Utilising a dual human transporter MDCKII-MDR1-BCRP cell line to assess efflux at the Blood Brain Barrier (BBB)

Authors: Nicola Colclough¹, Ravindra V. Alluri², James W. Tucker¹, Elnaz Gozalpour¹, Danxi Li³, Hongwen Du³, Wei Li³, Stephanie Harlfinger¹, Daniel J. O’Neill⁴, Graham G. Sproat⁴, Kan Chen⁵, Yumei Yan⁵, Dermot F. McGinnity¹

Address:

1. DMPK, Oncology R & D, AstraZeneca, Cambridge, United Kingdom
2. Clinical Pharmacology and Safety Sciences, BioPharmaceuticals R&D, AstraZeneca, Cambridge, United Kingdom
3. DMPK, Pharmaron, Beijing, China
4. Discovery Sciences, Biopharmaceuticals R&D, AstraZeneca, Cambridge, United Kingdom
5. DMPK Asia, Oncology R & D, AstraZeneca, Shanghai, China
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Corresponding author: Nicola Colclough, AstraZeneca, 1 Francis Crick Ave, Cambridge, United Kingdom, CB2 0AA

Email: Nicola.Colclough@astrazeneca.com

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Abbreviations

AZ: AstraZeneca; BBB: Blood Brain Barrier; BCA: Bicinchoninic acid; CNS: Central nervous system; DMEM: Dulbecco’s modified Eagle’s medium; DMSO: Dimethylsulphoxide; ER: Efflux ratio; FACS: Fluorescence-activated cell sorting; FBS: Fetal bovine serum; GFP: Green fluorescent protein; HBSS: Hank’s balanced salt solution; HPbCD: Hydroxypropyl-beta-cyclodextrin; IACUC: Institutional Animal Care and Use Committee; LC-MS/MS: Liquid chromatography mass spectroscopy mass spectroscopy; MDCK: Madin-Darby canine kidney; NIH: National Institutes of Health; NKI: Netherlands Cancer Institute; KO: Knock out; PCR:Polymerase chain reaction; PEG400: PEG400; SBEbeta-CD: Sulfobutyl-beta-cyclodextrin; TEER: trans-epithelial electrical resistance; TEG:DMA : Triethylene glycol: Dimethylacetamide; WT: Wild type
Abstract (max 250 words) 240

To facilitate the design of drugs readily able to cross the blood brain barrier (BBB) a MDCK cell line was established that over expresses both P-glycoprotein (Pgp) and Breast Cancer Resistance Protein (BCRP) the main human efflux transporters of the BBB. Proteomics analyses indicate BCRP is expressed at a higher level than Pgp in this cell line. This cell line shows good activity for both transporters (BCRP substrate dantrolene efflux ratio (ER) 16.3 ± 0.9, Pgp substrate quinidine ER 27.5 ± 1.2) and use of selective transport inhibitors enables an assessment of the relative contributions to overall ERs. The MDCKII-MDR1-BCRP ER negatively correlates with rat unbound brain/unbound plasma ratio, K$_{puu}$. Highly brain penetrant compounds with rat K$_{puu}$ ≥ 0.3 show ERs ≤ 2 in the MDCKII-MDR1-BCRP assay whilst compounds predominantly excluded from the brain, K$_{puu}$ ≤ 0.05, demonstrate ERs ≥ 20. A subset of compounds with MDCKII-MDR1-BCRP ER <2 and rat K$_{puu}$ < 0.3 were shown to be substrates of rat Pgp using a rat transfected cell line, MDCKII-rMdr1a. These compounds also showed ERs >2 in the human NIH MDCKI-MDR1 (high Pgp expression) cell line which suggests that they are weak human Pgp substrates. Characterisation of 37 drugs targeting the central nervous system (CNS) in the MDCKII-MDR1-BCRP efflux assay show 36 have ERs < 2. In drug discovery, use of the MDCKII-MDR1-BCRP in parallel with the NIH MDCKI-MDR1 cell line is useful for identification of compounds with high brain penetration.

Significance Statement (max 80 words) 53

A single cell line, that includes both the major human efflux transporters of the BBB, (MDCKII-MDR1-BCRP) has been established facilitating the rapid identification of efflux substrates and enabling the design of brain penetrant molecules. Efflux ratios using this cell line demonstrate a clear relationship with brain penetration as defined by rat brain K$_{puu}$.
Introduction (max 750 words) 737

There is a high need for new treatments for diseases of the brain (Gribkoff and Kaczmarek, 2017; 2018). In oncology, glioblastoma represents the most common primary brain carcinoma making up 16% of all primary brain cancers (Ostrom et al., 2013). Prognosis for patients with these intracranial malignancies, even with standard treatment, is poor. Brain metastases represent the most frequently observed intracranial tumors (Rick et al., 2019), commonly observed in breast and melanoma patients (Rick et al., 2019) and for non-small cell lung cancer between 30-50% of patients will develop brain metastases during the course of their disease (Moro-Sibilot et al., 2015). One reason for the high unmet need in this area is due to the significant challenge of designing drugs which can readily cross the BBB in addition to possessing the necessary potency and selectivity against the pharmacological protein target and pharmacokinetic properties (Bohn et al., 2016; Oberoi et al., 2016).

To maximise the likelihood of identifying successful treatments for intracranial tumors, candidate drugs must have the ability to cross the intact BBB to provide the necessary brain exposure to engage the pharmacological target to the required extent and duration (Sarkaria et al., 2018). As such it is important to ensure that molecules are not efficient substrates for the major human BBB xenobiotic efflux transporters. In drug discovery projects there is a need to rapidly identify and deprioritise such compounds. Structure activity relationships using efflux transporter substrate liability data must be established to facilitate the design of more brain penetrant molecules (Rankovic, 2015; Zeng et al., 2015). Typically, Madin-Darby canine kidney (MDCK) cells, transfected with either the human Pgp transporter (MDCK-MDR1) or human BCRP transporter (MDCK-BCRP), are utilised to this end (Di et al., 2013; Liu et al., 2018; Sato et al., 2021). Other cell lines are also utilised e.g. Epithelial-like pig kidney cell line (LLC-PK1)(Takeuchi et al., 2006; Sato et al., 2020), Caco2 and human CD34+ stem cell-derived endothelial cells (CD34+ECs)(Cecchelli et al., 2014; Moya et al., 2021). In the case of MDCK-MDR1 there are two commonly used cell lines that are commercially available (1) supplied by the Netherlands Cancer Institute (NKI) and (2) supplied by U.S. National Institutes of Health (NIH). These cell lines differ in the level of
expression of human Pgp transporter protein, with the NIH line reported to have 14 times more than the NKI cell line (Feng et al., 2019). The transfected MDCK cells, cultured into monolayers forming tight junctions, serve as a model of the BBB. Efflux transporter substrate liability is determined by the efflux ratio (ER) i.e. the apparent permeability rate constant for the compound crossing the cell monolayer in the basolateral (B) to apical direction (A) direction versus the apical (A) to basolateral (B) direction.

Schinkel et al. have shown that human Pgp and BCRP transporters can both be transfected into MDCKII cells forming a dual transfected BBB model enabling the assessment of the effects of both transporters in a single cell line (Poller et al., 2011). A similar dual transfected MDCKII cell line has been established in-house at AstraZeneca. MDCKII-MDR1 cells supplied by the NKI have been transfected with the BCRP transporter gene ABCG2 to form a MDCKII-MDR1-BCRP cell line. Using this cell line an efflux assay has been established which forms part of the strategy for the evaluation, design and discovery of brain penetrant compounds (Durant et al., 2018; Colclough et al., 2019). In this study the MDCKII-MDR1-BCRP cell line is characterised including an assessment of the relative contributions to efflux of Pgp and BCRP transporters for nine single and dual transporter substrates. In addition, the relationship between ER determined in this dual transporter transfected assay and in-vivo rat unbound brain to unbound plasma ratio, $K_{puu}$, has been investigated to facilitate interpretation of MDCKII-MDR1-BCRP efflux data in the context of in vivo (and inter-species) CNS penetration. This includes an assessment against brain $K_{puu}$ categories (Loryan et al., 2022) used at AZ (high $\geq 0.3$, moderate $0.3 > K_{puu} > 0.05$, low $\leq 0.05$) (Colclough et al., 2019). To better understand rat $K_{puu}$ data and the potential for human versus rat species differences a rat Pgp cell line has also been established in house (MDCKII-rMdr1a) and evaluated. The rat Mdr1a gene was transfected into canine Pgp Knock out (KO) MDCKII cells. Further, a range of CNS available drugs, clinically evaluated in the treatment of brain disease, have been measured in the MDCKII-MDR1-BCRP assay to aid BBB characterisation of compounds.
Materials and methods

Materials

Amantadine, biperiden, buspirone, caffeine, carbamazepine, citalopram, clomipramine, clozapine, dantrolene, dasatinib, desipramine, diazepam, diphenhydramine, doxepin, elacridar, erlotinib, flavopiridol, flumazenil, fluoxetine, haloperidol, hydroxyzine, imatinib, KO143, lamotrigine, mezoridazine, mirtazapine, momelotinib, nortriptyline, olanzapine, paroxetine, phenytoin, PK11195, prazosin, procyclidine, pyrimidone, quinidine, raclopride, risperidone, rosvuastatin, seligiline, spiperone, sulpiride, tacrine, talinolol, trazodone, venlafaxine, volinanserin, WAY 100635 and zosuquidar were synthesized in-house or purchased from Sigma-Aldrich or ICN Biomedicals inc. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Corning or Thermofischer scientific, Hank’s balanced salt solution (HBSS), fetal bovine serum (FBS) were purchased from Gibco, Sigma-Aldrich or Invitrogen. HEPES, Trypsin/EDTA, penicillin and streptomycin were purchased from Sigma-Aldrich or Solarbio. All other reagents used were of analytical grade.

Cells Lines

Human MDR1 transfected MDCKII cells (MDCKII-MDR1) were obtained from the Netherlands Cancer Institute (NKI-AVL) (NKI cell line). This cell line was transfected with human BCRP internally (AstraZeneca, Shanghai, China) to generate the MDCKII-MDR1-BCRP cell line. ABCG2 gene (NCBI reference sequence NM_004827.2) was cloned into pcDNA3.1/Hygro plasmid using restriction based cloning and confirmed using both PCR, restriction enzyme digestion in addition to full plasmid sequencing. ABCG2 was transfected into MDCKII-MDR1 cells using lipofectamine (Thermo Fisher Scientific) according to the manufacturers protocol. Cells with stable ABCG2 expression were selected with Hygromycin and single cell clones were isolated by dilution cloning. Clones were validated by mRNA...
analysis and functional studies were performed. The clone with the highest and most robust
BCRP activity was selected for efflux assays.

Human MDR1 transfected MDCKI cells (MDCKI-MDR1) were obtained from the
National Institutes of Health laboratories (NIH cell line).

MDCKII wild-type (MDCKII-WT cells) were obtained from NKI-AVL. Endogenous
canine Pgp in these cells was knocked out in-house using Crisper-Cas9 technology
generating MDCKII-KO cells. Briefly a sgRNA targeting Pgp exon 1 (5’
GAGTGCAGAGAAGAACTTC 3’) was cloned into a vector containing Cas9 and a GFP
cassette (azPGE02-Cas9-T2A-GFP). MDCKII cells were subsequently transfected with the
vector using lipofectamine 2000 (Thermo Fisher Scientific). After 48 hours GFP-positive cells
were sorted using FACS (Fluorescence-activated cell sorting) and individual clones
isolated by seeding cells at a density of 0.3 cells/well on 384 multi-well plates (Corning).

Following the isolation and validation of a single cell knockout clone (MDCKII-KO), rat Pgp
(Abcb1a (Rattus novegicus, NM_133401.1) cDNA (rMdr1a)) was stably knocked in to
MDCKII-KO cells using sleeping beauty transposase (Ivics et al., 1997). Single cell clones
were isolated to generate a stable MDCKII-rMdr1a cell line.

Caco2 cells were acquired from American Type Culture collection

Quantification of Pgp and BCRP using targeted proteomics

Membrane protein fractions were isolated in-house from MDCKII-MDR1-BCRP and
MDCKII-rMdr1a cells (~3-9 x 10^6) using ProteoExtract Native Membrane Protein Extraction
Kit (Calbiochem; Cat. No. 444810). Protein concentration in the isolated membrane fraction
was determined by the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific)
and samples (~1-2 mg/ml) were shipped to the Unadkat lab (UWRAPT), University of
Washington, Seattle and the Smith Lab, University of North Carolina at Chapel Hill (MDCKII-
MDR1-BCRP cell line analysed at both laboratories MDCKII-rMdr1a at one). Following
trypsin digestion, the amount of transporter protein in each sample was determined using LC-MS/MS (Prasad et al., 2013; Fallon et al., 2016; Billington et al., 2018). Human BCRP expression levels were determined using peptides VIQELGLDK, SSLLDVLAAR, ENLQFSAALR, human Pgp using peptides FYDPLAGK, IIDNKPSIDSYSK, NTTGALTTR, IATEAIENFR and rat Pgp using peptides NTTGALTTR, IATEAIENFR, QLNVQWLR. At the University of Washington human BCRP peptide SSLLDVLAAR and Pgp peptide NTTGALTTR alone were assessed. Results are reported as pmol of transporter protein/mg of membrane protein. Additional proteomic quantification method details are provided in the supplementary information section.

Cell culture

MDCKII-MDR1-BCRP, MDCKII-MDR1, NIH MDCKI-MDR1 and MDCKII-rMdr1a cells were cultured at 37°C in a 5% CO₂ atmosphere using Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum (FBS). For MDCKII-MDR1-BCRP, MDCKII-MDR1 and NIH MDCKI-MDR1 cells 0.1 mg/ml streptomycin and 100 IU/ml of penicillin was included in the cell culture. Cells were passaged using trypsin/EDTA after attaining approximately 85% confluence. For bi-directional transport studies, cells were seeded in 96 well-transwell plates (Corning) at cell density of 5.45 ×10⁵ cells/cm². Medium was replaced every other day and experiments were conducted between 4-7 days post seeding. For the MDCKII-rMdr1a assay cells were seeded in 96 well-transwell plates (Millipore) at a density of 2.7 x 10⁵ cells/cm² with experiments conducted between 5-7 days post seeding.

MDCKII-MDR1-BCRP, MDCKII-MDR1, NIH MDCKI-MDR1 and MDCKII-rMdr1a efflux assays

Prior to commencing efflux assays the integrity of the cell monolayer was confirmed using trans-epithelial electrical resistance (TEER) measurements using a Millipore Millicell
ERS-2 instrument across the cell monolayer. DMSO stocks (10mM) of test compounds were diluted in HBSS pH 7.4 to achieve a working solution of 0.1 µM. For the MDCKII-rMdr1a assay the working solution was 1 µM. Bidirectional transport assays were undertaken by adding the working solution to the apical chamber for A-B transport assessment and to the basolateral chamber for B-A transport assessment. The receiver wells contained HBSS pH 7.4. The efflux assays were run for 2 hours at 37°C without shaking. Samples were collected from donor wells at the start of the incubation (8 µL) and from the donor and acceptor wells (8 and 80 µL) at the end of the incubation. For the MDCKII-rMdr1a assay 5 µL was sampled from the donor wells and 50 µL from the acceptor wells. Donor samples were diluted 10-fold with HBSS pH 7.4 before dilution 4-fold (3-fold for MDCKII-rMdr1a) with acetonitrile containing internal standard. Acceptor samples were diluted 4-fold (3-fold for MDCKII-rMdr1a) directly with acetonitrile containing internal standard. Samples were analysed using LC-MS/MS analysis. Peak area ratios of analyte/internal standard were used for $P_{app}$ calculations. Cell monolayer integrity was checked at the end of the experiment using lucifer yellow transport to determine % leakage.

**Efflux assay data analysis**

The apparent permeability coefficient ($P_{app}$, cm/s) was calculated for each transport direction, A-B and B-A, using the following equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot C_0}$$  \hspace{1cm} (1)

where $dQ/dt$ is the permeation rate (nmol/min), $A$ is the surface area of the insert (0.143 cm$^2$ Corning, 0.11 cm$^2$ Millipore), and $C_0$ is the initial donor concentration (µM). The efflux ratio is calculated as the ratio of B-A $P_{app}$/A-B $P_{app}$.

**Contribution of Pgp and BCRP to efflux in the MDCKII-MDR1-BCRP cell line**
To assess the function of Pgp and BCRP in the MDCKII-MDR1-BCRP cells line bidirectional transport assays were run at 0.1 µM in the presence and absence of the Pgp inhibitor Zosuquidar at 10 µM and BCRP inhibitor KO143 at 1 µM (2 µM for MDCKII-rMdr1a assay). Similarly, studies were run in NKI MDCKII-MDR1 and NIH MDCKI-MDR1 cells in the presence and absence of Pgp inhibitor.

**Caco2 cell passive permeability**

The apparent cell permeability coefficients (Papp pH 7.4/7.4) of compounds were determined using the method outlined in (Colclough et al., 2021) but using Zosquidar, KO-143 and Benzbromarone as the MDR1, BCRP, and MRP transporter inhibitors respectively incubated with the Caco2 monolayers.

**Rat plasma and brain binding**

Fraction unbound in rat plasma (fu_{plasma}) was measured by equilibrium dialysis using the Thermoscientific™ Pierce™ rapid equilibrium device (RED) (Fischer Scientific, Loughborough, LE11 5RG). A cassette of 6-9 compounds at 1 mM in DMSO was spiked into rat plasma giving a concentration of 5 µM and dialysed with buffer at pH 7.4 and 37°C for 18 hours with shaking in a CO2 incubator. After incubation aliquots of both plasma and buffer from the device were taken and added to equal volumes of blank buffer and plasma respectively. Samples were then diluted in acetonitrile containing an internal standard prior to centrifugation and analysis of the supernatants by LC-MS/MS. The fu_{plasma} was calculated as the concentration from the buffer chamber divided by the concentration in the plasma chamber.

Rat fraction unbound in brain (fu_{brain}) was determined using the rat brain slice binding method detailed in reference (Friden et al., 2009). Note fu_{brain} = 1/Vu_{brain} where Vu_{brain} is the unbound brain volume of distribution.
Rat Brain/Plasma Ratios, $K_p$

Study conduct

Rat $K_p$ studies were approved by the Pharmaron Institutional Animal Care and Use Committee (IACUC) and conducted in compliance with AstraZeneca Global Standards and local regulatory requirements. Male Han Wistar rats were purchased from Vital River Laboratory Animal Technology Co. Ltd (China) (8-10 weeks old, 200-300 g) and housed in polycarbonate animal cages containing corncob bedding with acclimatization for a minimum of 3 days prior to study commencement. Animals were housed in a controlled temperature and humidity environment with 12 hours light/12 hours dark cycle and with free access to food and water.

Dose administration and sample collection

A cassette of up to 3 compounds in 1:1:1 TEG:DMA:water was dosed to 3 male Han Wistar rats via jugular vein infusion at 2 $\mu$mol/kg/h at 4 ml/kg for 4 hours. At 4 hours the rats were anesthetised with isoflurane by inhalation and blood collected in plastic tubes containing EDTA-K2 by heart puncture followed by exsanguination by cutting the heart. The rats were then decapitated, and the brain removed from the cranium. Plasma samples were prepared by centrifugation of the blood samples at 4,000g for 5 minutes at 4°C. The brains were weighed, and purified water added using the brain weight: water volume ratio 1:3 prior to homogenisation.

For studies assessing the effect of elacridar, a Pgp and BCRP inhibitor, on brain $K_p$, elacridar was given as an IV bolus at 5 mg/kg 2ml/kg in 10% DMA / 40% PEG400 / 30%HPbCD / 20% water to 3 rats 0.5 hours prior to an IV bolus dose of compound at 1 mg/kg 4 ml/kg in 5% DMSO / 95% SBEbeta-CD in water. Compound was given either as a discrete dose or in a cassette of 4 compounds with brain and plasma sampling at 1 hour.
post compound dose. Tissue sampling and bioanalysis were undertaken using the same method as for the IV infusion studies.

Sample analysis

Both plasma and brain homogenate samples were quantified using LC-MS/MS. Acetonitrile containing internal standard was added to brain or plasma samples v/v 4:1 for precipitation of protein. After centrifugation at 4°C, 2000g for 10 minutes the supernatant was diluted 3 times with water prior to injection on the LC-MS/MS. Calibration curves were established by serial dilution in 50% acetonitrile to cover all expected concentrations of the samples. The brain concentration was corrected for the residual blood by subtracting 0.8% of the plasma concentration from the total brain concentration (Fridén et al., 2010).

Statistical calculations

Data are presented as mean ± standard deviation where replicate measurements are undertaken. Ability of MDCKII-MDR1-BCRP efflux ratio to predict rat $K_{puu}$ is analysed using linear regression, logistical regression and receiver operating characteristic (ROC) curves using JMP 17.0.0. In statistical tests, a P value of <0.05 was considered statistically significant. Time dependent analysis of the MDCKII-MDR1-BCRP efflux assay used a cumulative sums technique ‘the Manhattan tool’ (Winiwarter et al., 2015).

Results

Assessing activity of BBB transporters in the MDCKII-MDR1-BCRP and MDCKII-MDR1 cell lines using efflux studies +/- transporter Inhibitors
In order to determine the activity of transporters in the MDCKII-MDR1-BCRP cell line nine known substrates of the human Pgp and/or BCRP transporters were incubated in the MDCKII-MDR1-BCRP and MDCKII-MDR1 efflux assays at 0.1 µM in the absence and presence of Pgp transporter inhibitor zosuquidar (10 µM) and/or BCRP inhibitor KO143 (1 µM) (Fig. 1a-i, Table 1). These transporter inhibitors are selective at the concentrations used (Dantzig et al., 2001; Allen et al., 2002). The BCRP inhibitor KO143 was used at a concentration of 1 µM to maximize BCRP inhibition whilst avoiding Pgp inhibition (Fig. 2). A substrate concentration of 0.1 µM was selected to best represent the physiologic conditions at the BBB and also to minimise potential for any saturation of transporter activity.

Pgp activity in the MDCKII-MDR1-BCRP cell line was confirmed using the Pgp selective substrate quinidine which gave a double-digit ER (ER 27.5) in the MDCKII-MDR1-BCRP efflux assay. Upon the addition of the Pgp inhibitor zosuquidar efflux was eliminated (Fig. 1a, Table 1) but, as expected, addition of the BCRP inhibitor Ko143 produced no change in the ER (Fig. 1a, Table 1). Similarly, Pgp selective substrate talinolol, demonstrated an ER (9.2) in the MDCKII-MDR1-BCRP assay that was significantly reduced by the Pgp inhibitor zosuquidar (3.9, Fig. 1b, Table 1) but not by the BCRP inhibitor KO143 (11.9) confirming Pgp activity (Fig. 1b, Table 1).

To confirm BCRP activity in the MDCKII-MDR1-BCRP cell line the BCRP selective substrate dantrolene was studied. Dantrolene showed a double-digit ER (16.3) in the MDCKII-MDR1-BCRP assay (Fig. 1c, Table 1) which was completely inhibited by addition of the BCRP inhibitor Ko143 alone (Fig. 1c, Table 1) confirming BCRP activity in the dual transfected cell line.

The MDCKII-MDR1-BCRP cell line has also been evaluated using a range of reported dual Pgp and BCRP efflux transporter substrates, namely dasatinib, erlotinib, flavopiridol, imatinib, momelotinib and prazosin, (Rautio et al., 2006; Marchetti et al., 2008; Chen et al., 2009; Zhou et al., 2009; Durmus et al., 2013; Poirier et al., 2014b; Liu et al., 2017) (Fig. 1d-i, Table 1). All give ERs in the MDCKII-MDR1-BCRP assay that are greater...
than that in the parent MDCKII-MDR1 assay by a factor ~2 to 23 (dasatinib ER 45 in MDCKII-MDR1-BCRP vs ER 19 in MDCKII-MDR1, erlotinib 7.3 vs 1.3, flavopiridol 31 vs 1.5, imatinib 22 vs 8.8, momelotinib 48 vs 2.1 and prazosin 15 vs 1.9 respectively, Table 1) consistent with an additional contribution to efflux from the BCRP transporter in the former cell line.

In the case of erlotinib, flavopiridol, momelotinib and prazosin published studies in single Pgp and BCRP transfected MDCKII cell lines (MDCKII-MDR1 and MDCKII-BCRP) suggest these compounds are weak Pgp substrates with low ERs in MDCKII-MDR1 but have larger ERs in the MDCKII-BCRP cell lines (Rautio et al., 2006; Marchetti et al., 2008; Zhou et al., 2009; Durmus et al., 2013). These 4 compounds in the MDCKII-MDR1-BCRP assay show marked inhibition of efflux by the BCRP inhibitor KO143 alone (erlotinib ER 7.3 (-KO143) vs ER 1.0 (+KO143), flavopiridol 31 vs 2.7, momelotinib 48 vs 8.4 and prazosin 15 vs 2.5, respectively, Fig. 1d-g, Table 1) while erlotinib, flavopiridol and momelotinib are also inhibited by the Pgp inhibitor zosuquidar alone but by a smaller magnitude and prazosin not at all (erlotinib ER 7.3 (-zosuquidar) vs ER 4.3 (+zosuquidar), flavopiridol 31 vs 22, momelotinib 48 vs 36 and prazosin 15 vs 16 respectively Fig. 1d-g, Table 1). However, both Pgp and BCRP inhibitors zosuquidar and Ko143 are required to completely eliminate efflux in the MDCKII-MDR1-BCRP assay (erlotinib ER (-zosuquidar/Ko143) 7.3 vs ER 0.4 (+zosuquidar/Ko143), flavopiridol 31 vs 0.6, momelotinib 48 vs 0.6 and prazosin 15 vs 0.5 respectively, Fig. 1d-g, Table 1). These results indicate that while the four compounds are substrates for both transporters, BCRP makes the larger contribution to overall efflux in the MDCKII-MDR1-BCRP cell line consistent with the pattern reported in the literature using single transfected MDCKII cells (Rautio et al., 2006; Marchetti et al., 2008; Zhou et al., 2009; Durmus et al., 2013).

Dasatinib and imatinib represent dual efflux transporter substrates that demonstrate a more equivalent Pgp and BCRP substrate liability as determined in single Pgp and BCRP transfected MDCKII cell lines (MDCKII-MDR1 and MDCKII-BCRP) (Chen et al., 2009; Zhou
et al., 2009). Dasatinib and imatinib generate ERs in the MDCKII-MDR1 assay, similar to or greater than quinidine (dasatinib ER 19, imatinib 8.8 and quinidine ER 9.2, Fig. 1h-i, Table 1). This is consistent with dasatinib and imatinib being more efficient Pgp substrates than erlotinib, flavopiridol, momelotinib and prazosin (Fig. 1d-g, Table 1). In the dual transporter transfected cell assay addition of zosuquidar alone or Ko143 alone each result in reduced ERs (dasatinib ER 45 vs 44 or 36, imatinib 22 vs 14 or 9.1 respectively, Fig. 1h-i, Table 1) but importantly both efflux inhibitors are required to completely eliminate efflux (dasatinib ER 45 vs 0.7, imatinib 22 vs 0.4 respectively, Fig. 1h-i, Table 1). Indeed for dasatinib the extent of reduction of the ER in the MDCKII-MDR1-BCRP assay by either efflux transporter inhibitor alone is relatively small, revealing the remaining significant efflux activity of the uninhibited transporter (Table 1). The extent of efflux inhibition seen with dasatinib and imatinib in the MDCKII-MDR1-BCRP assay using single inhibitors suggest a more equal balance of Pgp to BCRP substrate liability than observed for erlotinib, flavopiridol, momelotinib and prazosin where BCRP is more dominant (Table 1). This is in keeping with published observations in the single transfected cell lines (Chen et al., 2009; Zhou et al., 2009).

Assessing Pgp activity of BBB transporters in the NIH MDCKI-MDR1 cell lines using efflux studies +/- Pgp transporter Inhibitor

The same nine known substrates of the human Pgp and/or BCRP transporters used in the evaluation of the MDCKII-MDR1-BCRP and MDCKII-MDR1 cell lines were assessed in the highly human Pgp expressing NIH MDCKI-MDR1 cell line efflux assay (Table 1). As expected, given the higher Pgp expression level in the NIH MDCKI-MDR1 cell line compared to NKI MDCKII-MDR1, larger ERs for Pgp substrates were generally observed in the NIH vs NKI derived cell line (quinidine ER 24 vs 9.2, talinolol 20 vs 3.2, erlotinib, 5.6 vs 1.3, flavopiridol 18 vs 1.5, momelotinib 9.5 vs 2.1, prazosin 12.5 vs 1.9, dasatinib 20 vs 19 and imatinib 30 vs 8.8). Elimination of efflux upon addition of the Pgp inhibitor zosuquidar further confirmed Pgp activity in the NIH MDCK-MDR1 cell line (Table 1).
Relationship between MDCKII-MDR1-BCRP efflux ratio and rat brain $K_{puu}$

In order to facilitate interpretation of MDCKII-MDR1-BCRP efflux in the context of brain penetrance, the relationship between ER and rat brain $K_{puu}$ was determined. Studying a set of 58 compounds measured in the AZ in-house rat infusion $K_{puu}$ model, representing a diverse range of chemical structures and physicochemical properties across 18 drug projects, reveals a strong negative correlation between ER in the MDCKII-MDR1-BCRP assay and $K_{puu}$ ($K_{puu} = -0.77 \times \text{MDCKII-MDR1-BCRP ER} - 0.61$, $R^2 = 0.72$, Fig. 3). All compounds with a measured $K_{puu} \geq 0.3$ have an ER $\leq 2$. Likewise, all compounds with a measured ER $\geq 20$ have a $K_{puu} \leq 0.05$. However, only 11/27 (41%) of compounds with an ER $\leq 2$ had a $K_{puu} \geq 0.3$ and only 9 /19 (47%) of compounds with a measured $K_{puu} \leq 0.05$ had an ER $\geq 20$. This clearly demonstrates all compounds with high efflux (ER $\geq 20$) have low brain penetrance and all compounds require absence of efflux (ER $\leq 2$) in this cell line to achieve high levels of brain penetrance, but the absence of efflux in the MDCKII-MDR1-BCRP assay does not identify all compounds as high penetrance, a portion will be moderately penetrant ($0.3 > K_{puu} > 0.05$).

Analysis of MDCKII-MDR1-BCRP efflux ratio data against rat $K_{puu}$ expressed categorically ($K_{puu} \geq 0.3$, $0.3 > K_{puu} > 0.5$, $K_{puu} \geq 0.05$) also showed that the MDCKII-MDR1-BCRP ER model is a useful predictor of brain exposure (supplementary Fig. S1(A) and S1 (B)) Using logistical regression and ROC curve analysis MDCK-MDR1-BCRP ER data is seen to be predictive of the rat $K_{puu}$ categories.

Relationship between MDCKII-rMdr1a efflux ratio and rat brain $K_{puu}$

In order to assess rat versus human species differences a subset of the 58 compounds in Fig. 3 covering a range of rat $K_{puu}$ from 0.008 to 0.41 were selected and ER measured in a rat Pgp transfected cell line MDCKII-rMdr1a at 1 μM +/- the Pgp inhibitor
zosuquidar (Table 2). The seven compounds studied in the MDCKII-rMdr1a assay are all substrates of the rat Pgp transporter as indicated by the efflux ratios >2 and reduction in ER upon addition of zosuquidar. This is consistent with observed rat $K_{puu}$ values of < 1.

One of the seven compounds (AZ4) was also studied in-vivo, investigating the increase in rat brain $K_{puu}$ in the presence and absence of the dual Pgp and BCRP efflux transporter inhibitor elacridar (Fig. 4). This confirmed that the compound was also a substrate of rat efflux transporters in-vivo. While this compound had given a modest efflux ratio in the in-vitro MDCKII-rMDR1a assay of 2.5, there was a marked 14-fold increase in in-vivo rat brain $K_{puu}$ with elacridar pre-dosing.

Comparing efflux ratios in the rat MDCKII-rMdr1a assay with the human MDCKII-MDR1-BCRP assay for the 4 compounds with moderate or high category rat $K_{puu}$ i.e. $K_{puu}$ >0.05 in Table 2 reveals an apparent species difference (AZ4 rat MDCKII-rMdr1a ER 2.5 vs human MDCKII-MDR1-BCRP ER 0.7, AZ5 4.0 vs 0.9, AZ6 3.4 vs 1.5, AZ7 4.3 vs 0.9 respectively) with the data indicating the compounds are rat but not human Pgp substrates. However, when the compounds are evaluated in the more sensitive human NIH MDCKI-MDR1 assay it is apparent that these compounds are human Pgp substrates but too weak to be detected in the NKI derived MDCKII-MDR1-BCRP cell line (AZ4 NIH-MDCKI-MDR1 ER 11 vs MDCKII-MDR1-BCRP ER 0.7, AZ5 9.8 vs 0.9, AZ6 27 vs 1.5, AZ7 7.8 vs 0.9 respectively).

**Characterisation of CNS drugs in the MDCKII-MDR1-BCRP, NIH MDCK1-MDR1 Efflux and Caco2 Permeability Assays**

To further inform use of the MDCKII-MDR1-BCRP efflux assay in identifying compounds likely to have good brain penetration a set of 37 CNS drugs were evaluated in the assay at 0.1 μM. Fig. 5 (and supplementary Table S1) shows that of the 37 CNS drugs evaluated all but one (97%) gave efflux ratios < 2 with 29, the majority (73%), < 1. This
indicates that absence of efflux in this cell line is necessary, with few exceptions, to ensure sufficient levels of exposure in the brain. In the NIH MDCKI-MDR1 assay 95% of the 37 drugs tested had ER <10 with most in the low single digits (32/37 ER <5) and 57% showed no efflux (ER ≤ 2) (Fig. 5 and supplementary Table S1).

All the CNS drugs studied in the Caco2 permeability assay gave Caco2 \(P_{\text{app}}\) values ≥ 28 1E-6 cm/s indicating high passive permeability (Fredlund et al., 2017; Colclough et al., 2019) with the exception of sulpiride (supplementary Table S1) which is a substrate for the active uptake transporter OCT which is present in the BBB (Dos Santos Pereira et al., 2014).

**Efflux transporters expression levels in the MDCKII-MDR1-BCRP and MDCKII-rMdr1a cell lines**

Assessment of transporter expression levels in the MDCKII-MDR1-BCRP and MDCKII-rMdr1a cell lines has been undertaken via proteomic analysis using stable isotope labelled peptide standards as surrogates by two external laboratories. In the MDCKII-MDR1-BCRP cell line transporter levels were BCRP 12-118 pmol/mg protein and Pgp 3-12 pmol/mg protein (supplementary Table S2). In the MDCKII-rMdr1a cell line Pgp was measured as 2 – 12 pmol/mg (supplementary Table S2). Despite the high variability of determinations across the peptides and laboratories the results indicated BCRP is expressed at a higher level than the Pgp transporter in the MDCKII-MDR1-BCRP cell line.

**MDCKII-MDR1-BCRP cell line stability**

The MDCKII-MDR1-BCRP cell line showed functional stability with consistent dasatinib (dual Pgp and BCRP transporter) efflux ratios over 19 months (supplementary Fig. S2).
Discussion (max 1500 words) 1603

Efflux transporter assays are key to enable evaluation of the CNS properties of molecules and for designing brain penetrant drugs where it is important to minimise BBB efflux substrate liability. To this end we have established a dual transfected MDCK cell line in which both the major human BBB efflux transporters Pgp and BCRP are over expressed (MDCKII-MDR1-BCRP). Combining both transporters into one cell line results in a single assay format enabling an early assessment of potential BBB penetration with the ability to include discrete transporter inhibitor arms to assess relative contributions from either Pgp and/or BCRP. The relative contributions of BCRP to Pgp efflux observed in the MDCKII-MDR1-BCRP cell line for dual substrates show a similar pattern to that observed when comparing efflux ratios in single NKI transfected lines MDCKII-MDR1 and MDCKII-BCRP (Rautio et al., 2006; Marchetti et al., 2008; Chen et al., 2009; Zhou et al., 2009; Durmus et al., 2013; Poirier et al., 2014b) indicating similar relative activity levels.

The reduction in ER observed when both transporter inhibitors are added to the MDCKII-MDR1-BCRP assay appears disproportionally greater than the sum effect from adding single transporter inhibitors alone, particularly for substrates such as dasatinib and imatinib (dasatinib ER 45 vs 44 (+zosuquidar) vs 36 (+Ko143) vs 0.7 (+zosuquidar/+Ko143), imatinib 22 vs 14 vs 9.1 vs 0.4 respectively). A similar apparent disproportionate effect of Pgp and BCRP inhibition has been observed in in vivo mouse KO studies for a range of dual transporter substrates, including dasatinib and imatinib, where total brain levels in double KO mice appears significantly higher than the sum of individually inhibited transporters would suggest (dasatinib 5 mg/kg IV dose sample at 2 hrs - Bcrp1(-/-) mice have same brain level as WT mice, Mdr1a/b(-/-) mice have brain levels 6 fold higher than WT mice and Mdr1a/b(-/-)Bcrp1(-/-) mice have brain levels 18 times higher. Imatinib 5 mg/kg sub cut dose sample at 2 hrs – Blood to plasma ratio (Kp) WT mice Kp 0.02, Bcrp1(-/-) mice Kp 0.02, Mdr1a/b(-/-) mice Kp 0.11, Mdr1a/b(-/-)Bcrp1(-/-) mice Kp 1.55) (Chen et al., 2009; Zhou et al., 2009; Kodaira et al., 2010). Kodaira et al demonstrated that this phenomenon can be
explained by the high intrinsic efflux activities of each transporter relative to passive diffusion and their contribution to the net efflux at the BBB without the need to invoke any interaction between the transporters. It is also noted from these KO/inhibitor studies with dasatinib and imatinib that Pgp drives efflux more compared to BCRP in mice in contrast to the MDCKII-MDR1-BCRP cell line where BCRP makes a bigger contribution. Similarly using dual transporter substrates erlotinib, flavopiridol and prazosin, BCRP was observed to contribute to a greater extent than Pgp to the overall efflux in the MDCKII-MDR-BCRP assay (erlotinib ER 7.3 vs 4.3 (+zosuquidar) vs 1.0 (+Ko143), flavopiridol 31 vs 22 vs 2.7, prazosin 15 vs 16 vs 2.5 respectively) compared to reported transporter in-vivo mouse knock-out (KO) studies where Pgp was observed to be the more dominant transporter (erlotinib WT mice Kp 0.14, Bcrp1(-/-) mice Kp 0.14, Mdr1a/b(-/-) mice Kp 0.41, Mdr1a/b(-/-)Bcrp1(-/-) mice Kp 0.58; flavopiridol WT mice Kp 0.92, Bcrp1(-/-) mice Kp 1.22, Mdr1a/b(-/-) mice Kp 1.45, Mdr1a/b(-/-)Bcrp1(-/-) mice Kp 6.34; prazosin WT mice Kp 0.33, Bcrp1(-/-) mice Kp 0.37, Mdr1a/b(-/-)Bcrp1(-/-) mice Kp 6.34) (Zhou et al., 2009; Kodaira et al., 2010; de Vries et al., 2012). This difference likely reflects a higher expression level of Pgp versus BCRP in mouse BBB (Pgp 14.1 fmol/ug protein versus Bcrp 4.4 fmol/ug protein(Kamiie et al., 2008)) compared to the MDCKII-MDR1-BCRP cell line where BCRP has been shown to be the more highly expressed transporter via proteomic analysis.

Proteomic analysis of our MDCKII-MDR1-BCRP cell line has been undertaken in two different laboratory studies using peptides surrogates for the transporter levels. These studies indicate that the expression level of BCRP is higher than Pgp (BCRP 12-118 pmol/mg protein, Pgp 3-12 pmol/mg protein). Literature reports for the human BBB suggest BCRP levels may be up to 1.5 times higher than Pgp levels (Shawahna et al., 2011; Uchida et al., 2011; Al-Majdoub et al., 2019). However, the mass spectrometry-based quantification proteomic analysis technique can show variability (Wegler et al., 2017) which we have also observed in our expression data highlighting the difficulty in comparing absolute transporter expression levels. A double transfected MDCKII cell line containing both human Pgp and BCRP has previously been developed by Poller et al., 2011. This cell line has been
evaluated using the dual Pgp BCRP transporter substrates topotecan, sorafenib and sunitinib. With these 3 substrates Poller et al demonstrated a qualitatively similar pattern of relative contribution of BCRP versus Pgp transporters in their MDCKII-MDR1-BCRP efflux assay (using ERs +/- Pgp and BCRP inhibitors) to mouse in-vivo brain penetration data using WT and Pgp and/or BCRP KO mice. This contrasts with the pattern observed with the MDCKII-MDR1-BCRP cell line reported in this study and suggests that the Poller cell line may have lower BCRP levels relative to Pgp levels than the cell line in this study although no quantification of transporters in the dual transfected cell line was reported.

More recently a double (Pgp and BCRP) transfected cell line has been developed by (Robinson et al., 2019) using HEK239 cells to study drug resistance to chemotherapeutic agents in cancer cells. Using substrates of Pgp and BCRP the authors demonstrated, using fluorescence flow cytometry and cytotoxicity assays, that both transporters must be inhibited to completely block substrate transport and overcome resistance. This observation is consistent with our own findings using the MDCKII-MDR-BCRP cell line with dual substrates. In contrast to MDCKII cells the HEK293 cells do not form epithelial mono-layers with tight junctions and as such the authors note that the HEK293 model is more applicable to drug resistant cancer cells than the BBB (Robinson et al., 2019). Further, HEK293 cells cannot polarise, unlike MDCKII cells and the endothelial cells of the BBB (König et al., 2000; van Beest et al., 2006; Worzfeld and Schwaninger, 2016). The MDCKII-MDR1-BCRP ERs demonstrate a good correlation with rat $K_{puu}$ data generated using a diverse range of compounds and indicate that to achieve high levels ($K_{puu} \geq 0.3$) of brain penetration molecules should not have any efflux in this cell assay (ER $\leq 2$, Fig. 3). This is supported by similar observations with an evaluated set of 37 CNS drugs. However, the correlation between ER and rat $K_{puu}$ also reveals that it is not sufficient to solely achieve this target in the MDCKII-MDR1-BCRP assay as a significant percentage (59%) of such molecules with ER $\leq 2$ generate moderate category rat $K_{puu}$ values (0.3 to 0.05). Using a rodent Pgp transfected cell line on a subset of such compounds allowed confirmation that these
molecules are rodent Pgp efflux substrates explaining the lower than unity rat $K_{puu}$. Yet this does not necessarily imply a species difference since measurements of the same compounds in the more sensitive NIH MDCKI-MDR1 cell line reveals that these compounds are also human Pgp substrates. This data indicates that higher Pgp expression levels such as seen in the NIH MDCKI-MDR1 cells are required to detect weaker Pgp substrates. This enhanced sensitivity of the NIH-MDCKI-MDR1 cell line to Pgp substrates is consistent with other studies where the NIH MDCKI-MDR1 cell line has been compared to the parent NKI MDCKII-MDR1 line (Liu et al., 2018; Feng et al., 2019).

For a set of CNS drugs in the NIH MDCKI-MDR cell line most of the CNS compounds studied had efflux ratios ≤ 5 with over half being truly non-substrates (ER≤ 2) however 4 of the 37 CNS drugs had higher ERs most notably citalopram, respiridone, spiperone and mezoridazine (ER 7.4 – 43) with rat $K_{puu} < 1$ (citalopram rat $K_{puu}$ 0.28, respiridone rat $K_{puu}$ 0.27 (Culot et al., 2013), 0.04-0.08 (de Lange, 2013), mezoridazine rat $K_{puu}$ 0.02-0.10 using $K_p$ from reported thioridazine metabolite PK profile and AZ $f_{up}$ and $f_{ub}$ (Daniel et al., 2000), spiperone brain/blood rat $K_{puu}$ 0.19 using AZ $f_{ub}$ with reported rat data (Liu et al., 2018)). These latter compounds reflect that not only absolute ER and hence $K_{puu}$ values are important but moreover the relative brain exposure to potency against the pharmacological target.

The majority (97%) of CNS drugs studied have high Caco2 $P_{app}$ values (> 10 1E-6 cm/s (Fredlund et al., 2017; Colclough et al., 2019)) demonstrating that good passive permeability is desirable when seeking high brain exposure. Sulpiride is the noted exception with poor passive permeability (Caco2 Papp 1.2 1E-6 cm/s) and minimal efflux liability (MDCKII-MDR1-BCRP ER 1.6, NIH MDCKI-MDR1 ER 3.0) giving a low rat $K_{puu}$ (0.05 (Culot et al., 2013)). For sulpiride it has been reported that OCT transporter active uptake may contribute to increasing brain exposure (Dos Santos Pereira et al., 2014).

In conclusion the MDCKII-MDR1-BCRP efflux assay can be used to identify substrates for both the major human BBB efflux transporters in a single assay so aiding the
design of compounds with good brain penetration as well as enabling the evaluation of the 
CNS properties of molecules. Combined with inhibitor arms the assay allows identification of 
the relative contributions of the transporters to efflux. Based on the relationship with rat $K_{puu}$ 
data and efflux profiles of CNS drugs elimination of efflux in the MDCK-MDR1-BCRP assay 
(ER$\leq$ 2) is required if high brain penetration is sought while ERs $\geq$ 20 provides a useful 
benchmark for minimal brain penetration (e.g., if required to minimise on target CNS 
engagement for safety mitigation). Use of the MDCKII-MDR1-BCRP assay in parallel with 
the NIH MDCKI-MDR1 assay ensures weak Pgp transporter substrates with moderate $K_{puu}$ 
are identified and can aid the design of highly brain penetrant molecules.

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Data Availability Statement

The data supporting the findings of this study are available within the paper, its Supplemental Data and on request from the corresponding author.
Author contributions

Participated in research design: Alluri, Chen, Colclough, Gozalpour, Harlfinger, McGinnity, Yan

Conducted experiments: Alluri, Du, Gozalpour, Li, Li, O'Neil, Sproat, Tucker, Wei,

Contributed new reagents or analytical tools: Chen, O'Neil, Sproat, Yan

Performed data analysis: Alluri, Colclough, Harlfinger

Wrote or contributed to the writing of the manuscript: Alluri, Colclough, Harlfinger, McGinnity, O'Neil, Sproat
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Kodaira H, Kusuhara H, Ushiki J, Fuse E, and Sugiyama Y (2010) Kinetic analysis of the cooperation of P-glycoprotein (P-gp/Abcb1) and breast cancer resistance protein (Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and


transporters ABCB1, ABCG2, and ABCC2 on erlotinib hydrochloride (Tarceva) disposition in \textit{in vitro} and \textit{in vivo} pharmacokinetic studies employing Bcrp1\textsuperscript{-/-}/Mdr1a/1b\textsuperscript{-/-} (triple-knockout) and wild-type mice. \textit{Molecular Cancer Therapeutics} \textbf{7}:2280-2287.


Footnotes

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For reprint requests: Nicola Colclough, AstraZeneca, Cambridge, United Kingdom, CB2 0AA, Nicola.Colclough@astrazeneca.com
Figure Legends

Figure 1 - Efflux ratios (ER) and apparent permeability coefficients (Papp) A to B and B to A following incubation of substrates A to I at 0.1 µM alone, with 10 µM zosuquidar, with 1 µM Ko143 or both 10 µM zosuquidar and 1 µM Ko143 in MDCKII-MDR1-BCRP and MDCKII-MDR1 cell lines.

(A) quinidine and (B) talinolol selective MDR1 substrates (Kusuhara et al., 1997; Hilgendorf et al., 2005; Oswald et al., 2011; Hellinger et al., 2012; Takano et al., 2016; Mukkavilli et al., 2017) (C) dantrolene selective BCRP substrate (Lee et al., 2015; Liu et al., 2017; Feng et al., 2019), (D) erlotinib, (E) flavopiridol, (F) momelotinib (G) prazosin (H) dasatinib (I) imatinib dual MDR1 BCRP substrates (Rautio et al., 2006; Marchetti et al., 2008; Chen et al., 2009; Zhou et al., 2009; Durmus et al., 2013; Poirier et al., 2014a; Liu et al., 2017). Error bars St Dev. N=3

Figure 2 – Effect of concentration of efflux inhibitor K0143 on % transporter activity in the MDCKII-MDR1-BCRP efflux assay using (A) Pgp selective substrate quinidine at 0.1 µM (A to B EC50 6.1 µM 95% confidence interval 4.0 to 8.6 µM, B to A EC50 11 µM 95% confidence interval 8.3 to 14 µM) and (B) BCRP selective substrate dantrolene at 0.1 µM (A to B EC50 0.16 µM 95% confidence interval 0.11 to 0.23 µM, B to A EC50 0.061 µM 95% confidence interval 0.029 to 0.096 µM). Error bars show standard deviation for n=3.

Figure 3 – Plot of rat brain K_{puu} versus MDCKII-MDR1-BCRP ER for n=58 molecules from 18 drug discovery /development projects with diverse small molecule chemistry. Shapes indicate discrete project series. Red lines ER 20 and K_{puu} 0.05 - poor BBB penetration category cut off; green lines ER 2 and K_{puu} 0.3 - good BBB penetration category cut off. ER data with > 20% recovery. Only rat Kp data used where %CV ≤ 50% for n=3. Log Rat K_{puu} = - 0.77 log MDCKII-MDR1-BCRP ER – 0.61, R2 = 0.72
**Figure 4** – AZ4 Rat brain $K_{\text{puu}}$ A: at 1h post drug dosing at 1 mg/kg as iv bolus (mean $K_{\text{puu}}$: $0.084 \pm 0.022$, $n = 24$). B: at 1h post drug dosing at 1 mg/kg as iv bolus including pre-dosing (0.5 h) of Elacridar (Pgp and BCRP inhibitor) at 5 mg/kg as iv bolus (mean $K_{\text{puu}}$: $1.23 \pm 0.217$, $n = 24$). Cassette and discrete dosing data included.

**Figure 5** – Histogram of efflux ratios of CNS drugs at 0.1 μM in MDCKII-MDR1-BCRP and NIH MDCKI-MDR1 cell assays. Details in supplementary Table S1.
Table 1 – Efflux ratios of a range of human efflux transporter substrates incubated at 0.1 µM alone or with 10 µM zosuquidar or 1 µM Ko143 or both in NKI derived MDCKII-MDR1-BCRP, NKI MDCKII-MDR1 and NIH MDCKI-MDR1 cell lines.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ER MDCKII-MDR1-BCRP</th>
<th>ER NKI MDCKII-MDR1</th>
<th>ER NIH MDCKI-MDR1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No inhibitor</td>
<td>+Zosuquidar</td>
<td>+Ko143</td>
</tr>
<tr>
<td>Quinidine</td>
<td>27.5 ± 1.2</td>
<td>0.6 ± 0.0</td>
<td>36.8 ± 4.6</td>
</tr>
<tr>
<td>Talinolol</td>
<td>9.2 ± 1.1</td>
<td>3.9 ± 0.1</td>
<td>11.9 ± 1.7</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>16.3 ± 0.9</td>
<td>18.3 ± 1.2</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>7.3 ± 1.3</td>
<td>4.3 ± 0.5</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>31.2 ± 3.2</td>
<td>21.7 ± 1.6</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Momelotinib</td>
<td>48.2 ± 4.1</td>
<td>36.4 ± 2.6</td>
<td>8.4 ± 1.1</td>
</tr>
<tr>
<td>Prazosin</td>
<td>14.6 ± 1.9</td>
<td>16.3 ± 0.9</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>45.3 ± 4.2</td>
<td>43.7 ± 7.1</td>
<td>36.1 ± 5.8</td>
</tr>
<tr>
<td>Imatinib</td>
<td>21.7 ± 1.2</td>
<td>14.0 ± 2.0</td>
<td>9.1 ± 0.7</td>
</tr>
</tbody>
</table>

ER values average of n=3, +/- SD
**Table 2** - Efflux ratios of a subset of compounds from Fig. 3 in rat Pgp transfected MDCKII-rMdr1a cells at 1 µM plus and minus Pgp inhibitor 10 µM zosuquidar. Comparison with $K_{puu}$ and efflux ratios in human transporter transfected MDCKII-MDR1-BCRP and NIH MDCKI-MDR1 at 0.1 µM.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Project Code</th>
<th>Rat $K_{puu}$</th>
<th>Rat MDCKII-Mdr1a (-inhibitor) ER</th>
<th>Rat MDCKII-Mdr1a (+inhibitor) ER</th>
<th>Hu MDCKII-MDR1-BCRP (0.1 µM) ER</th>
<th>Hu NIH MDCKI-MDR1 (0.1 µM) ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ1</td>
<td>B</td>
<td>0.008</td>
<td>10.86 ± 4.44</td>
<td>1.87 ± 0.94</td>
<td>31.78 ± 2.64</td>
<td>58.66 ± 11.76</td>
</tr>
<tr>
<td>AZ2</td>
<td>M</td>
<td>0.012</td>
<td>14.01 ± 4.18</td>
<td>2.05 ± 0.15</td>
<td>18.46 ± 3.18</td>
<td>45.79 ± 0.79</td>
</tr>
<tr>
<td>AZ3</td>
<td>I</td>
<td>0.049</td>
<td>4.51 ± 1.77</td>
<td>1.53 ± 0.39</td>
<td>7.54 ± 4.14</td>
<td>63.3 ± 8.4*</td>
</tr>
<tr>
<td>AZ4</td>
<td>D</td>
<td>0.11</td>
<td>2.49 ± 0.53</td>
<td>0.74 ± 0.11</td>
<td>0.73 ± 0.15</td>
<td>10.75 ± 1.72</td>
</tr>
<tr>
<td>AZ5</td>
<td>D</td>
<td>0.16</td>
<td>4.00 ± 1.58</td>
<td>1.18 ± 0.45</td>
<td>0.94 ± 0.32</td>
<td>9.81 ± 1.26</td>
</tr>
<tr>
<td>AZ6</td>
<td>D</td>
<td>0.24</td>
<td>3.44 ± 1.29</td>
<td>1.05 ± 0.54</td>
<td>1.52 ± 0.72</td>
<td>27.15 ± 13.33</td>
</tr>
<tr>
<td>AZ7</td>
<td>D</td>
<td>0.41</td>
<td>4.29 ± 0.89</td>
<td>1.57 ± 0.55</td>
<td>0.90 ± 0.33</td>
<td>7.75 ± 0.88</td>
</tr>
</tbody>
</table>

ER data given as mean ± SD, n≥3, * n=2
Figure 1
Figure 2
Figure 3
Figure 4

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5
Supplemental Information

Utilising a dual human transporter MDCKII-MDR1-BCRP cell line to assess efflux at the Blood Brain Barrier (BBB)

Nicola Colclough, Ravindra V. Alluri, James W. Tucker, Elnaz Gozalpour, Danxi Li, Hongwen Du, Wei Li, Stephanie Harlfinger, Daniel J. O’Neill, Graham G. Sproat Kan Chen, Yumei Yan, Dermot F. McGinnity

Figure 1S: (A) Logistical regression analysis of categorical rat Kpuu vs. Log MDCKII-MDR1-BCRP ER, (B) Receiver operating characteristic (ROC) plot analysis

Figure S2: Plot showing log MDCKII-MDR1-BCRP efflux ratio for dasatinib over a period of 19 months illustrating stability of the cell line.

Table S1: Efflux ratios of CNS drugs in MDCKII-MDR1-BCRP and NIH MDCKI-MDR1 assays at 0.1 µM and Caco2 Papp intrinsic permeability.

Table S2: P-gp and BCRP transporter expression levels (pmol/mg total protein) in the MDCKII-MDR1-BCRP and MDCKII-rMdr1a cell lines determined by quantitative proteomics (LC-MSMS method).

Supplemental Method: Quantification of Pgp and BCRP using targeted proteomics.
Figure S1 – (A) Logistical fit of Rat Kpuu category (green Kpuu ≥ 0.3, blue 0.3 > Kpuu > 0.05, red Kpuu ≤ 0.05) versus Log MDCKII-MDR1-BCRP ER with whole model test analysis. (B) Receiver operating characteristic (ROC) plot and area under the curve using identical data set.
Figure S2 - Plot showing log MDCKII-MDR1-BCRP efflux ratio for dasatinib over a period of 19 months illustrating the stability of the cell line over time. Green triangles and blue line = individual data, red line = Manhattan line (cumulative sums technique (Winiwarter et al., 2015)).
**Table S1-** Efflux ratios of CNS drugs in MDCKII-MDR1-BCRP and NIH MDCKI-MDR1 assays at 0.1 µM and Caco2 Papp intrinsic permeability.

<table>
<thead>
<tr>
<th>CNS Drug</th>
<th>NIH MDCKI-MDR1 ER</th>
<th>MDCKII-MDR1-BCRP ER</th>
<th>Caco2 Papp 1E-6 cm/s</th>
<th>Rat Kp uu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seligiline</td>
<td>0.78 ± 0.05</td>
<td>1.04 ± 0.01</td>
<td>51.8 ± 10.9</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.88 ± 0.06</td>
<td>0.75 ± 0.08</td>
<td>57.4 ± 9.6</td>
<td>0.67, 0.68*</td>
</tr>
<tr>
<td>Hydroxyzine</td>
<td>0.94 ± 0.37</td>
<td>0.34 ± 0.04</td>
<td>66.2 ± 5.4</td>
<td>0.86*</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>0.96 ± 0.09</td>
<td>0.62 ± 0.05</td>
<td>60.2 ± 4.8</td>
<td>0.72, 0.88*, 0.61*</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.96 ± 0.09</td>
<td>0.82 ± 0.07</td>
<td>66.7 ± 2.7</td>
<td>1.07*, 0.49*</td>
</tr>
<tr>
<td>Amantadine</td>
<td>0.98 ± 0.04</td>
<td>0.92 ± 0.05</td>
<td>64.9 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>Ractopride</td>
<td>1.08 ± 0.06</td>
<td>0.93 ± 0.05</td>
<td>73.4 ± 19.6</td>
<td></td>
</tr>
<tr>
<td>Tacrine</td>
<td>1.12 ± 0.05</td>
<td>0.53 ± 0.05</td>
<td>77.8 ± 19.5</td>
<td>0.78*, 0.78*</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>1.13 ± 0.08</td>
<td>0.35 ± 0.06</td>
<td>61.8 ± 8.7</td>
<td>1.10*</td>
</tr>
<tr>
<td>Doxepin</td>
<td>1.15 ± 0.11</td>
<td>0.39 ± 0.03</td>
<td>66.0 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>1.16 ± 0.28</td>
<td>0.65 ± 0.15</td>
<td>70.0 ± 12.7</td>
<td>1.05*</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>1.26 ± 0.16</td>
<td>0.96 ± 0.07</td>
<td>64.3 ± 9.1</td>
<td></td>
</tr>
<tr>
<td>Buspirone</td>
<td>1.28 ± 0.19</td>
<td>0.36 ± 0.09</td>
<td>66.4 ± 14.0</td>
<td>0.61</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>1.28 ± 0.09</td>
<td>0.74 ± 0.64</td>
<td>35.0 ± 9.2</td>
<td></td>
</tr>
<tr>
<td>Trazodone</td>
<td>1.34 ± 0.21</td>
<td>0.71 ± 0.02</td>
<td>45.7 ± 12.6</td>
<td></td>
</tr>
<tr>
<td>Clozapine</td>
<td>1.35 ± 0.17</td>
<td>0.58 ± 0.37</td>
<td>80.3 ± 20.0</td>
<td>1.10*</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>1.38 ± 0.10</td>
<td>0.79 ± 0.02</td>
<td>78.9 ± 5.6</td>
<td>0.47*</td>
</tr>
<tr>
<td>WAY 100635**</td>
<td>1.72 ± 0.72</td>
<td>1.17 ± 0.04</td>
<td>67.0 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>1.75 ± 0.27</td>
<td>0.89 ± 0.05</td>
<td>49.8 ± 8.9</td>
<td>1.0*</td>
</tr>
<tr>
<td>Flumazenil</td>
<td>1.75 ± 0.06</td>
<td>1.20 ± 0.16</td>
<td>70.6 ± 19.1</td>
<td></td>
</tr>
<tr>
<td>Phenytoin</td>
<td>1.91 ± 0.23</td>
<td>0.63 ± 0.11</td>
<td>69.9 ± 5.0</td>
<td>0.48, 0.42*</td>
</tr>
<tr>
<td>PK11195**</td>
<td>2.02 ± 0.35</td>
<td>0.81 ± 0.04</td>
<td>56.7 ± 18.9</td>
<td></td>
</tr>
<tr>
<td>Olanzapine</td>
<td>2.37 ± 1.22</td>
<td>0.98 ± 0.06</td>
<td>85.7 ± 14.6</td>
<td></td>
</tr>
<tr>
<td>Procyclidine</td>
<td>2.38 ± 0.29</td>
<td>0.43 ± 0.03</td>
<td>67.9 ± 7.6</td>
<td></td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>2.43 ± 0.13</td>
<td>0.31 ± 0.01</td>
<td>46.8 ± 6.7</td>
<td>1.00*</td>
</tr>
<tr>
<td>Biperiden</td>
<td>2.52 ± 0.36</td>
<td>0.44 ± 0.05</td>
<td>78.0 ± 10.8</td>
<td></td>
</tr>
<tr>
<td>Paroxetine</td>
<td>2.67 ± 0.25</td>
<td>0.43 ± 0.01</td>
<td>44.5 ± 7.2</td>
<td>0.53*</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>2.71 ± 0.72</td>
<td>0.55 ± 0.10</td>
<td>58.2 ± 11.3</td>
<td>0.46</td>
</tr>
<tr>
<td>CNS Drug</td>
<td>NIH MDCKI-MDR1 ER</td>
<td>MDCKII-MDR1-BCRP ER</td>
<td>Caco2 Papp 1E-6 cm/s</td>
<td>Rat Kpuu</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>2.84 ± 0.08</td>
<td>0.26 ± 0.04</td>
<td>78.9 ± 15.6</td>
<td></td>
</tr>
<tr>
<td>Sulpiride</td>
<td>2.98 ± 0.22</td>
<td>1.58 ± 0.21</td>
<td>1.2 ± 0.1</td>
<td>0.05*</td>
</tr>
<tr>
<td>Volinanserin**</td>
<td>3.10 ± 0.33</td>
<td>1.16 ± 0.08</td>
<td>77.6 ± 11.4</td>
<td></td>
</tr>
<tr>
<td>Desipramine</td>
<td>3.94 ± 0.38</td>
<td>0.67 ± 0.21</td>
<td>66.2 ± 13.6</td>
<td></td>
</tr>
<tr>
<td>Primidone</td>
<td>5.06 ± 0.67</td>
<td>1.11 ± 0.03</td>
<td>28.5 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Citalopram</td>
<td>7.44 ±1.04</td>
<td>1.24 ± 0.28</td>
<td>67.8 ± 10.0</td>
<td>0.28</td>
</tr>
<tr>
<td>Respiridone</td>
<td>9.64 ± 0.28</td>
<td>1.80 ± 0.74</td>
<td>67.5 ± 21.7</td>
<td>0.27*</td>
</tr>
<tr>
<td>Spiperone</td>
<td>18.1 ± 3.1</td>
<td>1.87 ± 0.01</td>
<td>62.3 ± 10.4</td>
<td></td>
</tr>
<tr>
<td>Mesoridazine***</td>
<td>42.7 ± 14.0</td>
<td>5.16 ± 0.37</td>
<td>64.1 ± 11.8</td>
<td></td>
</tr>
</tbody>
</table>

Data given as mean ± St Dev, n ≥ 3

* Data from (Fridén et al., 2009) **Not a marketed drug, used in scientific research ***Withdrawn from market –side effects, irreg heart beat, QT prolongation

ǂ Data from (Culot et al., 2013)
Table S2 P-gp and BCRP transporter expression levels (pmol/mg total protein) in the MDCKII-MDR1-BCRP and MDCKII-rMdr1a cell lines (mean ± st. dev.) determined by quantitative proteomics (LC-MS/MS method).

<table>
<thead>
<tr>
<th>Transporter Peptide (Species)</th>
<th>Smith Lab, University of North Carolina, n=2, (pmol/mg protein)*</th>
<th>University of Washington Research Affiliate Programme of Transporters (UWRAPT), n=3, (pmol/mg protein)</th>
<th>Smith Lab, University of North Carolina, n=2, (pmol/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCRP SSLLDVLAAR (H,M,D,R)</td>
<td>118 ± 4.7</td>
<td>11.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>BCRP VIQELGLDK (H,M)</td>
<td>71.0 ± 8.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCRP ENLQFSAALR (H,M,D,R,Ms)</td>
<td>21.8 ± 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-gp IIDNKPSIDSYSK (H,M,D)</td>
<td>12.2 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-gp FYDPLAGK (H,M)</td>
<td>5.5 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-gp IATEAIENFR (H,M,D,R[a&amp;b],Ms)</td>
<td>7.0 ± 1.3</td>
<td>6.4 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>P-gp NTTGALTTR (H,M,D,R[a],Ms)</td>
<td>2.7 ± 1.2</td>
<td>4.8 ± 0.3</td>
<td>2.3 ± 1.2</td>
</tr>
<tr>
<td>P-gp QLNVQWLR (M,R[a],Ms)</td>
<td></td>
<td>12.0 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

H, M, D, R[a&b], Ms = Human, Monkey, Dog, Rat, Mouse respectively *LOQ 0.1 pmol/mg protein.
Supplemental Method: Quantification of Pgp and BCRP using targeted proteomics

Membrane protein fractions were isolated in-house from MDCKII-MDR1-BCRP and MDCKII-rMdr1a cells (~3-9 x 10^6) using ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem; Cat. No. 444810). Protein concentration in the isolated membrane fraction was determined by the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) and samples (~1-2 mg/ml) were shipped to the Unadkat lab (UWRAPT), University of Washington, Seattle and the Smith Lab, University of North Carolina at Chapel Hill (MDCKII-MDR1-BCRP cell line analysed at both laboratories MDCKII-rMdr1a at one).

University of Washington Protein Sample digestion and analysis by LC-MS/MS

AstraZeneca supplied 550 μL of MDCKII-MDR1-BCRP membrane protein fraction (total protein concentration 2.19 mg/ml). In addition, membrane protein from a pool of human liver tissue was used as a biological quality control sample. Transporter abundance data from this pool of human liver tissue must be ± 20% of published values.

1. In triplicate, 80 μl of the sample was combined with 10 μl of 250 mM dithiothreitol, 40 μl of 100 mM ammonium bicarbonate buffer (pH 7.8), 20 μl of 10% sodium deoxycholic acid and 10 μl of 10 mg/ml human serum albumin, mixed well and incubated at 95°C for 10 min with mild shaking at 300 rpm.

2. Samples were cooled to room temperature, 20 μl of 500 mM iodoacetamide was added, vortexed and incubated for 30 min at room temperature in the dark.

3. To each sample, 0.5 ml of ice-cold methanol, 0.1 ml of chloroform and 0.4 ml of water was added and vortexed.

4. Samples were centrifuged at 16,000 g for 5 min at 4°C, the upper layer and lower layer was aspirated and the pellet was left in fume hood for drying. After 10 min, the pellet was washed with 0.5 ml of ice-cold methanol and then centrifuged at 16,000 g for 5 min at 4°C. Methanol was aspirated and the pellet was left to dry in a fume hood for 30 min.

5. The pellet was resuspended with 60 μl of 50 mM ammonium bicarbonate buffer.
6. 20 µl of 0.16 µg/µl trypsin was added to each sample (approx. 1:25 trypsin: protein ratio (w/w)) and incubated for 18 h at 37 °C with mixing at 300 rpm. The reaction was quenched on the dry ice.

7. Digestion was stopped by adding 20 µl chilled heavy internal standard (dissolved in 80% acetonitrile, with 0.1% formic acid) and 10 µl of blank solvent (80% acetonitrile with 0.1% formic acid).

8. Samples were centrifuged for 5 min at 4,000xg and 4 °C and the supernatant was analysed by LC-MS/MS.

Unique surrogate peptides were selected for quantification of each transporter (Pgp - NTTGALTTR, BCRP - SSLLDVLAAR). Surrogate peptides were used as calibrators and with corresponding labelled peptides, at [13C615N2]-lysine and [13C615N4]-arginine residues, used as internal standards.

The calibrators, ranging from 0.3 to 88 fmol (on-column), were prepared by spiking 80 µl of 50 mM ammonium bicarbonate buffer with 10 µl of unlabelled surrogate peptide standard and 20 µl of labelled peptide standard. Quality control samples at low, medium and high concentrations were prepared in the same manner.

Surrogate peptides were quantified using an AB Sciex 6500 TQS tandem mass spectrometer coupled with a Water’s Acquity UPLC system operated in electrospray positive ionisation mode. The mass spectrometry conditions were curtain gas: 20 psi; ion spray voltage: 5500 V, source temperature: 350°C, gas 1: 50 psi, gas 2: 30 psi; and cell exit potential: 12 V.

Approximately, 8 µg of trypsin digest (5µL) was injected onto the column (Acquity UPLC HSS T3 1.8µm 100Å, 100 x 2.1 mm; Waters) fitted with a security guard column (C18, 4 x 2 mm; Phenomenex) and eluted at 0.3 ml/min with a gradient mobile phase consisting of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The linear gradient was 0 to 3 minutes: 97% A 3% B, 3 to 10 minutes: 87% A 13% B, 10 to 20 minutes: 75% A 25% B, 20 to 22 minutes: 66.7% A 33.3% B, 22 to 23 minutes: 50% A 50% B, 23 to 24 minutes: 20% A 80% B, 24 to 28 minutes: 97% A 3% B.
LC-MS/MS data were processed by integrating the peak areas generated from the reconstructed ion chromatograms for the analyte peptides and respective heavy internal standards using Analyst Software 1.6.2 (Milford, MA). The peak response from two transitions of each peptide was averaged (after confirming that they were correlated) for quantification of samples, standards, and quality controls. Acceptance criteria: Calibration standards and quality control samples must be back calculated to within ± 20% of nominal concentrations. For sponsor samples, the mean, SD & %CV of the three replicates following normalisation against the sample protein concentration was reported.

In addition to the transport proteins requested by the sponsor, surrogate peptides for human serum albumin and Na⁺K⁺ATPase were monitored, but not quantified. Albumin (added to samples prior to protein enrichment) was used to measure the trypsin digestion efficiency. Na⁺K⁺ATPase was used as a plasma membrane marker.

University of North Carolina Sample digestion and analysis by LC-MS/MS

AstraZeneca supplied 1000 μL of MDCKII-MDR1-BCRP membrane protein fraction (total protein concentration 0.8 mg/ml) and 1000 μL of MDCKII-rMdr1a membrane protein fraction (total protein concentration 1.13 mg/ml). HLM QC (BD Gentest from BD Biosciences, Woburn, MA [now Corning Gentest]) was analysed in duplicate with the main study batch. 20 μg of total membrane/microsomal protein was used in each digestion with the protein solution being evaporated to dryness at the beginning so that the volumes in each digestion reaction were consistent.

After evaporation of sample protein solution to dryness in a ThermoSavant SpeedVac 100 μL of 50 mM ammonium bicarbonate, 10 μL of 40 mM dithiothreitol, 10 μL of β-casein (0.5 μg/10 μL) (an indicator of digestion and a chromatography retention time marker) and 13.3 μL of 10 % sodium deoxycholate were added to each tube. Samples were denatured and reduced for 40 min at 60 °C in an Isotemp Thermal Mixer (Fisher Scientific, Pittsburg, PA) shaking at 500 rpm. After
cooling to room temperature, 10 µL of 135 mM iodoacetamide was added and tubes were incubated for 30-40 min in the dark at room temperature. One pmol of each heavy labelled peptide was then added from heavy labelled peptide mixes that were routinely prepared and used in the lab (concentrations of peptides in the mixes was 1 pmol/10 µL). Trypsin was then added (10 µL; 0.1 µg/µL in 50 mM acetic acid) to each sample to give a trypsin:protein ratio of 1:20 (w/w). Samples were then vortexed and digested at 37 °C for 16 h (overnight) using the Isotemp Thermal Mixer shaking at 300 rpm. The reaction was stopped by the addition of 10 % trifluoroacetic acid, such that the volume added was 10 % of the total reaction volume. This was 20.3 µL for each digest. A sodium deoxycholate precipitate formed. The samples were then hand vortex mixed and centrifuged at 13.3K x g for 5 min to pellet the precipitate. The supernatant was transferred to fresh tubes (eppendorf Protein LoBind, 0.5 mL) and the samples were evaporated for 10 min in the ThermoSavant SpeedVac to remove excess acetonitrile that may have been present from the heavy labelled peptide mixes. It was thought that the acetonitrile could decrease the efficiency of the subsequent solid phase extraction (SPE) step.

The SPE cartridges (10 mg/mL; polymeric reversed phase) were conditioned with methanol and purified water. Sample was added and the cartridges were washed with water. Elution was with 60 % acetonitrile/40 % formic acid 0.1 % into eppendorf 0.5 mL LoBind tubes. The eluate was evaporated to dryness and reconstituted in 50 µL modified mobile phase A (water/acetonitrile/formic acid 98/2/0.1, i.e. 2 % acetonitrile). Following centrifugation at 13.3K x g for 5 min the supernatant was transferred to deactivated vial inserts for analysis by nano LC-MS/MS.

Analysis was performed on a nanoACQUITY (Waters, Milford, MA) coupled to a SCIEX QTRAP 5500 hybrid mass spectrometer (Framingham, MA) equipped with a NanoSpray III source. Control was by Analyst 1.5 software (SCIEX) and nanoACQUITY UPLC Console. Mobile phase A consisted of 1 % acetonitrile and 0.1 % formic acid. Mobile phase B was 100 % acetonitrile. An injection volume of 0.1 µL (0.04 µg or 0.2 % of the sample) was loaded onto a Symmetry® C18 trap column, 2G-VM, 180 µm x 20 mm, 5 µm particle size (Waters) (part #
186006527) at a trapping flow of 15 µL/min of mobile phase A for 1 min. Peptides were eluted from the trap column and separated at a flow rate of 1.3 µL/min on a BEH130 C18 column, 150 µm x 100 mm, 1.7 µm particle size (Waters) (part # 186003550). Separation conditions were 100 % A at start, to 58 % A at 24 min, 5 % A at 24.5 min for 3 min and 100 % A at 28 min for 7 min (total run time was 35 min). The analytical column temperature was set to 35 °C.

MS/MS analysis was conducted in the positive mode with ion spray voltage at 4000. An uncoated PicoTip emitter (20 µm inner diameter, 10 µm tip; New Objective, Woburn, MA) was used to produce the nanospray. An MRM acquisition method for the transporter peptides (2 MRM for unlabelled peptide and 2 for labelled) and 2 x β-casein peptides (2 MRM per peptide [unlabelled only]) was used to obtain data for the samples on the mass spectrometer. Individual peptide MRM collision energies for all peptides had been optimized during method development using crude (unlabelled) peptides (occasionally the heavy labelled peptides had been used) and employing Skyline software (v2.6, University of Washington) for initial prediction of MRM lists. Equality of MRM response between the heavy labelled and unlabelled peptides was assumed. Human BCRP expression levels were determined using peptides VIQELGLDK, SSLLDVLAAR, ENLQFSAALR, human Pgp using peptides FYDPLAGK, IIDNKPSIDSYSK, NTTGALTTR, IATEAIENFR and rat Pgp using peptides NTTGALTTR, IATEAIENFR, QLNVQWLR. NaK ATPase was used as a membrane marker. MRM data processing was by MultiQuant 2.0.2 (SCIEX). Concentrations were calculated for each individual peptide using area ratios of unlabelled (endogenous) to labelled MRM responses (2 unlabelled and 2 labelled MRM summed per peptide).
References

