Multidrug resistance-associated protein 2 (MRP2) and oxaliplatin transport

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Abstract

The platinum-based drug oxaliplatin is currently used in the clinical treatment of gastrointestinal cancer. Efflux membrane transporters have been reported to play roles in determining the cellular accumulation and activity of platinum-based drugs, but there have been few studies of oxaliplatin. We hypothesized that the efflux transporter multidrug resistance protein-2 (MRP2), encoded for by the *ABCC2* gene, may play a role in determining cellular sensitivity to oxaliplatin by transporting oxaliplatin-derived platinum.

Human MRP2-expressing inside-out membrane vesicles prepared from Sf9 insect cells were used for studies of oxaliplatin transport. A HEK293 cell line stably transfected to over-express the human *ABCC2* gene (HEK-MRP2 cells) and its isogenic HEK293 parenteral (HEK-P) cell line, and a panel of seven human gastrointestinal cancer cell lines, were used to study the role of MRP2 in determining the cellular accumulation and activity of oxaliplatin, as assessed by inductively coupled plasma mass spectrometry and MTT growth inhibition assays.

Membrane vesicle studies showed that MRP2 mediated the ATP-dependent active membrane transport of oxaliplatin-derived platinum [Vmax = 2680 pmol per mg of protein per 10 min (95%CI of 2010 to 3360 pmol per mg of protein per 10 min); Km = 301 μM (95%CI of 163 to 438 μM)]. Studies of oxaliplatin stability in membrane vesicle incubation buffer (oxaliplatin stability half-life = 2.24 hrs with 95% CI of 2.08 to 2.43 hrs) suggested that intact oxaliplatin and its early degradation product, $[Pt(DACH)oxCl]^-$, were likely substrates for MRP2-mediated active transport. HEK293 cell line studies showed that cellular accumulation of oxaliplatin-derived platinum and sensitivity to oxaliplatin-induced growth inhibition were both reduced in HEK-MRP2 cells by up to two-fold compared to HEK-P cells, but these

deficits were reversed by inhibition of MRP2 with myricetin. Studies of human gastrointestinal cancer cell lines demonstrated that MRP2-mediated deficits in cellular accumulation of oxaliplatin-derived platinum were observed in HepG2 and PANC-1 cells (0.14- and 0.34-fold difference relative to HEK-P cells), which had high expression levels of *ABCC2* mRNA comparable to the HEK-MRP2 cells (1360- and 139-fold difference in relative to HEK-P cells). Myricetin increased cellular platinum accumulation of HepG2 and PANC-1 by 5- and 2.8-fold, respectively, and their sensitivity to oxaliplatin-induced growth inhibition by 3.4- and 4-fold, respectively. In HepG2 cells, siRNA-mediated knockdown of *ABCC2* expression increased cellular accumulation of oxaliplatin-derived platinum by approximately 3-fold compared to control-siRNA treated HepG2 cells. Inhibiting MRP2 sensitised MRP2-overexpressing gastrointestinal cancer cells to growth inhibition induced by clinically achievable concentrations of oxaliplatin (3.75 to 11.25 μM).

In conclusion, MRP2-mediates the active transport of oxaliplatin-derived platinum. Furthermore, MRP2 was identified as a targetable factor, limiting the cellular accumulation and anticancer activity of oxaliplatin in human gastrointestinal cancer.

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Table of Contents

List of Figures

List of Tables

Abbreviations

°C	Degree Celsius
5-FU	5-fluorouracil
A260	Absorption at a wavelength of 260 nm
A280	Absorption at a wavelength of 280 nm
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
BCA	Bicinchoninic acid
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
C.I.	Confidence interval
Cmax	Maximum plasma concentration
Ct	Cycle threshold
CTR1	Copper transporter 1

DACH	Diaminocyclohexane
DAPI	4’,6-diamino-2-phenylindole
DDI	Drug-drug interaction
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
EGFR	Epidermal growth factor receptor
FBS	Foetal bovine serum
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GSSG	Glutathione disulphide
h	Hour
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
IC-50	Concentration at half-maximal inhibitory effect
i.p.	Intraperitoneal
i.v.	Intravenous
kDa	Kilodaltons
KD	Knockdown
kg	Kilo-gram
Km	Concentration at half Vmax

KO	Knockout
LTC4	Leukotriene C4
LV	Leucovorin
M	Molar concentration
μM	Micromolar concentration
mM	Millimolar concentration
μg	Micro-gram
mg	Milli-gram
μl	Micro-litres
ml	Milli-litres
mol	Mole
MDCK	Madin-Darby canine kidney
MDR	Multidrug resistance
MEM	Minimal essential medium
min	Minutes
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OCT	Organic cation transporter
OCTN	Organic cation/carnitine transporter
Ng	Nano-gram
nM	Nanomolar concentration
nmol	Nanomole
NBD	Nucleotide binding domain
pmol	Picomole

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pen-strep	Penicillin-streptomycin
PFA	Paraformaldehyde
PFS	Progression-free survival
P-gp	P-glycoprotein
ppb	Parts-per-billion
Pt	Platinum
RNA	Ribonucleic acid
r^2	Coefficient of determination
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
SLC	Solute carrier
TMD	Transmembrane domain
Vmax	Maximum velocity

Chapter 1. Introduction

1.1. Gastrointestinal cancer

1.1.1. *Cancer burden worldwide and in New Zealand*

Cancer has become one of the leading causes of death in developed countries while the burden of cancer is also uprising in developing countries (1). According to GLOBOCAN 2012 estimates, the worldwide estimated new cancer cases and cancer deaths during 2012 were approximately 14.1 million and 8.2 million, respectively, while 32.6 million people were living with cancer (2). According to WHO estimates for 2012, out of 56 million deaths occurred globally during 2012, 38 million were due to non-communicable diseases (NCD), and cancer was the second most leading cause (21.7%) of these deaths following cardiovascular diseases accounting for 46% of NCD deaths (3).

In New Zealand, cancer is also an alarmingly increasing health issue; GLOBOCAN 2012 estimates reported that the estimated age-standardized rate (ASR per 100,000 population) of cancer incidence was highest in Australia/New Zealand region (1), and according to cancer registration and mortality data published in 2014 by Ministry of Health, New Zealand, cancer was the most leading cause of death for both male and female populations in New Zealand during 2011, responsible for nearly 30% of all deaths (4).

1.1.2. *Gastrointestinal cancer status in New Zealand and worldwide*

According to GLOBOCAN 2012 estimates, the five most common cancers were lung cancer (accountable for 12.9% of total newly diagnosed cases), followed by breast (11.9%), colorectal (9.7%), prostate (7.9%), and stomach (6.8%) cancers respectively (2). In regards to mortality caused by cancer, the mortality due to lung cancer was highest

with 19.4% of total cancer mortality followed by liver (9.1%), stomach (8.8%), colorectal (8.5%), and breast cancer (6.4%) (2). In New Zealand, the most common type of cancer was prostate cancer for males or breast cancer for females, accounting for 27.3% and 28.7% of total registered cancer cases respectively followed by colorectal cancer (14.8% in males and 10.8% in females) and melanoma (14.0% in males and 10.1% in females) while the type of cancer responsible for the highest deaths due to cancer was lung cancer with 18.9% of total deaths due to cancer followed by colorectal (13.4%), breast (7.2%), prostate (6.6%) and pancreatic (4.8%) cancers (4). Therefore, gastrointestinal cancers including colorectal, stomach, liver or pancreatic cancer are among the most commonly occurring cancer types, as well as causing a high percentage of total cancer deaths globally and in New Zealand. To understand the extent of the gastrointestinal cancer burden globally and in New Zealand, incidence and mortality of stomach, colorectal, liver or pancreatic cancer in worldwide and in New Zealand are mentioned further below.

Gastric or stomach cancer accounted for almost a million new cases per year, which was 6.8% of total new cancer cases, and caused around 720,000 deaths, which was 8.8% of total cancer deaths globally (2). It was ranked as the fifth most common cancer and third leading cause of death due to cancer (2). In New Zealand, gastric cancer was not listed in the five most common types of cancer, but accounted for 1.86% (391) of a total of 21,050 registered cancer cases and 3.33% (296) of a total of 8,891 total cancer deaths (4).

Colorectal cancer is the third most commonly diagnosed cancer with an estimated 1.4 million new colorectal cancer cases (9.7% of total cases) and 694,000 deaths (8.5% of cancer deaths) during 2012 worldwide (1). Among the regions all over the world, the incidence rate of colorectal cancer was highest in Australia/New Zealand region (1). In New Zealand, colorectal cancer was also the second most common type of cancer registered, with 3,030 registered cases accounting for 14.4% of total cases, responsible

for the second highest mortality rate (16.2 per 100,000 population accounting for 13.4% of total cancer deaths) according to cancer statistics in 2011 (4).

Liver cancer was ranked as the sixth most common type of cancer worldwide (782,000 new cases per annum accounting for 5.6% of total new cancer cases) and 9.1% of total cancer deaths (745,000 cancer deaths per annum) was due to liver cancer which was at the second highest cancer mortality rate worldwide (1, 2). However, liver or hepatic cancer was not one of the most common cancer in New Zealand; 1.55% of newly registered cancer cases (327 cases out of total 21,050 new cases) and 2.7% of total cancer mortality (240 out of 8,891 cancer deaths) were due to liver cancer in 2011 (4).

Pancreatic cancer was responsible for 2.4% (338,000 cases per annum) of total new cancer cases worldwide with the seventh highest mortality rate, accounting for 4% (331,000 deaths per annum) of total cancer deaths (2). In New Zealand, pancreatic cancer was accountable for the fifth highest cancer mortality rate (429 of total 8,891 cancer deaths or 4.8% of total cancer deaths), and was in the 10 most common types of cancer (2.16% of total registered cancer cases) in 2011 (4).

According to the statistics mentioned above, gastrointestinal cancers account for the majority of total cancer incidence and mortality worldwide and in New Zealand, hence current treatment approaches for gastrointestinal cancers and their effectiveness would be worthwhile to be explored so that further studies can be conducted for better treatment outcomes in patients.

1.1.3. *Current chemotherapy regimens for gastrointestinal cancer treatment*

1.1.3.1. Overview of cancer treatment regimens and chemotherapy

Chemotherapy is the treatment of cancers using anticancer drugs which are usually given to patients intravenously or orally before surgery to reduce the tumor size and help surgical removal of tumor easier and more successful and/or after surgery to kill remaining or non-resectable tumor cells and prevent the recurrence of cancers or help remission of cancers. Chemotherapy is also given as a primary course of cancer therapy for advanced stage III/IV cancers which are hard to be surgically removed or treated in order to deter the tumor growth and spread, and to relieve the symptoms of patients. Chemotherapy is given to cancer patients in cycles which are defined as certain periods of chemotherapy followed by periods of rest which allows the body to recover. Each cycle of chemotherapy usually lasts for a few weeks (5, 6).

1.1.3.2. Anticancer drugs used for gastric cancer chemotherapy

Current anticancer drugs used in the chemotherapy treatment of gastric cancer as a single agent or in combination of drugs are 5-FU (fluorouracil) with or without leucovorin (folinic acid), capecitabine (Xeloda®), cisplatin, epirubicin (Ellence®), oxaliplatin (Eloxatin®), paclitaxel (Taxol®), docetaxel (Taxotere®), S-1 (an oral fluoropyrimidine), etoposide and irinotecan (Camptosar®(7, 8). The common combination chemotherapy regimens used for gastric cancer include epirubicin, cisplatin and fluorouracil (ECF), epirubicin, oxaliplatin and fluorouracil (EOF), epirubicin, cisplatin and capecitabine (Xeloda®) (ECX), epirubicin, oxaliplatin, and capecitabine (EOX), capecitabine plus oxaliplatin (XELOX) and epirubicin, leucovorin, 5-FU and etoposide combination called ELFE regimen (7, 8).

1.1.3.3. Chemotherapy drugs used for colorectal cancer

Oxaliplatin has been used in combination with other anticancer drugs as the first-line treatment regimen of metastatic colorectal cancer; different oxaliplatin-based combination chemotherapy regimens used for colorectal cancer include FOLFOX (oxaliplatin, 5-fluorouracil with folinic acid), XELOX (oxaliplatin with capecitabine), and FOLFOXIRI (oxaliplatin, 5-fluorouracil, folinic acid, and irinotecan) (9-11). Oxaliplatin-containing chemotherapy is also used as adjuvant chemotherapy after surgical removal of locally advanced colorectal cancer (12, 13).

1.1.3.4. Chemotherapy drugs used for liver cancer

Common anticancer drugs used in combination chemotherapy for liver or hepatic cancer include epirubicin, cisplatin and infusional 5-fluorouracil (ECF regimen); doxorubicin, cisplatin and capecitabine regimen; cisplatin, interferon-alpha, doxorubicin and 5-fluorouracil chemotherapy; and oxaliplatin-based combined chemotherapy regimens such as GEMOX (gemcitabine with oxaliplatin), FOLFOX4 (oxaliplatin, 5-fluorouracil with folinic acid) and XELOX (oxaliplatin with capecitabine) (14-21). Recently, sorafenib. a multi-kinase inhibitor. has been approved for the treatment of advanced hepatocellular carcinoma, the most common type of hepatic cancer (22-25).

1.1.3.5. Chemotherapy drugs used for pancreatic cancer

Gemcitabine is commonly used for pancreatic cancer therapy, either as a single agent or in combination with erlotinib or capecitabine (26-28). FOLFIRINOX (oxaliplatin with 5-fluorouracil, folinic acid, and irinotecan) and GEMOXEL (gemcitabine, oxaliplatin, and capecitabine) have also been shown to be more effective and safe with higher median

overall survival rates in patients with metastatic pancreatic cancer compared to standard gemcitabine therapy (29).

Therefore, the topic of this thesis, and one of the anticancer drugs commonly used in the combination chemotherapy for the treatment of gastrointestinal cancers including stomach, liver, pancreatic and colorectal cancer, is oxaliplatin.

1.2. Oxaliplatin

Oxaliplatin is a third-generation platinum-based anticancer drug, and oxaliplatin-based combination therapies have been used for the treatment of colorectal and other gastrointestinal malignancies (30). In contrast to first- and second-generation platinum anticancer drugs, cisplatin and carboplatin, oxaliplatin has different carrier ligand [1,2-diaminocyclohexane (DACH)] and leaving-group (oxalate), hence it has a different anticancer activity and is effective against cisplatin- and carboplatin-resistant cancer (31-33). The structures of cisplatin, carboplatin and oxaliplatin are as shown in Figure 1.1.

1.2.1. *Clinical use*

Oxaliplatin has been used in combination with other anti-cancer drugs as the first-line treatment regimen of metastatic colorectal cancer. The different oxaliplatin-based combination chemotherapy regimens used for colorectal cancer include FOLFOX (oxaliplatin, 5-fluorouracil with folinic acid), XELOX (oxaliplatin with capecitabine), and FOLFOXIRI (oxaliplatin, 5-fluorouracil, folinic acid, and irinotecan) (9-11). Oxaliplatin-containing chemotherapy is also used as adjuvant chemotherapy after surgical removal of locally advanced colorectal cancer (12, 13). Furthermore, oxaliplatin-based chemotherapy is an important treatment of other gastrointestinal cancers including pancreatic, gastric and hepatocellular cancers (14, 29, 34-37). FOLFIRINOX (oxaliplatin with 5-fluorouracil, folinic acid, and irinotecan) and GEMOXEL (gemcitabine,

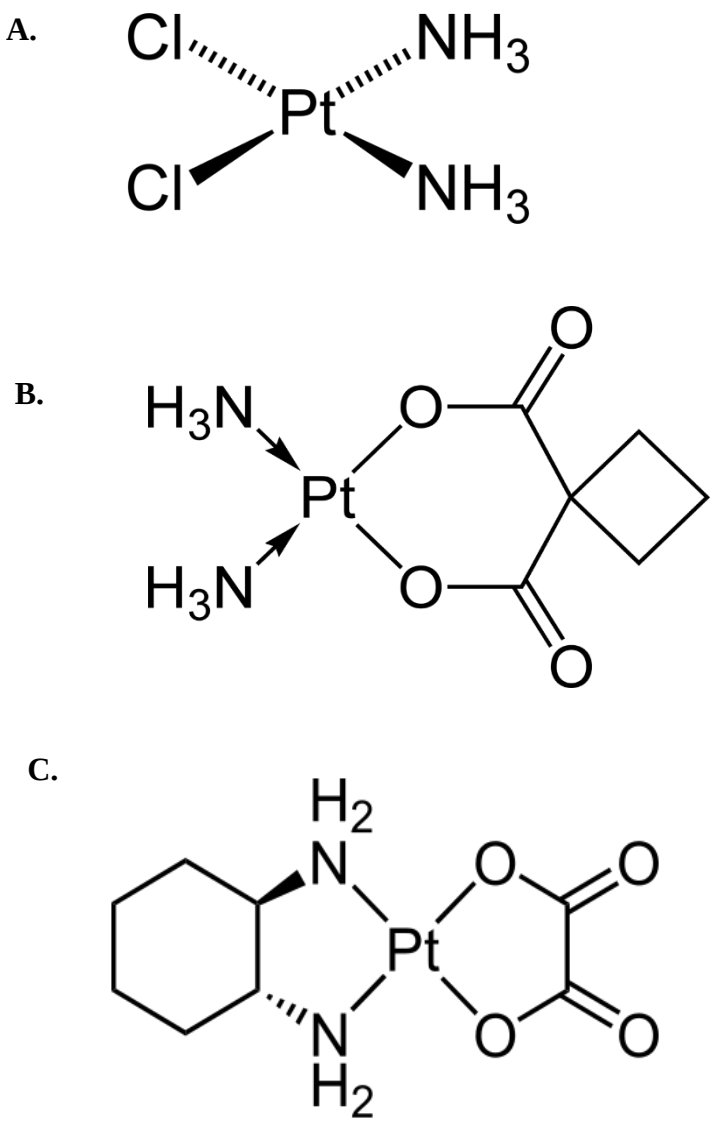


Figure 1.1 Chemical structures of first-, second- and third-generation platinum-based anticancer drugs, namely cisplatin (A), carboplatin (B), and oxaliplatin (C).

oxaliplatin, and capecitabine) were shown to be more effective and safe with higher median overall survival rate in patients with metastatic pancreatic cancer compared to standard gemcitabine therapy (29). Recently, combination chemotherapy consisting of oxaliplatin with capecitabine or S-1 (orally administered prodrugs of 5-fluorouracil) has been proved to be effective and safe for the treatment of advanced gastric cancer (36-38). Moreover, oxaliplatin-based combined chemotherapy regimens such as GEMOX (gemcitabine with oxaliplatin), FOLFOX4 (oxaliplatin, 5-fluorouracil with folinic acid) and XELOX (oxaliplatin with capecitabine) have been available for the treatment of hepatocellular carcinoma (14-16).

1.2.2. *Mechanism of action*

Oxaliplatin induces cytotoxic effects mainly by formation of cytotoxic platinum-DNA adducts similar to cisplatin and carboplatin, however with different platinum ligands and cytotoxic mechanisms (33, 39). Oxaliplatin-derived platinum forms platinum-DNA adducts mainly with guanines, resulting in intrastrand cross-links formation, and subsequently inhibiting DNA replication, transcription, cell cycle arrest and cell death (39). Oxaliplatin also induces other DNA damage such as interstrand cross-links and DNA-protein crosslinks, which are responsible for small proportion of total DNA damage (39). Some molecular mechanisms involved in oxaliplatin-induced cellular cytotoxicity are different from cisplatin; oxaliplatin-DNA adducts are not recognized by mismatch repair (MMR) complex which detects cytotoxic adducts induced by cisplatin and plays a role in determining whether cells enter to cell cycle for cell division and growth or cells proceed to apotosis and death depending on the degree of DNA damage (31, 32, 40-42). Thus, certain cancer cells with loss of MMR such as colorectal cancer cells are not sensitive to cisplatin and carboplatin, but are substantially sensitive to oxaliplatin which makes oxaliplatin to be clinically important in the

treatment of cisplatin- and carboplatin-resistant cancers (31, 32, 40-42). In addition, oxaliplatin has more effective cytotoxicity compared to cisplatin; oxaliplatin at the same concentration as cisplatin exerts similar or higher cytotoxic effects with fewer amount of DNA lesions (39, 43). Oxaliplatin-derived DACH-Pt-DNA adducts are bulky and more hydrophobic compared to cisplatin-derived *cis*-diammine-Pt-DNA adducts which could lead to slower rate of Pt-DNA reactivity but more efficient DNA damage and cytotoxicity of oxaliplatin than cisplatin (32, 39, 43).

1.2.3. *Biotransformation and pharmacokinetics*

Oxaliplatin consists of non-leaving group, 1, 2-diaminocyclohexane (DACH), and hydrolysable leaving group, oxalate, which accounts for water solubility of oxaliplatin (44-46). Oxaliplatin is administered to cancer patients intravenously; hence its bioavailability is 100% (44-46). Oxaliplatin, or oxaliplatin-derived platinum species, is distributed at high concentration in kidney, spleen, intestine and liver and red blood cells within 2 h after administration (47-49). Renal excretion is the main route of elimination for oxaliplatin, accounting for approximately 54% of total oxaliplatin administered with only 2% in faeces (48).

Oxaliplatin undergoes rapid non-enzymatic biotransformation to form reactive intermediates (48, 50). In the plasma, oxaliplatin reacts with water, chloride and sulphur-containing plasma protein such as glutathione, cysteine and methione which replace oxalato group in oxaliplatin to form the reactive platinum-containing species such as $Pt(DACH)(OH)_2$, $Pt(DACH)Cl_2$, Pt(DACH)Cl(OH), Pt(DACH)(methionine) and Pt(DACH)(glutathione) (39, 45, 51). During the transformation of oxaliplatin to $Pt(DACH)Cl_2$ or Pt(DACH)Cl(OH), an anionic intermediate of $[Pt(DACH)oxCl]^-$ in the ring opened form is formed (45, 46). The biotransformation pathways of oxaliplain is summarised in Figure 1.2. These intermediates

derived from biotransformation of oxaliplatin can undergo two pathways; they can either become inactive (48), or can induce cytotoxic effects . Within first hours after administration to patients, these intermediates bind to erythrocytes, albumin, globulins and other plasma proteins, become pharmacologically inactive and can rapidly be cleared from the circulation via renal excretion (48, 52). These intermediates not bound to proteins can enter into the cells and induce cytotoxic effects. However, more studies and evidence are needed to determine the significance of each metabolite derived from oxaliplatin biotransformation and whether these metabolites play a role in the oxaliplatin-induced cytotoxic effects.

Oxaliplatin biotransformation rate depends on the nuelophilic properties and concentration of reactants (53). In the presence of sulfhydryl-containing compounds, the reactivity of oxaliplatin was reported to be increased at a concentration-dependent manner, and in the presence of 10mM of glutathione, the half-life of oxaliplatin was less than 15 min (53). It is worth to note the kinetics of oxaliplatin biotransformation in the presence of glutathione since cytotoxic effects of oxaliplatin may be reduced after forming the oxaliplatin-glutathione complexes. Oxaliplatin-glutathione complex may be pharmacologically inactive or transported out of the cells by membrane transporters such as multidrug-resistance associated proteins (MRPs) which transport glutathione or glutathione adducts, resulting in reduction of cellular oxaliplatin levels (53-56). Previous *in vitro* studies reported that in the physiological solution of 0.9% saline, oxaliplatin was degraded to Pt(DACH)Cl_2 with the half-life of ~10h (57), and in the presence of Ca/Mg ions in the physiological saline solution, the rate of oxaliplatin degradation to Pt(DACH)Cl_2 was accelerated with the half-life of oxaliplatin being shortened from 9.1 h to 2.2 h (58). However, Han et al. has recently reported that clinical pharmacokinetics or metabolism of oxaliplatin in the presence or absence of Ca/Mg ions in the infusion were not different, and so did the acute neurotoxicity (59). It is note-worthy to explore the rate of oxaliplatin degradation in the presence of Ca/Mg ions in the

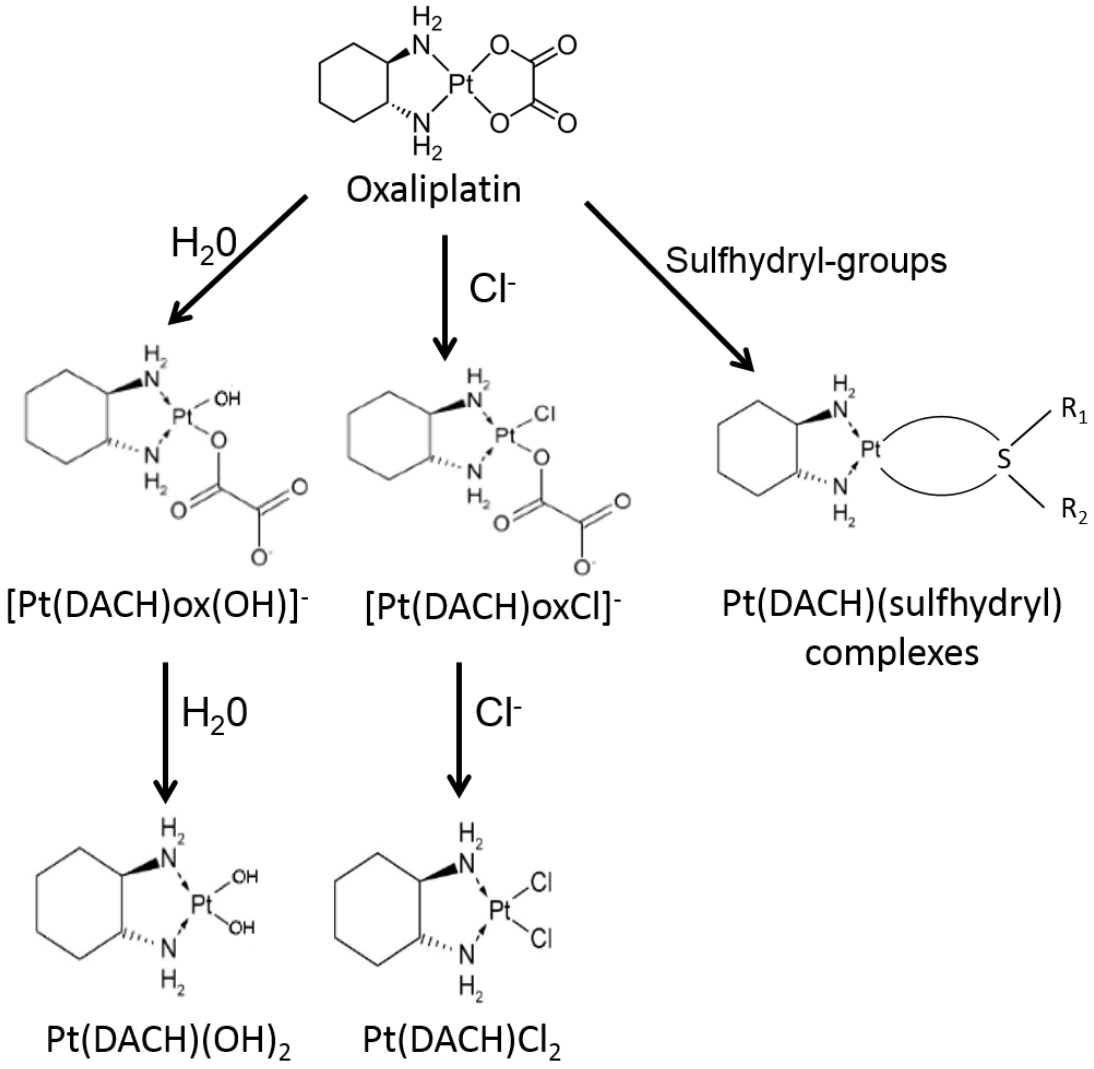


Figure 1.2 Biotransformation pathways of oxaliplatin

physiological saline since Ca/Mg infusions were used in combination with oxaliplatin chemotherapy to reduce neuropathy induced by oxaliplatin (60-62).

The pharmacokinetics of the unbound platinum in plasma ultrafiltrate of patients after receiving oxaliplatin treatment is triphasic, characterised by the short initial α and β distribution phases lasting for 0.28 h and 16.3 h, respectively, followed by a long terminal elimination phase lasting for 273 h (48). In patients receiving oxaliplatin treatment at the dose of 130 mg/m^2 every 3 weeks or 85 mg/m^2 every 2 weeks, the maximal plasma concentration (Cmax) of oxaliplatin after 2 h infusion was ranging from 0.681 μg/mL to 1.21μg/mL (48). Han et al. has recently reported that most of the unbound platinum in plasma of oxaliplatin-treated patients receiving either 130 mg/m^2 every 3 weeks or 85 mg/m^2 every 2 weeks was in the form of intact oxaliplatin at steady-state oxaliplatin concentrations during 2 h infusion ranging from 3.75 μM to 11.25 μM (59). Total body exposure to oxaliplatin [presented by area under the curve (AUC) of platinum concentration in plasma ultrafiltrate versus time] on cycle 1 after 2 h infusion of oxaliplatin at 130 mg/m^2 every 3 weeks was 11.9 μg.h/mL and that o 85 mg/m^2 every 2 weeks was 4.25 μg.h/mL, respectively (48). Han et al. has reported that the mean AUC of intact oxaliplatin and free platinum were 15.6 and 20.2 μmol/L*h, respectively (59). The mean volume of distribution of platinum ranged from 349 L to 812 L (48). The estimated clearance of ultrafilterable platinum was ranging from 9.34 to 10.1 L/h at 130 mg/m^2 to 18.5 L/h at 85 mg/m^2 (48). Platinum accumulation in the plasma ultrafiltrate following multiple dosing at 130 mg/m2 every 3 weeks or 85 mg/m2 every 2 weeks was found to be not significant (48).

1.2.4. *Clinical toxicities of oxaliplatin*

Oxaliplatin chemotherapy induce toxicities most commonly in haematopoietic, gastrointestinal and neurological systems, characterised by neutropenia, thrombocytopenia,

nausea, vomiting, diarrhoea and peripheral neuropathy (30, 63). The severity of haematopoietic and gastrointestinal toxicities are usually mild to moderate, and resolved after discontinuation of the treatment (30, 49, 63). The oxaliplatin-induced peripheral neuropathy is the major dose-limiting toxicity, and usually commences during or within a few days after oxaliplatin infusions (30, 49, 63). Oxaliplatin-induced neuropathy can be categorised into acute and chronic neuropathy (63-65). Majority of patients suffer from acute neuropathy; the symptoms include paresthesias and/or dysesthesias of extremities, mouth or throat usually triggered by cold, and mostly resolve within hours or days. The possible mechanism behind the acute neuropathy is that oxaliplatin or oxalate interact with voltage-gated sodium channels directly or indirectly via chelation of calcium and magnesium (66-68). Chronic neuropathy is usually progressive with the continuation of the treatment due to cumulative body exposure to oxaliplatin, and the proposed mechanism is neuronal atrophy and/or apoptosis resulting from the dose-dependent oxaliplatin accumulation in dorsal root ganglia. Grade 2 or more severe neuropathy is found in approximately 40–50% of patients receiving oxaliplatin, while grade 3 or 4 neuropathy occur in 10–20% of patients after receiving a cumulative dose of around 800 mg/m^2 (64, 65). The neuropathy is improved in majority of patients after discontinuation of oxaliplatin therapy, however a significant percent of patients still have some neuropathic symptoms up to a few years after discontinuation of the therapy (63-65). Signs and symptoms of chronic sensory neuropathy include loss of vibration sensation, numbness or pins and needles sensation in fingers and toes, reduced proprioception, loss of peripheral tendon reflexes, and sensory ataxia (63-65). However, oxaliplatin does not cause significant renal toxicity and hearing impairment which are commonly affected by cisplatin and carboplatin (30, 49, 63, 64).

1.2.5. *Limitations of oxaliplatin-based chemotherapy*

The clinical use of oxaliplatin-based chemotherapy is limited by poor efficacy and high toxicity in a proportion of treated patients, who exhibit disease progressive or severe adverse drug reactions early after the commencement of therapy (30). Oxaliplatin-based combination chemotherapy regimens were shown to improve the overall survival time and disease-free survival time in colorectal, liver, pancreatic and gastric cancers, however the maximal time of overall survival and disease-free survival were limited to 19.5 months and to 9 months, respectively (9-11, 13, 14, 16, 30, 34, 35, 37, 69, 70). This limited survival time in cancer patients receiving oxaliplatin-based chemotherapy could be due to either intrinsic or acquired tumour resistance, or due to the limitations in the amount of drug reaching the tumour such as reduction in the dose of oxaliplatin administered to the patients owing to the toxicity of drugs developed in patients, and the variability in pharmacokinetic parameters such as absorption, distribution, metabolism (biotransformation) and excretion which limits the bioavailability of oxaliplatin (71, 72).

1.2.6. *Tumour resistance to oxaliplatin*

To improve the clinical use of oxaliplatin, several studies have been done to explore and understand the mechanisms of oxaliplatin resistance, yet oxaliplatin resistance is not fully understood due to the complicated and multi-factorial nature of drug resistance phenomenon (71). Several factors are involved in the oxaliplatin resistance mechanisms and include reduced drug intake, increased drug efflux, altered membrane permeability, detoxification of drug with glutathione, alterations in DNA damage repair system and altered apoptosis which prevent cell death induced by drug. Reduced cellular uptake or increased cellular efflux of oxaliplatin results in reduced level of oxaliplatin accumulated inside the cells and subsequently, reduced Pt-DNA adduct formation, hence cellular transport of oxaliplatin is an

important determinant of oxaliplatin resistance (30, 31, 73-79). Second important mechanism involved in cellular oxaliplatin resistance is inactivation or detoxification of oxaliplatin with glutathione in which glutathione reacts with oxaliplatin to form glutathione-oxaliplatin adduct which is no longer cytotoxic; increased cellular glutathione level has been shown to be associated with increased oxaliplatin resistance and vice versa (77, 80, 81). Another mechanism shown to be involved in oxaliplatin resistance is altered DNA damage repair system; oxaliplatin induces formation of platinum-DNA adducts resulting in the DNA damage and cell death, and increased DNA damage repair leads to increased cell survival and consequently, increased cellular resistance to oxaliplatin. Excision repair cross-complementation group 1 (ERCC1), a crucial excision nuclease enzyme involved in nucleotide excision repair (NER), may play a role in oxaliplatin resistance. Increased activity or expression levels of ERCC1 has been shown to be associated with increased resistance to oxaliplatin-containing chemotherapies in colorectal cancer (73, 82-84), ovarian cancer (75) and gastric cancer (85-87). KRAS mutation has also been shown to be involved in determining oxaliplatin sensitivity of colorectal cancer (CRC) cells via downregulation of ERCC1, and KRAS mutation has potential to be a predictive marker for oxaliplatin response in CRC patients (88, 89). Altered apoptosis in cells is another mechanism behind oxaliplatin resistance; factors involved in altered cellular susceptibility to apoptosis induced by oxaliplatin are mutations of apoptotic protein p53 (83, 90) and anti-apoptotic factors including nuclear factor-κβ (NFκβ) (91) and taxol-resistant gene 1 (Txr1) (92). Tumour microenvironmental conditions, such as hypoxia, may also be involved in oxaliplatin resistance (93). Among these mechanisms, this thesis focussed on reduced cellular accumulation of oxaliplatin as a fundamental mechanism of oxaliplatin resistance.

1.2.7. *Cellular transport of oxaliplatin*

The transport of platinum drugs across the cellular membrane may be influenced by several factors, such as membrane stability, membrane potentials, affinity of substrates, types of platinum species entering into the cells and temperature (94-97). Previously, platinum drugs, for instance cisplatin, were believed to enter into the cells through the lipid bilayer of cellular membrane mainly by passive diffusion since cisplatin uptake was not saturable with increasing concentration and time, nor inhibited by its structural analogs (95, 98, 99). However, other studies based on cisplatin-resistant cell lines have postulated energy-dependent active transport mechanisms may also contribute to reduced cisplatin accumulation found in these cell lines (98, 100-102). Findings from these studies lead to the discovery of membrane transporters involved in oxaliplatin uptake such as organic cation transporters (OCTs) and copper influx transporter (CTR1) (103-107). In addition to the drug uptake mechanisms, drug export or efflux transporters such as copper efflux transporters (ATP7A and ATP7B) (108-111), multidrug and toxin extrusion transporters (MATE1 and MATE2) (105, 112), and ATP-binding cassette proteins (MRPs) maybe also important in cellular transport of oxaliplatin. The details upon how these membrane transporters play a role in the cellular transport and resistance of oxaliplatin will be discussed in section 1.4. Information about the structure, function and roles of membrane transporters in cancer pharmacology are also important for better understanding of the mechanisms behind the transporter-mediated oxaliplatin accumulation and resistance, and will be discussed in the next section.

1.3. Membrane transporters

1.3.1. *Overview of membrane transporters*

Physiologically important molecules such as proteins, glucose, minerals, organic ions, cellular metabolites, toxic substances and xenobiotics enter into or exit out of the cells through the lipid-bilayer of cellular membrane by passive diffusion mechanism, or by carrier-mediated transport mechanisms using different forms of energy (113). Therefore, carrier proteins or membrane transporter proteins play a role in the regulation of several physiological functions of the cells, including uptake of required nutrients; removal of metabolites, drugs and toxins from the cells as an homeostatic regulation of the cytoplasmic concentration; and the establishment of electrochemical gradients across biological membranes (113, 114). The transporters also transport metal ions required for the function of normal cells, such as copper transporters in neurons for the synthesis of neuropeptides (115). They may also participate in the export or secretion of biological active agents such as hormones (113, 114). Membrane transporters are also important pharmacologically since the membrane transport of drugs include paracellular transport by passive diffusion via tight junctions and transcellular transport which is further divided into passive diffusion, endocytosis and carrier-mediated transport (71, 116, 117).

Membrane transporter proteins are classified into 5 different types which are channels/pores, electrochemical potential-driven transporters, primary active transporters, group translocators and electron carriers (113). Alternatively, transporter proteins can be classified into two main groups, influx and efflux transporters, depending on their functions. The influx transporters mediate the uptake of molecules including ions, nutrients and drugs into the cells such as enterocytes, hepatocytes, renal tubular cells, and

tumour cells while the efflux transporters mediate the export of these molecules (118, 119).

Membrane transporter proteins important in the cancer pharmacology include the ATP-binding cassette (ABC) family, solute carrier (SLC) family, ion pumps and channels (120, 121). Due to the presence of the biological transporters in the membrane of intestinal epithelial cells, hepatocytes and renal tubular epithelial cells, they may regulate the absorption, metabolism, distribution and excretion of the drugs in the body; thus they become important determinants of the pharmacokinetic properties of the drug, such as plasma concentration, bioavailability, drug exposure, clearance and drug excretion (116, 122, 123). These transporters are also located in other tissues such as blood-brain barrier, mammary glands, placenta and testis-blood barrier, and thus they can also determine drug distribution into these tissues, and toxicity to normal tissues (119, 122). Since transporters are also present on the target cellular membrane, they may determine the drug uptake and efflux of target cells such as tumour cells, hence influencing the drug concentration inside target cells, drug-target interactions and drug sensitivity and efficacy (120, 121). Therefore, in addition to their physiological functions, the membrane transporters are important in both pharmacokinetics (PK) and pharmacodynamics (PD) of drugs in terms of drug disposition, efficacy and cellular sensitivity to anticancer drugs in the aspect of cancer pharmacology. Among all the membrane transporter proteins, ATP-binding cassette (ABC) transporters involved in multidrug resistance (MDR) mechanism of cancer are the most widely studied in cancer pharmacology (124).

1.3.2. *Important ATP-binding cassette (ABC) transporters in cancer pharmacology*

ATP-binding cassette (ABC) transporters are primary active transport proteins that use energy released from the hydrolysis of ATP by ATPase activity to transport substrates

against a chemical gradient (71, 120). To date, according to the Transporter Classification Database (TCDB), 53 human ABC proteins have been identified, and each has been grouped into seven subfamilies (ABCA–ABCG) (125). The structures of ABC transporters are as shown in Figure 1.3 (117, 120). ABC transporters operate in 4 main steps; firstly, substrate binding to the transporter's transmembrane domain (TMD) leads to the opening of ATP or nucleotide binding domain (NBD); secondly, high-affinity binding of ATP to NBD results in the release of energy for conformational changes of the TMD and translocation of substrate to other side of membrane; thirdly, ATP is hydrolysed to ADP and phosphate triggers the conformational changes in NBD; and as a last step, release of ADP and phosphate from NBD restores the transporters to original conformation with NBD in open-shaped dimer for the next transport cycle (126).

Different ABC transporters carry physiologically important substrates such as long-chain fatty acids, cholesterol, bile salts, leukotriene C4 and prostaglandins, thus abnormalities in the expression and/or functions of these transporters are associated with certain diseases in human (112). For instance, mutation in the *ABCC2* (ATP-binding cassette sub-family C member 2) transporter gene is associated with Dubin-Johnson's Syndrome, which is characterized by intermittent jaundice due to the defective excretion of conjugated bilirubin (127). ABC transporters are widely distributed in the human body including in the liver, intestine, kidney and in blood-tissue barriers such as blood-brain barrier and testis (116, 119, 122, 128-132). Hence, ABC efflux transporters play a role in excreting the waste products and toxins from the body via bile, urine and faecal matters as well as in protecting the important organs such as brain from toxic materials by pumping these materials back into bloodstream (116, 119, 122, 128-132).

Furthermore, ABC transporters play a role in the drug resistance phenomenon in cancer patients. ABC transporters are highly expressed in many human cancers including

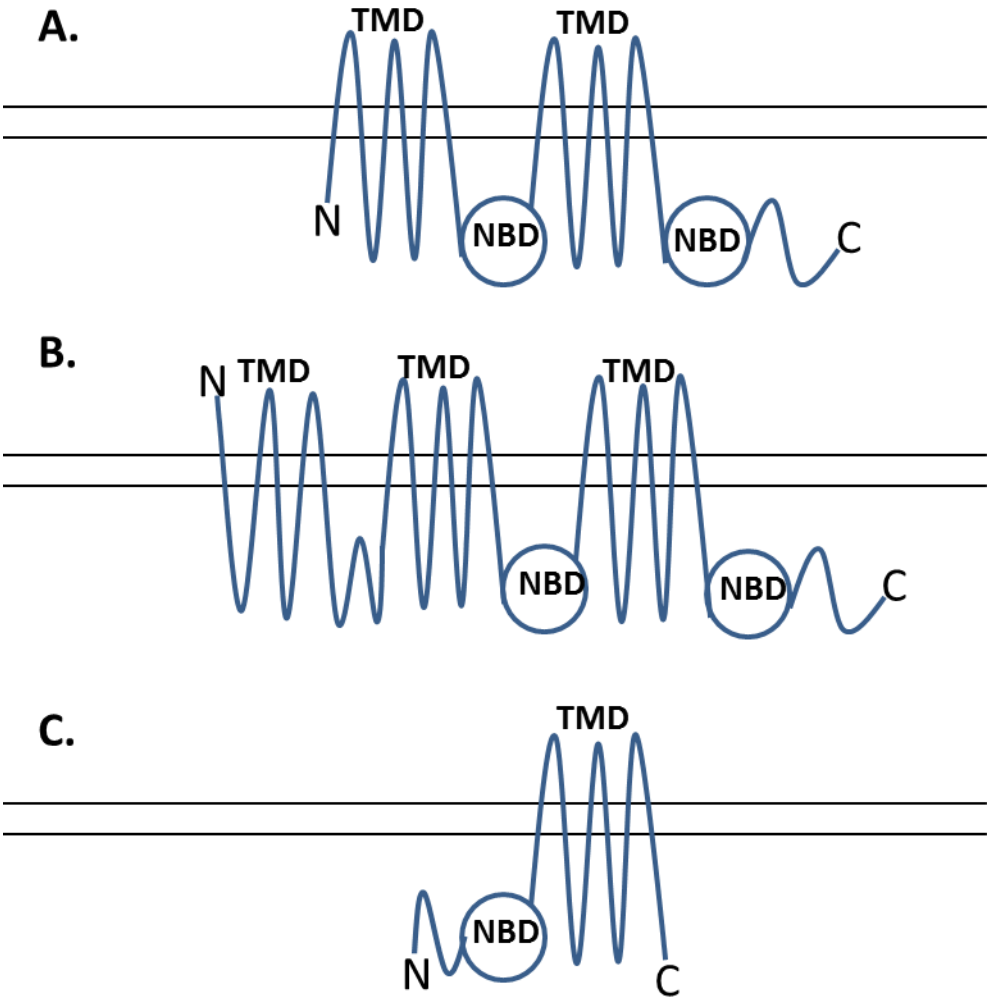


Figure 1.3 Structures of important ABC membrane transporters.

Structures of multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 4 (MRP4) and multidrug resistance-associated protein 5 (MRP5) are as shown in A, multidrug resistance-associated protein 1 (MRP1), multidrug resistance-associated protein 2 (MRP2) and multidrug resistance-associated protein 3 (MRP3) as in B, and breast cancer resistance protein (BCRP) in C. TMD, transmembrane domain; NBD, nucleotide binding domain; C, C-terminal; and N, N-terminal. Horizontal black lines represent lipid bilayer of cellular membrane. [The figure was redrawn with the permission from "Chan LM, Lowes S, Hirst BH. Eur J Pharm Sci. 2004 Jan;21(1):25-51" attached in Appendix 2.]

solid tumours and haematological malignancies. The high expression levels of these transporters are correlated with the poor response of patients to certain anti-cancer drugs in particular cancers (122, 133). These findings have shed the light on the role of ABC transporters in cancer chemotherapy as the important determinants of pharmacokinetic properties and efficacy of certain chemotherapeutic agents in patients. Several *in vitro* studies have proved this fact by demonstrating that ABC transporters partly influence the sensitivity of certain cancer drugs via affecting the drug uptake or accumulation inside the target cells (134-152). Some important ABC transporters in cancer pharmacology will be discussed here.

1.3.2.1. ABCB1 (MDR1, P-gp)

The first identified ABC transporter was MDR1 (multidrug resistance protein 1), or P-glycoprotein (P-gp), which is encoded for by *ABCB1* gene (120, 153). It has been demonstrated that MDR1 is highly expressed in solid tumours, including colon, renal and breast cancer, and its expression is correlated with the treatment failure and multidrug resistance in these cancers (117, 124). MDR1 commonly transports hydrophobic drugs with neutral or positive charge and a wide range of anti-cancer drugs including anthracyclines (e.g., doxorubicin), vinca alkaloids (e.g., vincristine), taxanes (e.g., paclitaxel), and novel anti-cancer drugs such as geldanamycin, imatinib, but ABCB1 does not appear to transport cisplatin (117, 118, 154).

MDR1 (P-gp) present in enterocytes can pump drugs back into the lumen of intestinal tract, limiting the drug absorption and the oral bioavailability of its substrates including anti-cancer drugs such as vinca alkaloids, doxorubicin and paclitaxel (119). Several studies using *mdr1a, mdr1b* knockout mice lack of ABCB1 proteins and clinical studies in human using ABCB1 inhibitors such as cyclosporin A and elacridar showed that the

oral bioavailability of anti-cancer drugs including paclitaxel, etoposide, and topotecan increased significantly after inhibiting the MDR1 transporter (116). MDR1 proteins present on the apical membrane of the hepatocyte transport its substrates from portal circulation into bile, and are involved in the biliary excretion and hepatic clearance of anti-cancer drugs that are mainly excreted by the hepatobiliary system, such as paclitaxel, doxorubicin and irinotecan (117, 119). As ABCB1 has a role in the drug-drug interactions of anti-cancer drugs, administration of other ABCB1 substrates including digoxin, loperamide can competitively inhibit the excretion of anti-cancer drugs, hence reducing the clearance and increasing the toxic effects of anti-cancer drugs. Toxic effects that are increased include neuropathy and bone marrow depression, as well as renal and liver toxicity due to accumulation of drugs in the kidney and liver (119, 155). Therefore, MDR1 proteins are important determinants of the pharmacokinetics of anti-cancer drugs, and hence, the drug disposition, clearance and toxicity in cancer chemotherapy.

Several studies have shown that chemoresistant cancer cells may have high level of expression of ABCB1, suggesting that ABCB1 or MDR1 confers the chemoresistance of cancer including liver, kidney, colon and haemotological malignancies such as leukaemia (118, 120, 122, 156). *In vitro* gene-drug relationship studies have demonstrated that expression of ABCB1 gene in different tumour cell lines is negatively correlated with potency of multiple drugs including taxanes, doxorubicin and vinca alkaloids (118). Genomic profiling studies in acute myeloid leukemia patients have demonstrated over-expression of ABC family proteins, especially the ABCB1 gene, is significantly associated with multidrug resistance in these cancer patients (133). In blood cancers including leukemia, lymphoma and multiple myeloma, ABCB1 expression levels are often initially low, but are up-regulated after chemotherapy (120).

MDR1 is located on the luminal surface of endothelial cells of brain capillaries that form the blood brain barrier (BBB), and prevents the entry of drugs into the central nervous system (CNS) (118, 119, 156) . It has been recently shown that the CNS penetration of several anti-cancer drugs usually used to treat the primary or secondary metastatic brain tumours such as gefitinib, dasatinib, and GDC-0941, a phosphatidylinositol 3-Kinase Inhibitor, is greatly increased in triple knockout [*Mdr1a/b(-/-), Bcrp1(-/-)*] mice deficient in both MDR1 and BCRP1, another important efflux in the BBB, suggesting that MDR1 together with BCRP1 in the BBB prevent the drugs from reaching the site of action and hence reduce the drug efficacy and result in worse clinical outcomes for patients (157-159).

1.3.2.2. ABCCs (MRPs)

The multidrug resistance-associated protein (MRP) family, encoded for by ABCC genes, has 9 MRP proteins (MRP1-9) encoded by ABCC1–6 and ABCC10–12 respectively (117, 120, 160). All the MRPs can translocate organic anionic compounds, glutathione and substrates conjugated to glutathione, indicating that they are important for detoxifying the endogenous metabolites as well as in detoxification of chemotherapy drug metabolites (117, 118, 120). Here, the roles of MRPs in anti-cancer drug pharmacology will be highlighted using MRP1 and MRP2 as examples.

MRP1 (multidrug resistance-associated protein 1) is responsible for the transport of hydrophobic drugs with neutral and negative charge, including vinca alkaloids, anthracyclines such as doxorubicin, methotrexate, which are conjugated with glutathione or glucuronic acid (120, 160). Being located on the basolateral surface of cellular membrane, MRP1 is involved in the export the drug substrates into blood, thereby protecting cells from drug toxicity (117, 120, 124). On exposure to vincristine at the

therapeutic doses, triple knockout [*mdr*1a/1b, *mrp*1] mice deficient in the P-gp and MRP1 developed severe bone marrow damage and intestinal mucosal damage, showing that MDR1 and MRP1 are important determining factors of the PK and toxicity of several anti-cancer drugs (161). MRP1 has been found to be overexpressed in small cell lung cancers, leukemia, oesophageal carcinomas, non-small cell lung cancers and prostate cancer, and its overexpression has been found to be correlated with drug resistance and clinical outcome in these cancer as well as in breast cancer and childhood neuroblastoma (120, 124). Studies based on triple knockout [*mdr*1a/1b, *mrp*1] mice showed these mice developed a hypersensitivity to a wide range of drugs including paclitaxel , anthracyclines, and vincristine, demonstrating that the MRP1 together with MDR1 play a role in the efficacy of these drugs and in the multi-drug resistance of certain cancers (162). The mRNA expression levels of MRP1 and MDR1 were found to be higher in residual tumors and recurrent tumours after chemotherapy compared to untreated primary tumors, and high expression levels were correlated with the tumour resistance to doxorubicin (163).

MRP2 (multidrug resistance-associated protein 2) transports organic anionic drugs such as vinca alkaloids, taxanes, anthracyclines and cisplatin, and their conjugates including glutathiones and sulphates (164). MRP2 transporter encoded by *ABCC2* is physiologically expressed in most of the tissues including luminal surface of enterocytes (116), canalicular membrane of hepatocytes (165-167) and luminal surface of renal proximal tubules (168, 169) which can determine absorption, metabolism and excretion of xenobiotics, and also in physiological tissue barriers such as blood-brain barrier (170), blood-testis barrier (171) and placenta (172). MRP2 is expressed in the apical surface of cellular membrane at important physiological barrier sites and functioning in exporting substances out of the cells, hence playing a role in bioavailability and disposition of drugs

administered into humans and body defence against toxins and drugs by eliminating drugs and toxic substances from the body through excretion into bile and urine. MRP2 expression was shown to be associated with the renal and liver toxicity of cisplatin as well as the liver toxicity of methotrexate since cisplatin and methotrexate are substrates of MRP2 (122, 128, 173-175).

1.3.2.3. BCRP (ABCG-2)

Another subtype of ABC transporter is breast cancer resistance protein (BCRP) encoded by *ABCG2* gene (120). BCRP transports both positively or negatively charged hydrophobic molecules including drug substrates such as mitoxantrone, SN38 (a metabolite of irinotecan), imatinib, topotecan, methotrexate, and doxorubicin (116, 120, 124). BCRP has been shown to be associated with cellular resistance to anticancer drugs such as doxorubicin, mitoxantrone, methotrexate, topotecan, SN38, and flavopiridol (176). Overexpression of BCRP is found to be associated with chemoresistance in breast, gastric, colorectal, liver and blood cancers (124, 176). BCRP is expressed on the apical membrane of intestine, thus it is involved in the drug efflux into gut lumen and limits the oral bioavailability of its substrates, particularly topotecan and irinotecan while BRCPs on hepatocytes also increase the clearance and reduce the toxicity of drugs including methotrexate (116). Being located on BBB, it functions together with MDR1 to prevent the CNS penetration and efficacy of certain anti-cancer drugs such as imatinib, dasatinib and GDC-0941 (157-159).

1.3.2.4. Inhibitors of ABC transporters in combating cancer drug resistance

Targeting specific ABC transporters that are potentially associated with multidrug resistance or resistance to specific drug in cancer chemotherapy may improve the treatment outcomes in cancer patients; hence inhibitors of specific ABC transporters have

been discovered and developed to be used in combination with anticancer drugs in future. The proposed mechanisms of actions for the inhibitions of transporters include competitive inhibitions of compounds with substrates at the substrate binding site, inhibiting ATPase activity of transporters and induction of changes in membrane composition or permeability (177, 178). MDR1 inhibitors such as verapamil, cyclosporin A, valspodar and elacridar have been investigated in preclinical and clinical studies (119, 179-185). Compounds identified as BCRP inhibitors include some MDR1 inhibitors such as elacridar, cyclosporine A and tariquidar, BCRP-specific inhibitors such as fumitremorgin C (FTC) and Ko143, tyrosine kinase inhibitors such as gefitinib, antivirals such as lopinavir and natural compounds like curcumin (186). MRP1 inhibitors discovered are leukotriene receptor antagonists such as MK571, organic anionic transport inhibitors such as probenecid, MDR1 inhibitors such as verapamil and cyclosporine A, natural compounds such as agosterol A and flavonoids including genistein and myricetin and antimalarial drugs (for example, chloroquine) (178, 187). Potential MRP2 inhibitors for clinical use include MK571, flavonoids such as myricetin, benzbromarone, MDR1 inhibitors such as cyclosporine A and some BCRP inhibitors such as lopinavir (177, 188, 189). However, the outcomes of preclinical and clinical studies of inhibitors are still disappointing since there has been the development of drug toxicities which may be due to several reasons; inhibiting ABC transporters, which play a role in protecting the body from toxicity via efflux of toxic substances, may increase the toxicity of anticancer drugs; overlapping of substrate specificity in all these inhibitors may lead to additive effects of inhibitions of transporters and exaggeration of toxic effects in the body (177, 178).

1.4. Role of membrane transporters in oxaliplatin uptake and resistance

1.4.1. *Organic cation transporters (OCTs)*

Several studies have shown that OCTs, the solute carrier (*SLC22*) family members, are involved in the uptake of platinum drugs including oxaliplatin (104-106, 190). OCTs have wide tissue distribution in human body, predominantly in intestinal, hepatic and renal epithelial cells, thus OCTs play a role in pharmacokinetics of platinum drugs (96). Some studies showed that oxaliplatin is a substrate of OCT2 and OCT1 while some found that its intracellular accumulation is contributed by OCT2 and OCT3 (104-106). Using overexpressed cell lines, human OCT2 has been shown to take part in the uptake of oxaliplatin and contribute to the cellular accumulation and sensitivity to oxaliplatin (104, 105, 191). However, the dose-limiting toxicity of oxaliplatin is neurotoxicity, not nephrotoxicity, thus the association of transporter proteins expressed in neurons with the oxaliplatin accumulation in these sites seems more important. Recently, it has been shown that organic cation/carnitine transporters novel 1 and 2 (OCTN1 and OCTN2 respectively), the *SLC22* family members, are expressed in rat dorsal root ganglion (DRG) tissues and mediate oxaliplatin transport, consequently oxaliplatin cellular accumulation and cytotoxicity (192). OCT2 has also been shown to be expressed in human DRG tissues and to be involved in the oxaliplatin neurotoxicity in mice (193).

1.4.2. *Copper influx transporter (CTR1)*

Copper influx transporter, CTR1, which is ubiquitously found in human body tissues and plays as a physiological transporter in regulating the intracellular copper level, may also be involved in the cellular transport of oxaliplatin (194, 195). Platinum accumulation in $CTR1^{-/-}$ mouse embryonic fibroblasts was reduced up to 35% compared to wild type fibroblasts on exposure to cisplatin and carboplatin whereas oxaliplatin accumulation is

not influenced by CTR1 at higher concentrations (196). Overexpression of human CTR1 in cisplatin-resistant cell lines resulted in increased cellular accumulation of platinum after exposure to oxaliplatin (107). Using $CTR1^{+/+}$ and $CTR1^{-/-}$ mouse embryonic fibroblasts and xenografts, Larson et al. showed that mammalian CTR1 plays a role in cellular sensitivity to cisplatin, carboplatin and oxaliplatin via influencing uptake of these drugs (103). Liu et al. have recently shown that cellular platinum accumulation is significantly enhanced in rat Ctr1-overexpressing HEK293 cells compared to its isogenic vector-transfected control cells and so does the oxaliplatin-induced cytotoxic effect (197). However, the study by Ivy and Kaplan has shown that CTR1 is not the major transport protein involved in the uptake of platinum drugs including cisplatin, hence the role of CTR1 in the transport of oxaliplatin is still questionable and needs further investigation (198).

1.4.3. *Copper efflux transporters (ATP7A and ATP7B)*

Copper efflux transporters (ATP7A and ATP7B) are found to be involved in cellular resistance and transport of platinum drugs including oxaliplatin (108-111). ATP7A expressed in most of the tissues except liver and ATP7B expressed in liver, kidney, placenta and to a smaller proportion in the brain are physiologically important copper transporters in human bodies; functional mutations of ATP7A and ATP7B result in inherited neuronal degenerative diseases with abnormal copper metabolic disorders such as Menkes disease and Wilson's disease, respectively (115). ATP7A-overexpressing cancer cells showed higher resistance to oxaliplatin with higher levels of cellular platinum accumulation, hence ATP7A most probably sequestered platinum into cytoplasmic vesicles and induced cellular resistance to oxaliplatin (108, 199). However, in human Menkes' disease fibroblasts overexpressing ATP7A (MeMNK) or ATP7B

(MeWND), cellular platinum accumulation levels, DNA platinum accumulation levels and sensitivity to oxaliplatin were increased compared to control cells, thus oxaliplatin might be sequestered into the cytoplasmic vesicles and released into the nuclear DNA resulting in the cytotoxic effects (109). Recently, it has been shown that oxaliplatin can activate the ATPase present in ATP7A or ATP7B, resulting in the translocation of copper and platinum ions, hence this finding indicates ATP7A or ATP7B is involved in the oxaliplatin transport (110). Moreover, increased level of ATP7B has been shown to be associated with poor outcome to oxaliplatin-based chemotherapy in colorectal cancer patients receiving oxaliplatin-based treatment (200).

1.4.4. *Multidrug and toxin extrusion transporters (MATE1 and MATE2)*

Multidrug and toxin extrusion transporters (MATE1 and MATE2) play a role in oxaliplatin transport (105, 112). Platinum accumulation in HEK293 cells with high expression levels of MATE1 or MATE2 is found to be higher compared to empty vector transfected control cells, indicating MATE1 and MATE2 are involved in oxaliplatin transport (105, 112). MATE1 encoded by *SLC47A1* gene is expressed in canalicular membrane of liver cells and luminal membrane of renal proximal tubules while MATE2 encoded by *SLC47A2* is present in luminal surface of renal proximal tubules (201, 202). Since the major factors hindering the clinical use of oxaliplatin are neurotoxicity and tumour resistance to oxaliplatin, the role of MATEs in oxaliplatin transport is not extensively studied.

1.4.5. *Multidrug resistance-associated proteins (MRPs)*

MRP efflux membrane transporters belonging to an ATP-binding cassette subfamily C (ABCC) are implicated in platinum drug transport (164, 203). MRP transporters are widely distributed in human body including major excretory sites such as liver and

kidney and other physiological barrier sites such as intestines, blood-brain barrier and blood-testis barrier (116, 131, 132, 204). Elevated expression level of MRP1 or MRP4 has been shown to be correlated with oxaliplatin resistance (74). Inhibition of MRP1 and reduction of cellular glutathione level with verapamil in gallbladder cancer cells increased cellular sensitivity to oxaliplatin and reduced tumour growth in mice (205). High expression level of MRP2 has been shown to be associated with the cellular resistance to oxaliplatin using MRP2 over-expressing cell lines, human cancer cell lines with high MRP2 expression level and cisplatin-resistance, however data is limited and inconsistent (74, 77, 78, 206). Positive clinical-association studies linking *MRP2* genotype and/or expression level with poor patient responses to oxaliplatin (207-210) have added further to the urgency for an understanding of MRP2-mediated platinum transport mechanism. MRP2 being an important efflux transporter of cisplatin and potentially of oxaliplatin, background knowledge concerned with MRP2's structure, functions and role in cancer pharmacology, especially in platinum-based anticancer drugs will be discussed next.

1.5. Multidrug resistance-associated protein 2 (MRP2)

The MRP2 protein is encoded for by *ABCC2* gene, and is a member of ATP-binding cassette subfamily C (ABCC) or MRP (Multidrug resistance-associated protein) family. It is an integral ~190 kDa protein consisting of 1545 amino acids (165, 167, 211, 212). The *ABCC2* gene is located on chromosome10q24 and consists of 32 exons with the length of 69 kilo base pairs (212, 213). The predicted structure of MRP2 consists of 17 transmembrane (TM) segments arranged in three transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) [Figure 1.3].

1.5.1. *Physiological role of MRP2*

MRP2 is located on the apical site of cellular membrane and expressed in physiologically important tissues and organs such as hepatocytes (165, 167, 214), proximal renal tubular cells (168, 169) and human small intestine (160) which can determine absorption, metabolism and excretion of xenobiotics, and also in physiological tissue barriers such as blood-brain barrier (170), blood-testis barrier (171) and placenta (172). The function of MRP2 is to transport glutathione and glutathione-conjugated substances out of the cells, and being located in the canalicular membrane of hepatocytes and luminal surface of renal tubules, MRP2 is involved in the excretion of glutathione, substances conjugated with glutathione, sulphate, glucoronate and cystines such as bilirubin glucuronides, 17β-glucuronosyl estradiol and cysteinyl leukotriene (leukotriene C4), and organic anionic drugs such as vinca alkaloids, taxanes, anthracyclines and cisplatin in the presence of glutathiones (160, 164, 204). The importance of MRP2 in the human body can be clearly observed in patients with Dubin-Johnson Syndrome which is an autosomal recessive disorder characterized by ABCC2 gene mutation, absence of functional MRP2 protein in liver of patients and intermittent jaundice along with other symptoms due to the defective excretion of conjugated bilirubin (215). The discovery of two mutant rat strains, the Eisai hyperbilirubinemic rat (EHBR) and GY/TR$^-$ mutant, which do not express Mrp2 in their livers and have hyperbilirubinemia and other symptoms due to defective ATP-dependent transport of conjugated bilirubin across the canalicular membrane of hepatocytes, highlights the importance of Mrp2 in the physiology of the body (166, 167). Mrp2 expression was shown to be associated with the renal and liver toxicity of cisplatin as well as the liver toxicity of methotrexate since cisplatin and methotrexate are substrates of Mrp2 (122, 128, 173-175). Therefore, being expressed in the apical surface of cellular membrane at important physiological barrier sites and functioning in exporting

substances out of the cells, MRP2 plays a role in body defence against toxins and drugs by eliminating drugs and toxic substances from the body through excretion into bile and urine as well as in controlling bioavailability and disposition of drugs administered into humans.

1.5.2. *Role of MRP2 in cancer pharmacology*

MRP2 expression level is found to be elevated in human cancers such as colorectal, ovarian, lung and nasopharyngeal cancers (127, 143, 149, 151, 214, 216). MRP2 is shown to be involved in the transport of anticancer drugs including cisplatin, doxorubicin, docetaxel, epirubicin, etoposide, irinotecan, methotrexate and vincristine (204). MRP2 function and/or expression is found to be associated with chemosensitivity of different anti-cancer drugs including cisplatin, etoposide, vinca alkaloids and methotrexate (217, 218). MRP2 expression is higher in cancerous regions of human colorectal tissues compared to non-cancerous regions, and such high expression level of MRP2 is correlated with reduced sensitivity to cisplatin (143). Increased level of MRP2 expression has been shown to be associated with reduced cellular accumulation of cisplatin and/or increased cellular resistance to cisplatin and/or reduced toxicity of cisplatin using MRP2 over-expressing cell lines, human cancer cell lines with high MRP2 expression level and cisplatin-resistance, animal models or clinical tumor samples (173, 212, 216-225). *ABCC2* expression was significantly higher in colorectal tissues having mild to moderate dysplasia or cancerous lesions compared to unaffected tissues, suggesting that MRP2 might be involved in early stages of colorectal cancer (CRC) development and altered *ABCC2* expression level might be useful as a biomarker or prognostic marker for CRC (226).

In cancer patients with Dubin-Johnson syndrome where ABCC2 gene is mutated and functional MRP2 protein is absent in canalicular membrane of hepatocytes, excretion of possible MRP2 anticancer drugs and consequently, efficacy and toxicity of these drugs might be affected, hence a few studies for such cases have been reported (227, 228). A patient with Dubin–Johnson syndrome who was treated with high-dose methotrexate for large B-cell lymphoma developed reversible renal toxicity due to three-fold decrease in elimination rate of methotrexate, and genetic analysis revealed that the patient had a heterozygous mutation of ABCC2 gene (412A>G) which resulted in the transport activity loss of MRP2 protein on functional analysis of gene mutation (228). This suggested that MRP2 played a role in the elimination of methotrexate and subsequently in the renal toxicity of methotrexate. Another study reported that a malignant pleural mesothelioma patient with Dubin–Johnson syndrome receiving cisplatin/pemetrexed combination therapy had no severe toxic effects of drugs where the drug toxicities reported were only grade 1-2, and showed a partial response without delay in the drug excretion, suggesting that MRP2 might not be involved in the excretion of pemetrexed (227). Studying the cancer patients with Dubin-Johnson syndrome can assist the researchers and clinicians in understanding the roles of MRP2 in pharmacokinetics and drug disposition of possible MRP2 substrates and in improving the treatment regimens for cancer patients with Dubin-Johnson syndrome.

Single nucleotide polymorphisms (SNPs) or genetic variants of MRP2 gene (*ABCC2*) have been studied, and among several *ABCC2* SNPs, three common SNPs reported are C-24T SNP which is a C to T substitution in the promoter region, G1249A SNP where G is substituted with A at exon 10 location, resulting in an exchange of Val with Ile amino acid at codon 417, and C3972T SNP occurring at exon 28 location with a substitution of C with T which results in the silent mutation at codon 1324 (229, 230). These SNPs can

be associated with defects in MRP2 expression or functional activity (229, 230). In lung cancer patients receiving platinum-based therapy, -24C homozygous genotype was found to be associated with a response to platinum treatment, and C3972T SNPs (C to T substitution) with increased risk of grade 3 or 4 thrombocytopenia toxicity, suggesting that C-24T SNPs and C3972T SNPs might have an effect on treatment response and on toxicities in lung cancer patients treated with platinum-based anticancer drugs respectively (231). One study reported that *ABCC2* SNP (C-24T) was associated with reduced disease-free survival and overall survival in lung cancer patients receiving platinum-based chemotherapy (232) whereas another report showed that there was a significant association between *ABCC2* C-24T SNP and response to platinum-based therapy (233). Another *ABCC2* SNP (G1249A) known to be associated with ABCC2 mRNA level changes and ABCC2 substrate specificity was shown to be associated with poor response of colorectal cancer patients to FOLFOX-4 chemotherapy as well as with the shorter survival rate of patients (207). *ABCC2* G1249A SNP was also found to be associated with response to platinum-based chemotherapy in ovarian cancer patients (234). Therefore, these three common *ABCC2* SNPs have potentials to be applied in personalised therapy of cancer patients receiving anticancer drugs which are most likely MRP2 substrates, however more studies are needed to be done to investigate the functions and significance of these SNPs. However, the impact of *ABCC2* SNPs on MRP2 protein functional activity remains controversial because of potential complex effects of multiple SNPs and haploypes, and difficulties intepreting functional studies of expressed variant proteins.

1.5.3. *Role of MRP2 in oxaliplatin pharmacology*

The role of MRP2 in tumour resistance and/or clinical outcome in gastrointestinal cancer patients who were receiving platinum-based chemotherapy was studied previously (143, 209, 235, 236). MRP2 expression level was found to be increased in tumour tissue samples from patients with hepatocellular carcinoma (237, 238), colorectal carcinoma (143, 209) and pancreatic cancer (235). Colorectal tumor tissue samples from patients with recurrence while receiving the treatment of 5-fluorouracil/leucovorin (FL) and oxaliplatin (FOLFOX-4) expressed high level of MRP2 compared to matched normal tissue samples, and increased overall survival occurred in patients with MRP2-negative tumor samples despite the difference being not statistically significant (209). *ABCC2* (MRP2 gene) polymorphism (G1249A) was shown to be associated with increased overall survival in patients with colorectal cancer receiving oxaliplatin-containing chemotherapy (207). *ABCC2* polymorphisms (rs3740066, rs1885301, rs4148396 and rs717620) were reported to be associated with increased risk of neurotoxicity from FOLFOX therapy (208). In a cohort of pancreatic cancer patients receiving gemcitabine or gemcitabine with cisplatin therapy, MRP2 G40A GG genotype showed weak association with low overall survival rate and significant association with poor tumor response to chemoradiotherapy (239). These studies suggested that MRP2 might play a role in the chemoresistance or susceptibility of patients to toxicity of oxaliplatin or clinical outcome in gastrointestinal cancer patients receiving oxaliplatin-based chemotherapy, yet the findings were still unclear. Hence, it will be interesting to further investigate the role of MRP2 in the cellular resistance mechanisms of human gastrointestinal cancer to oxaliplatin.

There are *in vitro* studies showing the association of MRP2 with the cellular resistance to cisplatin in human liver cancer (219, 238), cellular resistance to doxorubicin and

vincristine in HepG2 cells (221), and cellular resistance to cisplatin in human ovarian cancer cell line (A2780) (220, 222). On the other hand, there have been a limited number of *in vitro* studies investigating the role of MRP2 in cellular resistance to oxaliplatin using human gastrointestinal cancer cell lines with the contradictory findings; hence the role of MRP2 in the cellular resistance of oxaliplatin in human gastrointestinal cancers is still questionable. Shen and colleagues showed that MRP2 expression was significantly increased in oxaliplatin-resistant colorectal cancer cell line (HCT-116/L-OHP), and siRNA-mediated transient silencing of *ABCC2* in HCT-116 cells resulted in increased cellular sensitivity to oxaliplatin and increased cellular platinum accumulation (240). Liu et al. determined the expression levels of ABC transporter proteins such as P-gp (P-glycoprotein encoded by *ABCB1*), MRP1 (multidrug-resistance protein 1) and MRP2 in oxaliplatin-resistant colon cancer cell lines (SW620/L-OHP and Lovo/L-OHP) compared to non-resistant cell lines, and it was found that only MRP2 expression level detected by western blot was upregulated in the resistant cell lines whereas no significant change was observed for the levels of P-gp and MRP1, suggesting the involvement of MRP2 in oxaliplatin resistance of human colon cancer cells (206). Another study by Beretta and group showed that oxaliplatin-resistant ovarian cancer cells (IGROV-1/OHP) had lower levels of cellular platinum accumulation with increased expression levels of MRP1 and MRP4 compared to non-resistant IGROV-1 cells whereas MRP2 and MRP3 were not detectable in both non-resistant and resistant cell lines (74). Recently, another group has demonstrated that MRP1 and MRP2 inhibitor called Gü83 increased cellular platinum accumulation in human ileocecal colorectal adenocarcinoma cell line HCT-8 and its oxaliplatin-resistant variant HCT-8ox by nearly 3-fold and 2-fold respectively, supporting the hypothesis that MRP2 contributes to the cellular transport of oxaliplatin and ultimately to the cellular accumulation level of oxaliplatin (77). Another study

investigating the role of ABC transporters in synergistic anticancer activity of FOLFOX therapy reported that 5-FU pre-incubation increased cellular expression level of *ABCC2*, and MRP2 expression level was associated with increased cellular sensitivity to oxaliplatin, but with increased resistance to DACH, oxalate and combination of oxalate and $Pt(DACH)Cl_2$ (210). Taken together, these limited findings have suggested that MRP2 may play a role in the cellular transport of oxaliplatin and cellular resistance of human gastrointestinal cancer to oxaliplatin, thus the role of MRP2 in cellular platinum accumulation and cellular sensitivity to oxaliplatin in human cancer cells will be worth to be studied.

1.5.4. *Potential implications of MRP2 in oxaliplatin therapy*

ABCC2 SNPs, namely C-24T, C3972T and G1249A, are likely to be associated with defects in MRP2 expression or functional activity, and have potentials to be applied in personalised therapy of cancer patients. In lung cancer patients receiving platinum-based therapy, -24C homozygous genotype was found to be associated with a better response to treatment, and C3972T SNPs (C to T substitution) with increased risk of grade 3 or 4 thrombocytopenia toxicity, suggesting that C-24T SNPs and C3972T SNPs might have an effect on treatment response and on toxicities in lung cancer patients treated with platinum-based anticancer drugs respectively (231). A few studies reported that there was an association between *ABCC2* SNP (C-24T) and treatment outcomes in lung cancer patients treated with platinum-based anticancer drugs (232, 233). Another *ABCC2* SNP, G1249A, was also reported to be associated with poor response of colorectal cancer patients to FOLFOX-4 chemotherapy (207). Thus, *ABCC2* SNPs, C-24T and G1249A, might be a predictive biomarker for the response of cancer patients to platinum-based therapy, including oxaliplatin-based therapy, and be useful for optimising the dose and

regimens of platinum-based anticancer therapy in cancer patients. However, Sprowl et al. reported that different levels of nephrotoxicity and treatment outcomes in cancer patients receiving cisplatin therapy were not correlated with different SNPs or genetic variants of *ABCC2* (241). In accordance with this study, some other studies also demonstrated that *ABCC2* genetic variants were not correlated with treatment outcomes in ovarian cancer patient receiving platinum-based chemotherapies or with oxaliplatin-induced neurotoxicity in metastatic colorectal cancer patients (242, 243). Up till now, the data regarding the *ABCC2* SNPs and platinum-based anticancer therapies are limited and controversial, hence more studies should be done in future for better understanding of its roles in chemotherapy.

The level of MRP2 expression in tumours of cancer patients receiving oxaliplatin-based therapy may have potential effects on treatment outcomes in those patients. Patients with recurrence during the course of FOLFOX-4 treatment showed high expression levels of MRP2 in tumour tissue samples (209). MRP2 expression was increased in cancerous colorectal tissues compared to matched normal tissues, and was not correlated with overall survival or disease-free survival of patients, however patients showing recurrence during the treatment with FOLFOX-4 had high MRP2 expression levels in their cancerous tissues (209). Therefore, individual variability in genotypes of *ABCC2* and in expression levels of MRP2 of tumours could be linked to inter-patient variations of treatment outcomes and responses to oxaliplatin-containing therapy, and may be applicable in personalised medicine of cancer patients treated with oxaliplatin-based therapy.

MRP2 inhibitors have potentials to be used in combination with anticancer drugs to enhance the anticancer effects of drugs for better treatment outcomes in cancer patients

(244). Potential MRP2 inhibitors discovered include MK571, flavonoids such as myricetin, benzbromarone, MDR1 inhibitors such as cyclosporine A and some BCRP inhibitors such as lopinavir (177, 188, 189). However, most of these compounds are non-specific MRP2 inhibitors, and hence broad spectrum of inhibitions on other transporters could lead to enhanced toxic effects of anticancer drugs and the effectiveness of MRP2 inhibitions is still questionable (189).

1.6. Hypothesis and aims of thesis

Oxaliplatin, a platinum-based anticancer drug, and its combination therapies, have been widely used in the clinical treatment of colorectal and other gastrointestinal cancers (30). However, clinical use of oxaliplatin-based chemotherapy is limited by its poor efficacy and high toxicity occurring in some patients. To exert the cytotoxic effects in cells, oxaliplatin must enter into the cells and react with DNA, resulting in the formation of DNA-platinum adducts which induce cell death and cell cycle arrest (245). As oxaliplatin is a highly hydrophilic compound (44, 246) and chemically transforms into charged intermediates in extracellular biological fluids (247), its capacity to cross cell membrane by passive diffusion is inherently very poor. Recent evidence has accumulated pointing to alternative transport mechanisms other than passive diffusion involving membrane transporter proteins whereby platinum-based drugs like oxaliplatin move into and out of cells (96, 248). Several recent studies have reported possible oxaliplatin uptake/efflux transporters such as copper transporter protein 1 (CTR1) (197), organic cation/carnitine transporter N1 (OCTN1) (192), ATP7A/7B (108, 109) and MATE1/2 (105, 112). Several preclinical (cell-based and animal-based) and clinical studies have shown that MRP2 is associated with cisplatin resistance and with reduced cellular accumulation of cisplatin, suggesting MRP2 is an efflux transporter of cisplatin, as reviewed in (248). However,

very few studies have directly addressed transport of oxaliplatin or oxaliplatin-derived platinum by MRP2 (77, 206). Recently, some studies have suggested the association of *ABCC2* genotype and/ or expression level with patient responses to oxaliplatin (207, 208). Hence, the current study tested the hypothesis that MRP2 transports oxaliplatin, or platinum species derived from oxaliplatin, and thereby may control the amount of oxaliplatin-derived platinum accumulating in cells and their sensitivity to oxaliplatin-induced growth inhibition.

In this thesis, firstly, MRP2-mediated oxaliplatin transport mechanism was examined using MRP2-expressing membrane vesicles. It was crucial to determine directly whether MRP2 transports oxaliplatin prior to investigating the involvement of MRP2 in determining the cellular transport of oxaliplatin. No membrane vesicular transport studies had explored the MRP2-mediated oxaliplatin transport so far as far as we were appear. The transporter function of MRP2 in membrane vesicles used in this study was first verified using 5(6)-carboxy-2,'7'-dichlorofluorescein (CDCF), a substrate of MRP2. Then, MRP2-mediated oxaliplatin transport was measured as platinum accumulation in membrane vesicles using ICP-MS based platinum analysis method. MRP2-mediated transport of oxaliplatin-derived platinum was characterised by conducting time-course and concentration-course studies, and MRP2 inhibitor studies using myricetin as a model MRP2 inhibitor. Mechanisms of MRP2-mediated oxaliplatin transport was also be explored by investigating the stability of oxaliplatin in the glutathione-containing reaction buffer used in the vesicle transport assay.

The thesis then studied the role of MRP2 in platinum accumulation and oxaliplatin sensitivity at a cellular level was examined using MRP2 overexpressing HEK293 cells (HEK-MRP2) and its isogenic control cell line (parental HEK293 cells or HEK-P). This

cell-based study was done to determine the role of MRP2 in conferring cellular oxaliplatin resistance. Published data regarding the role of MRP2 in cellular transport of oxaliplatin and sensitivity to oxaliplatin was very limited to date. There was only one study showing that MRP2 inhibitor increased platinum-DNA levels in both parental and oxaliplatin-resistant human colorectal cancer cell lines (HCT-8 and HCT-8ox respectively), which suggested that oxaliplatin or its derivatives were MRP2 substrates and MRP2 had a potential role in determining the cellular and DNA platinum accumulation levels. However, the expression levels of MRP2 in these cell lines were not examined and the role of MRP2-mediated oxaliplatin transport in cellular sensitivity to oxaliplatin was inconclusive (77). Another study demonstrated that MRP2 expression detected with western blot method was up-regulated in the oxaliplatin-resistant human colon cancer cell lines (SW620/L-OHP and LoVo/L-OHP), indicating the involvement of MRP2 in oxaliplatin resistance in human colon cancer cells. However, neither MRP2 inhibition studies nor cellular platinum accumulation were not conducted in this study and hence the role of MRP2-mediated oxaliplatin transport in cellular oxaliplatin resistance was uncertain (248). HEK293 is human embryonic kidney cell line with cellular structure and functions being more comparable to in-vivo conditions, hence HEK293 cell model was chosen as a model in this study. The genotypic and phenotypic characteristics of MRP2 in HEK-MRP2 and HEK-P cell lines were also verified since these cell lines were not characterized before. Cellular platinum accumulation levels of HEK-P and HEK-MRP2 after exposure to oxaliplatin, and cellular sensitivity of HEK-P and HEK-MRP2 to oxaliplatin-induced cytotoxicity were determined. Effect of a model MRP2 inhibitor myricetin on the cellular accumulation of platinum and cellular sensitivity to oxaliplatin-induced cytotoxicity was lastly examined.

Lastly, this thesis examined the role of MRP2 in determining cellular oxaliplatin transport and cellular resistance to oxaliplatin in a panel of human gastrointestinal cancer cells lines. The purposes of this study were to understand the extent of the MRP2 involvement in oxaliplatin transport and oxaliplatin resistance occurred in human cancer cells and to generate the date which would assist investigators and clinical researchers to improve the efficacy of oxaliplatin therapy in gastrointestinal malignancies. Firstly, human gastrointestinal cancer cell lines were characterised in regards to *ABCC2* (MRP2 gene) expression level, MRP2 functional activity, cellular platinum accumulation and sensitivity to oxaliplatin. Cellular platinum accumulation and sensitivity to oxaliplatin-induced cytotoxicity of human cancer cells were measured and the role of MRP2 in these cellular mechanisms was further explored using chemical inhibition of MRP2 with myricetin, a model MRP2 inhibitor, and genetic inhibition of MRP2 with siRNA-mediated transient silencing of *ABCC2*.

Chapter 2. Materials and methods

2.1. Chemicals

Chemicals including chemical compounds, reagents, buffers and solutions used in this study together with their sources and suppliers were as listed below in Table 2.1.

Table 2.1 Chemicals used in this study with their sources

Chemicals	Sources (suppliers)
0.9% sodium chloride intravenous infusion BP 5% glucose intravenous infusion BP	Baxter Healthcare (Auckland, NZ)
70% nitric acid Ethanol Sodium hydroxide (NaOH) solution	Thermo Fisher Scientific (NZ)
Adenosine diphosphate (ADP)	Genomembrane (Yokohama, Japan)
Adenosine triphosphate (ATP)	Genomembrane (Yokohama, Japan)
Alexa Flour 594-labeled anti-mouse IgG Ab	Invitrogen (Carlsbad, CA, USA)
Anti-MRP2 antibody [M2 III-6] (catalogue #, ab3373)	Abcam (Melbourne, VIC, AU)
Bovine serum albumin (BSA)	ICPbio (Auckland, NZ)
CDCF (5(6)-Carboxy-2´,7´- Dichlorofluorescein)	Sigma-Aldrich (St Louis, MO, USA)
CDCFDA (5(6)-carboxy-2,'7'- dichlorofluorescein diacetate)	Sigma-Aldrich (St Louis, MO, USA)
Collagen I, Rat tail	BD biosciences (Auckland, NZ)
Disodium phosphate (Na_2HPO_4)	Scharlau (Barcelona, Spain)
DMF (Dimethyl formamide)	Sigma-Aldrich (St Louis, MO,USA)
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich (St Louis, MO,USA)
Dulbecco's Modified Eagle Medium (DMEM)	Life Technologies (Auckland, NZ)
Eagle's Minimum Essential Medium (MEM)	Life Technologies (Auckland, NZ)
Fetal bovine serum (FBS)	Life Technologies (Auckland, NZ)
Glutathione	Genomembrane (Yokohama, Japan)

Table 2.1 Continued

Goat serum	Invitrogen (Carlsbad, CA, USA)
HEPES buffer [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)]	Thermo Fisher Scientific (NZ)
HPLC-grade methanol	Sigma-Aldrich (St Louis, MO,USA)
Membrane vesicle incubation buffer	Genomembrane (Yokohama, Japan)
Methanol	Millipore (Bedford, USA)
Milli-Q grade water	Millipore (Bedford, USA)
MK571	Sigma-Aldrich (St Louis, MO, USA) Cayman Chem (MI, USA)
MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)	Sigma-Aldrich (St Louis, MO, USA)
Myricetin	Sigma-Aldrich (St Louis, MO, USA) Cayman Chem (MI, USA)
Oxaliplatin powder	Actavis (Auckland, NZ)
Paraformaldehyde powder	Sigma-Aldrich (St Louis, MO, USA)
Penicillin-Streptomycin (10,000 U/mL)	Life Technologies (Auckland, NZ)
Platinum standard for ICP	Sigma-Aldrich (St Louis, MO, USA)
Potassium phosphate (KH_2 PO_4)	Millipore (Bedford, USA)
Pt (R,R-DACH) Cl_2	Sigma-Aldrich (St Louis, MO, USA)
Sodium chloride (NaCl) Potassium chloride (KCl)	Sigma-Aldrich (St Louis, MO, USA)
Sodium dodecyl sulphate (SDS)	SERVA (NY, USA)
Thallium standard for ICP	SPEX CertiPrep (NJ, USA)
Trifluromethanesulfonic acid ("triflic acid")	Sigma-Aldrich (St Louis, MO, USA)
Triton-X-100	AppliChem (Darmstadt, Germany)
VECTASHIELD Mounting Medium with DAPI (6-diamidino-2-phenylindole)	Vector Laboratories (Burlingame, CA, USA)

Membrane vesicle incubation buffer solution consisted of 50 mM MOPS-Tris, 70 mM KCl, 7.5 mM $MgCl_2$, and stopping buffer solution contained 40 mM MOPS-Tris and 70 mM KCl. Both of the buffer solutions were from Genomembrane (Yokohama, Japan).

Oxaliplatin powder (Actavis, New Zealand) was dissolved in 5% glucose solution, sonicated and filtered with a 0.22 μm Millipore filtration system (Bedford, USA) and stored at -20°C.

Stock solutions of 5(6)-carboxy-2,'7'-dichlorofluorescein diacetate (CDCFDA) and myricetin at the concentrations of 1000 times higher than working solutions were prepared by dissolving in DMF (dimethylformamide) and were stored at -20°C.

A stock solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) at 10 mg/ml was prepared in Milli-Q grade water and stored at 4 ° C.

2.2. Membrane vesicular transport assay

The details of membrane vesicles and experimental conditions for the membrane vesicular transport assay were as described in section 3.2.2 and 3.2.3.

2.3. Cell lines and cell culture

2.3.1. *HEK293 (human embryonic kidney-293) cell lines*

Isogenic HEK293 parental (HEK-P) and HEK293 transfected to stably over-express *MRP2* (HEK-MRP2) cell lines were kindly provided by Professor PietBorst (Division of Molecular Biology and Centre for Biomedical Genetics, the NetherlandsCancer Institute, Amsterdam, Netherlands).

2.3.2. *Human gastrointestinal cancer cell lines*

A panel of human gastrointestinal cancer cell lines, namely HCT116, HT-29, SW620, WiDR, MIA Paca-2, PANC-1 and Hep G2 cell lines, used in this project was obtained from Auckland Cancer Society Research Centre (ACSRC). Apart from WiDR which was from Dr Martin Ford (Glaxo-Wellcome, Stevenage, UK), all cell lines were originated from ATCC. These cell lines were validated and maintained at ACSRC.

2.3.3. *Cell culture*

All the cell lines were stored at -80°C in liquid nitrogen tank, and cultured according to the manufacturer's protocol. Cell lines including HEK293, HCT116, HT-29, SW620, WiDR, MIA Paca-2 and PANC-1 were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 units of penicillin/streptomycin per ml, at 37°C under 5% CO2 with 95% humidified air. For Hep G2 cell line, it was grown in the same conditions as other cell lines apart from

the use of different growth medium which was Eagle's Minimum Essential Medium (MEM). Cells with passage number greater than 20 were discarded.

In this study, HepG2 and PANC-1 cells were found to grow at slower rates, hence the seeding density for these two cell lines for passaging and maintenance of cells was kept at 350,000 cells in T25 flask and 1000,000 cells in T75 flask while other cell lines were seeded at 250,000 cells in T25 flask and 750,000 cells in T75 flask.

2.4. Growth inhibition assay (MTT assay)

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was used as the main assay to measure viable cells in the experiments. In this assay, the tetrazolium salt is reduced by dehydrogenase enzymes in the mitochondria of living cells to produce purple formazan crystals, which were then dissolved in dimethyl sulfoxide (DMSO) solution. The absorbance or optical density (OD) of the solution was measured using a spectrophotometer at 540 nm wavelength with OD values representing the number of viable cells.

To evaluate the cellular sensitivity of different cell lines to oxaliplatin-induced cytotoxicity, cells were exposed to oxaliplatin at different concentrations for 2h followed by measurement of viable cells with MTT assay. Cells were seeded at 5000 cells/well in a collagen-coated 96-well plate and allowed to attach to the well surface over 24h incubation in normal drug-free growth medium. After attachment, cells were exposed to oxaliplatin at different concentrations and incubated for the desired time at 37^0C with 5% CO_2/95% air. To terminate drug exposure, the medium containing drug was removed and replaced with the drug-free growth medium. Cells were then allowed to grow to reach the optimal density which was until 72 hours since cells were first seeded and then MTT assay was performed to determine cell viability.

In case of MRP2 inhibition studies, the experiments were conducted in the same procedure apart from the fact that cells were exposed to myricetin for 30 min followed by 2h exposure to oxaliplatin with myricetin.

The percent cell viability at different concentrations was calculated using the OD measured and normalised to the mean OD value for untreated controlled cells, taken as 100 percent. The percent viability values were used to plot the nonlinear concentration-response (inhibition) curve, and the IC-50 values (50% reduction in the cell viability compared with untreated controlled cells) were determined from the curve fit using GraphPad Prism version 6. The IC-50 values for oxaliplatin in cells, and relative resistance (RR), which is the ratio of the IC-50 values of cells to that of HEK-P, were calculated in order to examine the sensitivity of cells to oxaliplatin-induced cytotoxicity. The experiments were repeated independently at least three times and the IC-50 values were calculated from the nonlinear dose-response (inhibition) curve fits using the mean percent cell viability values for each drug concentration from each individual experiment.

2.5. Platinum accumulation analysis with inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the cellular or vesicular concentrations of oxaliplatin-derived platinum after exposure to oxaliplatin. For measurement of cellular platinum accumulation, cells were seeded at 250,000 cells per well (350,000 cells per well for HepG2 and PANC-1) in a collagen-coated 6-well plate, and grown in the normal growth medium until the cells become around 80% confluent. And then, cells were incubated with oxaliplatin at designated concentrations dissolved in the incubation buffer for designated time points, and then the reaction was stopped by washing cells with ice-cold PBS (phosphate buffered saline), followed by drying cells for 30 min. For measurement of membrane vesicular

accumulation of platinum, the membrane vesicular transport assay was conducted according to section 2.2.2, and the processed vesicular samples were collected.

Cellular or membrane vesicular samples collected were then digested with 70% nitric acid for 2 h, and then the protein content of cells were determined using a modified tyrosine nitration assay (249). Cell or vesicular lysates were further digested by leaving samples at room temperature overnight and then heating at 95°C for 2 h. The digests were then diluted in milliQ water containing thallium 50 ppb as an internal standard. Platinum counts of samples were measured using a Varian 820MS ICP-MS (Agilent Technologies Inc., Santa Clara, CA, USA) at LabPLUS (Auckland, New Zealand), and then normalized for thallium internal standard counts. The platinum concentration of each sample was calculated from the platinum to thallium count ratios using the standard curve method. To generate the standard curve, the solutions of desired platinum concentrations were made using the same matrix as in cellular samples spiked with platinum stock of known concentration, and were included in the same ICP-MS run with the samples. Quality control (QC) of 3 different concentrations which were the lower, middle and upper concentrations over the standard curve range were included, and the accuracy and precision of QCs were calculated to determine the reliability of each run. The ICP-MS analysis run was accepted only if the standard curve was linear, and the QCs and at least 3 concentration values of the standard curve were within 15 % precision values and within 100 ± 15 % accuracy values. The method of platinum analysis in the cellular samples using the ICP-MS was developed and validated before the run of cellular samples. The sensitivity, accuracy and 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 guidance for the validation of bioanalytical method. The LOD and LLOQ of

the method were found to be 0.3 ppb and 1 ppb of platinum respectively as determined from different ICP-MS runs.

2.6. Fluorescent probe-based assay for validation of MRP2 function

2.6.1. *Membrane vesicular MRP2 functional validation assay*

To validate the MRP2-mediated membrane transport activity, MRP2-expressing and control membrane vesicles were exposed to a model fluorescent probe, CDCF (5(6)-carboxy-2',7'-dichlorofluorescein) [Sigma-Aldrich, St Louis, MO, USA] at a concentration of 5 μM for 5 min in the presence or absence of ATP. The details of the assay were mentioned in section 3.2.4

2.6.2. *Cellular MRP2 functional validation assay*

Cellular CDCF accumulation was measured to evaluate the transporter activity of MRP2 in HEK293 and human cancer cell lines. The non-fluorescent permeable precursor form of CDCF, CDCFDA [5(6)-carboxy-2,'7'-dichlorofluorescein diacetate], was used in this assay. Cells at the density of 50,000 cells per well (80,000 cells for HepG2 and PANC-1) were seeded in the collagen coated 24-well plates. When cells reached 80-90% confluence, the CDCF assay was performed as follows. A 500 μl aliquot of 10 μM CDCFDA dissolved in 10 mM HEPES-HBSS medium was put into each well and cells were exposed to CDCFDA for 90 min at 37°C. Cells were then washed twice with 10 mM HEPES-HBSS medium, treated with 0.1% Triton dissolved in 10 mM HEPES-HBSS medium for 10 min at room temperature (RT) which was covered with the aluminium foil to prevent the degradation of fluorescence by outside light. The cell lysate samples were then collected in a black 96-well plate for the reading of fluorescence intensity with fluorimeter at 485/528 nm with 20 nm wavelength. The protein levels of the samples

were measured using a modified tyrosine nitration assay after the cells were digested with 70% nitric acid for 2 h (250). The cellular accumulation of CDCF in fluorescence units (FU) per mg of protein was calculated using the fluorescence intensity of lysate measured as arbitrary fluorescence units and the amount of protein of cell lysate measured as mg of protein.

CDCF efflux was also measured in HEK293 cell lines to evaluate the cellular MRP2 functional activity. After 90 min of incubation with CDCFDA, the medium was collected in the black 96-well plate and the fluorescence intensity of the medium was measured with fluorimeter at 485/528 nm with 20 nm wavelength to measure the amount of CDCF effluxed from the cells into the medium.

2.7. High-pressure liquid chromatography (HPLC) analysis

In the vesicular transport assays, vesicles were incubated with the membrane vesicle incubation buffer containing 50 mM MOPS-Tris, 70 mMKCl, 7.5 mM $MgCl_2$ and 2mM glutathione during exposure to oxaliplatin. Therefore, the rate of degradation of oxaliplatin in the buffer solution over the period of 24 hours was studied using the validated HPLC-UV detection method of oxaliplatin in the solution (58). The details of the method were as described in section 3.2.6.

2.8. Immunocytochemistry (ICC)

Fluorescent immunocytochemistry (ICC) was undertaken to detect cellular MRP2 protein expression using a primary antibody, anti-MRP2 (1:100; ab3373 from Abcam), and a secondary antibody, Alexa Flour 594-labeled anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). Cells were cultured in the chamber slides until they reached the confluence of 60-70%. The growth medium was then replaced with pre-warmed PBS which was left for 5 min followed by fixation of cells with 4% paraformaldehyde for 15 min at RT (room temperature). Then, cells were permeabilised with 0.2% Triton X-100 in PBS for 15 min. Blocking of the non-specific protein binding was achieved by incubating cells with blocking solution (PBS containing 0.2% Triton X-100, 3% goat serum, and 2% bovine serum albumin) for 1 h at RT. After blocking, cells were incubated with the primary antibody, anti-MRP2 (ab3373 from Abcam) prepared in immunobuffer (PBS containing 0.2% Triton X-100 and 3% goat serum) with the dilution ratio of 1:100 for overnight at 4^0C. After that, the cells were washed for 3-4 times with PBS containing 0.2% Triton X-100 followed by incubation of cells with the secondary antibody (Alexa Flour 594-labeled anti-mouse IgG, Invitrogen, Carlsbad, CA, USA) diluted in the immunobuffer (dilution ratio was 1:500) for 3 h at 4^0C with the cover to protect from light. Cells were then washed with PBS and cover-slipped with VECTASHIELD Mounting Medium with DAPI (6-diamidino-2-phenylindole) to stain nucleic acid (Vector Laboratories, Burlingame, CA, USA). Images were captured using an Olympus FV1000 confocal laser scanning microscope (Olympus Inc., Tokyo) attached to a Nikon digital camera and then analysed using Nikon Elipse Net (Nikon, Melville, NY, USA) and ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.9. Real-time quantitative polymerase chain reaction (real-time qPCR)

2.9.1. *RNA extraction*

Extraction of RNA from cell lines including HEK293 and human gastrointestinal cancer cell lines was done using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Briefly, cells were grown in the 6-well plates with the culture conditions as mentioned in the section 2.3.3 until they became 80-90% confluent. Then, cells were trypsinised and collected as cell pellets which were treated with the cell lysis buffer to lyse the cell membranes. Cell lysates were then passed for several times through a 20-gauge needle attached to an RNase-free syringe in order to homogenise it. Then, 1 volume of 70% ethanol was added to the homogenized lysate and was mixed by pipetting it for several times. After that, the sample (maximum volume of 700 µl) was added to an RNeasy spin column in a 2 ml collection tube which was then centrifuged for 15 s at 8000 x g (10,000 rpm). The filtrate collected in the tube was discarded. The addition of buffer, spinning and discarding of the filtrate were repeating using 2 more buffers supplied in the kit. As a final step, the RNeasy spin column was placed in a new 1.5 ml tube, and 40µl of RNase-free water was added directly to the spin column membrane followed by centrifuging of the tube for 1 min at 8000 x g (10,000 rpm) to elute the RNA collected in the spin column membrane.

The purity and quantity of RNA in the sample were determined by measuring the absorbance of the sample at the wavelengths of 260 nm (A260) and 280 nm (A280)using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Australia). The RNA samples having the A260/A280 ratio of 1.9 to 2.1 were accepted to be pure. The concentration of RNA in the samples (ng/µl) was calculated by multiplying the A260

readings with 40, assuming that 1 unit of absorbance at 260 nm corresponded to 40 ng of RNA per µl of the sample.

2.9.2. *cDNA synthesis from RNA samples*

cDNA (complementary DNA) was synthesized from total RNA previously extracted from each cell line sample by reverse transcription of total RNA samples in which RNA was primed with the oligonucleotides, or primers, catalysed by reverse transcriptase enzyme to produce cDNA using a RT^2HT First strand kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Briefly, to eliminate the genomic DNA contamination of RNA samples before reverse transcription reaction, the RNA samples of 400 ng were incubated with genomic DNA elimination buffer solution that contained DNase enzyme for 5 min at 42° C in Bio-Rad thermacycler which was then placed on ice immediately for at least 1 min. After that, reverse-transcription master mix was prepared from the reverse transcription buffer solution, the reverse transcriptase enzyme mixture and the mixture of primers and external RNA control provided in the kit, and 10 µl of the mixture was added to the tube containing 10 µl of RNA-genomic DNA elimination mixture prepared previously. After mixing the solution by pipetting for a few times, the reverse transcription reaction was commenced by incubating the mixture at 42° C for 15 min, and then terminated by incubation of mixture at 95° C for 5 min using the Bio-Rad thermacycler. As the last step, 91 µl of RNase-free water was added to it and mixed by pipetting for a few times to get 111 µl of the final reaction mixture that was placed on ice and then stored at -20° C for real-time qPCR amplification in future.

2.9.3. *Real-time qPCR*

To measure the level of expression of the membrane transporter genes including *ABCC2* in the samples, real-time qPCR was performed and mRNA levels of these interested

genes were quantified using the fluorescent dye SYBR Green-based method with the appliance of ABI PRISM 7900HT Sequence Detection Systems and SDS 2.3 software (Applied Biosystems). 102 µl of cDNA previously synthesised from RNA samples of each cell line sample was mixed with SYBR Green qPCR master mix solution containing Hot Start DNA *Taq* Polymerase, PCR buffer, dNTP (deoxy-nucleotide phosphates) mixture, SYBR Green dye and ROX passive reference dye according to the manufacturer's protocol (Qiagen, Valencia, CA) to make the final qPCR reaction mixture. For the analysis using customised PCR array, 10 µl of the final reaction mixture was added to each of 96 wells in the customized RT^2 human transporter array qPCR plate (catalogue number CAPH12045-PAHS-070Z, Qiagen, Valencia, CA) where the forward and reverse primers of 88 different genes containing human transporter genes, housekeeping and control genes were pre-loaded in 4 replicates for 4 different samples in the 384-well format.

For the conventional PCR analysis, the primers of genes of interest were purchased from Invitrogen, Carlsbad, CA, USA, and stored at -20 °C. 5 µl of forward and reverse primers for each gene and 10 µl of qPCR reaction mixture containing cDNA samples and SYBR Green reaction mixture were placed in 384-well PCR plate. The sequences of gene-specific primers used in the PCR analysis were as shown in Table 2.3.

The PCR run was set-up according to the manufacturer's instructions; the PCR cycler conditions were as shown in Table 2.4. The Ct (cycle threshold which is the cycle number required for the fluorescent signal to reach the threshold) values of each cDNA sample for each gene measured were obtained from the amplification plots generated by SDS software based on the detected fluorescence levels over the cycle numbers. The average Ct values of house-keeping genes were calculated. For each cDNA sample, the Ct values

of gene of interest were also determined, and the delta Ct value was calculated by subtracting the average Ct value of house-keeping genes from the Ct values of gene of interest.

Table 2.2 RT-PCR primer sequences of ABCC1-5, ABCB1 and house-keeping genes

Gene	Accession no.	Primer sequence 5´-3´		Amplicon size (bp)
ABCC1	NM_004996	Forward	ACCATGCTGCTTGCTACCTT	413
		Reverse	ACTTGTTCCGACGTGTCCTC	
ABCC2	NM_000392	Forward	CCCTGTCCCTAGGGCTTTTT	214
		Reverse	TGCGTCTGGAACGAAGACTC	
ABCC3	NM_003786	Forward	GACGGCACGACACAAGGC	458
		Reverse	CAGCATGTTGAGGGGAAGTCT	
ABCC4	NM_005845	Forward	GTGTACCAGGAGGTGAAGCC	429
		Reverse	GAGCGTGCAAAAAGTCAGCA	
ABCC5	NM_005688	Forward	CGGCCGATGCCGCTATAAA	423
		Reverse	GCCCAGCACTAACAACAAGC	
ABCB1	NM_000927	Forward	TCGTAGGAGTGTCCGTGGAT	495
		Reverse	ATGTGCCACCAAGTAGGCTC	
Beta-actin	NM_001101	Forward	GAGCACAGAGCCTCGCCTTT	494
		Reverse	AGAGGCGTACAGGGATAGCA	
GAPDH	NM_002046	Forward	CTCTGCTCCTCCTGTTCGAC	121
		Reverse	GCGCCCAATACGACCAAATC	
HPRT1	NM_000194	Forward	AGGACTGAACGTCTTGCTCG	388
		Reverse	ATCCAACACTTCGTGGGGTC	

Table 2.3 Real-time qPCR cycler conditions

Number of cycles	Duration	Temperature (°C)	Purpose of the stage
1	10 min	95	Hot Start DNA *Taq* Polymerase is activated
40	15 s	95	Perform the PCR
	1 min	60	Perform the fluorescence data collection

2.10. Transient knockdown of MRP2 mediated by *ABCC2*-small interfering RNA (*ABCC2*-siRNA)

A reverse-transfection method, previously shown to be an efficient method for knockdown of MRP2 gene expression in the HepG2 cell line, was used in this study (251, 252). The details of the siRNA-mediated knockdown of *ABCC2* expression in HepG2 experiments were mentioned in section 5.2.7.

2.11. Statistical analysis

The data were presented as mean value with standard deviation (mean ± S.D), or mean value with 95% confidence intervals (95% CI), and were analysed using Prism 6 software (GraphPad, San Diego, CA, USA). Data were analyzed visually and by descriptive statistics. Linear and non-linear regression analyses were applied as appropriate using Prism 6 software. To test whether the mean values were statistically significant or not, 95% CI, unpaired Student's t-test, one way or two way ANOVA with Tukey's multiple comparison post tests were applied as appropriate. *P*-value of <0.05 was considered to indicate the results were statistically significant.

In membrane vesicular studies, trends in kinetic data were analysed by nonlinear regression fits, and statistical significance of differences between groups was analyzed by one-way or two-way ANOVA tests followed by Tukey's multiple comparison post-tests.

In HEK293 cell line studies, the tests used for statistical analysis of data were two-way ANOVA followed by Tukey's multiple comparison tests, Mann-Whitney's test, or unpaired t-test.

In human gastrointestinal cancer cell line studies, one-way or two-way ANOVA followed by Tukey's multiple comparison tests or unpaired Student's t-test were used appropriately to test the statistical significance of differences among datasets.

Chapter 3. Membrane vesicular studies

3.1. Introduction

Oxaliplatin, a third-generation platinum-based anticancer drug, and its combination therapies, have been used for treating colorectal and other gastrointestinal malignancies (30). However, oxaliplatin-based chemotherapy is limited by poor efficacy and high toxicity in a proportion of treated patients who exhibit disease progressive or severe adverse drug reactions early after the commencement of therapy (9, 10, 13, 69). The pharmacological basis of these variable clinical responses to oxaliplatin is currently unclear. Prior to inducing cytotoxicity in tumour or normal cells, oxaliplatin must transit through cell membranes before accessing and reacting with DNA, forming DNA-platinum adducts and inducing cell death and cell cycle arrest (245). As oxaliplatin is highly hydrophilic (44, 246) and chemically transforms into charged intermediates in biological solutions (247), its inherent capacity for crossing cell membranes by passive diffusion may be limited. Deficient intracellular platinum accumulation has been proposed as a major mechanism involved in cellular resistance to oxaliplatin (74, 76, 79, 96). Moreover, recent evidence has pointed to alternative transport mechanisms involving membrane transporter proteins whereby oxaliplatin moves into and out of cells (105, 108, 110, 155, 192, 248, 253-256). As this field is relatively new, it seems likely that many interactions involving oxaliplatin and membrane transporter proteins remain to be characterised.

The role of multidrug resistance-associated protein 2 (MRP2) in the membrane transport of oxaliplatin-derived platinum is currently unclear. MRP2 is an integral 109 kDa protein, also known as also known as canalicular multispecific organic anion transport (cMOAT),

and is encoded for by the *ABCC2* gene (204). The MRP2 protein consists of two ATP-binding domains and 17 transmembrane regions in its amino acid sequence, and functions in the transport of substrates across cell membranes using energy derived from ATP hydrolysis (204). The MRP2 protein is expressed at major physiological barriers, including the biliary canalicular membranes of hepatocytes and apical membranes of renal proximal tubular cells, where it functions in the excretion of a wide range of structural diverse endogenous and exogenous small molecular weight compounds into the bile and urine, respectively (204). MRP2 is also known for its expression by tumour cells and tissues and contributions to multidrug resistance (204), and for its functional genetic variations that contribute to altered drug handling (257). Early work suggested that MRP2 may be an efflux transporter of cisplatin (212, 219), and further evidence for interactions between MRP2 and cisplatin subsequently came from studies of recombinant cell lines, preclinical tumour models and clinical-association studies (217, 220, 222, 223, 258). However, there have been no studies directly addressing oxaliplatin or platinum derived from oxaliplatin is transported by MRP2 to date as far as we were aware. Recent reports of positive clinical-association studies linking ABCC2 genotype and/ or expression level with patient responses to oxaliplatin (207, 208) have added further to the urgency for understanding of this fundamental transport mechanism.

These considerations led us to undertake the present study in which we sought to determine if MRP2 could transport platinum derived from oxaliplatin *in vitro*. Human MRP2 protein expressing inside-out orientated membrane vesicles, prepared from Sf9 insect cells transiently transfected with the *ABCC2* gene were used for these studies. Inside-out membrane vesicular model has been widely used to investigate whether a substrate or drug of interest is transported by an efflux transporter of interest (218, 259-263). Membrane vesicle preparations have advantages for studies of drug efflux

transporter mechanisms (261), such as little or no other mammalian endogenous membrane transporters expressed on the insect cell membrane, providing more specificity for characterisation of transporter activity, and experimental control over free drug concentrations at the cytoplasmic transporter protein substrate binding sites, in a way that is not possible in whole cells. Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure membrane vesicle accumulation of platinum in this study. ICP-MS was highly sensitive and specific for detecting platinum in biological matrices (264, 265) but did not distinguish intact oxaliplatin from other forms of oxaliplatin-derived platinum that may have become associated with membrane vesicles during their incubation with oxaliplatin. As oxaliplatin degrades in aqueous solutions containing chloride ions (57, 247) or glutathione (53, 56), it could not be assumed that oxaliplatin had remained intact in membrane vesicle incubation buffer or was the form of platinum transported by MRP2 even though short incubation times of between 5 to 20 minutes were used. Therefore, the stability and degradation of oxaliplatin in membrane vesicle incubation buffer was studied using a validated HPLC-UV method (57).

A fluorescent probe, 5(6)-carboxy-2,'7'-dichlorofluorescein (CDCF) which has been used as a robust MRP2 substrate in a number of MRP2-related transporter studies, was used to validate the transport activity of the test system. This fluorescent substrate allowed the use of a fluorescent plate reader, removing the need for radio-labeled compounds and radioactive facilities (266-270). Inhibitors are often used to confirm the involvement of a membrane transporter in the transport of a substrate. Flavonoids, such as myricetin, have been reported to be MRP1 and MRP2 substrates and to exert inhibitory effects on these transporters (266, 271, 272). Myricetin was thus used as a model inhibitor of vesicular MRP2 as most of the other well-known MRP2 inhibitors, such as MK571 and

probenecid, are sulphur-containing compounds which may result in an increased rate of oxaliplatin degradation (46, 51, 53, 55, 247, 273).

The hypothesis of our study is that MRP2 is involved in the transport of oxaliplatin, contributing to the cellular resistance of human cancer cells to oxaliplatin.

The main objective of the studies described in this chapter was to determine whether MRP2 (Multidrug Resistance-Associated Protein 2) transports platinum derived from oxaliplatin using a non-cellular inside-out membrane vesicle model.

The aims of the experiments conducted in this chapter were:

1. To verify the function of MRP2 in MRP2 vesicles used in this study
2. To utilise the commercially available membrane vesicles (MRP2 and control vesicles) in this study
3. To characterise the MRP2-mediated transport of oxaliplatin-derived platinum by conducting time-course and concentration-course studies, and MRP2 inhibitor studies
4. To study the effect of a model MRP2 inhibitor called myricetin on the MRP2-mediate platinum transport, and
5. To explore the mechanisms of MRP2-mediated oxaliplatin transport by investigating the stability of oxaliplatin in the glutathione-containing reaction buffer used in the vesicle transport assay.

3.2. Materials and methods

3.2.1. *Chemicals*

The details of the chemicals, their sources and how the stock solutions were prepared were described in section 2.1.

3.2.2. *Membrane vesicles*

MRP2-expressing and control inside-out membrane vesicles (5 mg total protein/ml) prepared from Sf9 (*Spodoptera frugiperda*) insect cells, transiently transfected with the human *ABCC2* gene encoding MRP2 protein or an empty vector control, respectively, along with membrane vesicle incubation buffer solution containing 50 mM MOPS-Tris, 70 mM KCl and 7.5 mM $MgCl_2$ and stopping buffer solution containing 40 mM MOPS-Tris and 70 mM KCl were obtained from GenoMembrane, Co., Ltd (Yokohama, Japan). Vesicles were stored at -80°C until use.

3.2.3. *Membrane vesicle transport assay*

Membrane vesicular transport assays were performed according to the manufacturer's protocol with some modifications. Briefly, MRP2-expressing and control vesicles (40 µg protein/8 µL) were incubated with 11.6 µL of membrane vesicle incubation buffer in an Eppendorf tube at 37°C for 5 min. Then, a pre-warmed reaction mixture containing the test substrate, ATP or AMP (4mM) and glutathione (2 mM) was added to a final volume of 40 µL. After the designated incubation time, the reaction was stopped by adding 200 µL of ice-cold stopping buffer solution containing 40 mM MOPS-Tris and 70 mM KCl and mixing. The reaction mixture was then placed into pre-wet 96-well glass-fibre filter plates (MultiScreenHTS-FB plate, Merck-Millipore, MA, USA) fitted onto a suction filtration device (MultiScreenHTS Vacuum Manifold, Merck-Millipore, MA, USA)

connected to a vacuum pump. The wells were filtered and washed twice with ice-cold stopping buffer while under vacuum. The bottom of the plate was then thoroughly wiped, followed by the addition of 10% SDS (100 µL) to each well for 30 min to lyse the membrane vesicles trapped on the filter plate and elute the encapsulated test substrate. The filter plate was then centrifuged (1000 rpm for 1 min) twice to collect the dissolved membrane vesicles and eluted substrate into an underlying 96-well plate.

3.2.4. *Validation of MRP2 function in membrane vesicles*

To validate the MRP2-mediated membrane transport activity, MRP2-expressing and control membrane vesicles were exposed to a model fluorescent probe, CDCF (5 µM) for 5 min in the presence or absence of ATP. After incubation and filtration, the membrane vesicle CDCF accumulation was measured as follows. Dissolved membrane vesicles were collected in a black fluorometer plate and treated with 0.1N NaOH (100 µl) for 5 min. The fluorescent intensity of the solution was then measured at 495 nm excitation and 529 nm emission wavelengths with filters of 485 and 535 nm wavelengths, respectively, using a microplate multi-mode plate reader (Synergy HT, BioTek Instruments, Inc., USA). CDCF solution alone (4 µL 50 µM CDCF dissolved in 36 µL membrane vesicle incubation buffer) was also added to the wells of filter plate, treated with 100 µL 10% SDS solution for 30 min, centrifuged and treated with 100 µL 0.1N NaOH solution to give the total fluorescent intensity of 200 pmol CDCF. A background fluorescent reading was obtained by measuring the fluorescence intensity of the wells containing 100 µL 10% SDS solution and 100 µL 0.1N NaOH. All the fluorescent readings were corrected by subtracting this background reading. The amount of CDCF in samples (pmol) was calculated from the ratio the fluorescence intensity of the sample divided by the fluorescence intensity of the 200 pmol standard, multiplied by 200 pmol.

3.2.5. *Platinum accumulation measurement with ICP-MS (Inductively Coupled Plasma Mass Spectrometry)*

Membrane vesicle accumulation of platinum derived from oxaliplatin was measured as described in section 2.5. The ATP-dependent component of platinum accumulation was derived by subtracting the amount of platinum accumulating in the absence of ATP from that in the presence of ATP at each respective oxaliplatin exposure time and concentration.

3.2.6. *HPLC studies*

HPLC-UV detection method which was previously validated in our lab was used to detect oxaliplatin, Pt(DACH)Cl2, glutathione and degradation products in membrane vesicle incubation buffer. Briefly, 50 µl of the sample to be analysed was injected into the analytical column and the peaks of oxaliplatin and its metabolites including Pt(*R,R*-DACH)Cl2 were detected and analysed using the HPLC-UV detection system where the details of the system and the apparatuses used were shown in Table 3.1. The mobile phase used was 6% methanol which was prepared by adding 60ml of HPLC-grade methanol in 1 L of Milli-Q water with the pH adjusted to 2.5 with 10% triflic acid (v/v). The flow rate of the mobile phase in the column was set at 0.5 ml per min. The UV detection was at the wavelength of 210 nm with a bandwidth of 10 nm using the reference wavelength of 550 nm with a bandwidth of 50 nm. Before and after each run, the column was washed by running the solution consisting of 60:40 methanol and water for at least 30 minutes followed by Milli-Q water for at least 30 minutes. After washing before the run, the column was then equilibrated with the mobile phase for at least 30 minutes.
For stability studies, 100 µM oxaliplatin was incubated in membrane vesicle incubation buffer (pH 7.4, 37°C) containing 50 mM MOPS-Tris, 70 mM KCl and 7.5 mM $MgCl_2$

with or without 2 mM glutathione, and samples were immediately injected on the HPLC column at designated incubation times up to 24 hours. Oxaliplatin concentration was determined from the peak area. Other compounds were detected semi-quantitatively based on the retention times of authentic standards.

3.2.7. *Statistical analysis*

Data were analyzed as described in section 2.11.

Table 3.1 Details of the apparatuses used for the HPLC-based platinum analysis system

Apparatus	Characteristics of apparatus	Company
HPLC online system	Hewlett Packard HP1200	HP, Wilmington, DE, USA
Analytical column	Waters μBondapak C18 3.9 x 300 mm column	Waters, Massachusetts, USA
Guard column	N/A	Phenomenex, Torrance, CA, USA
UV detector	Millipore Waters Lambda-max model 480 LC Spectrophometer	Millipore, Lane Cove, Australia
HPLC operational software	HP4500 Chemstation	Agilent technologies, Avondale, USA
Chromatogram Intergration and analysis	HP1200 Agilent ChemStation Offline Software B.04.01	Agilent technologies, Avondale, USA

3.3. Results

3.3.1. *Effect of oxaliplatin on CDCF accumulation*

To investigate the effect of oxaliplatin on the activity of MRP2 in membrane vesicles, MRP2 and control membrane vesicles were incubated with 5 μM of 5(6)-carboxy-2,'7'-dichlorofluorescein (CDCF) in the presence of ATP or AMP for 5 minutes, or pre-incubated with 400 μM oxaliplatin for 5 minutes followed by co-incubation with 5 μM CDCF for 5 minutes in the presence of ATP. Experiments were conducted using a rapid filtration method described in the method section 3.2.3. CDCF accumulation in membrane vesicles was then measured using fluorescence.

The accumulation of CDCF was significantly higher in MRP2 membrane vesicles in the presence of ATP than in the presence of AMP or in control vesicles ($P < 0.0001$; Tukey's multiple comparisons post-tests following two-way ANOVA) [Figure 3.1 and Table 3.1]. In the presence of ATP, MRP2 membrane vesicles accumulated 1170 ± 122 pmol of CDCF per mg of protein, which was 7.7-fold higher than the amount of CDCF accumulated in MRP2 membrane vesicles in the presence of AMP (145 ± 133 pmol of platinum per mg of protein, $P < 0.0001$; Tukey's multiple comparisons post-tests following two-way ANOVA). In comparison, in control vesicles, the accumulation of CDCF was lower and appeared unaltered by the presence of ATP, with the mean values of 119 and 72.0 pmol of CDCF per mg of protein in the presence of ATP or AMP, respectively.

Oxaliplatin inhibited the ATP-dependent accumulation of CDCF by MRP2 expressing membrane vesicles without altering the accumulation of CDCF by control membrane vesicles. In the presence of oxaliplatin of 400 μM, the ATP-dependent CDCF

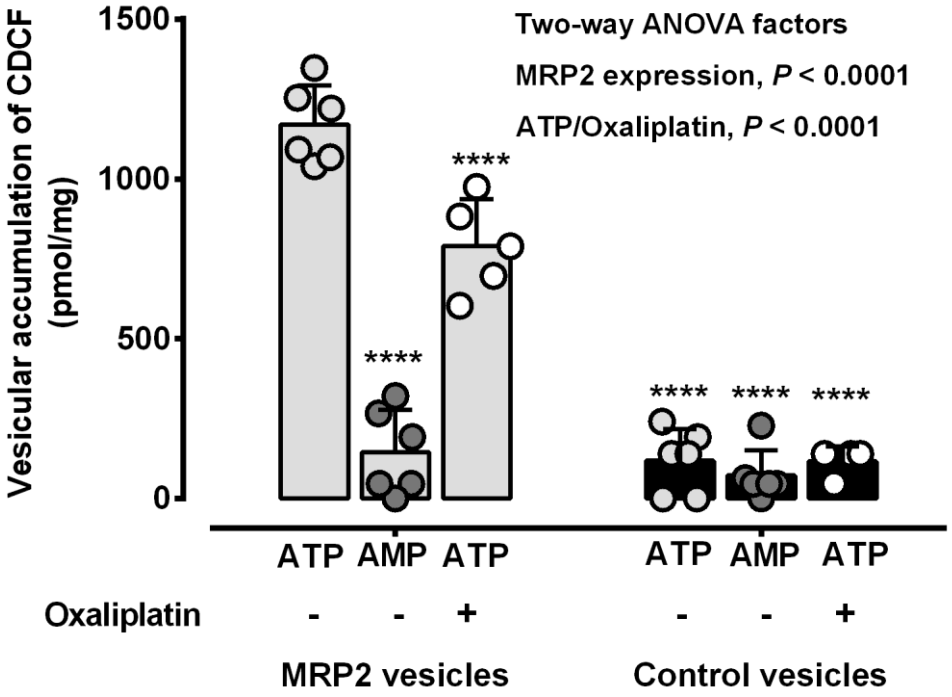


Figure 3.1 Oxaliplatin inhibition of ATP-dependent accumulation of CDCF by MRP2 vesicles

MRP2 and control membrane vesicles were incubated with 5 μM CDCF for 5 minutes in the presence of ATP or AMP, or pre-incubated with 400 μM oxaliplatin for 5 minutes followed by co-incubation with 5 μM CDCF for 5 minutes in the presence of ATP. Membrane vesicle accumulation of CDCF was then measured using fluorescence. Data were presented as the mean (bar) and standard deviation (error bar) of individual values (circles) pooled from two independent experiments. Data were analysed by two-way ANOVA and Tukey's multiple comparisons post-tests. Data showed higher accumulation of CDCF in MRP2-expressing membrane vesicles in the presence of ATP than in the presence of AMP or in control vesicles (**** $P < 0.0001$; Tukey's multiple comparison post-tests following two-way ANOVA). Oxaliplatin inhibited the ATP-dependent accumulation of CDCF by MRP2 expressing membrane vesicles without altering the accumulation of CDCF by control membrane vesicles. Bars: grey, MRP2-expressing membrane vesicles; black, control membrane vesicles. Circles: light grey, in the presence of ATP; dark grey, in the presence of AMP; white, in the presence of oxaliplatin.

Table 3.2 Oxaliplatin inhibition of ATP-dependent accumulation of CDCF by MRP2 vesicles (tabulated data)

	Vesicular accumulation (pmol/mg of protein)					
Vesicles	**MRP2**			**Control**		
ATP/AMP	**ATP**	**AMP**	**ATP**	**ATP**	**AMP**	**ATP**
Oxaliplatin	-	-	+	-	-	+
Mean	1170	145	789	119	72.0	116
Standard deviation	122	133	147	99.4	79.5	46.4
***P*-value[#]**		<0.001	<0.001	<0.001	<0.001	<0.001

P-values for comparison to MRP2 with ATP (Tukey's multiple comparison post-tests following two-way ANOVA)

accumulation in MRP2 expressing membrane vesicles was reduced by 1.48-fold (1170 ± 122 vs 789 ± 147 pmol of CDCF per mg of protein; P <0.0001, Tukey's multiple comparisons post-tests following two-way ANOVA). In the control membrane vesicles, oxaliplatin did not affect the CDCF accumulation even in the presence of ATP (119 ± 99.4 vs 116 ± 46.4 pmol of CDCF per mg of protein).

3.3.2. *Accumulation of oxaliplatin-derived platinum*

3.3.2.1. Time-dependence

To investigate the role of MRP2 in the transport of oxaliplatin-derived platinum, MRP2 and control membrane vesicles were incubated with 100 µM of oxaliplatin in the presence of ATP or AMP for 5, 10 or 20 min, followed by measurement of the levels of platinum associated with the vesicles by ICP-MS (Figure 3.2 and Table 3.2).

The membrane vesicle accumulation of platinum increased with increasing time of oxaliplatin exposure (P < 0.0001, two-way ANOVA). Platinum accumulation in MRP2 vesicles in the presence of ATP increased approximately linearly at a rate of 119 ± 18.5 pmol per mg of protein per min oxaliplatin exposure time, from 75.7 ± 31.2 pmol per mg of protein after 5 min, to 971 ± 110 pmol per mg of protein after 10 min before reaching 1920 ± 672 pmol per mg of protein after 20 min exposure (P<0.0001). Platinum accumulation in MRP2 membrane vesicles in the presence of AMP, and in control membrane vesicles with ATP or AMP, also increased approximately linearly with increasing oxaliplatin incubation time (P<0.05) but with slower overall rates of platinum accumulation than that observed in MRP2 membrane vesicles in the presence of ATP.

After 5 min incubation with oxaliplatin, there were no differences in the levels of platinum accumulation in MRP2 or control membrane vesicles, or in the presence of ATP

or AMP. Platinum levels in MRP2-expressing membrane vesicles were 75.7 ± 31.2 and 34.1 ± 35.1 pmol per mg of protein in the presence of ATP or AMP, respectively. Platinum levels in control membrane vesicles were 18.2 ± 24.5 and 37.7 ± 29.2 pmol per mg of protein in the presence of ATP or AMP, respectively. None of these differences were statistically significant (two-way ANOVA).

After 10 minutes incubation with oxaliplatin, higher levels of platinum had accumulated in MRP2-expressing membrane vesicles in the presence of ATP than in the presence of AMP or in control membrane vesicles. The level of platinum accumulation in MRP2-expressing membrane vesicles in the presence of ATP (971 ± 110 pmol per mg of protein) was 4-fold higher than that in MRP2-expressing membrane vesicles in the presence of AMP (241 ± 96.8 pmol per mg of protein; $P < 0.001$, Tukey's multiple comparison post-test following two-way ANOVA). The level of platinum accumulation in MRP2-expressing membrane vesicles in the presence of ATP (971 ± 110 pmol per mg of protein) was approximately 16-fold higher than that in control membrane vesicles either with ATP (60.4 ± 72.4 pmol per mg of protein; $P < 0.001$, Tukey's multiple comparison post-test following two-way ANOVA) or with AMP (51.3 ± 68.4 pmol per mg of protein; $P < 0.001$, Tukey's multiple comparison post-test following two-way ANOVA).

After 20 minutes incubation with oxaliplatin, higher levels of platinum again had accumulated in MRP2-expressing membrane vesicles in the presence of ATP than with AMP or in control membrane vesicles. The level of platinum accumulation in MRP2-expressing membrane vesicles in the presence of ATP (1920 ± 672 pmol per mg of protein) was 4.5-fold higher than that in MRP2-expressing membrane vesicles in the presence of AMP (428 ± 340 pmol per mg of protein; $P < 0.0001$, Tukey's multiple comparison post-test following two-way ANOVA). The level of platinum accumulation

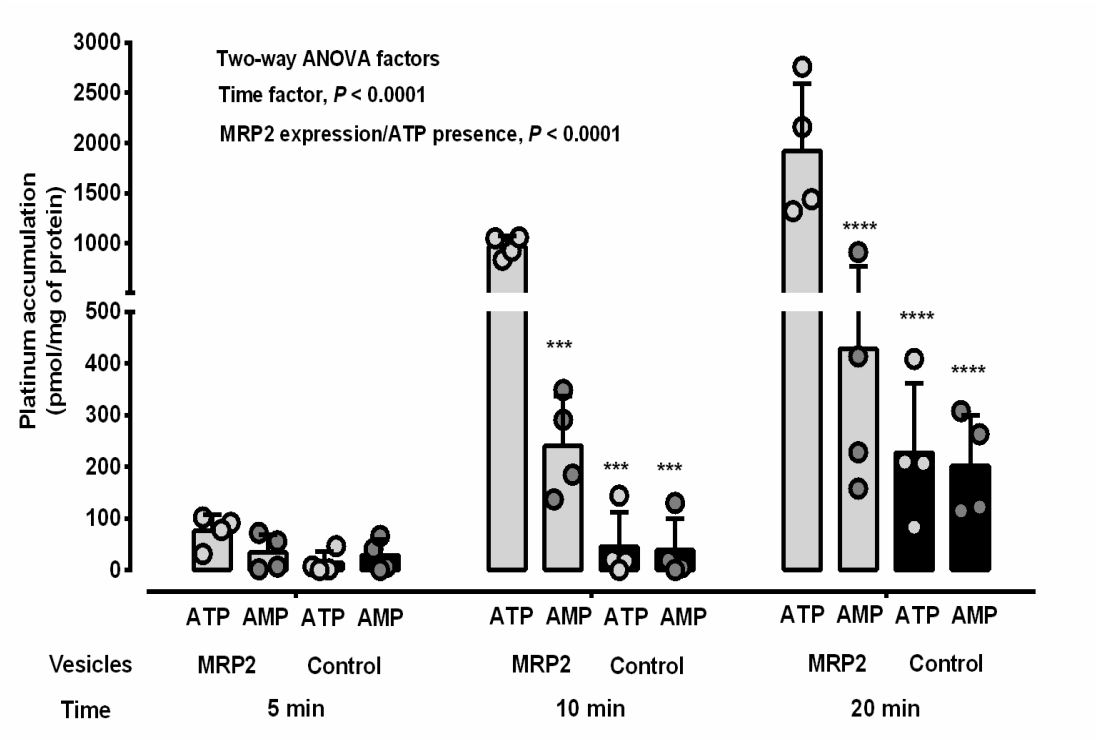


Figure 3.2 Accumulation of oxaliplatin-derived platinum in membrane vesicles: dependence upon oxaliplatin exposure time, presence of ATP and MRP2 expression

MRP2 and control membrane vesicles were incubated with 100 μM oxaliplatin in the presence of ATP or AMP for 5, 10 or 20 min followed by measurement of the levels of platinum accumulated in the vesicles by ICP-MS. Data were presented as the mean (bar) and standard deviation (error bar) of individual values (open circles) pooled from two independent experiments. Data were analysed using two-way ANOVA and Tukey's multiple comparison post-tests. Data showed increased membrane vesicle accumulation of platinum with increasing time of oxaliplatin exposure ($P < 0.0001$, two-way ANOVA). After five minutes oxaliplatin exposure, there were no differences in the levels of platinum accumulation in MRP2 or control membrane vesicles, or in the presence or of ATP or AMP. However, after 10 or 20 minutes oxaliplatin exposure, higher levels of platinum accumulated in MRP2 membrane vesicles in the presence of ATP compared to in the presence of AMP or in control vesicles (*** $P < 0.001$ and **** $P < 0.0001$; Tukey's multiple comparison post-tests following two-way ANOVA). Bars: light grey, MRP2 expressing membrane vesicles with ATP; dark grey, MRP2 expressing membrane vesicles with AMP; black, control membrane vesicles.

Table 3.3 Accumulation of oxaliplatin-derived platinum in membrane vesicles: dependence upon oxaliplatin exposure time, presence of ATP and MRP2 expression (tabulated data)

	Platinum accumulation (pmol/mg of protein)											
Time	**5 min**				**10 min**				**20 min**			
Vesicles	**MRP2**		**Control**		**MRP2**		**Control**		**MRP2**		**Control**	
ATP/AMP	**ATP**	**AMP**	**ATP**	**AMP**	**ATP**	**AMP**	**ATP**	**AMP**	**ATP**	**AMP**	**ATP**	**AMP**
Experiment 1	102	55.7	1.29	40.5	836	291	144	130	1440	158	83.3	308
	91.6	72.1	46.3	66.1	926	349	19.9	18.4	1320	414	409	263
Experiment 2	77.8	6.81	6.95	6.53	1070	185	17.4	5.54	2760	912	207	122
	31.3	1.7			1050	137			2160	228	209	115
Mean	75.7	34.1	18.2	37.7	971	241	60.4	51.3	1920	428	227	202
Standard deviation	31.2	35.1	24.5	29.9	110	96.8	72.4	68.4	672	340	135	98.2
***P*-value[#]**		NS	NS	NS		<0.001	<0.001	<0.001		<0.0001	<0.0001	<0.0001

#*P*-values for comparison to MRP2 with ATP (Tukey's multiple comparison post-tests following two-way ANOVA).

in MRP2-expressing membrane vesicles in the presence of ATP (1920 ± 672 pmol per mg of protein) was approximately 8.5-fold higher than that in control membrane vesicles either with ATP (227 ± 135 pmol per mg of protein; $P < 0.0001$, Tukey's multiple comparison post-test following two-way ANOVA) or with AMP (202 ± 98.2 pmol per mg of protein; $P < 0.0001$, Tukey's multiple comparison post-test following two-way ANOVA).

3.3.2.2. Concentration-dependence

To investigate the concentration-dependence of MRP2-mediated transport of oxaliplatin-derived platinum, MRP2-expressing membrane vesicles were incubated with oxaliplatin at different concentrations in the presence of ATP or AMP for 10 min followed by measurement of the levels of platinum accumulated in the membrane vesicles by ICP-MS (Figure 3.3 and Table 3.3).

Platinum accumulation in MRP2 vesicles increased with increasing oxaliplatin exposure concentration ($P < 0.0001$, two-way ANOVA), and in the presence of ATP ($P < 0.0001$, two-way ANOVA). In the presence of ATP, averaged platinum accumulation values increased progressively from 100 to 23.5, 155, 508, 802, 1500 and 1780 pmol per mg of protein with increasing oxaliplatin exposure concentrations from 6.25 to 12.5, 25, 50, 100, 200 and 400 µM, respectively. In the presence of AMP, averaged platinum accumulation values increased from 14.1 to 20.4, 211 and 301 pmol per mg of protein with increasing oxaliplatin exposure concentration from 6.25 to 25, 100 and 400 µM, respectively.

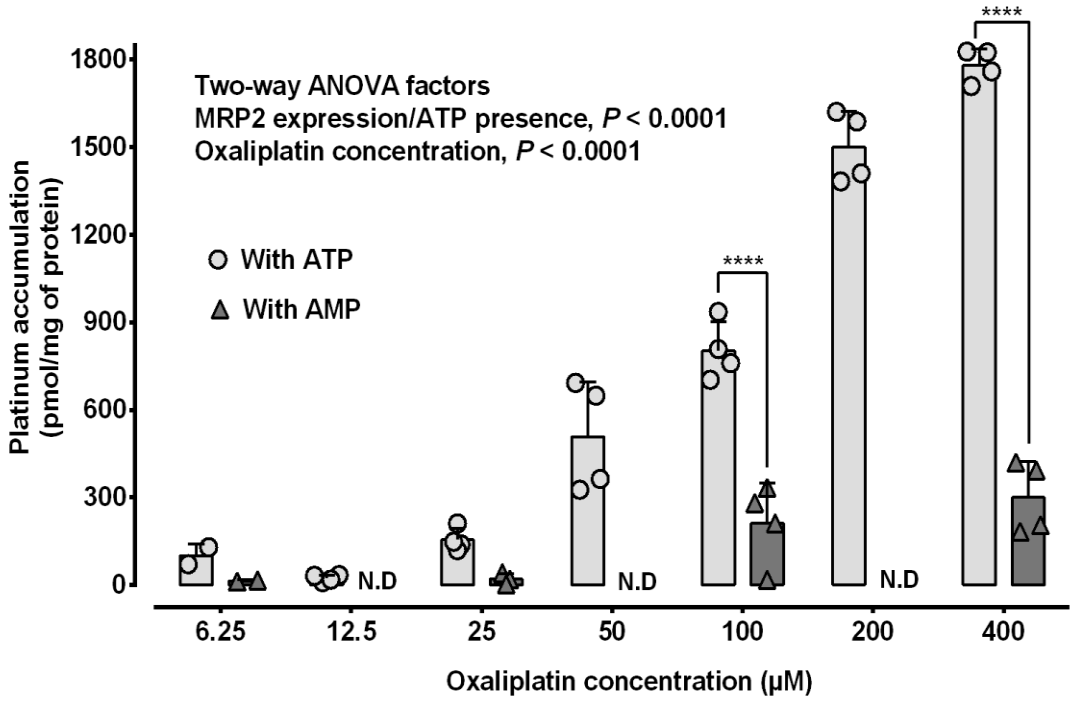


Figure 3.3 Accumulation of oxaliplatin-derived platinum in MRP2-expressing membrane vesicles: Concentration- and ATP-dependence

MRP2-expressing membrane vesicles were incubated with oxaliplatin at different concentrations in the presence of ATP or AMP for 10 min followed by measurement of the levels of platinum accumulated in membrane vesicles by ICP-MS. Data were presented as the mean (bar) and standard deviation (error bar) of individual values (symbols) combined from two independent experiments. Data were analysed by two-way ANOVA followed by Tukey's multiple comparison post-tests. Data showed increased membrane vesicle accumulation of platinum with increasing oxaliplatin exposure concentration ($P < 0.0001$, two-way ANOVA), and in the presence of ATP ($P < 0.0001$, two-way ANOVA). At 6.25 and 25 μM oxaliplatin exposure concentrations, trends for higher platinum accumulation in the presence of ATP did not reach statistical significance. At oxaliplatin exposure concentrations of 100 and 400 μM, higher levels of platinum accumulated in the presence of ATP than in the presence of AMP ($P<0.0001$; Tukey's multiple comparison post-tests following two-way ANOVA; ****). N.D (not done). Bars and symbols: light grey/circles, with ATP; dark grey/triangles, with AMP.

Table 3.4 Accumulation of oxaliplatin-derived platinum in MRP2 expressing membrane vesicles: Concentration- and ATP-dependence (tabulated data)

	Platinum accumulation (pmol/mg of protein)													
Oxaliplatin concentration (μM)	**6.25**		**12.5**		**25**		**50**		**100**		**200**		**400**	
ATP/AMP	**ATP**	**AMP**	**ATP**	**AMP**	**ATP**	**AMP**	**ATP**	**AMP**	**ATP**	**AMP**	**ATP**	**AMP**	**ATP**	**AMP**
Experiment 1	129	11.8	9.52		139	40.3	692		760	18.2	1410		1830	182
	71.4	16.3	34.1		120	18.8	649		936	212	1380		1830	420
Experiment 2			18.8		211	2.16	326		809	334	1590		1760	394
			31.5		148		363		703	281	1620		1710	206
Mean	100	14.1	23.5		155	20.4	508		802	211	1500		1780	301
Standard deviation	40.7	3.18	11.5		39.4	19.1	190		99.3	138	122		58.5	124
***P*-value[#]**	N.S				N.S				<0.0001				<0.0001	

#*P*-values were for the comparison of accumulation values with and without ATP at each respective oxaliplatin concentration (Tukey's multiple comparison post-tests following two-way ANOVA).

After oxaliplatin exposure at concentrations of 6.25 µM and 25 µM, there were trends for higher platinum accumulation in MRP2-expressing membrane vesicles in the presence of ATP but these did not reach statistical significance. Averaged platinum accumulation values at an oxaliplatin exposure concentration of 6.25 µM were 7.1-fold higher in the presence of ATP compared to with AMP (100 versus 14.1 pmol per mg of protein) although this difference did not reach statistical significance (Tukey's multiple comparison post-test following two-way ANOVA). Averaged platinum accumulation values at an oxaliplatin exposure concentration of 25 µM were 7.6-fold higher in the presence of ATP compared to with AMP (155 versus 20.4 pmol per mg of protein) although this difference also did not reach statistical significance (Tukey's multiple comparison post-test following two-way ANOVA).

After oxaliplatin exposure at concentrations of 100 and 400 µM, there was significantly higher platinum accumulation in MRP2-expressing membrane vesicles in the presence of ATP than in the presence of AMP. Averaged platinum accumulation values at an oxaliplatin exposure concentration of 100 µM were 3.8-fold higher with ATP compared to with AMP (802 ± 99.3 versus 211 ± 138 pmol per mg of protein ($P < 0.0001$; Tukey's multiple comparison post-test following two-way ANOVA). Averaged platinum accumulation values at an oxaliplatin exposure concentration of 400 µM were 5.9-fold higher with ATP compared to with AMP (1780 ± 58.5 versus 301 ± 124 pmol per mg of protein; $P < 0.0001$; Tukey's multiple comparison post-test following two-way ANOVA).

3.3.2.3. Kinetic analysis

To investigate the kinetics of the MRP2-mediated ATP-dependent transport of oxaliplatin-derived platinum, rates of ATP-dependent MRP-mediated membrane

vesicle accumulation of platinum were derived by subtracting the rate of platinum accumulation in the presence of AMP from the rate of platinum accumulation in the presence of ATP, for each oxaliplatin exposure concentration. Values for the rate of platinum accumulation in the presence of AMP, for the oxaliplatin exposure concentrations of 12.5, 50 and 200 µM, were calculated by interpolation from the adjacent measured values. The derived rates of ATP-dependent MRP-mediated platinum transport were then plotted against oxaliplatin exposure concentration and fitted to a non-linear model (Figure 3.4 and Table 3.4).

The rate of MRP2-mediated ATP-dependent transport of oxaliplatin-derived platinum increased non-linearly with increasing oxaliplatin concentration in approximation with Michaelis-Menten kinetics. A Michaelis-Menten model had a regression fit to the data with an r^2 of 0.954. The rate of platinum accumulation increased nonlinearly with increasing oxaliplatin exposure concentration approaching a plateau value of 2680 (95% CI, 2010 to 3360) pmol per mg of protein per 10 min (Figure 3.4). The oxaliplatin concentration achieving half-maximal rate for platinum accumulation was 301 µM (95% CI, 163 to 438 µM).

3.3.2.4. Effects of MRP2 inhibitors

To investigate effects of MRP2 inhibitors, MRP2-expressing and control membrane vesicles were incubated with 100 µM oxaliplatin and ATP, with or without myricetin (10, 30, 100 and 300 µM) or MK571 (100 µM) for 10 minutes followed by measurement of the accumulated levels of platinum by ICP-MS (Figure 3.5 and Table 3.5). Previously, both myricetin and MK571 had been shown to inhibit MRP2 transporter activity at the concentrations selected for use in these experiments (266, 271, 274-276). Myricetin did not contain sulfhydryl groups that could bind platinum and thereby potentially confound

these transporter inhibition experiments. On the other hand, MK571 contained sulfhydryl groups, however the incubation time was limited only for 10 minutes, suggesting that the amount of oxaliplatin metabolised from the reaction with sulfhydryl groups would not be high.

Platinum accumulation was higher in MRP2-expressing membrane vesicles in the absence of MRP2 inhibitors compared to in the presence of MRP2 inhibitors ($P = 0.0153$, two-way ANOVA) or control membrane vesicles ($P = 0.0039$, two-way ANOVA). After 10 min exposure to 100 µM of oxaliplatin in the presence of ATP without MRP2 inhibitors, platinum accumulation was 1490 ± 865 pmol of platinum per mg of protein, which was between 1.6- to 22.8-fold higher than the levels of platinum accumulation in MRP2-expressing membrane vesicles in the presence of MRP2 inhibitors ($P = 0.0153$, two-way ANOVA), and between 3.0- to 8.7-fold higher than the levels of platinum accumulating in control membrane vesicles ($P = 0.0039$, two-way ANOVA).

In MRP2-expressing membrane vesicles, platinum accumulation was decreased by concurrent treatment with myricetin or MK571. The inhibitory effect exerted by myricetin on the MRP2-mediated accumulation of platinum derived from oxaliplatin appeared to be increased with increasing myricetin concentration. After 10 min exposure to 100 µM of oxaliplatin without myricetin in the presence of ATP, platinum accumulated in MRP2-expressing membrane vesicles was 1490 ± 865 pmol of platinum per mg of protein. With co-treatment of 10, 30, 100 and 300 µM myricetin, platinum accumulation in MRP2 vesicles was diminished by 39 % (916 ± 197 pmol of platinum per mg of protein; $P > 0.05$), 55 % (672 ± 685 pmol of platinum per mg of protein; $P < 0.05$), 68% (479 ± 324 pmol of platinum per mg of protein; $P < 0.001$) and 85 % (219 ± 161 pmol of platinum per mg of protein; $P < 0.001$), respectively (P values, Tukey's multiple comparison post-tests following two-way ANOVA). With MK571 co-treatment

at 100 µM, platinum accumulation in MRP2 vesicles was reduced to 96 % (65.4 ± 14.5 pmol of platinum per mg of protein; $P < 0.01$, Tukey's multiple comparison post-test

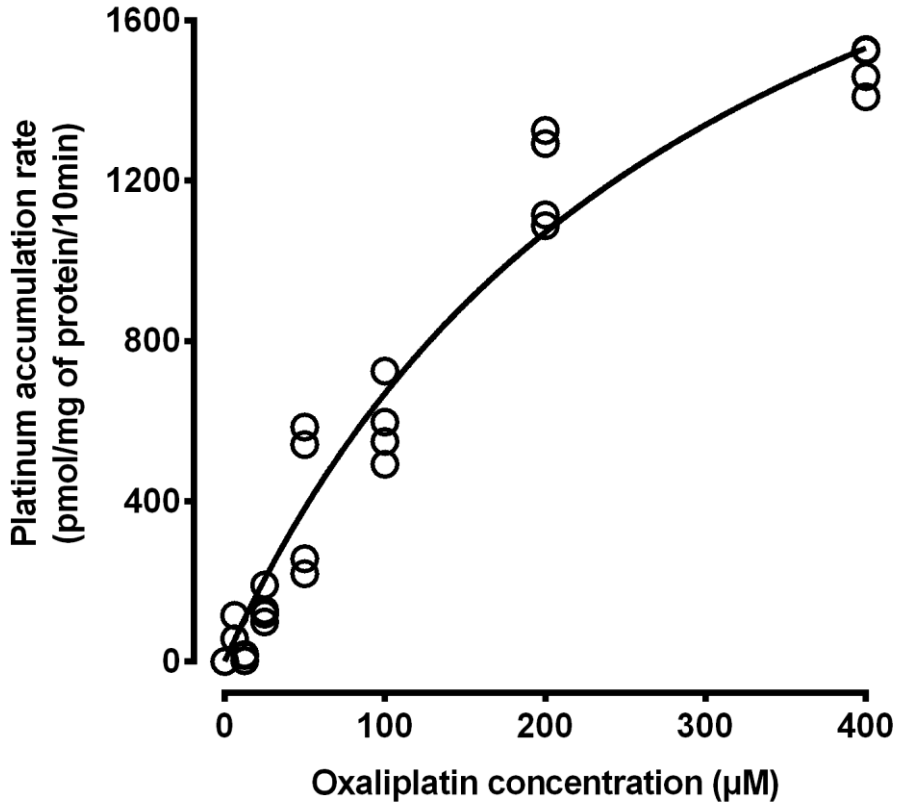


Figure 3.4 Kinetic analysis of MRP2-mediated ATP-dependent transport of oxaliplatin-derived platinum

Rates of MRP-mediated ATP-dependent of accumulation of oxaliplatin-derived platinum were calculated from data shown in Figure 3.3 and Table 3.2 by subtraction of the rate of platinum accumulation in the presence of AMP from the rate of platinum accumulation in the presence of ATP, at each oxaliplatin exposure concentration. Values for the rate of platinum accumulation in the presence of AMP, for the oxaliplatin exposure concentrations of 12.5, 50 and 200 μM, were calculated by interpolation from the adjacent measured values. Data were presented as individual values (circles) from two independent experiments, and a regression fit to the data (solid line) using a non-linear model whose parameters are shown in the accompanying table. Data showed that the rate of MRP2-mediated ATP-dependent transport of oxaliplatin-derived platinum increased nonlinearly with increasing oxaliplatin exposure concentration in approximation (r^2 = 0.954) with Michaelis-Menten kinetics with a Vmax of 2680 pmol per mg of protein per 10 min (95%CI, 2010 to 3360) and Km of 301 μM (95%CI, 163 to 438).

Table 3.5 Kinetic analysis of MRP2-mediated ATP-dependent transport of oxaliplatin-derived platinum (tabulated data)

Platinum accumulation (pmol/mg of protein)							
Oxaliplatin concentration (μM)	**6.25**	**12.5**	**25**	**50**	**100**	**200**	**400**
Experiment 1	115	17.9	119	585	549	1110	1530
	57.4		99.1	542	725	1090	1520
Experiment 2		2.56	191	219	598	1290	1460
		15.3	128	256	492	1330	1410
Mean	86.2	11.9	134	401	591	1210	1480
Standard deviation	40.7	8.21	39.7	190	99.3	123	56

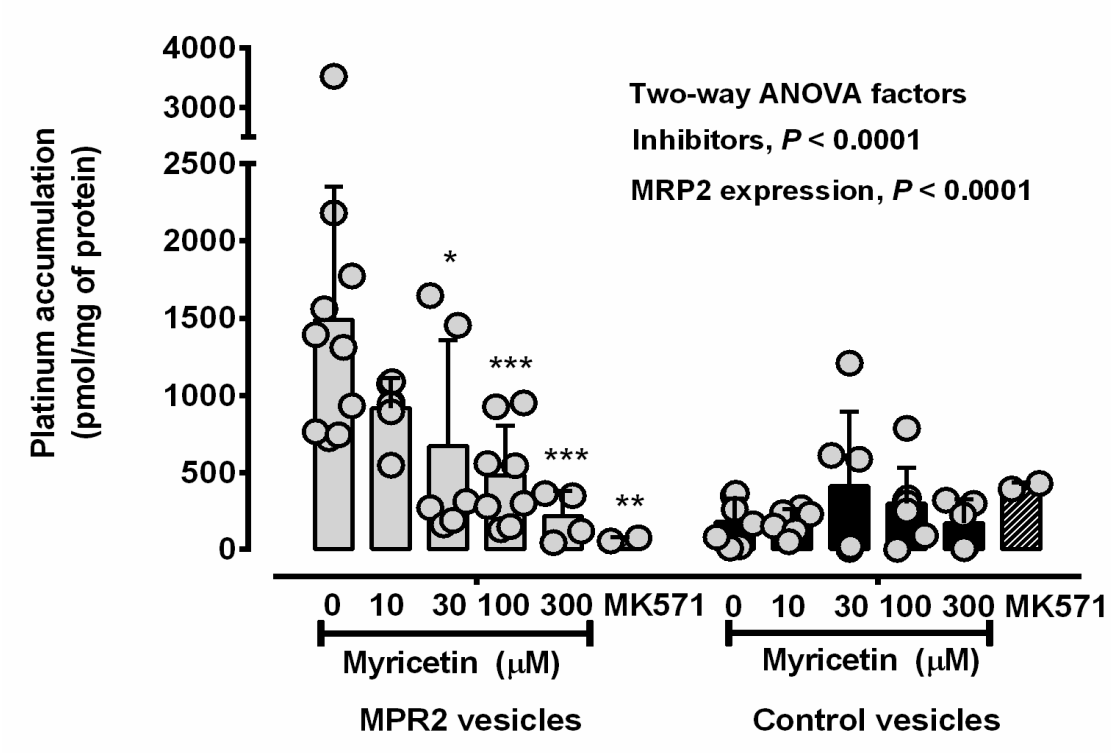


Figure 3.5 Effects of MRP2 inhibitors on the accumulation of oxaliplatin-derived platinum in membrane vesicles

MRP2-expressing and control membrane vesicles were incubated with 100 μM oxaliplatin and ATP, with or without myricetin (10, 30, 100 and 300 μM) or MK571 (100 μM) for 10 minutes followed by measurement of the accumulated levels of platinum with ICP-MS. Data were presented as the mean (bar) and standard deviation (error bar) of individual values (open circles) combined from at least two independent experiments. Data were analysed by two-way ANOVA followed by Tukey's multiple comparison post-tests. Data showed higher platinum accumulation in MRP2-expressing membrane vesicles in the absence of MRP2 inhibitors than in the presence of MRP2 inhibitors (P=0.0153, two-way ANOVA) or in control membrane vesicles (P=0.0039, two-way ANOVA). In MRP2-expressing membrane vesicles, platinum accumulation was decreased by concurrent treatment with myricetin at 30, 100 and 300 μM and by MK571 (100μM) (*, P<0.05; **, P <0.01; ***, P < 0.001; Tukey's multiple comparison post-tests following two-way ANOVA). The inhibitory effect of myricetin on MRP2-mediated platinum accumulation increased with increasing myricetin concentration. Platinum accumulation in control membrane vesicles was unchanged by MRP2 inhibitors.

Table 3.6 Effects of MRP2 inhibitors on the accumulation of oxaliplatin-derived platinum in membrane vesicles (tabulated data)

Myricetin concentration (µM)	Membrane vesicle platinum accumulation (pmol per mg of protein; mean ± SD)			
	MRP2 vesicles	*P*-value*	Control vesicles	*P*-value*
0 (Oxaliplatin 100 µM alone)	1490 ± 865	-	178 ± 150	-
10	916 ± 197	N.S	177 ± 83.6	N.S
30	672 ± 685	<0.05	491 ± 492	N.S
100	479 ± 324	<0.001	338 ± 214	N.S
300	219 ± 161	<0.001	172 ± 153	N.S
Oxaliplatin 100 µM + MK571 100µM	65.4 ± 14.5	<0.01	410 ± 25.2	N.S

* *P*-values were from Tukey's multiple comparison post-tests following two-way ANOVA for comparisons with oxaliplatin alone.

following two-way ANOVA). Neither myricetin nor MK571 exerted significant inhibitory effects on platinum accumulation in control membrane vesicles.

3.3.3. *Stability of oxaliplatin in membrane vesicle incubation buffer*

To investigate the stability of oxaliplatin in membrane vesicle incubation buffer, HPLC was used to detect glutathione, oxaliplatin and its dichloro degradation product ($Pt(DACH)Cl_2$) in membrane vesicle incubation buffer by UV detection. Representative chromatograms are shown in Figure 3.6. Injection of authentic standards in membrane vesicle induction buffer onto the HPLC column showed chromatographic retention times for glutathione, oxaliplatin and $Pt(DACH)Cl_2$ of 6.5 min, 12.5 min and 10.5 min, respectively. Injection of blank membrane vesicle incubation buffer showed little or no chromatographic peaks from components of the membrane vesicle incubation buffer interfering with detection of glutathione, oxaliplatin or $Pt(DACH)Cl_2$.

To generate incubation samples, oxaliplatin (100 μM) was added to membrane vesicle incubation buffer, and then incubated at 37°C and pH 7.4. Samples of incubation fluid were taken at various times during incubation for analysis by HPLC-UV chromatography. In Figure 3.6, representative chromatograms are shown comparing the incubation of oxaliplatin in membrane vesicle incubation buffer containing glutathione with membrane vesicle incubation buffer not containing glutathione. After 20 minutes incubation, HPLC-UV chromatograms of samples incubated with or without glutathione appeared similar, and showed only slight reductions in the oxaliplatin peaks and no new chromatographic peaks. With further incubation time, the oxaliplatin peaks reduced progressively in size, more rapidly in the presence of glutathione than without glutathione. In addition, with further incubation, new chromatographic peaks appeared corresponding to $Pt(DACH)Cl_2$,

which appeared in the membrane vesicle incubation buffer not containing glutathione, and an unknown compound with a chromatographic peak at 9.5 min, which appeared in the membrane vesicle incubation buffer containing glutathione.

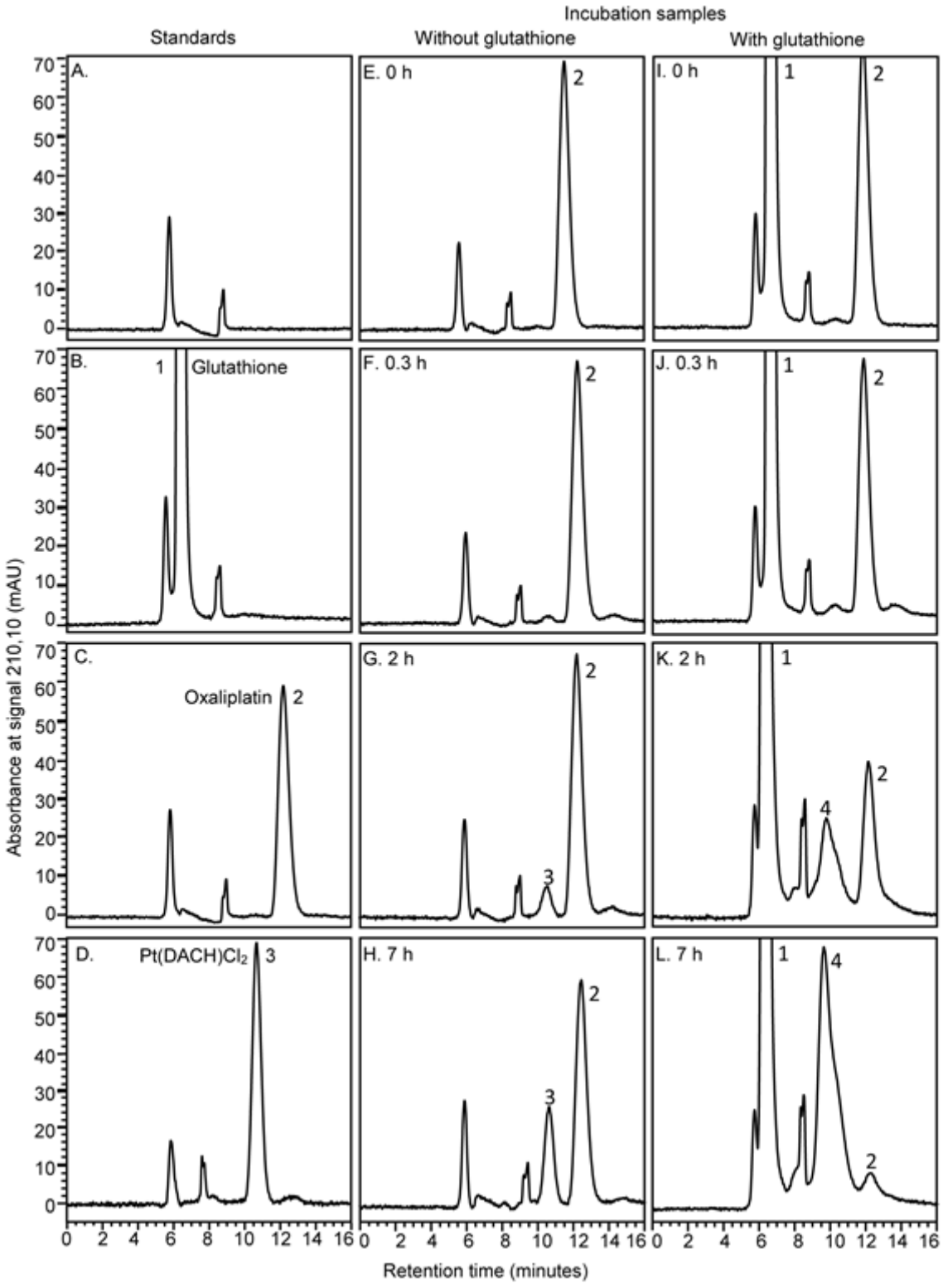


Figure 3.6 HPLC-UV chromatograms of standards and samples of membrane vesicle incubation buffer

(A-D) Retention times of glutathione (B, peak 1, 6.5 min), oxaliplatin (C, peak 2, 12.5 min) and $Pt(DACH)Cl_2$ (D, peak 3, 10.5 min) were determined by injection of authentic standards in membrane vesicle incubation buffer. Injection of blank membrane vesicle incubation buffer alone is shown in (A).

(E-L) Oxaliplatin (100 μM) was added to membrane vesicle incubation buffer with (I-L) or without glutathione (2 mM) (E-H), then incubated at 37°C and pH 7.4. Samples of incubation fluid were taken after 0 (E,I), 0.3 (F,J), 2 (G,K) and 7 hours (H,L) incubation and analysed by HPLC-UV. Data were presented as representative chromatograms that were reproducible from at least two independent experiments.

Data showed chromatographic separation of glutathione, oxaliplatin and $Pt(DACH)Cl_2$ without interfering peaks from components of membrane vesicle incubation buffer (A-D). After 20 minutes incubation, chromatograms for samples with (J) or without glutathione (F) appeared similar and showed only slight reductions in the oxaliplatin peaks without any new chromatographic peaks. With further incubation time, the oxaliplatin peak reduced progressively in size, more rapidly with glutathione (K,L) than without glutathione (G,H). In addition, new peaks appeared with further incubation corresponding to $Pt(DACH)Cl_2$ (G,H; peak 3, 10.5 min) and an unknown compound that appeared in the presence of glutathione (K,L; peak 4, 9.5 min).

To further investigate the stability of oxaliplatin in membrane vesicle incubation buffer, the kinetics of the loss of oxaliplatin in membrane vesicle buffer containing glutathione were studied. Oxaliplatin (100 μM) was incubated in membrane vesicle incubation buffer containing 2 mM glutathione at 37°C and pH 7.4. Samples of membrane vesicle incubation buffer were taken at different time points for analysis by HPLC-UV chromatography. Oxaliplatin chromatographic peak areas were expressed as a percentage of oxaliplatin chromatographic peak area at the start of the incubation (0 minutes) and plotted versus time (Figure 3.7). A non-linear one-phase exponential decay model was fitted to the data. The curve fit was used to interpolate values for estimating the amount of loss of oxaliplatin occurring after 10 minutes incubation time. Oxaliplatin was lost from membrane vesicle incubation buffer containing glutathione with a degradation half-life of 2.24 hrs (95% CI, 2.08 to 2.43 hrs). After 10 minutes incubation, 95.0% (95% CI, 95.4 to 94.6%) of the oxaliplatin that was added to the membrane vesicle incubation buffer had still remained intact.

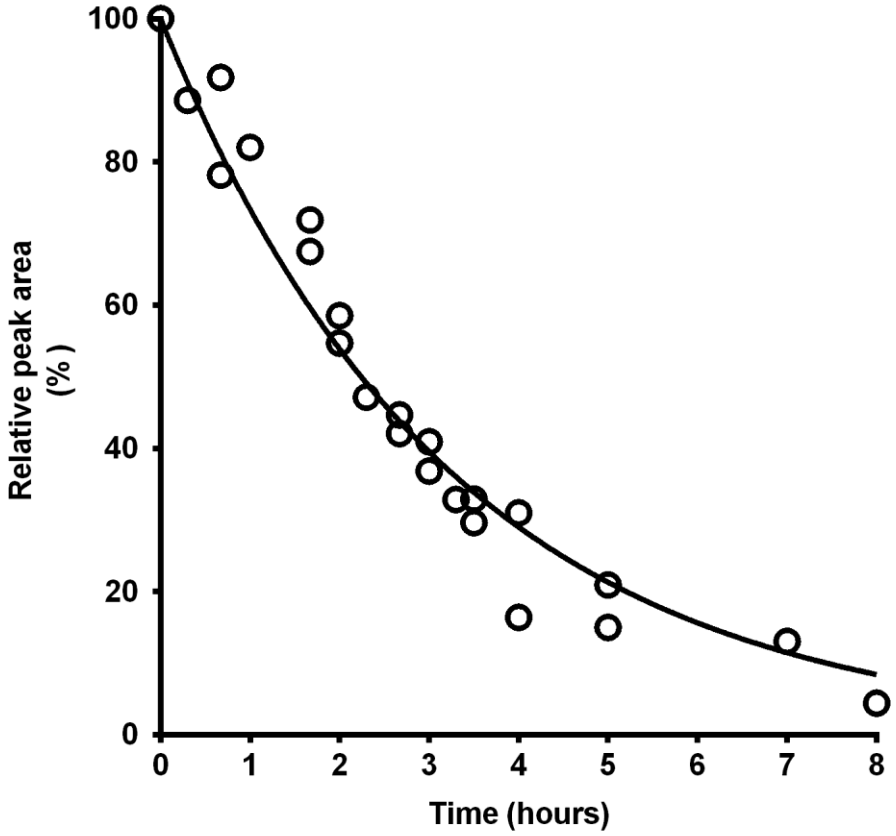


Figure 3.7 Kinetic analysis of oxaliplatin degradation in membrane vesicle incubation buffer

Oxaliplatin (100 μM) was incubated in membrane vesicle incubation buffer containing 2 mM glutathione at 37°C and pH 7.4. Samples of membrane vesicle incubation buffer were taken at different time points for analysis by HPLC-UV chromatography. Oxaliplatin chromatographic peak areas were expressed as a percentage of oxaliplatin chromatographic peak area at the start of the incubation (0 minutes). Data were presented as individual values pooled from two independent experiments, and a regression fit to those data of a one-phase exponential decay model, whose parameters are shown in the accompanying table. The curve fit was used to interpolate values for estimating the amount of oxaliplatin loss occurring after 10 min incubation. Data showed the loss of oxaliplatin in membrane vesicle incubation buffer containing glutathione occurring with a degradation half-life of 2.24 hrs (95% CI, 2.08 to 2.43 hrs). After 10 minutes incubation, 95.0% (95% CI, 95.4 to 94.6%) of the oxaliplatin that was added to the membrane vesicle incubation buffer had still remained intact.

Table 3.7 Kinetic analysis of oxaliplatin degradation in membrane vesicle incubation buffer (Tabulated data)

Time (hour)	Relative peak area (% of initial peak area)	
	Oxaliplatin with glutathione	
	Experiment 1	Experiment 2
0.00	100	100
0.30	88.6	
0.67	91.8	78.1
1.00	82.0	
1.67	71.9	67.5
2.00	54.7	58.6
2.30		47.1
2.67	44.7	42.1
3.00	40.9	36.8
3.30		32.8
3.50	32.9	29.6
4.00	31.0	16.4
5.00	20.9	15.0
7.00	13.0	
8.00		4.43

3.4. Discussion

The experiments described in this chapter have generated evidence in support of the involvement of MRP2 in mediating the membrane transport of oxaliplatin-derived platinum. Like other ABC transporters, MRP2 is thought to transport substrates across cell membranes via induction of protein conformational changes from high-affinity binding of substrate, ATP binding and hydrolysis, and dimerization of nucleotide binding domains, which thereby allow a change from a protein with an internally-accessible high-affinity substrate binding site to one with an externally-accessible lower-affinity substrate binding site (277). Our studies used inside-out membrane vesicles expressing human MRP2 protein to investigate the membrane transport of platinum during *in vitro* exposure to oxaliplatin in the presence or absence of ATP, known MRP2 substrates and inhibitors, and in comparison to control vesicles expressing no MRP2 protein. Membrane vesicle accumulation of platinum was found to be higher with expression of MRP2 compared to control vesicles, and in the presence of ATP compared to the absence of ATP with membrane vesicles expressing MRP2. The rate of platinum membrane transport, mediated by MRP2 and ATP, increased non-linearly with increasing oxaliplatin exposure concentration approaching a plateau level, suggesting saturation of oxaliplatin binding sites on the MRP2 protein. Known MRP2 inhibitors (myricetin or MK571) were shown to inhibit the membrane vesicle accumulation of platinum, and the membrane vesicle accumulation of the known MRP2 substrate (CDCF) mediated by MRP2 and ATP was shown to be inhibited by oxaliplatin, suggesting competition by oxaliplatin for the MRP2 substrate binding site. Taken together, these findings provide experimental evidence for oxaliplatin interacting with MRP2 protein and being transported across membranes by MRP2-mediated active transport processes.

Several interesting findings were made about the time-course of platinum accumulation in membrane vesicles in this study. Firstly, platinum accumulation was found to increase in a linear fashion with increasing oxaliplatin exposure time in both MRP2-expressing and control vesicles, and irrespective of ATP. Mechanisms other that ATP-dependent active transport mediated by MRP2 must have been involved in the platinum accumulation that occurred in control membrane vesicles and MRP2 vesicles in the absence of ATP. Oxaliplatin is known for irreversibly binding to sulfur residues in proteins with a half-life of approximately one hour (44, 52). Oxaliplatin may have become bound to membrane vesicle proteins other than MRP2 in this manner, thereby explaining the linear component of platinum association with membrane vesicles that was independent of ATP and MRP2. A second interesting observation was that of a specific time-dependent pattern of platinum accumulation resulting from the ATP-dependent and MRP2-mediated transport process. No differences were found in the membrane vesicle accumulation of platinum between MRP2-expressing and control membrane vesicles, or in the presence of ATP or AMP, after 5 min incubation with oxaliplatin. However, after 10 or 20 min incubation with oxaliplatin, membrane vesicle platinum accumulation was higher by 4- to 16-fold in MRP2-expressing vesicles in the presence of ATP compared to the presence of AMP or compare to control membrane vesicles. These findings suggested that the MRP-mediated ATP-dependent transport of oxaliplatin-derived platinum was delayed in its onset until after the first 5 min of incubation with oxaliplatin. This finding differed from reports of other membrane vesicle studies of other substrates showing almost instantaneous active transport, e.g., 30 sec in case of [^{3}H]leukotriene C4 transport in MRP2-expressing or MRP1-expressing membrane vesicles (217, 218, 263, 268), or radio-labeled substrates in MRP2 vesicles (278, 279). Explanations for the delayed onset in MRP-mediated ATP-dependent transport of oxaliplatin-derived platinum are unclear

and require further study. However, the MRP2 transporter itself was active at this early time point as shown by the ATP-dependent accumulation of CDCF in MRP2-expressing membrane vesicles at 5 min. Alternatively, the delayed onset of MRP2-mediated transport of oxaliplatin-derived platinum may have been due to time-dependent binding of platinum to the transporter protein or time-dependent formation of oxaliplatin-derived degradation products from reactions with chloride or glutathione (39,40), which were present in the membrane vesicle incubation buffer (278, 280, 281). Either of these processes could have explained the delayed onset in MRP2 mediated transport of oxaliplatin-derived platinum.

Previous studies have shown that oxaliplatin is unstable in aqueous solutions containing chloride or glutathione. In chloride-containing solutions, oxaliplatin degrades via oxalate ligand displacement reactions with chloride ions forming a monochloro oxalate ring opened intermediate $[Pt(DACH)oxCl]^-$ and $Pt(DACH)Cl_2$ (57, 247). Reactions between oxaliplatin and glutathione result in the formation of Pt(DACH) glutathione chelates (56). As the membrane vesicle incubation buffer used in this study contained both chloride and glutathione (50 mM MOPS-Tris, 70 mM KCl, 7.5 mM $MgCl_2$ and 2 mM glutathione , pH 7.4), it could not be assumed that oxaliplatin had remained intact under the membrane vesicle incubation conditions or that intact oxaliplatin was the form of platinum actively transported by MRP2. HPLC-UV studies of oxaliplatin stability and degradation in membrane vesicle incubation buffer shed some light on likely candidate substrates for MRP2-mediated transport. Oxaliplatin degraded slowly during its incubation in membrane vesicle buffer, with a degradation half-life of 2.3 hours. Approximately 95% and 90% of the added oxaliplatin had remained intact in the membrane vesicle incubation buffer after 10 and 20 min incubation, respectively, which were the incubation periods used for membrane vesicle transport assays.

Chromatographic peaks corresponding to $Pt(DACH)Cl_2$ or unknown degradation products compounds appeared only after several hours incubation. Although these findings have not identified the substrate for MRP2-mediated transport of oxaliplatin-derived platinum with any certainty, they suggest that intact oxaliplatin may be the substrate transported by MRP2 as it was the most abundant form of platinum present under the experiment conditions in the membrane vesicle incubation buffer. Alternatively, $[Pt(DACH)oxCl]^-$ is formed early during oxaliplatin degradation in chloride-containing solutions (247) and MRP2 is known for preferring anionic substrates (212, 282-284).

MRP2 is physiologically expressed on the apical membranes of hepatocytes, proximial tubules in kidney and enterocytes in intestines where its functions are in the biliary and renal excretion of endogenous and exogenous compounds (204). Previously, using the genetically modified mice, MRP2 expression was shown to be associated with the liver toxicity of cisplatin and methotrexate which are substrates of MRP2 (173, 175). Hence, MRP2 may contribute to the renal and biliary excretion of oxaliplatin, which could be further investigated in MRP2 gene knockout rodent models (285, 286). Variations in the expression level or in the DNA base sequence of *MRP2* were shown to be correlated with resistance to oxaliplatin-induced antitumour effects using human tumour samples or experimental cancer cell lines (74, 206-210). Further studies using MRP2-overexpressing cell lines or human cancer cell lines should be done to determine the functional role of MRP2 in limiting tumour cell accumulation of platinum and sensitivity to oxaliplatin, and the potential of inhibiting MRP2 as a therapeutic strategy for re-sensitizing resistant tumours to chemotherapy treatment.

In conclusion, the studies described in this chapter have demonstrated the ATP-dependent membrane transport of platinum derived by oxaliplatin mediated by MRP2 which was

never shown before. Intact oxaliplatin and its anionic monochloro oxalate ring-opened early degradation product are likely candidates as substrates for MRP2-mediated active transport of oxaliplatin-derived platinum.

Chapter 4. HEK293 cell line studies

4.1. Introduction

In the previous chapter, studies of MRP2 over-expressing and control membrane vesicles were described demonstrating the MRP2- mediated active membrane transport of platinum derived from oxaliplatin. However, the significance of this MRP2-mediated platinum transport process to the cellular accumulation of oxaliplatin, and to oxaliplatin effects on cell growth, was unclear from these membrane vesicle studies. Cell-based models have been used in previous *in vitro* studies of the role of transporters in the pharmacology of anti-cancer drugs (248). Unlike membrane vesicles, cell-based models can be used to study the significance of drug transport mechanisms for determining cellular sensitivity to anti-cancer drugs. Both drug-resistant cancer cell lines and genetically manipulated cell lines over-expressing the membrane transport of interest have been used (122). Cell lines genetically modified to over-express membrane transporter genes give investigators an opportunity to study the activities of the transporter of interest with experimental controls for the effects of other membrane transport mechanisms. HEK293 cells genetically modified to over-express the membrane transporter of interest have been used previously in the transporter studies since they are easy to manipulate and have high efficiency for successful transfection and are easy to grow and maintain (192, 217, 287, 288). Several studies have previously used MRP2 over-expressing cell lines to study its transport mechanisms and substrate specificities (79, 210, 217, 218, 289-292). In the current study, the role of MRP2 in determining the cellular accumulation of platinum and sensitivity of cells to oxaliplatin-induced growth

inhibition was examined using a HEK293 cell line genetically modified to over-express MRP2 (HEK-MRP2) and compared to its untransfected parental cell line (HEK-P cells).

Although there have been several studies associating MRP2 expression with reduced cellular accumulation and cellular resistance to cisplatin using various models, such as MRP2 over-expressing cell lines, human cancer cell lines, animal models and clinical tumor samples (173, 212, 216-225), similar data for oxaliplatin is limited and inconsistent (74, 77, 78, 206, 207, 209, 210). Previous studies have linked cellular resistance to vincristine, etoposide and cisplatin with high expression of MRP2. The expression level of the *MRP2* gene was found to be increased in cisplatin-resistant human cancer cell lines which showed reduced cellular accumulation of cisplatin (212, 293, 294). One study showed that the silencing of *MRP2* in human ovarian cancer cell line increased the cellular sensitivity to cisplatin, as well as cellular accumulation of cisplatin (222). Cui et al. showed cellular resistance to etoposide, cisplatin and doxorubicin was related to the over-expression of MRP2 using MRP2 over-expressing cell lines, including HEK-MRP2 and MDCKII-MRP2 (217). Silencing of *MRP2* in human liver, ovarian and nasopharyngeal cancer cell lines resulted in reduced IC-50 values for the model cytotoxic MRP2 substrates, cisplatin, vincristine and doxorubicin, indicating that MRP2 was associated with cellular resistance to these drugs (216, 221, 222, 225). Several previous studies have used 5(6)-carboxy-2,'7'-dichlorofluorescein (CDCF) as a model substrate to measure the transport activity of MRP2 (268, 280, 295-299). In most of the studies, 5(6)-carboxy-2,'7'-dichlorofluorescein diacetate (CDCFDA) is used since CDCFDA (non-fluorescent precursor of CDCF) can passively diffuse into the cells and is metabolised in the cells to the fluorescent CDCF which is not permeable and can only be transported out of the cells by the MRP2 transporter.

The main objective of the experimental work described in this chapter was to investigate the role of MRP2 in determining the cellular accumulation of oxaliplatin-derived platinum and sensitivity to oxaliplatin-induced growth inhibition using a cell-line stably transfected to overexpress the ABCC2 gene encoding the MRP2 membrane transporter protein (HEK-MRP2 cells). As this cell line had not been well characterized previously, its phenotypic characteristics in comparison to its isogenic untransfected parental line (HEK-P cells) was studied initially, followed by a comparison of HEK-MRP2 and HEK-P cells for their accumulation of oxaliplatin-derived platinum and sensitivity to oxaliplatin-induced growth inhibition, and the inhibitory effects of myricetin on these processes. Exploratory studies investigating the expression of other membrane transporter genes in HEK-MRP2 and HEK-P cells were also undertaken.

4.2. Materials and methods

4.2.1. *Chemicals*

The sources of chemicals used in this study and the preparation details of the stock solutions were as mentioned in section 2.1.

4.2.2. *Cell culture*

HEK293 parental (HEK-P) and human MRP2 over-expressing HEK293 (HEK-MRP2) cell lines used in this study. The sources and cell culture conditions of these cell lines were described in section 2.3.

4.2.3. *Drug treatments*

4.2.3.1. Growth inhibition assays

For the assessment of effects on cell growth of drugs of interest, HEK293 cells were exposed to drugs for designated times, followed by replacement of the drug-containing

medium with the normal growth medium until 72 h after the initial exposure to the drugs. Varying concentrations of drugs and incubation periods were used for different experimental designs as summarized below in Table 4.1.

4.2.3.2. Cellular platinum accumulation studies

For comparison of cellular platinum accumulation, HEK-MRP2 and HEK-P cells were exposed to 200 µM oxaliplatin for increasing incubation periods up to 120 min. The cells were processed after each incubation period for the measurement of oxaliplatin-derived platinum accumulation using ICP-MS.

For studies of the effect of myricetin on the cellular platinum accumulation, HEK293 cells were pre-incubated with myricetin 60 µM for 30 min, followed by co-incubation with myricetin 60 µM and oxaliplatin 200 µM for 1 h or 2 h. As a control, cells were exposed to oxaliplatin 200 µM alone for 1 h or 2 h.

Table 4.1 Drug treatments and incubation times used in growth inhibition experiments

Experiment design	Drug name	Drug concentration	Incubation periods
MRP2 characterisation	Vincristine	0.1 to 300 nM	72 h
	Etoposide	0.1 to 100 µM	
	Cisplatin	0.1 to 1000 µM	
Cellular sensitivity to oxaliplatin	Oxaliplatin	0 to 1000 µM	5 min
		0 to 300 µM	2 h
		0 to 300 µM	72 h
Cytotoxic effects of myricetin alone	Myricetin	0 to 100 µM	2.5 h
Cytotoxic effects of myricetin and oxaliplatin	Myricetin	0 to 100 µM	30 min preincubation and 2 h coincubation
	Oxaliplatin	10 µM	2 h coincubation
Effects of myricetin on IC-50s of oxaliplatin	Myricetin	60 µM	30 min preincubation and 2 h coincubation
	Oxaliplatin	0 to 100 µM	2 h coincubation

4.2.4. *Growth inhibition assay*

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was used to measure the number of viable cells in these experiments, and the details of MTT assay was mentioned in section 2.4. Prior to the growth inhibition experiments, seeding density experiments for each cell line were undertaken to determine the optimal seeding density for further experiments and to check the linearity of OD values detected over the seeding density changes (Figure 4.1). Drug concentrations and incubation times were varied depending on the studies as shown in Table 4.1.

4.2.5. *Platinum accumulation by ICP-MS*

Cells were seeded at 250,000 cells/well in a collagen-coated 6-well plate, and grown in the normal growth medium until the cells become around 80% confluent. The cells were then incubated for the desired time with oxaliplatin dissolved in the incubation buffer. The details of the assay conditions and procedure were as described in section 2.5. In these experiments, the linearity of the calibration curve as measured by r^2 values ranged from 0.983 to 0.999.

The precision and accuracy values for the QCs in all experiments varied from 0.3 % to 15.5 % and from 99.9 % to 87 % of their true values, respectively. The limit of detection (LOD) and lower limit of quantification (LLOQ) were 0.3 ppb and 1 ppb of platinum, respectively. Details of the incubation times and drug concentrations are given in section 4.2.3.

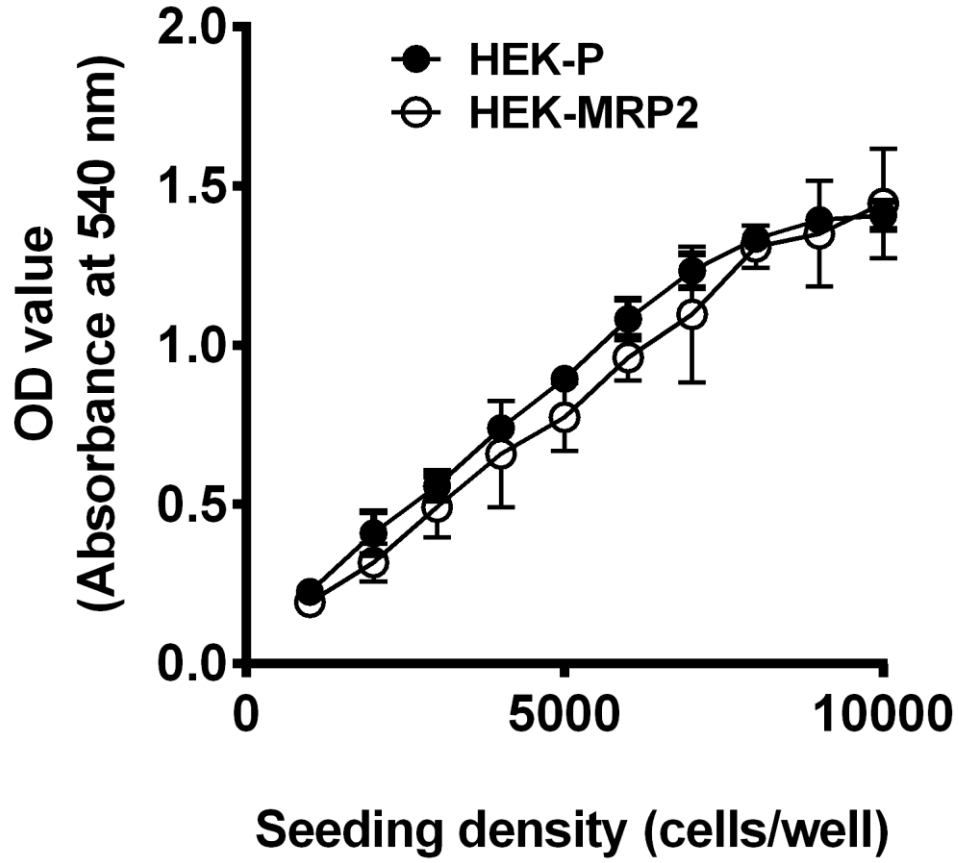


Figure 4.1 Linearity of OD values with increase in seeding density up to 8000 cells/well

HEK293 cells were seeded at a range of different densities (1000 to 10000 cells/well) for four days before the measurement of cellular viability with the MTT assay. The changes in OD values of absorbance detected at 540 nm wavelength over the different seeding densities of cells are shown. OD values increased linearly as the cellular seeding density was increased from 1000 cells/well to 8000 cells/well for both HEK-MRP2 and HEK-P cells. Symbols: closed, HEK-P; open, HEK-MRP2.

4.2.6. *CDCF [5(6)-carboxy-2,'7'-dichlorofluorescein] accumulation and efflux assays*

CDCF was used as a MRP2 substrate to evaluate the transporter activity of MRP2 in HEK-MRP2 and HEK-P cell lines. The non-fluorescent permeable precursor form of CDCF, CDCFDA [5(6)-carboxy-2,'7'-dichlorofluorescein diacetate] was used and the details of the assays could be found in section 2.6.2.

4.2.7. *Immunocytochemistry (ICC)*

Fluorescent immunocytochemistry was undertaken to detect cellular MRP2 protein expression using a primary antibody, anti-MRP2 (1:100; ab3373 from Abcam), and a secondary antibody, Alexa Flour 594-labeled anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). The details were as mentioned in section 2.8.

4.2.8. *Real-time quantitative polymerase chain reaction (qPCR)*

Extraction of RNA from HEK293 cells was undertaken using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol as described in section 2.9.1. Total RNA obtained was then primed with the oligonucleotides, or primers, catalysed by a reverse transcriptase enzyme to produce cDNA (complementary DNA) using a RT^2HT First strand kit (Qiagen, Valencia, CA) according to the manufacturer's protocol as described in section 2.9.2. To measure the mRNA expression levels of 88 membrane transporter genes (including *ABCC2*) in the samples, real-time qPCR was performed using a customized human membrane transporter PCR array (Qiagen, Valencia, CA) with mRNA levels of genes of interest quantified with the fluorescent dye SYBR Green-based method using the ABI PRISM 7900HT Sequence Detection System and SDS 2.3 software (Applied Biosystems) as described in section 2.9.3. The relative mRNA expression levels of *ABCC2* were presented as ΔCt values, and the normalised

mRNA expression levels of all 88 transporter genes were presented as $2^{\wedge -\Delta Ct}$ values, where $\Delta Ct = Ct_{\text{gene of interest}} - \text{Average } Ct_{\text{house-keeping genes}}$; house-keeping genes included glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein, large, P0 (*RPLP0*). The lower ΔCt values represented higher expression levels; whereas the higher $2^{\wedge -\Delta Ct}$ values presented higher expression levels. The experiment was repeated twice.

4.2.8 Statistical analysis

Data were analyzed as described in section 2.11.

4.3. Results

4.3.1. *Characterisation of HEK-MRP2 cells*

A HEK293 cell line stably transfected with the gene encoding human MRP2 (*ABCC2*) (HEK-MRP2 cells) and untransfected parental HEK293 cells (HEK-P cells) were used to study the role of MRP2 in oxaliplatin transport at a cellular level. As this HEK-MRP2 cell line had not been well-characterised previously, its functional expression of MRP2 was studied in comparison to HEK-P cells.

4.3.1.1. MRP2 immunostaining

To detect the expression of MRP2 protein, cells were grown on coverslips, then incubated with a primary anti-MRP2 antibody, followed by a secondary antibody before imaging with fluorescence microscopy and confocal laser scanning microscopy. Immunostaining of MRP2 protein was detected in the cytoplasmic and membrane regions of HEK-MRP2 cells, but not in HEK-P cells (Figure 4.2).

4.3.1.2. ABCC2 mRNA real-time quantitative PCR

To measure *ABCC2* mRNA expression levels, RNA was extracted from HEK-P and HEK-MRP2 cells, converted to cDNA, then analyzed by real-time quantitative PCR using a human membrane transporter PCR array and the SYBR Green fluorescent dye method. Reference gene corrected $2^{\wedge-\Delta Ct}$ values for *ABCC2* from replicate experiments were higher in HEK-MRP2 cells (1.73 and 1.26) than in HEK-P cells (0.001 and 0.001), indicating higher expression of *ABCC2* mRNA in HEK-MRP2 than in HEK-P cells with the fold-difference of 1730 and 1260 respectively.

4.3.1.3. CDCF accumulation and efflux

To measure the functional activity of MRP2 protein in HEK-P and HEK-MRP2 cells, their accumulation and efflux of a fluorescent model MRP2 substrate, CDCF, was measured. Cells were exposed to a CDCFDA (pro-drug of CDCF) for 90 min, followed by measurement of CDCF accumulation and efflux using fluorescence. CDCF accumulation was 0.468-fold lower in HEK-MRP2 compared to HEK-P cells ($P < 0.0001$; Mann Whitney test) and its efflux into the incubation buffer was 2.1-fold higher compared to HEK-P cells ($P < 0.0001$; Mann Whitney test) [Table 4.2].

4.3.1.4. Sensitivity to cytotoxic MRP2 substrates

To compare the sensitivity of HEK-MRP2 and HEK-P cells to known cytotoxic MRP2 substrates, cells were exposed to vincristine, etoposide or cisplatin at a range of concentrations for 72 h before measurement of the number of viable cells by the MTT assay. HEK-MRP2 cells were less sensitive to growth inhibition induced by vincristine, etoposide and cisplatin compared to HEK-P cells (Table 4.2). The IC-50 values for vincristine-induced cytotoxicity of HEK-MRP2 and HEK-P cells were 0.200 ± 0.229 *vs*

0.096 ± 0.114 nM, respectively. The IC-50 values for etoposide-induced cytotoxicity of HEK-MRP2 and HEK-P cells were 101 ± 108 *vs* 54.6 ± 66 nM, respectively. The IC-50 values for cisplatin-induced cytotoxicity of HEK-MRP2 and HEK-P cells were 5.40 ± 1.90 *vs* 3.25 ± 1.48 µM, respectively. Resistance ratios for vincristine, etoposide and cisplatin (geometric mean, (95% CI)) were 2.27 (1.10-4.68), 2.05 (1.57-2.68) and 1.70 (1.28-2.27), respectively, indicating that HEK-MRP2 cells were approximately 2-fold more resistant to cytotoxic MRP2 substrates compared to HEK-P cells.

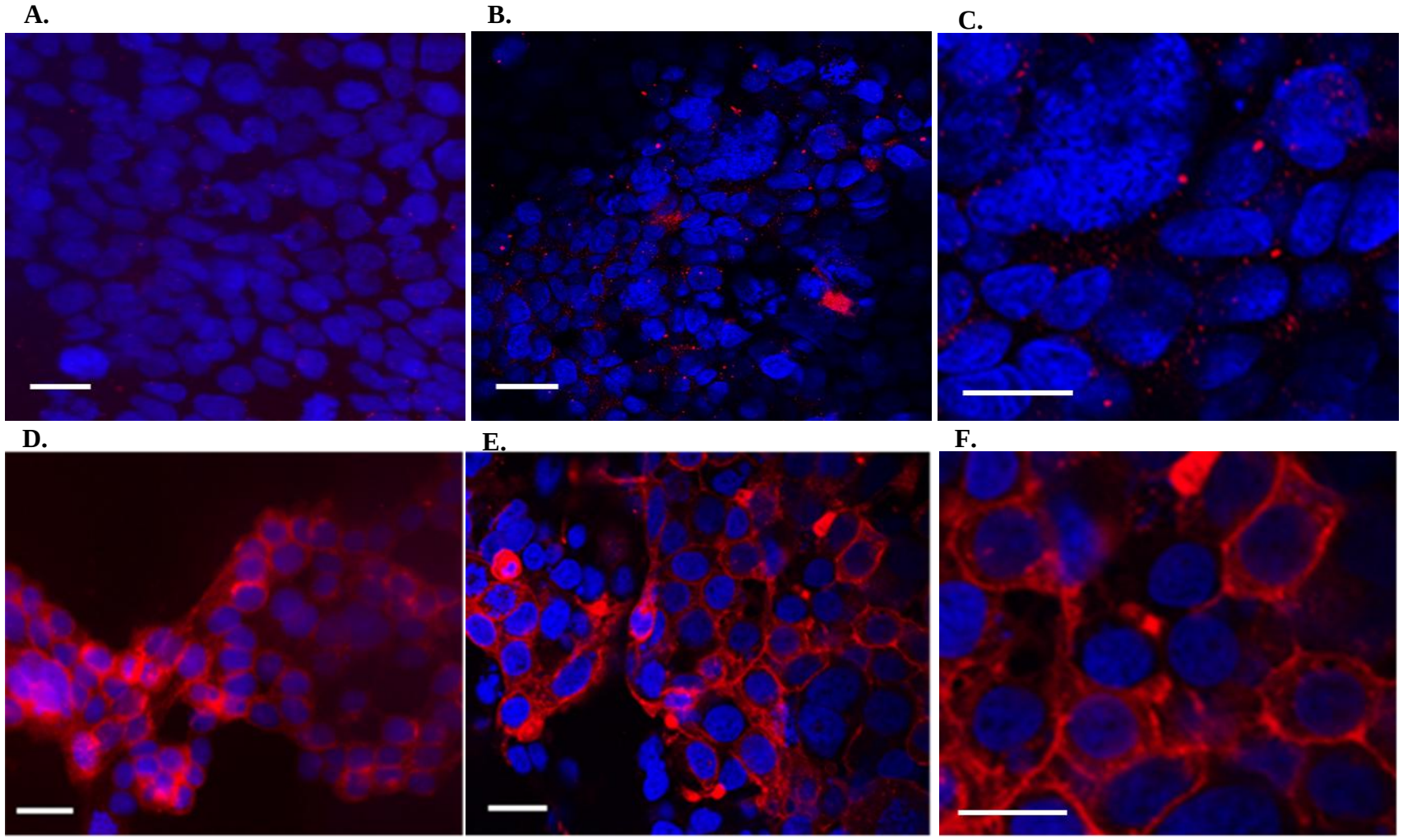

A.
B.
C.
D.
E.
F.

Figure 4.2 MRP2 immunocytochemistry: comparison of HEK-MRP2 and HEK-P cells.

HEK-MRP2 (lower panels) and HEK-P cells (upper panels) were grown on coverslips, then immunostained for MRP2 protein (red) and counterstained with DAPI (blue) before imaging by fluorescence microscopy (A, D) and confocal laser scanning microscopy (B, C, E, F). Images showed detection of MRP2 protein in the cytoplasmic and membrane regions of HEK-MRP2 cells, but not in HEK-P cells. Images are representative of 3 independent experiments. Bars: 50 μm.

Table 4.2 Characterisation of MRP2 in HEK-MRP2 cells and its isogenic control HEK-P cells [1]

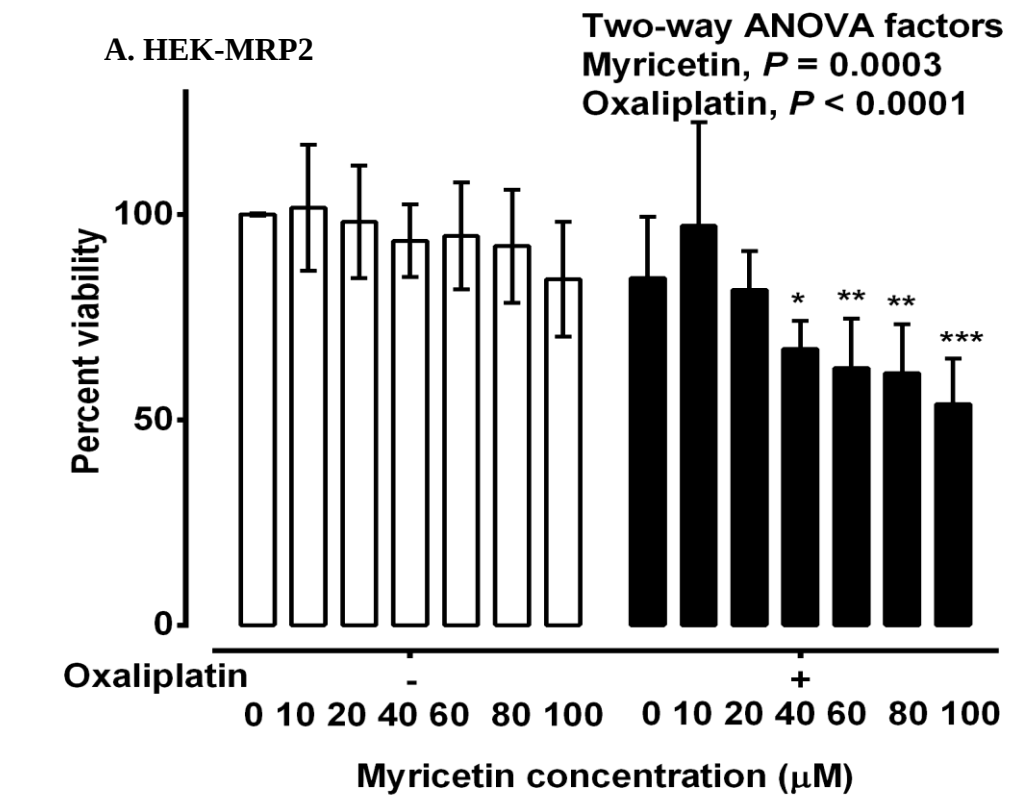
Characteristics of MRP2		n	HEK-MRP2	HEK-P	Ratio (95% CI)	*P*-value
MRP2 protein expression		2	+++ to ++++	0 to +	N/A	N/A
ABCC2 mRNA expression ($2^{\wedge -\Delta Ct}$ values)		2	1.73, 1.26	0.001, 0.001	1730, 1260	N/A
CDCF	accumulation (FU per mg of protein)	3	184 ± 79.3	410 ± 74.5	0.468 (0.326-0.610)	<0.0001*
	efflux (FU per ml of incubation buffer)	3	596 ± 167	290 ± 95.2	2.10 (0.208-0.063)	<0.0001*
IC-50 values for MRP2 substrates	Vincristine (nM)	4	0.200 ± 0.229	0.096 ± 0.114	2.27 (1.10-4.68)	<0.05
	Etoposide (nM)	5	101 ± 108	54.6 ± 66	2.05 (1.57-2.68)	<0.05
	Cisplatin (μM)	4	5.40 ± 1.90	3.25 ± 1.48	1.70 (1.28-2.27)	<0.05

[1] Data shown are mean ± standard deviation of values pooled from independent experiments.

* Mann-Whitney test comparing the values of HEK-P and HEK-MRP2

4.3.2. *Lack of effect of myricetin alone on cell growth*

To investigate the possible effects of myricetin alone on the growth of HEK-MRP2 and HEK-P cells, cells were exposed to increasing concentrations of myricetin (10 to 100 μM) for 2.5 h with or without oxaliplatin (10 μM for 2 h), followed by incubation in drug-free medium for three days before measurement of the number of viable cells by the MTT assay at three days after treatment. Myricetin had little or no effect on the growth of HEK-P cells, either alone or in the presence of oxaliplatin ($P = 0.979$, two-way ANOVA) (Figure 4.3 and Table 4.3). Myricetin alone had little or no effect on the growth of MRP2 cells but increased the growth inhibitory effect of oxaliplatin in a concentration-dependent manner ($P < 0.0001$, two-way ANOVA). Oxaliplatin inhibited the growth of both HEK-MRP2 and HEK-P cells ($P < 0.0001$, two-way ANOVA).


A. HEK-MRP2
Two-way ANOVA factors
Myricetin, P = 0.0003
Oxaliplatin, P < 0.0001
Percent viability
100
50
0
*
**
**
***
Oxaliplatin
-
+
0 10 20 40 60 80 100
0 10 20 40 60 80 100
Myricetin concentration (μM)

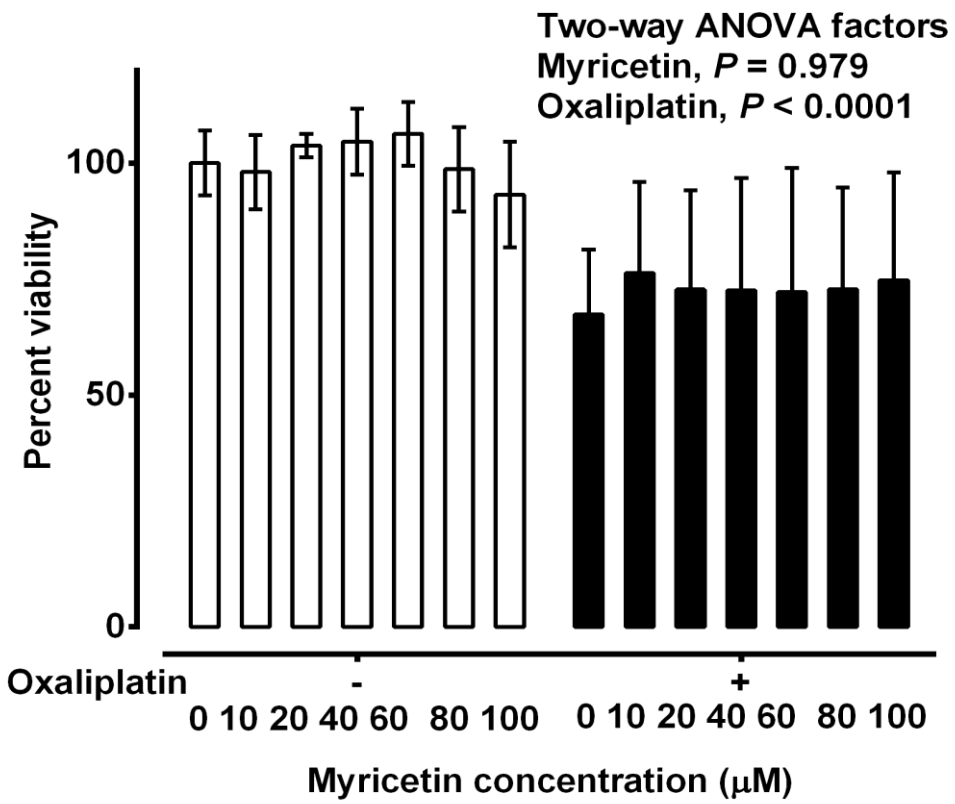

B. HEK-P
Two-way ANOVA factors
Myricetin, P = 0.979
Oxaliplatin, P < 0.0001
Percent viability
100
50
0
Oxaliplatin
-
+
0 10 20 40 60 80 100
0 10 20 40 60 80 100
Myricetin concentration (μM)

Figure 4.3 Lack of effect of myricetin alone on growth of HEK-MRP2 and HEK-P cells

HEK-MRP2 (A) and HEK-P (B) cells were exposed to myricetin (10-100 µM for 2.5 h) with or without oxaliplatin (10 µM for 2 h) followed by incubation in drug-free medium for three days before measurement of number of viable cells by MTT assay. Data were presented as the mean (bar) and standard deviation (error bar) of values pooled from at least three independent experiments. Data showed that myricetin alone had no effect on the growth of HEK-MRP2 or HEK-P cells, and that myricetin enhanced oxaliplatin-induced growth inhibition in HEK-MRP2 cells, but not in HEK-P cells. The *P* values shown as figures are from two-way ANOVA and those shown as * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$) are from Tukey's post-tests following two-way ANOVA. White bars: myricetin alone; black bars, myricetin with oxaliplatin.

Table 4.3 Lack of effect of myricetin alone on growth of HEK-P and HEK-MRP2 cells (tabulated data)

Myricetin concentration (μM)	Percent cell viability (% compared to control cell viability) [Mean ± SD, n ≥ 3]			
	HEK-P		HEK-MRP2	
	Without oxaliplatin	With oxaliplatin 10 μM	Without oxaliplatin	With oxaliplatin 10 μM
0	100 ± 7.00	67.4 ± 13.9	100 ± 0.287	84.7 ± 14.9
10	98.1 ± 8.05	76.3 ± 19.7	102 ± 15.4	97.3 ± 25.2
20	104 ± 2.51	72.7 ± 21.4	98.3 ± 13.8	81.7 ± 9.42
40	105 ± 7.15	72.6 ± 24.2	93.7 ± 8.85	67.3 ± 6.90
60	106 ± 6.85	72.2 ± 26.7	94.9 ± 13.1	62.9 ± 12.1
80	98.7 ± 9.17	72.8 ± 21.9	92.4 ± 13.8	61.4 ± 12.0
100	93.2 ± 11.4	74.8 ± 23.3	84.3 ± 14.0	53.9 ± 11.0

4.3.3. *Cellular accumulation of oxaliplatin-derived platinum*

4.3.3.1. Comparison of HEK-MRP2 and HEK-P cells

To evaluate the role of MRP2 in determining cellular accumulation of platinum derived from oxaliplatin, HEK-P and HEK-MRP2 cells were exposed to oxaliplatin (200 μM) for up to 2 h before measurement of platinum accumulation by ICP-MS. Cellular accumulation of platinum in HEK-MRP2 and HEK-P cells increased with increasing time of incubation with oxaliplatin ($P < 0.0001$, two-way ANOVA) [Figure 4.4 and Table 4.4]. Platinum accumulation was significantly lower in HEK-MRP2 cells compared to HEK-P cells ($P < 0.0001$, two-way ANOVA) by approximately 2-fold. At each time point, cellular accumulation of platinum was lower in HEK-MRP2 compared to HEK-P cells, but these differences reached statistical significance only at 20, 30, 60 and 120 min ($P < 0.01$, Tukey's multiple comparison post-tests following two-way ANOVA).

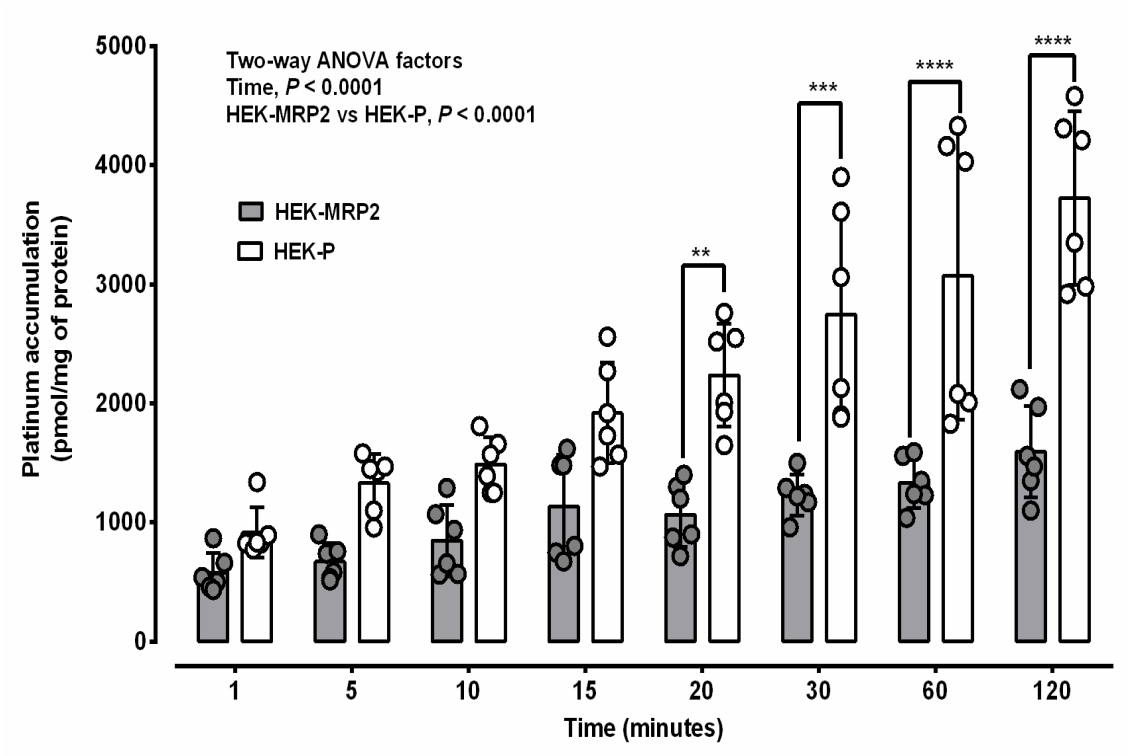


Figure 4.4 Cellular accumulation of oxaliplatin-derived platinum: comparison of HEK-MRP2 and HEK-P cells

HEK-MRP2 and HEK-P cells were incubated with oxaliplatin (200 μM) for up to 120 min before measurement of cellular platinum accumulation by ICP-MS. Symbols are the mean (bar), standard deviation (error bar) and individual values (open circles) pooled from three independent experiments. Data showed lower cellular platinum accumulation in HEK-MRP2 cells than in HEK-P cells. *P* values shown as numbers are from two-way ANOVA and those shown as ** ($P < 0.01$), *** ($P < 0.001$) and **** ($P < 0.0001$) are from Tukey's multiple comparison post-tests following two-way ANOVA. Bars and symbols: white, HEK-P; dark grey, HEK-MRP2.

Table 4.4 Cellular accumulation of oxaliplatin-derived platinum: comparison of HEK-MRP2 and HEK-P cells

Duration of drug exposure (min)	Cellular platinum accumulation (pmol/mg of protein) [Mean ± SD, n=3]		P-value*
	HEK-MRP2	HEK-P	
1	578 ± 163	916 ± 211	N.S
5	672 ± 152	1330 ± 244	N.S
10	848 ± 300	1490 ± 230	N.S
15	1130 ± 434	1920 ± 422	N.S
20	1070 ± 272	2240 ± 434	< 0.001
30	1230 ± 175	2750 ± 894	< 0.0001
60	1340 ± 211	3070 ± 1210	< 0.0001
120	1600 ± 384	3730 ± 728	< 0.0001

**P*-values were calculated from the data analysis with two-way ANOVA followed by Tukey's multiple comparison post-tests, comparing the respective values of HEK-P and HEK-MRP2.

4.3.3.2. Effects of myricetin

To further investigate the role of MRP2 in determining the cellular accumulation of oxaliplatin-derived platinum, the effects of inhibition of MRP2 with myricetin were studied. HEK-P and HEK-MRP2 cells were treated with oxaliplatin (200 µM) in the presence or absence of myricetin for 1 or 2 h followed by measurement of platinum accumulation by ICP-MS. Platinum accumulation was significantly increased by myricetin ($P < 0.0001$, two-way ANOVA) in HEK-MRP2 cells but not in HEK-P cells (Figure 4.5 and Table 4.5). Myricetin significantly increased platinum accumulation in HEK-MRP2 cells by up to 2.7-fold during 1 or 2 h exposure to oxaliplatin ($P < 0.0001$, Tukey's multiple comparison post-tests following two-way ANOVA). As previously shown, platinum accumulation was nearly 2-fold higher in HEK-P cells compared to HEK-MRPs cells ($P < 0.0001$, Tukey's multiple comparison post-tests following two-way ANOVA), but was unchanged by myricetin.

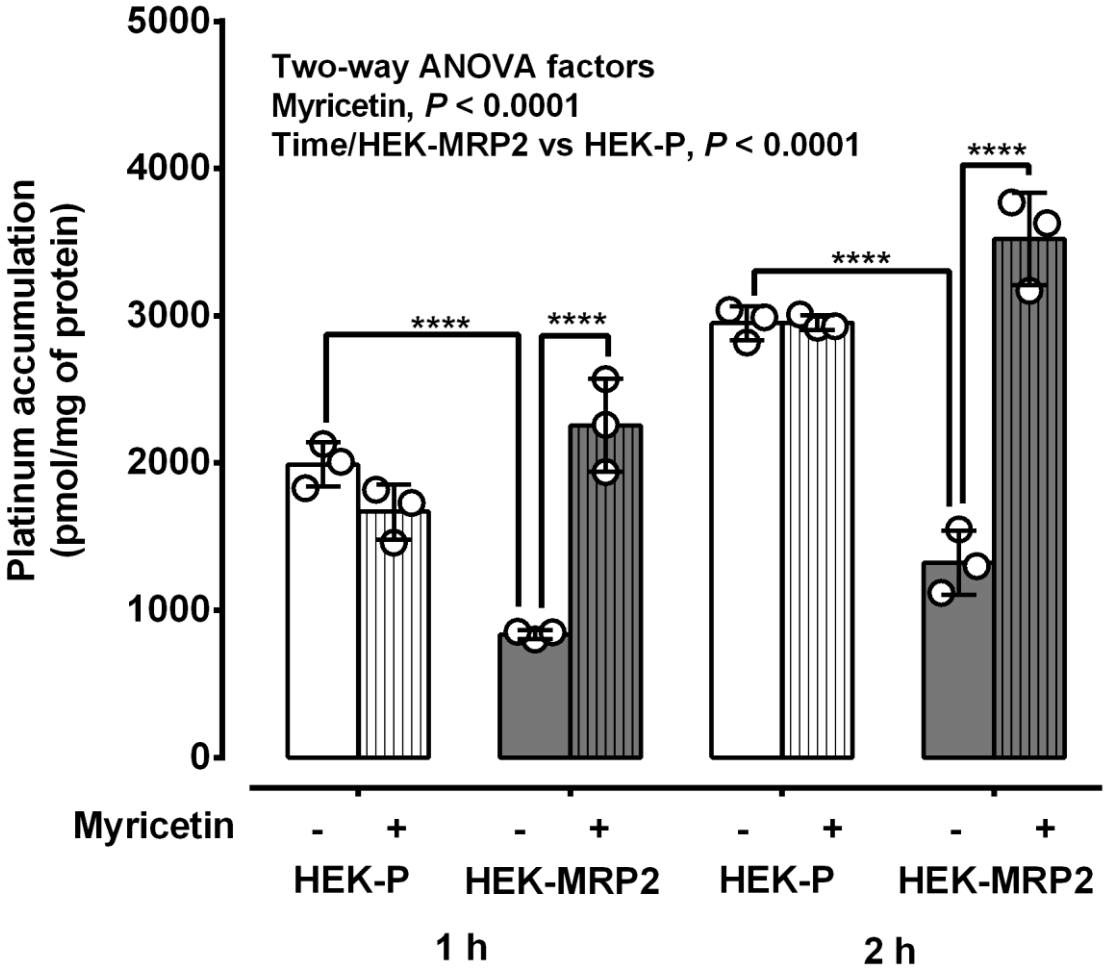


Figure 4.5 Increased accumulation of oxaliplatin-derived platinum induced by myricetin in HEK-MRP2 cells but not in HEK-P cells

HEK-MRP2 and HEK-P cells were incubated with oxaliplatin alone (200 μM) or with myricetin (60 μM) for 1 or 2 h before measurement of platinum accumulation by ICP-MS. Data showed that myricetin increased platinum accumulation in HEK-MRP2 but not in HEK-P cells. Symbols are the mean (bar) and standard deviation (error bar) of individual values (circles) from three independent experiments. *P* values shown as numbers are from two-way ANOVA and those shown as **** ($P < 0.0001$) are from Tukey's multiple comparison post-tests following two-way ANOVA. White bars, HEK-P; dark grey bars, HEK-MRP2; no pattern; without myricetin; striped pattern; with myricetin.

Table 4.5 Increased accumulation of oxaliplatin-derived platinum induced by myricetin in HEK-MRP2 cells but not in HEK-P cells

	Cellular platinum accumulation (pmol/mg of protein) [Mean ± SD, n=3]			
	1 h incubation		2 h incubation	
	HEK-P	HEK-MRP2	HEK-P	HEK-MRP2
Oxaliplatin 200 µM	1990 ± 151	836 ± 29.8	2950 ± 115	1320 ± 216
Oxaliplatin 200 µM with myricetin 60 µM	1670 ± 187	2260 ± 315	2950 ± 49.3	3520 ± 314

4.3.4. *Oxaliplatin-induced growth inhibition*

4.3.4.1. Comparison of HEK-MRP2 and HEK-P cells

To investigate the role of MRP2 in determining cellular sensitivity to oxaliplatin-induced growth inhibition, HEK-P and HEK-MRP2 cells were exposed to increasing concentrations of oxaliplatin for 5 min, 2 h or 72 h before measurement of the number of viable cells by the MTT assay at 3 days after the start of treatment. HEK-MRP2 cells were less sensitive to oxaliplatin-induced growth inhibition than HEK-P cells (Figure 4.6 and Table 4.6). IC-50 values for oxaliplatin-induced growth inhibition were 1.66-, 2.72- and 2.54-fold higher in HEK-MRP2 cells compared to HEK-P cells for 5 min, 2 h and 72 h drug exposure, respectively (P <0.01 paired Student's t-tests).

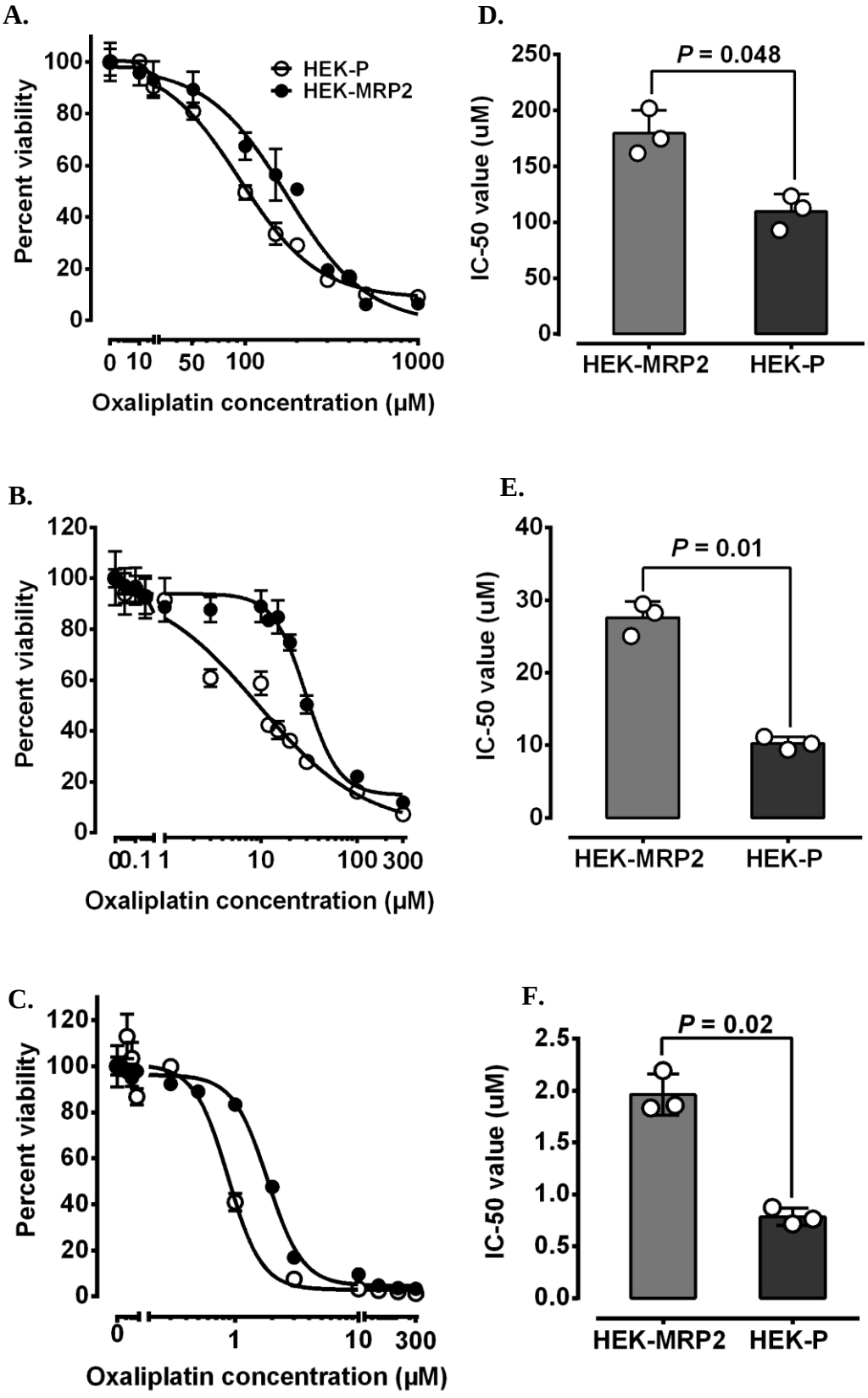

A.
Percent viability
HEK-P
HEK-MRP2
Oxaliplatin concentration (μM)
D.
IC-50 value (uM)
P = 0.048
HEK-MRP2
HEK-P
B.
Percent viability
Oxaliplatin concentration (μM)
E.
IC-50 value (uM)
P = 0.01
HEK-MRP2
HEK-P
C.
Percent viability
Oxaliplatin concentration (μM)
F.
IC-50 value (uM)
P = 0.02
HEK-MRP2
HEK-P

Figure 4.6 Oxaliplatin-induced growth inhibition: comparison between HEK-MRP2 and HEK-P cells at different drug exposure times

HEK-MRP2 and HEK-P cells were treated with increasing concentrations of oxaliplatin (μM) for 5 min (A,D), 2 h (B,E) or 72 h (C,F) followed by incubation in drug-free medium before the measurement of the number of viable cells by the MTT assay at 72 h after the start of treatment. The left panel shows representative growth inhibition curves (symbols, mean (circles) and standard error of mean (error bar) of at least four replicates). The right panel shows growth inhibition IC-50 values derived from three independent experiments (symbols; mean (bar), standard deviation (error bar) and individual values (circles)). Data showed less growth inhibition induced by oxaliplatin in HEK-MRP2 compared to HEK-P. *P* values are from paired t-tests. Bars: dark grey, HEK-MRP2; black, HEK-P.

Table 4.6 Oxaliplatin-induced growth inhibition: comparison between HEK-MRP2 and HEK-P cells at different drug exposure times

Exposure time	IC-50 (μM) [Mean ± SD, n=3]		*P*-value	Relative resistance ratio
	HEK-P	HEK-MRP2		
5 min	110 ± 15.4	180 ± 20.5	0.01	1.66
2 h	10.3 ± 0.877	27.6 ± 2.27	0.002	2.72
72 h	0.783 ± 0.081	1.96 ± 0.201	0.004	2.54

4.3.4.2. Effects of myricetin

To further investigate the role of MRP2 in determining cellular sensitivity to oxaliplatin-induced growth inhibition, HEK-MRP2 and HEK-P cells were exposed to increasing concentrations of oxaliplatin for 2 h with or without myricetin (60 µM for 2.5h) followed by the measurement of the number of viable cells by MTT assay 3 days after drug exposure. Myricetin increased oxaliplatin-induced growth inhibition in HEK-MRP2 cells, but not in HEK-P cells (Figure 4.7 and Table 4.7). IC-50 values for oxaliplatin-induced growth inhibition were decreased by up to 2.3-fold in HEK-MRP2 cells ($P = 0.0005$, unpaired Student's t-test). Compared to HEK-MRP2 cells, IC-50 values for oxaliplatin-induced growth inhibition were lower in HEK-P cells ($P = 0.0001$, paired Student's t-test) but unchanged by myricetin ($P = 0.61$, paired Student's t-test).

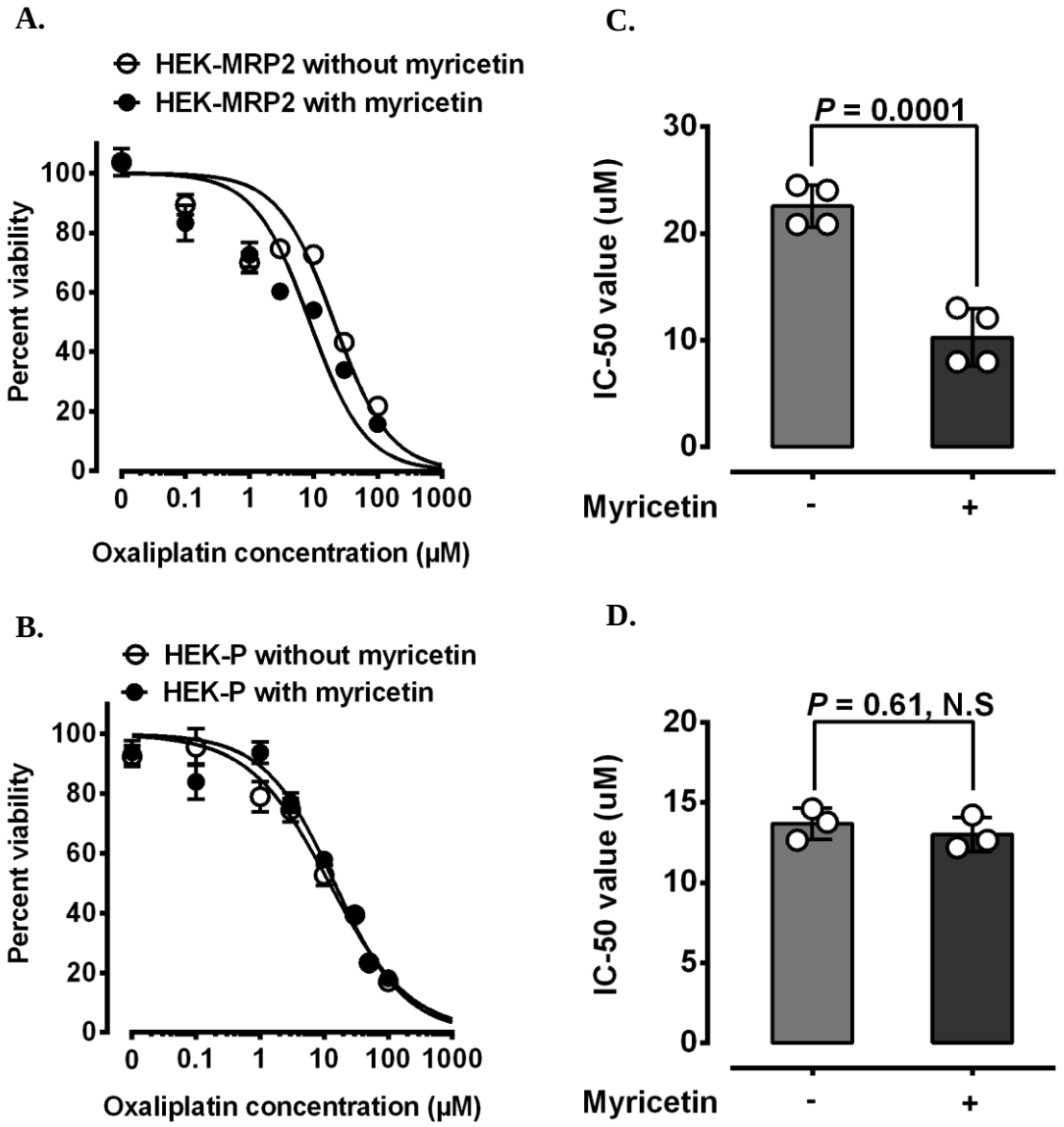


Figure 4.7 Increased oxaliplatin-induced growth inhibition induced by myricetin in HEK-MRP2, but not in HEK-P cells

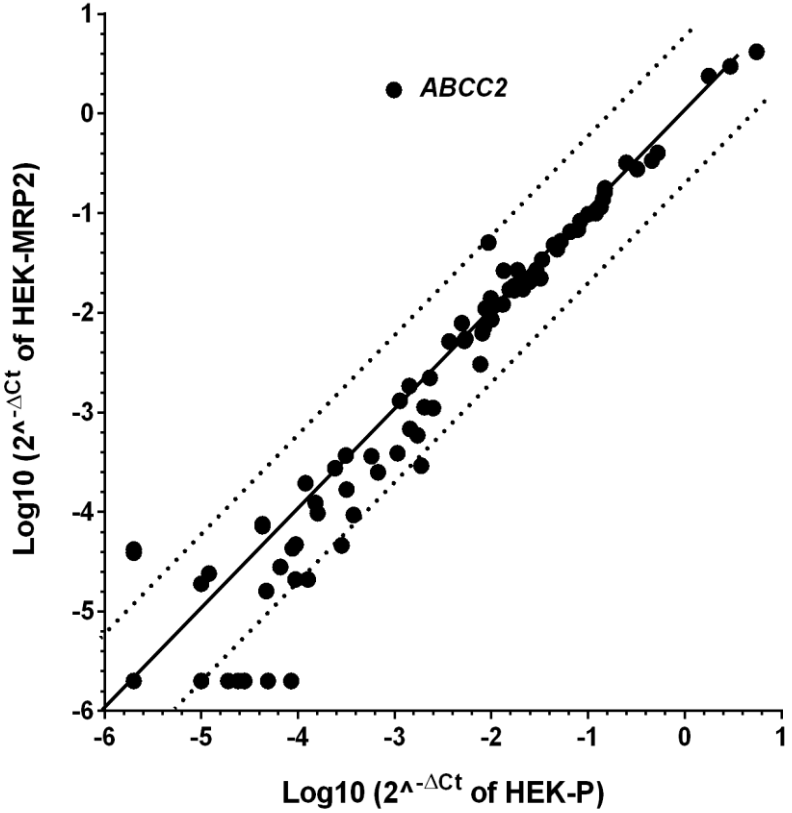
HEK-MRP2 (A, C) and HEK-P (B, D) cells were treated with oxaliplatin alone (increasing concentrations for 2 h) or with myricetin (60 μM for 2.5h) followed by incubation in drug-free medium for three days before measurement of the number of viable cells by MTT assay. The left panel (A,B) shows representative growth-inhibition curves (symbols, mean (circles) and standard error of mean (error bar) of at least 10 replicates. The right panel (C,D) shows growth inhibition IC-50 values derived from four independent experiments (symbols, mean (bar); standard deviation (error bar) and individual values (open circles)). IC-50 values are also shown in the inserted table. Data showed that myricetin enhanced oxaliplatin-induced growth inhibition in HEK-MRP2, but not in HEK-P cells. *P* values are from paired Student's t-tests. Bars: dark grey, HEK-MRP2; black, HEK-P.

Table 4.7 Increased oxaliplatin-induced growth inhibition induced by myricetin in HEK-MRP2, but not in HEK-P cells

	IC-50 value (μM) [Mean ± S.D, n = 4]	
	HEK-P	**HEK-MRP2**
Oxaliplatin 200 μM	13.7 ± 0.98	22.6 ± 1.98
Oxaliplatin 200 μM with myricetin 60 μM	13.0 ± 1.06	10.3 ± 2.67

4.3.5. *Comparative membrane transporter gene expression in HEK-MRP2 and HEK-P cells*

To compare membrane transporter gene expression in HEK-MRP2 and HEK-P cells, RNA was extracted from the cells, converted to cDNA, and then the expression level of 88 membrane transporter genes were measured by quantitative real-time PCR using a customized human membrane transporter PCR array and the SYBR Green fluorescent dye method. Correlation plots of reference gene corrected $2^{\wedge-\Delta Ct}$ values, from two biological replicate experiments, showed similar levels of expression for each membrane transporter gene, except for ABCC2, which was expressed markedly higher in HEK-MRP2 than in HEK-P cells (Figure 4.8 and Appendix 1).


ABCC2
Log10 (2^-ΔCt of HEK-MRP2)
Log10 (2^-ΔCt of HEK-P)
1
0
-1
-2
-3
-4
-5
-6
-6
-5
-4
-3
-2
-1
0
1

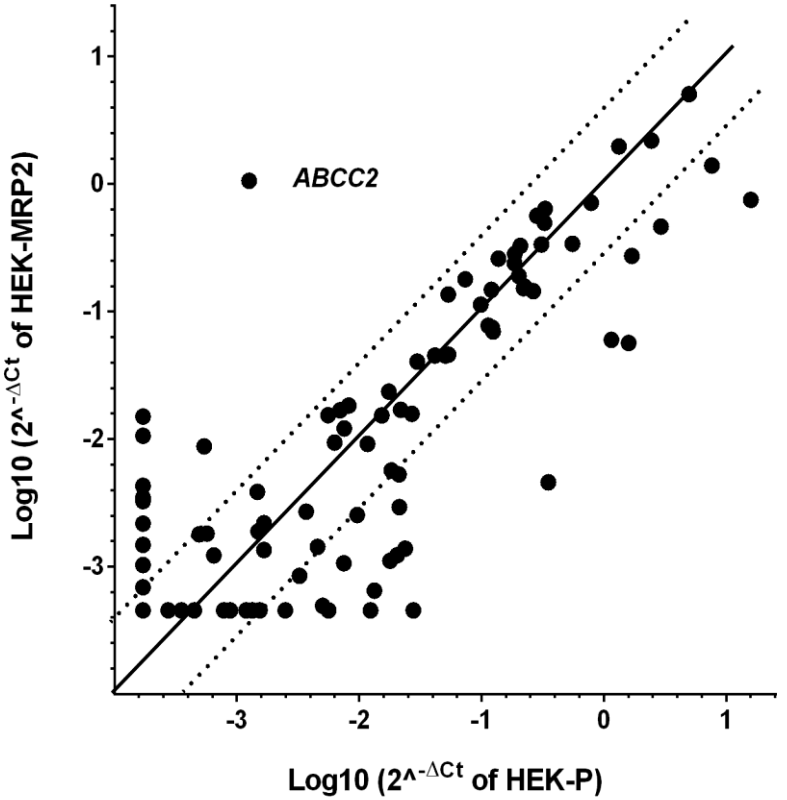

ABCC2
Log10 (2^-ΔCt of HEK-MRP2)
Log10 (2^-ΔCt of HEK-P)
1
0
-1
-2
-3
-3
-2
-1
0
1

Figure 4.8 Comparative membrane transporter gene expression in HEK-MRP2 and HEK-P cells

RNA was extracted from HEK-MRP2 and HEK-P cells, converted to cDNA, and then the expression levels of *ABCC2* and 87 other membrane transporter genes were measured by real-time quantitative PCR using a customized human membrane transporter PCR array and the SYBR Green fluorescent dye method. Data showed increased *ABCC2* gene expression in HEK-MRP2 cells, but similar levels of expression of other membrane transporter genes in HEK-MRP2 and HEK-P cells. Filled circles represent reference gene-corrected $2^{\wedge -\Delta Ct}$ values for the expression of individual membrane transporter genes from two independent experiments (error bar, range). Solid line, line of unity; dotted lines, upper and lower 3-fold threshold levels.

4.4. Discussion

The studies described in this chapter firstly aimed to characterise the phenotypic characteristics of a cell-line stably transfected to overexpress the *ABCC2* gene encoding the MRP2 membrane transporter protein, in comparison to a non-transfected parental line. HEK-MRP2 cells showed greater MRP2 protein and *ABCC2* mRNA expression than HEK-P cells. The efflux of CDCF in HEK-MRP2 cells was greater leading to a lower accumulation of CDCF, and the sensitivity to the cytotoxic effects of known MRP2 substrates (vincristine, etoposide and cisplatin) was less compared to HEK-P cells. An exploratory analysis of the comparative expression of the mRNA transcripts of 88 membrane transporter genes in HEK-MRP2 and HEK-P cells showed no major differences other than markedly increased expression of *ABCC2* in HEK-MRP2 cells. These results indicated that the stable transfection of the *ABCC2* gene in HEK-MRP2 cells resulted in functional overexpression of MRP2 protein in a manner that altered the transport and effects of MRP2 substrates, compared to HEK-P cells. The approximately two-fold differences in CDCF accumulation and efflux, and in IC-50 values of cytotoxic MRP2 substrates, between HEK-MRP2 and HEK-P cells were smaller than that conferred by *ABCC2* gene transfection in other studies (216, 217, 221, 222, 225) but were statistical significant. Ideally a HEK293 cell line stably transfected with an empty vector would have been used for comparisions to the HEK-MRP2 cell line but this was not available. Taken together, these findings suggest that this HEK-MRP cell line is a valid *in vitro* experimental model of moderate overexpression of the *ABCC2* gene for use in subsequent studies of oxaliplatin.

With regard to their accumulation of oxaliplatin-derived platinum and sensitivity to oxaliplatin-induced growth-inhibition, HEK-MRP2 cells accumulated approximately two-fold less platinum during their exposure to oxaliplatin as compared to HEK-P cells.

HEK-MRP2 cells were also two-fold less sensitive to the growth inhibitory effects of oxaliplatin as compared to HEK-P cells. These two fold-differences in platinum accumulation and oxaliplatin sensitivity were similar in magnitude to the approximately two fold-differences in CDCF accumulation and efflux, and IC-50 values for vincristine, etoposide and cisplatin, between HEK-MPR2 and HEK-P cells. Taken together, these findings suggested that the overexpression of MRP2 in HEK293 cells may have limited their accumulation and sensitivity to oxaliplatin.

To further investigate the role of MRP2 in determining platinum accumulation and growth inhibition of oxaliplatin, the effects of myricetin were studied. Myricetin is a non-sulfhydryl competitive inhibitor of MRP2 and other MRP membrane transporters (266, 271). Concurrent treatment of HEK-MRP2 cells with oxaliplatin and myricetin increased their accumulation of platinum and sensitivity to oxaliplatin-induced growth inhibition by approximately two-fold. In contrast, concurrent treatment of HEK-P cells with myricetin and oxaliplatin did not alter their accumulation of platinum or sensitivity to oxaliplatin-induced growth inhibition. These findings provide further evidence for the involvement of MRP2 in the reduction of platinum accumulation and sensitivity to oxaliplatin in HEK-MRP2 cells.

However, studies using genetically modified cell lines transfected to overexpress a membrane transporter gene of interest may be confounded by unintended changes in the expression of other membrane transporter genes. This possibility was investigated by comparing membrane transporter gene expression in HEK-MRP2 and HEK-P cells by a quantitative real-time PCR array that measured the expression of 88 different membrane transporter genes. The *ABCC2* gene was the only gene significantly overexpressed in HEK-MRP2 as compared to HEK-P cells; whereas all other genes were neither significantly increased nor decreased in their expression.

In addition, *in vitro* studies evaluating the effects of transporter inhibitors on the sensitivity of cells to cytotoxic drugs may be confounded by possible cytotoxic effects of the inhibitor itself. However, myricetin alone had no effect on the growth of HEK-MRP2 or HEK-P cells, indicating that there were no unintended confounding effects of gene transfection or cytotoxic effects of myricetin in these experiments.

In conclusion, these studies showed a reduced cellular accumulation of oxaliplatin-derived platinum and a decreased sensitivity to oxaliplatin-induced growth inhibition in cells that were genetically modified to stably overexpress the *ABCC2* gene and confirmed to have moderately increased functional overexpression of MRP2 protein. Inhibition of MRP2 with myricetin reversed these deficits in platinum accumulation and oxaliplatin sensitivity conferred by the overexpression of MRP2.

Chapter 5. Human gastrointestinal cancer cell line studies

5.1. Introduction

The previous chapter described studies of HEK-MRP2 and HEK-P cell lines showing that MRP2 was involved in determining their accumulation of oxaliplatin, detected as platinum associated with cells by ICP-MS, and their sensitivity to oxaliplatin-induced growth inhibition. Further evidence supporting this role of MRP2 came from inhibitor studies using myricetin, where myricetin reversed the MRP2-mediated reduction of cellular platinum accumulation and increased sensitivity to oxaliplatin-induced growth inhibition. In this current chapter, human gastrointestinal cancer cell lines were used to further explore the role of MRP2 in cellular platinum accumulation and their sensitivity to oxaliplatin.

Oxaliplatin-based combined chemotherapy has been used for the treatment of patients with gastrointestinal cancers including gastric, colorectal, hepatocellular and pancreatic cancers. In combination with other anti-cancer drugs, oxaliplatin forms as the first-line treatment regimen for metastatic colorectal cancer. Oxalipltin-based combination chemotherapy regimens used for colorectal cancer include FOLFOX (oxaliplatin, 5-fluorouracil with folinic acid), XELOX (oxaliplatin with capecitabine), and FOLFOXIRI (oxaliplatin, 5-fluorouracil, folinic acid, and irinotecan) (9-11). Oxaliplatin-containing chemotherapy is also used as adjuvant chemotherapy after surgical removal of locally advanced colorectal cancer (12, 13). Furthermore, oxaliplatin-based chemotherapy is being used for the treatment of other gastrointestinal cancers including pancreatic, gastric and hepatocellular cancers (14, 29, 34-37). FOLFIRINOX (oxaliplatin with 5-fluorouracil, folinic acid, and irinotecan) and GEMOXEL (gemcitabine, oxaliplatin, and

capecitabine) were shown to be more effective and safe with higher median overall survival rate in patients with metastatic pancreatic cancer compared to standard gemcitabine therapy (29, 69). Recently, oxaliplatin in combination with capecitabine or S-1 (orally administered prodrugs of 5-fluorouracil) has been proved to be effective and safe for the treatment of advanced gastric cancer (36-38). Moreover, oxaliplatin-based combined chemotherapy regimens such as GEMOX (gemcitabine with oxaliplatin), FOLFOX4 and XELOX are used for the treatment of hepatocellular carcinoma (14-16).

Several studies were previously conducted investigating the association of MRP2 expression with tumour resistance and/or clinical outcome in gastrointestinal cancer patients who were receiving platinum-based chemotherapy (143, 209, 235, 236). MRP2 expression level was found to be increased in tumour tissue samples from patients with hepatocellular carcinoma (237, 238), colorectal carcinoma (143, 209) and pancreatic cancer (235). Colorectal tumor tissue samples from patients with recurrence while receiving the treatment of 5-fluorouracil/leucovorin (FL) and oxaliplatin (FOLFOX-4) expressed high level of MRP2 compared to matched normal tissue samples, and they had decreased overall survival as compared to patients with MRP2-negative tumor samples despite the difference being not statistically significant (209). A *ABCC2* (MRP2 gene) polymorphism (G1249A) was shown to be associated with increased overall survival in patients with colorectal cancer receiving oxaliplatin-containing chemotherapy (207). Hepatocellular tumor samples with overexpression of MRP2 showed lower levels of tumor necrosis in patients receiving cisplatin-based chemotherapy (238). In a cohort of pancreatic cancer patients receiving gemcitabine or gemcitabine with cisplatin therapy, MRP2 G40A GG genotype showed an association with low overall survival rate and poor tumor response to chemoradiotherapy (239). These studies suggested that MRP2 might play a role in the chemoresistance and/or clinical outcome in gastrointestinal cancer

patients receiving platinum-based chemotherapy such as oxaliplatin and cisplatin, yet its contributions in these regards are still unclear. Hence, it will be interesting to further investigate the role of MRP2 in the cellular resistance mechanisms of human gastrointestinal cancer to oxaliplatin.

There were *in vitro* studies showing the association of MRP2 with the cellular resistance to cisplatin in human liver cancer (300), cellular resistance to doxorubicin and vincristine in HepG2 cells (221), and cellular resistance to cisplatin in human ovarian cancer cell line (A2780) (220, 222). On the other hand, there have been a limited number of *in vitro* studies investigating the role of MRP2 in cellular resistance to oxaliplatin using human gastrointestinal cancer cell lines with contradictory findings (74, 206, 240). Hence the role of MRP2 in the cellular resistance of oxaliplatin in human gastrointestinal cancers is still a open question. Shen and colleagues showed that MRP2 expression was significantly increased in oxaliplatin-resistant colorectal cancer cell line (HCT-116/L-OHP), and siRNA-mediated transient silencing of *ABCC2* in HCT-116 cells resulted in increased cellular sensitivity to oxaliplatin and increased cellular platinum accumulation (240). Liu et al. determined the expression levels of ABC transporter proteins such as P-gp (P-glycoprotein encoded by *ABCB1*), MRP1 (multidrug-resistance protein 1) and MRP2 in oxaliplatin-resistant colon cancer cell lines (SW620/L-OHP and Lovo/L-OHP) compared to non-resistant cell lines, and it was found that only MRP2 expression level detected by western blot was upregulated in the resistant cell lines whereas no significant change was observed for the levels of P-gp and MRP1 (206). Another study by Beretta and group showed that oxaliplatin-resistant ovarian cancer cells (IGROV-1/OHP) had lower levels of cellular platinum accumulation with increased expression levels of MRP1 and MRP4 compared to non-resistant IGROV-1 cells whereas MRP2 and MRP3 were not detectable in both non-resistant and resistant cell lines (74). Taken together, these limited

findings have suggested that MRP2 may play a role in the cellular transport of oxaliplatin and cellular resistance of human gastrointestinal cancer to oxaliplatin.

With this background, the experimental work described in this chapter aimed, firstly, to identify human gastrointestinal cancer cell lines as *in vitro* models of human gastrointestinal cancer with MRP2-mediated deficits in oxaliplatin accumulation. The work further aimed to examine the effects of inhibition of MRP2 activity and knockdown of *ABCC2* gene expression on the cellular accumulation of oxaliplatin-derived platinum and sensitivity to oxaliplatin-induced growth inhibition in human gastrointestinal cancer cell lines.

5.2. Materials and methods

5.2.1. *Chemicals*

The sources and the preparations of chemicals used in this study were described in details in section 2.1.

5.2.2. *Cell culture*

A panel of human gastrointestinal cancer cell lines including liver (HepG2), pancreatic (MiaPACA-2, PANC-1) and colorectal (WiDR, SW620, HCT116 and HT29) cancer cell lines were used in this study. The origin, histopathology features, cell culture conditions and growth rates of cell lines were summarised in Table 5.1. The details of cell culture conditions used in this study could be found in section 2.3.

Table 5.1 Characteristics and cell culture conditions for human gastrointestinal cancer cell lines used in the current study

Cell line	Disease model	Origin of cell line	Cell culture conditions	Doubling time (hour)	Reference
HepG2	Human epithelial hepatocellular carcinoma	15 yr old Caucasian male liver	Eagle's Minimum Essential Medium with 10% FBS	27 to 35	(300-302)
PANC-1	Human pancreatic epitheloid carcinoma	56 yr old Caucasian male pancreas	Dulbecco's Modified Eagle's Medium with 10% FBS	31 to 52	(303-305)
MiaPACA-2	Human epithelial pancreatic carcinoma	65 yr old male pancreas	Dulbecco's Modified Eagle's Medium with 10% FBS	17 to 40	(303, 305)
HCT116	Human colorectal epithelial carcinoma	48 yr old male colon primary tumor Dukes'D	Eagle's Minimum Essential Medium with 10% FBS	17 to 30	(306, 307)
HT29	Human colorectal epithelial adenocarcinoma	44 yr old female colon primary tumor Dukes' C	Eagle's Minimum Essential Medium with 10% FBS	24 to 28	(307, 308)
SW620	Human colorectal epithelial adenocarcinoma	51 yr old male colon lymph node (metastatic site) Dukes' C	Dulbecco's Modified Eagle's Medium with 10% FBS	22 to 34	(309-311)
WiDR	Human colorectal epithelial adenocarcinoma	Adult female colon	Eagle's Minimum Essential Medium with 10% FBS	15	(312, 313)

5.2.3. *Growth inhibition assay*

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was used to measure the number of viable cells in experiments conducted in this project. Details of the MTT assay are in section 2.4. Experiments were repeated independently at least three times.

5.2.4. *Cellular platinum accumulation analysis with inductively coupled plasma mass spectrometry (ICP-MS)*

Cells were seeded at 250,000 cells per well (350,000 cells per well for HepG2 and PANC-1) in a collagen-coated 6-well plate, and grown in the normal growth medium until the cells become around 80% confluent. Then, cells were incubated with 50 µM oxaliplatin dissolved in the incubation buffer for 2 h, after which cells were washed with ice-cold PBS followed by processing of the cells for ICP-MS measurement of platinum accumulation. The protein level of cellular lysate was measured with a modified tyrosine nitration assay as described in section 2.5, and the cellular platinum accumulation level in pmol of platinum per mg of protein was calculated. Details of these experimental procedures are in section 2.5.

In experiments mentioned in this chapter, the linearity of the ICP-MS platinum standard curve of the experiments was defined by r^2 values ranging from 0.9901 to 0.9999. The precision and accuracy of the QCs in the experiments ranged from 0.02% to 14% and within 100 ± 14.5%, respectively. Based on the experiment results, the limit of detection (LOD) and lower limit of quantification (LLOQ) were defined to be 0.3 ppb and 1 ppb of platinum, respectively.

5.2.5. *CDCF accumulation assays*

CDCF [5(6)-carboxy-2,'7'-dichlorofluorescein] accumulation was used to measure the MRP2 transporter activity in the HepG2 cell line. Cells were exposed to a non-fluorescent permeable pro-drug form of CDCF, 5(6)-carboxy-2,'7'-dichlorofluorescein diacetate (CDCFDA). HepG2 cells were seeded at 80,000 cells per well in collagen coated 24-well plates. The procedure of CDCF accumulation assay was as in section 2.6.2.

5.2.6. *Real-time quantitative polymerase chain reaction (qPCR)*

RNA was extracted from cell lines using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol as described details in the section 2.9.1. cDNA (complementary DNA) was synthesized from total RNA, previously extracted from each cell line sample, by reverse transcription of total RNA samples in which RNA was primed with the oligonucleotides, or primers, catalysed by reverse transcriptase enzyme to produce cDNA using a RT^2 HT First strand kit (Qiagen, Valencia, CA) according to the manufacturer's protocol as described details in the section 2.9.2. To measure the relative mRNA expression levels of the membrane transporter genes including *ABCC2* in samples, real-time qPCR was performed using cDNA samples and mRNA levels of these interested genes were quantified in human cancer cell lines using a customised PCR array (catalogue number CAPH12045-PAHS-070Z, Qiagen).

For *ABCC2*-siRNA knockdown studies in HepG2 cells, mRNA expression levels of *ABCC1-5* (MRP1-5 genes), *ABCB1* (MDR1 gene) and house-keeping genes including *beta-actin*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) were quantified using gene-specific primers purchased from Invitrogen. Fluorescent dye SYBR Green-based method was applied for real-time qPCR run with the appliance of ABI PRISM 7900HT Sequence Detection

Systems and SDS 2.3 software (Applied Biosystems) as described details in the section 2.9.3.

The expression levels of genes of interest (*ABCC1-5* and *ABCB1*) in each sample were represented as $2^{\wedge -\Delta Ct}$ values of corresponding genes where ΔCt values were calculated by subtracting the average Ct value of house-keeping genes including *beta-actin*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) from the Ct value of gene of interest. Finally, fold-changes in the expression levels of genes of interest in siRNA-treated cells compared to untreated control cells were then calculated by dividing $2^{\wedge -\Delta Ct}$ values of former to that of latter for each gene. The basal mRNA expression levels of ABC genes and house-keeping genes in untreated HepG2 control cells were measured with customised PCR array method and also with conventional real-time qPCR method using primers whereas the latter method was used to measure the mRNA expression levels of interested genes in siRNA treatment studies.

5.2.7. *siRNA-mediated knockdown of ABCC2 expression*

A reverse-transfection method was used for the knocking down expression of *ABCC2* of HepG2 in this study. *ABCC2*-siRNAs (a set of 3 different Stealth RNAi™ siRNAs predesigned for targeting human *ABCC2*), control-siRNA (Stealth RNAi™ negative control siRNA), Opti-MEM I reduced serum medium and Lipofectamine™ RNAiMAX were purchased from Invitrogen, Carlsbad, CA, USA. Briefly, siRNA was mixed with Opti-MEM I Medium without serum in the well of the tissue culture plate, and lipofectamine RNAiMAX was added to each well containing the diluted siRNA molecules which were mixed gently and incubated for 20 minutes at room temperature. HepG2 cells diluted in complete growth medium without antibiotics at the density of

50,000 cells per 500 µl were added to each well containing siRNA-lipofectamine RNAiMAX complexes. A final volume of 600 µl with the final RNA concentration of 10 nM was obtained, and cells were mixed with the reagents gently by rocking the plate back and forth. Cells were then incubated for another 48 hours at 37°C in a CO_2 incubator. After 48 h incubation of cells with siRNAs, RNA was extracted to measure expression levels of desired genes with real-time qPCR method as described previously. For the growth inhibition assays, after 48 h incubation of cells with siRNAs, cells were exposed to 5 µM oxaliplatin for two hours followed by replacing the medium with normal growth medium with antibiotics for three days until measurement of the number of viable cells by MTT assay. For CDCF accumulation assays and platinum accumulation and cytotoxicity assays, the siRNA-containing medium was replaced with normal growth medium containing antibiotics, and cells were incubated for another 24 or 48 h at 37°C in a CO_2 incubator followed by performing the assays as necessary. For functional validation of *ABCC2* silencing with CDCF accumulation assays, cells were incubated with 10 µM CDCFDA in 10 mM HEPES-HBSS medium for 90 min followed by the measurement of fluorescence intensity of cell lysates with details as mentioned in section 5.2.4. For cellular platinum accumulation assay, cells were exposed to 50 µM oxaliplatin for two hours followed by measurement of platinum associated with the cells with ICP-MS as described before in section 5.2.3.

5.2.8. *Statistical analysis*

Data analysis was done as mentioned in section 2.11.

5.3. Results

5.3.1. *ABCC2 mRNA expression and oxaliplatin accumulation in human gastrointestinal cancer cell lines*

A panel of seven human gastrointestinal cancer cell lines, including the HepG2, MiaPACA-2, PANC-1, WiDR, SW620, HCT116 and HT29 cancer cell lines, were studied to identify *in vitro* models of human gastrointestinal cancer with MRP2-mediated deficits in oxaliplatin accumulation. Levels of *ABCC2* mRNA expression were analyzed by quantitative real-time PCR array analysis. Cells were treated with oxaliplatin (50 μM) for two hours before measurement of platinum accumulation by ICP-MS. The HepG2 and PANC-1 cell lines showed high expression levels of *ABCC2* mRNA and low accumulation of platinum comparable to the HEK-MRP2 cell line. The HT29, SW620, WiDR, MiaPACA-2 and HCT116 cell lines showed high platinum accumulation levels and low expression levels of *ABCC2* mRNA comparable to the HEK-P line (Table 5.2).

Table 5.2 Classification of human gastrointestinal cancer cell lines by their accumulation of oxaliplatin-derived platinum and ABCC2 expression with reference to HEK-P and HEK-MRP2 cell lines

	Cell lines	Cellular platinum accumulation		$2^{-\Delta Ct}$ values for *ABCC2* expression			
		pmol/mg protein[1]	Fold-difference[2]	Exp 1	Exp 2	Average	Fold-difference[2]
HEK-MRP2-like cell-lines (Low platinum accumulation; high *ABCC2* expression)	HEK-MRP2	177 ± 35.8	0.39	1.73	1.26	1.50	1500
	HepG2	63.3 ± 24.0	0.14	0.708	2.02	1.36	1360
	PANC-1	156 ± 24.7	0.34	0.014	0.264	0.139	139
HEK-P-like cell-lines (High platinum accumulation; low ABCC2 expression)	HEK-P	455 ± 123	1	0.001	0.001	0.001	1
	HT29	360 ± 65.5	0.79	0.002	0.001	0.002	2
	SW620	433 ± 162	0.96	0.006	-	0.006	6
	WiDR	481 ± 200	1.07	-	0.002	0.002	2
	MiaPACA-2	499 ± 147	1.11	0.001	0.044	0.023	23
	HCT116	526 ± 248	1.18	0.039	0.008	0.024	24

[1]Mean ± SD (n=4); [2]Relative to HEK-P cell line

5.3.2. *Effect of myricetin on oxaliplatin accumulation and growth inhibition*

Cells were exposed to oxaliplatin (50 µM for 2 h) with or without myricetin (60 µM for 2.5 h) followed by measurement of cellular platinum accumulation with ICP-MS. Cells were incubated with oxaliplatin at different concentrations for 2 h with or without myricetin (60 µM for 2.5 h) then incubated in drug-free medium for three days before measurement of the number of viable cells by MTT assay.

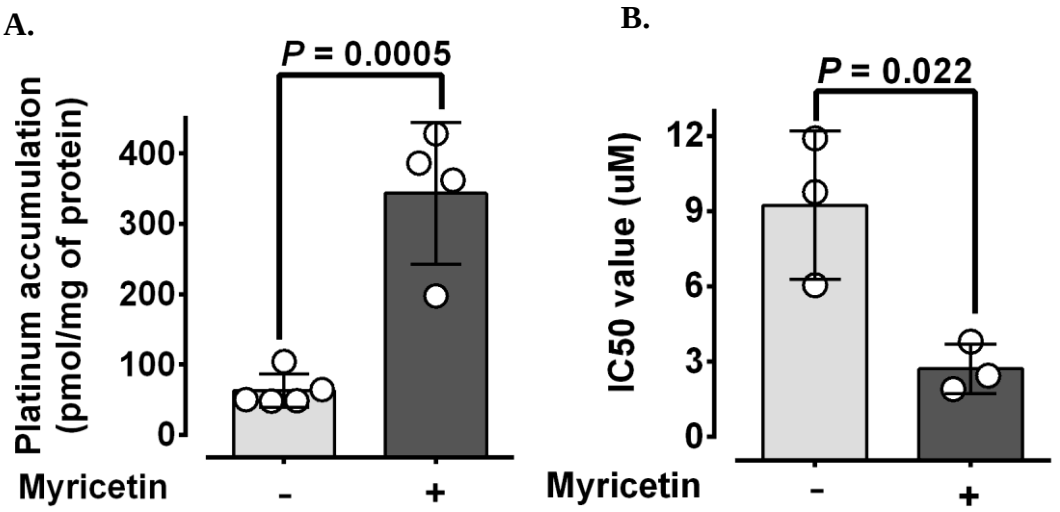
In HepG2 cells, myricetin significantly increased cellular platinum accumulation by 5-fold from 63.3 ± 24.0 to 343 ± 101 pmol/mg protein (P = 0.0005), and significantly reduced oxaliplatin-induced growth inhibition IC50 values by 3.4-fold from 9.24 ± 2.97 to 2.72 ± 0.978 µM (P = 0.022) [Figure 5.1].

In PANC-1 cells, myricetin significantly increased cellular platinum accumulation by 2.8-fold from 156 ± 34.7 to 298 ± 52.9 pmol/mg protein (P = 0.004), and significantly reduced oxaliplatin-induced growth inhibition IC-50 values by 4-fold from 28.5 ± 5.89 to 7.10 ± 3.56 µM (P = 0.006) [Figure 5.2].

In MiaPACA-2 cells, myricetin did not alter cellular platinum accumulation (499 ± 146 versus 397 ± 185 pmol/ mg protein, P = 0.42) but it reduced oxaliplatin-induced growth inhibition IC50 values by 2-fold (15.8 ± 3.58 versus 7.72 ± 3.78 µM, P = 0.02).

In HT29 cells, myricetin did not alter cellular platinum accumulation (321 ± 66.2 versus 192 ± 17.2 pmol/mg protein, P = 0.16) or oxaliplatin-induced growth inhibition IC50 values (33.1 ± 13.3 versus 33.9 ± 9.94 µM, P = 0.93).

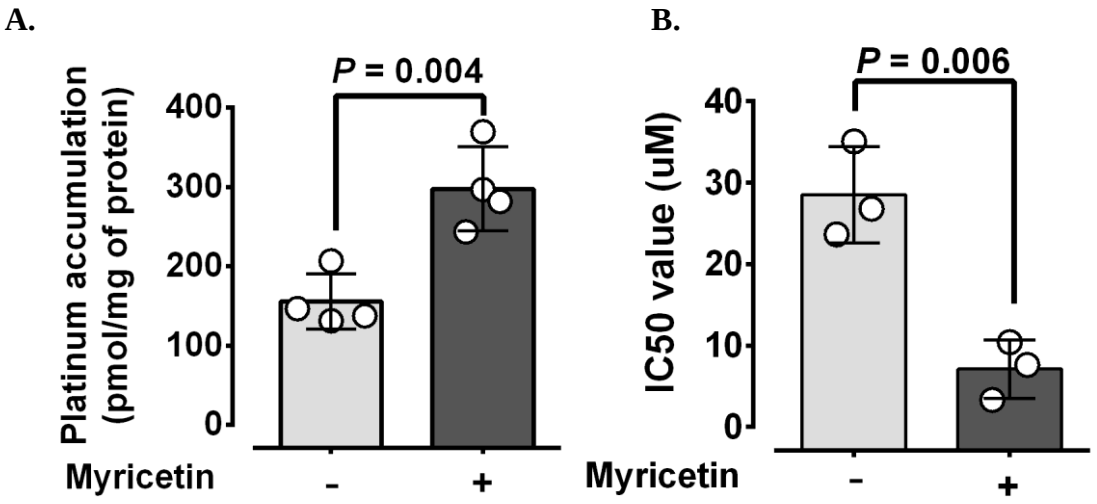
In HCT116 cells, myricetin did not alter cellular platinum accumulation (332 ± 103 versus 232 ± 82.5 pmol/mg protein, P = 0.40) or oxaliplatin-induced growth inhibition IC50 values



	Cellular platinum accumulation (pmol/mg of protein)		IC-50 values (μM)	
Myricetin	-	+	-	+
Mean	63.3	343	9.24	2.72
Standard deviation	24.0	101	2.97	0.978
***P*-value**		0.0005		0.022

Figure 5.1 Effect of myricetin on oxaliplatin accumulation and growth inhibition in HepG2 cells

(A) Cellular accumulation of oxaliplatin-derived platinum. HepG2 cells were treated with oxaliplatin (50 μM) for two hours, with or without myricetin (60 μM) for 2.5 hours, before the measurement of platinum accumulation by ICP-MS. Myricetin increased cellular accumulation of oxaliplatin-derived platinum in HepG2 cells. (B) Oxaliplatin-induced growth inhibition. HepG2 cells were treated with a range of different concentrations of oxaliplatin for two hours, with or without myricetin (60 μM) for 2.5 hours, then cultured in drug-free medium for three days before measurement of the number of viable cells by MTT assay. Myricetin decreased oxaliplatin-induced growth inhibition in HepG2 cells. Symbols are the mean and standard deviation of individual values (circles) from at least three independent experiments. The *P* values shown as numbers are from unpaired Student's t-test. Bars: light grey, without myricetin; dark grey, with myricetin.



	Cellular platinum accumulation (nmol/mg of protein)		IC-50 values (μM)	
Myricetin	-	+	-	+
Mean	156	298	28.5	7.10
Standard deviation	34.7	52.9	5.89	3.56
***P*-value**		0.004		0.006

Figure 5.2 Effect of myricetin on oxaliplatin accumulation and growth inhibition in PANC-1 cells

(A) Cellular accumulation of oxaliplatin-derived platinum. PANC-1 cells were treated with oxaliplatin (50 μM) for two hours, with or without myricetin (60 μM) for 2.5 hours, before the measurement of platinum accumulation by ICP-MS. Myricetin increased cellular accumulation of oxaliplatin-derived platinum in PANC-1 cells. (B) Oxaliplatin-induced growth inhibition. PANC-1 cells were treated with a range of different concentrations of oxaliplatin for two hours, with or without myricetin (60 μM) for 2.5 hours, then cultured in drug-free medium for three days before measurement of the number of viable cells by MTT assay. Myricetin decreased oxaliplatin-induced growth inhibition in PANC-1 cells. Symbols are the mean and standard deviation of individual values (circles) from at least three independent experiments. The *P* values shown as numbers are from unpaired Student's t-test. Bars: light grey, without myricetin; dark grey, with myricetin.

(19.5 ± 8.34 versus 20.0 ± 7.20 µM, $P = 0.92$).

In SW620 cells, myricetin did not alter cellular platinum accumulation (326 ± 163 versus 213 ± 135 pmol/mg protein, $P = 0.53$) or oxaliplatin-induced growth inhibition IC50 values (69.4 ± 30.2 versus 103 ± 46.8 µM, $P = 0.28$).

In WiDR cells, myricetin did not alter cellular platinum accumulation (328 ± 105 versus 227 ± 76.7 pmol/mg protein, $P = 0.39$) or oxaliplatin-induced growth inhibition IC50 values (163 ± 53.9 versus 147 ± 64.6 µM, $P = 0.65$).

5.3.3. *Relationship of oxaliplatin growth inhibition values to clinical oxaliplatin plasma concentrations*

To examine the clinical relevance of the *in vitro* effects of MRP2 and myricetin on the sensitivities of human gastrointestinal cancer cell lines to oxaliplatin, IC-50 growth inhibition values were plotted with clinically-achievable plasma oxaliplatin concentration (Figure 5.3). Previously, others in this laboratory had shown that most of the unbound platinum in plasma of oxaliplatin-treated patients was in the form of intact oxaliplatin at steady-state oxaliplatin concentrations during 2 h infusion ranging from 3.75 µM to 11.25 µM (59). During two hour exposure to oxaliplatin, HepG2 cells showed IC-50 values of 9.24 ± 2.97 µM which lied within the range. In contrast, the other cell lines exhibited IC-50 values higher than clinical oxaliplatin plasma concentrations. During exposure to oxaliplatin with myricetin, IC-50 values of MiaPACA-2, PANC-1 and HepG2 were significantly decreased by between 2- to 4-fold. Myricetin decreased IC-50 values of MiaPACA-2 and PANC-1 to be within the clinically-achievable oxaliplatin plasma concentration range, and the sensitivity of HepG2 cells to oxaliplatin concentrations to be lower than the clinically-achievable range (Figure 5.3).

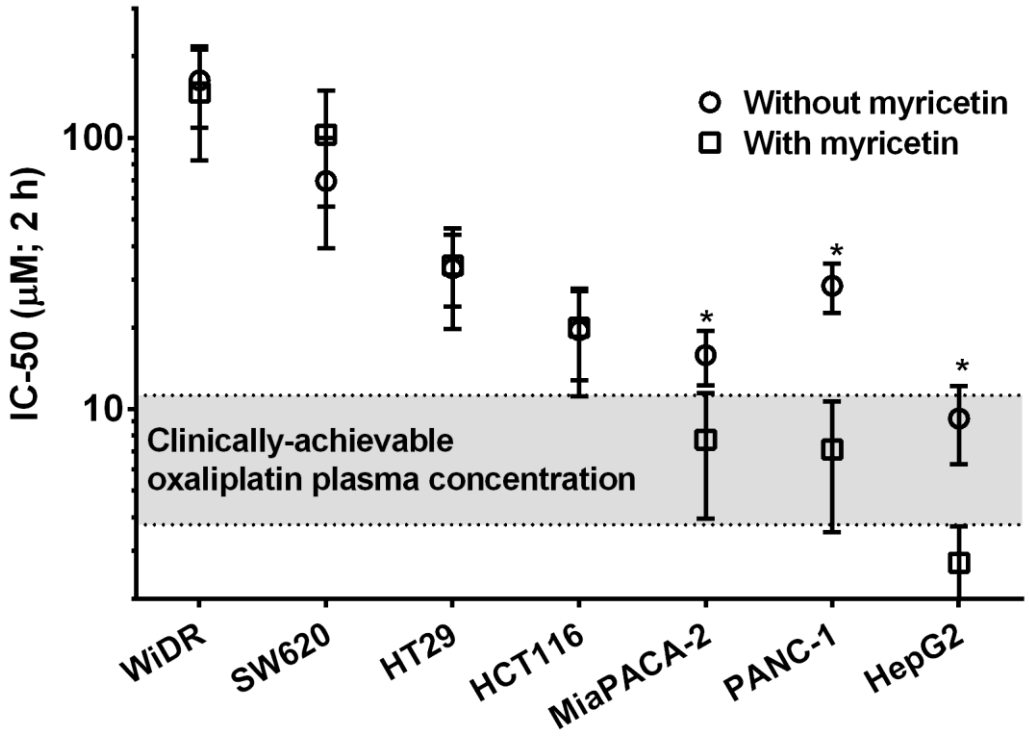


Figure 5.3 Effect of myricetin on oxaliplatin-induced growth inhibition in human gastrointestinal cancer cell lines: oxaliplatin growth inhibition IC-50 values related to clinical oxaliplatin plasma concentrations

A panel of seven human gastrointestinal cancer cell lines, including the WiDR, SW620, HT29, HCT116, MiaPACA-2, PANC-1 and HepG2 lines, were treated with a range of different concentrations of oxaliplatin for two hours, with or without myricetin (60 μM) for 2.5 hours, then cultured in drug-free medium for three days before measurement of the number of viable cells by MTT assay. IC-50 values were determined from the percent viability versus oxaliplatin concentrations curves. Symbols are the mean and standard deviation of IC-50 values from at least three independent experiments. The shaded area represents the reported range of clinically achievable oxaliplatin plasma concentrations in patients during 2 h infusion of oxaliplatin which was from 3.75 to 11.25 μM (59). Myricetin sensitized some gastrointestinal cancer cell types to clinically achievable concentrations of oxaliplatin. The * represent *P* value < 0.05 from unpaired Student's t-test. Symbols: circle, without myricetin; square, with myricetin.

5.3.4. *ABCC2-siRNA mediated knockdown in HepG2 cells*

HepG2 cells were used for *ABCC2* gene expression knockdown studies because of their high basal expression level of *ABCC2* and low levels of accumulation of oxaliplatin-derived platinum. Effects of siRNA-mediated knockdown of *ABCC2* expression on mRNA expression of *ABCC2*, other ABC transporters and housekeeping genes; CDCF accumulation; accumulation of oxaliplatin-derived platinum; and cell growth were studied (Figure 5.4).

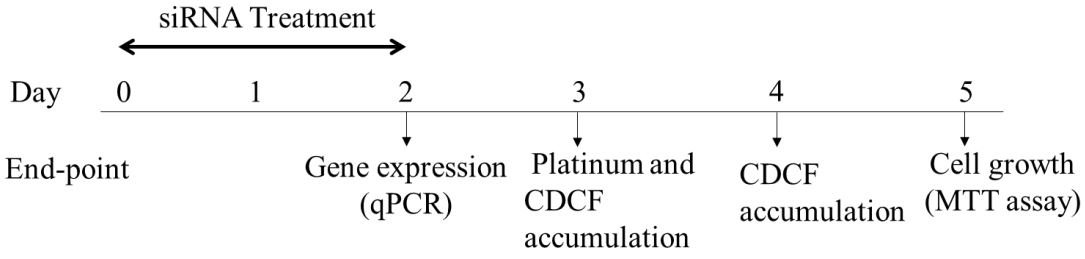


Figure 5.4 Experimental design of *ABCC2* expression knockdown studies

5.3.4.1. Effects on expression of ABCC2 and other genes

Basal mRNA expression levels of *ABCC2* were higher as compared to *ABCC1, ABCC3, ABCC4, ABCC5* and *ABCB1* but comparable to the expression levels of house-keeping genes (Figure 5.5). Averaged reference gene corrected $2^{\wedge-\Delta Ct}$ values for *ABCC2* mRNA expression in HepG2 cells, measured independently by customised PCR array analysis and conventional real-time qPCR, were 1.36 and 0.52 respectively, indicating high level of *ABCC2* expression. In contrast, *ABCC1*, *ABCC3*, *ABCC4*, *ABCC5* and *ABCB1* were expressed at lower levels, with reference gene corrected $2^{\wedge-\Delta Ct}$ values ranging from 0.0003 to 0.065.

After treatment with *ABCC2*-siRNA, the *ABCC2* mRNA expression level in HepG2 cells was significantly reduced with less or no effects on the expression levels of *ABCC1*,

ABCC3, *ABCC4*, *ABCC5*, *ABCB1* and house-keeping genes (Figure 5.5). *ABCC2* expression levels in HepG2 cells treated with *ABCC2*-siRNA-1, -2 and -3, were reduced by 32500-, 22000-, and 5020-fold, respectively. In contrast, control-siRNA treatment reduced *ABCC2* expression by 24.6-fold. *ABCC1*, *ABCC3*, *ABCC4*, *ABCC5* and *ABCB1* expression levels were reduced after siRNA treatment by between 0.122- to 1930-fold. The mRNA expression levels of house-keeping genes were reduced by siRNA treatment by between 0.387- to 71.7-fold.

5.3.4.2. Effects on CDCF accumulation

To investigate the effect of *ABCC2*-siRNA treatment on MRP2 functional activity, the cellular accumulation of a model MRP2 substrate, CDCF, was studied. Basal CDCF accumulation in HepG2 cells was lower as compared to that in HEK-MRP2 and HEK-P cells. After 48 h incubation of HepG2 cells with *ABCC2*-siRNAs, siRNA-containing medium was replaced with normal medium for another 24 or 48 h followed by incubation of cells with 10 μM CDCFDA (a pro-drug of CDCF) for 90 min and measurement of cellular CDCF accumulation by fluorescence. *ABCC2*-siRNA treatment increased cellular CDCF accumulation in HepG2 cells measured at 24 or 48 h post-siRNA treatment ($P = 0.0023$ or $P < 0.0001$ respectively, one-way ANOVA). Cellular CDCF accumulation in HepG2 cells treated with *ABCC2*-siRNA-1 was increased significantly with the highest fold-change compared to cells untreated with siRNA reagents; the fold-increases for cellular CDCF accumulation measurement at 24 or 48 h post-siRNA treatment were 1.64 and 2.23 respectively ($P < 0.05$ and $P < 0.0001$ respectively, unpaired t-tests) [Figure 5.6].

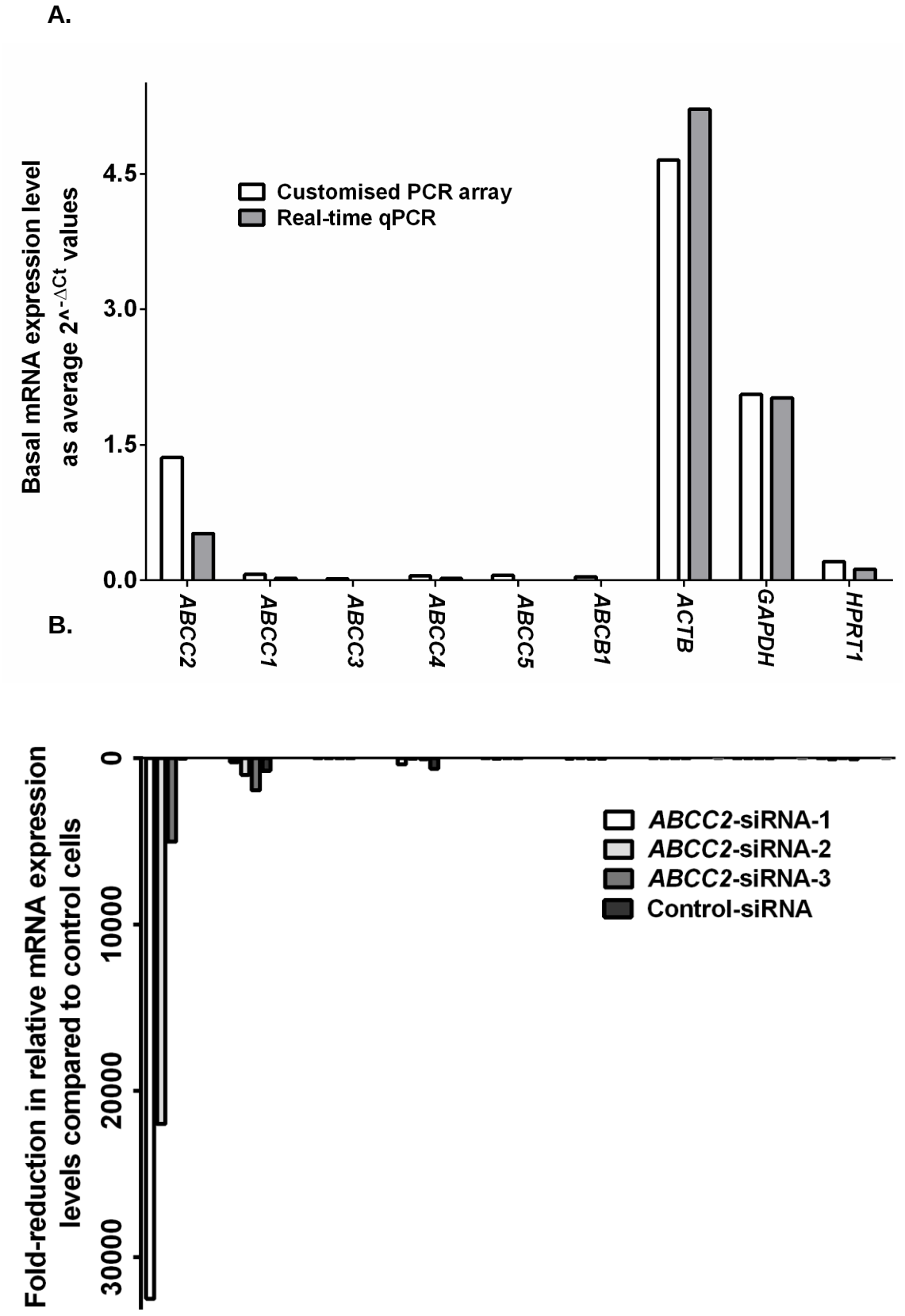
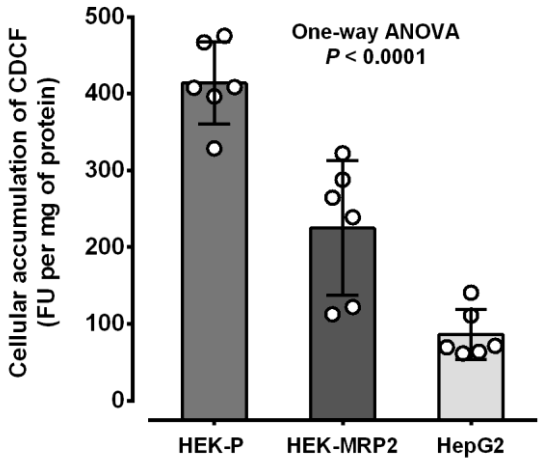

A.
Basal mRNA expression level as average 2^-ΔCt values
4.5
3.0
1.5
0.0
Customised PCR array
Real-time qPCR
ABCC2
ABCC1
ABCC3
ABCC4
ABCC5
ABCB1
ACTB
GAPDH
HPRT1
B.
Fold-reduction in relative mRNA expression levels compared to control cells
0
10000
20000
30000
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3
Control-siRNA

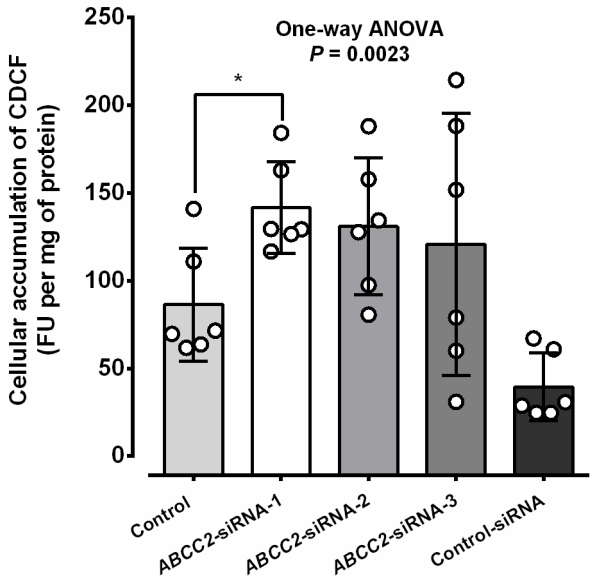
Figure 5.5 ABCC2 gene expression and knockdown in HepG2 cells.

(A) Basal expression of *ABCC2 r*elative to that of *ABCC1, ABCC3, ABCC4, ABCC5 ABCB1*, beta-actin, GAPDH and HPRT1 in HepG2 cells. Gene expression was measured by real-time qPCR array analysis (white) and conventional real-time qPCR measurement (dark grey) using the SYBR Green fluorescent dye method. Data are presented as reference gene corrected $2^{\wedge -\Delta Ct}$ values averaged from two independent experiments. (B) Effects of *ABCC2*-siRNA-1, *ABCC2*-siRNA-2, *ABCC2*-siRNA-3 and control-siRNA on the expression of *ABCC1-5, ABCB1*, beta-actin, GAPDH and HPRT1 mRNA. Gene expression was measured by real-time qPCR using the SYBR Green fluorescent dye method. Data are presented as the fold-reduction in reference gene corrected $2^{\wedge -\Delta Ct}$ mRNA expression levels from control HepG2 cells averaged from three independent experiments.

B.


Cellular accumulation of CDCF
(FU per mg of protein)
500
400
300
200
100
0
One-way ANOVA
P < 0.0001
HEK-P
HEK-MRP2
HepG2

C.


Cellular accumulation of CDCF
(FU per mg of protein)
250
200
150
100
50
0
One-way ANOVA
P = 0.0023
*
Control
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3
Control-siRNA

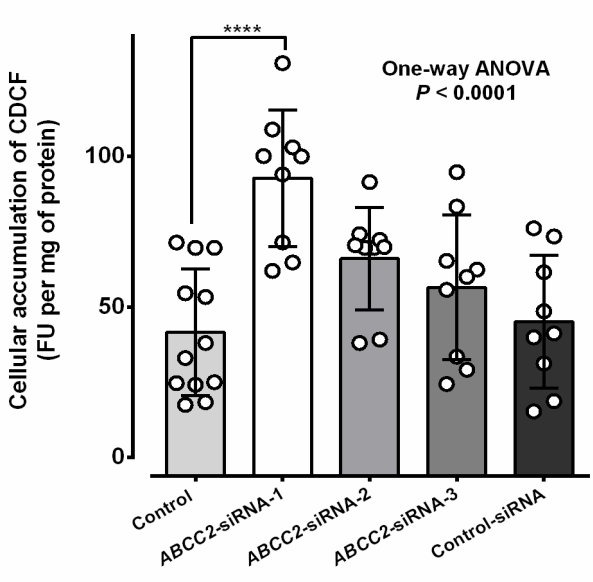

Cellular accumulation of CDCF
(FU per mg of protein)
100
50
0
****
One-way ANOVA
P < 0.0001
Control
ABCC2-siRNA-1
ABCC2-siRNA-2
ABCC2-siRNA-3
Control-siRNA

Figure 5.6 CDCF accumulation in HepG2 cells: comparison to HEK-MRP2 and HEK-P cells and effect of *ABCC2*-siRNA knockdown

(A) Basal accumulation of CDCF in HepG2, HEK-MRP2 and HEK-P cells was measured as fluorescence after 90 min incubation of the 80%-90% confluent cells with 10 µM CDCFDA. Basal accumulation of CDCF in HepG2 cells was lower as compared to HEK-MRP2 and HEK-P cells. (B,C) HepG2 cells were treated with siRNAs for 48 h followed by incubation in drug-free medium for 24 h (B) or 48 h (C) before 90 min incubation with 10 µM CDCFDA and measurement of cellular accumulation of CDCF by fluorescence. *ABCC2*-siRNA knockdown increased CDCF accumulation in HepG2 cells. Data are presented as the mean (bar) and standard deviation (error bar) of individual values (circles) from two independent experiments. *P*-values shown as numbers represent are from one-way ANOVA and those shown as * ($P<0.05$) and **** ($P<0.0001$) are from unpaired t-tests.

5.3.4.3. Effects on accumulation of oxaliplatin-derived platinum

ABCC2-siRNA knockdown significantly increased the accumulation of oxaliplatin-derived platinum in HepG2 cells by approximately 3-fold and 2-fold as compared to treatment with control-siRNA and untreated controls respectively ($P < 0.0001$; one-way ANOVA). Platinum accumulation in untreated control HepG2 cells was 46.9 ± 9.3 pmol per mg of protein while that of cells treated with control-siRNA were 59.8 ± 8.8. In contrast, platinum accumulation in HepG2 cells treated with *ABCC2*-siRNA-1 was significantly higher (135 ± 45.0 pmol per mg of protein) [Figure 5.7].

5.3.4.4. Effects on cell growth

To investigate effects of siRNA reagents on cell growth, HepG2 cells were treated with siRNAs for 48 h, then with or without oxaliplatin (5 μM) for 2 h, before incubation in drug-free medium for three days and measurement of the number of viable cells by MTT assay. Both control-siRNA and *ABCC2*-siRNA significantly inhibited cell growth in the presence or absence of oxaliplatin ($P < 0.0001$, two-way ANOVA) [Figure 5.8].

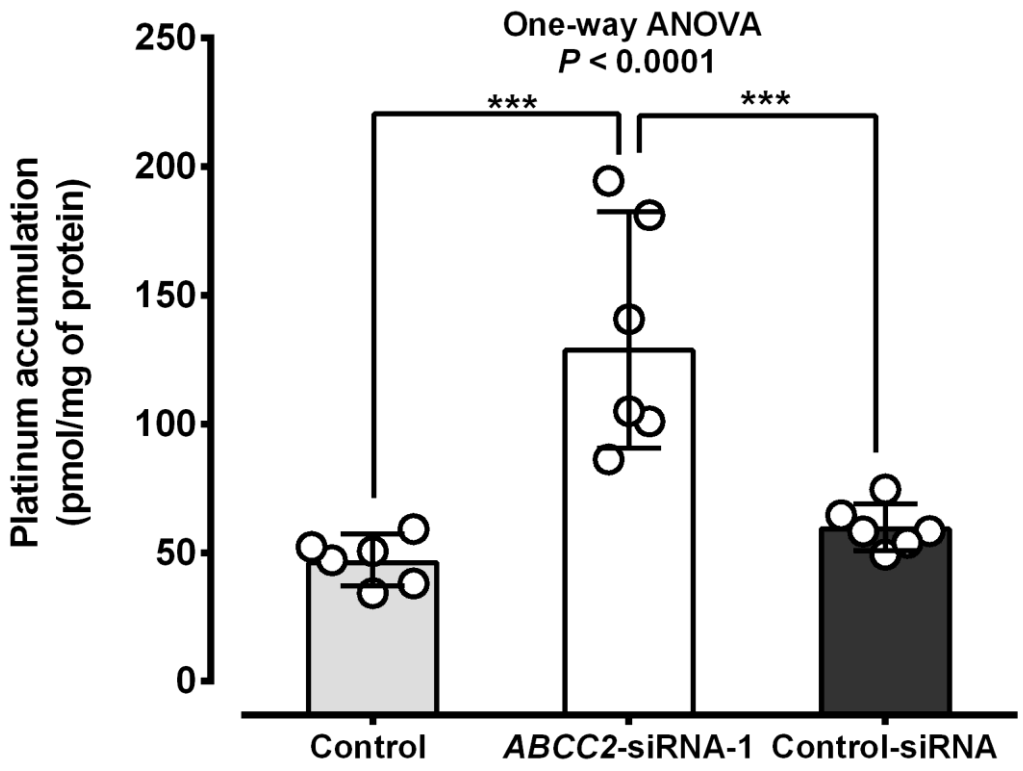


Figure 5.7 Platinum accumulation in HepG2 cells: effect of *ABCC2*-siRNA knockdown

HepG2 cells were treated with *ABCC2*-siRNA-1 or control-siRNA for 48 h followed by incubation in drug free medium for 24 h before treatment with oxaliplatin (50 μM) for 2 h and measurement of cellular accumulation of platinum by ICP-MS. Data were presented as the mean (bar) and standard deviation (error bar) of individual values (circles) pooled from three independent experiments. *ABCC2*-siRNA knockdown significantly increased platinum accumulation in HepG2 cells by 2-fold as compared to untreated controls and treatment with control-siRNA. The *P*-value shown as the number is from one-way ANOVA and those show as *** ($P < 0.001$) are from Tukey's multiple comparison tests following one-way ANOVA. Bars: light grey, untreated control; dark grey, control-siRNA; white, *ABCC2*-siRNA-1.

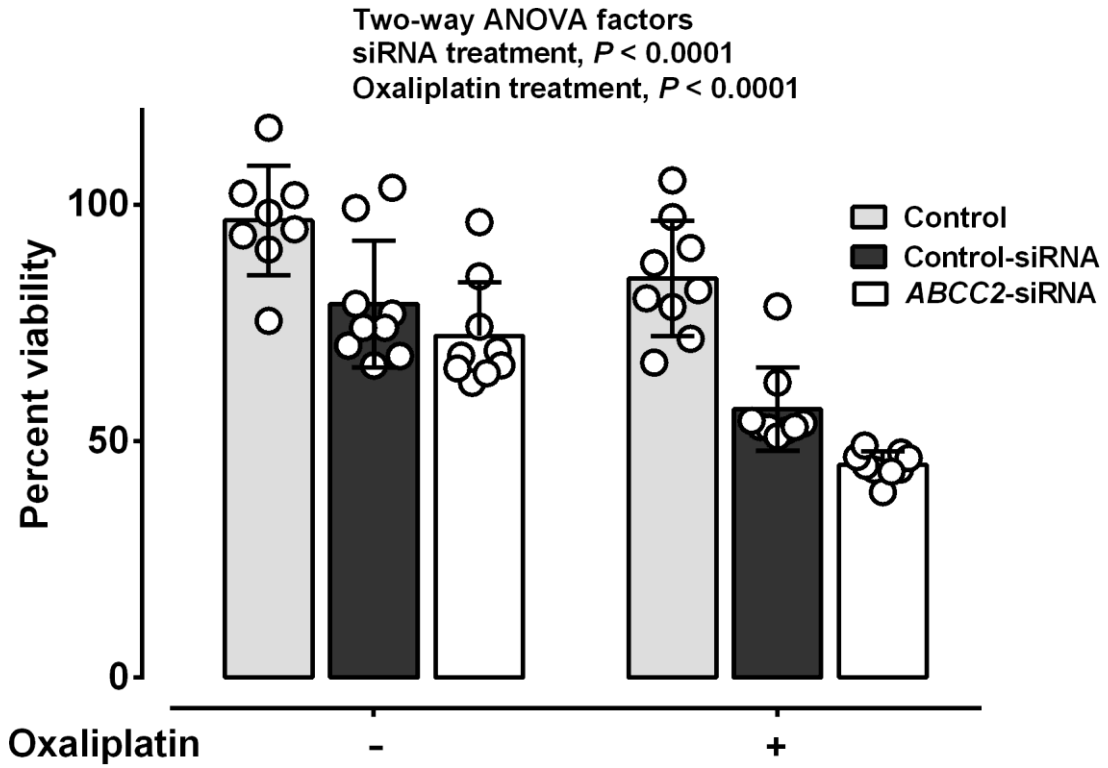


Figure 5.8 Non-selective inhibition of growth of HepG2 cells by siRNA reagents

HepG2 cells were treated with siRNAs for 48 h, then with or without oxaliplatin (5 μM) for 2 h before incubation in drug-free medium for three days and measurement of the number of viable cells by MTT assay. Data were presented as the mean (bar) and standard deviation (error bar) of individual values (circles) pooled from two independent experiments. Treatment with *ABCC2*-siRNA or control-siRNA and oxaliplatin inhibited the growth of HepG2 cells. The *P*-values shown as figure are from two-way ANOVA. Bars: light grey, untreated control; dark grey, control-siRNA; white, *ABCC2*-siRNA-1.

5.4. Discussion

In the experiments presented in this chapter, seven human gastrointestinal cancer cell lines were used to investigate whether MRP2 was involved in determining the cellular accumulation of oxaliplatin-derived platinum and sensitivity to oxaliplatin-induced inhibition of growth in human gastrointestinal cancer. Only a limited number of previous *in vitro* studies have investigated the role of MRP2 in cellular accumulation and activity of oxaliplatin using human gastrointestinal cancer cell lines with contradictory findings. The role of MRP2 in determining the accumulation and antitumour activity of oxaliplatin in human gastrointestinal cancers was still unclear. In these experiments presented in this chapter, human gastrointestinal cancer cell lines were firstly characterised in regards to their *ABCC2* expression level and platinum accumulation. The HepG2 and PANC-1 cell lines showed the highest expression levels of *MRP2* and the lowest cellular platinum accumulation levels. The remaining cell lines showed lower levels of MRP2 expression and higher levels of cellular platinum accumulation. Therefore, HepG2 and PANC-1 cells lines were identified as potential *in vitro* models of human gastrointestinal cancer with *MRP2*–mediated deficits in oxaliplatin accumulation, and were selected on this basis for further study.

Further evidence for the involvement of MRP2 in determining the cellular accumulation of oxaliplatin-derived platinum and sensitivity to oxaliplatin-induced growth inhibition in human gastrointestinal cancer cells came from experiments in HepG2 and PANC-1 cell lines using myricetin as a MRP2 inhibitor and, in HepG2 cells, using siRNA-mediated knockdown of *ABCC2* gene expression. Myricetin significantly increased cellular platinum accumulation and sensitivity to oxaliplatin-induced growth inhibition in HepG2 and PANC-1 cells. Treatment of HepG2 cells with *ABCC2*-siRNA-1, -2 and -3 efficiently

reduced mRNA levels of *ABCC2* and the functional activity of MRP2 measured by cellular CDCF accumulation level. In contrast, *ABCC2*-siRNA gene knockdown had less or no effect on the mRNA expression of other ABC and housekeeping genes. Cellular platinum accumulation level was increased in HepG2 cells after treatment with siRNA-1 compared to untreated controls or treatment with control-siRNA. Because the siRNA reagents nonspecifically inhibited the growth of HepG2 cells, it was not possible to demonstrate whether or not *ABCC2* knockdown sensitized HepG2 cells to oxaliplatin-induced growth inhibition. All these findings together provide further evidence for the contribution of MRP2-mediated oxaliplatin transport across the cellular membrane to the reduced cellular accumulation of oxaliplatin-derived platinum and reduced cellular sensitivity to oxaliplatin-induced growth inhibition in some human gastrointestinal cancer cells.

Five of the seven gastrointestinal cell lines studied appeared to lack MRP2-mediated deficits in oxaliplatin accumulation. The HT29, SW620, WiDR, MiaPACA-2 and HCT116 lines accumulated between 2.3- and 8.3-fold more platinum during their exposure to oxaliplatin as compared to the HepG2 and PANC-1 lines. Their *ABCC2* mRNA expression levels were between 5.8- and 680-fold lower as compared to the HepG2 and PANC-1 lines. Unlike the HepG2 and PANC-1 lines, myricetin had no effect on cellular platinum levels, and little or no effect on oxaliplatin-induced growth inhibition in the HT29, SW620, WiDR, MiaPACA-2 and HCT116 lines. These findings suggest that some, but not all, gastrointestinal cancers have MRP2-mediated deficits in oxaliplatin accumulation and sensitivity. Only a subset of gastrointestinal cancer would appear potentially responsive to therapeutic strategies based on targeting MRP2 to increase oxaliplatin sensitivity and accumulation. Therefore, identifying those individuals with

tumours with MRP2-mediated deficits in oxaliplatin accumulation would be an essential first step in the application of an MRP2-targeted therapeutic strategy.

A panel of 7 human gastrointestinal cancer cell lines used in this study showed a range of different cellular sensitivities to oxaliplatin-induced cytotoxicity and cellular platinum accumulation levels. Some cell lines with relatively low cellular sensitivities to oxaliplatin such as WiDR and SW620 had relatively high cellular platinum accumulation levels. Hence, no strong correlation could be concluded between cellular platinum accumulation level and cellular sensitivity to oxaliplatin for this set of human cancer cell lines. This could be explained by the fact that cellular resistance to oxaliplatin observed in gastrointestinal cancer cells involves complex molecular mechanisms apart from reduced cellular accumulation of oxaliplatin. These mechanisms include alterations in DNA damage repair system, altered apoptosis which prevent cell death induced by drug and epigenetic mechanisms. Oxaliplatin induces formation of platinum-DNA adducts resulting in the DNA damage and cell death, and increased DNA damage repair leads to increased cell survival and consequently, increased cellular resistance to oxaliplatin. Increased activity or expression levels of ERCC1 (excision repair cross-complementation group 1), a crucial excision nuclease enzyme involved in nucleotide excision repair (NER), has been shown to be associated with increased resistance to oxaliplatin-containing chemotherapies in colorectal cancer (73, 82-84) and gastric cancer (85-87). KRAS mutation has been shown to be involved in determining oxaliplatin sensitivity of colorectal cancer (CRC) cells via downregulation of ERCC1 (88, 89). Changes in cellular apoptotic pathways could also be involved in cellular oxaliplatin resistance; mutations of apoptotic protein p53 (83, 90) and anti-apoptotic factors including nuclear factor-κβ (NFκβ) (91) and taxol-resistant gene 1 (Txr1) (92) have been shown to be involved in altered cellular susceptibility to apoptosis induced by oxaliplatin. Tumour

microenvironmental conditions, such as hypoxia, may also be involved in cellular oxaliplatin resistance (93). Epigenetic mechanisms such as altered DNA methylation, histone modifications and altered expression of micro RNAs could play a role in cellular resistance of oxaliplatin although the evidences are still small (313). In addition to the fact that several important mechanisms are involved in the cellular resistance of human gastrointestinal cancer cell lines to oxaliplatin, different cell lines generally have different cellular expression and functional levels of these molecular pathways and mechanisms. Hence, it has become difficult to provide the firm conclusion regarding the correlation between cellular platinum accumulation levels and cellular sensitivity to oxaliplatin-induced cytotoxicity in human gastrointestinal cancer cell lines.

In summary, deficit in the cellular accumulation of oxaliplatin-derived platinum and sensitivity to oxaliplatin-induced growth inhibition were observed in HepG2 and PANC-1 cells. These deficits were reversed by a model MRP2 inhibitor, myricetin, consistent with MRP2 having contributed to the cellular platinum accumulation defect and lower oxaliplatin sensitivity of the HepG2 and PANC-1 cancer cell lines. Moreover, siRNA-mediated knockdown of *ABCC2* expression in HepG2 cells increased their cellular accumulation of platinum, confirming the mechanism of MRP2-mediated cellular transport of oxaliplatin in HepG2 cells. However, the cytotoxic effects of siRNAs of HepG2 cell growth confounded the examination of the involvement of MRP2 in determining cellular sensitivity to oxaliplatin-induced growth inhibition. These findings support a role of MRP2 in determine cellular responses of human gastrointestinal cancer cells to oxaliplatin and further investigation in future.

Chapter 6. General discussion

6.1. Introduction

In this final chapter, the important findings of the thesis will be summarised and discussed with regard to their significance and implications for understanding the pharmacology of oxaliplatin and its clinical use in the treatment of gastrointestinal cancer. The first topic of discussion concerns the role of MRP2 in mediating the active membrane transport of oxaliplatin-derived platinum and the identification of potential oxaliplatin-derived substrates involved in this MRP2-mediated transport process. The pharmacological and clinical relevance of the changes in cellular platinum accumulation and sensitivity to oxaliplatin mediated by MRP2 observed in the cellular models are then considered, followed by discussion on the potential significance of MRP2 in determining the disposition and toxicity of oxaliplatin. Finally, potential treatment strategies for gastrointestinal cancer patients receiving oxaliplatin-based chemotherapy based on these new insights into MRP2 mediated platinum transport, and implications of these MRP2-related platinum transport mechanisms for personalised therapy of human gastrointestinal cancer are elaborated upon.

6.2. Summary of the findings

Three *in vitro* models, including MRP2-expressing membrane vesicles, HEK293 and human gastrointestinal cancer cell lines were used to study the involvement of MRP2 in determining oxaliplatin transport, cellular accumulation and cellular growth inhibition.

The membrane vesicle studies demonstrated that MRP2 mediated the ATP-dependent membrane transport of platinum derived from oxaliplatin [Vmax = 2680 pmol per mg of protein per 10 min (95%CI of 2010 to 3360 pmol per mg of protein per 10 min); Km =

301 µM (95%CI of 163 to 438 µM)], which has not been shown before to our knowledge. Studies of oxaliplatin stability in membrane vesicle incubation buffer (oxaliplatin stability half-life = 2.24 hrs with 95% CI of 2.08 to 2.43 hrs) suggested that intact oxaliplatin and its early degradation product, $[Pt(DACH)oxCl]^-$, were likely substrates for MRP2-mediated active transport.

The HEK293 cell line studies showed cellular accumulation of oxaliplatin-derived platinum and sensitivity to oxaliplatin-induced growth inhibition were both decreased by up to two-fold in HEK-MRP2 cells that were genetically modified to stably overexpress the *ABCC2* gene and confirmed to have moderately increased functional overexpression of MRP2 protein compared to the parental cell line (HEK-P). Inhibition of MRP2 with myricetin reversed these deficits in platinum accumulation and oxaliplatin sensitivity conferred by the overexpression of MRP2.

Studies of human gastrointestinal cancer cell lines demonstrated MRP2-mediated deficits in cellular accumulation of oxaliplatin-derived platinum were observed only in HepG2 and PANC-1 cells (0.14- and 0.34-fold difference in relative to HEK-P cells), which had high expression levels of *ABCC2* mRNA comparable to the HEK-MRP2 cells (1360- and 139-fold difference in relative to HEK-P cells). Myricetin increased cellular platinum accumulation of HepG2 and PANC-1 by 5- and 2.8-fold, respectively, and their sensitivity to oxaliplatin-induced growth inhibition by 3.4- and 4-fold, respectively. This provided further evidence for the role of MRP2 in the cellular efflux of platinum derived from oxaliplatin. Moreover, siRNA-mediated knockdown of *ABCC2* expression in HepG2 cells increased their cellular accumulation of platinum by approximately up to 3-fold compared to control-siRNA treated HepG2 cells, confirming the likely mechanism of MRP2-mediated cellular efflux of oxaliplatin or a platinum metabolite in these cells.

Inhibiting MRP2 sensitised MRP2-overexpressing gastrointestinal cancer cells to growth inhibition induced by clinically achievable concentrations of oxaliplatin (3.75 to 11.25 μM). These findings support a role for MRP2 in determining the therapeutic response to oxaliplatin treatment in some human gastrointestinal cancers but further investigations are required to confirm this in patients.

6.3. MRP2-mediated active transport of oxaliplatin

A novel finding of this research was that MRP2 transported oxaliplatin-derived platinum in an ATP-dependent manner. Transport studies based on MRP2-overexpressing inside-out membrane vesicle models suggested that MRP2 mediated oxaliplatin-derived platinum transport across the membrane in the presence of ATP. Membrane vesicle accumulation of platinum was found to be higher with expression of MRP2 compared to control vesicles, and in the presence of ATP compared to the absence of ATP in MRP2-overexpressing membrane vesicles. The rate of platinum membrane transport, mediated by MRP2 and ATP, increased non-linearly with increasing oxaliplatin exposure concentration approaching a plateau level, suggesting saturation of the transport process. MRP2 inhibitors (myricetin or MK571) inhibited the membrane vesicle accumulation of platinum. The membrane vesicle accumulation of the known MRP2 substrate (CDCF) mediated by MRP2 and ATP was shown to be inhibited by oxaliplatin, suggesting competition by oxaliplatin for the MRP2 substrate binding site. Taken together, these findings have provided experimental evidence for oxaliplatin interacting with MRP2 protein and being transported across membranes by MRP2-mediated active transport processes.

MRP2-mediated oxaliplatin transport was likely to be involved in determining the cellular accumulation of oxaliplatin in HEK293 and human gastrointestinal cancer cell

lines. Cellular accumulation of oxaliplatin-derived platinum was reduced in MRP2-overexpressing HEK293 cells and in HepG2 and PANC-1 cells which had high expression level of *ABCC2* mRNA comparable to HEK-MRP2 cells. Such deficits were reversed by myricetin in HEK-MRP2 cells and in HepG2 and PANC-1 cells, and by the siRNA-mediated transient silencing of *ABCC2* in HepG2 cells. Therefore, these findings suggested the possible mechanism of MRP2-mediated cellular efflux of oxaliplatin or oxaliplatin-derived platinum metabolites in human gastrointestinal cancer cells with high *ABCC2* expression level.

Three in-vitro studies investigating the association of MRP2 with the cellular accumulation of oxaliplatin or cellular resistance to oxaliplatin using oxaliplatin-resistant human cancer cell lines have been reported to date (74, 77, 206). Liu and group found that MRP2 expression measured by western blot was increased in oxaliplatin resistant human colon cancer cell lines, namely SW620/L-OHP and LoVo/L-OHP, compared to parental cell lines, suggesting the involvement of MRP2 in oxaliplatin resistance of human colon cancer cells (206). Beretta et al. reported that an oxaliplatin-resistant variant of the human ovarian carcinoma cell line (IGROV-1/OHP) showed reduced cellular platinum accumulation (nearly 6-fold) and increased expression levels of MRP1 and MRP4 in comparison to its parental cell line (IGROV-1). However, MRP2 was not detected in either parental or resistant cell lines (74). More recently, the MRP1 and MRP2 inhibitor (Gü83) was reported to increase cellular platinum accumulation in human ileocecal colorectal adenocarcinoma cell line HCT-8 and its oxaliplatin-resistant variant HCT-8ox by nearly 3-fold and 2-fold, respectively, supporting the hypothesis that MRP2 contributes to the cellular transport of oxaliplatin and ultimately to the cellular accumulation of oxaliplatin (77). However, the data was relatively limited and inconclusive about the role of MRP2 in cellular platinum accumulation and cellular

resistance to oxaliplatin. There has been no study investigating the direct involvement of MRP2 in membrane transport of oxaliplatin or oxaliplatin-derived platinum species using membrane vesicles. The current study was the first to investigate the MRP2-mediated transport mechanism of oxaliplatin using membrane vesicles and the involvement of MRP2 in cellular oxaliplatin accumulation using MRP2-over-expressing cell lines and human gastrointestinal cancer cell lines. This was also the first study to show that MRP2 mediates the membrane transport of oxaliplatin-derived platinum, and high expression level of *ABCC2* is associated with reduced cellular accumulation of oxaliplatin-derived platinum.

6.4. Relevance of changes in cellular platinum accumulation and IC-50 values of cellular models

Cellular platinum accumulation in HEK-MRP2 cells was 2.3-fold less than control HEK-P cells and was increased by 2.6-fold with myricetin treatment to a level similar to that observed in HEK-P. HEK-MRP2 were 2.7-fold more resistant to oxaliplatin compared to HEK-P cells and were resensitised to oxaliplatin-induced growth inhibition effects by myricetin; oxaliplatin's IC-50 for HEK-MRP2 was reduced by 2.2-fold by myricetin treatment. Hence, MRP2-over-expression was associated with 2- to 3-fold reductions in cellular platinum accumulation and cellular sensitivity to oxaliplatin-induced growth inhibition, and such deficits in HEK-MRP2 cells were reversed to levels similar to those observed in oxaliplatin-sensitive HEK-P cells by myricetin. In the panel of seven human cancer cell lines examined, the effects of myricetin on reversing MRP2-mediated transport and resistance to oxaliplatin were only observed in PANC-1 and HepG2 cell lines, both of which showed a high expression level of *ABCC2*. In the PANC-1 cell line, myricetin increased platinum accumulation by 2.8-fold and cellular sensitivity by 4-fold.

In the HepG2 cell line, myricetin increased the cellular platinum accumulation by 5.4-fold and the cellular sensitivity to oxaliplatin by 3.4-fold. Interestingly, the siRNA-mediated transient silencing of *ABCC2* in HepG2 cells caused similar changes as myricetin, increasing cellular platinum accumulation by 2.9-fold and cellular sensitivity to oxaliplatin-induced growth inhibition by 1.9-fold compared to untreated HepG2 cells. These findings suggested that defective cellular platinum accumulation was a determinant of cellular sensitivity to oxaliplatin in certain cell types, and that a high level of *ABCC2* expression and inhibition of MRP2 were associated with 2- to 5-fold overall changes in cellular platinum accumulation and sensitivity to oxaliplatin-induced growth inhibition.

It was of interest to explore the pharmacological and clinical significances of these 2- to 5-fold changes in cellular platinum accumulation and sensitivity to oxaliplatin-induced growth inhibition conferred by high *ABCC2* expression level and MRP2 inhibition. Comparison of the IC-50 growth inhibition values for oxaliplatin in the seven human gastrointestinal cancer cell lines with previously reported clinically-achievable steady-state plasma oxaliplatin concentrations (ranging from 3.75 to 11.25 μM) (59), indicated that myricetin treatment reduced the IC-50 values for oxaliplatin of MiaPACA-2 and PANC-1 to within this concentration range, and that of HepG2 to below this concentration range. This has suggested that the relatively small 2- to 5-fold oxaliplatin-induced growth inhibitory effects induced by myricetin could be clinically very relevant in achieving more effective anti-tumour activity for oxaliplatin within the clinically-achievable oxaliplatin plasma concentration range. Hence, these findings indicated that MRP2 may play a role in cellular sensitivity of certain human cancer types to oxaliplatin via determining cellular platinum accumulation levels and changes in the latter induced by MRP2 inhibition, although small in magnitude, may be clinically significant. However, these *in vitro* studies have obvious limitations and the clinical relevance of

these findings should be further determined using *in vivo* models; for example, the establishment of tumours in mice using human gastrointestinal cancer cell lines (e.g., HepG2 & PANC-1), followed by investigation of their response to oxaliplatin with or without myricetin using parameters such as tumour growth delay and response to treatment. Additional pre-clinical studies might include patient tumour biopsies evaluated for MRP2 expression levels and used to establish xenograft models for further assessment of MRP2 involvement in cellular mechanisms of oxaliplatin resistance.

6.5. Potential oxaliplatin-derived substrates for MRP2 transport

The membrane vesicle transport studies presented in this thesis suggested that intact oxaliplatin and/or $[Pt(DACH)oxCl]^-$ were possible substrates for MRP2-mediated transport of oxaliplatin-derived platinum. In these studies, ICP-MS was used to measure membrane vesicle accumulation of platinum and, although highly sensitive and specific for platinum (264, 265), it does not distinguish between intact oxaliplatin and other platinum degradation products that may become associated with the membrane vesicles during their incubation with oxaliplatin. As oxaliplatin is known to degrade in aqueous solutions containing chloride ions (58, 247) or glutathione (51, 53), it could not be assumed that oxaliplatin had remained intact in the incubation buffer or was the platinum species transported by MRP2 (even with the use of short incubation times of 5 - 20 min). However, stability studies in membrane vesicle incubation medium indicated that oxaliplatin was the main form of platinum present under the experimental conditions used for oxaliplatin transport. $Pt(DACH)Cl_2$ and oxaliplatin-glutathione chelates appeared in the incubation buffer long after the end of the incubation period used for the transport studies. These findings suggested that intact oxaliplatin itself or early degradation products such as [Pt(DACH)oxCl] were likely substrates for MRP2.

Previous studies have suggested that oxaliplatin undergoes degradation via oxalate ligand displacement reactions with chloride ions forming a monochloro oxalate ring opened intermediate $[Pt(DACH)oxCl]^-$ and $Pt(DACH)Cl_2$ (57, 247) which may further react with water to form monoaquated and diaquated complexes, $[Pt(DACH)(H_2O)Cl]^+$ and $[Pt(DACH)(H_2O)2]^{2+}$, respectively, which may be cytotoxic and react with the DNA (45, 46, 314). Several studies have shown that oxaliplatin can also react with thiol-containing cytoplasmic compounds such as glutathione and cysteine to become inactive complexes (53, 56). Therefore, possible oxaliplatin-derived substrates for MRP2 include oxaliplatin-glutathione conjugates and intermediates of oxaliplatin metabolism such as $[Pt(DACH)oxCl]^-$, $Pt(DACH)Cl_2$, $[Pt(DACH)(H_2O)Cl]^+$ and $[Pt(DACH)(H_2O)_2]^{2+}$. Since MRP2 has been known for transporting glutathione and glutathione-conjugate substrates (219, 315, 316), oxaliplatin-glutathione conjugates may well be MRP2 substrates. However, an HPLC-UV-based analysis of oxaliplatin degradation during an 8 h incubation in membrane vesicle incubation buffer containing both chloride and glutathione indicated that oxaliplatin degraded slowly with a half-life of 2.3 h with approximately 95% and 90% remaining intact after 10 and 20 min incubation, respectively, (the incubation periods used for the membrane vesicle transport assays). Chromatographic peaks corresponding to $Pt(DACH)Cl_2$ or unknown degradation products appeared only after several hours incubation. These findings suggested that intact oxaliplatin, rather than an oxaliplatin-glutathione conjugate, was the major substrate transported by MRP2. However, $[Pt(DACH)oxCl]^-$ also remains as a possible substrate as it is formed early during oxaliplatin degradation in chloride-containing solutions and MRP2 is known for preferring anionic substrates (212, 282-284).

Clarification of whether intact oxaliplatin or a degradation or conjugation product is the major substrate for MRP2-mediated transport is important for further understanding the

role of MRP2 in cytotoxicity and resistance, and in particular for future studies where modification of oxaliplatin's structure may generate new analogues with greater efficacy in resistant cancers. The novel MRP2-over-expressing membrane vesicular transport assay for platinum drug transport studies established in this thesis could be used in future studies to identify the possible substrates of MRP2-mediated transport; for instance, different oxaliplatin-derived platinum complexes such as $[Pt(DACH)oxCl]^-$, $Pt(DACH)Cl_2$, $[Pt(DACH)(H2O)Cl]^+$ and $[Pt(DACH)(H2O)2]^{2+}$ which are possible substrates in MRP2-mediated transport of oxaliplatin-derived platinum could be incubated with MRP2-over-expressing membrane vesicles separately and platinum accumulation in membrane vesicles could be measured with ICP-MS. The membrane vesicular transport assay system using the MRP2-over-expressing membrane vesicles and the direct measurement of vesicle-associated platinum accumulation after oxaliplatin exposure established in this thesis is the first time to be reported. Previous MRP2-mediated transport studies used the MRP2-over-expressing membrane vesicles and a radiolabelled MRP2 substrate, leukotriene, $[^3H]LTC_{4,}$ as a surrogate marker to measure the effect of cisplatin on MRP2 transport (217, 218) or exposed the vesicles to radiolabelled cisplatin and measured the vesicle-associated radioactivity (278). There has been no report on the direct measurement of vesicular platinum accumulation levels using ICP-MS. The direct measurement of vesicular platinum accumulation after exposure of membrane vesicles to oxaliplatin and other platinum-based drugs using ICP-MS-based platinum analysis has advantages over the previously reported indirect measurement of drug accumulation; the ICP-MS-based platinum measurement is safer since there is no radioactivity-related hazard involved in the experiments, and is more accurate, directly reflecting the vesicular accumulation of platinum derived from oxaliplatin instead of indirect measurement of vesicular accumulation of MRP2 substrates. In future, using

membrane vesicles genetically modified to express the membrane transporters of interest, the vesicular transport of platinum-based drugs including oxaliplatin, cisplatin and carboplatin and of platinum complexes derived from the biotransformation of oxaliplatin or other platinum drugs could be studied.

6.6. Significance of MRP2 in drug disposition and toxicity of oxaliplatin

MRP2 protein is physiologically expressed in important tissue barrier sites including luminal surface of enterocytes (116), canalicular membrane of hepatocytes (165-167) and luminal surface of renal proximal tubules (168, 169) which can determine the absorption, distribution and excretion of xenobiotics. MRP2 is expressed in the apical surface of cellular membrane and functions by exporting substances out of cells. It appears to play a role in the body's defence against toxins and drugs by limiting their oral bioavailability and distribution in the body and aiding their excretion into bile and urine (204). This study has demonstrated that MRP2 transports and limits the cellular accumulation of oxaliplatin or a platinum derivative. Hence, MRP2 may play a role in determining the toxicity of oxaliplatin in patients by controlling platinum accumulation in healthy body tissues and also its excretion via bile and urine.

Inter- and intra-patient variability in total body exposure to oxaliplatin (represented by platinum AUC_{0-48}) in patients receiving 130 mg/m^2 every 3 weeks or 85 mg/m^2 every 2 weeks were reported to be 33% and 5%, respectively (48). Oxaliplatin is covalently bound to plasma proteins and rapidly cleared from the circulation by covalent binding to tissues and renal excretion (48). Oxaliplatin is distributed at high concentration in kidney, spleen, intestine and liver, with 50% of administered drug accumulating in the red blood cells within 2 h after administration (47-49). Renal excretion is the main route of elimination for oxaliplatin, accounting for approximately 54% of total oxaliplatin

administered with only 2% in faeces (48). Inter-patient variations in oxaliplatin exposure may be due to variations in distribution and clearance which is influenced by the drug metabolism and excretion. These processes presumably will be influenced by the expression levels or functional activities of membrane transporters involved in oxaliplatin transport including MRP2 at important physiological barrier sites such as intestine, liver and kidney in patients (123, 317, 318). Large variations in the volume of distribution and the tissue distribution of oxaliplatin have been observed in patients (47-49) which may also be related to the tissue expression levels of membrane transporters such as MRP2 involved in oxaliplatin transport (123, 155, 317, 318). However, further population pharmacokinetic studies using blood, urine and fecal samples from gastrointestinal cancer patients receiving oxaliplatin and MRP2 genotyping could help determine whether variations in pharmacokinetic parameters are associated with MRP2 expression levels.

Neurotoxicity in peripheral nerves is a notorious side-effect of oxaliplatin and several studies have shown that this is associated with increased oxaliplatin accumulation in dorsal root ganglions (DRGs) (62, 192, 319, 320). The MRP2 gene expression levels in DRGs are lower than in brain and spinal cord, suggesting that low MRP2 expression in DRGs may contribute to increased cellular oxaliplatin accumulation and neurotoxicity (321, 322). However the data is limited and it would be worthwhile to extensively study MRP2 expression and MRP2-mediated transport of oxaliplatin in DRGs. For example using a nude mouse model, basal mRNA and protein expression levels of MRP2 in DRGs could be measured using PCR and IHC or western blot, respectively, and correlated with DRG platinum accumulation and/or Pt-DNA adducts using ICP-MS. Platinum accumulation in DRGs could also be undertaken after oxaliplatin treatment with or without myricetin.

Oxaliplatin has been reported to induce hepatotoxicity in some patients (11, 12, 323, 324). MRP2 protein is highly expressed in the apical membrane of hepatocytes exporting substrates into the bile (165-167) and previous studies have reported that in patients with Dubin-Johnson syndrome where MRP2 mutation occurs, there is disruption in bilirubin excretion and liver function leading to hyperbilirubinaemia and cirrhosis of the liver (211, 213). Hence, MRP2-mediated platinum transport could play a role in the excretion of oxaliplatin via liver, and subsequently, in oxaliplatin-induced liver toxicity. The significance of MRP2 in oxaliplatin excretion and toxicity, could be explored using animal models for Dubin-Johnson syndrome, such as EHBR or TR- rats (325, 326) or MRP2-knockout mice (173). Oxaliplatin pharmacokinetic and platinum accumulation studies in liver, kidney and DRGs in these animal models would help answer these questions.

6.7. Potential treatment strategy for human gastrointestinal cancer patients receiving oxaliplatin chemotherapy

This research has provided evidence for MRP2's involvement in the transporter-mediated oxaliplatin resistance in human cancer cells. Therefore MRP2 could possibly be used as a target to improve the efficacy of oxaliplatin-based treatment in human gastrointestinal cancer patients. For example, the use of non-toxic plant-derived MRP2 inhibitors, such as myricetin, may be a suitable approach for circumventing oxaliplatin's resistance in gastrointestinal cancer patients. However, this mechanism of resistance is likely to occur only in a subset of cancer cell types, and it would appear necessary for the tumour to be screened for up-regulation of MRP2 before co-treatment with myricetin. It should also be kept in mind that MRP2 is highly expressed in liver (165-167) and moderately expressed in other physiological barriers, such as renal tubules (168, 169) and intestinal epithelial

cells (160), and thus off-target effects of a MRP2 inhibitor on oxaliplatin's pharmacokinetics would have to be considered, particularly in the liver where increased platinum accumulation due to MRP2 inhibition could induce or exaggerate liver toxicity in patients (323, 324, 327). Similar alterations in the pharmacokinetics of other anticancer drugs, such as 5-FU, leucovorin and irinotecan, would also have to be considered when given in combination with a MRP2 inhibitor, such as myricetin. Further *in vitro* and *in vivo* studies should be carried out to explore these issues and confirm the potential effectiveness of myricetin in human gastrointestinal cancers.

In addition, myricetin increased both cellular platinum accumulation and sensitivity to oxaliplatin only in HepG2 and PANC-1 among seven human gastrointestinal cancer cell lines examined, suggesting that this mechanism will be restricted to certain subsets of cancer cell types. It may be necessary for cancer patients to be screened first to determine whether MRP2 could be a possible determinant of oxaliplatin therapeutic efficacy to select individual patients for testing whether myricetin could be effective for increasing the efficacy of oxaliplatin.

6.8. Implications of MRP2 in personalized therapy of human gastrointestinal cancer

Previous studies of colorectal cancer patients receiving 5-fluorouracil/leucovorin and oxaliplatin (FOLFOX-4) have reported that an *ABCC2* SNP (G1249A) was associated with reduced overall survival (207), and that patients with recurrence during the course of FOLFOX-4 treatment showed high expression levels of MRP2 in tumour tissue samples (209). The *ABCC2* SNP (G1249A) was known to be associated with ABCC2 mRNA level changes and ABCC2 substrate specificity, and was associated with a poor response to FOLFOX-4 chemotherapy, as well as with a shorter survival rate (207). In the other study, MRP2 expression was increased in cancerous colorectal tissues compared to

matched normal tissues, and was not correlated with overall survival or disease-free survival of patients. However, patients showing a recurrence during FOLFOX-4 treatment had high MRP2 expression levels in their cancerous tissues (209). Thus, individual variability in *ABCC2* genotype and in MRP2 expression levels in tumours may be linked to inter-patient variations in response to oxaliplatin therapy and treatment outcomes.

This current study indicated that MRP2 mediated the active transport of oxaliplatin-derived platinum, thus limiting cellular platinum accumulation and sensitivity in human gastrointestinal cancer cells with high *ABCC2* expression levels. This mechanism could also potentially explain the reported clinical correlations between MRP2 tumour expression and poor clinical outcomes in patients receiving oxaliplatin-based therapy. However, further animal studies should be undertaken to confirm this mechanism. For example, immunohistochemistry (IHC)-based MRP2 expression level measurements and ICP-MS-based platinum accumulation assessment in tumour tissue samples from nude mice bearing tumours of human gastrointestinal cancer cell lines such as HepG2, PANC-1, HCT116 and HT29 could be done. Further clinical studies should also be carried out using IHC-based screening of MRP2 expression level, genotyping of *ABCC2*, PCR-based *ABCC2* expression measurement and ICP-MS-based platinum accumulation assessment in tumour samples from gastrointestinal cancer patients receiving oxaliplatin-based chemotherapy.

It would appear to be important to identify patients exhibiting a poor response to oxaliplatin via MRP2-mediated deficient platinum accumulation so that oxaliplatin-based treatment regimens could be tailored to the individual patient depending on their MRP2 status to achieve the most effective treatment outcomes. For patients with resistance to

oxaliplatin due to MRP2, the oxaliplatin dose might need to be adjusted and combined with MRP2 inhibitors such as myricetin to optimize its anti-tumour activity. To select such patients, MRP2 expression levels, genetic variants of *ABCC2*, MRP2 functional activity and oxaliplatin accumulation in tumours may need to be determined. MRP2 expression levels in tumour samples could be assessed with IHC (209), and genetic variants of *ABCC2* could be determined with SNP genotyping using patients' blood samples (232-234). Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) using the contrast agent named gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) is currently in clinical trials to evaluate the functional status of liver and possible liver metastases or lesions in cancer patients (328-331) and Gd-EOB-DTPA recently found to be transported by MRP2 has been in trials for the potential *in-vivo* functional assessment of MRP2 in liver (332). Therefore, DCE-MRI using Gd-EOB-DTPA contrast agent could be applied to measure the MRP2 functional activity in patients. Oxaliplatin accumulation levels in tumour samples during the treatment course could be measured using inductively coupled plasma mass spectrometry (ICP-MS)-based quantitative analysis of platinum in tumour samples or novel laser ablation ICP-MS (LA-ICP-MS) for scanning of platinum distribution and quantitative measurement of platinum in tissue sections or biopsy . Recent novel innovations such as combined LA-ICP-MS/APCI-MS (atmospheric pressure chemical ionization mass spectrometry) which can localise and identify molecular identity of platinum species remained in tissues could also be applied (333). Identification of platinum-DNA adducts or DNA-bound platinum in tumour tissue samples using ICP-MS analysis could also be applied to determine the platinum accumulation levels in tumours and to assess the anti-tumour activity of oxaliplatin in patients (334).

6.9. Overall conclusions

In conclusion, the experimental work described in this thesis has demonstrated that MRP2 mediates the active membrane transport of oxaliplatin-derived platinum in a manner that depended upon ATP in membrane vesicles. Intact oxaliplatin and its anionic mono-chloro oxalate ring-opened intermediate, seemed likely substrate candidates for MRP2-mediated transport. MRP2 was a targetable factor, limiting oxaliplatin accumulation in, and growth inhibition of, some gastrointestinal cancer cell types by 2- to 5-fold. Inhibition of MRP2 sensitised MRP2-overexpressing gastrointestinal cancer cells to clinically achievable plasma concentrations of oxaliplatin. Taken together, these studies have provided *in vitro* evidence in support of a therapeutic strategy to target MRP2 for increasing oxaliplatin accumulation and the chemo-sensitivity of gastrointestinal cancer. Further studies of this approach and of MRP2-mediated transport of platinum are now warranted.

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Appendix

Appendix 1.

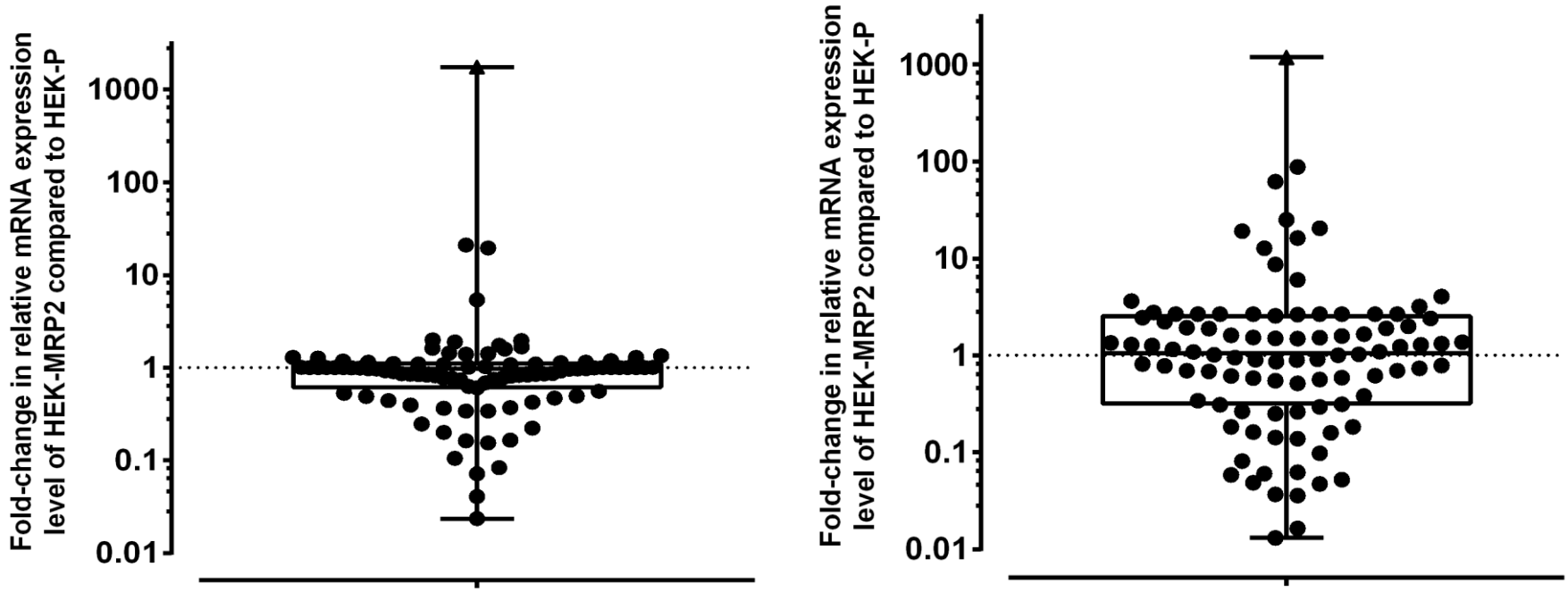

Figure A.1. Analysis of relative mRNA expression levels of membrane transporters genes in HEK-MRP2 and HEK-P.

Data presented are fold-changes in the expression levels of individual transporter genes by dividing the $2^{-\Delta Ct}$ values of each gene in HEK-MRP2 with that of HEK-P from two independent experiments. The triangle-shaped symbol represents for *ABCC2* (MRP2 gene) and the circle-shaped symbols for other transporter genes. *ABCC2* gene expression in HEK-MRP2 is outstandingly higher compared to HEK-P.

Table A.1. Relative mRNA expression levels of membrane transporter genes in HEK-P and HEK-MRP2 cells presented as $2^{\wedge -\Delta Ct}$ values and fold-change.

Data presented are from Experiment 1.

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
Gene of interest	MRP2	ABCC2	1.7275	0.0010	1730
Possible Oxaliplatin efflux transporters	MRP1	ABCC1	0.0269	0.0292	0.92
	MRP4	ABCC4	0.0214	0.0253	0.84
	ATP7A	ATP7A	0.0119	0.0108	1.10
	ATP7B	ATP7B	0.0030	0.0077	0.39
	MATE1	SLC47A1	0.0086	0.0100	0.85
Possible Oxaliplatin uptake transporters	OCT1	SLC22A1	0.0003	0.0002	1.14
	OCT2	SLC22A2	0.0000	0.0001	0.02
	OCT3	SLC22A3	0.0000	0.0001	0.49
	OCTN1	SLC22A4	0.0007	0.0014	0.47
	OCTN2	SLC22A5	0.0206	0.0249	0.83
	CTR1	SLC31A1	0.0435	0.0479	0.91
ATP-binding casettes (ABC) efflux transporters	ABCA1	ABCA1	0.0004	0.0003	1.18
	ABCA2	ABCA2	0.0051	0.0037	1.40
	ABCA3	ABCA3	0.0264	0.0134	1.97
	ABCA4	ABCA4	0.0001	0.0000	1.67
	ABCA5	ABCA5	0.0062	0.0081	0.77
	ABCA9	ABCA9	0.0001	0.0000	1.74

Table A.1. Continued

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
ATP-binding casettes (ABC) efflux transporters	ABCA12	ABCA12	0.0000	0.0003	0.16
	ABCA13	ABCA13	0.0000	0.0000	19.50
	MDR1	ABCB1	0.0139	0.0099	1.41
	MDR3	ABCB4	0.0004	0.0006	0.63
	ABCB5	ABCB5	0.0000	0.0000	0.20
	ABC14	ABCB6	0.0268	0.0187	1.43
	BSEP	ABCB11	0.0000	0.0000	1.00
	MRP3	ABCC3	0.0000	0.0001	0.42
	MRP5	ABCC5	0.0238	0.0287	0.83
	MRP6	ABCC6	0.0038	0.0007	0.37
	MRP7	ABCC10	0.0052	0.0053	0.99
	MRP8	ABCC11	0.0001	0.0002	0.81
	MRP9	ABCC12	0.0000	0.0000	1.00
	ABC42	ABCD1	0.0070	0.0084	0.84
	ABC43	ABCD3	0.0978	0.1003	0.97
	ABC41	ABCD4	0.0222	0.0323	0.69
	ABC27	ABCF1	0.0835	0.0831	1.00
	BCRP	ABCG2	0.0022	0.0023	0.96
	ABCG8	ABCG8	0.0000	0.0000	0.34

Table A.1. Continued

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
Vacuolar ATPase transporters	ATP6C	ATP6V0C	0.1638	0.1493	1.10
Solute carrier family	GLUT1	SLC2A1	0.0479	0.0443	1.08
	GLUT2	SLC2A2	0.0000	0.0000	0.11
	GLUT3	SLC2A3	0.0171	0.0154	1.11
	NBAT	SLC3A1	0.0006	0.0017	0.34
	CD98	SLC3A2	0.0997	0.1200	0.83
	SGLT1	SLC5A1	0.0000	0.0000	0.08
	SGLT3	SLC5A4	0.0003	0.0019	0.15
	CCBR1	SLC7A11	0.0055	0.0054	1.01
	LAT1	SLC7A5	0.0683	0.0788	0.87
	LAT-2	SLC7A6	0.0235	0.0305	0.77
	LAT3	SLC7A7	0.0011	0.0025	0.44
	LAT2	SLC7A8	0.0122	0.0132	0.92
	BAT1	SLC7A9	0.0000	0.0001	0.17
	NTCP	SLC10A1	0.0002	0.0003	0.53
	ASBT	SLC10A2	0.0000	0.0000	1.00
	PEPT1	SLC15A1	0.0000	0.0000	0.07
	PEPT2	SLC15A2	0.0004	0.0011	0.36

Table A.1. Continued

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
Solute carrier family	MCT1	SLC16A1	0.3369	0.4572	0.74
	MCT7	SLC16A2	0.0110	0.0087	1.27
	MCT3	SLC16A3	0.0000	0.0000	1.90
	FLT	SLC19A1	0.0185	0.0172	1.08
	THTR1	SLC19A2	0.0168	0.0172	0.98
	THTR2	SLC19A3	0.0018	0.0014	1.29
	OAT1	SLC22A6	0.0000	0.0000	1.00
	OAT2	SLC22A7	0.0000	0.0001	0.22
	OAT3	SLC22A8	0.0000	0.0000	1.00
	OAT4	SLC22A9	0.0000	0.0000	0.04
	CTLN2	SLC25A13	0.0522	0.0524	1.00
	CNT1	SLC28A1	0.0000	0.0000	2.00
	CNT2	SLC28A2	0.0000	0.0000	1.00
	CNT3	SLC28A3	0.0000	0.0000	21.00
	ENT1	SLC29A1	0.0651	0.0656	0.99
	ENT2	SLC29A2	0.0183	0.0189	0.97
	SNAT2	SLC38A2	0.1384	0.1426	0.97
	SNAT5	SLC38A5	0.0001	0.0002	0.61
	OATP1A2	SLCO1A2	0.0001	0.0004	0.25

Table A.1. Continued

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
Solute carrier family	OATP1B1	SLCO1B1	0.0000	0.0000	1.00
	OATP1B3	SLCO1B3	0.0000	0.0000	1.00
	OATP2A1	SLCO2A1	0.0011	0.0020	0.55
	OATP2B1	SLCO2B1	0.0000	0.0000	1.00
	OATP3A1	SLCO3A1	0.0079	0.0050	1.58
	OATP4A1	SLCO4A1	0.0115	0.0101	1.15
	ABC17	TAP1	0.0173	0.0212	0.81
	ABC18	TAP2	0.0343	0.0334	1.03
	PORIN	VDAC1	0.3207	0.2487	1.29
	POR	VDAC2	0.4033	0.5244	0.77
Major Vault protein	VAULT1	MVP	0.0013	0.0011	1.15
House-keeping genes	Beta-actin	ACTB	4.1726	5.5464	0.75
	β2 microglobulin	B2M	0.1782	0.1493	1.19
	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	2.3748	1.7722	1.34
	Hypoxanthine phosphoribosyl transferase 1	HPRT1	0.1150	0.1356	0.85

Table A.1. Continued

	Common Name	Gene	$2^{-\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
House-keeping genes	Ribosomal protein, large, P0	RPLP0	2.9831	2.9599	1.01
Control genes	Human genomic DNA control	HGDC	0.0000	0.0000	1.00
	Reverse-transcription controls	RTC	0.0506	0.0094	5.40
	Positive PCR controls	PPC	0.2766	0.3222	0.86

Table A.2. Relative mRNA expression levels of membrane transporter genes in HEK-P and HEK-MRP2 cells presented as $2^{\wedge -\Delta Ct}$ values and fold-change.

Data presented are from Experiment 2.

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
Gene of interest	MRP2	ABCC2	1.259	0.001	1190
Possible Oxaliplatin efflux transporters	MRP1	ABCC1	0.152	0.220	0.69
	MRP4	ABCC4	0.024	0.017	1.34
	ATP7A	ATP7A	0.001	0.007	0.14
	ATP7B	ATP7B	0.001	0.018	0.06
	MATE1	SLC47A1	0.000	0.012	0.04
Possible Oxaliplatin uptake transporters	OCT1	SLC22A1	0.002	0.001	1.26
	OCT2	SLC22A2	0.001	0.000	6.01
	OCT3	SLC22A3	0.001	0.003	0.26
	OCTN1	SLC22A4	0.000	0.000	1.65
	OCTN2	SLC22A5	0.000	0.006	0.08
	CTR1	SLC31A1	0.001	0.024	0.06
ATP-binding casettes (ABC) efflux transporters	ABCA1	ABCA1	0.015	0.006	2.77
	ABCA2	ABCA2	0.136	0.053	2.56
	ABCA3	ABCA3	0.564	0.283	2
	ABCA4	ABCA4	0.012	0.008	1.61
	ABCA5	ABCA5	0.045	0.051	0.89
	ABCA9	ABCA9	0.009	0.001	16.21

Table A.2. Continued

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
ATP-binding casettes (ABC) efflux transporters	ABCA12	ABCA12	0.002	0.002	1.32
	ABCA13	ABCA13	0.001	0.000	8.68
	MDR1	ABCB1	0.077	0.113	0.68
	MDR3	ABCB4	0.006	0.018	0.31
	ABCB5	ABCB5	0.001	0.002	0.81
	ABC14	ABCB6	0.260	0.137	1.89
	BSEP	ABCB11	0.001	0.000	4.04
	MRP3	ABCC3	0.000	0.000	2.65
	MRP5	ABCC5	0.190	0.201	0.94
	MRP6	ABCC6	0.009	0.012	0.78
	MRP7	ABCC10	0.017	0.022	0.78
	MRP8	ABCC11	0.009	0.006	1.50
	MRP9	ABCC12	0.011	0.000	61.95
	ABC42	ABCD1	0.045	0.041	1.09
	ABC43	ABCD3	0.335	0.308	1.09
	ABC41	ABCD4	0.497	0.326	1.53
	ABC27	ABCF1	0.156	0.225	0.70
	BCRP	ABCG2	0.016	0.027	0.59
	ABCG8	ABCG8	0.002	0.001	3.18

Table A.2. Continued

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
Vacuolar ATPase transporters	ATP6C	ATP6V0C	2.192272	2.440609	0.90
Solute carrier family	GLUT1	SLC2A1	0.0696	0.1238	0.56
	GLUT2	SLC2A2	0.0005	0.0002	2.65
	GLUT3	SLC2A3	0.3280	0.2073	1.58
	NBAT	SLC3A1	0.0005	0.0012	0.38
	CD98	SLC3A2	0.2730	1.6905	0.16
	SGLT1	SLC5A1	0.0005	0.0009	0.51
	SGLT3	SLC5A4	0.0005	0.0004	1.01
	CCBR1	SLC7A11	0.3400	0.5531	0.61
	LAT1	SLC7A5	0.7129	0.7846	0.91
	LAT-2	SLC7A6	0.0748	0.1221	0.61
	LAT3	SLC7A7	0.0153	0.0153	1.00
	LAT2	SLC7A8	0.1481	0.1204	1.23
	BAT1	SLC7A9	0.0025	0.0096	0.26
	NTCP	SLC10A1	0.0005	0.0008	0.58
	ASBT	SLC10A2	0.0005	0.0002	2.65
	PEPT1	SLC15A1	0.0005	0.0002	2.65
	PEPT2	SLC15A2	0.0183	0.0082	2.23

Table A.2. Continued

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
Solute carrier family	MCT1	SLC16A1	0.6386	0.3309	1.93
	MCT7	SLC16A2	0.0005	0.0050	0.10
	MCT3	SLC16A3	0.0005	0.0002	2.65
	FLT	SLC19A1	0.0053	0.0211	0.25
	THTR1	SLC19A2	0.1136	0.0984	1.15
	THTR2	SLC19A3	0.0014	0.0046	0.31
	OAT1	SLC22A6	0.0033	0.0002	19.05
	OAT2	SLC22A7	0.0043	0.0002	25.08
	OAT3	SLC22A8	0.0005	0.0013	0.34
	OAT4	SLC22A9	0.0035	0.0002	20.39
	CTLN2	SLC25A13	0.1443	0.2641	0.55
	CNT1	SLC28A1	0.0151	0.0002	88.01
	CNT2	SLC28A2	0.0018	0.0005	3.63
	CNT3	SLC28A3	0.0005	0.0002	2.65
	ENT1	SLC29A1	0.2835	0.1868	1.52
	ENT2	SLC29A2	0.2387	0.1856	1.29
	SNAT2	SLC38A2	5.0613	4.9526	1.02
	SNAT5	SLC38A5	0.0005	0.0002	2.65
	OATP1A2	SLCO1A2	0.0027	0.0037	0.73

Table A.2. Continued

	Common Name	Gene	$2^{\wedge -\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
Solute carrier family	OATP1B1	SLCO1B1	0.002	0.0002	12.74
	OATP1B3	SLCO1B3	0.000	0.0004	1.29
	OATP2A1	SLCO2A1	0.046	0.054	0.86
	OATP2B1	SLCO2B1	0.004	0.001	2.62
	OATP3A1	SLCO3A1	0.041	0.030	1.36
	OATP4A1	SLCO4A1	0.001	0.020	0.06
	ABC17	TAP1	0.000	0.002	0.18
	ABC18	TAP2	0.001	0.013	0.05
	PORIN	VDAC1	0.000	0.028	0.02
	POR	VDAC2	0.005	0.351	0.01
Major Vault protein	VAULT1	MVP	0.003	0.021	0.14
House-keeping genes	Beta-actin	ACTB	0.000	0.057	1.588654
	β2 microglobulin	B2M	0.005	0.060	1.149614
	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	1.394	7.607	0.18
	Hypoxanthine phosphoribosyl transferase 1	HPRT1	1.968	1.326	1.48

Table A.2. Continued

	Common Name	Gene	$2^{-\Delta Ct}$ values		Fold-change
			HEK-MRP2	HEK-P	
House-keeping genes	Ribosomal protein, large, P0	RPLP0	0.7499	15.884	0.05
Control genes	Human genomic DNA control	HGDC	0.0005	0.0002	2.65
	Reverse-transcription controls	RTC	0.1793	0.0735	2.44
	Positive PCR controls	PPC	0.4635	2.9233	0.16

Appendix 2.

Dear Barry H. Hirst,

I am a research student in the Department of Pharmacology and Clinical Pharmacology at The University of Auckland. I am writing up my research in a thesis entitled "**In-vitro studies of the contribution of MRP2 to oxaliplatin transport and cellular sensitivity to oxaliplatin".**

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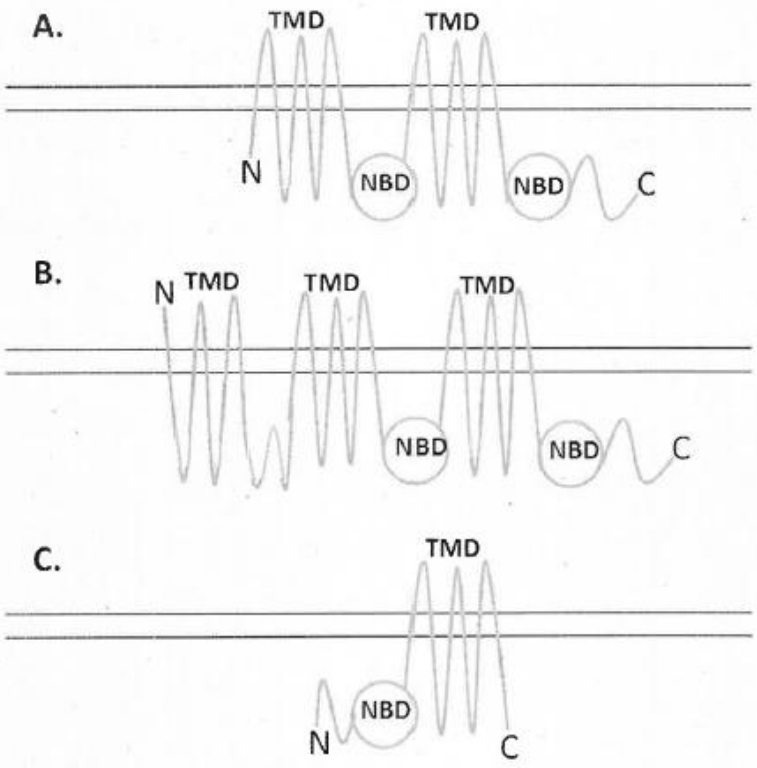
I have redrawn the figure as follow:



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