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Title Page

Title: The need for BCRP substrate and inhibition evaluation in drug discovery and development: why, when and how?

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Running Title Page

Running title: BCRP *in vitro* assays

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List of nonstandard abbreviations: BCRP, human breast cancer resistance protein; Bcrp1, rodent breast cancer resistance protein; DDI, drug-drug interactions; E3S, estrone-3-sulfate; ER, efflux ratio; IC_{50}, half maximal inhibitory concentration; LC-MS/MS, high-performance liquid chromatography

with tandem mass spectrometry; LLC-PK1, porcine kidney epithelial cell line (Lewis-lung cancer porcine kidney 1); L-MDR1, LLC-PK1 cells overexpressing MDR1; MDCKII, canine kidney epithelial cell line (Madin–Darby canine kidney); M-BCRP, MDCKII cells overexpressing BCRP; MDR1, multidrug resistance protein 1; MTX, methotrexate; PhIP, 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

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Abstract:

Whilst the multiplicity in transport proteins assessed during drug development is continuously increasing, the clinical relevance of the Breast Cancer Resistance Protein (BCRP, MXR) is still under debate. Our aim here is to rationalize the need to consider BCRP substrate and inhibitor interactions and to define optimum selection and acceptance criteria between cell-based and vesicle-based assays *in vitro*. Information on the preclinical and clinical pharmacokinetics (PK), drug-drug interactions and pharmacogenomics data were collated for 13 marketed drugs whose PK is reportedly associated with BCRP interaction. Clinical examples where BCRP impacts drug PK and efficacy appear to be rare and confounded by interactions with other transporters. Thirty-seven compounds were selected to be tested as BCRP substrates in a MDCKII cell-based assay and 18 in vesicles. Depending on the physico-chemical compound properties, we observe both *in vitro* systems to give false-negative read-outs. In addition, the inhibition potential of 19 compounds against BCRP was assessed in vesicles and in MDCKII cells, where we observed significant system and substrate-dependent IC_{50} values. Neither of the two test systems is therefore superior to each other. Instead, one system may offer advantages under certain situations (*e.g.* low permeability) and thus should be selected based on the physico-chemical compound properties. Finally, given the clinical relevance of BCRP, we propose that its evaluation should remain issue-driven: for low permeable, low bioavailable drugs, in particular when other more common processes do not allow a mechanistic understanding of any unexpected absorption or brain disposition, and for drugs with a low therapeutic window.

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Introduction:

The Breast Cancer Resistance Protein (BCRP) was discovered in multi-drug-resistant cancer cells, with the identification of chemotherapeutic agents as substrates. BCRP has a ubiquitous tissue distribution, thus, like MDR1 (multidrug resistance protein 1), its physiological role is to protect the body by limiting intestinal absorption and exposure of vital organs to xenobiotics (Polgar et al., 2008). Clinical examples have been reported where inter-individual differences in BCRP function correlated with increased toxicity (gefitinib-induced diarrhoea) or altered pharmacokinetics (PK) (rosuvastatin, sulfasalazine and topotecan) (Giacomini et al., 2010) (Table 1).

Sulfasalazine is a low permeable compound described as a BCRP substrate *in vitro* (Urquhart et al., 2008) (Table 1). Following oral administration to Bcrp knockout mice, a striking 111-fold increase in AUC was observed when compared to wild type mice (Zaher et al., 2006). However, several clinical studies have demonstrated that sulfasalazine PK in human is not as sensitive to BCRP efflux and has a large inter-individual variability in its PK. Thus its potential use as a BCRP probe substrate in the clinic is controversial (Adkison et al., 2010). Rosuvastatin, a BCRP substrate *in vitro*, has an estimated bioavailability of around 10-20%. Metabolism only plays a minor role in rosuvastatin elimination, as it is excreted mainly unchanged in bile (Table 1). Several clinical studies showed an impact of BCRP polymorphisms on its PK and pharmacodynamics (PD). The rise in exposure may be owing to the overlapped effects of increased oral bioavailability and decreased hepatic clearance (Ieiri et al., 2009). Clinical drug-drug interaction (DDI) studies with eltrombopag, ritonavir or cyclosporine A (CsA) increased rosuvastatin plasma exposure by up to 7-fold, but these perpetrators are also strong Organic Anion-Transporting Polypeptide (OATPs) inhibitors, a significant distribution and elimination pathway of rosuvastatin.

The FDA and EMA guidelines recommend to evaluate all investigational drugs *in vitro* to determine whether they are potential substrates of MDR1 and/or BCRP (EMA, 2010; FDA, 2012). Both agencies recognise however that drugs with high permeability and high solubility tend to be well

absorbed even if substrates of active transport and therefore can be exempt from *in vivo* evaluation of MDR1 or BCRP interactions (as victim). Both regulatory agencies also require evaluating new molecules as BCRP inhibitors. The rationale for this requirement is limited, and is thought to be largely due to the similarities with MDR1. A recent review on the effect of BCRP on human PK concluded that examples from human studies are rare and confounded by the functional redundancy of BCRP with other transporters such as MDR1 (*e.g.* topotecan), Organic Anion Transporters (OATs, *e.g.* methotrexate) or OATPs (*e.g.* rosuvastatin) (Schnepf and Zolk, 2013) (Table 1).

When assessing BCRP substrate or inhibition properties, different *in vitro* systems are used to study efflux transporters: systems overexpressing BCRP, such as transfected cell lines or membrane vesicles, and organotypic cell lines such as Caco-2 (Zamek-Gliszczynski et al., 2013). Different test systems may have limitations, depending on the type of study conducted. For instance, membrane vesicular assays may give false-negative results for highly lipophilic compounds due to high nonspecific binding (Xia et al., 2005; Giacomini et al., 2010). Likewise, polarized transcellular assays are not suitable for low permeable compounds, since they do not sufficiently penetrate the cells and therefore do not reach the export protein, potentially resulting in false-negative results. The existence of multiple binding sites on transport proteins could also add to the complexity in identifying substrate and inhibitor molecules in *in vitro* assay systems, leading to incorrect interpretation (Muenster et al., 2008; Giri et al., 2009). Careful selection of substrates and inhibitors is therefore critical for designing definitive studies, and may circumvent the current issues with very high experimental inter-laboratory variability in *in vitro* assays (Bentz et al., 2013).

The first objective of this paper was to define criteria that necessitate a need to assess BCRP substrate and inhibition in drug development. Whilst regulatory agencies and the International Transporter Consortium (ITC) continuously increase the number of transport proteins to be assessed during drug development, there is still debate on the clinical relevance of BCRP (Tweedie et al., 2013). In this regard, preclinical and clinical evidence for the clinical relevance of BCRP will be discussed.

The second objective of this work was to define the optimum choice of *in vitro* test system for BCRP assessment. Using in-house experimental conditions, we directly compare results between vesicle-based and cell-based assays for BCRP substrate and inhibition, with a view to identify which of these two assays are to best suited under which circumstances.

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Material and Methods

Material

Compounds used in the *in vitro* experiments were of a typical purity of ≥98% and were obtained from Sigma (Buchs, Switzerland), Fluka (Buchs, Switzerland) or Apin Chemicals (Abingdon, Oxfordshire, UK). The BCRP vesicles were from Solvo Biotechnology (Szeged, Hungary).

Cell origin and culture

The parent LLC-PK1 cell line is a porcine kidney epithelial cell line (Lewis-lung cancer porcine kidney 1). The parent MDCKII cell line is a canine kidney epithelial cell line (Madin–Darby canine kidney). LLC-PK1, MDCKII, L-MDR1 (LLC-PK1 cells transfected with human MDR1) and M-BCRP (MDCKII cells transfected with human BCRP) cell lines were obtained from Dr. A. Schinkel, The Netherlands Cancer Institute (Amsterdam, The Netherlands) and used under a license agreement. Control and BCRP-expressing membrane vesicles derived from transfected Sf9 cells were obtained from Solvo Biotechnology (Budapest, Hungary).

MDCKII and M-BCRP cells were grown in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin. L-MDR1 cells were grown in M-199 medium with phenol red containing 10% fetal bovine serum and 100 units/mL penicillin and 100 µg/mL streptomycin. Cells were split every three or four days by trypsinization. All cells were cultivated at 37°C in a humidified 5% CO_2 cell culture incubator. Standard tissue culture flasks were from Falcon and 96-well plates were from Millipore. All cells were seeded on permeable inserts (Millipore, 0.11 cm2 area, pore size 0.4 µm, low density) and transport measurements were performed at day 3 after seeding.

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Transcellular 96-insert plate automated in vitro experiment

The method used has been reported previously (Poirier et al., 2014). Tightness of the cell monolayer was controlled via the permeability of the extracellular marker lucifer yellow (10 µM). The assays were automated and performed on a robot (Tecan freedom Evo 200 Base) with integrated incubator (Liconic Instruments Storex Incubator). The medium was removed from apical (100 µl) and basolateral (240 µl) compartments and replaced on the receiver side by culture medium without phenol red, with or without inhibitor. The transcellular transport measurement was initiated by adding the test compound (final concentration 1 µM, except PhIP (2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) (2 µM) dissolved in culture medium together with the extracellular marker lucifer yellow to the donor side. The inhibitor (elacridar 1 µM, Ko143 1 µM or test compound) was added to both sides. The transport experiment was performed in both directions in triplicates. The plates containing the inserts were incubated at 37°C and 5% CO_2 under continuous shaking (100 rpm). Samples were taken from the donor and the opposite (acceptor) side after 3.5 hours incubation. Concentrations of substrate in both compartments were determined by scintillation counting for radioactive compounds or by LC-MS/MS. The extracellular marker (lucifer yellow) was quantified using a Tecan Ultra Evolution Reader at 430/535 nm (Ex/Em). Triplicate inserts were used for each condition. Experiments showing lucifer yellow permeation superior to 1%/h were rejected. Digoxin (MDR1), or PhIP (BCRP) were included on each 96-insert plate as a positive control.

BCRP vesicles 96-filter plate in vitro experiment

Buffers preparation

The assay buffer (10 mM Hepes-Tris, 100 mM KNO_3, 10 mM $Mg(NO_3)_2$, 50 mM sucrose) and the wash buffer (10 mM Hepes-Tris, 100 mM KNO_3, 50 mM sucrose) were filtered (through a sterile 0.2 µm filter for the assay buffer). On the day of the assay, a 200 mM MgAMP solution (250 mM Na_2AMP and 1 M $MgCl_2$) was prepared as well as 12 mM MgATP, 12 mM MgAMP, and radiolabeled substrate solutions by diluting their respective stocks in assay buffer. The final

concentration of MgATP (or MgAMP) and radiolabeled substrate in each well during the assay were 4 mM and 1 µCi/mL, respectively. The amount of membrane vesicles in each well during the assay was 25 µg. The final volume dosed in each well was 75 µL of which 25 µL was from either MgATP or MgAMP and 50 µL was from the assay buffer containing membrane vesicles and the radiolabeled substrate.

Pre-incubation

Membrane vesicles were thawed rapidly at 37°C and then placed on ice. Assay buffer (50 µL) containing membrane vesicles and radiolabeled substrate (final concentration 1 µM, except methotrexate 2 µM) was dispensed to a 96-well standard plate. Next, 50 µL of 12 mM MgATP (or MgAMP) without or with inhibitor was added to a second 96-well standard plate (MgATP/MgAMP plate). Both plates were incubated at 37°C for 5 min under shaking conditions. During this time the filtering apparatus (MultiScreen HTS vacuum manifold from Millipore, Billerica, MA) was set-up. The 96-filter plate (MultiScreen HTS-FB, Millipore, Billerica MA; Catalog #:MSFBN6B50) was wetted with 100 µL/well of wash buffer. The wetted filter plate was then incubated at room temperature for 1 min. The wash buffer was then filtered under vacuum.

Incubation

25 µL/well of the MgATP/MgAMP plate were added to corresponding wells of the membrane plate, thereby initiating the transport phase of the assay. The membrane plate was incubated at 37°C for 2 min under shaking conditions (400 rpm), and then 150 µL of ice cold wash buffer was added to each well. The samples from each well were then transferred to the 96-filter plate. Samples were filtered under vacuum and washed five additional times with 200 µL/filter of wash buffer. The filtering apparatus was turned on after each addition of wash buffer and turned off when the filter went dry and before adding additional wash buffer. Once the wash phase ended, 5 µL of assay buffer containing membrane vesicles and radiolabelled substrate (start mix) was dispensed to three unused filters of the 96-filter plate. The bottom of the 96-filter plate was blotted gently with filter paper and dried with a

hair dryer for several minutes. The collection plate was removed from the 96-filter plate and the bottom dried with a hair dryer for an additional 10-20 min being careful to not overheat the plastic part of the plate. The filters are considered dry when they turn from translucent to opaque. The cassette adapter (TopCount adapter for MultiScreen HTS white, Millipore, Billerica, MA; Catalog #: MSTPCWH50) was snapped on the bottom of the 96-filter plate. 100µL of MicroScint 20 scintillation cocktail (PerkinEimer, Shelton, CT) was added to each well. The top of the 96-filter plate was sealed with clear sealing tape (TopSeal-A: 96-well microplates, PerkinElmer). The plate was shaken for several minutes and radioactivity was counted using the TopCount NXT instrument from Perkin Elmer. The settings on the TopCount were as follows: 2 min/well for sample count.

Analytic

Analytical standards were prepared during the sample incubation as part of the assay. At the end of the experiment, samples were quenched with 3 volumes of acetonitrile containing the internal standards. Analyses of non-labeled compounds were performed by high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS). Briefly, fast reverse phase liquid chromatography was conducted on a Shimadzu 10ADvp pump system coupled with a CTC PAL HTS autosampler. The injection volume, mobile phase composition, analytical column and gradient profile were optimized for each compound. Mass spectrometric detection was performed on an AB Sciex API4000 or QTrap4000 equipped with a TurboIonspray source. Detection by tandem mass spectrometry was based on precursor ion transition to the strongest intensity product ion. Key instrumental conditions were optimized to yield best sensitivity. Typical run time was 1.5 min. The calibration range was typically 1 nM to 4 µM for each analyte. AB Sciex Analyst 1.4.2 software was used for data analysis. Concentration of compound in the samples was calculated from the peak area ratio between the analyte and the internal standard.

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Data evaluation

Transcellular transport (cell-based assay)

For the transcellular transport, the following equation was used for data evaluation:

$$P_{app} = \frac{1}{A * C_0} * \frac{dQ}{dt}$$

Where P_{app}, A, C_0, and dQ/dt represent the apparent permeability, the filter surface area, the initial concentration, and the amount transported per time period, respectively. P_{app} values were calculated on the basis of a single time point. Transport efflux ratios (ER) were calculated as follows:

$$ER = \frac{P_{app}BA}{P_{app}AB}$$

Where $P_{app}BA$ is the permeability value in the basolateral-to-apical direction, and $P_{app}AB$ the permeability value in the apical-to-basolateral direction. The average passive permeability value, $P_{app}i$, was calculated as follow:

$$P_{app}i = \frac{P_{app}ABi + P_{app}BAi}{2}$$

Where $P_{app}ABi$ and $P_{app}BAi$ represent the apparent permeability in the apical-to-basolateral and basolateral-to-apical direction, respectively, in presence of inhibitor.

Vesicle-based assay

All tested conditions within an experiment were run in triplicate. The disintegrations per minute (dpm) obtained from each filter were converted to pmol/mg protein/min as follows:

$$\text{pmol/mg protein/min} = \frac{(\text{dpm}) \times (\text{substrate concentration } (\mu\text{M})) \times (\text{volume}(\mu\text{L}))}{(\text{total dpm}) \times (\text{mg of membrane vesicles}) \times (\text{time(min)})}$$

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Substrate concentration refers to the radiolabeled plus unlabeled concentration of substrate added to each well; the volume refers to the total volume in each well during the transport assay (*i.e.* 75 µL); total dpm refers to the total disintegrations per minutes added to each well at the start of the transport assay; mg of membrane vesicles refers to the amount of membrane vesicles added to each well (*i.e.* 25 µg); time refers to the duration of the transport assay (*i.e.* 2 min).

The net uptake for each condition was calculated using the mean value of the triplicate filters as follows:

$$\text{net uptake (pmol/mg/min)} = \text{triplicate mean ATP (pmol/mg/min)} - \text{triplicate mean AMP (pmol/mg/min)}$$

The fold uptake for each condition was calculated using the mean value of the triplicate filters as follows:

$$\text{fold uptake} = \frac{\text{triplicate mean ATP (pmol/mg/min)}}{\text{triplicate mean AMP (pmol/mg/min)}}$$

IC_{50} estimation

A modified Hill's equation was used to estimate the IC_{50}s:

$$X = \frac{X_{max} - X_{min}}{1 + \left(\frac{[I]}{IC_{50}}\right)^{S}} + X_{min}$$

Where X is either the ER for cell-based assay or the net uptake for the vesicle-based assay, and X_{max} is the maximum value without inhibitor, X_{min} the value with Ko143 (maximum inhibition), [I] the nominal inhibitor concentration in µM and s the slope factor (or Hill coefficient). Origin® v7 (OriginLab Corporation, Northampton, MA) was used to perform the non-linear fitting of the data and evaluation of IC_{50} and s parameters.

Cut-off criteria

The cut-off criteria to categorized compounds as substrate or non-substrate for both the transcellular cell-based assay and the vesicle-based assay, were refined based on the results (see discussion) and previously published status (substrate / non-substrate).

BCRP clinical relevance – literature search

The data presented in Table 1 was gathered from recent reviews on BCRP, the American and European DDI regulations, the Metabolism and Transport Drug Interaction Database from the University of Washington and the latest published material (EMA, 2010; Poguntke et al., 2010; Meyer zu Schwabedissen and Kroemer, 2011; FDA, 2012; Schnepf and Zolk, 2013; University of Washington, 2013). The selection of drugs were not intended to offer an exhaustive list, but rather aim to represent a characteristic sample in terms of absorption, distribution, metabolism and elimination (ADME) properties, pharmaceutical class and regulators' concerns. The parameters collected were ADME/PK properties (enzymes and transporter substrate properties, bioavailability and any remarkable feature), passive permeability, BCRP substrate property *in vitro*, *in vivo* studies in Bcrp1 knockout mice, results of BCRP pharmacogenetic clinical studies and finally results of DDI interaction studies potentially linked to BCRP.

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Results

BCRP clinical relevance

Table 1 is an overview of drugs whose PK and/or PD has been reported to be linked to BCRP (for reviews see (Poguntke et al., 2010; Meyer zu Schwabedissen and Kroemer, 2011; Schnepf and Zolk, 2013)). The main drugs under scrutiny are statins and chemotherapy agents, such as tyrosine kinase inhibitors and camptothecins.

If the absorption, distribution or elimination of a drug is solely or mainly dependent on one specific pathway, the risk for DDI increases and the specific underlying mechanism can be more easily identified. Therefore, to put the importance of BCRP in the selected clinical examples into context, information on the involvement of other pathways (drug metabolizing enzymes and other transporters) is also described (Table 1). Rosuvastatin, pitavastatin, pravastatin and topotecan are mainly excreted unchanged, whereas the other drugs listed are metabolised via CYPs, aldehyde oxidases (methotrexate), carboxylesterases (irinotecan) or bacterial azoreductases (sulfasalazine). All have been identified *in vitro* as substrates of BCRP, as well as MDR1, OATs or OATPs (Table 1).

The significance of transporters *in vivo* is related to drug permeability; the most significant impact of efflux transport activity is when drugs have a low bioavailability and low permeability (in terms of fold interaction) along with a narrow therapeutic index (in terms of safety) (Giacomini et al., 2010; FDA, 2012; Schnepf and Zolk, 2013). For highly permeable molecules, the transporter effect will be minimal. As detailed in Table 1, all drugs listed have a reported low to medium permeability except for imatinib (highly permeable). Methotrexate, pitavastatin, gefitinib and imatinib all have a bioavailability above 40% whilst for the remaining drugs listed, their bioavailability is below 40%.

All selected BCRP substrates, apart from gefitinib, showed increases either in plasma exposure, brain distribution, and/or decreases in total or biliary clearance in Bcrp1 knockout mice (Table 1). The strongest impact was on sulfasalazine, with a 111-fold increase in plasma AUC. However in human

genetically controlled studies, sulfasalazine PK was not as sensitive to BCRP efflux as that observed in mice, with only a 2 to 4-fold increase in AUC. Indeed, for most of the drugs listed, the PK changes observed in Bcrp knockout mice did not translate into similar effects in the clinic. One exception is rosuvastatin, which showed clinical AUC increases of between 2 and 4-fold in genetically controlled studies, and this is similar to the effects seen in Bcrp1 knockout mice (Table 1). Other clinical studies reported impact on bioavailability (topotecan), PD (methotrexate) and toxicity (irinotecan). Imatinib, which is highly permeable with high bioavailability, was not impacted in Bcrp1 knockout mice or individuals with functional impairment of BCRP. Finally, apart from gefitinib, all drugs whose PK was altered in patients with certain BCRP single-nucleotide polymorphisms (SNPs) showed a bioavailability below 30%.

In contrast to MDR1-mediated clinical DDI studies of which there are more than 60 cases reported, only 4 DDI studies have been reported potentially linked to BCRP (Table 1: methotrexate, sulfasalazine, rosuvastatin and topotecan) (Poirier et al., 2014). We are not aware at the time of publication of any other clinical example. There were no reported changes in the PK of sulfasalazine when co-administered with pantoprazole, a known inhibitor of BCRP *in vitro*, and only a 3.2-fold increase in AUC when co-administered with high doses of curcumin. These findings are surprising, given the preclinical observations (111-fold change in AUC) and effect of BCRP polymorphism (up to 4-fold change in AUC) with sulfasalazine. For the other 3 drugs, changes in the PK parameters or toxicity were observed when co-administered with marketed drugs (omeprazole, pantoprazole, eltrombopag, ritonavir and CsA) or probe inhibitors (elacridar). An important consideration is that the perpetrator drugs mentioned above are not pure inhibitors of BCRP, and are recognised inhibitors of multiple transport proteins and enzymes.

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BCRP substrate in vitro assay

Cell-based assay (M-BCRP)

Thirty-seven compounds were selected to be tested as BCRP substrates in M-BCRP cells. Six compounds were selected as negative controls: CsA and ritonavir, previously reported as non BCRP substrates (Gupta et al., 2004; Xia et al., 2007), propranolol, metoprolol and verapamil, highly permeable drugs with no records as BCRP substrates, and metformin, a low permeability drug with questionable BCRP substrate data (Hemauer et al., 2010). Propranolol, metoprolol and verapamil showed ER in M-BCRP cells of around 1, which was insensitive to Ko143 and equivalent in MDCKII (parental cells), the same was observed for fumitremorgin C (FTC) (Table 2). The ER of CsA and ritonavir in M-BCRP cells was higher than 2 but equivalent to the ER in MDCKII cells and also not sensitive to Ko143. Therefore these two compounds were categorized as non-substrates.

Most of the other test compounds were selected as they have been identified previously as BCRP substrates (references given in Table 4) except FTC and MK571, which are rather described so far as inhibitors. Twenty compounds had a Ko143-sensitive ER above 2 in M-BCRP cells and markedly greater than the ER in MDCKII cells (Table 2). Daunorubicine and rifampicin ER in M-BCRP cells were significantly above 2 and around 2 in MDCKII cells which is characteristic of BCRP substrates. However, their ER in M-BCRP cells were insensitive to Ko143. This can be due to differences in binding sites and substrate-dependent inhibition (Muenster et al., 2008; Giri et al., 2009). Those examples well illustrate that not only the ER value in M-BCRP cells should be taken into consideration, but the impact of Ko143 and the ER in the control cells in order to avoid false-negative or false-positive results (Figure 1).

Erythromycin also appears as a false negative in the cell-based assay. The ER of erythromycin in M-BCRP cells was above 2 (8.0) but insensitive to Ko143 (7.6) and equivalent in MDCKII cells (10.8, Table 2) pointing towards an involvement of the canine P-gp expressed in those cells. Janvilisri and co-workers identified erythromycin as a BCRP substrate working with cells in suspension (no

transcellular transport assessment) with a 1h erythromycin pre-incubation (Janvilisri et al., 2005). The difference in experimental conditions could explain the divergence in results; erythromycin appears here not to be a BCRP substrate.

For ten of the compounds tested, all samples from the receiver compartments were below the limit of quantification (LOQ), corresponding to a permeability of around 15 nm/s when using the LOQ as nominal concentration. Thus, doxorubicin, estrone-3-sulfate (E3S), vincristine, lamivudine, methotrexate (MTX), rosuvastatin, metformin, tamoxifen, nitrofurantoin and sulfasalazine appear not permeable in MDCKII cells and therefore the transcellular cell-based assay is not a suitable method for testing these compounds for BCRP substrate interaction.

All confirmed BCRP substrates were also found to be MDR1 substrates, except simvastatin (Supplemental Table 1).

Vesicle-based BCRP assay

From the 37 compounds tested in M-BCRP cells, 18 were selected to be tested in membrane vesicles isolated from insect cells overexpressing human BCRP, based on their availability with a radiolabel.

The negative controls metoprolol, propranolol and metformin all exhibited a fold uptake of 1.0, with an insignificant net uptake (Table 3). The fold uptake of verapamil was 1.3 with a net uptake of 15.1 pmol/mg/min, however it was not affected by Ko143.

Fluvastatin, pitavastatin, MTX, topotecan, rosuvastatin and E3S all showed a fold uptake higher than 2 with high net uptake that was decreased in the presence of Ko143. They can be clearly identified as BCRP substrates in vesicles. Mitoxantrone and vincristine, known BCRP substrates, showed a fold uptake between 1.3 and 2, but net uptake greater than 40 pmol/mg/min which was significantly decreased by Ko143. Similarly, cimetidine, diadzein and prazosin, also known BCRP substrates, showed a fold uptake between 1.3 and 2 but in contrast, their net uptake was below 20 pmol/mg/min and not affected by Ko143.

PhIP, sunitinib and lamivudine, all reported BCRP substrates, showed a fold uptake below 1.3 and net uptake below the limit of quantification. They were categorized as non BCRP substrates.

L-MDR1 assay

In many research organisations new molecules are routinely screened for human MDR1-mediated transport during lead optimisation using cells (LLC-PK1 or MDCKII) transfected with human MDR1. The permeability results from this early MDR1 screen may provide a useful guide for selecting the correct *in vitro* tool (cells *vs.* vesicles) for studies on other efflux transporters such as BCRP at a later stage in development. To test the validity of this approach, all 37 compounds that we tested for BCRP interaction above, were retrospectively tested in L-MDR1 cells (LLC-PK1 cells transfected with MDR1). The results are reported in detail (Supplemental Table 1) and are part of summary Table 4.

In Table 4, compounds have been grouped according to the results obtained from the two different BCRP methods (cells and vesicle-based). Overall, the permeability results from all the compounds tested in M-BCRP and L-MDR1 assays show a good alignment. Compounds that were significantly permeable in L-MDR1 cells were also permeable (and thus suitable) in the M-BCRP cell based assay, except for doxorubicin, which had a measurable permeability in L-MDR1 cells of 15 nm/s (very low) but was below the limit of detection in M-BCRP cells (Table 4). Conversely, compounds not permeable in L-MDR1 cells were also not permeable in M-BCRP cells, except for coumestrol, which was not permeable in L-MDR1 cells.

BCRP inhibition in vitro assay

The inhibition potential of 19 compounds was tested in vesicles using 2 different substrates – E3S and MTX – and in M-BCRP cells using PhIP as substrate. Fexofenadine, metformin and vincristine were selected as negative controls (Xia et al., 2005). The inhibition potential of typical model substrates, E3S and MTX, were tested on each other, and 8 compounds were selected as well-known BCRP inhibitors, especially Ko143 and FTC (Xia et al., 2005). The inhibition potential of omeprazole,

pantoprazole, eltrombopag, ritonavir, CsA and elacridar was also determined in order to bridge the *in vitro* findings with the clinical observations reported in Table 1 for those respective inhibitors.

In all 3 systems, Ko143 was the most potent inhibitor with IC_{50} values that are 10-fold lower than elacridar (Table 5). Xia and co-workers compared IC_{50} data from vesicle-based and cell-based assays for Ko143, elacridar, zosuquidar, MK571, ritonavir, verapamil, CsA and fexofenadine (Xia et al., 2005). When using E3S uptake into human BCRP vesicles, their IC_{50} values are in the same range as those reported here, and the compounds ranked similarly. FTC, a mycotoxin, is one of the most potent BCRP inhibitors. Its inhibition potential on E3S and MTX in BCRP vesicles has been previously reported (respectively 0.28 µM and 0.30 µM (Chen et al., 2003; Kawahara et al., 2010)) and is identical to our measured value. Our IC_{50} values for imatinib, topotecan, rapamycin, eltrombopag, pantoprazole and omeprazole are also very comparable to published results (Breedveld et al., 2004; Houghton et al., 2004; Ozvegy-Laczka et al., 2004; Gupta et al., 2006; Allred et al., 2011). Reported here for the first time is the inhibitory potential of E3S, methotrexate, vincristine and metformin on BCRP.

Overall, the 3 test conditions (E3S and MTX in vesicles and PhIP in cells) ranked compounds similarly. The IC_{50} fold differences between E3S versus MTX in vesicles and PhIP in M-BCRP cells versus MTX in vesicles is reported in the last columns of Table 5. There were four main outliers: topotecan, MK571, E3S and eltrombopag. Topotecan and E3S inhibited MTX uptake into BCRP vesicles, but no inhibition was detected in M-BCRP cells when tested up to the highest soluble concentration in buffer. The potency of MK571 on MTX uptake by BCRP vesicles was 21-fold higher than on E3S uptake and 478-fold higher than on PhIP transcellular transport in M-BCRP cells. Eltrombopag was 12-fold more potent in vesicles compared to cell-based assay.

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Discussion

New chemical entities (NCEs) as BCRP substrate

Why and when to test for BCRP substrate property?

The evidence for the clinical importance of BCRP can be seen as inconsistent and is often confounded by the involvement of multiple elimination pathways as for the clinical DDI between topotecan and elacridar (Table 1). The basis of the association with BCRP over MDR1 is linked to a study in Mdr1 knockout mice, and that topotecan is described as a low affinity MDR1 substrate (Jonker et al., 2000; Kruijtzer et al., 2002). However, *in vitro* data shows that topotecan is a human MDR1 substrate with a comparable ER (> 4.2, Supplemental Table 1) as BCRP (> 6.4, Table 2), under identical experimental conditions. Moreover, although elacridar is a potent BCRP inhibitor (0.15 µM, Table 5), it is 6 times more potent against MDR1 (0.025 µM (Poirier et al., 2014)). Thus there is reasonable evidence to suggest that the mechanism of the DDI between topotecan and elacridar is not purely mediated by BCRP, but is also linked to inhibition of MDR1. It is also worth noting that elacridar is not a marketed drug, and therefore the practical risk of a DDI with elacridar is negligible.

A significant amount of discordance is apparent when comparing the observations in Bcrp knockout mice with that of clinical findings; the most significant of which is for sulfasalazine (Table 1). The fold effect on sulfasalazine exposure observed in knockout mice (111-fold AUC) does not translate to human: sulfasalazine PK was not affected in individuals with impaired BCRP function nor when co-administered with pantoprazole. However, in a recent clinical study, curcumin, an *in vitro* inhibitor of BCRP but also of aldose reductases and several drug metabolizing enzymes, increased sulfasalazine AUC by 3 fold (Du et al., 2006; Volak et al., 2008; Kusuhara et al., 2012). After oral administration, sulfasalazine is metabolised by bacterial azo-reductases in the lumen of the colon and rectum (Yamasaki et al., 2008). It might therefore be a possibility that the mechanism of the sulfasalazine/curcumin interaction is not only an inhibition of BCRP but also inhibition of the

bacterial degradation of sulfasalazine in the intestine. This would also explain the lack of interaction seen between pantoprazole and sulfasalazine mentioned earlier.

Furthermore, methotrexate and rosuvastatin are clinical victim drugs that are associated with OAT and OATP-mediated DDI's, respectively. Therefore BCRP inhibition solely cannot be responsible of these DDI's, particularly in cases where the precipitant is CsA, a potent and promiscuous inhibitor of multiple metabolic enzymes and transport proteins. Both FDA and EMA also acknowledge that a highly soluble and highly permeable drug may be exempt from MDR1 and BCRP testing *in vivo*. Most drugs whose PK is altered in BCRP pharmacogenetic studies have indeed a low permeability and a low bioavailability.

Based on the above findings, we propose not to go for a systematic evaluation of BCRP substrate properties, but rather on a more case-by-case strategy that is based on physicochemical and PK properties of the test compound. Hence BCRP substrate properties should be evaluated for compounds (Figure 1) with low permeability and low bioavailability, and where other more common interactions have been excluded from effect on absorption and/or brain disposition (*e.g.* MDR1 substrate), and for drugs with a low therapeutic window.

How to test for BCRP substrate property?

Choice of in vitro system

All permeable molecules, previously described as BCRP substrates, could be correctly categorized using M-BCRP cells as experimental system (Table 4). However, certain compounds were not permeable and therefore not suited for testing in the cell-based assay. There was a high rate of false negatives observed with the vesicles (40%). Initial cell-based screening assays (*e.g.* L-MDR1) are predictive of the passive permeability in a cellular system, and could be easily used as a selection criterion to choose between cell-based and vesicle-based assays for subsequent experiments (Figure 1). Given the higher risk of false negatives in vesicles, cell-based assays should remain the first choice if the drug shows acceptable permeability in cells.

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Cut-off criteria

The regulatory agencies provide cut-off criteria to identify BCRP substrates in a cell-based assay (ER ≥ 2) although they would accept other thresholds, based on prior experience with the cell system used. Tables 2 and 3 clearly indicate that all compounds, previously known as BCRP substrates, showed ER ≥ 2 and non-substrate < 2. This threshold seems appropriate for the M-BCRP used under our experimental conditions. Care has to be taken to exclude false-positives by including parental cells and selective inhibitors.

Establishment of a vesicle-based assay cut-off is more challenging, and must be based on a consistent and significant number of compounds tested under the same conditions. The results as presented in Table 3 indicate that a threshold of 2 in the fold uptake can clearly discriminate substrates (Figure 1). However, a number of recognised BCRP substrates presented a fold uptake between 1.3 and 2.0 (thus being false negatives) yet had a significant net uptake (above 30 pmol/mg/min). It is therefore more appropriate to consider the fold uptake with a reduce threshold to 1.3, in combination with a net uptake above 30 pmol/mg/min and sensitive to inhibition by Ko143. When applying these latter criteria, the false-negative rate was decreased (from 53% to 40%) without increasing the false-positive rate.

NCEs as BCRP inhibitors

Why and when to test for BCRP inhibition properties?

We have discussed in the above section that all the current clinical evidence associated with BCRP is ambiguous in its interpretation. In addition, should a BCRP-specific substrate / inhibitor be identified, the clinical relevance is as yet unclear. Therefore as a further refinement to the current regulatory recommendations, our proposal is to delay the assessment of BCRP inhibition until late-stage development (i.e. phases III, IV, before filing or even post approval) where it is still not anticipated to

impact greatly on any DDI risk, but serves to only to fulfil the DDI information package for completeness and regulatory compliance. However, given the above discussion on the limited BCRP-mediated DDI evidence, the results should always be put into context of other potential and more relevant mechanisms.

How to test for BCRP inhibition properties ?

Choice of in vitro system

BCRP inhibition has already been described as being substrate-dependent (Muenster et al., 2008; Giri et al., 2009). In vesicles, MK571 was 21-fold more potent on MTX than on E3S (Table 5). The observed fold difference between IC_{50} performed using MTX in vesicles and PhIP in M-BCRP cells may also be partly due to substrate-dependency, and not just because of the difference in the *in vitro* tool. E3S, MK571, eltrombopag and topotecan have limited permeability in the transcellular system used in this study, are therefore unable to reach the BCRP binding site in that system, hence the important shift in IC_{50} estimated using vesicles or M-BCRP. The situation might be different if other transcellular systems are used, such as Caco2 cells or double transfected cells, if the respective uptake transporters for the test substrate are expressed. Permeability of both substrate and inhibitor should be fundamental in selecting the *in vitro* tool to test for efflux inhibition. The selection of the model substrate is also crucial: ideally multiple substrates should be used, including the expected clinical victim drug.

Cut-off criteria

To quantitatively assess the risk of clinical DDI, the EMA and FDA recommend considering $[I_1]/IC_{50}$, ($[I_1]$, plasma concentration) and $[I_2]/IC_{50}$ ($[I_2]$, gut concentration) with respectively 0.1 and 10 as reasonable cutoffs. While several groups and institutions have worked on the predictability of $[I]/IC_{50}$ for MDR1-mediated DDI, there is no report on the relevance of those thresholds for BCRP (Ellens et al., 2013; Poirier et al., 2014). It can be partly explained by the lack of clinical interactions that are clearly linked to BCRP.

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Using the lowest BCRP IC_{50} from Table 5, and for all examples referenced in the last column of Table 1, all $[I_1]/IC_{50}$ would indeed be above 0.1 and $[I_2]/IC_{50}$ above 10. However, for all perpetrators, equivalent risks would be identified using IC_{50} data against other transport proteins (MDR1, OATPs), and the consequent clinical study performed (Supplemental Table 2). The pantoprazole/sulfasalazine co-administration would have been marked as a potential DDI ($[I_2]/IC_{50}$ = 38, $[I_1]/IC_{50}$ = 0.6) whereas no significant effect was observed (Adkison et al., 2010).

General conclusion

If an NCE is to be tested for BCRP substrate or inhibition properties, a careful selection of assay system is necessary in order to circumvent certain limitations that are inherent to each method and to avoid false readouts. The cell-based and the vesicle-based assays each offer advantages and should be selected based on physico-chemical properties of the NCE. Therefore a thorough understanding of the limitations of both tools is needed for their optimal use. Similar to other transport proteins, substrate-dependent inhibition has been reported for BCRP. This could be due to either differential binding to one or multiple binding site(s) or in the case of the cellular assay, involvement of other transporters modulating the intracellular concentration available at the binding site of BCRP. Further studies are needed to assess these properties of BCRP. Inhibition potency is in such cases mostly dependent on the substrate used *in vitro*.

Why and when a NCE should be tested for BCRP substrate or inhibition property is a matter of debate. Clinical examples are rare and in cases where BCRP is involved, other transporters or metabolizing enzymes might also contribute to the interaction. We propose to place BCRP assessment as a second line evaluation, testing first for drug transporters and metabolic enzymes for which relevant clinical examples of DDIs are more frequent and better defined. For those mechanisms the *in vitro* results can be put into perspective to *in vivo* using a number of clinical reported interactions to calibrate the *in vitro* assays. BCRP evaluation would then remain occasional: for low permeable, low

bioavailable drugs, specifically when other more common processes do not allow a mechanistic understanding of unexpected absorption and/or brain disposition, and for drugs with a low therapeutic window.

Authorship Contributions

Participated in research design: Poirier, Portmann, Cascais, Ullah and Funk

Conducted experiments: Portmann, Cascais and Bader

Contributed new reagents or analytic tools: Bader and Walter

Performed data analysis: Poirier, Portmann, Ullah and Funk

Wrote or contributed to the writing of the manuscript: Poirier, Walter, Ullah and Funk

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Legend for Figure

Figure 1: Proposed testing strategy for BCRP substrate properties of new chemical entities based on physicochemical properties, permeability in cell-based assays, and calibrated threshold of cell-based or membrane vesicles-based assays.

Tables

Table 1: Summary of the ADME properties, *in vitro* passive permeability, *in vitro* BCRP substrate properties, and details of pre-clinical and clinical PK studies for drugs associated with BCRP interaction.

Drug	**General ADME – PK properties**[c]	**passive permea-bility** [b]	**BCRP substrate *in vitro***	**PK changes in Bcrp1(-/-) mice**	**PK changes in BCRP pharmacogenetic clinical studies**	**PK changes in clinical DDI studies**
Methotrexate (chemotherapy)	MDR1 , OATs & aldehyde oxidase substrate (Norris et al., 1996) F ~ 70% low therapeutic window	low [a]	Y (Poguntke et al., 2010)	1.7 fold increase in AUC (Vlaming et al., 2011)	impact on PD (psoriasis patients), no toxicity, no information on PK (Warren et al., 2008)	delayed elimination + severe intoxication (+dual MDR1/BCRP inhibitors: omeprazole, pantoprazole (Santucci et al., 2010))
Sulfasalazine (inflammatory bowel diseases)	bacterial azo reductase substrate (Yamasaki et al., 2008) OATP2B1 substrate (Kusuhara et al., 2012) F~3-12 % / high protein binding	low [a]	Y (Urquhart et al., 2008)	111 fold increase in AUC (Zaher et al., 2006)	c.34GG and c.421AA/CC/CA: <2 to 4 fold AUC increases; inconsistent findings between studies (Adkison et al., 2010; Schnepf and Zolk, 2013)	- no significant association with PK (+dual MDR1 / BCRP inhibitor: pantoprazole) (Adkison et al., 2010) - 3.2 max fold AUC increase (+BCRP and enzymes inhibitor: curcumin) (Kusuhara et al., 2012)
Statins (hyperlipidemia) – all good OATP substrates (Poirier et al., 2007)						
Rosuvastatin	minor role of metabolism F ~ 10-20%	low [a]	Y (Hirano et al., 2005)	2 fold decrease in total plasma CL (Kitamura et al., 2008)	c.421CA/AA: 2-2.4 fold AUC increases (Zhang et al,. 2006 ;Ieiri et al., 2009; Keskitalo et al., 2009b)	- 1.6 max fold AUC increase (+ dual OATP/BCRP inhibitors: eltrombopag, ritonavir) (Allred et al., 2011) - 7 fold increase (+multiple transporters and enzymes inhibitor: CsA) (Schnepf and Zolk, 2013)

Atorvastatin	CYP3A4 substrate F ~ 12%	low [a]	borderline (Keskitalo et al., 2009b)	NR	c.421AA: 1.7 fold AUC increase (Keskitalo et al., 2009b)	NR
Fluvastatin	MDR1[a] & CYP2C9 substrate - extensive metabolism & biliary excretion F ~ 29% / high protein binding	low [a]	Y (Hirano et al., 2005; Xia et al., 2005)	NR	c.421AA: 2 fold AUC increase (Keskitalo et al., 2009a)	NR
Simvastatin lactone	CYP3A4 (major) & 2C8 substrate – significant metabolism F ~ 5%	medium [a]	Y (Xia et al., 2005)	NR	c.421AA: 2.1 fold AUC increas (Keskitalo et al., 2009a)	NR
Pitavastatin	MDR1 substrate [a] minor role of metabolism F ~ 50%	medium [a]	Y (Hirano et al., 2005)	no impact on total plasma CL / 10-fold decrease in biliary excretion clearance (Hirano et al., 2005)	c.421AA/CC/CA: no significant association with PK (Ieiri et al., 2007)	NR
Pravastatin	minor role of metabolism	low (Caco2 (Marino et al., 2005))	Y (Hirano et al., 2005)	NR	c.421AA/CC/CA: no significant association with PK (Keskitalo et al., 2009a)	NR
<u>tyrosine kinase inhibitors</u> (chemotherapy)						
Gefitinib	MDR1 substrate [a] extensive metabolism by CYP3A54 (major) & CYP2D6 F ~ 60%	medium [a]	Y (Xia et al., 2005; Li et al., 2007)	no impact on brain distribution (Agarwal et al., 2010)	c.421CA 1.4 fold increase of C_{trough} no impact on AUC or C_{max} (Li et al., 2007)	NR
Sunitinib	MDR1[a] & CYP3A4/5 substrate F unknown	medium [a]	Y (Tang et al., 2012)	- 3.7 fold increase in AUC (Mizuno et al., 2012) - no impact on plasma exposure but on brain distribution (Tang et al., 2012; Schnepf and Zolk, 2013)	c.421CA/AA 1.7-3 fold AUC increase (Mizuno et al., 2012)	NR

Imatinib	MDR1[a] & CYP3A4/5 substrate F ~ 100%	high [a]	Y (Xia et al., 2005)	no impact on plasma exposure but on brain distribution (Schnepf and Zolk, 2013)	c.421CA: no significant association with PK (Gardner et al., 2006)	NR
camptothecins (chemotherapy)						
Irinotecan	MDR1 substrate [a] F~10% considered as a prodrug (of SN-38), carboxylesterases substrate	low [a]	Y (Xia et al., 2005)	NR	c.421CA, c.34GA: no significant association with PK – some report of related myelosuppression (Meyer zu Schwabedissen and Kroemer, 2011)	NR
Topotecan	MDR1 substrate [a] F~40% (Gelderblom et al., 2003) not extensively metabolized (Schnepf and Zolk, 2013)	low [a]	Y (Xia et al., 2005)	3.6 fold increase in AUC (Yamagata et al., 2007)	c.421CA: increase in F from ~30% to ~40%, no significant change in AUC, very small sample size (Sparreboom et al., 2005)	increase in F from ~40% to ~100% (+dual MDR1 / BCRP inhibitor: elacridar) (Schnepf and Zolk, 2013)

a: data from L-MDR1 cells in presence of inhibitor (Poirier et al., 2014) ; b: low : < 30 nm/s – medium 30-100 nm/s – high > 100 nm/s ; c: from (Hartman and Limbird, 2001) and (University of Washington, 2013) if not stated otherwise

ADME: Absorption, Distribution, Metabolism and Elimination; AUC: Area Under The Plasma Concentration Curve; CL: clearance; CsA: Cyclosporine A; CYP: cytochromes P450; DDI: Drug-Drug Interactions; F: apparent oral bioavailability in human; IV: Intra-Venous; OATPs: Organic Anion-Transporting Polypeptide; OATs: Organic Anion Transporter; NR: Not Reported; Y: substrate (yes)

Table 2: BCRP substrate *in vitro* results of drugs tested in M-BCRP cells at 1 µM

	M-BCRP cells data										MDCKII cells data				
Compound name	Mean P_{app} AB	SD	Mean P_{app} BA	SD	**M-BCRP ER**	SD	**Average P_{app}i**	SD	ERi	SD	**MDCKII ER**	SD	Average P_{app}	SD	BCRP substrate
FTC	253	11	246	49	**1.0**	0.2	**234**	40	1.4	0.1	**1.0**	0.2	294	48	N
verapamil	197	60	209	37	**1.1**	0.4	**204**	56	1.6	0.2	**1.1**	0.3	178	44	N
metoprolol	275	31	333	4	**1.2**	0.1	**275**	25	1.2	0.1	**1.0**	0.0	271	42	N
propranolol	177	20	224	9	**1.3**	0.2	**200**	44	1.5	0.2	**1.0**	0.3	204	47	N
cyclosporine A	39	1	77	7	**2.0**	0.2	**60**	29	2.4	0.2	**1.9**	0.3	59	28	N[$]
ritonavir	22	1	102	10	**4.6**	0.5	**72**	61	7.9	1.1	**4.6**	0.9	73	53	N[$]
erythromycin	8	2	68	3	**8.0**	1.5	**36**	30	7.6	0.2	**10.8**	0.3	41	37	N[$]
sunitinib	72	8	197	9	**2.7**	0.3	**92**	7	1.1	*	**1.2**	*	100	17	Y
simvastatin	22	3	86	7	**4.0**	0.7	**52**	11	1.2	0.4	**0.8**	0.1	45	4	Y
gefitinib	53	8	237	31	**4.5**	0.9	**106**	33	1.6	0.4	**1.5**	0.1	108	31	Y
zidovudine	13	2	59	7	**4.5**	0.7	**42**	19	0.7	*	**0.8**	0.2	56	26	Y
prazosin	73	14	332	10	**4.5**	0.9	**136**	19	1.3	0.1	**0.9**	0.3	149	21	Y
imatinib	76	10	371	37	**4.9**	0.8	**170**	90	1.2	0.9	**1.5**	*	229	81	Y
MK571	22	1	105	7	**4.9**	0.4	**51**	12	0.7	0.2	**0.6**	0.1	63	4	Y
coumestrol	<23	1	117	9	**>5.1**	0.4	**31**	7	1.4	0.2	**1.3**	0.2	27	6	Y
SN38	13	1	72	2	**5.7**	0.6	**36**	6	0.8	0.1	**1.1**	0.2	34	6	Y
daunorubicine	22	7	128	18	**5.8**	2.0	**79**	59	4.8	1.1	**2.3**	*	80	50	Y[#]
cimetidine	6	2	35	7	**6.1**	2.7	**8**	2	1.0	0.3	**1.2**	0.2	15	3	Y
topotecan HCl †	<9	1	61	13	**>6.4**	1.4	**13**	2	1.1	0.3	**1.4**	*	18	7	Y
genistein	39	*	265	33	**6.9**	*	**69**	22	1.7	0.5	**1.1**	0.5	62	10	Y
mitoxantrone	27	*	197	56	**7.3**	*	**56**	67	0.5	0.7	**1.3**	0.5	37	7	Y
rifampicin	31	11	68	10	**7.3**	2.9	**67**	59	7.9	1.5	**2.2**	0.9	50	27	Y[#]
daidzein	22	1	311	36	**14.1**	1.6	**84**	15	0.9	0.2	**1.8**	0.7	63	17	Y
pitavastatin	5	1	70	8	**15.3**	1.8	**18**	4	0.7	0.1	**0.7**	0.2	34	6	Y
PhIP	32	7	509	12	**15.8**	3.5	**234**	16	1.2	0.3	**1.0**	0.2	252	12	Y
irinotecan	4	1	65	4	**16.9**	3.6	**23**	15	3.5	1.4	**2.0**	0.4	21	9	Y
fluvastatin	<2	1	99	10	**>43**	4.1	**26**	5	0.7	0.1	**1.1**	0.3	35	10	Y

ER: Efflux Ratio; ERi: Efflux Ratio in presence of inhibitor; FTC: fumitremorgin C; M-BCRP: MDCKII cells transfected with human BCRP; N: not substrate (No); P_{app} (in nm/s): apparent permeability either from apical to basolateral (AB) or from basolateral to apical (BA); $P_{app}i$ (in nm/s): apparent permeability in presence of inhibitor; SD: standard deviation; Y: substrate (Yes); †: tested at 0.5 µM (toxic at 1 µM); *: no SD (one or two triplicate(s) were discarded due to high LY or disproportionate recovery); #: ER in M-BCRP ≥ 2 significantly different in MDCKII however insensitive to Ko143 (1 µM); $: ER in M-BCRP ≥ 2 but equivalent in MDCKII and insensitive to Ko143 (1 µM); When P_{app}AB could not be estimated as associated samples were below the limit of quantification, a value was calculated using the limit of detection and is indicated as "<" to this value, equally the ER is estimated to be ">" to the calculated ratio. Ten more compounds were tested but failed in this high throughput screening set-up due to an apparent low permeability (all samples below the limit of detection, mostly corresponding to an average P_{appi} < 15 nm/s): doxorubicine, E3S, vincristine, lamivudine, methotrexate (MTX), rosuvastatin, metformin, tamoxifen, nitrofurantoin, sulfasalazine.

Table 3: BCRP substrate *in vitro* results of drugs tested in BCRP membrane vesicles at 1 µM

Compound name	fold uptake	fold uptake + inhibitor	net uptake (pmol/mg/min)	SD	net uptake + inhibitor (pmol/mg/min)	SD	BCRP substrate
metoprolol	1.0	0.9	0.0	0.3	0.0	0.2	N
propranolol	1.0	0.8	0.1	1.4	0.0	2.5	N
metformin	1.0	0.7	0.4	1.0	0.0	1.7	N
PhIP	1.0	1.0	-1.2	24.0	-0.1	18.4	N
sunitinib	1.1	1.3	3.7	0.8	8.5	3.6	N
lamivudine	1.2	0.7	0.2	0.8	0.0	0.3	N
verapamil	1.3	1.2	15.1	1.0	8.5	2.3	N
mitoxantrone	1.3	1.2	84.7	21.3	49.4	19.3	Y
cimetidine	1.5	1.7	3.9	1.9	5.8	4.0	N
daidzein	1.6	1.5	16.0	1.5	17.7	1.8	N
prazosin	1.8	1.2	6.8	2.9	2.0	1.2	N
vincristine	1.9	1.1	44.9	15.5	7.1	0.6	Y
fluvastatin	2.0	1.0	23.3	13.1	1.1	-	Y
pitavastatin	2.3	1.2	12.4	4.9	1.7	1.9	Y
methotrexate	2.8	1.2	18.5	2.8	1.8	3.4	Y
topotecan HCl	4.6	1.1	22.4	2.7	0.9	0.6	Y
rosuvastatin	7.6	1.1	66.3	7.1	1.2	2.7	Y
E3S	10.0	1.6	84.9	10.7	5.3	3.4	Y

E3S: estrone-3-sulfate; N: not substrate (No); SD: standard deviation; Y: substrate (Yes)

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Table 4: Summary of L-MDR1, M-BCRP and BCRP membrane vesicles results together with BCRP substrate published status

	L-MDR1 cell-based assay			M-BCRP cell-based assay					
Compound name	L-MDR1 ER	Average $P_{app}i$	MDR1 substrate	M-BCRP ER	Average $P_{app}i$	**M-BCRP substrate**	**BCRP substrate (vesicle)**	**BCRP published result**	Ref
YY : BCRP substrates suitable for both *in vitro* systems									
fluvastatin	>7.3	26	Y	>43	26	**Y**	**Y**	**Y**	[3]
mitoxantrone	>5.8	38	Y	7.3	56	**Y**	**Y**	**Y**	[1]
pitavastatin	5.0	32	Y	15.3	18	**Y**	**Y**	**Y**	[1]
topotecan	>4.2	17	Y	>6.4	13	**Y**	**Y**	**Y**	[1]
Y : BCRP substrates not tested in vesicles									
coumestrol	-	-	-	>5.1	31	**Y**	nt	**Y**	[5]
daunorubicine	>35.0	97	Y	5.8	79	**Y**[#]	nt	**Y**	[1]
gefitinib	8.1	84	Y	4.5	106	**Y**	nt	**Y**	[1]
genistein	2.1	145	Y	6.9	69	**Y**	nt	**Y**	[2]
imatinib	14.2	162	Y	4.9	170	**Y**	nt	**Y**	[1]
irinotecan	29.1	21	Y	16.9	23	**Y**	nt	**Y**	[1]
MK571	6.7	52	Y	4.9	51	**Y**	nt	nr	
rifampicin	29.6	41	Y	7.3	67	**Y**[#]	nt	**Y**	[2]
simvastatin	1.6	52	N	4.0	52	**Y**	nt	**Y**	[1]
SN38	7.1	37	Y	5.7	36	**Y**	nt	**Y**	[1]
zidovudine	2.2	56	Y	4.5	42	**Y**	nt	**Y**	[1]
YN and -N : BCRP substrates false negative in vesicles									
cimetidine	12.7	35	Y	6.1	8	**Y**	**(N)**	**Y**	[1]
daidzein	3.7	163	Y	14.1	84	**Y**	**(N)**	**Y**	[5]
lamivudine	-	-	-	-	-	-	**(N)**	**Y**	[1]
PhIP	5.2	300	Y	15.8	~200	**Y**	**(N)**	**Y**	[1]
prazosin	4.2	287	Y	4.5	136	**Y**	**(N)**	**Y**	[1]
sunitinib	4.1	96	Y	2.7	92	**Y**	**(N)**	**Y**	[4]
NN : non BCRP substrates									
CsA	37.1	68	Y	2.0	60	**N**[$]	nt	**N**	[6]
erythromycin	>17.0	20	Y	8.0	36	**N**[$]	nt	**Y**[†]	[2-8]
FTC	1.3	284	N	1.0	234	**N**	nt	nr	
metoprolol	1.4	279	N	1.2	275	**N**	**N**	nr	
propranolol	1.7	284	N	1.3	200	**N**	**N**	nr	
ritonavir	56.5	76	Y	4.6	72	**N**[$]	nt	**N**	[7]
verapamil	3.0	197	Y	1.1	204	**N**	**N**	nr	
-Y, -N and -nt : non permeable compounds, not suitable for cell-based assay									
E3S	-	-	-	-	-	**-**	**Y**	**Y**	[1]
methotrexate	-	-	-	-	-	-	**Y**	**Y**	[1]
rosuvastatin	-	-	-	-	-	-	**Y**	**Y**	[2]
vincristine	-	-	-	-	-	-	**Y**	nr	
metformin	-	-	-	-	-	-	**N**	nr	
sulfasalazine	-	-	-	-	-	-	nt	**Y**	[1]
tamoxifen	-	-	-	-	-	-	nt	nr	
nitrofurantoin	-	-	-	-	-	-	nt	**Y**	[1]
doxorubicin	>4.5	14	Y	-	-	**-**	nt	**Y**	[1]

CsA: cyclosporine A; E3S: estrone-3-sulfate; ER: Efflux Ratio; FTC: fumitremorgin C; L-MDR1: LLC-PK1 cells transfected with human MDR1; M-BCRP: MDCKII cells transfected with human BCRP; N: not substrate

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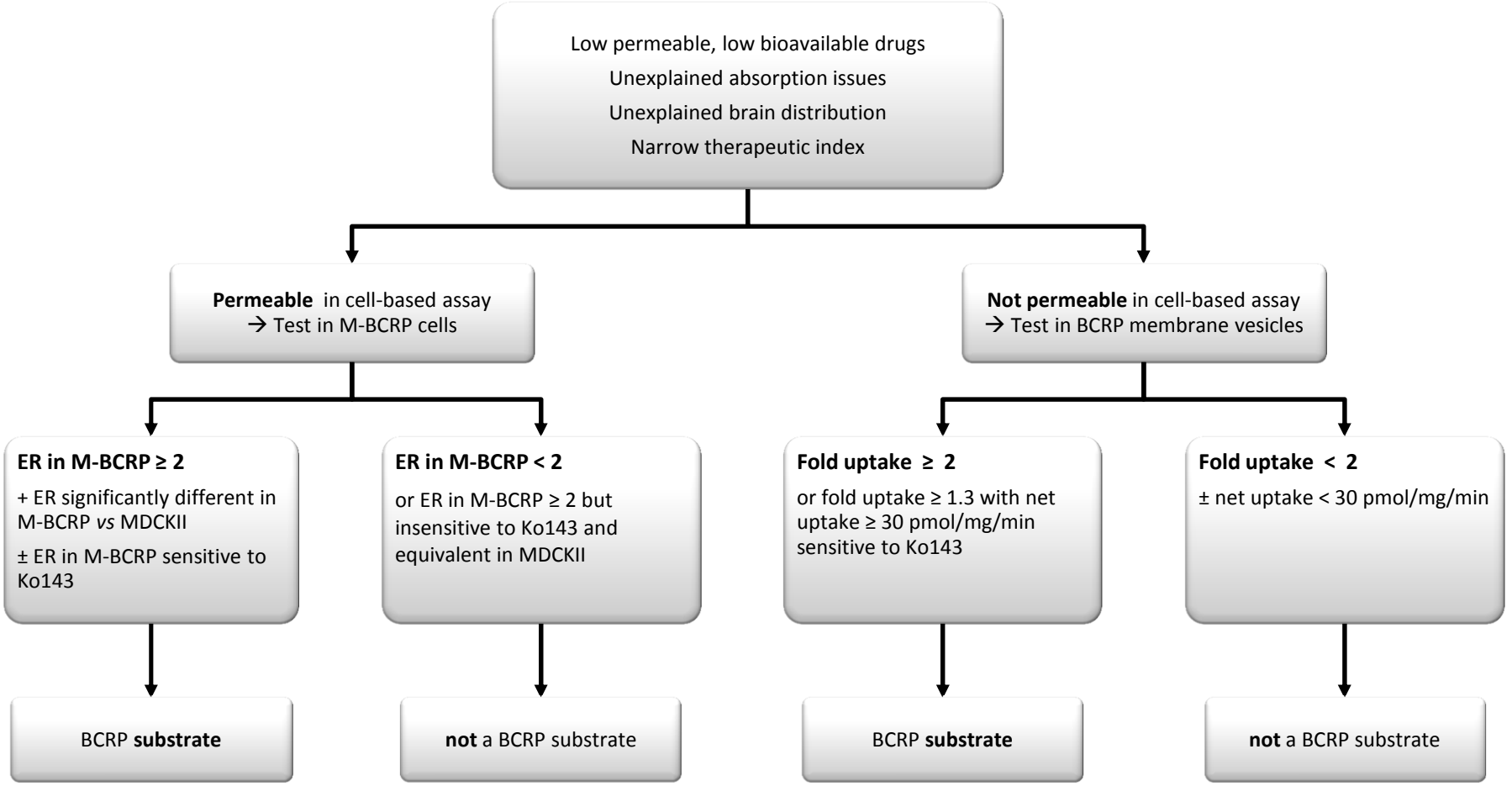
(No); (N): false negative results; nr: not reported; nt: not tested; $P_{app}i$ (in nm/s): apparent permeability in presence of inhibitor; SD: standard deviation; Y: substrate (Yes); #: ER in M-BCRP $\geq$ 2 significantly different in MDCKII however insensitive to Ko143 (1 µM); $: ER in M-BCRP $\geq$ 2 but equivalent in MDCKII and insensitive to Ko143 (1 µM); †: substrate identified in cells in suspension, not transcellular transport; -: not permeable in cell-based assay; Ref: literature reference for the BCRP substrate status; [1] (Xia et al., 2005); [2] (Polgar et al., 2008); [3] (Hirano et al., 2005); [4] (Mizuno et al., 2012); [5] (Enokizono et al., 2007); [6] (Xia et al., 2007); [7] (Gupta et al., 2004); [8] (Janvilisri et al., 2005)

Table 5: BCRP IC_{50} (in µM) measured in membrane vesicles using either estrone-3-sulfate (E3S, 1 µM) or methotrexate (MTX, 2 µM) as model substrates and measured in M-BCRP cells using PhIP (2 µM) as model substrate.

Compound name	substrate (system): ^{3}H-E3S (vesicles)	substrate (system): ^{3}H-MTX (vesicles)	substrate (system): ^{14}C-PhIP (M-BCRP)	E3S/MTX IC_{50} fold **substrate-dependency**	M-BCRP/vesicles IC_{50} fold **system-dependency**
Ko143	0.015 ± 0.005	0.052 ± 0.001	0.020 ± 0.001	0.3	0.4
elacridar	0.15 ± 0.04	0.60 ± 0.13	0.16 ± 0.07	0.3	0.3
imatinib	0.16 ± 0.02	0.34 ± 0.05	0.46 ± 0.04	0.5	1.4
FTC	0.25 ± 0.02	0.45 ± 0.08	0.20 ± 0.01	0.6	0.4
topotecan HCl	0.67 ± 0.21	1.32 ± 0.25	> 150	0.5	114
MK571	1.66 ± 0.45	0.078 ± 0.025	37.3 ± 6.0	21	478
eltrombopag	3.4 ± 0.1	3.1 ± 1.3	35.7 ± 3.3	1.1	12
rapamycin (sirolimus)	4.7 ± 0.5	5.3 ± 0.7	1.5 ± 0.3	0.9	0.3
ritonavir	7.2 ± 1.6	8.6 ± 2.0	19.5 ± 1.8	0.8	2.3
pantoprazole	11.2 ± 1.4	9.7 ± 2.2	16.5 ± 1.4	1.1	1.7
zosuquidar	12.5 ± 2.3	14.4 ± 2.7	71 ± 19	0.9	4.9
omeprazole	13.4 ± 1.8	16.8 ± 10.4	25.7 ± 2.8	0.8	1.5
CsA	15.8 ± 3.1	4.6 ± 0.7	11.1 ± 2.7	3.4	2.4
E3S	NA	17.7 ± 1.5	> 300	NA	17
verapamil	80.8 ± 8.9	83.7 ± 23.7	149 ± 25	1.0	1.8
MTX	> 100	NA	> 500	NA	NA
vincristine	> 150	> 150	> 150	NA	NA
fexofenadine	> 300	> 300	> 300	NA	NA
metformin	> 300	> 300	> 300	NA	NA

CsA: cyclosporine A; E3S: estrone-3-sulfate; FTC: fumitremorgin C; M-BCRP: MDCKII cells transfected with human BCRP; MTX: methotrexate; NA: not applicable; >: when no inhibition was observed, the IC_{50} was considered higher than the highest soluble concentration in buffer (in µM)

Figure 1



The need for BCRP substrate and inhibition evaluation in drug discovery and development: why, when and how?

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Drug Metabolism and Disposition

Supplemental Table 1: MDR1 substrate *in vitro* results of drugs tested in L-MDR1 cells at 1 µM

Compound name	Therapeutic indication	Mean P_{app} AB	SD	Mean P_{app} BA	SD	**L-MDR1 ER**	SD	Mean P_{app} ABi	SD	Mean P_{app} BAi	SD	**Average $P_{app}i$**	SD	ERi	SD	MDR1 substrate
FTC	mycotoxin	285	12	364	8	**1.3**	0.1	273	50	294	12	**284**	34	1.1	0.2	N
metoprolol	antihypertensive	236	12	339	61	**1.4**	0.3	236	81	322	56	**279**	78	1.4	0.5	N
simvastatin	hypercholesterolemia	38	12	61	20	**1.6**	0.7	54	4	51	12	**52**	8	0.9	0.2	N
propranolol	antihypertensive	213	56	361	78	**1.7**	0.6	215	27	353	14	**284**	78	1.6	0.2	N
genistein	isoflavone	64	12	135	16	**2.1**	0.5	121	10	169	20	**145**	30	1.4	0.2	Y
zidovudine	antiretroviral	33	7	73	1	**2.2**	0.5	32	4	79	6	**56**	26	2.4	0.4	Y
verapamil	antianginal	131	22	385	30	**3.0**	0.5	170	41	224	86	**197**	67	1.3	0.6	Y
daidzein	isoflavone	88	8	330	58	**3.7**	0.7	87	13	239	67	**163**	96	2.7	0.9	Y
sunitinib *	chemotherapy	76	4	314	4	**4.1**	0.3	74	10	119	3	**96**	26	1.6	0.2	Y
prazosin	antihypertensive	116	13	494	95	**4.2**	0.9	226	13	349	22	**287**	69	1.5	0.1	Y
topotecan HCl *†	chemotherapy	<9	-	39	9	**>4.2**	1.0	13	4	21	4	**17**	6	1.6	0.6	Y
doxorubicin	chemotherapy	<19	-	83	8	**>4.5**	0.4	19	0	8	1	**14**	6	0.4	0.0	Y
pitavastatin	hypercholesterolemia	12	3	60	3	**5.0**	1.1	19	7	45	1	**32**	15	2.3	0.9	Y
PhIP	model substrate	104	16	542	8	**5.2**	0.8	248	11	352	31	**300**	18	1.4	0.1	Y
mitoxantrone	chemotherapy	<26	-	150	9	**>5.8**	0.3	28	0	48	7	**38**	12	1.7	0.3	Y
MK571	bronchodilator	16	1	106	9	**6.7**	0.8	40	2	64	3	**52**	13	1.6	0.1	Y
SN38	chemotherapy	11	4	77	9	**7.1**	2.8	28	1	46	2	**37**	11	1.7	0.1	Y
fluvastatin	hypercholesterolemia	<9	-	63	5	**>7.3**	0.6	13	2	38	5	**26**	14	3.0	0.7	Y
gefitinib	chemotherapy	42	3	344	33	**8.1**	1.0	55	7	112	7	**84**	32	2.0	0.3	Y
cimetidine	anti-ulcerative	20	11	260	63	**12.7**	7.4	17	5	53	11	**35**	21	3.0	1.0	Y
imatinib	chemotherapy	28	3	396	41	**14.2**	2.2	124	29	200	26	**162**	48	1.6	0.4	Y
erythromycin	antibiotic	<3	-	54	4	**>17.0**	1.3	15	5	25	6	**20**	8	1.7	0.7	Y
irinotecan	chemotherapy	2	0	49	17	**29.1**	12.3	16	4	26	2	**21**	6	1.6	0.4	Y
rifampicin	antibiotic	6	2	175	1	**29.6**	8.3	33	2	49	4	**41**	9	1.5	0.1	Y
daunorubicine	chemotherapy	<19	-	667	30	**>35.0**	1.6	85	12	109	5	**97**	15	1.3	0.2	Y
CsA	immunosuppressant	6	1	219	35	**37.1**	10.5	60	8	76	12	**68**	13	1.3	0.3	Y
ritonavir	antiviral	2	1	129	3	**56.5**	12.4	65	8	88	5	**76**	14	1.3	0.2	Y

CsA: cyclosporine A; ER: Efflux Ratio; ERi: Efflux Ratio in presence of inhibitor; FTC: fumitremorgin C; L-MDR1: LLC-PK1 cells transfected with human MDR1; N: not substrate (No); P_{app} (in nm/s): apparent permeability either from apical to basolateral (AB) or from basolateral to apical (BA); $P_{app}i$ (in nm/s): apparent permeability in presence of inhibitor; SD: standard deviation; Y: substrate (Yes); †: tested at 0.5 µM (toxic at 1 µM); *: recovery between 60 and 70%; When P_{app}AB could not be estimated

as associated samples were below the limit of quantification, a value was calculated using the limit of detection and is indicated as "<" to this value, equally the ER is estimated to be ">" the calculated ratio. Ten more compounds were tested but failed in this high throughput screening set-up due to an apparent low permeability (all samples below the limit of detection, mostly average P_{appi} < 15 nm/s): estrone-3-sulfate, lamivudine, sulfasalazine, vincristine, coumestrol, methotrexate, rosuvastatin, metformin, tamoxifen, nitrofurantoin.

Supplemental Table 2: Clinical DDI studies: victim and perpetrator name, perpetrator plasma and gut concentrations, perpetrator f_up, BCRP IC_{50} and associated [I]/IC_{50} ratios, perpetrator inhibition potential on other transport proteins

Victim drug	Perpetrator	Perpetrator unbound fraction and concentrations							PK or tox impact (See Table 1)	Ref	Perpetrator BCRP inhibition potential				Perpetrator inhibition potential on other protein		
		$[I_{1T}]$ (µM)	Ref	f_up	Ref	$[I_{1u}]$ (µM)	$[I_2]$ (µM)	Ref			lowest IC_{50} (µM)*	$[I_2]$ / IC_{50}	$[I_{1u}]$ / IC_{50}	$[I_{1T}]$ / IC_{50}	protein	IC_{50} (µM)	Ref
methotrexate i.v.	omeprazole	3.5	[9]	0.05	[9]	0.18	232	[1]	tox impact	[1]	13.4	17	0.0131	0.261	MDR1	17.7	[6]
methotrexate i.v.	pantoprazole	3	[9]	0.02	[9]	0.06	185	[2]	tox impact	[2]	9.7	19	0.0062	0.309	MDR1	17.9	[6]
sulfasalazine	pantoprazole	6	[9]	0.02	[9]	0.12	370	[4]	no PK impact	[4]	9.7	38	0.0124	0.619	MDR1	17.9	[6]
rosuvastatin	CsA	1.1	[12]	0.1	[9]	0.11	665	[12]	PK impact	[12]	4.6	144	0.0241	0.241	OATP1B1	0.4	[7]
rosuvastatin	eltrombopag	18	[5]	0.01	[9]	0.18	678	[5]	PK impact	[5]	3.1	219	0.0581	5.806	OATP1B1	2.7	[5]
rosuvastatin	ritonavir	15	[11]	0.02	[9]	0.31	2219	[11]	PK impact	[11]	7.2	309	0.0428	2.142	OATP1B1	1.3	[7]
topotecan	elacridar	0.28	[3]	0.02	[10]	0.0056	7097	[3]	PK impact	[3]	0.67	10592	0.0083	0.416	MDR1	0.025	[8]

*: lowest IC_{50} value as estimated in Table 5 (main manuscript); CsA: cyclosporine A; f_up: fraction unbound in plasma; $[I_{1u}]$: unbound plasma concentration; $[I_{1T}]$: total plasma concentration; $[I_2]$: intestinal concentration; OATP1B1: Organic Anion-Transporting Polypeptide 1B1; Ref: literature reference for the value/information in the corresponding left side column.
$[I_2]$ was calculated as dose/250 ml converted into µM, in case of multiple dosing per day, only single doses are considered.
If needed, $[I_{1T}]$ levels were adjusted to the dose assuming dose linearity.
$[I_{1u}]$ was calculated as $[I_{1T}]$ x f_up.
f_up was approximated to 0.01 if the published value was below; in case of conflicting values or concentration dependent protein binding, the most conservative value was kept *i.e.* the highest f_up value yielding the highest $[I_{1u}]$.

[1] (Beorlegui et al., 2000); [2] (Troger et al., 2002); [3] (Kruijtzer et al., 2002); [4] (Adkison et al., 2010); [5] (Allred et al., 2011); [6] (Pauli-Magnus et al., 2001); [7] in-house data; [8] (Poirier et al., 2014); [9]; Metabolism and Transport Drug Interaction Database from University of Washington - Available from: http://www.druginteractioninfo.org; [10] (Kallem et al., 2012); [11] (Kiser et al., 2008); [12] (Simonson et al., 2004)

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