The Effects of Clioquinol, Zinc and Copper on the Abundance and Function of Breast Cancer Resistance Protein in Human Brain Microvascular Endothelial Cells

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Thesis abstract

The blood-brain barrier (BBB) is a complex network of capillary endothelial cells that form a large semi-permeable interface between the brain parenchyma and blood perfusing the brain. The passage of most molecules is tightly regulated by this interface and it is therefore often referred to as the "bottleneck" in brain drug development as it is a major obstacle in the penetration of potential CNS therapeutics. These specific endothelia express a large number of transporter proteins that serve to meet the high energy demands of the brain and to prevent the entry of potentially harmful molecules present in the blood. One of these transporters is breast cancer resistance protein (BCRP), a ~74 kDa ATP-binding cassette (ABC) transporter that is highly expressed at the luminal surface of brain microvascular endothelial cells. Capable of transporting a vast range of endogenous molecules and drugs, BCRP plays a major role in preventing the penetration of many blood-borne molecules into the brain. Chemical inhibition or genetic deletion of BCRP has been repeatedly shown to increase the brain accumulation of many therapeutics into the brain. Therefore, approaches aimed at decreasing the expression and function of BCRP at the BBB may lead to increased brain accumulation of CNS targeted therapeutics. In addition to the trafficking of drugs, it has been demonstrated that the efflux of amyloid-beta (Aβ), a neurotoxic peptide that accumulates in the Alzheimer's disease (AD) brain, is mediated by BCRP. Therefore, Increased expression and function of BCRP favours the removal of Aβ from the brain, reduces the brain level of Aβ and thus slows down the progression of AD.

As BCRP is highly relevant in the field of CNS drug access and potentially AD pathogenesis, unravelling novel approaches to modulate its expression and function may improve CNS penetration of drugs or enhance the extrusion of Aβ. To achieve such an aim, a brain-endothelial targeted approach would be required without affecting the crucial function of BCRP in other barriers such as the gastrointestinal tract, liver and kidney. Interestingly, several studies have demonstrated the potential of metals, specifically Zn^{2+} and Cu^{2+}, to impact molecular pathways involved in regulating BCRP, such as peroxisome proliferator-activated receptor alpha (PPARα) and glycogen synthase kinase-3 beta (GSK3β), suggesting that there may be a link between metal ion availability and BCRP

expression. Recent studies in our laboratory have shown that clioquinol (CQ, a metal chaperone) together with Zn^{2+} and Cu^{2+} (the combination referred to as CZC) was able to increase the expression and transport function of P-glycoprotein (P-gp) in hCMEC/D3 cells, a human brain endothelial cell model. Therefore, the aims of this study were to assess the impact of CZC on the protein abundance and function of BCRP in hCMEC/D3 cells and to identify the mechanisms responsible for any effect of this combination.

In order to carry out these studies, it was necessary to develop and optimize specific assays to address the aims. The MTT assay was used to assess the effects of CZC on hCMEC/D3 cell viability. To assess the protein expression of BCRP in hCMEC/D3 cells following treatment with CZC, Western blotting, a commonly used technique to quantify proteins of interest was optimised and employed. Furthermore, to assess the impact of CZC on BCRP function, a substrate accumulation assay was developed.

hCMEC/D3 cells were treated with CQ, Zn^{2+} and Cu^{2+} (CZC) (0.5 µM, 0.5 µM, 0.1 µM, respectively) for 24 h and BCRP mRNA and protein abundance was determined by qPCR and Western blot, respectively. After a series of optimisation studies assessing specificity of bodipy prazosin (BP) and Ko143 as a substrate and inhibitor of BCRP, respectively, the impact of CZC on BP uptake was assessed. While CZC did not increase mRNA expression of BCRP, BCRP abundance was increased 1.8 ± 0.1-fold; this was associated with a 68.1 ± 3.3% reduction in the accumulation of BP in hCMEC/D3 cells.

The findings resulting from this research are the first to demonstrate the ability of BCRP abundance and function to be regulated by modulating cytosolic metal concentrations, in a model of the human BBB, which may be exploited to influence the CNS passage of therapeutics and endogenous substrates.

Publications during enrolment

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Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer reviewed journal. The core theme of the thesis is pharmaceutical science. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within Monash University under the supervision of A/Prof Joseph Nicolazzo and Dr Jennifer Short (co-supervisor).

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of chapter 3 my contribution to the work involved the following:

Thesis Chapter	3
Publication Title	A Combination of Clioquinol, Zinc and Copper Increases the Abundance and Function of Breast Cancer Resistance Protein in Human Brain Microvascular Endothelial Cells
Status	Published
Nature and % of student contribution	90%. Concept, collecting data and writing first draft
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Co-author(s), Monash student Y/N*	1) No 2) No

Student signature: Christopher Yap **Date:** **4/04/2021**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: A/Prof Joseph Nicolazzo **Date:** 05/04/2021

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Abbreviations

Abbreviation	
Aβ	Amyloid beta
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
BP	BODIPY prazosin
CFB	Clofibrate
CQ	Clioquinol
CZC	Clioquinol, Zn^{2+} and Cu^{2+}
P-gp	P-glycoprotein
MRP1	Multidrug resistance protein-1
Cu^{II}(Atsm)	diacetyl*bis(N*(4)-methylthiosemicarbazonato)-Cu^{II}
Cu^{II}(Gtsm)	glyoxal-bis(N4-methylthiosemicarbazonato)Cu^{II}

Chapter 1 - General introduction

1. The structure and function of the blood brain barrier (BBB)

The blood-brain barrier (BBB) is a complex network of capillary endothelial cells that form a large semi-permeable interface between the brain parenchyma and blood vessels perfusing the brain, spanning a surface area of 12-18 m^2 in the average human adult (Abbott 2010). BBB endothelia express a variety of functional proteins that, in conjunction with other cell supports, tightly regulate the passage of molecules between the brain and blood, making this interface essential for maintaining brain homeostasis but also giving rise to multidrug resistance (*Figure 1*). These structures and proteins, as a collective, are known as a neurovascular unit (NVU). Tight junction (TJ) proteins such as claudin, occludin and junctional adhesion molecules are expressed between brain endothelial cells which maintain cell-cell adhesion and prevent the paracellular diffusion of molecules, acting as a physical barrier. Astrocyte foot processes supply the endothelium with factors such as basic fibroblast growth factor (Sobue 1999) and regulate blood flow (Attwell 2010). Pericytes, which line the capillaries, have been implicated in the regulation of vesicular transport across the BBB, gene expression and astrocyte polarization (Armulik 2010).

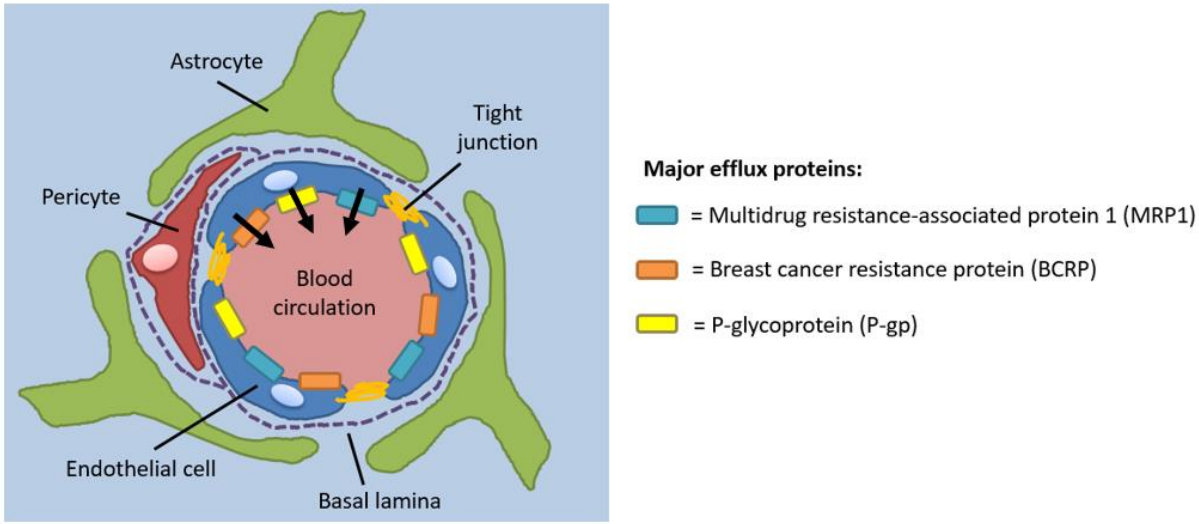

Figure 1 A schematic diagram of the BBB and peripheral structures.

The BBB is often referred to as the “bottleneck” in brain drug development as it is a major obstacle to the access of CNS therapeutics. Almost all large molecule therapeutics and ~98 % of small molecule drugs do not succeed in bypassing the BBB which significantly limits the number of effective treatments for many CNS disorders; a problem that exists to this day (Pardridge 2005). Specific proteins known as ATP-binding cassette (ABC) transporters play a major role in this multidrug resistance; P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP) are highly expressed at the BBB and many structurally diverse molecules are recognised as substrates to these transporters. Thus, the BBB plays an important role in defending the brain from potentially harmful blood borne molecules but with the duality of being a major obstacle for CNS drug development.

2. ATP-binding cassette (ABC) transporters and their role at the BBB

A major distinguishing factor between the specialized brain capillary endothelial cells that comprise the BBB and peripheral capillary endothelial cells is the comprehensive expression of ABC transporter proteins (Abbott 2010). These transport proteins are part of a superfamily of efflux transporters known as ATP-binding cassette (ABC) transporters and pose a large obstacle to the development of novel therapeutics required to cross the BBB. P-gp, BCRP and MRP1 have received a large research focus due to their high expression, relevance in drug disposition, and suspected role in neurodegenerative diseases. These transporters are capable of binding to and transporting a vast spectrum of structurally unrelated molecules. They are largely responsible for the restricted passage of blood-borne molecules into the brain which is problematic for brain targeted therapeutics. Each neuron is only 8 - 20 μm away from a blood microvessel and thus any drug targeted at neurons would immediately reach its site of action if this restrictive interface were overcome (Spencer 2007). Research on the expression, function, and regulation of ABC transporters at the BBB in health and disease is therefore crucial for the advancement of brain targeted therapeutics. Of these transporters, BCRP is the most highly expressed at the BBB in humans (Uchida 2011) and therefore warrants efforts to understand its regulation, function, and approaches to modulate these properties to open new avenues for CNS drug development.

3. BCRP and its role at the BBB

BCRP was first discovered by Doyle et al. in the multidrug-resistant human breast cancer cell line MCF-7/AdrVp, as the model displayed multidrug resistance (MDR) to various chemotherapeutics despite the absence of well-known MDR proteins P-gp and multidrug resistance protein-1 (Doyle 1998). BCRP is a ~74 kDa ATP-binding cassette (ABC) transporter that has been shown to function as a homodimer (Kage 2002) and is highly expressed at the luminal surface of brain microvascular endothelial cells (Weksler 2005). It can transport a vast range of endogenous substrates, xenobiotics, and pharmaceuticals, which have been extensively studied and summarized (Nicolazzo 2009). BCRP is also expressed in placental syncytiotrophoblasts, small intestine and colon epithelium, liver, and luminally in brain endothelial cells (Maliepaard 2012). The ever-growing list of substrates of BCRP are not just limited to chemotherapeutics such as prazosin, methotrexate, mitoxantrone and topotecan but even endogenous molecules such as estrone-3-sulfate (Figure 2). This pattern of expression and transport function suggests that BCRP plays a major protective role in many organs throughout the body. BCRP has also been implicated in the transport of amyloid beta (Aβ), an endogenous neurotoxic peptide whose build up in the brain plays a central role in the pathology of Alzheimer's disease (AD) (Xiong 2009, Tai 2009). The build-up of brain Aβ has been attributed to an overproduction of the peptide itself in familial AD due to genetic defects. This, however, comprises a small percentage of AD occurrences. In the majority of AD cases, termed "sporadic AD", it was discovered that the clearance of Aβ from the brain is impaired (Mawuenyega 2010). BCRP at the BBB plays a major role in multidrug resistance and may also protect the brain from Aβ burden.

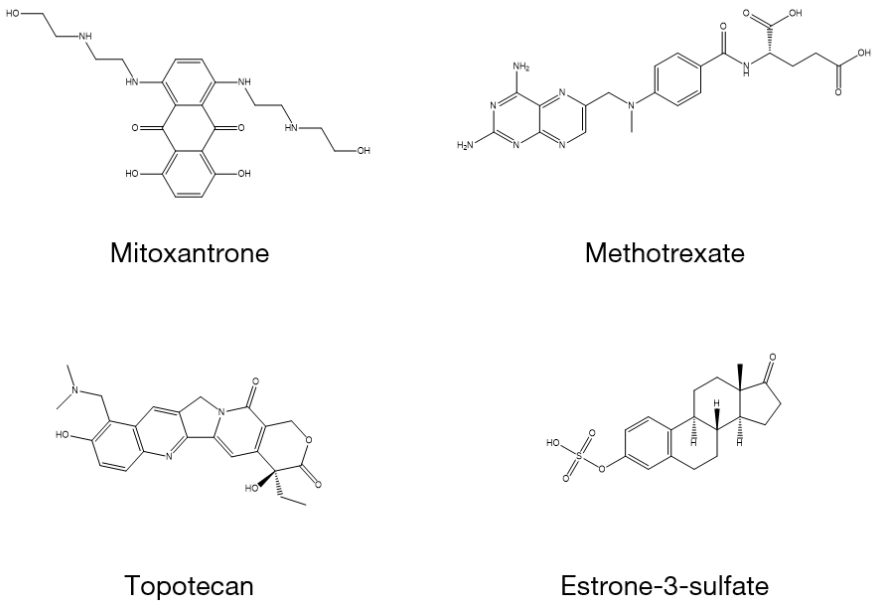

Figure 2 Molecular structures of known BCRP substrates include both synthetic and endogenous molecules.

4. BCRP and multidrug resistance (MDR)

4.1 Central nervous system (CNS) targeted therapeutics

Chemical inhibition or genetic deletion of BCRP has been repeatedly shown to increase the brain accumulation of many therapeutics. Pantoprazole mediated inhibition of BCRP or genetic knockout of BCRP led to a significant increase in the mouse brain accumulation of the anticancer drug, imatinib mesylate (Breedveld 2005). Similarly, CGP74588, a N-desmethyl derivative of imatinib, was found to have a significantly higher brain penetration in BCRP (−/−) mice relative to wild-type mice (Bihorel 2007). In another study, Nakanishi et al. showed that BCRP (−/−) mice displayed a higher brain to plasma ratio of various anti-epileptic drugs (AEDs) (Nakanishi 2013) such as phenytoin, lamotrigine and levtiracetam. However, as 6 AEDs were delivered simultaneously, it must be considered if competitive drug binding occurred and whether all AEDs tested were substrates of BCRP. Preclinical studies have also shown that chemically inhibiting BCRP function in mice increased brain penetration of imatinib (Breedveld 2006). From these reports, it appears that approaches aimed at decreasing the expression and function of BCRP at the BBB can influence the effectiveness of CNS targeted therapeutics.

4.2 BCRP in prevalent neurodegenerative diseases

Increasing BCRP expression at the BBB may be beneficial to slow down the progression of AD. Studies have demonstrated that Aβ is a substrate of BCRP. Using hCMEC/D3 cells, an immortalized human brain endothelial cell line, specific inhibitors of BCRP significantly increased the permeability of ^{125}I-A$\beta_{1\text{-}40,}$ a 40-peptide isoform of Aβ (Tai 2009), suggesting that BCRP plays a role in the prevention of blood-borne Aβ into the brain. To show that BCRP could actively transport Aβ *in vivo*, Cy5.5-labeled A$\beta_{1\text{-}40}$ was intravenously injected into wild-type mice and BCRP (−/−) mice where the brain accumulation was measured. BCRP (−/−) mice accumulated significantly more brain Cy5.5-labeled Aβ than their wild-type counterparts implicating BCRP in the efflux of Aβ across the BBB. Concordant with these results, another laboratory observed brain levels of intravenously injected Cy5.5-labeled A$\beta_{1\text{-}40}$ was much higher in BCRP (−/−) mice compared to wild-type mice (Shen 2010).

These findings are consistent with observations that BCRP was found to co-immunoprecipitate with Aβ in human AD brain microvessels (Xiong 2009). It should be noted that Hartz et al. found no evidence for BCRP-mediated transport of fluorescein-human $A\beta_{42}$ using isolated mouse brain capillaries. This may be due to species differences in efflux transporter expression where BCRP is more highly expressed at the human BBB compared to murine models (Uchida 2011). These results suggest that BCRP mediates the efflux of Aβ, and that modulation of BCRP expression and function could also impact Aβ accumulation in the brain, and therefore, AD pathogenesis.

5. The regulation of BCRP expression at the BBB

5.1 Overview of current identified regulators of BCRP at the BBB

As BCRP is highly relevant in the field of CNS-drug access and potentially AD pathophysiology, it is important to understand its mechanisms of regulation in order to improve CNS drug penetration or enhance the extrusion of Aβ. Peroxisome proliferator activated receptor-α (Hoque 2012, More 2016), constitutive androstane receptors (Wang 2010), estrogen receptors (Hartz 2010), glucocorticoid receptors (Narang 2008) and aryl hydrocarbon receptors (Wang 2011) have all been shown, upon activation or inhibition, to regulate BCRP in models of the BBB. It has also been observed both *in vitro* and *in vivo* that pharmacological inhibition of glycogen synthase kinase 3β (GSK3β) and consequent accumulation of cytosolic β-catenin leads to an increased transcript and function of BCRP at the BBB (Lim 2008, Harati 2013). Interestingly, several studies have repeatedly demonstrated the potential of metals, specifically Zn^{2+} and Cu^{2+}, to impact molecular pathways involved in regulating BCRP, PPARα and GSK3β in particular, suggesting that there may be a link between metal ion availability and BCRP expression (Ilouz 2002, Reiterer 2004, Shen 2008, Kang 2009, Crouch 2009, Lei 2017, McInerney 2018, Zaremba 2017 Zaremba 2019). In the following sections, the general roles of PPARα and GSK3β in the regulation of BCRP will be discussed. This will be followed by discussion of the biometals Zn^{2+} and Cu^{2+} and how their bioavailability has been shown to affect the activities of PPARα and GSK3β and, as a result, may govern BCRP expression and function.

5.2 The regulation of BCRP by peroxisome proliferator-activated receptor alpha (PPARα)

PPARs are transcription factors that are activated via ligands. Of the three isotypes of PPAR α, β and γ, PPARα has been reported to be expressed in the liver, kidneys, heart, skeletal muscle, and more recently, brain endothelial cells (Hoque 2012). PPARα is well known to be involved in lipid metabolism such as fatty acid oxidation, transport, and uptake, and possesses anti-inflammatory properties upon activation (Kersten 2000). To induce transcription of a target gene, PPARs will bind to a specific promoter region and then form a heterodimer with the retinoid X receptor. For this complex, either receptor can be activated to induce gene transcription, but a higher potency is

achieved if ligands for both receptors are bound (*Figure 3*). PPARα has been widely studied in liver hepatocytes due to its suggested role in certain diseases such as atherosclerosis, alcoholic liver disease and diabetes. Recent studies have demonstrated a role for PPARα in the regulation of BCRP at the BBB (Hoque 2012, Hoque 2015, More 2016). PPARα was first shown to upregulate BCRP in the hepatocytes of CD-1 mice treated with clofibrate (CFB); a PPARα agonist. While ~50% increases in mRNA and protein expression were observed in treated wild-type mice hepatocytes, these effects were abolished in the PPARα knockout mice (Moffit 2006). Another study showed similar increases of BCRP mRNA in C57BL mice hepatocytes using different PPARα agonists Wy14643 and GW7647 (Hirai 2007).

These data, in part, formed the basis of a study, the first of its kind, investigating the regulation of BCRP by PPARα using the hCMEC/D3 cell line *in vitro* model of the BBB (Weksler 2005). 24-hour treatments of PPARα ligands clofibrate (100 μM) or GW7647 (20 nM) were able to significantly increase protein abundance and function of BCRP (Hoque 2012). This study was the first to demonstrate direct BCRP regulation by PPARα in a human BBB cell model. Further studies were then conducted *in vivo* showing that isolated mouse brain capillaries exposed to CFB had significantly higher transport activity and induction of BCRP mRNA and protein in a much shorter timeframe than previously conducted *in vitro* studies (Hoque 2015). These studies demonstrate that PPARα directly regulates BCRP expression and function at the BBB.

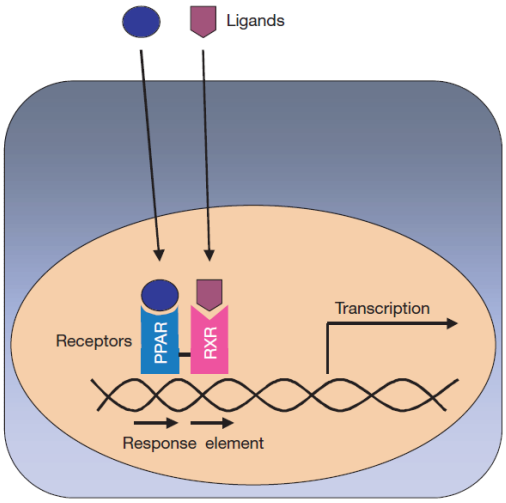

Figure 3 Basic summary of ligand activated PPAR activation resulting in gene transcription. This figure was adapted from Kersten et al. (2000).

5.3 The Wnt/β-catenin signalling pathway

GSK-3 is regulatory kinase which has, since its discovery, been shown to phosphorylate a variety of intracellular proteins involved in different pathways (Jope 2004). GSK-3 is involved in many important signalling pathways, one of which is the regulation of the Wnt/β-catenin pathway responsible for the expression and homeostasis of the BBB, including regulation of tight junction proteins and efflux transporters such as BCRP (Harati 2013, Lim 2008). Wnt ligands are endogenous glycoproteins which can activate the Frizzled (FzR) receptor involved in the stabilisation of the protein β-catenin. When the FzR receptor is activated, a destruction complex, containing GSK-3, which normally binds and ubiquitinates β-catenin is deactivated. The resulting accumulation of β-catenin then enters the nucleus and acts as a transcription factor for proteins. However, in the absence of Wnt ligand, the destruction complex remains active, and β-catenin is targeted for ubiquitination. Deactivation of the destruction complex can also be achieved if GSK-3 is inhibited.

A recent study using the hCMEC/D3 cell line showed that treatment with 0.5 μM of a GSK3 inhibitor, BIO, resulted in a significant increase in intracellular β-catenin. The authors then investigated the downstream effects of the treatment on ABC transporters and found that BCRP expression at both the mRNA and protein level were significantly upregulated (Lim 2008). Similarly, Harati et al. observed, *in vivo,* an increase in BCRP protein expression in fresh rat brain microvessels that had been injected with either GSK-3 inhibitor [(2-Z,3-E)-6-bromoindirubin-3-oxime] or BIO (Harati 2013) due to β-catenin stabilisation. Additionally, the authors observed a significant decrease in the uptake of prazosin, a substrate of BCRP, following GSK-3 inhibition when compared to control. Taken together, these results suggest that GSK-3 is a molecular regulator of BCRP at the BBB.

6. Biometals and their role in homeostasis of the brain and BBB

6.1 Overview of biometals and their role in brain homeostasis signalling

There has been recent interest in the metal ions Cu^{2+} and Zn^{2+} for their roles in critical cell functions and signalling pathways. They are considered biochemically functional metals (biometals) as they are integral for the activity of multiple essential cellular functions, especially in the brain. Studies have shown that Cu^{2+} and Zn^{2+}, which are found in high concentrations in the brain (Que 2008), are released during neurotransmission, establishing an important role for these metals in healthy brain function (Opazo 2013).

Intracellular Cu^{2+} is essential for basic cell function. This metal acts as a cofactor for numerous enzymes. For example, the cytosolic enzyme, superoxide dismutase, neutralizes toxic by-products of cellular activity such as free radicals. Cytochrome c oxidase, found in the mitochondria, requires Cu^{2+} for electron transfer in the production of ATP. Peptidylglycine α-amidating monooxygenase, involved in the synthesis of peptides, could not properly function without Cu^{2+} (Gaier 2013). Recent studies by Bica et al. demonstrated the beneficial effects of Cu^{2+} on neurons *in vitro*. The increase in bioavailable Cu^{2+} using the synthetic ionophore, glyoxal-bis(N4-methylthiosemicarbazonato)Cu(II) (Cu-gtsm), in PC12 cells robustly promoted neurite elongation, a sign of neuronal health. While a mechanism of action was not confirmed, the authors discovered that the Cu^{2+} chelating complex was able to inhibit phosphatases and suggested this as a potential link to the neuroprotective outcomes they observed (Bica 2014). Cu^{2+} has also been shown to be released by synaptic terminals at the hippocampus, with modulatory effects on the NMDA and $GABA_A$ receptors essential for proper memory function and synaptic plasticity (Peters 2011). It is interesting to note that the concentration of Cu^{2+} at the human hippocampus is higher than other brain regions, indicating a crucial role for Cu^{2+} in memory function (Dobrowolska 2008). Together these studies show an important role for Cu^{2+} in the regulation and homeostasis of critical cellular functions in the human body.

Zn^{2+} also plays a crucial role in cell protein function and acts as a cofactor to ~3000 human zinc proteins (Maret 2013). Primarily in the cerebral cortex, but also in the hippocampus, free Zn^{2+} is

stored within presynaptic vesicles also containing glutamate, termed ‘gluzingergic’, and these vesicular contents are released during neurotransmission (Frederickson 2001, Adlard 2010, Frederickson 2005). At the postsynaptic terminal, Zn^{2+} has been shown to modulate receptors such as the AMPA and NMDA receptors involved in synaptic plasticity and signalling (Takeda 2001). The importance of brain Zn^{2+} in neurotransmission was highlighted by a recent study investigating the zinc transporter-3 (ZnT3) protein. ZnT3 is responsible for the packaging of Zn^{2+} and movement of ‘gluzinergic’ vesicles to the presynaptic terminal. Using the ZnT3 knockout (KO) mouse, the authors observed the manifestation of learning and memory deficits in KO mice at 6 months of age. This was associated with significant decreases in crucial presynaptic and postsynaptic hippocampal proteins (Adlard 2010).

Thus, the important roles that Zn^{2+} and Cu^{2+} play in critical cellular function are clearly defined. However, it is also crucial to understand the effects these metals have on the BBB. High expression levels of metal transporters are present at the BBB such as divalent metal transporter-1 and copper transporter-1 which tightly regulate the influx of biometals between the blood and the brain (Choi 2009). It is well known that in CNS diseases such as AD, there is a maldistribution of metals in the brain and altered regulation of ABC transporters at the BBB, which have been summarised (Wolf 2012). Despite this, the relationship between metals and transporter expression at the BBB have not been thoroughly explored. Several studies have repeatedly demonstrated Zn^{2+} and Cu^{2+} to directly impact PPARα and GSK3β, which have been shown to regulate BCRP expression and function in models of the BBB (Hoque 2012, Zaremba 2018). Thus, there is a link between metal ion availability and the regulation of BCRP.

6.2 The link between biometals and the regulation of efflux transporters at the BBB

Studies have demonstrated the ability of Zn^{2+} and Cu^{2+} to impact pathways involved in regulating BCRP; PPARα and GSK3β. PPARα has been identified as a zinc finger transcription factor, where zinc is responsible for holding the receptor in an active conformation which is essential for binding to DNA. Reiterer et al. reported a significant decrease in PPARα target genes in Zn^{2+} deprived porcine vascular endothelial cells, an effect associated with decreased PPARα-DNA binding (Reiterer 2004), suggesting that Zn^{2+} was critical for PPARα activity. Kang et al. demonstrated that mice fed a Zn^{2+} deprived diet had significantly reduced PPARα expression and binding to DNA indicating lower PPARα signalling, effects which were rectified to some extent with Zn^{2+} supplementation (Kang 2009). Shen et al. showed that Zn^{2+} deprived porcine endothelial cells had reduced expression of PPARα mRNA and protein, an observation which was reversed by Zn^{2+} supplementation (Shen 2008). Zn^{2+} has also been shown to directly inhibit the enzymatic activity of pure GSK-3 (Ilouz 2002) which degrades β-catenin, a regulator of BCRP expression at the BBB (Xinquan 2014). Several studies have thus highlighted the importance of Zn^{2+} in PPARα signalling (*Figure 4*).

In addition to the multiple studies demonstrating a crucial role of Zn^{2+} in affecting pathways involved in BCRP regulation, a role for Cu^{2+} has also been suggested. Studies by Crouch et al. showed that the delivery of Cu^{2+} via the Cu^{II}(gtsm) compound inhibited GSK-3 in SH-SY5Y cells, an effect not observed when cells were treated with Cu^{II}(atsm), a similar compound that did not release Cu^{2+} (Crouch 2009). Cu^{2+} has also been shown to increase PPARα gene expression in liver, skeletal muscle and adipose tissue in rabbits with a copper enriched diet (45 mg/kg for 8 weeks) (Lei 2017). Therefore, the bioavailability of cytosolic Zn^{2+} and Cu^{2+} appear to be essential for PPARα and GSK-3 signalling which have been shown to regulate BCRP expression and function in models of the human BBB (*Figure 5*).

It has also recently been demonstrated that increasing cytosolic metal concentrations in BBB models was able to increase the expression or function of ABC transporters. Recent studies by our lab have shown that a 24-hour combination treatment of a metal ionophore (clioquinol) with Zn^{2+} and Cu^{2+} (0.5, 0.5 and 0.1 µM respectively) was able to significantly increase the expression and function of P-

gp in hCMEC/D3 cells (McInerney 2018). This was the first study, to our knowledge, to show a relationship between biometal availability and ABC transporter regulation. Other studies have shown an acute increase in P-gp and BCRP function when exposed to 0.1-10 μM $ZnCl_2$, further demonstrating a link between biometals and ABC transporter regulation (Zaremba 2017, Zaremba 2019). In summary, the stabilisation of β-catenin has been shown to impact the expression and function of BCRP in models of the BBB (Lim 2008, Harati 2013) and this was initiated via GSK-3 inhibition. Zn^{2+} and Cu^{2+} have been shown to inhibit GSK-3 activity (Ilouz 2002 and Crouch 2009 respectively). Thus, increasing cytosolic Zn^{2+} and Cu^{2+} could inhibit GSK-3 activity and result in the stabilisation of β-catenin subsequently increasing BCRP expression and function. Despite this, the impact of such metals on the expression of BCRP at the human BBB has not been investigated.

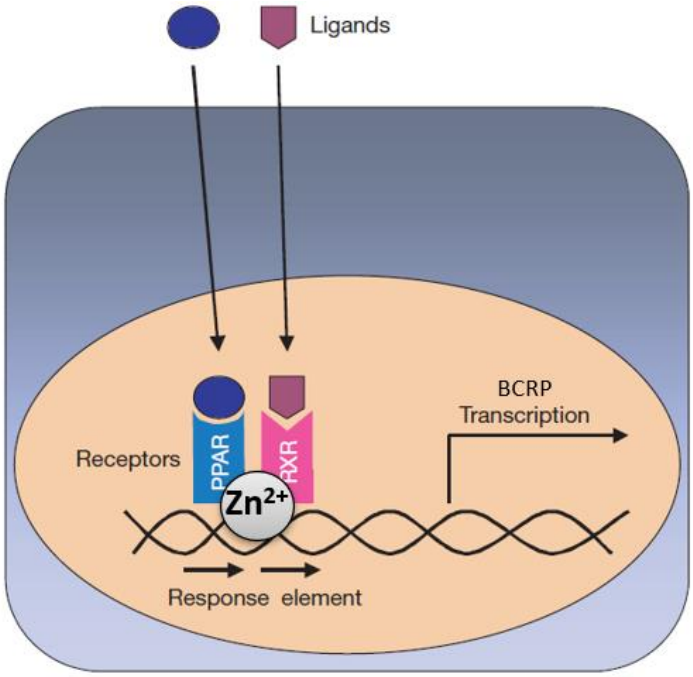

Figure 4 Zn^{2+} is required for PPARα complex activation. An increase in Zn^{2+} bioavailability may lead to increases in BCRP expression. This figure was adapted from Kersten et al. (2000).

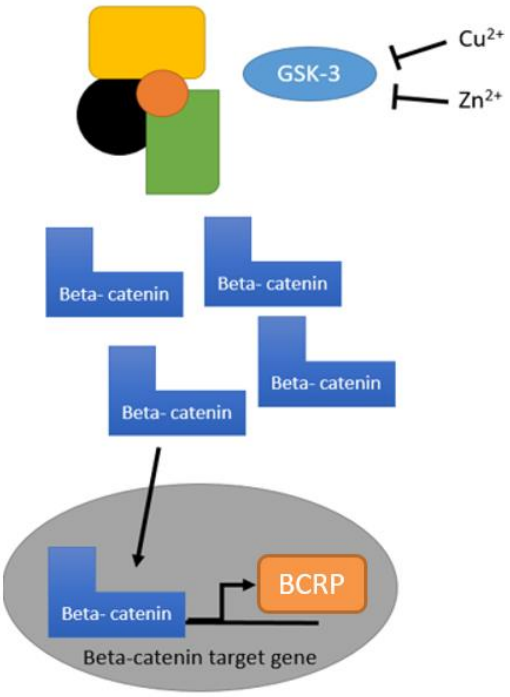

Figure 5 Inhibition of GSK-3 by contact with Cu^{2+} or Zn^{2+} deactivates the destruction complex that normally breaks down beta-catenin. Accumulation of cytosolic beta-catenin leads to increased transcription of target proteins including BCRP.

7. Hypothesis

It is hypothesized that modulating cytosolic metals in brain endothelial cells will impact the expression and function of BCRP, potentially via PPARα or GSK3β signalling pathways, which has consequences for increasing the brain access of CNS drugs and the clearance of Aβ in AD.

8. Research aims

The aim of this study was to assess the impact of clioquinol, Zn^{2+} and Cu^{2+} (CZC) on the protein abundance and function of BCRP in brain endothelial cells and identify the mechanisms responsible for any effect of this combination. As this was the first study to investigate this, experiments were conducted using hCMEC/D3 cells, an *in vitro* human model of the BBB which is easily cultured and is able to maintain a stable BBB phenotype (Weksler 2005, Weksler 2013). To address these aims, specific assays were developed and optimised. Briefly, to assess the protein expression of BCRP in hCMEC/D3 cells following treatment with CZC, Western blotting, a commonly used technique to quantify proteins of interest was employed. As an increase in protein abundance was expected, we used CFB, a known up-regulator of BCRP in hCMEC/D3 cells as a positive control. Using this technique, we observed the effects of CZC treatment on the protein abundance of BCRP. To assess if the observed changes in BCRP protein expression led to a change in function, a substrate accumulation assay was used. It was necessary to select a substrate specific to BCRP and to identify the optimal duration for accumulation in order to observe any changes in function caused by CZC. To determine if these changes by CZC were due to changes the genomic regulation of BCRP, the quantitative polymerase chain reaction (qPCR) assay was used to assess any changes in BCRP mRNA. The next chapter of this thesis will cover, in detail, the rationale and the steps taken to develop and validate the assays that were required to appropriately address the research aims.

Following this chapter, the experimental results of this study will be covered in detail. It is of importance to mention that this body of work is now a published journal article (Yap 2020). This is the first study to demonstrate that augmenting metal ion availability enhances protein abundance and function of BCRP at the BBB, which may be exploited to modulate the CNS access of therapeutics and endogenous substrates. With these studies, we hope to gain a better understanding of the relationship between biometals and the regulation of BCRP at the BBB, and to inspire others to further this research.

9. References

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Chapter 2 - Optimisation and validation of Western blot and substrate accumulation assays to measure breast cancer resistance protein expression and function in hCMEC/D3 cells

1. Introduction

The aims of this project were to assess the impact of clioquinol (CQ), zinc and copper (CZC) on the protein expression and function of breast cancer resistance protein (BCRP) using the hCMEC/D3 cell line as a model of human brain endothelial cells. As CZC treatment was expected to increase the protein abundance of BCRP, a known up-regulator, clofibrate (CFB), was used as a positive control (Hoque 2012). To measure protein expression and function, the western blot and a substrate accumulation assay were used, respectively. Thus, it was necessary to develop and optimise these methods to achieve valid results.

In order to determine the effects of CZC and CFB treatment on BCRP in hCMEC/D3 cells, it was essential to determine if the intended treatments and respective positive controls compromised cell viability. To do this, the MTT assay, a commonly used technique to assess the effects of interventions on cell viability, was used. MTT (3-(4,5-dimethylthiaazol-2-yl)-2,5-diphenyltetrazolium bromide), yellow in colour, is reduced by reductase enzymes in the mitochondria of cells to purple formazan. Typically, one or more treatments are delivered to cells, with 10% dimethyl sulfoxide (DMSO) used as a positive control to stimulate cell death. MTT is then added, and the absorbance of the purple formazan is measured where a higher level of absorbance indicates more functional mitochondria and therefore cell viability. This method was used to ensure that CZC and CFB treatments did not significantly affect normal cellular function which could complicate and invalidate data (McInerney 2018).

To assess the protein expression of BCRP in hCMEC/D3 cells, Western blotting, a commonly used technique to quantify proteins of interest was optimised. Next, to determine if any changes in BCRP protein expression in hCMEC/D3 cells led to an overall change in transporter function, a substrate accumulation assay was performed. This was necessary as an observed increase in BCRP protein may

not have necessarily resulted in changed function. BCRP, in particular, requires post translational modifications such as N-glycosylation, disulphide bond formation and phosphorylation for proper functional expression at the cell membrane (Wakabayashi 2009).

Western blotting is an analytical method capable of quantifying one or more specific proteins of interest and has been commonly used with hCMEC/D3 cells (Hoque 2012, Zaremba 2019). The sample is denatured chemically, by heat, or a combination of both and separated based on weight by electrophoresis (SDS-PAGE). The protein is then transferred to a membrane and quantified using primary and secondary antibodies. Several steps are necessary for optimising this process to achieve data consistency and linearity. First, it is essential that primary antibody specificity is confirmed using a positive control sample. For example, either a BCRP transfected cell line or human liver cancer cell line, such as HepG2, are suitable positive controls as they are known to contain human BCRP protein (Sukowati 2012). Next, it needs to be shown that the Western blot is able to generate signals that accurately reflect varying amounts of BCRP protein in hCMEC/D3 samples, as ultimately this signal is used to calculate fold changes in protein expression. To confirm this, a linear curve of known protein samples is processed by Western blotting and the resulting protein signals should be proportional (i.e. the signal generated by 10 µg should be approximately 2-fold greater in a 20 µg sample). These optimisations allow the assay to specifically detect the protein of interest and accurately reflect any changes in BCRP protein expression in hCMEC/D3 cells.

A substrate accumulation assay is typically used to determine the efflux capacity of a biological cell or tissue. This assay was used to evaluate the function of BCRP in hCMEC/D3 cells following treatment with CZC. BODIPY prazosin (BP) is a fluorescent substrate with high specificity for BCRP and was therefore used to measure transporter function (Qawasami 2014). Untreated hCMEC/D3 cells are able to prevent the accumulation of the substrate to a certain extent. If CZC increases BCRP protein expression and, therefore, function, a decreased accumulation of BP in the cell cytosol was predicted. For each assay, it is required to show that BCRP was functional for the duration of the experiment. To confirm this, Ko143, a potent inhibitor of BCRP was used (Allen 2002), and a significant increase in substrate accumulation was expected, indicative of reduced BCRP function.

This control step was important in order to attribute the effects of CZC to higher transport activity of BCRP as opposed to other efflux transporters or mechanisms of extrusion. However, Ko143 has been recently shown to have interactions with P-glycoprotein (P-gp), another highly expressed efflux transporter also known to transport BP, albeit to a lesser extent when compared to BCRP (Weidner 2015). It was therefore important to determine a concentration of Ko143 that was able to effectively inhibit BCRP transport activity without impacting P-gp. To do this, P-gp function needed to be measured using rhodamine 123 (R123), a substrate of P-gp, with cells exposed to an effective concentration of Ko143 (Allen 2002). To confirm P-gp functionality, PSC833, a potent P-gp inhibitor, was used to inhibit P-gp function and increase R123 accumulation (Newman 2017). These steps were necessary to ensure confidence in an assay that could assess the effects of an intervention on the function of BCRP in hCMEC/D3 cells.

The following work describes, in detail, the experiments carried out to fully optimise treatment conditions, the Western blot, and the substrate accumulation assays. These assays are used to investigate and complete the aims of this study in the following experimental chapter.

2. Materials and Methods

2.1 Materials

Tween-20, sodium chloride, R123, CQ, dimethyl sulfoxide, sodium dodecyl sulphate (SDS), MTT, ammonium persulfate (APS), Corning black polystyrene TC-treated 96 well plates, penicillin-streptomycin, tetramethylethylenediamine (TEMED), Trizma® base, sodium chloride, Triton® X-100, glycerol, deoxycholic acid (3α, 12α-dihydroxy-5β-cholan-24-oic acid), and glycine were all purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Odyssey blocking buffer and goat anti-mouse and donkey anti-rabbit antibodies were obtained from Millennium Science (Mulgrave, Victoria, Australia). Falcon® rat tail collagen, CFB and Hyclone Hank's Balanced Salt Solution (HBSS) were purchased from In Vitro Technologies (Carlsbad, CA). Anti-BCRP (rabbit monoclonal) and anti β-actin antibodies were purchased from Abcam (Boston, MA). 40% acrylamide/bis solution and extra thick blot paper were purchased from BIO-RAD (Hercules, CA). Nitrocellulose blotting membrane (0.2 μm), BODIPY® FL prazosin (BP) was purchased from ThermoFisher Scientific (Waltham, MA). Ko143 was purchased from ThermoFisher Scientific (Waltham, MA). Endothelial Basal Medium -2 (EBM-2) and EGM-2 SingleQuot Kit Supplement & Growth Factors were purchased from Lonza (Walkersville, MD). RNeasy Plus mini kit and HiPerFect Transfection reagent were purchased from Qiagen (Hilden, Germany). The Pierce BCA protein assay kit were obtained from Life Technologies (Rockford, IL). cOmplete Protease Inhibitor Cocktail was purchased from Roche Pharmaceuticals (Basel, Switzerland).

2.2 hCMEC/D3 cell culture

Cells were generously provided by Dr. Pierre-Olivier Couraud (INSERM, Paris, France) and used at passages 30 to 35 for all relevant experiments. hCMEC/D3 cells were cultured at 37°C, in 5% CO_2, and 95% humidified air, in EBM-2 supplemented with 0.01% (v/v) ascorbic acid, 0.01% (v/v) gentamicin/amphotericin, 0.01% (v/v) hydrocortisone, 0.025% (v/v) epidermal growth factor, 0.025% (v/v) insulin-like growth factor, 0.025% (v/v) vascular endothelial growth factor, 0.1% (v/v), b-splice variant fibroblast growth factor, 10 mM HEPES, and 1% (v/v) penicillin/streptomycin and 2.5% (v/v)

foetal bovine serum (FBS). Before seeding, flasks or plates were collagenated with Type I rat tail collagen in PBS (0.1 mg/mL) for 1 hour at 37 ° C.

2.3 Cell treatment protocols

hCMEC/D3 cells were seeded at 30,000 cells/cm^2 onto collagenated 96-well, 24-well, or 6-well plates for MTT, uptake, or Western blot experiments, respectively. For each experiment, media was aspirated from all wells and replaced with media containing treatments that were dissolved in either DMSO or Milli-Q water. For CFB treatments, a 75 mM stock in DMSO was diluted 1000-fold into serum containing culture medium for a final concentration of 75 µM. This concentration of CFB was chosen to induce BCRP protein expression, as per a previously described protocol (Hoque 2012). For CZC treatments, stocks of CQ, $ZnCl_2$ and $CuCl_2$ were diluted 1000-fold into serum-free culture medium from 0.5, 0.5 and 0.1 mM stocks, respectively. These concentrations were used as they have previously been confirmed to be non-toxic to hCMEC/D3 cells and induce the expression of P-gp (McInerney 2018). For controls, cells were treated with media containing 0.1% v/v DMSO. For CZC treatments only, FBS free media was required to prevent protein-CQ binding and thus respective controls also contained no FBS. The media was first aspirated, washed three times with 2 mL of warm PBS and then replaced with FBS free media containing the intervention or respective control. For CFB experiments, the media was aspirated and replaced with serum-containing media with either 0.1% v/v DMSO or 75 µM CFB. All experiments were conducted 24 hours after initial treatment when cells had achieved ~90% confluency and are summarised below (Table 1).

Table 1 Summary of hCMEC/D3 cell treatments to be assessed for cell viability (MTT assay) and protein expression of BCRP (Western blot).

Treatment	Aim	Time of treatment after seeding	Duration and concentration
Positive control CFB	To show that the Western blot can detect an upregulation of BCRP	24 hours	24 hours 75 µM
CQ, Zn^{2+} and Cu^{2+}	To observe the effects of metal ions on BCRP protein expression	24 hours	24 hours 0.5 µM CQ, 0.5 µM Zn^{2+} and 0.1 µM Cu^{2+}

2.4 Cell viability assay for intended treatments

Cells were seeded into collagen coated 96-well plates at 30,000 cells per cm^2 and were treated 24 hours after initial seeding with either 75 µM CFB or CZC, or their respective controls. Separate wells were treated with 10% v/v DMSO in culture media as a positive control to ensure that the assay was able to detect cellular death. Following 24 hour treatment, cells were washed with warm PBS and then incubated with 10 µL of an 8 mg/mL MTT reagent solution in blank EBM media for 3 hours to allow the cells to reduce the MTT. Next, the MTT reagent was carefully removed from the wells and then replaced with 150 µL of 100% v/v DMSO. This was undertaken to dissolve the formazan crystals resulting from mitochondrial function. After a further 30 minutes of incubation, the plates were agitated, and absorbance was read at 540 nm. Background absorbance, derived from wells being treated but without cells, was subtracted from all other wells before analysis. The absorbance readings of vehicle and treatments were average normalised against the average of the untreated cells and then expressed as a percentage of cell viability.

2.5 Western blot protein optimisation

The Western blot is a commonly used analytical method designed to detect proteins of interest in tissue homogenate or extract. Briefly, gel electrophoresis separates sample proteins by mass, and these separated proteins are then transferred to a nitrocellulose membrane. The membrane is then stained with antibodies specific to the protein of interest. In addition, a "housekeeping" protein is stained for normalisation in order to verify that changes in the expression of the protein of interest are not artefacts of differing protein loads. This technique was selected for the detection of BCRP protein in hCMEC/D3 cell lysates.

2.5.1 Validation of the bicinchoninic acid assay (BCA) to determine total protein concentration

Protein concentrations from cell lysis vary from well to well due to several factors such as small differences in cell confluency and lysing efficiency. This makes it very difficult to load the exact same mass of protein for every sample in a Western blot without first pre-determining the protein concentrations. Loading must be consistent for all samples in any Western blot comparing control samples to one or more experimental samples. Before each Western blot, it is therefore necessary to run a simple BCA assay on all samples and determine total cellular protein concentration to then load a consistent total protein amount into each well.

To justify the use of the BCA assay, validation was required to show that the assay was consistently accurate and precise. Six independently prepared bovine serum albumin (BSA) samples of low, medium and high concentrations (250, 750 and 2000 µg/mL, respectively) were analysed in parallel with duplicate standard curves with concentrations ranging from 125 to 2000 µg/mL. BSA used in the making of these standards was dissolved in RIPA buffer (1% w/v Tris-base, 1% w/v NaCl, 0.1% SDS, 0.5% w/v deoxycholic acid, 1% v/v Triton x100 and 10% v/v glycerol), the same solution that would be used for the lysis of hCMEC/D3 cells. In each well on a 96-well plate, 10 µL of standard was added to 200 µL of Pierce BCA protein assay kit working reagent. This solution contained BCA and $CuSO_4$. Cu(II) ions are reduced to the Cu(I) form by protein peptide bonds which in turn allows the

resulting Cu(I) ions to chelate with two BCA molecules. This product is purple in colour and its intensity is directly proportional to the amount of protein in each sample. To facilitate this reaction, the plate was incubated at 37 °C for 30 minutes and then allowed to cool to room temperature to halt further reaction. Absorbance values were obtained at 546 nm using a spectrophotometer (PerkinElmer, Waltham, MA) and then assessed for both accuracy and precision using the equations below. The average accuracy values of each range of protein concentration were required to fall between 90-110% and the average precision values should be less than 10%.

$$Accuracy = \frac{measured\ concentration}{theoretical\ concentration} \times 100$$

$$Precision = \frac{\delta}{mean} \times 100$$

Where δ = standard deviation.

2.5.2 BCRP detection in BCRP-transfected MDCK II cells

As a positive control and to ensure that the Western blot was able to detect human BCRP, lysates of BCRP-transfected Madin Darby Canine Kidney II (MDCK II) cells were initially employed in Western blot optimisation. Once the Western blot method was confirmed to successfully detect BCRP, hCMEC/D3 cells were then analysed using the same protocol to confirm the ability of the Western blot to detect BCRP with signals proportional to the amount of total protein used. Initially, a 1000-fold dilution of BCRP primary antibodies were used as this was the highest dilution recommended by the manufacturer and thus would give the greatest chance of observing a signal. A 15,000-fold dilution of secondary antibodies for BCRP and β-actin was used. To assess if the dilutions were within the limits of detection, protein loads of 5, 10, 20 and 40 µg were used. Two sets of samples were lysed with either IP Pierce lysis buffer or self-made RIPA buffer as preparing one's own reagent allows room for any desired adjustments compared to those that are simply purchased.

2.5.3 Western blot protocol

In preparation for lysis, hCMEC/D3 cells were washed with ice-cold PBS, scraped and then suspended in ice-cold PBS. The cell suspension was then centrifuged at 1,000 x g for 5 minutes. The

PBS was then carefully removed before the addition of 200 µL of RIPA buffer mixed with protease inhibitor. Cells were then gently agitated at 4 °C for 25 minutes. To remove cellular debris, lysates were centrifuged at 14,000 x g for 10 minutes. The resulting supernatant was then aliquoted and stored at -80 °C for future use.

After using the BCA assay to predetermine the protein concentrations, cell lysate samples were mixed via gentle aspiration with Laemmli buffer in a 5:1 ratio (20% w/w glycerol, 0.125 M Tris-HCl buffer, 10% v/v sodium dodecyl sulphate (SDS), 0.5% β-mercaptoethanol, 0.5% v/v bromophenol blue in Milli-Q water) and loaded into a polyacrylamide gel for electrophoresis. The Laemmli buffer coats the sample proteins with SDS which is highly negatively charged, allowing the proteins to be separated based purely on molecular weight, without the influence of the charged peptide residues. Furthermore, β-mercaptoethanol is a reducing agent responsible for the breaking of disulphide bonds which can inhibit protein denaturation. BCRP has been shown to be detected in its monomeric form at ~74 kDa in the presence of reducing agents, such as β-mercaptoethanol, rather than in its dimeric form (Kage 2002). As a molecular weight reference, 5 µL of Dual Xtra Precision Plus Protein Prestained Standards were loaded into two wells which would serve to aid protein identification. The polyacrylamide gel was then submerged in running buffer comprised of 25 mM Tris base, 190 mM glycine and 0.1% (w/v) SDS in Milli-Q water. Electrophoresis was then applied, using the Bio-Rad PowerPac™, to the gel at 60 volts (V) for 30 minutes, to delicately move the samples from the stacking to the separating gel. Lastly, 150 V was applied for 1 hour and 30 minutes to separate sample proteins by weight. After separation, the gel was soaked in transfer buffer (25 mM Tris buffer, 190 mM glycine and 20% methanol) for 20 minutes to remove various chemicals used during electrophoresis. The separated proteins in the gel were then transferred to a 0.2 µm nitrocellulose blotting membrane using a Bio-Rad Trans-Blot® Turbo™ Transfer System. The transfer was run at 25 V for 25 minutes. Once complete, the membrane was then briefly washed with Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (TBST). Next, to prevent non-specific antibody binding, the membrane was blocked with LI-COR® Odyssey® Blocking Buffer for 60 minutes. Afterwards, primary antibodies specific to BCRP and β-actin (a housekeeping gene) were diluted in 20 mL of

TBST (Rabbit anti-human and mouse anti-human respectively). The membrane was then incubated in this solution overnight (18 hours) at 4 °C. The following day, the membrane was washed for 5 minutes with ~25 mL of TBST four times, a step necessary to remove any excess primary antibodies in order to minimize background staining. Fluorescent secondary antibodies specific to the primary antibodies of either BCRP or β-actin (goat anti-mouse and donkey anti-rabbit respectively) were then diluted into 30 mL of TBST followed by the same washing procedure previously described. Each of the secondary antibodies were conjugated to fluorophores of different wavelengths (β-actin, green; BCRP, red) in order to assist in distinguishing between bands and to show that there was no indiscriminate binding. The membrane was then visualized using a LI-COR® Odyssey® scanner and densitometry was performed on the bands representing BCRP and β-actin using the Image J program.

2.5.4 Determining the optimal protein load of hCMEC/D3 cell lysates for Western blotting

Following confirmation of BCRP detection by Western blotting using BCRP-transfected MDCK II lysates, the method needed to be optimised as the assay showed highly saturated BCRP band signals at all protein loads. In order to properly assess changes in BCRP protein expression, it is ideal to discern a loading weight which generates a midrange signal. This is important to ensure that increases in protein expression do not saturate the reading and that decreases in protein do not result in a loss of signal. To do this, the dilution factors of the primary antibodies were increased. BCRP and β-actin primary antibody dilutions were increased to 10,000 and 100,000 respectively. A standard curve of protein loads (5, 10, 15, 20 and 30 µg) from untreated hCMEC/D3 cell lysates were processed using the Western blot. The signals obtained from the resulting bands were then analysed for direct proportionality (e.g. BCRP bands from 10 µg should be twice as dense than 5 µg).

2.5.5 Confirming cell system responsiveness using a known BCRP positive regulator

To show that the Western blot could detect increases in BCRP protein expression and that levels of BCRP could be enhanced, hCMEC/D3 cells were treated with a known up-regulator of BCRP. CFB has been demonstrated to upregulate BCRP protein expression in the hCMEC/D3 cell line (Hoque 2012, Hoque 2015, More 2016). Cells were seeded at 30,000 cell/cm^2 into 6-well plates. A day after

initial seeding, media from all wells was replaced with 75 µM CFB or vehicle (0.1% DMSO). Following the 24-hour treatment, cells were lysed, and the protein concentration was determined using the BCA assay. Then, 10 µg of cell lysate from CFB or control treated cells was processed using the Western blot method. Once this method showed BCRP protein upregulation, it was used for future studies using CZC.

2.6 Substrate accumulation assay to measure BCRP function in hCMEC/D3 cells

2.6.1 Selection of BCRP substrate and inhibitor

BP was first shown to be a substrate of BCRP when lower uptake was observed in BCRP overexpressing MCF-7 cells compared to parental MCF-7 cells (Litman 2000). Since then, BP has been commonly used as a measurement of BCRP transport function both *in vitro* and *in vivo* (Mahringer 2010, Hori 2004). Thus, to assess the effects of an intervention on BCRP function in hCMEC/D3 cells, BP was selected as the substrate. A limitation of this substrate, however, is that it has also been shown to be a potential substrate for P-gp (Kimchi-Sarfaty 2002) which is also expressed by hCMEC/D3 cells (Weksler 2005). It was therefore necessary to confirm that the transport of BP was not significantly affected by P-gp in hCMEC/D3 cells.

For accumulation studies, it is common practice to use an inhibitor for the transporter of interest. To demonstrate functionality, an effective concentration of inhibitor is delivered to an untreated sample and it is expected that substrate accumulation is significantly increased, an outcome indicative of decreased transporter function. For BCRP, Ko143 is the most typical inhibitor chosen given its high potency and affinity (Allen 2002)

2.6.2 Precision and accuracy validation of the BP standard curve

Accurate and precise handling of BP is crucial for the accumulation assay as its measurement is ultimately used to assess BCRP function. Thus, it was critical that the preparation of BP was of a satisfactory standard so that future results could be calculated using a standard curve to quantify unknown concentrations of BP in cell lysate. Five independently made BP samples of low, medium

and high (125, 500 and 1000 nM) concentrations diluted in 1% v/v Triton X-100, the same solution used to lyse cells in an accumulation assay, were analysed in parallel against a standard curve with concentrations ranging from 125 to 1000 nM. The fluorescence of BP was then measured at an excitation wavelength of 503 nm and emission wavelength of 513 nm. The average accuracy and precision of the samples were then calculated. The preparation of the samples was deemed to be accurate and precise if the average deviated no more than 10% according to the equation described in section 2.5.1.

2.6.3 Determining the optimal substrate accumulation time in the presence of Ko143

Once the BCRP inhibitor and substrate was chosen, it was essential to determine the appropriate concentrations and exposure time to allow for sufficient accumulation. For BP, 500 nM was chosen as a suitable concentration for *in vitro* studies (Qawasami 2014) whereas higher concentrations are typically used for *in vivo* studies (Hoque 2015, More 2016). hCMEC/D3 cells were incubated with 500 nM BP over specific timepoints (2, 5, 15, 30 and 60 minutes) with or without 500 nM Ko143. The timepoint at which the largest increase in BP accumulation caused by 500 nM Ko143 was detected was then selected as the optimal duration for future studies.

2.6.4 Efflux inhibitor cross-interaction studies

Ko143 is a potent BCRP inhibitor that was commonly presumed to be BCRP specific, until a study demonstrated otherwise (Weidner 2015). At concentrations of 1 µM or higher, Ko143 affects the transport activity of both P-gp and multidrug resistance-associated protein 1 (MRP-1), both of which are expressed in hCMEC/D3 cells. Studies have shown that Ko143 at concentrations of 500 nM or lower strongly inhibit BCRP without affecting other transporters (Allen 2002, Matsson 2009) and so 500 nM was the selected working concentration in all relevant experiments. To show that 500 nM Ko143 did not affect P-gp function, an accumulation assay using R123 (R123), a P-gp substrate, was used. The P-gp inhibitor, PSC833, was also used as a control for the inhibition of P-gp. Briefly, cells were incubated with transport buffer with or without 500 nM Ko143 or 300 nM PSC833 (an effective concentration of a potent P-gp inhibitor). Cells were then incubated with transport buffer containing 5

µM R123 (a P-gp substrate) with or without the previously mentioned inhibitors for 60 minutes. Finally, the cells were washed, lysed and then fluorescence of R123 measured at an excitation wavelength of 511 nm and emission wavelength of 534 nm. The concentration of R123 was determined using a standard curve of R123 diluted in 0.1% Triton X-100 v/v in transport buffer. The measurements from all replicates were normalised to protein count via the BCA assay.

Table 2 Summary of substrate accumulation assay optimisation experiments using BP (BCRP substrate), R123 (P-gp substrate), Ko143 (BCRP inhibitor) and PSC833 (P-gp inhibitor).

Aim	**Treatment**	**Experimental conditions and measured outcome**
To determine the optimal BP accumulation time where the largest inhibitory effect of Ko143 can be observed	hCMEC/D3 cells exposed to BP as the BCRP substrate and Ko143 as the inhibitor	Measured outcome: BP accumulation Transporter inhibited: BCRP Timepoint: 2, 5, 15, 30 and 60 minutes with 500 nM BP and 500 nM Ko143 with respective controls
To confirm that P-gp transport activity did not significantly affect the accumulation of BP by using a P-gp inhibitor (PSC833)	hCMEC/D3 cells exposed to BP alongside the blockade of P-gp by PSC833	Measured outcome: BP accumulation Transporter inhibited: P-gp 30 minutes exposure to 500 nM BP and 300 nM PSC833 with respective controls.
To demonstrate that BCRP transport activity did not significantly affect the accumulation of R123 by using a BCRP inhibitor (Ko143)	hCMEC/D3 cells exposed to R123 alongside the blockade of BCRP by Ko143	Measured outcome: R123 accumulation Transporter inhibited: BCRP 30 minutes exposure to 500 nM R123 and 500 nM Ko143 with respective controls.

2.7 Statistical analyses

All experiments were repeated using a minimum of 3 biological replicates. Results are displayed as mean ± S.D. Statistical analyses were performed using GraphPad Prism version 8.0.0 (San Diego, CA). A two-way analysis of variance (ANOVA) was applied to substrate accumulation time course assays. For Western blot positive control data, a two-tailed student's t-test was applied. For all other data, where relevant, a one-way ANOVA was applied.

3. Results

3.1 hCMEC/D3 cells tolerate CFB and low micromolar concentrations of CZC

Prior to commencing experimentation involving drug manipulations, it was essential to show that the interventions, at the concentrations used, did not significantly affect the viability of hCMEC/D3 cells. To show this, hCMEC/D3 cells were exposed to CZC (0.5, 0.5 and 0.1 µM respectively) or 75 µM CFB for 24 hours and cell viability was then assessed by the MTT assay. Cells treated with either control or 75 µM CFB showed no significant changes in cell viability following a 24 hour treatment. To confirm that this assay was able to detect toxicity, hCMEC/D3 cells were exposed to a highly toxic concentration of DMSO (10%) under the same experimental conditions where a 99.1 ± 0.4 % decrease in cell viability was observed (Figure 1). While experiments using CFB used serum-containing medium, it is important to note that CZC treatments were delivered using serum-free medium in order to reduce CQ-protein binding as this would interfere with CQ-metal binding. No significant changes in cell viability were observed in cells treated with vehicle or CZC (Figure 2). To confirm that this assay was able to detect toxicity, a highly toxic concentration of DMSO (10%) was exposed to hCMEC/D3 cells under the same experimental conditions and an 83.3 ± 3.9% decrease in cell viability was observed. A 24-hour treatment of CZC in serum free media (0.5, 0.5 and 0.1 µM respectively) was shown to be non-toxic to hCMEC/D3 cells, and this regimen was used for all subsequent studies.

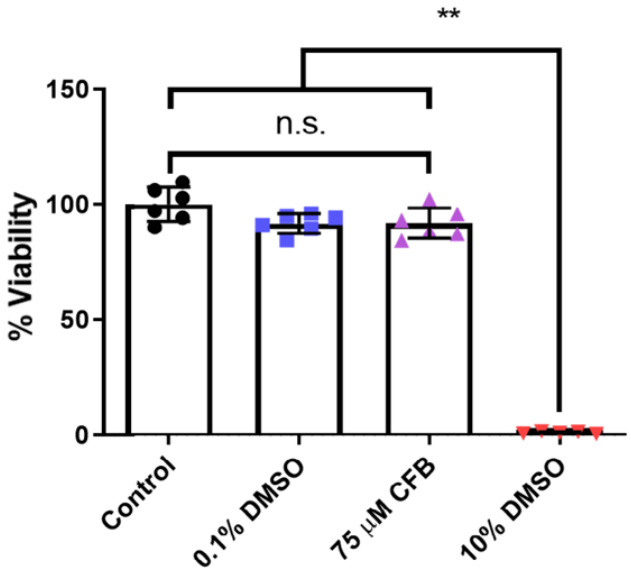

Figure 1 hCMEC/D3 cell viability following a 24 hour treatment with media (control), vehicle (0.1% DMSO), 75 µM clofibrate (CFB) and 10% DMSO, expressed as a percentage of the untreated cell control. Data shown as average ± SD, n = 5 – 6 biological replicates, one-way ANOVA with post-hoc Tukey's multiple comparison's test was used for statistical analysis, ** $p < 0.005$.

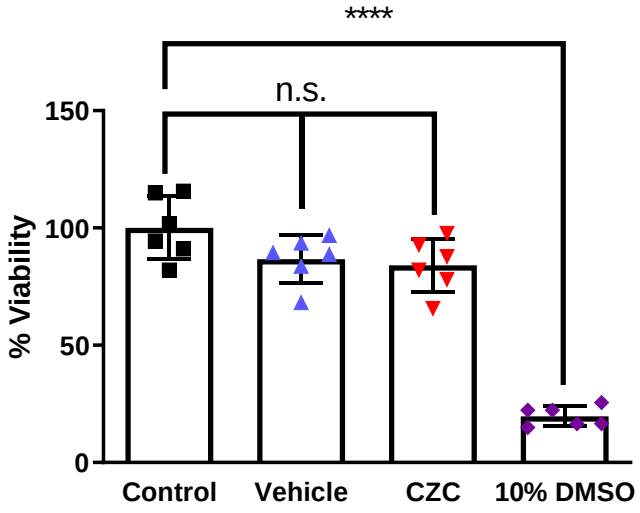

Figure 2 hCMEC/D3 cell viability following a 24 hour treatment with media (control), vehicle (0.1% DMSO), CZC (0.5, 0.5 and 0.1 µM of CQ, Zn^{2+} and Cu^{2+} respectively) and 10% DMSO, expressed as a percentage of the untreated cell control. Data shown as average ± SD. n = 6 (biological replicates), one-way ANOVA with post-hoc Tukey's multiple comparison's test was used for statistical analysis, **** $p < 0.0001$.

3.2 Western blot optimisation for the detection of BCRP protein expression in hCMEC/D3 cells

3.2.1 Validation of the bicinchoninic acid assay (BCA) to determine total protein concentration

The curve relating absorbance to known standard concentrations of BSA samples produced a linear regression with an R^2 value of 0.9946 (Figure 3). The calculated accuracy and precision results for the low, medium and high protein concentration quality control standards are provided in Table 2.

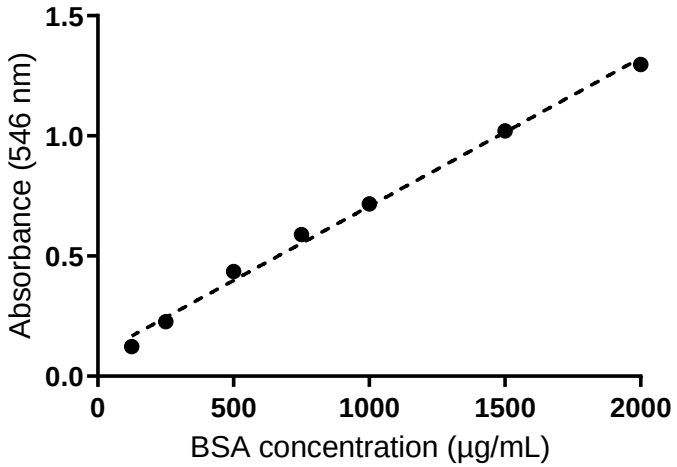

Figure 3 Absorbance readings from a standard curve of samples containing known concentrations of BSA diluted in RIPA buffer. Samples were incubated with BCA assay kit reagent at 37 °C for 30 minutes.

Table 3 Precision and accuracy values of samples containing known concentrations of BSA (250, 750 and 2000 µg/mL, 6 samples each) calculated using the standard curve generated in Figure 3.

Theoretical concentration (µg/mL)	**Calculated standard average (µg/mL)**	**Precision (%)**	**Accuracy ± SD (%)**
250	268.28	3.34	107.31 ± 3.07
750	803.61	3.12	107.15 ± 3.35
2000	2008.94	0.81	100.45 ± 0.81

The accuracy and precision for the measurement of low, medium and high protein concentrations of BSA were within satisfactory limits. Therefore, the Pierce BCA protein assay was considered to be a consistent and reliable method for determining protein concentrations of hCMEC/D3 cell lysate samples for subsequent Western blots.

3.2.2 BCRP primary antibody is able to detect BCRP protein in BCRP-transfected MDCK II cells

The first Western blot was aimed at optimising signals obtained from both BCRP and β-actin bands. In addition, two different cell lysis solutions were used: self-made RIPA buffer and Pierce™ IP Lysis Buffer (Figure 4). In order to generate an appropriate BCRP protein signal, an initial dilution factor of 1:1,000 for the BCRP primary antibody was chosen and a range of increasing hCMEC/D3 lysate protein loads was used. The densitometry values generated from BCRP and β-actin bands from samples lysed in either RIPA buffer or Pierce™ IP Lysis Buffer revealed that protein expression was not proportional to respective loading masses due to signal saturation (Figure 4). This suggested that the dilutions of either the secondary or primary antibodies were too high causing saturation of band signals. The results generated from the two lysis buffers did not appear to be substantially different from each other. Thus, RIPA buffer was chosen as its composition could be modified for future experiments if desired. The results were encouraging as bands with BCRP and β-actin bands were resolved at the expected molecular weights as indicated by the ladder.

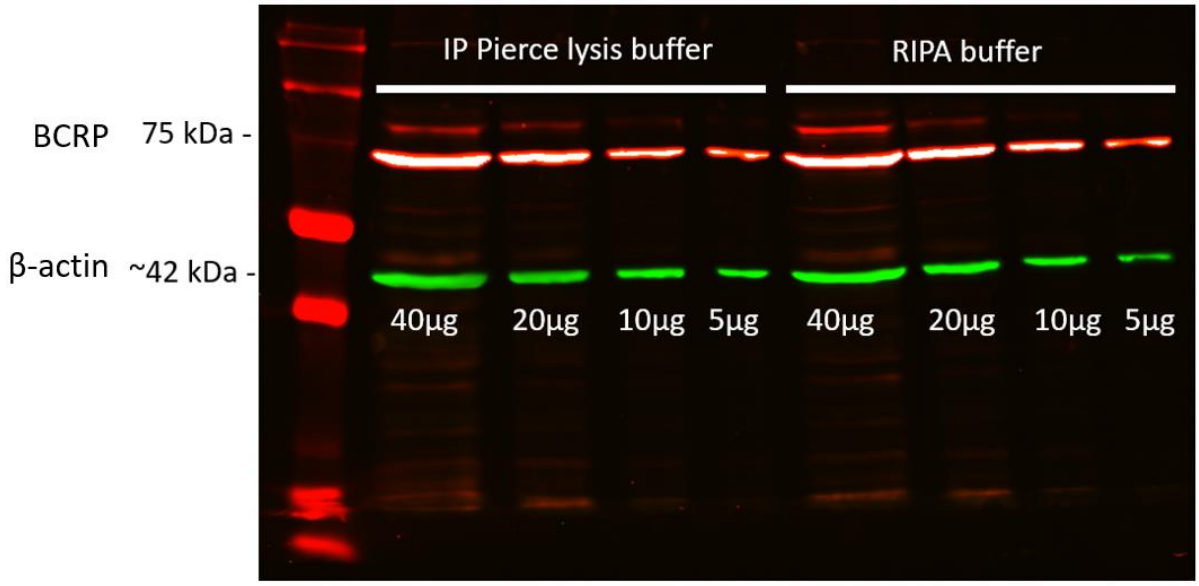

Figure 4 Western blot analysis of BCRP in BCRP-transfected MDCK II cells lysed with either RIPA buffer or Pierce™ IP Lysis Buffer. Actin bands fluoresce green and BCRP bands are shown to fluoresce red and are white in the center due to signal saturation at increasing total protein loads.

3.2.3 Determining the optimal protein load of hCMEC/D3 cell lysates for Western blotting

Once detection of human BCRP protein expression by Western blotting was confirmed, the next important step was to translate and apply this technique to hCMEC/D3 cells and identify an optimum protein load (Figure 5A). To do this, a standard curve of untreated hCMEC/D3 lysates was processed using the Western blot method (Figure 5B). It was expected that the generated signals from BCRP and β-actin would reflect the proportion of protein loaded (Figure 6). The ratio between BCRP and β-actin for all protein loads were ~3 except at 30 µg indicating saturation (Table 3). This indicated that the protein signal from protein loads of 5-20 µg were linear and proportional, which was considered ideal for quantitative analysis. The following experiments involved potential and known up-regulators of BCRP, with CFB expected to approximately double BCRP protein expression. Thus, 10 µg was chosen as the loading amount for all future Western blotting. Having determined the ideal hCMEC/D3 protein load, it was next important to show that BCRP protein expression was inducible using CFB, a previously described up-regulator of BCRP in hCMEC/D3 cells (Hoque 2012).

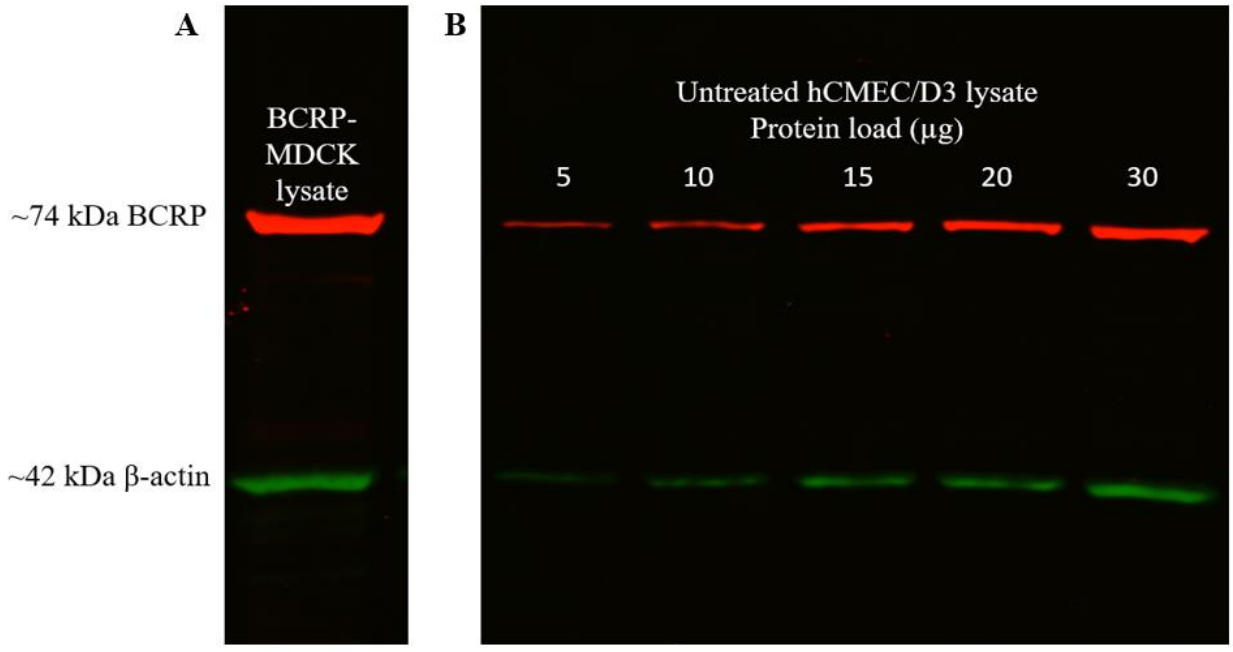

Figure 5 Western blot analysis of BCRP in BCRP-transfected MDCK II cells (A) and hCMEC/D3 cells (B). A standard curve of hCMEC/D3 cell protein was used to identify an ideal load to be used for future experiments.

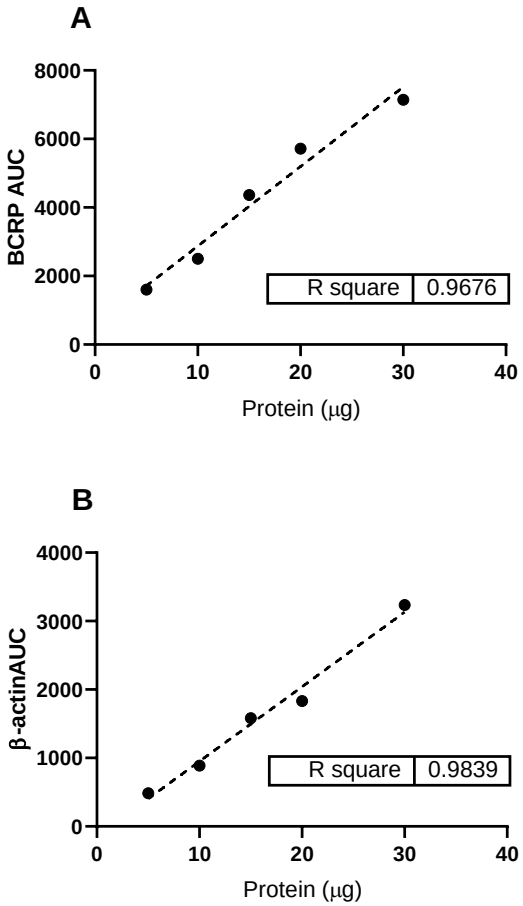

Figure 6 Quantitative densitometry analysis of (A) BCRP and (B) β-actin from a Western blot of hCMEC/D3 cells (Figure 5B). Data was normalized to total cellular protein determined via the bCA assay. R square values were generated using GraphPad Prism version 8.0.0 (San Diego, CA)

Table 4 Observed ratios of BCRP and β-actin band signals from a Western blot of hCMEC/D3 cells. This analysis was performed to observe the consistency between BCRP and β-actin at different protein loads.

Ratios between BCRP and β-actin signals	
Protein load (μg)	**BCRP:β-actin**
5	3.04
10	2.85
15	2.76
20	3.07
30	2.23

3.2.4 CFB upregulates BCRP protein expression in hCMEC/D3 cells

To show inducible BCRP protein expression in hCMEC/D3 cells using the Western blot method, cells were treated with CFB (Figure 7). Consistent with previous studies (Hoque 2010), CFB significantly upregulated BCRP protein expression in hCMEC/D3 cells, 1.5 ± 0.2-fold. This data showed that BCRP can be upregulated, confirming that CFB is a suitable positive control for future studies assessing the impact of CZC on BCRP protein expression.

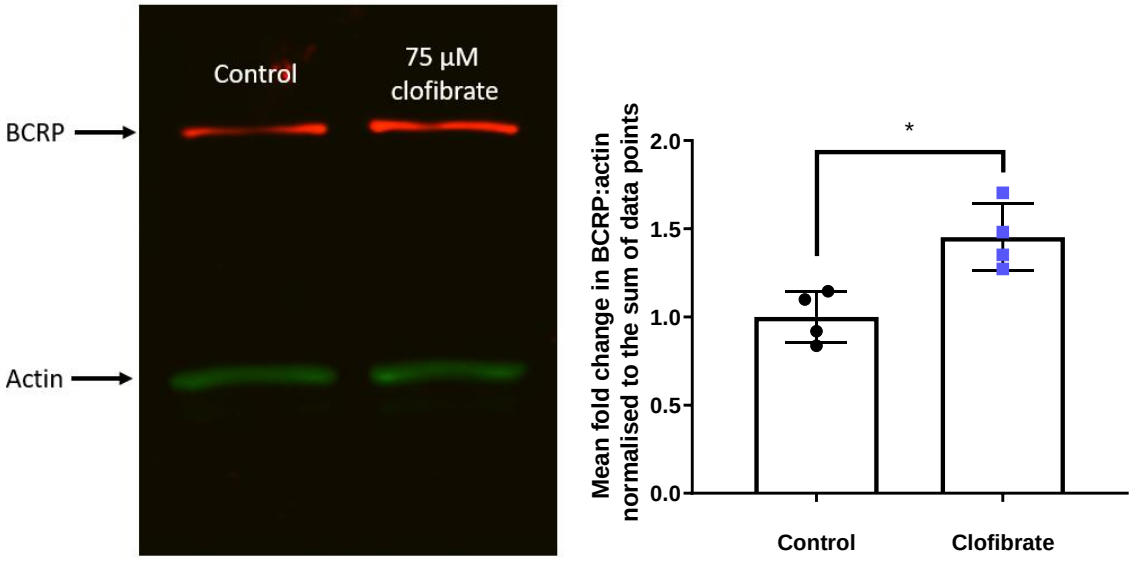

Figure 7 (Left) A representative Western blot showing BCRP and β-actin bands following a 24 hour treatment with 75 µM CFB. (Right) Densitometric analysis of the CFB dependent increase in BCRP. Data are expressed as mean ± SD. n = 4 biological replicates for control and treatment groups. Two-tailed t-test was used. * $p < 0.05$.

3.3 Development and optimisation of the accumulation assay to measure BCRP efflux function in the hCMEC/D3 cell line

To address the hypothesis that CZC can regulate the transport function of BCRP, an accumulation assay was developed as a means of measuring BCRP function. Described below are the experiments used to optimise this assay.

3.3.1 Precision and accuracy of the BP substrate standard curve

For each accumulation assay, it was necessary to prepare a standard curve of BP to calculate the amount BP accumulated in each cell lysate sample. The fluorescence readings from BP, or any light sensitive fluorescent substrate, will vary from experiment to experiment due to various factors such as temperature or different batches of cells. The standard curve of known BP standards diluted in 0.1% v/v Triton X-100 possessed an R-square value of 0.98 (Figure 8) and calculated measurements of high to low concentration samples were within satisfactory parameters (Table 5). The preparation of BP standards was deemed to be precise and accurate.

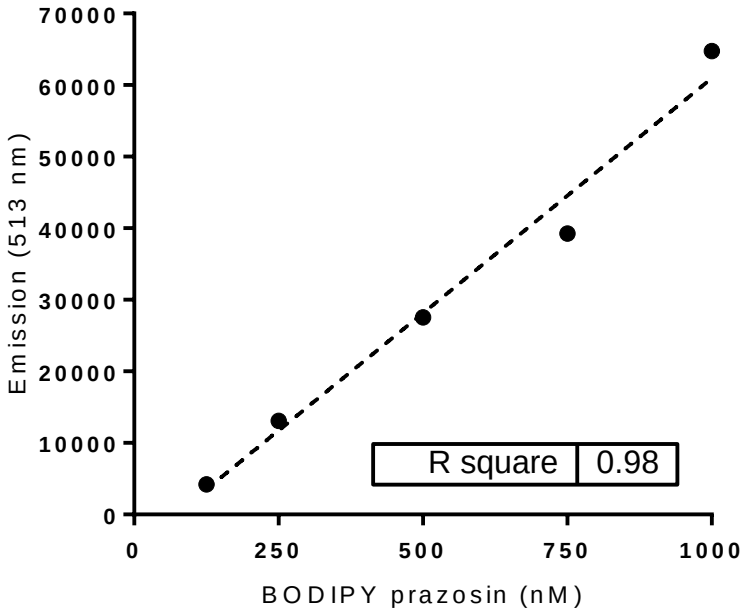

Figure 8 Emission (513 nm) from a standard curve of BP diluted in 0.1% v/v Triton X-100 relating known concentrations of BP to fluorescent emission values.

Table 5 Summary of precision and accuracy values of BP standards diluted in 0.1% v/v Triton X-100. Measurements were within the satisfactory requirements of ± 10%. n = 3.

Theoretical concentration (nM)	Sample average concentration (nM)	Precision (%)	Accuracy ± SD (%)
125	114.89	3.22	91.91 ± 0.20
500	483.95	4.16	96.79 ± 0.81
1000	1047.90	3.33	104.79 ± 1.11

3.3.2 Determination of the optimal uptake duration of BP by hCMEC/D3 cells in the presence of Ko143

The uptake of BP was measured at different timepoints over the course of 60 minutes with or without the presence of Ko143, a BCRP inhibitor (Figure 9). This experiment was designed to determine the timepoint where the uptake of BP would be most affected by pharmacological intervention. Significant differences in accumulation were observed at 15 and 30 minutes with similar fold changes in BP uptake, 1.67 ± 0.17 and 1.60 ± 0.29-fold, respectively. However, the total concentrations of accumulated BP in the 15-minute samples were low. Given that we expected a decrease in substrate accumulation following CZC treatment, using such a short time point may have resulted in data falling below the limit of detection. Thus, an accumulation time of 30 minutes was chosen for all future functional studies.

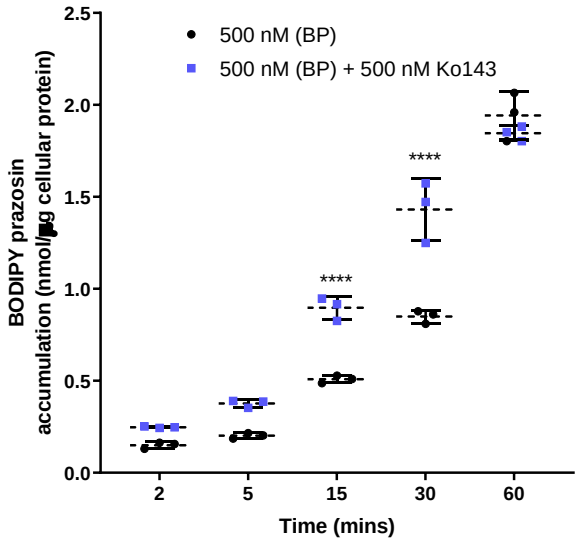

Figure 9 Uptake of BODIPY prazosin by hCMEC/D3 cells, normalised to total cellular protein, measured at timepoints over 2 hours with or without Ko143. Data shown as average ± SD, n = 3 biological replicates for each treatment and timepoint. Two-way ANOVA with post-hoc Tukey's multiple comparisons test was used, **** $p < 0.0001$.

3.3.3 Ko143 does not affect P-gp function at nanomolar concentrations

While Ko143 is a potent BCRP inhibitor, a recent study has shown that can affect other transporters like P-gp which are also largely expressed in hCMEC/D3 cells (Weidner 2015). As BP is a P-gp substrate, but to a lesser extent compared to BCRP, it was important to confirm that P-gp did not significantly affect BP accumulation, and that the concentration of Ko143 used did not affect P-gp function.

Firstly, to show that P-gp function was not affected by BCRP inhibition, the accumulation of 5 µM R123 in hCMEC/D3 cells was measured in the presence of 500 nM Ko143 (Figure 10A) where no significant changes were observed (89.2 ± 7.0 % of the control). P-gp was confirmed to be functional as R123 accumulation was significantly increased by 300 nM PSC833 to 130.3 ± 6.2 % of the control (Figure 10A). It was also demonstrated that a much higher concentration of 5 µM Ko143 (Figure 10B) did not affect P-gp function (95.8 ± 5.6% of the control).

To confirm that the accumulation of BP was not affected by P-gp, hCMEC/D3 cells were exposed to 500 nM BP and 0.3 µM PSC833 (Figure 10C). The accumulation of BP was not significantly affected by P-gp inhibition (122.0 ± 18.5% of the control). BCRP was confirmed to be functional as BP accumulation was significantly increased by 5 µM Ko143 to 214.9 ± 7.7% of the control (Figure 10C). Following these studies, 5 µM Ko143 was chosen for future experiments as under these conditions Ko143 was acting a highly effective BCRP inhibitor without affecting P-gp transport.

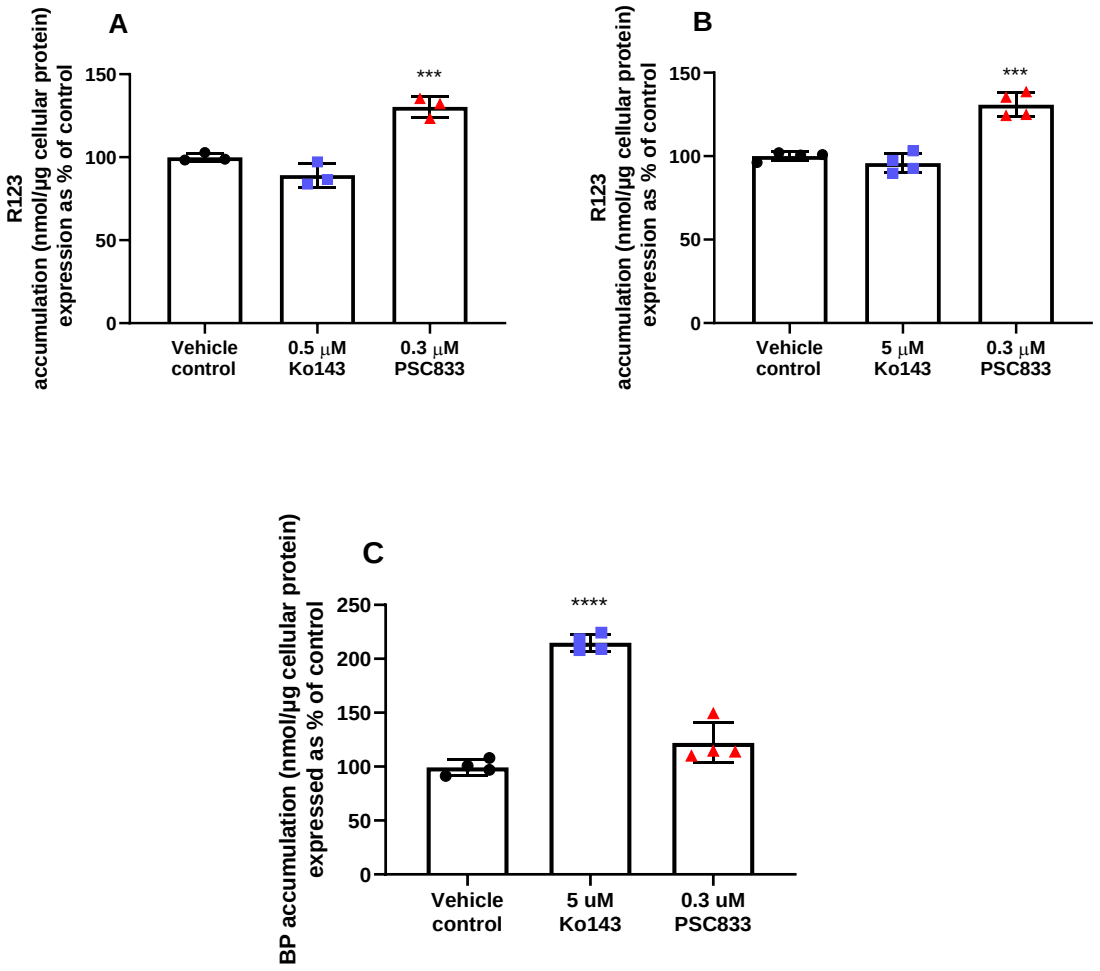

Figure 10 The accumulation of P-gp or BCRP substrate (5 µM R123 and 500 nM BP respectively) in the presence of P-gp or BCRP inhibitors (PSC833 and Ko143 respectively), normalized to total cellular protein, to investigate cross-transporter effects. Ko143 did not significantly affect R123 accumulation at (A) 0.5 µM or (B) 5 µM whereas 0.3 µM PSC833 increased accumulation. (C) 0.3 µM PSC833 did not affect BP accumulation, however BP accumulation was impacted by 5 µM Ko143 Data presented as mean ± SD, n = 4 biological replicates for each treatment group. A one-way ANOVA with post hoc Dunnett's test was used. **** $p < 0.01$, *** $p < 0.05$.

3.3.4 CZC do not quench the fluorescence of BP

We expected a decrease in BP accumulation following CZC treatment and therefore needed to confirm that CZC was not quenching the fluorescence of BP as this would be an artefact leading to a false positive result. The emission of samples containing the substrate diluted in the lysing medium of the experiment, 0.1% v/v Triton X-100, with or without CZC were measured (Figure 11). According to the two-tailed t-test, there was no significant difference in the readings between control and treatment. This was an important experiment as it confirmed the lack of interaction between clioquinol, Zn^{2+} and Cu^{2+} and BP, and that changes in accumulation were genuine and not artefacts of quenching.

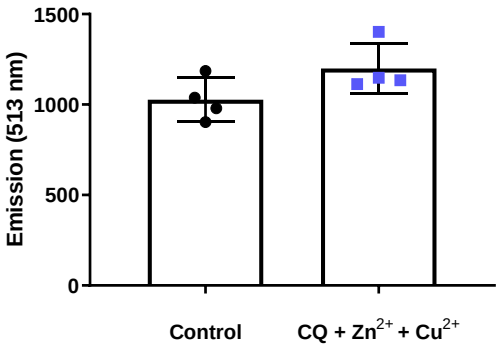

Figure 11 Emission values of samples containing BP with CZC ± vehicle control. Data are shown as mean ± SD. n = 4 biological replicates for each treatment. No statistical significance according to a two-tailed t-test.

4. Discussion

The purpose of this chapter was to assess cell viability after experimental manipulations and to develop methods to assess BCRP protein expression and function in hCMEC/D3 cells, which would subsequently be used in Chapter 3 to assess the aims outlined in the introductory chapter.

The MTT assay was used to measure cell viability after 24 hour treatments with either CFB or CZC. Consistent with previous studies performed in hCMEC/D3 cells, neither treatment significantly affected mitochondrial activity (Hoque 2012, McInerney 2018). As expected, cells treated with 10 % DMSO resulted in a markedly reduced cell viability. As the treatments did not appear to disrupt cellular homeostasis, steps to validate the Western blot and substrate accumulation assays were then carried out.

To validate the anti-BCRP (rabbit monoclonal) antibodies, BCRP-transfected MDCK II samples were analysed using a general Western blot protocol. Two different lysing reagents, self-made RIPA buffer or Pierce™ IP Lysis Buffer, were also used. Clear bands at ~74 kDa using either lysing reagent were observed, reflecting the literature reported molecular weight for a BCRP monomeric unit (Kage 2002). This was expected as β-mercaptoethanol, a reducing agent, was used which is known to break down the disulphide bond normally present in the homodimeric form of BCRP (~ 150 kDa) (Kage 2002). Thus, detection of BCRP was confirmed; validating the Western blot, primary antibody and RIPA buffer lysing reagent for detecting human BCRP. The signal of BCRP was very high due to the high abundance of protein in the overexpressing BCRP-transfected MDCK II cells. It was therefore necessary to determine the optimal conditions for BCRP protein detection in hCMEC/D3 cells.

To identify an appropriate protein load, BCRP primary dilutions were increased from 1:1,000 to 1:10,000 and a linear range of protein loads of hCMEC/D3 cells were processed by Western blotting. It was expected that the generated signals from BCRP and β-actin would reflect the proportion of protein loaded. Signals generated from BCRP and β-actin were linear and the ratio between the proteins was consistent, excluding 30 µg as saturation was occurring at this protein load. This, however, was not a concern as protein loads of 10, 15 and 20 µg produced a sufficient signal strength.

The 10 µg load was chosen as the signal was not saturated, allowing for a flexible assay where either increases or decreases in BCRP protein expression could be observed without moving into the lower and upper limits of protein detection. Next, to show that BCRP expression was inducible, hCMEC/D3 cells were treated with 75 µM CFB for 24 hours, a compound and duration of treatment known to induce BCRP protein expression in hCMEC/D3 cells (Hoque 2012). As expected, an upregulation in BCRP expression was observed; indicating that BCRP was manipulable in hCMEC/D3 cells.

Previous studies have demonstrated that 500 nM Ko143 is sufficient to inhibit BCRP, given that the IC_{50} of BCRP is approximately 20 nM (Allen 2002, Matsson 2009). To identify a suitable substrate accumulation time, BP accumulation was measured over a series of time points with or without 500 nM Ko143, a concentration previously shown to affect BCRP function at a previously described EC_{90} of 85 nM (Allen 2002). A significant increase in BP accumulation was observed at 15 and 30-minute timepoints. The latter was chosen as higher concentrations of BP were easier to discern and detect. At 60 minutes, the accumulation of BP in the presence of Ko143 was similar to the control. The maximum transport function of BCRP in hCMEC/D3 cells may have become saturated by exposure to excess concentrations of BP and so Ko143 no longer appeared to have an effect on BCRP function. Ko143 did not significantly increase BP accumulation at timepoints earlier than 15 minutes. It may be that at such short durations, differences in substrate accumulation are not easily revealed whereas at longer timepoints, the effects of Ko143 were quite apparent. Based on these observations, all functional studies were undertaken after 30 min incubation with BP.

While Ko143 is an extremely potent BCRP inhibitor which is frequently used for *in vivo* and *in vitro* studies, recent findings by Weidner et al. revealed that Ko143, at high concentrations, was able to stimulate P-gp ATPase activity (Weidner 2015). As P-gp is highly expressed in the hCMEC/D3 cells (Weksler 2005), and BP is a substrate of P-gp (Kimchi-Sarfaty 2002), it was important to ensure that Ko143, at concentrations of 500 nM and 5 µM, was not affecting P-gp function. It was demonstrated that neither 500 nM nor 5 µM Ko143 significantly affected the P-gp mediated uptake of R123, however as expected, R123 uptake was affected by the P-gp inhibitor, PSC833. Importantly, 300 nM PSC833, a concentration which inhibits P-gp function, did not significantly affect the accumulation of

500 nM BP indicating that at P-gp did not play a significant role in BP uptake under our experimental design. In another study using BCRP-transfected HEK293 cells exposed to mitoxantrone and Ko143 for 30 minutes, the highest accumulation of substrate occurred at 5 µM (Hori 2004). Therefore, future experiments used 5 µM Ko143 at the 30-minute timepoint to assess the effects of CZC on BCRP function.

Lastly, to ensure that our intended treatment of CZC did not interact with or alter the fluorescence of BP, the substrate to be used to measure BCRP function, a cross interaction study was done. This was necessary as BODIPY, the fluorescent molecule conjugated to prazosin, is extremely sensitive to heat and light and therefore vulnerable to quenching from multiple factors. Under the same experimental conditions, BP was incubated with 0.1 % v/v Triton X-100 alone and the resulting fluorescence was measured with or without CZC. No significant differences were observed suggesting that CZC did not interfere with the ability of the BODIPY molecule to produce a signal.

These studies show a detailed and logical approach to optimising some commonly used analytical methods in biology to be applied to the overall question of this thesis. The data show that the intended treatments and methods used are well tuned to the protein of interest and can accurately and robustly evaluate the effects of interventions on the protein expression and function of BCRP in hCMEC/D3 cells. Having undertaken these optimisation studies, it was possible to investigate the effects of a 24 hour treatment with CZC (0.5, 0.5 and 0.1 µM respectively) on the expression and function of BCRP in hCMEC/D3 cells, the subject of Chapter 3.

5. References

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Chapter 3 - A Combination of Clioquinol, Zinc and Copper Increases the Abundance and Function of Breast Cancer Resistance Protein in Human Brain Microvascular Endothelial Cells

1. Abstract

Modulating the abundance of the blood-brain barrier (BBB) efflux transporter breast cancer resistance protein (BCRP) has the potential to impact brain levels of drugs and endogenous substrates. Studies have demonstrated that the metal ionophore clioquinol (CQ) increases BBB abundance of P-glycoprotein (Pgp), an effect associated with increased endothelial cell levels of Cu^{2+}. This study therefore assessed whether human brain endothelial (hCMEC/D3) cell abundance and function of BCRP is modulated by CQ. hCMEC/D3 cells were treated with CQ, Zn^{2+} and Cu^{2+} (CZC) (0.5 µM, 0.5 µM, 0.1 µM, respectively) for 24 h and BCRP mRNA and protein abundance was determined by Western blot and qPCR, respectively. After a series of optimisation studies assessing specificity of bodipy prazosin (BP) and Ko143 as a substrate and inhibitor of BCRP, respectively, the impact of CZC on BP uptake was assessed. While CZC did not increase mRNA expression of BCRP, BCRP abundance was increased 1.8 ± 0.1-fold; this was associated with a 68.1 ± 3.3% reduction in accumulation of BP in hCMEC/D3 cells. This is the first study to demonstrate that augmenting metal ion availability enhances protein abundance and function of BCRP at the BBB, which may be exploited to modulate CNS access of therapeutics and endogenous substrates.

2. Introduction

The blood-brain barrier (BBB) is a complex network of capillary endothelial cells that form a large semi-permeable interface between the brain parenchyma and blood perfusing the brain (Abbott 2010). These cells express a large number of transporter proteins that serve to meet the high energy demands of the brain and to prevent the entry of potentially harmful molecules present in the blood. One of these transporters is breast cancer resistance protein (BCRP), a ~74 kDa ATP-binding cassette (ABC) transporter, that has been shown to function as a homodimer (Kage 2002) and is highly expressed at the luminal surface of brain microvascular endothelial cells (Cooray 2002). Capable of transporting a vast range of endogenous molecules and drugs (Nicolazzo 2009), BCRP plays a major role in preventing the penetration of many blood-borne molecules into the brain.

Chemical inhibition or genetic deletion of BCRP has been repeatedly shown to increase the brain accumulation of many therapeutics into the brain. For example, pantoprazole mediated inhibition of BCRP or genetic knockout of BCRP has been shown to significantly increase the brain uptake of imatinib mesylate (Breedveld 2005) and CGP74588, a N-desmethyl derivative of imatinib (Bihorel 2007). Therefore, approaches aimed at decreasing the expression and function of BCRP at the BBB may lead to increased brain accumulation of CNS targeted therapeutics. In addition to trafficking of drugs, BCRP has been suggested to play a role in the etiology of Alzheimer's disease (AD), with studies reporting that amyloid-beta (Aβ), a neurotoxic peptide that accumulates in the AD brain, is a substrate of BCRP. For example, following intravenous administration of Cy5.5-labeled Aβ to wild-type mice and BCRP (-/-) mice, significantly more Cy5.5-labeled Aβ accumulated in the brains of BCRP (-/-) mice than in the brains of their wild-type mice (Xiong 2009, Shen 2010). Concordant with these results, specific inhibitors of BCRP have been shown to significantly increase the permeability of 125I-$A\beta_{1-40}$ in hCMEC/D3 cells, an immortalized human brain endothelial cell line (Tai 2009). Taken together, these results suggest that BCRP mediates the efflux of Aβ, and that modulation of BCRP expression and function could also impact Aβ accumulation in the brain, and therefore, AD pathogenesis.

As BCRP is highly relevant in the field of CNS drug access and potentially AD pathogenesis, unravelling novel approaches to modulate its regulation can potentially improve CNS penetration of drugs or enhance the extrusion of Aβ. To achieve such an aim, a brain-endothelial targeted approach would be required, so as to not affect the crucial function of BCRP in other barriers such as the gastrointestinal tract, liver and kidney. Constitutive androstane receptors (Wang 2010), estrogen receptors (Hartz 2010), glucocorticoid receptors (Narang 2008) and aryl hydrocarbon receptors (Wang 2011) have all been shown, upon activation or inhibition, to regulate BCRP in models of the BBB. Similarly, it has been observed both *in vitro* and *in vivo* that pharmacological inhibition of glycogen synthase kinase (GSK3β) and consequent accumulation of cytosolic β-catenin leads to increased transcription and function of BCRP at the BBB (Lim 2008, Harati 2013). Multiple studies have also demonstrated that activation of peroxisome proliferator activated receptor α (PPARα) positively modulates BCRP expression, with exposure to PPARα agonists such as clofibrate (CFB) and GW7647 leading to increased protein abundance and function of BCRP in brain endothelial cells *in vitro* and *in vivo* (Hoque 2012, Hoque 2015, More 2017). Interestingly, several studies have repeatedly demonstrated the potential of metals, specifically Zn^{2+} and Cu^{2+}, to impact molecular pathways involved in regulating BCRP, such as PPARα and GSK3β, suggesting that there may be a link between metal ion availability and BCRP expression. For example, Reiterer et al. reported a significant decrease in PPARα target genes in porcine vascular endothelial cells that were Zn^{2+} deprived, and this was associated with decreased PPARα -DNA binding (Reiterer 2004), suggesting that Zn^{2+} was critical for PPARα activity. Shen et al. demonstrated that Zn^{2+} deprived porcine endothelial cells exhibited reduced expression of PPARα mRNA and protein, an observation which was reversed by Zn^{2+} supplementation (Shen 2008). In support, Kang et al. then reported that mice fed a Zn^{2+} deprived diet had significantly reduced PPARα expression and binding to DNA indicating lower PPARα signalling, effects which were rectified to some extent with Zn^{2+} supplementation (Kang 2009). Zn^{2+} has also been shown to directly inhibit the enzymatic activity of pure GSK-3 (Ilouz 2002) which degrades β-catenin, a regulator of BCRP expression at the BBB (Lim 2008, Harati 2013). In addition to the multiple studies demonstrating a crucial role of Zn^{2+} in affecting pathways involved in BCRP regulation, a role for Cu^{2+} has also been suggested. Studies by Crouch et al.

showed that the delivery of Cu^{2+} via the $Cu2^{2+}$ releasing compound glyoxal-bis(N4-methylthiosemicarbazonato)Cu(II) (Cu-gtsm) inhibited GSK3 in neuronal cells (Crouch 2009) and Cu^{2+} has also been shown to increase PPARα gene expression in liver, skeletal muscle and adipose tissue in rabbits (Lei 2017). Therefore, the biometals Zn^{2+} and Cu^{2+} appear to be essential for molecular pathways, specifically PPARα and Wnt/b-catenin signalling, that have been implicated in regulating BCRP expression at the BBB. Despite this, the impact of such metals on the expression of BCRP at the human BBB has not been investigated.

Recent studies in our laboratory have shown that clioquinol (CQ, a metal chaperone) together with Zn^{2+} and Cu^{2+} (CZC) was able to increase the expression and transport function of P-glycoprotein (P-gp) in hCMEC/D3 cells (McInerney 2018). CQ is a well-known ionophore that has been reported to bind to divalent metal cations such as Zn^{2+} and Cu^{2+} and deliver these metals into neurons, where they are able to interact with a number of signalling pathways described above, that are important for neuronal health (Crouch 2009). Due to these beneficial effects on neurons, CQ has been shown to have cognitive-enhancing effects associated with reducing the brain levels of Aβ in a transgenic mouse model of AD (Cherny 2001). Given that P-gp, like BCRP, has been shown to mediate the BBB efflux of Aβ (Citrrito 2005), we previously suggested that part of the Aβ-lowering effects of CQ, and therefore its disease-modifying potential, could be attributed to its ability to increase the abundance and function of P-gp at the BBB (McInerney 2018). While the mechanism involved in this CQ-mediated upregulation in P-gp was not reported, it is interesting to note that recent studies have shown an increase in P-gp and BCRP function when exposed to 0.1-10 mM $ZnCl_2$ for a short period of only 90 min (Zaremba 2017, Zaremba 2019), suggesting a possible direct effect of Zn^{2+} on protein function. With this in mind, the aim of this study was to assess the impact of CZC on the protein abundance and function of BCRP in brain endothelial cells and identify the mechanisms responsible for any effect of this combination. hCMEC/D3 cells were treated with non-toxic concentrations of CZC or CFB, a compound previously reported to increase BCRP abundance, and therefore used as a positive control (Hoque 2012). The effects of CZC treatment on BCRP function and expression were then assessed via substrate accumulation and uptake studies, western blotting and qPCR, the last to

determine if any observed effects were transcriptionally based. These studies are the first to explore the impact of biometal manipulation on the expression and function of BCRP at the BBB, an outcome important in modulating the barrier characteristics of the BBB.

3. Materials and methods

3.1 Materials

Tween-20, sodium chloride, rhodamine-123, dimethyl sulfoxide, sodium dodecyl sulphate (SDS), thiazolyl blue tetrazolium bromide (MTT), ammonium persulfate (APS), Corning black polystyrene TC-treated 96 well plates, penicillin-streptomycin, tetramethylethylenediamine (TEMED), Trizma® base, sodium chloride, Triton® X-100, glycerol, deoxycholic acid (3α, 12α-dihydroxy-5β-cholan-24-oic acid), 4',6-diamidino-2-phenylindole (DAPI) and glycine were all purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Falcon® rat tail collagen, CFB and Hyclone Hank's Balanced Salt Solution (HBSS) were purchased from In Vitro Technologies (Carlsbad, CA). Anti-BCRP (rabbit monoclonal) and anti β-actin antibodies were purchased from Abcam (Boston, MA), and the Odyssey blocking buffer and goat anti-mouse and donkey anti-rabbit antibodies were obtained from Millennium Science (Mulgrave, Victoria, Australia). 40% acrylamide/bis solution and extra thick blot paper were purchased from BIO-RAD (Hercules, CA). Nitrocellulose blotting membrane (0.2 mm) and BODIPY prazosin (BP) were purchased from ThermoFisher Scientific (Waltham, MA). Ko143 and a portion of BP was purchased from ThermoFisher Scientific (Waltham, MA). Endothelial Basal Medium-2 (EBM-2) and EGM-2 SingleQuot Kit Supplement & Growth Factors were obtained from Lonza (Walkersville, MD). RNeasy Plus mini kit and HiPerFect Transfection reagent were purchased from Qiagen (Hilden, Germany). Taqman primer/probes for human BCRP and GAPDH and the Pierce BCA protein assay kit were obtained from Life Technologies (Rockford, IL). cOmplete Protease Inhibitor Cocktail was purchased from Roche Pharmaceuticals (Basel, Switzerland).

3.2 Methods

3.2.1 hCMEC/D3 cell culture

Cells were generously provided by Dr. Pierre-Olivier Couraud (INSERM, Paris, France) and used at passages 30 to 34 for all relevant experiments and were cultured at 37 °C, 5% CO_2, and 95% humidified air in EBM-2 supplemented with vascular endothelial growth factor, epidermal growth factor, fibroblast growth factors, hydrocortisone, ascorbate, gentamicin, and 2.5% foetal bovine serum (FBS). Before seeding, flasks or plates were collagenated with Type I rat tail collagen in PBS (0.1 mg/mL) for 1 h at 37 °C.

3.2.2 Cell viability assay

Cells were seeded into collagen coated 96-well plates at 20,000 cells per cm^2 and were treated 24 h after initial seeding with either 75 mM CFB, a known upregulator of BCRP protein abundance in hCMEC/D3 cells, or CZC (CQ 0.5, $ZnCl_2$ 0.5 and $CuCl_2$ 0.1 µM, respectively) including their respective controls. These concentrations of CQ, Zn^{2+} and Cu^{2+} were used in this study based on our previous study demonstrating that this combination was both nontoxic to hCMEC/D3 cells and lead to a substantial increase in P-gp abundance and function (McInerney 2018). Separate wells were treated with 10% v/v DMSO in culture media as a positive control to confirm visualisation of cell death. Following 24 h treatment, cells were washed with warm PBS and then incubated with 10 mL of an 8 mg/mL MTT reagent solution in blank media for 3 h to allow the cells to reduce the MTT, after which, wells were washed 3 times with warm PBS. Next, the MTT reagent was carefully removed from the wells and then replaced with 150 mL of DMSO, to dissolve the generated formazan. After a further 30 min incubation, the plates were agitated, and absorbance was read at 540 nm using the Enspire fluorescence spectrophotometer (PerkinElmer, Waltham, MA). Background absorbance, derived from wells treated in the absence of cells, was subtracted from all other wells before analysis. The absorbance readings of vehicle and treatments were normalized against the average of the untreated cells and then expressed as a percentage of cell viability.

3.3.3 Cell treatment procedures

hCMEC/D3 cells were seeded at 20,000 cells/cm^2 onto collagenated 24-well or 6-well plates for functional studies, or Western blot and qPCR experiments, respectively. For each experiment, media was aspirated from all wells and replaced with media containing treatments which were dissolved in either DMSO or Milli-Q® water. For CFB treatments, a 75 mM stock in DMSO was diluted 1000-fold into serum containing culture medium. For CZC treatments, stocks of CQ, $ZnCl_2$ and $CuCl_2$ were diluted 1000-fold into serum-free culture medium from 0.5, 0.5 and 0.1 mM stocks, respectively. For controls, cells were treated with media containing 0.1% v/v DMSO. For treatments requiring FBS free media, media was first aspirated, washed 3 times with 2 mL of warm PBS and then replaced with FBS free media containing the intervention or its respective controls. All experiments were conducted 24 h after initial treatments where cells had achieved ~90% confluency.

3.3.4 RNA extraction and Quantitative Real-Time PCR (qPCR)

To identify changes in BCRP mRNA mediated by the interventions, qPCR was performed on RNA isolated from treated hCMEC/D3 cells. Cells were seeded and treated as described previously either with CZC or CFB as a positive control. Following treatment, cells were washed twice with ice cold PBS. Cells were then lysed and RNA was isolated using the RNeasy Plus Mini Kit. The concentration (absorbance at 260 nm) and purity (260 nm/280 nm absorbance ratio) of RNA samples were assessed using a spectrophotometer. Each sample was prepared with 12.5 mL of iScript 2x probes RT-PCR reaction mix, 0.5 mL of iScript reverse transcriptase, 0.695 mL of Taqman primer/probe, 100 ng of RNA (in 5 mL), and 6.305 mL of nuclease-free water. Measurement of gene expression by quantitative analysis was carried out in a CFX96 system (Bio-Rad, Hercules, CA). Thermocycling was performed at 50 °C for 10 min, 95 °C for 5 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 30 s. The threshold cycles (Ct) were calculated automatically using the CFX manager software. To determine the relative gene expression of BCRP mRNA in treated cells compared to control, the old-change method ($2^{-\Delta\Delta}$Ct) was employed using GAPDH and β-actin as housekeeping genes (Livak 2001).

3.3.5 Western blot analysis

Following treatment, hCMEC/D3 cells were washed twice with 2 mL of ice- cold PBS. The PBS was then aspirated followed by the addition of 200 µL of RIPA buffer (1% w/v Tris-base, 1% w/v NaCl, 0.1% SDS, 0.5% w/v deoxycholic acid, 1% v/v Triton x100 and 10% v/v glycerol) mixed with cOmplete Protease Inhibitor Cocktail. Cells were then gently agitated at 4 °C for 25 minutes. To remove cellular debris, lysates were centrifuged at 14,000 x g for 10 minutes. The resulting supernatant was then aliquoted and stored at -80 °C for future use. Cell lysate samples were mixed in a 5:1 ratio with laemmli buffer (comprising 20% w/w glycerol, 0.125 M Tris-HCl buffer, 10% v/v sodium dodecyl sulphate (SDS), 0.5% β-mercaptoethanol, and 0.5% v/v bromophenol blue in Milli-Q water) and loaded into a polyacrylamide gel for electrophoresis alongside at least one lane of Dual Xtra Precision Plus Protein Prestained Standards (Hercules, CA) as a molecular weight reference. Samples were always loaded into 10% acrylamide 1mm hand cast gels at 10 µg which was predetermined by the BCA assay comparing absorbance of samples to a standard curve generated with increasing concentrations of bovine serum albumin (BSA). The samples were then separated by electrophoresis in running buffer comprised of 25 mM Tris base, 190 mM glycine and 0.1% w/v SDS in Milli-Q water for 60 volts (V) for 30 minutes and then at 150 V was for 90 minutes using the PowerPac™ HC High-Current Power Supply (Hercules, CA). After separation, the gel was taken and left to soak in transfer buffer (comprising of 25 mM Tris buffer, 190 mM glycine and 20% v/v methanol) for 20 minutes to remove various chemicals used during electrophoresis and to fix the protein samples. The samples were then transferred to a 0.2 µm nitrocellulose blotting membrane using a Bio-Rad Trans-Blot ® Turbo™ Transfer System (Hercules, CA). The transfer was run at 25 V for 25 minutes. Once complete, the membrane was then briefly washed with Tris-buffered saline (TBS) containing 0.1% v/v Tween 20 (pH = 7.6). Next, to prevent non-specific antibody binding, the membrane was blocked with LI-COR ® Odyssey ® Blocking Buffer for 90 minutes. Afterwards, primary antibodies specific to BCRP and β-actin (a housekeeping protein) were diluted in 20 mL of TBST 10,000 and 100,000-fold, respectively. The membrane was then incubated in this solution overnight (18 hours) at 4 °C. The following day, the membrane was washed four times with 10 mL of

TBS-T for 5 minutes. Next, secondary antibodies specific to the primary antibodies of either BCRP or β-actin were then diluted 15,000-fold into 30 mL of TBST and applied to the membranes followed by the same washing procedure previously mentioned. The membrane was then visualized using a LI-COR ® Odyssey ® scanner and densitometry was performed on the bands representing BCRP and β-actin using the Image J program (NIH, Bethesda). Biological replicates were processed across multiple gels and thus required a method of normalisation. Normalisation by sum of replicates (Degasperi 2014) was used to pool Western blot data from different gels together.

3.3.6 Functional studies

Prior to assessing any impact of CZC on BCRP function, it was important to determine an experimental timepoint following BP addition to hCMEC/D3 cells where BCRP function was detectable. hCMEC/D3 cells were therefore incubated with BP for specific timepoints (2, 5, 15, 30 and 60 minutes) with or without Ko143, a potent and commonly used BCRP inhibitor.[32] The timepoint at which the largest increase in BP accumulation occurred as a result of Ko143 exposure was then selected as the optimum duration for all future studies. Once determining this timepoint, it was important to confirm that the uptake of BP was not impacted by P-gp function given that we have previously demonstrated that CZC can increase P-gp function (McInerney 2018). Therefore, the uptake of BP at the selected concentration and exposure timepoint determined above was assessed in the presence of PSC833, a known inhibitor of P-gp. By confirming that the uptake of BP is not affected by PSC833, it would be possible to associate any modulation in BP uptake to alterations in BCRP function as a result of CZC treatment and not to modulation in P-gp function. Finally, as P-gp function has been shown to be affected by Ko143 (Allen 2002), and we wanted to ensure that any impact of Ko143 was a result of BCRP inhibition and not P-gp inhibition, the uptake of the P-gp substrate, rhodamine123 (R123), in the presence of Ko143 was also assessed. To undertake these experiments, the following methods were employed. Two days following the initial seeding of the cells, media was aspirated from all wells and washed twice with warm PBS. Next, 500 µL of transport buffer (10 mM HEPES in HBSS, pH = 7.4) with or without 500 nM Ko143 or 300 nM PSC833 was then delivered to the appropriate wells and gently agitated at 37 °C and 5% CO 2 for 15 minutes. All

the appropriate wells were then replaced with 500 µL of transport buffer containing 500 nM of BP or 5 µM R123 with or without 500 nM Ko143 or 300 nM PSC833 (depending on the experimental question detailed above) and incubated with gentle agitation at 37 ° C and 5% CO 2 for 2, 5, 15, 30 and 60 minutes (for time course assays) or for 30 minutes (interaction assays). All wells were then washed 3 times with ice-cold transport buffer followed by the addition of 150 µL of 1% v/v Triton X-100. The cells were then left to lyse for 20 minutes at 4°C, allowing any BP or R123 that had accumulated during the incubation period to escape the cells. The fluorescence of BP or R123 was then measured using the Enspire fluorescence spectrophotometer (PerkinElmer, Waltham, MA) at an excitation wavelength of 503 nm and emission wavelength of 512 nm (for BP) or 511nm and 534 nm (for R123). The mass of BP or R123 released from cell lysates was quantified by comparing sample lysate fluorescence to that of standard solutions containing a known amount of the same substrate prepared in 1% v/v Triton X-100 in Milli-Q water. The fluorescence reading from wells containing only 1% v/v Triton X-100 was subtracted from the measured fluorescence readings from cell lysates. The calculated masses were then normalised to the total cellular protein count using the Pierce BCA protein assay (compared against BSA solution standards which were also prepared in 1% v/v Triton X-100 in Milli-Q water). Once the conditions for this accumulation assay were developed and it was clear that the assay was assessing only BCRP function, the impact of a 24 hour treatment of CZC on the hCMEC/D3 cellular accumulation of BP was assessed over a 30 minute period with or without 500 nM Ko143.

3.3.7 Statistical analyses

All experiments were repeated using a minimum of 3 biological replicates. Results are displayed as mean ± S.D. Where appropriate, the two-tailed t tests or a one-way or two-way analysis of variance (ANOVA) with the relevant post hoc tests were performed. Data were analysed using the IBM SPSS statistics 22 software (Armonk, NY).

4. Results

4.1 Identification of non-toxic concentrations of CZC in hCMEC/D3 cells determined by the MTT assay

Before commencing with mechanistic studies, it was essential to demonstrate that the concentrations of CZC used did not significantly affect the viability of hCMEC/D3 cells. To show this, hCMEC/D3 cells were exposed to relevant concentrations of CZC for 24 hours in serum free media and cell viability was then assessed by use of the MTT assay. No significant changes in viability were observed in cells treated with vehicle or the CZC treatment relative to untreated control cells (Chapter 2, Figure 2). To confirm that this assay was able to detect toxicity, hCMEC/D3 cells were exposed to a toxic concentration of DMSO (10%) under the same experimental conditions and a dramatic 83.3 ± 3.9% decrease in cell viability was observed. As a 24 hour treatment of CZC in serum free media (0.5, 0.5 and 0.1 µM respectively) was shown to be non-toxic to hCMEC/D3 cells, this was used for all subsequent studies.

4.2 CZC upregulates BCRP protein abundance in hCMEC/D3 cells

As it was hypothesized that treatment with CZC would increase BCRP protein abundance, it was first important to show that BCRP protein abundance could be increased using a known inducer of BCRP. As a positive control, cells were therefore treated with 75 µM clofibrate, a PPAR-α agonist previously shown to upregulate BCRP protein abundance in hCMEC/D3 cells (Hoque 2012), for 24 hours. This treatment significantly upregulated BCRP protein abundance in hCMEC/D3 cells 1.5 ± 0.2-fold relative to control (Figure 1a), confirming that it was possible to induce abundance of BCRP in hCMEC/D3 cells. Interestingly, the increased protein abundance of BCRP induced by CFB was not associated with increased mRNA expression of BCRP after a 24 hour treatment (Figure 1b).

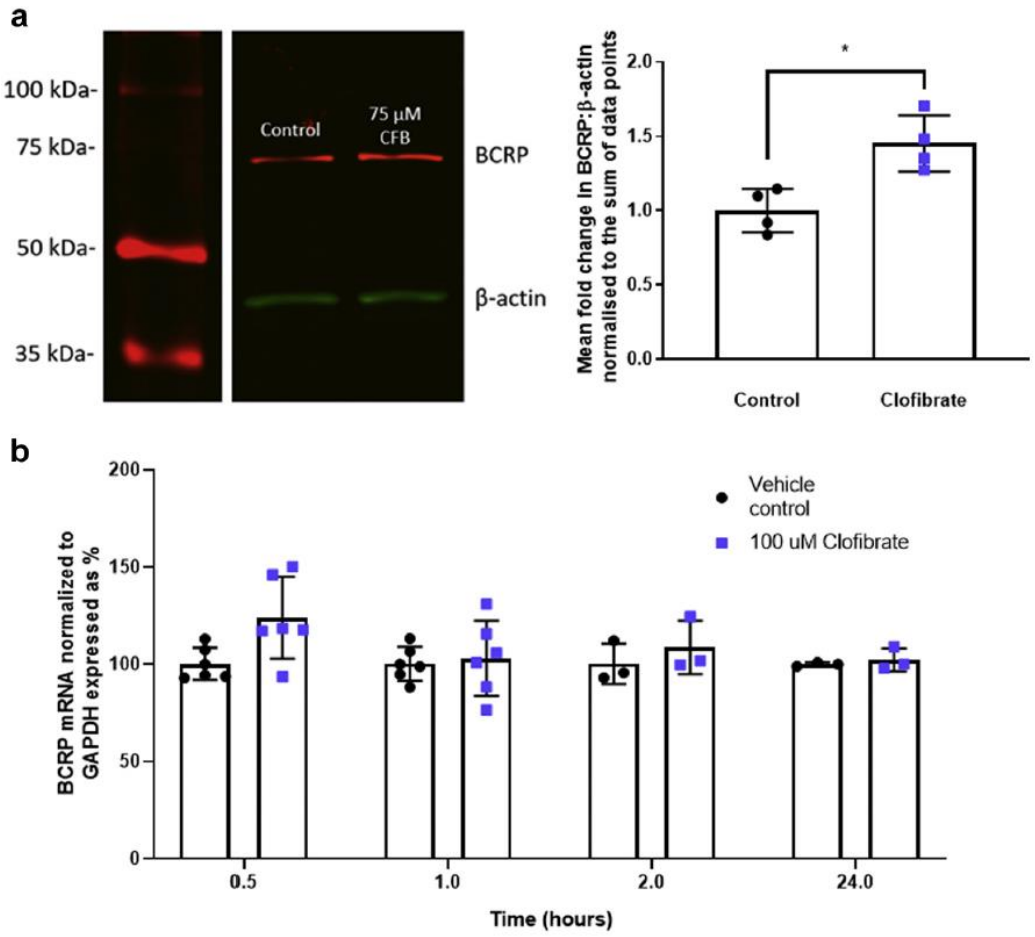

Fig. 1 (a) Western blot image depicting BCRP abundance in hCMEC/D3 cells following a 24 h treatment with 75 mM clofibrate (CFB) and densitometric analysis of the CFB dependent increase in BCRP abundance relative to control (as seen in Chapter 2 Figure 7). (b) mRNA expression of BCRP in the presence of 100 mM CFB for increasing exposure times. Data are expressed as mean ± SD (n = 4 biological replicates for protein and n = 3-6 biological replicates for mRNA). *p < 0.05 compared to control using a two-tailed t-test.

To ascertain whether any possible increase in protein abundance at 24 hours was associated with increased mRNA expression of BCRP at earlier timepoints, qPCR was conducted after shorter exposure times. As observed at 24 hours, shorter treatments with CFB did not result in increased mRNA expression of BCRP. Next, hCMEC/D3 cells were treated with CZC for 24 hours and analysed by western blotting to determine if the treatment was able to induce BCRP protein abundance. Consistent with our hypothesis, CZC treatment significantly upregulated the protein abundance of BCRP 1.8 ± 0.1-fold compared to control (Figure 2a). To ascertain if this increased abundance in BCRP was a result of CZC affecting transcriptional regulation of BCRP, qPCR experiments were conducted after multiple exposure times with CZC. As observed with CFB

treatment, transcript levels of BCRP were not affected at any of the timepoints assessed (Figure 2b), suggesting that the increase in protein abundance induced by CZC was not a result of genomic regulation.

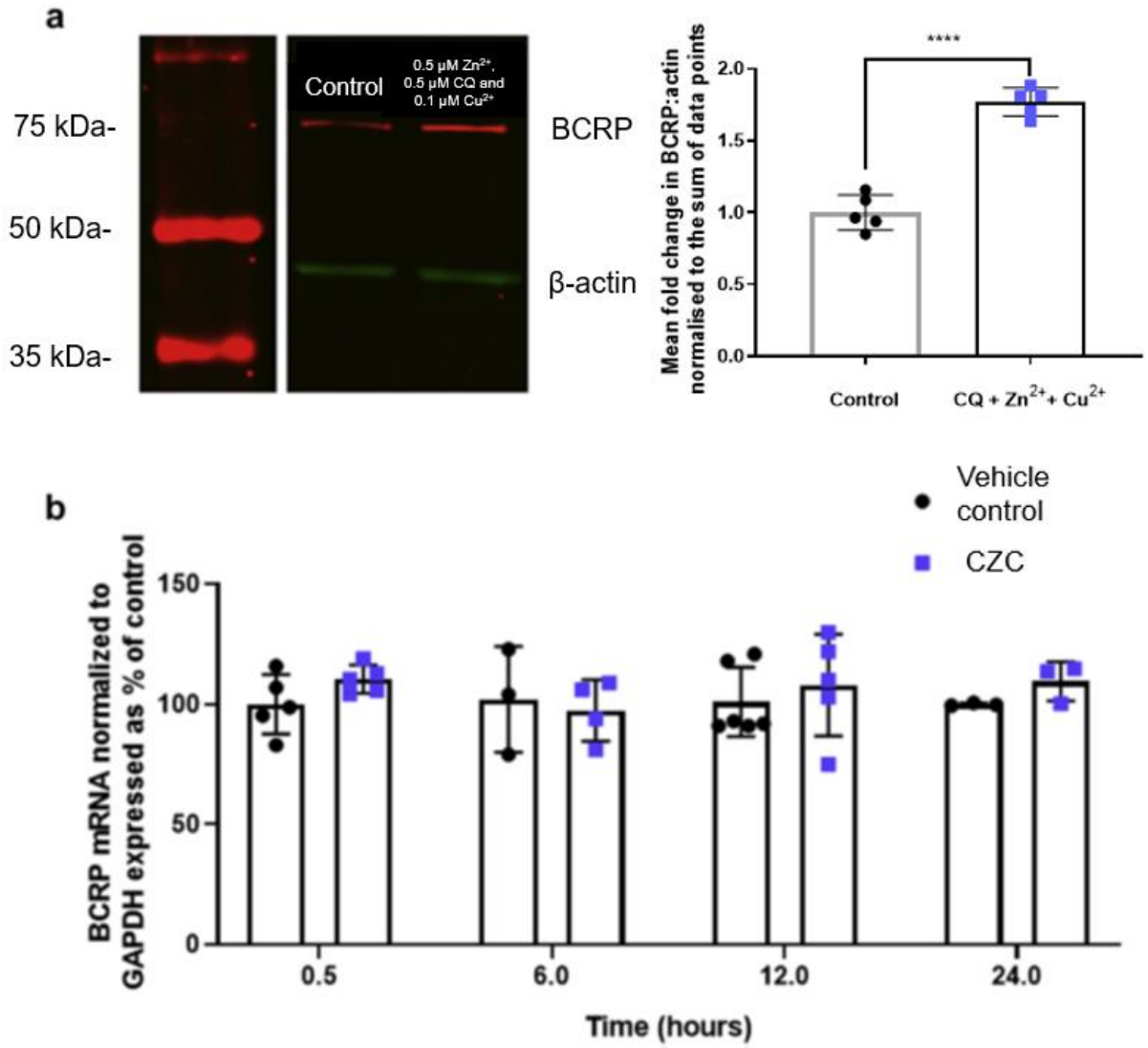

Fig. 2. (a) Western blot image depicting BCRP abundance in hCMEC/D3 cells following a 24 h treatment with CZC (0.5, 0.5 and 0.1 μM of CQ, Zn^{2+} and Cu^{2+}, respectively) and densitometric analysis of the CZC-dependent increase in BCRP abundance relative to control. (b) mRNA expression of BCRP in the presence of CZC for increasing exposure times. Data are expressed as mean ± SD (n = 4 biological replicates for protein and n = 3-6 biological replicates for mRNA). ****$p < 0.0001$ compared to control using a two-tailed t-test.

4.3 Identifying optimal accumulation duration of BP uptake in hCMEC/D3 cells

The accumulation of BP was measured at different timepoints over the course of 60 minutes in the presence and absence of 500 nM Ko143, a potent BCRP inhibitor, to identify the timepoint at which the accumulation of BP would be most affected by pharmacological intervention. Significant differences in the hCMEC/D3 accumulation of BP were observed after 15 and 30 minute exposure times in the presence and absence of Ko143, with similar fold changes in BP accumulation of 1.7 ± 0.2 and 1.6 ± 0.3-fold, respectively (Chapter 2 Figure 9). So as to maximise the sensitivity of the assay and allow for the greatest accumulation of BP, while still retaining the ability to modulate BCRP function, a 30 minute timepoint was chosen for all subsequent BP accumulation studies.

4.4 Ko143 and BP Are a Specific Inhibitor and Substrate, Respectively, of BCRP

While Ko143 is a potent BCRP inhibitor, a recent study has shown that it can affect other transporters, such as P-gp (Weidner 2015), which is also expressed in hCMEC/D3 cells. It was therefore important to demonstrate that Ko143 did not affect P-gp function, particularly given that we have demonstrated CZC can enhance P-gp function (McInerney 2018). Furthermore, BP is a substrate of P-gp, albeit to a lesser extent compared to BCRP, and so it was important to confirm that the uptake of BP in hCMEC/D3 cells at the concentration used in this study was only being affected by BCRP and not P-gp.

Firstly, to show that P-gp function was not affected by Ko143, the accumulation of 5 µM R123 in hCMEC/D3 cells was measured in the presence and absence of 500 nM Ko143 (Chapter 2 Figure 10a). There was no significant change to the uptake of R123 in the presence of Ko143 (89.2 ± 7.0 % of the control), even though P-gp was confirmed to be functional as R123 accumulation was significantly increased by 300 nM PSC833 (Chapter 2, Figure 10a). It was also demonstrated that a much higher concentration of 5 µM Ko143 did not affect P-gp function (95.8 ± 5.6% of the control) (Chapter 2, Figure 10b).

Secondly, to confirm that the accumulation of BP was not affected by P-gp, and we were indeed measuring BCRP function, hCMEC/D3 cells were exposed to 500 nM BP in the presence of 0.3 µM

PSC833, a concentration shown to modulate P-gp efflux of R123. While BP uptake was affected by Ko143 at 5 µM (214.9 ± 7.7% relative to the control), the accumulation of BP was not significantly affected by P-gp inhibition (122.0 ± 18.5% relative to the control) (Chapter 2, Figure 10c). These studies therefore demonstrated that at the concentration of BP and Ko143 used, the function of BCRP (and not P-gp) was being assessed, and therefore, any impact of CZC on BP uptake was a result of modified BCRP function.

4.5 Treatment with CZC Decreases the Accumulation of BP in hCMEC/D3 cells

To show that CZC increased the transport function of BCRP, the validated functional assay developed above was employed. Cells treated with CZC for 24 hours exhibited a significantly lower accumulation of BP (68.1 ± 3.3% of the control), suggesting that BCRP function was enhanced by CZC (Figure 3). Cells treated with 5 µM Ko143 significantly increased the accumulation of BP by 146.4 ± 25.3% of the control. While Ko143 did not completely rescue the reduced accumulation of BP mediated by CZC, the accumulation of BP for the CZC/Ko143 combination was approximately 2-fold higher compared to CZC treatment alone, suggesting a partial reversal of BCRP function. These results demonstrate that the increase in BCRP abundance is associated with increased BCRP-mediated efflux of BP.

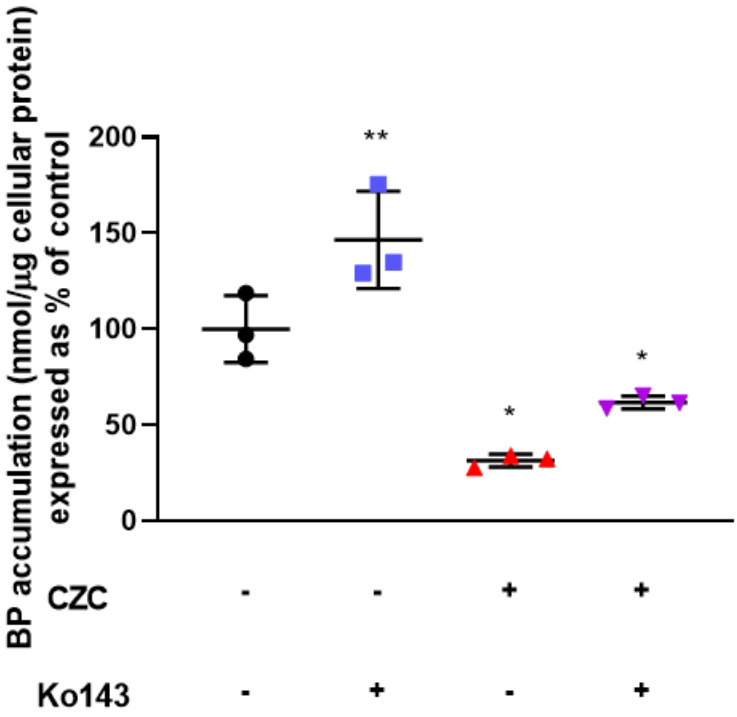

Fig. 3 Accumulation of BP after 30-minute exposure to 500 nM BP, normalized to total cellular protein count and expressed as % relative to the control, in hCMEC/D3 cells treated with CZC (0.5, 0.5 and 0.1 µM of CQ, Zn^{2+} and Cu^{2+}, respectively) and/or 5 µM Ko143 in serum free media for 24 h. Data are expressed as mean ± SD. n = 3 for each treatment group. One-way ANOVA with post-hoc Tukey's multiple comparisons test was used for statistical analysis. * $p < 0.05$ and ** $p<0.01$ compared to control.

5. Discussion

BCRP function at the BBB is not only crucial in limiting drug access to the CNS but has been more recently implicated in the BBB efflux of Aβ. Therefore, modulating BCRP expression and function at the BBB may help improve the brain access of CNS targeted drugs and could serve as a novel approach to reduce β-amyloid burden in the brain, being of importance for AD pharmacotherapy. In recent years, significant research efforts have been made to understand the molecular mechanisms that regulate BCRP expression, some of which are modulated by Zn^{2+} and Cu^{2+}, such as PPARα and the GSK-3β signalling pathways (Reiterer 2004, Shen 2008, Kang 2009, Ilouz 2002, Crouch 2009, Lei 2017). The purpose of this study was to investigate the effects of modulating cytosolic levels of Zn^{2+} and Cu^{2+}, facilitated by CQ, on BCRP expression and function using a human model of the BBB. Previous work by our laboratory has shown that the combination treatment of CZC was able to

significantly upregulate P-gp in hCMEC/D3 cells (McInerney 2018), and in those studies, it was demonstrated that cytosolic levels of Cu^{2+} were increased by this CZC treatment.

As predicted, and similar to what we have reported for P-gp, a 24 h treatment with CQ and the two biometals Zn^{2+} and Cu^{2+} was able to increase the abundance of BCRP in hCMEC/D3 cells. Whether a similar effect on BCRP abundance would be observed when hCMEC/D3 cells are treated with Zn^{2+}, Cu^{2+} or CQ alone requires further investigation, however, our previous experiments with these treatments in isolation had no impact on P-gp abundance in hCMEC/D3 cells (McInerney 2018). Similarly, as observed by Hoque et al., treatment with CFB was also able to increase BCRP levels (Hoque 2012), and we were able to show a similar extent of upregulation using this positive control. Our results are the first to demonstrate that this biometal combination together with the metal chaperone is able to increase the abundance of a key efflux transporter, confirming a crucial role of biometals in the defence characteristics of the BBB. After observing increased BCRP expression and function as a result of CZC treatment, we sought to further investigate the mechanism of action by assessing transcript levels of BCRP. Given that (i) we have previously reported that treatment of hCMEC/D3 cells with this CZC treatment leads to increased Cu^{2+} levels (McInerney 2018), (ii) Cu^{2+} has been shown to inhibit GSK-3 and increase PPARα gene expression in other cell types (Crouch 2009, Lei 2017), and (iii) these pathways have been shown to regulate mRNA BCRP expression at the BBB (Harati 2013, Lim 2008, Hoque 2012), it was expected that the increase in BCRP abundance mediated by CZC would be associated with increased mRNA expression of BCRP. Interestingly, no increase in mRNA expression of BCRP was observed with CZC treatment at any of the timepoints assessed, suggesting that the positive effects of CZC on BCRP in hCMEC/D3 cells are likely mediated via a non-transcriptional mechanism. Our results showing a lack of increased BCRP mRNA following CFB treatment were not in line with that previously reported (Hoque 2012), however, this is possibly a result of the more narrow passage range used in our study (P30-34) compared to the previously-published study using a passage range of P28-39. It is possible that the use of later passages of hCMEC/D3 cells may exhibit differential transcriptional regulation of BCRP and/or greater sensitivity to CFB given that passages beyond 35 have not been validated (Weksler 2013).

This suggestion of pharmacological interventions impacting on BCRP function in a non-transcriptional regulatory manner is in line with recent reports. For example, BCRP function at the BBB has been shown to be acutely affected by 17-β-estradiol where transport activity of BCRP was modulated with no change in genomic regulation (Hartz 2010). More recent studies have also demonstrated rapid changes to P-gp, BCRP and multidrug resistance associated protein 1 efflux capacity in both rat brain capillaries and hCMEC/D3 cells as a result of Zn^{2+} exposure which was attributed to activation of serum and glucocorticoid-inducible kinase 1 (Zaremba 2017, Zaremba 2019). However, in these published studies, while there was a change to transporter function, no alteration in protein abundance was observed. In our studies, the increase in transporter function was associated with increased protein abundance, and given there was no increase in mRNA expression, it could be suggested that the increase in BCRP abundance induced by CZC treatment could be a result of CZC hindering BCRP protein clearance. Indeed, it has been demonstrated that BCRP can be cleared through proteasomal degradation (Wakabayashi 2007) and that inhibition of proteasomal degradation in brain endothelial cells can impact BCRP function (Mahringer 2010). Furthermore, it has been reported, albeit not in brain endothelial cells, that CQ has proteasomal inhibitory activity both in myeloma cells (Mao 2009) and in human breast cancer cells (Daniel 2005), and that this is dependent on the ability of CQ to traffic Cu^{2+} into cells (Zhai 2010). It is possible, therefore, that the increase in Cu^{2+} induced by CQ treatment (as we have demonstrated previously) inhibits proteasomal activity in hCMEC/D3 cells, reducing degradation of BCRP, which leads to increased BCRP abundance and function. Further studies would be required to demonstrate that this is the mechanism by which CZC is increasing BCRP abundance in hCMEC/D3 cells.

Having demonstrated that CZC was able to increase BCRP abundance, it was then crucial to confirm that this led to a functional change in BCRP, although a number of cellular accumulation optimisation experiments were necessary before assessing this phenomenon. While we demonstrated that 30 min was the most appropriate time to assess the impact of intervention on BCRP function using BP uptake, there were some additional optimisation experiments required before assessing the impact of CZC on BCRP function. Ko143 is an extremely potent BCRP inhibitor which is frequently used for *in*

vivo and *in vitro* studies. Our studies demonstrated that Ko143 significantly increased BP accumulation after 15 and 30 min incubations, after which the effect of Ko143 was no longer evident. It is possible at this later time point that an equilibrium between the intracellular and extracellular concentrations of BP was established (as the medium was not being replenished) and therefore, the inhibitory activity of Ko143 was not detectable. For this reason, all future intervention studies were undertaken at 30 min, where BCRP modulation was detectable. Recent findings by Weidner et al. revealed that Ko143, at high concentrations, was able to stimulate P-gp ATPase activity (Weidner 2015). As BP is reportedly also a substrate of P-gp (Kimchi-Sarfaty 2002), it was important to ensure that Ko143, at a concentration of 500 nM and 5 mM, was not affecting P-gp function. It was demonstrated that neither 500 nM, nor 5 mM Ko143 significantly affected the P-gp mediated uptake of R123, whilst R123 uptake was, as expected, affected by the P-gp inhibitor, PSC833. Furthermore, it was essential to ensure that the uptake of BP was not being modulated by P-gp as we have previously reported that CZC can increase P-gp function in hCMEC/D3 cells. Therefore, the uptake of BP was assessed in the presence and absence of 300 nM PSC833, a concentration we demonstrated to inhibit P-gp function. At a concentration of 500 nM BP, the uptake of BP was not affected by PSC833, indicating that P-gp does not play a significant role in BP uptake within our experimental design. Therefore, we felt confident to conclude that any impact of CZC on BP uptake at a concentration of 500 nM and at a 30 min timepoint reflected a modulation in BCRP function. Using these experimental conditions, we demonstrated, in line with an increase in BCRP abundance, that BCRP function was significantly enhanced by CZC, suggesting that this combination could be used to increase the efflux capacity of this important BBB transporter. Furthermore, inclusion of the BCRP inhibitor Ko143 rescued, albeit partially, the effect of CZC on BP accumulation, confirming the effects of CZC on BP accumulation were BCRP-mediated. It is possible that the reduction in BP accumulation could also be attributed to modified passive permeability of BP as a result of CZC treatment. However, given that the reduction in CZC-induced BP accumulation was reversed, albeit not completely, by the BCRP inhibitor Ko143, CZC mediated modulation of BCRP activity is considered to contribute significantly to the reduced accumulation of BP.

With our previous observations that CZC increases cytosolic Cu^{2+} levels in hCMEC/D3 cells, these results are the first to demonstrate the ability of BCRP abundance and function to be regulated by modulating cytosolic metal concentrations, in a model of the human BBB. Interestingly, CQ has been shown to reduce brain Aβ burden in mouse models of AD (Cherny 2001, Grossi 2009) and while there have been a multitude of mechanisms proposed, it is possible that CQ may have increased brain clearance of Aβ through enhancing the P-gp and BCRP mediated efflux of this neurotoxic peptide. Modulating brain endothelial cell biometal levels may therefore represent a novel approach to increase BBB efflux of Aβ, opening new options for influencing the progression of AD. Furthermore, these studies also demonstrate that modifying brain endothelial cell biometal levels has the potential to impact CNS drug access. Strategies aimed at reducing brain endothelial metal levels may be exploited to attenuate BCRP abundance and function, potentially providing a new approach to increase brain access of therapeutics whose CNS access is limited by BCRP function.

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Summary, implications, and future directions

The purpose of this study was to assess the impact of CQ, Zn^{2+} and Cu^{2+} (CZC) on the protein abundance and function of BCRP in hCMEC/D3 cells and to identify the mechanisms responsible for any effect of this combination. Specific assays were developed and optimized in order to carry out the necessary experiments to address these aims. hCMEC/D3 cells were treated with CZC (0.5 µM clioquinol, 0.5 µM Zn^{2+}, 0.1 µM Cu^{2+}) for 24 h and BCRP mRNA and protein abundance was determined by Western blot and qPCR, respectively. After a series of optimisation studies assessing specificity of bodipy prazosin (BP) and Ko143 as a substrate and inhibitor of BCRP, respectively, the impact of CZC on BP uptake was assessed. CZC increased BCRP abundance 1.8 ± 0.1-fold which was associated with a 68.1 ± 3.3% reduction in accumulation of BP in hCMEC/D3 cells. This effect was partially reversed in the presence of Ko143, a potent inhibitor of BCRP, showing CZC mediated modulation of BCRP activity was contributing significantly to the reduced accumulation of BP. Interestingly, CZC did not increase mRNA expression of BCRP suggesting that genomic regulation did not play a role in the change of BCRP abundance. These results are the first to demonstrate that modulating cytosolic metal concentrations with CZC increases the abundance and function of BCRP, confirming a crucial role of biometals in the defence characteristics of the BBB.

Attempts at elucidating the mechanism by which CZC increased BCRP expression and function revealed that the transcriptional regulation of BCRP was unchanged. It is possible that the treatment inhibited the proteosomal activity linked to the rate of BCRP turnover which would in turn lead to an apparent increase in BCRP abundance. Further studies using a known inhibitor of proteolytic activity as a positive control would be required to demonstrate that this is the mechanism by which CZC is increasing BCRP abundance in hCMEC/D3 cells. It would also be prudent to show that increasing the abundance of BCRP in this manner still results in an increase of function. Recent studies by our lab have shown an increase in P-gp protein expression by CZC treatment, although the exact mechanism has not yet been thoroughly investigated. It may also be that the clearance rate of P-gp has been reduced alongside BCRP and a role for biometals in regulating efflux transporter turnover rate might

be established. The next steps in these studies should assess if the observed effects of CZC on BCRP expression and function translate to an *in vivo* model. To assess the function of BCRP in a clinically relevant manner, studies should observe the effects of CZC treatment on the transport of CNS targeted therapeutics such as irinotecan or imatinib which are known substrates of BCRP. Should the effects of CZC be similar to our *in vitro* studies, one would expect reduced brain accumulation of these drugs as a result of increased BCRP function. Follow up studies should assess the impact of modulating BCRP function on Aβ clearance using an AD mouse model such as APP/PS1.

The passage of most molecules into the brain are tightly regulated by the BBB, earning the BBB the title of the "bottleneck" in brain drug development as it is a major obstacle to the access of CNS therapeutics. BCRP is highly expressed at the BBB and has been repeatedly shown to play a major role in multidrug resistance through the binding and efflux of structurally distinct molecules. Thus, control over the function of BCRP at the BBB may serve to improve CNS drug delivery. In addition, BCRP has recently been implicated in the transport of Aβ, an endogenous neurotoxic peptide found at abnormally high concentrations in the AD brain. An increased production of Aβ has been linked to familial AD. However, in the more common sporadic AD, brain Aβ accumulation has been linked to a reduced rate of clearance of Aβ from the AD brain compared to healthy individuals. Therefore, in addition to increasing the access of CNS therapeutics, manipulating the function and expression of BCRP at the BBB can serve to alleviate Aβ burden from the AD brain which opens novel pathways for targeting AD progression. The results in this study have revealed that this is indeed possible by regulating cytosolic metal concentrations in a cell model of human brain endothelium to regulate BCRP abundance and function.