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Therapeutic drugs modulate ATP-Binding Cassette transporter-mediated transport of amyloid beta_(1–42) in brain microvascular endothelial cells

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Abstract

Deposition of amyloid- β peptide ($A\beta_{(1-42)}$) is a hallmark of Alzheimer's disease. Clearance of $A\beta_{(1-42)}$, across the blood-brain barrier (BBB), is mediated by ATP-binding Cassette (ABC) efflux transporters. Many therapeutic drugs inhibit ABC transporters, but little is known of the effect of therapeutic drugs on $A\beta_{(1-42)}$ transport across BBB endothelial cells. The effects of selected, widely prescribed, therapeutic drugs on ABCB1, ABCC5 and ABCG2 activities were determined by measuring intracellular levels of calcein, GS-MF, and Hoechst 33342 respectively in primary porcine brain endothelial cells (PBECs). The ability of ABCB1, ABCC5 and ABCG2 to transport $A\beta_{(1-42)}$ was determined using fluorescent $A\beta_{(1-42)}$. The ability of the ABCB1, ABCC5 and ABCG2 inhibitor telmisartan to modify transcellular $A\beta_{(1-42)}$ transport was investigated using PBEC monolayers housed in Transwell® inserts.

Treatment of PBECs with ABC transporter inhibitory drugs (indomethacin, olanzapine, chlorpromazine, telmisartan, pantoprazole, quinidine, sulfasalazine and nefazodone) increased $A\beta_{(1-42)}$ intracellular accumulation. Inhibition of ABCB1, ABCC5 and ABCG2 by telmisartan increased $A\beta_{(1-42)}$ transport in the apical to basal direction and reduced its transport in basal to apical direction in PBEC monolayers. ABCB1, ABCC5 and ABCG2 mediate the efflux transport of $A\beta_{(1-42)}$ in BBB endothelial cells. Inhibition of ABC transporters by therapeutic drugs, at plasma concentrations, could decrease $A\beta_{(1-42)}$ clearance from brain, across BBB endothelial cells into blood, and potentially influence levels of the $A\beta_{(1-42)}$ peptide within the brain.

Keys words: ABCB1, ABCG2, ABCC5, amyloid-beta, Alzheimer's disease, therapeutic drugs, porcine brain endothelial cells.

1. Introduction

The blood-brain barrier (BBB) is the regulatory interface between the brain and systemic circulation that governs the internal environment of this privileged site by tightly controlling the trafficking of molecules between the peripheral and central compartments. ATP-binding cassette (ABC) efflux transporters play a fundamental role in determining the permeability characteristics of the BBB and function to restrict penetration of molecules into the brain, whilst also mediating the clearance transport of molecules out of the brain. The ABCB1 (P-glycoprotein), ABCG2 (breast cancer resistance protein) and ABCC5 (multidrug resistance-associated protein-5) transporters are expressed in BBB endothelial cells (Cantrill et al., 2012; Kubo et al., 2015; Shubbar and Penny, 2018) and limit penetration of numerous therapeutic drugs and endogenous compounds across the BBB (King et al., 2001; Schinkel et al., 1996).

Importantly, widely prescribed therapeutic drugs from multiple pharmacological classes significantly inhibit ABC transporter activity and/or modulate the expression of ABC transporters and influence penetration of transporter substrates across the BBB (Sugimoto et al., 2013; Vautier et al., 2008).

Alzheimer's disease is characterised by accumulation of amyloid- β peptide ($A\beta_{(1-42)}$) peptide (Rank et al., 2002) leading to memory loss, a decline in cognitive function, confusion and difficulty in completing familiar tasks (Gravina et al., 1995). The level of $A\beta_{(1-42)}$ in the healthy brain is a consequence of the balance between production and processing of amyloid precursor protein (APP), entry of plasma $A\beta_{(1-42)}$ into the brain across the BBB, chiefly via the receptor for advanced glycation end products (RAGE), and $A\beta_{(1-42)}$ clearance from brain by the low density lipoprotein-related protein receptor 1 (LRP1) (Deane et al., 2003; Demattos et al., 2002; Shibata et al., 2000).

There is also a growing body of evidence that the efflux transporters ABCB1 (Cirrito et al., 2005; Kuhnke et al., 2007; Lam et al., 2001), ABCG2 (Deo et al., 2014; Tai et al., 2009; Xiong et al., 2009) and ABCC1 (Krohn et al., 2011) mediate clearance transport of amyloid- β peptide. Interestingly, studies suggest that raised $A\beta_{(1-42)}$ levels in the brain are a consequence of defective clearance of the peptide across the BBB (Zlokovic, 2005) and that there is an inverse relationship between ABCB1 expression and the deposition of amyloid- β peptide in the brain (Cirrito et al., 2005; Vogelgesang et al., 2002). Whilst the drug efflux transporters ABCB1 and ABCG2 are known to transport amyloid- β peptide, no studies yet report the ability of ABCC5, which is also expressed in BBB endothelial cells (Kubo et al., 2015; Warren et al., 2009), to transport this peptide. Furthermore, studies of the effects of

therapeutic drugs, at plasma concentrations, on ABC transporter-mediated transport of amyloid- β peptide are lacking.

Therefore, in the current study, we sought to clarify the role of ABCC5, in addition to the roles of ABCB1 and ABCG2, in amyloid- β peptide transport and to establish the effects of known inhibitory drugs on accumulation of A $\beta_{(1-42)}$ in primary porcine brain endothelial cells (PBECS) and on the transendothelial transport of A $\beta_{(1-42)}$ across an *in vitro* model of the BBB.

2. Materials and Methods

2.1. Materials

Fluorescent amyloid- β peptide (FAM-A $\beta_{(1-42)}$) was purchased from Anaspec Inc. (USA). Dulbecco's phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), Neutral Red powder, heparin sodium salt from porcine intestinal mucosa, trypsin-EDTA solution, verapamil hydrochloride, Ko143, Hoechst 33342, penicillin/streptomycin, puromycin dihydrochloride, indomethacin, olanzapine, chlorpromazine hydrochloride, telmisartan, pantoprazole sodium hydrate, quinidine, sulfasalazine and nefazodone hydrochloride were purchased from Sigma-Aldrich (UK). Calcein-AM and HEPES were purchased from Invitrogen (Paisley, Scotland), CMFDA was purchased from Abcam (Cambridge, UK), Collagen type I (rat tail) and fibronectin (human) were purchased from SLS Ltd (Nottingham, UK). DMEM (low glucose, no pyruvate, no glutamine, and no phenol red), DMEM (high glucose, no pyruvate) and foetal bovine serum were purchased from Life Technologies Ltd (Paisley, UK). Cell culture plastic ware was obtained from Greiner Bio-one (Stonehouse, UK) and 12-well Transwell® inserts and plates were obtained from Corning (Deeside, UK). Plasma-derived serum was from First Link (Birmingham, UK) and the Bradford protein assay reagent was obtained from Bio-Rad (Hemel Hempstead, UK).

2.2. Isolation and culture of primary porcine brain endothelial cells

Fresh brains from male and female Landrace cross Large White pigs, aged 22-24 weeks, 105-110 kg, were obtained from a local abattoir. Isolation of porcine brain microvessels was based on the method described by Skinner et al. (Skinner et al., 2009). Microvessels were seeded into 6-well plates pre-coated with 100 μ g/ml type I rat tail collagen and 7.5 μ g/ml

human fibronectin and maintained in growth medium composed of DMEM (low glucose without phenol red) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, 2 mM glutamine, 10 % (v/v) plasma derived serum and 125 µg/ml heparin in a humidified atmosphere at 37 °C with 5 % CO₂ for 24 h. PEBCs were treated for 48 h with puromycin dihydrochloride, 4 µg/ml, maintained in growth medium and astrocyte-conditioned medium (ACM) (1:1) for 6 days then subcultured into 96-well plates or onto 12-well Transwell® inserts.

2.3. Culture of astrocytes

The rat astrocyte cell line CTX-TNA2 line (passages 10–15) was maintained in DMEM supplemented with 10 % (v/v) foetal bovine serum with 100 U/ml penicillin and 100 mg/ml streptomycin at a seeding density of 1×10^6 cells per 75 cm² flask. When cells reached confluency, astrocyte-conditioned medium was collected and stored at -20 °C until use.

2.4. Measurement of the effect of ABCB1, ABCG2 and ABCC5 inhibition on intracellular accumulation of A β _(1–42)

Functional activity of ABCB1, ABCG2 and ABCC5 was measured as described previously (Cantrill et al. 2012; Gutmann et al. 1999; Shubbar and Penny 2018).

PBECs in 96-well plates were incubated in assay medium (DMEM low glucose without phenol red supplemented with 2 mM L-glutamine) containing either 10 µM verapamil hydrochloride (ABCB1 inhibitor) (Hawthorne and Dively, 2011), 1 µM Ko143 (ABCG2 inhibitor) (Poller et al., 2010) or 25 µM Mk571 (ABCC5 inhibitor) (Poller et al., 2008) for 30 min at 37 °C. A stock solution of fluorescent amyloid- β (FAM-A β _(1–42)), was prepared by dissolving 500 µg lyophilized FAM-A β _(1–42) in 40 µl of 0.25 M sodium hydroxide and PBS added to produce a concentration of 100 µM. FAM-A β _(1–42) was added to PBECs to a final concentration of 1 µM and plates incubated for 2 h at 37 °C. Cell monolayers were washed twice with cold PBS and the intracellular fluorescence of FAM-A β _(1–42) measured using an excitation wavelength of 494 nm and an emission wavelength of 521 nm using a Safire multiplate reader (Tecan, Germany). Subsequently, the protein content of each well was measured using the Bradford assay (Bradford, 1976). FAM-A β _(1–42) was quantified as pmol/µg protein and data are presented as percentage of non-treated control.

2.5. Measurement of the effect of ABCB1, ABCG2 and ABCC5 inhibition on efflux of intracellular A β ₍₁₋₄₂₎

Porcine brain endothelial cells in 96-well plates were incubated in assay medium containing 1 μ M FAM-A β ₍₁₋₄₂₎ for 2 h at 37 °C. Cells were then washed twice with PBS and incubated in assay medium alone or assay medium containing either 10 μ M verapamil hydrochloride, 0.5 μ M Ko143 or 25 μ M Mk571 for 15 min at 37 °C. Cell monolayers were washed three times with cold PBS and fluorescence intensity of FAM-A β ₍₁₋₄₂₎ measured spectrofluorometrically as described above and data are presented as percentage of non-treated control.

2.6. Measurement of cell viability

The effect of therapeutic drugs on PBEC viability was determined using the Neutral Red assay (Repetto et al., 2008). PBECs in 96-well plates were treated with either 5 μ M indomethacin, 0.65 μ M olanzapine, 10 μ M chlorpromazine hydrochloride, 1.5 μ M telmisartan, 5.5 μ M pantoprazole sodium hydrate, 7 μ M quinidine, 2.5 μ M sulfasalazine and 4 μ M nefazodone hydrochloride in treatment medium (DMEM low glucose without phenol red supplemented with 1 % (v/v) FBS and 2 mM L-glutamine) for 24 h at 37 °C in 5% CO₂. Following treatment, the medium was removed and replaced with assay medium containing Neutral Red, 40 μ g/ml. Cells were incubated for 2 h at 37 °C with 5% CO₂, washed twice with PBS, 150 μ l of destain solution (1 % (v/v) acetic acid in 50 % (v/v) ethanol) added to each well and plates agitated rapidly for 15 min on a rocking platform. Fluorescence intensity was measured using an excitation wavelength of 530 nm and an emission wavelength of 645 nm and data are presented as percentage of non-treated control.

2.7. Measurement of the effect of therapeutic drugs on ABCB1, ABCG2 and ABCC5 functional activity

Porcine brain endothelial cells in 96-well plates were treated with 5 μ M indomethacin, 0.65 μ M olanzapine, 10 μ M chlorpromazine hydrochloride, 1.5 μ M telmisartan, 5.5 μ M pantoprazole sodium hydrate, 7 μ M quinidine, 2.5 μ M sulfasalazine and 4 μ M nefazodone hydrochloride in 100 μ l treatment medium for 2 h at 37 °C in 5 % CO₂. Treatment medium,

50 µl, containing either calcein-AM, 0.5 µM (to measure ABCB1 activity), Hoechst 33342, 1 µM, (to measure ABCG2 activity) or CMFDA, 4 µM, (to measure ABCC5 activity) was added and following a 30 min incubation at 37 °C PBECs were washed twice with cold PBS and the intracellular fluorescence of calcein (excitation 484 nm; emission 530 nm), Hoechst 33342 (excitation 370 nm; emission 450 nm) and GS-MF (excitation 492 nm; emission 516 nm) measured. Cell monolayers were then washed twice with cold PBS and the protein content measured using the Bradford assay (Bradford, 1976). Relative fluorescence units (RFU) recorded in each individual well were normalised for protein content and functional activity is represented as % RFU/µg protein compared to non-treated control.

2.8. Measurement of the effect of therapeutic drugs on intracellular accumulation of Aβ₍₁₋₄₂₎

Porcine brain endothelial cells were treated with treatment medium containing 5 µM indomethacin, 0.65 µM olanzapine, 1 µM chlorpromazine hydrochloride, 1.5 µM telmisartan, 5.5 µM pantoprazole sodium hydrate, 7 µM quinidine, 2.5 µM sulfasalazine and 4 µM nefazodone hydrochloride for 2 h at 37 °C in 5 % CO₂. Cell monolayers were washed twice with cold PBS and the intracellular fluorescence of FAM-Aβ₍₁₋₄₂₎ measured as described above.

2.9. Determination of the roles of ABC transporter proteins in the transendothelial transport of Aβ₍₁₋₄₂₎

Porcine brain endothelial cells were maintained on polycarbonate Transwell® inserts (surface area 1.2 cm²; pore size 0.4 mm) according to Cantrill et al. and Skinner et al. (Cantrill et al., 2012; Skinner et al., 2009). Cells were subcultured into 12-well Transwell® inserts, pre-coated with 100 µg/ml type I rat tail collagen and 7.5 µg/ml human fibronectin, at a density of 125,000 cells/insert. Inserts were placed into 12-well plates containing CTX-TNA2 astrocytes at 80,000 cells/well and incubated for three days at 37 °C in 5 % CO₂. Control, blank, inserts contained no PBEC monolayers. Cells were maintained in growth medium:ACM, (1:1), 500 µl in the apical compartment and 1,500 µl in the basal compartment. After 3 days, PBECs were transferred to new 12-well plates and maintained in growth medium:ACM, (1:1) for 48 h. Subsequently, the medium was replaced with DMEM

containing 1 % penicillin/streptomycin, 2 mM L-glutamine, 125 μ M heparin, 312.5 μ M 8-4-chlorophenylthio-cAMP, 17.5 μ M RO-20-1724 and 55 nM hydrocortisone and cells were maintained at 37 °C in 5 % CO₂ for 24 h.

Monolayer integrity, as determined by transendothelial electrical resistance (TEER), was measured daily using an Evom VoltOhmMeter (Word Precision Instruments, Inc., Sarasota, FL, USA). The restrictive nature of cell monolayers was assessed by measuring the apparent permeability (P_{app}) of Lucifer Yellow, as described by Artursson et al. (Artursson, 1990).

Cell monolayers were pre-treated with verapamil hydrochloride (10 μ M), Ko143 (0.5 μ M), Mk571 (25 μ M) and telmisartan (1.5 μ M) in assay medium for 1 h at 37 °C. The medium was then replaced with Hank's balanced salt solution (HBSS) supplemented with 5.5 mM glucose and appropriate transporter inhibitor. To measure apical to basal transport of amyloid- β peptide, FAM-A $\beta_{(1-42)}$ at 1 μ M was included in the HBSS buffer the donor apical compartment of the Transwell[®] insert. To measure basal to apical transport, FAM-A $\beta_{(1-42)}$ was included in the donor basal compartment. Following a 2 h incubation at 37 °C with 5 % CO₂ FAM-A $\beta_{(1-42)}$ samples were removed from the donor and receiver compartments and Lucifer Yellow fluorescence measured with an excitation and emission wavelengths of 370 nm and 450 nm respectively.

2.10. Statistical analysis

All experiments were carried out at least four times, unless stated. Data are expressed as mean $\pm$ standard deviation and SPSS v20 (IBM) was used to carry out unpaired t-tests. A P-value of less than 0.05 is considered significant.

3. Results

3.1 ABCB1, ABCG2 and ABCC5 reduce intracellular accumulation of A $\beta_{(1-42)}$ in blood-brain barrier endothelial cells

ABCB1, ABCG2 and ABCC5 functional activities have been demonstrated previously in porcine brain endothelial cells utilized in the present study (Shubbar and Penny 2018).

Studies were subsequently carried out to investigate the effect of ABC efflux transporters on intracellular accumulation of FAM-A $\beta_{(1-42)}$ in PBECs.

Pharmacological inhibition of ABCB1 resulted in a significant increase in intracellular accumulation of FAM-A $\beta_{(1-42)}$, with similar findings observed with inhibition of ABCC5 and ABCG2 (Fig. 1). Cellular uptake of amyloid- β peptide into blood-brain barrier endothelial cells is reported to be mediated by the receptor for advanced glycation end products (RAGE) and clearance by the low density lipoprotein-related receptor 1 (LRP1) (Shibata et al. 2000a; Demattos et al. 2002; Deane et al. 2003). To clarify the involvement of facilitated uptake of FAM-A $\beta_{(1-42)}$ in PBECs, FAM-A $\beta_{(1-42)}$ accumulation was also studied at 4 °C. There was a significant reduction in FAM-A $\beta_{(1-42)}$ accumulation at 4 °C compared to the level of accumulation at 37 °C (Fig. 1).

Inset Fig 1 here

3.2. ABCB1, ABCG2 and ABCC5 mediate efflux of intracellular A $\beta_{(1-42)}$ from blood-brain barrier endothelial cells

Amyloid $\beta_{(1-42)}$ is less soluble than the more common amyloid- $\beta_{(1-40)}$ peptide and the form primarily associated with plaques within the brain. ABCB1 has been reported to efflux A $\beta_{(1-42)}$ (Lam et al., 2001) whilst ABCG2 has been shown to efflux the more soluble A $\beta_{(1-40)}$ peptide (Do et al., 2012). However, to date, no studies report the ability of ABCG2 and ABCC5 to transport the A $\beta_{(1-42)}$ peptide. Therefore, we investigated the ability of ABCG2 and ABCC5 (and ABCB1) to efflux intracellular A $\beta_{(1-42)}$ out of PBECs.

Treatment of PBECs, preloaded with FAM-A $\beta_{(1-42)}$, with the ABCG2 inhibitor Ko143 resulted in significantly higher levels of FAM-A $\beta_{(1-42)}$ retained in cells compared to levels in non-treated control cells (Fig. 2). Similarly, inhibition of the ABCC5 and ABCB1 efflux transporter resulted in significantly higher levels of FAM-A $\beta_{(1-42)}$ retained in cells compared to control cells (Fig. 2).

The ABCB1, ABCC5 and ABCG2 are primary active efflux transporters that utilise ATP hydrolysis to energise substrate transport. To confirm efflux of FAM-A β ₍₁₋₄₂₎ from PBECs was energy dependent, efflux of intracellular FAM-A β ₍₁₋₄₂₎ was analysed at 4 °C. Intracellular levels of FAM-A β ₍₁₋₄₂₎ were significantly higher in cells incubated at 4 °C, compared to control cells incubated at 37 °C (Fig. 2).

Inset Fig 2 here

3.3. Effect of therapeutic drugs on the viability of porcine brain endothelial cells

Our studies aimed to investigate the effects of plasma concentrations of therapeutic drugs on ABC transporter activity. Therefore, initial studies were carried out to establish the effects of the therapeutic drugs on the viability of PBECs using the Neutral Red assay. Exposure of PBECs to therapeutic drugs over a 24 h period had no significant effect on cell viability (Fig. 3).

Inset Fig 3 here

3.4. Inhibition of ABCB1, ABCG2 and ABCC5 functional activity by therapeutic drugs

The effect of selected therapeutic drugs on ABCB1, ABCG2 and ABCC5 transporter activity was assessed by measuring intracellular accumulation of fluorescent probe substrates.

Exposure of PBECs to indomethacin, olanzapine, chlorpromazine, telmisartan, quinidine and nefazodone resulted in a significant increase in intracellular calcein accumulation, indicative of significant inhibition of ABCB1 (Fig.4 A). However, pantoprazole and sulfasalazine had no significant effect on ABCB1 activity when compared to non-treated control cells (Fig.4 A). All drugs employed in this study significantly inhibited ABCG2

activity (Fig. 4 B) whilst all drugs, with the exception of quinidine, significantly inhibited ABCC5 activity (Fig. 4 C).

Inset Fig 4 here

3.5. Inhibition of ABC transporters by therapeutic drugs increases intracellular accumulation of A β ₍₁₋₄₂₎

Studies were then conducted to determine the effects of therapeutic drug-mediated inhibition of ABC transporters on intracellular accumulation of FAM-A β ₍₁₋₄₂₎ in PBECs. Treatment of cells with all drugs studied significantly increased intracellular accumulation of FAM-A β ₍₁₋₄₂₎ (Fig. 5), consistent with the drug-dependent significant inhibition of ABC-transporter mediated efflux of fluorescent probe substrates (Fig. 4).

Inset Fig 5 here

3.6. Inhibition of ABC transporters modulates transendothelial transport of A β ₍₁₋₄₂₎

The role of ABCB1, ABCG2 and ABCC5 in the bidirectional transendothelial transport of FAM-A β ₍₁₋₄₂₎ across BBB endothelial cells was investigated using an *in vitro* model of the BBB composed of PBEC monolayers grown on Transwell® inserts. The TEERs achieved in this study increased over time and were consistently high, reaching $1200 \pm 100 \Omega \cdot \text{cm}^2$ at the point at which cell monolayers were used (day 6 of culture). Further studies investigated the integrity of the *in vitro* BBB by measuring the P_{app} of the paracellular probe Lucifer Yellow. The P_{app} of Lucifer Yellow across PBEC monolayers, $7.7 \times 10^{-7} \pm 0.2 \times 10^{-7} \text{ cm/sec}$, was 18-fold less than the P_{app} across control cell-free Transwell® inserts, $1.4 \times 10^{-5} \pm 0.1 \times 10^{-5} \text{ cm/sec}$.

The angiotensin II receptor antagonist telmisartan significantly inhibits ABCB1-, ABCG2- and ABCC5-mediated transport of fluorescent probe substrates (Fig. 4 A, B, C). This therapeutic drug also inhibits ABC transporter-mediated efflux of FAM-A $\beta_{(1-42)}$, resulting in increased intracellular accumulation of the peptide (Fig. 5). Therefore, the effect of the widely-used ABCB1 inhibitor verapamil, the ABCG2 inhibitor Ko143 and the ABCC5 inhibitor Mk571, and the effect of telmisartan, which inhibits all three transporters, on transcellular transport of FAM-A $\beta_{(1-42)}$ was investigated in PBEC monolayers grown on Transwell® inserts.

There was a significant increase in the transendothelial transport of FAM-A $\beta_{(1-42)}$ from the apical to basal compartment (analogous to transport from blood to brain) when PBEC monolayers were treated with verapamil, Ko143, Mk571 and telmisartan (Fig. 6 A).

Conversely, there was a significant decrease in transport of FAM-A $\beta_{(1-42)}$ in the basal to apical direction (analogous to clearance of A $\beta_{(1-42)}$ from brain to blood) following treatment of PBECs with verapamil, Ko143, Mk571 and telmisartan (Fig. 6 B).

Inset Fig 6 here

4. Discussion

Aggregation of A $\beta_{(1-42)}$ monomers into fibrils and deposition into plaques in brain is a hallmark of Alzheimer's disease. Therefore it is important to obtain a better understanding of the mechanism(s) by which A $\beta_{(1-42)}$ is cleared, across the BBB, from brain to systemic circulation. In the current study we employed a well characterised *in vitro* model of the BBB (Cantrill et al., 2012; Shubbar and Penny, 2018; Skinner et al., 2009) to investigate the roles of ABCB1, ABCG2 and ABCC5 in A $\beta_{(1-42)}$ clearance.

Our previous studies employing this PBEC-based BBB model demonstrated expression and function of ABCB1, ABCC5 and ABCG2 (Cantrill et al., 2012; Shubbar and Penny, 2018).

The current PBEC model demonstrated extremely high TEERs and low Lucifer yellow P_{app} , indicative of highly restrictive monolayers, consistent with previous scientific reports (Cantrill et al., 2012; Lelu et al., 2017; Smith et al., 2007).

There is evidence, both from *in vitro* and *in vivo* studies, that ABCB1 is involved in $A\beta_{(1-42)}$ (Cirrito et al., 2005; Kuhnke et al., 2007; Lam et al., 2001) and $A\beta_{(1-40)}$ transport (Ito et al., 2006; Tai et al., 2009). To date, no studies yet report ABCB1-mediated $A\beta_{(1-42)}$ transport in a PBEC model of the BBB, although Tai et al 2009 report ABCB1-mediated transport of [^{125}I] $A\beta_{(1-40)}$ in hCMEC/D3 brain endothelial cells. In the current study inhibition of ABCB1 resulted in increased accumulation of $A\beta_{(1-42)}$ in PBECs and decreased efflux of intracellular $A\beta_{(1-42)}$, indicating ABCB1-mediated transport of $A\beta_{(1-42)}$, consistent with published observations (Tai et al., 2009).

ABCG2-mediated transport of the more soluble $A\beta_{(1-40)}$ peptide, but not the $A\beta_{(1-42)}$ peptide, has been reported in the literature (Do et al., 2012). In the current study, similar findings were observed with ABCG2 as with ABCB1, in that inhibition of ABCG2 resulted in increased $A\beta_{(1-42)}$ accumulation and decreased efflux of intracellular $A\beta_{(1-42)}$, indicating $A\beta_{(1-42)}$ is transported by ABCG2. This is consistent with studies reporting inhibition of ABCG2 decreased efflux of [^{125}I]-labelled- $A\beta_{(1-40)}$, compared with control cells (Do et al., 2012; Tai et al., 2009), and with studies demonstrating higher levels of Cy5.5-labeled $A\beta_{(1-40)}$ in brains of Abcg2-null mice than in wild-type controls (Xiong et al., 2009).

In addition to ABCB1 and ABCG2, ABCC5 is expressed in PBECs (Kubo et al., 2015; Shubbar and Penny, 2018). In the current study, consistent with the findings of ABCB1- and ABCG2-mediated transport of $A\beta_{(1-42)}$, inhibition of ABCC5 also resulted in increased $A\beta_{(1-42)}$ accumulation and decreased efflux of intracellular $A\beta_{(1-42)}$, demonstrating $A\beta_{(1-42)}$ is an ABCC5 substrate. This is the first study to report ABCC5-mediated transport of $A\beta_{(1-42)}$, however reports demonstrate increased cerebral $A\beta_{(1-42)}$ levels in abcc1 deficient mice in an animal model of Alzheimer's disease (Kuhnke et al., 2007). The findings of the present study are also consistent with reports that, in mouse primary brain endothelial cells, and in microglia, $A\beta_{(1-42)}$ and $A\beta_{(1-40)}$ are substrates of the abca7 lipid transporter (Fu et al., 2016; Lamartinière et al., 2018).

Therapeutic drugs belonging to numerous pharmacological categories inhibit ABCB1, ABCG2 and ABCC5. Use of therapeutic drugs, particularly chronic usage, therefore has the potential to modulate BBB ABC transporter function and permeation of ABC transporter substrates across the BBB.

In this study we investigated the effects of widely prescribed therapeutic drugs reported to inhibit ABC transporters at plasma concentrations, namely indomethacin (Hvidberg et al., 1972), olanzapine (Bergemann et al., 2004), chlorpromazine (Wang et al., 2006), telmisartan (Stangier et al., 2000), pantoprazole (Suzuki et al., 2009), quinidine (Karbwang et al., 1992), sulfasalazine (Adkison et al., 2010) and nefazodone (Störmer et al., 2001), on ABCB1-, ABCG2- and ABCC5-mediated $A\beta_{(1-42)}$ transport. At the concentrations employed these drugs had no significant effect on PBEC viability. Verapamil (Garrigos et al., 1997), olanzapine (Bergemann et al., 2004), chlorpromazine (Wang et al., 2006) and sulfasalazine (Shukla et al., 2009) have been reported to act as competitive inhibitors, and given the polyspecificity of ABC transporters, it is likely that the remaining drugs act by a similar mechanism.

Indomethacin significantly inhibited ABCB1 and ABCG2 activities, as reported previously (Nozaki et al., 2007; Yu et al., 2009). This is the first study to show indomethacin significantly inhibits ABCC5 activity, although this finding is supported somewhat by a study which demonstrates inhibition of ABCC1 and ABCC4 by indomethacin (Reid et al., 2003).

The antipsychotic drugs olanzapine and chlorpromazine significantly inhibited ABCB1 and ABCG2 functional activity in PBECs. This finding is in agreement with the study of Wang et al reporting both drugs inhibit ABCB1 (Wang et al., 2006), however a later study reported chlorpromazine, but not olanzapine, inhibited ABCG2 (Wang et al., 2008). As far as we are aware, this is the first study to report olanzapine and chlorpromazine inhibit ABCC5.

The proton pump inhibitor pantoprazole had no significant effect on ABCB1 activity at 5.5 μ M. This finding is in contrast with studies in the L-MDR1 cell line which report pantoprazole inhibited ABCB1, but at a concentration of 17 μ M, which is higher than reported plasma levels (3 to 9 μ M) (Pauli-Magnus et al., 2001). However, pantoprazole significantly inhibited ABCG2 activity in PBECs, consistent with the studies of Suzuki et al. (Suzuki et al., 2009). Whilst pantoprazole inhibits ABCC5 in PBECs in the current study, no studies yet report the effect of pantoprazole on ABCC5 activity. In the current study, pantoprazole significantly increases intracellular accumulation of GS-MK, an ABCC5 substrate. However, pantoprazole had no significant effect on intracellular accumulation of calcein, an ABCB1 substrate, indicating calcein is not an ABCC5 substrate.

Treatment of PBECs with quinidine significantly inhibited ABCB1, consistent with numerous studies (Fromm et al., 1999; Glavinas et al., 2011). In the current study, quinidine

also significantly inhibited ABCG2, but not ABCC5. This is the first finding that quinidine inhibits ABCG2, whilst no studies have reported the effect of quinidine on ABCC5.

In the current study, sulfasalazine exhibited no significant inhibitory effect on ABCB1 activity. As far as we are aware, no other studies have investigated the effect of sulfasalazine on ABCB1. However, in PBECs, ABCG2 and ABCC5 were significantly inhibited by sulfasalazine. This is the first study to demonstrate these findings, although Dahan and Amidon reported sulfasalazine is a substrate of ABCG2 and ABCC1 (Dahan and Amidon, 2009).

Nefazodone significantly inhibited ABCB1 in PBECs, consistent with the literature (Störmer et al., 2001). Nefazodone also significantly inhibited ABCG2 and ABCC5 activities in PBECs and, to date, no studies yet report these effects.

The current study found telmisartan significantly inhibited ABCB1 and ABCG2 activities, which reflect the findings of Weiss et al. (Weiss et al., 2010). In PBECs, telmisartan also significantly inhibited ABCC5 activity. Whilst there are no published reports of the effect of telmisartan on ABCC5 activity, Weiss et al. 2010 reported telmisartan inhibited ABCC2 activity (Weiss et al., 2010).

Telmisartan not only inhibited the functional activities of ABCB1, ABCG2 and ABCC5 to a greater extent than the other therapeutic drugs employed, but treatment with telmisartan caused the greatest increase in intracellular FAM-A $\beta_{(1-42)}$ accumulation. Therefore, we subsequently investigated the ability of telmisartan to influence ABCB1-, ABCG2- and ABCC5-mediated transcellular transport FAM-A $\beta_{(1-42)}$ in PBEC monolayers.

Telmisartan treatment significantly increased FAM-A $\beta_{(1-42)}$ transport from the apical (equivalent to blood) to basal (equivalent to brain) compartment and significantly reduced FAM-A $\beta_{(1-42)}$ transport from the basal to apical compartment. In control studies, verapamil (ABCB1 inhibitor), Ko143 (ABCG2 inhibitor) and Mk571 (ABCC5 inhibitor) had similar effects on transendothelial transport of FAM-A $\beta_{(1-42)}$ in PBEC monolayers, increasing apical to basal permeability and decreasing basal to apical permeability. The effects of telmisartan on FAM-A $\beta_{(1-42)}$ transport are consistent with telmisartan-dependent inhibition of ABCB1-, ABCG2- and ABCC5-mediated FAM-A $\beta_{(1-42)}$ transport. These results are supported by the study of Kuhnke et al. which report transcellular transport of FTC-conjugated A $\beta_{(1-42)}$ from the basal to apical compartment was reduced by treatment with the ABCB1 inhibitor cyclosporine in LLC-MDR1 cells (Kuhnke et al., 2007). The findings of the current study are partially supported by the study of Tai et al. 2009 which reports, in the hCMEC/D3 human

brain endothelial cell line, transcellular transport of [125 I]A $\beta_{(1-40)}$ from the apical to basal compartment increased following treatment with ABCB1 and ABCG2 inhibitors (tariquidar, vinblastine, FTC), whilst transport in the basal to apical direction was unchanged following inhibitor treatment (Tai et al., 2009). It is worth noting the latter study employed A $\beta_{(1-40)}$ while the current study used A $\beta_{(1-42)}$.

The current study demonstrates widely prescribed therapeutic drugs significantly inhibit the major efflux transport systems expressed in BBB endothelial cells. The A $\beta_{(1-42)}$ transport characteristics of the BBB are governed by RAGE-mediated uptake of plasma A $\beta_{(1-42)}$ across the BBB into the brain, in conjunction with LRP1 and ABC transporter-mediated clearance of A $\beta_{(1-42)}$ from brain to blood. The potential consequences of BBB ABC efflux transporter-based drug/amyloid- β peptide interactions via competitive inhibition, are reduced clearance of the peptide from the brain which may potentially influence brain levels of A $\beta_{(1-42)}$.

5. Conclusion

The current study has demonstrated the effects of individual therapeutic drugs on BBB endothelial cell ABC transporter-mediated transport of A $\beta_{(1-42)}$. However, many individuals receive treatment regimens containing multiple therapeutic drugs, in which one or more of the drugs may inhibit BBB endothelial cell ABC transporter activities. Therefore, combined drug therapy also has the potential to influence ABC transporter-mediated A $\beta_{(1-42)}$ clearance across the BBB. Interestingly, studies suggest that raised A $\beta_{(1-42)}$ levels in the brain are a consequence of defective clearance across the BBB (Zlokovic, 2005) and that there is an inverse relationship between ABCB1 expression and the deposition of amyloid- β peptide in the brain (Cirrito et al., 2005; Vogelgesang et al., 2002). Furthermore, processing of APP by β - and γ -secretases, and degradation of A $\beta_{(1-42)}$ by insulin degrading enzyme and neprilysin influence brain A $\beta_{(1-42)}$ levels. Therefore, potential interactions between therapeutic drugs and these enzymes could also influence the dynamics of A $\beta_{(1-42)}$ handling at the BBB. In summary, the current study is the first to demonstrate ABCG5-mediated transport of A $\beta_{(1-42)}$ in endothelial cells and adds to our understanding of how widely prescribed therapeutic drugs could modulate amyloid- β peptide clearance at the blood-brain barrier.

Conflict of interest

The author(s) declare(s) that they have no conflicts of interest to disclose.

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Figure legends

Fig. 1. Pharmacological inhibition of ABCB1, ABCG2 and ABCC5 increases cellular accumulation of A β ₍₁₋₄₂₎ in PBECs. Cells were pre-treated for 30 min at 37 °C with 10 μ M verapamil hydrochloride, 0.5 μ M Ko143 or 25 μ M Mk571, or pre-incubated at 4 °C for 30 min. Cells were then incubated with 1 μ M FAM-A β ₍₁₋₄₂₎ at 37 °C or 4 °C for 2 h, washed and intracellular FAM-A β ₍₁₋₄₂₎ levels measured. Data were analysed using unpaired Student's t-test and are presented as the mean $\pm$ S.D. (n=4), *P<0.05.

Fig. 2. Inhibition of ABCB1, ABCG2 and ABCC5 reduces efflux of intracellular A β ₍₁₋₄₂₎ in PBECs. Cells were preloaded with FAM-A β ₍₁₋₄₂₎ for 2 h then washed and treated for 15 min at 37 °C with 10 μ M verapamil hydrochloride, 0.5 μ M Ko143 or 25 μ M Mk571, or pre-incubated 4 °C for 15 min. Cells were washed and intracellular FAM-A β ₍₁₋₄₂₎ levels measured. Data were analysed using unpaired Student's t-test and are presented as the mean $\pm$ S.D. (n=4), *P<0.05, ** P $\leq$ 0.001.

Fig. 3. Therapeutic drugs do not affect the viability of porcine brain endothelial cells. PBECs were incubated for 24 h at 37 °C with 5 μ M indomethacin, 0.65 μ M olanzapine, 1 μ M chlorpromazine

hydrochloride, 1.5 μ M telmisartan, 5.5 μ M pantoprazole sodium hydrate, 7 μ M quinidine, 2.5 μ M sulfasalazine or 4 μ M nefazodone hydrochloride and cell viability measured using the Neutral Red assay. Data were analysed using unpaired Student's t-test and are presented as the mean $\pm$ S.D. (n=4).

Fig. 4. Inhibition of ABCB1, ABCG2 and ABCC5 functional activity by therapeutic drugs. Intracellular accumulation of calcein, ABCB1 substrate (A), Hoechst 33342, ABCG2 substrate (B) and GS-MF, ABCC5 substrate (C) in control cells and cells pre-treated for 2 h at 37°C with 5 μ M indomethacin, 0.65 μ M olanzapine, 1 μ M chlorpromazine hydrochloride, 1.5 μ M telmisartan, 5.5 μ M pantoprazole sodium hydrate, 2.5 μ M sulfasalazine or 4 μ M nefazodone hydrochloride. Data were analysed using unpaired Student's t-test and are presented as the mean $\pm$ S.D. (n=4). *P<0.05, ** P $\leq$ 0.001.

Fig. 5. Inhibition of ABCB1, ABCG2 and ABCC5 functional activity by therapeutic drugs increases intracellular levels of fluorescent A $\beta_{(1-42)}$. Intracellular accumulation of FAM-A $\beta_{(1-42)}$ was measured in control cells and cells pre-treated for 2 h at 37°C with 5 μ M indomethacin, 0.65 μ M olanzapine, 1 μ M chlorpromazine hydrochloride, 1.5 μ M telmisartan, 5.5 μ M pantoprazole sodium hydrate, 2.5 μ M sulfasalazine or 4 μ M nefazodone hydrochloride. Data were analysed using unpaired Student's t-test and are presented as the mean $\pm$ S.D. (n=4). *P<0.05, ** P $\leq$ 0.001.

Fig. 6. Inhibition of ABCB1, ABCG2 and ABCC5 functional activity modulates transendothelial transport of fluorescent A $\beta_{(1-42)}$. PBEC monolayers were pre-treated for 2 h at 37°C with 10 μ M verapamil hydrochloride, 0.5 μ M Ko143, 25 μ M Mk571 or 1.5 μ M telmisartan and fluorescent A $\beta_{(1-42)}$ was quantified in (A) the receiver basal compartment and (B) receiver apical compartment. Data were analysed using unpaired Student's t-test and are presented as the mean $\pm$ S.D. (n=4). *P<0.05.

Figure 1

Figure 2

Figure 3

Figure 4

(A)

(B)

(C)

Figure 5

Figure 6

(A)

(B)