# IDENTIFICATION AND CHARACTERIZATION OF TRANSPORTERS IN HUMAN GLIOMAS

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# ABSTRACT

Functional overexpression of the ATP binding cassette (ABC) transporters at the cell surface is thought to be responsible for clinical multidrug resistance (MDR) in tumours of the brain. Inhibition of ABC transporters by existing inhibitors has proven to be inconclusive.

This research program hypothesized an alternative location for the ABC transporters in glioblastoma cells and also proposed to develop stationary phases for the identification of ABC transporters inhibitors.

Expression profile investigation of P-glycoprotein (PGP), multidrug resistant protein 1 (MRP1), multidrug resistant protein 2 (MRP2) and the breast cancer resistant protein (BCRP) in glioblastoma multiforme cell lines and clinical patient specimens suggested varying levels of expression. Localisation studies by confocal microscopy confirmed cell surface expression and also indicated that BCRP was localised at the nucleus of the T98 and LN229 cells. Immunoblots of LN229 nuclear extracts indicated ~ 2 fold higher expression of BCRP as compared to cytoplasmic extracts. Immunohistochemistry studies with clinical samples confirmed the nuclear and perinuclear location of BCRP. $IC_{50}$ value for Mitoxantrone (MTX); a BCRP substrate was calculated as 0.29 ± 0.020 μM for the LN229 cell line, and pre-treatment with the cell impermeant fumitremorgin C

(FTC, 5 μM) slightly reduced the $IC_{50}$ value to 0.16 ± 0.087 μM. This refractoriness to FTC is in contrast with the literature showing a ~ 6-fold reduction in $IC_{50}$ value of MTX upon pre-treatment with FTC in human breast cancer MCF-7 cell line with ectopic expression of BCRP. The results supported the notion that the nuclear presence of endogenously expressed BCRP actively extrudes MTX, and that because FTC is not able to inhibit the nuclear BCRP, significant reduction in the $IC_{50}$ was not observed. The results suggest that the treatment of clinical MDR should be expanded to include inhibition of ABC transporters functioning at the nuclear membrane.

Cellular membrane affinity chromatography columns were developed for the study of the MRP1, MRP2 and BCRP using *Spodoptera frugiperda* (Sf9) cells that had been stably transfected with human Mrp1, Mrp2 or Bcrp cDNA. The resulting columns and a control column were characterized using frontal affinity chromatography using [$^3$H]-etoposide as the marker ligand and etoposide, benzbromarone and MK571 as the displacers on the CMAC($Sf9_{MRP1}$) column, etoposide and furosemide on the CMAC($Sf9_{MRP2}$) column and etoposide and fumitremorgin C on the CMAC($Sf9_{BCRP}$) column. The binding affinities obtained from the chromatographic studies were consistent with the data obtained using non-chromatographic techniques and the results indicate that the immobilized MRP1, MRP2 and BCRP transporters retained their ability to selectively bind known ligands. The results indicated that the CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$) columns can be used for the study of binding to the MRP1,

MRP2 and BCRP transporters and that membranes from the Sf9 cell line can be used to prepare CMAC columns.

This study expands our knowledge of the ABC transporters and makes a case for the finding that nuclear efflux proteins play a pivotal role in the overall MDR phenotype in CNS tumours. Also the CMAC columns developed and characterised provide a tool to study the binding of potential therapeutic candidates to ABC proteins.

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## ABBREVIATIONS

| | |
|---|---|
| ABC | ATP binding cassette |
| ATP | adenosine tri phosphate |
| BBB | blood brain barrier |
| Bmax | Maximum number of binding sites |
| BCA | bicinchoninic acid assay |
| BCRP | breast cancer resistant protein |
| BSA | bovine serum albumin |
| CMAC | cellular membrane affinity chromatography |
| CNS | central nervous system |
| CpG | C phosphate G |
| CSF | cerebrospinal fluid |

| | |
|---|---|
| CT | computer tomography |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| ECL | enhanced chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | epidermal growth factor receptor |
| EGTA | ethylene glycol tetraacetic acid |
| EMEM | Eagle's minimum essential medium |
| FTC | fumitremorgin C |

| | |
|---|---|
| GBM | glioblastoma multiforme |
| GSH | glutathione |
| HER | human epidermal growth factor receptor |
| HHV-6 | human herpesvirus 6 |
| HIV | human immunodeficiency virus |
| HMGA2 | high mobility AT hook 2 |
| IAM-PC | immobilized artificial membrane phospahtidyl choline |
| IM | integral domain |
| LSC | liquid scintillation counter |
| LTC4 | leukotrine C4 |
| LTD4 | leukotrine D4 |
| LTE4 | leukotrine E4 |

| | |
|---|---|
| MDR | multiple drug resistance |
| MRI | magnetic resonance imaging |
| MRP1 | multi drug resistance protein 1 |
| MRP2 | multi drug resistance protein 2 |
| MRP3 | multi drug resistance protein 3 |
| MSD | membrane spanning domain |
| MTS | MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] |
| MXR | mitoxantrone resistant receptor |
| NBD | nucleotide binding domain |
| p53 | protein 53 |
| PBS | phosphate buffer solution |
| PDGF | platelet derived growth factor |

| | |
|---|---|
| Pgp | P-glycoprotein |
| PTEN | phosphatase and tensin homologue |
| PVDF | polyvinylidene fluoride |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| SDS | sodium dodecyl sulphate |
| siRNA | small interfering ribonucleic acid |
| SV40 | simian virus 40 |
| TMD | trans membrane domain |
| TMS | trans membrane spanning |
| WHO | world health organization |

# Chapter 1

# 1. General Introduction

## 1.1 Background

Glioblastoma multiforme (GBM) is the most common form of malignant primary brain tumours in adults. Classified as a Grade IV astrocytoma, GBM develops from the lineage of glial cells, called astrocytes, which support nerve cells. GBM develops primarily in the cerebral hemispheres but can develop in other parts of the brain, brainstem, or spinal cord (Woodworth et al. 2013). GBM has four distinct genetic subtypes that respond differently to aggressive therapies, making treatment extremely difficult and challenging. GBM are almost always refractory to chemotherapy. This refraction is the result of the multi-drug resistance (MDR) phenotype due to the over-expression of the ATP-binding cassette (ABC) efflux transporters.

ABC transporters are members of a protein superfamily that is one of the largest families of proteins. ABC transporters are trans-membrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carry out certain biological processes including translocation of various substrates across membranes (Choi, Yu 2013). They transport a wide variety of substrates across extra- and intracellular membranes, including metabolic products, lipids and sterols, and

drugs. Proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding cassette (ABC) domain(s). ABC transporters are involved in tumour resistance, cystic fibrosis and a range of other inherited human. In addition to extruding a wide variety of anti-neoplastic agents, the ABC transporters are known to efflux a broad range of ligands like protease inhibitors and anti-malarial drugs, thereby decreasing their bioavailability.

ABC transporters are known to play a crucial role in the development of multidrug resistance. In multidrug resistance, patients that are on medication eventually develop resistance not only to the drug they are taking but also to several different types of drugs. This is caused by several factors, one of which is increased excretion of the drug from the cell by ABC transporters. Malignant gliomas are frequently chemoresistant and this resistance seems to depend on their interaction with several ABC drug efflux transporters that are overexpressed by the endothelial or epithelial cells. Resistance may also involve the tumour cells themselves. Although ABC drug efflux transporters in tumour cells confer multidrug resistance (MDR) on several other solid tumours, their role in gliomas is unclear.

Given the widespread tissue distribution and substrate recognition, it is imperative to investigate the role of the ABC transporters in GBM and elicit their role in clinical MDR.

## 1.2 Introduction to Cancer

Cancer is a broad group of diseases involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumours, and invade nearby parts of the body. Cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream. Not all tumours are cancerous; benign tumours do not invade neighbouring tissues and do not spread throughout the body. There are over 200 different known cancers that afflict humans.

The causes of cancer are diverse, complex, and only partially understood. Many things are known to increase the risk of cancer, including tobacco use, dietary factors, certain infections, exposure to radiation, lack of physical activity, obesity, and environmental pollutants. These factors can directly damage genes or combine with existing genetic faults within cells to cause cancerous mutations. Approximately 5 – 10% of cancers can be traced directly to inherited genetic defects. (Siegel, Naishadham & Jemal 2012) Many cancers can be prevented by not smoking, eating more vegetables, fruits and whole grains, eating less meat and refined carbohydrates, maintaining a healthy weight, exercising, minimizing sunlight exposure, and being vaccinated against some infectious diseases.

Cancer can be detected in a number of ways, including the presence of certain signs and symptoms, screening tests, or medical imaging. Once a possible cancer is detected it is diagnosed by microscopic examination of a tissue sample. Cancer is usually treated with chemotherapy, radiation therapy and surgery. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment. While cancer can affect people of all ages, and a few types of cancer are more common in children, the risk of developing cancer generally increases with age.

According to the epidemiological data from Cancer Research, UK it was reported that around 7.5 million people worldwide would die from cancer. It is believed that one in three people develop cancer in their lifetime. The progression of cancer can be described as a malignant tumour, which is the irregular and uncontrolled growth of cells that continually divide, then attack and damage surrounding tissues. The spread and growth of malignant tumours is rapid and life threatening if not treated in the early stages.

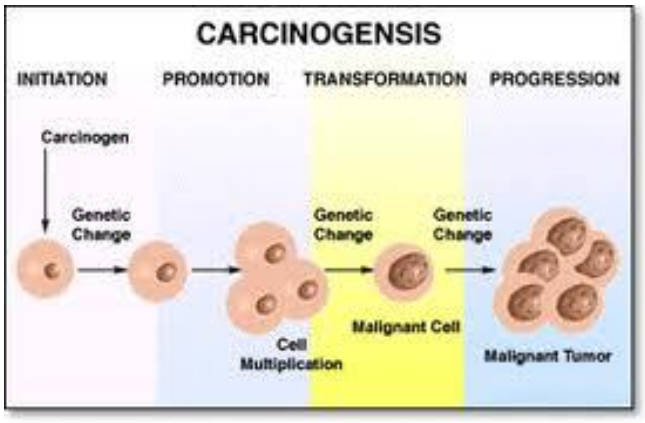


***Figure 1. Stages of Carcinogenesis showing initiation, promotion, transformation and progression.***

Carcinogenesis is the term that describes the progression of cancer within the body. This is a multi-step process that entails mutation, amplification and deletion of DNA strands in essential genes of the body. There are four stages of carcinogenesis: initiation, promotion, transformation and progression as depicted in Figure 1. Initiation is the quickest and requires a carcinogen to bind to the DNA within the cells, resulting in damage of the strand and cause a number of irreversible mutations. The second stage of carcinogenesis involves amplified proliferation of the initiated cells. This stage of carcinogenesis can be reversed and occurs over many years before continuing to the final stage. Therefore the final stage of the progression is an extended part of the promotion stage and causes more proliferation. This results in an alteration of the genotype and phenotype of the cells causing metastasis and malignancy. The initiation of

carcinogenesis is due to the carcinogens, which are compounds; people are exposed to everyday in the environment, which modify the DNA sequences. (Wasserman, Zambetti & Malkin 2012)

Carcinogenesis or oncogenesis or tumourigenesis is literally the creation of cancer. It is a process by which normal cells are transformed into cancer cells. It is characterized by a progression of changes at the cellular, genetic and epigenetic level that ultimately reprogram a cell to undergo uncontrolled cell division, thus forming a malignant mass. (Weber et al. 2013)

Cell division is a physiological process that occurs in almost all tissues and under many circumstances. Under normal circumstances, the balance between proliferation and programmed cell death, usually in the form of apoptosis, is maintained by tightly regulating both processes to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer disrupt these orderly processes by disrupting the programming regulating the processes.

Carcinogenesis is caused by mutation of the genetic material of normal cells, which upsets the normal balance between proliferation and cell death. This results in uncontrolled cell division and the evolution of those cells by natural selection in the body. The uncontrolled and often rapid proliferation of cells can lead to

benign tumours; some types of these may turn into malignant tumours. Benign tumours do not spread to other parts of the body or invade other tissues, and they are rarely a threat to life unless they compress vital structures or are physiologically active, for instance, producing a hormone. Malignant tumours can invade other organs, spread to distant locations and become life-threatening.

More than one mutation is necessary for carcinogenesis. In fact, a series of several mutations to certain classes of genes is usually required before a normal cell will transform into a cancer cell.

Cancer is fundamentally a disease of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, genes that regulate cell growth and differentiation must be altered. Genetic and epigenetic changes can occur at many levels, from gain or loss of entire chromosomes, to a mutation affecting a single DNA nucleotide, or to silencing or activating a microRNA that controls expression of 100 to 500 genes. There are two broad categories of genes that are affected by these changes. Oncogenes may be normal genes that are expressed at inappropriately high levels, or altered genes that have novel properties. In either case, expression of these genes promotes the malignant phenotype of cancer cells. Tumour suppressor genes are genes that inhibit cell division, survival, or other properties of cancer cells. Tumour suppressor genes are often disabled by cancer-

promoting genetic changes. Typically, changes in many genes are required to transform a normal cell into a cancer cell.

There is a diverse classification scheme for the various genomic changes that may contribute to the generation of cancer cells. Many of these changes are mutations, or changes in the nucleotide sequence of genomic DNA. There are also many epigenetic changes that alter whether genes are expressed or not expressed. Aneuploidy, the presence of an abnormal number of chromosomes, is one genomic change that is not a mutation, and may involve either gain or loss of one or more chromosomes through errors in mitosis.

Large-scale mutations involve the deletion or gain of a portion of a chromosome. Genomic amplification occurs when a cell gains many copies (often 20 or more) of a small chromosomal region, usually containing one or more oncogenes and adjacent genetic material. Translocation occurs when two separate chromosomal regions become abnormally fused, often at a characteristic location. A well-known example of this is the Philadelphia chromosome, or translocation of chromosomes 9 and 22, which occurs in chronic myelogenous leukemia, and results in production of the BCR-abl fusion protein, an oncogenic tyrosine kinase. (Xu et al. 2012)

Small-scale mutations include point mutations, deletions, and insertions, which may occur in the promoter of a gene and affect its expression, or may occur in the gene's coding sequence and alter the function or stability of its protein product. Disruption of a single gene may also result from integration of genomic material from a DNA virus or retrovirus, and such an event may also result in the expression of viral oncogenes in the affected cell and its descendants.

Epimutations include methylations or demethylations of the CpG islands of the promoter regions of genes, which result in repression or de-repression, respectively of gene expression. Epimutations, can also occur by acetylation, methylation, phosphorylation or other alterations to histones, creating a histone code that represses or activates gene expression, and such histone epimutations can be important epigenetic factors in cancer. In addition, carcinogenic epimutation can occur through alterations of chromosome architecture caused by proteins such as HMGA2 (High mobility AT hook 2). A further source of epimutation is due to increased or decreased expression of microRNAs (miRNAs). For example extra expression of miR-137 can cause downregulation of expression of 491 genes, and miR-137 is epigenetically silenced in 32% of colorectal cancers. (Sun et al. 2013)

Treatment options are limited owing to the nature of the disease. The progression is fast and the damage often irreparable. Surgical resection and radiation therapy are used to debulk the tumour. Once the tumour is debulked, single agent or adjuvant chemotherapy is employed to manage the remaining cell mass.

Amongst all the malignancies, tumours of the brain are the most aggressive and fatal. Owing to the fact that brain is by far the most important organ in the human body and is responsible for various important functions of the human body.

### 1.2.1 Glioblastoma multiforme

Tumours of the CNS are varied and include neoplasms from the neuroepithelial, meningeal, germ cell and sellar origins. Glioblastoma multiforme (GBM) are neuroepithelial tumours that result from astrocytes and/or glial cells. Glioblastomas are usually highly malignant — a large number of tumour cells are reproducing at any given time, and they are nourished by an ample blood supply. Dead cells may also be seen, especially toward the centre of the tumour (Deutsch et al. 2013). Because these tumours come from normal brain cells, it is easy for them to invade and live within normal brain tissue. However, glioblastoma rarely spreads elsewhere in the body.

Because glioblastomas can grow rapidly, the most common symptoms are usually caused by increased pressure in the brain. These symptoms can include headache, nausea, vomiting, and drowsiness. Depending on the location of the tumour, patients can develop a variety of other symptoms such as weakness on one side of the body, memory and/or speech difficulties, and visual changes. For unknown reasons, GBM occurs more commonly in males.

Most glioblastoma tumours appear to be sporadic, without any genetic predisposition. No links have been found between glioblastoma and smoking, consumption of cured meat, or electromagnetic fields. Alcohol consumption may be a possible risk factor. Glioblastoma has been associated with the viruses SV40 (Simian virus 40), HHV-6 and cytomegalovirus. (Chi et al. 2012) There also appears to be a small link between ionizing radiation and glioblastoma. Some also believe that there may be a link between polyvinyl chloride and glioblastoma. There is an association of brain tumour incidence and malaria, suggesting that the anopheles mosquito, the carrier of malaria, might transmit a virus or other agent that could cause glioblastoma or that the immunosuppression associated with malaria could enhance viral replication. Also HHV-6 (Human herpesvirus 6) reactivates in response to hypersensitivty reactions from drugs and environmental chemicals.

#### *1.2.1.1 Epidemiology*

The incidence of primary brain tumours worldwide is approximately seven per 100,000 individuals per year, accounting for ~2% of primary tumours and 7% of the years of life lost from cancer before the age of 70. The malignant astrocytomas, the anaplastic astrocytoma and glioblastoma multiforme, are the most common glial tumours, with an annual incidence of 3 to 4 per 100,000 populations. (Aldape et al. 2003)

#### *1.2.1.2 Classification*

Glial tumours are divided into two main categories: astrocytic and oligodendroglial (Benjamin, Capparella & Brown 2003). Both can be either low grade or high grade. High-grade (malignant) glial neoplasms can arise either alone (primary glioblastoma) or from a pre-existing low-grade tumour (secondary glioblastoma); in secondary glioblastoma, low-grade tumour may be immediately adjacent to highly malignant disease. All gliomas, particularly the astrocytic neoplasms are histologically, genetically, and therefore therapeutically heterogeneous (Huse, Holland 2010).

Glial tumours are graded pathologically, on the basis of the most malignant area identified, according to either the World Health Organization (WHO) system or the St. Anne–Mayo system, both of which are based on the presence or absence of nuclear atypia, mitosis, microvascular proliferation, and necrosis (Vogelstein, Kinzler 2004).

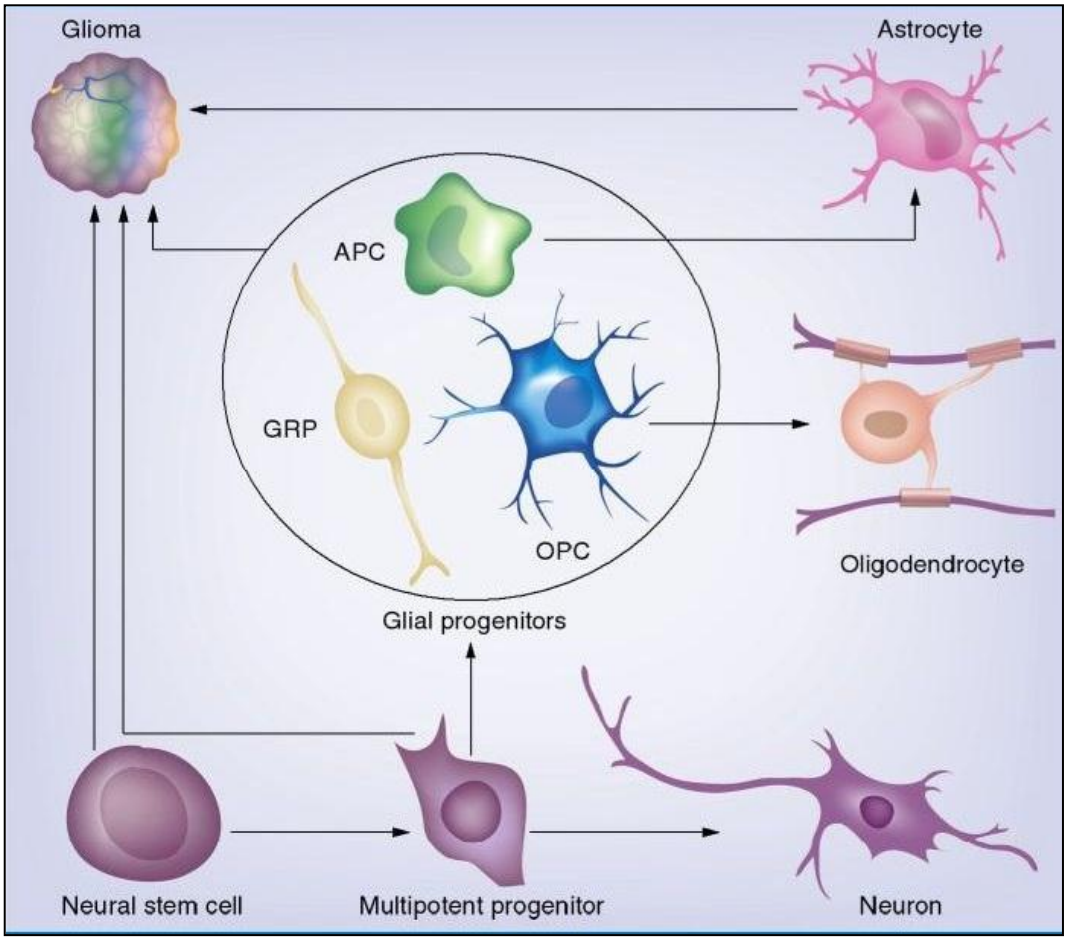


***Figure 2: Classification of glioblastomas***

Gliomas can occur anywhere in the brain but usually affect the cerebral hemispheres. The male-to-female ratio among affected patients is about 3:2. The peak age at onset for anaplastic astrocytomas is in the fourth or fifth decade, whereas glioblastomas usually present in the sixth or seventh decade. Most malignant astrocytomas are sporadic, but they can occasionally complicate genetic syndromes such as neurofibromatosis type 1, neurofibromatosis type 2, Li–Fraumeni syndrome, and Turcot's syndrome (Zelcer et al. 2003).

Primary glioblastomas tend to occur in older patients (mean age, 55 years), whereas secondary glioblastomas tend to occur in younger adults (45 years of age or less). The difference between these two can be recognized radiographically, when regions of non-enhancing tumour are evident in secondary glioblastomas, as well as pathologically, when a surgical specimen contains low-grade disease. The two types of glioblastoma arise through different molecular pathways. Primary glioblastomas are associated with a high rate of overexpression or mutation of the epidermal growth factor receptor, p16 deletions, and mutations in the gene for phosphatase and tensin homologues (PTEN). (Tachibana et al. 2000)

Secondary glioblastomas have genetic alterations involving the p53 (protein 53) gene and overexpression of platelet-derived growth factor A and its receptor,

platelet-derived growth factor receptor a. These two pathways clearly demonstrate that the glioblastoma phenotype can arise by at least two mechanisms.

#### *1.2.1.3. Clinical presentation*

Brain tumours can cause either focal or generalized neurologic symptoms. Generalized symptoms reflect increased intracranial pressure and consist of headache and, when the illness is severe, nausea, vomiting, and sixth-nerve palsy. Focal symptoms and signs, such as hemiparesis and aphasia, reflect the intracranial location of the tumour. (Kleihues, Ohgaki 1999) The frequency and duration of symptoms vary with the type of tumour. Headache occurs in about half of all patients with brain tumours. Typically, the headache is diffuse, but it can accurately indicate the hemisphere in which the tumour is located. Generally, the headache is more noticeable on awakening in the morning and, even without treatment, dissipates within a few hours. (Han et al. 2010) The headache can occasionally be unilateral and throbbing and can mimic migraine or even cluster headaches. Seizures occur at presentation in 15 to 95 percent of patients with brain tumours, depending on the type of tumour. Typically, the seizures are focal but may become generalized and cause loss of consciousness.

Signs and symptoms from glioblastoma multiforme usually result from infiltration or compression of normal brain by tumour, oedema, and sometimes haemorrhage. Cerebrospinal fluid (CSF) pathways or vascular flow can also be compromised, leading to further deficits and increased intracranial pressure. The most common findings include headaches, seizures, focal neurologic deficits, and changes in mental status.

The most common differential diagnoses include brain metastases, primary central nervous system lymphoma, enhancing low-grade glioma, and non-neoplastic disorders such as abscess, multiple sclerosis, progressive multifocal leukoencephalopathy, cerebral infarction, and vascular malformation (Grossman, Batara 2004).

#### *1.2.1.4. Diagnosis*

The only test needed to diagnose a brain tumour is cranial magnetic resonance imaging (MRI). Computed tomography (CT) can miss structural lesions, particularly in the posterior fossa, or non-enhancing tumours such as low-grade gliomas. Therefore, if a brain tumour is a diagnostic consideration, MRI with gadolinium enhancement is the test of choice; a normal contrast-enhanced MRI scan essentially rules out the possibility of a brain tumour. An angiogram can also

be employed where a special dye is injected into the arteries that feed the brain, making the blood vessels visible on X-ray. This test helps locate blood vessels in and around the brain tumour. Biopsy where a surgeon typically removes a small tissue sample from the tumour to further study the type of the tumour is used a confirmatory procedure. (Gajewicz et al. 2003)

#### *1.2.1.5. Pathogenesis*

At least two distinct molecular pathways lead to the development of glioblastoma multiforme. When this tumour arises from a pre-existing low-grade astrocytoma, it is referred to as a secondary glioblastoma. The low-grade astrocytomas often have mutations in the p53 tumour-suppressor gene and overexpression of platelet-derived growth factor (PDGF).

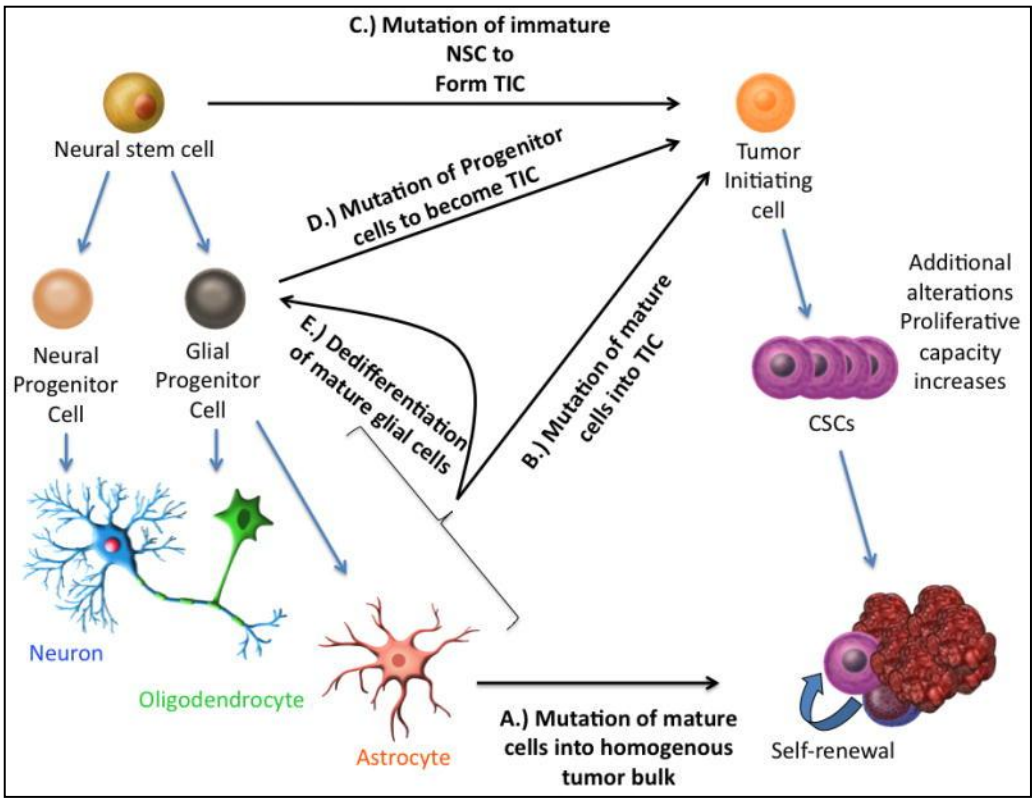


***Figure 3. Pathogenesis of glioblastomas***

Primary glioblastomas demonstrate loss of the tumour-suppressor gene PTEN or amplification or overexpression of epidermal growth factor receptor (EGFR) without p53 mutation (Grossman, Batara 2004). Multiple theories about the pathogenesis of gliomas exist as shown in Figure 3. Astrocytes arising from glial progenitor cells that morph from neural stem cells result in homogenous tumour bulk. Neural stem cells may also mutate into tumour initiating cells as shown in C.) Tumour initiating cells may also result from progenitor cells as well as dedifferentiated glial cells.

### *1.2.1.6. Treatment*

There are no curative treatment options for GBM. The standard approach is to surgically resect the tumour as much as possible. Surgery has a critical role in the management of patients with newly diagnosed glioblastoma multiforme. Stereotactic biopsies are generally reserved for tumours located in deep tissue or in patients with extensive lesions where meaningful debulking cannot be obtained. Surgical resection is followed by radiotherapy. Radiation remains the most effective therapy for post-operative patients with glioblastoma multiforme. One of the recent advanced surgical techniques is gamma knife radio surgery and stereotactic radiosurgery using cyberknife or other linear accelerator based systems.

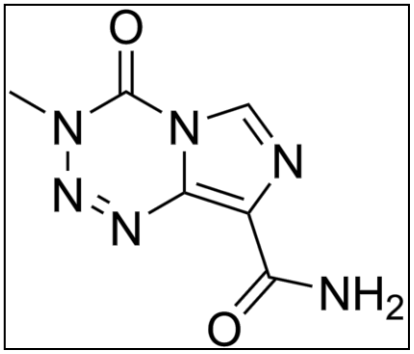


***Figure 4. Structure of Temozolamide; a chemotherapeutic agent for the treatment of gliomas***

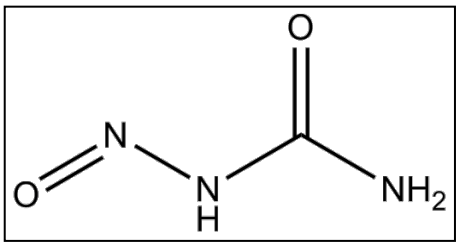


***Figure 5. Structure of Nitrosourea; used in chemotherapy of gliomas***

Chemotherapy is often administered in an adjuvant therapy to patients who have no remaining detectable cancer but are at high risk for recurrence. In this situation, the impact of chemotherapy is optimized till the total-body tumour burden is minimal. However effective chemotherapy is limited due to drug resistance. Nitrosoureas are used to manage newly diagnosed GBM. Recently temozolomide has been used as the primary choice of treatment, as it was shown to significantly improve the survival from 12.1 months to 14.6 months (Du, Searle 2009).

### 1.2.2. Multidrug resistance

The phenomenon where cells become resistant to structurally unrelated drugs is classically termed as multi drug resistance (MDR) (Lemos et al. 2009). MDR can be innate or acquired during chemotherapy.

There are two general classes of resistance to anticancer drugs: those that impair delivery of anticancer drugs to tumour cells and those that arise in the cancer cell itself due to genetic and epigenetic alterations that affect drug sensitivity. Impaired drug delivery can result from poor absorption of orally administered drugs, increased drug metabolism or increased excretion, resulting in lower levels of drug in the blood and reduced diffusion of drugs from the blood into the tumour mass. Recent studies have emphasized the importance of the tumour vasculature and an appropriate pressure gradient for adequate drug delivery to the tumour (Liu et al. 2009). In addition, some cancer cells that are sensitive to chemotherapy as monolayer cells in culture become resistant when transplanted into animal models. This indicates that environmental factors, such as the extracellular matrix or tumour geometry, might be involved in drug resistance (Decleves et al. 2006). Cancer cells grown in culture as three-dimensional spheroids, mimicking their in vivo geometry, have also been shown to become resistant to cancer drugs.

Cellular mechanisms of drug resistance have been intensively studied, as experimental models can be easily generated by in vitro selection with cytotoxic agents. Cancer cells in culture can become resistant to a single drug, or a class of drugs with a similar mechanism of action, by altering the drug's cellular target or by increasing repair of drug-induced damage, frequently to DNA (Decleves et al. 2006). After selection for resistance to a single drug, cells might also show cross-resistance to other structurally and mechanistically unrelated drugs. This might explain why treatment regimens that combine multiple agents with different targets are not more effective.

Multidrug resistance can also result from activation of co-ordinately regulated detoxifying systems, such as DNA repair and the cytochrome P450 mixed-function oxidases (Dauchy et al. 2008).

Resistance can also result from defective apoptotic pathways. This might occur as a result of malignant transformation; for example, in cancers with mutant or non-functional p53 (Huang et al. 2009). Alternatively, cells might acquire changes in apoptotic pathways during exposure to chemotherapy, such as alteration of ceramide levels or changes in cell-cycle machinery, which activate checkpoints and prevent initiation of apoptosis (Liu et al. 2008).

An important principle in multidrug resistance is that cancer cells are genetically heterogeneous (Boutin et al. 2009). Although the process that results in uncontrolled cell growth in cancer favours clonal expansion, tumour cells that are exposed to chemotherapeutic agents will be selected for their ability to survive and grow in the presence of cytotoxic drugs (Calcagno et al. 2010). These cancer cells are likely to be genetically heterogeneous because of the mutator phenotype. So, in any population of cancer cells that is exposed to chemotherapy, more than one mechanism of multidrug resistance can be present. This phenomenon has been called multifactorial multidrug resistance.

Finally, one of the main reasons of the MDR phenotype is the over-expression of the ATP-binding cassette efflux transporters.

ABC transporters are known to play a crucial role in the development of multidrug resistance (MDR). In MDR, patients that are on medication eventually develop resistance not only to the drug they are taking but also to several different types of drugs. This is caused by several factors, one of which is increased excretion of the drug from the cell by ABC transporters. For example, the ABCB1 protein (P-glycoprotein) functions in pumping tumour suppression drugs out of the cell. Pgp also called MDR1, ABCB1, is the prototype of ABC transporters and

also the most extensively-studied gene. Pgp is known to transport organic cationic or neutral compounds. A few ABCC family members, also known as MRP, have also been demonstrated to confer MDR to organic anion compounds. The most-studied member in ABCG family is ABCG2, also known as BCRP (breast cancer resistance protein) confer resistance to most of Topoisomerase I or II inhibitors such as topotecan, irinotecan, and doxorubicin.

It is unclear exactly how these proteins can translocate such a wide variety of drugs, however one model (the hydrophobic vacuum cleaner model) states that, in P-glycoprotein, the drugs are bound indiscriminately from the lipid phase based on their hydrophobicity.

### 1.2.3. ATP binding cassette transporters

The ATP-binding cassette (ABC) superfamily of proteins contains a number of membrane bound ATP driven transporters that pump drugs, drug metabolites and endogenous compounds out of cells (Liu et al. 2009). Although at present there are 49 known human ABC family members belonging to 7 different subfamilies; P-glycoprotein (Pgp), multi drug resistance protein 1 (MRP1), multi drug

resistance protein 2 (MRP2) and the breast cancer resistant protein (BCRP) have gained significant attention, owing to their widespread tissue distribution and role in multiple drug resistance.

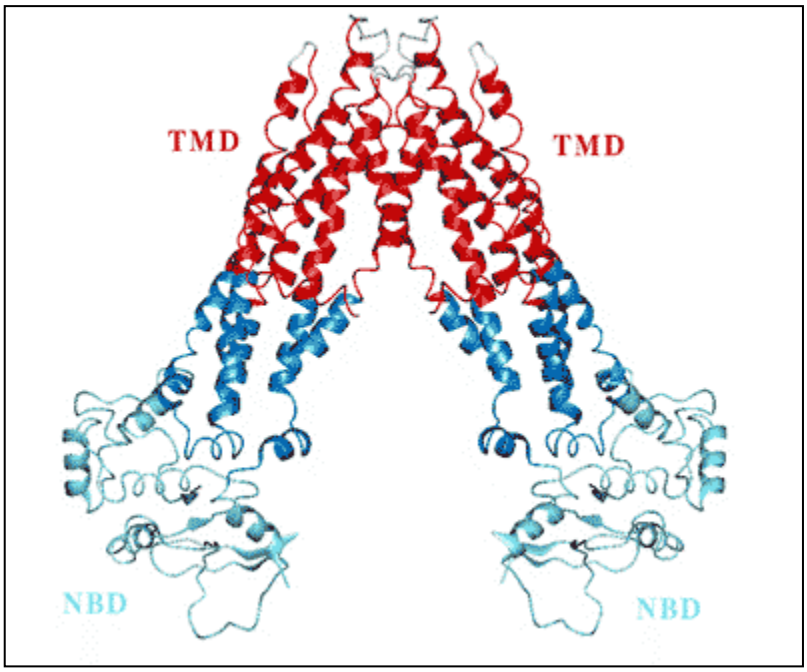


***Figure 6. A typical ABC protein containing two transmembrane domains (TMDs). Each domain consisting of α-helices which 'criss cross' the phospholipid bilayer, forming between between six to eleven (usually six), membrane-spanning regions. All ABC proteins also contain either two ATP-binding domain(s), which are also called nucleotide-binding domains (NBDs) and are located on the cytoplasm side of the phospholipid bilayer.***

ABCs are mostly unidirectional, meaning they only function in one direction. In bacteria, they mainly pump essential compounds such as sugars, vitamins, metal ions, etc. into the bacteria. These transporters are called importers. In eukaryotes, ABCs mainly move compounds from within the cytoplasm to the outside of the cell or into a membrane bound organelle (endoplasmic reticulum, mitochondria, peroxisomes, etc.). These transporters are called exporters. ABC transporters are largely dispersed throughout the genome and have an extremely wide variety of uses. For example lipid flippases work by flipping a lipid from the inner phospholipid membrane to the other phospholipid membrane.

ABC transporters utilize the energy of ATP hydrolysis to transport various substrates across cellular membranes. They are divided into three main functional categories. In prokaryotes, importers mediate the uptake of nutrients into the cell. The substrates that can be transported include ions, amino acids, peptides, sugars, and other molecules that are mostly hydrophilic. The membrane-spanning region of the ABC transporter protects hydrophilic substrates from the lipids of the membrane bilayer thus providing a pathway across the cell membrane. Eukaryotes do not possess any importers. (Gottesman, Pastan 1993) Exporters or effluxers, which are both present in prokaryotes and eukaryotes, function as pumps that extrude toxins and drugs out of the cell. In gram-negative bacteria, exporters transport lipids and some polysaccharides from the cytoplasm to the periplasm. The third subgroup of ABC proteins do not function as transporters, but are rather involved in translation and DNA repair processes.

The common feature of all ABC transporters is that they consist of two distinct domains, the transmembrane domain (TMD) and the nucleotide-binding domain (NBD). The TMD, also known as membrane-spanning domain (MSD) or integral membrane (IM) domain, consists of alpha helices, embedded in the membrane bilayer. It recognizes a variety of substrates and undergoes conformational changes to transport the substrate across the membrane. The sequence and architecture of TMDs is variable, reflecting the chemical diversity of substrates that can be translocated. The NBD or ATP-binding cassette (ABC) domain, on the other hand, is located in the cytoplasm and has a highly conserved sequence. The NBD is the site for ATP binding. In most exporters, the N-terminal transmembrane domain and the C-terminal ABC domains are fused as a single polypeptide chain, arranged as TMD-NBD-TMD-NBD. An example is the E. coli hemolysin exporter HlyB. Importers have an inverted organization, that is, NBD-TMD-NBD-TMD, where the ABC domain is N-terminal whereas the TMD is C-terminal, such as in the E. coli MacB protein responsible for macrolide resistance.

The structural architecture of ABC transporters consists minimally of two TMDs and two ABCs. Four individual polypeptide chains including two TMD and two NBD subunits may combine to form a full transporter such as in the E. coli BtuCD importer involved in the uptake of vitamin B12. Most exporters, such as in the multidrug exporter Sav1866 from Staphylococcus aureus, are made up of a

homodimer consisting of two half transporters or monomers of a TMD fused to a nucleotide-binding domain (NBD). (Dawson, Locher 2007) A full transporter is often required to gain functionality. Some ABC transporters have additional elements that contribute to the regulatory function of this class of proteins. In particular, importers have a high-affinity binding protein (BP) that specifically associates with the substrate in the periplasm for delivery to the appropriate ABC transporter. Exporters do not have the binding protein but have an intracellular domain (ICD) that joins the membrane-spanning helices and the ABC domain. The ICD is believed to be responsible for communication between the TMD and NBD.

#### 1.2.3.1. P-glycoprotein

Pgp was discovered by Juliano and Ling (Tolcher et al. 1996) and is possibly the best-studied ABC drug efflux transporter to date. Its most striking property is the diversity in structure of substrates that can be transported, including a vast number of drugs applied for a range of therapeutic applications. Many cytotoxic anticancer drugs are transported by Pgp, which was first identified because it was overexpressed in cell lines made resistant to such cytotoxic drugs (Tolcher et al. 1996). Owing to the broad substrate specificity of Pgp, the cells displayed cross-resistance to many different cytotoxic drugs, hence the name multidrug resistance.

There are few common structural denominators for transported Pgp substrates. They are usually organic molecules ranging in size from less than 200 Da to almost 1900 Da. Many contain aromatic groups, but non-aromatic linear or cyclic molecules are also transported. Most of the efficiently transported molecules are uncharged or basic in nature, but some acidic compounds can also be transported (Teodori et al. 2006). The only common denominator identified so far in all Pgp substrates is their amphipathic nature (Lee et al. 2002). This may have to do with the mechanism of drug translocation by Pgp: it has been proposed that intracellular Pgp substrates first have to insert into the inner hemi-leaflet of the cell membrane, before being 'flipped' to the outer hemileaflet, or being extruded directly into the extracellular medium by Pgp. Only amphipathic molecules would have the proper membrane insertion properties (Teodori et al. 2006).

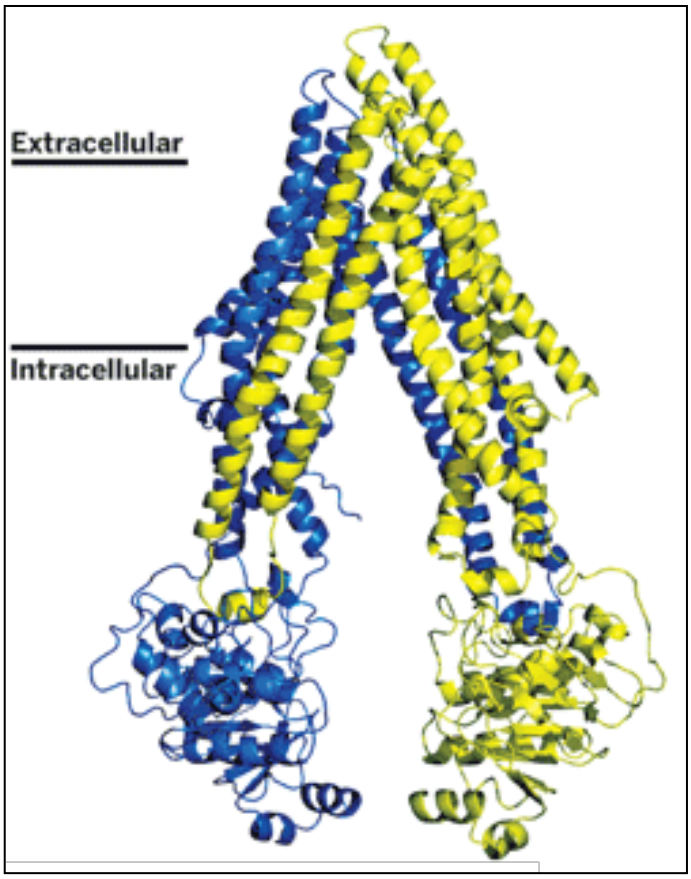


***Figure 7. Structure of Pgp***

As most Pgp substrates are quite hydrophobic, in principle they can diffuse passively across biological membranes at a reasonable rate. For cell-biological and pharmacological studies, this means that in the absence of active transport, Pgp substrates will cross membranes and penetrate into tissues and pharmacological compartments. It also means that a contribution of active transport by Pgp will only result in noticeable distribution effects if the rate of

active transport for a certain compound is substantial relative to the passive diffusion rate. If not, the pump activity will be overwhelmed by the passive diffusion component.

#### *1.2.3.1.1. Inhibitors of Pgp*

Certain compounds can also inhibit transport of cytotoxic and other substrates by Pgp. Many of the initially identified inhibitors, like the calcium channel blocker verapamil or the immunosuppressive agent cyclosporin A, turned out to be themselves transported substrates of Pgp, suggesting that they act as competitive inhibitors (Rao, Scarborough 1994).

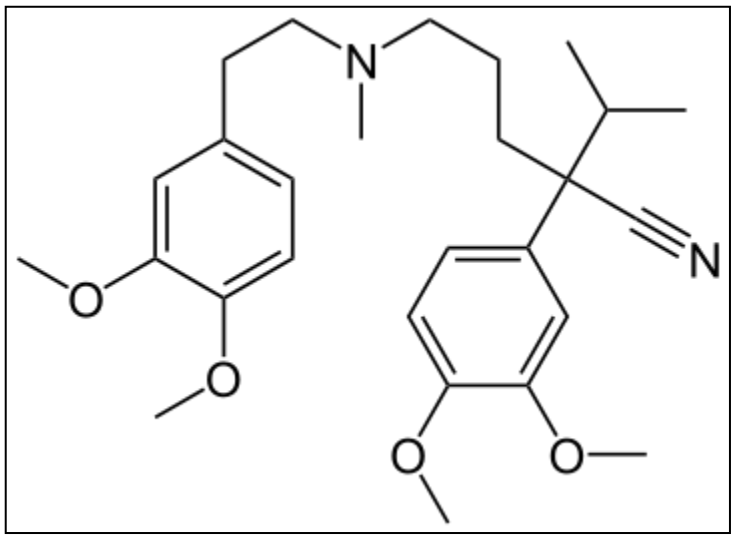


***Figure 8. Structure of Verapamil***

Initial thoughts on clinical application of Pgp inhibitors were focused on reversing MDR in chemotherapy-resistant tumour cells that contain significant amounts of Pgp, but later findings indicated that such inhibitors might also be useful to modulate the general pharmacological behaviour of drugs in the body. The Pgp inhibitors that were initially recognized, such as verapamil, are actually relatively poor Pgp inhibitors in vivo, and they frequently have their own pharmacodynamic effects that put severe restrictions on the plasma levels that can be safely achieved in patients (Iannetti, Spalice & Parisi 2005).

PSC 833 is a cyclosporin A analogue that does not have the immunosuppressive effect of cyclosporin A, and can be given at quite high dosages to patients and (Chen et al. 1997). PSC 833 is a high-affinity, but slowly transported substrate for Pgp, which probably acts as an effective inhibitor because its release from Pgp is very slow. Although PSC 833 is an efficient Pgp inhibitor, it does have the complication that it is also an inhibitor of cytochrome P450 3A4 (CYP3A4), one of the main drug-metabolizing enzymes in the body. Consequently, when administered to patients, next to inhibiting Pgp, it may have additional effects on the clearance of drug substrates that are degraded by CYP3A4. Many cytotoxic anticancer drugs that are Pgp substrates, such as etoposide and doxorubicin, are also extensively degraded by CYP3A4. Therefore, co-administration with PSC

833 can intensify the toxic side effects of these anticancer drugs, necessitating a dose reduction for safe treatment of the patient. The main dose-limiting toxicity of PSC 833 itself in patients is ataxia (Evers et al. 2000), (Baer et al. 2002), (Kolitz et al. 2004).

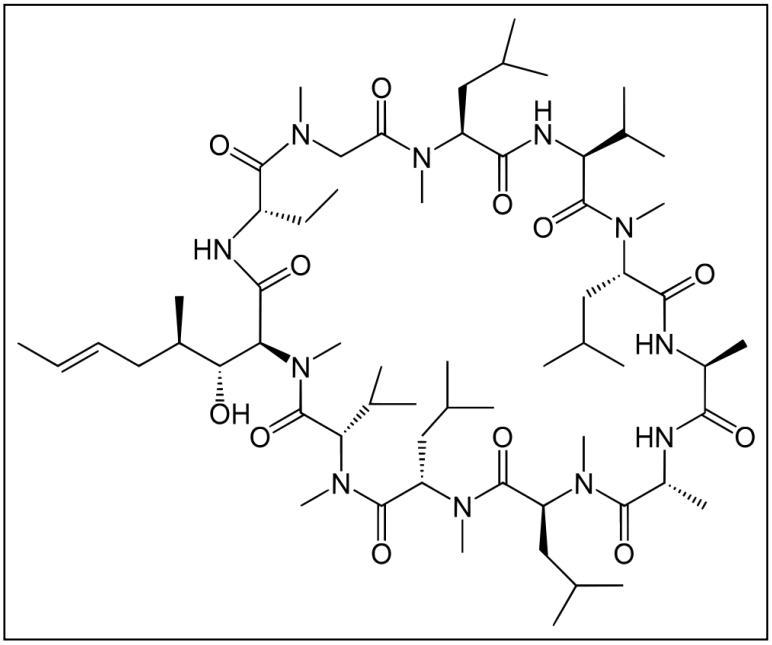


***Figure 9: Structure of Cyclysporine A***

LY335979 is another specifically developed, highly effective Pgp inhibitor (Dantzig et al. 1996). Like PSC-833, it improves chemotherapy response in mice with transplanted Pgp-containing tumours, and it does not clearly affect the plasma clearance of intraperitoneally administered doxorubicin or etoposide. However, this was only tested with intravenously or intraperitoneally

administered LY335979. Whether LY335979 could be orally active is as yet unclear (Dantzig et al. 1999).

#### *1.2.3.1.2. Functions of Pgp*

Pgp is mainly present in epithelial cells in the body, where it localizes to the apical membrane (Evers et al. 1996). As a consequence, transported Pgp substrates are translocated from the basolateral to the apical side of the epithelium. This can have dramatic consequences for the pharmacological behaviour of substrate drugs.

#### *1.2.3.1.3. Role in blood brain barrier*

Endothelial cells of the small blood capillaries in the brain are closely linked to each other by tight junctions, and they cover the entire wall of these blood vessels. As a consequence, all compounds that are not small enough to diffuse between the cells have to cross the endothelial cell in order to translocate from the blood compartment into the surrounding brain tissue. Since most Pgp substrates are quite hydrophobic, in principle they have the possibility to passively diffuse across the endothelial cell membranes and thus enter the brain at a reasonable

rate. However, owing to a high level of Pgp in the luminal membrane of the endothelial cells (Zhang et al. 2004) substrate drugs entering the endothelial cells from the blood are immediately pumped back into the blood. As a consequence, the net penetration of substrate drugs and other substrate compounds from the blood into the brain tissue can be dramatically decreased. In the absence of Pgp in the blood–brain barrier, the brain penetration of Pgp substrate drugs can increase up to 10- to 100-fold, with sometimes dramatic consequences for the clinical applicability and toxicity of compounds.

The brain is a critical organ, and potentially very sensitive to all kinds of toxic and other pharmacodynamic actions of exogenous compounds. It is quite obvious that Pgp must have evolved in part to protect the brain from damaging effects of xenotoxins that can be taken up with food, or are perhaps generated by pathogenic organisms in the intestine.

#### *1.2.3.1.4. Role in excretion*

Pgp is very abundant in the bile canalicular membrane of hepatocytes, and in the apical membrane of small and large intestinal epithelium (Chieli et al. 1993). One function for Pgp in these locations is extrusion of substrate drugs and other compounds from the liver hepatocyte into the bile, and from the intestinal

epithelium into the intestinal lumen. As many compounds can readily enter the hepatocytes and intestinal epithelial cells from the blood compartment, this would result in a net excretory function for Pgp. Extruded substrates would ultimately leave the body via the faeces, so overall this would result in plasma clearing and detoxifying function.

#### *1.2.3.1.5. Role in oral drug bioavailability*

As Pgp is abundant in the intestinal epithelium, one obvious potential function is that it could restrict the rate at which substrate compounds present in the intestinal lumen enter the bloodstream. Many drugs are Pgp substrates, so this could mean that Pgp might restrict the bioavailability of many orally administered drugs. In Pgp knockout animals, the oral bioavailability of the anticancer drug paclitaxel was markedly increase. Later experiments by various groups confirmed that the oral bioavailability of many other drugs is limited by Pgp activity as well. For pharmacotherapy purposes, oral bioavailability is a very important factor. Oral drug administration is highly preferred because it is cheap, relatively safe, and patient friendly. However, if a drug has low oral bioavailability, the plasma level of the drug may not attain sufficiently high levels to have therapeutic effect. Also, low drug bioavailability is frequently associated with variable drug uptake, and this can be a problem if the drug has a narrow therapeutic concentration window. Thus, Pgp activity can be a major problem for therapeutic applications of drugs.

For this reason, it is of interest for pharmaceutical companies to check at an early stage whether a drug under development is a good Pgp substrate, because this could mean that it is not readily orally available. Depending on the therapeutic category of the drug this could limit its ultimate clinical use.

#### *1.2.3.1.6. Role in pharmacotherapy*

Because of the impact of Pgp on a variety of important pharmacological and toxicological processes, it is of great interest to consider whether the available efficient Pgp inhibitors could be used to optimize various aspects of pharmacotherapy. Efforts to increase the penetration of anticancer drugs into multidrug resistant tumours containing Pgp is a very important aspect, especially in chemotherapy treatment of tumours like GBM that are positioned behind the blood–brain barrier, and therefore poorly accessible to most anticancer drugs. For other diseases of the central nervous system (CNS) it might be desirable to improve the brain parenchyme penetration of drugs. As a proof of principle to show that it is possible to extensively inhibit Pgp in the blood–brain barrier, a number of studies were performed. Indeed, it turned out to be feasible to largely block Pgp in the blood–brain barrier in mouse models, but only when highly efficacious Pgp inhibitors were used (Hopper et al. 2001), (Stouch, Gudmundsson 2002), (Ekins et al. 2000).

### 1.2.3.2. Multidrug resistant protein 1

MRP1 was first identified in a cell line made highly resistant to doxorubicin and subsequent analysis showed that it conferred MDR against a range of anticancer drugs (Hidalgo, Li 1996). Substrate anticancer drugs include Vinca alkaloids, anthracyclines, epipodophyllotoxins, mitoxantrone and methotrexate. Unlike Pgp, MRP1 does not confer high levels of resistance to paclitaxel or bisantrene in cells. In further contrast to Pgp, MRP1 functions mainly as a co-transporter of amphipathic organic anions. It can transport hydrophobic drugs or other compounds that are conjugated or complexed to the anionic tripeptide glutathione (GSH), to glucuronic acid, or to sulphate (Spiegl-Kreinecker et al. 2002). In fact, efficient export of several non-anionic anticancer drugs by MRP1 is dependent on a normal cellular supply of GSH (van Asperen et al. 1997), and it is likely that MRP1 exports drugs such as vincristine and etoposide by co-transport with reduced GSH (Ozben 2006), (Jungsuwadee et al. 2009). Non-organic heavy metals like arsenite and trivalent antimony are also transported by MRP1, in all likelihood complexed to GSH (van Zanden et al. 2007).

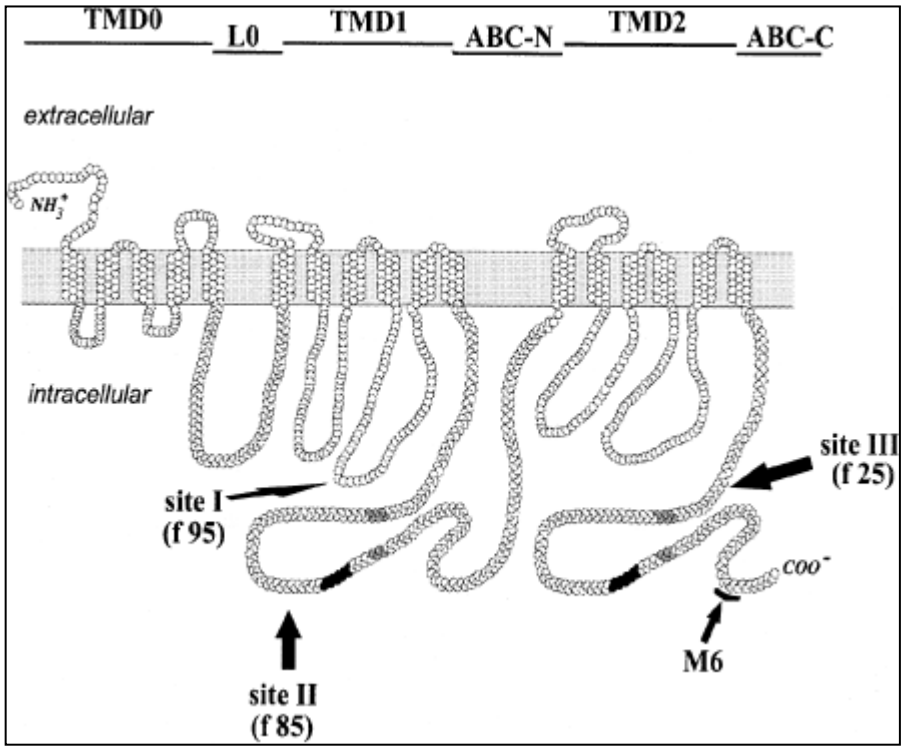


***Figure 10. Structure of MRP 1. The upper line indicates the arrangement of domains of MRP1 (L0 indicates the cytoplasmic loop connecting TMD0 and TMD1; ABC-N and ABC-C indicate the cytoplasmic ABC domains at the N- and the C-half of protein respectively). Arrows indicate cleavage at Site I, Site II and Site III at which Vi-induced cleavage reactions generate fragments of 95 kDa (f95), 85 kDa (f85) and 25 kDa (f25) respectively. Half-shaded regions of the polypeptide chain denotes the conserved Walker A and Walker B motifs, shaded regions show the ABC-signature motifs.***

In contrast to Pgp, MRP1 localizes to the basolateral membrane of epithelial cell layers, and its substrates are therefore transported towards the basolateral side of epithelia. Knockout mice lacking Mrp1 are viable and fertile, but they do show deficiencies in LTC4-mediated inflammatory reactions, suggesting that secretion of LTC4 is an important physiological function of MRP1 (Boumendjel et al. 2005), (Wu et al. 2005).

#### *1.2.3.2.1. Pharmacological functions of Multidrug resistant protein 1*

Even though MRP1 localises predominantly to the basolateral membrane of epithelial cells, it still has important pharmacological and toxicological functions. Wijnholds *et al*. showed that Mrp1 knockout mice are more sensitive to the toxicity of intravenously administered etoposide in the oropharyngeal mucosal layer and testicular tubules (Wijnholds et al. 2000). This can be explained by the fact that these cells are shielded from blood-borne toxins by epithelia that have the basolateral membrane facing the blood circulation. Moreover, Mrp1 in the basolateral membrane of choroid plexus epithelial cells can mediate a substantial clearance of etoposide from the cerebrospinal fluid, indicating that this compartment is also protected by Mrp1 (Filipits et al. 2005). Other studies demonstrated that a combined deficiency of Mdr1a and Mdr1b, Pgps and Mrp1 in knockout mice resulted in a dramatically increased sensitivity to intraperitoneally administered vincristine, but also to etoposide, whereas a Pgp deficiency alone

resulted in a 16- and 1.75-fold increased sensitivity to this drug, respectively (Meaden et al. 2002). In this case, greatly increased vincristine toxicity was observed in bone marrow and the gastrointestinal mucosa, suggesting that these compartments are normally extensively protected by the Pgp and/or Mrp1 transporters.

At the cellular level, the endogenous expression of MRP1 can already contribute substantially to the basal resistance of cell lines to a range of cytotoxic anticancer drugs, as was demonstrated *in vitro* with murine fibroblast and embryonic stem cell lines deficient for murine Mrp1 and/or Mdr1a and Mdr1b (Deeley, Cole 1997). A marked increase in sensitivity to epipodophyllotoxins, vinca alkaloids, anthracyclines, topotecan, SN-38, and arsenite was found in these lines due to Mrp1 deficiency. Such contributions can explain the markedly increased drug sensitivity of bone marrow and intestinal epithelial cells deficient for Mrp1, and especially when drug-transporting Pgp is also absent.

#### *1.2.3.2.2. Inhibitors of Multidrug resistant protein 1*

So far, it has been much more difficult to find good small molecule inhibitors for MRP1 than for Pgp, especially ones that work in intact cells. This probably has to do with the preference of MRP1 for anionic compounds as substrates and

inhibitors: most anionic compounds enter cells poorly, so it may be difficult to obtain sufficient intracellular concentrations of the inhibitor for efficacious inhibition. A variety of inhibitors of MRP1 have been described. Some examples are the LTC4 analogue MK571, LTC4 itself, S-decylglutathione, sulfinpyrazone, benzbromarone and probenecid (Boumendjel et al. 2005), (Bakos et al. 2000), (Bakos et al. 1998).

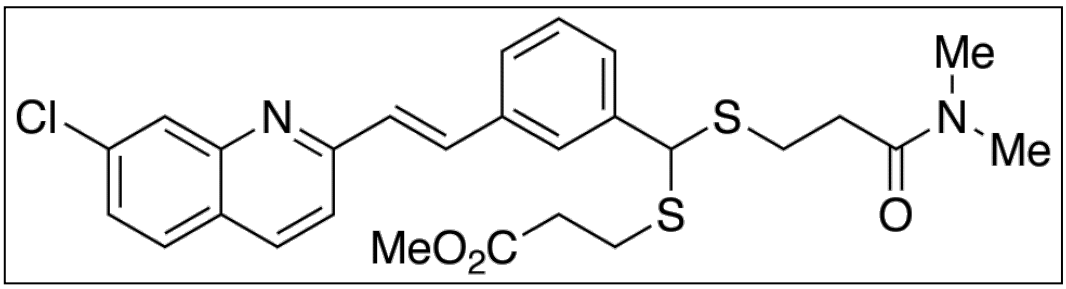


***Figure 11: Structure of MK571, an inhibitor of MRP1.***

Pgp inhibitors like cyclosporin A and PSC 833, with reasonable cellular penetration, inhibit MRP1, but only with low affinity and poor specificity (Qadir et al. 2005). For specific *in vivo* inhibition of MRP1 the general organic anion transporter inhibitors sulfinpyrazone, benzbromarone and probenecid are also not very suitable, as they extensively affect organic anion uptake systems as well (Bobrowska-hägerstrand et al. 2003), (Norman 1998). Moreover, these compounds need to be used at relatively high concentrations. For the aim of specifically inhibiting MRP1 activity *in vivo*, for instance to improve anticancer

chemotherapy, better MRP1 inhibitors will have to be developed, with reasonable specificity and cellular penetration properties, and low cytotoxicity.

#### 1.2.3.3. Multidrug resistant protein 2

The *in vivo* function of MRP2 was well studied before the identification of the MRP2 gene, as the MRP2 gene is fully deficient in two mutant rat strains (TR−/GY and EHBR), and in patients that suffer from the Dubin–Johnson syndrome (Hashimoto et al. 2002). Affected individuals suffer from a recessively inherited conjugated hyperbilirubinemia, which can result in observable jaundice, but overall the clinical phenotype of this disease is relatively mild. The cause of the defect is the absence of MRP2 from the hepatocyte canalicular membrane, where it normally mediates the hepatobiliary excretion of mono- and bis-glucuronidated bilirubin. The MRP2 gene was identified based on its similarity to MRP1, and absence of its expression in MRP2-deficient rats and humans (Evers et al. 1998).

Identification of substrates transported by MRP2 was based on many different approaches: analysis of compounds transported into bile of normal rats, but not of Mrp2 mutant rats; differential uptake of compounds into inside-out bile canalicular membrane vesicles isolated from normal and Mrp2 mutant rats;

transfection or transduction of human MRP2 or rat or rabbit Mrp2 cDNA into cell lines followed by analysis of drug resistance, accumulation of compounds into cells or isolated inside-out membrane vesicles, and transepithelial transport of compounds. In one study an MRP2 cDNA antisense construct was used to suppress endogenous MRP2 levels in the HEPG2 hepatocyte cell line (Morrow, Smitherman & Townsend 2000). It turns out there are many similarities between the compounds transported by MRP2 and MRP1, but there is not a complete overlap. Anticancer drugs transported by MRP2 include methotrexate, doxorubicin, epirubicin, mitoxantrone, vincristine, vinblastine, CPT-11/irinotecan, SN-38, cisplatin and etoposide (Klopman, Shi & Ramu 1997), (Evers et al. 2000), (Tada et al. 2002), (Vlaming et al. 2006). Like MRP1, MRP2 is primarily an organic anion transporter, so it seems likely that weakly basic drugs are co-transported with GSH by MRP2. This was strongly suggested by a study of vinblastine transport in MRP2-transduced polarized cells, which occurs stoichiometrically with GSH transport. Moreover, depletion of cellular GSH by treatment with l-buthionine sulfoximine (l-BSO) resulted in decreased substrate transport and drug resistance in MRP2-overexpressing cells (Liu et al. 2001). Mrp2 is involved in the hepatobiliary excretion of GSH conjugates of inorganic arsenic, after administration of arsenite (H3AsO3) to rats (Kala et al. 2000). MRP2 (and probably MRP1) may therefore also be involved in cellular and organismal protection against arsenic trioxide (As2O3), a compound recently introduced for the treatment of relapsed acute promyelocytic leukemia by extrusion of As-GSH conjugates. The observation that cellular GSH levels

correlate inversely with the sensitivity to arsenic trioxide is in line with this possibility, although direct detoxification of As by GSH complexation may be as relevant (Liu et al. 2001).

Other well-defined substrates of MRP2 include many amphipathic anionic drugs and endogenous compounds, including GSH-, glucuronide-, and sulfate conjugates. Some examples are reduced and oxidized GSH, LTC4, LTD4, LTE4, estradiol-17β(β-d-glucuronide) (E217G), p-aminohippurate, S-glutathionyl 2,4-dinitrobenzene (GS-DNP), (S-glutathionyl-)sulfobromophthalein, glucuronidated SN-38, pravastatin, and the organic anion transport inhibitor sulfinpyrazone. Moreover, some food-derived (pre-) carcinogens and glucuronide conjugates thereof are also transported by Mrp2 (Cui et al. 1999), (Morikawa et al. 2000), (Horikawa et al. 2002), (Huisman et al. 2002).

#### *1.2.3.3.1. Pharmacological functions of Multidrug resistant protein 2*

Unlike MRP1, but similar to Pgp, MRP2 localizes to the apical membrane of polarized cell lines in which it is expressed (Nies et al. 1998). Moreover, *in vivo* MRP2 is found in a range of tissues important for the pharmacokinetics of substrate drugs, namely, next to the bile canalicular membrane of hepatocytes in the luminal membrane of the small intestinal epithelium and in the luminal

membrane of the proximal tubules of the kidney (Dresser, Leabman & Giacomini 2001), (Harris et al. 2001). In human jejunum, MRP2 mRNA levels as measured by RT-PCR were amongst the highest of all tested ABC transporters (Berggren et al. 2007).

The role of MRP2 in hepatobiliary excretion has been amply demonstrated, but MRP2 in intestine and kidney could also contribute to direct intestinal and active renal excretion of substrate compounds, and intestinal MRP2 could limit oral uptake of compounds.

In humans MRP2 has been detected in the apical syncytiotrophoblast membrane of term placenta (Meyer zu Schwabedissen et al. 2005). Similar to Pgp present at these locations, MRP2 might limit the brain and foetal penetration of a range of substrate compounds present in the maternal plasma. Overall, there is fairly extensive overlap between MRP2 and Pgp tissue distribution, so it is likely that these two proteins have considerable overlap in pharmacological and toxicological protective functions, although with different sets of substrates.

#### *1.2.3.3.2. Inhibitors of Multidrug resistant protein 2*

The selection of currently available small molecule inhibitors of MRP2 that can be used in intact cells is quite limited. Many of the anionic transported substrates of MRP2 act as competitive inhibitors when applied in *in vitro* systems where MRP2 is present in an inside-out orientation. Some examples are LTC4, phenolphtalein glucuronide and fluorescein methotrexate (Gutmann et al. 2000), (Ogasawara, Takikawa 2001), (Breen et al. 2004). However, such compounds frequently do not penetrate most normal cells to a sufficient extent to obtain useful levels of inhibition. MRP2 inhibitors that have been demonstrated to work to a greater or lesser extent in intact cells include cyclosporin A, sulfinpyrazone, benzbromarone, probenecid, PSC 833, PAK-104P and furosemide (Westley et al. 2006), (Bakos et al. 2000), (Chen et al. 2003). It is clear that compounds that specifically inhibit MRP2 are rare, as all the compounds identified so far have considerable activity against MRP1 and Pgp.

### 1.2.3.4. Breast Cancer Resistant Protein

Breast cancer resistant protein (BCRP) was first discovered in a doxorubicin-resistant MCF-7 breast cancer cell line (MCF-7/AdrVp) (Doyle et al. 1998). Transfection of BCRP cDNA demonstrated that BCRP itself could confer

resistance to mitoxantrone, doxorubicin and daunorubicin, and that it acted by energy-dependent extrusion of its drug substrates (Gupta et al. 2006). Because the gene was isolated from a breast cancer cell line, it was called the breast cancer resistance protein BCRP. BCRP cDNA sequences were also cloned by Miyake *et al*. (Miyake et al. 1999) and Allikmets *et al*. (Allikmets et al. 1998), who called the gene MXR (mitoxantrone resistant receptor) and ABCP (placental ABC protein), respectively.

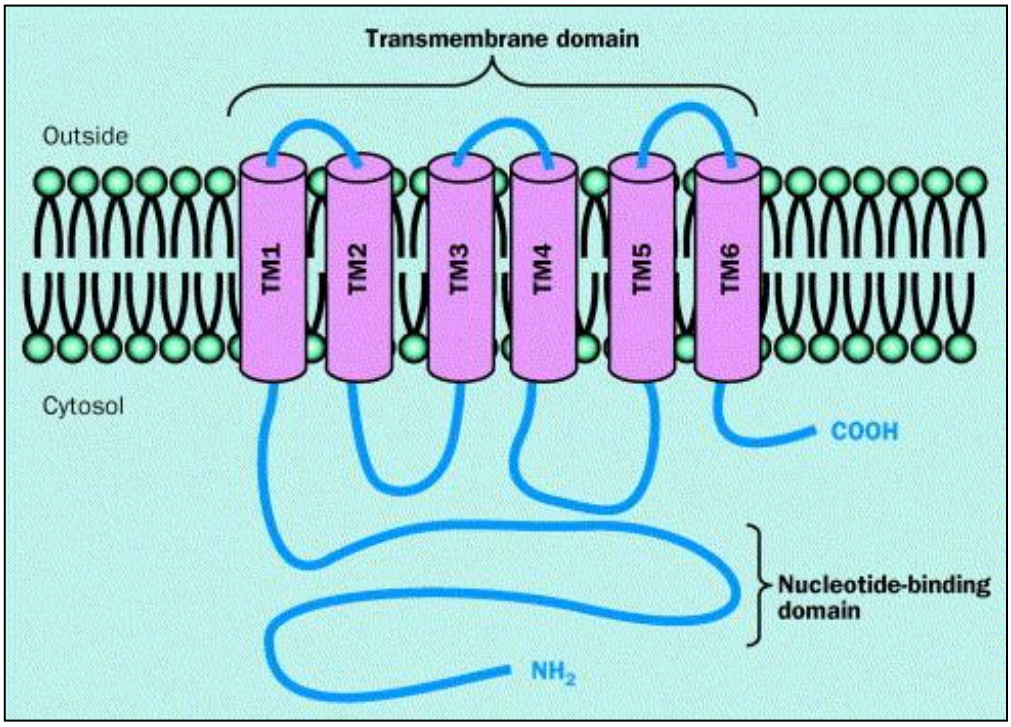


***Figure 12. Structure of BCRP consists of two half-transporters. It is proposed that the transmembrane domains form a channel-like structure serving to translocate substrates. TM = transmembrane helix.***

Human BCRP encodes a 655 amino acid ABC protein, containing a single N-terminal ATP binding cassette, followed by 6 putative transmembrane segments (TMSs). Based on structural and sequence homology, BCRP belongs to the ABCG gene family.

There is considerable, but varying overlap in anticancer drug substrate specificity between BCRP on the one hand and Pgp, MRP1, and MRP2 on the other hand. Cell lines selected for resistance to mitoxantrone, topotecan, doxorubicin, SN-38 and flavopiridol all overexpressed BCRP, strongly suggesting that these drugs are transported substrates for BCRP (Dahan, Amidon 2009), (Mahringer, Delzer & Fricker 2009).

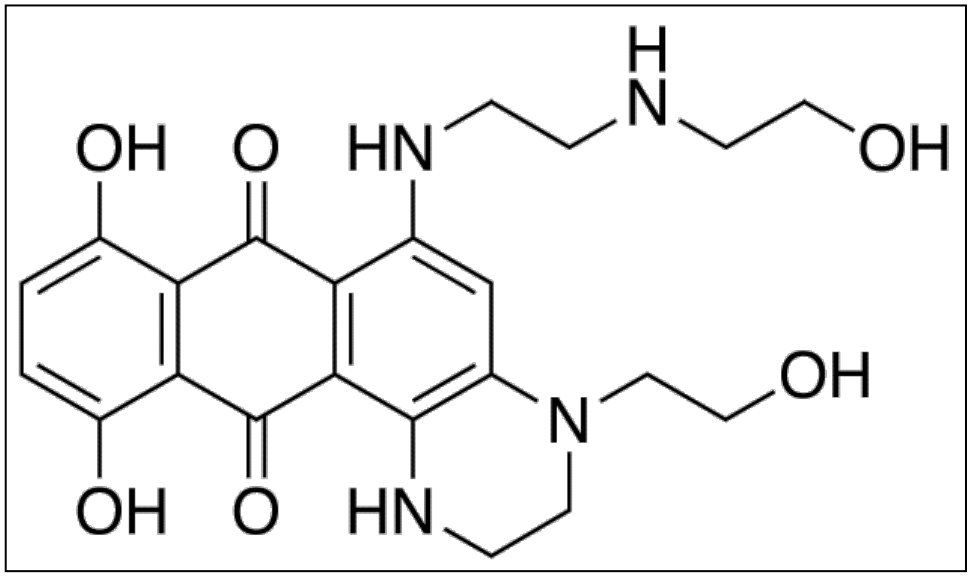


***Figure 13: Structure of Mitoxantrone; a BCRP substrate.***

Drug-selected BCRP-overexpressing cell lines displayed high cross-resistance to daunorubicin, epirubicin, 9-aminocamptothecin, bisantrene, etoposide and teniposide (Sargent et al. 2001), (Kamiyama et al. 2006), (Plasschaert et al. 2004), (Shiozawa et al. 2004), (Solazzo et al. 2009). In some selected cell lines, BCRP conferred resistance to anthracyclines, whereas in other cell lines there was hardly any resistance to anthracyclines compared to mitoxantrone resistance, which was consistently high. Moreover, topotecan resistance was generally quite high, but low in some BCRP-overexpressing lines. Honjo *et al*. recognized that two BCRP-overexpressing lines (MCF-7/AdVp3000 and S1-M1-80, each with high anthracycline resistance) could efflux rhodamine 123 efficiently, whereas all other BCRP-overexpressing lines tested could not (Honjo et al. 2001).

Sequence analysis revealed that the rhodamine-extruding lines contained a mutant BCRP, deviating from the 'wild-type' BCRP at arginine 482, which was replaced with either threonine (in the MCF-7/AdVp3000 line) or glycine (in the S1-M1-80 line). The MCF-7/AdVp3000 line had been the source of the first cloned BCRP cDNA (Doyle et al. 1998), which therefore represented a mutant BCRP. Further experiments confirmed that the wild-type (R482) BCRP could efficiently extrude mitoxantrone, but not rhodamine 123 or doxorubicin. In contrast, both R482T and R482G mutants efficiently extruded all three compounds. In the parental cell lines, only the wild-type BCRP sequence was found, indicating that in some lines the extended drug selection must have resulted in selection of mutant BCRPs that were more efficient in conferring resistance to the selecting drug.

### *1.2.3.4.1. Inhibitors of Breast cancer resistant protein*

GF120918, a highly efficient Pgp inhibitor that can be used in animals and patients, is also an effective BCRP inhibitor (Maliepaard et al. 2001b). GF120918 is not a specific BCRP inhibitor, but for some clinical applications this may be an advantage, as two multidrug transporters, BCRP and Pgp, each with their own potentially adverse activity, can be blocked at the same time.

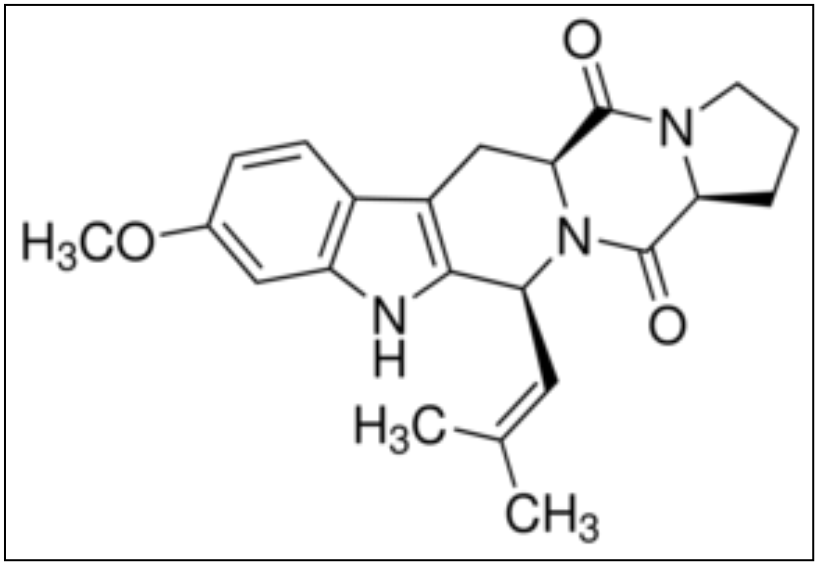


***Figure 14: Structure of fumitremorgin C; a potent BCRP inhibitor***

In a systematic search for drug resistance-reversing compounds, fumitremorgin C (FTC), a tremorgenic mycotoxin produced by the fungus Aspergillus fumigatus, was found to effectively reverse drug resistance and increase cellular drug accumulation in BCRP-expressing cells (Rabindran et al. 2000). FTC inhibited BCRP *in vitro* at concentrations well below those toxic to cultured cells, and had little effect on Pgp- or MRP1-mediated drug resistance. This relative specificity makes FTC very useful for cell pharmacological studies of BCRP, but its neurotoxic effects limit its application in animals or patients. Van Loevezijn *et al*. tested 42 tetracyclic indolyl diketopiperazine analogues of FTC as inhibitors of human BCRP. The most potent analogues (Ko132 and Ko134) have comparable or greater activity than FTC (van Loevezijn et al. 2001). The HER tyrosine kinase inhibitor, CI1033, also inhibits BCRP, although with moderate affinity. CI1033 also appears to be a transported substrate for BCRP (Erlichman et al. 2001).

#### *1.2.3.4.2. Tissue distribution of Breast cancer resistant protein*

Very high BCRP RNA expression was found in human placenta, but in murine placenta the expression of Bcrp1 was quite moderate. In contrast, mice displayed highest expression of Bcrp1 RNA in kidney, where humans appear to have low BCRP expression (Tanaka et al. 2004). The immunohistochemical studies by Maliepaard *et al*. used two independent monoclonal antibodies, BXP-21 and BXP-34, recognizing human BCRP (Maliepaard et al. 2001b). BCRP was

detected in the placental syncytiotrophoblast plasma membrane facing the maternal bloodstream, in the bile canalicular membrane of the liver hepatocytes and in the luminal membrane of villous epithelial cells in the small and large intestine, locations shared with Pgp. BCRP was further found in the apical side of part of the ducts and lobules in the breast, and in the venous and capillary endothelial cells of practically all tissues analysed, but not in arterial endothelium (Maliepaard et al. 2001a). A recent study by Scharenberg *et al*. demonstrated a very similar distribution of BCRP expression in the human hematopoietic compartment, with high expression in putative hematopoietic stem cells, and much decreased expression in committed progenitor cells, which only comes up again in natural killer cells and erythroblasts, but not in other differentiated lineages (Scharenberg, Harkey & Torok-Storb 2002).

## 1.3 Cellular membrane affinity chromatography

It is clear from the above discussion that the ABC transporters play a very important role in clinical MDR in human GBM and also have a profound effect on reducing the oral bioavailability of substrate drugs. Also there are limited if any, tools available for screening lead candidates for possible transport by the ABC transporters. Cellular membrane affinity chromatography (CMAC) can be used to immobilize membrane fragments expressing target proteins. These

columns can be used to study binding parameters of ligands with target proteins and also to screen chemical libraries for drug discovery.

The approach utilized in this work is based upon affinity chromatography. The mechanism of affinity separations is most often described by a "lock and key" analogy. Retention depends on complementarity of a ligand molecule (the "key") and a specific binding site (the "lock") with high affinity for the ligand. Typically the molecule containing the binding site is a protein, although some nucleic acid interactions may also be useful. The ligand can be a small molecule, a carbohydrate, another protein, a prosthetic group, a specific amino acid sequence in a protein, or a specific nucleotide sequence. There are virtually an unlimited number of affinity separations that can be used, the only requirement being a strong and specific binding interaction that is in some way reversible.

In some cases the ligand is covalently bound to the stationary media. The affinity adsorbent thus constructed can be used to specifically scavenge proteins or other macromolecules containing a binding site from a sample which may be a complex mixture containing many other non-binding molecules. This strategy has been used to isolate proteins with binding sites for specific cofactors, enzyme inhibitors, cell-surface carbohydrates, and nucleic acid sequences, among other possibilities.

In other cases a protein with affinity for a specific ligand or sequence is covalently bound to the media and used to scavenge the ligand, or a molecule to which the ligand is covalently attached, from solution. This approach has much in common with the fundamental processes of drug action, absorption, distribution, excretion and receptor activation which are dynamic in nature and have similarities with the basic mechanisms involved in chromatographic distribution. Indeed, the same basic intermolecular interactions (hydrophobic, electrostatic, hydrogen bonding) determine the behaviour of chemical compounds in both biological and chromatographic environments.

The relationships between pharmacological and chromatographic processes have been emphasized by the inclusion of a wide variety of proteins as active components of chromatographic systems, in the creation of protein-based stationary phases. The extreme complexity of biological systems limits rational design of an individual chromatographic system that would directly mimic a total biological system. However, construction of the right protein-based stationary phase can readily yield a great amount of diversified, precise and reproducible data about key aspects of that system. The possibilities are only limited by the ability to create and use unique phases. In addition, the ability of enzymes and carrier proteins to act as chiral selectands has been extensively used in drug development, as well as adapted for use in the chromatographic sciences.

An example of the utility of the latter approach is the creation of chiral stationary phases (CSPs) through the immobilization of biopolymers on chromatographic backbones. These CSPs have been based upon enzymes and carrier proteins and used primarily in liquid chromatographic systems. The use of biopolymeric based CSPs has been particularly valuable in the quantification and separation of small chiral compounds on an analytical scale.

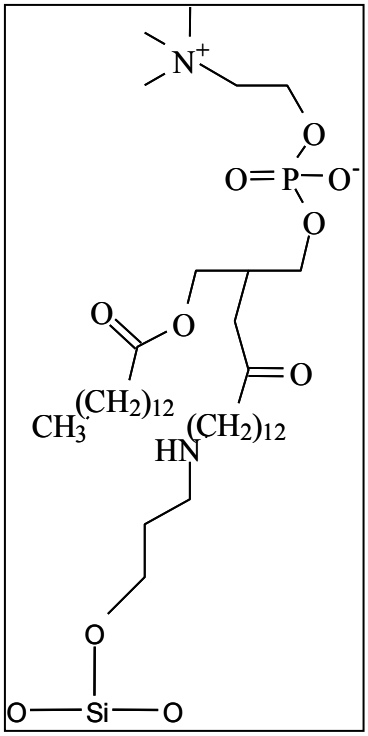


***Figure 15. The chemical structure of the monolayer that is covalently bound to the silica on the IAM-PC particles (12 micron, 300 Å pores).***

In general, two approaches have been used in the construction of biopolymer-based CSPs – covalent immobilization and entrapment/adsorption. The enzymes α- chymotrypsin (ACHT), trypsin (TRYP) and cellobiohydrolase I have been covalently attached to silica, ACHT and TRYP have been entrapped in the interstitial spaces of an immobilized artificial membrane stationary phase (IAM), while ACHT has been adsorbed on LiChrospher phases. The carrier protein phases have been synthesized using entrapment followed by cross-linking as in the case of α1 acid glycoprotein (AGP) or through covalent immobilization used when immobilizing serum albumin.

Biopoylmeric based CSP's have also found a use in pharmacological applications. The immobilization of proteins has also been widely studied and a variety of covalent and non-covalent immobilization techniques have been developed. In addition, the experimental approaches to the isolation and purification of receptors from biological matrices have been extensively studied and discussed.

It has been demonstrated in recent work that these techniques can be used to immobilize transporter proteins such as the P-glycoprotein transporter (Pgp), carrier proteins such as human serum albumin (HSA) and receptor proteins such as nicotinic acetylcholine receptors. The nicotinic receptor and Pgp were immobilized via hydrophobic insertion into the interstitial spaces of an immobilized artificial membrane (IAM) stationary phase (Figure 15). The IAM is comprised of silica particles (12 μm id with 300 Å pores) to which phospholipid

analogues, with functional head groups, have been covalently coupled in a monolayer.

### 1.3.1. Principles of cellular membrane affinity chromatography

CMAC utilizes affinity chromatography as its principle. The mechanism of affinity separations is a complimentary relationship between a ligand and its substrate. The molecule containing the binding site is a protein. The ligand is a small molecule. There are various affinity separations that can be used; the only requirement is that the binding should be specific and reversible. The receptor of interest is immobilized via hydrophobic insertion into the interstitial spaces of an immobilized artificial membrane (IAM) stationary phase. The IAM is comprised of silica particles (12 μm id with 300 Å pores) to which phospholipid analogues, with functional head groups, have been covalently coupled in a monolayer. The specific feature of IAM beads is its resemblance to a hydrophobic environment that allows immobilization of membrane proteins and receptors.

The stationary phases can be characterized using frontal chromatographic experiments. Binding affinities (expressed as dissociation constants, $K_d$) are determined using the stationary phases and in some cases a mixtures of ligands are resolved according to their relative affinities.

In frontal affinity chromatography a larger sample volume is required than zonal elution. A marker ligand is placed in the mobile phase and passed through the column. The frontal regions are composed of the relatively flat initial portion of the chromatographic traces, which represent the nonspecific and specific binding of the marker to the cellular membranes and the target (Figure 16). The saturation of the target by the marker produces a steep rise in the chromatographic trace, which ends, or plateaus, when the target is saturated.

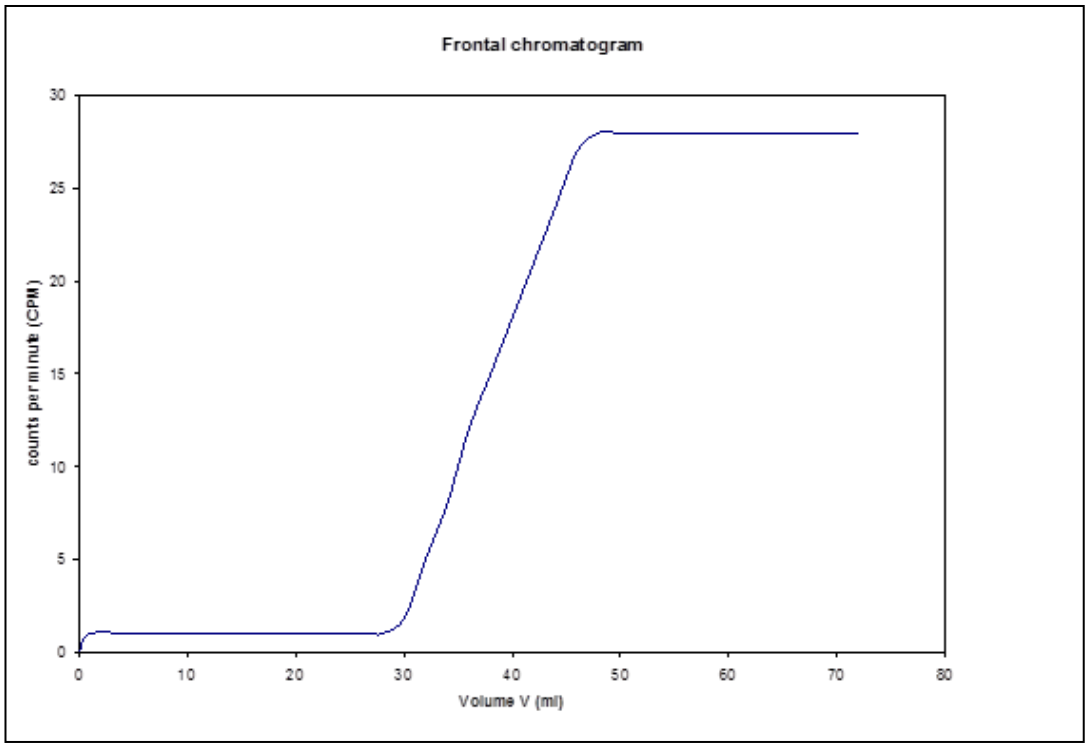


***Figure 16. Schematic of a frontal chromatogram showing a saturation binding curve of the marker ligand when placed in the mobile phase and passed through the chromatographic column. The plot looks like the familiar sigmoidal dose-response curve or is described as a rectangular hyperbola or a binding isotherm curve.***

Frontal affinity chromatography can be used to calculate the dissociation constant $K_d$ and the number of active binding sites Bmax.

The $K_d$ value is used to describe how tightly a ligand (such as a drug) binds to a protein. Such binding is usually non-covalent, i.e., no chemical bonds are made or broken. Since the binding is usually described by a two-state process

$$\mathrm{P} + \mathrm{L} \leftrightarrow \mathrm{C}$$

the corresponding dissociation constant is defined

$$K_d = \frac{[\mathrm{P}]\,[\mathrm{L}]}{[\mathrm{C}]}$$

where [P], [L] and [C] represent the concentrations of the protein, ligand and bound complex, respectively. The Kd has the units of concentration (M), and corresponds to the concentration of ligand [L] at which the binding site on the protein is half occupied, i.e., when the concentration of protein with ligand bound [C] equals the concentration of protein with no ligand bound [P]. The smaller the dissociation constant, the more tightly bound the ligand is; for example, a ligand with a nanomolar (nM) dissociation constant binds more tightly than a ligand with a micromolar (μM) dissociation constant.

Kd and Bmax values can be obtained from frontal chromatographic data using one of two models, a) linear regression model or b) non-linear regression model. (Gottschalk et al., 2002)

For the linear regression model, the association constants of the competitive ligands (CL), $K_{CL}$, as well as the number of the active and available binding sites of immobilized receptors, Bmax, were calculated using the following equations [(1) and (2)]

$$(V_{max}-V)^{-1}=(1+[M]K_M)(V_{min}[B_{max}]K_M)^{-1}+(1+[M]K_M)^2(V_{min}[B_{max}]K_MK_{CL})^{-1}[drug]^{-1} \quad \textbf{(1)}$$

$$(V-V_{min})^{-1}=(V_{min}[B_{max}]K_{CL})^{-1}+(V_{min}[B_{max}])^{-1}[M] \quad \textbf{(2)}$$

where, $V$ is the retention volume of the marker ligand ($M$); $V_{max}$ the retention volume of $M$ at the lowest concentration and in the absence of drugs; $V_{min}$ the retention volume of $M$ when the specific interaction is completely suppressed. The value of $V_{min}$ is determined by running $M$ in a series of concentration of drugs and plotting $1/(V_{max}-V)$ versus 1/[CL] extrapolating to infinite [CL]. From the above plot and a plot of $1/(V-V_{min})$ versus [$M$], dissociation constant values, $K_d$,

for *M* and CL can be obtained as can the number of active binding sites on the immobilized protein [$B_{max}$].

In the non-linear regression model the relationship between displacer concentration and retention volume can be established using Equation 3 and can be used to determine the $K_d$ value of the displacer and the number of active binding sites, $B_{max}$.

$$[X] (V - V_{min}) = B_{max} [X] (K_{dx} + [X])^{-1} \quad \textbf{(3)}$$

where: V is retention volume of drug (displacer); $V_{min}$, the retention volume of drug (displacer) when the specific interaction is completely suppressed (this value can be determined by running the drug with a high concentration of displacer).

An example of a non-linear regression model is shown in Figure 17, where [X] (V - $V_{min}$) is plotted vs [X]. The plateau represents the $B_{max}$ where all the sites are saturated and 50% of the Bmax represents the $K_d$.

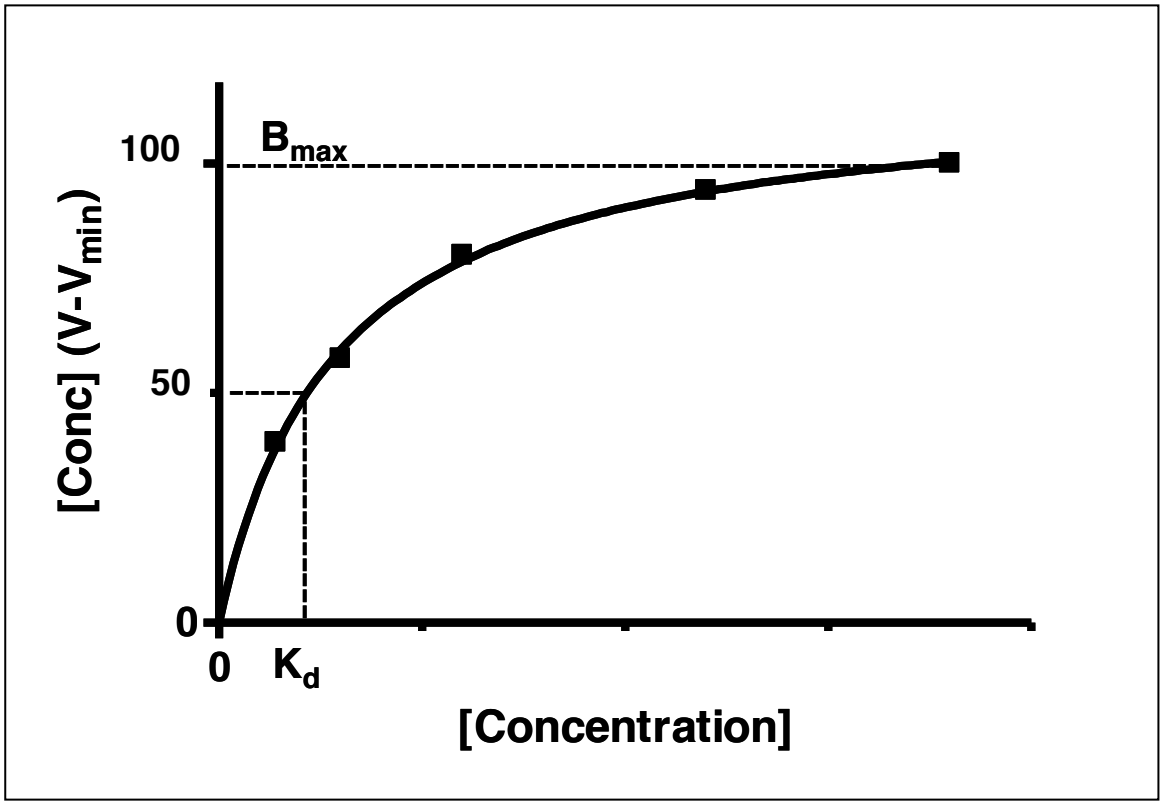


***Figure 17. A representative non-linear regression plot of [concentration] M vs. [conc](V-$V_{min}$) that results from the data obtained from frontal chromatography which is used to derive the dissociation constant $K_d$ and the number of active binding sites $B_{max}$.***

In affinity chromatography various isotherm equations may arise according to the chemical system considered. For the interaction of the solute and the immobilized ligand, non-equilibrium conditions must be taken into account. The association constants measured between the solute and the immobilized ligand are hardly comparable to the solvated conditions. Thus the trend is then to produce

immobilized ligands with affinity properties as close as possible to those of the free ones, to decrease the nonspecific interactions of the support itself. Frontal affinity chromatography decreases the nonspecific interactions between the solute and the immobilized ligand as it saturates the column. Thus for measuring the equilibrium constants of complicated systems frontal elution is a powerful tool, since the amount of interacting biopolymers/compounds at equilibrium is easily related to the chromatographic data.

## 1.4. Aims and objectives

ABC transporters like Pgp, MRP1, MRP2 and BCRP are capable of transporting a wide range of compounds. The transported ligands include commonly used anti-cancer agents, anti-malarial drugs, and anti-HIV drugs like the protease inhibitors. Active efflux of the aforementioned substrates leads to insufficient intracellular concentration, thereby leading to decreased or no therapeutic effect. Innate and over-expression of these ABC transporters in cancer patients leads to clinical MDR.

GBM is the third most common cause of death by cancer in the world. GBM patients almost always develop resistance to commonly used chemotherapy regimen. Hence the initial aim of this research programme was to understand the

expression profile of ABC transporters in human GBM cell lines. Biological and cellular techniques like immuno-blotting and confocal laser microscopy were to be used to assess the expression and localization profiles of the ABC transporters in GBM *in vitro* models. It was proposed to investigate functional activity by using efflux and cell proliferation assays and to use immuno-histochemistry to study the clinical significance of the ABC proteins in healthy and GBM autopsy specimens.

One of the most widely accepted approaches to circumvent clinical MDR has been to co-administer specific ABC inhibitors with anti-cancer agents, thereby ensuring sufficient intracellular concentrations. The challenge however had been to identify specific inhibitors.

The secondary aim of the programme was to develop stationary phases with immobilized ABC receptors. These stationary phases would be characterized by frontal displacement chromatography and used to study binding interactions of known ligands to the ABC proteins. It was envisioned that these columns would be an important aid in discovering potential ABC protein inhibitors.

# Chapter 2

## 2. Identification of ABC transporters in human astrocytomas and glioblastoma multiforme cell lines

### 2.1. Introduction

As discussed in Chapter 1, malignant astrocytomas and glioblastomas are often refractory to chemotherapy. Several factors like poor drug penetration contribute to the MDR phenotype. A particular problem is active expression of the ABC efflux transporters at the blood brain barrier and this poses a formidable challenge for effective chemotherapy. In addition to their presence at the BBB, ABC transporters have also been reported to be present in tumor cells. However their expression profile in human astrocytomas and glioblastoma is contradictory. While significant Pgp expression was reported in glioblastoma cell lines (Calatozzolo et al. 2005), no Pgp presence was reported by Matsumoto *et al* (Matsumoto et al. 1991). Similar results have been reported for MRP2 (Matsumoto, Tamiya & Nagao 2005) and BCRP (Carcaboso et al. 2010).

The poor prognosis of glioma patients is partly based on the minor success obtained from chemotherapeutic treatments. Resistance mechanisms at the tumor cell level may be, in addition to the blood–brain barrier, involved in the intrinsic chemo-insensitivity of brain tumors. Considerable expression of P-gp was

relatively rare in glioma cells, in contrast to MRP1, which was constitutively overexpressed in cells derived from astrocytomas as well as glioblastomas. Also, normal astrocytes cultured in vitro expressed high amounts of MRP1 but no detectable P-gp. Given the widespread distribution of ABC transporters and role in multidrug resistance, it was important to establish the absence or presence of the ABC transporters in the astrocytoma and glioblastoma cell lines selected in this study.

## 2.2. Aims and Objectives

The key main therefore at the outset of the research programme was to establish the expression profile of Pgp, MRP1, MRP2 and BCRP in human astrocytoma and glioblastoma cell lines. The plan was to carry this out by using Western blotting.

## 2.3. Materials and Methods

### 2.3.1. Chemicals

All chemicals were obtained from Sigma Aldrich (St-Louis, MO, USA) unless stated otherwise.

### 2.3.2. Cell culture and optimization of cell growth conditions

Glioblastoma and astrocytoma cell lines in the NIH cell bank were chosen for this experiment. Cell lines LN229, T98, A172, 1321N1, U87, U118, and U138 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cryogenically frozen cells were removed from the -80$^{o}$C freezer and kept for thawing in a water bath controlled at 37$^{o}$C. Initially all cell lines were grown in Dulbecco's modified Eagle's medium (D-MEM). LN229, T98, A172 and 1321N1 cell lines responded well to the selected medium hence they were cultured in Dulbecco's modified Eagle's medium (D-MEM). The U87, U118 and U138 cell lines were cultured in Eagle's minimum essential medium (E-MEM) as prescribed by ATCC, with 4.5 g glucose /L (Quality Biological Inc., Gaithersburg, MD, USA). This was determined to be the optimum amount of glucose required for healthy cell growth. In order to prevent bacterial contamination, both mediums were supplemented with penicillin (25 U / ml), streptomycin (25 μg / ml), pyruvic acid and 5% v/v feotal calf serum (Thermo Scientific, Waltham, MA, USA) was added to the medium as growth supplement. 5% v/v was found to be the optimum concentration as cells became contaminated at 10 % v/v serum. Cells were maintained at 37 $^{o}$C in a humidified incubator with 5% $CO_2$.

### 2.3.3. Preparation of whole cell lysates and optimization

In order to ensure significant and accurate protein expression in immunoblotting analyses, it is important for the cells to be lysed at their healthiest form. Fully confluent cells grown in T175 flasks were lysed in the beginning. Cell morphology analysis of the cells under an electron microscope suggested that the cells were not in their optimum health. This was further corroborated by the Trypan blue dye cell exclusion methods. It was determined that cells at 70% confluence would be the most suitable as they were in their linear growth phase. Cells at approximately 70 – 80% confluence were lysed on ice for 20 min in Tris-HCl [10 mM, pH 7.4] containing 0.15 M NaCl, 5mM EDTA, 1% v/v Triton X-100, 1:500 dilution of a stock solution of protease inhibitor cocktail, 0.1M sodium ortho-vanadate and 0.1 M DTT. 20 minutes of lysis time was determined as the upper limit as more lysis time resulted in sub-optimal protein expression. Protease inhibitors were added to prevent proteases from denaturing the target protein. Triton X-100 was selected as the detergent of choice as the expression of protein was found to be significantly expressed. Insoluble material was removed by centrifugation (17,000 × g for 20 min at 4$^{o}$C) and the supernatant was collected and stored at -80 $^{o}$C until further analysis.

### 2.3.4. Western Blot analysis

Western blotting is an analytical technique used to detect a specific protein in an extract or a lysate. The protein of interest is separated by gel electrophoresis by the length of the polypeptide. Once separated, the protein is then transferred to a nitrocellulose or PVDF (poly-vinyl difluoride) membrane, where they are probed by antibodies specific to the target antigen.

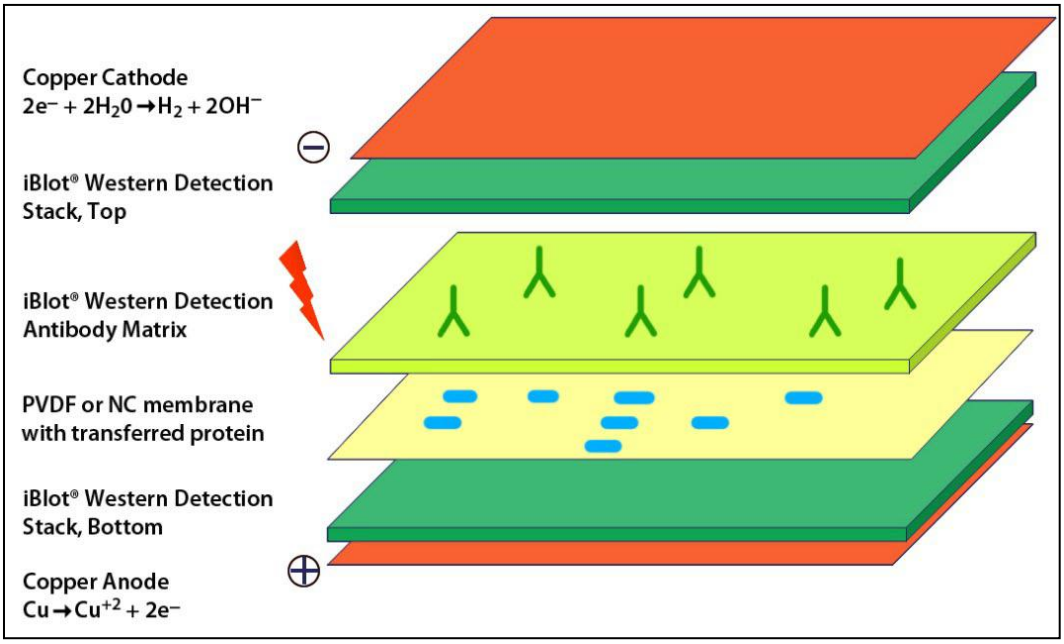


***Figure 18. iBlot protein transfer. Protein samples are loaded onto the appropriate polyacrylamide gel, as current flows from the copper cathode to the anode; proteins are transferred from the polyacrylamide gel on to the PVDF membrane based on their size.***

**Protein concentration optimization**

In order to determine the appropriate concentration of the protein to be loaded for western blotting experiments, an optimization experiment was conducted by loading β-actin protein lysate on a 4-12% gradient SDS-polyacrylamide gel. The proteins were transferred onto a PVDF membrane using the iBlot system. Upon resolution it was observed (Figure 19) higher concentrations of 150, 125 and 100 µg produced a significantly large band at the expected 42 kDa size but multiple bands were also observed. The protein signal was found to be very week at lower concentrations of 50-5 µg. Given these findings, 70 µg was chosen as the optimum concentration to be loaded for all subsequent western blotting experiments.

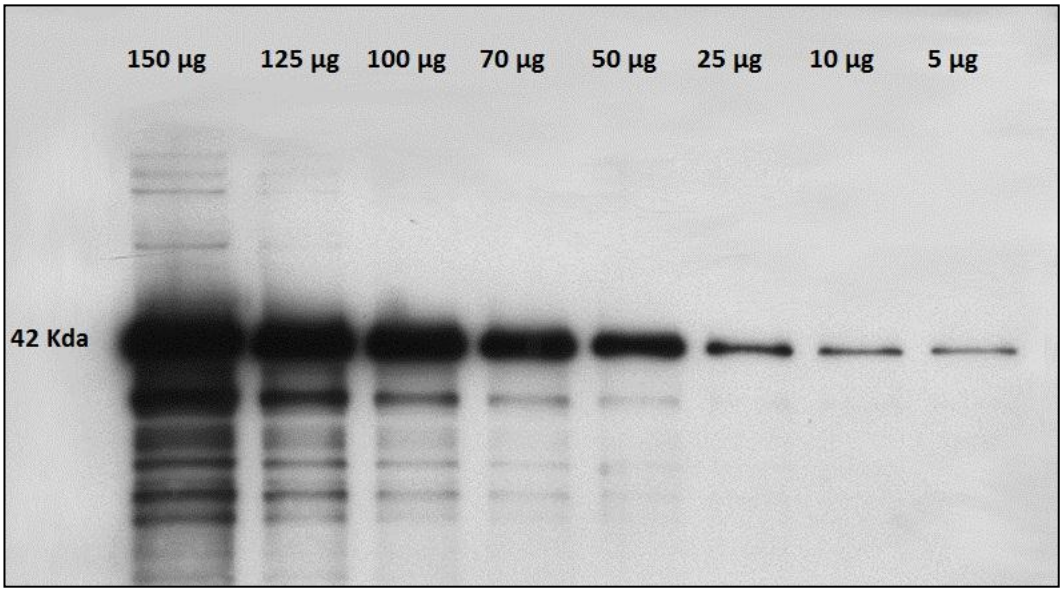


***Figure 19: Optimization of loading protein concentration. Housekeeping protein β-actin was loaded on a 4-12% SDS-polyacrylamide gel in the concentrations of 150 µg to 5 µg. Based on the clarity of the resolution of the protein, 70 µg protein was chose as the concentration for subsequent experiments.***

**Blocking and washing optimization**

Blocking of non-specific binding is an important step in western blotting. Sufficient blocking by a non-specific protein is required in order to ensure the primary antibody specifically binds to the target epitope. This also provides a secondary screen by preventing non-specific binding. It was determined that 3 cycles of washing the membranes with 1X PBS were required in order to produce clear and distinct protein bands as shown in Figure 19. Insufficient washing and blocking led to immunoblots with dark background as shown in Figure 20.

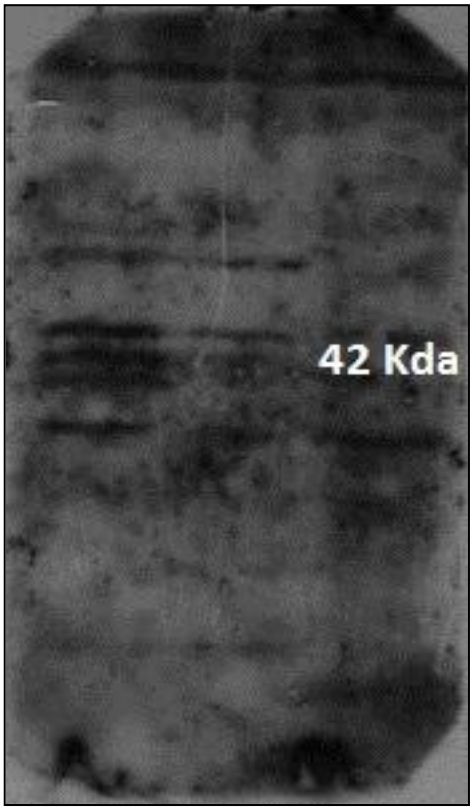


***Figure 20. Optimization of non-specific blocking and washing. 70 µg β-actin cell lysate was loaded on a 4-12% SDS-polyacrylamide gel. The dark immunoblot is indicative of in-sufficient blocking.***

**Primary antibody optimization**

In order to get the best protein resolution, it is imperative to use the appropriate concentration of primary target epitope antibody. Optimization experiments for all antibodies used in this study were done using known positive controls and a range of chosen antibody. As shown in Figure 21, higher concentration of the primary antibody resulted in large 'running' bands.

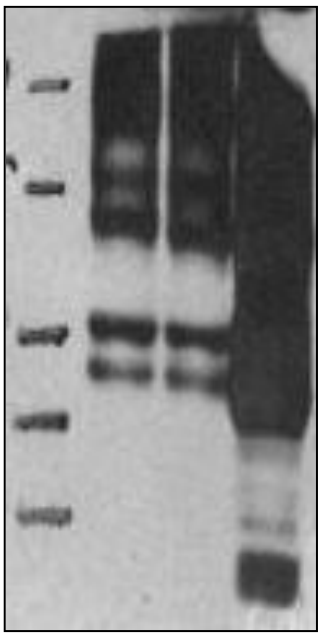

***Figure 21: Primary antibody optimization. Triplicate loads of 70 µg cell lysate from LCC6-MDR1(+) cell line were loaded onto a 4-12% SDS-polyacrylamide gel. The immunoblot was probed with mouse monoclonal anti-Pgp (clone C219) at the concentration of 1:1000 dilution resulting in multiple and thick bands.***

In the study described in this chapter, the protein concentration of the lysates was measured with the BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) using BSA (bovine serum albumin) as standard. An equal amount of 3X Laemlli sample buffer was added, and the samples (70 μg) were separated by electrophoresis using precast 4-12% gradient SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride membranes using iBLOT (Invitrogen).

The membranes were blocked with 5% v/v dry non-fat milk in Tris-buffered saline with 0.1% v/v Tween-20 and then probed with mouse monoclonal anti-Pgp (clone C219, 1:500 dilution; Alexis Biochemicals, Plymouth Meeting, PA, USA), rat monoclonal anti-MRP1 (clone MRPr1, 1:500 dilution, Alexis Biochemicals), mouse monoclonal anti-MRP2 (clone $M_2$I-4, 1:500 dilution, Alexis Biochemicals), mouse monoclonal anti-BCRP (clone BXP-21, 1:500 dilution; Alexis Biochemicals) and mouse monoclonal anti-β-actin (clone AC-15, 1:5000 dilution; Abcam, Cambridge, MA, USA). This was followed by appropriate horseradish peroxidase-conjugated secondary antibodies; ECL Anti-mouse IgG (1:5000 dilution; GE Healthcare, Piscataway, NJ, USA) in the case of Pgp, MRP2, BCRP and β-Actin. MRP1 blots were incubated with ECL Anti-rat IgG (1:5000 dilution; GE Healthcare). The ECL Western blotting detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for visualization.

## 2.4. Results and Discussion

The aim of this study was to establish the expression profile of Pgp, MRP1, MRP2 and BCRP in the selected glioma cell lines. While Pgp, MRP1 and MRP2 have an estimated molecular weight of 170-200 kDa, BCRP and β-actin have much lower molecular weights; 72 and 42 kDa respectively. Since the size range of the target proteins was rather large, a 4-12 % gradient poly-acrylamide gel was chosen for the electrophoretic separation. Proven, well-documented and widely accepted antibodies were chosen for the immunobloting stage of the study. The anti-Pgp C219 clone has been shown to detect a 170 kDa band (Weber, Eckert & Müller 2006), (Ambroziak et al. 2010) which corresponds to Pgp. This antibody does not cross-react with MRP1 or MRP2. The anti-MRP1, MRPr1 clone detects a band of 190 kDa (Calatozzolo et al. 2005), (Rajagopal, Simon 2003). For MRP2, the anti-MRP2, $M_2$I-4 clone antibody was used which has been shown to detect the MRP2 band of ~200 kDa (Stross et al. 2009). The BXP-21 clone was used to detect the BCRP band at 72 kDa as shown by Maliepaard *et al*. (Maliepaard et al. 2001a).

While most cell lines expressed Pgp, MRP1 and MRP2 in varied proportions, BCRP was found to be expressed in all the cell lines (Figure 22). Significant expression of Pgp was observed in the 1321N1, U118 and U138 cell lines. T98 and U87 cell lines showed prominent expression of MRP1 and BCRP. MRP2

expression was observed in the T98 and the A172 cell lines. The membrane was re-probed by β-actin to ensure equal loading.

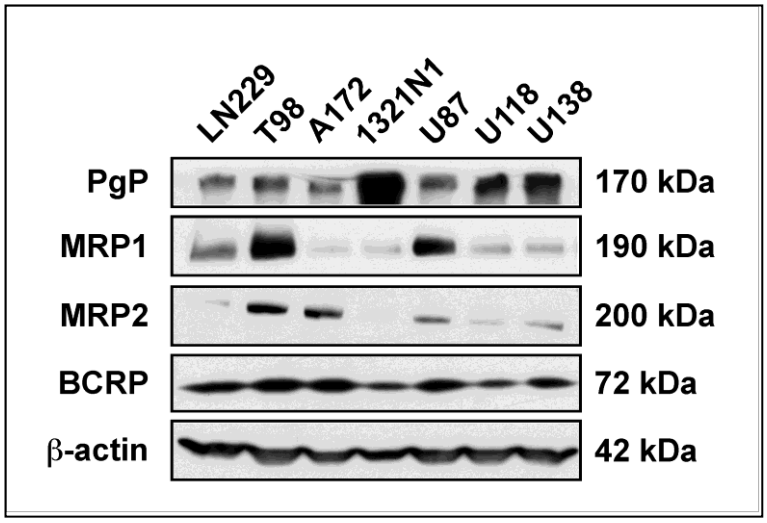


***Figure 22. Expression of Pgp, MRP1, MRP2 and BCRP in various glioblastoma cell lysates by Western blotting. Proteins extracted from glioblastoma cell lines were separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Results shown are from a single experiment and are representative of 3 different protein extractions.***

## 2.5. Conclusion

The aim of this study was to study the expression profiles of ABC transporters in the selected glioma cell lines. Immunoblotting was used as the technique of

choice as it provides a specific representation of the target protein. Established antibodies were chosen for this study and were optimized for the cell lines by a series of primary antibody dilution experiments. Importantly, the selected glioma and astrocytoma cell lines were shown to express Pgp, MRP1, MRP2 and BCRP. While Pgp was found to be expressed in all the chosen cell lines, 1321N1, U118 and U138 showed the most expression of Pgp. MRP1 was significantly expressed in T98 and U87. While not a lot of MRP2 was found in the glioma cell lines, T98 and A172 showed some MRP2 expression. Interestingly a uniform expression of BCRP was found in all glioma cell lines. Having confirmed the presence of the ABC transporters in the glioma cell lines, it was then important to ascertain the functionality of the efflux transporters. Vinblastin and mitoxantrone efflux assays were developed to assess the functional profile of Pgp, MRP1, MRP2 and BCRP using known inhibitors as vinblastine is a known substrate for Pgp, MRP1 and MRP2 and mitoxantrone is a well-established substrate for BCRP.

# Chapter 3

## 3. Efflux Assay To Investigate The Functionality of the ABC Transporters Identified in the Glioma Cell Lines.

### 3.1 Introduction

Having confirmed the presence of Pgp, MRP1, MRP2 and BCRP in the selected glioma and astrocytoma cell lines, it was important to determine if the aforementioned transporters were functional. To study the functionality of the efflux transporters, the innate efflux property of the transporters was used. Efflux transporter-expressing cells actively pump substrates out of the cell, which results in a lower rate of substrate accumulation, lower intracellular concentration at steady state, or a faster rate of substrate elimination from cells loaded with the substrate. Transported radioactive substrates or labeled fluorescent dyes can be directly measured, or in an indirect set up, the modulation of the accumulation of a probe substrate can be determined in the presence of a test drug or an inhibitor. Radio labeled vinblastin was chosen as the substrate for this study, as it is widely transported by Pgp, MRP1 and MRP2. Mitoxantrone was used as a substrate for BCRP. Known inhibitors of ABC transporters like benzbromarone, MK571 and sulfinpyrazone were used to study the differential efflux of vinblastin. Fumitremorgin-C was used as a BCRP inhibitor. Activity of the multidrug transporters was reflected by the difference between the amounts of substrate accumulated in the presence and the absence of inhibitor.

## 3.2 Aims and objectives

The aim of this study was to investigate the efflux mechanism of Pgp, MRP1, MRP2 and BCRP in the presence and absence of known inhibitors, using a whole cell based efflux assay.

## 3.2 Materials and Methods

$25\times10^5$ cells/well were seeded in 12 well plates, pre-coated with poly-L-lysine (0.0001% solution v/v in 1XPBS; Sigma Aldrich). The plates were coated with poly-L-lysine to ensure cell adherence. The 0.0001% v/v concentration did not have any effect of healthy cell growth. Cells were allowed to adhere and grow for 24 h. Cells were washed twice with 1XPBS and pre-treated with specific inhibitors or vehicle (DMSO; Sigma Aldrich) for 1h. The inhibitors used for this study were benzbromarone, MK571, sulfinpyrazone, indomethacine and FTC. The concentrations that were used: 0.5 μM, 5 μM, 10 μM, 50 μM, 100 μM, 250 μM and 500 μM. 20 nM [$^3$H]-vinblastine sulphate (Amersham; specific activity 5.90 Ci / mmol) and 20 nM [$^3$H]-mitoxantrone (Amersham; specific activity 4.20 Ci/mmol) was added to the wells and the cells were incubated for 30 min. The extra-cellular medium was collected. Counts were calculated in liquid scintillation counter (LSC) vials with 3 ml LSC fluid; Ecoscint A (National Diagnostics). The

wells were washed once with 500 µL 1XPBS and lysed by adding 500 µL 1 mM NaOH in 1XPBS for 30 min and the intra-cellular medium was collected. Radioactivity in both the extra and intra-cellular media was counted by a liquid scintillation counter (Beckman LC61000) in LSC vials with 3ml LSC fluid; Ecoscint A (National Diagnostics).

## 3.3 Results and Discussion

Findings from Chapter 2 indicated significant expression of ABC transporters in selected GBM and astrocytoma cell lines. Pgp and BCRP expression was uniform across all the cell lines, while MRP1 was significantly expressed in T98 and the U87 cell lines. MRP2 was found to be expressed in T98 and A172 cell lines. Before investigating the localization of the transporters it was thought to be important to understand the functionality of the transporters. A radio-labelled whole cell based efflux assay was developed to study the export of known substrates in absence and presence of known inhibitors. [$^3$H]-vinblastine sulphate was used as a substrate for Pgp, MRP1 and MRP2 as it is a known substrate for Pgp (Chanmahasathien et al. 2011), (Laska et al. 2002), MRP1 (Saito et al. 2001), MRP2 (Evers et al. 2000). For BCRP, [$^3$H]-mitoxantrone was used as it has been previously validated as a substrate for BCRP (Takara et al. 2012). 20 nM of both [$^3$H]-vinblastine-sulphate and [$^3$H]-mitoxantrone was used for the experiments.

| Inhibitor / Cell Line | Benzbromarone | MK571 | Indomethacin | Sulfinpyrazone | Fumitremorgin-C |
|---|---|---|---|---|---|
| **1321N1** | 28.81 ± 0.05 µM | - | | - | 16 ± 0.90 µM |
| **A172** | 7.27 ± 1.56 µM | 6 ± 0.68 µM | 86 ± 1.59 µM | 4.53 ± 1.59 µM | 22 ± 0.10 µM |
| **LN229** | 13.60 ± 0.72 µM | 19 ± 0.80 µM | - | - | 7 ± 0.02 µM |
| **T98** | 19.80 ± 0.01 µM | 11.70 ± 1.2 µM | 782 ± 1.21 µM | 8.98 ± 0.71 µM | 28 ± 0.46 µM |
| **U118** | 42.71 ± 0.09 µM | - | - | 979 ± 0.66 µM | 72 ± 0.38 µM |
| **U138** | 11.27 ± 0.87 µM | - | - | - | 48 ± 0.97 µM |
| **U87** | 56.03 ± 1.31 µM | 4.8 ± 22 µM | - | 553 ± 1.73 µM | 76 ± 1.22 µM |

***Table 1. $IC_{50}$ values of ABC transporter modulators determined by [$^3$H]-vinblastine efflux assay and [$^3$H]-mitoxantrone efflux assay. n=3. (-) indicates no efflux activity was observed.***

As seen in Table 1, $IC_{50}$ values indicated significant inhibition of efflux by the inhibitors. This data indicates that the ABC transporters present in the GBM and astrocytoma cell lines are functional.

## 3.4 Conclusions

This study confirmed that the ABC transporters in the GBM and astrocytoma cell lines were indeed functional. Significant difference was observed in efflux of know substrates in absence and presence of known ABC transporter inhibitors. Having confirmed the presence and functionality of the ABC transporters, the next step was to investigate the localization of the transporters.

# Chapter 4

# 4. Study of localization of ABC transporters by confocal immunofluorescence

## 4.1. Introduction

Results from the Western blot analysis indicated a significant presence of Pgp, MRP1, MRP2 and BCRP in the selected GBM cell lines. The functional efflux assay showed that the ABC proteins were indeed active and played a crucial role in the MDR phenotype exhibited by the GBM cell lines. The next step towards characterizing the ABC proteins in the GBM cell lines was to study their localization profile. Confocal laser microscopy was used to investigate the location of Pgp, MRP1, MRP2 and BCRP in selected GBM cell lines.

## 4.2. Aims and Objectives

The aim of the next step in the research programme therefore was to study the localization of Pgp, MRP1, MRP2 and BCRP in selected glioma cell lines by using confocal laser microscopy.

## 4.3. Materials and Methods

Selected glioma cell lines were harvested at 90% confluency and cell number was determined by a trypan blue exclusion method. 25,000 cells were plated on a LabTek 4 chamber slide with cover CC2 sterile glass slide (Nalge Nunc International # 154917). Following 24 h incubation at 37$^{o}$C, 5% $CO_2$, the medium was replaced with sera free medium and the cells were incubated for a further 2 h to equilibrate the protein signal and eliminate any stimulatory or inhibitory effects of sera. The cells were washed with 2XPBS and treated with 4% v/v formaldehyde at room temperature for 10 min to fix the cells. After 2 washes with 2 X PBS, the cells were permeablised with 0.2% v/v Triton X-100 for 5 min to expose the target epitope. The cells were then treated with 8% v/v BSA (bovine serum albumin) at room temperature for 1 h, to block any non-specific binding to the primary antibody. The primary monoclonal antibody dilutions, 1:50 [Pgp mouse(C219), MRP1 rat(MRPr1), MRP2 mouse($M_2$I-4) and BCRP mouse(BXP21)], were made in 1% v/v BSA and the cells were probed at 4$^{o}$C, for 12 h. The cells were washed to remove any unbound antibodies and probed with the appropriate secondary antibody. Goat anti-mouse conjugated to Alexa Fluor 488 in case of Pgp, MRP2 and BCRP and goat anti-rat conjugated to Alexa Fluor 568 in case of MRP1. At the end of the incubation period the cells were washed twice with 2XPBS and mounted with Prolong Gold stain with DAPI for nuclear staining. The slides were then analysed by a Zeiss Meta 510 confocal microscope.

## 4.4. Results and Discussion

Confocal microscopy is an optical imaging technique often used in cellular studies to investigate the localization of target proteins. A derivative of traditional fluorescence microscopy; confocal microscopy has a clear advantage, as the microscope uses point illumination as compared to flooding the entire specimen, thus decreasing the signal to noise ratio to almost negligible amounts (Shotton 1989).

Indirect staining was used in the present study as this adds another screen to eliminate non-specific binding of either the primary or secondary antibody, as only the antigen-antibody complex is illuminated by the fluorophore conjugated secondary antibody. Care was taken to eliminate non-specific signals at every step with adequate washes with 2 X PBS between every staining step. 4 well chamber slides were chosen and the last well of each slide was not probed by the primary antibody to provide the negative control for imaging.

Of all the glioma cells lines that were characterized by Western blotting, five were chosen based on the expression profile of the ABC transporters. Comparing the signal intensity of the Pgp, MRP1, MRP2 and BCRP blots with that of the internal standard; β-actin, it was inferred that Pgp levels were uniform all across

the cell lines, MRP1 and MRP2 were abundant in U87, LN229, T98, 1321N1, A172 and BCRP was expressed in high amounts in the T98 and LN229 cell lines.

Results from confocal immuno-fluorescence confirmed previous findings from immuno-blotting. Pgp was found to be present in 1321N1 (Figure 23), A172 (Figure 24) and U87 (Figure 25) cell lines, localized on the membrane and in the cytosol. MRP1 and MRP2 were present in LN229, T98, U87, 1321N1 and A172. As shown in Figure 26, there was co-localization of MRP1 and MRP2 in LN229 cells, further confirming the specificity of the method and indicating towards the over-lap in the localization of the ABC transporters.

BCRP was found to be present in the LN229 and T98 cells as expected. The positive signal was observed in the cytoplasm but primarily in the nucleus (Figure 27 and 28). Although by definition and indeed according to published work the ABC transporters are essentially membrane efflux transporters, localized primarily on the surface of the cells and in the plasma membrane whereby the most efficiently facilitate the extrusion of their substrates (Ambudkar et al. 1999). However, there have been isolated reports about BCRP being found in the nucleus of chorion leave cells (Yeboah et al. 2008) and in the mitochondria in some instances (Solazzo et al. 2009). The exact physiological role that BCRP might play in the nucleus is unclear, although it could act as a reservoir for Pkt

translocation of the protein or like in the case of hematopoetic stem cells, act like a side population of ABCG2 (Zhou et al. 2001).

The primary aim of confocal immunofluorescence was to confirm the absence or presence of Pgp, MRP1, MRP2 and BCRP in the selected glioma cell lines. While most of the findings were consistent with previously reported immuno-blotting data, the localization of BCRP in the nucleus of the T98 and LN229 cells may indicate a secondary site for efflux.

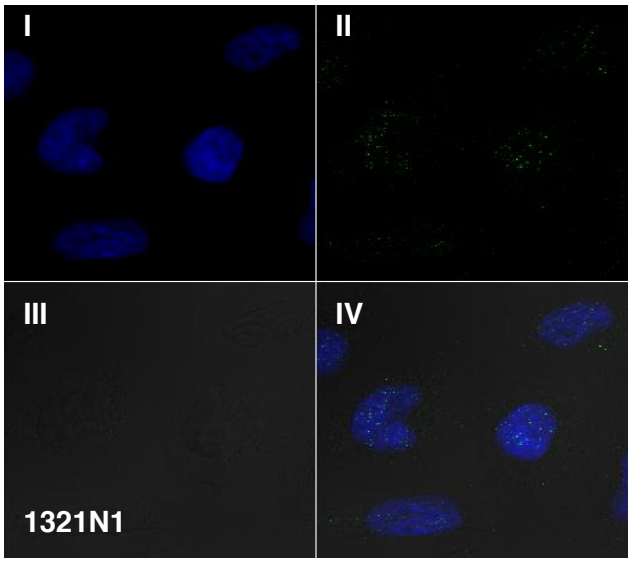


***Figure 23. Confocal immuno-fluorescence staining of 1321N1 cells using Pgp mouse(C219) antibody. Stained with ALEXA Fluor 488 conjugated anti mouse. (I) Nuclear staining with DAPI, (II) Pgp staining, (III) Cell morphology control, (IV) Merge. Image magnification = 500X***

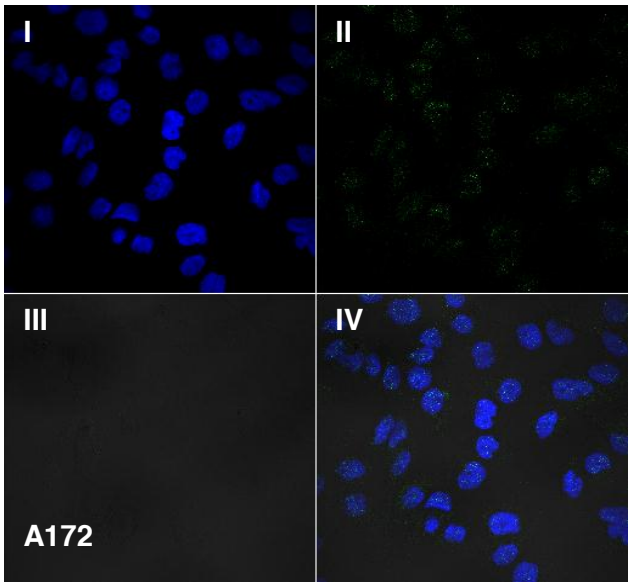


***Figure 24. Confocal immuno-fluorescence staining of A172 cells using Pgp mouse(C219) antibody. Stained with ALEXA Fluor 488 conjugated anti mouse. (I) Nuclear staining with DAPI, (II) Pgp staining, (III) Cell morphology control, (IV) Merge. Image magnification = 10X***

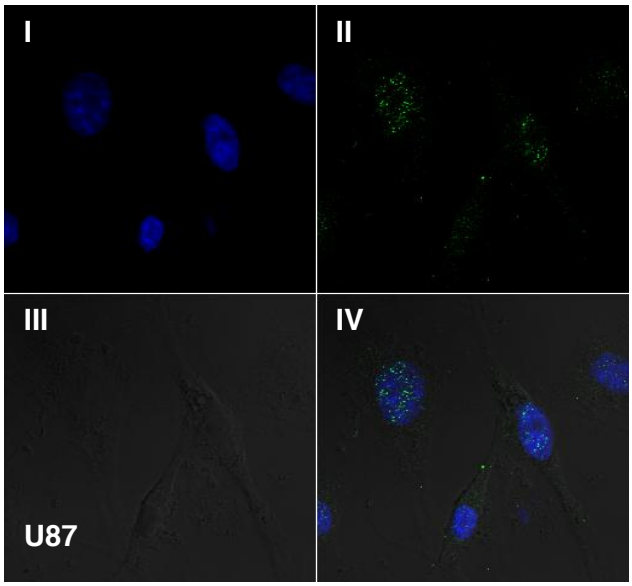


***Figure 25. Confocal immuno-fluorescence staining of U87 cells using Pgp mouse (C219) antibody. Stained with ALEXA Fluor 488 conjugated anti mouse. (I) Nuclear staining with DAPI, (II) Pgp staining, (III) Cell morphology control, (IV) Merge. Image magnification = 500X***

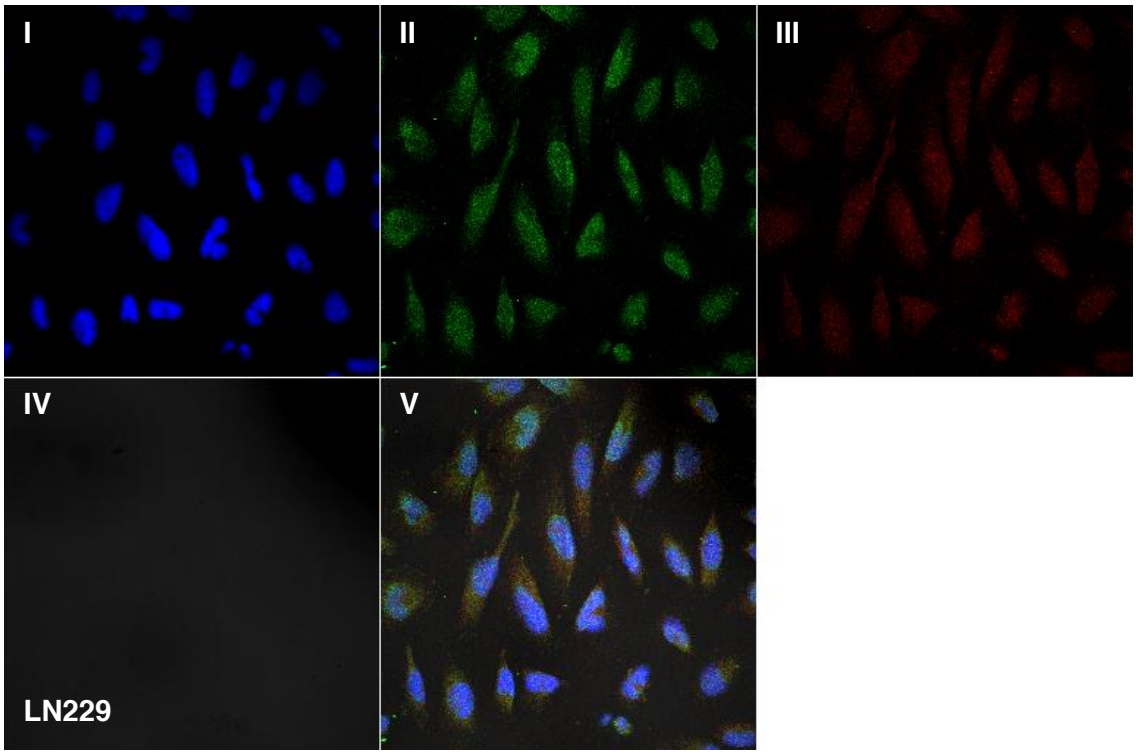


***Figure 26. Co-localisation of MRP1 and MRP2 in LN229, glioma cells. (I) LN229 cells stained with nuclear stain DAPI, (II) LN229 cells stained with MRP2 mouse($M_2$-III4), (III) LN229 cells stained with MRP1 rat(MRPr1), (V) Morphology control, indicating the healthy and intact morphology of the LN229 cells and (IV) co-localization of MRP1 and MRP2. Image magnification = 10X***

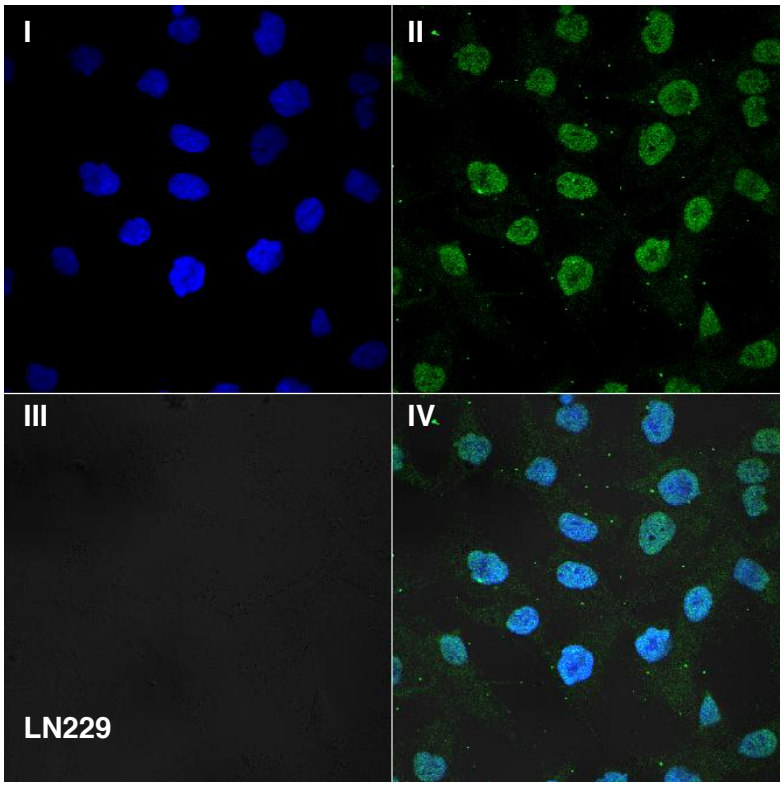


***Figure 27. Confocal immuno-fluorescence staining of LN229 cells using BCRP mouse(BXP-21) antibody. Stained with ALEXA Fluor 488 conjugated anti mouse. (I) Nuclear staining with DAPI, (II) Pgp staining, (III) Cell morphology control, (IV) Merge. Image magnification = 10X***

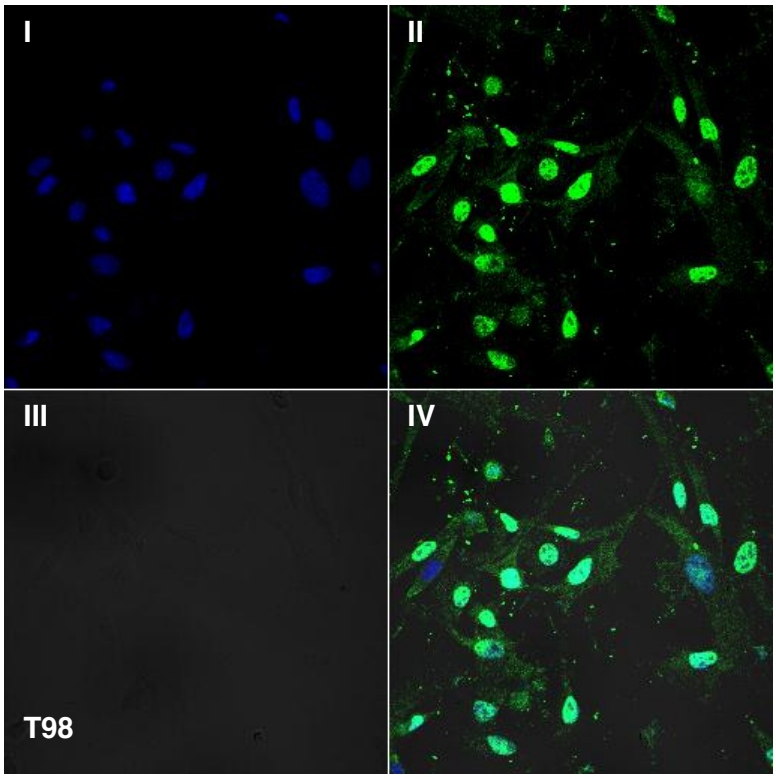


***Figure 28. Confocal immuno-fluorescence staining of T98 cells using BCRP mouse (BXP-21) antibody. Stained with ALEXA Fluor 488 conjugated anti mouse. (I) Nuclear staining with DAPI, (II) BCRP staining, (III) Cell morphology control, (IV) Merge. Image magnification = 10X***

## 4.5. Conclusion

Findings from confocal microscopy confirmed data from western blotting in Chapter 2. Interestingly, in addition to cytoplasmic expression of Pgp, MRP1, MRP2 and BCRP, significant expression of BCRP was observed in the nucleus of the LN229 and T98 cells. In order to further investigate this finding, LN229 cells were chosen for subcellular fractionation BCRP investigation using immuno-blotting and confocal microscopy with specific nuclear markers.

# Chapter 5

# 5. Analysis of nuclear expression of breast cancer resistant protein

## 5.1 Introduction

At this point in the research programme, the presence of BCRP in the selected glioma cell lines had been confirmed by whole cell Western blot analysis and confocal microscopy had been used to indicate a significant BCRP localization at the nucleus of the LN229 cells. Given the unusual nature of this finding, it was thought that it would be useful to confirm it by using sub-cellular fractionation Western blotting.

## 5.2 Aims and Objectives

Accordingly, the aim of this phase of the research programme was to study the expression of BCRP in native and BCRP silenced LN229 cells using sub cellular fractionation immuno-bloting and confocal microscopy.

## 5.3 Materials and Methods

### *5.3.1. Cell culture*

LN229 and MCF-7 cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in Dulbecco's modified Eagle's medium (D-MEM) and Eagle's minimum essential medium (E-MEM) respectively, with 4.5 g glucose / L (Quality Biological Inc., Gaithersburg, MD, USA), supplemented with penicillin (25 U/ml), streptomycin (25 µg/ml), pyruvic acid and 5% v/v foetal calf serum (Thermo Scientific, Waltham, MA, USA). Cells were maintained at 37$^{o}$C in a humidified incubator with 5% $CO_2$.

### *5.3.2 Preparation of whole cell lysates*

Cells at approximately 70 – 80% confluence were lysed on ice for 20 min in Tris-HCl [10 mM, pH 7.4] containing 0.15 M NaCl, 5mM EDTA, 1% v/v Triton X-100, 1:500 dilution of a stock solution of protease inhibitor cocktail, 0.1M sodium ortho-vanadate and 0.1M DTT (Sigma Aldrich, St-Louis, MO, USA). Insoluble material was removed by centrifugation (17,000×g for 20 min at 4$^{o}$C) and the supernatant was collected and stored at -80$^{o}$C until further analysis.

### *5.3.3. Subcellular fractionation*

Nuclear and cytoplasmic lysates were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit according to the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA).

### *5.3.4. Western Blot analysis*

The protein concentration of the lysates was measured with the BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) using BSA as standard. An equal amount of 3X Laemlli sample buffer was added, and the samples (70 µg) were separated by electrophoresis using precast 4-12% gradient SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride membranes using iBLOT (Invitrogen). The membranes were blocked with 5% v/v dry non-fat milk in Tris-buffered saline with 0.1% v/v Tween-20 and then probed with mouse monoclonal anti-BCRP (clone BXP-21, 1:500 dilution; Alexis Biochemicals, Plymouth Meeting, PA, USA) and mouse monoclonal anti-β-actin (1:5000 dilution; Abcam, Cambridge, MA, USA) as a loading control. The quality of the cytoplasmic and nuclear extracts was

confirmed by probing the blots for the cytoplasmic marker NFκB p65 using mouse monoclonal anti-NFκB p65 (clone F-6, 1:5000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) and the nuclear marker Brg-1 using mouse monoclonal anti-Brg-1 (clone G-7, 1:5000 dilution; Santa Cruz Biotechnology). This was followed by appropriate horseradish peroxidase-conjugated secondary antibodies. The ECL Western blotting detection system (Santa Cruz Biotechnology) was used for visualization.

### *5.3.5. BCRP siRNA transfection*

Subconfluent LN229 and MCF-7 cells were transfected with a pool of three BCRP siRNA oligonucleotides; s18056, s18057 and s18058 (Ambion, Austin, TX, USA) and a siRNA non-targeting control, 4390844 (Ambion), using forward transfection technique with Lipofectamine RNAiMAX™ (Invitrogen) according to the manufacturer's instructions. Briefly, 15 nM siRNA was mixed with 35 μL Lipofectamine RNAiMAX™ and was allowed to form a siRNA-Lipofectamine complex for 20 min at room temperature. Cells were harvested, re-suspended in appropriate media without penicillin-streptomycin and seeded with the siRNA-Lipofectamine complex. After 48-72 hr, the cells were assayed for BCRP knockdown.

### *5.3.6. Confocal microscopy*

LN229 cells ($25\times10^3$ cells/well) and MCF-7 cells ($15\times10^3$ cells/well) were seeded on a 4-well LabTek II CC2 chamber slide system with lid (Nalge Nunc International, Roskilde, Denmark) and allowed to grow for 24 h. The medium was replaced with serum-free medium for 2 h, after which the cells were washed twice with phosphate-buffered saline (PBS) and fixed with 4% v/v formaldehyde (Sigma Aldrich) at room temperature for 10 min. After fixing, the cells were washed twice with PBS, permeabilized with 0.2% v/v Triton X-00 (Sigma Aldrich) for 5 min, washed again with PBS, and then blocked with 8% v/v BSA at room temperature for 1 h. The mouse monoclonal anti-BCRP (clone BXP-21; 1:500 dilution) and rabbit polyclonal anti-emerin (1:1000; Abcam) were diluted with 1% v/v BSA and added to the cells for 12 h at $4^oC$. After three PBS washes, Alexa Fluor 488 conjugated goat anti-mouse and Alexa Fluor 568 conjugated goat anti-rabbit antibodies were added to the slides and the cells were incubated at room temperature for 1 h. The cells were washed three times with PBS, mounted in Prolong Gold antifade (Invitrogen) and cured for 24 h at room temperature in the dark. Images were taken using a Zeiss Meta 510 confocal microscope.

## 5.4. Results and Discussion

### *5.4.1. Subcellular fractionation analysis of breast cancer resistant protein*

Nuclear and cytoplasmic fractions from the LN229 and MCF-7 cells were probed for BCRP. The LN229 and MCF-7 cells were also probed BCRP and emerin to study any nuclear co-localization, since emerin is a nucleus specific lamin protein. Expression of BCRP was determined in the LN229 cells by Western blotting using a BCRP specific monoclonal antibody; clone BXP-21 (Maliepaard et al. 2001a). The human derived breast adenocarcinoma cell line, MCF-7, was used as positive control as it has been previously demonstrated that BCRP is expressed in this cell line (Burger et al. 2004). The results from the whole cell lysates demonstrated that BCRP was expressed in both the LN229 and MCF-7 cell lines (Figure 29A). Densitometric calculations using β-actin as the loading control indicated that there were no significant differences in the relative expression of BCRP in the LN229 and MCF-7 cell lines.

Cytoplasmic and nuclear extracts were obtained from the whole cell lysates of the LN229 and MCF-7 cells by subcellular fractionation, and the fractions were probed for BCRP by Western blot analysis. The results indicate that BCRP was expressed in both the cytoplasmic and nuclear fractions of LN229 cells (Figure

29B). A direct comparison of the intensities of the BCRP bands indicates that the nuclear expression of BCRP was ~2-fold greater than in the cytosolic fraction. In contrast, BCRP was readily observed in the cytosolic fractions prepared from MCF-7 cells, but was not detected in the nuclear extracts of these cells (Figure 29B).

The expression of BCRP in LN229 and MCF-7 cells was further probed using a pool of siRNAs targeted against the BCRP gene. siRNA-mediated BCRP gene knockdown resulted in a significant reduction (~80%) in the relative expression of BCRP transcript in LN229 and MCF-7 cell lines when compared to cells transfected with a control non-targeting siRNA (Figure 29C). To investigate the effect of gene silencing on the subcellular compartmentalization of BCRP, cytoplasmic and nuclear extracts were prepared from LN229 and MCF-7 cells that were transfected with either control or BCRP siRNAs. There was significant reduction in BCRP levels in the nuclear fraction of LN229 cells treated with BCRP siRNAs, while BCRP could no longer be detected in the cytoplasmic fraction of these cells (Figure 29D). BCRP was not detected in either the cytoplasmic or nuclear fraction of the MCF-7 cells depleted of BCRP (Figure 29D).

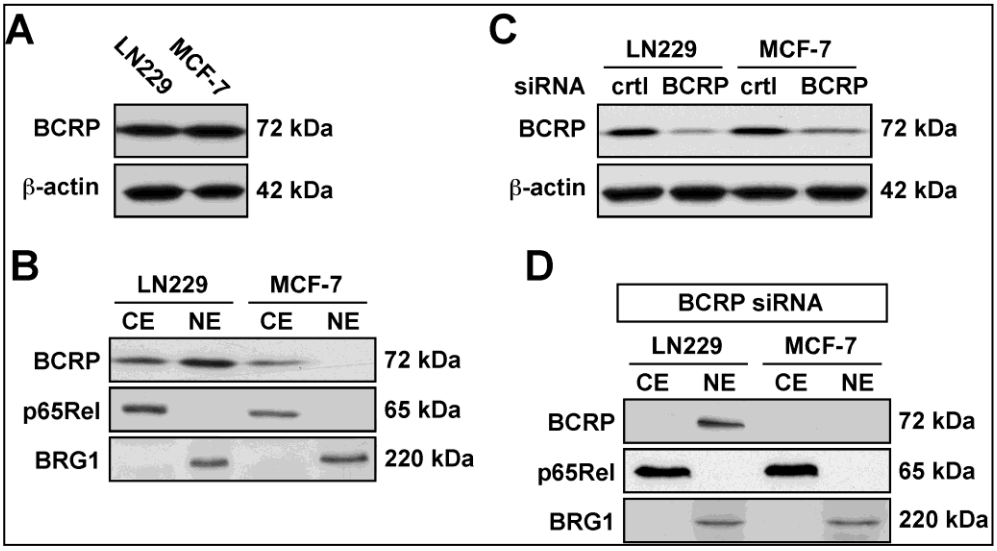


***Figure 29. Detection of BCRP by immunoblot analysis. (A) The human brain tumor cell line LN229 and the MCF-7 breast cancer cells were lysed and processed for Western blot analysis. The blots were probed for BCRP and β-actin expression using specific primary antibodies. The molecular mass markers (in kDa) are shown on the right. (B) Cytosolic and nuclear extracts of LN229 and MCF-7 cells were resolved by SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis for the detection of BCRP. The membranes were reprobed with antibodies against the cytosolic marker, p65Rel [a subunit of NFκB], and the nuclear marker Brg1 to confirm the quality of the subcellular fractionation. (C) LN229 and MCF-7 cells were transfected with a siRNA non-targeting control or BCRP siRNAs for 24 h, after which cells were lysed and processed for the detection of BCRP and β-actin by immunoblot analysis. (D) Cytosolic and nuclear extracts were prepared from control and BCRP siRNA-treated LN229 and MCF-7 cells followed by Western blot analysis using the indicated antibodies. Similar results were obtained in three independent experiments.***

### *5.4.2. Confocal microscopy analysis of breast cancer resistant protein co-localization with emerin*

Indirect immunofluorescence confocal laser microscopy was used to further investigate the localization profile of BCRP in the LN229 and MCF-7 cell lines (Figure 30). Significant cytosolic localization of BCRP was observed in both cell lines (Figure 30A, upper panels). Co-staining of emerin indicated significant nuclear localization of BCRP in the LN229 cells, but not MCF-7 cell line (Figure 30A, middle and bottom panels). These results are in agreement with the data from the Western blot studies. Vertical sections in the *x-z* and *y-z* planes of confocal images were generated to confirm BCRP co-localization with emerin (Figure 30B). The *x-z* plane generated from fixed LN229 cells showed staining consistent with their co-localization (depicted in yellow). Using the same approach, no detectable nuclear expression of BCRP was observed in MCF-7 cells, as there was a clear demarcation of BCRP and emerin staining with no co-localization. Cross-sectional quantification of the images indicated the signal intensity to be uniform in both sets of images.

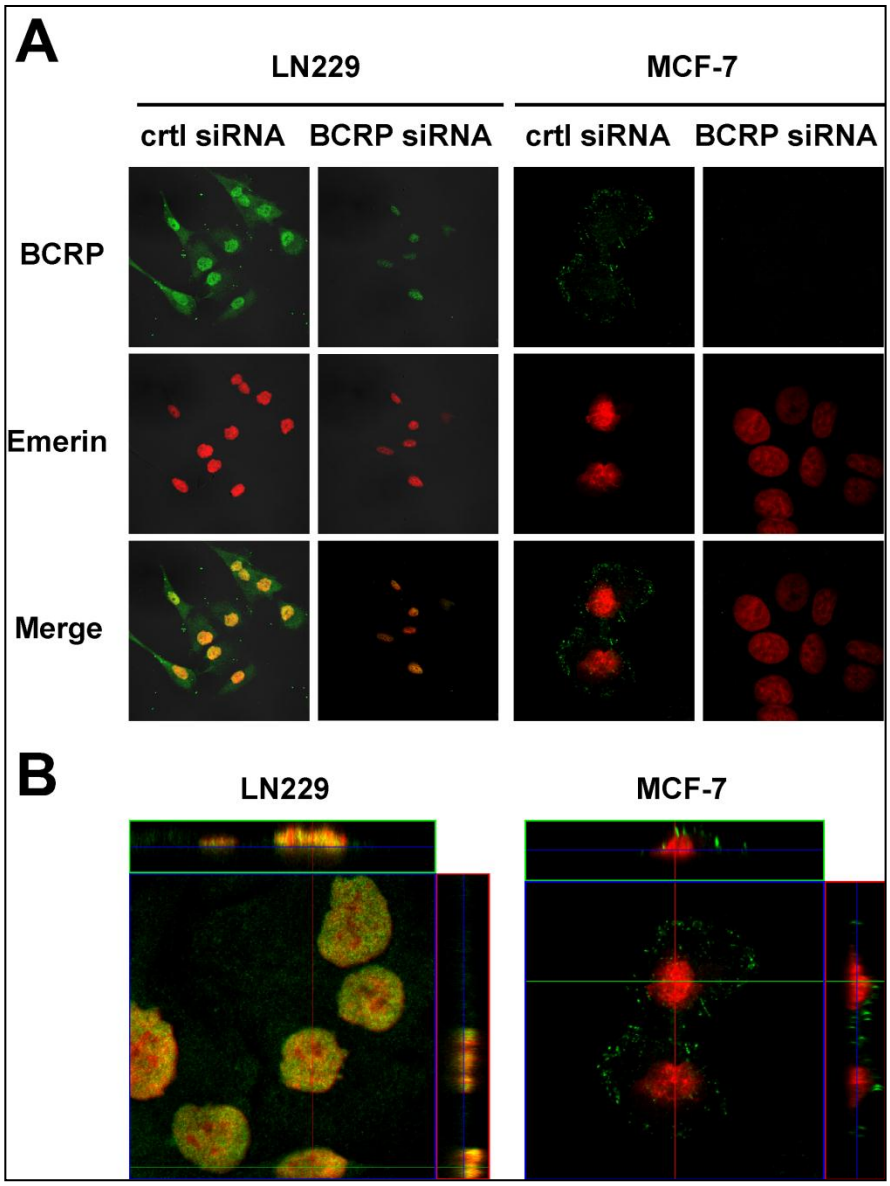


***Figure 30. Cellular distribution of BCRP by confocal laser microscopy. LN229 cells and MCF-7 cells were transfected with control and BCRP siRNAs for 24 h. Cells were then fixed and immunolabeled with monoclonal anti-BCRP (green, upper panels) and polyclonal anti-emerin (red, middle panels) antibodies. Merge images are shown in the bottom panels. Areas of nuclear co-localization between BCRP and emerin appear in yellow in LN229 cells, which were absent in MCF-7 cells. Similar results were obtained in three independent experiments. Magnification 40X, (A) XY plane; (B) XZ plane.***

## 5.5. Conclusion

The results demonstrated that in the human-derived LN229 GBM cell line BCRP is expressed in both the cellular and nuclear membranes, with nuclear expression predominating. The data from the Western blot, confocal laser microscopy and siRNA knockdown studies confirm the presence of BCRP in the nucleus. Clearly this could have significant functional consequences, which would have to be investigated.

# Chapter 6

# 6. Functional analysis of nuclear breast cancer resistant protein

## 6.1. Introduction

Subcellular fractionation and siRNA transfection studies had confirmed the nuclear presence of BCRP in the LN229 GBM cells and Z-section confocal imaging further consolidated the finding, showing significant co-localization of BCRP with the nuclear specific marker; emerin. While the exact purpose and reason for this was unknown, it was hypothesized that the nuclear location of BCRP acts as a secondary efflux site for substrates. In order to investigate this line of thought, it was important to assess the functional role of nuclear BCRP in the LN229 cells. It was thought that this could be investigated by an MTS cell proliferation assay. The non-radioactive MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] cell proliferation assay is such an assay, which could be used to study the role of BCRP in the LN229 GBM cells. It is a colorimetric technique to assess the viability and proliferation of cells in a 96 well system. It relies on the ability of mitochondria in live cells to convert the MTS dye into a purple colour formazan, which has an absorbance maximum at 490-500 nm.

## 6.2. Aims and Objectives

The aim of this part of the study was to investigate the functional role of nuclear BCRP in the effect of mitoxantrone on the LN229 GBM cells.

## 6.3. Materials and Methods

### *6.3.1. Cell culture*

LN229 cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in Dulbecco's modified Eagle's medium (D-MEM) with 4.5 g glucose / L (Quality Biological Inc., Gaithersburg, MD, USA), supplemented with penicillin (25 U/ml), streptomycin (25 μg / ml), pyruvic acid and 5% feotal calf serum (Thermo Scientific, Waltham, MA, USA). Cells were maintained at 37 $^{o}$C in a humidified incubator with 5% $CO_2$.

### *6.3.2. BCRP siRNA transfection*

Subconfluent LN229 cells were transfected with a pool of three BCRP siRNA oligonucleotides; s18056, s18057 and s18058 (Ambion, Austin, TX, USA) and a siRNA non-targeting control, 4390844 (Ambion), using forward transfection technique with Lipofectamine RNAiMAX™ (Invitrogen) according to the manufacturer's instructions. Briefly, 15 nM siRNA was mixed with 35 μL Lipofectamine RNAiMAX™ and was allowed to form a siRNA-Lipofectamine complex for 20 min at room temperature. Cells were harvested, re-suspended in appropriate media without penicillin-streptomycin and seeded with the siRNA-Lipofectamine complex. After 48-72 h, the cells were assayed for BCRP knockdown.

### *6.3.3. MTS assay*

The non-radioactive CellTiter 96® Aqueous cell proliferation assay was performed according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, cells were seeded in 96-well plates at a density of $3.5\times10^3$ cells/well and cultured for 24 h. Stock solutions of MTX (Sigma Aldrich) and Fumitremorgin C (FTC, Sigma Aldrich) were prepared in DMSO, each at 1 mM. FTC is a fungal toxin that has been shown to reverse resistance to doxorubicin,

MTX, and topotecan in BCRP-expressing cells at micromolar concentrations. MTX was diluted in cell culture medium with a maximum of 0.5% v/v DMSO to each well. The concentrations of MTX were 0.5, 5, 50, 500, 1000, 2500 and 5000 nM. After 48 h incubation, 20 µl of 0.5 mg/ml of the tetrazolium compound MTS was added to each well and further incubated for 1 h. The absorbance was measured at 490 nm with a micro-plate reader (Thermo Scientific). Change in growth rate was calculated as follows: [$A_{490nm}$ of treated cells/$A_{490nm}$ of control cells].

## 6.4. Results and Discussion

The functional activity of BCRP was studied in LN229 cells using MTX, a commonly used chemotherapeutic agent that acts as a DNA intercalating agent, and FTC, a specific, selective, and potent BCRP inhibitor (Rabindran et al. 2000). MTX had been identified as a specific substrate of BCRP efflux activity (Kodaira et al. 2010) and previous studies had demonstrated that the cytotoxicity of MTX is directly related to intracellular concentration (Jansen, Yip & Louis 2010). In the current study described here, the growth inhibitory activity of 500 nM MTX was determined in LN229 cells transfected with either a negative, non-silencing control siRNA or BCRP siRNA with and without the addition of 5 µM FTC (Figure 31B). The addition of FTC in LN229 cells transfected with control siRNA produced a ~20% increase in MTX-mediated growth inhibition ($n=3$, $p < 0.001$)

as assessed by the MTS assay. BCRP gene knockdown resulted in a ~50% and ~70% increase in MTX-dependent growth inhibition, respectively, when MTX was added alone ($n=3$, $p < 0.001$) or combined with FTC ($n=3$, $p < 0.001$). The same trend was observed with the calculated MTX $IC_{50}$ values, which were reduced from 570 ± 1.4 nM to 77 ± 0.9 nM by the addition of FTC and to 5.2 ± 1.1 nM in LN229 cells pre-treated with BCRP siRNA. When the MTS assay was repeated using the MCF-7 cell line, the addition of MTX reduced proliferation by 45% relative to control and co-treatment with FTC increased this effect to 75%. The silencing of BCRP in the MCF-7 cells produced no significant differences in the cytotoxic potency of MTX when the drug was utilized alone or in combination with FTC (Figure 31). This effect was comparable to that of FTC-treated MCF-7 cells transfected with siRNA negative control.

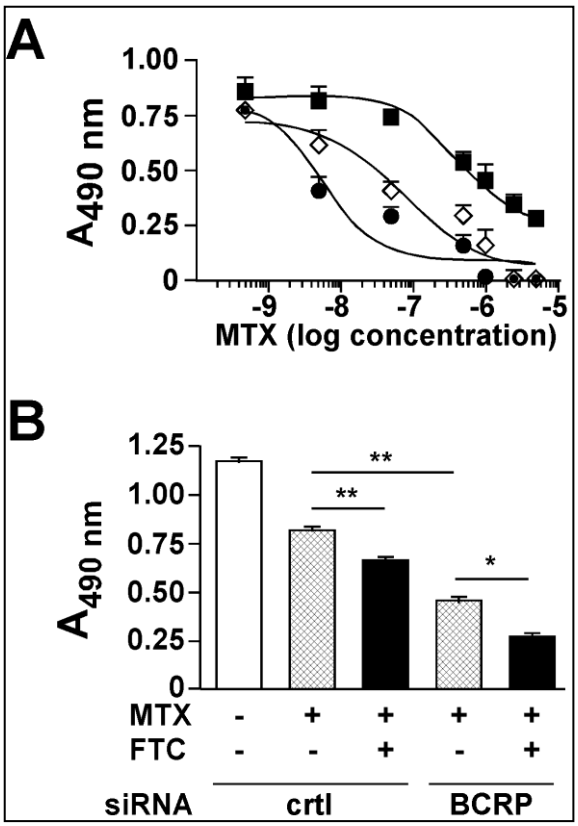


***Figure 31. Role of BCRP in LN229 cell proliferation. (A) LN229 cells were incubated with the indicated concentrations of mitoxantrone (MTX) in the absence (square) or the presence of 5 µM fumitremorgin C (FTC) (diamond) for 48 h. A cell proliferation colorimetric assay based on MTS reduction was then performed. In some instance, BCRP siRNA-treated LN229 cells were incubated with MTX followed by the MTS cell proliferation assay (open triangle). (B) LN229 cells were transfected with control or BCRP siRNA for 24 h followed by the addition of 500 nM MTX in the absence or presence of 5 µM FTC for 48 h. Values are means ± SEM from 3 independent experiments performed in triplicate. Statistical analysis by ANOVA with Dunnett's Multiple comparison test was performed, where $^{**}P < 0.01$ vs. vehicle treated controls and $^{*}P < 0.05$ vs. MTX-treated cells transfected with BCRP siRNAs.***

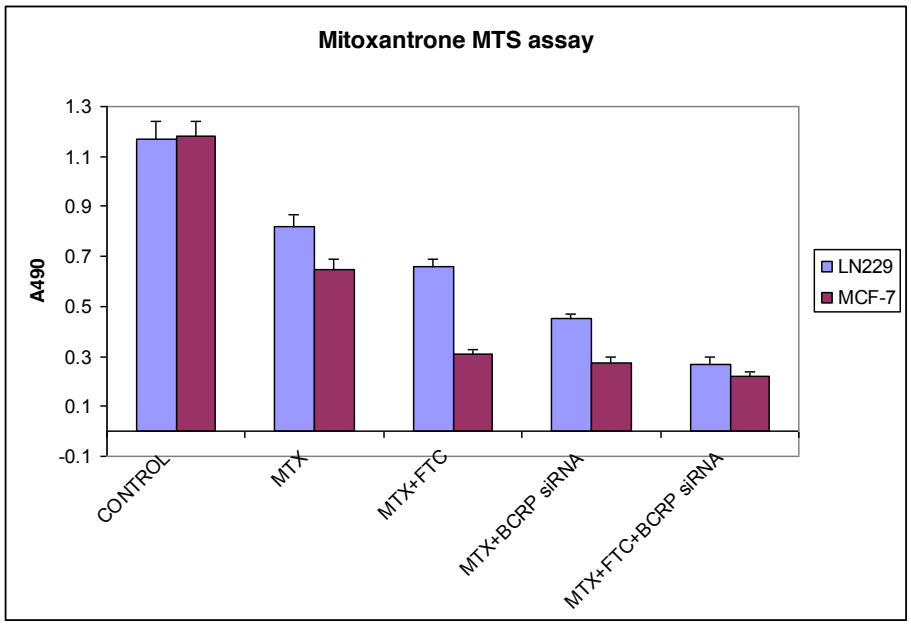


***Figure 32. Comparative study of effect of MTX on BCRP(+) and BCRP(-) LN229 and MCF-7 cells. LN229 and MCF-7 cells were transfected with control or BCRP siRNA for 24 h followed by the addition of 500 nM MTX in the absence or presence of 5 µM FTC for 48 h. Values are means ± SEM from 3 independent experiments performed in triplicate. Statistical analysis by ANOVA with Dunnett's Multiple comparison test was performed, where $^{a}P < 0.01$ vs. vehicle treated controls, $^{b}P < 0.01$ vs. MTX treatment and $^{c}P < 0.05$ vs. MTX-treated cells transfected with BCRP siRNAs.***

## 6.5. Conclusion

The functional consequence of the nuclear expression of BCRP was investigated by examining the cytotoxic effect of MTX after competitive inhibition of BCRP transport activity by FTC and siRNA reduction of BCRP expression. In LN229 cells cytotoxicity was investigated by assessing growth inhibition using the MTS assay using the MCF-7 cell line as the positive control as only cytosolic expression of BCRP was observed in the MCF-7 cell line. The data from the studies indicated that FTC-mediated inhibition of MTX transport produced a 20% increase in growth inhibition, BCRP gene knockdown increased this effect to 50%, and the combination of the two approaches increased the MTX-dependent growth inhibition by 70%. These results differed from the effects observed with the MCF-7 cell line where the co-administration of MTX and FTC decreased cellular proliferation by 75%, relative to a 45% reduction observed with MTX alone, while the effect of BCRP siRNA was similar to that observed with FTC and there was no additive effect produced by combining the two approaches. These results indicated that the nuclear expressed BCRP plays a protective role in the LN229 cell line and that both a reduction in BCRP expression and its competitive inhibition were required to reach the optimum cytotoxic effect of either one of these treatments in MCF-7 cells.

# Chapter 7

# 7. Cellular membrane affinity chromatography

## 7.1. Introduction

ABC transporters have also been found to be functionally expressed in the human GI tract where they limit the bioavailability of their substrates. Most drug discovery programmes seek to screen lead candidates for transport by ABC proteins like P-glycoprotein (Pgp) using the Caco-2 efflux assay. These cell-based assays are time consuming and labour intensive. Also, no definitive assays for multidrug resistance protein 1 (MRP1), multidrug resistance protein 2 (MRP2) and breast cancer resistant protein (BCRP) had been developed. It was apparent that the development of high through-put cellular membrane affinity chromatography columns (CMAC), prepared by immobilizing membrane fragments from cell lines, stably transfected by Pgp, MRP1, MRP2, BCRP and control vectors would enable, screening of potential inhibitors of the ABC proteins.

## 7.2. Aims and Objectives

Therefore it was sought to synthesize cellular membrane affinity chromatography columns by immobilizing membrane fragments from Sf9 insect cells transfected by Pgp, MRP1, MRP2 and BCRP encoding vectors. The stationary phases were characterized using displacement frontal affinity chromatography using known inhibitors.

## 7.3. Materials and methods

### *7.3.1. Chemicals*

[$^{3}$H]-Etoposide, was obtained from Moravek Biochemicals (Brea, CA, USA). MK571 and fumitremorgin C was obtained from Calbiochem (San Diego, CA, USA). Etoposide, benzbromarone, (R)-verapamil, (S)-verapamil, furosemide, HEPES, NaCl, β-mercaptoethanol, Tris-HCl, protease inhibitor cocktail (Catalog number P8340), adenosine triphosphate (ATP), glycerol, ethylene glycol-bis(2-aminotheylether)-N,N,N'N'-tetra-acetic acid (EGTA), glutathione (GSH), CHAPS and trizma were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The immobilized artificial membrane phospahtidyl choline stationary

phase (IAM-PC, 12 $\mu$m, 300 Å) was purchased from Regis Technologies Inc. (Morton Grove, IL, USA).

### *7.3.2. Sf9 membranes*

Sf9-Human MRP1 Membranes (Catalog number 453456), Sf9-Human MRP2 Membranes (Catalog number 453332) and the Sf9-Human BCRP Membranes (Catalog number 453270) isolated from Sf9 cell line transfected with Human Mdr1 cDNA, Human Mrp1 cDNA, Human Mrp2 cDNA and Human BCRP cDNA genes using a baculovirus system and Control Membranes (Catalog number 453200) isolated from non-transfected, native Sf9 cell line were obtained from BD Biosciences (Woburn, MA, USA)

### *7.3.3 Preparation of CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$), CMAC($Sf9_{BCRP}$) and CMAC(Sf9) columns*

750 µg protein were solubilised in 5ml TMEP buffer (50mM Tris-HCl, 50mM glycerol, 2mM EGTA, 2mM β-mercaptoethanol, 1:500 protease inhibitor cocktail and 1% w/v CHAPS; pH 7.0) for 12h at 4$^{o}$C. After the solubilisation, the membranes were incubated with 100mg IAM-PC stationary phase for 1h at room

temperature on an orbital shaker. The membrane-IAM-PC complex was dialysed in dialysis buffer (20mM HEPES, 500mM NaCl and 1mM EDTA; pH 8.0) overnight at $4^{O}$C. Following dialysis, the CMAC stationary phases were packed into Tricorn 5/20 columns (GE Healthcare, UK) yielding a 75 mm x 5 mm (i.d) chromatographic beds.

### *7.3.4 Chromatographic studies*

The cellular membrane affinity columns were placed in a frontal affinity chromatography system and competitive displacement studies were carried out using previously described techniques (Moaddel, Wainer 2009). Briefly the system consisted of a manual FPLC injection valve, 50-ml superloop both obtained from (Amersham Biotechnology, Columbia, MD), CMAC column, LC-10AD HPLC pump (Shimadzu Inc.) and an on-line radioactive/scintillation flow detector (IN/US, Tampa, FL, USA). Solutions of the marker and test ligands were prepared in the running buffer; Tris-HCl [10 mM, pH 7.5] containing 1 mM $MgCl_2$, and 5 ml samples were placed in the superloop, pumped across the CMAC column at a flow rate of 0.2 ml/min and monitored through a 250 µl flow cell with the radioflow detector. The scintillation flow rate was 0.6 ml / min while the split ratio was 100. The breakthrough volume of the marker was calculated using the retention times at the midpoint of the chromatographic curves and the effect on the breakthrough volumes produced by increasing displacer

concentrations was used to calculate the dissociation constant ($K_i$) of the displacer as previously described (Moaddel, Wainer 2009). The concentration of the marker ligand, [$^3$H]-etoposide, was 1nM, and the concentrations of the displacer ligands etoposide and benzbromarone were 10 μM, 25 μM, 50 μM, 100 μM and 250 μM, the concentrations of MK571 were 1 μM, 5 μM, 10 μM, 25 μM and 5 0μM, furosemide was injected in the concentrations of 1 μM, 2.5 μM, 5 μM, 15 μM and 30 μM and fumitremorgin C was passed through the column in the concentration of 500 nM, 1 μM, 2.5 μM, 5 μM and 10 μM.

## 7.4 Data analysis

Binding affinities, expressed as $K_i$ values, were calculated using non-linear regression with a rectangular hyperbolic curve as previously described (Moaddel, Wainer 2009), using Prism 4 software (Graph Pad Software Inc., San Diego, CA, USA) running on a personal computer.

## 7.5 Results and Discussion

### *7.5.1. CMAC($Sf9_{MRP1}$)*

The membranes were immobilized on the IAM stationary phase to create the CMAC($Sf9_{MRP1}$) and CMAC(Sf9) columns. The binding activity of the immobilized MRP1 protein was established using [$^3$H]-etoposide as the marker ligand. This compound, also known as VP-16, has been identified as a substrate for the human MRP1 (Bobrowska-Hägerstrand et al. 2007), (Moaddel, Wainer 2009) and has been previously used in competitive inhibition studies (Gaj et al. 1998). When 1 nM [$^3$H]-etoposide was placed in the running buffer, the resulting frontal chromatographic trace obtained on the CMAC($Sf9_{MRP1}$) column contained an initial flat region, followed by a vertical breakthrough and a plateau region, Figure 33, Curve A. These results indicate that the marker bound in a specific and saturable manner on the CMAC column (Moaddel, Wainer 2009). The addition of 10 μM etoposide produced a significant reduction in the retention volume of the marker indicating that the competitive displacement experiments could be conducted on the CMAC($Sf9_{MRP1}$) column, Figure 33, Curve B. When the same experiments were conducted on the CMAC(Sf9) column, a frontal chromatographic trace was also observed, Figure. 34, Curve A, indicating that [$^3$H]-etoposide also bound specifically and non-specifically to proteins and other components of the membranes obtained from the non-transfected Sf9 cells.

However, the addition to the mobile phase of up to 100 µM etoposide produced no significant reduction in the retention volume of the marker, Figure 34, Curve B, indicating that etoposide could be used as a marker for specific competitive binding experiments on the CMAC($Sf9_{MRP1}$) column. This was consistent with previous studies which have demonstrated that specific binding to a target protein can be studied in the presence of additional specific and non-specific interactions with the cellular membrane by the use of control columns (Moaddel, Wainer 2009).

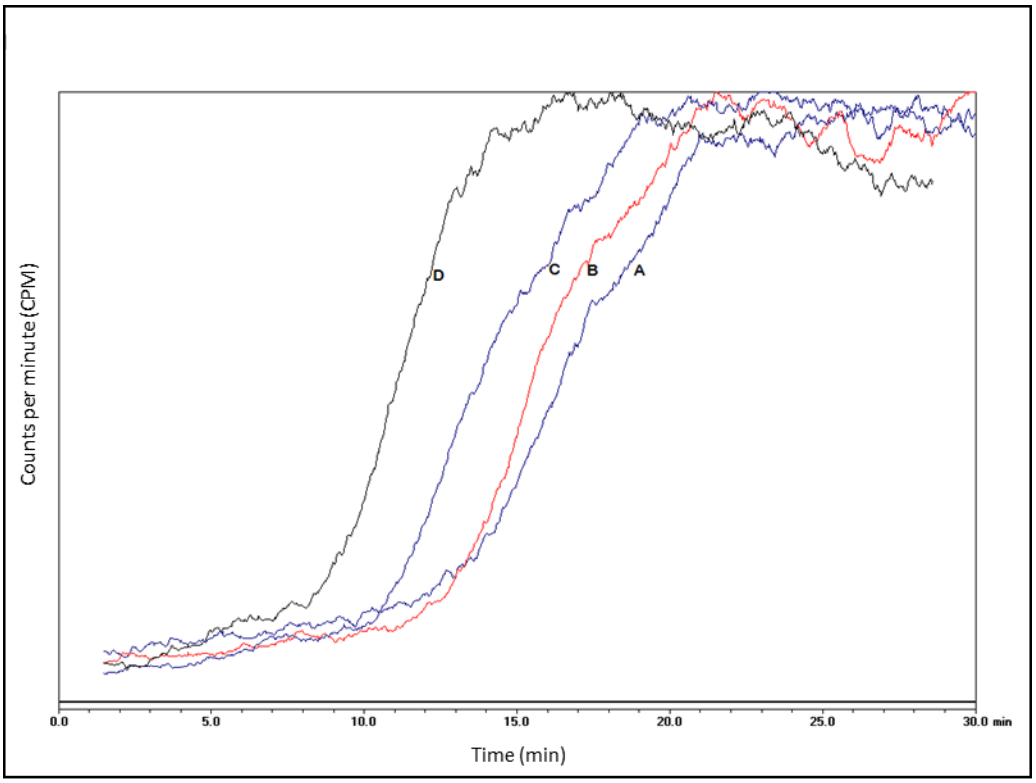


***Figure 33. Chromatographic traces produced by [$^3$H]-etoposide on the CMAC($Sf9_{MRP1}$) column, where A = [$^3$H]-etoposide alone, B = with 10 µM etoposide, C = 10 µM (R)-verapamil and D = 10 µM (S)-verapamil.***

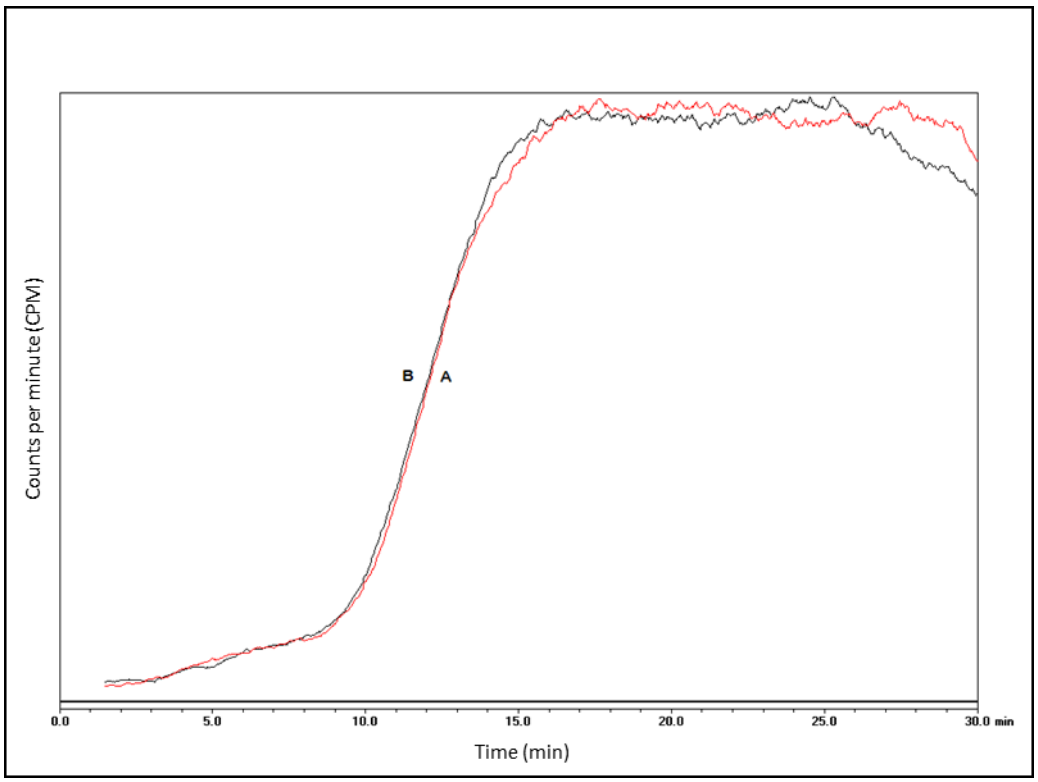


***Figure 34. Chromatographic traces produced by (A).[$^3$H]-etoposide 1 nM and (B).1nM [$^3$H]-etoposide with 10 µM etoposide on the CMAC(Sf9) column.***

In order to confirm that specific binding to the MRP1 protein binding could be studied on the CMAC($Sf9_{MRP1}$) column, the effect of the enantiomers of verapamil; (*R*)-verapamil and (*S*)-verapamil, on the retention of [$^3$H]-etoposide was determined. Since (*R*)-verapamil and (*S*)-verapamil have equivalent

physicochemical properties, any difference in their effect on the retention of [$^3$H]-etoposide will be due to specific interactions between the immobilized membranes and the individual isomers.

Verapamil was also chosen on the basis of the report by Gaj, *et al*. (Gaj et al. 1998) which demonstrated that (*S*)-verapamil was a more efficient MRP1 inhibitor than (*R*)-verapamil in human nasopharyngeal KB cell lines over-expressing MRP1. In the study by Gaj, *et al*., 10 μM concentrations of each of the verapamil enantiomers were used to determine the changes in the $IC_{50}$ values associated with the anti-proliferative activities of etoposide, vincristine and doxorubicin. In each instance, the presence of (*S*)-verapamil produced a greater decrease in the $IC_{50}$ value of the anticancer agent than (*R*)-verapamil. When etoposide was the anti-proliferative agent, the observed $IC_{50}$ was decreased from 28 μM to 5 μM by (*S*)-verapamil and to 15 μM by (*R*)-verapamil, a 3-fold difference in enantioselectivity.

In this study, the addition of 10 μM (*S*)-verapamil to the mobile phase reduced the retention time of [$^3$H]-etoposide by 5.4 min, while the equivalent concentration of (*R*)-verapamil reduced the retention by 2.6 min, Figure. 33, Curves C and D, respectively, Table 2. The results indicated that the affinity of (*S*)-verapamil is ~2-fold greater than that of (*R*)-verapamil, which is consistent with the data reported by Gaj, *et al* (Gaj et al. 1998). Both verapamil enantiomers produced a

larger displacement than 10 µM etoposide, which reduced the retention of the marker by 0.9 min, Figure 33A, Curve B, Table 2. This is also consistent with the previously reported difference in $IC_{50}$ values of 48 µM for etoposide (Wong et al. 2009) and 5 to 13 µM for racemic verapamil (Wong et al. 2009) (Dekkers et al. 1998). When the same experiments were conducted on the CMAC(Sf9) column, no specific displacements of [$^3$H]-etoposide were produced by the compounds indicating that the competitive displacements observed on the CMAC($Sf9_{MRP1}$) column were due to interactions at the expressed MRP1 protein.

| **Displacer** | **Retention time (Td) (min)** | **ΔT = T(o) – T(d) (min)** | **$IC_{50}$ ($\mu$M)** |
|---|---|---|---|
| **etoposide** | 15.4 | 0.9 | NC |
| **(R)-verapamil** | 13.8 | 2.6 | 8.6 |
| **(S)-verapamil** | 11.0 | 5.4 | 4.1 |

***Table 2. The displacement of 1 nM [$^3$H]-etoposide by the addition of 10 µM etoposide, (R)-verapamil and (S)-verapamil to the running buffer represented as ΔT calculated as ΔT = T(o) – T(d), where T(o) is (15.4 min) the retention time of [$^3$H]-etoposide with no displacer in the running buffer and T(d) is the retention time of [$^3$H]-etoposide after the addition of the displacer. NC = not calculated.***

In a previous study utilizing a CMAC(α3β4) it was demonstrated that relative agonist activities ($EC_{50}$ values) could be ascertained by a single competitive displacement experiment (Moaddel, Bullock & Wainer 2004). The experimental approach involved the determination of the change in the retention volume (Δml) of a characterized marker ligand produced by the addition of a test compound to the mobile phase, where Δml = retention volume of the marker alone - retention volume of the marker in the presence of the test compound. When known agonists with established $EC_{50}$ values (standards) were included in the experimental set, the relative $EC_{50}$ values of the test compounds could be determined from the relationship between Δml (test) and Δml (standard).

The relationship between the Δml and $EC_{50}$ values is based upon the approach developed by Cheng and Prusoff (Cheng, Prusoff 1973) in which the functional inhibition ($IC_{50}$ values) of enzymes by competitive inhibitors was equated to the binding affinities ($K_i$ values) of these inhibitors. The analysis can be used to determine relative $IC_{50}$ values between different inhibitors if the inhibitors have identical mechanisms of action and the assays are performed under the same conditions. The data obtained in this study was analysed in the same manner in order to calculate the relative $IC_{50}$ values for (*S*)-verapamil and (*R*)-verapamil, although the change in retention time (ΔT) was used in place of Δml. The calculated $IC_{50}$ values were 4.1 μM and 8.6 μM, respectively. The magnitudes of the $IC_{50}$ values was consistent with the previously reported $IC_{50}$ value for racemic verapamil of 5-7 μM (Dekkers et al. 1998) and 13.4 μM (Wong et al. 2009) and

the observation that the inhibitory effect of (*S*)-verapamil was ~ 2-fold stronger than (*R*)-verapamil was also consistent with the enantioselecitivity reported by Gaj, *et al* (Gaj et al. 1998). It was of interest to note that the experiments conducted by Gaj, *et al*. involved the inhibition of etoposide transport by 10 μM concentrations of the verapamil enantiomers.

The application of the CMAC($Sf9_{MRP1}$) column in the determination of binding affinities, $K_i$ values, to the immobilized MRP1 protein was confirmed using competitive binding experiments in which increasing concentrations of displacer ligands were added to the mobile phase and the effects on the retention of the marker ligand used to calculate the affinity of the displacer to the immobilized protein (Moaddel, Wainer 2009). Increasing concentrations of the MRP1 ligands etoposide, benzbromarone and MK571 (Figure 35), produced significant reductions in the retention of [$^3$H]-etoposide.

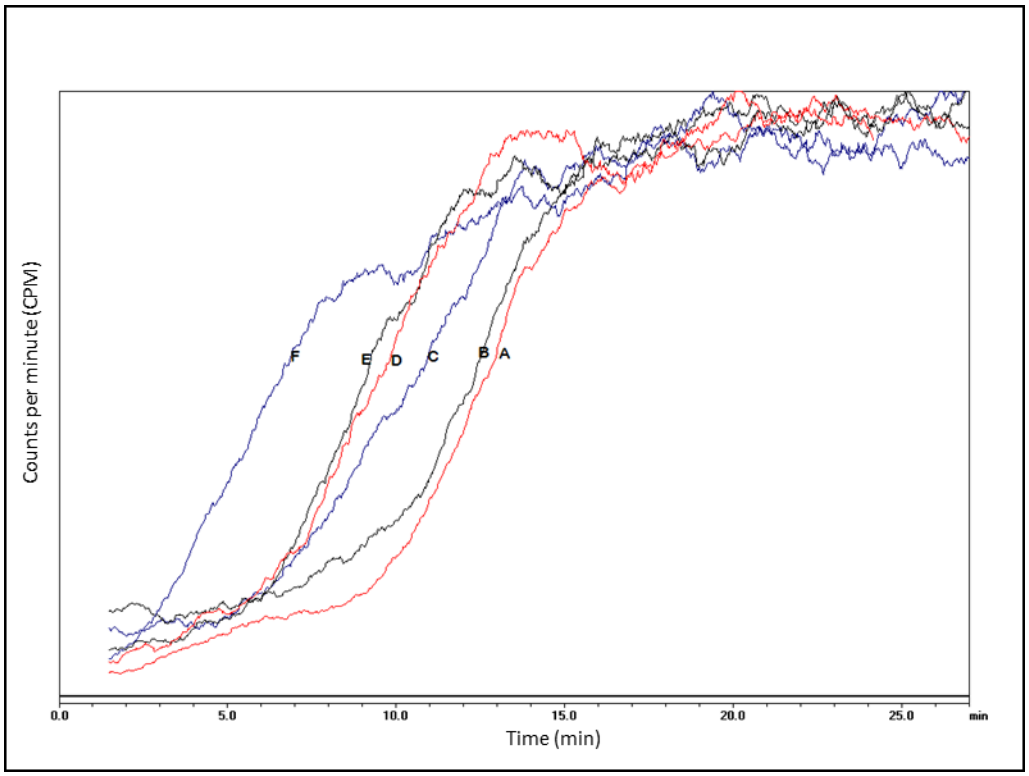


***Figure 35. Chromatographic traces produced by the displacement of [$^3$H]-etoposide on the CMAC (Sf9$_{MRP1}$) column by the addition of MK571, where curve A = [$^3$H]-etoposide alone, B = after addition of 1 µM MK571, C = 5µM, D =10µM, E = 25 µM, F = 50 µM.***

The $K_i$ values were calculated and were consistent with previously reported values calculated using non-chromatographic methods, Table 3. The results indicated that the CMAC approach could be used to determine binding affinities to the MRP1 protein.

| | $K_i$ ($\mu$M) | $IC_{50}$ ($\mu$M) |
|---|---|---|
| **CMAC($Sf9_{MRP1}$)** | | |
| **Etoposide** | 23.8 ± 6.6 | 48 (Wong et al. 2009) |
| **Benzbromarone** | 21.0 ± 2,2 | 4 (Bobrowska-Hagerstrand et al. 2003) |
| **MK571** | 4.8 ± 1.9 | 1.2 (Wu et al. 2005) |
| **CMAC($Sf9_{MRP2}$)** | | |
| **Etoposide** | 17.9 ± 9.2 | 48 (Wong et al. 2009) |
| **Furosemide** | 18.0 ± 5.1 | 24 (Wong et al. 2009) |
| **CMAC($Sf9_{BCRP}$)** | | |
| **Etoposide** | 2.9 ± 1.5 | 8 (Allen et al. 2003) |
| **Fumitremorgin-C** | 1.7 ± 1.2 | 3 (Dahan, Amidon 2009) |

***Table 3. The binding affinities ($K_i$ values) of selected MRP1, MRP2 and BCRP ligands calculated from competitive displacement binding studies performed using the CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$) columns. See text for experimental details.***

### *7.5.2 CMAC ($Sf9_{MRP2}$)*

The membranes were immobilized on the IAM stationary phase to create the CMAC($Sf9_{MRP2}$) column, and when 1 nM [$^3$H]-etoposide was placed in the running buffer the expected frontal chromatographic trace was observed. Competitive displacement studies were conducted using etoposide and furosemide, a specific MRP2 competitive inhibitor (Wong et al. 2009), (Figure 36), and the calculated $K_i$ values were consistent with previously reported values calculated using non-chromatographic methods, Table 3. The results indicated that the CMAC approach could be used to determine binding affinities to the MRP2 protein.

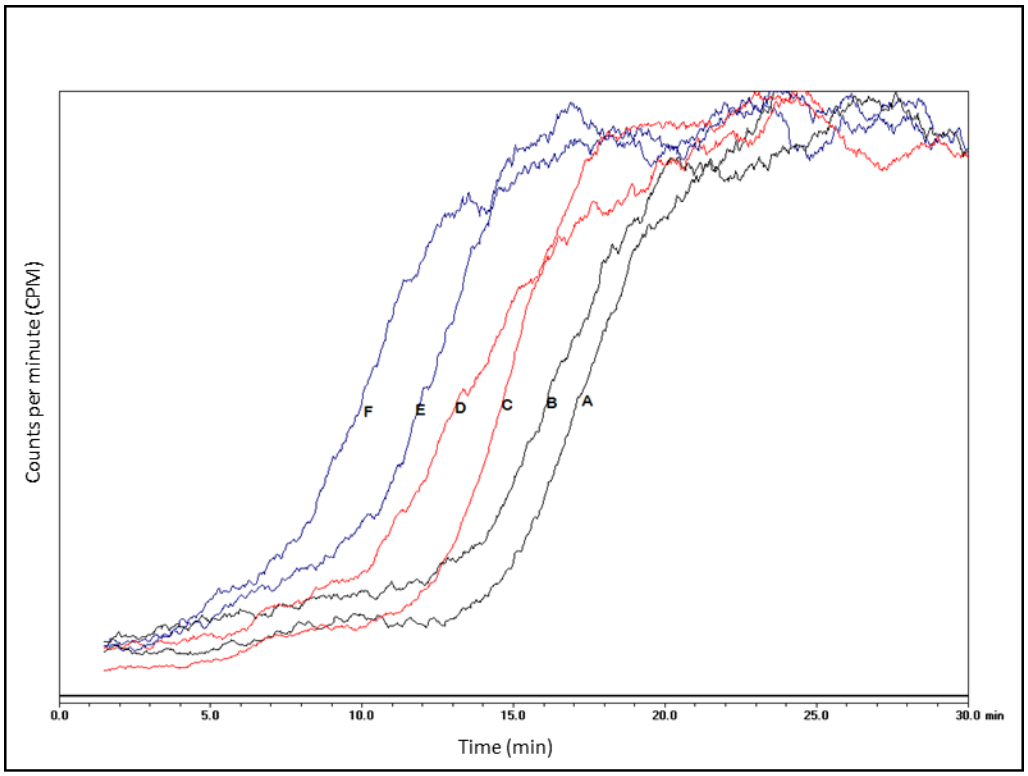


***Figure 36. Chromatographic traces produced by the displacement of [$^3$H]-etoposide on the CMAC(Sf9$_{MRP2}$) column by the addition of etoposide, where curve A = [$^3$H]-etoposide alone, B = after addition of 5 µM etoposide, C =10µM, D = 25 µM, E = 50 µM, F = 75 µM.***

### *7.5.3 CMAC ($Sf9_{BCRP}$)*

The membranes were immobilized on the IAM stationary phase to create the CMAC($Sf9_{BCRP}$) column, and when 1 nM [$^3$H]-etoposide was placed in the running buffer the expected frontal chromatographic trace was observed. Competitive displacement studies were conducted using etoposide and fumitremorgin C, a specific BCRP competitive inhibitor (Rabindran et al. 2000) (Figure 37), and the calculated $K_i$ values were consistent with previously reported values calculated using non-chromatographic methods, Table 3. The results indicated that the CMAC approach could be used to determine binding affinities to the BCRP protein.

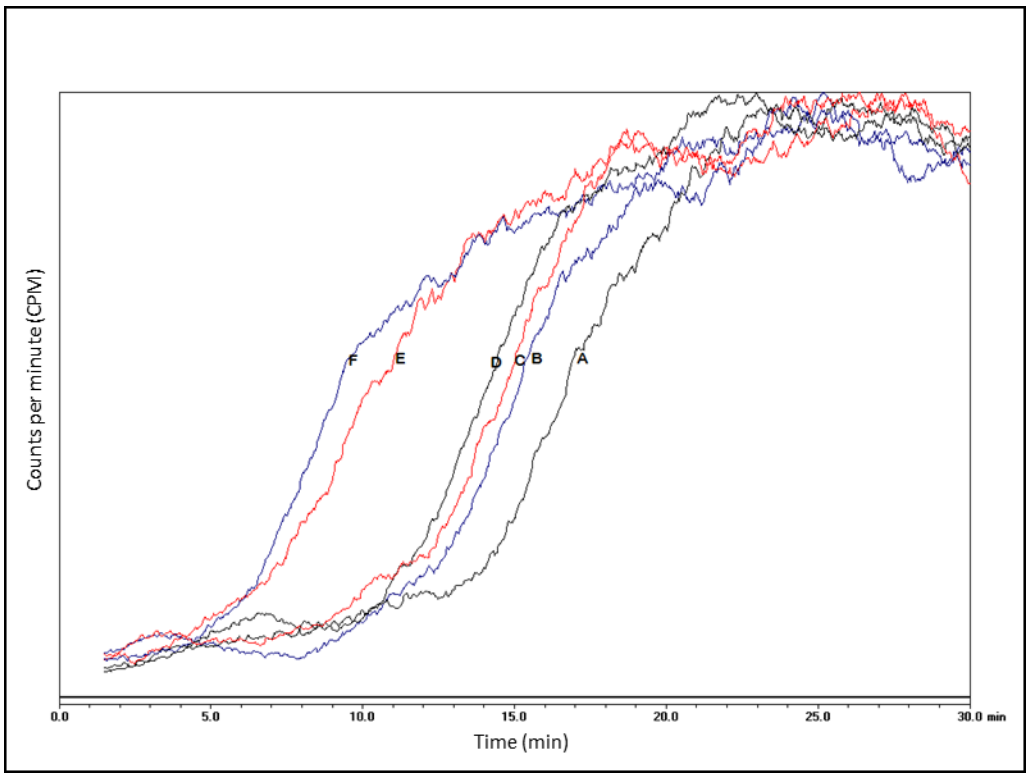


***Figure 37. Chromatographic traces produced by the displacement of [$^3$H]-etoposide on the CMAC(Sf9$_{BCRP}$) column by the addition of fumitremorgin-C, where curve A = [$^3$H]-etoposide alone, B = after addition of 500 nM fumitremorgin-C , C = 1 µM, D = 2.5 µM, E = 5 µM and F = 10 µM.***

The data from this study indicated that membranes from stably transfected Sf9 cell lines expressing the MRP1, MRP2 or BCRP transporters hae been successfully immobilized on the IAM stationary phase to create a series of cellular membrane affinity chromatography columns; CMAC(Sf9),

CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$). The results demonstrated that the resultant columns could be used to study the interactions of small molecules with the expressed transporters and to determine $K_i$ values, relative $IC_{50}$ values and enantioselectivities. The successful development and characterization of the Sf9 columns represented the first CMAC columns produced using membranes obtained from an insect cell line. Since the Sf9 and related insect cell lines are popular and highly productive expression systems, these results represent a potential expansion of the CMAC approach.

The CMAC(Sf9) columns produced in this study were stable for up to one month and could be used for ~100 samples during this period. As has been previously discussed, there was no attempt to purify the immobilized membranes and the amount of immobilized protein on the column, and thereby the column to column reproducibility, was determined using the number of active binding sites, $B_{max}$ (Moaddel, Wainer 2009). The calculated $B_{max}$ values had less than 10% variation between columns for the two CMAC ($Sf9_{MRP1}$) columns, indicating that the columns can be reproduced.

Currently, there are several approaches that are used to screen substrates / inhibitors for interactions with Pgp, MRP1, MRP2 and BCRP. MRP1 transport, for example, has been studied using isolated membrane vesicles (Renes et al. 1999), (Renes et al. 2000) inside-out vesicles (Wu et al. 2005), substrate toxicity

(Dahan, Amidon 2009) and intracellular accumulation of transported substrate (Wong et al. 2009). Similar studies with membrane vesicles (Smeets et al. 2004) and inside-out vesicles (Zimmermann et al. 2008) were also carried out for the MRP2 and BCRP transporters (Lagas et al. 2008). Thus most of the standard methods employed to screen compounds involve culture of native or transfected cells and isolation of membranes.

The data from this study indicated that the CMAC columns produced and characterized in this study represent a potential alternative approach to the screening of compounds for their binding to and activity at MRP1, MRP2 and BCRP. However, while the average time needed for the frontal experiment was 25 min, the average washout period was 2 h, which limited the potential use of these columns in large screening programs. It had previously demonstrated that CMAC-based screening is hampered by the long washout periods required with the IAM stationary phase and that this problem can be overcome by moving to an open tubular chromatographic format (Moaddel, Bullock & Wainer 2004). This is illustrated by the studies with the CMAC(Pgp) columns in which moving to an open tubular format reduced the total time required for the frontal experiment and washout to 30 min and resulted in a throughput that was equivalent to the rate obtained with the Caco-2 screen (Moaddel et al. 2006). The demonstration that the Sf9 membranes used in this study can be immobilized on the IAM support to create functional CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$) and CMAC($Sf9_{BCRP}$)

columns suggested that these membranes can also be immobilized in the open tubular format.

## 7.6 Conclusion

The data from this study indicated that membranes from insect cell lines like the Sf9, expressing a target protein can be immobilized on an IAM (immobilized artificial membrane) stationary phase. The resultant columns could be used to study the interactions of target proteins with specific ligands using displacement frontal chromatography. Binding affinities so calculated have been shown to correlate well with reported literature values calculated using cell based assay systems. Moreover the CMAC($Sf9_{MRP1}$), CMAC($Sf9_{MRP2}$), CMAC($Sf9_{BCRP}$) and the CMAC(Sf9) columns reported in this study can be constructed in 48 hours as the cellular membranes are commercially available with the target protein expressed. The columns are fairly easy to assemble, reproducible and efficient. The CMAC columns reported here are an initial step in the development of screens for lead candidates as these efflux proteins play a crucial role in oral drug bioavailability and drug-drug interactions. The functional over-expression of MRP1, MRP2 and BCRP in neoplastic tumours also makes these screens essential in the development of anticancer agents.

# Chapter 8

# 8. Analysis of ABC transporters in clinical specimens

## 8.1. Introduction

Having confirmed the presence, localization and functionality of Pgp, MRP1, MRP2 and BCRP in the GBM and astrocytoma cell lines; the final step of the research programme was to investigate the clinical implications of the above-mentioned findings. Literature suggests strong evidence of expression profiles of ABC transporters in various tissues (Warren et al. 2009). Considerable work has been done as regards as investigating the clinical significance of the presence of ABC transporters (Dauchy et al. 2009) but definitive evidence of nuclear expression profile is hardly found.

## 8.2. Aims and objectives

In order to investigate the expression of ABC transporters in clinical specimens, healthy brain and GBM autopsy specimens were analysed for Pgp, MRP1, and BCRP by western blot analyses. Immunohistochemistry was used to gain a better understanding of the localization of the BCRP in both healthy brain and GBM autopsy and biopsy specimens.

## 8.3. Materials and Methods

### *8.3.1 Preparation of whole tissue lysates*

100 mg of tissue was lysed on ice for 20 min in Tris-HCl [10 mM, pH 7.4] containing 0.15 M NaCl, 5mM EDTA, 1% v/v Triton X-100, 1:500 dilution of a stock solution of protease inhibitor cocktail, 0.1M sodium ortho-vanadate and 0.1M DTT (Sigma Aldrich, St-Louis, MO, USA). Insoluble material was removed by centrifugation (17,000×g for 20 min at 4$^{o}$C) and the supernatant was collected and stored at -80$^{o}$C until further analysis.

### *8.3.2 Western Blot analysis*

Western blotting is an analytical technique used to detect a specific protein in an extract or a lysate. The protein of interest is separated by gel electrophoresis by the length of the polypeptide. Once separated, the protein is then transferred to a nitrocellulose or PVDF (poly-vinyl difluoride) membrane, where they are probed by antibodies specific to the target antigen. In the study described in this chapter, the protein concentration of the lysates was measured with the BCA protein assay kit (Thermo Scientific, Waltham, MA, USA) using BSA (bovine serum albumin)

as standard. An equal amount of 3X Laemlli sample buffer (Laemmli 1970) was added, and the samples (70 μg) were separated by electrophoresis using precast 4-12% gradient SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride membranes using iBLOT (Invitrogen). The membranes were blocked with 5% v/v dry non-fat milk in Tris-buffered saline with 0.1% v/v Tween-20 and then probed with mouse monoclonal anti-Pgp (clone C219, 1:500 dilution; Alexis Biochemicals, Plymouth Meeting, PA, USA), rat monoclonal anti-MRP1 (clone MRPr1, 1:500 dilution, Alexis Biochemicals), mouse monoclonal anti-BCRP (clone BXP-21, 1:500 dilution; Alexis Biochemicals) and mouse monoclonal anti-β-actin (clone AC-15, 1:5000 dilution; Abcam, Cambridge, MA, USA). This was followed by appropriate horseradish peroxidase-conjugated secondary antibodies; ECL Anti-mouse IgG (1:5000 dilution; GE Healthcare, Piscataway, NJ, USA) in the case of Pgp, BCRP and β-Actin. MRP1 blots were incubated with ECL Anti-rat IgG (1:5000 dilution; GE Healthcare). The ECL Western blotting detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for visualization.

### *8.3.3 Immunohistochemistry*

Immuno-histochemical staining was carried out using an established avidin-biotin complex indirect immune-peroxidase method as described (Bain, McLachlan & LeBlanc 1997), (van Tellingen et al. 2003). Routine biopsy specimens were

retrospectively obtained from the Department of Pathology at Cooper University Hospital Institutional Review Board. The specimens were cryostat sectioned at 7 μm onto poly-L-lysine coated slides, air-dried and stored at -70$^{o}$C. Immuno-histochemical stains were performed on a fully automated Benchmark Ultra IHC stainer (Ventana Medical Systems, Tuscon, AZ, USA) to visualize BCRP using the mouse monoclonal anti-BCRP (clone BXP-21, 1:100 dilution)

## 8.4 Results and Discussion

The aim of this study was to confirm the expression profile of Pgp, MRP1 and BCRP in human healthy brain and GBM autopsy tissue specimens. Owing to the large variation in the size of the target proteins in this study, a 4-12 % gradient poly-acrylamide gel was chosen for the electrophoretic separation. The anti-Pgp C219 clone has been shown to detect a 170 kDa band (Weber, Eckert & Müller 2006), (Ambroziak et al. 2010) which corresponds to Pgp. The anti-MRP1, MRPr1 clone detects a band of 190 kDa (Calatozzolo et al. 2005), (Rajagopal, Simon 2003). The BXP-21 clone was used to detect the BCRP band at 72 kDa as shown by Maliepaard *et al*. (Maliepaard et al. 2001b). Significant and uniform levels of Pgp and MRP1 were observed in the healthy brain specimens as indicated in Figure 38 A. While BCRP was expressed in all six specimens, variation was observed in half the population. Equal loading of protein was ensured by normalizing to β-actin. The reverse trend was observed in the

immunoblots of the GBM tissue specimens in Figure 38 B. Expression of Pgp and MRP1 was inconsistent among the six samples, whereas BCRP was presence in a uniform manner.

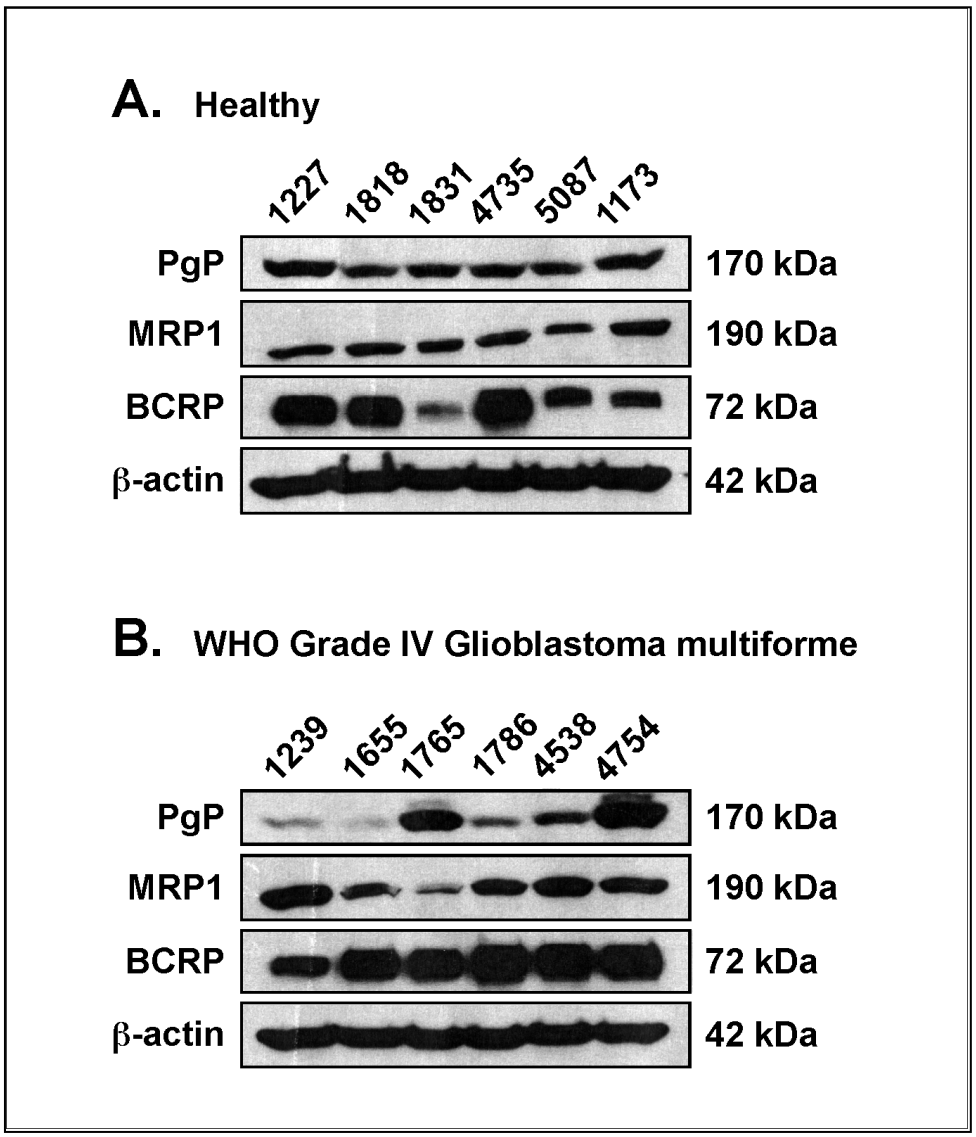


***Figure 38. Western Blot analysis of ABC transporters in healthy and WHO Grade IV Glioblastoma multiforme autopsy specimens. (A) Expression of Pgp, MRP1, MRP2 and BCRP in healthy brain autopsy specimens, (B) Expression of Pgp, MRP1, MRP2 and BCRP in WHO Grade IV Glioblastoma multiforme brain autopsy specimens.***

The next stage of this study was to investigate the expression pattern of BCRP in autopsy and biopsy brain specimens by immunohistochemistry. Other groups have investigated the expression of BCRP in various tissues and indicated subcellular localization of BCRP. The study of Diestra *et al*. (Diestra et al. 2002) clearly showed that BCRP is expressed in 129 out of 150 tumour samples including 5 out of 5 GBM tissue specimens. More recently, BCRP has been shown to be localised only in microvessel endothelium of human control brain (Aronica et al. 2005). The results from this immuno-histochemical study indicated that BCRP was indeed expressed in microvessel endothelium and in the extranuclear compartment of glial cells in a human control brain tissue biopsy (Figure 39A). When tissue from a human breast tumour biopsy specimen was examined, a marked staining of BCRP was observed in the cytosolic/membraneous compartment, but no nuclear staining was found (Figure 39B). Immuno-histochemical staining of a tissue biopsy sample from a human GBM tumour indicated the presence of BCRP in the extra-nuclear compartment and, most importantly, nuclear BCRP expression was observed in a subpopulation of tumour cells (Figure 39C).

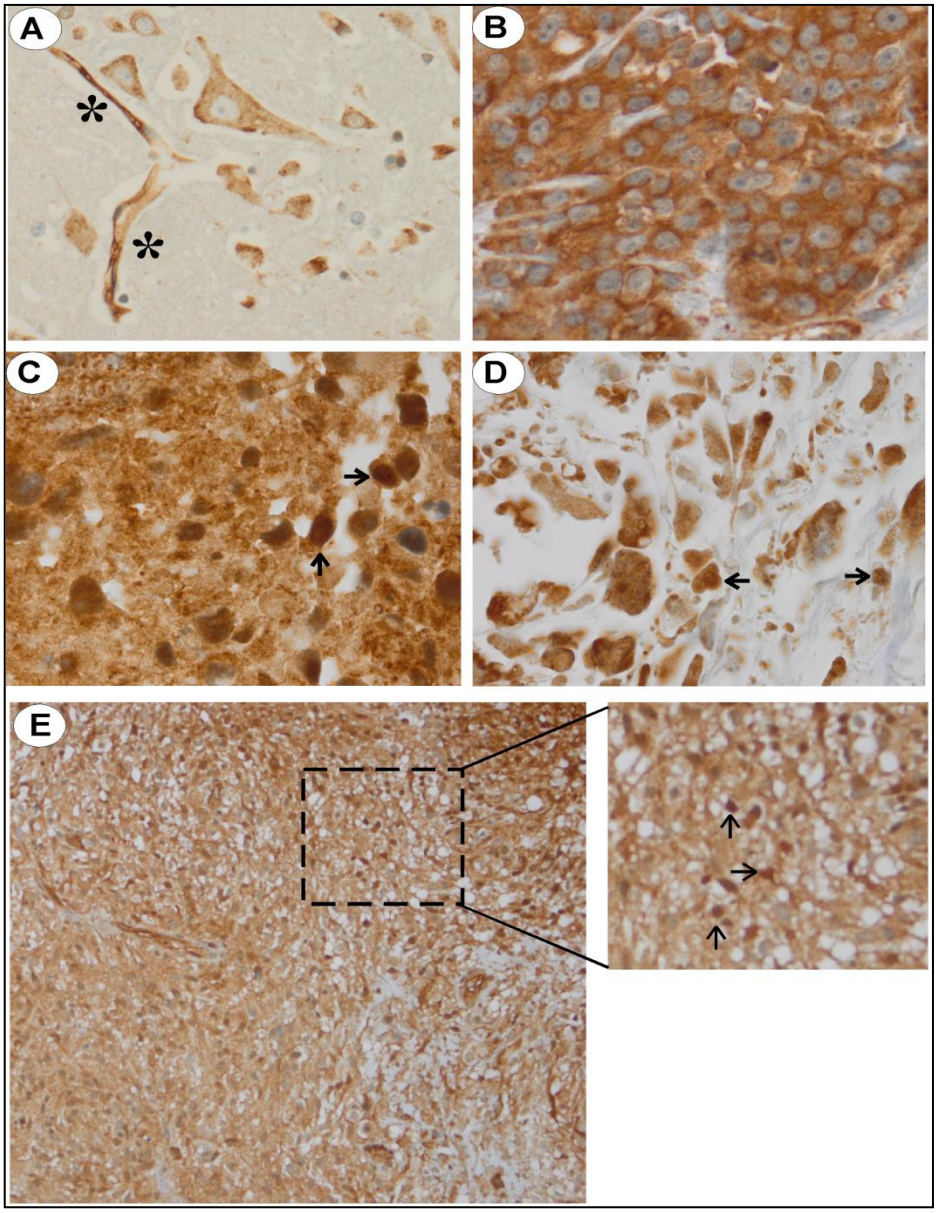


***Figure 39. Expression and localisation of BCRP in human brain tumour biopsy and autopsy specimens. Immunohistochemistry analysis of BCRP in (A) Healthy specimen number 1227 with staining of brain endothelial cells (denoted as *) and significant cytoplasmic expression without nuclear expression of BCRP in brain glial cells; (B) breast cancer specimen showing significant cytoplasmic expression and no nuclear expression of BCRP; (C) Biopsy GBM specimen and (D) autopsy GBM specimen number 1786 showing significant cytoplasmic and nuclear expression of BCRP. (E) Biopsy GBM specimen at low (200X) and high (400X) magnification (inset) demonstrating nuclear BCRP staining in a subpopulation of cells. Arrows denote nuclear staining of BCRP.***

## 8.5. Conclusion

Presence of Pgp, MRP1 and BCRP in healthy brain tissue and observed up-regulation of BCRP in the GBM tissue specimens corroborates with the findings from the cellular studies. Significant presence of BCRP in the GBM autopsy specimens as confirmed by western blotting might indicate a key role in drug resistance. Nuclear expression of BCRP in GBM cells, which was reported in Chapters 4 and 5, was confirmed in select tissue specimens. GBM autopsy specimens indicated a strong presence of BCRP both in cytoplasm and nucleus. These findings indicate the importance of BCRP as a target at the nuclear level for drug resistant GBM.

# Chapter 9

# 9. Conclusion and suggestion for future work

Effective chemotherapy of GBM is limited due to a multitude of factors. Many anticancer agents fail to cross the blood brain barrier (BBB) as they are not lipophilic and those that do penetrate the BBB are often effluxed by ABC transporters such as Pgp, MRP1, MRP2 and BCRP. One clinical strategy to overcome transporter-mediated MDR is to co-administer ABC transporter inhibitors with the objective of increasing intracellular concentrations of the chemotherapeutic agents and achieving required therapeutic exposure. This approach is predicated on the assumption that ABC transporter proteins are located primarily on cellular membranes and that the primary goal is to increase the cytosolic concentrations of the cytotoxic agents. However, the data from this research programme suggest that overcoming a BCRP-related MDR phenotype may be a more complex task. In this study, it was demonstrated the presence of BCRP in the nuclear extracts of a number of human derived glioblastoma and astrocytoma cell lines. This finding is consistent with previous reports showing that BCRP can be found in intracellular compartments, such as the mitochondria (Takada et al. 2005) and the perinuclear region (Lemos et al. 2009). In this research programme it was reported for the first time that BCRP being localises specifically to the nucleus of GBM cells. In-silico analysis of human BCRP protein sequence (Swiss-Prot entry: Q9UNQ0) indicated the presence of a cluster of four lysine residues at position 357–360. This motif (357KKKK360), present in all three BCRP variants, has been identified as a putative nuclear localisation

sequence (NLS), which tags a protein for nuclear import through interaction with specific receptors at nuclear pores (Nardozzi, Lott & Cingolani 2010). Interestingly, BCRP is the target of phosphorylation at Thr362 by Pim-1 kinase (Xie et al. 2008), which could have a profound impact on its cellular redistribution. Moreover, the protein Ser/Thr kinase, Akt, regulates the cell surface expression of BCRP by promoting its phosphorylation (Takada et al. 2005). Whether nuclear BCRP import is dependent of the phosphorylation on Thr362 is unclear. Alternatively, phosphorylation of a yet unknown putative site may upregulate importin-dependent BCRP nuclear import. Such phosphorylation may elicit conformational change within BCRP to enable better or reduced access to the nuclear import machinery. It is tempting to speculate that nuclear import of BCRP could be mediated, at least in part, through cell type- or region-specific activation of a discrete pool of kinase(s) acting on BCRP. The data from the immuno-histochemical studies of human tissue biopsies indicate that the nuclear expression of BCRP is a clinically relevant issue. The results also shed some light on the mechanisms associated with the development of the ABC transporter-associated MDR phenotype. In a recent review, Moitra and co-workers (Moitra, Lou & Dean 2011) present four potential models of this phenomenon: (i) conventional model in which a population of the tumour cells have the MDR phenotype before initiation of chemotherapy; (ii) cancer stem cell model in which a small population of tumour stem cells express the MDR phenotype before chemotherapy; (iii) acquired-resistance stem cell model in which chemotherapy induces genetic changes that produce the MDR phenotype; (iv) intrinsic resistance

model in which the tumour has intrinsic resistance to chemotherapy. The identification of BCRP nuclear expression in a subpopulation of tumour cells in a human GBM biopsy specimen supports the first two models. It is believed that the nuclear expression of BCRP has functional consequences with regard to the removal of cytotoxic drugs from the nucleus. Variants of BCRP have been shown to respond differentially to its substrates (Kondo et al. 2004), and, therefore, discrete variations in BCRP transcript sequences may contribute to cell type specific differences in the magnitude of BCRP's export function (Ni et al. 2010). Moreover, the presence of Pgp on the nuclear membrane indicates that removal of MTX from the nucleus is presumably mediated by nuclear BCRP (this study), Pgp (Baldini et al. 1995) or other proteins involved in the nuclear cytoplasmic trafficking and compartmentalisation of drugs. In conclusion, the expression of BCRP in the nuclei of glioblastoma and astrocytoma cell lines represents a potentially new mechanism of MDR. Insertion of BCRP within the nuclear envelope may alter the conformation and/or function of the protein as well as the efficacy of chemotherapeutic drugs relative to the transporter located at the plasma membrane. Interaction with the various components of cellular membranes has been suggested as the source of functional differences in β2 adrenergic receptors expressed in different cell lines (Audet, Bouvier 2008). The presence of BCRP and other ABC transporters on the nuclear membrane poses new therapeutic challenges in the treatment of MDR tumours and indicate that the development of specific inhibitors of nuclear ABC transporters may be required. Indeed the substrate and inhibitor overlap among the ABC transporters poses

additional problem. There are hardly if any definitive screening techniques which can aid in defining selective ABC transporter inhibitors. The immobilization of Sf9 insect cells expressing Pgp, MRP1, MRP2 and BCRP proteins was a step in this direction. These stationary phases were shown to successfully identify receptor specific substrates and inhibitors and open new avenues for screening of larger libraries of test compounds.

# Chapter 10

## 10. References

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# Chapter 11

# 11. APPENDICES

## 11.1 Papers

Bhatia, P., Kolinski, M., Moaddel, R., Jozwiak, K. & Wainer, I. 2008, "Determination and modelling of stereoselective interactions of ligands with drug transporters: A key dimension in the understanding of drug disposition", *Xenobiotica,* vol. 38, no. 7-8, pp. 656-675.

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## 11.2 Book Chapter

Bhatia, P. & Moaddel, R. 2012, *Stereoselective transport of drugs*. In: Jozwiak, K., Lough, W.J. Lough & Wainer, I.W. 2012, *Drug Stereochemistry: Analytical Methods and Pharmacology*,

## 11.3 Oral presentations

Bhatia, P., Moaddel, R., & Wainer, I.W. 2009, *Immobilized Membrane based affinity chromatography for studying ABC transporter-drug interactions*.
13th International Meeting on Recent Developments in Pharmaceutical Analysis 2009, 9-12 September, Milan, Italy.

Bhatia, P., Moaddel, R., & Wainer, I.W. 2009, *Identification and characterization of drug transporters in human gliomas*.
20th International Symposium on Pharmaceutical and Biomedical Analysis 2009, 1-4 March, Agra, India.

## 11.4 Poster presentations

Bhatia, P., Bernier, M., & Wainer, I. W. 2013. *Nuclear localization of the breast cancer resistant protein (BCRP/ABCG2) presents a novel target for chemotherapy in drug resistant glioblastoma multiforme*.
Neuroscience 2010, 13-17 November, San Diego, CA.

Moaddel, R., Bhatia, P., Frazier, C., Ravichandran, S., Dossou, S., & Wainer, I.W. 2012. *Enantioselective Retention on Cellular Membrane Affinity*

*Chromatography Columns: Using Chirality to Probe Small Molecule-protein Interactions*.

ITP 2012, 30 September-12 October, Baltimore, USA.

Bhatia, P., Moaddel, R., & Wainer, I.W. 2009. *Identification and characterization of ABC transporters in human gliomas*.

14th National Institute on Aging, Annual Scientific Retreat 2009, 25-27 March, Linthicum, MD, USA.

## 11.5 Conferences

101st Annual AACR meeting, 2010, 17-21 April, Washington DC, USA.

Organizing secretary for the 20th International Symposium on Pharmaceutical and Biomedical Analysis 2009, 1-4 March, Agra, India.