

Phytoestrogens Modulate Breast Cancer Resistance Protein Expression and Function at the Blood-Cerebrospinal Fluid Barrier

Manjit Kaur, and Raj K. S. Badhan

Aston University, Aston Research Centre for Healthy Ageing, School of Life and Health Sciences, Birmingham, UK.

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ABSTRACT - PURPOSE: Breast cancer resistance protein (BCRP/ABCG2) is a drug efflux transporter expressed at the blood cerebrospinal fluid barrier (BCSFB), and influences distribution of drugs into the central nervous systems (CNS). Current inhibitors have failed clinically due to neurotoxicity. Novel approaches are needed to identify new modulators to enhance CNS delivery. This study examines 18 compounds (mainly phytoestrogens) as modulators of the expression/function of BCRP in an *in vitro* rat choroid plexus BCSFB model. **METHODS:** Modulators were initially subject to cytotoxicity (MTT) assessment to determine optimal non-toxic concentrations. Reverse-transcriptase PCR and confocal microscopy were used to identify the presence of BCRP in Z310 cells. Thereafter modulation of the intracellular accumulation of the fluorescent BCRP probe substrate Hoechst 33342 (H33342), changes in protein expression of BCRP (western blotting) and the functional activity of BCRP (membrane insert model) were assessed under modulator exposure. **RESULTS:** A 24 hour cytotoxicity assay (0.001 μ M-1000 μ M) demonstrated the majority of modulators possessed a cellular viability $IC_{50} > 148 \mu$ M. Intracellular accumulation of H33342 was significantly increased in the presence of the known BCRP inhibitor Ko143 and, following a 24 hour pre-incubation, all modulators demonstrated statistically significant increases in H33342 accumulation ($P < 0.001$), when compared to control and Ko143. After a 24 hour pre-incubation with modulators alone, a 0.16-2.5 -fold change in BCRP expression was observed for test compounds. The functional consequences of this were confirmed in a permeable insert model of the BCSFB which demonstrated that 17- β -estradiol, naringin and silymarin (down-regulators) and baicalin (up-regulator) can modulate BCRP-mediated transport function at the BCSFB. **CONCLUSION:** We have successfully confirmed the gene and protein expression of BCRP in Z310 cells and demonstrated the potential for phytoestrogen modulators to influence the functionality of BCRP at the BCSFB and thereby potentially allowing manipulation of CNS drug disposition.

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INTRODUCTION

The blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) are the two primary barriers across which endogenous nutrients and hormones and exogenous chemicals must transverse in order to gain access to the central nervous system (CNS). The BBB and BCSFB are often termed the gatekeepers to the CNS and play an obvious role in controlling the neuronal microenvironment. In recent years the importance of the BCSFB as a site for drug metabolism and in governing the composition of cerebrospinal fluid (CSF) has gained more attention due to its significance in normal physiology and disease pathology (1). The BCSFB is formed from the epithelial cells of the choroid plexus (CP) facing the CSF, which are sealed by the tight junctions (2).

The choroid plexuses are located within the brain ventricles: one in each lateral ventricle, the third ventricle and fourth ventricle. The CP epithelium is a dynamic tissue which has a greater perfusion rate compared to the cerebral blood flow and weighing only 2 g (3-6). It secretes CSF at a rate of 25 mL/hour in humans (7) which helps to maintain brain extracellular fluid (ECF) and CSF homeostasis (2, 8) as well as providing mechanical support to the brain, removal of metabolic products (9) and as a route for the distribution of nutrients, neurotransmitters and hormones across the CNS (10, 11) (12, 13).

Corresponding Author: Raj K. S. Badhan; Aston University, Aston Research Centre for Healthy Ageing, School of Life and Health Sciences, Birmingham, UK; Email: r.k.s.badhan@aston.ac.uk

A key component of the protective mechanism of the BCSFB is the expression of a variety of ATP-Binding Cassette (ABC) transporter proteins (14-18), which play a significant role in drug transport across the BCSFB. A key member of the ABC family of transporters is the breast cancer resistance protein (BCRP/ABCP/MXR). BCRP is the second member of the G-subfamily of transporter proteins, and derives its name from the result of its almost simultaneous identification in the drug-resistant breast cancer cell line MCF-7/AdrVp (19), human placenta (ABCP) (20) and in a mitoxantrone-resistant cell line (MXR) (21). BCRP mRNA encodes for a 655-amino acid, 72 kDa protein which forms a single nucleotide binding domain (NBD) and six transmembrane domains (TMD), hence is often termed a half-transporter. The endogenous function of BCRP is varied, and can be found expressed in stem cells (22-24), the apical membrane of enterocytes, the liver canaliculi, the proximal tubules of the kidneys, the BBB and BCSFB, the blood-testes barrier and the blood-placental barrier and blood-retinal barriers (25-29). The localisation at key secretory organs results in BCRP imparting a 'protective' pharmacokinetic function for human physiology and limits access of drugs to various tissues.

Much work has focused on the role of BCRP in governing drug penetration across the BBB and rodent knock-out systems have implicated BCRP playing a more complicated role in cooperation with other transporter proteins to limit BBB penetration of drugs, e.g. topotecan (30). Furthermore position-emission tomography studies with tariquidar (31), gefitinib (32, 33), sorafenib (34), CEP-32496 (35) have confirmed the importance of BCRP at the BBB. At the BCSFB, only a handful of reports have

demonstrated expression in the choroid plexus of mice (36), rats (37) and humans (38). The localisation of BCRP at the CSF side suggests that it would facilitate but not restrict the transport to the CSF (37, 38).

In vitro and *in vivo* inhibition of BCRP is of great interest for improving the pharmacokinetics of therapeutics. The first series of reported inhibitors included cyclosporine A, pantoprazole and verapamil but demonstrated limited clinical efficacy due to significant toxicity and interactions with CYP3A (39, 40). Second generation inhibitors included topotecan, irinotecan, gefitinib and SN-38 but again demonstrated limited clinical application due to a range of cytotoxicity concerns and high potential for drug-drug interactions (39, 41). Third generation inhibitors represent molecules that have been recently developed and include the fungal toxin derived fumitremorgin C (FTC) (42, 43), GF120918, Ko143 (43-45) but again widespread clinical use is limited due to an uncertain safety profile (43). Therefore, there is an urgent need to identify novel inhibitors of BCRP with a tolerable safety profile.

To this end, a novel class of compounds that have gained interest as potential candidates are phytoestrogens, of which flavonoids are the most chemical group. Flavonoids are polyphenolic compounds found in fruit, vegetables and many herbal products. In the U.S. the average daily intake of flavonoids has been estimated to be at 1 g (46). In epidemiological and animal studies, flavonoids have been suggested to possess anti-inflammatory (47, 48), anti-oxidative, antithrombogenic (49, 50), anti-tumour (51) and anti-viral (52, 53) effects.

Numerous studies have demonstrated a direct interaction of flavonoids with BCRP, resulting in the modulation of its activity (Table 1).

Table 1. Various cell lines studied to investigate at the interaction of flavonoids with BCRP.

Cell line	Flavonoid	Reference
MCF-7	Apigenin, genistein, naringenin, quercetin, biochanin A, hesperitin, silymarin and fistein	(54-58)
NCI-H60	Apigenin, naringenin, quercetin, biochanin A, hesperitin, silymarin and fistein	(56, 57)
BT-474	Apigenin and genistein	(54, 58)
T47-D	Apigenin and genistein	(54)
Caco-2	Apigenin, genistein, naringenin, quercetin, biochanin A, hesperitin, silymarin and fistein	(59-62)
K562	Rutin, hesperidin and silymarin	(55)

The impact of flavonoids/modulators on CNS drug disposition is highlighted in Figure 1. Under 'normal' physiological conditions BCRP would play a role in the efflux of substrates from the brain endothelial microvasculature back into the systemic circulation and thereby limiting brain ISF exposure. At the BCSFB BCRP would potentially enhance the disposition of drugs into the CSF, with potential CSF-ISF mixing before exiting through CSF drainage routes. This overall balance of drug disposition would therefore favour the CSF. Where flavonoids/modulators are thought to either directly inhibit or down-regulate BCRP protein expression, the effects would be reversed with enhanced BBB penetration and brain ISF disposition coupled with diminished delivery across the BCSFB and into the CSF (due to the lack of CSF penetration enhancement provided by BCRP under normal conditions). Under circumstances where BCRP is induced, the balance of disposition would favour CSF delivery provided by the enhanced additional transport in the blood-CSF as a result of increased abundance of BCRP at the BCSFB.

In the current study, we identified and investigated the genomic and protein expression of BCRP in immortalised rat choroid plexus cells and the potential for modulation of BCRP-mediated transport at the BCSFB using reported phytoestrogenic and non-phytoestrogenic modulators of BCRP expression and function to ascertain whether such modulators can be employed as agents to alter the disposition of transporter substrates at the BCSFB.

MATERIALS AND METHODS

Materials

Dulbecco's modified essential media with glucose (DMEM), fetal bovine serum (FBS), amphotericin B, penicillin/streptomycin and gentamycin were obtained from Biosera (Sussex, UK); Resveratrol and Ko143 from Santa Cruz Biotechnology (Texas, USA); Curcumin from Cayman Chemical (Cambridge, UK); Rat tail I collagen solution from First Link (Birmingham, UK) and all other chemicals were sourced from Sigma (Dorset, UK). GenElute Total RNA extraction kits were purchased from Sigma (Dorset, UK); My Taq™ one-step RT-PCR kit and Easy Ladder I obtained from Bioline (London, UK). All PCR primers were designed synthesised by IDTDna (Leuven, Belgium); Optiblot SDS-page gel and western blot reagents obtained from Abcam (Cambridge, UK); ABCG2 (M-70), beta-actin (C4), broad

range markers, goat anti-rabbit IgG-FITC and protease inhibitor cocktail were obtained from Santa Cruz Biotechnology (Texas, USA). Stock solutions of all test compounds were prepared in dimethylsulfoxide (DMSO) and stored at -20°C until use.

Cell culture

The rat choroid plexus cell line Z310 was a kind gift from Dr. Wei Zheng (Purdue University, West Lafayette, USA). The cells were maintained in the medium as described by Zheng *et al* (63) with minor modifications. In brief, cells were grown in DMEM supplemented with 4.5 g/L glucose, 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2.5 mg/mL gentamycin, amphotericin B and epidermal growth factor (EGF) to a final concentration of 10 ng/mL in a humidified atmosphere of 5% CO₂ at 37°C. Cells were used at passages 224-260.

Reverse-transcriptase polymerase chain analysis

RNA was extracted according to manufacturer's instructions. In brief, cells were seeded at cell density of 5x10⁴ per well in a 6-well plate. Cells were lysed and transferred into a GenElute Total RNA mini prep kit filtration column and centrifuged at 12,000 x g for 2 minutes. The lysate was transferred into a fresh column and centrifuged. The column was washed twice with wash solution and 50 µL of the elution solution was added into the binding column and centrifuged. The concentration of total extracted RNA was quantified by a Nanodrop system (Thermo Scientific, Nanodrop 1000).

RNA amplification was conducted using the My Taq One-Step RT-PCR kit according to the manufacturer's instructions. The PCR primers used were as follows: TTR (accession number: NM_022712) forward primer 5'-ATTAGAGCTTGGTGCATGCCT, reverse primer 5'-GGCCACAACCTCACTGGACTT; β-actin (accession number: NM_031144) forward primer 5'-CCCGCGAGTACAACCTTCTT and reverse primer 5'-AACACAGCCTGGATGGCTAC; BCRP (accession number: NM_181381) forward primer 5' - TGTGAGCCCTACAACAACCC, reverse primer 5' - AAAAAGCCTCCACCGTCCTC. The thermal cycle was run as recommend by the manufacturer's kit and the amplified products were separated and visualised using a 1% agarose gel stained with ethidium bromide.

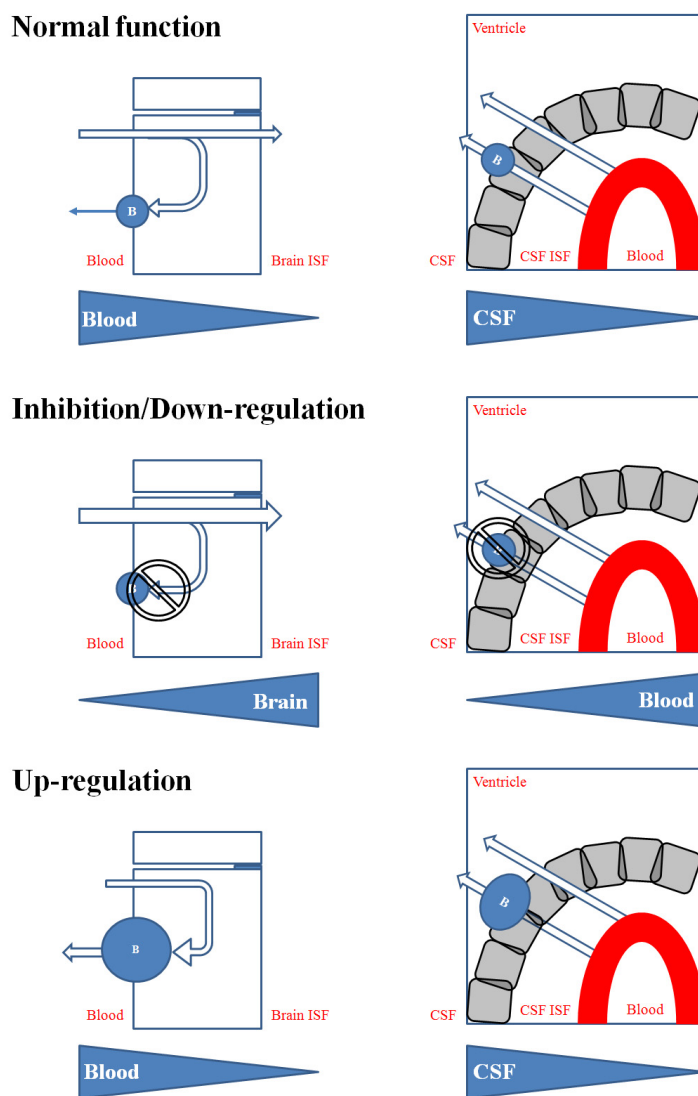


Figure 1. Balance of drug disposition at the BBB and BCSFB during normal physiological function of BCRP (top), when BCRP is inhibited or down-regulated (middle) and when BCRP is up-regulated (bottom). Arrows indicate the direction of flux; blue triangles indicate the overall balance of disposition; B: BCRP; CSF: cerebrospinal fluid; ISF: interstitial fluid.

Immunofluorescence

Approximately 15,000 cells were seeded onto glass cover slips for 48 hours and subsequently washed with phosphate buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT), washed a further three times with PBS and blocked with 5 % goat serum in Tris buffer saline tween-20 (TBST) for 30 minutes at RT. The blocking buffer was removed and incubated with primary antibody (ABCG2 M-70) for 2 hours at RT. Cells were washed again with the PBS and incubated with goat anti-rabbit IgG-FITC in the dark for 1.5 hours at RT and mounted with DAPI-containing mounting media.

Confocal Laser Scanning Microscopy

The cover slips were analysed using an upright confocal microscope (Leica SP5 TCS II MP) and visualised with a 40x oil immersion objective. All images were acquired using an argon laser at 494 nm to visualise FITC and a helium-neon laser to visualise DAPI at 461 nm.

Modulators

A total of 18 modulators (Table 2) consisting primarily of flavonoids, were dissolved in DMSO and stored at -20°C until required.

Table 2. Modulators of BCRP used in this study

Modulator Class	Name	Impact on BCRP
Diarylheptanoid	Curcumin	SD Rats (Inhibition) (64); FVB Mice (Inhibition: Increase in Cmax and AUC ₀₋₈) (65); Humans (Inhibition: Increase in Cmax and AUC ₀₋₂₄) (64); MCF-7 (Induction) (66)
Flavone	Apigenin	MCF-7 MX100 and NCI-H460 MX20 (Inhibition) and K562/BCRP (Inhibition) (67)
Flavone	Baicalin	Caco-2 (Substrate) (68)
Flavone	Chrysin	Caco-2 (Inducer-mRNA) (66)
Flavone	Flavone	Caco-2 (Inducer-mRNA) (66)
Flavone	α -naphthoflavone	Aryl-Hydrocarbon Receptor [AhR] antagonist (BCRP regulator) (69)
Flavone	6,2,4-Trimethoxy-flavone (TMF)	Aryl-Hydrocarbon Receptor [AhR] antagonist (BCRP regulator) (70)
Flavonol	Fisetin	MCF-7 MX100 and NCI-H460 MX20 (67)
Flavonol	Quercetin	Caco-2 (Inducer-mRNA) (66); HEK293/BCRP (71); MCF7/MX, K562/BCRP (Inhibition) (72)
Flavonol	Reservatrol	Aryl-Hydrocarbon Receptor [AhR] antagonist (BCRP regulator) (73); Caco-2 and MCF-7 (Inducer-Protein) (66).
Flavonol glycoside	Rutin	Membrane vesicles (Inhibition) (74)
Flavonolignan	Silymarin	MCF-7 MX100 and NCI-H460 MX20 (Inhibition) (67); Caco-2 (Inducer-Protein) (66)
Flavanone	Hesperidin	Unknown
Flavanone	Hesperetin	MCF-7 MX100 and NCI-H460 MX20 (Inhibition) (67)
Flavanone glycoside	Naringin	MCF-7 MX100 and NCI-H460 MX20 (Inhibition) (67)
O-methylated isoflavone	Biochanin A	MCF-7/MX100 (Inhibition) (67, 75)
Polycyclic aromatic hydrocarbon	Benzo(a)pyrene (BAP)	Aryl-Hydrocarbon Receptor [AhR] agonist (BCRP regulator) leading to BCRP modulation (76)
Oestrogens	17- β -Estradiol	Estrogen receptor agonist (BCRP regulator) and SD Rats (Inhibition) leading to pronounced BCRP downregulation (77)

Cytotoxicity assay

5x10⁴ cells per well were seeded into the 96-well plate and allowed to attach for 24 hours. The media was subsequently removed and cells washed with PBS, followed by the addition of 200 μ L of growth media containing modulators per well across a seven-fold log concentration range (0.001-1000 μ M) and incubated for 24 hours at 37°C in a 5% CO₂ air humidified environment. Thereafter 20 μ L of 5 mg/mL 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in PBS was added to each well and incubated at 37°C, 5% CO₂ in an air humidified environment for 4 hours. After incubation the medium was removed and 100 μ L of DMSO was added. Plates were left in dark for 5-15 minutes and the UV-absorbance of the formazan product was determined at 595 nm. Each concentration was assayed in eight wells and run in three independent experiments and results

expressed as percentage cytotoxicity relative to a non-modulator control (exposed to 0.5% DMSO only).

Hoechst 33342 intracellular accumulation assay

To assess the impact of modulators on the intracellular accumulation of a BCRP substrate, a fluorescent BCRP substrate, Hoechst 33342 (H33342), was utilised in a 96-well plate format accumulation assay. 2×10^4 cells were seeded into each well of 96-well plate and allowed to attach for 48 hours. The media was removed and cells were washed twice with 100 μ L warm Hanks balances salt solution (HBSS) and pre-incubated with an optimal concentration of modulator (as determined from the MTT assay) for 24 hours in cell culture media with a no-modulator control (0.5% DMSO). After 24 hours the cells were washed with ice cold HBSS and incubated with 25 μ M H33342 alone, for one hour in HBSS supplemented with 10mM HEPES. For comparison to the known specific BCRP inhibitor Ko143, cells were pre-incubated for only 1 hour before incubating with H33342.

At the end of the incubation period, media was removed and cells were washed with ice cold HBSS and 100 μ L of the lysis buffer (50 mM Hepes, 200 mM NaCl, 5% glycerol and 0.5% triton-X) was added into each well and the plates shaken on an orbital plate shaker (500 rpm for 2 minutes) before being incubated in the dark for 30 minutes. The wells were read on fluorescent plate reader SpectraMax MX5 reader (Molecular Devices LLC, Sunnyvale, CA) with an excitation wavelength of 355 nm and emission of 460 nm. Each modulator was assayed in eight wells and run in three independent experiments.

The impact of modulator fluorescence interfering and overlapping with the fluorescence of H33342 was assessed in a cell-free system using identical modulator and H33342 concentrations used in the intracellular accumulation assay on a 96-well plate. 100 μ L of each modulator and H33342 were added to wells (quadruplicate) of a 96-well plate and fluorescence measured with an excitation wavelength of 355 nm and emission of 460 nm.

Modulation of BCRP protein expression

To assess the impact of modulator exposure on BCRP protein expression, cells were grown in a 25cm² flasks for 2-3 days or until confluent and treated with 25 μ M of modulator (unless otherwise stated) for 24 hours. Cells were washed with warm PBS and whole cell lysate were

obtained by trypsinised with 0.5% trypsin-EDTA. The cell suspension was centrifuged at 1500 rpm for 10 minutes and supernatant was discarded. Freshly prepared ice cold RIPA buffer (1xTBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 0.01% PMSF solution, sodium orthovanadate and protease inhibitor cocktail) was added. The lysate was homogenized by ultra-sonication on ice. Cell debris was removed by centrifugation for 30 minutes at 16,000 x g and 4°C.

The supernatant was collected and protein content was determined by a bicinchoninic acid assay (BCA). Approximately, 75 μ g of protein was loaded into each well and transferred onto a PVDF membrane. The membrane was incubated overnight at 4°C with a 1:500 dilution of BCRP primary antibody (ABCG2-M70). The blot was then incubated with a 1:7500 dilution of a HRP-conjugated secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase) on a shaker for 2 hours at RT. For detection of the loading control (β -actin) a 1:5000 dilution of a HRP-conjugated beta-actin antibody (C4) was used. Bands were visualised by a laboratory made enhanced chemiluminescence (ECL) solution (90 mM *p*-coumaric acid, 250 mM luminol, 1M tris and 30% H₂O₂) before development.

***In vitro* BCSFB model: modulation of BCRP transport function**

Directional transport experiments were conducted in Z310 cells to evaluate the functional activity of BCRP in Z310 cells and to assess the impact of pre-incubation of cells with BCRP modulators on the apical-to-basolateral (CSF-to-blood) transport of the BCRP substrate sulfasalazine. The porous polyester membrane attached to the inner chamber of a 1.12 cm² permeable insert (ThinCert[®]) was pre-coated with 0.01% collagen for 3-4 hours. The inserts were washed twice with PBS and 1 mL of cell suspension containing 2.0×10^5 cells in cell culture media supplemented 1 μ M dexamethasone (78) was added to the insert chamber and 1.5 mL of cell culture media added to the outer well. The cell monolayer was formed 4-6 days post-seeding and was confirmed by the formation of apical 'CSF' for 24 hours, TEER value of $60 \pm 5 \Omega \cdot \text{cm}^2$ (63, 78-83) and < 1 % lucifer yellow permeation.

Transendothelial electrical resistance was measured using chop-stick electrodes and corrected for background resistance (collagen-coated inserts without cells) and by the surface area of the insert (1.12 cm²). Lucifer yellow (LY)

permeability assays were conducted using 100 μM of LY (prepared in HBSS) added apically and incubated for 60 minutes at 37 °C before 100 μL samples were removed from the basolateral well. The permeation of LY was assessed using a fluorescent plate reader (SpectraMax MX5 reader: Molecular Devices LLC, Sunnyvale, CA), with an excitation wavelength of 485 nm and emission of 530 nm.

For transport studies the media was replaced with fresh maintenance media supplemented with 25 μM of the modulator and incubated for 24 hours. Thereafter inserts were washed with warm serum free maintenance media (SFM) and incubated with SFM for 30 minutes to equilibrate. Following this equilibration period, 1 mL fresh SFM was spiked with 10 μM sulfasalazine for all wells (with no modulators) and for control wells, 1 μM Ko143, added to the insert well and 1.5 mL of warm SFM added to the outer well. Samples were taken from the outer well at 0, 30, 60, 90, 120, 150, 180 and 210 minutes, replaced with fresh warm SFM and sulfasalazine concentrations analysed by HPLC analysis and corrected for volume replacement.

The apical-to-basolateral apparent permeability coefficient was calculated according to equation 1:

$$P_{\text{app,AB}} = \frac{dQ}{dt} \times \frac{1}{AC_0} \quad (1)$$

where $P_{\text{app,AB}}$ (cm/s) is the apparent permeability coefficient, dQ/dt the amount of drug permeated per unit of time and is calculated from the regression line of time points of sampling, A (cm^2) is the insert surface area (1.12 cm^2) available for permeation and C_0 the initial drug concentration in the donor compartment.

High-performance liquid chromatography analysis

The isocratic HPLC method utilised within this study was derived from the studies of Elmasry *et al.*, (2011). The HPLC analyses (Shimadzu, LC-2010A HT) of the samples were performed on a reversed-phase C18 column (Phenomenex Luna 5- μm) with a mobile phase consisting of 70:29:1 methanol:millQ water:acetic acid, with a flow rate of 1 mL/min and a retention time of 7 minutes. In our studies a linear correlation was reported up to

50 μM ($r^2=0.998$) for sulfasalazine prepared in HBSS transport buffer and detected at 365 nm. The lowest limits of quantification were 0.025 μM .

STATISTICAL ANALYSIS

Unless otherwise stated, three independent experiments were carried out for each test compound. Statistical significance was evaluated by one-way ANOVA or paired two-tail Students t-test using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Calculations of IC_{50} were determined using a four-parameter logistic sigmoidal fitting function within GraphPad Prism. Unless otherwise states, data is reported as mean \pm standard deviation (SD). A significance level (P-value) of < 0.05 was considered as statistically significant.

RESULTS

Expression and localisation of BCRP in Z310 cells

The immortalised rat choroid plexus Z310 cell line has previously been used as an *in vitro* tool to study drug transport at BCFSB (37, 82). To date, only one study has confirmed the presence of BCRP in Z310 cells (37) and based upon this observation, we investigated the presence of BCRP in Z310 cells. Reverse-transcriptase PCR confirmed the presence of BCRP in Z310 cells with an expected product size of 146 base pairs alongside the presence of key choroid plexus phenotypic markers transthyretin (TTR) and β -actin (BA) loading control (Figure 2A). Furthermore, Z310 were successfully shown to be localised in Z310 cells when grown on cover slips and subjected to immunofluorescence staining (Figure 2B).

Cytotoxicity studies

A 24 hour MTT assay across a seven-fold log concentration range (0.001 μM -1000 μM) showed no significant cytotoxicity for the majority of the modulators studied up to 100 μM , with the lowest cellular viability IC_{50} attributed to α -naphthoflavone ($\text{IC}_{50}=1.4$ μM) with all remaining modulators demonstrated an IC_{50} in excess of 148 μM (Figure 3).

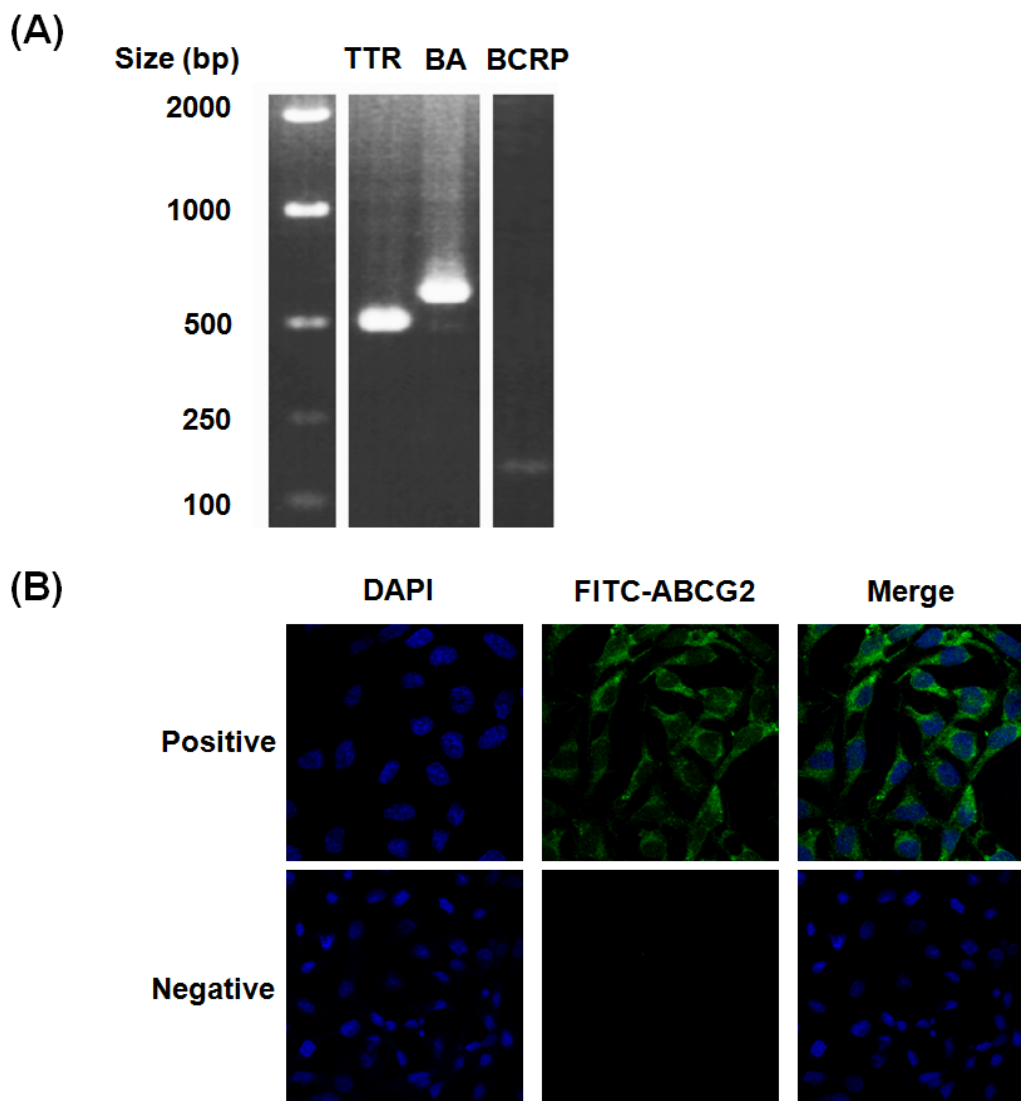


Figure 2. (A) Amplicon products for TTR, loading control (BA) and BCRP; (B) Localisation of BCRP in Z310 cells. Cells were grown on the coverslips for 2-3 days. Z310 cells were fixed with 4% paraformaldehyde and stained for BCRP using the ABCG2-M70 primary antibody and goat anti-rabbit IgG-FITC secondary antibody (Green). Cell nuclei were visualized using DAPI (Blue). Negative excludes ABCG2-M70 primary antibody but includes FITC secondary, whereas positive includes primary and secondary antibody incubation for BCRP detection.

Hoechst 33342 intracellular accumulation assay

The functional activity of BCRP was evaluated by measuring the accumulation of H33342, a fluorescent BCRP substrate, following incubation of H33342 in the presence of modulators and Ko143. Exposure of cells to the BCRP specific inhibitor Ko143, resulted in a significant increase ($P < 0.001$) in intracellular H33342 accumulation when compared to control (no inhibitor) and with all modulators demonstrating a highly significant increase ($P < 0.0001$) in H33342 accumulation compared to control (Figure 4). All modulators demonstrated background-equivalent fluorescence

emission with no overlapping fluorescence with H33342 except for baicalin, fisetin and α -naphthoflavone. Furthermore biochanin-a, chrysin, quercetin, rutin and silymarin demonstrated significantly higher intracellular H33342 accumulation when compared to Ko143 (Figure 4).

For the non-phytoestrogenic compounds (BAP, curcumin and 17- β -estradiol), significantly higher RFUs compared to Ko143 were observed for 17- β -estradiol only ($P < 0.001$), whilst BAP and curcumin demonstrated significantly higher RFU compared to control only ($P < 0.0001$).

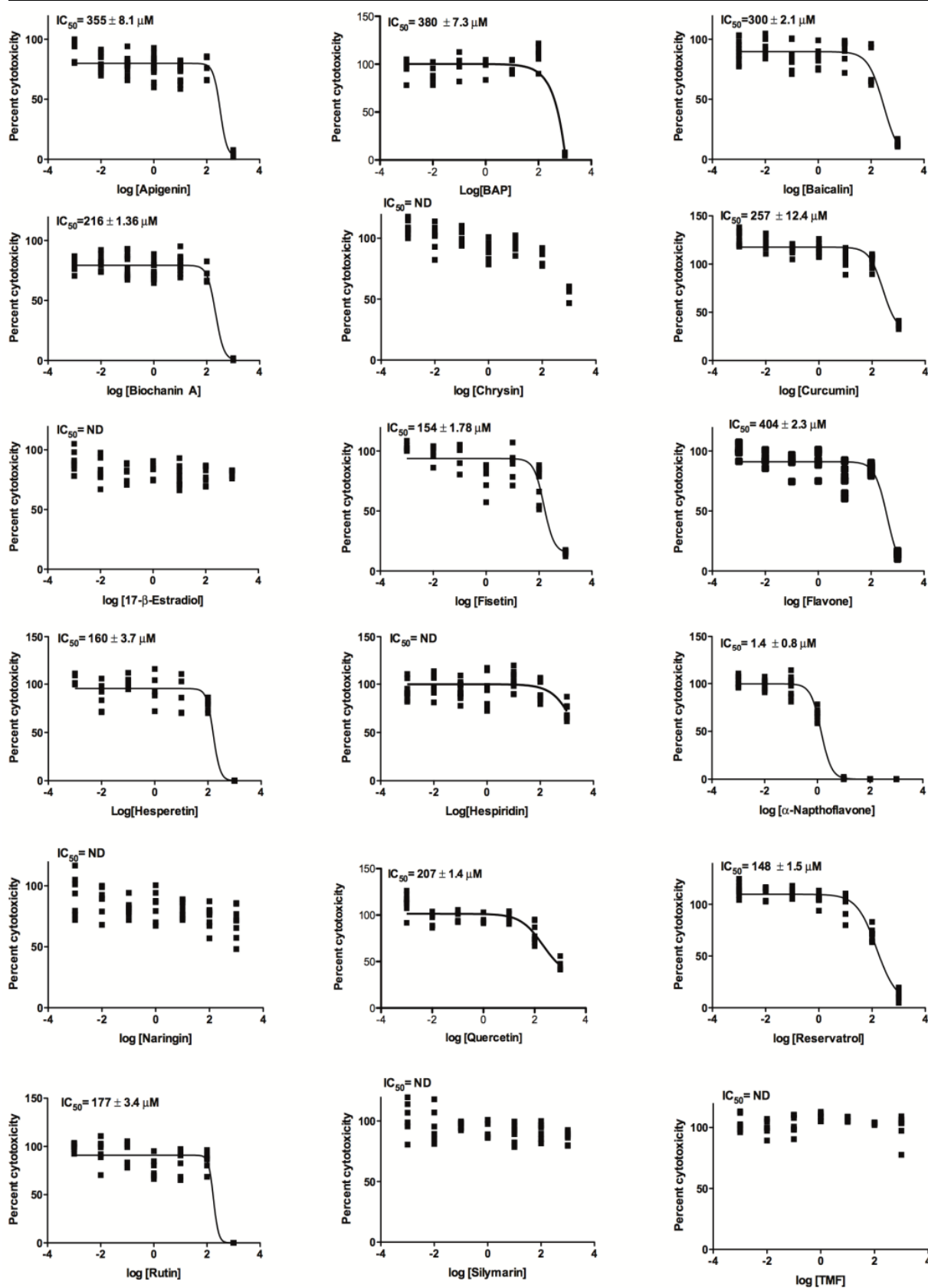


Figure 3. Cytotoxicity of modulators of BCRP on the Z310 cells line. Z310 cells were grown on a 96-well plate for 48 hours and exposed to modulators over a concentration range of 0.001-1000 μM for 24 hours. Data for each modulator is reported as scatter points with up to 8 replications per compound in three independent experiments. ND: not determined.

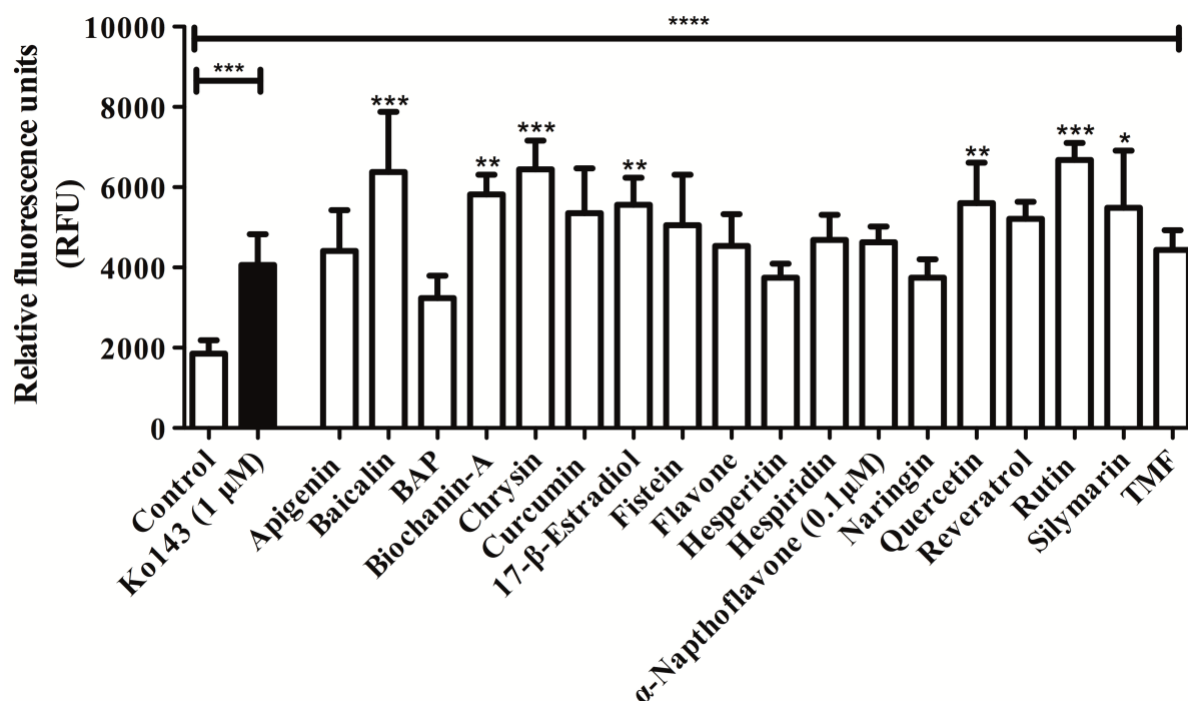


Figure 4. H33342 accumulation assay for BCRP function in Z310 cells. Cells were grown in a 96 well plate for 48 hours and washed with warm HBSS supplemented and incubated for 24 hours with media containing, unless otherwise stated, 25 μ M of test compound. Subsequently cells were incubated with media containing H33342 for 1 hour and lysed. Data is represented as mean \pm SD of three independent experiments with n=6-8 wells for each modulator in each experiment. Significant differences between Ko143 and modulators are indicated above the appropriate error bars. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

Modulation of BCRP protein expression

To investigate the effect of different modulators on BCRP protein expression, Z310 cells were incubated with modulators for 24 hours and western blotting employed to assess any change in BCRP protein expression. BCRP was successfully demonstrated to be expressed in Z310 cells with an expected size of 72 kDa (Figure 5A). A significant increase in BCRP protein was observed for flavone (2.65 \pm 0.12 fold), baicalin (2.42 \pm 0.19 fold), hesperidin (2.43 \pm 0.09 fold) and BAP (1.61 \pm 0.17 fold) (Figure 5). Furthermore, a significant down-regulation in BCRP was observed for naringin (0.16 \pm 0.07 fold) and silymarin (0.22 \pm 0.09 fold), quercetin (0.29 \pm 0.08 fold) and 17- β estradiol (0.49 \pm 0.11 fold) (Figure 5A).

In vitro BCSFB model: modulation of BCRP transport function

In the presence of 1 μ M of the BCRP specific inhibitor Ko143, a significant increase in the apical-to-basolateral transport of sulfasalazine was observed across all time points ($P < 0.05$) (Figure 6a). Similarly modulators resulting in

down-regulation of BCRP in western blots analysis (section 3.5), namely 17- β -estradiol (Figure 6c), naringin (Figure 6e) and silymarin (Figure 6f) also demonstrated a significant ($P \leq 0.05$) increase in sulfasalazine apical-to-basolateral transport at the end of the study period. Baicalin (Figure 6b) and flavone (Figure 6d) demonstrated an up-regulation of BCRP protein expression (Figure 5) but only baicalin demonstrated a significant decrease ($P \leq 0.05$) in the apical-to-basolateral transport of sulfasalazine at earlier time points (60 minutes to 150 minutes) when compared to control.

The apparent permeability ($P_{app,AB}$) of sulfasalazine was found to be $1.32 \times 10^{-6} \pm 0.12 \times 10^{-6}$ cm/s, and this was significantly increased to $2.11 \times 10^{-6} \pm 0.09 \times 10^{-6}$ cm/s when exposed to the specific BCRP inhibitor Ko143 (Table 3), demonstrating the functional activity of BCRP in Z310 cells. Furthermore, naringin, silymarin and 17- β -Estradiol also resulted in significant increases in sulfasalazine apical-to-basolateral permeability; $3.83 \times 10^{-6} \pm 0.34 \times 10^{-6}$ cm/s, $3.33 \times 10^{-6} \pm 0.61 \times 10^{-6}$ cm/s and $2.01 \times 10^{-6} \pm 0.23 \times 10^{-6}$ cm/s respectively (Table 3).

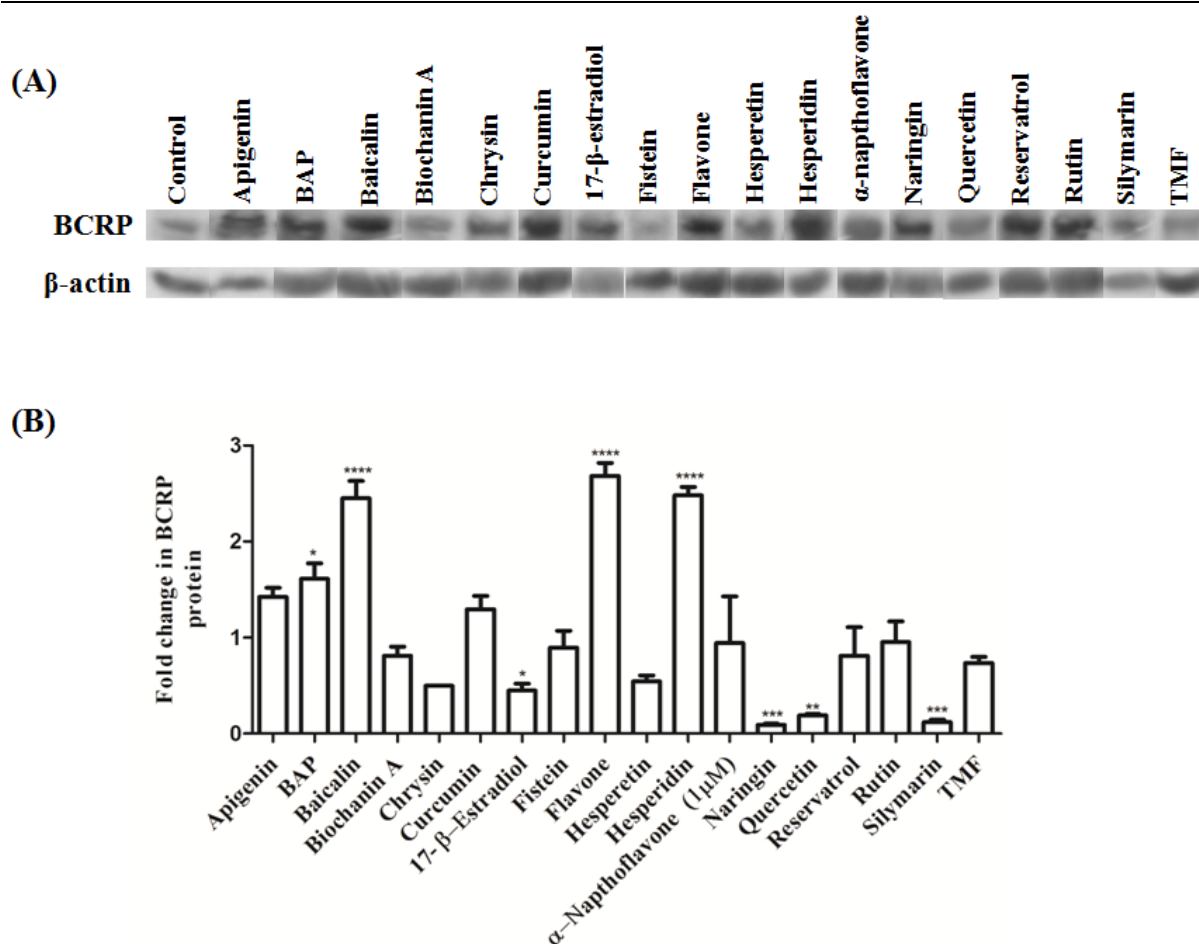


Figure 5. (A) Western blot results of modulator incubation with Z310 cells. Modulators were prepared in media to a final concentration of 25 μM (0.1 μM for α -naphthoflavone) and incubated with cells for 24 hours followed by extraction of whole cell lysate and separation of protein on an 8% SDS-Polyacrylamide gel (75 $\mu\text{g}/\text{lane}$), incubated with antibodies and detection by ECL methods. (B) Fold-change in BCRP protein expression. Data is represented as mean \pm SD of three independent experiments with statistically significant differences between control and modulator exposed conditions indicated as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

DISCUSSION

The regulation of drug disposition into the brain and wider CNS is tightly governed by the impermeable nature of the BBB and BCSFB coupled with an increasingly important complement of drug transporter proteins which tightly regulate the brain and CNS microenvironment (2, 3, 7-12, 84). The expression and impact of BCRP is now clearly recognised at the BBB and BCSFB (39, 40, 43-45, 85-88). Despite the existence of inhibitors of BCRP which have demonstrated an ability reverse the BCRP efflux phenotype *in vitro* and, to a limited extent *in vivo* (41, 42, 47-50), there yet remains a paucity in the identification of safe and effective modulators of BCRP function.

A novel class of compounds that have gained interest as potential transporter modulation candidates are phytoestrogens, of which flavonoids are the most abundant class. In this context, this study was designed to examine the impact of, primarily, flavonoid exposure on the expression and function of BCRP at the BCSFB using the rat choroid plexus Z310 cells lines which has previously been shown to express P-gp, MRP1 (80, 89) and BCRP (37).

This present study has confirmed the expression of BCRP in Z310 cells using reverse-transcriptase polymerase chain reaction, with an amplicon product of the expected size (146 base pairs), immunofluorescence confocal microscopy (Figure 2) and western blot analysis (Figure 5A) demonstrating the expression and localisation of BCRP in the rat choroid plexus, consistent with

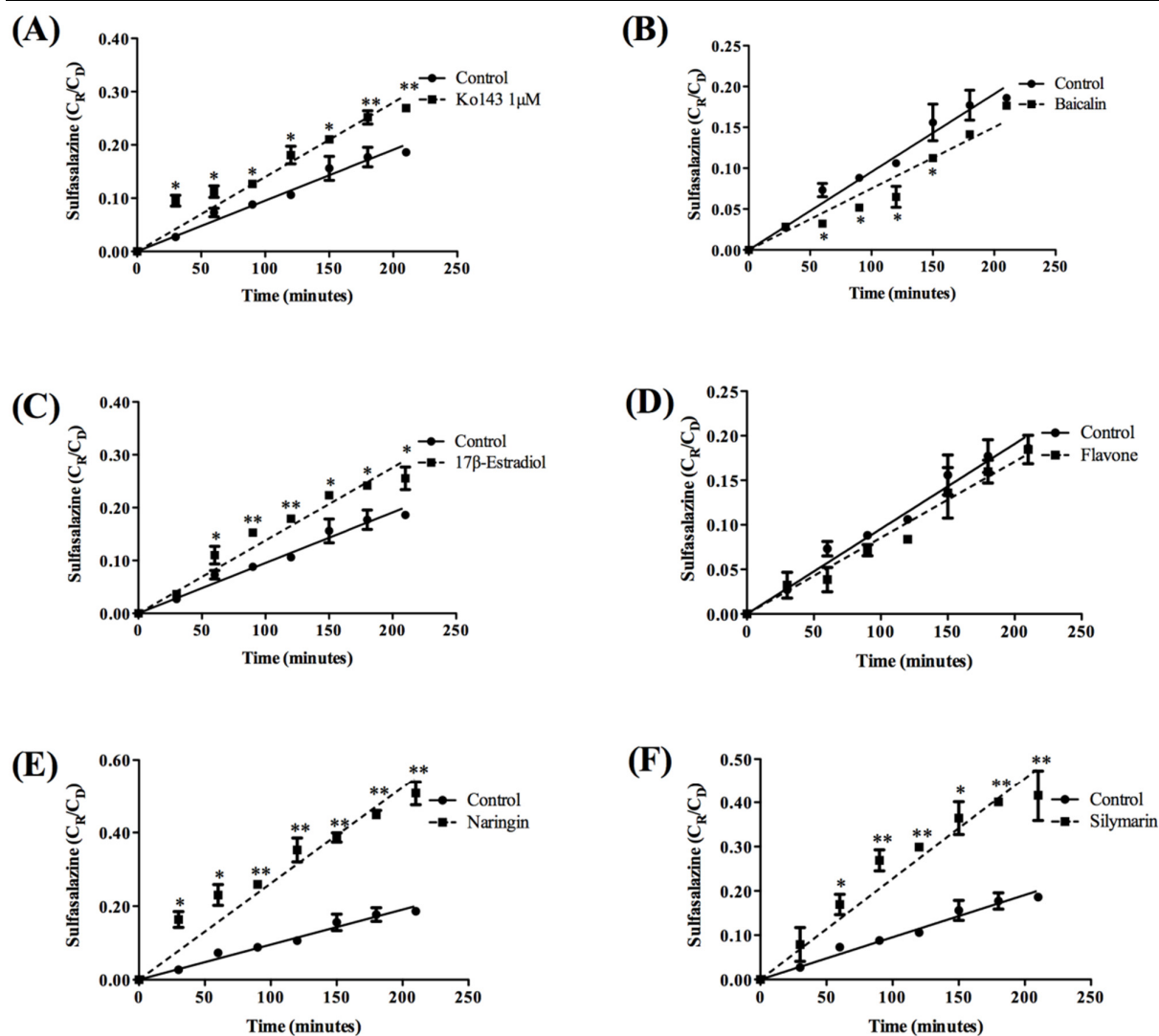


Figure 6. Fraction of sulfasalazine transported across an *in vitro* BCSFB model in the absence and presence of BCRP modulators. Cells were grown on permeable insert and transport studies were performed on day 8 (TEER $\geq 60 \Omega \cdot \text{cm}^2$) using 25 μM of modulators. Data is represented as mean \pm SD of three independent experiments and reported as the ratio of receiver concentration (C_R) and initial donor concentration (C_D). Statistically significant differences between control and modulator exposed conditions are indicated as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

reported expression and localisation studies in rats (90) (37) and (91). Furthermore BCRP was demonstrated to be functionally active through both a fluorescent substrate (H33342) intracellular accumulation assay in the presence of the known specific BCRP inhibitor (Ko143) (Figure 4) and demonstrated a significantly increased H33342 ($P < 0.001$) intracellular accumulation (2.1 \pm 0.5 fold). Furthermore a monolayer-based permeable insert BCSFB model demonstrated an increased apical-to-basolateral transport of the BCRP substrate sulfasalazine in the presence Ko143 (Figure 6a).

We have, for the first time, demonstrated the cytotoxicity profile of a range of potential BCRP

modulators at the BCSFB. For all test compounds studied, the calculated cellular viability IC_{50} values were in excess of 148 μM (Figure 3) and all possessed a dose-dependent cytotoxicity profile.

Whilst there is limited data on the cytotoxicity of flavonoids and their derivatives on CNS-derived tissues, many of the flavonoids employed within this study have been utilised in a range of cell systems with reported cell viabilities and cytotoxicities which concur with our observations and thus may enable limited comparisons to be made for any potential cytotoxic effects (Table 4).

Table 3. Calculated apparent permeability (apical-to-basolateral) for sulfasalazine in the absence and presence of modulators of BCRP

	Apparent permeability	
	$P_{app,AB}$ (10^{-6} cm/s)	SD (10^{-6} cm/s)
Sulfasalazine	1.32	0.12
+Ko143	2.11 **	0.09
+Flavone	1.24	0.19
+Naringin	3.83 ***	0.34
+Silymarin	3.33 ***	0.61
+Baicalin	1.10	0.08
+17- β -Estradiol	2.01 *	0.23

$P_{app,AB}$ was calculated for sulfasalazine alone, in the presence of Ko143 and following pre-incubation for 24 hours with modulators. Statistically significant differences between sulfasalazine alone and modulator exposed conditions are indicated as * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

Despite paucity in flavonoid cytotoxicity data at the BCSFB and other neural tissues, our cellular viability IC_{50} values and cytotoxicity profiles reported are in agreement with reported observations in a wide range of *in vitro* cell systems and demonstrate both potentially limited toxicity at sub-100 μ M exposure and, in principal, the ability for the flavonoids to penetrate into a range of different cell types.

Interestingly α -naphthoflavone demonstrates a low IC_{50} of 1.4 μ M, which in contrast to other compounds used, was significantly lower. Whilst there is a paucity of brain-derived cell viability IC_{50} values, α -naphthoflavone is routinely used at sub- μ M concentrations as a weak AhR agonist (0.01 μ M-15 μ M) (69, 101) and our reported cellular viability IC_{50} is within this range studied.

The expected *in vivo* exposure is therefore an important element in attempting to discern any potential *in vivo* cytotoxicity. In a microdialysis study brain concentrations of naringin were determined in rodents following a 100 mg/kg dose with a reported C_{max} of 0.82 μ g/mL (1.4 μ M) (102). In a further study hesperetin was administered as an IV bolus dose (50mg/kg) to rats and detected in the brain biophase with a C_{max} of approximately 0.15 μ g/mL (0.45 μ M) (103). In relation to exposure of flavonoids in the CSF, data is limited but it has been suggested the concentrations are within the range of 1-5 μ M (104). Despite this paucity, it is therefore apparent that the expected *in vivo* exposure would be within below the IC_{50} of many of the modulators studied.

Table 4. Reported cytotoxicity of flavonoids

Flavonoid	Extent of toxicity	Cell Line
Apigenin	Limited cytotoxicity up to 100 μ M	HFIIE (92)
	Limited cytotoxicity up to 100 μ M	Rat C6 (92)
Biochanin A	No significant cytotoxicity between 2-100 μ M during a 72-hour exposure	MCF-10A and NIH-3T3 (93)
	Dose-dependent cytotoxicity	SK-BR-3 (94)
	No toxicity up to 100 μ M	RAW264.7 (94)
	IC_{50} =50 μ M	HT-9 (94)
Fistein	27.6 % reduction in viability at 120 μ M for 24 hours	COLO205 (95)
Hesperidin	IC_{50} =195 μ M	Caco-2 (96)
	IC_{50} =230 μ M	CEM/ADR5000 (96)
	IC_{50} =95 μ M	CCRF_CEM (96)
	Limited cytotoxicity at 100 μ M with a viability of 53%	SNU-668 (97)
	IC_{50} =150 μ M	MSTO-211H (98)
Quercetin	No significant cytotoxicity between 20-80 μ M	RAW264.7 cells (99)
Rutin	Limited cytotoxicity up to 270 μ M with a viability of 80% at 810 μ M	HTC (100)

BCRP has a highly diverse range of substrates and has been extensively reviewed elsewhere (105-107). In this study we chose to utilise a commonly used probe substrate for BCRP, H33342 (108, 109), and take advantage of its fluorescent properties to assess the intracellular accumulation of H33342 (110) Using the known specific inhibitor for BCRP, Ko143 (43, 111), our accumulation results demonstrated a highly significant ($P < 0.001$) increase in the intracellular accumulation of H33342 (Figure 4), indicating the specific inhibition of BCRP-associated H33342 efflux and concurring with other reports demonstrating the specific nature of BCRP inhibition when using Ko143 (43, 111). when compared to control cells, the exposure of 25 μM modulators to Z310 cells for 24 hours resulted in a statistically significant increase in H33342 accumulation ($P \leq 0.0001$) for all modulators studied. Furthermore, when compared to Ko143 a range of flavonoids demonstrated significantly increased intracellular accumulation of H33342 ($P < 0.05$: silymarin; $P < 0.01$: biochanin A, and quercetin; $P < 0.001$: chrysin and rutin). Although baicalin, fisetin and α -naphthoflavone also demonstrated significantly increased intracellular accumulation of H33342, this should be viewed with caution when considering the inherent fluorescence emission observed overlapping with that of H33342. Furthermore 17- β -estradiol, a known potent BCRP downregulator (77), also demonstrated significantly increased intracellular accumulation of H33342

As the mechanism of inhibition is not a direct competitive one, due to the pre-incubation period followed by a wash-out period in fresh incubation media, these effects suggest the modulators studied are able to impart an effect that is potentially unrelated to an effect on the substrate binding sites. To this end, we undertook a western blotting procedure to assess any discernible change in BCRP protein expression following similar 24 hour incubation with modulators.

Our results highlighted that out of the 18 modulators studied, only 8 showed a statistically significant change in BCRP protein expression, with baicalin, hesperidin and flavone showing a BCRP up-regulation effect and naringin, quercetin and silymarin showing a down-regulation effect (Figure 5). Of the non-phytoestrogenic compounds the positive control used within this study (17- β -estradiol) demonstrated the expected trend of down-regulation of BCRP. This down regulation could be the result of interference with 17- β -estradiol signalling pathways by ER α and ER β . Imai *et al*

(2005) (112) demonstrated that 17- β -estradiol significantly reduced the expression of BCRP in MCF-7 cells at low nanomolar concentrations (3 nmol/L) for 1,2 and 4 days (2-,5- and 10-fold down regulation respectively). Furthermore, Hartz *et al* (2010) (77) found that the protein expression of BCRP was down regulated in the presence of 17- β -estradiol in rat brain capillaries.

Of note is a 2.5-fold or greater change in BCRP expression when exposed to flavone, baicalin and hesperidin and a 0.16-0.49 fold decrease in BCRP expression when exposed to naringin, silymarin, quercetin and 17- β estradiol (Figure 5).

In addressing the observed changes in BCRP expression, a study by Ebert *et al* (66) reported that 25 μM and 50 μM quercetin increased BCRP protein expression in human intestine carcinoma cells (Caco-2) by 2.6 and 5.3-fold following a 72-hour incubation. On an mRNA level Ebert *et al* (66) also demonstrated a 19-37-fold increase in BCRP mRNA when exposed to 50 μM of chrysin, quercetin, resveratrol and flavone.

Similarly, our studies demonstrated a significant down-regulation of BCRP expression with silymarin, but in Caco-2 cells Ebert *et al* (66) demonstrated increased expression. Modulators, specifically flavonoids, have therefore been demonstrated to have the capacity to alter BCRP expression at the protein levels, although the differences in modulation between our study and those reported by Ebert *et al* (66) in Caco-2 cells may signify cell specificity in the change in protein expression and/or may suggest a time-dependant effect.

To be in a position to gauge the functional consequences of any change in BCRP expression we conducted a transport study using a permeable insert system. The assay was developed by pre-incubating cells, grown on a permeable insert system, for 24 hours with the modulator followed by a wash-out period and monitoring the transport of the BCRP substrate sulfasalazine (in the absence of any modulators) for the duration of the transport study. We chose the BCRP specific inhibitor Ko143, and the modulators baicalin, 17- β -estradiol, flavone, naringin and silymarin to investigate and sulfasalazine as known BCRP substrate (114, 115). Our initial studies were conducted with Ko143 and we observed a statistically significant increase in apical-to-basolateral sulfasalazine flux across all time points when compared to control inserts (Figure 6a) concurring with the presence of BCRP protein as indicated in the western blot analysis (Figure 5a) and the functional direction of efflux transport

(basolateral-to-apical) and hence the inhibition of BCRP (increased apical-to-basolateral flux) at the BCSFB.

Baicalin demonstrated a significant increase in protein expression (2.42 ± 0.19 fold) and was therefore chosen as a potential up-regulator of BCRP expression. In the transport studies a significant decrease ($P < 0.05$) in apical-to-basolateral flux (approximately 10%) for a portion of the assay period (60-150 minutes) was observed, demonstrating the increasing basolateral-to-apical transport of sulfasalazine and hence diminishing the overall apical-to-basolateral flux. However, when examining the impact of flavone on BCRP, no significant differences were observed in apical-to-basolateral flux for the duration of the study. This was surprising as flavone demonstrated the greatest up-regulation of BCRP protein in western blots (Figure 5) and may indicate time-dependant protein decay following the up-regulation phenomena.

When investigating the impact of the down-regulators 17- β -estradiol, naringin and silymarin, we observed statistically significant differences across all time-points with a 8%, 40% and 26% increase in sulfasalazine transport, for 17- β -estradiol (Figure 6c), naringin (Figure 6e) and silymarin (Figure 6f). This was also translated into similar significant increases in the $P_{app,AB}$ for these compounds (Table 3).

Of interest is the translational effect of down-regulation in BCRP protein when exposed to naringin and silymarin (0.16 ± 0.07 fold and 0.22 ± 0.09 fold change in protein expression, respectively, figure 5) and the resultant effect on 'CSF-to-blood' sulfasalazine transport where $P_{app,AB}$ for naringin and silymarin was increased by 2.9- and 2.5-fold, respectively. This effect clearly highlights the potential impact of prolonged exposure of flavonoids to BCRP may have on substrate transport and how this may influence the disposition of transporter substrate at the BCSFB and wider CNS.

Given the paucity in reported data on the interaction of flavonoids of BCRP expression/function at the BCSFB, our western blot and functional transport studies concur with other similar results from Hartz *et al* (2010) (77) who demonstrated that 17- β -estradiol is able to down-regulate BCRP at the BBB.

The question remains however, how effective will flavonoids be *in vivo* and will they reach sufficient levels to initiate such a response? The dietary intake of flavonoids provides the body with flavonoids which exist predominantly *in*

planta as glycoside conjugates and which often possess limited absorption into the systemic circulation (116). Upon contact with lactase phloridzin hydrolase (LPH) at the brush border of the enterocyte, the aglycone is formed and is absorbed by passive diffusion (117). The final form found in the circulation is often the sulfate, glucuronide and/or methylated metabolites as a result of the action of sulfotransferases (SULT), uridine-5'-diphosphate glucuronosyltransferases (UGTs) and catechol-*O*-methyltransferases (COMT) (116). It is therefore unlikely that the conjugated form of the flavonoids would naturally be capable of crossing the BCSFB or BBB.

Numerous studies have demonstrated that the aglycone is capable of transferring across the BBB. Naringenin and hesperetin (30 μ M) have been demonstrated to be able to penetrate the BBB in two brain endothelial cell lines (mouse b.END5 and rat RBE4) and an *in vitro* model of the BBB (ECV304 cells co-cultured with C6 glioma cells), with a significant level of uptake into b.END5 and RBE4 cells (hesperetin: 140 and 146 ng/mg protein, respectively; naringenin: 177 and 127 ng/mg protein, respectively) (118), with the aglycone form of flavonoids demonstrating significantly greater penetration across the BBB compared to the conjugated form [aglycone: naringenin (P_{app} =350 nm/s) and hesperetin (P_{app} =290 nm/s); conjugated (P_{app} =113-182 nm/s)]. In a further study the aglycone form of [3 H]-naringenin was able to be detected in most brains regions using *in situ* perfusion studies in rats (119).

Furthermore, in an *in vivo* study by Rangel-Ordonez *et al* (2010) (120), a standard extract of *Ginkgo biloba* (extract EGb761) was given to rats at a single dose of 600 mg/kg. Following this dose, only kaempferol and isorhamnetin were detected in brain tissues (293 ng/g brain). Furthermore, high concentrations of quercetin were detected in the hippocampus, stratum and cerebellum, with levels exceed 1000 ng/g brain protein. Peng *et al.*, (1998) (121) demonstrated that the aglycone form of naringenin was detected in the cerebral cortex of rats following an IV bolus dose.

It is clear that the aglycone form is capable of penetrating the BBB and therefore the delivery of such aglycone (and unconjugated) flavonoids are important. This has been demonstrated through approaches involved nanoparticulate delivery systems encapsulating often poorly soluble aglycone forms of flavonoids (Table 5).

It is therefore envisaged that, at the BCSFB, the aglycone form of flavonoids would be able to

gain access to the choroidal epithelial cells to elicit a modulator effect on BCRP expression, with the potential benefit of a nanoparticulate carrier system enhancing the retention of the aglycone form within the circulation.

Our studies have demonstrated that flavonoids can be utilised as potential modulators of BCRP function at the BCSFB, but the mechanism of the observed changes are important in the understanding of how flavonoids can be adapted to be useful. BCRP has been reported to be modulated by a number of nuclear receptors including peroxisome proliferator-activated receptor α (PPAR α) in human immortalised hCMEC/D3 cells (132, 133), nuclear factor kB (NF-kB) in primary (134) and immortalised (hCMEC/D3) (135) brain endothelial cells, pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (136, 137), estrogen receptors in rodent brain capillaries (77) (138) and recently the aryl hydrocarbon receptor (AhR) (66, 139-142).

The regulation of BCRP has clearly been demonstrated in a range of tissues but a complete understanding of its role at neural tissues is still lacking. The exact mechanism by which BCRP regulation is precisely controlled therefore warrants further study, particularly considering there is evidence to suggest that flavonoids are capable of permeating the BBB and/or the BCSFB, for example quercetin (119, 143); catechin and epicatechin (144); hesperetin, naringenin, epicatechin and their glucuronides (118) and kaempferol and isorhamnetin (120) are capable of permeating the BBB and/or the BCSFB and therefore have the potential to interact with any of these regulatory elements.

A potentially novel application of the modulation of the regulatory control of drug transporter proteins was recently reported by Hartz *et al* (2010) (145) using an Alzheimer's disease rodent model. Using this model the expression of P-glycoprotein at the BBB was found to be reduced, leading to the accumulation of the neurotoxic peptide β -amyloid in neural tissues. Hartz *et al* (2010) (145) was able to demonstrate that the restoration of P-glycoprotein expression through, PXR activation, resulted in the reduction of brain levels of β -amyloid. Thus, further work is required to ascertain the exact mechanism of regulatory control of BCRP during prolonged exposure to flavonoid and how this could be adapted to enhance the distribution of therapeutic agents into the brain and wider CNS.

CONCLUSION

We have, for the first time, successfully demonstrated the ability of flavonoids to modulate the protein expression of BCRP at the BCSFB. Furthermore, our studies have demonstrated that flavonoids are generally expected to show limited cytotoxicity at the BCSFB with naringin and silymarin possessing the characteristics of potent down-regulators of BCRP protein at the BCSFB which translated into a significant increase in CSF-to-blood passage of drug, potentially providing an opportunity to alter the CNS disposition patterns of drugs. It is important to further elucidate the mechanism of such regulatory changes, whether genomic via nuclear receptors or non-genomic in nature, and furthermore the *in vivo* impact on BCRP substrate transports the BCSFB.

Table 5. Encapsulation and targeting of phytoestrogens to the CNS

Phytoestrogen nanoparticles	Payload	Species
PLGA	Quercetin and catechin (122)	<i>In vitro</i>
	Quercetin and etoposide (123)	Rats
Nanographene oxide	Quercetin (124)	<i>In vitro</i>
Non-phytoestrogen nanoparticles		
PLA (125)		Rats
Chitosan (126)		Rats
Albumin nanoparticles (127)		Mice
Functionalised nanoparticles		
Gelatin-soloxane TAT-peptide (128, 129)		Mice
Ritonavir loaded TAT-conjugated PLA nanoparticles (130)		Mice
Transferrin anchored pegylated albumin nanoparticles (131)		Rats

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