ATP-BINDING CASSETTE SUBFAMILY C (ABCC) TRANSPORTER 1 (ABCC1) AND 4 (ABCC4) INDEPENDENT OF THEIR DRUG EFFLUX ABILITY AFFECTS BREAST CANCER BIOLOGY

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Thesis Summary

Breast cancer treatment has been a challenge to date, due in part to cancer cells acquiring drug resistance. One of the mechanisms by which resistance can occur is the overexpression of drug efflux pumps such as ATP-binding cassette, subfamily C (ABCC) transporter 1 (ABCC1) and 4 (ABCC4), which are members of ABC transporters. Recently research has shown that these proteins may be implicated in cancer biology independent of cytotoxic drug efflux, but so far little is known about this in regards to breast cancer.

ABCC1 and ABCC4 protein levels in MDA-MB231 and MCF-7, human breast cancer derived cell lines were measured by Western blot. The role of ABCC1 and ABCC4 in cell proliferation and migration were evaluated by colony formation, MTT and scratch assays in the presence of various ABCC inhibitors – MK571, Indomethacin, Reversan, Ceefourin 1 and Ceefourin 2, inhibitors of ABCC4. Similarly the effect on proliferation, migration and invasion was monitored following knock down of ABCC1 and ABCC4 with ABCC1- and ABCC4- specific siRNAs, or overexpression using transfection with pcDNA3.1 plasmids containing the *ABCC1* and *ABCC4* genes. The potential correlation between ABCC1 and/or ABCC4 and the expression of G-protein-coupled receptor 55 (GPR55), or extracellular signal-regulated kinase (ERK), or the extracellular efflux of cyclic adenosine monophosphate (cAMP), prostaglandin E_2 (PGE_2), sphingosine-1-phosphate (S1P) and cyteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) were investigated by Western blot and enzyme-linked immunosorbent assays.

This thesis demonstrates that the expression levels of ABC transporters varies between breast cancer cell lines. Our results suggest that ABCC1 may be more involved in mediating breast cancer cell proliferation than ABCC4 and in contrary ABCC4 may be involved in mediating breast cancer cell invasion more than ABCC1. There is also an indication that both ABCC1 and ABCC4 are implicated in breast cancer migration. In addition, potential correlation between ABCC1 and/or ABCC4 with cAMP or S1P efflux looks promising but further investigation is required.

Taken together this thesis shows that ABCC1 and ABCC4 may be implicated in breast cancer development and progression. Further investigations are needed to validate our current results, but ABCC1 and ABCC4 could be potential therapeutic targets for breast cancer.

Keywords: MDA-MB231, MCF-7, ABCC inhibitor, siRNA, transfection.

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List of Abbreviations

15-PGDH	15-hydroxyprostaglandin dehydrogenase
ABC	ATP-binding cassette transporters
ABCA	ATP-binding cassette subfamily A transporters
ABCB	ATP-binding cassette subfamily B transporters
ABCC	ATP-binding cassette subfamily C transporters
ABCD	ATP-binding cassette subfamily D transporters
ABCE	ATP-binding cassette subfamily E transporters
ABCF	ATP-binding cassette subfamily F transporters
ABCG	ATP-binding cassette subfamily G transporters
ADP	Adenosine diphosphate
ALD	Adrenoleukodystrophy
ALOX5	Arachidonate 5-lipoxygenase
AML	Acute myeloid leukaemia
Angpt2	Angiopoietin2
Angptl4	Angiopoietin-like-4
Apaf-1	Apoptotic protease activating factor 1
APP	Amyloid precursor protein
APS	Ammonium persulphate
AraC	Cytarabine
ATP	Adenosine triphosphate
BACE1	β-site amyloid precursor protein (APP) cleaving enzyme 1
BCA	Bicinchoninic acid
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BCRP	Breast cancer resistance protein
bFGF	Basic fibroblast growth factor
BM	Basement membrane
BME	Basement membrane extract
BSA	Bovine serum albumin
C1P	Cer-1-phosphate
cAMP	Cyclic adenosine monophosphate
CDase	Ceramidase
Cdk	Cyclin dependent kinase
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane regulator gene
cGMP	Cyclic guanosine monophosphate
CML	Chronic myeloid leukaemia
cMOAT	Canalicular multiple organic anion transporter
CNS	Central nervous system
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
cPLA2	Cytosolic phospholipases A2
CRISPR	Clustered regularly interspaced short palindromic repeats
CTC	Circulating tumour cell
CXCR1	Chemokine receptor 1
CXCR4	Chemokine receptor 4
CysLT1	Cysteinyl leukotriene receptor 1
DDR	DNA damage response
DHCA	Dihydroxycholestanoic acid
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DPBS	Dulbecco's phosphate buffered saline
dsRNA	Double-stranded RNA
E.coli	*Escherichia coli*
$E_2 17\beta G$	17-β-D-estradiol-glucoronide
ECD	Extracellular domain
ECF	Energy coupling factor
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eEF3	Eukaryotic elongation factor 3
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
EREG	Epiregulin
ERK	Extracellular signal–regulated kinase
FAK	Focal adhesion kinase
FBS	Foetal Bovine Serum
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange-factor
GPCR	G protein-couple receptor
GPR55	G-protein receptor 55
GRB2	Growth receptor-bound protein 2
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HDAC	Histone deacetylases
HDACi	Histone deacetylase inhibitors
HER2	Human epidermal growth factor receptor 2
hMLH1	Hypermethylation of the human mutL homog 1
HRP	Horseradish peroxidase
ID1	Cell differentiation 1
ID3	Cell differentiation 3
IGF1R	Insulin-like growth factor 1 receptor
IL-11	Interleukin-11
IL-1β	Interleukin-1 β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IM	Inner membrane
IMS	Industrial methylated spirit
LB	Luria-Bertani
LOX	Lysyl oxidase
LPI	Lysophosphatidylinositol
LPS	Lipopolysaccharide
LSB	Laemmli sample buffer
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MGMT	O6-methlguanine DNA methyltransferase

miRNA	MicroRNA
MMP-1	Matrix metalloproteinase 1
MMP-10	Matrix metalloproteinase 10
MMP-2	Matrix metalloproteinase 2
MMP-3	Matrix metalloproteinase 3
MMP-9	Matrix metalloproteinase 9
mRNA	Messenger RNA
MRP	Multidrug resistance protein
MTT	Thiazolyl blue tetrazolium bromide
MTX	Methotrexate
NBD	Nucleotide binding domain
NER	Nucleotide excision repair system
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSB	Non-specific binding
OD	Optical density
OM	Outer membrane
PCR	Polymerase chain reaction
PDCD4	Programmed cell death protein 4
PEI	Polyethylenimine
p-ERK	Phosphorylated ERK
PFA	Paraformaldehyde
PGA_2	Prostaglandin A_2
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PGJ_2	Prostaglandin J_2
P-gp	Glycoprotein
PGT	Prostaglandin transporter
pH	Negative logarithm of the hydrogen ion concentration
PHB2	Prohibitin 2
Pi	Phosphate
PI3K	Phosphoinositide 3-kinase
PKCδ	Protein kinase C delta
PMA	Phorbol 12-myristate 13-acetate
PMEA	9-(2-phosphonylmehoxyethly) adenine
pNpp	Para-Nitrophenylphosphate
PNS	Peripheral nervous system
PPARγ	Peroxisome proliferator-activated receptor-γ
PPi	Inorganic pyrophosphate
pRB	Retinoblastoma protein
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
PXE	Pseudoxanthoma elasticum
qPCR	Quantitative polymerase chain reaction
RFC	Reduced folate carrier
RNA	Ribonucleic acid
RNAi	RNA interference
RRM	Recombination repair mechanisms
RS	Regulatory sequence
RT-PCR	Reverse transcription polymerase chain reaction
S1P	Sphingosine-1-phosphate
SBP	Substrate binding protein
SDF-1	Stromal cell-derived factor-1
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
shRNA	Short hairpin RNA

siRNA	Small interfering ribonucleic acid
SK1	Sphingosine kinase 1
SK2	Sphingosine kinase 2
SL	Sphingolipid
SOS	Son of sevenless
SP1	Specificity protein 1
STAT	Signal transducer and activator of transcription
STII	Heat-stable enterotoxin II
SUR1	Sulfonylurea 1
SUR2	Sulfonylurea 2
TA	Total activity
TAM	Tumour-associated macrophages
TBS-T	Tris buffered saline – Tween 20
TEMED	Tetramethlylenediamine
TGFβ	Transforming growth factor-β
THCA	Trihydroxycholestanoic acid
TIC	Tumour-initiating cell
TM	Transmembrane
TMB	3,3',5,5'-Tetramethylbenzidine
TMD	Transmembrane domain
TNF-α	Tumor necrosis factor-α
TRAF2	Tumor necrosis factor receptor-associated factor 2
TRAIL	Tumour necrosis factor related apoptosis-inducing ligand
TSB	Tris sucrose buffer
UGT	Uridine 5'-diphospho-glucuronosyltransferase
uPA	Urokinase-type plasminogen activator
uRAR	Urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VLCFA	Very long chain fatty acid
X-ALD	X-linked adrenoleukodystrophy

Units

%	Percentage
µl	Microlitre
µm	Micrometre
µM	Micromolar concentration
g	Gram
kb	Kilobase
kDa	Kilo Dalton
L	Litre
M	Molar concentration
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar concentration
ng	Nanogram
nm	Nanometre
nM	Nanomolar concentration
ºC	Degrees celsius
rpm	Revolutions per minute
V	Volts
v/v	Volume per volume
w/v	Weight per volume
µg	Microgram

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Chapter 1 – Introduction

1.1 Nature of Cancer

The term cancer also referred to as malignant tumours and neoplasms is a generic term that describes a large group of diseases that can affect any part of the body. One major feature of cancer is the uncontrolled growth and reproduction of a cell due to abnormal cell internal regulatory mechanisms that can often cause the abnormal cells to grow beyond their usual boundaries. Tissues are made up of normal cells that behave in a controlled, specified and coordinated unit (World Health Organisation, 2018). However, when normal cells start to accumulate genetic and epigenetic changes, they become disorganised among the cell population and proliferate in a disorderly manner, forming tumours. Hence the cells break free from the tight network of controls that coordinate the homeostasis balance between cell death and cell proliferation (Hanahan et al., 2000; Herceg et al., 2007). These tumours can invade adjoining parts of the body and spread to other organs. This process is often termed as metastasis and is a major cause of death from cancer. There are a wide variety of disorders that can occur depending on the type of tissue. Furthermore, some tumours are made up of different cells producing different abnormal biological processes (Weinberg, 2013; World Health Organisation, 2018).

In 2012, approximately 14 million new cases of cancer were diagnosed making cancer one of the leading causes of mortality and morbidity worldwide (World Health Organisation, 2018). In 2015, cancer was the second leading cause of death globally and was responsible for 8.8 million deaths (World Health Organisation, 2018). In addition an estimated 9.6 million deaths were recorded in 2018 and over the next two decades the number of new cases is expected to rise by about 70% (World Health Organisation, 2018). As a result, cancer is one of the major research topics among scientists. Although many genes/proteins/pathways have been implicated in the development of cancer, the precise process by which cells become malignant is still unclear and that further research is required to be able to understand the pattern of disease progression and the course of carcinogenesis so as to produce effective actions to reduce or eliminate the risk of cancer (Buhle, 2012).

1.1.1 Development of cancer

The regulation of the growth of normal cells is maintained by feedback mechanisms that may inhibit or stimulate cell division (Hanahan et al., 2011; Barnum et al., 2014). In tissues, aged cells are constantly being replaced with new ones, so as to maintain the health and function

of the tissue (Lloyd, 2013). However, some cells such as neurons, lack the ability to form new cells or are unable to repair even after tissue damage (Buhle, 2012).

In contrast, when cells lose their ability to divide in an orderly and controlled manner, the body's normal regulatory mechanisms do their best to solve the problem. If they cannot, the overall number of cells increases very fast, and as a result a tumour is formed. In addition these cells tend to lose the ability to differentiate (Buhle, 2012). Hence, cancer can arise from normal cells transforming into tumour cells in a multistage process from a pre-cancerous lesion to a malignant tumour (World Health Organisation, 2018).

In some cases, cancer cells originally growing in one part of the body (primary cancer) can break away and travel to other parts of the body to form secondary cancer (metastases) (DeNittis, et al., 2002).

1.1.2   Types of tumours

A tumour is a lump of cells produced from abnormal cells that divides uncontrollably to produce more abnormal cells. Depending on the degree of aggressive growth, a tumour can be classified as either benign or malignant (Weinberg, 2013).

Benign tumours are tumours that keep the cell morphology thus retaining their similarity to the tissue of origin and they do not invade new sites of growth. In addition, benign tumours do not spread to other parts of the body and usually grow very slowly (Cancer research UK, 2017; Buhle, 2012). Furthermore, benign tumours do not cause cancer but may cause problems when they put pressure on nearby vital structures such as blood vessels and nerves. On the contrary, malignant tumours are cancerous in that they are invasive having the tendency to stop differentiating whilst rapidly dividing to produce new cancer cells and they are more likely to move to other sites of the body in the bloodstream or through the lymph (Weinberg, 2013). A summary of the main differences between benign and malignant tumours is show in table 1.1.

Table 1.1. Summary of the differences between benign and malignant (adapted from Buhle, 2012).

Benign	Malignant
• Encapsulated • Non invasive • Highly differentiated • Mitoses rare • Slow growth • Little or no anaplasia • No metastases	• Non encapsulated • Invasive • Tendency toward de-differentiation • Mitoses may be relatively common • Usually rapid growth • Anaplastic to varying degrees • May Metastasise

1.1.3   Localisation of cancers

The type of tissue involved (histology) and the site of the primary tumour determine the name of the cancer. There are four main groups of normal body tissue namely epithelial, connective and muscle, hematopoietic and nerve tissue (Weinberg, 2013).

All the external body surfaces and all internal cavities and spaces are covered and protected with epithelial tissue. The epithelial tissues account for the growth of most human tumours. Cancers that arise from epithelial tissues are called carcinomas (Table 1.2). In the western world, more than 80% of cancer related deaths are due to carcinomas (Buhle, 2012, Weinberg, 2013).

Table 1.2. Carcinomas (adapted from Weinberg, 2014).

(A) Tissue sites of more common types of adenocarcinoma	(B) Tissue sites of more common types of squamous cell carcinoma	(C) Other types of carcinomas
lung colon breast pancreas stomach oesophagus prostate endometrium ovary	skin nasal cavity oropharynx larynx lung oesophagus cervix	small-cell lung carcinoma large-cell lung carcinoma hepatocellular carcinoma renal cell carcinoma transitional-cell carcinoma (of the urinary bladder)

The other malignant tumours formed, are from nonepithelial tissues, which, are classified into three major groups.

The first group of nonepithelial cancers are formed from various connective tissues with a common origin in the mesoderm of the embryo are called the sarcomas (Table 1.3). A variety of mesenchymal cell types account for this type of cancer. The second group of nonepithelial cancers are those formed from various cell types that form the blood forming (hematopoietic) tissues and cells of the immune system (Table 1.3). The third and last group of nonepithelial cancers are formed from cells of various parts of the peripheral and central nervous system that consist of two types of cells, glial cells and neurons. These types of nonepithelial cancers are called neuroectodermal (Table 1.3). It is of note that some types of tumours do not fall under any of the four major groups mentioned above, such as melanomas (Weinberg, 2013).

Table 1.3. Various types of more common sarcomas, hematopoietic and neuroectodermal malignancies (adapted from Weinberg, 2013).

Sarcomas	Hematopoietic malignancies	Neuroectodermal malignancies
osteosarcoma	acute lymphocytic leukaemia	glioblastoma multiforme
liposarcoma	acute myelogenous leukaemia	astrocytoma
leiomyosarcoma	chronic myelogenous leukaemia	meningioma
rhabdomyosarcoma	multiple myeloma	neurinoma
malignant fibrous histiocytoma	non-Hodgkin's lymphoma	retinoblastoma
fibrosarcoma	Hodgkin's disease	neuroblastoma
synovial sarcoma		ependymoma
angiosarcoma		oligodendroglioma
chondrosarcoma		medulloblastoma

1.1.4 Progressive development of cancers

It has been shown that the development of a malignant tumour is a complex and multi-step process whereby abnormal cells progressively become more and more invasive and aggressive. Some tumours contain a large number of cells that have proliferated uncontrollably but look almost similar to those of normal tissue, this type of growth is called hyperplastic. On the other hand, there are cases whereby one type of normal cells will replace cells of another type at a site not normally found by that type of cell. These type of cells also show minimal differences from normal, are termed metaplasia. Cells that are abnormal cytologically, no longer look similar to normal cells of the tissue is called dysplastic. Dysplastic cells have variable shape and size with increased mitotic activities. They no longer behave as normal differentiated cells of the tissue thus causing major changes in the overall tissue architecture. Tumour cells that are in the transitional state between completely benign and those that are premalignant are considered to be dysplastic. When the growth of abnormal cells starts to break through underlying tissues, these cells become malignant cells that can be life threatening and such growth are called neoplasms (Weinberg, 2013).

1.1.5 Monoclonal and polyclonal growth

Tumours can be monoclonal as well as polyclonal in origin. Most tumours are monoclonal which are made up of cells that have a common genetic marker, indicating they come from a single initially mutated cell. However, polyclonal tumours are made up of subpopulation of cells that have different genetic markers from each other (Figure 1.1) (Weinberg, 2013). There is some debate about whether certain tumours are monoclonal or polyclonal. From the evidence acquired so far it is certain that at least 24 different human tumours can have polyclonal tumour origin such as tumours of the breast (Going et al., 2001), bladder (Cheng et

al., 2002), colon (Ulintz et al., 2018), prostate (van Etten et al., 2016), stomach (Nomura et al., 1998) and cervix (Guo et al., 2000). However, initial transformed cells through secondary or tertiary genetic changes can cause tumours to acquire cytogenetically different clones. Therefore, this heterogeneity in tumour of the same diagnostic type contributes to differences in response to treatment and clinical behaviour. Together with the initial clone and subclones, progenitor cancer cells can also be found in tumours, thus causing the tumour to have a range of cells with different genetic states of differentiation and mutation. These populations can differ in sensitivity to radiotherapy, chemotherapy and other treatment, thus making clinical management difficult (Croce, 2008). In fact, to distinguish whether a tumour is monoclonal or polyclonal is very important because for improving cancer risk assessment, for determining a more accurate therapeutic approaches, for developing precise mathematical models of tumour development and for acquiring a more specific understanding of the initial developments in carcinogenesis (Croce, 2008; Parsons, 2008).
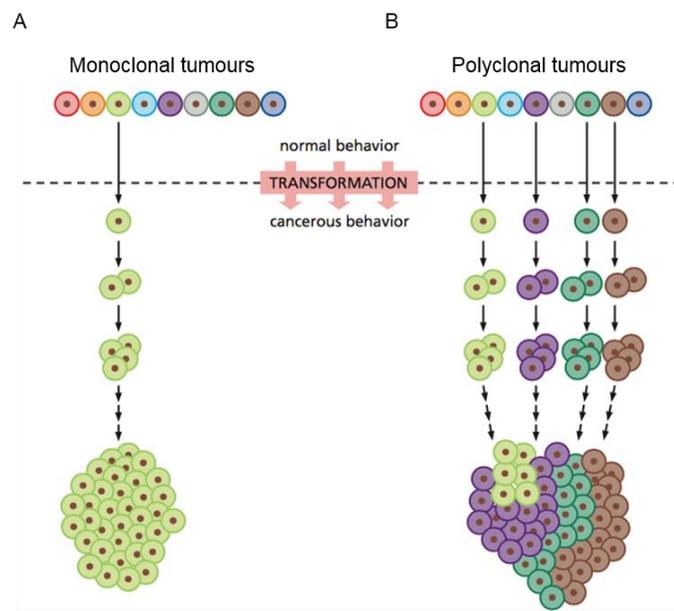
Figure 1.1. Schematic representation of the initial events in carcinogenesis. The cells are coloured to represent a cell lineage marker independent of the carcinogenic process. According to monoclonal tumor origin (A) cells that begin to form tumour originate from a single cell displaying initial genetic lesions. This cell will further be mutated thus producing a clone of cells with multiple mutations that favours propagation and may develop into a malignancy. Therefore, tumours can be consider as clones of cells with different subsets of genetic lesions and may contain additional genetic lesions as well. According to polyclonal tumor origin (B), two or more cells or clones of cells with genetic lesions come together to begin the formation of tumour. Irrespective of whether the tumour a rise from monoclonal or polyclonal tumour origins, the concept regarding the need for the accumulation of mutations, the types of the genetic lesions and subsequent clonal selection are equally applicable to both schemes (Parsons, 2008; Weinberg, 2013).

1.1.6   Known or suspected causes of cancers

Reports have established that lung cancer (1.76 million deaths), liver cancer (782,000 deaths), colorectal cancer (862,000 deaths), stomach cancer (783,000 deaths) and breast cancer (627,000 deaths) are the most common causes of cancer death (World Health Organisation, 2018). Agents that are able to cause cancers are known as carcinogens. There are many factors that have been found and suspected to cause cancers (Weinberg, 2013), often it is an interaction between a person's genetic factors and the external agents including; chemical carcinogens such as components of tobacco smoke, arsenic (a drinking water contaminant), asbestos and aflatoxin (a food contaminant); physical carcinogens such as ionising and ultraviolet radiation; biological carcinogens such as infections from certain bacteria, viruses or parasites (Weinberg, 2013).

Furthermore, the risk of development of cancer rises with ageing, most likely due to an accumulation of risk over time combined with the tendency for cells to repair themselves becoming less effective with ageing (Weinberg, 2013).

1.2   Oncogenes and tumour suppressor genes

Alterations in oncogenes, tumour suppressor genes and microRNA genes can cause cancer and usually occur in somatic cells, although mutation can occur in germ-line cells and predispose a person to familial or heritable cancer. Moreover, the development of a malignant tumour is rarely due to a single genetic change but is due to a multistep process of sequential changes in several, tumour suppressor genes, oncogenes or microRNA genes in cancer cells (Chow, 2010).

1.2.1   Oncogenes

Certain genes known as oncogenes have the ability to cause cancer, transforming normal cells to malignant cells. These genes are often expressed in high levels or mutated in abnormal cells (Buhle, 2012). Oncogenes code for proteins that control cellular growth and division involved in cell cycle regulation typically causing cells to proceed from G (gap) phases to either chromosome segregation (mitosis) or chromosome replication (S phase) (Figure 1.2). Examples include signalling molecules that link the receptors to the replication initiator through various pathways, proteins that interact with DNA to start replication, receptors at the cell surface that binds to growth factors (Croce, 2008; Chow, 2010).

Figure 1.2. Cell cycle control by tumour suppressors and oncogenes. Checkpoints are represented as thick red bars. The stages of the cell cycle included G1: Gap 1, S: DNA synthesis, G2: Gap 2, and M: mitosis. Tumour suppressors such as p53 and pRB act to maintain checkpoints (arrows) whereas oncogenes such as *Ras* and *Myc* allow for checkpoints to be overcome (stop lines) (Chow, 2010).

In their normal state, the proteins that control these critical processes are coded by genes known as proto-oncogenes. However, when proto-oncogenes undergo certain changes, they become oncogenes and cause an increase in activity that leads to the formation of tumour. Once a tumour starts to form, the tumour cells will progress through all the phases of the cell cycle, instead of stopping within a G phase as it should normally do, leading to uncontrolled cell growth. Furthermore oncogenes can inhibit cells from programmed cell death (apoptosis) (Chow, 2010).

1.2.1.1   Proto-oncogene to oncogene

Proteins that normally interchange between active and inactive states, occasionally, can be permanently mutated. Ras proteins are a group of proteins that belong to the large family of small GTPases which in response to numerous extracellular stimuli are activated such as in response to growth factor stimulation. Activation causes Ras proteins to bind to a number of effector proteins, causing the activation of several signalling cascades within the cell that are responsible for adhesion, growth, cytoskeletal integrity, differentiation, migration and survival (Goodsell, 1999; Rajasekharan et al., 2013). In normal cells, Ras protein signalling is tightly

controlled as a small change in Ras signalling may lead to cells becoming malignant (Rajalingam et al., 2007; Rajasekharan et al., 2013). It is estimated that about 20% of all human tumours have a Ras gene that is mutated (Rajalingam et al., 2007).

The Ras proteins function as binary molecular switches that alternate between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound states. The stimulation of guanine nucleotide exchange-factors (GEFs) promotes conversion of the Ras stable, inactive GDP-bound form to active GTP-bound form. The conversion back to the inactive form is controlled by GTPase-activating proteins (GAPs). GEFs and GAPs are large, multi-domain proteins that can efficiently interact with other proteins, regulatory molecules and lipids and control the level of active and inactive Ras to respond to and regulate multiple signal inputs (Rajalingham et al., 2007; Simanshu et al., 2017).

Mutation in Ras proteins have been recognised for many years (Simanshu et al., 2017). During the 'on state', these proto-oncogenes cause a proliferation-stimulating signal that if mutated to an oncogene, will permanently keep Ras active, regardless of the signals the cell receives. Hence a dysregulated Ras signalling can cause the cell to become cancerous (Chow, 2010; Rajasekharan et al., 2013). There are 5 types of Ras oncogenes identified so far (Table 1.4) (Rajasekharan et al., 2013).

Table 1.4. Classification of Ras oncogenes (adapted from Rajasekharan, et al., 2013).

Gene	Abbreviation	Location	Common mutation
H-ras	Harvey rat sarcoma	11p15.5	G12V, G12S, G12A, G13D, Q 61R
K-ras	Kirsten rat sarcoma	12p12.1	G12D,G12S,G12R,G12A,G13D,G12V,G12C
N-ras	Neuroblastoma Ras	1p13.2	G12D, Q61K
M-ras	Muscle Ras	3q22.3	G22V, Q71L
R-ras	Related Ras	19q13.33	-

One of the key regulators of MAPK pathway is Ras (Figure 1.3). The MAPK pathway is activated when growth factors binds to protein tyrosine kinase receptors, which dimerise and cross-phosphorylates tyrosine residues in the cytosolic domains. The tyrosine phosphorylation activates son of sevenless (SOS), a set of genes encoding guanine nucleotide exchange factors for Ras proteins, which binds to the phosphotyrosine via Growth

receptor-bound protein 2 (GRB2) adaptor proteins and also to plasma membrane. Consequently when Ras protein is activated, Raf1 kinase is recruited to phosphorylate raf1 hence causing a downstream signalling. The phosphorylation of Raf1 (a MAP3K) will in turn activate and phosphorylate MEK, a duel specific tyrosine/threonine kinase (a MAP2K). Phosphorylated MEK, further activates and phosphorylates ERK1 and ERK2 (MAPKs) which in turn phosphorylates ELK1 transcription factor causing immediate early response genes such as the gene that encode c-Fos and c-Jun protein to transcribe immediately. Hence it is well established that mutation in Ras protein is a prognostic factor which constitutively activates Ras-MAPK pathway causing cancer to occur. However, due to the complex networks of the Ras-MAPK pathway, it is very difficult to target Ras-MAPK pathway. Therefore, understanding the mechanism of how Ras promotes the formation of tumours will help to discover other therapeutic approaches (Fernandez-Medarde et al., 2011; Rajasekharan et al., 2013).
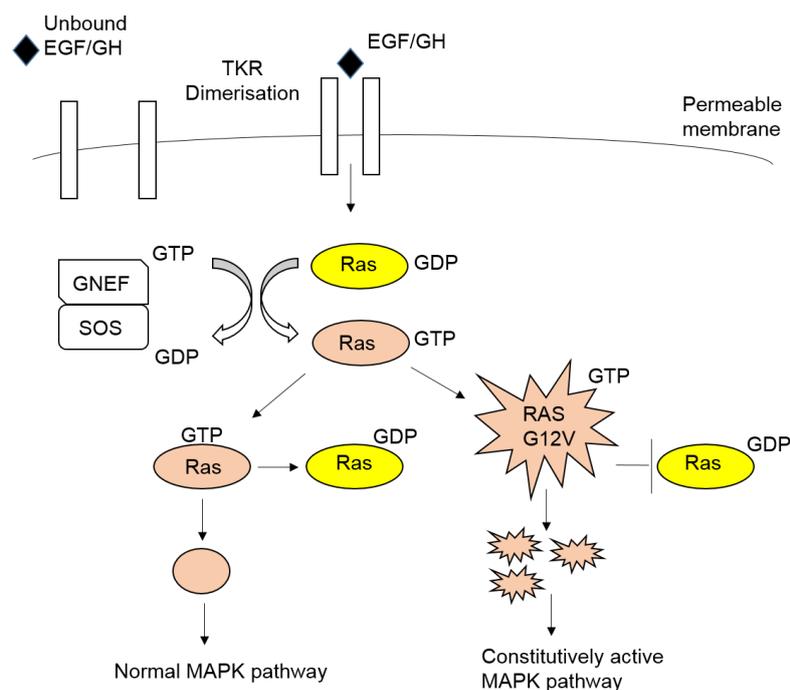
Figure 1.3. Normal and mutant Ras circuit connecting MAPK pathway. Epidermal growth factor receptor (EGFR) dimerises upon receiving the external signals (EGF/Growth factor (GH)) causing autophosphorylation of tyrosine residues that leads to the activation of the tyrosine kinase activity. The removal of GDP from Ras is facilitated by Growth factor receptor-bound protein 2 (GRB2) associates with Son of seven (SOS) and docking protein. Moreover, phosphorylation of MEK, ERK and Raf occurs when Ras binds with GTP. Ras is kept at its active state due point mutations (G12V) in exon 1 of it gene which is essential in the activation of MAPK pathway (Rajasekharan et al., 2013).

Interestingly, mutation in oncogenic Ras and other components of Ras mitogen-activated protein kinase (Ras-MAPK) signalling pathway have been reported in a number of cancer cells and tissues, indicating for tumorigenesis to occur deregulation of Ras-dependent signalling is essentially required. In addition, mutation in H-Ras, K-Ras and N-Ras are often detected in tumours (Fernandez-Medarde et al., 2011; Rajasekharan et al., 2013).

A chromosomal translocation is another type of genetic alteration that converts a proto-oncogene to an oncogene. This is where pieces of broken chromosomes reattach randomly, causing either a change in the regulation of the protein expression or the production of a fusion protein containing the C-terminus from one protein and the N-terminus from another protein (Figure 1.4). *BCR/ABL* is an example of an oncogene that is formed by chromosomal translocation. The *BCR/ABL* oncogene codes for a protein with the N-terminus of Bcr (breakpoint cluster region) and the C-terminus of Abl, a tyrosine kinase that relays proliferative signals. Hence, when the fusion protein is formed, Abl is switched on permanently, causing unregulated cell cycling (Pérez-Caro, et al., 2007; Chow, 2010; Ross, et al., 2014). Furthermore, specific breakpoints in the chromosomal translocation generates three forms of BCR/ABL protein, p210 BCR/ABL, p230 BCR/ABL and p185 BCR/ABL which is associated with chronic myelogenous leukaemia (CML), neutrophilic leukaemia and acute lymphoblastic leukaemia, respectively. However, due to lack of an appropriate in vivo model, the signalling pathway and the molecular mechanism of *BCR/ABL* is still unclear, hence targeting the BCR/ABL pathway for therapeutic approaches is made difficult. Therefore, better understanding of the BCR/ABL pathway, is needed and may open a new path for other therapeutic approaches and drug development (Pérez-Caro et al., 2007; Ross et al., 2014).

Another way oncogenes are formed is simply by proto-oncogene existing in multiple copies in the cells, leading to an increased expression (Figure 1.4). The *c-MYC*, a transcription factor, has about 8 to 30 copies of the proto-oncogenes in each HL60 cells, a promyelocytic leukaemia cell line. The increased expression of *c-MYC* will in turn increase expression of its transcriptional targets, many of which help to drive the cell cycle forward (Chow, 2010).
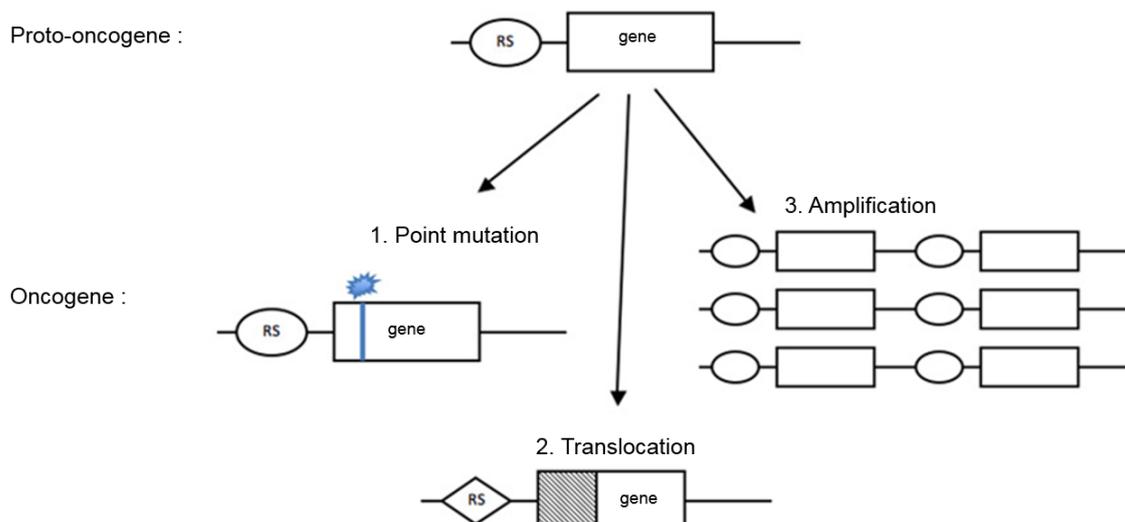
Figure 1.4. A schematic diagram representing three major types of genetic alterations leading to oncogene activation. A horizontal black line and a rectangle in the centre represents region of DNA and a proto-oncogene (gene) respectively, and to the left of the gene is a regulatory sequence (RS). The activation of the proto-oncogene leads to three different genetic alterations. The first alteration is a point mutation, which is shown by a blue star and a blue, vertical stripe on the rectangle representing the proto-oncogene. The second alteration is a translocation. The translocation of the proto-oncogene altered the sequences of both the original RS and part of the proto-oncogene as is represented by one-third of the proto-oncogene rectangle is shade grey, and the oval-shaped RS is changed with a diamond shape. The third alteration is amplification. During amplification both the proto-oncogene and the RS has been duplicated within a region of DNA. Three DNA regions with this same duplicated pattern are shown, one on top of the other. (Chow, 2010).

1.2.2   Tumour suppressor genes

In contrast to proto-oncogenes and oncogenes, tumour suppressor genes code for proteins that prevent cell proliferation and division or even initiate apoptosis (Giacinti et al., 2006). Examples includes proteins that are necessary for apoptosis initiation, inhibitors of cell cycle progression and factors required in maintenance of cell cycle checkpoints (Chow, 2010; Reisman et al., 2012). Genetic mutations in tumour suppressor genes restrict the regulatory protein from inhibiting cell proliferation leading to tumorigenesis. So far there are more than 100 dominant oncogenes and around 30 tumour suppressor genes (Table 1.5) (Naga Deepthi et al., 2011).

Table 1.5. Tumour suppressor genes involved in human cancers and diseases (Thomas et al., 2012).

Gene	Chromosomal location	Cellular location	Mode of action	Example of cancers
Rb	13 q 14	Nucleus	Transcriptional regulator	Retinoblastoma, osteosarcoma, carcinomas of breast, prostate, bladder and lung
p53	17 p 13.1	Nucleus	Transcription factor /regulator	Most human cancers, breast, brain, sarcomas, leukaemia
APC	5 q 21	Cytoplasm	Unknown	Carcinomas of colon, stomach and pancreas
WT1	11 p 13	Nucleus	Transcription factor	Nephroblastoma
DCC	18 q 21	Membrane	Cell adhesion molecule	Carcinomas of colon and stomach
NF-1	17 q 11	Cytoplasm	p21, Ras, GTPase activator	Schwannomas
NF-2	22 q 12	Inner membrane	Cytoskeleton membrane link	Schwannomas, meningiomas
pVHL	3 p 25	Cytoplasm	Inhibits transcriptional elongation	Renal cell carcinoma

Rb, retinoblastoma protein; p53, Tumour protein p53; APC, adenomatous polyposis coli; WT1, wilms tumour protein; DCC, deleted in colorectal carcinoma; NF-1, neurofibromatosis type 1; NF-2, neurofibromatosis type 2; pVHL, von Hippel-Lindau tumour suppressor.

The first tumour suppressor gene to be identified and play a critical role in cell cycle regulation was the *RB1* gene that codes for retinoblastoma protein (pRb). The pRb protein have 16 potential sites of cyclin dependent kinase (Cdk) phosphorylation and is an important regulator of G1 progression. The Rb pathway is shown in figure 1.5. Initially in G1 stage, pRb is tightly bound to the E2F transcription factor and is in a hypophosphorylated state. When pRb is phosphorylated by Cdk complexes, which is a sequential process initiated by Cdk4 and Cdk6, E2F is released and the cell can start DNA synthesis. This causes the formation of active Cdk2/cyclin E complexes that help the continuation of pRb phosphorylation, which as a result promote pRb-E2F separation so that E2F can be transcriptionally active, an obligation for the cell to move from G1 into S phase. Cyclin A is expressed as cells progress into S phase and it becomes the main cyclin linked with Cdk2. Moreover, the activity of cdc2 complexed with cyclin B phosphorylates proteins regulated during mitosis, thus is a requirement for the cells to progress from G2 to mitosis. In addition, pRb phosphorylation is maintained throughout S and G2, and during mitosis pRb is rapidly dephosphorylated. Also, during G1 when pRb is hypophosphorlated, E2F is again sequestered (Thomas et al., 2012). Therefore, the function of pRb is simply to stop the expression of genes needed for progression into the S phase of the cell cycle. However, when it is inactivated often due to genetic alterations involving either

deletions or frameshift in the RB1 gene, causing defective protein expression and premature introduction of stop codon, cells starts to divide uncontrollably. There are some instances when other components in the pathway in which pRB functions are defective such as activation of the positive component as CDKs and G1 cyclins or inactivation of negative components as p53 and CDK inhibitors (Chow, 2010; Di Fiore et al., 2013).
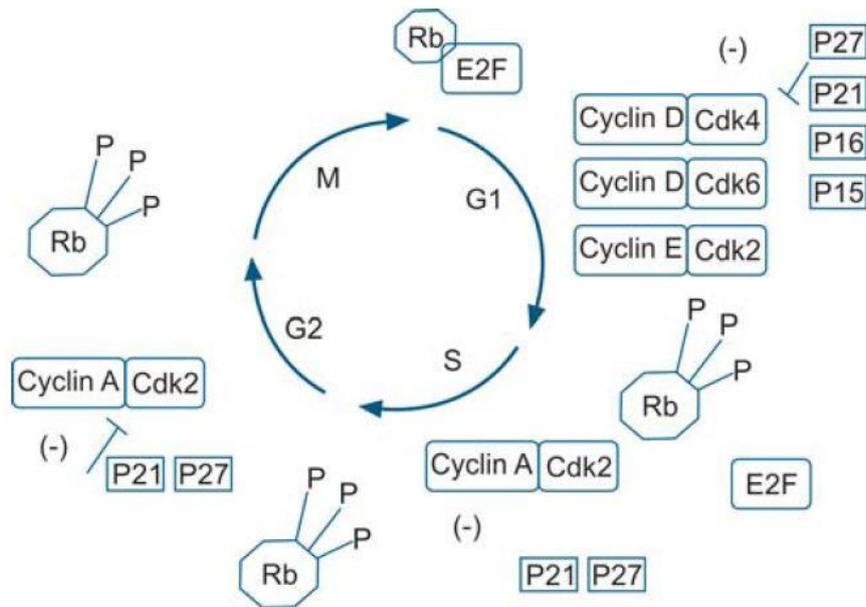
Figure 1.5. The Rb pathway. In the G1 phase, Rb is linked to E2F transcription factor in a hypophosphorylated state. The phosphorylation of Rb by Cdk4 and Cdk6 causes the release of E2F from Rb to initiate DNA synthesis. The phosphorylation of Rb continuous due to the formation of Cdk2/cyclin E complexes hence causing the cells to move from G1 into S phase. As cells moves in the S phase, cyclin A is formed and becomes the main cyclin linked with Cdk2. As the cells moves from G2 to mitosis, Cdk1 complexed with cyclin B phosphorylates proteins regulated during mitosis. Moreover, throughout S and G2 phases, the phosphorylation of Rb is maintained but is rapidly dephosphorylated during mitosis (Thomas et al., 2012).

Another commonly mutated gene in human tumours is the tumour suppressor gene, p53 (Chow, 2010; Naga Deepthi et al., 2011; Rivlin et al., 2011; Lai et al., 2014). The function of the p53 protein is to inhibit proliferation and promote apoptosis in response to DNA damage in cells, hence it plays a very important role in managing the cell cycle, the G1 to S cell cycle checkpoint. The p53 protein holds the cell long enough for the DNA repair proteins to fix the damage and once the damage is fixed the cell is then allowed to continue the cell cycle. On the contrary, if the DNA damage proves to be irreparable, the p53 protein will initiate apoptosis (Chow, 2010; Naga Deepthi et al., 2011). However, the inactivation of p53 gene due to genetic changes will allow a cell to continue to divide even in the presence of DNA damage.

Furthermore, for tumorigenesis to occur both the paternal and maternal copies of a gene coding a tumour suppressor must be modified (Chow, 2010; Buhle, 2012).

The mutation of p53 gene codes a stable p53 mutant protein that apart from losing its tumour suppressive activities has acquired oncogenic functions that help cells to grow and survive without limitations. Interestingly, it was found that when cells become cancerous during the multistep process of malignant transformation, at each phase, p53 gene mutation was shown to contribute differentially to tumour promotion, initiation, metastasis and aggressiveness (Rivlin et al., 2011).

1.2.3   MicroRNA genes

MicroRNA genes consist of a single RNA strand of about 21 to 23 nucleotides that mediate gene expression. Although MicroRNA do not encode proteins, they have the ability to cause degradation of messenger RNA (mRNA) or block protein translation by annealing to the mRNA that has a nucleotide sequence that complements the sequence of the microRNA (Iorio et al., 2005; MacFarlane et al., 2010). Example of microRNA are miR-15a and miR-16-1 which in chronic lymphocytic leukaemia are often down-regulated (Calin et al., 2002). Furthermore, depending on their targets in a specific tissue, a microRNA gene can be an oncogene if its target is a tumour suppressor gene and vice versa. In addition, the demethylation of CpG islands in the promoter regions of the gene or the deregulation and amplification of a transcription factor may lead to up regulation of microRNA (Croce, 2008).

1.3   Cancer metastasis

In many physiological events such as embryogenesis, angiogenesis, inflammation, neurogenesis and wound healing, cell migration and invasion are important steps but they are also implicated in the pathophysiology of many diseases such as cancer (Bozzuto et al., 2010).

Cancer metastasis is a process in which cancer cells disseminate from the primary tumour through lymphatic vessels and blood vessels to settle and grow at a different site other than the primary tumour site (Guan, 2015). About 10% of deaths due to cancer arise from primary tumours, the remaining approximately 90% of death from cancer are due to cancerous growth occurring at a distant site from where the primary tumour was originally found (Valastyan et al., 2011; Gao et al, 2013; Weinberg, 2014; Orimo, 2014; Guan, 2015;; Pachmayr et al., 2017). Most cancer research so far has focused primarily on the development of agents that can inhibit tumour growth and on methods that can detect tumours at the early stage. Therefore,

if tumours are diagnosed early and before metastasis occurs, most solid tumours can be cured or managed. Whereas cancers that are metastatic at diagnosis are usually difficult to manage and cure (Guan, 2015; Chambers et al., 2015). Research into the field of metastasis is lagging mainly due to the complexity of the metastatic process.

1.3.1   Invasion metastasis cascade

Cancer metastasis is a complex succession of a series of cell-biological processes known as the invasion metastasis cascade (Figure 1.6) whereby cells in primary tumours invade locally through surrounding extracellular matrix (ECM) and stromal cell layers, intravaste into blood and lymphatic vessels, survive and arrest in the circulatory system, extravasate into a new microenvironment to form micrometastases and, with enough nutrients and the right conditions these micrometastases will colonise the tissue, to form a macroscopic metastasis. Colonisation is the most challenging and complex step because the new area (tissue) where the cancer cells have landed does not provide them with survival factors and the collection of familiar growth factors that their original place of survival has. As a result, the metastasising cells may rapidly die or remain as micrometastases that often cannot be clinically detected. Furthermore, only a few micrometastases in cancer patient will have the ability to eventually grow several millimetres, large enough to be clinically detectable. (Leber et al., 2008; Bozzuto et al., 2010; Valastyan, 2011; van Zijl et al., 2011; Weinberg, 2014; Orimo, 2014; Pachmayr et al., 2017).
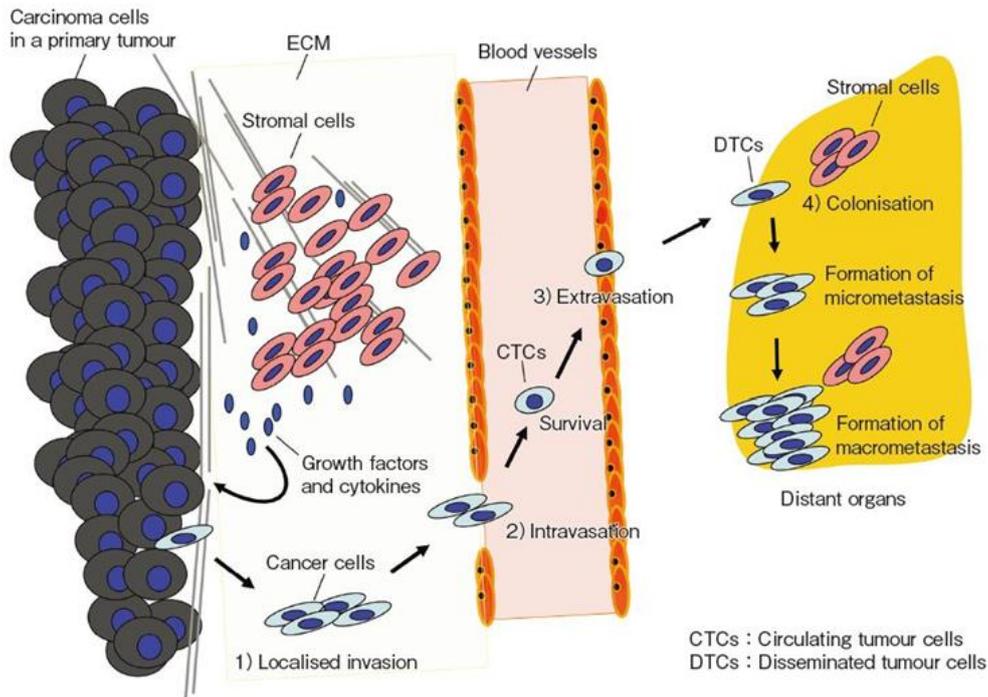
Figure 1.6. The invasion metastasis cascade. A multi-step cell-biological process consisting of a series of cellular events including (1) cancer cells invades into surrounding tissue (local invasion), (2) enters the blood and lymphatic vessels (intravasation), (3) circulating tumour cells (CTCs) survive and exit from the bloodstream (extravasation), and (4) form micro and macroscopic metastases in distant organs (colonisation) (Orimo, 2014).

1.3.1.1 Local invasion

Local invasion involves the migration of tumour cells from the confined primary tumour into the surrounding tumour-associated stroma and thereafter into the adjacent normal tissue. In order to invade the underlying connective tissue, tumour cells must invade the basement membrane (BM) and the lamina propria and upon doing this, tumour cells change from highly differentiated to undifferentiated cells and develop mechanisms to detach from the primary tumour. Such transformation enabling tumour cells to accomplish migration and invasion is called epithelial-mesenchymal transition (EMT) (Figure 1.7) (Le et al., 2010; van Zijl et al., 2011; Guan, 2015).
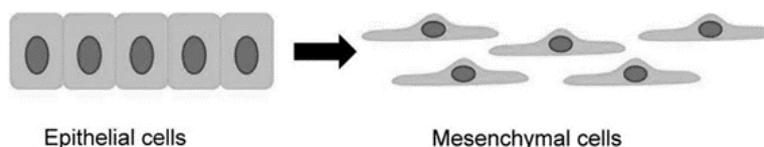
Figure 1.7. Epithelial-to-mesenchymal transition (EMT). The epithelial cells undergo phenotypic changes to take on mesenchymal-like characteristics (Scully et al., 2012).

During this process, transcription factors like Twist, Snail, Slug and ZEB-1/-2 downregulate E-cadherin, a key protein for epithelial cell-cell adhesion, causing the epithelium to be more permeable. Certain microRNAs such as those belonging to the miR-200 family have the ability to post-transcriptionally suppress expression of the ZEB1 and ZEB2 hence promoting an epithelial phenotype. In response, ZEB1 and ZEB2 transcriptionally repress miR-200 microRNAs, reinforcing the residence of cells in either the epithelial or mesenchymal state. Moreover, in response to migratory stimuli, tumour cells are able to migrate as they acquire morphological protrusions. These membrane extensions which contain actin serve as a traction site for cells (van Zijl et al., 2011; Valastyan et al., 2011; Pachmayr et al., 2017).

Once the tumour cells have detached from the primary tumour, tumour cells undergo phenotypic changes to take on mesenchymal-like characteristics allowing them to migrate and invade through the basement membrane. Hence, tumour cells can activate the proteolytic urokinase-type plasminogen activator system (uPA/uPAR) and secrete proteases like matrix metalloproteinase (MMP-1, MMP-2 and MMP-9) (Harlozinska, 2005; Bozzuto et al., 2010) to further degrade the BM and the extracellular matrix (ECM). In addition, the MMP-expressing cells also help cancer cell proliferation by releasing growth factors (Leber et al., 2008; Valastyan et al., 2011; Pachmayr et al., 2017).

The loss of BM allows the tumour cells to enter the stromal compartment where they are confronted with various cell types including dendritic cells, macrophages, neutrophils and lymphocytes. Furthermore, the stromal cells reacts to tumour invasion with inflammation which further increases the aggressive behaviours of tumour cells. For example, the secretion of interleukin-6 (IL-6) by adipocytes present in the local microenvironment can stimulate breast cancer invasiveness. In addition, breast cancer cells secrete IL-4 which generate cathepsin protease activity in tumour-associated macrophages (TAMs) that further increase tumour cell invasiveness. The interactions between tumour cells and the nearby stroma is bi-directional in that the tumour cells activate the formation of an inflamed stroma which in response reinforce the malignant traits of the tumour cells thus setting up a likely self-amplifying positive feedback loop. In summary the entry of tumour cells in the stroma, increases the tumour cells opportunity to directly enter the systemic circulation to travel to distant sites (Valastyan et al., 2011; Pachmayr et al., 2017).

1.3.1.2   Intravasation

During intravasation, tumour cells have the ability to cross the endothelial and pericyte barriers of the microvessels of the lymphatic or blood vessels due to molecular changes such as during

transcriptional programme, cytokine transforming growth factor-β (TGFβ) switches cells from cohesive to single cell motility thus facilitating intravasation (Giampieri et al., 2009). Many of the mechanisms that help facilitate intravasation, involve stimulation of vascular endothelial growth factors (VEGFs) that encourage new blood vessels to form within the local microenvironment, a process known as neoangiogenesis (Leber et al., 2008). Furthermore, the activation of uPA/uPAR due to the suppression of tumour suppressor programmed cell death protein 4 (PDCD4) stimulates intravasation. Urokinase receptor (uPAR) was found to be inversely correlated with PDCD4 expression and the activation of uPAR mediated by specificity protein 1 (SP1) can be suppressed by PDCD4. In addition the secretion of proteases like MMP-1, -2 and -9 also enhance intravasation by promoting angiogenesis (Chiang et al., 2015; Pachmayr et al., 2017). It has been reported that epiregulin (EREG) and cyclooxygenase-2 (COX-2) facilitate breast cancer cell intravasation due to their ability to reduce VEGF, hence causing blood vessels to become more permeable and to stimulate neoangiogenesis (Valastyan et al., 2011; Pachmayr et al., 2017).

1.3.1.3   Survival in the circulatory system

Once tumour cells have successfully entered the blood vessels, they can travel throughout the circulatory system. However because of their diameter (20 – 30 μm) larger than the diameter of capillaries (~ 8 μm), a large number of tumour cells are prone to arrest in various capillaries as soon as they enter the circulatory system. Conversely, the circulating tumour cells (CTCs) must overcome a number of stresses before they can reach distant organ sites. When CTCs are in the circulatory system they lack the integrin-dependent adhesion to ECM components which is critical for normal cell survival. In normal cells, the absence of such anchorage cause the cells to undergo anoikis (Valastyan et al., 2011; Guan, 2015), a form of apoptosis but in CTCs, a number of signalling events such as the control of glucose uptake, the pentose phosphate pathway and the tryrosine kinase TrKB are stimulated to suppress anoikis responses, and thus help metastatic progression of the CTCs (Valastyan et al., 2011; Pachmayr et al., 2017).

Furthermore, apart from withstanding the stresses imposed by matrix, CTCs must overcome cells of the immune system especially the natural killer cells and the hemodynamic shear forces in the circulatory system (Harlozinska, 2005; Valastyan et al., 2014). Such threats are easily overcome in that the CTCs mediate the expression of Tissue-Factor and/or L- and P-selectin in order to interact with blood platelets to form relatively large emboli, thus protecting themselves from immune cells and the shear forces (Guan, 2015). Therefore enabling the tumour cells to disseminates and arrest at distant organ sites (Valastyan, et al., 2011).

1.3.1.4   Arrest at distant organ site and extravasation

Travelling CTCs in the circulatory system can disseminate to a wide variety of distant target organ sites but depending on individual tumour types only form metastatic cells to certain organ sites. This theory has been well explained in the Paget's 'seed and soil' theory which states that a specific tumour cell (the seed) will only metastasise and be detectable at a particular suitable organ or location (the soil) where they can survive and proliferate (Valastyan et al., 2011; Jiang et al., 2015; Pachmayr et al., 2017).   Consistent with the 'seed and soil' theory many cancers have an increased tendency to survive and grow at certain specific organ sites. For example, gastrointestinal cancers appear to frequently metastasise to the liver and lung, whereas prostate and breast cancers favour metastasis to the bone. Furthermore, certain organs such as the bone, lung, brain and liver have become the main secondary sites for most cancer metastasis (Valastyan et al., 2011). One factor which may help tumour cells to have specific affinity for certain organs might be the release of chemokines specific for each organ and the tumour cells have appropriate chemokine receptors that helps tumour cells to target the organ. Chemokine receptor 4 (CXCR4) on tumour cells such as breast cancer cell, are attracted to organs expressing high levels of CXCL12 ligand such as the lung, brain, liver and bone (Leber et., 2008; Valastyan et al., 2011; Jiang et al., 2015) . Conversely, the mechanisms for many cancer types choosing to settle and grow at specific organ sites is still widely being investigated by scientists. Such findings could help initiate new strategies to combat cancer metastasis (Valastyan et al., 2011; Pachmayr et al., 2017).

Once CTCs arrest in the micro vessels of the distant organs, the formation of a microcolony eventually break the vessel wall thereby allowing the CTCs to enter into the new microenvironment, a process called extravasation (Valastyan et al., 2011). In order, for the CTCs to penetrate in tissues, the CTCs must overcome the low intrinsic microvessel permeability by secreting factors to induce vascular hyper-permeability and to change these distant microenvironments. Therefore, the facilitation of extravasation of breast cancer cells in the lungs to disrupt the pulmonary vascular endothelial cell-cell junctions is from the secretion of pleiotropically acting factors COX-2, EREG, MMP-1 and MMP-2 as well as protein angiopoietin-like-4 (Angptl4) (Pudua et al., 2008; Valastyan et al., 2011).   Other factors secreted by various types of cancer cells to help extravasation in the lung are MMP-3, MMP-10, angiopoietin2 (Angpt2), VEGF and placental growth factors (Weis, et al., 2004; Huang et al., 2009; Valastyan et al., 2011).

1.3.1.5   Micrometastasis formation

For metastasis to form, the extravasated cancer cells must survive and adapt to the new microenvironment which is usually different from the site of primary tumour formation in terms of ECM constituents, types of stromal cells, cytokines, available growth factors and the morphology of the tissue itself. Furthermore, for cancer cells to survive in their new microenvironments, a series of systemic signals are released by the tumour cells preparing and making the metastatic site more receptive to the arrival of the cancer cells (Psaila et al., 2009). Signals such as those that include lysyl oxidase (LOX) help to upregulate fibronectin in specific organs from resident tissue fibroblasts which in turn shift VEGF-receptor-1-positive (VEGFR1+) hematopoietic progenitor cells from the bone marrow to the metastatic sites by the interaction between the integrin α4β1 receptor found on the hematopoietic progenitor cells and the deposited fibronectin (Erler et al., 2009). Furthermore, the secretion of MMP-9 by these hematopoietic progenitor cells does not only stimulate various integrins but also activate insulated molecules in the ECM such as the stromal cell-derived factor-1 (SDF-1) (Psaila et al., 2009). The evidence so far established that in order for tumour cells to initially survive at the new microenvironments, tumour cells must use complex mechanisms to change these microenvironments (Valastyan et al., 2011).

1.3.1.6   Metastatic colonisation

The initial survival of cancer cells in their new microenvironment often does not indicate that the cancer cells will proliferate to form large macrometastases a process known as metastatic colonisation. Many persist as micrometastases and eventually become dormant while others slowly degrade over periods of weeks and months (Valastyan et al., 2011). Many of the micrometastases, lack the ability to activate the Src pathways, integrin β1 and focal adhesion kinase (FAK) which are essential to make the foreign microenvironment more hospitable (Barkan et al., 2008; Shibue et al., 2009). Alternatively, the micrometastases may continuously grow but their growth is suppressed by apoptosis. However, the mechanism to explain such high rate of apoptosis is not well understood but it may be due to failure to initiate neoangiogenesis by the tumour cells (Valastyan et al., 2011).

A number of genes have been identified whose expression facilitates the metastatic colonisation of breast cancer cells to lung (Minn et al., 2005), bone (Kang et al., 2003), liver (Tabaries et al., 2011) or brain (Bos et al., 2009). One example is breast cancer cells facilitate osteoclastic cytokine IL-11 to form osteolytic bone metastases whereby IL-11 disrupts the

normal physiological signals between osteoclasts and osteoblasts (Kang et al., 2003; Valastyan et al., 2011).

Another factor that may determine the success of metastatic colonisation is that metastatic cells must possess a high self-renewal capacity. It was found that only a small population of cells in a tumour may possess high self-renewal capacity. These cells are known as tumour-initiating cells (TICs). Therefore, it is those cells, the TICs that must disseminate from the tumour to distant organs sites to be able to form macrometastases (Valastyan et al., 2011). Furthermore, the promotion of tumour cells to TICs is facilitated by a group of molecules known as the EMT-promoting transcriptional factors (TFs), such as Twist, Snail and ZEB1 (Leber et al., 2008; Valastyan et al., 2011; Pachmayr et al., 2017). In addition, the metastatic colonisation of the lung and breast cancers are also modulated by the homeobox transcriptional factor NKx2-1 (Winslow et al., 2011) and the inhibitors of cell differentiation (ID) transcriptional factor family, ID1 and ID3 respectively (Gupta et al., 2007). Moreover, there are many regulators identified that have a pleiotropic function in the invasion metastasis cascade such as the microRNA miR-31 (Le, et., 2010), which inhibits breast cancer metastasis by simultaneously slowing metastatic colonisation, local invasion, and one or more early post-intravasation events (Valastyan et al., 2011).

In conclusion, the continuous change in genetic and/or epigenetic phenotypes as well as the interaction of cancer cells with stromal cells, helps cancer cells to complete an intricate cell biological process to the formation of macrometastases at distant organ sites which are responsible for most cancer deaths (Valastyan et al., 2011). Therefore an in-depth understanding of the mechanism underlying the invasion metastases cascade is critical in finding new therapeutic strategies to inhibit metastatic growth.

1.4 Breast cancer metastasis

1.4.1 Detection of breast cancer metastasis

One of the most common types of cancer is breast cancer and it accounts for the highest cancer mortality in women (Lage, 2003; Scully et al., 2012; Martin et al., 2014; Nounou et al., 2015). Breast cancer mortality is not only due to primary tumour itself but mainly due to breast cancer metastases to distant organ sites (Scully, et al., 2012; Ugenskiene, et al., 2012). Currently, there are a number of methods used to detect breast cancer metastasis such as radiological evaluations, clinical manifestations of the spread to distant organs, serum tumour markers and biopsies of affected organs (Scully, et al., 2012; Nounou et al., 2015). With early

diagnosis using mammographic screening, the mortality rate from metastasis has decreased but the methods mentioned above are often inefficient at accurately predicting the clinical outcome of the disease and at identifying metastasis at the earliest stage. Therefore in order to improve the efficiency of diagnosis, the analysis of CTCs to detect metastasis has to be used. CTCs are tumour cells found largely circulating in the bloodstream of cancer patients and originate from the primary tumour. In addition, CTCs have critical roles in cancer metastasis, hence their analysis will help predict metastatic progression and relapse more accurately. Furthermore, research also found that CTCs correspond with survival and clinical outcome of cancer patients. One study indicated that before treatment, patients with metastatic breast cancer with more than five CTCs in 7.5 ml of blood are subject to shorter overall survival and progression-free. Moreover, after neoadjuvant chemotherapy, the detection of one CTC in 7.5 ml of blood could correlate to metastatic relapse. There are a number of methods used to detect CTCs and are showing promising results but in order to improve quality and efficacy, a need to normalise the techniques is required (Scully et al., 2012).

1.4.2   Treatment of breast cancer metastasis

The survival of metastatic breast cancer patients has significantly improved in the recent few years but the metastatic disease has proven to be a challenge. Targeted therapy and standard chemotherapy are the two types of treatment for breast cancer metastasis (Scully et al., 2012).

Hormone therapy, antiangiogenic therapy and immunological therapy are considered to be targeted therapies. In hormone therapy the aim is to inhibit the enzyme aromatase to reduce estrogen or simply to block the estrogen receptor (ER). In post-menopausal women the only source of endogenous estrogen is from the conversion of adrenal androgen to endogenous estrogen by aromatase. In post-menopausal women with metastases the initial hormone therapy use is tamoxifen, which is an agent that blocks the ER causing tumour regression. Furthermore, aromatase inhibitors used are anastrozole, exemestane and letrozole which have a better therapeutic index than tamoxifen, as first-line therapy for post-menopausal women with metastases (Scully et al., 2012; American Cancer society, 2017).

In addition, breast cancer patients that show a high expression of human epidermal growth factor receptor 2 (HER2) are often treated with trastuzumab (Maughan et al., 2010), a monoclonal antibody that selectively binds to the extracellular domains of HER2 to decrease tumour growth. More often, trastuzumab is combined in chemotherapy with reports indicating an improved response rate, overall survival rate and time-to-regression in metastatic breast

cancer patients. Other monoclonal antibodies targeting HER2 include pertuzumab and trastuzumab-MCC-DM1 (trastuzumab emtansine), all show encouraging results in metastatic breast cancer therapy (Scully et al., 2012; Nounou, 2015).

Furthermore angiogenesis is crucial in the formation of metastases at distant organ thus inhibiting new blood vessel growth is necessary to limit the metastases formation. A monoclonal antibody derived from murine VEGF known as bevacizumab, is used to limit the supply of nutrients and oxygen to the cancer cells, hence inhibiting endothelial growth by blocking all VEGF-A isoform only, thus preventing blood vessels from growing to the cancer cells. To increase progression-free survival duration, bevacizumab is often used in combination with other chemotherapeutic agents but may cause serious side effects such as haemorrhage, high blood pressure and heart failure in breast cancer patients (Nielsen et al., 2010; Sully et al., 2012; Gampenrieder et al., 2017).

On the other hand, in standard chemotherapy, a number of cytotoxic drugs are used, including anthracyclines (doxorubicin and epirubicin), taxanes (paclitaxel and docetaxel) and 5-fluorouracil which are first, second and third lines of therapy respectively (Nicolini et al., 2006). However, many of these cytotoxic drugs have severe side effects such as the use of anthracycline have been link to cardiac dysfunction (Mitry et al., 2015). Therefore, newer cytotoxic agents such as ixabepilone and epothilones (Luu et al., 2011) have been developed which show greater efficacy in metastatic breast cancer patients who were originally treated with taxanes and anthracyclines (Alvarez, 2010; Scully et al., 2012; American cancer society, 2017).

Many factors and pathways have rendered breast cancer metastasis to be a very complex process where a lot needs to be elucidated to fully understand the process which, at the same time could provide new therapeutic strategies. Additionally, more efficient methods are needed to predict and detect breast cancer metastasis at the earliest stage possible to increase the effectiveness of treatment.

1.5   Drug resistance in cancer cells

In the treatment of diseases, over a period of time, the disease can acquire a phenomenon known as drug resistance, a similar concept to bacterial resistant to certain antibiotics. Similarly, during chemotherapeutic treatment of cancers, cancer cells can become resistant to the drugs (Leonard et al., 2003; Housman et al., 2014). Depending on the response of tumour cells to the initial therapy, cancer resistance can be divided in to two categories:

primary drug resistance, which exists before any given treatment, and acquired drug resistance, which appears after initial treatment (Yasui et al., 2004; Bozzuto et al., 2010; Zahreddine et al., 2013). However, regardless of primary or acquired resistance, there are many genetic and epigenetic processes taking place inside the cancer cells to give rise to cancer drug resistance, hence at certain points during treatment, patients can develop resistance (Zahreddine et al., 2013). There are many mechanisms by which cancer cells can become resistant to chemotherapy, namely drug inactivation, drug target alteration, epithelial-mesenchymal transition (EMT), cell death inhibition, DNA damage repair, drug efflux and epigenetics (Figure 1.8) (Yasui et al., 2004; Housman et al., 2014; Cree et al., 2017).
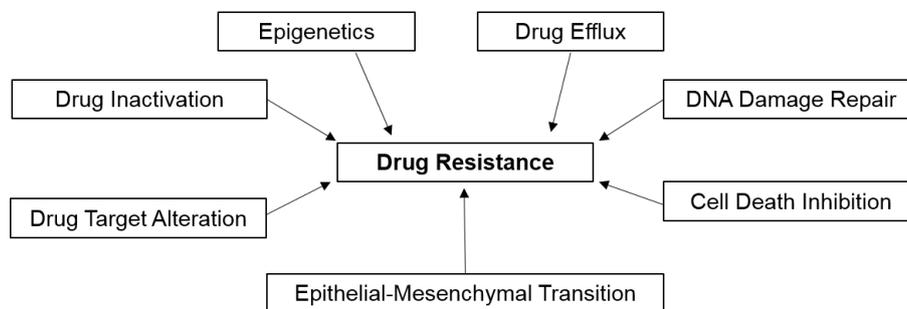
Figure 1.8. Mechanisms through which cancer cells can adopt to promote drug resistance. Cancer cells can acquired drug resistance via one or in combination with other mechanism that trigger a series of signal transduction pathways (Housman et al., 2014).

1.5.1 Drug inactivation

Many anticancer drugs need to undergo metabolic activation in order to be clinically effective during treatment. Therefore drugs must undergo complex mechanisms of interactions with different proteins to either, complex, modify or partially degrade the drugs, all in the aim of significantly activating the drugs. However, during cancer treatment, cancer cells can develop resistance to anticancer drugs by lowering drug activation (Housman et al., 2014). For instance, cytarabine (AraC), a nucleoside drug, used to treat acute leukaemia (AML) must be activated to its active form, AraC-triphosphate through a series of phosphorylation steps that involve enzymes such as deoxycytidine kinase. To decrease the activation of AraC, mutation or downregulation in this pathway can lead to AraC resistance (Zahreddine et al., 2013; Housman et al., 2014; Mansoori et al., 2017).

A group of detoxifying enzymes that normally engaged in protecting cellular macromolecules from electrophilic compounds known as Glutathione S-transferase (GST), also may promote drug resistance by inhibiting the mitogen-activated protein kinase (MAPK) pathway and through direct detoxification. In cancer cells these enzymes are overexpressed causing an increase in detoxification of the anticancer drugs which leads to less cancer cells toxicity (Housman et al., 2014; Mansoori et al., 2017).

Furthermore, platinum-based chemotherapy can be inactivated by thiol glutathione and methallothionein which promotes detoxification hence causing cancer cells to acquire resistance to platinum based anticancer drugs such as cisplatin, oxaliplatin, and carboplatin (Zahreddine, et al., 2013; Housman, et al., 2014). Another way in which cancer cells can acquire drug resistance is via changes to apoptosis-related proteins such as the tumour suppressor protein p53. In response to chemotherapy, p53 promotes apoptosis but when this gene is mutated, cancer cells acquire drug resistance. In addition, inactivation of p53 effectors such as caspase-9 and its cofactor, apoptotic protease activating factor 1 (Apaf-1) can also develop drug resistance (Housman et al., 2014).

Finally, Uridine 5'-diphospho-glucuronosyltransferase (UGT) is a group of enzymes that catalyse the glucuronic acid component of UDP-glucuronic acid to a hydrophobic molecule in a reaction known as glucuronidation. This reaction mediates the production of inactive hydrophilic glucuronides with substrates such as bile acids, steroids and xenobiotics. UGT genes such as *UGT1* and *UGT2* genes facilitate the first line metabolic defence against pathogenic substrates in many tissues such as in the breast, gut and prostate. DNA methylation can negatively regulate *UGT1A1* expression causing irinotecan, a topoisomerase I inhibitor to be functional when the gene is silenced (Gagnon et al., 2013). Unfortunately, resistance to irinotecan and other drugs occurs when *UGT1A1* expression increases due to epigenetic changes (Housman et al., 2014; Takano et al., 2017). In conclusion, drug inactivation is an important mechanism through which cancer cells acquire drug resistance which needs further investigation.

1.5.2 Drug target alteration

The expression levels of a drug target can be altered due to modification or mutation, hence affecting the efficacy of the drug. Such changes in drug target can lead to drug resistance. Topoisomerase II is an enzyme that obstructs super coiled or under-coiled DNA from forming. Many anticancer drugs target topoisomerase II by blocking the action of topoisomerase II thus leading to inhibition of DNA synthesis and promotion of DNA damage and eventually apoptosis

and cell death. However, in certain cancer cell lines, the topoisomerase II gene is mutated thus the efficacy of the topoisomerase II inhibitor decreases leading to drug resistance (Housman et al., 2014). Moreover, signalling kinases, such as the EGFR family, Ras, Src, Raf and Mitogen-activated protein kinase (MAPK) are being targeted by many anticancer drugs. However, these drugs lose their efficacy once mutation in these kinases occur leading to overexpression of the genes or over activation. One example HER2, receptor tyrosine kinase is often targeted in chemotherapy but due to its overexpression in certain breast cancer patients, drug resistance is inevitable after long term use of the inhibitors (Housman et al., 2014; Cree et al., 2017).

Another way which drug response can be altered in cancer cells is through modification of the level of enzyme expression. On the other hand, androgen receptor was found to be genomically amplified in certain prostate cancers, thus rendering anticancer drugs such as bicalutamide and leuprolide ineffective in androgen deprivation therapy (Heinlein et al., 2004; Housman et al., 2014).

Changes in signal transduction processes that control drug activation, may alter drug targets leading to drug resistance. For example the efficacy of trastuzumab in the treatment of HER2-postive breast cancers as a single agent is often low, where some patients do not respond to treatment and even though the efficacy is a lot higher in combination with other anticancer drugs, the continuous use of the drug leads to drug resistance and relapse. Moreover, activation of PI3K/Akt pathway, co-expression of growth factor receptors, cell cycle inhibition and loss of Phosphatase and tensin homolog (PTEN) function is thought to contribute to the mechanism of resistance (Berns, et al., 2007; Pohlmann et al., 2009; Luque-Cabal et al., 2016). In the trastuzumab-resistant cell line, significant increases in the levels of insulin-like growth factor 1 receptor (IGF1R) confirmed that inhibition of IGF1R increases the efficacy of trastuzumab. Therefore, targeting both, IGF1R and HER2 at the same time may enhance treatment in HER2-positive breast cancer patients (Browne et al., 2011; Housman et al., 2014; Sanabria-Figueroa et al., 2015).

In the treatment of breast cancer, tamoxifen resistance may occur via alterations in signalling mechanisms. Tamoxifen is an oestrogen receptor (ER) antagonist that inhibits the effect of oestrogen on these receptors. Moreover, interaction of ER signalling with other growth signalling pathways may promote drug resistance such as in breast cancer with HER2 overexpression whereby inhibiting the activity of tamoxifen is reversed to promote tumour growth (Shou et al., 2004). Furthermore, in the treatment of ER-positive breast cancer with tamoxifen, high expression levels of EGFR and HER2 were detected which continues to

increase when tumour became resistant (Massarweh et al., 2008; Chang, 2012; Ali et al., 2016). Finally, an in-depth understanding of the mechanism of drug target alteration will enable the treatment of drug resistant cancers more effective.

1.5.3 DNA damage repair

During chemotherapy, cancer cells trigger a mechanism of DNA damage response to counteract the DNA damage done by the anticancer drugs. Cisplatin, a platinum based drug promotes apoptosis by producing harmful DNA crosslinks, but cancer cells become resistant to platinum based drugs by activating the homologous recombination and nucleotide repair mechanisms. Therefore, in order to increase the efficacy of the therapy, inhibitors of the DNA repair pathway must be used in combination with anticancer drugs that promotes DNA damaging (Housman et al., 2014).

Furthermore, cancer cells intricately have many DNA damage response (DDR) mechanisms such as the homologous recombination repair mechanisms (RRM) and the nucleotide excision repair system (NER) which can compensate other DDR mechanisms that are either impaired or dysregulated by either epigenetic silencing or mutation in certain genes involved in the DDR mechanisms. Therefore, for effective chemotherapy, both the over-active and the under-active DDR mechanisms must be identified so as to target these DDR mechanisms to leave the cancer cells more vulnerable to DNA damaging drugs (Mansoori et al., 2017).   One enzyme that has been targeted in the DDR pathway for therapy is the O6-methlguanine DNA methyltransferase (MGMT). The aim of the drugs are to induce guanine O6 alkylation which makes the cancer cells undergo cell death. However, targeting the MGMT has proved to be a challenge as the drugs on clinical trial such as O6-benzylguanine also affect healthy cells (Verbeek et al., 2008; Fan et al., 2013). Therefore, identifying specific proteins in the DDR pathways and producing specific drugs to that protein will help to sensitise cancer cells to cytotoxic drugs whilst leaving healthy cells unharmed.

1.5.4 Cell death inhibition

Apoptosis and autophagy are two critical mechanisms that promote cell death. Apoptosis promotes cell death through either an intrinsic pathway that requires the mitochondria and involves proteins such as caspase-9, B-cell lymphoma 2 (BCL-2) family proteins and Akt, and an extrinsic pathway that involve death receptors on the cell surface. Furthermore, the activation of both pathways activates caspase-3 which causes apoptosis (Housman et al., 2014; Mansoori et al., 2017).

Drugs targeting the apoptosis pathways are very important in ensuring high efficacy in therapy. Proteins such as Akt, BCL-2 family proteins, signal transducer and activator of transcription (STAT) proteins and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are high overexpressed and highly active, making them potential drug targets. Moreover, through the activation of caspase-8, recombinant form of tumour necrosis factor related apoptosis-inducing ligand (TRAIL) and agonistic antibodies to these receptors can promote apoptosis (Mohammad et al., 2015; Rathore et al., 2017). In clinical trials, the use of TRAIL with other cytotoxic drugs shows significant results but its use alone is ineffective. In addition, several other drugs that may improve the efficacy of therapy includes the histone deacetylase inhibitors (HDACi), protease inhibitors, BCL-2 protein inhibitors and kinase inhibitors (Housman et al., 2014).

Cell survival can be promoted through a process that involves recycling of damaged or unnecessary cellular components and lysosomal degradation known as autophagy, which in turn maintains intracellular homeostasis. Therefore in chemotherapy, cancer cells will attempt to maintain intracellular balance by eliminating cellular stress and dysfunctional organelles triggered by anticancer drugs, hence keeping cancer cells alive (Mohammad et al., 2015). Moreover, drugs such as chloroquine and its derivatives causes the pH of the lysosomes to increase which result in inactivation of the digestive enzymes in the lysosomes. Chloroquine is very effective in rendering cancer cells vulnerable to cytotoxic drugs. Hence, chemotherapy is made more effective when fluorouracil is combine with chloroquine (Sasaki et al., 2010). Moreover, the inhibition of autophagy in ER-positive cancer cells by hydroxychloroquine, a chloroquine derivatives, enhances the sensitivity of ER inhibitors such as tamoxifen (Cook et al., 2014). In general, chloroquine is particularly useful in sensitising autophagy drug resistant cancers to other anticancer drugs (Manic et al., 2014; Housman et al., 2014). In conclusion, targeting the apoptosis and autophagy pathways is crucial in increasing the efficacy of therapy.

1.5.5 Epithelial-Mesenchymal Transition (EMT)

As mentioned above, tumour cells become metastatic by employing a mechanism that ensures the transition of epithelial cells to mesenchymal cells known as EMT. During EMT, the expression of cell adhesion receptors which help in cell-cell attachment are reduced while those that promote cell motility are increased. The release of chemokines and cytokines by tumour cells or cells in the microenvironment also mediate cell motility. In addition the MMPs help tumour cells to migrate, promoting metastasis. Since, EMT is a very important mechanism in cancer metastasis, its involvement in cancer drug resistance may prove to be a critical target to reduce drug resistance (Housman et al., 2014).

The degree of EMT and the differentiation levels of tumour account for the factors that play an important role in the development of drug resistance during EMT. For example, tumours that express high levels of β1 integrins in HER2-positive breast cancer are more resistant to inhibitors such as trastuzumab (Lesniak et al., 2009; Hou et al., 2016). Moreover, during the differentiation processes of cancer cells to facilitate EMT, drug resistance may occur. For example, the highly expressed αvβ1 integrin in colon cancer interacts with stromal cell adhesion molecules to positively mediate the expression of transforming growth factor β (TGFβ), which is essential for EMT (Bates et al., 2005). Other factors such as β3 integrin, src (Galliher et al., 2006), FAK (Shibue et al., 2009), VEGF, Flt-1 (Bates et al., 2005), and selectin (Bendas et al., 2012) play an important role in mediating the process of EMT and cell survival in cancer. Furthermore, research suggest that stromal cells may play a crucial role in causing drug resistance in cancer. Such finding was observed when inhibitors of B-Raf, an intermediate kinase initiated by receptor tyrosine kinases, were used in melanoma cells which develop drug resistance when co-cultured with fibroblast (Straussman et al., 2012; Du et al., 2016).

Although there are various factors that are involved in cancer metastasis and EMT, cell differentiation in metastasis cancer is not uniform due to a heterogeneous cell population (Heerboth et al., 2015). Hence, this account for some patients to be more responsive to treatment than the others. Furthermore, EMT may bring about drug resistance in some or all the cells in the tumour by producing signals that increase survival (Housman et al., 2014). Since, EMT is a very complex mechanism, a thorough investigation is necessary to understand its involvement in drug resistance so that factors which play a critical role in promoting drug resistance can be targeted to improve cancer treatments.

1.5.6   Drug efflux

The efflux of drugs from cancer cells is one of the most complex mechanisms cancer cells use to promote drug resistance. Whereas many other methods of developing resistance can be overcome by changing to a different drug regime, drug efflux mechanisms in response to one drug can lead to resistance to other drugs. This phenomenon is often known as multidrug resistance (MDR) where a cell becomes resistant to mechanistically and structurally unrelated drugs, including drugs they have not previously been treated with. Thus MDR in cancer cells is almost identical to antibiotic resistance (Leonard et al., 2003; Bozzuto et al., 2010). It was found that drug efflux by several membrane proteins within the ATP-binding cassette (ABC) transporter family proteins are responsible for the multidrug resistance phenomenon (Bozzuto et al., 2010; Zahreddine et al., 2013). The first MDR ABC transporter to be identified was P-

glycoprotein (P-gp/ABCB1) in 1976 by Juliano and Ling, which is capable of exporting a wide range of drugs (Jaramillo et al., 2018). Since then several other members of the ABC superfamily have also been found to contribute to drug efflux, such as the multidrug resistance protein 1 (MRP1/ABCC1) (Cole et al., 1994) (Cole, 2014) and the breast cancer resistance protein (BCRP/ABCG2) (Nakanishi et al., 2012; Sun et al., 2012). Many of the ABC transporters efflux a variety of xenobiotics, including anthracyclines, vinca alkaloids, kinase inhibitors, epipodophyllotoxins and taxanes and have a wide substrate specificity (Liu et al., 2010). Thus, during chemotherapy, many of the ABC transporters are activated as an act of defence to protect the cancer cells. Therefore, MDR mediated by ABC transporters is one of the biggest challenges clinically in overcoming tumour progression. Often, certain ABC transporters in cancer cells are highly overexpressed rendering the cancer cells more resistant to anticancer drugs such as docetaxel, cisplatin, topotecan and doxorubicin to name a few, which correlate with higher patient mortality. Therefore, inhibiting certain ABC transporters may help in increasing the efficacy of treatment but at the same time can affect the normal functions of cells (Morgan et al., 2012). Moreover, continuous use of maximum dose of chemotherapy causes tumour remission but due to selection of ABC transporter overexpressing cells may also boost chemotherapy resistance in newly formed metastases (Kryczka et al., 2018). Thus further investigation is required to fully understand the complex role ABC transporters play in cancer progression. The ABC transporters is further discussed in the ABC transporter sections below.

1.5.7   Epigenetics

The heritable changes in gene expression and phenotype that are not due to change in nucleotide sequence are termed epigenetics. Epigenetic mechanisms are important in normal development and cell functions including chromosome x inactivation, differentiation, genomic imprinting and gene silencing (Lim et al., 2010; Ghasemi et al., 2017). However in cancer cells, epigenetic modification through DNA methylation and histone modification are important mechanisms that are found to cause drug resistance in cancer treatment (Housman et al., 2014). DNA methylation is a process in which a methyl group is specifically bound to the base cytosine in the dinucleotide 5'-CpG-3'. The CpG is a base cytosine linked to a base guanine by a phosphate bond in the DNA nucleotide sequence. Usually, in the human genome, most CpG dinucleotides are methylated and the unmethylated CpGs are grouped together in a cluster known as CpG islands which are in the promoter region of many important genes. In cancer cells, the methylation of CpG islands often leads to silencing of gene expression such as the tumour suppressor genes (Lim et al., 2010; Christmann et al., 2017). On the other hand, histone modifications through histone acetylation and deacetylation opens or closes the

conformation of chromatin respectively. However, the normal regulation of chromatin in cancer cells is mutated (Vaissiere et al., 2008; Housman et al., 2014; Christmann et al., 2017). For example hypermethylation of tumour suppressor genes (Herman, 1999) and hypomethylation of oncogenes (Ehrlich, 2009) are often silenced or overexpressed respectively. Therefore the mechanisms of epigenetic are usually reversible which makes them ideal drug targets to overcome drug resistance.

The DNA damage repair can be influenced by epigenetic mechanisms. The most common method which cancer cells used to silence tumour suppressor genes is through DNA hypermethylation. However, transcription factors and DNA repair genes can be affected by hypermethylation that may indirectly affect downstream targets causing tumorigenesis and genetic error (Lattanzio et al., 2015). For example during the hypermethylation of the human mutL homog 1 (hMLH1) gene promoter, the DNA mismatch repair processes is impaired causing cancer development (Fleisher et al., 2001). Furthermore, drug resistance due epigenetic mechanisms are also found in breast cancer cells. For example, a lack of reduced folate carrier (RFC) expression and an essential change in drug uptake promoted methotrexate resistance in MDA-MB231 breast cancer cells (Worm et al., 2001).

Additionally, epigenetics are critical in the cancer progression and formation of cancer stem cells which often acquire drug resistance. Moreover, epigenetic modifications may play an important role in tumour metastasis and tumour formation hence targeting them would be effective in counteracting drug resistance (Housman et al., 2014). Finally, it is very important in understanding epigenetics modifications that leads to drug resistance so as to identify regulators that trigger the modification and thus target theses regulators to improve cancer treatments.

1.5.8   Strategies implemented to overcome drug resistance

New synthetic analogues of existing drugs such as vinca alkaloid derivatives have been development to try to circumvent drug resistance. In most cases this approach has been successful but other toxicity has been a problem (Cree et al., 2017).

Another approach is combining therapies in the aim of targeting two or more independent pathways so as to increase the likelihood of hitting two or more targets on the same pathway. Thus, increasing the efficacy of therapy while reducing tumours ability to acquire drug resistance (Yardley, 2014; Cree et al., 2017; Kydd et al., 2017).

Furthermore, another approach suggested is sequential combinations of drugs to reduce toxicity and at the same time increase efficacy (Vijayaraghavalu et al., 2013). However, due to a lack of molecular understanding of their efficacy, relatively few sequential combinations have entered clinical practice. Additionally, the development of DNA and RNA sequencing technologies have now facilitated the prediction of sequential combination of drugs effect (Cree et al., 2017).

A mechanistic approach to combination and sequence strategies known as synthetic lethality is an important new concept in drug development (Shaheen et al., 2011; Weidle et al., 2011). Consequently, sufficient knowledge of the molecular makeup of individual cancers is needed to ensure the approaches to synthetic lethality is a success. In addition, companion diagnostic development strategies and drug development must be coordinated and that careful testing in a variety of preclinical setting is needed before moving on to humans (Cree et al., 2017).

Lastly, another approach to circumvent anticancer drug resistance is to use immunotherapy which could benefit in the treatment for certain cancers such as lung cancer and melanoma (Keller et al., 2017; Cree et al., 2017). As mentioned earlier, the activation of certain pathways as a result of not only genetic and epigenetic events but also due to extracellular signals, can cause cancer cells to acquire drug resistance during treatment. Therefore, identifying signalling pathways that might promote drug resistance will inspire more systematic approach as well as categorising patients into groups depending on their response to the drugs. Also such approach can help design combination strategies to act simultaneously on resistant pathways and on multiple cancer cells (Cree et al., 2017).

1.6   ATP-binding cassette (ABC) transporters

1.6.1   Historical background

ATP-binding cassette (ABC) transporters have a diverse physiological functions and are considered to be one of the largest of all protein families. ABC transporters are present in all species ranging from microorganism to human, having wide variety of physiological roles and are important in economy and medicines. In microorganisms, ABC transporters play a key role in antifungal and antibiotic resistance and in humans, may get mutated causing a variety of genetic diseases including Tangier disease, cystic fibrosis, obstetric cholestasis and are also responsible for drug resistance in cancers (Higgins, 1992 and 2001; Coy, 2012).

The study of the ABC transporters all started from the studies on nutrient uptake in bacteria particularly *Escherichia coli* and *Salmonella typhimurium* in the 1970s. Researchers found that bacteria use multiple systems for the uptake of most nutrients including the phosphotransferase systems which incorporate transporters that depend on electrochemical gradient and the shock-sensitive system which incorporate transporters that uses energy from ATP hydrolysis. Furthermore, several genes encoding such transporters lead by the maltose permease of *Escherichia Coli* and histidine permease of *Salmonella typhimurium* were cloned in the early 1980s (Higgins, 2001; Theodoulou et al., 2015).

It was later found in 1986 that bacterial transporters had ATP binding subunits which grouped a large superfamily of proteins and their structures were proposed to have four domains. Most of these domains belonged to the active transport system with a few maybe able to couple to ATPase activity for other biological processes such as DNA repair. In the same period, the gene encoding the human multidrug resistance protein, P-glycoprotein/ABCB1 was discovered and was the first example of this family of transporters in eukaryotic cells. Although, in 1986 saw the discovery of the large family of proteins that has ATP-binding subunits, the name ATP binding cassette (ABC) transporters became apparent in 1990. These discoveries, highlighted the importance of the ABC transporters as a diverse family of proteins involved in a variety of physiological and biochemical processes (Higgins, 2001; Theodoulou et al., 2015). A brief overview of the key events in ABC transporter research is shown in figure 1.9.
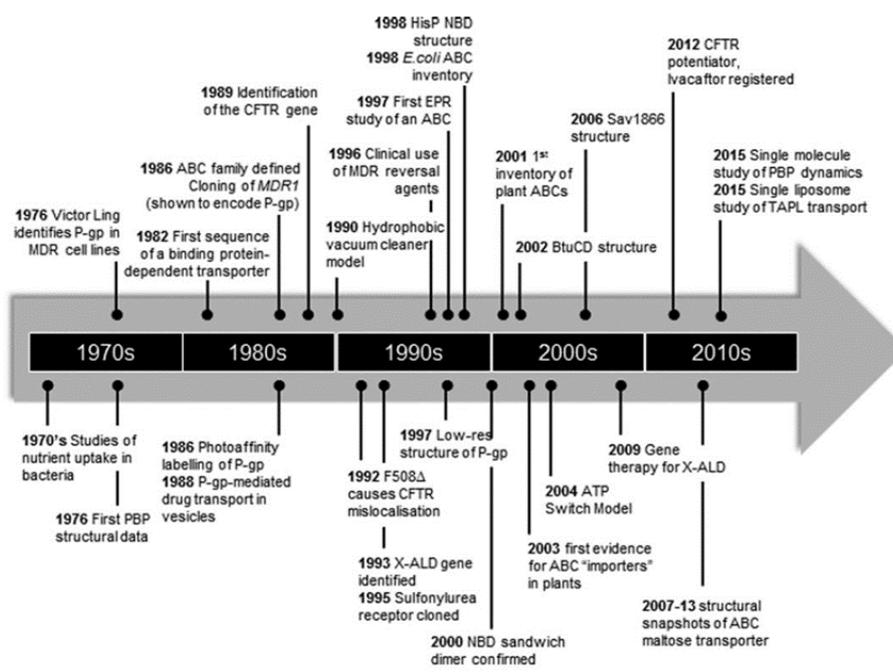
Figure 1.9. Timeline showing some of the past key events in ABC transporter research (Theodoulou et al., 2015).

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1.6.2 Structure of ABC transporters

Although ABC transporters have a diverse transport mechanism, many of them share similar domain architectures. The core structure of ABC transporters are made up of a pair of ATP-binding domains, also known as nucleotide binding domain (NBD) and two sets of transmembrane domains (TMDs). Upon, dimerisation of the two TMDs, the translocation pathway is formed and the conformation of the TMDs is controlled by the NBDs through hydrolysis-induced separation and ATP-induced dimerisation (Dean et al., 2001; Higgins, 2001; Procko et al., 2009).

So far, based on TMDs folds, there are seven types of ABC transporters discovered (Figure 1.10), of which three can only transport substrates from outside to inside of the cells known as Type I and Type II importers, and energy coupling factor (ECF) transporters (Type III importers) (ter Beek et al., 2014; Rice et al., 2014; Lewinson et al., 2017). These ABC importers are only found in prokaryotes. While type IV and V ABC transporters are known as the ABC exporters which are found in both prokaryotes and eukaryotes (Locher, 2009; ter Beek et al., 2014) and type VI and VII are found in bacterial cells (Crow et al., 2017; Greene et al., 2018; Hicks et al., 2018).
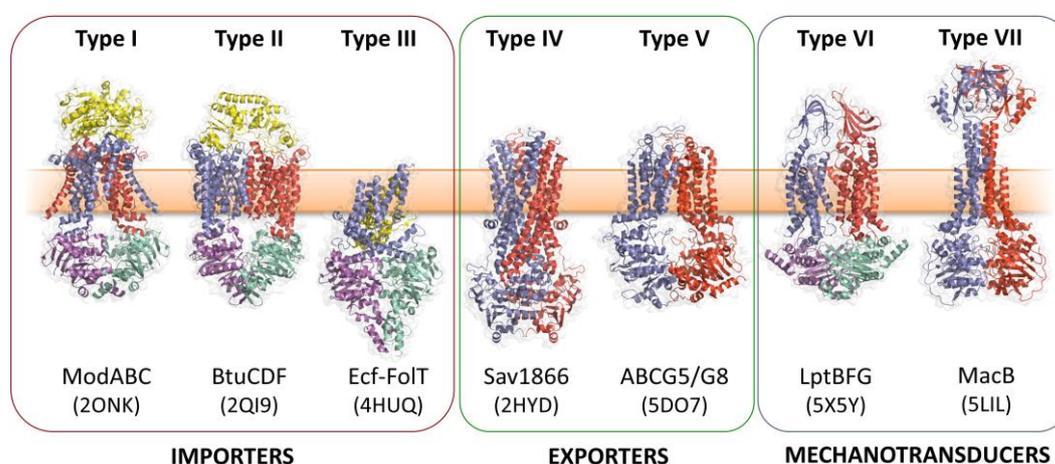
Figure 1.10. Schematic diagram showing the available crystal structures of seven types of ABC transporters. The protein data bank identifiers are in bracket below the name of the transporters. A single colour code present each protein chain. There are three types of importers (Type I, II and III (Energy coupling factor (ECF)) (Example: molybdate transporter (ModABC), vitamin B12 transporter (BtuCDF), folate importer (ECF-FolT) respectively). two types of exporters (Type IV and V) (Example: multidrug exporter (Sav1866) and sterol transporter (ABCG5/G8) repectively) and two types of mechanotransducers (Type VI and VII) (Example: lipopolysaccharide extractor (LptBFG) and, enterotoxin and macrolide transporter (MacB) respectively) (Greene et al., 2018).

In prokaryotes, the two TMDs and the two NBDs of the ABC transporters are often assembled as separate protein subunits. Therefore, the two TMDs and NBDs can be identical or different proteins (Wilkens, 2015). Furthermore, where the TMDs differ to the NBDs, usually the two NBDs are similar in structure and in most cases the two TMDs are also similar in structure with the exception of the two TMDs in ECF transporters (Xu et al., 2013; ter Beek et al., 2014). In other cases, in particular in prokaryotic transporter have two and occasionally three protein domains fused together such as the fusion of the two NBDs and the fusion of the TMDs with the NBDs (ter Beek et al., 2014; Wilkens, 2015). See figure 1.11 for a schematic diagram of the different core structures of ABC transporters. On the other hand, type IV exporters either exist as a dimer of two polypeptides, each of which made up of a TMD and an NBD such as in the bacterial exporters (Dawson et al., 2006), or one polypeptide chain comprising all domains as in eukaryotic exporters (Locher, 2009 ter Beek et al., 2014) and type V exporters are known as 'half transporters' comprising of one NBD fused to one TMD to form a single polypepide chain consisting of six transmembrane helices (Lee et al., 2016; Taylor et al., 2017). The third group of ABC transporters are the mechanotransducers, type VI and VII.

Moreover, the Type I and II ABC importers require an additional substrate binding proteins (SBPs) are to help facilitate the transport of substrate by capturing the substrate on the trans-side and distributing it to the TMDs (Figure 1.11). The SBPs can either be found as a separate protein subunit or fused with a TMD to form a multidomain subunit (Higgins, 2001; Locher, 2009). Additionally, apart from the core domains many ABC transporters in prokaryotes and eukaryotes have additional subunits or domains such as extra TMDs or regulatory domains (Figure1.11) (Theodoulou et al., 2015).
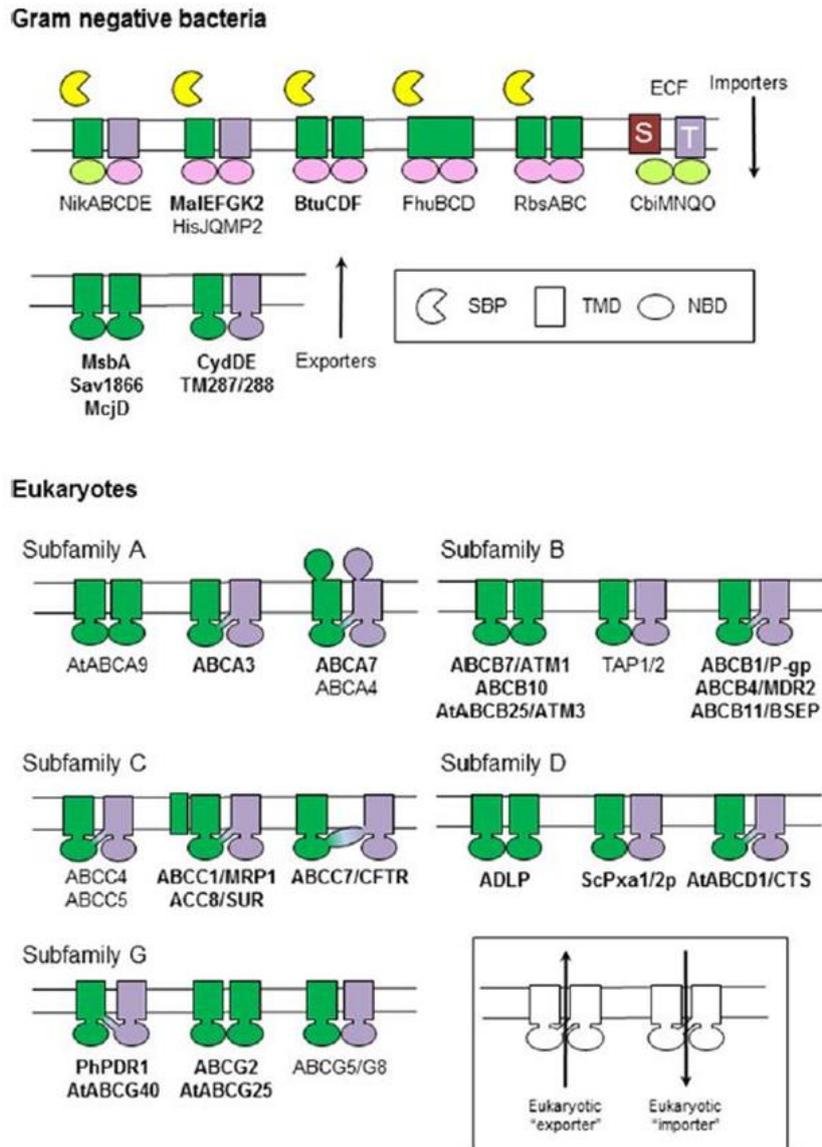
Figure 1.11. A schematic diagram of the different core structures of ABC transporters. The ABC importers in prokaryotes (Example in Gram-negative bacteria) consist of two TMDs linked to two NBDs and a SBP subunit. The ECF transporters comprise of a substrate-specific integral membrane protein (S) and a transmembrane protein (T). The ABC exporters in all bacteria are homodimeric or heterodimeric, in which a TMD is fused with an NBD. In eukaryotes, the ABC transporters are divided into eight distinct subfamilies (A-H) (See section 1.6.4). Each subfamilies, has some representative of different arrangement of core and additional domains. However, subfamilies E, F and H are not present in plants, fungi and mammals (Theodoulou et al., 2015).

1.6.2.1   Nucleotide binding domain (NBD)

As mentioned earlier all ABC transporters have two NBDs which bind and hydrolyse ATP. A core of about 200 amino acids make each NBD which also consist of two subdomains, the

RecA-like domain and the α-helical domain which is exclusive to ABC transporters. The NBDs consist of seven highly conserved motifs (Figure 1.12) (ter Beek et al., 2014; Wilkens, 2015).

Figure 1.12. A view along an axis perpendicular to the membrane plane from the trans-side onto the NBDs. The TMDs and SBP are not shown. A colour code show the different highly conserved sequence motifs and domains: red, A-loop; magenta, Walker A; orange, Walker B; cyan, ABC motif; blue, D-loop; green, H-loop; yellow, Q-loop; faded grey, regulatory C-terminal domain; light blue, RecA-like domain; green, α-helical domain. The ATP analogue Adenylyl-imidodiphosphate (AMP-PNP) is represented in sticks (ter Beek et al., 2014).

1.6.2.2   Transmembrane domain (TMD)

The TMDs of ABC transporters comprise a translocation pathway which facilitates the movement of substrates across the membrane (ter Beek et al., 2014).

Type I ABC importers consist of two TMDs each made up of five transmembrane (TM) helices. The TMDs are either homodimers or similar in structure. Furthermore, in many Type I importers there is an additional N-terminal helix thus making the total number of TM helices to 12 or 8 in some cases. The interface between the two TMDs produces the translocation pathway (Locher, 2009; ter Beek et al., 2014; Rice et al., 2014).

Type II ABC importers consist of two similar TMDs each made up of 10 tightly packed TM helices. In addition the helices in one TMD do not cross over to the other TMD, rather the helices line up to each other in a TMD. Furthermore, in each TMD has two group of helices pack together to found two subdomains which are position in opposite direction with respect to the membrane. Thus the two TMDs form a translocation pathway at the interface (Locher, 2009; ter Beek et al., 2014; Rice et al., 2014).

ECF-type ABC importers, consist of two TMDs, termed the T-component (EcfT subunit) and the S-component. Their function and structure are not similar. The T-component has five TM helices and in other transporters may have between four to eight TM helices (Eitinger et al., 2011) while S-component usually has six TM helices and sometimes an additional N-terminal helix (Xu et al., 2013). The S-component has a high binding affinity for transported substrates (Berntsson et al., 2012). However, unlike the other ABC importers, the translocation pathway in ECF-type ABC importers rely on the S-component that uses another access mechanism (ter Beek et al., 2014).

Type IV ABC exporters consist of two TMDs each comprised of six TM helices. The two TMDs can either be similar in structure or identical, often the TMDs are fused with the NBDs and there is a crossover of two helices from each TMD to the other TMD. Moreover, both TMDs are embedded deep in the cytoplasm causing the NBDs to be located very far from the membrane. Therefore the translocation pathway is more likely to be located at the interface of the dimerisation of the TMDs (Higgins, 2001; Procko et al., 2009; ter Beek et al., 2014).

As mentioned above, type V ABC exporters are 'half transporters' in that they consist of only one TMD and one NBD. Example of a protein belonging to this group is ABCG2. The TMD is made up of six TM helices. Similar to other nucleotide-free ABC exporters and importers, type V ABC exporters have also adopted an inward conformation. Since they are 'half transporters' their functional form is a homodimer where by the TM helices from one subunit do not cross over to the TMD of the other 'half transporter'. Moreover, the regions between TM helix 5 and TM helix 6 form distinct α-helical structures in the extracellular domain (ECD) (Lee et al., 2016; Taylor et al., 2017).

Type VI ABC transporters are lipopolysaccharide (LPS) exporters (Hicks et al., 2018). An LPS exporter is an oligomeric complex consisting of Lpt protein B, F, G and C subunits which are all bound to the inner membrane (IM) and Lpt D/E heterodimer which is bound in the out membrane (OM). The subunits in the IM are connected to the subunit in the OM by periplasmic LptA subunit thus form a teramer (Botos et al., 2016; Hicks et al., 2018).

Type VII ABC transporters such MacB is a macrolide antibiotic-specific transporter in *E. Coli*. MacB consist of four TM helices, an N-terminal NBD and a large periplasmic domain linking TM helix 1 to TM helix 2 (Xu et al., 2009; Crow et al., 2017). MacB is a homodimeric protein binds MacA adaptor protein and Tolc exit duct to form a tripartite efflux pump to efflux antibiotics and heat-stable enterotoxin II (STII) out of the bacterial cell (Xu et al., 2009; Crow et al., 2017; Fitzpatrick et al., 2017; Greene et al., 2018)

1.6.3   Mechanism of ABC transporters

The mechanism by which ABC transporters are able to transport substrate across membrane is very complicated due partly to structural diversity in ABC transporters. Furthermore, the transport mechanism is different between the different types of ABC transporters (Locher, 2009; Procko et al., 2009). Also to note, that between members of the ABC transporters that have similar structures may adopt a different transport mechanism (ter Beek et al., 2014). However, as more studies have been done on both prokaryotic and mammalian systems, scientists have begun to predict the mechanism of ABC transporters.

Generally, a conformational change in NBDs and TMDs by ATP binding and hydrolysis facilitates the movement of substrate across the membrane. Furthermore, there are two ATPase active sites at the interface but is still unclear whether one or two ATP molecules are required to hydrolyse one substrate at a time. Moreover, whether the binding of ATP molecules to the NBDs is sequential or simultaneous is still a debate (Higgins, 2001; Procko et al., 2009).

More recently, scientists has proposed a model for the transport mechanism of ABC transporters known as the ATP switch model which, explained that substrate movement depends on the low and high affinity states of substrate binding coupled with ATP catalytic cycle. In summary, the ATP-switch model proposed four steps to explain the transport mechanism of ABC transporter as first substrate binding, followed by ATP binding and substrate translocation and finally ATP hydrolysis to return the protein domains to substrate binding conformation (Mamo et al., 2017).

1.6.3.1   Type I ABC importers

Without the nucleotides, the two NBDs are apart and the two TMDs are open from the cytoplasmic side of the membrane. The binding of the substrate to SBP trigger a conformational changes in the NBDs and in the TMDs which allows the NBDs to come close

together to facilitate the binding of ATP (pre-translocation state). In the catalytic intermediate state, the two TMDs come close together on the cytoplasmic side of the membrane to form an outward facing cavity. At the same time the SBP is still attached and prevent other substrate from passing through the cavity and the periplasm. After the release of the substrate into the cavity, the SBP is in an open conformational state. The substrate binds to a specific site on the TMD. To complete the transport cycle, ATP hydrolysis release a phosphate (Pi) and ADP which causes the TMDs to return into the inward conformation with the cavity facing the cytoplasm, thus release the substrate in to the cytoplasm (Figure 1.13) (Locher, 2009; ter Beek et al., 2014; Rice et al., 2014).

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Figure 1.13. Schematic presentation of the transport mechanism of Type I ABC importers. Substrate binds to the SBP and move to the transporter. Hence causing the NBDs to move closer to each other to allow ATP binding and eventually NBDs closure. As a results, the TMDs are force to form an outward facing conformation that lead to SBP opening. Once the SBP opens, substrate is release in to the cavity and bind on to the TMD. Finally, ATP hydrolysis cause the TMDs to form an inward facing conformation which release the substrate in to the cytoplasm (ter Beek et al., 2014).

1.6.3.2 Type II ABC importers

Depending on the presence of the substrate and nucleotides, different conformations are adopted by the NBDs, TMDs and periplasmic SBP. When the SBP and the nucleotide are not present, the transporter adopts an outward facing conformation in which the TMDs are open toward the periplasmic side of the membrane thus creating an outward facing cavity. Furthermore, a transporter complex is formed when ATP together with SBP bind to the transporter to give a translocation pathway where by both ends are sealed by the TMDs. The substrate binds non-specifically to the cavity (Locher, 2009; ter Beek et al., 2014; Rice et al., 2014).

To release the substrate to the cytoplasm, the binding of ATP causes ATP hydrolysis to convert to Pi and ADP, which causes the NBD dimer to open up and the TMDs to adopt an inward facing conformational changes via the coupling helices with the cavity facing the cytoplasm. Once the substrate is force out of the cavity in to the cytoplasm, the translocation pathway is distorted as the complex changes into an asymmetric state. In addition, the tight binding of SBP requires ATP binding to release the SBP (Figure 1.14) (Locher, 2009; ter Beek et al., 2014; Rice et al., 2014).

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Figure 1.14. Schematic representation of the transport mechanism of Type II ABC importer. The substrate binds to the SBP which binds to the transporter in an outward conformation. The binding of ATP causes a conformational change in the TMDs forming a cavity which the substrate binds non-specifically. ATP hydrolysis further causes the TMDs to form an inward conformation and the substrate is release in to the cytoplasm. The transporter then adopt an asymmetrical state which may be required to prevent leakage of small molecules (ter Beek et al., 2014).

1.6.3.3   ECF-type ABC importers

Due to research in ECF-type ABC importer still being at a relatively early stage, the toppling of the S-component and the coupling mechanism between nucleotide binding, hydrolysis and release in the NBDs are still not clear. However, the knowledge acquired from the crystal structure, has shown that the T-component has two long X-shaped α-helices which interacts with NBDs at one side and the S-component at the other side. The orientation of the S-component may be affected by the scissor-like movement of the helices and during the catalytic cycle. After release of substrate in the cytoplasm, the S-component spontaneously assume a conformational change where by the substrate binding site is facing outward in the periplasm (Figure 1.15) (ter Beek et al., 2014; Rice et al., 2014).

Figure 1.15. Schematic representation of the transport mechanism of ECF-type ABC importers. Not much is known about the transport mechanism of ECF-type ABC importers as the crystal structures obtained so far are limited. However, it is assumed that the S-component (orange) with the substrate binding site at one end topple over and rotate into the membrane to transport the substrate from the periplasm and release the substrate into the cytoplasm (ter Beek et al., 2014).

1.6.3.4 Type VI ABC transporters

In the inner membrane, the subcomplex of LptF, LptG and dimeric LptB are responsible to transport LPS from the inner membrane to the outer membrane along the periplasmic bridge. The TptB dimer hydrolyse ATP to form a complex with and change the transmembrane LptF/G dimer conformation. Unlike other ABC transporters, the substrate is extracted directly from one conformation of the LptBFG complex (Figure 1.16) (Botos et al., 2016; Hicks et al., 2018).
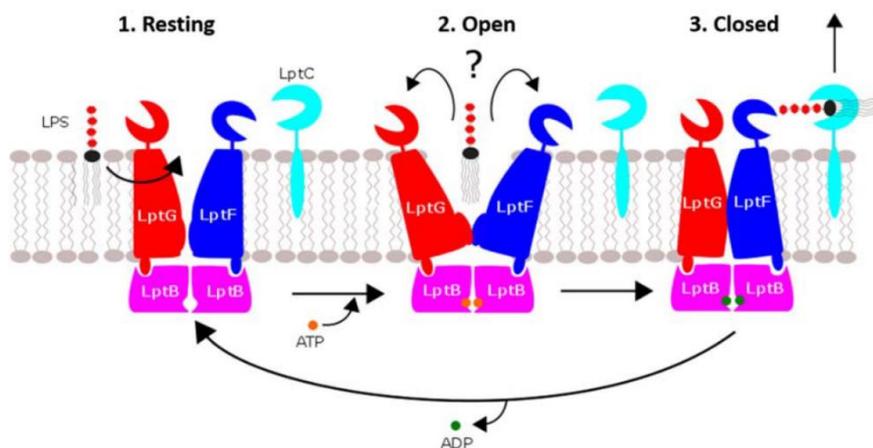
Figure 1.16. The mechanism of LPS transporter complex. In the resting state, the LptB nucleotide-binding sites are unoccupied, and the LptF/G subunits have an inward conformation. Upon binding of ATP to LptB, a conformational change in LptF/G dimer is induced forming an outward facing cavity to receive the LPS substrate that is still attached in the inner membrane. Once the substrate is inside the cavity, the hydrolysis of ATP by LptB causes the LptF/G cavity to close again. As a result the substrate is forced out of the inner membrane into the periplasm (Hicks et al., 2018).

1.6.3.5   Type VII ABC transporters

The inner membrane ABC transporter, MacB, operates in assembly with the periplasmic adaptor protein MacA and TolC exit duct, to form the MacAB-TolC ABC-type tripartite multidrug efflux pump (Crow et al., 2017; Fitzpatrick et al., 2017).

According to the mechanotransmission mechanism, an opening in MacB located between periplasmic domains and stalk may have caused substrates to enter the interior cavity (Figure 1.17A). Once the substrate is in the interior cavity, ATP binding causes the MacB periplasmic domain to close, forcing the substrate under pressure to move through the MacA gate ring which acts as a one-way valve (Fitzpatrick et al., 2017). When the substrate has been expelled out into the extracellular space, the pressure on either side of the gate equalised thus causing the gate ring to return to its closed resting state, which prevent substrates from moving back. The system is reset due to ATP hydrolysis to drive more substrates from the periplasm to the extracellular space (Crow et al., 2017; Greene et al., 2018) (Figure 1.17B).

Figure 1.17. Mechanotransmission mechanism for the MacAB-TolC tripartite efflux pump. (A) Crystal structures of nucleotide-free MacB (Left) and ATP-bound MacB (Right). Blue, TM helices; red, NBD; purple, periplasmic domain. (B) A schematic diagram showing the proposed mechanism of MacAB-TolC efflux pump. Once the substrate enters the interior cavity, ATP binding causes the MacB to close. The substrate is then forced out through MacA gate ring into the extracellular space under pressure. Once the substrate has expelled out, the

gate ring return to its closed resting state. ATP hydrolysis reset the system to drive more substrates out of the periplasm (Crow et al., 2017).

1.6.3.6 ABC exporters

In ABC exporters, the NBDs are connected to the TMDs in a single protein. ABCG2, 1, 4, 5 and 8 and ABCB2, 3, 5 and 10 all are homo/hetero dimers. However, with limited crystal structures of ABC exporters and only two different states which have been identified, outward facing and inward facing conformational states. The coupling mechanism between substrate binding, TMDs conformational changes and the ATP binding and hydrolysis are still not clear (ter Beek et al., 2014).

Generally, in the presence of no nucleotide, the NBDs are apart causing the TMDs to adopt an inward facing conformation, forming an inward facing cavity where the substrate is likely to bind to. The cavity is relatively large with multiple binding sites of irregular polarity which provide a very wide and efficient substrate recognition (Sarkadi et al., 2006). Furthermore, when ATP binds to the NBDs, the TMDs undergo a conformational change and adopt an outward facing conformational change where the substrate is expelled outside the cell. Once the substrate is released the ATP hydrolysis releases Pi and ADP causing the TMDs to return back to the inward facing conformation ready to accept another substrate to be transported across the membrane (Figure 1.19) (Higgins, 2001; Procko et al., 2009).

Figure 1.18. Schematic representation of the transport mechanism of ABC exporter. The NBDs have an inward open conformation. Substrate enter in the cavity formed by the TMDs binds to the TMDs. The binding of ATP molecules to the NBDs causes the TMDs to adopt an outward facing conformation where by the substrate is move out of the cavity into the extracellular space. ATP hydrolysis release Pi and ADP which causes the TMDs to move back to the original resting conformation which is an inward facing conformation (Procko et al., 2009).

1.6.4   Human ABC transporters

There are at present 49 human ABC genes identified and these are divided into seven subfamilies from ABCA to ABCG (Table 1.6) (Vasiliou et al., 2009) based on structural organisation of domain, similarity in gene structure and sequence homology in the NBDs and TMDs (Lorkowski et al., 2002; Mamo et al., 2017). A list of human ABC proteins, their chromosomal location and function is shown in Table 1.8. (Vasiliou et al., 2009). In addition, certain genes of the ABC transporters are mutated causing a variety of genetic diseases. Mutations in these genes are linked to nineteen known genetic diseases and the most common ones include Stargardt's macular dystrophy, progressive familial intrahepatic cholestasis, cystic fibrosis and adrenoleukodystrophy (Table 1.7) and currently at least 15 ABC transporters are responsible for multidrug resistance in cancers, particularly multidrug resistance protein 1 (MRP1), P-glycoprotein (P-gp, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) (Higgins, 2001; Gillet et al., 2011; Coy, 2012; Jaramillo et al., 2018).

Table 1.6. Human ABC transporter subfamilies (Adapted from Vasiliou et al., 2009).

Subfamily name	Aliases	Number of genes	Number of pseudogenes
ABCA	ABC1	12	5
ABCB	MDR	11	4
ABCC	MRP	13	2
ABCD	ALD	4	4
ABCE	OABP	1	2
ABCF	GCN20	3	2
ABCG	White	5	2
Total		**49**	**21**

ABC1, ATP-binding cassette 1; MDR, multi-drug resistance; ALD, adrenoleukodystrophy; OABP, organic anion-binding protein.

Table 1.7. ABC transporters with known genetic disorders and their substrates (Adapted from Coy, 2012).

Name	Tissue Location	Substrate	Disease
ABCA1	Ubiquitous	Phospholipid, Cholesterol	Tangier disease
ABCA4	Retina	N-retinylidene phosphatidyl-ethanolamine	Stargardt disease, retinitis pigmentosa, macular degeneration
ABCB4/ MDR3	Liver hepatocytes	Phosphatidylcholine	Progressive Familial Intrahepatic Cholestasis (PFIC)-3
ABCB11/ BSEP	Liver hepatocytes	Bile salts	PFIC-2
ABCC2/ MRP2	Liver, intestine, kidney	Bilirubin glucuronides, GSH and GSSG, glucuronide, GSH, and sulphate conjugates	Dubin-Johnson syndrome
ABCC6/ MRP6	Kidney, liver	small peptides	Pesudo-xanthoma elasticum
ABCC7/ CTFR	Lung, intestine, cholangiocyte, sweat glands	Chloride channel	Cystic Fibrosis
ABCD1	Many	Long-chain fatty acids	Adrenoleukodystrophy
ABCG5/ ABCG8	Liver, intestine	Cholesterol, plant sterols	Sitosterolemia

1.6.4.1 Subfamily A of the ABC family (ABCA)

So far there are 12 members of ABCA subfamily of transporters, most of which are involved in lipid trafficking in many diverse organs and cell types. Some of the ABCA transporters are considered to be among the largest ABC transporters with more than 2100 amino acids in

length. Mutations in some of these genes have led to severe genetic disorders such as Tangier disease T1 and harlequin ichthyosis. Furthermore, ABCA transporters could be responsible for the resistance of several cancers against anticancer drugs (Dean et al., 2001; Albrecht et al., 2007; Vasiliou et al., 2009).

1.6.4.2   Subfamily B of the ABC family (ABCB)

There are 11 members of ABCB subfamily of transporters, four of which are full transporters in that they have two TMDs and two NBDs within a single polypeptide chain, and seven half transporters in that they have only one TMD and one NBD in a single chain, but then form dimers. ABCB family transporters are the only ABC subfamily transporters that have both full and half transporters. Many ABCB transporters are involved in multidrug resistance in cancer cells. ABCB genes can be mutated causing diseases such as lethal neonatal syndrome, coeliac disease and ankylosing spondylitis (Dean et al., 2001; Stefkova et al., 2003; Vasiliou et al., 2009).

1.6.4.3   Subfamily D of the ABC family (ABCD)

To date, there are four ABCD subfamily transporters often known as the Adrenoleukodystrophy (ALD) or peroxisomal transporters. The ABCD transporters are half transporters which can either form homodimers or heterodimers to be functional (Stefkova et al., 2003; Vasiliou, et al., 2009). Furthermore, ABCD transporters are involved in the transport of long and very long chain fatty acids (VLCFA) or their CoA-derivatives into peroxisome and bile acid intermediates di- and tri-hydroxycholestanoyl-CoA (DHCA and THCA) and branched chain acyl-CoA (Morita et al., 2012; Kawaguchi et al., 2016). Moreover, mutation in certain ABCD transporters are implicated in disease such as hepatosplenomegaly and X-linked adrenoleukodystrophy (X-ALD) (Table 1.7) (Dean et al., 2001; Stefkova et al., 2003; Vasiliou, et al., 2009; Morita et al., 2012; Kawaguchi et al., 2016).

1.7.4.4   Subfamily E of the ABC family (ABCE)

There is only one member of the ABCE subfamily of proteins known as the ABCE1 (OABP). The structure of archaeal ABCE1 is made up of two ATP-binding domains linked together by a hinge region to facilitate orientation, a pivotal point of the ATP-binding domains and an iron-sulphur binding domain consisting of eight cysteine residues that mediate two diamagnetic $[4Fe-4S]^{2+}$ clusters present at the N-terminal region of ABCE proteins but no transmembrane domain (Navarro-Quiles et al., 2018). Hence, ABCE1 is unlikely to function as a transporter. it

Furthermore, research found that the inhibition of protein synthesis by the activation of ribonuclease L in a critical pathway involving viral interferon action is often interfered by ABCE1 which, resist the activity of ribonuclease L hence, ABCE1 helps to promote interferon activity. Currently no disease has been associated with ABCE1 transporter (Dean et al., 2001; Stefkova et al., 2003; Vasiliou et al., 2009).

1.6.4.5   Subfamily F of the ABC family (ABCF)

ABCF subfamily is similar to ABCE subfamily, all have a pair of ATP-binding domains which are not fused to transmembrane domains. Hence, similar to ABCE, ABCF subfamiy are also unlikely to be transporters (Kerr, 2004). ABCF subfamily consist of eukaryotic Elongation factor 3 (eEF3) protein which is important in *Saccharomyces cerevisiae* for polypeptide elongation (Skogerson et al., 1976; Murina et al., 2018), ribosome-associated protein GCN20 is associated with uncharged tRNAs on eukaryotic ribosome for sensing starvation (Vazquez de Aldana et al., 1995; Murina et al., 2018), ABC50 promotes translation initiation in eukaryotic cells (Paytubi et al., 2009) and both New1 and Arb1 have been proposed to be implicated in the stimulation of ribosomal biogenesis in eukaryotic cells (Dong et al., 2005; Murina et al., 2018). Research found that tumour necrosis factor-α (TNF-α) cause the upregulation of ABCF genes and thus ABCF transporters may play a role in inflammatory processes. In addition, similar to ABCE1 transporter, no disease have been associated with ABCF transporters (Dean et al., 2001; Stefkova et al., 2003; Vasiliou et al., 2009).

1.6.4.6   Subfamily G of the ABC family (ABCG)

ABCG subfamily of transporters consist of five members, all of which are reverse half transporters in that they have an NBD at the N-terminus and a TMD at the C-terminus (the second half of a heterodimer) (Vasiliou et al., 2009). ABCG1 efflux cholesterol in macrophages and its presence in other cells may help to mediate cellular lipid homeostasis (Lorkowski et al., 2002). ABCG2 functions as a multidrug resistance transporter (Woodward et al., 2011). The highly expressed ABCG3 in the spleen and thymus, may be involved in the transport of specific hydrophobic compounds or peptides from lymphocytes (Lorkowski et al., 2002; Vasiliou et al., 2009). ABCG5 and ABCG8 have shown to encourage biliary excretion and reduce intestinal absorption of sterols. Moreover, mutations in either ABCG5 or ABCG8 can cause sitosterolaemia (Table 1.7) (Dean et al., 2001; Lorkowski et al., 2002; Stefkova et al., 2003; Vasiliou et al., 2009; Woodward et al., 2011).

Table 1.8. A list of ABC human transporters, their chromosomal location and function
(Adapted from Dean et al., 2001).

Symbol	Alias	Chromosomal location	Expression	Function
ABCA1	ABC1	9q31.1	Ubiquitous	Cholesterol efflux onto HDL
ABCA2	ABC2	9q34	Brain	
ABCA3	ABC3, ABCC	16p13.3	Lung	Drug resistance
ABCA4	ABCR	1p22.1-p21	Rod photoreceptors	
ABCA5		17q24		N-retinylidiene-PE efflux
ABCA6		17q24	Muscle, heart, testes	
ABCA7		19p13.3	Liver	
ABCA8		17q24	Spleen, thymus	
ABCA9		17q24	Ovary	
ABCA10		17q24	Heart	
ABCA12		2q34	Muscle, heart	
ABCA13		7p-11-q11	Stomach	
			Low in all tissues	
ABCB1	PGY1, MDR	7p21	Adrenal, kidney, brain	Multidrug resistance
ABCB2	TAP1	6p21		Peptide transport
ABCB3	TAP2	6p21	All cells	Peptide transport
ABCB4	TGY3	7p21.1	All cells	PC transport
ABCB5		7p14	Liver	
ABCB6	MTABC3	2q36	Ubiquitous	Iron transport
ABCB7	ABC7	Xq12-q13	Mitochondria	Fe / S cluster transport
ABCB8	MABC1	7q36	Mitochondria	
ABCB9		12q24	Mitochondria	
ABCB10	MTABC2	1q42	Heart, brain	
ABCB11	SPGP	2q24	Mitochondria	Bile salt transport
			Liver	
ABCC1	MRP1	16p13.1	Lung, testes, PBMC	Drug resistance
ABCC2	MRP2	10q24	Liver	Organic anion efflux
ABCC3	MRP3	17q21.3	Lung, intestine, liver	Drug resistance
ABCC4	MRP4	13q32	Prostate	Nucleoside transport
ABCC5	MRP5	3q27	Ubiquitous	Nucleoside transport
ABCC6	MRP6	16q13.1	Kidney, liver	
CFTR	ABCC7	7q31.2	Exocrine tissues	Chloride ion channel
ABCC8	SUR	11p15.1	Pancreas	Sulphonylurea receptor
ABCC9	SUR2	12p12.1	Heart, muscle	
ABCC10	MRP7	6p21	Low in all tissues	
ABCC11	MRP8	16q11-q12	Low in all tissues	
ABCC12	MRP9	16q11-q12	Low in all tissues	
ABCD1	ALD	Xq28	Peroxisomes	VLCFA transport regulation
ABCD2	ALDL1, ALDR	12q11-q12	Peroxisomes	
ABCD3	PXMP1, PMP70	1p22-p21	Peroxisomes	
ABCD4	PMP69, P70R	14q24.3	Peroxisomes	

ABCE1	OABP, RNS4I	4q31	Ovary, testes, spleen	Oligoadenylate binding protein
ABCF1	ABC50	6p21.33	Ubiquitous	
ABCF2		7q36	Ubiquitous	
ABCF3		3q25	Ubiquitous	
ABCG1		21q22.3	Ubiquitous	Cholesterol transport?
ABCG2	ABC8, White	4q22	Placenta,	Toxin efflux, drug
ABCG4	ABCP, MXR,	11q23	intestine	resistance
ABCG5	BCRP	2p21	Liver	
ABCG8	White2	2p21	Liver, intestine	Sterol transport
	White3		Liver, intestine	Sterol transport

PBMC, peripheral blood mononuclear cells; VLCFA, very long chain fatty acids. Since the project focus on the ABCC subfamily of transporters, all the members of ABCC transporters are highlighted in yellow.

1.6.4.7 Subfamily C of the ABC family (ABCC)

The ABCC subfamily of ABC transporters is one of the largest subfamilies of ABC transporters comprising thirteen ABCC subfamily members and nine of these transporters are sometimes called the multidrug resistance proteins (MRPs) 1-9 because they have been found to efflux chemotherapeutic compounds or their metabolites from cancer cells, at least *in vitro*, and as a result may play a role in mediating tumour cells multidrug resistance (Table 1.9) (Chen et al., 2011; Sodani et al., 2012). The remaining members of ABCC subfamily include cystic fibrosis transmembrane regulator gene (CFTR / ABCC7) which is a regulated chloride ion channel (Sheppard et al., 1999), two cell surface receptors [sulfonylurea 1 and sulfonylurea 2 (SUR1 / ABCC8 and SUR2 / ABCC9)] which are the ATP-sensing subunits of a potassium ion channel complex that regulates the potassium ion permeability (Bienengraeber et al., 2004) and are not known to transport any substrates and one truncated protein (pseudogene) that does not transport substrates (ABCC13) (Sodani et al., 2012) . The nine MRPs range from 1325 to 1545 amino acids (Liu et al., 2010) and are further divided into two groups according to their structural features. MRP4, MRP5, MRP8 and MRP9 (ABCC4, ABCC5, ABCC8 and ABCC9) have a typical type IV ABC efflux transporter structure comprising of two TMDs (TMD_1 and TMD_2) each containing six transmembrane α-helices linked to two NBDs (NBD1 and NBD2) and are called the short MRPs (Table 1.9 and Figure 1.19). Conversely MRP1, MRP2, MRP3, MRP6 and MRP7 (ABCC1, ABCC2, ABCC3, ABCC6 and ABCC7), have an additional TMD (TMD_0) which contains only five transmembrane α-helices that is attached to the N-terminal of a typical ABC transporter via a cytoplasmic linker (L_o), thus are known as the long MRPs (Table 1.9 and Figure 1.19) (Dallas et al., 2006; Slot et al, et a., 2011; Sodani et al., 2012; Zhang et al., 2015). In addition, the membrane localisation whether apical or basolateral and the tissue distribution of the MRPs are important as they help to distinguish the

physiological and pharmacological functions of each MRPs (Figure 1.20) (Chen et al., 2010). Furthermore, the elimination of drugs and their metabolites in normal tissues are also modulated by some MRPs. Therefore, MRPs can facilitate the transport of a wide variety of molecules across membranes (Table 1.9) and play an important role in many physiological and pathological functions (Tian et al., 2005; Liu et al., 2010; Chen et al., 2011). Some MRPs share substrate specificity by transporting similar substrates. However, the way in which one MRP transports a common substrate is significantly different to how another MRP transport the same substrate. In addition, one or more of the MRPs also play an important role in cellular efflux of physiologically important organic anions such as cAMP and leukotriene C_4 (Chen et al., 2011; Slot et al., 2011).

Finally, as with other ABC transporters, mutation of several MRPs is linked to human genetic diseases such as mutations in ABCC2 / MRP2 can cause Dubin–Johnson syndrome, a conjugated hyperbilirubinemia (Lee et al., 2006) and mutations in ABCC6 / MRP6 causes Pseudoxanthoma elasticum, a connective tissue disorder (Hu et al., 2003).
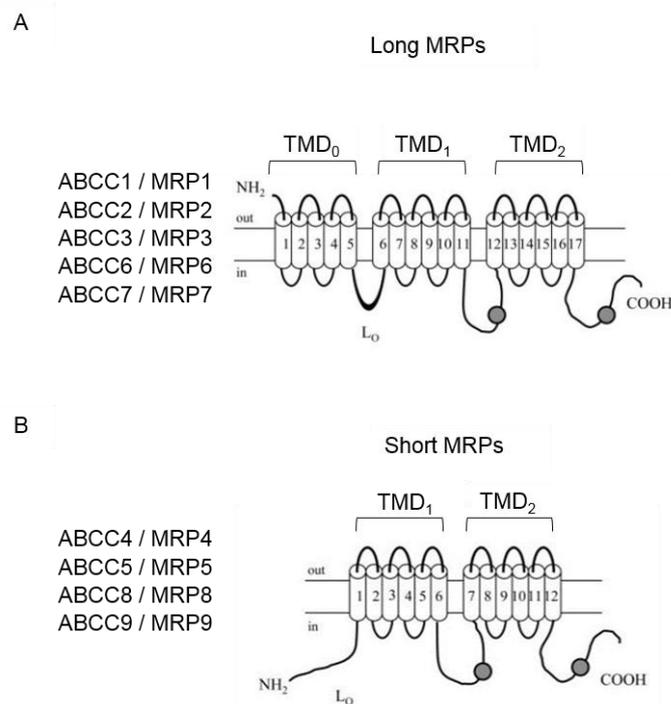
Figure 1.19. Predicted structures of MRPs. (A) Long MRPs consist of the core structure of a typical ABC transport that is two TMDs, each of which contains six transmembrane α-helices and two NBDs. On top of the core structure, long MRP have an additional transmembrane domain (TMD$_0$) comprise of five transmembrane α-helices, which is attached to the N-terminal via a cytoplasmic linker (L$_o$) (MRP1, MRP2, MRP3 MRP6 and MRP7) and (B) Short MRPs consist of only the core structure of a typical ABC transporters (MRP4, MRP5, MRP8 and MRP9) (Dallas et al., 2006).

Figure 1.20. A schematic diagram showing the cellular localisation of the MRPs. Epithelial and endothelial cells can be polarised, forming distinct apical and basolateral membranes of different biochemical properties. MRPs are located either on the apical or the basolateral membrane of polarised cells depending on the tissues where it is found. MRP1, 3, 6, 5 and 4 are located on the basolateral membrane whereas MRP 2, 8 and depending on the tissue, MRP4 also can be found on the apical membranes of polarised cells. The membrane localisation of MRP7 and MRP9 remain to be elucidated (Chen et al., 2011).

1.7   Substrates and functions of the human ABCCs / MRPs

1.7.1   The Long ABCCs / MRPs

1.7.1.1   ABCC1 / MRP1

ABCC1 was the first ABCC proteins to be cloned in 1992 from a doxorubicin-selected multidrug resistant human lung carcinoma cell line H69AR (Tian et al., 2005; Cole, 2014; Zhang et al., 2015). In these lung cancer cells, the *ABCC1* mRNA is a 1531 amino acids protein with a molecular weight of 170 kDa. However, due to phosphorylation and N-glycosylation, the ABCC1 in mammalian cells exhibit a molecular weight of 190kDa. Therefore, the ABCC1 protein band in gels is often quite diffuse as it depends on the cell type in which it is expressed in (Slot et al., 2011). In normal cells, ABCC1 plays an important role in the disposition of drug and xenobiotics and also helps to protect certain tissues from harmful cytotoxic chemicals. Therefore, ABCC1 is widely expressed in various tissues including skeletal, testis, cardiac muscles, macrophages, lung, kidney and placenta and is found on the basolateral membrane of polarised cells (Figure 1.20). In addition, ABCC1 is also found in certain blood-organ interfaces such as the blood brain barrier and the blood-testis barrier which helps to reduce the penetration of cytotoxic agents (Chen et al., 2011; Sodani et al., 2012). ABCC1 is localised on the basolateral membrane of polarised cells except in the placenta and blood brain barrier (Jaramillo et al., 2018).

ABCC1 can transport glutathione (GSH), oxidised glutathione (GSSG) and a number of glucoronate, GSH and sulphate conjugated drugs (Hammond et al., 2007) (Table 1.9). In addition, ABCC1 also transports a wide variety of physiological substrates ranging from organic anions conjugates to glucuronates to sulphates such as the GSH-conjugated cysteinyl

leukotriene C_4 (LTC_4), unconjugated bilirubin, 17-β-D-estradiol-glucoronide ($E_2$17βG), prostaglandin (PG) A GSH conjugates and sulphate bile acids (Leier et al., 1994; Loe et al., 1996; Jedlitschky et al., 1996; Cole, 2014) (Table 1.9). Moreover, ABCC1 has a high affinity for LTC_4 which confers the specific role of ABCC1 in immune responses linked with cellular excretion of LTC_4 (Liu et al., 2010; Cole, 2014). A point to note is that although the amino acid sequence of the human ABCC1 is more than 90% similar to all other species, there is a marked differences between the substrate specificity. For example, ABCC1 in human confers resistance to anthracycline drugs but not in other species (Chen et al., 2011).

GSH is a cellular tripeptide antioxidant that has a critical role in protecting cells from harmful effect of oxidative stress. In a number of cases xenobiotic metabolism is conjugated with GSH to form GS-X conjugates before exported by ABCC1 (Chen et al., 2011). Therefore, with compiling evidences that GSH is a substrate of ABCC1 has confirmed that ABCC1 plays a crucial modulatory role in redox homeostasis and cellular oxidative stress (Krause et al., 2007). A range of xenobiotics and some stimulatory compounds that are not ABCC1 substrates can stimulate the transport of GSH in ABCC1 (Chen et al., 2011; Slot et al., 2011; Cole 2014). The transport of GSH by ABCC1 can be enhanced by vinca alkaloid vincristine and at the same time GSH can stimulates vincristine transport (Loe et al., 1996; Mao et al., 2000). As a result GSH appear to be co-transported with the drug (Chen et al., 2011; Slot et al., 2011). Finally, ABCC1 has many substrates which are conjugated organic anions and in some cases these conjugated organic anions are being transported due to GSH or is enhanced by GSH (Cole, 2014). For example, GSH stimulates the transport of estrone sulphate (Leslie et al., 2003) and the conjugates 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol-*O*-glucuronide (Leslie et al., 2001). Furthermore, nonreducing tripeptides, in which the cysteine residue is modified with a short chain alkyl moiety (example S-methyl GSH) or has been replaced with another amino acids such as leucine can also stimulate drug transport by ABCC1 (Loe et al., 1996; Leslie et al., 2003; Chen et al., 2011).

In tumour cells, ABCC1 can confer resistance to many antineoplastic drugs such as doxorubicin, daunorubicin etoposide, vincristine, saquinavir, mitoxantrone, vinca alkaloids and methotrexate (MTX) (Table 1.9) (Chen et al; 2011; Sodani et al., 2012; Zhang et al 2015). ABCC1 can also transport certain tyrosine kinase inhibitors that modulate a number of signal transduction pathways (Hegedus et al., 2002). High expression of ABCC1 has been found in numerous cancer types such as in non-small cell lung cancer (Fang et al., 2018), leukaemia (van der Kolk et al 2000; Beck et al., 2001), oesophageal carcinomas (Nooter et al., 1998), prostate cancer (Sullivan et al., 2000) and breast cancer (Filipits et al., 2005), and high expression of ABCC1 was related to shorter overall survival and relapse-free survival in breast

cancer (Filipits et al., 2005). Additionally, negative correlation between clinical outcome and the ABCC1 expression in cancers has been found (Roundhill et al., 2015; Jaramillo et al., 2018).

1.7.1.2   ABCC2 / MRP2

ABCC2 was first cloned from rat hepatocytes in 1996 using strategies that took advantages of its structural similarity to human ABCC1 and was originally named hepatocellular canalicular multiple organic anion transporter (cMOAT) (Slot et al., 2011; Zhang et al 2015). The amino acids of ABCC2 have 49% similarity with ABCC1 but its expression pattern is different (Tian et al., 2005; Chen et al., 2011; Sodani et al., 2012). ABCC2 is expressed in the apical membrane of polarised cells from a variety of tissues mainly in hepatocyte, small intestine and renal proximal tubules (Fanayte et al., 2004. Nies et al., 2007). In addition, ABCC2 mRNA is also present in the gallbladder, peripheral nerves, placental trophoblasts and CD4^{+} lymphocytes (Chen et al., 2011; Sodani et al., 2012; Zhang et al., 2015).

ABCC2 can transport a wide variety of substrates and has very similar substrate specificities to ABCC1. Thus, in the presence of GSH, ABCC2 can also mediate transport of some hydrophobic compounds but has a lower affinity to GSH conjugates than that of ABCC1 (Tian et al., 2005; Chen et al., 2011). For example, LTC_4 and N-ethylmaleimide glutathione has a significantly lower affinity for ABCC2 than that of ABCC1 (Bakos et al., 2000) but the affinity to ABCC2 for both bilirubin mono glucuronide and bilirubin bis glucuronide is higher for ABCC2 (Konno et al., 2003; Tian et al., 2005). Moreover, ABCC2 has the ability to confer resistance to cisplatin most likely in the presence of GSH which ABCC1 cannot (Borst et al., 2000; Sodani et al., 2012).

ABCC2 can transport a wide range of conjugated endogenous metabolites such a GSH, LTC_4, GSSG, bilirubin conjugates, leukotriene E_4 (LTE_4), leukotriene D_4 (LTD_4), and the glucuronide conjugates of triiodo-L-thyronine and estrodiol (Table 1.9) (Bakos et al., 2000; Tian, et al 2005). Additionally, ABCC2 can also transport organic anions including glucuronide, sulphate and GSH conjugates (Nies et al., 2007: Chen et al., 2011).

ABCC2 appear to have an important physiological role in eliminating endogenous metabolites and xenobiotics and their metabolites. Therefore, there has been an extensive amount of interest in understanding the role of ABCC2 in drug-drug interactions. Currently, the activity of ABCC2 can be inhibited by a wide range of structurally unrelated compounds such as MK571, a leukotriene receptor antagonist (Tian et al., 2005; Roy et al., 2009). Moreover, when ABCC2

overexpressing cells were treated with montelukast, a drug used to treat asthma, the elimination of saquinavir and paclitaxel was reduced (Roy et al., 2009; Chen et al., 2011). In addition, indomethacin, cyclosporine A, rifampicin, glibenclamide and antibiotics such as fusidate and azithromycin are some other modulators of ABCC2 (Chen et al., 2011).

ABCC2 expression has been reported in several human cancer cell lines of the renal, gastric, colorectal and lung as well as from the kidney, lung, ovary, colon and breast (Narasaki et al., 1997; Sandusky et al., 2002; Roundhill et al., 2015). A variety of anticancer drugs, including cisplatin, paclitaxel, MTX, irinotecan and vincristine have been reported to be transported by ABCC2 *in vitro* (Table 1.9) (Borst et al., 2000; Chen et al., 2011; Roundhill et al., 2015). Furthermore, overexpression of ABCC2 have been detected in few cisplatin- and doxorubicin-resistant cell lines and recent reports have shown that the efficacy of cisplatin-based chemotherapy in the treatment of patient with hepatocellular carcinoma is dependent upon the expression level of ABCC2 (Chen et al., 2011; Sodani et al., 2012).

Mutations in the ABCC2 genes can cause an inherited autosomal recessive disorder known as Dubin-Johnson syndrome characterised by impaired secretion of anionic conjugates from hepatocytes into the bile, chronic conjugated hyperbilirubinemia and deposition of brown pigments in the liver (Tian et al., 2005; Lee et al., 2006; Slot et al., 2011). Several mutations including missense, deletion, splice site and nonsense of the ABCC2 genes have severely reduced or eliminated the level of ABCC2 protein in the canalicular membrane (Lee et al., 2006; Nies et al., 2006; Chen et al., 2011). Many of the studies on Dubin-Johnson syndrome have been based from well-established animal model of the disease such as the Groninger Yellow transporter rat strains model and the Abcc2 deficient Eisai hyperbilirubinuria rat model (Hirohashi et at., 1998; Koopen et al., 1998; Gerk et al., 2002).

1.7.1.3   ABCC3 / MRP3

ABCC3 was first detected in human and rat hepatocytes and shared the highest structural similarity to ABCC1 (58% amino acid identity) (Tian et al., 2005; Borst et al., 2007; Chen et al., 2011; Sodani et al., 2012). Like ABCC1, ABCC3 is also localised on the basolateral membranes of polarised cells (Slot et al., 2011; Roundhill et al., 2015; Zhang et al., 2015). The expression of ABCC3 is mainly detected in the adrenal glands, small intestine, colon, gallbladder, placenta and pancreas, and to a lower extend in the stomach, prostate, liver, spleen, brain, tonsils, kidney and lungs (Tian et al., 2005; Dallas et al., 2006; Chen et al., 2011; Sodani et al., 2012).

Low expression of ABCC3 is detected in the normal human liver and is mainly located to the basolateral membranes of the hepatocytes surrounding the portal tracts and bile duct epithelial cells (Borst et al., 2007; Chen et al., 2011). However, in Dubin-Johnson syndrome and cholestatic patients, the expression of ABCC3 is upregulated often due to downregulation of ABCC2 in liver canalicular membranes (Scheffer et al., 2002a; Borst et al., 2007). Therefore, the function of ABCC3 may compensate for the downregulation of ABCC2 in the liver (Konig et al., 1999). Indirect approaches using *in vitro* assays such as in the determination of substrate specificity, tissue localisation and upregulation in pathological conditions have used to study the physiological function of ABCC3 (Chen et al., 2011). In addition, Abcc3 knockout mouse model have provided direct investigation of the role of Abcc3 in the transport of xenobiotics and endogenous compounds (Belinsky et al., 2005; Zelcer et al., 2006; Chen et al., 2011). Therefore, the studies on the physiological function of ABCC3 using the mice models have suggested that during toxic accumulation of hepatotoxic bile salts, ABCC3 may protect the liver from cholestasis (Dallas et al., 2006; Teng et al., 2007).

A variety of amphipathic anions including monoanionic bile acids and glucoronate conjugates are transported by ABCC3, but unlike ABCC1 and ABCC2, ABCC3 have a very low affinity for GSH conjugates even though ABCC3 has high structural similarity to ABCC1 (Kool et al., 1999a; Borst et al., 2007). Furthermore, ABCC3 does not require GSH to transport drug substrates (Zelcer et al., 2001; Borst et al., 2007). Additionally, studies using mice models have suggested that ABCC3 plays an important role in the basolateral disposition of certain glucuronide conjugates and bile acids (Kool et al., 1999a; Lagas et al., 2010).

High expression levels of ABCC3 in primary ovarian cancer (Ohishi et al., 2002; Ween et al., 2015), human hepatocellular carcinomas (Nies et al., 2001) and adult acute lymphoblastic leukaemia (Plasschaert et al., 2005) have been detected. Such overexpression of ABCC3 in adult acute myeloid leukaemia (Benderra et al., 2005), and in childhood and adult acute lymphoblastic leukaemia (Plasschaert et al., 2005) and pancreatic carcinoma (Konig et al., 2005) was associated with poor event-free survival and overall survival. Therefore, ABCC3 expression was predicted to be a prognostic factor. However, high expression level of ABCC3 in neuroblastoma was associated with improved overall survival (Henderson et al., 2011; Roundhill et al., 2015). Compared to ABCC1 and ABCC2, ABCC3 transport fewer anticancer substrates such as teniposide, etoposide and MTX (Zelcer et al., 2001; Borst et al., 2007).

1.7.1.4   ABCC6 / MRP6

The *ABCC6* gene is located next to *ABCC1* gene on chromosome 16 and encodes a 1503

amino acids proteins (Zhang et al., 2015). ABCC6 was initially detected in epirubicin resistant human leukemic cells during the amplification of the 3'-end of the *ABCC6* gene (Borst et al., 2000) and has 45% structurally similarity to ABCC1 (Tian et al., 2005; Chen et al., 2011). The kidney and liver have the highest level of ABCC6 protein expression whereas other tissues such in the small intestine, pancreas, lung, skeletal muscle, eye and skin have low levels of ABCC6 protein expression (Kool et al., 1999b; Scheffer et al., 2002b; Beck et al., 2005; Matsuzaki et al 2005). In addition, ABCC6 is widely distributed in various epithelial cells of endocrine and exocrine tissues, mucosal cells of intestine, acinar cells in the pancreas, follicular epithelial cells of the thyroid, and the canalicular and lateral plasma membrane of hepatocytes and is localised in the basolateral membrane of polarised cells (Madon et al., 2000; Beck et al., 2005; Slot et al., 2011; Zhang et al., 2015).

Studies using animal models such as the membrane vesicles prepared from ABCC6-transfected Chinese hamster ovary cells and from insect cells have shown that the *in vitro* transport of the GSH conjugates such as S-glutathione, N-ethylmaleimide and LTC_4 are mediated by ABCC6 but failed to transport glucuronide conjugates such as $E_217\beta G$ (Belinsky et al., 2002; Ilias et al., 2002; Dallas et al., 2006; Chen et al., 2011). These observation indicate that ABCC6 is a lipophilic anionic transporter (Belinsky et al., 2002; Sodani et al., 2012) but further studies are necessary to fully understand the physiological and pharmacological functions of ABCC6.

ABCC6 can confer low levels of cellular resistance to a variety of anticancer drugs *in vitro* such as doxorubicin, cisplatin, etoposide and daunorubicin (Belinsky et al., 2002; Tian et al 2005; Chen et al., 2011), although whether this is relevant *in vivo* is unclear. The gene expression of ABCC6 has been detected in both adult and child acute lymphoblastic leukaemia and may be related to overall survival and event free survival (Plasschaert et al., 2005; Roundhill et al., 2015). However, due to limited studies done to investigate the multidrug resistance profile of ABCC6 further studies are needed to fully understand if or how ABCC6 is involved in multidrug resistance (Chen et al., 2011; Zhang et al., 2015).

Mutation in *ABCC6* gene have been associated with genetic abnormalities of the heritable connective tissue disorder that affect the elastic tissues mainly in the eyes, skin and cardiovascular system called pseudoxanthoma elasticum (PXE) (Matsuzaki et al., 2005; Tian et al., 2005; Sodani et al., 2012). Patient with PXE surfer myocardial infarction, visual impairment and blood vessel rupture (Belinsky et al., 2002; Hu et al., 2003; Chen et al., 2011). Furthermore, abnormalities of the elastic fibers are due to calcification of elastic structures and the accumulation of miniralised elastotic material in the skin causing loss of vision, baggy skin

and calcification of the blood vessels (Li et al., 2009; Chen et al., 2011). Research has also suggested that supplementation of inorganic pyrophosphate (PPi) may prevent soft tissue mineralisation PXE (Jansen et al., 2013; Favre et al., 2017) but the mechanism by which ABCC6 uses PPi to prevent abnormal mineralisation in physiological conditions is still unknown (Favre et al., 2017). Since ABCC6 is mainly expressed in the liver and kidney and with low expression in other tissues, many theories have been formed to explain the unexpected association of *ABCC6* gene mutation with PXE (Kool et al., 1999b; Scheffer et al., 2002b; Beck et al., 2005; Matsuzaki et al 2005).

1.7.2   The short ABCCs / MRPs

1.7.2.1   ABCC4 / MRP4

ABCC4 gene was identified in a T-lymphoid cell line in 1996 and was characterised as a transporter of 9-(2-phosphonylmehoxyethly) adenine (PMEA), a nucleoside monophosphate antiviral agent (Borst et al., 2000; Chen et al., 2010; Sodani et al., 2012). Currently, there are no genetic diseases associated with *ABCC4* gene mutation but *ABCC4* gene is highly polymorphic (Slot et al., 2011). In addition, selective defect in ADP storage in platelet δ-granules have been linked to absence of ABCC4 which may cause abnormal bleeding and bleeding times (Jedlitschky et al., 2010; Slot et al., 2011). ABCC4 shares a 41% amino acid identity with ABCC1 (Chen et al., 2010). ABCC4 is expressed at very high levels in the prostate, but is found in all normal tissues (Borst et al., 2007; Chen et al., 2011). Compared to other ABCCs that are localised to either the apical or basolateral membranes in polarised cells, ABCC4 is localised to the apical membrane in some polarised cells, whereas it is at the basolateral membrane in others. For example, ABCC4 is found at the luminal side of brain capillary endothelium cells (Nies et al., 2004; Russel et al., 2008) and renal proximal tubule cells (Russel et al., 2008), and the basolateral membrane in polarised cells such as in the hepatocytes, choroid plexus epithelium cells and prostate tubuloacinar cells (Borst et al., 2007; Russel et al., 2008; Chen et al., 2011; Sodani et al., 2012). Moreover, ABCC4 can also be localised to both apical and basolateral side of cells such as the colonic epithelial cells (Russel et al., 2008). ABCC4 can transport a wide range of endogenous and xenobiotic organic anionic compounds and helps protect crucial tissues against the cytotoxic effects and conferring resistance to a variety of cytotoxic compounds (Sodani et al., 2012; Zhang et al., 2015).

Apart from ABCC4 mediating the disposition of drugs and their metabolites, ABCC4 also regulates the transport of several important endogenous metabolites that are involved in the signalling pathways of critical processes such as inflammation, cell differentiation and

apoptosis (Chen et al., 2011). Among the first ABCC4 substrates identified were cAMP and cGMP (Chen et al., 2001; Sodani et al., 2012). The affinity of ABCC4 for cAMP and cGMP is relatively low which questioned whether ABCC4 is sufficient in regulating the intracellular levels of these cyclic nucleotides. (Van Aubel et al., 2002; Wielinga et al., 2003; Borst et al., 2007). However, a large amount of evidence has suggested that the cyclic nucleotide signalling is highly compartmentalised meaning that instead of regulating the whole cell concentration of cAMP and cGMP, ABCC4 may be more involved in regulating local microdomains (Russel et al., 2008; Chen et al., 2010; Sodani et al., 2012). Other substrates of ABCC4 include sulphated bile acids, GSH, LTC_4 and GSH-conjugated leukotriene B_4 (LTB_4) (Tian et al., 2005; Rius et al., 2008; Chen et al., 2010) and several eicosanoids such as prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2) which are inflammation and pain mediators and also is involved angiogenesis, development of tumour and response to cytotoxic chemotherapy (Reid et al., 2003b; Chen et al., 2011; Kochel et al 2015 and 2017).

Additionally, high expression of ABCC4 have been detected in some invasive tumours including neuroblastoma (Norris et al., 2005; Henderson et al., 2011; Huynh et al., 2012; Roundhill et al., 2015) and epithelial ovarian carcinoma (Bagnoli et al., 2013; Roundhill et al., 2015) and is associated with poor patient survival. In contrast, a low expression level of ABCC4 in prostate tumours is associated with poor event free survival (Ho et al., 2008; Chen et al., 2011; Montani et al., 2013). Furthermore, together with PMEA and other antiviral agents, ABCC4 can also efflux a wide range of anticancer agents including methotrexate (MTX), thiopurine analogs and topotecan (Wielinga et al., 2002; Russel et al., 2008; Chen et al., 2002 and 2011). Moreover, in order for thiopurine analogs and most nucleotide-based chemotherapeutic drugs to exert their pharmacologic activity, they must undergo intracellular phosphorylation (Wielinga et al., 2002; Chen et al., 2011; Slot et al. 2011). Therefore, ABCC4 effluxes the phosphorylated compounds rather than the parent compounds and as a result may cause treatment failure (Zhang et al., 2015). Also, studies have shown that an upregulation of COX-2 via the PGE_2-dependent pathway in lung cancer may cause an overexpression of ABCC4 (Maeng et al., 2014; Zhang et al., 2015). With inconclusive evidences provided thus far on the role of ABCC4 in clinical drug resistance further studies are needed to elucidated the drug resistance profile of ABCC4 (Sodani et al., 2012).

1.7.2.2   ABCC5 / MRP5

ABCC5 was first identified during a database screening of sequence tags (Kool et al., 1997) and shared 38% amino acid identity with ABCC1 (Chen et al., 2011). The expression level of ABCC5 is relatively low in most normal tissues but highly expressed in tissues such as in

heart, skeletal muscle, cornea and brain (Dazert et al., 2003; Nies et al., 2004; Dallas et al., 2006; Karla et al., 2009; Sodani et al. 2012; Zhang et al., 2015). However, due partly to lack of suitable antibodies for definitive immunolocalisation studies, the characterisation of ABCC5 tissue distribution is partially described (Borst et al., 2007; Slot et al., 2011). Similar to ABCC1, ABCC5 is expressed on the pyramidal neurons, subcortical white matter astrocytes, luminal side of brain capillary endothelial cells as well as in smooth muscle cells of various tissues in the human genitourinary system but the physiological role of ABCC5 in these tissues has not been elucidated (Chen et al., 2011; Slot et al., 2011).   Furthermore, ABCC5 is localised in the basolateral membrane of polarised cells (Nies; et al., 2004; Borst et al., 2007; Chen et al., 2011; Slot et al., 2011; Sodani et al., 2012).

ABCC4 and ABCC5 are structurally similar in that they both lack the TMD_0 but there is a difference between the two proteins in that at the N-terminus, ABCC5 has an extra 95 amino acids compare to ABCC4. The exact function of this additional segment is still not yet understood (Haimeur et al., 2004; Sodani et al., 2012).

Thus far, the functional studies of ABCC5 using ABCC5 knockout mice model have produced limited understanding into the functional activity of the protein (Chen et al., 2011). Like ABCC4, ABCC5 can mediate the transport of cAMP and cGMP and it has higher affinity for cGMP than for cAMP (Adachi et al., 2002; Wielinga et al., 2003; Tian et al., 2005; Chen et al., 2011; Sodani et al., 2012). In addition, the cGMP in pial arteriolar smooth muscle cells (Xu et al., 2003) and in anterior pituitary cells (Andric et al., 2006) is selectively transported by ABCC5. Consequently, ABCC5 can transport a variety of organic anions, including certain purine analogues such as thioguanine and PMEA (Wielinga et al., 2002; Tian et al., 2005; Chen et al., 2011) and certain monophosphate nucleotide metabolites such as cAMP and cGMP (Jedlitschky et al., 2000; Chen et al., 2011; Zhang et al., 2015). Hence, like ABCC4, ABCC5 is a cyclic nucleotide organic anion transporter (Jedlitschky et al., 2000; Reid et al., 2003a; Borst et al., 2007). Other substrates of ABCC5 include unmethylated thionucleotides and other organic anions such as GSH and S-(2, 4-dinitrophenyl) glutathione (Chen et al., 2011; Slot et al., 2011).

Elevation of ABCC5 protein expression has been found in pancreatic, lung, colon and breast cancer samples as well as in ischemic heart (Oguri et al., 2000; Dazert et al., 2003; Pratt et al., 2005; Nambaru et al., 2011). Further *in vitro* studies have also detected *ABCC5* gene expression in doxorubicin and cisplatin treated non-small cell lung cancer (Oguri et al., 2000; Pratt et al., 2005; Zhang et al., 2015). Therefore, ABCC5 could efflux a wide variety of anticancer drugs, including cisplatin, doxorubicin, pyrimidine analogues such as 5-fluorouracil,

gemcitabine, and cytosine arabinoside, purine analogues such as 6-thioguanine and 6-mercaptopurine, and antifolate drugs such MTX. However, ABCC5 does not provide resistance to vinca alkaloids such as vincristine (Borst et al., 2000; Wielinga et al., 2002 and 2005; Pratt et al., 2005; Chen et al., 2011). The evidences thus far have suggested the role of ABCC5, hence further studies are required in elucidating the exact role of ABCC5 (Chen et al., 2011; Slot et al., 2011; Zhang et al., 2015).

1.7.2.3   ABCC11 / MRP8

Through expressed sequence tag database mining and a gene prediction program, high expression of ABCC11 was first identified in breast cancer (Bera et al., 2001; Chen et al., 2011; Toyoda et al., 2010; Sodani et al., 2012) and among the members of the ABCC subfamily, ABCC11 is unusual in that no orthologous genes have been found in mammals except for primates (Slot et al., 2011). The expression level of *ABCC11* gene transcript is highest in the brain, placenta, liver, testes and breast (Yabuuchi et al., 2001; Chen et al., 2011; Sodani et al., 2012). Furthermore, the variation in the level of *ABCC11* gene transcript may partly be due to the discovery of multiple splice form of the *ABCC11* gene transcript (Yabuuchi et al., 2001). Hence, no definite correlation have been found between *ABCC11* gene transcript levels with ABCC11 protein expression levels in tissues (Chen et al., 2011; Sosonkina et al., 2011). Therefore, to investigate the relation between ABCC11 tissue expression levels and the multiple splice form of *ABCC11* gene, further protein expression studies are needed. In addition, ABCC11 has been suggested to be associated with the efflux of neuromodulatory steroids such dehydroepiandrosterone 3-sulphate as it has been detected in axons of the human central nervous system (CNS) and peripheral nervous system (PNS) and is localised to apical membranes of polarised epithelial cells (Chen et al., 2011; Sodani et al., 2012; Zhang et al., 2015).

Through *in vitro* vesicular transport assay, using membrane vesicles showed that ABCC11 can transport a wide variety of compounds including lipophilic anions including natural and synthetic glutathione conjugates such as *S*-(2,4-dinitrophenyl) glutathione, estradiol glucuronide and LTC_4, cyclic nucleotides such as cAMP and cGMP, folic acids, glucuronidated steroids and sulphated conjugates such as estrone 3-sulphate and hydroepiandrosterone 3-sulphate (Table 1.9) (Chen et al., 2005 and 2011; Martin et al., 2010; Baumann et al., 2014).

The ABCC11 drug resistance profile has been determined using ABCC11 transfected pig kidney epithelial (LLC-PK1) cells. Thus according to the system, ABCC11 was found to efflux antimetabolites such as MTX, 5-fluorouracil, 5-fluoro-5-deoxyuridine, 5-fluro-2-deoxyuridine,

PMEA and cytosine arabinoside (Table 1.9) (Guo et al., 2003; Dallas et al., 2006 Oguri et al., 2007; Chen et al., 2011). Clinically, high expression level of ABCC11 has been detected in breast cancer (Bera et al., 2001; Sodani et al., 2012; Yamada et al., 2013) and gastric cancer (Matsumoto et al., 2014; Zhang et al., 2015). Furthermore, overexpression of ABCC11 has been associated with low overall survival in acute myelogenous leukaemia (AML) patients, indicating that ABCC11 may be a potential biomarker for AML treatment outcome (Guo et al., 2009). Consequently, the distribution, activity and expression of ABCC11 in human tissues still need to be elucidated. Therefore, further studies are required to investigate the possibility that ABCC11 may have a role in clinical multidrug resistance (Chen et al., 2011; Slot et al., 2011; Sodani et al., 2012; Zhang et al., 2015; Roundhill et al., 2015).

A physiological function of ABCC11 was revealed from a single nucleotide polymorphism in *ABCC11* gene, 538G>A (Gly180Arg) that causes the production of either wet or dry ear wax (Yoshiura et al., 2006; Nakano et al., 2009; Ohashi et al., 2011). The single nucleotide polymorphism, 538G>A, appear to interfere with the stability and glycosylation of ABCC11 by causing a non-conservative arginine substitution of Gly180 in the TMD1 of ABCC11 (Ota et al., 2010; Slot et al., 2011; Toyoda et al., 2016a and 2016b). Consequently, the ABCC11 polymorphism does not transport cAMP hence this may account for the production of dry ear wax (Chen et al., 2011). Furthermore, the 538A/A genotype is common in East Asian population causing dry ear wax whereas the 538G/A and 538G/G genotypes are common in European and African population causing wet ear wax (Yoshiura et al.,2006; Ohashi et al., 2011; Harker et al., 2014; Toyoda et al., 2016b). In addition, individual with 538G/A and 538G/G genotypes also suffer a condition known as axillary osmidrosis where fetid sweat are secreted from the armpit and in other cases individuals may have colostrums secretion and may potentially develop breast cancer. However, such conditions are rarely noticed in individual with 538A/A genotype (Nakano et al., 2009; Martin et al., 2010; Ota et al., 2010; Toyoda et al., 2010, 2016a and 2016b). Moreover, the ABCC11 protein with 538A/A genotype lacks N-linked glycosylation and, due to disruption of plasma membrane trafficking possibly because of misfolding, may cause 538A/A genotype ABCC11 polymorphism to undergo proteosomal degradation (Chen et al., 2011; Toyoda et al., 2016b).

1.7.2.4   ABCC12 / MRP9

ABCC12 is located just 20 kb apart from ABCC11 on chromosome 16q12.1 in a head-to-tail orientation (Yabuuchi et al., 2001; Chen et al., 2011; Slot et al., 2011; Sodani et al., 2012; Zhang et al., 2015). ABCC12 shares similar sequence identity (47%) and similarity (56%) to that of ABCC11, thus *ABCC12* gene may be formed due to a gene duplication event (Yabuuchi

et al., 2001; Chen et al., 2011). However, one major difference between ABCC11 and ABCC12 is that ABCC12 has got two TMDs each with four membrane spanning regions and only one NBD. Any standard ABC transporter requires two NBDs for the transporter to be functional but ABCC12 does not require the second NBD to be activated instead a premature stop codon truncates the protein. Hence producing only one TMDs which is the carboxyl half of the protein (Bera et al., 2002). Furthermore, ABCC12 has got 44% amino acid identity and 55% sequence protein identity to that of ABCC5 (Bera et al., 2002; Chen et al., 2011). Moreover, ABCC12 is an unusual ABCC in that it does not have an N-linked glycosylation (Ono et al., 2007; Sodani et al., 2012) and the expression of ABCC12 is predominantly localised in the endoplasmic reticulum rather to the plasma membrane (Ono et al., 2007; Chen et al., 2011; Sodani et al., 2012).

Moreover, due to multiple ABCC12 gene transcripts that encode different size of proteins, the tissue distribution and activity of ABCC12 is not well defined (Yabuuchi et al., 2001). Conversely, some studies have reported that one *ABCC12* gene transcript of 4.5 kb in length is expressed in normal breasts, testis and breast cancer and another *ABCC12* gene transcript of 1.3 kb in length is expressed in the ovaries, brain and skeletal muscle (Bera et al., 2002; Sodani et al., 2012).

However, so far the substrate specificity of ABCC12 is still unknown hence its functional characteristics in terms of its physiological role and whether it is implicated in clinical multidrug resistance in cancer chemotherapy have not been reported. Therefore, further studies are needed to better understand the functional characteristics of ABCC12 (Chen et al., 2011; Slot et al., 2011; Sodani et al., 2012; Zhang et al., 2015).

Name	Alternate name / Symbol	Amino acids	Tissue location	Expression level	Major drug substrates	Physiological substrates	Inhibitors
MRP1	ABCC1	1531	All major tissues	Differs in various organs and cell lines	Folate-based antimetabolites (MTX, edatrexate, ZD1694), anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin), plant alkaloids (etoposide, vincristine, vinblastine, paclitaxel, irinotecan, SN-38), antiandrogens (flutamide, hydroxyflutamide), saquinavir, ritonavir, indinavir, ciprofloxacin, difloxacin, grepafloxacin, berberine, pirarubicin, sodium arsenite, sodium arsenate, potassium antimonite, potassium antimony tartrate, leucovorin, various conjugates	LTC_4, $E_217\beta G$, sulphated bile acids, folic acid, bilirubin, PGA GSH conjugate, GSH, GSSG, N-acetyl-Leu-Leu-norleucinal	Probenecid, sulfinpyrazone, indomethacin, verapamil, quercetin, genistein, biochanin A, kaempferol, schisandrin B, cyclosporine, PAK-104P, steroid analogues, MK-571, ONO-1078, sulphonylurea, glibenclamide, delavirdine, efavirenz, nevirapine, abacavir, emtricitabine, lamivudine, tenofovir, LY475776, LY402913
MRP2	ABCC2 / cMOAT	1545	Liver, kidney, intestine, brain		Conjugates, cisplatin, etoposide, vinca alkaloids, anthracyclines, camptothecins, MTX, olmesartan, saquinavir, lopinavir, ritonavir, indinavir, ezetimibe, temocaprilat, atorvastatin, phloridzin, quercetin 4'-β-glucoside, genistein-7-glucoside, fexofenadine, NNAL, cefoperazone, cefbuperazone, cefpiramide, ceftriaxone, carboxydichlorofluroscein	LTC_4, GSH, GSSG, bilirubin conjugates, LTD_4, LTE_4	MK-571, probenecid, furosemide
MRP3	ABCC3 / MOAT-D	1527	Small intestine, pancreas, colon, placenta, adrenal gland	Low levels in the liver, brain, kidney and prostate	Etoposide, teniposide, dinitrophenyl S-glutathione, acetaminophen glucuronide, vincristine, MTX	LTC_4, $E_217\beta G$, cholate, glycocholate, taurocholate	Etoposide, MTX, delavirdine, efavirenz, nevirapine, emtricitabine, lamivudine, and tenofovir
MRP4	ABCC4 / MOAT-B	1325	Kidneys, prostate, testis, ovary, lung, hepatocytes, intestine, pancreas	Low levels in other tissues	MTX, 6-thioguanine, PMEA, 6-mercaptopurine, topotecan, irinotecan, SN-38, cyclophosphamide, ifosfamide, ceftizoxime, cefazolin, cefotaxime, cefmetazole, azathioprine	cGMP, cAMP, DHEAS, $E_217\beta G$, PGE_1, PGE_2, GSH and GSH conjugated bile acid, LTB_4 and LTC_4	MK-571, MTX, celecoxib, rofecoxib, diclofenac, sulfinpyrazone, benzbromarone, verapamil, indomethacin
MRP5	ABCC5 / MOAT-C	1437	Most tissues	Low levels	6-Mercaptopurine, 6-thioguanine, PMEA, heavy metals, 5-fluorouracil, MTX, PMEA, daunorubicin, gemcitabine, Ara-C, azathioprine, doxorubicin	cGMP, cAMP, S-(2,4 dinitrophenyl) glutathione	Probenecid, sulfinpyrazone, benzbromarone, MK-571

MRP6	ABCC6 / MOAT-K / PXE	1503	Liver, kidneys	Low levels in other tissues	N-ethylmaleimide S-glutathione, dinitrophenol glutathione, etoposide, doxorubicin, cisplatin, daunorubicin, teniposide, actinomycin-D	LTC_4, S-(2,4 dinitrophenyl) glutathione	Indomethacin, probenecid, benzbromarone
MRP7	ABCC10	1492	Most tissues	Very low levels	Docetaxel, paclitaxel, vincristine, vinblastine, gemcitabine, Ara-C, epothilone-B	$E_2$17βG, LTC_4	MK-571, LTC_4, glycolithocholate 3-sulphate, cyclosporine
MRP8	ABCC11	1382	Normal breast, testis	Low levels in the liver, brain and placenta	5-Fluorouracil, 5'-fluoro-5'-deoxyuridine, 5'-fluoro-2'-deoxyuridine, PMEA, MTX, Ara-C	cGMP, cAMP, LTC_4, DHEAS, bile acids, estrone 3-sulphate, $E_2$17βG, folic acids, S-(2,4 dinitrophenyl) glutathione	Unknown
MRP9	ABCC12	1359	Breast cancer, normal breast, testis, brain, skeletal muscle, ovary	Low levels	Unknown	Unknown	Unknown

Table 1.9. The tissue distribution, substrates and inhibitors of the MRPs (Adapted from Liu et al., 2010 and Chen et al., 2010).

cMOAT, canalicular multispecific organic anion transporter; DHEAS, dehydroepiandrosterone-3-sulphate; $E_2$17βG, 17-β-D-oestradiol glucuronide; GSH, glutathione; GSSG, oxidized glutathione; MRP, multidrug resistance protein; LT, leukotriene; MTX, methotrexate; NNAL, 4 (methylnitrosamino)-1-(3-pyridyl)-1-butanol; PG, prostaglandin; PMEA, 9-(2-phosphonylmethoxyethyl) adenine; Ara-C, arabinofuranosyl cytidine

1.8   ABC transporters and cancer hallmarks

Tumorigenesis in humans is a multistep process that brings about genetic changes causing normal cells to progressively transform into highly malignant derivatives. Furthermore, under pathological conditions, cells at organ sites may reveal lesions which represent the evolution of normal cells into a series of premalignant states to form invasive cancers. In cancer cells, the signaling pathways responsible for maintaining normal cell proliferation and homeostasis are dysregulated. Moreover, within specific organs more than 100 distinct types of cancer and subtypes of tumours can occur (Hanahan et al., 2000).

Subsequently, the malignant growth of cancer cells is governed by six essential alterations in cell physiology and, they are sustaining proliferative signaling, resisting cell death, evading growth suppressors, inducing angiogenesis, activating invasion and metastasis, and enabling replicative immortality (Figure 1.21) (Hanahan et al., 2000 and 2011).

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Figure 1.21. The hallmarks of cancer. The essential capabilities cancers must acquire during their development (Hanahan et al., 2011).

ABC transporters are well known for their role in mediating chemoresistance in cancer cells. Recently many studies have suggested that ABC transporters might be implicated in the cancer hallmarks (Fletcher et al., 2010 and 2016). To date, little is known about their direct contribution to cancer progression and development irrespective of their drug-efflux abilities (Fletcher et al., 2010 and 2016; Adamska et al., 2018). In recent years, several studies have

suggested that the biological functions of ABC transporters are more complex than their role in drug resistance. Since ABC transporters transport many active signalling molecules and hormones as their substrates, it has been suggested that these substrates can activate cancer cells as well as regulate the tumour environment so as to help cancer cells to grow and develop (Adamska et al., 2018). In addition, signalling lipid molecules such as sphingosine-1-phosphate (S1P), prostaglandins and leukotrienes have a profound role in tumour biology and are known to be substrates of certain ABC transporters. Therefore, mediating the efflux of these molecules may also be important in cancer progression and development (Fletcher et al., 2010).

1.8.1 The role of ABC transporters in apoptosis and proliferation

In a specific organ site, the number of cells are tightly regulated by proliferation and apoptosis. In many cancers, this tight regulation is abolished and cancer cells become resistance to apoptosis and grow uncontrollably to form tumours (Hanahan et al., 2000 and 2011). During chemotherapy, ABC transporters efflux a number of cytotoxic drugs thus causing cancer cells to become resistant to apoptosis. Apart from the drug effluxing ability of ABC transporters, several studies have shown that ABC transporters may also help cancer cells to survive (Fletcher et al., 2010 and 2016). For example, as a single agent, valspodar, an ABCB1 inhibitor can promote apoptosis and cell cycle arrest in human leukaemia cells *in vitro* (Lehne et al., 1999 and 2002). However, another study showed that in some cell lines the cause of apoptosis by valspodar is independent of the inhibition of the transporter (Lopes et al., 2003). Moreover, the activation of ABCB1 in normal and cancer cells in response to apoptotic stimuli have shown to inhibit cell apoptosis (Pallis et al., 2000).

Studies have found that in neuroblastoma, ABCC1 helps cell survival and spontaneous cell death was observed both in mouse xenograft model and in human neuroblastoma cell lines *in vitro* when ABCC1 gene was silenced (Peaston et al., 2001; Kuss et al., 2002). Therefore, consistent with these observations, the expression level of ABCC1 gene is often used as an independent prognostic factor in neuroblastoma (Haber et al., 2006).

Studies found that cancer cell proliferation is directly mediated by bioactive phospholipids and it was found that the transport of lysophosphatidylinositol (LPI) by ABCC1 activates GPR55 in an autocrine manner to trigger signalling cascade that induce cell proliferation (Begicevic et al., 2017; Adamska et al., 2018).

The expansion of tumour populations are well attributed to increase in proliferation of tumour cells (Hanahan et al., 2000 and 2011; Fletcher 2010). Studies have found that the proliferation of vascular smooth muscle cells *in vivo* and *in vitro* was significantly reduced when ABCC4 gene was knocked down (Sassi et al., 2008). Furthermore, the knock down of ABCC1 gene inhibited cell proliferation in neuroblastoma cell line xenografts (kuss et al., 2002). In addition, the knockdown of ABCB1 gene significantly reduced tumour expansion in colon cancer xenograft model and inhibited the proliferation of a colon cancer cell line *in vitro* (Katoh et al., 2008). Additionally, ABCB1 together with ABCA5 may regulate the differential state of colon tumours, hence suggesting that ABCB1 and ABCA1 may have a role in tumour development (Ohtsuki et al., 2007; Dvorak et al., 2017). Retrovirus that encoded ABCG2 gene largely increased the proliferation of retinal progenitors but the proliferation was suppressed when the ABCG2 gene was silenced by small interfering RNA (siRNA) (Bhattacharya et al., 2007). Moreover, the suppression of ABCG2 was found to decrease cell proliferation and G0/G1-phase cell arrest in cancer cells through the up regulation of p21 and down regulation of cyclin D3 (Chen et al., 2010).

ABCC4 is the main transporter for cAMP, which is a very active molecule involved in cell differentiation, proliferation and apoptosis. A study found that when ABCC4 was pharmacologically inhibited or genetically silenced in acute myeloid leukaemia cells (AML), cell proliferation was inhibited, cell differentiation and apoptosis were induced (Cospel et al., 2011 and 2014). In another study, inhibition of ABCC4 expression in non-small cell lung cancer impaired cell proliferation. This suggested that ABCC4 could mediate cell cycle progression via phosphorylation of pRB protein hence promote cell proliferation (Zhao et al., 2014). Moreover, downregulation of ABCC4 expression in pancreatic cell lines was associated with a decrease in cell proliferation *in vitro*, hence suggesting ABCC4 promotes cell proliferation (Zhang et al., 2012). In another study, the knockdown of ABCC4 gene in gastric cancer cell lines was linked to a decreased in cell growth and suggested that ABCC4 may mediate gastric cancer cell growth by manipulating the S and the G2/M phase cell cycle regulators (Chen et al., 2014).

In response to oxidative stress, GSH is activated to maintain redox status of cells in homeostasis. However, activation of GSH mediates several signalling processes that regulates cell apoptosis, proliferation and immune response. Furthermore, the transport of GSH, GSSG and GSH conjugates (GS-X) are mediated by several members of the ABC transporters including ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC7, ABCG2 and ABCB10. Thus, suggesting that these ABC transporters are involved in regulating oxidative

stress and cell detoxification such as in breast and colorectal cancers (Dovarak et al., 2017; Begicevic et al., 2017; Adamska et al., 2018).

1.8.2 The Role of ABC transporters in cell migration, invasion and metastasis

The vast majority of cancer death is due to cancer metastasis whereby cancer cells develop the ability to disseminate to distant organ sites and start growing to found new tumours (van Zijl et al., 2011; Valastyan et al., 2011; Orimo et al., 2014; Chamber et al., 2015; Guan et al., 2015). Currently little is known about the role of ABC transporters in metastasis but several studies have been done to try to understand the role of ABC transporters in cell migration and invasion (Fletcher et al., 2010).

In normal cells, several ABC transporters have been found to be implicated in cell migration (Fletcher et al., 2010 and 2016). In Abcc1 gene knockout mice, a decrease in peripheral dendritic cell migration to lymph nodes was observed and the dendritic cells with Abcc1 gene knockdown had restricted chemotactic response *in vitro* (van de Ven et al., 2006). Moreover, the addition of LTC_4 and LTD_4 exogenously, both are ABCC1 substrates, were found to promote cell migration *in vivo* and *in vitro* even in the absence of ABCC1. Thus suggesting that ABCC1 in mouse dendritic cells may be involved in autocrine signalling which regulates dendritic migration to lymph nodes by transporting LTC_4 (Robbiani et al., 2000; Fletcher et al., 2010). Other studies has found that other ABC transporters such as ABCB1 may be involved in dendritic cell migration as one study showed that the migration of dendritic cells from human skin explants were inhibited by the inhibitor verapamil and ABCB1-specific neutralising antibodies (Randolph et al., 1998; Fletcher et al., 2010). However, the roles of various ABC transporters across species is different thus making it very difficult to compare the role of ABC transporters across species such as the role of ABCC4 in murine and human dendritic migration (van de Ven et al., 2008 and 2009). Consequently, the migration of human dendritic cells are more influenced by ABCC4 than ABCC1 as a study conducted showed that inhibition of dendritic cell migration by sildenafil or by ABCC4 gene knockdown, attenuated their migration from human skin explants (van de Ven et al., 2008 and 2009).

Similarly, the roles of ABC transporters in cell migration was also observed in normal as well as in cancer cell lines. A study assessing the migration of MCF-7 breast cancer cells in matrigel invasion and transwell migration assays were significantly supressed when ABCB1 gene was knockdown by siRNA (Miletti-Gonzalez et al., 2005). Further studies have also shown that after suppressing CD147, a major stimulator of metalloproteinases (MMPs) and has been proposed to be a poor prognosis marker in some cancers, in MCF-7/ADR breast cancer cells,

have led to an increased in chemosensitivity to ABCB1 substrates, while an overexpression of CD147 enhanced ABCB1 efflux ability (Li et al., 2007; Buzzoto et al., 2010). In addition, a drug-resistant human melanoma cancer cell line with high expression levels of ABCB1 was found to be more invasive than the parental cell line and that the knockdown of ABCB1 gene by siRNA consequently decreases cell invasion *in vitro* (Colone et al., 2008). In the canine kidney cell line MDCK and in the rat brain endothelial cell line RBE4, the expression of ABCB1 also promote increased in cell migration (Barakat et al., 2008). Moreover, another study which used wound closure assays to assess the cell migration of neuroblastoma cells in vitro was significantly reduced when both ABCC1 and ABCC4 were suppressed (Henderson et al., 2011; Fletcher et al., 2016). In a study the suppression of ABCA1 was found to inhibit ovarian cancer cell proliferation and migration (Ween et al, 2015). In another study, the suppression of ABCE1 by siRNA in small lung cancer cells inhibited cell proliferation, uneasiness and induce cell apoptosis, thus suggesting ABCE1 may be involved in small lung cancer tumorigenesis (Huang et al., 2010).

Finally, the role of ABC transporters in cancer metastasis remain to be determined but several studies has shown that ABC transporters may be involved in cancer metastasis. For example, a study on human prostate carcinoma cell line found that the expression levels of mRNA, MMP-2, MMP-9, basic fibroblast growth factor (bFGF) and ABCB1 were high in highly metastatic cells compared to those in poorly metastatic parental cells line. This suggested that ABCB1 mediated both metastasis and drug resistance (Greene et al., 1997; Buzzoto et al., 2010). Furthermore, another study found that high expression of ABCB1 in human hepatoma cells *in vitro* was correlated with an increase in invasive properties but not in low ABCB1 expressing human hepatoma cells (Buzzoto et al., 2010).

CD44 is a glycoprotein, a cell surface adhesion receptor, that is highly expressed in several cell types and in many cancers. Subsequently, CD44 has been shown to play a critical role in cancer development and progression, mediating metastasis, cell adhesion and tumorigenesis (Buzzoto et al., 2010; Senbanjo et al., 2017; Chen et al., 2018). Furthermore, a study found that the interaction of CD44 and ABCB1 is very important in modulating cancer cell migration, invasion along with multidrug resistance (Miletti-Gonzalez et al., 2005).

A study which investigated the role of ABCB1 in the invasion potential of drug-resistant (M14 ADR, ABCB1-positive) and drug-sensitive (M14 WT, ABCB1-negative) human melanoma cells were found to activate ERK1/2 and p38 MAPK pathways that stimulated the interaction of ABCB1 with CD44 causing an increased in proteolytic activity and in metalloproteinase (MMP-2, MMP-3 and MMP-9) mRNAs. In addition, when ABCB1 gene was knockdown, CD44

was not able to promote cell migration and invasion. As a result, ABCB1 was suggested to be an important component in cell migration as well as in the activation of ERK1/2 and p38 MAPK signalling (Colone et al., 2008).

In addition, ABCC1 and ABCC4 have both been found to have a role in cancer development and progression in neuroblastoma. Suppression of both ABCC1 and ABCC4 in human neuroblastoma cell line inhibited tumour cell migration and cell proliferation. However, an overexpression of ABCC3 inhibits cell migration and proliferation. Therefore, this indicates that low levels of both ABCC1 and ABCC4 were correlated with a decrease in cell migration and proliferation whilst an increase in differentiation (Porro et al., 2010; Henderson, et al., 2011).

Furthermore, in a breast cancer study, higher ABCC1 expression was detected in metastatic lymph nodes compared to the corresponding primary tumour (Fletcher et al., 2010). In another study using melanoma cancer cell lines, the cell lines derived from metastases were found to have higher expression of ABCC1 and ABCC4 genes compared to those derived from primary tumours (Fletcher et al., 2010). Therefore, in order to fully elucidate the role of ABC transporters in cancer metastases more direct lines of investigation will be required (Fletcher et al., 2010).

1.8.3   The biological role of ABC transporters in cancer stem cells

Pluripotent stem cells also known as the embryonic stem cells have the ability to self-renewal and form differentiated cells and tissues (Fletcher et al., 2010; Chagastelles et al., 2011). The expression of ABC transporters is significantly higher in both normal and cancer stem cells compared with their more differentiated progeny. Hence, this may suggest that stems cells are more protected from damage caused by xenobiotic substances and as such may have critical therapeutic implications with cancer stem cells, as the pluripotent cells in a tumour population may acquire a drug resistant phenotype, as a result may help to re-establish tumour in a similar manner to stem cell-driven recovery of normal tissue after chemotherapy (Dean et al., 2005; Fletcher et al., 2010; Moitra et al., 2015; Begicevic et al., 2017). However, it is not yet clear whether the cancer cell phenotype is influenced by the expression of ABC transporters or by other genetic changes during tumorigenesis (Fletcher et al., 2010; Begicevic et al., 2017).

The involvement of ABC transporters in the regulation of stem cell differentiation is still not clear as current genetic evidence shows that the maintenance of normal stem cell compartment does not require any ABC transporters and that mice models deficient in Abcg2, Abcb1 or Abcc1 genes as well as deficient in both Abcb1 and Abcg2 genes did not affect their

development. Therefore, the regulation of cellular differentiation or stem cell maintenance may not require ABC transporters (Zhou et al., 2002 and 2003). However, because of the high expression of a large number of ABC transporters present in stem cells, it is suggested that the role of ABC transporters might have an important role in stem cell growth, differentiation, survival and maintenance which still needs to be elucidated (Fletcher et al., 2010; Begicevic et al., 2017; Adamska et al., 2018).

Subsequently, ABC transporters has been associated with differentiation in several overexpression studies. For example, a study has demonstrated that ABCG2 is implicated in the efflux of androgens in prostate stem cells (Sabnis et al., 2017; Begicevic et al., 2017). Furthermore, under normal conditions ABC transporters are not required to maintain an undifferentiated state but may be required when upregulated in cancer (Fletcher et al., 2010). For several tumour types a correlation between poor patient outcome and a low degree of differentiation with the presence of transcriptional programmes was found to resemble closely to those seen in embryonic stem cells. Moreover, the impaired *MYC* gene expression can also induced such transcriptional programmes (Wong et al., 2008; Ben-Porath et al., 2008).

The PI3K/Akt pathway activation is very important for stem cells pluripotency maintenance. Hence the viability and maintenance in certain cancer stem cells such as in prostate cancer, breast cancer and brain tumour, required the activation of the PI3K/Akt pathway (Zhou et al., 2007; Hambardzumyan et al., 2008; Dubrovska et al., 2009, Zinzi et al., 2014). Furthermore, in a study, LY294002, a PI3K specific inhibitor was used to inhibit osteosarcoma cancer stem cells cell cycle inducing apoptosis while at the same time causing the inhibition of ABCG2, ABCB1 and ABCC1 in the stem cells. Thus suggesting that ABCG2, ABCB1 and ABCC1 may be involved in cancer stem cells differentiation and maintenance (Imai et al., 2012; Zinzi et al., 2014; Begicevic et al., 2017). In addition, in melanoma stem cells, ABCB5 was found to regulate cellular differentiation and a study found that ABCB5 activates pro-inflammatory interleukin 1β/ interleukin 8 (IL1β/IL8) and chemokine receptor 1 (CXCR1) signallings to control the pro-inflammatory signallings and thus to maintain melanoma stem cells and promote tumour growth (Wilson et al., 2014; Begicevic et al., 2017).

Therefore, due to high expression of ABC transporters in tumours, it is very important to elucidate their exact role in tumour development apart from their drug efflux abilities or whether they are simply present but does not have an effect in tumour development (Fletcher et al., 2010).

1.8.4 Substrates of ABC transporters with relevance to cancer

ABC transporters transport a wide range of physiological substrates, several of these substrates are signalling molecules with established roles in tumour biology. For many of these substrates, ABC transporters are either the most important or the only known efflux mechanism. These substrates include cyclic nucleotides, platelet activating factor (PAF), cholesterol metabolites, leukotrienes, prostaglandins and sphingosine-1-phosphate (S1P) (Table 1.10) (Fletcher et al., 2010).

Table 1.10. Selected ABC transporters with their cancer-related substrates and their expression in cancer stem cell-like populations (Adapted from Fletcher et al., 2010 and Begicevic et al., 2017).

ABC transporters	Cancer-related cellular substrates	Expression in cancer stem cell-like populations
ABCA		
ABCA1	S1P and cholesterol	Ovarian cancer
ABCA2	Cholesterol	Lung cancer and AML
ABCA3	Phospholipids	Neuroblastoma
ABCB		
ABCB1	PAF	Lung cancer, glioma, ovarian, breast, renal and AML
ABCB5	ND	Melanoma
ABCC		
ABCC1	LTC_4, PGA_2, 15d-PGJ2, PGE_2 and S1P	Squamous cell carcinoma lines, lung cancer cell lines, glioma and AML
ABCC2	LTC_4, PGD_2, PGA_1 and PGE_2	ND
ABCC3	LTC_4 and 15-deoxy-Δ12,14-prostaglandin J2	ND
ABCC4	LTB_4, LTC_4, PGA_1, PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, TXB_2, cAMP and cGMP	Osteocarcinoma
ABCC5	cAMP and cGMP	ND
ABCC6	LTC_4	ND
ABCC10	LTC_4	ND
ABCC11	LTC_4, cAMP and cGMP	ND
ABCG		
ABCG2	cGMP	Lung cancer, AML, oesophageal carcinoma, glioma, neuroblastoma, squamous cell carcinoma cell lines, melanoma, ovarian cancer and nasopharyngeal carcinoma cell lines

ABC, ATP binding cassette; cAMP, cyclic AMP; cGMP, cyclic GMP; LT, leukotriene; ND, not determined; PAF, platelet activation factor; PG, prostaglandin; S1P, sphingosine-1-phosphate. Several substrates for ABCC1–4 are effluxed as either glutathione or glucuronide conjugates, or in a glutathione-dependent manner.

1.8.4.1   Cyclic nucleotides

Cyclic nucleotides are substrates of ABCC4, ABCC5, ABCC11 and ABCG2 and are important secondary messengers downstream of G protein-couple receptors (GPCR) that may be implicated in cancer biology (Jedlitschky et al., 2000; Wielinga et al., 2003; Guo et al., 2003; de Wolf et al. 2007; Russel et al., 2008; Sassi et al. 2008; Fletcher et al., 2010). However, due to their low affinities in cells, their biological relevance in the development of cancer is still not clear (Borst et al., 2004 and 2007), but recent studies have found that a decrease in extracellular and an in increased in intracellular cAMP was noticed when Abcc4 gene was downregulated *in vitro*, therefore the transport of cyclic nucleotide by ABCC4 may be of physiological significant in some systems (Lin et al. 2008; Fletcher et al., 2010).

1.8.4.2   Platelet activating factor (PAF)

PAF is a bioactive lipid that has been found to be a substrate of ABCB1 and is involved in various tumour-associated signals and functions such as in breast cancer angiogenesis (Montrucchio et al., 1998; Ragger et al., 2001). Furthermore a study found that the activation of its extracellular GPCR by PAF induced a secondary signalling pathway that caused an upregulation of the anti-apoptotic proteins, BCL-2 and BCL-XL in a nuclear factor-kB (NF-kB) dependent way leading to the inhibition of tumour cell apoptosis (Heon Seo et al., 2006). In addition, PAF has been shown to facilitate metastasis in colorectal cancer (Denizot et al., 2005) and melanoma (Melnikova et al., 2006) as well as to enhance cell proliferation and motility, and contribute to angiogenesis in breast cancer (Bussolati et al. 2000).

1.8.4.3   Cholesterol metabolites

Many ABCA transporters are implicated in the regulation of lipid metabolism and transport (Albrecht et al., 2007). Cellular membranes are composed of cholesterol, an essential component of the lipid rafts which not only is important in bringing together cooperating membrane-bound proteins for optimal activation of phosphorylation cascades but is also important in mediating the division and rapid growth of tumour cells. Furthermore, several cancers have an impaired cholesterol metabolism. For example, one study found a lack of

circulating cholesterol for tumour growth and cell survival in the prostate cancer xenografts in immunodeficient mice. Additionally, a reduction in apoptosis, an increased in phosphorylation of Akt and tumour growth in prostate cancer were associated with an upregulation of cholesterol (Zhuang et al., 2005). Moreover, the rate of low density lipoprotein uptake and the availability of intracellular fatty acids, such as arachidonic acids can be influenced by intracellular cholesterol levels and cholesterol trafficking respectively (Hughes-Fulford et al., 2001). Hence activating many important signalling intermediates. However, the direct link between the regulation of ABC transporters in effluxing lipid signalling intermediates and regulating cholesterol metabolism to its effect on tumour phenotype is unclear and that further studies are required (Fletcher et al., 2010).

1.8.4.4 Leukotrienes

Leukotrienes are eicosanoids which are chronic inflammatory mediators synthesised from arachidonate 5-lipoxygenase (ALOX5) pathway (Peters-Golden et al., 2007). The binding of leukotrienes to extracellular GPCRs activates signalling cascades that cause the activations of several signalling molecules including leukocyte invasive behaviour (Perter-Golden et al., 2007), β-catenin which is essential in promoting cell proliferation and survival (Nielsen et al., 2005; Mezhybovska et al., 2006; Ihara et al., 2007; Yang et al., 2017), and cause neutrophils to secret pro-angiogenic factors (Tazzyman et al., 2009). Furthermore, leukotriene biosynthetic enzymes are upregulated in various cancers such as in pancreatic cancer (Hennig et al., 2002; Moore et al., 2017) and studies have shown that successful inhibition of ALOX5 in animal models of lung cancer (Poczobutt et al., 2016), pancreatic cancer (Henning et al., 2002), prostate cancer (Sarveswaran et al., 2014) and chronic myeloid leukaemia (CML), and the knockdown of *Alox5* gene in mouse model of BCR-ABL-induced CML, impaired the function of leukemic stem cells thus preventing the development of leukaemia (Chen et al., 2009; Fletcher et al., 2010).

The efflux and release of LTC_4 from leukotriene producing cells are greatly mediated by ABCC1 (Fletcher et al., 2010; Chen et al., 2010; Cole, 2014). Moreover, the release of LTC_4 was significantly reduced in the bone marrow-derived mast cells from Abcc1 deficient mice, thus exhibited a lack of response to leukotriene-inducing inflammatory stimuli (Fletcher et al. 2010; Cole et al., 2014). Additionally, the mediation of the transport of LTC_4 by ABCC1 has been found to be important in facilitating dendritic cell and T lymphocyte migration (van de Ven et al., 2006). Apart from ABCC1, many other ABCC subfamily of transporters including ABCC2, ABCC3, ABCC10 and ABCC11 are able to efflux LTC_4 while ABCC4 is able to efflux both LTC_4 and LTB_4 (Cui et al., 1999; Rius et al., 2008; Chen et al., 2002). In a study, LTB_4

was found to activate cell proliferation and induce ERK1/2 phosphorylation (Knab et al., 2014). Currently the therapeutic targets in leukotriene signalling are enzymes involved in leukotriene receptors and leukotriene synthesis, but with the implications of ABCC transporters in leukotriene signalling could provide additional or alternative therapeutic targets (Fletcher et al., 2010).

1.8.4.5 Prostaglandins

Some cancers proliferate and strive better in an inflammatory microenvironment which, also helps in promoting angiogenesis and metastasis. Prostanoids are members of eicosanoids which are mediators of chronic inflammation produced from arachidonic acid via the cyclooxygenase (COX) pathway (Figure 1.22) (Dannenberg et al., 2003; Fletcher et al., 2010).
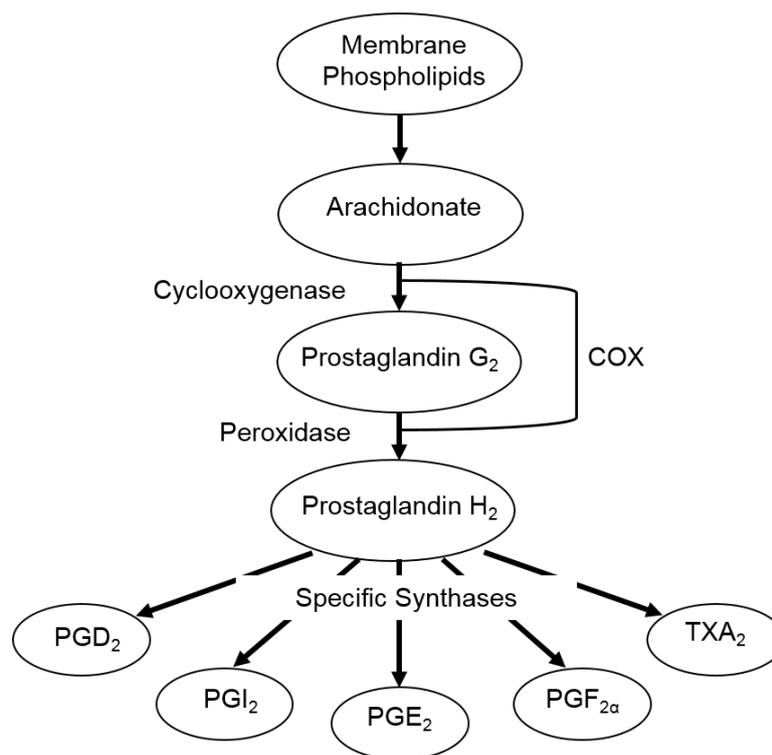
Figure 1.22. Prostaglandin biosynthesis. Arachidonic acid is first produced in a reaction catalysed by phospholipase A_2. Then molecular oxygen is inserted into arachidonic acid in a catalytic reaction involving COX enzymes to produce an unstable intermediate prostaglandin G_2 (PGG_2) which is quickly converted to prostaglandin H_2 (PGH_2). After, the PGH2 reacts with specific isomerases to form different isoforms of prostaglandins (PGD_2, PGE_2, PGI_2 and $PGF_{2\alpha}$) and thromboxane A_2 (TxA_2) (Dannenberg et al., 2003).

The importance of prostaglandin-mediated pathways in cancer has been well established from genetic evidence and clinical observation from mouse models of various tumour types including lung, colorectal, breast and liver cancers (Knab et al., 2014; Diakos et al., 2014). Numerous evidences has shown that COX is a critical enzyme in the production of prostaglandin and has two isoforms, COX-1 and COX-2. Both isoforms are equally important whereby COX-1 is essentially expressed in many tissues and COX-2 is a major therapeutic target as is often expressed in premalignant and malignant tissues (Dannenberg et al., 2003; Fulton et al., 2006; Greenhough et al., 2009). A study found that ABCC4 expression and function in lung cancer is regulated by COX-2 via the prostaglandin E synthase dependent pathway with an elevated PGE_2 production (Maeng et al., 2014). Furthermore, other components of prostaglandin synthesis including the various prostaglandin receptors and the other downstream enzymes may form potential therapeutic targets (Chell et al., 2006; Reader et al., 2011; O'Callaghan et al., 2015; Majumder et al, 2018).

The downward signalling of prostaglandin activates extracellular GPCRs in an autocrine or paracrine manner which, later activates other signalling pathways including the PI3K/Akt pathway (Tessner et al., 2004; Callaghan et al., 2015), which can lead to various responses such as the activation of β-catenin signalling and angiogenic factors such as vascular endothelial growth factor (VEGF) to promote a progenitor-like phenotype and to stimulate angiogenesis respectively (Castellone et al., 2005; Xie et al., 2018), all to promote cell survival, proliferation, motility, angiogenesis and metastasis (Vivanco et al., 2002; Ding et al., 2003; Faes et al., 2015). In addition, prostaglandin signalling might promote inflammatory cells and impair the differentiation of dendritic cells as a way to stimulate tumour cells to escape immunosurveillance (Sombroek et al., 2002; Mantovani et al., 2008).

Subsequently, many studies have concentrated on the receptors and enzymes of prostaglandin synthesis but the role of prostaglandin efflux is not well understood, particularly in cancers (Fletcher et al., 2010). One way prostaglandin is effluxed out of cells is through passive diffusion but such process is very inadequate as prostaglandin transporters (PGTs) and other transporters help the re-uptake of prostaglandin which later undergo intracellular oxidation by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Schuster, 2002; Tai et al., 2002; Nomura et al., 2004; Majumder et al., 2018). Several ABCC transporters have been found to facilitate the efflux of prostaglandin and are a major transport mechanism of prostaglandin in cancer cells where often these transporters are highly expressed (Fletcher et al., 2010). In addition, PGE_2, an isoform of prostaglandin, is a substrate of ABCC4 and is implicated in cancer progression such as inhibition of apoptosis and induction of angiogenesis and proliferation (Reid et al., 2003b; Knab et al., 2014; Cole, 2014; Kochel et al., 2015 and

2016). Studies have found that in human colorectal cancer specimen and cell lines, the expression of ABCC4 is high but the expression of prostaglandin transporter (PGT) is low, thus suggesting that with an increased prostaglandin efflux and a decreased re-uptake, enhanced prostaglandin signalling could occur (Holla et al., 2008). Furthermore, in another study found that the suppression of ABCC4 decreases metastases of triple negative breast cancer cells and was suggested to be correlated with the accumulation of PGE_2 in the tumour microenvironment (Kochel et al., 2017).

Additionally, cyclopentenone prostaglandins, a subset of prostaglandin, consisting of 15-deoxy-12,13-didehydro-14,15-didehydro-PGJ_2 (15d-Δ12,14-PGJ2), 12,13-didehydro-PGJ_2 (Δ12-PGJ_2) and PGA_2, are actively transported into cells and mediate a variety of gene expressions. Ultimately, 15d-Δ12,14-PGJ_2 and PGA_2, have been found to activate intracellular nuclear receptors such as peroxisome proliferator-activated receptor-Υ (PPARΥ) but not GPCR to trigger various responses to inhibit proliferation as well as tumorigenesis and induce apoptosis (Negishi et al., 2002; Piva et al., 2005; Musiek et al., 2005; Koyani et al., 2014). However, majority of the efflux of glutathione-conjugated 15d-Δ12,14-PGJ_2 and PGA_2 is via ABCC1 and ABCC3 (Evers et al., 1997; Paumi et al., 2003; Cole et al., 2014) . Moreover, a study found that ABCC1 and ABCC3 expressions may influenced the activities of 15d-Δ12,14-PGJ_2 by preventing the cytotoxicity of breast cancer cells. Therefore, ABC transporters are very important components in prostaglandin signalling in that they transport pro-tumorigenic prostaglandin to activate their extracellular GPCRs and at the same time efflux anti-tumorigenic cyclopentenone prostaglandins to prevent the intracellular activation of their downstream signalling, all in all to help promote tumorigenesis (Fletcher et al., 2010).

1.8.4.6   Sphingosine-1-phosphate (S1P)

S1P together with ceramide (Cer), sphingosine (Sph), and Cer-1-phosphate (C1P) are bioactive molecules representing a major class of lipids known as sphingolipids (SLs) that play a critical role in regulating cell signal transduction, differentiation, cell growth and apoptosis (Lahiri et al., 2007; Bartke et al., 2009; Pralhada Rao et al., 2013). The synthesis of S1P involve a series of catalytic reactions whereby ceramide is first converted to sphingosine using ceramidase (CDase) and after phosphorylated by sphingosine kinases (SK1 and SK2) to form S1P (Figure 1.23) (Lahiri et al., 2007; Bartke et al., 2009; Fletcher et al., 2010).

Figure 1.23. The metabolism of sphingolipids. The bioactive sphingolipids metabolites are highlighted in red. Ceramide can be produced by the de novo pathway that involve a series of catalytic reactions involving serine palmitoyl transferase (SPT), (dihydro) ceramide synthase (CerS, or Lass), and dihydroceramide desaturase (DES), or via the hydrolysis of sphingomyelin (SM) by sphingomyelinases (SMases). Ceramide can also be a precursor for the synthesis of SM catalysed by SM synthase (SMS) and involving the hydrolysis of phosphatidylcholine (PC) to diacylglycerol (DAG), the formation of glucosylceramide by glucosylceramide synthase (GCS), or galactosylceramide, and ceramide-1- phosphate (C1P) by catalystic reactions involving ceramide kinase (CK). Ceramide can be hydrolysed by ceramidases (CDases) to form sphingosine, which can be further converted to sphingosine-1-phosphate (S1P) by sphingosine kinase (SK). S1P can react with S1P lyase to form lipids and ethanolamine-1-phosphate. Moreover, dephosphorylation of Cer1P or S1P or C1P phosphatase C1PP and Sphphosphate phosphatase SPPase, respectively produce ceramide. (Ogretmen, 2006; Bartke et al., 2009).

Furthermore, S1P can mediate its action intracellularly such as the mediation of pro-apoptotic precursor ceramide and SK1 to inhibit apoptosis and the induction of calcium release from endoplaspic reticulum but currently little is known about the intracellular targets of S1P (Strub et al., 2010; Berdyshev et al., 2011). However, recently evidences found that S1P can bind and change the function of certain intracellular proteins including TNF receptor-associated factor 2 (TRAF2), histone deacetylases (HDACs), protein kinase C delta (PKCδ), prohibitin 2 (PHB2) and β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) (Maceyka et al., 2011). On the other hand, through five high-affinity GPCRs, S1P has been found to mediate important biological actions extracellularly including cell migration, differentiation,

proliferation and cytoskeletal organisation (Taha et al., 2004; Ishii et al., 2004; Young et al., 2006; Strub et al., 2010).

Consequently S1P has also been found to promote angiogenesis by encouraging the formation of blood vessels and stimulation of endothelial cells migration, possibly mediated through extracellular S1P receptors, Edg-1 and Edg-2 (Kimura et al., 2000). Studies using SK1 and SK2 double knockout mice and S1P receptor-knockout mice have shown a deficiency in the formation of blood vessel thus emphasising that S1P is important in angiogenesis (Liu et al., 2000; Mizugishi et al., 2005; Argraves et al., 2010). Another study has shown that the tumour progression in mouse xenograft and allograft was extensively diminished after treatment with a S1P-specific monoclonal antibody, possibly by blocking S1P signalling pathways that facilitates tumour angiogenesis, proliferation and metastasis. As a result S1P is a key component in promoting tumour angiogenesis (Visentin et al., 2006). Apart from facilitating angiogenesis, S1P also helps tumour cells to escape apoptosis and encourage tumour cell proliferation, invasion, metastasis as well as protecting tumour cells from apoptosis (Kimura et al., 2000; Maceyka et al., 2002; Visentin et al., 2006; Huang et al., 2011). In addition, S1P has been found to be a key component in mediating the invasiveness of cancer cells such as in human glioblastoma cancer cell lines (van Brocklyn et al., 2003; van Brocklyn, 2007; Mahajan-Thakur et al., 2017). Moreover, the production of PGE_2 and the expression of COX-2 have been suggested to be mediated by S1P (Pettus et al., 2003; Hsu et al., 2015; Rumzhum et al., 2015; Cheng et al., 2016).

Evidence also has pointed that a reduction in S1P efflux was observed in Abca1-deficient astrocytes thus confirming that potent S1P efflux may be mediated by ABCA1 (Sato et al., 2007; Takabe et al., 2008). ABCA1 is also associated in S1P efflux in epithelial ovarian cancer and its expression was associated with poor outcome of ovarian cancer. Thus suggesting that the transport of S1P by ABCA1 may promote angiogenesis in ovarian cancer (Hedditch et al., 2014). Additionally, when ABCC1 gene was knockdown in mast cells, the efflux of S1P was substantially reduced thus suggesting that ABCC1 is a major transporter for the efflux of S1P in mast cells that may influence migration of mast cells to antigen (Mitra et al., 2006; Alvarez et al., 2007). ABCG2 has also been implicated in S1P transport. In a study, using MCF-7 breast cancer cells, it was found that estradiol stimulated the production of S1P whose efflux was mediated by ABCC1 and ABCG2. However, the efflux of estradiol-induced S1P was suppressed when ABCC1 and ABCG2 genes were inhibited by either gene silencing or pharmacological inhibitors (Takabe et al., 2010; Reitsema et al., 2014). In another study, the export of S1P by ABCC1 lead to a decrease in survival of mice and patients with breast cancer (Yamada et al., 2018). A study also found that ABCC4 is responsible for the efflux of S1P from

platelets (Vogt et al., 2018).   Apart from ABC transporters as the efflux mechanisms for S1P, other transporters such as SPNS2 transporter have also been identify to transport S1P. Nonetheless the implication of these mechanisms to tumour biology remains to be determined (Fletcher et al., 2010; Bradley et al., 2014; Reitsema et al., 2014).

1.9   Project aims and objectives

The most common cancer in women is breast cancer, affecting 1.38million women worldwide (Martin, et al., 2014). Following the recent research that has shown the role of ABCC1 and ABCC4 have in neuroblastoma development and progression (Henderson, et al., 2011) as well as the role of ABCC4 in lung cancer and leukaemia progression (Copsel, et al., 2011; 2014; Zhao, et al., 2014), the aim of the project was to investigate whether ABCC1 and ABCC4 have an important role in breast cancer development or progression independent of cytotoxic efflux.

Several studies of ABCC1 in breast cancers have shown that a high expression level of ABCC1 leads to a reductions in both overall survival and time relapse. Furthermore, there is a link between patient relapse-free survival and ABCC1 expression. In addition, patients treated with anthracycline-based therapy regime show a correlation between ABCC1 and progression-free survival but not in patients treated without anthracyclines alone (Lage, 2003). Thus for early-stage breast cancer, ABCC1 expression can be used as a negative prognostic marker. Studies also have found that ABCC1 may play an important role in breast cancer as high level of expression of ABCC1 in breast cancer lymph node metastases were reported. Furthermore, certain drugs such as methotrexate used to treat metastatic breast cancer can be mediated by ABCC1 (Munoz, et al., 2007). However, more work is needed to elucidate the exact role and mechanism of ABCC1 in breast cancer (Lage, 2003).

In the case of ABCC4, substantial studies have been carried out showing high expression levels of ABCC4 in certain cancers. One study found that when anticancer and antiviral drugs were transported in human cervix cancer cells, ABCC4 was upregulated after COX-1 down regulation (Radilova, et al., 2009). Recently, ABCC4 was found to be one of the most commonly up regulated transcript in prostate cancer (Montani, et al., 2013). Moreover, there are studies showing that ABCC4 is involved in the regulation of gastric cancer and pancreatic cancer (Chen, et al. 2014; Carozzo, et al., 2015). Furthermore, Norris, et al., 2005 have confirmed that ABCC4 is a marker of poor prognosis in neuroblastoma. Recently, ABCC4 was shown to be expressed in breast cancer cells (Kochel, et al., 2015) but there is a limited amount of work done on ABCC4 in breast cancer. Therefore, work still needs to be done to determine the exact mechanism of ABCC4 in cancers.

Since ABCC1 and ABCC4 transport a wide variety of bioactive substrates that mediates various signalling pathways that regulate cell proliferation, migration, metastasis, invasion,

differentiation and apoptosis as previously mentioned. This thesis hypothesises that ABCC1 and ABCC4 are involved in breast cancer proliferation, migration and invasion.

Research was conducted *in vitro* using breast cancer cell lines MCF-7 and MDA-MB231 cells to carry out a series of cellular functional assays such as colony formation assay to determine cell proliferation, scratch assay to determine cell migration, MTT assay to determine cell viability, transwell invasion assay to determine cell invasion capacity and Western blotting and reverse transcription polymerase chain reaction (RT-PCR) to detect RNA expression of ABC transporter proteins.

Both MCF-7 and MDA-MB231 breast cancer cells are invasive with MCF-7 considered to be luminal-type breast cancer with the presence of progesterone, oestrogen and human epidermal growth factor 2 (HER2) receptors. However, MDA-MB231 is considered to be a basal-type breast cancer without progesterone, oestrogen and HER2 receptors (Triple negative).

The effects of ABCC1 and ABCC4 inhibitors were investigated, as well as the effect of knockdown or overexpression of these proteins. Finally preliminary investigations were undertaken to investigate possible mechanisms by which these proteins might affect cancer progression.

The results obtained will be a starting point to suggest these proteins might have a role in breast cancer cell migration and/or proliferation and thus open up new possible therapeutic targets for breast cancer.

Chapter 2 – Materials and methods

2.1   Cell lines

2.1.1   HeLa cells

The HeLa cells are a human epithelial carcinoma cell line from a fatal cervical carcinoma obtained from Henrietta Lacks, in 1951, who was suffering from cervical cancer. HeLa sticks to surfaces thus are adherent cells and *in vitro* they maintain contact inhibition (Fertey et al., 2011).

2.1.2   MDA-MB231 cells

The MDA-MB231 is a human breast cancer cell line acquired at M.D. Anderson Cancer Centre in 1973 from a patient. The MDA-MB231 cells looks like spindle shaped epithelial cells. The cell line has an invasive phenotype *in vitro* and is adherent cells (Kenny et al., 2007).

2.1.3   MCF-7 cells

The MCF-7 is a human breast cancer cell line obtained from malignant adenocarcinoma breast tissue that was first isolated from a 69-year old woman in 1970. The MCF-7 cells got their name from the institute, Michigan Cancer Foundation-7, in Detroit. The MCF-7 cells are adherent cells capable of forming epithelial and domes like cells when grown *in vitro* (Kenny et al., 2007).

2.1.4   HEK293T cells

The HEK293T is a human embryonic kidney cell line first isolated from human embryonic kidney and later was used to produce effectively glycosylated human recombinant proteins, adenovirus and other viral vectors. As a result, the HEK293T cell line is often transfected to express recombinant proteins and used for virology studies (Dalton et al., 2014). The HEK293T cells are adherent cells.

2.2   Basic cell culture laboratory techniques

All cell culture works were carried out in a sterile environment in a cell culture hood (HERAsafe) and cells were incubated in an incubator at temperature of 37°C with 5% CO_2. The work surfaces, the surrounding area and equipment used were thoroughly disinfected with 70% Industrial methylated spirit (IMS) (ThermoFisher) before and after use. Any spillage was quickly wiped with 70% IMS.

2.2.1   Cell passaging

2.2.1.1   Buffers and reagents

Prewarmed DMEM (1X) (Dulbecco's Modified Eagle's Medium) (Lonza) with 4.5 g/L glucose and L-glutamine was supplemented with 10% v/v FBS (Foetal Bovine Serum) (Gibco® by Life Technologies™), 100 U/ml Penicilin and 0.1 mg/ml streptomycin (Sterile filtered Penicillin-Streptomycin solution 100x, Biowest).

Sterile filtered DPBS (Dulbecco's Phosphate Buffered Saline) (Biowest) (GE Healthcare Life Sciences).

0.25% Trypsin-EDTA (1X) (Gibco® by Life Technologies™).

2.2.1.2   Procedure for passaging cells

After prewarming culture medium at 37°C in a water bath, cells in T-75 flask were checked under microscope (10X) to confirm at least 80% confluency. Culture medium in the flask was discarded into a waste bottle and 5 - 6 ml of DPBS was added in the flask. The flask was gently swirled and DPBS was discarded. Cells were then trypsinised with 2 ml of trypsin-EDTA placed in an incubator (37°C, 5% CO_2) for 1 - 2 minutes or until cells were fully dislodged from the surface. 6 ml of fresh culture medium was added in the flask to inhibit trypsin. The cell suspension was then centrifuged at 230 x g and resuspended in 10 ml fresh culture medium. 10 ml of fresh culture medium was added to a new flask. Then 2 ml of suspension cells were added to the new flask (1:5 split) and was gently swirled to ensure even spreading of cells in the flask. Cells were then incubated at 37°C with 5% CO_2. Cells usually took 2 - 3 days to reach at least 80% confluency. Different cell line grows at a different rate, therefore cells were constantly monitored to ensure cells grow healthily.

2.2.2   Freezing cells

2.2.2.1   Buffers and reagents

Cryoprotective Medium (Basal Eagle Medium with Hanks' BSS and 15% dimethylsulphuroxide without L-glutamine) (Lonza).

2.2.2.2   Cryopreserving cultured cells

After adding culture medium to trypsinised cells, the suspension cells were pipetted in a centrifuge tube and centrifuged at 60 x g for 5 minutes. The supernatant was discarded and a ratio of 1:1 of fresh cell culture medium and cryoprotective medium were added to the cell and mixed thoroughly by pipetting up and down. The cell suspension was then aliquoted into cryovials (1 ml) and placed in the cryo-freezing container ("Mr. Frosty"), which was then placed at -80°C. After two days the cryovials were removed from the cryo-freezing container and kept at -80°C.

2.2.3   Thawing frozen cells

10 ml of prewarmed culture medium was added to a T-75 tissue culture flask. The frozen cells were thawed at 37°C and the cell suspension was quickly added to prewarmed culture medium, which was then placed in the incubator (37°C, 5% CO_2). Since the cryoprotective medium contains DMSO that may dissolve the cell membrane, the cell culture medium was replaced up to 3 times with fresh culture medium so as to completely remove the DMSO to obtain optimum cell recovery.

2.2.4   Cell counting

The cell suspension was mixed with trypan blue (0.2% v/v) at a ratio of 1:4. 10 µl of the cell mixture was loaded in a chamber of the hemocytometer under the coverslip and placed under the microscope at a magnification of x10. The unstained cells (live cells) in the 4 corner squares were counted (those that fall on the top and right edges of the squares were counted). Once a total cell count was obtained cell concentration was calculated from the following formula (Equation 1).

Total cells per ml = Total cells counted $\times \frac{\text{dilution factor}}{\text{\# of squares}} \times 10{,}000$ cells per ml (Equation 1)

2.3   Using ABCC inhibitors in cell proliferation and migration assays

2.3.1   Colony formation assay

The colony formation assay is an *in vitro* cell survival assay that determines the ability of a cell to grow in to a colony after being treated with cytotoxic agents or any kind of inhibitors. There should be at least 50 cells clustered together to make a colony (Franken et al., 2006).

2.3.1.1   Buffers and reagents

Fixing reagent – 4% w/v paraformaldehyde in DPBS (PFA) (Fisher Scientific).
Staining reagent – 0.1% w/v crystal violet in water (Acros Organic).

Table 2.1. ABC inhibitors used.

Inhibitors	Inhibits ABCC1	Inhibits ABCC4	Concentration	supplier	Reference
MK571	✓	✓	50 µM and 100 µM	Sigma® life science	Tivnan et al., 2015
Indomethacin	X	✓	10 µM and 30 µM	Sigma® life science	Bai, et al., 2004
Reversan	✓	X	10 µM and 30 µM	Tocris bioscience	Tivnan et al., 2015
Ceefourin 1	X	✓	10 µM and 30 µM	Abcam Biochemicals®	Cheung, et al., 2014
Ceefourin 2	X	✓	10 µM and 30 µM	Abcam Biochemicals®	Cheung, et al., 2014

2.3.1.2   Colony formation assay

After cells were trypsinised with trypsin-EDTA, prewarmed fresh cell culture medium was added to the trypsinised cells. The cell suspension was then centrifuged at 230 x g for 5 minutes and resuspended in fresh cell culture medium. The cell suspension was passed through a cell strainer (40 µm nylon mesh) (Fisher Scientific) to ensure cells were single, not clumps. The strained cells were then counted and 100 cells were seeded per well of a 6-well plate, which contained 3 ml of fresh culture medium per well. The cells were incubated at 37°C for 24 hours. After 24 hours, the cells in each well were treated with each inhibitor at various

concentrations (Table 2.1) from a stock solution prepared in DMSO. Each condition was repeated at least 3 times.

After 7 days of incubation at 37°C, the culture medium was removed and 1 ml of 4% w/v PFA was added to each well and were left at room temperature for 20 minutes. The 4% w/v PFA was then removed and 0.5 ml of 0.1% w/v crystal violet was added and left at room temperature for 5 minutes. The crystal violet was then removed and the wells were washed thoroughly with water and were left to dry in open air at room temperature. The colonies formed were counted manually using a transparent film and a fine marker.

2.3.2   Scratch assay

One assay commonly adapted and modified to study mammalian cell proliferation and migration in response to a variety of experimental conditions such as chemical exposure *in vitro* is the scratch assay. The Scratch assay is a straightforward assay whereby a scratch (a gap) in the cell monolayer is created and the cell motility and growth are monitored to determine the rate of migration of cells towards the centre of the gap. Different experimental conditions will alter the rate of cells closing the gap (Liang, et al., 2007).

In our lab, the scratch assay was performed using the Cell-IQ (CM Technologies – Intelligent cell analysis). The Cell-IQ is an advanced incubator which has a time lapse imaging system where by image positions were set at certain time intervals for a period of time and images were automatically taken at that time interval set. A patented cell secure plate lid was used to keep cultures from contamination. Furthermore, to avoid CO_2 fluctuations when incubator door is being opened or closed, CO_2 is directly fed into the culture plate.

2.3.2.1   Cell-IQ scratch assay

Cells were seeded in a 24-well plate from 1 x 10^5 to 6 x 10^5 cells to determine which cell seeding density is best to reach 100% confluency after 24 hours incubation. After identifying the cell seeding density of a cell line, that seeding density was used throughout the assay for that cell line.

The cells were incubated at 37°C for 24 hours after which a scratch on the monolayer of cells was carefully performed using a 10 µl pipette tip. The culture medium was removed from the wells and the cells were carefully washed with sterile DPBS as much as possible not to disturb the scratch and the monolayer of cells. After washing, fresh prewarmed culture medium was

carefully added to the cells. The cells were then treated with inhibitors as detailed in table 2.1. Each condition was repeated at least 3 times.

Distilled water was added in between the wells and the plate was sealed with insulation tape together with the patented cell secure plate lid, the plate was placed in Cell-IQ as indicated in the instruction guide. Three image positions were allocated for each well, with images being taken every 1 hour for 24 hours. After 24 hours, imaging was stopped manually and the image results were analysed using the Cell-IQ analysis software.

2.3.3   MTT (Thiazolyl blue tetrazolium bromide) Assay

The MTT assay is an *in vitro* cell proliferation and viability assay used to measure the cell proliferation or cell metabolic activities in response to external factors such as exposure to chemical reagents. The principle behind MTT assay is that under defined conditions the dehydrogenase enzymes, NAD(P)H-dependent cellular oxidoreductase enzymes may reflect cell viability. The yellow tetrazolium MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is reduced by these enzymes to form insoluble formazan, which is purple in colour (Figure 2.1). To solubilise the insoluble formazan, DMSO is added and a purple solution is produced. Depending on light absorbance of this solution, will indirectly measure how viable the cells are (Riss, et al., 2013).
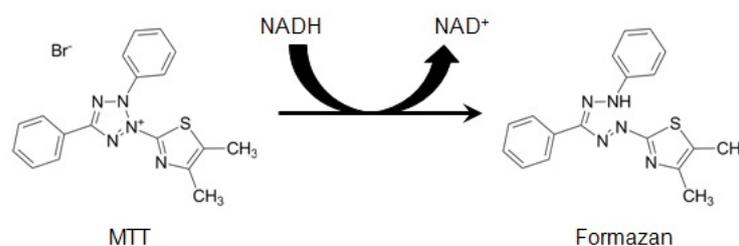
Figure 2.1. Structures of MTT and colored formazan product. MTT is being converted into a purple coloured formazan product with an absorbance of 570 nm by the active metabolism involving NADH in viable cells. The intensity of the colour is an indication of the amount of viable cells present. Cells, which have died, are unable to convert MTT into formazan (Riss, et al., 2013).

2.3.3.1   Buffers and reagents

Thiazolyl Blue Tetrazolium Bromide (Sigma® life science) – 5 mg/ml in DPBS.

2.3.3.2   MTT assay

15000 cells (HeLa and MCF-7) or 6000 cells (MDA-MB231) were seeded per well of a 24-well plate and for each well, 400 µl of prewarmed fresh culture medium was added. The cells were incubated at 37°C for 4 hours after which cells were treated with inhibitors as detailed in table 2.1.

The cells were then left to incubate for 6, 12, 24, 48 or 72 hours at 37°C. Once incubation time was complete, 40 µl of MTT reagent was added to each well. The cells were incubated for 1 hour at 37°C. The culture medium was removed from the wells and the wells were left to air dry at 37°C for 5 mins. After the wells were dried, 400 µl of DMSO (Fisher Scientific) were added to each well and the plate was left to incubate at 37°C for 10 mins. 100 µl of each sample were transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm using multiskan Go microplate reader (ThermoScientific).

2.3.4   Determining cell viability and growth by manual counting

In order to distinguish between dead and live cells, trypan blue is often used, as it stains dead cells leaving live cells unstained. Such method is often used to determine cell viability. However, trypan blue staining does not distinguish the cells that are alive but lost cell function to healthy cells. Therefore, in this project trypan blue staining was used as a second cell viability assay to MTT assay.

2.3.4.1   Buffers and reagent

Trypan blue solution (0.4% w/v) (Sigma Life Science) was diluted in DPBS at a ratio of 1:1.

2.3.4.2   Trypan Blue cell viability assay

15000 cells (HeLa and MCF-7) or 6000 cells (MDA-MB231) were seeded per well of a 24-well plate. Each well contained 400 µl of culture medium. The cells were incubated for 4 hours after which the cells were treated in duplicates with a concentration of an inhibitor as detailed in table 2.1, and each condition was repeated at least 3 times. The cells were left to incubate at 37°C for 6, 12, 24, 48 or 72 hours.

Once the incubation time was over, the culture medium in each wells was discarded and the wells were washed twice with DPBS and the cells were detached with trypsin-EDTA. A cell

suspension was created with culture medium after which was centrifuged at 230 x g for 5 minutes room temperature. The supernatant was discarded and the cells were suspended in culture medium and ready to count according to the cell counting protocol (Section 2.2.4).

2.4 Cell transfection

2.4.1 Standard siRNA transfection of mammalian cells

Gene silencing refers to mechanisms other than genetic modification whereby mRNA translation or transcription of a target gene, is being suppressed or interrupted. Gene silencing can be obtained by small RNA molecules known as miRNA and siRNA (Shah, et al., 2012).

In order to ensure efficient gene silencing and reproducible transfection, INTERFERin, a powerful siRNA transfection reagent was used. At 1 nM siRNA, INTERFERin can provide more than 90% silencing efficiency in a wide variety of cells, hence reducing off-targets effects as stated from the manufacturer's guide.

The ABCC1 and ABCC4 genes were suppressed through silencing RNA (siRNA) knockdown using INTERFERin *in vitro* siRNA transfection reagent.

2.4.1.1 Buffers and reagents

OptiMEM – reduced serum medium (Gibco® by Life Technologies)
INTERFERin ™ transfection reagent (Polyplus Transfection®)
ABCC1 siRNA (SASI_Hs01 00155530) (Sigma)
ABCC1 siRNA (SASI_Hs01 00155531) (Sigma)
ABCC4 siRNA (SASI_Hs02 00324134) (Sigma)
ABCC4 siRNA (SASI_Hs02 00324135) (Sigma)
Sigma-mission siRNA universal negative control (Sigma)

The stock concentration of the siRNAs were at 100 μM in OpiMEM.
The working concentration of siRNAs were 1 nM per well of a 24-well plate.
All dilutions were done in OptiMEM.

2.4.1.2 Gene knockdown using siRNA

On day one, 25000 cells were seeded per well of a 24-well plate according to the INTERFERin

manual. The cells were seeded in 1 ml of fresh culture medium and the plate was swirled gently for homogenous coverage of cells. The next day, transfection complexes were made by adding siRNA to 100 µl of OptiMEM after which 2 µl of INTERFERin was then added and the mixture was immediately mixed by vortexing for about 10 secs to achieve a final concentration of 1 nM of siRNA in cell culture. The transfection complexes were allowed to form on incubation at room temperature for 10 - 15 minutes. After incubation, 100 µl of the transfection complexes were added to the wells and the plate was swirl for even dispersing of the RNAi complexes. The plate was incubated for 72 hours. Constant monitoring of cells was necessary and a change of fresh culture medium was required when high cell death was observed. On day five, the transfected cells were harvested and stored at -20°C or were used for further experiments.

2.4.2 Amplification of plasmid DNA

Plasmids; pcDNA3.1 empty vector, pcDNA3.1 – ABCC1, pcDNA3.1 – ABCC4 (Kind gift from Prosfessor Susan Cole, Queen's University, Kingston, Ontario, Canada).

2.4.2.1 Bacterial media

Luria-Bertani (LB) broth - For liquid broth 20 g LB (Melford Bioscience) dissolved in 1 L distilled water and autoclaved. For selective media, ampicillin stored at -20°C at a concentration of 100 mg/ml was added to a final concentration of 100 µg/ml.

For LB agar a packet of *E.coli* fast-media (X-gal agar) (Invivogen) was dissolved in distilled water according to manufacturer's instructions. Once the LB agar was made, it was supplemented with 100 µg/ml ampicillin and equal volume of agar was pipetted into petri dishes and left to set. The agar plates were stored upside down at 4°C.

2.4.2.2 *E.coli* transformation

An aliquot of 500 µl of DH5-alpha *E.coli* chemically competent cells was thawed on ice and mixed gently by flicking and/or inverting tube with 1 µl of desired plasmid. The competent cells were then left on ice undisturbed for 20 minutes before heat shock at 42°C for 30 seconds without mixing. After heat shock, the competent cells were left on ice for 2 minutes without mixing. 500 µl of LB media without antibiotics was added immediately to the competent cells and incubated for 1 hour at 37°C with 200 rpm shaking. The mixture was spread on LB-

ampicillin agar plates and incubated at 37°C for 24 hours after which they were kept for a maximum of 3 months at 4°C.

2.4.2.3   Inoculating a liquid bacterial culture

10 ml of LB medium was poured in a universal tube with ampicillin added to a final concentration of 100 µg/ml. A colony of the transformed *E.coli* used to inoculate the culture which was then incubated for 8 - 12 hours at 37°C with 200 rpm shaking.

2.4.2.4   Plasmid DNA Mini prep

The plasmid isolation from transformed *E.coli* cells were obtained by using the GeneJET Plasmid Miniprep kit (ThermoFisher Scientific) according to manufacturer's instructions.

2 ml of overnight culture of *E.coli* competent cells containing the desired plasmid was subject to centrifugation at 6800 x g in a microfuge for 30 seconds. The pellet was then resuspended completely in 250 µl of the resuspension solution. 250 µl of the lysis solution was added to the resuspended cell pellet and mixed thoroughly by inverting the tube 4 - 6 times until the solution becomes viscous and slightly clear. 350 µl of the neutralisation solution was then added to the solution and mixed immediately and thoroughly by gently inverting the tube 4 - 6 times. The solution was centrifuged for 5 minutes at 14000 x g in a microfuge to pellet chromosomal DNA and cell pellet and the supernatant obtained was carefully transferred to the supplied GeneJet spin column. The column was centrifuged for 1 minute at 14000 x g and the flow-through was discarded and the column was placed back into the same collection tube. 500 µl of the wash solution was added into the column and centrifuged for 1 minute. The flow-through was discarded and the column was placed back into the same collection tube. The wash step was repeated one more time after which column was centrifuged an additional 1 minute at 14000 x g to remove residual wash solution. The column was after transferred into a fresh 1.5 ml microfuge tube and 50 µl of distilled water was added to the centre of the column. The column was then incubated for 2 minutes at room temperature and centrifuged for 2 minutes at 14000 x g. The eluted plasmid DNA was aliquoted and stored at -20°C.

2.4.2.5   Plasmid DNA Max prep

2.4.2.5.1   Buffers and reagents

Solution 1 – 50 mM Tris-hydrochloric (Tris-HCl) pH 8.0 and 10 mM ethylenediaminetetraacetic acid (EDTA) were mixed in distilled water and stored at 4°C. Upon use, RNase at final concentration of 50 µg/ml was added and the solution was sterile filtered using 0.45 µm syringe filters.

Solution 2 – 200 mM sodium hydroxide (NaOH) and 1% w/v SDS were mixed in distilled water and stored at room temperature. Upon use, the solution was sterile filtered using 0.45 µm syringe filters.

Solution 3 – 3 M potassium acetate was dissolved in distilled water and pH 5.5 was adjusted using glacial acetic acid. Upon use, the solution was sterile filtered using 0.45 µm syringe filters.

2.4.2.5.2.   Maxi prep procedure

200 µl of an inoculated overnight culture was added to 200 ml of LB medium with ampicillin at working concentration of 100 µg/ml was incubated for 18 - 22 hours at 37°C with 200 rpm shaking. The cells were centrifuged for 20 minutes at 2550 x g at 4°C. The supernatant was discarded and the cell pellet was resuspended in 5 ml of solution 1 by thoroughly vortexing. Once the cells were resuspended, 5 ml of solution 2 was added to the cell suspension and incubated for 5 minutes at room temperature. After incubation, 5 ml of solution 3 was added to the suspension by vortexing, followed by incubation for 30 minutes at 4°C. The suspension was then poured into a 50 ml tube and centrifuged for 20 minutes at 4000 rpm in Beckman table top centrifuge at 4°C. A sterile forceps was then used to fish off the fat layer swimming on top of the supernatant. The supernatant was sterile filtered using 0.45 µm syringe filters into a new 50 ml tube. 15 ml of 100% isopropanol was added to the supernatant and mixed thoroughly. The tube was then left to incubate on ice for 15 minutes. After incubation on ice, the supernatant was centrifuged for 20 minutes at 4000 rpm in Beckman table top centrifuge at 4°C. The supernatant obtained was discarded and the pellet resuspended and washed in 10 ml of 70% ethanol. The suspended pellet in 70% ethanol was centrifuged for 10 minutes at 4000 rpm in Beckman table top centrifuge at 4°C and the supernatant discarded. Subsequently the pellet was washed one more time and the pellet was left to dry at 37°C

ensuring no ethanol remains. The dry pellet was suspended in 1 ml of distilled water and aliquoted and stored at -20°C.

2.4.2.6.   Plasmid DNA quantification

The plasmid DNA obtained from plasmid DNA mini prep and plasmid DNA max prep were quantified using the Nanodrop™ 1000 spectrophometer (ThermoFisher Scientific) according to manufacturer's instructions. The concentration of plasmid DNA obtained were all expressed in µg/µl.

2.4.2.7   Creating bacterial glycerol stocks for long term storage of plasmid DNA

500 µl of the overnight culture was mixed gently with 500 µl of 50% v/v glycerol (diluted in distilled water and autoclaved) in a 2 ml cryovial. The glycerol stock tube was frozen at -80°C.

2.4.3   Overexpression of gene using Polyethyleneimine (PEI) as a transfection reagent in mammalian cells

PEI is a stable polymer with cationic nitrogen atoms that can form polyplexes with anionic DNA. PEI forms a protective shell around the DNA molecule. Upon endocytosis by the cells, the DNA molecule is released into the cytoplasm. The addition of sodium chloride can help DNA delivery as culture osmolality increases (Sou, et al., 2013).

2.4.3.1   Buffers and reagents

Fresh prewarmed DMEM was supplemented with 2.5% (v/v) FBS and 100 U/ml Penicilin and 0.1 mg/ml streptomycin (Sterile filtered Penicillin-Streptomycin solution 100x, Biowest).

Plasmids; pcDNA3.1 – empty vector, pcDNA3.1 – ABCC1, pcDNA3.1 – ABCC4

The quantity of DNA in the plasmid DNA solutions was measured using Nanodrop™ 1000 spectrophotometer (Thermo Scientific) in µg/µl.

PEI – stock concentration 23 mM was made by making a concentration of PEI at 1 mg/ml (23 mM solution) in 20 ml of water in a 50 ml tube and the pH was adjusted to 7.0 with HCl. The solution was then diluted 2.3-folds to a concentration of 10 mM. The 10 mM solution of PEI was filter sterilised and aliquoted to store at -20°C.

Sterile DPBS/EDTA was made by adding sterile EDTA to DPBS for a final concentration of 2 mM.

2.4.3.2   Overexpression of genes using PEI as a transfection reagent

Cells were seeded in 6-well plate at a cell seeding density so as to reach 60 - 70% confluency at the time of transfection (24 hours) (HEK293T cells: seeding density 300,000 cells). After 24 hours, culture medium was replaced with culture medium containing 2.5% (v/v) FBS and the plate was incubated for 3 hours at 37°C.

A PEI nitrogen to DNA phosphate ratios of 10:1, 15:1 and 20:1 were used in previous studies in our lab and found 15:1 to give >70% transfection efficiency. Therefore, 18 µl of PEI (10 mM) was added to 80 µl of pcDNA plasmids (4 µg) (Both the PEI and DNA were first diluted in sterile water to form the working solution). The transfection complex was mixed with a pipette and 100 µl of OptiMEM was then added to the complex. The transfection complex was immediately added drop wise to the cells and the plate was gently tilted to ensure evenly distribution of transfection complex. The culture medium was replaced with culture medium containing 10% (v/v) FBS after 24 hours of incubation at 37°C. Cells were left to incubate for 48, 72 and 96 hours. After 48, 72 and 96 hours post transfections, cells were harvested and stored at -20°C.

2.4.4   Overexpression of genes using Lipofectamine transfection reagent in mammalian cells

Lipofectamine is a cationic lipid that can facilitate the delivery of DNA and siRNA into cells. Cationic lipids are made up of a positively charged head group and two hydrocarbon chains. The positively charged head group is responsible for the facilitation of DNA condensation and the interaction between the lipid and the phosphate backbone of the nucleic acid. Often, cationic lipids are formed with a helper lipid or neutral co-lipid, followed by microfluidisation or extrusion that can form a positive surface charge in a unilamellar liposomal structure when in water (ThermoFisher Scientific, 2016).

The interaction of the nucleic acid and the cell membrane is mediated by the positive surface charge of the liposomes as a result forming the liposome / nucleic acid transfection complex with the negatively charged cell membrane. Hence by endocytosis the transfection complex

can enter the cell and once inside the cell, the complex have to diffuse through the cytoplasm and enter the nucleus for gene expression (ThermoFisher Scientific, 2016).

2.4.4.1   Buffers and reagents

Lipofectamine 3000 reagent kit consist of the Lipofectamine 3000® reagent and P3000 reagent (Invitrogen – ThermoFisher Scientific).

2.4.4.2   Overexpression of genes using plasmid DNA and the Lipofectamine 3000® transfection reagent

The transfection of plasmid DNA using the Lipofectamine 3000® reagent was performed using the Lipofectamine 3000® reagent kit (Invitrogen – Thermofisher Scientific). According to the manufacturer's protocol, on day one, cells were seeded in a 24-well plate so that it reached 70% confluent at transfection. On day two, DNA / Lipofectamine complex was made. 25 µl of optiMEM was added to tube A and tube B. As the transfection efficiency of Lipofectamine differs from one cell line to another, an optimisation volume of 0.75 µl and 1.5 µl of Lipofectamine 3000 were initially used. Therefore, either 0.75 µl or 1.5 µl of Lipofectamine 3000 reagent was added to tube A and mixed thoroughly. To tube B, 0.5 µg of plasmid DNA and 1 µl of P3000 reagent was added and mixed thoroughly. Once the contents of both tubes have been mixed respectively, the mixture of tube B was added and mixed to the mixture of tube A. The Lipofectamine plasmid DNA complex mixture was then left to incubate at room temperature for 10 - 15 minutes.

In the meantime, the culture medium in the wells were replaced with 500 µl of fresh culture medium. After 10 - 15 minutes incubation of the Lipofectamine plasmid DNA complex mixture, 50 µl of the complex mixture was added to each well. The plate was swirled gently to ensure even distribution of complex mixture over cells. The cells were incubated for two days after which the cells were either harvested for Western blot analysis or for experiments.

The volume of reagents specified above to form the Lipofectamine plasmid DNA complex mixture is for one well of a 24-well plate.

2.5 Cell invasion

The movement of cells from one area to another in response to a chemical signal is known as cell migration and is responsible for a variety of functions such as cell differentiation, wound repair, the metastasis of tumours and embryonic development (Kramer et al., 2013).

On the other hand, cell invasion requires cell to migrate through by enzymatically degrade a basement membrane extract (BME) barrier or an extracellular matrix (ECM) and relocate into a new location. Cell invasion is common process in normal cells such as in response to inflammation and in tumour cells such as in the process of metastasis. Since cell invasion is involved in a wide variety of biological systems, it is very important to understand its underlying mechanisms (Kramer et al., 2013).

2.5.1 Buffers and reagents

Prewarmed DMEM (1X) (Dulbecco's Modified Eagle's Medium) (Lonza) with 4.5 g/L glucose and L-glutamine was supplemented with 10% v/v FBS (Fetal Bovine Serum) (Gibco® by Life Technologies™) and 100 U/ml Penicilin and 0.1 mg/ml streptomycin (Sterile filtered Penicillin-Streptomycin solution 100x, Biowest).

Prewarmed DMEM (1X) (Dulbecco's Modified Eagle's Medium) (Lonza) with 4.5 g/L glucose and L-glutamine was supplemented with 0.5% v/v FBS (Fetal Bovine Serum) (Gibco® by Life Technologies™) and 1% v/v P/S (Sterile filtered Penicillin-Streptomycin solution 100x) (Biowest).

Serum free cell culture medium - DMEM (1X) (Dulbecco's Modified Eagle's Medium) (Lonza) with 4.5 g/L glucose and L-glutamine.

Corning Matrigel basement membrane matrix (Corning) (Aliquoted and stored at -20°C).

Differential Quik Stain Kit (Modified Giemsa) – Solution A (fixative), Solution B (Blue) and Solution C (Red) (Polysciences, Inc).

2.5.2 Transwell invasion assay

On day one, the Matrigel was thawed overnight at 4°C. On day two, the 24-well plate transwells inserts (pore size 8 μm, translucent) (Greiner bio-one) were coated with 30 μl of ice cold

Matrigel mixed with cold serum free medium at a ratio of 1:3. The coated transwell inserts were left to set at 37°C for 2 hours. It is to note that pipettes, tips and tubes were pre-cooled when handling the Matrigel.

Transfected cells were left to reach 70 - 80% confluency before the cells were prepared for the invasion assay. 50,000 cells were seeded in each transwell inserts suspended in 300 µl of 0.5% v/v FBS culture medium. 800 µl of chemoattractant (10% v/v FBS cell culture medium or 0.5% v/v FBS cell culture medium) was added to the wells and the transwell inserts were placed in each of the wells that contain a chemoattractant. The transwells inserts were incubated for 24 hours at 37°C in an incubator.

After 24 hours, the culture medium in the transwell inserts were discarded and a cotton swab moistened with DPBS was used to gently but putting firm pressure to rub the inside of the transwell inserts and this process was repeated with a second cotton swab.

The cells that have moved to the lower surface of the membrane of the transwell inserts were stained with Differential Quik stain. First, the transwell inserts were placed in solution A for 10-15 minutes after which were placed in solution C and solution B for 5 - 10 minutes each. The transwell inserts were then rinsed twice in water. A cotton swab moistened with DPBS was used to gently but putting firm pressure to wipe again the inside of the transwell inserts to rid of any cells left. The transwell inserts were left to dry at room temperature.

To determine the percentage cell invasion, the invaded cells were counted under a light microscope at a magnification of x40. The invaded cells were counted from randomly selected five fields of view for each transwell inserts and the mean number of invaded cells were obtained. The % invasion was calculated according to equation 2.

$$\% \text{ invasion} = \frac{\text{mean number of transfected cells invading through the coated transwell inserts}}{\text{mean number of untreated cells invading through the coated transwell inserts}} \times 100 \quad \text{(Equation 2)}$$

2.6   Protein sample preparation and analysis

2.6.1   Cell Membrane preparation

2.6.1.1   Buffers and reagents

Homogenisation buffer (50 mM Tris-HCl pH 7.4, 250 mM Sucrose and 0.25 mM calcium chloride ($CaCl_2$)).

Tris sucrose buffer (TSB) comprised 50 mM Tris-HCl pH 7.4 and 0.25 mM sucrose.

100x Protease inhibitor mix contained 15 mg/ml benzamidine, 1 mg/ml leupeptin and 0.1 mg/ml pepstatin.

2.6.1.2   Cell membrane preparation using nitrogen cell cavitation

Cell pellets harvested and stored at -80°C, were resuspended in 20 ml of homogenisation buffer and 200 µl of 100x protease inhibitor. All the subsequent steps were done on ice. The cell suspension was then placed in the cell disruption vessel for nitrogen cavitation at 500 psi for 15 minutes (Model 4639 Parr Cell Disruption Vessels (Parr®)). The vessel was left on ice. Pressure was released and samples were then collected and centrifuged for 10 minutes at 560 x g at 4°C (centrifuge 5810R (Eppendorf)). The supernatants were collected and the pellet, which contained unbroken cells and debris, was discarded. The supernatants were then pipetted in ultra-clear centrifuge tubes (14 x 95 mm - Beckman Coulter) and weighed to ensure equal weight of supernatant in each tube. The supernatants were centrifuged in i70 motor for 20 minutes at 38000 x g at 4°C (Optima™ L-100K Ultracentrifuge (Beckman Coulter)). After centrifugation the supernatants were carefully discarded so as not to disturb the pellet, which is the membrane proteins. Membrane pellets were resuspended in TSB (0.5 - 1 ml). A needle syringe (0.45 mm x 13 mm) was used to resuspend the membrane proteins in TSB. The membrane proteins samples were then aliquoted and stored at -80°C. Samples were kept on ice during the membrane preparations.

2.6.2   Whole cell lysate

2.6.2.1   Buffers and reagents

Ice-cold lysis buffer (0.15 M sodium Chloride (NaCl), 0.05 M Tris, pH 8.0, 1% (v/v) Triton-X100

1 mM EDTA, 1% (v/v) 100x Protease Inhibitors)

2.6.2.2   Whole cell lysis

Cells harvested were placed on ice in tubes. 300 - 400 µl of ice-cold lysis buffer was added to the cells. The cells were resuspended by vortexing. The cell suspension obtained was kept on ice and vortexed every 10 minutes for 1 hour. Cells suspension were then pipetted in eppendorf tubes and centrifuged for 15 minutes at 15600 x g at 4°C (Eppendorf centrifuge 5415R) and the supernatant which is the whole cell lysate was carefully aliquoted and stored at -20°C or was mixed with sample buffer for loading on sodium dodecyl sulphate (SDS) gels.

2.6.3   BCA (Bicinchoninic acid) protein assay

A BCA protein assay was used to quantify total protein in both membrane preparation and whole cell lysates, since this assay is tolerant of detergents (ThermoFisher).

2.6.3.1   Buffers and reagents

Pierce TM BCA Protein Assay Kit (Thermo Scientific) contains;
BCA Reagent A - Sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide.
BCA Reagent B - 4% w/v cupric sulphate

Albumin Standard Ampules, 2 mg/ml - Bovine serum albumin (BSA) at 2 mg/ml in 0.9% w/v saline and 0.05% w/v sodium azide.

2.6.3.2   Protein quantification

A BCA assay was carried out to determine the amount of protein present in each sample. BSA concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1.0, 1.2, 1.5 mg/ml were prepared by dilution with distilled water and 10 µl of each concentrations were transferred to each well of a 96-well plate in triplicates. Protein samples were diluted 1/3, 1/10 and 1/30 in distilled water. 10 µl of each protein samples, diluted and undiluted were transferred to each well of the 96-well plate in triplicates. The BCA reagent A and B were mixed in a ratio of 50:1 and 200 µl of mixture was added to all wells. The plate was then incubated for 30 minutes at 37°C and absorbance values were measured at 570 nm using multiskanGo microplate reader

(ThermoScientific). A BSA protein standard curve was generated to determine the quantity of proteins in each sample, expressed in µg/µl according to manufacturer's protocol.

2.6.4 SDS PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis

2.6.4.1 Buffers and reagents

5x Laemmli sample buffer (LSB) was made using 144 mM Tris pH 6.5, 29% v/v glycerol, 38% w/v SDS, 4% v/v β-mercaptoethanol and 0.05% v/v bromophenol blue. The solution was kept at room temperature.

1x SDS gel running buffer was made by diluting 100 ml of 10x SDS Tris buffer (0.25 M Tris, 1.92 M glycine and 1% w/v SDS) in 900 ml of distilled water.

2.6.4.2 SDS PAGE gel preparation

The SDS PAGE gel was prepared as detailed in tables 2.2 and 2.3 below. The tables represent the different buffers and their volumes that were mixed together to make two 8% separating and two 5% stacking mini-gels respectively. 10% w/v ammonium persulphate (APS) and tetramethlylenediamine (TEMED) were added last because TEMED with APS catalyses acrylamide polymerisation.

Table 2.2. Preparation of 8% acrylamide separating gel.

	8% Separating/ Resolving gel Volume to prepare for 2 gels	10% Separating/ Resolving gel Volume to prepare for 2 gels
Distilled water	5.4 ml	4.9 ml
Acrylamide : Bis-Acrylamide 37.5:1	2 ml	2.5 ml
1.5 M Tris pH 8.8 buffer	2.5 ml	2.5 ml
10% w/v SDS	100 µl	100 µl
10% w/v APS	100 µl	100 µl
TEMED	10 µl	10 µl

Table 2.3. Preparation of 4% acrylamide stacking gel.

4% Stacking gel	Volume to prepare for 2 gels
Distilled water	2.4 ml
Acrylamide : Bis-Acrylamide 37.5:1	0.5 ml
0.5M Tris pH 8.8 buffer	1 ml
10% w/v SDS	40 µl
10% w/v APS	40 µl
TEMED	5 µl

Once the separating gel mixture was made, it was poured in between two glass plate in a gel casting system and allowed to set. The stacking gel was then added to the top of separating gel and a comb was inserted and removed when the gel solidified.

5x LSB sample buffer was added to protein samples and they were loaded into the wells of the gels. 4 µl of prestained protein molecular weight marker (20 – 120 kDA) (Thermo Scientific) was also loaded in a well of the gel. Using the Bio-Rad PROTEAN 3 cell the SDS PAGE was first run at 120 V and once the dye front reached the top of the separating gel, the voltage was increased to 180 V for ~1 hour or until the dye front reached the bottom of the gel.

2.6.5   Western Blotting

2.6.5.1   Buffers and reagents

1x Transfer buffer was made by diluting 100 ml of 10x Transfer buffer (0.25 M Tris and 1.92 M glycine) with 200 ml of methanol and 700 ml of distilled water.

Tris buffered saline – Tween 20 (TBS-T) washing buffer was made by diluting 50 ml of 20x Tris-Buffered Saline (0.5 M Tris and 3 M NaCl) and 1 ml of Tween 20 with 950 ml of distilled water.

Blocking buffer was made by dissolving 5% w/v BSA in TBS-T. 0.1% w/v sodium azide was added as a preservative. The solution was stored at 4°C. Alternatively (GPR55 analysis) blocking buffer comprised 5% w/v skimmed milk in TBS-T, which was used only once.

For ABCC1, ABCC4, ABCB1 and ABCG2 analysis, samples were run on 8% acrylamide gel and were transferred on to PVDF membrane blots (0.45 µm) (Immobilon® – P). For GPR55 analysis, samples were run on 10% acrylamide gel and were transferred on to PVDF membrane. For ERK and ERK1/2 analysis, samples were run on 8% acrylamide gel and were transferred on to nitrocellulose membrane blots (0.45 µm) (ThermoFisher Scientific).

Table 2.4. Primary antibodies used.

Antibody	Type of antibody	Working dilution in blocking buffer	Company
Primary	anti-ABCC1 (rat) – (EPR4658(2)) – C-terminal	1:1000	Abcam®
	anti-ABCC4 (rabbit) (M$_4$ I- 10)	1:100	Abcam®
	anti-ABCG2 (mouse) (Bxp21): sc-58222	1:50	Santa Cruz Biotechnology Inc.
	anti-ABCB1 (mouse)	1:200	ThermoFisher Scientific
	anti-GPR55 (rabbit)	1:100	Abcam®
	anti-p-ERK (mouse) (E-4): sc-7383	1:200	Santa Cruz Biotechnology Inc.
	anti-ERK1/2 (mouse) (E-4): sc-514302	1:100	Santa Cruz Biotechnology Inc.
	anti-α-tubulin (mouse)	1:1000	Sigma
	anti-β-actin (mouse)	1:5000	Sigma

Table 2.5. Secondary antibodies used.

Antibody	Type of antibody	Working dilution in TBS-T	Company
Secondary	anti-rat HRP	1:5000	Sigma
	anti-mouse HRP	1:4000	Cell signalling Technology®
	anti-rabbit HRP	1:3000	Cell signalling Technology®

-SuperSignal ™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific)

-Li-Cor pen

2.6.5.2   Western blot transfer and detection of proteins

Following SDS PAGE, the separating gel was removed from the stacking gel. Fibre pads, filter paper (Bio-Rad) and PVDF membrane (0.45 um) (pre-activated with methanol) were soaked in 1x transfer buffer to allow for equilibration.

After the cassette was locked, it was placed into a colour-coded electrophoresis blotting cell. The blotting cell and an ice pad was placed in the Bio-Rad PROTEAN 3 cell. The cell was then filled with 1x transfer buffer to the top and the transfer was run at 100 V for 1 hour.

After blotting, the PVDF membrane was removed from the cassette and transferred to a container of 15 ml of blocking buffer. The membrane was blocked on a rocker for 1 hour at room temperature after which the membrane in blocking buffer was left to block overnight at 4°C. The following day, the blocking buffer was replaced and the membrane was incubated in blocking buffer containing the desired diluted primary antibody for 1 hour at room temperature on a rocker or overnight at 4°C. The primary antibody was removed by washing the membrane 3 times with 15 ml of TBS-T for 5 minutes each wash. The membrane was then incubated for 1 hour at room temperature in TBS-T containing the desired diluted HRP conjugated secondary antibody. . The secondary antibody was removed by washing the membrane blot 5 times with 15 ml of TBS-T for 5 minutes each wash. The membrane blot was then incubated for 1 to 2 minutes in 1:1 mixture of peroxide to luminol from SuperSignal™ West Pico PLUS chemilunescent substrate kit (Thermo Scientific). The membrane blot was placed in the Li-Cor C-Digit blot scanner and the acquisition of images were analysis using Image Studio Lite software.

2.7   Gene Expression analyses – Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a very common technique to study gene expression. First, the RNA molecules has to be extracted and isolated from cell culture samples. After the RNA molecules are converted to their complementary DNA (cDNA) sequences by reverse transcriptase. Then the newly synthesised cDNA is amplified by quantitative polymerase chain reaction (qPCR) procedures.

2.7.1   RNA isolation

RNA was isolated from samples using Bioline ISOLATE RNA mini kit. Following the manufacturer's protocol, cells were resuspended by pipetting in 450 µl lysis Buffer R (Supplied). The cell suspension was then incubated for 3 minutes at room temperature. The supernatant was transferred to a spin column R1 placed in a collection tube and centrifuged at 10,000 x g for 2 minutes. The spin column was discarded and 1 volume of 70% ethanol (450 µl) was thoroughly mixed with the filtrate. After, the mixture was transferred to a spin column R2 in a new collection tube. 700 µl of wash buffer BR (supplied) was added to the mixture which was then centrifuged at 10,000 x g for 1 minute. This time, the collection tube

was discarded and the spin column R2 was placed in a new collection tube, centrifuged at 10,000 x g for 3 minutes. After centrifuged, the collection tube was again discarded and the spin column R2 was placed in a 1.5 ml PCR tube. To elute RNA, 50 µl of RNase/DNase-free water was added to the spin column R2 and centrifuged at 6000 x g for 1 minute. The quantity of the isolated RNA obtained was measured using NanoDrop^{TM} 1000 spectrophotometer (ThermoFisher Scientific) and samples were stored at -80°C.

2.7.2   Reverse transcription of RNA samples

The RNA samples obtained were reverse transcribed to cDNA using the Precision nanoScript ^{TM} 2 Reverse Transcription kit (Primerdesign). Following the manufacturer's protocol, 600 ng of all the RNA samples were diluted in a volume of 9 µl with RNase/DNase-free water. All samples were kept on ice. After 1 µl of Oligo-dT primer was added to each sample and then incubated at 65°C for 5 minutes on a thermostatically controlled hot block. The samples were immediately transferred to the ice and 10 µl mixture of 5 µl of nanoScript2 4X buffer, 1 ul of dNTP mix 10 mM, 3 µl of RNase/DNase free water was added to each sample. The samples were then placed in a thermocycler which was set for 20 minutes at 42°C, 10 minutes at 75°C and hold at 4°C. The cDNA samples obtained were diluted 1 in 10 with RNase/DNase-free water and stored at -20°C.

2.7.3   Quantitative polymerase chain reaction (qPCR)

The cDNA samples were amplified in qPCR using the Thermo Scientific PiKoReal 96 Real-Time PCR system. For one reaction, a master mix containing 10 µl of PrecisionPlus^{TM} 2X qPCR Mastermix with Sybr green with inert blue dye (Primerdesign), 1 µl of the reverse primer, 1 µl of the forward primer and 3 µl of RNase/DNase free water was prepared. 5 µl of cDNA were first added to each well of a 96-well Piko PCR plate in triplicates followed by 15 µl of the master mix ensuring that no bubbles are formed on the samples in the wells. A PCR film is then placed on the PCR plate which was then placed in the PikoReal 96 Real-Time PCR machine (Thermo Scientific) to determine the expression levels of the targeted genes in the samples. The expression levels were normalised to human β-actin and human GAPDH as the house keeping genes. The RNase/DNase-free water was used as the negative control. The primers used are shown in table 2.6.

The thermal cycling condition were as follows;

1.   10 minutes at 95°C.

2. 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.
3. Melt curve analysis: 5 seconds at 95°C, 60 seconds at 65°C and continuous for 97°C.
4. Cooling: 40°C for 10 seconds.

The qPCR data were analysed using the double delta Ct analysis.

Table 2.6. Primers used for qPCR.

Primers	Company	Primer sequence
Human β-actin	Invitrogen, Thermo Fisher Scientific	**F**: 5′-CTGGAACGGTGAAGGTGACA-3′ **R**: 5′-AAGGGACTTCCTGTAACAATGCA-3′
Human GAPDH	Invitrogen, Thermo Fisher Scientific	**F**: 5′-TGCACCACCAACTGCTTAGC-3′ **R**: 5′-GGCATGGACTGTGGTCATGAG-3′
Human ABCC1	Invitrogen, Thermo Fisher Scientific	**F**: 5′-CGACATGACCGAGGCTACATT-3′ **R**: 5′-AGCAGACGATCCACAGCAAAA-3′
Human ABCC4	Invitrogen, Thermo Fisher Scientific	**F**: 5′-TGTGGCTTTGAACACAGCGTA-3′ **R**: 5′-CCAGCACACTGAACGTGATAA-3′

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; F, Forward nucleotide; R, Reverse nucleotide.

2.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a plate-based assay technique used for detecting and quantifying proteins, peptides, antibodies, antigens and hormones. They can be used to detect the presence of antigens antibodies.

2.8.1 Measurement of cyclic AMP (cAMP) in conditioned medium from siRNA transfected breast cancer cells by ELISA

After the cells were transfected with siRNAs, the transfected and non-transfected cells were stimulated with 100 µM forskolin for 2 hours at 37°C. The cell culture medium was then replaced with fresh cell culture medium and 3 wells of non-transfected cells were treated with 50 µM MK571, the cells were incubated for 18 hours at 37°C. The supernatants (conditioned media) were centrifuged at 1000 x g to pellet out any floating cells and collected and stored at -80°C or used immediately for determination of cAMP by using the cAMP ELISA kit (Enzo Life Sciences Inc).The cells were also harvested for protein quantification. All reagents used were brought to room temperature for at least 30 minutes prior to opening. The cAMP standards,

non-acetylated format were prepared as shown in table 2.7. Each Standard was vortexed thoroughly.

Table 2.7. Dilutions for making cAMP standards, non-acetylated version.

Standard (Std.)	Cell culture medium Vol. (µl)	Vol. added (µl)	cAMP Conc. (pmol/ml)
1	900	100, standard stock	200
2	750	250, std. 1	50
3	750	250, std. 2	12.5
4	750	250, std. 3	3.12
5	750	250, std. 4	0.78

To determine the concentration of cAMP efflux from breast cancer cells, the cAMP ELISA was performed according to the manufacturer's instructions. Briefly, 100 µl of cell culture medium was added to the non-specific binding (NSB) and the 0 pmol/ml standard (Bo) wells. 50 µl of Assay Buffer 2 was then added to the NSB wells. After 100 µl of the standards and samples were added to the appropriate wells in duplicates. 50 µl of the blue conjugate was added to each well except the total activity (TA) and the blank wells followed by the addition of 50 µl of the yellow antibody into each wells except the blank, TA and NSB wells. The plate was then sealed and incubated at room temperature of a plate shaker at 500 rpm for 2 hours. After incubation, the contents of the wells were discarded and the wells were washed thoroughly 3 times with the wash buffer diluted in distilled water (1:20 dilution). To remove any remaining wash buffer, the plate was firmly tapped on paper towel and 5 µl of the blue conjugate was added to the TA wells followed by 200 µl of the substrate solution to each of the wells. The plate was the sealed and incubated for one hour at room temperature without shaking. After one hour incubation, 50 µl of the stop solution was added to each well to stop the reaction and the absorbance was measured at 405 nm using multiskan Go microplate reader (ThermoScientific).

Once the absorbance values were obtained, the mean optical density (OD) of each samples was calculated by subtracting the mean OD of the blank from the mean OD of each sample. The data was then input on Graphpad Prism (7.0) and the concentration of cAMP was obtained from a standard curve generated from a four parameter (4PL) logistic fitting curve as detailed by the manufacturer's instructions.

2.8.2 Measurement of prostaglandin E₂ (PGE₂) in conditioned medium from siRNA transfected breast cancer cells by ELISA

After the cells were transfected with siRNAs, the transfected and non-transfected MCF-7 and MDA-MB231 cells were stimulated with 10 µg/ml lipopolysaccharides (LPS) for 24 hours at 37°C and with 80 nM phorbol 12-myristate 13-acetate (PMA) for 1 hour at 37°C respectively. The cell culture medium was replaced with fresh cell culture medium and 3 wells of non-transfected cells were treated with 50 µM MK571, the cells were incubated for 18 hours at 37°C . The supernatants (conditioned media) were centrifuged at 1000 x g to pellet out any floating cells and collected and stored at -80°C or used immediately for determination of PGE₂ by using the PGE₂ ELISA kit (Enzo Life Sciences Inc).The cells were also harvested for protein quantification. All reagents used were brought to room temperature for at least 30 minutes prior to opening. The PGE₂ standards were prepared as shown in table 2.8. Each Standard was vortexed thoroughly.

Table 2.8. Dilutions for making PGE₂ standards.

Standard (Std.)	Cell culture medium Vol. (µl)	Vol. added (µl)	PGE₂ Conc. (pg/ml)
1	980	20, standard stock	1000
2	500	500, std. 1	500
3	500	500, std. 2	250
4	500	500, std. 3	125
5	500	500, std. 4	62.5
6	500	500, std. 5	31.25
7	500	500, std. 6	15.63
8	500	500, std. 7	7.81

To determine the concentration of PGE₂ efflux from breast cancer cells, the PGE₂ ELISA was performed according to the manufacturer's instructions. Briefly, 100 µl of cell culture medium was added to the non-specific binding (NSB) and the 0 pmol/ml standard (Bo) wells. 50 µl of the Assay Buffer was then added to the NSB wells. After 100 µl of the standards and samples were added to the appropriate wells in duplicates, following which 50 µl of the blue conjugate was added to each wells except the total activity (TA) and the blank wells followed by the addition of 50 µl of the yellow antibody into each wells except the blank, TA and NSB wells. The plate was then sealed and incubated overnight (18 - 24 hours) at 4°C. After incubation, the contents of the wells were discarded and the wells were washed thoroughly 3 times with the wash buffer diluted in distilled water (1:20 dilution). To remove any remaining wash buffer, the plate was firmly tapped on paper towel and 5 µl of the blue conjugate was added to the TA wells followed by 200 µl of the para-Nitrophenylphosphate (pNpp) substrate solution to

each of the wells. The plate was then sealed and incubated for one hour at 37°C without shaking. After one hour incubation, 50 μl of the stop solution was added to each well to stop the reaction and the absorbance was measured at 405 nm using multiskan Go microplate reader (ThermoScientific).

Once the absorbance values were obtained, the mean optical density (OD) of each sample was calculated by subtracting the mean OD of the blank from the mean OD of each sample. The data was then input on Graphpad Prism (7.0) and the concentration of PGE$_2$ was obtained from a standard curve generated from a four parameter (4PL) logistic fitting curve according to the manufacturer's instructions.

2.8.3   Measurement of sphingosine-1-phosphate (S1P) in conditioned medium from siRNA transfected breast cancer cells by ELISA

After the cells were transfected with siRNAs the cell culture medium was replaced with fresh cell culture medium and 3 wells of non-transfected cells were treated with 50 μM MK571, the cells were incubated for 18 hours at 37°C . The supernatants (conditioned media) were centrifuged at 1000 x g to pellet out any floating cells and collected and stored at -80°C or used immediately for determination of S1P by using the human S1P ELISA kit (Abbexa Ltd.).The cells were also harvested for protein quantification. The standard was reconstituted in 1 ml of the Sample/Standard diluent buffer with gentle agitation for about 15 minutes prior to carrying out serial dilution to generate the S1P standards as shown in table 2.9. Each Standard was vortexed thoroughly.

Table 2.9. Dilutions for making S1P standards.

Standard (Std.)	Sample/Standard diluent buffer Vol. (μl)	Vol. added (μl)	S1P Conc. (ng/ml)
1	300	300, standard stock	100
2	300	300, std. 1	50
3	300	300, std. 2	25
4	300	300, std. 3	12.5
5	300	300, std. 4	6.25
6	300	300, std. 5	3.125

To determine the concentration of S1P efflux from breast cancer cells, the S1P ELISA was performed according to the manufacturer's instructions. Briefly, the pre-coated ELISA plate was washed twice with the wash buffer diluted in distilled water (1:25 dilution). The remaining wash buffer was removed from the plate by firmly tapping the plate on paper towel before

which 100 μl of the prepared standards, Sample/Standard diluent buffer and samples were added into the appropriate wells. The plate was then sealed and incubated for 90 minutes at 37°C. After incubation, the contents of the wells were discarded and the wells were washed thoroughly twice with the diluted wash buffer (1:25 dilution) and tapped firmly on paper towel to remove any remaining wash buffer. 100 μl of the biotin conjugated antibody diluted in the antibody diluent buffer (1:100 dilution) was added to each well and the plate was again sealed and incubated for 60 minutes at 37°C. The plate was then washed 3 times and the wells allowed to soak in the diluted wash buffer for 1 - 2 minutes in between washes before which 100 μl of the streptavidin-HRP conjugate diluted in HRP diluent (1:100 dilution) was added to each well. The plate was again sealed and incubated for a further 30 minutes at 37°C. After incubation, the plate was washed 5 times with the diluted wash buffer and the wells were allowed to soak in the diluted wash buffer for 1 - 2 minutes in between washes. 90 μl of the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to each well and the plate was covered and incubated in the dark for 15 - 20 minutes at 37°C. When an apparent gradient can be seen in the standard wells, 50 μl of the stop solution was added to each well to stop the reaction and the absorbance was measured at 450 nm using multiskan Go microplate reader (ThermoScientific).

Once the absorbance values were obtained, the mean optical density (OD) of each sample was calculated by subtracting the mean OD of the blank from the mean OD of each sample. The data was then input on Graphpad Prism (7.0) and the concentration of S1P was obtained from a standard curve generated from a Log-log fitting curve as recommended by the manufacturer's instructions.

2.8.4   Measurement of cysteinyl (Cys) leukotrienes (LTC$_4$, LTD$_4$ and LTE$_4$) in conditioned medium from siRNA transfected breast cancer cells using ELISA

After the cells were transfected with siRNAs, the MCF-7 and MDA-MB231 cells were stimulated with 10 mM acetylsalicylic acid (aspirin) for 2 hours at 37°C after which the cell culture medium was replaced with fresh cell culture medium and 3 wells of non-transfected cells were treated with 50 μM MK571, the cells were incubated for a further 5 hours at 37°C. The supernatants (conditioned media) were centrifuged at 1000 x g to pellet out any floating cells and collected and stored at -80°C or used immediately for determination of LTC$_4$ by using the Cys LTs ELISA kit (Enzo Life Sciences Inc).The cells were also harvested for protein quantification. All reagents used were brought to room temperature for at least 30 minutes prior to opening. The Cys LTs standards, were prepared as shown in table 2.10. Each Standard was vortexed thoroughly.

Table 2.10. Dilutions for making Cys LTs standards.

Standard (Std.)	Cell culture medium Vol. (µl)	Vol. added (µl)	Cys LTs Conc. (pg/ml)
1	900	100, standard stock	2500
2	500	500, std. 1	1250
3	500	500, std. 2	625
4	500	500, std. 3	312.5
5	500	500, std. 4	156.3
6	500	500, std. 5	78.1

To determine the concentration of Cys LTs efflux from breast cancer cells, the Cys LTs ELISA was performed according to the manufacturer's instructions. Briefly, 100 µl of cell culture medium was added to the non-specific binding (NSB) and the 0 pmol/ml standard (Bo) wells. 50 µl of the Assay Buffer was then added to the NSB wells. After 100 µl of the standards and samples were added to the appropriate wells in duplicates, following which 50 µl of the blue conjugate was added to each wells except the total activity (TA) and the blank wells followed by the addition of 50 µl of the yellow antibody into each wells except the blank, TA and NSB wells. The plate was then sealed and incubated on a plate shaker (500 rpm) for 2 hours at room temperature. After incubation, the contents of the wells were discarded and the wells were washed thoroughly 3 times with the wash buffer diluted in distilled water (1:20 dilution). To remove any remaining wash buffer, the plate was firmly tapped on paper towel and 5 µl of the blue conjugate diluted in the Assay Buffer (1:10 dilution) was added to the TA wells followed by 200 µl of the pNpp substrate solution to each of the wells. The plate was then sealed and incubated for two hour at 37°C without shaking. After two hours incubation, 50 µl of the stop solution was added to each well to stop the reaction and the absorbance was measured at 405 nm using multiskan Go microplate reader (ThermoScientific).

Once the absorbance values were obtained, the mean optical density (OD) of each sample was calculated by subtracting the mean OD of the blank from the mean OD of each sample. The data was then input on Graphpad Prism (7.0) and the concentration of Cys LTs was obtained from a standard curve generated from a four parameter (4PL) logistic fitting curve as recommended by the manufacturer's instructions.

2.9   Statistical analysis

Statistical analysis of data was carried out using GraphPad Prism (7.0). For multiple comparisons with one independent variables, a one-way ANOVA was used with Dunnett's

post-hoc test. For multiple comparisons with two independent variables, a two-way ANOVA was used with Dunnett's post-hoc test. A value of $p < 0.05$ was considered significant.

Chapter 3 – Analysis of the effects of small molecule ABC inhibitors on breast cancer cell lines *in vitro*

3.1 Objectives

In order to first assess the possible role of ABCC1 and ABCC4 in breast cancer biology in terms of cell proliferation and migration, a series of small molecule ABC inhibitors were selected as shown in table 3.1. These ABC inhibitors were used to test their effect on cell proliferation and migration of the cancer cells.

Table 3.1. Small molecule ABC inhibitors used in this study

Inhibitors	Inhibits ABCC1	Inhibits ABCC4	supplier	Reference
MK571	✓	✓	Sigma® life science	Tivnan et al., 2015
Indomethacin	X	✓	Sigma® life science	Bai, et al., 2004
Reversan	✓	X	Tocris bioscience	Tivnan et al., 2015
Ceefourin 1	X	✓	Abcam Biochemicals®	Cheung, et al., 2014
Ceefourin 2	X	✓	Abcam Biochemicals®	Cheung, et al., 2014

The concentrations of each ABC inhibitors where based upon commonly used values in the literature (Fujino et al., 2007; Koley et al., 2012; Barrington et al., 2015) and in our lab. The ABC inhibitors were first diluted in dimethyl sulfoxide (DMSO) to produce a stock solution and after serial diluted with water to the working concentrations so as to obtain a less than 1% concentration of DMSO in growth medium so as to ensure no toxic effect attributed from DMSO. Therefore, no vehicle (DMSO) control was tested.

The breast cancer cell lines used were MCF-7 and MDA-MB231 breast cancer cells. Both are invasive, however, MCF-7 is considered to be luminal-type breast cancer with progesterone, oestrogen and human epidermal growth factor 2 (HER2) receptor positive while MDA-MB231 is consider to be basal-type breast cancer with progesterone, oestrogen and HER2 receptors negative.

As a positive control cancer cell line, the HeLa cervical cancer cell line was used as it was the first cultured cancer cell line used in laboratories to test the therapeutic efficacy of anticancer agents in cancers and to study the biology of cancer. Furthermore, various cancer cell lines have been derived from the HeLa cervical cancer cell line (Gillet et al., 2013). It was previously established in our lab that HeLa cells expressed both ABCC1 and ABCC4.

This chapter described the effect of the various ABC inhibitors on HeLa, MCF-7 and MDA-MB231 cancer cell lines with respect to cell proliferation and migration. To assess the effect of the various ABC inhibitors on cell proliferation we performed the colony formation assay and the MTT assay as well as the trypan blue growth assay. The scratch assay was performed to assess the cancer cell migration.

3.2　Determining the expression levels of ABC transporters by Western blot

The ABCB1 (P-glycoprotein), ABCC1 (MRP1) and ABCG2 are the most well studied members of the ABC transporters and are involved in multidrug resistance in a number of drug resistance tumours (Kovalev, et al., 2013; Begicevic et al., 2017). Together with ABCC4, these proteins also transport a variety of physiological, active substrates that may be implicated in cancer cell biology (Fletcher et al., 2010; Adamska et al., 2017).

Therefore, the expression levels of ABCB1, ABCC1, ABCC4 and ABCG2 were determined in HeLa, MCF-7 and MDA-MB231 cells by western blotting. Membrane protein samples were obtained by using the nitrogen cell cavitation and differential centrifugation, whereas the whole cell lysate samples were extracted in lysis buffer containing detergent. Figures 3.1 and 3.2 show the expression levels of the proteins in the membrane protein samples and in the whole cell lysate samples respectively.
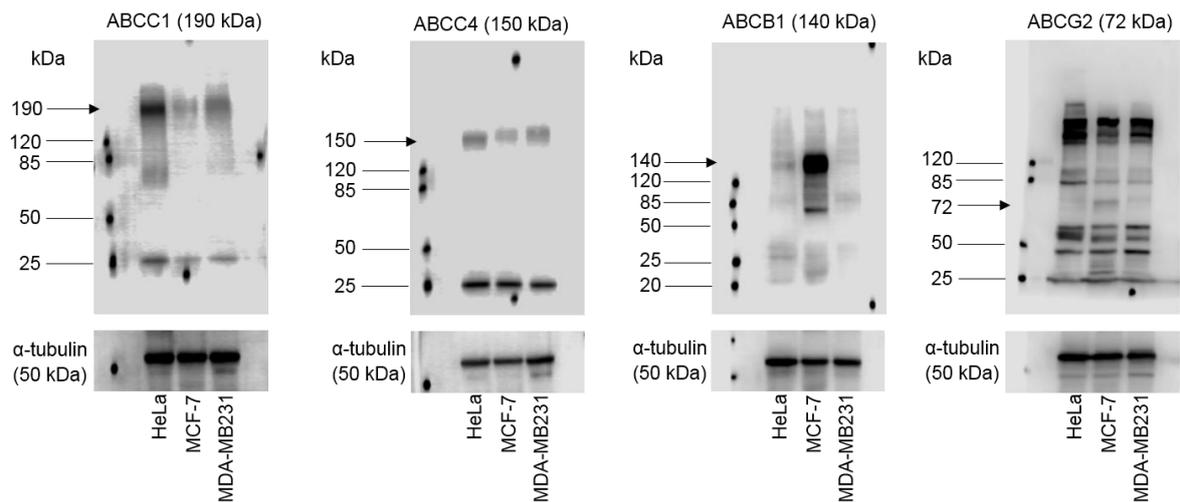
Figure 3.1. Western blot analysis of ABCC1, ABCC4, ABCB1 and ABCG2 protein expression derived from whole cell lysate samples from HeLa, MCF-7 and MDA-MB231 cell lines. 80 µg of total protein was loaded in each well and α-tubulin was used as the loading control. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa). The PVDF membrane blots for ABCC1, ABCC4, ABCB1 and ABCG2 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer), anti-ABCC4 derived in rat (1:100 in blocking buffer), anti-ABCB1 derived in mouse (1:200 in blocking buffer) and anti-ABCG2 derived in mouse (1:50 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T), anti-rat HRP (1:5000 in TBS-T) and anti-mouse HRP (1:4000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. Western blot analysis was repeated at least 3 times and similar protein expression levels were obtained.

Figure 3.2. Western blot analysis of ABCC1, ABCC4, ABCB1 and ABCG2 protein expression derived from membrane protein samples from HeLa, MCF-7 and MDA-MB231 cell lines. 80 µg of total protein was loaded in each well and α-tubulin used as the loading control. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa). The PVDF membrane blots for ABCC1, ABCC4, ABCB1 and ABCG2 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer), anti-ABCC4 derived in rat (1:100 in blocking buffer), anti-ABCB1 derived in mouse (1:200 in blocking buffer) and anti-ABCG2 derived in mouse (1:50 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T), anti-rat HRP (1:5000 in TBS-T) and anti-mouse HRP (1:4000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. Western blot analysis was repeated at least 3 times and similar protein expression levels were obtained

The expression level of ABCC1 and ABCC4 is highest in HeLa cells followed by MDA-MB231 cells and MCF-7 cells (Figure 3.1 and 3.2). High expression level of ABCB1 was only seen in MCF-7 whole cell lysate sample (Figure 3.1). However, no expression of ABCB1 was detected in HeLa and MDA-MB231 cells. Moreover, ABCG2 was only detected in MCF-7 cells with high expression level found in the MCF-7 membrane protein sample (Figure 3.1 and 3.2).

Since these experiments were looking at native expression levels within cancer cell lines, the relative expression of these proteins may be very small and thus hard to detect. However, the experiments were repeated at least 3 times and the results obtained were reproducible as confirmed by densitometry (Data not shown), it is suggested that different cancer cell lines express different levels of ABC transporters.

3.3   Effects of small molecule ABC inhibitors on cancer cell colony formation

The colony formation assay, is an *in vitro* cell survival assay that determines the ability of a cell to grow in to a colony after being treated with cytotoxic agents or any kind of inhibitors. The cancer cells were exposed to the inhibitors to examine the ability of cells to proliferate from a single cell to form at least 50 cells clustered together which was considered to be a colony. Various concentrations of ABC inhibitors were used. After 7 days of exposure of cells to the ABC inhibitors, the cells were fixed with 4% w/v PFA and after stained with 0.1% w/v crystal violet. The plates were then washed thoroughly with water and left at room temperature to air dry. Purple spots of colonies could be visually seen with the naked eye and were manually counted (Figure 3.3). A graph of number of colonies formed for each cell lines were plotted against the ABC inhibitors used as shown in figures 3.4, 3.5 and 3.6. The untreated group is referred to cells not exposed to any inhibitors or vehicle (DMSO).

As can be seen in figures 3.4A and 3.6A, the growth of HeLa and MDA-MB231 cells was significantly inhibited by 100µM of MK571, an inhibitor of both ABCC1 and ABCC4. However MK571 did not inhibit the growth of MCF-7 cells (Figure 3.5A). Furthermore, treatment with Reversan, an ABCC1 inhibitor shows a decrease in colony formation in a concentration-dependent manner in all three cell lines (Figures 3.4C, 3.5C and 3.6C). Although Reversan was originally reported as an ABCC1 specific inhibitor (Burkhart et al., 2009, Henderson et al., 2011) it has since been shown to also inhibit ABCB1 (Tivnan et al., 2015) In contrast, indomethacin, Ceefourin 1 and Ceefourin 2 which all are ABCC4 inhibitors did not significantly inhibit cell growth in HeLa cancer cell lines (Figure 3.4B, 3.4D and 3.4E), MCF-7 cancer cell lines (Figure 3.5B, 3.5D and 3.5E) or MDA-MB231 cancer cell line (Figures 3.6B, 3.6D and 3.6E).
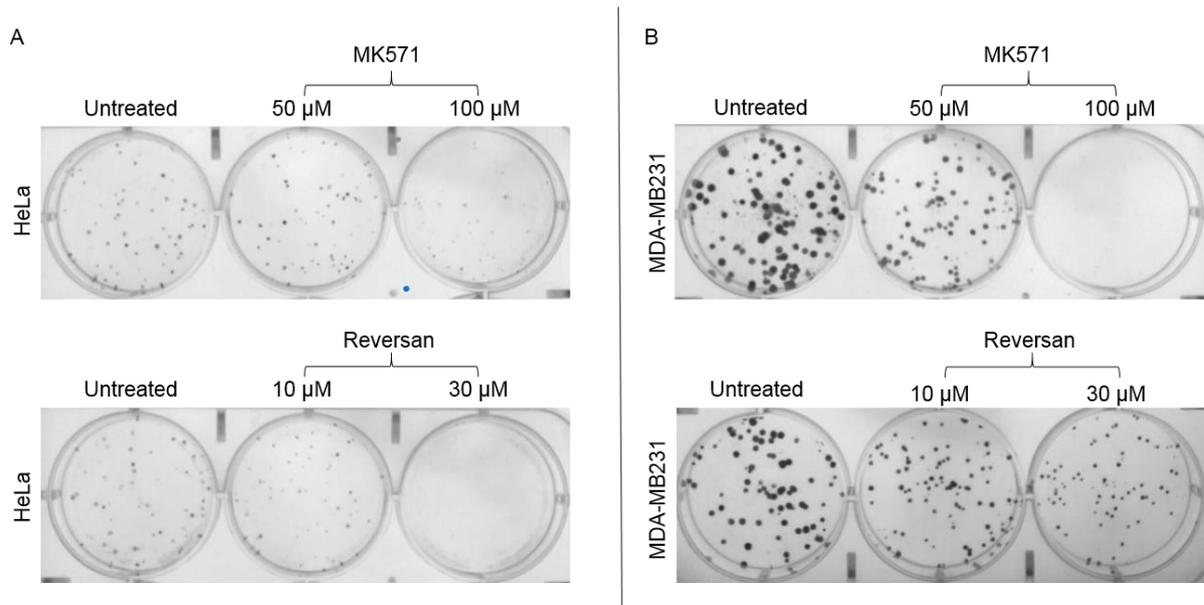
Figure 3.3. The effect of ABCC1 and ABCC4 inhibitors on the colony formation. Representative images of colony formation assay wells for (A) HeLa cells and (B) MDA-MB231 cells treated with MK571 (50 μM and 100 μM) or Reversan (10 μM and 30 μM) (Scale - each well was 34.8 mm diameter).
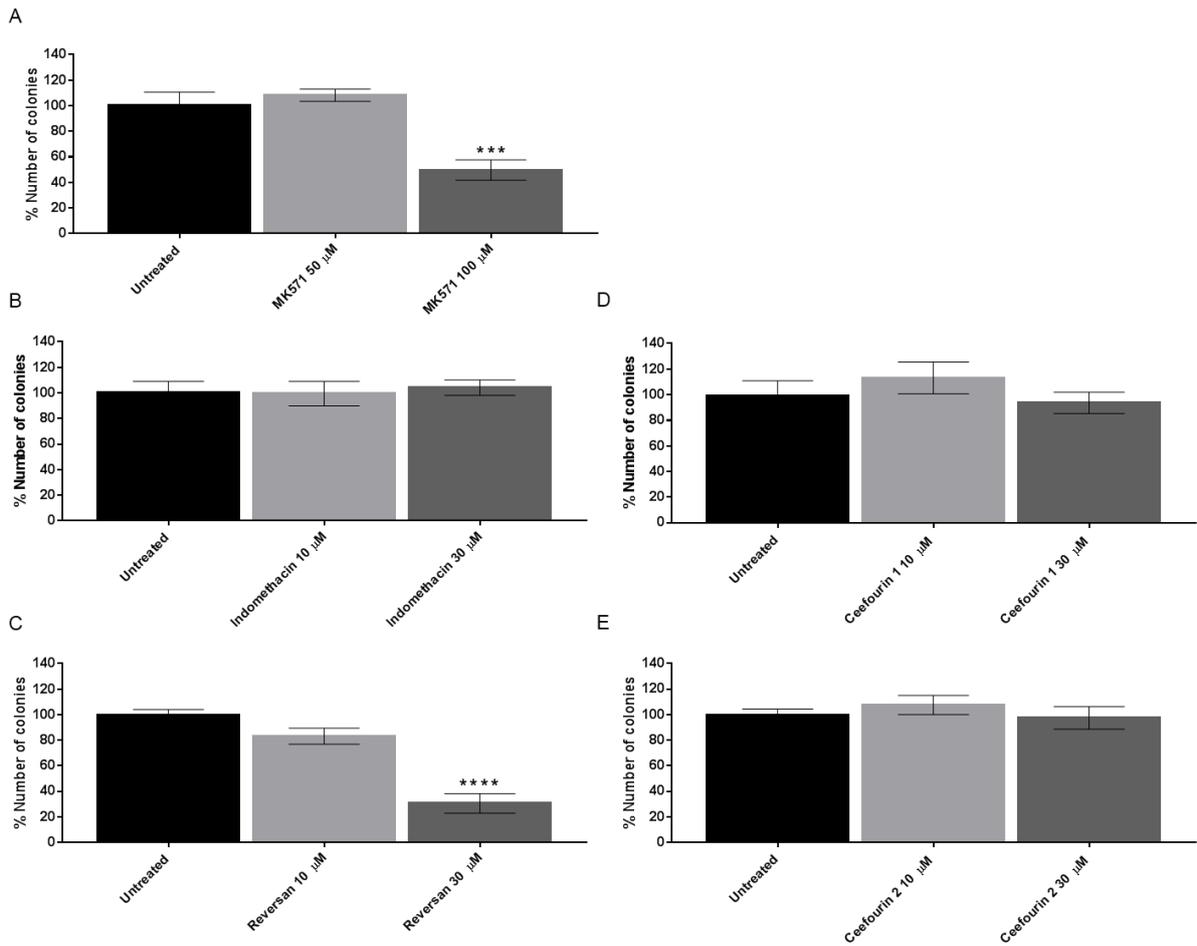
Figure 3.4. The impact of ABCC1 and ABCC4 inhibitors on the number of colonies formed in HeLa cancer cell line. The HeLa cells were treated with (A) MK571 (50 µM and 100 µM), (B) Indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (D) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM). Cells were incubated at 37°C for 7 days after which were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and were counted manually. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. *** $P < 0.001$ and **** $P < 0.0001$. P values were derived from one-way ANOVA vs untreated.

Figure 3.5. The impact of ABCC1 and ABCC4 inhibitors on the number of colonies formed in MCF-7 cancer cell line. The MCF-7 cells were treated with (A) MK571 (50 μM and 100 μM), (B) Indomethacin (10 μM and 30 μM), (C) Reversan (10 μM and 30 μM), (D) Ceefourin 1 (10 μM and 30 μM) and (E) Ceefourin 2 (10 μM and 30 μM). Cells were incubated at 37°C for 7 days after which were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and were counted manually. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. * *P* < 0.05 and *** *P* < 0.001. *P* values were derived from one-way ANOVA vs untreated.
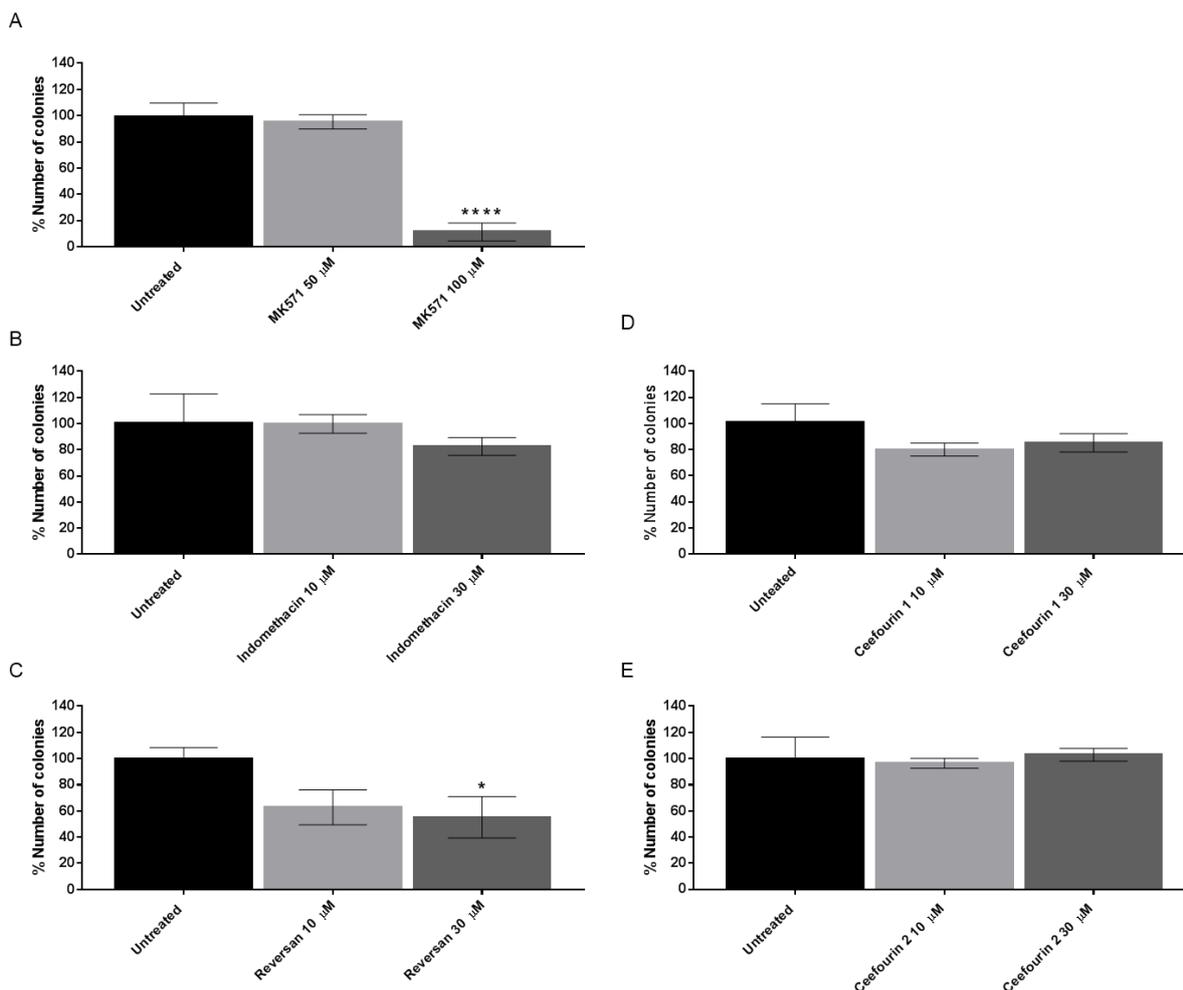
Figure 3.6. The impact of ABCC1 and ABCC4 inhibitors on the number of colonies formed in MDA-MB231 cancer cell line. The MDA-MB231 cells were treated with (A) MK571 (50 µM and 100 µM), (B) Indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (D) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM). Cells were incubated at 37°C for 7 days after which were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and were counted manually. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. * $P < 0.05$ and **** $P < 0.0001$. P values were derived from one-way ANOVA vs untreated.

In addition, I observed that some ABC inhibitors, in particular, MK571 and Reversan affected not just the number of colonies but also the size of colonies formed. Therefore, the size of colonies formed was measured by using Image J. Briefly, images of each well were selected on Image J and inverted. The size of the bright spots which represented the colonies were measured from the inverted images. The results showed a concentration-dependent decrease in colony size for HeLa (Figure 3.7A and 3.7C), MCF-7 (Figure 3.8A and 3.8C) and MDA-MB231 cells (Figure 3.9A and 3.9C) treated with MK571 or Reversan. However, no significant reduction in the size of colonies was seen in HeLa (Figure 3.7B, 3.7D and 3.7E), MCF-7 (Figure 3.8B, 3.8D and 3.8E) and MDA-MB231 (Figure 3.9B, 39.D and 3.9E) cancer cell lines

after treatment with indomethacin, Ceefourin 1 or Ceefourin 2 when compared to the untreated cells (No inhibitor or vehicle added).
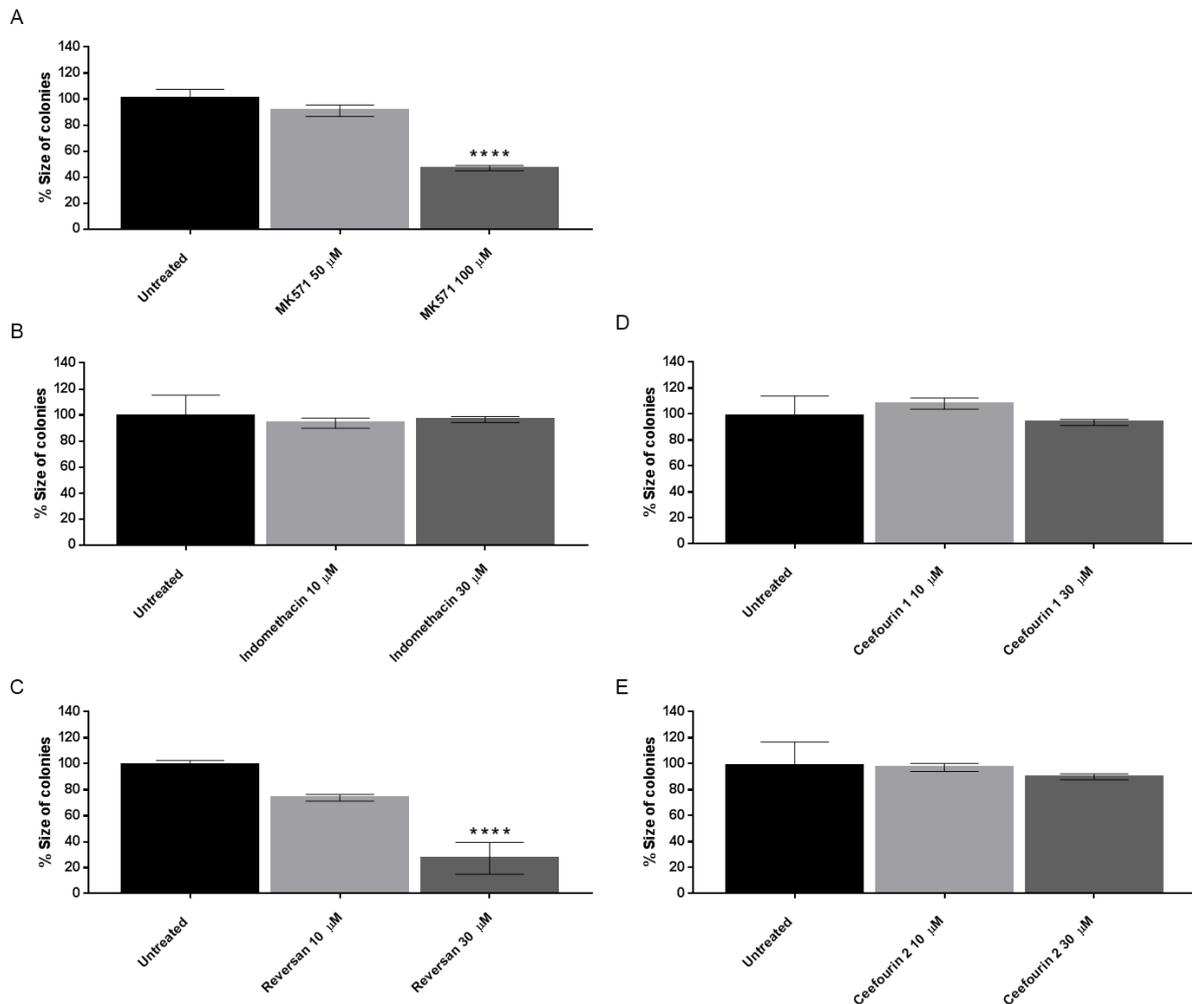
Figure 3.7. The impact of ABCC1 and ABCC4 inhibitors on the size of colonies formed in HeLa cancer cell line. The HeLa cells were treated with (A) MK571 (50 µM and 100 µM), (B) Indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (d) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM). Cells were incubated at 37°C for 7 days after which were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and images of the wells were taken and the size of colonies were analysed using Image J. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. **** $P < 0.0001$. P values were derived from one-way ANOVA vs untreated.

Figure 3.8. The impact of ABCC1 and ABCC4 inhibitors on the size of colonies formed in MCF-7 cancer cell line. The MCF-7 cells were treated with (A) MK571 (50 µM and 100 µM), (B) Indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (D) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM). Cells were incubated at 37°C for 7 days after which were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and images of the wells were taken and the size of colonies were analysed using Image J. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. * $P < 0.05$. P values were derived from one-way ANOVA vs untreated.

Figure 3.9. The impact of ABCC1 and ABCC4 inhibitors on the size of colonies formed in MDA-MB231 cancer cell line. The MDA-MB231 cells were treated with (A) MK571 (50 μM and 100 μM), (B) Indomethacin (10 μM and 30 μM), (C) Reversan (10 μM and 30 μM), (D) Ceefourin 1 (10 μM and 30 μM) and (E) Ceefourin 2 (10 μM and 30 μM). Cells were incubated at 37°C for 7 days after which were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and images of the wells were taken and the size of colonies were analysed using Image J. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. **** $P < 0.0001$. P values were derived from one-way ANOVA vs untreated.

3.4 Effects of small molecule ABC inhibitors on cell viability using MTT assay

To determine whether ABC inhibitors cause direct cytotoxicity to cells which may eventually lead to cell death, the MTT assay was performed. The results are as shown in figures 3.10, 3.11 and 3.12.

Upon addition of MTT reagent to cells, the MTT reagent is converted into a purple coloured formazan product by viable cells with active metabolism. Therefore, when cells die, the conversion of MTT into formazan is no longer apparent, thus the colour formation serves as a convenient and useful indicator of only viable cells. However, the exact cellular mechanism of MTT reduction into formazan is still not well understood but may involve the NAD(P)H-dependent oxidoreductases (Berridge et al., 2005).
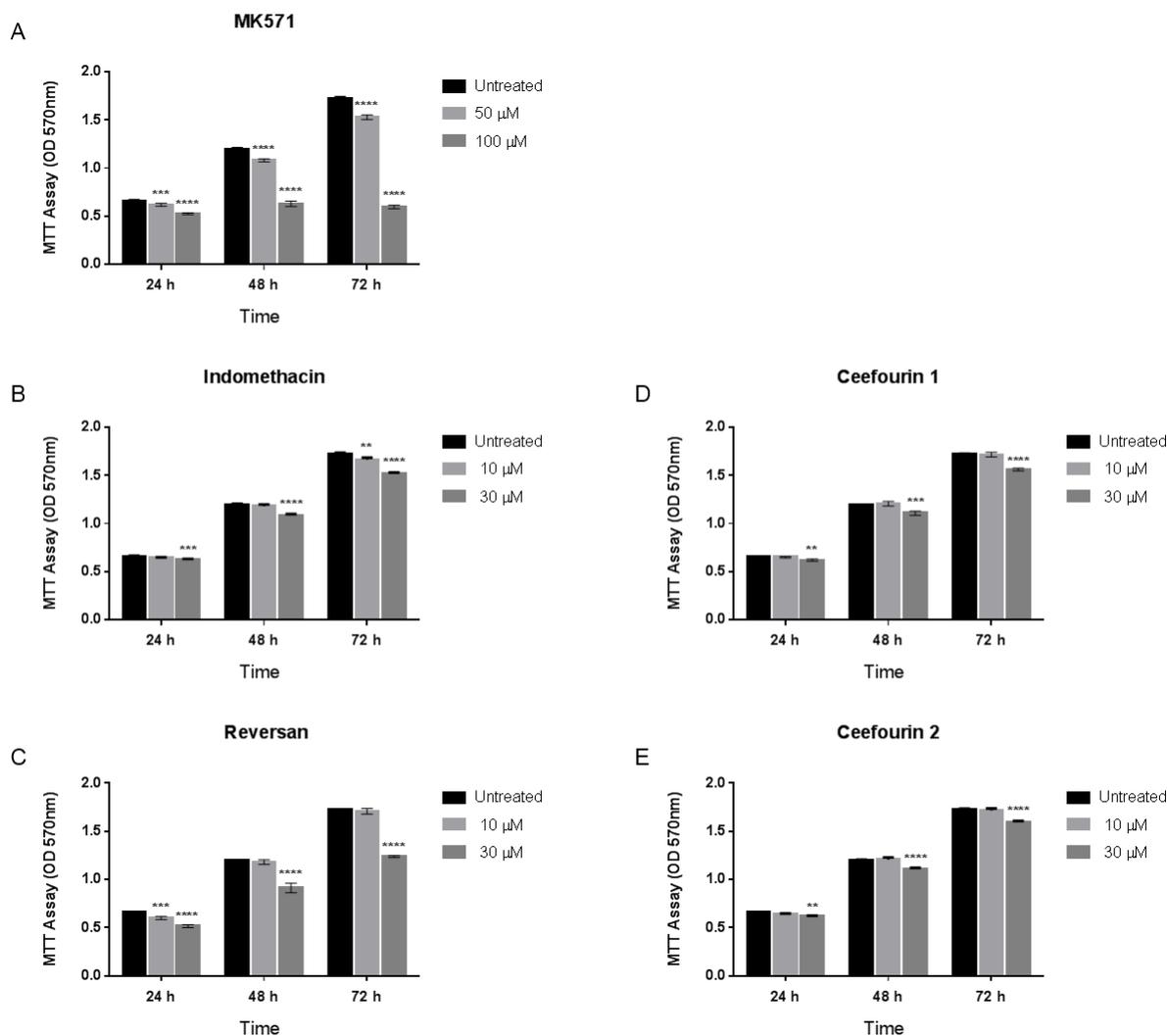
Figure 3.10. Cell viability of HeLa cells in the presence of ABC inhibitors. HeLa cells were seeded in 24-well plates at a seeding density of 1.5 x 10^4 cell/well. The cell viability was assessed at 24-hourly intervals for 72 hours after addition of ABC inhibitors. Briefly, was added to each well. After each specific time point, 40 µl of MTT reagent (5 mg/ml) was added to the cells and left to incubate for a further 1 hour, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. (A) MK571 (50 µM and 100 µM), (B) indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (D) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM) compared to untreated HeLa cells. Data represent the mean ± standard error of the mean from at least three independent

experiments. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. P values were derived from two-way ANOVA vs untreated.

As can be seen from figure 3.10A, the ability of HeLa cells to reduce MTT was significantly decreased when HeLa cells were exposed to 50 µM and 100 µM MK571 for 24, 48 and 72 hours. This may indicate a reduction in cell viability. For other ABC inhibitors, a reduction in cell viability was seen when HeLa cells were exposed to 30 µM indomethacin (figure 3.10B), 30 µM Reversan (figure 3.10C), 30 µM of ceefourin 1 (Figure 3.10D) and 30 µM ceefourin 2 (figure 3.10E) for 24, 48 and 72 hours.
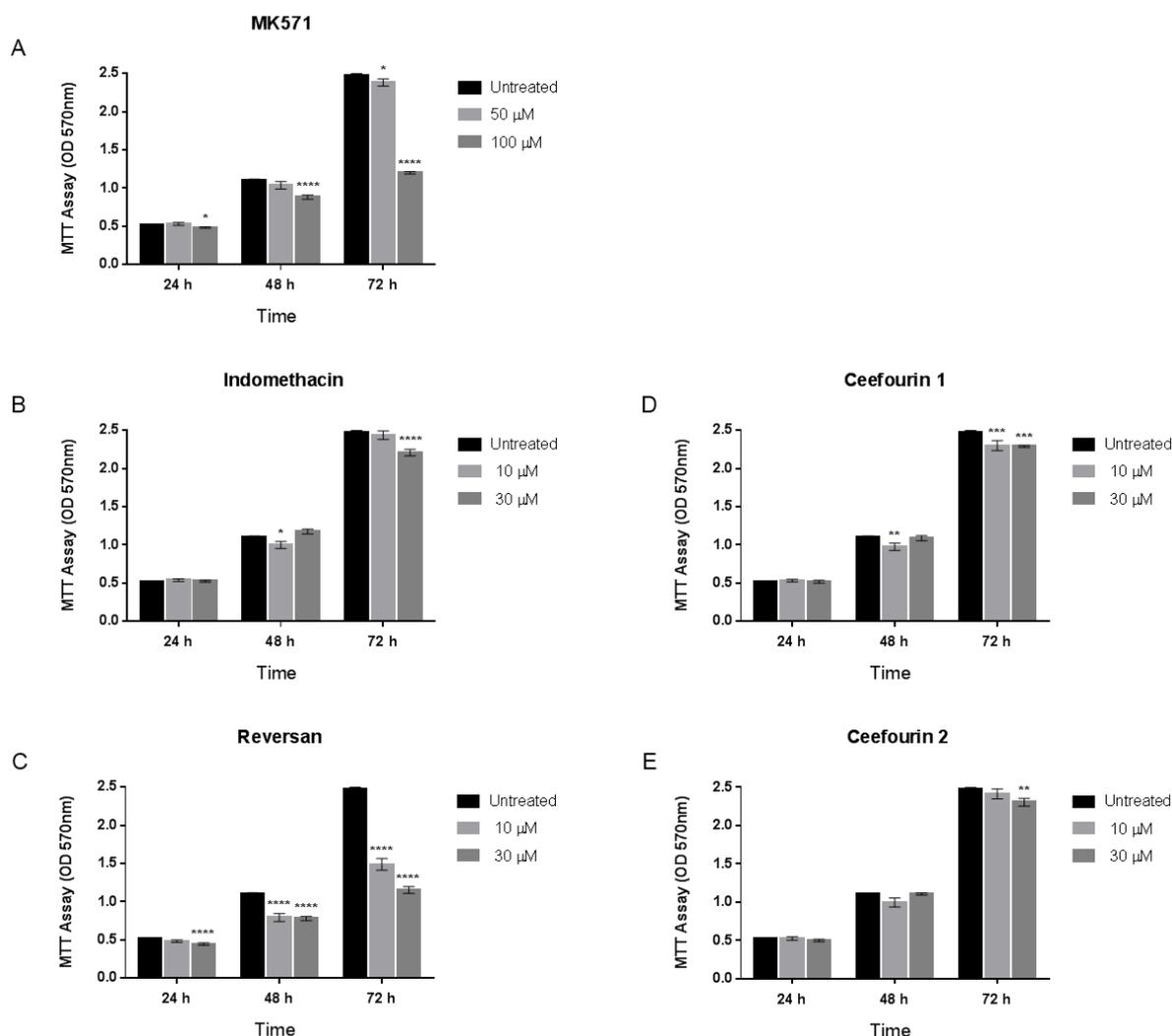
Figure 3.11. Cell viability of MCF-7 cells in the presence of ABC inhibitors. MCF-7 cells were seeded in 24-well plates at a seeding density of 1.5 x 10^4 cell/well. The cell viability was assessed at 24-hourly intervals for 72 hours after addition of ABC inhibitors. Briefly, was added to each well. After each specific time point, 40 µl of MTT reagent (5 mg/ml) was added to the cells and left to incubate for a further 1 hour, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. **(**A) MK571 (50 µM and 100 µM), (B) indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (D) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM) compared to untreated MCF-7 cells. Data represent the mean ± standard error of the mean from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. P values were derived from two-way ANOVA vs untreated.

In MCF-7 cells, after exposure to 100 µM MK571 for 48 and 72 hours, the cell viability was significantly reduced (Figure 3.11A). After exposure of 30 µM indomethacin for 72 hours, a significant decrease in cell viability was recorded (Figure 3.11B). Furthermore, 10 µM and 30 µM Reversan decreased the ability of MCF-7 cells to reduce MTT, after both 48 and 72 hours exposure (Figure 3.11C).

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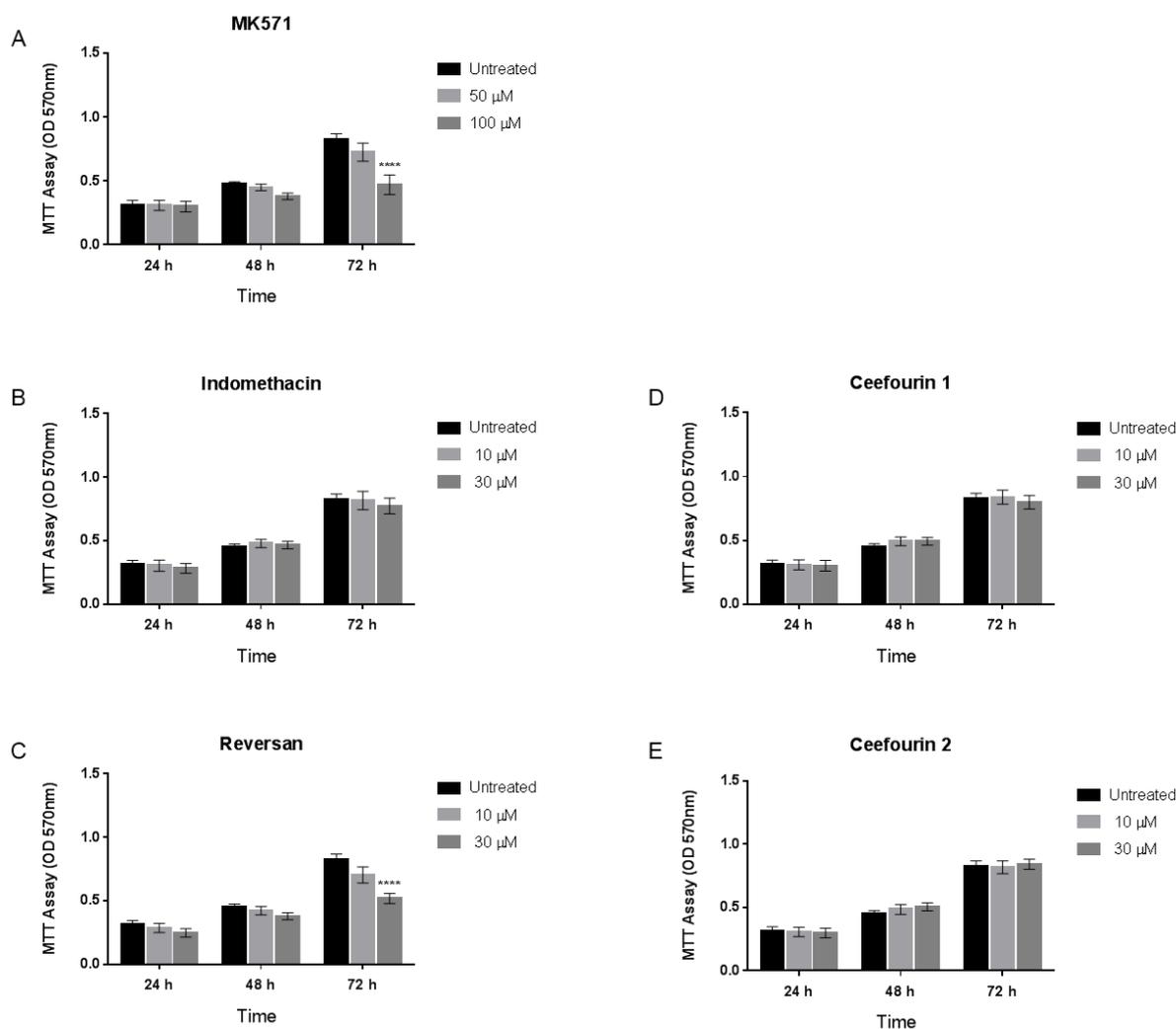
Figure 3.12. Cell viability of MDA-MB231 cells in the presence of ABC inhibitors. MDA-MB231 cells were seeded in 24-well plates at a seeding density of 6 x 10^3 cell/well. The cell viability was assessed at 24-hourly intervals for 72 hours after addition of ABC inhibitors. Briefly, was added to each well. After each specific time point, 40 µl of MTT reagent (5 mg/ml) was added to the cells and left to incubate for a further 1 hour, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. **(**A) MK571 (50 µM and 100 µM), (B) indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (D) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM) compared to untreated MDA-MB231 cells. Data represent the mean ± standard error of the mean from at least three independent experiments. **** *P* < 0.0001. *P* values were derived from two-way ANOVA vs untreated.

When 1.5 x 10^4 MDA-MB231 cells were seeded per well of 24-well plate, 100% confluency was observed after 24 hours. Therefore, a lower cell seeding density of 6 x 10^3 cells was trialled.

Consequently, when MDA-MB231 cells were exposed to 100 µM MK571 and 30 µM Reversan for 72 hours, a decrease in cell viability was recorded (Figures 3.12A and 3.12B respectively). However, when MDA-MB231 cells were exposed to indomethacin (Figure 2.12B), Ceefourin 1 (Figure 3.12D) and Ceefourin 2 (Figure 3.12E), the cell viability was not affected when compared to the untreated cells.

When comparing the results from all cell lines, exposure to MK571 or Reversan appear to affect cell viability for all three cell lines. However exposure to ABCC1 inhibitors, indomethacin, Ceefourin 1 and Ceefourin 2 only significantly affected cell viability of HeLa and MCF-7 cells at the 72 hour time point and did not affect MDA-MB231 cell at all. The long exposure time with the ABC inhibitors may cause cell cytotoxicity. Other possible issues with the longer incubation times include, the cells may have passed their log phase growth and the cells reach confluency, the cell metabolism may slow down thus may affect the rate of MTT reduction. Hence the linearity between absorbance and cell number may no longer exist. Furthermore with a longer incubation time, other changes in the culture conditions such as a reduction in essential nutrients such as glucose, or a change in pH of the culture medium may affect the ability of cells to reduce MTT (Riss et al., 2016). Subsequently, the cells ability to reduce MTT after exposure to ABC inhibitors were assessed at lower time points of 6 and 12 hours. The results are as shown in figures 3.13, 3.14 and 3.15.
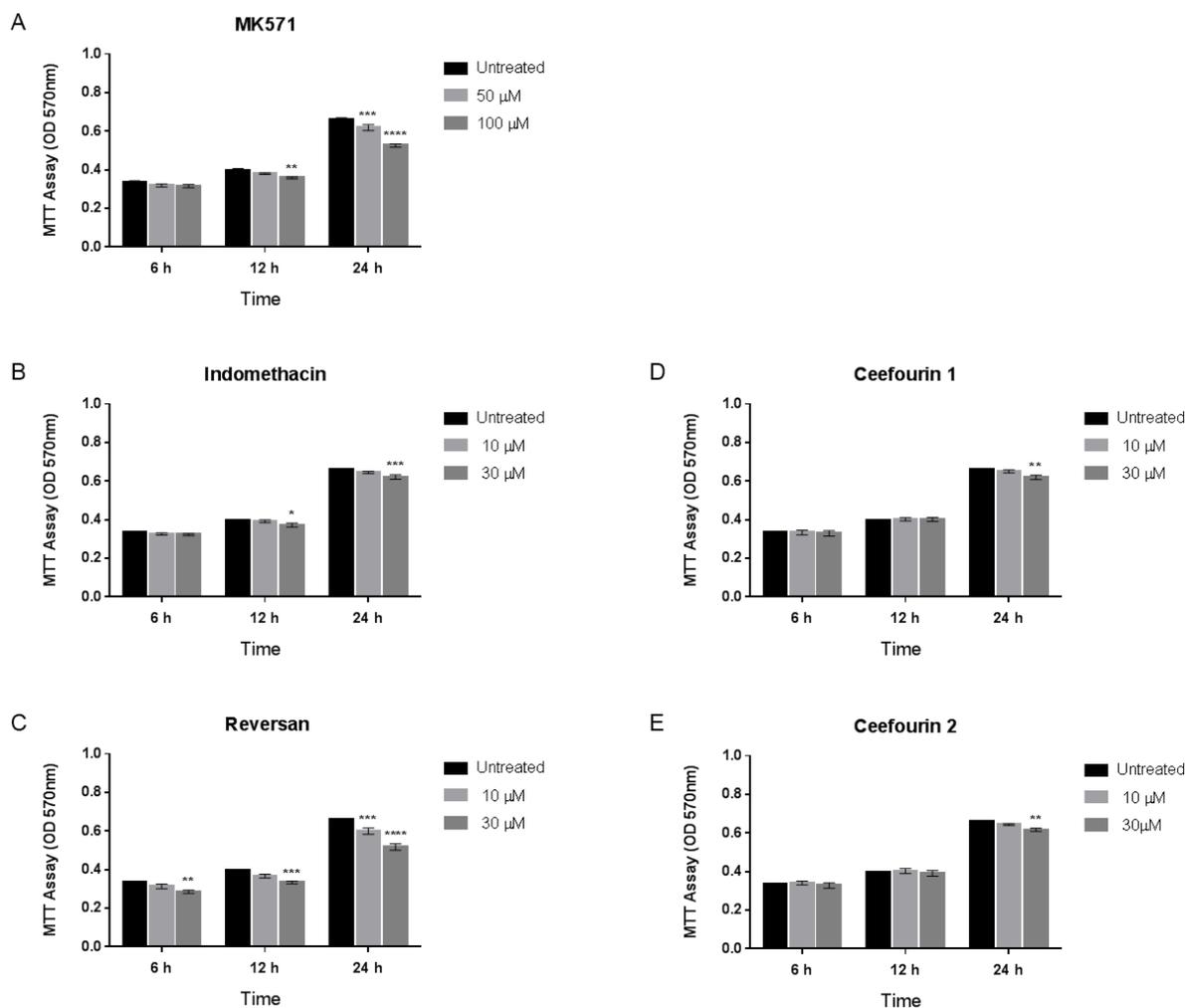
Figure 3.13. Cell viability of HeLa cells in the presence of ABC inhibitors for shorter periods of time. HeLa cells were seeded in 24-well plates at a seeding density of 1.5×10^4 cell/well. The cell viability was assessed at 6, 12 and 24 hours after addition of ABC inhibitors. After each specific time point, 40 µl of MTT reagent (5 mg/ml) was added to the cells and left to incubate for a further 1 hour, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. **(**A) MK571 (50 µM and 100 µM), (B) indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (D) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM) compared to untreated HeLa cells. Data represent the mean ± standard error of the mean from at least three independent experiments. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. P values were derived from two-way ANOVA vs untreated.

As can be seen from figure 3.13A, when HeLa cells were exposed to 100 µM MK571 for 12 hours, significant reduction in cell viability was observed. Similar effect was seen when HeLa cells were exposed to 30 µM indomethacin for 12 hours (Figure 3.13B). Furthermore, Reversan at a concentration of 30 µM, affected HeLa cell viability after 6 and 12 hours treatment (Figure 3.13C). However, exposure to Ceefourin 1 and Ceefourin 2 for 6 and 12 hours, did not affect HeLa cell viability (Figures 3.13D and 3.13E respectively).
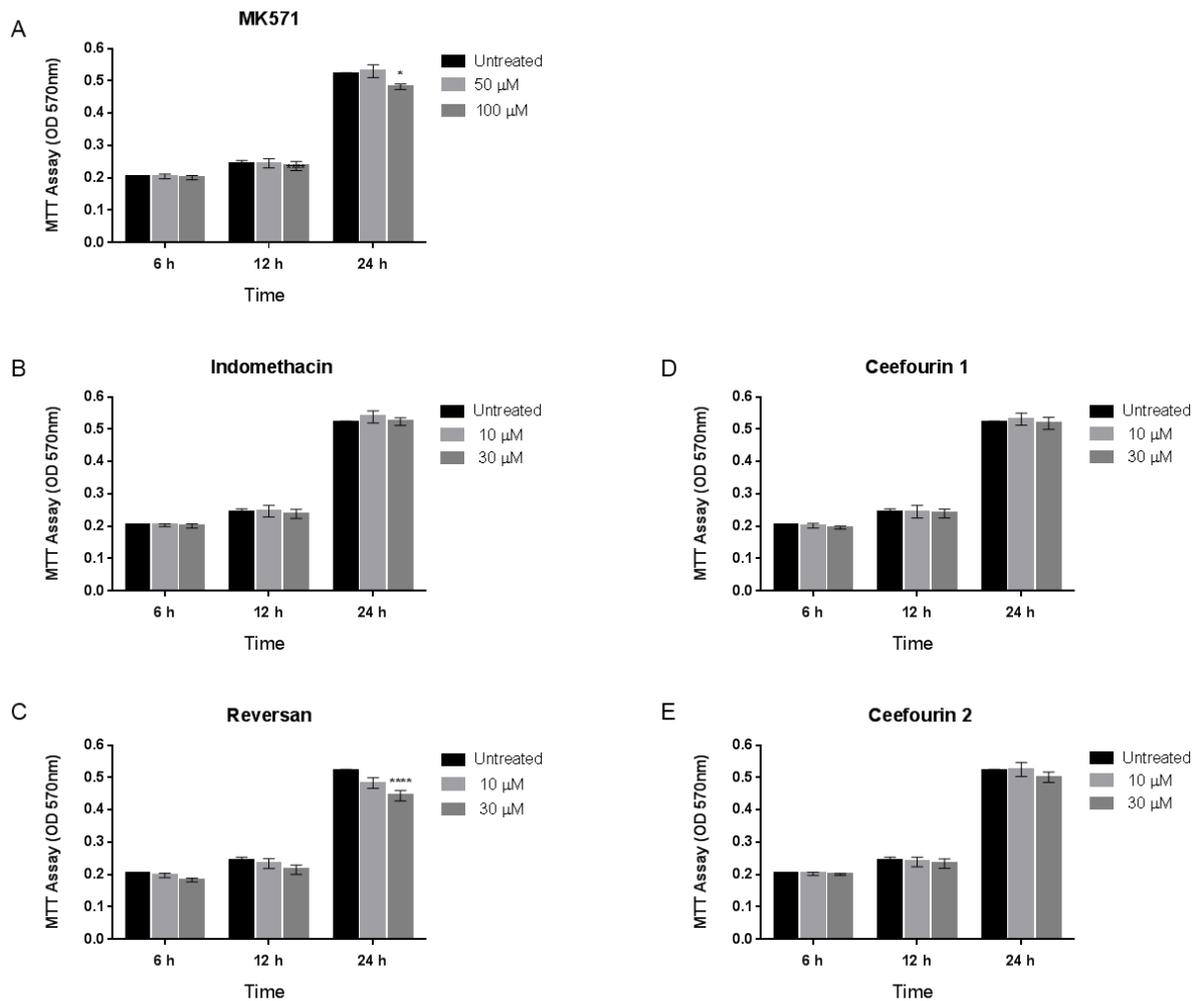
Figure 3.14. Cell viability of MCF-7 cells in the presence of ABC inhibitors for shorter periods of time. MCF-7 cells were seeded in 24-well plates at a seeding density of 1.5 x 10^4 cell/well. The cell viability was assessed at 6, 12 and 24 hours after addition of ABC inhibitors. After each specific time point, 40 µl of MTT reagent (5 mg/ml) was added to the cells and left to incubate for a further 1 hour, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. **(**A) MK571 (50 µM and 100 µM), (B) indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (D) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM) compared to untreated MCF-7 cells. Data represent the mean ± standard error of the mean from at least three independent experiments. * $P < 0.05$ and **** $P < 0.0001$. P values were derived from two-way ANOVA vs untreated.

Figure 3.15. Cell viability of MDA-MB231 cells in the presence of ABC inhibitors for shorter periods of time. MDA-MB231 cells were seeded in 24-well plates at a seeding density of 6 x 10^3 cell/well. The cell viability was assessed at 6, 12 and 24 hours after addition of ABC inhibitors. After each specific time point, 40 µl of MTT reagent (5 mg/ml) was added to the cells and left to incubate for a further 1 hour, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. **(**A) MK571 (50 µM and 100 µM), (B) indomethacin (10 µM and 30 µM), (C) Reversan (10 µM and 30 µM), (D) Ceefourin 1 (10 µM and 30 µM) and (E) Ceefourin 2 (10 µM and 30 µM) compared to untreated MDA-MB231 cells.

Exposure of ABC inhibitors to MCF-7 and MDA-MB231 cells did not affect cell viability either at 6 or 12 hour time points as compared to the untreated cells (Figure 3.14 and Figure 3.15). Therefore, at a shorter time scale the ABC inhibitors did not restrict the ability of cells to reduce MTT thus did not affect cell viability. Hence at a shorter time scale the ABC inhibitors were suggested to be nontoxic to these cells.

The MTT assay is an enzyme-based colourimetric method used as a measure of cell viability which relies on a reductive colouring reagent and dehydrogenase in a viable cell. Therefore the MTT assay only account for metabolically active viable cells (Riss et al., 2016). Moreover, the reduction of MTT by the viable cells, produce insoluble formazan product that precipitate inside as well as near the cell surface and also in the cell culture medium. Therefore, in order to solubilise the formazan product to read absorbance on a plate reader, the cell culture medium must first be completely removed and DMSO added to dissolve the formazan product (Yadav et al., 2014; Riss et al., 2016). However, when pipetting out cell culture medium from each well, complete removal of cell culture medium may not be attain and the amount of cell culture medium left in the wells may vary from well to well. Moreover, the results of the MTT assay only account for viable cells and may not specifically account for cell proliferation as an unviable cell may still proliferate (Riss et al., 2016). Thus the reading obtained may not be accurate. Hence, in order to validate the results from the MTT assay, the trypan blue dye exclusion assay was carried out.

Basically, trypan blue is a negatively charged molecules that only interacts with cells with damaged membranes and not cells with undamaged membranes. Therefore, cells with damaged membranes are usually dead cells will take up trypan blue. As a result, trypan blue stains dead cells, but is excluded from healthy cells (Tran et al., 2011; Aslanturk, 2018). Moreover, when performing the trypan blue dye exclusion assay, certain factors must be considered such as some cells that have been damaged by cytotoxic agents may have their membrane intact for several days before the membrane is lost, hence within that time interval the lethally damaged cell may continue to proliferate. Consequently this is consider to be one of the downside of trypan blue staining is that it cannot be used to determine between the healthy viable cells from the cells that are alive but lost their cell functions (Aslanturk, 2018). Nevertheless, the trypan blue dye exclusion assay is an inexpensive straight forward assay and the results confirmed the same pattern of cell growth/viability as shown from the MTT assay (Data not shown).

3.5   Effects of small molecule ABC inhibitors on cell migration

One of the straightforward cell migration assays to study cancer cell migration in response to a variety of experimental conditions, is the scratch assay (Kramer et al., 2013). Simply cells were seeded a day before the assay to obtained 100% confluency. A gap was carefully created in the cell monolayer using with a 10 µl pipette tip and the cell motility and growth were monitored to determine the rate of migration of cells towards the centre of the gap.

The plate was placed in the Cell-IQ, where the selected image positions were taken every hour for 24 hours. The data were analysed using the Cell-IQ software and some examples of analysed images are as shown in figure 3.16. Graphs showing the percentage wound closure at 6, 12 and 24 hours for the HeLa, MCF-7 and MDA-MB231 cells were plotted as shown in figures 3.17, 3.18 and 3.19 respectively.
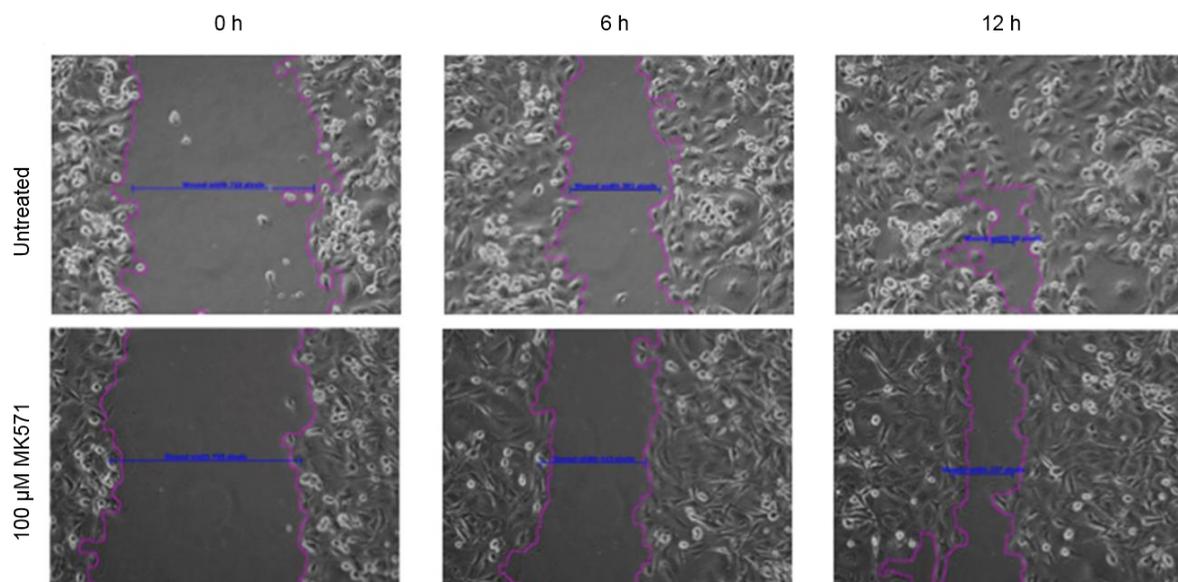
Figure 3.16. Representative images of MDA-MB231 cells migration in the presence of MK571. The cell migration assay was performed using the Cell-IQ. Three image positions were allocated in each well of a 24 well-plate, with images being taken every 1 hour for 24 hours. After 24 hours, imaging was stopped manually and the image results were analysed using the Cell-IQ analysis software. The control is untreated cells. Images were taken at a magnification of x10 objective.
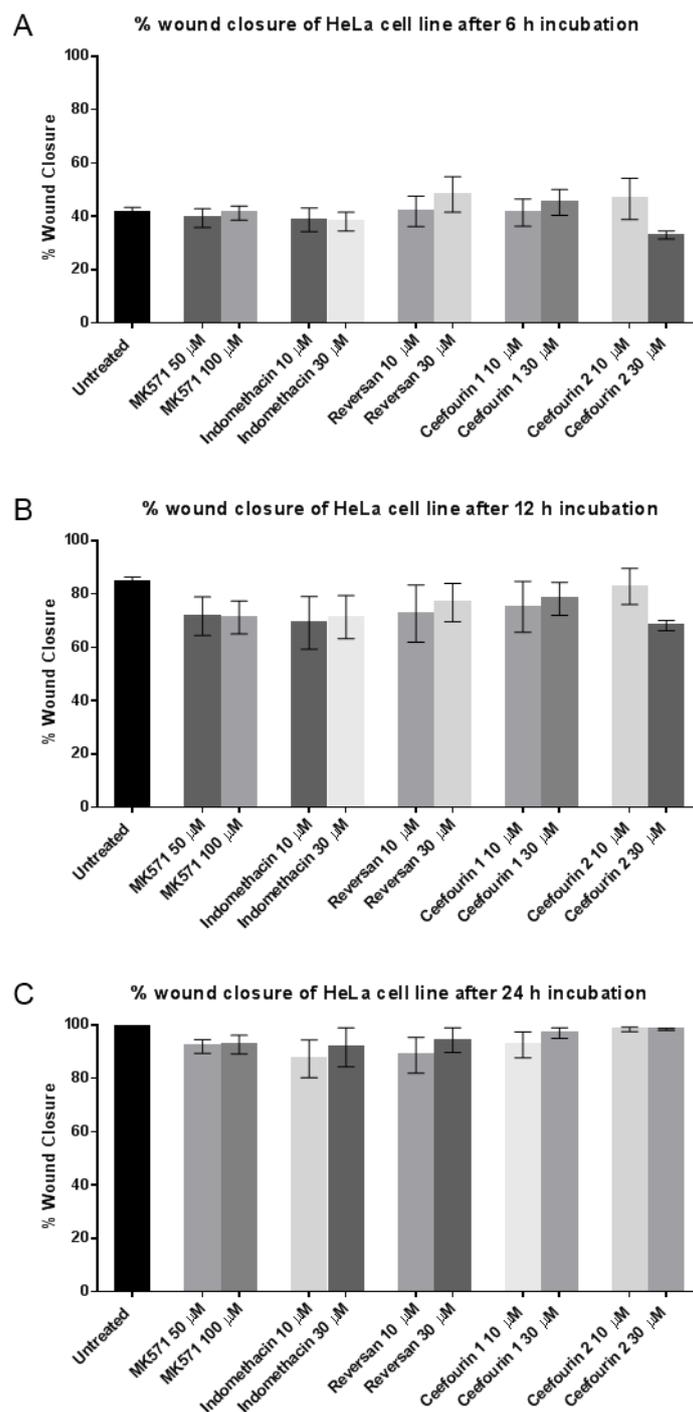
Figure 3.17. Average scratch closure of HeLa cells after treatment with ABC inhibitors. (A) 6 h, (B) 12 h and (C) 24 h incubation respectively, in the present of MK571 (50 µM and 100 µM), indomethacin (10 µM and 30 µM), Reversan (10 µM and 30 µM), Ceefourin 1 (10 µM and 30 µM) and Ceefourin 2 (10 µM and 30 µM). Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

As can be seen from figure 3.17, the ABC inhibitors did not affect cell migration of HeLa cells as compared to the untreated HeLa cells.
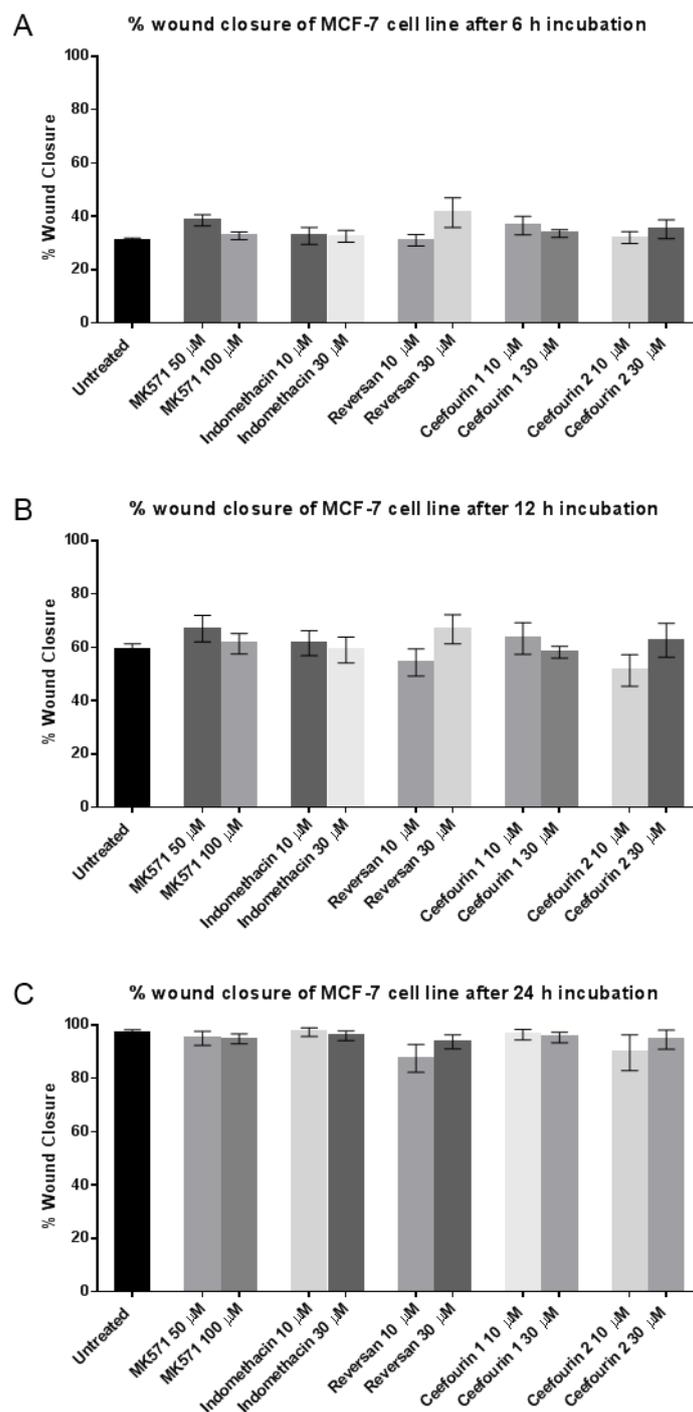
Figure 3.18. Average scratch closure of MCF-7 cells after treatment with ABC inhibitors. (A) 6 h, (B) 12 h and (C) 24 h incubation respectively, in the present of MK571 (50 μM and 100 μM), indomethacin (10 μM and 30 μM), Reversan (10 μM and 30 μM), Ceefourin 1 (10 μM and 30 μM) and Ceefourin 2 (10 μM and 30 μM). Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

Similarly, the ABC inhibitors did not affect cell migration of MCF-7 cells as compared to the untreated MCF-7 cells (Figure 3.18).
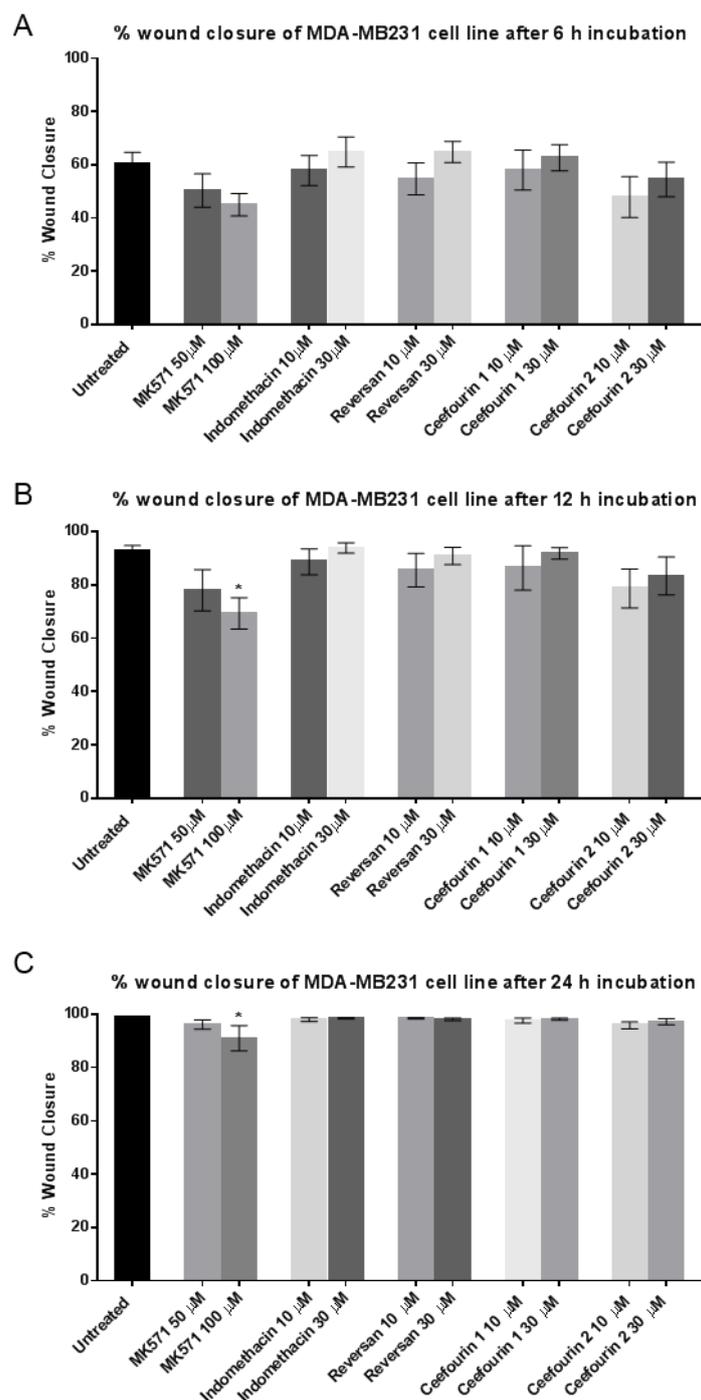
Figure 3.19. Average scratch closure of MDA-MB231 cells after treatment with ABC inhibitors. (A) 6 h, (B) 12 h and (C) 24 h incubation respectively, in the present of MK571 (50 µM and 100 µM), indomethacin (10 µM and 30 µM), Reversan (10 µM and 30 µM), Ceefourin 1 (10 µM and 30 µM) and Ceefourin 2 (10 µM and 30 µM). Data are expressed as the mean ± standard error of the mean from at least three independent experiments. * $P < 0.05$. P values were derived from one-way ANOVA vs untreated.

In the case of MDA-MB231 cells, 100 µM MK571 was found to significantly inhibit cell migration of MDA-MB231 cells (Figures 3.19B and 3.19C). However the significant inhibition

by other ABC inhibitors were not apparent especially at a longer incubation period (24 hours incubation) (Figure 3.19C). Subsequently, the cells may have adapted to the altered external environment and the cells may have activated other ABC transporters to compensate for the inhibition of ABCC1 and ABCC4.

Furthermore, the results observed is due to cell migration rather than cell proliferation as the results obtained from the MTT assays indicated that at a shorter time scale, the ABC inhibitors did not affect cell proliferation as compared to the untreated cells. Hence, these results suggested that ABCC1 and ABCC4 may help to promote cell migration of MDA-MB231 cells more than in HeLa and MCF-7 cells. However, further investigation is required to confirm the statement.

3.6   Summary

- ABCC1 and ABCC4 are most highly expressed in HeLa cells, second highest in MDA-MB231 cells, and lowest in MCF-7 cells.

- Other ABC multidrug efflux pumps often implicated in cancer, ABCB1 and ABCG2, were only expressed at significant levels in MCF-7 cells.

- MK571, which inhibits ABCC1 and ABCC4, has been found to affect cell proliferation of HeLa, MCF-7 and MDA-MB231 cells.

- Reversan which inhibits ABCC1 (Burkhart et al., 2009, Henderson et al., 2011) was also found to affect cell proliferation of all cell lines tested.

- Inhibition of cell proliferation was larger in HeLa cells, followed by MDA-MB231 cells and MCF-7 cells. Since this matches the relative levels of ABCC1 and ABCC4 expression in these cell lines, the decrease in cell proliferation may be due to inhibition of ABCC1 and ABCC4.

- Since only Reversan but not MK571 affected the cell proliferation of MCF-7 cells, it might suggest that ABCC1 is more implicated in cell proliferation than ABCC4. However, as Reversan has also been shown to inhibit ABCB1 (Tivnan et al., 2015) and MCF-7 cells were the only ones expressing relatively high levels of ABCB1, this could be an alternative explanation.

- Only MK571 affected the cell migration of MDA-MB231 cells. The other ABCC1 and ABCC4 inhibitors did not have significant effect on cell migration. Since MK571 can inhibit both ABCC1 and ABCC4 perhaps it is this dual inhibition which has the greatest effect on migration.
  - Alternatively, MK571 is also a leukotriene antagonist, inhibiting the binding of LTD_4 to cysteinyl leukotriene receptor 1 (CysLT1) (Gekeler et al., 1995). The binding of LTD_4 to CysLT1 activates β-catenin signalling promoting cancer cell survival and inhibiting apoptosis, thus, inducing cell proliferative response (Nielsen et al., 2005; Mezhybovska et al., 2006; Perters-Golden et al., 2007).

In conclusion, since none of the inhibitors used are totally specific to ABCC1 and ABCC4 it makes it difficult to interpret the results. Therefore, in chapter 4, ABCC1 and ABCC4 knockdowns were used to test these proteins involvement in breast cancer biology.

Chapter 4 – Analysis of the effects of gene knockdown of ABC transporters in breast cancer cell lines *in vitro*

4.1   Objective

Due to the fact that the ABC inhibitors used were not specific as they can inhibit more than one ABC transporters in addition to other targets, the results obtained from chapter 3 are not conclusive. Therefore, this chapter looks to target each ABC transporter, ABCC1 and ABCC4 by knocking down their gene expression and carrying out functional cellular assays using the gene knockdown cells to investigate if ABCC1 and ABCC4 promote breast cancer cell proliferation, cell migration and/or cell invasion.

Gene silencing describe general processes where by the translation or transcription of the mRNA of the target gene is being interrupted or supressed by mechanisms other than genetic modification often known as RNA interference (RNAi). The RNAi is a post transcriptional sequence-specific gene silencing process that occurs in response to the initiation of double-stranded RNA (dsRNA) into a cell to produce highly specific suppression of gene expression. The RNAi is mediated by either the small interfering RNAs (siRNAs) or microRNA (miRNA) (Mocellin et al., 2004; Sandy et al., 2005; Shah et al., 2012).

In this project, siRNAs were used to knock down ABCC1 and ABCC4 genes respectively in MCF-7 and MDA-MB231 breast cancer cell lines. Furthermore, in order to increase the confidence in the experimental results, two different siRNAs for each transporters were used, each targeting a different specific sequence within the gene sequence of interest. In addition, a negative siRNA was used to determine sequence-specific silencing from non-specific effects. Hence to ensure the results obtained from the siRNA knockdown is due to the effect of the specific ABC siRNAs and not just the process for carrying out the knockdown. Moreover, the untreated cells were used to monitor and compare the baseline cell viability, phenotype and target gene level to the siRNA treated cells.

As a mode of transfection, INTERFERin, a siRNA transfection reagent was used. It was recommended as according to the manufacturer recommendation, 1 nM siRNA with INTERFERin can provide more than 90% silencing efficiency in a wide variety of cells, hence reducing off-target effects.

In this chapter, I investigated whether ABCC1 and/or ABCC4 is/are implicated in cell proliferation, migration and invasion by first conducting single siRNA knock down of *ABCC1*

and *ABCC4* genes in MCF-7 and MDA-MB231 breast cancer cell lines. The siRNA knockdown cells were then used in cellular functional assays, including the colony formation assay to assess cell growth, MTT assay to assess cell proliferation, scratch assay to assess cell migration and transwell invasion assay to assess cell invasion.

Furthermore, since some ABC transporters transport similar active physiological substrates thus the inhibition of one ABC transporter is usually compensated by the activation of another ABC transporters (Efferth et al., 2006; Samant et al., 2015; Martins et al., 2016). We therefore wanted to investigate whether ABCC1 and ABCC4 work together to compensate each other, hence both ABCC1 and ABCC4 were knocked down in the breast cancer cell lines. The ABCC1 and ABCC4 combination siRNA knockdown breast cancer cells were then used to test whether the effect of combination knockdown of ABCC1 and ABCC4 will cause a greater decrease in cell proliferation, migration and invasion in breast cancer cells.

4.2   Analysis of the efficiency of single gene knock down of ABC transporter in breast cancer cell lines

4.2.1   Protein expression analysis by Western blot

Before starting the cellular functional assays using the siRNA knockdown cells, we tested the efficiency of siRNA transfection. All the siRNA transfection of cells were done in 24-well plate.

Cells were seeded the day before transfection to reach 30 - 50% confluency at the time of transfection. On the day of transfection, transfection complexes of 1 nM siRNA, INTERFERin and OptiMEM were made according to the manufacturer's protocol. The transfection complexes were allowed to form on incubation at room temperature for 10 - 15 minutes. After incubation, 100 µl of the transfection complexes were added to the wells and the plate was swirl for even dispersing of the RNAi complexes. The plate was incubated for 72 hours after which the transfected cells were harvested. The efficiency of siRNA transfection was determined by Western blot analysis as shown in figures 4.1 and 4.2.
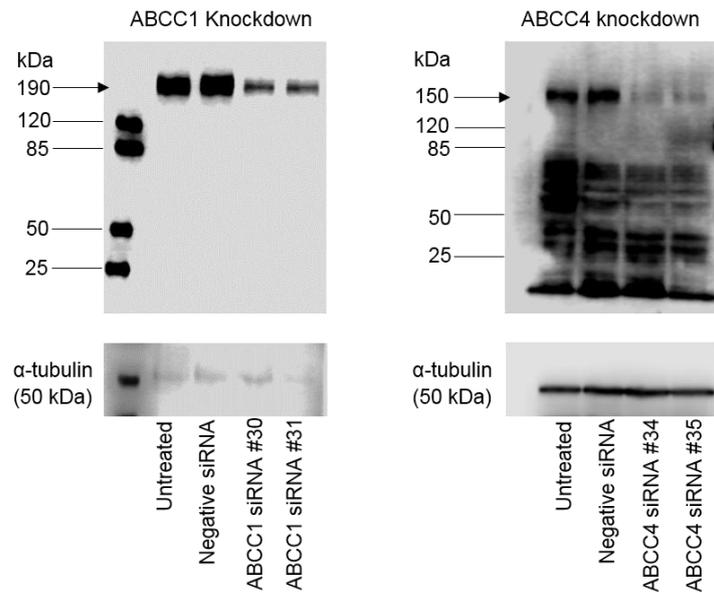
Figure 4.1 Western blot analysis of ABCC1 and ABCC4 protein expression following transfection of MCF-7 breast cancer cells with ABCC1- and ABCC4- specific siRNA molecules. ABCC1-specific siRNA molecules: siRNA #30, siRNA #31, and ABCC4-specific siRNA molecules: siRNA #34, siRNA #35 were compared with a negative siRNA as a negative control to offset any non-specific silencing. After treatment of MCF-7 cells with specific siRNA molecules, the MCF-7 transfected cells were left to incubate for 72 hours. Protein lysate of the MCF-7 transfected cells were obtained and the efficiency of silencing was analysed in Western blot analysis. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 20 µl of samples were loaded in each lanes. α-tubulin was used as the loading control. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. Western blot analysis was repeated at least three times and successful silencing of ABCC1 and ABCC4 protein expression in MCF-7 transfected cells were obtained each time.

Figure 4.2. Western blot analysis of ABCC1 and ABCC4 protein expression following transfection of MDA-MB231 breast cancer cells with ABCC1- and ABCC4- specific siRNA molecules. ABCC1-specific siRNA molecules: siRNA #30, siRNA #31, and ABCC4-specific siRNA molecules: siRNA #34, siRNA #35 were compared with a negative siRNA as a negative control to offset any non-specific silencing. After treatment of MDA-MB231 cells with specific siRNA molecules, the MDA-MB231 transfected cells were left to incubate for 72 hours. Protein lysate of the MDA-MB231 transfected cells were obtained and the efficiency of silencing was analysed in Western blot analysis. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 20 µl of samples were loaded in each lanes. α-tubulin was used as the loading control. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. Western blot analysis was repeated at least three times and successful silencing of ABCC1 and ABCC4 protein expression in MDA-MB231 transfected cells were obtained each time.

As can be seen from figure 4.1, the Western blot analysis indicate that the protein expression levels of ABCC1 and ABCC4 were successfully decreased by siRNA knockdown in MCF-7 cells when compared to the untreated MCF-7 cells and the MCF-7 cells treated with a negative siRNA. Similarly, Western blot analysis indicated that the expression levels of ABCC1 and ABCC4 proteins were successfully decreased by siRNA transfection in MDA-MB231 cells when compared to the untreated MDA-MB231 cells and the MDA-MB231 cells treated with a negative siRNA (Figure 4.2). Furthermore, it seem that both ABCC1-specific siRNA molecules, siRNA #30 and siRNA #31 give a similar degree of *ABCC1* gene silencing in both

MCF-7 and MDA-MB231 cells. In contrast, ABCC4-specific siRNA #34 results in a higher degree of *ABCC4* gene silencing than ABCC4-specific siRNA #35 in MDA-MB231 cells.

Subsequently, all cells treated with the INTERFERin / siRNA complexes, the culture medium were not change during the whole duration of transfection as low cell cytotoxicity was observed compared to untreated cells. Hence the cell culture medium containing the INTERFERin / siRNA complexes was not changed within 4 to 6 hours after transfection. Moreover, the MCF-7 and MDA-MB231 breast cancer cell lines are considered to be difficult to transfect cell lines but we have successfully managed to knockdown ABCC1 and ABCC4 in MCF-7 and MDA-MB231 cells using INTERFERin as the transfection reagent.

4.2.2 mRNA expression analysis using RT-PCR

To determine the efficiency of gene silencing by measuring the mRNA levels of *ABCC1* and *ABCC4* in siRNA knockdown breast cancer cells, the reverse transcription polymerase chain reaction (RT-PCR) was performed. Briefly, MCF-7 and MDA-MB231 breast cancer cells were transfected with ABCC1- and ABCC4-specific siRNAs. After 72 hours incubation, the transfected breast cancer cells were harvested and RNA isolation was performed. The RNA concentration of purified samples were determined using the NanoDrop spectrophotometer. A 600 ng total RNA from each RNA sample was reverse transcribed to cDNA using random primers and the Precision nanoScript ^{TM} 2 Reverse Transcription kit (Primerdesign) according to manufacturer's protocols and the cDNA samples obtained were diluted 1 in 10 with RNase/DNase-free water and stored at -20ºC. Subsequently, the cDNA samples were amplified in quantitative PCR (qPCR) reactions, which were performed in triplicates using the PikoReal 96 Real-Time PCR system. Specific forward and reverse ABCC1 and ABCC4 primers from Invitrogen, Thermo Fisher Scientific were used and the *ABCC1* and *ABCC4* mRNA expression levels were normalised to the level of *β-actin* and *GAPDH* mRNAs. The relative *ABCC1* and *ABCC4* mRNA levels were analysed using the double delta CT ($2^{-\Delta\Delta CT}$) method (Livak et al., 2001; Rao et al., 2013). The relative *ABCC1* and *ABCC4* mRNA expression levels in ABCC1 and ABCC4 knockdown MCF-7 and MDA-MB231 breast cancer cells are shown in figures 4.3 and 4.4 respectively.
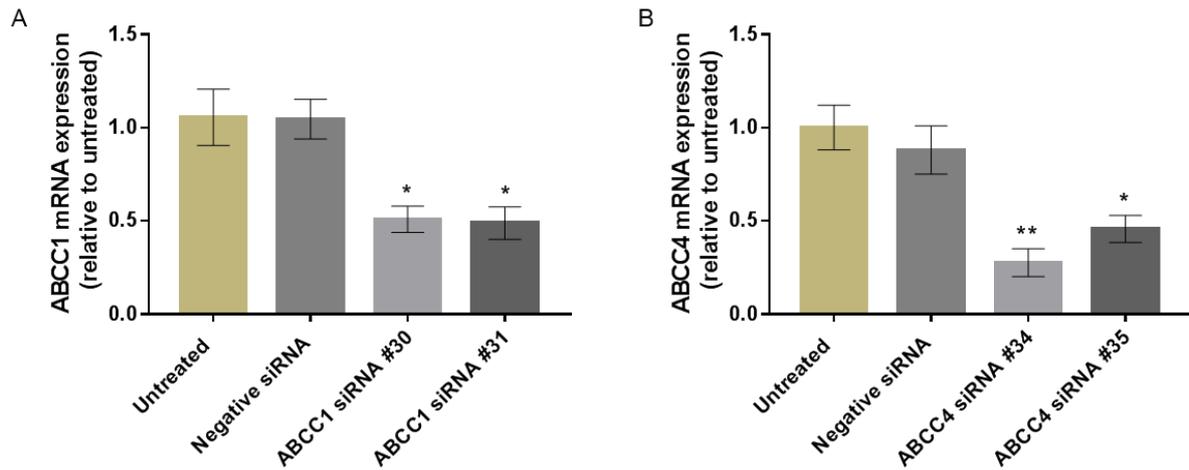
Figure 4.3. *ABCC1* and *ABCC4* **mRNA expression levels following siRNA knockdown in MCF-7 cells.** Briefly, MCF-7 cells were transfected with ABCC1- and ABCC4-specific siRNAs. After 72 hours incubation, the transfected breast cancer cells were harvested and RNA isolation was performed. cDNA was synthesed from 600 ng total using random primers and the Precision nanoScript™ 2 Reverse Transcription kit (Primerdesign) according to manufacturer's protocols and the cDNA samples obtained were diluted 1 in 10 with RNase/DNase-free water and stored at -20°C. Subsequently, quantitative PCR (qPCR) reactions were performed in triplicates using the PikoReal 96 Real-Time PCR system. Specific forward and reverse ABCC1 and ABCC4 primers were used and the *ABCC1* and *ABCC4* mRNA expression levels were normalised to the level of *β-actin* and *GAPDH* mRNAs. The RNase/DNase-free water was used as a negative control and the untreated MCF-7 cells as the control without any siRNA treatment. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$. *P* values were derived from one-way ANOVA vs untreated.
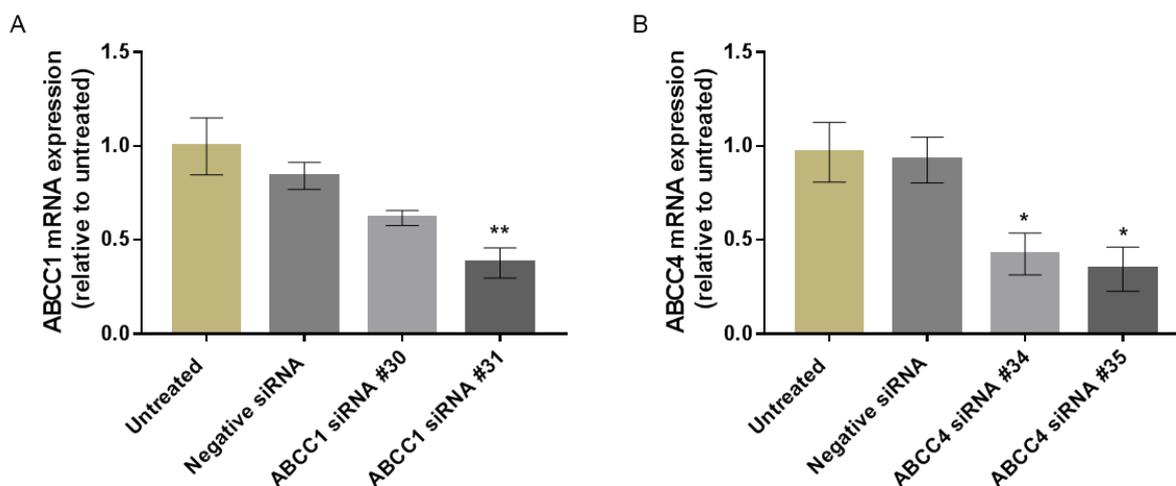
Figure 4.4. *ABCC1* and *ABCC4* mRNA expression levels following siRNA knockdown in MDA-MB231 cells. Briefly MDA-MB231 were transfected with ABCC1- and ABCC4-specific siRNAs. After 72 hours incubation, the transfected breast cancer cells were harvested and RNA isolation was performed. cDNA was synthesed from 600 ng total using random primers and the Precision nanoScript™ 2 Reverse Transcription kit (Primerdesign) according to manufacturer's protocols and the cDNA samples obtained were diluted 1 in 10 with RNase/DNase-free water and stored at -20°C. Subsequently, quantitative PCR (qPCR) reactions were performed in triplicates using the PikoReal 96 Real-Time PCR system. Specific forward and reverse ABCC1 and ABCC4 primers were used and the *ABCC1* and *ABCC4* mRNA expression levels were normalised to the level of *β-actin* and *GAPDH* mRNAs. The RNase/DNase-free water was used as a negative control and the untreated MDA-MB231 cells as the control without any siRNA treatment. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$. P values were derived from one-way ANOVA vs untreated.

As can be seen from figures 4.3A and 4.3B the mRNA expression levels of *ABCC1* and *ABCC4* were significantly decreased in MCF-7 cells following specific siRNA knockdowns when compared to the negative siRNA or untreated MCF-7 cells.

Similarly, the expression levels of ABCC1 was significantly decreased in MDA-MB231 cells treated with ABCC1 siRNA #31. However, whilst MDA-MB231 cells treated with ABCC1 siRNA #30 showed a decrease in ABCC1 expression, this was not statistically significant when compared to the untreated MDA-MB231 cells (Figure 4.4A). In the case of ABCC4 knockdown in MDA-MB231 cells, treatment with both ABCC4 siRNAs, showed a significant decrease in ABCC4 expression level when compared to the untreated MDA-MB231 cells (Figure 4.4B).

Consequently, the analysis of the qPCR data which correlated with the Western blot analysis suggested that the knockdown of ABCC1 and ABCC4 in MCF-7 and MDA-MB231 breast

cancer cells by ABCC1- and ABCC4-specific siRNA molecules was a success. Hence, similar siRNA transfection procedure was performed for all future cellular functional experiments.

4.3   Effects of single gene knockdown of ABC transporters on cancer cell colony formation

Cells transfected with siRNA following the INTERFERin manufacturers' protocol were seeded for the colony formation assay to assess the ability of a single cell to grow in to a colony after ABCC1 and ABCC4 had been knocked down. Graphs showing the number of colonies formed from MCF-7 and MDA-MB231 cell lines are shown in figure 4.5.
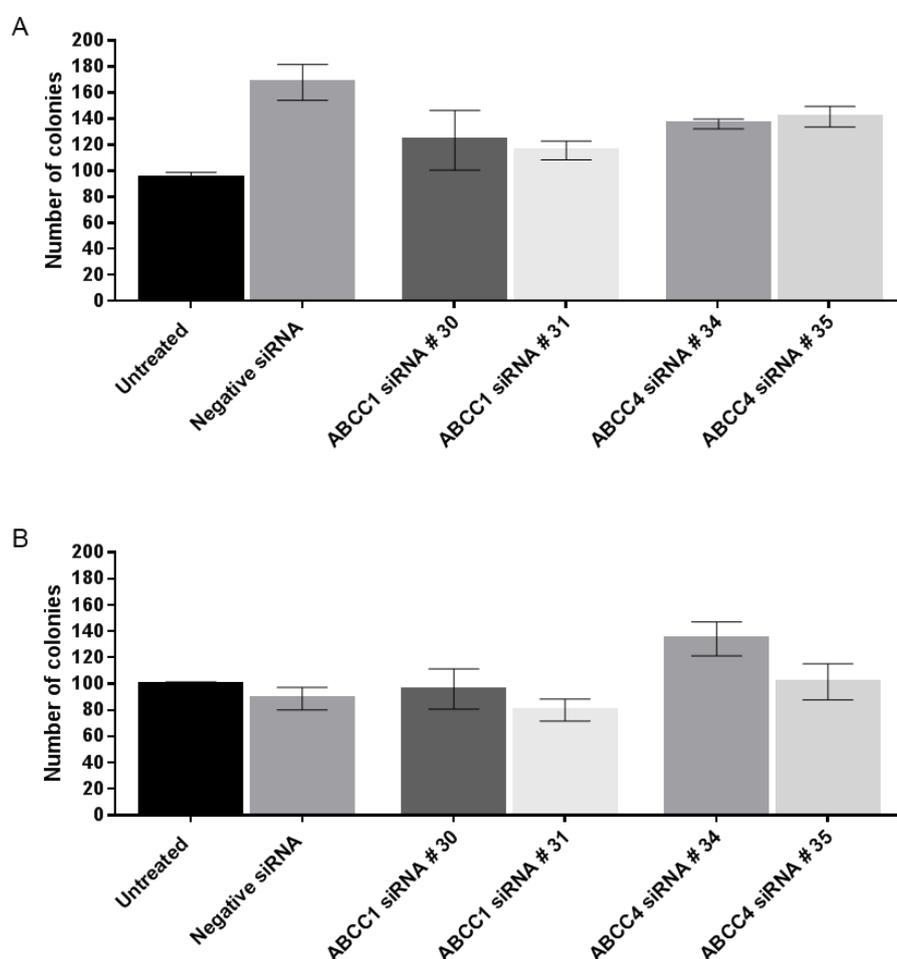
Figure 4.5. The impact of suppression of *ABCC1* and *ABCC4* gene expression on colony formation. (A) MCF-7 and (B) MDA-MB231 breast cancer cells were transfected with 1 nM of siRNA and the clonogenic capacity of the breast cancer cells upon depletion of ABCC1 and ABCC4 was assayed after 7 days. The transfected breast cancer cells were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and were counted manually. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

As can be seen in figures 4.5A and 4.5B, the ability of MCF-7 and MDA-MB231 cells to form colonies did not decrease after ABCC1 and ABCC4 depletion. In fact, ABCC1 and ABCC4 suppression in MCF-7 cells may have increased the number of colonies formed (Figure 4.5A) while only the suppression of ABCC4 in MDA-MB231 cells may have increased the number of colonies formed. Furthermore, the suppression of ABCC1 in MDA-MB231 cells did not affect the clonogenic capacity of MDA-MB231 cells (Figure 4.5B).

In general, the RNAi mechanism of suppression of gene expression is usually transient and lasts for 96 to 120 hours after which the gene expression will start to recover (Mocellin et al., 2004). Consequently, since the colony formation assay last for 7 days, it is suggested that the suppression of ABCC1 and ABCC4 may have recovered. Therefore, a shorter incubation period, for example 3 days may be suggested. Moreover, introducing plasmids which express the siRNA may produce a stable RNAi transfection. In addition, some labs uses short hairpin RNAs (shRNAs) expressed from plasmids or use viral transduction methods including lentivirus, adenovirus and retrovirus based vectors (Mocellin et al., 2004; Sandy et al., 2005).

Initially, the ABCC1 and ABCC4 suppression may have overexpressed other ABC transporters as a compensatory effect hence causing the transfected cells to overcome the impact of ABCC1 and ABCC4 suppression.

4.4 Effects of single gene knockdown of ABC transporters on cancer cell viability

To study the impact of ABCC1 and ABCC4 suppression in breast cancer cells have on cell viability and cell cytotoxicity, the MTT assay was performed. Subsequently, transfected cells were seeded in 24-well plate and the cell viability was assessed after 6, 12 24, 48 and 72 hours. Graphs showing the absorbance values of each cell lines are shown in figures 4.6 and 4.7.

A
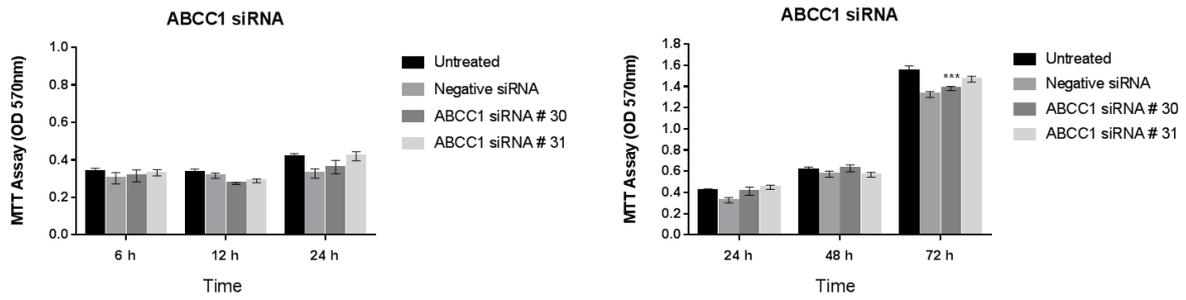
B
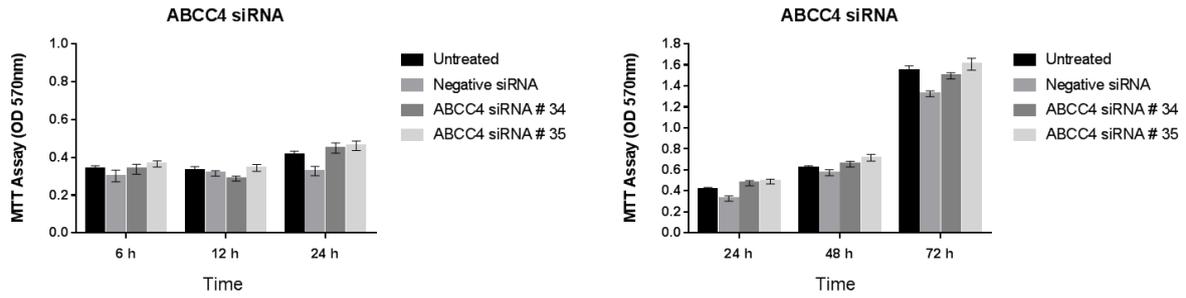
Figure 4.6. Cell viability of ABCC1 knockdown MCF-7 cells and ABCC4 knockdown MCF-7 cells. (A) ABCC1 knockdown MCF-7 cells and (B) ABCC4 knockdown MCF-7 cells were seeded in 24-well plates at a seeding density of 1.5×10^4 cell/well. An MTT assay was performed. The cell viability was assessed after 6, 12, 24, 48, 72 hours. Briefly, 40 µl of MTT (5 mg/ml) was added to each well. After an additional one hour of incubation, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. *** $P < 0.001$. P values were derived from two-way ANOVA vs untreated.
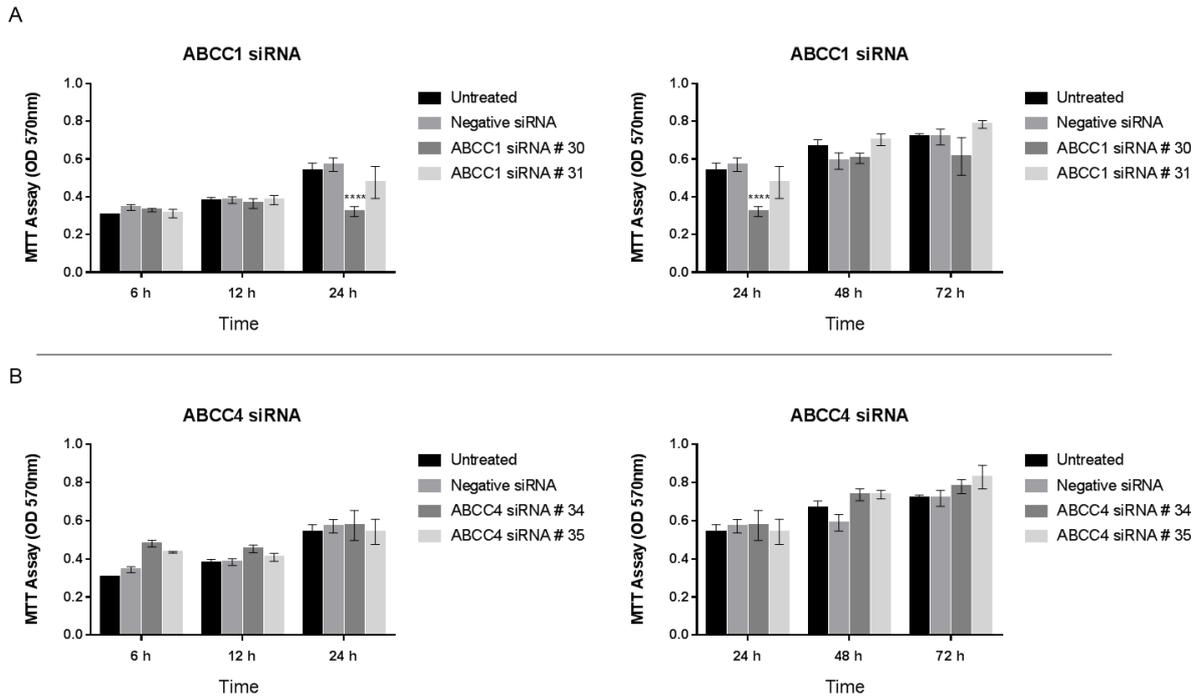
Figure 4.7. Cell viability of ABCC1 knockdown MDA-MB231 cells and ABCC4 knockdown MDA-MB231 cells. (A) ABCC1 knockdown MDA-MB231 cells and (B) ABCC4 knockdown MDA-MB231 cells were seeded in 24-well plates at a seeding density of 1.5 x 10^4 cell/well. An MTT assay was performed. The cell viability was assessed after 6, 12, 24, 48, 72 hours. Briefly, 40 µl of MTT (5 mg/ml) was added to each well. After an additional one hour of incubation, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. * *** $P < 0.0001$. P values were derived from two-way ANOVA vs untreated.

According to the results obtained from the MTT assay, the depletion of ABCC1 and ABCC4 in MCF-7 cells did not affect cell viability when compared to the negative siRNA or untreated cells (Figures 4.6A and 4.6B). However, the suppression of ABCC1 in MDA-MB231 cells by siRNA #30 significantly decreased cell viability only after 24 hours (Figure 4.7A). One explanation could be that since the expression level of ABCC1 in MDA-MB231 cells is higher than in MCF-7 cells, therefore the knockdown of ABCC1 in MDA-MB231 cells may be more apparent usually at a shorter time scale compared to siRNA knockdown in MCF-7 cells.

4.5 Effects of single gene knockdown of ABC transporters on cancer cell migration

Upon depletion of ABCC1 and ABCC4 in MCF-7 and MDA-MB231 breast cancer cells in 24-well plate that was left for 72 hours incubation according to the manufacturer's instructions, a cell monolayer was formed and a scratch assay was performed. Simply, a gap was carefully

created in the cell monolayer usually with a 10 µl pipette tip and the cell motility were monitored to determine the rate of migration of cells towards the centre of the gap.

The plate was placed in the Cell-IQ, where the selected image positions were taken every hour for 24 hours. The data collected were analysed using the Cell-IQ analysis software. The percentage wound closure at 6, 12 and 24 hours for the MCF-7 and MDA-MB231 cells were plotted against the siRNAs as shown in figures 4.8 and 4.9 respectively.
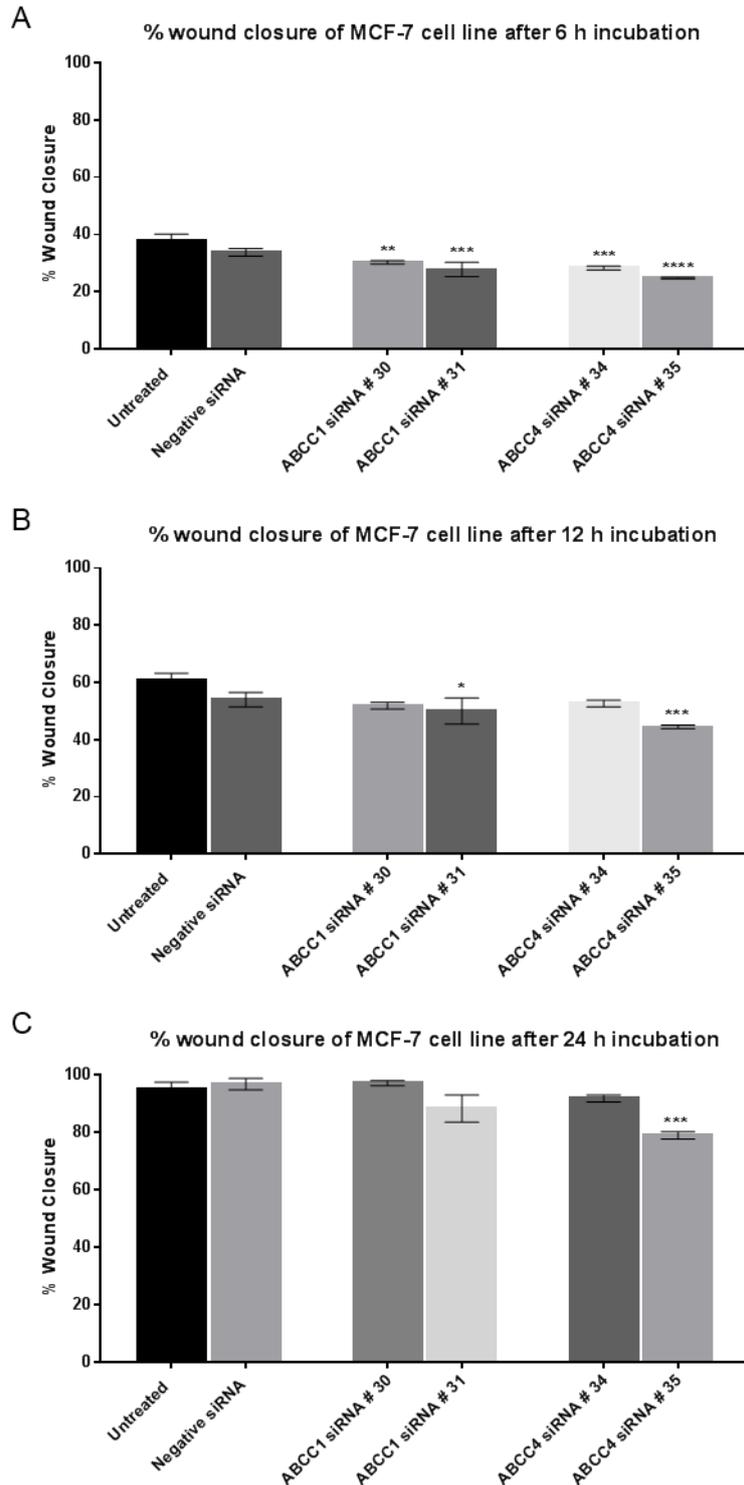
Figure 4.8. Average scratch closure of ABCC1 knockdown MCF-7 cells and ABCC4 knockdown MCF-7 cells. MCF-7 cells transfected with ABCC1 and ABCC4 siRNAs were left to incubate for 72 hours to ensure efficient transfection, a cell monolayer was formed and a scratch with a 10 µl pipette tip was made in the cell monolayer. The plate was placed in the Cell-IQ where the selected image positions were captured every hour for 24 hours. The transfected cells were incubated for (A) 6, (B) 12 and (C) 24 hours respectively. The captured images were analysed in the Cell-IQ analysis software. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. *$P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$. P values were derived from one-way ANOVA vs untreated.
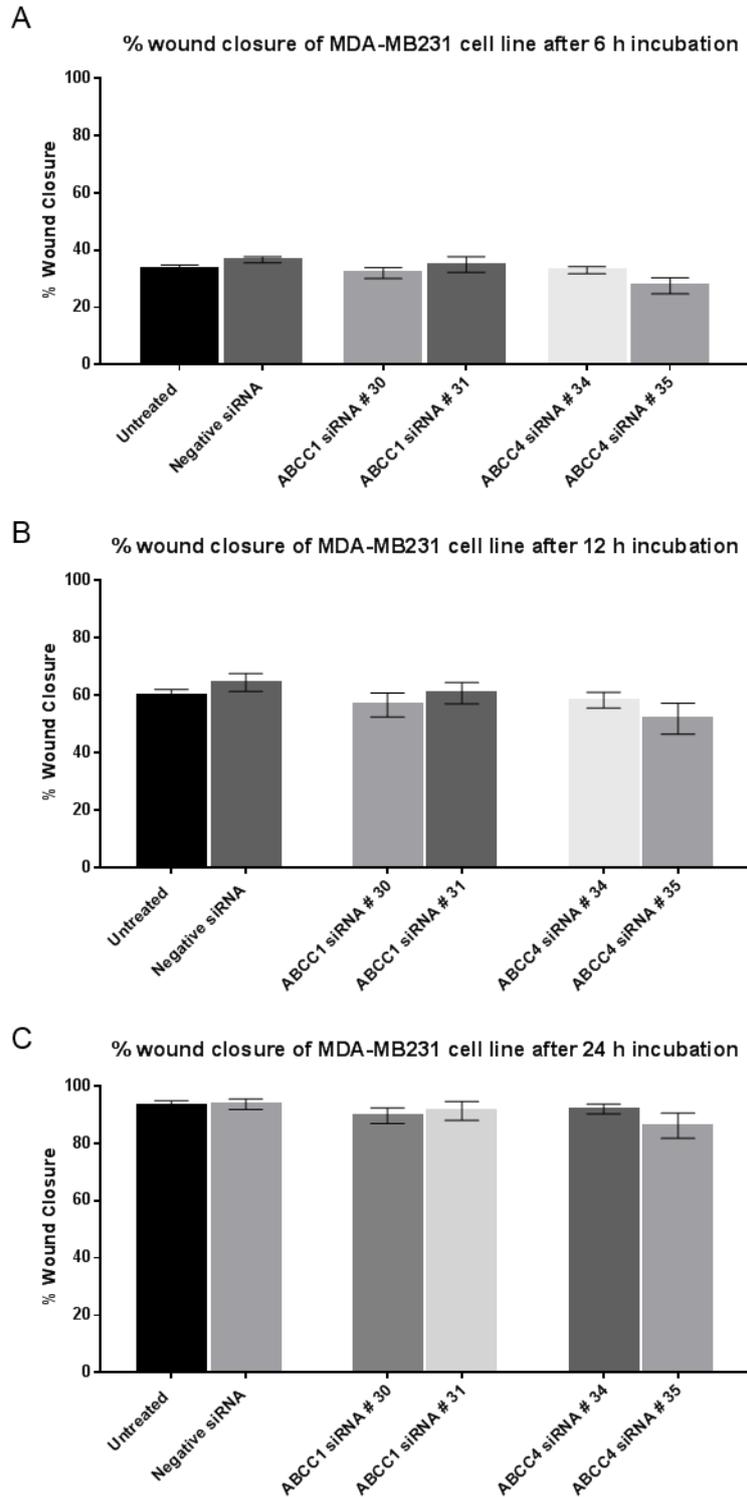
Figure 4.9 Average scratch closure of ABCC1 knockdown MDA-MB231 cells and ABCC4 knockdown MDA-MB231 cells. MDA-MB231 cells transfected with ABCC1 and ABCC4 siRNAs were left to incubate for 72 hours to ensure efficient transfection, a cell monolayer was formed and a scratch with a 10 μl pipette tip was made in the cell monolayer. The plate was placed in the Cell-IQ where the selected image positions were captured every hour for 24 hours. The transfected cells were incubated for (A) 6, (B) 12 and (C) 24 hours respectively. The captured images were analysed in the Cell-IQ analysis software. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

Upon depletion of ABCC1 and ABCC4 in MCF-7 cells, the cell motility was assessed by performing the scratch assay. The suppression of either *ABCC1* or *ABCC4* gene expression levels in MCF-7 cells significantly decreased cell migration when compared to the untreated cells after 6 hours incubation (Figure 4.8A). However, it seem that the suppression levels of ABCC1 and ABCC4 decreased with time. This could be the fact that the transfection mechanism is transient, thus the *ABCC1* and *ABCC4* genes slowly recovers with time. Consequently after 24 hour incubation the decrease in cell migration of ABC siRNA knockdown MCF-7 cells were only affected by one ABCC4 siRNA (ABCC4 siRNA #35) (Figure 4.8C).

Furthermore, the suppression of *ABCC1* and *ABCC4* gene expression in MDA-MB231 cells may be weak as compared to MCF-7 cells. Hence, the depletion of ABCC4 by only one of the two ABCC4 siRNAs (ABCC4 siRNA #35) caused a decrease in cell migration of the MDA-MB231 cells after 6, 12 and 24 hours incubation (Figure 4.9A, 4.9B and 4.9C) but the decrease was not significant when compared to the untreated MDA-MB231 cells. These results suggested that the knockdown of ABCC1 and ABCC4 decreased cell migration in MCF-7 cells but not in MDA-MB231 cells.

4.6 Effects of single gene knockdown of ABC transporters on cancer cell invasion

To determine whether the of suppression of *ABCC1* and *ABCC4* gene expression levels in MCF-7 and MDA-MB231 breast cancer cells have an effect on not just migration but ability to invade, a transwell invasion assay was performed, which is an *in vitro* system often used to study cell invasion of malignant and normal cells. First, 24-well plate transwell inserts were coated with ice cold Corning Matrigel at a ratio of 1:3 with serum free cell culture medium to provide a condition to assess cancer cell invasive capacity *in vitro*. The Corning Matrigel Matrix blocks the pores of the membrane transwells to serve as a reconstituted basement membrane *in vitro*. Hence the only cells that have the ability to penetrate the Corning Matrigel Matrix and through the membrane pores are those that secrete proteases that enzymatically degrade the Corning Matrigel Matrix and these cells are often termed invasive cells. Non-invasive cells should not be able to migrate through the membrane (Kramer et al., 2013).

In an optimisation process to identify the optimum cell seeding density and the thickness of the Corning Matrigel Matrix to be used, we found a seeding density of 50,000 cells and a volume of 30 µl of Corning Matrigel Matrix were optimal for each transwell insert. The cells were suspended in 0.5% v/v FBS cell culture medium and, 0.5% v/v and 10% v/v FBS cell

culture medium were used as the chemoattractant. The cells were incubated for 24 hours after which the cells that migrated across the membranes pores were fixed and stained with Differential Quik stain according to manufacturer's protocol. To determine the percentage cell invasion, the migrated cells which were stained were counted from five randomly selected fields of view under a light microscope at a magnification of x40. Graphs of percentage invasion of ABCC1 knockdown and ABCC4 knockdown MCF-7 and MDA-MB231 cells were plotted as shown in figures 4.10 and 4.11 respectively.

A

% Invasion of ABCC1 knockdown MCF-7 cells

B

% Invasion of ABCC4 knockdown MCF-7 cells
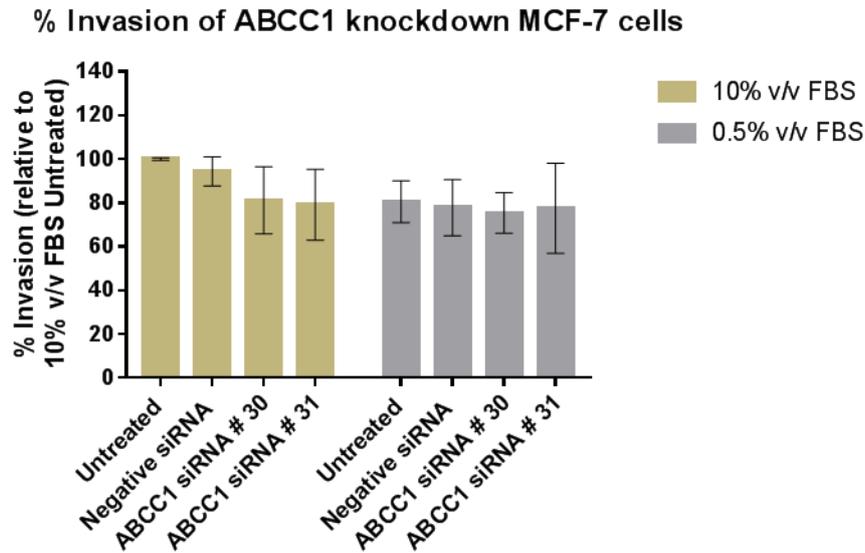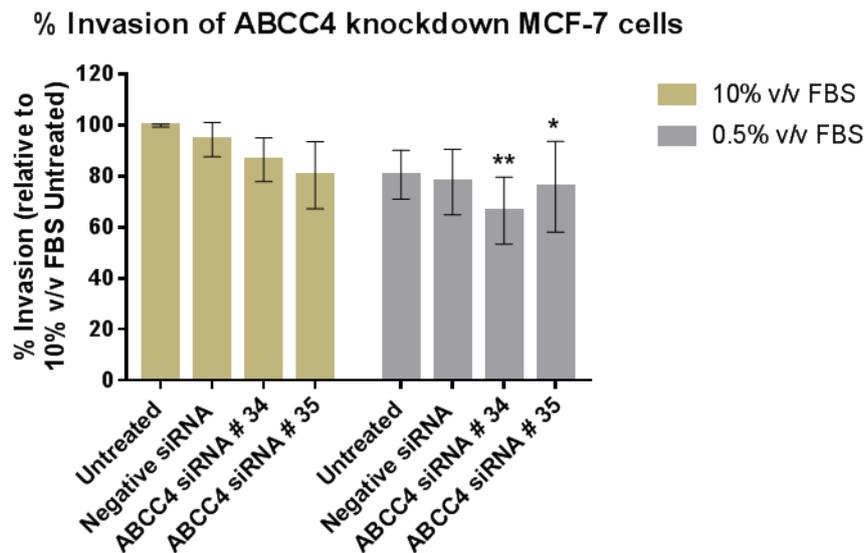
Figure 4.10. Effect of ABCC1 or ABCC4 knockdown on MCF-7 cell invasion. Percentage invasion of (A) ABCC1 knockdown MCF-7 cells and (B) ABCC4 knockdown MCF-7 cells across a reconstituted basement membrane *in vitro*. Upon depletion of ABCC1 and ABCC4 in MCF-7 cells, the ABCC1 knockdown MCF-7 cells and the ABCC4 knockdown MCF-7 cells were seeded for transwell invasion assay. The transwells were first coated with Corning Matrigel Matrix and a cell suspension of the ABCC1 knockdown MCF-7 cells and ABCC4 knockdown MCF-7 cells in 0.5% v/v FBS cell culture medium were seeded in the transwell inserts that were then placed in wells of a 24-well plate containing 10% v/v or 0.5% v/v FBS cell culture medium. After 24 hours incubation, invasive cells that have crossed the membrane were fixed and stained with Differential Quik stain according to manufacturer protocol. Stained cells were counted using a light microscope (x40) from five different fields of view. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. *$P < 0.05$ and ** $P < 0.01$. *P* values were derived from two-way ANOVA vs 10% v/v FBS untreated.

A

% Invasion of ABCC1 knockdown MDA-MB231 cells

Legend:
- 10% v/v FBS
- 0.5% v/v FBS

B

% Invasion of ABCC4 knockdown MDA-MB231 cells
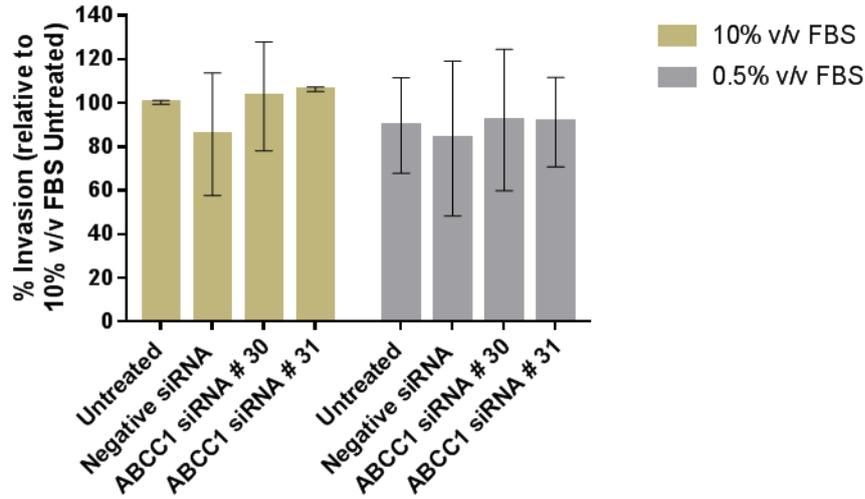
Legend:
- 10% v/v FBS
- 0.5% v/v FBS
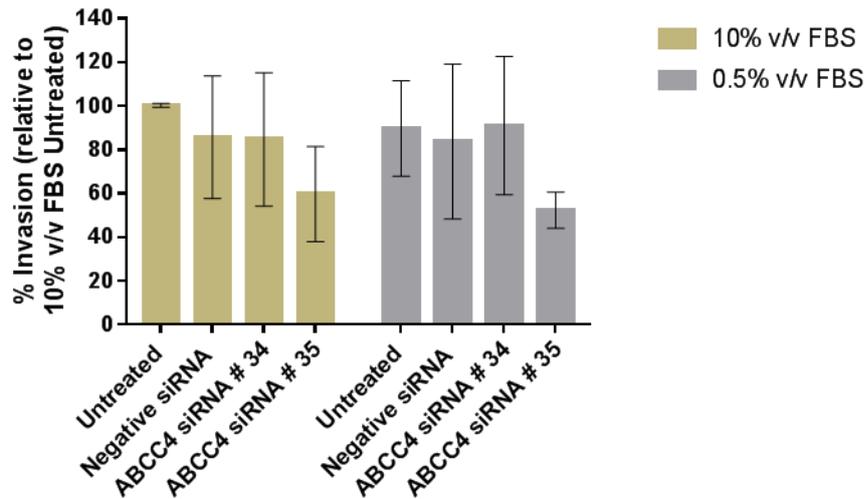
Figure 4.11. Effect of ABCC1 or ABCC4 knockdown on MDA-MB231 cell invasion. Percentage invasion of (A) ABCC1 knockdown MDA-MB231 cells and (B) ABCC4 knockdown MDA-MB231 cells across a reconstituted basement membrane *in vitro*. Upon depletion of ABCC1 and ABCC4 in MDA-MB231 cells, the ABCC1 knockdown MDA-MB231 cells and the ABCC4 knockdown MDA-MB231 cells were seeded for transwell invasion assay. The transwells were first coated with Corning Matrigel Matrix and a cell suspension of the ABCC1 knockdown MDA-MB231 cells and ABCC4 knockdown MDA-MB231 cells in 0.5% v/v FBS cell culture medium were seeded in the transwell inserts that were then placed in wells of a 24-well plate containing 10% v/v or 0.5% v/v FBS cell culture medium. After 24 hours incubation, invasive cells that have crossed the membrane were fixed and stained with Differential Quik stain according to manufacturer protocol. Stained cells were counted using a light microscope (x40) from five different fields of view. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. *$P < 0.05$ and ** $P < 0.01$. P values were derived from two-way ANOVA vs 10% v/v FBS untreated.

In general, Foetal bovine serum (FBS) was used as the chemoattractant and cancer cells usually migrate from a low concentration to a high concentration of chemoattractant. Consequently, the ABCC1 and ABCC4 knockdown MCF-7 and MDA-MB231 cells were suspended in 0.5% v/v FBS cell culture medium and the chemoattractant was either 0.5% v/v or 10% v/v FBS cell culture medium. Therefore, higher numbers of cells were expected to migrate when the chemoattractant was 10% v/v FBS cell culture medium compared to 0.5% v/v cell culture medium.

The invasive ability of ABCC1 knockdown MCF-7 cells (siRNA #30 and #31) were impaired, as a lower percentage of cells moved across the membrane pores to the chemoattractant 10% v/v FBS cell culture medium when compared to that of the untreated MCF-7 cells. However, when the chemoattractant was 0.5% v/v FBS cell culture medium, no significant inhibition of cell invasion was observed (Figure 4.10A).

Moreover, the depletion of ABCC4 in MCF-7 cells (siRNA #34 and #35) show no significant decrease in migration across the membrane pores to 10% v/v FBS cell culture medium (Figure 4.10B). Interestingly, the suppression of *ABCC4* gene expression in MCF-7 cells by ABCC4 siRNA #34 and #35 caused an impaired cell invasive ability of ABCC4 knockdown MCF-7 cells to migrate to 0.5% v/v FBS cell culture medium relative to 10% v/v FBS untreated MCF-7 cells (Figure 4.10B).

In the case of ABCC1 knockdown in MDA-MB231 cells, the depletion of ABCC1 in MDA-MB231 cells did not inhibit cell migration across the membrane pores to either 10% v/v or 0.5% v/v FBS cell culture medium (Figure 4.11A). Furthermore, the suppression of *ABCC4* gene expression levels in MDA-MB231 cells by ABCC4 siRNA #35 caused a decrease in cell invasion across the membrane pores to either 10% v/v or 0.5% v/v FBS cell culture medium (Figure 4.11B). However, the results obtained may not be reliable due to large degree of variation in data resulting in large error bars especially for MDA-MB231 cells.

Nevertheless, results may indicate that depletion of ABCC4 in breast cancer cells may affect cell invasion more than the depletion of ABCC1 regardless of the chemoattractant used.

4.7   Analysis of the efficiency of ABCC1 and ABCC4 combination knock down in breast cancer cell lines

In order to efficiently knockdown both ABCC1 and ABCC4 in breast cancer cells, we investigated the efficiency of combination siRNA transfection between sequential siRNA

knockdown and simultaneous siRNA knockdown methods. A total of 1 nM of siRNAs (ABCC1 and ABCC4 siRNAs) were used in each siRNA transfection. Therefore, in sequential siRNA knockdown, 1 nM of ABCC1 siRNA were used to transfect the cells and 24 hours later 1 nM of ABCC4 siRNA were added. Therefore, transfected cells were exposed to a total of 1 nM of siRNAs at any one time. Hence, before 1 nM of ABCC4 siRNA was added to the cells, the cell culture medium was removed and replaced with fresh cell culture so that the cells were exposed to 1 nM of siRNA. The transfected cells were incubated for 72 hours after which they were harvested. In the case of simultaneous siRNA knockdown, 0.5 nM of each siRNAs (ABCC1 and ABCC4 siRNAs) were added to the cells at the same time. Similarly, the transfected cells were incubated for 72 hours after which they were harvested. The efficiency of sequential and simultaneous siRNA transfections were determined by Western Blot analysis (Figure 4.12). The combination of siRNAs were chosen randomly, hence siRNA #30 was combined with siRNA #34 and siRNA #31 was combined with siRNA #35.
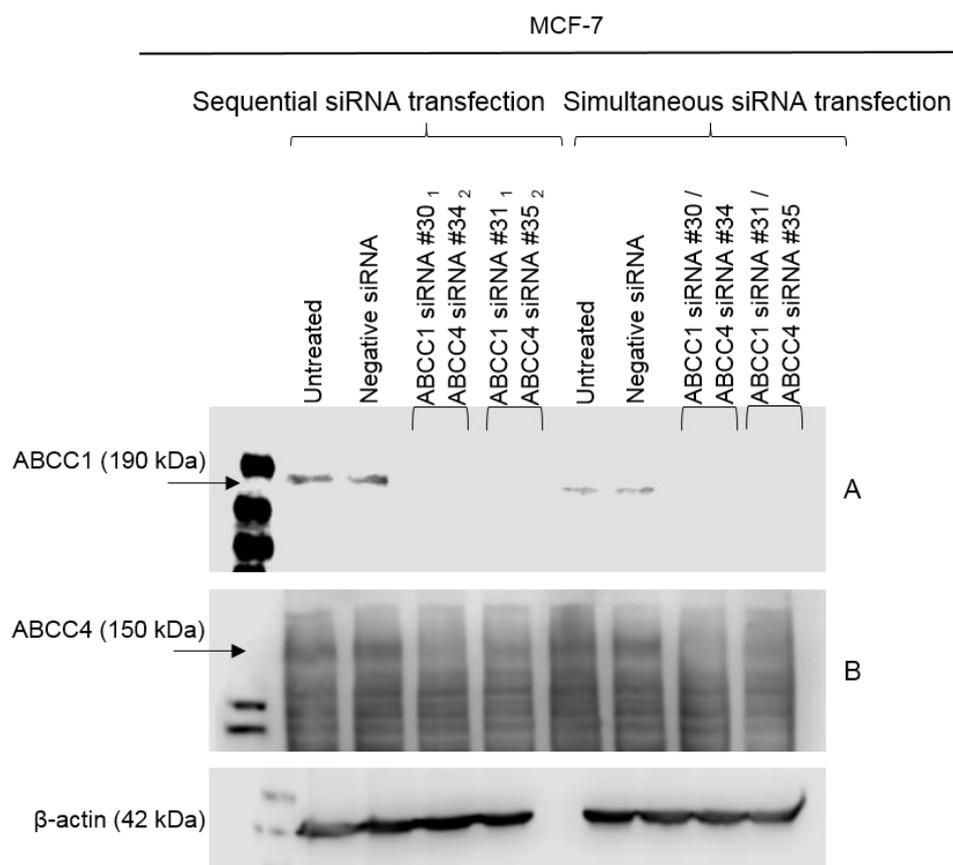
Figure 4.12. Western blot analysis of ABCC1 and ABCC4 protein expression following combination knockdown experiments. MCF-7 breast cancer cells were transfected with ABCC1- and ABCC4-specific siRNA molecules in combination (Combination: ABCC1 siRNA #30 and ABCC1 siRNA #34; ABCC1 siRNA #31 and ABCC4 siRNA #35). A negative siRNA was used as a negative control to offset any non-specific silencing. In sequential siRNA transfection, 1 nM of ABCC1 siRNA was exposed to MCF-7 cells first. After 24 hours incubation, the cell culture medium was replaced with fresh cell culture medium and 1nM of ABCC4 siRNA was exposed to MCF-7 cells. The transfected MCF-7 cells were incubated for 72 hours after which the transfected MCF-7 cells were harvested for Western blot analysis. For simultaneous siRNA transfection, 0.5 nM of ABCC1 siRNA and 0.5 nM of ABCC4 siRNA were exposed to MCF-7 cells at the same time. The transfected MCF-7 cells were incubated for 72 hours after which the transfected MCF-7 cells were harvested for Western blot analysis. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 20 µl of samples were loaded in each lanes. β-actin was used as the loading control. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. Western blot analysis was repeated at least three times and successful silencing of ABCC1 and ABCC4 protein expression in MCF-7 transfected cells were obtained each time.

As can be seen from the ABCC1 Western blot (Figure 4.12 Western blot A), the ABCC1 protein expression is reduced in both protein lysates obtained from MCF-7 cells treated in sequential and simultaneous siRNA transfections when compared to protein lysate from untreated and negative siRNA treated MCF-7 cells. Moreover, it seem that the ABCC1 protein expression levels from the untreated and negative siRNA treated MCF-7 cells under sequential siRNA transfection condition were higher than those under simultaneous siRNA transfection condition. This may be because the untreated and the negative siRNA treated MCF-7 cells in the sequential siRNA condition were left to incubate along side the sequential transfected cells and since the cells were exposed to the first siRNA for 24 hours and after the second siRNA, the cells were all left to incubate for 96 hours while those in simultaneous siRNA transfection condition were altogether incubated for 72 hours. Therefore, the longer incubation time means more cell growth thus the higher quantity of protein. In addition, low cell cytotoxicity was also observed.

Although the Western blot for ABCC4 (Figure 4.12 Western blot B) may not be clear due to high non-specific background, in the lanes for the protein lysates obtained from the untreated and negative siRNA treated MCF-7 cells, a dark band (at 150 kDa mark) can be seen compared to the lanes for protein lysates obtained from combination siRNA treated MCF-7 cells.

Consequently, the efficiency of combination siRNA between sequential and simultaneous siRNA transfection methods were only tested in MCF-7 cells. Therefore, since both sequential and simultaneous siRNA transfection worked, we decided to use simultaneous siRNA transfection as the preferred combination siRNA transfection method for experiments as less time is need for successful transfection. In addition, since the efficiency of combination siRNA using the sequential and simultaneous siRNA transfections methods were only tested in MCF-7 cells, we suspected that there is a high probability that the combination siRNA transfection will work in MDA-MB231 cells. Therefore, MDA-MB231 cells were exposed to ABCC1- and ABCC4-specific siRNA molecules in combination by using simultaneous siRNA transfection method for experiments and the Western blot analysis is shown in figure 4.13.

MDA-MB231

ABCC1 (190 kDa) →   A

ABCC4 (150 kDa) →   B

α-tubulin (50 kDa)
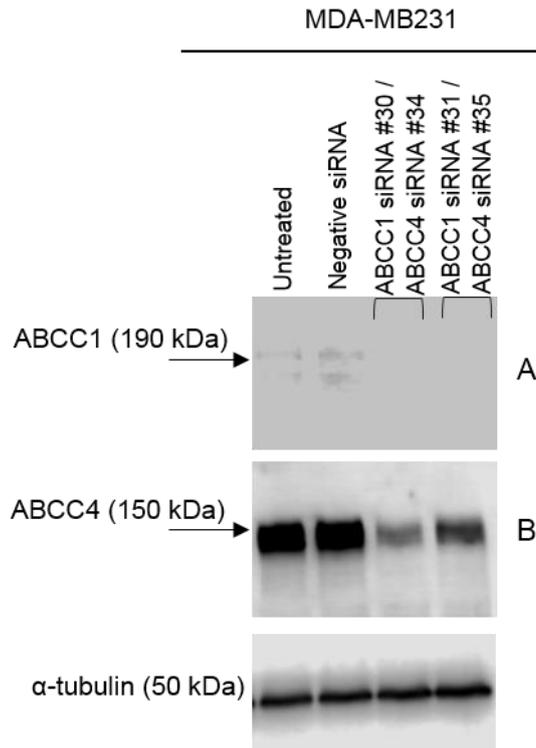
Figure 4.13. Western blot analysis of ABCC1 and ABCC4 protein expression following combination siRNA knockdown. MDA-MB231 breast cancer cells to ABCC1- and ABCC4-specific siRNA molecules in simultaneous combination (Combination: ABCC1 siRNA #30 and ABCC1 siRNA #34; ABCC1 siRNA #31 and ABCC4 siRNA #35). A negative siRNA was used as a negative control to offset any non-specific silencing. 0.5 nM of ABCC1 siRNA and 0.5 nM of ABCC4 siRNA were exposed to MDA-MB231 cells at the same time. The transfected MDA-MB231 cells were incubated for 72 hours after which the transfected MDA-MB231 cells were harvested for Western blot analysis. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 20 μl of samples were loaded in each lanes. α-tubulin was used as the loading control. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. The Western blot analysis was repeated at least three times and similar transfection efficiency were obtained each time.

As can be seen from figure 4.13. Exposure of MDA-MB231 cells to ABCC1 and ABCC4 siRNAs simultaneously, successfully decrease both ABCC1 (Figure 4.13 Western blot A) and ABCC4 (Figure 4.13 Western blot B) protein expressions in MDA-MB231 transfected cells when compared to the untreated MDA-MB231 cells and the MDA-MB231 cells treated with negative siRNA. In addition, ABCC4 knockdown was partial when compared to ABCC1 knockdown.

Therefore, the simultaneous siRNA transfection method was adapted as the method to expose MCF-7 and MDA-MB231 breast cancer cells to both ABCC1 and ABCC4 siRNAs at the same time. The combination siRNA transfected breast cancer cells were used for cellular functional experiments.

4.8 Effects of combination gene knockdown of ABC transporters on cancer cell colony formation

Upon combined depletion of ABCC1 and ABCC4 in breast cancer cells, the transfected cells were seeded for the colony formation assay to assess the ability of a cell to grow in to a colony after both ABCC1 and ABCC4 has been knockdown. Graphs of number of colonies formed for each cell lines are shown in figures 4.14.
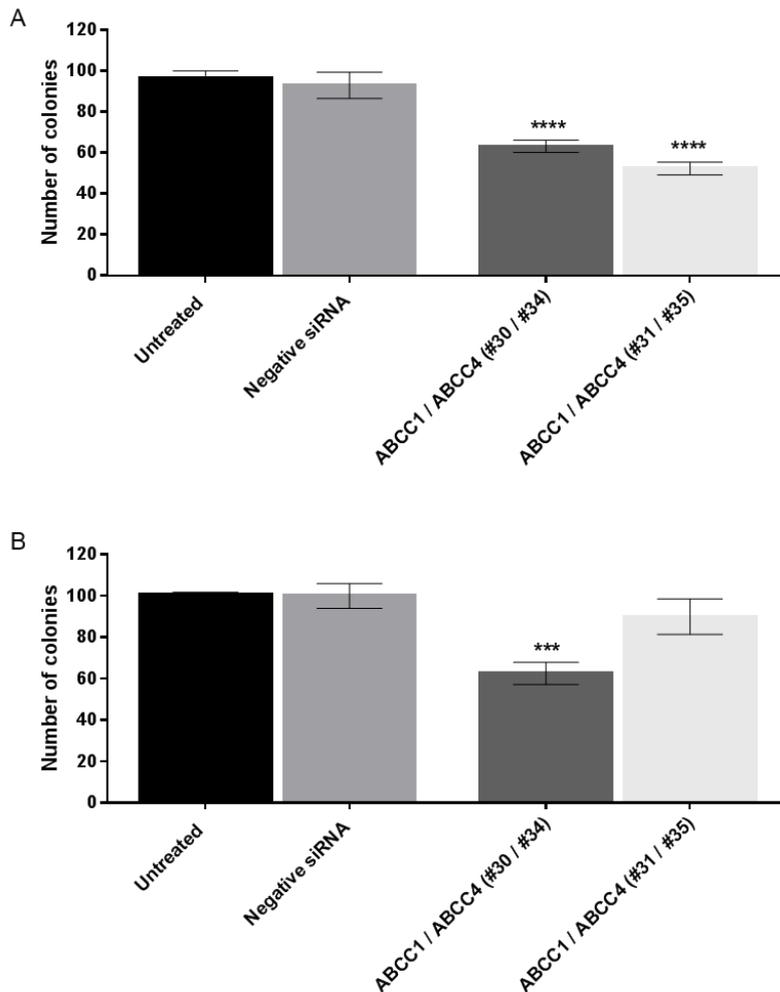
Figure 4.14. The impact of combination suppression of *ABCC1* and *ABCC4* gene expression levels on colony formation. (A) MCF-7 and (B) MDA-MB231 breast cancer cells were transfected with 1 nM of total siRNAs and the clonogenic capacity of the breast cancer cells upon depletion of ABCC1 and ABCC4 was assayed after 7 days. The transfected breast cancer cells were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and were counted manually. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. *** $P < 0.001$ and **** $P < 0.0001$. *P* values were derived from one-way ANOVA vs untreated.

The combination knockdown of *ABCC1* and *ABCC4* genes in MCF-7 cells inhibited the clonogenic capacity of MCF-7 cells to form cell colonies (Figure 4.14A). Moreover, both combination siRNA knockdowns (ABCC1 siRNA #30 and ABCC4 siRNA #34; ABCC1 siRNA #31 and ABCC4 siRNA #35) in MCF-7 cells significantly reduced the number of colonies formed when compared to the untreated MCF-7 cells. Similar effect of reduction in clonogenic capacity of MDA-MB231 cells was seen with one combination of siRNA knockdown (ABCC1 siRNA #30 and ABCC4 siRNA #35). The other combination siRNA knockdown (ABCC1 siRNA #31 and ABCC4 siRNA #35) in MDA-MB231 cells did not significantly affect the clonogenic capacity of MDA-MB231 cells when compared to the untreated MDA-MB231 cells (Figure

4.14B). These results indicate that ABCC1 and ABCC4, together may mediate breast cancer cell growth.

4.9 Effects of combination gene knockdown of ABC transporters on cancer cell viability

To study the impact of combine suppression of *ABCC1* and *ABCC4* gene expression levels in breast cancer cells have on cell viability and cell cytotoxicity, the MTT assay was performed. Graphs showing the absorbance results are as shown in figures 4.15 and 4.16.
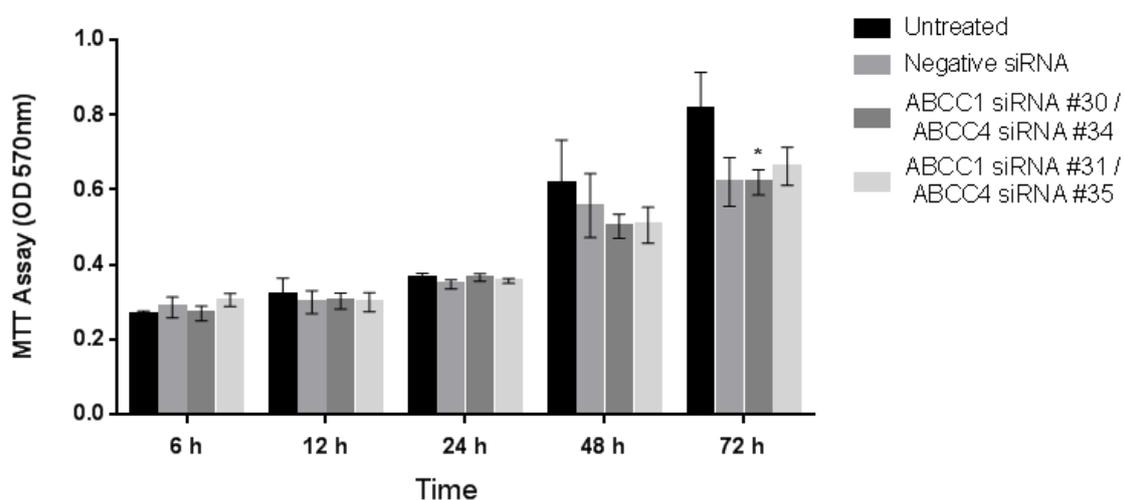
Figure 4.15. Cell viability of combination ABCC1 and ABCC4 knockdown MCF-7 cells. The combination ABCC1 and ABCC4 knockdown MCF-7 cells, the untreated MCF-7 cells were seeded in 24-well plates at a seeding density of 1.5×10^4 cell/well. An MTT assay was performed. The cell viability was assessed after 6, 12, 24, 48 and 72 hours. Briefly, 40 µl of MTT (5 mg/ml) was added to each well. After an additional 1 hour of incubation, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample from each well was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. * $P < 0.05$. *P* values were derived from two-way ANOVA vs untreated.

Figure 4.16. Cell viability of combination ABCC1 and ABCC4 knockdown MDA-MB231 cells. The combination ABCC1 and ABCC4 knockdown MDA-MB231 cells, the untreated MDA-MB231 cells were seeded in 24-well plates at a seeding density of 1.5×10^4 cell/well. An MTT assay was performed. The cell viability was assessed after 6, 12, 24, 48 and 72 hours. Briefly, 40 µl of MTT (5 mg/ml) was added to each well. After an additional 1 hour of incubation, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample from each well was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

The combined suppression of ABCC1 and ABCC4 in MCF-7 cells did not affect cell viability after 6, 12 and 24 hours incubation but may have an effect after 48 and 72 hours incubation as a decrease in cell viability was seen (Figure 4.15). Although decreased growth was observed for double knockdowns at 48 and 72 hours, this was also seen for the negative control siRNA suggesting a non-specific effect. No significant changes in cell viability were observed after combined depletion of ABCC1 and ABCC4 in MDA-MB231 cells (Figures 4.16). These results may indicate that combined depletion of ABCC1 and ABCC4 in MCF-7 cells have a greater effect on cell viability than the combine depletion of ABCC1 and ABCC4 in MDA-MB231 cells. However, it should be noted that transfection efficiency in MDA-MB231 cells may not be as high as in MCF-7 cells.

4.10 Effects of combination gene knockdown of ABC transporters on cancer cell migration

The effect of the double knockdown of ABCC1 and ABCC4 on cell migration were next investigated using the scratch assay. The data collected were analysed using the Cell-IQ analysis software. The percentage wound closure at 6, 12 and 24 hours for the combination

ABCC1 and ABCC4 knockdown MCF-7 and MDA-MB231 cells are as shown in figures 4.17 and 4.18 respectively.
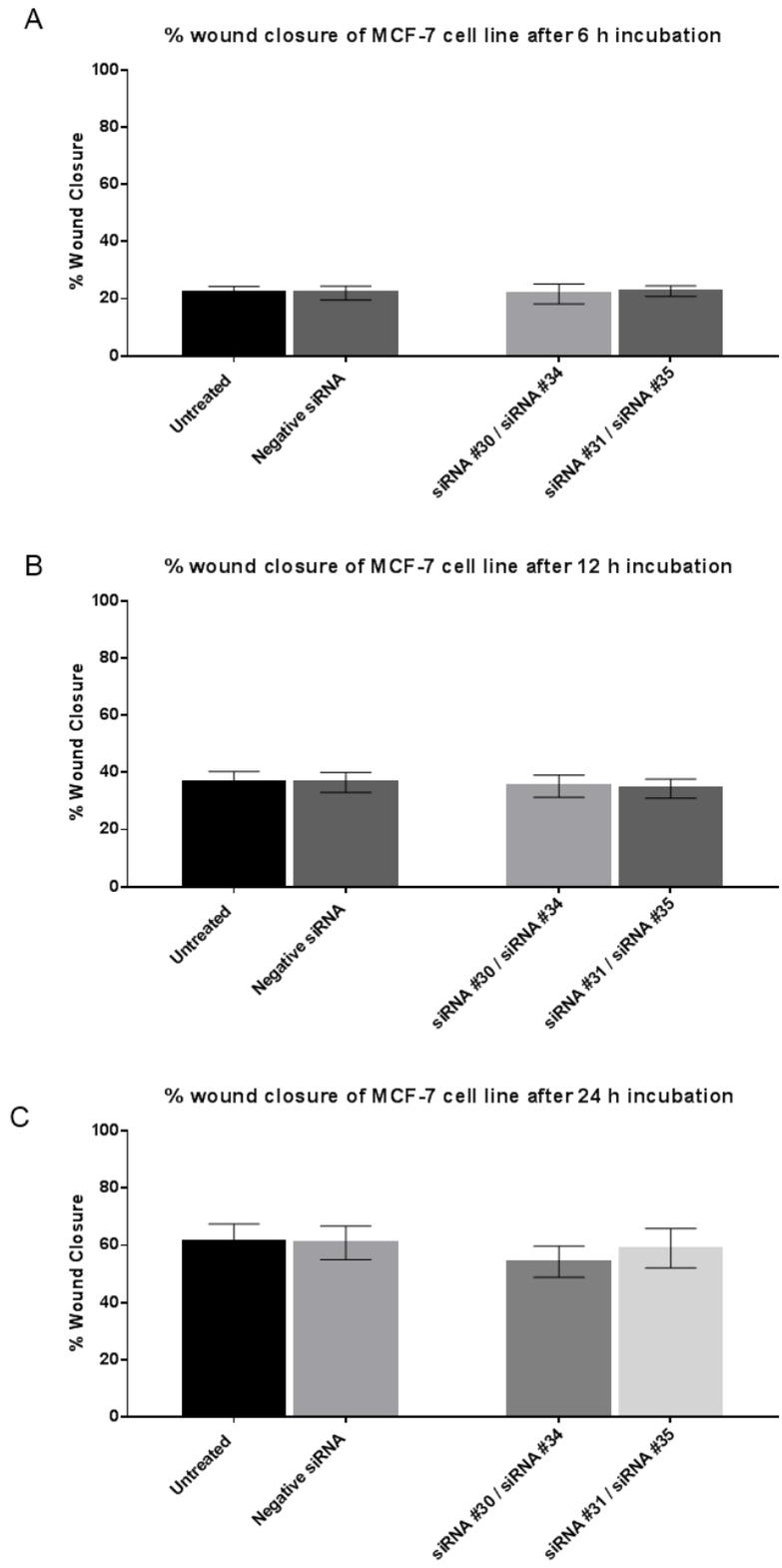
Figure 4.17. Average scratch closure of combination ABCC1 and ABCC4 knockdown MCF-7 cells. The MCF-7 cells transfected with a combination of ABCC1 and ABCC4 siRNAs, were left to incubate for 72 hours. A cell monolayer was formed and a scratch was made in the cell monolayer with a 10 µl pipette tip. The plate was placed in the Cell-IQ where the selected image positions were captured every hour for 24 hours. The captured images were analysed using the Cell-IQ analysis software. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.
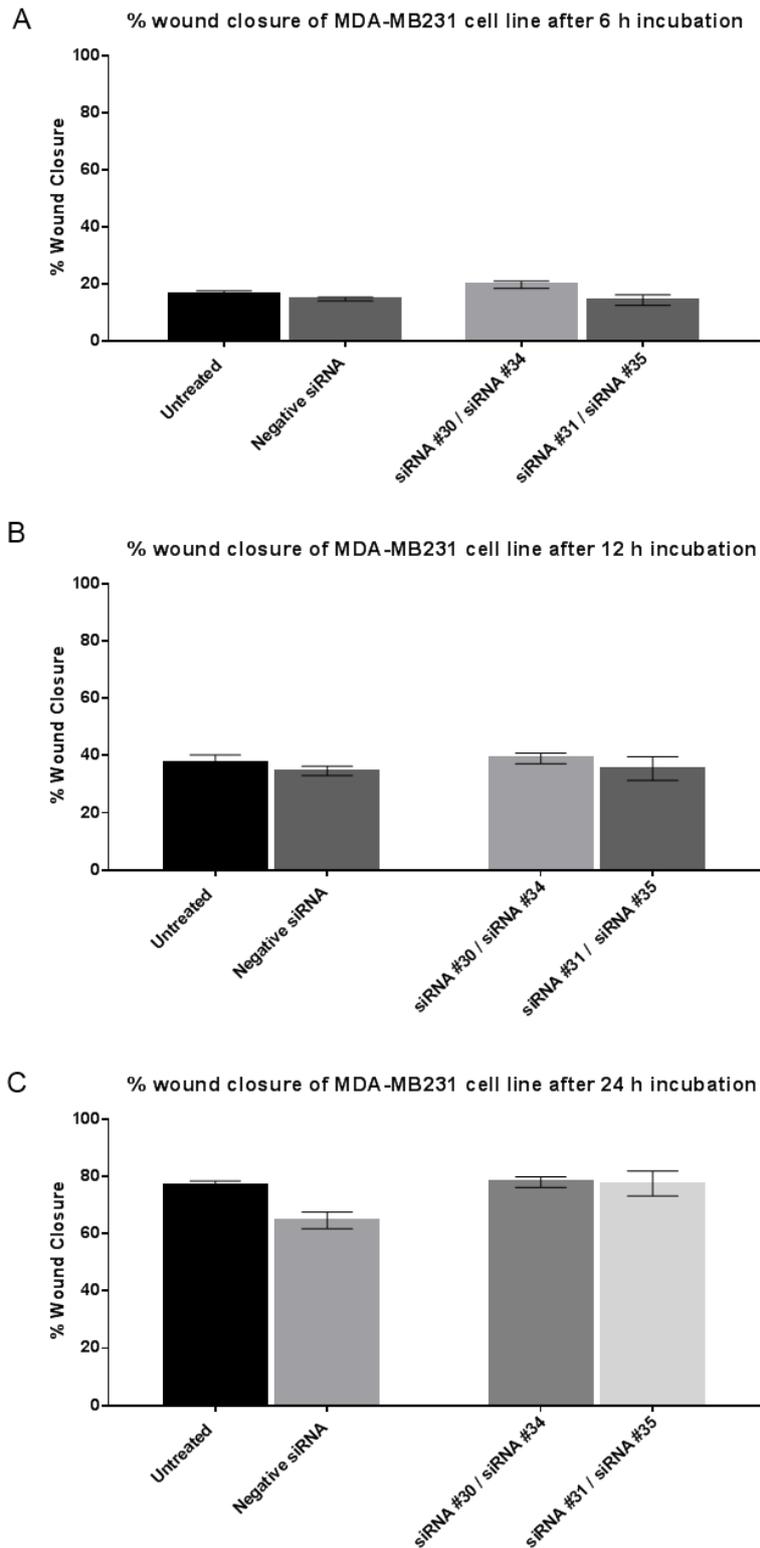
Figure 4.18. Average scratch closure of combination ABCC1 and ABCC4 knockdown MDA-MB231 cells. The MDA-MB231 cells transfected with a combination of ABCC1 and ABCC4 siRNAs, were left to incubate for 72 hours, according to the INTERFERin transfection instructions. A cell monolayer was formed and a scratch was made in the cell monolayer with a 10 µl pipette tip. The plate was placed in the Cell-IQ where the selected image positions were captured every hour for 24 hours. The captured images were analysed using the Cell-IQ analysis software. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

As can be seen from figure 4.17, combined suppression of *ABCC1* and *ABCC4* gene expression levels in MCF-7 cells did not inhibit cell migration. Similarly, the combined depletion of ABCC1 and ABCC4 in MDA-MB231 cells did not affect cell migration (Figure 4.18). At present, it is not clear why this may be the case, since a single knockdown of either ABCC1 or ABCC4 in the breast cancer cells significantly inhibited cell migration. In theory the combine suppression of *ABCC1* and *ABCC4* gene expression levels in breast cancer cells would be expected to cause significant decrease in cell migration beyond that caused by a single gene knock down. Therefore, further investigation may be required.

4.11   Effects of combination gene knockdown of ABC transporters on cancer cell invasion

Finally, the invasive ability of MCF-7 and MDA-MB231 breast cancer cells were investigated using the cell invasion assay upon combined depletion of ABCC1 and ABCC4 in the breast cancer cells. Graphs of percentage invasion following combination ABCC1 and ABCC4 knockdown in MCF-7 and MDA-MB231 cells are shown in figures 4.19 and 4.20 respectively.
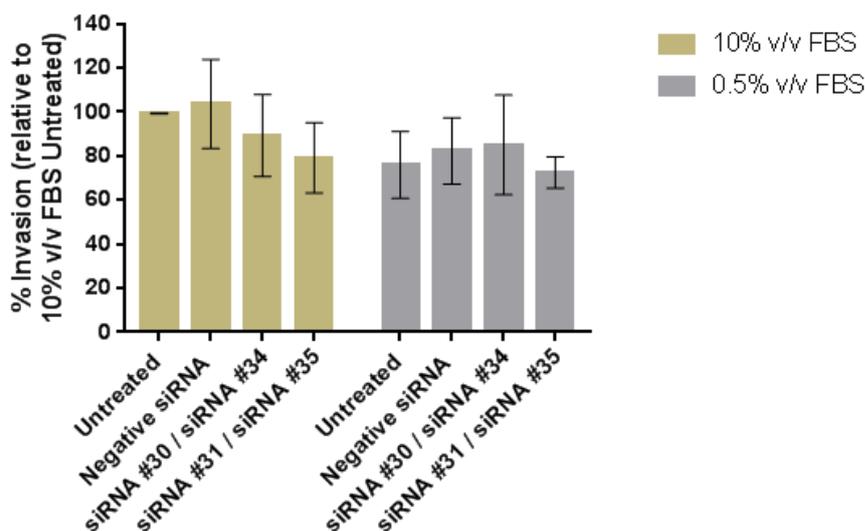
Figure 4.19. Effect of double knockdown of ABCC1 and ABCC4 on MCF-7 cell invasion. Percentage invasion of combination knockdown ABCC1 and ABCC4 in MCF-7 cells across a reconstituted basement membrane *in vitro*. Upon combined depletion of ABCC1 and ABCC4 in MCF-7 cells, the transfected MCF-7 cells were seeded for transwell invasion assay. The transwells were first coated with Corning Matrigel Matrix and a cell suspension of MCF-7 transfected cells in 0.5% v/v FBS cell culture medium were seeded in the transwell insert that was then placed in wells of plate wells containing 10% v/v or 0.5% v/v FBS cell culture medium. After 24 hours incubation, invasive cells that have crossed the membrane were fixed and stained with Differential Quik stain according to manufacturer protocol. Stained cells were counted using a light microscope (x40) from five randomly selected fields of view. Data are

expressed as the mean ± standard error of the mean from at least three independent experiments.
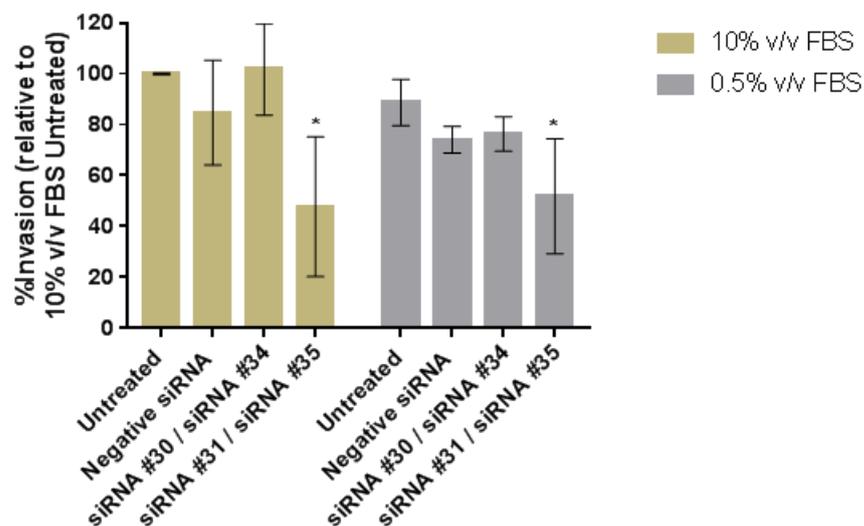
Figure 4.20. Effect of double knockdown of ABCC1 and ABCC4 on MDA-MB231 cell invasion. Percentage invasion of combination knockdown ABCC1 and ABCC4 in MDA-MB231 cells across a reconstituted basement membrane *in vitro*. Upon combined depletion of ABCC1 and ABCC4 in MDA-MB231 cells, the transfected MDA-MB231 cells were seeded for transwell invasion assay. The transwells were first coated with Corning Matrigel Matrix and a cell suspension of MDA-MB231 transfected cells in 0.5% v/v FBS cell culture medium were seeded in the transwell insert that was then placed in wells of plate wells containing 10% v/v or 0.5% v/v FBS cell culture medium. After 24 hours incubation, invasive cells that have crossed the membrane were fixed and stained with Differential Quik stain according to manufacturer protocol. Stained cells were counted using a light microscope (x40) from five randomly selected fields of view. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. * $P < 0.05$. *P* values were derived from two-way ANOVA vs 10% v/v FBS untreated.

While there are some trends with both cell lines suggesting the double knockdowns might cause decreased invasion in both cells (Figures 4.19 and 4.20), a significant decreased in invasion was observed in MDA-MB231 cells after double knocked down with siRNA #31 and #35 (Figure 4.20). However the data shows a large degree of variation resulting in large error bars.

4.12   Summary

- ABCC1 and ABCC4 expression levels in MCF-7 and MDA-MB231 cells were successfully knocked down using ABCC1- and ABCC4-siRNA specific molecules and

INTERFERin as the transfection reagent as confirmed by Western blot analysis and gene expression analysis using RT-PCR.

- The single knockdown of *ABCC1* and *ABCC4* genes did not inhibit the ability of cells to form colonies as compared to the untreated cells. This could suggest that the knockdown of ABCC1 or ABCC4 have no effect on breast cancer cell growth. However, the knockdown of ABCC1 or ABCC4 may be compensated for by other transporters.

- A combination knockdown of ABCC1 and ABCC4 did decrease the number of colonies formed. Hence suggesting that ABCC1 and ABCC4 affect the clonogenic capacity of breast cancer cells synergistically.

- Although there were some suggestions that ABCC1 knockdown has more of an effect on proliferation than ABCC4. However, no significant effects were seen in the combined knockdown.

- The knockdown of ABCC1 and ABCC4 in MCF-7 cells affected cell migration more than the knockdown of ABCC1 and ABCC4 in MDA-MB231 cells. This was especially apparent within the first 6 hours.

- ABCC1 knockdown MCF-7 and ABCC4 knockdown MCF-7 cells have an impaired invasion ability compared to the untreated MCF-7 cells. In comparison, only the knockdown of ABCC4 in MDA-MB231 cells decreased cell invasion. This could suggest that ABCC4 may be involved in cell invasion more than ABCC1.

- Surprisingly, the combination knockdown of ABCC1 and ABCC4 showed no effect on either MCF-7 or MDA-MB231 cell migration using the scratch assay. Also although there were some apparent differences, a significant decreased in invasion was observed in MDA-MB231 cells after double knocked down with siRNA #31 and #35.

- It is important to note that the method of siRNA transfection is transient and it is a knockdown not a knockout.
    - Gene expression usually recovers after 96 to 120 hours after transfection.
    - Since the MDA-MB231 cells may have a higher native expression level of both ABCC1 and ABCC4, even after knockdown they may still have sufficient levels of the proteins.

- The efficiency of transfection was not measured in terms of the proportion of cells that were knocked down in comparison to those that were unaffected. MDA-MB231 cells may be more difficult to transfect than MCF-7 cells, this may be an important consideration.
- Although the double knockdowns did show decreased expression of both proteins on Western blot, this is a bulk measurement and does not prove if both are knocked down with in the same cell.

- The results obtained are preliminary but do suggest that ABCC1 may be involved in cell proliferation, while ABCC4 is implicated in cell migration and invasion.
  - To validate the results obtained a more reliable and efficient method to knockdown MCF-7 and MDA-MB231 cells may be required such as the use of plasmids which express siRNA, or short hairpin RNAs (shRNAs) expressed from plasmids, which can undergo selection to obtain stable cell lines with knockdown of the target protein. Alternatively a more efficient delivery method such as lentivirus, retrovirus and adenovirus based vectors could be beneficial. Alternatively, CRISPR/Cas9 could be trialled to achieve an effective knockout of these genes.

Chapter 5 – Analysis of the effects of gene overexpression of ABC transporters on breast cancer cell lines *in vitro*

5.1 Objective

In chapter 4, the main focus was to knock down *ABCC1* and *ABCC4* genes in breast cancer cells and use these knockdown cells to carry out functional cellular assays to determine whether ABCC1 and ABCC4 have an impact on breast cancer cell proliferation, cell migration and/or cell invasion. In contrast, this chapter, looks to target ABCC1 and ABCC4 in breast cancer cells overexpressing the genes by using specific plasmid DNA. Consequently, the overexpressed ABCC1 and ABCC4 breast cancer cells were used to investigate whether the overexpression of ABCC1 and ABCC4 have an impact in breast cancer cell proliferation, cell migration and/or cell invasion.

In general, plasmids are small double-stranded DNA fragments that can carry genes and can replicate independently from chromosomal DNA. In the field of recombinant DNA technology, plasmids are most commonly used to facilitate the study and manipulation of genes as most plasmids are relatively small with 3000 – 6000 base pairs and are easily replicated in *E. coli.* A typical plasmid has a DNA replication region, a region for the insert of exogenous DNA fragments and an antibiotic-resistance gene (Addgene, 2018).

Using transfection of cell lines with plasmids encoding ABCC1 and ABCC4, it was investigated whether ABCC1 and/or ABCC4 is/are implicated in cell proliferation, migration and invasion by first conducting single overexpression of *ABCC1* and *ABCC4* genes in MCF-7 and MDA-MB231 breast cancer cell lines. The overexpressed ABCC1 and ABCC4 breast cancer cells were then used in cellular functional assays, including the colony formation assay to assess cell growth, MTT assay to assess cell proliferation, scratch assay to assess cell migration and transwell invasion assay to assess cell invasion.

Furthermore, since some ABC transporters transport similar active physiological substrates (Liu et al., 2010; Chen et al., 2010) we hypothesised that the overexpression of ABCC1 and ABCC4 in combination in breast cancer cells may cause a greater significant impact in breast cancer biology than that caused by the overexpression of ABCC1 or ABCC4 alone when compared to the untreated breast cancer cells and those treated with empty vector plasmid. We therefore wanted to investigate whether ABCC1 and ABCC4 work together when they are both overexpressed in a breast cancer cell lines to affect cell proliferation, migration and invasion.

5.2   Plasmid DNA verifications

The plasmid DNAs were a kind gift from Professor Susan Cole's lab. Briefly, the plasmid DNA was transformed into competent cells using the protocol for standard heat-shock transformation of chemically competent bacteria. The transformed bacteria were then grown overnight on agar plate and a colony of bacteria carrying a specific plasmid DNA was selected and inoculated in an overnight liquid LB bacterial culture. Subsequently, plasmid DNA isolation and purification were performed using Thermo Scientific GeneJET Plasmid Miniprep kit. The purified plasmid DNAs were quantified using NanoDrop ™ 1000 spectrophotometer (ThermoFisher Scientific) and samples were stored at -20ºC.

Large quantities of the plasmid DNAs were amplified in *E.Coli* and isolated and purified using 100% kit free plasmid maxiprep. Verification of the plasmid DNAs were performed by transfecting HEK293T kidney embryonic cells with the plasmid DNAs using polyethylenimine (PEI) and running a Western blot analysis on whole cell lysates (Figure 5.1).
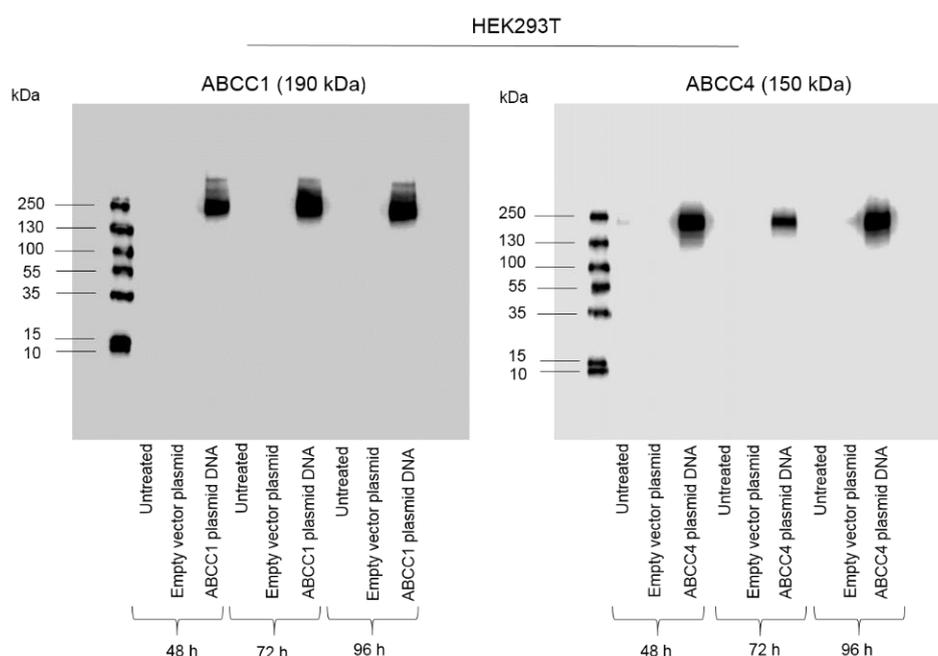
Figure 5.1. Western blot analysis of ABCC1 and ABCC4 protein expression levels derived from whole cell lysate samples from ABCC1 and ABCC4 overexpressed HEK293T cells. HEK293T cells were transfected using PEI at a ratio of 15:1 to plasmid DNA (empty vector plasmid DNA, ABCC1 plasmid DNA and ABCC4 plasmid DNA). The transfected HEK293T cells were incubated for specific time points of 48, 72 and 96 hours. The transfected HEK293T cells were harvested and Western blot analysis was performed. The the molecular weight standards were loaded in lane 1 (Thermo Scientific PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa) and 16 µl of samples were loaded in each lanes. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit

(1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system.

The Western blot analysis indicated that transfection of HEK293T cells with ABCC1 or ABCC4 plasmid DNA led to a marked overexpression of the target protein (Figure 5.1). The native ABCC1 / ABCC4 expression levels in HEK293T cells transfected with empty vector plasmid or untreated HEK293T cells are relatively low hence they were not detected with the exposure used. Polyethyleneimine (PEI) was used as it is a relatively cheap transfection reagent that can produce high transient transfection efficiency in most cell lines (Sou et al., 2013).

5.3   Optimisation of plasmid DNA transfection conditions using Polyethyleneimine (PEI) as a transfection reagent

Polyethyleneimine (PEI) is a cationic polymer that is often used in both small and large-scale transient expression in mammalian cells (Longo et al., 2013) as it is a very cost effective transfection reagent providing 40 to 90% transfection efficiency (Sou et al., 2013). PEI works by forming nucleic acid-polymer complexes which are positively charged. Through electrostatic interaction, the positively charged nucleic acid-polymer complexes interact and adhere to the negatively charged cell membrane and releases the complexes in to the cells via endocytosis (Gibco Life Technologies, 2016).

Therefore, because PEI is a very cost effective transfection reagent that produces highly stable complexes and reproducible results as well as high transfection efficiency in most cell lines, PEI was used as the transfection reagent to transfect cells with the plasmid DNAs. However, different cell lines may require different conditions for optimal transfection. Hence, optimisation of plasmid DNA transfection condition using PEI was performed. Briefly, the cell seeding density of each cell line was determined so as to obtain 60 – 70% cell confluency at the time of transfection. The cells were starved for 3 hours at 37°C and transfection complex of PEI nitrogen to DNA phosphate ratios of 10:1, 15:1 and 20:1 were used to transfect the cells. After 24 hours of transfection, the low FBS (2.5% v/v) cell culture medium was replaced with 10% v/v FBS cell culture medium and cell were left to incubate for 48, 72 and 96 hours. After 48, 72 and 96 hours post transfections, cells were harvested. Subsequently, the protein expression levels of ABCC1 and ABCC4 in the transfected cells were analysed by Western blot analysis (Figure 5.2). All PEI transfections were performed in 6-well plates.
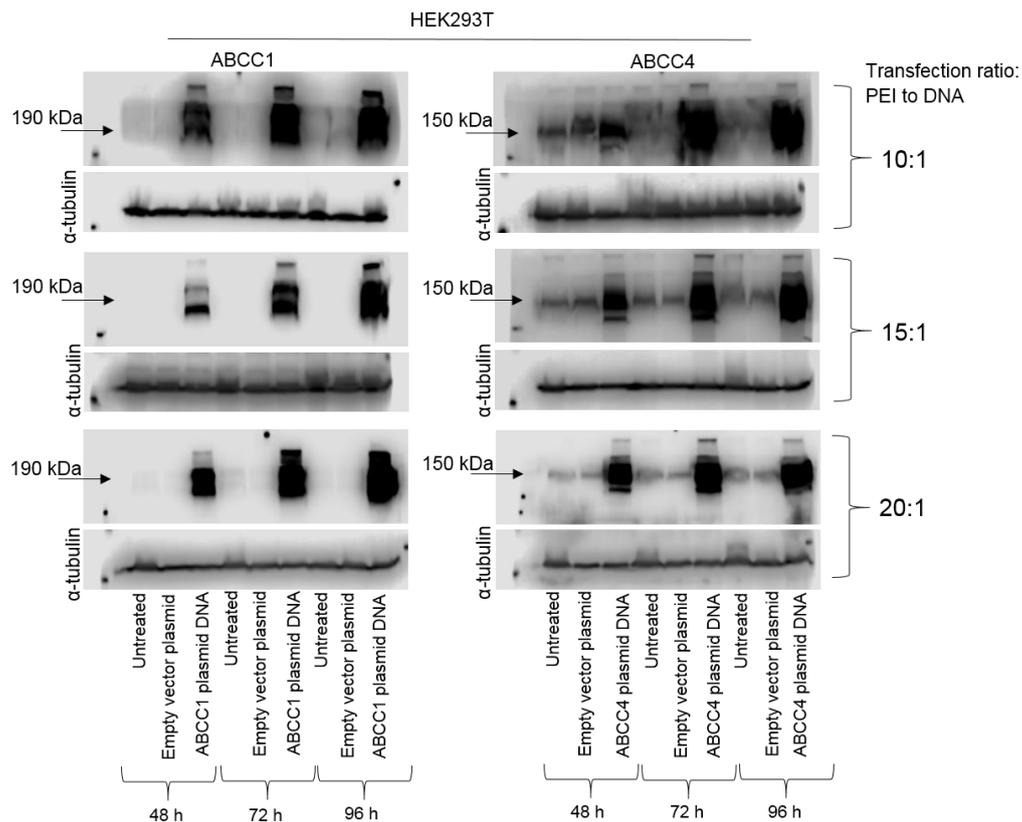
Figure 5.2. Protein expression levels of ABCC1 and ABCC4 in HEK293T cells following PEI transfection with ABCC1 and ABCC4 plasmid DNA respectively. HEK293T cells were transfected with PEI at a ratio of 10:1, 15:1, 20:1 to plasmid DNA (empty vector plasmid, ABCC1 plasmid DNA and ABCC4 plasmid DNA). The transfected HEK293T cells were incubated at specific time points of 48, 72 and 96 hours. At each specific time point, transfected HEK293T cells were harvested and Western blot analysis was performed. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 12 µl of samples were loaded in each lanes. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system.

As an expression tool for recombinant protein in many experimental set up, HEK293T cell line was used. Moreover, HEK293T cells are quick and easy to grow in the lab and produce high transfection efficiency and large amount of protein (Thomas et al., 2005). Therefore, as can be seen from figure 5.2, it is apparent that high levels of ABCC1 and ABCC4 were seen in protein lysate from HEK293T cells transfected with ABCC1 and ABCC4 plasmid DNA respectively as compared to the untreated and the HEK293T cells treated with empty vector plasmid DNA. Consequently, the results suggested that at PEI to DNA ratio of 10:1 and after 96 hours post transfection was the optimal PEI transfection condition as relatively high protein

expression levels of ABCC1 and ABCC4 were obtained in protein lysate from HEK293T cells transfected with ABCC1 and ABCC4 plasmid DNA respectively.

The optimisation of PEI transfection was also performed in HeLa cells as shown in figure 5.3.
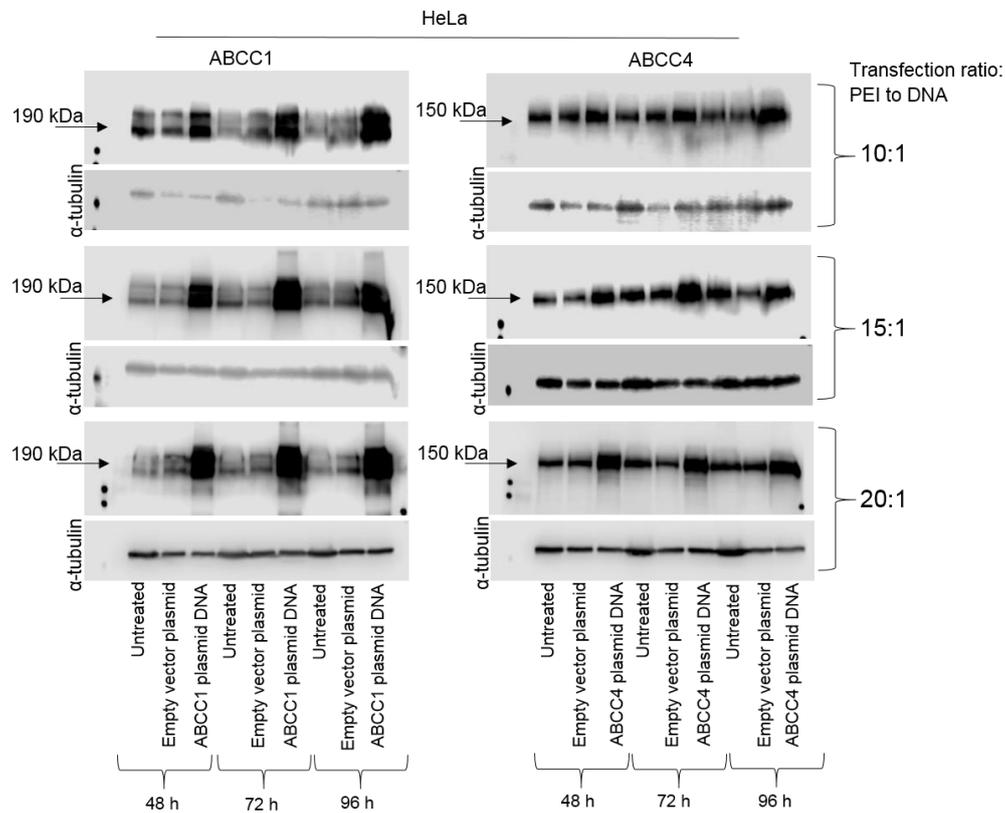
Figure 5.3. Protein expression levels of ABCC1 and ABCC4 in HeLa cells following PEI transfection with ABCC1 and ABCC4 plasmid DNA respectively. HeLa cells were transfected with PEI at a ratio of 10:1, 15:1, 20:1 to plasmid DNA (empty vector plasmid, ABCC1 plasmid DNA and ABCC4 plasmid DNA). The transfected HeLa cells were incubated at specific time points of 48, 72 and 96 hours. At each specific time point, transfected HeLa cells were harvested and Western blot analysis was performed. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 12 µl of samples were loaded in each lanes. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system.

In the case of PEI transfection in HeLa cells, overexpression of ABCC1 in HeLa was obtained after transfection with ABCC1 plasmid DNA at PEI to DNA ratios of 10:1, 15:1 and 20:1 when compared to the untreated HeLa cells and the HeLa cells treated with empty vector plasmid

DNA (Figure 5.3). However, high protein level of ABCC4 in HeLa cells treated with ABCC4 plasmid DNA was significant only at PEI to DNA ratio 20:1. Hence, the results suggested that at PEI to DNA ratio of 20:1 and after 96 hours post transfection was the optimal PEI transfection condition as relatively high protein expression levels of ABCC1 and ABCC4 in HeLa transfected cells were obtained.

Due to the high efficiency of PEI transfection in HEK293T and HeLa cells, attempts to optimise the transfection of ABCC1 and ABCC4 plasmid DNA in MCF-7 and MDA-MB231 breast cancer cells using PEI to try to overexpress the endogenous ABCC1 and ABCC4 were performed and results were analysed using Western blot analysis as shown in figures 5.4 and 5.5.
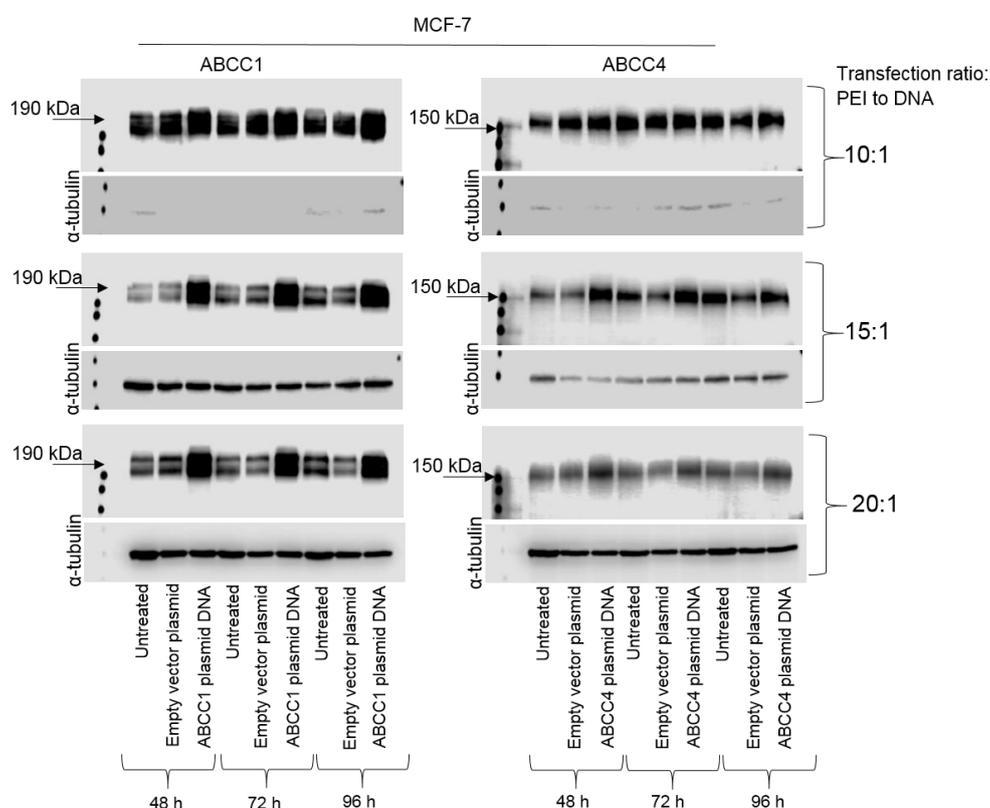
Figure 5.4. Protein expression levels of ABCC1 and ABCC4 in MCF-7 cells following PEI transfection with ABCC1 and ABCC4 plasmid DNA respectively. MCF-7 cells were transfected with PEI at a ratio of 10:1, 15:1, 20:1 to plasmid DNA (empty vector plasmid, ABCC1 plasmid DNA and ABCC4 plasmid DNA). The transfected MCF-7 cells were incubated at specific time points of 48, 72 and 96 hours. At each specific time point, transfected MCF-7 cells were harvested and Western blot analysis was performed. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 12 µl of samples were loaded in each lanes. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor

C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system.

Figure 5.5. Protein expression levels of ABCC1 and ABCC4 in MDA-MB231 cells following PEI transfection with ABCC1 and ABCC4 plasmid DNA respectively. MDA-MB231 cells were transfected with PEI at a ratio of 10:1, 15:1, 20:1 to plasmid DNA (empty vector plasmid, ABCC1 plasmid DNA and ABCC4 plasmid DNA). The transfected MDA-MB231 cells were incubated at specific time points of 48, 72 and 96 hours. At each specific time point, transfected MDA-MB231 cells were harvested and Western blot analysis was performed. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa or 10 to 170 kDA) and 12 μl of samples were loaded in each lanes. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system.

Significant overexpression of ABCC1 in MCF-7 cells was obtained after transfection with ABCC1 plasmid DNA at PEI to DNA ratios of 15:1 and 20:1 when compared to the untreated MCF-7 cells and the MCF-7 cells treated with empty vector plasmid DNA (Figure 5.4). However, protein expression level of ABCC4 in MCF-7 cells treated with ABCC4 plasmid DNA was relatively stable at PEI to DNA ratio 10:1, 15:1 and 20:1 when compared to the untreated

and the MCF-7 cells treated with empty vector plasmid DNA. Hence, the results suggested that using PEI as a transfection reagent to transfect MCF-7 cells with ABCC4 plasmid DNA may not be as effective as when it was used to transfect MCF-7 cells with ABCC1 plasmid DNA. Furthermore, PEI transfection in MDA-MB231 cells with ABCC1 and ABCC4 plasmid DNAs did not cause any increase in protein expression levels of ABCC1 and ABCC4 in MDA-MB231 transfected cells when compared to the untreated MDA-MB231 cells and the MDA-MB231 cells treated with empty vector plasmid DNA at all the transfection condition of PEI to DNA ratio 10:1, 15:1 and 20:1 (Figure 5.5).

Therefore, the results suggested that at transfection condition of PEI to DNA ratios of 15:1 and 20:1 were equally efficient in transfecting MCF-7 cells with ABCC1 plasmid DNA but PEI as a transfection reagent was inefficient in transfecting MCF-7 cells with ABCC4 plasmid DNA. Moreover, PEI was equally inefficient in transfecting MDA-MB231 cells with ABCC1 and ABCC4 plasmid DNAs.

Although, PEI is a very cost effective transfection reagent that produces highly stable complexes and reproducible results as well as high transfection efficiency in most cell lines and was able to transfect MCF-7 cells with low cell cytotoxicity, PEI has proved to be inefficient in transfecting MDA-MB231 cells. Therefore, Lipofectamine 3000® Transfection Reagent, which can also produce high transfection efficiency was suggested.

5.4   Optimisation of plasmid DNA transfection conditions using the Lipofectamine® 3000 reagent as a transfection reagent

The Lipofectamine 3000® Transfection Reagent is a cationic lipid transfection reagent that can facilitate the delivery of DNA and siRNA into cells. Cationic lipids are made up of one or two hydrocarbon chains attached to a positively charged head group. The charged head group promote DNA condensation and mediate the interaction between the phosphate backbone of the nucleic acid and the lipid. The positive surface charge of the liposomes facilitate the fusion of the liposome / nucleic acid transfection complex with the negatively charged cell membrane causing the transfection complex to enter the cell via endocytosis (Gibco Life Technologies, 2016).

Therefore, because The Lipofectamine 3000® reagent has proven to produce simple and fast delivery of plasmid DNA in cells, have a high transection efficiency, works effectively with a wide range of cell types and the results produce are consistent (Gibco Life Technologies, 2016), the Lipofectamine 3000® reagent was selected as an alternative transfection reagent

to transfect cells with the plasmid DNAs. Moreover, as MCF-7 cells and in particular MDA-MB231 cells have proven to be difficult to transfect using PEI, the Lipofectamine 3000® reagent, could be a reliable transfection reagent with a higher transfection efficiency with improved cell viability in difficult-to-transfect cell types (Gibco Life Technologies, 2016). Like with any transfection reagent, optimisation of plasmid DNA transfection condition using The Lipofectamine 3000® reagent was performed.

The transfection of plasmid DNA using the Lipofectamine 3000® reagent was performed using the Lipofectamine 3000® reagent kit (Invitrogen – Thermofisher Scientific). According to the manufacturer's instructions, the plasmid DNA-lipid complex was made up of either 0.75 µl or 1.5 µl of the Lipofectamine 3000® reagent as proposed volume for optimisation transfection condition, 0.5 µg of total plasmid DNA and 1 µg of P3000® reagent (provided from the Lipofectamine 3000® reagent kit) for transfection in 24-well plate. The plasmid DNA-lipid complex was incubated at room temperature for 10-15 minutes and after added to the cells. To improve cell viability, the cell culture medium was replaced with fresh cell culture medium 24 hours after transfection. Subsequently, the transfected cells were incubated for two days, after which the transfected cells were harvested and the protein expression levels of ABCC1 and ABCC4 in the MCF-7 and MDA-MB231 transfected cells were analysed in Western blot analysis and results are as shown in figures 5.6 and 5.7 respectively.
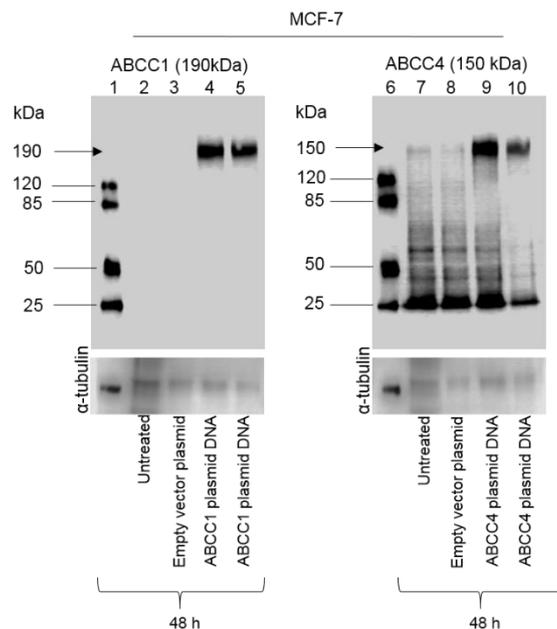
Figure 5.6. Protein expression levels of ABCC1 and ABCC4 in MCF-7 cells following the Lipofectamine® 3000 transfection with ABCC1 and ABCC4 plasmid DNA respectively. According to the manufacturer's instructions, MCF-7 cells were seeded a day before the day of transfection with the optimal cell seeding density for MCF-7 cells was 1.1×10^5 cells per well of 24-well plate so as to reach 70% confluency the day of transfection. Briefly, the plasmid DNA-lipid complex for one well was made by mixing one tube containing 25 µl of optiMEM, 0.5 µg of plasmid DNA and 1 µl of the P3000® Reagent (provided in Lipofectamine 3000® reagent kit) with another tube containing 25 µl of optiMEM and either 0.75 µl or 1.5 µl of Lipofectamine 3000® reagent for optimisation transfection. The plasmid DNA-lipid complex was incubated at room temperature for 10-15 minutes and after added to the cells. Subsequently the transfected cells were incubated for two days after which the transfected cells were harvested and the protein expression levels of ABCC1 and ABCC4 in the MCF-7 transfected cells were analysed in Western blot analysis. The molecular weight standards were loaded in lanes 1 and 6 (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 20 µl of samples were loaded in each lanes. Samples in lanes 2, 3 and 9 were treated with 0.75 µl of Lipofectamine 3000® reagent and samples in lanes 5, 8 and 10 were treated with 1.5 µl of Lipofectamine 3000® reagent. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system.

As can be seen from figure 5.6, the Western blot analysis indicated that the Lipofectamine 3000® reagent efficiently transfected MCF-7 cells with ABCC1 and ABCC4 plasmid DNAs as the protein lysate from MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNA respectively showed higher protein expression levels than those of the untreated and the MCF-7 cells treated with empty vector plasmid DNA. It is to note that MCF-7 cells do express ABCC1 and in this case the blot for ABCC1 expression (Figure 5.6) did not show any expression of ABCC1

in untreated and in empty vector plasmid DNA treated cells, this may be because the relative level of protein expressions were relatively low hence they were not detected with the exposure used. Moreover, using 0.75 µl of Lipofectamine 3000® reagent was able to produce relatively higher protein expression levels of ABCC1 and ABCC4 in ABCC1 and ABCC4 plasmid DNA treated MCF-7 cells than when 1.5 µl of Lipofectamine 3000® reagent was used. Hence, the results suggested that the optimal volume of the Lipofectamine 3000® reagent to use for one transfection in a well of a 24-well plate was 0.75 µl. Therefore, 0.75 µl of Lipofectamine 3000® reagent was used as the optimal volume for overexpression of ABCC1 and ABCC4 in MCF-7 cells for downward cellular functional assays.

Figure 5.7. Protein expression levels of ABCC1 and ABCC4 in MDA-MB231 cells following the Lipofectamine® 3000 transfection with ABCC1 and ABCC4 plasmid DNA respectively. According to the manufacturer's instructions, MDA-MB231 cells were seeded a day before the day of transfection with the optimal cell seeding density for MDA-MB231 cells was 1.5×10^5 cells per well of 24-well plate so as to reach 70% confluency the day of transfection. Briefly, the plasmid DNA-lipid complex for one well was made by mixing one tube containing 25 µl of optiMEM, 0.5 µg of plasmid DNA and 1 µl of the P3000® Reagent (provided in Lipofectamine 3000® reagent kit) with another tube containing 25 µl of optiMEM and either 0.75 µl or 1.5 µl of Lipofectamine 3000® reagent for optimisation transfection. The plasmid DNA-lipid complex was incubated at room temperature for 10-15 minutes and after added to the cells. Subsequently the transfected cells were incubated for two days after which the transfected cells were harvested and the protein expression levels of ABCC1 and ABCC4 in the MCF-7 transfected cells were analysed in Western blot analysis. The molecular weight standards were loaded in lanes 1 and 6 (Thermo Scientific Prestained Protein Molecular

weight marker, 20 to 120 kDa) and 20 µl of samples were loaded in each lanes. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system.

The Western blot analysis indicated that the use of Lipofectamine 3000® reagent as a transfection reagent may not be efficient in transfecting MDA-MB231 cells with ABCC1 and ABCC4 plasmid DNAs as the protein lysate from MDA-MB231 cells treated with ABCC1 and ABCC4 plasmid DNA alone showed no significant increase in protein expression levels than those of the untreated and the MDA-MB231 cells treated with empty vector plasmid DNA (Figure 5.7). However, the protein expression levels of ABCC1 and ABCC4 in the protein lysate from ABCC1 and ABCC4 plasmid DNA treated MDA-MB231 cells may indicate a slight increase when compared to those from the untreated and the MDA-MB231 cells treated with empty vector plasmid DNA.

To further optimised plasmid DNA transfection conditions using Lipofectamine 3000 in MDA-MB231 cells, MDA-MB231 cells were transfected again with ABCC1 and ABCC4 plasmid DNAs respectively using either 0.75 µl, 1.5 µl as well as 2.25 µl of Lipofectamine 3000® reagent in one transfection. The use of a higher volume of Lipofectamine 3000® reagent (2.25 µl) was suggested as assumption that a higher volume may produce higher transfection efficiency. The results are as shown in figure 5.8.
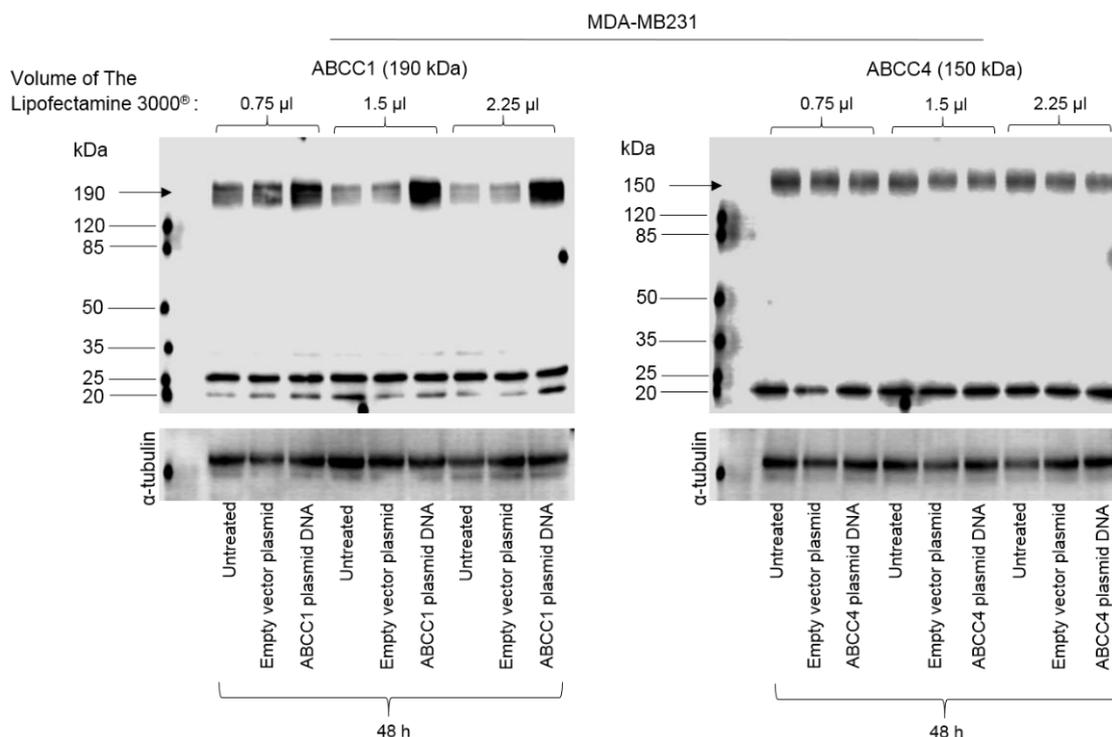
Figure 5.8. Comparison of the protein expression levels of ABCC1 and ABCC4 in MDA-MB231 cells following the Lipofectamine® 3000 transfection with ABCC1 and ABCC4 plasmid DNA respectively. According to the manufacturer's instructions, MDA-MB231 cells were seeded a day before the day of transfection with the optimal cell seeding density for MDA-MB231 cells was 1.5×10^5 cells per well of 24-well plate so as to reach 70% confluency the day of transfection. Briefly, the plasmid DNA-lipid complex for one well was made by mixing one tube containing 25 µl of optiMEM, 0.5 µg of plasmid DNA and 1 µl of the P3000® Reagent (provided in Lipofectamine 3000® reagent kit) with another tube containing 25 µl of optiMEM and either 0.75 µl, 1.5 µl or 2.25 µl of Lipofectamine 3000® reagent for optimisation transfection. The plasmid DNA-lipid complex was incubated at room temperature for 10-15 minutes and after added to the cells. Subsequently the transfected cells were incubated for two days after which the transfected cells were harvested and the protein expression levels of ABCC1 and ABCC4 in the MCF-7 transfected cells were analysed in Western blot analysis. The molecular weight standards were loaded in lanes 1 and 6 (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 16 µl of samples were loaded in each lanes. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system.

As can be seen from figure 5.8, using either 0.75 µl, 1.5 µl or 2.25 µl of Lipofectamine 3000®

reagent was able to produce relatively higher protein expression levels of ABCC1 in ABCC1

plasmid DNA treated MDA-MB231 cells when compared to those from the untreated and the

MDA-MB231 cells treated with empty vector plasmid. As previously shown in figure 5.7,

transfecting MDA-MB231 cells with ABCC1 and ABCC4 plasmid DNAs may not be efficient

as the protein lysate from MDA-MB231 cells treated with ABCC1 and ABCC4 plasmid DNA showed no significant increase in protein expression levels when compared to those of the untreated and the MDA-MB231 cells treated with empty vector plasmid DNA.

These results indicate that MDA-MB231 cells may prove to be a difficult to transfect cell type that even the use of Lipofectamine 3000® reagent may not give a high transfection efficiency. Although the cell viability was not affected during transfection, a lot of factors such as experimental parameters, nature of the plasmid and the method of delivery must be considered to obtain a more efficient transfection. Furthermore, the transfection in one cell batch may varies to another cell batch as using Lipofectamine 3000® reagent produce transient transfection. Thus consideration to use a more effective alternative such as viral mediated transfection or switch to stable cell lines may solve the problem.

Subsequently as shown in figure 5.7, protein expression level of ABCC4 in the protein lysate from ABCC4 plasmid DNA treated MDA-MB231 cells may indicate a slight increase when compared to those from the untreated and the MDA-MB231 cells treated with empty vector plasmid DNA, we decided to still use Lipofectamine 3000® reagent as a transfection reagent to transfect MDA-MB231 cells. Therefore, the volume of the Lipofectamine 3000® reagent used to try to obtain ABCC1 and ABCC4 overexpression in MDA-MB231 cells respectively so as to use the transfected MDA-MB231 cells for downward cellular functional experiments was 1.5 µl per well of 24-well plate.

5.5   Effects of single overexpression of ABC transporters on cancer cell colony formation

Cells transfected with plasmid DNAs following the Lipofectamine 3000® manufacturers' protocol were seeded for the colony formation assay to assess the ability of a single cell to grow in to a colony after ABCC1 and ABCC4 had been overexpressed. Graphs showing the number of colonies formed from MCF-7 and MDA-MB231 cell lines are shown in figures 5.9A and 5.9B respectively.
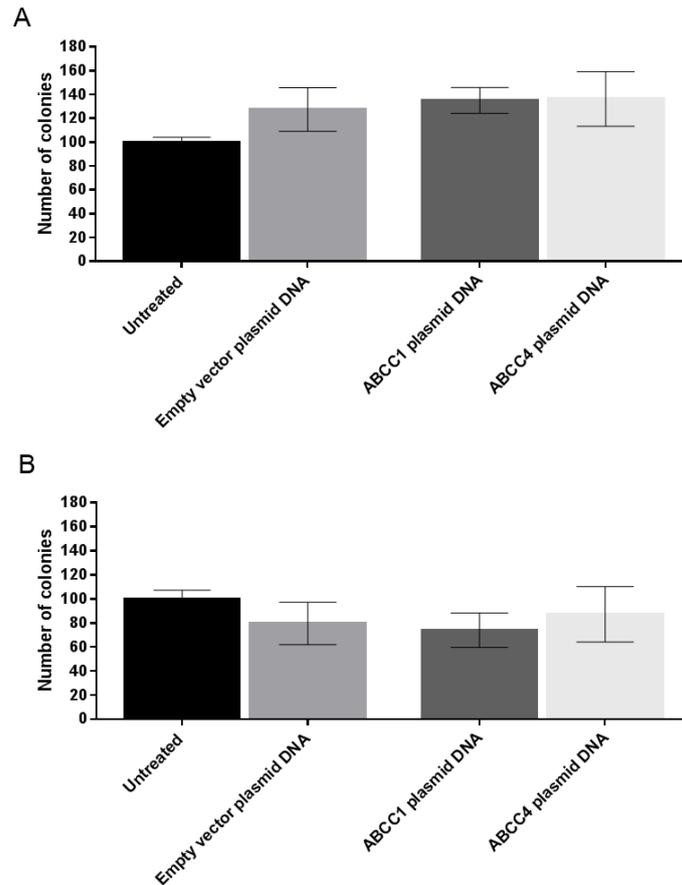
Figure 5.9. The impact of overexpression of *ABCC1* and *ABCC4* gene expression in (A) MCF-7 and (B) MDA-MB231 cells on colony formation. The MCF-7 and MDA-MB231 breast cancer cells were transfected with 0.5 µg of plasmid DNA using the Lipofectamine 3000® reagent and the clonogenic capacity of the breast cancer cells upon overexpression of ABCC1 and ABCC4 was assayed after 7 days. The transfected breast cancer cells were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and were counted manually. The untreated cells are consider the control without any treatment. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

As can be seen in figure 5.9A, the ability of MCF-7 cells to form colonies did increase after ABCC1 and ABCC4 overexpression but the increase was not statistically significant when compared to that of the untreated MCF-7 cells. In fact, the colonies formed from the MCF-7 cells treated with empty vector plasmid DNA did also increased when compared to that of the untreated MCF-7 cells. In contrary, an opposite effect was seen in MDA-MB231 cells, in that the MDA-MB231 cells treated with ABCC1 and ABCC4 plasmid DNA respectively showed no significant decrease in the number of cell colonies formed when compared to that of the untreated MDA-MB231 cells (Figure 5.9B). MDA-MB231 cells treated with empty vector plasmid DNA also showed a small inhibition in clonogenity when compared to that of the untreated MDA-MB231 cells. Similarly, MDA-MB231 cells treated with empty vector plasmid

should not affect the clonogenic capacity as in principle, empty vector plasmid DNA should not contain any DNA fragment. Therefore, it would suggest that any effects seen may simply be caused by the process of transfection.

5.6 Effects of single overexpression of ABC transporters on cancer cell viability

The impact of overexpression of ABCC1 and ABCC4 in breast cancer cells on cell viability and cell cytotoxicity was assessed by performing the MTT assay. Subsequently, transfected cells were seeded in 24-well plate and after 6, 12, 24, 48 and 72 hours the viability of cells was examined (Figures 5.10 and 5.11).
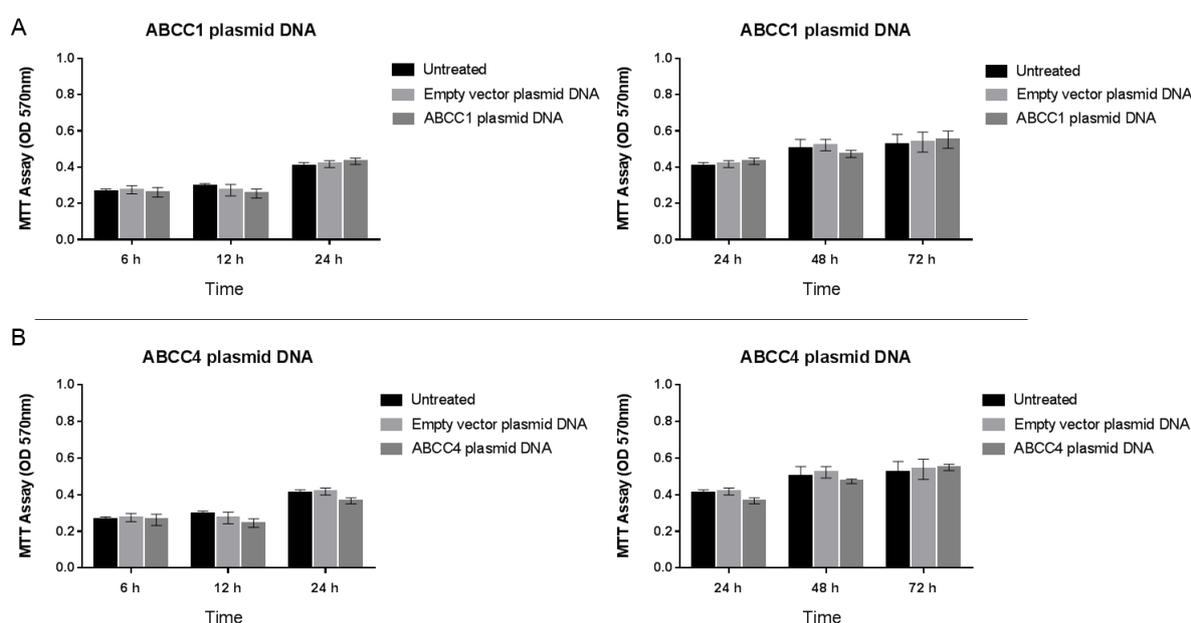
Figure 5.10. Cell viability of MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs respectively. The ABCC1 overexpressed MCF-7 cells, the ABCC4 overexpressed MCF-7 cells and the untreated MCF-7 cells (Control) were seeded in 24-well plates at a seeding density of 1.5×10^4 cell/well. An MTT assay was performed. The cell viability was assessed after 6, 12, 24, 48 and 72 hours. At each specific time point, 40 µl of MTT (5 mg/ml) was added to each well. After an additional one hour of incubation, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.
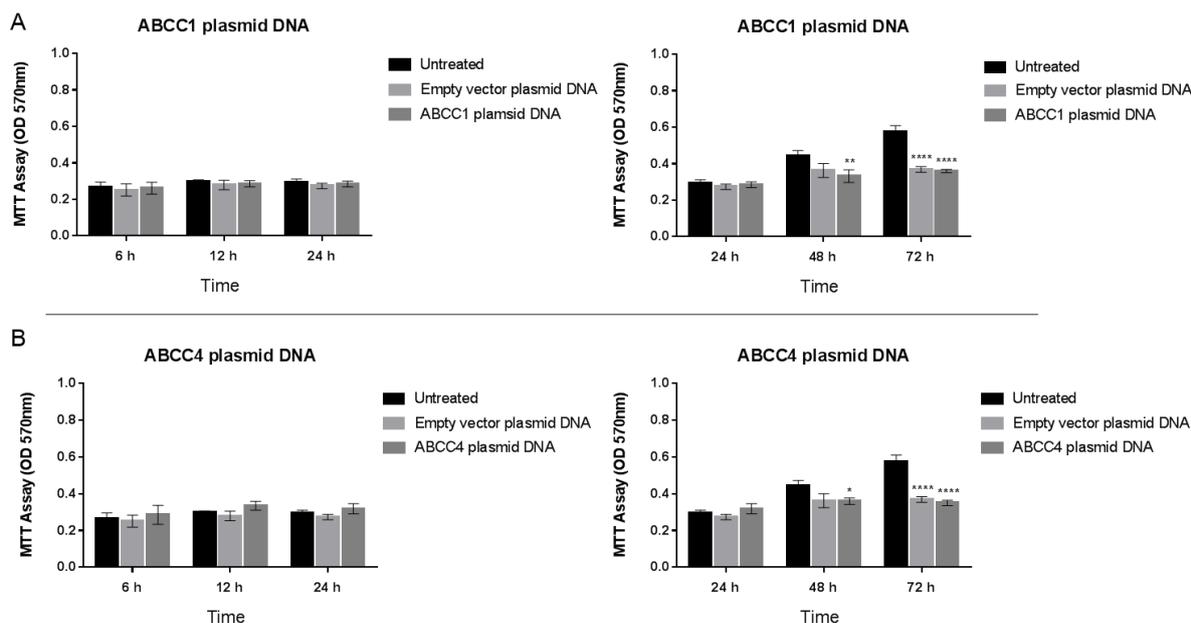
Figure 5.11. Cell viability of MDA-MB231 cells treated with ABCC1 and ABCC4 plasmid DNAs respectively. The ABCC1 overexpressed MDA-MB231 cells, the ABCC4 overexpressed MDA-MB231 cells and the untreated MDA-MB231 cells (Control) were seeded in 24-well plates at a seeding density of 1.5×10^4 cell/well. An MTT assay was performed. The cell viability was assessed after 6, 12, 24, 48 and 72 hours. At each specific time point, 40 µl of MTT (5 mg/ml) was added to each well. After an additional one hour of incubation, the cell culture medium was removed from each well and 400 µl of DMSO was added. 100 µl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. $*P < 0.05$, $** P < 0.01$ and $**** P < 0.0001$. P values were derived from 2-way ANOVA vs untreated.

As can be seen from figures 5.10A and 5.10B, the cell viability of MCF-7 cells were not affected at any specific time points when they were treated with ABCC1 and ABCC4 plasmid DNAs when compared to those of untreated and MCF-7 cells treated with empty vector plasmid DNA. Therefore, the overexpression of ABCC1 and ABCC4 in MCF-7 cells did not cause any significant increase in cell viability. Similarly, the cell viability of MDA-MB231 cells were also not affected at 6, 12 and 24 hour time points when they were treated with ABCC1 or ABCC4 plasmid DNAs alone when compared to those of untreated and MDA-MB231 cells treated with empty vector plasmid (Figure 5.11A). However, an unexpected significant decrease in cell viability was seen for the transfected MDA-MB231 cells after 48 and 72 hours (Figure 5.11B). This was observed for both ABCC1 and ABCC4 transfections as well as transfection with the empty vector. These results may indicate that transfection with ABCC1 or ABCC4 in MDA-MB231 cells did not increase in cell viability as was seen in MCF-7 cells. However, the significant decrease in cell proliferation observed in transfected MDA-MB231 cells at longer time scales (48 and 72 hours) may suggest that the plasmids and/or transfection process may

cause cell cytotoxicity. Nevertheless, these results suggested that overexpression of ABCC1 and ABCC4 in MCF-7 and MDA-MB231 cells may not have a direct effect on cell viability.

5.7   Effects of single overexpression of ABC transporters on cancer cell migration

Upon overexpression of ABCC1 and ABCC4 in MCF-7 and MDA-MB231 breast cancer cells in 24-well plate with ABCC1 and ABCC4 plasmid DNAs respectively. The transfected cells were incubated for 48 hours post transfection according to the Lipofectamine 3000® reagent transfection instructions. By 48 hours post transfection, a cell monolayer was formed and a scratch assay was performed to monitor cell migration.

The percentage wound closure at 6, 12 and 24 hours for the MCF-7 and MDA-MB231 cells are shown in figures 5.12 and 5.13 respectively.
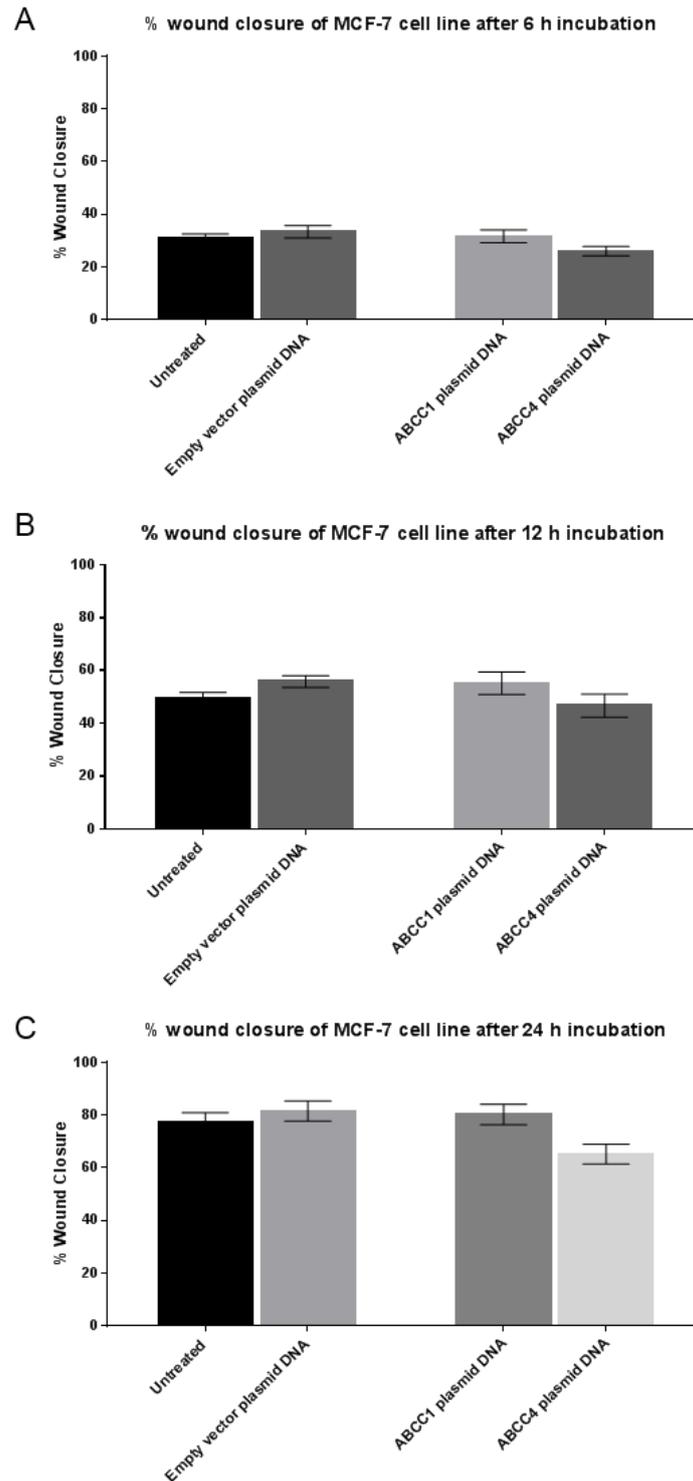
Figure 5.12. Percentage wound closure of MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs alone. After transfection of MCF-7 cells with ABCC1 and ABCC4 plasmid DNAs alone, the transfected cells were left to incubate for 48 hours. Consequently, a cell monolayer was formed and a scratch with a 10 µl pipette tip was made in the cell monolayer. The plate was placed in the Cell-IQ where the selected image positions were captured every hour for 24 hours. The captured images were analysed in the Cell-IQ analysis software. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

A

% wound closure of MDA-MB231 cell line after 6 h incubation

B

% wound closure of MDA-MB231 cell line after 12 h incubation

C

% wound closure of MDA-MB231 cell line after 24 h incubation
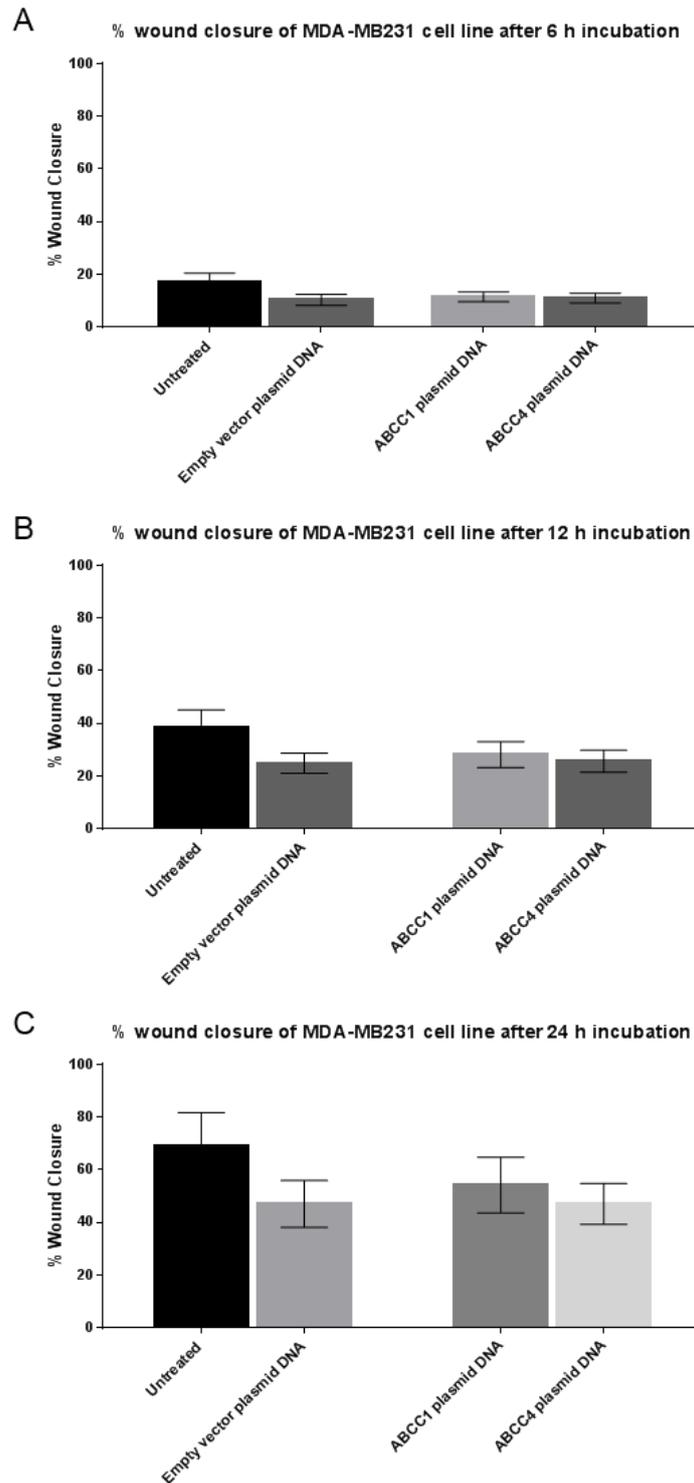
Figure 5.13. Percentage wound closure of MDA-MB231 cells treated with ABCC1 and ABCC4 plasmid DNAs alone. After transfection of MDA-MB231 cells with ABCC1 and ABCC4 plasmid DNAs alone, the transfected cells were left to incubate for 48 hours. Consequently, a cell monolayer was formed and a scratch with a 10 μl pipette tip was made in the cell monolayer. The plate was placed in the Cell-IQ where the selected image positions were captured every hour for 24 hours. The captured images were analysed in the Cell-IQ analysis software. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

The transfection of MCF-7 with ABCC1 or ABCC4 plasmid DNA did not significantly affect the cell motility when compared to the untreated and the MCF-7 cells treated with empty vector plasmid DNA (Figure 5.12A, 5.12B and 5.12C). In contrast, transfection of MDA-MB231 cells with ABCC1, ABCC4 or empty vector plasmid DNAs caused a decreased in cell motility (Figure 5.13A, 5.13B and 5.13C). Again this is most likely linked to the effect that the transfection procedure has on these cells.

5.8   Effects of single gene knockdown of ABC transporters on cancer cell invasion

To determine whether the overexpression of ABCC1 and ABCC4 in MCF-7 and MDA-MB231 breast cancer cells have an effect on cell invasion, the transwell invasion assay was performed.

Similar to the setup for assessing cell invasion of MCF-7 and MDA-MB231 cells treated with ABCC1 and ABCC4 siRNAs, 5.0×10^4 cells suspended in 0.5% v/v FBS cell culture medium were seeded in each transwell. The chemoattractant used were 0.5% v/v and 10% v/v FBS cell culture medium. The transfected cells were incubated for 24 hours after which the transfected cells that migrated across the membranes pores were fixed and stained with Differential Quik stain (Polysciences, Inc.) according to manufacturer protocol. The percentage cell invasion, was determined by counting the stained migrated cells from 5 randomly selected fields of view under a light microscope at a magnification of x40. Graphs of percentage invasion of MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs alone were plotted against the plasmid DNAs as shown in figure 5.14.

% Invasion of plasmid DNA transfected MCF-7 cells
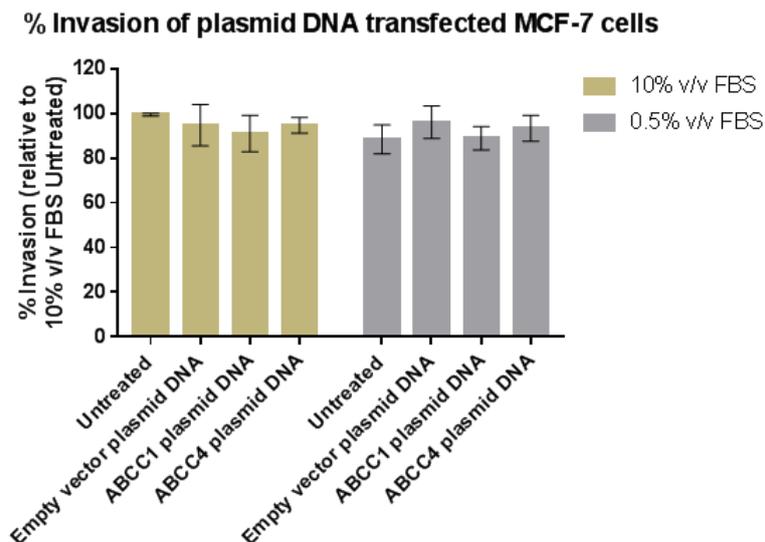
Figure 5.14. Effect of MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs alone on cell invasion across a reconstituted basement membrane *in vitro*. Upon treatment of MCF-7 cells with ABCC1 and ABCC4 plasmid DNAs alone, the cell invasion was assessed by performing the transwell invasion assay. The transwells were first coated with Corning Matrigel Matrix and a cell suspension of the transfected MCF-7 cells in 0.5% v/v FBS cell culture medium were seeded in the transwells. Cell culture medium containing 10% v/v or 0.5% v/v FBS were used as the chemoattractant. After 24 hours incubation, invasive cells that have crossed the membrane pores were fixed and stained. Stained cells were counted using a light microscope (x40) from five different fields of view. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

Normally, more cells are expected to migrate to 10% v/v FBS cell culture medium compared to 0.5% v/v FBS cell culture medium. However, as can be seen from figure 5.14, MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs alone did not affect cell invasion when compared to untreated and MCF-7 cells treated with empty vector plasmid DNA. These results may indicate that an overexpression of ABCC1 and ABCC4 may not affect cell invasion.

Furthermore, in the case of MDA-MB231 cells, we did not perform transwell invasion assay on these cells because the others assays (Colony formation, MTT and scratch assays) indicated a reduction in cell viability when the MDA-MB231 cells were treated with the plasmid or the transfection procedure DNAs that may be due to the nature of the plasmid more than to do with the transporters.

5.9 Analysis of the efficiency of co-overexpression of ABC transporters in breast cancer cell lines

5.9.1 Protein expression analysis by Western blot

One of the major factors contributing to failure in chemotherapy is due to multidrug resistance that occur because of the overexpression of specific ABC transporters in the cancer cells and tumours (Sun et al., 2012). Furthermore, the current finding indicate that these ABC transporters may also be implicated in cancer cell proliferation, migration and invasion (Adamska et al., 2018). Therefore, the hypothesis is if more than one ABC transporters are overexpressed, not only will cause higher multidrug resistance (Mlejnek et al., 2017) but also will increase the cancer cell proliferation, migration and invasion more than if just only one ABC transporter is being overexpressed.

Therefore, this section looks at whether co-overexpression of ABCC1 and ABCC4 in MCF-7 breast cancer cells has an effect on cancer cell proliferation, migration and invasion. Only MCF-7 cells were included because the transfection of MDA-MB231 cells was not very effective in overexpression of the target proteins, and the transfection procedure itself appeared to affect the properties of the cells. An analysis of the efficiency of co-overexpression of ABCC1 and ABCC4 in the breast cancer cells was performed by Western blot analysis. Initially, breast cancer cells were transfected with plasmid DNAs using the Lipofectamine 3000® reagent. According to the manufacturer's instructions, the plasmid DNA-lipid complex was made up of either 0.75 µl, 1.5 µl or 2.25 µl of the Lipofectamine 3000® for optimisation transfection condition, 0.5 µg of total plasmid DNA (0.25 µg of ABCC1 and ABCC4 plasmid DNAs) and 1 µl of P3000® reagent (provided from the Lipofectamine 3000® reagent kit). Subsequently, the transfected cells were incubated for two days. The transfected cells were harvested and a Western blot analysis was performed to determine the protein expression levels of ABCC1 and ABCC4 in the MCF-7 transfected cells (Figures 5.15).
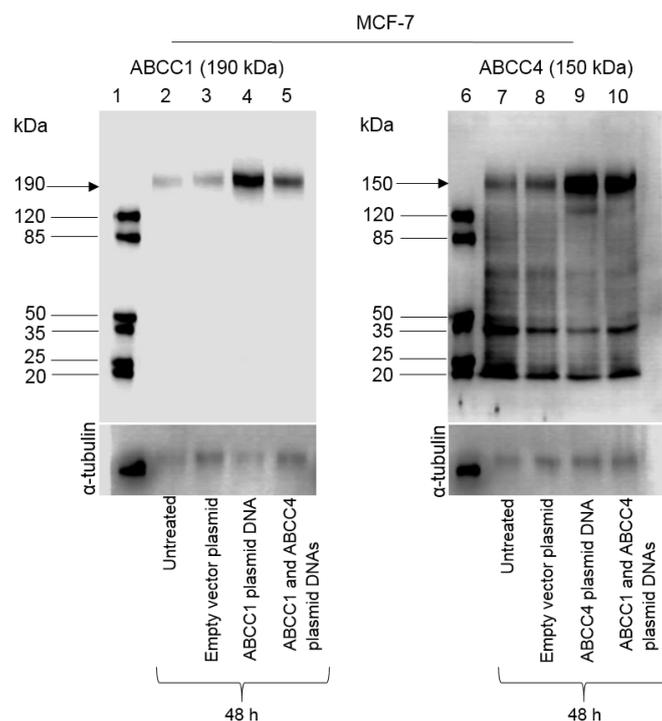
Figure 5.15. Western blot analysis of co-overexpression of ABCC1 and ABCC4 in MCF-7 cells following the Lipofectamine® 3000 transfection with ABCC1 and ABCC4 plasmid DNAs in combination. The plasmid DNA-lipid complex for one well was made by mixing one tube containing 25 µl of optiMEM, 0.5 µg of total plasmid DNAs and 1 µl of the P3000® Reagent (provided in Lipofectamine 3000® reagent kit) with another tube containing 25 µl of optiMEM and 0.75 µl of the Lipofectamine 3000® reagent. The transfected cells were incubated for two days and were harvested for protein analysis. The molecular weight standards were loaded in lanes 1 and 6 (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa) and 20 µl of samples were loaded in each lanes. The PVDF membrane blots for ABCC1 and ABCC4 proteins were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ABCC1 derived in rabbit (1:1000 in blocking buffer) and anti-ABCC4 derived in rat (1:100 in blocking buffer) respectively. The secondary antibodies used were anti-rabbit HRP (1:3000 in TBS-T) and anti-rat HRP (1:5000 in TBS-T). The level of protein expressions were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system.

Initially, 0.75 µl of Lipofectamine® 3000 reagent was found to give a higher protein expression levels in single overexpression of ABCC1 and ABCC4 alone in MCF-7 cells. Therefore, only 0.75 µl of Lipofectamine® 3000 reagent was used in co-expression of ABCC1 and ABCC4 in MCF-7 cells. Therefore, as can be seen from figure 5.15, clearly indicated that co-overexpression of ABCC1 and ABCC4 was achieved in MCF-7 cells as the protein expression levels of ABCC1 and ABCC4 in the protein lysate from MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs in combination was higher than those of the untreated and the MCF-7 cells treated with empty vector plasmid DNA. Therefore, similarly, co-overexpression of ABCC1 and ABCC4 in MCF-7 cells was performed using 0.75 µl of Lipofectamine® 3000 reagent for all downward cellular functional experiments.

5.10 Effects of co-overexpression of ABC transporters on cancer cell colony formation

Initially, MCF-7 breast cancer cells were treated with ABCC1 and ABCC4 plasmid DNAs in combination. The colony formation assay was performed to assess the ability of a single cell to grow in to a colony after co-overexpression of ABCC1 and ABCC4. The number of colonies formed from MCF-7 cell lines are shown in figure 5.16.
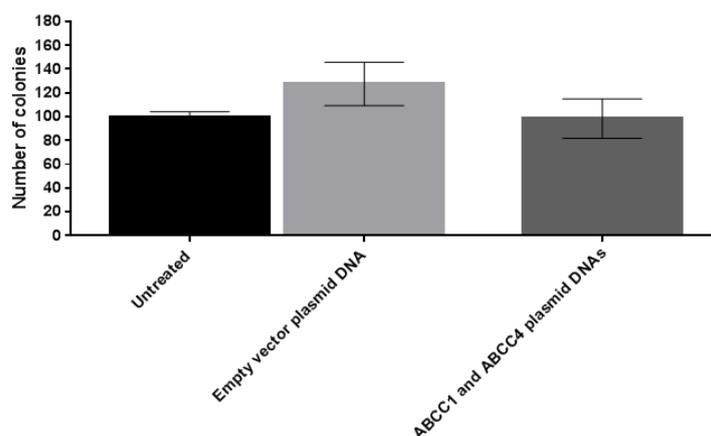
Figure 5.16. The impact of co-overexpression of ABCC1 and ABCC4 in MCF-7 breast cancer cells on colony formation. MCF-7 breast cancer cells were transfected with 0.5 µg of total plasmid DNAs using the Lipofectamine 3000® reagent and the clonogenic capacity of the breast cancer cells upon co-overexpression of ABCC1 and ABCC4 was assayed after 7 days. The transfected breast cancer cells were fixed and stained with PFA and crystal violet respectively. Stained cell colonies were visible and were counted manually. The untreated cells are consider the control without any treatment. Data are expressed as the mean ± standard error of the mean from at least three independent experiments.

As can be seen from figure 5.16, co-overexpression of ABCC1 and ABCC4 in MCF-7 cells did not affect colony formation when compared to those of the untreated and the MCF-7 cells treated with empty vector plasmid DNA.

5.11 Effects of co-overexpression of ABC transporters on cancer cell viability

MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs in combination was also used to assess the effect of co-overexpression of ABCC1 and ABCC4 have on cell viability and cell cytotoxicity by performing the MTT assay. Graphs showing the absorbance results are shown in figure 5.17.
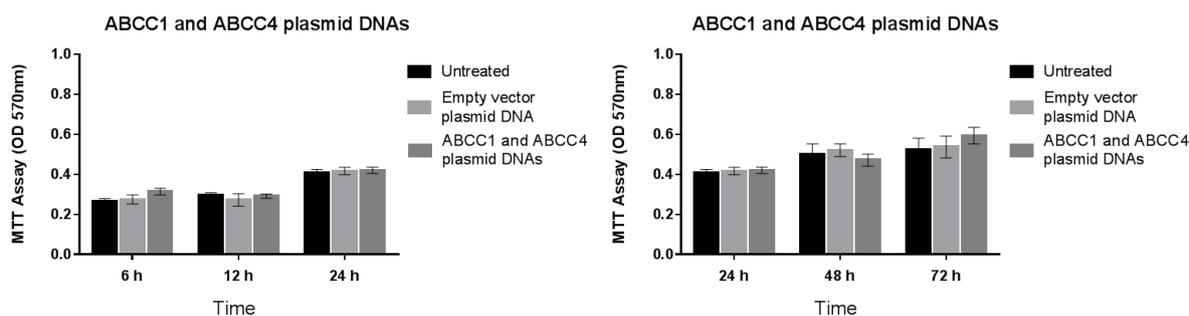
Figure 5.17. Cell viability of MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs in combination. Breast cancer cells treated with ABCC1 and ABCC4 plasmid DNAs in combination were seeded in 24-well plates at a seeding density of 1.5×10^4 cell/well. An MTT assay was performed. The cell viability was assessed after 6, 12, 24, 48 and 72 hours. At each specific time point, 40 μl of MTT (5 mg/ml) was added to each well. After an additional 1 hour of incubation, the cell culture medium was removed from each well and 400 μl of DMSO was added. 100 μl of each sample was transferred into a 96-well plate in triplicates. The absorbance values were read at 570 nm. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. *$P < 0.05$ and ****$P < 0.0001$. P values were derived from two-way ANOVA vs untreated.

As can be seen from figure 5.17, the cell viability of MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs in combination were not affected at any specific time points when compared to those of the untreated and the MCF-7 cells treated with empty vector plasmid DNA. Therefore, the co-overexpression of ABCC1 and ABCC4 in MCF-7 cells did not cause any significant increase in cell proliferation.

5.12   Effects of co-overexpression of ABC transporters on cancer cell migration

To assess the impact of co-overexpression of ABCC1 and ABCC4 in MCF-7 cells, the scratch assay was performed. The percentage wound closure at 6, 12 and 24 hours for the MCF-7 cells were plotted against the plasmid DNAs as shown in figure 5.18.

A

% wound closure of MCF-7 cell line after 6 h incubation

B

% wound closure of MCF-7 cell line after 12 h incubation

C

% wound closure of MCF-7 cell line after 24 h incubation
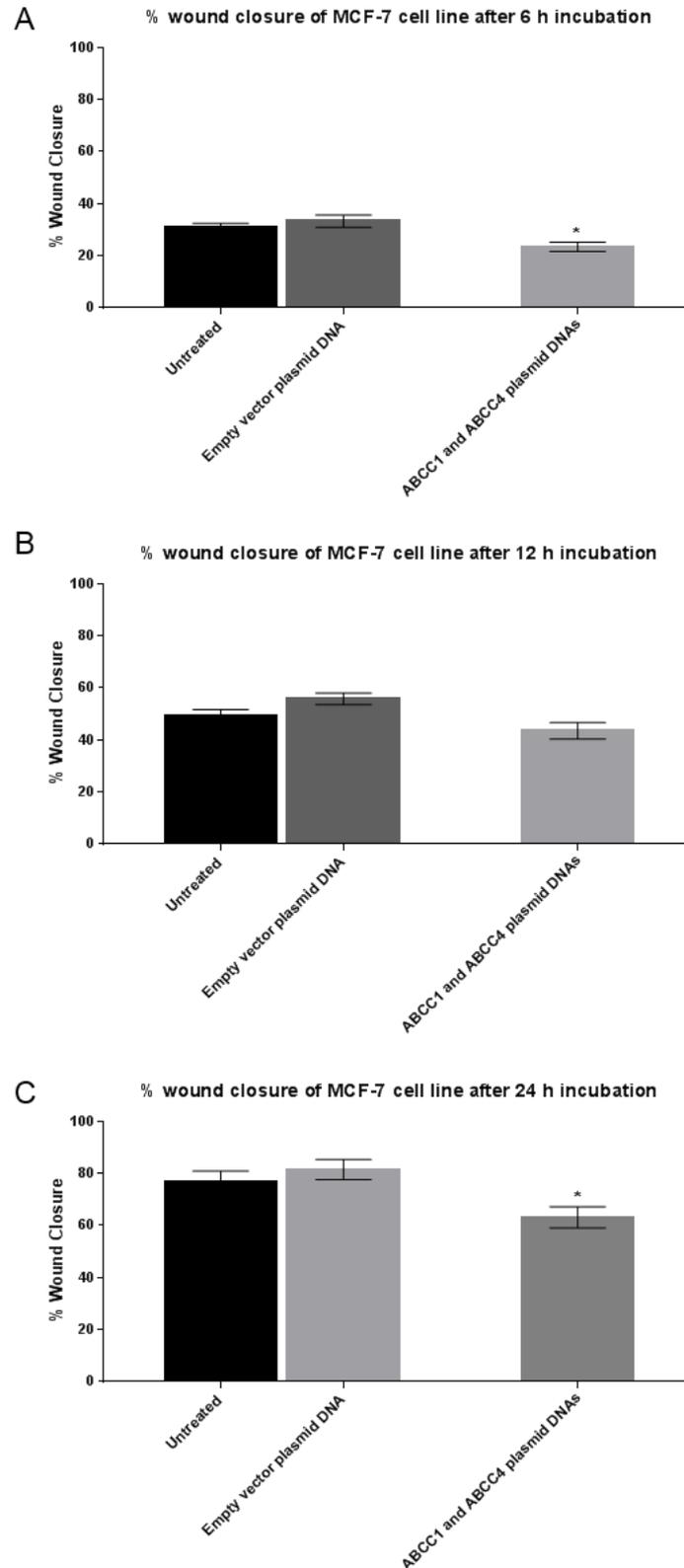
Figure 5.18. Percentage wound closure of MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs in combination. The transfected MCF-7 cells were left to incubate for 48 hours. Consequently, a cell monolayer was formed and a scratch using a 10 µl pipette tip was made in the cell monolayer. The plate was placed in the Cell-IQ where the selected image positions were captured every hour for 24 hours. The captured images were analysed in the Cell-IQ analysis software. Data are expressed as the mean ± standard error of the mean from

at least three independent experiments. *P < 0.05. P values were derived from one-way ANOVA vs untreated.

The cell motility of MCF-7 cells transfected with ABCC1 and ABCC4 plasmid DNAs in combination were assessed by performing the scratch assay. The co-overexpression of ABCC1 and ABCC4 in MCF-7 cells caused a decrease in cell migration after 6 and 24 hours incubation when compared to the untreated and the MCF-7 cells treated with empty vector plasmid DNA (Figures 5.18A and 5.18C respectively).

5.13   Effects of co-overexpression of ABC transporters on cancer cell invasion

The invasiveness of MCF-7 breast cancer cells treated with ABCC1 and ABCC4 plasmid DNAs in combination was investigated by performing the transwell invasion assay. Graph of percentage invasion of MCF-7 cells transfected with ABCC1 and ABCC4 in combination is shown in figure 5.19.
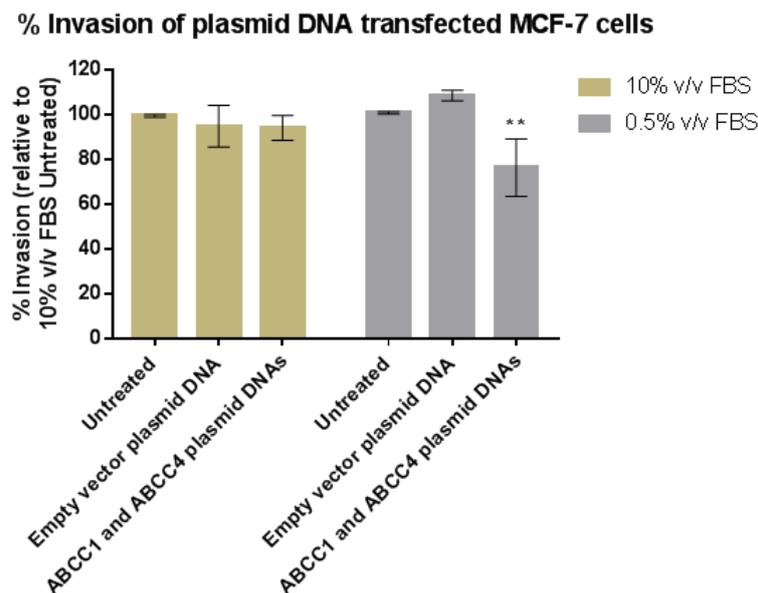
Figure 5.19. Effect of MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs in combination on cell invasion across a reconstituted basement membrane *in vitro*. Upon treatment of MCF-7 cells with ABCC1 and ABCC4 plasmid DNAs in combination, the transwell invasion assay was performed to assess cell invasion. The transwells were first coated with Corning Matrigel Matrix and a cell suspension of the transfected MCF-7 cells in 0.5% v/v FBS cell culture medium were seeded in the transwells. Cell culture medium containing 10% v/v or 0.5% v/v FBS were used as the chemoattractant. After 24 hours incubation, invasive cells that have crossed the membrane pores were fixed and stained. Stained cells were counted using a light microscope (x40) from five different fields of view. Data are expressed as the mean ± standard error of the mean from at least three independent experiments. ** $P < 0.01$. P values were derived from one-way ANOVA vs 10% v/v FBS untreated.

The MCF-7 cells treated with ABCC1 and ABCC4 plasmid DNAs in combination did not affect cell invasion when the chemoattractant was 10% v/v FBS cell culture medium (Figure 5.19). It seem a bit odd as an increased in cell invasion was expected. Moreover, a significant decreased in cell invasion was seen in the ABCC1 and ABCC4 combined transfected MCF-7 cells when the chemoattractant was 0.5% v/v FBS cell culture medium. Although the possible explanation is unknown at the moment, the results may suggest that co-overexpression of ABCC1 and ABCC4 may have no effect in cell invasion.

5.14   Summary

- Effective overexpression of both ABCC1 and ABCC4 in HEK293T cells and HeLa cells could be effectively achieved by transfection with pcDNA3.1 plasmids containing the gene of interest, using the transfection reagent PEI.

- The transfection of ABCC1 and ABCC4 plasmid DNA in MCF-7 and MDA-MB231 cells using PEI as a transfection reagent was only achievable in MCF-7 cells at certain PEI to DNA ratios (15:1 and 20:1). In contrast, the use of PEI in transfecting MDA-MB231 cells was not effective and proved to be a challenge, as other factors such as the amount of DNA used may be considered.

- Lipofectamine® 3000 transfection reagent was used as an alternative. The transfection of MCF-7 cells using the Lipofectamine® 3000 transfection reagent was efficient but was not particularly efficient in transfecting MDA-MB231 cells even after optimising the volume of the Lipofectamine® 3000 transfection reagent used.

- For MCF-7 cells, the co-expression of ABCC1 and ABCC4 could be achieved using the Lipfectamine® 3000 transfection reagent.

- The overexpression of ABCC1 and ABCC4 alone or in combination in MCF-7 cells did not have any significant effect on cell colony formation, proliferation, migration and cell invasion.

- The transfection procedure alone caused a decrease in proliferation of MDA-MB231 cells. This was not associated with ABCC1 or ABCC4, as the same effect was observed with empty vector controls.

- It was hypothesised that an overexpression of ABCC1 and ABCC4 in breast cancer cells would cause an increase in cell proliferation, cell migration and cell invasion when compared to the untreated and those treated with empty vector plasmid DNA. Furthermore, we hypothesised that the co-overexpression of ABCC1 and ABCC4 will further increase the effects. However, the results do not indicate this.

- It is important to note that the method of overexpression is transient. Thus the gene expression usually returns to normal after 96 to 120 hours after transfection and that similar to the siRNA transfection, the efficiency of transfection was not measured in terms of the proportion of cells that overexpressed the target proteins in comparison to those that were unaffected. In addition, as MDA-MB231 cells may be more difficult to transfect than MCF-7 cells, this may be an important consideration.

- The results obtained are preliminary and improvement in the method of transfection such as the use of a different plasmid construct, the use of a more efficient delivery method such as lentivirus, retrovirus and adenovirus based vectors could be beneficial and since a stable expression of the proteins are required, consideration in generating a stable cell line might be very useful.

Chapter 6 – Analysis of possible mechanisms by which ABCC1 and ABCC4 affect cancer cell growth and development

6.1 Objective

Since ABC transporters such as ABCC1 and ABCC4 transport many active signalling molecules and hormones as their substrates, it has been suggested that these substrates can activate cancer cells as well as regulate the tumour environment so as to help cancer cells to grow and develop (Adamska et al., 2018). Therefore mediating the efflux of these substrates by ABC transporters may also be important in cancer progression and development (Fletcher et al., 2010).

In order to investigate the potential correlation between ABCC1 and/or ABCC4 and their bioactive substrates and/or other proteins, MCF-7 and MDA-MB231 breast cancer cells were siRNA transfected with ABCC1- and ABCC4-specific siRNA molecules and the protein expression of selected proteins (GPR55, p-ERK and ERK1/2) were determined by Western blot. GPR55 was studied as in previous studies it was found to play an important role in the modulation of breast cancer cell migration (Ford et al., 2010) and proliferation (Pineiro et al., 2011). In addition, GPR55 was also found to be a receptor for l-α-lysophosphatidylinositol (LPI) which is also a substrate for ABCC1 (Pineiro et al., 2011; Ruban et al., 2014). The extracellular signal-regulated kinase (ERK) signalling pathway is important in mediating a wide range of cellular process such as cell differentiation, proliferation, survival, migration and metastasis in cancer cells (Roberts et al., 2007).

Secondly, specific ELISAs were used to determine the concentration of cyclic adenosine monophosphate (cAMP), prostaglanding E_2 (PGE$_2$), sphingosine-1-phosphate (S1P) and cysteinyl (Cys) leukotrienes (LTC$_4$, LTD$_4$ and LTE$_4$) produced by cells with or without siRNA knockdown of ABCC1 and ABCC4 and cells treated with MK571. ABCC4 has been previously shown to export cAMP and cGMP (Wielinga et al., 2003) The cAMP signalling pathway controls a diverse range of cellular processes such as cell differentiation, apoptosis and proliferation (Cospel et al., 2011). Similarly, PGE$_2$ also was found to promote cancer cell survival, migration, angiogenesis, invasion and proliferation (Greenhough et al., 2009), and has previously been shown to be a substrate for ABCC4 (Reid et al., 2003b). S1P may also be important in promoting cancer cell survival, migration, proliferation and metastasis (Maceyka et al., 2012), and is a proposed substrate for both ABCC1 and ABCC4 (Takabe et al., 2010; Vogt et al., 2018) In addition Cys LTs control inflammatory responses (Nielsen et al., 2005; Mezhybovska et al., 2006) and in cancer, LTD$_4$ was found to affect the expression

of many proteins such as β-catenin that helps to promote cell migration, invasion and proliferation (Yang et al., 2017). Both ABCC1 and ABCC4 efflux LTC_4 (Tian et al., 2005; Rius et al., 2008; Chen et al., 2010; Liu et al., 2010; Cole et al., 2014), and ABCC4 has also been shown to transport LTB_4 (Rius et al., 2008).

6.2 Determining the expression levels of bioactive proteins by Western blot

The expression levels of GPR55, ERK1/2 and p-ERK were determined in HeLa, MCF-7 and MDA-MB231 cells by Western blot. Membrane protein samples were obtained by using the nitrogen cell cavitation and differential centrifugation, whereas the whole cell lysate samples were extracted in lysis buffer containing detergent. Figures 6.1 and 6.2 show the expression levels of GPR55 in the membrane protein samples and in the whole cell lysate samples.
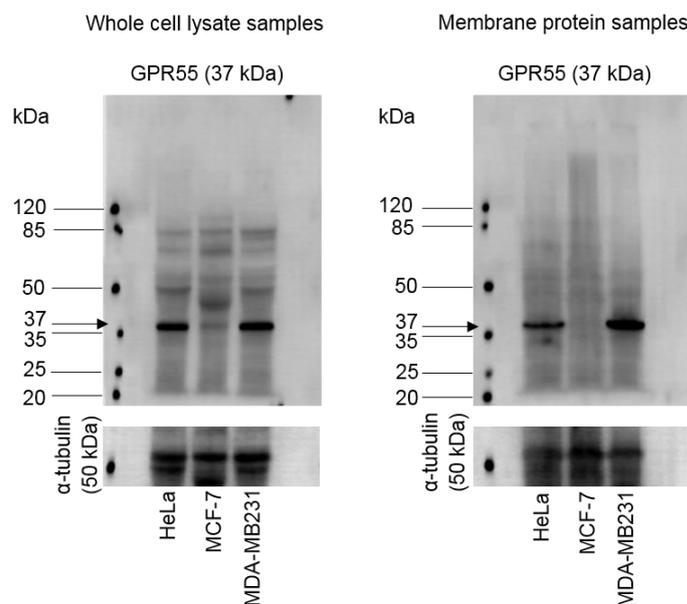
Figure 6.1. Western blot analysis of protein expression of GPR55 derived from whole cell lysate samples and membrane protein samples from HeLa, MCF-7 and MDA-MB231 cell lines. 80 μg of total protein was loaded in each well of a 10% v/v SDS gel and α-tubulin was used as the loading control. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa). The PVDF membrane blots were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibody, anti-GPR55 derived in rabbit (1:100 in blocking buffer). The secondary antibody used was anti-rabbit HRP (1:3000 in TBS-T). The level of protein expression of GPR55 was detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. Western blot analysis was repeated at least three times and similar protein expression levels were obtained.

Figure 6.2. Western blot analysis of protein expression of ERK1/2 and p-ERK derived from whole cell lysate samples from HeLa, MCF-7 and MDA-MB231 cell lines. 80 µg of total protein was loaded in each well and α-tubulin was used as the loading control. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa). The nitrocellulose membrane blots were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ERK1/2 derived in mouse (1:100 in blocking buffer) and anti-p-ERK derived in mouse (1:200 in blocking buffer). The secondary antibody used was anti-mouse HRP (1:4000 in TBS-T). The level of protein expression of ERK1/2 and p-ERK were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. Western blot analysis was repeated at least three times and similar protein expression levels were obtained.

The protein expression level of GPR55 was highest in MDA-MB231 cells followed by HeLa cells and barely detectable in MCF-7 cells (Figure 6.1). Furthermore, the protein expression level of ERK1/2 (Figure 6.2) was highest in MCF-7 cells followed in MDA-MB231 cells then in HeLa cells as confirmed by densitometry (Data not shown). Although the Western blot for detection of p-ERK may not be clear due to high non-specific background, at the expected position on the Western blot clearly shows that the expression level of p-ERK may be equally detectable in HeLa and MCF-7 cells but very low expressed in MDA-MB231 cells. Since these experiments were looking at native expression levels within cancer cell lines, the relative expression of these proteins may be very small and thus hard to detect. However, since the results obtained were reproducible, it is suggested that different cancer cell lines express different levels of these proteins.

6.3 The effect of ABCC1 or ABCC4 knockdown on GPR55 and ERK expression

After MCF-7 and MDA-MB231 cells were siRNA transfected with ABCC1- and ABCC4-specific siRNA molecules, the transfected cells were harvested and protein lysates were obtained in lysis buffer containing detergent. Since the expression level of GPR55 was barely detectable in MCF-7 cells (Figure 6.2), the relative expression levels of GPR55 was only analysed in ABCC1 and ABCC4 knockdown MDA-MB231 cells by Western blot as shown in figures 6.3. Furthermore the relative expression levels of ERK1/2 and p-ERK in ABCC1 and ABCC4 knockdown MCF-7 and MDA-MB231 cells were also determine by Western blot analysis as shown in figure 6.4.
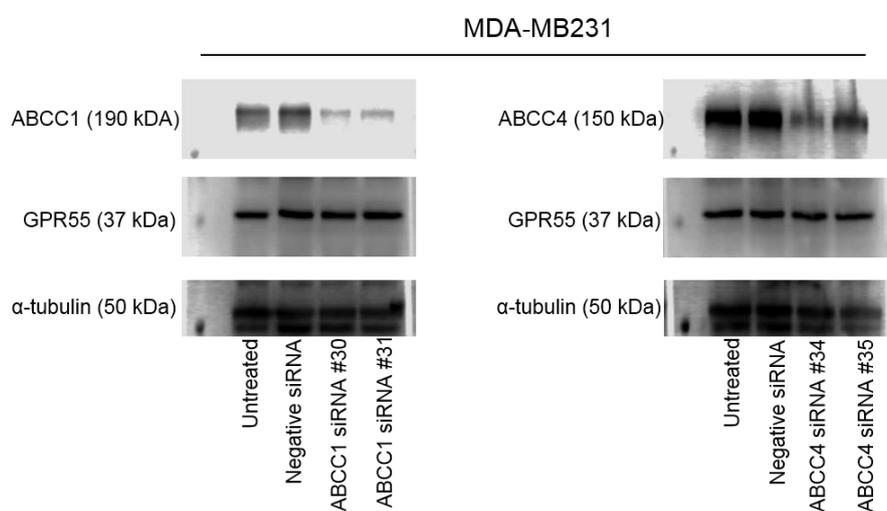
Figure 6.3. Western blot analysis of protein expression of GPR55 in ABCC siRNA transfected MDA-MB231 cells. 20 µl of protein lysate was loaded in each well of a 10% v/v SDS gel and α-tubulin was used as the loading control. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa). The PVDF membrane blots were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibody, anti-GPR55 derived in rabbit (1:100 in blocking buffer). The secondary antibody used was anti-rabbit HRP (1:3000 in TBS-T). The level of protein expression of GPR55 was detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. Western blot analysis was repeated at least three times and similar protein expression levels were obtained.

ABCC1

ABCC1 (190 kDa)

ERK 1/2 (44/42 kDa)

α-tubulin (50 kDa)

MCF-7

ABCC1 (190 kDA)

ERK 1/2 (44/42 kDa)

α-tubulin (50 kDa)

MDA-MB231

Untreated
Negative siRNA
ABCC1 siRNA #30
ABCC1 siRNA #31

ABCC4

ABCC4 (150 kDa)

ERK 1/2 (44/42 kDa)

α-tubulin (50 kDa)

MCF-7

ABCC4 (150 kDa)

ERK 1/2 (44/42 kDa)

α-tubulin (50 kDa)

MDA-MB231

Untreated
Negative siRNA
ABCC4 siRNA #34
ABCC4 siRNA #35

Figure 6.4. Western blot analysis of protein expression of ERK1/2 and p-ERK in ABCC siRNA transfected MCF-7 and MDA-MB231 cells. 20 μl of protein lysate was loaded in each well and α-tubulin was used as the loading control. The molecular weight standards were loaded in the first lane (Thermo Scientific Prestained Protein Molecular weight marker, 20 to 120 kDa). The nitrocellulose membrane blots were blocked in blocking buffer (5% BSA in TBS-T) overnight and incubated in appropriate primary antibodies, anti-ERK1/2 derived in mouse (1:100 in blocking buffer) and anti-p-ERK derived in mouse (1:200 in blocking buffer). The secondary antibody used was anti-mouse HRP (1:4000 in TBS-T). The level of protein expression of ERK1/2 and p-ERK were detected using the Li-Cor C-Digit blot scanner and the images were analysed using Image Studio Lite software imaging system. Western blot analysis was repeated at least three times and similar protein expression levels were obtained.

As can be seen from figure 6.3, the knockdown of ABCC1 and ABCC4 was achieved in MDA-MB231 cells. However the knockdown of ABCC1 and ABCC4 did not affect the level of expression of GPR55. Similarly, the knockdown of ABCC1 and ABCC4 in MCF-7 and MDA-MB231 cells did not affect the expression levels of ERK1/2. Past studies have demonstrated that ABCC1 is involved in an autocrine loop where by the production of LPI is pumped out of cells by ABCC1 and act on GPR55 receptors to initialise a downstream cascade (Pineiro et al., 2011; Arifin et al., 2016). Moreover, LPI was also found to activate ERK1/2 (Pineiro et al., 2011). Detection of p-ERK in the knockdown samples proved tricky with high level of background and requires further optimisation.

6.4 Determining the concentration of bioactive substrate secreted by ABCC siRNA transfected breast cancer cells by ELISA

6.4.1 Cyclic adenosine monophosphate (cAMP)

For measurements of extracellular cAMP in conditioned medium, the transfected and control cells were stimulated with 100 µM forskolin for 2 hours at 37 °C (Xie et al., 2011). After replacement with fresh cell culture medium (conditioned medium), three wells of non-transfected cells were treated with 50 µM MK571 and the cells were incubated for 18 hours at 37 °C. The conditioned media were collected for determination of cAMP by using the cAMP ELISA kit (Enzo Life Sciences Inc) which is a competitive immunoassay (Figure 6.5).
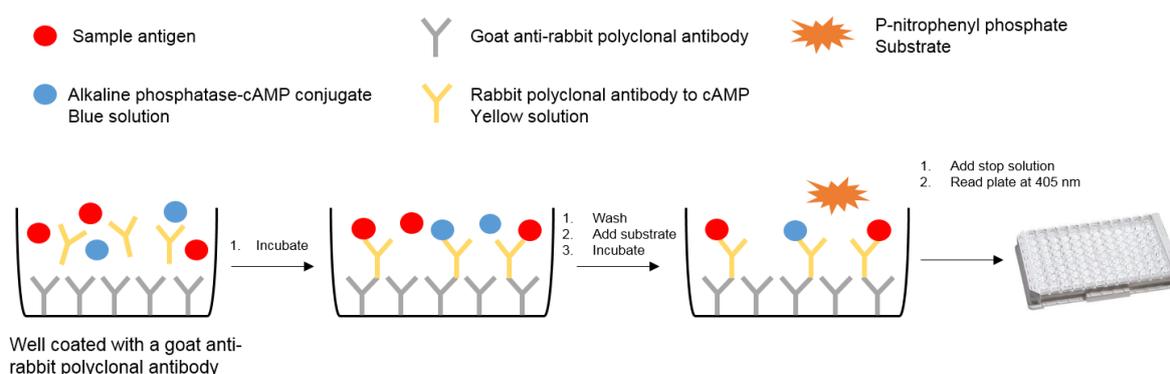
Figure 6.5. Schematic diagram showing competitive cAMP ELISA. The concentration of cAMP produced from the samples were determined by using the cAMP ELISA kit from Enzo Life Sciences Inc. Briefly, the cAMP standards (non-acetylated format) and the samples were added to the appropriate wells in duplicates followed by the blue conjugate and the yellow antibody into the appropriate wells. The plate was then sealed and incubated at room temperature of a plate shaker at 500 rpm for 2 hours. After incubation, the contents of the wells were discarded and the wells were washed thoroughly three times with the wash buffer diluted in distilled water (1:20 dilution). After complete removal of any remaining wash buffer, the blue conjugate was added to the appropriate wells followed by the substrate solution to each of the wells. The plate was then sealed and incubated for one hour at room temperature. After one hour incubation, the stop solution was added to each well and the absorbance was measured at 405 nm using multiskan Go microplate reader (ThermoScientific).

Once the absorbance values were obtained at 405 nm, the mean optical density (OD) of the standards and each samples were calculated and subtracted from the mean OD of Blank. The data was then input on Graphpad Prism (7.0), a standard curve was generated from a four parameter (4PL) logistic fitting curve, as recommended by the manufacturers (Figure 6.6A), and the concentration of cAMP from the samples were obtained as shown in figure 6.6B.

A cAMP standard curve
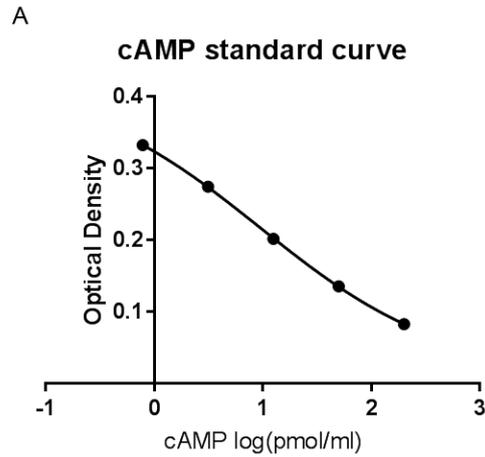
B Concentration of cAMP secreted by siRNA knockdown MDA-MB231 cells
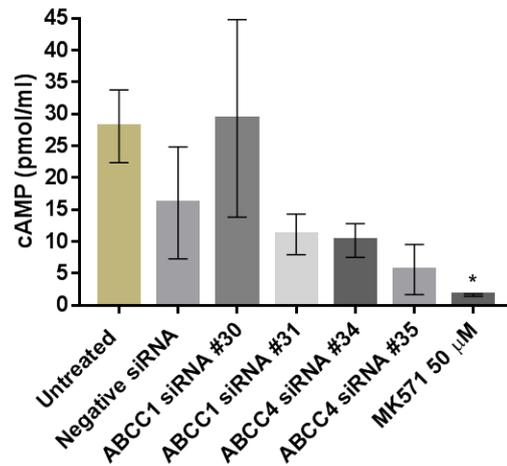
Figure 6.6. Measurement of extracellular cAMP in conditioned medium. (A) cAMP standard curve obtained from cAMP standards OD values. (B) Concentration of extracellular cAMP in conditioned medium secreted by ABCC siRNA transfected MDA-MB231 cells and MDA-MB231 cells treated with MK571. The absorbance values were read at 405 nm. The mean optical density (OD) for each samples were calculated by subtracting the mean OD for blank from the mean OD for each samples. Once the cAMP standard curve was generated, the concentration of extracellular cAMP from the samples were obtained from extrapolating from the cAMP standard curve using a four parameter (4PL) logistic fitting curve analysis. Data are expressed as the mean ± standard error of the mean from two independent experiments.

Table 6.1. Optical density values from the cAMP ELISA on MCF-7 cell samples.

Cancer cell line	Sample	Mean OD of sample	Mean OD of sample – Mean blank OD (0.1611)
MCF-7	Untreated	0.1660	0.0049
	Negative siRNA	0.1654	0.0043
	siRNA #30	0.1822	0.0211
	siRNA #31	0.1519	-0.0093
	siRNA #34	0.1544	-0.0067
	siRNA #35	0.1871	0.0260
	MK571 50 µM	0.1906	0.0295

The absorbance values were read at 405 nm. The OD values for the blank were obtained from the wells where only p-nitrophenyl phosphate (pNpp) substrate and stop solution were added. The mean optical density (OD) for each samples were calculated by subtracting the mean OD for blank from the mean OD for each samples.

The measurement of extracellular cAMP was performed on a 96-well plate coated with affinity purified antibody. Since only one plate was provided, fitting all the samples in triplicates was not possible on the same plate. Therefore, samples from three independent experiments for MCF-7 cells were selected but only two independent experiments for MDA-MB231 cells. The standard curve obtained (Figure 6.6A) was as expected according to the product manual (Enzo Life Sciences).

The concentration of extracellular cAMP from MDA-MB231 cells are shown in figure 6.6B. Treatment with ABCC1 siRNA #31 and ABCC4 siRNA #34 and #35 gave lower extracellular cAMP concentrations than the untreated MDA-MB231 cells. This difference was not statistically significant, but with further replicates this might change. The decrease was statistically significant for MDA-MB231 cells treated with MK571. Since cAMP is a substrate of ABCC4 (Russel et al., 2008), the results may indicate that a decrease in cell migration, invasion and to a lesser extent cell proliferation was observed when ABCC4 was knocked down or inhibited in MDA-MB231 cells, could be caused by less cAMP being pumped out of the cells.

In contrast to the results observed for MDA-MB231 cells, as can be seen from table 6.1 the mean OD of some samples from MCF-7 cells were negative and some were well below the cAMP standard curve (Figure 6.6A), hence the concentrations of extracellular cAMP were not able to be extrapolated from the curve. Only one sample treated with siRNA #35 and one sample treated with MK571 were able to be extrapolated from the cAMP standard curve (Data not shown). Since the ELISA is a competitive immunoassay, this suggests high binding of extracellular cAMP to the plate, hence high concentration of extracellular cAMP produced from

MCF-7 cells. Consequently, the samples from MCF-7 cells may need to be diluted before running another cAMP ELISA.

6.4.2 Prostaglandin E$_2$ (PGE$_2$)

For measurements of PGE$_2$ in conditioned medium, the control and siRNA transfected MCF-7 and MDA-MB231 cells were stimulated with 10 µg/ml lipopolysaccharides (LPS) for 24 hours at 37°C and with 80 nM phorbol 12-myristate 13-acetate (PMA) for one hours at 37°C respectively (Kochel et al., 2017). The cell culture medium was then replaced with fresh cell culture medium (conditioned medium) and three wells of non-transfected cells were treated with 50 µM MK571, and the cells were incubated for 18 hours at 37°C. The conditioned media were collected for determination of PGE$_2$ by using the PGE$_2$ competitive ELISA kit (Enzo Life Sciences Inc).

A

PGE$_2$ standard curve

B
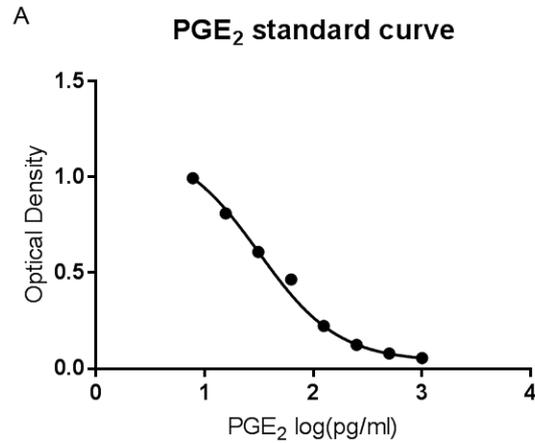
Concentration of PGE$_2$ secreted from siRNA knockdown MCF-7 cells
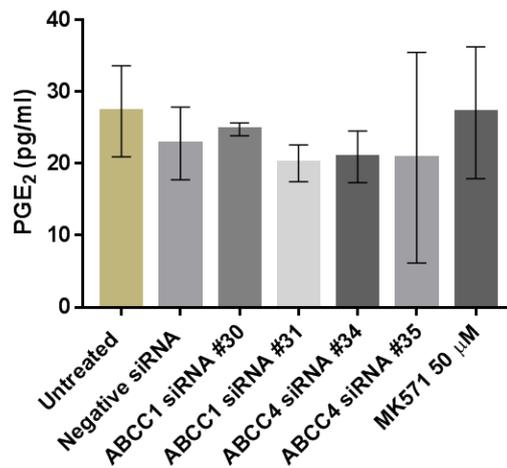
Figure 6.7. Measurement of Prostaglandin E$_2$ (PGE$_2$) in conditioned medium. (A) PGE$_2$ standard curve obtained from PGE$_2$ standards OD values. (B) Concentration of PGE$_2$ in conditioned medium secreted by ABCC siRNA transfected MCF-7 cells and MCF-7 cells treated with MK571. The absorbance values were read at 405 nm. The mean optical density (OD) for each samples were calculated by subtracting the mean OD for blank from the mean OD for each samples. Once the PGE$_2$ standard curve was generated, the concentration of PGE$_2$ from the samples were obtained from extrapolating from the PGE$_2$ standard curve using a four parameter (4PL) logistic fitting curve analysis. Data are expressed as the mean ± standard error of the mean from two independent experiments.

Table 6.2. Optical density values from the PGE$_2$ ELISA using MDA-MB231 cell samples.

Cancer cell line	Sample	Mean OD of sample	Mean OD of sample – Mean blank OD (0.1410)
MDA-MB231	Untreated	0.1375	-0.0035
	Negative siRNA	0.1288	-0.0123
	siRNA #30	0.1338	-0.0072
	siRNA #31	0.1204	-0.0206
	siRNA #34	0.1343	-0.0067
	siRNA #35	0.1298	-0.0112
	MK571 50 µM	0.1355	-0.0055

The absorbance values were read at 405 nm. The OD values for the blank were obtained from the wells where only p-nitrophenyl phosphate (pNpp) substrate and stop solution were added. The mean optical density (OD) for each samples were calculated by subtracting the mean OD for blank from the mean OD for each samples.

The standard curved obtained (Figure 6.7A) was similar to what was suggested from the product manual (Enzo Life Sciences).

The extracellualar concentration of PGE$_2$ from MCF-7 cells treated with ABCC siRNAs and MK571 are shown in figure 6.7B. It can be seen that PGE$_2$ levels were not affected by the siRNA knockdown when compared to the concentration of PGE$_2$ from untreated MCF-7 cells. Notably there are large error bands, which may be improved by further replicates, but there are no suggestions of any effects on extracellular PGE$_2$ levels.

The mean OD of some samples from MDA-MB231 cells (Table 6.2) were negative and some were well below the PGE$_2$ standard curve (Figure 6.7A), hence the concentrations of PGE$_2$ were not able to be extrapolated from the curve. Since the ELISA is a competitive immunoassay, the very low OD values of the MDA-MB231 samples may indicate high binding of PGE$_2$ from the conditioned medium to the plate, hence suggesting that MDA-MB231 cells efflux high amount of PGE$_2$ out of the cells. Therefore in order to determine the concentration of PGE$_2$ from the samples from MDA-MB231 cells, these samples may need to be diluted so that their OD values fall within the standard curve.

6.4.3 Sphigosine-1-phosphate (S1P)

For measurements of S1P in conditioned medium, the transfected and non-transfected cells were incubated in fresh cell culture medium (conditioned medium) with three wells of non-transfected cells treated with 50 µM MK571 and the cells were incubated for 18 hours at 37°C. The conditioned media were collected for determination of S1P by using the S1P ELISA kit (Abbexa Ltd) which is a sandwich immunoassay.
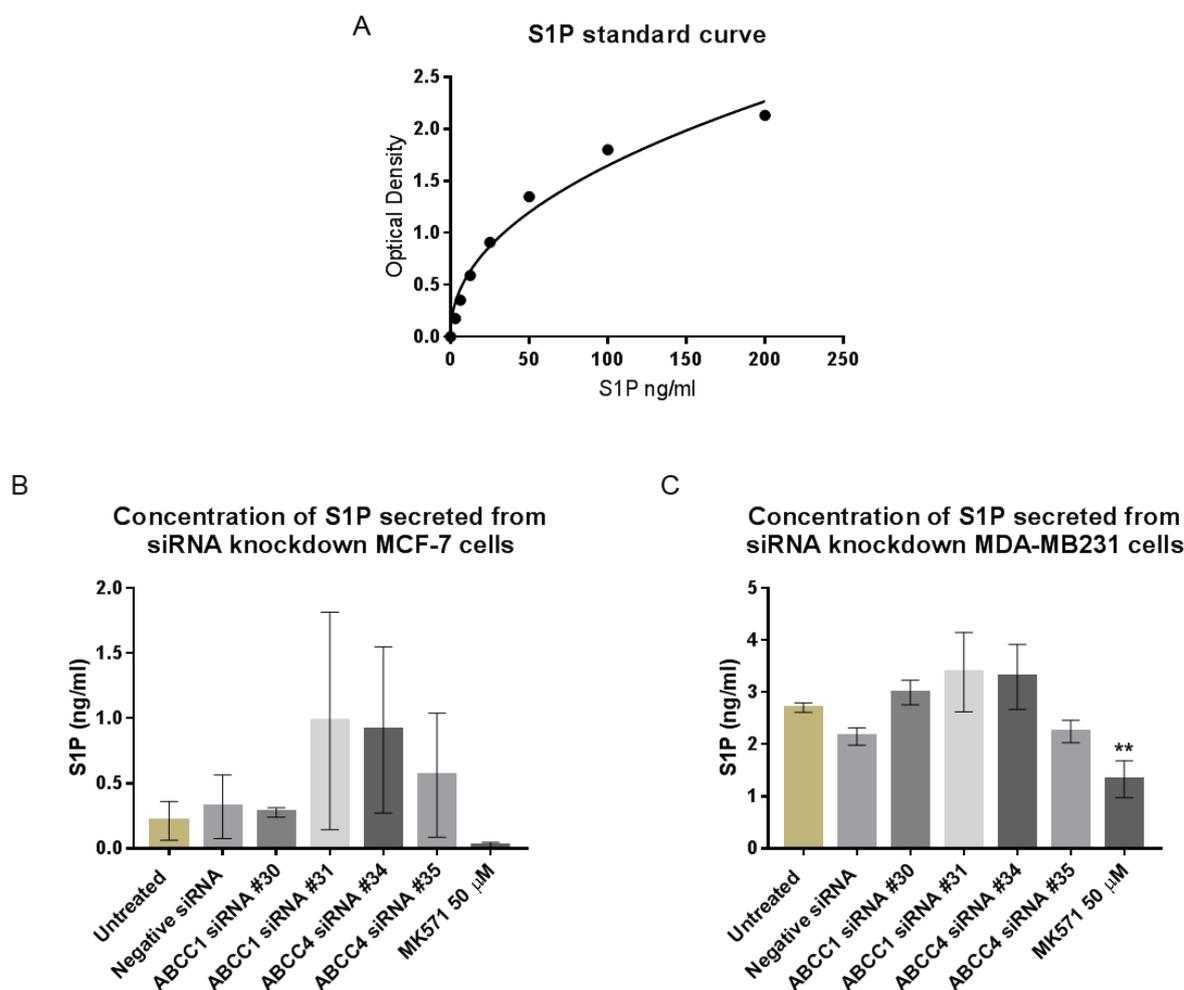
Figure 6.8. Measurement of S1P in conditioned medium. (A) S1P standard curve obtained from S1P standards OD values. (B) Concentration of S1P in conditioned medium secreted by ABCC siRNA transfected MCF-7 cells and MCF-7 cells treated with MK571. (C) Concentration of S1P in conditioned medium secreted by ABCC siRNA transfected MDA-MB231 cells and MDA-MB231 cells treated with MK571. The absorbance values were read at 450 nm. The mean optical density (OD) for each samples were calculated by subtracting the mean OD for blank from the mean OD for each samples. Once the S1P standard curve was generated, the concentration of S1P from the samples were obtained from extrapolating from the S1P standard curve using a log-log fitting curve analysis. Data are expressed as the mean ± standard error of the mean from two independent experiments for MCF-7 samples and three independent experiments for MDA-MB231 samples. * $P < 0.05$. P values were derived from one-way ANOVA vs untreated.

The S1P standard curve obtained (Figure 6.8A) was as expected according to the product manual (Abbexa Ltd). Subsequently, the mean OD of samples from MCF-7 and MDA-MB231 cells, all fit within the range the S1P standard curve, and hence the concentrations of S1P were able to be extrapolated from the curve. The MCF-7 cells gave very low absorbance readings, since this ELISA is a sandwich immunoassay the low OD values of the MCF-7 samples indicate low levels of S1P in the conditioned medium, and suggest that MCF-7 cells

efflux low amounts of S1P (Figure 6.8B). The large error bars likely reflect this low signal as well as only two replicates. In contrast, MDA-MB231 cells efflux 2-3 – fold more S1P than MCF-7 cells (Figure 6.8C). Knockdown of ABCC1 or ABCC4 did not seem to have a significant effect on extracellular levels of S1P, however treatment with MK571, which inhibits both ABCC1 and ABCC4, caused a significant decrease in efflux of S1P when compared to that of the untreated MDA-MB231 cells.

6.4.4 Cysteinyl leukotrienes (LTC$_4$, LTD$_4$ and LTE$_4$)

For measurements of cysteinyl leukotrienes in conditioned medium, the control and siRNA transfected cells were stimulated with 10 mM acetylsalicylic acid (Aspirin) for 2 hours at 37°C (Mortaz et al., 2007) after which the cell culture medium was replaced with fresh cell culture medium (Conditioned medium) and three wells of non-transfected cells were treated with 50 µM MK571, the cells were incubated for a further 5 hours at 37°C.. The conditioned media were collected for determination of cysteinyl leukotrienes by using the cysteinyl leukotrienes competitive ELISA kit (Enzo Life Sciences Inc).
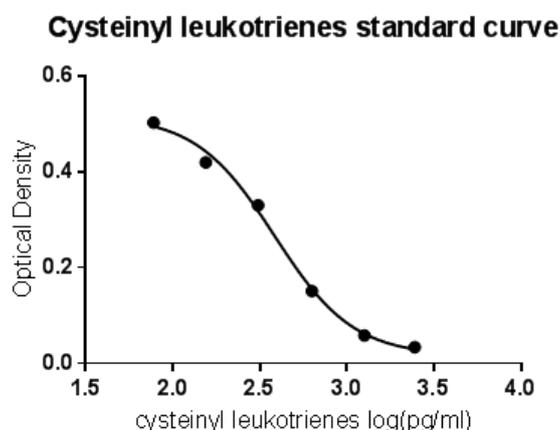
Figure 6.9. Cysteinyl leukotrienes standard curve obtained from cysteinyl leukotrienes standards OD values. The absorbance values were read at 450 nm. The mean optical density (OD) for each standards were calculated by subtracting the mean OD for blank from the mean OD for each samples. Once the cysteinyl leukotrienes standard curve was generated, the concentration of cysteinyl leukotrienes from the samples were obtained from extrapolating from the cysteinyl leukotrienes standard curve using a four parameter (4PL) logistic fitting curve analysis.

Table 6.3. Optical density values and concentration of the cysteinyl leukotrienes in the samples.

Cancer cell line	Sample	Mean OD of sample	Mean OD of sample – Mean blank OD (0.1410)	Concentration of LTC$_4$ (pg/ml)
MCF-7	Untreated	0.6953	0.5798	
	Negative siRNA	0.7172	0.6017	
	siRNA #30	0.6794	0.5639	
	siRNA #31	0.6861	0.5706	
	siRNA #34	0.6840	0.5685	
	siRNA #35	0.6949	0.5794	
	MK571 50 µM	0.2480	0.1325	712.853
MDA-MB231	Untreated	0.7213	0.6058	
	Negative siRNA	0.7150	0.5995	
	siRNA #30	0.7298	0.6143	
	siRNA #31	0.7157	0.6002	
	siRNA #34	0.7302	0.6147	
	siRNA #35	0.7077	0.5922	
	MK571 50 µM	0.2451	0.1296	726.106

The absorbance values were read at 405 nm. The OD values for the blank were obtained from the wells where only p-nitrophenyl phosphate (pNpp) substrate and stop solution were added. The mean optical density (OD) for each samples were calculated by subtracting the mean OD for blank from the mean OD for each samples. The data was then input on Graphpad Prism (7.0) a standard curve was generated from a four parameter (4PL) logistic fitting curve. The concentration of the samples were then extrapolated from the standard curve.

Figure 6.9 shows the standard curve, which was as expected according to the manufacturer's handbook (Enzo Life Sciences).

As can be seen from table 6.3 the mean OD of samples from ABCC siRNA transfected MCF-7 and MDA-MB231 cells were all higher and over the range of the standard curve, hence the concentration of cysteinyl leukotrienes from these samples was not able to be extrapolated from the standard curve (Figure 6.9). As the ELISA is a competitive ELISA, higher OD values mean less cysteinyl leukotrienes from the conditioned medium, as a result may need to incubate the cells in smaller volume of medium to increase the concentration of the cysteinyl leukotrienes, if any are present. Furthermore, only samples from cells treated with MK571 produced mean ODs that fall within the range of the standard curve thus the concentrations of cysteinyl leukotrienes were able to extrapolate from the standard curve. However, since the conditioned medium contained MK571 and MK571 is a selective leukotriene LTD$_4$ receptor antagonist, it may be that MK571 itself is binding to the cysteinyl leukotrienes antibody and hence result in high false positive.

6.5 Summary

- The protein expression level of GPR55 was highest in MDA-MB231 cells followed by HeLa cells and barely expressed in MCF-7 cells.

- The protein expression level of ERK1/2 was highest in MCF-7 cells and may be slightly higher in MDA-MB231 cells than in HeLa cells. In addition, in all the cell line the protein expression level of ERK1 was lower than that for ERK2. Moreover, the protein expression level of p-ERK may be equally expressed in HeLa and MCF-7 cells but not in MDA-MB231 cells

- The protein expression levels of GPR55 and ERK1/2 were not affected by the knockdown of ABCC1 and ABCC4. Therefore these results suggest that there is no correlation between ABCC1 and ABCC4 with GPR55, ERK1/2 and p-ERK.

- Specific ELISA to detect cAMP, PGE_2, S1P and cysteinyl leukotrienes in conditioned medium from ABCC siRNA transfected MCF-7 and MDA-MB231 cells and those treated with MK571 were performed. MCF-7 cells efflux higher levels of cAMP than MDA-MB231 cells. However, MCF-7 cells efflux lower levels of both PGE_2 and S1P than MDA-MB231 cells.

- Since only two separate experiments were performed, the knockdown of ABCC4 in MDA-MB231 cells and those treated with MK571 efflux so far may indicated a lower amount of cAMP out of the cells when compared to that of the untreated and may indicated that neither the knockdown of ABCC1 or ABCC4 or MK571 treatment affected the efflux of PGE_2 out of MCF-7 cells. Hence, more replicates are need to validate these findings.

- The efflux of S1P by MDA-MB231 cells treated with MK571 were significantly reduced when compared to that of the untreated.

- Both cells lines efflux low levels of cysteinyl leukotrienes. Large concentration values of cysteinyl leukotrienes were obtained from cells treated with MK571. Since MK571 is a selective leukotriene LTD_4 receptor antagonist, it may have a binding affinity for the cysteinyl leukotrienes antibody that may result in high false positive.

Chapter 7 – Discussion

Breast cancer is one of the most common types of cancer affecting women (Lage, 2003; Scully et al., 2012; Martin et al., 2014; Nounou et al., 2015). Breast cancer mortality is mainly due to breast cancer metastases to distant organ sites as well as the primary tumour itself (Scully, et al., 2012; Ugenskiene, et al., 2012).

Cancer cells can become resistant to the drugs during chemotherapeutic treatment (Leonard et al., 2003; Housman et al., 2014). Often due to either drugs becoming inactive, drug efflux, changes in drug target and cancer cells acquiring epithelial-mesenchymal transition (EMT), becoming resistance to death, acquiring the ability to repair DNA damage, and modification in epigenetics (Yasui et al., 2004; Housman et al., 2014; Cree et al., 2017).

The efflux of drugs out of cancer cells is one of the most complex mechanisms of drug resistance, and often leads to resistance to more than one drug. A phenomenon known as multidrug resistance (MDR) (Leonard et al., 2003; Bozzuto et al., 2010). Several members of the ATP-binding cassette (ABC) transporter family of proteins have been found to be responsible for the multidrug resistance phenomenon (Bozzuto et al., 2010; Zahreddine et al., 2013).

Research investigating the role of ABC transporters in multidrug resistance is well established. However, recently many studies have suggested that ABC transporters might be implicated more generally in the cancer hallmarks (Fletcher et al., 2010 and 2016). To date, little is known about their direct contribution to cancer progression and development irrespective of their drug-efflux abilities (Fletcher et al., 2010 and 2016; Adamska et al., 2018). Since ABC transporters transport many active signalling molecules and hormones as their substrates, it has been suggested that these substrates can activate cancer cells as well as regulate the tumour environment so as to help cancer cells to grow and develop (Adamska et al., 2018). Therefore, mediating the efflux of these substrates may also be important in cancer progression and development (Fletcher et al., 2010).

The focus of this thesis was to investigate whether ABCC1 and ABCC4 are implicated in the progression and development of breast cancer. As mentioned previously, ABCC1 and ABCC4 transport a wide variety of bioactive substrates that have been found to mediate various signalling pathways that regulates cell proliferation, migration, metastasis, invasion, differentiation and apoptosis. Hence, this thesis, looked to address the hypothesis that ABCC1

and ABCC4 are involved in breast cancer proliferation, migration and invasion irrespective of their drug efflux abilities.

In this thesis, MCF-7 and MDA-MB231 breast cancer cells were treated with ABCC inhibitors, the expression of ABCC1 and ABCC4 were knocked down using specific siRNAs as well as overexpression by transient transfection with ABCC1- and ABCC4- specific plasmid DNA. The treated cells were used *in vitro* to carry out cellular functional assays such as colony formation assay to determine cell proliferation, scratch assay to determine cell migration, MTT assay to determine cell viability, transwell invasion assay to determine cell invasion capacity and Western blotting and reverse transcription polymerase chain reaction (RT-PCR) to detect expression of proteins.

This chapter, looks to discuss and evaluate the results obtained, identify any potential limitations and weaknesses encountered during the study and propose any alternative methods. Furthermore, the results obtained were further evaluated to see if they are related to the literature and how they may agree or disagree with previously published knowledge. Subsequently from these findings, future work is proposed that may be undertaken to better understand the role of ABCC1 and ABCC4 in breast cancer.

7.1 Different expression levels of ABC transporters in different breast cancer cell lines

Multi-drug resistance whether acquired or constitutive in cancer therapy is often attributed to a group of proteins known as the ABC transporters which are membrane drug transporters which are often overexpressed in different cancer types. Their enhanced expressions often correlates with poor prognosis. The ABCB1 (P-glycoprotein), ABCC1 (MRP1) and ABCG2 are the most well studied members of the ABC transporters and are involved in multidrug resistance in a number of drug resistant tumours (Kovalev, et al., 2013; Begicevic et al., 2017). Together with ABCC4, these proteins also transport a variety of physiological, active substrates that may be implicated in cancer cell biology (Fletcher et al., 2010; Adamska et al., 2017).

In our study, the expression of ABCB1, ABCG2, ABCC1 and ABCC4 in HeLa, MCF-7 and MDA-MB231 cells were analysed by Western blot. According to our finding, high expression of ABCB1 was found in MCF-7 cells followed by HeLa cells and barely detectable in MDA-MB231 cells. The result indicated that ABCB1 is expressed in MCF-7 cells and may not be expressed in MDA-MB231 cells which supports the findings of Mutoh et al., (2006) who found

oestrogen mediates the down regulation of ABCB1 in oestrogen positive human breast cancer cells. Mutoh et al., (2006) also compared the level of expression of ABCB1 between different cancer cell lines and used MDA-MB231 cells as one of the controls since MDA-MB231 cells does not express ABCB1. In contrast to our findings another previous study found that the expression of ABCB1 was not detectable in drug sensitive MCF-7 cells but when MCF-7 cells were exposed to doxorubicin, ABCB1 was overexpressed (Wang et al., 2015). Our results clearly show expression of ABCB1 in drug sensitive MCF-7 cells, so perhaps this is simply a matter of the sensitivity of the different antibodies and detection systems.

As for the level of expression of ABCG2, it was highly expressed in MCF-7 cells but not detectable in HeLa or MDA-MB231 cells. A previous study also found that MCF-7 cells express ABCG2 and that its expression is mediated during epithelial-mesenchymal transition (EMT). Moreover, the study also found that MDA-MB231 cells do express ABCG2 (Yin et al., 2008).

The expression levels of both ABCC1 and ABCC4 were highest in HeLa cells followed by MDA-MB231 cells and lowest in MCF-7 cells. A previous study by Kochel et al., (2017) also found that ABCC4 was highly expressed in MDA-MB231 cells but low expression in MCF-7 cells. The expression level of ABCC1 and ABCC4 was lower in MCF-7 cells than in MDA-MB231 cells maybe because MDA-MB231 cells are basal cell type compared to MCF-7 cells which are luminal cell type. It is possible that MDA-MB231 cell are more invasive due to their basal cell type and perhaps this is linked to the expression levels of ABCC1 and ABCC4.

ABCC1 expression has previously been found to be a negative prognostic marker for early breast cancer (Munoz et al., 2007) and that there is a strong correlation between the expression level of ABCC1 and overall survival and time to relapse (Nooter et al., 1997; Munoz et al 2007). In addition, a study found high expression of ABCC1 in breast cancer lymph node metastases and suggest that ABCC1 may be implicated in breast cancer metastasis (Zochbauer-Muller et al., 2001; Munoz et al., 2007). Moreover, high expression of ABCC1 has been found in numerous other cancer types and has been linked to poor clinical outcome such as in neuroblastoma (Haber et al., 2006), intrahepatic cholangiocarcinoma (Srimunta et al., 2012), non-small cell lung cancer (Fang et al., 2018), leukaemia (van der Kolk et al., 2000; Beck et al., 2001), oesophageal carcinomas (Nooter et al., 1998), prostate cancer (Sullivan et al., 2000).

Similarly, high expression of ABCC4 in some invasive tumours has been associated with poor patient survival such as epithelial ovarian carcinoma (Bagnoli et al., 2013; Roundhill et al., 2015) and neuroblastoma (Norris et al., 2005; Henderson et al., 2011; Huynh et al., 2012;

Roundhill et al., 2015), gastric cancer (Zhang et al., 2009), pancreatic cancer (Zhang et al., 2012). In contrast, a low expression level of ABCC4 in prostate tumours is associated with poor event free survival (Ho et al., 2008; Chen et al., 2011; Montani et al., 2013). However, the association of ABCC4 to poor prognosis in breast cancer is still not clear but the expression of ABCC4 in breast cancer was found to mediate the malignant behaviour in breast cancer (Kochel et al., 2015).

Although many studies have been carried out on these transporters, studying them can be a challenge as they are expressed in relatively low levels. To effectively study these proteins often the cells have been transfected with specific plasmids that express the gene of the protein or they have been exposed to high levels of drugs to acquire resistance so that the level of the protein is over expressed such as in doxorubicin resistant cancer cell lines and etoposide resistant cancer cell lines (Doyle et al., 1998 and 2003; Benderra et al., 2000; Alpsoy et al., 2014; Balaji et al., 2016; Gao et al., 2016). However the initial aim of the work in this study was to investigate the role of the native expression of these transporters since both ABCC1 and ABCC4 expression could be readily detected in MCF-7 and MDA-MB231 cells.

7.2 ABCC1 may be more involved in mediating breast cancer cell proliferation than ABCC4

A colony formation assay was done to determine the cell ability to divide (proliferate) from a single cell to form a colony. It was found that Reversan, an ABCC1 and ABCB1 inhibitor (Burkhart et al., 2009; Henderson et al., 2011; Tivnan et al., 2015) inhibits colony formation in a concentration dependent manner, for all three types of cells HeLa, MCF-7 and MDA-MB231 cells. Furthermore, MK571, a non-specific ABCC1 inhibitor that can also inhibit ABCC4, selectively inhibited colony formation in HeLa and MDA-MB231 cells but not significantly in MCF-7 cells. Notably, the level of expression of ABCC1 and ABCC4 were higher in HeLa and MDA-MB231 cells than in MCF-7 cells, hence this may suggest a high expression of ABCC1 and ABCC4 could correlate with an increase in colony formation. Only Reversan but not MK571 affected the cell proliferation of MCF-7 cells, which might suggest that ABCC1 is more implicated in cell proliferation than ABCC4. However, as Reversan has also been shown to inhibit ABCB1 (Tivnan et al., 2015) and MCF-7 cells were the only ones expressing relatively high levels of ABCB1, this could be an alternative explanation. A study found that ABCB1 promotes cell survival in acute myeloblastic leukaemia independent of its drug cytotoxic efflux ability and modulate apoptosis (Pallis et al., 2000). Another, important observation was that MK571 at 50 µM did not cause any significant inhibition relative to untreated cells as opposed to when cells were treated with 100 µM. Further observations confirmed that Reversan and

MK571 not only inhibited the formation of colonies but also reduced the size of colonies thus suggesting that the inhibition of ABCC1 (and ABCB1) inhibits cell proliferations (Henderson, et al., 2011).

On the other hand, ABCC4 specific inhibitors, Ceefourin 1 and Ceefourin 2 (Cheung, et al., 2014), did not inhibit colony formation in any of the three types of cell lines indicating that inhibition of ABCC4 may not be involved in cell proliferation. Indomethacin, a non-specific inhibitor of ABCC4 (Draper, et al., 1997) also did not affect cell proliferation. Similarly both MK571 and Reversan inhibited cell viability. Hence, from these findings, it can be inferred that ABCC4 may not have as important a role in cell proliferation as ABCC1 does.

The trypan blue dye exclusion assay was carried out to validate the results obtained from the MTT assay. Although it is a straightforward, inexpensive assay, it cannot distinguish between healthy viable cells from cells that have an intact membrane but are not healthy. Moreover, the cells were counted manually using a hemocytometer that may account for about 10% counting error and various factors such as loss of cells in cell suspension and inconsistency in the volume of cell suspension when loading the chamber and the density of cell suspension may vary between cell samples can give rise to large deviation between counts that eventually leads to large error bars (Aslanturk et al., 2018). Nonetheless, the results obtained from the trypan blue dye exclusion assay, agree to some extent to the MTT assay, in that it also indicated that MK571 and Reversan significantly decrease cell proliferation. This agrees with the results of the colony formation assay that ABCC1 may have a larger role in breast cancer cell proliferation than ABCC4 does.

As the ABC inhibitors used were not specific and they can inhibit more than one ABC transporters in addition to other targets, we decided to knockdown *ABCC1* and *ABCC4* genes using ABCC1- and ABCC4- siRNA specific molecules in both MCF-7 and MDA-MDA breast cancer cell lines so as to try to validate the results obtained with ABC inhibitors.

In our findings, the depletion of ABCC1 and ABCC4 did not decrease the number of colonies formed in either MCF-7 or MDA-MB231 cells. However combination knockdown of *ABCC1* and *ABCC4* genes in MCF-7 cells inhibited the clonogenic capacity of MCF-7 cells to form cell colonies. Similar effect of reduction in clonogenic capacity of MDA-MB231 cells was seen with one combination of siRNA knockdown (ABCC1 siRNA #30 and ABCC4 siRNA #35). These results indicate that ABCC1 and ABCC4, together may mediate breast cancer cell growth and that ABCC1 and ABCC4 may have a combined effect affecting the clonogenic capacity of breast cancer cells.

In a previous study of colony formation by neuroblastoma cells, the knockdown of ABCC1 and ABCC4 did cause a decrease in the number of colonies formed and a combined knockdown of ABCC1 and ABCC4 decreased the number of colonies formed even more (Henderson et al., 2011). Whilst the single knockdowns in our study did not show significant effects, the combined knockdown shows similar effects. Reasons for this difference could include differing efficiencies of siRNA knockdown and/or different expression levels of ABCC1 and ABCC4 to start with. Furthermore, Henderson et al., 2011 also found that the number of neuroblastoma colonies formed was reduced after cells were treated with Reversan, which agrees well with our findings.

The study of cancer cell viability using MTT assays showed little effect upon ABCC1 or ABCC4 knockdown, either individually or in combination. This contrasts with the findings by Zhao et al., (2014) who found that ABCC4 is required for cell proliferation in non-small cell lung cancer. However they used shRNA lentiviral vector to mediate the knockdown in the cells, which may have been more efficient and thus may have a significant impact on the results obtained (Zhao et al., 2014). Furthermore, another study found that the downregulation of ABCC4 expression in Panc-1 and BxPC-3 human pancreatic cancer cell lines using a lentivirus expressing an ABCC4 shRNA also significantly decreased colony formation and proliferation *in vitro* (Zhang et al., 2012). In another study, the proliferation of MGC80-3 gastric cancer cells was significantly inhibited with lentivirus ABCC4 shRNA (Chen et al., 2014).

In general, the RNAi mechanism of suppression of gene expression is usually transient and lasts for 96 to 120 hours after which the gene expression will start to recover (Mocellin et al., 2004). Originally it was speculated that since the colony formation assay last for 7 days, it is suggested that the suppression of ABCC1 and ABCC4 may have recovered. Therefore, a shorter incubation period, for example 3 days may be suggested. However, in the neuroblastoma study by Henderson et al., (2011), colony formation assays were also carried out for 7 days, and in the non-small lung cancer study by zhao et al., (2014) the assays were run for 8 days.

Different methods were used for the transfections however, with Henderson et al., (2011) using Lipofectamine 2000 as the transfection reagent, whereas INTERFERin was used in the current study. Even though the Western blot data did show a knockdown of ABCC1 and ABCC4 in both MCF-7 and MDA-MB231 cells when compared to the untreated cells, and according to the manufacturers' recommendation, 1nM siRNA with INTERFERin can provide more than 90% silencing efficiency in a wide variety of cells, the percentage or the number of

cells that was actually being transfected is not known in each transfection. Further optimisation of the knockdown process might be required such as using a different volume of INTERFERin or increasing the concentration of siRNA per transfection. Consideration to use other transfection reagent such as Lipofectamine 3000, or the use of plasmids which express siRNA, or short hairpin RNAs (shRNAs) expressed from plasmids, which can undergo selection to obtain stable cell lines with knockdown of the target protein. Alternatively, a more efficient delivery method such as lentivirus, retrovirus and adenovirus based vectors could be beneficial. Moreover, CRISPR/Cas9 could be trialled to achieve an effective knockout of these genes. CRISPR/Cas9 is a gene-editing technology that requires an endonuclease, CRISPR-associated protein 9 (Cas9) to break a double-stranded DNA hence allowing the genome to be modify and for efficiency gene editing, a guide RNA is required to match a desired target gene (Redman et al., 2016).

Moreover, the suppression of ABCC1 and ABCC4 may have upregulated other ABC transporters as a compensatory effect for the down regulation of ABCC1 and ABCC4. Several ABC transporters have overlapping substrate specificity and it is known that they can in certain circumstances compensate for each other. A study found that the treatment of vinblastine and methotrexate (MTX) with ABCA2 RNAi partially decreased the expression of ABCA2 but at the same time increased ABCA3 expression in childhood T-cell acute lymphoblastic leukaemia. A similar observation was seen when ABCA3 was suppressed which caused an increased in ABCA2 expression (Efferth et al., 2006).

Attempts to overexpress ABCC1 and ABCC4 in MCF-7 and MDA-MB231 cells were a challenge especially in MDA-MB231 cells transfection. The Lipofectamine® 3000 transfection reagent was used as the transfection reagent and efficient transfection was obtained in MCF-7 cells but not in MDA-MB231 cells even after optimising the volume of the Lipofectamine® 3000 transfection reagent used. Again perhaps lentivirus delivered overexpression might be a way forward for overexpression studies.

Therefore, according to our findings, single and co-overexpression of ABCC1 and ABCC4 in MCF-7 and MDA-MB231 cells did not affect colony formation and cell proliferation when compared to untreated. In Henderson et al., (2011) overexpression of both ABCC1 and ABCC4 in neuroblastoma cell lines was achieved effectively, and this showed an increase in cell migration and colony (Henderson et al., 2011). In our study, the overexpression of ABCC1 and ABCC4 in MCF-7 and MDA-MB231 cells were expected to cause an increase in colony formation but were not the case. Again, this may be the method of transfection was not very efficient as Henderson et al., (2011) used a retroviral vector carrying a plasmid with the gene

of interest to efficiently transfect the cells. Moreover, although no cell cytotoxicity was observed during transfection, speculation that the size of the plasmid DNA vector might affect the efficiency of transfection, as there may be a low percentage of cell that have actually been transfected. A study found that the size of plasmid DNA molecules may affect gene transfer capacity (Kreiss et al., 1999) and that the introduction of plasmid DNA molecules may impaired the cells normal metabolic functioning (Metabolic load) (Glick, 1995).

Finally, the method of overexpression of ABCC1 and ABCC4 in MCF-7 and MDA-MB231 cell is transient. Similar to the siRNA transfection, the efficiency of transfection was not measured in terms of the proportion of cells that were overexpressed in comparison to those that were unaffected. Hence, alternative transfection methods such as to generate a stable cell lines expressing the gene of interest may be an important consideration.

7.3   ABCC1 and ABCC4 may be involved in mediating breast cancer cell migration

Another aspect commonly used to assess cancer development is cell migration. In normal physiological process, cell migration is a complex process that is important for embryonic development, gastrulation (Keller, 2005), nervous system development (Hatten, 1999), placental development, and immune cell trafficking (Wilson et al., 2010) and wound healing (Kramer, et al., 2013; Valster, et al., 2005). However, cell migration can also contribute to pathological processes such as tumour formation and metastasis (Valastyan et al., 2011; Pachmayr et al., 2017). Tumour metastasis is one of the major problem in the treatment of cancer and it is associated with low survival of patients and poor prognosis (Kramer, et al., 2013). Thus, we assessed cell migration using the scratch assay.

Cell migration was monitored using the scratch assay. It should be noted that the results obtained from the scratch assay do represent cell migration and not cell proliferation as according to the MTT assay, the cell proliferation was not affect by any of the treatments over the short periods of time used for the scratch assay.

Only MK571, a non-specific inhibitor of ABCC1 and ABCC4, inhibited cell migration in the MDA-MB231 cell line but not in MCF-7 cell line. In contrast, the other inhibitors did not affect cell migration as measured by the scratch assay. As MK571 can inhibit both ABCC1 and ABCC4, perhaps it is this dual inhibition which has the greatest effect on migration. However, as MK571 is also a leukotriene antagonist, it may also inhibit the binding of LTD_4 to cysteinyl leukotriene receptor 1 (CysLT1) (Gekeler et al., 1995). Hence down regulating β-catenin

signalling which is important in promoting cancer cell survival and inhibiting apoptosis (Nielson et al., 2005; Mezhybovska et al., 2006; Peters-Golden et al., 2007).

Moreover, ABCC1 suppression and ABCC4 suppression in MCF-7 cells inhibited cell migration more than the knockdown of ABCC1 and ABCC4 in MDA-MB231 cells. Analysis of the scratch assays with MCF-7 knockdown cells suggested that ABCC4 inhibition may play a larger role in breast cancer cell migration than ABCC1. Surprisingly, when both ABCC1 and ABCC4 were knocked down in combination, the MCF-7 or MDA-MB231 cell migration were not affected. The overexpression of ABCC1 or ABCC4 in MCF-7 and MDA-MB231 cells also did not affect cell migration. The reasons for these conflicting results are not totally clear, however a more efficient transfection method may help.

Nevertheless, our findings indicate that ABCC1 and ABCC4 may be important in breast cancer cell migration as was seen in the ABC inhibitor experiments and that ABCC4 may have a greater effect on cell migration than ABCC1 as was seen in MCF-7 cells in the siRNA experiments. Our findings contrast to the results obtained with neuroblastoma cells by Henderson, et al., 2011 who found that Reversan an ABCC1 inhibitor significantly inhibited neuroblastoma cell migration. In addition, the knockdown of ABCC1 significantly decreased cell migration in neuroblastoma more than the knockdown of ABCC4 and that an exposure of ABCC1- and ABCC4- specific siRNA molecules in combination in neuroblastoma cells caused even more significantly decreased in cell migration (Henderson et al., 2011). Interestingly, it has been shown that the migration of human dendritic cells are more influenced by ABCC4 than ABCC1 as a study conducted showed that inhibition of dendritic cell migration by sildenafil or by ABCC4 gene knockdown, attenuated their migration from human skin explants (van de Ven et al., 2008 and 2009). Therefore, maybe the roles of ABCC1 and ABCC4 are different between cells / cancer types.

The scratch assay is a widely used experiment to evaluate cell migration as it is easy to setup, analyse and is cheap. In addition, the scratch assay is a convenient assay to study cell-cell interaction and cell-matrix interactions on cell migration, which can mimic cell migration *in vivo*, such as the metastases of cancer cells from one region of the body to another (Liang, et al., 2007; Orimo, 2014). However, one major drawback is that the scratch is unevenly thick as usually the scratch is made manually with a pipette tip and variation in gap width is critical. In addition, after wounding, some cells may be left floating in the gap even after washing, often these cells reattached to the plate and migrate and proliferate in to the wounded area which may lead to unreliable results. Furthermore, wounding in an uncontrolled manner may cause cell damage. To ensure invariable wound sizes with defined edges, the electrical cell-substrate

impedance sensing (Lo et al., 1995) or laser ablation (Tamada et al., 2007) of the cells in a defined area may be useful. However both systems might leave cells in the wounding area after the electric ablation (Poujade, et al., 2007). One suggestion that could be used to measure cell migration is to monitor the movement of single cells. The single cell motility assay could be performed to measure the migration of a single cell on colloidal gold particle coated surfaces (Takaishi et al., 1995). In principle, cells are seeded on cell culture plates coated with colloidal gold particles and when the cells move, they phagocytose the gold particles leaving a white track that can be photographed and the area that has been cleared by the moving cell can be measured, thus allowing the detection of cell pathway in real time and the determination of the absolute speed of migration of a single cell (Kramer et al., 2013).This assay has been used to monitor the migratory paths of pancreatic cancer cells (Lin et al., 2005), squamous cell carcinomas of the head and neck (Sommer et al., 2011) as well as in breast cancer cells (van Golen et al., 2000). Another way that could be used to measure the movement of a single cell is to track the individual cells in time-lapse experiments using a videomicroscopy (Jaqaman et al., 2008; Meijering et al., 2012). Essentially, the migration paths of the selected cells are recorded and analysed to determine the rate and direction of migration at a given time point as well as the total distance move by the cells. Other possible approaches to monitor cellular migration could include transwell migration assay (Boyden chamber assay), cell exclusion zone assay, spheroid migration assay, fence assay (ring assay), capillary chamber migration assays (microfluidic chamber assay) and microcarrier bead assay (Kramer, et al., 2013).

7.4   ABCC4 may be involved in mediating breast cancer cell invasion more than ABCC1

Approximately 90% of death from cancer are due to cancer metastasis whereby cancerous growth occurring at a distant site from where the primary tumour was originally found (Valastyan et al., 2011; Gao et al., 2013; Weinberg, 2014; Orimo, 2014; Guan, 2015; Pachmayr et al., 2017). Early detection of tumours is essentially in combating cancer and stopping metastatic spreading. However, cancers that are metastatic at diagnosis are usually highly fatal and incurable (Guan, 2015; Chambers et al., 2015). Therefore, the need for novel therapeutic strategies are essential to prevent cancer metastasis. As the metastatic process is a multistage process, the mechanism of metastasis is still not clear hence is still a challenge to produce anti-metastatic drugs.

Although cancer metastasis is a multistage processes, the initial invasion of cancer cells into the surrounding tissue at the primary tumour site is critical. Therefore, understanding the

molecular mechanism of cancer cells acquiring an invasive phenotype is essential so as to come up with novel cancer metastasis treatment strategies (Kramer et al., 2013).

As cancer invasion is an essential part of cancer development and progression, we decided to investigate whether ABCC1 and ABCC4 are implicated in fascilitating cell invasion in breast cancer using the transwell invasion assay. Results showed that the suppression of ABCC1 and ABCC4 in MCF-7 cells decreased cell invasion.

In comparison, only the knockdown of ABCC4 in MDA-MB231 cells decreased cell invasion. Since the MDA-MB231 breast cancer cell line is considered to be more invasive than the MCF-7 breast cancer cell line and the expression level of ABCC1 and ABCC4 are higher in MDA-MB231 cells than in MCF-7 cells, it is suggested that ABCC4 may be involved in mediating breast cancer cell invasion more than ABCC1. However, past studies have found that the efflux of sphingosine-1-phosphate (S1P) by ABCC1 also influences cell invasion as well as cell proliferation in MCF-7 breast cancer cells (Takabe et al., 2010; Yamada et al., 2018).

Since the transwell invasion assay measures the number of invasive cells that have degraded the matrix, moved through the extracellular matrix layer and attached to the underside of the membrane inserts (Valster et al., 2005; Kramer et al., 2013; Justus et al., 2014), to determine the thickness of the basement membrane extract of the ECM and the cell seeding density are very important as they vary between cell lines. Furthermore, the transwell invasion assay is an endpoint assay. In our experiment, the cells that have stayed on top of the membrane were initially removed with a cotton swab and the invasive cells at the bottom of the membrane were fixed, stained and counted under the microscope. However, the procedure of removal of cells that have stayed on top of the membrane is tricky and this can lead to errors. Moreover, counting cells under a light microscope can be very tedious as for each well, five areas were selected for cell counting and also if invasive cells at the bottom of membrane are overlapping and close to each other, made cell counting difficult. As a result, this may account for the large variations in the results obtained that leads to large error bars. Therefore, for the results to be more reliable, careful removal of cell at the top of the membrane is needed, and once the stained invasive cells have been counted, the stained invasive cells can be lysed and quantified in a plate reader.

Other alternative invasion assay that may be considered are the platypus invasion assay (No platypus in the assay), 3D cell tracking assay, vertical gel invasion assay, spheroid confrontation assay, gelatin degradation assay, spheroid gel invasion assay and spheroid/monodispersed cell invasion assay (Kramer et al., 2013).

7.5 Possible signalling cascade involving ABCC1 and ABCC4 in mediating breast cancer progression and development

To date, the direct contribution of ABC transporters in mediating cancer progression and development irrespective of their drug-efflux abilities is still not clear (Fletcher et al., 2010 and 2016; Adamska et al., 2018). Since ABC transporters transport many active signalling molecules and hormones as their substrates, it has been suggested that these substrates can activate cancer cells as well as regulate the tumour environment hence contributing to cancer development and progression (Adamska et al., 2018). In addition, signalling lipid molecules such as sphingosine-1-phosphate (S1P), prostaglandins and leukotrienes have a profound role in tumour biology and are known to be substrates of certain ABC transporters. Therefore, mediating the efflux of these molecules may also be important in cancer progression and development (Fletcher et al., 2010).

GPR55, an orphan G-protein-coupled receptors (GPCR) has been proposed to be involved in an autocrine loop that includes ABCC1 exporting lysophosphatidylinositol (LPI), important for mediating cancer cell proliferation (Figure 7.1) (Pineiro et al., 2011; Yamashita et al., 2013). GPR55 signalling cascade involves ERK (Pineiro et al., 2011), and the activation of ERK has also been found to mediate migration of pancreatic carcinoma cells (Stahle et al., 2003).

Initially, the expression levels of GPR55, ERK1/2 and p-ERK were compared in HeLa, MCF-7 and MDA-MB231 cells by Western blot. The protein expression level of GPR55 was highest in MDA-MB231 cells followed by HeLa cells and barely detectable in MCF-7 cells. Our findings support the finding of Ford et al., 2010 who performed a quantitative RT-PCR analysis to determine the expression of GPR55 in MCF-7 and MDA-MB231 cells. Ford et al., 2010 found that the level of expression of GPR55 was around 30-fold higher in MDA-MB231 cells when compared to that in MCF-7 cells.

Furthermore, ERK1/2 expression was highest in MCF-7 cells and may be slightly higher in MDA-MB231 cells than in HeLa cells. In addition, p-ERK expression may be equally detectable in HeLa and MCF-7 cells but very low expressed in MDA-MB231 cells. Similar level of expression of ERK1/2 and p-ERK was seen in a study by Choi et al., 2009, who also found that the expression of ERK1/2 and p-ERK was higher in MCF-7 than in MDA-MB231 cells. However, in contrast another previous study found that the expression of ERK1/2 was higher in MDA-MB231 cells but lower in MCF-7 cells. In addition, they also found that MCF-7 cells has a higher p-ERK expression than in MDA-MB231 cells (Taherian et al., 2011). Since these

experiments were looking at endogenous expression levels of the proteins, the level of expression of these proteins may be hard to detect.

Furthermore, ABCC1 and ABCC4 in MCF-7 and MDA-MB231 cells were knocked down and the expression level of GPR55 and ERK were assessed by Western blot. However, the protein expression levels of GPR55 and ERK1/2 were not affected by the knockdown of ABCC1 and ABCC4.

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Figure 7.1. An autocrine loop involving ABCC1, LPI and GPR55 for regulating cancer cell proliferation. LPI is synthesised intracellularly by cPLA2. Once LPI is made, it is pump out of the cells by ABCC1. LPI can then bind to GPR55 on the cell surface that in turn activate downward signalling pathways such as the induction of calcium mobilisation, the activation of Akt and ERK1/2 in these cells which are involve in mediating cell proliferation. ABCC1, ATP-binding cassette transporter C1; LPI, lysophosphatidylinositol; GPR55, G-protein receptor 55; cPLA2; cytosolic phospholipases A2; Akt, protein kinase B; ERK1/2, mitogen-activated protein kinase 1/2 (Pineiro et al., 2011).

It is plausible that knockdown of ABCC1 (or ABCC4) should not have a direct effect on the expression of GPR55. Knockdown of ABCC1 should decrease levels of LPI exported, but this does not mean it will affect the expression of GPR55, just the signalling through it. If the

signalling is affected though it might be expected that an effect on downstream ERK signalling might be observed. No effect on ERK was observed in our study, however i) GPR55 is able to signal via more than one signalling pathway, ii) other GPCRs also utilise ERK and iii) the antibody for detecting phosphorylated ERK gave very high background signals making it difficult to detect.

To investigate which other substrates of ABCC1 and/or ABCC4 might play a role, the extracellular levels of cAMP, PGE_2, S1P and cysteinyl leukotrienes were analysed by ELISA.

According to our study, the suppression of ABCC4 in MDA-MB231 cells and those treated with MK571, an ABCC1 and ABCC4 inhibitor inhibited the efflux of cAMP out the cells. cAMP is a substrate for ABCC4 (Russel et al., 2008) but not ABCC1. cAMP is involved in the regulation of differentiation, proliferation and apoptosis (Kim et al., 2001; Guillemin et al., 2002). Moreover, a study on acute myeloid leukaemia (AML) found that the intracellular cAMP is regulated by ABCC4 which in turn caused inhibition of cell proliferation but promoted cell differentiation (Copsel et al., 2011). This cAMP efflux may be an important factor by which ABCC4 affects breast cancer development. Our results, also suggested that MDA-MB231 cells efflux lower levels of cAMP than MCF-7 cells. A prevous study also found that the level of activity of cAMP in MCF-7 cells was higher than that in MDA-MB231 cells (Dezong et al., 2014).

PGE_2 has been reported to regulate cell migration and proliferation while inhibiting immune surveillance and apoptosis in cancer cells (Dannenberg et al., 2003; Greenhough et al., 2009; Knab et al., 2014). A previous study found that high expression of ABCC4 together with low level of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and low expression of prostaglandin transporter (PGT) promote the accumulation of PGE_2 in the tumour microenvironment hence stimulate cancer cell proliferation, metastasis and cell survival that may contribute to the poor prognosis of triple-negative breast cancer (Kochel et al., 2016 and 2017). However, our results from the ELISA indicated that the knockdown of ABCC1 or ABCC4 in MCF-7 cells or MK571 treatment had no effect on the efflux of PGE_2 out of the cells, suggesting PGE_2 was not involved. However, it was not possible to measure the effect in MDA-MB231 cells since the levels of PGE_2 exceeded the standard curve. Therefore, more experiments would be needed to explore this further. Moreover, our results also may indicate that MCF-7 cells efflux a low concentration of PGE_2 than MDA-MB231 cells which agrees with a study done by Timoshenko et al., 2003, who investigated the role of prostaglandin E_2 receptors in migration of murine and human breast cancer cells.

S1P is a ligand of five specific GPCRs $S1P_{1-5}$ (S1PRs) and has been found to mediate important biological actions including cell migration, differentiation, proliferation and cytoskeletal organisation, angiogenesis, lymphocyte trafficking, mast cell function and vascular maturation (Saba et al., 2004; Taha et al., 2004; Ishii et al., 2004; Young et al., 2006; Alvarez et al., 2007; Strub et al., 2010). ABCC1 has been shown to be a major transporter of S1P in mast cells that may influence migration of mast cells to antigen (Mitra et al., 2006; Alvarez et al., 2007). ABCC4 has also been implicated in efflux of S1P release from platelets (Vogt et al., 2018). In a previous study, using MCF-7 breast cancer cells, it was found that estradiol stimulated the production of S1P by sphingosine kinase 1 (SphK1). The export of S1P was facilitated by ABCC1 and ABCG2. Once S1P had been effluxed out of the cell, S1P bound and activated S1P receptors that increase epidermal growth factor receptor (EGFR) transactivation in a matrix metalloprotease-dependent manner and stimulate ERK1/2 to further activate downstream signalling cascade that are responsible for breast cancer proliferation, invasion and progression (Figure 7.2) (Takabe et al., 2010).

In this current study, the efflux of S1P by MDA-MB231 cells treated with MK571 were significantly reduced when compared to that of the untreated MDA-MB231 cells. Although the individual knockdowns did not alter S1P levels, MK571 is able to inhibit both ABCC1 and ABCC4 and this combined effect may be larger. Further experiments with a more efficient method of knockdown will allow this question to be addressed.

Figure 7.2. A schematic diagram showing the potential mechanism involving ABCC1 and S1P in promoting breast cancer proliferation, invasion and progression. Binding of E_2 to ER-α and not to GPR30 stimulate the production of S1P by SphK1. The export of S1P by ABCC1 and ABCG2, binds and activates S1PR that in turn stimulate ERK1/2 signalling cascade which is important in mediating breast cancer cell proliferation, invasion and progression. ABCC1, ATP-binding cassettes transporter C1; S1P, sphingosine-1-phosphate; E_2, Estradiol; GPR30, G-protein receptor 30; ER-α, estrogen receptor α; SphK1, sphingosine kinase 1; ERK1/2, mitogen-activated protein kinase 1/2 (Takabe et al., 2010).

In our study, we also found that the MCF-7 samples showed low concentrations of S1P exported out of the cells compared to MDA-MB231 cells. This contrasts with Takabe et al., 2010 who found that MCF-7 cells export relatively high levels of S1P into the cell culture medium, more than MDA-MB231 cells. Furthermore, another study have confirmed that the export of S1P by ABCC1 activates SphK1 and the accumulation of S1P in the tumour microenvironment in breast cancer have enhanced important biological processes involved in cancer progression (Yamada et al., 2018).

According to our ELISA results, large concentration of cysteinyl leukotrienes were obtained from cells treated with MK571. Since MK571 is a selective leukotriene LTD_4 receptor antagonist, it may have a binding affinity for the cysteinyl leukotrienes antibody that may result in high false positive. Alternatively, it could be that inhibiting the LTD_4 receptor results in some kind of feedback loop to increase the production of cysteinyl leukotrienes. Many other ABCC subfamily of transporters including ABCC2, ABCC3, ABCC10 and ABCC11 are able to efflux LTC_4 (Cui et al., 1999; Rius et al., 2008; Chen et al., 2005 and 2010). ABCC4 is able to efflux both LTC_4 and LTB_4 which are important mediators of inflammation (Cui et al., 1999; Rius et

al., 2008; Chen et al., 2005 and 2010). Since in our study, only ABCC1 and ABCC4 were being knocked down, other ABCC transporters may still be capable in exporting Cysteinyl leukotrienes, thus the concentration of cysteinyl leukotrienes in the conditioned medium may not be affected by the knock down of ABCC1 and ABCC4 as indicated in our results. Moreover, in pancreatic cancer cells, LTB_4 was found to induce ERK1/2 phosphorylation that in turn activate cell proliferation (Tong et al., 2002; Knab et al., 2014).

Although the ELISA is a straightforward assay that is easy to follow and requires little hands-on-time, each step is very critical and involved careful pipetting of small volumes of a number of reagents to the plate at different times, thus the degree of errors and contaminations between reagents are very high hence may lead to large degree of variation in data resulting in large error bars as this may be the case in our experiments. Some of our data fall outside the standard curve so was difficult to extrapolate the concentration, therefore, the samples might need to be diluted or concentrated. Moreover, since the ELISA was done only one time, a repeat might be useful to validate the results obtained thus far.

7.6   Future work

- Use flow cytometry to analyse the percentage of cells that have been transfected with ABCC1- and ABCC4-specific siRNA molecules in a cell population. The results will determine the efficiency of transfection and allow optimisation or use a different transfection reagent or methods.

- Consider generating stable cell lines for knockdown and/or overexpression of ABCC1 and ABCC4.

- Perform immunofluorescence staining on cell lines to determine the level of expression of specific proteins of interest and their location in the cell.

- Repeat ELISA analysis, including dilution/concentration of samples so that all signals lie within the standard curves.

7.7   Overall conclusion

This thesis demonstrate that the expression levels of ABC transporters varies between breast cancer cell lines. The involvement of ABCC1 and ABCC4 in mediating breast cancer cell proliferation, migration and invasion were investigated. Our results suggested that ABCC1

may be more involved in mediating breast cancer cell proliferation than ABCC4 and in contrast ABCC4 may be involved in mediating breast cancer cell invasion more than ABCC1. Furthermore, our findings also indicate that breast cancer cell migration may be influenced by the expression of ABCC1 and ABCC4.

Subsequently, understanding the mechanism by which ABCC1 and ABCC4 are involved in breast cancer development and progression is very important. Since ABC transporters transport many active signalling molecules and hormones that are also implicated in cancer progression, studying these molecules would be very useful in determining any possible mechanism. Although in this thesis, only cAMP, PGE_2, S1P and cysteinyl leukotrienes were being examined, results looks promising and further investigation in to these molecules may be required to obtain a clear correlation with ABCC1 and ABCC4.

Taken together our findings have shown that ABCC1 and ABCC4 may be implicated in breast cancer development and progression. However, further investigations are need to validate our current results. Nevertheless, our results suggest that ABCC1 and ABCC4 could be a potential therapeutic target for breast cancer.

8. References

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