Analysing the factors that regulate expression of blood-brain barrier drug transporter proteins

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy (PhD) in the Faculty of Biology, Medicine and Health

2021

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List of abbreviations

ABC	ATP-binding cassette
ACM	Astrocyte-conditioned medium
AhR	Aryl hydrocarbon receptor
ATP	Adenosine 5-triphosphate
BBB	Blood-brain barrier
BCRP	Breast resistant cancer protein
BECs	Brain endothelial cells
BSA	Bovine serum albumin
BMEC	Brain microvessel endothelial cell
Calcein-AM	Calcein acetoxymethyl ester
cAMP	Cyclic adenosine monophosphate
CAPE	Caffeic acid phenethyl ester
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CAR	Constitutive androstane receptor
CCRP	Cytoplasmatic CAR retention protein
cGMP	Cyclic guanosine monophosphate
CITCO	6-(4-chlorophenyl)-imidazo(2,1-b)thiazole-5-carbaldehyde
cm	Centimetre
CMFDA	Chloromethylfluorescein diacetate
CNS	Central nervous system
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CYP	Cytochrome P450
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DX	Dexamethasone
EDTA	Ethylenediaminetetraacetic acid
EP-1	Prostaglandin E2 receptor 1
ERK	Extracellular signal-regulated kinase
ET	Endothelin receptor.
FBS	Foetal bovine serum
FXR	Farnesoid X receptor
GS-MF	Glutathione methyl fluorescein
GR	Glucocorticoid receptor
GRIP1	Glutamate receptor-interacting protein 1
x g	Gravity
h	Hour or hours
HBSS	Hank’s balanced salt solution
hCMEC/D3	Human cerebromicrovascular endothelial cell line D3
HC	Hydrocortisone
HEPES	4-(2-hydroxyethyl)-1-1piperazineethanesulfonic acid
HNF4α	Hepatocyte nuclear factor 4α
HNK	Honokiol
hsp90	Heat shock protein 90
H33342	Hoechst 33342
Iκ-Bα	Inhibitor of nuclear factor kappa B alpha

IKK-α	Inhibitor of nuclear factor kappa-B kinase subunit alpha
IKK-β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1	Interleukin-1
IL-1R1	Interleukin-1 receptor type 1
IL-1RA	Interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK4	Interleukin-1 receptor-associated kinase 4
JAM	Junctional adhesion molecule
JNK	c-Jun N-terminal Kinase
KDa	Kilo Dalton
l	Litre
LBD	Ligand-binding domain
L-SFN	L-sulforaphane
LXR	Liver X receptor
mA	Milli Ampere
MAPK	Mitogen-activated protein kinases
MKK/MEK/ MAPKK	Mitogen-activated protein kinase kinase
MEKK/MAP3K/ MAPKKK	Mitogen-activated protein kinase kinase kinase
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minutes
μM	Micromolar
mM	Millimolar
MF	Mifepristone
MRP	Multidrug resistance-associated protein
MSD	Membrane spanning domain
MyD88	Myeloid differentiation primary response 88
NBDs	Nucleotide-binding domains
NF-κB	Nuclear factor kappa B
NIK	Nuclear factor-kappa-B-inducing kinase
nm	Nanometre
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NcoR1	Nuclear receptor corepressor 1
OATs	Organic anion transporters
Oatps	Organic anion transport polypeptides
OCT	Organic cation transporter
p38	p38 mitogen-activated protein kinases.
p50/p100/p105	Nuclear factor kappa B subunit
PBECs	Porcine brain endothelial cells
PBS	Phosphate buffered saline
PCN	Pregnene-16α-carbonitrile
PDS	Plasma derived serum
PKC	Protein Kinase C
PKCβ1	Protein Kinase C beta 1
PKCβ2	Protein Kinase C beta 2
PLA2	Phospholipases A2

PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
PVDF	Polyvinyldene fluoride
PXR	Pregnane X receptor
rpm	Revolutions per minute
mRNA	Messenger ribonucleic acid
RAF	Rapidly accelerated fibrosarcoma
RelA/RelB/c-Rel	RELA proto-oncogene, NF-kB subunit
RF	Rifampicin
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide
SRC1	Steroid receptor coactivator-1
TACE	Tumour necrosis factor-α-converting enzyme
TAK1	Mitogen-activated protein kinase kinase kinase 7
TBS-T	Tris-buffered saline Tween-20
TCPOBOP	1,4-bis-(2-(3,5-dichloropyridyloxy)) benzene
TEER	Transendothelial electrical resistance
TLR4	Toll-like receptor 4
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TJ	Tight junction
TMDs	Transmembrane domains
TRAF6	Tumour necrosis factor receptor associated factor 6
TRIS	Trizma base
UDP	Uridine
v	Volume
VDR	Vitamin D receptor
VEGF	Vascular endothelial cell growth factor
w	Weight
ZO	Zonula occludens

Abstract

The University of Manchester

Yu Siong Ho

Doctor of Philosophy

Thesis title: Analysing the factors that regulate expression of blood-brain barrier drug transporter proteins

21/12/2020

The blood-brain barrier (BBB) is a highly selective physical and enzymatic barrier formed by the endothelial cells of cerebral microvessels. It helps to regulate and restrict the entry of endogenous and exogenous compounds into the central nervous system. A number of ATP-binding cassette (ABC) transporters, including ABCB1, ABCG2 and ABCC5, are expressed in BBB brain endothelial cells and efflux an array of substances into the systemic circulation, impairing the pharmacotherapy of brain disorders.

Regulation of ABC transporter expression and activity is highly complex and little is known of the precise regulatory mechanisms in BBB endothelial cells compared to other systems, for example liver, with expression and activity influenced by therapeutic drugs, xenobiotics and pathophysiological conditions, for example inflammation. The nuclear receptors pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR) and retinoid x receptor (RXR) are major regulators of ABC efflux transporter activities yet their roles in regulating ABC transporters in BBB is not well understood. Therefore, the objectives of this thesis were to: (i) identify optimal culture conditions for an *in vitro* BBB model comprising primary porcine brain endothelial cells (PBECs) (ii) characterise ABCB1, ABCG2 and ABCC5 expression and activity in PBECs (iii) determine the expression profile of GR, PXR, CAR and RXR nuclear receptors in PBECs (iv) determine the role of GR, PXR, CAR, RXR nuclear receptor signalling pathways on the expression and activity of ABCB1, ABCG2 and ABCC5 in PBECs (v) determine the effects of the inflammatory mediator interleukin-1β (IL-1β) on expression and activity of ABCB1, ABCG2 and ABCC5 in PBECs and (vi) determine the effects of anti-inflammatory glucocorticoids on expression and activity of ABCB1, ABCG2 and ABCC5 in PBECs.

Western blotting confirmed expression of ABCB1, ABCG2 and ABCC5 transporters and PXR, CAR, RXR and GR receptors in PBECs. PBECs possessed higher levels of ABCB1, ABCG2 and ABCC5 activities when maintained in Dulbecco's Modified Eagle medium (DMEM) compared to DMEM:Ham's F12 (1:1) or M199 medium.

Activation of PXR with rifampicin or GR with dexamethasone and hydrocortisone significantly up-regulated ABCB1, ABCG2 and ABCC5 transport activities and expression, which were abrogated by co-treatment with PXR agonist (rifampicin) and antagonist (L-sulforaphane) or GR agonist (dexamethasone, hydrocortisone) and antagonist (mifepristone). Both CAR agonist (CITCO) and inverse agonist (meclizine) significantly down-regulated ABCB1, ABCG2 and ABCC5 transporter activities and expression. Expression of PXR, CAR and RXR at the protein level was not significantly affected by treatment of PBECs with PXR and CAR agonists and antagonists/inverse agonist, but was significantly up-regulated by treatment with GR agonists, demonstrating distinct PXR/CAR and GR pathways are involved in the regulation of ABC transporter activity and expression.

Interleukin-1β significantly up-regulated the protein expression of PXR, CAR and RXR and the activity and expression of ABCB1, ABCG2 and ABCC5. The use of inhibitors of the NF-κB pathway, namely caffeic acid phenethyl ester, honokiol and SN50 failed to revert the IL-1β-induced up-regulation of ABCB1 ABCG2 and ABCC5 transporter activities, but significantly down-regulated the protein expression of ABCB1, ABCG2, ABCC5 and of the nuclear receptors PXR, CAR, RXR and GR. Furthermore, inhibition of the MAPK JNK and ERK1/2 with SP600125 and FR180204 respectively, significantly abrogated the IL-1β-mediated upregulation of ABCB1, ABCG2 and ABCC5 transport activity.

In summary, this thesis has shed light, for the first time in some cases, on the signalling pathways involved in regulating BBB transporter (ABCB1, ABCG2 and ABCC5) and nuclear receptor (PXR, RXR, CAR and GR) expression and activity, and demonstrates PBECs can prove valuable in deciphering how xenobiotics, drugs and endogenous mediators regulate the BBB's xenobiotic-sensing/detoxification mechanisms. The findings of this thesis have further advanced the knowledge of the different factors that govern the activity of ABCB1, ABCG2 and ABCC5 transporters expressed in BBB. The further exploration of these signalling pathways is crucial to facilitate therapeutic drugs to cross the BBB in treating wide range of neurological disorders which are currently known to be incurable.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Yu Siong Ho

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The Author

Yu Siong Ho graduated with a 1st class Bachelor of Science (Hons) with Honours in Biotechnology from the University of Tunku Abdul Rahman, Malaysia, in 2010. In 2016, he obtained an MSc in Medical Sciences from University of Tunku Abdul Rahman. During these studies he gained expertise in designing *in vitro* disease models, extracting natural compounds, formulating bioactive compounds and analysing drug-cell interactions based on cellular metabolomic, proteomic and genomic assays. One of his formulated extraction methods has earned him a patent in 2016, and a gold award for the Best Research Invention in 2018, awarded at 29th International Invention & Innovation Exhibition (ITEX) in Kuala Lumpur Convention Centre, Malaysia.

Acknowledgments

First and foremost, I would like to take this golden opportunity to express my deepest sincere gratitude to my supervisor Dr Jeffrey Penny for his thoughtful criticism of this thesis and manuscript, on-going guidance, invaluable suggestion, enduring support, kindness, and patience in teaching me the techniques of every related lab work and the correct mind-set in conducting this project. Indubitably, his wisdom and intelligence never cease to inspire me to move on upon shuttered doors. Next, I would like to express my appreciation to Dr Pablo Torres-Vergara for taking time out of his busy schedule to evaluate and provide invaluable advice and guidance to me regarding the progress of my project and manuscript preparation. I would like to thank my co-supervisor Dr Constantinos Demonacos as well for his thoughtful advice and ideas in conducting my research. Furthermore, not to forget Living Cost Support Fund from the University of Manchester, The CISN hardship fund from churches together in Britain and Ireland and The Leche Trust for their generous financial support in the 4th year of my PhD.

I also wish to express my appreciation to the University of Manchester for providing all the laboratory facilities needed for completing this research work. Special thanks to all the scientific officers for their help in providing the necessary training for the use of lab equipment. I feel very grateful for their assistance, advice, and keen sense of humour so necessary for going through the challenges of research.

Besides, my heartfelt gratitude is also dedicated to my friends and lab-mates, Maryam Shubbar, Atheer Al-Zurfi, Jessica Carter, Carlson Carvalho, Amenah Alrabeea, Sabrina, Sukamto and Debbie Fischer, for their kindness, generosity, encouragement, friendship, cooperation, unaccountable guidance, and their willingness to share their experience with me and the support given to go this far in this project. It has been my greatest privilege in life to be blessed by their acquaintance and companionship through the ups-and-downs of my life.

Furthermore, my faithful thankfulness and love dedicated to my wonderful supportive family. I will always be strong because I know that they have unbreakable faith in me. Their unfading love and incessant support is one of my greatest motivations throughout the journey of this project.

Once again, a deep sincere thanks to all who had contributed throughout my preparation of this thesis and those who have helped me, one way or another. All of them have become my motivation and made me more confident to complete my project.

Above all, I thank God for being with me all the way, throughout the times of my highs and lows, as well as the twist and turn along the road to the completion of my work. His amazing grace and love that never ends. I can do all things through Him who strengthens me.

Publications

Article:

Torres-Vergara P, Ho YS, Espinoza F, Nualart F, Escudero C, Penny J. 2020. The constitutive androstane receptor and pregnane X receptor in the brain. Br J Pharmacol., 177(12): 2666 - 2682.

Conference Abstracts:

Yu Siong Ho, Pablo Torres-Vergara and Jeffrey Penny. Analysing the factors that regulate expression of blood-brain barrier drug transporter proteins. Annual meeting of the British Pharmacology Society, December 2019, Edinburgh International Conference Centre, UK. Proceedings at
https://bpspubs.onlinelibrary.wiley.com/doi/abs/10.1111/bph.15035

Yu Siong Ho, Pablo Torres-Vergara and Jeffrey Penny. Analysing the nuclear receptors that regulate expression of blood-brain barrier drug transporter proteins. Annual Postgraduate Research Showcase, September 2018, Whitworth Hall, University of Manchester, UK.

Yu Siong Ho and Jeffrey Penny. Analysing the roles of nuclear receptors on blood-brain barrier drug efflux transporter proteins. Annual Postgraduate Research Showcase, September 2017, Michael Smith building, University of Manchester, UK.

Chapter 1

Introduction

1.1 Background of blood-brain barrier

The adult human brain possesses close to 100 billion neurones and controls a plethora of functions including motor control (Hall & Guyton, 2011), perception (Jennifer, 2014), arousal (Kandel et al., 2000), homeostasis (Guyton & Hall, 2006), learning and memory (Hall & Guyton, 2011) and motivation (Armony, 2013). However, this organ is susceptible to a multitude of untreatable disorders at every stage of life (McEwen & Morrison, 2013). Neurodegenerative disorders are among the most serious health issues causing enormous economic burden to our society. Analysis conducted in 2010 revealed that there were approximately 45 million cases of brain disorders in the UK, costing £115 billion *per annum* (Fineberg, 2013). According to the recent data compiled by Neurological Alliance in 2019, it was estimated that in 2018, there were 16.5 million cases of individuals having neurological condition(s) in England alone, equating to at least 1 in 6 people living with one or more neurological condition(s), costing the NHS more than £4.4 billion (Neuro Numbers 2019). Despite the unprecedented development of promising drugs, an ability to treat brain disorders is still greatly impaired by the blood-brain barrier (BBB) (Oller-Salvia et al. 2016).

The blood-brain barrier is a dynamic physical barrier associated with brain capillaries, functions to maintain brain homeostasis, regulate brain nutrient supply and prevent brain toxicity by restricting entry of deleterious molecules and cells from the blood (Persidsky et al., 2006; Stamatovic et al., 2008; Baeten and Akassoglou, 2012). The permeability of the BBB is limited due to expression of tight junction proteins between adjacent endothelial cells of the microvascular capillaries. Tight junctions allow the passage of selected ions and small, polar molecules, forcing the majority of molecular traffic to cross the BBB through transcellular pathways (Begley & Brightman, 2003; Wolburg et al., 2009; Cardoso et al., 2010). Solute permeability across the BBB is in agreement with Lipinski's "rule of five", where molecules with a weight of ≤ 400 – 500 Da, an oil/water partition coefficient (LogP) of ≤ 5, polar surface area between 60 and 90 Å, hydrogen bonding of ≤ 5 H-bond donors and ≤ 10 H-bond acceptors and molecular flexibility with the number of rotatable single bonds ≥ 10 may cross the BBB (Lipinski, 2004, Pajouhesh & Lenz, 2005; Mikitsh & Chacko, 2014; Fong, 2015). For example, some gases and low molecular weight lipophilic molecules (≤ 400-500 Da) can easily cross the BBB through passive diffusion. On the other hand, relatively small polar molecules, such as glucose and amino acids, must be transported across endothelial cells via specific solute

transporters, such as the GLUT1 facilitative glucose transporter (Fischer, Gottschlich & Seelig, 1998; Tsuji, 2005).

ATP-binding Cassette (ABC) efflux transporters are major contributors to the highly restrictive function of the BBB (Miller 2015a). The ATP-binding cassette transporters, ABCB1 (MDR1, P-glycoprotein), ABCG2 (BCRP, breast cancer resistance protein) and ABCC5 (MRP-5, multidrug resistance-associated protein-2) expressed in the BBB are involved in the transport of endogenous physiological compounds, including hormones and numerous metabolites. (Schinkel & Jonker, 2012; Tamima et al., 2014; Fletcher et al., 2016). However, therapeutic drugs are also substrates for these efflux transporters and consequently the latter can interfere with drug delivery to the CNS and pose a huge challenge for the treatment of some brain disorders (Montesinos et al., 2014; Pawlik et al., 2005; Eckford & Sharom, 2006; Ebert et al., 2005; Dankers et al., 2012; Wielinga et al., 2003; Jedlitschky et al., 2000). The combination of highly restrictive tight junction complexes and ABC efflux transporters impact drug penetration to such an extent it has been estimated that approximately 98% of therapeutic agents developed for treatment of neurological diseases failed to cross the BBB (Ghose et al., 1999; Lipinski et al., 2004; Pardridge, 2015).

Currently, ABCB1, ABCG2 and ABCC5 are among the ATP-binding cassette transporters that are reported to be highly expressed in human BBB, however the detailed mechanisms regulating the expression and activities of ABCB1, ABCG2 and ABCC5 efflux transporters are well established in hepatocyte (Hunter et al., 2017; Gabbia et al., 2018) but not in the BBB. Therefore, an investigation of the regulatory pathways controlling the expression and activities of efflux transporters in the BBB is essential. Herein, the expression of efflux transporters in BBB endothelial cells and the role of nuclear receptors in regulating their expression are discussed.

1.2 Structural components of blood-brain barrier

The blood-brain barrier is composed of highly specialised endothelial cells, which are surrounded by pericytes, basal membrane, astrocyte end-feet processes and neurones (Figure 1.1). These different cell types interact with one another and form the so-called neurovascular unit (Muoio, Persson & Sendeski, 2014). This neurovascular unit represents a dynamic

interface that completely separates the brain compartment from the blood compartment, while maintaining a rigorous system of exchange between both compartments (Takano et al., 2006).

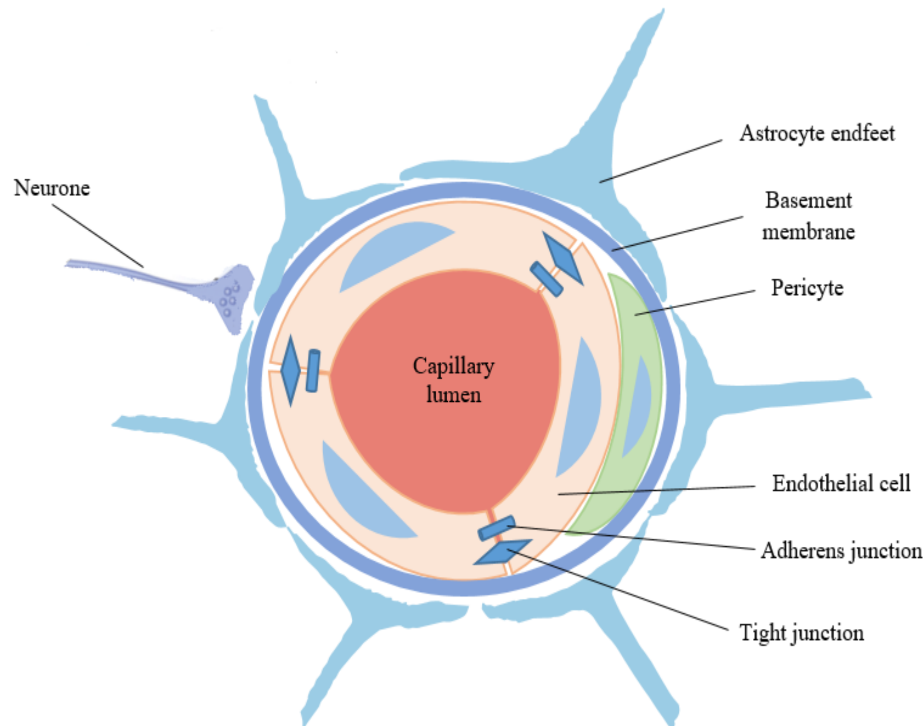


Figure 1.1: Structural representation of different BBB components.
The BBB is composed of specialised endothelial cells sealed together by tight junctions and adherens junctions. The endothelial cells are surrounded by basement membrane, pericytes, astrocyte end-feet and neurones.

1.2.1 Capillary endothelial cells

Capillary endothelial cells are the basic component of the BBB and play a pivotal role in regulating the transport of solutes into, and out of, the brain. The endothelial cells are locked together by adherens junctions and tight junctions which confer high electrical resistance and highly selectively permeable to ions and small charged molecules (Balbuena et al., 2011; Fricker & Mahringer, 2014). Adherens junctions consist of VE-cadherin connected by catenins to the cytoskeleton, while tight junctions are formed by claudins, occludins, zona occludens (ZO) and junctional adhension molecules (JAMs) that almost completely seal the paracellular route (Watabe-Uchida et al., 1998; Vorbrodt & Dobrogowska, 2003; Vincent et al., 2004). The highly restrictive nature of the tight junctions means that, in order to cross the BBB, many ions and molecules cross the BBB through the transcellular pathway using ion channels, receptors,

solute transporters and active efflux transporter expressed in the plasma membrane of the endothelial cells.

1.2.2 Astrocytes

Astrocytes are an essential component of the BBB. The projections from astrocytes, known as astrocyte end-feet, cover approximately 99 % of the abluminal surface of the capillary basement membrane and form a fine lamellae network, which together with the parenchymal basal lamina, form the external layer of the BBB known as the glia limitans (Abbott et al. 2006; Mathiisen et al., 2010; Kacem et al. 1998). The main role of the glia limitans is to provide biochemical support to endothelial cells and regulate inflammatory events, while at the same time acting as a physical barrier against the entry of unwanted cells or molecules (Meshulam et al., 2012). The glial limitans also functions to induce BBB properties in endothelial cells and refine BBB characteristics by promoting the formation and maturation of tight junctions, elevating trans-endothelial electrical resistance (TEER) and upregulating the expression and localisation of endothelial transporters (Daneman & Prat, 2015). *In vitro* studies have identified numerous astrocyte-derived secreted factors including glial cell line-derived neurotrophic factor (GDNF) (Miyazaki & Asanuma, 2016), brain-derived neurotrophic factor (BDNF) (Miyazaki & Asanuma, 2016), nerve growth factor (NGF) (Gray and Patel, 1992), basic fibroblast growth factor (bFGF) (Gray and Patel, 1992), ciliary neurotrophic factor (CNTF) (Gray and Patel, 1992), transforming growth factor-β (TGFβ) (Tran et al., 1999), angiopoetin 1 (Lee et al., 2003) and vascular endothelial growth factor (VEGF) (Alvarez et al., 2013; Wong et al., 2013). Furthermore, given their myriad roles in regulating CNS homeostasis, astrocytes are identified as potential candidates in the involvement of neurologic disorders (Sofroniew & Vinters, 2010).

1.2.3 Basement membrane

The blood-brain barrier contains a basement membrane located between endothelial cells and astrocyte end-feet or pericytes (Figure 1). The basement membrane consists of extracellular matrix proteins such as collagen IV, elastin, fibronectin, laminins, nidogen/entactin, vitronectin and proteoglycans (perlecan, agrin and heparan sulfate proteoglycans) (Salmivirta et al., 2002; Tilling et al., 2002; Ljubimova et al., 2006; Kose et al., 2007; Correale & Villa, 2009; Rauch et al., 2011; Thomsen et al., 2017). The basement membrane is crucial for the overall development and stability of the BBB (Engelhardt &

Sorokin, 2009) since it provides physical support to the different cellular components of the BBB and regulates cell-to-cell communication and interactions (Thomsen et al., 2017).

1.2.4 Pericytes

Pericytes are perivascular cells derived from the vascular smooth muscle cell lineage. Studies reveal pericytes have roles in regulating blood flow in cerebral capillaries, differentiation of the BBB, angiogenesis and phagocytosis (Armulik et al., 2011; Winkler et al., 2011; Baeten and Akassoglou, 2012; Sagare et al., 2013; Hall et al., 2014). *In vivo* studies have demonstrated that a lack of pericytes may lead to aberrant alignment in tight junctions, an increase in endothelial intercellular space, upregulation of the endothelial transcytotic pathway, alteration in endothelial cell gene expression and loss of astrocyte end-feet polarisation (Paik et al., 2004; Bell et al., 2010; Daneman et al., 2010; Armulik et al., 2011). Other studies suggest pericytes function to induce barrier formation in endothelial cells, control tight junction protein expression during development, and increase junctional integrity, transendothelial electrical resistance and the secretion of basal membrane components. It is clear that barrier properties are maintained by the synergistic effect between pericytes and astrocytes, however their contributions in BBB integrity may vary during different developmental stages (Dohgu et al., 2005; Dore-Duffy et al., 2006; Brachvogel et al., 2007; Daneman et al., 2010).

1.2.5 Neurones

Neurones influence in the formation of the BBB and they have been found to regulate the extent and shape of astrocyte networks. Both astrocytes and neurones play a synergistic effect in regulating brain homeostasis, stabilising tight junctions and inducing barrier formation in the BBB (Banerjee & Bhat, 2007).

It is suggested that neurones do not interact directly with capillary endothelial cells, but they are in close proximity to the vessel. They have been reported to have an inductive effect that may directly influence BBB permeability (Mizee & Vries, 2013; Fricker & Mahringer, 2014). However, the exact role of neurones in the formation and maintenance BBB properties of endothelial cells is not fully elucidated.

1.3 Transport mechanisms at the blood-brain barrier

The highly restrictive tight junctions, along with the lack of fenestrations and reduced pinocytosis in the endothelial cells of BBB severely restrict the movement of ions and molecules across the BBB. This is in contrast to peripheral capillary endothelial cells which allow the movement of molecules through both transcellular and paracellular pathways (Wolburg & Lippoldt, 2002; Hawkins & Davis, 2005). The different transport routes in the BBB are illustrated in Figure 1.2.

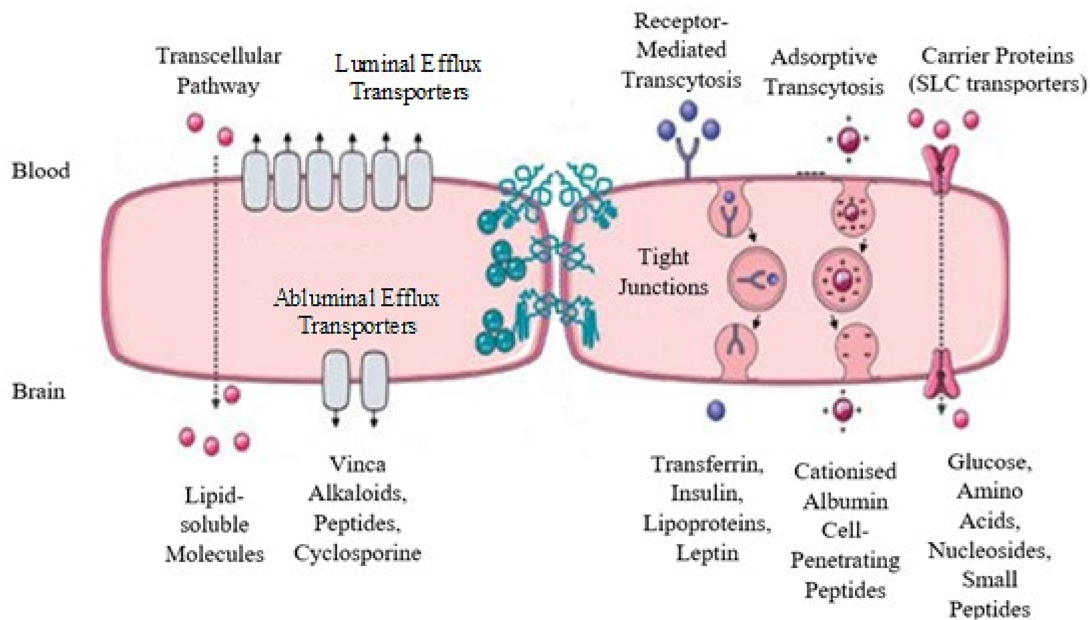


Figure 1.2: Different transport routes and mechanisms across the blood-brain barrier and the localisation of transporter proteins and tight junctions.
Lipophilic molecules may passively diffuse through the surface lipid of the cell membrane via a transcellular pathway while the transport of hydrophilic molecules through a paracellular pathway is restricted by the presence of the tight junctions. Receptor-mediated transcytosis facilitates transport of essential polar macromolecules (peptides, proteins) and adsorptive-mediated transcytosis appears to be induced by positively charged molecules resulting in the transport into the brain. Transporter proteins (ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters) can transport essential polar molecules into the brain. Arrows indicate the direction of substrate transport. Figure taken from Figueiredo et al. (2016).

Small lipophilic molecules and gases, such as oxygen and carbon dioxide, passively diffuse across the plasma membrane of capillary endothelial cells. Essential lipid-insoluble compounds, including small polar solutes (glucose, amino acids and small peptides) cross the BBB via specialised transporter proteins belonging to the solute carrier, or SLC class of transporters (Yang & Hinner, 2015; Hediger et al., 2013). Larger molecules, such as peptides and proteins, have relatively low BBB permeability and are primarily transported by two

classes of vesicular transport, adsorptive-mediated transcytosis (Kumagai et al., 1987) and receptor-mediated transcytosis (Duffy & Pardridge, 1987; Descamps et al., 1996) In addition, brain capillary endothelial cells express high levels of several members of the ATP-binding cassette (ABC) efflux transporter family, which restrict the penetration of a variety of toxic metabolites (Miller, 2015a) and therapeutic drugs (Löscher & Potschka, 2005) into the brain.

The BBB endothelial cells express a variety of neurotransmitter-metabolizing enzymes such as aminopeptidases, andendopeptidases, catechol O-methyltransferase (COMT), cholinesterases, GABA transaminase and monoamine oxidases (MAO), along with several xenobiotic and drug-metabolizing enzymes, particularly cytochrome P4501A and P4502B (Minn et al.,1991; De Leon, 2003; Granberg et al., 2003; Haseloff et al., 2005; Ueno, 2009; Wang et al., 2011). The activities of these enzymes therefore enhance the barrier property with respect to the entry of many neurotoxic and neuroactive compounds.

1.3.1 Passive diffusion

Passive diffusion through the BBB, which involves neither transporter proteins nor energy consumption (Alam et al., 2010), is the most common mode by which small lipophilic substances, such as alcohol, nicotine and caffeine, move from the blood into the brain via the transcellular pathway (Fischer et al., 1998). In addition, many low molecular weight and lipid soluble anaesthetic drugs such as heroin, procaine, thiopental, tetrahydrocannabinol and physostigmine can cross the BBB easily (Lewis, 1992; Thal et al., 2012).

1.3.2 Solute carrier superfamilies

Solute carriers play an important role in transporting mainly organic anions and cations across the BBB. They are divided into two SLC superfamilies. The SLCO/SLC21A superfamily consists of organic anion transporting polypeptides (OATPs), while the SLC22A superfamily consists of organic anion transporters (OATs) and organic cation transporters (OCTs) (Kusuhara & Sugiyama, 2005; He et al., 2009). Both SLC superfamilies are ATP-independent transporters, therefore cannot transport a compound against the concentration gradient. They are multispecific transporters and are expressed in the luminal and abluminal plasma membranes of BBB endothelial cells (Ronaldson & Davis, 2015).

1.3.2.1 SLCO/SLC21A superfamily

The SLCO/SLC21A superfamily consists of OATPs which are an important group of membrane solute carriers in brain capillary endothelial cells. They form sodium-independent transport systems with partially distinct and partially overlapping substrate preferences (Roth, Obaidat & Hagenbuch, 2012). The substrates for OATPs are mainly hydrophobic organic anions, including a wide spectrum of amphipathic organic compounds, such as thyroid hormones, steroid conjugates, bile salts, organic dyes, anionic oligopeptides, numerous drugs and other xenobiotic substances (Hagenbuch & Meier, 2003; Hagenbuch & Meier, 2004; Ronaldson & Davis, 2013). Currently, there are a total of 14 SLCO/SLC21 members identified in rodents and humans (Hagenbuch & Stieger, 2013; Fricker & Mahringer, 2014). Out of the 14 members, OATP1A2, OATP1A4 and OATP1C1 are reported to be expressed in human BBB endothelial cells (Hagenbuch & Stieger, 2013; Tamima et al., 2014).

OATP1A2 is predominantly expressed at the luminal membrane, but also reported to be expressed at the abluminal membrane of human brain capillary endothelial cells (Ronaldson & Davis, 2015). It has a broad substrate specificity that includes antibiotics (erythromycin and ciprofloxacin), fexofenadine, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors (rosuvastatin, pitavastatin), and HIV-1 protease inhibitors (saquinavir, lopinavir, darunavir) and has an important role to play in drug delivery across the BBB and the elimination of organic metabolites from the brain (Dalvi et al., 2014).

OATP1A4 is reported to be expressed at both luminal and abluminal membrane of BBB endothelial cells and is suggested to mediate the transfer of organic anions between the brain and the blood compartments (Hagenbuch & Meier, 2004; Roth et al., 2012).

OATP1C1 is expressed on both the abluminal and luminal membranes of brain capillary endothelial cells (Roth, Obaidat & Hagenbuch, 2012; Tamima et al., 2014) and demonstrates a high degree of multi-specificity but has a rather limited number of substrates, which include estradiol-17β-glucuronide, cerivastatin, estrone-3-sulfate, thyroid hormone T3, thyroxine and reverse triiodothyronine (Hagenbuch & Meier, 2004; Kusuhara & Sugiyama, 2005). Among these substrates, OATP1C1 shows especially high transport affinity for thyroxine and reverse triiodothyronine and it is suggested that OATP1C1 plays a crucial role in the delivery and disposition of thyroid hormones in the brain (Kusuhara & Sugiyama, 2005; Roth, Obaidat & Hagenbuch, 2012).

1.3.2.2 SLC22A superfamily

Members of the SLC22 superfamily include organic anion transporters (OATs), organic cation transporters (OCTs) and organic cation/carnitine transporters (OCTN2) (Roth, Obaidat & Hagenbuch, 2012). Among these members of the SLC22 superfamily, OAT3, OCT1, OCT2 and OCTN2 have been demonstrated to be expressed in human BBB endothelial cells (Uchida, et al., 2011; Fromm & Kim, 2011; Okura, Kato & Deguchi, 2014).

Organic anion transporters (OATs) are polyspecific transporters which act as anion exchangers. They mediate the transport of small and hydrophilic organic anions, including biogenic amines, steroid hormone conjugates, various toxins and drugs (Fromm & Kim, 2011). Of the 10 OAT family members, OAT3 is abundantly expressed at both luminal and abluminal of rodent and human BBB endothelial cells. OAT3 has a Na^+-independent mechanism and plays an important role as an efflux transporter for endogenous products of hormone metabolism (indoxylsulfate homovanillic acid), neurotransmitter (dopamine, norepinephrine, serotonin and histamine), therapeutic drugs (benzylpenicillin, p-aminohippurate, probenecid) and other amphipathic organic anions (estrone sulfate, Dehydroepiandrosterone sulphate and estradiol 17β—glucoronide) (Koepsell & Endou, 2004; Kusuhara & Sugiyama, 2005; Tamima et al., 2014; Dalvi et al., 2014).

Organic cation transporters (OCTs) are considered to be liver-specific transporters that are expressed at low levels in brain. Studies have reported that OCT1 and OCT2 are expressed at the luminal side of the rodent and human BBB (Tamima et al., 2014; Okura, Kato & Deguchi, 2014). OCT1 and OCT2 have overlapping substrate specificities for small organic cations such as tetraethylammonium, methyl 4-phenylpiridinium, quinine and metformin (Koepsell et al., 2003; Koepsell & Endou, 2004; Tamima et al., 2014).

OCTs facilitate passive diffusion of a wide range of structurally unrelated small and hydrophilic organic cations down their electrochemical gradients by Na^+-independent and electrogenic mechanisms. Such transport may occur in either direction independent of Na^+/pH (Koepsell & Endou, 2004; Roth, Obaidat & Hagenbuch, 2012). Most substrates for OCT1 and OCT2 are weak organic bases and substrates include monoamine neurotransmitters,

catecholamines, tetaraethylammonium, N-methylnicotineamide, quinidine and quinine (Hagenbuch & Meier, 2003; Kusuhara & Sugiyama, 2005; Tamima et al., 2014).

OCTN2 is expressed at both luminal and abluminal of bovine, porcine, rodent and human BBB endothelial cells and has been characterised as a sodium-dependent carnitine transporter with high affinity for carnitine, which facilitates the brain uptake of carnitine (Miecz et al., 2008). Other substrates of OCTN2 include the zwitterionic β-lactam antibiotic cephaloridine, L-methionine and L-lysine. In addition, OCTN2 functions as a polyspecific and Na^+-independent organic cation uniporter and, in the absence of Na^+, OCTN2 translocates cations including TEA, choline, verapamil and pyrilamine (Ohashi et al., 1999; Koepsell et al., 2003).

1.3.3 ATP-Binding Cassette (ABC) efflux transporters

ATP-binding cassette (ABC) efflux transporters are the most prevalent ATP-dependent efflux transporters expressed in BBB endothelial cells. These efflux transporters, located in the luminal, blood-facing membrane, act to prevent the entry of structurally diverse xenobiotics, amphiphilic and lipophilic molecules from the circulation into the brain. Conversely, those located in the abluminal, CNS-facing membrane act to facilitate the transport of solute into the brain (Miller, 2015a). In humans, 49 *ABC* genes have been reported which encode 44 functional transporters (Mahringer & Fricker, 2016). The ABC efflux transporter superfamily contains seven sub-families, ABCA to ABCG, with members of the B, C and G sub-families expressed in human BBB endothelial cells. The most widely studied are ABCB1 (P-glycoprotein, P-gp, MDR1), ABCG2 (breast cancer resistance protein, BCRP) and ABCC1-5 (multidrug resistance-associated protein-1-5, MRP1-5) which are confirm to be expressed in human BBB (Figure 1.3) (Warren et al., 2009; Cui et al., 2015; Mahringer & Fricker, 2016).

The functions of ABC efflux transporters in restricting the entry of molecules into the BBB directly poses a barrier to a remarkably broad range of therapeutic drugs, including chemotherapeutics and many antibiotics, anti-inflammatory, anti-neoplastic and immunosuppressive drugs; which further complicates the development of drug therapy for CNS diseases (Klaassen and Aleksunes, 2010; Miller, 2015b; Qosa et al., 2015). Therefore, increased understanding of the signalling pathways that govern expression of ABC efflux

transporters in the BBB may help improve modification of transporter expression and aid CNS drug delivery.

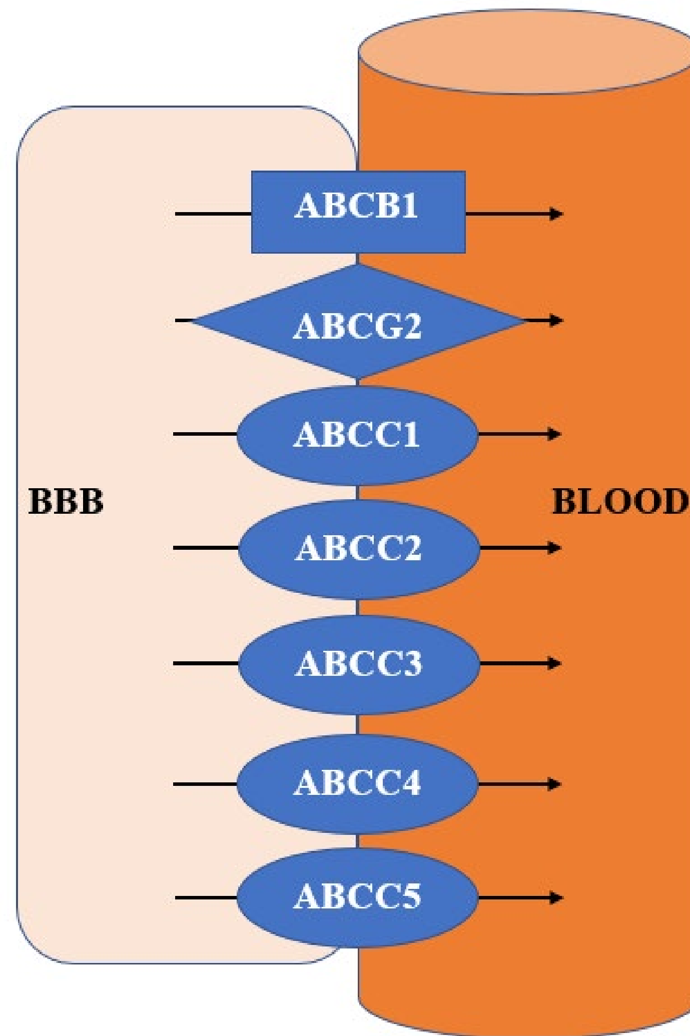


Figure 1.3: Localisation of ABC efflux transporters at the human BBB endothelium. The arrows indicate the direction of substrate transport. ABCG2 is the most highly expressed ABC transporter at human BBB followed by ABCB1 and ABCCs family. The expression of ABCG2 is 1.34-fold higher than ABCB1. Figure was compiled according to information from Nies et al. (2004); Löscher and Potschka (2005); Bendayan et al. (2006); Warren et al. (2009); Hartz and Bauer (2011); Ronaldson and Davis (2013); Miller (2015a); Mahringer and Fricker, 2016; Qosa et al. (2016); Morris et al. (2017) and Gomez-Zepeda et al. (2020).

1.3.3.1 ABCB1 (P-glycoprotein, MDR1)

In humans, ABCB1 (P-glycoprotein, Pgp, MDR1), encoded by the *MDR1* gene, consists of an *N*-glycosylated, 1280-amino acid transmembrane protein. It has a molecular weight of approximately 170 kDa and is expressed as a single chain with two homologous parts

(Aller et al., 2009). Each of these parts contains six transmembrane (TM) domains and a nucleotide-binding domain with an ATP binding site as shown in Figure 1.4. ABCB1 was the first efflux transporter discovered and was initially identified in Chinese hamster ovary cells selected for resistance to colchicine (Juliano and Ling, 1976).

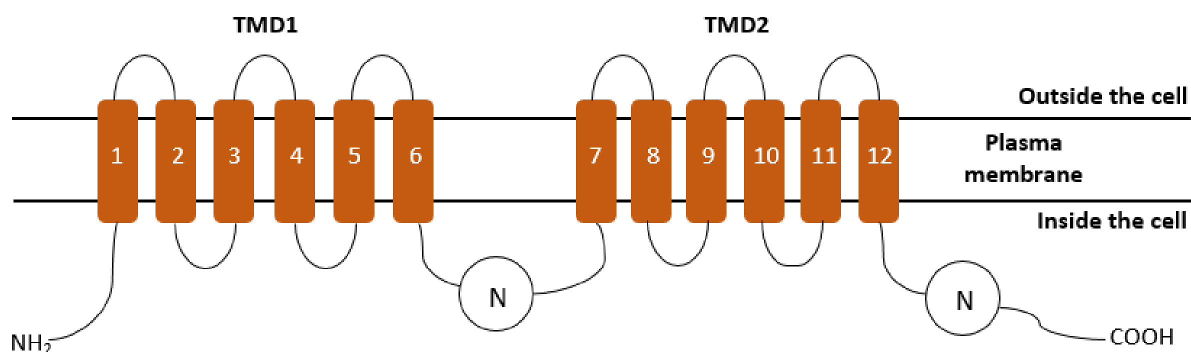


Figure 1.4: Transmembrane structure of ABCB1. N: nucleotide-binding domain; TMD: transmembrane domain.

ABCB1 is highly expressed in human, porcine, mouse, rat and bovine BBB (Virgintino et al., 2002; Warren et al., 2009; Shawahna et al., 2011). It has been reported to localise in the luminal (apical) membrane of bovine and human endothelial cells of the BBB (Tsuji et al., 1992; Ohnishi et al., 1995; Shawahna et al., 2011), as well as in both luminal and abluminal of rat BBB (Stewart et al., 1996; Beaulieu et al., 1997). Thus far, only one study reported the expression of ABCB1 in the in both luminal and abluminal of human BBB (Bendayan et al., 2006) endothelial cells of the BBB (Figure 1.3). Its broad substrate specificity and its localisation at the luminal plasma membrane of the capillary endothelial cell membrane make it a primary determinant of drug entry into the brain (Mahringer & Fricker, 2016). The most striking property of ABCB1 is its ability to prevent, or limit, the CNS entry of a diverse range of lipophilic and amphiphilic compounds including an array of therapeutic drugs, such as antibiotics (erythromycin), anti-HIV drugs (atazanavir, saquinavir, indinavir, ritonavir), anticancer agents (doxorubicin, etoposide, vincristine), immunosuppressive drugs (cyclosporine), analgesics (morphine) and antihistamines (fexofenadine) (Kim, 2002; Borst & Schinkel, 2013; Qosa et al., 2015; Fletcher et al., 2016). Hence, ABCB1 plays an important role in both neuroprotection and pharmacoresistance.

Interestingly, to date, no definitive chemical structure for ABCB1 substrates has been determined. However, most substrates are hydrophobic and usually contain amphiphilic or lipophilic groups, hydrogen bonding groups and electronegative groups (Lam et al., 2001; Gleeson, 2008). However, a number of substrates have been identified that contain aromatic groups, or are non-aromatic linear or circular in nature, and that are basic, uncharged, zwitterionic or cationic charged (phosphatidylcholine analogues, methotrexate) (Teodori et al. 2006). In general, uncharged or weakly basic compounds are more efficiently transported than acidic compounds. For example, several sphingolipids, phospholipids, aldosterone and amyloid-β are ABCB1 substrates (Schinkel & Jonker 2012).

As shown in Table 1.1, ABCB1 expression is regulated by multiple nuclear receptors and signalling molecules. The most common nuclear receptors are pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR), peroxisome proliferator-activated receptors (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR) and vitamin D receptor (VDR) (Narang et al., 2008; Wang et al., 2011; Chan et al., 2011; Elali & Hermann, 2012; Durk et al., 2014; Mahringer & Fricker, 2016). Signalling molecules found to directly affect ABCB1 expression are interleukin-1β (Alasmari et al., 2018), TNF-α (Miller 2010), glutamate (Bauer et al., 2008b), nuclear factor erythroid 2–related factor 2 (Nrf2) (Wang, X. et al., 2014), and by numerous pathways including the mitogen-activated protein kinase (MAPK) pathway and nuclear factor-κB (NF-κB), (Tamima et al., 2014), protein kinase C pathway (Miller, 2010), Rho pathways (Zhong et al., 2010b) and the sphingolipid signalling pathway (Miller, 2014). Currently, the detailed mechanisms that regulate ABCB1 expression have not been fully elucidated in the BBB, and this thesis will investigate this further.

Table 1.1: Regulation of ABC efflux transporter protein expression by nuclear receptors, pro-inflammatory cytokines and transcription factors in blood–brain barrier endothelial cells of rodent (R), porcine (P) and human (H). Up-arrows denote increased transporter protein expression, horizontal arrows denote no change in expression and down-arrows denote decreased transporter protein expression.

Regulator \ ABC efflux transporter	ABCB1	ABCG2	ABCC5
GR	R ↑	R ↑	R ↔
PXR	R P H ↑ ↑ ↑	R P H ↑ ↑ ↑	
CAR	R P H ↑ ↑ ↑	R P ↑ ↑	R ↔
AhR	R P H ↑ ↑ ↔	R P H ↑ ↑ ↔	
PPARs	R ↑	R ↑	
VDR	R ↑		
LXR	R ↑		
FXR			
Inflammation TNF−α (Short-term exposure 6 h)	R P ↑ ↑	R H P ↑ ↑ ↑	
Inflammation TNF−α (long-term exposure 24 – 48 h)	R P ↑ ↑	R H P ↓ ↓ ↓	
Inflammation IL-1β	H P ↔ ↓	H P ↓ ↓	
Inflammation IL-6	H ↔	H ↓	
Oxidative stress (Nrf2)	R ↑	R ↑	M ↔
Glutamate	R H ↑ ↑	R P H ↑ ↓ ↓	

1.3.3.2 ABCG2 (Breast cancer resistance protein, BCRP)

ABCG2 (breast cancer resistance protein, BCRP) belongs to the G subfamily of the ABC superfamily. It is a product of the *ABCG2* gene and consists of 665 amino acids with a 1111molecular weight of approximately 75 kDa. In BBB endothelial cells ABCG2/abcg2 expression has been detected at the luminal membrane of human (Cooray et al., 2002; Zhang et al., 2003); rat (Hori et al., 2004a; Roberts et al., 2008), mouse (Cisternino et al., 2004; Tachikawa et al., 2005) and porcine (Eisenblatter et al., 2003) endothelial cells of the BBB (Figure 1.3). ABCG2 is referred to as a half ABC transporter possessing six membrane-spanning domains and one nucleotide-binding domain (Figure 1.5). It is predicted to function as a homodimer or even oligomer, but evidence also suggests that BCRP may also functions as a monomer (Mitomo et al., 2003; Hazai & Bikadi, 2008; Ni et al., 2010).

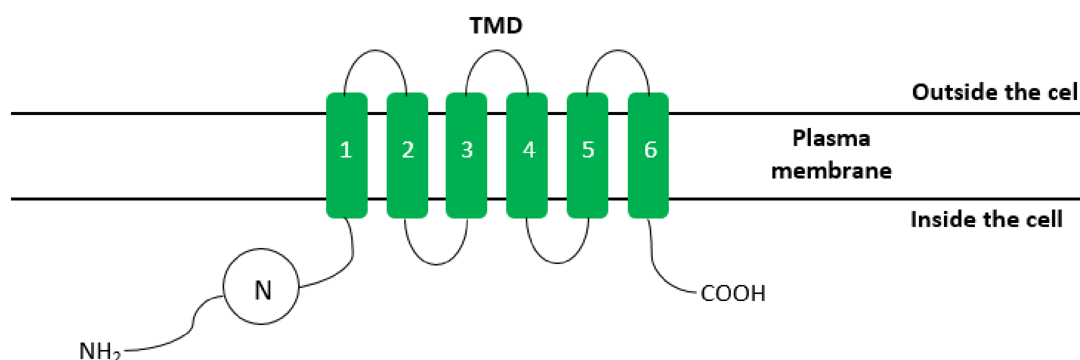


Figure 1.5: Transmembrane structure of ABCG2. N: nucleotide-binding domain; TMD: transmembrane domain.

ABCG2 substrates include a broad range of structurally and functionally diverse molecules including anticancer drugs (methotrexate, daunorubicin, doxorubicin and mitoxantrone), antibiotics (erythromycin, ciprofloxacin and ofloxacin) and antiretroviral drugs (acyclovir, zidovudine), endogenous substances, particularly sulphated and glucuronide conjugates (estradiol-17β glucuronide, estrone 3-sulfate), steroid hormones, estrones, folic acid, flavonoids and bile acids (Mao & Unadkat, 2005; Schinkel & Jonker, 2012; Tamima et al., 2014). Similar to ABCB1, the broad substrate specificity of ABCG2 makes it an important determinant of drug entry into the brain. Interestingly, ABCG2 and ABCB1 demonstrate significant overlap in substrate specificity hence ABCG2 and ABCB1 are able to take over

each other's function and even amplify the action of each other to exert a synergistic effect in limiting BBB permeability (Tamima et al., 2014; Qosa et al., 2015).

ABCG2 has been reported to be regulated by GR, PXR, CAR, PPAR and AhR (Narang et al., 2008; Wang et al., 2011; Miller, 2015b; Mahringer & Fricker, 2016; Kaur & Badhan, 2017). ABCG2 expression is also altered during inflammation *via* TNF-α (Poller et al., 2010), oxidative stress *via* Nfr2 (Grewal et al., 2017) and by glutamate (Yousif et al., 2012) (Table 1.1). To date, the detailed signalling pathways that regulate ABCG2 expression have not been comprehensively studied in the BBB, and this thesis will investigate this further.

1.3.3.3 ABCC (Multidrug resistance-associated protein, MRP)

ABCC transporters (Multidrug resistance-associated proteins, MRPs) belong to the ABC efflux transporter subfamily encoded by *ABCC* genes. ABCC isoforms are classified into 13 members according to their membrane topology (ABCC1-13) (Tamima et al., 2014). Based on their predicted domain structure the ABCC subfamily has been assigned to one of two clusters. the first cluster being the ABCC1, ABCC2, ABCC3, ABCC6, ABCC7 composed of two nucleotide-binding domains and three membrane spanning domains (MSD0, 1 and 2) with 17 transmembrane α-helices, five in TMD0 and six each in TMDs 1 and 2 (Figure 1.6). The second cluster being the ABCC4, ABCC5, ABCC8 are composed of 2 x 6 transmembrane domains and 2 nucleotide binding sites (Figure 1.7).

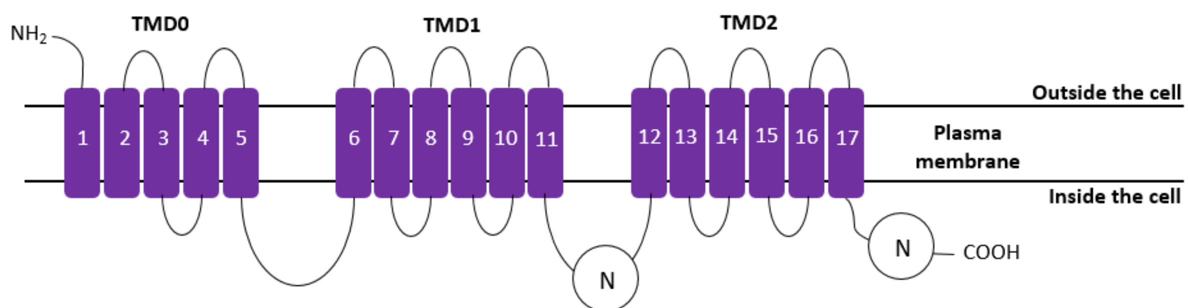


Figure 1.6: Transmembrane structure of ABCC1, 2, 3, 6, 7. N: nucleotide-binding domain; TMD: transmembrane domain.

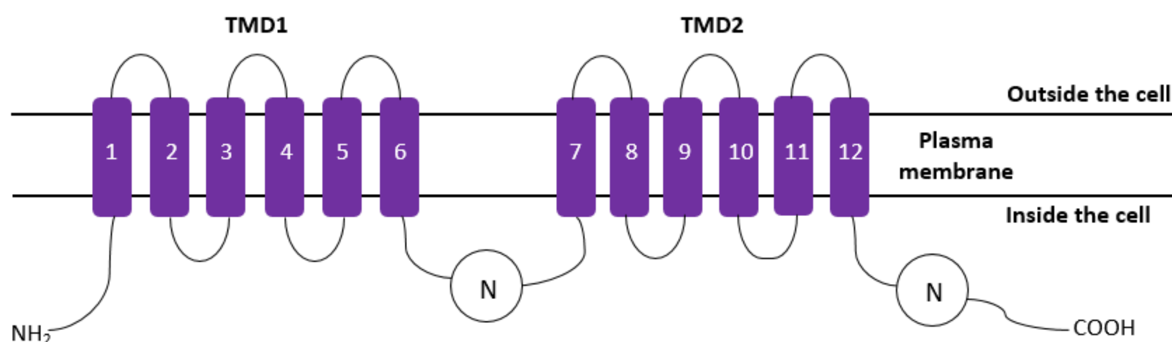


Figure 1.7: Transmembrane structure of ABCC4, 5, 8. N: nucleotide-binding domain; TMD: transmembrane domain.

The substrates for ABCC subfamily members are typically amphiphilic organic anions with an approximate molecular weight between 300 and 1,000 Da. Leukotriene C4 (LTC4) has remained the most characteristic substrate for most ABCC subfamily members and exhibits a high affinity for ABCC1, ABCC4 ABCC5.

ABCC1 is encoded by the *ABCC1* gene and consists of 1531 amino acids with a molecular weight of approximately 190 kDa (Haimeur et al., 2004) and is reported to be expressed at the luminal domain of human (Nies et al., 2004; Zhang et al., 2004), bovine (Zhang et al., 2004; Kusch-Poddar 2005; Warren et al., 2009) and mouse (Soontornmalai et al., 2006) and abluminal of rat (Roberts et al., 2008) and mouse (Soontornmalai et al., 2006) membrane of endothelial cells (Figure 1.3). ABCC1 displays significant substrate overlap with ABCB1, particularly hydrophobic molecules, with a wide range of therapeutic drugs such as antibiotics, anticancer drugs and anti-HIV drugs (Mahringer & Fricker, 2016). However, ABCC1 preferentially transports organic anionic conjugates, such as GSH-conjugates of leukotrienes and prostaglandins, along with glutathione (GSH) and glutathione disulfide (GSSG), and also sulfate- and glucuronide conjugates (estradiol-17β glucuronide, estrone 3-sulfate) and some unconjugated anticancer (daunorubicin, etoposide, vincristine), antibiotics (difloxacine) and antiretroviral drugs (saquinavir, ritonavir, indinavir) (Leslie et al., 2001; Dallas et al., 2006; Schinkel & Jonker, 2012; Qosa et al., 2015). ABCC1 expression is reported to be regulated by nuclear receptor LXR, TNF-α and NF-κB signalling pathways (Ronaldson & Bendayan, 2008; Elali & Hermann, 2012; Kooij et al., 2011).

ABCC2, encoded by the *ABCC2* gene, is comprised of 1545 amino acids having 49% identity with ABCC1 and a molecular weight of approximately 190 kDa. It is predominantly localised to the luminal membrane of capillary endothelial cells in fish, porcine, mouse, rat and human BBB (Miller et al., 2000; Fricker et al., 2002; Miller et al., 2002; Potschka et al., 2003; Wang X., 2014; Luna-Munguia et al., 2015) (Figure 1.3). ABCC2 shares similar substrate specificity with ABCC1 and transports many of the same conjugated and unconjugated organic anions but the affinities of the two transporters for a given anion can differ significantly. For example, ABCC1 has a much higher (10-fold) affinity for LTC4, but mono- and bisglucuronosyl bilirubin are better substrates for ABCC2.

The expression of ABCC2 is regulated by the nuclear receptors PXR, CAR, AhR and FXR (Bauer et al. 2008a; Wang et al., 2011; Miller, 2015b) as well as TNF-α (Hartz et al., 2006), Nfr2 (Grewal et al., 2017) and glutamate (Luna-Munguia et al., 2015).

ABCC3 is encoded by the *ABCC3* gene, consists of 1527 amino acids, has a molecular weight of 170-kDa and has 58% identity with ABCC1. ABCC3 expression has been detected in cultured bovine (Zhang et al., 2000), mouse and porcine brain microvessel endothelial cells (Warren et al., 2009). ABCC3 has a similar, but narrower, substrate profile compared to ABCC1 and ABCC2, and transports a broad range of endogenous compounds and xenobiotics, mostly conjugated organic anions, with considerable, but not complete overlap in drug substrates with ABCC1 and ABCC2 (Kool et al., 1999; Zeng et al., 1999; Tian et al., 2006). Some notable differences exist. For example, in comparison to ABCC1 and ABCC2, ABCC3 has lower affinity for glutathione conjugates such as LTC4, but demonstrated higher affinity for glucuronide conjugates (Zeng et al., 2000). Typical substrates of ABCC3 include endogenous compounds (estradiol-17β-glucuronide, glucuronide E217βG, leukotriene C4, monovalent bile salts such as cholate and glycocholate), chemotherapeutic agents, and other drugs and drug conjugates such as acetaminophen-glucuronide, morphine-3-glucuronide, fexofenadine, etoposide and teniposide (Grant et al., 2008; Kool et al., 1999; Zelcer et al., 2001; Zhang et al., 2003).

ABCC4 is encoded by the *ABCC4* gene, is composed of 1325 amino acids, has a molecular weight of 150 kDa and is localised in both the luminal and abluminal membranes of rat (Roberts et al., 2008; Leggas et al., 2004), bovine (Zhang et al., 2004), human (Nies et al., 2004; Bronger et al., 2005), porcine (Warren et al., 2009) brain endothelial cells and the

abluminal of bovine (Zhang et al., 2004) brain endothelial cells (Figure 1.3). ABCC4 was found to be the most abundantly expressed ABCC transporter in mouse brain endothelial cells (Warren et al., 2009). Functionally, ABCC4 is characterized as an organic anion transporter, particularly important in the transport of cyclic nucleotides (guanosine 3′,5′-monophosphate (cGMP) and adenosine 3′,5′-monophosphate (cAMP)) and nucleobase analogues (azidothymidine, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG)) (Ohtsuki and Terasaki, 2007). In addition, ABCC4 exhibits a uniquely broad substrate specificity for glucuronide and glutathione conjugates (leukotrienes C4 and B4), lipid mediators (prostanoids and conjugated steroids) and unconjugated bile acids (cholyltaurine, cholylglycine, and choline) (Borst et al., 2004; Belinsky et al., 2007). In comparison to ABCC1, ABCC2 and ABCC3, ABCC4 possess distinct membrane topology and does not interact with typical substrates of ABCC1, ABCC2 and ABCC3, such as vincristine, etoposide, or daunorubicin (McAleer et al., 1999; Jedlitschky et al., 2000). However, research into the expression and regulatory mechanism of ABCC4 in the BBB is still in its infancy (Schinkel & Jonker, 2012).

ABCC5 is encoded by the *ABCC5* gene, consists of 1437 amino acids, has a molecular weight of 161 kDa (Suzuki et al., 2000) and has been reported to be localised to the luminal membrane of bovine and human BBB endothelial cells (Zhang et al., 2004; Nies et al., 2004; Bronger et al., 2005) whereas low levels of ABCC5 expression has been found in the abluminal membrane of rat BBB endothelial cells (Roberts et al., 2008) (Figure 1.3). In comparison to other ABCC members, ABCC5 was reported to the most highly expressed transporter in rat (Warren et al., 2009), bovine, (Warren et al., 2009; Zhang et al., 2004) porcine (Warren et al., 2009) and human (Nies et al., 2004; Warren et al., 2009) brain endothelial cells. ABCC5 is closely related to ABCC4 and has been reported to efflux organic anions including the anionic dye fluorescein diacetate (McAleer et al. 1999), the cyclic nucleotides cGMP and cAMP (Jedlitschky et al. 2000), a number of nucleoside monophosphate analogues, and some glutathione S-conjugates (Wijnholds et al. 2000). Similar to ABCC4, ABCC5 does not interact with vincristine, etoposide, or daunorubicin (McAleer et al., 1999; Jedlitschky et al., 2000), but both transporters are important regulators of cAMP and cGMP tissue levels (Schinkel & Jonker, 2012). However, the substrate profile of ABCC5 seems to be much narrower than ABCC4.

As with ABCC4, the precise mechanisms regulating expression of ABCC5 in BBB is relatively understudied compared to ABCB1 and ABCG2. Thus far, Ambroziak et al., 2010

reported both CAR and GR ligands have no effect on ABCC5 mRNA expression in immortal-ised rat brain endothelial cell line (Table 1.1). Thus far, the endotoxins lipopolysaccharide (LPS) known to trigger inflammatory pathway by inducing the expression of TNF-α, IL-1β and IL-6 is found to upregulate ABCC5 mRNA and protein expression (Lickteig et al., 2007; Gibson et al., 2012) in BV-2 microglia cells. The nuclear factor erythroid 2-related factor 2 (Nrf2) which plays a role in oxidative was shown to have no effect on ABCC5 mRNA and protein expression *in vivo* mice brain (Ghanem et al., 2015) (Table 1.1).

1.4 Regulation of ABC efflux transporter expression and activity in the blood-brain barrier

Regulation of the expression and activity of ABC efflux transporters is via several different signalling pathways. Most of these signalling pathways are triggered by either surface receptors or nuclear receptors in response to exposure to xenobiotics. In addition, inflammatory stress, oxidative stress and glutamate are also implicated in regulation of ABC efflux transporter expression (Figure 1.8). At present, compelling data pinpoint the crucial role of nuclear receptors in regulating expression of ABC efflux transporters through numerous intracellular messengers and transcription factors.

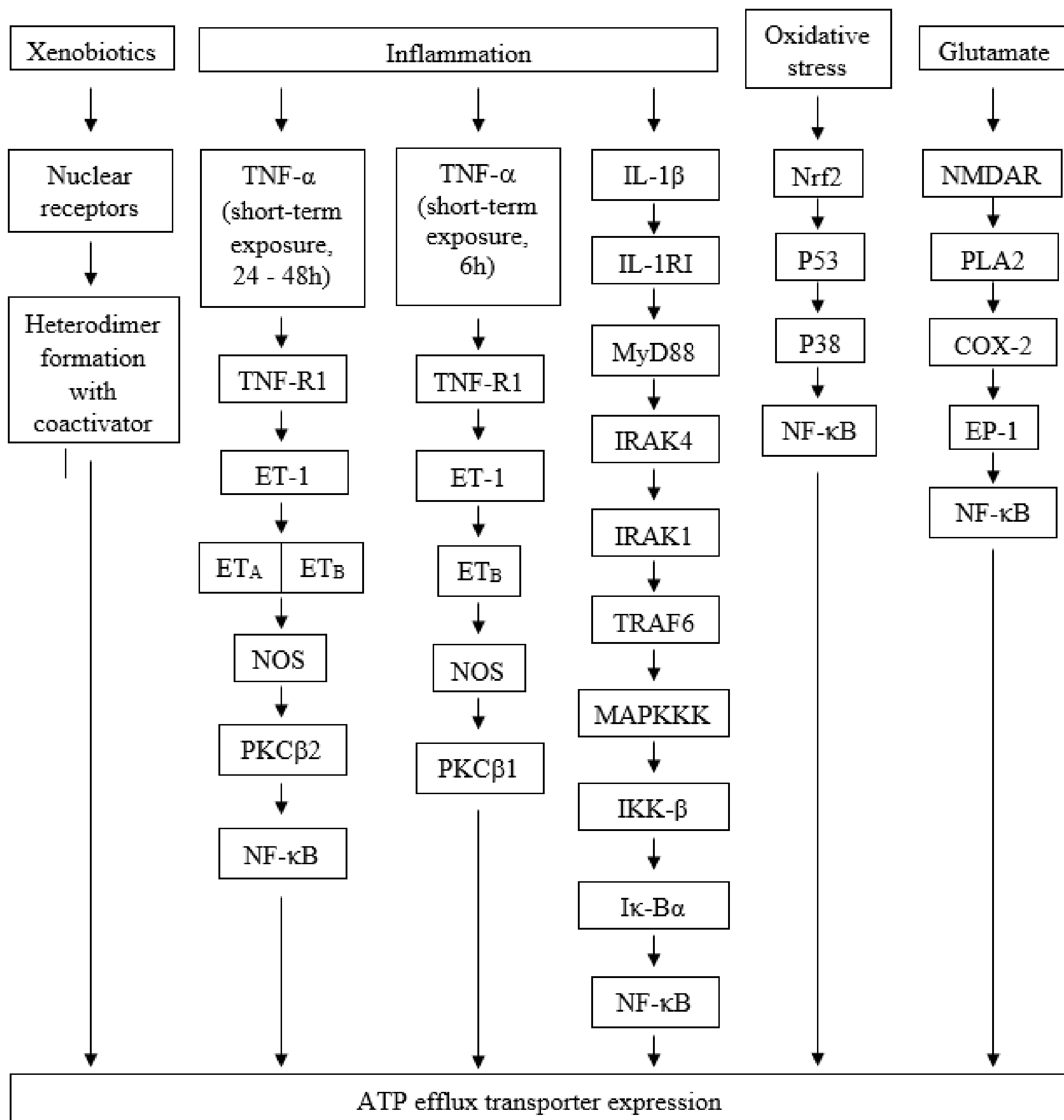


Figure 1.8: Regulation of ABC efflux transporter (ABCB1, ABCG2, ABCC1 and ABCC2) protein expression in blood-brain barrier endothelial cells by different signalling pathways that triggered by inflammation, oxidative stress and glutamate.

iNOS, inducible nitric oxide synthase; TNF-a, Tumour necrosis factor; TNF-R1, tumour necrosis factor-receptor 1; PKCb1, Protein Kinase C beta1; PKCb2, Protein Kinase C beta2; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR4, Toll-like receptor 4; TACE, tumour necrosis factor-α-converting enzyme; IL-1β, Interleukin 1 beta; IL-1RI, interleukin-1 receptor type I; MyD88, Myeloid differentiation primary response 88; IRAK4, Interleukin-1 receptor-associated kinase 4; IRAK1, Interleukin-1 receptor-associated kinase 1; TRAF6, Tumour necrosis factor receptor associated factor 6; MAPKKK, Mitogen Activated Protein (MAP) kinase kinase kinase; IKK-β, inhibitor of nuclear factor kappa-B kinase subunit beta; Iκ-Bα, inhibitor of nuclear factor kappa B; NMDAR, N-methyl-D-aspartate receptor; PLA2, Phospholipases A2; COX-2, cyclooxygenase-2; EP-1, Prostaglandin E2 receptor 1; ET, endothelin receptor.

1.4.1 Nuclear Receptor-mediated regulation of ABC efflux transporter proteins

Nuclear receptors belong to the superfamily of DNA-binding transcription factors responsible for regulating gene expression following exposure to exogenous and endogenous molecules, thereby producing a protective response (Novac & Heinzel, 2004).

Nuclear receptors can be classified into two main families, steroid and orphan receptors, based on their ligand-binding specificities (Germain, et al., 2006; Li & Wang, 2010a). The steroid or endocrine receptors, such as the glucocorticoid receptors (GR) mediate the actions of steroid hormones, and regulate numerous cellular activities including development, metabolism and immune responses (Tirona and Kim, 2005; Germain et al., 2006; De Bosscher et al., 2010). Orphan receptors are classified into two types, true orphan receptors which are those identified through molecular sequencing with unknown endogenous ligands or functions; adopted orphan receptors are those receptors that were cloned without prior knowledge of their endogenous ligand profiles or function and where their endogenous ligands have subsequently been identified (Germain et al., 2006; Benoit et al., 2006). Several adopted orphan receptors include, pregnane X receptor (PXR), constitutive androstane receptor (CAR), Arylhydrocarbon-receptor (AhR), peroxisome proliferator-activated receptor (PPARα) and vitamin D receptor (VDR). These receptors are key regulators for transcription of genes encoding phase I and phase II metabolising enzymes and ABC efflux transporters (Benoit et al., 2006; Li & Wang, 2010; Chan et al., 2013).

In general, xenobiotics initially permeate through the plasma membrane and bind to a nuclear receptor located within the cytosol, causing dissociation of the receptor from a histone deacetylase-containing complex. The ligand-bound nuclear receptor migrates into the nucleus and forms a heterodimer with a coactivator in the nucleus. The heterodimer in turn binds to enhancer and promoter regions of genes that encode ABC efflux transporters and initiates the assembly of a transcription complex. To date, direct interaction between ligand-bound nuclear receptors and promoter regions of transporter genes is the only known mechanism by which nuclear receptors activate ABC efflux transporters and up-regulate their expression (Olefsky, 2001; Miller, 2015b; Qosa et al., 2015). PXR, CAR, VDR, LXRs, FXR and PPARs are the nuclear receptors known to bind to the same co-activator, RXR, to yield a heterodimer (Germain et al., 2006; Miller, 2015b). The general pathway for activation of a nuclear receptor is shown in Figure 1.8.

1.4.1.1 Steroid receptor

The glucocorticoid receptor (GR/NR3C1) is a steroid receptor that regulates a plethora of physiological processes such as metabolism, cardiovascular function, cognition, reproduction and the immune function (Barnes, 1998; Sapolsky, Romero & Munck, 2000).

The GR receptor responds to both steroidal (cortisol, dexamethasone) and nonsteroidal GR modulators (sulfonamide and budesonide) (Tirona & Kim, 2005; De Bosscher, et al., 2010; Edman et al., 2014; Gauvreau et al., 2015). To date, it is not known if the glucocorticoid responsive element (GRE) is located in the promoter regions of genes encoding ABC efflux transporter proteins. However, the presence of the GRE in the promoter region of *CAR* and *PXR* genes suggests that GR is able to indirectly affect the expression of drug efflux transporters by altering PXR and CAR expression (Pascussi et al., 2000; De Bosscher et al., 2010; Chan et al., 2013). Thus far, studies have shown that dexamethasone, a ligand for GR and PXR, increased expression of abcb1 and abcg2 in the rodent BBB, whereas the addition of the GR antagonist RU486 partially blocked expression. GR has also been shown to directly induce the expression of PXR through ligand-activated GR. The results indicate that dexamethasone may be capable of inducing abcb1 and abcg2 expression either through direct interaction with both GR and PXR or through GR-mediated upregulation of PXR (Narang et al., 2008; Miller, 2015b).

1.4.1.2 Orphan nuclear receptors

1.4.1.2.1 Pregnane X receptor (PXR)

The Pregnane X receptor (PXR) is an orphan nuclear receptor belonging to the superfamily of nuclear hormone receptors. It is primarily expressed in barrier and excretory tissue, including brain, kidney, liver and intestine (Kliewer, Goodwin & Willson, 2002; Francis et al., 2003). PXR is an important regulator for a plethora of physiological processes, including glucose and cholesterol homeostasis, bile acid clearance, and steroid hormone balance (Ihunnah et al., 2011).

PXR was the first xenobiotic sensor reported to regulate expression of ABC efflux transporters, and it is also referred to as "a master xenobiotic sensor" since many ABC efflux transporter substrates, including endogenous substrates such as 5β-cholestane-3α,7α,12α-triol and steroids; as well as exogenous metabolites of environmental pollutants, drugs, toxicants

and herbal compounds, interact with PXR (Geick et al., 2001; Dussault and Forman, 2002; Bauer et al., 2004; Oladimeji and Chen, 2018). Studies have shown that dosing rats with the PXR ligands pregnenolone-16-carbonitrile (PCN) and dexamethasone caused an increase in the expression and activity of abcb1 and abcc2 as well as phase II glutathione S-transferase-pi in BBB endothelial cells. However, PCN is not a PXR ligand in human and porcine BBB endothelial cells (Bauer et al., 2004; Bauer et al., 2008a; Miller, Bauer, and Hartz, 2008). Conversely, the PXR ligands rifampicin and hyperforin have been shown to up-regulate the expression of ABCB1 and ABCG2 in both human and porcine BBB endothelial cells, but not in rodent BBB endothelial cells (Ott et al., 2009). Thus far, *in vitro* studies have demonstrated the regulation of ABCB1 by PXR in primary cultures of brain endothelial cells or immortalised brain endothelial cells derived from rodent (Lombardo et al., 2008; Narang et al., 2008), bovine (Perloff et al., 2007), porcine (Ott et al., 2009) and human brain (Chan et al., 2011).

At the BBB, upon ligand activation, PXR translocates to the nucleus and binds to RXR to form a heterodimer. The PXR-RXR heterodimer causes the dissociation of co-repressor proteins from PXR and allows the recruitment of co-activator proteins to initiate the transcription process (Kliewer et al. 1998; Harmsen et al. 2007, Hariparsad et al. 2009). The heterodimer complex interacts with DNA response elements in promoter regions and initiates the transcription and expression of genes encoding the ABCB1 and ABCG2 transporters (Table 1.1), as well as phase I (cytochrome P450) and phase II (glutathione S-transferases, UDP-glucuronosyl-transferases and sulfotransferases) drug metabolising enzymes (Geick et al., 2001; Synold, Dussault & Forman, 2001; Kast et al., 2002; Wang and LeCluyse, 2003a; Urquhart et al. 2007). The postulated pathway for PXR activation is shown in Figure 1.9. Thus far, studies on whether ABCC5 is PXR-dependent/independent were rather elusive and conflicting.

Currently, the list of PXR ligands is still growing (Appendix A) and includes therapeutic drugs (chemotherapeutic agents, anticonvulsive agents, rifampicin, paclitaxel, and HIV protease inhibitors), environmental pollutants and pesticides (chlordane, trans-nonachlor and phenols), bile acids (cholic acid), endogenous steroids (glucocorticoids, progesterone and 5a-pregnane-3,20-dione) and natural herbal-derived compounds (colupulone and hyperforin) (Dussault, et al., 2001; Chang and Waxman, 2006; Di Masi et al., 2009; Chan et al., 2013).

Species differences in response to exposure to ligands have been reported, and may be explained by differences in receptor sequence/structure. For example, the PXR ligand-binding domain demonstrates only 76 % sequence homology between rodent and human (Wang & LeCluyse 2003a) whereas there is 87 % sequence homology between porcine and human (Moore etal., 2002). As a result, the ligand activation profile of human and rodent PXR differ significantly (Moore et al. 2002, Chang & Waxman 2006), whilst the activation profiles of porcine and human PXR are similar (Wang & LeCluyse 2003a). Owing to the multi-specificity of PXR, the interaction between drug ligands and PXR can significantly affect the pharmacokinetics and pharmacodynamics of a huge number of drugs.

At present, the precise mechanisms of PXR and ligand interactions, and the subsequent effects on ABC efflux transporter expression, are more well established in liver than in the BBB, and the postulated pathway of PXR-mediated gene transcription in the BBB is illustrated in Figure 1.9. Hence, it is important to gain a better understanding of these regulatory mechanisms which may have important clinical implications for the effective treatment of CNS disorders.

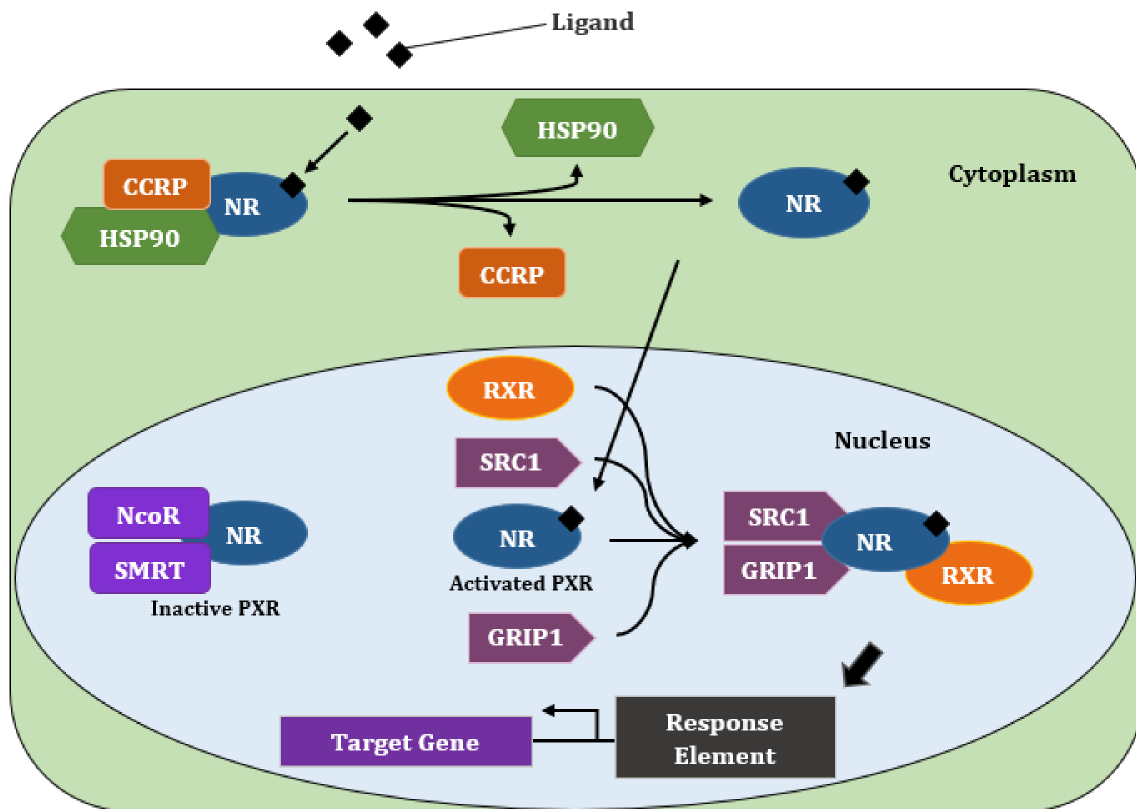


Figure 1.9: Schematic illustration of the possible mechanisms of direct ligand-mediated activation of nuclear receptor, NR (PXR/CAR) in blood-brain barrier endothelial cells. CCRP: cytoplasmic retention protein; EGFR: epidermal growth factor receptor; GRIP1: glucocorticoid receptor interacting protein 1; HSP90: heat-shock protein 90; P: phosphate group; PB: phenobarbital; PP2A: protein phosphatase 2A; NcoR: nuclear receptor co-repressor 1; RXR: retinoid x receptor; SMRT: silencing mediator of retinoid and thyroid receptors; SRC-1: steroid receptor coactivator 1/nuclear receptor coactivator 1.

In the absence of ligand, nuclear receptor (NR) is sequestered in the cytoplasm by binding to heat-shock protein (hsp) 90 and cytoplasmic retention protein (CCRP). Upon ligand binding, the protein complex dissociates and NR translocates to the nucleus to form a heterodimer with RXR. Subsequently, two coactivators, GRIP1 and SRC1 are recruited and bind to the CAR-RXR heterodimer. The resulting protein complex binds to its response element to initiate gene transcription.

Alternatively, NR may also be present in the nucleus, in the absence of ligands, where it is constantly silenced by recruitment of corepressors SMRT and NcoR1 instead of coactivators GRIP1 and SRC1.

On exposure to ligand, the latter binds to NR resulting in the release of bound corepressors (SMRT and NcoR1), formation of heterodimer with RXR and recruitment of coactivators to initiate gene transcription process. (Kawamoto et al., 1999; Dussault et al., 2002; Mäkinen et al., 2003; Li et al., 2003; Orans et al., 2005; Li et al., 2009; Di Masi et al., 2009; Timsit and Negishi, 2014).

1.4.1.2.2 Constitutive androstane receptor (CAR)

The constitutive androstane receptor (CAR) is an orphan nuclear receptor belonging to the superfamily of nuclear hormone receptors. It is also referred to as "a master xenobiotic sensor" and can be activated by a wide range of endogenous ligands and xenobiotics (Pascussi et al., 2008; Di Masi et al., 2009). CAR plays a pivotal role in regulating the expression of phase I and phase II metabolising enzymes and membrane transporters, thereby coordinating xenobiotic clearance (Urquhart et al., 2007; Chen et al., 2012).

CAR has been demonstrated to act as a positive regulator for ABCB1 and ABCG2 but no effect on ABCC5 expression (Table 1.1) (Kast et al., 2002; Burk et al., 2005; Jigorel et al., 2006; Ambroziak et al., 2010). Abcb1, abcc2 and abcg2 have been reported to be up-regulated in rat and mouse brain capillaries exposed to the CAR ligands phenobarbital and TCPOBOP (1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene) (Wang, X. et al., 2010), whilst exposure of porcine and immortalised human brain endothelial cells to the human CAR ligand CITCO (6-(4-chlorophenyl)-imidazo[2,1-b]thiazole-5-carbaldehyde) resulted in up-regulation of the expression and activities of ABCB1 and ABCG2 (Chan et al., 2011; Lemmen et al., 2013a). However, TCPOBOP failed to induce abcb1 and abcg2 expression in cultures of primary porcine brain capillary endothelial cells (Lemmen et al., 2013a). Similarly, Moore et al. (2000), and Honkakoski et al. (2003) also reported that TCPOBOP does not activate human CAR. In contrast CITCO does not activate rodent CAR (Maglich et al. 2002). Although nuclear receptors are involved in regulating expression of ABC efflux transporters, little is known of the precise pathways involved in BBB endothelial cells.

In contrast to other orphan nuclear receptors, CAR activation can occur in two ways, either by directly binding with ligand (Figure 1.9), as is observed with CITCO and the antimalarial artemisinin, consistent with other nuclear receptors, or indirectly (Figure 1.10). For example, phenobarbital and bilirubin do not directly bind to the receptor, but they activate

CAR by recruiting protein phosphatase 2A (PP2A) to dephosphorylate CAR thereby leading to translocation of CAR to the nucleus (Kawamoto et al., 1999; Huang et al., 2003; Xie et al., 2003; Yamamoto et al., 2003; Simonsson et al., 2006; Merrell et al., 2008). However, the precise details of the mechanism of indirect CAR activation in BBB endothelial cells remains unclear, and the postulated pathway for indirect CAR activation in the BBB is shown in Figure 1.10.

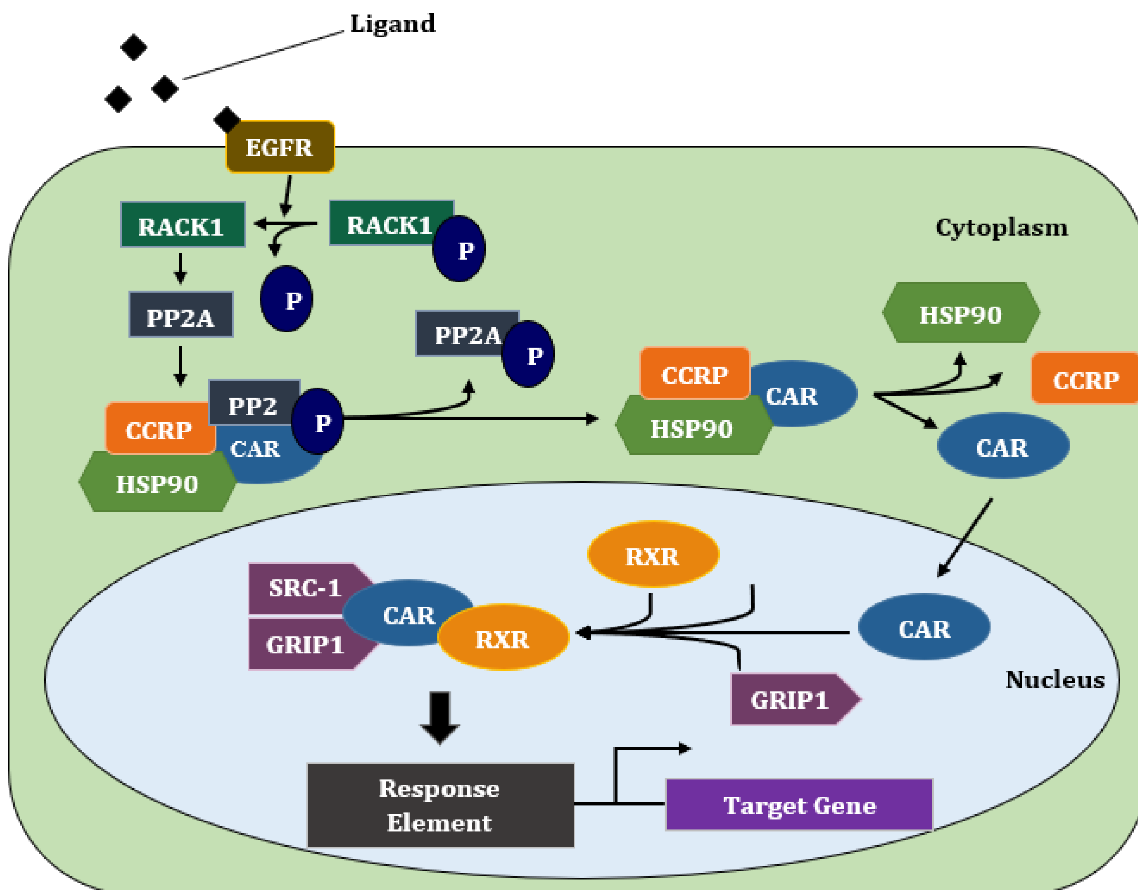


Figure 1.10: Schematic illustration of the possible mechanisms of indirect activation of nuclear receptor CAR in blood-brain barrier endothelial cells.
CCRP: cytoplasmic retention protein; EGFR: epidermal growth factor receptor; GRIP1: glucocorticoid receptor interacting protein 1; HSP90: heat-shock protein 90; P: phosphate group; PP2A: protein phosphatase 2A; PP2: Protein phosphatase 2; RACK1: receptor for activated C kinase 1; RXR: retinoid x receptor; SRC-1: steroid receptor coactivator 1/nuclear receptor coactivator 1.

Unlike PXR, nuclear translocation for CAR is less well characterised. Several studies using immortalised liver and human brain endothelial cell lines demonstrated spontaneous accumulation of CAR in the nucleus without exhibiting significant nuclear translocation in

response to exposure to CITCO ligand (Timsit and Negishi 2007; Chan et al., 2011). However, Li et al., (2009) were able to demonstrate nuclear translocation of CAR using CITCO in primary cultures of human hepatocytes. The study of Chan et al., (2011) suggested that human CAR (hCAR) is widely distributed in the cytosol and nucleus, thus the activation of CAR by CITCO in the nucleus is sufficient to induce gene transcription without a dependence upon translocation from the cytoplasm to the nuclear. Apart from the unique pattern of nuclear translocation, CAR also exhibits constitutive transcription activity in the absence of ligand (Timsit & Negishi 2007) and it has been suggested that, in the absence of ligand, CAR possesses an active structural conformation and is able to recruit co-activators independent of ligand (Stanley et al. 2006).

CAR interacts with a wide array of compounds (Appendix A), including bile acids (6-ketolithocholic acid, cholic acid), endogenous metabolites (androstanol and androstenol), antiretroviral drugs (nevirapine, efavirenz and abacavir) and hydrocarbons (2,3,3',4',5',6-hexachlorobiphenyl and 6-(4-chlorophenyl)imidazo) (Swales et al., 2005; Kodama & Negishi, 2006; Pascussi et al., 2008). The activities of CAR and PXR are closely related and overlap, since both regulate expression of genes encoding phase I (cytochrome P450) and phase II drug metabolising enzymes (glutathione S-transferases, UDP-glucuronosyl-transferases and sulfotransferases) (Huang et al., 2003; Urquhart et al., 2007; Tolson and Wang, 2010). Similar to PXR, CAR exhibits species differences in terms of interactions with drugs and ligands and studies have shown that the CAR ligand-binding domain in porcine and human share 84 % sequence homology, compared to only 70 % sequence homology between human and mouse (Timsit & Negishi 2007; Gray, Peacock & Squires, 2009).

In the indirect activation of CAR, the ligand binds to the EGF receptor and causes the dephosphorylation of RACK1 and the recruitment of PP2A to dephosphorylate the CAR-HSP90-CCRP protein complex leading to the dissociation of CAR from HSP90 and CCRP and subsequent nuclear translocation of CAR. In the nucleus, CAR heterodimerizes with RXR and recruits two coactivators, glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator-1 (SRC1) to form a protein complex that subsequently binds to its response element to initiate gene transcription (Kawamoto et al., 1999; Kobayashi et al., 2003; Mäkinen et al., 2003; Yoshinari et al., 2003; Li et al., 2009; Mutoh et al., 2013; Timsit & Negish, 2014).

1.4.1.2.3 Retinoid X receptor (RXR)

The retinoid X receptor (RXR) is an obligatory component for the formation of nuclear receptor heterodimers which subsequently regulate gene expression. Endogenous ligands of RXR include 9-cis retinoic acid, all-trans retinoic acid, endogenous fatty acids and docosahexaenoic acid (DHA) (Heyman et al., 1992; Lala et al., 1996; De Urquiza et al., 2000). Three isoforms of RXR (RXRα, NR1B1; RXRβ, NR1B2; RXRγ, NR1B3) are known to be expressed in rat and mouse brain capillaries (Heyman et al., 1992; Germain et al., 2006; Akanuma et al., 2008; Arfaoui et al., 2013). These partnering receptors are implicated in the regulation of expression of several drug transporters in the BBB. Thus far, PXR, CAR, VDR, FXR, PPARs and LXR are among the nuclear receptors that form heterodimers with RXR after translocating to the nucleus. The heterodimers then bind directly to response elements located in the promoter regions genes, including genes encoding ABCB1 and ABCG2, however little is known of the role of RXR in regulating ABCC5 expression (Kliewer et al., 1992; Lehmann et al., 1998; Costet et al., 2000; Murthy et al., 2002, Pfrunder et al., 2003; Maher et al., 2005; Maher et al., 2006; Urquhart, Tirona & Kim, 2007; Saeki et al., 2008; Bauer et al., 2008a; Wang, Y.X., 2010; Hoque, et al., 2012; Miller, 2014; More et al., 2016). Therefore, heterodimerisation with RXRs is the foremost step in the activation and binding of nuclear receptors to the promoter regions of their target genes (Mangelsdorf et al., 1991; Kliewer et al., 1992; Mader et al., 1993; Germain et al., 2006) and studies show a lack of RXR significantly reduces expression of PXR and CAR target genes (Honkakoski et al., 1998; Frank et al., 2003).

RXR-binding partners can be categorised into permissive or non-permissive binding partners. When permissive partners, such as PPARs, LXRs, FXR, PXR, and CAR, are bound to RXR, the heterodimer can be activated by either RXR ligands or partnering receptor ligands and produce a synergic activation (Westin et al., 1998). For example, the CAR-RXR heterodimer can be activated by either CAR ligands (CITCO) or RXR ligands (9-cis-Retinoic acid) leading to the activation of the transcription process (Mackowiak and Wang et al., 2016). When RXR is bound to non-permissive binding partners, such as the retinoid acid receptor (RAR), vitamin D receptor (VDR) and thyroid hormone receptor (T_3R) the heterodimer can only be activated by ligands specific to the partnering receptors. For example, the T_3R-RXR heterodimer can only be activated by T_3R ligands (thyroid hormone) leading to initiation of the transcription process (Mackowiak and Wang et al., 2016). (Kurokawa et al., 1994; Forman et

al., 1995; Willy et al., 1995; Mandrekar-Colucci & Landreth, 2011). To date, the complex role of RXR in mediating expression of ABC drug efflux transporters is poorly understood.

1.4.2 Pro-inflammatory cytokines

Inflammatory stress is associated with various CNS disorders, such as neurodegenerative diseases, neuroinflammation, stroke, trauma and infections. All these conditions can trigger an inflammatory response that affects BBB permeability (Rosenberg, 2012). Pro-inflammatory cytokines for example tumour necrosis factor-α (TNF-α), interleukin-1*β* (IL-1β) and interleukin-6 (IL-6), and inflammogens (lipopolysaccharide (LPS)) are mediators of the inflammatory response and bind to surface receptors of brain endothelial cells and alter the expression and activity of ABC efflux transporters (Bauer et al., 2007; von Wedel-Parlow et al., 2009).

Dysfunction of the BBB has been described in many systemic and CNS inflammatory diseases (Erickson & Banks, 2018) and plays a central role in the pathological mechanism of many neurological diseases (Zhao et al., 2015; Liebner et al., 2018). TNF-α and IL-1β are the two most studied pro-inflammatory cytokines in neuroinflammation associated with CNS pathologies (Sochocka, Diniz & Leszek, 2017; Liebner et al., 2018). Furthermore, TNF-α and IL-1β production is induced by systemic inflammation (Hoogland et al., 2015). Elevated levels of these two cytokines enhance BBB permeability and cause chronic neurodegenerative diseases (Sochocka, Diniz & Leszek, 2017; Liebner et al., 2018). Both TNF-α and IL-1β amplify the immune response by inducing chemokine expression which serves to recruit immune cells to the site of inflammation. The positive effect of inflammation is that the migrating cells can help to rapidly eliminate invading pathogens, whilst the negative effect is that inflammation can be harmful to neurons, oligodendrocytes and other cells of the CNS. In general, TNF-α and IL-1β tend to have neuroprotective effects at low concentrations, whereas they cause neuronal death at higher levels (Simard and Rivest, 2005).

Interleukin-1β is a key factor for the development of chronic metabolic diseases including diabetes mellitus (i.e. type 2 diabetes), gout and metabolic syndrome (Dinarello, 2011). IL-1β also plays an important role in psychiatric diseases such as depression (Howren et al., 2009) and schizophrenia (Watanabe et al., 2010) and in a broad spectrum of other neurological conditions including infections, trauma, stroke, and epilepsy, as well as chronic

neurologic diseases such as muscular atrophy, Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease (Walsh et al., 2014). BBB disruption or impairment of ABC efflux transporters is associated with progression of these diseases (Muldoon et al., 2013). To date, many studies have focussed on the effect of TNF-α on ABCB1 in model systems including male guinea pig brain endothelial cells (Iqbal et al., 2012), rat brain capillaries (Bauer et al., 2007; Hartz et al., 2006; Theron et al., 2003), porcine brain endothelial cells (von Wedel-Parlow et al., 2009) and primary rat astrocytes (Ronaldson and Bendayan, 2006), and on ABCG2 (Poller et al., 2010; von Wedel-Parlow et al., 2009) and ABCC1 (Ronaldson and Bendayan, 2008). However, far less research has been carried out to investigate the effects of IL-1β on ABC efflux transporter expression and activity in BBB endothelial cells. Thus, given the fact that IL-1β is a key factor in disease development, this thesis will investigate the effects of IL-1β on ABC efflux transporter expression and activity in porcine primary brain endothelial cells.

1.4.2.1 interleukin-1

Interleukin-1β belongs to the IL-1 family that contains 3 proteins, IL-1α, IL-1β, and the IL-1 receptor antagonist (IL-1RA) and IL-1α, IL-1β, and IL-1RA are the products of 3 separate genes located on the long arm of the chromosome 2 (Dinarello, 1996; Dinarello, 1991; Dinarello and Wolff, 1993). IL-1α and IL-1β share less than 30% amino acid homology, but they bind to the same receptor, interleukin-1 receptor type 1 (IL-1RI) and have similar biological activities. IL-1 receptor antagonist is an endogenous antagonist that possesses no agonist activity and blocks IL-1α- and IL-1β-mediated processes by binding to IL-1RI but not inducing intracellular signalling (Auron and Webb, 1994; Mantovani et al., 1996).

1.4.2.2 The effect of porcine interleukin-1β on BBB integrity

Interleukin-1β treatment has been demonstrated to significantly increase the paracellular permeability of primary rat brain endothelial cell monolaters by disrupting the distribution and overall structure of the tight junctional proteins (Wang, Y. et al., 2014; Harazin et al., 2018). It has also been shown that the expression levels of the tight junction proteins claudin-5, occludin, and ZO-1 were closely correlated with monolayer integrity with levels of these proteins highest when permeability was lowest (Wang, Y. et al., 2014). Interleukin-1β-induced inflammation is accompanied by a break-down in the barrier function of both *in vivo* and *in vitro* BBB models. For example, injection of IL-1β into rat brain parenchyma results in

the loss of occludin and ZO-1 expression at endothelial cells (Bolton et al., 1998). Similarly, IL-1β treatment disrupts monolayer integrity by reducing transendothelial electrical resistance (TEER), increasing the paracellular permeability and downregulating expression of ZO-1 and occludin in human, mouse and rat brain endothelial cells (Wang, Y. 2014; Blamire et al., 2000; Laflamme et al., 1999; Coisne and Engelhardt, 2011; Rigor et al., 2012; Labus et al., 2014). Currently, is stated that IL-1β is one of the major pro-inflammatory factors involve in the BBB disruption (Ding et al., 2020). Disruption of BBB integrity as a result of endothelial dysfunction of tight junctions and transporters lead to debilitating CNS disorders, such as Alzheimer's disease (Zhong, 2008; Altman and Rutledge, 2010), Parkinson's disease (Barcia, 2005), amyotrophic lateral sclerosis (Arhart, 2010), multiple sclerosis (Alexander, 2011), human immunodeficiency virus (HIV)-1-associated neurocognitive disorder (Banks et al., 2006), cognitive impairment (Bowman et al., 2018) and traumatic brain injury (Morel, 2008). Hence, since IL-1β is a key factor in CNS inflammatory disease development, it is crucial to investigate the signalling pathway of IL-1β on BBB endothelial cell function.

1.4.2.3 The interleukin-1β pathway

In the event of IL-1β-mediated inflammatory pathway, IL-1 receptor type 1 (IL1-R1) was activated by IL-1β and binds to Myeloid differentiation primary response 88 (MYD88) (Yamasaki et al., 1995). MyD88 then binds and activates IL-1 receptor associated kinase 4 (IRAK4) via its death domain (Kawagoe et al., 2008; Ferrao et al., 2014), IRAK4 phosphorylates and activates IRAK1. The latter subsequently leads to the recruitment and oligomerisation of TNF receptor-associated factor 6 (TRAF6) (Arron et al.,2002; Kawagoe et al., 2008; Ferrao et al., 2014). TRAF6 forms a complex with mitogen-activated protein kinase kinase kinase (MAPKKK) and stimulates two distict signalling pathways – the activation of transcription factors Nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinases (MAPKs). The three most commonly MAPKs in IL-1β pathway are p38, cJun N-terminal kinase (JNK) and the extracellular signal-regulated protein kinase (ERK1/2) (Dunne & O'Neill, 2003; O'Neill, 2002; Kawagoe et al., 2008; Dinarello, 2009). MAPK and NF-κB are the most common factors triggering the inflammatory pathway downstream of IL-1β (Risbud and Shapiro, 2014; Liu et al., 2017).

1.4.2.3.1 NF-κB dependent Interleukin-1β-mediated inflammatory pathway

NF-κB is a sequence-specific transcription factor that controls the synthesis of a wide variety of molecules involved in orchestrating the inflammatory response (Karin & Ben-Neriah, 2000; Karin, 1999; Krasnow et al., 2017; Gosselin and Rivest, 2008). It consists of five structurally related members, including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel, which mediates transcription of target genes by binding to a specific DNA element in the form of various homo- or heterodimers (Hayden & Ghosh, 2012; Sun, 2012; Lin et al., 1998).

The NF-κB-dependent inflammatory pathway consists of canonical and noncanonical pathways as illustrated in Figure 1.11. In canonical pathway, it mediates the activation of NF-κB1 p50, RELA and c-REL. In the event of IL-1β-mediated inflammatory pathway, TRAF6 forms a complex with Mitogen-activated protein kinase kinase kinase 7 (TAK1) (Sun et al., 2004), which in turn phosphorylates and activates IKKβ, leading to IKK activation. IKK is comprised of 3 subunits, IKKα, IKKβ and IKKγ and only the IKKβ subunit is common in canonical NF-κB activation (Baeuerle, 1998; Delhase et al., 1999). Consequently, activated IKKβ phosphorylates Iκ-Bα, which targets the latter for polyubiquitination and degradation by proteasomes (Baeuerle, 1998; Baeuerle and Baltimore, 1996). NF-κB is a p50/p65 heterodimer that is normally sequestered in the cytoplasm forming an inactive complex with the inhibitor Iκ-Bα. The degradation of Iκ-Bα frees NF-κB in the cytoplasm and allows the nuclear translocation of canonical NF-κB family members to translocate into the nucleus where it can bind its consensus DNA sequence in the form of various dimeric complexes, including RELA–p50, c-REL–p50, and p50–p50 and induce gene transcription. Following its degradation, Iκ-Bα is rapidly re-synthesized to act as an endogenous inhibitory signal for NF-κB. NF-κB proteins are sequence-specific transcription factors that control the synthesis of a wide variety of molecules involved in orchestrating the inflammatory response (Karin & Ben-Neriah, 2000; Karin, 1999; Krasnow et al., 2017; Gosselin and Rivest, 2008; Dinarello, 2009). As far as we are concerned, the detailed mechanism of NF-κB in regulating the expression of ABC efflux in BBB remains inconclusive.

In the non-canonical NF-κB pathway, TRAF6 forms a complex with TAK1 and phosphorylates and activates Mitogen-activated protein kinase kinase kinase 14/ NFκB-inducing kinase (NIK) (Xiao et al., 2001; Liu et al., 2017). NIK phosphorylates and activates

IKKα, which in turn phosphorylates carboxy-terminal serine residues of p100, triggering selective degradation of the C-terminal IκB-like structure of p100 and leading to the generation of free NF-κB in the cytoplasm and allow the nuclear translocation of NF-κB complex p52/RelB and the activation of target genes (Sun et al., 1994; Tucker et al. 2004; Zarnegar et al., 2008).

Functionally, canonical NF-κB is involved in almost all aspects of the inflammatory response, whereas the noncanonical NF-κB pathway appears to be involved as a supplementary signalling axis that cooperates with the canonical NF-κB pathway in the regulation of specific inflammatory responses (Liu et al., 2017).

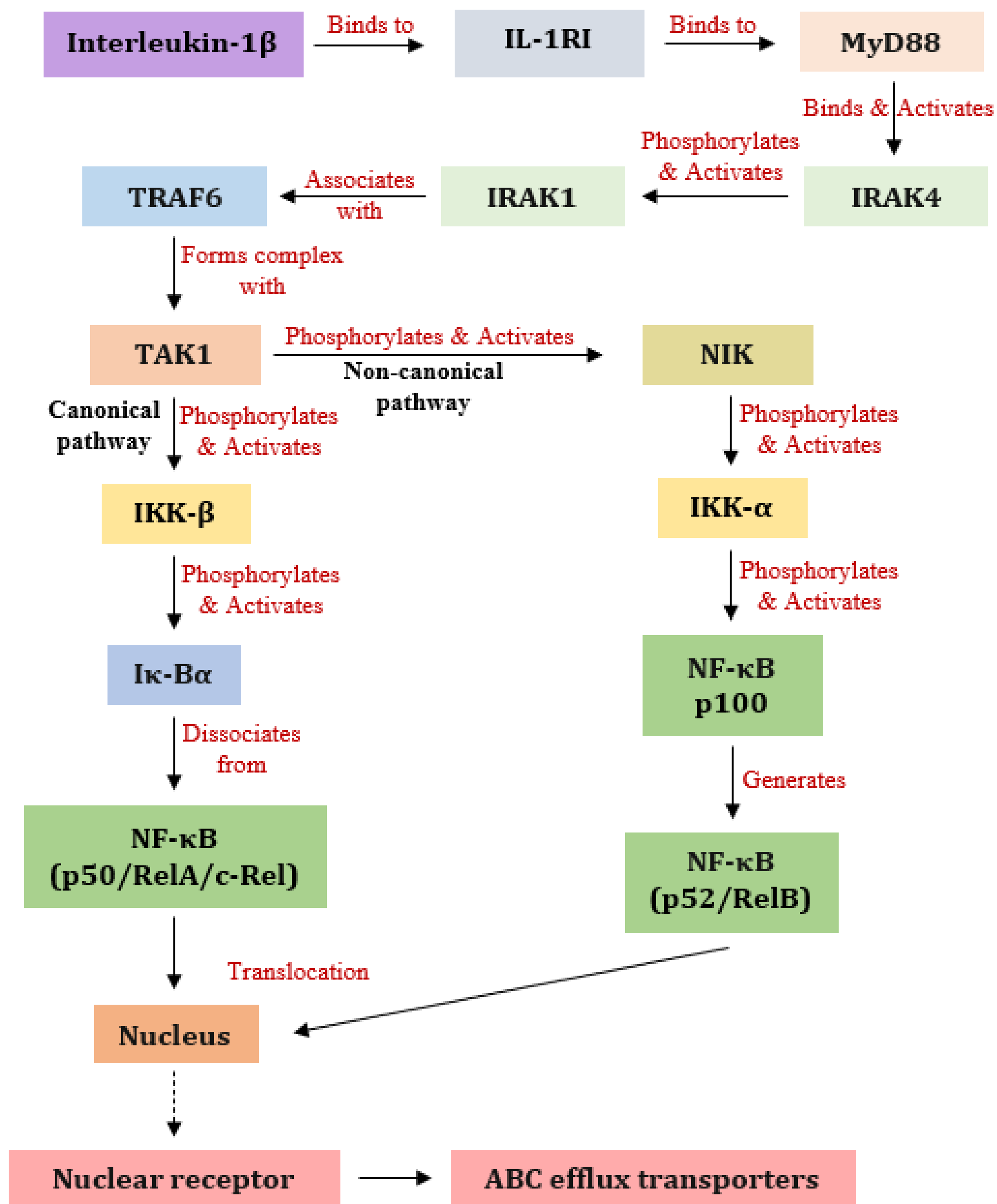


Figure 1.11: Schematic illustration of the possible NF-κB-dependent inflammatory pathway that regulates ABC efflux transporter expression in blood-brain barrier endothelial cells.
IL-1RI, interleukin-1 receptor type I; MyD88, Myeloid differentiation primary response 88; IRAK4, Interleukin-1 receptor-associated kinase 4; IRAK1, Interleukin-1 receptor-associated kinase 1; TRAF6, Tumour necrosis factor receptor associated factor 6; MAP3K, Mitogen Activated Protein (MAP) kinase kinase kinase; TAK1, Mitogen-activated protein kinase kinase kinase 7; NIK, Nuclear factor-kappa-B-inducing kinase; IKK-α, Inhibitor of nuclear factor kappa-B kinase subunit beta; IKK-β, Inhibitor of nuclear factor kappa-B kinase subunit beta; Iκ-Bα, inhibitor of nuclear factor kappa B alpha; NF-κB, Nuclear factor kappa B; dotted arrow: unknown pathway.

1.4.2.3.2 MAPK-dependent Interleukin-1β pathway

In the event of activation of the IL-1β-mediated inflammatory pathway, as illustrated in Figure 1.12, TRAF6 forms a complex with Mitogen-activated protein kinase kinase kinase (MAP3K) (Sun et al., 2004). Activation of MAPK pathway is mediated by a three-tiered kinase model comprised of MAP3K, Mitogen-activated protein kinase kinase (MAP2K) and Mitogen-activated protein kinase (MAPK) through sequential protein phosphorylation. MAP3K is a family of serine/threonine kinases (Ser/Thr). There are a total of 21 kinases identified to function as MAP3K, RAF, MEKK1 and TAK1 are among the members of MAP3K activated by TRAF6 in MAPK-mediated inflammatory pathway (Symons et al., 2006; Winter-Vannet al., 2007; Ozbabacan 2014). The activated RAF, MEKK1 and TAK1 in turn phosphorylate and activate MKK1/2, MKK4/7 and MKK3/6 respectively (Zhang et al., 2014; Dhanasekaran and Reddy, 2008; Natsvlishvili et al., 2015). MKK1/2, MKK4/7 and MKK3/6 belong to the member of MAP kinase kinase (MAPKK), they are responsible for the phosphorylation and activation of ERK1/2, JNK and p38 (Roskoski, 2012a,b; Pouysségur and Lenormand, 2016). ERK1/2, JNK and p38 are the major subgroups of MAP kinase (MAPK), activated MAPKs translocate to the nucleus to phosphorylate transcription factors coordinate the expression of downstream target genes in response to inflammation (Kirkwood et al., 2007; Huang, 2009; Malik et al., 2017; Sabio & Davis, 2014). Currently the detailed MAPK-dependent interleukin-1β pathway in regulating ABC efflux transporters in BBB involving ERK1/2, JNK and p38 remains largely elusive.

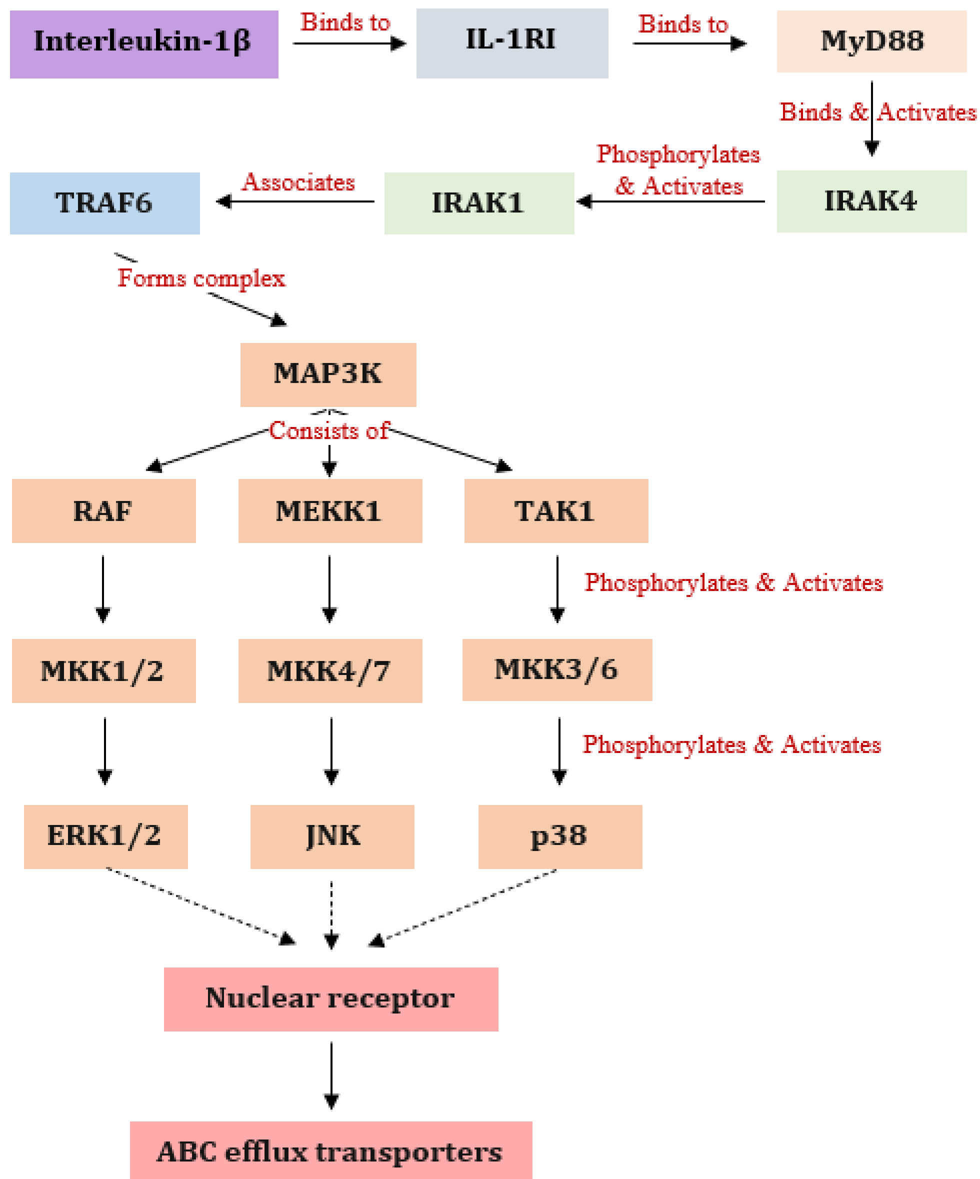


Figure 1.12: Schematic illustration of the possible MAPK-dependent inflammatory pathway that regulates ABC efflux transporter expression in BBB endothelial cells.
IL-1RI, interleukin-1 receptor type I; MyD88, Myeloid differentiation primary response 88; IRAK4, Interleukin-1 receptor-associated kinase 4; IRAK1, Interleukin-1 receptor-associated kinase 1; TRAF6, Tumour necrosis factor receptor associated factor 6; MAP3K, Mitogen Activated Protein (MAP) kinase kinase kinase; RAF, Rapidly accelerated fibrosarcoma; MEKK1, Mitogen-activated protein kinase kinase kinase 1; TAK1, Mitogen-activated protein kinase kinase kinase 7; MKK1/2, Mitogen-activated protein kinase kinase 1/2; MKK4/7 Mitogen-activated protein kinase kinase 4/7; MKK3/6, Mitogen-activated protein kinase kinase 3/6; ERK1/2, Extracellular signal-regulated protein kinase 1/2; JNK, c-Jun N-terminal kinase; p38, p38 mitogen-activated protein kinases; dotted arrow: unknown pathway.

1.4.2.4 The effect of interleukin-1β on ABC efflux transporter expression

Despite the fact that IL-1β is one of the most important pro-inflammatory cytokines, the detailed mechanisms of the IL-1β pathway with respect to the modulation of ABC efflux transporters in BBB has not been widely studied and clearly elucidated. Several studies have reported the possible role of IL-1β in the regulation of ABC efflux transporters in BBB in inflammatory conditions however the studies are contradictory and inconclusive. For example, IL-1β has been reported to either increase (Ronaldson and Bendayan, 2006; Torres-Vergara and Penny 2018; Zuloaga et al., 2012), decrease (von Wedel-Parlow et al., 2009; Iqbal, 2012) or have no effect (Poller et al., 2010) on ABCB1 transporter activity and expression in BBB endothelial cells. The reported effect of IL-1β on ABCG2 is somewhat more consistent, with both von Wedel-Parlow et al. (2009) and Poller et al. (2010) demonstrating IL-1β treatment significantly reduce ABCG2 transporter activity and expression in porcine and human BBB endothelial cells respectively, however, Xiong et al. (2009) reported IL-1β treatment had no effect on ABCG2 transporter activity and expression in human primary epithelial cells. This apparent contradiction is not surprising because inflammation is a complex process.

1.4.3 Post-translational modification of ABC efflux transporters

Post-translational modifications (PTMs) are regulators that significantly influence the ABC transporters' function, expression, efficiency, structure, fate, interactions, and more via a series of molecular mechanisms. Among the over 400 types of PTMS identified to date, the most common types of PTMs known to play a role in the regulation of ABC transporters are phosphorylation (addition of a phosphoryl functional group), acetylation (addition of an acetyl functional group), ubiquitination (addition of small protein), glycosylation (addition of sugar chains), SUMOylation (addition of Small Ubiquitin-like Modifier proteins) and palmitoylation (addition of lipids) on solvent accessible amino acid residues (Walsh et al., 2005; Hunter, 2007; Shaikh et al., 2013; Duan and Walther, 2015; Korkuc & Walther, 2017).

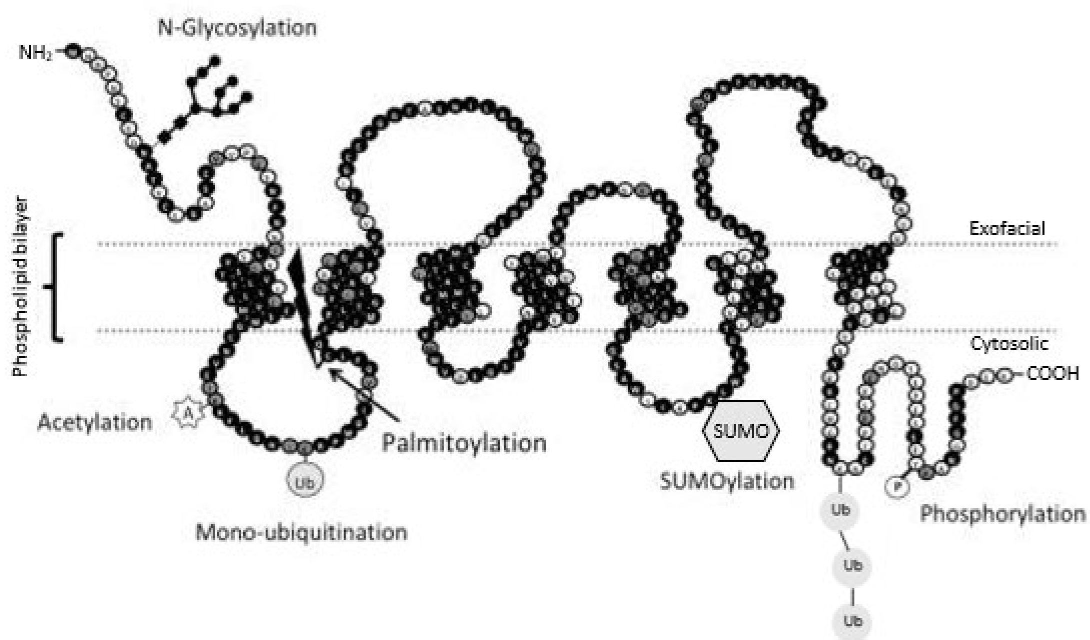


Figure 1.13: Localisation of common post-translational modifications on a general transporter structure (Czuba et al., 2018).

1.4.3.1 Phosphorylation

Protein phosphorylation is a reversible PTM that involves the attach of a terminal phosphate monoester group to a free hydroxyl group of primarily serine, threonine and tyrosine residues or to a lesser extent on histone, lysine, and arginine residues. The addition of phosphate group is catalysed by different kinases and phosphatases, leading to different biological outcomes (Bradshaw, 2010).

Phosphorylation of ABCC1 at T249 was shown to increase the efflux of doxorubicin and estradiol 17β-D-glucuronide in human breast cancer cells, MCF7 (Stolarczyk et al., 2012). Serine/threonine kinase Pim-1 has been reported to positively regulate both ABCB1 and ABCG2 transporters. Phosphorylate ABCB1 at ser683 by Pim-1 was shown to increase its expression by inhibiting its proteolytic degradation and increase the efflux of doxorubicin in OVCAR-8-Pgp cells (Xie et al., 2010). Whereas phosphorylation of ABCG2 by Thr-362 was shown to modulate the dimerization/oligomerisation of ABCG2 and its plasma membrane localisation and promote docetaxel resistance in prostate cancer cells (Xie et al., 2007). Protein kinase C (PKC) isoenzymes have also been commonly reported to alter the ABCB1 transporter activity, depending on which isoenzyme phosphorylates the ABCB1. Study shows that

phosphorylation of ABCB1 by PKCα led to an inhibitory effect on its ATPase activity and its drug binding affinity to azidopine, while Inhibition of PKCα by safingol enhanced drug accumulation and sensitivity in MCF-7 DOXR cells to vinblastine and enhance toxicity of Vinca alkaloids and anthracyclines (Sachs et al., 1995).

1.4.3.2 N-glycosylation

N-glycosylation is the process of enzymatically adding an oligosaccharide to extracellular asparagine residues and is often signalled by the motif N-X-S/T, where X is any amino acid except proline (P) or aspartic acid (D).

Glycosylation of ABCB1 promotes its stability in the membrane by preventing it from proteolytic degradation (Xie et al., 2010). Similarly, glycosylation of ABCG2 was shown to promote stability of ABCG2 while disruption of *N*-linked glycosylation results in a reduced ABCG2 protein expression level (Wakabayashi-Nakao et al., 2009). N-glycosylation can influence the substrate binding affinity and overall transport mechanisms of ABCCs transporters. N-glycosylated at N746 and N754 in ABCC4 affect its ability to transport prostaglandin E2, but not estradiol glucuronide (Miah et al., 2016). N-glycosylation of ABCC1 at N19, N23 appears to enhance its substrate biding affinity and its capacity in transporting substrates (Miah et al., 2016). Furthermore, glycosylation of ABCC1 facilitates the localisation of the transporter in cisplatin-resistant cancer cell lines (Liang et al., 2003). Defective in the glycosylation of ABCC1 and ABCC4 lead to upregulation of both transporter expression and activity and associated with resistance to platinum compounds in ovarian carcinoma cell lines (Beretta et al., 2009).

1.4.3.3 Acetylation

The proteomics data by Lundby et al. (2012) and co-workers suggest multiple ABC transporters may be acetylated; yet the functional significance is unknown for most. Two unique peptides with three acetylated residues were attributed to ABCA5 at K614, K620, and K1438. The mitochondrial transporters ABCB7, ABCB8, and peroxisomal transporter ABCD3, were also shown to be acetylated along with some uncharacterized proteins. In addition, ABCG2 was modified at K171, a residue that was conserved in the human sequence and located in the first multi-spanning domain (Lundby et al., 2012).

1.4.3.4 Palmitoylation

Protein palmitoylation is a widespread reversible lipid modification that involves the enzymatic modification of one or more cysteine thiols on a substrate protein to form a thioester with a palmitoyl group for rapid regulation of the function of many cellular proteins (Dietrich & Ungermann, 2004; Guan and Fierke, 2011).

Studies on palmitoylation on ABC transporters are relatively scarce, thus far, only cholesterol and lipid efflux transporter are reported. Palmitoylation of ABCA1 transporter is vital for its rapid (de)-association in the plasma membrane, and consequently for its functional status. Palmitoylation of four cysteine residues on ABCA1 at the N-terminal region – C3 and C23– and two in the linker region –C1110 and C1111 significantly increased the efflux of phospholipids and cholesterol by ~50% and increased cell surface expression by ~90% (Singaraja et al., 2009).

ABCG1 is palmitoylated in its N-terminal domain at C26, C150, C311, C390, and C402. However, unlike ABCA1, where multiple palmitoylation residues are critical to expression, only C311 appears to have a functional role for ABCG1. Palmitoylation of ABCG1 appeared to enhance cholesterol efflux by altering trafficking of the transporter from the endoplasmic reticulum to the plasma membrane. However, other ABCG family members such as ABCG5 and ABCG8 has been shown not affected by either palmitoylation or depalmitoylation (Wang et al., 2008; Li et al., 2013; Gu et al., 2013).

1.4.3.5 Ubiquitination

Ubiquitination is a process of attaching ubiquitin by forming an isopeptide bond between a Gly residue of ubiquitin and a specific Lys residue of a target protein. The ubiquitin-proteosome is a physiological pathway that targets the degradation of unstable proteins via the proteasome via the three-step mechanism. Initially, E1 (ubiquitin-activating enzyme) activates ubiquitin (a small, 8 kDa polypeptide) to generate a high-energy thiol ester intermediate, E1-S∼ubiquitin. The E2 (ubiquitin-carrier proteins) transfers the activated ubiquitin moiety from E1, by the addition of high-energy thiol ester intermediate, E2-S∼ubiquitin, to the substrate that is specifically bound to E3 (ubiquitin-protein ligase), to generate a third high-energy thiol ester intermediate, E3-S∼ubiquitin. Multiple cycles of conjugation of ubiquitin to the NH_2-terminal amino group of the substrate generate a polyubiquitin chain which is recognized by

the downstream 26S proteasome complex (Pickart, 2001; Glickman & Ciechanover, 2002). The linkages of ubiquitination can alter the biological outcomes of a protein, for example, Lys-48–linked polyubiquitination commonly serves as the earmark for proteasome-mediated degradation (Yau & Rape, 2016). On the other hand, Lys-63–linked polyubiquitination is used for multiple purposes, including the regulation of the endosomal recycling and degradation events near the plasma membrane (MacGurn et al., 2012).

Ube2r1 and FBXO15 are the two main regulators that mediate ABCB1 ubiquitination. Ube2r1 and FBXO15 knockdown supress ubiquitination and significantly upregulated cell-surface ABCB1 expression and enhanced its efflux activity (Katayama et al., 2013). Inhibition of MAPK pathways using U0126 (ERK inhibitor) down-regulate ABCB1 expression by promoting its degradation ABCB1 via ubiquitin-proteasome pathway leading to enhanced intracellular accumulation of rhodamine123 and paclitaxel (Katayama et al., 2007). The detailed mechanism of ubiquitination was illustrated in ABCC2 and ABCB11 in which ubiquitination was facilitated by clathrin-mediated endocytosis to promote internalisation of ABCC2 and ABCB11 leading to the disappearance from the cell surface (Aida et al., 2014).

In ABCG2, ubiquitination is commonly occurred in ABCG2 F208S and S441N variant proteins, which is lacking N-linked glycosylation and recognised as a misfolded protein in the endoplasmic reticulum to be targeted for ubiquitination and proteasomal degradation (Nakagawa et al., 2011). Furthermore, disruption of N-linked glycosylation in ABCG2 also leads to increase misfolded ABCG2 proteins and enhance the chance of undergoing ubiquitin–proteasome proteolytic pathway (Nakagawa et al., 2009).

The ubiquitin-mediated proteasomal degradation of ABC transporters is a new aspect of pharmacogenomics and also serves to enhance ER protein quality control to reduce genetic polymorphism in ABC transporters (Nakagawa et al., 2008).

1.4.3.6 SUMOylation

Similar to ubiquitin, sumoylation is a reversible modification that involves the enzymatic addition of a small protein SUMO (Small Ubiquitin-like MOdifier) to an accessible lysine. Unlike ubiquitination, modification by SUMO does not cause degradation, but rather facilitates the formation of an intracellular holding pool that is available for the rapid response

to central nervous system signalling. The process of sumoylation requires an E1 activating enzyme (SAE1/SAE2), an E2 conjugating enzyme (Ubc9) and possibly a member from the family of E3 ligases (Hay, 2005). Attachment of SUMO requires a lysine residue that is located within a SUMO consensus motif, ΨKXE. The motif is somewhat flexible, since Ψ represents a large hydrophobic residue, K is the lysine to which SUMO-1 is conjugated, X can be any amino acid, and E is glutamic acid (Rodriguez et al., 2001).

The studies of sumoylation on ABC transporters are limited, thus far only ABCC2 is reported to contain ΨKXE motif. This lysine motif is obligatory for Ubc9 binding and the subsequent conjugation of SUMO to ABCC2. Suppression of endogenous Ubc9 by small interfering RNA resulted in a selective 30% reduction in Abcc2 protein expression in the post-nuclear supernatant (Minami et al., 2009).

1.5 Types of *in vitro* models for characterisation of ABC transporter at the blood-brain barrier

Efforts to generate a BBB model based on the *in vitro* culture of brain endothelial cells started in the early 1970s with isolations of brain capillaries (Joo et al., 1973; Mrsulja et al., 1976). Over the years, rat, bovine, porcine and human brains have been commonly used to isolate and establish primary cultures of brain endothelial capillary cells. The growing popularity of primary cells over immortalised cell lines is due to the fact that immortalised cell lines lack restrictive barrier characteristics and demonstrate extremely low junctional tightness as reflected in low TEER and high BBB permeability (Gumbleton and Audus 2001; Barar et al. 2016). Importantly, the key *in vivo* feature of a restrictive barrier is more commonly retained in primary endothelial cells in culture. Despite the complex, time-consuming isolation procedures and the possibility of contamination with other cells, like astrocytes and pericytes, primary cultures can better reflect the *in vivo* BBB, especially when low passage cultures are used (Rubin et al., 1991, Franke et al., 2000, Zhang et al., 2006, Jeliazkova-Mecheva and Bobilya, 2003, Avdeef, 2011). Although *in vivo* models are the ideal model, when it comes to the primary study of physiological and pharmacological events, the resources and time invested to obtain reliable results, as well as ethical concerns of using higher species, limit their applications. For those reasons, *in vitro* models are a good alternative for this type of research.

A good quality and reliable primary *in vitro* model must closely mimic the *in vivo* BBB characteristics and meet the following criteria: highly restrictive paracellular pathway reflected by high transendothelial electrical resistance (TEER) and low permeability, demonstration of BBB marker enzymes (alkaline phosphatase and γ-glutamyl transpeptidase), expression of tight junction proteins including occludins, claudins, and junctional adhesion molecules (JAMs), expression of functional efflux transporters, and lastly, that a sustainable and large quantity of endothelial cells can be obtained in a single isolation procedure to ensure multiple studies can be carried out. (Deli et al., 2005; Rubin and Staddon, 1999; Nitta et al., 2003; Reichel et al., 2003; Hamm et al., 2004; Hawkins et al., 2006; Hawkins and Egleton, 2007; Begley, 2004).

1.5.1 Mouse model

Mouse primary brain endothelial cells are the least popular model as the yield of endothelial cells is low compared to rat, bovine, porcine and human. However, the advantages of using a mouse BBB model is the availability of transgenic and gene-targeted animals, oligoprobes, and a wide range of antibodies (Deli et al., 2005; He et al., 2014; Helm et al., 2016). Primary mouse brain endothelial cells are reported to display classic BBB characteristics including expression of occludin, claudin, zonula occludens and Junction Adhesion Molecules (Coisne et al., 2005; Stamatovic et al., 2003; Steiner et al., 2011; Bernard-Patrzynski et al., 2019; Johnsen et al., 2018) and the BBB marker enzymes alkaline phosphatase and γ-glutamyl-transpeptidase (Tatsuta et al., 1992; Weidenfeller et al., 2005; Garberg et al., 2005; Yu et al., 20011; Johnsen et al., 2018). Generally, cell monolayer permeability of sucrose is reported to be 1.0 - 4.5 × 10^{-6} cm/s (Coisne et al., 2005; Brown et al., 2007; Yang et al., 2017), however, TEER values are usually 100 – 300 $\Omega.cm^2$, with the highest TEER reported at 800 $\Omega.cm^2$ (Coisne et al., 2005; Brown et al., 2007; Yang et al., 2017). TEER measurements of this magnitude are rarely reported in studies on mouse primary endothelial cells and are generally much lower compared to bovine and porcine models.

In isolated mouse brain endothelial cells ABCB1 is the most highly expressed transporter, followed by ABCG2 and members of ABCC subfamily (ABCC1, ABCC3, ABCC4, ABCC5, ABCC6) (Kamiie et al., 2008; Daneman et al., 2010; Warren et al., 2009; Chun et al., 2011).

1.5.2 Rat model

Similar to the mouse system, the yield of rat brain endothelial cells is low, only 1–2 million endothelial cells per rat brain can be isolated. The rat model has been shown to express the tight junction proteins occludin, claudin-1, -3, -5, -11, -12, -19, ZO-1 and ZO-2 (Calabria et al., 2006; Molino et al., 2014; Xue et al., 2013; Watson et al., 2013; Walter et al., 2015; Cardoso et al., 2012), along with BBB marker enzymes alkaline phosphatase and γ-glutamyl transpeptidases (Blasig et al., 2001; Walter et al., 2015). The BBB sucrose permeability is around 2 - 10 × 10^{-6}cm/s (Stanness et al., 1999; Blasig et al., 2001; Garciaa-Garcia et al., 2005; Krizanac-Bengez et al., 2006) and rat models generally display low to medium TEER, around 100–400 Ω.cm^2 (Imamura 2011; Wilhem et al., 2011; Walter et al., 2015; Cardoso et al., 2012 Molino et al., 2014; Liu et al., 2014). However, only a small number of studies have reported TEER values of 500 – 999 Ω.cm^2 (Veszelka et al., 2013; Watson et al., 2013). Barrier properties are better than those observed in the mouse models, but they do not reach the level of the best bovine or porcine monolayers. Similar to mouse models, relative expression levels of ABC efflux transporters in rat brain endothelial cells, from high to low, are ABCB1, ABCG2 and ABCC subfamily members (ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6) (Enerson and Drewes, 2006; Warren et al., 2009; Wang et al., 2011; Hoshi et al., 2013).

1.5.3 Bovine model

The gray matter of a bovine brain will invariably yield an average of around 100 million viable cells. Bovine brain endothelial cells are generally seeded into culture at 50,000 cells/cm^2 and reach confluence on day 7 or 8 and form a tight monolayer on approximately day 10 or 11 (Colgan et al., 2007). Bovine brain endothelial cells will begin to undergo noticeable morphological and functional changes after day 16, losing some of their BBB characteristics (Raub et al., 1992). Reports demonstrate expression of the tight junction protein occludin, claudin-1, claudin-5, ZO-1 and ZO-2 and the junction adherens protein JAM-1 (Hamm et al., 2004; Culot et al., 2008; Helm et al., 2010; Fauquette et al.,2012). Alkaline phosphatase and γ-glutamyl transpeptidases have also been detected (Mizuguchi et al., 1994; Deracinois et al., 2012; Hakkarainen et al., 2014). Although there is a high yield of endothelial cells from bovine brain, cell monolayers display significantly lower TEER and higher permeability compared to porcine models (Nakhlband and Omidi, 2011). The sucrose permeability in monolayers of bovine brain cells ranged between 4 – 30 x 10^{-6} cm/s (Reardon and Audus, 1993; Abbruscato and Davis, 1999; Cecchelli et al., 1999; Nakhlband and Omidi, 2011) with average TEER

values of 800 Ω.cm^2 (Cecchelli et al., 1999; Gaillard et al., 2001; Nakhlband and Omidi, 2011), whilst some studies reported higher TEER at between 1,500 – 2,500 Ω.cm^2 (Zenker et al., 2003; Helm et al., 2010; Helms et al., 2014). Among the most highly expressed ABC efflux transporters are ABCB1, ABCG2, ABCC1, 3, 4, 5, 6 (Warren et al., 2009; Gaillard et al., 2001; Helm et al., 2014), however, the bovine model was shown to lack functional activity of some ABC and SLC-transporters (Bohara et al., 2014; Garberg et al., 2005; Wang, Dentler and Borchardt, 2001; Grube et al., 2018). Furthermore, substantial variations in TEER have been reported between, and even within, laboratories utilising the same model (Wang, Dentler and Borchardt, 2001; Garberg, 2005; Grube et al., 2018; Bohara et al., 2014; Hakkarainen et al., 2014). For example, many reports have shown variation of TEER values of individual batches of bovine brain endothelial cells, with TEER values in the range of 92 to 857 Ω.cm^2 (Gaillard et al., 2001) and 327 Ω.cm^2 to 2,555 Ω.cm^2 (Helms et al., 2014). Intra-laboratory variations are evident in a series of publications where TEER varies from high values of around 800 Ω.cm^2 to around 150–300 Ω.cm^2, (Eisenblatter and Galla, 2002; Schaddelee et al., 2003; van der Sandt et al., 2001a; van der Sandt et al., 2001b). To date, BBB models based on primary endothelial cells of bovine origin are labour intensive and reproducibility between, and even within labs, is a disadvantage.

1.5.4 Human model

Isolation of primary human brain endothelial cells is difficult to achieve high yield due to limited access to healthy human brain tissue on a regular basis, and also not usually available because of ethical reasons (Bernas et al., 2010; Subileau et al., 2009; Helm et al., 2016; He et al., 2014). In several studies of primary human brain endothelial cells *in vitro*, the expression of several tight junctional proteins are claudin-3, -5, occludin, ZO-1, ZO-2 and JAM2 (Zenker et al., 2003; Bernas et al., 2010; Mabondzo et al., 2010; Urich et al., 2012), along with alkaline phosphatase and γ-glutamyl transpeptidase (Muruganandam et al., 1997; Stins et al., 2001). However, this model has been reported to have extremely low TEER ranging from as low as 10 Ω.cm^2 up to 400 Ω.cm^2 (Stins et al., 2001; Jong et al., 2001; Megard et al., 2002; Zenker et al., 2003; Urich et al., 2012) and a very high sucrose permeability between 3 – 33 x 10^{-6} cm/s (Megard et al., 2002; Mabondzo et al., 2010; Urich et al., 2012). In terms of relative expression levels, a proteomic analysis of human brain endothelial cells demonstrated ABCG2 is most highly expressed followed by ABCB1 and ABCC subfamily members (ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6) (Nies et al., 2004; Dauchy et al., 2008; Warren et al.,

2009; Shawahna et al., 2011; Uchida et al., 2011; Hoshi et al., 2013; Al-Majdoub et al., 2019). Despite expressing all the key features of the BBB *in vivo*, establishing a human model faces serious limitations, such as constraints in access to human brain tissue, ethical issues, high variation of permeability, low TEER values and less robustness compared to bovine and porcine models (Bernas et al., 2010; Prat et al., 2000; Subileau et al., 2009; Megard et al. 2002). In addition, human brain is difficult to acquire on a regular basis, often making routine establishment of a BBB model based on primary human brain endothelial cells and wide-scale studies impractical. Instead, efforts have been made to create alternative models based on immortalised human brain endothelial cells, namely the hCMEC/D3 cell line, which is the most widespread and well characterised of the published cell lines.

hCMEC/D3 cell monolayers display TEER values of 30 – 500 $\Omega.cm^2$ (Weskler et al., 2005; Vu et al., 2009; Hatherell et al., 2011; Urich et al., 2012; Forster et al., 2008) with values as high as 800 – 900 $\Omega.cm^2$ reported (Schreibelt et al., 2007). The permeability of sucrose in hCMEC/D3 cell monolayers is 12 – 37 x 10^{-6} cm/s (Weskler et al., 2005; Poller et al., 2008; Urich et al., 2012) and expression of ZO1, ZO2, occludin, claudin-3, -5, -12, JAM-1 and JAM-A have been documented (Weskler et al., 2005; Afonso et al., 2008; Coureuil et al., 2009; Paolinelli et al., 2013 and Forster et al., 2008). Expression levels of ABC efflux transporters in hCMEC/D3 cells have been shown to vary with Dauchy et al. (2008) reporting ABCG2 mRNA levels to be approximately seven-fold higher than ABCB1 levels, whereas in the studies of Lindner et al., (2012) and Dauchy et al. (2008), ABCG2 mRNA levels were approximately seven-fold lower than ABCB1 mRNA levels. In addition, differences in expression of members of the ABCC subfamily have been reported with Poller et al., (2008) demonstrating expression of ABCC1 – 5, and Dauchy et al., (2008) and Lindner et al., (2012) demonstrating expression of ABCC1 - 6.

These findings strongly suggest significant differences in expression patterns of genes encoding ABC transporters in the hCMEC/D3 cell line. Furthermore, expression levels of CAR and PXR which are highly expressed in primary human brain endothelial cells are barely detectable in this cell line (Dauchy et al. 2009). In addition, tight junction proteins occludin and claudin-5 in hCMEC/D3 cells are also lower than in primary human cerebral endothelial cells (Urich et al., 2012).

Whilst, hCMEC/D3 cells represents a sustainable, reproducible model of the human BBB that can be easily grown without losing its BBB phenotype, which is amenable to study mechanisms of drug transport this immortalised cell line still falls short of the ideal restrictive BBB.

1.5.5 Porcine model

The BBB models derived from primary porcine brain endothelial cells have been reported to express the tight junction proteins occludin, claudin-5, ZO-1 and ZO-2 (Matthes et al., 2011; Bornhorst et al., 2012; Mulac et al., 2012; Malina et al., 2009) as well as BBB-associated marker proteins γ-glutamyl transpeptidase and alkaline phosphatase (Cantrill et al., 2012). This model generally develops very high TEER reaching 500 to 1500 $\Omega.cm^2$ (Franke et al., 2000; Lohmann et al., 2002; Nitz et al. 2003; Smith et al., 2007; Matthes et al., 2011; Katayun et al., 2012; Mulac et al., 2012; Malina et al., 2009; Lemmen et al., 2013a; Nakhlband and Omidi, 2011; Bornhorst et al., 2012; Simeone et al., 2017) and has been reported to reach 2500 $\Omega.cm^2$ (Kroll et al., 2009; Cantrill et al., 2012). The high TEER translates into low permeability of small molecules with sucrose permeabilities ranging from 0.1 – 10.0 x 10^{-6} cm/s (Nakhlband and Omidi, 2011; Matthes et al., 2011; Katayun et al., 2012; Mulac et al., 2012; Bornhorst et al., 2012; Simeone et al., 2017).

In comparison to human, bovine and other rodent models, porcine models have received the most attention due to the ease of brain acquisition, high yield of brain endothelial cells, cost effectiveness and less ethical issues. From each porcine brain (4- to 6-month old pig), approximately 50 million endothelial cells can be isolated, which potentially provides for numerous experiments based on a cell seeding density of 0.2 to 0.25 x 10^6 cells per cm^2 (Gumbleton and Audus, 2001). In comparison to bovine model, the optimised *in vitro* porcine BBB model is relatively simple to prepare, reliable and reproducible, with monolayers taking 5 to 6 days to reach confluency, compared with 10 to 12 days with the bovine model (Zhang et al., 2006; Smith et al., 2007).

Thus far, the BBB features in PBEC models have been validated in many studies at the cellular and molecular levels. PBEC models generally retain many of the important key features of the BBB and the rate at which the BBB phenotype of primary porcine brain endothelial cells is lost in culture is relatively slow compared to the more rapid rate in rat and

bovine brain endothelial cells. Furthermore, cell viability can be maintained following up to 12 months' cryopreservation (Smith et al., 2007; Zhang et al., 2006).

The porcine anatomy, physiology, genome and disease progression reflect human biology more closely than many established laboratory animals, such as rats or mice (Walters et al.,2011). A recent quantitative proteomics comparison of isolated brain capillaries from different species showed that endothelial cells from porcine brain capillaries express a range of BBB phenotype ABC transporters, with the ABCG2:ABCB1 ratio closer to that of monkey and human than is observed in rodent brain capillaries (Kubo et al., 2015). Among the highly expressed ABCC subfamily members, ABCC1, ABCC4 and ABCC5 are observed to be highly expressed in both porcine and human brain capillaries also (Eisenblatter et al., 2003; Zhang et al., 2006; Warren et al., 2009; Kubo et al., 2015). Furthermore, in the studies of sequence similarity using BLASTN and BLASTP analysis at NCBI and UniProt, the nucleotide sequence of ABCB1, ABCG2 and ABCC5 in porcine brain capillaries shared 89%, 84%, 89% identities respectively with that of human; while the protein sequence of porcine ABCB1, ABCG2 and ABCC5 shared 84%, 84% and 95% identity respectively to that of human (Fung & Gottesman, 2009 & Guo et al., 2016).

In term of nuclear receptors, the "wild-type" PXR transcripts discovered in both pig and human were highly expressed in liver, small intestine, and colon, and have common metabolic pathways, including drug responses (Lamba et al. 2004). However, CAR is less well studied in porcine. Furthermore, sequence similarity using NCBI BLASTN and UniProt BLASTP, the nucleotide sequence of PXR, CAR and GR in porcine brain capillaries shared 85%, 86% and 87% respectively with that of human; while the protein sequence of porcine PXR, CAR and GR shared 85%, 83% and 90% identity respectively to that of human (Pollock et al., 2007).

As an overall, considering the high TEER, low permeability, high yield of brain endothelial cells in PBEC model, together with the close physiological and anatomical relation between the human and pig brain, especially both have similar expression profile and high sequence similarity of nuclear receptors and ABC efflux transporters; the porcine model can serve as a reliable and easily reproducible model for different *in vitro* BBB studies. The quality, simplicity and robustness of the porcine BBB model make it an attractive and the most suitable *in vitro* BBB model to be employed in this thesis.

1.6 Aim and Objectives

1.6.1 Aim

Neurological disorders are the persistent problem and enhance the efficacy and penetration of drug into the brain is of extreme importance. Among the multiple factors involved in the pathophysiology of neurological disorders are the ATP-binding cassette (ABC) transporters. ABCB1, ABCG2 and ABCC5 are among the clinically relevant transporters that highly expressed in brain endothelial cells of the BBB.

Dysfunction/downregulation of ABCB1 and ABCG2 transporters, at expression and/or activity level, has been associated with many neurological diseases, including Creutzfeldt-Jakob's (Vogelgesang et al., 2006), Alzheimer's disease (Kania et al., 2011; Zhang et al., 2018) and Parkinson's disease (Westerlund et al., 2008; Fernandes et al., 2018) which leads to protein aggregation in the brain. Furthermore, ABC transporters are strikingly associated with the pharmacoresistance to central nervous system (CNS) acting drugs. For example, ABCB1 and ABCG2 contributed to the therapeutic failure of drugs used in the treatment of brain tumour (Ginguené et al., 2010; Fallacara et al., 2019; Salaroglio et al., 2019) and amyotrophic lateral sclerosis (Milane et al., 2007; Milane et al., 2009; Mohamed et al., 2017); whilst ABCB1, ABCG2, ABCC1, ABCC2 and ABCC5 were reported to reduce the therapeutic efficacy of drugs used in the treatment epilepsy (Ferreira et al., 2018; Deng et al., 2019; Fei et al., 2019), HIV (Kis et al., 2010; Zheng et al., 2020) and ischemic stroke (Patak et al., 2011; DeMars et al., 2017). In comparison to ABCB1 and ABCG2, ABCC5 is considerably less well studies though it is the most highly expressed transporter at the BBB in ABCCs family.

Hence, unveiling the signalling pathways in the regulation of these ABC transporters can add substantial knowledge in the discovery of new treatments, which can have a significant impact on the prevention of neurological disorders. To date, PXR and CAR are considered master regulators of hepatic ABC efflux transporter activities (Wang & LeCluyse, 2003; Rigalli et al., 2019), both are highly expressed in the liver, small intestine and colon (Pollock et al., 2007; Nannelli et al., 2010; Pavek et al., 2014; Hudson et al., 2017; Cai et al., 2021). Their mechanism of actions are tightly regulated by the GR in hepatocytes (Pascussi et al., 2000; Pascussi et al., 2003; El-Sankary et al., 2000; Duret et al., 2006; Cooper et al., 2008; Hunter et al., 2017; Smutny et al., 2020).

At present, the precise mechanisms of PXR, CAR and GR in regulating ABC efflux transporter expression are more well established in liver, small intestine and colon than in the BBB. Several studies have reported the upregulation of ABCB1, ABCG2, ABCC1 and ABCC2 by PXR and CAR has been reported in human (Chan et al., 2011), mouse (Bauer et al., 2006), rat (Bauer et al., 2004; Bauer et al., 2008a; Laura et al., 2008; Laura et al., 2008; Lombardo et al., 2008; Narang et al., 2008; Wang et al., 2010), bovine (Perloff et al., 2007) and pig brain capillaries (Ott et al., 2009; Lemmen et al., 2013a; Lemmen et al., 2013b). Thus far, GR has been reported to upregulate PXR and abcb1 and abcg2 in mouse and rat brain capillaries (Narang et al., 2008; Chan et al., 2013). To date the interaction between PXR, CAR, RXR and GR in regulating ABC efflux transporters in higher mammalians remain elusive.

Currently, the amount of research carried out in the study of factors that influence the of ABC transporters expression at the BBB *in vitro* and *in vivo* model is highly dependent on rodent models and human immortalised cell lines which is not the best representation of phenotypical features of human BBB. As a consequence, most of the knowledge of the roles of receptors and transcription factors in the regulation of ABCB1, ABCG2 and ABCCs expressed in BBB is based on assumptions that are not entirely accurate due to cell- and species- related differences in the activation of signalling pathways involved in the regulation of ABC transporters.

As porcine brain endothelial cell-based models have received increasing attention due to their phenotypical similarities with its human counterpart in both nuclear receptors and ABC transporter expression profiles, this model expresses a phenotype consistent with that observed in primary and *in vivo* human brain endothelial cells, and has been successfully used for cell biology studies (Skinner et al., 2009) and as a highly restrictive BBB model in pharmaceutical research (Cantrill et al., 2012; Shubbar and Penny, 2018).

The overall aim of this project is to establish the signalling pathway of nuclear receptors in regulating the activity and protein expression of ABC efflux transporters and to determine how this signalling pathway influenced by the presence of pro-inflammatory cytokine, IL-1β in disease condition in primary porcine brain endothelial cells. The findings of this study would further advance the understanding of the ABC efflux transporter regulatory mechanisms and this knowledge is crucial in manipulating the tightness of BBB in order to facilitate therapeutic drugs to cross the BBB in the treatment of wide-range of brain disorders.

1.6.2 Objectives

1. Identification of optimal culture conditions for growing primary porcine brain endothelial cells in order to establish a fully functional *in vitro* BBB model for this study.
2. Characterisation of the transporter activity of ABC efflux transporters (ABCB1, ABCG2 and ABCC5), including the expression profile of ABC efflux transporters (ABCB1, ABCG2 and ABCC5) and nuclear receptors (GR, PXR, CAR and RXR) in primary porcine brain endothelial cells in order to ensure the established *in vitro* BBB features the key characteristics of an *in vivo* BBB.
3. Evaluate the regulatory roles of nuclear receptors (GR, PXR, CAR, RXR) on the activity and expression of ABC efflux transporters (ABCB1, ABCG2 and ABCC5) in primary porcine brain endothelial cells, in order to understand how BBB responds to therapeutic drugs and prevent their entry into the brain.
4. Determine how the pro-inflammatory cytokines alter the regulatory roles of nuclear receptors (GR, PXR, CAR, RXR) on the ABC efflux transporters (ABCB1, ABCG2 and ABCC5) activity and expression in primary porcine brain endothelial cells. This study serves to unveil the changes of the regulatory mechanisms of BBB in disease condition.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

The materials and reagents used are summarised in Appendix B.

2.2 Methods

2.2.1 Culturing of the CTX-TNA2 immortalised cell line and harvesting of astrocyte-conditioned medium

The immortalised rat astrocyte CTX-TNA2 cell lines from passage 1 – 5 and passage 15 - 20 were used to produce astrocyte-conditioned medium (ACM), based on the procedure described by Cantrill et al. (2012). The cells were grown in T75 cell culture flasks (Greiner Bio One, Germany), using high glucose Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, UK) supplemented with 10 % (v/v) heat-inactivated foetal bovine serum (FBS) (GIBCO, UK) and 100 U penicillin/streptomycin mixture (Sigma-Aldrich, UK). The cells were incubated at 37 °C in a humidified atmosphere under 5 % CO_2. Once the cells reached 40 % confluency, the astrocyte-conditioned medium was harvested and filtered through a 0.22 µm low-protein binding polyethersulfone membrane filter disk (Merck, Ireland), and stored at -20 ºC for future use. The ACM harvested from cells from passage 1 – 5 is denoted as ACM-1, and ACM harvested from cells from passage 15 – 20 is denoted as ACM-2.

2.2.2 Isolation and purification of primary porcine brain endothelial cells

Fresh brains from male and female Landrace cross Large White pigs, aged 22 – 24 weeks, weighing 105 – 110 kg, were obtained from a local abattoir. The isolation of porcine brain microvessels was performed based on the method described by Skinner et al. (2009) with some minor modifications. Briefly, porcine brains without bruises obtained from a local abattoir were kept in high glucose DMEM (GIBCO, UK) supplemented with 1X penicillin/streptomycin (Sigma-Aldrich, UK) until further processing under sterile conditions.

Each brain was washed thoroughly in ice-cold Dulbecco's phosphate buffer saline (PBS) (Sigma-Aldrich, UK) supplemented with 1X penicillin/streptomycin. The meninges and visible blood vessels were removed using curved forceps. The white matter of each hemisphere was carefully removed and the grey matter was transferred into high glucose DMEM supplemented with 10 % (v/v) FBS, 1 % (v/v) penicillin/streptomycin and 1M HEPES, pH 7.5 (GIBCO, UK). The grey matter was then chopped into small pieces and passed through a 50 ml sterile syringe into a sterile container containing high glucose DMEM supplemented with 10 % (v/v) FBS, 1 %

(v/v) penicillin/streptomycin and 1 M HEPES, pH 7.5. The ratio of medium to brain tissue was 1:2.

Approximately 40 ml of brain extract was transferred into a Dounce tissue grinder (Wheaton, USA) and homogenised with a loose-fitting pestle for 15 strokes and with a tight-fitting pestle for 15 strokes. The resulting homogenate was filtered through a 150 µm pore nylon mesh (Plastok, UK), the filtrate collected and subsequently filtered again through a 60 µm pore nylon mesh (Plastok, UK).

The 60 µm pore nylon mesh filters containing microvessels were placed into 150 mm diameter petri dishes containing digest mix (M199 medium (Sigma, UK) supplemented with 210 U/mg collagenase (Worthington, UK), 114 U/mg trypsin (Worthington, UK), 91 U/mg DNase I (Worthington, UK), 10 % (v/v) FBS (Biowest, USA) and 1 % (v/v) penicillin/streptomycin and incubated for 1 h at 37 ºC with gentle shaking.

Thereafter, the filters containing capillaries were washed thoroughly by continuous pipetting to detach the capillaries adhered to each filter. The resulting digestion mix containing the capillaries was centrifuged at 150 x g for 5 min in a Hettich Universal 320 centrifuge (Hettich, Germany). The supernatant was discarded, the microvessel pellet resuspended in high glucose DMEM supplemented with 10 % (v/v) FBS, 1% (v/v) penicillin/streptomycin and 1M HEPES, pH 7.5, and centrifuged at 150 x g for 5 min. This step was carried out three times and the final pellet was resuspended in cryopreservation media (90 % (v/v) FBS and 10 % (v/v) DMSO). Microvessels were either maintained in culture (see section 3.2.3.) or aliquoted into cryogenic vials and stored at -80 ºC in a cryopreservation unit (Nalgene, USA) for 24 h before being transferred to liquid nitrogen (−196 °C) for long term storage until use.

2.2.3 Coating of plates for cell culture

Rat collagen type I (Corning, USA) solution was prepared by diluting the collagen solution in 20 mM acetic acid to yield a working solution at 100 µg/ml. One millilitre of the solution was added into individual wells of a 6-well plate (Greiner Bio One, Germany) to fully cover the surface of each well. The plates were maintained in a microbiological safety hood for 2 h in order to allow coating of the plastic surface under sterile conditions. After 2 h, the collagen solution was removed and the wells were rinsed twice with PBS (Sigma-Aldrich, UK).

Each individual well of the 6-well plate was subsequently treated with 1 ml of 7.5 μg/ml human fibronectin (Corning, USA) prepared in sterile deionised water. The plate was maintained overnight at 4 °C and another one hour of incubation at room temperature. The 6-well plate was rinsed twice with PBS prior to the seeding of PBECs. A similar protocol was applied for the coating of 96-well plates (SARSTEDT, Germany) and CELLview 10-well microscopy chambers (Greiner Bio One, Germany) but the final volumes for each well was 32 μl.

2.2.4 Culture of primary porcine brain endothelial cells

One vial containing cryopreserved porcine brain microvessels suspension was removed from liquid nitrogen and quickly thawed in a 37 °C water bath. The microvessels were transferred into a centrifuge tube containing 9 ml of PBEC growth medium, composed of phenol red-free low glucose DMEM (GIBCO, UK) supplemented with 1% (v/v) penicillin/streptomycin (Sigma-Aldrich, UK), 2 mM glutamine (GIBCO, UK), 10 % (v/v) plasma derived serum (First Link, UK) and 125 μg/ml heparin (Sigma-Aldrich, UK). The microvessels were centrifuged at 180 x g for 5 min, the supernatant was discarded and the pellet was re-suspended in 6 ml of PBEC growth medium. The microvessels were then transferred into a 6-well plate (Greiner Bio One, Germany) with a volume of 1 ml in each well, and incubated at 37 °C in a humidified atmosphere under 5 % CO_2 for 24 h.

The PBEC growth medium was replaced with PBEC growth medium containing 4μg/ml puromycin dihydrochloride (Sigma-Aldrich, UK) (puromycin dihydrochloride was directly dissolved in PBEC growth medium) to remove contaminating cells, and plates incubated for 48 h at 37 °C in a humidified atmosphere under 5 % CO_2. The puromycin-containing medium was then discarded and replaced with 1:1 mixture of PBEC growth medium/astrocyte-conditioned medium. The cells were incubated until confluent at 37 °C in a humidified atmosphere under 5 % CO_2.

2.2.5 Sub-culturing of primary porcine brain endothelial cells

On the sixth day of culture, the cells were sub-cultured into collagen-fibronectin coated 96-well plates (Greiner Bio One, Germany) or CELLview 10-well microscopy chambers (SARSTEDT, Germany). Cell monolayers grown in the 6-well plate (Greiner Bio One, Germany) were washed twice with PBS and treated with trypsin-EDTA (Sigma-Aldrich, UK). The plate was incubated for 10 min at 37 ºC under 5 % CO_2 to allow the detaching of PBECs.

After 10 min, the resulting cell suspension was centrifuged at 180 x g for 5 min. The supernatant was discarded and the cell pellet was suspended in PBEC growth medium/ACM (1:1). Cell concentration was determined using a haemocytometer and 25,000 cells/well, in a volume of 200 µl, was seeded into individual wells of a 96-well plate or CELLview 10-well microscopy chambers pre-coated with rat collagen type I and human fibronectin.

2.2.6 Measurement of protein concentration using the Bradford assay

Protein concentration in whole cell lysates was measured by the Bradford assay in a 96-well format using an adapted protocol provided by Bio-Rad (Bio-Rad, UK). The working Bradford reagent was prepared by diluting the concentrated dye solution with deionised water at a ratio of 1:4 (v/v). A calibration curve of bovine serum albumin (BSA) (Sigma-Aldrich, UK) was established by preparing a serial dilution of a 10 mg/ml stock solution BSA in PBS (Sigma-Aldrich, UK) in the linear range of 0.125 – 1.5 mg/ml. A volume of 10 µl from each BSA dilution or whole cell lysate was mixed with 200 µl of working Bradford reagent in a 96-well plate (Greiner Bio One, Germany). The samples were incubated at room temperature for 5 min and the absorbance was measured at 595 nm using a Hidex sense microplate reader (Hidex, Finland).

The protein concentration of cell monolayers in 96-well plates was measured by the Bradford assay following the protocol from Bio-Rad. A calibration curve of BSA was established in the linear range of 10 – 40 µg/ml by mixing 160 µl of each diluted protein standard prepared in PBS with 40 µl of concentrated Bradford reagent in the 96-well plate. For cell monolayers, 160 µl of PBS were added to each well, followed by 40 µl of concentrated Bradford reagent. The samples were mixed carefully by pipetting and incubated at room temperature for 5 min. The absorbance was measured at 595 nm using a Hidex sense microplate reader (Hidex, Finland).

2.2.7 Treatment of primary porcine brain endothelial cells with selected compounds

On the seventh day of culture, cell monolayers in 6-well plates, 96-well plates and CELLview 10-well microscopy chambers were treated with different compounds. Briefly, the growth medium was removed and cell monolayers were washed twice with warm PBS. Next, phenol red-free low glucose DMEM supplemented with 1 % (v/v) FBS and 2 mM L-glutamine, was added to each well, 1000 µl for 6-well plate, 100 µl for both 96-well and CELLview 10-

well microscopy chambers. Cell monolayers were equilibrated for at least 1 hour. All the compounds used in cell treatment were diluted, from their respective stock solutions prepared in either dimethylsulfoxide or distilled water, to the desired concentrations in the above medium and the final volume was 2000 µl in 6-well plate and 200 µl in 96-well and CELLview 10-well microscopy chambers.

The final concentration of DMSO used in all the drug treatments never exceeded 0.1 % (v/v), and the cell viability and transporter activity were not affected at this concentration.

2.2.8 Evaluation of cell viability using the neutral red assay

Cells were seeded onto collagen-fibronectin coated flat-bottom 96-well plates and cultured according to the protocol in section 2.2.4. After the cells were treated with selected compounds, as outlined in figure legends, cell monolayers were washed with PBS and incubated with 200 µl of warm phenol red-free DMEM supplemented with 2 mM L-glutamine for 30 min at 37 °C.

Neutral red reagent (Sigma-Aldrich, UK) was diluted to a concentration of 40 µg/mL with phenol red-free DMEM supplemented with 2 mM L-glutamine and filtered through a PES 0.22 µm disk membrane filter (Millipore, UK). Briefly, cell monolayers were washed with warm PBS and 100µL of Neutral red was added to each well and the 96-well plate was incubated for 2 h at 37°C in a humidified atmosphere with 5 % CO_2. After incubation, the neutral red solution was removed and cell monolayers washed with warm PBS. Destain solution, 50 % (v/v) absolute ethanol, 49 % (v/v) deionised water and 1 % (v/v) acetic acid, at a volume of 150 µL was added into each well in the 96-well plate and plates shaken for 15 min at room temperature.

Samples were analysed in a Hidex sense microplate reader (Hidex, Finland) with an excitation wavelength of 484 nm and emission wavelength of 530 nm. All experiments were carried out at least three times and the cell viability, expressed as percentage viability of non-treated control cells, was calculated according to the following equation:

$$\%\, Cell\ viability = \left(\frac{A(treatment) - A\,(Blank)}{A\,(Control) - A\,(Blank)}\right) \mathrm{X}\ 100$$

Where A is the absorbance obtained at excitation wavelength of 530 nm and emission wavelength of 645 nm

2.2.9 Determination of ABCB1 activity in primary porcine brain endothelial cells

The efflux activity of ABCB1 was determined by measuring the intracellular accumulation of the fluorescent probe calcein. Calcein-AM (Sigma, UK), a substrate for ABCB1, is the membrane permeable acetoxymethyl ester of calcein that is converted to membrane impermeable calcein by intracellular hydrolases.

PBECs were maintained in 96-well plated as described in section 2.2.4. On the day of the experiment, the cell monolayers were washed twice with warm PBS and equilibrated at 37 ºC in a humidified atmosphere incubator under 5 % CO_2 for 30 min with phenol red-free low glucose DMEM supplemented with 2 mM L-glutamine (150 µl for cells not treated with ABCB1 inhibitor verapamil (control condition) and 100 µl for cells exposed to verapamil). A stock solution of verapamil hydrochloride (Sigma, UK), 20 mM, was prepared in sterile, deionised water and diluted in phenol red-free low glucose DMEM to 30 µM. Fifty microlitres of 30 µM verapamil was added to the appropriate wells to yield a final concentration of 10 µM and the cells were incubated at 37 ºC for another 30 min. Subsequently, 50 µl of 2 µM calcein-AM solution, prepared by dissolving in phenol red-free low glucose DMEM, was added to each well to yield a final concentration of 0.5 µM. The cells were incubated at 37 ºC for another 30 min. The cell monolayers were washed twice with 200 µl ice-cold PBS and 160 µl of ice-cold PBS added to each well. Fluorescence emitted by calcein was analysed using a Hidex sense microplate reader (Hidex, Finland), with an excitation wavelength of 484 nm and an emission wavelength of 530 nm. To allow normalisation of fluorescence data with protein content, 40 µl of Bradford reagent was added into each well containing 160 µl PBS. The samples were mixed thoroughly by pipetting and the absorbance was read using a Hidex sense microplate reader, at a wavelength of 595 nm. The relative fluorescence values (RFUs) were divided by respective protein content of each well and values are represented as RFU/µg protein. Normalised values of the control condition (i.e. no inhibitor) were set to 100% and the normalised values for experimental conditions are represented as a percentage of the control. Routinely, at least five independent experiments were carried out with at least six replicates in each experiment.

2.2.10 Determination of ABCG2 activity in primary porcine brain endothelial cells

The efflux activity of ABCG2 was determined based on the intracellular accumulation of the fluorescent probe Hoechst 33342 (Sigma, UK), an ABCG2 substrate. PBECs were maintained in 96-well plated as described in section 2.2.4.

On the day of the experiment, the cell monolayers were washed twice with warm PBS and equilibrated at 37 ºC in a humidified atmosphere incubator under 5 % CO_2 for 30 min with phenol red-free low glucose DMEM supplemented with 2 mM L-glutamine (150 µL for cells not treated with ABCG2 inhibitor Ko143 (Sigma, UK) (control condition) and 100 µL for cells treated with ABCG2 inhibitor Ko143). A stock solution of Ko143 hydrochloride (Sigma, UK), 10 mM, was prepared in sterile, deionised water and diluted in phenol red-free low glucose DMEM to 1.5 µM. Fifty microlitres of 1.5 µM Ko143 was added to the appropriate wells to yield a final concentration of 0.5 µM, and the cells were incubated at 37 °C for 30 min.

A stock solution of Hoechst 33342 was prepared in phenol red-free low glucose DMEM at a concentration of 20 µM. Subsequently, 50 µL of 20 µM Hoechst 33342 solution was added to each well to yield a final concentration of 5 µM, and the monolayers were incubated for a further 30 min as described in section 2.2.9.

The cell monolayers were washed twice with 200 µl ice-cold PBS and 100 µl of ice-cold PBS was added to each well. Fluorescence emitted by Hoechst 33342 was analysed using a Hidex sense microplate reader (Hidex, Finland) with an excitation wavelength of 370 nm and an emission wavelength of 450 nm. To allow normalisation of fluorescence data with protein content, the PBS was removed and 160 µl of fresh PBS were added to each well with 40 µl of Bradford reagent. The samples were mixed thoroughly by pipetting and the absorbance was read using a Hidex sense microplate reader at a wavelength of 595 nm. The relative fluorescence values (RFUs) were divided by respective protein content of each well and values are represented as RFU/µg protein. Normalised values of the control condition (i.e. no inhibitor) were set to 100% and the normalised values for experimental conditions are represented as a percentage of the control. Routinely, at least five independent experiments were carried out with at least six replicates in each experiment.

2.2.11 Determination of ABCC5 activity in primary porcine brain endothelial cells

The efflux activity of ABCC5 was determined by measuring intracellular accumulation of the fluorescent probe CMFDA (Abcam, UK), an ABCC5 substrate. PBECs were cultured in 96-well plated as described in section 2.2.4

On the day of the experiment, the cell monolayers were washed twice with warm PBS and equilibrated at 37 ºC in a humidified atmosphere incubator under 5 % CO_2 for 30 min with phenol red-free low glucose DMEM supplemented with 2 mM L-glutamine (150 µl for cells not treated with ABCC5 inhibitor Mk571 (Sigma, UK) (control condition) and 100 µL for cells treated with ABCC5 inhibitor Mk571). A stock solution of Mk571 (Sigma, UK), 10 mM, was prepared in sterile, deionised water and diluted in phenol red-free low glucose DMEM to 75 µM. Fifty microlitres of 75 µM Mk571 was added to the appropriate wells to yield a final concentration of 25 µM, and the cells were incubated at 37 °C for 30 min.

A stock solution of CMFDA was prepared in phenol red-free low glucose DMEM at a concentration of 16 µM. Subsequently, 50 µL of 16 µM CMFDA solution was added to each well to yield a final concentration of 4 µM, and the monolayers were incubated for a further 30 min as described in section 2.2.9.

The cell monolayers were washed twice with 200 µl ice-cold PBS and 100 µl of ice-cold PBS added to each well. Fluorescence emitted by CMFDA was analysed using a Hidex sense microplate reader (Hidex, Finland) with an excitation wavelength of 492 nm and an emission wavelength of 516 nm. To allow normalisation of fluorescence data with protein content, the PBS was removed and 160 µl of fresh PBS were added to each well with 40 µl of Bradford reagent. The samples were mixed thoroughly by pipetting and the absorbance was read using a Hidex sense microplate reader at a wavelength of 595 nm. The relative fluorescence values (RFUs) were divided by respective protein content of each well and values are represented as RFU/µg protein. Normalised values of the control condition (i.e. no inhibitor) were set to 100 % and the normalised values for experimental conditions are represented as a percentage of the control. Routinely, at least five independent experiments were carried out with at least six replicates in each experiment.

2.2.12 Western blotting

2.2.12.1 Preparation of primary porcine brain endothelial cell lysate

Porcine brain endothelial cells were grown in 6-well plated as described in section 2.2.4 and treated with selected compounds as described in section 2.2.7. Confluent monolayers of PBECs in 6-well plates were treated with 200 µl of Cellytic M lysis buffer (Sigma-Aldrich, UK) containing 0.1 % (v/v) protease inhibitor cocktail (Sigma-Aldrich, UK) and incubated on shaker for 15 min at room temperature. Lysates were collected into ice-cold 2 ml micro-centrifuge tubes and maintained on ice for 10 min to allow lysis of cells. Lysate were then centrifuged at 15,000 x g for 15 min at 4 ºC in a Thermo Scientific Heraeus Fresco 17 micro centrifuge (Thermo Scientific, UK). The supernatant was collected in ice-cold micro-centrifuge tubes and the protein concentration quantified by the Bradford assay with a calibration curve containing bovine serum albumin as a standard, as described in section 2.26. The samples were stored at -80 °C until use.

2.2.12.2 Preparation of rat liver homogenate

Rat liver was freshly obtained and homogenised on ice in ice-cold PBS supplemented with 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, UK) in a ratio of 3 ml/g tissue. Homogenate was pipetted as 1 ml aliquots into ice-cold micro-centrifuge tubes and immediately stored at -20 °C. Thawed liver homogenate was transferred into hypotonic solution (100 mM Tris (Sigma-Aldrich, UK), 1 M $MgCl_2$ (Sigma-Aldrich, UK), 1 M KCl (Sigma-Aldrich, UK)) supplemented with 2 % (v/v) mercaptoethanol (Sigma-Aldrich, UK) and 1% (v/v) protease inhibitor cocktail at 1 ml/0.25 g liver homogenate. The sample was mixed thoroughly by pipetting, incubated on ice for 15 min and then centrifuged at 900 x g for 10 min at 4 °C. The supernatant was collected in ice-cold micro-centrifuge tubes and protein concentration determined by the Bradford assay with a calibration curve containing bovine serum albumin as a standard as described in section 2.26. The samples were stored at -80 °C.

2.2.12.3 SDS-PAGE

Proteins were separated according to their molecular weight by sodium dodecyl sulphate polyacrylaminde gel electrophoresis (SDS-PAGE) using a Mini-Protean TetraCell (Bio-Rad, UK) system. The SDS-PAGE was carried out on a discontinuous system comprised of a 4 % (w/v) polyacrylamide stacking gel and different concentrations of polyacrylamide resolving gels, depending on the molecular weight of the target protein analysed. For ABCB1

and ABCC5 analyses, a 6 % (w/v) polyacrylamide resolving gel was prepared, for ABCG2 and GR analyses, a 8 % (w/v) polyacrylamide resolving gel was prepared, and for PXR, CAR and RXR analyses, a 10 % (w/v) polyacrylamide resolving gel was prepared.

Before preparing each gel, the glass casting plates were cleaned with 70 % (v/v) ethanol and air-dried. The glass plates were inserted and locked into a casting frame with the short plate facing the front of the frame. The casting frame was placed into a casting stand and the bottom of the gel cassette was sealed with a gasket to avoid leakage.

The stacking and resolving gels were prepared according to Table 2. The bis-acrylamide, Tris buffer and deionised H_2O components were gently and thoroughly mixed in a glass beaker. Ammonium persulphate (APS, Sigma-Aldrich, UK) and TEMED (Sigma-Aldrich, UK) were subsequently added and the gel mixture swirled gently and immediately poured into the cast. The resolving gel solution was poured to a level 1 cm below the comb teeth and was overlaid with isopropanol (Fisher Scientific, UK). The gel was left to polymerise for 45 min at room temperature. The isopropanol was discarded, the gel surface was rinsed with deionised water and the latter removed by capillary action using filter paper. The stacking gel solution was poured on to the resolving gel to the top of the short plate. The comb was inserted immediately to form the loading wells. The stacking gel was left to polymerise for 45 min at room temperature.

Table 2.1: Composition of stacking and resolving gels for SDS-PAGE

	Stacking gel 4 % (ml)	**Resolving gel 6 % (ml)**	**Resolving gel 8 % (ml)**	**Resolving gel 10 % (ml)**
Deionised H_2O	6.1	5.4	4.7	4.1
Bis-acrylamide 30 % (Sigma-Aldrich, UK)	1.3	2.0	2.7	3.3
Tris buffer (Sigma-Aldrich, UK)	2.5 (0.5 M, pH 6.8)	2.5 (1.5 M, pH 8.8)	2.5 (1.5 M, pH 8.8)	2.5 (1.5 M, pH 8.8)
SDS (Sigma-Aldrich, UK)	0.1	0.1	0.1	0.1
APS 0.1 % (w/v) (Sigma-Aldrich, UK)	0.05	0.05	0.05	0.05
TEMED (Sigma-Aldrich, UK)	0.01	0.005	0.005	0.005

The gel cassettes were secured firmly in a clamping frame with the short plate facing inward and placed into an electrophoresis tank. The combs were gently removed and the wells were rinsed with 1X running buffer (25 mM Tris (Sigma-Aldrich, UK), 200 mM glycine (Sigma-Aldrich, UK), 0.1 % (w/v) SDS (Sigma-Aldrich, UK)). Running buffer was poured into the internal chamber of the assembled electrophoresis module, to the top of the tall plate, whilst the outer chamber was filled with running buffer to the level of "2-gel mark" label. Then equal quantities of lysate protein, 15 μg for ABCG2 and GR, 40 μg for PXR, RXR and CAR and 80 μg for ABCB1 and ABCC5, were mixed with 6X Laemmli sample buffer at a ratio of 5:1, and loaded into the wells. Electrophoresis was carried out at a constant voltage of 120 V until the bromophenol blue marker reached approximately 1 cm above the bottom of the gel.

2.2.12.4 Electrotransfer of proteins

After electrophoresis, proteins were electrotransferred to a Hybond-P 0.45 μm PVDF membrane (GE Healthcare Life Sciences, USA) using a Mini Trans-Blot cell system (Bio-Rad, UK). PVDF membrane was cut to an appropriate size, activated in methanol for 10 s then transferred into distilled deionised water for 5 min. Prior to protein transfer, the PVDF membrane was equilibrated in chilled transfer buffer composed of 10 mM CAPS (Merck, UK), pH 11 for 10 min and fibre pads and filter paper were pre-soaked in transfer buffer.

The gel was gently transferred onto the PVDF membrane and sandwiched between filter paper, and then between fibre pads. Air bubbles were gently removed and the casts were assembled and inserted into a blotting cell system, which had been filled with ice-cold CAPS transfer buffer. Electrotransfer was carried out on ice at 400 mA for 2 h.

2.2.12.5 Immunological detection and semi-quantitative analysis of protein expression

After electrotransfer of proteins, the PVDF membrane was incubated for 1 h at room temperature in 5 % (w/v) skimmed milk diluted in TBS-T (150 mM NaCl (Sigma-Aldrich, UK), 10 mM Tris (Sigma-Aldrich, UK), 0.05 % (v/v) Tween-20 (VWR, UK). The membrane was washed three times with TBS-T for 10 min and incubated overnight at 4 ºC with anti-ABCB1 polyclonal antibody, 1:500 (Proteintech, UK) for detection of ABCB1, BXP-53 anti-ABCG2 monoclonal antibody, 1:1000 (Abcam UK) for detection of ABCG2, anti-MRP5 polyclonal antibody, 1:200 (Abcam UK) for detection of ABCC5, anti-pregnane X receptor

polyclonal antibody 1:500 (Abcam UK) for detection of PXR, anti-constitutive androstane receptor polyclonal antibody,1:500 (Abcam UK) for detection of CAR, recombinant anti-retinoid X receptor monoclonal antibody, 1:500 (EPR7106, Abcam UK) for detection of RXR, recombinant anti-glucocorticoid receptor monoclonal antibody, 1:1000 (EPR19621, Abcam UK) for detection of GR, recombinant anti-vinculin monoclonal antibody 1:5000 (EPR8185, Abcam UK) for detection of vinculin and anti-β-actin monoclonal antibody, 1:5000 (Sigma, UK) for detection of β-actin.

The primary antibody was removed and the membrane was washed twice with TBS-T for 10 min. Subsequently, the membrane was incubated with secondary antibody at a dilution of 1:5,000 in TBS-T: IgG horseradish peroxidase (HRP)-conjugated mouse-anti rabbit secondary antibody (Santa Cruz biotechnology, Germany) for the detection of Vinculin, ABCB1, CAR, RXR and GR; IgG horseradish peroxidase (HRP)-conjugated mouse-anti goat (Santa Cruz biotechnology, Germany), for the detection of ABCC5 and PXR; IgG horseradish peroxidase (HRP)-conjugated mouse-anti rat (Santa Cruz biotechnology, Germany) for the detection of ABCG2 and IgG horseradish peroxidase (HRP)-conjugated sheep anti-mouse antibody (General Electric Healthcare, UK) for the detection of β-actin.

Following the incubation with secondary antibodies, the protein-antibody conjugates were detected through enhanced chemiluminescence. The membranes were washed three times with TBS-T for 10 min and the excess buffer was drained with a piece of filter paper. The membrane was then incubated at room temperature for 1 min with 1:1 ratio of with Radiance Plus Luminol/enhancer solution and Radiance Plus Peroxide Chemiluminescent Detection Reagent (Azure biosystem, USA). After 1 min, the excess reagent was removed, the membranes were sandwiched between two acetate sheets and placed into a ChemiDoc imaging system (Bio-Rad, UK) for detection of the protein bands.

Images were analysed using the ImageJ software (NIH, USA) and the values obtained from the densitometric analysis of the target protein band were normalised to the values from the β-actin or vinculin loading control. Results are presented as a fold change compared to untreated control and every experiment was repeated at least three times.

2.2.13 Immunocytochemical detection of CAR and PXR in primary porcine brain endothelial cells

Porcine brain endothelial cells were grown in collagen/fibronectin coated CELLview 10-well microscopy chambers as described in section 2.2.4 and treated with the selected compounds according to the protocol described in section 2.2.7. Cell monolayers were fixed with 4 % (w/v) formaldehyde in PBS for 10 min at room temperature, then washed twice with cold PBS and the formaldehyde was quenched with 0.1 M Tris buffer, pH 7.5 for 5 min. The cells were then permeabilised with 0.2 % (v/v) Triton X-100 in PBS for 10 min at room temperature. After three 5 min washes with PBS, cells were blocked with 10 % (v/v) normal goat serum (Abcam, UK) in PBST (PBS + 0.1% Tween 20) at room temperature for 1 h.

For detection of CAR and PXR, fixed cells were incubated for 1 h at 4 °C with rabbit anti-PXR polyclonal antibody and rabbit anti-CAR polyclonal antibody (Abcam, UK) 1:50 dilution in 5 % (v/v) normal goat serum in PBST. Cell monolayers were then rinsed three times with PBS for 5 min each and incubated with goat anti-rabbit IgG H&L (heavy and light chains) (Alexa Fluor 188) preadsorbed secondary antibody (Abcam, UK), 1:200 dilution in 5 % (v/v) normal goat serum in PBST for 1 h at room temperature in the dark. After three 5 min washes with PBST, the cells were stained with 5 µg/mL DAPI (Sigma, UK) (diluted in PBS) incubated for 15 min at room temperature. Slides were mounted with Prolong Diamond antifade solution (Life Technologies, UK) and cured overnight at 4 °C. Negative controls were performed in parallel, where the cells were treated with a 5 % (v/v) normal goat serum in PBST without primary antibody. The slides were stored in the dark at 4°C before imaging.

Samples were analysed in an IX83 Inverted deconvolution microscope (Olympus, Japan) using a 100X/1.35 UplanApo objective through FITC and DAPI filters. Images were captured with a R6 Qimaging CCD camera with a Z optical spacing of 0.2 µm controlled by the Metamorph v7.8.4.0 software (Olympus, Japan). Raw images were deconvolved using the Huygens Pro software (SVI) (Scientific Volume Imaging, Netherland) and further analysed with ImageJ software (National Institute of Health, USA).

2.2.14 Statistical Analysis

All the data are presented as mean ± SD of a minimum of three independent experiments (n numbers are indicated in legends). Cell viability and transporter assay data

were analysed by Student's parametric unpaired t-test using Microsoft Excel for comparing two groups or parametric one-way ANOVA followed by a Tukey's post hoc test, for comparing multiple groups, using Graphpad Prism software version 7.00 (GraphPad Software, Inc., California, USA). The data for Western blot and immunohistochemistry were normalized to the control group in order to control unwanted sources of variation, and were thus analysed non-parametrically with either the Mann-Whitney test (two groups) or Kruskal–Wallis test with Dunn’s post hoc analysis (more than two groups), using Graphpad Prism software version 7.00.

Chapter 3

Isolation, culture and characterisation of primary porcine brain endothelial cells

3.1 Isolation and characterisation of primary porcine brain endothelial cells

3.1.1 Isolation and culture of primary porcine brain endothelial cells

Primary porcine brain endothelial cells (PBECs) were successfully isolated based on the protocol outlined in section 2.2. Cells started to emerge from the capillaries and expanded to form a monolayer of cells after treated with puromycin, 4 μg/ml for 48 h, at the second day of culture. At day seven, the cells formed a confluent monolayer and developed an elongated spindle-like morphology, a characteristic of brain microvascular endothelia as shown under phase-contrast microscopy, Figure 3.1.

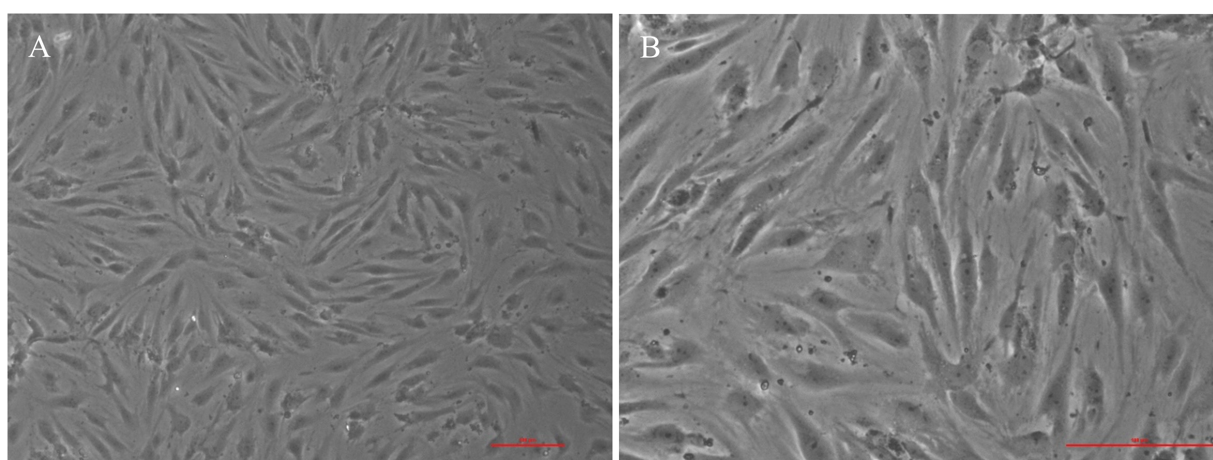


Figure 3.1: Monolayers of primary porcine brain endothelial cells.
Confluent monolayers of PBECs in culture at day 7. Porcine brain microvessels (1 ml suspension) were seeded in a collagen/fibronectin coated 6-well plate and maintained in PBEC growth medium (phenol red-free low glucose DMEM supplemented with 1% penicillin/streptomycin, 2 mM glutamine, 10 % plasma derived serum and 125 μg/ml heparin) for 24 h. The cells were treated with 4 mg/ml puromycin for 48 h and the medium was replaced with PBEC growth medium/ACM. The cells were then grown for a further 3 days and imaged using an inverted light microscope. Panel (A) 10X magnification, panel (B) 40X magnification, scale bar: 100 mm. Representative images of cells isolated from a pool of 15 brains.

3.1.2 Determination of ABCB1 transporter activity and protein expression in primary porcine brain endothelial cells

The transport activity of ABCB1 in PBECs was determined by measuring the accumulation of intracellular calcein. ABCB1-mediated transport is sensitive to inhibition, by verapamil, and the latter was used to assess whether the transport of calcein-AM in PBECs was mediated by ABCB1. Calcein-AM is a substrate for ABCB1 that is cleaved by intracellular esterases into calcein which is membrane impermeant and trapped inside the cells.

PBECs pre-treated with verapamil demonstrated a significant increase ($P < 0.005$) in intracellular fluorescence of over 7-fold, compared to untreated control cells, Figure 3.2. This confirms that the isolated PBECs express functionally active ABCB1.

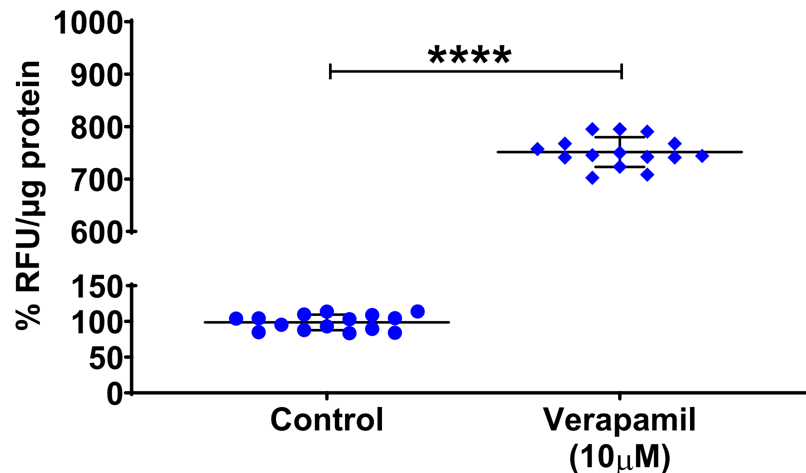


Figure 3.2: Inhibitory effect of verapamil on ABCB1 functional activity in primary porcine brain endothelial cells
ABCB1 functional activity was determined by the intracellular accumulation of calcein in control cells and cells pre-treated with ABCB1 inhibitor verapamil. Verapamil is a highly specific and low cytotoxic ABCB1 inhibitor. Data are expressed as mean ± standard deviation of five independent experiments, with at least three replicates in each experiment. Statistical significance was determined using Student's t-test. RFU: relative fluorescent units. ****: $P < 0.0001$.

In order to determine ABCB1 expression at the protein level in primary cultures of PBECs, Western blot analyses were performed on whole cell lysates obtained from PBEC monolayers, as described in section 2.2.12. Rat liver served as positive control. Western blotting using the anti-ABCB1 polyclonal antibody directed to ABCB1 indicated cross reactivity of a protein with an apparent molecular weight of 150 kDa (Figure 3.3).

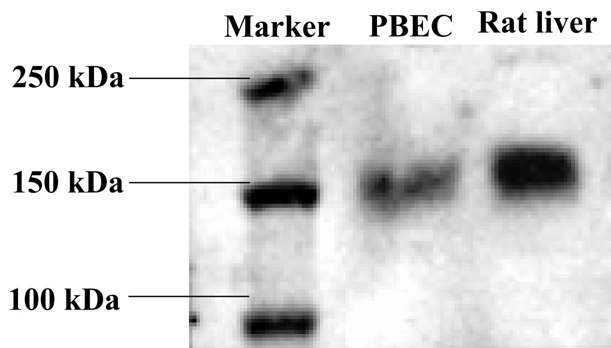


Figure 3.3: ABCB1 protein expression in primary porcine brain endothelial cells and rat liver.
Rat liver serves as positive control. Representative image from 5 independent experiments.

3.1.3 Determination of ABCG2 transporter activity and protein expression in primary porcine brain endothelial cells

To assess the functional activity of ABCG2 (breast cancer resistance protein) in PBECs, the cellular uptake of the fluorescent probe Hoechst 33342, a substrate for ABCG2, was measured. ABCG2-mediated Hoechst 33342 transport is sensitive to inhibition by Ko143, and this agent was employed in order to assess whether transport of H33342 was mediated by ABCG2 in the PBECs employed in this thesis.

Intracellular accumulation of Hoechst 33342 in cells pre-treated with Ko143 increased significantly ($P < 0.0001$) by 200 %, i.e. over 2.0-fold, compared to untreated control cells (Figure 3.4). This indicates the ABCG2 transporter is functionally active in the isolated PBECs.

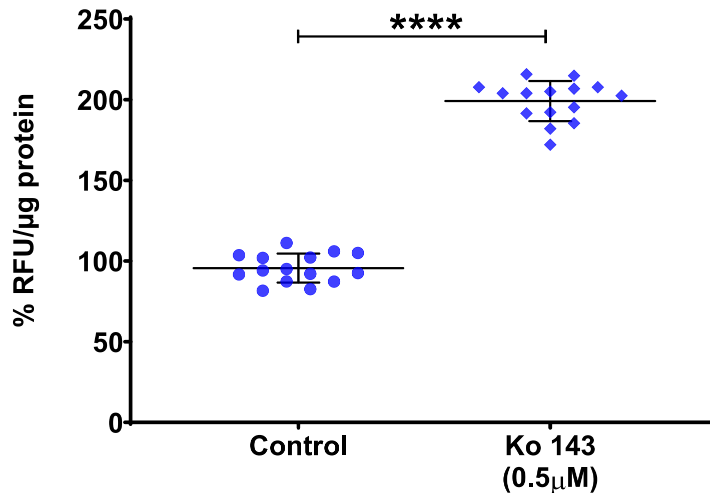


Figure 3.4: Inhibitory effect of Ko 143 on ABCG2 functional activity in primary porcine brain endothelial cells.
ABCG2 functional activity was determined by the intracellular accumulation of Hoechst 33342 in control cells and cells pre-treated with ABCG2 inhibitor Ko 143. Ko 143 is a potent ABCG2 specific inhibitor. Data are expressed as mean ± standard deviation of five independent experiments, with at least three replicates in each experiment. Statistical significance was determined using Student's t-test. RFU: relative fluorescent units. ****: $P < 0.0001$.

In order to assess whether ABCG2 is expressed at the protein level in primary cultures of PBECs, Western blot analyses were performed on whole cell lysates of PBEC monolayers as described in section 2.2.12. Rat liver was used as positive control. Western blotting using the BXP-53 monoclonal antibody directed to ABCG2 indicated cross reactivity of a protein with an apparent molecular weight of 75 kDa (Figure 3.5).

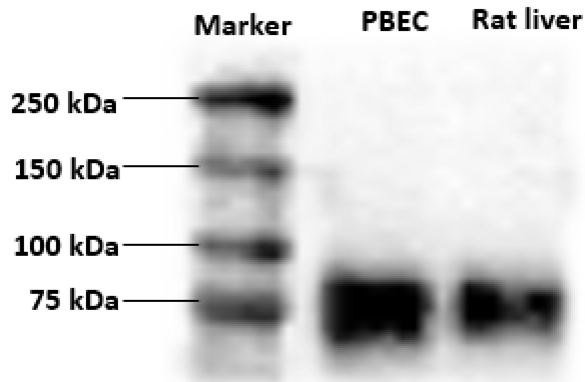


Figure 3.5: ABCG2 protein expression in primary porcine brain endothelial cells and rat liver.
Rat liver serves as positive control. Representative image from 5 independent experiments.

3.1.4 Determination of ABCC5 transporter activity and protein expression in primary porcine brain endothelial cells

The functional activity of ABCC5 was determined by measuring the intracellular accumulation of glutathione methylfluorescein (GS-MF), a fluorescent probe substrate for ABCC5. ABCC5-mediated transport is sensitive to MK571 inhibition; therefore, this agent was used to assess whether GS-MF transport was mediated by ABCC5 in PBECs.

Cells pre-treated with MK571 showed a significant increase ($P < 0.0001$) in intracellular GS-MF by almost 2.5-fold compared to untreated control cells (Figure 3.6). This demonstrated that the isolated PBECs express functionally active ABCC5.

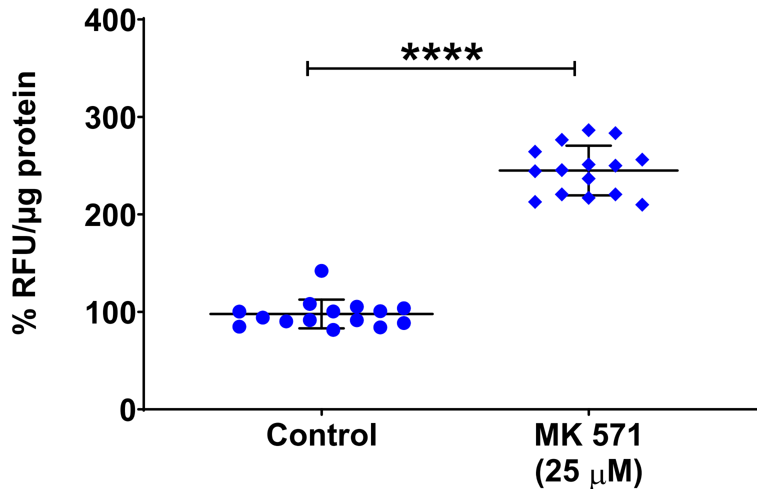


Figure 3.6: Inhibitory effect of MK 571 on ABCC5 functional activity in primary porcine brain endothelial cells.
ABCC5 functional activity was determined by the intracellular accumulation of GS-MF in control cells and cells pre-treated with ABCC5 inhibitor MK571. MK571 is a highly specific and most commonly used ABCC5 inhibitor. Data are expressed as mean ± standard deviation of five independent experiments, with at least three replicates in each experiment. Statistical significance was determined using Student's t-test. RFU: relative fluorescent units. ****: $P < 0.0001$.

In order to determine the expression of ABCC5 at the protein level in primary cultures of PBECs, Western blot analyses were performed on whole cell lysates obtained from PBEC monolayers as described in section 2.2.12. CTX-TNA2 rat astrocyte cell lysate served as positive control. Western blotting using the anti-ABCC5 antibody directed to ABCC5 indicated cross reactivity of a protein with an apparent molecular weight of 161 kDa (Figure 3.6).

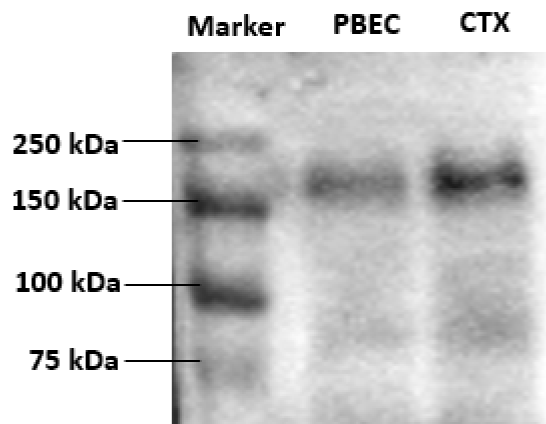


Figure 3.7: ABCC5 protein expression in primary porcine brain endothelial cells and CTX-TNA2 rat astrocyte.
CTX-TNA2 rat astrocyte serves as positive control. Representative image from 5 independent experiments.

3.1.5 Determination of ABCB1, ABCG2 and ABCC5 transporter activity in primary porcine brain endothelial cells using three different types of culture media.

The three most commonly used media to culture brain endothelial cells in the scientific literature are DMEM, DMEM-F12 (i.e. 50 % DMEM and 50 % F12) and M199. In this study, PBECs were grown in three different types of media in order assess whether medium has any effect on drug efflux transport activity in PBECs.

PBECs pre-treated with verapamil in all three media demonstrated a significant increase ($P < 0.0001$) in intracellular accumulation of calcein compared to their respective controls, Figure 3.8. The highest level of verapamil inhibition was observed in PBECs cultured in DMEM with over 7.0-fold increase, followed by DMEM-F12 (over 4.0-fold increase) and M199 (over 1.2-fold increase). These findings suggest PBECs maintained in DMEM growth medium exhibit higher levels of verapamil-sensitive ABCB1 activity than PBECs maintained in either DMEM-F12 or M199.

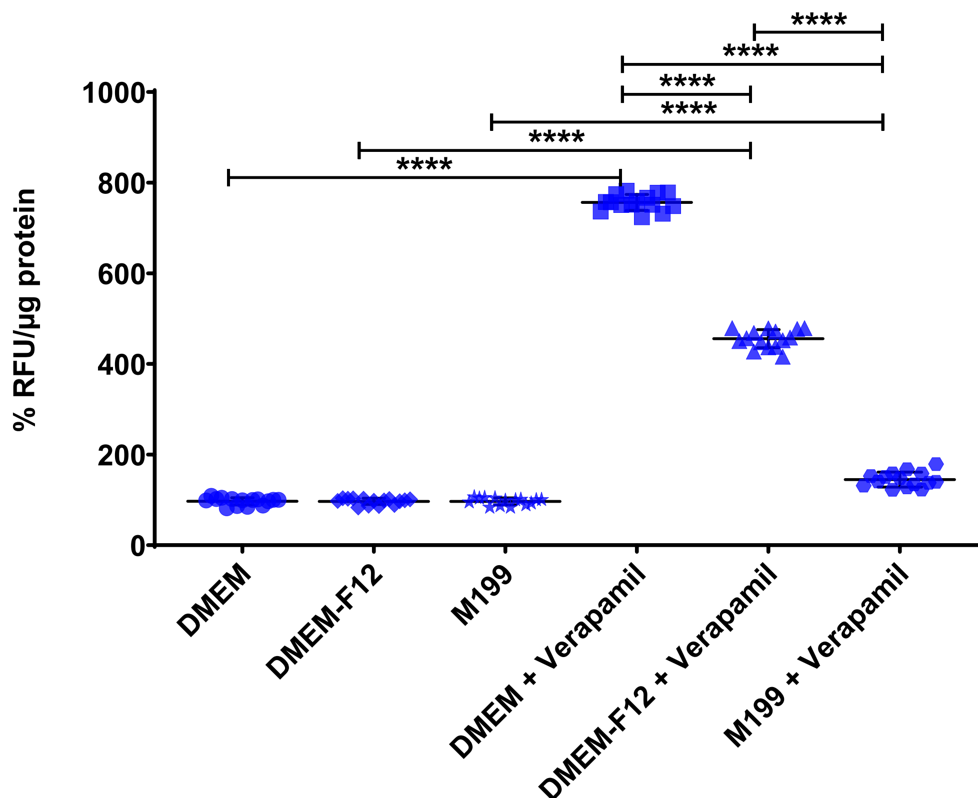


Figure 3.8: Effects of three different media on ABCB1 functional activity in porcine brain endothelial cells.
Intracellular accumulation of calcein was measured in control cells (media without ABCB1 inhibitor, verapamil) and treated cells (media containing ABCB1 inhibitor, verapamil). Data are expressed as mean ± standard deviation of five independent experiments, with at least three replicates in each experiment. Statistical significance was determined using One-Way ANOVA with Tukey as post hoc test. RFU: relative fluorescent units. ****: P < 0.0001.

The findings for the effect of media on ABCG2 transporter activity were similar to those observed for ABCB1. Intracellular accumulation of Hoechst 33342 in cells pre-treated with Ko143 increased significantly (P < 0.0001) compared to their respective control cells when PBECs were grown in all three culture media (Figure 3.9). The highest level of Ko143-mediated inhibition was observed when cells were maintained in DMEM (1.8-fold), followed by DMEM-F12 (over 1.5-fold) and M199 (over 1.3-fold) (Figure 3.9). These findings revealed that both ABCB1 and ABCG2 transporter activities are susceptible to the medium used to culture PBECs.

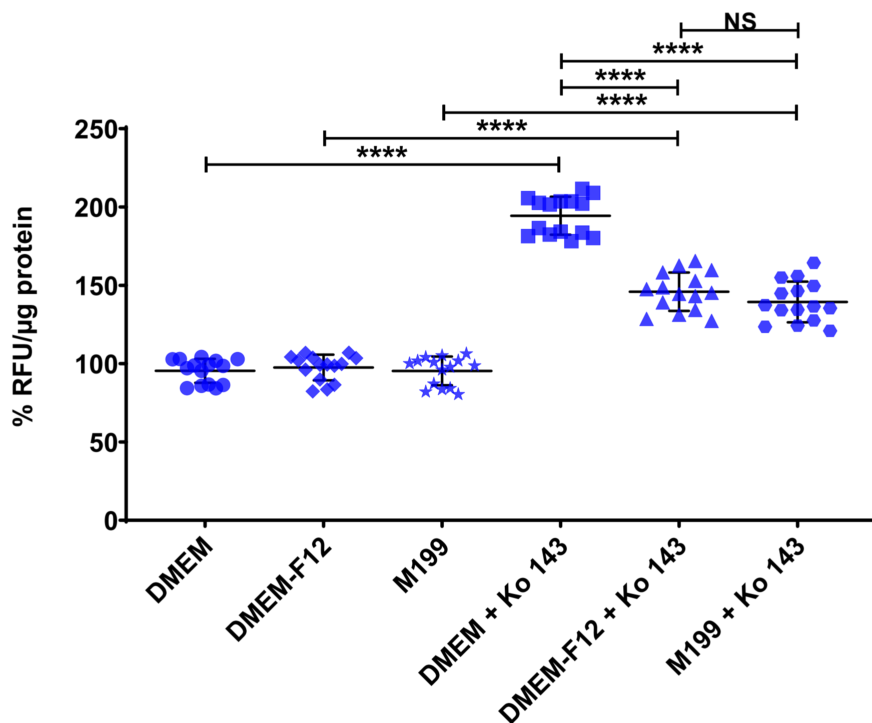


Figure 3.9: Effects of three different media on ABCG2 functional activity in porcine brain endothelial cells.
Intracellular accumulation of Hoechst 33342 was measured in control cells (media without ABCG2 inhibitor, Ko 143) and treated cells (media containing ABCG2 inhibitor, Ko 143). Data are expressed as mean ± standard deviation of five independent experiments, with at least three replicates in each experiment. Statistical significance was determined using One-Way ANOVA with Tukey as post hoc test. RFU: relative fluorescent units. ****: P < 0.0001, NS: not significant.

3.1.6 Determination of ABCB1, ABCG2 and ABCC5 transporter activity in primary porcine brain endothelial cells using astrocyte-conditioned medium from early and late passage astrocyte (CTX-TNA2) cells

Astrocyte-conditioned medium (ACM) was derived from the culture of CTX-TNA2 cells and was mixed 1:1 with DMEM to produce PBEC growth medium. In this study, two different types of ACM were used in order to assess their influence on the drug efflux transporter activity in PBECs. ACM-1 is the ACM obtained from the culture of early passage astrocyte cell line at between passage 1 to 5; while ACM-2 was obtained from late passage astrocyte cell line at between passage 15 to 20.

As can be seen in Figure 3.10, in PBECs treated with either ACM-1 and ACM-2 there was a significant increase in the intracellular accumulation of calcein (P < 0.0001) when cells were treated with the ABCB1 inhibitor verapamil; 7.4-fold for ACM-1 and 6.4-fold for ACM-

2. Furthermore, there was significantly ($p < 0.0001$) greater verapamil-sensitive ABCB1 transporter activity demonstrated by cells treated with ACM-1 than ACM-2, suggesting greater ABCB1 activity in cells that were maintained in ACM derived from CTX-TNA2 cells of passages 1 – 5.

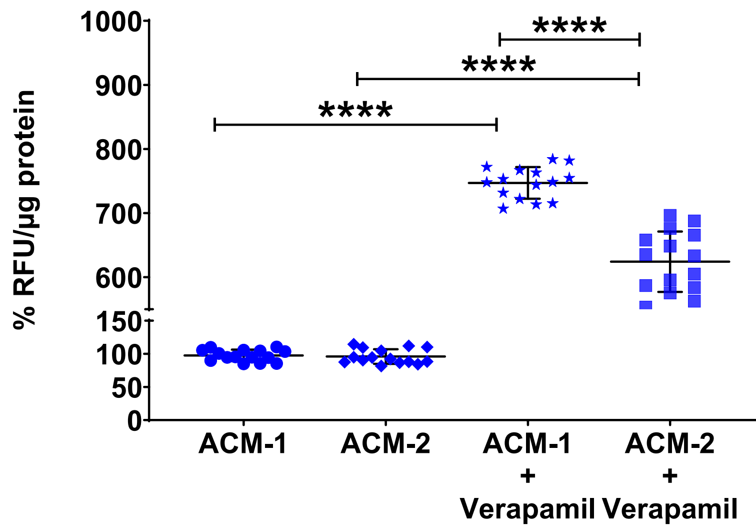


Figure 3.10: Effects of astrocyte-conditioned media (harvested from CTX-TNA2 cells at early and late passage) on ABCB1 functional activity in porcine brain endothelial cells. Intracellular accumulation of calcein was measured in control cells (DMEM medium containing ACM without ABCB1 inhibitor, verapamil) and treated cells (DMEM medium containing ACM and ABCB1 inhibitor, verapamil). Data are expressed as mean ± standard deviation of five independent experiments, with at least three replicates in each experiment. Statistical significance was determined using One-Way ANOVA with Tukey as post hoc test. RFU: relative fluorescent units. ****: $P < 0.0001$, ACM-1: astrocyte-conditioned medium from early passage CTX-TNA2 cells (passage 1 - 5), ACM-2: astrocyte-conditioned media from late passage CTX-TNA2 cells (passage 15 - 20).

Further studies were carried out to assess the effect of ACM on Ko143-sensitive ABCG2-mediated H33342 transport. In comparison to untreated control cells, cells treated with Ko143, that had been maintained in ACM-1 and ACM-2 culture conditions, exhibited a significant increase ($P < 0.0001$) in the intracellular accumulation of H33342, with increases of 2.0-fold in ACM-1 and 1.5-fold in ACM-2, Figure 3.11. As was observed with the ABCB1 transporter, there was significantly greater ($P < 0.0001$) Ko143-sensitive ABCG2 transporter activity demonstrated by cells treated with ACM-1 than ACM-2, suggesting greater ABCG2 activity in cells that were maintained in ACM derived from CTX-TNA2 cells of passages 1 – 5.

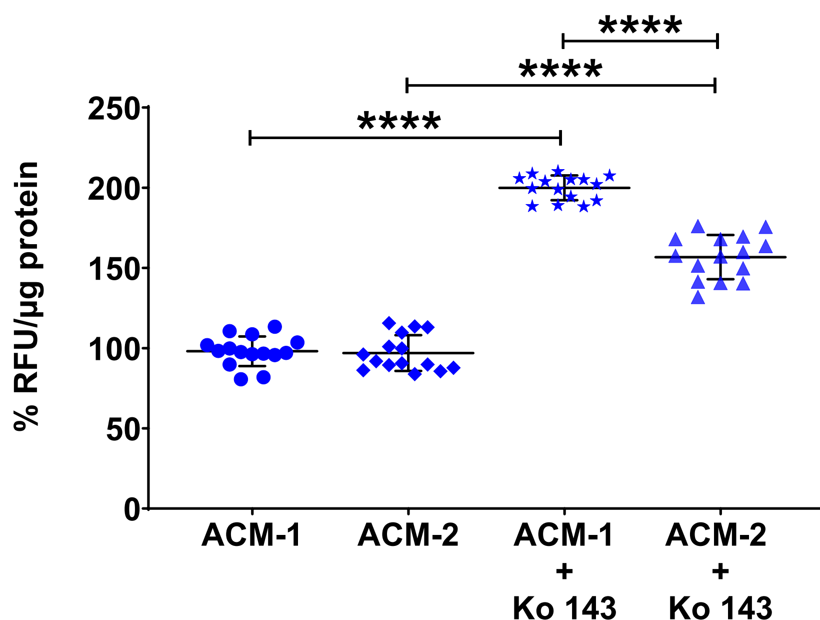


Figure 3.11: Effects of astrocyte-conditioned media (harvested from CTX-TNA2 cells at early and late passage) on ABCG2 functional activity in porcine brain endothelial cells. Intracellular accumulation of Hoechst 33342 was measured in control cells (DMEM medium containing ACM without ABCG2 inhibitor, Ko 143) and treated cells (DMEM medium containing ACM and ABCG2 inhibitor, Ko 143). Data are expressed as mean ± standard deviation of five independent experiments, with at least three replicates in each experiment. Statistical significance was determined using One-Way ANOVA with Tukey as post hoc test. RFU: relative fluorescent units. ****: P < 0.0001, ACM-1: astrocyte-conditioned medium from early passage CTX-TNA2 cells (passage 1 - 5), ACM-2: astrocyte-conditioned media from late passage CTX-TNA2 cells (passage 15 - 20).

The ABCC5 efflux transporter has also been shown to be expressed in PBECs, so similar studies were carried out to ascertain the effect of astrocyte-conditioned media, from early and late passage CTX-TNA2 cells, on ABCC5 activity. Similar findings were observed with ABCC5 activity as are reported for ABCB1 and ABCG2. In both culture conditions treatment of PBECs with the ACBC5 inhibitor Mk 571 significantly increased (P<0.0001) intracellular accumulation of GS-MF by 2.5-fold (ACM-1) and 1.5-fold (ACM-2) compared to their respective untreated control cells, Figure 3.12. Similarly, there was significantly greater (P < 0.0001) MK 571-sensitive ABCC5 transporter activity demonstrated by cells treated with ACM-1 than ACM-2, suggesting greater ABCC5 activity in cells that were maintained in ACM derived from CTX-TNA2 cells of passages 1 – 5.

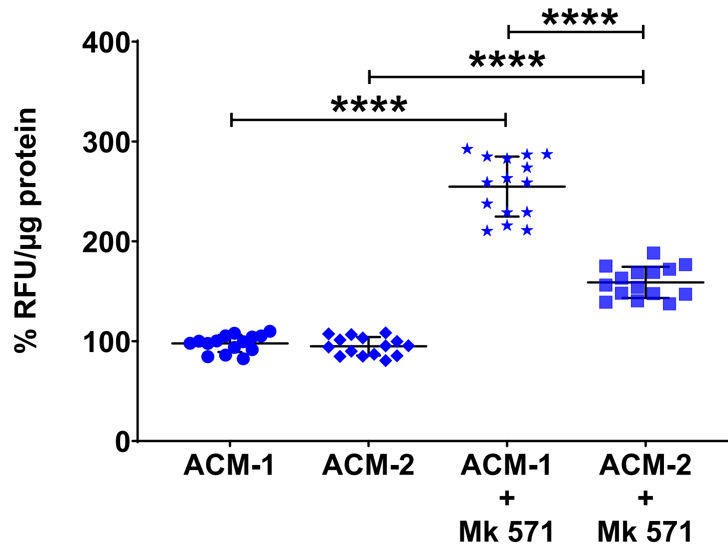


Figure 3.12: Effects of astrocyte-conditioned media (harvested from CTX-TNA2 cells at early and late passage) on ABCC5 functional activity in porcine brain endothelial cells. Intracellular accumulation of GS-MF was measured in control cells (DMEM medium containing ACM without ABCC5 inhibitor, MK571) and treated cells (DMEM medium containing ACM and ABCC5 inhibitor, MK571). Data are expressed as mean ± standard deviation of five independent experiments, with at least three replicates in each experiment. Statistical significance was determined using One-Way ANOVA with Tukey as post hoc test. RFU: relative fluorescent units. ****: $P < 0.0001$, ACM-1: astrocyte-conditioned medium from early passage CTX-TNA2 cells (passage 1 - 5), ACM-2: astrocyte-conditioned media from late passage CTX-TNA2 cells (passage 15 - 20).

3.1.7 Determination of the specificity of ABCB1, ABCG2 and ABCC5 fluorescent probe substrates

In order to evaluate whether the fluorescent probes applied in this study were specific to each transporter, we investigated the effect of verapamil, Ko143 and Mk 571 on the intracellular accumulation of calcein, H33342 and GS-MF. These studies illustrated that verapamil specifically, and significantly, inhibited intracellular accumulation of calcein, with a 7.4-fold increase observed following verapamil treatment (Figure 3.13 A). However, verapamil treatment had no significant effect on the intracellular accumulation of H33342 or GS-MF. Treatment of PBECs with Ko143 specifically and significantly inhibited intracellular accumulation of H33342 by 2.0-fold and had no significant effect on calcein and GS-MF accumulation, Figure 3.13 B.

Whilst MK 571 was found to significantly increase intracellular accumulation of GS-MF (i.e. significantly inhibit ABCC5-mediated transport of GS-MF) by 2.3-fold, Figure 3.13

C, treatment of PBECs with MK 571 also significantly increased intracellular calcein accumulation (Figure 3.13 C), suggesting that calcein, or the membrane permeable calcein-AM, is an ABCC5 substrate. Importantly, in this thesis, when measuring ABCB1 activity, the ABCB1-specific inhibitor verapamil is used to determine verapamil-sensitive ABCB1-mediated transport.

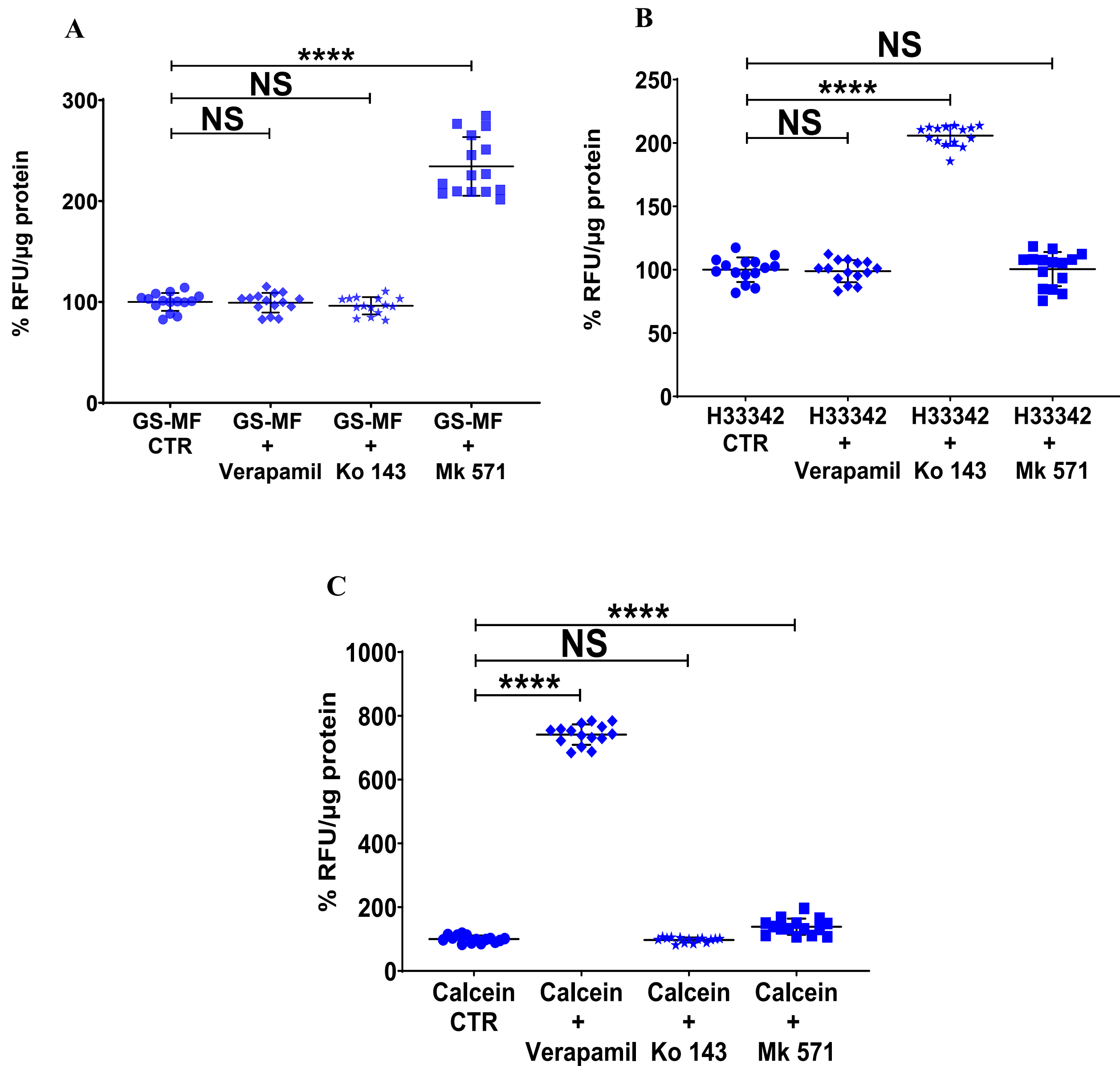


Figure 3.13: Effects of different transporter inhibitors on the intracellular accumulation of calcein, H33342 and GS-MF in primary porcine brain endothelial cells.
The specificity of each inhibitor and substrate was tested for ABCB1, ABCG2 and ABCC5. Data are expressed as mean ± standard deviation of five independent experiments, with at least three replicates in each experiment. Statistical significance was determined using One-Way ANOVA with Tukey as post hoc test. RFU: relative fluorescent units. ****: $P < 0.0001$; NS: not significant.

3.1.8 Expression of PXR, CAR, RXR and GR in primary porcine brain endothelial cells

The nuclear receptors PXR, CAR, RXR and GR are among the main nuclear receptors promoting the expression and activity of ABCB1, ABCG2 and ABCC5. To characterise the PBECs we use as a model of the blood-brain barrier, we determine whether PXR, CAR, RXR and GR are expressed in primary culture of PBECs. Western blotting was conducted on whole lysates obtained from PBEC monolayers as described in method section 2.2.12. Rat liver served as positive control.

Immunoblotting with anti-PXR, anti-CAR, anti-RXR and anti-GR antibodies demonstrated that PXR (Figure 3.14 A), CAR (Figure 3.14 B), RXR (Figure 3.14 C) and GR (Figure 3.14 D) protein are highly expressed in PBECs with apparent molecular weights of 50 kDa, 40 kDa, 51 kDa and 86 kDa respectively.

These findings strongly support the validity of using a PBEC-based BBB model to study the role of key receptors in the regulation of ABC transporter activity and expression.

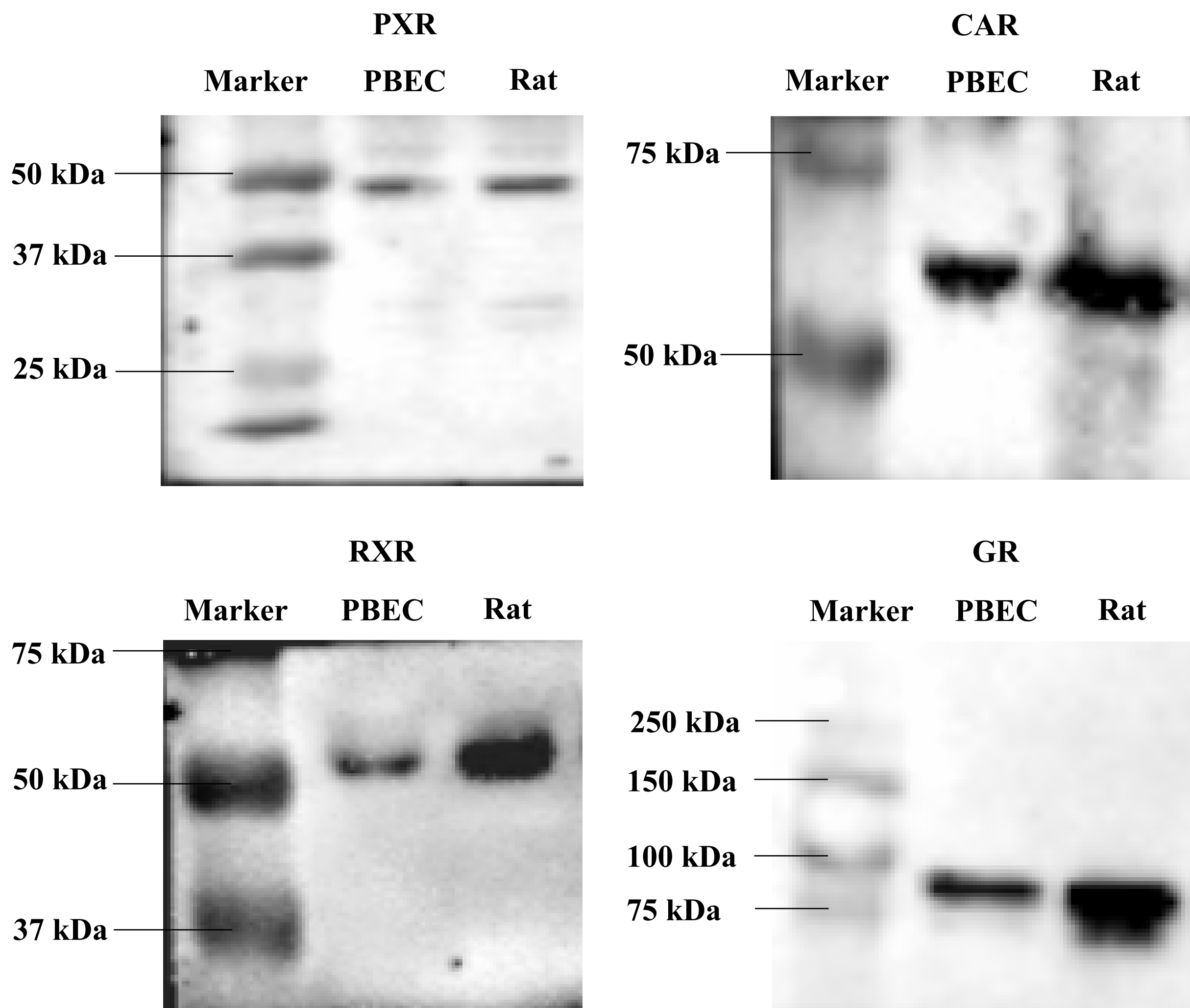


Figure 3.14: Western blot analysis of (A) PXR, (B) CAR, (C) RXR and (D) GR protein expression in primary porcine brain endothelial cells and rat liver.
Rat liver serves as positive control. Representative image from 5 independent experiments.

3.1.9 Discussion

3.1.9.1 Isolation and culture of primary porcine brain endothelial cells

In this study, porcine brain endothelial cells were successfully isolated based on the proposed method by Rubin et al., (1991) with slight modifications in reference to the other protocols (Cantrill et al. 2012; Patabendige et al. 2013; Torres-Vergara and Penny, 2018).

The incorporation of puromycin in the culture of PBECs had successfully eradicated contaminating cells, such as astrocytes and pericytes. This is due the fact that puromycin is an ABCB1 substrate and ABCB1 is only highly expressed in PBECs, making them not susceptible to the cytotoxicity of puromycin (Perriere et al. 2005; Nielsen et al., 2017).

In the current study the highly purified PBECs generated from puromycin treatment formed a confluent cell monolayer and developed an elongated spindle-like morphology, a characteristic of brain microvascular endothelia. The morphology of PBECs observed in this thesis is consistent with the findings of Cantrill et al. (2009), Nakhlband and Omidi (2011) and Nielsen et al. (2017), who also demonstrated purified PBECs exhibited spindle-like morphology. The strengths of the present method are that it combines simplicity and reproducibility with optimum cell yield and purity, making the resulting PBEC model robust, reliable and flexible, with good preservation of BBB features present *in vivo*.

3.1.9.2 Determination of ABC efflux transporter activity and protein expression in primary porcine brain endothelial cells

In the present study, primary porcine brain endothelial cells expressed three major functionally active ATP-binding cassette transporters, ABCB1, ABCG2 and ABCC5. This finding is consistent with many other studies reporting the similar findings using primary porcine brain endothelial cells (von Wedel-Parlow et al., 2009; Ott et al., 2009; Mahringer et al., 2009; Cantrill et al., 2012; Shubbar and Penny, 2018).

The ABCB1 activity in this PBEC model was determined by calcein-acetoxymethyl ester (calcein-AM) assay, which has been widely used to assess the functionality of the transporter in *in vitro* brain endothelial cells (Ott et al., 2009; Mahringer et al., 2009; Idbal, Gibb and Matthews, 2011). In this thesis, the inhibition of ABCB1 activity by verapamil resulted in a > 7.5-fold increase in cellular accumulation of calcein. Similar findings are

observed by Cantrill et al. (2012) and Steglich et al. (2012) reported a 6-fold increase in intracellular accumulation of calcein in PBECs, whilst Smith et al. (2007) observed a 7.5-fold increase in rhodomine-123 intracellular accumulation in PBECs pre-treated with verapamil.

In the assay of ABCG2 transport activity, treatment with the ABCG2 inhibitor Ko143 significantly elevate the intracellular accumulation of Hoechst 33342 by over 2-fold, confirming the studies of von Wedel-Parlow et al., (2009) and Mahringer et al., (2009) using PBEC model. von Wedel-Parlow et al., (2009) reported an approximate 1.5-fold increase in intracellular accumulation of both H33342 and mitoxanthrone using fumitremorgin C as ABCG2 inhibitor, a less potent analogue of Ko143 (Allen et al., 2002). Mahringer et al. (2009) reported an increase in intracellular accumulation of mitoxanthrone by a factor of 2 using Ko143. Over 2-fold increase of intracellular accumulation of H33342 has also been reported in other cell types, such as kidney (Kwatra et al., 2010) and lung cells (Galettiet al., 2015) using Ko143.

ABCC5 was shown to be functionally active in the PBECS employed in this study with the use of the ABCC5 inhibitor MK571. MK571 treatment significantly increased intracellular levels of the ABCC5 substrate GS-MF by 2.4-fold, which is similar to the value reported by the study of Shubbar and Penny (2018). Gutmann et al. (1999) demonstrated a 3-fold increase in GS-MF levels in PBECs treated with MK571, whilst other studies report MK571 increased intracellular accumulation of GS-MF by approximately 2-fold in the immortalised hBCEC cell line (Ketabi-Kiyanvash et al., 2007).

Initial studies demonstrated functional activity of ABCB1, ABCG2 and ABCC5 in PBECs in this thesis. Subsequent studies sought to substantiate these findings, and Western blotting was conducted to detect the expressions of the three transporters. These studies confirmed previous findings employing PBECs-based BBB model, who have reported expression of ABCB1 and ABCG2 (von Wedel-Parlow et al. 2009; Cantrill et al. 2012; Lemmen et al., 2013a). ABCC5 transporter protein expression has rarely been reported, thus far our studies are consistent with those of Shubbar and Penny reporting the expression of ABCC5 at protein level in PBECs using Western blotting (Shubbar and Penny, 2018) and QTRAP mass spectrometer (Kubo et al., 2015). Warren et al. (2009) reported relatively high level of ABCC5 mRNA expression in both PBECs and human brain endothelial cells.

3.1.9.3 Determination of ABC efflux transporter activity in primary porcine brain endothelial cells in different types of culture media

Throughout the literature, two culture methods are commonly adopted to establish the in vitro culture of PBECs. The first culture method is the use of M199 medium supplemented with 10% (v/v) serum to promote proliferation of PBECs, with serum-free DMEM-F12 used to induce PBEC phenotypic functions (Panzenboeck et al., 2002; Eisenblatter et al., 2003; Nitz et al., 2003; Mahringer et al., 2009; von Wedel-Parlow et al., 2009; Nakhlband et al., 2011; Bornhorst et al., 2012; Mulac et al., 2012; Lemmen et al., 2013a,b). The second culture method is the use of DMEM supplemented with 10% (v/v) serum to induce proliferation of PBECs, while DMEM with 1% (v/v) serum is used to promote PBEC phenotypic functions (Skinner et al., 2009; Cantrill et al., 2012; Kafa et al., 2015; Shubbar and Penny, 2018; Torres-Vergara and Penny, 2018).

In this study, PBECs were initially grown in each of the three media, DMEM, DMEM-F12 and M199, all the three media were supplemented with 10 % (v/v) plasma derived serum (PDS) to promote proliferation, then all the three culture media were changed from supplementation with 10 % (v/v) PDS to supplementation with 1 % (v/v) foetal bovine serum (FBS) to promote PBEC phenotypic functions. FBS, derived from the growing and developing foetus, possesses higher levels of growth factors, e.g. PDGF and VEGF, and other vasoactive/proliferative factors, compared to PDS (which is derived from the plasma of animals rather than the foetus) hence FBS is able to support the basic needs of cell growth at a reduced level of 1 % (Abbott et al., 1992).

DMEM with 1 % (v/v) FBS gave rise to the highest ABCB1 and ABCG2 transporter activity, followed by DMEM-F12 with 1 % (v/v) FBS and M199 with 1 % (v/v) FBS. This is the first study to report the effect of different media on ABC transporter activity. Hence, it is currently unknown why DMEM with 1 % (v/v) FBS contributed to the highest ABCB1 and ACBG2 activities. Each of the three media have been used interchangeably throughout the literature. In comparison to DMEM-F12 and M199, DMEM lacks many non-essential amino acids, vitamins, and other chemical components (Appendix B.1) (Dulbecco and Freeman, 1959), the differences in chemical composition in DMEM might be the possible reason for it to promote phenotypic functions of PBECs and hence, demonstrating enhanced ABCB1 and ABCG2 transporter activity. To the best of our knowledge, no study has any detailed explanation of how the differences in medium composition may alter brain endothelial cell

phenotypic functions. The most commonly used method to promote PBEC phenotypic functions is serum-deprived medium as demonstrated in the studies of Nakhlband et al. (2011); Bornhorst et al. (2012); Mulac et al. (2012) and Lemmen et al. (2013a,b). Indeed, it has been reported that changes in medium composition and application of different culture media play a key role in manipulating differentiation efficacy in embryonic stem cells (Kilberg et al., 2016), human induced pluripotent stem cells (Yamazaki et al., 2016) and neural stem cells (Lundqvist et al., 2013). Whereas, studies from Wang, X. et al. (2014) and Saito et al. (2017) demonstrated that utilising nutrient-depleted medium favoured cellular differentiation over proliferation in neural and intestinal stem cells. Hence, the lack of certain vitamins, non-essential amino acids and other components in DMEM may probably influence cellular functions in PBECs.

According to the chemical compositions of DMEM-F12 (Appendix B.2) and M199 (Appendix B.3) M199 contained the higher number of vitamins, amino acids and other chemical components compared to DMEM-F12 (Morgan and Campbell, 1955; Morgan, et al., 1950; Smith et al., 1960; Morton, 1970). The high nutrient composition in M199 might not be the suitable medium to promote PBECs functional characteristics, as a result, the cells expressed lower levels of functionally active ABCB1 and ABCG2, which thus gave rise to lower ABCB1 and ABCG2 transport activity. In contrast, PBECs cultivated in DMEM-F12 demonstrated slightly higher transporter activity compared to M199. DMEM-F12 lacks several amino acids and vitamins, and other components, present in M199 (Dulbecco and Freeman, 1959; Ham, 1965), which may therefore induce phenotypic changes in PBECs to express relatively higher levels of ABCB1 and ABCG2 transport activity. However, further studies need to be done to evaluate how media compositions induce changes in PBEC genotypic and phenotypic functions.

3.1.9.4 Determination of ABC efflux transporter activity in primary porcine brain endothelial cells using ACM from early and late passage astrocyte cell lines

Astrocytes cover a significant part of the endothelial surface (Kacem et al. 1998) and play a crucial role in modulating BBB permeability, enzymatic and transport functionalities of the BBB (Allt and Lawrenson 2000; Kuo and Lu 2011). Many studies have demonstrated factor(s) produced by astrocytes are important for the establishment of a fully functional BBB *in vitro*. Therefore, astrocyte-conditioned medium (ACM) was used in this study to optimise the physiological characteristics of the PBEC model. The application of ACM has been shown to be the major modulator of BBB and regulate the tight junction, permeability, enzymatic and

transport functionalities of the BBB. For example, ACM treatment led to significant increased TEER and decreased paracellular permeability in primary bovine (Raub et al., 1992; Rubin et al. 1991) human (Cecchelli et al., 1999; Megard et al., 2002; Kuo and Lu, 2011) and porcine brain endothelial cells model (Torok et al., 2003; Butt et al., 1990; Fischer et al., 2000; Zhang et al., 2006; Smith et al., 2008; Thomsen et al., 2015). Astrocyte-conditioned medium has also been reported to increase expression of the tight junctional protein ZO-1 in primary bovine brain endothelial cells (Rubin et al. 1991), and enhanced expression of BBB marker enzyme, alkaline phosphatase, in porcine brain endothelial cells (Smith et al., 2008). Only a limited number of studies have reported that ACM induce ABCB1 and ABCG2 mRNA expression in immortalised human and rat brain endothelial cell line (Puech et al., 2018; Hori et al., 2004a). It is widely known that ACM confers immense benefit on the growth and functional activities of PBECs, however, no study has characterised the ACM compositions, thus far. It has, however, been suggested that ACM contains astrocyte-derived secreted factors including glial cell line-derived neurotrophic factor (GDNF) (Miyazaki & Asanuma, 2016), brain-derived neurotrophic factor (BDNF) (Miyazaki & Asanuma, 2016), nerve growth factor (NGF) (Gray and Patel, 1992), basic fibroblast growth factor (bFGF) (Gray and Patel, 1992), ciliary neurotrophic factor (CNTF) (Gray and Patel, 1992), transforming growth factor-β (TGF β) (Tran et al., 1999), angiopoetin 1 (Lee et al., 2003) and vascular endothelial growth factor (VEGF) (Alvarez et al., 2013; Wong et al., 2013) which all together contribute to the maintenance of BBB genotypic and phenotypic features in PBEC model (Rubin et al., 1991, Cantrill et al., 2012).

Despite the growing number of studies on astrocytes and their secretome in the past few years, little is known about how cell passaging might influence the astrocytes' phenotype, genotype and secretome compositions. A few studies have focused on how astrocyte passaging impacts neuronal growth and behaviours, while to our knowledge, no studies yet report the influence of astrocyte cell passage on brain endothelial cell phenotype. The present study demonstrated that PBECs treated with ACM-1 collected from CTX-TNA2 rat astrocyte cell line from passage 1 – 5 showed higher ABCB1 an ABCG2 activity compared to cells treated with ACM-2 collected from astrocytes from passage 15 – 20. This finding suggests that although the immortalised CTX-TNA2 astrocyte cell line used in this thesis has an unlimited lifespan, continuous passaging and proliferation over an extended period may eventually contribute to changes in their phenotype and genotype, with these changes reflected in the secretome and subsequently on the functional activity of PBECs.

The findings of this thesis are in line with the study of Miranda et al. (2012) who demonstrated that proliferation of neural progenitor cells treated with ACM collected from primary astrocytes isolated from 13-month old mice was less than when neural progenitor cells were incubated with ACM derived from astrocytes isolated from 3-month old mice. Another study from Miranda et al. (2012) showed that in comparison to neurones co-cultured with low passage immortalised astrocytes (passage 2 - 8), neurones co-cultured with high passage immortalised astrocytes (passage 17 - 28) exhibited reduced cellular viability, decreased proliferation rates, lower mitochondria ability to buffer high concentrations of cytoplasmic Ca^{2+} and demonstrated qualitatively more depolarised mitochondria in the presence of neurotoxicant 1,3-dinitrobenzene (Maurer et al, 2016; Turnquist et al., 2016).

The typical ageing signs of astrocytes include changes/reduction in their phenotypic markers such as glutamine synthetase (GS), 2',3'-Cyclic-nucleotide 3'-phosphodiesterase, GFAP and S100B activity and expression; decline in cellular functions such as Apolipoprotein E secretion, Wnt production, sensitivity to phosphodiesterase inhibitor, resistance to CD95-induced death and plasticity, and impaired cellular homeostasis and proliferation (Vernadakis et al., 1992; Grove et al., 1995 Baskin et al.,1997; Saas et al., 1999; Kuijlaars et al., 2016; Cohen et al., 2017; Limbad et al., 2020). In this thesis, passage 15 – 20 is considered high passage, because studies from Katakowski et al. (2009), Maurer et al. (2016) and Turnquist et al. (2016) showed that CTX-TNA2 cell exhibited abrupt phenotypic changes in terms of increased cell area, compromised mitochondrial membrane potential, decreased viability, antioxidant function and a gradual loss of the ADAM17 enzyme, epidermal growth factor expression and overall neuroprotective effect towards neurones at between passage 9 – 16. Hence, the most probable reason for reduced ABCB1 and ABCG2 transporter activity in PBECs treated with ACM from high passage CTX-TNA2 cells is altered composition of the astrocyte secretome. However, future research is needed to characterise the changes in astrocyte secretome in ACM harvested from both high and low passage CTX-TNA2 cells and how these changes may affect the genotypic and phenotypic of PBEC culture.

The present study shows that increasing passage number as an in vitro proxy for “ageing” of immortalised astrocytes. To our knowledge, ours is the first study showing the influence of immortalised astrocyte cell passage on ABC transporter function of PBECs. The use of ACM has been criticised due to its unknown, or non-standardised composition, at each

harvesting, which could potentially influence the reproducibility of each experiment performed (Franke et al., 2000, Zhang et al., 2006). However, from the findings in this study, the levels of verapamil-sensitive ABCB1 activity, Ko143-sensitive ABCG2 activity and MK 571-sensitive ABCC5 are consistent at each harvesting of ACM using CTX-TNA2 cells in between the passage number of 1 – 5.

3.1.9.5 Determination of the specificity of ABC efflux transporter fluorescent probe substrates

In this study, fluorescent marker substrates, calcein-AM, H33342 and CMFDA were used to access the functional activity of ABCB1, ABCG2 and ABCC5 transporters respectively. However, in order to determine whether the fluorescent marker substrates exhibit exclusive specificity for each transporter, substrate specificity studies were carried out.

Calcein-AM has long been used to measure ABCB1 functional activity based on the calcein accumulation assay and was demonstrated to be a reliable assay across different cells, such as brain endothelial cells (Steglich et al., 2012; Ott et al., 2009), epithelial cells (Pasquier et al., 2013; Eneroth et al. 2001), fibroblast (Hollo et al., 1994), cancer cells (Pasquier et al., 2013) and immune cells (Srinivas et al. 1998). From these studies, calcein-Am has been known as ABCB1 substrate, owing to the inability of calcein to cross biological barriers after the cleavage from the acetoxymethyl portion by cytoplasmic esterases, the calcein-AM assay measures intracellular calcein accumulation.

In this study, treatment with verapamil and Mk571, but not Ko143, led to intracellular accumulation of calcein, confirming that calcein-AM used in our model is substrate for both ABCB1 and ABCC5. The findings of this thesis agree with studies that demonstrated calcein is not a substrate for ABCG2 (Karaaszi et al., 2001; Litman et al, 2000; Weksler et al., 2005; Szabo et al., 2018). However, treatment with the ABCC inhibitor Mk571 only contributed to a relatively small increase of intracellular accumulation of calcein at 1.5-fold compared to that observed with verapamil treatment at over 7.0-fold. Hence, measurement of verapamil-sensitive ABCB1-mediated calcein-AM transport is an acceptable way to measure ABCB1 activity. Similar studies also reported that calcein-AM is not exclusively an ABCB1 substrate, but also for substrate for ABCC1 and ABCC2 (Srinivas et al., 1998; Eneroth et al., 2001; Bauer et al., 2003; Litman et al., 2001; Weksler et al., 2005; Molinas et al., 2012). For example, the use of ABCC inhibitors, such as MK571, indometacin and probenecid only contributed to a

minor increase in intracellular accumulation of calcein (≤ 2-fold) in brain endothelial cells (Bauer et al., 2003) and epithelial cells (Eneroth et al., 2001), hence ABCCs only have a small influence on the calcein-AM assay in PBECs.

Hoechst 33342 has been widely used as an ABCG2 specific substrate in different cells, including PBCECs (von Wedel-Parlow et al., 2009), kidney cells (Kwatra et al., 2010; Weidner et al., 2015), lung cells (Galettiet al., 2015), cancer cells (Scharenberg et al., 2002; Sinha et al., 2019) and bronchial epithelial cells (Paturi et al., 2010). Similarly, in this study, Hoechst 33342 was proven to a specific substrate for ABCG2, since the inhibition of ABCB1 and ABCC5 did not contribute to an increase in intracellular accumulation of Hoechst 33342. This finding also agrees with Scharenberg et al. (2002), Szabo et al. (2018) and Jenkinson et al, (2012) who demonstrated that Hoechst 33342 is not a substrate for ABCCs.

Several studies have reported that Hoechst 33342 is a substrate for both ABCB1 and ABCG2 in PBECs (Lemmen et al., 2013a,b), kidney cells (Jenkinson et al, 2012), ovarian carcinoma cells (Muller et al., 2007; Scharenberg et al., 2002), squamous carcinoma (Nerada et al., 2016) and Caco-2 (Lingam et al., 2017). However, in this thesis, no significant increase in intracellular Hoechst 33342-associated fluorescence was observed when PBECs were treated with the ABCB1 inhibitor verapamil. Therefore, to date, there is debate whether Hoechst 33342 is an ABCB1 substrate.

CMFDA is a fluorescent probe that freely passes through the cell's plasma membrane and is rapidly converted into the membrane-impermeant fluorescent product GS-MF. GS-MF has been reported to be an ABCC5 substrate in many studies including in MRP5-transfected HEK293 cells (McAleer et al.. 1999; Pratt et al., 2006; Nehmann et al., 2009), HEKc10 cells (Aszalos and Taylor, 2009), MRP5-transfected MDCK cells (Wijnholds et al., 2000; Luna-Tortós et al., 2010) and PBECs (Shubbar and Penny, 2018). Whilst this thesis found treatment of PBECs with the ABCC5 inhibitor Ko143 significantly increased intracellular accumulation of fluorescent GS-MF, no such accumulation was observed when PBECs were treated with ABCB1 and ABCG2 inhibitors, which is in agreement with Shubbar and Penny, (2018). Other studies using cell lines transfected with ABC transporter cDNA reported that GS-MF is not be a substrate for ABCB1, ABCG2, ABCC1 and ABCC2 (Forster et al., 2008; Luna-Tortos et al., 2009; Luna-Tortós et al., 2010; Strouse et al., 2013).

Taken together, these studies have verified a lack of substantial overlap in substrate specificity between the ABCB1, ABCG2 and ABCC5 transporters thereby demonstrating the reliability of the three substrates in measuring transporter functional activity in this thesis.

3.1.9.6 Expression of nuclear receptors in primary porcine brain endothelial cells

The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are 'orphan' nuclear receptors and key components of the pathways involved in regulation of ABCB1 and ABCG2 expression through the cooperation of the retinoid X receptor (RXRα) (Miller 2010, Urquhart et al. 2007) or indirect regulation by the glucocorticoid receptor (GR) (Pascussi et al., 2000a; De Bosscher et al., 2010; Chan et al., 2013). Therefore, initial studies set out to determine if PXR, CAR, GR and RXRα are expressed in primary porcine brain endothelial cells employed in our laboratory.

In this thesis PXR expression at the protein level has been confirmed in PBECs. This finding is consistent with those studies reporting expression in rat brain capillaries (Narang et al., 2008), hCMEC/D3 cells (Zastre et al. 2009; Chan et al., 2011) and primary human brain endothelial cells (Ghosh et al., 2017).

Expression of both CAR and RXR at the protein level in PBECs has also been demonstrated in this thesis. These findings are in agreement with studies reporting the expression of CAR at the protein level in hCMEC/D3 cells (Chan et al., 2011) and PBECs (Shubbar and Penny, 2018). RXR expression in PBECs in this study is consistent with the findings reported in rat and mouse brain capillaries (Arfaoui et al., 2013; De Urquiza et al., 2000), in conditionally immortalised rat brain capillary endothelial cells (TR-BBB13) (Akanuma et al., 2008) and in primary human brain endothelial cells (Ghosh et al., 2017).

Expression of the GR in PBECs at the protein level has been confirmed in this thesis. This is in line with studies which demonstrated GR protein expression in primary human brain endothelial cells (Ghosh et al., 2017), bovine brain endothelial cells (Gaillard et al., 2001) and mouse brain endothelial cells (Kleinschnitz et al., 2011; Salvador et al., 2014).

3.1.9.7 Characterisation of primary porcine brain endothelial cells

The preliminary studies carried out in this Chapter demonstrated PBECs expressed functionally active ABC transporters (ABCB1, ABCG2 and ABCC5) and nuclear receptors (PXR, CAR, RXR and GR). Furthermore, it is worth noting that in each batch of isolation, 15 brains were pooled together in the isolation of PBECs. In this thesis, more than a dozen PBEC isolations were carried out, utilising over a hundred porcine brains, and in all batches of PBEC isolations, there was a high degree of consistency and reproducibility in the functional activity of ABCB1, ABCG2 and ABCC5, and the expression of PXR, CAR, RXR and GR. The findings of this thesis demonstrate PBECs as a robust and reproducible *in vitro* BBB model to investigate the factors modulating the ABC transporters activity and expression at BBB.

Further characterisation of PBECs based on transendothelial electrical resistance (TEER), permeability and enzyme (Alkaline phosphatase and γ-glutamyl transpeptidase) assays are not carried out in this thesis, because all these assays had been extensively studied in this PBEC model by Skinner (2009), Cantrill (2012), Pablo (2015) and Maryam (2018) in Dr. Jeffrey Penny´s group. The outcomes of the assays demonstrated in all these studies are consistent throughout over 10 years of research. The TEER of PBEC monolayers is reported in between 1,200 - 1,800 $\Omega \cdot cm^2$ (Cantrill, 2012; Maryam, 2018), this high TEER value indicates the restriction of the tight junction complexes and low permeability. Next, the apparent permeability (Papp) of Lucifer Yellow through PBEC monolayers was reported in between $5.7 \times 10^{-7} \pm 0.46 \times 10^{-7}$ - $7.7 \times 10^{-7} \pm 0.2 \times 10^{-7}$ $cm.s^{-1}$ (Cantrill, 2012; Maryam, 2018). Lastly, the enzyme activity of γ-glutamyl transpeptidase is shown to be in the range of 62.54 ± 27.61 - 84.7 ± 27.4 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ (Pablo, 2015; Maryam, 2018) and alkaline phosphatase activity in the range of 1004 ± 302.5 - 1170 ± 184.8 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ (Pablo, 2015; Maryam, 2018). Alkaline phosphatase and γ-glutamyl transpeptidase are regarded as phenotypic marker enzymes associated with brain endothelial cells and they function to sustain the BBB phenotypic features in *in vitro* with *in vivo* (Sobue et al. 1999).

The monolayer culture established in this study is comparable to 2D (co-culture of PBECs with astrocytes) and 3D (triple-culture of PBECs, astrocytes and pericytes) cultures demonstrated in the study of Thomsen et al. (2015). In the study of Thomsen et al. (2015), Monolayer, 2D and 3D cultures were shown to have the same level of TEER (800 - 1100 $\Omega \cdot cm^2$), but 2D culture demonstrated significant lower permeability (1.0×10^{-6} $cm.s^{-1}$) when

compared with monolayer and 3D cultures (2 – 3 x 10^{-6} cm.s^{-1}). In terms of tight junction protein, all three types of culture exhibited the same level expression of occluding but 2D demonstrated the highest level of claudin-5. No significant difference in ABCB1 and ABCG2 protein expression observed in all three types of cultures. Hence, this demonstrated that monolayer culture established in this study is robust and suitable to be used to study BBB functions.

3.1.9.8 Limitations of the study

In this study, PBECs have been successfully isolated and characterised which express an array of ABC efflux transporters (ABCB1, ABCG2 and ACBC5) and receptors (PXR, CAR, GR and RXR) and therefore represent a valuable model in which to study the role of key receptors in regulating pharmacologically important drug transporters in the blood-brain barrier. The medium DMEM supplemented with ACM harvested from cells of early passage appeared to be the most suitable medium to establish *in vitro* BBB model to study ABC transporter activity; however, there are several limitations identified in this study. For example, in this study, the suitability of the medium is tested only on ABC transporter activity; hence, it may not be sufficient to claim that the culture medium is suitable to study the overall function of BBB, other assays, such as permeability, TEER and tight junction protein expression need to be carried out in the future. Furthermore, the components present in ACM secretome harvested in between early and late passage of cells that affect ABC transporter activity are currently unknown; hence, it is worth identifying those essential components present in ACM that may affect the ABC transporter activity in PBECs.

Another limitation in this study is the use of puromycin in culture to remove contaminating cells and enhance PBEC purity, integrity and phenotype (Cantrill et al. 2012). Several studies demonstrated that puromycin has significant influence on BBB functional activity. It was reported that puromycin treatment for 48 h significantly increased TEER values and reduced permeability in rat (Perrière et al., 2005) and porcine brain endothelial cells (Cantrill et al. 2012; Patabendige et al., 2013). Puromycin is considered the substrate of ABCB1 which is able to restrict cellular uptake of the cytotoxic puromycin, while contaminating cells such as pericytes, smooth muscle cells, astrocytes and fibroblasts (Greenwood 1992) that are more vulnerable, tend to be eliminated by puromycin treatment (Perrière et al., 2005). Hence, there is a concern that incorporation of puromycin in PBEC culture may influence the outcome of ABC transporter activity and expression in subsequent

assays. The study from Alms et al. (2014) demonstrated that puromycin significantly upregulated ABCB1 transporter activity in primary human, porcine and rat brain endothelial cells, although increased ABCB1 protein expression was only shown in immortalised rat brain endothelial cells. However, Perrière et al. (2005) reported that increased ABCB1 protein expression was not detected, compared to control, in primary rat brain endothelial cells when the cells were treated with puromycin for 4 and 13 days. Thus far, no studies have demonstrated the significant influence of puromycin on ABC transporter activity and expression in response to drug treatments. In this study, the incubation of puromycin lasted for 48 hr, and the cells were maintained in culture without puromycin for 5 days, which is likely sufficient to eliminate the effect of puromycin on PBECs. However, further studies are needed to prove that the influence of puromycin is completely eliminated after 5-day of culture and will not interfere with subsequent drug assays in the study of ABC transporters.

In addition, the influence of the presence of other media components such as serum and antibiotics on nuclear receptors and ABC transporters has rarely been reported. Thus far, penicillin-streptomycin was found to activate the PXR/RXR pathway in liver cells (Ryu et al., 2017). Another study reported that media free of steroid, serum and antibiotic are essential to maintain PXR in the deactivated form (Saradhi et al., 2005). Similarly, Chilton et al. (1990) also reported increased responsiveness of glucocorticoid receptor to glucocorticoid in CD4+ T-Cell clonal lines grown in serum-free media (Chilton et al., 1990). In this study, we have incorporated the use of penicillin-streptomycin and a low level of serum (1% FBS) in the medium, which might contribute to the increase basal expression of ABC transporters (ABCB1, ABCG2 and ABCC5) and nuclear receptors (PXR, CAR, GR and RXR) in this study. However, further research needs to be conducted in this model to provide clear evidence.

Chapter 4

The role of nuclear receptors in regulating ABC transporter expression and activity

4.1 Effect of nuclear receptor ligands on transporter activity and expression

4.1.1 Background

Nuclear receptors are the main determinant of tissue responses against exposure to xenobiotics, and a key response is modulating the expression of ABC efflux transporters. The nuclear receptors pregnane x receptor (PXR) and constitutive androstane receptor (CAR) are master regulators of ABC efflux transporters (Rigalli et al., 2019). In human, they are more widely expressed in liver and intestine compared to brain (Lamba et al., 2004; Miki et al., 2005). Hence, extensive research has been primarily focused on the regulatory mechanism of PXR and CAR on ABC efflux transporters and metabolising enzymes involved in drug disposition in liver (Merrell et al., 2008; Maher et al., 2006; Pfrunder et al., 2003; Schrenk et al., 2001) and intestine (Martin et al., 2008; Naruhashi et al., 2011; Korjamo et al., 2005).

Recently, several studies have demonstrated the significant influence of PXR and CAR on ABCB1 and ABCG2 expression in the BBB (Chan et al., 2011; Lemmen et al., 2013a, 2013b; Ott et al., 2009; Wang, X. et al., 2010; Slosky et al., 2013). However, little is known of the detailed mechanism by which PXR and CAR regulate BBB efflux transporter functional activity, as well as the influence of xenobiotics and other nuclear receptors, such as the role of glucocorticoid receptor (GR) in regulating PXR and CAR expression. Hence, a better understanding of how nuclear receptors regulate BBB transporters may help improve therapeutic drug delivery into the CNS. Therefore, the aim of this part of the project was to study the effect of GR on the expression of PXR, CAR and RXR, and to determine the effects of PXR, CAR and RXR on the expression and activity of ABCB1, ABCG2 and ABCC5.

4.1.2 Pregnane X Receptor (PXR) ligands

4.1.2.1 Effect of PXR agonist and antagonist on ABCB1, ABCG2 & ABCC5 transporter activities and protein expression

Treatment of PBECs for 24 h with PXR agonist rifampicin (RF), at a concentration of 10 µM, significantly decreased the intracellular accumulation of calcein (ABCB1 substrate) ($p < 0.01$) (Figure 4.1 A) and GS–MF (ABCC5 substrate) ($p < 0.05$) (Figure 4.1 C) by over 30 % and intracellular accumulation of Hoechst 33342 (ABCG2 substrate) ($p < 0.01$) (Figure 4.1 B) by over 25 %. These results indicate treatment of PBECs with rifampicin significantly increased ABCB1, ABCG2 and ABCC5 transport activities.

In order to determine whether the changes in transporter activities are caused by inherent PXR activation, L-sulforaphane (L-SFN), a PXR antagonist was used. In PBECs treated with L-SFN, at a concentration of 5 μM for 24 h, there was a significant decrease in ABCB1 (Figure 4.1 A) and ABCC5 (Figure 4.1 B) transport activities, as demonstrated by significant increases ($p < 0.0001$) in intracellular accumulation of calcein and GS-MF by 50.2 % ± 15.9 % and 66.5 % ± 19.7 % respectively; however, no effect was observed on ABCG2 activity (Figure 4.1 C). Interestingly, co-treatment with rifampicin and L-sulforaphane significantly enhanced ($p < 0.0001$) the accumulation of calcein, Hoechst 33342 and GS–MF by 11.5-fold, 6.5-fold and 8.9-fold respectively. None of the treatments tested affected cell viability as determined by the neutral red assay (Appendix D.2).

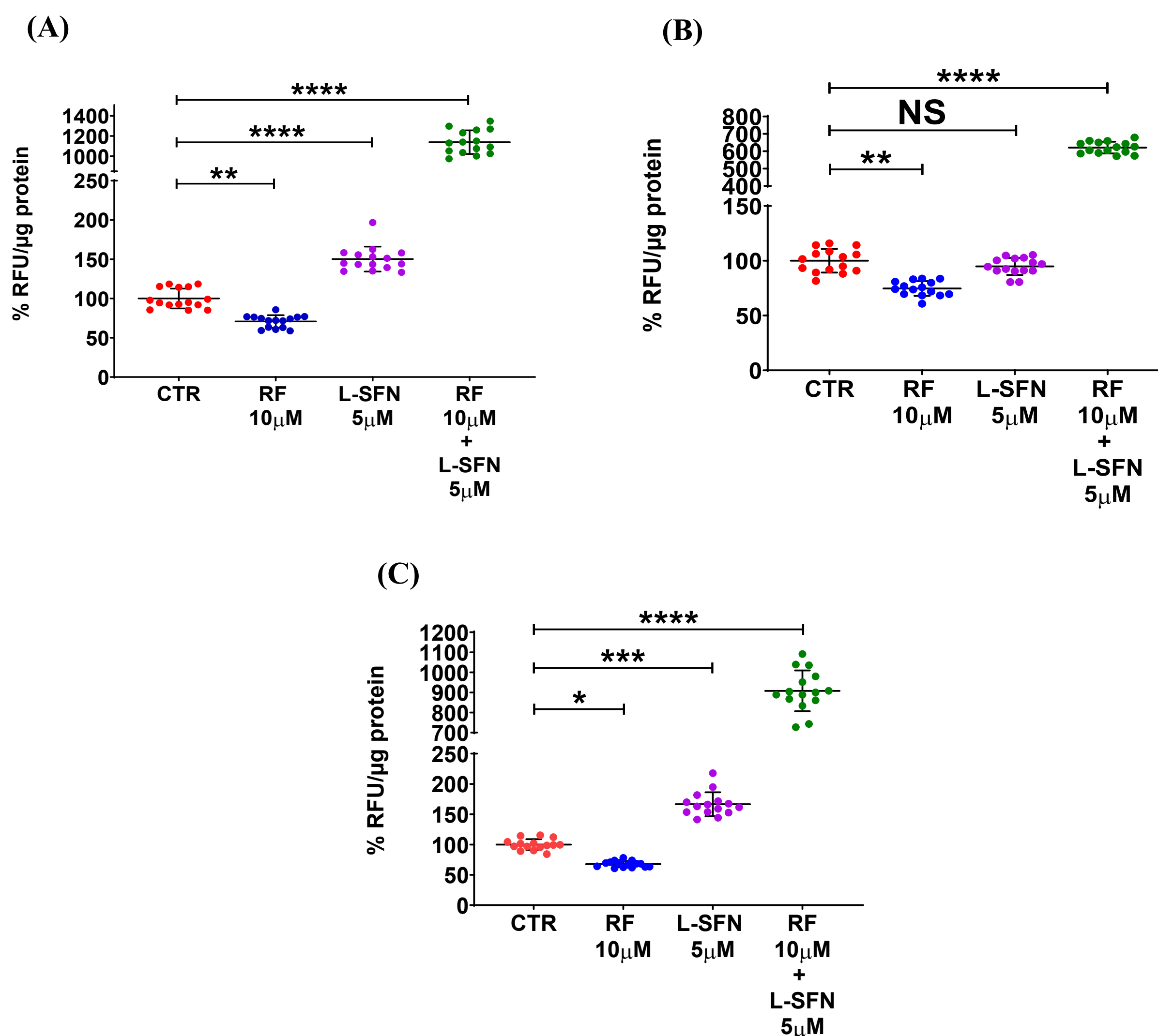


Figure 4.1: Effects of PXR agonist (rifampicin, RF) and antagonist (L-sulforaphane, L-SFN) on ABCB1, ABCG2 and ABCC5 functional activity in PBECs.
Intracellular accumulation of (A) calcein - ABCB1 substrate, (B) Hoechst 33342 - ABCG2 substrate, (C) GS–MF - ABCC5 substrate were measured in control cells and cells treated with 10 µM RF and 5 µM L-SFN as single treatment or co-treatment for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, NS: non-significant. The concentrations used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.2).

In order to determine whether the outcome presented after CITCO treatment is related to direct interaction with ABCB1, i.e., inhibition of ABCB1, ABCG2 and ABCC5 activity, a new experiment was conducted by reducing the 24 h incubation time to 30 min. Under these experimental conditions exposure to 10 µM rifampicin and 5 µM L-sulforaphane did not result

in any significant changes (p > 0.05) to the intracellular accumulation of calcein (Figure 4.2 A), Hoechst 33342 (Figure 4.2 B) and GS-MF (Figure 4.2 C) when compared to control. On the other hand, co-treatment with rifampicin and L-sulforaphane significantly enhanced (p < 0.0001) the accumulation of calcein by 2.3-fold (Figure 4.2 A), and Hoechst 33342 (Figure 4.2B) and GS–MF (Figure 4.2 C) by over 1.6-fold.

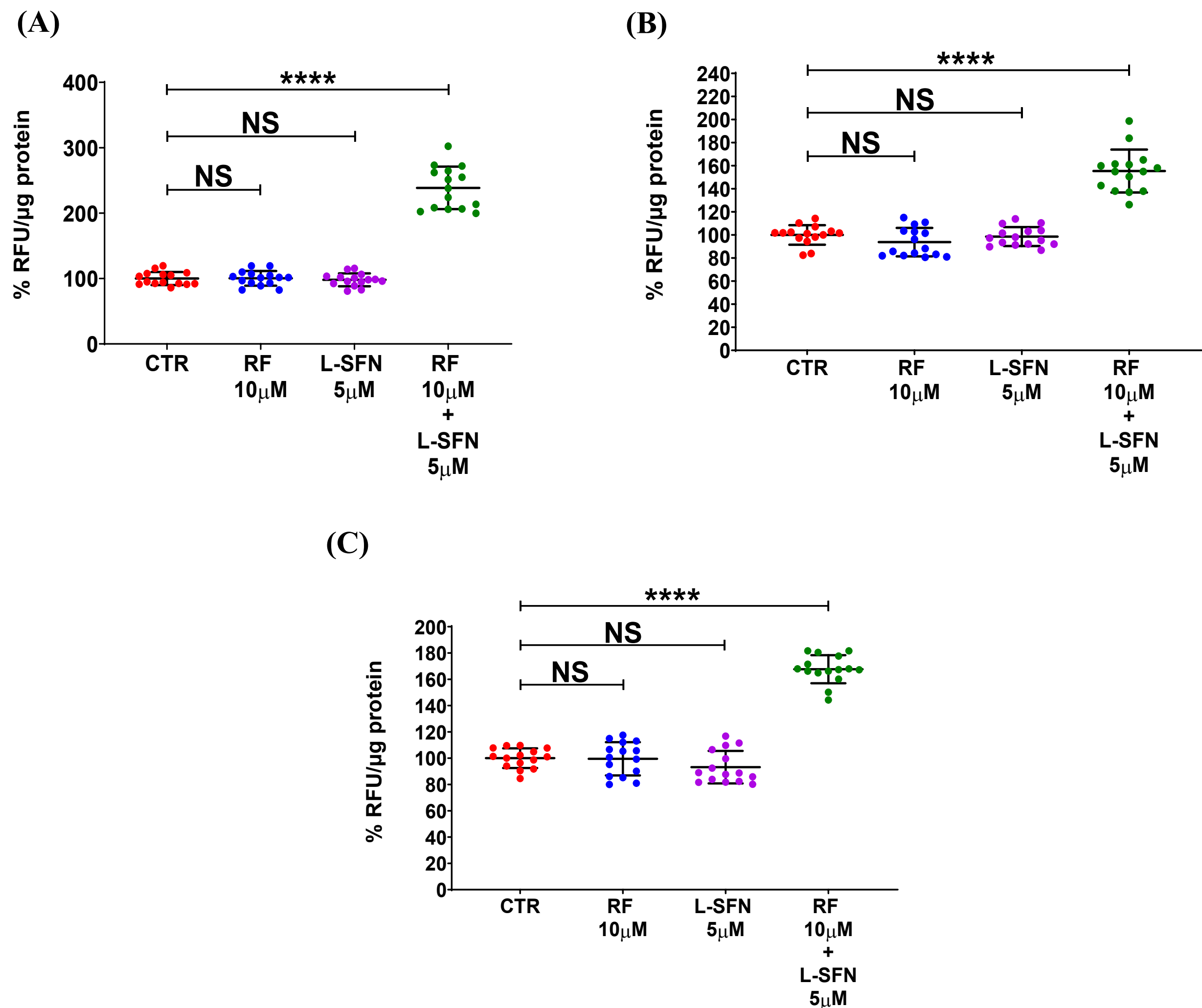
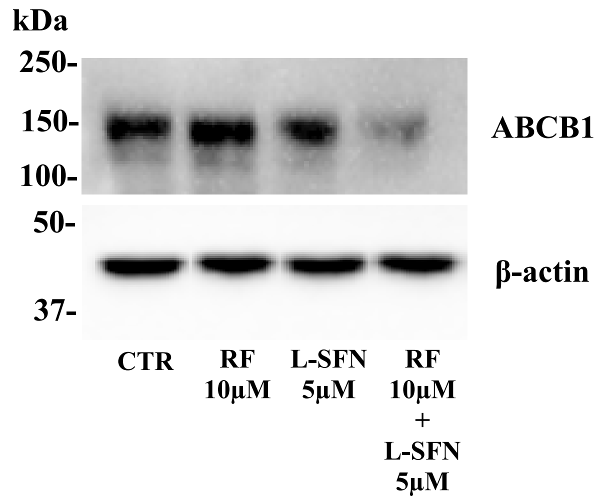


Figure 4.2: Effects of short-term exposure to PXR agonist (rifampicin, RF) and antagonist (L-sulforaphane, L-SFN) on ABCB1, ABCG2 and ABCC5 functional activity in PBECs.

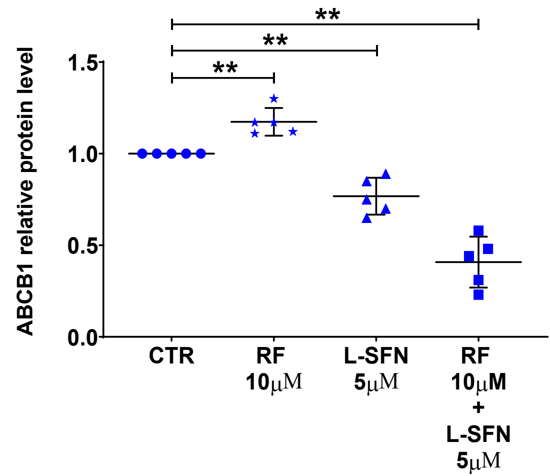
Intracellular accumulation of (A) calcein - ABCB1 substrate, (B) Hoechst 33342 - ABCG2 substrate, (C) GS–MF - ABCC5 substrate were measured in control cells and cells treated 10 µM RF and 5 µM L-SFN for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant. The concentrations used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.2).

The regulatory role of PXR on the functional activity of ABCB1, ABCG2 and ABCC5 transporters was confirmed by Western blotting. Western blotting (Figure 4.3 A - C) and densitometric analysis (Figure 4.3 G-I) revealed expression of ABCB1 (Figure 4.3 A, D), ABCG2 (Figure 4.3 B, E) and ABCC5 (Figure 4.3 E, F) transporters were significantly upregulated ($p < 0.01$) following treatment of PBECs with rifampicin by 17.4 % ± 7.6 %, 36.4 % ± 19.9 % and 50.6 % ± 16.6 % respectively. Treatment with L-sulforaphane alone significantly downregulated ($p < 0.01$) the expression of ABCB1 and ABCC5 by approximately 25 %, whilst ABCG2 expression was not significantly affected. Furthermore, the rifampicin-mediated upregulation of protein expression for all the three transporters was significantly reduced ($p < 0.01$) by 59.2 % ± 13.9 % (ABCB1), 67.4 % ± 17.3 % (ABCG2) and 77 % ± 13.9 % (ABCC5) when PBECs were co-treated with rifampicin (PXR agonist) and L-sulforaphane (PXR antagonist).

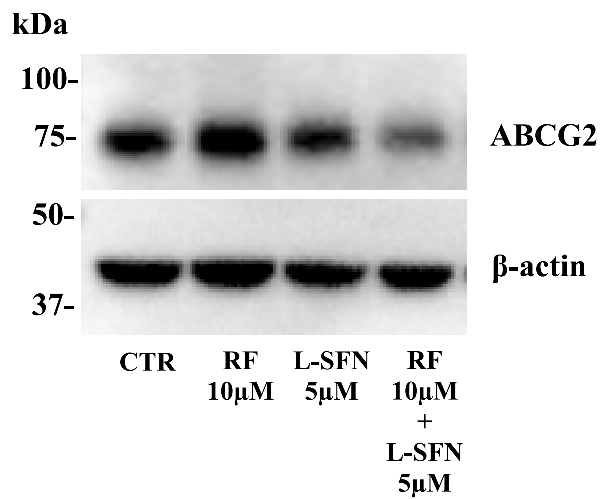
(A)



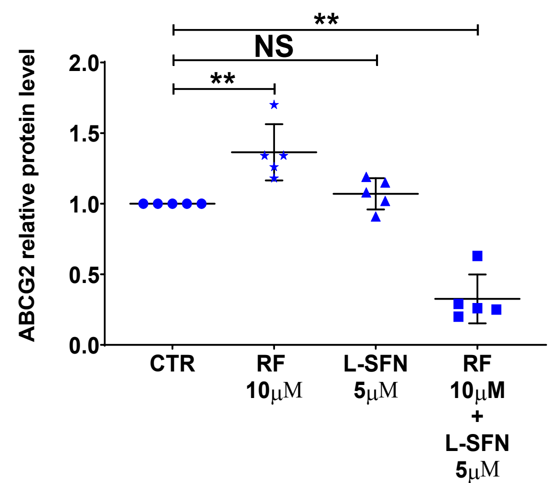
(D)



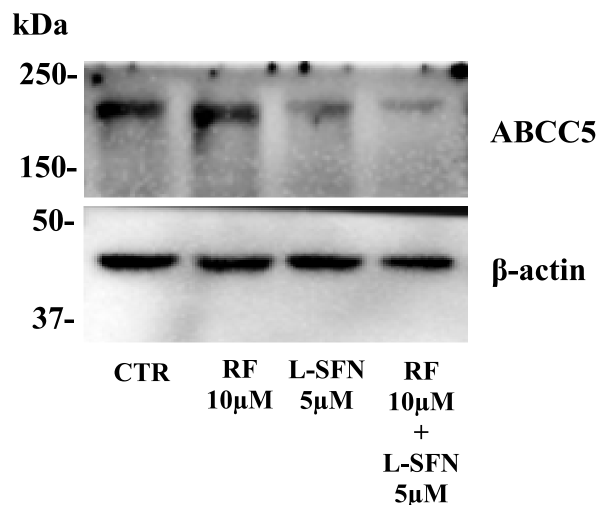
(B)



(E)



(C)



(F)

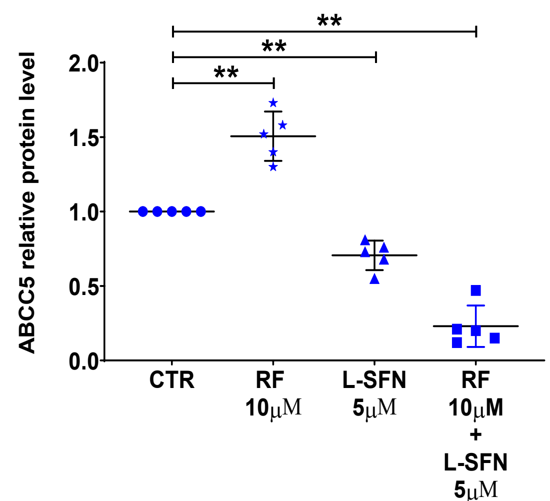


Figure 4.3: Effects of PXR agonist (rifampicin, RF) and antagonist (L-sulforaphane, L-SFN) on ABCB1, ABCG2 and ABCC5 protein expression in PBECs.
Cells were treated with 10 µM RF and 5 µM L-SFN as single treatment or co-treatment for 24 h. Panel (A), (B) and (C) are representative Western blot images of ABCB1, ABCG2 and ABCC5 respectively. Panel (D), (E) and (F) are densitometric quantification of the relative protein expression levels of ABCB1, ABCG2 and ABCC5 respectively. Densitometric data are normalised to β-actin and are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. **: $P < 0.01$, NS: non-significant.

4.1.2.2 Effects of PXR agonist and antagonist on PXR and RXR protein expression

Thus far, no studies report the effect of PXR agonist, rifampicin (RF), and antagonist, L-sulforaphane (L-SFN), on the expression of PXR and RXR at protein level. Therefore, Western blotting studies were carried out to determine the effect of rifampicin and L-sulforaphane on PXR and RXR protein expression in PBECs. Exposure to either rifampicin (10 µM) or L-sulforaphane (5 µM), or co-treatment with both compounds for 24 h did not significantly modify the protein expression of PXR (Figure 4.4 A, C) and RXR (Figure 4.4 B, D) compared to non-treated control cells.

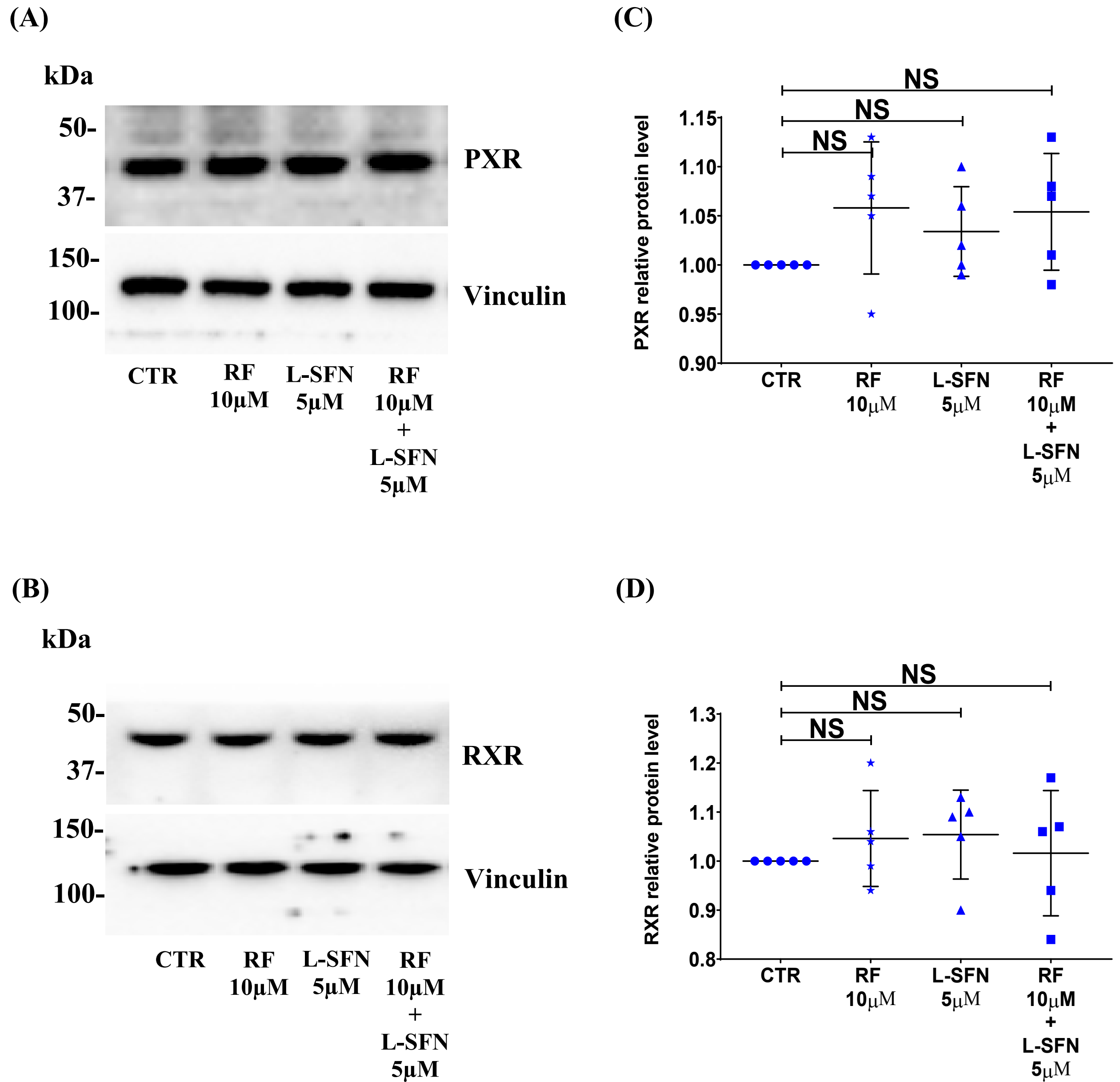
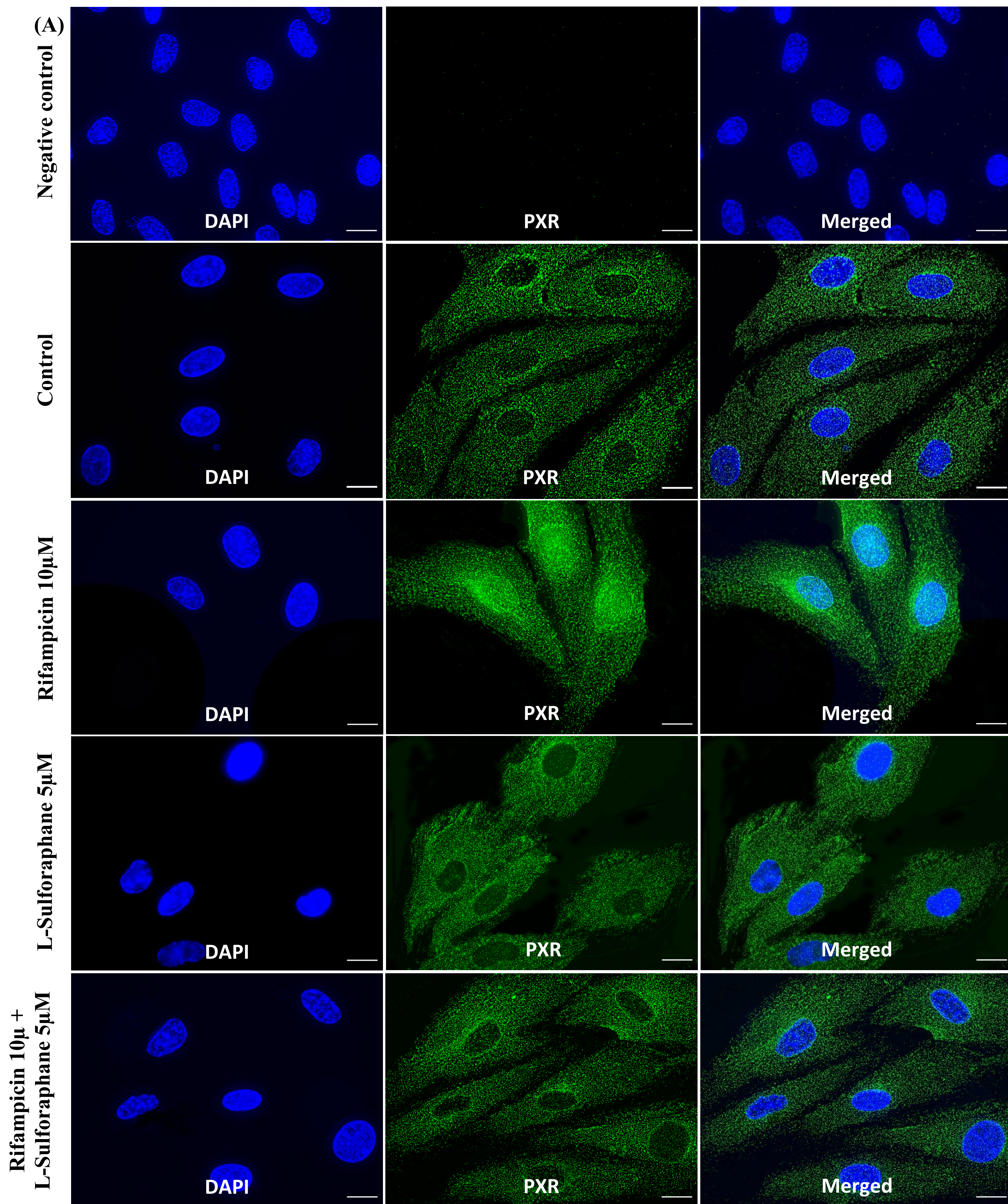


Figure 4.4: Effects of PXR agonist (rifampicin, RF) and antagonist (L-sulforaphane, L-SFN) on PXR and RXR protein expression in PBECs.

Cells were treated with 10 µM RF, 5 µM L-SFN and their co-treatments for 24 h. Panel (A) and (B) are representative Western blot images of PXR and RXR respectively. Panel (D) and (E) are densitometric quantification of the relative protein expression levels of PXR and RXR respectively. Densitometric data are normalised to vinculin and are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. CTR: control, NS: non-significant.

4.1.2.3 Effects of PXR agonist and antagonist on PXR protein localisation

Immunocytochemistry studies were carried out to determine subcellular distribution of PXR. The localisation of PXR fluorescent signal (green) is predominantly in the cytoplasm, rather than the nucleus, in the control condition (Figure 4.5 A). Exposure to 10 µM rifampicin for 24 h caused a dramatic increase in the fluorescent intensity associated with the nucleus, indicating the translocation of PXR from cytoplasm into nucleus. However, the rifampicin-mediated PXR nuclear translocation was completely abrogated by the co-treatment with 5 µM L-sulforaphane for 24 h. The negative control revealed no apparent non-specific binding by the secondary antibody. In order to determine whether there were any quantitative differences between PXR translocation induced by rifampicin and L-sulforaphane, quantitative immunofluorescence analysis was performed in blindly selected regions from the cytoplasm and nucleus using digital image analysis in 50 cells. The quantitative analysis in Figure 4.5 B confirmed that rifampicin caused a significant 4.0-fold increase ($p < 0.01$) in PXR nuclear to cytosolic fluorescent intensity compared to control cells. Co-incubation of rifampicin with L-sulforaphane counteracted the rifampicin-mediated nuclear translocation of PXR with the PXR nuclear to cytosolic fluorescent intensity level reverting to a similar level observed in the control condition. Treatment of PBECs with L-sulforaphane alone did not significantly alter the PXR nuclear to cytosolic fluorescent intensity.


(A)
Negative control
Control
Rifampicin 10µM
L-Sulforaphane 5µM
Rifampicin 10µ +
L-Sulforaphane 5µM
DAPI
PXR
Merged

`
(B)

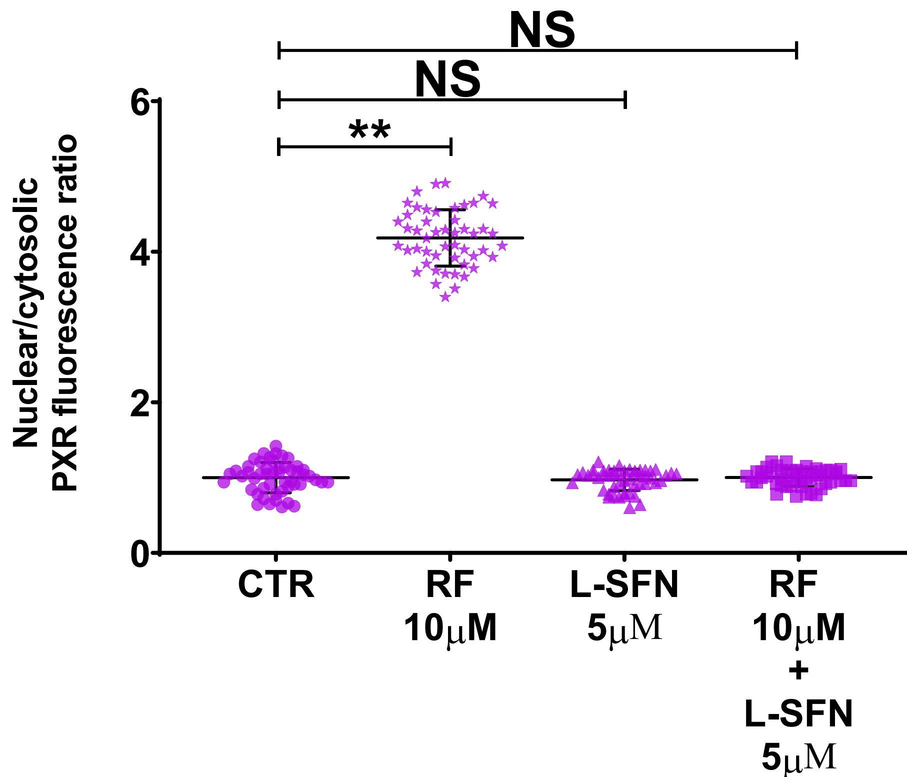


Figure 4.5: Effects of PXR agonist (rifampicin, RF) and antagonist (L-sulforaphane, L-SFN) on PXR activation/nuclear translocation in PBECs.

Cells were treated with 10 μM RF and 5 μM L-SFN as single treatment or co-treatment for 24 h. PXR protein was localised with anti-PXR polyclonal antibody (1:20) and Alexa Fluor® 188-conjugated secondary antibody (1:500). Nucleus was visualised with DAPI staining. For negative control, only the Alexa Fluor® 188-conjugated secondary antibody and DAPI staining were applied. (A) Representative deconvolution microscopy image of immunocytochemical localisation of PXR (green) and the nucleus (blue) in PBECs. (B) Quantitative analysis was performed in blindly selected regions of cytoplasm and nucleus, the average PXR fluorescence ratio (nuclear to cytosolic) normalised to control were obtained from five independent experiments. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD (n = 5). *: $P < 0.01$, NS: non- significant.

4.1.3 Constitutive androstane receptor (CAR) ligands

4.1.3.1 Effect of CAR agonist and antagonist on ABCB1, ABCG2 and ABCC5 transporter activities and protein expression

Treatment of PBECs with 5 µM CAR agonist, CITCO (CT), for 24 h significantly increased ($p < 0.0001$) the intracellular accumulation of calcein (ABCB1substrate) (Figure 4.6 A), Hoechst 33342 (ABCG2 substrate) (Figure 4.6 B) and GS–MF (ABCC5 substrate) (Figure 4.6 C) by over 6.8-fold, 1.8-fold and 2.4-fold respectively. In order to assess whether the changes in transporter activities are caused by CAR activation, meclizine (MC), a CAR inverse agonist was used. Treatment of PBECs with 10 µM meclizine alone significantly increased ($p < 0.0001$) the intracellular accumulation of calcein, Hoechst 33342 and GS–MF by over 5.0-fold, 1.5-fold and 2.1-fold respectively. Surprisingly, co-incubation with CITCO and meclizine significantly reduced ($p < 0.0001$) ABCB1, ABCG2 and ABCC5 activities as shown by increased accumulation of calcein, Hoechst 33342 and GS–MF of 53-fold, 8.4-fold and 11-fold respectively. None of the treatments applied in this study significantly reduced cell viability as determined by neutral red assay (Appendix D.3).

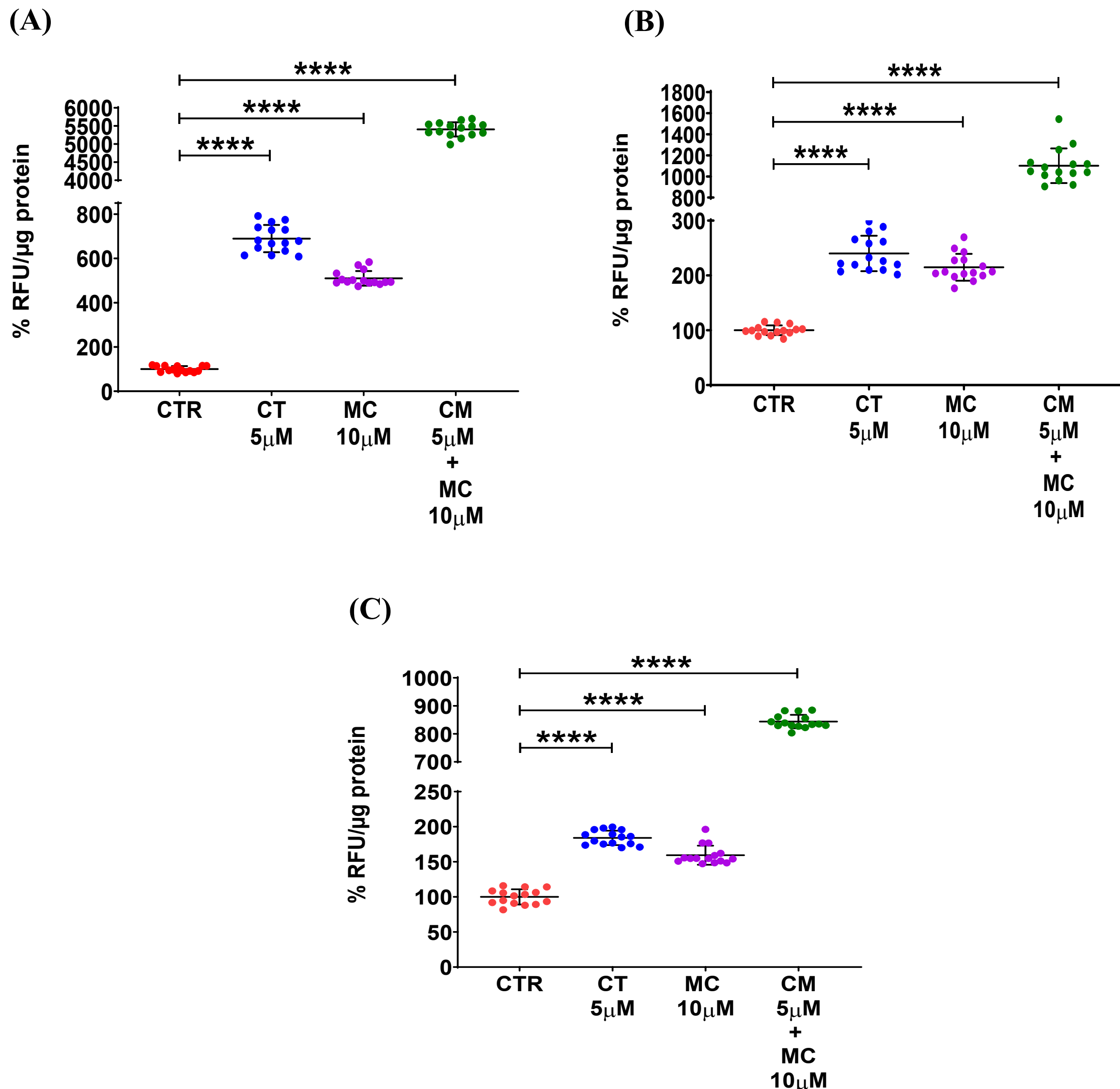


Figure 4.6: Effects of CAR agonist (CITCO, CT) and inverse agonist (meclizine, MC) on ABCB1, ABCG2 and ABCC5 functional activity in PBECs.
Intracellular accumulation of (A) calcein - ABCB1 substrate, (B) Hoechst 33342 - ABCG2 substrate, (C) GS–MF - ABCC5 substrate were measured in control cells and cells treated with 5 µM CT and 10 µM MC as single treatment or co-treatment for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$. The concentrations used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.3).

In order to determine whether the outcome presented after CITCO treatment is related to direct interaction with ABCB1, i.e. inhibition of ABCB1, ABCG2 and ABCC5 activity, a new experiment was conducted by reducing the 24 h incubation time to 30 min. Under these

experimental conditions exposure to 5 μM CITCO resulted in a significant 2.3-fold increase ($P < 0.0001$) in the intracellular accumulation of calcein (Figure 4.7 A), and over 1.4-fold increase in intracellular accumulation of Hoechst 33342 (Figure 4.7 B) and GS-MF (Figure 4.7 C). Treatment of 10 μM meclizine resulted in significant 2.8-fold increase ($P < 0.0001$) in intracellular accumulation of calcein (Figure 4.7 A), and a significant over 1.3 and 1.4-fold increase ($P < 0.0001$) in intracellular accumulation of Hoechst 33342 (Figure 4.7 B) and GS-MF (Figure 4.7 C) respectively. Interestingly, co-treatment with CITCO and meclizine significantly enhanced ($p < 0.0001$) the accumulation of calcein by 10.5-fold, and Hoechst 33342 and GS–MF by over 2.0-fold.

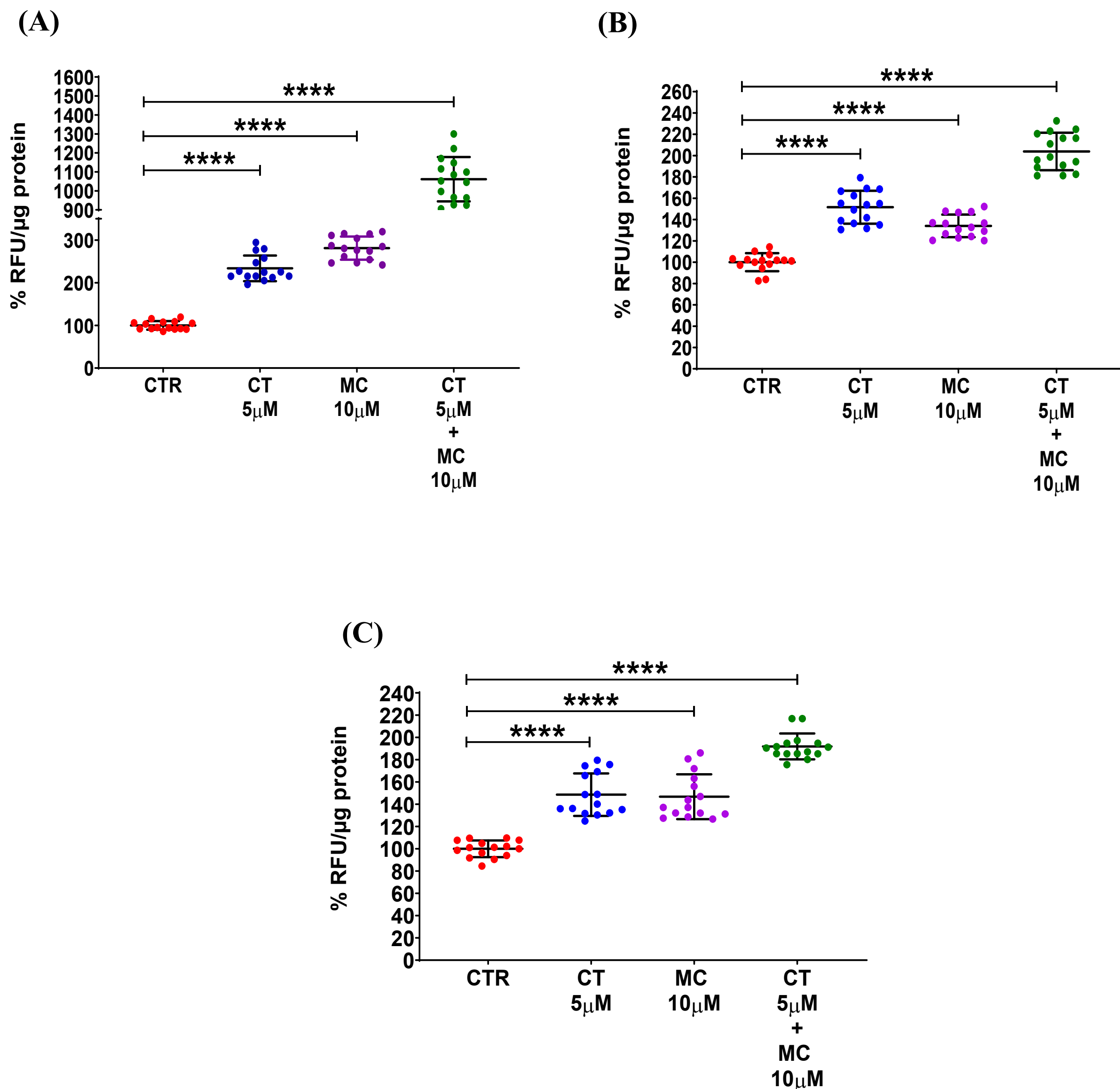
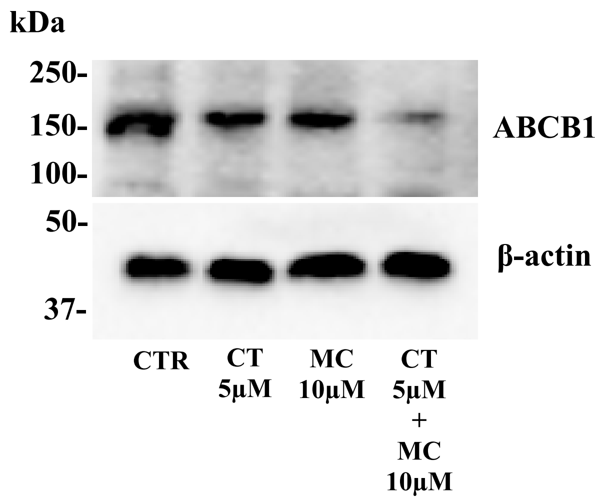


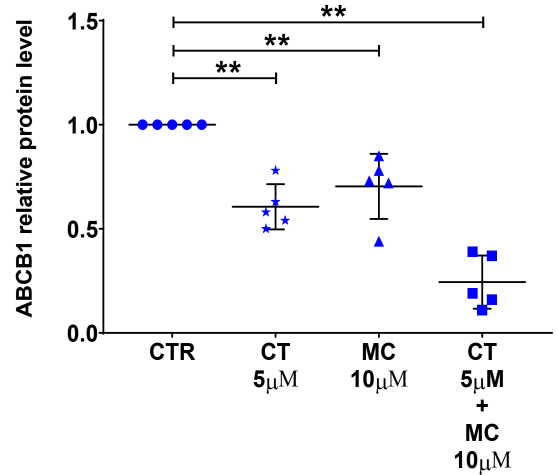
Figure 4.7: Effects of short-term exposure to CAR agonist (CITCO, CT) and inverse agonist (meclizine, MC) on ABCB1, ABCG2 and ABCC5 functional activity in PBECs. Intracellular accumulation of (A) calcein - ABCB1 substrate, (B) Hoechst 33342 - ABCG2 substrate, (C) GS–MF - ABCC5 substrate were measured in control cells and cells treated with 5 µM CT and 10 µM MC as single treatment or co-treatment for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of at least five independent experiments, with at three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$. The concentrations used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.3).

To confirm the regulatory role of CAR on ABCB1, ABCG2 and ABCC5 transporters, Western blotting of whole cell lysates (Figure 4.8 D - F) followed by densitometric analysis (Figure 4.8 G - I) confirmed the significant downregulation ($p < 0.01$) of ABCB1 (Figure 4.8 A, D), ABCG2 (Figure 4.8 B, E) and ABCC5 (Figure 4.8 C, F) protein expression by 39.4 % ± 10.9 %, 17.4 % ± 4.4 % and 38.4 % ± 8.7 % respectively in PBECs treated with CITCO and 29.6 % ± 15.6 %, 25.8 % ± 12 % and 42 % ± 13.7 % respectively in PBECs treated with meclizine (Figure 4.8 B, C). Interestingly, with the co-incubation of CITCO and meclizine, the protein expression for ABCB1, ABCG2 and ABCC5 were dramatically reduced in a significant manner ($p < 0.01$) by 75.6 % ± 12.8 %, 58.8 % ± 6.2 % and 64.6 % ± 5.4 % respectively.

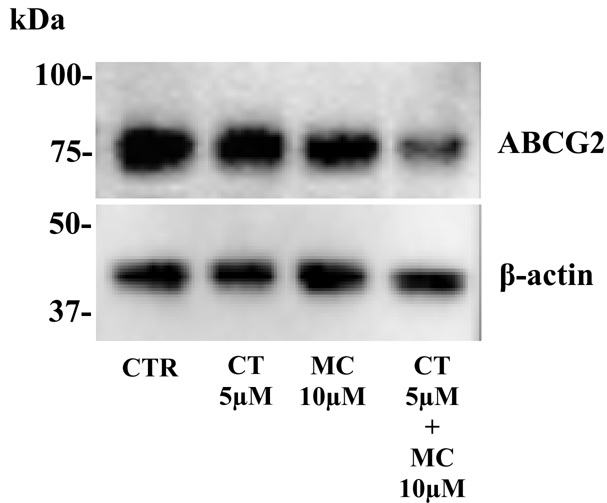
(A)



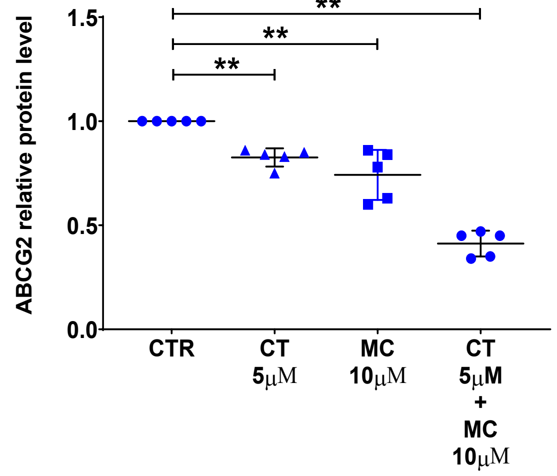
(D)



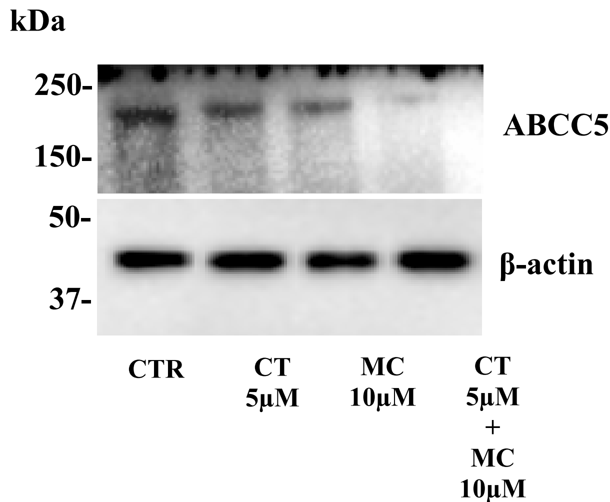
(B)



(E)



(C)



(F)

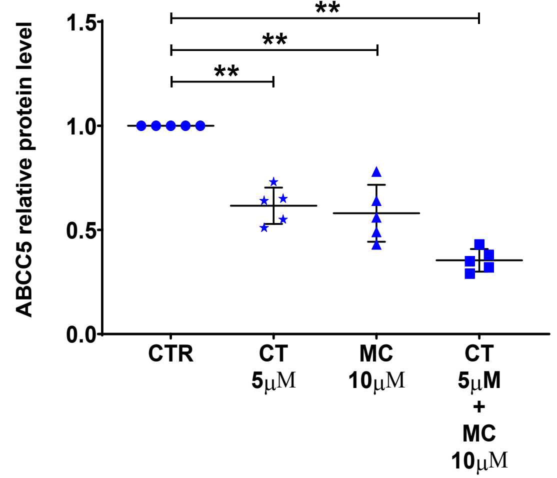


Figure 4.8: Effects of CAR agonist (CITCO, CT) and inverse agonist (meclizine, MC) on ABCB1, ABCG2 and ABCC5 protein expression in PBECs.
Cells were treated with 5 μM CT and 5 μM MC as single treatment or co-treatment for 24 h. Panel (A), (B) and (C) are representative Western blot images of ABCB1, ABCG2 and ABCC5 respectively. Panel (D), (E) and (F) are densitometric quantification of the relative protein expression levels of ABCB1, ABCG2 and ABCC5 respectively. Densitometric data are normalised to β-actin and are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. **: $P < 0.01$, NS: non-significant.

4.1.3.2 Effects of CAR agonist and inverse agonist on CAR and RXR protein expression

To date, no studies report the effect of CAR agonist, CITCO (CT), and inverse agonist, meclizine (MC), on the expression of CAR and RXR at the protein level. Hence, Western blotting studies were carried out to investigate the effect of CITCO and meclizine on CAR and RXR protein expression in PBECs. Treatment of cells with either CITCO (5 μM) or meclizine (10 μM), or co-incubation with both drugs for 24 h, did not significantly modify the expression of CAR (Figure 4.9 A, C) and RXR (Figure 4.9 B, D) compared to non-treated control cells.

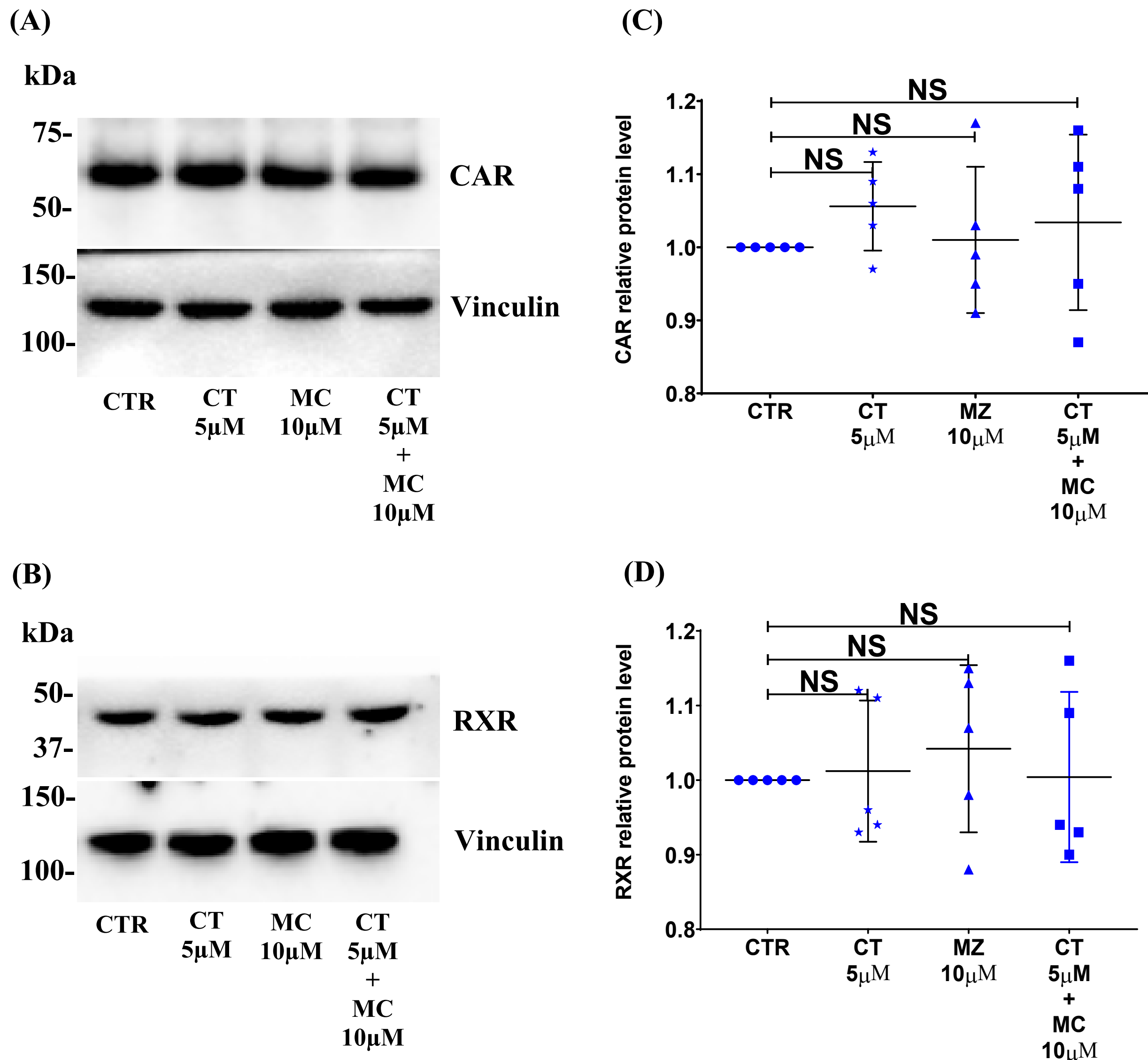


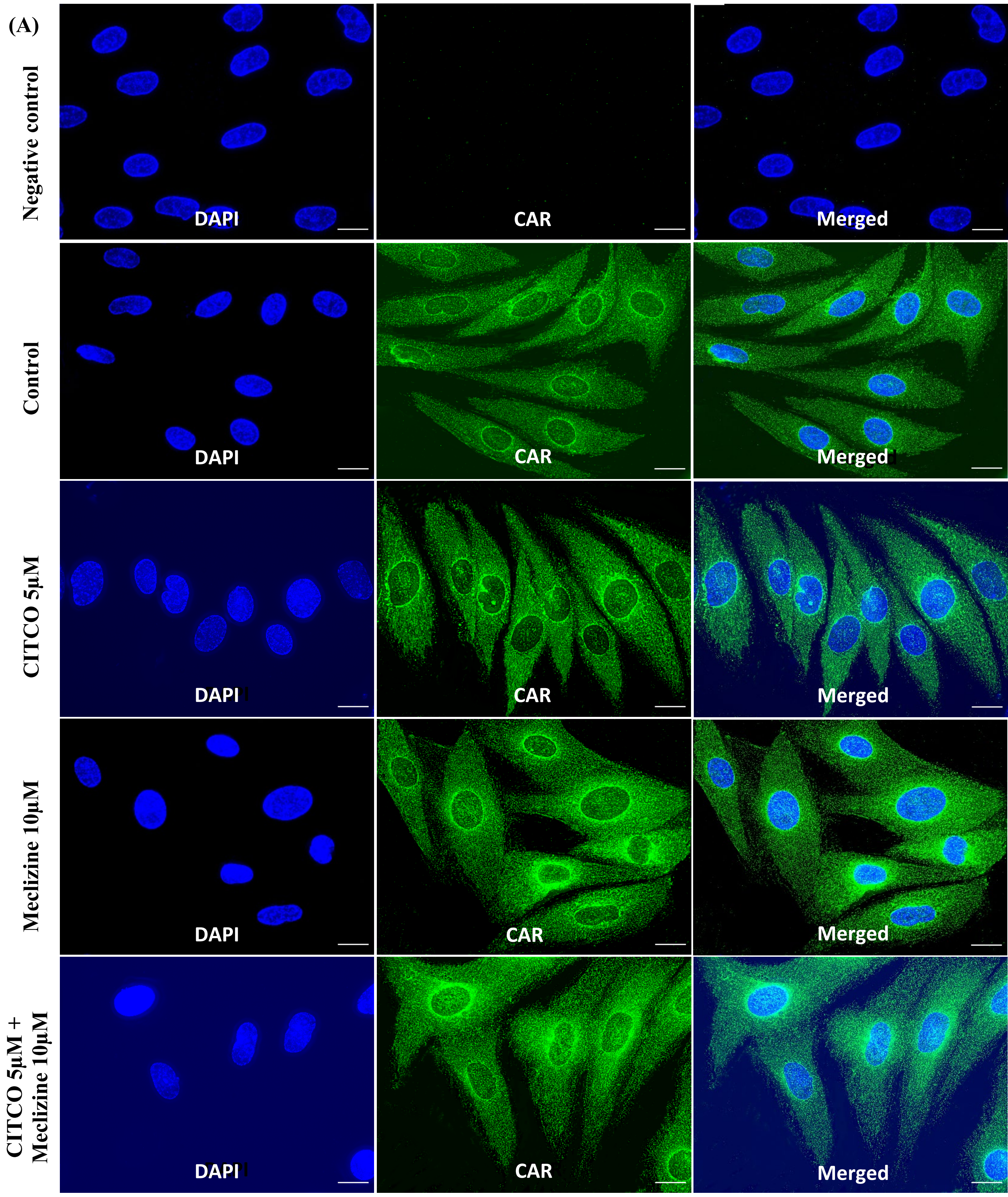
Figure 4.9: Effects of CAR agonist (CITCO, CT) and inverse agonist (meclizine, MC) on CAR and RXR protein expression in PBECs.

Cells were treated with 5 µM CT and 10 µM MC as single treatment or co-treatment for 24 h. Panel (A) and (B) are representative Western blot images of PXR and RXR respectively. Panel (D) and (E) are densitometric quantification of the relative protein expression levels of PXR and RXR respectively. Densitometric data are normalised to vinculin and are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. CTR: control, NS: non-significant.

4.1.3.3 Effects of CAR agonist and inverse agonist on CAR protein localisation

Immunocytochemistry studies were carried out to examine the subcellular distribution of CAR. The CAR fluorescent signal (green) was widely distributed in both nucleus and cytoplasm in the non-treated control condition (Figure 4.10 A). Exposure to either 5 µM

CITCO or 10 μM meclizine, or co-treatment of both compounds for 24 h did not alter this pattern. The negative control revealed no apparent non-specific binding by the secondary antibody. In order to determine whether there were any quantitative differences between control and treated cells, quantitative immunofluorescence analysis (Figure 4.10 B) was performed in blindly selected regions from the cytoplasm and nucleus using digital image analysis in 50 cells. The quantitative analysis confirmed that no significant differences were found in the CAR nuclear to cytosolic fluorescent intensity ratio between CITCO and/or meclizine-treated cells and non-treated control cells.


(A)
Negative control
DAPI
CAR
Merged
Control
DAPI
CAR
Merged
CITCO 5µM
DAPI
CAR
Merged
Meclizine 10µM
DAPI
CAR
Merged
CITCO 5µM +
Meclizine 10µM
DAPI
CAR
Merged

(B)

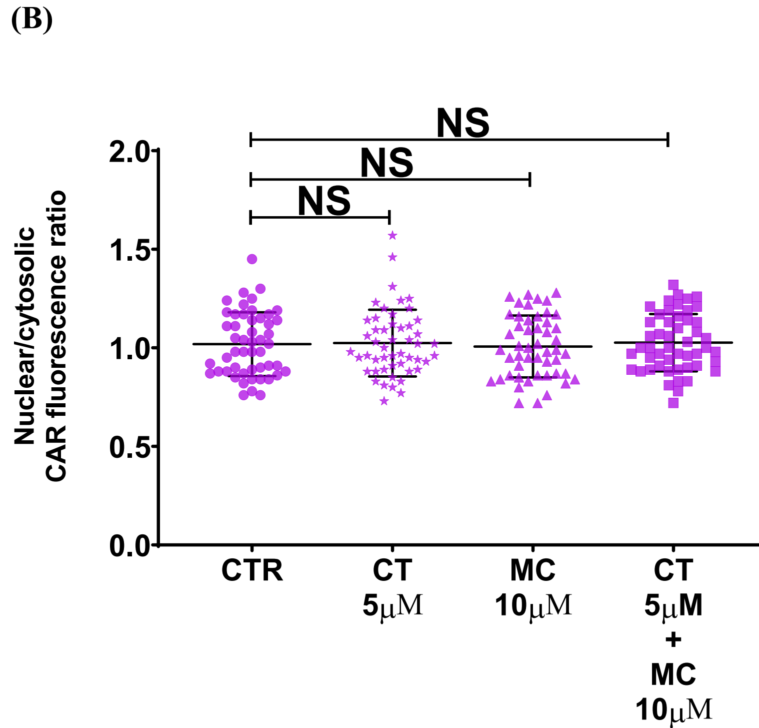


Figure 4.10: Effects of CAR agonist (CITCO, CT) and inverse agonist (meclizine, MC) on CAR activation/nuclear translocation in PBECs.

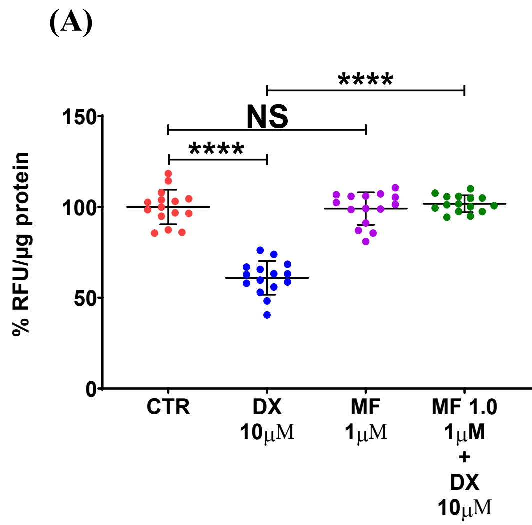
Cells were treated with 5 µM CT and 10 µM MC as single treatment or co-treatment for 24 h. CAR protein was localised with anti-CAR polyclonal antibody (1:20) and Alexa Fluor® 188-conjugated secondary antibody (1:500). Nucleus was visualised with DAPI staining. For negative control, only the Alexa Fluor® 188-conjugated secondary antibody and DAPI staining were applied. (A) Representative deconvolution microscopy image of immunocytochemical localisation of CAR (green) and the nucleus (blue) in PBECs. (B) Quantitative analysis was performed in blindly selected regions of cytoplasm and nucleus, the average CAR fluorescence ratio (nuclear to cytosolic) normalised to control were obtained from five independent experiments. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD (n = 5). *: $P < 0.01$, NS: non- significant.

4.1.4 Glucocorticoid receptor (GR) ligands

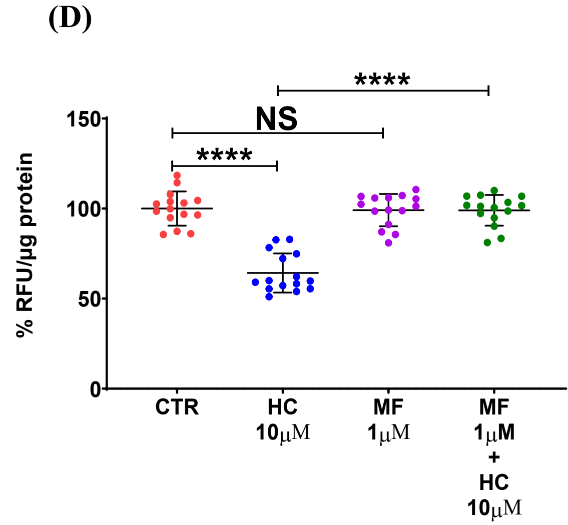
4.1.4.1 Effect of glucocorticoid receptor agonist and antagonist on ABCB1, ABCG2 & ABCC5 transporter activities and protein expression

In order to determine whether the GR is implicated in the regulation of ABC efflux transporter activity and expression, GR agonists dexamethasone (DX) and hydrocortisone (HC), and antagonist, mifepristone (MF) were used. PBECs treated with 10 μM of dexamethasone (Figure 4.11 A - C) and hydrocortisone (Figure 4.11 D - F) for 24 h displayed significantly decreased ($p < 0.0001$) intracellular accumulation of calcein (ABCB1substrate) (Figure 4.11 A, D), Hoechst 33342 (ABCG2 substrate) (Figure 4.11 B, E) and GS–MF (ABCC5 substrate) (Figure 4.11 C, F) by over 30 %, 20 % and 25 % respectively. However, in co-treatment studies, GR antagonist, mifepristone, was able to completely abrogate both the dexamethasone and hydrocortisone-mediated reduction in ABCB1, ABCG2 and ABCC5 transport activities, with activities of all three transporters reverting to control values. These findings suggest that the agonist-induced increase in ABCB1, ABCG2 and ABCC5 activities are mediated by GR activation. Treatment with mifepristone alone had no significant effect on the activity of all three transporters. The treatments used in these studies had no significant effect on cell viability, as demonstrated by the neutral red assay (Appendix D.4).

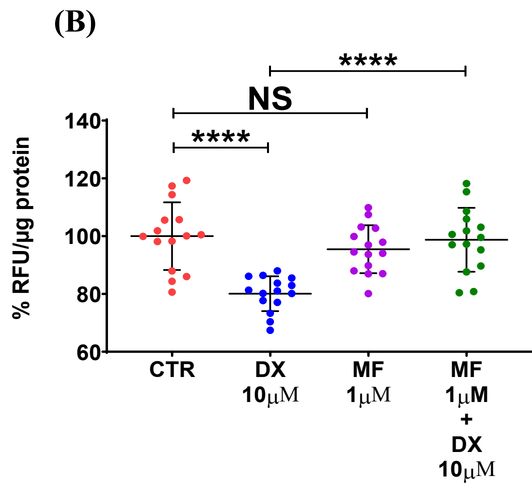
(A)



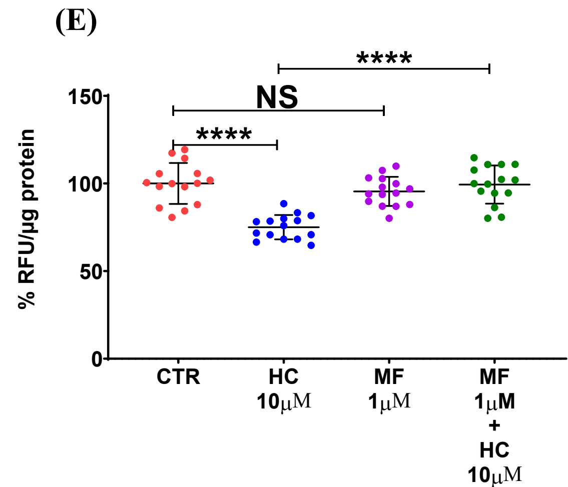
(D)



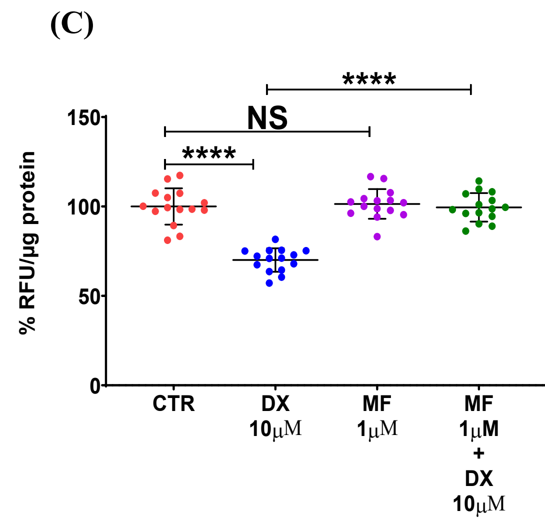
(B)



(E)



(C)



(F)

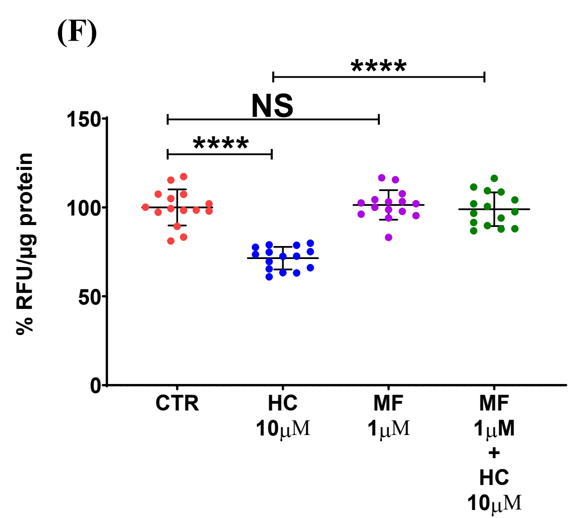
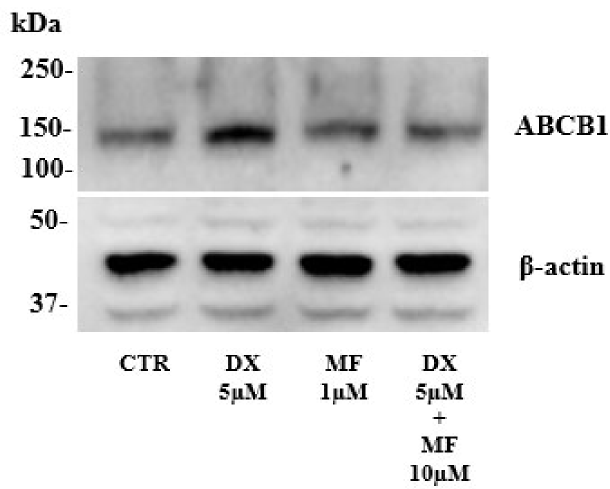
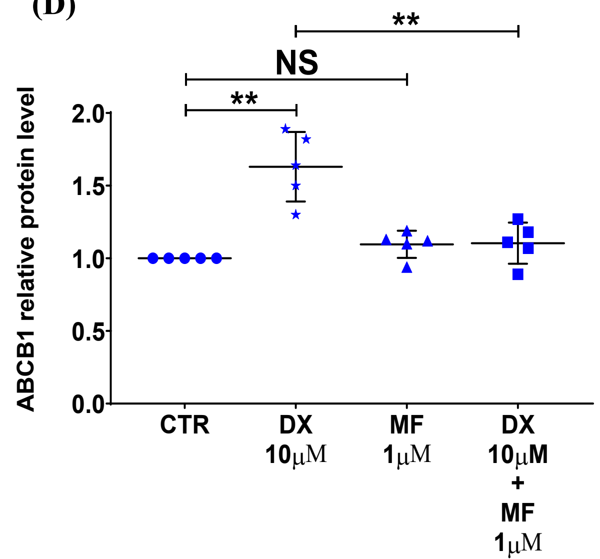


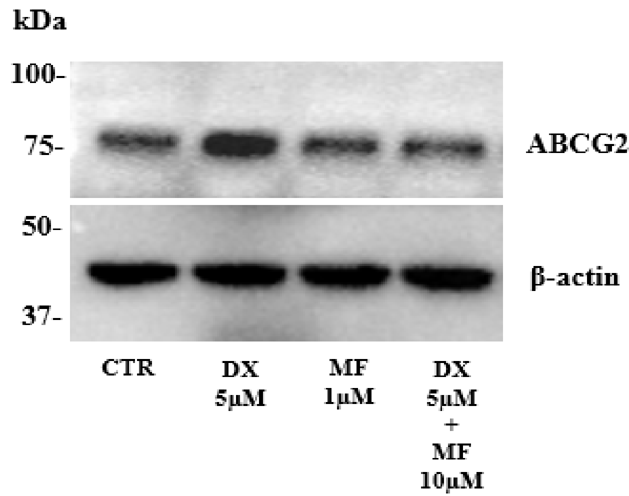
Figure 4.11: Effects of GR agonist (dexamethasone, DX and hydrocortisone, HC) and antagonist (mifepristone, MF) on ABCB1, ABCG2 and ABCC5 functional activity in PBECs.

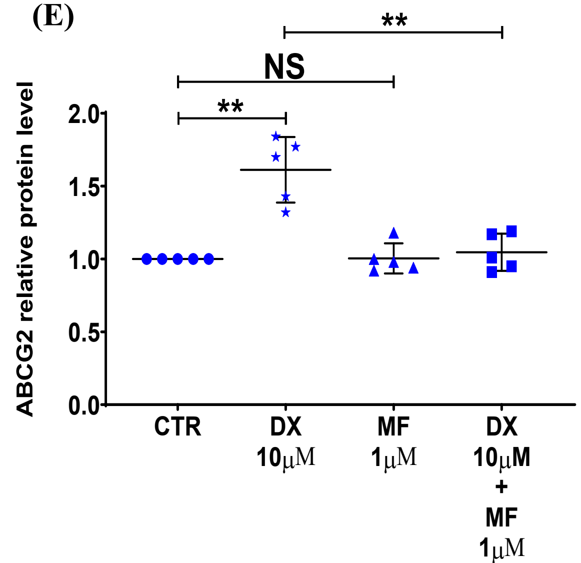
Intracellular accumulation of (A, D) calcein - ABCB1 substrate, (B, E) Hoechst 33342 - ABCG2 substrate, (C, F) GS–MF - ABCC5 substrate were measured in control cells and cells treated with either 10 µM DX or 10 µM HC as single treatment or co-treatment with 1 µM MF for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant. The concentrations used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.4).

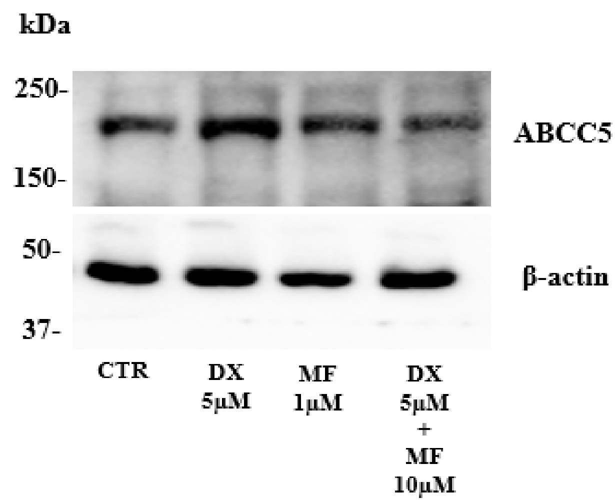
To determine whether the regulatory role of the GR on transporter activities was paralleled with changes in protein expression, Western blotting studies of whole cell lysates was performed. Western blotting (Figure 4.12 A - C) and densitometric analysis (Figure 4.12 D - F) showed the expression of the ABCB1 (Figure 4.10 A, D), ABCG2 (Figure 4.12 B, E) and ABCC5 (Figure 4.12 C, F) transporters were significantly upregulated ($p < 0.01$) by approximately 50 % following treatment of dexamethasone. Mifepristone alone did not modify the expression of any of the three transporters, but it significantly attenuated the upregulation induced by dexamethasone, reverting activities to a similar level to those observed in the control condition.


(A)
kDa
250-
150-
100-
50-
37-
ABCB1
β-actin
CTR
DX 5μM
MF 1μM
DX 5μM + MF 10μM


(D)
ABCB1 relative protein level
2.0
1.5
1.0
0.5
0.0
**
NS
**
CTR
DX 10μM
MF 1μM
DX 10μM + MF 1μM


(B)
kDa
100-
75-
50-
37-
ABCG2
β-actin
CTR
DX 5μM
MF 1μM
DX 5μM + MF 10μM


(E)
ABCG2 relative protein level
2.0
1.5
1.0
0.5
0.0
**
NS
**
CTR
DX 10μM
MF 1μM
DX 10μM + MF 1μM


(C)
kDa
250-
150-
50-
37-
ABCC5
β-actin
CTR
DX 5μM
MF 1μM
DX 5μM + MF 10μM

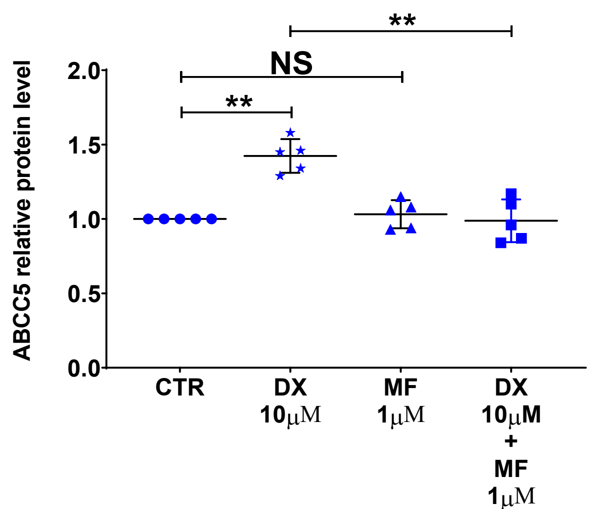
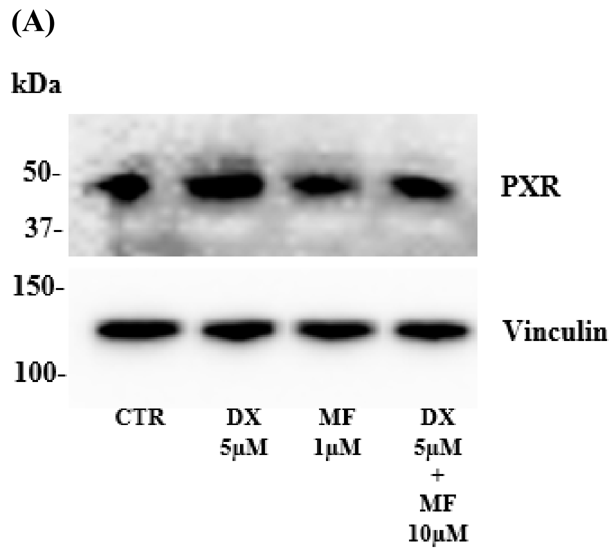
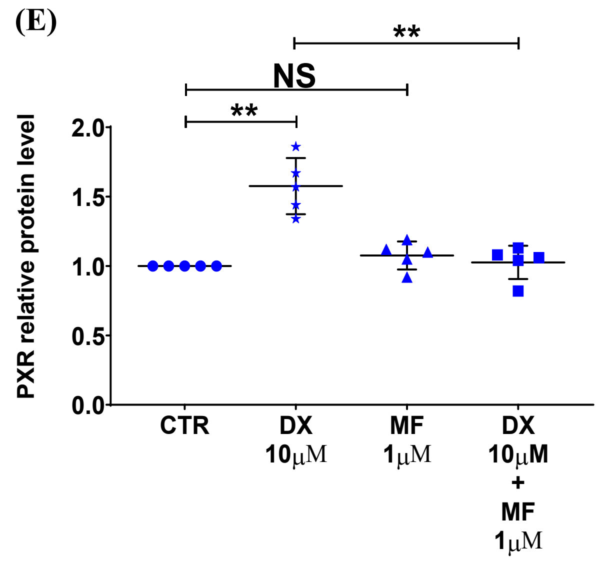

(F)
ABCC5 relative protein level
2.0
1.5
1.0
0.5
0.0
**
NS
**
CTR
DX 10μM
MF 1μM
DX 10μM + MF 1μM

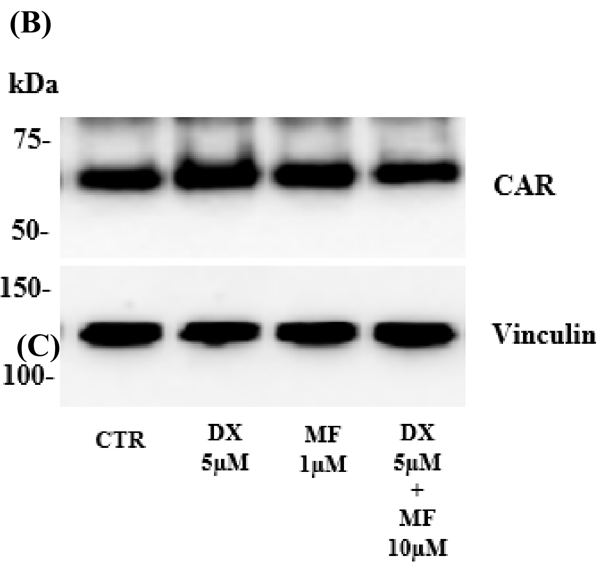
Figure 4.12: Effects of GR agonist (dexamethasone, DX) and antagonist (mifepristone, MF) on ABCB1, ABCG2 and ABCC5 protein expression in PBECs.
Cells were treated with 10 μM dexamethasone and 1 μM mifepristone (MF) as single treatment or co-treatment for 24 h. Panel (A), (B) and (C) are representative Western blot images of ABCB1, ABCG2 and ABCC5 respectively. Panel (D), (E) and (F) are densitometric quantification of the relative protein expression levels of ABCB1, ABCG2 and ABCC5 respectively. Densitometric data are normalised to β-actin and are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. CTR: control, **: $P < 0.01$, NS: non-significant.

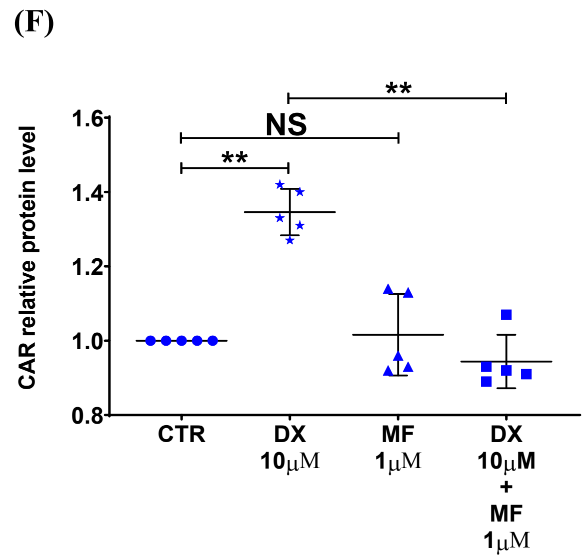
4.1.4.2 Effects of GR agonist and antagonist on PXR, CAR, RXR and GR protein expression

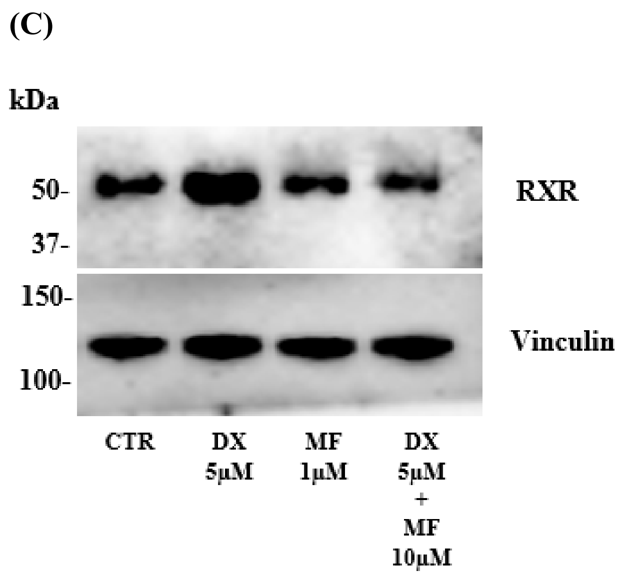
To assess the influence of the GR on nuclear receptor protein expression, Western blotting studies were carried out to determine the effect of GR agonist (dexamethasone) and antagonist (mifepristone) on the expression of PXR, CAR, RXR and GR in PBECs. Exposure to dexamethasone significantly upregulated ($p < 0.01$) the protein expression of PXR (Figure 4.13 A, E), CAR (Figure 4.13 B, F), RXR (Figure 4.13 C, G) and GR (Figure 4.13 D, H) nuclear receptors by 57.6 % ± 20.2 %, 34.6 % ± 6.3 %, 52.6 % ± 26.8 % and 40.0 % ± 15.5 % respectively compared to control cells. The expression of these four nuclear receptors was not affected by the treatment of mifepristone alone. However, co-treatment with dexamethasone and mifepristone completely attenuated the dexamethasone-induced protein expression for all four nuclear receptors.

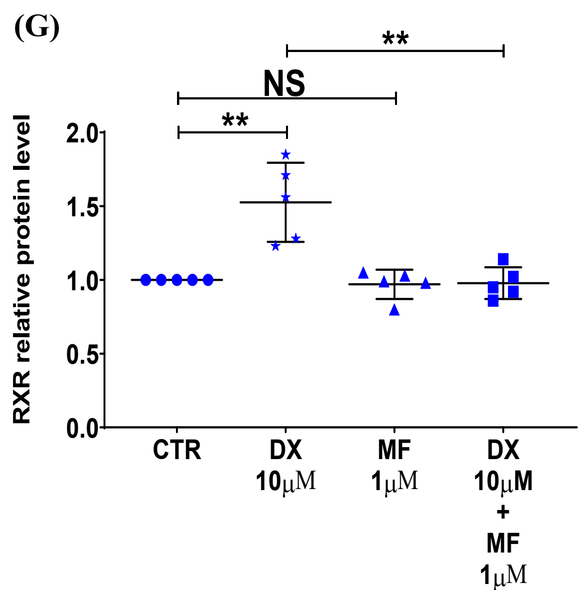

(A)
kDa
50-
37-
150-
100-
PXR
Vinculin
CTR
DX 5μM
MF 1μM
DX 5μM + MF 10μM


(E)
**
NS
**
PXR relative protein level
2.0
1.5
1.0
0.5
0.0
CTR
DX 10μM
MF 1μM
DX 10μM + MF 1μM


(B)
kDa
75-
50-
150-
100-
CAR
Vinculin
(C)
CTR
DX 5μM
MF 1μM
DX 5μM + MF 10μM


(F)
**
NS
**
CAR relative protein level
1.6
1.4
1.2
1.0
0.8
CTR
DX 10μM
MF 1μM
DX 10μM + MF 1μM


(C)
kDa
50-
37-
150-
100-
RXR
Vinculin
CTR
DX 5μM
MF 1μM
DX 5μM + MF 10μM


(G)
**
NS
**
RXR relative protein level
2.0
1.5
1.0
0.5
0.0
CTR
DX 10μM
MF 1μM
DX 10μM + MF 1μM

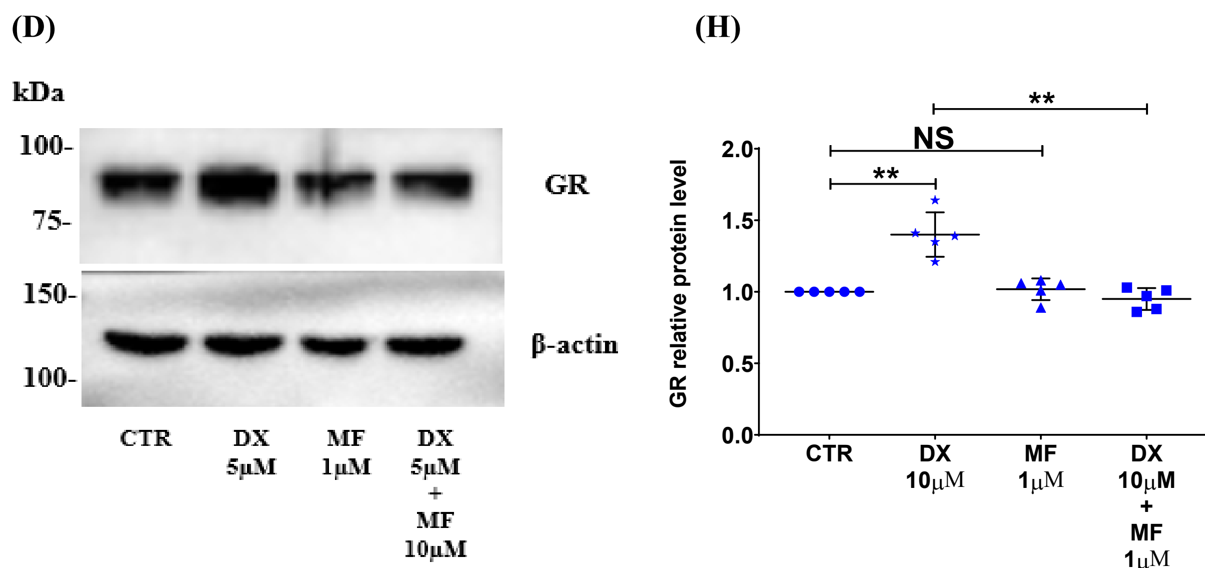


Figure 4.13: Effects of GR agonist (dexamethasone, DX) and antagonist (mifepristone, MF) on PXR, CAR, RXR and GR protein expression in PBECs.
Cells were treated with 10 µM dexamethasone and 1 µM mifepristone (MF) as single treatment or co-treatment for 24 h. Panel (A), (B), (C) and (D) are representative Western blot images of PXR, CAR, RXR and GR respectively. Panel (E), (F), (G) and (H) are densitometric quantification of the relative protein expression levels of PXR, CAR, RXR and GR respectively. PXR, CAR and RXR densitometric data are normalised to vinculin, while GR data is normalised to β-actin and all data are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. CTR: control, **: P < 0.01, NS: non-significant.

The findings contained in this chapter show, for the first time in many cases, the important roles major nuclear receptors have in regulating the expression and activity of key drug efflux transporters associated with blood-brain barrier endothelial cells. Importantly, these findings highlight the potential roles of PXR, CAR, RXR and GR in influencing drug transport, and consequently drug delivery, to the central nervous system.

4.1.5 Discussion

ABCB1 and ABCG2 are the two most prominent ABC efflux transporters that influence whole body pharmacokinetics and drug delivery to the CNS. Consequently, alteration of transporter activity and expression could significantly impact treatment of CNS disorders. Despite extensive research, there are still only extremely limited approaches available to overcome the blood–brain barrier and reach sufficiently high concentrations of chemotherapeutics inside the brain (Agarwal et al., 2011; Vogelgesang et al., 2011). The underlying mechanisms regulating ABC transporter expressions and activity are very well established in liver and intestine, but not in the blood–brain barrier (BBB), thus this thesis contributes to the understanding of interplay between different nuclear receptors and key ABC transporters at the BBB. In this study, we employed an *in vitro* model of the BBB, comprised of primary PBECs which have reported expression and function of ABCB1, ABCG2 and ABCC5 (Skinner et al., 2009; Cantrill et al., 2012; Torres-Vergara and Penny, 2018; Shubbar & Penny, 2018). In this thesis we incorporated the study of ABCC5 expression and activity, which is relatively understudied and yet the most highly expressed ABCC subfamily member in porcine (Warren et al., 2009; Shubbar & Penny, 2018) and bovine brain endothelial cells (Zhang et al., 2000), as well as the third most highly expressed ABC transporter after ABCB1 and ABCG2 in all human brain regions (Niles et al., 2004; Dauchy et al., 2008; Warren et al., 2009).

The nuclear receptors CAR and PXR, have been called "master xenobiotic sensors" because of their ability to recognise a wide array of endogenous metabolites and xenobiotics (Timsit and Negishi 2007). These two nuclear receptors, CAR and PXR, are potential regulators of ABC transporter expression, but their expression is reported to be influenced by the activation and inhibition of GR (Hunter et al., 2017) in liver. To date there is a lack of detailed knowledge about the specific events that regulate nuclear receptor expression and activity and their subsequent transcriptional modulation of target genes. This thesis reveals novel findings of the effect of ligands on the expression of PXR, CAR, RXR and GR at protein level in blood-brain barrier endothelial cells, which have not been reported elsewhere.

4.1.5.1 The effects of agonist and antagonist on the Pregnane X Receptor (PXR)

In this thesis, expression of PXR at the protein level has been confirmed in PBECs, which is consistent with other studies using the same model (Ott et al., 2009; Lemmen et al.,

2013a; Shubbar and Penny, 2018). In an attempt to study the influence of PXR on ABCB1, ABCG2 and ABCC5 transport activity and protein expression, PXR agonist, rifampicin (RF) was used. Rifampicin significantly induced both upregulation of ABCB1, ABCG2 and ABCC5 transport activity and protein expression, which is in agreement with the findings of Ott et al. (2009) and Lemmen et al. (2013a) who demonstrated rifampicin enhanced ABCB1 and ABCG2 transport activity and protein expression in PBECs. To confirm this enhancement is mediated by PXR activation, we employed L-sulforaphane (L-SFN), a specific PXR antagonist. Our experiments revealed that L-SFN not only attenuated the rifampicin-mediated increase in ABCB1, ABCG2 and ABCC5 transport activity and expression, but also significantly reduced endogenous transport activity and protein expression. Here we confirmed that the activity of PXR can be pharmacologically manipulated by a selective inhibitor to prevent ABC transporter induction. Our findings showed treatment with L-SFN alone weakly, but significantly, reduced ABCB1 and ABCC5, but not ABCG2, endogenous transport activity and expression. In the event of co-treatment with rifampicin and L-SFN, there was a significantly amplified inhibitory effect of L-SFN on transporter activity and expression for ABCB1 ABCC5 and ABCG2; however, the possibility of rifampicin amplifying the inhibitory effect of L-SFN couldn’t be ruled out.

The findings of this thesis contradict those of Lemmen et al. (2013a), who demonstrated co-treatment with rifampicin and L-SFN partially abrogated the rifampicin-mediated increase in ABCB1 and ABCG2 protein expression. The 24 h of co-treatment of both rifampicin and L-SFN is served to measure the genotypic changes in PBECs, while in order to establish if rifampicin and L-SFN acted as substrate/inhibitor that directly interfere with transporter activity, as measured by the fluorescent substrate accumulation assay, PBECs were incubated for a short period of 30 min with either rifampicin, L-SFN or both. The findings show for the first time that neither rifampicin nor L-SFN alone acted as substrate/inhibitor, but rifampicin and L-SFN in combination were able to significantly decrease the activity of ABCB1 ABCC5 and ABCG2. Interestingly, this outcome demonstrated that L-SFN did not abrogate the agonist effect of rifampicin as reported in other studies, but both L-SFN and rifampicin showed unusual synergistic effect to not only exert direct inhibition on ABC transporter activity but reducing their protein expression levels.

In this case, it is not clear how rifampicin is able to switch from apparently have the characteristic of an agonist to an antagonist in the presence of L-SFN. However, it cannot be

considered as unexpected, since there is evidence of rifampicin being an ABCB1 inhibitor in other *in vitro* and *in vivo* models (Fardel et al., 1995, Zong and Pollack, 2003). Furthermore, a single-dose pharmacokinetic study performed in human has also reported the inhibitory effect of rifampicin (Oswald et al., 2006). Hence, rifampicin may act as a PXR inhibitor in co-treatment situations, however, further investigation needs to be carried out to unveil the underlying mechanism of rifampicin as an PXR inhibitor.

This thesis is also the first study to report the effect of rifampicin and L-SFN on ABCC5 at the level of transport activity and protein expression, with findings consistent with the effects of rifampicin and L-SFN on ABCB1. Thus far, many studies have reported the effect of rifampicin and L-SFN on ABCC5 at the mRNA expression level using different cell lines. The findings of our study corroborated those of Pfrunder et al. (2003) and Schrenk et al. (2001), who demonstrated rifampicin induced ABCC5 mRNA expression in Caco-2, TC-7 and HepG2 cells. The down-regulation of ABCC5 transport activity and protein expression in response to L-SFN or co-treatment of L-SFN and rifampicin in our studies contradict the findings of Jeong et al. (2015) and Udasin et al. (2016), who reported L-SFN had no effect on ABCC5 mRNA expression in human proximal tubular cells and primary keratinocytes. This apparent discrepancy may be explained by the fact that the studies used different cell-based models with cells derived from different tissues which may lead to differential responses when exposed to a specific ligand. Furthermore, even though ABCC5 mRNA is highly expressed in the brain, it has been difficult to detect at the protein level (Cooray et al., 2002; Scheffer et al., 2000; Robert et al., 2008), which may be due to inefficient translation resulting in low levels of the ABCC5 protein (Scheffer et al., 2000). Furthermore, levels of mRNA and protein expression do not always correlate because of post-translational regulatory mechanisms (Greenbaum et al., 2003).

Further investigations were carried out to discover the effect of PXR activation by PXR agonist (rifampicin) and antagonist (L-SFN) on protein expression and cellular localisation. Both rifampicin and L-SFN did not exert any effect on PXR expression. This finding confirms that regulation of PXR protein expression is independent of the PXR agonist rifampicin and antagonist L-SFN. Thus far, no study has reported the effect of rifampicin and L-SFN on PXR at the protein level, and the findings in this thesis are not consistent with those at the mRNA level carried out by Ott et al. (2009) and Lemmen et al. (2013a) who demonstrated rifampicin increased PXR mRNA in PBECs. However, our study is in line with other studies demonstrating rifampicin treatment did not alter hPXR mRNA levels in LS180, Caco-2 and

TC-7 cell lines (Pfrunder et al., 2003), nor in human hepatocytes (Pascussi et al., 2000a). There are several possible factors that may contribute to these apparent discrepancies, including the use of cells from different species, cell isolation procedures, culturing conditions and use of supplements for cell-based models, amongst others. Interestingly, the lack of detailed explanations for such discrepancies is noticeable in the Discussions of papers in the scientific literature. However, in saying this, the study of Messina et al., (2013) has reported differential actions of PXR ligands amongst different tissues.

It must also be noted that mRNA level is not always a true representation of protein expression. Furthermore, Maglich et al. (2002) reported that PXR is under the control of a complex autoregulatory mechanism. Thus, complex transcriptional, post-transcriptional, and/or translational mechanisms that are currently unknown could be involved in regulating PXR. The findings of this thesis suggest that the PXR agonist rifampicin and antagonist L-SFN are not necessarily involved in the regulation of PXR gene expression.

The immunocytochemistry findings in this thesis demonstrate a ligand-dependent nuclear translocation of PXR in rifampicin treated cells, confirming the findings of Ott et al. (2009) and Lemmen et al. (2013a), who demonstrated PXR activation and accumulation in the perinuclear region and the nucleus in PBECs treated with PXR agonist. The nuclear localisation of activated PXR suggests it has a role to play in the induction of ABC transporters via gene expression. Furthermore, the data in this thesis represent the first evidence that even though L-SFN alone did not affect PXR expression at the protein level nor nuclear translocation of the receptor, it abrogated rifampicin-induced PXR nuclear translocation. This phenomenon is expected as L-SFN has been reported to be a PXR antagonist that efficiently represses PXR activities in the mammalian two-hybrid assay (Zhou et al., 2007). The outcomes of our studies therefore support ligand-dependent PXR nuclear translocation in blood-brain barrier endothelial cells which has already been well studied in liver and intestine. Inactive PXR is generally bound to a protein complex comprised of Hsp90and CCRP in the cytosol (Squires et al., 2004), however, unlike CAR, which does not require direct ligand interaction for its activation, PXR must bind to a ligand in order to be released from the protein complex and translocate to the nucleus where it forms a heterodimer with RXR (Ihunnah et al., 2011). This heterodimer binds the respective DNA response elements of the target genes, initiating their transcription. However, the detailed mechanisms that govern the translocation of ligand-activated PXR from the cytosol to the nucleus are still not well understood. The precise

molecular mechanism of ligand-receptor binding of L-SFN and subsequent PXR inhibition are still elusive, particularly since a recent human clinical study suggested that L-SFN was not an effective antagonist of the PXR *in vivo* (Poulton et al., 2013).

In summary, in this thesis we provide evidence that PXR-induced increases in ABC transporter expression and activity are the result of PXR nuclear translocation and not modulation of PXR protein expression.

4.1.5.2 The effects of agonist and inverse agonist on the Constitutive androstane receptor (CAR)

Several reports, including studies in our laboratory, report CAR is expressed at the protein level in PBECs (Nannelli et al., 2010; Lemmen et al., 2013b; Shubbar and Penny, 2018). Furthermore, treatment with CITCO, a CAR agonist, has been reported to increase ABCB1 and ABCG2 transport activity and protein expression in hCMEC/D3 cells (Chan et al., 2011) and PBECs (Lemmen et al., 2013b). However, these findings were not observed in our study, instead, CITCO acted as a potent CAR inverse agonist, and significantly reduced the transport activity and protein expression of ABCB1, ABCG2 and ABCC5.

The inverse agonist nature of CITCO has also been reported previously whereby studies report that CITCO is one of the few ligands that demonstrates dual action upon binding to CAR, i.e. it can act as either agonist or inverse agonist, recruiting either coactivators or corepressors respectively (Jyrkkarinne et al., 2012; Lempiainen et al., 2005). One of the possible explanations of this dual role is that CAR has a large ligand-binding pocket that is able to recognise a wide spectrum of ligands. CITCO appears to be a small ligand, and the binding of this small ligand to the large ligand-binding pocket does not result in significant changes to its conformation (Lempiainen et al., 2005). As a result, CAR is able to interact with either co-activators or co-repressors, thus, the net effect of CITCO on CAR activity is likely to be dependent on the ratio between co-activator and co-repressor proteins within the cell which influences the conformation and activity state of CAR (Makinen et al., 2003; Lempiainen et al., 2005; Jyrkkarinne et al., 2012). It may be possible that the PBECs we used in this study contained a high ratio of co-repressors to co-activators thereby allowing CITCO to act as an inverse agonistic.

Similar to the findings obtained with PXR (above), co-treatment of PBECs with CITCO and meclizine induced a potent synergistic effect resulting in significant down-regulation of ABCB1, ABCG2 and ABCC5 activity and protein expression. This finding is in contrast to those of Chan et al. (2011) and Lemmen et al. (2013b), which demonstrate meclizine partially reduced CITCO-mediated increase in ABCB1 and ABCG2 activity and expression in hCMEC/D3 cells and PBECs. This is an expected finding from their studies, because CITCO was shown to be a potent CAR agonist whilst meclizine was shown to be a CAR inverse agonist, that alone had no individual effect on ABCB1 protein expression in hCMEC/D3 cells. In this thesis, treatment with meclizine alone significantly reduced the endogenous transport activity and protein expression of ABCB1, ABCG2 and ABCC5. The ability of meclizine to reduce the basal activity of CAR has been demonstrated by Huang et al. (2004) and Li et al. (2008), whereby meclizine served as a potent CAR inverse agonist that effectively reduced CAR transcriptional activity by approximately 50% in CAR reporter gene-transfected HepG2 cells. Cells with constitutive localisation of CAR in the nucleus tend to have high basal transcription activity, Moore et al. (2002). Hence, meclizine, an effective CAR inverse agonist may block the interaction between CAR and co-activator proteins and promote interaction of co-repressor binding, thereby inhibiting CAR transcription activity and reducing its overall basal transcription activity.

In this thesis, initial control studies were carried out to establish whether CITCO and meclizine potentially directly interact with the ABCB1, ABCG2 and ABCC5 transporters, thereby reducing transporter activity. PBECs were incubated for 30 min with either CITCO or meclizine or both. When PBECs were treated with either CITCO or meclizine, or both, for a short period of time, the compounds appeared to significantly inhibit transport activity of all three ABC transporters. This finding constitutes the first evidence of CITCO and meclizine directly impacting ABCB1, ABCG2 and ABCC5 transporter activities.

In this thesis CITCO and meclizine significantly reduced ABCC5 transporter activity and protein expression, consistent with the findings observed for ABCB1 and ABCG2. This thesis is the first to report the effects of CAR ligands CITCO and meclizine on ABCC5 and the first to report that ABCC5 is under the regulatory control of CAR in the BBB.

The findings of this thesis are however in line with those of Maher et al. (2006) that show the CAR ligands phenobarbital and polychlorinated biphenyl 99 significantly repressed

ABCC5 mRNA expression in rat hepatocytes *in vivo*. In contrast, Merrell et al. (2008) reported phenobarbital induced ABCC5 mRNA expression in rat hepatocytes *in vivo*, while Ambroziak et al., 2010 reported phenobarbital has no effect of on ABCC5 mRNA in immortalised rat brain endothelial cell line.

Discrepancies in findings in relation to the effects of CAR ligands on ABCC5 can be attributed to differences in cell/tissue types, methods of isolation and tissue handling. Interestingly, it is also claimed that ABCC5 expression/activity is regulated independently of nuclear receptor action in rat liver, and that regulation is not part of the coordinated regulation of drug metabolising enzymes and efflux transporters that is often observed with other ABC transporters (Maher et al., 2006). However, the detailed mechanism of the regulatory role of CAR on ABCC5 transporter activity and expression is relatively under-researched and further studies are required. Furthermore, post-transcriptional modifications, which include phosphorylation, acetylation, SUMOylation and ubiquitination, have been reported to significantly affect the functionality of nuclear receptors (Sugatani et al., 2012), and it is feasible these processes will influence CAR-mediated ABCC5 expression.

Based on the outcomes of fluorescent immunocytochemistry, CITCO and meclizine did not significantly affect CAR protein expression and nuclear translocation. This is consistent with the study of Chan et al. (2011) who, using hCMEC/D3 immortalised brain endothelial cells, demonstrated CITCO had no significant effect on CAR nuclear translocation, despite CITCO treatment significantly increasing CAR-mediated expression of the *ABCB1* target gene.

In contrast, other studies have demonstrated that in primary cells, inactive CAR is expressed in the cytosol, bound to the Hsp90/CCRP protein complex, in the same way to PXR, prior to ligand activation (Yoshinari et al., 2003, Kobayashi et al., 2003) and that CAR translocates into the nucleus upon activation in a ligand-dependent manner in mouse and human primary hepatocytes (Kawamoto et al., 1999; Kawamoto et al., 2000; Li et al., 2009) and in primary rat hepatocytes transfected with human CAR (Maglich et al., 2003). Studies have also reported that in immortalised cell lines, cellular factors, such as the Hsp90/CCRP protein complex that holds CAR in the cytoplasm, are missing or malfunctioning, therefore CAR is constitutively nuclear-localised and activated in these cells even in the absence of xenobiotic stimulation, in HepG2 (Kawamoto et al., 1999; Guo et al., 2007; Zelko et al., 2001), rat liver RL-34 (Kanno et al., 2005), Hep3B (Guo et al., 2007) and HEK293 cells (Zelko et al.,

2001). However, these studies only demonstrated distinct CAR localisation between primary and immortalised liver cells and not brain endothelial cells. Thus far, one study has reported constitutive CAR localisation in the nucleus in primary rat hepatocytes (Shizu et al., 2017). This provide evidence that constitutive CAR nuclear localisation is not limited to only immortalised cell lines.

Unlike PXR and other classical nuclear receptors, CAR activation and nuclear translocation is more complicated due largely to the fact that CAR can be activated by both direct ligand binding and/or by an indirect mechanism at multiple stages. For example, CITCO and the antimalarial agent artemisinin activate CAR through direct ligand binding whilst phenobarbital and bilirubin activate CAR through an indirect mechanism (Yamamoto et al., 2003; Simonsson et al., 2006; Merrell et al., 2008). Furthermore, following ligand activation of CAR, many studies observed that, nuclear translocation does not necessarily lead to activation of CAR-mediated target gene transcription, on the other hand, activation CAR-mediated target gene transcription does not necessarily rely on CAR nuclear translocation (Li & Wang, 2010; Zelko & Negishi. 2000; Sueyoshi and Negishi, 2001). Nuclear translocation of dephosphorylated CAR is the key process that activates transcription of target genes. However, CAR activation, and subsequent gene transcription, is tightly regulated in the nucleus and is rather complicated due to the fact that the receptor possesses many potential phosphorylation sites which may determine nuclear receptor function (Ohno et al., 2014; Negishi, 2017; Shizu et al., 2018; McMahon et al., 2019). Given the inverse agonistic nature of both CITCO and meclizine in this thesis, and the available findings thus far, it is reasonable to speculate that CAR is constitutively localised in nucleus and CITCO and meclizine serve as inverse agonists that actively supressed the endogenous transcription activity of CAR.

The role of the retinoid X receptor (RXR) in the regulation of nuclear receptor function is well established, however, surprisingly, studies on the influence of RXR on ABCB1, ABCG2 and ABCC5 expression are scarce. In this thesis, the expression of RXR at the protein level was unaffected by both PXR and CAR agonist and antagonist/inverse agonist. It is true that RXR only serves as a binding partner to both CAR and PXR in their active state. Once RXR binds to either CAR or PXR, it will stabilise the interaction of PXR/CAR to co-activator and initiate the transcription of target genes (Pettersson et al., 2008; Chen et al., 2010; Tian et al., 2018; Gwag et al., 2019). The data presented in this thesis also corroborate studies showing that the PXR activators clotrimazole and rifampicin do not affect RXR total protein expression

in primary human hepatocytes (Pascussi et al., 2000a). Similarly, treatment with the CAR agonist, Phenobarbital did not alter RXR expression at the protein level (Kawamoto et al., 1999). This is also the first study to demonstrate that RXR is unaffected by both PXR and CAR ligands in brain endothelial cells and, in its capacity as a nuclear receptor binding partner, it may have a rather indirect effect on ABC transporter activity and expression.

4.1.5.3 The effects of agonist and antagonist on the Glucocorticoid receptor (GR)

In the present study, the GR ligands hydrocortisone and dexamethasone significantly increased ABCB1, ABCG2 and ABCC5 transport activity and protein expression. To verify whether hydrocortisone and dexamethasone-induced upregulation of ABC transport activity is GR-dependent, a specific GR inhibitor, mifepristone was used. The hydrocortisone and dexamethasone-induced increase in transport activity was completely abolished by the concomitant treatment of PBECs with mifepristone. This signifies the involvement of GR in modulating ABC transport activity. This finding is consistent with studies reporting similar outcomes for both ABCB1 and ABCG2 transport activity in porcine (Drees et al., 2005; von Wedel-Parlow et al., 2009; Torres-Vergara and Penny, 2018) rat (Miller, 2015b; Narang et al., 2008) and primary guinea pig (Iqbal et al., 2011) brain endothelial cells. To confirm the regulatory effect of GR on ABC transporter activity, the effect of dexamethasone on ABCB1, ABCG2 and ABCC5 protein expression levels was determined. Treatment of PBECs with dexamethasone significantly increased ABCB1, ABCG2 and ABCC5 at the protein level, whilst co-treatment with dexamethasone and mifepristone completely abolished the dexamethasone-induced increase. This finding is consistent with those of Narang et al. (2008) and Miller et al. (2015b) that demonstrated dexamethasone significantly increased both protein expression and transporter activity of ABCB1 and ABCG2 in primary rat brain endothelial cells, while this effect was significantly reduced by co-treatment with mifepristone.

As was observed with ABCB1 and ABCG2, treatment of PBECs with hydrocortisone and dexamethasone significantly increased ABCC5 expression and activity, and this increase was abolished by co-treatment with mifepristone. Thus far, no study has reported the effect of hydrocortisone and dexamethasone at ABCC5 transport activity and protein expression levels. Among the ABCC family, hydrocortisone was reported to induce ABCC1, ABCC3 and ABCC4 transporter activity in PBECs (von Wedel-Parlow et al., 2009) and lung cancer cells (Pułaski et al., 2005). Manceau et al. (2012a) reported similar findings, showing the induction

effect of dexamethasone on ABCC5 mRNA expression in CCRF-CEM cells; conversely, other studies demonstrated dexamethasone had no effect on ABCC5 mRNA expression in HepG2 cells (Schrenk et al., 2001), rat hepatocyte *in vivo* (Maher et al., 2006), GPNT cells (immortalised rat brain endothelial cell line) (Ambroziak et al., 2010) and syncytiotrophoblasts (Manceau et al., 2012b). Such discrepancies may be attributable to the fact that the cells are derived from different sources. As mentioned previously, ABCC5 mRNA levels may not truly reflect ABCC5 protein expression levels, and previous studies have shown ABCB1 activity was not accurately reflected by ABCB1 mRNA levels (Haslam et al., 2008; Ott et al., 2009). Thus far, the complex transcriptional, post-transcriptional, and/or translational characteristics of ABCC5 remain elusive.

In order to examine whether regulation of ABC transporter expression and activity was mediated by GR directly, and exclusively, or indirectly via PXR, CAR and RXR, the effect of dexamethasone on GR, PXR CAR and RXR protein expression was analysed. In this thesis, dexamethasone significantly increased the expression of the GR, PXR CAR and RXR nuclear receptors at the protein level, however, co-treatment with mifepristone and dexamethasone completely abrogated the effect of dexamethasone. Mifepristone alone had no significant effect on expression of PXR, CAR and RXR, suggesting that the induction of PXR, CAR and RXR is mediated by the activation of GR. This study confirmed that the GR is able to regulate expression of PXR, CAR and RXR, which in turn regulates the activity and expression of ABC-transporters. The mechanism of GR-induced upregulation of PXR, CAR and RXR protein expression by dexamethasone has been widely reported in liver, especially in rat (Zhang, C. et al, 2012; Anglicheau et al., 2003) and human hepatocyte (Zhang et al, 2015; Pascussi et al., 2000a; Pascussi et al., 2000b) however only a limited number of studies, using mouse and rat brain endothelial cells, report GR-induced expression of PXR protein (Narang et al., 2008; Chan et al., 2013).

In the context of this thesis, it is noteworthy to point out that the nuclear receptors PXR, CAR and RXR were upregulated in parallel with ABCB1, ABCG2 and ABCC5. The dynamic effect of dexamethasone was attenuated by the GR antagonist mifepristone. This suggests that dexamethasone induces expression and activity of the efflux transporters through a direct interaction with GR. The precise mechanism by which GR positively regulates expression of both PXR and CAR is not well documented, but the most feasible explanation is that dexamethasone mediates increased expression of PXR and CAR through interaction with a

potential glucocorticoid response element (GRE) located in the promoter region of the genes encoding these two nuclear receptors. However, although Gerbal-Chaloin et al., 2002, Wang et al. (2003b) and Pascussi et al., (2003a) identified a GRE located in the promoter region of the human CAR gene, no consensus GRE has been identified in the PXR promoter region. Overall, the findings of our study are consistent with other findings demonstrating similar regulatory cascades involving GR-mediated regulation of PXR and CAR protein expression. Our findings are also consistent with those confirming dexamethasone-induced GR, PXR, CAR, CAR and RXR mRNA and protein expression in transfected CV-1 cells (Kliewer et al., 1998; Lehmann et al., 1998), human hepatocytes (Pascussi et al., 2000a, b, 2001), rat brain endothelial cell (Narang et al., 2008), human retinal pigment epithelial cell (Zhang, Y. et al., 2012), T lymphoblastoid cell and hepatoma cells (Juan et al., 2011; Manceau et al., 2013), and dexamethasone-induced transcriptional activity using a reporter gene assay in GR transgenic cells (Novotna and Dvorak, 2014; Korhonova et al., 2015).

In addition to the GR-PXR/CAR signalling pathway, Pascussi et al. (2000a; 2000b), Wang & Negishi (2005) and Narang et al. (2008) described low concentrations (≤ 0.1 µM) of dexamethasone activated the GR-PXR pathway; whereas, Pascussi et al. (2000a,b, 2001) and Huss & Kasper, (2000) found that higher concentrations (≥ 10 µM) directly activated PXR in hepatocytes, suggesting that dexamethasone is a potent PXR agonist. However, on the contrary, a few studies showed that dexamethasone, in the range of 10 µM, is either a weak (Lehmann et al., 1998; Moore et al. 2000) or not a PXR ligand (Zhang, Y. et al., 2012). A recent study, that utilised ligand-binding domain constructs of PXR from different species, demonstrated that dexamethasone was not able to significantly activate both human and porcine constructs, but that the steroid did activate the mouse counterpart (Kublbeck et al., 2015). In this thesis, 10 µM dexamethasone was used because this is the concentration that demonstrated significant induction of ABCB1, ABCG2 and ABCC5 transporter activities at 24 h. This is in comparison to Narang et al. (2008) who showed significant induction of ABCB1 and ABCG2 transporter activities at 0.25 µM at 24 h in PBECs. Furthermore, in our study, the use of a supramicromolar concentration of dexamethasone did not act as PXR agonist as mentioned by the above studies, because the co-treatment with mifepristone completely abrogated the dexamethasone-induced expression of ABCB1, ABCG2 and ABCC5 transporters, as well as PXR protein expression. However, it is important to reiterate that contradictory findings for the regulatory responses of transporters and nuclear receptors to corticosteroids, such as dexamethasone, may occur in a species-dependent and tissues-specific manner. It is well known that dexamethasone is not a

CAR ligand (Sueyoshi et al., 1999; Pascussi et al., 2001; Wang & Negishi, 2005; Qatanani et al., 2005). This has been confirmed in this thesis. However, it is intriguing that if dexamethasone is not acting as PXR and CAR agonist, the increased expression of PXR and CAR will not likely to induce target gene expression in the absence of ligands. This is also observed in the study of Pascussi et al., (2000a; 2001; Pascussi et al., 2003b) and Wang et al., 2003b who demonstrated PXR and CAR activated target gene expression (CYP3A4) in the absence of an agonist. There are several possible explanations for these outcomes. First, it is probable that PXR and CAR are able to activate transporter expression even in the absence of a specific agonist (basal expression), or a natural ligand is present in the culture medium or endogenous activator compounds synthesised in the cells to maintain PXR and CAR in an activated form. Secondly, GR may involve in the recruitment of specific PXR/CAR coactivator proteins or function as coactivator itself to facilitate direct CAR/RXR and PXR/RXR binding to the response elements of ABC transporter genes. This principle has been demonstrated by Wang et al. (2003b) and Dvorak et al., 2007 who reported that GR may function as a coactivator to facilitate direct CAR/RXR and PXR/RXR binding to a target gene expression of CYP2B6. In addition, Cooper et al. (2013) showed that GR may also recruit coactivators to the transcriptional assembly, thus promoting PXR stimulation of target gene transcription. Thirdly, GR may facilitate the translocation of PXR and CAR into nucleus. For example, Pascussi et al., (2000a,b) reported that in the presence of activated GR the nuclear accumulation of PXR and CAR in the absence of any exogenous xenobiotic/ligands increased. Lastly, GR is able to directly activate the transcription of transporter expression, provided the gene encoding the ABC transporter contains a GRE. Thus far, a GRE has been reported in the *abcb1* promoter in Chinese hamster lung cells and in the *ABCB1* promoter of the U2-OS human osteosarcoma cell-line (Egan and Scotto, 1996).

Similar to PXR and CAR, GR has been reported to bind to RXR to allow the transduction of specific signals in response to specific ligands (Kliewer et al., 1992; Thompson et al., 2001). In this study, RXR expression was significantly increased by GR ligand, dexamethasone, but not by PXR and CAR ligands. This signifies the unique pathway of GR in regulating RXR expression. This is also the first study to report the influence of GR ligand on RXR expression. However, based on the result, it is impossible to ensure that the increase in RXR expression would have significant effect on ABC expression and activity, since both PXR and CAR are concomitantly upregulated by GR ligand. Several studies reported that increased expression of RXR would lead to the enhancement of the transcriptional activity of PXR and

CAR (Pascussi et al., 2000a). Study also showed that the use of RXR ligand, bexarotene leads to upregulation of ABCB1 transporter expression (Kuntz et al., 2015), in contrast, deficient of RXR in transgenic mice demonstrated a decrease in the basal expression of target gene, cytochrome P450 (CYP3A) (Wan et al., 2000; Cai, 2002). Despite the importance of RXR in regulating the target gene expression of PXR, CAR and GR, there are still significant lack of clarification of how RXR ligands participate in regulating ABC transporter expression and activity.

Taken together, the results in this thesis suggest that more than one pathway may be involved in GR-mediated regulation ABC transporters, where GR works synergistically in the regulation of ABC transporters. However, functional cross-talk between the GR- and PXR-signalling pathways is speculative at the moment. Together these results provide evidence for the involvement of GR-PXR and GR-CAR pathways in the regulation of drug transporters at the BBB where GR, PXR, CAR and RXR are active. Further experiments are thus needed to explore the precise mechanisms by which dexamethasone induces ABC efflux transporter expression, both by the GR-PXR cascade and by other signalling pathways.

4.1.5.4 Limitations of the study

In this study the agonist rifampicin and inverse agonist CITCO exhibited dual functions as agonist and antagonist/inverse agonist. More specific agonist and antagonist/inverse agonist should be applied in the future to accurately determine regulatory function of nuclear receptors on ABC transporters. Furthermore, the antagonist and inverse agonist used in this study significantly downregulate the basal activity and expression of ABC transporters; in the future, it is worth considering what factors contributed to the high basal activity and expression of ABC transporters, this might be due to the effect of puromycin and penicillin/streptomycin in the culture medium.

Another limitation identified in this study is that in immunocytochemistry studies the same cell number was used as in other assays, which is 25,000 cells/well, however the random photos captured for cells in different area of the chamber slides in 5 independent experiments only totalled up to over 100 cells were analysed. Therefore, more cells need to be captured in the future to accurately represent the outcome of protein localisation.

Chapter 5

The effects of inflammatory conditions on ABC transporter expression and activity

5.1 Effect of pro-inflammatory and anti-inflammatory compounds on transporter activity and expression

5.1.1 Background

Pro-inflammatory mediators are associated with many systemic and CNS inflammatory diseases (Erickson & Banks, 2018) that lead to immunological alterations (Howren et al., 2009, Dowlati et al., 2010) and chronic psychiatric and neurodegenerative disease (Zhao et al., 2019; Liebner et al., 2018). Pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β are key elements in development and progression of CNS neuroinflammation (Sochocka, Diniz & Leszek, 2017; Liebner et al., 2018).

In comparison to any other cytokine family, the IL-1 family is primarily associated with acute and chronic inflammation. IL-1β has emerged as a therapeutic target for an extensive number of local and systemic inflammatory conditions (Dinarello, 2012; Kaneko et al., 2019) and is known to be associated with various pathological states, including stroke, psychiatric and neurodegenerative disorders (Denes et al., 2011; Dinarello, 2011; Tsai, 2017).

The administration of multiple therapeutic drugs to control disease symptoms, especially in a comorbidity situation is unavoidable. However, many therapeutic drugs used in the treatment of chronic diseases are potential substrates of ABC efflux transporters. Consequently, the potential modulation of ABC efflux transporter expression and activity under inflammatory conditions has added another element to the factors responsible for drug resistance and high variability in the pharmacological response observed in patients receiving drug-based therapies. Hence, it is extremely important to study the influence of inflammatory cytokines on the regulation of ABC efflux transporters at the BBB in order to understand how drug disposition in the brain could be affected in chronic inflammatory conditions.

5.1.1.1 Effect of IL-1β and NF-κB inhibitors on ABCB1 transporter activity and expression

Treatment of PBECs for 24 h with IL-1β at a concentration of 10 μM significantly decreased ($p < 0.0001$) the intracellular accumulation of calcein by 52.32 % ± 7.8 % (Figure 5.1) suggesting an increase in ABCB1 activity under pro-inflammatory conditions. On the contrary, treatment of PBECs with the NF-κB inhibitors (i.e. compounds that block the IL-1β signalling pathway which would be expected to have an anti-inflammatory effect), Honokiol

(Figure 5.1 a) and CAPE (Figure 5.1 B) significant decreased ($p < 0.0001$) ABCB1 activity, i.e. increased the intracellular accumulation of calcein by 47.3 % ± 10 % and 53.7 % ± 9.0 % respectively. SN50 (Figure 5.1 C) however, had no significant effect on ABCB1 activity. In co-treatment conditions with IL-1β and each of the three NF-κB inhibitors, none of the latter reversed the effect of the cytokine (Figure 5.1 A, B, C). None of the compounds affected cell viability over the concentrations tested using neutral red assay (Appendix D.6).

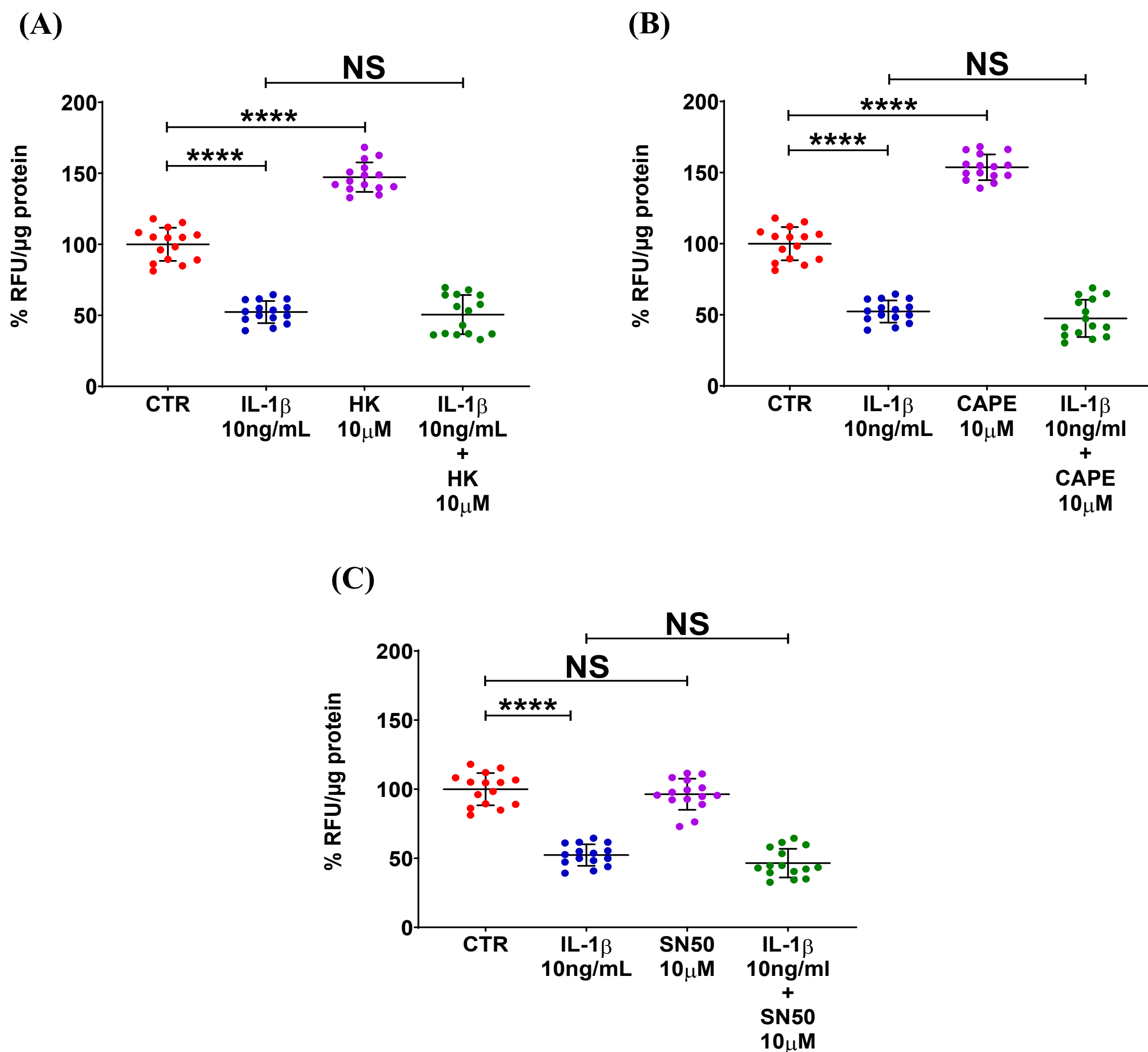


Figure 5.1: Effects of IL-1β and NF-κB inhibitors (honokiol, HK; caffeic acid phenethyl ester, CAPE and SN50) on ABCB1 functional activity in PBECs.
Cells were treated with 10 µM of (A) HK, (B) CAPE or (C) SN50 as single treatment or co-treatment with 10 µM IL-1β for 24 h. Intracellular accumulation of calcein - ABCB1 substrate was measured to determine ABCB1 transporter activity. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $p < 0.0001$, NS: non-significant. The concentrations of IL-1β and NF-κB

inhibitors used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.6).

The effect of IL-1β and NF-κB inhibitor on ABCB1 activity was further assessed at the protein level by using Western blotting. Exposure of PBECs to IL-1β significantly ($p < 0.01$) up-regulated the expression of ABCB1 by 1.51-fold (Figure 5.2), confirming the findings obtained in the calcein-AM functional assay above. As expected, treated with the NF-κB inhibitor honokiol alone significantly ($p < 0.01$) down-regulated ABCB1 expression to 0.71-fold compared to control, consistent with calcein-AM functional assay above. However, in the co-treatment studies (i.e. co-incubation with IL-1β and honokiol) honokiol counteracted the effect of IL-1β by reducing the IL-1β-mediated up-regulation of ABCB1 expression, to control level. This particular outcome does not tie in with the calcein-AM functional assay above.

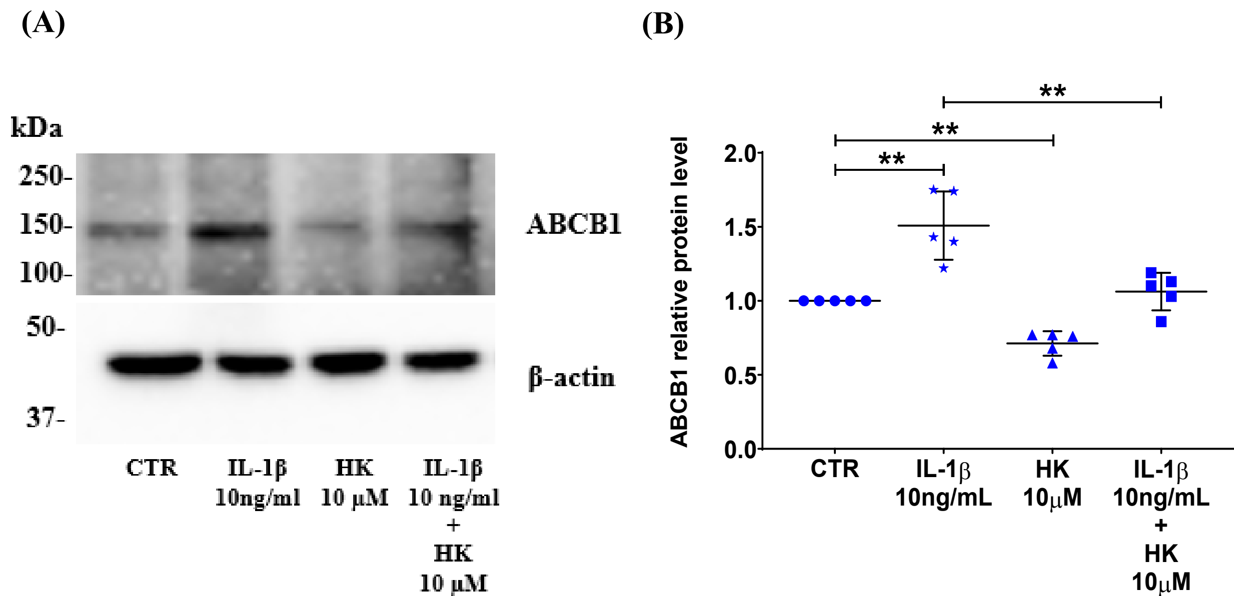


Figure 5.2: Effects of IL-1β and NF-κB inhibitor (Honokiol, HK) on ABCB1 protein expression in PBECs.

Cells were treated with 10 μM IL-1β and 10 μM HK as single treatment or co-treatment for 24 h. Panel (A) is representative Western blot image of ABCB1, panel (B) is densitometric quantification of the relative protein expression levels of ABCB1. Densitometric data are normalised to β-actin and are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. CTR: control, **: $p < 0.01$.

5.1.1.2 Effect of IL-1β and NF-κB inhibitors on ABCG2 transporter activity and expression

As was observed with ABCB1 activity above, treatment of PBECs for 24 h with 10 µM IL-1β significantly decreased ($p < 0.0001$) the intracellular accumulation of Hoechst 33342 (Figure 5.3) suggesting increased ABCG2 activity. However, treatment with the NF-κB inhibitors Honokiol (Figure 5.3 A) and CAPE (Figure 5.3 B) resulted in a significant decrease in ABCG2 activity indicated by significantly increased intracellular accumulation of Hoechst 33342 by 139.2 % ± 21.7 % and 131.3 % ± 16.9 % respectively compared to control level. SN50 (Figure 5.3 C) did not affect ABCB1 activity. When PBECs were co-treated with IL-1β and each of the three NF-κB inhibitors the latter did not reverse the effects of the cytokine (Figure 5.3 A, B, C).

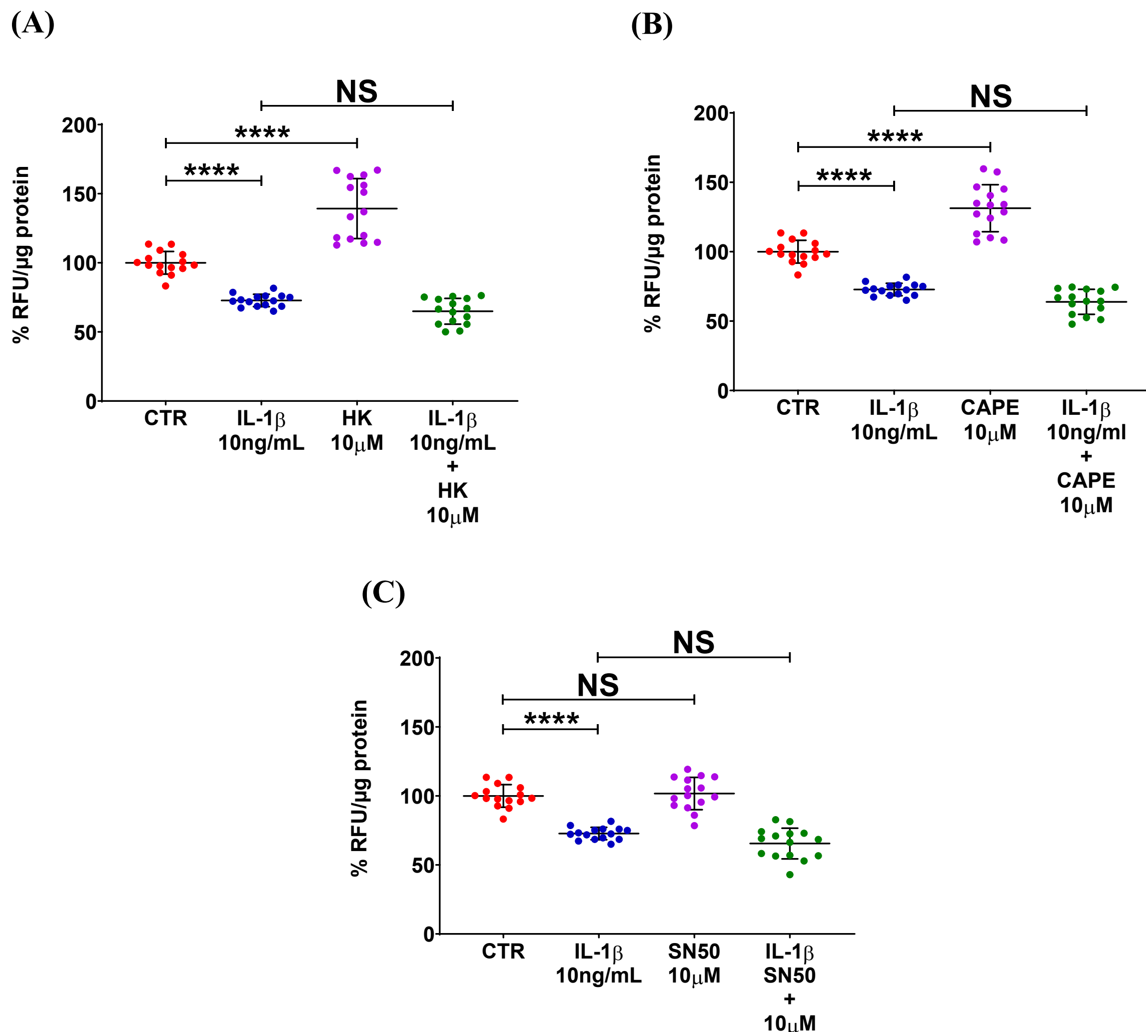


Figure 5.3: Effects of IL-1β and NF-κB inhibitors (honokiol, HK; caffeic acid phenethyl ester, CAPE and SN50) on ABCG2 functional activity in PBECs.
Cells were treated with 10 µM of (A) HK, (B) CAPE or (C) SN50 as single treatment or co-treatment with 10 µM IL-1β for 24 h. Intracellular accumulation of Hoechst 33342 - ABCG2

substrate was measured to determine ABCG2 transporter activity. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $p < 0.0001$, NS: non-significant. The concentrations of IL-1β and NF-κB inhibitors used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.6).

The effects of IL-1β and NF-κB inhibitors on ABCG2 activity was confirmed at the protein level using Western blotting. As expected, exposure to IL-1β significantly ($p < 0.01$) increased the expression of ABCG2 by 1.4-fold (Figure 5.4), which is in keeping with the findings obtained in the Hoechst 33342 functional assay above. Treatment with honokiol alone significantly decreased ($p < 0.01$) ABCG2 protein expression down to 0.7-fold of control level, which matches the findings of the Hoechst 33342 functional assay above. Interestingly, in the co-treatment studies, honokiol and IL-1β appeared to abrogate the effect of each other, reverting ABCG2 expression to a similar level observed in the control condition, which is not consistent with the findings of Hoechst 33342 functional assay above.

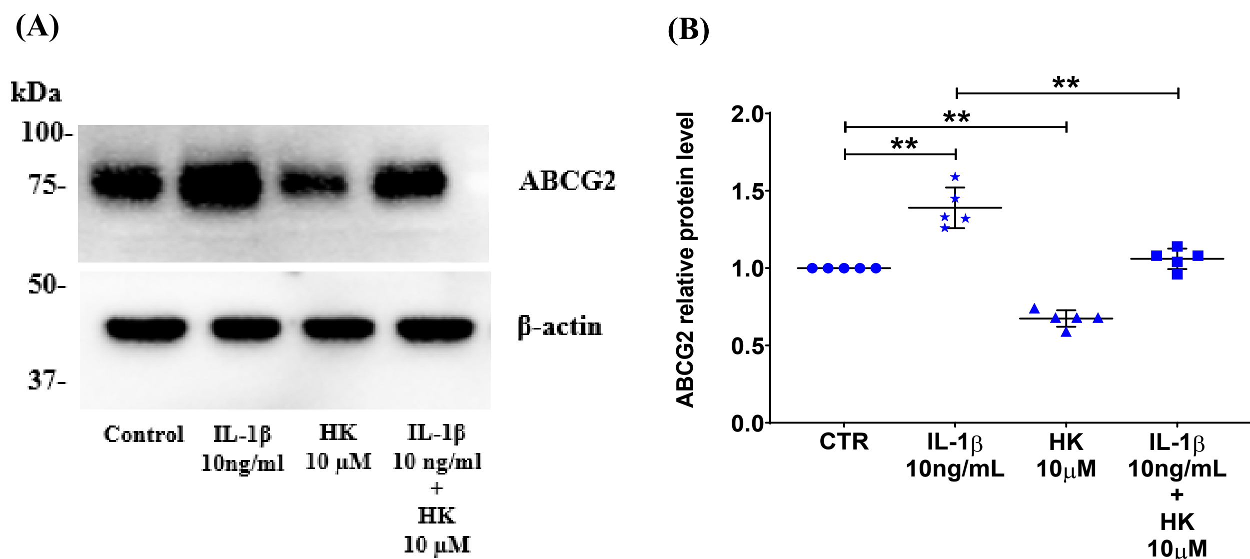


Figure 5.4: Effects of IL-1β and NF-κB inhibitor (Honokiol, HK) on ABCG2 protein expression in PBECs.
Cells were treated with 10 µM IL-1β and 10 µM HK as single treatment or co-treatment for 24 h. Panel (A) is representative Western blot image of ABCG2, panel (B) is densitometric quantification of the relative protein expression levels of ABCG2. Densitometric data are normalised to β-actin and are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. CTR: control, **: $p < 0.01$.

5.1.1.3 Effect of IL-1β, and NF-κB inhibitors on ABCC5 transporter activity and expression

A 24 h treatment of PBECs with IL-1β significantly reduced ($p < 0.0001$), intracellular GS-MF accumulation (i.e. significantly increased ABCC5 activity). As expected, honokiol (Figure 5.5 A) and CAPE (Figure 5.5 B), but not SN50 (Figure 5.5 C), significantly decreased ABCC5 activity compared to control. In the co-treatment studies involving cytokine and NF-κβ inhibitors, none of the latter abrogated the effects of IL-1β, with ABCG2 activity in all co-treatment conditions not significantly different to ABCC5 activity with IL-1β alone. This finding is in accord with the outcomes observed above for both ABCB1 and ABCG2 activities.

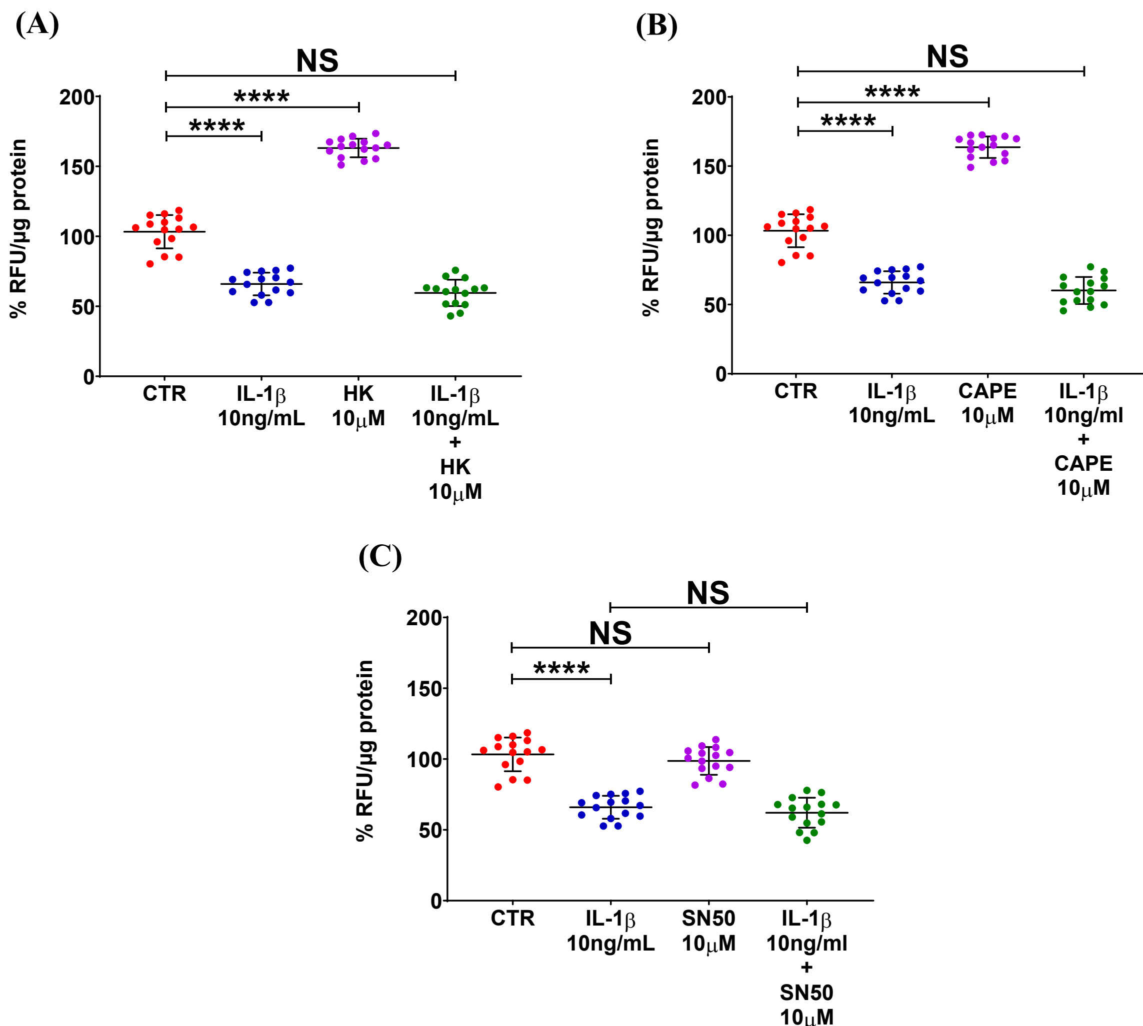


Figure 5.5: Effects of IL-1β and NF-κB inhibitors (honokiol, HK; caffeic acid phenethyl ester, CAPE and SN50) on ABCC5 functional activity in PBECs.
Cells were treated with 10 µM of (A) HK, (B) CAPE or (C) SN50 as single treatment or co-treatment with 10 µM IL-1β for 24 h. Intracellular accumulation of GS-MF - ABCC5 substrate was measured to determine ABCC5 transporter activity. Data were analysed using One-Way

ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $p < 0.0001$, NS: non-significant. The concentrations of IL-1β and NF-κB inhibitors used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.6).

In the ABCC5 protein expression studies, similar outcomes were obtained as observed with ABCB1 and ABCG2. Treatment of IL-1β significantly ($p < 0.01$) enhanced the expression of ABCC5 by 1.6-fold (Figure 5.6), which is in agreement with the findings obtained in the CMFDA functional assay above. Treatment with honokiol significantly reduced ($p < 0.01$) ABCC5 protein expression down to 0.5-fold, which is in line with the CMFDA functional assay. However, the outcome of the co-treatment studies is contrary to the CMFDA functional assay, demonstrating IL-1β and honokiol nullified the effect of each other by reverting the ABCC5 expression to control condition.

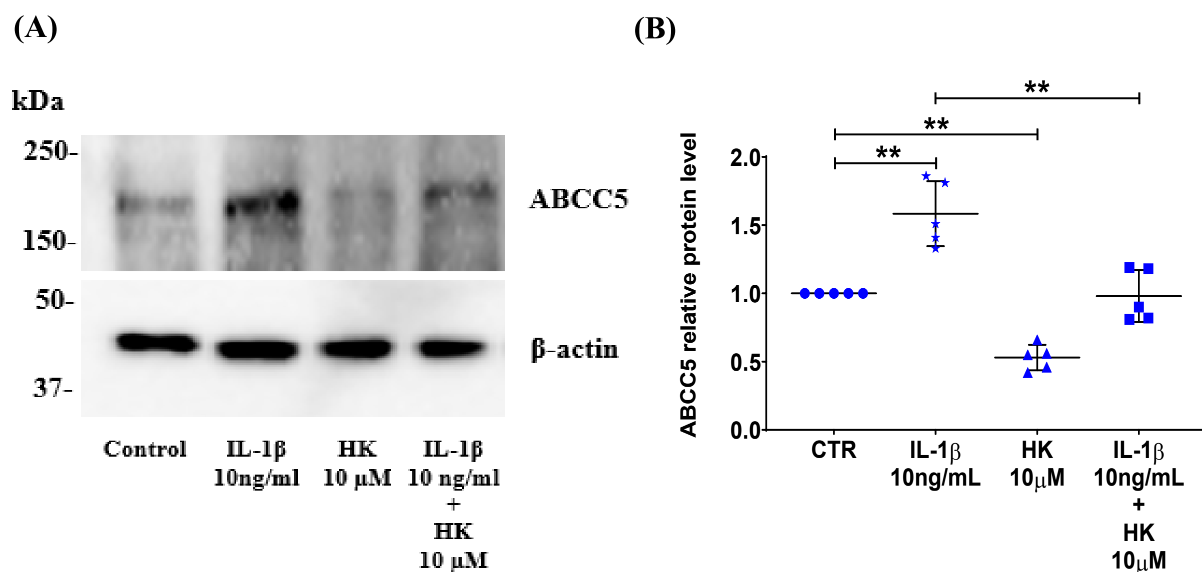


Figure 5.6: Effects of IL-1β and NF-κB inhibitor (Honokiol, HK) on ABCC5 protein expression in PBECs.
Cells were treated with 10 μM IL-1β and 10 μM HK as single treatment or co-treatment for 24 h. Panel (A) is representative Western blot image of ABCC5, panel (B) is densitometric quantification of the relative protein expression levels of ABCC5. Densitometric data are normalised to β-actin and are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. CTR: control, **: $p < 0.01$.

In order to determine whether NF-κB inhibitors treatment is related to direct interaction with ABCB1, i.e. inhibition of ABCB1, ABCG2 and ABCC5 activity, a new experiment was conducted by reducing the 24 h incubation time to 30 min. The resulting treatments did not cause any significant effect ($p > 0.05$) on all three ABC transporter activity (Figure 5.7).

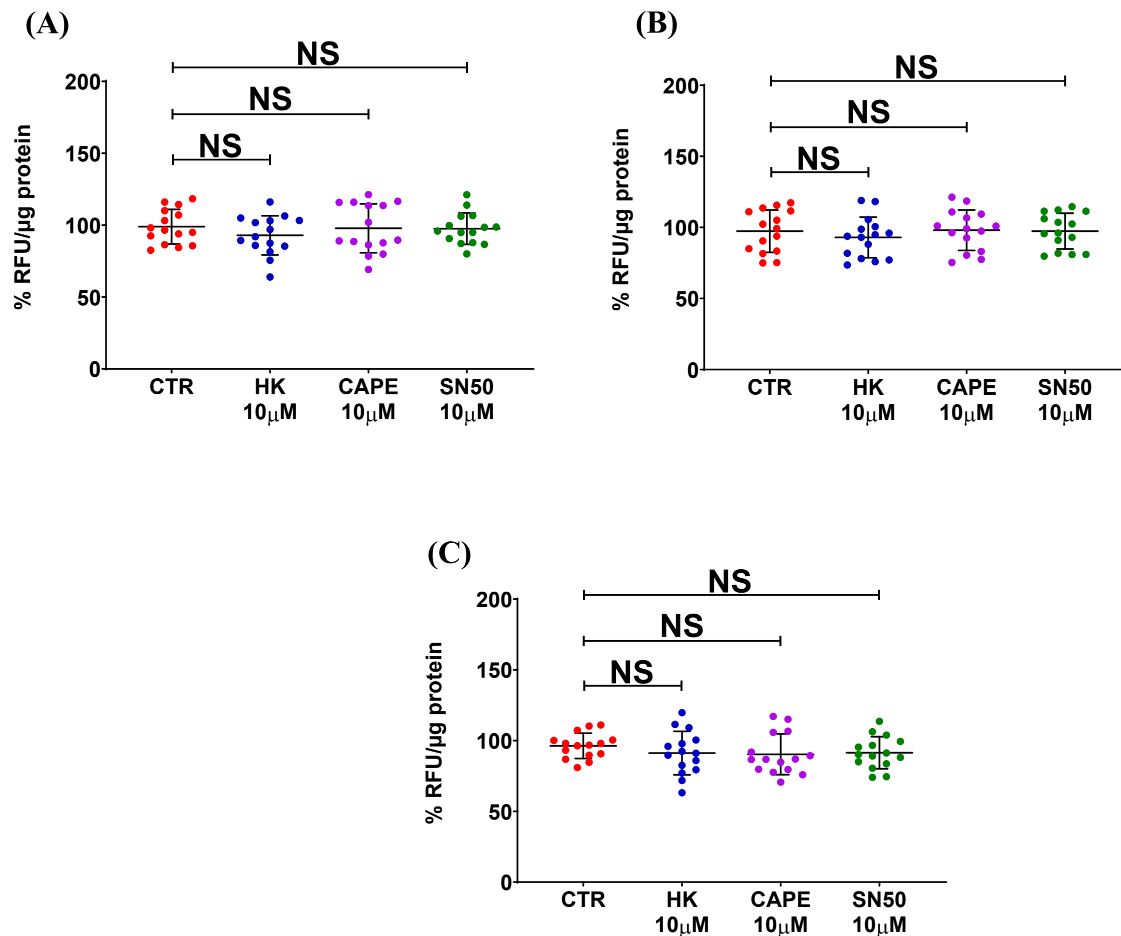
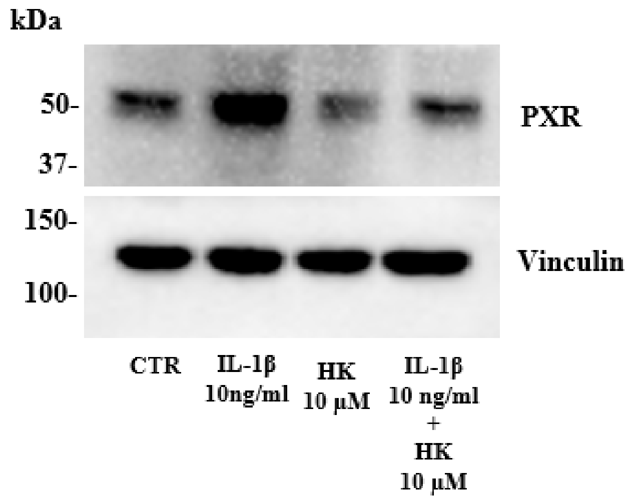


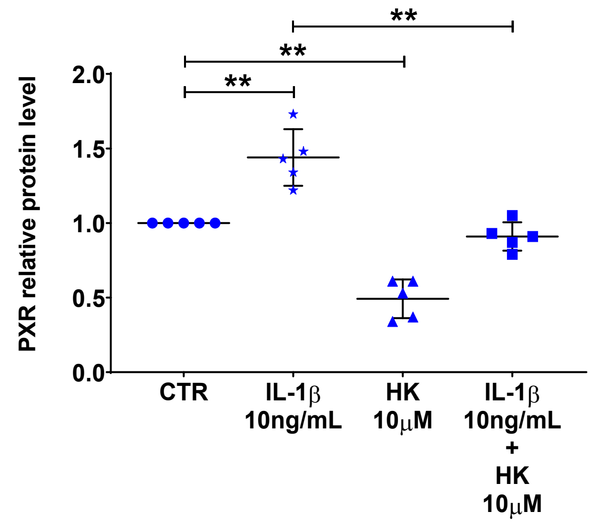
Figure 5.7: Effects of short-term exposure of NF-κB inhibitors (honokiol, HK; caffeic acid phenethyl ester, CAPE and SN50) on ABCB1, ABCG2 and ABCC5 functional activity in PBECs.

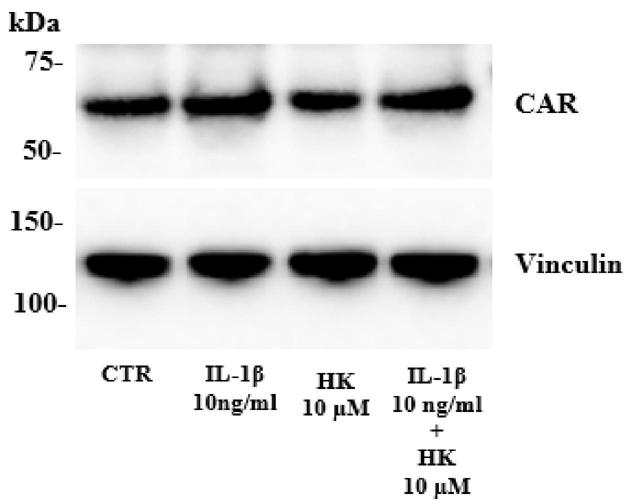
Intracellular accumulation of (A) calcein - ABCB1 substrate, (B) Hoechst 33342 - ABCG2 substrate, (C) GS–MF - ABCC5 substrate were measured in control cells and cells treated with 10 µM of HK, CAPE and SN50 for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. NS: non-significant. The concentrations used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.6).

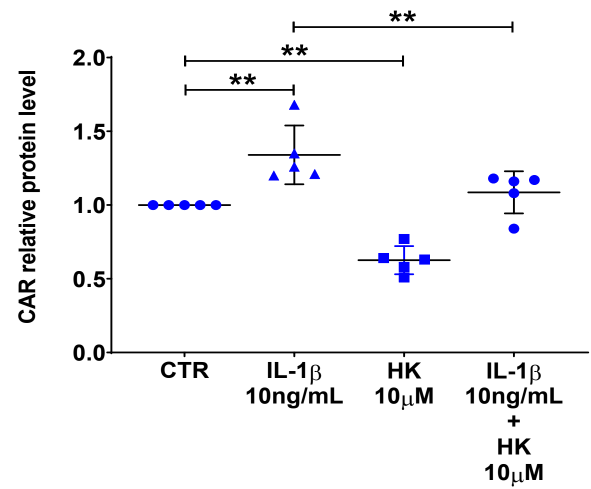
5.1.1.4 Effects of IL-1β, and NF-κB inhibitor on nuclear receptor protein expression

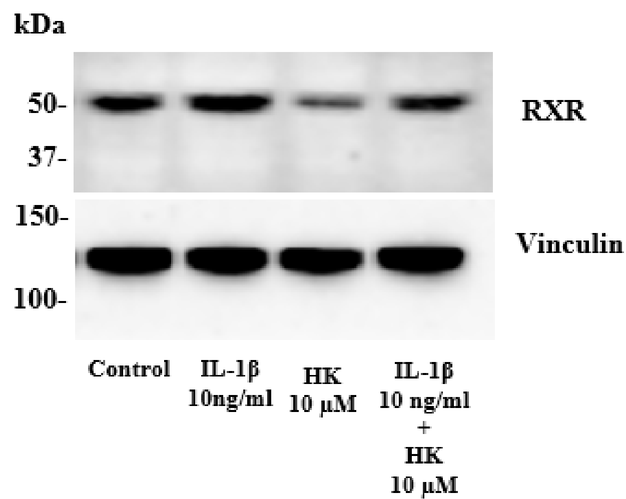
In order to determine whether nuclear receptors are involved in the regulation of ABC transporter expression caused by treatment with IL-1β and NF-κB inhibitors, Western blotting was employed to measure PXR, CAR, RXR and GR protein expression. IL-1β significantly upregulated ($p < 0.01$) protein expression of PXR (Figure 5.8 A, E), CAR (Figure 5.8 B, F), RXR (Figure 5.8 C, G) and GR (Figure 5.8 D, H), whereas honokiol treatment had the opposite effect, significantly down-regulating expression. Co-incubation of PBECs with both IL-1β and honokiol did not significantly modify PXR, CAR, RXR and GR protein expression. The findings of the effects of IL-1β and honokiol on nuclear receptor protein expression in this study mirror the findings observed with the effects of IL-1β and honokiol on ABC transporter protein expression, as outlined above.

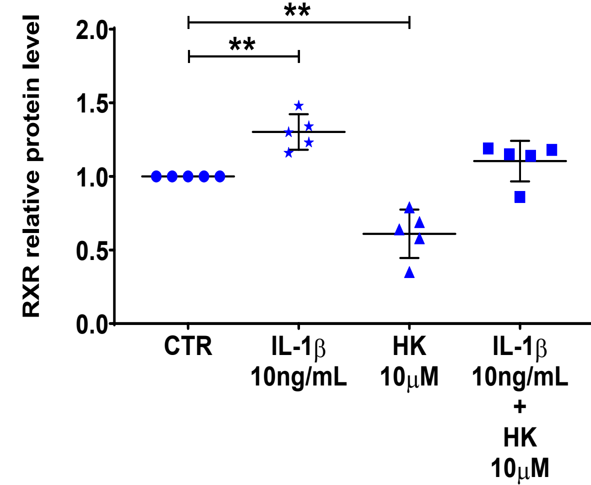

(A)
kDa
50-
37-
150-
100-
PXR
Vinculin
CTR
IL-1β 10ng/ml
HK 10 µM
IL-1β 10 ng/ml + HK 10 µM


(E)
PXR relative protein level
2.0
1.5
1.0
0.5
0.0
**
**
**
CTR
IL-1β 10ng/mL
HK 10µM
IL-1β 10ng/mL + HK 10µM


(B)
kDa
75-
50-
150-
100-
CAR
Vinculin
CTR
IL-1β 10ng/ml
HK 10 µM
IL-1β 10 ng/ml + HK 10 µM


(F)
CAR relative protein level
2.0
1.5
1.0
0.5
0.0
**
**
**
CTR
IL-1β 10ng/mL
HK 10µM
IL-1β 10ng/mL + HK 10µM


(C)
kDa
50-
37-
150-
100-
RXR
Vinculin
Control
IL-1β 10ng/ml
HK 10 µM
IL-1β 10 ng/ml + HK 10 µM


(G)
RXR relative protein level
2.0
1.5
1.0
0.5
0.0
**
**
**
CTR
IL-1β 10ng/mL
HK 10µM
IL-1β 10ng/mL + HK 10µM

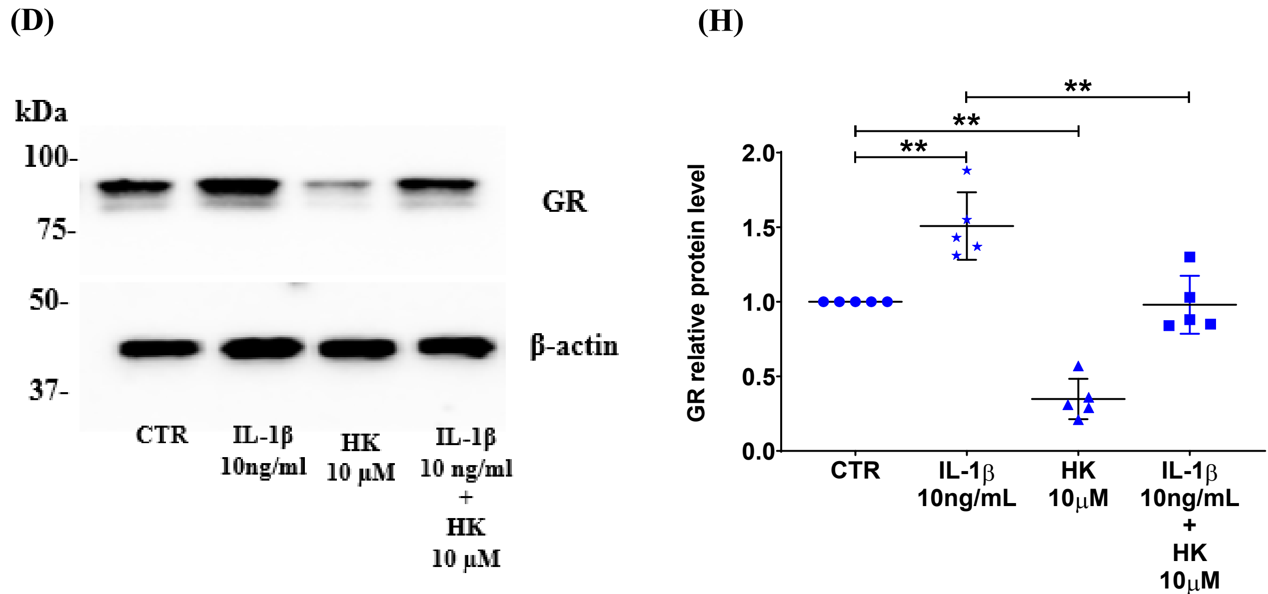


Figure 5.8: Effects of IL-1β, and NF-κB inhibitor (honokiol, HK) on PXR, CAR, RXR and GR protein expression in PBECs.

Cells were treated with 10 µM of IL-1β and HK as single treatment or co-treatment for 24 h. Panel (A), (B), (C) and (D) are representative Western blot image of PXR, CAR, RXR and GR respectively. Panel (E), (F), (G) and (H) are densitometric quantification of the relative protein expression levels of PXR, CAR, RXR and GR respectively. PXR, CAR and RXR densitometric data are normalised to vinculin, while GR data is normalised to β-actin and all data are presented as a percentage of untreated control cells. Data were analysed using non-parametric Mann–Whitney test and are presented as mean ± SD of at least five independent experiments. CTR: control, **: $p < 0.01$, NS: not significant.

5.1.1.5 Effect of IL-1β, and inhibitors of the MAP Kinase pathway on ABCB1 transporter activity and expression

Treatment of PBECs for 24 h with IL-1β at a concentration of 10 μM significantly decreased ($p < 0.0001$) the intracellular accumulation of calcein to 58.8 % ± 10.9 % of control (Figure 5.9) suggesting increased ABCB1 activity in pro-inflammatory conditions. Treatment with the p38 inhibitor SB239063 alone had no significant effect on ABCB1 activity (Figure 5.9 A) and upon co-treatment with IL-1β and the p38 inhibitor SB239063, the latter had no significant effect on the IL-1β-mediated increase in ABCB1 activity (Figure 5.9 A).

However, when PBECs were co-treated with IL-1β and either the JNK inhibitor SP600125 (Figure 5.9 B) or the ERK1/2 inhibitor FR180204 (Figure 5.9 C) the inhibitors completely supressed the effect of IL-1β with ABCB1 activity reverting back to the level observed in control. When treating PBECs with the JNK inhibitor SP600125 (Figure 5.9 B) and the ERK1/2 inhibitor FR180204 (Figure 5.9 C) alone (i.e. in the absence of IL-1β) a significant decrease ($p < 0.0001$) in ABCB1 activity (i.e. a significant increase in the intracellular accumulation of calcein) of over 1.6-fold was observed. These findings signify that JNK and ERK1/2 are involved in regulation of ABCB1 activity. None of the compounds employed in these studies affected the cell viability over the concentrations tested using the neutral red assay (Appendix D.7).

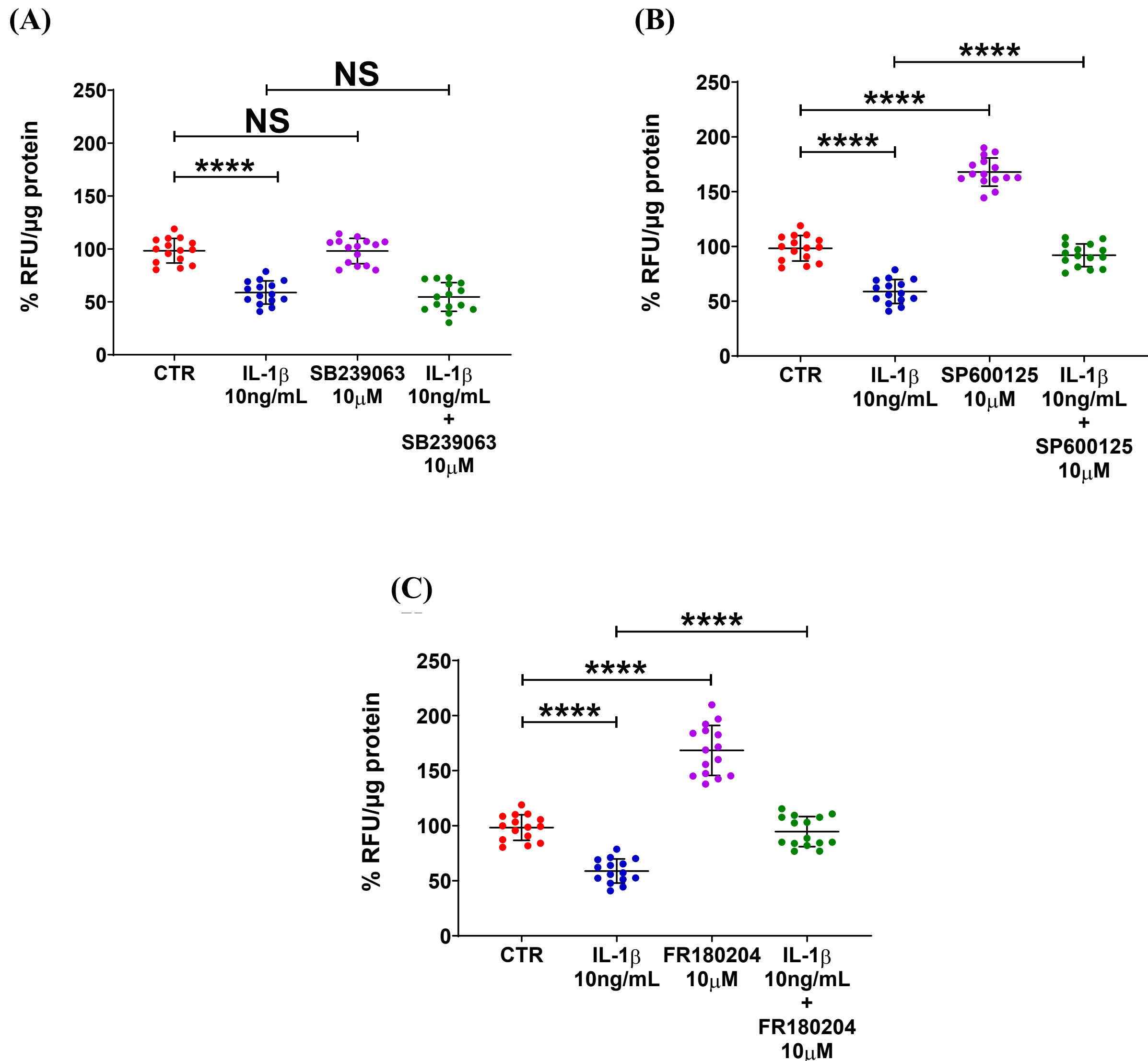


Figure 5.9: Effects of IL-1β and MAPK inhibitors (SB239063, SP600125 and FR180204) on ABCB1 functional activity in PBECs.

Cells were treated with 10 µM of (A) SB239063, (B) SP600125 and (C) FR180204 as single treatment or co-treatment with 10 µM IL-1β for 24 h. Intracellular accumulation of calcein - ABCB1 substrate was measured to determine ABCB1 transporter activity. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $p < 0.0001$, NS: non-significant. The concentrations of IL-1β and MAPK inhibitors used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.7).

5.1.1.6 Effect of IL-1β, and inhibitors of the MAP Kinase pathway on ABCG2 transporter activity and expression

Similar to the findings observed with studies on ABCB1 activity, treatment of PBECs for 24 h with 10 μM IL-1β significantly decreased ($p < 0.0001$) the intracellular accumulation of Hoechst 33342 (Figure 5.10) suggesting increased ABCG2 activity. Also, as was observed with ABCB1 activity, treatment of PBECs with the p38 inhibitor SB239063 failed to abrogate the IL-1β-mediated increase in ABCG2 activity (Figure 5.10 a) whilst treatment with SB239063 alone had no significant effect on ABCB1 activity. However, co-treatment of PBECs with IL-1β and either the JNK inhibitor SP600125 (Figure 5.10 b) or the ERK1/2 inhibitor FR180204 (Figure 5.10 c) completely abrogated the effect of IL-1β, with ABCG2 activity at the control level.

When treating PBECs with the JNK inhibitor SP600125 (Figure 5.10 b) or the ERK1/2 inhibitor FR180204 (Figure 5.10 c) alone (i.e. in the absence of IL-1β) significant decreases ($p < 0.001$) in ABCG2 activity (i.e. increase in the intracellular accumulation of Hoechst 33342) were observed.

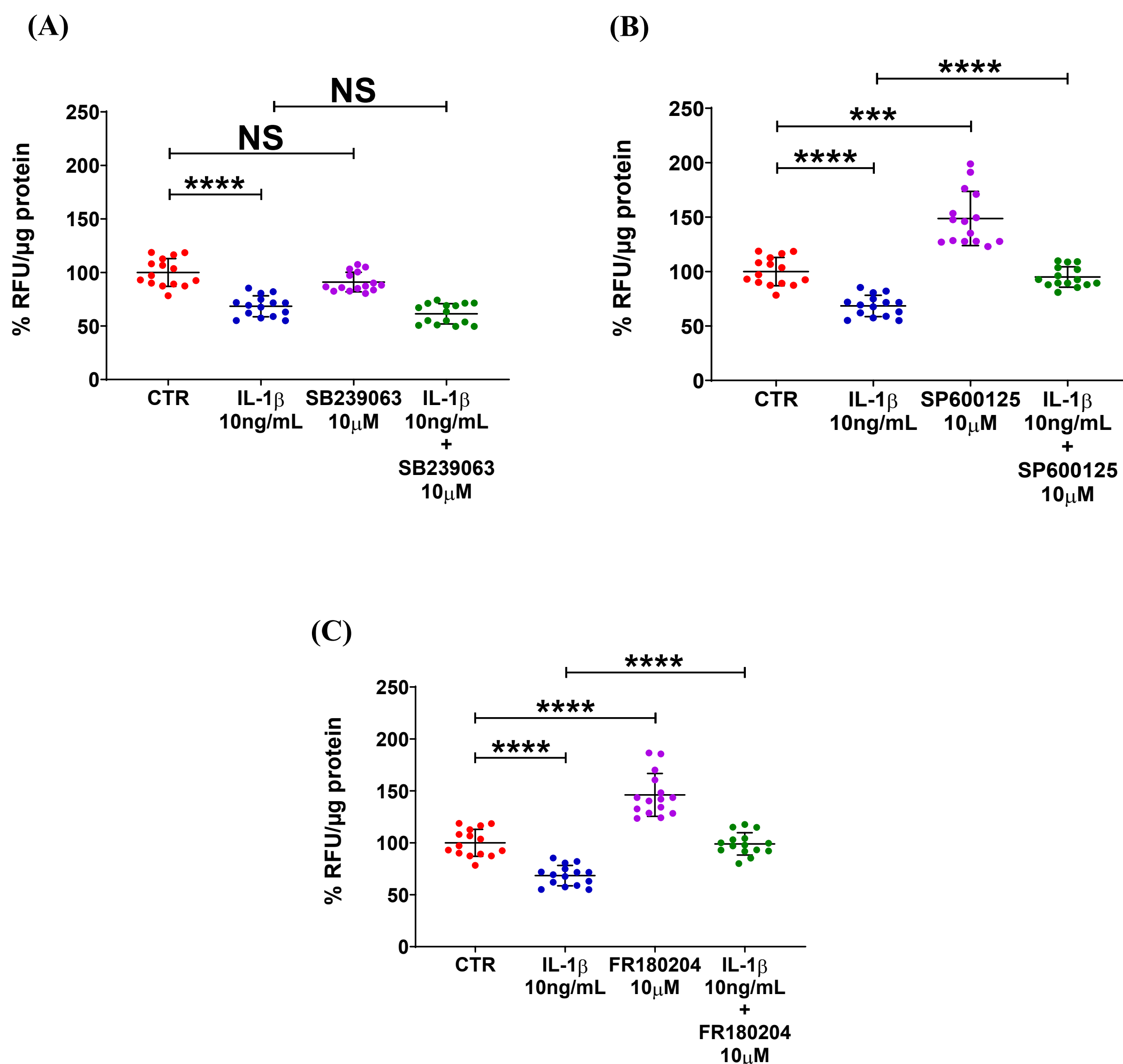


Figure 5.10: Effects of IL-1β and MAPK inhibitors (SB239063, SP600125 and FR180204) on ABCG2 functional activity in PBECs.

Cells were treated with 10 µM of (A) SB239063, (B) SP600125 and (C) FR180204 as single treatment or co-treatment with 10 µM IL-1β for 24 h. Intracellular accumulation of Hoechst 33342 - ABCG2 substrate was measured to determine ABCG2 transporter activity. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $p < 0.0001$, ***: $p < 0.001$, NS: non-significant. The concentrations of IL-1β and MAPK inhibitors used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.7).

5.1.1.7 Effect of IL-1β, and inhibitors of the MAP Kinase pathway on ABCC5 transporter activity and expression

A 24 h treatment of PBECs with IL-1β significantly reduced ($p < 0.0001$) intracellular GS-MF accumulation (i.e. significantly increased ABCC5 activity) (Figure 5.11). In the co-treatment studies, the JNK inhibitor SP600125 (Figure 5.11 B) or ERK1/2 inhibitor FR180204 (Figure 5.11 C) completely nullified IL-1β-mediated increase in ABCC5 activity, however, treatment with the p38 inhibitor SB239063 (Figure 5.11 A) failed to abrogate the effects of IL-1β. When PBECs were treated with each of the three individual MAPK inhibitors alone, significant decreases in ABCC5 activity when compared to control were observed with JNK inhibitor SP600125 ($p < 0.001$) (Figure 5.11 B) and ERK1/2 inhibitor FR180204 ($p < 0.001$) (Figure 5.11 C) but not with SB239063 (Figure 5.11 A). This finding is consistent with the outcomes observed for both ABCB1 and ABCG2 transporters, indicating that both JNK and ERK are involved in the regulation of expression of the three ABC transporters.

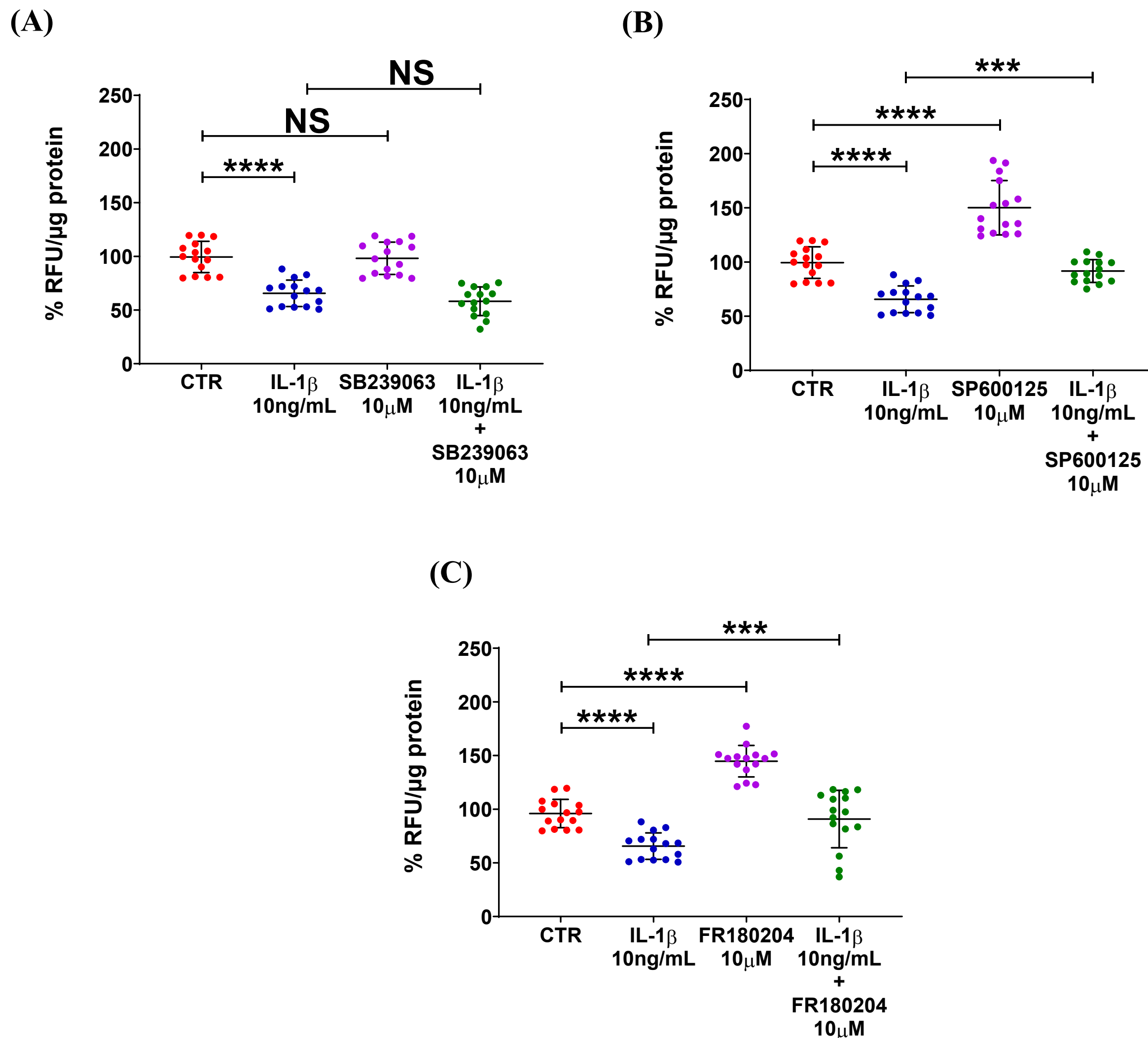


Figure 5.11: Effects of IL-1β and MAPK inhibitors (SB239063, SP600125 and FR180204) on ABCC5 functional activity in PBECs.
Cells were treated with 10 µM of (A) SB239063, (B) SP600125 and (C) FR180204 as single treatment or co-treatment with 10 µM IL-1β for 24 h. Intracellular accumulation of GS-MF - ABCC5 substrate was measured to determine ABCC5 transporter activity. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $p < 0.0001$, ***: $p < 0.001$, NS: non-significant. The concentrations of IL-1β and MAPK inhibitors used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.7).

In order to determine whether NF-κB inhibitors treatment is related to direct interaction with ABCB1, i.e. inhibition of ABCB1, ABCG2 and ABCC5 activity, a new experiment was conducted by reducing the 24 h incubation time to 30 min. The resulting treatments did not cause any significant effect ($p > 0.05$) on all three ABC transporter activity (Figure 5.12).

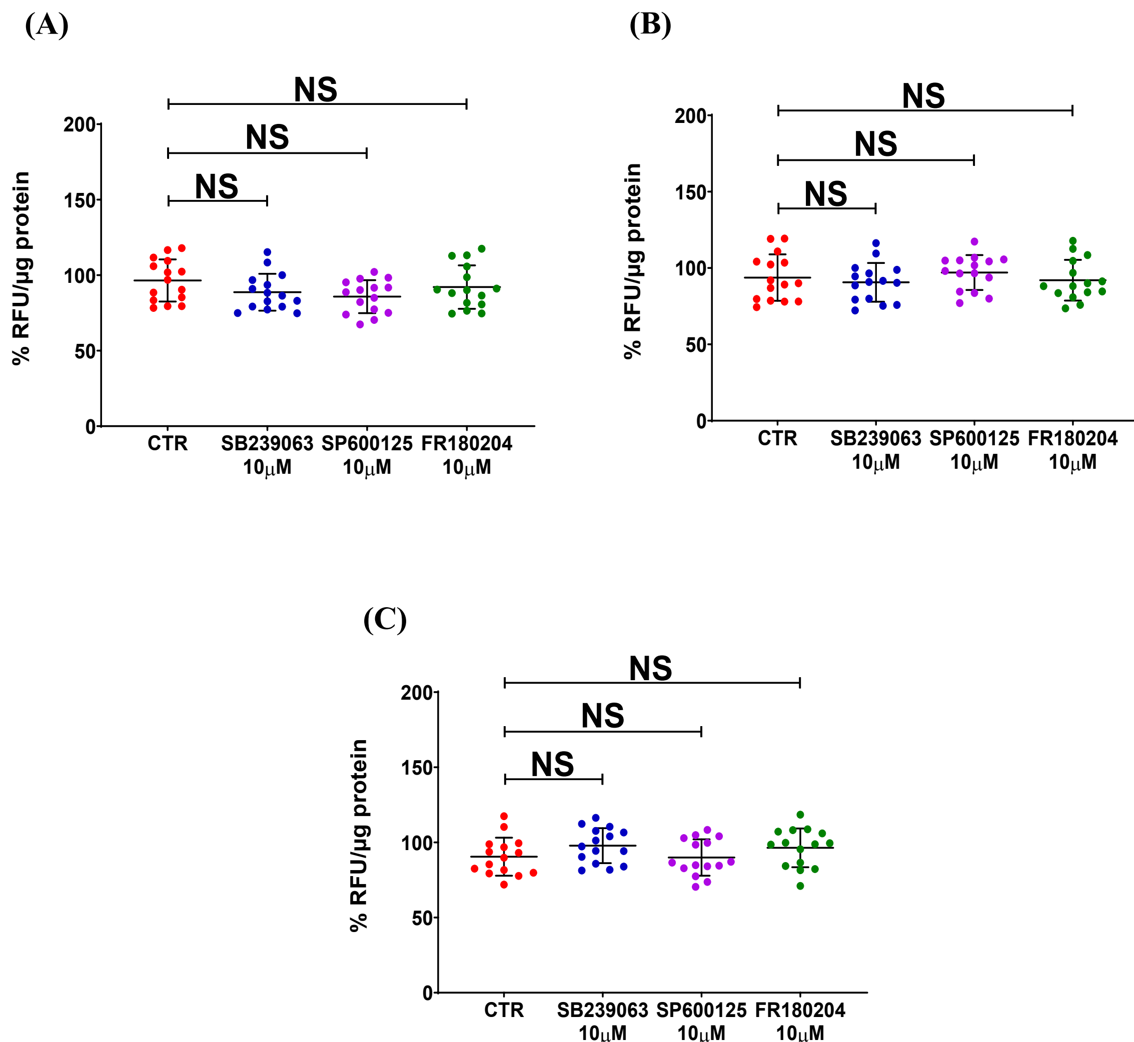


Figure 5.12: Effects of short-term exposure MAPK inhibitors (SB239063, SP600125 and FR180204) on ABCB1, ABCG2 and ABCC5 functional activity in PBECs.
Intracellular accumulation of (A) calcein - ABCB1 substrate, (B) Hoechst 33342 - ABCG2 substrate, (C) GS–MF - ABCC5 substrate were measured in control cells and cells treated with 10 µM of SB239063, SP600125 and FR18020 for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. NS: non-significant. The concentrations used in these studies were all non-toxic, as determined by the neutral red assay (Appendix D.7).

The findings contained in this chapter show the important role of IL-1β has in regulating the expression and activity of key drug efflux transporters associated with blood-brain barrier endothelial cells. Importantly, these findings highlight for the first time that NF-κB and MAPK are involved in IL-1β pathway, hence, inhibiting either NF-κB or MAPK completely abrogated the effect of the IL-1β on drug efflux transporters. The results further demonstrate the key drug

efflux transporters in BBB are sensitive to the influence of both pro-inflammatory and anti-inflammatory compounds and affecting drug delivery to the central nervous system.

5.1.2 Discussion

Inflammation induced by pro-inflammatory cytokines can significantly affect the expression of ABC transporters through the activation of the NF-κB signalling pathway at the BBB (Ronaldson et al., 2010; Maeng et al., 2007; Yu et al., 2011), cancer cells (Bentires-Alj et al., 2003; Verma et al., 2013) and kidney cells (Thevenod et al., 2000).

The downstream effect of TNF-α, which results in the activation of NF-κB and ABC transporter activity, has been widely studied (Ronaldson et al., 2010; Ros et al., 2001). On the other hand, despite IL-1β being one of the most important pro-inflammatory cytokines, the cascade of molecular mechanisms that mediate IL-1β-induced changes in expression and activity of ABC transporters has yet to be precisely defined.

5.1.2.1 The effect of IL-1β and NF-κB inhibitors on ABC transporter activity and expression

In this study, IL-1β significantly enhanced the transporter activity and protein expression of ABCB1, ABCG2 and ABCC5 in PBECs. Thus far, the effect of IL-1β has only been reported on ABCB1, ABCG2, ABCC1 and ABCC4 at BBB *in vitro*, and the results are rather conflicting across different studies. For example, studies reported IL-1β upregulated ABCB1 functional activity and protein expression in primary porcine (Torres-Vergara and Penny, 2018) and human (Zuloaga et al., 2012) brain endothelial cells, which are consistent with the findings outlined in this thesis. However, other studies report different findings with von Wedel-Parlow et al. (2009) reporting reduced ABCB1 and ABCG2 activity and protein expression using primary porcine brain endothelial cells and Iqbal et al., (2012) reporting reduced ABCB1 activity and protein expression in guinea pig brain endothelial cells. Studies with human model systems are sparse, with only one study to date demonstrating IL-1β had no effect on ABCB1 activity and protein expression but significantly reduced ABCG2 activity and protein expression in hCMEC/d3 cells (Poller et al., 2010).

Significantly less research into the effects of inflammatory cytokines has been carried out on members of the ABCC family compared to members of the ABCB and ABCG families. To date only one study has reported IL-1β increased mRNA expression of ABCC1 but no effect was observed on ABCC4 mRNA expression in PBECs (von Wedel-Parlow et al., 2009).

The intracellular mechanisms by which pro-inflammatory cytokines regulate ABC transporter functional activity and expression remain ambiguous, although there is evidence IL-1β exerts most of its effects via activation of NF-κB, the most prominent transcription factor activated by pro-inflammatory cytokines. This may suggest the involvement of the canonical NF-κB signalling pathway in inducing the expression and activity of ABC transporters.

With these scientific findings as background, it was proposed that pharmacological inhibition of NF-κB action would be an interesting way to reveal the involvement of this nuclear factor in the IL-1β-mediated up-regulation of ABC transporters. Three potent NF-κB inhibitors were selected for this study, honokiol, CAPE and SN50. Both honokiol and CAPE are potent NF-κB inhibitors that prevent phosphorylation and degradation of IκBα by inhibiting IKK activity (Lee et al., 2010; Maria et al., 2012; Zhu, X. et al., 2014; Nabekura et al., 2015; Xie et al., 2015). SN50 inhibits NF-κB action by interfering with its translocation into the nucleus (Gupta et al., 2010; Wu et al., 2020). In co-treatment studies employing IL-1β and the NF-κB inhibitors honokiol, CAPE and SN50, all inhibitors failed to abrogate the IL-1β-induced upregulation of ABCB1, ABCG2 and ABCC5 activity. Honokiol, CAPE and SN50 have been widely reported to significantly inhibit the IL-1β-induced inflammatory pathway to ameliorate inflammatory diseases in different *in vivo* and *in vivo* models (Luo et al., 2020; Wu et al., 2017; Wijesuriya and Lappas, 2018; Chen et al., 2013; Yang et al., 2014; Lee et al., 2016; Dai et al., 2020; Mamik et al., 2011; Tiwari et al., 2011; Choi et al., 2013). However, as far as we are aware, there are no published reports on the effects of both IL-1β and NF-κB inhibitors on the ABCB1, ABCG2 and ABCC5 transporters. Therefore, the findings contained in this thesis constitute the first experimental evidence of a lack of effect of NF-κB inhibitors on IL-1β-mediated increase in ABC transporter activity.

The effect of honokiol on ABC transporter activity was further verified in protein expression assay. Interestingly, it was found that honokiol effectively abrogated the IL-1β-induced upregulation of ABCB1, ABCG2 and ABCC5 protein expression. There are several possible explanations to account for the findings of a reduction in ABC transporter protein expression but no significant change in transporter activity. These include phosphorylation/dephosphorylation of transporters by PKC isozymes (Terlouw et al., 2003; Hartz et al., 2006; Bauer et al., 2007; Stolarczyk et al., 2011; Mayati et al., 2017; Crawford et al., 2018) and interaction of transporters with regulatory proteins, for example, Phosphoinositide 3-kinases (PI3Ks), clathrin, adaptin and Rab5 or membrane proteins, for

example, caveolin (Sachs et al., 1999; Kipp and Arias, 2002; Terlouw et al., 2003; Barakat et al., 2007; Barakat et al., 2008; Storch et al., 2007; Zhong et al., 2010a; Crawford et al., 2018). PKCs are more commonly reported to affect different aspects of drug transporter regulation, such as transcriptional or translational mechanisms controlling transporter expression, membrane insertion or internalization processes and phosphorylation status of transporters. The involvement of PKCs in insertion and retrieval of ABC transporters depends on its isoforms, and the pathway involves the interaction between PI3K, MAPK (p38 and ERK1/2) and PKCs (Boaglio et al., 2012; Schonhoff et al., 2016). For example, PKCε activation enhances the retrieval of ABCC2 (Beuers et al., 2003; Schonhoff et al., 2013; Answer et al., 2014); while PKCδ activation promotes the insertion of ABCC2 to the plasma membrane induced by cAMP (Schonhoff et al., 2008; Park et al., 2012; Answer et al., 2014).

Furthermore, one important process whereby transporter activity can be maintained despite a reduction in gene expression is recruitment of transporters from pre-existing intracellular pools into the plasma membrane, (Kipp et al., 2001; Kipp and Arias, 2002; Bauer et al., 2007; Miller 2010). Indeed studies report the cell's trafficking machinery can significantly alter ABC transporter activity by rapid insertion and removal of transporters in plasma membrane (Roston et al., 2012; Harris et al., 2018; Monzner et al., 2019; Geisler and Hegedus, 2020) in order to relocalise, reshuffle, redistribute them which has been initially discovered in liver (Kipp and Arias, 2002), renal (Miller, 2002; Terlouw et al., 2003) and cancer cells (Kim et al., 1997; Storch et al., 2007; Molinari et al., 2002). Presently, it is not known to what extent transporter trafficking takes place in brain endothelial cells and whether this can be triggered in the event of inflammation. Another factor worth considering is that the treatment duration conducted in this study is 24 h, thus it is uncertain whether chronic dosing that extended over 24 h would influence the protein expression level. Future studies are needed to take into consideration the time-dependent effect.

Treatment of PBECs with honokiol and CAPE alone resulted in significant inhibition of endogenous ABCB1, ABCG2 and ABCC5 transporter activities. Whilst honokiol demonstrated to downregulate endogenous ABCB1, ABCG2 and ABCC5 transporter expression. The inhibition of endogenous ABC transporter activity has also been reported in several studies. For example, honokiol was shown to reduce endogenous ABCB1 and ABCC1 activity and expression in numerous cancer cell lines (Xu et al., 2006; Han and Anh, 2012; Thulasiraman and Johnson, 2016; Nabekura et al., 2018) and CAPE has been reported to down-

regulate endogenous ABCB1, ABCC1 and ABCC2 transporter activity in both kidney (Wortelboer et al., 2005) and cancer cell lines (Takara et al., 2007; Teng et al., 2020).

In this thesis, treatment of PBECs with the NF-κB inhibitor SN50 alone had no significant effect on the endogenous activities of ABCB1, ABCG2 and ABCC5, which is consistent with studies demonstrating a lack of effect of SN50 on endogenous ABCB1 activity and expression in both kidney (Heemskerk et al., 2010; Ashraf et al., 2011) and primary mouse brain endothelial cells (Hayashi et al., 2006). SN50 is a synthetic peptide used almost exclusively as a NF-κB inhibitor. In contrast, both honokiol and CAPE are complex naturally occurring compound that involved in multiple cellular signalling pathways, for example, honokiol is able to exert anti-oxidation (Caballero et al., 2020), anti-cancer (Cheng et al., 2016), anti-apoptosis (Liu et al., 2019), neuroprotection (Morroni et al., 2018), anti-anxiety and anti-depression (Sulakhiya et al., 2015) activities; whilst CAPE demonstrated anti-tumour (Gupta et al., 2017), neuroprotection (Turan et al., 2020), anti-oxidant (Tolba et al., 2014) and immunomodulatory properties (Armitcu et al., 2015). This involvement in multiple intrinsic signalling pathways is the most likely reason why treating PBECs with either honokiol or CAPE alone inhibited activity of endogenous ABCB1, ABCG2 and ABCC5 transporters, a finding that was not observed with the more NF-κB-specific inhibitor, SN50.

Another possible reason for the differential outcomes observed between honokiol/CAPE and SN50 in ABC transporter activities is the different pathway exhibited by them. In this thesis, the honokiol- and CAPE-mediated inhibition of endogenous ABCB1, ABCG2 and ABCC5 transporter activities can be explained by the fact that both possess similar mechanisms in that they inhibit phosphorylation and activation of IKKα/β (Tse et al., 2005; Ahn et al., 2006; Sheu et al., 2008; Zhu, X. et al., 2014; Chen et al., 2014; Chen et al., 2016; Wang, L.C. et al., 2010; Bezerra et al. 2012), thereby blocking the inflammatory pathway at the IKKα/β stage. This subsequently leads to reduced phosphorylation and degradation of IκBα (Tse et al., 2005; Ahn et al., 2006; Sheu et al., 2008; Toyoda et al., 2009; van de Laar et al., 2010; Wang, L.C. et al., 2010; Lee et al., 2010; Shvarzbeyn and Huleihel, 2011; Bezerra et al. 2012; Chen et al., 2014; Cho et al., 2014; Zhu, X. et al., 2014; Liu et al., 2018; Yang et al., 2014; Chen et al., 2016; Li et al., 2017; Takakura et al., 2018) subsequently preventing the phosphorylation (Tse et al., 2005; Toyoda et al., 2009; Bezerra et al. 2012; Chen et al., 2014; Liu et al., 2014; Yang et al., 2014; Takakura et al., 2018; Liang et al., 2019), activation, nuclear translocation (Natarajan et al., 1996; Wang, L.C. et al., 2010; Shvarzbeyn and Huleihel, 2011;

Cho et al., 2014; Li et al., 2017; Zhu, X. et al., 2014) and DNA binding activity of p65 NF-κB (Natarajan et al., 1996; Márquez et al., 2004; Choi and Choi, 2008; Ha et al., 2009; Li et al., 2017). Unlike honokiol and CAPE, SN50 has no effect on ABC transporter activities and it is reported to have no effect on cytokine-induced phosphorylation of IKKα/β (Barma et al., 2009; Wu et al., 2020) and phosphorylation and degradation of IκBα (Lin et al., 1995; Kilgore et al., 1997; Uberti et al., 2004; Gross and Worms, 2005; Barma et al., 2009; Cho et al., 2011;Wu et al., 2020), in fact SN50 blocks the inflammation pathway downstream of IKK phosphorylation and IκBα degradation (Gross and Worms, 2005; Sinha et al., 2004), as a result, it effectively inhibited phosphorylation (Saika et al., 2005; Marquardt et al., 2015; Guan et al., 2019), nuclear translocation (Lin et al., 1995; Kilgore et al., 1997; Uberti et al., 2004; Saika et al., 2005; Cho et al., 2011; Chian et al., 2014; Pathak et al., 2014; Vulpis et al., 2017; Wu et al., 2020), DNA binding activity and transcriptional activation of p65 NF-κB (Mitsiades et al., 2002; Birkenkamp et al., 2004; Gross and Worms, 2005; Xie and Shaikh, 2006; Wu et al., 2020).

In attempt to establish if HK, CAPE and SN50 acted as substrate/inhibitor that directly inhibit transporter activity, as measured by the fluorescent substrate accumulation assay, PBECs were incubated for a short period of 30 min with either HK, CAPE or SN50. The findings show for the first time that neither of the three NF-κB inhibitors acted as substrate/inhibitor to ABCB1 ABCC5 and ABCG2.

5.1.2.2 The effect of inhibitors of IL-1β and NF-κB on nuclear receptor expression

The results presented in this thesis reinforce the need of characterising the signalling pathways involved in the regulation of ABC transporters during inflammation.

Most of the cited reports approached the issues from a deterministic perspective rather than mechanistic. Therefore, this study attempted to study the expression of the PXR, CAR, RXR and GR nuclear receptors, which are key regulators of ABC transporters in response to inflammation. In the current study PXR, CAR, RXR and GR were shown to be expressed in PBECs, with expression significantly upregulated upon treatment with IL-1β. Furthermore, treatment with honokiol completely abrogated the IL-1β-mediated increase in PXR, CAR, RXR and GR protein expression which is clearly reflected in the decrease of ABCB1, ABCG2 and ABCC5 transporter protein expression. This study has therefore clearly shown that IL-1β, and honokiol, regulate ABC transporter expression via a nuclear receptor-dependent pathway.

Thus far, studies of the interaction between honokiol and nuclear receptors are relatively few and the findings relate to kidney, intestine, liver and macrophages rather than blood-brain barrier endothelial cells. According to the available studies, honokiol functions as a nuclear receptor ligand, for example, honokiol is able to enhance ABCB1 transporter activity and protein expression in the presence of PXR (Nabekura et al., 2018). Also, honokiol is regarded as an RXR ligand capable of activating the transcriptional activity of RXRα in luciferase reporter assays and playing a subsidiary/synergistic role in the activation of RXR heterodimers (Kotani et al., 2010; Kotani et al., 2012; Latkolik, 2017). Furthermore, honokiol has been reported to increase expression of GRα mRNA and protein (Wang et al., 2018). Among the studies listed above, only Nabekura et al. (2018) established the link that activation of PXR and RXR by honokiol eventually led to an increase in ABCB1 transporter activity and protein expression (in intestinal cells). Even though the findings of our studies contradict the above studies, they are in line with several studies reporting honokiol down-regulated ABCB1 transporter activity and protein expression in cancer cells (Xu et al., 2006; Han and Van Anh, 2012; Wang et al., 2007). Similarly, findings of studies on the role of CAPE in the regulation of ABCB1, ABCCs transporters are also contradictory (Nabekura et al., 2018; Gou et al., 2016; Teng et al., 2020; Takara et al., 2007), whilst SN50 has been consistently shown to have no individual effect on ABCB1 activity and expression (Heemskerk et al., 2010; Hayashiet al., 2006). The discrepancies observed with honokiol may be largely explained by cell type and also the levels of nuclear receptors or coregulators expressed in the cells. In addition, honokiol has been reported to be gene specific or dependent on the surrounding regulatory proteins of the promoter region of target genes (Kotani et al., 2012).

Several studies have demonstrated that IL-1β induced upregulation of NF-κB to promote PXR and GR protein expression, and subsequently led to increases in their target gene expression (Mosaffa et al., 2009; Malekshah et al., 2011; Malekshah et al., 2012). IL-1β was also shown to induce GR nuclear translocation, DNA binding activity, transcription and sensitivity towards glucocorticosteroids (Rakasz et al., 1993; Falus et al., 1995; Costas et al., 1996; Verheggen et al., 1996). This result is in line with our studies showing IL-1β upregulated both ABC transporters and nuclear receptors expression. Pradhan et al. (2010) have demonstrated the crucial cooperative interaction between NF-κB and nuclear receptor to promote target gene expression, for example NF-κB was shown to interact with the estrogen receptor (ER) leading to upregulation of ABCG2 expression. ER is required to facilitate the binding of p65 to the NF-κB response element located near the estrogen response element (ERE)

in the gene promoter, while recruitment of NF-κB to the gene is required to stabilise the occupancy of ER at the functional ERE. The cooperative binding of ER and p65 at adjacent response elements leads to a major increase in *ABCG2* protein expression. Hence, it is possible that the cooperative effort between NF-κB and PXR, CAR, RXR and GR leads to the upregulation of ABC transporter activity and protein expression observed in this study. In this thesis, inhibition of NF-κB using honokiol led to down-regulation of ABC transporter and nuclear protein expression, which could clearly demonstrate the role of NF-κB in regulating ABC transporters during inflammatory events in the blood-brain barrier.

5.1.2.3 The effect of inhibitors of IL-1β and MAP Kinase pathway on ABC transporter activities

Apart from NF-κB which is involved in IL-1β-mediated inflammation, recent reports indicate that endothelial mitogen-activated protein kinase (MAPK) activation can lead to inflammation via downstream activation of canonical NF-κB signalling (Bottero et al., 2006; Baker et al., 2011; Roth Flach et al., 2015; Espinoza-Sánchez et al., 2019) and MAPK has been reported to be activated by the pro-inflammatory cytokines IL-1β and TNF-α (Kim and Choi, 2015; Verma and Datta, 2012). Many studies have shown that activation of MAPK, especially ERK, p38 and JNK signalling, drives activation and nuclear translocation of NF-κB in human brain microvascular endothelial cells (Bai et al., 2015), mouse brain endothelial cells (Zhou et al., 2018) and in human and rat BBB *in vivo* (Yuan et al., 2020) in inflammatory conditions. On the other hand, inhibiting p38, JNK and ERK prevents NF-κB phosphorylation and nuclear translocation, diminishing NF-κB-driven transcriptional activity and attenuating expression of NF-κB target genes (Carter et al., 1999; Li et al., 2002; Saha et al., 2007; Zhu, F. et al., 2014; Bai et al., 2015; Zhou et al., 2018; Yuan et al., 2020).

In order to further characterise the downstream events of IL-1β signalling involving MAPK, the three highly selective MAPK inhibitors SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) and FR180204 (ERK inhibitor) were used. In this thesis, SP600125 (JNK inhibitor) and FR180204 (p38 inhibitor), but not FR180204 (ERK inhibitor) effectively nullified the IL-1β-mediated increase in ABC transporter activity, demonstrating that ERK and JNK are involved in IL-1β-mediated regulation of ABC transporter activity. This finding is in line with other reports demonstrating that activation of ERK1/2 and JNK in inflammation is involved in the up-regulation of ABCB1 activity, mRNA and protein expression in mouse brain endothelial cells and astrocytes (Hayashi et al., 2006; Hartz et al., 2008), lung cancer cells (Shinoda et al.,

2005), lymphoma cell lines (Tomiyasu et al., 2013b) and lymphoblastic leukemia cells (Tomiyasu et al., 2013a). A similar effect has also been observed with ABCG2, in which activation of ERK1/2 and JNK pathway up-regulated ABCG2 gene expression in lymphoblastic leukaemia cells (Tomiyasu et al., 2013a) and in a colon cancer cell line (Zhu et al., 2012). Studies reporting the effect of MAPK signalling on members of the ABCC family are rare. Thus far, activation of the ERK signalling pathway leading to up-regulation of ABCC1 mRNA and transport activity has been reported in lymphocyte and lymphoblastic leukaemia cell lines (Azreq et al., 2012).

MAPK, which is involved in the activation of NF-κB, is located upstream of the transcription factor. Intriguingly, in this study, inhibiting MAPK using JNK and p38 inhibitors is able to nullify the effect of IL-1β mediated increase in ABC transporter activities but not in the case of inhibiting NF-κB using NF-κB inhibitors (honokiol, CAPE and SN50). The conflicting outcomes between both NF-κB and MAPK inhibitors on ABC transporter activities may be due to the implications of regulation of ABC transporter expression, degradation, trafficking, phosphorylation, and protein-protein interaction as mentioned in Section 5.1.2.1. are relatively understudied and unexplored. Hence, future study of the detailed mechanisms of MAPK and NF-κB in protein modification, protein localisation and protein-protein interaction may probably help to explain the up/downregulation of ABC transporter activity independent of their protein expression.

In this study, treating PBECs with SB203580 alone had no effect on the activities of the ABCB1, ABCG2 and ABCC5 transporters; however, treating cells with either SP600125 or FR180204 alone significantly reduced transport activities of all the three transporters, similar to what was observed following treatment with the NF-κB inhibitors Honokiol and CAPE. These findings prove that SP600125 (JNK inhibitor) and FR180204 (ERK 1/2 inhibitor) are involved in reducing endogenous activity of ABC transporters. In an attempt to determine if the reduction in transporter activity associated with treatment with MAPK inhibitors is related to direct interaction with ABC transporters, i.e. inhibition of ABCB1, ABCG2 and ABCC5 activity, transport studies were carried out where the time cells were incubated with SP600125 alone and FR180204 alone was reduced from 24 h to 30 min. Under this short-term exposure to MAPK inhibitors, SB203580, SP600125 and FR180204 did not result in significant changes in ABCB1, ABCG2 and ABCC5 transporter activities, indicating that both MAPK inhibitors used in this study did not directly interfere with the transporters themselves, i.e. they are not

substrates or inhibitors of the three ABC transporters. Since both SP600125 and FR180204 are not substrates/inhibitors of the ABCB1, ABCG2 and ABCC5 transporters, SP600125 and FR180204 associated reduction in ABC transporter activities is probably exerted through a decrease in transporter protein expression, however, it was not possible to investigate this due to lack of time.

The findings of this study are consistent with the other studies reporting SB203580 did not affect the phosphorylation of p38, ABCB1 protein expression and endogenous transport activity in colon cancer cell lines (Katayama et al., 2007) and rat brain capillary endothelial cells (Wang, X. et al., 2014). The results of SP600125 (JNK inhibitor) downregulated the endogenous ABCB1, ABCG2 and ABCC5 transporter activities in this thesis is consistent with studies that show SP600125 inhibited JNK activity (Zhou et al., 2006), down-regulated ABCG2 mRNA and protein expression and reduced ABCG2 endogenous activity in colon cancer cells (Zhu et al., 2012) and down-regulated ABCB1 protein expression and activity in human bronchial epithelial cell (Wang et al., 2015). To date, the ability of FR180204 (ERK1/2 inhibitor) to modulate ABC transport activity have not been reported, hence our studies constitute the first experimental evidence FR180204 in downregulating the endogenous ABCB1, ABCG2 and ABCC5 transporter activities. Thus far, U0126 and PD98059, two of the most studied ERK inhibitors, are reported to down-regulate ABCB1, ABCG2 and ABCC1 mRNA and protein expression and reduce endogenous activity in colon cancer cells (Katayama et al., 2007; Zhu et al., 2012).

Collectively, the data presented in this thesis suggest that the MAPK and NF-κB signalling pathways are key pathways responsible for modulating expression and activity of transporter-based detoxification systems in blood-brain barrier endothelial cells in response to exposure to pro-inflammatory cytokine.

5.1.2.4 Limitations of the study

A limitation of this study is the use of non-specific NF-κB inhibitors, Honokiol and CAPE, which have been reported to be implicated in multiple cellular signalling pathway. Honokiol has been demonstrated to exhibit anti-angiogenic, anti-tumour, anti-inflammatory and antioxidative activities by triggering a wide-range of cellular signalling pathways involving VEGF (Wen et al., 2015), protein kinase B/Akt (Bai et al., 2003), NADPH oxidase, myeloperoxidase, cyclooxygenase glutathione (GSH) peroxidase, NF-κB (Liou et al.,

2003; Dikalov et al., 2008; Sheu et al., 2008), inducible nitric oxide synthase (iNOS), TNF-a (Son et al., 2000; Matsuda et al., 2001), protein kinase C (PKC) (Wang et al., 1998) and MAP kinase (p38, JNK, ERK) (Bai et al., 2003, Yang et al., 2003; Shtil et al., 2001; Kuo et al., 2002; Callaghan et al., 2013; Thulasiraman et al., 2016).

On the other hand, CAPE has been discovered to possess various activities, such as anti-angiogenic (Song et al., 2002; Basini et al., 2012), anti-tumour (Lin et al., 2010), antiapoptotic (Chen et al., 2001; Watabe et al., 2004), antimicrobial (Velazquez et al., 2007; Lee et al., 2013), antioxidant (Russo et al., 2002; Jo et al., 2013; Kim and Jang, 2014), anti-inflammatory (Tambuwala et al., 2018), and cytotoxicity activities. Also, CAPE has been reported to be involved in a wide-range of cellular signalling pathways, including PI3K/AKT (Yang et al., 2014; Khan et al., 2018), NF-κB p65 subunit nuclear translocation and DNA binding activity (Ilhan et al., 2004; Márquez et al., 2004; Jung et al., 2008; Cao et al., 2011; Shen et al., 2013; Khan et al., 2018), IκB-α phosphorylation, (Márquez et al., 2004; Toyoda et al., 2009; Lin et al., 2010; Yang et al., 2014; Khan et al., 2018), MAPK family proteins p38, ERK1/2 and JNK (Watabe et al., 2004; Kwak & Seok-Jai, 2012), IL-1β, TNF-α, PKC, IFN-γ, IL-2, IL-6 and IL-8 (da Cunha et al., 2004; Toyoda et al., 2009; Cao et al., 2011; Kwak & Seok-Jai, 2012; Shen et al., 2013; Jo et al., 2013; Zhao et al., 2014).

Among the signalling molecules regulated by honokiol and CAPE, many studies have reported that some of the signalling molecules have significant effects on the activity and expression of ABCB1, ABCG2, ABCC1 and ABCC2, and those signalling molecules include IL-1β (von Wedel-Parlow et al., 2009; Poller et al., 2010; Iqbal, 2012; Zuloaga et al., 2012; Torres-Vergara and Penny 2018; Alasmari et al., 2018), TNF-α (Heemskerk et al., 2010; Miller 2010; Poller et al., 2010), Glutamate (Bauer et al., 2008b; Yousif et al., 2012), vascular endothelial growth factor (Hawkins et al., 2010; Banković et al., 2013; de Gooijer et al., 2021), nuclear factor erythroid 2–related factor 2 (Wang, X. et al., 2014; Grewal et al., 2017), PI3K/AKT (Wu et al., 2020; Zhang et al., 2020), MAPK (JNK) (Zhou et al., 2006; Zhu et al., 2012; Wang et al., 2015), MAPK (ERK1/2) (Katayama et al., 2007; Zhu et al., 2012), NF-κB (Ronaldson & Bendayan, 2008; Kooij et al., 2011; Elali & Hermann, 2012; Tamima et al., 2014), inducible nitric oxide synthase (Heemskerk et al., 2007), protein kinase A (Idriss et al., 2000; Wojtal et al., 2006; Wimmer et al., 2008; Stolarczyk et al., 2011), protein kinase C (Conseil et al., 2001; Kameyama et al., 2008; Xie et al., 2008; Schonhoff et al., 2013; Answer et al., 2014; Mayati

et al., 2015; Michaelis et al., 2015; Miklos et al., 2015; Schonhoff et al., 2016), cyclooxygenase-2 (Saikawa et al., 2004; Arunasree et al., 2008; Surowiak et al., 2008; Kalalinia et al., 2011; Liu et al., 2013; Pagliarulo et al., 2013; Chae et al., 2015) and NADPH oxidase (Hartz et al., 2008).

At the present time, it is only possible to assume that some of these molecular signals may be involved in reducing the basal activity and expression of ABCB1, ABCG2 and ABCC5. Furthermore, several signalling molecules, such as PI3K, MAPK and PKCs regulated by honokiol and CAPE are actively involved in post-translational processes, such as insertion and retrieval of ABC transporters within the plasma membrane which may influence ABC transporter activity independent of their protein expression. This may be important when studying the influence of NF-κB inflammatory pathways on ABC transporter activity and expression.

Apart from the multiple regulatory pathways influenced by honokiol and CAPE, honokiol was found to be both ABCB1 substrate and inhibitor in Caco-2 cells (Zeng et al., 2011), and functions (like verapamil) by either directly inhibiting ABCB1 activity or stimulating ATPase activity to accelerate the consumption of ATP and eventually deplete the levels of ATP (Han et al., 2012). In comparison to honokiol, CAPE is not an ABCB1 substrate, but functions as verapamil to directly inhibit ABCB1, ABCC1 and ABCC2 activity by stimulating ATPase activity (Wortelboer et al., 2005; Takara et al., 2007; Nabekura et al., 2018; Teng et al., 2020). However, in this thesis, honokiol and CAPE did not exert direct inhibition of ABC transporters in our 30 min transporter activity assay. Amid the non-specificity of both honokiol and CAPE as NF-κB inhibitor, in the future analysis, a highly specific NF-κB inhibitor such as SN50, which is neither an inhibitor of ABC transporters nor involved in multiple signalling pathways can be used in order to accurately present the effect of pro-inflammatory cytokines on ABC transporter activity and protein expression.

The effect of MAP kinase in the regulation of ABC transporters has yet to be elucidated in this study, as only transporter activity assays were carried out, it is unclear as whether MAP kinase is involved in regulating nuclear receptors. Future analysis is needed to clarify the detailed signalling pathway.

Chapter 6

General Conclusions and Future Work

6.1 General conclusions

ABC drug efflux transporters are important component of BBB that plays an important role in transporting both endogenous and xenobiotics, out of the CNS (Miller, 2015b; Puecha et al., 2018). Although these transporters are crucial in providing neuroprotection, their wide range substrate specificities grant them the potential to block the entry of many therapeutic drugs into CNS (Miller, 2015b; Cannon et al., 2020). As a consequence, these ABC efflux transporters significantly affect the bioavailability, therapeutic efficacy and pharmacokinetics of a variety of drugs thereby contributing to the development of pharmacoresistance (Miller, 2015b; Mahringer and Fricker, 2016; Leandroa et al., 2019). An understanding of ABC transporter regulatory network at the blood–brain barrier, could lead to novel strategies to improve pharmacotherapy of numerous brain disorders, such as Alzheimer's disease, epilepsy, or brain cancer.

The aim of this thesis is to establish an optimum culture condition for the growth of PBECs as the in vitro blood-brain barrier model, to investigate the regulatory mechanism of nuclear receptors on ABC transporters and investigate the pro-inflammatory and anti-inflammatory effect on ABC transporters. The *in vitro* model employed in this study has been developed and refined by Dr. Jeffrey Penny´s group over the years. This *in vitro* model retains most of the *in vivo* BBB phenotype, including demonstration of elongated spindle-like morphology expected of brain endothelial cells in culture, shows high TEER and low permeability, and the expression of tight junction proteins (ZO-1 and occludin), endothelial marker enzymes (y-glutamyl transpeptidase and alkaline phosphatase), key ABC transporters and nuclear receptors responsible for regulating ABC transporter expression (Skinner et al. 2009; Cantrill et al., 2012; Shubbar and Penny 2018; Torres-Vergara and Penny 2018).

The first part of the thesis focused on the optimisation and characterisation of PBECs culture. Among the three different types of media investigated, DMEM proved to be the most suitable culture medium to promote functional activity of ABC transporters. The employed model also shown to express functional ABCB1, ABCG2 and ABCC5 transporters, as well as PXR, CAR, RXR and GR nuclear receptors.

In the second part of the thesis, ABCB1, ABCG2 and ABCC5 activity and expression are significantly influenced by the activation and inhibition of PXR and CAR using

the pharmacological inducers rifampicin (PXR agonist) and CITCO (CAR agonist), and inhibitors L-sulforaphane (PXR antagonist) and meclizine (CAR inverse agonist). More importantly, dexamethasone treatment demonstrated that GR is integrally involved in the regulation of ABCB1, ABCG2 and ABCC5 in an PXR or CAR-dependent pathway, which further confirmed the existence of a functional cross talk between the GR- and PXR/CAR signalling pathways.

The third part of the thesis presented the inflammatory pathway of IL-1β in the regulation of ABC transporters. It was shown that IL-1β-induced upregulation of ABCB1, ABCG2 and ABCC5 transporters in an NF-κB and MAPK-dependent pathway. Use of the NF-κB inhibitors, honokiol and CAPE successfully attenuated IL-1β-induced upregulation of ABCB1, ABCG2 and ABCC5 protein expression, but not their transport activities, suggesting the involvement cellular machinery and trafficking that alter the transporter activities independent of protein expression. On the other hand, blocking MAPK with JNK and ERK inhibitors successfully abrogated the IL-1β-induced upregulation of ABCB1, ABCG2 and ABCC5 transport activities, suggesting the involvement JNK and ERK in IL-1β inflammatory pathway.

In conclusion, the outcome of this study further advance our understanding of the complex regulatory network between GR, PXR, CAR and RXR that are actively participated in the regulation of ABCB1, ABCG2 and ABCC5 transporter activity and expression at BBB. Furthermore, this complex regulatory network of nuclear receptors are subjected to the influence of pro-inflammatory and inflammatory compounds, which in turn affecting the ABC transport activity and expression. Our findings constitute the first evidence that GR is integrally involved in the regulation of ABCB1, ABCG2 and ABCC5 transporter activity and expression, and also the first to report that ABCC5 is tightly regulated by nuclear receptors and inflammation.

The outcomes of this thesis have laid the groundwork of how ABC transporters can be regulated by nuclear receptors and also by pro-inflammatory cytokines in disease conditions. This knowledge is crucial in improving future drug design and delivery method to facilitate therapeutic drug to cross BBB, which is the only way to enhance drug concentration in the brain and its efficacy in the treatment of brain diseases.

6.2 Future work

The *in vitro* model employed in this thesis is suitable to be used to further characterise the detailed signalling pathway of other nuclear receptors that may probably involve in modulating ABC transporters.

Overall in this study, it is clear that nuclear receptors PXR, CAR, RXR and GR are actively involved in the regulation of ABC transporter activity and protein expression, and their activities are influenced by proinflammatory cytokines.

This study has shown that PXR, CAR and RXR protein expression are significantly affected by GR ligands but not PXR and CAR ligands. However, further studies are needed to investigate whether activation of GR would lead to increased PXR and CAR transcription activity or whether GR has a direct influence on ABC transporter activity and protein expression.

Given the significant influence of inflammation on nuclear receptors and ABC transporters in this study, it would be interesting to expand these studies to investigate a wider range of pro-inflammatory cytokines and anti-inflammatory compounds at different time points. Furthermore, the influence of IL-1β and NF-κB/MAPK inhibitors on protein localisation of NF-κB and MAPK should be carried out in order to further confirm the involvement of both NF-κB and MAPK in regulating the expression level of ABC transporters in the event of inflammation.

Based on the outcomes of this study, protein expression is not always a true representation ABC transporter activity and future studies will investigate the roles of post-translational modification and translocation on ABC transporter activity. In particular, protein kinase C (PKC) is commonly involved in the phosphorylation of ABC transporters and studies will investigate the activity of PKC and determine the degree of transporter phosphorylation in PBECs. In term of translocation of ABC transporters, it is of interest to compare the protein expression levels in plasma membrane, cytoplasm and nucleus in order to determine the localisation of ABC transporters, as insertion into or retrieval from the plasma membrane may significantly affect substrate accumulation in cells. Hence, future studies of ABC transporter activity, protein expression, post-translational modification and translocation of ABC

transporters would offer a more complete picture of how ABC transporter activities are regulated.

Appendices

Appendix A – List of human PXR and CAR ligands

Table A.1: List of specific human PXR and CAR ligands

Human PXR		Human CAR	
Angonist		Angonist	
Synthetic drugs	**References**	**Synthetic drugs**	**References**
Artemisinin	Burk et al., 2005; Persson et al., 2006; Faucette et al., 2007; Burk et al., 2012	Amiodarone	Lynch et al., 2013
Avasimibe	Sahi et al., 2003	Artemisinin	Burk et al., 2005; Faucette et al., 2007; Burk et al., 2012; Kublbeck et al., 2011
Betamethasone	Persson et al., 2006	Atorvastatin	Kobayashi et al., 2005 contradict with Haslam et al., 2008
BK8644	Drocourt et al., 2001	Carbamazepine	Faucette et al., 2007
Bosentan	van Giersbergen et al., 2002	Carvedilol	Lynch et al., 2013
Carbamazepine	Geik et al., 2001; Luo et al., 2002; Persson et al., 2006; Faucette et al., 2007	Cerivastatin	Kobayashi et al., 2005
Chlorpromazine	Faucette et al., 2007	Chlorpromazine	Swales et al., 2005; Faucette et al., 2007
Clotrimazole	Bertilsson et al., 1998; Lehmann et al., 1998; Moore et al., 2000; Geik et al., 2001	CITCO	Maglich et al., 2003; Huang et al., 2003; Kublbeck et al., 2011
Colupulone	Teotico et al., 2008	Clotrimazole	Moore et al., 2000; Jyrkkarinne et al., 2008; Kublbeck et al., 2011; Lynch et al., 2012
Cyclophosphamide	Faucette et al., 2007	Cyclophosphamide	Faucette et al., 2007
Dexamethasone	Lehmann et al., 1998; Moore et al., 2000a; Geik et al., 2001; Persson et al., 2006;	Flexible diaryl compounds (FL81)	Kublbeck et al., 2011
Etoposide	Schuetz et al., 2002	Fluvastatin	Kobayashi et al., 2005
Isradipine	Drocourt et al., 2001	Nefazodone	Lynch et al., 2013
Lansoprazole	Persson et al., 2006	Nicardipine	Lynch et al., 2013
Lovastatin	Lehmann et al., 1998; Faucette et al., 2007	Reserpine	Faucette et al., 2007
Metyrapone	Faucette et al., 2007	Simvastatin	Kobayashi et al., 2005
Mevastatin	Raucy et al 2002; Faucette et al., 2007	Sulfonamides	Kublbeck et al., 2008
Mifepristone (RU486)	Kliewer et al., 1998; Moore et al., 2000a; Lehmann et al., 1998	Telmisartan	Lynch et al., 2013
Nicardipine	Drocourt et al., 2001; Faucette et al., 2007	Thiazolidin-4-ones	Kublbeck et al., 2008

Nifedipine	Bertilsson et al., 1998; Blumberg et al., 1998; Geik et al., 2001; Faucette et al., 2007	Tolnaftate	Lynch et al., 2013
Omeoprazole	Persson et al., 2006; Faucette et al., 2007	Troglitazone	Faucette et al., 2007
Paclitaxel	Synold et al., 2001		
Pantoprazole	Persson et al., 2006		
Phenobarbital	Luo et al., 2002; Lemaire et al., 2004; Persson et al., 2006		
Phenytoin	Luo et al., 2002; Persson et al., 2006		
Pregnenolone-16α-carbonitrile	Moore et al., 2000a; Geik et al., 2001		
6,16α-dimethylpregnenolone	Geik et al., 2001		
Primaquine	Persson et al., 2006		
Rabeprazole	Persson et al., 2006		
Reserpine	Geik et al., 2001; Faucette et al., 2007		
Rifampicin	Bertilsson et al., 1998; Lehmann et al., 1998; Jones et al., 2000; Moore et al., 2000a; Geik et al., 2001; Chrencik et al., 2005; Lemaire et al., 2004; Persson et al., 2006		
Simvastatin	Faucette et al., 2007		
Spironolactone	El-Sankary et al., 2000		
SR12813	Moore et al., 2000a; Watkins et al., 2001		
Sulfadimidine	Luo et al., 2002		
Tamoxifen (4-hydroxy-tamoxifen)	Desai et al., 2002		
Taxol	Luo et al., 2002		
TCPOBOP	Moore et al., 2000a		
Topiramate	Nallani et al., 2003		

Topotecan	Schuetz et al., 2002 contradict with Haslam et al., 2008		
Troglitazone	Jones et al., 2000; Faucette et al., 2007		
T0901317	Xue et al., 2007		
Verapamil	Persson et al., 2006		
Warfarin	Persson et al., 2006		
Zearalenone	Mnif et al., 2007		
Steroids	**References**	**Steroids**	**References**
Androstanol	Moore et al., 2000	5β-pregnane-3,20-dione	Moore et al., 2000; Maglich et al., 2003
Corticosterone	Blumberg et al., 1998; Kliewer et al., 1998; Geik et al., 2001		
Estradiol (17β-Estra-diol)	Blumberg et al., 1998; Xue et al., 2007; Del-fosse et al., 2015		
Ethynylestradiol (17α-ethinylestradiol)	Delfosse et al., 2015		
Progesterone	Blumberg et al., 1998; Kliewer et al., 1998		
Zearalenone	Mnif et al., 2007		
5β-pregnane-3,20-di-one	Kliewer et al., 1998; Lehmann et al., 1998; Geik et al., 2001; Lemaire et al., 2004		
5β-Cholestan-3α,7α,12α-triol	Goodwin et al., 2003		
17α-hydroxypregne-nolone	Lehmann et al., 1998		
17α-hydroxyprogester-one	Lehmann et al., 1998		
Nonsteroidal com-pounds	**References**	**Nonsteroidal com-pounds**	**References**
Ferutinine	Mnif et al., 2007	Diethylstilbestrol	Kublbeck et al., 2011
Indomethacin	Persson et al., 2006		
HIV drugs	**References**	**HIV drugs**	**References**
Abacavir	Svärd et al., 2010	Abacavir	Svärd et al., 2010; Chan et al., 2013

Amprenavir	Gupta et al., 2008; Chan et al., 2013	Efavirenz	Faucette et al., 2007; Li et al., 2009; Chan et al., 2013
Atazanavir	Gupta et al., 2008; Chan et al., 2013	Nevirapine	Faucette et al., 2007; Li et al., 2009; Chan et al., 2013
Darunavir	Chan et al., 2013	Fosamprenavir	Svärd et al., 2010
Efavirenz	Hariparsad et al., 2004; Faucette et al., 2007; Svärd et al., 2010; Chan et al., 2013	Lopinavir	Svärd et al., 2010; Chan et al., 2013
Fosamprenavir	Svärd et al., 2010	Tipranavir	Svärd et al., 2010
Indinavir	Gupta et al., 2008		
Lopinavir	Gupta et al., 2008; Svärd et al., 2010; Chan et al., 2013		
Nelfinavir	Svärd et al., 2010		
Nevirapine	Faucette et al., 2007		
Ritonavir	Dussault et al., 2001; Luo et al., 2002; Tipra-navir Gupta et al., 2008; Svärd et al., 2010; Chan et al., 2013;		
Saquinavir	Gupta et al., 2008		
Natural Products	**References**	**Natural Products**	**References**
Coumestrol	Blumberg et al., 1998	Baicalein	Carazo et al., 2015
Cis-guggulsterone	Owsley & Chiang, 2003; Brobst et al., 2004		
Trans-guggulsterone	Owsley & Chiang, 2003; Brobst et al., 2004	Chrysin	Yao et al., 2011
Kava extract (Desmeth-oxyyangonin)	Ma et al., 2004	Ellagic acid	Yao et al., 2011
Kava extract (Dihydro-methysticin)	Ma et al., 2004	Galangin	Carazo et al., 2015
Forskolin	Ding & Staudinger, 2005		
Hyperforin	Moore et al., 2000b; Watkins et al., 2003; Persson et al., 2006		
Schisandrin	Mu et al., 2006		
Plasticizer	**References**	**Plasticizer**	**References**
Mono(2-ethylhexyl) phthalate	Hurst and Waxman, 2004	Octicizer (Strong)	Lynch et al., 2013
		Triaryl phosphates	Jyrkkarinne et al., 2008
		di(2-ethylhexyl) phthalate	DeKeyser et al., 2009

Chemicals	References	Insecticide/pesticides	References
Polychlorinated biphenyls	Wahlang et al., 2014	Carbamates (benfuracarb)	Abass et al., 2012
		Pyrethroids (permethrin, cypermethrin)	Kublbeck et al., 2011
Bile acids	**References**	Organochlorines (Methoxychlor & o,p′-DDT)	Kublbeck et al., 2011
Cholic acid	Staudinger et al., 2001; Ma et al., 2008		
Chemicals	**References**		
Polychlorinated biphenyls	Wahlang et al., 2014		
Antagonist		**Indirect activators**	
Synthetic drugs	**References**	**Synthetic drugs**	**References**
Ecteinascidin-743	Synold et al., 2001	Phenobarbital (Weak)	Moore et al., 2000; Kublbeck et al., 2011
Ketoconazole	Takeshita et al., 2002	Phenytoin (Weak)	Wang et al., 2004; Kublbeck et al., 2011
Trabectedin	Synold et al., 2001		
A-792611	Healan-Greenberg et al., 2008; Chan et al., 2011		
Natural products	**References**	**Natural products**	**References**
Sulforaphane	Zhou et al., 2007; Lemmen et al., 2013	6,7-dimethylesculetin	Huang et al., 2004
		Inverse agonist	
		Synthetic drugs	**References**
		Clotrimazole	Moore et al., 2000; Maglich et al., 2003; Huang et al., 2003; Jyrkkarinne et al., 2008; Lynch et al. 2012
		Meclizine	Huang et al., 2003; Chan et al., 2011; Kublbeck et al., 2011; Lemmen et al., 2013 Pb bbb
		PK11195	Li et al., 2008; Kublbeck et al., 2011; Lynch et al., 2012 Kanno et al., 2014
		S07662	Kublbeck et al., 2011
		CINPA1	Cherian et al., 2015
		Steroids	**References**

		5α-Androstan-3α-ol (Androstanol)	Moore et al., 2000; Sakai et al., 2009; Dau et al., 2013
		5α-Androst-16-en-3α-ol (Androstenol)	Burk et al., 2005; Sakai et al., 2009; Dau et al., 2013
		Estradiaol (17β-Estradiaol)	Dau et al., 2013
		Ethinylestradiol (17α-Ethinylestradiol)	Jyrkkärinne et al., 2005; Dau et al., 2013; Kanno et al., 2014

Appendix B- Materials

Materials	Company
Acetic acid	Fisher scientific , UK
Acrylamide/ bisacrylamide 30% filtered solution	Sigma-Aldrich Chemical Co, UK
Anti-ABCB1 polyclonal antibody (22336-1-AP)	Proteintech, Manchester, UK
Anti-β-actin monoclonal antibody (A5441-.2ML)	Sigma-Aldrich Chemical Co, UK
Anti-CAR antibody (Ab83850)	Abcam, UK
Anti-glucocorticoid receptor antibody (ab183127)	Abcam, UK
Anti-MRP5 antibody (ab115327)	Abcam, UK
Anti-PXR (ab81970)	Abcam, UK
Anti-RXR alpha antibody [EPR7106] (ab125001)	Abcam, UK
Anti-vinculin (ab129002)	Abcam, UK
Astrocyte cell line CTX-TNA2 line	ECACC, UK
Bovine serum albumin	Sigma-Aldrich Chemical Co, UK
Bradford reagent	Bio-Rad laboratories Ltd, UK
BXP-53 anti-ABCG2 monoclonal antibody (ab24115)	Abcam, UK
Calcein acetoxymethyl ester (Calcein-AM)	Invitrogen, Paisley, Scotland
Caffeic Acid Phenethyl Ester	Stratech Scientific Ltd, UK
CAPS (3-(cyclohexylamino)-1-propane sulfonic acid	MERCK, Germany
Cellytic M	Sigma-Aldrich Chemical Co, UK
Collagen type I (rat tail)	SLS Ltd, UK
CELLVIEW cell culture slide	Greiner Bio-One, UK
CITCO	Santa Cruz Biotechnology, UK
Collagenase type 3	CLS-3 Lorne Laboratories Ltd, UK
CMFDA (5-Chloromethylfluorescein diacetate)	Abcam, UK
Dexamethasone	Sigma-Aldrich Chemical Co, UK
Dimethylsulfoxide	Sigma-Aldrich Chemical Co, UK
Dulbecco’s Modified Eagle Medium (DMEM)	Life Technologies Ltd, UK
DMEM (high glucose, no pyruvate)	Sigma-Aldrich Chemical Co, UK
Dulbecco’s phosphate buffered saline	Sigma-Aldrich Chemical Co, UK
Deoxyribonuclease I (DP)	Lorne Laboratories Ltd, UK
Ethanol	Fisher scientific , UK
Fibronectin (human)	SLS Ltd, UK
Foetal Bovine Serum	Invitrogen, Paisley, Scotland
FR180204	Stratech Scientific Ltd, UK
Heparin sodium salt from porcine intestinal mucosa	Alfa Aesar, US
HEPES sterile solution	Thermo Fisher Scientific, US
Hoechst 33342	Sigma-Aldrich Chemical Co, UK
Honokiol	Stratech Scientific Ltd, UK
Hybond P PVDF membranes for Western blot	GE Healthcare Life Sciences, UK
Hydrochloric acid	Fisher scientific , UK
Hydrocortisone	Sigma-Aldrich Chemical Co, UK
HRP mouse anti-goat conjugated secondary antibody	Santa Cruz Biotechnology, UK
HRP mouse-anti rabbit conjugated secondary antibody	Santa Cruz Biotechnology, UK
HRP mouse anti-rat conjugated secondary antibody	Santa Cruz Biotechnology, UK
HRP sheep anti-goat conjugated secondary	GE Healthcare Life Sciences, UK

antibody	
HRP sheep anti-mouse conjugated secondary antibody	GE Healthcare Life Sciences, UK
Interleukin-1β recombinant from porcine	R & D System, USA
Ko143	Tocris Bioscience, UK
Laemmli sample buffer 6 X	Alfa Aesar, UK
L-glutamine solution	Invitrogen, Paisley, Scotland
L-sulforaphane	Sigma-Aldrich Chemical Co, UK
M199 medium	Sigma-Aldrich Chemical Co, UK
Meclizine	Alfa Aesar, UK
Methanol	Sigma-Aldrich Chemical Co, UK
2- mercaptoethanol	Sigma-Aldrich Chemical Co, UK
MK 571 sodium salt	Sigma-Aldrich Chemical Co, UK
Neutral Red dye	Sigma-Aldrich Chemical Co, UK
Penicillin/streptomycin mixture solution	Sigma-Aldrich Chemical Co, UK
Plasma Derived serum	First Link, UK
Potassium chloride	Sigma-Aldrich Chemical Co, UK
Protease inhibitor cocktail	Sigma-Aldrich Chemical Co, UK
Puromycin dihydrochloride	Sigma-Aldrich Chemical Co, UK
Radiance Plus	Cambridge Bioscience Limited
Rifampicin	Sigma-Aldrich Chemical Co, UK
SB203580	Stratech Scientific Ltd, UK
SN50	Eurogentec Ltd, UK
SP600125	Stratech Scientific Ltd, UK
TEMED	Sigma-Aldrich Chemical Co, UK
T-flask 75 cm2	Greiner Bioscience, UK
Tissue culture 6-well plates	Greiner Bioscience, UK
Tissue culture 96-well flat-bottomed plates	Sarstedt, Germany
Trypan blue	Bio-Rad laboratories Ltd, UK
Trizma base	Sigma-Aldrich Chemical Co, UK
Trypsin-EDTA solution	Sigma-Aldrich Chemical Co, UK
Trypsin (TRL)	Lorne Laboratories Ltd, UK
Tween 20	Sigma-Aldrich Chemical Co, UK
Verapamil	Sigma-Aldrich Chemical Co, UK
Universal container 7 ml, 25 ml and 50 ml	Greiner Bioscience, UK

Appendix C- Media formulation

Table C.1: DMEM formulation

Components	**Concentration (mg/L)**
Amino Acids	
Glycine	30.0
L-Arginine hydrochloride	84.0
L-Cystine 2HCl	63.0
L-Glutamine	292.0
L-Histidine hydrochloride-H_2O	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-Lysine hydrochloride	146.0
L-Methionine	30.0
L-Phenylalanine	66.0
L-Serine	42.0
L-Threonine	95.0
L-Tryptophan	16.0
L-Tyrosine	72.0
L-Valine	94.0
Vitamins	
Choline chloride	4.0
D-Calcium pantothenate	4.0
Folic Acid	4.0
Niacinamide	4.0
Pyridoxine hydrochloride	4.0
Riboflavin	0.4
Thiamine hydrochloride	4.0
i-Inositol	7.2
Inorganic Salts	
Calcium Chloride ($CaCl_2$.2H2O)	264.0
Ferric Nitrate ($Fe(NO_3)_3.9H_2O$)	0.1
Magnesium Sulfate ($MgSO_4$.7H2O)	200.0
Potassium Chloride (KCl)	400.0
Sodium Bicarbonate ($NaHCO_3$)	3700.0
Sodium Chloride (NaCl)	6400.0
Sodium Phosphate monobasic ($NaH_2PO_4.2H_2O$)	141.0
Other Components	
D-Glucose (Dextrose)	1000.0
Sodium Pyruvate	110.0

Table C.2: DMEM/F12 formulation

Components	Concentration (mg/L)
Amino Acids	
Glycine	18.75
L-Alanine	4.45
L-Arginine hydrochloride	147.5
L-Asparagine-H_2O	7.5
L-Aspartic acid	6.65
L-Cysteine hydrochloride-H_2O	17.56
L-Cystine 2HCl	31.29
L-Glutamic Acid	7.35
L-Glutamine	365.0
L-Histidine hydrochloride-H_2O	31.48
L-Isoleucine	54.47
L-Leucine	59.05
L-Lysine hydrochloride	91.25
L-Methionine	17.24
L-Phenylalanine	35.48
L-Proline	17.25
L-Serine	26.25
L-Threonine	53.45
L-Tryptophan	9.02
L-Tyrosine disodium salt dihydrate	55.79
L-Valine	52.85
Vitamins	
Biotin	0.0035
Choline chloride	8.98
D-Calcium pantothenate	2.24
Folic Acid	2.65
Niacinamide	2.02
Pyridoxine hydrochloride	2.0
Riboflavin	0.219
Thiamine hydrochloride	2.17
Vitamin B12	0.68
i-Inositol	12.6
Inorganic Salts	
Calcium Chloride ($CaCl_2.2H_2O$)	116.6
Cupric sulfate ($CuSO_4.5H_2O$)	0.0013
Ferric Nitrate ($Fe(NO_3)_3.9H_2O$)	0.05
Ferric sulfate ($FeSO_4.7H_2O$)	0.417
Magnesium Chloride (anhydrous)	28.64
Magnesium Sulfate ($MgSO_4.7H_2O$)	48.84
Potassium Chloride (KCl)	311.8
Sodium Bicarbonate ($NaHCO_3$)	2438.0

Sodium Chloride (NaCl)	6995.5
Sodium Phosphate dibasic (Na_2HPO_4) anhydrous	71.02
Sodium Phosphate monobasic ($NaH_2PO_4.2H_2O$)	62.5
Zinc sulfate ($ZnSO_4.7H_2O$)	0.432
Other Components	
D-Glucose (Dextrose)	3151.0
Hypoxanthine Na	2.39
Linoleic Acid	0.042
Lipoic Acid	0.105
Putrescine 2HCl	0.081
Sodium Pyruvate	55.0
Thymidine	0.365

Table C.3: M199 formulation

Components	Concentration (mg/L)
Amino Acids	
Glycine	50.0
L-Alanine	25.0
L-Arginine hydrochloride	70.0
L-Aspartic acid	30.0
L-Cysteine hydrochloride-H_2O	0.1
L-Cystine 2HCl	26.0
L-Glutamic Acid	75.0
L-Glutamine	100.0
L-Histidine hydrochloride-H_2O	21.88
L-Hydroxyproline	10.0
L-Isoleucine	40.0
L-Leucine	60.0
L-Lysine hydrochloride	70.0
L-Methionine	15.0
L-Phenylalanine	25.0
L-Proline	40.0
L-Serine	25.0
L-Threonine	30.0
L-Tryptophan	10.0
L-Tyrosine disodium salt dihydrate	58.0
L-Valine	25.0
Vitamins	
Ascorbic Acid	0.05
Biotin	0.01
Choline chloride	0.5
D-Calcium pantothenate	0.01
Folic Acid	0.01
Menadione (Vitamin K3)	0.01
Niacinamide	0.025
Nicotinic acid (Niacin)	0.025
Para-Aminobenzoic Acid	0.05
Pyridoxal hydrochloride	0.025
Pyridoxine hydrochloride	0.025
Riboflavin	0.01
Thiamine hydrochloride	0.01
Vitamin A (acetate)	0.1
Vitamin D2 (Calciferol)	0.1
alpha Tocopherol phos. Na salt	0.01
i-Inositol	0.05
Inorganic Salts	
Calcium Chloride ($CaCl_2$) (anhyd.)	200.0

Ferric nitrate ($Fe(NO_3).9H_2O$)	0.7
Magnesium Sulfate ($MgSO_4$) (anhyd.)	97.67
Potassium Chloride (KCl)	400.0
Sodium Chloride (NaCl)	6800.0
Sodium Phosphate monobasic ($NaH_2PO4.H_2O$)	140.0
Other Components	
Adenine sulfate	10.0
Adenosine 5'-phosphate	0.2
Adenosine 5'-triphosphate	1.0
Cholesterol	0.2
D-Glucose (Dextrose)	1000.0
Deoxyribose	0.5
Glutathione (reduced)	0.05
Guanine hydrochloride	0.3
Hypoxanthine Na	0.354
Ribose	0.5
Sodium Acetate	50.0
Thymine	0.3
Tween 80	20.0
Uracil	0.3
Xanthine-Na	0.3

Appendix D: Neutral red cell viability assay data

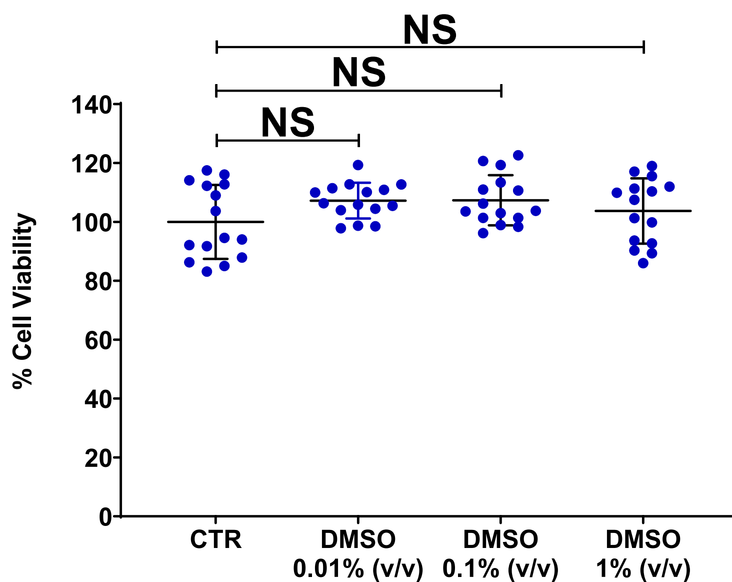


Figure D.1: Effect of solvent on cell viability
Cell viability was determined using Neutral Red assay. The uptake of neutral red dye was measured in control cells (without DMSO) and cells treated with different concentrations of DMSO. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. CTR: control, NS: non-significant.

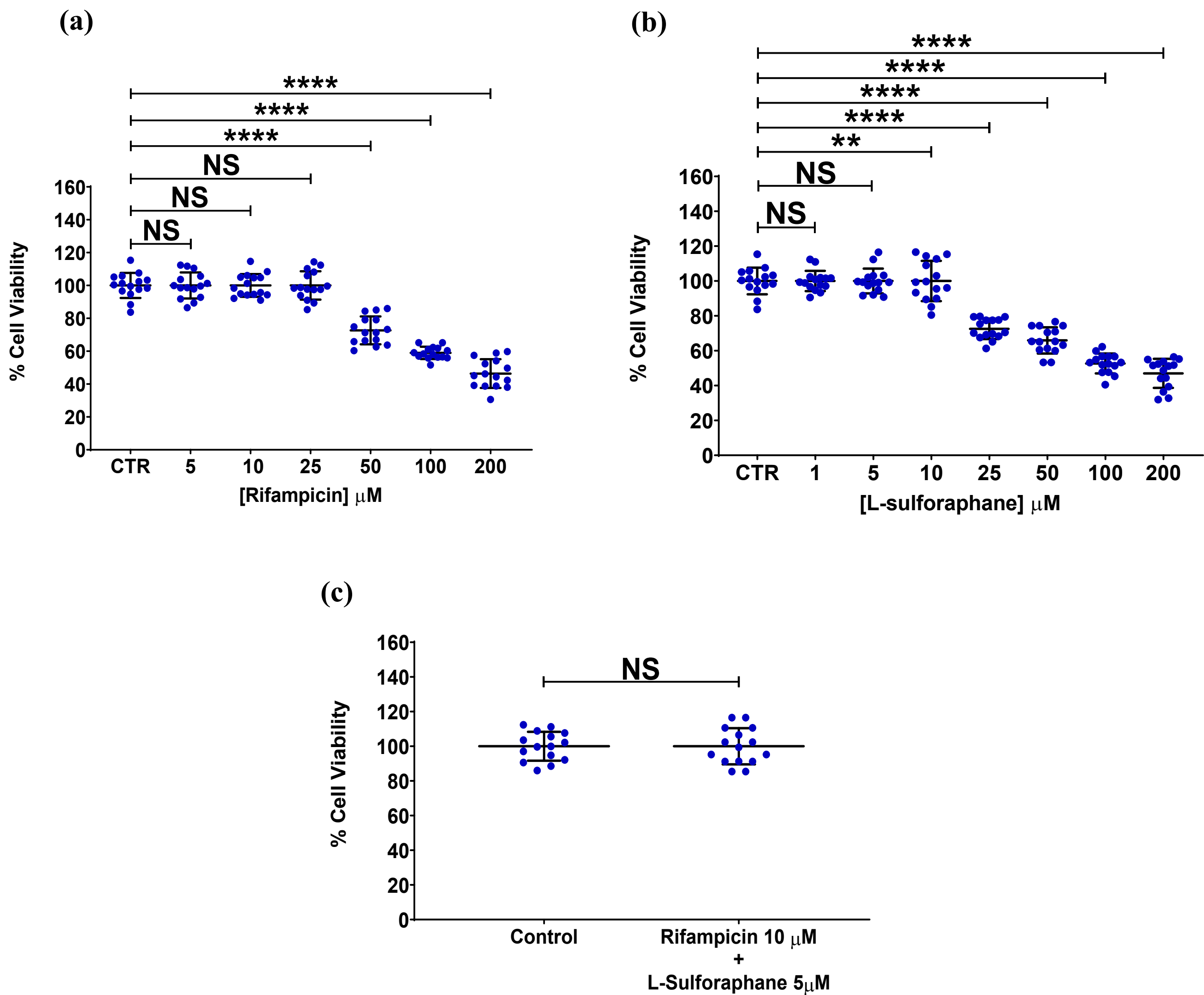


Figure D.2: Effects of pregnane X receptor (PXR) ligands on cell viability

Cell viability was determined using Neutral Red assay. The uptake of neutral red dye was measured in control cells (without treatment) and cells treated with different concentrations of (a) rifampicin, (b) L-sulforaphane and (c) co-treatments of rifampicin and L-sulforaphane. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. CTR: control, ****: $P < 0.0001$, **: $p < 0.01$, NS: non-significant.

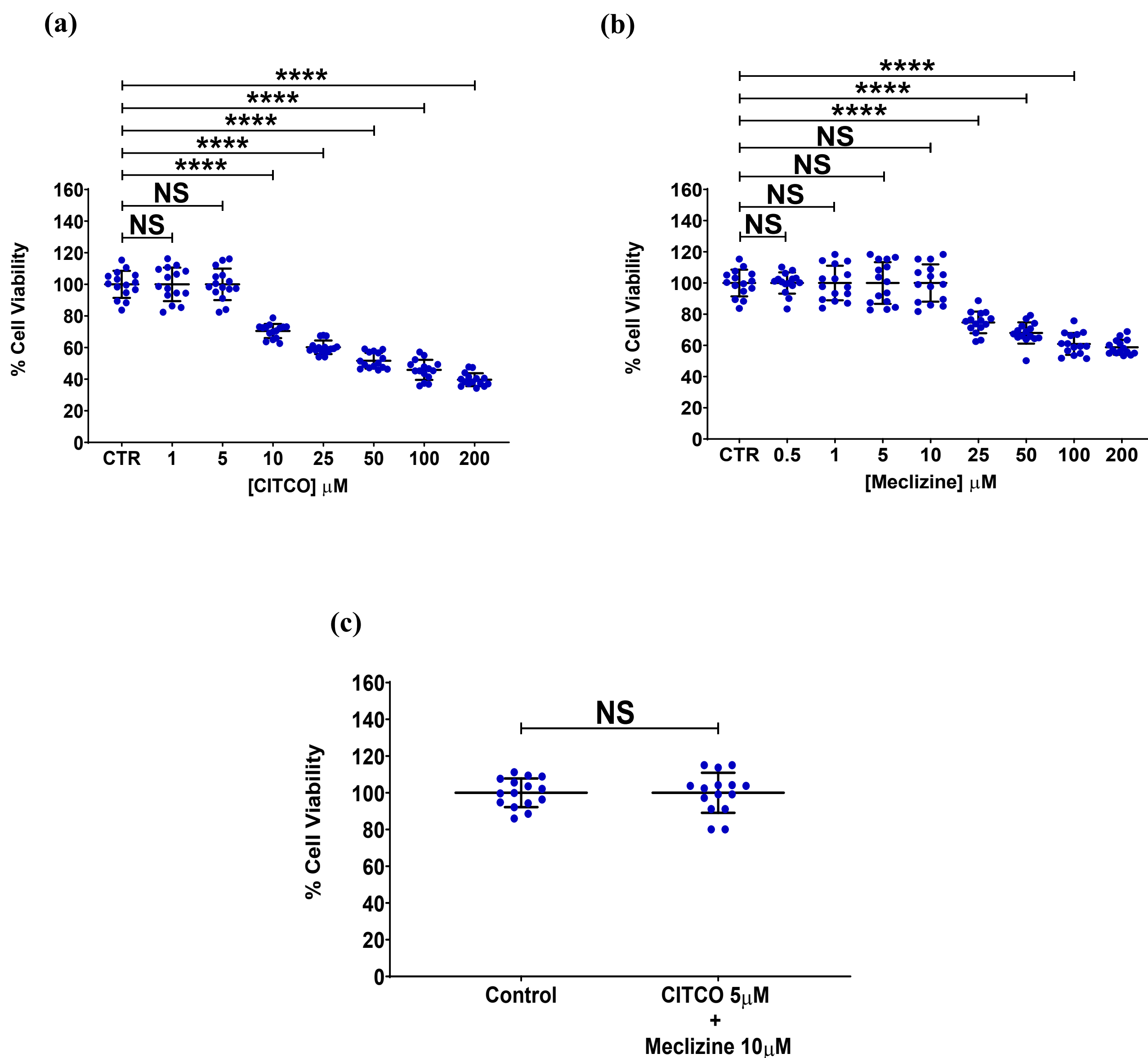
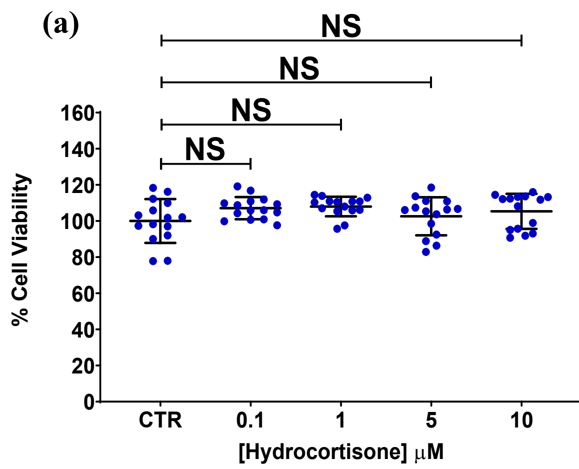
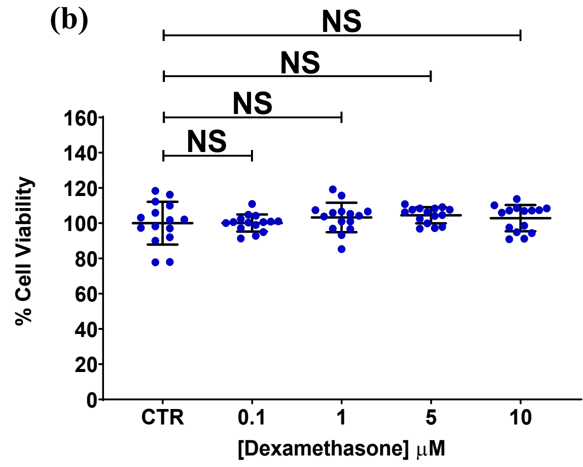
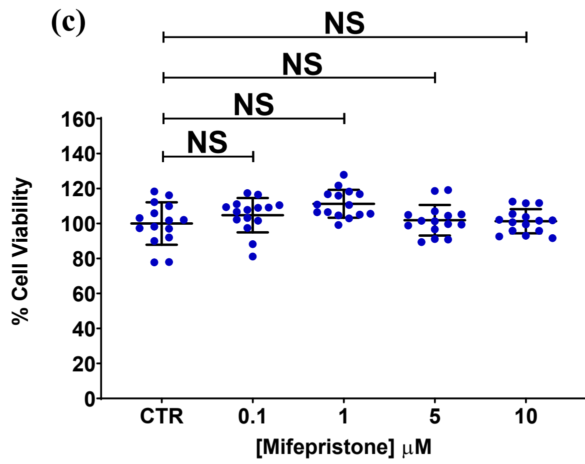
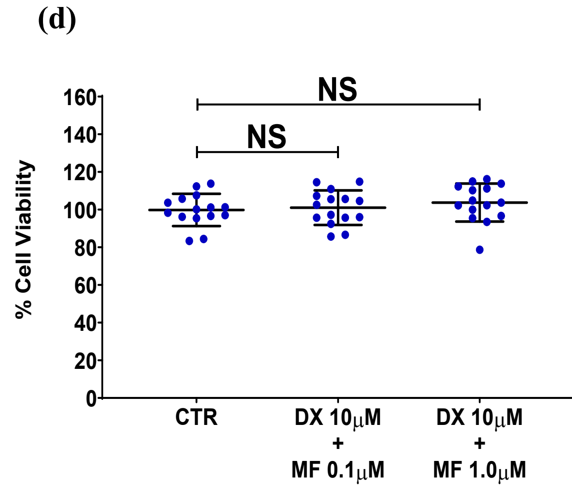


Figure D.3: Effects of constitutive androstane receptor (CAR) ligands on cell viability
Cell viability was determined using Neutral Red assay. The uptake of neutral red dye was measured in control cells (without treatment) and cells treated with different concentrations of (a) CITCO, (b) meclizine and (c) co-treatments of CITCO and meclizine. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. CTR: control, ****: $P < 0.0001$, **: $p < 0.01$, NS: non-significant.


(a)
NS
NS
NS
NS
160
140
120
100
80
60
40
20
0
% Cell Viability
CTR
0.1
1
5
10
[Hydrocortisone] μM


(b)
NS
NS
NS
NS
160
140
120
100
80
60
40
20
0
% Cell Viability
CTR
0.1
1
5
10
[Dexamethasone] μM


(c)
NS
NS
NS
NS
160
140
120
100
80
60
40
20
0
% Cell Viability
CTR
0.1
1
5
10
[Mifepristone] μM


(d)
NS
NS
160
140
120
100
80
60
40
20
0
% Cell Viability
CTR
DX 10μM + MF 0.1μM
DX 10μM + MF 1.0μM

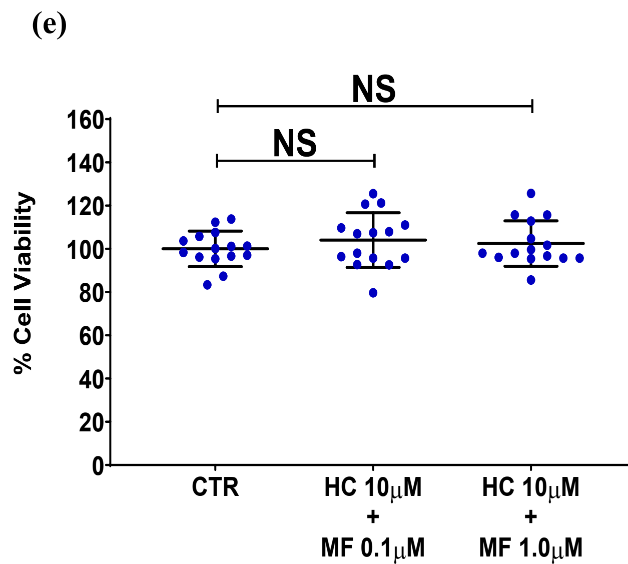

(e)
NS
NS
160
140
120
100
80
60
40
20
0
% Cell Viability
CTR
HC 10μM + MF 0.1μM
HC 10μM + MF 1.0μM

Figure D.4: Effects of glucocorticoid receptor ligands on cell viability
Cell viability was determined using Neutral Red assay. The uptake of neutral red dye was measured in control cells (without treatment) and cells treated with different concentrations of (a) hydrocortisone, (b) dexamethasone, (c) mifepristone, (d) co-treatments of dexamethasone (DX) and mifepristone (MF), and (e) co-treatments of hydrocortisone (HD) and mifepristone (MF). Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. CTR: control, NS: non-significant.

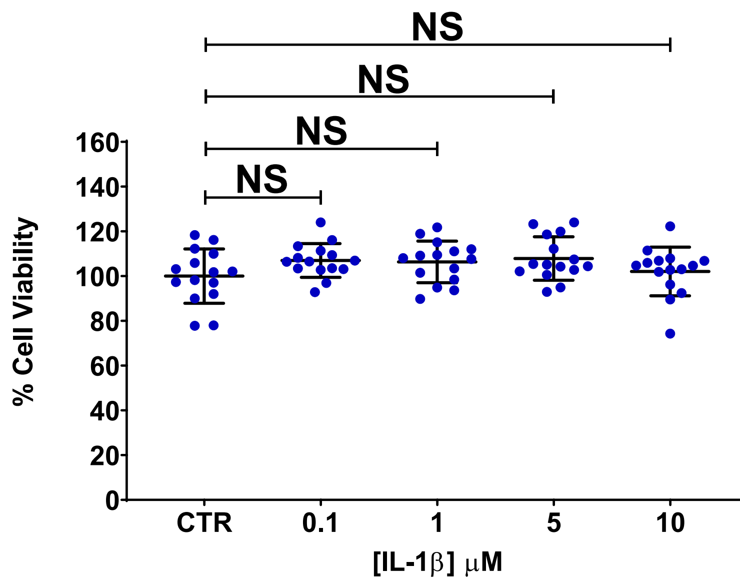


Figure D.5: Effects of pro-inflammatory cytokine on cell viability
Cell viability was determined using Neutral Red assay. The uptake of neutral red dye was measured in control cells (without treatment) and cells treated with different concentrations of IL-1β. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. CTR: control, NS: non-significant.

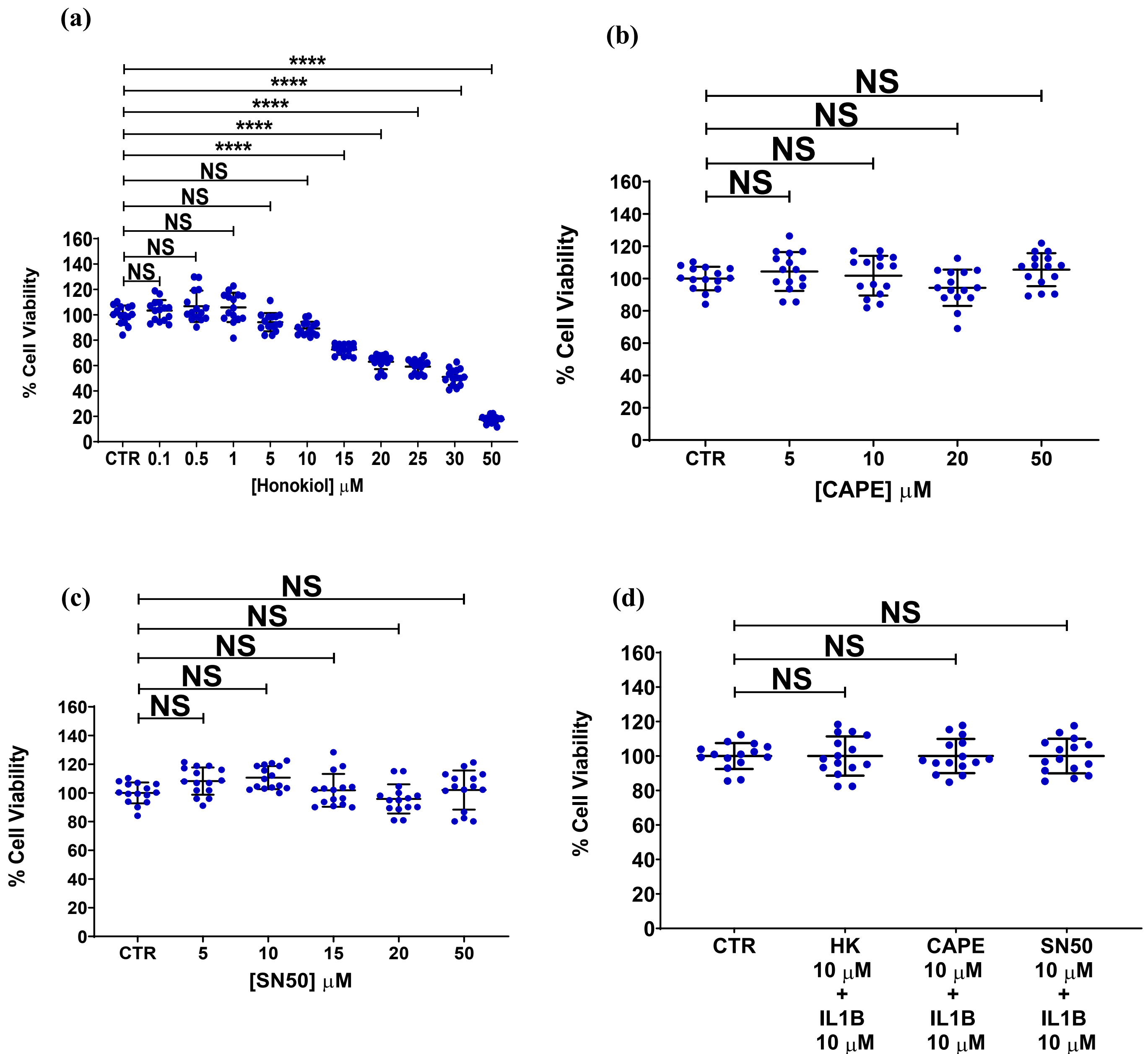


Figure D.6: Effects of NF-κB inhibitors on cell viability
Cell viability was determined using Neutral Red assay. The uptake of neutral red dye was measured in control cells (without treatment) and cells treated with different concentrations of (a) honokiol, (b) CAPE, (c) SN50, (d) co-treatments of honokiol (HK), CAPE or SN50 with IL-1β. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. CTR: control, ****: $P < 0.0001$, NS: non-significant.

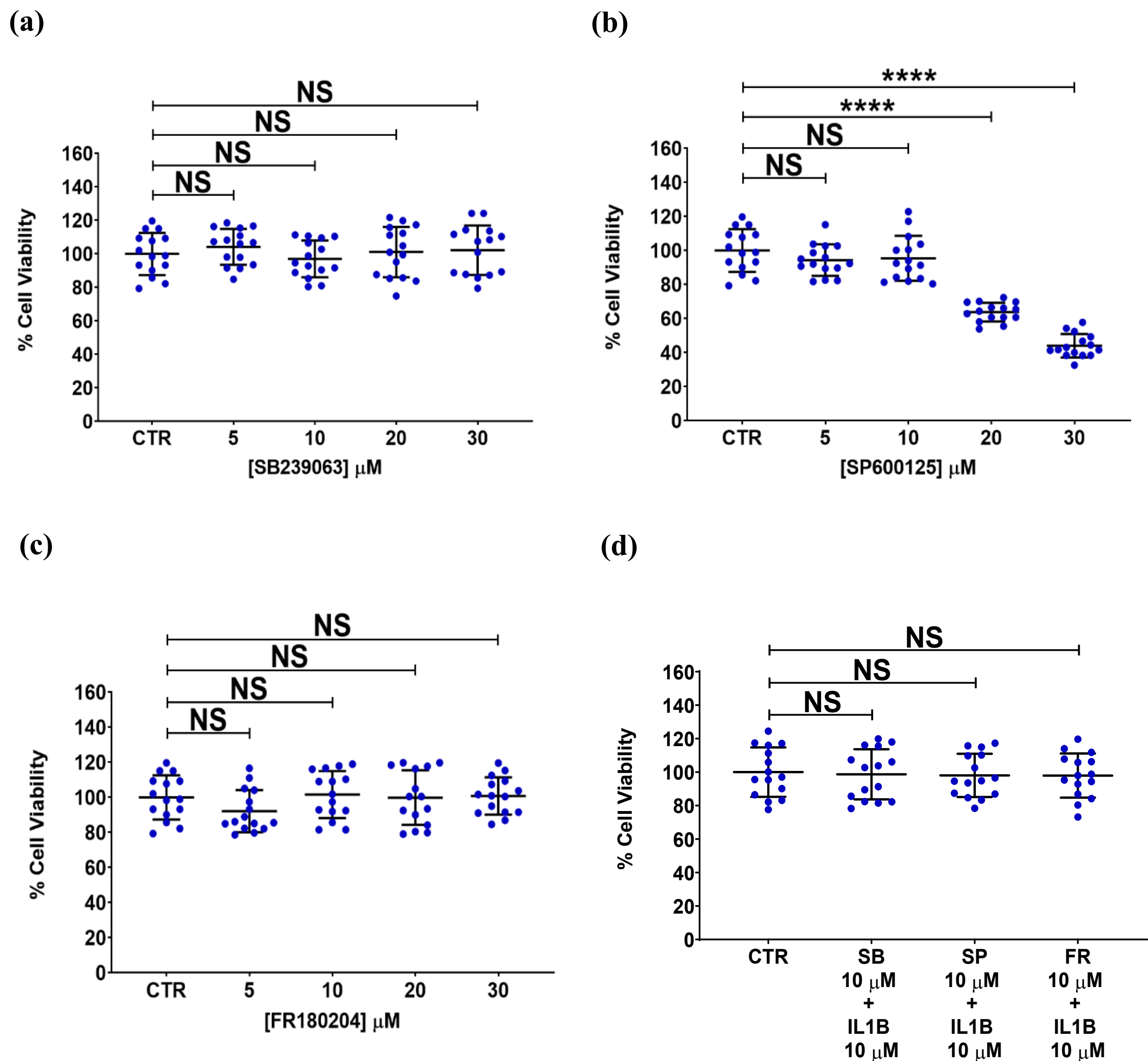


Figure D.7: Effects of MAPK inhibitors on cell viability

Cell viability was determined using Neutral Red assay. The uptake of neutral red dye was measured in control cells (without treatment) and cells treated with different concentrations of (a) SB239063, (b) SP600125, (c) FR180204 and (d) co-treatments of SB239063, SP600125 or FR180204 with IL-1β. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. CTR: control, ****: P < 0.0001, NS: non-significant.

Appendix E: Determination of ABCB1 transporter activities

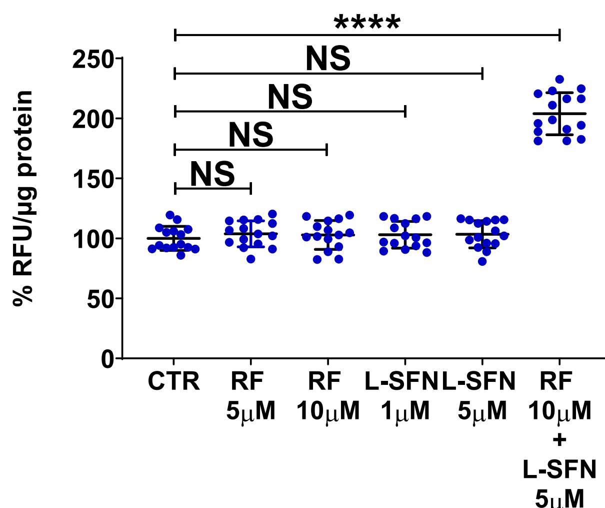


Figure E.1: Effects of pregnane X receptor (PXR) ligands on ABCB1 transporter activity at 30 min.

Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentrations of rifampicin (RF), L-Sulforaphane (L-SFN) or co-treatment of RF and L-SFN for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

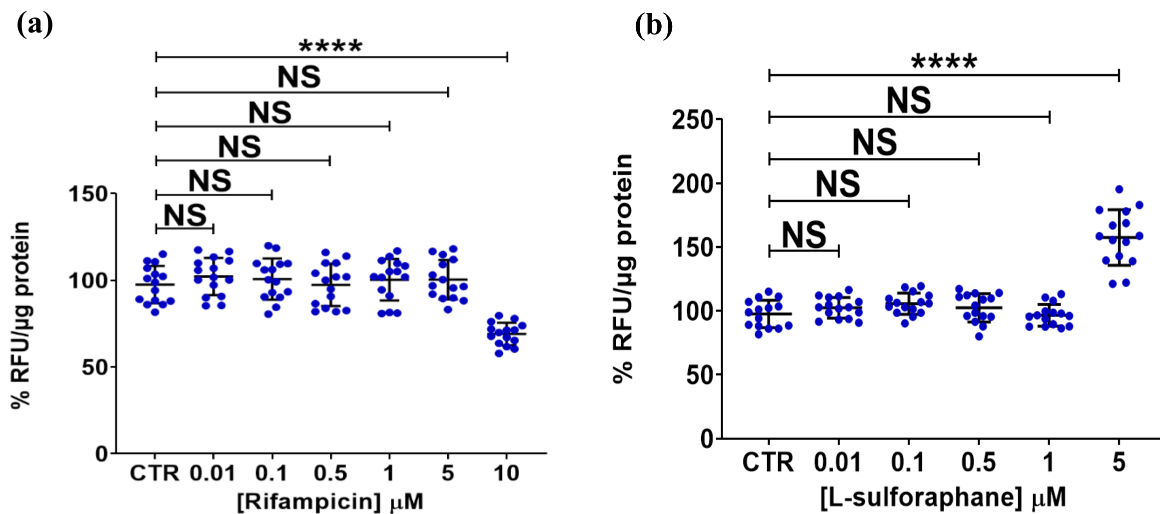


Figure E.2: Effects of pregnane X receptor (PXR) ligands on ABCB1 transporter activity at 24 h.

Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentrations of rifampicin (RF) and L-Sulforaphane (L-SFN) for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

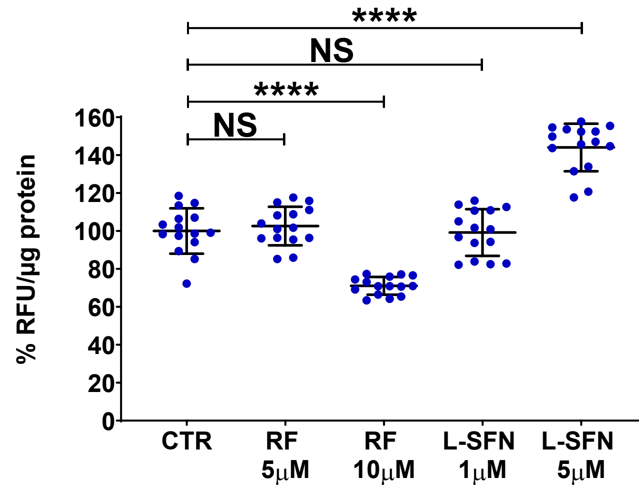


Figure E.3: Effects of pregnane X receptor (PXR) ligands on ABCB1 transporter activity at 72 h
Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentrations of rifampicin (RF) and L-Sulforaphane (L-SFN) for 72 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

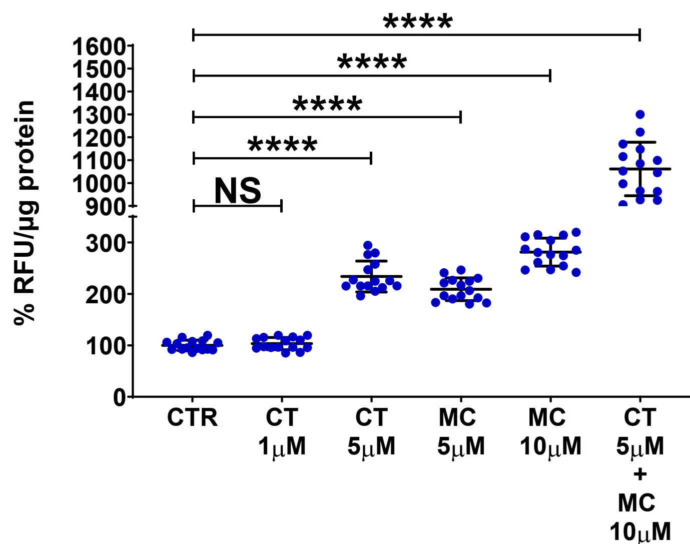


Figure E.4: Effects of constitutive androstane receptor (CAR) ligands on ABCB1 transporter activity at 30 min
Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentrations of CITCO (CT), meclizine (MC) or co-treatment of CT and MC for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three

replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

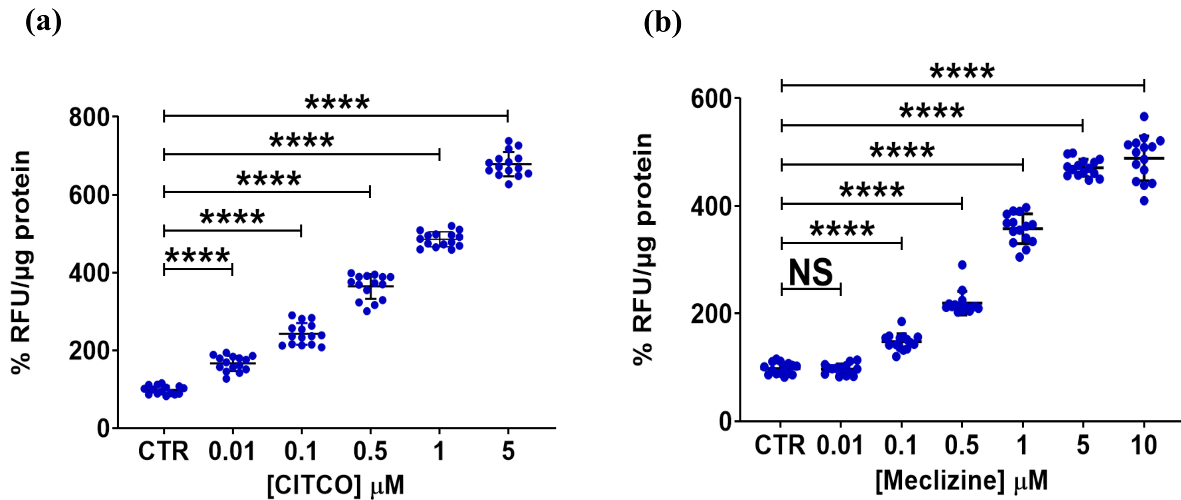


Figure E.5: Effects of constitutive androstane receptor (CAR) ligands on ABCB1 transporter activity at 24 h

Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentrations of CITCO (CT) and meclizine (MC) for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

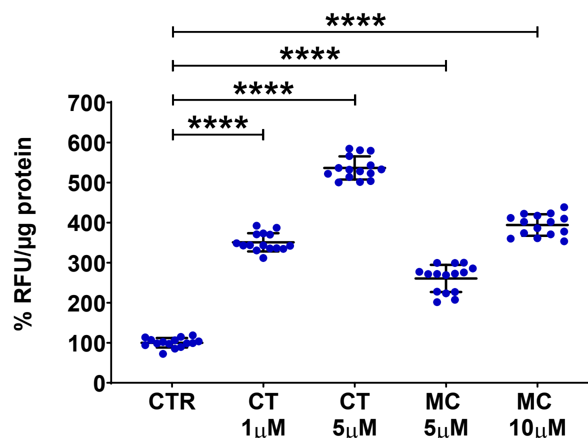
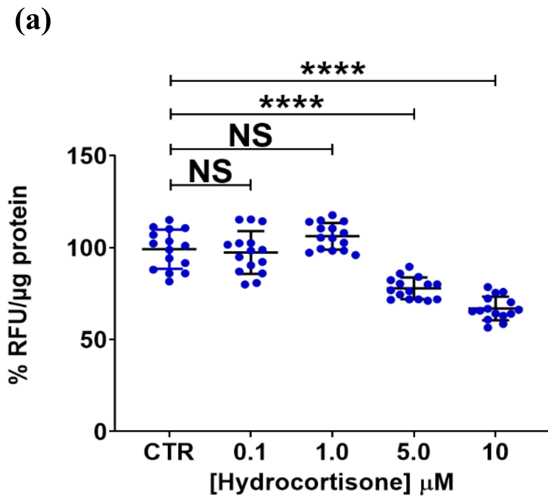
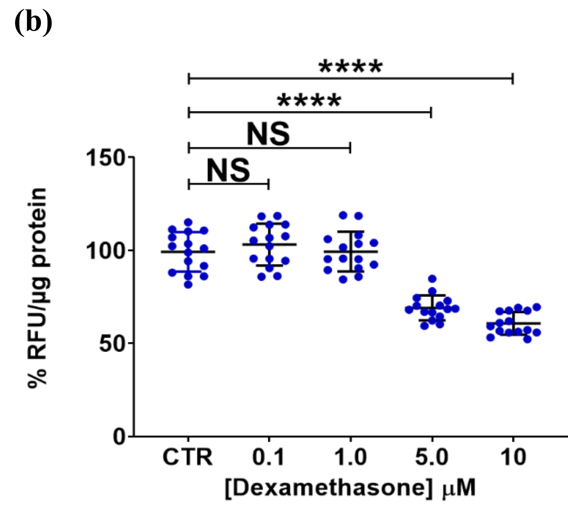
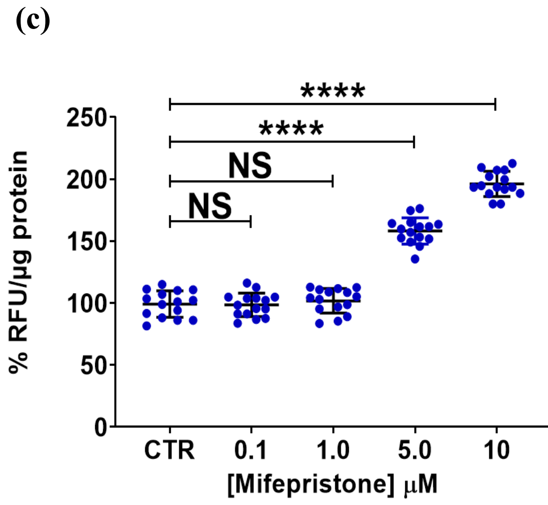


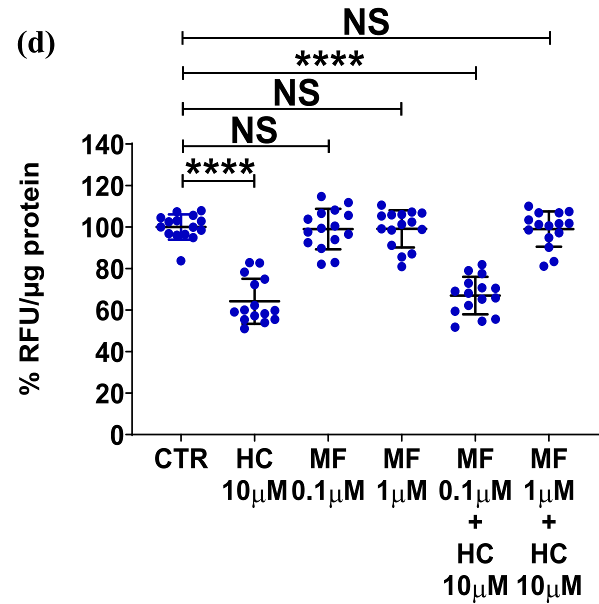
Figure E.6: Effects of constitutive androstane receptor (CAR) ligands on ABCB1 transporter activity at 72 h

Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentrations of CITCO (CT) and meclizine (MC) for 72 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001.


(a)
****
****
NS
NS
150
100
50
0
% RFU/µg protein
CTR
0.1
1.0
5.0
10
[Hydrocortisone] µM


(b)
****
****
NS
NS
150
100
50
0
% RFU/µg protein
CTR
0.1
1.0
5.0
10
[Dexamethasone] µM


(c)
****
****
NS
NS
250
200
150
100
50
0
% RFU/µg protein
CTR
0.1
1.0
5.0
10
[Mifepristone] µM


(d)
NS
****
NS
NS
****
140
120
100
80
60
40
20
0
% RFU/µg protein
CTR
HC 10µM
MF 0.1µM
MF 1µM
MF 0.1µM + HC 10µM
MF 1µM + HC 10µM

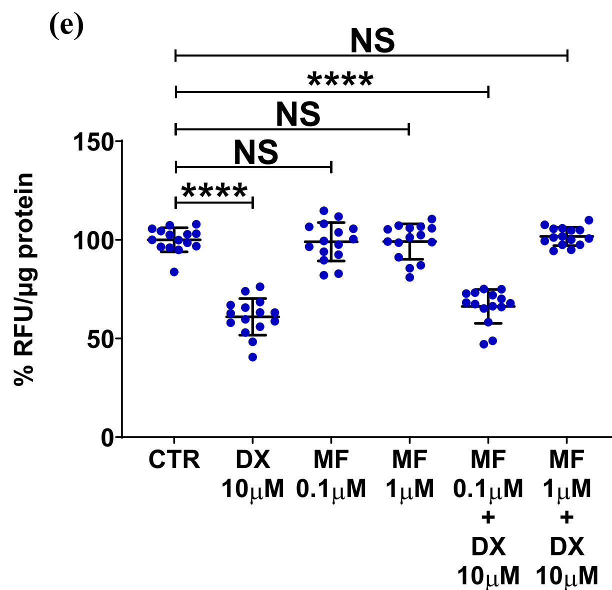

(e)
NS
****
NS
NS
****
150
100
50
0
% RFU/µg protein
CTR
DX 10µM
MF 0.1µM
MF 1µM
MF 0.1µM + DX 10µM
MF 1µM + DX 10µM

Figure E.7: Effects of glucocorticoid (GR) ligands on ABCB1 transporter activity at 24 h Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentrations of (a) hydrocortisone (HC), (b) dexamethasone (DX), (c) mifepristone (MF), (d) co-treatment of HC and MF and (e) co-treatment of DX and MF for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

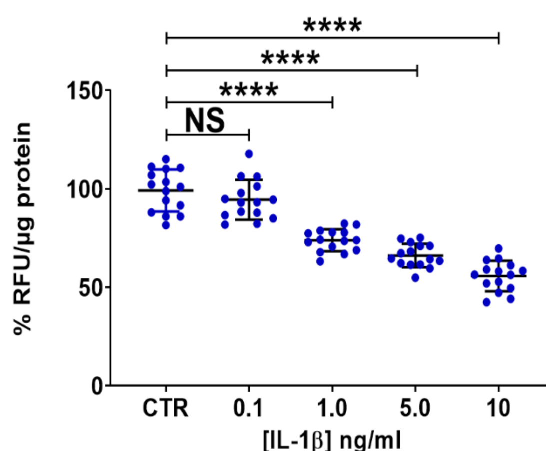


Figure E.8: Effects of pro-inflammatory cytokine on ABCB1 transporter activity at 24 h Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentration of IL-1β for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

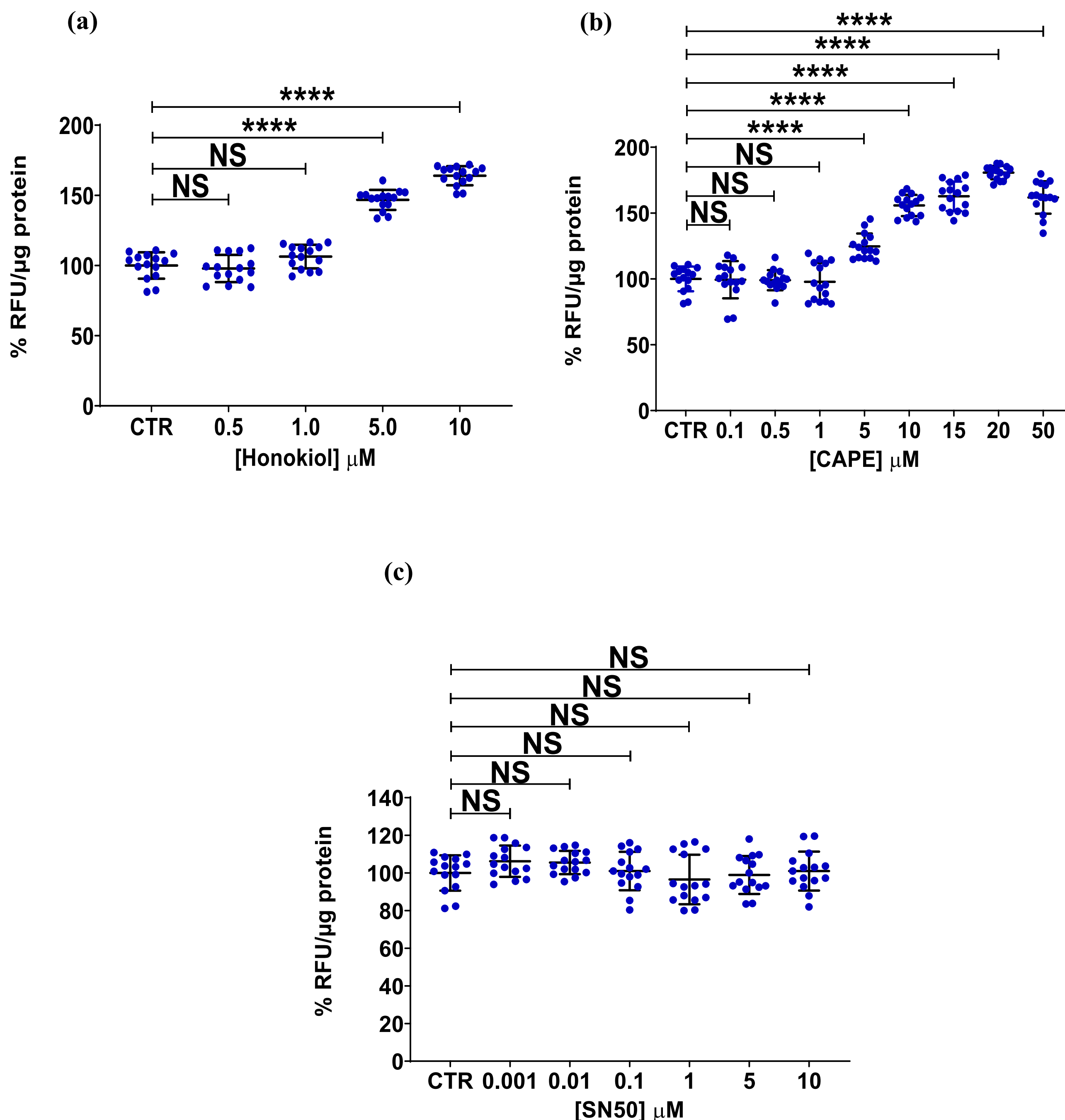


Figure E.9: Effects of NF-κB inhibitors on ABCB1 transporter activity at 24 h
Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentration of (a) honokiol, (b) CAPE and (c) SN50 for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

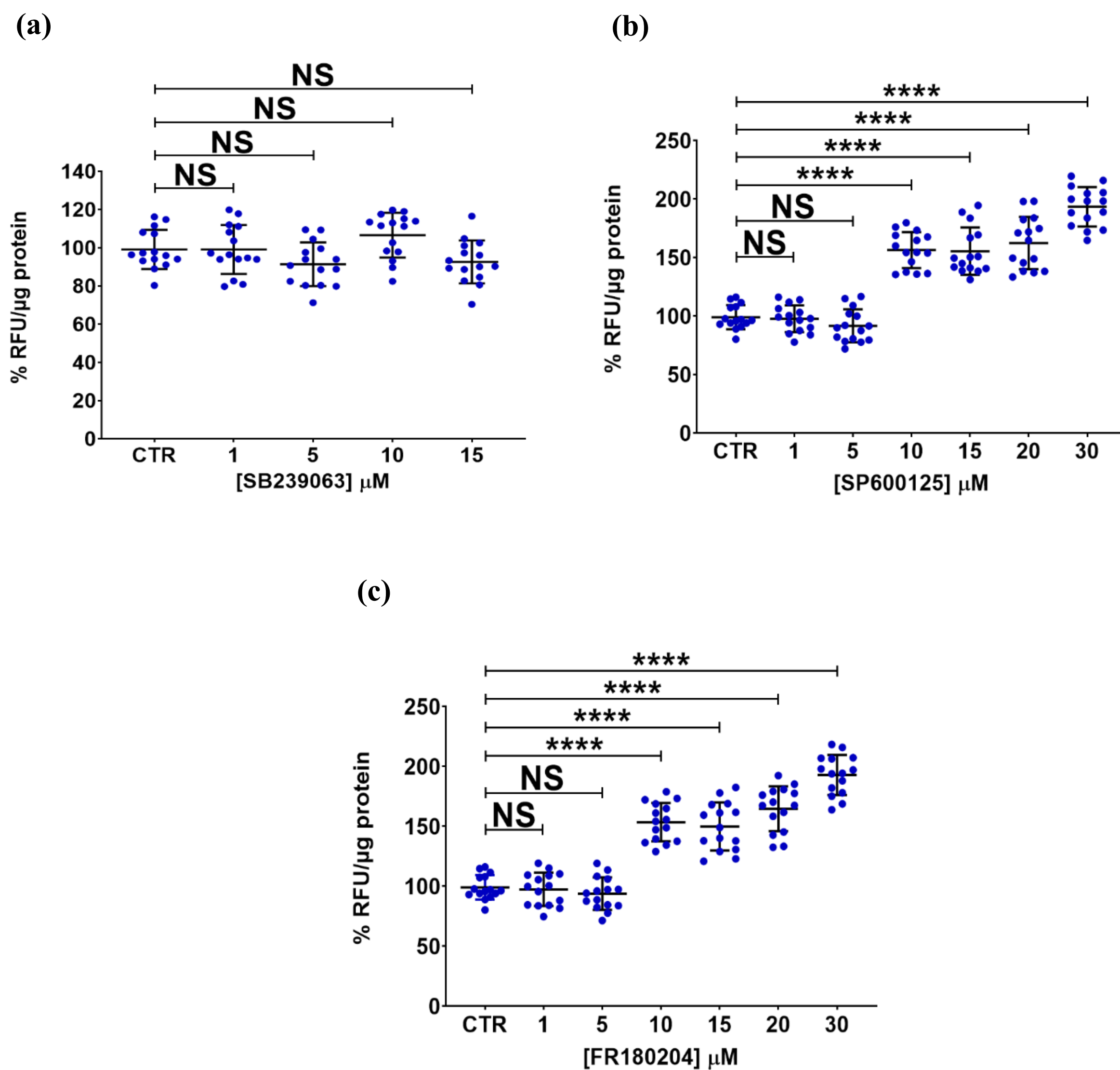


Figure E.10: Effects of MAPK inhibitors on ABCB1 transporter activity at 24 h
Intracellular accumulation of calcein - ABCB1 substrate was measured in control cells and cells treated with different concentration of (a) SB239063, (b) SP600125 and (c) FR180204 for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

Appendix F: Determination of ABCG2 transporter activities

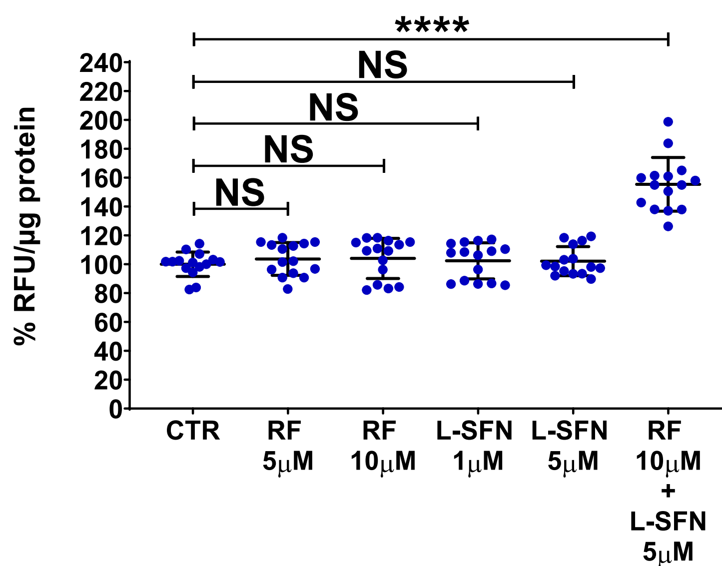


Figure F.1: Effects of pregnane X receptor (PXR) ligands on ABCG2 transporter activity at 30 min

Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of rifampicin (RF), L-Sulforaphane (L-SFN) or co-treatment of RF and L-SFN for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

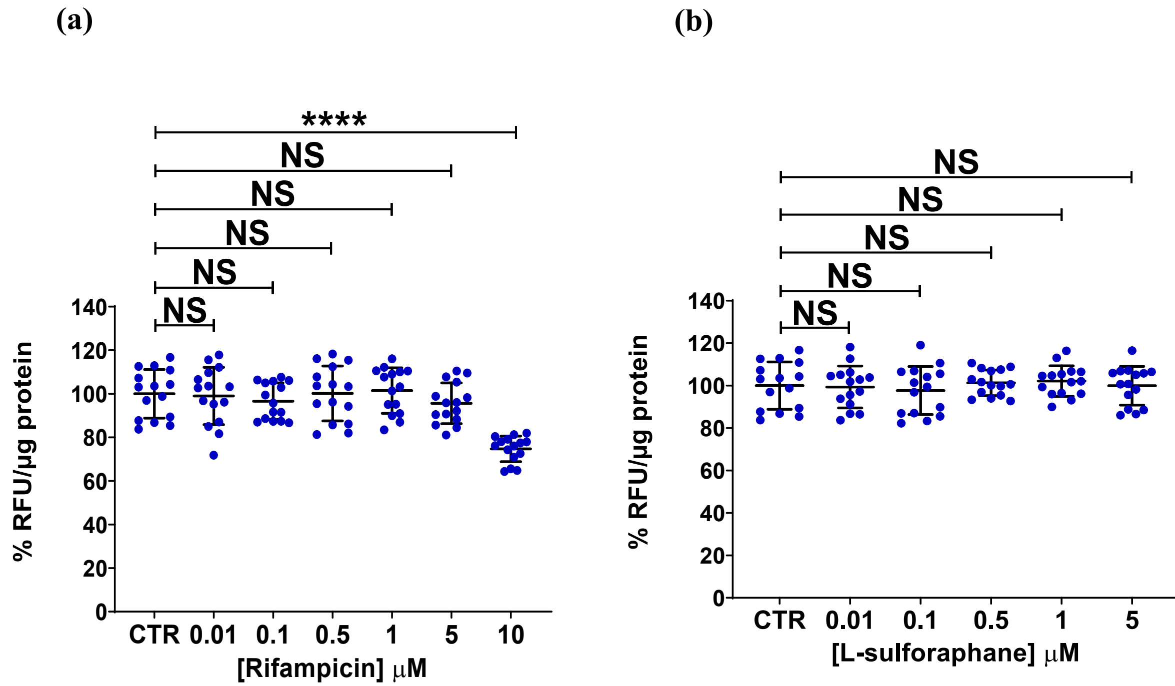


Figure F.2: Effects of pregnane X receptor (PXR) ligands on ABCG2 transporter activity at 24 h

Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of rifampicin (RF) and L-Sulforaphane (L-SFN) for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

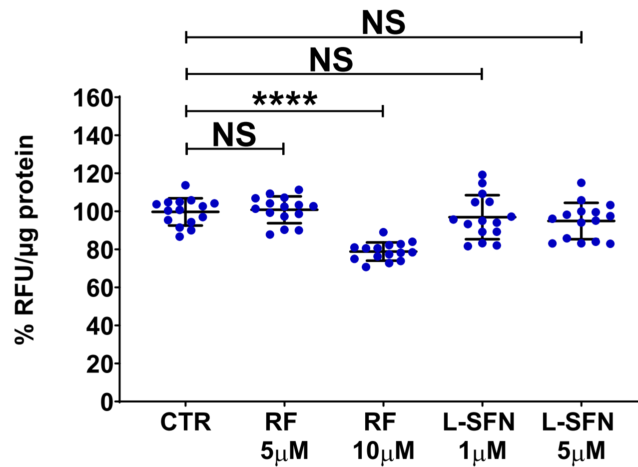


Figure F.3: Effects of pregnane X receptor (PXR) ligands on ABCG2 transporter activity at 72 h

Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of rifampicin (RF) and L-Sulforaphane (L-SFN) for 72 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

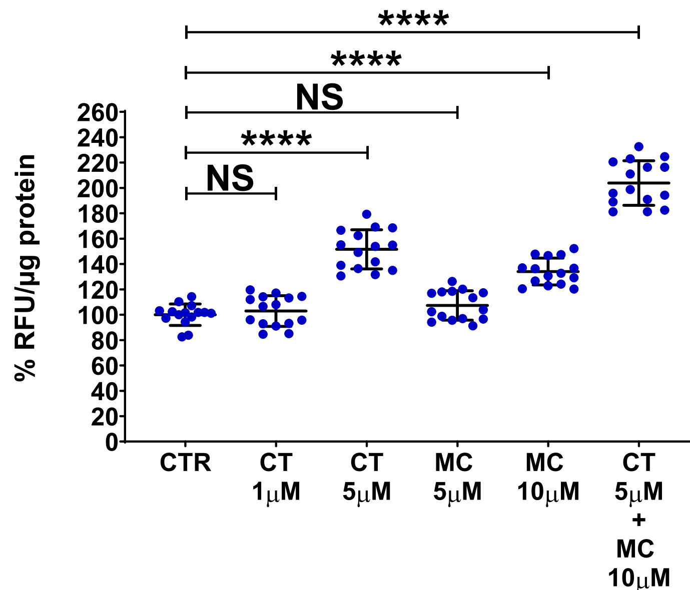


Figure F.4: Effects of constitutive androstane receptor (CAR) ligands on ABCG2 transporter activity at 30 min

Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of CITCO (CT), meclizine (MC) or co-treatment of CT and MC for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

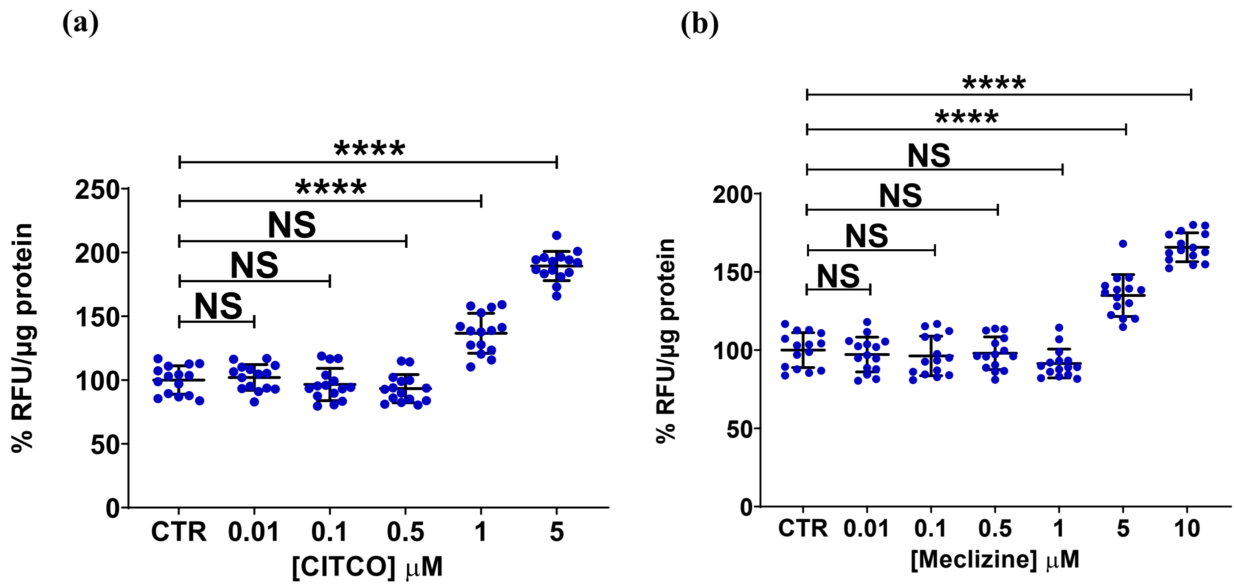


Figure F.5: Effects of constitutive androstane receptor (CAR) ligands on ABCG2 transporter activity at 24 h

Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of CITCO (CT) and meclizine (MC) for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

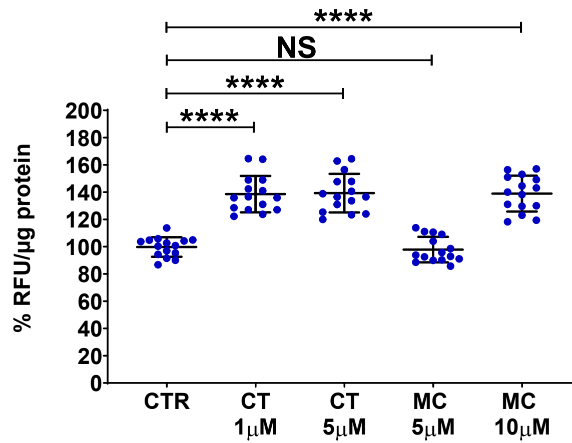


Figure F.6: Effects of constitutive androstane receptor (CAR) ligands on ABCG2 transporter activity at 72 h

Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of CITCO (CT) and meclizine (MC) for 72 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001.

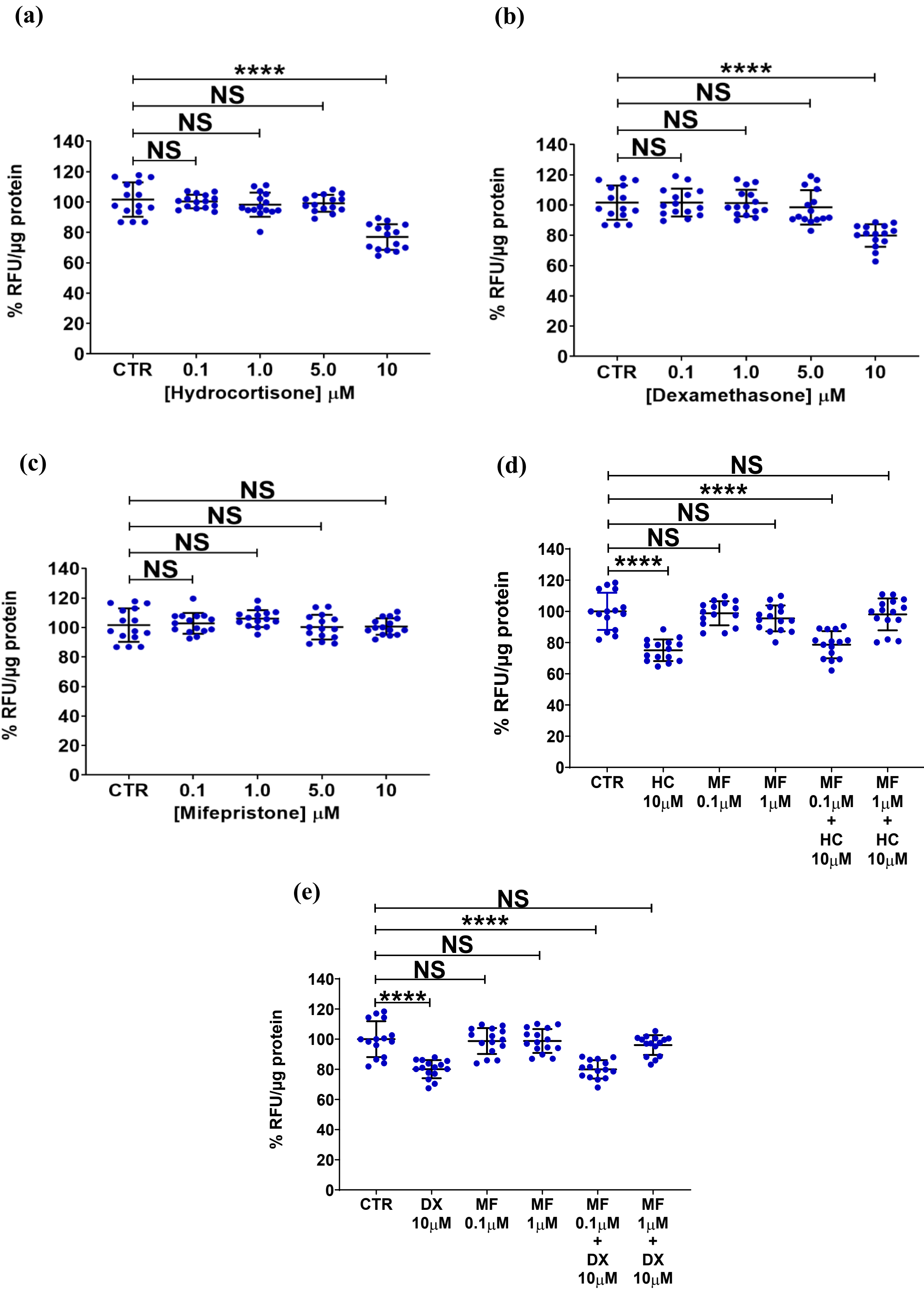

(a)
****
NS
NS
NS
% RFU/µg protein
140
120
100
80
60
40
20
0
CTR
0.1
1.0
5.0
10
[Hydrocortisone] µM
(b)
****
NS
NS
NS
% RFU/µg protein
CTR
0.1
1.0
5.0
10
[Dexamethasone] µM
(c)
NS
NS
NS
NS
% RFU/µg protein
CTR
0.1
1.0
5.0
10
[Mifepristone] µM
(d)
NS
****
NS
NS
****
% RFU/µg protein
CTR
HC 10µM
MF 0.1µM
MF 1µM
MF 0.1µM + HC 10µM
MF 1µM + HC 10µM
(e)
NS
****
NS
NS
****
% RFU/µg protein
CTR
DX 10µM
MF 0.1µM
MF 1µM
MF 0.1µM + DX 10µM
MF 1µM + DX 10µM

Figure F.7: Effects of glucocorticoid (GR) ligands on ABCG2 transporter activity at 24 h Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of (a) hydrocortisone (HC), (b) dexamethasone (DX), (c) mifepristone (MF), (d) co-treatment of HC and MF and (e) co-treatment of DX and MF for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

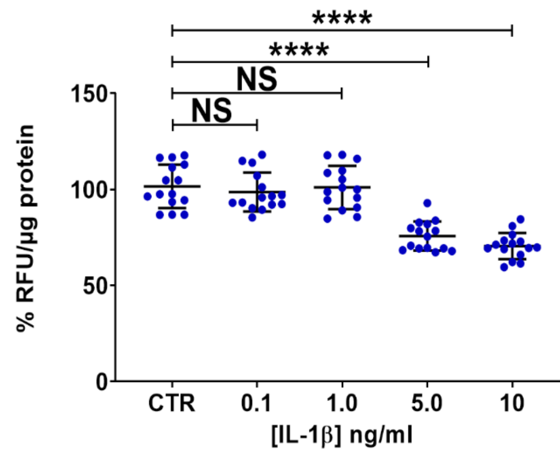


Figure F.8: Effects of pro-inflammatory cytokine on ABCG2 transporter activity at 24 h Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of IL-1β for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

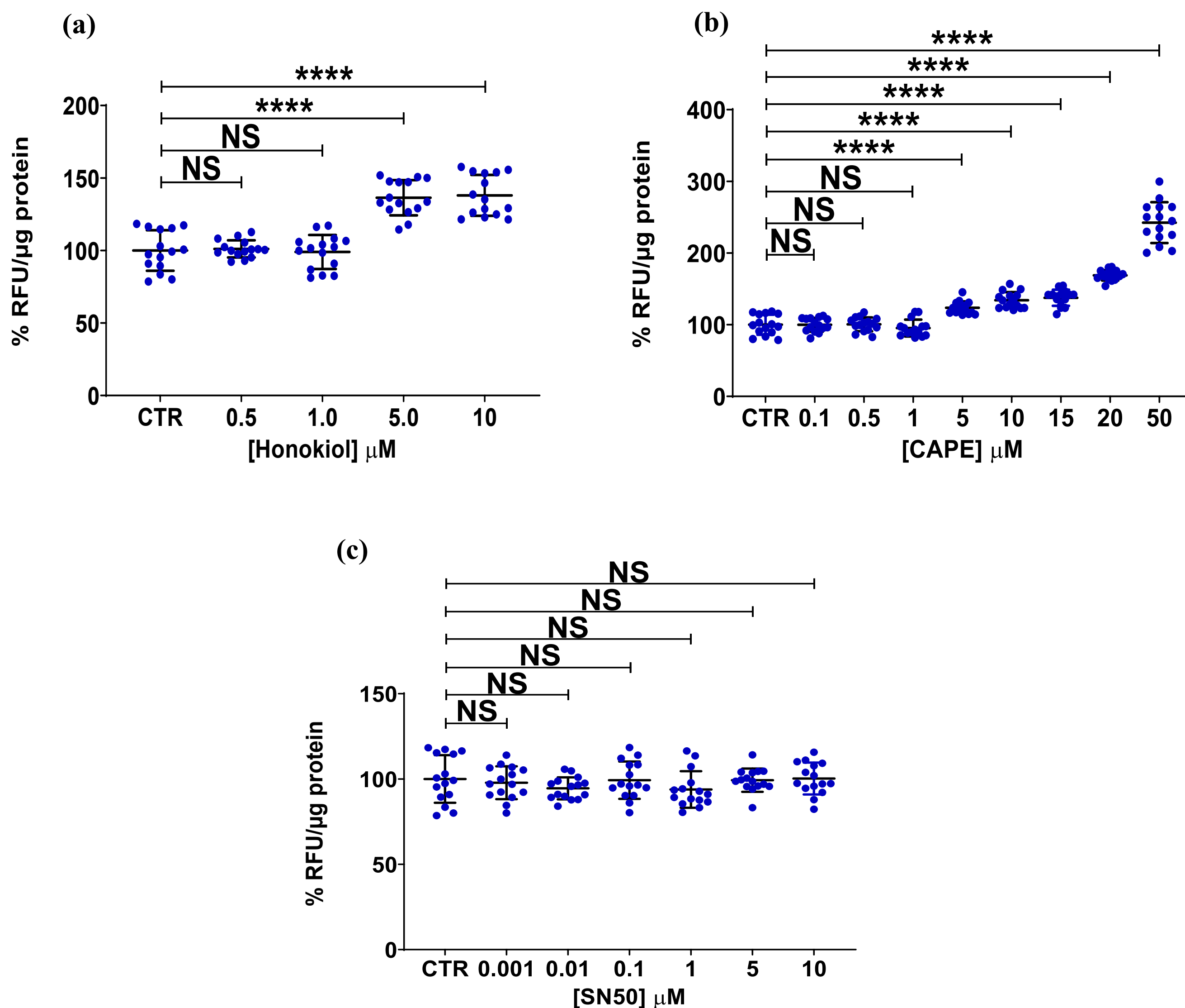


Figure F.9: Effects of NF-κB inhibitors on ABCG2 transporter activity at 24 h
Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of (a) honokiol, (b) CAPE and (c) SN50 for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

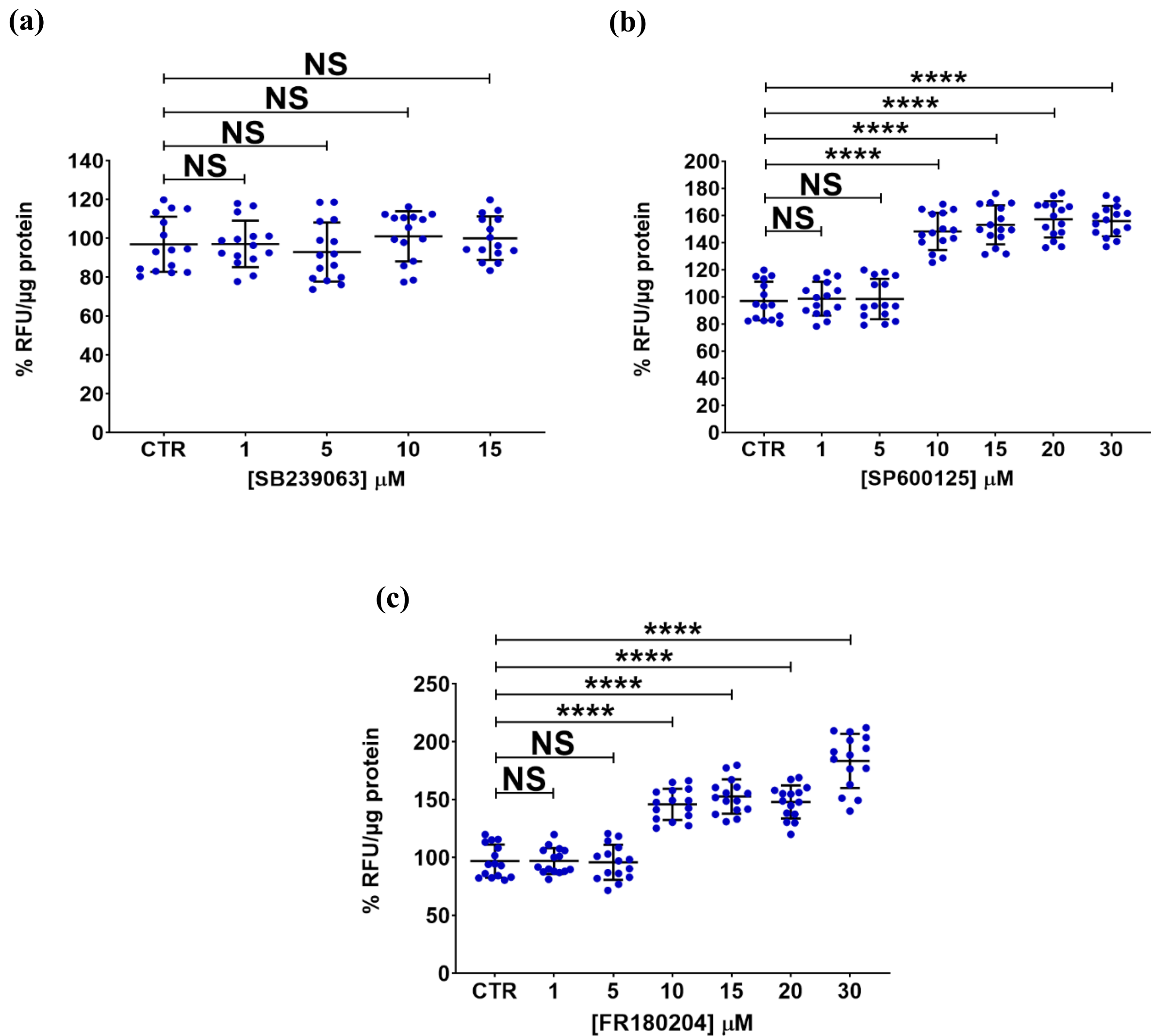


Figure F.10: Effects of MAPK inhibitors on ABCG2 transporter activity at 24 h
Intracellular accumulation of Hoechst 33342 – ABCG2 substrate was measured in control cells and cells treated with different concentrations of (a) SB239063, (b) SP600125 and (c) FR180204 for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

Appendix G: Determination of ABCC5 transporter activities

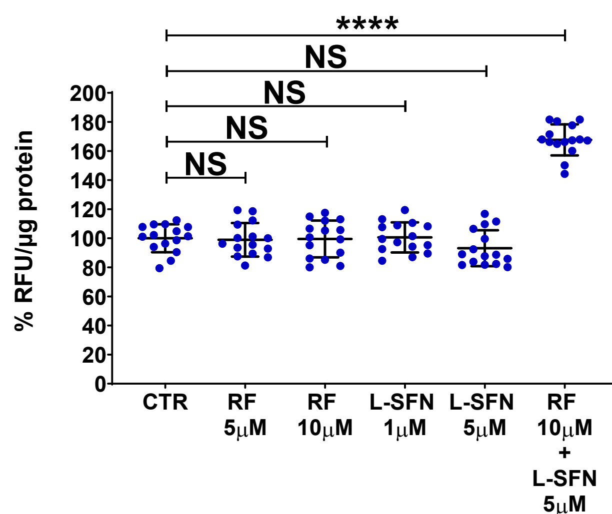


Figure G.1: Effects of pregnane X receptor (PXR) ligands on ABCC5 transporter activity at 30 min

Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of rifampicin (RF), L-Sulforaphane (L-SFN) or co-treatment of RF and L-SFN for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

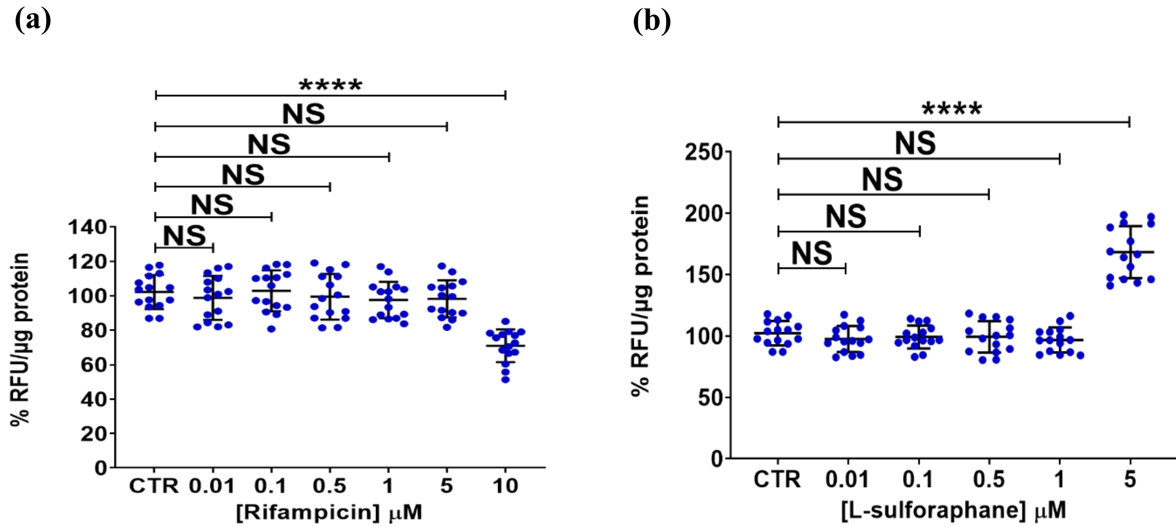


Figure G.2: Effects of pregnane X receptor (PXR) ligands on ABCC5 transporter activity at 24 h

Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of rifampicin (RF) and L-Sulforaphane (L-SFN) for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

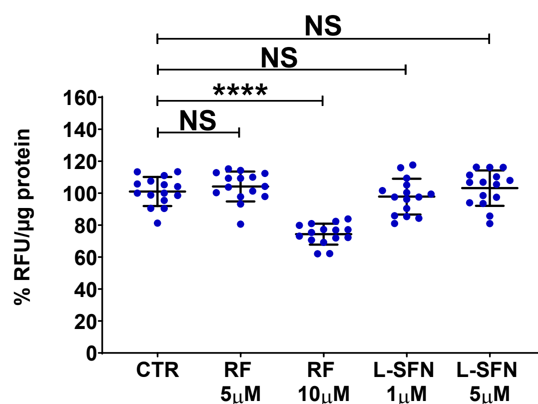


Figure G.3: Effects of pregnane X receptor (PXR) ligands on ABCC5 transporter activity at 72 h

Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of rifampicin (RF) and L-Sulforaphane (L-SFN) for 72 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

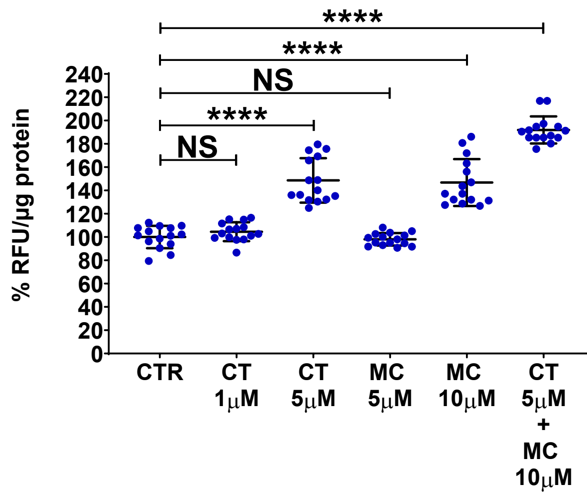


Figure G.4: Effects of constitutive androstane receptor (CAR) ligands on ABCC5 transporter activity at 30 min

Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of CITCO (CT), meclizine (MC) or co-treatment of CT and MC for 30 min. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

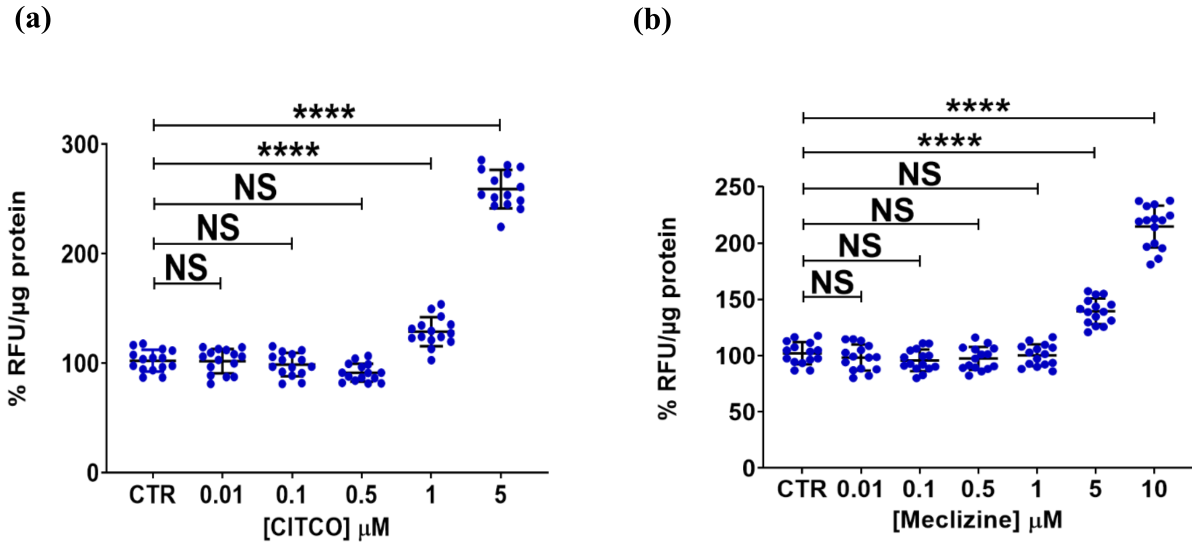


Figure G.5: Effects of constitutive androstane receptor (CAR) ligands on ABCC5 transporter activity at 24 h

Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of CITCO (CT) and meclizine (MC) for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

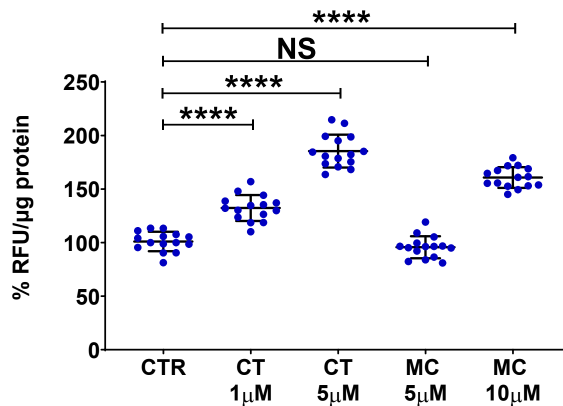
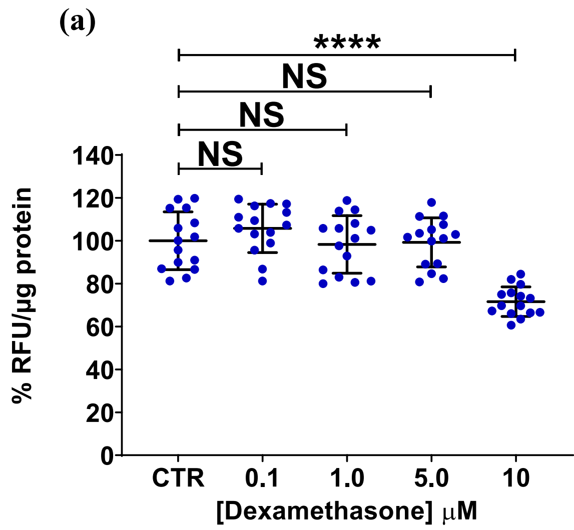
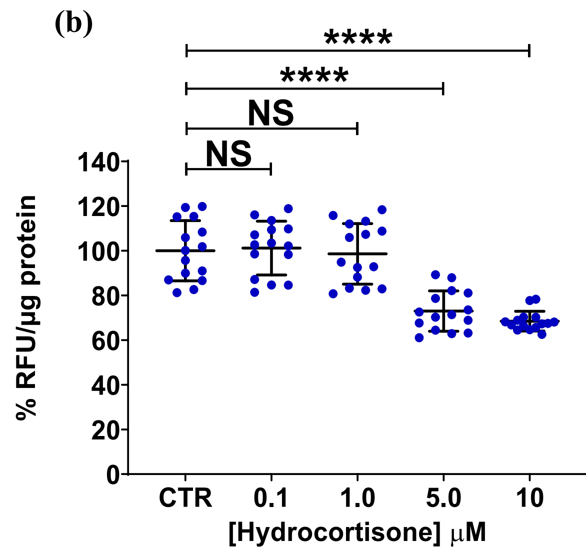
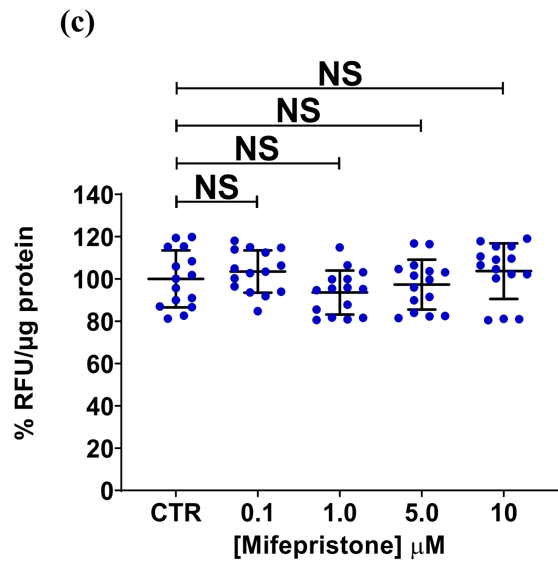


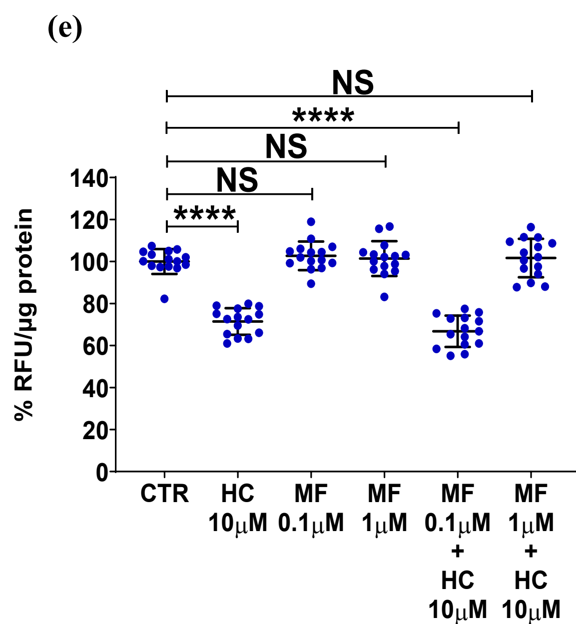
Figure G.6: Effects of constitutive androstane receptor (CAR) ligands on ABCC5 transporter activity at 72 h

Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of CITCO (CT) and meclizine (MC) for 72 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$.


(a)
****
NS
NS
NS
% RFU/µg protein
140
120
100
80
60
40
20
0
CTR
0.1
1.0
5.0
10
[Dexamethasone] µM


(b)
****
****
NS
NS
% RFU/µg protein
140
120
100
80
60
40
20
0
CTR
0.1
1.0
5.0
10
[Hydrocortisone] µM


(c)
NS
NS
NS
NS
% RFU/µg protein
140
120
100
80
60
40
20
0
CTR
0.1
1.0
5.0
10
[Mifepristone] µM


(e)
NS
****
NS
NS
****
% RFU/µg protein
140
120
100
80
60
40
20
0
CTR
HC 10µM
MF 0.1µM
MF 1µM
MF 0.1µM + HC 10µM
MF 1µM + HC 10µM

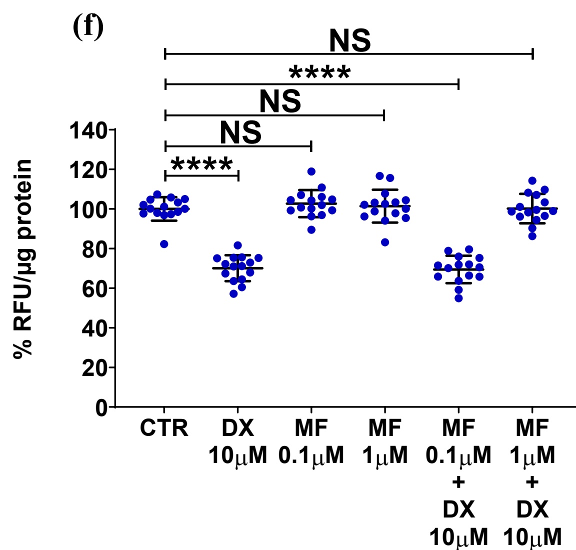

(f)
NS
****
NS
NS
****
% RFU/µg protein
140
120
100
80
60
40
20
0
CTR
DX 10µM
MF 0.1µM
MF 1µM
MF 0.1µM + DX 10µM
MF 1µM + DX 10µM

Figure G.7: Effects of glucocorticoid (GR) ligands on ABCC5 transporter activity at 24 h Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of (a) hydrocortisone (HC), (b) dexamethasone (DX), (c) mifepristone (MF), (d) co-treatment of HC and MF and (e) co-treatment of DX and MF for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

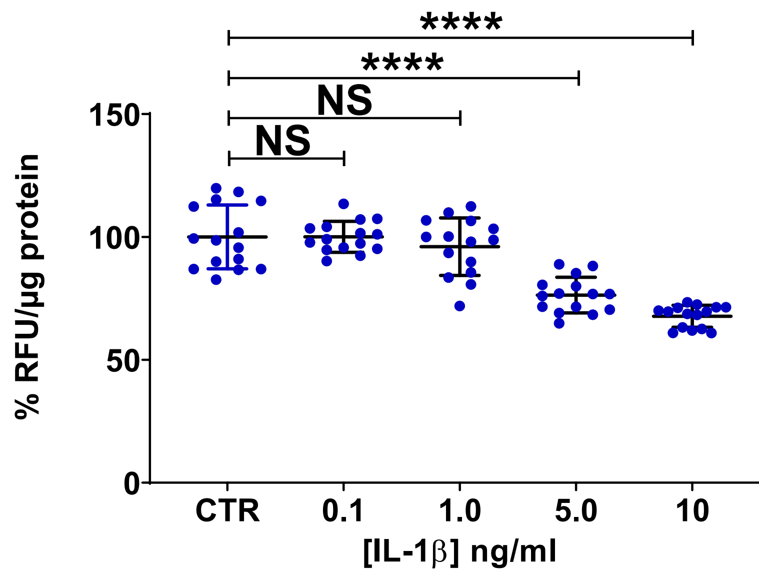


Figure G.8: Effects of pro-inflammatory cytokine on ABCC5 transporter activity at 24 h Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of IL-1β for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

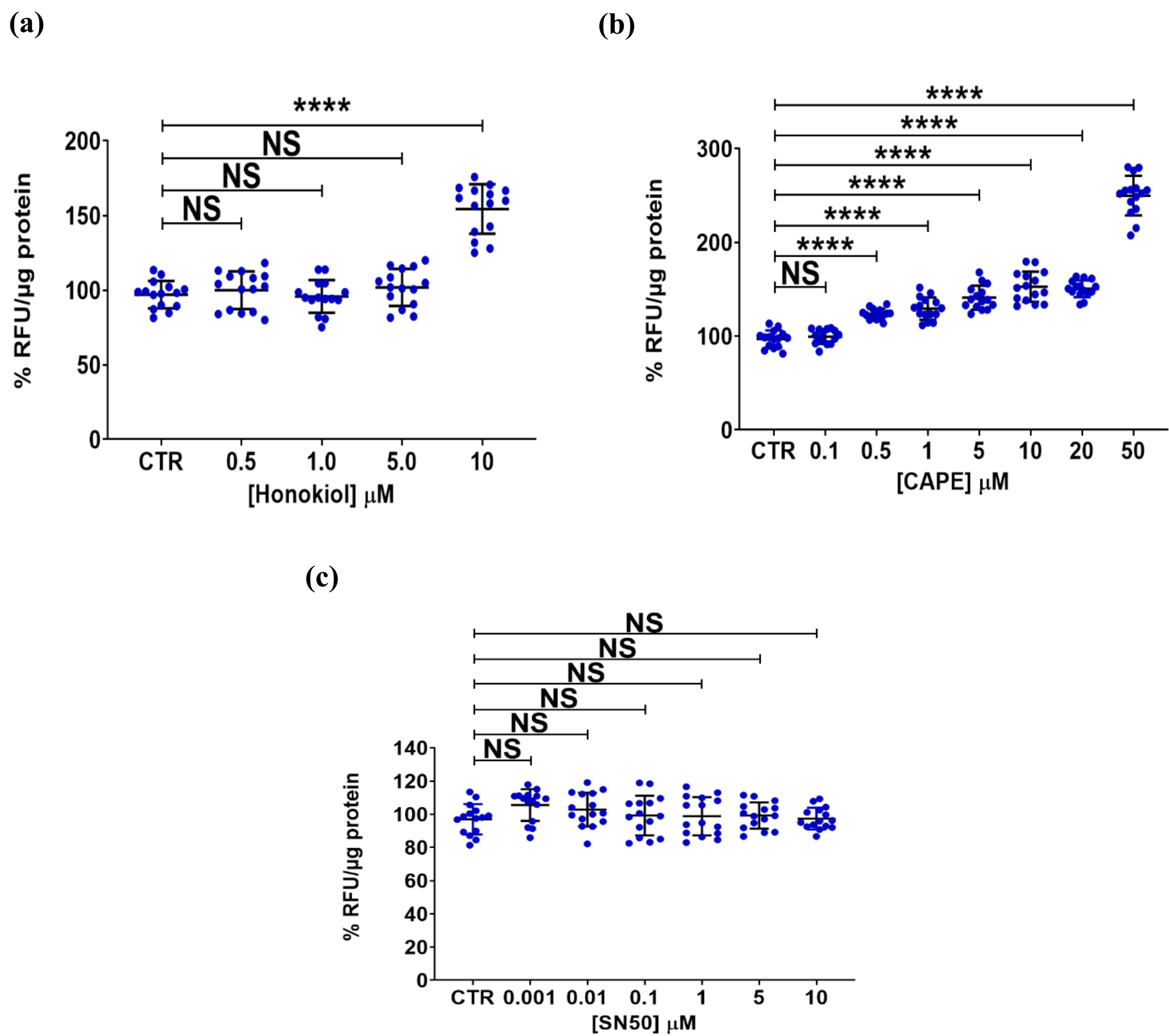


Figure G.9: Effects of NF-κB inhibitors on ABCC5 transporter activity at 24 h
Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of (a) honokiol, (b) CAPE and (c) SN50 for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: $P < 0.0001$, NS: non-significant.

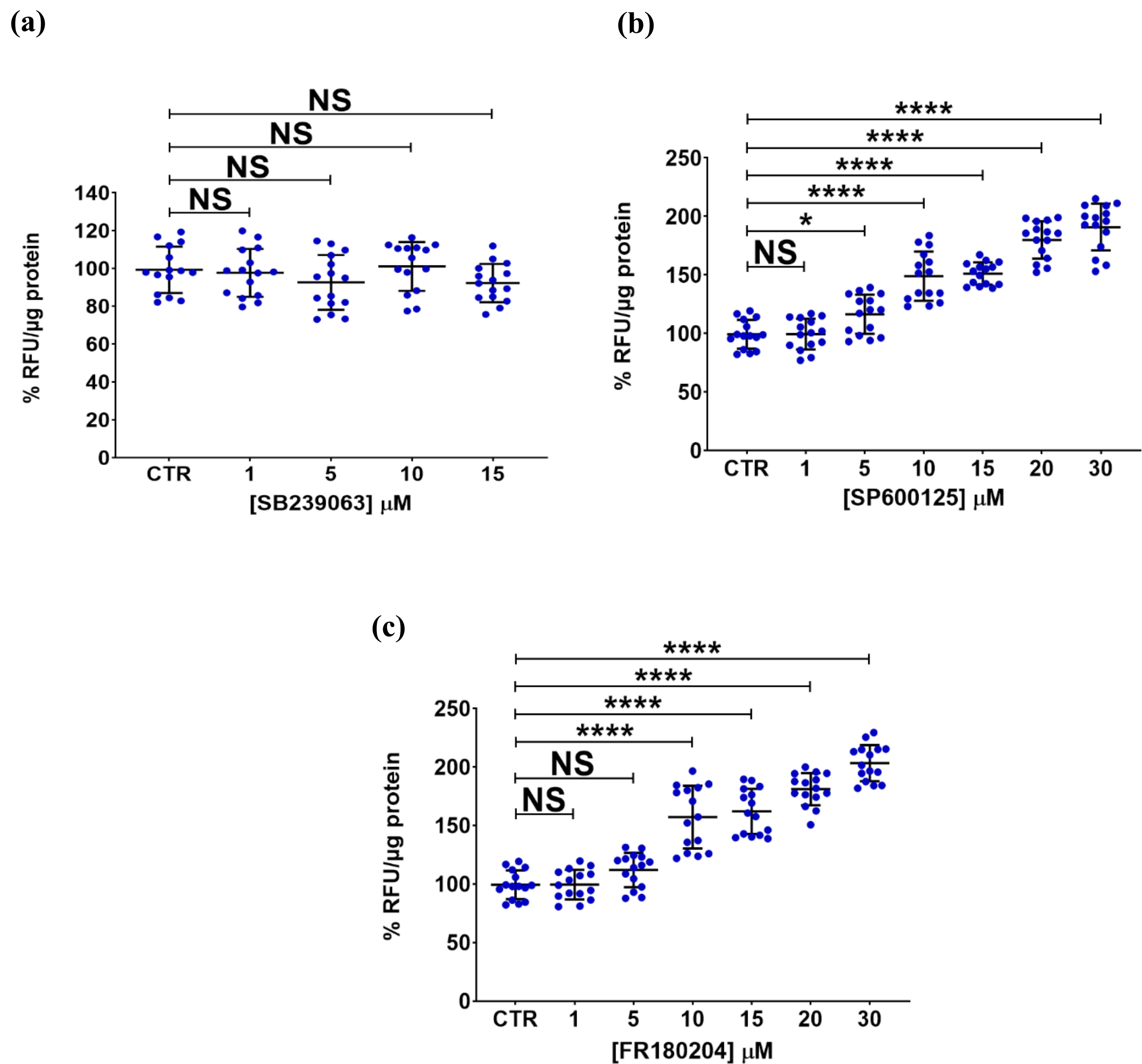


Figure G.10: Effects of MAPK inhibitors on ABCC5 transporter activity at 24 h
Intracellular accumulation of GS–MF - ABCC5 substrate was measured in control cells and cells treated with different concentrations of (a) SB239063, (b) SP600125 and (c) FR180204 for 24 h. Data were analysed using One-Way ANOVA with Tukey as post hoc test and are presented as mean ± SD of five independent experiments, with at least three replicates in each experiment. RFU: relative fluorescence units. CTR: control, ****: P < 0.0001, NS: non-significant.

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