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Phytochemical mediated-modulation of the expression and transporter function of breast cancer resistance protein at the blood-brain barrier: an in-vitro study

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ABSTRACT

Clinical translation of BCRP inhibitors have failed due to neurotoxicity and novel approaches are required to identify suitable modulators of BCRP to enhance CNS drug delivery. In this study we examine 18 compounds, primarily phytochemicals, as potential novel modulators of AhR-mediated regulation of BCRP expression and function in immortalised and primary porcine brain microvascular endothelial cells as a mechanism to enhance CNS drug delivery. The majority of modulators possessed a cellular viability $IC_{50} > 100\mu M$ in both cell systems. BCRP activity, when exposed to modulators for 1 hour, was diminished for most modulators through significant increases in H33342 accumulation at < 10 μM with 2,6,4-trimethoflavone increasing H33342 intracellular accumulation by 3.7-6.6 fold over 1-100 μM. Western blotting and qPCR identified two inducers of BCRP (quercetin and naringin) and two down-regulators (17-β-estradiol and curcumin) with associated changes in BCRP efflux transport function further confirmed in both cell lines. siRNA downregulation of AhR resulted in a 1.75 ± 0.08 fold change in BCRP expression, confirming the role of AhR in the regulation of BCRP. These findings establish the regulatory role AhR of in controlling BCRP expression at the BBB and confirm quercetin, naringin, 17-β-estradiol, and curcumin as novel inducers and down-regulators of BCRP gene, protein expression and functional transporter activity and hence potential novel target sites and candidates for enhancing CNS drug delivery.

KEYWORDS

Breast cancer resistance protein; central nervous system; brain; flavonoid; aryl hydrocarbon receptor.

ABBREVIATIONS

ABC: ATP-binding cassette; AhR: aryl hydrocarbon receptor; BBB: blood-brain barrier; BCRP: breast cancer resistance protein; BCSFB: blood-CSF-barrier; CNS: central nervous system; PhA: pheophorbide A; TEER: trans-endothelial electrical resistance.

1. INTRODUCTION

The blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) are the two principal barriers to drug permeation to the central nervous system (CNS). CNS drug delivery is often hindered by the complex anatomy and physiology of the brain and CNS [1].

Although this restriction to drug permeation is governed by a complex network of cellular communication mechanisms, the expression of a variety of ATP-Binding Cassette (ABC) transporter proteins [2, 3] significantly enhances the barrier phenotype and contributes to a 'gatekeeper' effect, limiting access of drugs to the brain and CNS biophase. An important member of the ABC family of transporters is the breast cancer resistance protein (BCRP/ABCP/MXR) [4-6]. BCRP is a 655-amino acid, 72 kDa protein containing a single nucleotide binding domain (NBD) with six transmembrane domain (TMD) regions and is localised to a range of sanctuary site tissues. The transcriptional regulation of BCRP at the BBB, as with many ABC transporters, is thought to be governed by a range of nuclear hormone receptors [7, 8] and the interference of these signalling pathways under physiological and pathophysiological conditions provides a new approach to modulate BCRP function at the CNS barrier [9-11]. The aryl hydrocarbon receptor (AhR) has been reported to be highly expressed in brain microvessels [12-14]. The prototypical ligand for the induction of AhR activity are dioxins such as the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and induction of AhR activity by TCDD was first studied in hepatic cells [15]. AhR is therefore an important regulatory element in controlling the homeostatic balance of xenobiotic transporter/clearance pathways, but is also a potentially important target for modulating the expression of drug transporter proteins such as BCRP at the BBB, with a view to enhancing the delivery of therapeutic agents into the CNS[14]. Inhibitors of BCRP have been developed and studied in human trials with poor clinical efficacy because of significant toxicity and drug-drug interactions [16-18]. Recently phytochemicals have gained interest as potential candidates for

drug transporter inhibition, but which also demonstrate a relatively wide safety/toxicity profile. Phytochemicals, of which flavonoids are the most widely cited, are polyphenolic compounds that are widely ingested in the diet of humans and which can be found in fruit, vegetables and herbal products. The dietary ingestion of flavonoids results in the exposure of the gastrointestinal system to the glycoside conjugate form of the flavonoids, which often possess limited absorption into the systemic circulation [19]. Within the stomach and small-intestine, these glyosidic forms come into contact with lactase phloridzin hydrolase (LPH), which results in an aglycone form of the flavonoids which is then absorbed by passive diffusion [20]. It is therefore unlikely that the conjugated form of the flavonoids would naturally be capable of crossing the BBB.

Recently we demonstrated that phytochemicals were capable of modulating the expression and function of BCRP at the BCSFB [21]. The aim of the present study is threefold: (i) investigate the impact of phytochemical modulators on the genomic and protein expression of BCRP in immortalised porcine brain microvascular endothelial cells; (ii) assess the functional consequences of modulation of BCRP efflux transport in a primary porcine brain microvascular endothelial cells; (iii) investigate the role of phytochemicals in modulating AhR-mediated transcriptional regulation of BCRP.

2. RESULTS

2.1 Expression and localisation of BCRP in PBMEC/C1-2 and primary cells

The expression of BCRP in both cell lines was confirmed by reverse-transcriptase PCR with an expected amplicon product of 653 bp (Figure 1A). Furthermore localisation of BCRP was observed in PBMEC/C1-2 grown on coverslips (Figure 1B) and primary PBMEC grown on permeable inserts (Figure 1C).

2.2 Modulator induced cellular toxicity

To investigate the cellular toxicity of modulators towards the PBMEC/C1-2 cells, a MTT cellular toxicity assay was conducted whereby cells were exposed to a 7-fold log concentration range of modulators, 0.001 μM-1000 μM, for 24 hours. Modulators demonstrated a range of toxicities towards PBMEC/C1-2 cells (Figure 2). The lowest IC_{50} (1.5 ± 2.7 μM) was observed for α-napthoflavone. Similarly, baiclain, 17-β-estradiol, hesperidin and hesperetin demonstrated low micromolar IC_{50} with the majority of modulators demonstrating minimal toxicities in the range of 100-1000 μM (Figure 2). The cellular toxicity of modulators towards the primary PBMEC was also investigated using MTT toxicity assay. Cells were exposed to 25 μM and 100 μM of modulators for 24 hours. Similar trends were observed as for PBMEC/C1-2 with α-napthoflavone showing significant cytoxicity at 25 μM and 100 μM in addition to 25 μM of curcumin (31.9 ± 9.8 % viability) and 17-β-estradiol (28.3 ± 7.2 % viability) (Figure 3). Additionally, a number of modulators demonstrated minimal toxicities up to 100 μM and included apigenin, biochanin A, chrysin, fistein, flavone, I3C, naringin, quercetin, resveratrol, rutin, silymarin and TMF (Figure 3). Modulator concentrations of 25 μM were utilised for subsequent assays, except for curcumin, 17-β-estradiol, α-napthoflavone, baicalin, hesperetin and hesperedin (1 μM upper limit)

2.3 Hoechst 33342 intracellular accumulation assay

The functional activity of BCRP was evaluated by measuring the intracellular accumulation of a fluorescent BCRP substrate (H33342) in PBMEC/C1-2 cells following co-incubation in the presence of modulators and the specific BCRP inhibitor Ko143. Studies were conducted to assess the potential for inhibtion of BCRP efflux fuction (1-hour incubation) and for modulation of BCRP-protein expression (24-hour incubation). All modulators were screened for their inherent auto-fluorescence at the $\lambda_{ex/em}$ for H33342 and three were excluded (baicalin, fisetin and α-napthoflavone).

Our results demonstrated a concentration dependant increase in H33342 for most modulators studied, across a concentration range of 1-100 μM, when incubated for 1 hour (Figure 4A). Furthermore, the fold change in H33342 intracellular accumulation was equal to or greater than that observed for Ko143 (represented by the grey shaded area in figure 4A) for apigenin (1-100 μM), hesperidin (100 μM), I3C (1-100 μM) and 2,6,4-trimethoxyflavone (TMF) (1-100 μM) and leading to an increase in H33342 accumulation by 1.8-2.3 mean fold range, 2.4 ± 0.5 fold change, 2.3-4.1 mean fold range and 3.7-6.6 mean fold range respectively when compared to the absence of modulators.

Time dependent functional activity of BCRP was also evaluated following incubation of H33342 in the presence of modulators for a 24 h period. Our results demonstrated that incubation with biochanin A (10-100 μM), chrysin (1-100 μM), curcumin (0.1-1 μM),17-β-estradiol (0.01-1 μM), I3C (10 μM and 100 μM), silymarin (1-100 μM) and TMF (10-100μM) resulted in a significant increase in intracellular H33342 accumulation when compared to control (Figure 4B) and in a concentration-dependant manner. Furthermore, a significant reduction in H33342 accumulation was observed with hesperetin and hesperidin (0.5-0.8 mean fold change), naringin (0.37-0.48 mean fold change) and quercetin (0.75-0.46 mean fold change) when compared to control across all concentration studied, primarily in a concentration-dependant manner and generally to a similar extent as that observed with the BCRP inducer TBhQ (Figure 4B).

2.4 Modulation of BCRP expression

To assess the impact of modulators on the protein expression of BCRP, PBMEC/C1-2 cells were exposed to modulators for 24 hours and changes in protein expression were assessed through western blotting. BCRP protein (monomer) was confirmed with a product size of 72 kDa (Figure 5A). Significant induction ($p \leq 0.05$) of BCRP protein was observed for

biochanin A (0.42 ± 0.10 fold), hesperetin (0.51 ± 0.14 fold), hespiridin (0.78 ± 0.21 fold), naringin (1.2 ± 0.05 fold) and quercetin (1.25 ± 0.07 fold) (Figure 5B and C). Furthermore, at the transcription level, these changes were confirmed with quercetin and naringin significantly ($p < 0.0001$) up-regulating *BCRP* expression by 1.63 ± 0.28 and 1.36 ± 0.71 fold (Figure 6A).

Significant ($p \leq 0.001$) down-regulation of BCRP protein was observed with curcumin (1.75 ± 0.22 fold), 17-β-estradiol (1.23 ± 0.19 fold) and less so with TMF (0.56 ± 0.10 fold) ($p \leq 0.01$) (Figure 6A) and confirmed with similar changes in *BCRP* gene expression (curcumin: 1.78 ± 0.05 and 17-β-estradiol: 1.54 ± 0.05) (Figure 6A). Confirmation of BCRP induction was evident with TBHQ (positive control) yielding a 1.29 ± 0.12 fold increase in BCRP protein expression.

2.5 AhR mediated regulation of BCRP in PBMEC/C1-2 cells

To confirm the role of AhR in the transcriptional regulation of BCRP, gene silencing of *AhR* (AhR targeting siRNA) and antagonism of AhR activity (CH223191[22]) was conducted to assess changes in both AhR and BCRP expression by qPCR approaches. Using both a chemical antagonist of AhR and *AhR*-targetted siRNA, we demonstrated successful down-regulation of BCRP and AhR in PBMEC/C1-2 cells. CH223191 at 1 μM and 10 μM resulted in no significant reduction in *AhR* expression but a significant 2.31 ± 0.08 fold down-regulation with siRNA ($p \leq 0.01$) (Figure 6B). BCRP gene expression was also quantified in the same samples that were treated with CH223191 and demonstrated down-regulation when exposed to 1 μM ($p \leq 0.001$) and 10 μM ($p \leq 0.05$) leading to a 1.12 ± 0.09 and 0.79 ± 0.12 fold change respectively. In samples exposed to dicer AhR siRNA, *BCRP* gene expression was also significantly down-regulated ($p < 0.01$) leading to a 1.75 ± 0.08 fold change (Figure 6B).

2.6 Phytochemical mediated modulation of *AhR* and *BCRP* gene expression

To assess whether phytochemicals were able to modulate the expression of *AhR*, siRNA techniques were used to silence *AhR* and assess subsequent changes in *AhR* expression following incubation with modulators in PBMEC/C1-2 cells.

In the absence of siRNA, *AhR* was significantly increased ($p \leq 0.0001$) when exposed to BCRP protein inducers quercetin (0.62 ± 0.31 fold) and naringin (0.84 ± 0.08 fold), relative to control samples (Figure 6C). However for BCRP protein down-regulates curcumin and 17-β-estradiol, no significant differences in *AhR* was detected (Figure 6C).

When cells were exposed to siRNA, *AhR* gene expression was significantly reduced (compared to control samples), when exposed to BCRP protein inducers quercetin (1.26 ± 0.06 fold) ($p \leq 0.0001$) and naringin (0.64 ± 0.08 fold) ($p \leq 0.05$). Additionally, significant differences existed when compared the absence and presence of siRNA ($p < 0.0001$). Furthermore, *AhR* gene expression was significantly reduced when exposed to BCRP protein down-regulators curcumin (1.06 ± 0.09 fold) ($p \leq 0.01$) and 17-β-estradiol (0.97 ± 0.09 fold) ($p \leq 0.01$ (Figure 6C). However, no significant difference was detected when comparing samples in the absence or presence of siRNA (Figure 6C).

In the absence of *AhR*-targetting siRNA, *BCRP* was significantly increased ($P \leq 0.0001$) for BCRP protein inducers, namely quercetin (1.63 ± 0.28 fold) and naringin (1.36 ± 0.71 fold), relative to control samples (Figure 6D). Similarly, BCRP protein down-regulators demonstrated significant decrease in *BCRP*, curcumin (1.78 ± 0.05 fold) ($P \leq 0.0001$) and 17-β-estradiol (1.54 ± 0.05 fold) ($P \leq 0.01$) (Figure 6D). When *AhR* was silenced, *BCRP* was reduced compared to control samples for BCRP protein inducers, quercetin (0.18 ± 0.12 fold) and naringin (0.41 ± 0.09 fold) and were not significantly different from control (absence of modulators) (Figure 6D) but were significantly different from results obtained for *BCRP*

expression in the absence of siRNA. For BCRP protein down-regulators, *BCRP* expression increased when compared to samples in the absence of siRNA for curcumin (0.62 ± 0.10 fold) and 17-β-estradiol (0.22 ± 0.21 fold) but were not significantly different from control (absence of modulators) (Figure 6D). Furthermore, when compared to samples in the absence of siRNA significant differences between –siRNA and +siRNA samples existed for curcumin ($P \leq 0.01$) and naringin ($P \leq 0.05$) (Figure 6D).

2.7 Activation of AhR by modulators in H1L6.1c2 cells

To confirm the activation of AhR by modulators, a chemically-activated luciferase (CALUX) assay was employed using the stably transfected luciferase H1L6.1c2 cell line to screen modulators known to modulate BCRP protein expression in PBMEC/C1-2 cells, relative to the maximum activation from incubation with omeprazole (data not shown).

Modulators identified as 'strong' AhR activators (>75 % maximum activation relative to omeprazole) included hesperidin (76.09 % ± 4.3 %), naringin (75.54 % ± 1.8 %) and quercetin (73.75 % ± 4.6 %). Modulators with 'minimal' activation of AhR included biochanin A, curcumin, 17-β-estradiol, reservatrol and rutin. All other flavonoids demonstrated a greater than 25 % but less than 75 % maximum luciferase induction (when considering the SD) (Figure 7).

2.8 Phytochemical mediated modulation of BCRP transporter function

The function of BCRP in a representative *in vitro* BBB model was assessed by measuring the transport of PhA in the absence and presence of Ko143, a known BCRP inhibitor. Our results demonstrated that 1-hour incubation with Ko143 (1 μM) significantly increased the apical-to-basolateral (AB) flux and decreased the basolateral-to-apical (BA) flux of PhA during our transport studies ($p < 0.05$) in both cell systems (Table 1). The calculated efflux ratio (ER) for PBMEC/C1-2 and PBMEC were 5.6 and 11.2, respectively, in the absence of Ko143 and 1.7 and 1.6 in the presence of Ko143.

The functional assessment of BCRP following modulator exposure was evaluated in the presence of BCRP down-regulators (curcumin and 17-β-estradiol). Our results demonstrated that 24 hour incubation of curcumin and 17-β-estradiol significantly increased the apical-to-basolateral (AB) passive permeability ($Papp_{,AB}$) (Table 1) of PhA in both PBMEC/C1-2 and PBMEC (Figure 8A). Incubation with curcumin resulted in a 5-fold increase in AB flux for PBMEC/C1-2 but a larger 6.2-fold increase in AB flux for PBMEC (Figure 8B). For 17-β-estradiol a 4.1-fold increase in AB flux for PBMEC/C1-2 and 6.9 fold increase in AB flux for PBMEC (Figure 8B). Furthermore, our results demonstrated that 24-hour incubation with naringin and quercetin (25 μM) increased the basolateral-to-apical (BA) flux of PhA (Table 1) by 1.3-fold and 1.07-fold in PBMEC/C1-2 and PBMEC cells and 1.4-fold and 1.20 fold in PBMEC/C1-2 and PBMEC for quercetin (Figure 8C).

3. DISUCSSION

3.1 Phytochemical toxicity at the BBB

Our results demonstrated that the majority of modulators exhibited IC_{50} values in excess of 100 μM in PBMEC/C1-2 (Figure 2) and limited toxicity at 25 μM in PBMEC (Figure 3). However, a number of modulators demonstrated low IC_{50} values ranging from 1.5 - 63 μM and included flavones such as baiclain, flavone, α-napthoflavone and flavonones such as hesperidin and hesperetin, 17-β-estradiol and curcumin. In most instances, the concentration employed within the study were below the cytotoxicity IC_{50}, and when considering that brain concentrations of flavonoids are typically < 50 μM [23-25], the inherent toxicity towards the BBB endothelium would be minimal. Furthermore, whilst difficult to compare with reported studies, the IC_{50} values determined for modulators were found to be within the same order of magnitude as published reports from, often, non-cerebral origin cell lines [26-31].

3.2 Intracellular accumulation of Hoechst 33342

The fluorescent properties of H33342 make it an ideal tool to use during the study of its intracellular accumulation when modulating the function of BCRP, and has widely been used as a BCRP substrate. Our results demonstrated both inhibition of BCRP efflux function (Figure 4A) and potentially genomic/proteomic level alteration of BCRP efflux function following prolonged incubation (Figure 4B). Furthermore, the results demonstrated that TMF is a potent modulator of BCRP function with a 3.70 ± 0.39 fold change in H33342 accumulation compared to control at the lowest concentration studied, 1 μM (Figure 4A). Of the published reports available, TMF has been identified as potent inhibitor of BCRP efflux activity at the BBB having also been identified in BCRP-transduced human leukaemia K562 cells [32]. Furthermore, of the published reports available, curcumin has been reported to inhibit BCRP function in HEK293 cells without altering protein levels over 72-hours of incubation nor inhibiting the ATPase function of the NBD [33]. Furthermore, it has recently been recommended that curcumin be used as the 'best' *in vivo* inhibitor of BCRP [34]. Our results also demonstrated that 17-β-estradiol increased H33342 accumulation in a concentration dependant manner with the greatest increase observed at 1 μM, 1.51 ± 0.21 fold ($p \leq 0.01$), and has been widely reported inhibitor of BCRP function [9, 35].

During prolonged incubation the majority of phytochemicals demonstrated some level of concentration-dependant modulatory effects leading to statistically significant increases or decreases in H33342 accumulation compared to control (Figure 4B). Of note were naringin (10-100 μM), quercetin (10-100 μM), hesperetin and hesperidin (1 μM) which resulted in a reduction in H33342 accumulation to within the reported by the positive control inducer THBQ and suggesting these modulations possess BCRP down-regulatory properties.

These finding indicate that modulators may play a role in directly inhibiting BCRP at the BBB during short-term incubations. The exact mechanism of BCRP inhibition is not clear. But it has been reported that glycosylated flavonoids have anti-BCRP activity due to their water solubility [36]. Modulators lacking significant inhibitory activity may be hindered by their low lipophilicity and reduced permeability. This may explain why we observed no inhibitory effects for resveratrol and rutin which are glycosides and have lower lipophilicity compared with their respective aglycones [37]. Furthermore, the binding of flavonoids to the nucleotide-binding domain of BCRP has been identified as being important the inhibition process [32, 38], leading to inhibition of the ATPase function and hence halting of the conformational changes required to transport substrates across the cell membrane. Additionally QSAR analyses have demonstrated a strong structure-inhibition relationship between BCRP and flavonoids. Flavonoids with a hydroxyl group at position 5, double bond between position 2 and 3 and methoxyl moiety at position 3 or 6 [39] show preference for binding to BCRP and inhibiting the functional transport. This may explain why the greatest intracellular accumulation of H33342 was observed with TMF, as O-methylation at position 2, 4 and 6 increases the local hydrophobicity and hence signifies TMF mediated anti-BCRP activity.

It should be noted that H33342 is also a substrate of P-glycoprotein, and concentration-dependant differences in intracellular H33343 accumulation, such as those identified with apigenin and TMF, may be attributed to inhibition of P-gp. Nevertheless, the H33342 intracellular accumulation studied aided in identifying potential modulators of BCRP function, which were further validated at a genomic and proteomic level through qPCR and western blotting analysis.

Furthermore, the mechanism of BCRP modulation following prolonged incubation was further assessed in subsequent studies investigated both genomic and proteomic level effects coupled with the role of the nuclear hormone receptor AhR, in BCRP regulation.

3.3 Modulation of BCRP protein expression

BCRP protein (Figure 5) and gene (Figure 6) expression was successful confirmed in both PBMEC/C1-2 and primary PBMEC cells. Whilst previous reports have identified phytochemicals as being able to modulate BCRP protein in non-cerebral cell lines [40], we identified two phytochemicals capable of inducing BCRP expression at the BBB, namely narinign and quercetin (Figure 5C) and two phytochemicals capable of down-regulation BCRP expression at the BBB, curcumin and 17-β-estradiol (Figure 5C), which also demonstrates some similar correlations with our 24-hour H33342 intracellular accumulation studies (Figure 4B), despite the differing end-point analytic metrics.

Furthermore, previous studies have reported similar observations and confirm our choice of naringin[21, 41], quercetin[40], curcumin[34] and 17-β-estradiol [35,9] as viable BCRP modulators and support our identification and selection of BCRP-modulatory phytochemicals.

3.4 AhR mediated regulation of BCRP in PBMEC/C1-2 cells

The relationship between AhR and its transcriptional regulation of BCRP was also identified by Tompkins et al [42], who reported that activation of BCRP expression in human colon adenocarcinoma-derived LS174T cells is regulated by AhR. To identify whether AhR would be a potentially novel target for modulation of BCRP function we assessed the role of Aryl Hydrocarbon Receptor (AhR) in regulator BCRP expression at the BBB. Through pharmacological antagonism and gene silencing (siRNA) of AhR, BCRP gene expression was significantly downregulated under all treatment conditions (Figure 6B) with statistically significant differences in AhR and BCRP expression under CH223191 and siRNA treatment when compared to control (untreated: fold-change = 0). Previously, only one study has

reported the modulation of BCRP through AhR dependent manner in Caco-2 cells [43], and taken together our results confirm that AhR plays a significant role in the transcriptional regulation of BCRP.

3.5 Phytochemical mediated modulation of *AhR* and *BCRP* gene expression

Having confirmed the role *AhR* plays in regulating the expression of *BCRP*, the identified up- and down-regulators of BCRP gene/protein expression were assessed against *AhR* silenced cells and it was confirmed that silencing of *AhR* abolished the induction/down-regulation effect elicited by phytochemicals in the absence of siRNA (Figure 6D). For the up-regulators, the lack of statistically significant differences between control and + siRNA for changes in BCRP expression highlight a 'normalisation' of BCRP gene expression as it returns to baseline and hence no significant difference in gene expression compared to control. Similarly curcumin and 17-β-estradiol were identified as down-regulators of *BCRP* and when *AhR* is silenced *BCRP* expression recovers to 0.51 ± 0.09 and 0.29 ± 0.19 of control and is not significantly different from control, again suggesting a return to baseline expression for BCRP (Figure 6D).

At the BBB this confirms that AhR has a significant role to play in the regulation of BCRP, and is supported by a recent study by Wang et al [14]. Furthermore, the modulators identified as up- or down-regulators of BCRP expression may, in part, act to impact directly upon the activity of AhR, such as nuclear translocation [44 45], as a possible mechanism which can alter the gene expression of BCRP.

3.6 Activation of AhR by modulators in H1L6.1c2 cells

We selected the stably transfected H1L6.1c2 cell lines along with an associated luciferase assay based on the CALUX assay developed by He et al [46] to assess whether modulators were capable of modulating AhR by activating a luciferase response following a period of 24 h incubation in H1L6.1c2 cells. All modulators demonstrated some degree of activation of

AhR, with hesperidin, naringin and quercetin identified as 'strong' activators, and the majority of modulators yield a reduction in H33342 intracellular accumulation being identified as 'minimal' activators of AhR activity (Figure 7). It has been reported that quercetin significantly increased AhR activity by inducing CYP1A1 in human hepatoma HepG2 cells, whereas rutin a glycoside of quercetin failed to induce AHR and activation of CYP1A1 [47] when incubated with similar concentration (10-50 μM) [47]. Additionally, other studies have also confirmed AhR activation by similar modulators identified in our studies. For example quercetin has been shown to activate AhR mediated CYP1A1 mRNA expression in Caco-2 cells [48, 49] and MCF-7 cells [50] at concentrations of 0.5-10 μM. Another study also reported that chrysin, baiclain, galangin and genistein induced the luciferase activity in stably transfected Hepa-1 cells whereas quercetin, emodin and apigenin demonstrated inhibitory effect on AHR induction relative to TCDD and act in a concentration dependant manner [51]. Taken together, this demonstrates that phytochemicals are capable of activating the AhR regulator pathway which can (in the case of the identified modulators) directly impact upon the gene and protein expression BCRP.

3.7 Phytochemical mediated modulation of BCRP transporter function

Whilst gene or protein changes in BCRP expression may suggest at a possible change in the functional expression of BCRP, without conducting transport studies using a BBB monolayer model, the consequences of any gene/protein level changes in BCRP cannot be assessed in a functional sense. Across both PBMEC/C1-2 and primary cell culture model we identified similar behaviours of induction or down-regulation of BCRP expression and the functional consequences of this was confirmed using the permeable insert models. Our results confirmed that BCRP is expressed in PBMEC/C1-2 cells and primary porcine brain microvascular endothelial cells.

By comparing both immortalised and primary, the impact on BCRP transporter function in the low TEER PBMEC/C1-2 model can be compared with the higher TEER primary PBMEC model. In addressing the low TEER values for PBMEC/C1-2, these cells have been reported to retain several BBB properties such as the presence of glucose transporter (GLUT-1), γ-glutamyltranspeptidase (γ-GT) and apolipoprotein A-1 along with the presence of the von Willebrand factor (vWF), lectin binding receptors for UEA-1 and uptake of acetylated LDL [52]. Furthermore the induction of cellular tightness by astrocytic factors also contributes to the barrier function [53] typically giving TEER values similar to that of the human immortalised hCMEC/d3 BBB cell line (30-60 $\Omega.cm^2$) [54, 55]. Therefore, the model presents a viable one with which to study BBB phenotype [56, 57] and are augmented by the observations in primary PBMEC, the findings of which suggest that 17-β-estradiol and curcumin are viable down-regulators and quercetin and naringin are viable inducers of BCRP expression and efflux function. This has clear implications for modulating the efflux role of BCRP at the BBB towards either clearing agents from the brain biophase back into the systemic blood or forcing equilibrium towards enhanced brain delivery of therapeutic compounds. Further work is required to address the potential in-vivo translation of this effect in the presence of the plasma biophase (and associated plasma proteins) which may hinder phytochemical BBB penetration. However, although brain concentration of many phytochemicals are lacking in the literature, curcumin has been reported at brain tissues concentrations of 0.4 ± 0.01 μg/g [58] and 2.9 nmol/g [59] following a 100 mg/kg dose in mice; C_{max} of 13 ± 12 ng/g [60] following a 23 mg/kg oral dose; C_{max} of 5.87 μg/brain (~ 2.92 μg/g) following an oral dose of 500 mg/kg [61]. Following a 5 mg/kg intravenous dose of nanoparticle formulated curcumin, regional brain tissue concentration of curcumin ranged from 40-165 ng/g (~0.5 μM) [62]. For naringin, a C_{max} of 1-2 μg/mL (~2-4 μM) was reported following a 30 and 100 mg/kg intravenous dose in rats [63]. Furthermore, for quercetin concentrations of 22.91± 3.35 μg/mL (~45 μM) have been

reported in rat brains following an oral dose of 50mg/kg [64]. Furthermore, this increased to 33.28 ± 1.27 μg/mL following the administration of a nanoformulation of quercetin [64]. Although the majority of pharmacokinetics studies assessing plasma or brain concentrations of phytochemical modulators have utilised oral routes, the BBB would be limited due to poor absorption through the gastrointestinal tract [20, 65] and the use of nanoparticle formulation systems would be a viable route with which to parenterally deliver the identified phytochemical modulators to the BBB at brain vasculature concentrations capable of modulating BCRP expression and function.

4. CONCLUSIONS

Drug delivery to the CNS has become a major challenge due to the presence of the BBB and associated insidious network of drug efflux transporters, which together acts as a physical and metabolic barrier for the transport of therapeutic agents into the wider CNS. Phytochemical modulators, primarily flavonoids, were demonstrated to possess limited cellular toxicity towards immortalised and primary PBMEC and functional inhibition the efflux activity of BCRP, with TMF identified as a potentially potent flavonoid-based inhibitor of BCRP activity. Furthermore, quercetin and naringin were identified as inducers with curcumin and 17-β-estradiol as down-regulators of BCRP gene and protein. The role of AhR in both directly regulating BCPR expression and mediating modulator-induced changes in transcriptional regulation of BCRP function was also identified. Therefore, phytochemicals can be considered to be a potentially useful class of compounds possessing multifaceted activity towards the goal of negating BCRP efflux at the BBB or enhancing the efflux potential for the removal of agents from the CNS.

5. MATERIALS AND METHODS

5.1 Materials

Dulbecco's modified essential media with glucose (DMEM), fetal bovine serum (FBS), 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); premium fetal bovine serum was obtained from Fisher Scientific (Loughborough, 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^{TM} one-step RT-PCR kit and Easy Ladder I obtained from Bioline (London, UK). All RT-PCR primers and siRNA duplexes were designed synthesised by IDTDna (Leuven, Belgium); Luciferase Assay System was obtained from Promega (Wisconsin, USA); Optiblot SDS-page gel and western blot reagents obtained from Abcam (Cambridge, UK); qPCR gene primers were custom synthesised by PrimerDesign (Southampton, UK); Anti-BCRP antibody was obtained from Sigma (Dorset, UK), beta-actin (C4), broad range markers, goat anti-rabbit IgG-FITC, RIPA buffer 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.

5.2 Cell culture: immortalised porcine BMEC

The immortalised porcine brain microvascular endothelial cell line PBMEC/C1-2 was a kind gift from Dr P. Friedl [66]. PBMEC/C1-2 were grown in conditioning media obtain from rat C6 astrocytes. C6 cells were obtained from Cell Line Services (Germany). The cells were resuspended in a T25 flask containing C6 media (Hams F12 50%, IMDM 50%, 7.5% NCS, 7mM L-glutamine, 5μg/mL transferrin, 0.5 U/mL heparin and 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulphate). Cells were grown at 37°C in a humidified

atmosphere of 5% CO_2 for 24 h and media changed after 24 h. Thereafter the cells were passaged 3-4 days post seeding (at 70-80% confluency) by washing with pre-warmed PBS followed by the addition of 1 mL of trypsin-EDTA to the flask.

The flask was then placed in an incubator for 5 min and cell suspension was resuspended in 5 mL of growth media. Cell suspensions were then transferred to a 15 mL centrifuge tube and centrifuged at 1500 rpm for 5 min and the pellet was resuspended in 2 mL of the media and transferred to a T75. The media was aspirated every other day, sterile filtered (0.22 μm) and stored at 4°C for further use.

This media was labelled as astrocyte conditioning media (ACM). Subsequently, ACM was mixed in a 1:1 with C6 media to develop the maintenance media for PBMEC/C1-2 [52, 56, 66, 67] and this was termed C12 media.

5.3 Cell culture: primary porcine BMEC

Porcine brain endothelial cells were isolated according to the methods described by Patabendige et al (2013) [68]. Porcine brains were acquired from a local abattoir (Long Compton Abbatoir, Oxford, UK) within 1-hour of sacrifice. The brains were transferred to the laboratory in sterile box containing L-15 media supplemented with 1 % v/v penicillin/streptomycin on ice. Each brain was separated into respective hemispheres and thoroughly washed with ice-cold PBS media supplemented with 1 % v/v penicillin/streptomycin. The meninges and blood vessels were removed along with the choroid plexus and capillaries located within brain sulci. The hemispheres were then placed in a clean beaker containing PBS supplemented with HEPES (10 mM) and penicillin/streptomycin sulphate. White matter was then carefully removed and grey matter dissected and transferred to a beaker containing MEM supplemented with HEPES (10 mM) and 1% v/v penicillin/streptomycin. The grey matter tissue was then chopped into small 1 cm^3 sections using a sterile scalpel before being transferred into a 50 mL syringe and passed

into a T75 containing 50 mL of MEM supplemented with HEPES (10 mM) and 1 % v/v penicillin /streptomycin. 15 mL of this brain extract was transferred into a homogeniser (Dounce Homogeniser, Jencons, UK) and 25 mL of MEM supplemented with HEPES (10 mM) and 1 % v/v penicillin/streptomycin sulphate was added to the homogeniser. The brain extract was then homogenised gently for 15 strokes with a loose pestle (Type B) followed by 15 strokes with tight pestle (Type A). The resulting homogenate was then transferred to a sterile T175 and process repeated for the remaining tissue.

200 mL of homogenate was then filtered through a 150 µm pore nylon mesh and the filtrate collected and subsequently filtered again through a 60 µm pore nylon mesh (Plastok Associates Ltd, Wirral, UK). The filters were removed and placed into separate 15 cm Petri dishes containing 80 mL of digest mix (M199 containing collagenase (223 U/mg), trypsin (211 U/mg), DNase I (2108 U/mg), 10 % v/v FCS and 1 % v/v penicillin/streptomycin). Filters were then incubated at 37°C for 1 hour in an orbital shaker and labelled as '150s' and '60s'.

Thereafter the filters were thoroughly washed and the digest mix transferred to 50 mL centrifuge tubes labelled '150s' and '60s' and centrifuged at 4°C for 5 minutes at 240 g. The pellet was resuspended in 10 mL of MEM supplemented with HEPES (10 mM) and 1 % v/v penicillin /streptomycin sulphate and centrifuged again at 4 °C for 5 minutes at 240 g. This was repeated 3 times. The final pellets were resuspended in cryopreservation media (90 % FBS and 10% DMSO) and cryovials maintained at -80°c for 24 hours before being transferred to liquid nitrogen (-196°C) for long term storage until use.

5.4 Development of *in-vitro* BBB permeable insert models

For PBMEC/C1-2, 1 x10^5 cells/cm^2 were seeded onto collagen coated (5 µg/cm^2) 12-well inserts (Greiner ThinCert®) and maintained in a 1:1 mixture of C12 media and astrocyte conditioning media (ACM), termed C12-ACM, and supplemented with 1 µg/mL fibronectin,

to support the development of an appropriately tight endothelial monolayer. Cells were used in transport studies when the trans-endothelial electrical resistance (TEER) was > 50 Ω.cm^2 [53, 66, 69]. ACM was obtained from the supernatant of rat C6 glioma cells grown in C12 media in T75 flasks. The supernatant was sterile filtered (0.22 μm filter) and stored at 4 °C.

For primary PBMEC 1 x10^5 cells/cm^2 ('60s') were seeded into permeable inserts (Greiner ThinCert™, pore size 1 μm) that had been pre-coated with 300 μg/mL collagen for 3-4 hours in a laminar air hood, and maintained in basic growth media to attach for 24 hours. To enhance the formation of the monolayer, the cell culture media was switched on day 4 to serum free media supplemented with 250 μM CPT-cAMP, 17.5 μM RO20-1724 and 500 nM of hydrocortisone [70, 71] and TEER values used to assess the development of a tight monolayer with an acceptable cut-off of > 800 Ω.cm^2 [68].

For both cell systems, monolayer formation was deemed acceptable using the TEER value criteria alongside < 1 % lucifer yellow (LY) permeation across monolayers. For TEER measurements, a chop-stick electrodes (EVOM, World Precision Instruments, USA) was used and corrected for background resistance (coated inserts without cells) and by the surface area of the insert (1.12 cm^2). The LY permeability assays were conducted in inserts apically exposed to 100 μM of LY prepared in HBSS, for 60 minutes at 37 °C. The basolateral permeation 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.

5.5 Cell culture: murine H1L6.1c2

The stably transfected AhR-responsive luciferase reporter gene cell line (H1L6.1c2) was obtained as a gift from Professor M. Denison [72]. Cells were grown to confluency in 75 cm^2 flasks in α-MEM supplemented with 10 % (v/v) premium fetal bovine serum.

5.6 Reverse-transcriptase polymerase chain reaction

PBMEC/C1-2 and primary cells were seeded at cell density of 5 x10^4 per well in a 6-well plate. Cells were subsequently and transferred into a GenElute Total RNA mini prep kit filtration column and centrifuged at 12,000g for 2 minutes. The lysate was transferred into a new column and centrifuged at 12,000g for 2 minutes. 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 using a Nanodrop system (Thermo Scientific, Nanodrop 1000).

RNA amplification was conducted using the My Taq One-Step RT-PCR kit according to the manufactures instructions. The PCR primers used were as follows: β-actin (accession number: **AY550069**) forward primer 5'- AAGCCAACCGTGAGAAGATG and reverse primer 5'- CAACTAACAGTCCGCCTAGAAG; BCRP (accession number: **NM_214010**) forward primer 5'- TCCGACCACCATGACAAATC, reverse primer 5'- GTACACCGAGCTCTTCTTCTTC. 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.

5.7 Immunofluorescence detection of BCRP

For PBMEC/C1-2 20,000 cells were seeded onto gelatin coated glass cover slips and allowed to adhere for 48 hours. For primary PBMEC, 1.0 x10^5 cells/cm^2 were seeded onto collagen (300 µg/mL)-fibronectin (7.5 µg/mL) coated 12-well permeable insets (Greiner ThinCert®) and grown for 4 days. The cover slips were subsequently washed with phosphate buffered saline (PBS), fixed with methanol at -20 °C for 20 minutes before being blocked with 1 % BSA in PBS for 30 minutes. Cells were subsequently incubated with Anti-BCRP antibody (1:400) for 2 hours at 37 °C and goat anti-rabbit IgG-FITC (1:500) for 45 minutes at room temperature before being mounted with DAPI-containing mounting media. Cover slips were

subsequently analysed in an upright confocal microscope (Leica SP5 TCS II MP) and visualised with a 40x oil immersion objective. Images were acquired using an argon laser at 494 nm to visualise FITC and a helium–neon laser to visualise DAPI at 461 nm.

5.8 Selection of modulators

Modulators were selected based on our previous screening with choroid epithelial cells [21] and included: apigenin, baicalin, biochanin A, chrysin, curcumin, 17-β-estradiol, fisetin, flavone, hesperidin, hesperetin, indole-3-carbinol (I3C), α-napthoflavone, naringin, quercetin, reservatrol, rutin, silymarin and 6,2,4-trimethoxyflavone (TMF).

5.9 Modulator induced cellular toxicity

For PBMEC/C1-2, 6 x10^4 cells per well were seeded into the 96-well plate and allowed to attach for 24 hours followed by incubation with modulators (0.001-1000 μM) for 24 hours. Thereafter 20 μL per well of a 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added and incubated at 37°C 5% CO_2 for 4 hours followed by the addition of 100 μL per well of DMSO and the UV-absorbance of the formazan product determined at a UV-absorbance of 595 nm. Results were expressed as percentage cytotoxicity relative to a control (exposed to 0.5% DMSO only) and the IC50 was subsequently calculated using a sigmoidal dose response function within the Graphpad Prism version 5.0 (GraphPad Software, Inc. USA). For PBMEC, two concentrations were selected based on the outcome of the studies with C12 cells.

5.10 Hoechst 33342 intracellular accumulation assay

The potential for modulators to alter the activity of BCRP was then assessed by measuring changes in the intracellular accumulation of H33342 in the absence and presence of modulators. 2 x10^4 cells PBMEC/C1-2 cells were seeded onto clear-bottomed 96-well plates for 24 hours. To assess the potential for direct inhibition of BCRP function, cells were pre-treated with 25 μM of modulators (unless or otherwise stated) and incubated at 37 °C for 1

hour. Cells were also incubated with 1 μM Ko143 for 1 hour (used as a positive inhibitor control comparator) or 25 μM *tert*-butyl hydroquinone (TBHQ) (used as a positive inducer control comparator)[40, 73]. Following pre-incubation with modulators or Ko143, media was replaced with fresh media containing 10 μM H33342 and 25 μM of modulators (unless otherwise indicated) and incubated for a further 30 minutes. Thereafter cells were washed twice with ice cold PBS and lysed at -80°C for 20 minutes before being resuspended in 100 μL of water and fluorescence measured with dual-scanning microplate spectrofluorometer (Spectra Max Gemini XS, molecular devices, Sunnyvale, California) at an excitation wavelength of 355 nm and emission wavelength of 460 nm.

To assess the potential for long-term BCRP modulation (possibly through transcriptional/translation effects), cells were pre-incubated with 25 μM of modulators (unless otherwise stated) for 24 hours in PBMEC media at 37°C. Following pre-incubation, the cells were treated as described above.

5.11 Modulation of BCRP protein expression

In an attempt to assess the impact of modulators on BCRP protein expression and confirm observations from the H33342 intracellular accumulation assay, PBMEC/C1-2 cells were grown to confluency and treated with 25 μM of modulator (unless otherwise stated) for 24 hours. Cells were subsequently treated with 0.5% trypsin-EDTA to obtain whole cell lysates. The obtained cell suspension was centrifuged at 2,000 rpm for 5 minutes and the pellet was incubated with 40 μL radioimmuniprecipitation (RIPA) lysis buffer (Santa Cruz Biotechnology, Texas, USA) before being homogenised by ultra-sonication on ice. The resultant cell debris was then removed by centrifugation at 16,000g and 4 °C for 30 minutes and the supernatant protein content determined by a bicinchoninic acid assay (BCA).

70 μg of protein was subsequently loaded into each well of a 8 % pre-cast SDS-PAGE gel (Optiblot, Abcam) and gel electrophoresis conducted according to the manufacturer's

instructions (Optiblot Electrophoresis Kit, Abcam). Proteins were transferred onto a PVDF membrane and blotted before being blocked with 5 % milk powder in TBST for 1 hour at room temperature and incubated with anti-BCRP (Sigma-Aldrich, UK) for 24 hours at 4°C (1:4000) and goat anti-rabbit IgG conjugated horseradish peroxidase (1:7500). β-actin loading control was detected by HRP-conjugated beta-actin antibody (C4) (1:7500). All bands were subsequently visualised by an incubation with an enhanced chemiluminescence (ECL) solution (90mM *p*-coumaric acid, 250mM luminol, 1M tris and 30% H_2O_2) before X-ray film development. The functional consequences of changes in BCRP expression were subsequently assessed in an *in-vitro* permeable insert model.

5.12 Quantitative polymerase chain analysis

RNA obtained from PBMEC/C1-2 were reverse transcribed using a Nanoscript reverse transcription kit (PrimerDesign, UK). Gene detection analysis was conducted using a SYBER green Precision Master Mix (PrimerDesign, UK) and a custom designed gene detection kit for *BCRP* (PrimerDesign, UK). The house keeping gene used was *HPRT1* (accession number: **DQ845175**) forward primer 5'- GGTCAAGCAGCATAATCCAAAG and reverse primer 5'-CAAGGGCATAGCCTACCACAA). To assess modulator-mediated genomic changes *BCRP*, modulators showing up- or down-regulation of BCRP protein were selected and incubated with PBMEC/C1-2 cells (seeded at cell density of 5 $x10^4$ per well in a 6-well plate) for 24-hours prior to RNA extraction and qPCR analysis. *BCRP* expression in PBMEC/C1-2 and primary cells was also confirmed through non-modulator control wells (0.5 % DMSO). Results are presented as *BCRP* normalised RNA levels (arbitrary units) normalised to *HPRT1* using relative with a $2^{\wedge ddCT}$ method used to describe the fold-change compared to untreated samples.

5.13 AhR mediated regulation of BCRP in PBMEC/C1-2

Dicer siRNA duplexes were custom synthesised by IDTDna for targeting of porcine *AhR* (NM_001303026); sense: 5'-ACACAUUGAAAUAGGUGCCUUAUUCUU-3' and antisense: 3'-TGUGUAACUUUAUCCACGGAAUAAG-5'. Cell density and transfection efficacy was optimised using a fluorescently tagged siRNA control duplex (TYE® 563). PBMEC/C1-2 were seeded into a 12-well plate at a seeding density of 100,000 cells/well and allowed to attach for 24 hours. Cells were subsequently washed and siRNA duplex prepared with the cationic transfection reagent TransIT (Mirus Bio, Madison, USA) according to manufacturer's instruction and incubated with cells (10 nM final concentration) for 12-hours. In addition to the use of siRNA downregulate *AhR*, chemical downregulation was assessed by incubating cells with the *AhR* antagonist CH223191 at a concentration of 1 μM and 10 μM for 24 hours. Subsequent changes in *AhR* and *BCRP* gene expression following siRNA silencing or CH223191 downregulation were assessed using qPCR. The modulation of *BCRP* and *AhR* gene expression was further assessed following the incubation of modulators identified as possible BCPR inducers/down-regulators from western blotting studies, for 24 hours following silencing of *AhR*.

5.14 Luciferase activation assay

To assess the potential of modulators to activate AhR pathways, H1L6.1c2 cells were seeded into 96-well plates at a seeding density of 75,000 cells/well and allowed to attach overnight. The activation of AhR was confirmed by exposure to a 7-fold log concentration range (0.0001-1000 μM) of the known AhR activator omeprazole for 24 hours. Cell were washed with warm PBS before 20 μL 1X Lysis Buffer (Promega) was added to each well and the plates shaken for 20 minutes (1000 rpm) on an orbital plate shaker. 20 μL of the cell lysate was then transferred into wells of an opaque 96-well plate and 100 μL of the Luciferase Assay Reagent was added per well. The luminescence was read immediately and at 10

seconds intervals for a duration of 60 seconds (SpectraMax MX5 reader: Molecular Devices LLC, Sunnyvale, CA). Modulators were prepared at a fixed optimal concentration (determined from the H33342 intracellular accumulation assay) and the assay conducted as described above. Results are presented as percent activation relative to the maximum response from omeprazole.

5.15 Functional expression of BCRP in an *in vitro* permeable insert BBB model

For assessing the functional consequences of a change in BCRP protein expression, modulators were incubated for 24 hours in fresh maintenance media in both compartments, prior to the addition of fresh SFM containing 10 μM pheophorbide (PhA) only into the apical compartment and SFM into the basolateral compartment. For assessing the ability to inhibit BCRP function, modulators (or 1 μM Ko143) were preincubated with monolayers in SFM for 30 minutes in both apical and basolateral compartments. Thereafter the media was replaced in the apical compartment with SFM containing modulator (or Ko143) and 10 μM PhA, and SFM only into the basolateral compartment. For assessing BCRP inducers, PhA was added into the basolateral compartment. Samples were taken from the inner (inducers) or outer (inhibitor/downregulator) well at 0, 30, 60, 90, 120, 150 and 180 minutes and replaced with fresh warm SFM with PhA concentrations analysed using a fluorescent plate reader (SpectraMax MX5 reader, Molecular Devices LLC, Sunnyvale, CA) with an excitation wavelength of 410 nm and emission of 675 nm.

The apparent permeability coefficient (P_{app}) was calculated according to equation 1:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0} \qquad (1)$$

where 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 available for permeation and C_0 the initial drug concentration in the donor compartment.

5.15 Statistical Analysis

All statistical analyses were performed in Graphpad Prism (La Jolla, California, USA). One-way ANOVA and t-tests were carried out to determine the differences between the mean values. For all multi-well based assay replicates of at least 6 were used in three independent experiments. For western blot and transport studies replicates of at least three were used and repeated in three independent experiments. IC_{50} and EC_{50} metrics were calculated using sigmoidal fit functions within Graphpad Prism. A significance p-value of < 0.05 was considered as statistically significant.

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REFERENCES

1. Abbott, N. J. Dynamics of CNS barriers: evolution, differentiation, and modulation. *Cell Mol Neurobiol* **2005,** *25*, (1), 5-23.

2. Ek, C. J.; Wong, A.; Liddelow, S. A.; Johansson, P. A.; Dziegielewska, K. M.; Saunders, N. R. Efflux mechanisms at the developing brain barriers: ABC-transporters in the fetal and postnatal rat. *Toxicology Letters* **2010,** *197*, (1), 51-59.

3. Kusuhara, H.; Sugiyama, Y. Efflux transport systems for drugs at the blood-brain barrier and blood-cerebrospinal fluid barrier (Part 2). *Drug discovery today* **2001,** *6*, (4), 206-212.

4. Doyle, L. A.; Yang, W.; Abruzzo, L. V.; Krogmann, T.; Gao, Y.; Rishi, A. K.; Ross, D. D. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* **1998,** *95*, (26), 15665-70.

5. Allikmets, R.; Schriml, L. M.; Hutchinson, A.; Romano-Spica, V.; Dean, M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer research* **1998,** *58*, (23), 5337-9.

6. Miyake, K.; Mickley, L.; Litman, T.; Zhan, Z.; Robey, R.; Cristensen, B.; Brangi, M.; Greenberger, L.; Dean, M.; Fojo, T.; Bates, S. E. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer research* **1999,** *59*, (1), 8-13.

7. Wang, H.; Negishi, M. Transcriptional regulation of cytochrome p450 2B genes by nuclear receptors. *Current drug metabolism* **2003,** *4*, (6), 515-25.

8. Xu, C.; Li, C. Y.; Kong, A. N. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Archives of pharmacal research* **2005,** *28*, (3), 249-68.

9. Mahringer, A.; Fricker, G. BCRP at the blood-brain barrier: Genomic regulation by 17β-estradiol. *Molecular Pharmaceutics* **2010,** *7*, (5), 1835-1847.

10. Hartz, A.; Bauer, B. ABC transporters in the CNS–an inventory. *Curr. Pharm. Biotechnol* **2011,** *12*, 656-673.

11. Bauer, B.; Yang, X.; Hartz, A. M.; Olson, E. R.; Zhao, R.; Kalvass, J. C.; Pollack, G. M.; Miller, D. S. In vivo activation of human pregnane X receptor tightens the blood-brain barrier to methadone through P-glycoprotein up-regulation. *Molecular pharmacology* **2006,** *70*, (4), 1212-9.

12. Dauchy, S.; Dutheil, F.; Weaver, R. J.; Chassoux, F.; Daumas-Duport, C.; Couraud, P. O.; Scherrmann, J. M.; De Waziers, I.; Decleves, X. ABC transporters, cytochromes P450 and their main transcription factors: expression at the human blood-brain barrier. *Journal of neurochemistry* **2008,** *107*, (6), 1518-28.

13. Dauchy, S.; Miller, F.; Couraud, P. O.; Weaver, R. J.; Weksler, B.; Romero, I. A.; Scherrmann, J. M.; De Waziers, I.; Decleves, X. Expression and transcriptional regulation of ABC transporters and cytochromes P450 in hCMEC/D3 human cerebral microvascular endothelial cells. *Biochem Pharmacol* **2009,** *77*, (5), 897-909.

14. Wang, X.; Hawkins, B. T.; Miller, D. S. Aryl hydrocarbon receptor-mediated up-regulation of ATP-driven xenobiotic efflux transporters at the blood-brain barrier. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2011,** *25*, (2), 644-52.

15. Poland, A.; Glover, E.; Kende, A. S. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. *The Journal of biological chemistry* **1976,** *251*, (16), 4936-46.

16. Suzuki, H.; Sugiyama, Y. Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *European journal of pharmaceutical sciences* **2000,** *12*, (1), 3-12.

17. Allen, J. D.; van Loevezijn, A.; Lakhai, J. M.; van der Valk, M.; van Tellingen, O.; Reid, G.; Schellens, J. H.; Koomen, G. J.; Schinkel, A. H. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Molecular cancer therapeutics* **2002,** *1*, (6), 417-25.

18. Breedveld, P.; Beijnen, J. H.; Schellens, J. H. Use of P-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs. *Trends in pharmacological sciences* **2006,** *27*, (1), 17-24.

19. Donovan, J. L.; Manach, C.; Faulks, R. M.; Kroon, P. A., Absorption and metabolism of dietary secondary metabolites. In *Plant Secondary Metabolites. Occurrence, Structure and Role in the Human Diet*, Crozier, A.; Clifford, M. N.; Ashihara, H., Eds. Blackwell Publishing: Oxford, UK, 2006; pp 303–351.

20. Day, A. J.; Canada, F. J.; Diaz, J. C.; Kroon, P. A.; McLauchlan, R.; Faulds, C. B.; Plumb, G. W.; Morgan, M. R.; Williamson, G. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS letters* **2000,** *468*, (2-3), 166-70.

21. Kaur, M.; Badhan, R. K. Phytoestrogens Modulate Breast Cancer Resistance Protein Expression and Function at the Blood-Cerebrospinal Fluid Barrier. *J Pharm Pharm Sci* **2015,** *18*, (2), 132-54.

22. Xiong, H.; Callaghan, D.; Jones, A.; Bai, J.; Rasquinha, I.; Smith, C.; Pei, K.; Walker, D.; Lue, L.-F.; Stanimirovic, D.; Zhang, W. ABCG2 is up-regulated in Alzheimer's brain with cerebral amyloid angiopathy and may act as a gatekeeper at the blood-brain barrier for Aβ(1-40) peptides. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2009,** *29*, (17), 5463-5475.

23. Peng, H. W.; Cheng, F. C.; Huang, Y. T.; Chen, C. F.; Tsai, T. H. Determination of naringenin and its glucuronide conjugate in rat plasma and brain tissue by high-performance liquid chromatography. *Journal of chromatography. B, Biomedical sciences and applications* **1998,** *714*, (2), 369-74.

24. Youdim, K. A.; Dobbie, M. S.; Kuhnle, G.; Proteggente, A. R.; Abbott, N. J.; Rice-Evans, C. Interaction between flavonoids and the blood-brain barrier: in vitro studies. *Journal of neurochemistry* **2003,** *85*, (1), 180-92.

25. Youdim, K. A.; Qaiser, M. Z.; Begley, D. J.; Rice-Evans, C. A.; Abbott, N. J. Flavonoid permeability across an in situ model of the blood-brain barrier. *Free Radic Biol Med* **2004,** *36*, (5), 592-604.

26. Bai, H.; Jin, H.; Yang, F.; Zhu, H.; Cai, J. Apigenin induced MCF-7 cell apoptosis-associated reactive oxygen species. *Scanning* **2014,** *36*, (6), 622-31.

27. Choudhury, D.; Ganguli, A.; Dastidar, D. G.; Acharya, B. R.; Das, A.; Chakrabarti, G. Apigenin shows synergistic anticancer activity with curcumin by binding at different sites of tubulin. *Biochimie* **2013,** *95*, (6), 1297-309.

28. Li, X.; Liu, Y.; Wu, T.; Jin, Y.; Cheng, J.; Wan, C.; Qian, W.; Xing, F.; Shi, W. The Antiviral Effect of Baicalin on Enterovirus 71 In Vitro. *Viruses* **2015,** *7*, (8), 4756-71.

29. Kong, Y.; Ma, W.; Liu, X.; Zu, Y.; Fu, Y.; Wu, N.; Liang, L.; Yao, L.; Efferth, T. Cytotoxic activity of curcumin towards CCRF-CEM leukemia cells and its effect on DNA damage. *Molecules (Basel, Switzerland)* **2009,** *14*, (12), 5328-38.

30. Santostefano, M.; Merchant, M.; Arellano, L.; Morrison, V.; Denison, M. S.; Safe, S. alpha-Naphthoflavone-induced CYP1A1 gene expression and cytosolic aryl hydrocarbon receptor transformation. *Molecular pharmacology* **1993,** *43*, (2), 200-6.

31. Wu, M. L.; Li, H.; Wu, D. C.; Wang, X. W.; Chen, X. Y.; Kong, Q. Y.; Ma, J. X.; Gao, Y.; Liu, J. CYP1A1 and CYP1B1 expressions in medulloblastoma cells are AhR-independent and have no direct link with resveratrol-induced differentiation and apoptosis. *Neuroscience letters* **2005,** *384*, (1-2), 33-7.

32. Neuhaus, J.; Risau, W.; Wolburg, H. Induction of blood-brain barrier characteristics in bovine brain endothelial cells by rat astroglial cells in transfilter coculture. *Annals of the New York Academy of Sciences* **1991,** *633*, 578-80.

33. Chearwae, W.; Shukla, S.; Limtrakul, P.; Ambudkar, S. V. Modulation of the function of the multidrug resistance-linked ATP-binding cassette transporter ABCG2 by the cancer chemopreventive agent curcumin. *Molecular cancer therapeutics* **2006,** *5*, (8), 1995-2006.

34. Chearwae, W.; Shukla, S.; Limtrakul, P.; Ambudkar, S. V. Modulation of the function of the multidrug resistance–linked ATP-binding cassette transporter ABCG2 by the cancer chemopreventive agent curcumin. *American Association for Cancer Research* **2006,** *5*, (8), 1995-2006.

35. Hartz, A. M. S.; Mahringer, A.; Miller, D. S.; Bauer, B. 17-β-Estradiol: a powerful modulator of blood–brain barrier BCRP activity. *Journal of Cerebral Blood Flow & Metabolism* **2010,** *30*, (10), 1742-1755.

36. Imai, Y.; Tsukahara, S.; Asada, S.; Sugimoto, Y. Phytoestrogens/flavonoids reverse breast cancer resistance protein/ABCG2-mediated multidrug resistance. *Cancer research* **2004,** *64*, (12), 4346-52.

37. Dreiseitel, A.; Oosterhuis, B.; Vukman, K. V.; Schreier, P.; Oehme, A.; Locher, S.; Hajak, G.; Sand, P. G. Berry anthocyanins and anthocyanidins exhibit distinct affinities for the efflux transporters BCRP and MDR1. *British journal of pharmacology* **2009,** *158*, (8), 1942-50.

38. Fleisher, B.; Unum, J.; Shao, J.; An, G. Ingredients in Fruit Juices Interact with Dasatinib Through Inhibition of BCRP: A New Mechanism of Beverage–Drug Interaction. *Journal of Pharmaceutical Sciences* **2015,** *104*, (1), 266-275.

39. Pick, A.; Müller, H.; Mayer, R.; Haenisch, B.; Pajeva, I. K.; Weigt, M.; Bönisch, H.; Müller, C. E.; Wiese, M. Structure–activity relationships of flavonoids as inhibitors of breast cancer resistance protein (BCRP). *Bioorganic & Medicinal Chemistry* **2011,** *19*, (6), 2090-2102.

40. Ebert, B.; Seidel, A.; Lampen, A. Phytochemicals induce breast cancer resistance protein in Caco-2 cells and enhance the transport of benzo[a]pyrene-3-sulfate. *Toxicological sciences : an official journal of the Society of Toxicology* **2007,** *96*, (2), 227-36.

41. Zhang, S.; Yang, X.; Morris, M. E. Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. *Molecular pharmacology* **2004,** *65*, (5), 1208-1216.

42. Tompkins, L. M.; Li, H.; Li, L.; Lynch, C.; Xie, Y.; Nakanishi, T.; Ross, D. D.; Wang, H. A novel xenobiotic responsive element regulated by aryl hydrocarbon receptor is involved in the induction of BCRP/ABCG2 in LS174T cells. *Biochemical pharmacology* **2010,** *80*, (11), 1754-1761.

43. Tan, K. P.; Wang, B.; Yang, M.; Boutros, P. C.; Macaulay, J.; Xu, H.; Chuang, A. I.; Kosuge, K.; Yamamoto, M.; Takahashi, S.; Wu, A. M.; Ross, D. D.; Harper, P. A.; Ito, S. Aryl hydrocarbon receptor is a transcriptional activator of the human breast cancer resistance protein (BCRP/ABCG2). *Molecular pharmacology* **2010,** *78*, (2), 175-85.

44. Mukai, R.; Shirai, Y.; Saito, N.; Fukuda, I.; Nishiumi, S.; Yoshida, K.; Ashida, H. Suppression mechanisms of flavonoids on aryl hydrocarbon receptor-mediated signal transduction. *Archives of biochemistry and biophysics* **2010,** *501*, (1), 134-41.

45. Li, L.; Stanton, J. D.; Tolson, A. H.; Luo, Y.; Wang, H. Bioactive terpenoids and flavonoids from Ginkgo biloba extract induce the expression of hepatic drug-metabolizing enzymes through pregnane X receptor, constitutive androstane receptor, and aryl hydrocarbon receptor-mediated pathways. *Pharmaceutical research* **2009,** *26*, (4), 872-82.

46. Kubo, Y.; Ohtsuki, S.; Uchida, Y.; Terasaki, T. Quantitative Determination of Luminal and Abluminal Membrane Distributions of Transporters in Porcine Brain Capillaries by Plasma Membrane Fractionation and Quantitative Targeted Proteomics. *J Pharm Sci* **2015,** *104*, (9), 3060-8.

47. Vrba, J.; Kren, V.; Vacek, J.; Papouskova, B.; Ulrichova, J. Quercetin, Quercetin Glycosides and Taxifolin Differ in their Ability to Induce AhR Activation and CYP1A1 Expression in HepG2 Cells. *Phytotherapy Research* **2012,** *26*, (11), 1746-1752.

48. Kannan, P.; Telu, S.; Shukla, S.; Ambudkar, S. V.; Pike, V. W.; Halldin, C.; Gottesman, M. M.; Innis, R. B.; Hall, M. D. The "Specific" P-Glycoprotein Inhibitor Tariquidar Is Also a Substrate and an Inhibitor for Breast Cancer Resistance Protein (BCRP/ABCG2). *ACS Chemical Neuroscience* **2011,** *2*, (2), 82-89.

49. Bauer, M.; Karch, R.; Zeitlinger, M.; Stanek, J.; Philippe, C.; Wadsak, W.; Mitterhauser, M.; Jäger, W.; Haslacher, H.; Müller, M.; Langer, O. Interaction of (11)C-Tariquidar and (11)C-Elacridar with P-glycoprotein and Breast Cancer Resistance Protein at the Human Blood-Brain Barrier. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **2013,** *54*, (8), 10.2967/jnumed.112.118232.

50. Deeley, R. G.; Westlake, C.; Cole, S. P. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. *Physiological reviews* **2006,** *86*, (3), 849-99.

51. Januchowski, R.; Wojtowicz, K.; Andrzejewska, M.; Zabel, M. Expression of MDR1 and MDR3 gene products in paclitaxel-, doxorubicin- and vincristine-resistant cell lines. *Biomedicine & Pharmacotherapy* **2014,** *68*, (1), 111-117.

52. Neuhaus, W.; Lauer, R.; Oelzant, S.; Fringeli, U. P.; Ecker, G. F.; Noe, C. R. A novel flow based hollow-fiber blood-brain barrier in vitro model with immortalised cell line PBMEC/C1-2. *J Biotechnol* **2006,** *125*, (1), 127-41.

53. Neuhaus, W.; Stessl, M.; Strizsik, E.; Bennani-Baiti, B.; Wirth, M.; Toegel, S.; Modha, M.; Winkler, J.; Gabor, F.; Viernstein, H.; Noe, C. R. Blood-brain barrier cell line PBMEC/C1-2 possesses functionally active P-glycoprotein. *Neuroscience letters* **2010,** *469*, (2), 224-8.

54. Weksler, B. B.; Subileau, E. A.; Perriere, N.; Charneau, P.; Holloway, K.; Leveque, M.; Tricoire-Leignel, H.; Nicotra, A.; Bourdoulous, S.; Turowski, P.; Male, D. K.; Roux, F.; Greenwood, J.; Romero, I. A.; Couraud, P. O. Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2005,** *19*, (13), 1872-4.

55. Hatherell, K.; Couraud, P. O.; Romero, I. A.; Weksler, B.; Pilkington, G. J. Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation Transwell models. *Journal of neuroscience methods* **2011,** *199*, (2), 223-9.

56. Neuhaus, W.; Plattner, V. E.; Wirth, M.; Germann, B.; Lachmann, B.; Gabor, F.; Noe, C. R. Validation of in vitro cell culture models of the blood-brain barrier: tightness characterization of two promising cell lines. *J Pharm Sci* **2008,** *97*, (12), 5158-75.

57. Novakova, I.; Subileau, E. A.; Toegel, S.; Gruber, D.; Lachmann, B.; Urban, E.; Chesne, C.; Noe, C. R.; Neuhaus, W. Transport rankings of non-steroidal antiinflammatory drugs across blood-brain barrier in vitro models. *PLoS One* **2014,** *9*, (1), e86806.

58. Pan, M. H.; Huang, T. M.; Lin, J. K. Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug metabolism and disposition: the biological fate of chemicals* **1999,** *27*, (4), 486-94.

59. Perkins, S.; Verschoyle, R. D.; Hill, K.; Parveen, I.; Threadgill, M. D.; Sharma, R. A.; Williams, M. L.; Steward, W. P.; Gescher, A. J. Chemopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev* **2002,** *11*, (6), 535-40.

60. Cheng, K. K.; Yeung, C. F.; Ho, S. W.; Chow, S. F.; Chow, A. H. L.; Baum, L. Highly Stabilized Curcumin Nanoparticles Tested in an In Vitro Blood–Brain Barrier Model and in Alzheimer's Disease Tg2576 Mice. *The AAPS Journal* **2013,** *15*, (2), 324-336.

61. Suresh, D.; Srinivasan, K. Tissue distribution & elimination of capsaicin, piperine & curcumin following oral intake in rats. *Indian J Med Res* **2010,** *131*, 682-91.

62. Chiu, S. S.; Lui, E.; Majeed, M.; Vishwanatha, J. K.; Ranjan, A. P.; Maitra, A.; Pramanik, D.; Smith, J. A.; Helson, L. Differential distribution of intravenous curcumin formulations in the rat brain. *Anticancer research* **2011,** *31*, (3), 907-11.

63. Tsai, T.-H. Determination of Naringin in Rat Blood, Brain, Liver, and Bile Using Microdialysis and Its Interaction with Cyclosporin A, a P-Glycoprotein Modulator. *J Agr Food Chem* **2002,** *50*, (23), 6669-6674.

64. Bagad, M.; Khan, Z. A. Poly(n-butylcyanoacrylate) nanoparticles for oral delivery of quercetin: preparation, characterization, and pharmacokinetics and biodistribution studies in Wistar rats. *International journal of nanomedicine* **2015,** *10*, 3921-35.

65. Ravindranath, V.; Chandrasekhara, N. In vitro studies on the intestinal absorption of curcumin in rats. *Toxicology* **1981,** *20*, (2-3), 251-7.

66. Teifel, M.; Friedl, P. Establishment of the permanent microvascular endothelial cell line PBMEC/C1-2 from porcine brains. *Exp Cell Res* **1996,** *228*, (1), 50-7.

67. Neuhaus, W.; Trzeciak, J.; Lauer, R.; Lachmann, B.; Noe, C. R. APTS-labeled dextran ladder: a novel tool to characterize cell layer tightness. *J Pharm Biomed Anal* **2006,** *40*, (4), 1035-9.

68. Patabendige, A.; Skinner, R. A.; Morgan, L.; Joan Abbott, N. A detailed method for preparation of a functional and flexible blood–brain barrier model using porcine brain endothelial cells. *Brain Research* **2013,** *1521*, (0), 16-30.

69. Lauer, R.; Bauer, R.; Linz, B.; Pittner, F.; Peschek, G. A.; Ecker, G.; Friedl, P.; Noe, C. R. Development of an in vitro blood–brain barrier model based on immortalized porcine brain microvascular endothelial cells. *Il Farmaco* **2004,** *59*, (2), 133-137.

70. Hoheisel, D.; Nitz, T.; Franke, H.; Wegener, J.; Hakvoort, A.; Tilling, T.; Galla, H. J. Hydrocortisone reinforces the blood-brain properties in a serum free cell culture system. *Biochemical and biophysical research communications* **1998,** *247*, (2), 312-5.

71. Rubin, L. L.; D.E.Hall; S.Porter; K.Barbu; C.Cannon; H.C.Horner; M.Janatpour; C.W.Liaw; K.Manning; J.Morales; L.I.Tanner; K.J.Tomaselli; F.Bard. A cell culture model of the blood-brain barrier. *The Journal of Cell Biology* **1991,** *115*, (6), 1725-1735.

72. Han, D.; Nagy, S. R.; Denison, M. S. Comparison of recombinant cell bioassays for the detection of Ah receptor agonists. *BioFactors* **2004,** *20*, (1), 11-22.

73. Ebert, B.; Seidel, A.; Lampen, A. Identification of BCRP as transporter of benzo[a]pyrene conjugates metabolically formed in Caco-2 cells and its induction by Ah-receptor agonists. *Carcinogenesis* **2005,** *26*, (10), 1754-63.

Figure Legend

Figure 1. Expression and localisation of BCRP in cells. (A) Amplicon products for loading control (ACTB) and BCRP; (B) localisation of BCRP in PBMEC/C1-2 and (C) localisation of BCRP in and PBMEC ('60s' fraction). Cells were grown on the coverslips for 2-3 days (PBMEC/C1-2) or permeable inserts (PBMEC), fixed with methanol at -20 °C for 20 minutes and stained for BCRP using the anti-ABCG2 primary antibody and goat anti-rabbit IgG-FITC secondary antibody (Green). Cell nuclei were visualized using DAPI (Blue). Negative: excludes anti-ABCG2 antibody but includes FITC secondary; Positive: includes primary and secondary antibodies. Unless otherwise states an objective lens of 40x was used.

Figure 2. Cellular cytotoxicity of modulators of BCRP on PBMEC/C1-2. Cells were seeded and grown on a 96-well plate for 48 hours prior to exposure to modulators over a concentration range of 0.001-1000 μM for 24 hours. Subsequently 20μl of MTT was added into each well and the plate incubated for 4 hours. The media was aspirated and 100 μl of DMSO was added before the absorbance of each well was read at 595 nm. Data presented for each modulator is reported as result from up to 8 replications per compound in three independent experiments. ND: not determined.

Figure 3. Cellular cytotoxicity of modulators of BCRP on primary PBMEC. Cells were seeded and grown on a 96-well plate for 48 hours prior to exposure to modulators at 25 μM and 100 μM for 24 hours. Subsequently 20μl of MTT was added into each well and the plate incubated for 4 hours. The media was aspirated and 100 μl of DMSO was added before the absorbance of each well was read at 595 nm. Data presented for each modulator is reported as result from up to 8 replications per compound in three independent experiments.

Figure 4. H33342 accumulation assay for BCRP function in PBMEC/C1-2 cells. Cells were grown in a 96 well plate for 48 hours and subsequently washed with HBSS. Cells were then incubated for 1 hour (A) or 24 hours (B) with media containing 0.01-1 μM or 1-100 μM (as

indicated) of test compound. At the end of the incubation period, cells were washed with ice cold HBSS and incubated with media containing H33342 for 1 hour. Cells were lysed and the intracellular accumulation of H33342 assessed by measuring its absorbance on a fluorescent plate reader (Excitation and emission wavelength of 350 nm and 490 nm respectively). Data is represented as intracellular H33342 accumulation (normalised to control) and reported as mean ± SD, with control being equivalent to a value of 1.0 on the y-axis. Grey shaded areas represent the fold-change range with Ko143 (1 μM) (upper panel) or TBHQ (25 μM) (lower panel) for comparison. Statistical significance was conducted relative to control, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$. # indicates phytochemicals not analysed due to their auto-fluorescence properties.

Figure 5. (A) Western blot protein expression of β-actin and BCRP. (B) Western blot results of modulator incubation with PBMEC/C1-2. (C) Fold-change in BCRP protein expression. Modulators were exposed to cells for 24-hours in culture prior to extraction of protein. Modulator concentrations of 25 μM were utilised, except for curcumin, 17-β-estradiol, α-napthoflavone, baicalin, hesperetin and hesperedin (1 μM). Whole cell lysate (70 μg/lane) were separated on an 8% SDS-Polyacrylamide gel and proteins transferred onto a PVDF membrane and incubated with a 1:4000 dilution of the BCRP primary antibody (anti-ABCG2) overnight followed by a 1:7500 dilution of a HRP-conjugated secondary antibody for 2 hours before development using a ECL method. For detection of the control (β-actin) membranes were incubated with a 1:7500 dilution of HRP-conjugated β-actin primary antibody overnight and detected by the ECL method. Data is represented as mean ± SD of three independent experiments with statistically significant differences between control (fold change = 0) and modulator exposed conditions indicated as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$, N=4.

Figure 6: Modulation of *AhR* and *BCRP* gene expression

PBMEC/C1-2 cells were seeded onto a 24-well plate and incubated with (A) modulators alone, (B) siRNA or CH223191 (1 and 10 μM) and quercetin (25 μM), naringin (25 μM), curcumin (1 μM) and 17-β-estradiol (1 μM) for 24 h to assess changes in *AhR* (C) or *BCRP* (D). RNA was extracted and changes in gene expression determined using qPCR and calculated relative to normalised control samples (absence of modulator, siRNA or CH223191). Significant differences between control and siRNA or CH223191 exposed samples are indicated above the appropriate error bars. Significant differences between each untreated/control condition and each modulator condition is indicated by stars above the error bars. Significant difference in the absence and presence of siRNA are indicated above identifier lines. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001 and **** P ≤ 0.0001. N=3.

Figure 7: The activation of AhR by phytochemical modulators.

H1L6.1c2 cells were seeded to a 96-well plate and incubated with modulators for 24 h and CALUX assay was performed. Modulator concentrations of 25 μM were utilised, except for curcumin, 17-β-estradiol, α-napthoflavone, baicalin, hesperetin and hesperedin (1 μM). Using the luminescence quantified from the maximum activation of AhR by omeprazole (data not shown), the activation of AhR by modulators was reported as percentage maximum omeprazole induction. To simplify the analysis, activation between 1-25 % was classified as 'minimal' with values of greater than 75 % being classified as 'strong' activators of AhR. Eight replicates were used per modulators and replicated across three independent experiments.

Figure 8: Cumulative transport of PhA across an *in-vitro* BBB model.

Cells were grown on permeable inserts and transport studies were performed on day 4 in the absence or presence of Ko143 (1 μM) or modulators (down-regulators: 1 μM curcumin and 1 μM 17-β-estradiol; up-regulators: 25 μM naringin and 25 μM quercetin). The apical

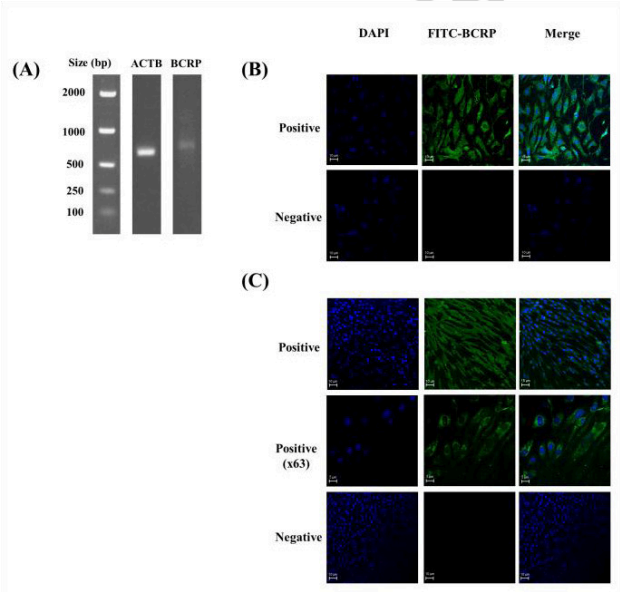
compartment was selected as the donor compartment in all studies except for up-regulators, where the basolateral was selected as the donor compartment. Statistically significant differences between control and Ko143/modulators samples at each data point are indicated. * $P \leq 0.05$. N=5.

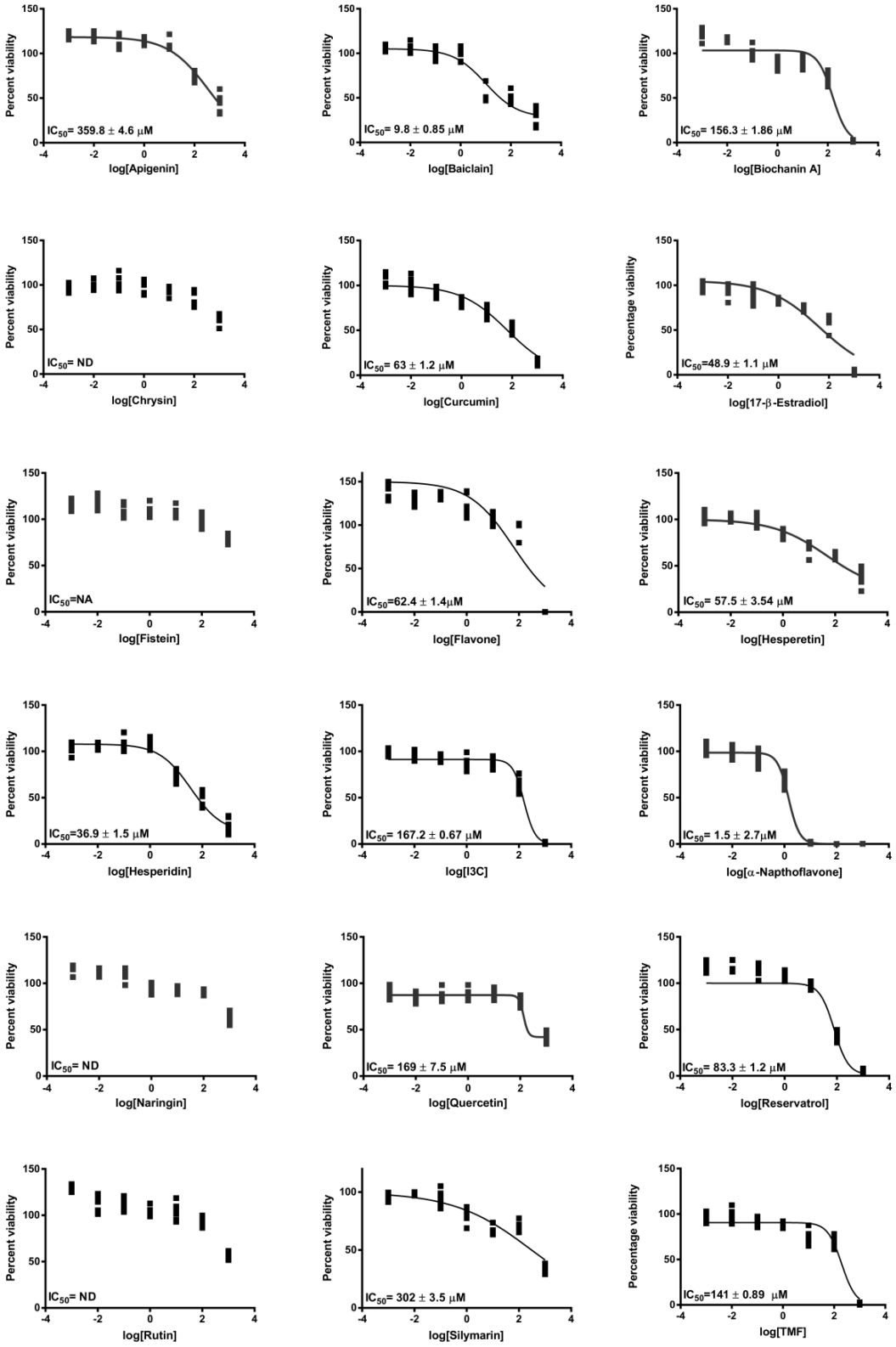
Tables

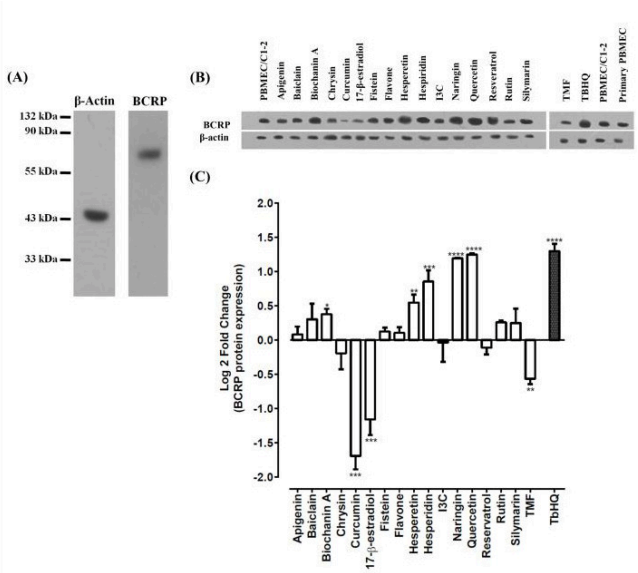
Table 1. Apparent permeability of PhA in the absence and presence of modulators of BCRP

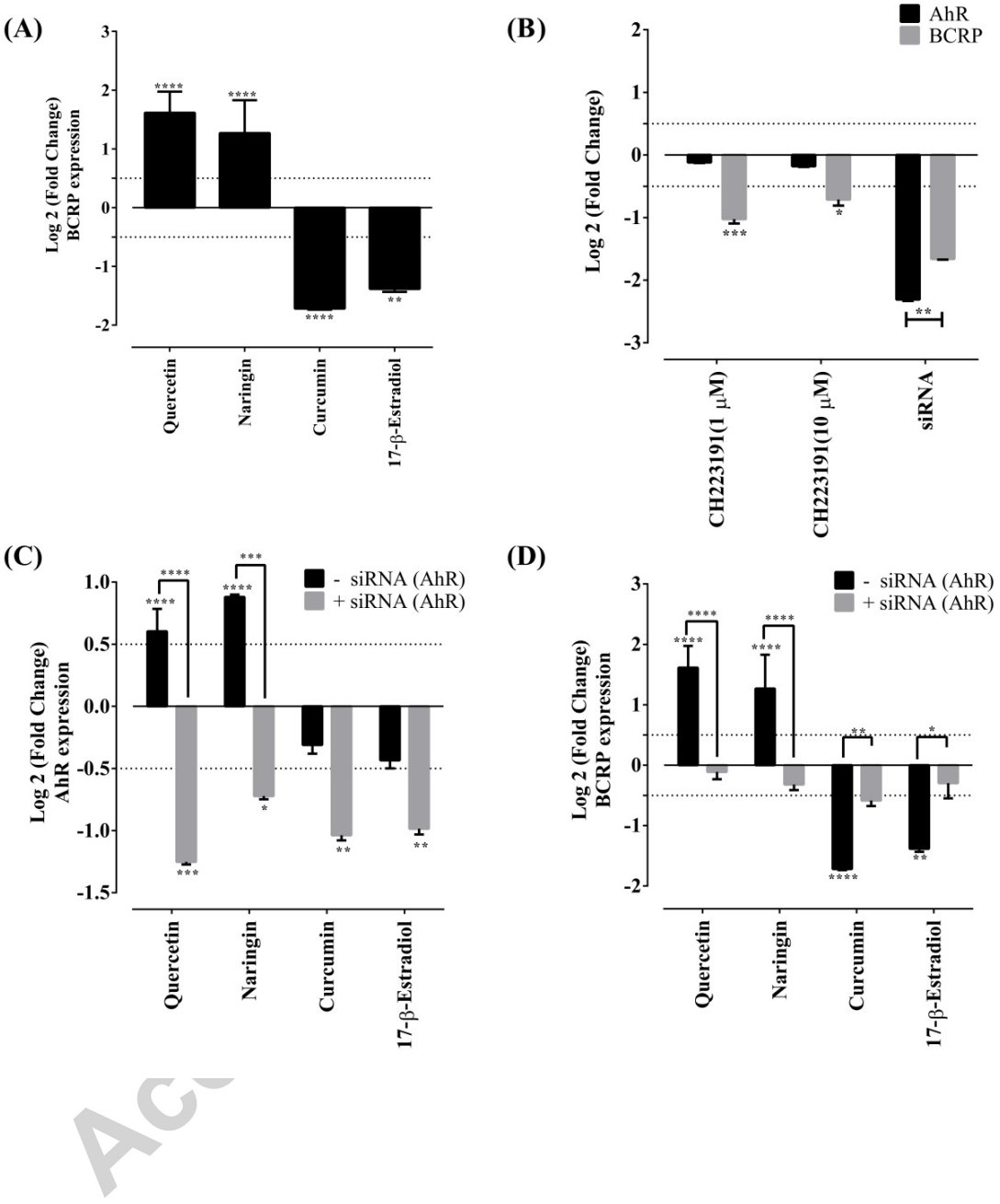
	PBMEC/C1-2	Primary PBMEC
	P_{app} ($x10^{-6}$ cm/s)	
PhA (AB)	7.86 ± 0.23	4.82 ± 0.47
PhA (BA)	44.13 ± 0.31 ***	54.12 ± 1.08 ***
+Ko143 (AB)	17.85 ± 0.32	14.75 ± 0.89
+Ko143 (BA)	30.77 ± 0.58 ***	24.75 ± 1.21 **
+17-β-Estradiol (AB)	32.74 ± 0.34 ***	33.18 ± 3.25 **
+Curcumin (AB)	40.08 ± 0.65 ***	29.88 ± 0.89 ***
+Quercetin (BA)	63.21 ± 0.54 **	66.18 ± 1.98 *
+Naringin (BA)	59.23 ± 0.29 **	58.26 ± 1.23

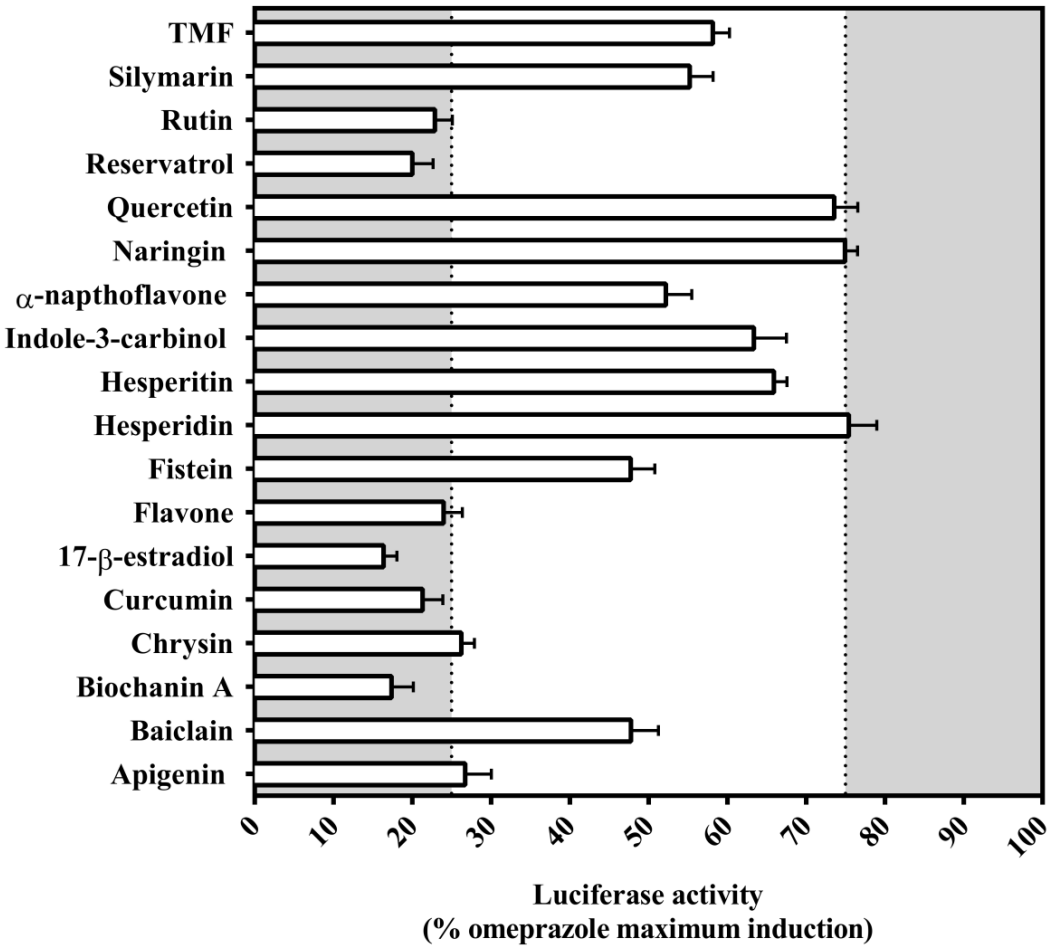
P_{app} measured in the apical to basolateral flux direction. BA indicates studies where the donor compartment is the basolateral and the flux is determined in the basolateral to apical direction. Statistical differences are indicated in superscript and are in comparison to associated PhA transport in AB or BA directions : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

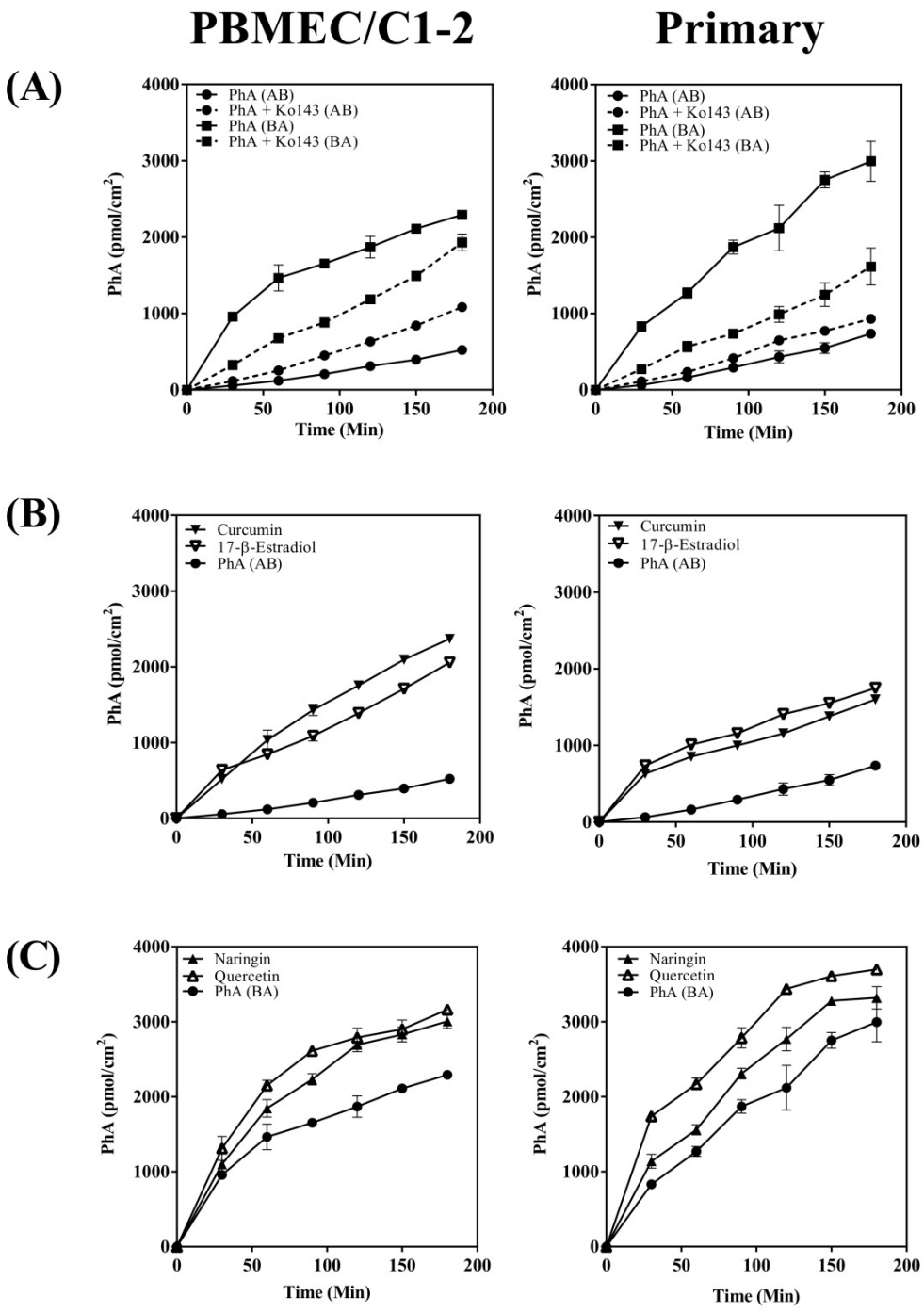



Percent viability
IC50= 359.8 ± 4.6 μM
log[Apigenin]
Percent viability
IC50= 9.8 ± 0.85 μM
log[Baiclain]
Percent viability
IC50= 156.3 ± 1.86 μM
log[Biochanin A]
Percent viability
IC50= ND
log[Chrysin]
Percent viability
IC50= 63 ± 1.2 μM
log[Curcumin]
Percentage viability
IC50=48.9 ± 1.1 μM
log[17-β-Estradiol]
Percent viability
IC50=NA
log[Fistein]
Percent viability
IC50=62.4 ± 1.4μM
log[Flavone]
Percent viability
IC50= 57.5 ± 3.54 μM
log[Hesperetin]
Percent viability
IC50=36.9 ± 1.5 μM
log[Hesperidin]
Percent viability
IC50= 167.2 ± 0.67 μM
log[I3C]
Percent viability
IC50= 1.5 ± 2.7μM
log[α-Napthoflavone]
Percent viability
IC50= ND
log[Naringin]
Percent viability
IC50= 169 ± 7.5 μM
log[Quercetin]
Percent viability
IC50= 83.3 ± 1.2 μM
log[Reservatrol]
Percent viability
IC50= ND
log[Rutin]
Percent viability
IC50= 302 ± 3.5 μM
log[Silymarin]
Percentage viability
IC50=141 ± 0.89 μM
log[TMF]




(A)
Log 2 (Fold Change) BCRP expression
Quercetin
Naringin
Curcumin
17-β-Estradiol
(B)
AhR
BCRP
Log 2 (Fold Change)
CH223191(1 μM)
CH223191(10 μM)
siRNA
(C)
- siRNA (AhR)
+ siRNA (AhR)
Log 2 (Fold Change) AhR expression
Quercetin
Naringin
Curcumin
17-β-Estradiol
(D)
- siRNA (AhR)
+ siRNA (AhR)
Log 2 (Fold Change) BCRP expression
Quercetin
Naringin
Curcumin
17-β-Estradiol


TMF
Silymarin
Rutin
Reservatrol
Quercetin
Naringin
α-napthoflavone
Indole-3-carbinol
Hesperitin
Hesperidin
Fistein
Flavone
17-β-estradiol
Curcumin
Chrysin
Biochanin A
Baiclain
Apigenin
0
10
20
30
40
50
60
70
80
90
100
Luciferase activity
(% omeprazole maximum induction)


PBMEC/C1-2
Primary
(A)
PhA (AB)
PhA + Ko143 (AB)
PhA (BA)
PhA + Ko143 (BA)
PhA (pmol/cm2)
Time (Min)
(B)
Curcumin
17-β-Estradiol
PhA (AB)
(C)
Naringin
Quercetin
PhA (BA)

Highlights

- Naringin and quercetin induced BCRP expression and function
- Curcumin and 17-β-estradiol down-regulated BCRP expression and function
- 2,6,4-trimethoflavone is a potent inhibitor of BCRP function