Received: 30 January 2021 | Accepted: 1 February 2021

DOI: 10.1002/prp2.740

ORIGINAL ARTICLE

Optimization of dose and route of administration of the P-glycoprotein inhibitor, valspodar (PSC-833) and the P-glycoprotein and breast cancer resistance protein dual-inhibitor, elacridar (GF120918) as dual infusion in rats

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Abstract

Transporters can play a key role in the absorption, distribution, metabolism, and excretion of drugs. Understanding these contributions early in drug discovery allows for more accurate projection of the clinical pharmacokinetics. One method to assess the impact of transporters *in vivo* involves co-dosing specific inhibitors. The objective of the present study was to optimize the dose and route of administration of a P-glycoprotein (P-gp) inhibitor, valspodar (PSC833), and a dual P-gp/breast cancer resistance protein (BCRP) inhibitor, elacridar (GF120918), by assessing the transporters' impact on brain penetration and absorption. A dual-infusion strategy was implemented to allow for flexibility with dose formulation. The chemical inhibitor was dosed intravenously via the femoral artery, and a cassette of known substrates was infused via the jugular vein. Valspodar or elacridar was administered as 4.5-hour constant infusions over a range of doses. To assess the degree of inhibition, the resulting ratios of brain and plasma concentrations, Kp's, of the known substrates were compared to the vehicle control. These data demonstrated that doses greater than 0.9 mg/hr/kg valspodar and 8.9 mg/hr/kg elacridar were sufficient to inhibit P-gp- and BCRP-mediated efflux at the blood-brain barrier in rats without any tolerability issues. Confirmation of BBB restriction by efflux transporters in preclinical species allows for subsequent prediction in humans based upon the proteomic expression at rodent and human BBB. Overall, the approach can also be applied to inhibition of efflux at other tissues (gut absorption, liver clearance) or can be extended to other transporters of interest using alternate inhibitors.

KEYWORDS
blood-brain barrier, BCRP, Breast cancer resistance protein, chemical and genetic knock out, dantrolene, efflux transporter, elacridar, glyburide, IVIVC, Kp, loperamide, P-glycoprotein, P-gp, quinidine, rat, valspodar, verapamil

Abbreviations: ADME, absorption distribution metabolism excretion; CO_2, carbon dioxide; HBSS, Hank's balanced salt solution; IVIVE, *in vitro* to *in vivo* extrapolation; LC-MS/MS, liquid chromatography tandem mass spectrometry; PK, pharmacokinetics.

1 | INTRODUCTION

Transporters can affect nearly every aspect of ADME.[1] Drug transporter activity and abundance can vary across species and tissues, specifically rodents (murine and rat) have a higher expression of P-glycoprotein, P-gp, and breast cancer resistant protein, BCRP, at the blood-brain barrier, but in non-human primate and human, the trend is reversed,[2] accurate pharmacokinetic prediction therefore requires an understanding of the relative contributions by the transporters of interest. For example, P-gp and BCRP are promiscuous and highly expressed in tissues including the intestine[3–5] and brain endothelium.[5–8] Various models can be adopted to account for the potential impact of these transporters on absorption[9] and/or brain penetration,[8–10] but all approaches require knowledge of the *in vivo* activity or establishment of a mechanistic *in vitro-in vivo* correlation (IVIVC). Quantifying the correlation between single transfected cell lines, MDCK-MDR1 and MDCK-BCRP, against the *in vivo* function and expression of efflux transporters at the rodent blood-brain barrier allows for a prediction of brain penetration in rodent but also for scaling to human based upon proteomics.[2,8]

Two primary methods are used to assess the *in vivo* impact of transporters: pharmacokinetics of a potential substrate can be assessed in genetic knockout animals and compared to that found in the wild-type strain,[11–13] or the pharmacokinetics can be obtained in wild-type animals in the presence or absence of a chemical inhibitor.[5,14–17] The advantage of the latter approach is that wild-type animals can be used to evaluate a wide-range of transporters provided that suitable chemical inhibitors are identified. However, quite often these chemical inhibitors have severe negative side effects, lack sufficient potency to elicit inhibition *in vivo*, or lack selectivity toward a single efflux transporter.

Brain penetration is commonly reported as Kp, the ratio of total brain concentration to total plasma concentrations. Taking the active free fraction into account, the Kpuu, or free brain to free plasma is widely used in terms of PK/PD. The *in vivo* efflux ratio for each compound is calculated as the ratio of Kp with and without inhibitor,[18] since the free fraction of both plasma and brain will be negated by the *in vivo* efflux ratio. The *in vivo* efflux ratio is a function of the transporter-mediated reduction in brain penetration of a small molecule and is a measurement of the functional inhibition of P-gp or BCRP and is critical in understanding and predicting subsequent BBB penetration.

The present work established protocols for characterizing the impact of P-gp and Bcrp *in vivo*. For P-gp inhibition, valspodar (PSC833), a macrocylic compound derived from cyclosporin D, has shown concentration-dependent selectivity against P-gp *in vitro* compared to other common efflux transporters.[19] On the other hand, elacridar (GF120918) inhibits both P-gp and BCRP.[15,20–22] Formulation, dose, and route of administration were optimized to obtain a feasible dose that inhibited the transporters of interest yet was safe and well tolerated in rats. Inhibition was demonstrated by an increase in the Kp (ratio of exposures in brain and plasma), or increase in absorption following oral dosing of known substrates. Once the rodent chemical knockout protocol was established, its implementation allowed for the assessment of BBB restriction attributed to P-gp or BCRP thereby confirming *in vitro* results. This information is critical when applying the species differences in proteomic transporter expression to scale Kp to human.[2,10]

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Commercially available compounds were used in the studies. Substrates for P-gp: loperamide HCl, quinidine, verapamil HCl, substrates for BCRP: dantrolene Na, 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP), glyburide, and the permeability markers/non-substrates: atenolol and minoxidil were acquired from Sigma-Aldrich (St Louis, MO). Loperamide, quinidine, and verapamil were selected as the prototypical commercial substrates for P-gp, dantrolene for BCRP substrate, glyburide as dual P-gp and BCRP substrate, based upon extensive in-house in vitro and in vivo studies as well as previously published chemical and genetic knockout studies. Atenolol and minoxidil are non-substrates and were used to monitor the tight junctions in epithelial cells of the rodent BBB. The P-gp and BCRP dual inhibitor, elacridar, was also acquired from Sigma-Aldrich. The P-gp inhibitor, valspodar, was acquired from Sigma-Aldrich and Tocris Bioscience (Minneapolis, MN). Transporter compound specificity has been a challenge, historically. Many compounds inhibit and/or are transported by numerous drug transporters and/or CYP enzymes. The substrates and inhibitors were selected based upon study design and *in vitro* and *in vivo* concentrations.

2.2 | In vivo

2.2.1 | Procurement of rodents

Sprague Dawley male rats aged eight to ten weeks with femoral artery and right jugular vein catheterization were purchased from Charles River Laboratories (Shrewsbury, MA). A two-channel vascular access button for rat (cat # VABR2B/22, Instech Laboratories (Plymouth Meeting, PA) was used to connect the femoral artery (for inhibitor) and jugular vein (for cassette) catheters. Catheters were flushed 5–7 days after surgery with 0.9% saline with 20 U/ml heparin and then every 5–7 days throughout study to maintain patency.

2.2.2 | Dose route and formulations

In Sprague Dawley rats, vehicle or valspodar was administered over a range of doses (8, 2.7, 0.9, 0.3, and 0.09 mg/hr/kg) as a 4.5-hour constant infusion (10 ml/kg) formulated in Cremophor (EL):ethanol: 0.9% saline (0.65:0.35:9 w/w/v).[23] Probe substrates for P-gp and BCRP were dosed as a cassette using a 4-hour constant infusion, with

each compound at a final concentration of 0.02 mg/ml (0.05 mg/hr/kg) in 20% captisol. A 4-hour infusion time was selected based upon technician time limitations, formulation stability, and to ensure catheter patency. Within the constraints of the routine 4-hour brain penetration study (Kp), multiple IV bolus injections were also evaluated. Valspodar was dosed at 5 mg/kg, 1 ml/kg 30 minutes prior to start of test article, and a second bolus injection (3–5 mg/kg) 2 hours later.

Similarly, vehicle or elacridar was dosed over a range of doses (8.9, 0.9, 0.09, and 0.009 mg/hr/kg) as a 4.5-hour constant infusion (10 ml/kg) formulated in dimethylacetamide (DMAC): polyethylene glycol (PEG)-400; 30% hydroxypropyl-beta-cyclodextrin (HPCD); saline (1:4:3:2 v/v/v/v).[24,25] Probe substrates for P-gp and BCRP were dosed in a cocktail approach as a 4-hour constant infusion with a final concentration of 0.02 mg/mL (0.05 mg/hr/kg) in 20% captisol. Formulations were made fresh daily.

2.2.3 | In life collection

Plasma and brain samples were collected at 4 hours following initiation of constant infusion pump for cassette compounds. Brains were not perfused but were washed well with saline; weight was recorded. Whole-blood samples were collected into ethylenediaminetetraacetic acid (EDTA) vacutainer tubes and spun within 30 minutes of sampling, and plasma was removed and frozen at −80°C until bioanalytical analysis.

2.2.4 | Bioanalytical

Brains were homogenized using a ratio of one-part tissue (g) to two parts volume of blank plasma (ml). Analytical standards were prepared in blank plasma via a serial dilution scheme. All standards, samples, and control blanks were aliquoted into the extraction plate and normalized at a ratio of 1:1 to contain an equal mixture of plasma and brain homogenate. The samples were extracted via protein precipitation using acetonitrile containing internal standard (IS). Supernatants were transferred and diluted into water then injected for analysis by LC-MS/MS. 0.1% formic acid in water and 0.1% formic acid in acetonitrile were used as mobile phases A and B, respectively. The column used for elacridar was an ACE EXCEL 3 C18-PFP 2.1 × 50 mm. The preferred column used for valspodar was an ACE EXCEL 3 CN2.1 × 50 mm.

2.3 | In vitro

2.3.1 | In vitro cell lines

Madin-Darby canine kidney (MDCK) cells which were singly transfected to express human P-gp (MDR1) efflux transporter proteins were acquired from Absorption Systems (Exton, PA, and originated at NIH, Bethesda MD). MDCK cells overexpressing human BCRP (MDCKII-BCRP-LV and respective control MDCKII-Mock-LV cell lines) were acquired from Solvo Biotechnology (Szeged, Hungary). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (PEST) in a humidified incubator at 37°C with 5% CO_2. The culture medium was changed three times weekly, and cell growth was observed by light microscopy. The MDCK-MDR1 or MDCK-BCRP cell lines were seeded into Corning transwells (cat #3392) for 7 or 5 days at 0.28 or 0.4 million cells/mL, respectively. Medium was changed once to twice per week. Monolayer confluence was monitored pre- and post-2-hour incubation by transepithelial electrical resistance (TEER) measurement. A two-hour incubation time is common across laboratories (generally range from 1.5 to 3 hours) and a time course was not conducted for these studies; however, some compound-specific variability in the *in vitro* efflux ratio may result from a single time point not reflective of equilibrium. Aliquots of test article DMSO stock were added to Hanks balanced salt solution (HBSS) containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), at pH 7.4; the nominal concentration was 1.0 µM, and the assays were conducted in triplicate. The in-house protocol utilized 1% bovine serum albumin (BSA) in the receiver wells to negate non-specific binding. The samples were withdrawn at preselected time points (t = 0 and 120 minutes) from the receiver and donor compartments and matrix-matched with either equal volume of dH2O or 1%BSA in buffer and crashed with acetonitrile containing IS. Samples were injected onto a high-throughput liquid chromatography instrument (RapidFire, Agilent) using trap and elute chromatography with a phenyl cartridge. The samples were quantified with a triple-quadrupole mass spectrometer (API5500, Sciex). The peak area ratio (PAR) of test article/IS was used for all calculations. Monolayer confluence and active efflux were confirmed with commercially available compounds such as the low-permeability compound, bestatin, the P-gp substrate, loperamide, or the BCRP substrates, daidzein or PhIP.

2.3.2 | Plasma protein and brain binding

Valspodar or elacridar was spiked into a 2000 µl aliquot of plasma or brain homogenate (1 part rat brain homogenate: 7 parts PBS buffer) at a final concentration of 0.5 µM. For highly non-specific or protein-bound compounds, the PBS buffer was also spiked at a final concentration of 0.5 µM of test article to alleviate the non-specific binding to the device or plate and also ensure equilibrium is reached during the incubation time. Three hundred microliters of the respective plasma samples were added to donor chambers in the rapid equilibrium dialysis device (RED, Thermo Fisher, Waltham, MA). Five hundred microliter aliquots of 100 mM potassium phosphate 150 mM NaCl, pH 7.4 (phosphate buffer), were added to the corresponding receiver sides of the RED device inserts. Test articles were incubated in rat and human plasma at 37°C for 4 hours with shaking (200 rpm) in the presence of 5% CO_2. A separate stability experiment was conducted in parallel

with identical mixtures of test article in plasma as described above and was incubated at 37°C for 4 hours. Detailed plasma protein binding studies using multiple concentrations and time points were considered outside of the scope and not needed considering the existing literature; therefore, a time course was not conducted. A four-hour time point was selected to screen proprietary compounds and to confirm literature values of protein binding.

Aliquots of 50 µl of were removed from the RED donor/plasma compartment (for the protein binding experiment) and from the corresponding plasma stability experiment within 10 minutes of addition and after 4 hours to provide the 100% control for the plasma stability experiment. These aliquots were transferred to an individual 96-well plate containing 50 µl of the phosphate buffer containing IS. An additional 50 µl aliquot from each RED device receiver compartment was removed after 4 hours, and these were transferred into individual 96-well plate wells containing 50 µL of blank plasma mixture and 200 µl of IS solution. All samples were vortexed for 30 seconds and centrifuged at 4000 rpm for 10 minutes.

Aliquots of 50 µl of brain homogenate were removed from the RED donor compartment. These aliquots were transferred to an individual 96-well plate containing 50 µl of the phosphate buffer and 200 µl of 1:1 methanol/acetonitrile containing IS. An additional 50 µL aliquot from each RED device receiver compartment was removed after 6 hours and these were transferred into individual 96-well plate wells containing 50 µL of blank brain homogenate mixture and 200 µl of IS. For brain binding, a six-hour time point was selected based upon in-house data to screen proprietary compounds and to confirm literature values. All samples were vortexed for 30 seconds and centrifuged at 4000 rpm for 10 minutes. All brain homogenate and buffer samples were matrix-matched.

Sample supernatants (50 µl) were transferred to a new 96-well plate containing 200 µL of 0.1% formic acid in 90/10 water/acetonitrile and vortexed gently. The plasma protein binding, plasma stability assays, and brain homogenate samples were analyzed in triplicate. All standards and samples were matrix-matched and went through the same preparation procedure as the samples. All samples were directly injected into the LC/MS/MS system for sample analysis by peak area ratio (PAR).

3 | RESULTS

3.1 | Tolerability

Clinically dose limiting, negative side effects (ataxia) of valspodar have been well documented.[26] Similar side effects in rats including lethargy and increased urination were seen with IV bolus doses at 10 mg/kg or higher and with repeat doses of 5 mg/kg or higher without proper washout (less than 2 h between doses). Maximum doses of 5 mg/kg with repeat of 3 mg/kg limited the side effects of valspodar and yielded similar increases in brain penetration to the constant infusion studies. Overall, the side effects were mitigated with a constant-infusion dose up to 8 hours,however, some instances were observed with the highest, 8 mg/hr/kg dose. No side effects were observed at the 0.9 mg/hr/kg and 2.7 mg/hr/kg doses up to 8 hours. For elacridar, no negative side effects were observed under any study conditions up to 8-hour duration.

3.2 | Brain and plasma exposure

By starting the constant infusion of the inhibitors 30 minutes prior to the administration of the test articles, steady state in plasma was achieved by 2.5 hours for both elacridar and valspodar (based upon 3x the half-life). The in-house data are consistent with simulations using literature values of clearance and volume.[23,27]

Valspodar exposures were linked to efficacy by the *in vivo* efflux ratio and were shown to be most effective when the total plasma exposures were greater than 3500 ng/mL in plasma and 4000 ng/g in brain, based upon the titration curve and multiple IV bolus doses (Figure 1). Citing literature[23] and in-house values for protein binding in rat plasma (6–10% free), the free plasma concentrations at steady state from a minimal efficacious dose, 0.9 mg/hr/kg (constant infusion or multiple IV bolus) fell between 245 and 411 nM, respectively. A maximum soluble dose of 8 mg/hr/kg resulted in a free plasma exposure of 2592 ng/ml (2,135 nM); please see Supplemental Table S1 for additional details. The *in vitro* inhibition potential for valspodar on P-gp ranged from 10 nM to 1 µM depending on cell type and substrate.[19,26] In-house, the inhibition of loperamide, a common P-gp substrate, by valspodar yielded an IC_{50} of 100 nM in the MDCK-MDR1 (NIH, Absorption Systems) cell line. The *in vitro* potency data of valspodar suggested that the lowest efficacious dose of 0.9 mg/hr/kg constant infusion or multiple IV bolus should have sufficient exposure to inhibit P-gp at the BBB and gut with a 245 nM free plasma concentration.

Elacridar was shown to be the most efficacious at the highest possible dose, limited by solubility (Figure 2). A constant infusion dose of 8.9 mg/hr/kg yielded greater than 2000 ng/ml total drug in plasma with greater than 45,000 ng/g total drug in brain. Elacridar is well known to be highly bound to rat plasma proteins, 1–2% free, and was confirmed in-house.[27] Steady state total plasma was found to be around 3500 nM (35–70 nM free); please see Supplemental Table S2 for additional details. The *in vitro* potency of elacridar has been well documented to inhibit a range of substrates with IC_{50}s from 300 to 600 nM in MDCK-BCRP or Caco-2 cells, respectively. The *in vitro* data suggested that the achieved steady state free plasma exposures may not fully inhibit BCRP-mediated efflux *in vivo* as summarized in Figure 3.

The *in vitro* potency data of valspodar suggested that the lowest efficacious dose of 0.9 mg/hr/kg constant infusion should have sufficient exposure to inhibit P-gp at the BBB and gut with a 245 nM free plasma concentration (Figure 4A). Using a bolus approach, a 5 mg/kg dose followed by a 3 mg/kg dose two hours later produced

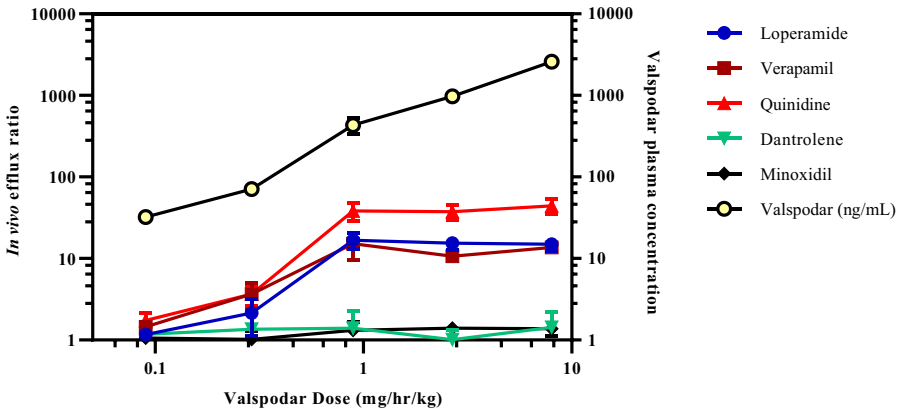

FIGURE 1 Inhibition of rat P-gp at the BBB using valspodar. The P-gp inhibitor, valspodar (PSC833) dosed from 0.09 to 8 mg/hr/kg as constant infusion over 4.5 hours shows a linear dose dependent increase in free plasma exposure (mean, rat plasma binding =92%) from 32 ng/ml to 2,592 ng/ml. When co-administered with established P-gp and BCRP substrates, valspodar, dosed above 0.9 mg/hr/kg does not result in an increase in brain exposure (as measured by the in vivo efflux ratio, brain/plasma ratio with inhibitor: brain/plasma ratio without inhibitor) for the selected P-gp substrates, loperamide, verapamil and quinidine suggesting complete chemical inhibition at the blood-brain barrier. The valspodar dose of 0.9 mg/hr/kg yielded a found free plasma concentration of 431.2 +/- 95.2 ng/ml which equates to 245 nM, well above the 100 nM invitro IC_{50}. Notably, the formulation and concentrations do not impact the brain concentrations for non- or BCRP-mediated substrates, minoxidil and dantrolene, respectively, suggesting the tight junctions of the BBB were not impacted by the formulation for inhibitor, nor did valspodar inhibitor BCRP at any concentration

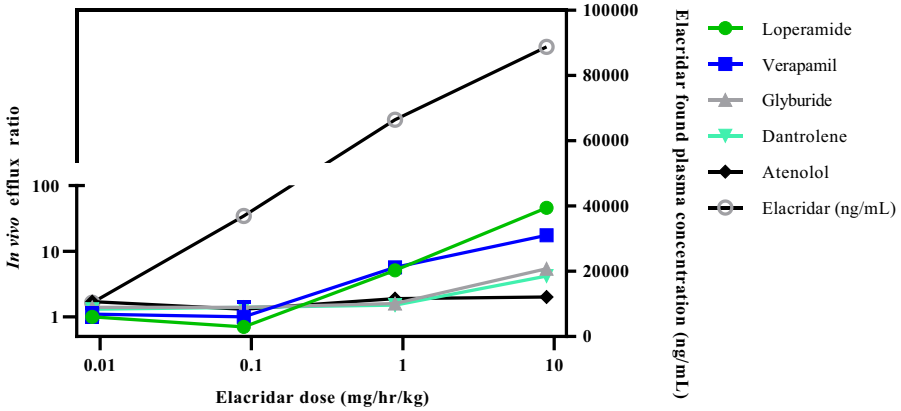

FIGURE 2 Inhibition of P-gp and Bcrp at the rat BBB with elacridar. The multiple transporter inhibitor, elacridar (GF120918) dosed as constant infusion from 0.009 mg/hr/kg to the maximum soluble, 8.9 mg/hr/kg over 4.5 hours showed linear PK with a found total plasma exposure (mean) ranging from 1.6 ng/ml to 3267 ng/ml which equates to 0.025 to 49 ng/ml free and maximum 53 nM free using 1.5% rat plasma free fraction. At the highest concentrations, we observed inhibition of P-gp and Bcrp, as shown by probe substrates loperamide and verapamil (P-gp); glyburide and dantrolene (BCRP) in a concentration dependent manner resulting in increased in brain exposure which was quantified by the *in vivo* efflux ratio, ranging from 4.2 to 46 at 53 nM free elacridar. Although possibly limited by solubility and the dose range from 0.009 to 8.9 mg/hr/kg, no plateau was observed in *in vivo* efflux ratio as was seen with valspodar. The formulation and range of elacridar did not impact the blood-brain barrier as seen with the low permeability molecule, atenolol

comparable inhibition and plasma/brain exposure to that of the constant infusion dosed above 0.9 mg/hr/kg. However, the highest soluble dose of elacridar may not fully inhibit BCRP at the rat BBB based upon in vitro values (Figure 4B).

3.3 | Oral bioavailability and clearance

In addition to the BBB, P-gp and BCRP are also expressed in the gut and biliary canalicular compartment. Their role in oral absorption and clearance has been well established. The constant-infusion approach of valspodar and elacridar yielded exposures consistent with published results of P-gp and BCRP efflux in the rodent gut, as shown by a notable increase in plasma C_{max} (2–10-fold; Figures 5 and 6). Chemical inhibition also allowed for an assessment of clearance and biliary efflux. The inhibition of P-gp showed a direct impact on the PK profile of loperamide with a 5–10-fold increase in plasma C_{max} in addition to the 3.5- and 2.6-fold increase in C_{max} and AUC of fexofenadine, respectively (Figure 5). No impact on elimination was observed, nor any increase in liver concentrations at 8 hours post-oral administration. The inhibition of BCRP by elacridar impacted the PK of sulfasalazine and topotecan as well. Sulfasalazine PK was

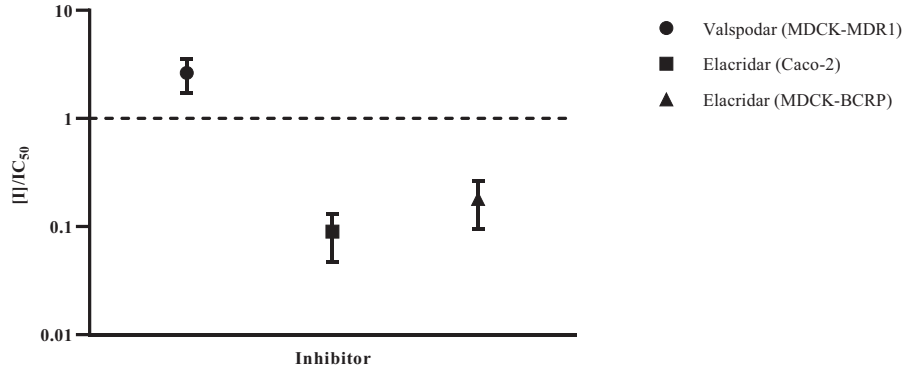

FIGURE 3 *In vivo* exposure in relation to *in vitro* inhibition. The respective free plasma concentration in rat, [I] divided by the *in vitro* IC_{50} for valspodar in MDCK-MDR1 cells (circle), elacridar in Caco-2 cells (square) and in MDCK-BCRP cells (triangle). A line of equivalency, where the free plasma concentration is equal to the IC_{50}, is set at 1. The concentration of valspodar in rat plasma is clearly higher than the *in vitro* IC_{50} suggesting that significant if not complete inhibition was achieved at the rat BBB. In contrast, due to the limit of solubility with the elacridar formulation, perhaps incomplete inhibition was achieved based upon *in vitro* IC_{50} values. Notably, the published IC_{50} s for elacridar vary significantly across cell lines, labs, and substrates. This range of invitro inhibition makes a direct comparison from *invitro* to *invivo* challenging. Future studies using a wider range of substrate and inhibitors to establish an inclusive database, particularly with *invivo* data (chemical as shown here and genetic) would clarify the interpretation around BCRP

impacted by the inhibition of BCRP in the gut and the biliary space, as shown by the 2-fold increase in Cmax, 4-fold increase in liver concentrations, and a significant increase in half-life up to 8 hours post-dose (Figure 6). Topotecan clearance was not impacted as shown by the calculated $t_{1/2}$ life and liver concentrations, but a noted 5-fold increase in plasma C_{max} was observed suggesting the PK is limited by oral absorption but not by hepatic, biliary efflux of BCRP. These results were in agreement with previous literature.[3,7,17,25,28,29] Elacridar, the dual-efflux inhibitor of P-gp, and BCRP has also been shown to inhibit OATPs as well (OATP1B1, -1B3, -2B1) which further strengthens the need for assessing tissue-to-plasma ratios when evaluating transporter impact on PK.

4 | DISCUSSION

For small molecule neuroscience programs, estimating the brain penetration early in drug discovery allows for increased efficiency in screening, structure activity relationship (SAR), PK/PD, and finally human dose prediction. Tools are available for this estimation, ranging from simple *in vivo* preclinical studies in a range of species (and measuring Kp) to expanded knowledge of laboratory-specific *in vitro* screens (single or multiple transfected cell lines expressing efflux transporters, P-gp and BCRP, are most common) and then establishing an IVIVC.

To quantify the *in vivo* efflux ratio in rats, we chose to optimize transporter-specific inhibitors due to commercial availability compared to genetic knockout and ease of use. We evaluated three dosage regimens: oral, IV bolus, and IV constant infusion. Although previously published studies often used oral administration due to ease of use, the main drawbacks are gut solubility and first pass metabolism which significantly limited the free plasma exposure of the inhibitors which likely did not fully inhibit transport activity.[26] Our objective was to increase the plasma exposure above previous levels to maximize the inhibitory effect at the BBB. IV bolus and constant infusion were optimized within the constraints of Biogen's routine Kp study design using constant infusion for 4 hours in rats.

The multiple IV bolus approach allows for ease of use and simple experimental setup; the tolerability challenges may not align with visual endpoint-specific PK/PD or toxicology readouts. The side effects of valspodar have limited its use as a clinical P-gp inhibitor and was a concern in the preclinical rodent study designs as well.[26] A constant infusion approach using valspodar, a P-gp specific inhibitor, was optimized to increase plasma exposure above previously published levels and *in vitro* IC_{50}s and reduce known tolerability issues.[19,26] In the Smith publication (1998), the authors hypothesized that the tolerability and protein binding of valspodar would limit the compounds use as a preclinical P-gp inhibitor. Smith et al nicely summarized the need for an increase in free plasma exposure to the overcome the incomplete inhibition *in vitro* as they saw a 23-fold decrease in *in vitro* efficacy from serum binding. Applying the constant infusion approach, valspodar dosed above 0.9 mg/hr/kg yielded a functional saturation point where additional exposure of the inhibitor did not increase brain penetration of the tested commercial P-gp substrates, loperamide, quinidine, and verapamil, suggesting the maximum efficacious dose of valspodar is between 0.9 and 2.7 mg/hr/kg.

P-gp (Mdr1a/b) genetic knockout rodents (mice and rats) are available but published data are limited making direct functional comparison of our chemical knockout protocol to the gold-standard genetic knockout challenging. Fortunately, a high level of substrate overlap and binding sites has been observed between mouse, rat, and human P-gp or BCRP, respectively, allowing for cross-species comparisons.[30,31] Some data exist for loperamide in genetic knockout rats and quinidine, verapamil in genetic knockout mice. Loperamide's *in vivo* efflux ratio of 17, from co-administration of valspodar, is comparable to, albeit on the lower end of the published genetic knockout

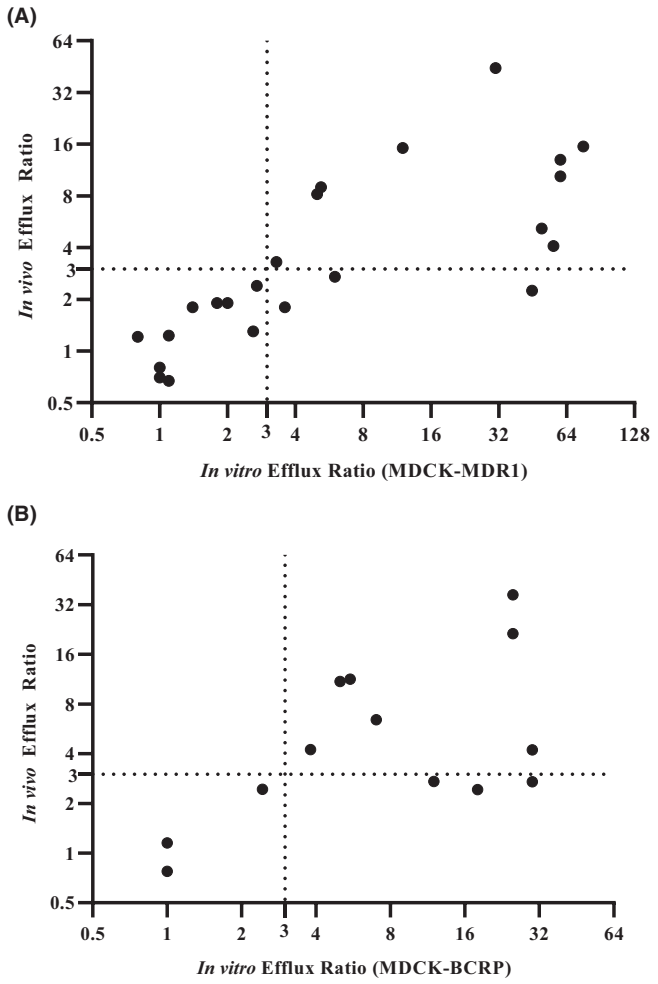

FIGURE 4 (A, B) *In vitro* to *in vivo* correlation of P-glycoprotein. Utilizing the constant infusion approach to inhibiting active P-gp or Bcrp-mediated efflux at the rat blood-brain barrier (BBB) thereby calculating the *in vivo* efflux ratio (Kp inhibitor / Kp WT), the *in vitro* to *in vivo* correlation (IVIVC) has been shown to be linear with the efflux ratios derived from MDCK-MDR1 (A; NIH/Absorption Systems) and MDCK-BCRP (B; Solvo Biotechnology) cell lines using commercial and proprietary compounds from in-house data. These data confirmed previously published MDR1 results from Kalvass, Trapa, et al using different methods. Compounds shown here were screened *in vitro* against both efflux transporters, P-gp and BCRP and any dual substrates were not used in this comparison, therefore IVIVC is compared per transporter. Overall the dynamic range of the engineered cell lines appears to be greater than the functional efflux at the rat BBB, as defined by the *in vivo* efflux ratio. The constant infusion tool inhibiting rat P-gp and Bcrp allowed for internal calibration of IVIVC to better inform assay cut-off values, SAR, confirmation of *in vitro* values, and prediction of brain penetration

data (Mdr1a[32]; which showed a 22–65-fold increase in CNS partitioning following an oral dose. Quinidine and verapamil had a maximum *in vivo* efflux ratio of 44 and 15 when co-administered with valspodar in rat, respectively, which aligns nicely with the published mouse genetic KO data showing a 12.4- and 16.4-fold increase in Kp_{brain}, respectively.[33] The protein expression and binding of efflux transporters is comparable between rat and mouse increasing the confidence in this comparison across species. These data suggest that the chemical knockout using a constant infusion of valspodar is comparable to the Mdr1a/b genetic knockout and may be a good tool when genetic knockout is not available.

The *in vivo* efflux ratio also increased with escalating doses of the dual P-gp and BCRP inhibitor, elacridar; however, increased brain penetration of P-gp and BCRP substrates could be limited by elacridar's solubility and a plateau was not reached (Figure 2). Additional incremental concentrations near the maximum soluble dose could have shown a cleaner plateau. At the highest dose of elacridar, the brain penetration increased for P-gp substrates, loperamide and verapamil, with *in vivo* efflux ratios of 46 and 17.5, respectively, a slight increase compared to valspodar suggesting that P-gp was fully inhibited by elacridar. In addition, the dual substrate glyburide had an *in vivo* efflux ratio of 5.4, and the BCRP-specific substrate, dantrolene had an *in vivo* efflux ratio of 4.2. These values showed the increase in brain penetration is comparable to published genetic knockout data showing a 3.3- to 3.9-fold increase in brain penetration for dantrolene[34] and a 2.5-fold increase in placental tissue concentration of glyburide due to BCRP knockout.[35] The genetic knockout data, although limited, increased our confidence that the functional P-gp-mediated efflux at the rat BBB was significantly inhibited to a level equal the genetic knockout, and perhaps almost equal for BCRP. In addition, compared to other chemical inhibitor studies using elacridar, the constant infusion dose of elacridar yielded a 10x higher plasma exposure than published oral administration doses.[15] These studies show that the use of a chemical inhibitor such as valspodar and elacridar can be easily used when a genetic knockout model is not available or appropriate.

The optimization of chemical inhibitors for P-gp and BCRP allowed for the quantitative comparison of the *in vitro* cell lines, MDCK-MDR1 and MDCK-BCRP, and the *in vivo* translation of these drug transporters at the rat BBB, illustrated in Figure 4A,B. The IVIVC for P-gp correlated in a predictable manner[18] (Kalvass et al., 2007) and suggests that the *in vitro* efflux ratio is linear to *in vivo* functional efflux. This information can help establish *in vitro* cutoff values. For example, in the MDCK-MDR1 test system, Figure 4A, illustrates that for efflux ratio less than 3, there is a minimal amount (<2-fold) of functional P-gp-mediated efflux in rats. This IVIVC can guide project teams on the potential impact of substrates with weak to moderate P-gp efflux when evaluating compounds for neuroscience projects.[10]

Similarly, for compounds that are solely restricted by BCRP, the IVIVC of BCRP-mediated efflux at the rodent BBB can be compared to the *in vitro* MDCK-BCRP cell line as in Figure 4B. Overall, the *in vitro* efflux ratio of the BCRP substrates tested correlate well with their *in vivo* efflux ratio (with/without elacridar). Some tested compounds do not align, and BCRP-mediated efflux appears to be underestimated by the *in vivo* efflux ratio. Some possible explanations of this disconnect would be incomplete chemical inhibition due to the limited solubility of elacridar, multiple binding sites, low expression of rat BCRP, involvement of multiple transporters other than P-gp (P-gp was screened out *in vitro*) and BCRP, steady state not reached in 4 hours, and/or possible residual blood contamination of harvested rat brains.

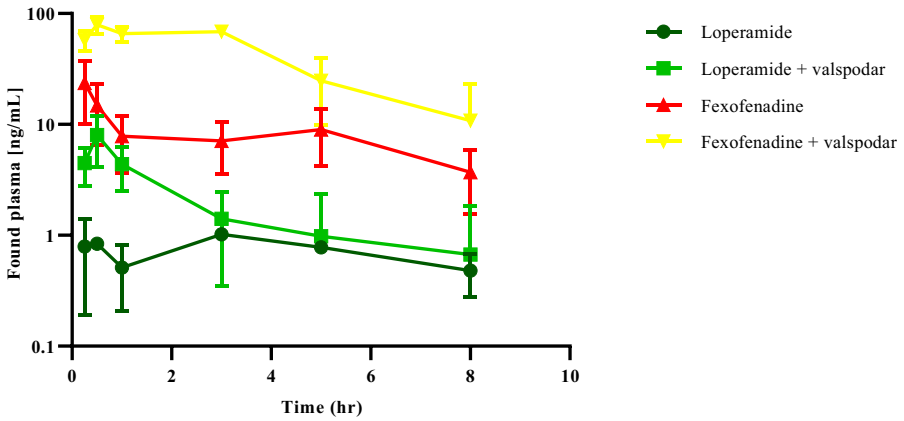

FIGURE 5 Inhibition of P-glycoprotein in the gut. A single oral administration of P-gp substrates, loperamide or fexofenadine with a constant infusion of the P-gp-selective inhibitor, valspodar, for the duration of the study have shown significant impact on their pharmacokinetic profiles, most likely due to improved absorption though alterations to clearance cannot be excluded given the design

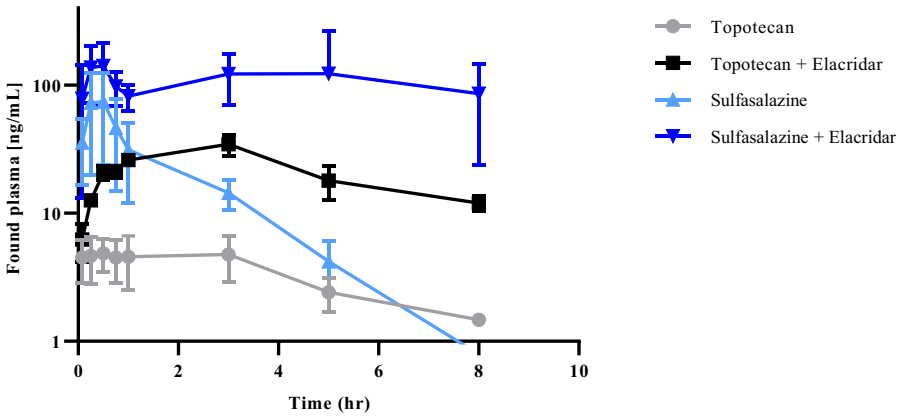

FIGURE 6 Inhibition of Bcrp in the gut and biliary compartments. Single oral administration of BCRP substrates, topotecan or sulfasalazine with a constant infusion of the inhibitor, elacridar, over the duration of the study have shown significant impact on their pharmacokinetic profiles. Oral exposure for both molecules increased when dosed with the inhibitor from 2-to 10-fold. In addition, the elimination rate of sulfasalazine was also significantly impacted which suggested that the clearance of this molecule is also dependent on active Bcrp-mediated efflux in the biliary compartment. The constant infusion dose allowed for systemic steady state of the inhibitor, elacridar, over the course of the study informing on rate-limiting steps in absorption and clearance

The IVIVC of efflux ratios between MDCK-MDR1 or MDCK-BCRP cell lines to the BBB can finally be established and inform scaling to higher species, guide project teams and support SAR. In addition, the same novel protocol applied to the rodent BBB can be adapted to evaluate the functional efflux at other tissues, oral/gut absorption, and biliary elimination by efflux. These novel protocols can be further applied with other inhibitors to possibly diagnose and tease-out the rate limits of CYP- or transporter-mediated clearance in rats.

The optimization of chemical inhibitors for P-gp and BCRP at the BBB should also translate to the application to other tissues as well. Intestinal absorption of P-gp or BCRP substrates have been shown to be a rate-limiting factor, impacting oral bioavailability and a possible cause of clinical DDI.[3,7,17,25,28,29] In addition, active efflux in the biliary canaliculi can increase the elimination of many substrates of P-gp and BCRP. An added benefit of the constant infusion dosing scheme and maintenance of inhibitor steady state over a traditional oral PK study allows for quantification of rate-limiting efflux in the gut and clearance. Identification of rate-limiting efflux can be useful in a discovery setting to guide SAR around liabilities and improvements in pharmacokinetics. Rate-limiting transport in oral bioavailability and/or hepatic clearance was identified with the P-gp substrates, loperamide and fexofenadine, as well as the BCRP substrates topotecan and sulfasalazine.

In summary, the dose and route of administration of two chemical inhibitors were optimized to quantify the pharmacokinetic impact and distribution of substrates for the efflux transporters, P-gp and BCRP. Through the establishment of the chemical inhibition protocol using constant infusion, we conducted a systematic study to quantify the *in vivo* efflux ratio of commercially available probe substrates for P-gp and BCRP at the BBB. The inhibition protocol has been applied to proprietary compounds to confirm active efflux as the main restriction agent at the rat BBB, key to predicting the human brain penetration (Kp, Kpuu) by transporter proteomics. We have also shown that the inhibition protocol which was established for the BBB can be extended to other tissues as well.

ETHICS STATEMENT

Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. This article does not contain any studies involving human participants performed by any of the authors.

ACKNOWLEDGMENTS

The authors thank Tony Peterson for his support in valspodar formulation experiments, Elvana Veizaj and Roger Rusesabagina for introduction of dual-cannulation methodologies, Collin McGladrigan for his support of *in vitro* inhibition, Doug Burdette and Guangqing Xiao for discussions around study design. All studies were financially supported by Biogen Inc (Cambridge, MA).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

AUTHOR CONTRIBUTIONS

Participated in research design: Rowbottom, Tuczkewycz, Pietrasiewicz. Conducted experiments: Tuczkewycz, Qiu, Grater, Pietrasiewicz, Kapadnis, Rowbottom. Contributed new reagents or analytic tools: Pietrasiewicz, Tuczkewycz. Performed data analysis: Rowbottom, Pietrasiewicz, Trapa. Wrote or contributed to the writing of the manuscript: Rowbottom, Pietrasiewicz, Tuczkewycz, Grater, Trapa.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available in the supplementary material of this article. Data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Rowbottom C, Pietrasiewics A, Tuczewycz T, et al. Optimization of dose and route of administration of the P-glycoprotein inhibitor, valspodar (PSC-833) and the P-glycoprotein and breast cancer resistance protein dual-inhibitor, elacridar (GF120918) as dual infusion in rats. *Pharmacol Res Perspect*. 2021;9:e00740. https://doi.org/10.1002/prp2.740