non-enzymatic biotransformation into intermediates [59, 67]. The first step of oxaliplatin activation is its reaction with water, chloride and sulphur-containing plasma proteins such as glutathione, methionine and cysteine. It replace this oxalate group in oxaliplatin into reactive intermediates such as Pt (DACH)$(OH)_2$, Pt(DACH)Cl_2, Pt(DACH)Cl(OH), $[Pt(DACH)]_2$ (methionine) and $[Pt(DACH)]_2$ (glutathione) [68-70]. The active platinum-containing intermediates can either become inactive [71, 72] or can induce cytotoxic effects [51]. The biotransformation pathways of oxaliplatin are summarised in Figure 1.2.

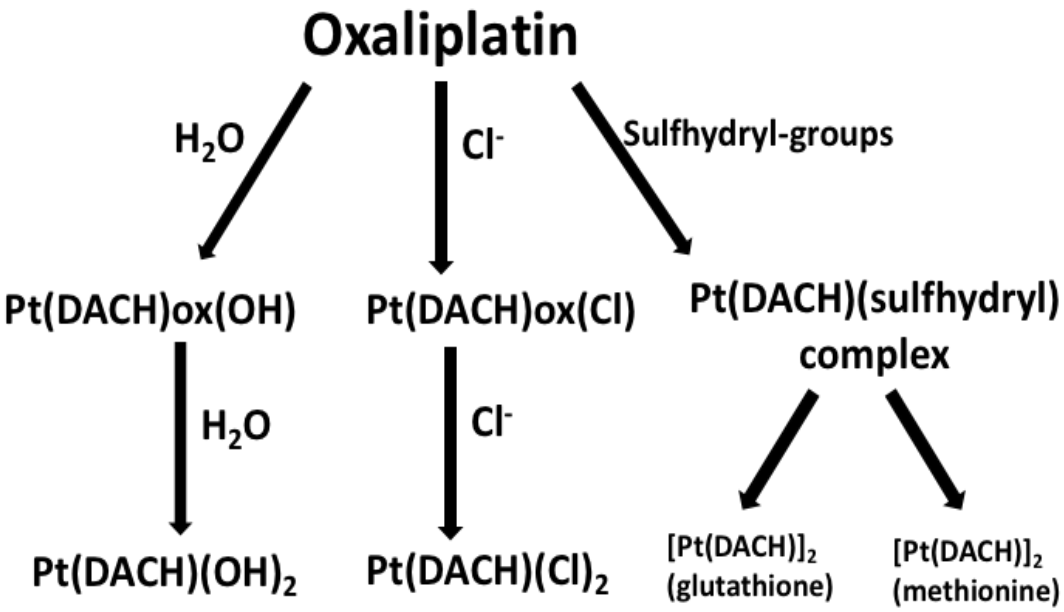


Figure 1.2 Schematic representation of biotransformation of oxaliplatin.

Within the first hours after administration, oxaliplatin binds to albumin, erythrocytes and other plasma proteins inside the body. Only about 15% of the drug remains in the systemic circulation after two hours of infusion and the remaining 85% is rapidly distributed into the tissues or is eliminated in the urine. Approximately 54% of total administered oxaliplatin is eliminated by renal excretion and around 2% is excreted by faeces [72]. The oxaliplatin intermediates that did not bind to proteins can induce cytotoxic effects in the cells. However, more evidence is required to verify the significance of each intermediate derived from oxaliplatin biotransformation and its role in oxaliplatin-induced cytotoxicity. Patients with mild or moderate renal impairment did not alter the maximum oxaliplatin exposure. However, the platinum exposure increased with increase in renal dysfunction. The clearance of platinum has no effect in patients suffering from hepatic dysfunction [73].

Metabolism of oxaliplatin can also depend on the concentration of other reactants inside the cells. The degradation rate of oxaliplatin in the presence of sulfhydryl-containing compounds increased in a concentration-dependent manner and in the presence of 10 mM glutathione, the half-life of oxaliplatin was less than 15 minutes [74]. The cytotoxic effects of oxaliplatin may be reduced in the presence of glutathione after forming oxaliplatin-glutathione complexes, which can inactivate oxaliplatin or help in effluxion out of the cells through membrane transporters such as multidrug resistance-associated proteins (MRPs), resulting in decreased cellular accumulation of oxaliplatin [75]. *In vitro* studies reported that in the presence of 0.9% saline solution, oxaliplatin degraded to $Pt(DACH)Cl_2$ with a half-life of 10 hr and in the presence of Ca/Mg ions in saline solution, degradation of oxaliplatin to $Pt(DACH)Cl_2$ was escalated with a half-life of 9 h to 2.2 h [76]. Hence, it would be interesting to further study the oxaliplatin degradation rate in the presence of Ca/Mg ions in saline as Ca/Mg infusion was used in the oxaliplatin combination chemotherapy to reduce neuropathy [77].

The pharmacokinetic data of the unbound platinum in plasma ultrafiltrate after receiving oxaliplatin is triphasic and contains two short initial distribution phases (α and β) with half-lives ($t_{1/2}$) of 0.28 hr – 0.43 hr and 16.3 hr – 16.8 hr respectively, depending on the administered dose (85 mg/m^2 or 130 mg/m^2), followed by a long terminal elimination phase (χ) with a half-life of 273 h – 391 hr [59]. In patients receiving oxaliplatin at a dose of 85 mg/m^2 every two weeks or 130 mg/m^2 every three weeks, the maximal plasma concentration (C_{max}) of oxaliplatin after 2 hr infusion was 0.681 μg/ml – 1.21

μg/ml [72]. Clinically, it has been reported that in patients receiving oxaliplatin at a dose of 85 mg/m^2 every two weeks or 130 mg/m^2 every three weeks, most of the unbound platinum in plasma was in the form of intact oxaliplatin and their concentration ranges from 3.75 μM to 11.25 μM during a 2 hr infusion [76]. The area under the curve (AUC) of platinum concentration in plasma filtrate versus time on cycle 1 after a 2 hr infusion of oxaliplatin at 85 mg/m^2 every two weeks was 4.25 μg.h/ml and at 130 mg/m^2 every three weeks was 11.9 μg.h/ml. The estimated clearance of ultrafiltrable platinum ranged from 9.34 – 10.1 L/h at 130 mg/m^2 and 18.5 L/h at 85 mg/m^2 [72].

1.1.7. Cellular transport of oxaliplatin

Oxaliplatin is a clinically important platinum-based drug for treating advanced cancer, but its molecular pharmacokinetics and tumour distribution are not well understood. Recently, several factors influence transport mechanisms of oxaliplatin to cross the cellular membrane, such as membrane stability, different types of transporter, proteins or substrates on the membrane, membrane permeability, type of platinum species and surrounding temperature [63, 78, 79]. Also, the accumulation of drugs are probably dependent on lipophilicity and speciation of the drugs [63]. Pt drugs mainly enter into the cells via two mechanisms: (a) Passive diffusion and (b) Active uptake of drugs regulated by a number of membrane transporters. Since drugs share same physiological characteristics with certain endogenous substrates, a number of membrane transporters are recognised as drug transporters. These drug transporters play an important role in determining drug pharmacokinetics, efficacy, drug reaction and their accumulation inside the cells. These membrane or drug transporters are functionally classified as uptake and efflux transporters, based on the movement of the substrate relative to the cell membrane [80-82]. Different membrane transporters such as organic cation transporters (OCTs) and copper influx transporters (CTR1) help in the oxaliplatin uptake inside the cells [83-85]. In addition to drug uptake mechanisms, a few other membrane transporters are involved in oxaliplatin export or efflux from cells, such as copper efflux transporters (ATP7A and ATP7B) [86-88], multidrug and toxin extrusion transporters (MATE1 and MATE2) [85, 89] and ABC transporter proteins (MRPs). All these membrane transporters play important roles in transport-mediated oxaliplatin accumulation and resistance in the cells. The details on structure, function and their role in oxaliplatin transport and resistance are discussed in section 1.3.

1.1.8. Clinical studies

Oxaliplatin-based chemotherapy has been used as an integral part of the treatment of colorectal cancer as well as for other GI cancers including liver, pancreatic and gastric cancers. Oxaliplatin has been used in combination with other anti-cancer drugs like 5-FU, folinic acid (leucovorin) or capecitabine as a first-line treatment regimen for metastatic colorectal cancer [49, 90]. It is widely used in combination with 5-FU and irinotecan in advanced pancreatic cancer [91].

Recently, there has been growing interest in incorporating adjuvant treatment as the combination of fluorouracil, irinotecan, oxaliplatin and leucovorin (FOLFIRINOX) and gemcitabine. It has been observed that with the use of adjuvant therapy, the immune response against pancreatic cancer has been enhanced [33]. Moreover, oxaliplatin-based combined chemotherapy such as GEMOXEL (gemcitabine, oxaliplatin and capecitabine) and FOLFIRINOX were shown to be more effective with a higher median overall survival rate in patients with metastatic pancreatic cancer compared to gemcitabine therapy [31]. It has been observed that objective response rate (ORR) reported in phase II trials from single-agent oxaliplatin is about 20% and in a phase III trial is about 13% [92]. However, after combining oxaliplatin with other chemotherapeutic agents like fluoropyrimidines (5-FU), the ORR rate increased to 50% for phase II trials and for phase III trials, it went up to 15% [92, 93].
Several different types of drugs are licensed for the treatment of pancreatic cancer. Gemcitabine is a first-line drug for pancreatic cancer treatment. Single-agent gemcitabine has modest activity compared to the combination of gemcitabine with other chemotherapy [30, 94], which has been shown in two clinical trials. Recently, an oxaliplatin-based FOLFIRINOX regimen has shown significant effects in prolonging overall survival (OS) rates in patients with metastatic pancreatic cancer [33]. Indeed, the use of FOLFIRINOX compared to gemcitabine showed significant improvement in median survival, quality of life and health of metastatic patients. Currently, FOLFIRINOX or its combination with gemcitabine and gemcitabine-nab-paclitaxel is considered a standard treatment with an increase in survival rate of up to 10% [33]. Unfortunately, molecular interactions between oxaliplatin, 5-FU, radiation and gemcitabine are not elucidated yet.

In colorectal cancer clinical trials, unlike other platinum drugs such as cisplatin or carboplatin that did not show any significant activity, oxaliplatin demonstrated

antitumoural activity alone or in combination with 5-FU [95]. According to Phase III trials in metastatic colorectal cancer by Giacchetti et al. and de Gramont et al., adding oxaliplatin to 5-FU and leucovorin significantly increased the objective response rate (ORR) (16/22.3% vs 53/50.7%) and progression-free survival rate (PFS) (6.1/6.2 vs 8.7/9.0 months) [37, 96]. The FOLFOX4 (leucovorin, 5-FU, oxaliplatin) evolved as a standard regimen for colorectal cancer after these clinical studies [37]. According to another clinical study, FOLFOX4 regimen has shown to be more efficient than FOLFIRI (leucovorin, 5-FU, irinotecan) with increased disease-free survival and OS than FOLFIRI [59]. The IROX (irinotecan and oxaliplatin) showed similar ORR and better OS than FOLFIRI and irinotecan alone [97]. The FOLFOX4 regimen had significantly lower rates of other toxicity profiles like vomiting, diarrhoea, neutropenia and dehydration [59]. As compared to FOLFIRI, the combination of oxaliplatin and FOLFIRI (FOLFOXIRI) improved ORR and OS [38, 98]. The next generation of clinical studies investigated the combination of oxaliplatin and capecitabine (XELOX) [99-101]. Several randomised Phase II and III trials showed similar PFS and OS for both XELOX and FOLFOX [102]. Randomised data have shown that combination of FOLFOX with bevacizumab or anti-EGFR antibodies increased the patients' response rate [59].

1.1.9. Toxicities of oxaliplatin

The clinical use of oxaliplatin-based chemotherapy is limited by high toxicity profiles in treated patients who exhibit severe adverse drug reactions early after the commencement of therapy [59]. Most common toxicities induced by oxaliplatin chemotherapy in haematopoietic, gastrointestinal and peripheral systems are characterised by neutropenia, thrombocytopenia, anaemia, nausea, vomiting, acute neurotoxicity and diarrhoea. The adverse reactions of oxaliplatin in haematopoietic and gastrointestinal systems are mild to moderate [57, 64, 95]. Oxaliplatin has moderately myelotoxic effects on progenitor cells in the bone marrow [57]. Repeated infusion of oxaliplatin can also cause anaemia and secondary immune thrombocytopenia [59].

Neurotoxicity is considered to be a primary dose-limiting toxicity of oxaliplatin [95]. There are two different types of a distinct pattern of peripheral sensory neuropathy, acute neuropathy and chronic neuropathy [57, 59]. Acute neuropathy is induced by affecting the voltage-gated sodium channels involving calcium whereas chronic neuropathy is associated with atrophy and mitochondrial dysfunction in dorsal root

ganglion cells due to platinum accumulation [57, 59]. One of the *in vivo* studies reported that cisplatin and carboplatin were associated with higher concentrations of platinum in peripheral nerves, but showed less neurotoxicity than oxaliplatin [64]. Although accumulation of cisplatin is higher than oxaliplatin in dorsal root ganglion cells of Wistar rats, the nerve damage caused by oxaliplatin is severe than cisplatin [64]. Most of the patients suffer from acute neuropathy during or shortly after oxaliplatin infusion and this is characterised by paresthesia, dysesthesia or allodynia affecting extremities, lips and oropharyngolaryngeal areas. Chronic neuropathy usually progresses with the continuation of the treatment due to cumulative exposure to oxaliplatin. Approximately 40-50% patients receiving oxaliplatin reported grade 2 or severe neuropathy and around 15% of patients reported grades 3 and 4 neuropathy after receiving a cumulative dose of about 800 mg/m2 oxaliplatin [103, 104]. Major symptoms of chronic neuropathy include loss of vibration sensation, numbness or needle sensation in fingers and toes, loss of peripheral tendon reflexes, reduced proprioception, and sensory ataxia [57]. Unlike other platinum-based drugs, oxaliplatin does not cause significant renal toxicity and ototoxicity, which are the common side effects caused by cisplatin and carboplatin [52, 57, 59].

1.1.10. Oxaliplatin resistance mechanism

Many GI cancers, including pancreatic adenocarcinoma and colorectal cancer are among the most chemoresistant cancers due to the broad heterogeneity of genetic mutation or decreased accumulation of anticancer drugs. The therapies targeting cancer-associated molecular pathways have not given satisfactory results [105]. The development of drug resistance is the main cause of lack of efficiency of oxaliplatin-based therapy for GI cancer. In the past few decades, studies have been undertaken to understand the mechanism of oxaliplatin resistance to improve the clinical efficacy of oxaliplatin, yet oxaliplatin resistance is not well understood due to the complicated and multifactorial nature of the drug resistance phenomenon.

Drug resistance is the major limiting factor for the successful treatment of cancer with most cytotoxic anticancer drugs. Most of the oxaliplatin resistance mechanisms are pharmacodynamic in nature and result from adaption within the tumour cells. Cancer patients who responded to oxaliplatin-based therapies had higher tumour Pt concentrations than those who failed, which indicates that Pt accumulation is also an important factor for clinical efficacy [106]. Therefore, reduced cellular uptake or increased efflux of oxaliplatin results in a reduced level of oxaliplatin accumulation,

which indirectly causes oxaliplatin resistance [54, 59, 107-110]. Several other factors can contribute to oxaliplatin resistance, including alteration in DNA repair systems, repairing Pt-DNA lesions, detoxification of drug with glutathione, altered membrane permeability and altered apoptosis pathway that prevents cell death [111].

The mechanism of oxaliplatin resistance can be categorised into pre-target (those interfering with oxaliplatin transport), on-target (repair of Pt-DNA lesions), post-target (alteration in cellular events after Pt-DNA adducts) and off-target (alteration in signalling pathways that are not directly involved by oxaliplatin but interfere in oxaliplatin-induced apoptosis) [112]. One of the important mechanisms involved in cellular oxaliplatin resistance is reduced cellular uptake and increased cellular efflux of oxaliplatin, which implicate the regulation of oxaliplatin accumulation. One *in vitro* study reported that OCT1-deficient primary mouse hepatocytes showed decreased oxaliplatin accumulation [113]. Multiple other studies using cell lines with overexpressed OCT1 and OCT2 have provided evidence of increased accumulation of oxaliplatin. In contrast, oxaliplatin accumulation was reduced by exposure to OCT inhibitors [114].
The second important mechanism involved in oxaliplatin resistance is inactivation or detoxification of oxaliplatin with glutathione. Elevated levels of glutathione-S-transferase-π (GST-π) increased oxaliplatin coupling to GSH, resulting in a glutathione-oxaliplatin adduct that is no longer cytotoxic. Increased cellular glutathione levels also increased oxaliplatin resistance [109]. Pt-glutathione adduct was reported to be excreted out of the cells by MRP2. Another member of the antioxidant defence system is thioredoxin (Trx), similar to glutathione, which regulates the oxidation-reduction environment of cells [60]. Trx is mostly involved in transcription factors, apoptosis and DNA synthesis, and is reduced by thioredoxin reductase (TrxR). In clinical samples, positive correlation has been observed between TrxR levels and platinum resistance. The platinum drugs, cisplatin and oxaliplatin, show inhibition of TrxR [60].

Another main mechanism involved in oxaliplatin resistance is altered DNA damage repair, which leads to increased cell survival and thus resistance to oxaliplatin. The Pt-DNA adducts are recognised by several protein families, including high mobility proteins 1 and 2 (HMG1 and HMG2), nucleotide excision repair (NER) protein and mismatch repair (MMR) proteins. HMG1 and HMG2 recognise intrastrand DNA adducts between adjacent guanines, subsequently inducing apoptosis [111]. Also, the

HMG1 expression is correlated to oxaliplatin cytotoxicity [48]. Several translesion DNA polymerases (POLβ, POLη, POLζ) ensure DNA replication in the absence of DNA repair. The expression of these translesion DNA polymerases was correlated to oxaliplatin cytotoxicity inversely and removal of these DNA polymerases can increase sensitivity to oxaliplatin [115-117]. Nucleotide excision repair (NER), is the primary pathway used by cancer cells for the removal of Pt-DNA adducts and DNA damage repair. The process involves the recognition of the lesion by DNA excision repair proteins (ERCC1, ERCC2). One of the studies in NCI-60 cells revealed a significant association between ERCC1 expression and cisplatin resistance, and a significant association between ERCC2 expression and oxaliplatin resistance [48]. The ERCC1 expression has also been shown to be associated with oxaliplatin resistance in colorectal cancer [118, 119]. Single nucleotide polymorphisms (SNP) in ERCC1 and ERCC2 genes have been also associated with clinical response to oxaliplatin. The ERCC1 SNP rs11615, leads to high protein expression and the ERCC2 SNP rs13181 leads to higher DNA repair capacity and lower tumour response and poor overall survival in colorectal cancer patients treated with oxaliplatin [120].

Altered apoptosis in cancer cells is another mechanism responsible for oxaliplatin resistance. After DNA platination, DNA damage activates different cellular signal pathways that eventually lead to apoptosis. The inactivation of tumour suppressor p53 is one of the predominant mechanisms of oxaliplatin resistance [121, 122]. Mutation in anti-apoptotic factors, including nuclear factor κβ (NF-κβ), contributes to oxaliplatin resistance [123]. Tumour microenvironmental conditions like hypoxia may also contribute to oxaliplatin resistance [124].

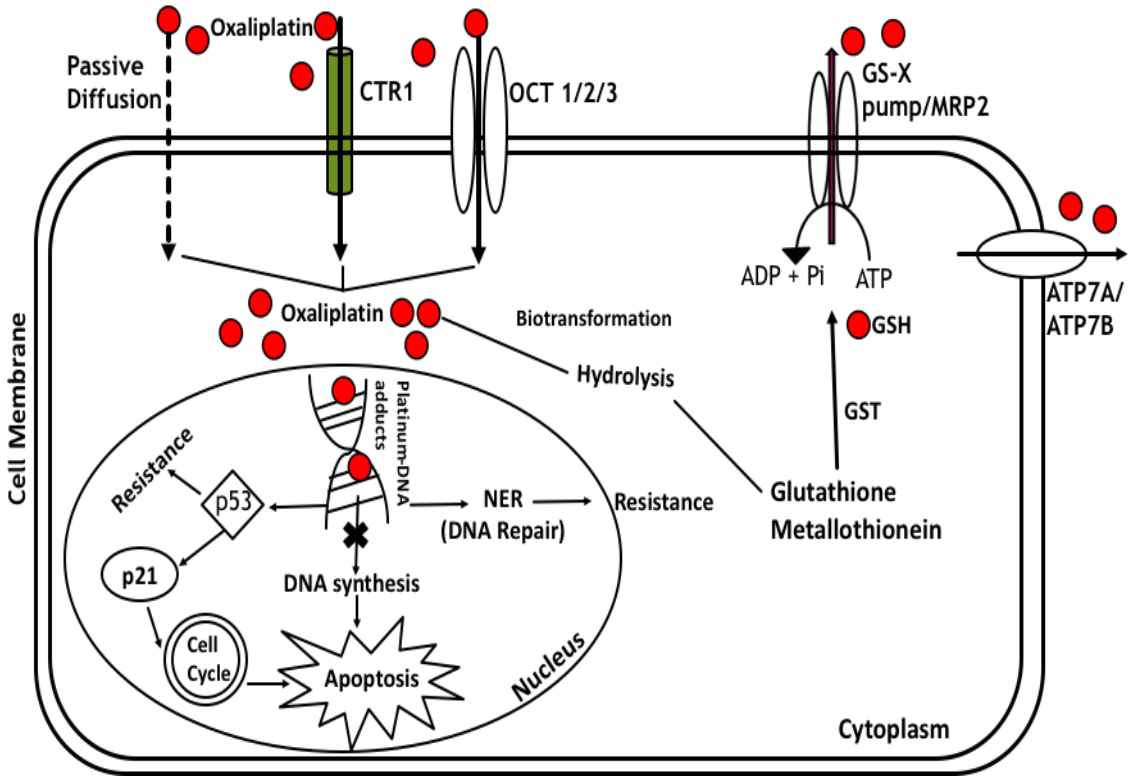


Figure 1.3 Schematic representation of the mechanism of action of oxaliplatin and resistance to the drug.

1.3. Membrane transporters

1.3.1. Overview of membrane transporters

In the past few decades, the importance of membrane transporters in drug disposition, response, therapeutic efficiency and adverse drug reactions has increased significantly. Plasma membrane transporters play an important role in the uptake and efflux of physiologically important molecules, such as cellular metabolites, organic ions, proteins, minerals, toxic substances and xenobiotics through the lipid bilayer of cellular membranes by passive diffusion using a different form of energy [125-127]. Membrane transporter proteins are classified into five different types, including electrochemical potential-driven transporters, primary active transporters, electron carriers and channels or pores [125]. Depending on the functions of membrane transporters, transporter proteins are categorised into influx and efflux transporters. The major role of influx transporters is to uptake molecules including ions, minerals, nutrients, drugs and xenobiotics into the cells such as enterocytes, hepatocytes, renal tubules, epithelia of the intestine, liver, kidney, and into the endothelium of the blood-brain barrier and tumour cells. The efflux transporters mediate the export of these molecules out of the cells [126, 128, 129].

The two major superfamilies of membrane transporters, which have been annotated in the human genome, are ATP-binding cassette (ABC) and solute carrier (SLC) transporters [126]. Numerous studies have suggested that these membrane transporters play important roles in pharmacology, affecting the entry and extrusion of drugs in and out of cells, drug disposition, efficacy and adverse drug reactions. Clinically, transporter-based drug-drug interaction (DDI) studies have suggested that these transporters often work together with drug metabolising enzymes in drug absorption and elimination [126]. Thus, these membrane transporters become key determinants of the pharmacokinetic properties of the drugs, which provide considerable information on drug bioavailability, plasma concentration, exposure, clearance and excretion [126, 130, 131]. Some of these membrane transporters are also located in other major tissues like the central nervous system (CNS), blood-brain barriers, placenta and testis-blood barriers, and limit drug disposition into these tissues and thus prevent toxicity [126]. In an aspect of cancer pharmacology, the study of membrane transporters is important in determining the pharmacodynamics (PD) and pharmacokinetics (PK) of drugs in terms of drug disposition, efficacy and their sensitivity inside cancer cells.

Membrane transporters play a key role in the absorption and disposition of numerous drugs, xenobiotics and endogenous compounds throughout the body. In addition, they act as a natural barrier and efflux these molecules out of cells, causing multidrug resistance. Over the period of discovery of membrane transporters and their functions, it has been observed that a number of pharmaceutical drugs were substrates, inhibitors and inducers of multiple drug transporters, and many drugs were in fact both substrates and inhibitors of membrane transporters [132]. The list of important membrane transporters involved in pharmacokinetic DDI are listed in table 1.1. The transporter proteins and their details are listed in table 1.1 that are responsible for transport function.

Table 1-1 List of ABC membrane transporters that are considered relevant to drug disposition.

Transporter Protein (gene)	**Substrates**	**Inhibitors**	**Localisations**	**Regulatory recommendation**
MDR1/P-gp, ABCB1 (ABCB1)	Digoxin, loperamide, berberine, irinotecan, doxorubicin, vinblastine, paclitaxel, fexofenadine	Cyclosporine, quinidine, tariquidar, verapamil	Intestinal enterocytes, kidney proximal tubule, hepatocytes (canalicular), brain endothelial cells	Has a role in absorption, disposition, excretion and clinical DDI.
MDR3/ABCB4 (ABCB4)	Phosphatidylcholine, paclitaxel, digoxin, vinblastine.	Verapamil, cyclosporine	Hepatocytes (canalicular)	Has a role in disposition and clinical DDI.
BSEP/SPGP, cBAT, ABCB11 (ABCB11)	Taurocholic acid, pravastatin, bile acids	Cyclosporin A, rifampicin, glibenclamide	Hepatocytes (canalicular)	Has a role in excretion and clinical drug-drug interactions. In addition, has clinically relevant genetic polymorphisms.

MRP2/ABCC 2, cMOAT (ABCC2)	Glutathione and glucuronide conjugates, methotrexate, etoposide, mitoxantrone, valsartan, olmesartan, glucuronidated SN-38	Cyclosporine , delaviridine, efavirenz, emtricitabine	Hepatocytes (canalicular) , kidney (proximal tubule, luminal), enterocytes (luminal)	Has a role in absorption, disposition, excretion and clinical DDI. Has clinically relevant genetic polymorphisms.
MRP3/ABCC 3 (ABCC3)	Oestradiol-17β-glucuronide, methotrexate, fexofenadine, glucuronate conjugates	Delavirdine, efavirenz, emtricitabine	Hepatocytes (sinusoidal), intestinal enterocytes (basolateral)	Has a role in disposition.
MRP4/ABCC 4 (ABCC4)	Adefovir, tenofovir, cyclic AMP, dehydroepiandrosterone sulphate, methotrexate, topotecan, furosemide, cyclic GMP, bile acids plus glutathione	Celecoxib, diclofenac	Kidney proximal tubule (luminal), choroid plexus, hepatocytes (sinusoidal), platelets	Has a role in disposition and excretion.
BCRP/MXR (ABCG2)	Mitoxantrone, methotrexate, topotecan, imatinib, irinotecan, statins, sulphate conjugates, porphyrins	Oestrone, 17β-oestradiol, fumitremorgin C	Intestinal enterocytes, hepatocytes (canalicular) , kidney proximal tubule, brain endothelial cells, placenta, stem cells, mammary glands (lactating)	Has a role in excretion and clinical DDI. In addition, has clinically relevant genetic polymorphisms.

1.3.1.1. ABC Superfamily

In humans, there are seven families of ABC transporter genes, which are grouped by the letters ABCA to ABCG, encoding 49 individual ABC transporters and involving in several diseases, including genetic disorders [133, 134]. The ABC transporters are primarily located in the plasma membrane, where they mainly efflux a wide range of endogenous substrates from the cells, including drugs, conjugated bile salts, steroid hormones and unconjugated bilirubin [132]. Therefore, any changes in the expression or functions of transporters cause certain diseases in human. For example, mutation in ABCC2 (ATP-binding cassette sub-family C member 2) transporter gene is associated with Dubin-Johnson's Syndrome, which is characterised by intermittent jaundice due to the defective excretion of conjugated bilirubin [135]. ABC transporters are widely distributed in the human body, including the liver, kidney, renal tubule, intestine and blood-tissue barriers. Therefore, ABC transporters play a key role in excreting waste products, toxins and xenobiotics from the body through bile, urine and faeces, as well as protecting the essential organs such as CNS and brain from toxic products by extruding these waste products into the bloodstream [130, 136].

The ABC protein contains two transmembrane domains (TMD1 and TMD2), each fused at a C-terminus to a nucleotide-binding domain (NBD1 and NBD2). These two domains may be fused as a single protein or combined as homo- or heterodimers to form functional transporters. Some members of the ABCC family contain an extra TMD at the N-terminus known as TMD0, which is connected by the cytoplasmic loop L0 to the TMD [137]. The transmembrane domain is characterised by a pore-like structure that forms a channel across the membrane. These transmembrane domains are regulated by ATP binding and its hydrolysis. The ATP hydrolysis provides energy for the conformational change in ATP binding domains and in turn helps the movement of substrates across membranes against their concentration gradient [133, 134].

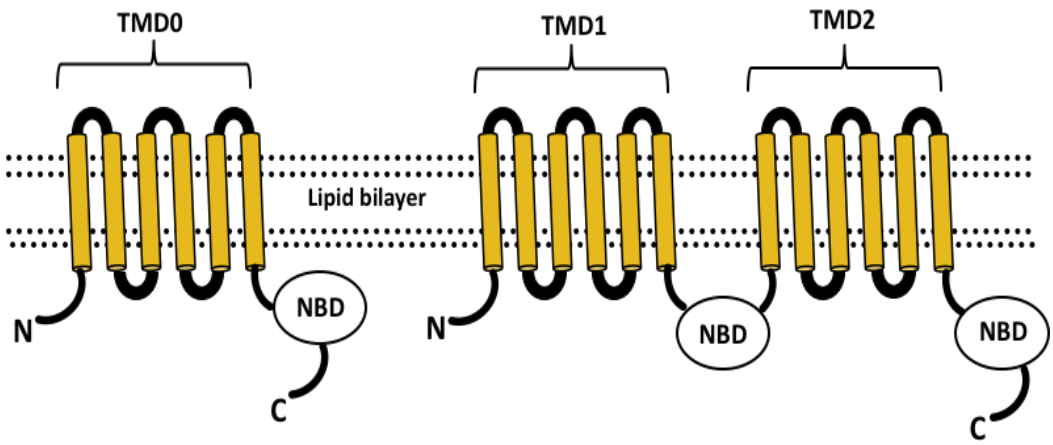


Figure 1.4 General structure of ABC membrane transporters.

In this figure, TMD is a transmembrane domain, NBD is a nucleotide-binding domain, C represents C-terminal and N is N-terminal. Horizontal dotted lines represent the lipid bilayer of the cellular membrane.

The ABC transporters operate in four main steps. Firstly, binding of a substrate to the TMD unit. As soon as the substrate binds to the TMD unit, this leads to the opening of NBD unit. Secondly, ATP binds to the NBD unit. The high-affinity of binding of ATP to the NBD unit results in the release of energy, which causes conformational changes to the TMD unit and translocates the substrate to the other side of the membrane. Thirdly, hydrolysation of ATP. Hydrolysis of ATP to ADP and inorganic phosphate triggers conformational changes in NBD. Lastly, restoration of transporters to their original shape. The release of ADP and phosphate from the NBD unit restores the transporters to their original conformation with NBD in an open-shaped dimer for the next transport cycle [137].

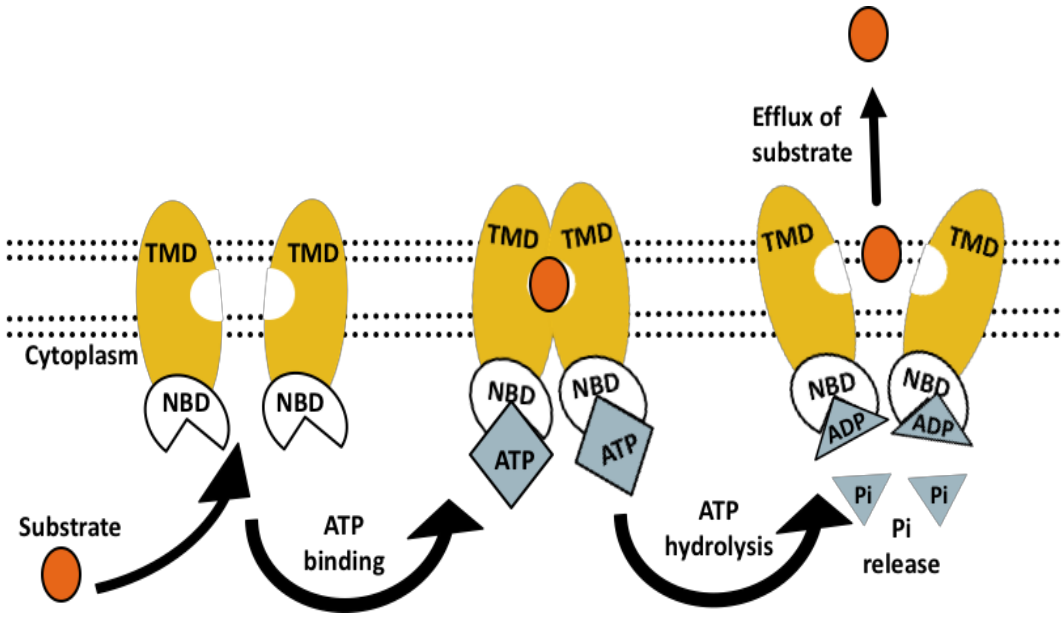


Figure 1.5 Function of ABC transporters.

Schematic representation of mechanism of transport of substrate by ABC transporters. ABC transporters exhibit conformational change upon substrate binding and ATP hydrolysis drives the transport of substrate.

The ABC drug transporters are divided into three classes. The first type of ABC transporters, which is widely studied, is the ABCB family containing MDR1 (multidrug-resistant transporter 1; ABCB1) and ABCB11. The second type is the ABCC family containing MRPs (ABCC1 to ABCC6). Multidrug resistance-associated proteins (MRPs) are members of the ATP binding cassette (ABC) efflux transporter family, which effectively efflux various drugs and molecules to protect the cells from toxins [133, 138]. The third most well-known drug transporter family is the ABCG family containing BCRP (ABCG2). Clinically, all these ABC transporters are the most relevant transporters and are discussed below.

ABCB1 (MDR1, P-gp)

The first identified ABC transporter is from the ABCB family, i.e., MDR1 (multidrug-resistant transporter 1, ABCB1; also known as P- glycoprotein, P-gp), which is encoded by ABCB1 gene [139]. P-gp is localised at the apical surface of epithelial cells of hepatocytes [140, 141]. P-gp mostly transports hydrophobic drugs with neutral or positive charge and anticancer drugs including taxanes (paclitaxel), vinca alkaloids (vincristine), an anthracycline (doxorubicin), imatinib and irinotecan [142]. Several

studies, including Mdr1a and Mdr1b knockout mice that lack Abcb1 proteins and clinical studies in humans using ABCB1 inhibitors such as cyclosporin A and elacridar, showed that oral bioavailability of anticancer drugs like paclitaxel, etoposide and topotecan significantly increased after inhibiting the activity of the MDR1 transporter [130, 142]. As P-pg is expressed in the apical surface of the hepatocyte, it is involved in the transport of substrates or drugs from portal circulation into bile and causes biliary excretion and hepatic clearance of the anticancer drugs such as irinotecan, doxorubicin and paclitaxel [128, 142]. Administration of P-gp substrate, including digoxin and loperamide, can inhibit the excretion of anticancer drugs, hence reducing their clearance and increasing their toxic effects of [132]. Therefore, P-gp plays a role in DDI of anticancer drugs and in is an important determinant of the drug disposition, pharmacokinetics, clearance and toxicity of anticancer drugs in cancer chemotherapy.

ABCCs (MRPs)

The second most important ABC transporters are known as the multidrug resistance-associated protein (MRP) family, which is encoded by ABCC genes; nine MRP proteins (MRP1 to MRP9) encoded by ABCC1-6 and ABCC10-12 respectively [139]. All the MRPs are responsible for the transport of anionic compounds, glutathione, substrates conjugated with glutathione and metabolites of substrates. Thus, MRPs play an important role in detoxification of chemotherapeutic drugs and their metabolites [128, 129, 139].

Multidrug resistance-associated protein 1-3 (MRP1, MRP2, MRP3) with N-linked glycosylation occurs on the fourth extracellular loop. They also have an additional N-terminal extension consisting of five putative transmembrane segments. In total, this group has 17 transmembrane segments. MRP1 is localised mainly in the basolateral cell surface and is expressed mostly in the lungs, kidney, peripheral blood cells and liver [143]. MRP1 translocate neutral or anionic compounds and are capable of carrying complex hydrophobic substrates, including doxorubicin and methotrexate, which are conjugated with glutathione, glucuronic acid or sulphate [139, 142, 144]. MRP1 is involved in the translocation of drugs into blood, thereby protecting the cells from drug toxicity. Therefore, MRP1 is important in determining the PK and toxicity of several anticancer drugs. Overexpression of MRP1 in small cell lung cancer, non-small cell lung cancers, leukaemia, oesophageal carcinoma, prostate cancer and breast cancer is correlated with the resistance of several anticancer drugs [139, 145].

The MRP1 and MRP2 transport mechanism is similar and helps with the transport of mostly anionic drugs and their metabolites [143]. MRP2 transports drugs like anthracyclines, taxanes, vinca alkaloids and platinum-based drugs such as cisplatin and their conjugates, including glutathione and sulphates. Physiologically, MRP2 is expressed in tissue barrier sites such as the blood-brain barrier, blood-testis barrier and placenta [139, 145], as well as in the surface of the canalicular membrane of hepatocytes, luminal surface of renal proximal tubules and small intestine [142], where it functions in absorption, metabolism and excretion of substrates and toxic substances. Thus, MRP2 plays a major role in the body's defence against drugs and toxins by controlling bioavailability and disposition of drugs, and excretion of toxic substances in bile and urine.

MRP3 is considered as closely related to MRP1, which is expressed in the liver, intestines, adrenal gland, pancreas and kidneys. MRP3 is known to confer resistance to a variety of chemotherapeutic drugs such as etoposide and methotrexate [142].

MRP4 was the first ABC transporter reported to translocate nucleoside-monophosphates. It is expressed in the lungs, kidneys, bladder and prostate as well as to some extent the small intestine [142]. MRP4 mediates ATP-dependent accumulation of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP).

ABCG2 (BCRP)

The last drug transporters group is known as the breast cancer resistance protein (BCRP or ABCG2) and was originally isolated from the P-gp inhibitor doxorubicin and verapamil [146]. BCRP is considered as a half-transporter with six transmembrane segments and single N-terminal NBD unit. Normally, last extracellular loop is N-glycosylated [147]. Like P-gp and MRP2, BCRP transports a wide range of substrates like mitoxantrone, camptothecin, methotrexate, doxorubicin and SN38 (a metabolite of irinotecan) [145]. BCRP is mainly expressed in the apical membrane of the intestine and is involved in the efflux of anticancer drugs like doxorubicin, mitoxantrone, methotrexate, SN38 and flavopiridol [130]. Thus, it helps in maintaining the oral bioavailability, clearance and toxicity of these drugs inside the body. As BCRP is expressed in the blood-brain barrier, along with P-gp, it prevents the penetration and efficacy of certain drugs such as imatinib in the CNS [148-150]. Overexpression of

BCRP is associated with chemoresistance in breast, colorectal, liver, gastric and blood cancers [145].

1.3.1.2. SLC Superfamily

Based on the amino acids, the SLC superfamily is classified into 52 different families. The members of the SLC superfamily are responsible for the transport of a wide range of molecules, including amino acids, peptides, sugars, inorganic ions, organic anions and cations, electrolytes, metal ions and neurotransmitters [132, 151]. Important members of the SLC superfamily responsible for the transport of drugs are SLCO, SLC22 and SLC47. Pharmacologically, most relevant SLCO family members include OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3); SLC22 family members include OCT1 (SLC22A1), OCT2 (SLC22A2), OAT1 (SLC22A6) and OAT3 (SLC22A8); and SLC47 family members include MATE1 (SLC47A1) and MATE2-K (SLC47A2). The SLCO and SLC22 family members are responsible for the uptake of drugs and SLC47 members facilitate the efflux of drugs. All these SLC transporters transport a number of important chemotherapeutic agents and are relevant to the study of clinical transporter-mediated DDI. The members of the SLCO and SLC22 family have 12 TMDs (78). In the next section, the role of the ABC and SLC superfamily in oxaliplatin transport is discussed.

Table 1-2 List of SLC membrane transporters that are considered relevant to drug disposition.

Transporter Protein(gene)	Substrates	Inhibitors	Localisations	Regulatory recommendation
OATP1B1/OATP-C, OATP2, LST-1 (SLCO1B1)	Bromosulphophthalein, oestrone-3-sulphate, oestradiol-17β-glucuronide, statins, repaglinide, valsartan, olmesartan, bilirubin glucuronide, bilirubin, bile acids	Saquinavir, ritonavir, lopinavir, rifampicin, cyclosporine	Hepatocytes (sinusoidal)	Has a role in disposition and excretion, clinical DDI and relevant in polymorphisms.
OATP1B3/OATP-8 (SLCO1B3)	Bromosulphophthalein, cholecystokinin 8, statins, digoxin, fexofenadine, telmisartan glucuronide, telmisartan, valsartan,	Rifampicin, cyclosporine, ritonavir, lopinavi	Hepatocytes (sinusoidal)	Has a role in disposition and excretion.

	olmesartan, oestradiol-17- β-glucuronide, bile acids			
OCT1 (SLC22A1)	Tetraethylammonium, N-methylpyridinium, metformin, oxaliplatin	Quinine, quinidine, disopyramide	Hepatocytes (sinusoidal), intestinal enterocytes	Has a role in disposition and excretion, clinical DDI and relevant in polymorphisms.
OCT2 (SLC22A2)	N-Methylpyridinium, tetraethylammonium, metformin, pindolol, procainamide, ranitidine amantadine, amiloride, oxaliplatin, varenicline	Cimetidine, pilsicainide, cetirizine, testosterone, quinidine	Kidney proximal tubule, neurons	Has a role in disposition and excretion, clinical DDI and relevant in polymorphisms.
OAT1 (SLC22A6)	Para-aminohippurate, adefovir, cidofovir, zidovudine, lamivudine, zalcitabine, acyclovir, tenofovir, ciprofloxacin, methotrexate	Probenecid, novobiocin	Kidney proximal tubule, placenta	Has a role in disposition and excretion and clinical DDI.
OAT3 (SLC22A8)	Oestrone-3-sulphate, non-steroidal anti-inflammatory drugs, cefaclor, ceftizoxime, furosemide, bumetanide	Probenecid, novobiocin	Kidney proximal tubule, choroid plexus, blood-brain barrier	Has a role in disposition and excretion and clinical DDI.
MATE1 (SLC47A1)	Metformin, N-methylpyridinium, tetraethylammonium	Quinidine, cimetidine, procainamide	Kidney proximal tubule, liver (canalicular membrane), skeletal muscle	Has a role in disposition and excretion and clinical DDI.
MATE2-K (SLC47A2)	Metformin, N-methylpyridinium, tetraethylammonium	Cimetidine, quinidine, pramipexole	Kidney proximal tubule	Has a role in disposition and excretion.

1.3.1.3. Ion pumps (ATPase) family

ATPase or ion pumps are the ATP-dependent active ion transporter family that pumps ions such as Na^+, K^+, H^+, Ca^+, and Cu^+ out of the cell [152]. Ion pumps help in generating and maintaining electrochemical ion gradients across the membrane. These ion gradients are associated with the disposition and cellular accumulation of drugs as well as sensitivity to anticancer drugs [139]. Ion pumps work in synergy with SLC transporters to translocate nutrients, ions and drugs. Little investigation has been done in terms of ion pumps; however, the activity of a variety of chemotherapeutic agents is likely to depend on the functions of ion pumps. In the past few decades, research has focused on a few families of ATPases, such as vacuolar-H^+-ATPase and copper export pump ATP7A and ATP7B. The expression of ATP7A and ATP7B is highly correlated with cytotoxic drugs like platinum-based drugs in various cancer cell lines [139]. These studies demonstrated that expression of ATP7A and ATP7B might be used as a predictive marker of chemoresistance for cisplatin and oxaliplatin [111, 153].

1.3.2. Role of membrane transporters on oxaliplatin transport

The clinical activity of oxaliplatin is dependent on factors regulating its cellular accumulation and resistance by virtue of the expression of different membrane transporters in tumour cells. Oxaliplatin can enter or exit the cells via different membrane transporters. Members of the ABC, SLC and ATPase membrane transporter superfamilies are responsible for chemosensitivity and chemoresistance of oxaliplatin.

1.3.2.1. Copper transporter (CTR1)

Copper influx transporter, CTR1, encoded by the SLC31A1 gene and localised in the plasma membrane, regulates Cu^+ cell homeostasis and has been implicated in the cellular accumulation of platinum-based compounds including oxaliplatin. Overexpression of CTR1 in cancer cell lines have shown stimulated cellular oxaliplatin uptake and cytotoxicity [154]. One study using $CTR1^{+/+}$ and $CTR1^{-/-}$ mouse embryonic fibroblast and xenografts, showed that CTR1 physiologically forms a pore through the plasma bilayer to import Cu^+ and enables cellular accumulation of cisplatin, carboplatin and oxaliplatin [48]. Another *in vivo* study showed that cellular platinum accumulation significantly increased in rat Ctr1 overexpressing HEK293 cells compared to control cells, and also induced cytotoxic effects [155]. Additionally, increased expression of CTR1 contributed to neurotoxicity of oxaliplatin in neural cells of the dorsal root

ganglia of rats [156]. The involvement of the copper transporters, CTR2 has not been implicated in the cellular transport of oxaliplatin.

1.3.2.2. ATPase P-type family (ATP7A and ATP7B)

The P-type ATPase family, ATP7A and ATP7B, regulates the efflux of copper at the cellular level. There is no clear evidence that copper efflux transporters directly efflux the platinum drugs, but their expression levels directly modulate the oxaliplatin accumulation into the cells [157]. ATP7A is expressed in intestine, endothelial and aorta cells, whereas ATP7B is mainly expressed in liver cells and both ATP7A and ATP7B are expressed in brain, kidney, lung, placenta and mammary gland cells [158]. A functional mutation in ATP7A and ATP7B result in inherited neuronal degenerative disorders, Menkes disease and Wilson's disease respectively, both with abnormal copper metabolism [158]. In the human fibroblast cell line Me32a with stable expressing ATP7A (MeMNK) and ATP7B (MeWND), cellular platinum accumulation levels, DNA-platinum adducts and sensitivity to oxaliplatin were enhanced compared to control cells, which indicates that ATP7A and ATP7B most probably sequestered oxaliplatin into the cytoplasmic vesicles, preventing them from reaching DNA targets and inducing cellular resistance to oxaliplatin [87, 157]. Compared with the parental Me32a cells, platinum uptake, oxaliplatin-induced DNA adducts and cytotoxicity were increased or unaltered in MeWND [157, 159]. Moreover, the presence of ATP7A prevents neurotoxicity of oxaliplatin in rat dorsal root ganglion tissue [160]. Clinically, increased levels of ATP7B have been associated with poor outcome in colorectal cancer patients treated with oxaliplatin-based chemotherapy [123]. This data indicates that both ATP7A and ATP7B are involved in efflux of oxaliplatin.

1.3.2.3. Multidrug and toxin extrusion transporters (MATE1 and MATE2)

The multidrug and toxin extrusion transporter, MATE1 (encoded by SLC47A1) and MATE2 (encoded by SLC47A2) have been implicated in regulating oxaliplatin transport [85, 89]. MATE1 is expressed in the canalicular membrane of liver cells and the luminal membrane of renal proximal tubules and MATE2 is mainly expressed in the luminal membrane of renal proximal tubules [161, 162]. Cellular studies of overexpressing MATE1 cell models have shown that oxaliplatin is a substrate of MATE1 [85]. Moreover, oxaliplatin has been shown to be a substrate of one of the isoforms of MATE2, known as MATE2-K. The reduced neurotoxicity of oxaliplatin compared to cisplatin has been considered to be dependent on tissue-specific expression of MATE2-K [85, 89]. At present, no clinical evidence is available associating MATE1 and MATE2 transporters with oxaliplatin pharmacokinetics or toxicity.

1.3.2.4. Organic cation transporters (OCTs)

Various evidence has shown that organic cation transporters (OCTs) of the SLC superfamily are associated with the uptake of platinum-based drugs including oxaliplatin [85, 114, 163, 164]. OCTs have wide tissue distribution in the human body and are expressed in intestinal, hepatic and renal epithelial cells; thus, OCT plays a key role in the pharmacokinetics of platinum drugs. However, due to conflicting preclinical results, the role of OCTs in oxaliplatin transport and pharmacokinetics is not well understood. Studies have shown that using cell lines with overexpressing OCT1 (SLC22A1 gene) and OCT2 (SLC22A2 gene) have provided evidence that oxaliplatin is a substrate of OCT1 and OCT2. In addition, in the presence of an OCT inhibitor, oxaliplatin accumulation is reduced [114]. Studies have also reported that oxaliplatin is not a substrate of OCT1 [85, 89]. Moreover, lack of alteration of pharmacokinetics in OCT1-deficient mice vs wild-type mice shows that multiple factors can regulate platinum elimination [113]. Recently, it has been shown that in patients with metastatic colorectal cancer treated with FOLFOX chemotherapy, OCT2 is involved in oxaliplatin accumulation and OCT2, along with OAT2, has been proposed to mediate cellular uptake of 5-FU [165]. Some studies showed that oxaliplatin is a substrate of human OCT3 (SLC22A3 gene) [85, 89, 114]. In addition, it has been shown that organic cation/carnitine transporters novel 1 and 2, OCTN1 (SLC22A4 gene) and OCTN2 (SLC22A5 gene) expressed in rat dorsal root ganglion tissues, contribute to oxaliplatin accumulation and cytotoxicity [166]. This evidence suggests that involvement of OCTs

may account for oxaliplatin accumulation at a tumour or drug disposition throughout the body.

1.3.2.5. Multidrug resistance-associated proteins (MRPs)

Efflux transporters of the ABC superfamily, mainly the MRPs family, have been shown to contribute to platinum drug transport [167]. MRP transporters are widely distributed in the human body, mainly in excretory sites including the liver, renal tubule, kidney and intestines as well as in barrier sites such as the blood-brain barrier and blood-testis barrier [130, 168-170]. Studies have shown that increased levels of MRP1 (ABCC1 gene) or MRP4 (ABCC4 gene) is associated with oxaliplatin resistance [171]. Moreover, inhibition of MRP1 and reduction of cellular glutathione level with verapamil increased the oxaliplatin sensitivity with reduced tumour growth in mice [172]. MRP2 (encoded by ABCC2 gene) mostly efflux drugs conjugated with glutathione [173] and glutathione conjugation is the major pathway for platinum detoxification, which leads to the formation of inactive metabolites and ultimately leads to platinum resistance by efflux of platinum glutathione conjugates by MRP2 [48]. The expression level of MRPs has been shown to be associated with cellular resistance to oxaliplatin in different human oxaliplatin-resistance cancer cells [109, 110, 171, 174]. In another study, modulation of MRP-mediated drug transporters has been implicated in explaining the synergistic action of FOLFOX combination chemotherapy. The presence of 5-FU in FOLFOX regimen was found to increase the expression of MRP2, which was associated with hypersensitivity to oxaliplatin [175]. Recently, it has been shown that MRP2 confers oxaliplatin transport with the help of ATP as a source of energy in a membrane vesicle study [176]. Taken together, these studies suggest that MRP2 is an important efflux transporter of oxaliplatin. The background knowledge of MRP2 and its function and role in oxaliplatin resistance is discussed in the next section.

Overall, the clinical impact of drug transporters in oxaliplatin accumulation and efflux have important roles in determining pharmacokinetics, efficacy and adverse drug reactions.

1.3.2.6. ABC transporters and multidrug resistance (MDR)

MDR is the main reason for treatment failure in various cancers. Studies have shown that the expression levels of ABC transporters regulate various drugs' accumulation and overexpression of ABC transporters leads to MDR [145, 179]. Some of the ABC transporters are highly expressed in human cancers including liver, pancreatic, colorectal and breast cancer. This high expression of ABC transporters is associated with the drug resistance phenomenon in cancer patients and cause multidrug resistance (MDR). MDR can be associated with several reasons in cancer patients, such as activation of DNA repair mechanisms, alteration in apoptotic signalling pathways, activation of drug-metabolising enzymes (cytochrome P450), reduced drug influx and increased drug efflux activity [177, 178]. One of the main reasons is overexpression of several ABC transporters leading to the efflux of various chemotherapeutic drugs out of cells and thus MDR. The high expression of these transporters is correlated with the poor response of patients to the anticancer drugs in certain cancers [139]. Several *in vitro* studies have demonstrated that ABC transporters alter the sensitivity of anticancer drugs at least partly by decreasing cellular accumulation of those drugs. All these findings substantiated that ABC transporters play a key role in cancer chemotherapy as an important determinant of pharmacokinetics and efficacy of certain chemotherapeutic drugs.

At least 15 ABC transporters have been implicated to confer drug resistance and the major membrane transporters that are responsible for MDR belong to the ABC superfamily, including P-gp, MRP1, MRP2, BCRP and CFTR (cystic fibrosis transmembrane conductance regulator) [179, 180]. Any modulation in these transporters results in drug resistance as well as a genetic disease because of the inherent substrate pumping ability associated with the transporters. From the above section, we can conclude that expression levels of ABC transporters and other membrane transporters can regulate the oxaliplatin accumulation inside the cells.

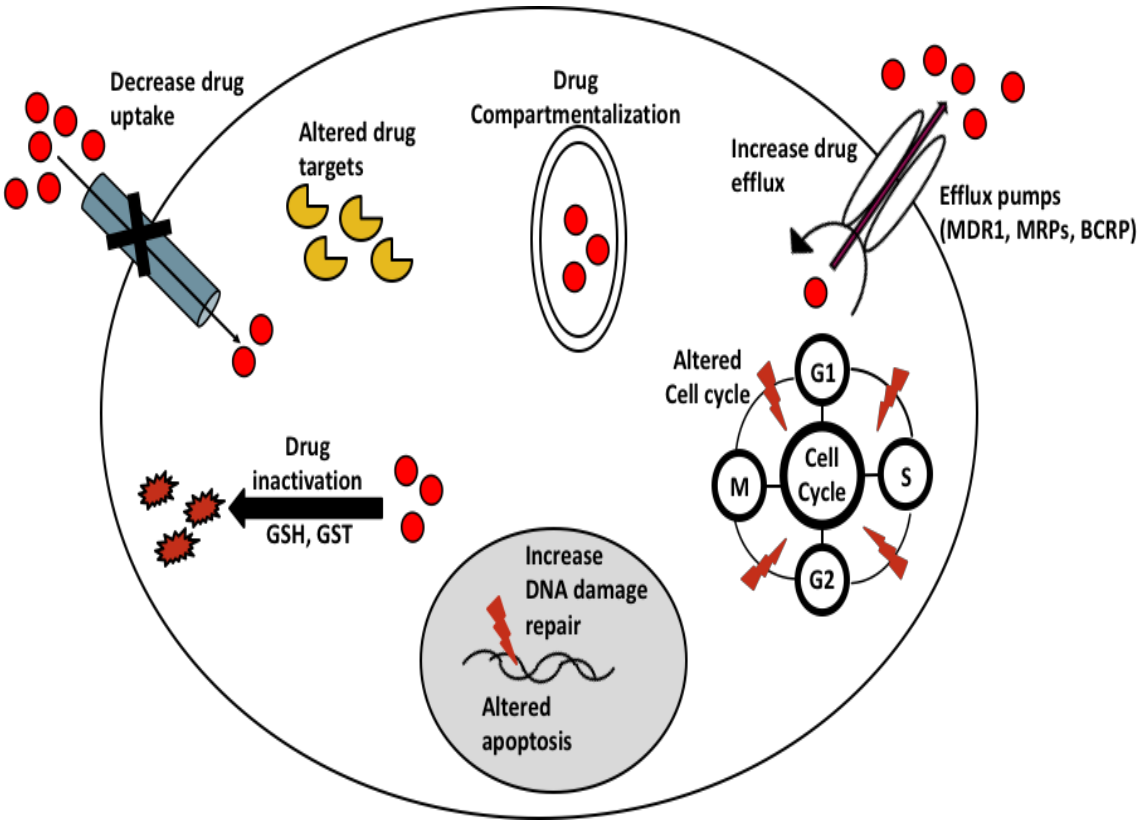


Figure 1.6 List of different mechanisms of drug resistance in cancer cells.

The various mechanisms cancer cells develop to reduce chemotherapeutic efficacy. These mechanisms include increased efflux of drugs, decreased uptake of drugs, intracellular drug sequestration, specific drug inactivation, changes in gene expression involved in apoptosis, and altered cell cycle.

In some cases, cancer cells show intrinsically higher expression of ABC transporters, even in the absence of the chemotherapeutic agents. This phenomenon is known as intrinsic resistance. There are many factors that lead to intrinsic resistance against a variety of chemotherapeutic drugs in tumour cells, such as genetic mutation, the nature of the tissues and tumour microenvironment [44]. However, overexpression of efflux transporters can be induced due to the presence of anticancer drugs. The overexpression of these transporters could be possible because of the mutation in MDR gene. This phenomenon is known as acquired resistance. Moreover, modulation of a cellular detoxification process such as glutathione conjugation could also lead to acquired resistance as it enables the cells to efflux chemotherapeutic drugs faster and thereby reduces the therapeutic effects [179].

Several *in vitro* and *in vivo* findings indicate that the efflux activity of ABC transporters mediates MDR. The importance of ABC transporters in MDR is demonstrated by numerous anticancer drugs that have been identified as substrates, including taxols, vinca alkaloids, anthracyclines, epipodophyllotoxins and tyrosine kinase inhibitors [180]. Recently, it has been observed that the MRP2 transporter transports oxaliplatin-associated platinum. This result led us to focus on inhibitions of MRP2 activity; modulating MRP2 transporter could improve the response to oxaliplatin-based chemotherapy and patients' outcomes by increased drug accumulation. Therefore, this thesis focuses on modulation of MRP2 to circumvent oxaliplatin chemoresistance in GI cancer cells.

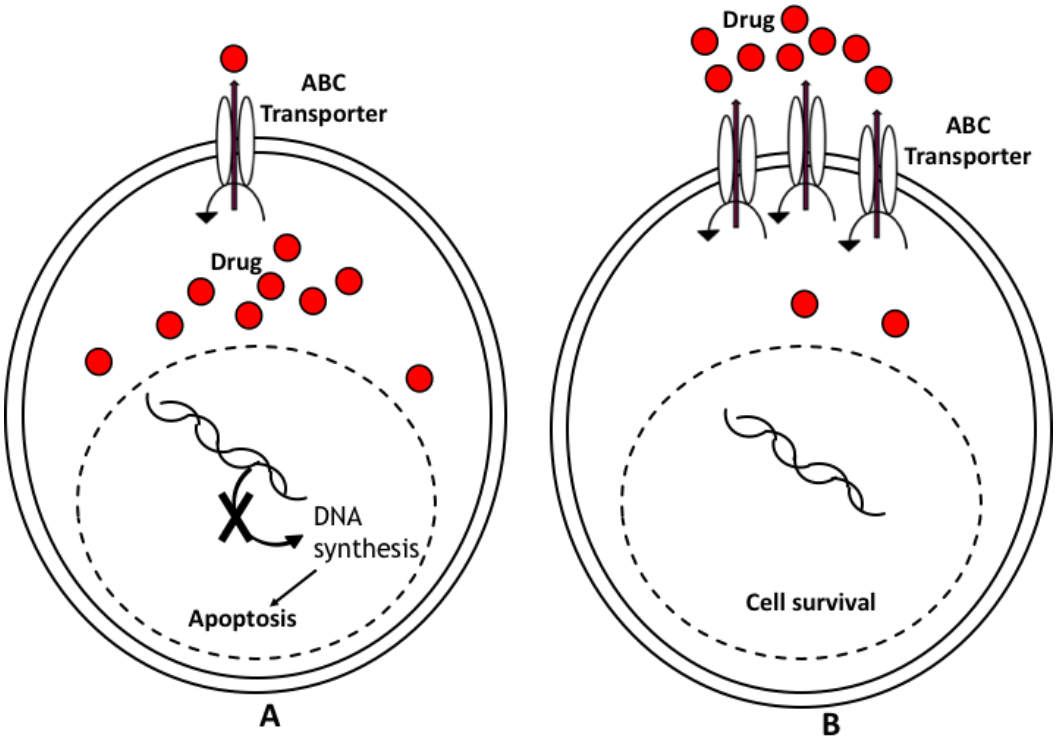


Figure 1.7 Comparison between (A) a drug sensitive cell and (B) a multidrug resistant cell.

In drug-sensitive cells, cellular accumulation of drugs is high and in a multidrug resistance cells, overexpression of ABC transporters increases drug efflux and reduces intracellular drug concentration.

1.4. Multidrug resistance-associated protein 2 (MRP2)

MRP2, also known as canalicular multiple specific organic anion transporters 1 (cMOAT) or ATP-binding cassette subfamily C member 2 (ABCC2), is a membrane transporter protein that is encoded by the ABCC2 gene and is a member of the ABC superfamily. The ABCC2 gene is located on chromosome 10q24 and consists of 32 exons with a length of 69 kb pairs [181, 182]. The basic structure consists of 17 transmembrane domains and 2 nucleotide-binding domains. It consists of 1545 amino acids with an additional 200 amino acids in the amino-proximal domain [183]. MRP2 is a membrane protein the size of around 190 kDa expressed on the apical membranes of canalicular cells, including the liver [184], small intestine, kidney renal proximal tubules and gallbladder [185], and functions in biliary transport. MRP2 is mainly involved in the excretion of small organic anions [186].

1.4.1. The physiological role of MRP2

MRP2 transporter proteins have been found in a variety of tissues, suggesting a physiological role for MRP2. Drug efflux pumps like MRP2 have essential functions in anticancer drug resistance. In humans, MRP2 is found in the apical site of hepatocytes, renal proximal tubule and small intestine as well as in physiological tissue barriers such as the blood-brain barrier, blood-tissue barrier and placenta [187]. Localisation of MRP2 suggests that MRP2 is involved in absorption, metabolism and extrusion of drugs, xenobiotics and toxins. Protection can be achieved by elimination of the toxins in the intestine or by active excretion in the liver, kidney or intestine. Therefore, MRP2 plays both a direct excretory role of drugs and limits the uptake of the xenobiotics. An essential physiological function of MRP2 is its role in detoxification of substrates in cells by transporting a variety of compounds and xenobiotics. The function of MRP2 is to transport glutathione and glutathione-conjugated substances out of the cells.

MRP2 is especially expressed on the apical membrane of polarised cells, which govern the significant process of drug absorption, distribution and excretion, and is also expressed at physiological barriers such as the blood-brain barrier [188] and placenta [189]. The function of MRP2 is to transport a wide range of both conjugated and unconjugated anionic compounds into the bile ducts, such as glutathione (GSH), glutathione-conjugates, glucuronides and 17β- glucuronosyl estradiol [144, 169, 190, 191] and cysteinyl leukotriene (leukotriene C4). Many organic anionic anticancer drugs

are transported by MRP2, including anthracyclines, vincristine, methotrexate and cisplatin [147]. The weakly basic drugs, such as vinblastine, are generally transported with GSH by MRP2 [192].

In humans, mutations in the MRP2 gene causes Dubin-Johnson syndrome (DJS), which is an autosomal recessive inherited disorder characterised by conjugated hyperbilirubinemia, resulting in a noticeable jaundice condition [182]. The function of MRP2 has been highlighted from studies using two mutant rat strains of Groningen Yellow GY/TR^{--} Wistar and Eisai hyperbilirubinemic rats (EHBR). These rat strains are Mrp2 deficient and are characterised by hyperbilirubinemia [193, 194] and defective ATP-dependent transport of conjugated bilirubin across the canalicular membrane of hepatocytes, which shows the importance of Mrp2 in the physiology of the body [187]. The expression of MRP2 is associated with renal and liver toxicity of anticancer drugs like cisplatin and methotrexate.

Therefore, MRP2 plays an important role in the body's defence against drugs and xenobiotics by eliminating them from the body through excretion into bile or urine and helps in controlling the bioavailability and disposition of drugs in the body.

1.4.2. The significance of MRP2 transporter in cancer

MRP2 protein expression is observed in human cancers, including colorectal, ovarian, lung and other gastrointestinal cancer cells [135, 195, 196]. MRP2 has also been detected in clinical specimens of renal, gastric, colorectal and hepatocellular cancers [44]. *In vitro* and *in vivo* results demonstrated that MRP2 effluxes a wide range of chemotherapeutics used clinically for the treatment of cancer, including cisplatin, doxorubicin, docetaxel, etoposide, irinotecan, methotrexate and vincristine [169].

MRP2 functional activity is associated with sensitivity of different anticancer drugs, including cisplatin, methotrexate, etoposide and vinca alkaloids [44]. The MRP2 expression is higher in a wide variety of platinum-based drug-resistant human cancer cells, including bladder, prostate, colon, ovarian, adrenocortical and melanoma [181, 184, 196, 197]. Moreover, increased levels of MRP2 expression has been associated with reduced cellular accumulation of cisplatin, which in turn reduced the toxicity of cisplatin in MRP2 overexpressed human cancer cell lines [181, 198]; therefore concluding that MRP2 plays an important role during chemotherapy in cancer cells.

In one of the studies, it was observed that MRP2 mRNA is expressed in both human pancreatic cancer and normal pancreatic tissues and expression levels of MRP2 was 1.2 to 30-fold higher in pancreatic cancer tissues [196]. Moreover, *in vitro* studies showed 1.5-fold higher expression levels of MRP2 mRNA in CDDP (combination of gemcitabine and cisplatin) drug-resistant pancreatic cell line in contrast to parent cells. Thus, expression of MRP2 plays a vital role in human pancreatic cancer. The expression of MRP2 is higher in human colorectal cancer tissues compared to non-cancer tissues, suggesting that the MRP2 gene can be a potential biomarker for colorectal cancer [199, 200]. High expression of MRP2 is associated with reduced sensitivity to platinum-based therapy [181, 201-204]. Many studies have shown that ABCC2 mRNA levels are significantly upregulated in colorectal cancer tissues compared to noncancerous regions from the same patients, and normal colorectal mucosa showed very low or no ABCC2 mRNA expression [205]. These results suggest that MRP2 is often upregulated in various tumour types and results in increased drug efflux, generating drug resistance to chemotherapeutic agents [206].

1.4.3. The significance of MRP2 polymorphisms in cancer

In contrast to the other ABC transporters like P-gp polymorphisms, less data has been reported regarding genetic variants in the MRP2 transporter gene (ABCC2). Dubin–Johnson syndrome (DJS), a disease leading to impaired hepatobiliary secretion of organic anions from hepatocytes into the bile, conjugated hyperbilirubinaemia, and deposition of melanin-like pigment in the liver, is caused by a rare missense mutation (2302C>T) in the MRP2 gene, located in the C motif in first NBD [44, 207]. Furthermore, causative missense, nonsense and splice site and deletion mutations have been reported in the MRP2 gene associated with DJS [207].

A lot of other single nucleotide polymorphisms (SNPs) or genetic variants have been studied in ABCC2. Three most commonly reported ABCC2 SNPs are C24T SNP, which is a C to T substitution in the promotor region, G1249A SNP, where G is substituted with A at exon 10 location, and C3972T SNP occurring at exon 28 location where C is substituted with T, resulting in a silent mutation at codon 1324 [208]. All these SNPs are associated with defects in MRP2 expression or functional activity. One of the studies reported that the frequently studied ABCC2 C24T SNP has no effect on the expression of ABCC2 mRNA in human duodenal enterocytes [209], but a

significant association was reported between C24T SNP and lung cancer patients [210]. Another study reported that C24T SNP was associated with reduced disease-free survival and OS in lung cancer patients receiving platinum-based chemotherapy [211]. The ABCC2 G1249A SNP was associated with the poor response of colorectal cancer patients to FOLFOX4 chemotherapy and shorter survival rate in patients [212]. Moreover, ABCC2 G1249A SNP was associated with poor response to platinum-based chemotherapy in ovarian cancer patients, whereas another study had shown no association between G1249A SNP and progression-free survival (PFS) and OS of ovarian cancer patients receiving platinum-based chemotherapy [213]. The ABCC2 C397T SNP with an increased risk of grade 3 or 4 thrombocytopenia toxicity was reported to be associated with toxicities in lung cancer patients treated with platinum-based chemotherapy [210].

The impact of ABCC2 SNPs on MRP2 functional activity remains controversial due to the contradictory findings as well as the complex functionality of multiple SNPs. However, based on this evidence, we can suggest that ABCC2 SNPs play a significant role in cancer treatment and have the potential to be applied in personalised therapy of cancer patients receiving drugs that are the substrates of MRP2.

1.4.4. The significance of MRP2 in oxaliplatin resistance

The MRP2 transporters' significance in MDR or in clinical outcomes in gastrointestinal cancer patients receiving platinum-based chemotherapy were studied previously. MRP2 expression was found to be increased in tumour tissues from patients with hepatocellular carcinoma, pancreatic cancer and colorectal cancer [196, 200, 212, 214, 215]. The use of platinum-based chemotherapy showed significant effects in gastrointestinal cancer tumours, including pancreatic cancer with overexpressed MRP2 levels [196, 200].

Many *in vitro* studies have shown the association of MRP2 with the cellular accumulation of cisplatin in human liver cancer and ovarian cancer [202]. *In vitro* studies showed the association of MRP2 with the cellular resistance to cisplatin in human liver cancer [214]. In contrast to other ABC transporters, high expression levels of MRP2 on both mRNA and protein levels have been observed in the platinum drug-resistant melanoma cancer cell line MeWo CIS 1. Moreover, overexpressed MRP2 levels reduced the formation of platinum-induced intra-strand cross-links in the nuclear

DNA and decreased the level of platinum DNA [Pt- d (GpG)] adducts in platinum-resistant melanoma cells [201]. These findings suggested that MRP2 might play a role in the cellular transport of platinum-based drugs. In pancreatic cancer patients receiving gemcitabine and cisplatin therapy, MRP2 G40A GG genetic variants showed low OS and significant association with poor response to chemotherapy [216]. Another study determined the expression level of ABC transporters in oxaliplatin-resistant colon cancer cell lines SW620/L- OHP and LoVo/L- OHP. According to this study, it has been observed that the only expression level of MRP2 in the resistant cell lines was upregulated, whereas no significant changes were observed for P- gp and MRP1 [217]. It has been detected that cellular accumulation of oxaliplatin is increased nearly two-fold in the presence of the MRP1 and MRP2 inhibitor called Gü83 in human ileocecal colorectal adenocarcinoma cell line HCT8 and its oxaliplatin-resistant variant, HCT8 ox [218]. Therefore, this indicates that MRP2 contributes to the cellular transport of oxaliplatin and the cellular accumulation level in cancer cells. Studies showed that MRP2 expression was increased in cancerous colorectal tissues compared to normal tissues and was not correlated with OS or disease-free survival of patients. However, patients showing recurrence during the course of FOLFOX4 chemotherapy showed high expression levels of MRP2 in tumour tissues [212]. Another study reported that 5-FU preincubation in FOLFOX therapy in colorectal cancer patients increased the expression level of MRP2 and the MRP2 expression level was associated with increased cellular sensitivity to oxaliplatin and increased resistance to DACH, oxalate and Pt(DACH)Cl_2 platinum adducts [175].

Based on the various *in vitro* and clinical studies, we can conclude that MRP2 transporter plays a major role in cellular resistance to oxaliplatin in human GI cancer cells. Thus, there is a strong association between MRP2 expression levels and cellular resistance of oxaliplatin in human GI cancer. Therefore, it will be worthwhile to determine the cellular platinum accumulation and oxaliplatin sensitivity in GI cancer cells after modulating MRP2 transporter.

1.5. Approaches to overcome MDR in cancer

From the previous sections, we can conclude that GI cancer a lethal malignancy and one of the leading causes of cancer-related death among both males and females throughout the world. The current regimens for treating liver, pancreatic and colorectal cancer commonly include surgery followed by chemotherapy, mainly platinum-based chemotherapy. Despite the development of various new treatment strategies like immune therapy and targeted therapy, the overall survival rate has improved only slightly. One of the major challenges for GI cancer treatments is the development of intrinsic or acquired MDR to chemotherapy. Overcoming MDR is still an elusive challenge in clinical oncology to date. Studies have confirmed that overexpression of ABC transporters like P-gp, MRP1, MRP2 and BCRP is the major limiting factor in the efficacy of chemotherapy drugs. Overexpression of MRP2 results in the development of MDR in human malignancies including liver, pancreatic, colorectal cancer. Therefore, overcoming MRP2-based MDR using MRP2 inhibitors and small interfering RNAs (siRNA) will be a significant strategy for GI cancer patients. This section summarises several novel strategies to overcome MDR in GI cancer, including potent and specific MRP2 inhibitors and gene editing technologies.

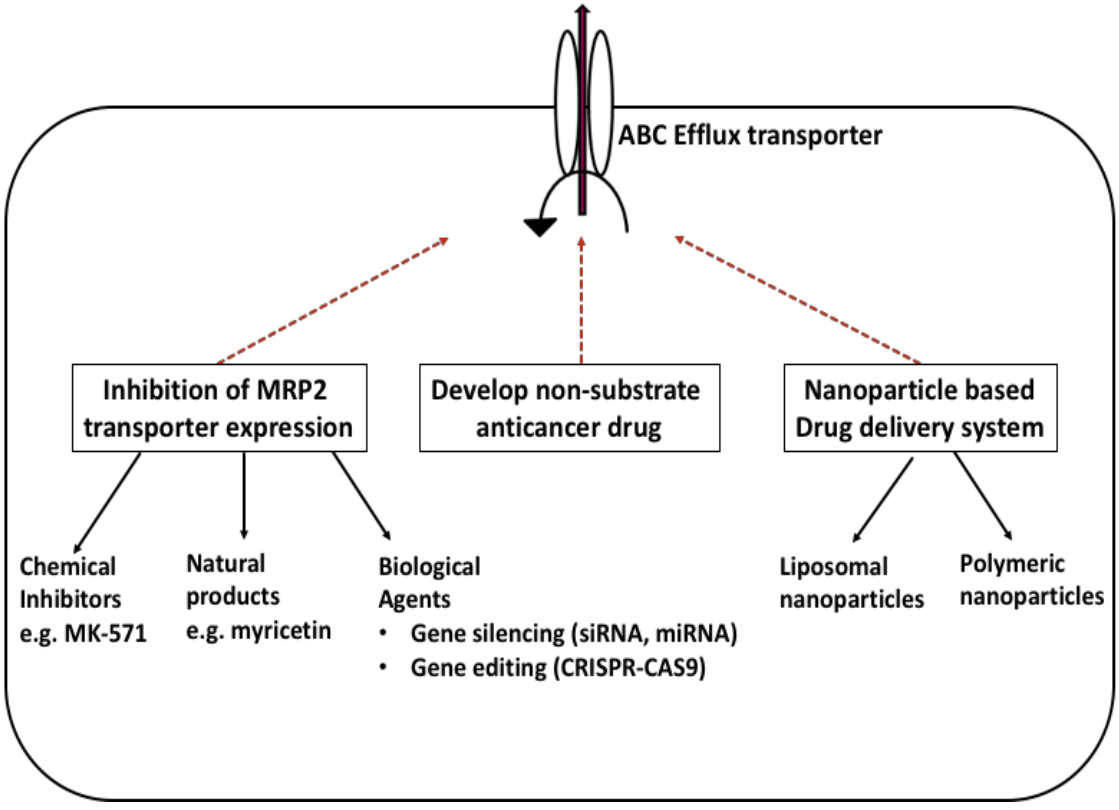


Figure 1.8 Different approaches to overcome drug resistance mediated by ABC transporters.

1.5.1. Inhibition of ABC transporters

A member of the ABC transporter family, MRP2 confers cellular resistance to many anticancer drugs, including oxaliplatin. To date, many potent MRP2 inhibitors have been discovered, such as leukotriene D4 receptor agonist MK571, glutathione conjugates, GF120918, Ko143, chlorprothixene, thioridazine, loperamide, prazosin, haloperidol, bromosulfalein, quercetin, myricetin, and curcumin, which are described as very effective in *in vitro* and membrane vesicle studies [219-221]. However, some have limited effective *in vivo* results due to their broad spectrum of inhibition on other transporters, low bioavailability or toxic effects [219]. These inhibitors have the potential to be used in combinations with anticancer drugs to enhance the effects of drugs [173].

1.5.2. Gene therapeutic approaches

Over the past few decades, genome-editing technology has been developed that provides an opportunity for researchers to manipulate the target gene in a broad range of cell types. This new revolutionary technology includes RNA interference (RNAi), zinc-finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 systems. All this technology is based on the application of engineered nucleases that consist of sequence-specific DNA-binding domains [222, 223]. The ZFNs and TALENs technologies require protein-DNA interactions to recognise the target sequence while RNAi and CRISPR-Cas9 technologies act through RNA-DNA interactions. The RNAi and CRISPR-Cas9 technologies have become more a widely used application in gene silencing and drug development as the synthesis of RNA and its entry into the cell is easier than protein domains [224, 225]. In my thesis, I have used an RNAi application to knockdown MRP2 gene (ABCC2) expression in GI cancer cells to reinforce sensitivity to oxaliplatin.

RNA interference (RNAi) mechanism has become one of the major discoveries for the treatment of many diseases, including cancer. In 1998, Fire and Mello et al., first demonstrated the gene suppression by RNAi in *Caenorhabditis elegans* [226]. Currently, RNAi is considered as an important tool for drug development and personalised cancer therapy, which can knock down the expression of targeted genes with high specificity, selectivity and minor side effects [227]. The RNAi mechanism is mediated mainly by small interfering RNA (siRNA), vector-based short hairpin RNA

(shRNA), endogenous microRNA (miRNA), non-protein-coding transcripts based non-coding RNAs (ncRNAs) and pyknons [224]. The double-stranded siRNA has become a more suitable method in gene silencing as well as in drug development as it has the ability to silence heterologous and endogenous gene expression of many disease-related genes, and siRNA can be synthesised easily.

1.5.3. Mechanism of action of siRNA

A definite endogenous siRNA is a short, double-stranded RNA (dsRNA) nearly 20 nucleotides long with two nucleotide overhangs on the 3' end of both strands. The long dsRNA, coded by a certain gene or from an exogenous source, is cleaved into smaller double-stranded nucleotides as siRNA by a ribonuclease III-like enzyme known as Dicer. The double-stranded siRNA binds with a RNA-induced silencing complex (RISC), which includes a helicase that splits the double-stranded siRNA into passenger and guide strands. The passenger strand is then degraded and released. The guide strand along with RISC, pairs with a complementary sequence of targeted mRNA and induces cleavage by Argonaut and rapidly silences the expression of a specific gene [228].

1.5.4. Advantages and disadvantages of siRNA in cancer therapy

There are many advantages of siRNA compared to other methods. Firstly, siRNA is safer than other methods because it acts on the post-translational step of gene expression, thereby avoiding the risk of mutation by not interacting with DNA. Therefore, it is less toxic. Secondly, it is highly specific compare to other anticancer drugs. An effectively designed siRNA drug can specifically silence the cancer genes [229]. The greatest advantage of siRNA is that it can target and suppress the expression of any class of genes. There are only a few chemical inhibitors and monoclonal antibodies that can inhibit certain proteins and many of them are not specific [230]. Compared to dominant negative mutants, siRNA is easy to synthesise. Combining siRNA technology with oxaliplatin-based chemotherapy may provide a rationale for the optimal combination for patient and their treatment regimens.

The most commonly used therapeutic application of RNAi in cancer therapy is silencing targeted cancer-related genes and their regulators. The main advantage of RNAi in cancer therapy is that RNAi can effectively suppress the growth of a tumour with relatively low cost and high specificity. RNAi is also useful for addressing the problem

related to drug resistance. For instance, siRNA targeting VEGF inhibitors in combination with bevacizumab reversed the resistance to bevacizumab drug [231]. In recent years with the development of clinically relevant delivery methods, a number of RNAi-based drugs have been successfully used by *in vivo* models mainly focusing on cancer [224]. After the discovery of RNAi, synthetic siRNAs were used as a potential approach not only to cancer therapy, but also to antiviral therapy, stem cell therapy, and treatment related to cardiovascular disease and diabetes [224].

However, siRNA is unstable under some physiological conditions. After entering the body, it is easily degraded by nucleases and filtered by the kidney [232]. Moreover, siRNA is not readily taken up by cells because it is an anionic hydrophilic double-stranded small RNA. One of the major limitations of siRNA therapy is the off-target effects that occur by siRNA by recognition of other mRNAs with partial homology [233]. Despite specific gene suppression, there are many specific and non-specific methods through which siRNA can cause effects other than gene silencing. The 5' end of either the siRNA passenger or guide strand plays an important role in the transcript silencing and directing off-target effects [234]. The end of siRNA that is complementary to the 3' UTR of mRNA results in unintended transcripts as well as widespread effects on miRNA processing [235]. Nonspecific off-target effects of siRNA mainly result in the activation of innate immune responses and production of cytokines [229, 236]. High levels of siRNA result in inhibition of interferon (IFN) pathways. siRNA induces the expression of toll-like receptors (TLRs), which results in recognition of viral dsRNA or viral proteins.

1.5.5. Potential siRNA drug delivery

To minimise the barriers to siRNA therapy, chemical modification with proper drug delivery methods are required to transport siRNA to their target site with minimal adverse effects. With the development of more efficient delivery systems, siRNA could be used as personalised drug for specific patients in cancer treatment [237, 238]. Currently for cancer therapy, siRNA delivery systems are divided into following main categories: chemical modification, nanoparticle delivery systems like lipid-based and polymer-based nanoparticles, exosomes and conjugated therapy. Nanoparticle-based delivery is the most common method for the delivery of siRNA as it provides high structural and functional stability, enhanced cell entry and endosome escape, resist clearance, and generates low toxicity and immunogenicity.

Chemical Modification

The 5'- or 3'-terminal of the nucleobase of siRNA can be chemically modified. The most common modification introduced at the 2' position of the ribose, including 2'- O'-methylpurines and 2'-deoxy-2'-fluoropyrimidines, enhances siRNA stability by preventing degradation by endonucleases [230].

Lipid-based nanoparticle (Liposomes)

Recently, liposomes have been used as efficient molecules for siRNA delivery with minimal toxicity and reduced degradation [239]. Liposomes are considered as an effective *in vitro* siRNA transfection with greater entrapment power to transfer into the cell, as liposomes are typically less than 100 nm and have a natural tendency to interact well with cell membranes [224]. Cationic-based liposomes, e.g., dioleoyl-phosphatidylethanolamine and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), are most effective for the delivery of negatively charged siRNA [229]. However, because of the increased cytotoxicity of a cationic liposome, neutral lipids are desired. 1,2-Dioleoyl-*sn*-glycerol-3-phosphocholine (DOPC) is a neutral lipid that effectively reduces cellular toxicity [240]. Moreover, to improve the stability of nanoparticles, polyethene glycol (PEG) is mostly used on the surface of nanoparticles. Coating liposomes with PEG increases the half-life of siRNA [241].

Polymer-mediated nanoparticles

Polymer-based nanoparticles are small, solid and biodegradable particles that cause efficient delivery of anticancer drugs. Commonly used carriers of polymer-mediated nanoparticles are synthetic polypeptides, polyethyleneimine, and carbohydrate-based polymers, such as chitosan [224]. Polymer-based nanoparticles include polycaprolactone (PCL), poly (D, L-lactide) (PLA) and poly (D, L-lactide-co-glycolide) (PLGA). SNALPs (stable nucleic acid-lipid particles) are lipid-based nanoparticles that encapsulate siRNA and have been used to therapeutically deliver siRNA into the target cells. SNALPs accumulate at the sites of cancer cells due to enhanced permeability and retention (EPR) effect and endocytosised easily and delivered siRNA successfully at the cancer sites [242].

Exosomes

Exosomes are natural biocarriers with phospholipid bilayer vesicles of 40-120 nm in diameter and originate from endosomes. The exosome is like an extracellular vesicle that contains a series of specific transmembrane proteins that guide them to the target cells. Exosomes are suitable for carrying soluble drugs and have few off-target effects [224].

Conjugate siRNA delivery

The most common method for delivering siRNA *in vitro* is by directly conjugating delivery materials to siRNA. The commonly used conjugated materials are small drugs, peptides, lipids, proteins and polymers [229].

1.5.6. siRNA delivery targeting MDR

As discussed in the earlier sections, overexpression of membrane transporters is the major reason for MDR. Although many potent ABC transporter-specific inhibitors have been discovered to overcome MDR, some of these inhibitors have failed to provide clinical benefits. The RNAi-based gene editing therapy can selectively transport siRNA to the cancer cells both *in vitro* and *in vivo*, and avoid MDR arising from chemotherapy through the strategy of codelivery of siRNA and chemotherapy. This approach could maximise the chemotherapeutic effects of anticancer drugs with a minimal chemotherapy dose, produce a better therapeutic response and increased survival rates [243, 244]. The RNAi-based siRNA method can silence the target gene responsible for MDR to increase drug accumulation in chemotherapy-resistant cells [245]. The primary mechanisms of chemoresistance in most cancer cells with MDR include pump and non-pump resistance. Pump resistance is induced by membrane proteins like MRP2, whereas non-pump resistance can be induced by activation of any cellular pathways to prevent cell death [224]. Overexpression of the member of ABC transporter family, MRP2, is believed to correlate a with poor oxaliplatin-based chemotherapy response. Furthermore, codelivery of siRNA targeting MRP2 gene (ABCC2) and oxaliplatin could be a promising approach to reverse MDR in GI patients with high expression of MRP2.

1.6. Hypothesis and aim of the thesis

The platinum-based anticancer drug oxaliplatin, and its combination therapies, are important in the clinical treatment of colorectal cancer and other gastrointestinal malignancies [59]. However, oxaliplatin chemotherapy has poor efficacy in a proportion of treated patients for reasons that are currently unclear. One of the major obstacles to the effective treatment of GI cancer is the development of MDR to chemotherapy. The main mechanism of MDR is decreased cellular accumulation and cellular efflux of anticancer drugs by ABC transporters. Various *in vitro* and *in vivo* studies have shown that overexpression of different types of ABC transporters in many cancers has been correlated with poor treatment outcome in patients. Clinical studies have demonstrated that high expression of MRP2 in GI cancer, including liver, pancreatic and colorectal cancer, is associated with reduced sensitivity of platinum-based chemotherapy in patients [212, 246]. It has been suggested that the main reason for poor efficacy is associated with the reduced cellular accumulation of oxaliplatin [154, 258]. There is a possibility that oxaliplatin clinical activity is dependent on factors regulating its accumulation by virtue of their expression in GI tumour cells or by biological barriers affecting drug disposition. Studies have reported that several membrane transporters like CTR1, OCTs, ATP7A, ATP7B, MATE1, MATE2 regulate oxaliplatin accumulation into the cells [85, 87, 89, 155, 157, 166]. Also a recent study has suggested that MRP2 is an efflux transporter of oxaliplatin [176]. According to Khine et al., MRP2 transports oxaliplatin and its anionic monochloro oxalate ring-opened degradation product. We hypothesise that MRP2 may be a candidate membrane transporter protein for enabling gastrointestinal cells to resist oxaliplatin-induced antitumour activity by limiting their accumulation of oxaliplatin-derived platinum.

Many approaches have been used to circumvent the problem of MDR. Inhibition of MRP2 transporters by various inhibitors has been demonstrated to reverse MDR and increase the sensitivity of cells to chemotherapy. A previous study showed that the MRP2 inhibitor increased platinum-DNA levels in oxaliplatin-resistant human colorectal cancer cells, suggesting that inhibiting MRP2 had a potential role in the cellular accumulation of oxaliplatin; however, cellular sensitivity to oxaliplatin was inconclusive. Moreover, some of the clinical trials of MRP2 transporter inhibitors showed limited effect due to their broad spectrum of inhibition on other transporters, low bioavailability or toxic effects of inhibitors [219]. This has led to a search for

alternative therapeutic experimental strategies for overcoming MDR in GI tumours overexpressing MRP2 transporters. These include gene-editing technology applying RNAi techniques.

In the RNAi method, the synthetic RNA molecules, i.e., double-stranded, small interfering RNA (siRNA) molecules, can specifically silence the gene of interest in cancer cells. After the first successful application of RNAi technology in an animal model *in vivo* [247], RNA-based drugs were used clinically with efficient results [242]. Moreover, siRNA-based technology has also been shown to overcome MDR by inhibition of ABCB1 [225]. Hence, we hypothesise that the RNAi-based technology, ABCC2-specific siRNA, will successfully silence the MRP2 gene and its expression with minimal toxicity. Moreover, after silencing the MRP2 gene, it will increase cellular accumulation of oxaliplatin in GI cancer cells, which will enhance the sensitivity to oxaliplatin-based therapy, thereby becoming effective in inhibiting cancer growth by restoring oxaliplatin drug resistance in GI cancer. Therefore, the GI tumour cells will become more sensitive to oxaliplatin-based chemotherapy by silencing MRP2. Application of siRNA in combination with other treatment such as chemotherapy may represent an effective treatment for GI cancer.

As far as we know, no studies have been conducted that determined the effects of ABCC2 silencing by siRNA on cellular accumulation of platinum and sensitivity to oxaliplatin in GI cancer cells. In this thesis, the MRP2 gene (ABCC2) was knocked down using three different subsets of ABCC2-specific siRNAs in GI cancer cells with high expression of MRP2, including liver, pancreatic and colorectal cancer cell lines. For this research we used HepG2, PANC-1 and Caco-2 cells. In clinical studies, the expression of MRP2 is higher in cancer tissues compared to non-cancer tissues [199, 200]. Previous studies reported that HepG2, PANC-1 and Caco-2 have high MRP2 expression level [248, 249]. To mimic clinical situation we used these cell lines, which is suitable for our translational research. The mRNA level of ABCC2 in MRP2-silencing cells were first verified using real-time quantitative qPCR. These results determined the percentage of MRP2 gene knockdown in GI cancer cell lines. The MRP2-mediated transport of substrate in this study was verified using MRP2-specific substrate, 5(6)-carboxy-2, ‘7’-dichlorofluorescein (CDCF) and myricetin as an MRP2

inhibitor. We then determined the oxaliplatin-derived platinum accumulation via MRP2-mediated oxaliplatin transport in MRP2-silencing cells using an ICP-MS-based platinum analysis method after exposure to oxaliplatin. We also studied the cellular sensitivity of MRP2-silencing cells to oxaliplatin-induced cytotoxicity to determine the role of MRP2 in conferring cellular oxaliplatin resistance. The expression levels of MRP2 in MRP2-silencing cells were determined using a cell surface staining method. We determined the rate of oxaliplatin-induced apoptosis in GI cancer cell lines with different concentrations of oxaliplatin after inhibiting MRP2 expression using both chemical inhibition of MRP2 with myricetin and gene editing inhibition of MRP2 with siRNA-mediated transient silencing of ABCC2. Lastly, this thesis determines the transport activity of MRP2 transporters in the presence of oxaliplatin using an ATPase assay. The main purpose of this study is to reverse MRP2-mediated oxaliplatin resistance using the siRNA-mediated transient gene knockdown method and investigate the role of MRP2 in oxaliplatin transport and oxaliplatin resistance in human GI cancer cells by determining MRP2 expression at the mRNA level and functional level, MRP2 transport activity, cellular platinum accumulation, and oxaliplatin-induced sensitivity and apoptosis rate.

The specific aims of this thesis were:

i. To determine MRP2 ATPase activity by interaction with oxaliplatin using a cell-free system (Chapter 3).

ii. To inhibit MRP2 expression by using siRNA and determine the cellular platinum accumulation, chemosensitivity to oxaliplatin and anti-cancer activity in an MRP2-overexpressing human liver cancer cell model (Chapter 4).

iii. To explore the effects of silencing MRP2 by siRNA on cellular platinum accumulation and chemosensitivity to oxaliplatin in human pancreatic and colorectal cancer cell lines (Chapter 5).

iv. To determine the oxaliplatin-induced apoptosis rate in human GI cancer cell lines after inhibiting the MRP2 transporter with an MRP2-specific inhibitor myricetin (Chapter 6).

Chapter 2 Materials and methods

2.1. Chemicals and equipment

The materials and reagents, including chemical compounds, buffers and solutions that were used in this research are listed in table 2-1.

Table 2-1. List of chemicals used in this study with their sources

Chemicals	Suppliers
70% Nitric acid	Thermo Fisher Scientific (NZ)
Absolute Ethanol	Thermo Fisher Scientific (NZ)
Absolute Methanol	Thermo Fisher Scientific (NZ)
Anti-MRP2 antibody [M2 III-6] (catalogue # ab3376) (dilution: 1:100 ratio in 2% BSA)	Abcam (Melbourne, VIC, AU)
Alexa Fluor 488-labeled anti-mouse IgG Ab (catalogue # ab150120) (dilution: 1:1000 ratio in 2% BSA)	Abcam (Melbourne, VIC, AU)
Apoptosis Kit (catalogue # V13245)	Invitrogen (Carlsbad, CA, USA)
ABC Transporter Control membrane vesicles (catalogue # GM0003)	GenoMembrane (Life Technologies)
SB-MRP2-PREDEASY-ATPase Assay kit (catalogue # SBPE03-10RXN)	Solvo biotechnology (Sigma-Aldrich)
Bovine serum albumin (BSA)	Sigma-Aldrich (St Louis, MO, USA)
cDNA synthesis kit (catalogue # 04896866001)	Roche life science (NZ)
CDCFCD (5(6)-Carboxy-2', 7' – Dichlorofluorescein) (catalogue # 21884-100MG)	Sigma-Aldrich (St Louis, MO, USA)
Fetal bovine serum (FBS)	MediRay (NZ)
LC480 LightCycler Master Kit (catalogue # 04707516001)	Roche life science (NZ)
L-Glutamine (200 mM; 100 ml)	Life Technologies
Lipofectamine RNAiMAX (catalogue # 13778075)	Life Technologies (Thermo Fisher Scientific, NZ)
Mouse IgG2a Isotype Control (catalogue # MA1-10419) (dilution: 1:400 ratio in 2% BSA)	Invitrogen (Carlsbad, CA, USA)
MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) Formazan powder (catalogue # M5655-500MG)	Sigma-Aldrich (St Louis, MO, USA)
Myricetin (catalogue # M6760-25MG)	Sigma-Aldrich (St Louis, MO, USA)
Opti-MEM 1 (catalogue # 31985-070)	Life Technologies (Thermo Fisher Scientific, NZ)
Oxaliplatin powder	Actavis (Auckland, NZ)
Paraformaldehyde powder (catalogue # P6148-500g)	Sigma-Aldrich (St Louis, MO, USA)
Penicillin-Streptomycin (10,000 U/mL; 100 mL)	Life Technologies (Thermo Fisher Scientific, NZ)

Platinum standard	Sigma-Aldrich (St Louis, MO, USA)
Primers for qRT-PCR	Integrated DNA Technologies (IDT, Iowa, USA)
RNA isolation (RNeasy mini) kit	Qiagen (Germany)
Roswell Park Memorial Institute (RPMI) 1640 Medium	Life Technologies (NZ)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (St Louis, MO, USA)
Saponin (catalogue # 47036-50g)	Sigma-Aldrich (St Louis, MO, USA)
Stealth ABCC2-siRNAs (catalogue # 1299001)	Life Technologies (Thermo Fisher Scientific, NZ)
Stealth siRNA Negative control, Mediun GC (catalogue # 12935-300)	Life Technologies (Thermo Fisher Scientific, NZ)
Synth-a-Freeze Medium	Life Technologies (Thermo Fisher Scientific, NZ)
Thallium standard	SPEX CertiPrep (NJ, USA)
Trypan Blue Stain (0.4%)	Life Technologies (NZ)

Table 2-2. List of equipment used in this study with their sources

Equipment	Suppliers
Inverted phase contrast microscope	Zeiss Primovert (Fisher scientific, NZ)
15 ml and 50 ml centrifuge tubes	In vitro Technologies (NZ)
25 ml and 75 ml vented cap tissue culture flasks	In vitro Technologies (NZ)
1 ml, 5 ml, 10 ml and 25 ml sterile disposable pipettes	In vitro Technologies (NZ)
Microtiter plate reader	Tecan Spark 10M (Mannedorf, Switzerland)
0.22 µM filter and a 10 ml syringe	Thermo Fisher Scientific (NZ)
Haemocytometer (Improved Neubauer)	Boeco (Germany)
6, 12 and 96-well plate	In vitro Technologies (NZ)
Centrifuge 5418 R	Eppendorf (North Ryde, NSW, AU)
Flow cytometer	Beckman Coulter MoFlo™ XDP (NZ)
LightCycler 480	Roche Life Science (NZ)
Varian 820MS ICP-MS	Agilent Technologies Inc. (Santa Clara, CA, USA)

12 mM MTT (3-(4, 5-dimethylthiazil-2-yl)-2, 5-diphenyl tetrazolium bromide) stock solution was prepared by adding 1 ml of sterile PBS to 5 mg of MTT and stored at 4°C.

CDCFCD and myricetin stock solution was prepared at the concentration of 1000 times higher than working solution by dissolving in DMSO and these were stored at -20°C.

Oxaliplatin (Actavis, New Zealand) stock solution at 5 mg/ml was prepared by dissolving 100 mg powder into 20 ml MiliQ grade water followed by sonication and

filtered with a 0.22 μm Millipore filter. The stock solutions were immediately aliquot and stored at -20°C. The stock solutions were discarded one month after preparation.

2.2. Cell lines and cell culture

All the cell culture procedures were carried out using sterile solutions and equipment following sterile techniques in a class I biological safety cabinet.

2.2.1. Human gastrointestinal cancer cell lines

A panel of human gastrointestinal cancer cell lines, including HepG2, PANC-1 and Caco-2 cell lines with a high expression of MRP2 were selected. The cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Once the cells were received from ATCC, they were stored in liquid nitrogen Dewar and maintained according to ATCC protocol. The general information about cell lines is listed in table 2-3.

Table 2-3. Characteristics of human gastrointestinal cancer cell lines used in the experiment

Cell lines	General description	Culture properties	Morphology (Tissue)
HepG2	Human liver hepatocellular carcinoma	Adherent	Epithelial (liver)
PANC-1	Human pancreatic epithelioid carcinoma	Adherent	Epithelial (pancreas/duct)
Caco-2	Human colorectal adenocarcinoma	Adherent	Epithelial-like (Colon)

2.2.2. Revival of the cells from the frozen culture

The cryovials containing frozen cell stocks were quickly thawed in a 37°C water bath after removal from liquid nitrogen. The cell suspension was transferred to a sterile 15 ml centrifuge tube, which was already prefilled with prewarmed complete RPMI medium. After centrifugation at 500 × *g* for 5 min, the supernatant was aspirated and cell pellets were resuspended in 1 ml of complete medium and transferred into a T_{25} flasks (BD Falcon, Auckland, NZ) containing prewarmed complete RPMI medium supplemented with 10% (v/v) foetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin and 100 units/mL streptomycin. Cells were maintained and allowed to grow in a humidified atmosphere of 5% carbon dioxide at 37°C.

2.2.3. Cell maintenance and subculturing

The cells were monitored at regular intervals and were subcultured when they were 70% to 80% confluent as they reached the end of the exponential phase. The cells were split by washing the cell flask with prewarmed PBS followed by addition of TrypLE™ Express, approximately 2 ml for T_{25} flask and 4 ml for the T_{75} flask, to detach the cells from the flask. The cells were incubated for 5 to 10 min, depending on the cell lines, in the 37°C incubator. In this study, HepG2 cells required long incubation time for 10 mins, whereas the incubation time for PANC-1 and Caco-2 cell lines were around 5 to 8 min. To stop trypsinisation, approximately 4 to 8 ml (twice the volume of dissociation reagent) of prewarmed complete RPMI medium was added. Cell suspensions were transferred into 15 ml centrifuge tubes. After centrifugation at 500 × *g* for 5 mins, the supernatant was aspirated and the cell pellet was resuspended in 1ml of complete RPMI medium. Cells were counted using a haemocytometer (refer 2.2.4) and the appropriate volume of cells was dispersed into the new cell flask with prewarmed complete medium and allowed to grow in a humidified incubator at 37°C and 5% CO_2.

The cell lines were seeded at 250,000 cells in a T_{25} flask and 750,000 cells in the T_{75} flask. For HepG2 and PANC-1, seeding density for passaging and maintenance was kept at 400,000 cells in a T_{25} flask and 1,000,000 cells in a T_{75} flask. Cells with a passage number greater than 20 were discarded.

2.2.4. Cell counting using a haemocytometer

The number of viable cells was determined using a haemocytometer. A volume of 10 µl of cell suspension was mixed with 10 µl of 0.4% Trypan Blue stain, which selectively stains dead cells blue over live cells. A volume of 10 µl of cell mixture (10 µl of cell suspension and 10 µl of Trypan Blue stain) was placed in a haemocytometer counting chamber. Cells were counted within four large quadrants under the inverted microscope. The cell density was calculated using the following formula.

Concentration (cells/ml) = Average number of cells * dilution factor * 10,000

After calculating cell density, cell lines were seeded in a new cell culture flask. Cells were grown to 70% to 80% confluence and were either split or used in experiments.

2.3. Transfections of siRNA

An siRNA targeting ABCC2 gene was transfected into gastrointestinal cell lines using Invitrogen transfection reagent. A set of three different ABCC2 stealth RNAi™ siRNAs, Lipofectamine™ RNAiMAX and Opti-MEM I reduced serum medium were used in this study. A non-targeting negative stealth siRNA (scrambled) was used as a negative control. All the reagents were purchased from Invitrogen, Carlsbad, CA, USA. The cells were seeded at a density of 1.5×10^5 cells per well in 12-well plates. Cells were transfected with different ABCC2 siRNA subtypes (siRNA-1, siRNA-2 and siRNA-3) at 16 - 30 nM concentration for 48 hours using Invitrogen lipofectamine RNAiMAX. For efficient silencing without toxicity, siRNA and Lipofectamine RNAiMAX reagent ratio was maintained at 1:0.6. After 48 hr, transfected cells were used for functional, cellular platinum accumulation and the cytotoxicity assay. HepG2 and Caco-2 cells were transfected using a reverse transfection method. For PANC-1 cells, we used conventional forward transfection method. The details of the siRNA transfection methods are described in each chapter. The sequences of validated steath ABCC2 siRNAs are listed below:

a. ABCC2-siRNA-1: 5’-GAU CAU GAA UGA GAU UCU UAG UGG A-3’
   5’-UCC ACU AAG AAU CUC AUU CAU GAU C-3’
b. ABCC2-siRNA-2: 5’-CCA GCA AAG GCA AGA UCC AGU UUA A-3’
   5’-UUA AAC UGG AUC UUG CCU UUG CUG G-3’
c. ABCC2-siRNA-3: 5’-ACC AAG ACA UUA GUG AGC AAG UUU G-3’
   5’-CAA ACU UGC UCA CUA AUG UCU UGG U-3’

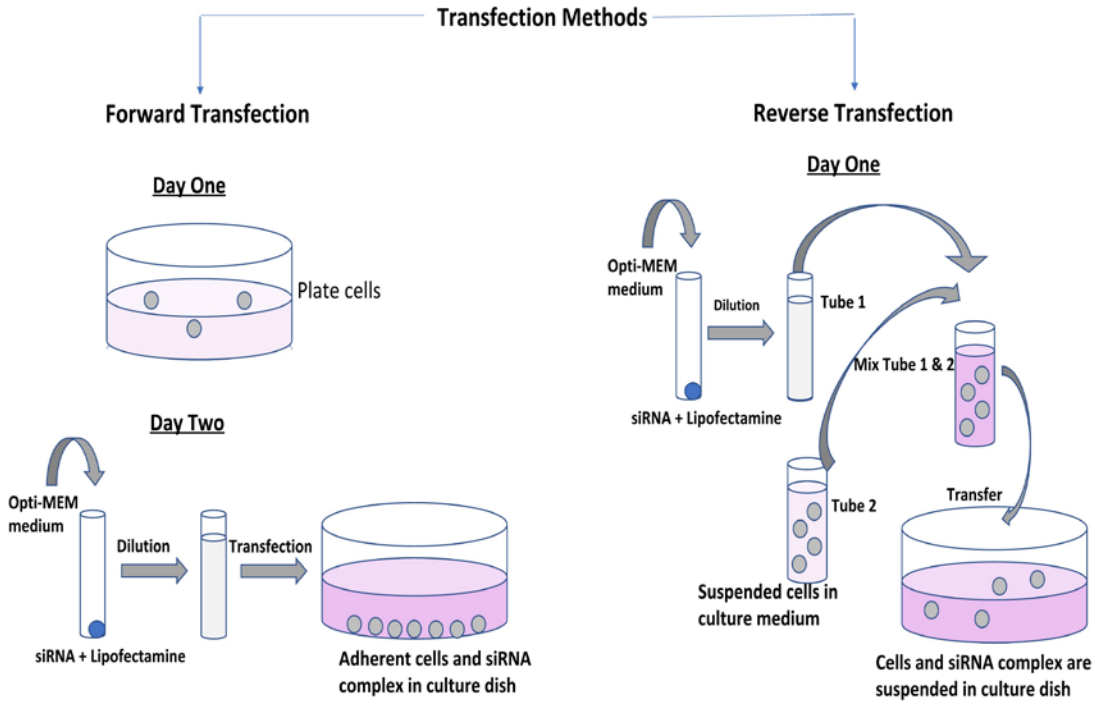


Figure 2.1. Schematic diagram of transfection method with siRNA and Lipofectamine.

In conventional forward transfection, siRNA lipofectamine complex is diluted in medium then added to preplated adherent cells. In reverse transfection, siRNA lipofectamine complex is diluted in medium then mixed with the cell culture medium followed by transfer to a culture dish.

2.4. Quantitative real-time polymerase chain reactions (qRT-PCR)

2.4.1. RNA Extraction

Total RNA was extracted from gastrointestinal cell lines and transfected cells using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Cells were seeded in 12-well plates until they were confluent to approximately 80%. Cells were then trypsinised and centrifuged at 300 × *g* for 5 min. The cell pellets were collected and lysed by pipetting the cell pellet with 350 µl of lysis buffer mixture. The cell lysate was mixed with one volume of 70% ethanol by pipetting the solution. The mixture (including any precipitate) was transferred into an RNeasy spin column placed in a 2 ml collection tube and spun for 15 sec at 8,000 × *g* (10,000 rpm). The filtrate collected in the tube was aspirated. The washing steps were repeated using two more buffers at the specific conditions mentioned in the manufacturer's protocol. Around 40 µl of RNase-free water was added into the RNeasy spin column placed in a new 1.5 ml collection tube. The RNA was eluted by centrifugation at 8,000 × *g* (10,000 rpm) for 1 min and was kept at -20°C for further quantification.

The quantity of eluted RNA in the cell sample was checked using a Qubit® 2.0 Fluorometer (Invitrogen). The Qubit assay kits were provided with assay reagent, dilution buffer and prediluted standards. Qubit working solution was prepared by mixing assay reagent and dilution buffer in a 1:200 ratio. The working solution was aliquoted in 2 ml eppendrof tubes. Thereafter, 10 µl of each Qubit standards were added to tubes containing working solution for a total volume of 200 µl. Then 1 µl of RNA sample was added to appropriate tubes containing Qubit working solution for a total volume of 200 µl. Each tube was vortexed for 2-3 sec and incubated at room temperature for 2 min. After incubation, the concentration of each sample was observed on the Qubit 2 fluorometer. The signal is usually stable for 3 hr.

2.4.2. cDNA synthesis

cDNA was synthesised from the total RNA, which was previously extracted from the cell lines using the transcriptor first strand cDNA synthesis kit (Roche Life Science) according to the manufacturer's instructions. Before starting the experiment, the extracted RNA was stored on ice. After that, the reverse transcription master mix was prepared from reverse transcription buffer, RNase inhibitor, oligonucleotides and transcriptor reverse transcriptase enzyme provided in the kit. The extracted total RNA was added to the transcription master mixture along with RNase free water to make a

total volume of 20 µl of sample. After mixing the solution by pipetting for a few minutes, the reverse transcription reaction was started by incubating the reaction at 50°C for 60 min followed by inactivation of reaction at 85°C for 5 mins. The reaction was terminated by placing the tube on ice. At this point, the tube was stored in the freezer for qPCR reaction or at -20°C for longer periods.

2.4.3. Real-time qPCR

The expression levels of all the targeted genes were measured using real-time qPCR, and genes were quantified using a LC480 LightCycler (Roche Applied Science). Forward and Reverse primers (listed in table 2-4) for each targeted gene containing transporter genes and housekeeping were purchased from IDT (Integrated DNA Technology, USA) and stored at -20°C. qPCR master mix was prepared from LightCycler-FastStart DNA Master SYBR Green 1 Master Mix, DNA Taq Polymerase and gene-specific primers at 180 nM. For running the PCR reaction, 15 µl of the qPCR master mix was loaded in each of the 96 wells in the array PCR plate. Then, 5 µl of synthesised cDNA was added in the reaction mixture in each well accordingly. The PCR run was set up according to the manufacturer's instructions and a melting point of the primers.

The reaction conditions were as follows: 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, at 58°C for 30 s, and 72°C for 30 s. The results were analysed using the comparative threshold cycle method. The mean Ct value was determined from the amplification plots generated by "LightCycler 480 software" to detect the mRNA expression of each targeted gene. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control to normalise the target gene expression using the $2^{-\Delta\Delta Ct}$ method.

Table 2-4. Primers used for RT-PCR

Target Genes	Forward Primers (5'-3')	Reverse Primers (5'-3')	Reference
ABCC1	AGTGGAACCCCTCTCTGTTTAAG	CCTGATACGTCTTGGTCTTCATC	[250]
ABCC2	AATCAGAGTCAAAGCCAAGATGCC	TAGCTTCAGTAGGAATGATTTCAGGAGCAC	[250]
ABCC3	TCCTTTGCCAACTTTCTCTGCAACTAT	CTGGATCATTGTCTGTCAGATCCGT	[250]
ABCC4	TGATGAGCCGTATGTTTTGC	CTTCGGAACGGACTTGACAT	[250]
ABCC5	AGAGGTGACCTTTGAGAACGCA	CTCCAGATAACTCCACCAGACGG	[250]
ABCG2	CCGCGACAGTTTCCAATGACCT	GCCGAAGAGCTGCTGAGAACTGTA	[250]
hCTR1	TCACCATCACCCAACCACTT	TCTTAAAGCCAAAGTAGAAGGTCA	[251]
ATP7A	ATGATGAGCTGTGTGGCTTG	TGCCAACCTGAGAAGCAATAG	[175]
ATP7B	TACCCATTGCAGCAGGTGTC	ACTTGAGCTGCAGGGATGAG	[175]
GSTP1	GCCCTACACCGTGGTCTATT	TGTAGATGAGGGAGATGTATTTGC	[251]
GAPDH	GCACCGTCAAGGCTGAGAAC	GCCTTCTCCATGGTGGTGAA	[252]

Primer sequences used in this study are ordered from IDT. GAPDH primer sequences listed table 2-4 were used as reference genes for normalisation of target gene expression.

2.5. Flow cytometry determination of CDCF accumulation

To evaluate the transport activity of MRP2 in human gastrointestinal cancer cell lines, MRP2-expressing cell lines were exposed to a fluorescent probe, CDCF (5(6)-carboxy-2',7'-dichlorofluorescein). CDCF is an MRP2-specific substrate used in this research to validate MRP2-mediated membrane transport activity [253]. The non-fluorescent cell-permeable probe CDCFDA (5(6)-carboxy-2',7'-dichlorofluorescein diacetate) was used in this assay, which de-esterified intracellularly and turned into fluorescent CDCF.

2.5.1. Determination of time to reach steady state

The cells grown in a T_{75} flask with about 80% to 90% were washed twice with PBS and trypsinised (see section 2.2.3). TrypLE was neutralised with RPMI complete medium and centrifuged at 500 × *g* for 5 min. Media was discarded and cell pellets were resuspended in foetal bovine serum-free and phenol red-free RPMI or PBS. Cell density was determined as per section 2.2.4. Cells were transferred to a 15 ml centrifuge tube and resuspended in the above media or PBS to achieve a cell density of approximately 5 × 10^5 cells/ml. For a steady-state substrate accumulation study, six different time points were observed in duplicate.

The cells in the 15 ml centrifuge tubes were kept at room temperature. The accumulation of CDCFDA was performed by incubating half of the cell samples with 100 µM myricetin and one cell sample with 0.1% DMSO at 37°C for 10 min. The cells incubated with DMSO represent a control sample. Thereafter, 2 - 5 µM of CDCFDA were added to the cell samples. The samples were gently vortexed and incubated for 5, 10, 15, 20, 60 and 40 min accordingly at 37°C. Samples were wrapped in aluminium foil or kept inside a box for minimal light exposure to prevent fluorescence bleaching. After incubation, the accumulation was stopped by immediately placing the samples on ice and adding 3 ml of ice-cold PBS. The cells were centrifuged at 500 × *g* for 5 min at 4°C and again resuspended with ice-cold PBS. The cells were washed twice with PBS to wash off the excess fluorescent probe. After washing with ice-cold PBS the cells were reconstituted in 500 µl of ice-cold PBS and placed into ice immediately. The intracellular level of CDCF was analysed using the Moflo XDP flow cytometer (Beckman Coulter, Auckland) equipped with a standard laser for excitation at 488 nm and a bandpass filter at 525 nm was used to detect CDCF fluorescence.

Table 2-5. Time points to accumulate CDCF substrate

Samples	Time points (min)		
	HepG2	PANC-1	Caco-2
1	0	0	0
2	5	10	10
3	10	15	15
4	20	20	20
5	40	40	40
6	60		

2.5.2. MRP2 functional validation assay

Gastrointestinal cell lines and their corresponding transfected cells were trypsinised and resuspended in PBS at a density of 5×10^5 cells/ml as per the steps mentioned in section 2.5.1. The accumulation of CDCF by transfected cells was performed by adding non-fluorescent MRP2 substrate CDCFDA into 1 ml of cells of density 5×10^5 cells/ml and incubated for the appropriate time (time to achieve steady state as per the previous experiment in section 2.5.1). After incubation, the reaction was stopped with ice-cold PBS and cells were washed twice to remove the excess substrate. Finally, cells were resuspended in 500 µl PSB and kept on ice until analysis in the flow cytometry with a standard laser for excitation at 488 nm and a bandpass filter at 525 nm to detect fluorescence by accumulation of CDCF.

Cells shown in forward scatter and side scatter were gated and acquired through the fluorescence signal. The amount of fluorescence was plotted as a histogram within the gate. Mean fluorescence intensity was determined using Kaluza Flow cytometry software analysis (Beckman Coulter).

2.6. Cellular Accumulation of platinum by inductively coupled plasma spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the cellular accumulation of oxaliplatin-derived platinum in transfected cells. The cells were seeded at 250,000 cells per well (350,000 cells per well for HepG2 and PANC-1) in a 6-well plate and transfected and allowed to grow in RPMI complete medium to become around 80% to 90% confluent. The cells were then incubated with oxaliplatin drug at different concentration ranges for 2 hr. The incubation reaction was terminated by washing the cells with ice-cold PBS three times followed by drying the cells for 30 min.

2.6.1. Determination of protein concentration

The air-dried cells were digested with 300 µl of 70% nitric acid at room temperature with constant agitation for 2 hr. During this time, BSA standard solutions were prepared to analyse the protein concentration in samples. The selected concentration ranges were 2800 µg/ml, 1400 µg/ml, 700 µg/ml, 350 µg/ml, 175 µg/ml and 87.5 µg/ml. 300 µl of BSA standards and lysate samples were transferred to a 96-well plate and the protein concentration was determined by measuring the absorbance at 358 nm. The samples in the 96-well plate were used for determination of platinum by ICP-MS.

2.6.2. Platinum accumulation assay

The cell samples were transferred into 5 ml screw-capped polypropylene vials and kept overnight at room temperature for digestion. The cell lysate samples were further digested by heating at 95°C for 30 min. 200 µl of digested samples were then diluted with 1.8 ml of 50 ppb thallium (internal standard) in ICP-MS tubes. The platinum counts of the samples were measured using Varian 820 ICP-MS (Agilent Technologies Inc., Santa Clara, CA, USA). Then the platinum concentration of each sample was calculated from platinum to thallium count ratio using a standard curve method. Platinum counts of samples were measured and normalised for thallium internal standard counts. The standard curve was generated by preparing desired platinum concentrations solutions in the same matrix as used in cellular samples spiked with known platinum stock concentration and were included in the same IC-MS run with the samples.

2.6.3. Method validation for platinum quantification by ICP-MS

The validation of elements was determined according to the US FDA guidelines for bioanalytical method validation.

2.6.3.1 Linearity – Platinum standard curve in the cell lysate

The linearity of the method was determined by preparing different concentrations of the platinum standard from a platinum stock standard solution of 1,000,000 ppb in the cell lysate. The platinum stock solution was further diluted in a cell lysate to produce 200 µl of the desired concentration range. The final concentrations of 200 µl platinum standards were further diluted with 1750 µl of 50 ppb thallium internal standard (IS) and transferred to screw-capped 5ml PP vials for ICP-MS analysis. The serial dilutions of platinum standards are shown in table 2-6:

Table 2-6. Preparation of platinum standard in cell lysate with Thallium Internal Standard

Final Pt concentration for ICPMS analysis (µM)	**The volume of HNO3 and cell lysate (µL)**	**50 µl of [Stock Pt] to add (µM)**	**The volume of thallium spiked MiliQ water to add (µL)**
1	200	40	1750
0.75	200	30	1750
0.5	200	20	1750
0.25	200	10	1750
0.1	200	4	1750
0.05	200	2	1750

The equation of the linearity was calculated using linear regression analysis. Slope, intercept and regression coefficient values were calculated using standard formulas or with the aid of Microsoft EXCEL.

Linear Regression (Coefficient of Determination)

$$r^2 = \left[\frac{\sum_i\{(x_i - x)(y_i - y)\}}{\{[\sum_i(x_i - x)^2][\sum_i(y_i - y)^2]\}^{1/2}}\right]^2$$

x_i = Expected Concentration $\quad\quad$ x = Expected mean value

y_i = Measured and calculated mean value in ratio $(\frac{\text{Pt}}{\text{Thallium}})$

y = MV of y_i

2.6.3.2. Accuracy and precision

The accuracy and precision of the method were established by preparing three replicates of quality control (QC) samples, referred to as lower QC, middle QC and upper QC over the standard curve range.

The actual concentration of each replicate was calculated against the standard curve. The transformation was calculated follows:

$$\text{Pt concentration found (in ppb)} = \frac{\text{Yi}-\text{b}}{\text{a}}$$

Where:

Y_i = The response ratio of platinum to thallium (IS)

b = intercept point of the regression line

a = slope of the line

$$\% \text{ Revcovery (Accuracy)} = \frac{\text{Concentration Pt found}}{\text{Theoretical Pt concentration}} X\ 100\%$$

The precision of the method was measured with respect to the coefficient of variation (CV) of three replicates results of platinum across the concentration range.

$$CV\ \% = \frac{\mathrm{S}}{MV_n} X\ 100$$

$$S = \sqrt{\frac{1}{1-n} X \sum_{i=1}^{n} (x_i - MV_n)^2}$$

Where:

n = No. of values

x_i = Value of single value in series

The ICP-MS analysis run was acknowledged only if the standard curve was linear and the QC samples over the standard curve were within 85% to 115% accuracy values. According to the different ICP-MS runs, the LOD and LLOQ of the method were found to be 0.3 ppb and 1 ppb of platinum respectively.

2.7. Cell viability Assay (MTT assay)

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was the main assay used in this research to measure viable cells. This assay converts a water-soluble MTT tetrazolium salt into an insoluble purple-coloured formazan precipitate with the help of mitochondrial succinate dehydrogenase enzymes in viable cells, to generate reducing equivalents like NADH and NADPH. The formazan crystals were then dissolved in an organic solvent like DMSO and the quantity of formazan (which is directly proportional to the number of viable cells) was measured by recording absorbance or optical density (OD) at 540 nm using a multiwall spectrophotometer. A reference wavelength of 680 nm was used.

The cellular sensitivity of transfected gastrointestinal cell lines to the platinum-derived oxaliplatin cytotoxicity was measured by the MTT assay in 96-well plates. The cells were transfected with siRNA before being seeded in 96-well plates at a density of 5,000-8,000 cells per well and were grown over 24 hr in a normal drug-free medium so that the cells were attached to the well surface. After 24 hr incubation, cells were incubated with oxaliplatin for 2 hr at various concentrations. After drug exposure, the medium containing drug was removed and replaced with drug-free growth medium. The cells were then incubated until 72 hr (to reach the optimal density) from the addition of oxaliplatin. Cell viability was quantified by measuring photometric absorbance at 540 nm in a multiwell plate reader. The experiments were done independently at least three times.

The cell viability percentage was calculated using OD values measured at different concentrations and normalised to the OD values for untreated controlled cells, which are taken as 100%. The cell viability percentage at different concentrations were used to plot the nonlinear response curve (inhibition) and the IC_{50} values were determined using GraphPad Prism 7 software. The IC_{50} values represent the percentage half reduction in the cell viability compared with untreated controlled cells. The IC_{50} values for oxaliplatin in transfected cells were measured to observe the sensitivity of the siRNA transfected cells to oxaliplatin-induced cytotoxicity.

2.8. Apoptosis Assay

Apoptosis is a regulated process of cell death important in the protective mechanism of removing damaged cells as well as in the mechanism of tumour suppression [254]. Cells undergoing apoptosis are characterised by morphological and biochemical changes, including shrinkage of cytoplasm, nuclear condensation and fragmentation, loss of membrane asymmetry and separation of cellular components into apoptotic bodies [255]. Apoptosis is mainly triggered by two main mechanisms: the intrinsic pathway and the extrinsic pathway. In the intrinsic pathway, the death signal is triggered by various intracellular factors, including DNA damage, starvation and oxidative stress, which results in mitochondrial protein leakage, which in turn induces apoptosis through activation of different caspase proenzyme families. The extrinsic apoptotic pathway is mainly triggered by extracellular death factor, which activates cell surface receptors and induces apoptosis by activation of various caspase proenzyme families.

In this study, we used Invitrogen Alexa Fluor® 488 annexin V kit to analyse the apoptosis induced by oxaliplatin in gastrointestinal cell lines. In the apoptotic cells, phosphatidylserine (PS), which was located on the cytoplasmic surface of the cell membrane in normal cells, is translocated to the outer side of the plasma membrane [256]. The annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for PS labelled with a fluorophore in this kit and detects apoptotic cells by binding to PS [257]. This kit contains annexin V conjugated with fluorophores, the Alexa Fluor® 488 dye and red-fluorescent propidium iodide (PI). PI is a nucleic acid binding dye that stains dead cells with red fluorescence. The Alexa Fluor® 488 annexin V kit detects apoptosis rate in cells by flow cytometry utilising Annexin V to detect early apoptosis and propidium iodide to detect late apoptosis. The cell populations, which were treated and non-treated with different oxaliplatin concentrations, were distinguished in different cell lines using flow cytometry with 488 nm of laser for excitation.

2.8.1. Apoptosis assay in the presence of inhibitor

Cells were seeded at a density of 400,000 – 600,000 cells per well in a 6-well plate. After overnight incubation, cells were pretreated with and without 60 μM of myricetin for 30 min. Thereafter, cells were incubated with oxaliplatin for 2 hr. Oxaliplatin of 25 μM, 50 μM and 100 μM were added to cells incubated with myricetin. Therefore, the cell samples were coincubated with both myricetin and oxaliplatin for 2 hr. After 2 hr of coincubation with myricetin and oxaliplatin, the medium was replaced with complete growth medium and cells were allowed to incubate for 48 hr at 37°C in the 5% CO_2 incubator. The cells were harvested after the incubation period and washed with ice-cold PBS. The kit was provided with 5× annexin-binding buffer. 1× annexin-binding buffer was prepared by adding 1 ml of 5× annexin-binding buffer to 4 ml of deionised water. The working solution of PI (100 μg/ml) was prepared by adding 5 μl of the 1 mg/ml PI stock solution to 45 μl of 1× annexin-binding buffer. After washing the cells with ice-cold PBS, the cells were trypsinised and centrifuged at 500 × *g* for 5 min at 4°C. The cells were resuspended in 100 μl of 1× annexin-binding buffer. To analyse the oxaliplatin-induced apoptosis, 4 μl of Alexa Fluor 488 annexin V and 1 μl of 100 μg/ml PI working solution were added to each 100 μl of cell suspension. The cells were incubated at room temperature for 15 min. To terminate the reaction, after incubation the cells were transferred on ice and 400 μl of 1× annexin-binding buffer were added and samples were mixed gently and kept on ice until analysed by flow cytometry. The stained cells were analysed by flow cytometry by measuring the fluorescence emission at 530 nm and 575 nm using 488 nm excitation.

2.8.2. Apoptosis cells in transfected cells

Cells were seeded at a density of 250,000 – 350,000 cells per well and transfected with ABCC2-siRNAs and control-siRNA at 20 nM for 48 hr. After transfection, cells were incubated with different concentrations of oxaliplatin for 2 hr at 37°C in a 5% CO_2 incubator. After incubation, cells were washed twice with ice-cold PBS. The cells were then trypsinised and centrifuged at 500 × *g* for 5 min at 4°C. The sample preparation is similar to the methods mentioned in section 2.8.1.

2.8.3. Annexin V apoptosis detection assay

The staining with both Annexin V and PI is used for identification of early and late apoptosis. Viable cells with intact membranes exclude PI and Annexin V staining. Therefore, viable cells are considered as both Annexin V and PI negative (Annexin V^-/PI^-). Cells that are in early apoptosis are Annexin V positive and PI negative (Annexin V^+/PI^-), cells that are in late apoptosis or death are both Annexin V and PI positive (Annexin V^+/PI^+), and cells that are dead or damaged are permeable to PI considered as PI positive (Annexin V^-/PI^+).

The performance quality control (QC) samples were prepared by incubating cells with only Annexin V, only PI and without any staining. The Annexin V FITC assay was set up in MoFlo Beckman Coulter summit and Kaluza software manually. Then the QC samples and samples were run in flow cytometry with acquisition criteria of 12,000 events for each tube. The data were analysed in Kaluza software. The Annexin V FITC assay includes the following plots and gates for untreated and treated samples:

I. FSC-A vs SSC-A with gate for cells
II. Annexin V FITC vs PI with gates for the following populations:
    a. Annexin V^-/PI^-
    b. Annexin V^+/PI^-
    c. Annexin V^+/PI^+
    d. Annexin V^-/PI^+

The summary of assay results with statistics for samples were calculated in Kaluza software.

2.9. ATPase Assay

MRP2 transporter mediates the transport of substrate against a concentration gradient with the help of energy derived from ATP-hydrolysis, which is coupled to substrate translocation. Therefore, ATPase assays for ABC transporters help to determine the transport activity of MRP2 and can be used to monitor the effects of oxaliplatin on MRP2 transporter activity. The details of the effects of platinum-based drug oxaliplatin on MRP2 transporter activity are mentioned in a later chapter 3.

2.10. Cell Surface Staining

The MRP2 surface expression studies were conducted in GI cancer cell lines and their respective knockdown cells using immunofluorescence staining followed by flow cytometry. The anti-MRP2 antibody has been used for specific detection of surface MRP2 in the cells, which is targeted to an internal epitope of the MRP2 protein. Direct immune staining was carried out using anti-mouse IgG H&L (Alexa Fluor 488) as a secondary antibody. The secondary antibody is a fluorophore, which is directly attached to an anti-MRP2 primary antibody and generates a fluorescence signal. The non-specific binding of primary and secondary antibodies can lead to false positives. To avoid this, we used host-matched IgG2a isotype control in cell surface staining experiment. The isotype control antibody targeted to an irrelevant antigen, but of the same isotype as the MRP2 primary antibody. The main roles of the isotype control antibody are to quantify the background signal caused by non-specific antibody interaction with cellular proteins and determine the non-specific binding of the secondary antibody. The MRP2 expression fluorescence in GI cell lines was measured by subtracting the signal from isotype control with the MRP2 fluorescence signal. The surface staining was quantified using flow cytometry as it is more quantitative and sensitive in detecting subtle changes.

2.10.1. MRP2 surface expression in GI cell lines

The GI cells (HepG2, PANC-1 and Caco-2) were grown in T_{75} flasks until it was 90% confluent, trypsinised, counted and resuspended in RPMI medium, according to the procedure mentioned in section 2.2.3 and 2.2.4. The cell density of 1 x 10^6 cells per ml were aliquoted into a 2 ml Eppendorf tube and kept on ice. Most of the experimental procedure was conducted on ice, unless otherwise stated. The cell samples were centrifuged at 500 × *g* for 5 min at 4°C and cell pellets were washed with 1 ml of PBS

and spun down again at 500 × *g* for 5 min at 4°C. The cell pellets were then resuspended in freshly prepared 1% paraformaldehyde in PBS and incubated for 15 min on ice. After fixing the cells with paraformaldehyde, the cells were washed twice with PBS 0.2% Tween-20 as washing buffer and centrifuged at 500 × *g* for 5 min. The cells were then permeabilised with a permeabilising agent by adding 100 µl of 0.2% saponin and incubating for 30 min at room temperature. After incubation, the cells were washed twice with washing buffer and centrifuged at 500 × *g* for 5 min. The cells were blocked with 100 µl of 5% BSA in PBS for 15 min at room temperature to block non-specific binding sites. The cells were washed twice with washing buffer and centrifuged at 500 × *g* for 5 min. The cell pellets were then resuspended in 2.5 µg/ml of the primary anti-MRP2 antibody in 2% BSA in PBS and 2.5 µg/ml of IgG2a isotype control in 2% BSA in PBS. The samples were incubated with primary and isotype control antibody for 60 min at room temperature in the dark. The cells were washed three times with washing buffer after incubation by centrifugation at 500 × *g* for 5 min. Meanwhile, the Alexa Fluor 488-labeled secondary antibody was diluted in 1:1000 ratio in 2% BSA in PBS and the cells were resuspended in this solution. The cells were again incubated for 60 min at room temperature in the dark. After incubation, the cells were washed three times with washing buffer by centrifugation at 500 × *g* for 5 min. The cell pellets were resuspended in 200 µl of ice-cold 1% paraformaldehyde in PBS and stored on ice in the dark until analysis. For the best results, the cell samples were analysed on flow cytometry as soon as possible.

2.10.2. Flow cytometry data analysis

The MRP2 cell surface staining was determined using flow cytometer and data was analysed with Kaluza software. The cells were gated and forward and side scatter parameters were used to exclude cell doublets, cellular debris, dead and non-viable cells. Mean fluorescence intensity was measured with the blue laser channel using 488 nm excitation and 519 nm emission. The flow cytometry histogram was produced using Kaluza software and mean fluorescence intensity was calculated based on isotype control. The mean fluorescence intensity (MFI) was calculated as a % of control-siRNA and based on the below equation:

$$\left[\frac{\text{MFI (Test sample)} - \text{MFI (Isotype control)}}{\text{MFI (controi} - \text{siRNAl)} - \text{MFI (Isotype control)}}\right] \times 100$$
$$= \text{MFI as \% of control} - \text{siRNA}$$

MFI = Mean fluorescence intensity

2.11. Flow cytometry analysis

For all the flow cytometry experiments, samples were analysed using Beckman Coulter Kaluza software. The flow cytometry experiments were analysed using gating strategies to exclude cell doublets, cellular debris and non-viable cells. The histograms were designed according to the respective experiments and based on forward and side scatter parameters. Forward scatter measures cell size and side scatter measures cell intracellular complexity or shape and increases with greater particle density within cells. The total cell population was plotted using forward and side scatters parameters. The non-viable or dead cells appear on the left or upper-left side of the cell population in the forward vs side scatter histogram (FSC vs SSC) due to their lower forward scatter and high side scatter. These populations were excluded from subsequent analysis by forming a gate “A” around the viable cells only.

To measure the fluorescence intensity from single cells only, doublets were gated out using the doublet exclusion method [258]. For this method, we plotted a histogram of forward scatter area vs height signal (FSC-A vs FSC-H) and side scatter area vs height signal (SSC-A vs SSC-H). The area of the signal for the forward or side scatters increased proportionally to the height of the signal in single cells. From the viable

population “A”, a SSC-A vs SSC-H plot was created and a gate “B” was designed in the cell population. From the population B, the same process is repeated using FSC-A vs FSC-H plot to create a “C” gate. Thus, the population under gate C was considered as a single cell and for all the samples, mean fluorescence was measured for the cell population under gate C.

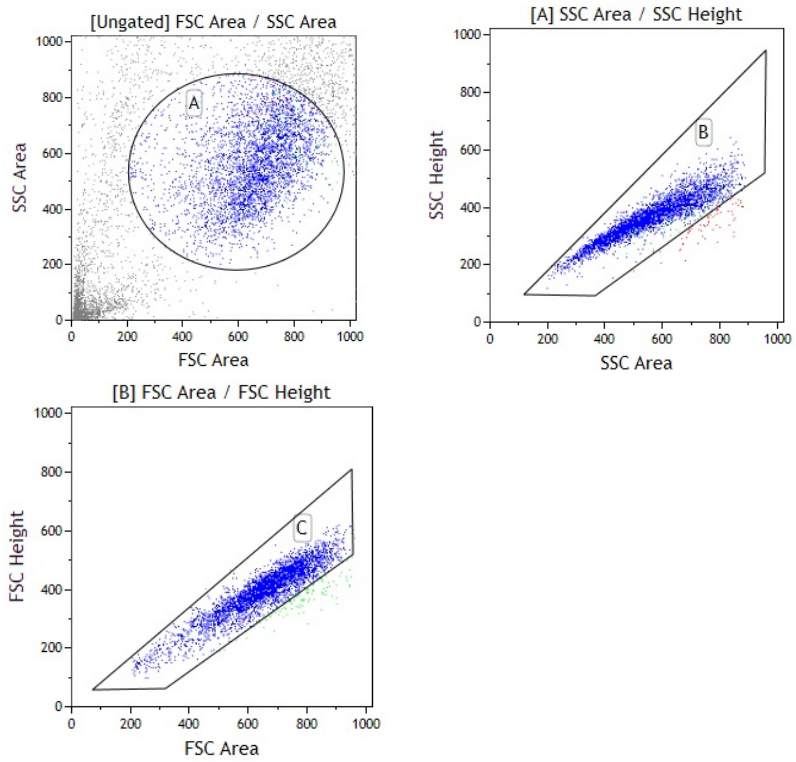


Figure 2.2. Graphical representation of flow cytometry data and use of gating to restrict the analysis of single cell population.

2.12. Statistical analysis

All the data were analysed using Prism 7 software (GraphPad, San Diego, CA, USA) and were presented as a mean value with a standard error of the mean (mean ± S.E.M). All the results were from two to three independent experiments unless otherwise stated in each experiment. To determine whether the observed values were statistically significant or not, analysis of variance (ANOVA) with post-hoc tests were applied. A p-value <0.05 was considered to be statistically significant.

In transfected cells, the statistical analysis was done by one-way ANOVA. For IC_{50} calculation, normalised mean absorbance values were obtained from three independent experiments and non-linear regression analyses were applied to determined IC_{50} values of oxaliplatin using Prism software.

For the Apoptosis assay and cell surface staining, the samples were analysed using Kaluza software. Thereafter, multiple comparisons between control and different treatment groups were carried out using one-way ANOVA with Dunnett's post-hoc test.

Chapter 3 Oxaliplatin and MRP2 ATPase interaction

3.1. Introduction

Development of intrinsic or acquired drug resistance to different chemotherapeutic drugs drastically degrades the outcomes of chemotherapy of cancer [180]. One of the major mechanisms of drug resistance is overexpression of ATP-binding cassette (ABC) transporters, which extrude drugs and xenobiotics out of the tumour cells [132, 139, 178]. The ABC transporters implicated in drug resistance include P-glycoprotein (Pgp), multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP) [179]. The Pgp was the first discovered and is the most widely studied ABC transporter protein in clinical multidrug resistance [138]. The other most commonly observed ABC transporter that participates in multidrug resistance is the multidrug resistance protein 1 (MRP1 or ABCC1) and breast cancer resistance protein (BCRP or ABCG2) [138]. Moreover, there are other ABC transporter proteins, including MRP2-5, which are capable of mediating the transmembrane movement of a large variety of substrates.

MRP2, a member of the MRP subfamily, plays a crucial role in organic anion transport in various tissues. MRP2 is highly expressed in the apical membrane of polarised cells, such as hepatocytes, the proximal renal tubule, and intestinal epithelia, where it uses ATP-hydrolysis energy to mediate unidirectional efflux of substrates [186]. In comparison to other MRP/ABCC transporters, only MRP2 localises exclusively to the apical membrane of polarised cells [259]. In cancer cells, MRP2 has been reported to be expressed in hepatocellular, colorectal, gastric, ovarian, lung and breast carcinomas [44, 139, 196, 210]. Like other ABC transporters, MRP2 uses ATP as a source of energy to transport substrate across cell membranes. The primary mechanism of MDR is that the ABC transporters actively extrude anticancer drugs from the cells. Therefore, the drug level inside the cancer cells is lower than the cell killing threshold. MRP2 mediates transport of various anticancer drugs with high affinity, including cisplatin, doxorubicin, methotrexate, mitoxantrone and irinotecan, as well as organic anions such as glucuronate, GSH-conjugated substrate (e.g. LTC4), reduced and oxidised GSH, and bromosulfophthalein [169, 190, 191, 206, 260]. One study has shown that GSH transport by MRP2 is stimulated at low concentrations of sulfinpyrazone while at high concentrations, it prevents the cotransport of GSH yet allows sulfinpyrazone to be transported alone. This study suggests that MRP2 has at least two substrate-binding

sites [260]. As mentioned earlier, MRP2 confers resistance to chemotherapeutic drugs, such as cisplatin, methotrexate, mitoxantrone and irinotecan. However, synergistic drug interactions due to MRP2 modulation is difficult to elucidate in cell-based models for several reasons, including drug metabolism, lack of potent inhibitors and involvement of other transporters (e.g. OATPs, Pgp, BCRP and other MRPs).

Recently, it has been observed that MRP2 transports oxaliplatin-derived platinum in an ATP-dependant manner in MRP2-overexpressing membrane vesicle [248]. Despite various functional implications, the ATP-binding and ATP-hydrolytic properties of MRP2 have not been well understood. Therefore, to mechanistically characterise the effects of oxaliplatin on MRP2 ATPase activities, this chapter describes the application of MRP2 ATPase assay for further investigation of oxaliplatin-MRP2 interactions using cell-free membrane preparations.

Previously, membrane-based methods were used to study the transport process before the discovery of ABC transporters. These methods are currently widely used in pharmaceutical research to determine the interaction of drugs and ABC transporters. Most commonly used membrane-based assays are the ATPase, the vesicular transport and the nucleotide-trapping assay [138].

The ATPase assay as a functional study is based on the principle that most substrates and inhibitors of ABC transporters modulate ATPase activity of the transporters. ABC transporters transport substrates across the cell membrane against the concentration gradient using ATP hydrolysis as a source of energy. Compounds interacting with the ABC transporter can induce or suppress its ATPase activity, which is reflected by the amount of inorganic phosphate (Pi) generated by ATP hydrolysis over a designated period in the assay. A colourimetric reaction can be used to detect the Pi that is yielded from ATP hydrolysis. The rate of Pi liberated can be used as a surrogate for the activity of the transporter ATPase [261]. In the ATPase assay, substrates and inhibitors of ABC transporters display distinct concentration-response curve patterns [138]. In general, transporter substrates exhibit either a “bell-shaped” curve or “sigmoidal” curve based on the binding sites present in transporters. The prevalent explanation of the different pattern of curves is the presence of two binding sites in transporters. In this phenomenon of the two-binding-site model, the transporter accommodates a high-affinity substrate transport site and a second low-affinity binding site, which may inhibit

transporter activity when it is saturated by substrates present at a high concentration [138]. The difference between the relative affinities to the two binding sites are substrate-specific, which determines the distinctive pattern of interaction for each substrate.

A bell-shaped curve is usually observed when a substrate induces ATPase activity at lower concentrations but inhibits ATPase activity at higher concentrations [138], and the relative affinities of the substrate for both binding sites are similar. For the sigmoid curve, ATPase activity increases with increasing concentration until it reaches a plateau because the relative affinity of the substrate for the second inhibitory site is lower than the transport site by at least several orders of magnitude [262].

Membrane-based ATPase assay containing ABC transporters show a basal ATPase activity. Substrates increase the baseline ATPase activity while inhibitors or slowly transported compounds inhibit the baseline ATPase activity. Therefore, the ATPase assays can determine whether a compound acts as a transporter substrate or an inhibitor. ABC transporters are effectively inhibited by sodium orthovanadate (Na_3VO_4), so the activity of the transporter is measured as the vanadate-sensitive of the total ATPase activity. The ATPase assay is composed of three different modes: (i) basal mode (test compound alone), (ii) inhibition mode (test compound + activator), and (iii) activation mode (test compound + inhibitor). In the basal mode, a membrane expressing MRP2 transporter is incubated with test drugs or compounds and the effect of the compound on basal vanadate-sensitive ATPase activity is measured. Stimulation of ATPase detected in the activation assay indicates that the test drug/compound is the transported substrate of the transporter. The inhibitor or slowly transported compounds do not stimulate the ATPase activity; their interactions with a transporter are detected using an inhibition assay as mentioned previously. The inhibition mode is carried out in the presence of an activator, and the inhibitor or slowly transported compounds are tested for their ability to reduce the stimulatory effect of drugs on the transporter. The activation mode is performed in the presence of inhibitor, which suppress the high baseline ATPase activity of membrane transporters. Thus, activation mode helps in identification of substrate functionality.

The major problem of using the ATPase assay for substrate identification is the possibility of false negative results. Some substrates are transported at a lower rate and

do not generate a noticeable amount of Pi, thus escaping detection by the assay. This drawback can be overcome by performing the inhibition mode of the assay. For the inhibition mode, the compound is coadministered with a known activating substrate and the modulatory effect of the test compound on the ATPase activity is triggered by the activating substrate. Then the slowly transported substrate will suppress the triggered ATPase activity by competing with the high yield substrate. Although the inhibition assay mode is useful for identification of slowly carried substrate, it will not distinguish between a slowly transported substrate and an inhibitor, which can also cause concentration-dependent suppression of ATPase activity triggered by the activating substrate [138].

The ATPase assay is commonly performed in membrane vesicles prepared from Sf9 insect cells transfected with a human or rat transporter. In this chapter, human MRP2 overexpressing Sf9 membrane stock was used in two different modes: (a) test drug only (basal mode) and (b) test drug and inhibitor (activation mode). The inhibitors have been demonstrated to lower the background activity of transporter membranes and improve the functionality of the membrane [263]. In this study, we used MRP2 inhibitors, benzbromarone and myricetin. Benzbromarone has been provided by the Solvo biotechnology ATPase assay kit as a validated Sf9/MRP2 ATPase reaction inhibitor. According to Solvo biotechnology (manufacturer's protocol), a known MRP2 substrate, sulfasalazine, stimulates the baseline vanadate-sensitive activity of MRP2 in the Sf9/MRP2 membrane preparations. Therefore, sulfasalazine is used as the activator of MRP2 ATPase activity. The basal mode was performed in the presence of oxaliplatin without coadministration of an activator to determine if the oxaliplatin results in stimulation effect on the baseline vanadate-sensitive ATPase activity of MRP2. This result would indicate whether the oxaliplatin is a substrate or inhibitor, or neither of them. The inhibition mode was usually performed in the presence of sulfasalazine, which is an MRP2 substrate known to stimulate ATPase activity in the Sf9/MRP2 membrane. The activation mode confirms the inhibitory activity of an inhibitor as well as detects the competitive inhibition caused by a slowly transported substrate. The activation mode was performed in the presence oxaliplatin coincubated with two inhibitors of MRP2, myricetin and benzbromarone, to determine if inhibitors suppress the basaline ATPase activity of Sf9/MRP2 membranes incubated with oxaliplatin. The quantification of ATPase activity was done by the amount of Pi generated in the presence of MRP2 membrane over the experimental period. The presence of Pi was

detected using a colourimetric detection method, and further, gave an absorption band in the range of 590 and 630 nm. In the ATPase assay, the data were represented as vanadate-sensitive ATPase activity, which was derived from the difference between the ATPase activities detected in the presence and absence of vanadate. During ATP hydrolysis in the assay, vanadate formed an inhibitory inorganic vanadate complex, i.e., transporter-MgADP-Vi where Vi is inorganic vanadate. This vanadate complex is stable and prevents any further hydrolysis activity by the transporter ATPase [264, 265].

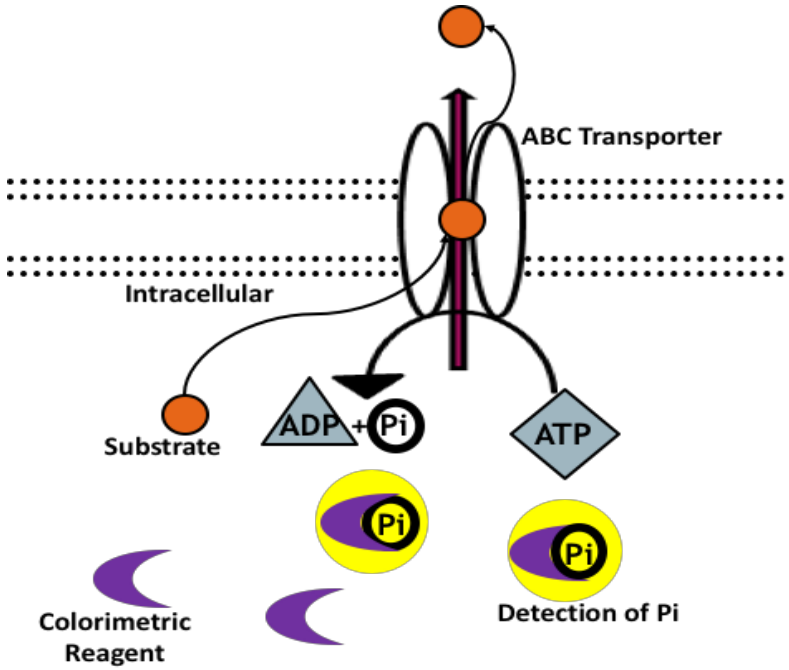


Figure 3.1. A schematic representation of the working principle of the ATPase assay.

In the ATPase assay, the transport process of ABC transporter requires ATP and quantification of the transport process is measured by the amount of inorganic phosphate (Pi) generated in the presence of the test compounds. Therefore, the presence of substrate stimulates the activity of ATP with the increased rate of ATP hydrolysation leading to increased Pi generation. Moreover, the presence of an inhibitor suppresses the stimulatory effect because of the decreased rate of ATP hydrolysation, leading to a low generation of Pi.

The main aim of this chapter is to determine the time and concentration-dependant effects of oxaliplatin on MRP2 ATPase activity by performing basal mode and activation mode studies in MRP2 overexpressing Sf9 membrane vesicles. This chapter confirms whether oxaliplatin is the substrate or inhibitor of the MRP2 transporter by using the ATPase assay. Since four control sets were used to determine any Pi contamination, non-enzymatic ATP hydrolysis and to characterise the intrinsic vanadate-insensitive ATPase activity, the experiments also provide a "clean" cell-free platform to further elucidate the interaction mechanisms of ATP, oxaliplatin and MRP2 transporters.

3.2. Materials and methods

3.2.1. Materials and equipment

The chemicals and materials used for this study are listed in table 3-1 with details of the manufacturer. Both control membrane vesicles and Sf9/MRP2 membrane vesicles which have been used in this study were ordered commercially. According to manufacturer Sf9/MRP2 membrane vesicles were prepared from purified plasma membranes isolated from an insect cell system (Sf9 cells infected with baculovirus) expressing human MRP2. The control membrane vesicles is used as a mock transfected membrane vesicles prepared from purified plasma membranes isolated from Sf9 insect cell.

Table 3-1. List of materials used for ATPase assay

Materials	Manufacturer
Circulating water Bath (temperature maintained at 37°C)	Digisystem Laboratory Instrument Inc.
Multichannel-pipette with corresponding tips	Eppendorf Research
96-well microtiter plates	Corning COSTAR 3585
Microplate reader	SPARK Tecan
2 ml, 15 ml tubes	Corning (Sigma-Aldrich)
Dimethyl sulfoxide (DMSO, A.C.S reagent spectrophotometric grade, >=99.9%)	Sigma-Aldrich (St Louis, MO, USA)
SB-MRP2-PREDEASY-ATPase Assay kit	SOLVO biotechnology (Sigma-Aldrich)
ABC Transporter Control membrane vesicles	GenoMembrane (Life Technologies)

3.2.2. Chemicals in the ATPase kit

The list of chemicals provided with the ATPase kit and their assay conditions are listed in table 3-2.

Table 3-2. List of chemicals provided with PREDEASY ATPase kit

Vial	Substance	Chemicals amount	Storage	Assay conditions
A	Membrane stock (5 mg/ml)	5 X 500 µl	-80°C	on ice
B	10x Medium	10 ml	-80°C	on ice
C	Phosphate solution (0.4 mM)	1000 µl	≤-20°C	on ice
D	Phosphate solution (0.8 mM)	1000 µl	≤-20°C	on ice
E	Na-Orthovanadate solution (600 mM)	500 µl	≤-20°C	on ice
F	Sulfasalazine solution (50 mM in DMSO)	500 µl	≤-20°C	RT
G	3x Developer	3 X 15 ml	≤-20°C	RT
H	10x Blocker	15 ml	≤-20°C	RT
I	Inhibitor solution (Benzbromarone, 5 mM)	500 µl	-80°C	RT
J	MgATP solution (0.2 M)	1000 µl	≤-20°C	on ice
K	Glutathione (300 mM)	1000 µl	-80°C	on ice

3.2.3. Details of controls

The controls used for the measurement of different components of signals in this experiment are suggested below.

Control 1 (Baseline ATPase activity): This control represents the basic level of Pi liberated in the presence of MRP2 membrane transporter and solvent for the drugs. The Pi released for control 1 wells is the result of Pi contamination, non-transporter related ATP cleavage and the baseline ATPase activity of the MRP2 transporter.

Control 2 (Na_3VO_4 insensitive ATPase activity): This control represents the Pi level resulting from Pi contamination and non-transporter related ATP cleavage.

Control 3 (ATPase activity of fully activated membranes): This is the positive control for the activation studies. The Pi liberated in the control 3 wells is the result of PI contamination, non-transporter related ATP cleavage and the maximal MRP2 transporter ATPase activity.

Control 4 (Na_3VO_4 insensitive ATPase activity of fully activated membranes): This control is used to obtain the full vanadate-sensitive ATPase activity by subtracting from control 3.

Control 5 (Inhibited ATPase activity of fully activated membranes): This is the positive control for the inhibition studies. This control represents if the known inhibitor of MRP2 transporters inhibits the ATPase activity of fully activated membranes. The inhibited vanadate-sensitive ATPase activity was obtained by subtracting control 4.

3.2.4. ATPase assay

3.2.4.1. Basal study

The solutions required for this assay were always prepared freshly by diluting the stock solution with phosphate-free MilliQ water. The oxaliplatin solutions were prepared at eight different concentrations with 2-fold serial dilution starting from 100 µM. The blocker and developer solution were prepared in 1× concentration and were kept at room temperature. The assay mix was prepared by mixing 10× medium with MilliQ water and glutathione (300 mM). The solution was then mixed properly and kept on ice for further use. The MgATP solution was prepared by diluting 50 µl of 0.2 M MgATP with 950 µl of assay mix. The membrane stock was then thawed in a 25°C water bath and appropriately homogenised by gentle pipetting. The membrane suspension was prepared by mixing membrane stock with assay mix gently by pipetting and kept on ice. The membrane suspension was divided into parts for the basal study as shown in Figure 3.2 (A).

For the basal study, a basic membrane suspension was prepared by adding 13 µl of MilliQ water in 5200 µl of membrane suspension solution. For the preparation of controls 1 and 2, 2400 µl of basic membrane suspension was mixed with 6 µl of MiliQ water and 6 µl 600 mM Na-orthovanadate solution, respectively.

For the inhibition study, activated membrane suspension was prepared by mixing 800 µl membrane suspension with 2 µl of 50 mM sulfasalazine. The control 3 solution was prepared by mixing 400 µl activated membrane suspension with 1 µl of MilliQ water. Then, control 4 was prepared by mixing 200 µl of activated membrane suspension with 0.5 µl 600 mM Na-orthovanadate solution.

The 96-well plate was placed on ice, and 40 µl of assay mix was added to the phosphate calibration well. Then, 40 µl of appropriate membrane suspensions was added to each well according to the plate setup as illustrated in figure 6, so that each well would contain 8 µg membrane protein. After that, 10 µl of appropriate phosphate solutions was added for the phosphate calibration, and 1 µl of Milli-Q water was added to the control wells (control 1,2,3,4). In the control 5 well, 1 µl inhibitor (5 mM Benzbromarone) was added. A series of oxaliplatin stock solutions were then added to the remaining wells to generate final concentrations ranging from 0.78 to 100 µM. Before the ATPase reaction, both 96-well plate and MgATP solution were prewarmed for 15 min at 37°C. The ATPase reaction was started by adding 10 µl of prewarmed MgATP solution to each reaction well except the phosphate calibration wells. The plate was then incubated at 37°C for another 10, 20 or 30 min. The ATPase reaction was terminated by adding 100 µl of developer solution. After incubation at room temperature for 2 min, 100 µl of blocker solution was added to each well. The plate was then incubated at 37°C for 30 min and then the absorbance was measured at a wavelength of 620 nm.

3.2.4.2. Activation study

The solutions required for the activation study is prepared according to the details mentioned in above section 3.2.4.1. For activation study, 100 µM and 25 µM of oxaliplatin were prepared. MRP2 inhibitors, myricetin and benzbromarone were used for activation assay. The plate layout figure 3.2 (B) were used for activation study. The control solutions were prepared and added in 96-well plate according to the methods mentioned in basal study. After adding the assay mix in each well, the series of different inhibitor solutions were added to the wells accordingly to generate final concentration of 60 µM and 40 µM of myricetin and 100 µM of benzbromarone. Thereafter, oxaliplatin solutions were added in wells to generate final concentration of 100 µM and 25 µM. The plate was mixed well and prewarmed for 15 min at 37°C. The ATPase reaction was started by adding 10 µl of prewarmed MgATP solution to each reaction well except the phosphate calibration wells and incubated for 20 min at 37°C. The ATPase reaction was terminated by adding 100 µl of developer solution. After incubation at room temperature for 2 min, 100 µl of blocker solution was added to each well. The plate was then incubated at 37°C for 30 min and then the absorbance was measured at a wavelength of 620 nm.

A.

	Controls				Oxaliplatin (µM)				Oxaliplatin (µM) + vanadate			
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank				100	100	100	100	100	100	100	100
B	4 nmol Pi				50	50	50	50	50	50	50	50
C	8 nmol Pi				25	25	25	25	25	25	25	25
D	Control 1				12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50
E	Control 2				6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25
F	Control 3				3.12	3.12	3.12	3.12	3.12	3.12	3.12	3.12
G	Control 4				1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56
H	Control 5				0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

B.

	Controls				Oxaliplatin (µM)				Oxaliplatin (µM) + vanadate				
	1	2	3	4	5	6	7	8	9	10	11	12	
A	Blank				100	100	100	100	100	100	100	100	No Inhibitors
B	4 nmol Pi				25	25	25	25	25	25	25	25	
C	8 nmol Pi				100	100	100	100	100	100	100	100	40 µM Myricetin
D	Control 1				25	25	25	25	25	25	25	25	
E	Control 2				100	100	100	100	100	100	100	100	60 µM Myricetin
F	Control 3				25	25	25	25	25	25	25	25	
G	Control 4				100	100	100	100	100	100	100	100	100 µM Benzbromarone
H	Control 5				25	25	25	25	25	25	25	25	

Legend

Blank	Assay mix + 10 µl MilliQ water
4 nmol Pi	Assay mix + 10 µl 0.4 mM Pi
8 nmol Pi	Assay mix + 10 µl 0.8 mM Pi
Control 1	Basic membrane suspension + 1 µl MilliQ water
Control 2	Basic membrane suspension + vanadate (1.2 mM) + 1 µl MilliQ water
Control 3	Activated membrane suspension + 1 µl MilliQ water
Control 4	Activated membrane suspension + vanadate (1.2 mM) + 1 µl MilliQ water
Control 5	Activated membrane suspension + 1 µl inhibitor
	Basic membrane suspension + 1 µl test drug
	Basic membrane suspension + vanadate (1.2 mM) + 1 µl test drug

Figure 3.2. Assay plate map for basal and activation study.

3.3. Results

The ATPase assay was performed by incubating Sf9/MRP2 membrane stock at 37°C for 10, 20 and 30 min in the presence of MgATP (10 mM), with and without sodium orthovanadate (Na_3VO_4). The concentration of 1.5 mM Na_3VO_4 is used throughout the experiment, as Na_3VO_4 at this concentration has been reported to inhibit the MRP2 ATPase activities completely.

A standard calibration curve for Pi concentration was generated in each experiment and a typical example is shown in figure 3.3. The data of ATPase assay are presented as the relative amount of Pi generated or relative vanadate-sensitive ATPase activity per mg protein per min. The experiments at a different time point were compared by normalising data to baseline values derived from the basal mode and represented the amount of Pi generated. The vanadate-sensitive ATPase activity is the primary parameter used for detection of substrate-transporter interactions. However, the amount of Pi generated at 20 min is slightly more than 10 min of reaction time.

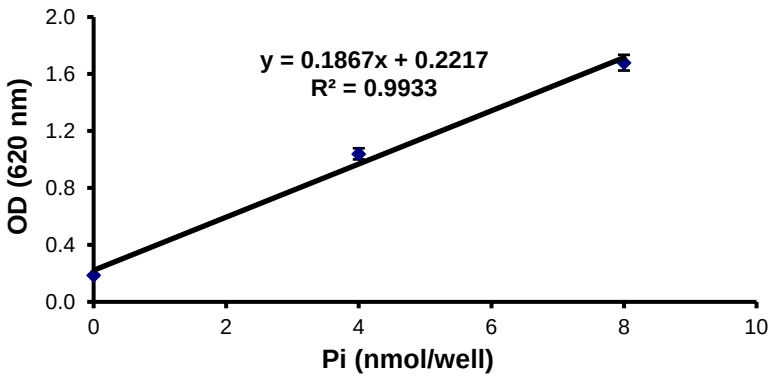


Figure 3.3. A typical standard curve for the Pi calibration.

In the ATPase assay, phosphate standards were prepared by diluting 10 µl of 0.4 mM and 0.8 mM monopotassium phosphate solution in the assay buffer in the 96-well microplate in a volume of 40 µl. The final amounts of inorganic phosphate (Pi) were in the range of 0 to 80 nmol. Data are expressed as means ± SEM (n=4). Linear curve-fitting and corresponding equations are shown.

3.3.1. Basal mode study

As shown in figure 3.4, the amount of Pi generated increased linearly with the increase in incubation time (10, 20 and 30 min). We observed that Pi is generated in the presence of Na_3VO_4. Hence, we can illustrate that MRP2 membrane contains a comparable level of vanadate-insensitive ATPase activity. Usually, a membrane preparation of ABC transporters includes a certain level of vanadate-insensitive ATPase activity [261]. The rate of Pi generated is higher in the absence of Na_3VO_4. Figure 3.4 depicted the vanadate-sensitive ATPase activities of the MRP2 membrane at different time points. The vanadate-sensitive ATPase activity was calculated by dividing the difference between the generated Pi in the absence and presence of Na_3VO_4 by the amount of membrane used. The baseline vanadate-sensitive ATPase activities appear to be linear within the 30 min time point.

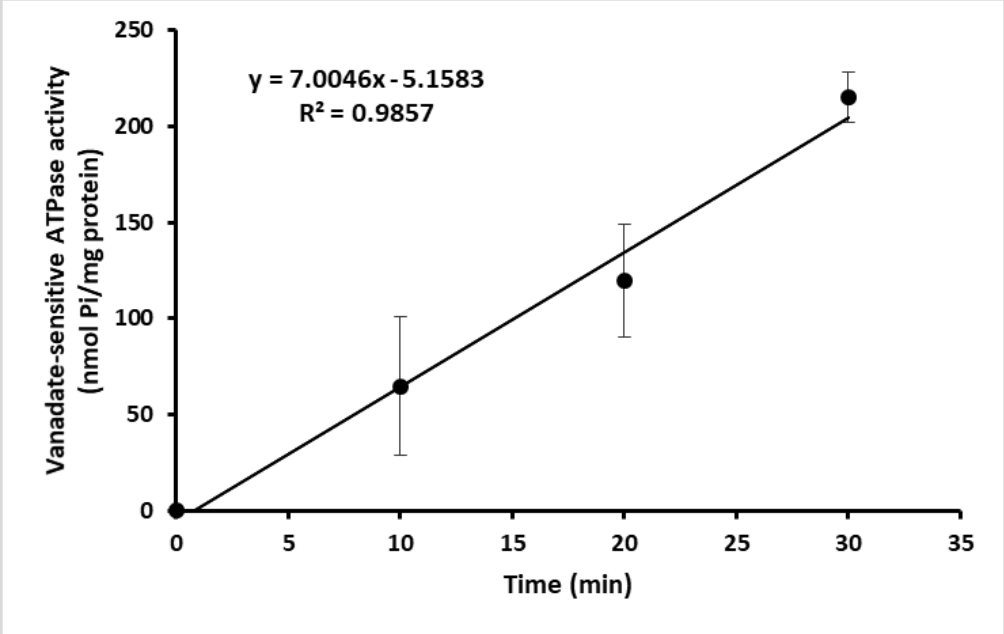


Figure 3.4. Vanadate-sensitive ATPase activity in basal mode at a reaction time of 10, 20 and 30 min.

Sf9/MRP2 membrane vesicles (8 µg/well) were incubated in the ATPase assay reaction buffer at 37°C for 10, 20 and 30 min. Membrane vesicles at the specified time points were quantified by colourimetric detection. The amount of Pi generated by MRP2 ATPase increased with an increase in reaction time. The data shows the amount of Pi generated in the presence or absence of 1.5 mM Na_3VO_4 in Sf9/MRP2. All data are represented as mean ± SEM, n=4.

3.3.2. Inhibition mode study

The effects of sulfasalazine on the ATPase activity in Sf9/MRP2 membrane protein were also tested in the assay. Sulfasalazine is known to stimulate the vanadate-sensitive ATPase activity in the MRP2 membrane vesicles and is used as an activator for the MRP2 ATPase assay. If the test compound is an inhibitor or slowly transported compound, the sulfasalazine as an activator would result in a shift in the IC_{50} (half-maximal inhibitory concentration) values of the test compound, and the test compound inhibits the sulfasalazine-induced activity by competitive binding to the transporter [266]. However, for some ABC transporters like BCRP, the ATPase assay performed for BCRP with activator concentration at the minimal activation unlike MRP2. The reason for using minimum concentration is because membrane expression BCRP generally exhibits a high baseline vanadate-sensitive ATPase activity suppressible by test compounds, unlike MRP2 [266]. Therefore, for inhibitors, the IC_{50} values for MRP2 ATPase are mostly calculated from an activated membrane with maximum concentration of sulfasalazine in inhibition mode.

Sulfasalazine at 125 μM was used as the activator for the ATPase assay performed in the control samples, in which the Sf9/MRP2 membranes were preactivated with sulfasalazine at 10, 20 and 30 min. The activated vanadate-sensitive ATPase reaction appeared to be increased with increase in reaction time.

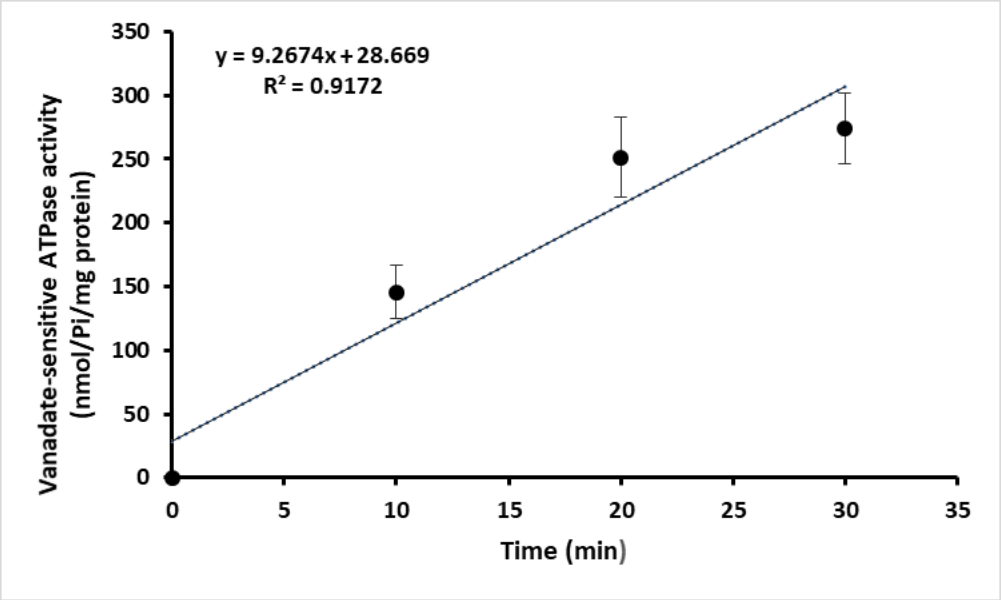


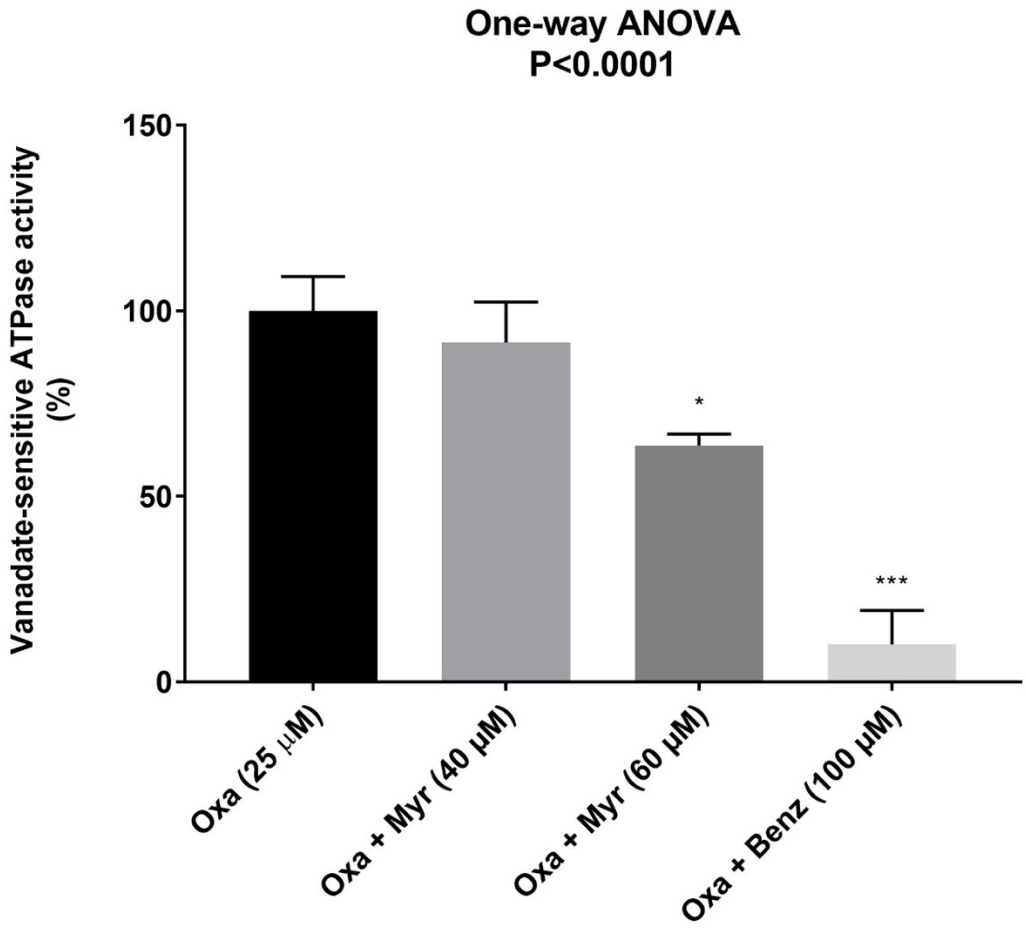
Figure 3.5. Vanadate-sensitive ATPase activity in inhibition mode at a reaction times of 10 min, 20 min and 30 min.

Sf9/MRP2 membrane vesicles (8 µg/well) were incubated in the ATPase assay reaction buffer containing 125 µM of sulfasalazine at 37°C for various time points. Membrane vesicles at the specified time points were quantified by colourimetric detection. The amount of ATPase activity increased with an increase in reaction time. Above data represent the amount of Pi generated in the presence or absence of 1.5 mM Na3VO4 in Sf9/MRP2. All data are represented as mean ± SEM, n=4.

3.3.3. Activation mode study

Benzbromarone is known to inhibit the vanadate-sensitive ATPase activity in the MRP2 membrane vesicle and therefore, is used as a suppressor of MRP2 ATPase activity. To study whether MRP2 inhibitors inhibit the oxaliplatin in vanadate-sensitive MRP2 ATPase activity, we have used myricetin and benzbromarone in ATPase reaction. In the presence of 25 µM of oxaliplatin, the vanadate sensitive ATPase activity significantly reduced by 40% and 90% after incubation with 60 µM myricetin and 100 µM benbromarone. As compare with the basal vanadate sensitive ATPase activity with 25 µM of oxaliplatin, the vanadate-sensitive ATPase activity in the presence of 60 µM myricetin and 100 µM benzbromarone was 63.7% ± 3.1% and 10.1% ± 9%. In the presence of 100 µM of oxaliplatin, the vanadate-sensitive ATPase activity reduced by almost 30% with 60 µM myricetin and significantly reduced by 90% with 100 µM benzbromarone. With low concentration of myricetin at 40 µM the vanadate sensitive ATPase activity has been reduced slightly but the results were not significant.

A.



B.

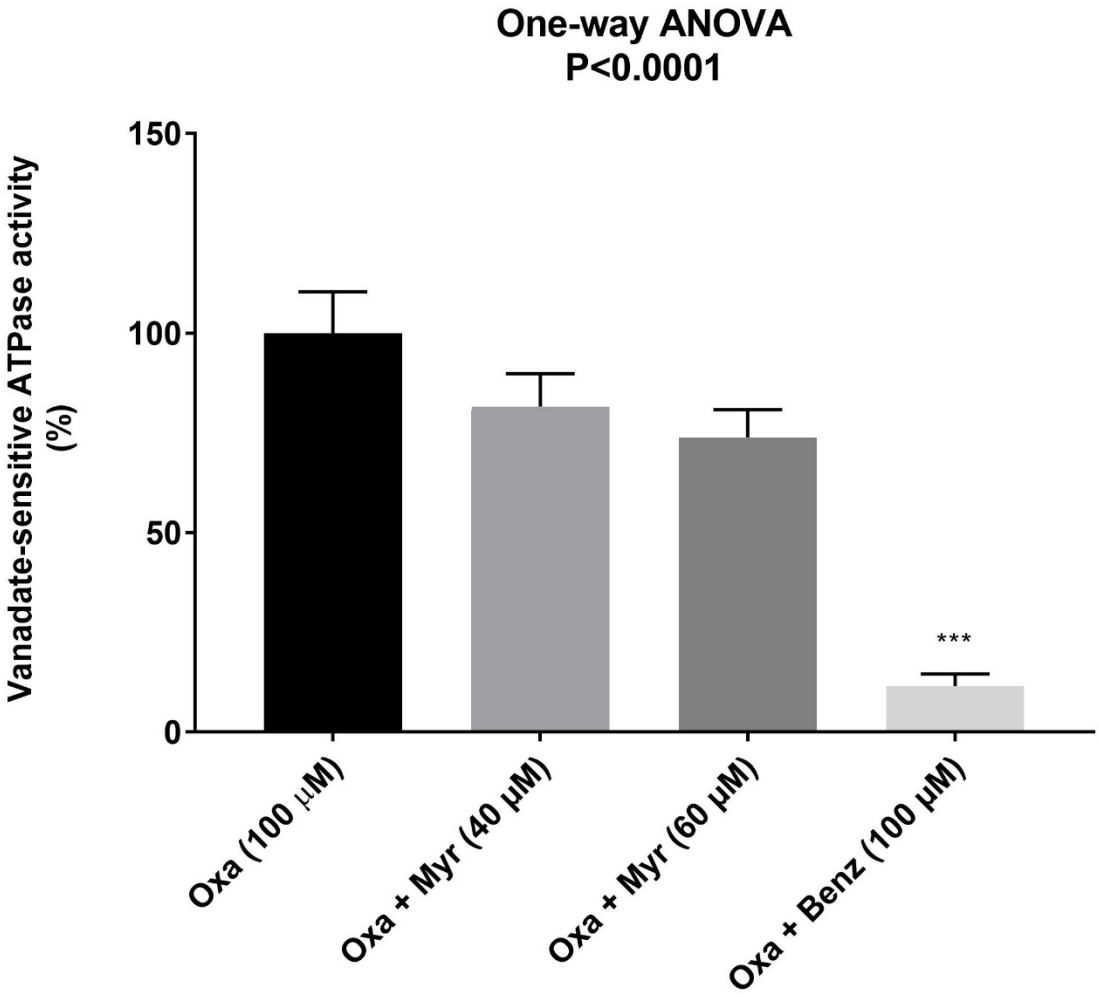


Figure 3.6. Vanadate-sensitive ATPase activity in activation mode in the presence of inhibitors at a reaction time of 20 min.

Sf9/MRP2 membrane vesicles were incubated with (A) 25 µM and (B) 100 µM of oxaliplatin in the assay buffer at 37°C for 20 min in the presence or absence of 1.5 mM Na_3VO_4 and inhibitors. The assay reaction was carried out in the presence of 40 µM and 60 µM of myricetin and 100 µM of benzbromarone. Membrane vesicles at the specified time point were quantified by colourimetric detection. The data represented the percentage of vanadate-sensitive ATPase activity as compare with basal vanadate-sensitive ATPase activity in the presence of oxaliplatin only. All data are represented as mean ± SEM, n=4. Asterisks are P values (*, P<0.05; ***, P<0.001) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all samples to basal vanadate-sensitive ATPase activity sample. Oxa, Myr and Benz represent oxaliplatin, myricetin and benzbromarone respectively.

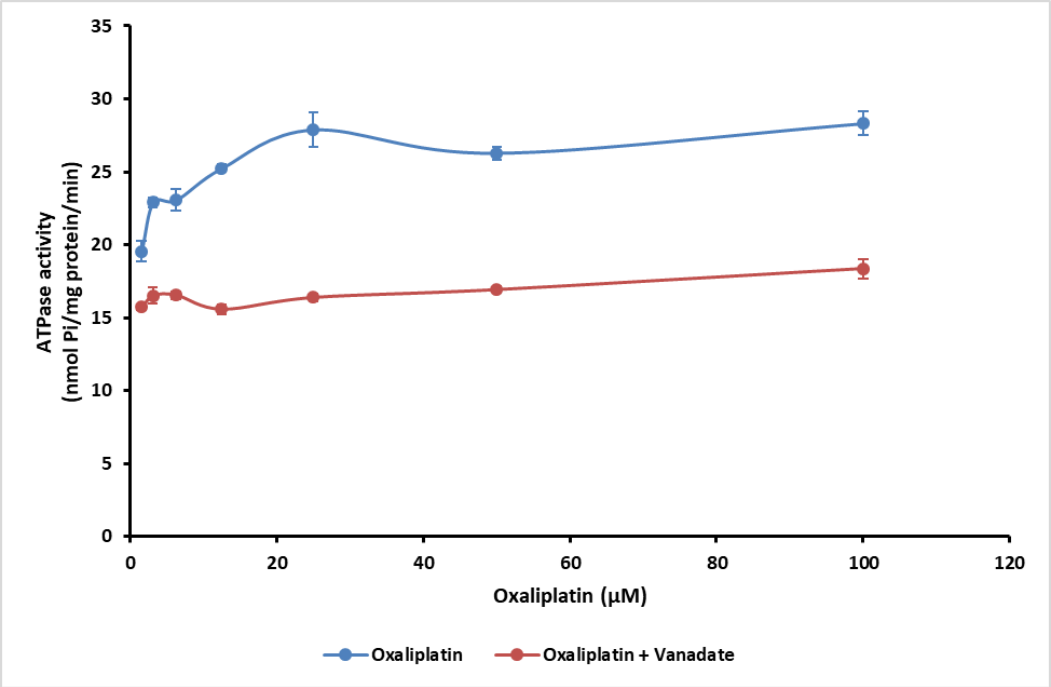
3.3.4. Oxaliplatin

After analysing the basal and inhibition modes of the control groups in Sf9/MRP2 vesicles, we studied the oxaliplatin in vanadate-sensitive MRP2 ATPase activity. As reported in a recent study, the MRP2-expressing membrane vesicle accumulated 19-fold more oxaliplatin-derived platinum during their incubation with oxaliplatin and ATP compared to the control membrane vesicles and in the absence of ATP in a membrane vesicular transport assay [176]. The rate of ATP-dependent MRP2-mediated transport of oxaliplatin increased non-linearly with the increase in oxaliplatin concentration with half-maximal platinum accumulation rate (K_m) at 301 μM of oxaliplatin [176]. According to this result, oxaliplatin appears to be the substrate for MRP2 transport. To further investigate whether oxaliplatin exhibits a stimulatory or inhibitory effect in MRP2-mediated transport, we performed a MRP2 ATPase assay.

Oxaliplatin of 0.78 μM to 100 μM was incubated with a Sf9/MRP2 membrane vesicle to investigate the effect of oxaliplatin on MRP2 ATPase activities. Oxaliplatin exerted a concentration-dependent stimulation effect on the baseline activated Pi generation and vanadate-sensitive ATPase activities for 10 min, 20 min and 30 min of ATPase activity. As from the baseline vanadate ATPase activity at 10 min, it was clear that oxaliplatin is the substrate of MRP2. At 10 min we did not get accurate quantification of ATPase results; therefore, we only proceeded for basal ATPase activity at 20 min and 30 min of reaction time. The effect of oxaliplatin in MRP2 membranes at 20 min is illustrated in figure 3.7, in which stimulation of basal vanadate-sensitive ATPase activity was observed starting from 1.5 μM, peaking at 3.1 μM and followed by a steady state with increasing oxaliplatin concentrations from 25 μM. As shown in figure 3.8, at 20 min of reaction time, the vanadate sensitive ATPase activity exhibited a sigmoidal stimulation response from an oxaliplatin concentration of 0.78 μM to 100 μM with an EC_{50} value of 8.3 ± 0.7 μM. The effect of oxaliplatin in Sf9 control membrane at 20 min is illustrated in figure 3.8. The resulting vanadate-sensitive ATPase activity in control membranes was not stimulated or inhibited by oxaliplatin. This result indicates that no significant effect in vanadate-sensitive ATPase activity was induced by oxaliplatin in the Sf9 control membranes. The stimulation of basal vanadate-sensitive ATPase activity at 30 min of reaction time was observed starting from 1.5 μM, peaking at 3.1 μM and followed by a steady state with increasing oxaliplatin concentrations from 12.5 μM (figure 3.9). The EC_{50} value for oxaliplatin-induced stimulation at 30 min of reaction time in the MRP2 membranes was 4.7 ± 0.9 μM.

According to the results, oxaliplatin stimulated the baseline ATPase activity in the MRP2 membrane, but there was no inhibitory or stimulatory effect observed on the vanadate-insensitive activity in the membrane. Oxaliplatin stimulates the basal vanadate sensitive ATPase activity at 20 min and 30 min reaction times. Therefore, no significant inhibitory effect in vanadate-sensitive ATPase activity was induced by oxaliplatin. The stimulatory effect in the MRP2 vanadate sensitive ATPase activity indicated that oxaliplatin is an MRP2 substrate that only shows a stimulatory effect.

A.



B.

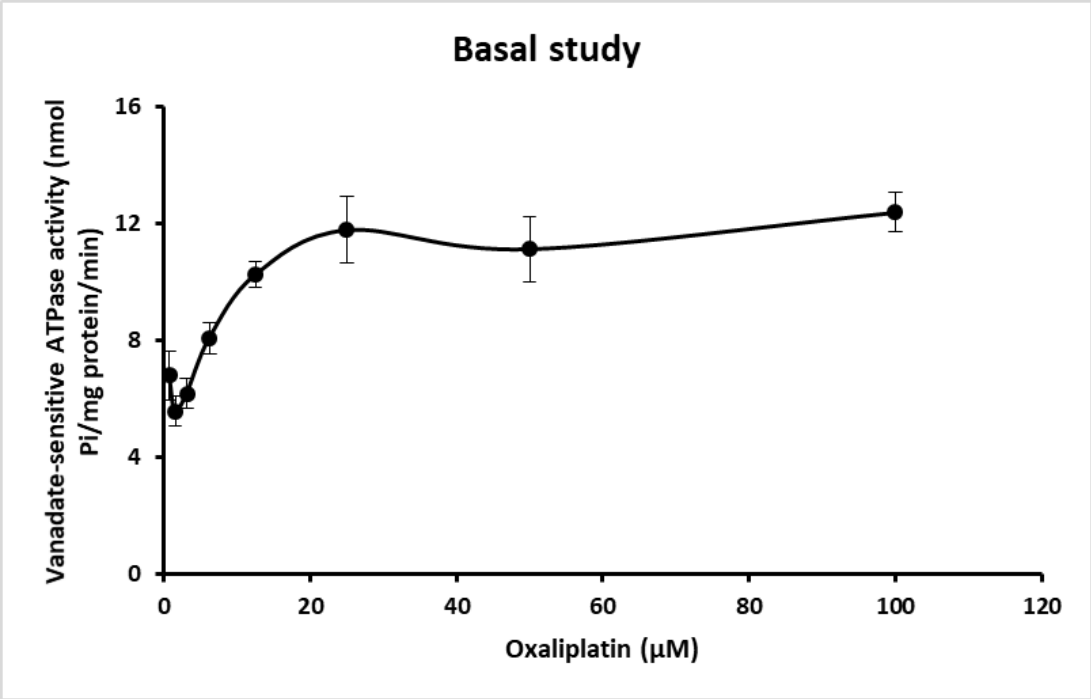


Figure 3.7. Effects of oxaliplatin on the ATPase activity of MRP2 membrane vesicle at 20 min.

Sf9/MRP2 membrane vesicles were incubated with different concentrations of oxaliplatin in the assay buffer at 37°C for 20 min in the presence or absence of 1.5 mM Na_3VO_4. The amount of Pi generated was quantified by colourimetric detection and calculated. (A) ATPase activity, (B) Vanadate-sensitive ATPase activates are presented. All data were expressed as mean ± SEM, n=4.

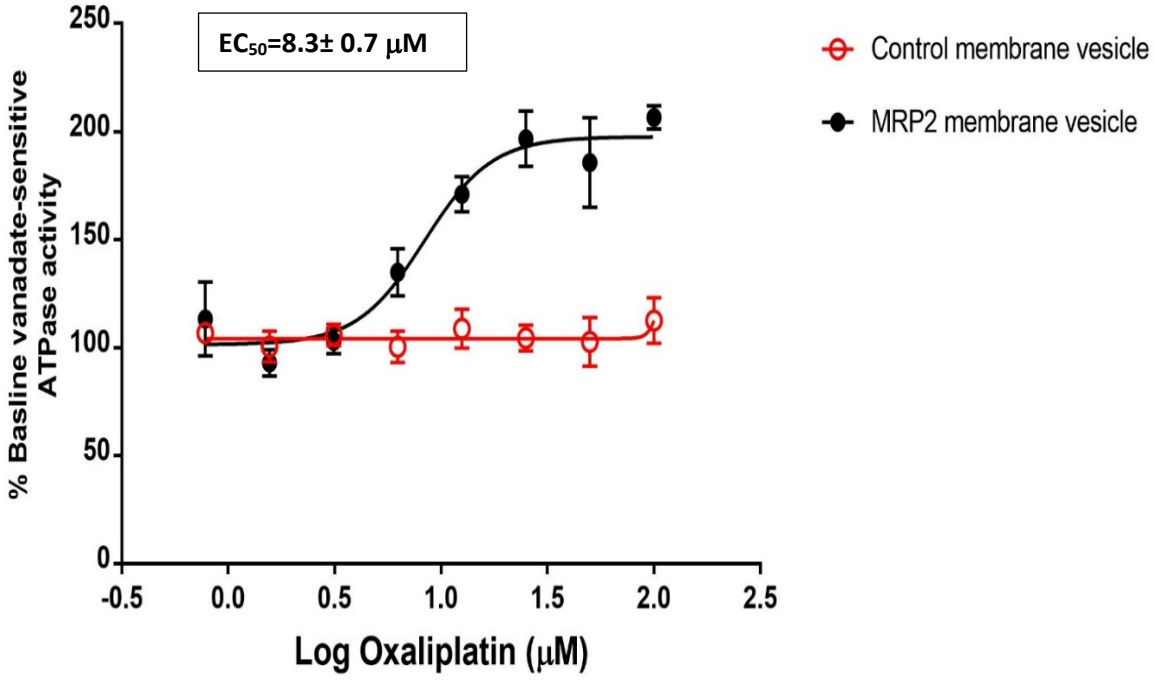
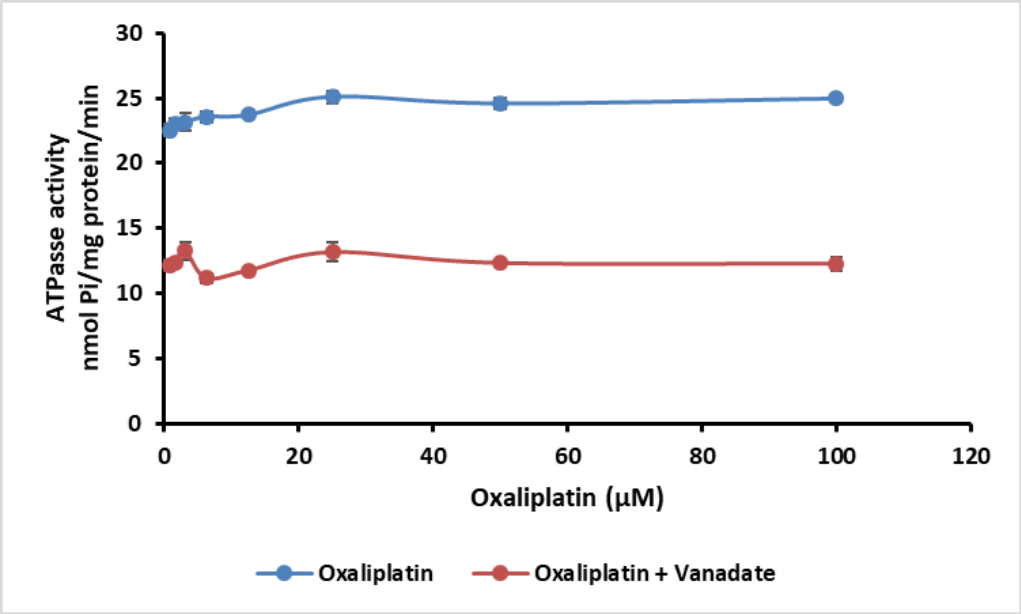


Figure 3.8. Effects of oxaliplatin on the ATPase activity of MRP2 and control membrane vesicle at 20 min.

Sf9/MRP2 membrane vesicles and control membrane vesicles were incubated with different concentrations of oxaliplatin (0.78 µM to 100 µM) in the assay buffer at 37°C for 20 min in the presence or absence of 1.5 mM Na_3VO_4. The amount of Pi generated was quantified by colourimetric detection. The dose-dependent oxaliplatin stimulation was calculated with EC_{50} value 8.3 ± 0.7 µM and the control Sf9 membrane vesicles were compared with MRP2 membrane vesicles for the basal mode. The data were normalised to control basal activity of the reaction. All data were expressed as mean ± SEM, n=4.

A.



B.

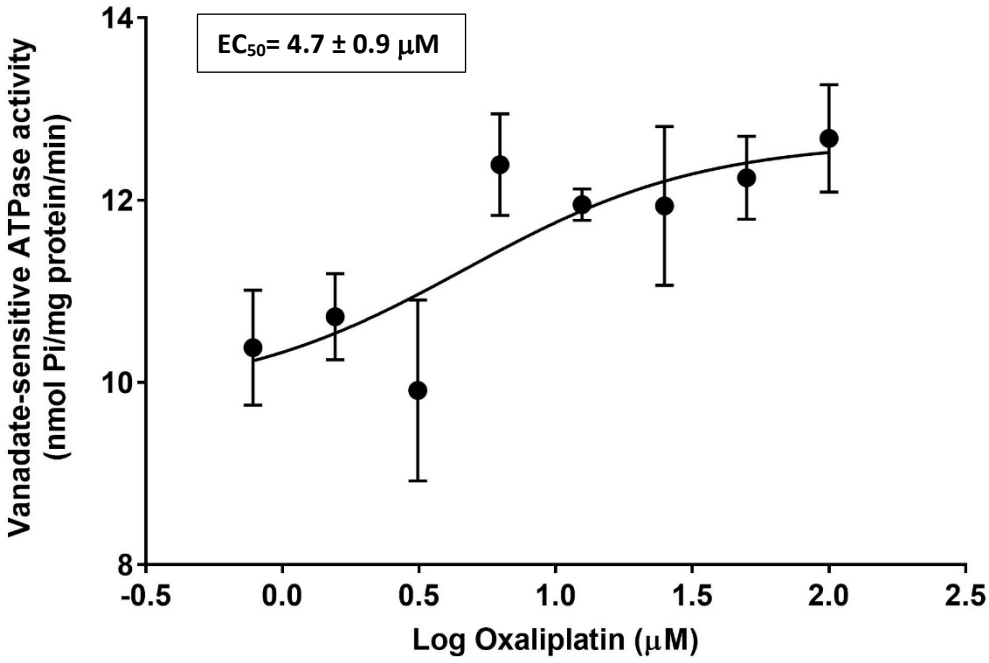


Figure 3.9. Effects of oxaliplatin on the ATPase activity of MRP2 membrane vesicle at 30 min.

Sf9/MRP2 membrane vesicles were incubated with different concentrations of oxaliplatin in the assay buffer at 37°C for 30 min in the presence or absence of 1.5 mM Na_3VO_4. The amount of Pi generated was quantified by colourimetric detection and calculated. (A) ATPase activity, (B) Vanadate-sensitive ATPase activates are presented. The dose-dependent oxaliplatin stimulation was calculated with EC_{50} value 4.7 ± 0.9 μM. All data were expressed as mean ± SEM, n=4.

3.4. Discussion

In this chapter, the establishment of the ATPase assay and its application to study the interaction between oxaliplatin anticancer drug and MRP2 are described. The ATPase assay provides information on the nature of the interaction of test compounds, whether oxaliplatin is a potential substrate or inhibitor of MRP2. The substrate relationship with MRP2 has been shown for oxaliplatin.

Oxaliplatin is a third generation of platinum-based drugs and also a substrate of MRP2. Oxaliplatin is rapidly absorbed and mainly metabolised to monocholoro-DACH platin, dichloro-DACH platin, diaquo-DACH platin, methionine, monocholorocreatinine and glutathione. Oxaliplatin is primarily excreted by urine (53.8%) rather than faecal elimination (2.1%). Renal clearance contributes to approximately half of the total removal of platinum, which indicates that glomerular filtration is a major mechanism of platinum elimination. These facts demonstrate an essential role of MRP2 efflux transporter in the intestine and liver. Thus, MRP2 transporter plays a significant role in anticancer drug disposition and in controlling drug bioavailability. MRP2 can have a substantial effect on the absorption, distribution, metabolism and excretion (ADME) of drugs, their efficacy, toxicity and drug-drug interactions [267].

The baseline vanadate sensitive ATPase activities were in the range of 6 – 12 nmol Pi/mg protein/min in Sf/9MRP2 membrane vesicles generated after 20 min of reaction time. As a comparison with 30 min of reaction time, the baseline vanadate-sensitive ATPase activities were in the range 9 - 12 nmol Pi/mg protein/min. Thus, we observed that with the change in the reaction time, there was no change in the baseline vanadate-sensitive ATPase activity. However, with an increase in reaction time, there was a slight increase in the rate of generated Pi. The baseline Pi generated was 11, 20 and 30 nmol Pi/mg protein for the 10 min, 20 min and 30 min reaction respectively. The baseline vanadate-sensitive ATPase activities appear to be linear within the 30 min time point. The baseline ATPase activity of Sf9/MRP2 membrane vesicles was stimulated by an increase in reaction time, indicating MRP2 membrane functionality. In the presence of sulfasalazine, the baseline vanadate-sensitive ATPase activity of MRP2 was stimulated. These results demonstrated that the ATPase assay utilising Sf9/MRP2 membrane vesicle could provide useful information on test compound interactions with MRP2.

The stimulatory effect of oxaliplatin on MRP2 ATPase activity indicates that oxaliplatin is either a substrate of MRP2 or a stimulant of MRP2 activity without necessarily being transported. As demonstrated in previous membrane vesicular transport studies, MRP2 confers transport of oxaliplatin [176]; therefore, we can confirm that oxaliplatin is a substrate of MRP2. Besides, oxaliplatin can be used as a stimulatory substrate and coupled with other compounds or drugs of interest that are the substrates or inhibitors of MRP2, to identify if the adjuvant drug treatment results in suppression or expression of oxaliplatin-induced vanadate-sensitive ATPase activity. In the presence of 60 µM myricetin and 100 µM benzbromarone, oxaliplatin-induced vanadate sensitive ATPase activities were significantly suppressed. These results demonstrated that MRP2 inhibitors, myricetin and benzbromarone inhibited the oxaliplatin-induced ATPase activity in MRP2 membrane. While at lower concentration of myricetin oxaliplatin-induced ATPase activity were suppressed but results were not significant. Thus, we can conclude that MRP2 inhibitor myricetin inhibited the ATPase activity in a dose-dependent manner and 60 µM of myricetin significantly inhibited the oxaliplatin-induced vanadate-sensitive MRP2 ATPase activity.

The EC_{50} values of oxaliplatin studied in the MRP2 ATPase assay were 8.3 ± 0.7 µM and 4.7 ± 0.9 µM for 20 min and 30 min of reaction time respectively. Oxaliplatin stimulated the baseline vanadate-sensitive ATPase activities of Sf9/MRP2 membrane vesicles. The stimulatory effect of oxaliplatin was demonstrated by a sigmoidal-shaped curve in the ATPase assay. Therefore, oxaliplatin is considered as a substrate that stimulates the ATPase activity of MRP2 membrane. From the dose-dependent oxaliplatin stimulation study, it was observed that MRP2 membrane has a possibility of two binding sites as the hillslope value was around 2. This result is consistent with the previous MRP2 ATPase result where MRP2 membrane has two binding sites [260]. Since stimulation effect of oxaliplatin was demonstrated by the sigmoidal-shaped curve, we postulate that the relative affinity of oxaliplatin for the second inhibitory site is lower than the activating or transport site. Hence, oxaliplatin has a stronger affinity for interacting with MRP2 at the transport site, which stimulates the MRP2 ATPase activity.

3.5. Conclusion

In summary, this chapter describes the establishment of an ATPase assay and its application to study the ATPase activity of the oxaliplatin in Sf9/MRP2 membrane vesicle. The ATPase assay identified a stimulatory effect of oxaliplatin in the basal mode, indicating that oxaliplatin is a substrate of MRP2 possibly with two binding sites. Furthermore, MRP2 inhibitors reduced the oxaliplatin-induced vanadate sensitive ATPase activity that confirmed oxaliplatin indeed a substrate of MRP2.

Chapter 4 Silencing MRP2 in HepG2 cells

4.1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and the leading cause of cancer-related death. Curative treatment involves surgical resection and liver transplantation, but these approaches are feasible for less than 20% of patients and in most cases, patients have progressed to an advanced stage even after complete surgical resection [268-271]. As a palliative treatment, chemotherapy is an important approach for metastatic HCC patients. However, the response rate with different chemotherapeutic regimens is limited by poor efficacy or development of resistance to chemotherapy [26, 272]. Hence, alternative chemotherapeutic strategies are necessary for the patients who are tolerant to chemotherapy.

Oxaliplatin, a third generation platinum-based chemotherapy that has less toxicity than cisplatin and carboplatin, is widely used as oxaliplatin-based combinatorial treatments for various cancers including gastric, colorectal, advanced HCC [273-275]. In phase II clinical studies, oxaliplatin-based combination therapies have shown promising antitumour effects in patients with advanced HCC [276, 277]. Recently, a number of different oxaliplatin-based chemotherapies such as oxaliplatin and gemcitabine (GEMOX), and oxaliplatin and fluoropyrimidines (FOLFOX4) have enhanced the efficacy of oxaliplatin by prolonging the survival of patients. However, these regimens have shown poor tumour responses in a proportion of treated patients [24, 25]. The pharmacological basis of these variable clinical responses to oxaliplatin-based chemotherapy is ambiguous.

In most cases, it has been observed that the expressions of ABC transporter proteins are responsible for drug resistance in gastrointestinal cancer patients receiving platinum-based chemotherapy [214, 278, 279]. The expression level of MRP2 was found to be increased in patients with hepatocellular carcinoma receiving platinum-based chemotherapy [214]. Moreover, some *in vitro* studies showed that overexpression of MRP2 confers resistance to platinum-based anticancer drugs like cisplatin to other cancer cell lines [280]. In recent years, studies have shown that single-nucleotide polymorphisms (SNPs) affect tumour responses and patients' outcomes [24]. Several SNPs have been identified in ABCC2, which are responsible for determining the platinum drug disposition [208, 281]. For example, polymorphism of ABCC2 -24C > T increased responses to platinum-based chemotherapy in non-small lung cancer [282],

while one study found that ABCC2 -24C > T SNP did not affect DNA-protein binding in platinum-resistant cells [281]. These studies suggested that MRP2 plays an important role in the chemoresistance to platinum-based anticancer drugs, mainly cisplatin, in gastrointestinal cancer patients. Recently, it has been reported that MRP2 confers transport of oxaliplatin-derived platinum by using MRP2 overexpressing membrane vesicles [176]. This evidence has shown an association between membrane transporter MRP2 and oxaliplatin. There have been few *in vitro* studies showing the association of MRP2 with the cellular resistance to oxaliplatin in human liver cancer cells.

This chapter aims to determine the contribution of MRP2 to oxaliplatin resistance by using an MRP2 expressing human liver cancer HepG2 cell line as a model. The overall purpose of our research is to determine whether silencing MRP2 by siRNA might increase platinum accumulation and reverse oxaliplatin resistance in HepG2 cells. RNA interference (RNAi) is considered to be a developed method for specific inhibition of gene expression by degrading the double-stranded RNA (dsRNA) induced by homologous mRNA and blocking the corresponding gene expression, thus results in specific post-transcriptional gene silencing (PTGS) [283]. Since RNAi has high specificity that effectively blocks the expression of a specific gene, siRNA-mediated gene silencing was used to knock down MRP2 HepG2 cell lines. Compared with "chemical knockdown" (by MRP2 inhibitors), siRNA gene silencing provides a better method for understanding the MRP2 gene functions in chemoresistance to oxaliplatin.

In our research, we have used stealth siRNA. According to Invitrogen, stealth siRNAs are stable for 72 hrs in serum and do not trigger an interferon response in cells. The main advantage of stealth siRNAs is that they provide effective knockdown with high specificity and greater stability compared to traditional siRNA. Moreover, studies have reported that standard siRNA can induce cellular stress response that results in cellular toxicity, whereas chemically modified stealth siRNA minimises the nonspecific cellular stress response.

The objective of the study in this chapter was to determine the sensitivity of liver cancer cells to oxaliplatin after modulation of MRP2 with siRNA.

The aims of the experiments conducted in this chapter were:

I. To knockdown the expression of MRP2 gene in HepG2 cells by using ABCC2-siRNA transfection.
II. To verify any off-target effects after transfection in other transporter genes and enzymes.
III. To measure the cellular accumulation of CDCF in MRP2-silencing HepG2 cells.
IV. To examine the cellular cytotoxicity of oxaliplatin in MRP2-silencing HepG2 cells.
V. To measure the cellular accumulation of oxaliplatin-derived platinum in MRP2-silencing HepG2 cells.
VI. To determine oxaliplatin-induced apoptosis in MRP2-silencing HepG2 cells at a different concentration of oxaliplatin.

4.2. Materials and methods

4.2.1 Chemicals

The details of chemicals, sources and their stock solutions preparation were described earlier in chapter 2 in section 2.1. The details of the apparatus used for this study is given in chapter 2, table 2-2.

4.2.2. Transfecting stealth siRNA into HepG2 cells

The transfection of stealth siRNA into human HepG2 hepatocellular carcinoma cells was done using Lipofectamine RNAiMAX (Cat. No. 14778-150). Lipofectamine RNAiMAX has a broad range of activity, specifically developed for highly efficient delivery of siRNA into mammalian cells with maximal knockdown effects. Reverse transfection was used for transfecting HepG2 cells. Three subsets of MRP2 specific siRNAs, ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 and one scramble siRNA (negative control siRNA) were used for our research. The siRNA concentration and assay times were adjusted to establish optimal knockdown of MRP2 gene in preliminary studies. Finally, 20 µM of ABCC2-siRNAs for 48 hr was used for transfection. Before mixing lipofectamine and ABCC2-siRNAs, the manufacturer recommends diluting RNAi duplexes and Lipofectamine RNAiMAX in Opti-MEM I Reduced Serum Medium (Cat. No. 31985-062). Antibiotics should not be added to the media during transfection as this may cause cell death.

In reverse transfection, the Lipofectamine RNAiMAX and ABCC2-siRNA complexes were prepared inside the wells. Thereafter, cells and medium were added. The transfection procedure was carried out in 12-well plates. For each well to be transfected, 60 pmol ABCC2-siRNAs were added in 100 µl serum-free Opti-MEM I Medium in the plate, followed by gentle mixing. Then, an aliquot of 2 µl of Lipofectamine RNAiMAX in 100 µl Opti-MEM I Medium was added to each well containing the diluted ABCC2-siRNA. The Lipofectamine RNAiMAX and ABCC2-siRNAs complexes were mixed gently and incubated for 15 min at room temperature. HepG2 cells were diluted in complete growth medium without antibiotics at a cell density of 150,000 cells per ml (cells should be 50% confluent after 24 hr plating). To each well with ABCC2-siRNA and Lipofectamine RNAiMAX complexes, 1 mL of diluted cells were added. This gave a total volume of 1.2 ml solution and a final RNA concentration of 50 nM. The plate was gently mixed back and forth to properly mix the complexes with diluted cells. The

cells were incubated for 24 hr at 37°C in 5% CO_2 with 95% humidified air before the transfection complexes were removed and replaced with complete growth medium. After 48 hr incubation of cells with ABCC2-siRNAs, HepG2 cells were used for the different experimental assays.

Table 4-1. Details of reagent amounts and volumes

Culture plate	Rel. surface area (cm^2)	The volume of plating medium	Cells density	ABCC2-siRNA amount (pmol)	Final siRNA duplex concentration (nM)	Lipofectamine RNAiMAX amount (µl)
12-well	4	1000 µl	150,000	60	50	2

4.2.3. Real-time quantitative PCR

4.2.3.1. RNA extraction and cDNA synthesis

After 48 hr incubation with ABCC2-siRNAs and control-siRNA, RNA from each sample was extracted to measure the expression level of the target gene using the real-time qPCR method. RNA was extracted from samples using a RNeasy MINI Kit (Qiagen, Valencia, CA) as described in section 2. Thereafter, from the total RNA, cDNA was synthesised by reverse transcription of total RNA samples in which RNA was primed with oligonucleotides and catalysed by a reverse transcriptase enzyme using Transcriptor First strand cDNA kit (Roche Applied Science) according to the manufacturer's protocol as described in section 2.4. To measure the relative gene expression of the target gene and other ABC transporter genes, real-time qPCR was performed using the cDNA samples.

4.2.3.2. Relative gene expression

For ABCC2-siRNAs knockdown studies, mRNA expression of target gene ABCC2 (MRP2 gene), other ABC transporter genes and housekeeping genes were quantified using real-time quantitative qPCR. For real-time qPCR, gene-specific primers were purchased from IDT (Integrated DNA Technology, USA). A list of primers used in this experiment is listed in table 3.3. Quantitative real-time PCR was performed at LC480 LightCycler (Roche Applied Science) using LightCycler-FastStart DNA Master SYBR Green 1 Master Mix (Roche Applied Science) and gene-specific primers at 180 nM. The reaction details were described in section 2.4.

The mRNA expression levels of genes of interest in each sample were calculated as relative gene expression and represented as $2^{-\Delta\Delta Ct}$ values of corresponding genes. Firstly, ΔCt values were calculated by subtracting the Ct values of house-keeping genes, i.e., glyceraldehyde 3-phosphate dehydrogenase (GAPDH), from the Ct values of the gene of interest. Finally, ΔΔCt values were calculated by subtracting the average ΔCt value of control-siRNA from the ΔCt values of the gene of interest. The MRP2 knockdown in the expression levels of genes of interest in ABCC2-siRNA treated cells compared to control-siRNA cells were then calculated by dividing the value obtained by subtracting the $2^{-\Delta\Delta Ct}$ values of the gene of interest from the $2^{-\Delta\Delta Ct}$ value of the control siRNA by that of $2^{-\Delta\Delta Ct}$ value of control siRNA for each gene.

4.2.4. Cell surface staining to MRP2

The MRP2 transporter surface expression was assessed by staining the MRP2 transporter in HepG2 cells and MRP2-silencing HepG2 cells with the anti-MRP2 primary antibody. The non-specific MRP2 staining was also determined using a host-species matched IgG2a isotype control. Both antibodies were conjugated with Alexa Fluor 488 secondary antibody.

4.2.4.1. MRP2 staining in HepG2 cells

HepG2 cells were grown in a T_{75} flask and cells were trypsinised and collected in an Eppendorf tube at a density of 1 x 10^6 per ml. The cell samples then allowed for fixation, permeabilisation, and blocking according to the procedure mentioned in section 2.10.1. The experiment was repeated twice independently.

4.2.4.2. MRP2 surface staining in siRNA knockdown HepG2 cells

To determine the MRP2 transporter expression in ABCC2-siRNA HepG2 cells, cells were first transfected with ABCC2-siRNAs and control-siRNA at a density of 350,000 cells per well in a 6-well plate for 48 hr. After transfection, 50×10^4 cells were collected for each transfected cell and washed with ice-cold PBS followed by centrifugation at $500 \times g$ for 5 min. The cells pellets were resuspended in 100 µl of 1% paraformaldehyde in PBS and incubated for 15 min on ice. Thereafter, the processes of permeabilisation, blocking, and staining with primary and secondary antibodies were carried out according to the procedure mentioned in section 2.10.1.

4.2.5. Cellular CDCF accumulation assay

For functional validation of MRP2 transporter in ABCC2-siRNA HepG2 cells, cells were first transfected for 48 hr. After transfection, the cells were allowed to incubate for another 24 hr at 37°C in a 5% CO_2 incubator. After that, ABCC2-siRNA and control-siRNA transfected cells were incubated with 2.5 µM of CDCF for 20 min followed by measurement of fluorescence intensity in flow cytometry as mentioned in section 2.5.2. Experiments were repeated three times independently.

4.2.6. Growth inhibition assay

The cytotoxicity of oxaliplatin in ABCC2-siRNAs and control-siRNA transfected HepG2 cells were determined by an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. The MTT assay was used to determine the number of viable cells after oxaliplatin treatment. 48 hr after transfection with siRNAs, HepG2 cells at the density of 8000 per well were seeded in 96-well plates. After 24 hr incubation, cells were exposed to oxaliplatin for 2 hr followed by replacing the drug solution with complete growth medium for three days. Further details of MTT assay are shown in section 2.7. Experiments were repeated three times independently.

4.2.7. Cellular platinum accumulation

Cells were seeded at 350,000 cells per well in a 6-well plate and transfected with ABCC2-siRNA and control-siRNA. 24 hr after transfection, the transfection medium was replaced with normal growth medium. HepG2 cells become around 80% confluent after a further 24 hr of growth. Cells were then were incubated with 25 µM oxaliplatin for 2 hr. After incubation with oxaliplatin, cells were washed with ice-cold PBS three times followed by processing of cells for the ICP-MS assay and the protein concentration measurement as described in section 2.6.1.

The ICP-MS procedure and method validation were carried out as described in section 2.6. The validation of elements, including accuracy, the precision of the method as well as the limit of detection (LOD) and lower limit of quantification (LLOQ), were determined according to the US FDA guidelines for bioanalytical method validation. The accuracy and precision of the method were established by preparing three replicates of quality control (QC) samples, referred to as lower QC, middle QC and upper QC, over the standard curve range. The ICP-MS analysis run was acknowledged only if the

standard curve was linear and the QC samples over the standard curve were within 15% precision values and accuracy should be between 85% to 115%. In this experiment, the linearity of the ICP-MS platinum standard curve of the experiment was defined by r^2 > 0.9995. The precision and accuracy of the QC samples in the experiment were 5.62% and 99.65% respectively. According to the different ICP-MS runs, the LOD and LLOQ of the method were found to be 0.3 ppb and 1 ppb of platinum respectively.

4.2.8. Apoptosis assay

Apoptosis induced by oxaliplatin in ABCC2-siRNAs and control-siRNA transfected HepG2 cells were measured by flow cytometry. HepG2 cells were seeded at a density of 350,000 cells per well and transfected with required ABCC2-siRNAs and control-siRNA for 48 hr. After transfection, the cells were treated with 25 μM and 100 μM of oxaliplatin for 2 hr. The sample preparation and analysis methods for flow cytometry detection of apoptosis percentage were described in detail in section 2.8.

4.2.9. Statistical analysis

Data analysis was carried out according to the details mentioned in section 2.11 and 2.12.

4.3. Results

4.3.1. Silencing effects of ABCC2-siRNAs on HepG2 cells

According to manufacturer protocol, reverse transfection was used for maximum transfection in HepG2 cells. The transfection conditions were initially optimised to maximise the knockdown effect by using reverse transfection. Cells were transfected with three independent siRNAs specific for ABCC2, and the reduction in ABCC2 mRNA expression was measured by real-time PCR.

Treatment of HepG2 cells using negative control siRNA did not cause any significant effects on gene expression, whereas transfection of ABCC2-specific siRNAs caused a significant decrease in the targeted gene. The ABCC2 mRNA expression after analysing real-time PCR results was decreased to 48%, 59% and 42% in ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3-transfected HepG2 cells, respectively. The expression of ABCC2 mRNA was significantly higher ($P < 0.01$) in negative control cells compared with ABCC2 siRNA-transfected cells (Figure 4.1).

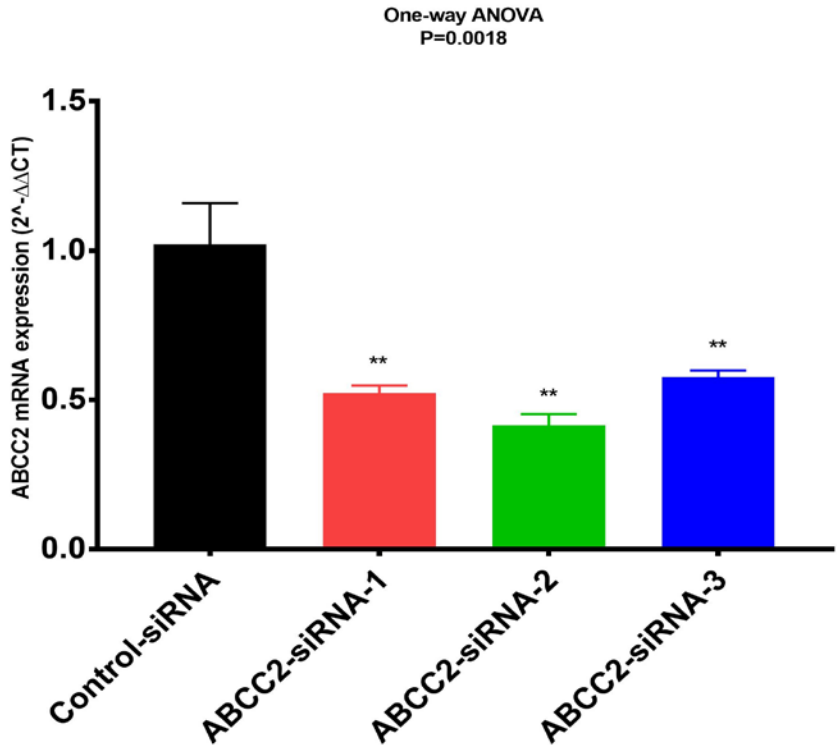


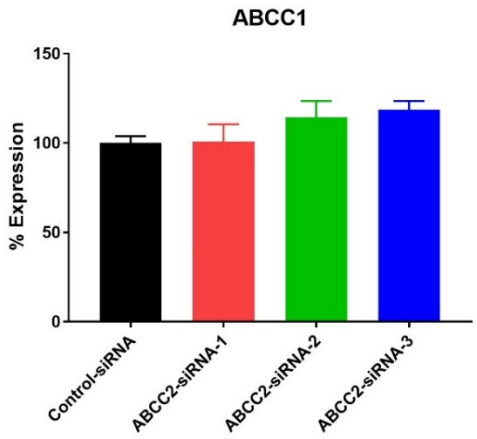
Figure 4.1. ABCC2 mRNA expression level in control and ABCC2-siRNAs knockdown HepG2 cells.

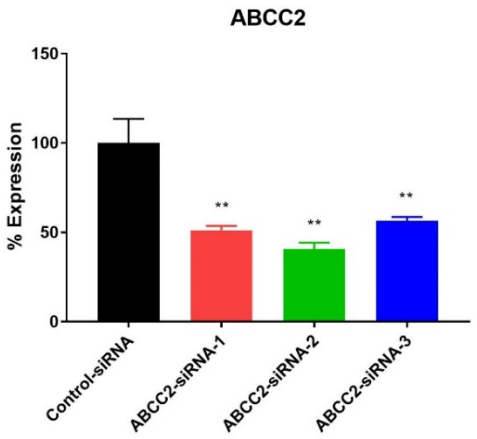
60 pmol of ABCC2-siRNA-1, ABCC2-siRNA-2, ABCC2-siRNA3 and control-siRNA were transfected in HepG2 cells. Relative ABCC2 mRNA expression was detected by real-time qPCR. Data are presented as relative gene expression $2^{-\Delta\Delta CT}$ averaged from three independent experiments. The bar represents the mean and S.E.M. of the mean values from at least three independent experiments. Asterisks are P values (, P<0.01) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=3). There were no significant difference between ABCC2-siRNA-1, siRNA-2 and siRNA-3.**

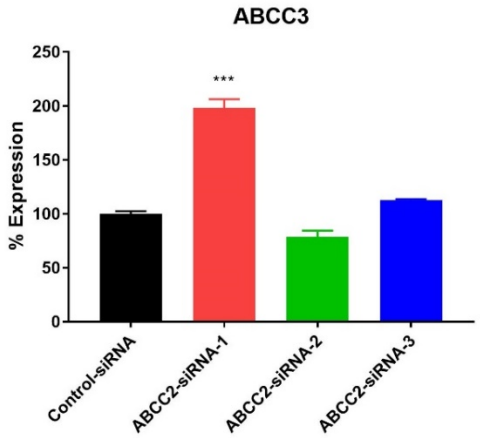
4.3.2. Off-target effects of ABCC2-siRNAs

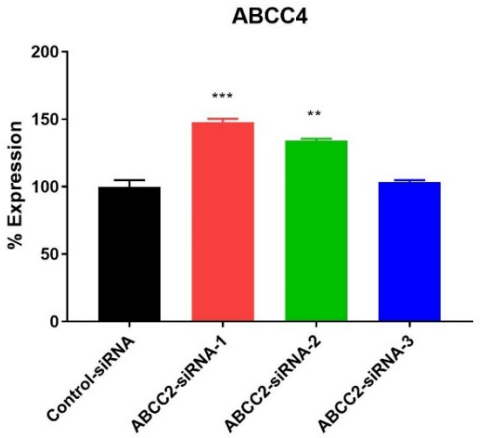
In the transfection method, siRNAs are not always specific and may lead to off-target effects. To investigate any effects in the mRNA expression of the other oxaliplatin-relevant transporters and enzymes, the expression of ABCC1, ABCC3, ABCC4, ABCC5, ABCG2, GSTP1, hCTR1, ATP7A, ATP7B mRNA were also measured by real-time PCR.

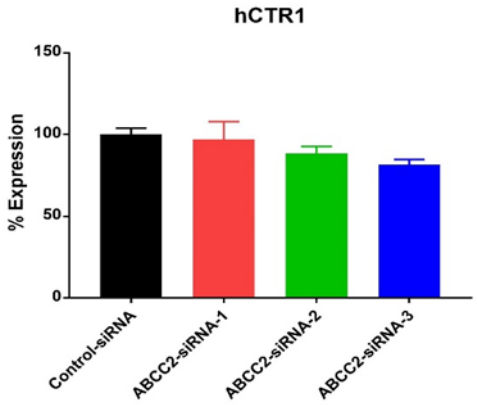
After ABCC2-siRNAs transfection in HepG2 cells, we examined the mRNA expression of other transporters, ABCC1, ABCC3, ABCC4, ABCC5, ABCG2, hCTR1, ATP7A, ATP7B and the enzyme GST1. As analysed by the comparative threshold cycle ($\Delta\Delta C_T$) method, no significant changes were observed. Generally, no major changes were observed in the expression of the other transporters; however, ABCC3, ABCC4, ABCC5, ABCG2 and ATP7A expression were increased to some extent in ABCC2-siRNA-1 compared with the negative control-siRNA. The expression of ABCC4 and ABCC5 were slightly upregulated in ABCC2-siRNA-2 transfected HepG2 cells. Therefore, after treatment with ABCC2-siRNAs, the ABCC2 mRNA expression level in HepG2 cells were reduced significantly, while minor or no significant off-target effects were detected for other genes including ABCC1, ABCC3, ABCC4, ABCC5, ABCG1, hCTR1, ATP7A, ATP7B and enzyme GST1.

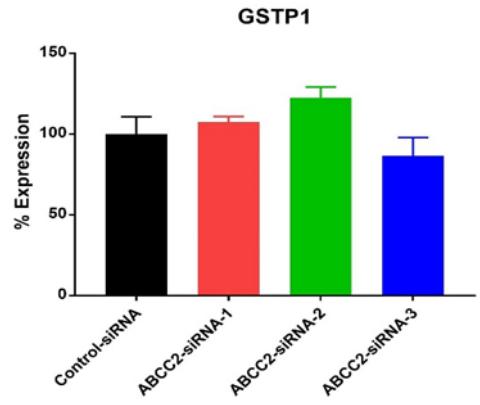

ABCC1
% Expression
150
100
50
0
Control-siRNA
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3


ABCC2
% Expression
150
100
50
0
**
**
**
Control-siRNA
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3


ABCC3
% Expression
250
200
150
100
50
0
***
Control-siRNA
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3


ABCC4
% Expression
200
150
100
50
0
***
**
Control-siRNA
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3


hCTR1
% Expression
150
100
50
0
Control-siRNA
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3


GSTP1
% Expression
150
100
50
0
Control-siRNA
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3

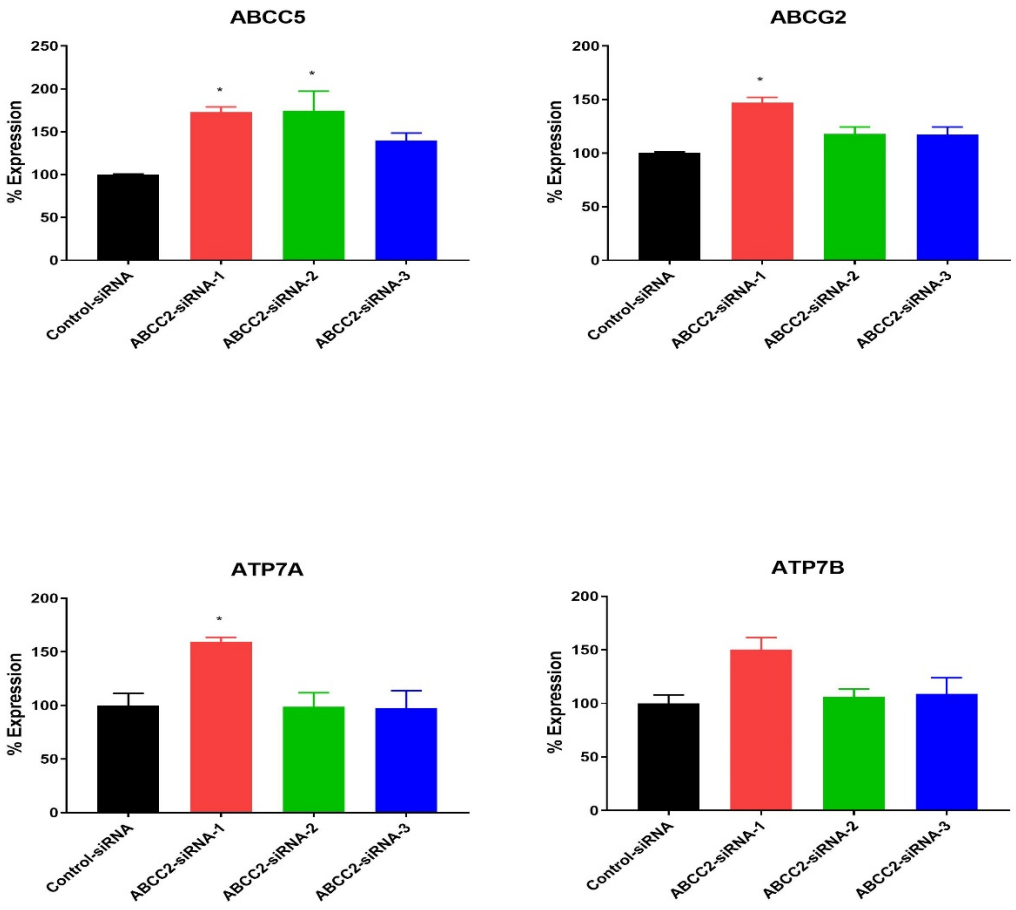


Figure 4.2. Percentage expression of mRNA in different genes after control and ABCC2-siRNA transfection in HepG2 cells.

mRNA expression at different transporters and enzymes were measured by real-time qPCR. Results were normalised by GAPDH mRNA expression and compared with the levels in control-siRNA HepG2 cells. ABCC2 mRNA expression was reduced in knockdown cells compared to control cells, whereas the mRNA expression levels of other transporters and enzymes in ABCC2-siRNAs knockdown HepG2 cells were negligible. Data are presented as relative gene expression $2^{-\Delta\Delta CT}$ averaged from two independent experiments (n=3).

4.3.3. MRP2 transporter surface expression

4.3.3.1. Cell surface staining to MRP2 transporter in HepG2 cells

The cell surface staining was assessed by staining the HepG2 cells with anti-MRP2 primary and control isotype IgG2a antibody. The mean fluorescence intensity was 0.34, 0.71 and 1.42 respectively for unstained, IgG2a and anti-MRP2 antibody stained HepG2 cells. Compared with isotype IgG2a stained HepG2 cells, the mean fluorescence intensity increased by 100% in anti-MRP2 antibody stained HepG2 cells (Representative histograms are shown in Appendix I), suggesting the presence of MRP2 transporter on the surface of HepG2 cells.

4.3.3.2. MRP2 surface staining after ABCC2-siRNA transfection in HepG2 cells

To determine if silencing the MRP2 gene decreased the surface expression of MRP2 in HepG2 cells, cell surface staining was conducted in the cells after transfecting the cells for 48 hr with ABCC2 and control-siRNAs. MRP2 cell surface expression in siRNA transfected HepG2 cells was compared with the fluorescence intensity in control-siRNA transfected cells. After 48 hr of transfection with siRNA, there was not much difference in MRP2 expression between HepG2 cells and control-siRNA HepG2 cells (Appendix I). Figure 4.3 shows that transfection with all three different subsets of ABCC2 siRNAs decreased the MRP2 surface expression. The MRP2 surface expression were 20%, 5% and 30% in ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 transfected HepG2 cells respectively. Therefore, silencing the MRP2 gene showed downregulation of MRP2 surface expression in HepG2 cells.

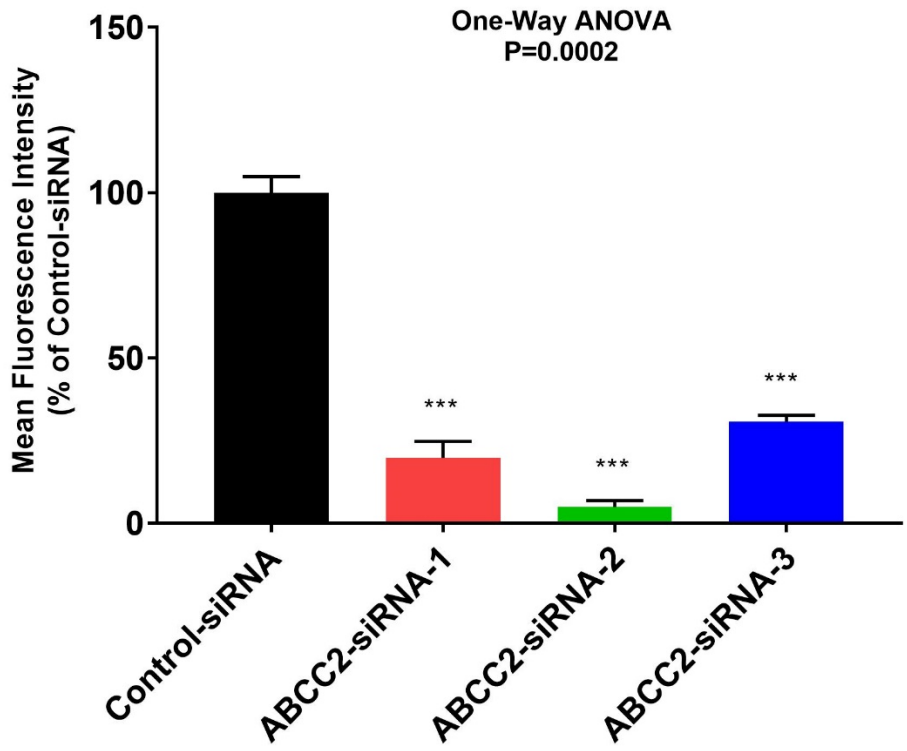


Figure 4.3. Cell surface expression of MRP2 in control and ABCC2-siRNA transfected HepG2 cells.

The data is presented as a mean percentage of fluorescence in the samples. MRP2 surface expression was detected with the MRP2 primary antibody and Alexa Fluor 488 secondary antibody, followed by flow cytometry to measure the fluorescence intensity. Results are mean ± SEM of replicate samples (n=3). Asterisks are P values (*, P<0.001) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample.**

4.3.4. Effects of silencing MRP2 on CDCF accumulation in HepG2 cells

Figure 4.4 shows that CDCF accumulation significantly increased after knockdown of ABCC2 expression. ABCC2-siRNA transfected HepG2 cells enhanced cellular CDCF accumulation measured at 48 hr post siRNA treatment ($P < 0.0001$, one-way ANOVA). The scramble siRNA treatment has no effect on accumulation of an MRP2 substrate, CDCF, in HepG2 cells, while silencing MRP2 led to an increase of cellular accumulation CDCF by 160% to 170% (Figure 4.4, $P < 0.001$). Results were represented as mean fluorescence intensity normalised with the control-siRNA fluorescence intensity.

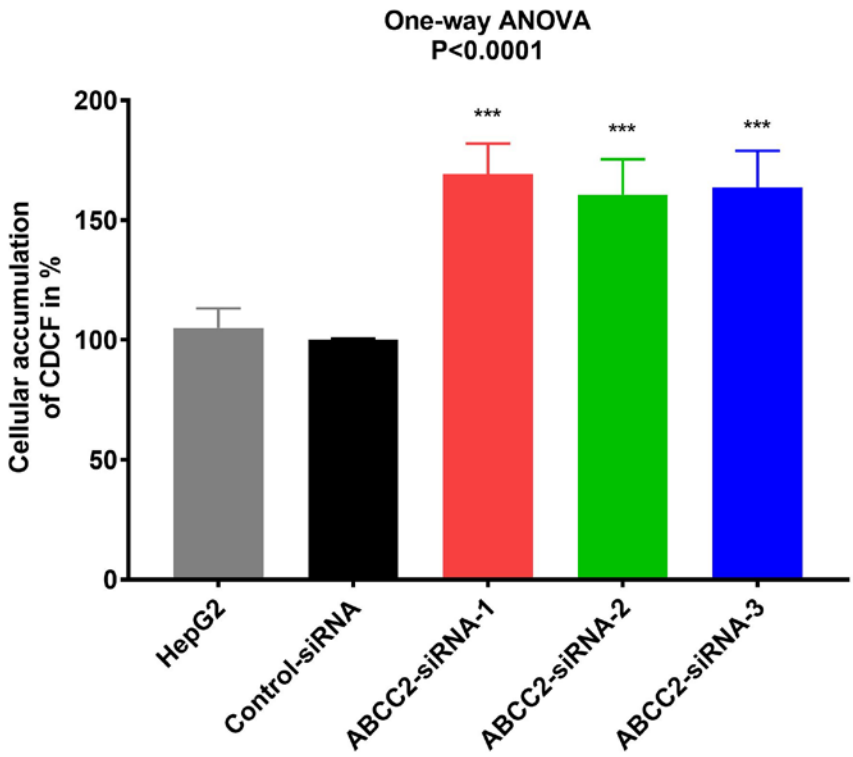


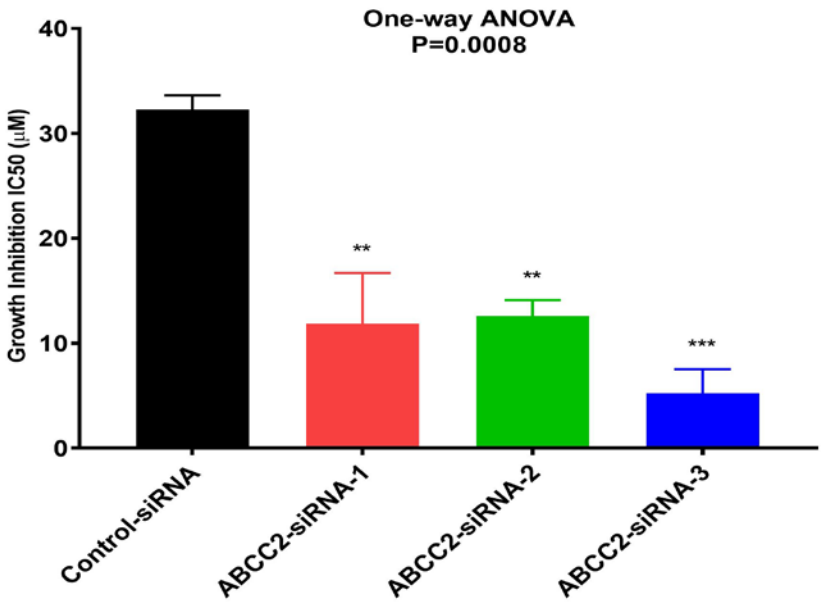
Figure 4.4. CDCF accumulation study in HepG2 cells.

CDCF accumulation in HepG2 cells and in control or ABCC2-siRNAs knockdown HepG2 cells for 20 min. Accumulation of CDCF in HepG2 and ABCC2-siRNA knockdown HepG2 cells were measured as fluorescence after 20 min incubation of 8 X 10^5 cells/ml with 5 µM CDCFDA. HepG2 cells were treated with siRNA for 48 hr before 20 min incubation with 5 µM CDCFDA and CDCF accumulation were measured by flow cytometry. ABCC2-siRNA knockdown increased CDCF accumulation in HepG2 cells. Data are presented as the mean and SEM of fluorescence percentage from three independent experiments. Asterisks are P values (*, P<0.001) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=4).**

4.3.5. Silencing MRP2 increased oxaliplatin cytotoxicity in HepG2 cells

The cytotoxic effects of oxaliplatin were compared between the scramble and ABCC2-siRNA transfected HepG2 cells by using a 72 hr MTT assay. IC_{50} values against oxaliplatin were 2- to 5-fold higher in HepG2 cells transfected with control siRNA compared with those transfected with MRP2-siRNAs. The IC_{50} value of oxaliplatin in control-siRNA HepG2 cells was 32.3 µM and for cells transfected with ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3, the IC_{50} values of oxaliplatin were 11.8 µM, 12.5 µM and 5.2 µM respectively (Figure 4.5). Based on the IC_{50} values, transfection of ABCC2-siRNAs enhanced the sensitivity of HepG2 cells to oxaliplatin. These results indicate that the ABCC2 silencing enhances oxaliplatin sensitivity in HepG2 cells.

A.



B.

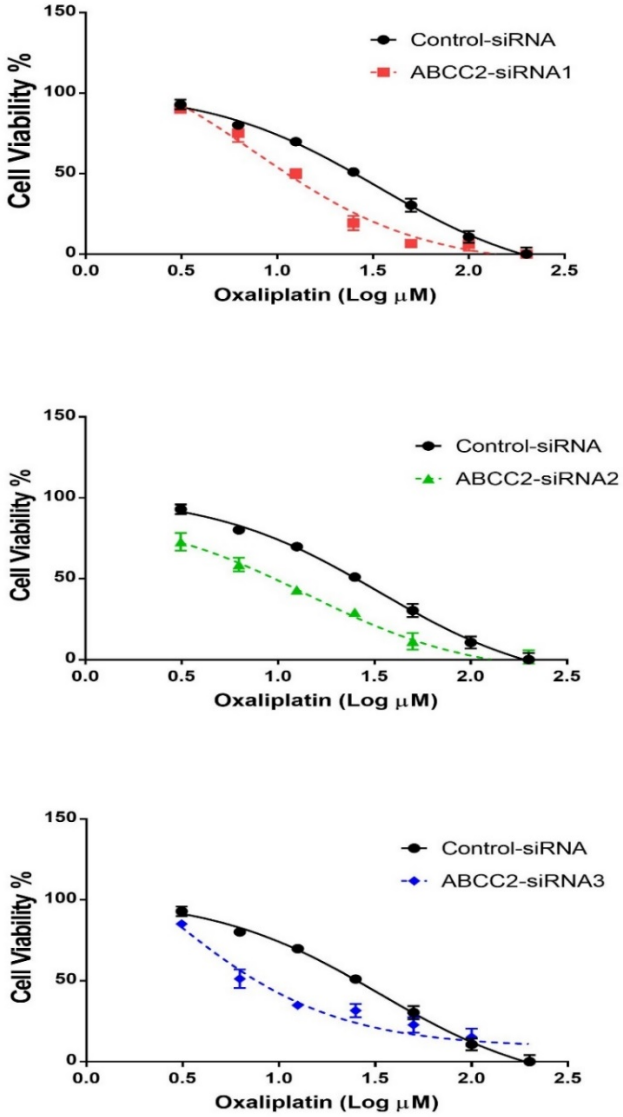


Figure 4.5. Oxaliplatin-induced growth inhibition in control-siRNA and ABCC2-siRNA knockdown HepG2 cells.

A) HepG2 cells were treated with ABCC2-siRNA and control-siRNA for 48 hr followed by incubation in a drug-free medium for 24 hr, then cells were treated with a range of different concentrations of oxaliplatin for 2 hr, then cultured in drug-free medium for 72 hr before measurement of the number of viable cells by MTT assay. In siRNA knockdown HepG2 cells, oxaliplatin sensitivities were increased by 1.5- to 5.1-fold compared with control-siRNA. Data are presented as the mean and SEM of IC_{50} of oxaliplatin in cells from three independent experiments. Asterisks are P values (, P<0.01; ***, P<0.001) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=3). B) Supplementary data of oxaliplatin sensitivities in control and ABCC2-siRNAs knockdown HepG2 cells. Each point represents the mean of quadruplicate values ± standard errors of the mean (n=4).**

4.3.6. Effects of silencing MRP2 on oxaliplatin-derived platinum accumulation

The MRP2-mediated oxaliplatin transport was further measured by determining the cellular accumulation of oxaliplatin-derived platinum in scramble and ABCC2-siRNA-transfected HepG2 cells.

Compared with the control, ABCC2-silencing significantly increased platinum accumulation by 186 ± 13% ($P<0.001$), 52 ± 2% ($P < 0.05$) and 155 ± 7% ($P < 0.001$) in ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3-treated HepG2 cells, respectively (Figure 4.6). The cellular platinum accumulation after 2 hr exposure to 25 μM oxaliplatin in control-siRNA HepG2 cells was 40.9 ± 5.9 pmol per mg protein. However, platinum accumulation in siRNA treated HepG2 cells were 117 ± 5.3 ($P < 0.001$), 62.4 ± 1.0 ($P < 0.05$), 104.7 ± 2.9 ($P < 0.001$) pmol per mg protein in ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 respectively. These results indicate that ABCC2-siRNA treatment plays a major role in the accumulation of oxaliplatin in the transfected HepG2 cells.

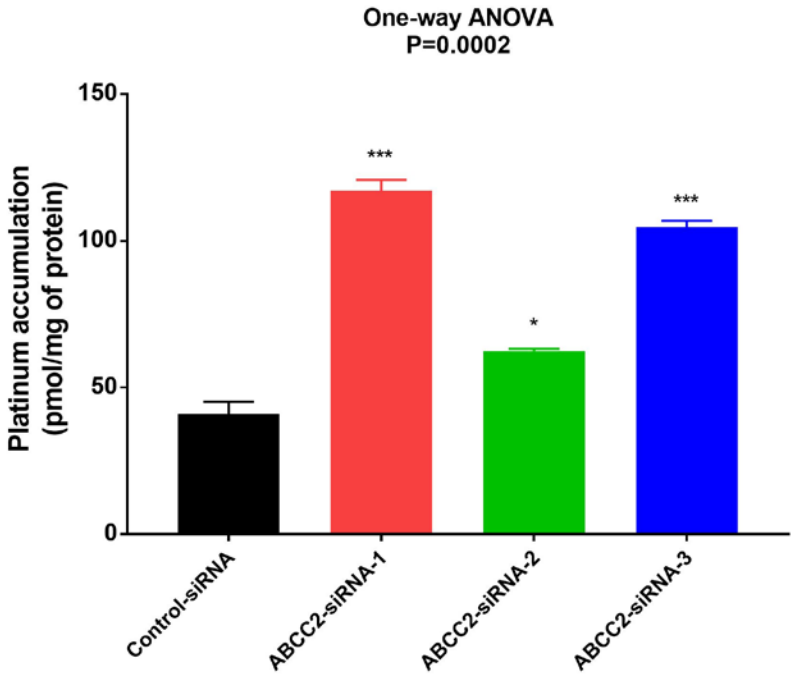


Figure 4.6. Platinum accumulation in control and ABCC2-siRNAs knockdown HepG2 cells.

HepG2 cells were treated with ABCC2-siRNAs and control-siRNA for 48 hr followed by incubation in drug-free medium for 24 hr, then treated with 25 µM of oxaliplatin for 2 hr and cellular accumulation of platinum was measured by ICP-MS. Data were presented as the mean and SEM values. ABCC2-siRNA-1, ABCC2-siRNA-2, ABCC2-siRNA-3 knockdown significantly increased platinum accumulation in HepG2 cells compared to treatment with control-siRNA. Asterisks are P values (*, P<0.05; ***, P<0.001) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=2).

4.3.7. Silencing MRP2 enhanced oxaliplatin-induced apoptosis in HepG2 cells

To further explore the relationship between MRP2 transporter and apoptosis in HepG2 cells induced by oxaliplatin, oxaliplatin-induced apoptotic effects were analysed by flow cytometry.

Fig 4.7 and table 4-3, 4-4 showed that oxaliplatin treatment increased the apoptosis rate with increased concentration of oxaliplatin. The increased apoptosis effect mainly resulted from late apoptosis (Annexin V^+/PI^+). A 2 hr treatment with 25 μM oxaliplatin significantly increased the early apoptosis rate from 26% to 36%. Compared with control values (26%), the rate of apoptosis induced by 25 μM oxaliplatin increased to 36%, 34% and 33% respectively in HepG2 cells transfected with ABCC2-siRNA-1, ABCC2-siRNA-2 and ABBC2-siRNA-3. After treatment with 100 μM oxaliplatin, the apoptosis percentage was 46.2%, 41.5% and 45.3% for ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 HepG2 cells respectively, whereas for control-siRNA, the apoptosis rate was 31%. The apoptosis percentage slightly increased in HepG2 cells without any oxaliplatin treatment between the control-siRNA and ABCC2-siRNAs, but no statistical significance was achieved for ABCC2-siRNA-1. These data suggest that knockdown of MRP2 gene effectively enhanced oxaliplatin-induced apoptosis in HepG2 cells.

Altogether, our data suggest that apoptotic cell death by oxaliplatin is significantly increased with increased oxaliplatin concentration. Knockdown of MRP2 gene using ABCC2-siRNAs transfection enhanced the oxaliplatin-induced apoptosis.

Table 4-2. Data analysis of apoptosis assay without any treatment

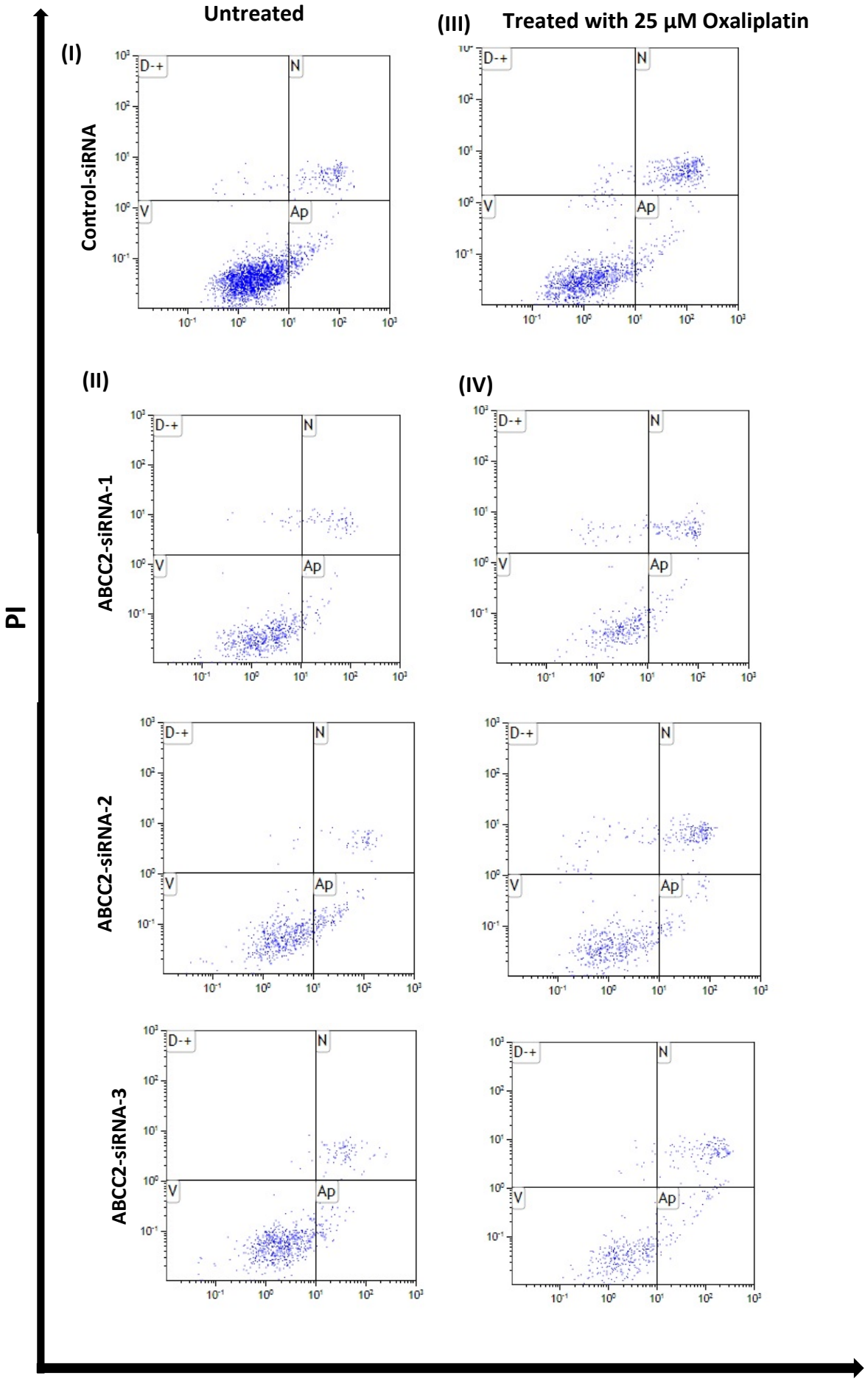
Treatment	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value (Total apoptosis)
Control-siRNA	**86.33 ± 0.32**	7.09 ± 1.33	5.57 ± 1.27	**12.66 ± 0.33**	
ABCC2-siRNA-1	**81.91 ± 0.21**	8.19 ± 1.04	8.16 ± 0.61	**16.35 ± 0.61**	0.227
ABCC2-siRNA-2	**72.13 ± 0.77**	12.13 ± 3.51	12.36 ± 2.79	**24.50 ± 1.44**	<0.001
ABCC2-siRNA-3	**75.34 ± 2.32**	7.31 ± 1.03	14.89 ± 1.41	**22.20 ± 1.75**	<0.001

Table 4-3. Data analysis of apoptosis assay with 25 µM oxaliplatin

Treatment	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value (Total apoptosis)
Control-siRNA	**69.60 ± 0.85**	8.19 ± 0.73	18.37 ± 1.23	**26.56 ± 1.29**	
ABCC2-siRNA-1	**53.76 ± 1.34**	10.89 ± 0.76	25.34 ± 0.76	**36.36 ± 1.26**	0.0001
ABCC2-siRNA-2	**57.78 ± 1.43**	11.79 ± 3.45	21.98 ± 2.64	**33.77 ± 0.92**	0.0072
ABCC2-siRNA-3	**59.65 ± 0.97**	9.34 ± 1.09	23.70 ± 0.91	**33.05 ± 1.36**	0.0052

Table 4-4. Data analysis of apoptosis assay with 100 µM oxaliplatin

Treatment	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value (Total apoptosis)
Control-siRNA	**61.53 ± 1.39**	7.67 ± 0.95	23.85 ± 1.95	**31.52 ± 2.59**	
ABCC2-siRNA-1	**44.05 ± 1.58**	9.90 ± 0.54	36.35 ± 1.94	**46.24 ± 1.49**	0.0001
ABCC2-siRNA-2	**46.37 ± 0.99**	10.80 ± 0.73	30.77 ± 1.58	**41.57 ± 1.11**	0.0030
ABCC2-siRNA-3	**41.55 ± 2.01**	13.73 ± 4.48	31.59 ± 3.21	**45.32 ± 1.28**	0.0001


Untreated
(III) Treated with 25 µM Oxaliplatin
(I)
Control-siRNA
(II)
(IV)
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3
D-+
N
V
Ap
PI
Annexin-V

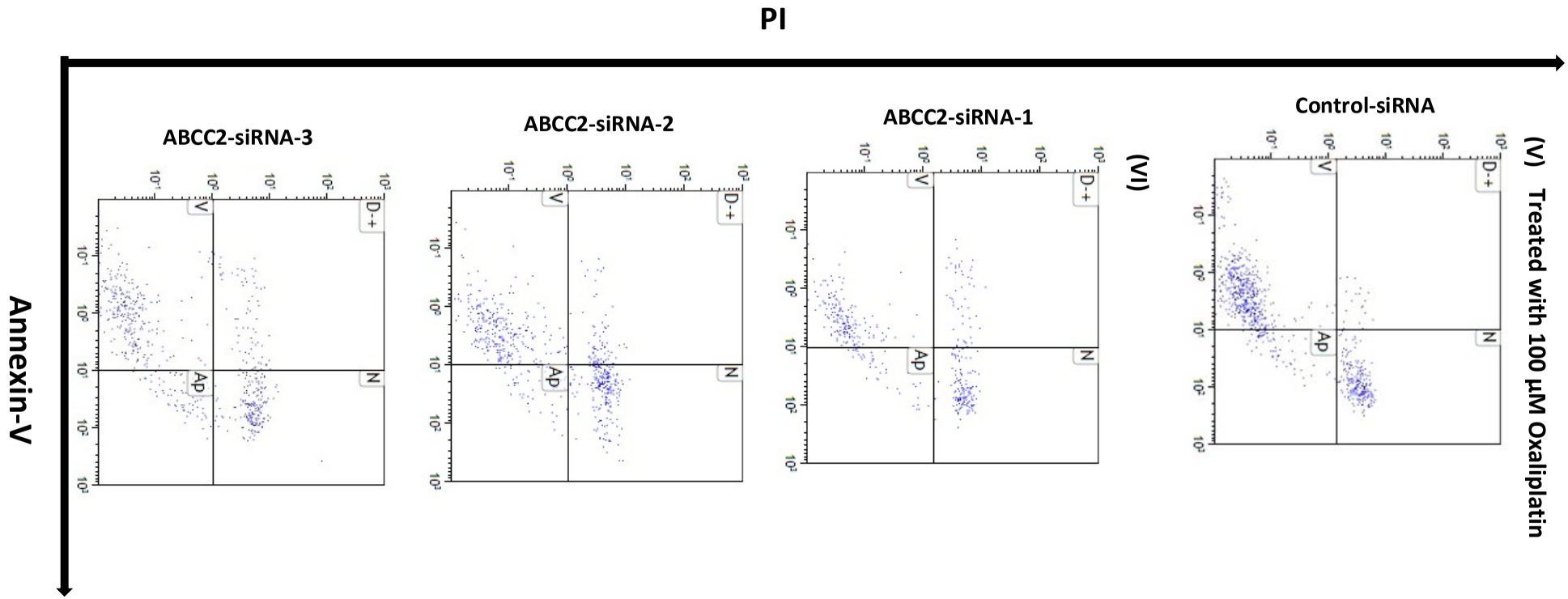
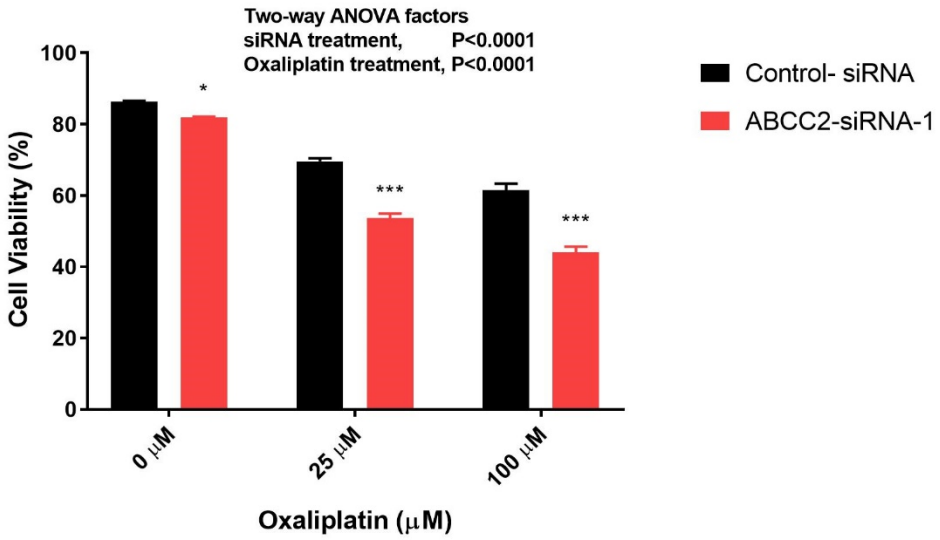


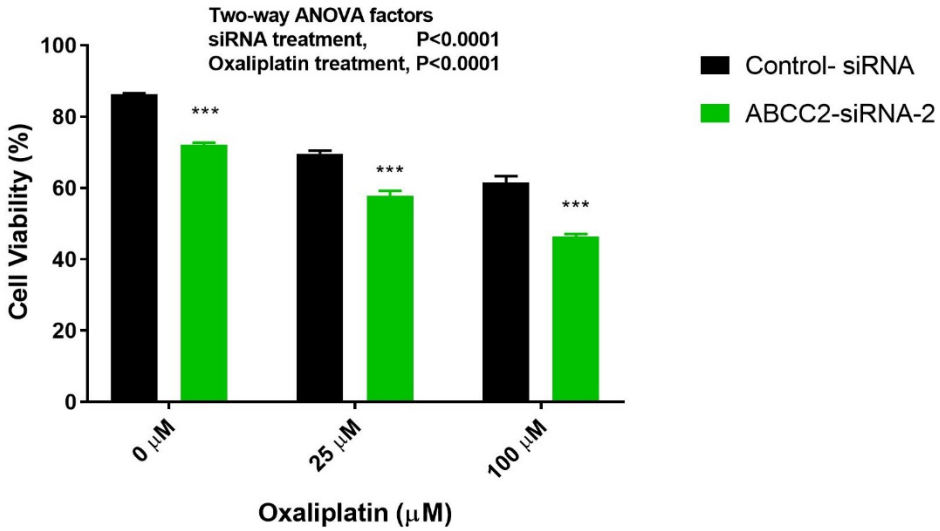
Figure 4.7. Apoptotic changes in HepG2 cells in the presence of oxaliplatin after siRNA transfection.

Detection of apoptosis by concurrent staining with Annexin-V and PI. HepG2 cells were transfected with three different subsets of ABCC2-siRNAs and control-siRNA and treated with oxaliplatin at different concentrations. Cells were subsequently stained with Annexin-V and PI and their fluorescence was measured by flow cytometry. Viable cells (V) are both Annexin-V and PI negative. At an early stage of apoptosis (Ap), the cells bind with only Annexin-V. At the late stage of apoptosis (N), the cells bind with both Annexin-V FITC and PI. The upper left quadrant (D-+) represent the debris cells or dead cells. (I) Control-siRNA transfected cells without any treatment, (II) MRP2-silencing HepG2 (ABCC2-siRNA-1, siRNA-2, siRNA-3) without any treatment, (III) Control-siRNA transfected cells with 25 µM oxaliplatin, (IV) MRP2-silencing HepG2 cells (ABCC2-siRNA-1, siRNA-2, siRNA-3) with 25 µM oxaliplatin, (V) Control-siRNA transfected cells with 100 µM oxaliplatin, and (VI) MRP2-silencing cells (ABCC2-siRNA-1, siRNA-2, siRNA-3) treated with 100 µM oxaliplatin.

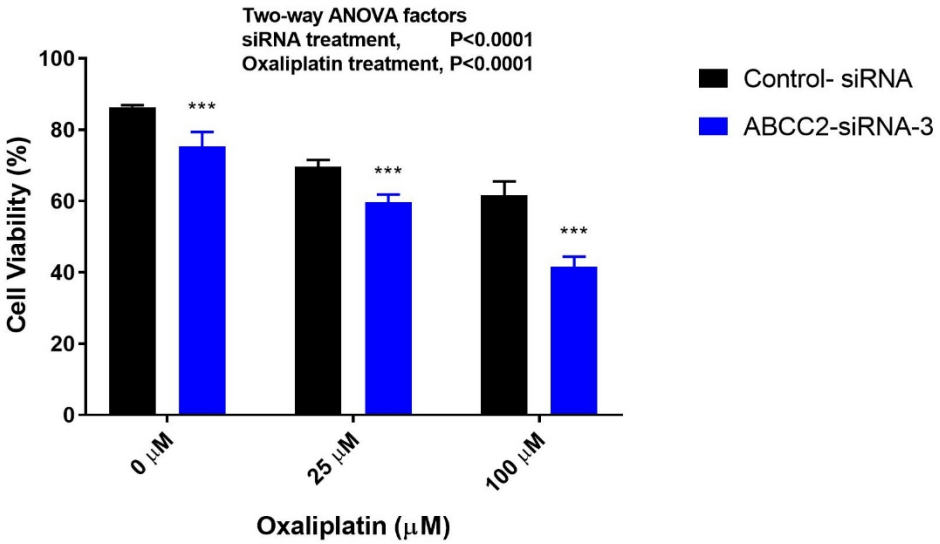
A.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell Viability (%)
100
80
60
40
20
0
*
***
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-1

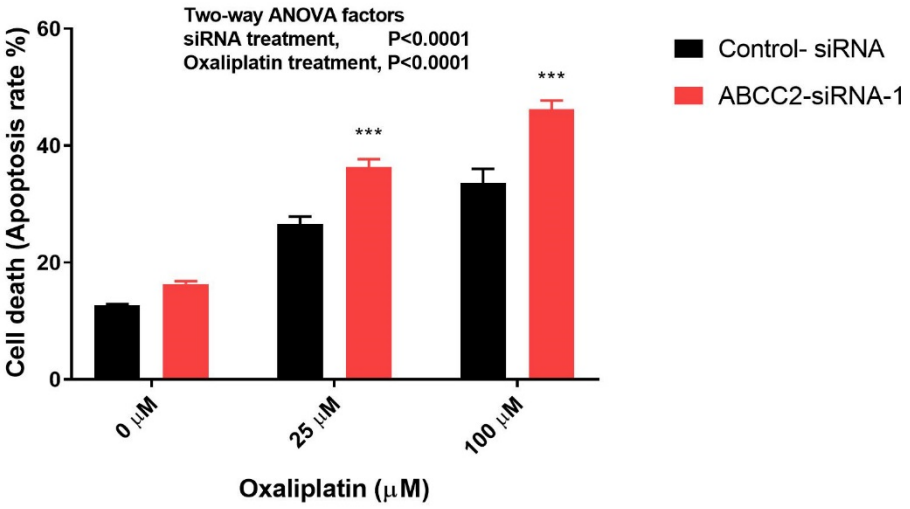
B.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell Viability (%)
100
80
60
40
20
0
***
***
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-2

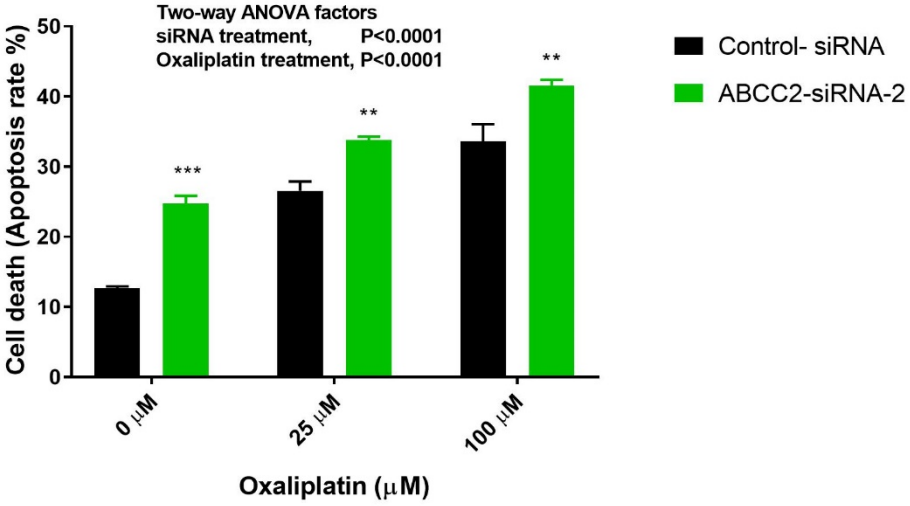
C.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell Viability (%)
100
80
60
40
20
0
***
***
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-3

D.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell death (Apoptosis rate %)
0
20
40
60
***
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-1

E.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell death (Apoptosis rate %)
0
10
20
30
40
50
***
**
**
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-2

F.

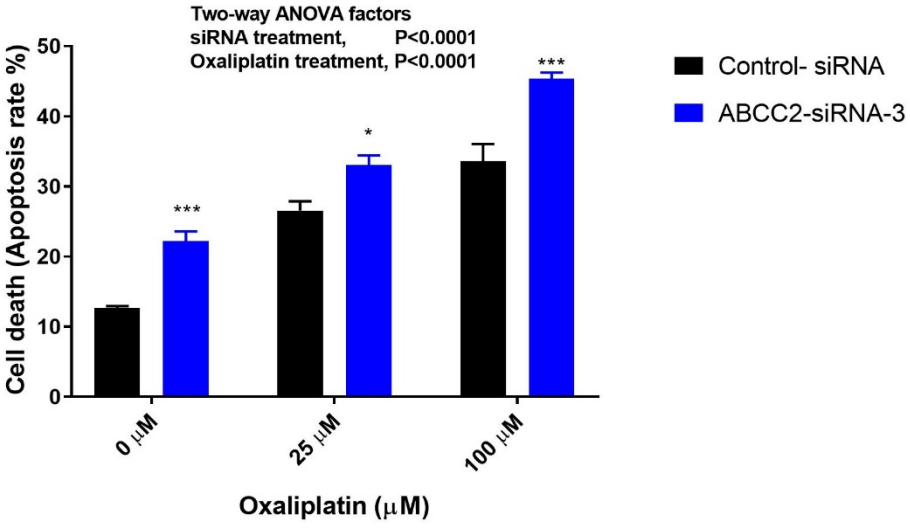

Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell death (Apoptosis rate %)
0
10
20
30
40
50
***
*
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-3

Figure 4.8. Oxaliplatin-induced apoptosis in control and ABCC2-siRNAs knockdown HepG2 cells.

HepG2 cells were treated with ABCC2-siRNA and control-siRNA for 48 hr followed by incubation in drug-free medium for 24 hr, then cells were treated with a range of different concentrations of oxaliplatin for 2 hr and stained with Annexin V/PI and rate of apoptosis was measured by flow cytometry. A, B and C represent viable cells percentage in control and ABCC2-siRNA-1, siRNA-2 and siRNA-3 transfected cells respecctively. D, E and F represent total apoptotic cells percentage in control and ABCC2-siRNA-1, siRNA-2 and siRNA-3 transfected cells respectively. Data are presented as mean and SEM of three independent experiments. Asterisks are P values (*, P<0.01; **, P<0.01; ***, P<0.001) from Sidak post-tests that followed a Two-way ANOVA (n=5).

4.4. Discussion

Gastrointestinal cancer is the most common cancer in both males and females; however, current therapeutic agents provide poor effective treatment [284]. Thus, there is a great need to define better therapeutic targets in the treatment of GI cancer. One of the major obstacles in successful cancer chemotherapy is the overexpression of one or more membrane transporters that protect cancer cells against the drug effects by active efflux of anticancer drugs out of the cytoplasm [285]. Researchers have demonstrated that expression of membrane transporters is usually higher in cancer cells than in normal cells [285-287]. One clinical study showed higher expression of membrane transporter ABCC2 in patients who were not responding to oxaliplatin, than patients who did respond to oxaliplatin chemotherapy [288]. Since overexpression of membrane exporters is one of the main mechanisms of multidrug resistance, current research is aimed at inhibiting or silencing the specific transporter for effective chemotherapy.

The purpose of this study was to investigate the sensitivity of oxaliplatin after silencing MRP2 in HepG2 cells. The previous study has provided direct evidence suggesting MRP2 mediates the efflux of oxaliplatin-derived platinum [176]. To the best of our knowledge, this is the first study that directly indicates the role of MRP2 and oxaliplatin sensitivity in human liver cancer HepG2 cells. The previous study provided MRP2 silencing data but focused on cisplatin sensitivity in human nasopharyngeal carcinoma cells [204]. Recently, Zheng et al., provided evidence that silencing MRP2 enhanced the sensitivity of adriamycin-resistant HepG2/ (ADM) cells to oxaliplatin, doxorubicin, 5-FU and vinblastine [289]. However, neither the cellular accumulation of oxaliplatin-derived platinum nor the off-target effects of MRP2 silencing by siRNA were assessed in Zhang et al.'s study. Thus, a more precise research design was required to assess the role of MRP2 in oxaliplatin transport and sensitivity in liver cancer cells.

In this study, three different ABCC2-siRNAs were transfected into HepG2 cell lines by reverse transfection techniques to downregulate MRP2 gene in hepatocellular carcinoma cells. After transfection, we sought to determine the cellular and functional studies in MRP2-silencing HepG2 cells. To validate the MRP2 transport activity, a fluorescent probe, 5(6)-carboxy-2, '7'-dichlorofluorescein (CDCF), was used as an MRP2 substrate in the functional study. Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the cellular accumulation of platinum in this study. ICP-MS is considered a highly sensitive tool for specific detection of metals like platinum in biological matrices [290]. From the kinetic analysis of oxaliplatin degradation in the

membrane vesicle incubation study, oxaliplatin degradation half-life is 2.24 hrs [176]. Therefore, the MRP2-silencing HepG2 cells were incubated with oxaliplatin for 2 hr in our research.

According to Khine et al., intact oxaliplatin and its anionic monochloro oxalate ring-opened early degradation product is the main substrate for MRP2-mediated active transport of oxaliplatin-derived platinum [176]. Therefore, the hypothesis of our study is that MRP2 contributes to oxaliplatin resistance at the cellular level in HepG2 cells and silencing MRP2 can significantly increase the cellular accumulation of oxaliplatin-derived platinum and/or reverse oxaliplatin drug resistance.

We observed a significant difference in ABCC2 mRNA expression level in the control and ABCC2-siRNA transfected HepG2 cells. The siRNA gene silencing was successful, reaching 42% to 59% gene-knockdown of ABCC2 in HepG2 cell line. In contrast, ABCC2-siRNA transfected cells had no significant effect on the mRNA expression of the other membrane transporters and enzymes related to platinum transports compared to control HepG2 cells. This result indicates that siRNA was successful in silencing ABCC2 expression with negligible off-target effects. However, we observed upregulation of ABCC3, ABCC4, ABCC5, ABCG2 and ATP7A gene in ABCC2-siRNA-1 transfected HepG2 cells and a slight increase in expression of ABCC4 and ABCC5 gene in ABCC2-siRNA-2 transfected HepG2 cells. One of the possible explanations is the use of a high concentration of siRNA. RNAi is often effective at minimal concentrations and using a high concentration of siRNA has been suggested to increase the unwanted side effects [291]. In this study, we increased the siRNA concentration to achieve effective MRP2 knock down in HepG2 cells. The upregulation of certain ABC genes in ABCC2-siRNAs transfected HepG2 cells suggested that the effects we observed were likely to be caused by using siRNA at high concentrations. The cell surface staining experiment showed that after siRNA gene silencing, not only was ABCC2 expression reduced at mRNA level, but also the MRP2 transporter surface expression was downregulated.

The functional activity of ABCC2 gene knockdown cells was confirmed by the MRP2-specific substrate CDCF uptake study. Our results showed that CDCF accumulation significantly increased in the HepG2 cells after siRNA transfection. This means that the silencing of ABCC2 increased the substrate accumulation and this represents the decreased activity of ABCC2 efflux pump. In the ICP-MS platinum accumulation study, we observed that platinum accumulation increased in the ABCC2 silencing

HepG2 cells compared with the control HepG2 cells. After 2 hr incubation with oxaliplatin in ABCC2 silencing and control HepG2 cells, platinum accumulation increased by 52% to 186% in transfected HepG2 cells. This finding suggests that accumulation of platinum mediated by ABCC2 was enhanced in ABCC2 silencing HepG2 cells.

To examine whether ABCC2 silencing has an influence on cancer cell susceptibility to oxaliplatin, IC_{50} values were determined in ABCC2-siRNAs transfected cells. We observed that IC_{50} values significantly decreased in the ABCC2 silencing cells compared to control cells. Clinically achievable oxaliplatin plasma concentrations during 2 hr infusion of oxaliplatin was from 3.75 to 11.25 μM [76]. After ABCC2 gene knockdown, the oxaliplatin IC_{50} doses were similar or lower than the possible plasma drug concentration range in HepG2 cells. This suggests that knockdown of ABCC2 expression during chemotherapy can reverse multidrug resistance.

Similarly, in the apoptosis assay, we observed a more apoptotic cell population in early apoptosis (Annexin V^+/PI^-) and late apoptosis (Annexin V^+/PI^+) although the rate of apoptosis increased with increased concentration of oxaliplatin in control HepG2 cells also. However, after knockdown of MRP2 in HepG2 cells, the apoptosis rate induced by oxaliplatin had almost increased by 10% to 15% in ABCC2-siRNAs transfected cells compared to control-siRNA transfected cells. These results correspond to our findings that oxaliplatin-induced apoptosis in knockdown HepG2 cells was elevated because of increased cellular accumulation of oxaliplatin-derived platinum in transfected HepG2 cells. Previously, it has been reported that introduction of MRP1-4 siRNA duplex decreased the corresponding membrane transporters' expression in HepG2/ADM cells with increased cell apoptosis to oxaliplatin [289]. We also found that modulation of MRP2 results from ABCC2-siRNA increased oxaliplatin-induced apoptosis.

In summary, our results show that knockdown of the ABCC2 transporter using RNAi is a novel approach in cancer chemotherapy. Targeting specific membrane transporters could decrease anticancer drug doses, thereby reducing the risks of side effects. However, these *in vitro* studies should be confirmed using *in vivo* studies and more studies related to other GI cancer cells with high expression of ABCC2 are required.

4.5. Conclusion

Silencing the ABCC2 membrane transporter using siRNA significantly increased platinum accumulation and increased the sensitivity of oxaliplatin-derived platinum anticancer drug in HepG2 cells.

Chapter 5 Silencing MRP2 in Caco-2 and PANC-1 cells

5.1. Introduction

Colorectal cancer (CRC) is the third most common cancer throughout the world in both males and females and is considered to be the second leading cause of cancer death [5, 292]. In New Zealand, along with prostate cancer, colorectal cancer is the most commonly diagnosed cancer in adults with around 3,000 new cases per year. New Zealand has one of the highest rates of colorectal cancer and death from colorectal cancer in the world [293]. The front-line drugs approved for the treatment of CRC with the best response and highest overall survival (OS) are 5-Fluorouracil (5-FU), irinotecan and oxaliplatin [294]. The combination of these chemotherapeutic drugs has been approved to treat CRC. The most popular combination therapies are FOLFIRI (folinic acid, 5-FU and irinotecan) [295] and FOLFOX (folinic acid, 5-FU and oxaliplatin) [37, 49, 296]. Clinically, FOLFOX has been reported to be more efficient than FOLFIRI in terms of increased disease-free survival and OS [49, 93, 297]. Moreover, the combination of cetuximab with oxaliplatin-based combination chemotherapy has shown greater efficiency compared with standard treatment with FOLFOX [294, 298]. Although a wide range of chemotherapeutic drugs and other targeted approaches have been developed to treat CRC, chemoresistance remains one of the major challenges for the successful treatment of CRC [298, 299]. The elucidation of the mechanisms responsible for chemoresistance in CRC is important for the successful treatment of CRC.

Similarly, pancreatic adenocarcinoma is one of the major leading causes of cancer death throughout the world with poor prognosis [284, 300] due to the fact that most pancreatic cancer patients are diagnosed at an advanced stage. Only a few patients are eligible for surgical resection. Therefore, chemotherapy remains the only treatment method for pancreatic cancer patients with non-resectable diseases [301, 302]. A fluorinated analogue of deoxycytidine, gemcitabine, is the main chemotherapeutic drug used as the first-line agent for the treatment of pancreatic cancer. However, gemcitabine treatment alone has not led to improved therapeutic efficiency. Combination regimens, such as gemcitabine and platinum agents like cisplatin, have also been used as standard regimens for patients with advanced pancreatic cancer; however, these treatments have not improved prognoses [35, 196, 303]. In recent years, oxaliplatin-based FOLFIRINOX (5-FU, leucovorin, irinotecan and oxaliplatin) and GEMOXEL

(gemcitabine, oxaliplatin and capecitabine) regimens have been commonly used to treat pancreatic cancer, which have improved the survival status of patients [31-33, 304, 305]. Although patients' performance status has been improved with FOLFIRINOX treatment, the therapeutic efficiency had not been improved due to the chemoresistance of the regimen [306, 307]. One of major mechanisms of chemoresistance development is the increased expression of ABC transporters like MDR1, MRP1, MRP2 or BCRP [142, 306, 308]. These transporters play a pivotal role in exporting xenobiotics as well as drugs out of cancer cells, thereby reducing their efficiency [142]. Overall, response to different chemotherapeutic regimens is limited due to the development of drug resistance in pancreatic cancer [142, 301, 309].

In addition, different molecular mechanisms, such as alteration in pathways controlling apoptosis that increase the cancer cells' survival and suppress apoptosis [310-312], changes in expression in insulin-like growth factor receptors (IGF-IR) [313], or mutations in nuclear factor-erythroid 2 p45 related factor 2 (Nrf2) can regulate the gene expression of MRP2 [314, 315]. The efflux transporter, MRP2, has been expressed both at mRNA and protein level in human colorectal cancer cells and in tumour tissue of colon cancer patients [184, 187, 200, 316]. Overall, response to chemotherapeutic regimens is limited due to development of drug resistance mainly by MRP2 transporter in CRC [317].

Different studies have reported the association of MRP2 transporter expression with tumour resistance in gastrointestinal cancer patients receiving platinum-based chemotherapy [196, 200, 212, 215, 278]. In addition, MRP2 protein expression was detectable in most pancreatic cancer patient tumour tissue samples, but not in normal tissues [196]. One of the clinical studies evaluated the association between SNPs of MRP2 and patients' outcomes with pancreatic cancer. According to this study, pancreatic cancer patients have shown significant association of MRP2 G40AA GG genotype receiving gemcitabine or gemcitabine with cisplatin therapy with low overall survival rate and poor tumour response [216]. These studies suggested that MRP2 plays an important role in the chemoresistance in pancreatic cancer patients receiving platinum-based chemotherapy. According to different studies, a significant correlation was observed between MRP2 gene polymorphism and CRC patients receiving oxaliplatin-based chemotherapy [212, 318]. Genetic polymorphism in the sequence of MRP2 gene can affect therapeutic response, toxicity and survival of patients. One of the

studies has shown significant association of MRP2 G1249A polymorphism and CRC patients receiving FOLFOX-4 chemotherapy [212]. This study demonstrated that MRP2 G1249A is a predictive marker for the survival of patients with stage II/III CRC treated with FOLFOX-4 therapy [212].

According to randomised controlled trials (RCTs) of gemcitabine monotherapy versus gemcitabine in combination with platinum compounds in pancreatic cancer patients, platinum-based combination chemotherapy showed an improved overall response rate (ORR) but failed to improve overall survival (OS) significantly [319]. Although several studies reported that oxaliplatin-based combination chemotherapy conferred a significant response to patients' survival rate [34, 320, 321], some studies showed that platinum-based chemotherapy failed to improved OS [320, 322, 323]. The reason for poor OS may be an association of MRP2 with increased resistance to platinum compounds. It is still undetermined whether MRP2 is associated with chemoresistance in pancreatic cancer patients receiving platinum-based combination chemotherapy. Hence, it will be interesting to further investigate the role of MRP2 in cellular resistance of oxaliplatin in pancreatic cancer.

Oxaliplatin-based chemotherapy has shown *in vitro* and *in vivo* antitumor activities in CRC [95]. The combination of oxaliplatin and 5-FU in the FOLFOX regimen significantly improved patients' response rate with metastatic CRC [37, 324, 325]. Other clinical studies demonstrated that administration of oxaliplatin to 5-FU improved the adjuvant treatment of stage III CRC by increasing OS and reducing the risk of recurrence [49, 326]. However, nearly half the patients receiving adjuvant chemotherapy failed to eradicate CRC and did not benefit from oxaliplatin [37, 325]. Oxaliplatin forms DNA-platinum adducts in DNA double strands, resulting in the inhibition of DNA replication and transcription, and induces apoptosis [48]. In contrast to another platinum-based chemotherapeutic agents, oxaliplatin-induced adducts are not recognised by the mismatch repair (MMR) system and are predominantly repaired by nucleotide excision repair (NER) and base excision repair (BER) [327, 328]. Therefore, the main reason for the failure of oxaliplatin-based chemotherapy may be because of enhanced DNA repair efficiency or decreased accumulation of oxaliplatin, which contributes to chemoresistance in GI cancer. The cellular accumulation of platinum-based chemotherapeutic agents is considered a significant factor in determining drug sensitivity. Previous studies reported that membrane transporters play an important

factor underlying the intracellular accumulation and efficacy of anticancer drugs [142, 329]. A recent study demonstrated that the membrane transporter MRP2 is involved in the cellular efflux of oxaliplatin [176]. Thus, in the context of CRC, the tumour expression of MRP2 may affect the response to oxaliplatin-based chemotherapy.

In the previous chapter, we demonstrated that silencing the MRP2 transporter increased cellular platinum accumulation and sensitivity to oxaliplatin in an MRP2-overexpressing liver cancer HepG2 cell line. Studies have linked cellular resistance to oxaliplatin with high expression of MRP2 in human liver cancer [330]. MRP2 has been reported to overexpress in a number of cisplatin-resistance cancer cell lines, including hepatocellular carcinoma, prostate cancer, colon cancer, ovarian cancer, adrenocortical cancer [181, 197, 280]. Overall, there are limited *in vitro* studies investigating the contribution of MRP2 in cellular resistance to oxaliplatin and the underlying mechanisms in CRC and pancreatic cancer. This chapter will elucidate the association between MRP2 and oxaliplatin chemoresistance in colorectal and pancreatic cancer cells. MRP2 has been reported to overexpress in platinum-resistant colorectal cancer cells. Given oxaliplatin is a favoured drug for treatment of CRC and pancreatic cancer, it is of clinical implication to further investigate the role of MRP2 in cellular resistance of oxaliplatin by silencing MRP2 using siRNA in colorectal and pancreatic cancer cells.

According to previous chapters, oxaliplatin is a substrate of MRP2 (Chapter 3) and silencing MRP2 significantly increases the cellular accumulation of oxaliplatin-derived platinum in hepatocellular cancer cells (Chapter 4). MRP2 is responsible for efflux of oxaliplatin in inside-out membrane vesicles [176]. In addition, the MRP2 expression level was high in pancreatic and colorectal cancer tumour samples. Therefore, we hypothesise that MRP2 contributes to oxaliplatin resistance at the cellular level in GI cancer and silencing MRP2 gene like in the previous chapter can increase oxaliplatin accumulation in Caco-2 and PANC-1 cell lines and/or reverse chemoresistance in GI cancer patients receiving oxaliplatin-based combination chemotherapy.

The main aim of the present study was to examine the expression profile of MRP2 after silencing the MRP2 gene with three sets of ABCC2-siRNAs and to evaluate the correlation between MRP2 expression and oxaliplatin resistance in colorectal and pancreatic cancer cells.

The aims of the experiments conducted in this chapter were:

I. To knockdown the expression of MRP2 gene in Caco-2 and PANC-1 cells after ABCC2-siRNA transfection.
II. To verify any off-target effects after transfection in other transporter genes and enzymes.
III. To measure the cellular accumulation of CDCF in MRP2-silencing Caco-2 and PANC-1 cells.
IV. To examine the cellular cytotoxicity of oxaliplatin in MRP2-silencing Caco-2 and PANC-1 cells.
V. To measure the cellular accumulation of oxaliplatin-derived platinum in MRP2-silencing Caco-2 cells.
VI. To determine oxaliplatin-induced apoptosis in MRP2-silencing Caco-2 and PANC-1 cells at a different concentration of oxaliplatin.

5.2. Materials and methods

5.2.1. Drugs and Chemicals

A solution of oxaliplatin (5 mg/ml) was freshly prepared in Milli-Q water and stock solutions were aliquot, stored at -20°C and discarded one month after preparation. The details of chemicals and reagents, sources and their stock solutions preparation were described in chapter 2, section 2.1.

5.2.2. Transfecting stealth siRNA into GI cancer cells

Like the previous chapter, the transfection of stealth siRNA into Caco-2 and PANC-1 was carried out using Lipofectamine RNAiMAX. Reverse transfection was used for transfecting Caco-2 cells. Forward transfection was used for transfecting PANC-1 cells. Three subsets of MRP2-specific siRNAs, ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 and control-siRNA (Stealth RNAi negative control siRNA) were used in this study. The siRNA concentration and assay times have been adjusted to establish optimal knockdown of MRP2 gene. Finally, 20 µM of ABCC2-siRNAs for 48 hr was used for transfection. Before mixing lipofectamine and ABCC2-siRNAs, RNAi duplexes and Lipofectamine RNAiMAX were diluted in Opti-MEM I Reduced Serum Medium. The transfection procedure was carried out in 12-well plate.

5.2.2.1. Reverse transfection in Caco-2 cells

In reverse transfection, the Lipofectamine RNAiMAX and ABCC2-siRNA complexes were prepared inside the wells. Thereafter, cells and medium were added. For each well to be transfected, 40 pmol of ABCC2-siRNAs were added to 100 µl of Opti-MEM I Medium without serum in the plate with gentle mixing. For each well, 2 µl of Lipofectamine RNAiMAX was added into 100 µl Opti-MEM I Medium with gentle mixing. The Lipofectamine RNAiMAX complex was added to each well containing the diluted ABCC2-siRNA. The Lipofectamine RNAiMAX and ABCC2-siRNAs complexes were mixed gently and incubated for 15 min at room temperature. Caco-2 cells were diluted in complete growth cell density of 150,000 cells in 1 ml. To each well of ABCC2-siRNA and Lipofectamine RNAiMAX complexes, 1 mL of diluted cells were added. This would give a total volume of 1.2 ml solution and a final RNA concentration of 34 nM. The plate was gently mixed back and forth to mix the complexes with diluted cells properly. The cells were incubated for 48 hr at 37°C in a 5% CO_2 incubator. The transfection complexes were removed after 24 hr and replaced with complete growth medium. After 48 hr incubation of cells with ABCC2-siRNAs, Caco-2 cells were used for the different experimental assays.

5.2.2.2. Forward transfection in PANC-1 cells

In forward transfection, firstly cells were plated in the wells and the transfection reagents are added the next day. The transfection procedure was carried out in 12-well plate. One day before transfection, the cells were plated in a 12-well plate in 1000 µl of growth medium without antibiotics so they would be around 30-50% confluent at the time of transfection. The next day, siRNA and Lipofectamine RNAiMAX complexes were prepared separately. For each well to be transfected, 30 pmol of ABCC2-siRNAs were added in 100 µl Opti-MEM I Reduced Serum Medium without serum and mixed gently. Then 2 µl of Lipofectamine RNAiMAX were added to 100 µl of Opti-MEM I Reduced Serum Medium with gentle mixing. The diluted siRNA complexes and diluted Lipofectamine RNAiMAX were combined together, mixed gently and incubated for 15 min at room temperature. The siRNA-Lipofectamine RNAiMAX complexes were added in each well containing cells. This would give a total volume of 1.2 ml solution and a final RNA concentration of 25 nM. The plate was gently mixed back and forth to mix the complexes properly. The cells were incubated for 48 hr at 37°C in a 5% CO_2 incubator. The transfection complexes were removed after 24 hr and replaced with

complete growth medium. After 48 hr incubation of cells with ABCC2-siRNAs, PANC-1 cells were used for the different experimental assays.

Table 5-1. Details of reagent amounts and volumes

Cell Line	Culture plate	Rel. surface area (cm^2)	The volume of plating medium	Cells density	ABCC2-siRNA amount (pmol)	Final siRNA duplex concentration (nM)	Lipofectamine RNAiMAX amount (µl)
Caco-2	12-well	4	1000 µl	150,000	40	34	2
PANC-1	12-well	4	1000 µl	100,000	30	25	2

5.2.3. Cell surface staining to MRP2

The MRP2 transporter surface expression in GI cancer cells was assessed by staining the MRP2 transporter in Caco-2 and PANC-1 cells with the anti-MRP2 primary antibody. The non-specific MRP2 staining was also determined using a host-species matched IgG2a isotype control. Both antibodies were conjugated with Alexa Fluor 488 secondary antibody.

To determine MRP2 surface expression in GI cancer cells, Caco-2 and PANC-1 cells were grown in a T_{75} flask and cells were trypsinised and collected in an Eppendorf tube at a density of 1×10^6 per ml. The cell samples then allowed for fixation, permeabilisation and blocking according to the procedure mentioned in section 2.10.1. The experiment was repeated twice independently.

For determining the MRP2 transporter expression in ABCC2-siRNA transfected GI cancer cells, cells were first transfected with ABCC2-siRNAs and control-siRNA at a density of 250,000 cells per well in a 6-well plate for 48 hr. After transfection, 50×10^4 cells were collected for each transfected cell and washed with ice-cold PBS followed by centrifugation at $500 \times g$ for 5 min. The cells pellets were resuspended in 100 µl of 1% paraformaldehyde in PBS and incubated for 15 min on ice. Thereafter, the process of permeabilisation, blocking, and staining with primary and secondary antibodies was carried out according to the procedure mentioned in section 2.10.1.

5.2.4. Real-time quantitative PCR

After 48 hr transfection with siRNAs, total RNA from each sample was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol as described in the section 2.4.1. Real-time qPCR was then performed to measure the relative gene expression of the target gene and other transporter genes.

For ABCC2-siRNAs knockdown studies, mRNA expression of the target gene ABCC2 (MRP2 gene), other transporter ABC transporter genes and housekeeping genes were quantified using real-time quantitative qPCR. Primers used in this experiment are listed in table 3-3. Quantitative real-time PCR was performed at LC480 LightCycler using LightCycler-FastStart DNA Master SYBR Green 1 Master Mix (Roche Applied Science) and gene-specific primers at 180 nM. The reaction details were described in section 2.4.3.

The mRNA expression levels of genes of interest in each sample were calculated as relative gene expression and represented as $2^{-\Delta\Delta Ct}$ values of corresponding genes. Firstly, ΔCt values were calculated by subtracting the Ct values of house-keeping genes, i.e., glyceraldehyde 3-phosphate dehydrogenase (GAPDH), from the Ct values of the gene of interest. Finally, ΔΔCt values were calculated by subtracting the average ΔCt value of control-siRNA from the ΔCt values of the gene of interest. The MRP2 knockdown in the expression levels of genes of interest in ABCC2-siRNA treated cells compared to control-siRNA cells were then calculated by dividing the value obtain by subtracting $2^{-\Delta\Delta Ct}$ values of gene of interest from $2^{-\Delta\Delta Ct}$ value of control siRNA by that of $2^{-\Delta\Delta Ct}$ value of control siRNA for each gene.

5.2.5. Cellular uptake of CDCF

After 48 hr incubation of Caco-2 and PANC-1 cells with ABCC2-siRNAs and control-siRNA, the functional activity of MRP2 transporter was determined in MRP2-silencing PANC-1 cells. After transfection, the cells were allowed to incubate for another 24 hr at 37°C in a 5% CO_2 incubator. After that, ABCC2-siRNAs and control-siRNA transfected cells were incubated with 5 µM of CDCF for 10 min followed by measurement of fluorescence intensity in flow cytometry as mentioned in section 2.5.2. Experiments were repeated three times independently.

5.2.6. Drug sensitivity assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was used to determine the cytotoxicity of oxaliplatin in ABCC2-siRNAs and control-siRNA transfected Caco-2 and PANC-1 cells. After 48 hr incubation with siRNAs, 5000 and 8000 cells per well were seeded in a 96-well plate for Caco-2 and PANC-1 respectively. The transfected cells were incubated for 24 hr in a normal drug-free medium. After 24 hr, incubated cells were exposed to oxaliplatin for 2 hr followed by replacing the medium with normal growth medium with antibiotics for three days. Further details of MTT assay are in section 2.7. Experiments were repeated two times independently.

5.2.7. Cellular accumulation of platinum

Cells were seeded at 250,000 cells per well in a 6-well plate and transfected with ABCC2-siRNA and control-siRNA. After 48 hr transfection, Caco-2 cells were grown in normal growth medium for 48 hr. Then cells were incubated with 25 µM and 100 µM oxaliplatin for 2 hr. After incubation with oxaliplatin, cells were washed with ice-cold PBS followed by processing of cells for the ICP-MS assay. The protein concentration of the cell lysates was measured using nitric acid as described in section 2.6.1. The ICP-MS procedure and method validation were carried out as described in section 2.6. The validation of elements including accuracy, the precision of the method, as well as the limit of detection (LOD) and lower limit of quantification (LLOQ) was determined according to the US FDA guidelines for bioanalytical method validation. The accuracy and precision of the method were established by preparing three replicates of quality control (QC) samples referred to as lower QC, middle QC and upper QC over the standard curve range. The ICP-MS analysis run was acknowledged only if the standard curve was linear and the QC samples over the standard curve were within 15% precision values and accuracy should be between 85% to 115%. In this experiment, the linearity of the ICP-MS platinum standard curve of the experiment was defined by r^2 = 0.99. The average precision and accuracy of the QC samples in the experiment were 6% and 99.56% respectively.

5.2.8. Oxaliplatin-induced apoptosis in the transfected cells

Apoptosis induced by oxaliplatin in ABCC2-siRNAs transfected Caco-2 and PANC-1 cells were measured by flow cytometry. Caco-2 and PANC-1 cells were seeded at a density of 250,000 cells per well in 6-well plate and transfected with the required ABCC2-siRNAs and control-siRNA for 48 hr. After transfection, the cells were treated

with 25 μM and 100 μM of oxaliplatin for 2 hr. The cells were washed with ice-cold PBS and cultured with complete growth medium for 48 hr at 37°C in a humidified atmosphere of 5% CO_2. The sample preparation and analysis methods for flow cytometry detection of apoptosis percentage were described in detail in section 2.8.

5.2.9. Data analysis

Flow cytometry data analysis was carried out according to the details mentioned in section 2.11. Statistical analysis was performed according to the details as mentioned in section 2.12.

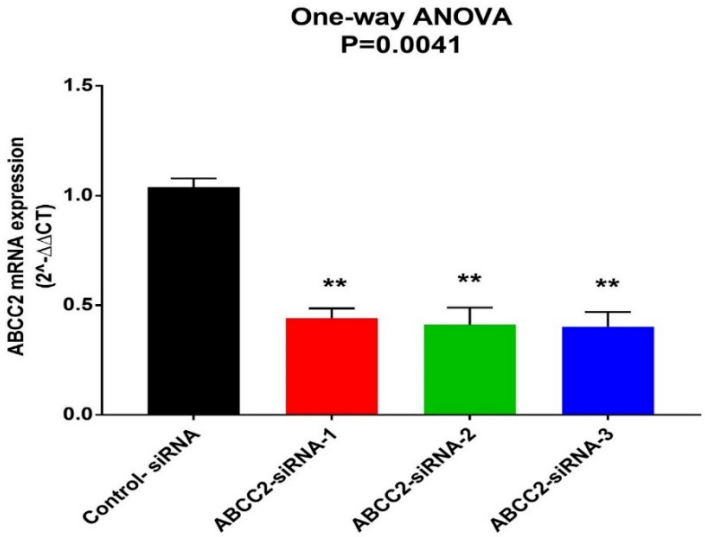
5.3. Results

5.3.1. MRP2 expression in ABCC2-siRNAs transfected GI cancer cells

The expression of MRP2 mRNA in mock and ABCC2-siRNA-transfected Caco-2 cells was confirmed by qRT-PCR. The expression of the mRNA transcripts of MRP2 gene was much lower in ABCC2-siRNA-transfected cells compared with the control-siRNA transfected cells. After analysing real-time PCR results, MRP2 knockdown percentage compared to control-siRNA transfected Caco-2 cells was around 56%, 59% and 60% in ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 respectively (Figure 5.1(A)).

For PANC-1 MRP2 mRNA expression, we found that control-siRNA PANC-1 cells ABCC2 expression was higher than the ABCC2-siRNA transfected cells. There was no significant change in MRP2 gene expression between PANC-1 and control-siRNA PANC-1 cells. Whereas, after analysing real-time PCR results, ABCC2 mRNA expression was decreased by 50%, 70% and 72% in PANC-1 cells transfected with ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 respectively (Figure 5.1(B)).

A.



B.

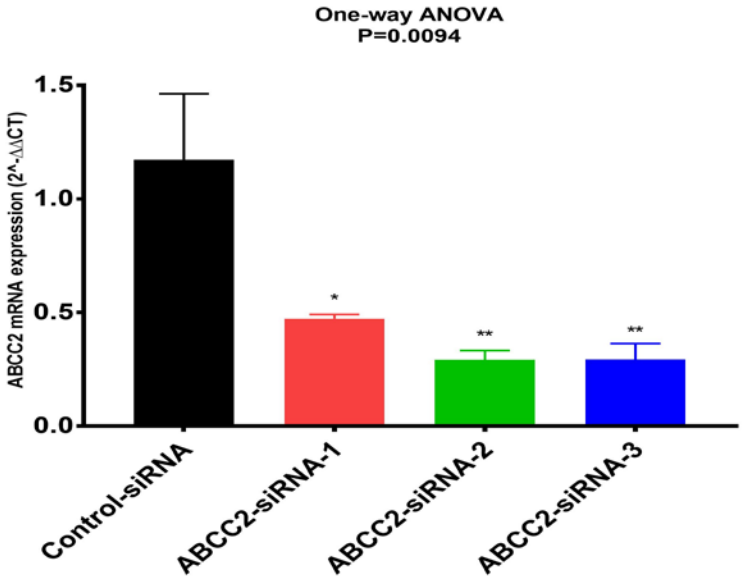


Figure 5.1. ABCC2 mRNA expression level in control and ABCC2-siRNAs knockdown Caco-2 and PANC-1 cells.

20 µM of ABCC2-siRNA-1, ABCC2-siRNA-2, ABCC2-siRNA-3 and control-siRNA were transfected in Caco-2 and PANC-1 cells. Relative ABCC2 mRNA expression was detected by real-time qPCR. Data are presented as relative gene expression $2^{-\Delta\Delta CT}$ averaged from three independent experiments for (A) Caco-2, and (B) PANC-1 cells. The bar represents the mean and S.E.M. of the mean values from at least three independent experiments. Asterisks are P values (*, P<0.05; **, P<0.01) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=3).

5.3.2. MRP2 transporter surface expression in Caco-2 cells

The cell surface staining was assessed by staining the Caco-2 cells with anti-MRP2 primary and control isotype IgG2a antibody. The fluorescence intensity was increased by 2-fold in Caco-2 cells stained with anti-MRP2 antibody as compared with cells stained with isotype control (shown in Appendix II). This represents the presence of MRP2 transporter on the surface of Caco-2 cells.

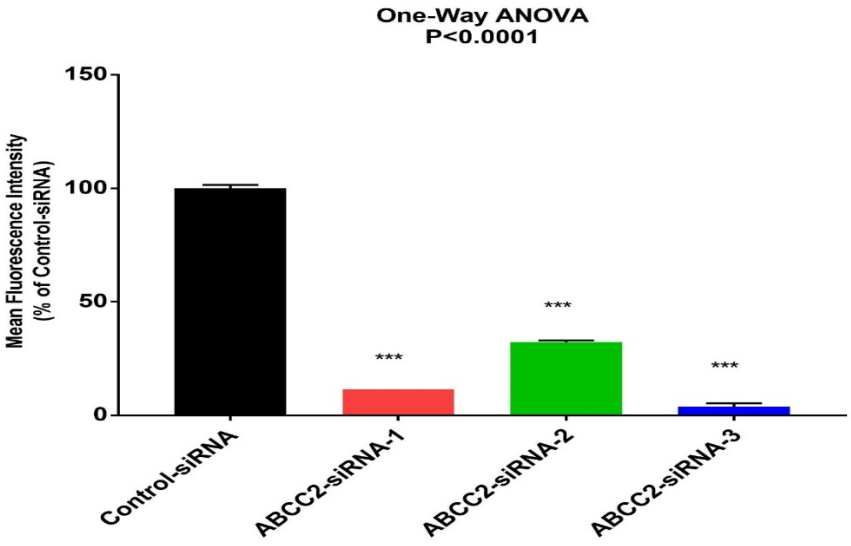
To determine if silencing the MRP2 gene decreased the surface expression of MRP2 in the cells, cell surface staining was conducted in the Caco-2 cells after transfecting the cells with ABCC2-siRNA and control-siRNA for 48 hr. Figure 5.2(A) shows MRP2 cell surface expression in ABCC2-siRNA transfected Caco-2 cells compared with the fluorescence intensity in control-siRNA transfected cells. Knockdown of MRP2 gene using siRNA1, siRNA2 and siRNA3 decreased MRP2 surface expression by 70-89% in Caco-2 cells. Therefore, silencing MRP2 gene showed downregulation of MRP2 surface expression in Caco-2 cells.

5.3.3. MRP2 transporter surface expression in PANC-1 cells

For PANC-1 cells also, the fluorescence intensity was increased by 1-fold in cells stained with anti-MRP2 antibody as compared with cells stained with isotype control (shown in Appendix III). This represents the presence of MRP2 transporter on the surface of PANC-1 cells.

After ABCC2-siRNAs transfection in PANC-1 cells, MRP2 cell surface expression in ABCC2-siRNAs transfected Caco-2 cells was compared with the fluorescence intensity in control-siRNA transfected cells. Knockdown of MRP2 gene using siRNA1, siRNA2 and siRNA3 decreased MRP2 surface expression by 50-60% compared with the control-siRNA PANC-1 cells. Thus, knockdown of MRP2 gene downregulated the surface expression of the MRP2 transporter in PANC-1 cells. Figure 5.2(B) shows MRP2 cell surface expression in siRNA transfected PANC-1 cells compared with the control-siRNA transfected cells in terms of fluorescence intensity.

A.



B.

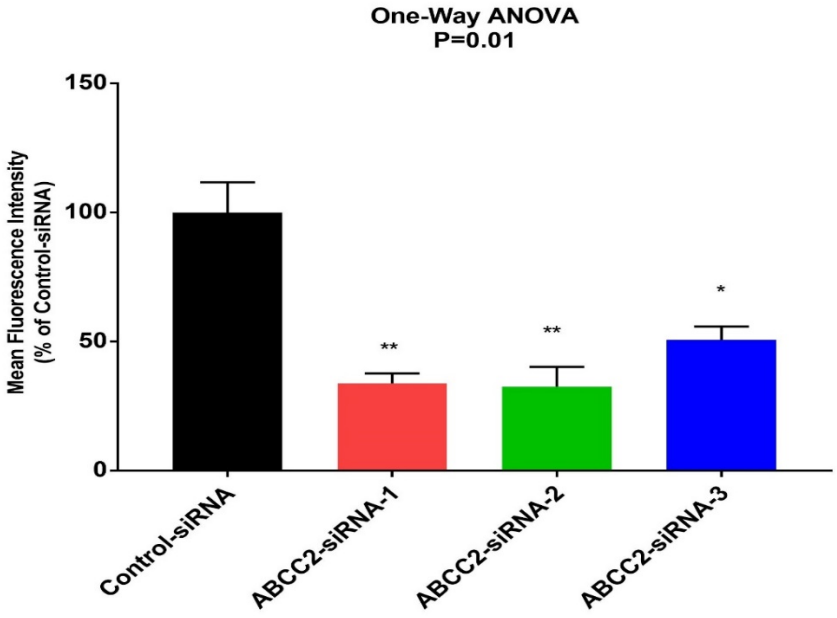


Figure 5.2. Cell surface expression of MRP2 in control and ABCC2-siRNAs transfected GI cancer cells.

The data is presented as a mean percentage of fluorescence in (A) Caco-2 and (B) PANC-1 cells. MRP2 surface expression was detected with the MRP2 primary antibody and Alexa Fluor 488 secondary antibody, followed by flow cytometry to measure the fluorescence intensity. Results are mean ± SEM of replicate samples. Asterisks are P values (*, P<0.05; **, P<0.01; ***, P<0.001) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=2).

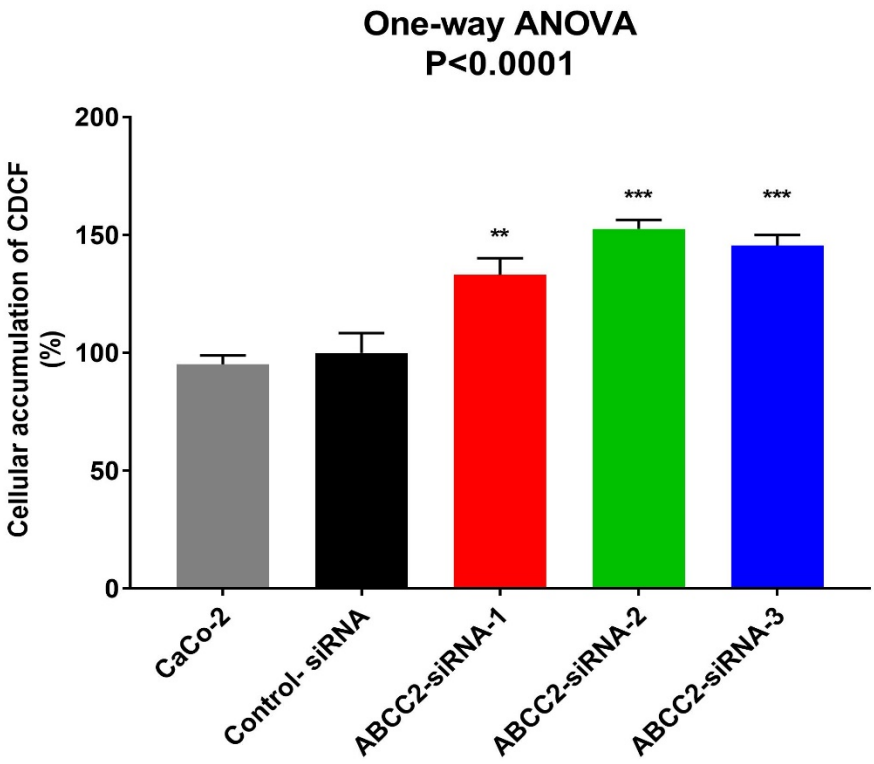
5.3.4. Functional Activity of MRP2 in ABCC2-siRNAs transfected GI cancer cells

For functional confirmation of MRP2 silencing in transfected cells, control-siRNA and ABCC2-siRNA transfected Caco-2 and PANC-1 cells were incubated with CDCFDA for 10 min at 37°C. Based on the flow cytometry results, after knockdown of MRP2 gene, CDCF (a well-defined MRP2 substrate) accumulation significantly increased.

All ABCC2-siRNAs transfected Caco-2 cells had high fluorescence intensity measured at 48 hr post siRNA treatment ($P < 0.0001$, one-way ANOVA). We observed that ABCC2-siRNA transfection in Caco-2 cells significantly increased the mean fluorescence intensity. Compared with control-siRNA transfected cells, mean fluorescence increased by 33 ± 10% ($P< 0.01$), 52 ± 9% ($P< 0.001$) and 45 ± 9% ($P< 0.001$) in ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 transfected Caco-2 cells respectively.

ABCC2-siRNA transfected PANC-1 cells enhanced cellular CDCF accumulation measured at 48 hr post siRNA treatment. Figure 5.3 (B) shows that ABCC2-siRNA transfection in PANC-1 cells significantly increased the mean fluorescence intensity. The mean fluorescence related to CDCF accumulation in control-siRNA transfected cells was 100% whereas, in ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 transfected PANC-1 cells, the mean fluorescence was increased by 53 ± 6% ($P<0.01$), 54 ± 10% ($P<0.01$) and 48 ± 4% ($P<0.01$) respectively. This experiment was repeated three times independently. The results were represented as mean fluorescence intensity normalised with the control-siRNA cells fluorescence intensity.

A.


One-way ANOVA
P<0.0001
Cellular accumulation of CDCF (%)
200
150
100
50
0
**
***
***
CaCo-2
Control- siRNA
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3

B.

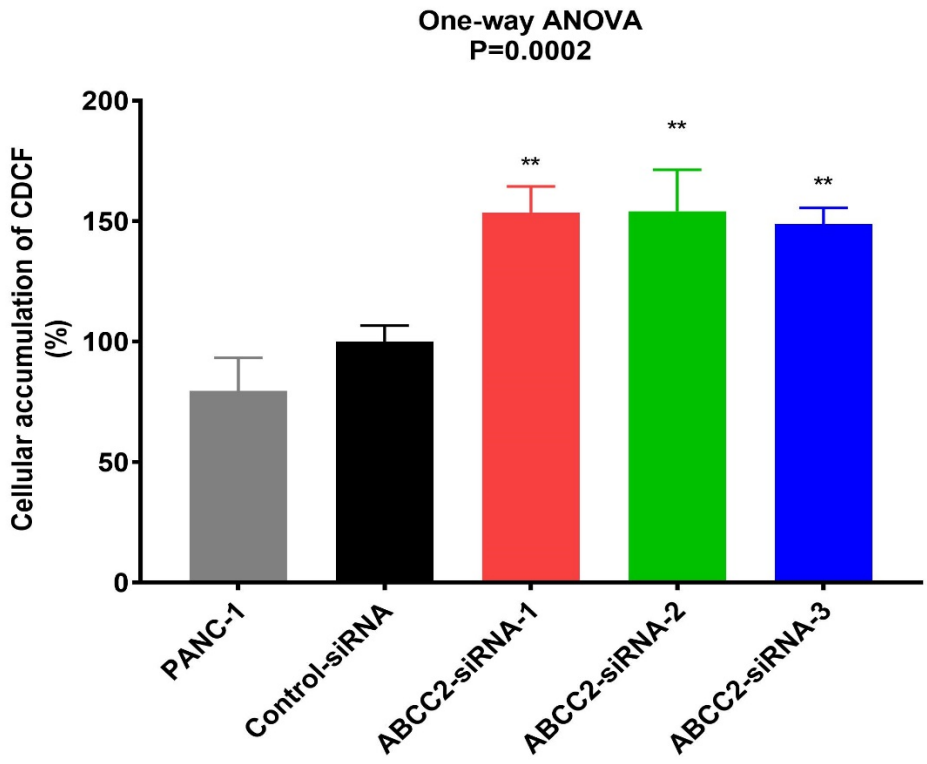


Figure 5.3. CDCF accumulation study in GI cancer cells.

CDCF accumulation in control or ABCC2-siRNAs knockdown GI cancer cells for 10 min. Accumulation of CDCF in (A) Caco-2 and ABCC2-siRNA knockdown Caco-2 cells, and (B) PANC-1 and ABCC2-siRNA knockdown PANC-1 cells were measured as fluorescence intensity after 10 min incubation with 2.5 µM CDCFDA. CDCF accumulation was measured by flow cytometry. ABCC2-siRNA knockdown increased CDCF accumulation in both Caco-2 and PANC-1 cells. Data are presented as the mean and SEM of fluorescence percentage from three independent experiments. Asterisks are P values (, P<0.01; ***, P<0.001) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=4).**

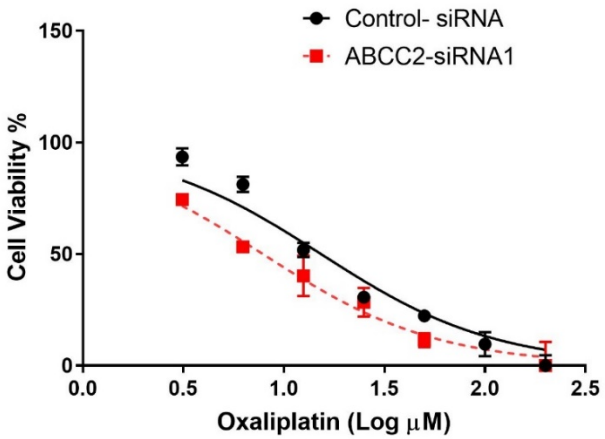
5.3.5. ABCC2 siRNA increased the sensitivity of oxaliplatin in GI cancer cells

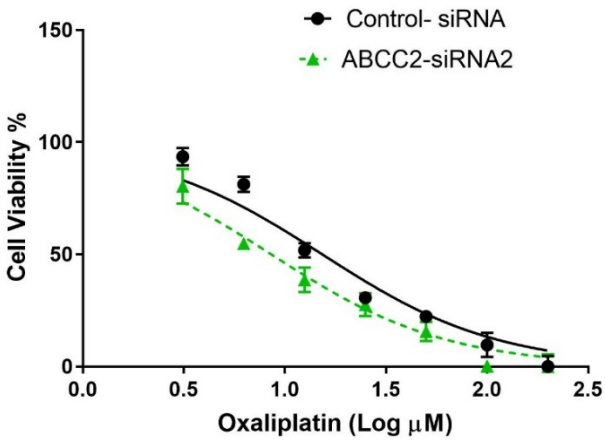
We determined the cytotoxicity of oxaliplatin in Caco-2 and PANC-1 cells after silencing MRP2 gene using an MTT assay. The MTT assay of cell viability after 2 hr exposure to oxaliplatin showed significant variation in the IC_{50} values between control and transfected PANC-1 cells.

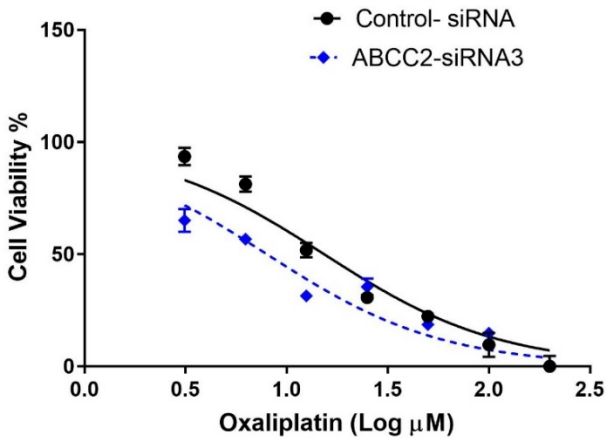
Oxaliplatin inhibited the growth of negative control and ABCC2-siRNAs transfected Caco-2 cells in a dose-dependent manner. As shown in figure 5.4, after 2 hr of treatment with oxaliplatin, the IC_{50} values were 7.5 µM, 8.4 µM, 7.0 µM for ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 Caco-2 cells respectively. Whereas the IC_{50} value for control transfected Caco-2 cells were 13.8 µM. These results indicate that transfection of siRNAs increased the sensitivity of Caco-2 cells to oxaliplatin. Thus, we can observe that silencing ABCC2 significantly reduced the concentration of oxaliplatin required to obtain 50% growth inhibition.

The IC_{50} values of oxaliplatin in PANC-1 cells transfected with control-siRNA, ABCC2-siRNA-1, ABCC2-siRNA-2 and ABCC2-siRNA-3 were 35 µM, 18.6 µM, 23.2 µM and 20.5 µM respectively (Figure 5.5). The IC_{50} values of oxaliplatin in control-transfected PANC-1 cells were significantly higher than in siRNA-transfected cells. Based on the IC_{50} values, transfection of ABCC2-siRNAs enhanced the sensitivity of PANC-1 cells to oxaliplatin. These results indicate that the ABCC2 silencing enhances oxaliplatin sensitivity in PANC-1 cells.

A.


Control- siRNA
ABCC2-siRNA1
Cell Viability %
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
Oxaliplatin (Log μM)


Control- siRNA
ABCC2-siRNA2
Cell Viability %
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
Oxaliplatin (Log μM)


Control- siRNA
ABCC2-siRNA3
Cell Viability %
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
Oxaliplatin (Log μM)

B.

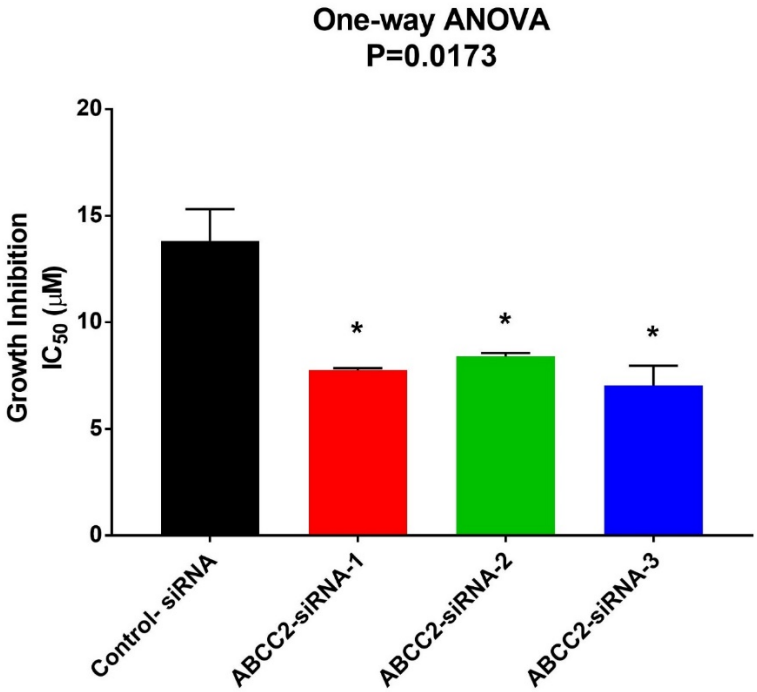
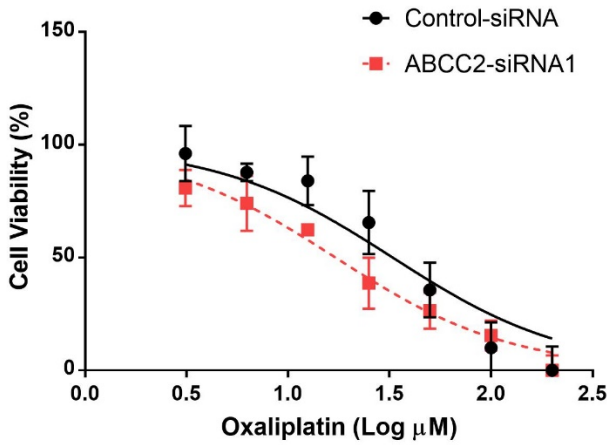
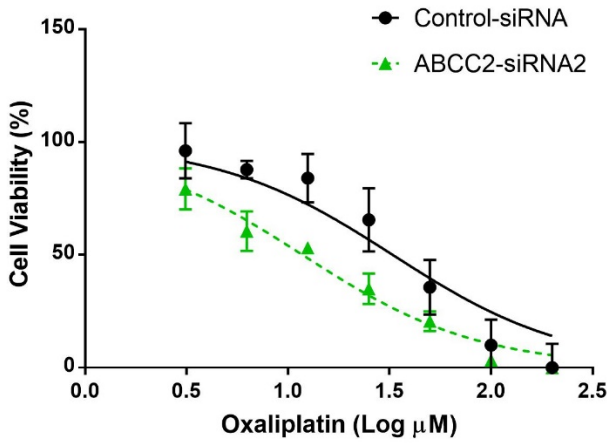


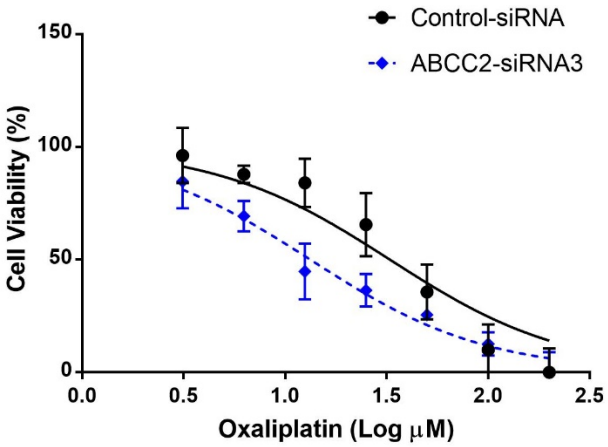
Figure 5.4. Oxaliplatin-induced growth inhibition in control-siRNA and ABCC2-siRNA knockdown Caco-2 cells.

Caco-2 cells were treated with ABCC2-siRNAs and control-siRNA for 48 hr followed by incubation in the drug-free medium for 24 hr, then cells were treated a range of different concentrations of oxaliplatin for 2 hr, then cultured in drug-free medium for 72 hr before measurement of the number of viable cells by MTT assay. A) Supplementary data of cell viabilities in control and ABCC2-siRNAs knockdown Caco-2 cells after oxaliplatin treatment. Each point represents the mean of quadruplicate sample (n=4). B) In siRNA knockdown Caco-2 cells, oxaliplatin sensitivities were increased compared with control-siRNA. Data are presented as the mean and SEM of IC_{50} of oxaliplatin in cells from two independent experiments. Asterisks are P values (*, P<0.05) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=2).

A.


Control-siRNA
ABCC2-siRNA1
Cell Viability (%)
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
Oxaliplatin (Log μM)


Control-siRNA
ABCC2-siRNA2
Cell Viability (%)
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
Oxaliplatin (Log μM)


Control-siRNA
ABCC2-siRNA3
Cell Viability (%)
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
Oxaliplatin (Log μM)

B.

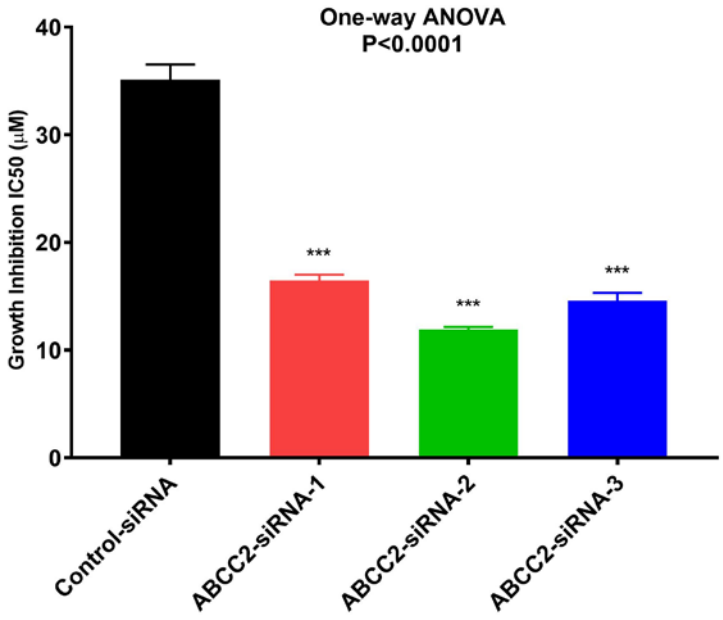


Figure 5.5. Oxaliplatin-induced growth inhibition in control-siRNA and ABCC2-siRNA knockdown PANC-1 cells.

PANC-1 cells were treated with ABCC2-siRNA and control-siRNA for 48 hr followed by incubation in the drug-free medium for 24 hr, then cells were treated a range of different concentrations of oxaliplatin for 2 hr, then cultured in drug-free medium for 72 hr before measurement of the number of viable cells by MTT assay. A) Supplementary data of oxaliplatin sensitivities in control and ABCC2-siRNAs knockdown PANC-1 cells. Each point represents the mean of quadruplicate samples (n=4). B) In siRNA knockdown PANC-1 cells, oxaliplatin sensitivities were increased compared with control-siRNA. Data are presented as the mean and SEM of IC_{50} of oxaliplatin in cells from three independent experiments. Asterisks are P values (*, P<0.001) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=3).**

Table 5-2. Oxaliplatin-induced growth inhibition: Comparison between control-siRNA and ABCC2-siRNAs transfected cells*

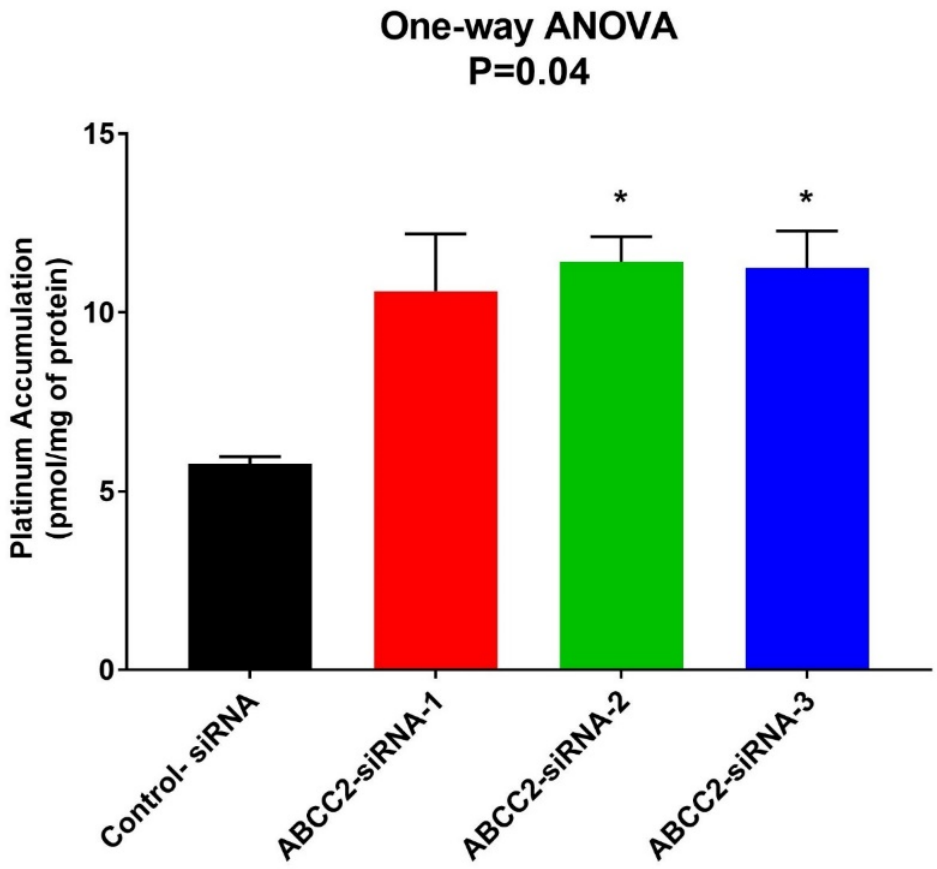
Cell lines	Transfected Cells	IC_{50} (Mean ± SEM)	P-value
Caco-2	Control-siRNA	13.82 ± 1.48	-
	ABCC2-siRNA-1	7.75 ± 0.15	0.018
	ABCC2-siRNA-2	8.4 ± 0.16	0.027
	ABCC2-siRNA-3	7.03 ± 0.93	0.012
PANC-1	Control-siRNA	35.13 ± 1.39	-
	ABCC2-siRNA-1	16.48 ± 0.53	<0.0001
	ABCC2-siRNA-2	11.91 ± 0.25	<0.0001
	ABCC2-siRNA-3	14.60 ± 0.72	<0.0001

***Data shown are mean ± SEM of values from independent experiments.**

5.3.6. Platinum accumulation rates in transfected Caco-2 cells after exposure to oxaliplatin

The transport activity of MRP2 after silencing the MRP2 gene was measured by determining the cellular accumulation of oxaliplatin-derived platinum in transfected Caco-2 cells. To determine the role of MRP2 in the membrane transport of oxaliplatin, negative control and ABCC2-siRNAs transfected Caco-2 cells were treated with oxaliplatin for 2 hr followed by measurement of platinum accumulation by ICP-MS. The cellular platinum accumulation rate after 2 hr of exposure to oxaliplatin (25 µM) in control-siRNA Caco-2 cells was 5.8 ± 0.1 pmol/mg of protein. Silencing of MRP2 gene resulted in a 2-fold increase in the rate of platinum accumulation in ABCC2-siRNA Caco-2 cells [ABCC2-siRNA-1, 10.34 ± 1.8 pmol/mg of protein (P=0.066); ABCC2-siRNA-2, 11.4 ± 0.7 pmol/mg of protein (P<0.05); ABCC2-siRNA-3, 11.2 ± 1 pmol/mg of protein (P<0.05)]. The platinum accumulation was significantly increased by ABCC2-siRNA-transfected cells compared to negative control-transfected cells. After 2 hr exposure to 25 µM oxaliplatin, the platinum accumulation in ABCC2-siRNA-1, ABCC2-siRNA-2, and ABCC2-siRNA-3 was increased by 80 ± 32%, 100 ± 12% and 95 ± 18% respectively compared to the control Caco-2 cells. The cellular platinum accumulation rate after 2 hr of exposure to 100 µM oxaliplatin in control-siRNA Caco-2 cells was 105.2 pmol/mg of protein. Silencing of MRP2 gene resulted in increase in the rate of platinum accumulation in ABCC2-siRNA Caco-2 cells [ABCC2-siRNA-1, 128.75 ± 2 pmol/mg of protein (P=0.22); ABCC2-siRNA-2, 152.5 ± 1.2 pmol/mg of protein (P<0.05); ABCC2-siRNA-3, 180.7 ± 17 pmol/mg of protein (P<0.01)]. The platinum accumulation in ABCC2-siRNAs was higher than the negative control Caco-2 cells. ABCC2-siRNA transfection inhibited the transport function of MRP2, which increased the platinum accumulation in MRP2 silencing Caco-2 cells. This result indicates that MRP2 plays a critical role in the uptake of oxaliplatin in the transfected cells.

A.


One-way ANOVA
P=0.04
Platinum Accumulation
(pmol/mg of protein)
15
10
5
0
*
*
Control- siRNA
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3

B.

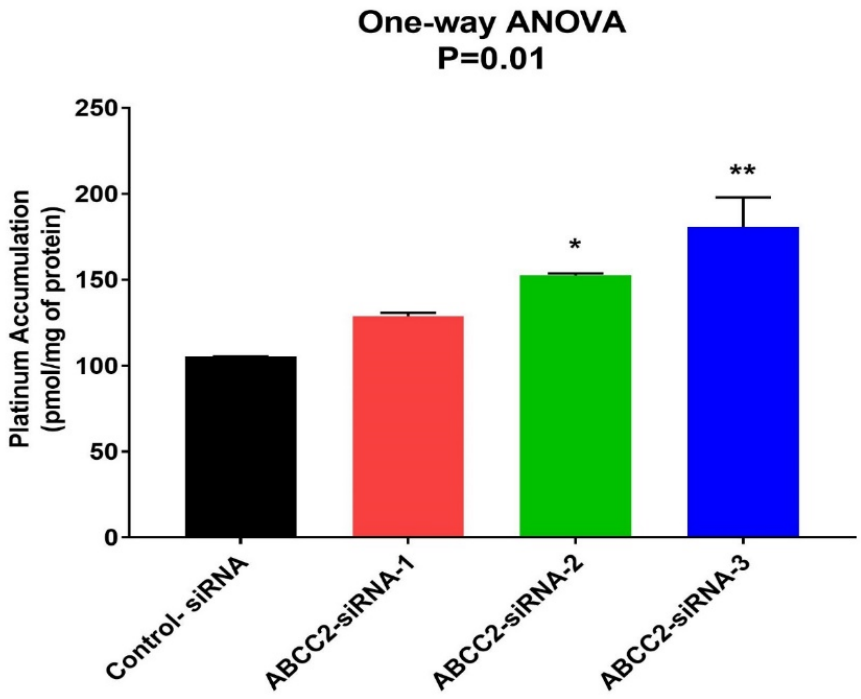


Figure 5.6. Platinum accumulation in control and ABCC2-siRNAs knockdown Caco-2 cells.

Caco-2 cells were treated with ABCC2-siRNAs and control-siRNA for 48 hr followed by incubation in the drug-free medium for 24 hr, then treated with (A) 25 µM and (B) 100 µM of oxaliplatin for 2 hr and cellular accumulation of platinum was measured by ICP-MS. Data were presented as the mean and SEM values. ABCC2-siRNA-1, ABCC2-siRNA-2, ABCC2-siRNA-3 knockdown significantly increased platinum accumulation in Caco-2 cells compared to treatment with control-siRNA. Asterisks are P values (*, P<0.05; **, P<0.01) from Dunnett's post hoc test that followed one-way ANOVA for comparisons of all ABCC2-siRNA samples to negative control sample (n=2).

5.3.7. Oxaliplatin-induced apoptosis rates in ABCC2-siRNAs transfected Caco-2 cells

After 48 hr transfection of ABCC2-siRNAs in Caco-2 cells, cells were treated with oxaliplatin at 25 μM or 100 μM for 2 hr and apoptotic effects were analysed by flow cytometry.

The results in table 5-4 and 5-5 showed that oxaliplatin treatment was able to increase the apoptosis rate. The increased apoptosis effect mainly resulted from early apoptosis (Annexin V^+/PI^-). Two-hour treatment with oxaliplatin significantly increased the early apoptosis rate. The increase in the late apoptosis (Annexin V^+/PI^+) was also noted. Flow cytometry results showed that compared to control-siRNA Caco-2 cells, the apoptosis rate increased by 7% to 15% after treatment with 25 μM oxaliplatin. After treatment with 100 μM oxaliplatin, the apoptosis percentages were 50.9%, 53.2% and 51.2% for ABCC2-siRNA-1, ABCC2-siRNA-2 and ABBC2-siRNA-3 Caco-2 cells respectively. The total apoptosis rate increased by almost 20% in MRP2 knockdown Caco-2 cells. The apoptosis percentage significantly increased in Caco-2 cells without any oxaliplatin treatment between the control-siRNA and ABCC2-siRNAs. These data suggest that knockdown of the MRP2 gene effectively elevated oxaliplatin-induced apoptosis in Caco-2 cells.

Altogether, our data suggest that apoptotic cell death by oxaliplatin significantly increased with increased oxaliplatin concentration. Knockdown of the MRP2 gene using ABCC2-siRNAs transfection enhanced the oxaliplatin-induced apoptosis. From the present data, it is apparent that silencing MRP2 elevated the apoptosis rate induced by oxaliplatin in transfected Caco-2 cells with an increased concentration of oxaliplatin.

Table 5-3. Data analysis of apoptosis assay without any treatment

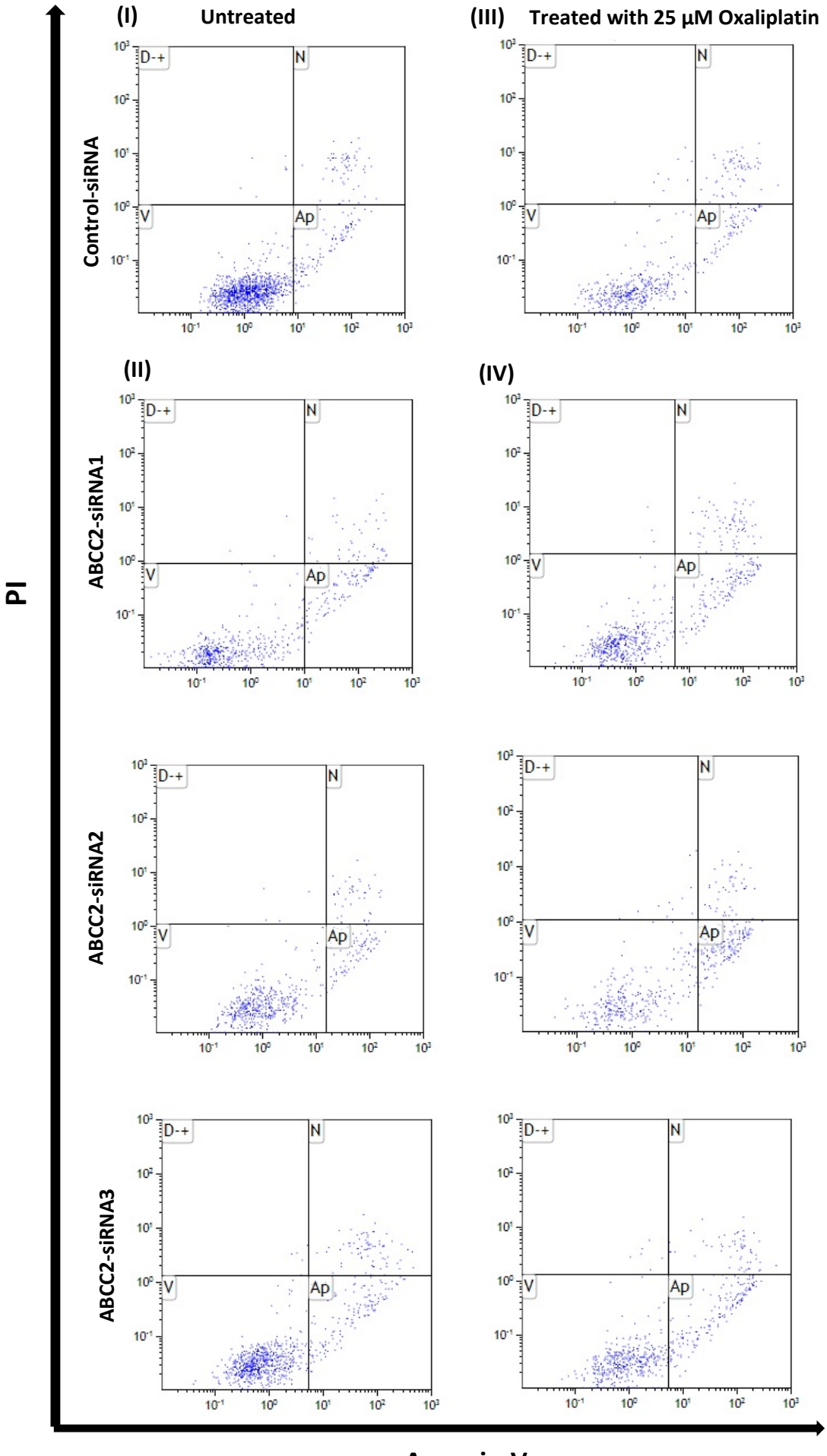
Treatment	Viable Cells (%)	Cell viability P-value	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value (Total Apoptosis)
Control-siRNA	87.9 ± 1.2	-	7.6 ± 0.4	4.1 ± 0.7	11.7 ± 1.7	-
ABCC2-siRNA-1	75.2 ± 1.7	0.0004	12.0 ± 2.3	11.3 ± 1.7	23.4 ± 1.8	0.02
ABCC2-siRNA-2	72.9 ± 1.2	0.0001	17.9 ± 1.2	8.4 ± 1.0	26.3 ± 1.2	0.003
ABCC2-siRNA-3	73.7 ± 1.6	0.0001	17.3 ± 1.5	8.3 ± 0.2	25.6 ± 1.5	0.005

Table 5-4. Data analysis of apoptosis assay with 25 µM oxaliplatin

Treatment	Viable Cells (%)	Cell viability P-value	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value (Total Apoptosis)
Control-siRNA	72.8 ± 0.4	-	14.8 ± 0.5	9.1 ± 1.4	23.9 ± 1.7	-
ABCC2-siRNA-1	63.0 ± 0.4	0.0007	20.9 ± 1.7	9.4 ± 0.5	30.3 ± 3.6	ns
ABCC2-siRNA-2	55.1 ± 0.5	0.0001	25.4 ± 3.6	13.1 ± 2.7	38.5 ± 3.1	0.0003
ABCC2-siRNA-3	62.7 ± 0.5	0.0005	21.2 ± 1.5	11.2 ± 1.3	32.4 ± 1.7	0.04

Table 5-5. Data analysis of apoptosis assay with 100 µM oxaliplatin

Treatment	Viable Cells (%)	Cell viability P-value	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value (Total Apoptosis)
Control-siRNA	63.0 ± 0.6	-	20.4 ± 0.7	13.3 ± 1.7	33.7 ± 2.3	-
ABCC2-siRNA-1	48.1 ± 1.7	0.0001	38.6 ± 2.7	12.3 ± 1.7	50.9 ± 2.0	0.0001
ABCC2-siRNA-2	45.8 ± 2.4	0.0001	44.2 ± 1.8	9.0 ± 1.2	53.2 ± 2.1	0.0001
ABCC2-siRNA-3	47.3 ± 4.3	0.0001	41.5 ± 4.2	9.7 ± 2.2	51.2 ± 3.7	0.0001


(I) Untreated
(III) Treated with 25 µM Oxaliplatin
(II)
(IV)
Control-siRNA
ABCC2-siRNA1
ABCC2-siRNA2
ABCC2-siRNA3
D-+
N
V
Ap
PI
Annexin-V

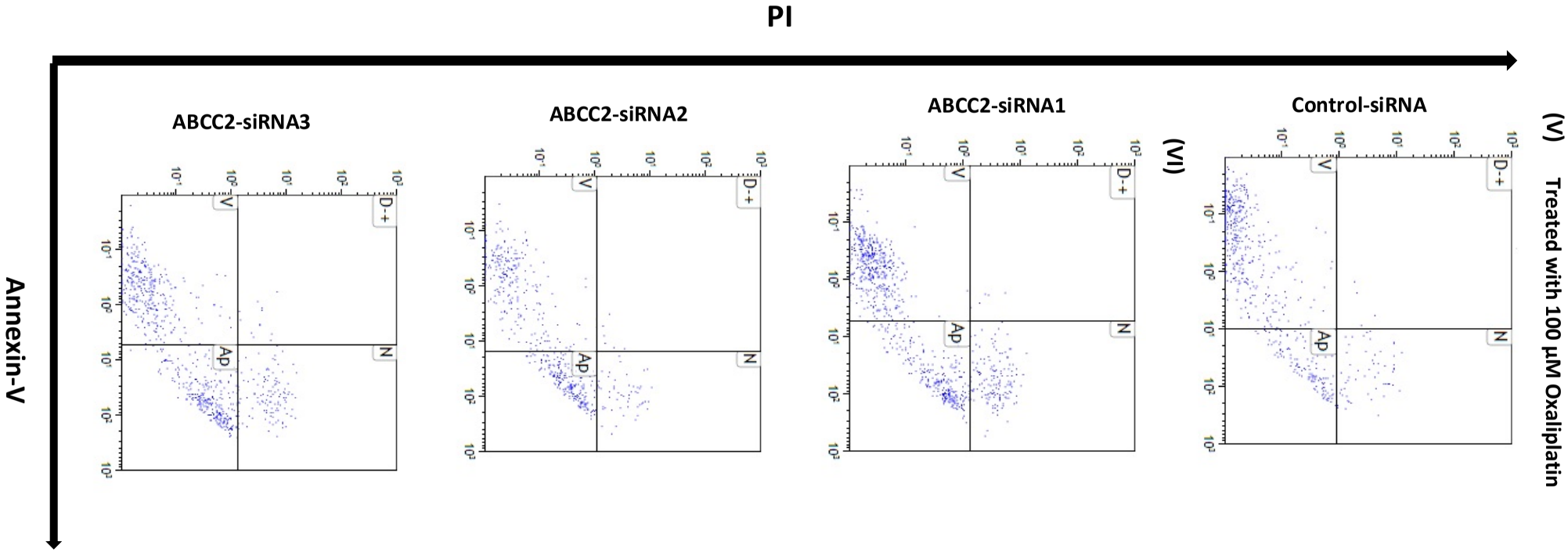
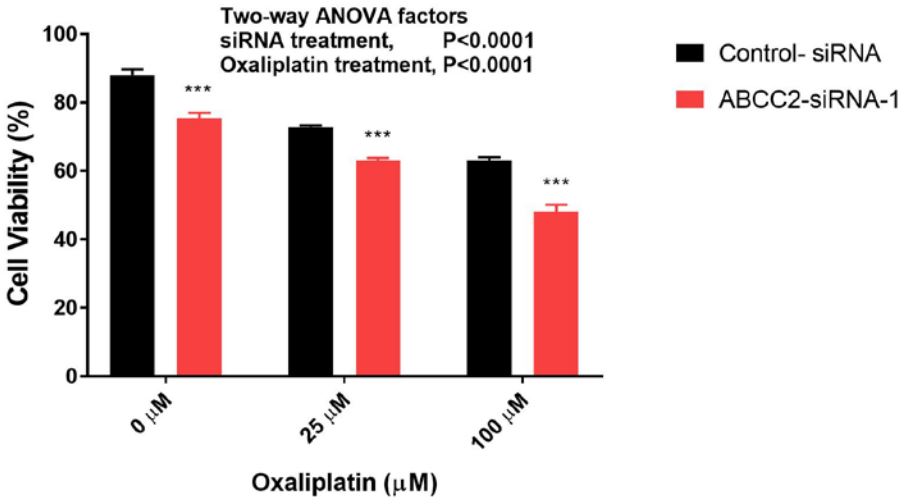


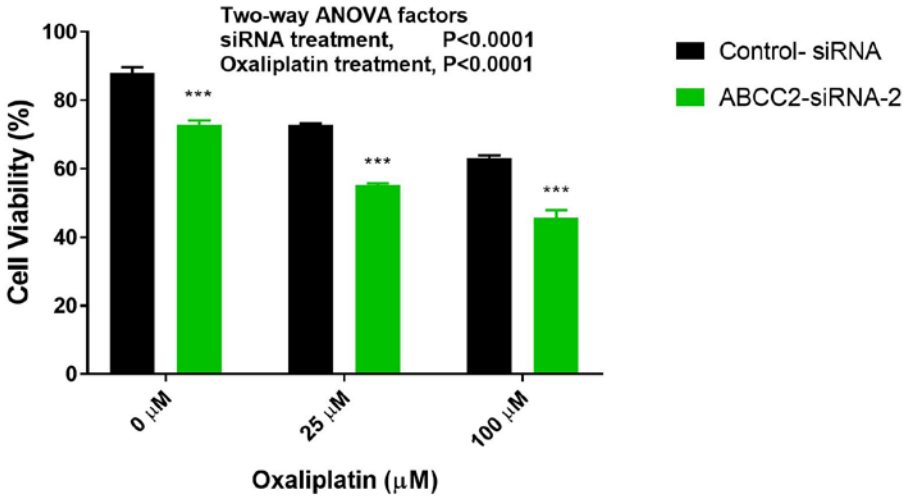
Figure 5.7. Apoptotic changes in Caco-2 cells in the presence of oxaliplatin after siRNA transfection.

Detection of apoptosis by concurrent staining with Annexin-V and PI. Caco-2 cells were transfected with three different subsets of ABCC2-siRNAs and control-siRNA and treated with oxaliplatin at different concentrations. Cells were subsequently stained with Annexin-V and PI and their fluorescence was measured by flow cytometry. Viable cells (V) are both Annexin-V and PI negative. At an early stage of apoptosis (Ap), the cells bind with only Annexin-V. At the late stage of apoptosis (N), the cells bind with both Annexin-V FITC and PI. The upper left quadrant (D-+) represents the debris cells or dead cells. (I) Control-siRNA transfected cells without any treatment, (II) MRP2-silencing Caco-2 without any treatment, (III) Control-siRNA transfected cells with 25 µM oxaliplatin, (IV) MRP2-silencing Caco-2 cells with 25 µM oxaliplatin, (V) Control-siRNA transfected cells with 100 µM oxaliplatin, and (VI) MRP2-silencing cells treated with 100 µM oxaliplatin.

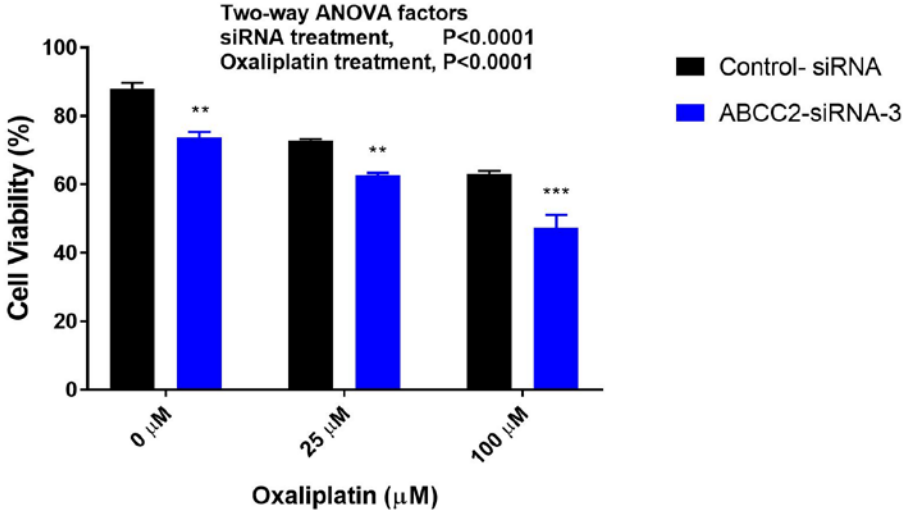
A.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell Viability (%)
100
80
60
40
20
0
***
***
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-1

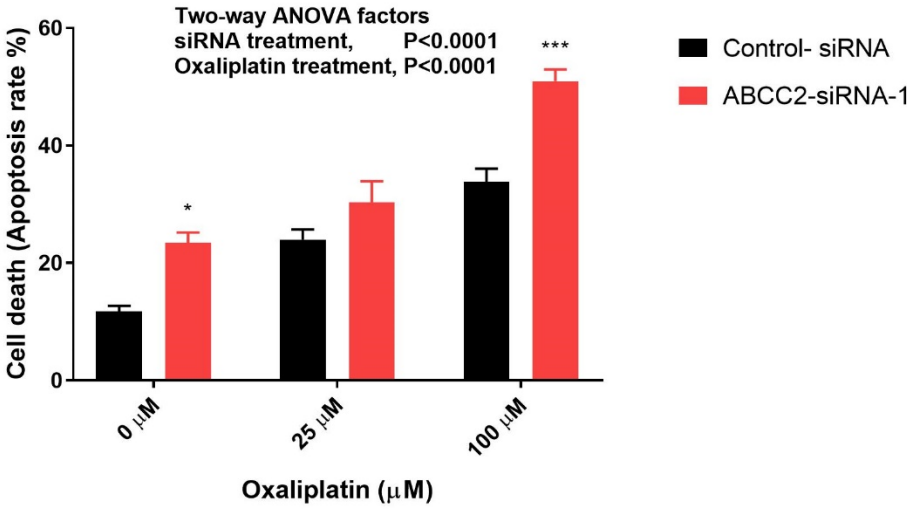
B.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell Viability (%)
100
80
60
40
20
0
***
***
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-2

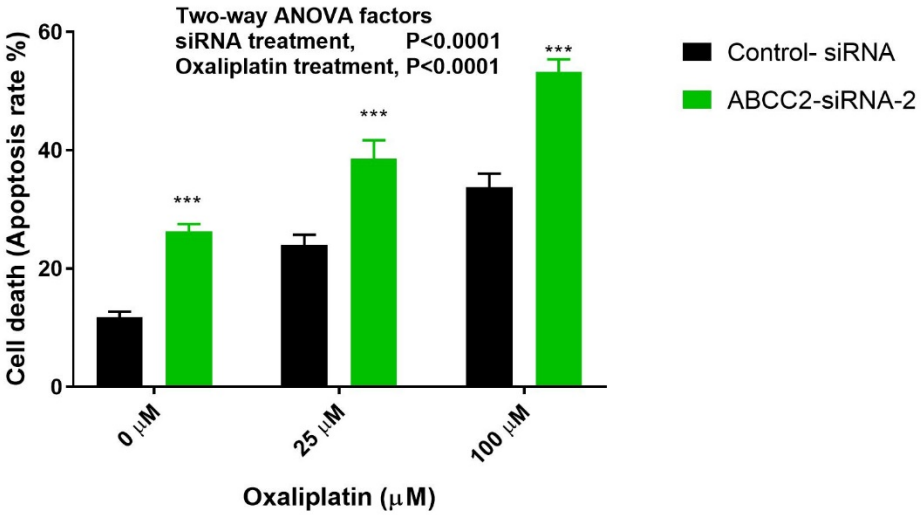
C.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell Viability (%)
100
80
60
40
20
0
**
**
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-3

D.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell death (Apoptosis rate %)
60
40
20
0
*
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-1

E.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell death (Apoptosis rate %)
60
40
20
0
***
***
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-2

F.

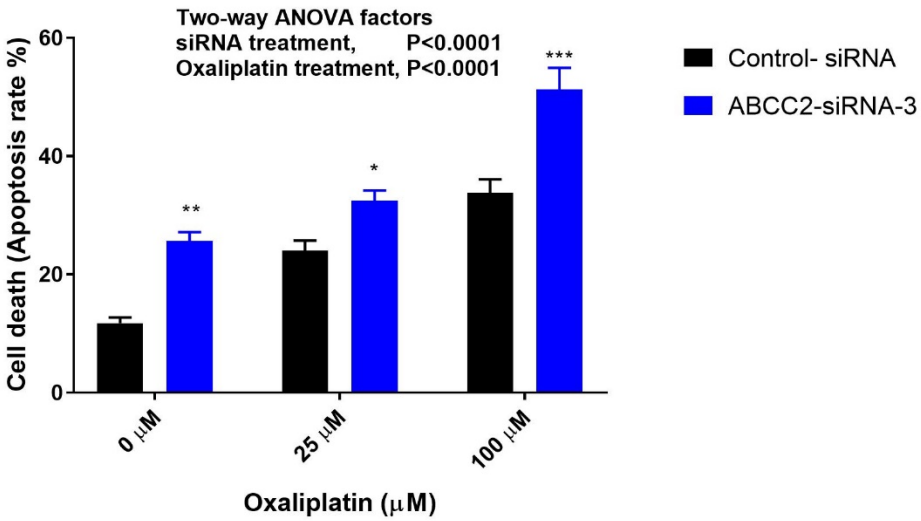

Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell death (Apoptosis rate %)
60
40
20
0
**
*
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-3

Figure 5.8. Oxaliplatin-induced apoptosis in control and ABCC2-siRNAs transfected Caco-2 cells.

Caco-2 cells were treated with ABCC2-siRNAs and control-siRNA for 48 hr followed by incubation in the drug-free medium for 24 hr, then cells were treated with a range of different concentrations of oxaliplatin for 2 hr and stained with Annexin V/PI and rate of apoptosis was measured by flow cytometry. A, B and C represent viable cells percentage in control-siRNA, ABCC2-siRNA-1, siRNA-2 and siRNA-3 transfected cells respectively. D, E and F represent total apoptotic cells percentage in control-siRNA, ABCC2-siRNA-1, siRNA-2 and siRNA-3 transfected cells respectively. Data are presented as mean and SEM of two independent experiments. Asterisks are P values (*, P<0.01; **, P<0.01; ***, P<0.001) from Sidak post-tests that followed a Two-way ANOVA (n=4).

5.3.8. ABCC2-siRNAs enhanced oxaliplatin-induced apoptosis in PANC-1 cells

The results in figures 5.9 and 5.10 show that oxaliplatin treatment was able to increase the apoptosis rate from 10% to 20% with a different concentration of oxaliplatin in ABCC2-siRNAs transfected PANC-1 cells. The increased apoptosis effect mainly resulted from late apoptosis (Annexin V^+/PI^+). After 2 hr of treatment with 25 µM oxaliplatin the apoptosis rate significantly increased by 12% to 15%. In the late apoptosis (Annexin V^+/PI^-), the rate of apoptosis increased. Flow cytometry results showed that compared to control-siRNA PANC-1 cells, for a cell transfected with ABCC2-siRNA-1, ABCC2-siRNA-2 and ABBC2-siRNA-3, the rate of apoptosis increased by 13%, 15% and 15% respectively, after treatment with 25 µM oxaliplatin. After treatment with 100 µM oxaliplatin, the apoptosis percentage was 36%, 42% and 33% for ABCC2-siRNA-1, ABCC2-siRNA-2 and ABBC2-siRNA-3 PANC-1 cells respectively. The apoptosis percentage slightly increased in PANC-1 cells without any oxaliplatin treatment between the control-siRNA and ABCC2-siRNAs. These data suggested that knockdown of MRP2 gene contributed to the sensitisation of the PANC-1 cells to oxaliplatin.

Altogether, our results suggested that apoptotic cell death by oxaliplatin is significantly increased with increased oxaliplatin concentration. Knockdown of MRP2 gene using ABCC2-siRNAs enhanced the oxaliplatin-induced apoptosis. About 20% and 15% increase in apoptotic cells resulted from siRNA transfection after treatment with 25 µM and 100 µM oxaliplatin respectively. We conclude that targeting the MRP2 gene increases the sensitivity of PANC-1 cells to oxaliplatin.

Table 5-6. Data analysis of apoptosis assay without any treatment

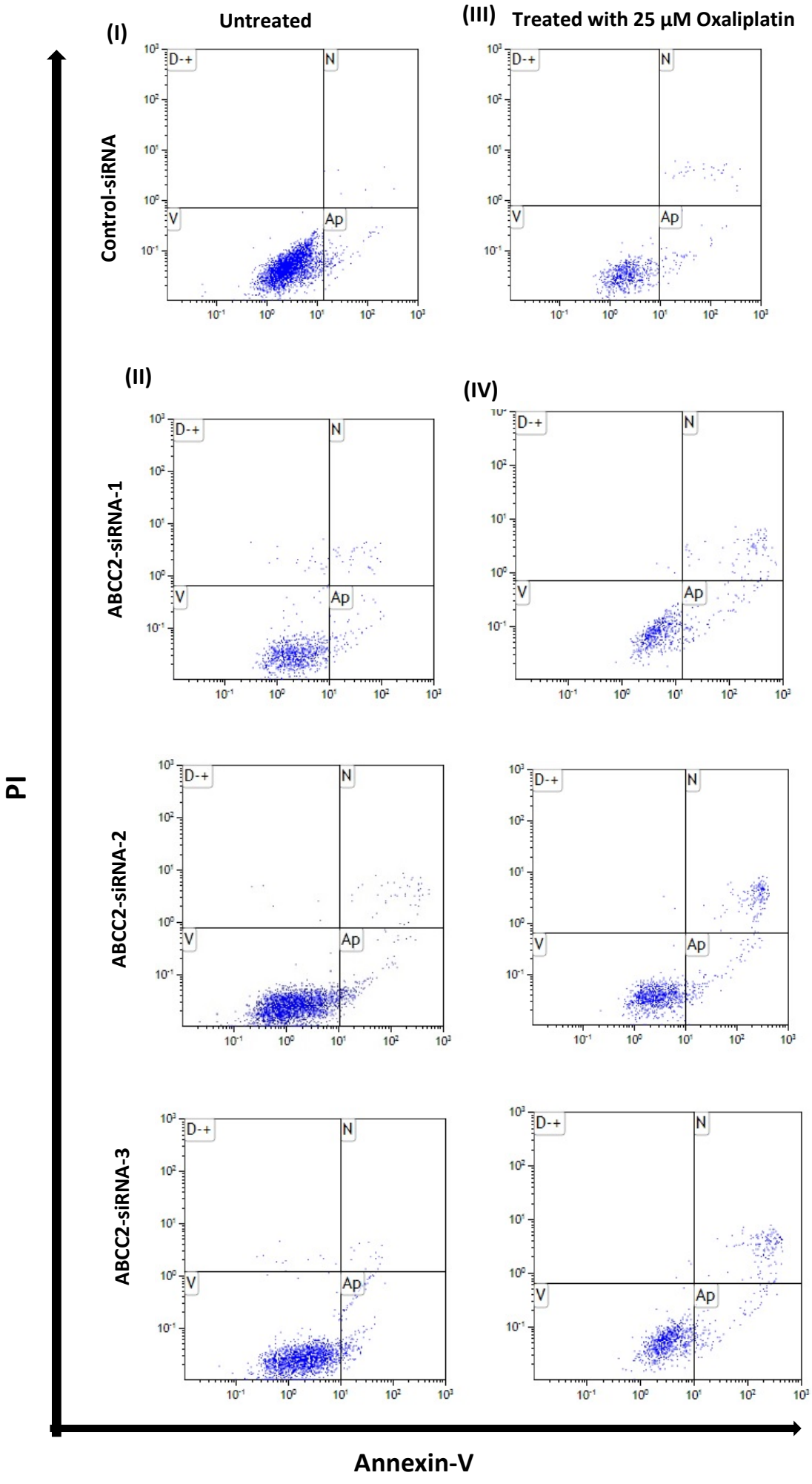
Treatment	Viable Cells (%)	Viability P-value	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value (Total Apoptosis)
Control-siRNA	96.1 ± 1.0	-	3.9 ± 0.9	0.1 ± 0.09	4.0 ± 0.8	-
ABCC2-siRNA-1	95.8 ± 1.5	ns	5.1 ± 1.6	4.2 ± 0.3	9.3 ± 1.4	0.04
ABCC2-siRNA-2	91.3 ± 1.6	ns	7.3 ± 0.7	2.9 ± 0.8	10.2 ± 0.09	0.01
ABCC2-siRNA-3	90.8 ± 0.6	0.04	8.9 ± 1.0	0.6 ± 0.1	9.6 ± 0.9	0.03

Table 5-7. Data analysis of apoptosis assay with 25 µM oxaliplatin

Treatment	Viable Cells (%)	Viability P-value	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value (Total Apoptosis)
Control-siRNA	87.2 ± 0.7	-	5.7 ± 0.5	3.4 ± 0.0	9.15 ± 0.5	-
ABCC2-siRNA-1	74.9 ± 0.5	0.0004	9.8 ± 1.7	12.3 ± 1.6	22.2 ± 3.4	0.0001
ABCC2-siRNA-2	75.1 ± 0.6	0.0005	7.6 ± 1.5	16.3 ± 2.6	24.0 ± 1.0	0.0001
ABCC2-siRNA-3	76.0 ± 1.6	0.0009	7.5 ± 2.0	15.9 ± 1.7	23.5 ± 0.3	0.0001

Table 5-8. Data analysis of apoptosis assay with 100 µM oxaliplatin

Treatment	Viable Cells (%)	Viability P-value	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value (Total Apoptosis)
Control-siRNA	72.4 ± 2.3	-	18.5 ± 0.8	9.8 ± 0.5	28.3 ± 1.3	-
ABCC2-siRNA1	63.6 ± 2.9	0.005	14.7 ± 2.2	21.8 ± 0.7	36.5 ± 1.5	0.002
ABCC2-siRNA2	58.5 ± 1.1	0.0001	11.3 ± 2.03	30.8 ± 2.5	42.1 ± 0.5	0.0001
ABCC2-siRNA3	62.8 ± 1.9	0.0029	10.45 ± 0.07	22.3 ± 0.9	32.8 ± 0.9	ns


(I)
Untreated
(III)
Treated with 25 µM Oxaliplatin
Control-siRNA
(II)
(IV)
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3
D-+
N
V
Ap
PI
Annexin-V

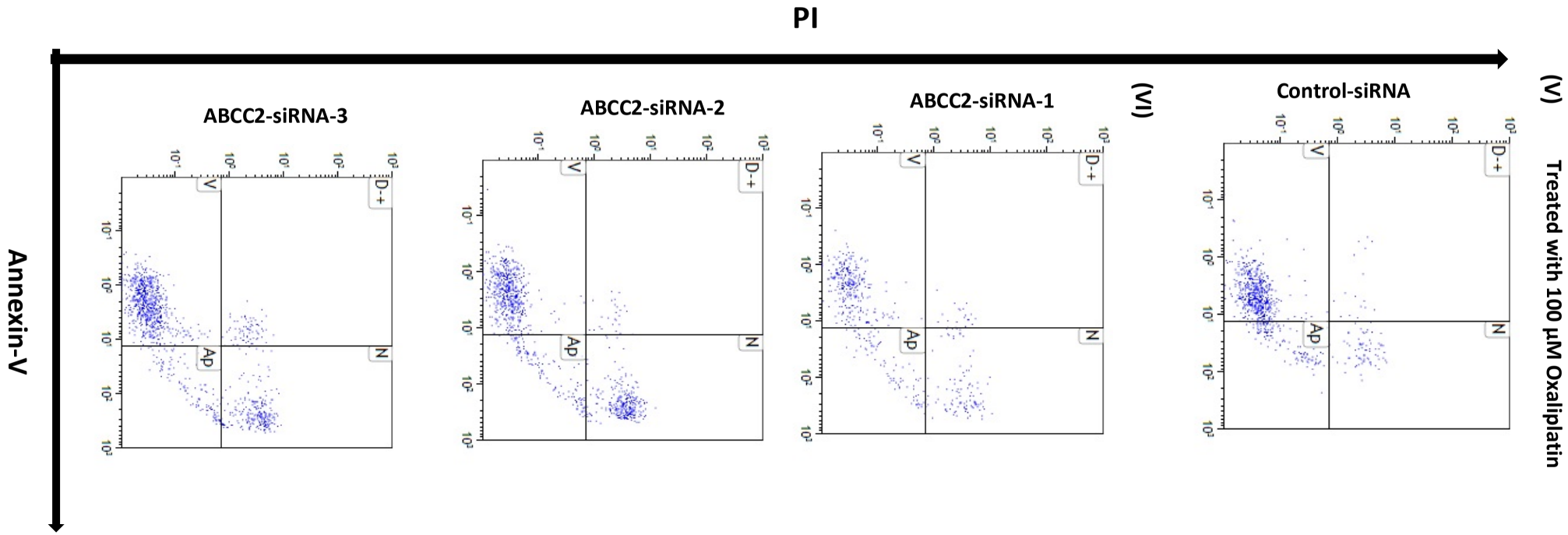
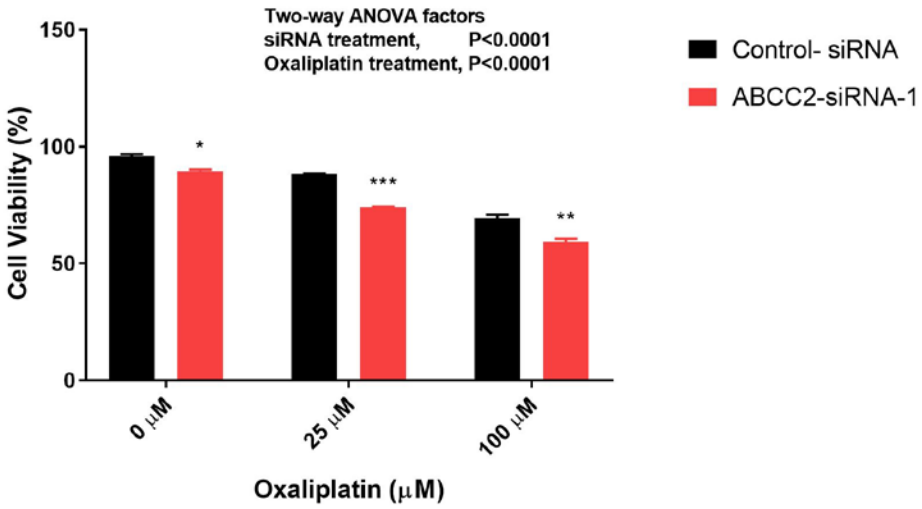


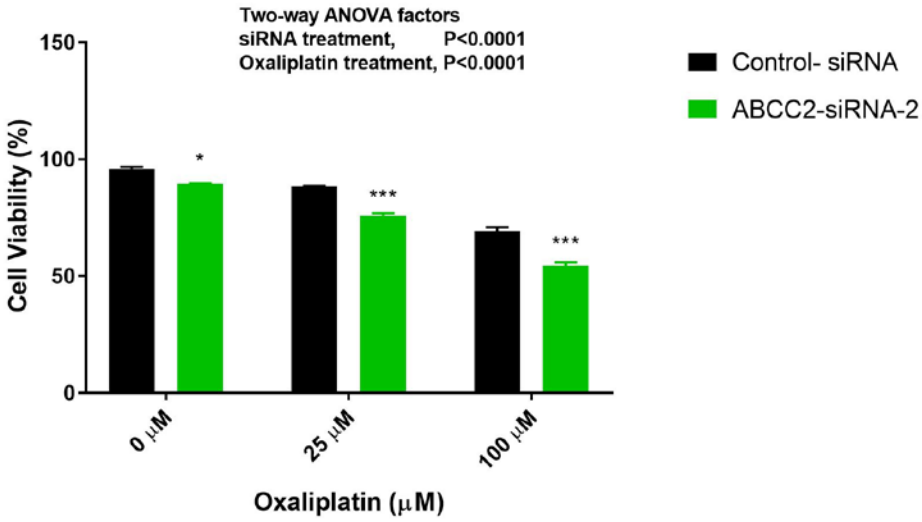
Figure 5.9. Apoptotic changes in PANC-1 cells in the presence of oxaliplatin after siRNA transfection.

Detection of apoptosis by concurrent staining with Annexin-V and PI. PANC-1 cells were transfected with three different subsets of ABCC2-siRNAs and control-siRNA and treated with oxaliplatin at different concentrations. Cells were subsequently stained with Annexin-V and PI and their fluorescence was measured by flow cytometry. Viable cells (V) are both Annexin-V and PI negative. At an early stage of apoptosis (Ap), the cells bind with only Annexin-V. At the late stage of apoptosis (N), the cells bind with both Annexin-V FITC and PI. The upper left quadrant (D-+) represents the debris cells or dead cells. (I) Control-siRNA transfected cells without any treatment, (II) MRP2-silencing cells without any treatment, (III) Control-siRNA transfected cells with 25 µM oxaliplatin, (IV) MRP2-silencing cells with 25 µM oxaliplatin, (V) Control-siRNA transfected cells with 100 µM oxaliplatin, and (VI) MRP2-silencing cells treated with 100 µM oxaliplatin.

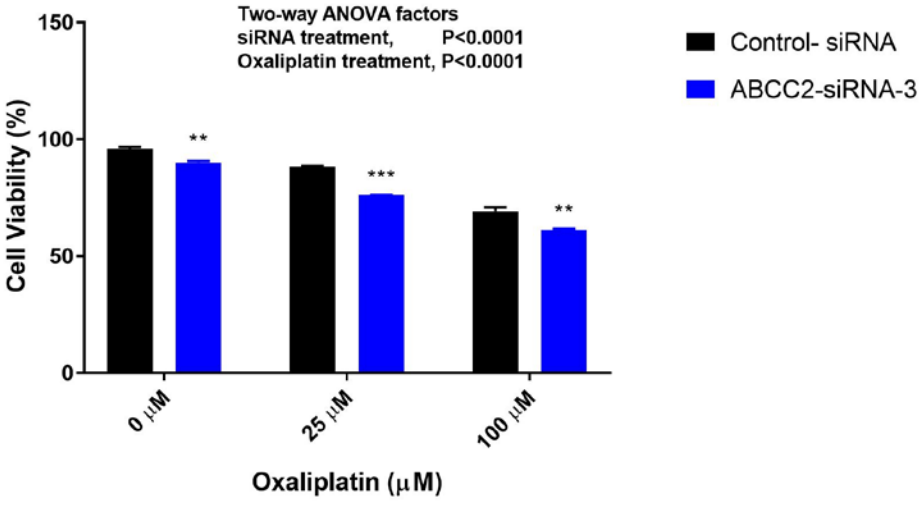
A.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell Viability (%)
150
100
50
0
*
***
**
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-1

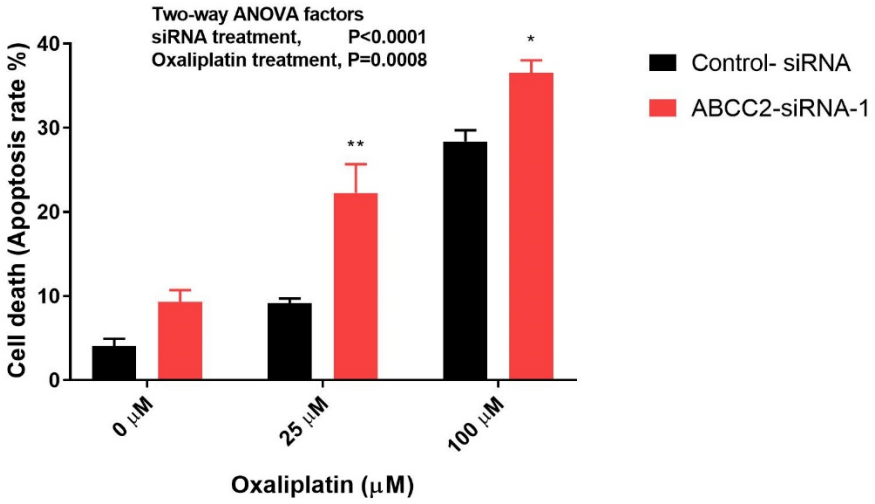
B.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell Viability (%)
150
100
50
0
*
***
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-2

C.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell Viability (%)
150
100
50
0
**
***
**
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-3

D.


Two-way ANOVA factors
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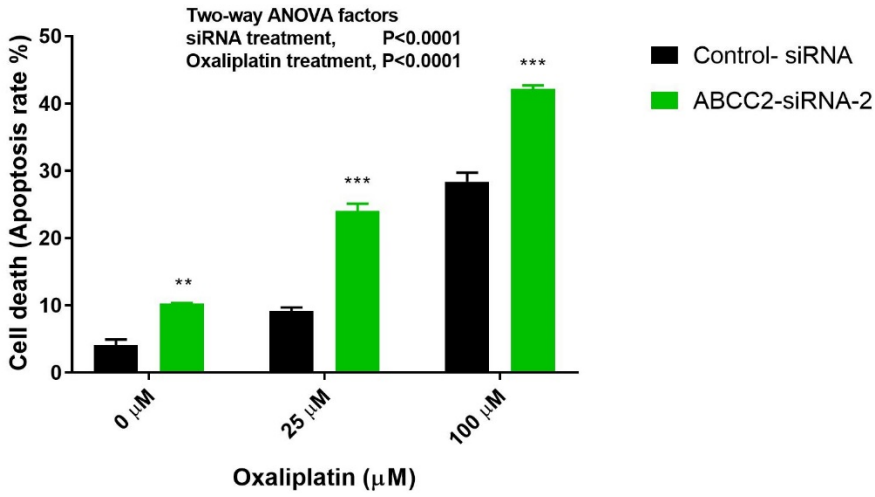
Oxaliplatin treatment, P=0.0008
Cell death (Apoptosis rate %)
0
10
20
30
40
**
*
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-1

E.


Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell death (Apoptosis rate %)
0
10
20
30
40
50
**
***
***
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-2

F.

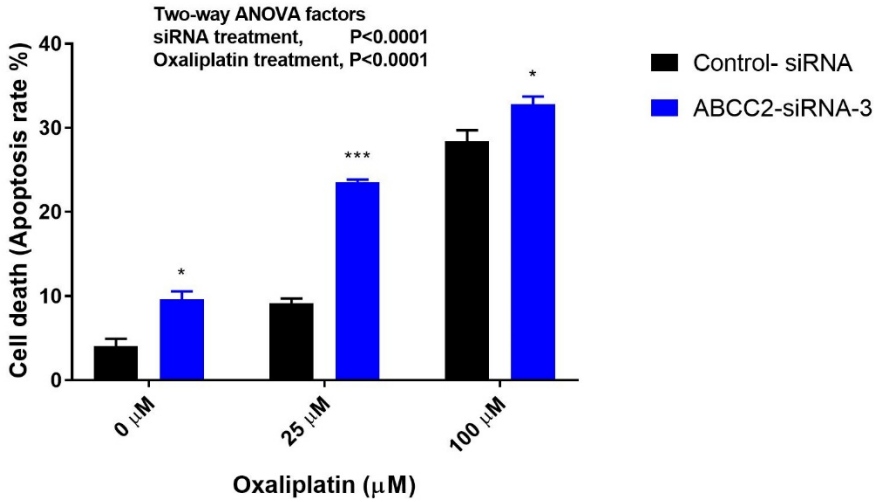

Two-way ANOVA factors
siRNA treatment, P<0.0001
Oxaliplatin treatment, P<0.0001
Cell death (Apoptosis rate %)
0
10
20
30
40
*
***
*
0 μM
25 μM
100 μM
Oxaliplatin (μM)
Control- siRNA
ABCC2-siRNA-3

Figure 5.10. Oxaliplatin-induced apoptosis in control and ABCC2-siRNAs knockdown PANC-1 cells.

PANC-1 cells were treated with ABCC2-siRNA and control-siRNA for 48 hr followed by incubation in the drug-free medium for 24 hr, then cells were treated with a range of different concentrations of oxaliplatin for 2 hr and stained with Annexin V/PI and rate of apoptosis was measured by flow cytometry. A, B and C represents viable cells percentage in control-siRNA, ABCC2-siRNA-1, siRNA-2 and siRNA-3 transfected cells respectively. D, E and F represents total apoptotic cells percentage in control-siRNA, ABCC2-siRNA-1, siRNA-2 and siRNA3 transfected cells respectively. Data are presented as mean and SEM of two independent experiments. Asterisks are P values (*, P<0.01; **, P<0.01; ***, P<0.001) from Sidak post-tests that followed a Two-way ANOVA (n=2).

5.4. Discussion

The antitumor activity of platinum-based drugs like cisplatin has been established by several clinical experiences. However, development of acquired and intrinsic resistance by the cancer cells limits the application of platinum-based drugs. To widen the anticancer spectrum of platinum agents, numerous platinum-based drugs have been tested. Notably, oxaliplatin in comparision with cisplatin has enhanced efficacy against CRC [111]. In combination with 5-FU, enhanced response rates compared with the treatment with 5-FU alone in CRC [331]. Oxaliplatin and its combination with other anticancer drugs are widely used for chemotherapy in the treatment of CRC patients; however, multidrug resistance is one of the major obstacles leading to the failure of chemotherapy in GI cancer, including colorectal and pancreatic cancer. Numerous mechanisms may be involved in oxaliplatin resistance, including increased expression of ABC transporter, alteration in the apoptotic pathway, increased DNA damage repair, changes in drug metabolism and changes in drug target [332]. As mentioned earlier, development of acquired drug resistance is one of the main reasons for ineffective drug treatment to GI cancer. The mechanisms controlling cellular efflux of oxaliplatin is rarely investigated. However, overcoming chemoresistance to oxaliplatin is important to increase the sensitivity of oxaliplatin to GI cancer.

A previous study investigated oxaliplatin as a substrate of MRP2 transporter [176]. Our findings suggest a role of MRP2 in chemoresistance to oxaliplatin-based chemotherapy in GI cancer. In this study, we addressed whether silencing the MRP2 gene using siRNAs could increase the sensitivity of oxaliplatin in human colorectal and pancreatic cancer cells. MRP2 has been expressed both at mRNA and protein level in the human colorectal carcinoma cell line Caco-2. The Caco-2 cells also detected different MRP2-mediated transport activity [184, 316, 333]. In addition, a significant association has been observed between MRP2 mRNA level and CRC chemosensitivity to platinum-based chemotherapy [288]. Moreover, it has been reported that the PANC-1 cell lines showed high expression level of MRP2 gene [196]. Therefore, we have used Caco-2 and PANC-1 cells as a potential *in vitro* model of GI cancers with MRP2-mediated transport of oxaliplatin deficit in cellular accumulation of oxaliplatin. In our study, after siRNA transfection in Caco-2 and PANC-1 cells, we have observed that MRP2 mRNA level was significantly decreased and the drug cytotoxicity assay showed the low IC_{50} value of oxaliplatin in all the ABCC2-siRNA transfected cells compared to the control. This study suggests that silencing MRP2 increases the sensitivity of oxaliplatin.

Our results indicate that ABCC2-siRNAs was successful in silencing MRP2 gene expression by reaching approximately 60% MRP2 gene knockdown in the Caco-2 cells, and 50% to 70% MRP2 gene knockdown in the PANC-1 cells. In our present work, we demonstrated the apoptotic effects of oxaliplatin on ABCC2-siRNA-transfected Caco-2 and PANC-1 cells. Our results indicate that oxaliplatin was able to induce apoptosis in a dose-dependent manner. The platinum-based chemotherapeutic drugs have proven to be beneficial in the treatment of colorectal cancer, but it also has been reported that nearly 50% of patients did not respond to a combination therapy of oxaliplatin and 5-FU treatment [37], and it failed to remove micrometastasis in nearly 30% of patients receiving combination therapy of oxaliplatin, 5-FU and leucovorin [39, 326]. Therefore, combining ABCC2-siRNA along with the oxaliplatin for colorectal cancer may increase the therapeutic efficiency. According to our study, silencing MRP2 in GI cancer cells increased the apoptosis rate even with less concentration of oxaliplatin. Notably, the low expression level of MRP2 transporter significantly correlates to the elevated rate of apoptosis. Thus, the administration of ABCC2-siRNA appeared to increase the sensitivity of both Caco-2 and PANC-1 cells to oxaliplatin. Furthermore, transfection of ABCC2-siRNAs also appeared to increase the MRP2-specific substrate cellular accumulation in Caco-2 and PANC-1 cells. This suggests that silencing the MRP2 gene decreased the activity of MRP2 efflux pump in GI cancer cells.

In further experiments, the knockdown of MRP2 increased the oxaliplatin-derived platinum accumulation in Caco-2 cells. These results suggested that increased accumulation of platinum elevated the cell apoptosis rate and increased sensitivity to oxaliplatin, eventually altering the drug resistance by inhibiting the activity of MRP2. Therefore, the present study revealed expression pattern of MRP2 and its function in siRNA-transfected Caco-2 cells to elucidate the mechanism of MRP2-induced oxaliplatin resistance. However, our study was only performed at a cellular level in tumour cells. Studies on animal models are yet to be performed to further confirm our results. It would be interesting to assess the distribution and cytotoxicity effects of oxaliplatin in xenograft models of CRC cells overexpressing MRP2 transporter, considering expression or activity of MRP2 transporter. Thus, the therapeutic effects of oxaliplatin are compromised in CRC patients with high expression levels of MRP2 transporter protein. This may be one of the reasons for the failure of adjuvant therapy containing oxaliplatin and 5-FU in specific patients with advanced colorectal cancer.

However, it is rather difficult to illustrate that only MRP2 is responsible for the chemoresistance to oxaliplatin. The overexpression of organic cation SLC transporters, like OCT1 and OCT2, in colorectal cancer cells have been implicated to mediate oxaliplatin uptake, which explains the efficacy of oxaliplatin in CRC [114]. Transporters like OCT1, OCT2, OCT3 and CTR1 were related to the influx of oxaliplatin and its cytotoxicity into cells, while ATP7A and ATP7B were related to its efflux and resistance [112, 114, 334, 335]. Moreover, genetic alteration in genes coding ABC/SLC transports, DNA damage repair machinery such as excision cross-complementing genes (ERCC1, ERCC2) and X-ray repair cross-complementing protein 1 (XRCC1), and conjugating enzymes glutathione S-transferases (GSTM1, GSTP1) also contribute to pharmacogenetics of oxaliplatin [336, 337]. *In vitro* studies have shown that enhanced expression of BCRP and MRP2 gene resulted in increased activity of oxaliplatin [175]. Excision nucleases, such as ERCC1 and ERCC2, play an important role in the DNA repair in cancer cells. Overexpression of ERCC1 has been found to be one of the major factors for the early failure of FOLFOX chemotherapy with shorter OS in patients [338-340]. Similarly, another gene, XRCC1 that contributed in DNA repair and polymorphism of this gene (CC genotype in rs25487) in patients receiving FOLFOX-4 chemotherapy has shown better OS [339]. Studies have shown that GSTP1 polymorphism responded to FOLFOX chemotherapy, while other studies failed to show any association between GSTP1 and oxaliplatin-based chemotherapy [339, 341, 342]. One of the recent studies reported that the iodide analogue of oxaliplatin, PtI_2(DACH), enhanced the cellular uptake of oxaliplatin and triggered cell apoptosis in colorectal cancer cells better than oxaliplatin [343].

The cellular resistance to oxaliplatin in the PANC-1 cells may involve other molecular mechanisms apart from reduced MRP2 substrate accumulation. These mechanisms include pancreatic cancer stem cells (CSCs), changes in DNA damage repair systems, alteration in the cellular apoptotic pathway, overexpression of ErbB2/HER2 oncogenic receptor, increased expression of ERCC1 or mutation in apoptotic protein p53 [107, 121, 122, 344-346]. Recently, it has been reported that pancreatic adenocarcinoma upregulated factor (PAUF) in pancreatic CSCs also contributes to multidrug resistance in pancreatic cancer cells [347]. Alteration in apoptosis that prevents cell death leads to increased cell survival and consequently, increased cellular resistance to oxaliplatin. Several other mechanisms can be involved in cellular resistance of oxaliplatin in

PANC-1 cells, but the evidence is still minimal. For example, according to a study demonstrated by Skrypek et. al., silencing ErbB2 induces pancreatic cancer cells' resistance to FOLFIRINOX chemotherapy via an upregulation of MRP2 transporter protein [348].

All these findings suggested that numerous other mechanisms could be involved in oxaliplatin resistance in pancreatic cancer; therefore, it is difficult to provide any firm conclusion regarding the correlation between oxaliplatin resistance and its sensitivity in the PANC-1 cells. In the past few decades, a number of studies including clinical trials have provided conflicting results regarding the impact of gene polymorphisms and their expression related to oxaliplatin resistance, the mechanism of action and toxicity. However, no clear recommendation has been suggested for oxaliplatin chemoresistance in GI cancers. Hence, identifying patients with a tumour with MRP2-mediated deficit in oxaliplatin accumulation would be an essential step in the application of MRP2-targeted therapeutic strategy using siRNA.

Based on our results, we studied the effect of MRP2 on chemoresistance to oxaliplatin by silencing the MRP2 gene in GI cancer cells. After knockdown of the MRP2 gene, both Caco-2 and PANC-1 cells enhanced oxaliplatin-induced apoptosis with increased sensitivity to oxaliplatin. Thus, silencing the MRP2 gene can be a potential novel therapeutic target in GI cancers. Therefore, according to the current study, the addition of ABCC2-siRNAs to oxaliplatin may enhance the effective treatment of oxaliplatin-based chemotherapy in GI cancer patients.

In summary, the present study demonstrated that transfection of ABCC2-siRNAs in GI cancer cells elevated the oxaliplatin-induced apoptosis. The combination of ABCC2-siRNA and oxaliplatin appears to significantly increase the cellular accumulation of oxaliplatin-derived platinum in Caco-2 and PANC-1 cells, therefore, enhances the cytotoxicity of oxaliplatin. Overall, this study indicates that ABCC2-siRNA can inhibit the MRP2-mediated drug transport function by inhibiting the MRP2 expression at mRNA and surface level. The current study suggests that the combination of ABCC2-siRNA to current regimens with oxaliplatin can be used as a novel approach to alternate MRP2-mediated drug resistance in pancreatic and colorectal cancer, which may enhance the therapeutic effects and thus, be beneficial for GI cancer patients.

5.5. Conclusion

Based on our present findings, clinical studies are now needed to evaluate the potential interest of MRP2 transporters in personalised medicine. Assessment of gene expression levels of the MRP2 gene could help physicians with the choice of appropriate chemotherapy regimens for GI cancer patients. Finally, inhibiting MRP2 transporters or downregulation of the MRP2 gene could be considered as a novel therapeutic approach to circumvent chemoresistance to oxaliplatin-based chemotherapy.

Chapter 6 Myricetin: a novel strategy to enhance oxaliplatin-induced apoptosis in GI cancer cells

6.1. Introduction

Studies have shown that ABC transporters are responsible for the efflux of drugs, which in turn decreases intracellular drug concentrations, causing failure of chemotherapy. Therefore, the effective approaches to sensitise drug-resistant cancer cells to the anticancer drug will inhibit the activity of ABC transporters by suppressing their expression or by coadministering inhibitors, such as synthetic inhibitors along with anticancer drugs. In previous chapters, we have observed that suppressing the activity of MRP2 transporter in GI cancer cells using ABCC2-siRNAs enhanced the sensitivity of oxaliplatin. However, cell type-specific delivery of functional siRNAs into cells is difficult. The critical hurdle for siRNA-based clinical applications is the delivery of siRNAs across the cell plasma membrane and most of the approaches described earlier have the disadvantage of delivery siRNAs to cells non-specifically [349]. Therefore, in this section we have used a different strategy to overcome MDR in GI cancer cells. This strategy includes the use of a small molecule as an MRP2 inhibitor to inhibit MRP2 transport activity.

The use of synthetic inhibitors was first developed to inhibit the activity of P-gp. The first-generation P-gp inhibitors, such as cyclosporine A, erythromycin, tamoxifen and verapamil, have low efficacy and high toxicity at tolerable doses. The highest dosage of these inhibitors was required to reverse MDR [350, 351]. The second-generation of P-gp inhibitors improved the efficacy with reduced side effects, such as valspodar (an analogue of cyclosporine A). Valspodar was 10 to 20-fold more effective than cyclosporine A in reversing MDR in cell lines and animal models [142, 352]. However, a high concentration of chemotherapeutic drugs was used along with second-generation inhibitors as these inhibitors led to drug metabolism and increased excretion of drugs. Consequently, end results led to a high risk of toxicity [353]. These ineffectiveness results led to the development of third-generation P-gp inhibitors, such as tariquidar and phenothiazines, which were more effective than previous inhibitors [354-356]. Tariquidar was combined with a non P-gp substrate carboplatin to treat terminated lung cancer [357], leading to confounded results with tariquidar.

The clinical use of most of the synthetic inhibitors of other ABC transporters was limited to *in vitro* experiments. MK571 is well-known third-generation inhibitor to inhibit the activities of MRPs including MRP2 in different cancer cell lines [219, 358, 359]. The use of synthetic inhibitors in *in vivo* systems has been barely investigated. The main reasons for limited use of inhibitors were related to toxicity, drug clearance and insufficient knowledge of the pharmacokinetic properties of the compounds [360-362]. MK571 may not even be suitable for *in vitro* oxaliplatin-MRP2 interaction studies because it can interact with oxaliplatin directly to form Pt(DACH) sulfhydryl complexes, potentially confounding MRP2 modulation effects.

Several other small molecules were developed to modulate ABC transporters and these molecules exhibit less cytotoxicity than synthetic inhibitors. Natural compounds extracted from plants, fruits, vegetables, herbs and animals as well as their derivatives by chemical modification are able to reverse MDR in ABC transporters or modulate the activity of efflux transporters and therefore exhibit less toxic effects than synthetic inhibitors [363, 364]. The well-known natural products used to inhibit or modulate the activity of transporters are curcumin, fumitremorgin C (FTC), myricetin, saponin and Sipholenol A [219, 364-366].

In this chapter, we have used myricetin as a natural flavonoid that inhibits the activity of MRP2 transporters. Myricetin is a common plant-derived flavonoid extracted mainly from members of the family Myricaceas (myricetin). Myricetin is mainly produced from taxifolin through the dihydromyricetin intermediate or directly produced from another flavonoid known as kaempferol [367]. This natural compound is very common in berries, vegetables, teas and wines produced from various plants. Myricetin has been shown to possess a wide range of activities that include antioxidant, anticancer, antidiabetic and anti-inflammatory activities [368]. It also plays an important role in maintaining the central nervous system.

In terms of potential health effects, myricetin protects DNA from oxidative damage and prevents the risk of cancer [369]. Myricetin is cytotoxic towards a number of human cancer cell lines, including hepatic, skin, pancreatic and colon cancer cells. Previous studies report that myricetin can induce cell cycle arrest and enhanced apoptosis in colon cancer cells [370]. Furthermore, myricetin has increased the sensitivity to vincristine in MDCKII cells by inhibiting the activity of MRP1 and MRP2 transporters [371]. It has been reported that the combination of myricetin and cisplatin increased its

cytotoxic efficacy in human cervical cancer cells compared with cisplatin alone [372]. This investigation suggests that myricetin combined with cisplatin has a potential clinical chemotherapeutic approach in human cervical cancer. Furthermore, recently it has been reported that one of the variants of myricetin, dihydromyricetin, reverses MRP2-mediated oxaliplatin resistance in colorectal cancer cells [373]. According to Wang et. al., the proliferation of oxaliplatin-resistant colorectal cancer cells, HCT116/L-OHP, was suppressed by dihydromyricetin and therefore, enhanced the sensitivity of oxaliplatin in colorectal cancer cells by downregulating the expression of MRP2. However, it remains to be determined whether myricetin can reverse platinum-based oxaliplatin chemoresistance in GI cancer. It will be interesting to determine the effect of myricetin on the MRP2-mediated oxaliplatin transport.

The main objective of the experimental work described in this chapter was to determine the cellular accumulation of MRP2 substrate and oxaliplatin-induced apoptosis rate after treating the GI cancer cells with the MRP2 inhibitor, myricetin. Similar to the previous studies, we have used CDCF as a model substrate to measure the transport activity of MRP2. In this study, CDCFDA was used, which is a non-fluorescent precursor of CDCF and it can passively diffuse into the cells and be metabolised in the cells to the fluorescent CDCF, which can only be transported out of the cells by the MRP2 transporter. The oxaliplatin-induced apoptosis rate was determined by Annexin V-FITC apoptosis staining assay.

The main aim of this chapter is:

I. To characterise the MRP2-mediated transport of the substrate, CDCF, by conducting a time-course study in the presence of myricetin in GI cancer cell lines.

II. To determine the rate of apoptosis induced by oxaliplatin in the presence and absence of myricetin in GI cancer cell lines.

6.2. Materials and methods

6.2.1. Chemicals

The details of chemicals, sources and their stocks solution preparation were described earlier in chapter 2, section 2.1. The details of the apparatuses used for this study is given in chapter 2, table 2-2.

6.2.2. Cell culture and maintenance

HepG2, Caco-2 and PANC-1 cell lines from ATCC were grown and maintained in RPMI 1640 medium supplemented with 10% FBS, 5 mg/ml L-glutamine and 5 mg/ml penicillin-streptomycin solution in an atmosphere of 5% CO_2. Cell viability was between 80%-90% and cell concentration was calculated using trypan blue exclusion.

6.2.3. Cellular CDCF accumulation study

MRP2 substrate, CDCF (5(6)-carboxy-2',7'-dichlorofluorescein) accumulation assay was used to measure the transport activity of MRP2 in HepG2 cell line. Firstly, the HepG2, PANC-1 and Caco-2 cells were incubated with 60 µM of myricetin. Thereafter, cells were incubated with 2.5 µM of CDCF for 5, 10, 20, 40 and 60 min at 37°C with minimal light exposure. This procedure was carried out at different time points to determine the time the substrate took to reach steady state. The details of the experimental procedure are discussed in section 2.5.1.

6.2.4. Oxaliplatin-induced apoptosis assay in the presence of myricetin

To determine the oxaliplatin-induced apoptosis in the presence of MRP2 inhibitor, the HepG2, PANC-1 and Caco-2 cells were seeded at a density of 200,000 cells per ml in 6-well plate and incubated with 60 µM myricetin and different ranges of oxaliplatin concentration. After seeding cells in a 6-well plate, cells were incubated with 60 µM myricetin for 30 min. Thereafter in the same cell culture treated with myricetin different concentrations of oxaliplatin (25 µM, 50 µM and 100 µM) was coincubation for 2 hr. The cells were washed with ice-cold PBS after incubation. The samples were prepared as per the procedure mentioned in section 2.8.1. Finally, oxaliplatin-induced apoptosis in the presence of inhibitor in stained cells was measured by flow cytometry.

6.2.5. Data analysis

Flow cytometry data were analysed according to the details mentioned in section 2.11 and statistical data analysis was carried out according to the details mentioned in section 2.12.

6.3. Results

6.3.1. Effects of myricetin on functional activity of MRP2 transporter in HepG2 cells

The cellular accumulation of MRP2 substrate, CDCF, was observed to investigate the effect of myricetin on MRP2 functional activity. Myricetin was used as an inhibitor of MRP2. Myricetin increased the efflux activity of MRP2 in HepG2 cells. The efflux activity of MRP2 was determined in the presence and/or absence of myricetin at different time points using CDCF, a known substrate of MRP2. A significant increase in CDCF accumulation in HepG2 cells by myricetin suggests decreased MRP2 efflux activity within 20 min.

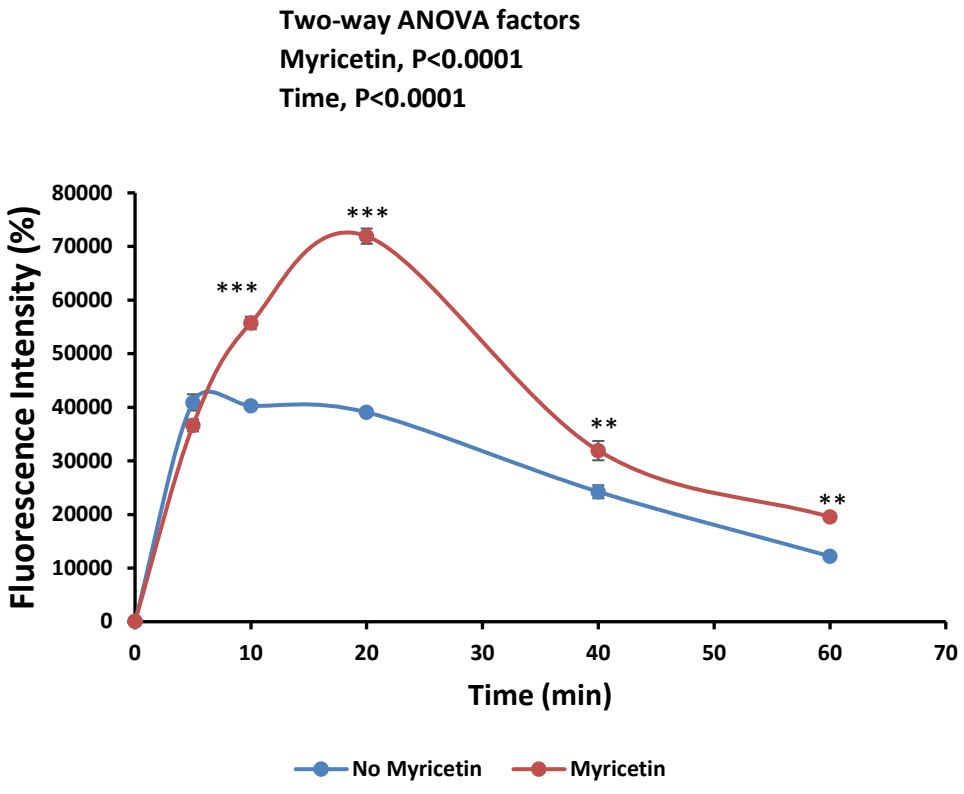


Figure 6.1. CDCF accumulation study in HepG2 cells.

CDCF accumulation at different time points (5, 10, 20, 40 and 60 min) in the presence and absence of myricetin. Data are presented as mean and SEM. Asterisks are P values (*, P<0.001) from Sidak multiple comparisons test that followed two-way ANOVA for comparisons of all cell samples (n=2).**

6.3.2. Oxaliplatin and myricetin combination enhanced apoptotic cell death in HepG2 cells

We examined the effect of the combination of myricetin and oxaliplatin in cell apoptosis in HepG2 cells. Drug combination groups used for the apoptosis assay were 60 μM myricetin plus 25 μM oxaliplatin and 60 μM myricetin plus 100 μM oxaliplatin, because with 25 μM and 100 μM, oxaliplatin showed increased cellular accumulation of oxaliplatin-derived platinum. Cells were treated with myricetin alone (60 μM), oxaliplatin alone (25 μM and 100 μM) and the combination of both, as mentioned above, for 2 hr and were then subjected to flow cytometry analysis. For cells only without any treatment, the apoptotic cell percentage was 9.7%. The percentage of apoptosis cells were almost the same as HepG2 cells only after exposure to 60 μM myricetin. The percentage of apoptosis cells were 20% and 23% after exposure to 25 μM and 100 μM oxaliplatin respectively. Similarly, with the myricetin and oxaliplatin combination treatment, the apoptosis cell percentages were 26.5% and 45% after exposure to a combination treatment of 60 μM myricetin plus 25 μM oxaliplatin and 60 μM myricetin plus 100 μM oxaliplatin respectively. Statistical analysis showed that the percentage of apoptosis in HepG2 cells in combination treatments were significantly higher than those treated with myricetin or oxaliplatin treatment alone. These results suggest that myricetin alone has no apoptotic effect in HepG2 cells, while increasing the dose of oxaliplatin increases the rate of apoptosis in HepG2 cells. From the results, we can suggest that myricetin obviously enhances the rate of apoptosis in HepG2 cells compared to oxaliplatin used alone.

Table 6-1. Data analysis of apoptosis assay with oxaliplatin treatment in HepG2 cells in the presence and absence of myricetin (60μM)

Treatment	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P- value* (Total Apoptosis)
Normal	**88.75 ± 0.38**	8.04 ± 0.6	1.70 ± 0.07	**9.74 ± 0.73**	
Myricetin (Myr) only	**81.87 ± 1.99**	2.50 ± 0.83	4.91 ± 1.64	**7.42 ± 1.91**	ns
Oxaliplatin (Oxa) (25 μM)	**75.3 ± 0.92**	13.37 ± 0.51	6.90 ± 0.98	**20.27 ± 0.47**	
Myr and Oxa (25 μM)	**66.82 ± 0.73**	15.57 ± 0.52	10.94 ± 0.98	**26.51 ± 0.46***	0.023
Oxa (100 μM)	**58.90 ± 1.54**	5.13 ± 1.08	17.80 ± 1.22	**22.93 ± 2.3**	
Myr and Oxa (100 μM)	**41.62 ± 0.18**	21.38 ± 1.64	23.43 ± 1.38	**44.81 ± 1.44*****	<0.0001

***P-value compared with the total apoptosis induced by oxaliplatin only.**

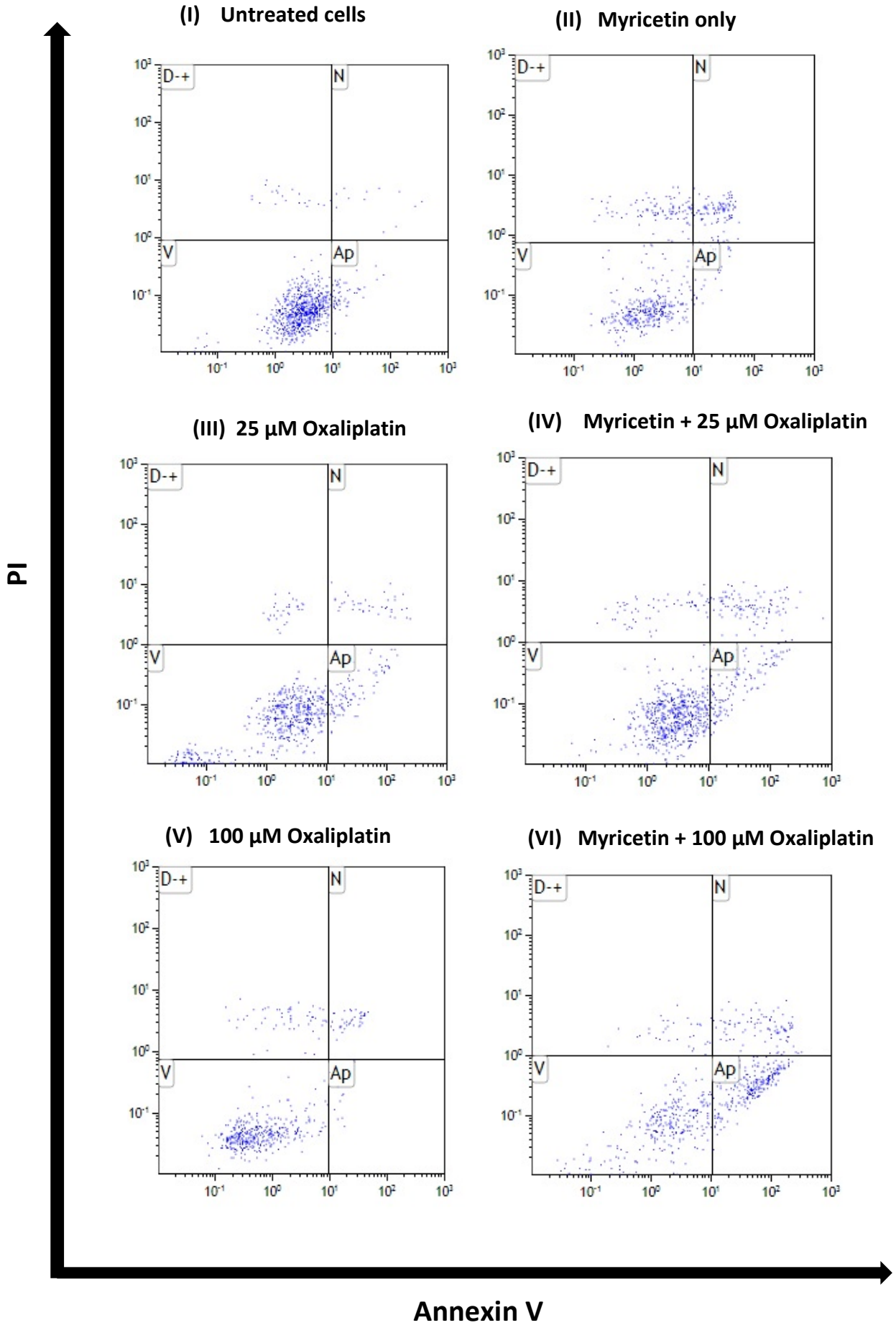
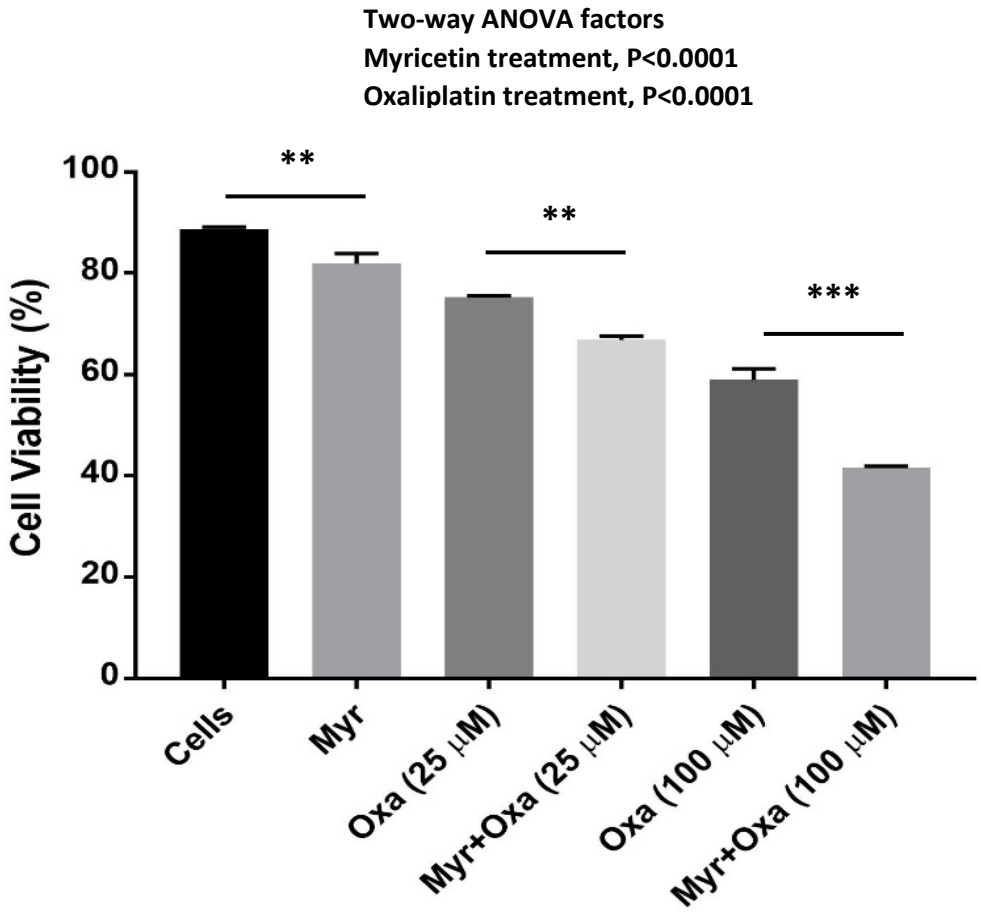

(I) Untreated cells
(II) Myricetin only
(III) 25 µM Oxaliplatin
(IV) Myricetin + 25 µM Oxaliplatin
(V) 100 µM Oxaliplatin
(VI) Myricetin + 100 µM Oxaliplatin
D-+
N
V
Ap
PI
Annexin V

Figure 6.2. Apoptotic changes in HepG2 cells and their treatment with myricetin and oxaliplatin.

Detection of apoptosis by concurrent staining with Annexin-V and PI. HepG2 cells were untreated or treated with myricetin and oxaliplatin at different concentrations. Cells were subsequently stained with Annexin-V and PI and their fluorescence was measured by flow cytometry. Viable cells (V) are both Annexin-V and PI negative. At an early stage of apoptosis (Ap), the cells bound with only Annexin-V. At the late stage of apoptosis (N), the cells bound with both Annexin-V FITC and PI. The last quadrant (D-+) represents the debris cells or dead cells. (I) Untreated HepG2 cells, (II) Cells treated with only myricetin, (III) Cells treated with 25 μM of oxaliplatin, (IV) Cells treated with myricetin and 25 μM oxaliplatin, (V) Cells treated with 100 μM oxaliplatin, and (VI) Cells treated with both myricetin and 100 μM oxaliplatin.

A.



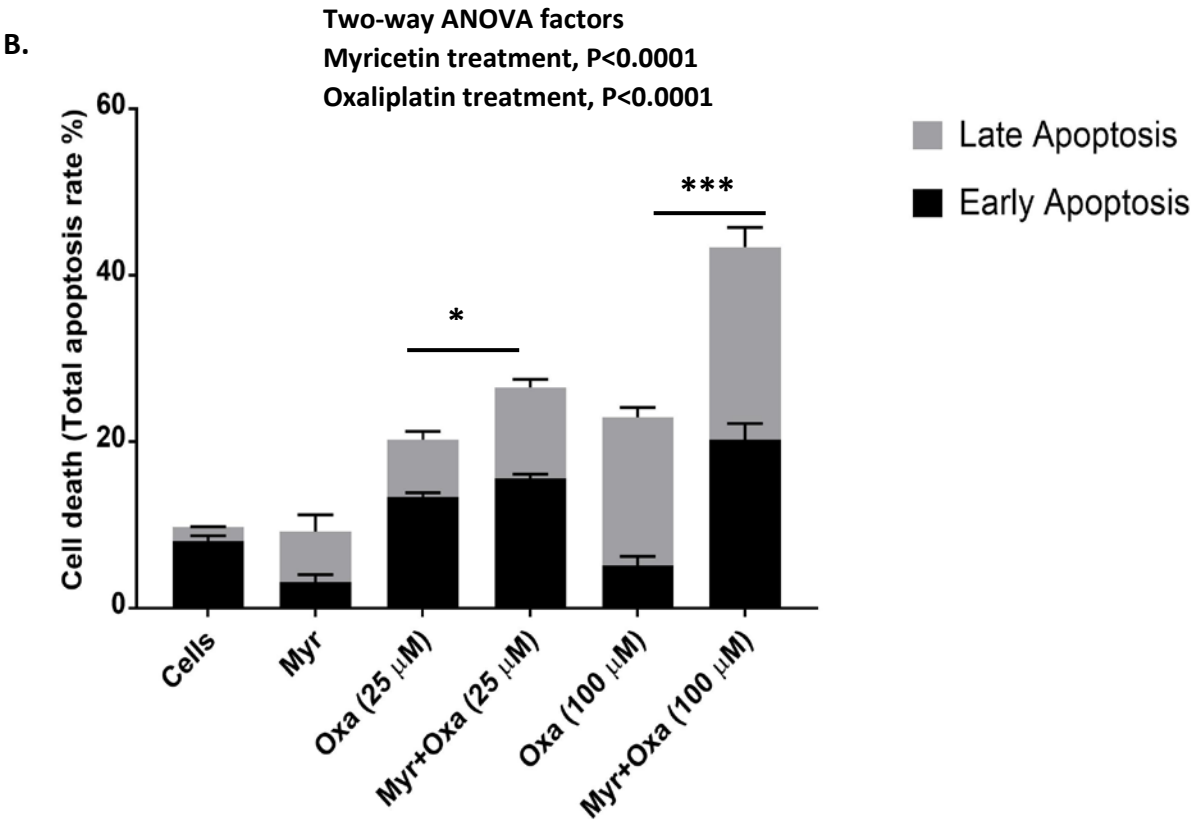


Figure 6.3. Oxaliplatin-induced apoptosis in HepG2 cells in the presence or absence of myricetin.

HepG2 cells were treated with 60 μM of myricetin (myr) for 30 min followed by coincubation with oxaliplatin (oxa) for another 2 hr. The cell viability and apoptosis rate were assessed by flow cytometry. A) Percentage cell viability after treatment of HepG2 with different concentrations of oxaliplatin (25 μM and 100 μM). B) Early and late apoptosis rate in HepG-2 cells after myricetin and oxaliplatin treatment. Data are presented as mean and SEM of two independent experiments. Asterisks are P values (*, P<0.05; **, P<0.01; ***, P<0.001) from Sidak multiple comparisons test that followed two-way ANOVA for comparisons of all cell samples (n=2).

6.3.3. Effects of myricetin on functional activity of MRP2 transporter in PANC-1 cells

The MRP2-specific substrate, CDCF, has been used to determine the functional activity of MRP2 transporters. The efflux activity of MRP2 was determined by measuring the fluorescence intensity of CDCF at a different time point in the presence and/or absence of myricetin. The fluorescence intensity increased with increase in time. After 10 min, no significant changes were observed in the fluorescence intensity. In PANC-1 cell lines, no significant changes were observed in terms of cellular accumulation of substrate in cells treated with myricetin. However, with an increase in incubation time, after 15 min, the CDCF cellular accumulation increased compared to cells without myricetin treatment.

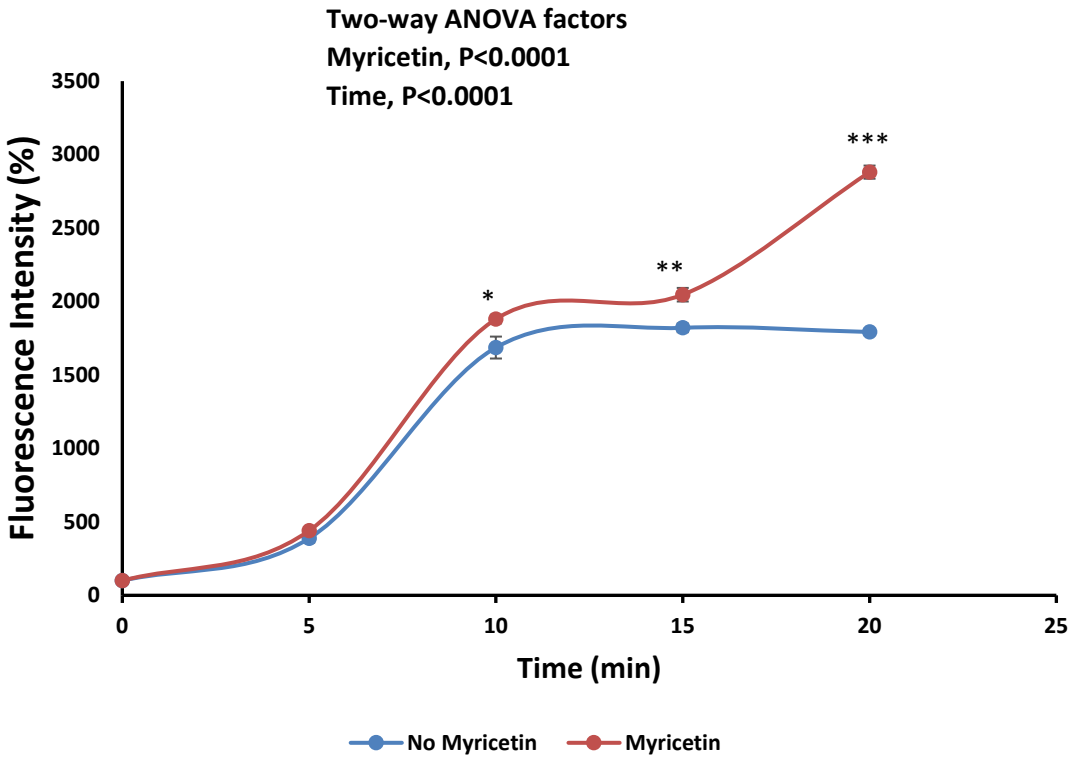


Figure 6.4. CDCF accumulation study in PANC-1 cells.

CDCF accumulation at different time points (5, 10, 15 and 20 min) in the presence and absence of myricetin. Data are presented as mean and SEM. Asterisks are P values (*, P<0.05; **, P<0.01; ***, P<0.001) from Sidak multiple comparisons test that followed two-way ANOVA for comparisons of all cell samples (n=2).

6.3.4. Oxaliplatin and myricetin combination enhanced apoptotic cell death in PANC-1 cells

To investigate the mechanism of sensitisation induced by oxaliplatin in the PANC-1 cells treated with myricetin, we examined the effect of the combination of myricetin and oxaliplatin in cell apoptosis in the PANC-1 cells. Drug combination groups used for the apoptosis assay were 60 μM myricetin plus 25 μM oxaliplatin, 60 μM myricetin plus 50 μM oxaliplatin and 60 μM myricetin plus 100 μM oxaliplatin. Cells were treated with myricetin alone (60 μM), oxaliplatin alone (25 μM, 50 μM and 100 μM) and the combination of both, as mentioned above, for 2 hr then subjected to flow cytometry analysis. For cells only without any treatment, the total apoptotic cell percentage was approximately 7%. The percentage of apoptosis cells was almost same as the PANC-1 cells only after exposure to 60 μM myricetin. The percentages of total apoptosis cells were 12%, 18% and 25% after exposure to 25 μM, 50 μM and 100 μM oxaliplatin respectively. Similarly, with the myricetin and oxaliplatin combination treatment, the total apoptosis cells percentages were 27%, 40% and 50% after exposure to combination treatment of 60 μM myricetin plus 25 μM oxaliplatin, 60 μM myricetin plus 50 μM oxaliplatin and 60 μM myricetin plus 100 μM oxaliplatin respectively. Hence, in the cells treated with myricetin, the total apoptosis rate was increased by 15% ($P<0.0001$), 22% ($P<0.0001$) and 25% ($P<0.0001$) after exposure to 25 μM, 50 μM and 100 μM oxaliplatin respectively, compared to cells treated with respective concentrations of oxaliplatin alone. Statistical analysis showed that percentages of apoptosis in the PANC-1 cells in combination treatment were significantly higher than those treated with myricetin or oxaliplatin alone. These results suggest that myricetin alone has no apoptotic effect in PANC-1 cells, while increasing the dose of oxaliplatin increases the rate of apoptosis. From the results, we suggest that oxaliplatin markedly increased both early and late apoptosis rates in the PANC-1 cells after treatment with both myricetin and oxaliplatin compared with the treatment with oxaliplatin alone, and this occurred in a dose-dependent manner. These results indicate that myricetin obviously enhanced cell apoptosis in the PANC-1 cells.

Table 6-2. Data analysis of apoptosis assay with oxaliplatin treatment in PANC-1 cells in the presence and absence of myricetin (60μM)

Treatment	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P-value* (Total Apoptosis)
Normal	**93.07 ± 1.07**	6.38 ± 0.84	0.54 ± 0.23	**6.92 ± 1.07**	
Myricetin only	**91.90 ± 0.95**	6.66 ± 0.92	1.34 ± 0.05	**8.00 ± 0.97**	ns
Oxa (25 μM)	**87.12 ± 0.31**	7.69 ± 0.11	4.99 ± 0.24	**12.68 ± 0.13**	
Myr and Oxa (25 μM)	**71.59 ± 1.58**	17.93 ± 1.92	9.67 ± 1.09	**27.6 ± 0.83****	0.004
Oxa (50 μM)	**82.03 ± 0.23**	10.47 ± 1.11	7.39 ± 1.40	**17.86 ± 0.28**	
Myr and Oxa (50 μM)	**59.5 ± 0.78**	19.72 ± 0.18	20.39 ± 0.43	**40.11 ± 0.61*****	<0.0001
Oxa (100 μM)	**73.97 ± 1.21**	13.37 ± 0.16	12.5 ± 0.89	**25.87 ± 1.05**	
Myr and Oxa (100 μM)	**49.98 ± 0.42**	24.99 ± 8.11	24.86 ± 7.75	**49.85 ± 0.36*****	0.0008

***P-value compared with the total apoptosis induced by oxaliplatin only.**

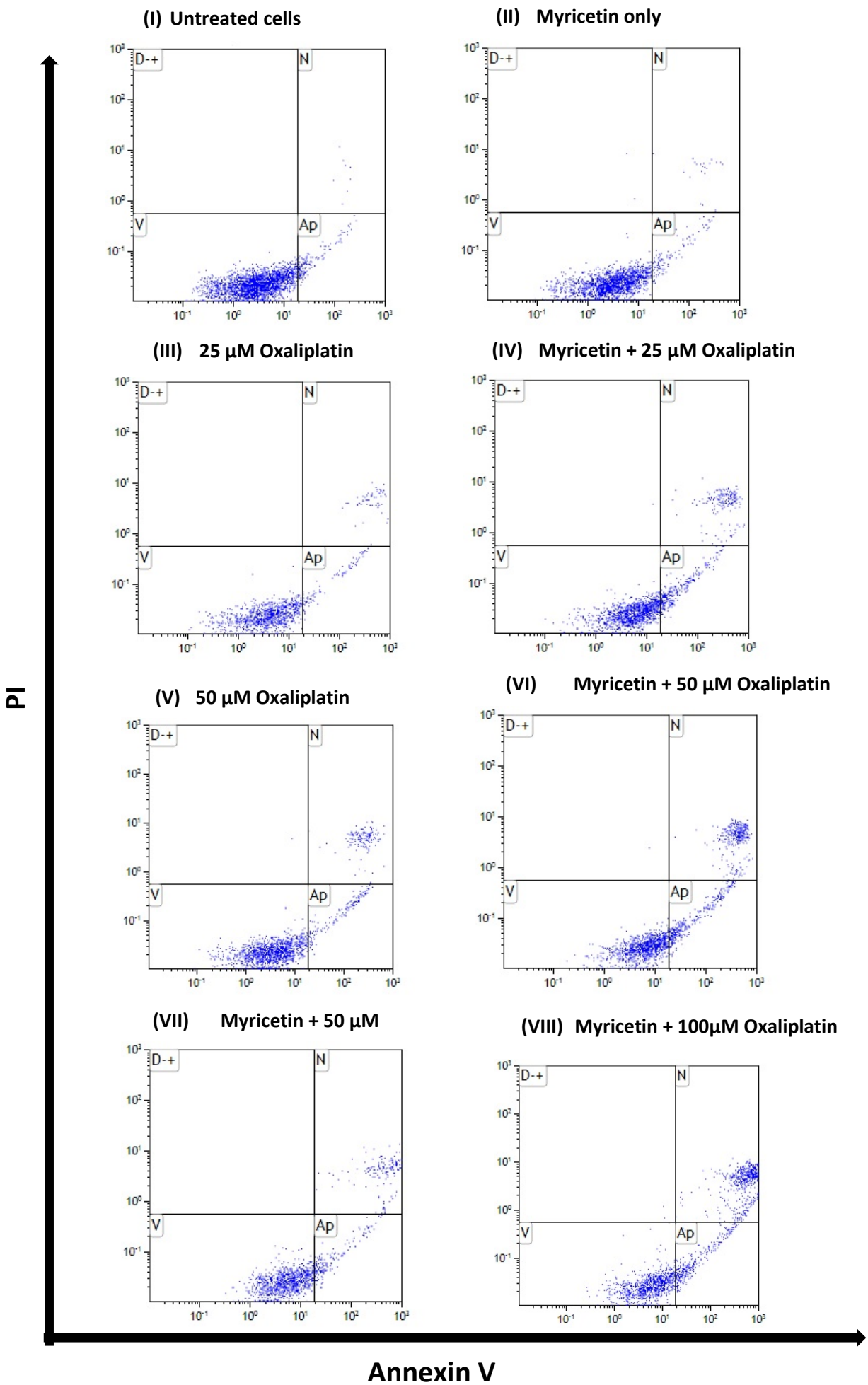
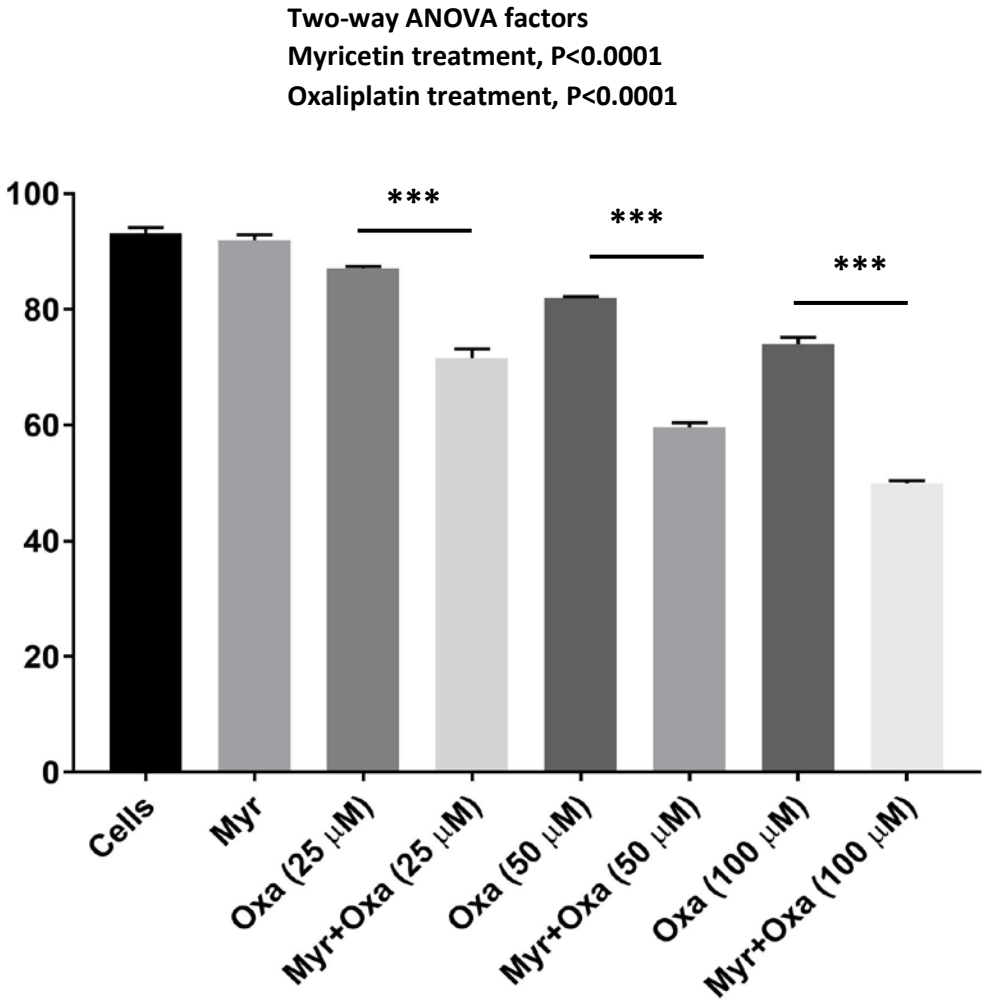

(I) Untreated cells
(II) Myricetin only
(III) 25 µM Oxaliplatin
(IV) Myricetin + 25 µM Oxaliplatin
(V) 50 µM Oxaliplatin
(VI) Myricetin + 50 µM Oxaliplatin
(VII) Myricetin + 50 µM
(VIII) Myricetin + 100µM Oxaliplatin
D-+
N
V
Ap
PI
Annexin V

Figure 6.5. Apoptotic changes in PANC-1 cells and their treatment with myricetin and oxaliplatin.

Detection of apoptosis by concurrent staining with Annexin-V and PI. PANC-1 cells were untreated or treated with myricetin, and oxaliplatin at different concentrations. Cells were subsequently stained with Annexin-V and PI and their fluorescence was measured by flow cytometry. Viable cells (V) are both Annexin-V and PI negative. At an early stage of apoptosis (Ap), the cells bound with only Annexin-V. At the late stage of apoptosis (N), the cells bound with both Annexin-V FITC and PI. The last quadrant (D-+) represents the debris cells or dead cells. (I) Untreated PANC-1 cells, (II) Cells treated with only myricetin, (III) Cells treated with 25 μM of oxaliplatin, (IV) Cells treated with myricetin and 25 μM oxaliplatin, (V) Cell treated with 50 μM oxaliplatin, (VI) Cells treated with myricetin and 50 μM oxaliplatin, (V) Cell treated with 100 μM oxaliplatin, and (VIII) Cells treated with myricetin and 100 μM oxaliplatin.

A.



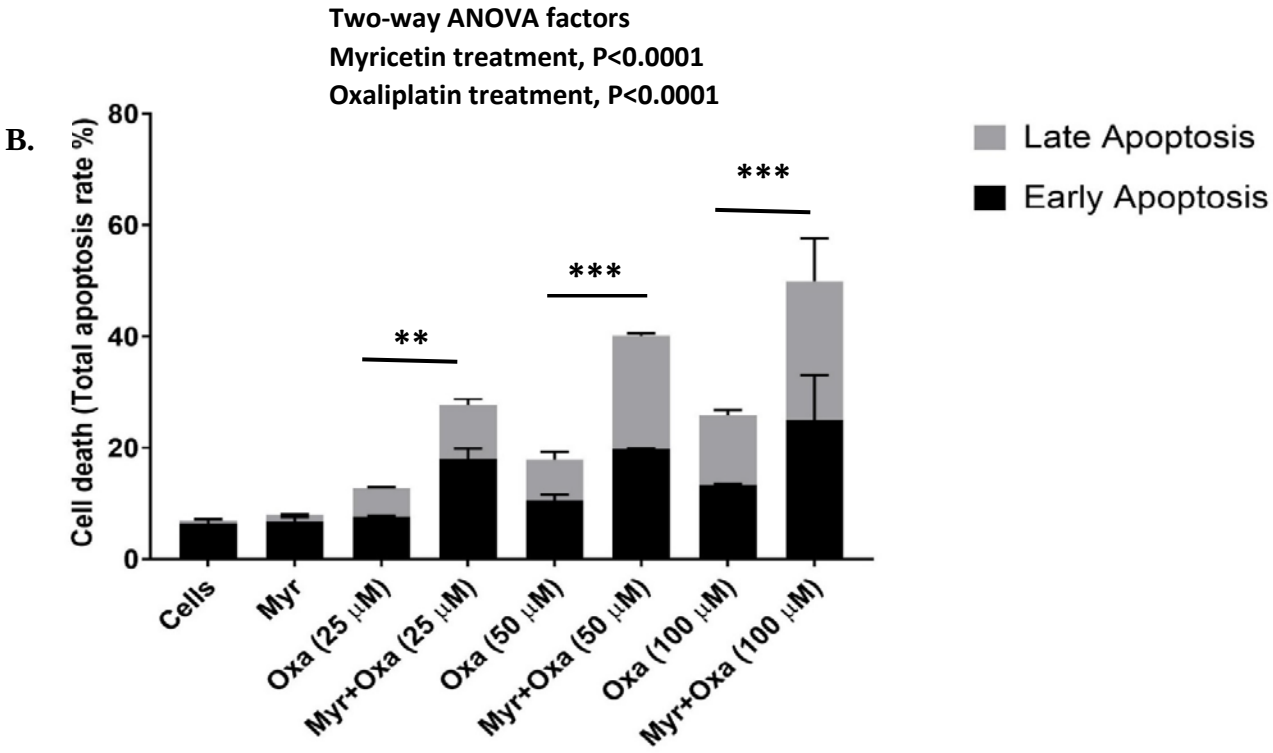


Figure 6.6. Oxaliplatin-induced apoptosis in PANC-1 cells in the presence or absence of myricetin.

PANC-1 cells were treated with 60 μM of myricetin (myr) for 30 min followed by coincubation with oxaliplatin (oxa) for another 2 hr. The cell viability and apoptosis rates were assessed by flow cytometry. A) Percentage of cell viability after treatment of PANC-1 with different concentrations of oxaliplatin (25 μM, 50 μM and 100 μM). B) Early and late apoptosis rates in PANC-1 cells after myricetin and oxaliplatin treatment. Data are presented as mean and SEM of two independent experiments. Asterisks are P values (*, P<0.001) from Sidak multiple comparisons test that followed two-way ANOVA for comparisons of all cell samples (n=2).**

6.3.5. Effects of myricetin on functional activity of MRP2 transporter in Caco-2 cells

The cellular accumulation of MRP2 substrate, CDCF, was observed to investigate the MRP2 functional activity in Caco-2 cells. Myricetin was used as an inhibitor of MRP2. The efflux activity of MRP2 was determined in the presence of myricetin at different time points using CDCF. The fluorescence intensity increased with the increase in time points and after 10 min, no changes were observed in the fluorescence intensity. The fluorescence intensity was higher in cells treated with myricetin compared to cells with no myricetin treatment. This result shows that myricetin was able to inhibit the functional activity of MRP2 in Caco-2 cells. After 10 min, the cellular substrate accumulation reaches steady state with no change; therefore, ABCC2-siRNA transfected cells were treated with CDCFDA for 10 min to determine the efflux effect of MRP2 in ABCC2-siRNAs transfected Caco-2 cells in chapter 5.

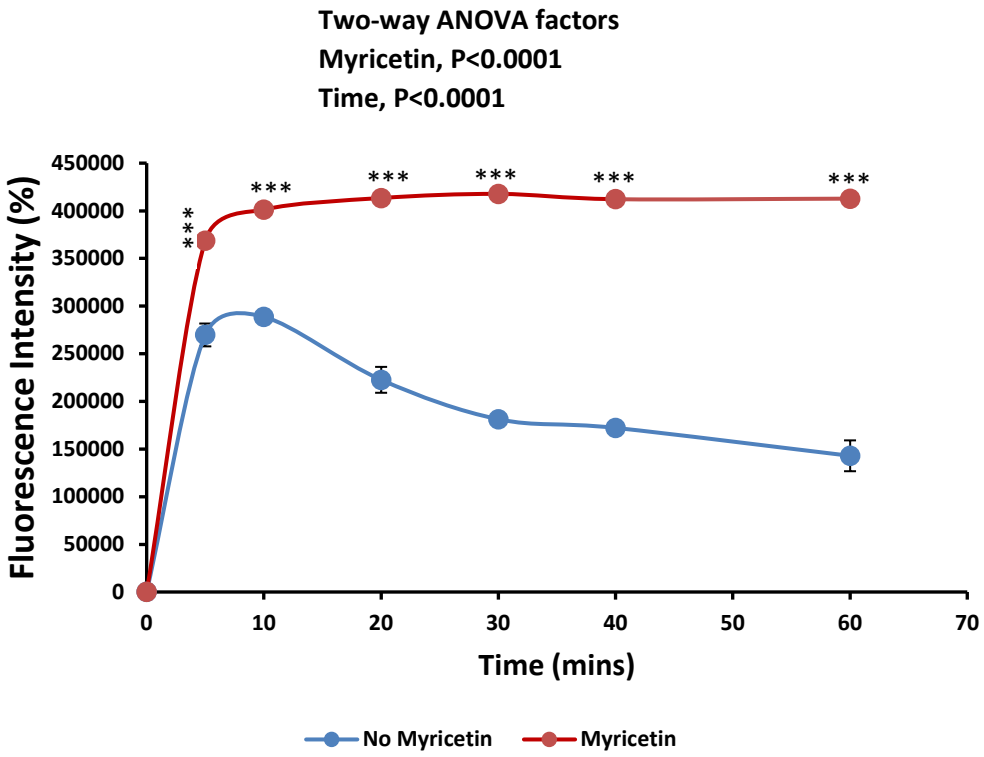


Figure 6.7. CDCF accumulation study in Caco-2 cells.

CDCF accumulation at different time points (5, 10, 20, 30, 40 and 60 min) in the presence and absence of myricetin. Data are presented as mean and SEM. Asterisks are P values (*, P<0.001) from Sidak multiple comparisons test that followed two-way ANOVA for comparisons of all cell samples (n=2).**

6.3.6. Effect of myricetin on oxaliplatin-induced apoptosis in Caco-2 cells

The mechanism of sensitisation induced by oxaliplatin in the Caco-2 cells treated with myricetin was examined by apoptosis assay. Drug combination groups used for the apoptosis assay were 60 μM myricetin plus 25 μM oxaliplatin and 60 μM myricetin plus 100 μM oxaliplatin. Cells were treated with myricetin alone (60 μM), oxaliplatin alone (25 μM and 100 μM) and a combination of both, as mentioned above, for 2 hr, then subjected to flow cytometry analysis. For cells only without any treatment, the total apoptotic cells percentage was 5.83%. The percentage of apoptosis cells was 14% in the Caco-2 cells after exposure to 60 μM myricetin. Therefore, after myricetin treatment the apoptosis rate was increased by 8% (P=0.006), which indicates that myricetin treatment only was slightly cytotoxic to Caco-2 cells. The percentages of apoptosis cells were 15% and 25% after exposure to 25 μM and 100 μM oxaliplatin respectively. Similarly, with the myricetin and oxaliplatin combination treatment, the apoptosis cells percentages were 25% and 36% after exposure to the combination treatment of 60 μM myricetin plus 25 μM oxaliplatin and 60 μM myricetin plus 100 μM oxaliplatin respectively. Statistical analysis shows that the percentages of apoptosis in the Caco-2 cells in combination treatments were significantly higher than those treated with myricetin or oxaliplatin treatment alone and the apoptosis rates were increased by 10% (P=0.0006) and 11% (P<0.0001). These results suggest that myricetin alone has a less cytotoxic effect in Caco-2 cells and its combination treatment with oxaliplatin enhanced the rate of total apoptosis. From these results, we can suggest that oxaliplatin markedly increased both early and late apoptosis rates in the Caco-2 cells after treatment with both myricetin and oxaliplatin compared with the treatment with oxaliplatin alone, and this occurred in a dose-dependent manner. These results indicate that myricetin obviously enhances the cell apoptosis in the Caco-2 cells.

Table 6-3. Data analysis of apoptosis assay with oxaliplatin treatment in Caco-2 cells in the presence and absence of myricetin (60 μM)

Treatment	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptosis (%)	P-value* (Total Apoptosis)
Normal	**93.68 ± 0.97**	5.58 ± 0.89	0.72 ± 0.12	**5.83 ± 0.87**	
Myricetin only	**81.64 ± 1.36**	6.04 ± 1.78	7.97 ± 0.79	**14.01 ± 2.79****	0.002
Oxa (25 μM)	**82.68 ± 0.78**	9.08 ± 0.82	6.01 ± 0.44	**15.09 ± 0.34**	
Myr and Oxa (25 μM)	**68.23 ± 1.70**	12.87 ± 0.31	12.08 ± 1.92	**23.80 ± 0.66*****	0.0002
Oxa (100 μM)	**70.61 ± 1.58**	14.93 ± 1.22	10.08 ± 0.52	**24.24 ± 1.06**	
Myr and Oxa (100 μM)	**54.50 ± 1.46**	15.19 ± 0.62	21.32 ± 1.49	**35.26 ± 1.64*****	<0.001

***P-value compared with the total apoptosis induced by oxaliplatin only.**

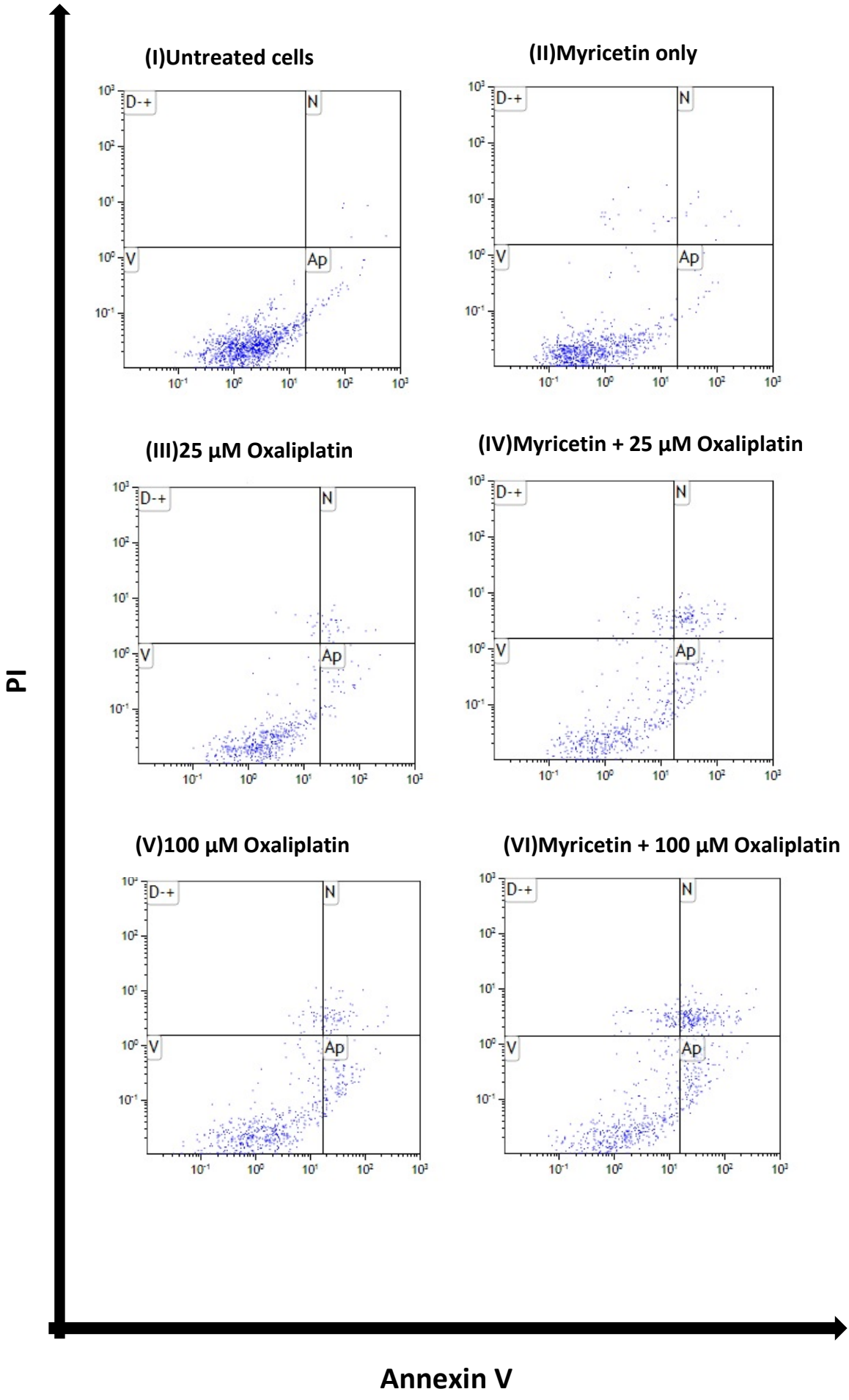
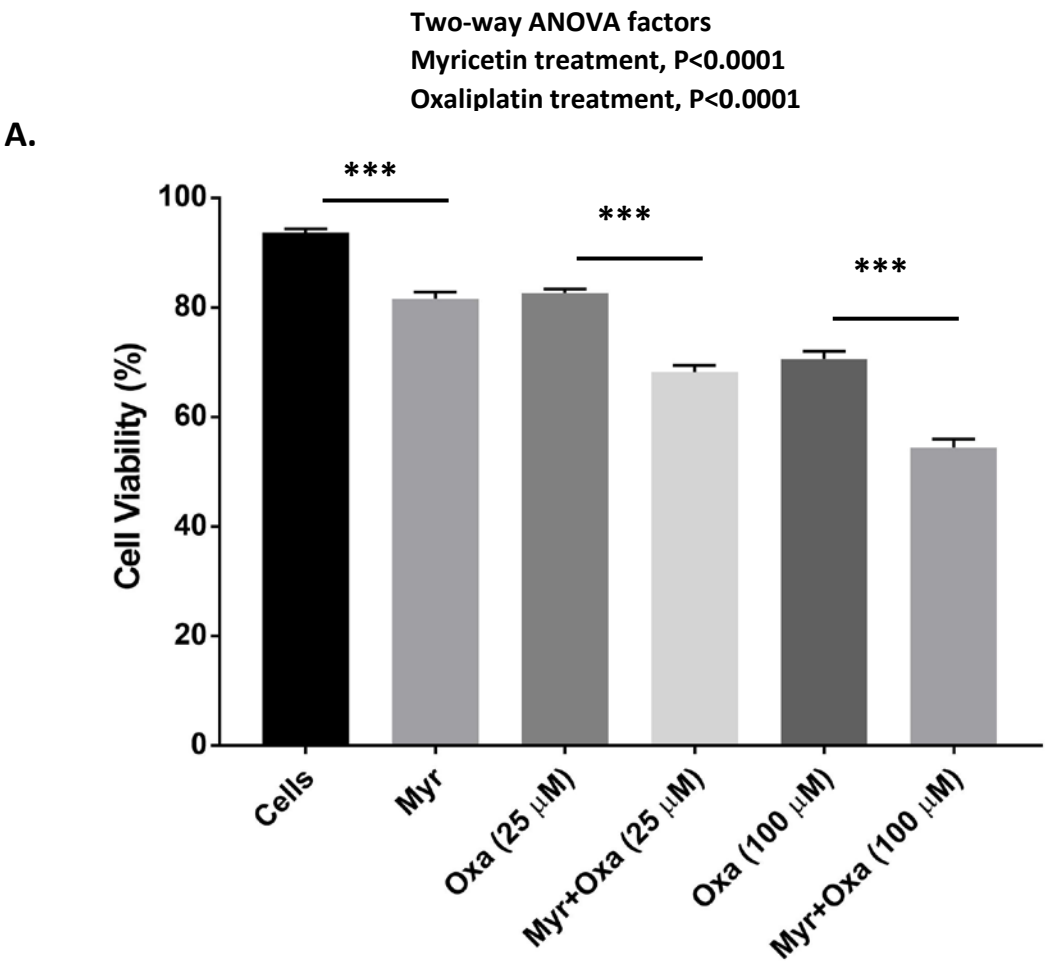

(I)Untreated cells
(II)Myricetin only
(III)25 µM Oxaliplatin
(IV)Myricetin + 25 µM Oxaliplatin
(V)100 µM Oxaliplatin
(VI)Myricetin + 100 µM Oxaliplatin
D-+
N
V
Ap
PI
Annexin V

Figure 6.8. Apoptotic changes in Caco-2 cells and their treatment with myricetin and oxaliplatin.

Detection of apoptosis by concurrent staining with Annexin-V and PI. Caco-2 cells were untreated or treated with myricetin, and oxaliplatin at different concentrations. Cells were subsequently stained with Annexin-V and PI and their fluorescence was measured by flow cytometry. Viable cells (V) are both Annexin-V and PI negative. At an early stage of apoptosis (Ap), the cells bound with only Annexin-V. At the late stage of apoptosis (N), the cells bound with both Annexin-V FITC and PI. The last quadrant (D-+) represents the debris cells or dead cells. (I) Untreated Caco-2 cells, (II) Cells treated with only myricetin, (III) Cells treated with 25 μM of oxaliplatin, (IV) Cells treated with myricetin and 25 μM oxaliplatin, (V) Cell treated with 100 μM oxaliplatin, (VI) Cells treated with myricetin and 100 μM oxaliplatin.



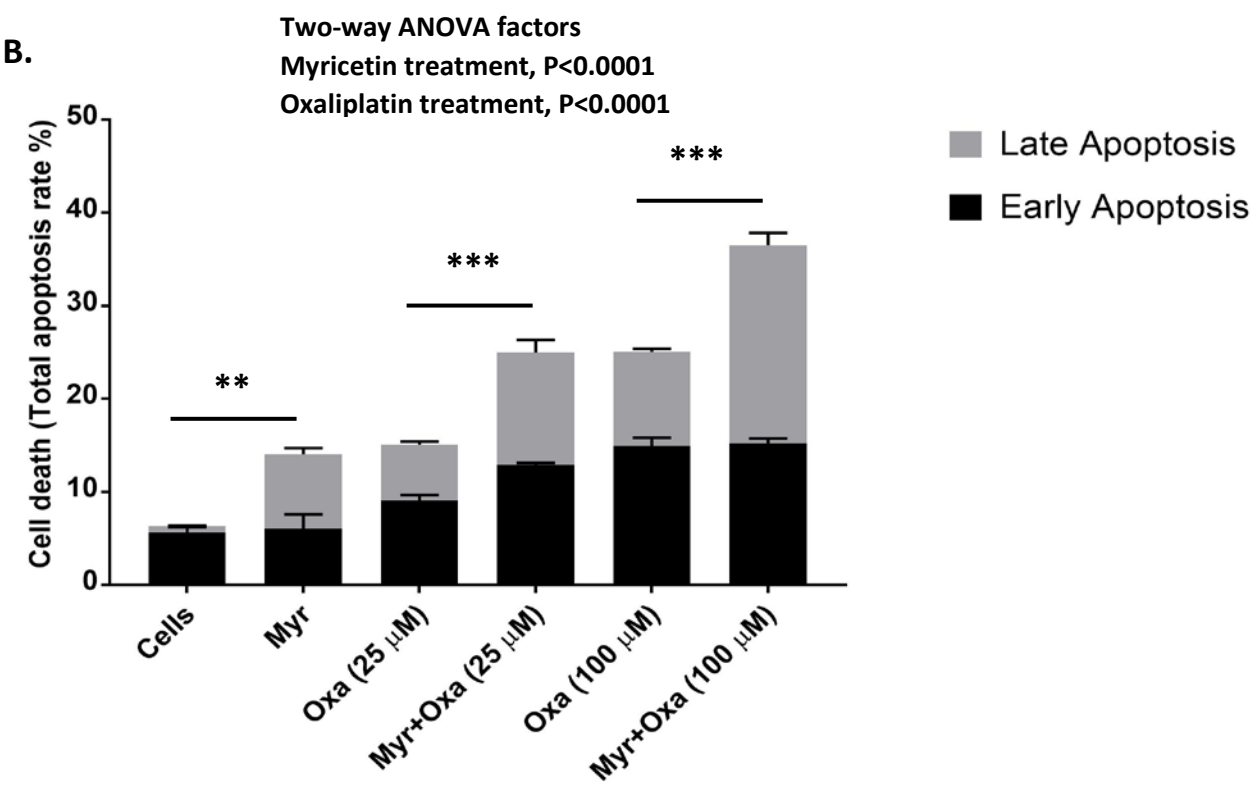


Figure 6.9. Oxaliplatin-induced apoptosis in Caco-2 cells in the presence or absence of myricetin.

Caco-2 cells were treated with 60 μM of myricetin (myr) for 30 min followed by coincubation with oxaliplatin (oxa) for another 2 hr. The cell viability and apoptosis rates were assessed by flow cytometry. A) Percentage of cell viability after treatment of Caco-2 with different concentrations of oxaliplatin (25 μM, 50 μM and 100 μM). B) Early and late apoptosis rate in Caco-2 cells after myricetin and oxaliplatin treatment. Data are presented as mean and SEM of three independent experiments. Asterisks are P values (, P<0.01; ***, P<0.001) from Sidak multiple comparisons test that followed two-way ANOVA for comparisons of all cell samples (n=4).**

6.4. Discussion

Small molecule inhibitors, either a synthetic inhibitor or a natural product like myricetin, have become an effective target for pharmacological inhibition of ABC transporters in cancer. Myricetin as a natural flavonoid inhibitor has been reported to induce cell death in a dose-dependent manner in human colon cancer [374]. *In vivo* studies done by others have shown that myricetin has antitumor activity in colon, hepatocellular, gastric, ovarian and pancreatic cancer [374-378]. According to one of the studies, in human colon cancer cells, myricetin not only induced cell apoptosis but also inhibited cell proliferation by inhibiting DNA polymerase [370]. Moreover, a combination of myricetin and platinum-based cisplatin enhanced the apoptosis in cervical cancer cells [376]. A recent study reported that myricetin induced apoptosis in cisplatin-resistant cancer cell lines [376]. According to these studies, we can conclude that myricetin could potentially be used to overcome GI cancer chemoresistance against platinum-based therapy. However, the combined effect of myricetin and oxaliplatin in GI cancers is not well studied.

In this chapter, we found that platinum-based oxaliplatin anticancer drugs caused a concentration-dependent stimulation of apoptosis in HepG2, Caco-2 and PANC-1 cells. According to a recent study, oxaliplatin-derived platinum accumulation was significantly increased by myricetin in HEK 293 cells overexpressing MRP2 transporters compared to the parental cells. The concurrent treatment of cells with oxaliplatin and myricetin increased platinum accumulation and sensitivity to oxaliplatin-induced growth inhibition by 2-fold [248]. This finding provides evidence for the involvement of myricetin in inhibiting the functional activity of MRP2 and increasing the sensitivity of oxaliplatin in MRP2-expressing cells. The increased expression of MRP2 in GI cancers has been studied in various cancer cells, both *in vitro* and *in vivo*. Studies have shown that myricetin exhibited greater cytotoxicity in combination with platinum-based cisplatin in ovarian cancer cell lines and myricetin alone was less cytotoxic to the normal cell lines [376]. According to our study, the use of 60 μM of myricetin alone has no significant apoptotic effect in HepG2 and PANC-1 cells, indicating that there were no cytotoxic effects of myricetin in liver and pancreatic cancer cell lines. While for Caco-2 cells, the apoptosis rate increased by 8% compared to the untreated cells. Hence, we can conclude that use of myricetin only is less cytotoxic compared to its combination with oxaliplatin.

The mechanism through which cancer cells develop resistance to chemotherapy is associated with increased resistance to apoptosis. In preclinical models, drugs that induce apoptosis have been reported to increase their sensitivity in tumour cells to chemotherapy and radiotherapy [379]. Myricetin was not toxic at a concentration of 60 μM, but it enhanced the cytotoxicity of oxaliplatin in GI cancer cells including HepG2, PANC-1 and Caco-2 cells by enhancing oxaliplatin-induced apoptosis. These results provided evidence that use of myricetin increased the sensitivity of oxaliplatin in GI cancer cells. Therefore, the oxaliplatin-induced apoptosis rate was increased in all the GI cancer cells.

We found that myricetin treatment increased the levels of intracellular CDCF in GI cancer cells and this inhibition of drug efflux occurred in a time-dependent manner. With the increase in time, the cellular accumulation of CDCF also increased and after a certain time-point, no significant changes were observed. Therefore, these results indicate that myricetin reversed drug resistance by suppressing MRP2 transporter functional activity. The previous study reported that cellular platinum accumulation increased by almost 2.8- and 5-fold in PANC-1 and HepG2 cells respectively after treating cells with 60 μM myricetin [248]. Moreover, after myricetin treatment, oxaliplatin-induced growth inhibition IC_{50} values significantly decreased in GI cancer cell lines, including PANC-1 and HepG2 cells [248]. Thus, we can conclude that myricetin enhanced the sensitivity of oxaliplatin in GI cancer cells. Similarly, our results depicted that oxaliplatin-induced apoptosis rate increased from almost 10% to 20% in HepG2, PANC-1 and Caco-2 cells after concurrent treatment of cells with myricetin and oxaliplatin compared to the cells treated with oxaliplatin alone. Therefore, myricetin can be used for clinical studies in GI tumours overexpressing MRP2 and receiving oxaliplatin-based therapy.

Our *in vitro* data provide strong preliminary evidence that inhibiting the activity of MRP2 by myricetin in GI cancer cells enhances oxaliplatin-induced apoptosis. Thus, use of myricetin is an essential step in the development of a therapeutic approach for the treatment of GI cancer cells' resistance to oxaliplatin-based chemotherapy.

6.5. Conclusion

In conclusion, our study has provided evidence that a natural potent MRP2 inhibitor, myricetin, increases oxaliplatin-induced total apoptosis in GI cancer cells.. Myricetin can be considered a potential therapeutic approach, which is more effective in reversing oxaliplatin resistance in GI cancer cells. Therefore, myricetin can be used for clinical benefits in GI cancer with high MRP2 expression.

Chapter 7 General Discussion

7.1. Summary of the results

Oxaliplatin and its combination regimens are of major importance in the clinical treatment of colorectal cancer and other gastrointestinal malignancies including gastric, oesophageal, pancreatic, rectal and other types of gastrointestinal cancer based on robust evidence from multiple randomised controlled trials [36, 37, 49, 304, 380-383]. However, tumour resistance has become a major obstacle in the treatment of colorectal cancer as well as other GI cancers, leading to short-lived tumour responses in some patients and progressive disease in others. To overcome oxaliplatin chemoresistance and enhance chemotherapy sensitivity, a novel strategy has been established by genetically or chemically silencing the MRP2 in the MRP2 expressing human GI cancer cell lines including HepG2, PANC-1 and Caco-2 cells. Besides, we have mechanistically characterised the effects of oxaliplatin on MRP2 ATPase activities and demonstrated that inhibition of MRP2 ATPase by chemical inhibitors lead to attenuated MRP2-mediated oxaliplatin transport in three GI cancer cell lines.

MRP2 transporters are mainly expressed in the apical part of the intestinal cells, including the canalicular membrane of hepatocytes, the apical membrane in the proximal renal tubules of the kidney and the endothelial cell of the brain capillaries [384]. Most of the anticancer drugs either act as substrates and/or inhibitors of the ABC transporters and this can have a significant effect on the absorption, distribution, metabolism and excretion (ADME) of the anticancer drugs and their toxicity and have the potential for adverse drug-drug interactions. Previously, it was reported that MRP2 transporter mediates transport of oxaliplatin as a substrate by using the energy derived from ATP hydrolysis in a membrane vesicle study [176]. Therefore, we anticipated that the ATPase activity of the MRP2 transporter reflects the transport activity and can be used to monitor the effects of oxaliplatin on transporter activity. The ATPase studies confirmed that oxaliplatin is the substrate of the MRP2 transporter. With increased concentration of oxaliplatin, it stimulates MRP2 ATPase activity with the EC_{50} value of 8.3 ± 0.7 μM. At lower concentrations, oxaliplatin had no effect, whereas at high concentrations, it reaches saturation stage where the MRP2 transporter showed no effect. Moreover, in the presence of MRP2 inhibitors, benzbromarone and myricetin the oxaliplatin-induced ATPase activity decreased. These results provide strong evidence that oxaliplatin is a substrate of MRP2 transporter.

The HepG2, PANC-1 and Caco-2 cell lines studies showed that sensitivity to oxaliplatin-induced growth inhibition and cellular accumulation of oxaliplatin-derived platinum were both increased in MRP2-silencing GI cells that were genetically modified by transiently knockdown the MRP2 gene. They were confirmed to have decreased surface expression of MRP2 transporter compared to the negative control cell line. Moreover, silencing the MRP2 gene in these cell lines also enhanced the oxaliplatin-induced apoptosis.

Inhibiting MRP2 expression in HepG2 cells increased the MRP2-specific substrate accumulation by 60% to 70% at the cellular level. The sensitivity of oxaliplatin increased in MRP2-silencing HepG2 cells with IC_{50} values in the range of 3 μM to 11 μM, compared to negative-control HepG2 cells with IC_{50} value 30 μM. Compared to the control cells, MRP2-silencing HepG2 cells significantly increased the oxaliplatin-derived platinum by 52% to 186%. Inhibition of MRP2 with myricetin increased oxaliplatin-induced apoptosis by 10% to 20%, though myricetin alone showed no significant apoptotic effect. Similarly, in siRNA knockdown HepG2 cells, with increasing concentration of oxaliplatin the rate of apoptosis increased and compared to the negative control HepG2 cells, the apoptosis rate enhanced by 10% to 15% in MRP2-silencing cells.

We observed that silencing the MRP2 transporter in HepG2 cells could effectively reverse MRP2-mediated resistance to oxaliplatin with increased sensitivity to oxaliplatin drug. Therefore, we anticipated that knockdown of the MRP2 gene in pancreatic and colorectal cancer cell lines could also have similar effects of sensitising PANC-1 and Caco-2 cells to MRP2 substrate and reversing oxaliplatin resistance. The siRNA transfection was successfully able to knock down the expression of MRP2 gene by 50% - 70% and 55% - 60% in PANC-1 and Caco-2 cells respectively. Thus, subsequent experiments focused on silencing the MRP2 gene with the aim of gaining some insight into the MRP2-mediated chemoresistance to oxaliplatin. Using a MRP2-specific substrate accumulation study, it was confirmed that knockdown of the MRP2 gene increased the cellular accumulation of MRP2 substrate CDCF by 120% - 160% and 130% - 150% in MRP2-silencing PANC-1 and Caco-2 cells respectively. It was observed from the growth inhibition assay that IC_{50} was significantly decreased to 20 μM in MRP2-silencing PANC-1 cells compared to control cells IC_{50}, which was 35 μM.

In the case of Caco-2 cells, the IC_{50} also reduced by almost half with the IC_{50} value of 7 μM in MRP2-silencing Caco-2 cells and 13.8 μM in control Caco-2 cells. Furthermore, the transport activity of MRP2 after silencing the MRP2 gene was measured by determining the cellular accumulation of oxaliplatin-derived platinum in Caco-2 cells. After 2 hr exposure to oxaliplatin, the platinum accumulation in MRP2-silencing Caco-2 cells was increased by 80% - 100%. It was evident from the flow cytometry results that MRP2-silencing PANC-1 and Caco-2 cells enhanced the oxaliplatin-induced apoptosis rate with an increase in apoptosis rate of 10% – 15%. Moreover, oxaliplatin-induced apoptosis rates were also increased after inhibiting these GI cancer cells with the MRP2-specific inhibitor, myricetin.

Overall, modulating the expression of the MRP2 transporter in HepG2, PANC-1 and Caco-2 cancer cells increased the sensitivity of oxaliplatin in these GI cancer cells. These findings conclude that inhibiting the expression of MRP2 in oxaliplatin-based therapy has a potential role in therapeutic response to oxaliplatin treatment in some human GI cancers.

7.2. Future studies

7.2.1. Animal (*in vivo*) study

In this thesis, we experimentally verified the association of oxaliplatin response with respect to the expression of MRP2 transporter by modulating MRP2 expression with RNAi in *in vitro* experiments. The combination of siRNA and oxaliplatin has increased the chemosensitivity of drugs in hepatocellular, pancreatic and colorectal cancer cells. Also, the combination of oxaliplatin and the MRP2 modulator, myricetin, enhanced the oxaliplatin-induced apoptosis rate in these cell lines. Based on our results, future research should focus on the modulation of MRP2 transporter expression in tumour xenograft models of hepatocellular, pancreatic and colorectal cancers receiving oxaliplatin drug. This could be established by subcutaneously injecting GI cancer cells into nude mice or by the orthotopic implantation of cancer cells into nude mice, followed by random allocation of animals into treatment groups such as control, oxaliplatin alone, MRP2 modulator alone or combination of oxaliplatin and MRP2 modulator. Alternately, the establishment of knockout mice by subcutaneously injecting GI cancer cells into nude mice in which MRP2 gene has already been knocked out. In these studies, *in vivo* chemosensitivity effects of the modulation of MRP2 in

combination with oxaliplatin could be assessed, together with their toxicity and pharmacokinetics.

7.2.2. Inhibition of other signalling pathways

The MRP2 transporter has been expressed in various cancer cell lines, including liver, pancreatic, lung, oesophageal and colorectal cancer. The molecular mechanisms of regulation of MRP2 protein expression were reported by the involvement of signalling pathways like Nrf2 activation [385], the PI3K/AKT signalling pathway [386], the nuclear factor-κβ (NF-κβ) pathway [387], and the p38 MAPK pathway [388].

Nuclear factor-erythroid 2 p45 related factor 2 (Nrf2) is a transcription factor that contains antioxidant response element (ARE) sequences in their promoter regions and is involved in transcriptional activation of genes. Studies have reported that regulation of the MRP2 gene is dependent on Nrf2 [315, 385]. Previously, it was reported that dihydromyricetin (DMY) downregulated the expression of MRP2, specifically by the Nrf2 signalling pathway in colorectal cancer cell lines [373]. This data strongly suggests a potential role of Nrf2 signalling in DMY-regulated MRP2 expression. However, the involvement of other signalling pathways in GI cancer-associated oxaliplatin chemoresistance is still uncertain. Myricetin possibly downregulates MRP2 surface expression by inhibiting the PI3K/AKT pathway, which is a regulator of the MRP2 transporter as reported in previous studies [386, 389]. Besides, myricetin is known to inhibit the activity of NF-κβ, which is an effector of PI3K/AKT pathway signalling [390]. However, it has not been demonstrated that myricetin directly involves NF-κβ inhibition, which modulates the activity of PI3K/AKT signalling. Therefore, it would be interesting to determine the PI3K/AKT signalling inhibition by myricetin and its association with surface and total protein downregulation of MRP2 transporter as the PI3K/AKT pathway has been implicated in the expression of MRP2.

7.2.3. Inhibition of other efflux transporters

Platinum-based oxaliplatin drugs are essential in the clinical treatment of GI cancer, especially colorectal and pancreatic cancer. Several transporters responsible for oxaliplatin transport are accountable for oxaliplatin response and sensitivity in GI tumours. Studies have reported clinical associations between the expression of oxaliplatin transporters and patient response to oxaliplatin. The MRP2 transporter is differentially expressed between colorectal cancer patients who responded to FOLFOX

chemotherapy [288]. Many oxaliplatin transporter candidate genes have been observed, including transporter proteins from the ABC transporters, SLC transporters and the ATPase membrane protein superfamily, which have shown oxaliplatin transport *in vitro* [48]. There are possibilities that the expression of other oxaliplatin transporter genes can interfere with oxaliplatin or oxaliplatin-based chemotherapy. However, out of all these transporters, which one contributes to determining clinical responses to oxaliplatin in GI cancer is still unknown. Current studies significantly support the involvement of ATP7A and ATP7B in platinum drug resistance and these can be used as potential biomarkers to predict the sensitivity of oxaliplatin-based chemotherapy in GI cancer [391]. There is a possibility that ATP7A and ATP7B mediate the resistance to platinum drugs through more than one mechanism, such as by sequestration of drugs, efflux of platinum drugs and/or modulating intracellular copper levels inside platinum-resistant cells [391]. However, the detailed mechanism is uncertain and deep insight is needed into the roles of ATP7A and ATP7B for future study. It would be relevant to determine other efflux transporters in the human genome as potential biomarkers that are expressed differentially in GI cancer patients given oxaliplatin-based chemotherapy.

7.2.4. Nanotechnology-based drug delivery

The delivery of oxaliplatin in the combination of MRP2 gene-specific siRNA or inhibitor is quite complicated, and the chance of degradation of siRNA is high due to its small size. The drug delivery systems in the form of nanoparticles or nano-liposomal formulations are effective. Encapsulating the siRNA or MRP2-specific inhibitor and anticancer drug and delivering them to the tumour sites is advantageous to enhance drug accumulation in the tumour tissues compared to conventional drug solutions due to enhanced permeability and retention (EPR) effect. In addition, it may prevent off-target side effects like myelosuppression, neurotoxicity or nephrotoxicity, which may arise from the inhibition of ABC transporters in bone marrow, BBB or kidney tissues [392].

The nano-formulation delivery system delivers both anticancer drugs and efflux-pump inhibitors to the tumour tissue more effectively and minimises its toxic side effects to healthy tissues [393]. The EPR effect is usually caused by the interendothelial gap formed in tumours, which increases leaky blood vessels that allow extravasation of nano-scaled particles to tumour sites and not to the healthy tissues. Therefore, the EPR effect allows the passive targeting of nanoliposomes. Problems like toxicity, low specificity and intracellular delivery can be avoided using nanoparticles loaded with

drugs and siRNA or inhibitors [394, 395]. Apart from targeting specific tumours, nanoparticles may modulate the pharmacokinetics of the encapsulated drug by extending its plasma half-life inside the body and reducing the distribution of drugs to healthy tissues. Many of the nanoparticle-based drugs like Doxil and DaunoXome, which have been approved by FDA, have shown significant antitumor activity in mice with colon cancer [396]. Hence, nanoliposome formulations of oxaliplatin and MRP2 modulators should be considered for future *in vitro* and *in vivo* studies. It would be interesting to observe the oxaliplatin sensitivity to GI tumours and its toxicity in healthy cells after delivering the nano-liposomal formulation of both oxaliplatin and MRP2 siRNA or inhibitor.

7.2.5. Development of experimental model for analysing oxaliplatin resistance in GI cancer

In our thesis, we have reported that silencing the MRP2 transporter increased the oxaliplatin response in GI cancer. However, the detailed mechanisms underlying the oxaliplatin chemoresistance in GI cancers are not well understood. To further clarify the mechanisms of oxaliplatin resistance, various research models need to be developed.

The main mechanism of resistance to oxaliplatin is due to the decrease in drug accumulation, increase in drug detoxification by glucuronidation, increase in DNA damage repair and alteration in the pathways involved in cell cycle or apoptosis [57, 397]. Nevertheless, the detailed mechanisms are not fully understood due to the lack of a proper experimental model. From the results of two-dimensional (2D) cell research, it is difficult to reflect cellular heterogeneity and behaviour of tissues *in vitro*. Recently, the use of three-dimensional (3D) experimental models have been implemented in cell cultures for drug discovery, especially for colorectal cancer cells, which help to clarify more detailed mechanisms of drug resistance [398].

The 3D experimental model could be established using the Matrigel or air-liquid interface (ALI) method. Previously, it was reported that Matrigel organoid from colorectal cancer patients recapitulated the same properties of an original tumour [399]. Another 3D culture system called ALI, which utilises double-layered collagen gel, can mimic the same tumour microenvironment as an *in vivo* study [400]. Recently, tumour ALI organoids established from human colorectal cancer tissues showed more resistance to 5-FU and irinotecan compared to colorectal cancer cell lines [401].

Another study reported that a combination treatment of Hedgehog signal inhibitors with 5-FU, irinotecan or oxaliplatin upregulated the sensitivity of tumour ALI organoids [402]. These findings indicate that ALI organoid 3D cultures from colorectal cancer patients may be useful for investigating drug resistance to chemotherapy in the tumour microenvironment. Therefore, there is a possibility that the 3D cell culture technology could be applicable to personalised oxaliplatin-based therapy for GI cancer in the near future.

7.3. Clinical implications

The clinical relevance of MRP2 transporters in oxaliplatin-based chemotherapy depends on their distribution in human tissue, their therapeutic index, and variability in the pharmacokinetics (PK) and pharmacodynamics (PD) properties of the oxaliplatin drug. Polymorphisms of the MRP2 gene are considered as key determinants associated with alterations in the function of MRP2 and thus, mediates the oxaliplatin transport. Alteration in MRP2 expression and/or function may have a variety of clinically relevant effects. Firstly, decrease in MRP2 function can impair normal hepatic function, including a capacity to excrete endogenous compounds. Secondly, alteration in MRP2 function can change absorption, distribution, metabolism and excretion of clinically important drugs, including oxaliplatin. Thirdly, alteration in MRP2 expression reported changes in pharmacokinetic drug-drug interactions and these interactions might result in bone marrow suppression and renal failure.

As discussed earlier, MRP2 is mainly expressed in the apical membrane of polarised epithelial cells of organs such as hepatocytes, intestine and kidney, which determine the absorption, distribution and excretion of substrates, as well as expressed in the BBB and placenta and functions by exporting the compounds out of cells. MRP2 transporter is known to transport anionic drug conjugates such as glucuronates, sulphates and glutathiones; therefore, MRP2 is considered to play a role in detoxification. Mutation in the MRP2 gene leads to disruption in liver function and bilirubin excretion, which ultimately leads to hyperbilirubinaemia [182, 403]. On the other hand, in some patients, oxaliplatin has been reported to induce hepatotoxicity [404, 405]. Therefore, MRP2-mediated oxaliplatin transport could lead to oxaliplatin-induced liver toxicity. Our study has demonstrated that oxaliplatin is a substrate of MRP2 and inhibiting the expression of MRP2 gene limits the cellular accumulation of oxaliplatin-derived platinum in GI cancer cells. Hence, MRP2 plays a vital role in determining the bioavailability and

toxicity of oxaliplatin in GI cancer patients by regulating platinum accumulation inside a cancer tumour. The significance of MRP2-mediated oxaliplatin transport, toxicity and excretion could be further studied using MRP2 gene knockout mice.

As in the MRP2 gene, several genetic variations have been reported that alter the oxaliplatin-based chemotherapy in patients. The absence of the MRP2 gene in the liver is associated with Dubin-Johnson syndrome (DJS) and many SNPs in the ABCC2 gene have been discovered that impair the expression or function of MRP2 transporter leading to DJS, including 4145A>G (1382Gln>Arg), 1066C>T (creates a stop codon), 2302C>T (768Arg>Trp). More than 50 SNPs have now been identified in MRP2, which are responsible for alteration in MRP2 function. Some clinically relevant polymorphisms that are associated with reduced MRP2 function or changes in mRNA or protein expression [406]. Most commonly studied SNPs are ABCC2 24C>T, 1249G>A and 3563T>A. In renal cell carcinoma, ABCC2 24C>T is associated with lower mRNA levels. Studies have reported the relationship between ABCC2 24C>T and the PK of methotrexate [406]. However, there has been no data reported regarding 24C>T SNP and the pharmacokinetics of oxaliplatin, although 24C>T is associated with reduced disease-free survival in lung cancer patients receiving platinum chemotherapy. Another SNP ABCC2 1249G>A was associated with reduced overall survival in colorectal cancer patients receiving FOLFOX-4 (5-FU/leucovorin and oxaliplatin) chemotherapy and patients with recurrence during FOLFOX-4 treatment showed a high expression of MRP2 in their tissue samples [212, 407]. Thus, ABCC2 1249G>A is associated with the change in ABCC2 mRNA levels and inadequate response to oxaliplatin-based chemotherapy. Another study reported that MRP2 expression increased in cancerous colorectal tissues compared to normal tissues with no changes in overall survival rate [407]. These variations in ABCC2 genotype in patients receiving oxaliplatin-based chemotherapy may be associated with MRP2 expression level with interpatient variations in response to oxaliplatin-based therapy. Hence, genetic variants in the MRP2 gene and association of SNPs with PK/PD profiles of oxaliplatin are clinically relevant for treatment with oxaliplatin-based chemotherapy. Therefore, further clinical studies should be done to confirm this mechanism by determining the MRP2 expression level at mRNA and protein level, genotyping of *ABCC2*, and measuring the cellular platinum accumulation in tumour samples from GI cancer patients receiving oxaliplatin-based chemotherapy.

Oxaliplatin binds to the plasma proteins and is mainly cleared from the body by covalent binding to tissues and renal excretion. Within 2 hr of administration, around 50% of oxaliplatin accumulated in the red blood cells and was mainly distributed at high concentrations in the liver, spleen, kidney and intestine [51, 65, 72]. Oxaliplatin is primarily excreted from the body via renal excretion, accounting for approximately 54% of total oxaliplatin administered and only 2% is excreted by faeces [72]. In patients receiving 130 mg/m^2 oxaliplatin every three weeks or 85 mg/m^2 oxaliplatin every two weeks, the inter and intrapatient variation in total body exposure to oxaliplatin (AUC_{0-48}) was reported to be 33% and 5% respectively [51, 65, 72]. These variations in oxaliplatin exposure may be due to the alteration in distribution and clearance, which is influenced by the drug metabolism and excretion. Also, indirectly, there is a possibility that these processes are influenced by the expression or functional activities of MRP2 transporter, which is expressed in physiological barrier sites such as the intestine, liver, spleen and kidney, and mediate oxaliplatin transport [72]. As reported in earlier studies, a wide range of variations has been observed in patients regarding both volume distribution and tissue distribution of oxaliplatin [51, 65, 72]. This discrepancy in variations may be related to the different tissue expression levels of MRP2 transporters that are involved in oxaliplatin transport. Moreover, further PK studies using blood, urine and faecal samples are required in GI cancer patients receiving oxaliplatin-based therapy, and variation in MRP2 genotype in these patients could help to determine the clinical association between MRP2 expression level and oxaliplatin-based therapy.

Oxaliplatin is widely used in the therapy of colorectal cancer, and oxaliplatin-based combination therapy is extensively used for other GI cancers, including pancreatic cancer. Our study has confirmed that oxaliplatin is a substrate of MRP2 and MRP2 is a targetable factor involved in oxaliplatin resistance in human GI cancer. Therefore, targeting MRP2 could improve the efficacy of oxaliplatin-based treatment in human GI cancer patients. However, we can only observe the mechanism of oxaliplatin resistance in a subset of cancer cell types, and it would be necessary to screen tumour upregulation of MRP2 before targeting the MRP2 transporter. Previous research had shown that the pharmacogenomics of oxaliplatin focused mainly on interindividual differences in oxaliplatin pharmacokinetics and genetic alterations in genes coding for ABC transporters such as MRP2, DNA damage repair mechanisms such as ERCC1, ERCC2, and conjugating enzymes glutathione S-transferases (GST) such as GSTP1, GSTT1 [337]. Other than MRP2, there are ABC transporters, such as MRP4, MRP5, BCRP,

which are potentially involved in the disposition of platinum compounds [175, 198, 408]. Increased expression of different ABC transporters can directly cause a decrease in oxaliplatin inactivation [409]. For example, in one of the studies, oxaliplatin-resistant colorectal cancer cell lines not only showed resistance to oxaliplatin but also showed cross-resistance to 5-FU, cisplatin, vincristine, etoposide, and epirubicin, with high expression of MRP2 transporter and no significant changes in P-gp or MRP1 [174]. It would be interesting to determine the synergistic action of oxaliplatin and other drug combinations in different drug transporters in cancer cells. Moreover, the involvement of different excision nucleases that play a major role in DNA adducts repairs, like ERCC1 and ERCC2, are also involved in oxaliplatin resistance. Thus, low ERCC1 and ERCC2 gene expression leading to decreased DNA repair should be a decisive predictive factor of therapeutic effects of oxaliplatin. Similarly, studies have reported that the gene encoding GST was associated with survival of colorectal cancer patients receiving oxaliplatin-based therapy [342, 410]. However, many other studies provided conflicting results regarding the association of genes related to oxaliplatin mechanism of action and toxicity [337]. Therefore, more studies could be done to study the pharmacogenomics of oxaliplatin, focusing mainly on oxaliplatin pharmacokinetics and genes involved in oxaliplatin mechanisms of action and toxicity to improve oxaliplatin-based treatment strategies in GI cancer.

Our current study implicated that oxaliplatin is a substrate of MRP2 and silencing the ABCC2 gene that encodes MRP2 increases cellular accumulation and sensitivity of oxaliplatin in human GI cancer cells. This mechanism can also be explained by further studying the clinical correlations between MRP2 tumour expression and clinical outcomes in patients receiving oxaliplatin-based therapy. For clinical research, the samples could be examined from patients with histopathologically confirmed primary GI cancer like liver cancer, pancreatic adenocarcinoma and colorectal cancer who are provided with the oxaliplatin-based regimen. The expression of MRP2 and its correlation with oxaliplatin response should be examined using immunohistochemical (IHC) analysis, which analyses MRP2 protein expression, PCR-based MRP2 expression at mRNA level, MRP2 gene genotyping, and assessment of oxaliplatin-derived platinum accumulation by ICP-MS in tumour samples from GI cancer patients receiving oxaliplatin-based chemotherapy. Identifying patients exhibiting poor response to oxaliplatin because of MRP2-mediated deficient platinum accumulation is a major step before starting oxaliplatin-based treatment, as based on the MRP2 expression, the

oxaliplatin-based regimens could be adjusted depending on the MRP2 expression in individual patients. GI cancer patients who are resistance to oxaliplatin due to MRP2 oxaliplatin-based regimen might need to combine with MRP2 modulators to increase the response of oxaliplatin. Therefore, screening patients based on MRP2 expression levels, MRP2 gene variants, MRP2 functional activity and oxaliplatin accumulation in tumours is essential for the most effective treatment outcomes. The MRP2 protein expression in tumour samples could be assessed by IHC, and expression of MRP2 at mRNA could be evaluated by qRT-PCR. The genetic variants of the MRP2 gene in patients could be determined with SNP genotyping using the patient blood samples [211, 282]. The patient's health could be surveyed by physical examination and measurement of possible tumour metastasis or lesions could be examined by CT, MRI scan or ultrasonography [411]. The oxaliplatin-derived platinum accumulation in tumour samples during the treatment course could be measured using ICP-MS-based quantitative analysis of platinum to measure platinum in tumour tissues.

The functional significance of MRP2 expressed in cancer cells could be the subcellular localisation of the MRP2 transporter, and it could be confirmed by IHC assay. In normal tissues, MRP2 localises in the apical membrane of polarised cells and is functionally expressed in these sites [412]. Polymorphisms in MRP2 that occur in cytoplasmic localisation of the MRP2 protein have been described and may reduce *in vivo* function [413]. The previous study showed that MRP2 confers resistance to the platinum-based drug only when expressed at the nuclear membrane [202]. Another study showed expression of MRP2 in both the cytoplasm and membrane of cancer cells [411]. These studies indicate that subcellular localisation of MRP2 and its functional significance plays a significant role in chemoresistance. MRP2 protein localised in the cell cytoplasm might not function as an efflux pump [414]; however, focusing on the subcellular localisation of MRP2 and functional and clinical significance of such localisation is required to elucidate the mechanism of chemoresistance induced by MRP2 in GI cancer patients.

Thus, high expression of the MRP2 gene limits oxaliplatin sensitivity in GI cancer. Therefore, screening tumour MRP2 expression levels to select patients for treatment with oxaliplatin alone or oxaliplatin in combination with MRP2 modulator could improve outcomes of oxaliplatin-based GI cancer treatment. Overall, MRP2 expression and oxaliplatin pharmacokinetics in GI cancers could be explored in animal models or

clinically to confirm the significance of MRP2 transporters in oxaliplatin-based chemotherapy.

7.4. Final conclusions

Overall, in conclusion, the experimental work in this thesis has demonstrated that MRP2 is a targetable factor and one of the main reason of oxaliplatin chemoresistance in oxaliplatin-based therapy by limiting its accumulation. Our studies have provided *in vitro* evidence that modulating the MRP2 transporter increased oxaliplatin-derived cellular platinum accumulation and enhanced the sensitivity of oxaliplatin and oxaliplatin-induced apoptosis rate in GI cancer cells. *In vivo* studies are warranted to verify these results prospectively. The findings of this study has laid a solid foundation for development of new therapeutic approaches and the exploration of effective oxaliplatin-based regimens for GI cancer.

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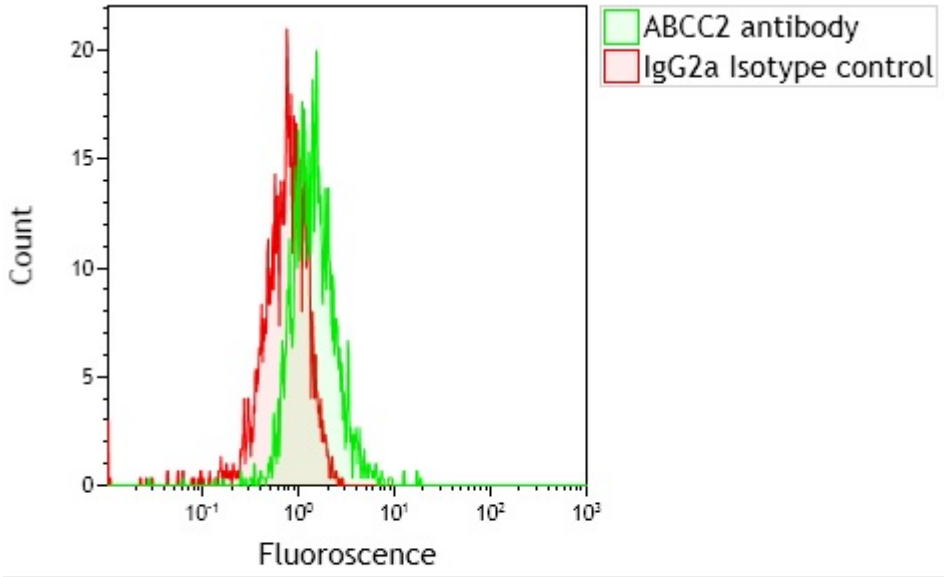
Appendices

Appendix I. MRP2 Surface staining flow cytometry histogram data in HepG2 cells

A.

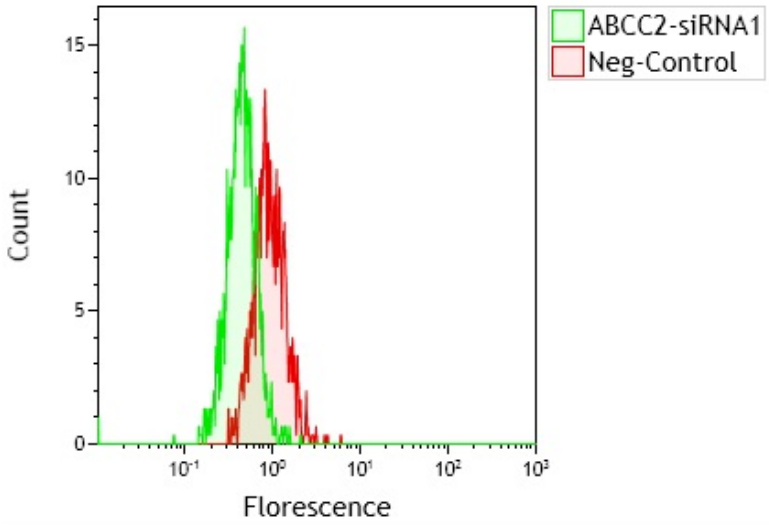


Marker	%Gated	X-Med	X-AMean	X-GMean
All	100.00	1.40	1.67	1.42
All	100.00	0.77	0.82	0.71

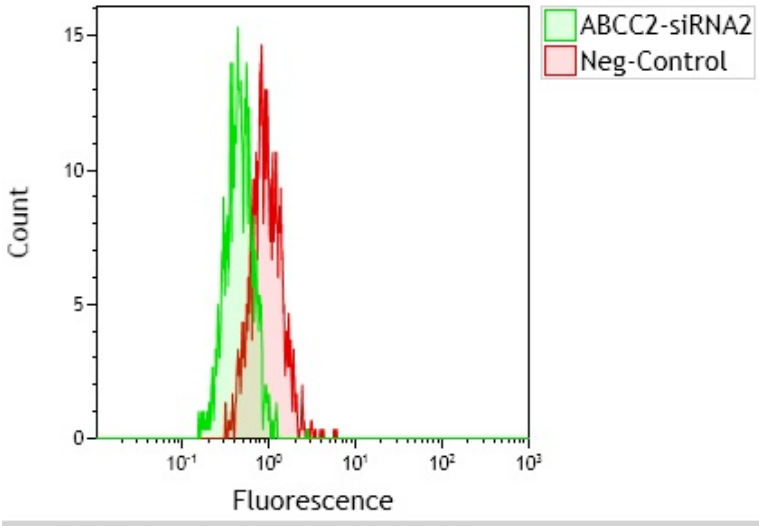
Figure A-1. Flow cytometry histogram of cell surface staining using the anti-MRP2 primary antibody and isotype control IgG2a on HepG2 cells.

Both primary antibody and isotype control were labelled with Alexa Fluor 488 secondary antibody. The X-axis is the fluorescence signal intensity in the FL1 blue laser channel displayed in a liner log scale. The y-axis represents the cell counts.

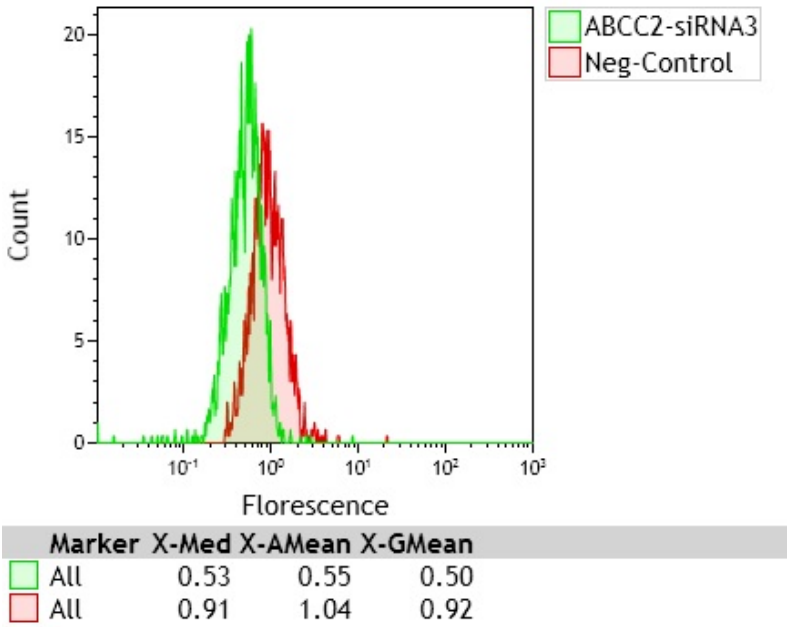
B.



Marker	%Gated	X-Med	X-AMean	X-GMean
All	100.00	0.45	0.48	0.44
All	100.00	0.90	1.00	0.92



Marker	X-Med	X-AMean	X-GMean
All	0.45	0.49	0.45
All	0.91	1.01	0.92



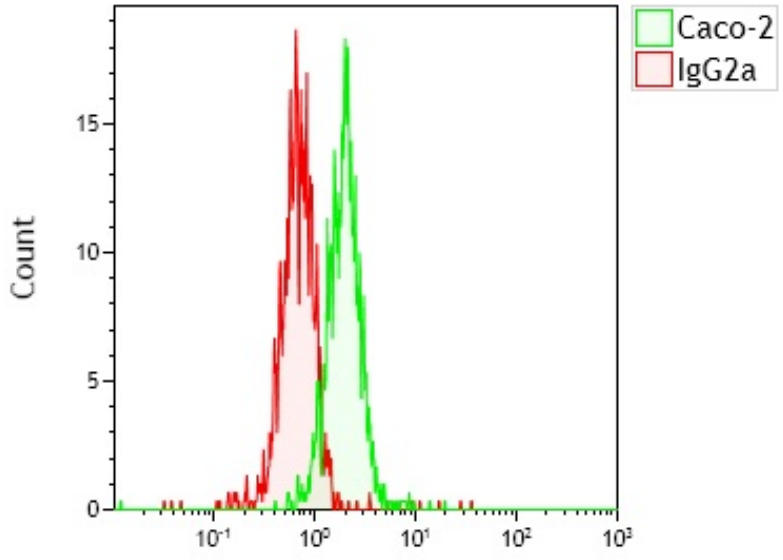
Marker	X-Med	X-AMean	X-GMean
All	0.53	0.55	0.50
All	0.91	1.04	0.92

Figure A-2. Flow cytometry histogram of cell surface staining using the anti-MRP2 primary antibody and isotype control IgG2a on siRNA transfected HepG2 cells.

The fluorescence signal of ABCC2-siRNAs and control-siRNA transfected HepG2 cells. The X-axis is the fluorescence signal intensity in the FL1 blue laser channel displayed in a liner log scale. The y-axis represents the cell counts.

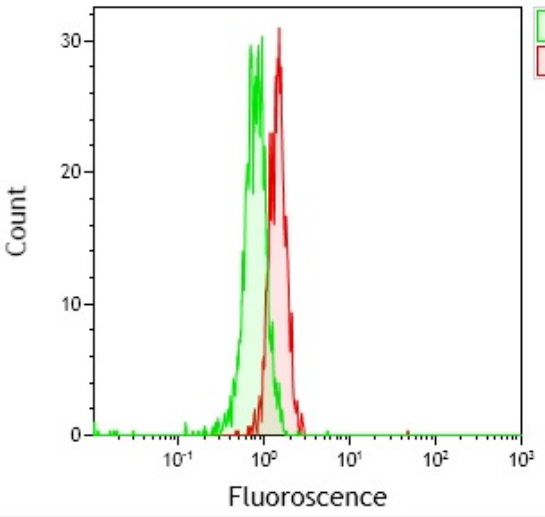
Appendix II. MRP2 surface staining flow cytometry histogram data in Caco-2 cells

A.

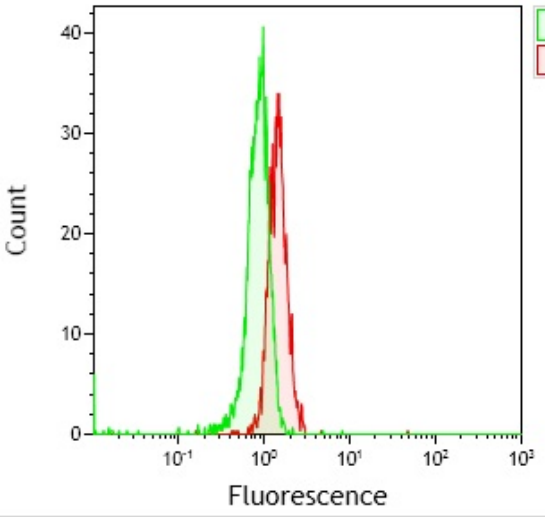


Marker	%Gated	X-Med	X-AMean	X-GMean
All	100.00	1.98	2.12	1.93
All	100.00	0.68	0.82	0.67

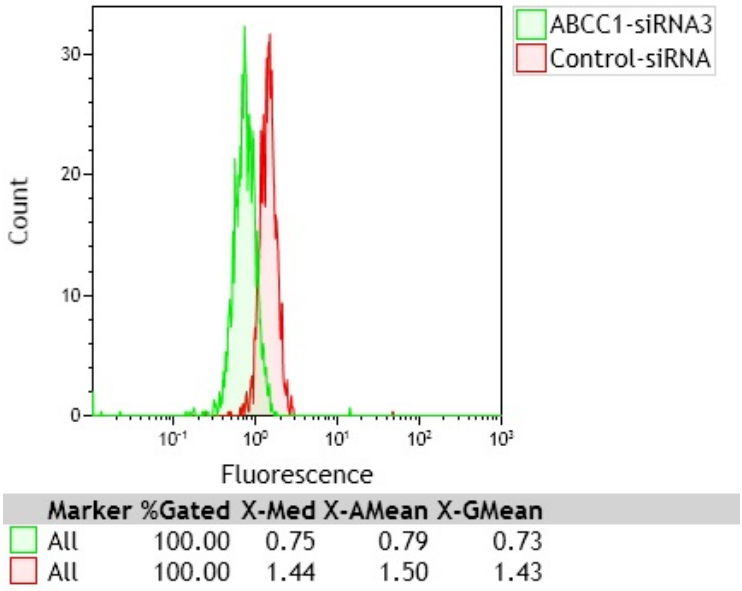
B.



Marker	%Gated	X-Med	X-AMean	X-GMean
All	100.00	0.79	0.81	0.76
All	100.00	1.45	1.51	1.43



Marker	%Gated	X-Med	X-AMean	X-GMean
All	100.00	0.87	0.88	0.81
All	100.00	1.44	1.52	1.43



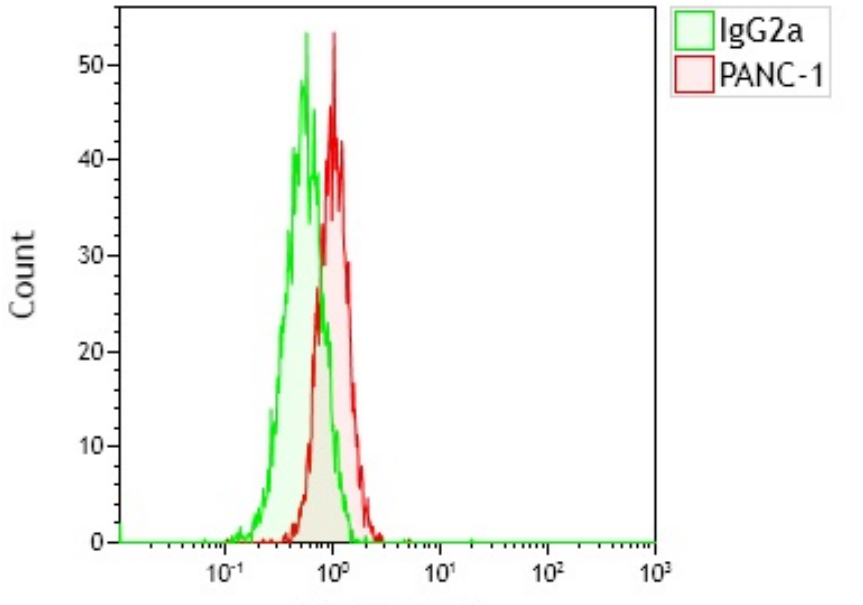
Marker	%Gated	X-Med	X-AMean	X-GMean
All	100.00	0.75	0.79	0.73
All	100.00	1.44	1.50	1.43

Figure A-3. Flow cytometry histogram of cell surface staining using the anti-MRP2 primary antibody and isotype control IgG2a on Caco-2 and siRNA transfected Caco-2 cells.

The graph represents the fluorescence intensity in (A) Caco-2 cells stained with MRP2 antibody and IgG2a stained cells; and (B) ABCC2-siRNAs transfected Caco-2 cell compared with control-siRNA cells. Both the primary antibody and isotype control were labelled with Alexa Fluor 488 secondary antibody. The x-axis is the fluorescence signal intensity in the FL1 blue laser channel displayed in a liner log scale. The y-axis represents the cell counts.

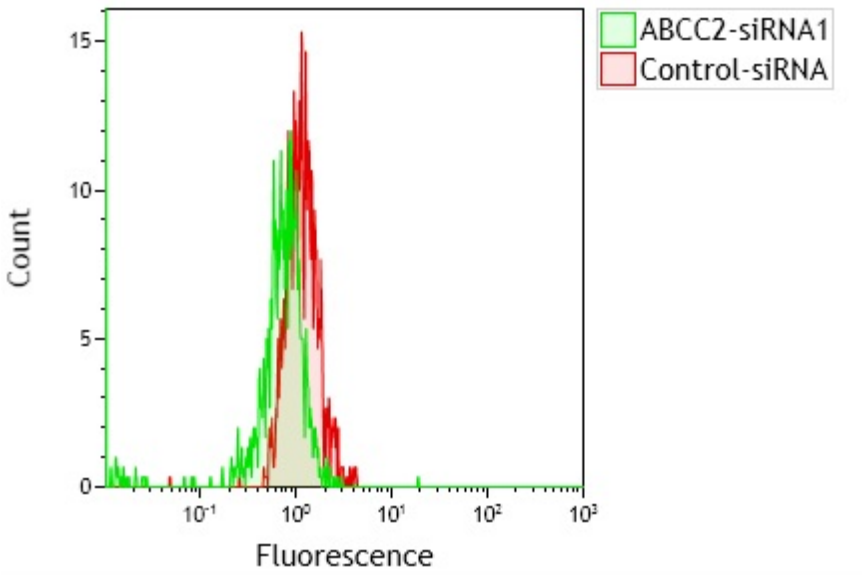
Appendix III. MRP2 surface staining flow cytometry histogram data in PANC-1 cells

A.

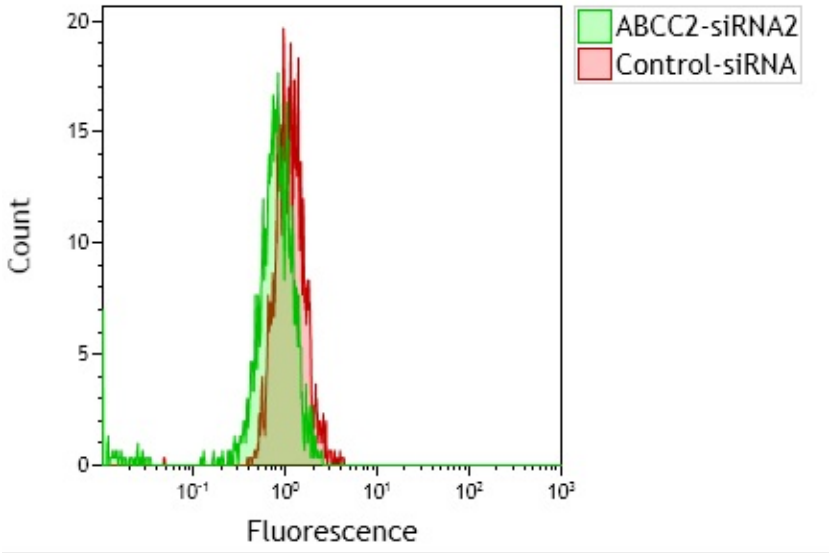


Marker	%Gated	X-Med	X-AMean	X-GMean
All	100.00	0.54	0.58	0.53
All	100.00	1.01	1.06	1.00

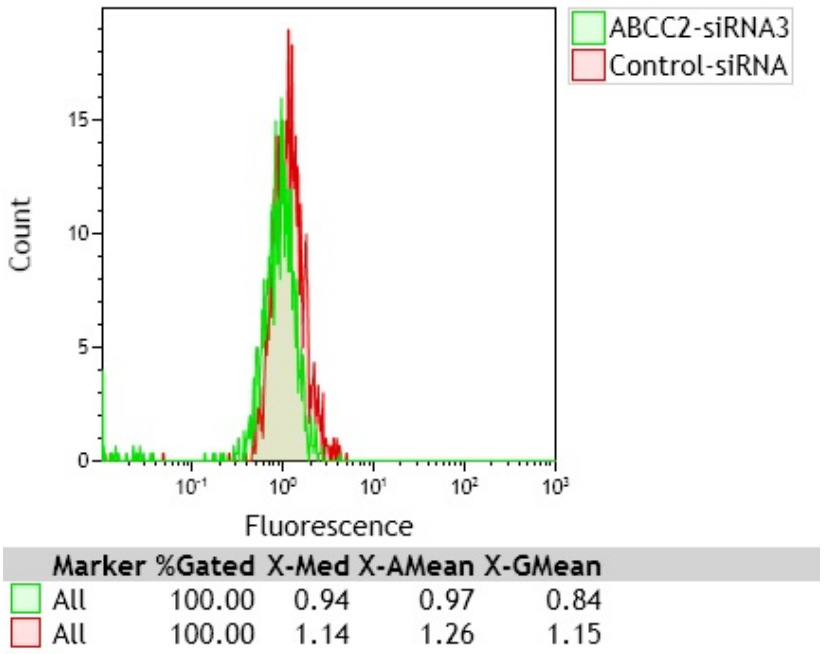
B.



Marker	%Gated	X-Med	X-GMean
All	100.00	0.75	0.61
All	100.00	1.14	1.15



Marker	X-Med	X-AMean	X-GMean
All	0.80	0.84	0.71
All	1.12	1.20	1.11



Marker	%Gated	X-Med	X-AMean	X-GMean
All	100.00	0.94	0.97	0.84
All	100.00	1.14	1.26	1.15

Figure A-4. Flow cytometry histogram of cell surface staining using the anti-MRP2 primary antibody and isotype control IgG2a on PANC-1 and siRNA transfected PANC-1 cells.

The graph represents the fluorescence intensity in (A) PANC-1 cells stained with MRP2 antibody and IgG2a stained cells; and (B) ABCC2-siRNAs transfected PANC-1 cell compared with control-siRNA cells. Both the primary antibody and isotype control were labelled with Alexa Fluor 488 secondary antibody. The x-axis is the fluorescence signal intensity in the FL1 blue laser channel displayed in a liner log scale. The y-axis represents the cell counts.