ABC Transporters and the creation of a tumour-conducive microenvironment in neuroblastoma

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Abstract

ABC transporters have long been implicated in the promotion of cancer survival due to their role in cytotoxic drug efflux and conferring MDR. The broad substrate specificity associated with ABC transporters, particularly in the ABCC subfamily, is however implicating ABC transporters in the promotion of carcinogenesis independent of drug efflux. Eicosanoids including PGE2 and cysteinyl leukotrienes are among the substrates transported by ABCC transporters and are main players in the body's natural inflammatory response and more importantly, tumour-promoting inflammation.

Neuroblastoma cell lines overexpressing the MycN oncogene are documented to heavily expand their ABC expression profile, with upregulations seen in both ABCC1 and ABCC4. This project aims to assess how the expression of certain ABCs like ABCC1 and 4 affects extracellular concentrations of eicosanoids by inhibiting their transport, at the same time assessing how ABC expression affects the migratory potential of cells and potentially implicating PGE2 and cysteinyl leukotrienes in the more aggressive phenotypes demonstrated by NB cell lines.

cDNA converted from the RNA of 5 different NB cell lines (3 MycN amplified, 2 non-MycN amplified) was used to run qPCR to determine their ABC transporter expression profile and explore the effects of MycN amplification on the expression of ABC transporters involved in eicosanoid efflux. Wound-healing assays were used to determine the migratory potential of different NB cell lines under pharmacological inhibition of transporters ABCB1, C1 and C4.

The effects of ABC inhibition on eicosanoid efflux using competitive ELISA was also examined to explore potential associations with ABC transporter expression and eicosanoid efflux.

KELLY and SK-N-BE(2)c cells showed the ABC expression patterns previously documented to be associated with MycN amplification, showing increases in ABCC1 and 4 and a downregulation of ABCC3. IMR32 cells did not follow this trend but had the most aggressive migratory phenotype under basal conditions. ABC inhibition had no effect on the migration of NB cells, regardless of MycN amplification.

The expression patterns of the ABC transporters considered in this project did not appear to have a major effect on the efflux of eicosanoids or cAMP, contradicting the original hypothesis that ABC-mediated eicosanoid efflux is a major player in enhancing the migration and invasiveness of NB cell lines, accounting for the more aggressive phenotype in MycN amplified cell lines. This does not conclude however that ABC transporter expression is not associated with NB cell behaviour as other carcinogenic hallmarks remain to be explored in this field. The redundancy in substrate specificity of some ABC transporters, namely ABCC1 and ABCC4 with their shared specificity to PGE2, needs to be

addressed in future studies with the use of multiple inhibitors to more accurately restrict efflux of eicosanoids and better demonstrate their impact on migration. When focusing on key inflammatory eicosanoids such as PGE2 and cysteinyl leukotrienes, ABCCC1 and C4 are the most important to target as they are the main mediators of eicosanoid efflux and are currently not well understood.

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Section 1: Introduction

1.1 : Cancer

A common misconception of cancer is that it is a singular disease. It is, in fact, a term that describes the behaviour and phenotype of a group of diseases that can stem from different locations and manipulate the expression of crucial genes, with vast differences in mortality rates depending on these factors. The term cancer is defined by a group of cells that have acquired a series of mutations that provide them with immortality and unlimited replicatory potential (Sarkar et al., 2013). This leads to uncontrolled cell division and eventually the formation of a tumour which, if left untreated, metastasises and spreads to other parts of the body via blood and lymphatic systems which is usually what causes mortality of patients (Huysentruyt & Seyfried, 2010). Unlimited replicative potential is one of six key hallmarks of cancer documented by Douglas Hanahan and Robert A. Weinberg that are crucial to their development. Others include evading growth suppressors, resisting cell death, sustaining proliferative signaling, inducing angiogenesis and invasion/metastasis (Hanahan & Weinberg, 2011).

Cancer is a global epidemic and is the leading cause of death worldwide, even regardless of economic stature (Bray et al., 2018). In 2012, an estimated 14.1 million new cancer cases were reported worldwide, with 8.2 million of these causing death (Mehrotra & Kaushik, 2018). The cause for increasing cancer incidence can largely be attributed to manmade factors such as smoking, air pollution, obesity and poor diet, and improper sleep (Chen et al., 2018). Treatment of cancer normally involves a series of chemotherapy, radiotherapy, and surgical removal if possible. The problem in finding a “one size fits all” cure for cancer is the multifaceted nature of the disease, across all groups. The evolutionary process that results in the development of a cancerous colony results in cells that are highly adaptable to adverse changes in their environment and thus become hard to eradicate (Wu, Wang, Ling & Lu, 2016). Despite this, cancer research is constantly becoming more sophisticated in terms of technology and approach. A combination of being able to detect cancer earlier and finding more effective treatments has contributed to improving survival rates of patients. Not only this, as cancer has been better understood overtime, it has become apparent that all cancers are diverse and behave differently. The understanding of researchers that different cancers need approaching in different ways has been crucial to developing more effective treatments, which has led to an increase in 1-year and 5-year net survival rate of all cancer types in developed countries (Arnold et al., 2019).

1.2 : Neuroblastoma: Epigenetics and Molecular Biology

Neuroblastoma is the most common extracranial tumour found in young children, particularly in those less than 1 year of age; accounting for 28% of all cancers in young infants (Heck, Ritz, Hung, Hashibe & Boffetta, 2009). The tumour consists of neuroectodermal cells that stem from primary neural crests contributing to the development of the autonomic nervous system (ANS), specifically the sympathetic nervous system and most commonly, the adrenal medulla (Bown, 2001).

An incidence of 10.2 cases per million children was reported in 2010, with 500 new cases being reported annually (Colon & Chung, 2011). The prevalence of neuroblastoma has resulted in it being the second highest cause of death in children, second only to accidents (Castel, Grau, Noguera & Martínez, 2007). The clinical diversity in the behaviour of neuroblastoma cases contributes to this. Tumours can originate from a range of locations throughout the body and thus cause different initial symptoms (Figure 1.1).

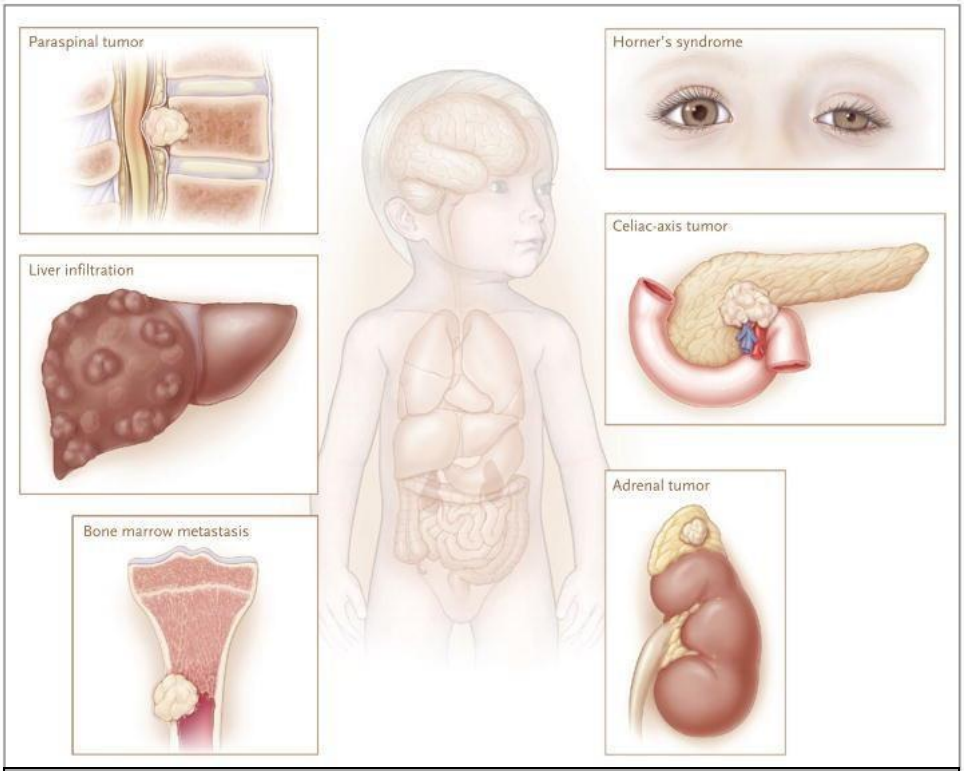


Figure 1.1: Clinical implications and features of neuroblastoma. Neuroblastoma can originate from any site of the sympathetic nervous system, with the majority stemming from the adrenal medulla. Tumours can also form and progress on the spinal cord causing neuronal dysfunction and ultimately paralysis. Horner's syndrome can occur when tumours originate from the neck and disrupt sympathetic nerves controlling eye movement and control. Low-risk cases found in early development can normally be surgically removed from the patient and have little to no long term effects. In cases of more aggressive and metastatic neuroblastoma, the tumours commonly spread to the lymph nodes and bone marrow via the hematopoietic system and invade local structures and organs leading to poor prognosis (Maris, 2010).

Evidence suggests that neuroblastoma is a largely sporadic disease, with only 2% of cases being genetically related. In the majority of such cases, heritable mutations in ALK (anaplastic lymphoma kinase) and/or PHOX2B (paired-like homeobox 2B) genes are a hallmark (Pugh et al., 2013). Sporadic cases of NB however are incredibly different, both genetically and phenotypically. Sporadic tumours are known to vary widely in gene expression patterns, accounting for the differences in NB susceptibility and patient prognosis seen between cases. The prognosis of a diagnosed patient is often

determined by the age at which NB is detected; moreover, the age of the patient at diagnosis can often predict the severity of the tumour. In cases where the patient was over 1 year of age, 75% presented at stage 4 (disseminated metastases) and were both chemoresistant and aggressive (Weinstein, 2003). These tumours are generally incurable and largely contribute to the high mortality rate associated with NB (Bown, 2001). Patients younger than 1 year of age, however, generally stand a much better chance of survival as tumours are commonly found at lower stages and are less aggressive, resulting in much higher cure rates (Bown, 2001). The stark contrast in patient prognosis and phenotypic behaviour between low and high-risk neuroblastoma make it a very difficult disease to accurately review with a general perspective. For example, there has been a steady increase in survival rates in patients with neuroblastoma, seeing an increase of 22% in 5-year survival rates (74%) (Maris, 2010). However, survival rates in the low-risk neuroblastoma group are ~92% (Maris, 2010), showing that generalised statistics cannot give a clear representation of the epidemiological outlook of neuroblastoma. Rather, these studies tend to separate their findings between high-risk and low-risk groups (Meany, 2019; Coughlan, Gianferante, Lynch, Stevens & Harlan, 2017).

A rather harrowing feature of neuroblastoma is the likelihood of relapse and the poor prognosis that accompanies this. Around 50-60% of high-risk cases relapse and the median time taken for this to occur is 13.2 months according to a study by the International Neuroblastoma Risk Group, which also found that risk of death is significantly higher in patients who relapse within 6-18 months (London et al., 2010). Even with the implementation of more aggressive therapies, survival at this stage is still only around 20% at 5 years (Colon & Chung, 2011).

1.2.1 : MycN amplification vs Non-MycN amplification

MycN is a proto-oncogene associated mainly with neuroblastomas and is sometimes dramatically amplified in some of these tumours, which has long been known to be associated with a much worse prognosis in comparison to those in which MYCN is not amplified (Tonini et al., 1997). Amplification of MycN correlates heavily to later-stage NB (3 and 4), with one study finding that 50% of 48 late-stage NB cases showed amplification, while 0 of 15 early-stage cases did (Brodeur, Seeger, Schwab, Varmus & Bishop, 1984). The reason for this is clear, as MycN amplification has been associated with major differences in the level of expression of a wide range of proteins that ultimately effect tumorigenicity. One example of this is the expression of proteins belonging to the ABC (ATP-Binding Cassette) superfamily (Porro et al., 2010). A 1997 study using rat models showed that subjects with aberrant MycN expression in the peripheral neural crest via the tyrosine hydroxylase promoter went on to develop neuroblastoma (Weiss, 1997).

The study also showed that targeted overexpression of MycN alone can indeed initiate neuroblastoma, but cannot directly cause neuroblast transformation and requires further mutations; as tumours with inhibited retinoblastoma and neurofibromin (vital tumour suppressors) expression showed reduced latency and increased penetrance (Huang & Weiss, 2013).

MycN is the most important prognostic marker in high-risk neuroblastoma and has a large influence on how severe an individual case of NB is deemed to be. In 1986, a staging system was designed to help stratify cases of neuroblastoma and ultimately decide which course of treatment is necessary for the best chances of survival (Brodeur et al., 1988). This system is called the International Neuroblastoma Staging System (INSS) and is split into 6 stages, 1, 2A, 2B, 3, 4 and 4S. Stage 1 represents the lowest risk cases, while stage 4 is the most aggressive and lethal. Stage 4S is pathologically poorly understood and different to tumours at other stages of the INSS. Stage 4S NB is defined by infants with small primary tumours that carry metastases in the skin, liver or bone marrow (Schleiermacher et al., 2003). These tumours represent approximately 7-10% of all NB cases and surprisingly have a high rate of spontaneous regression and good prognosis. A 2000 study by Nickerson et al even showed a 5-year overall survival rate of 92% in a study of infants with stage 4S NB (Nickerson et al., 2000; Schleiermacher et al., 2003).

Cases presenting MycN amplification are much more likely to be stratified into stages 3 or 4 due to its role in promoting tumour aggression and survival. It is primarily these later stage cases that present a much larger problem when trying to treat NB cases, due to their persistently invasive phenotype and adaptive capabilities.

1.2.2 : Current treatment and therapy for NB cases

The approach taken to treat NB cases depends largely on the stage and progression of the tumour. Treatments can range from observation only (seen in lower-risk cases) to intensive, multifaceted therapy.

Although surgical removal of early-stage tumours carries high success rates, many cases of low-risk neuroblastoma have shown spontaneous regression, particularly in prenatally diagnosed adrenal masses (Holgersen, Subramanian, Kirpekar, Mootabar & Marcus, 1996) and even in larger tumours (Kushner et al., 1996). A 2012 study by Nuchtern et al found that 81% of cases in a population of 84 patients with small localized adrenal lesions showed spontaneous regression, did not require treatment, and showed an overall 3-year disease-free survival rate of 97.7% (Nuchtern et al., 2012), (Louis & Shohet, 2015).

The main goal moving forward in NB therapies is to devise new treatments that are more specific, less toxic and free of long-term side effects. In North America, the process of NB therapy is split into 3 stages, induction, consolidation and maintenance therapy. The induction stage involves chemotherapy, stem cell collection and surgical resection. Consolidation therapy involves the use of a higher dose of chemotherapy and radiation therapy. Finally, maintenance therapy involves the use of immunotherapy and cis-Retinoic acid (Smith & Foster, 2018). Immunotherapy against NB is a relatively new treatment and uses monoclonal antibodies to target disialoganglioside GD2, a cell-surface lipid expressed on a variety of cancers including neuroblastoma, where its expression is ubiquitous and found on all primary tumours regardless of the stage (Salt & Modak, 2017). 13-cis-retinoic acid is often used after chemotherapy and is an effective differentiation agent, helping to prevent recurrence (Hämmerle et al., 2013).

Chemotherapy treatments in high-risk NB cases involve platinum, alkylating and topoisomerase agents such as vincristine, doxorubicin, cyclophosphamide and cisplatin (Smith & Foster, 2018). While potentially life-saving, the use of these agents can cause further long-term damage to patients. Cisplatin, for example, is a first-line anticancer treatment but can be severely nephrotoxic, with several mechanisms of renal damage being identified (Barton et al., 2017). Using radiation therapy in such young patients also causes long-term damage due to the interference with normal hormone production. This can lead to a wide range of issues including delayed puberty onset and abnormal ovulation in females (Mohan et al., 2019).

As with many cancers, treatment with traditional chemotherapeutic drugs is often ineffective in highly evolved tumours as they develop “multidrug resistance”, a phenomenon that has been found to have a huge influence on the prognosis of cancer patients. Neuroblastoma is no exception and in many cases is often very well adapted to dealing with anticancer drugs, mainly in those which are MycN amplified. Much of their multidrug resistance is attributable to the upregulation of ABCC1 seen in these cell types. The upregulation of ABCC1 greatly enhances the efflux of many frontline agents used in NB therapy. These ABCC1 substrates include etoposide, doxorubicin, vincristine and irinotecan (Fletcher, Williams, Henderson, Norris & Haber, 2016). The enhanced resistance of NB cells to these agents contributes to the poor prognosis associated with MycN amplified NB cases.

1.3 : ABC Transporters and their implication in tumour promotion

ABC transporters are ubiquitously expressed proteins across all documented species. Some species express greater numbers of ABCs than others (28 in *Saccharomyces*, 69 in *Escherichia*.Coli and 48 in humans) (Linton, 2007). This highlights their importance in cell physiology, particularly in signalling, communication and detoxification. Indeed, the primary role for the majority of ABCs transporters is the active movement of substrates across the cell membrane. In humans, mutations in genes expressing ABCs result in different diseases affecting a wide range of organs, some with devastating effects. For example, harlequin ichthyosis, a disease causing major deformities to the skin, is a result of either frameshift or nonsense substitution mutations in the gene encoding ABCA12[3]. While a mutation in ABCA1 is a hallmark of Tangiers disease, which results in a dramatic reduction in high-density lipoprotein (HDL) and can cause a wide range of symptoms ranging from cardiovascular disease to atherosclerosis. ABC transporters have a minimum of four core domains, including two transmembrane domains which form the binding site for substrates, and two nucleotide-binding domains (NBD) responsible for binding and hydrolysing adenine triphosphate (ATP), producing energy for substrate translocation across the membrane. The nucleotide-binding domains of ABC transporters are homologous throughout the superfamily and share characteristic motifs (Linton, 2007). Unlike NBDs, TMDs are highly heterogenic between different ABCs, accounting for their wide range of substrate specificity. The majority of ABC transporters contain TMDs with 6 transmembrane α-helices, but this can vary between 6-10 depending on the transporter (Wilkens, 2015). The structure and function of a typical ABC transporter is depicted in Figure 1.2.

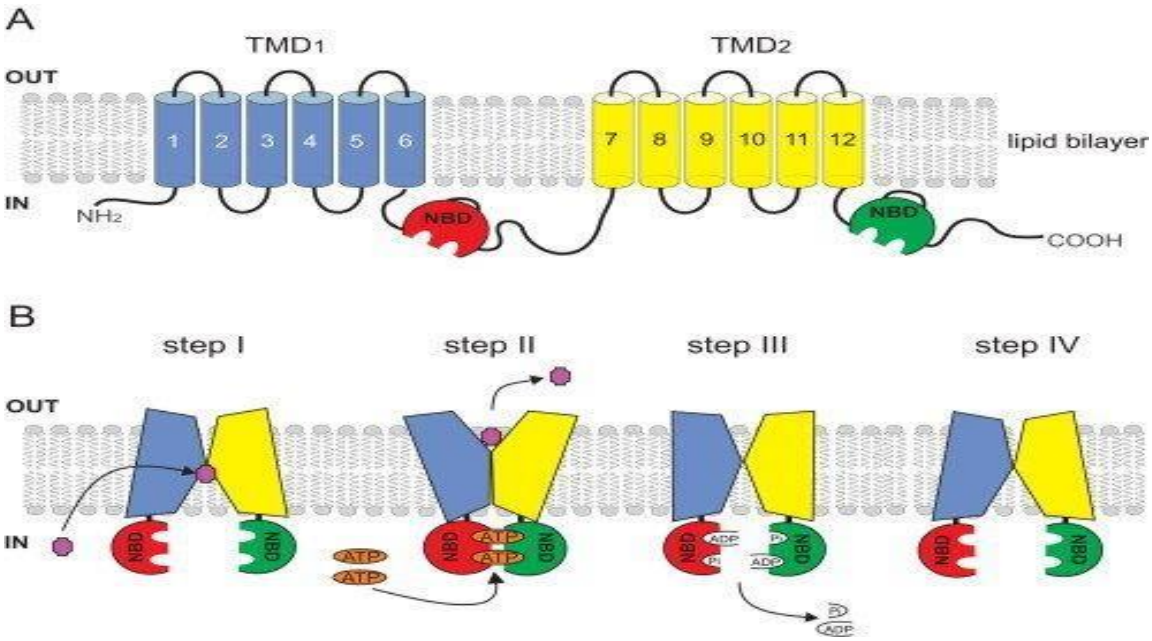


Figure 1.2: A) The typical structure of an ABC transporter, containing 2 TM domains (blue and yellow), each with 6 transmembrane segments and 2 NBDs (green and red). Different ABCs can contain a different number of TMDs. B) The process of substrate transport across a membrane by an ABC transporter, otherwise named the "ATP-switch mechanism" (Higgins & Linton, 2004). Transport is initiated when a substrate (purple) binds to a high affinity pocket created by the 2 TMDs (step I) causing conformational changes to the structure of the NBDs allowing binding of ATP (orange), causing the formation of a closed NBD dimer (step II), resulting in the hydrolysis of the bound ATP (step III). The subsequent release of the ATP metabolites ADP and phosphate allows the open-NBD dimer to form again (Dermauw & Van Leeuwen, 2014).

1.3.1 : ABC Transporters and multidrug resistance (MDR)

The vast majority of research implicating the role of ABC transporters in cancer progression focusses on their ability to indiscriminately export a wide range of chemotherapeutic drugs with no structural or function similarities (Rees, Johnson & Lewinson, 2009). The manipulation of ABC expression by cancer cells is a major contributing factor towards their ability to resist cell death, one of the 6 hallmarks of cancer as documented by Hanahan and Weinberg in 2011. Three ABC transporters account for most drug efflux in malignancies; P-Glycoprotein/ABCB1, multidrug resistance protein 1 (MRP1)/ABCC1 and breast cancer resistance protein (BCRP)/ABCG2. Targeting these transporters in tandem with standard chemotherapeutic treatment has been suggested as a potential method of treatment to increase the effectiveness of treatment against malignancies by increasing the penetration of cytotoxic drugs (Sun, Patel, Kumar & Chen, 2012). However, developing a clinically effective inhibitor of ABC transporters without causing off-target toxicity has proven problematic and no developed drugs have made it past clinical testing (Cui, Zhang, Chen & Liu, 2015). The original ABC-inhibitors on the market were drugs used for different purposes, drugs such as verapamil. Verapamil inhibits ABCB1 but is used to treat cardiovascular issues such as hypertension, angina and supraventricular tachycardia by blocking calcium ion channels. The result of using drugs such as verapamil as an ABC-inhibitor are off-target side effects as a result of their activity against its original targets. Drugs used in this project such as ceefourin and reversan are considered safe and non-toxic carrying few side effects, but currently are only used as scientific tools until they pass clinical testing.

1.3.1 : ABCB1/MDRP1/P-Glycoprotein

P-Glycoprotein is a 170kDa protein that is a major contributor to cytotoxic drug efflux in all cancers and was in fact the first ABC transporter to be discovered. It carries 12 transmembrane domains and 2 nucleotide binding domains, which largely contributes to the protein's ability to transport such a wide range of products (Aller et al., 2009).

This protein is expressed extensively in the intestinal epithelium, liver, kidneys and the brain and is vital for toxin clearance and filtration around the body.

1.3.2 : ABCC1/MRP1

The C subfamily of ABC transporters, also known as the multidrug resistance protein (MRP) subfamily, are one of the most heavily implicated sub-families of ABC transporters in cancer. MRP1 in particular is one of the most heavily implicated and well-researched ABC transporters in cancer research with ABCB1. It is a 190kDa protein containing 2 TMDs and NBDs encoded by the ABCC1 gene on

chromosome 16p13.1 (He, Li, R. Kanwar & Zhou, 2011). This ABC transporter is unique to others in the MRP subfamily as it contains a third membrane-spanning domain of around 200 amino acids with 5 predicted transmembrane segments (Rosenberg et al., 2001). ABCC1 transports a wide range of organic ions including many therapeutic agents with diverse physiological properties. Evidence has suggested that MRP1 may contribute to the development of drug resistance in various cancers such as prostate, breast and lung (Munoz, Henderson, Haber & Norris, 2007).

Key proinflammatory signaling lipids are one of the many substrates of ABCC1. A 1994 study using an inside-out membrane vesicle transport system found a major substrate of ABCC1 to be leukotriene C4 (LTC4) and its metabolites leukotriene D4 and E4 with high affinity (Leier et al., 1997). These inflammatory mediators bind to G-protein coupled receptors CysLTR1 and CysLTR2 resulting in a major inflammatory response in the host. Other arachidonic acid derivatives have also been found to be a substrate of ABCC1, including members of the prostaglandin family. Prostaglandin E2 is one of the prostaglandins found to be a substrate of ABCC1, but with a lower affinity (Cole, 2014).

ABCC1 is well documented to be upregulated in cancers where it contributes to MDR (e.g. prostate, breast and lung) (Munoz, Henderson, Haber & Norris, 2007). Non-small lung cell carcinoma is a good example of this. This form of lung cancer accounts for over 75% of lung cancers and ABCC1 is frequently seen to be amplified in many of these tumours after administration of chemotherapeutic drugs. The level of ABCC1 expression is also known to be a factor in the prognosis in neuroblastoma cases (Munoz, Henderson, Haber & Norris, 2007) and has been found to be one of many direct transcriptional targets of the MycN oncogene, resulting in gene overexpression in cell lines with amplified MycN.

1.3.3 : ABCC4/MRP4

ABCC4 is the shortest transporter of the ABCC subfamily and has a wide range of both endogenous and exogenous substrates. Similarly to ABCC1, ABCC4 is also implicated in NB prognosis, with its overexpression being strongly associated with decreased patient survival. ABCC4 is also a direct transcriptional target of MycN (Porro et al., 2010), and is commonly overexpressed in NB cell lines with MycN amplification (Yue et al., 2014). Despite also being associated with chemoresistance, ABCC4 has also been linked with conferring tumorigenesis independent of drug resistance. The wide range of substrates associated with the transporter contributes to this hypothesis. ABCC4 transports several eicosanoids with high affinity; namely PGE2, LTB4, LTC4 and thromboxane B2 (Yu, Huynh, Truong, Haber & Norris, 2015)

The expression profile of key ABC transporters involved in eicosanoid efflux is known to be influenced by MycN expression from Yu, Huynh, Truong, Haber & Norris, 2015). Table 1 summarises the trends in expression of these ABC transporters seen between MycN and non-MycN amplified NB cell lines.

ABC transporter	Prognostic significance on survival	Direct regulation by MycN	Function
ABCA1	Low	None	Sterol and lipid efflux
ABCB1	None	Positive	Cytotoxic drug and toxin efflux, MDR
ABCC1	High	Positive	MDR, leukotriene efflux
ABCC3	Low	Negative	Organic ion transport
ABCC4	High	Positive	MDR, PGE2 efflux
ABCG1	Low	Unknown	Lipid efflux

Table 1: Overview of the relationship between MycN amplification and expression pattern of numerous key ABC transporters and the effect their expression has on patient prognosis (adapted from Yu, Huynh, Truong, Haber & Norris, 2015).

1.4: Tumour-promoting inflammation, eicosanoid signalling and downstream effects on tumorigenicity

1.4.1 : Tumour microenvironment and inflammation

The term 'tumour microenvironment' refers to the supplementation and infrastructure surrounding a colony of tumour cells. The tumour microenvironment heavily influences how tumours behave in terms proliferation and migration; so much so that approaches to cancer research have been increasingly focusing on combatting tumours more passively, i.e. manipulating their microenvironment, rather than attempting to manipulate them directly (Pitt et al., 2016; Reisfeld, 2013). The benefit of this approach is the wide range of crucial components that make up the microenvironment that can be targeted (Figure 1.3). The composition of the microenvironment is complex and differs depending on the site of the tumour. All tumours however recruit non-cancerous

stromal cells such as immune cells (T-Cells, macrophages and neutrophils), cancer-associated fibroblasts (CAFs) and vascular endothelial cells for angiogenesis (Hirata & Sahai, 2017). CAFs are the most abundant of these cells within tumour colonies of most cancers and can initially inhibit cancer growth in their inactivated form (Alkasalias, Moyano-Galceran, Arsenian-Henriksson & Lehti, 2018). Eventually, however, fibroblasts become manipulated by tumour cells and develop into CAFs, which are now widely accepted to play a key role in promoting carcinogenesis via expression of pro-invasive chemokines and cytokines (Franco, Shaw, Strand & Hayward, 2010). Research suggests that this transformation is a result of the interaction of fibroblasts with transforming growth factor β (TGF-β) secreted by tumour cells (Guido et al., 2012). These manipulated fibroblasts express many distinguishing biomarkers which make them easily detectible such as α-smooth muscle actin, vimentin, desmin and fibroblast-activation protein (Yuan, Jiang, Sun & Chen, 2016).

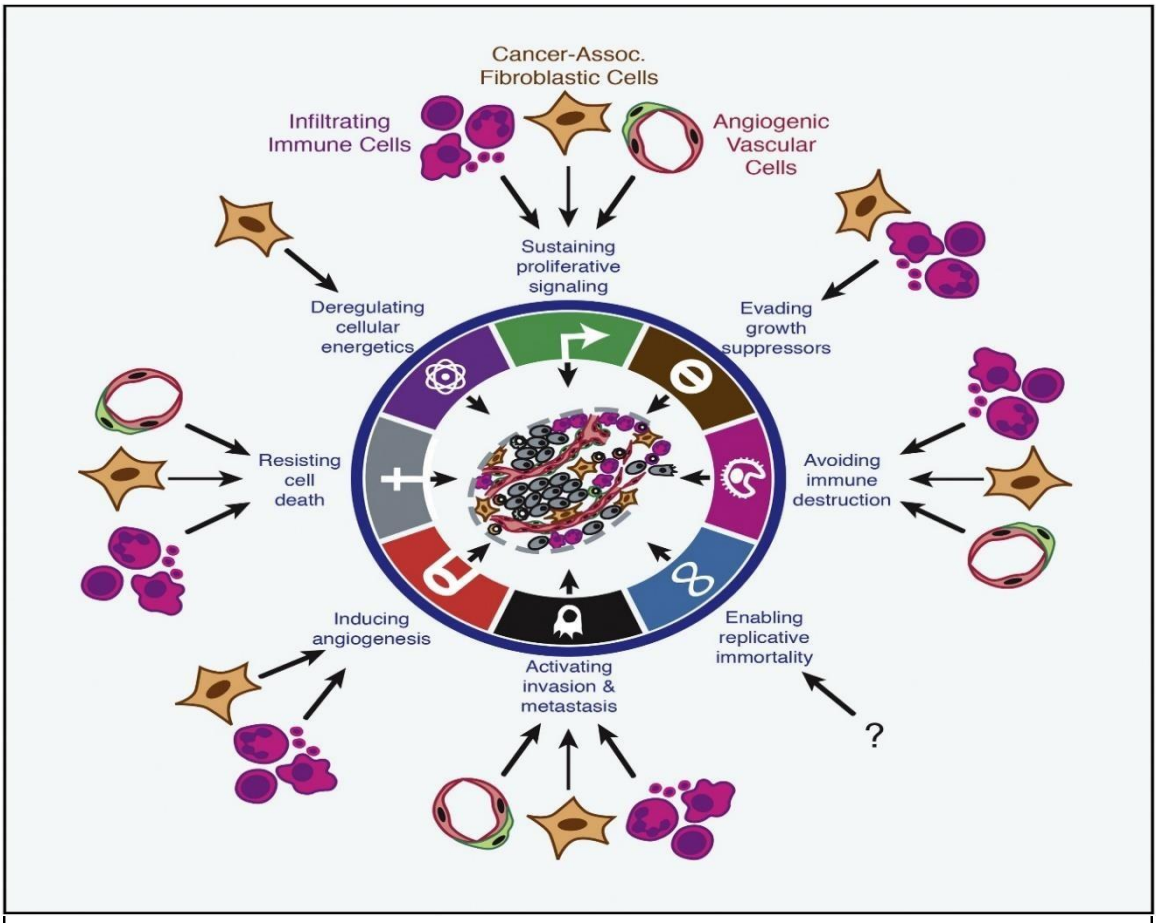


Figure 1.3: Stromal cells recruited to tumour sites can contribute to seven of the eight key hallmarks of all tumours. Although it is not yet determined whether stromal cells can promote replicative immortality of tumour cells, establishment and maintenance of the tumour microenvironment plays a significant role in tumour survival. The cell types depicted in figure 1 are a generalised representation of an otherwise complex arrangement of many different cells performing different roles. (Hanahan & Coussens, 2012)

1.4.2 : Cancer-Associated Fibroblasts (CAFs)

CAFs are becoming increasingly well known for their role in promoting carcinogenesis. They appear to have a major involvement in the synthesis, secretion and organisation of the extracellular matrix (ECM) which is responsible for providing tumour colonies with structural support to aid migration, proliferation, metastasis and immune suppression (Botti et al., 2013). The secretome of CAFs are complex and can suppress anti-tumour immunity in a number of ways, one of which is by recruiting immunosuppressive cells such as T-regulatory cells and TH2 cells (Ziani, Chouaib & Thiery, 2018). In addition to this, they are also documented to release stimulators of the cyclooxygenase-2 (COX-2) pathway such as IL-1β (Huang et al., 2019), which is one of the main players in eicosanoid production (Neeb et al., 2011).

1.4.3 : Inflammation caused by the efflux of eicosanoids

Inflammation is a natural process triggered by the reaction of a host's immune system to exogenous threats such as wounding or pathogens (Chen et al., 2017). Research has previously suggested that chronic inflammation is linked to cancer across multiple stages, such as tumour progression and even initiation (Perwez Hussain & Harris, 2007). Further studies have proceeded to strengthen this argument, for example, linking ulcerative colitis as a major risk factor in the development of colon cancer (Gupta *et al*., 2007). Inflammation has been implicated in cancer progression to such an extent that it is now considered as one of the hallmarks of cancer (Hanahan & Weinberg, 2011).

Eicosanoids are a major player in cancer-related inflammation (Greene, Huang, Serhan & Panigrahy, 2011). Prostaglandin E2 (PGE2) and cysteinyl leukotrienes (CysLTs) in particular contribute largely to the inflammatory stimulation that aids many cancers (Nakanishi & Rosenberg, 2012; Burke et al., 2016). These proinflammatory stimulators exert their effects via intercellular communication mediated by receptors on the cellular membrane. To achieve this however, the eicosanoids need to be transported out of the cell from which they were produced, and this is mediated by ABC transporters, implicating their role in promoting cancer-related inflammation (Fletcher, Haber, Henderson & Norris, 2010).

Arachidonic acid derivatives such as prostaglandins (particularly prostaglandin E2) and leukotrienes have largely been the main focal point of research into the association of inflammation and carcinogenesis. The biosynthesis of these inflammatory eicosanoids is driven by a family of myeloperoxidase enzymes called cyclooxygenase (COX). COX enzymes are known to exist in 3 isoforms, COX-1, 2 and 3. While the function of COX-3 remains largely unknown, COX-1 and 2 are

known to be the drivers of prostanoid production, catalysing their synthesis from unstable arachidonic acid metabolites (Rundhaug, Simper, Surh & Fischer, 2011). Despite sharing 60% homology, these catalytic proteins serve very different functions. COX-1 is consistently expressed in almost all tissues, playing a role in the homeostasis of renal blood flow and maintaining the integrity of the gastric mucosa, among other functions (Crofford, 1997). COX-2, however, is an inducible isoform that is heavily repressed under basal conditions (Crofford, 1997). Under normal circumstances, COX-2 is undetectable in most epithelial tissues with the exception of the brain and kidney (Rundhaug & Fischer, 2010). Only when exposed to inflammatory stimuli is COX-2 expressed. These findings have led to COX-2 being heavily implicated in a wide range of cancers, suggesting its upregulation and the resulting increase in prostanoid efflux, particularly PGE2, bares tumour-promoting tendencies.

1.4.4 : Prostaglandin E2

Eicosanoids consist of various families of signalling molecules derived from arachidonic acid and other polyunsaturated fatty acids. Derived from the Greek name “eicosa”, meaning twenty, eicosanoids represent all oxygenated 20-carbon essential fatty acids (Greene, Huang, Serhan & Panigrahy, 2011). Prostaglandins belong to the eicosanoid superfamily and play a large role in the inflammatory response. It is for this reason that prostanoids, particularly PGE2, have been implicated in many cancers. PGE2 among other prostanoids such as $PGF_2\alpha$, PGD_2, PGI_2 and thromboxane A_2 (TXA_2) is synthesised from membrane bound unstable fatty acids by the previously mentioned COX-1 and 2 enzymes from prostaglandin G2 (PGG2) (Nakanishi & Rosenberg, 2012). PGH2 has no significant role in mediating an inflammatory response and thus must be converted to more stable prostanoids via the action of specific prostaglandin synthases (Park, Pillinger & Abramson, 2006). To date, there are 3 terminal PGE2 synthases currently known, mPGES-1, 2 and cPGES (Figure 1.4).

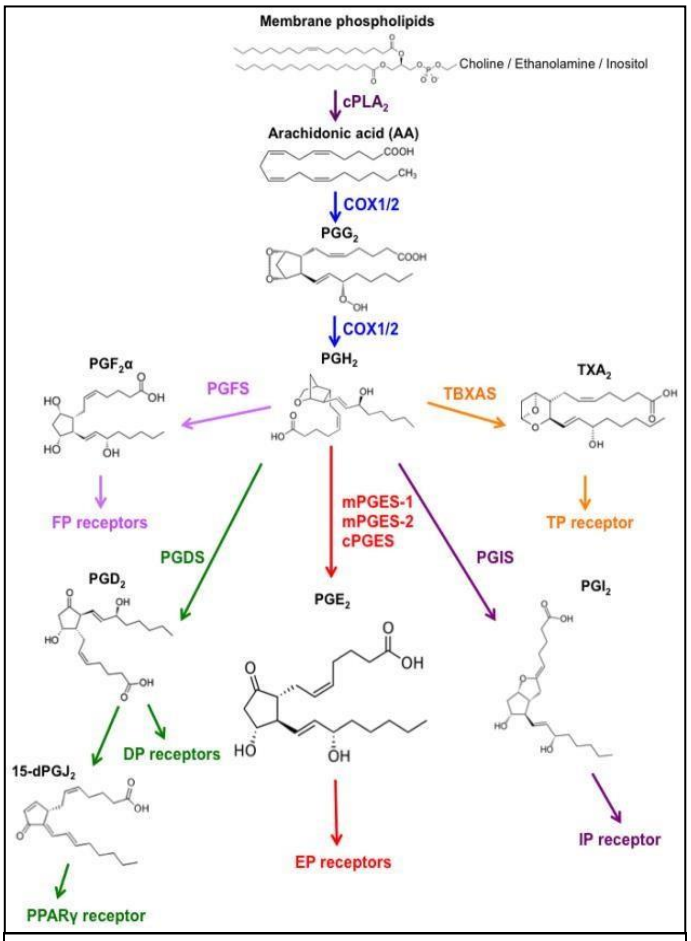


Figure 1.4: Schematic diagram of the prostaglandin synthesis pathway. Arachidonic acid can be synthesised from various membrane bound phospholipids such as choline, ethanolamine and inositol. Once PGE2 is synthesised from prostaglandin endoperoxide H2 (PGH2) via the action of specific synthases, it binds to 1 of 4 membrane bound Gα-coupled EP receptors, all causing different effects (Nakanishi & Rosenberg, 2012).

Under basal conditions, mPGES-1 is only constitutively expressed in certain tissues including the kidney, bladder and reproductive tissues and is virtually undetectable in others. However, upon activation by inflammatory stimuli, mPGES-1 can be upregulated by all tissues and can convert PGH2 synthesised by both COX-1 and 2, unlike the other PGE synthases.

Following synthesis, PGE2 must be rapidly exported by either passive diffusion or transport by ABCC4 or it is degraded by 15-dehydroxyprostaglandin dehydrogenase (15-PGDH) into 15-keto-prostaglandin E2 (Greenhough et al., 2009). Following export from the cell in which it is synthesised, PGE2 mediates an inflammatory response via binding to one of four types of EP receptors found on the membrane of surrounding cells. All these receptors are coupled to a Gα protein, and following activation, cause an amplified downstream effect on the target cell in which they are found. The 4 EP receptors are coupled to different Gα-subunits, which determines the response that is elicited upon binding of PGE2 to the receptor (Figure 1.5).

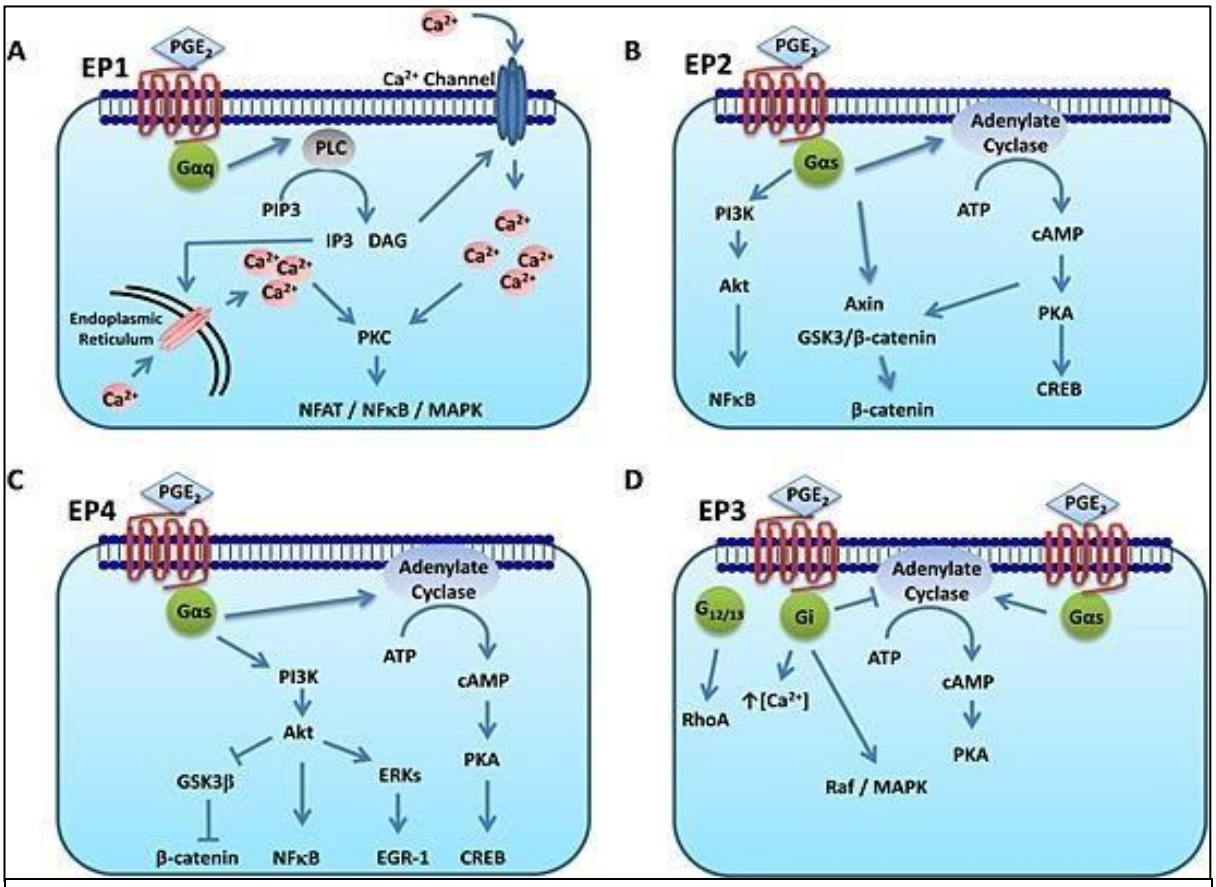


Figure 1.5: Downstream effects of PGE2 upon binding to one of the four EP receptors. A) Binding of PGE2 to EP1 receptors activates phospholipase C, ultimately causing a rapid influx of cytoplasmic calcium via channels on the endoplasmic reticulum and the cellular membrane due to elevation of inositol trisphosphate (IP3) and diacylglycerol (DAG). B,C) Stimulated EP2 and 4 receptors causes cleavage of Gαs protein and activates adenylate cyclase, causing elevated synthesis of cAMP from ATP. Cyclic AMP acts as a second messenger and can bind to protein kinase A (PKA), causing major downstream phosphorylation of serine and threonine residues. It can also activate the phosphoinositide 3-kinase (P13K) pathway, a pathway known to have major influence on characteristics involved in malignancies such as proliferation, differentiation and migration. D) Unlike the other EP receptors, EP3 couple multiple G-Proteins. Cleaving of the G_i subunit results in inhibition of adenylate cyclase, whereas cleaving of Gαs, also coupled to EP3 receptors, activates cAMP (O'Callaghan & Houston, 2015).

Levels of PGE2 and mPGES-1 are well documented to be elevated in certain cancers such as colon cancer (van Rees et al., 2003), nonsmall cell lung cancer (Yoshimatsu et al., 2001) and prostate cancer (Jain, Chakraborty, Raja, Kale & Kundu, 2008). The PGE2-induced activation of G-protein coupled receptors as seen in Figure 1.5 can contribute to various hallmarks of cancer; for example, inhibiting apoptosis via modulating B-cell lymphoma 2 (Bcl-2) expression (Islam, Shehzad & Lee, 2015), increasing proliferation and promoting invasiveness via transactivation of the epidermal growth factor (EGF) receptor (Pai et al., 2002) (Buchanan, Wang, Bargiacchi & DuBois, 2003), and stimulating angiogenesis via activation of ERK signaling promoting VEGF expression (Pai et al., 2001) (Larsson et al., 2015).

1.4.5 : Cysteinyl Leukotrienes

Leukotrienes are another member of the eicosanoid family produced de novo from membrane-bound arachidonic acid. These proinflammatory lipid mediators are implicated in asthma and allergic reactions and are predominantly produced by a host of cells involved in immunity. These cells include activated macrophages, leukocytes and mast cells (Hedi & Norbert, 2004).

1.4.6: Biosynthesis of CysLT's

The biosynthesis of leukotrienes begins with 5-lipoxygenase (5-LOX), a non-heme iron containing enzyme that, when activated by 5-LOX activating protein (FLAP), converts arachidonic acid to an unstable derivative, leukotriene A4, by oxygenating the 5^{th} carbon atom in the chain (Figure 1.6). FLAP works by binding to arachidonic acid and presenting it to the 5-LOX enzyme located on the nuclear membrane (Wang & DuBois, 2010). Under basal conditions, 5-LOX is not located on the nuclear membrane and must be translocated from either the cytosol or the nucleus depending on the cell it is found in. The translocation of 5-LOX requires calcium and can be stimulated by ATP, phosphatidylcholine, lipids and hydroperoxides (Hedi & Norbert, 2004). The translocation of 5-LOX is regulated by a series of phosphorylation of various serine residues by MAPK and ERK proteins. Conversion of 5-LOX to one of it's cysteinyl metabolites (LTC4, LTD4 or LTE4) leads to activation of one of 2 G-protein coupled CysLT receptors, CysLTR1 or CysLTR2. Despite being structurally very similar, the cysteinyl leukotrienes exert a range of different functional effects.

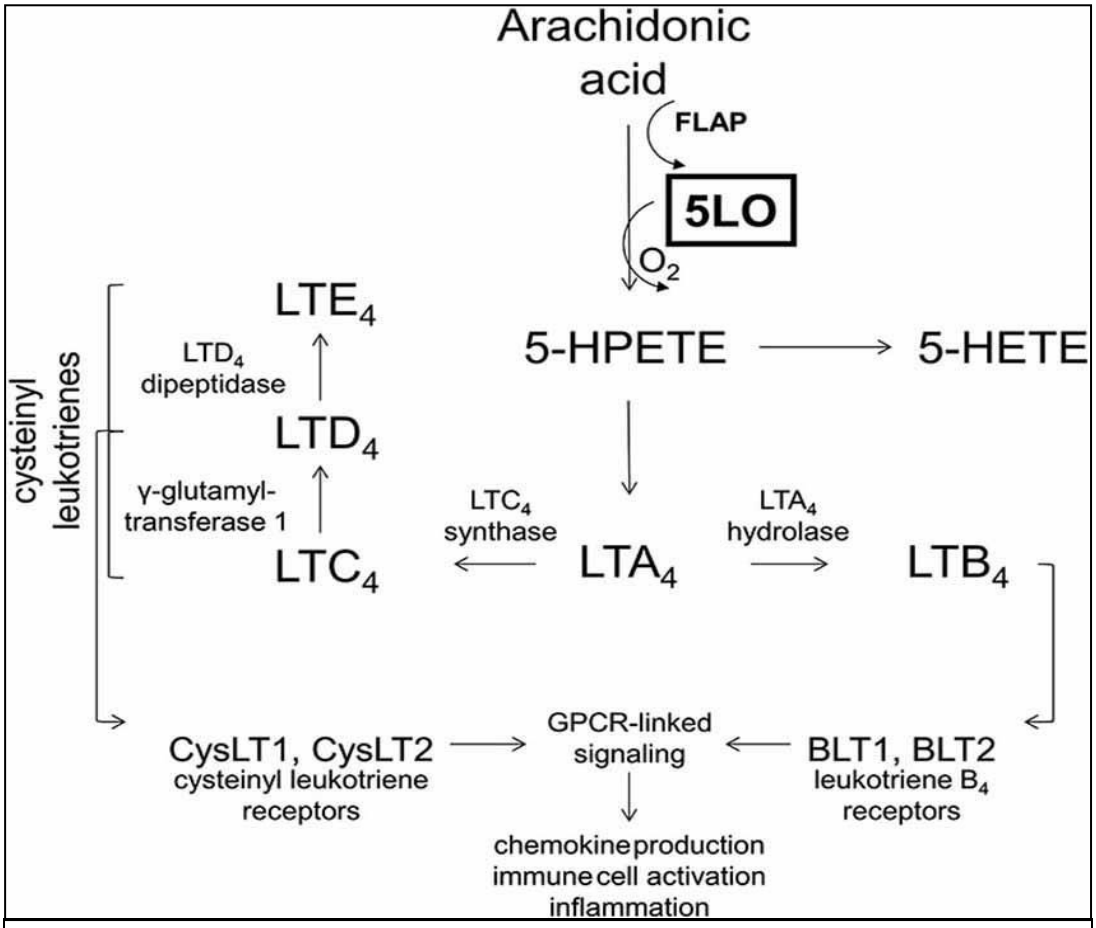


Figure 1.6: The 5-LOX pathway and downstream effects. Once 5-LOX is translocated to the nuclear membrane, the 5th carbon of the arachidonic acid is oxygenated and becomes arachidonic acid 5-hyperperoxide (5-HPETE). 5-LOX then catalyses the conversion of 5-HPETE to the allylic epoxide, LTA4. From here, LTA4 can be converted to one of 3 cysteinyl leukotrienes, all of which bind CysLT receptors causing G-Protein activation and downstream effects on tumorigenic factors such as cell proliferation and migration (Rådmark & Samuelsson, 2010).

1.4.7: CysLT receptors and their implication in cancer

Cysteinyl leukotrienes and their receptors are recognised to play roles in cancer-promoting inflammation in many cancers. Of these cancers, the most heavily implicated is colorectal adenocarcinomas. Dysregulated expression of CysLTR 1 and 2 in these tumours are a key hallmark in their survival and have an inverse relationship in their expression. CysLTR1 is upregulated while CysLT2 is downregulated, suggesting that CysLTR2 bares protective properties while CysLTR1 has the opposite effect. CysLT receptors are relatively unique as they are found on both the plasma and nuclear membranes of cells, the subcellular localization of CysLTR's also plays a critical role in the prognosis of CRC cases (Magnusson et al., 2010). Binding of substrates to CysLTR1 is known to activate various downstream pathways involved in carcinogenesis such as inducing P13K-Akt signalling resulting in activation of cyclin D1, COX-2 and c-myc genes via nuclear translocation of β-catenin, which are all modulators of proliferation (Burke et al., 2016).

Cysteinyl leukotrienes and the activation of their receptors are known to contribute to many hallmarks of cancer including the activation of migration and invasion, resistance to cell death and proliferative signalling. Activation of CysLTR1 by leukotriene D4 for example has been shown to decrease the expression of E-cadherin in HCT-116 cells, which plays a key role in cell adhesion and thus aids in the process of the epithelial-mesenchymal transition (Burke et al., 2016).

1.5 : Non-Steroidal Anti-inflammatory Drugs (NSAIDs)

NSAIDs are one of the most commonly used drugs in circulation and exert their effects by inhibiting the synthesis of prostaglandins via the COX pathway, most likely being more active against COX-2 (Cashman, 1996). Their introduction into pharmacology was orchestrated by an experiment in 1987 performed by Felix Hoffman, a German chemist who observed the reaction of salicylic acid with acetic anhydride which acetylated the salicylic acid to produce what we now know today as aspirin. Hoffman treated his father, who was suffering with rheumatoid arthritis, with the aspirin and observed a major improvement in symptoms (Zhang, Chen & Shang, 2018). There are a wide variety of NSAIDs being used every day, many with different pharmacological effects, despite the mechanism of action remaining the same. Aspirin and ibuprofen for example are the most well-known NSAIDs, are most commonly used for relief against inflammatory pains such as arthritis and headaches. A growing body of research over the last few decades have associated NSAIDs with possessing cancer-preventive properties. The discovery of this has stemmed from both epidemiological and experimental studies, which have found that NSAIDs can prevent neoplasia leading to a range of cancers including breast, prostate, colorectal, ovarian and cancers of the head and neck. The cancer-protective properties of NSAIDs only strengthen the implications of inflammation in cancer promotion, but this is a dangerous oversimplification (Wong, 2019). Despite NSAIDs being associated with prevention of certain cancers, they have also been linked to higher mortality rates in others (Brasky et al., 2011) (Choueiri, Je & Cho, 2013).

The specificity of different NSAID's to the COX enzymes varies depending on the administered drug. For example, aspirin is largely specific to COX-1, which is constitutively expressed in most tissues under basal conditions, whereas other NSAIDs such as ibuprofen, sulindac and diclofenac, inhibit both COX enzymes with similar specificities (Johnsen et al., 2005). The cancer-protective associations with NSAIDs only strengthens the hypothesis that the ABC-mediated efflux of key inflammatory eicosanoids could be a major player in promoting the progression of NB, especially in such cases where these ABCs are overexpressed.

1.6 : Regulation of ABC transporter-mediated eicosanoid efflux and association with NB prognosis

Previous studies examining the effects of ABC transporter inhibition on NB has implicated their expression in promoting carcinogenesis even in the absence of chemotherapeutic drugs, showing that ABCs can influence NB cell behaviour independently of their contribution to MDR. ABCC transporters in particular are a prime example of this (Henderson et al., 2011). Inhibition of ABCC1 has shown to halt the growth of NB cells independently of drug efflux in hMycN transgenic mice (Henderson et al., 2011), while ABCC4 inhibition has shown the same results in other cancers such as human leukaemia (Huynh, Norris, Haber & Henderson, 2012). Due to the ability of ABCC transporters to transport a wide range of endogenous substrates across the cell membrane, the cancer-protective effects observed following their pharmacological inhibition could be the result of the reduced efflux of substrates other than eicosanoids. This study aims to assess the association of key eicosanoids such as PGE2 and CysLTs in the progression of NB and determine if/which ABC transporters are implicated in promoting cellular migration. There is already published literature implicating proinflammatory eicosanoids in the promotion of migration. A good example of this is in skin dendritic cells (DCs). A 2008 study by van de Ven et al. investigated the role of ABCC4-mediated efflux of PGE2 in promoting migration of DCs and found that inhibition of the transporter with sildenafil caused a dose-dependent reduction in migration, which was significant in 20 and 60 µmol/L doses of the inhibitor (van de Ven et al., 2008). Migratory potential of mesenchymal stem cells (MSCs) have also been shown to be directly influenced by eicosanoids, specifically PGE2. A 2017 study showed this with the use of scratch assays on MSCs using serum-free media either with or without 1 µmol/L PGE2. The results showed significantly faster migration rates in MSCs treated with PGE2 compared to those without (Lu et al., 2017). Although evidence linking eicosanoid efflux and migration is currently sparse in NB, the previous literature in cell types such as DCs and MSCs make a promising case to explore this further to see if the effects can be replicated.

1.7 : Aims & Objectives

The role eicosanoids play in promoting cancer progression via downstream signaling following G-Protein activation is becoming increasingly apparent across a wide variety of cancers. In addition, their active efflux by various members of the ABC transporter family, also linked to multi drug resistance, is clear. With the use of specific ABC inhibition, this preliminary study aims to address several questions to provide an insight into the role of eicosanoids and their ABC-mediated efflux specifically in neuroblastoma cell lines:

A) How do the ABC expression profiles differ across different neuroblastoma cell lines and is this affected by MYCN status?

Determining the expression pattern of certain ABC transporters such as ABCC1 and ABCC4 via quantitative PCR can provide an early insight into the level of eicosanoid transport taking place in different cell lines.

B) How does activity of ABC transporters affect migration?

Measuring the aggression of different NB cell lines and comparing these findings to the ABC expression profile could possibly implicate certain ABC transporters in the promotion of carcinogenesis independently of MDR. Treating cell lines with specific ABC inhibitors and comparing scratch assay results to that of vehicle controls could provide evidence for the role eicosanoids play in promoting the aggressiveness of NB cell lines.

C) Does altered ABC expression affect the release of eicosanoids?

By quantifying the levels of eicosanoids released by different NB cell lines and comparing this to the levels of ABC expression, the means by which eicosanoids are exported by NB cells will be made clearer. This could also highlight synergistic relationships between ABC transporters in terms of eicosanoid transport. Quantifying the concentration of eicosanoids in the culture media of ABC-inhibited samples and comparing this to the levels found in vehicle controls could explain results seen in scratch assays and further implicate these eicosanoids in the promotion of migration in NB cell lines.

The ABC inhibitors used in this project were reversan (as an ABCC1 inhibitor), ceefourin (as an ABCC4 inhibitor) and tariquidar (as an ABCB1 inhibitor). All three of these inhibitors show a good selectivity for their respective transporter. Reversan is a pyrazolopyrimidine selective to both ABCC1 and ABCB1, though it is more selective to ABCC1 (Burkhart et al., 2009). Tariquidar acts specifically on ABCB1 at low concentrations but can also inhibit ABCG2 at higher concentrations (≥100 nM) (Kannan et al., 2010). Pharmacological inhibitors specific to ABCC4 have been difficult to identify, limiting the understanding of the transporter. Ceefourin is a relatively new, highly selective ABCC4 inhibitor that is more potent than the most widely used ABCC4 inhibitor MK-517 (Cheung et al., 2014). All 3 of these ABC inhibitors display little to no cellular toxicity but have not passed clinical trials.

Section 2: Materials and Methods

2.1 : Cell Culture and Maintenance

A total of 5 neuroblastoma cell lines were obtained for research as a gift from Dr C. Cooper and Dr S. Allison, University of Huddersfield. The cells gifted included 2 non-MycN amplified lines (SH-SY5Y and SK-N-SH) and 3 MycN amplified lines (KELLY, SK-N-BE(2)c and IMR32). The stocks were recovered from liquid nitrogen and seeded onto T25 flasks (Starstedt, Leicester, UK) in a medium consisting of DMEM (Sigma, Gillingham, UK) (89%), foetal bovine serum (FBS; 10%) (Thermo Fisher, Altrincham, UK) and L-Glutamine (1%) (Lonza, Leicestershire, UK). One exception was SK-N-SH cells, which were grown in a slightly different medium consisting of 50/50 DMEM/RPMI (Sigma, Gillingham, UK) with the same ratios of FBS and L- Glutamine. All cell culture procedures and sterile experiments were carried out within a class II laminar flow hood. To ensure further sterilisation, all apparatus used in these procedures (i.e. pipettes, tube racks etc.) were sprayed with 70% ethanol solution prior to entering the hood.

2.2 : Sub-culture

Upon reaching 80-90% confluence, cells were washed with 1x phosphate buffered saline (PBS) (Lonza, Leicestershire, UK) and passaged using a 0.5% trypsin-EDTA solution (ThermoFisher, Leicestershire, UK) to promote detachment from the surface of the flask. Trypsinised cells were diluted with fully-supplemented growth media and centrifuged to produce a pellet. The supernatant was discarded, and the pellet was resuspended in fresh media before seeding into requisite culture vessels.

2.3 : Wound-healing Assays

Scratch/wound-healing assays were used to assess the migratory potential of NB cell lines following ABC transporter inhibition. Cells were seeded onto 6-Well plates at a density of 300,000 cells per well. Upon reaching ~100% confluence, a uniform scratch wound was made using a P200 pipette tip attached to an aspirator to remove loose cells and debris. Following the creation of a wound, each well was treated with a specific ABC inhibitor. Reversan (Sigma, Gillingham, UK) ceefourin 1 (ABCAM, Cambridge, UK) and tariquidar (Sigma, Gillingham, UK) were used to treat NB cells at concentrations of 10µM to inhibit ABCC1, ABCC4 and ABCB1 respectively. Vehicle controls containing dimethyl sulfoxide (DMSO) (Sigma, Gillingham, UK) were used at concentrations equal to that of the respective drug, leaving 3 wells with an ABC inhibitor and 3 wells with a vehicle control per plate. The media used to culture cells after wound creation was made using only 1% FBS to restrict wound closure to migratory rate by limiting proliferation. Following treatment with inhibitors, 200x magnification

images were taken at a pre-marked location on the wound using a light microscope every 24 hours over a 3 day period (0hrs, 24hrs, 48hrs, 72hrs).

These images were analysed using ImageJ in order to determine the percentage cell-free area, indicative of cellular migration via wound closure. Using this percentage value, the wound area of each image was calculated and normalised to their respective 0 hour value. The mean and standard deviation of the 3 repeats for each treatment were calculated and plotted on a line graph.

2.4 : RNA extraction from NB cells

Before PCR assays could be performed, cDNA samples had to be collected after treatment after ABC inhibition. These were collected by growing extra T25 flasks and treating them once they had reached ~60% confluence to ensure that cells had room to migrate naturally. After 24 hours of ABC inhibition, cells were pelleted and stored at -80°C until RNA was extracted. RNA extraction was performed with the RNA Cell Miniprep System kit provided by Promega (Madison, USA). Following extraction, the purity and concentration of the RNA samples were calculated using a Nanodrop. RNA samples were also stored at -80°C until their conversion to cDNA.

2.5 : cDNA conversion

The concentrations of RNA samples deduced by the nanodrop were used to calculate how much of each sample was needed to produce 1µg cDNA for qPCR. The RNA was converted using the protocol and reagents provided in the Tetro cDNA Synthesis Kit purchased from Bioline (London, UK). The apparatus used for temperature regulation during conversion cycles was the DNA Engine Tetrad 2 Peltier Thermal Cycler (Biorad, California, USA). Converted cDNA samples were stored at -20°C until they were required.

2.6 : qPCR

The cDNA samples were acquired to run qPCR, which was used to determine the expression of a range of ABC transporters and receptors in NB cell lines. PCR was conducted using Taqman probes supplied by Thermofisher (Leicestershire, UK) (Table 2) and Precision Fast qPCR Master Mix with ROX (PrimerDesign, Southampton, UK). Every sample was ran in triplicate on a 96-well plate.

Gene	Product Code
ABCA1	Hs01059137_m1
ABCB1	Hs00184500_m1
ABCC1	Hs01561483_m1
ABCC3	Hs00978452_m1
ABCC4	Hs00988721_m1
ABCG1	Hs01555198_m1
CysLTR	Hs00272624_s1
GAPDH	Hs02758991_g1
18S	Hs0300331_g1
PPIA	Hs99999904_m1

Table 2: TaqMan probes used for qPCR and their respective product codes.

The genes of interest were amplified using StepOne software v2.3 and an Applied Biosystems StepOne plus Real-Time PCR thermocycler. The data generated from these systems was analysed to calculate fold changes in gene expression via the ΔΔCt method (Livak & Schmittgen, 2001). The expression levels were calculated relative to the geometric mean of the 3 housekeeper genes used (GAPDH, 18S and PPIA) and plotted onto a bar chart.

2.7 : ELISA assays

Media samples harvested from cultured cell lines treated with ABCB1, C1 and C4 inhibitors were collected after a 24-hour treatment period. The media was then assayed for concentrations of PGE2 using a competitive ELISA kit supplied by R&D Systems (Minneapolis, USA. Product ID: KGE004B). This assayed the competitive binding of sample PGE2 with horseradish peroxidase-labelled PGE2 with complimentary antibodies. Unlike the other ELISA assays performed in this project, the cells used in the preliminary PGE2 ELISA were cultured in a T25 flask until around 50% confluent which is when

1ml of media was harvested and frozen at -80°C until required for use. These samples were stored for no longer than 4 weeks.

The following ELISA assays used cell samples that had been cultured on 12-well plates in 1ml of media were cultured until they had reached a confluence of around 200,000 cells/well which is when the media was removed. The media harvested from these assays was stored at -80°C for no longer than 1 week to improve the accuracy of results by reducing the possibility of degradation of target molecules.

Following the results of the preliminary PGE2 ELISA, a repeat was performed in conjunction with a Bradford assay (protocol and reagents supplied by ThermoFisher, Leicestershire) to quantify protein levels of the samples. This allowed normalisation of PGE2 concentrations to the cell density of each sample. In this assay, cells were treated with the same ABC inhibitors, but also with 10ng/ml IL-1ß (Sigma, Gillingham, UK) for the same amount of time (24 hours) to stimulate the COX-2 pathway in NB cell lines.

The same samples were also tested for cAMP concentrations to assess the levels of activation of receptors EP2-4. Samples assayed for the cAMP ELISA were prepared by lysing cells for 20 minutes with 0.1M hydrochloric acid (ThermoFisher, Leicestershire, UK) and using a cell scraper to further break the cells and release the contents. The lysate was used for protein quantification via Bradford assay and run on a competitive ELISA kit supplied by ABCAM (product ID: ab133039).

The final ELISA assay involved quantifying levels of CysLT's in ABC-inhibited NB cell lines. The samples assayed for CysLT concentrations were cultured onto 12-well plates with 1µM arachidonic acid (Sigma, Gillingham, UK) to supplement 5-LOX. The supplemented NB cells were then cultured until they reached around 90% confluence, which is when they were treated with ABC inhibitors and a calcium ionophore (A23187) (Sigma, Gillingham, UK) to facilitate CysLT production. The kit used to assay CysLT concentration was also supplied by ABCAM (product ID: ab133042).

PGE2 ELISAs required samples to be diluted before being assayed. To minimize readings below the level of quantification (LoQ), the samples used in the PGE2 ELISA assays were diluted 3-fold, which was as concentrated as protocol guidelines allowed.

Section 3: Results

3.1 : Changes in ABC transporter profile across 5 NB cell lines

As a first step to infer how ABC activity might influence the migration of NB cell lines, it was essential first to determine their levels of expression using qPCR. The results also allow potential correlations between MycN amplification and the expression of different ABC transporters and opens discussion as to why these genetic modifications might promote carcinogenesis.

Figure 3.1.1 shows little change in ABC expression between non-MycN amplified cell lines i.e. SH-SY5Y and SK-N-SH. The expression of ABCC3 in KELLY cells was downregulated almost 20-fold relative to SH-SY5Y cells. ABCC3 was also found to be downregulated in SK-N-BE(2)c cells, but to a much lesser extent and with less variability in repeats. ABCC3 expression in IMR32's was amplified 13-fold, however, despite these cells being MycN amplified.

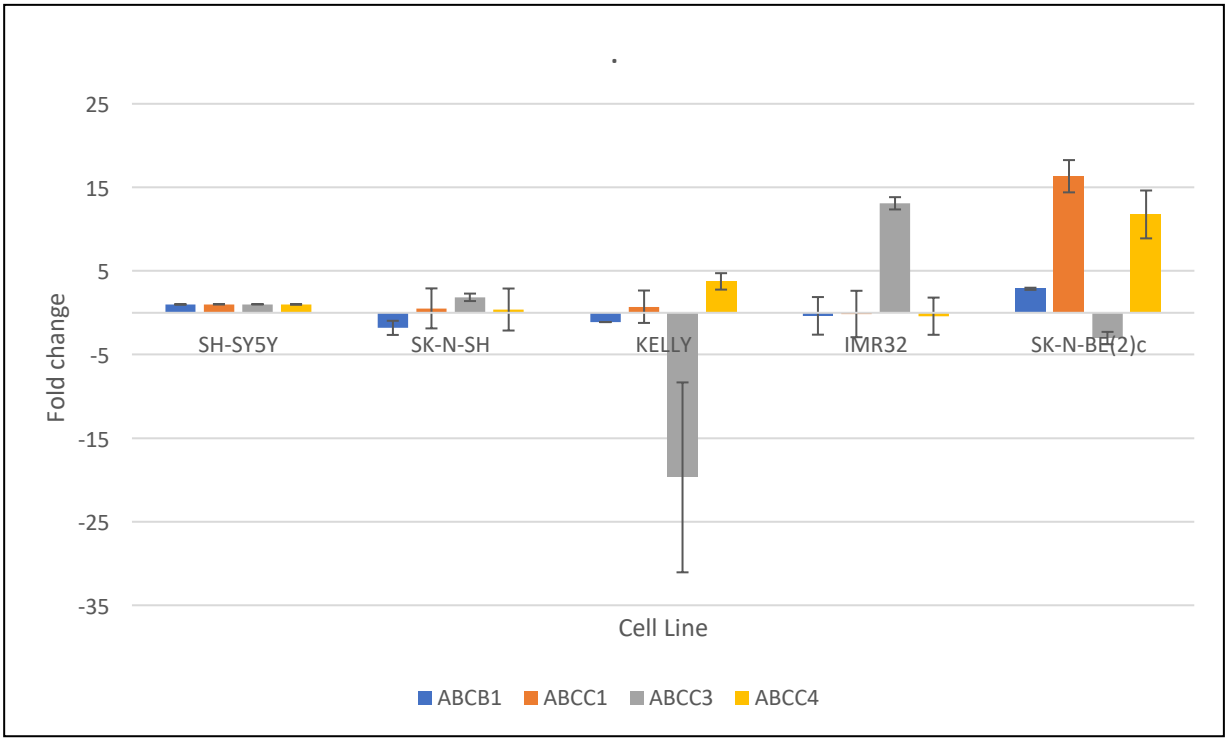


Figure 3.1.1: The expression patterns of ABCB1 and ABCC transporters across 5 neuroblastoma cell lines quantified by real time quantitative PCR relative to 3 housekeeper genes, GAPDH, 18S and PPIA. The fold change in expression of ABC transporters was calculated relative to the SH-SY5Y cells. Data are Mean +/- SD (N=2).

Expression of other ABC transporters was relatively similar between all 5 neuroblastoma cell lines, with the exception of ABCC1 and ABCC4 in SK-N-BE(2)c cells. The expression of these to transporters was upregulated around 16-fold and 11-fold respectively in these cells, while their expression in other cell lines remained somewhat unaffected. KELLY cells also had a relatively small upregulation of ABCC4 (4-fold increase relative to SH-SY5Y's).

The expression of lipid and sterol efflux transporters as shown in Figure 3.1.2 shows few major changes in expression of ABCA1 or ABCG1 among the neuroblastoma cell lines tested. Most notably, ABCA1 expression in SK-N-SH cells was amplified around 12-fold higher in comparison to that of SH-SY5Y cells. A small upregulation of both transporters was found in KELLYs and IMR32 cells had a small increase in ABCG1 gene expression. This experiment was only conducted once and would require repeats to confirm the legitimacy of the data.

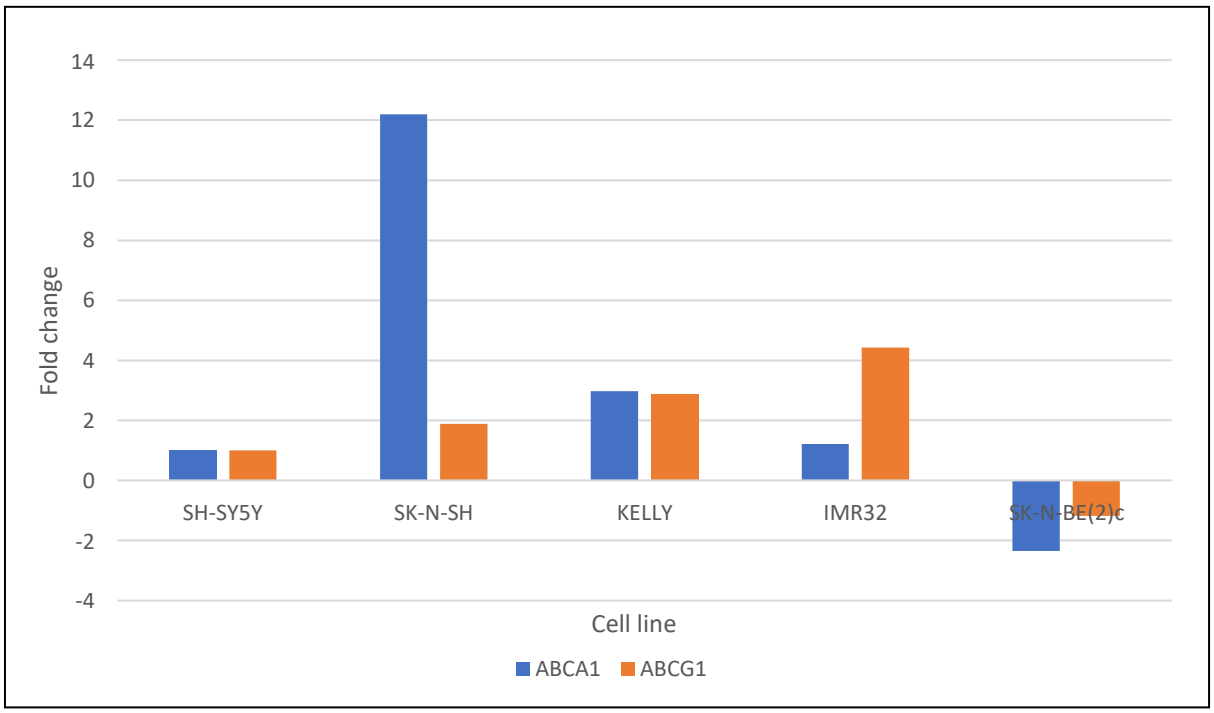


Figure 3.1.2: Gene expression patterns of ABCA1 and ABCG1 across 5 neuroblastoma cell lines. Fold changes of gene expression were calculated relative to SH-SY5Y cells. (N=1)

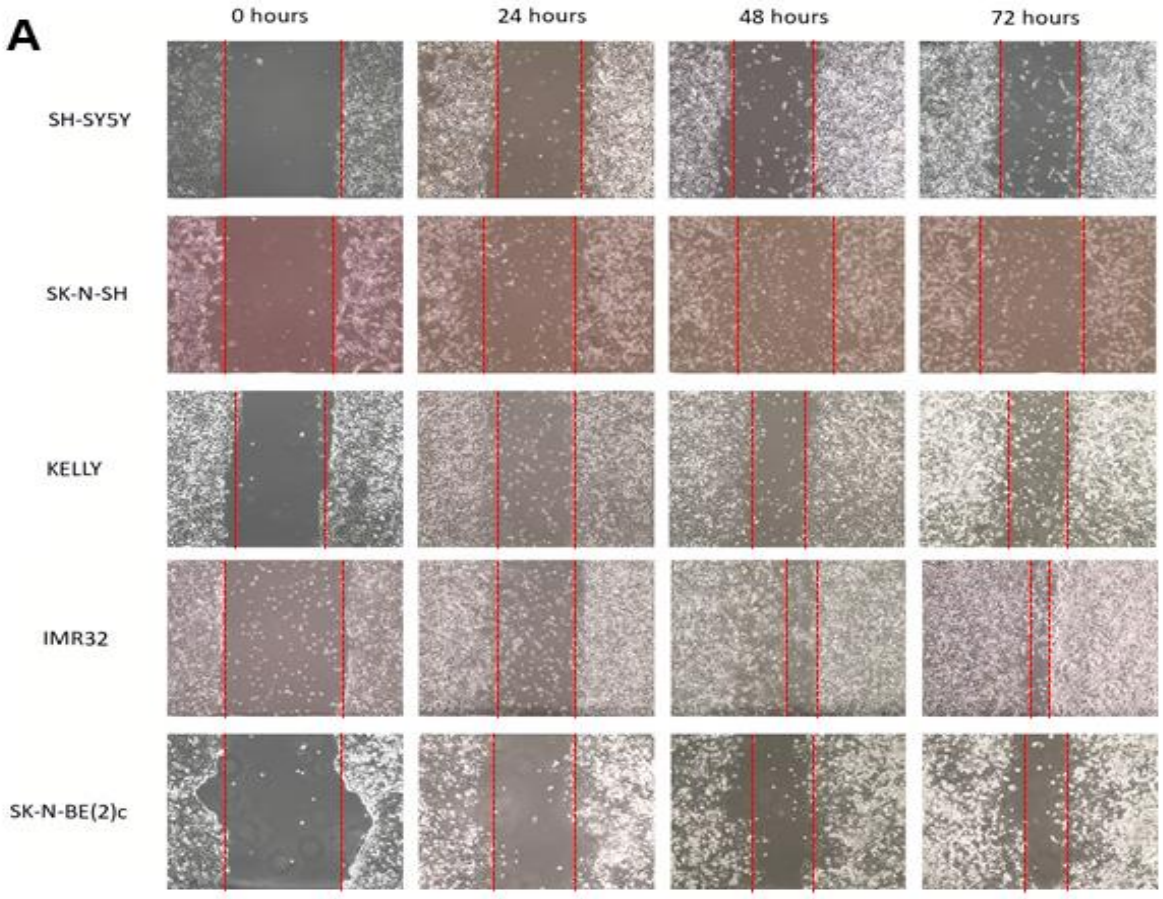
3.2 : How does ABC inhibition affect migration of NB cell lines?

To better determine how inhibition of ABC transporters affects tumour progression, wound-healing assays were used to assess how efficiently different NB cell lines cultured in serum-deficient media migrate following pharmacological inhibition of different ABC transporters. Firstly, a comparison was made of the basal migratory rate of the 5 NB cell lines when exposed only to the vehicle. This gave an insight into the possible effects of MycN on migratory potential of these cells.

Some wound-healing assay data taken from SH-SY5Y and KELLY assays was collected by undergraduate students Sewa Adebisi and Callum Robson under my supervision.

3.2.1 : Basal migration rates of NB cell lines

Figure 3.2.1 shows that IMR32 cells displayed the most migratory capacity over a 3 day period. This was followed closely by SK-N-BE(2)c cells and then by KELLY and SH-SY5Y's, which shared a similar mean rate of migration. SK-N-SH cells did not show much, if any, migration and average wound area remained almost constant over the 3-day period. Some cells responded better in low-serum culture than others. The viability of SK-N-SH cells began to decrease quicker than other cell lines and detached from the surface of the plates. Cells such as IMR32s and SK-N-BE(2)cs had little problem in the same media and appeared viable throughout the 72-hour period. There was likely a low level of proliferation during the assay, but with a 10-fold reduction in growth serum, the results displayed are more representative of migratory potential rather than proliferative.



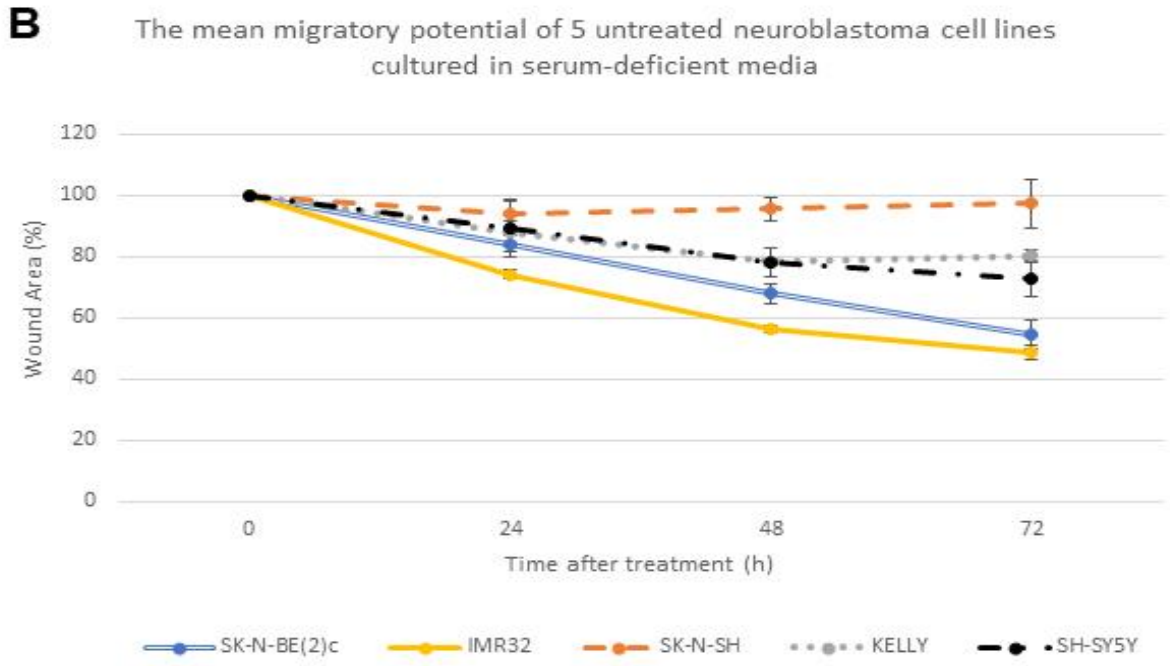


Figure 3.2.1: Basal migratory rate of vehicle-treated NB cell lines.
A) Images of wound healing assay best representing the mean migration rates of 5 different NB cell lines. Dashed red lines show generalised closure of wound area over the 72 hour period. B) The mean rate of migration/wound-healing seen across SH-SY5Y, SK-N-SH, KELLY, IMR32 and SK-N-BE(2)c cells in vehicle-control cells. Values are mean +/-SD. N=3.

3.2.2 : Migration of SH-SY5Y cells under the influence of ABC inhibitors

As seen in Figure 3.2.1B, SH-SY5Y cells migrated at a steady rate under basal (vehicle-treated) conditions. From Figures 3.2.2A-D, inhibition of ABCB1, ABCC1 and ABCC4 did not appear to influence cellular migration. This is seen particularly in Figure 3.2.2-B in cells treated with reversan. This data shows no mean difference in wound closure over 3 days between control and treatment groups. Inhibition of ABCB1 with tariquidar did show small differences in migration to the vehicle after 24 hours, with migration slowing slightly. However, viability of cells treated with tariquidar between 24 and 72 hours after treatment was visibly decreasing as cells were detaching from the wells and decreasing in confluence which in many cases which could account for this trend.

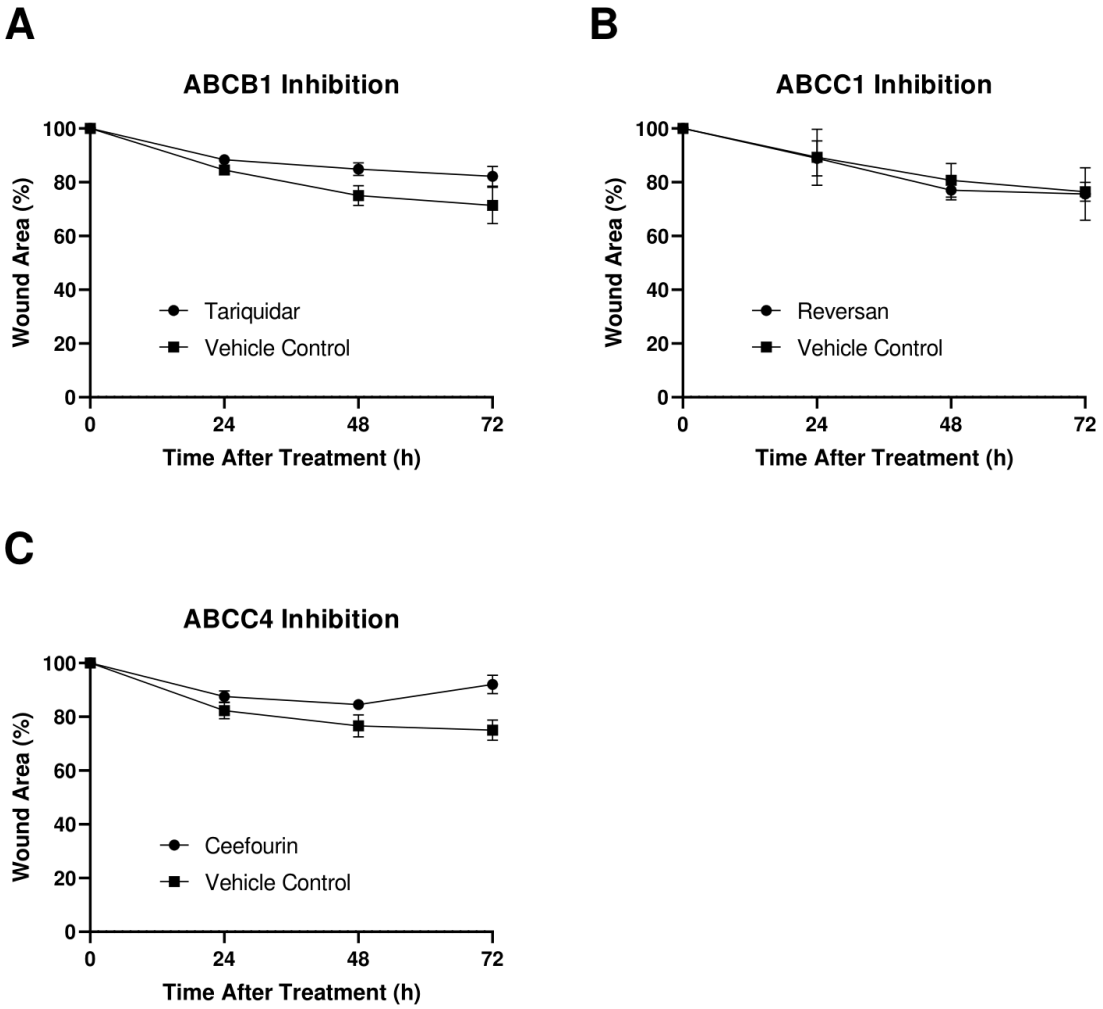


Figure 3.2.2: Migration of SH-SY5Y cells after treatment with inhibitors of ABCB1 (A) (N=2), ABCC1 (B) (N=3) and ABCC4 (C). Values are mean +/- SD. N=2.

3.2.3 : Migration of SK-N-SH cells under the influence of ABC inhibitors

Of all the cell lines assayed, SK-N-SH cells showed the least migratory potential (Figure 3.2.1B). Over the course of the assay the mean wound area showed little to no reduction, in many cases of both control and treatment groups, cell adhesion decreased rapidly after the wound was created, indicating a loss of viability. Treatment of SK-N-SH cells with reversan and ceefourin again made little difference to the migratory potential of cells when compared with the vehicle control groups (Figure 3.2.3). There is very little difference in the mean wound area of cultures at every time point over the course of 3 days with a relatively small spread of data.

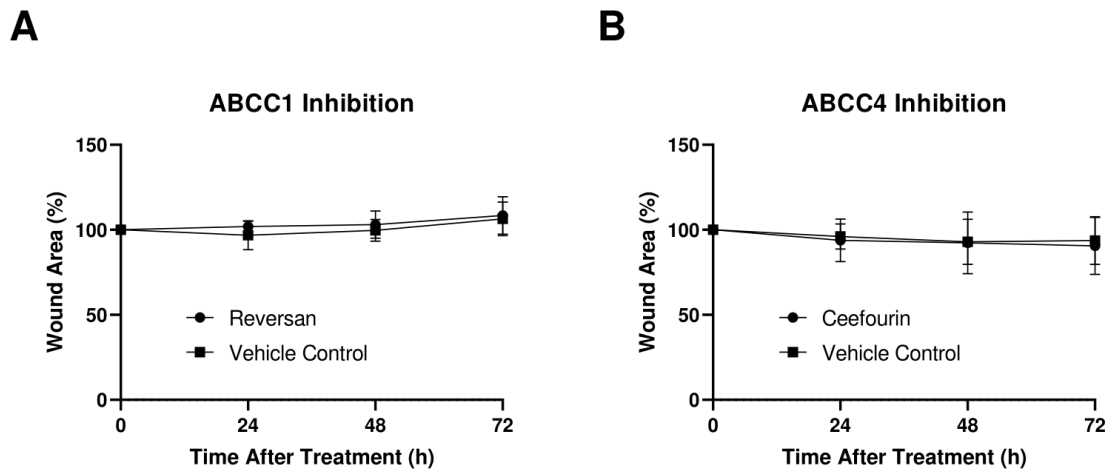


Figure 3.2.3: Migration of SK-N-SH cells after treatment with inhibitors of (N=3), ABCC1 (A) (N=3) and ABCC4 (B) (N=3). Values are mean +/- SD. N=3.

3.2.4 : Migration of KELLY cells under the influence of ABC inhibitors

KELLY cells showed a slow but steady rate of migration under basal conditions like SH-SY5Y cells (Figure 3.2.1B). Both treatment and control cultures in ABCC1 and ABCC4-inhibited KELLY groups showed a halt/slight decrease in wound closure between 48- and 72-hour time points after treatment and a steady rate of closure prior to this as shown by Figure 3.2.4A-B.

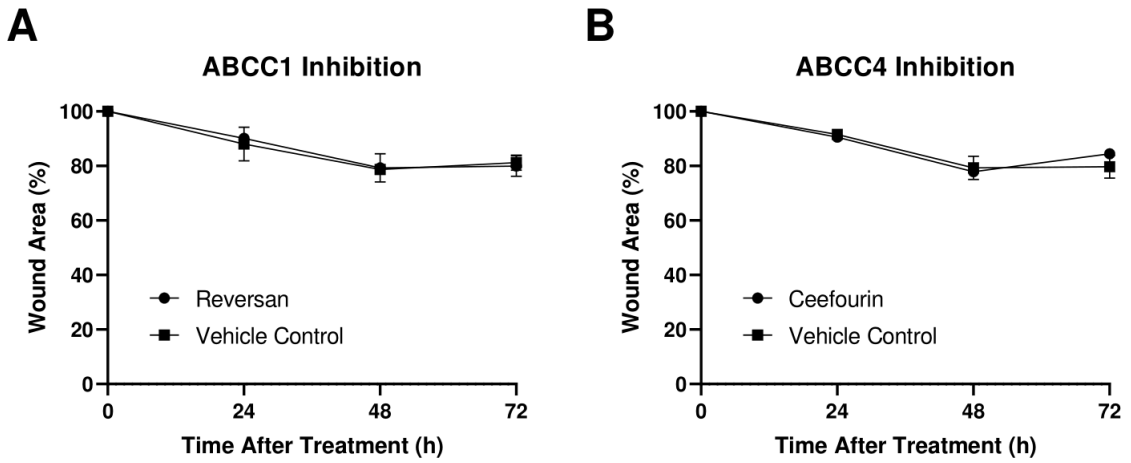


Figure 3.2.4: Migration of KELLY cells after treatment with inhibitors of ABCC1 (A) (N=2) and ABCC4 (B). Values are mean +/- SD. N=2.

Figure 3.2.5: Migration of IMR32 under the influence of ABC inhibitors

IMR32 cells displayed the most aggressive migratory phenotype (figure 3.2.1B), this trend was prominent even under the influence of ABC inhibitors. Inhibition of ABCB1, ABCC1 and ABCC4 appeared to have had minimal effect on IMR32 cells as they all showed almost identical migratory patterns compared to respective control groups (figure 3.2.5). This is seen most clearly in cells treated with reversan (figure 3.2.5B), where the data is almost inseparable and difficult to distinguish between treatment and control groups. Furthering this, all 3 ABC-inhibited groups showed the same pattern of migration over the 3 day period, with a sharp but steady rate of wound closure with little drop off in migration.

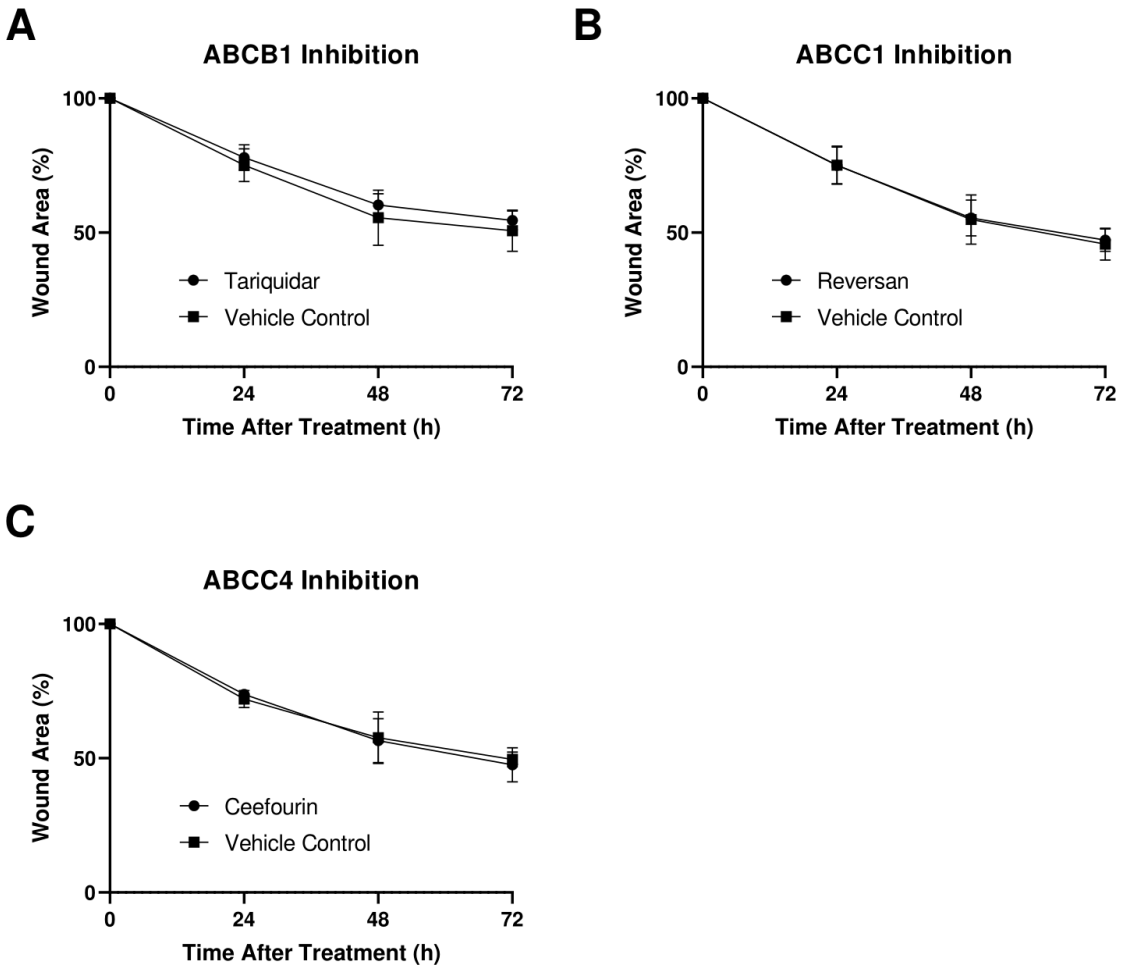


Figure 3.2.5: Migration of IMR32 cells after treatment with inhibitors of ABCB1 (A), ABCC1 (B) (N=3) and ABCC4 (C) (N=3). Values are mean +/- SD. N=3

Figure 3.2.6: Migration of SK-N-BE(2)c cells under the influence of ABC inhibitors

SK-N-BE(2)c cells also showed a sharp rate of migration under basal conditions, falling just short of that seen in IMR32 cells. Pharmacological inhibition of ABCB1, ABCC1 and ABCC4 in SK-N-BE(2)c cells showed to have no implications on migratory rate of cells when compared to respective vehicle-control groups (Figure 3.2.6), as seen in all other NB cell lines. The rate of wound closure seen in SK-N-BE(2)c's remained sharp and steady over the course of the assays.

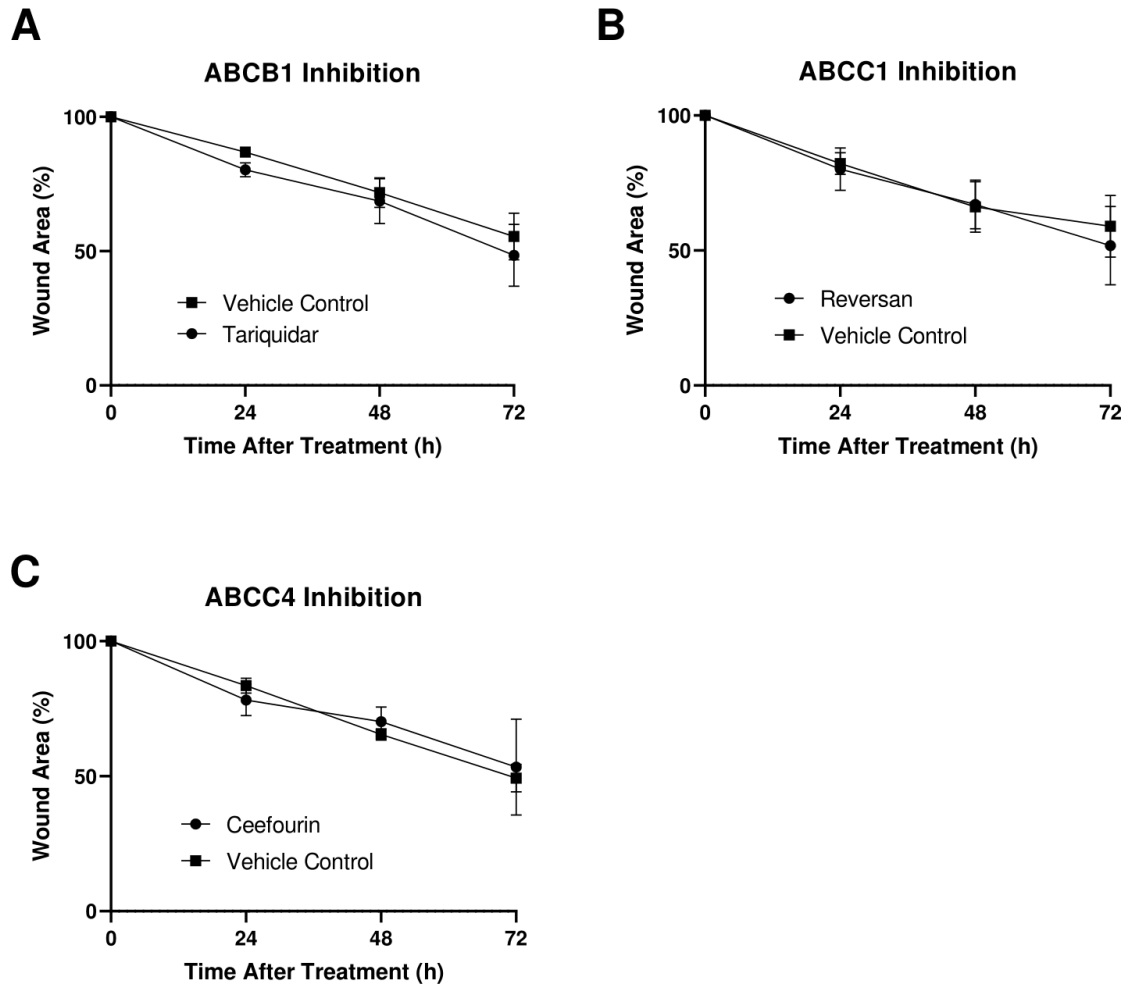


Figure 3.2.6: Migration of SK-N-BE(2)c cells after treatment with inhibitors of ABCB1 (A), ABCC1 (B) (N=2) and ABCC4 (C) (N=2). Values are mean +/- SD. N=2.

3.3 : How does ABC transporter inhibition affect efflux of eicosanoids?

The efflux patterns of inflammatory mediators such as PGE2 and cysteinyl leukotrienes by NB cells could potentially provide a link to their phenotypic behaviour *in vitro* and *in vivo* (Henderson et al., 2011). Levels of PGE2 release from different cells correlates to EP receptor activation which is well documented to drive hallmarks of carcinogenesis (Sun & Li, 2018). Assessing the concentrations of PGE2 in ABC was investigated to determine whether these efflux proteins might influence the release of these important inflammatory mediators.

3.3.1 : Preliminary PGE2 ELISA without proinflammatory stimulators or normalisation to cell density

A preliminary PGE2 ELISA revealed that PGE2 efflux decreased in response to ABCC4 inhibition across all three cell lines assayed (SH-SY5Y, IMR32 and SK-N-BE(2)c), so much so that the levels of PGE2 were below the LoQ in SH-SY5Y culture media under the influence of ceefourin (Figure 3.3.1A). IMR32 cells showed the largest efflux of PGE2 under basal conditions by a relatively small margin (Figure 3.3.1B), followed by SH-SY5Y cells while SK-N-BE(2)c culture media showed the lowest levels with under 20pg/ml (Figure 3.3.1C). IMR32 cells with inhibited ABCC1 transport showed a large increase in PGE2 efflux, far surpassing the levels found in the vehicle control group and recording the highest concentrations found in all cell lines, although there was substantial variability in the data.

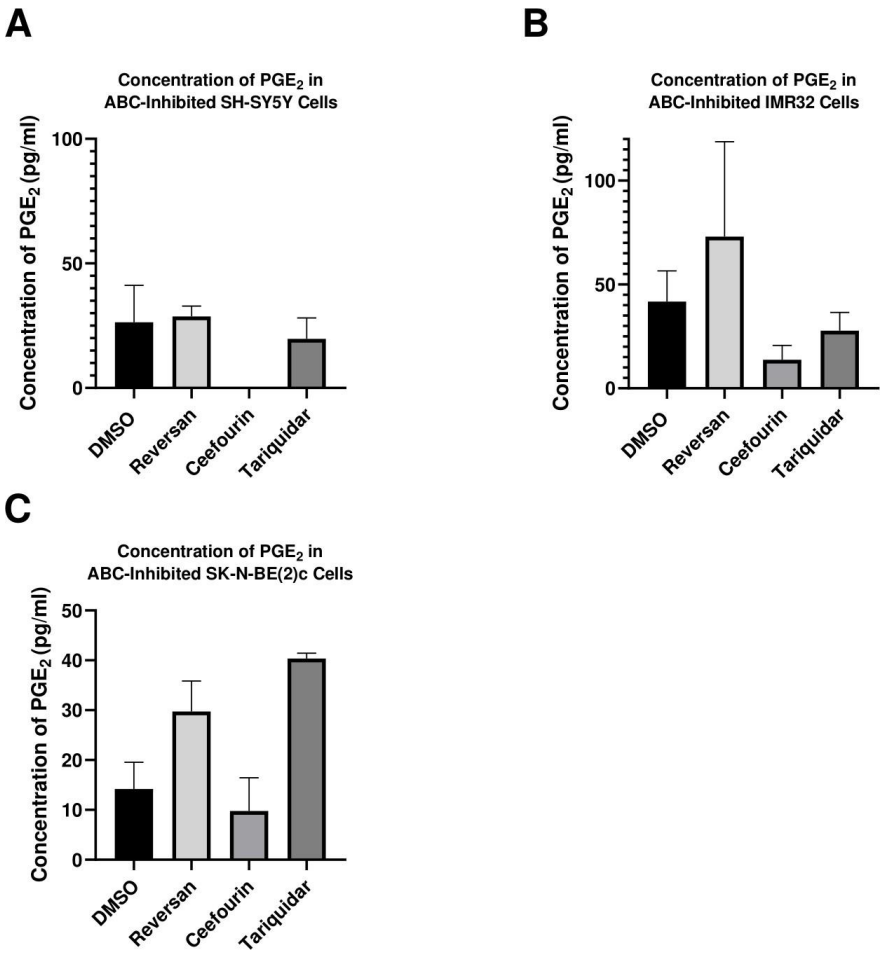


Figure 3.3.1: Preliminary analysis of PGE2 concentrations in ABC-Inhibited SH-SY5Y (A), SK-N-BE(2)c (B) and IMR32 (C) cells in the absence of stimulatory mediators or normalisation to protein concentration of samples via competitive ELISA assay. Values are mean +/- SD. N=1.

Despite showing the lowest levels of basal PGE2 efflux, SK-N-BE(2)c cells treated with ABCB1 inhibitor tariquidar showed a major increase, with over double the amount of PGE2 being quantified compared to the control. This was also the case in SK-N-BE(2)c cells treated with reversan but to a slightly lesser extent. The impact of tariquidar was not observed in IMR32 or SH-SY5Y cells.

3.3.2 : PGE2 efflux in ABC-inhibited cell lines when treated with IL-1ß and normalised to cell density

Given the low levels of PGE2 secretion observed in preliminary experiments, it was next examined whether PGE2 production and release could be stimulated using IL1-β, a pro-inflammatory cytokine, as previously described (Neeb et al., 2011). As shown in Figure 3.3.2, treatment with IL1β did not stimulate additional PGE2 release compared to untreated controls, in any of the cell lines tested with the exception of KELLY cells as seen in figure 3.3.2C.

Figure 3.2.3 shows that SH-SY5Y cells had a much lower rate of basal PGE2 efflux compared with other

cell lines with a mean concentration of under 10pg/ml/ug protein. Mean efflux of PGE2 in DMSO groups was similar in SK-N-SH and SK-N-BE(2)c cell lines at around 30pg/ml/ug protein. The IMR32 cells showed the second largest basal efflux at around 50pg/ml/ug protein. These values were dwarfed by the DMSO group for KELLY cells, which showed a mean PGE2 concentration of around 300pg/ml/ug protein, although with large variability.

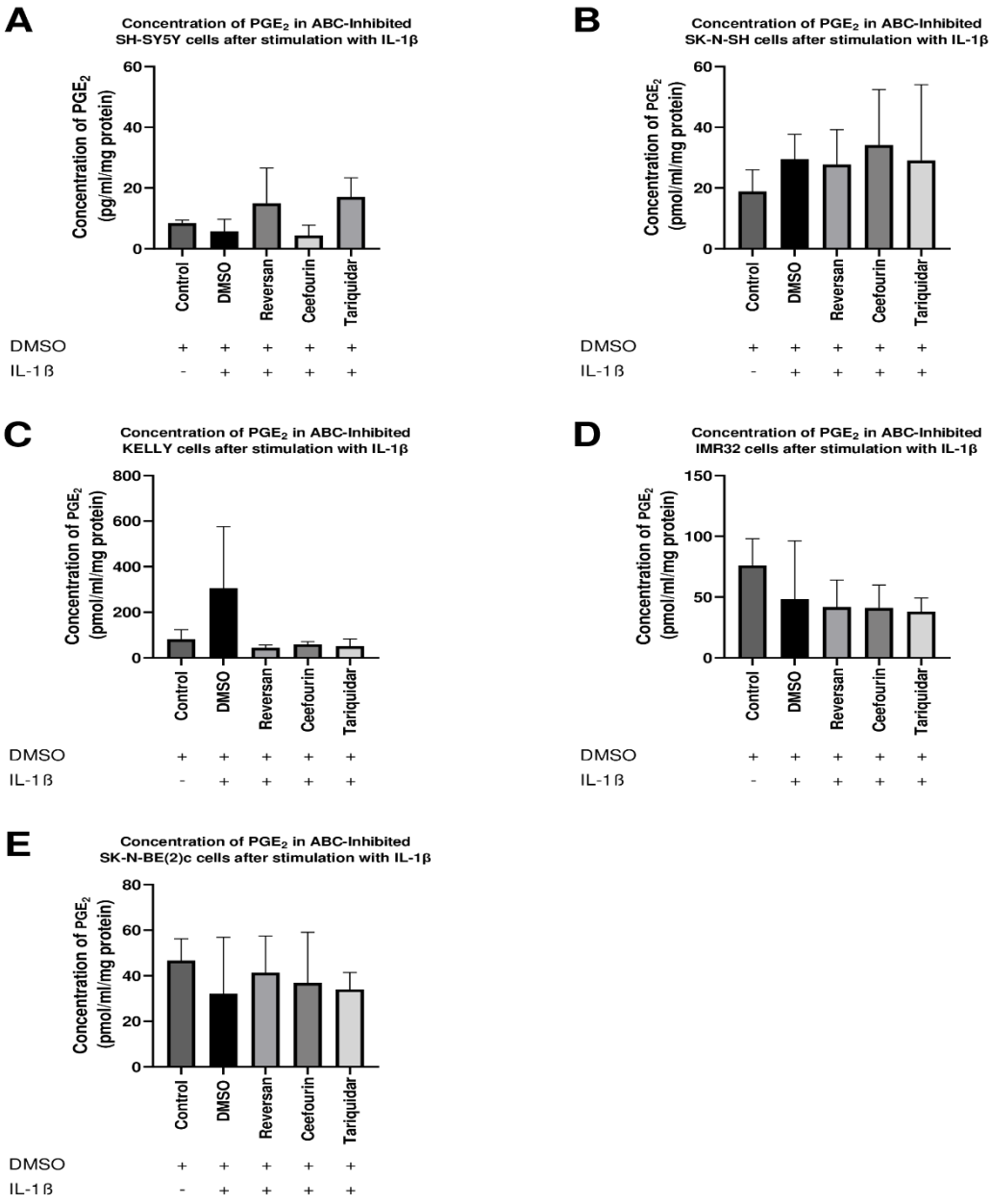


Figure 3.3.2: A measure of PGE2 efflux in ABC-inhibited SH-SY5Y (A), SK-N-SH (B), KELLY (C), IMR32 (D) and SK-N-BE(2)c (E) cells following 24-hour stimulation with IL-1ß. Values are mean +/- SD. N=1.

Reversan and tariquidar both showed to cause a large increase in PGE2 efflux relative to what was seen in the DMSO control in SH-SY5Y cells. Ceefourin treated cells however showed a marginally lower PGE2 output than the DMSO control. Treating SH-SY5Y cells with IL-1ß resulted in a 47.5% decrease in PGE2. Generally, the concentrations of PGE2 quantified in all groups of SH-SY5Y cells was incredibly low compared to the levels seen among other cell lines and lower than the levels seen in the preliminary PGE2 ELISA (Figure 3.3.1A).

The lowest levels of PGE2 in SK-N-SH cells was found in unstimulated cells with no IL-1ß, which showed a 36% decrease in mean PGE2 concentrations from the DMSO control. The ABCB1 and ABCC1 inhibitors appeared to have little effect on PGE2 efflux in SK-N-SH cells, as these groups showed similar concentrations to the DMSO control with a range of just 2pg/ml/ug protein separating these groups. Cells treated with ceefourin showed a slightly increased efflux of PGE2 compared to the control (15% increase).

As previously mentioned, KELLY cells showed a large efflux of PGE2 in the DMSO control relative to the other 4 cell lines, and in fact also relative to the other treatment groups, including the unstimulated control. The DMSO control was 273% higher than the IL-1ß negative group, which saw the second- highest PGE2 output of the 5 groups. Of the ABC-inhibited treatments, ceefourin caused the highest mean extracellular concentration of PGE2 by a small margin, followed by tariquidar (15% difference), with reversan-treated cells showing the lowest PGE2 efflux at 44.6pg/ml/ug protein, a 25.5% drop from the reversan group and a 50.5% decrease from the IL-1ß negative group.

IMR32 cells with no treatment interestingly showed an increase of 57% PGE2 efflux compared to the DMSO control, whereas treatment with ABC-inhibitors with IL-1ß suppressed efflux below levels seen in the DMSO control. There was almost no mean difference in PGE2 efflux between ceefourin and reversan-treated groups, with a difference of just 0.8pg/ml/ug protein. Tariquidar treated IMR32's showed the lowest mean efflux of PGE2 by a small margin.

Similarly to the IMR32's, SK-N-BE(2)c cells cultured without IL-1ß showed the highest PGE2 output of any treatment. However, the mean concentration of PGE2 found in the DMSO control was the lowest of any group. The difference in mean PGE2 concentrations between treatment groups was relatively small, with reversan-treated cells showing the highest efflux (29% increase from DMSO) followed by ceefourin (16% increase) and then tariquidar (6%).

The general trend of the data shown in Figure 3.3.2 appears to show no significant effect of ABC inhibition on the release of PGE2 in the NB cell lines assayed, despite the increase of PGE2 concentration seen in the vehicle control group in the KELLY cells. Given the lack of biological repeats and large variability seen within this set of data compared to other groups, further repeats would be required to check the validity of this result.

3.3.3 : Intracellular cAMP concentration in ABC-inhibited cell lines when treated with IL-1ß and normalised to cell density

Changes in cAMP levels can be stimulated in response to binding of PGs to their requisite receptors. Intracellular cAMP concentrations were therefore determined during the experiments outlined in the previous section.

The general concentrations of cAMP quantified from the competitive ELISA were very low across all cell lines (Figure 3.3.3).

The concentration of cAMP was seen to be highest in unstimulated SH-SY5Y cells lacking IL-1ß, this was the highest value recorded across all cell lines. This group had an 84.6% increase compared to the DMSO control. Reversan-treated SH-SY5Y cells also produced a higher mean concentration of cAMP than the DMSO control (50% mean increase), while concentrations in ceefourin and tariquidar groups dropped dramatically, particularly in cultures treated with ceefourin.

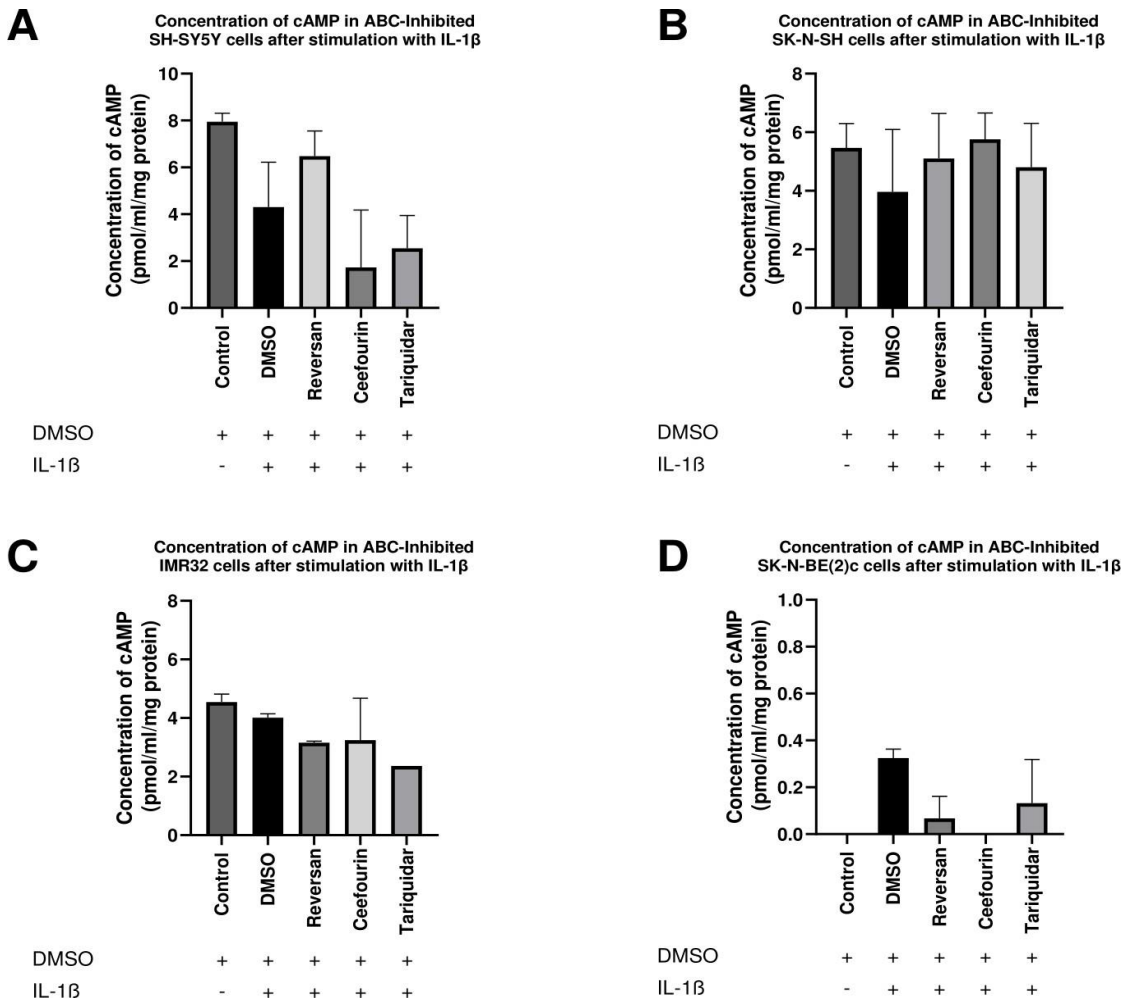


Figure 3.3.3: A measure of cAMP concentrations in SH-SY5Y (A), SK-N-SH (B), IMR32 (C) and SK-N-BE(2)c (D) cell lines. KELLY cells were also assayed but showed no quantifiable levels of cAMP in any samples. The cell density of the KELLY samples used in this assay were considerably lower than the other 4 cell lines which could account for this. Values are mean +/- SD. N=1.

As seen in SH-SY5Y cells, the concentration of cAMP in SK-N-SH cells was higher in unstimulated cells than in the DMSO control. In the SK-N-SH cells, all 3 ABC-inhibited groups showed higher concentrations of cAMP than the DMSO control. Ceefourin-treated cells showed the highest mean concentration (45% increase from DMSO control), followed by reversan (28% increase) and tariquidar (21% increase).

Unstimulated IMR32 cells yet again produced a higher mean concentration of cAMP compared to the DMSO control treated with IL-1ß. The mean concentrations of cAMP quantified in reversan and ceefourin-treated groups was very similar and was marginally lower than the DMSO control (19% and 21% reduction respectively). However, the values deduced from assaying ceefourin-treated cells

carried large variability. ABCB1-inhibited IMR32 cells recorded the lowest mean cAMP concentrations, showing a mean reduction of 41% compared to the DMSO control.

SK-N-BE(2)c cells showed extremely low concentrations of cAMP, to the extent that concentrations in ceefourin-treated cells and Unstimulated cells were unquantifiable. The DMSO control group produced the highest mean concentration of cAMP at just 0.32pmol/ml/mg protein. Reversan-treated SK-N-BE(2)c cells showed the lowest mean concentration of cAMP (80% reduction to DMSO control) while tariquidar repressed cAMP production by 60%.

3.3.4 : Efflux of cysteinyl leukotrienes in ABC-inhibited neuroblastoma cell lines

In addition to prostanoids, leukotrienes are both responsible for mediating cell activities associated with cancer cell biology (i.e. migration) and can be released from cells via the activity of certain ABC transporters (Savari, 2014) (Kooij, van Horssen, Bandaru, Haughey & de Vries, 2012). As such, efflux of these leukotrienes was also assessed after cells had been stimulated to produce CysLTs via the addition of arachidonic acid to the culture media for 48 hours and a calcium ionophore (A23187) for 5 minutes to enhance 5-LOX stimulation by increasing intracellular calcium concentrations.

As seen in Figure 3.3.4, There were treatments across all cell lines that produced levels of cysteinyl leukotrienes that were below the LoQ. Among the treatments used in SH-SY5Y cells, the only treatments that produced a quantifiable amount of CysLT's were reversan and ceefourin (+AA and A23187). Of these two treatments, mean CysLT concentrations were slightly higher in ceefourin-treated cells but with high variability of results, leaving no distinguishable difference.

The only treatment that produced a quantifiable level of CysLT in SK-N-SH cells was the ceefourin group, which gave a mean concentration of 70.3pg/ml media, one of the highest mean concentrations of CysLT's recorded across all cell lines.

Ceefourin-treated cells were the only group to produce quantifiable amounts of CysLT's again in KELLY cells, this time with a mean concentration of 54.8pg/ml media but with higher variability.

IMR32 cells produced slightly more CysLT's than the previously mentioned cells, with very low levels being detected in the vehicle control group (8.1pg/ml media) and slightly more in the reversan group (29.15pg/ml media). Treating IMR32 cells with tariquidar resulted in one of the largest mean concentrations of CysLTs recorded across any of the cell lines with a mean concentration of 104.86pg/ml with relatively low variability across the mean.

The data generated from the cysteinyl leukotriene ELISA is too preliminary to draw a firm conclusion on how ABC transporter expression affects the efflux of CysLTs. With no biological repeats and such a high degree of variability in the level of leukotrienes detected, drawing solid conclusions from this assay would risk overstating the data.

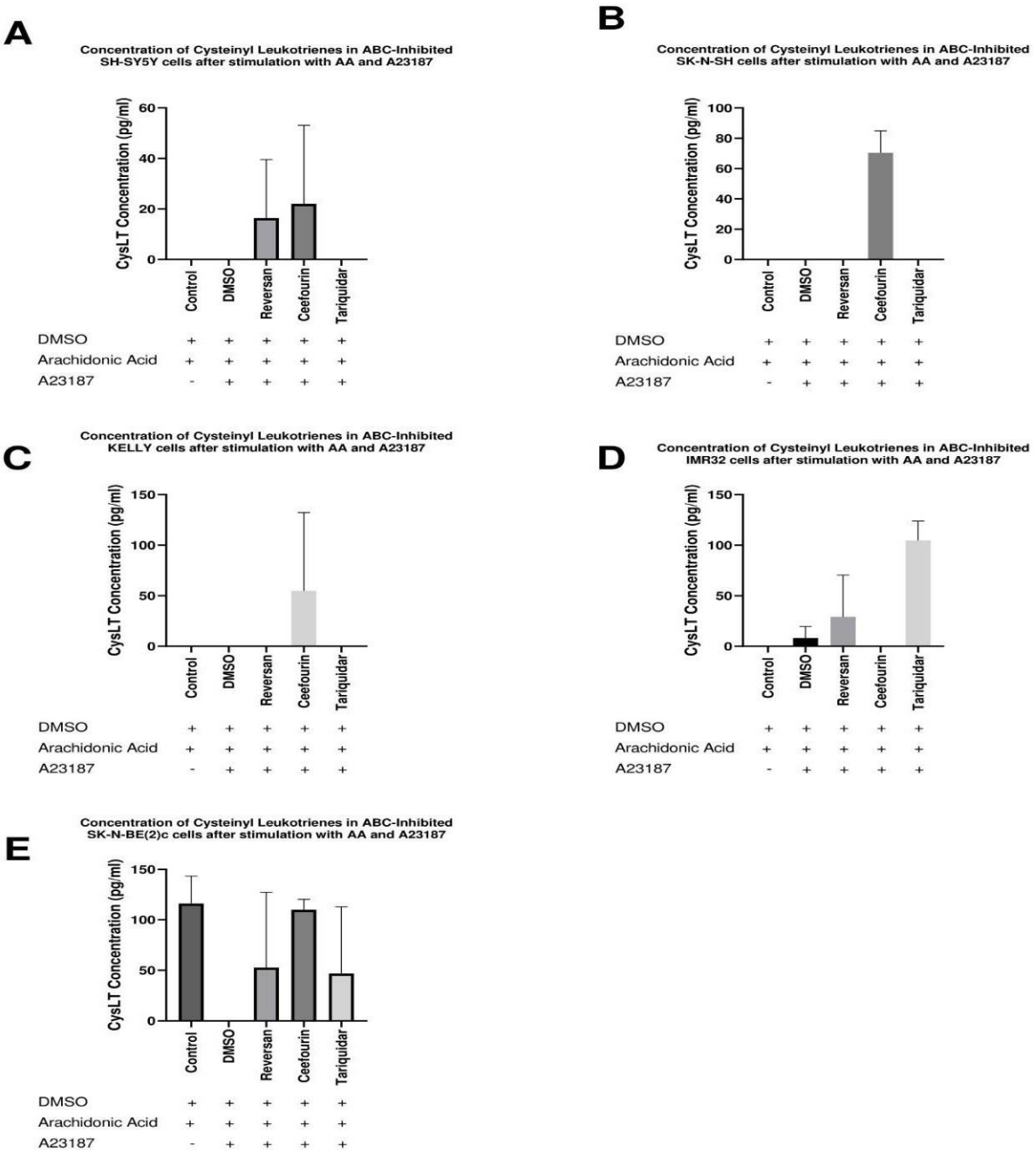


Figure 3.3.4: A measure of cysteinyl leukotriene concentrations in SH-SY5Y (B), SK-N-SH (C), KELLY (D), IMR32 (E) and SK-N-BE(2)c (F) cell lines. Concentrations were not normalised to protein density of samples, but all samples were grown to a similar confluence before being assayed. Each treatment sample was cultured in duplicate wells and assayed in duplicate, resulting in 4 repeats per sample. Values are mean (+/- SD). N=1.

The results of the CysLT ELISA seen in Figure 3.3.4 opened the door to questions about the levels of CysLTR expression in NB cell lines due to the low and inconsistent amounts found in their respective culture media. This led to a qPCR assay of CysLTR. In all 5 cell lines, the level of CysLTR expression was below level of quantification.

Section 4: Discussion

4.1 : Overview of results

Previous evidence has suggested a correlation between MycN amplification, ABC transporter expression and prediction of treatment outcome in NB (Huynh et al., 2012; Porro et al., 2010; Munoz, Henderson, Haber & Norris, 2007). The current work aimed to establish whether this previously documented association between MycN amplification and ABC expression could be established in a range of MycN and non-MycN amplified NB cell lines. In addition to this, the hypothesis that ABC transporter expression was associated with the efflux of prostaglandins and leukotrienes was explored, as the efflux of these eicosanoids can impact tumour proliferation and migration (Yu et al., 2015).

Of the cell lines examined, KELLY and SK-N-BE(2)c cells showed what was expected of a MycN amplified cell line, showing increased expression of ABCB1, C1 and C4 and a downregulation in ABCC3 when compared to SH-SY5Y cells, which are non MycN amplified. IMR32 cells, however, showed no such trends in ABC expression despite being MycN amplified and even showing the greatest migratory potential under basal conditions in the wound healing assays. This could suggest that independent of the substrates of ABCC1 and ABCC4 could have a bigger influence on how aggressively NB cells migrate and metastasise.

The results of the wound healing assays demonstrated clearly the relationship between MycN amplification and a more aggressive phenotype in NB cell lines, particularly IMR32 and SK-N-BE(2)c cell lines. The difference in migratory potential of these cell lines and non-MycN amplified cell lines SH-SY5Y and SK-N-SH provided an initial indication as to why MycN amplification is associated with a worse prognosis and lower rates of survival.

Contrary to this, the wound healing and ELISA assays suggested that inhibition of key eicosanoid transporters ABCC1 and ABCC4 had little effect on NB cell migration, regardless of their MycN status. These results cast a shadow on the original hypothesis that the migratory phenotype of NB cells is

mediated by ABC-driven eicosanoid efflux in MycN amplified cells (Murray et al., 2017; Porro et al., 2010; Kassmer, Rodriguez & DeTomaso, 2018). This is further discussed in the sections below.

4.2 : ABC transporter expression and comparison to previous findings

The gene expression profiles of the 5 NB cell lines examined showed some surprising results; particularly the upregulation of ABCC3 in MycN amplified IMR32 cells. Previous literature exploring the link between MycN amplification and ABC expression has found ABCC3 to be a direct transcription target of MycN in several amplified cell lines (Yu et al., 2015).

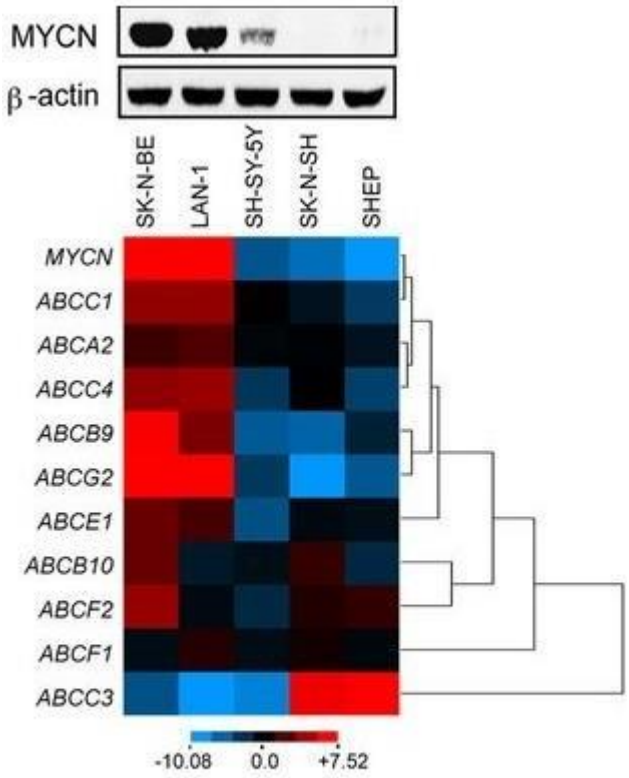


Figure 4.1: The correlation of MycN and ABC transporter gene expression in NB cell lines documented in a study by Porro et al., 2010.

One study by Porro et al (2010) exploring the correlation between MycN amplification and ABC expression found ABCC3 expression to be repressed in both MycN cell lines studied, SK-N-BE(2)c and LAN-1. These findings correlate with what was found in this project, which saw a 3-fold downregulation of the ABCC3 transporter in SK-N-BE(2)c cells relative to the non-MycN amplified SH-SY5Y's. In Porro's study, the expression of key ABCs involved in eicosanoid efflux (i.e. ABCB1, ABCC1 and ABCC4) in SK-N-SH cells remained relatively similar to that seen SH-SY5Y, however, ABCC3 expression in SK-N-SH expression was largely upregulated.

The trends shown by MycN amplified NB cell lines and ABC transporter expression described by Porro et al are followed by SK-N-BE(2)c and KELLY cells in this project as they both displayed upregulations in ABCC4 and downregulations in ABCC3, to different extents. ABCC1 expression remained

relatively unchanged in KELLY cells in comparison to SH-SY5Y's. IMR32 cells, however, which are thought to also be MycN amplified (Zaatiti et al., 2018), did not follow these trends in ABC transporter expression. The events leading up to tumour formation involves mutations and dysregulation of the

expression of many different proteins and transcriptional regulators, so the ABC expression profile seen in IMR32 cells could suggest that the expression of ABC transporters in NB cell lines can be influenced by factors other than MycN. This has been demonstrated in other studies. For example, Takwi et al (2014) have shown that constitutive androstane receptor (CAR), which modulates ABC transporter expression, can be controlled by the activity of the microRNA, miR-137 (Takwi et al., 2014). MicroRNAs have also been implicated in ABC transporter repression (i.e. Lu et al., 2017). It is therefore possible that, despite IMR32 cells being MycN amplified, other factors regulating the expression of ABCs can interfere with the changes otherwise associated with MycN status. Although, MycN amplification still appears to have a strong influence on their expression given the profile found in KELLY and SK-N-BE(2)c cells.

Despite the lower expression of ABCC1 and ABCC4 in IMR32 cells, MycN amplification is still observed to be associated with a more aggressive migratory phenotype. This is reinforced by the results seen in Figure 3.2.1B, which shows that IMR32 cells possessed the highest migratory potential of all 5 NB cell lines examined. These findings could suggest that the migratory potential of NB cells may be independent of ABC transporter expression but is still likely to be influenced by MycN status. These findings could be further explored using different methods of halting proliferation of cells such as exposure to UV light or pharmacological inhibition using streptomycin instead of culturing cells in serum-deficient media as this could have affected their ability to migrate.

4.3 : Migratory potential of NB cells and association with ABC expression

Examining the rate of migration of NB cells in the presence of ABC inhibitors only strengthened the hypothesis that expression of ABC transporters has no effect on migration, given that no cell lines assayed showed any change in mean migratory potential over a 3-day period when ABCB1, ABCC1 and ABCC4 were inhibited.

The apparent lack of affect that ABC transporter inhibition had on migration of NB cells could suggest that other factors play a more pivotal role in contributing to migratory potential. For example, histone deacetylase 6 (HDAC6), a member of the deacetylase family located in the cytoplasm, has been heavily implicated in promoting the migration of NB cells and is commonly overexpressed similarly to ABC transporters. This protein exerts its effects by reversibly regulating the acetylation of cytosolic proteins such as α-tubulin and cortactin, disrupting the extracellular matrix and making it easier for cells to disseminate (Zhang et al., 2014). It is also possible that HDAC6-induced migration is associated with

MycN amplification as they have been documented to interact and cause a downstream alteration of genes involved in carcinogenesis such as transglutaminase 2 (TGM2) (Liu et al., 2006).

Epigenetic factors have also more recently been implicated in NB cell biology, with pronounced epigenetic alterations more prominent in aggressive tumour types (Parodi et al., 2016). Methylation across these tumour types should therefore be considered, in addition to ample changes in gene expression, when considering phenotypes. This does not imply that migratory potential of NB cells is not influenced by ABC inhibition, but given the marginal differences seen in migration rates between inhibited groups and vehicle controls, it is likely that other factors could be playing more of a role.

4.4 : Is PGE2 efflux affected by ABC expression?

Preliminary data examining PGE2 concentrations in ABC-inhibited NB cell lines contradicted the data shown by the PCR analysis when considering previous literature, as IMR32 cells showed the highest efflux of PGE2 under basal conditions among all cell lines. Given that ABCC4 is the primary transporter for PGE2 (Kochel, Goloubeva & Fulton, 2016), the relatively low ABCC4 expression in IMR32 would suggest that PGE2 efflux should be reduced, however, it was the highest amongst SH-SY5Y, IMR32 and SK-N-BE(2)c cells in control groups. The PCR data also showed that IMR32 cells had lower expression of ABCC1 than both SH-SY5Y and SK- N-SH cells, showing that ABCC1 mediated efflux of PGE2 could not have compensated for the low levels of ABCC4. Some data could suggest that ABCC4 is playing a role in PGE2 efflux in these NB cells, given that small reductions in media PGE2 levels are observed following inhibition with ceefourin. However, these reductions are frequently too small or variable to be convincing without additional experimentation.

In addition, the influence of ABC transporter functional redundancy cannot be ruled out. Indeed, as PGE2 is a substrate for more than 1 ABC transporter (Cole, 2014), inhibiting the activity of just one may not reduce the efflux of PGE2 if other transporters are able to compensate for its loss (Cole, 2014). In order to determine this, additional experiments in which multiple ABC transporters were inhibited would need to be performed and compared against single inhibitor results.

Given the low levels of PGE2 release observed under basal conditions in the NB cell lines tested, stimulation of PGE2 production with IL-1β was examined in the belief that if greater levels of PGE2 were present, higher levels of efflux would be observed, thereby increasing the likelihood of observing an impact of ABC transporter inhibition. IL-1β is well documented to be present in the NB tumour microenvironment (Fultang et al., 2018). Treating cells with the proinflammatory cytokine IL-1β is known to stimulate the COX-2 pathway and lead to an increased production of eicosanoids including

PGE2 (Huang et al., 2019). The results shown after treating NB cell lines with IL-1β showed no clear effect on PGE2 concentration in the media, which could suggest that the experiment failed to elicit the expected increases in PGE2 production. Without measuring COX-2 activity directly, or indeed intracellular PGE2 levels, this cannot however be confirmed with any certainty.

There is some evidence in the data to suggest that ABC-inhibition had an effect on PGE2 efflux, particularly in figures 3.3.1-B and 3.3.2-C. Figure 3.3.1-B showed a large increase in PGE2 efflux in ABCC1-inhibited IMR32 cells. This could possibly demonstrate the synergistic relationship between ABCC1 and ABCC4. With PGE2 being a substrate of both transporters, it is possible that the inhibition of ABCC1 caused a larger efflux via the ABCC4 transporter, which is already known to have higher selectivity to PGE2 than ABCC1. The differences in PGE2 efflux between the groups, however, was not significant and therefore it is not possible to draw definitive conclusions. The data from figure 3.3.2C does indeed suggest that inhibition of ABC transporters caused a large decrease in PGE2 release in KELLY cells when compared to the DMSO vehicle control, however, the variability of data in the control group is too significant to merit this interpretation and repeat experiments would be required to build a firm conclusion. Considering these results, the inhibition of ABCC1 and ABCC4 generally appeared to have little effect on PGE2 efflux in NB cell lines. These findings further strengthen the hypothesis that ABC transporter expression has no effect PGE2 release. This also supports the results from scratch assays, given that PGE2 has previously been correlated with cell migration rates in other cancers (Mayoral, Fernández-Martínez, Boscá & Martín-Sanz, 2005; Kim, Lakshmikanthan, Frilot & Daaka, 2010).

The results shown in Figure 3.3.3 could potentially suggest that intracellular cAMP concentrations in MycN amplified NB cell lines are affected by the activity of ABC transporters. In both IMR32 and SK-N-BE(2)c cell lines, cAMP concentrations in all ABC-inhibited groups were below that of the vehicle control. A consequence of higher PGE2 release would be increased activation of EP receptors and therefore, increased production of cAMP as a key second messenger (O'Callaghan & Houston, 2015). cAMP concentrations in SH-SY5Y and SK-N-SH appear largely unaffected by ABC inhibition, although high variability in the data was observed. There is a possibility, however, that MycN amplified cell lines may have been affected. The variability in results seen in IMR32 cells was very low in DMSO and reversan treated groups, meaning the decrease in cAMP concentrations seen in ABCC1 inhibited cells could implicate the transporter in reduction of EP receptor activation, however this experiment would need to be repeated to further test the legitimacy of these findings.

The levels of cAMP found across SK-N-BE(2)c cells were extremely low, showing again that higher expression of ABCC1 and ABCC4 does not appear to influence cAMP concentrations. Again, this

correlates with other findings suggesting that the influence of ABC transporter activity on PGE2 release and the associated changes in cell behaviour (i.e. migration) that could be predicted. This would need to be explored further with more biological repeats to validate these findings however.

4.4: Production and Efflux of CysLT's in NB cell lines

The significance of CysLT's in tumour protection/promotion is currently better studied in intestinal carcinomas such as colorectal cancer (Savari, 2014; Burke et al., 2016) and thus their effects on neuroblastoma cell lines are not currently well documented. There currently is no assay available that can detect a specific leukotriene exclusively, so the ELISA kit used in this project detected for general CysLTs. Despite this, Figure 3.3.4 shows a complete lack of any cysteinyl leukotriene efflux under basal conditions in all NB cell lines with the exception of SK-N-BE(2)c cells supplemented with arachidonic acid but not with a calcium ionophore. Given that the expression of CysLTR was unquantifiable in all 5 cell lines, these findings are unsurprising and could suggest that cysteinyl leukotrienes do not play as much of a role in tumour-promoting inflammation as other inflammatory mediators such as PGE2. The results also suggest that treatment with a calcium ionophore does not increase production of CysLT's in NB cell lines, as has been documented in other cell types (Talahalli, Zarini, Sheibani, Murphy & Gubitosi-Klug, 2010; Przygodzki, Sokal & Bryszewska, 2005).

Other types of cancer however are well documented to benefit heavily from CysLT production and receptor activation. Colorectal cancer is the best example of this. Increased expression and activation of CysLTR1, a characteristic of aggressive colorectal adenocarcinomas, is well documented to aid carcinogenesis and is associated with increased cellular migration, proliferation and survival as a result of the nuclear translocation of Erk 1/2 (Burke et al., 2016).

Contrary to the findings of this study however, there have indeed been previous studies implicating cysteinyl leukotrienes in the survival and proliferation of NB cell lines. The results of a 2018 study by Sveinbjörnsson et al (2018) encouraged this hypothesis. This study also measured the concentration of CysLT's in cell culture media supplemented with arachidonic acid in SK-N-BE(2) and SH-SY5Y cells. The study also found that supplementation of leukotrienes B4 and D4 to SK-N-BE(2) cells increased % cell viability relative to a vehicle control after 96 hours via MTT assay.

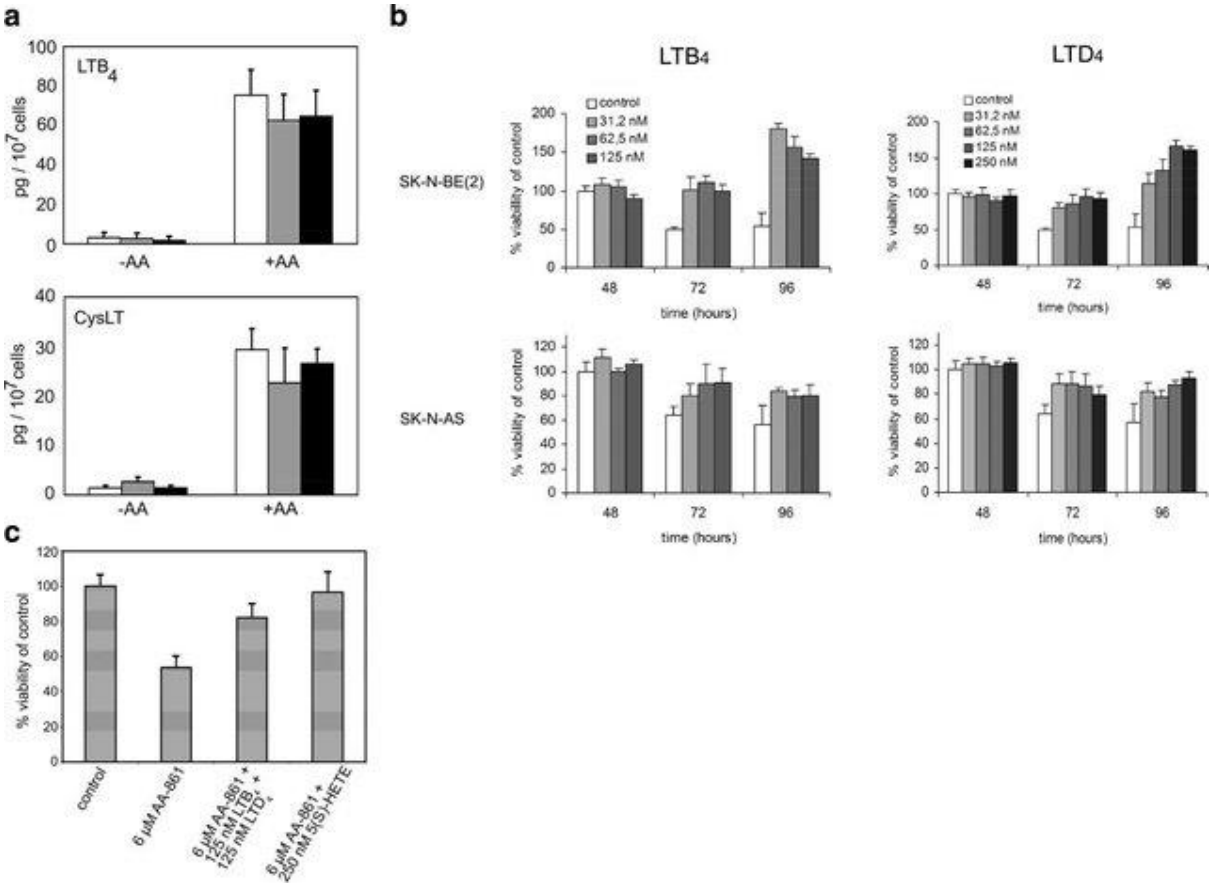


Figure 4.2: Sveinbjörnsson et al found a considerable increase in the production of LTB4 and CysLT in cell homogenates that had been treated with 2mM ATP, 2mM Ca^{2+} and 80µM arachidonic acid for 10 minutes at 37°C (reproduced from Sveinbjörnsson et al., 2018)

Figure 4.2-a shows little to no production of either LTB4 or CysLT's in SK-N-BE(2)c (Open bar), SK-N-AS (grey bar) or SH-SY5Y (black bar) cells when no arachidonic acid is supplemented. However, when supplemented with 80µM arachidonic acid, CysLT production increases dramatically in all 3 cell lines.

Interestingly, the concentration of CysLT's transported by SH-SY5Y cells was unquantifiable in all treatment groups with the exception of ABCC1 and C4 inhibited cells, even when treated with arachidonic acid. The concentrations of CysLT's in SH-SY5Y media in Sveinbjörnsson's study does not fall far off from the concentrations seen from the SK-N-BE(2)c cells. The reason for the difference seen in the 2 studies could be attributed to a number of factors. One reason, for example, could be the difference in approach taken to prepare ELISA samples. This study used culture media for the ELISA, whereas Sveinbjörnsson's study used cell homogenate, meaning that the concentrations of CysLT found in their samples was from both intra and extracellular origin. This means it is possible that the SH-SY5Y cells used in this study were in fact producing cysteinyl leukotrienes, but they were not transporting them out of the cell. Strangely, however, the extracellular concentrations of CysLT's increased in SH-SY5Y's when they were treated with reversan and ceefourin. Given that the main transporter of cysteinyl leukotrienes is ABCC1, this finding was surprising.

The concentration of arachidonic acid used to supplement cells in Sveinbjörnsson's study, however, was 80µM, whereas this study only used 10µM. This is likely a factor in the differences in the extracellular concentration of CysLT's between the 2 sets of results as the 5-lipooxygenase enzyme has 8 times more substrate to convert.

Section 5: Conclusions and future perspectives

The association between MycN amplification and trends in ABC transporter expression has not been shown to hold-true for all NB cell lines tested in this study. Furthermore, no relationship between ABC transporter expression and activity was found in relation to cellular migration or the release of PGE2 and CysLTs. This does not however, entirely rule-out the influence of ABC transporters on NB cell behaviour. An important follow up to this project would be to confirm the effects seen in the ELISA assays were on-target effects, caused directly by ABC-transporter inhibition. This could be achieved by repeating the experiments after either silencing the ABC transporters via use of siRNA or doing the opposite and overexpressing the transporters to see if the results can be mimicked.

Indeed, although changes in proliferation were originally to be included in the current work, time pressures did not allow this work to be finalised. As such, it remains possible that ABC transporter levels may indeed alter levels of proliferation in these cells. This should be assessed using a direct measure of proliferative activity. One such method would be the use of 5-ethynyl-2'-deoxyuridine (EdU) incorporation assays (Salic & Mitchison., 2008), which involves the use of Edu to bind to the

nuclei of proliferating cells and fluoresce when incorporated in conjunction with a dye such as Alexa Fluor (Krishan & Hamelik, 2010). Other angles of research such as cell invasion assays and cell death assays could also be explored. Cell invasion assays are similar to scratch assays used in this project but give a more accurate representation of cell motility and aggression as their interaction with the ECM can be observed.

The time constraints on this project also restricted repetition of crucial data sets, particularly in the ELISA assays. Limited replicates made it difficult to draw accurate conclusions from the data gathered and thus need to be explored further.

In addition, to provide a more complete assessment of MycN status and transporter levels, a wider range of NB cell lines should be assessed for their ABC transporter profile. This could include a variety of MycN amplified cell lines such as SK-N-DZ, LAN-1 and SiMA, and a number of non-MycN cell lines, namely SHEP and SK-N-AS cells.

Although the current study focussed on a single prostanoid (PGE2), in addition to CysLTs, ABC transporters have been described as transporting a wide-array of eicosanoids (Kassmer, Rodriguez & DeTomaso, 2018), which could influence NB cell behaviour. As such, a broader screening approach using LCMS-based lipidomic analysis (also following ABC transporter inhibition) may elucidate an ABC-transporter secretory profile not identified in the current, limited dataset. A similar approach has previously been reported, examining fatty acids specifically (Prasinou et al., 2017).

ABC transporter activity and eicosanoid release may well be one factor associated with the aggressive phenotype of high-risk NBs, yet both tumour microenvironment and other genetic (and epigenetic) factors are likely to combine to influence this. As such, further study in this area is warranted and may yet identify alternate targets in the treatment of aggressive tumours.

To better determine whether the results are a direct result of MycN amplification, a series of repeat experiments could be carried out under the same conditions i.e. under the effects of ABC-inhibition in the same cell lines but this time using siRNA to suppress MycN and observing whether the same effects are seen.

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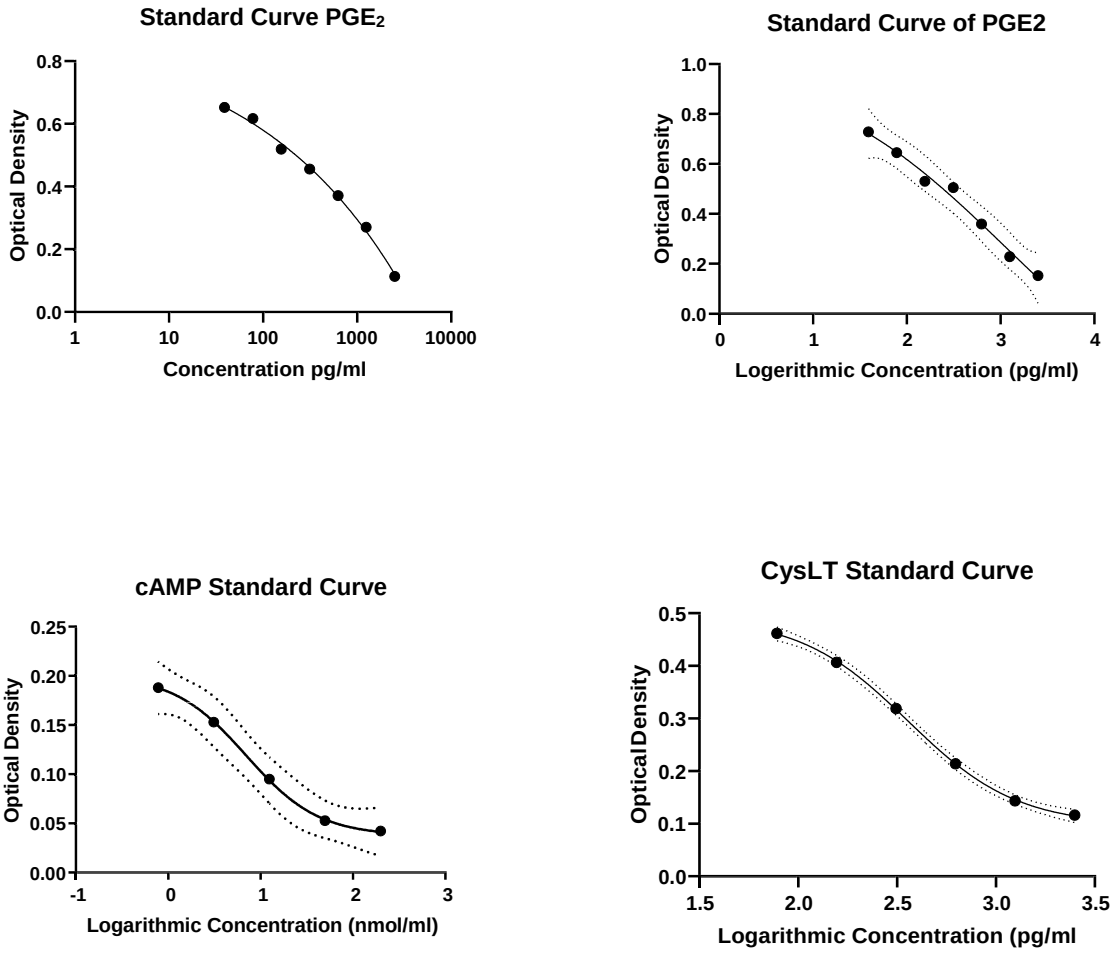
Appendix



Appendix 1: Standard curves used to interpolate unknown values generated from competitive ELISA assays.