In Vitro Assessment of the Drug–Drug Interaction Potential of Verinurad and Its Metabolites as Substrates and Inhibitors of Metabolizing Enzymes and Drug Transporters

V. Sashi Gopaul, Anna Vildhede, Tommy B. Andersson, Fredrik Erlandsson, Caroline A. Lee, Susanne Johansson, and Constanze Hilgendorf

ABSTRACT

Verinurad is a selective uric acid transporter 1 (URAT1) inhibitor in development for the treatment of chronic kidney disease and heart failure. In humans, two major acyl glucuronide metabolites have been identified: direct glucuronide M1 and N-oxide glucuronide M8. Using in vitro systems recommended by regulatory agencies, we evaluated the interactions of verinurad, M1, and M8 with major drug-metabolizing enzymes and transporters and the potential for clinically relevant drug–drug interactions (DDIs). The IC50 for inhibition of CYP2C8, CYP2C9, and CYP3A4/5 for verinurad was $>$14.5 μM, and maximum free plasma concentration (fu,max)/IC50 was <0.02 at the anticipated therapeutic Cmax, and therefore not considered a DDI risk. Verinurad was not an inducer of CYP1A2, CYP2B6, or CYP3A4/5. Verinurad was identified as a substrate of the hepatic uptake transporter organic anion-transporting polypeptide (OATP) 1B3. Since verinurad hepatic uptake involved both active and passive transport, there is a low risk of clinically relevant DDIs with OATP, and further study is warranted. Verinurad was a substrate of the efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), and renal transporter organic anion transporter 1 (OAT1), although it is not considered a DDI risk in vivo because of dose-proportional pharmacokinetics (P-gp and BCRP) and limited renal excretion of verinurad (OAT1). M1 and M8 were substrates of multidrug resistance-associated protein (MRP) 2 and MRP4 and inhibitors of MRP2. Apart from verinurad being a substrate of OATP1B3 in vitro, the potential for clinically relevant DDIs involving verinurad and its metabolites as victims or perpetrators of metabolizing enzymes or drug transporters is considered low.

SIGNIFICANCE STATEMENT

Drug transporters and metabolizing enzymes have an important role in the absorption and disposition of a drug and its metabolites. Using in vitro systems recommended by regulatory agencies, we determined that, apart from verinurad being a substrate of organic anion-transporting polypeptide 1B3, the potential for clinically relevant drug–drug interactions involving verinurad and its metabolites M1 and M8 as victims or perpetrators of metabolizing enzymes or drug transporters is considered low.

Introduction

Verinurad is a novel, selective inhibitor of URAT1 that was initially studied for the treatment of gout (Shiramoto et al., 2018; Fitz-Patrick et al., 2019; Terkeltaub et al., 2019). Verinurad is currently in development for chronic kidney disease (CKD) and heart failure with preserved ejection fraction (HFpEF), in which high levels of uric acid are observed and may constitute either a contributing cause or risk factor for heart failure. In humans, two major acyl glucuronide metabolites have been identified: direct glucuronide M1 and N-oxide glucuronide M8. Using in vitro systems recommended by regulatory agencies, we evaluated the interactions of verinurad, M1, and M8 with major drug-metabolizing enzymes and transporters and the potential for clinically relevant drug–drug interactions (DDIs). The IC50 for inhibition of CYP2C8, CYP2C9, and CYP3A4/5 for verinurad was $>$14.5 μM, and maximum free plasma concentration (fu,max)/IC50 was <0.02 at the anticipated therapeutic Cmax, and therefore not considered a DDI risk. Verinurad was not an inducer of CYP1A2, CYP2B6, or CYP3A4/5. Verinurad was identified as a substrate of the hepatic uptake transporter organic anion-transporting polypeptide (OATP) 1B3. Since verinurad hepatic uptake involved both active and passive transport, there is a low risk of clinically relevant DDIs with OATP, and further study is warranted. Verinurad was a substrate of the efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), and renal transporter organic anion transporter 1 (OAT1), although it is not considered a DDI risk in vivo because of dose-proportional pharmacokinetics (P-gp and BCRP) and limited renal excretion of verinurad (OAT1). M1 and M8 were substrates of multidrug resistance-associated protein (MRP) 2 and MRP4 and inhibitors of MRP2. Apart from verinurad being a substrate of OATP1B3 in vitro, the potential for clinically relevant DDIs involving verinurad and its metabolites as victims or perpetrators of metabolizing enzymes or drug transporters is considered low.

This work and development of this manuscript was supported by AstraZeneca.

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This article has supplemental material available at jpet.aspetjournals.org.
the diseases (Ramirez-Sandoval and Madero, 2018; Sato et al., 2019; Carnicelli et al., 2020a,b). Multiple clinical trials have studied the pharmacokinetics, pharmacodynamics, safety, and tolerability of verinurad (Shen et al., 2017; Hall et al., 2018; Lee et al., 2018). Based on its potent inhibition of URAT1 (25 nM) (Tan et al., 2017), pharmacokinetic properties in humans (Shen et al., 2017), as well as high permeability and solubility, the expected therapeutic dose of verinurad is low (<30 mg/day). Verinurad is currently being evaluated in two separate phase 2 studies in patients with CKD (NCT03990363) or heart failure (NCT04327024) at doses up to 24 mg once daily.

Verinurad shows dose-proportional exposure (maximum observed plasma concentration [Cmax] and area under the curve [AUC]) up to and including 40 mg following a single dose and up to and including 15 mg following multiple once-daily doses (Shen et al., 2017; Hall et al., 2018). Furthermore, there is minimal accumulation of verinurad (approximately 1.2-fold for Cmax and 1.3-fold for AUC) observed after once-daily dosing (Hall et al., 2018). Verinurad is primarily metabolized by multiple glucuronosyl transferases (UGT1A1, 1A3, 1A8, 2B4, 2B7, and 2B17) and cytochrome P450 CYP enzymes (1A8, 2B4, 2B7, and 2B17) and cytochrome P450 CYP enzymes CYP3A4 and CYP2C8 (Lee et al., 2018). The acyl glucuronide metabolites M1 (direct glucuronidation) and M8 (glucuronidation of N-oxide) (Fig. 1) are rapidly formed after the absorption of verinurad and circulate in the plasma at concentrations equimolar to verinurad but lack efficacy toward inhibition of URAT1 (Lee et al., 2018). M1 is formed by direct glucuronidation of verinurad via several UGTs. The formation of the N-oxide metabolite of verinurad (M4) is mediated by CYP3A4 in vitro, which is further metabolized by UGTs to form M8 (Lee et al., 2018). Additionally, M8 can be formed from M1 via CYP2C8. Therefore, as reported previously (Lee et al., 2018), the potential for comedication to alter the exposure of verinurad as a substrate of UGT and CYP enzymes is low due to the involvement of multiple parallel and sequential metabolic pathways. While verinurad is excreted via the feces, metabolites M1 and M8 are excreted renally.

Based on observations of enterohepatic recirculation, it is hypothesized that the disposition of verinurad and its metabolites involves active hepatic uptake and renal secretion (Lee et al., 2018). Given the importance of transporters in the absorption and disposition of a drug and its metabolites, this can contribute to clinically relevant drug–drug interactions (DDIs) (Varma et al., 2015; Zhang et al., 2018). In particular, the involvement of multiple mechanisms in the disposition of drugs may lead to mechanistically complex DDIs (Varma et al., 2015). For this reason, the evaluation of pharmacokinetic DDIs of new molecular entities requires multiple methodologies that combine in vitro, in silico, and clinical studies early in drug development and following the most recent guidelines issued by regulatory agencies (European Medicines Agency, 2013; Japanese Pharmaceuticals and Medical Devices Agency, 2018; Food and Drug Administration, 2020).

By virtue of its mechanism of action of URAT1 inhibition, verinurad shows a pharmacodynamic-related DDI when coadministered with allopurinol. The active metabolite of allopurinol, oxypurinol, is a substrate for URAT1 and oxypurinol reabsorption, and thereby exposure decreases when given with verinurad (Kankam et al., 2018).

Here, we adopt a comprehensive strategy using a range of in vitro systems to determine the in vitro interactions of verinurad and its major metabolites M1 and M8 as victims or perpetrators of drug transporters and metabolizing enzymes (CYPs and UGTs). The in vitro studies reported here were conducted with appropriate reference inhibitors and positive controls in line with the acceptance criteria for assay performance according to recently published guidance (European Medicines Agency, 2013; Japanese Pharmaceuticals and Medical Devices Agency, 2018; Food and Drug Administration, 2020). The clinical relevance of the in vitro findings is further contextualized within the framework of basic static equations to evaluate the potential for DDIs, following guidance provided by regulatory agencies to identify potential clinical DDIs and avoid adverse clinical consequences (European Medicines Agency, 2013; Japanese Pharmaceuticals and Medical Devices Agency, 2018; Food and Drug Administration, 2020).

Materials and Methods

Materials. Unlabeled verinurad (2-(3-(4-cyanonaphthalen-1-yl)pyridin-4-ylthio)-2-methylpropanoic acid) was synthesized at Piramal Pharma Solutions (Torican, Canada) following the synthetic route described previously (Ouk, 2013; Lee et al., 2018). 14C-verinurad ethanol solution (0.5 mCi/ml) with a specific activity of 55.2 mCi/mmol and radiochemical purity of 98.8% (3.2 mg/ml) was obtained from Moravek Biochemicals, Inc. (Brea, CA).

Verinurad acyl glucuronide (M1), verinurad N-oxide acyl glucuronide (M8), and the D6-stable isotope-labeled verinurad were synthesized at Moravek Biochemicals, Inc. (part of AstraZeneca, San Diego, CA), and the D6-stable isotope-labeled metabolites for M1 and M8 were synthesized by Syncom BV (Groningen, The Netherlands), as described previously (Lee et al., 2018). The identity of the materials

Fig. 1. Structures of verinurad and metabolites M1 and M8. MW, molecular weight.
was confirmed by liquid chromatography–mass spectrometry and nuclear magnetic resonance (NMR), and the purity was determined by quantitative NMR.

All other materials, such as solvents, buffer salts, or reference compounds, were of high-performance liquid chromatography or analytical grade.

**Plasma Protein Binding.** In vitro plasma protein binding (PPB) of verinurad was evaluated in pooled (n = 3) human plasma (BioIVT, Westbury, NY), human serum albumin (HSA; Sigma-Aldrich, St Louis, MO), and 1-acid glycoprotein (AGP, Sigma-Aldrich) in triplicate. Plasma and protein solutions were spiked with 4-O-verinurad to achieve concentrations of 1, 10, and 50 μM. Incubations were conducted at 37 °C for 18 hours to allow for equilibrium of free drug to be reached between the plasma and buffer chambers using the rapid equilibrium dialysis device (Thermo Fisher Scientific, Waltham, MA). Sample aliquots from each chamber were analyzed by liquid scintillation counting. Radioactivity concentrations in aliquots from both sample and buffer chamber were determined directly in 6 ml Ultima Gold-cocktail (Perkin-Elmer, Waltham, MA) using a Packard Tri-Carb 3100TR liquid scintillation counter (Perkin-Elmer).

Due to the difference in stability of the acyl glucuronide metabolites compared with the parent drug, the PPB of metabolites M1 and M8 was determined in pooled (n = 3) human mixed-sex plasma (BioIVT) by ultrafiltration using the Ultrafiltration Device with Ultracel YM-T membrane (Merck-Millipore, 4010). The stability of glucuronides M1 and M8 under assay conditions was verified prior to the PPB assay. M1 or M8 were added to 500 μL human plasma at a final concentration of 1 and 50 μM, with the final volume containing 1% of organic solvent. An aliquot of 50 μL taken immediately served as the T = 0 minute sample. The remaining solution was incubated at 37 °C for 30 minutes. A total of 200 μL plasma sample was loaded into the Ultrafiltration Device and centrifuged at 37 °C at 2000 g for 10 minutes. At the end of centrifugation, 50 μL filtrate was analyzed, and the remaining volume of filtrate in each collection tube was measured to determine the total volume of filtrate for recovery calculation. M1 and M8 concentrations in filtrate and stability samples were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Supplemental Table 1).

**Enzyme Systems.** Pooled human liver microsomes (HLMs; 150 donors, mixed sex) for in vitro enzyme inhibition studies were purchased from BD Biosciences (San Jose, CA, USA) and Corning Corporation (Corning, NY, USA). Fresh primary human hepatocytes (n = 3 different female donors) for in vitro induction studies were obtained from Xenogen (San Diego, CA, USA). Details of the incubation are provided in Supplemental Table 1. Hepatocytes were treated once daily for three consecutive days with either vehicle control (0.1% DMSO), verinurad (1, 10, or 100 μM), or one of three prototypical CYP inducers. Approximately 24 hours after the last treatment, the cells were incubated with enzyme marker substrates for 30 minutes at 37 °C to monitor the rate of formation of CYP isofrom–specific metabolites (LC-MS/MS; Supplemental Table 1). Additional hepatocytes from the same treatment groups were harvested with TRIzol to isolate RNA, which was analyzed by quantitative reverse-transcription polymerase chain reaction to assay the effect of verinurad on CYP1A2, CYP2B6, and CYP3A4 mRNA levels.

**Cells and Culture Conditions.** Cryopreserved hepatocytes for hepatic uptake studies were purchased from Triangle Research Laboratories (Research Triangle Park, NC). Drug transporter interaction studies were performed using cell lines or vesicles with either endogenous (Caco-2), stable, or transient expression of the transporter, and corresponding background control systems (empty vector-transfected cells). Origins of all in vitro systems are summarized in Supplemental Tables 4 and 5. The culture conditions followed established and validated protocols (Zhang et al., 2015; Yu et al., 2016; Fredlund et al., 2017).

**Determination of Intrinsic Permeability of Verinurad.** Intrinsic permeability of verinurad was tested at a concentration of 10 μM in Caco-2 cell monolayers, as previously described (Fredlund et al., 2017). Cells were cultivated for 2 weeks in Transwell filter plates (Corning Corporation, 0.4 μm pore size), and the study was performed in the apical-basolateral direction with a pH gradient of 6.5/7.4, reflecting the slightly acidic upper intestinal pH in the donor compartment. Samples from the receiver compartment were collected over a time interval of 120 minutes and analyzed with LC-MS/MS.

**Determination of Passive and Active Hepatic Uptake of Verinurad in Cryopreserved Human Hepatocytes.** The assay was conducted by QPS LLC (Newark, DE) as described previously (Kimoto et al., 2011, 2019). The hepatic uptake mechanism of verinurad was investigated at 0.5 and 5 μM in suspended cryopreserved human hepatocytes (final concentration of 1 × 10⁶ cells/ml). Hepatocytes were preincubated with a mixture of 25 μM rifampin SV and 25 μM prazosin (inhibitors of OATPs and OCT1) or buffer/solvent control at 37 °C for 10 minutes. Uptake was initiated by the addition of verinurad as the test compound or positive control substrate (rosuvastatin for active uptake and verapamil for passive uptake). At time points 0.5, 1, and 1.5 minutes, samples were filtered through a centrifuge tube containing a mix of mineral and silicone oil over a bottom layer of 2 M ammonium acetate. The tubes were immediately centrifuged for 30 seconds at 18,300 g to separate cells from the buffer and subsequently frozen down. Each tube was cut near the oil/base interface, and the cell pellet was resuspended in 50 μL water and 100 μL of the appropriate internal standard in acetonitrile. The samples were vortexed for 6 minutes at high speed to extract the pellet, which was then centrifuged at 3000 rpm for 10 minutes. Substrate concentration in the supernatant was determined using LC-MS/MS (Supplemental Table 1). The percent active uptake was quantified from the slope of uptake in the absence and presence of inhibitors. Each treatment group was conducted in quadruplicate.

**Evaluation of Verinurad, M1, and M8 as Substrates and Inhibitors of Human Drug Transporters.** Assays were conducted by Optivia Biotechnology (Menlo Park, CA), QPS LLC, Phar- maron (Beijing, China), and Cyprotex Discovery Ltd (Macclesfield, UK). In the vitro models used to study the transporter interactions are summarized in Table 1.
The methods for the substrate and inhibition assays have been described previously (Zhang et al., 2015; Yu et al., 2016). Furthermore, Supplemental Tables 4 and 5 summarize the experimental conditions used to characterize the substrate and inhibitory interaction of verinurad, M1, and M8 with human drug transporters. Transporter substrate studies included control systems and specific inhibitors to corroborate the significance of the observed in vitro substrate data. Transporter inhibition studies were conducted with index substrates at multiple concentrations of test drug (verinurad, M1, or M8) over a clinically relevant concentration range. All reactions were performed in triplicate.

**Determination of Verinurad as a Substrate of P-Glycoprotein and Breast Cancer Resistance Protein in Madin-Darby Canine Kidney II-MDR1 and Caco-2 Cells.** Madin-Darby Canine Kidney (MDCK) II-MDR1 and Caco-2 cells were grown to confluency in 24-well Transwell plates, washed in assay buffer, and preincubated at 37°C for 20–30 minutes. Verinurad or 14C-verinurad was either added to the apical or basolateral side and incubated with orbital shaking at 50–60 rpm. The appearance of verinurad in the opposite receiver chamber was measured for 120 minutes. 14C-Verinurad was quantified with radiometric detection on a 1450 Microbeta (PerkinElmer, CT), whereas unlabeled verinurad was quantified with LC-MS/MS (Supplemental Table 1).

**Determination of Verinurad as a Substrate of Human Solute Carrier Transporters.** The potential for verinurad to be a substrate of human drug transporters OCT1, OCT2, OAT1, OAT3, MATE1, MATE2-K, OATP1B1, and OATP1B3 was evaluated in MDCKII cells transiently transfected with the respective drug transporter of interest. OCTP2B1 interaction was studied in OATP2B1-transfected Chinese hamster ovary (CHO) cells.

**Cellular uptake of 14C-verinurad was studied over 5 minutes of incubation at 37°C with orbital shaking at 50–60 rpm.** Then, cells were quickly rinsed with an ice-cold phosphate-buffered solution, and a cell extraction solution (50:50 mixture of acetonitrile and water) was added. The intracellular 14C-verinurad concentration was quantified with radiometric detection.

**Determination of Verinurad, M1, and M8 as Substrates of Efflux Transporters Multidrug Resistance-Associated Proteins 2 and 4.** Studies of verinurad and metabolites M1 and M8 as substrates of efflux transporters multidrug resistance-associated protein (MRP) 2 and MRP4 were performed by Optivia Biotechnology using *Spodoptera frugiperda* (S9) insect vesicles expressing human MRP2 or MRP4. Briefly, samples were incubated with blocking buffer (40 mM MOps-Tris pH 7.0, 70 mM potassium chloride (KCl), and 0.5 mg/ml bovine serum albumin) at 37°C for 60 minutes with orbital shaking at 200 rpm. Blocking buffer was removed, and assay uptake buffer (50 mM MOps-Tris pH 7.0, 70 mM KCl, 7 mM magnesium chloride, and 3 mM 1-glutathione reduced) containing MRP2 or MRP4 vesicles was added. Verinurad, M1, M8, or positive control substrate with and without reference inhibitor were added and preincubated at 37°C with orbital shaking at 200 rpm for 10 minutes. To start uptake, adenosine triphosphate (ATP) or adenosine monophosphate (AMP) was added to the plates to a final concentration of 5 mM, and the plates were incubated for 5 (MRP2) or 10 minutes (MRP4). Incubations were stopped by the addition of an ice-cold wash buffer (50 mM MOps-Tris pH 7.0 and 70 mM KCl). The samples were transferred to glass fiber filtration plates to separate vesicles from the buffer by applying a vacuum. The filter plates were washed and allowed to dry under vacuum. Filter wells were punched out, and radiolabeled substrate was quantified with radiometric detection on a 1450 Microbeta. Unlabeled M1 and M8 were quantified by LC-MS/MS (Supplemental Table 1).
Determination of Verinurad, M1, and M8 as Inhibitors of Human Efflux Transporters Bile Salt Export Pump, MRP2, and MRP4. The potential for verinurad and metabolites M1 and M8 to inhibit the transport of probe substrates by human efflux transporters bile salt export pump (BSEP), MRP2, and MRP4 was determined using S9 vesicles expressing the respective transporter (Supplemental Table 5). The methods used were similar to those described for the substrate transport assays using S9 inverted membrane vesicles. Membrane vesicles were preincubated with the probe substrate and a concentration series of test compound or positive control inhibitor at 37°C with orbital shaking for 10 minutes. To start the uptake process, ATP or AMP was added to a final concentration of 5 mM, and the samples were incubated at 37°C with orbital shaking for 5 minutes for MRP2, 10 minutes for MRP4, and 15 minutes for BSEP. Uptake reactions were stopped by fast filtration using a glass fiber filtration plate and washing with wash buffer. The amount of substrate in the filters was quantified either by LC-MS/MS or with radiometric detection.

LC-MS/MS Analysis. LC-MS/MS analysis of verinurad, M1, M8, probe substrates (transporter assays), and enzyme-specific metabolites (enzyme inhibition and induction assays) varied according to the service contractor used. The system conditions used for sample analysis (e.g., type of column, flow rate, gradient profile, and analysis time) and transitions monitored for the analytes are summarized in Supplemental Table 1. LC-MS/MS analysis of verinurad, M1, and M8 in the in vitro assays was based on the methods described previously (Lee et al., 2018), wherein D3 internal standards of each analyte were available for analysis.

Data Analysis. For assays run in triplicate, mean and standard deviation are reported.

In Vitro Plasma Protein Binding in Human (Free Fraction). For the determination of the PPB of M1 and M8, the percentage of unbound drug metabolite was calculated as:

\[
\text{% Unbound} = \frac{\text{Concentration filtrate}}{\text{Concentration total}} \times 100
\]  

Enzyme Inhibition IC50 Calculations (CYP and UGT). Inhibition of metabolizing enzyme activity was expressed as the percentage decrease in the activity of a marker metabolite formation compared with noninhibited controls (100% activity). Mean enzyme activity (n = 3) for each test concentration was plotted against the log inhibitor concentration and fitted to an IC50 curve using GraphPad Prism or Excel XLFit (5.3.1.3):

\[
Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + \left(\frac{[IC_{50}]}{X}\right)^n}
\]

in which X is the logarithm of inhibitor concentration, Y is the percent of control activity and Y starts at the bottom and goes to the top with a sigmoid shape, and Top and Bottom are the Y values at the top and bottom plateau of the curve.

No inhibition (NI) represents no inhibition at the highest concentration tested, and IC50 >30 or >100 μM (for CYPs with metabolites and verinurad, respectively) and >1250 μM (for UGTs) represent inhibition at the highest concentration tested but not enough to obtain an accurate IC50 determination.

The TDI of each CYP450 enzyme in HLMs was calculated as percent NADPH-dependent activity shift after preincubation in the absence and presence of CYP-cotransporter NADPH as follows:

\[
\text{% TDI} = \left(1 - \frac{\% \text{ control activity}_{\text{NADPH}}}{\% \text{ control activity}_{\text{No NADPH}}}\right) \times 100
\]

Transporter Substrate Determinations. In bidirectional transport assays, efflux transporter substrates were characterized through efflux ratios (ERs) (Supplemental Table 4).

Permeability of reference substrate or test drug was estimated as apparent permeability (Papp):

\[
P_{\text{app}} = \frac{1}{\text{Area}} \times \frac{dM}{dt} \quad \text{(cm/s)}
\]

in which Area is the area of the filter (0.33 cm²), C_{D(0)} is the initial substrate concentration in the donor compartment, and dM/dt is the flux rate of test drug to the receiver compartment.

ER was expressed as a ratio of B → A over A → B P_{app}:

\[
ER = \frac{P_{\text{app}}(B \rightarrow A)}{P_{\text{app}}(A \rightarrow B)} \quad \text{(dimensionless)}
\]

In the human uptake transporter substrate assays, uptake in the test system (expressed the transporter of interest) was compared with uptake in the control system (did not express the transporter of interest) using unpaired Student’s t test with Holm-Sidak correction for multiple comparisons. A P value of <0.05 was considered indicative of active transporter-mediated uptake of the test article. Verification of transporter specificity in the human uptake transporter substrate assays was performed by adding a strong reference inhibitor. Percent inhibition by the reference inhibitor was calculated by:

\[
\% \text{ inhibition} = \left(1 - \frac{(\text{Net transporter} - \text{mediated uptake})_{\text{with inhibitor}}}{(\text{mean Net transporter} - \text{mediated uptake})_{\text{without inhibitor}}}\right) \times 100
\]

in which net transporter-mediated uptake was calculated by subtracting uptake in the control system from uptake in the test system.

IC50 Calculations for Transporters. To determine the IC50 of verinurad or its metabolites in uptake and efflux experiments, data were fitted by nonlinear regression to a sigmoidal variable slope IC50 model (GraphPad Prism or Excel XLFit [5.3.1.3, Eq. 201]):

\[
Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + \left(\frac{[IC_{50}]}{X}\right)^n}
\]

in which Y is the percent control activity/percent inhibition in the presence of the test article throughout the concentration range tested, Top is the percent control activity/percent inhibition in the absence of the test article, X is the inhibitor concentration, and n is a Hill coefficient. Bottom can be fixed to 0 when defined through background experiments with full inhibition.

To determine the IC50 in transcellular flux experiments (i.e., MDCKII-MDR1 and Caco-2 [BCRP] inhibition assays), percent control activity values were calculated using the below equation:

\[
\% \text{ control activity} = \frac{P_{\text{app}}(\text{+ test compound}) - P_{\text{app}}(\text{passive})}{P_{\text{app}}(\text{vehicle control}) - P_{\text{app}}(\text{passive})} \times 100
\]

in which P_{app(+ test compound)} is the P_{app} of substrate in the presence of test compound, P_{app(veilcle control)} is the mean P_{app} of substrate in the absence of test compound, and P_{app(passive)} is the mean P_{app} of substrate in the presence of the highest concentration of positive control inhibitor assuming 100% inhibition at this concentration. From this, percent control activity values in the presence of a range of test compound/inhibitor concentrations were plotted against nominal inhibitor concentration and fitted to the 4 Parameter Logistic model above.

To determine the IC50 in intracellular accumulation assays, the percent inhibition of the net transporter-mediated uptake was calculated according to eq. 6 and plotted against nominal inhibitor...
concentrations. IC_{50} values were subsequently calculated by fitting the data to eq. 7.

Risk Assessment and Margin Calculations for Transporter and Enzyme Inhibition. As recommended by regulatory agencies (European Medicines Agency, 2013; Japanese Pharmaceuticals and Medical Devices Agency, 2018; Food and Drug Administration, 2020), the degree of inhibition in humans was estimated by relating the observed IC_{50} to maximal total (I_{max} or C_{max}) or free drug concentration (I_{u,max} or C_{u,free}) (Table 2) and/or by calculating R values as defined by the US FDA 2020 guidance. The combined inhibitory potential of verinurad and its metabolites was calculated as a sum of the individual ratios of exposure in relation to in vitro inhibition constants.

The anticipated worst case therapeutic concentration values of verinurad, M1, and M8 were 0.238 and M8 (using a terminal half-life of 21.2 and 22.8 hours, respectively) between formulations (60%), and the expected accumulation ratio of M1 verinurad, accounting for the difference in dose, relative bioavailability daily (unpublished AstraZeneca data on met et al., 2018).

with severe renal impairment after a single dose of verinurad (Smith in dose and accounting for the 2.28-fold higher exposure seen in those with severe renal impairment after a single dose of verinurad (Smith et al., 2018). The C_{max} of verinurad was estimated from the observed C_{max} (36.3 ng/ml tested in phase 2 studies (NCT03990363 and NCT04327024). The C_{max} of verinurad was estimated from the observed C_{max} (36.3 ng/ml or 0.104 μM) in healthy volunteers receiving verinurad 12 mg once daily (unpublished AstraZeneca data on file), adjusting for differences in dose and accounting for the 2.28-fold higher exposure seen in those with severe renal impairment after a single dose of verinurad (Smith et al., 2018).

The C_{max} values of M1 and M8 were estimated from observed C_{max} values (91.0 and 97.2 ng/ml, respectively, or 0.173 and 0.161 μM, respectively) in subjects with severe renal impairment receiving a single dose of 15 mg verinurad, accounting for the difference in dose, relative bioavailability between formulations (60%), and the expected accumulation ratio of M1 and M8 (using a terminal half-life of 21.2 and 22.8 hours, respectively) after 12 mg once daily verinurad dosing (Smith et al., 2018).

Results

Permeability of Verinurad. The intrinsic permeability of verinurad in Caco-2 cells was 35.8 x 10^{-6} cm/s, with a P_{app} value higher than that for the high-permeability reference compound metoprolol (18.3 x 10^{-6} cm/s).

Plasma Protein Binding of Verinurad, M1, and M8. For the equilibrium dialysis assessment of verinurad, the average ratio in the drug chamber and sample chamber was 0.993 ± 0.008, indicating equilibrium was reached using the rapid equilibrium dialysis device. In human plasma, verinurad was highly bound (average 97.1%) to plasma protein between 1 and 50 μM with minimal differences observed with increasing concentration (Table 3). Binding of verinurad to HSA was >96.3% at all concentrations evaluated and was similar to the fraction bound in human plasma (Table 3). The extent of binding to AGP was <10% at all concentrations, indicating that binding in human plasma is primarily to serum albumin (Table 3).

Because of the difference in stability of the acyl glucuronide metabolites compared with the parent drug, the PPB of metabolites M1 and M8 was determined by ultra centrifugation. The protein binding experiments using ultracentrifugation showed that both M1 and M8 had good stability (>85%) over 60 minutes. The mean percent recovery of M1 was 93.6 ± 3.9% and 95.1 ± 3.92% at 1 and 50 μM, respectively, and for M8 was 86.6 ± 3.4% and 85.9 ± 2.2% at 1 and 50 μM, respectively. Our results showed that M1 was highly protein bound (average 92.1%), and M8 was moderately protein bound (average 66.2%) in human plasma at 1 and 50 μM (Table 3).

Passive and Active Hepatic Uptake of Verinurad. The mechanism of hepatic uptake of verinurad was determined based on the rate of its appearance in suspended human hepatocytes in the absence and presence of a mixture of 25 μM prazosin and 25 μM rifampicin SV (inhibitors of the major hepatic uptake transporters OCT1 and OATPs,

<table>
<thead>
<tr>
<th>Transports/Organ</th>
<th>Verinurad</th>
<th>M1 and M8</th>
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<tbody>
<tr>
<td>P-gp, BCRP in gastrointestinal tract</td>
<td>I_{u,max}/IC_{50} or K_{u} ≈ 10 (oral)</td>
<td>Not applicable to metabolites</td>
</tr>
<tr>
<td>P-gp, BCRP in liver and kidney</td>
<td>I_{max}/IC_{50} ≥ 0.1 (US FDA)</td>
<td>I_{max}/IC_{50} ≥ 0.1 (US FDA)</td>
</tr>
<tr>
<td>OATP1B1, OATP1B3 in liver</td>
<td>I_{u,max}/IC_{50} ≥ 0.02 (EMA)</td>
<td>I_{u,max}/IC_{50} ≥ 0.02 (EMA)</td>
</tr>
<tr>
<td>OAT1, OAT3, OCT2, MATE1, MATE2-K in kidney</td>
<td>I_{u,max}/IC_{50} or K_{u} ≥ 0.1 (considering first-pass concentration) (US FDA, JPMDA)</td>
<td>I_{u,max}/IC_{50} or K_{u} ≥ 0.1 (considering first-pass concentration) (US FDA, JPMDA)</td>
</tr>
<tr>
<td>OCT2, OCT1 in liver</td>
<td>I_{u,max}/IC_{50} or K_{u} ≥ 0.02 (EMA, JPMDA; MATEs)</td>
<td>I_{u,max}/IC_{50} or K_{u} ≥ 0.02 (EMA, JPMDA; MATEs)</td>
</tr>
<tr>
<td>BSEP in liver</td>
<td>No explicit guidance by regulatory agencies</td>
<td>No explicit guidance by regulatory agencies</td>
</tr>
<tr>
<td>MRP2, MRP4 in liver and kidney</td>
<td>I_{max}/IC_{50} or K_{u} ≥ 0.1 in analogy to OATP considerations (US FDA, JPMDA)</td>
<td>I_{max}/IC_{50} or K_{u} ≥ 0.1 in analogy to OATP considerations (US FDA, JPMDA)</td>
</tr>
<tr>
<td>CYP3A in gastrointestinal tract</td>
<td>I_{max}/K_{u} ≥ 0.1 (oral)</td>
<td>I_{max}/K_{u} ≥ 0.1 (oral)</td>
</tr>
<tr>
<td>CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A in liver</td>
<td>I_{u,max}/K_{u} ≥ 0.02 (all regulatory agencies)</td>
<td>I_{u,max}/K_{u} ≥ 0.02 (all regulatory agencies)</td>
</tr>
</tbody>
</table>

EMA, European Medicines Agency; FDA, Food and Drug Administration; fu, unbound fraction in plasma; I_{int}, intestinal luminal concentration; I_{max}, maximum free hepatic inlet concentration; I_{max}, maximum free plasma concentration; JPMDA, Japanese Pharmaceuticals and Medical Devices Agency; K_{u}, inhibitory constant.
respectively). The uptake profiles of verinurad with and without transporter inhibitors were different, indicating an active uptake mechanism similar to that of active control substrate rosuvastatin (Supplemental Table 6). The calculated percent active uptake of verinurad was 51% at 0.5 μM and 86% at 5 μM, indicating that hepatic uptake of verinurad involves both passive and active mechanisms. For the passive control substrate verapamil, there was no difference in the uptake slope with and without inhibitor (Supplemental Table 6).

**In Vitro Evaluation of Verinurad, M1, and M8 as Inhibitors of Human Metabolizing Enzymes.** From the panel of CYP enzymes and over the verinurad concentrations tested (0.016–100 μM), verinurad inhibited CYP2C8, CYP2C9, and CYP3A4/5, with IC₅₀ values of 14.5 μM, 25.6 μM, and 77.1/85.3 μM, respectively (Table 4). For the other CYPs examined, < 50% inhibition at the highest concentration tested was observed, with IC₅₀ values determined to be > 100 μM (Table 4). The IC₅₀ values of verinurad as an inhibitor of UGT1A1 and UGT2B7 were 192.7 μM and 1120 μM, respectively (Table 4). For M1, although inhibition of CYP1A2, CYP2B6, and CYP2C8 activity was observed at the highest concentration tested, the inhibition was insufficient to accurately determine IC₅₀ values (IC₅₀ > 30 μM; Table 4). Likewise, <20% inhibition of CYP1A2, CYP2A6, CYP2B6, and CYP2E1 activity by M8 was observed at the highest concentration tested, with IC₅₀ values >30 μM (Table 4). There was no evidence that M1 or M8 inhibited other CYP enzymes studied over the concentration range tested (0.1–30.0 μM; Table 4).

Preincubation with verinurad, M1, and M8 did not enhance inhibitory potency toward any CYP enzyme studied (CYP1A2, 2C8, 2C9, 2C19, 2D6, and 3A4/5); either <20% TDI was observed at the highest test concentration, or IC₅₀ values did not decrease upon preincubation. By contrast, preincubation of HLMs with reference inhibitors for CYP1A2 (10 μM furafylline), CYP2C8 (10 μM gemfibrozil-1-O-β glucuronide), CYP2C9 (2.5 μM tienilic acid), CYP2C19 (5 μM ticlopidine), CYP2D6 (5 μM paroxetine), and CYP3A4/5 (1.5 μM troleandomycin) resulted in TDI as expected. This indicates that verinurad, M1, and M8 are not time-dependent inhibitors of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5.

**In Vitro Evaluation of Verinurad as a CYP Inducer in Human Metabolizing Enzymes.** Treatment of cultured hepatocytes with up to 100 μM verinurad had less than 2-fold change on CYP1A2, CYP2B6, and CYP3A4 mRNA expression levels (Supplemental Table 7). There was a trend for a concentration-dependent increase in CYP3A4/5 activity in all three hepatocyte cultures with verinurad; however, verinurad was less than 20% (13.5% on average) as effective as rifampicin at inducing CYP3A4/5 activity at the highest concentration tested in all donors (Supplemental Table 8). This is below the 20% threshold from the US FDA (Food and Drug Administration, 2020) and collectively indicates low risk of induction.

**In Vitro Evaluation of Verinurad, M1, and M8 as Substrates of Human Drug Transporters.** Verinurad and its metabolites M1 and M8 were evaluated as substrates of drug transporters in various in vitro test systems (Supplemental Table 4). Functional transport activity in each test system was confirmed by transport studies using probe substrates and specific inhibitors. The resulting findings are summarized in Figs. 2 and 3 (efflux transporters), Fig. 4 (renal transporters), and Fig. 5 (hepatic uptake transporters).

**Transport of Verinurad, M1, and M8 by Ubiquitously Expressed Efflux Transporters P-gp, BCRP, MRP2, and MRP4.** In vitro, verinurad was a substrate of P-gp, with an ER > 2 at 1 and 10 μM in MDCKII-MDR1 cells. The efflux was inhibited by the reference inhibitor ketoconazole, whereas verapamil did not reduce the ER of 10 μM verinurad to <2, indicating a potential involvement of other efflux transporters alongside P-gp (Fig. 2A). In additional studies in MDCKII-MDR1 cells, verinurad had ERs of 1.80–2.53 at concentrations of 1–30 μM (Fig. 2B). The addition of the reference inhibitor elacridar decreased the ER of verinurad to <1 at 1 μM and 3 μM, and <1.5 at 10 μM and 30 μM (Fig. 2B), indicating that verinurad is a substrate of P-gp in vitro.

Verinurad was also identified as a substrate of BCRP, with an ER > 2 at concentrations of 0.3, 1, and 5 μM in Caco-2 cells; however, the ER remained >2 at 5 μM verinurad after the addition of the reference inhibitor chrysin (Fig. 2C). In a follow-up experiment in MDCKII-BCRP cells, the ER of verinurad was >2 at concentrations of 0.5 and 2 μM (Fig. 2D). The ER was reduced to <2 at both concentrations after the addition of reference inhibitors KO143 and elacridar (Fig. 2D), suggesting that verinurad is a substrate for human BCRP in vitro.

The evaluation of verinurad and metabolites M1 and M8 as substrates of MRP2 and MRP4 was conducted in S99 insect vesicles expressing human MRP2 or MRP4. ATP did not significantly increase uptake of verinurad compared with AMP controls (P > 0.05) in neither MRP2 (Fig. 3A) nor MRP4 vesicles (Fig. 3B), indicating that verinurad is not a substrate of MRP2 or MRP4 in vitro. In contrast, both M1 and M8 demonstrated significantly higher uptake in MRP2 vesicles in the presence of ATP compared with AMP (M1: P = 0.0001; M8: P < 0.0001) (Fig. 3, C and D). The reference inhibitor benzbro-maron clearly inhibited MRP2-mediated uptake of 1 μM of M1 by 99.7% and 1 μM of M8 by 99.2% (Fig. 3, C and D), confirming that they are substrates of MRP2 in vitro. Similarly, both M1 and M8 demonstrated concentration-dependent uptake in MRP4 vesicles in the presence of ATP compared with AMP (M1: P ≤ 0.0002; M8: P = 0.0124) (Fig. 3, E and F). The MRP4-mediated uptake of 1 μM of M1 and 1 μM of M8 was inhibited by benzbro-maron by 91.5% and 100.0%, respectively (Fig. 3, E and F), confirming that they are substrates of MRP4 in vitro.

**Transport of Verinurad by Human Renal Transporters.** In vitro uptake studies showed OAT1-dependent uptake of verinurad in MDCKII-OAT1 cells at concentrations of 0.1–10 μM, with statistically significant differences between MDCKII-OAT1 and control cells at the four highest concentrations (P < 0.05) (Fig. 4A), indicating that verinurad is a
substrate of OAT1 in vitro. In contrast, none of the tested concentrations of verinurad (0.1–10 μM) showed more than 17% increase in cellular accumulation in MCDKII-OAT3 cells compared with control cells (Fig. 4B), indicating that verinurad is not a substrate of OAT3 in vitro.

In MDCKII-MATE1 and MDCKII-MATE2-K cells, uptake of verinurad was not significantly higher than in control cells at concentrations of 0.3–5 μM (P > 0.05) (Fig. 4, C and D), indicating that verinurad is not a substrate of MATE1 and MATE2-K in vitro. Similarly, verinurad uptake was comparable in MDCKII-OCT2 cells and control cells at concentrations of 0.3–5 μM (Fig. 4E), indicating that verinurad is not a substrate of OCT2 in vitro.

**Transport of Verinurad by Human Hepatic Uptake Transporters.** Verinurad was found to undergo active hepatic uptake in suspended human hepatocytes, as there was a clear difference in uptake in the presence and absence of transporter inhibitors. It is worth noting that the inhibitors and concentrations used in the hepatocyte experiment primarily inhibit OCT1 and OATP transporters over other hepatic uptake transporters, including Na+/taurocholate cotransporting polypeptide and OAT2 (Bi et al., 2019). To elucidate the transporter(s) involved in the hepatic uptake, in vitro substrate studies were subsequently performed with OATP1B1, OATP1B3, OATP2B1, and OCT1 using MDCKII or CHO cells transfected with the respective transporter. Verinurad did not demonstrate OATP1B1-dependent uptake, with comparable uptake between MDCKII-OATP1B1–transfected cells and control cells at concentrations of 0.3–5 μM or in the presence of rifampicin (Fig. 5A). In contrast, verinurad was identified as an OATP1B3 substrate in vitro, with statistically significant differences between MDCKII-OATP1B3 cells and control cells at 1 μM (P = 0.012) and 5 μM (P = 0.0028) (Fig. 5B). The OATP1B3-mediated uptake of verinurad was inhibited by 100 μM rifampicin, further confirming the role of OATP1B3 in the hepatic uptake (Fig. 5B).

In CHO-OATP2B1 cells, uptake of verinurad was comparable with that in control cells at concentrations of 0.3–5 μM.
In Vitro Evaluation of Verinurad, M1, and M8 as Inhibitors of Human Drug Transporters. The potential for verinurad and metabolites M1 and M8 to inhibit the transport of probe substrates by efflux (P-gp, BCRP, BSEP, MRP2, and MRP4), renal (OAT1, OAT3, MATE1, MATE2-K, and OCT2), and hepatic uptake transporters (OCT1, OCT2, OATP1B1, and OATP1B3) was determined in transporter-expressing cells or vesicles (Table 1, Supplemental Table 5). The resulting findings are summarized in Table 5 (efflux transporters), Table 6 (renal transporters), and Table 7 (hepatic uptake transporters).

In vitro, verinurad demonstrated concentration-dependent inhibition of OAT1, OATP1B1, OATP1B3, and BSEP activity, with IC$_{50}$ values of 14.2, 19.4, 31.0, and 109 μM, respectively (Tables 5–7). In addition, inhibition of OAT3-mediated transport was noted at the highest concentration tested (IC$_{50}$ > 100 μM; Table 6). In contrast, verinurad did not inhibit the transport of probe substrates by P-gp, BCRP, OCT1, or OCT2 over the concentration range investigated (0.3–100 μM; Tables 5–7).

Metabolites M1 and M8 inhibited MRP2-, OAT3-, and OATP1B1-dependent transport in vitro, with IC$_{50}$ values of 1.13, 11.9, and 3.07 μM for M1 and 5.50, 30.4, and 19.8 μM for M8, respectively (Tables 5–7). M1 also inhibited BCRP and OATP1B3 with IC$_{50}$ values of 145 and 18.4 μM, respectively, whereas inhibition of these transporters by M8 was insufficient to accurately determine IC$_{50}$ values (IC$_{50}$ > 100 μM; Table 5 and 7). Less than 50% inhibition of OAT1 activity by M1 and M8 and of MATE1 and MATE2-K activity by M1 was also observed (IC$_{50}$ > 100 μM), whereas M8 did not inhibit the transport of probe substrates by either MATE1 or MATE2-K (Table 6). Likewise, M1 inhibited MRP4 at the highest concentration tested (IC$_{50}$ > 10 μM), whereas M8 did not demonstrate concentration-dependent inhibition of MRP4 over the concentration range investigated (0.1–10 μM; Table 5). Similar to verinurad, neither M1 nor M8 inhibited the active transport by P-gp, OCT1, and OCT2 (Tables 5–7).

Discussion

Verinurad is in development for CKD and HFP EF, which are associated with comorbidities and frequent polypharmacy. Assessment of the potential for clinically relevant DDIs with verinurad early in development is therefore essential. The present studies characterized the in vitro DDI potential of verinurad and metabolites M1 and M8 as victims and perpetrators of key metabolizing enzymes and drug transporters. Our results provide new insights on verinurad’s disposition in humans (Fig. 6) and inform phase 2 and 3 protocols and inclusion and exclusion criteria.
The inhibitory potencies of verinurad and metabolites M1 and M8 on major CYP enzymes were examined in vitro using drug substrates recommended by regulatory agencies and reported by the scientific community. For verinurad, IC50 values could only be obtained for CYP2C8, CYP2C9, and CYP3A4/5. However, the calculated ratios of intrinsic clearance values (R1) were <1.02 for systemic interactions with CYP2C8, CYP2C9, and CYP3A4/5, and the calculated R1, gut for CYP3A4/5 (4.6) was below the risk threshold of 11 for intestinal CYP3A4/5 interactions, indicating a low risk of clinically relevant DDIs with verinurad as a direct inhibitor of CYP2C8, CYP2C9, and CYP3A4/5. Neither M1 nor M8 produced direct inhibition of any metabolizing enzyme tested, and there was no evidence of TDI of CYP1A2, CYP2C8, CYP2C9, CYP2C9, CYP3A4/5, and CYP3A4/5, and the calculated R1, gut for CYP3A4/5 (4.6) was below the risk threshold of 11 for intestinal CYP3A4/5 interactions, indicating a low risk of clinically relevant DDIs with verinurad as a direct inhibitor of CYP2C8, CYP2C9, and CYP3A4/5. Neither M1 nor M8 produced direct inhibition of any metabolizing enzyme tested, and there was no evidence of TDI of CYP1A2, CYP2C8, CYP2C9,
CYP2C19, CYP2D6, or CYP3A4/5 with verinurad, M1, or M8. This was an interesting finding with respect to CYP2C8 and one that is in contrast with studies showing that some acyl glucuronides, such as those of clopidogrel, gemfibrozil, and delboeuvir, are direct and time-dependent inhibitors of CYP2C8 (Shitara et al., 2004; Ogilvie et al., 2006; Tornio et al., 2014; Backman et al., 2016; Kim et al., 2016; Sane et al., 2016; Ma et al., 2017). Although not recommended explicitly by regulatory agencies, we also assessed the systemic (hepatic) and intestinal UGT interactions for verinurad as described for CYP-mediated interactions and observed a low risk of clinically relevant DDIs with verinurad as a direct inhibitor of UGT1A1 and UGT2B7. Overall, there is a low potential for verinurad and metabolites M1 and M8 to inhibit CYP and UGT enzymes at clinically relevant concentrations.

In primary human hepatocytes, verinurad did not induce activity or mRNA expression of CYP1A2, CYP2B6, or CYP3A4/5 compared with prototypical inducers and was below the 20% threshold from the US FDA (Food and Drug Administration, 2020). Consistent with the absence of reports in the literature to implicate acyl glucuronides as CYP inducers, it is likely that M1 and M8 are not inducers of CYP1A2, CYP2B6, or CYP3A4/5 either.

Our collective studies showed that verinurad was a substrate of the efflux transporters P-gp and BCRP in vitro. However, the finding that efflux of verinurad was not reduced by verapamil in a similar manner as with ketoconazole in the MDCKII-MDR1 cell line means that we cannot exclude the potential contribution of canine P-gp to the lack of P-gp inhibitory effect of verapamil. Conceptually, P-gp and BCRP may

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**Fig. 4.** Transport of verinurad by human renal transporters. Uptake of verinurad in MDCKII-OAT1 (A), MDCKII-OAT3 (B), MDCKII-MATE1 (C), MDCKII-MATE2-K (D), and MDCKII-OCT2 (E) cells as compared with uptake in control cells. Data are mean and S.D. of triplicate samples. Unpaired Student’s t test was used to assess the difference in uptake in transporter-expressing and control cells. A p-value of <0.05 was considered statistically significant.
impact the oral bioavailability, tissue distribution, and hepatic and renal elimination of their substrates (Zhang et al., 2018). However, because of its high solubility and high intrinsic permeability, verinurad absorption is not impacted by intestinal efflux transporters, as reflected in its fraction absorbed of $\geq 64\%$ (Lee et al., 2018) and dose-proportional pharmacokinetics up to 40 mg after a single dose (Shen et al., 2017) and multiple once-daily doses (Hall et al., 2018). Collectively, this suggests that P-gp and BCRP are likely to have minimal impact on the risk of clinically relevant DDIs.

Hepatic disposition of verinurad involves a combination of active uptake and passive diffusion followed by metabolism and efflux (Fig. 6). Our in vitro data revealed that verinurad is a substrate of OATP1B3 but not of other OATP transporters unlike typical anionic compounds or carboxylic acid moieties, which are common substrates shared by multiple OATP-isosforms (Kalgutkar and Daniels, 2010). In vitro, verinurad uptake into hepatocytes showed almost equal active transport and passive diffusion at the lower concentration and largely active uptake at the higher concentration. This unexpected finding made it challenging to deduce the clinical role of active hepatic uptake in the disposition of verinurad. Therefore, the OATP DDI potential will be further elucidated in a clinical DDI study (study D5495C00013 [NCT04532918]).

Verinurad was also identified as a substrate of OAT1 in vitro. Metabolites M1 and M8 are the main components of the verinurad dose eliminated in urine, whereas $<2\%$ of verinurad is excreted unchanged renally (Lee et al., 2018), and the contribution is expected to be even lower in renally impaired patients. Therefore, OAT1 inhibition is not expected to significantly alter the pharmacokinetics of verinurad. In renal proximal tubular cells, CYP and UGTs can contribute to the formation of M1 and M8, which can then be effluxed by MRP2/4 into the urine (Fig. 6). Although urinary excretion decreases with reduced renal function, evidence from a single-dose renal impairment study supports the safety and tolerability of higher concentrations of M1 and M8 observed in severe renal impairment (Smith et al., 2018). Therefore, the risk of comedications that may alter uptake by OAT1 and change renal clearance of verinurad itself is low, but inhibitors or inducers of MRP2 may impact the pharmacokinetics of verinurad by altering the elimination of metabolites (Marschall et al., 2005; Oscarson et al., 2007).

In our in vitro transporter inhibition studies, verinurad did not inhibit P-gp, BCRP, OCT1, OCT2, or OAT3, whereas M1 and M8 did not inhibit P-gp, MRP4, OCT1, MATE1, MATE2K, or OCT2. Applying established static equations with the moderate inhibitory potencies observed for OATPs or OAT1 together with the estimated worst case $C_{\text{max}}$ of verinurad and metabolites based on the highest dose tested in phase 2 clinical studies, the overall risk for clinically relevant pharmacokinetic DDIs via inhibition of the efflux, renal, and hepatic transporters studied is considered low, except for the previously described pharmacological effect on oxypurinol.

![Fig. 5. Transport of verinurad by human hepatic uptake transporters. Cellular accumulation of verinurad in MDCKII-OATP1B1 (A), MDCKII-OATP1B3 (B), CHO-OATP2B1 (C), and MDCKII-OCT1 (D) cells as compared with control cells. Data are mean and S.D. of triplicate samples. Unpaired Student's t test was used to assess the difference in uptake in transporter-expressing and control cells. A p-value of $<0.05$ was considered statistically significant. BSP, bromosulfophthalein; CHO, Chinese hamster ovary.](https://jpet.aspetjournals.org/content/119/1/119/F5)
elimination through inhibition of URAT1, which is itself a renal transporter (Kankam et al., 2018).

A unique aspect of our studies is the assessment of in vitro DDI potentials of metabolites M1 and M8 as victims and perpetrators of drug transporters. Acyl glucuronides M1 and M8 were substrates of MRP2 and MRP4 in vitro. Efflux via MRPs contributes to the disposition of several glucuronide conjugates, for example, those of mycophenolic acid (Westley et al., 2006; Patel et al., 2013), atorvastatin (Rodrigues et al., 2020), and diclofenac (Zhang et al., 2016), in which MRP4 contributes

### TABLE 5
In vitro evaluation of inhibition of efflux transporters by verinurad and metabolites M1 and M8

<table>
<thead>
<tr>
<th>Transporter</th>
<th>IC50 (μM)</th>
<th>( \frac{I_{\text{u,max}}}{IC50} )</th>
<th>( \frac{I_{\text{u,max}}}{IC50} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verinurad</td>
<td>NI</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>M1</td>
<td>NI</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>M8</td>
<td>NI</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BCRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verinurad</td>
<td>NI</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>M1</td>
<td>145</td>
<td>0.00287</td>
<td>0.000228</td>
</tr>
<tr>
<td>M8</td>
<td>&gt;300</td>
<td>&lt;0.0138</td>
<td>&lt;0.00460</td>
</tr>
</tbody>
</table>

(33.8% inhibition at 300 μM)

Verinurad + M1 + M8

\(<0.00423\) \(<0.00069\)

BSEP<sup>a</sup>

| Verinurad   | 109       | 0.00218                       | 0.000642                      |

MRP2<sup>a</sup>

| M1          | 1.13      | 0.368                         | 0.0295                        |
| M8          | 5.50      | 0.0744                        | 0.0251                        |
| M1 + M8<sup>b</sup> | 0.442    | 0.0546                        |                               |

MRP4<sup>a</sup>

| M1          | >10       | <0.0416                       | <0.00333                      |
| M8          | >10       | <0.0409                       | <0.0138                        |

(38.3% inhibition at 10 μM)

M1 + M8<sup>b</sup>

| <0.0825      | <0.0172                        |

C<sub>max</sub>, maximum observed concentration; EMA, European Medicines Agency; FDA, Food and Drug Administration; \( I_{\text{u,max}} \), total maximum plasma inhibitor concentration; \( I_{\text{u,max}} \), maximum free plasma inhibitor concentration; NI, no inhibition.

<sup>a</sup>Estimated margin in analogy to efflux transporter inhibition concept in US FDA 2020 and EMA guidance, 10-fold total C<sub>max</sub> (US FDA): \( R = \frac{I_{\text{u,max}}}{IC50} \geq 0.1 \); 50-fold unbound C<sub>max</sub> (EMA): \( \frac{I_{\text{u,max}}}{IC50} \leq 0.02 \).

<sup>b</sup>The combined inhibitory potential of verinurad and its metabolites was calculated as a sum of the individual ratios of exposure in relation to in vitro inhibition constants.

### TABLE 6
In vitro evaluation of inhibition of human renal transporters by verinurad and metabolites M1 and M8

<table>
<thead>
<tr>
<th>Transporter</th>
<th>IC50 (μM)</th>
<th>( \frac{I_{\text{u,max}}}{K_i} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verinurad</td>
<td>NI</td>
<td>–</td>
</tr>
<tr>
<td>M1</td>
<td>NI</td>
<td>–</td>
</tr>
<tr>
<td>M8</td>
<td>NI</td>
<td>–</td>
</tr>
<tr>
<td>OAT1</td>
<td>14.2</td>
<td>0.000048</td>
</tr>
<tr>
<td>M1</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>M8</td>
<td>&gt;100</td>
<td></td>
</tr>
</tbody>
</table>

(47.1% inhibition at 100 μM)

(31.7% inhibition at 100 μM)

Verinurad + M1 + M8<sup>c</sup>

| <0.00219      |                               |

OAT3

| Verinurad   | >100      | 0.000068<sup>a</sup>          |                               |
| M1          | 11.9      | 0.00559                       |                               |
| M8          | 30.4      | 0.00914                       |                               |

Verinurad + M1 + M8<sup>c</sup>

| <0.00148      |                               |

MATE1

| M1          | >100      | <0.00033<sup>b</sup>          |
| M8          | NI        | –                             |

MATE2-K

| M1          | >100      | <0.00033<sup>a</sup>          |
| M8          | NI        | –                             |

C<sub>max</sub>, maximum observed concentration; \( K_i \), inhibitory constant; \( K_m \), Michaelis-Menten constant; NI, no inhibition.

<sup>a</sup>In inhibition studies, probe substrate concentration was sufficiently lower than its \( K_m \) such that IC<sub>50</sub> approximates to \( K_m \).

<sup>b</sup>Risk margin estimate based on highest drug concentration in the assay (which yielded < 50% inhibition).

<sup>c</sup>The combined inhibitory potential of verinurad and its metabolites was calculated as a sum of the individual ratios of exposure in relation to in vitro inhibition constants.
to basolateral hepatic efflux and MRP2 contributes to biliary excretion that can lead to enterohepatic recirculation after hydrolysis by β-glucuronidases and intestinal reabsorption. This reuptake route may explain the small secondary peak observed in the plasma exposure profile after verinurad dosing (Lee et al., 2018). Transporter inhibition by cyclosporine

### Table 7

<table>
<thead>
<tr>
<th>Transporter</th>
<th>IC₅₀ (µM)</th>
<th>Iₘₐₓ,max (µM) ᵃ</th>
<th>Iₘₐₓ,max/Kᵢᵇ</th>
<th>Rᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OATP1B1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verinurad</td>
<td>19.4</td>
<td>7.89</td>
<td>0.0233</td>
<td>1.023</td>
</tr>
<tr>
<td>M1</td>
<td>3.07</td>
<td>0.694</td>
<td>0.0322</td>
<td>1.032</td>
</tr>
<tr>
<td>M8</td>
<td>19.8</td>
<td>0.682</td>
<td>0.0207</td>
<td>1.021</td>
</tr>
<tr>
<td>Verinurad + M1 + M8ᵈ</td>
<td></td>
<td></td>
<td>0.0762</td>
<td>1.076</td>
</tr>
<tr>
<td><strong>OATP1B3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verinurad</td>
<td>31.0</td>
<td>7.89</td>
<td>0.00365</td>
<td>1.004</td>
</tr>
<tr>
<td>M1</td>
<td>18.4</td>
<td>0.694</td>
<td>0.00302</td>
<td>1.003</td>
</tr>
<tr>
<td>M8</td>
<td>&gt;100</td>
<td>0.682</td>
<td>&lt;0.00230</td>
<td>&lt;1.002</td>
</tr>
<tr>
<td>(40.6% inhibition at 100 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verinurad + M1 + M8ᵈ</td>
<td></td>
<td></td>
<td>0.0097</td>
<td>1.009</td>
</tr>
<tr>
<td><strong>OAT2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verinurad</td>
<td>&gt;100</td>
<td>7.89</td>
<td>&lt;0.00453</td>
<td>1.005</td>
</tr>
<tr>
<td>(33.1% inhibition at 100 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>OCT1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verinurad</td>
<td>NI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>NI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M8</td>
<td>NI</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Iₘₐₓ,max calculated based on US FDA 2020 guidance. Iₘₐₓ,max = Iₘₐₓ + (Iₘₐₓ x Kᵢ x dose(Qₚ))/B:P = 0.238 µM + [(1 x 1.1 x 0.1 x 68.9 mmol/L/1.5/0.6) = 7.654 = 7.89, in which dose = 24 mg = 68.9 µmol, Qₚ = 1.5 l/min, and B:P = 0.6. For metabolites, Iₘₐₓ,max is estimated from total Cₘₐₓ and Rb of 0.6-0.693 µM and 0.682 µM for M1 and M8, respectively.

ᵇ Kᵢ was estimated to equal IC₅₀ when substrate concentration was well below the reported Km or as IC₅₀/2 when substrate concentration was close to Km.

ᶜ R = 1 + (Iₘₐₓ × IC₅₀)/IC₅₀ · Iₘₐₓ ≥ 1.1.

ᵈ The combined inhibitory potential of verinurad and its metabolites was calculated as a sum of the individual ratios of exposure in relation to in vitro inhibition constants.

**Notes:**
- Cₘₐₓ: maximum observed concentration.
- FDA: Food and Drug Administration.
- Iᵤ: unbound fraction in plasma.
- Iₘₐₓ,max: maximum hepatic inlet concentration.
- Iₘₐₓ: total maximum plasma inhibitor concentration.
- IC₅₀: inhibitory constant.
- NC: not calculated.
- OATP1B1: organic anion transporting polypeptide 1B1.
- OATP1B3: organic anion transporting polypeptide 1B3.
- OAT2: organic anion transporting polypeptide 2.
- OCT1: organic cation transporter 1.
- UGTs: UDP-glucuronosyltransferases.
- CYP3A4: cytochrome P450 3A4.
- β-glucuronidase: enzyme that breaks down β-glucuronides.
- M₁, M₄: acyl glucuronide of verinurad.
- V: verinurad.
- M: metabolite.
- B: bile.
- P: plasma.
- H: hepatic.
- U: urine.
- R: renal.

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**Fig. 6.** The hypothesized mechanisms of disposition of verinurad and metabolites M1 and M8. After oral administration of verinurad, metabolism to form M4, M1, and M8 may occur in enterocytes, as CYP3A4/UGT enzymes are present. Verinurad and metabolite(s) enter the portal vein, and verinurad is taken up by the hepatocytes in the liver via passive diffusion and active transport by OATP1B3. In the hepatocytes, verinurad is metabolized two ways: 1) to M₄ (by CYP3A4) and then subsequently conjugated by UGTs to form M₈ and 2) to M₁ (by UGTs), which may be sequentially metabolized to M₈ by CYP2C8. In addition, P-gp and BCRP may contribute to hepatic efflux of verinurad to the bile. Metabolites that undergo enterohepatic recycling are effluxed to the bile via MRP2 and undergo complete deconjugation by β-glucuronidase to release the aglycone to enter the portal system to the liver. Metabolites effluxed by MRP4 to the systemic circulation can be eliminated by the kidney and excreted in the urine or can be taken up by the liver and undergo enterohepatic recycling. Verinurad is also eliminated by the kidney to a limited extent, possibly via OAT1-mediated uptake and P-gp-mediated efflux across the renal proximal tubular epithelial cells. Fₜ₞, fraction of the dose of inhibitor that is absorbed; M₁, acyl glucuronide of verinurad; M₄, N-oxide of verinurad; M₈, acyl glucuronide of verinurad-N-oxide; UGT, glucuronosyl transferases; V, verinurad.
decreases the AUC of mycophenolic acid by disrupting the enterohepatic recycling process (Westley et al., 2006). By the same analogy, the pharmacokinetics of verinurad may be altered by comediations that inhibit MRPs.

In our in vitro transporter inhibition studies, only MRP2 was inhibited by M1 and M8 in vitro with IC_{50} values 3- to 13-fold above projected therapeutic C_{max} respectively. Guidance from regulatory agencies do not provide a framework for assessing the risk for clinically relevant MRP2 inhibition; however, the potential for DDIs can be explored by applying the assessment approaches for other efflux transporters. For instance, both M1 and M8 meet the threshold for potential DDIs described by the US FDA for MATE based on a 10-fold margin for the free plasma concentration (Food and Drug Administration, 2020). Yet, both M1 and M8 were above (34- and 40-fold margin, respectively) the threshold for potential DDIs described by the EMA for P-gp based on a 50-fold threshold for free plasma concentration (European Medicines Agency, 2013). Additionally, the US FDA and JPMDA use a 10-fold margin for P-gp, and our in vitro data indicate that the total I_{max}IC_{50} for M1 is lower than this threshold, but for M8 it is higher (Japanese Pharmaceuticals and Medical Devices Agency, 2018; Food and Drug Administration, 2020). Therefore, metabolites M1 and M8 cannot be fully excluded as inhibitors of MRP2 based on risk assessment approaches for other efflux transporters. MRP2 substrates are often conjugated drug metabolites (Hillgren et al., 2013) whose clearance may be modulated by inhibition of MRP2 (Westley et al., 2006; Patel et al., 2013; Picard, 2013) but compensated by efflux through other MRP isoforms, such as MRP3 or MRP4 (Hillgren et al., 2013; Zhang et al., 2016). Overall, the clinical interpretation of our findings is complicated by an absence of a specific substrate of MRP2 to evaluate inhibition in vivo. Furthermore, there is neither evidence in the literature for clinically significant inhibition of MRP2 that includes MRP2 polymorphisms or specific pharmacokinetic-related substrates (Suzuki and Sugiyama, 2002; Niemi et al., 2006; Hillgren et al., 2013) nor specific guidance provided by regulatory agencies.

Besides the individual DDI risk assessments for verinurad, M1, and M8, we also assessed the combined inhibitory potential of verinurad and its metabolites by calculating additive R values (see Materials and Methods section for details). Except for OATP1B1, in which the FDA threshold for potential DDI risk was met, whereas the EMA threshold was not, our calculations indicated no further risk when characterizing the combined inhibitory effect of verinurad and its metabolites.

In conclusion, the systematic quantitative risk analysis of in vitro data conducted here indicates that apart from verinurad inhibiting URAT1 and being a substrate of OATP1B3, the potential for clinically relevant DDIs for verinurad and metabolites M1 and M8 as victims or perpetrators of metabolizing enzymes or drug transporters is low. A clinical study is ongoing to assess the relevance of OATP engagement in the pharmacokinetics of verinurad. Following recent US FDA guidance, a unique aspect of this report is the evaluation of the nonactive acyl glucuronide metabolites, which were identified as substrates and inhibitors of MRPs.

Acknowledgments

The authors thank Chad EImore of AstraZeneca for ensuring the purity of synthetic materials. Medical writing support was provided by Shaun W. Foley, BSc (Hons) CMPF, and editorial support was provided by Bethany King, BSc (Hons), all of Core Medica, London, UK, supported by AstraZeneca according to Good Publication Practice guidelines. The sponsor was involved in the study design, collection, analysis, and interpretation of data as well as data checking of information provided in the manuscript. Ultimate responsibility for opinions, conclusions, and data interpretation lies with the authors.

Authorship Contributions

Conducted experiments: Lee.


Wrote or contributed to the writing of the manuscript: Gopaul, Vildhede, Andersson, Erlandsson, Lee, Johansson, Hilsengord.

References


Picard N (2013) The pharmacokinetic interaction between myophenolic acid and cyclosporine revisited: a commentary on "Myophenolic acid glucuronide is transported by multidrug resistance-associated protein 2 and this transport is not inhibited by cyclosporine, tacrolimus or sirolimus." *Xenobiotica* 43:836–838.


