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Evaluation of Ketoconazole and its Alternative Clinical CYP3A4/5 Inhibitors as Inhibitors of Drug Transporters: The In Vitro Effects of Ketoconazole, Ritonavir, Clarithromycin and Itraconazole on 13 Clinically-Relevant Drug Transporters

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Running Title: Effect of clinical CYP3A4/5 inhibitors on drug transporters

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Abbreviations used are: AUC, area under the plasma drug concentration-time curve; C_{max}, maximum plasma concentration; DDI, drug-drug interaction; EMA, European Medicines Agency; FDA, U.S. Food and Drug Administration; HLM, human liver microsomes; K_m, substrate concentration that gives a reaction rate equal to half of V_{max}; LC-MS/MS, liquid chromatography tandem mass spectrometry; UW DIDB, University of Washington Metabolism and Transport Drug Interaction Database.

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ABSTRACT

Ketoconazole is a potent CYP3A4/5 inhibitor, and until recently, recommended by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) as a "strong" CYP3A4/5 inhibitor in clinical drug-drug interaction (DDI) studies. Ketoconazole sporadically causes liver injury or adrenal insufficiency. Because of this, the FDA and EMA recommended suspension of ketoconazole use in DDI studies in 2013. FDA specifically recommended use of clarithromycin or itraconazole as alternative strong CYP3A4/5 inhibitors for use in clinical DDI studies, but many investigators have also used ritonavir as an alternative. Although the effects of these clinical CYP3A4/5 inhibitors on other CYPs are largely established, reports on the effects on the broad range of drug transporter activities are sparse. In this study, the inhibitory effects of ketoconazole, clarithromycin, ritonavir and itraconazole (and its CYP3A4-inhibitory metabolites, hydroxy-, keto- and *N*-desalkyl itraconazole) towards 13 drug transporters (OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2, MATE1, MATE2-K, P-gp, BCRP, MRP2, MRP3 and BSEP) were systematically assessed in transporter-expressing HEK-293 cell lines or membrane vesicles. In vitro findings were translated into clinical context with the basic static model approaches outlined by the FDA in their 2012 draft guidance on DDIs. The results indicate that, like ketoconazole, the alternative clinical CYP3A4/5 inhibitors ritonavir, clarithromycin and itraconazole each have unique transporter inhibition profiles. None of the alternatives to ketoconazole provided a clean inhibition profile towards the 13 drug transporters evaluated. The results provide guidance for the selection of clinical CYP3A4/5 inhibitors when transporters are potentially involved in a victim drug's pharmacokinetics.

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INTRODUCTION

Ketoconazole is an orally available, synthetic, broad-spectrum antifungal of the imidazole class that was initially approved by the US Food and Drug Administration (FDA) in June 1981 for the treatment of systemic fungal infections (FDA, 2014). For many years, ketoconazole was known as a clinically-relevant CYP3A4/5 inhibitor, with early case reports of interactions appearing by 1982 and the first significant quantitative increase in triazolam exposure (~23-fold) published in 1994 (Varhe et al., 1994; University of Washington, 2015). Furthermore, the most recent drug-drug interaction (DDI) guidance documents issued by the FDA and European Medicines Agency (EMA) in 2012 recommended the use of ketoconazole as a "strong" CYP3A4/5 inhibitor in clinical DDI studies (EMA, 2012; FDA, 2012). However, within the same year of its 1981 approval, reports of ketoconazole-induced hepatitis were published, with an incidence of symptomatic hepatotoxicity of 0.008% reported by 1984 (Van Tyle, 1984). Most of these reactions were observed after several months of dosing and were typically reversible.

Accumulating evidence over subsequent decades showed that in a small number of healthy patients, the typical doses used in clinical DDI studies (200 – 400 mg) for short periods (e.g., 5 days) could cause liver injury or adrenal insufficiency (FDA, 2013a). Because of these observations, the FDA issued a Drug Safety Communication regarding ketoconazole on July 26, 2013 and the EMA recommended the complete suspension of marketing authorization on the same day (EMA, 2013; FDA, 2013b). These regulatory actions now require that clinical investigators desiring to define the maximal clinical impact of CYP3A4/5 inhibition on drug candidates use alternative CYP3A4/5 inhibitors. The FDA specifically recommended the use of clarithromycin or itraconazole as alternative strong CYP3A4/5 inhibitors for use in clinical DDI studies, but further noted that investigators may suggest other CYP3A4/5 inhibitors (FDA,

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2013a). In addition to itraconazole and clarithromycin, ritonavir has also been suggested by some authors as a possible alternative to ketoconazole (Greenblatt and Greenblatt, 2014; Greenblatt, 2015; Greenblatt and Harmatz, 2015), but excluded by others on the basis of non-specific CYP inhibition or induction (Ke et al., 2014; Liu et al., 2015).

Although the effects of these clinically used CYP3A4/5 inhibitors on other CYPs are largely established, the effects on various transporter activities could confound the results of clinical DDI studies for some CYP3A4/5 substrates. Whereas several studies have investigated the effects of ketoconazole and its alternatives on one or more drug transporters, the current literature demonstrates that none have comprehensively compared the inhibitory effects of these drugs and their major metabolites on a large number of clinically relevant drug transporters in the same study. Furthermore, even for those CYP3A4/5 inhibitors evaluated extensively in vitro there are large inter-laboratory differences in reported IC_{50} values which are dependent on test system, assays conditions and probe substrates, amongst other factors. For instance, the reported range of IC_{50} values for ketoconazole vary by 37-fold and 6.4-fold for the inhibition of P-gp and OATP1B1, respectively (Gnoth et al., 2011; Izumi et al., 2013; Matsson et al., 2013; Mikkaichi et al., 2014). Similarly, IC_{50} values for ritonavir vary by 85-fold and 117-fold for the inhibition of P-gp and OATP1B1, respectively (Keogh et al., 2006; Herédi-Szabó et al., 2013; Izumi et al., 2013). Therefore, this work was undertaken to systematically assess the inhibitory effects of ketoconazole, clarithromycin, ritonavir and itraconazole (and its CYP3A-inhibitory metabolites, hydroxy-itraconazole, keto-itraconazole and *N*-desalkyl itraconazole), on OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), OAT1 (SLC22A6), OAT3 (SLC22A8), OCT1 (SLC22A1), OCT2 (SLC22A2), MATE1 (SLC47A1), MATE2-K (SLC47A2), P-gp (ABCB1), BCRP (ABCG2), MRP2 (ABCC2), MRP3 (ABCC3) and BSEP (ABCB11).

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MATERIALS AND METHODS

Chemicals and Reagents. Methanol, estradiol-17β-glucuronide, estrone-3-sulfate, *p*-aminohippurate, verapamil, Ko143, cyclosporin, benzbromarone, cimetidine, rifampin, probenecid, quinidine, ritonavir, clarithromycin, itraconazole, and ketoconazole were purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H]-estrone-3-sulfate, [^{3}H]-estradiol-17β-glucuronide, [^{3}H]-*p*-aminohippuric acid, and [^{3}H]-taurocholic acid were purchased from Perkin Elmer (Oak Brook, IL), hydroxy-itraconazole, keto-itraconazole, and *N*-desalkyl itraconazole were purchased from Toronto Research Chemicals (Ontario, Canada). N-methylquinidine was purchased from Solvo Biotechnologies (Boston, MA), [^{14}C]-tetraethylammonium bromide was purchased from American Radiolabeled Chemicals (St. Louis, Mo.), and [^{14}C]-metformin was purchased from Moravek Biochemicals (Brea, CA). 10x Hanks balanced salt solution (HBSS) was purchased from Invitrogen (Waltham, MA).

Test systems. HEK-293 cells transfected with SLCO1B1 (OATP1B1), SLCO1B3 (OATP1B3), SLC22A6 (OAT1), SLC22A8 (OAT3), SCL22A1 (OCT1), and SLC22A2 (OCT2) or empty vector (HEK-293 control) were purchased from ATCC (Manassas, VA). HEK-293 cells transfected with SLC47A1 (MATE1), SLC47A2 (MATE2-K), and empty vector (HEK-293 control) were purchased from Corning Life Sciences (Tewksbury, MA). Membrane vesicles expressing ABCB1 (P-gp) and ABCG2 (BCRP) were purchased from Sigma-Aldrich (St. Louis, MO). Membrane vesicles expressing ABCB11 (BSEP), ABCC2 (MRP2), and ABCC3 (MRP3) were purchased from GenoMembrane (Yokohama, Kanagawa, Japan).

HEK-293 Cell culture. OATP1B1, OATP1B3, OAT1, OAT3, OCT1, and OCT2 cells were thawed from cryopreservation and directly seeded onto 96-well plates at a density of $7x10^{5}$ cells/mL in DMEM supplemented with fetal bovine serum (10%), L-glutamine (10%), and

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penicillin-streptomycin (0.89%) in 37°C, 95% relative humidity, and 5% CO_2. After 24 hours, OATP1B1 and OATP1B3 cells were supplemented with 0.220 mg/mL (2mM) sodium butyrate in cell culture medium to increase transporter expression *in vitro*. OAT1, OAT3, OCT1, and OCT2 media was replaced with supplemented DMEM. MATE1 and MATE2-K cells were thawed from cryopreservation and directly seeded onto 96-well plates at a density of $1x10^5$ cells/mL in DMEM supplemented with glucose, MEM non-essential amino acids (5%), fetal bovine serum (10%), and penicillin-streptomycin (0.89%). Cells were kept under the same conditions as described above and after 24 hours were supplemented with sodium butyrate (2 mM) to increase transporter expression.

HEK-293 inhibition assays. Inhibition of OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2, MATE1, and MATE2-K in transfected and control HEK-293 cells was carried out as previously described (Cui et al., 2001; Hagenbuch and Meier, 2004; Kimua, 2005; Feng et al., 2008) with some modifications. Briefly, cells were washed once with HBSS (pH 7.4) and then HBSS containing ketoconazole, itraconazole, hydroxy-itraconazole, keto-itraconazole, *N*-desalkyl itraconzole, clarithromycin, ritonavir, or the solvent control (DMSO) was added for 15 min. Inhibitor solutions were removed and replaced with HBSS containing the inhibitors (or solvent control, DMSO) and probe substrate for the designated time. Substrate solutions were removed and cells were washed once with 0.2% BSA in PBS and two times with 1x PBS. After the final wash, cells were lysed with 0.1N NaOH for liquid scintillation analysis. Inhibitor concentration ranges used are summarized in Supplemental Table 1. Probe substrates and inhibitors were as follows; OATP1B1 and OATP1B3: [^{3}H]-estradiol-17β-glucuronide and rifampin (50 nM and 10 μM, respectively), OAT1: [^{3}H]-*p*-aminohippurate and probenecid (1μM and 100 μM, respectively), OAT3:[^{3}H]-estrone-3-sulfate and probenecid (50 nM and 100 μM, respectively),

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OCT1: [^{14}C]-tetraethylammonium bromide and quinidine (5 μM and 300 μM, respectively), OCT2: [^{14}C]-metformin and quinidine (10 μM and 300 μM , respectively), MATE1 and MATE2-K: [^{14}C]-metformin and cimetidine (10 μM and 1000 μM , respectively). For all assays, following the 15 min preincubation with inhibitor, the inhibitor solution was aspirated and fresh inhibitor, plus the substrate was added and incubated for 2 min, with the exception of OAT1 and OCT1, which were incubated for 1 min and 15 min, respectively. For each inhibitor, three assays were performed in triplicate. IC_{50} values were determined from the average percent (%) inhibition values from each experiment ± the standard error of the measurement as calculated in GraFit (version 7.0.2).

Vesicle inhibition assays. Inhibition of efflux of probe substrates into membrane vesicles expressing P-gp, BCRP, MRP2, MRP3, and BSEP were carried out according to manufacturer's instructions with some modifications. Briefly, vesicle membrane suspensions were added to a 96-well plate stored on ice. Incubation media containing ketoconazole, itraconazole, hydroxy-itraconzaole, keto-itraconazole, *N*-desalkyl-itraconazole, clarithromycin, or ritonavir was added to the plate and incubated for 15 min. Substrate solutions containing either MgATP or MgAMP and probe substrate were added to the plate for the designated time. The incubation was ended by the addition of ice-cold wash mix. The sample solution was transferred to a filter plate and washed five times with wash mix. Plates were allowed to dry at room temperature for approximately 1 hr, after which scintillation cocktail was added to the filter plate wells and incubated for 1 hr prior to analysis by liquid scintillation. Inhibitor concentration ranges used are summarized in Supplemental Table 2. Probe substrates and inhibitors were as follows; P-gp: N-methylquinidine and verapamil (0.5 .μM and 60 μM,respectively), BCRP: [^{3}H]-estrone-3-sulfate and Ko143 (1 μM and 1 μM, respectively) MRP2 and MRP3: [^{3}H]-estradiol-17β-glucuronide

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and benzbromarone (50 nM and 100 µM, respectively) BSEP: [^{3}H]-taurocholic acid and cyclosporine (0.4 µM and 20 µM, respectively). Following the 15 min preincubation time with the inhibitor, the probe substrate was added for 3 min (P-gp), 1 min (BCRP), or 5 min (MRP2, MRP2, and BSEP). For each inhibitor, two assays were performed in triplicate. IC_{50} values were determined from the average percent (%) inhibition values from each experiment ± the standard error of the measurement as calculated in GraFit (version 7.0.2).

Analytical methods. Analyst Instrument Control and Data Processing Software (AB SCIEX, version 1.6.1) was used to analyze the unlabeled probe substrate in P-gp vesicle assays (N-methylquinidine, NMQ) for data collection and integration, which were then processed with Microsoft Excel 2007 (Microsoft, Redmond, WA). Calibration standards were employed to calculate concentration based on analyte/internal standard peak-area ratios with Analyst Instrument Control and Data Processing Software (AB SCIEX, version 1.6.1). A Shimadzu API 4000 mass spectrometer in positive mode (4500 V) was employed in tandem with a Waters Atlantis (dC18, 5 µm, 100 x 2.1 mm) column (at 40°C) and Luna C8 guard column (4.0 x 2.0 mm) for separation in a mobile phase of 0.2% formic acid in water and 0.2% formic acid in methanol and an injection volume of 1 µL. Mobile phase flow rate was 0.6 mL/min and the mass transitions used to identify NMQ were m/z = 339.1 and 339.3. Deuterated NMQ (d_3-NMQ) was used as an internal standard and identified with mass transitions of m/z = 342.1 and 342.3.

Analysis of all other samples in the HEK-293 inhibition and vesicle inhibition assays which were incubated with radioactive compounds was carried out with a MicroBeta^2 liquid scintillation counter (Perkin Elmer) according to manufacturer's instructions.

Data analysis. All data processing and statistical analyses were conducted with Microsoft Excel 2010 (Microsoft, Redmond, WA). As described above, IC_{50} values were determined from the

average percent (%) inhibition values from each experiment ± the standard error of the measurement. Non-linear fitting and determination of IC_{50} values were conducted with GraFit 7.0.2 (Erithacus Software Ltd., Horley, Surrey, UK). Calculation of R-values, $[I]_1$ $(C_{max,t})/IC_{50}$, Unbound C_{max}/IC_{50} and $[I]_2/IC_{50}$ values were calculated as described in the 2012 FDA guidance document (FDA, 2012). Equations are available in the *supplemental materials* (Supplemental Equations 1).

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RESULTS

IC_{50} determinations. A summary of the inhibitory effects of ketoconazole, clarithromycin, ritonavir, itraconazole (and its CYP3A4-inhibitory metabolites) towards OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2, MATE1 and MATE2-K in over-expressing HEK-293 cell lines are shown in Table 1, with individual bar graphs shown in *supplemental materials* (Supplemental Figures 1 – 8). Similarly, a summary of the inhibitory effects of these same compounds towards P-gp, BCRP, MRP2, MRP3 and BSEP in transporter-expressing membrane vesicles are also shown in Table 1, with individual bar graphs shown in *supplemental materials* (Supplemental Figures 9 – 11). Positive control inhibitors were used is each experiment in both test systems and the results are located in Supplemental Figure 12. At the substrate concentrations used in these assays (Supplemental Tables 1 and 2), which are low relative to their K_m values (i.e., ~0.05-10% of the K_m), the IC_{50} values are expected to be similar to the extrapolated K_i values assuming competitive inhibition.

Under the conditions employed in this study, clarithromycin, itraconazole and N-desalkyl itraconazole inhibited the fewest transporters to such an extent that IC_{50} values could be determined, whereas ketoconazole and ritonavir inhibited the most transporters evaluated in this study (Table 1). Nearly all transporters examined were inhibited by at least one drug or metabolite, with the notable exceptions of MRP2 and MRP3, which were not inhibited by any of the compounds, and MATE2-K, inhibited only by ritonavir.

In vitro to in vivo extrapolation with the FDA's basic or static models: To put these IC_{50} values into clinical context, the basic model approaches outlined by the FDA in their 2012 Guidance (FDA, 2012) and described in detail within the *supplemental materials* (Supplemental

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Equations 1) were utilized. These equations were applied as follows: 1) Hepatic uptake and efflux transporters: $[I]_1/IC_{50}$ for P-gp, BCRP, BSEP, MRP2, MRP3, OATP1B1, OATP1B3, and OCT1 (Figure 1A), 2) calculation of R-values for hepatic uptake transporters (i.e., OATP1B1 and 1B3) (Figure 1B), 3) Renal and/or blood-brain barrier uptake and efflux transporters: unbound C_{max}/IC_{50} for OAT1, OAT3, OCT1, OCT2, MATE1, MATE2-K, P-gp and BCRP (Figure 1C), 4) Intestinal efflux transporters: $[I]_2/IC_{50}$ (Figure 1D). The clinical C_{max} values that were used in the calculations are shown in Table 2, with calculated R-values, etc. shown in Table 3 and Figure 1A-D.

For hepatic P-gp, BCRP, BSEP, OATP1B1, OATP1B3, and OCT1 the majority of compounds examined for which IC_{50} values were determined have $[I]_1/IC_{50}$ values > 0.1 (Table 3, Figure 1). Particularly high $[I]_1/IC_{50}$ values (i.e., > 100-fold the 0.1 cut-off) include ketoconazole for OCT1 (21.0), ritonavir for P-gp (14.7), itraconazole for P-gp (24.1) and hydroxy-itraconazole for OCT1 (55.3). The sum of $[I]_1/IC_{50}$ values for itraconazole and its metabolites was >100-fold the cut-off value for P-gp, BSEP, and OCT1. Calculation of R-values for hepatic uptake predicted that only ketoconazole and clarithromycin would affect OATP1B1 and 1B3 (i.e., R-values >1.25), with values ranging from 1.4 – 3.5 (Table 3, Figure 1). For basic *in vitro* to *in vivo* extrapolation for inhibition of renal OAT1, OAT3, OCT1, OCT2, MATE1 and MATE2-K, ketoconazole was determined to have unbound C_{max}/IC_{50} values > 0.1 for all but OAT1 (Table 3, Figure 1). For the basic in vitro to in vivo extrapolation for inhibition of the intestinal efflux transporter P-gp, $[I]_2/IC_{50}$ values were approximately 30- and 50-times the cut-off value of 10 for ketoconazole and clarithromycin, respectively. Ritonavir and itraconazole had particularly high values toward P-gp at approximately 230- and 1200-times the cut-off value. For the basic *in vitro* to *in vivo* extrapolation for inhibition of P-gp and BCRP at the blood-brain-

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barrier, $[I]_{1,unbound}/IC_{50}$ values were greater than the 0.1 cut-off value only for ritonavir and itraconazole towards P-gp (Table 3, Figure 1).

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DISCUSSION

As previously reviewed by Ke and colleagues (2014), of the approximately 20 strong CYP3A4/5 inhibitors (i.e., midazolam AUCR ≥5), the majority are unsuitable as clinical alternatives to ketoconazole because 1) they are not approved drugs in the US, 2) are known to be non-specific CYP3A4/5 inhibitors, 3) have significant safety issues, 4) are used exclusively in combination with ritonavir, or 5) produce only moderate CYP3A4/5 inhibition. The remaining single-drug alternatives include clarithromycin, ritonavir and itraconazole (Ke et al., 2014; Greenblatt and Harmatz, 2015); therefore, the data presented here were limited to a comparison of these drugs with ketoconazole regarding their impact on the most important drug transporters. The major metabolites of itraconazole were also included because 40-50% of the overall impact on CYP3A4/5 after multiple dosing is attributable to these metabolites (Ke et al., 2014).

In the current study, experiments were designed in such a manner that would allow clinical investigators to make informed decisions regarding which ketoconazole alternatives would be least likely to cause transporter-based interactions in clinical DDI studies. In order to do this, an extensive query of the University of Washington Metabolism and Transport Drug Interaction Database (UW DIDB) was first undertaken (University of Washington, 2015). The DIDB was developed by the University of Washington's Department of Pharmaceutics, School of Pharmacy with input from pre-clinical and clinical pharmaceutical scientists and is a large curated collection of in vitro and in vivo human drug-drug interaction data. The DIDB integrates experimental conditions and results of DDI studies from peer-reviewed journal articles, FDA Prescribing Information and NDA Reviews. The goals of these data analyses were threefold: 1) to define the range of observed plasma C_{max} values for ketoconazole, clarithromycin, ritonavir and itraconazole (and its CYP3A4-inhibitory metabolites) observed in clinical DDI studies

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designed to quantify the effects of CYP3A4/5 inhibition on victim drugs, 2) to define the range of *in vitro* IC_{50} or K_i values determined for the transporters to be evaluated in this study (if reported), and 3) to define a range of *in vitro* concentrations to be used that would encompass these values.

To put the IC_{50} values reported in Table 1 into clinical context, the basic static model approach outlined by the FDA in their 2012 Guidance (FDA, 2012) was used for data analysis. The majority of compounds examined exhibited IC_{50} values where the $[I]_1/IC_{50}$ values >0.1 for hepatic P-gp, BCRP, BSEP, OATP1B1, OATP1B3, and OCT1; however, no clinically significant interactions were predicted for MRP2 or MRP3 for the majority of compounds examined. Notable exceptions include clarithromycin for BCRP, BSEP, and OCT1; itraconazole for OATP1B1 and 1B3, and keto-itraconazole for OATP1B1 and 1B3. Particularly high $[I]_1/IC_{50}$ values were observed with ketoconazole and hydroxy-itraconazole for OCT1. The sum of $[I]_1/IC_{50}$ values for ketoconazole and itraconazole and its metabolites was >100-fold the cut-off value for OCT1. Although few reports show that the pharmacokinetics of OCT1 substrates are altered by OCT1 inhibitors (Cho et al., 2014; Müller 2015), the high $[I]_1/IC_{50}$ values for OCT1 reported here may be clinically relevant for some substrates of OCT1. For instance, verapamil was found to have an IC_{50} value of 12.5 μM towards OCT1-mediated metformin transport, with an $[I]_1/IC_{50}$ value of only 0.048 (Ahlin et al., 2011; University of Washington, 2015). Yet, coadministration of verapamil with metformin lowered the area under the glucose concentration–time curve by ~240%, likely due to decreased OCT1-mediated hepatic uptake of metformin (Cho et al., 2014). The clinical relevance of itraconazole and ritonavir inhibition of P-gp appears to be limited to interactions that occur in the intestine (University of Washington, 2015).

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Calculation of R-values for hepatic uptake predicted that only ketoconazole and clarithromycin would affect OATP1B1 and 1B3 (i.e., R-values >1.25). Of the drugs considered as *in vivo* OATP1B1 or 1B3 substrates in the FDA's 2012 Guidance (FDA, 2012), only bosentan is a victim of a DDI with ketoconazole, with a 122% increase in exposure (University of Washington, 2015). However, because bosentan is also a CYP3A4/5 substrate, it is possible that much of the effect is due to inhibition of CYP3A4/5 in this case. Unlike ketoconazole, and consistent with its higher R-values for OATP1B1 and 1B3, clarithromycin causes clinically significant interactions with OATP substrates such as bosentan, glyburide, and pravastatin, amongst others (Jacobson 2004; Lilja et al., 2007; Markert et al., 2014; University of Washington, 2015). Although CYP3A4/5 plays some role in the metabolism of all of these substrates, clarithromycin is a weaker CYP3A4/5 inhibitor *in vivo* than ketoconazole, and the change in midazolam clearance with clarithromycin does not correlate with the change in bosentan clearance (Markert et al., 2014). Therefore, at least some of the interactions can be attributed to inhibition of OATP1B1 by clarithromycin.

In the basic model for inhibition of renal OAT1, OAT3, OCT1, OCT2, MATE1 and MATE2-K, ketoconazole was determined to have unbound C_{max}/IC_{50} values >0.1 for all but OAT1. However, a survey of the UW DIDB showed that of these transporters, only MATE1 has been mentioned as possibly being inhibited to a clinically significant extent by ketoconazole and only hepatic canalicular MATE1 inhibition is implicated in the interaction between ketoconazole and atecegaran metoxil (AZD0837) (Matsson et al., 2013; University of Washington, 2015). Ritonavir and itraconazole had unbound C_{max}/IC_{50} values >0.1 only for OCT1, whereas clarithromycin did not have any values >0.1. A survey of the UW DIDB did not reveal any mention of OCT1 interactions with ritonavir or clarithromycin in clinical studies

(University of Washington, 2015). Ketoconazole and hydroxy-itraconazole had a particularly high unbound C_{max}/IC_{50} value for OCT1. The sum of unbound C_{max}/IC_{50} values for itraconazole and its metabolites toward OCT1 is nearly 5-fold higher than the 0.1 cut-off value and 7-fold higher for ketoconazole. As mentioned above, inhibition of OCT1 could be implicated in interactions with drugs such as metformin.

For the basic model for inhibition of the intestinal efflux transporter P-gp, $[I]_2/IC_{50}$ values were approximately 30- and 50-times the cut-off value of 10 for ketoconazole and clarithromycin, respectively. Ritonavir and itraconazole had particularly high values toward P-gp at approximately 230- and 1200-times the cut-off value. Consistent with these findings, the clinical relevance of clarithromycin, ketoconazole, itraconazole and ritonavir inhibition of intestinal P-gp has been described for substrates with minimal CYP3A4/5 contribution, including fexofenadine, quinidine and digoxin, among others (Jalava et al., 1997; Kaukonen et al., 1997; Gurley et al., 2008; Tateishi et al., 2008; Kirby et al., 2012; University of Washington, 2015). Intestinal BCRP was not predicted to be inhibited by clarithromycin, whereas ketoconazole, ritonavir and itraconazole had calculated $[I]_2/IC_{50}$ values toward BCRP that were approximately 25-, 8- and 30-fold the cut off value of 10. However, a survey of the UW DIDB did not find any mention of BCRP with these drugs in clinical studies (University of Washington, 2015). However, as reported by the International Transporter Consortium, clinically relevant inhibition of drug efflux at the blood-brain-barrier is unlikely to occur (Kalvass et al., 2013).

One limitation to the methods used to determine these IC_{50} values is the potential for nonspecific binding of the inhibitors in the test system. This is acknowledged in recent industry and academic publications, but there is currently no standardized or agreed upon methodology within the field for accurately determining the unbound concentrations of inhibitor in the system.

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As such, the experimental design for these assays utilized a consistent design including a pre-incubation with the inhibitor (as described in *Materials and Methods*) and is similar to recent publications (Brouwer et al., 2013; Izumi et al., 2013; Izumi et al., 2015; Shitara et al., 2013; Taub et al., 2011; Zamek-Gliszczynski et al., 2013).

The data presented here attempt to put the *in vitro* inhibitory potencies into a clinically-meaningful context inasmuch as the inhibitory potencies were evaluated in relation to the anticipated clinical exposures in various tissues throughout the body. The results indicate that, like ketoconazole, the alternative clinical CYP3A4/5 inhibitors ritonavir, clarithromycin and itraconazole each have unique transporter inhibition profiles. Unfortunately, none of the alternatives to ketoconazole provided a clean inhibition profile towards the 13 drug transporters evaluated. While many of these findings may not result in clinically-significant drug interactions, several transporters are potentially inhibited at clinically-relevant concentrations. In clinical studies designed to evaluate the extent of CYP3A4/5 metabolism ($f_{m,CYP3A4/5}$) on the clearance of a drug candidate, it is oftentimes assumed that the extent of interaction with other mechanisms of clearance (*i.e.* other metabolic enzymes and/or transporters) is minimal unless reported otherwise (*e.g.* ritonavir inhibition of P-gp intestinal efflux) (FDA, 2012). However, if the pharmacokinetic profile of a drug candidate (e.g. absorption, distribution or elimination) has the potential to be influenced by a transporter then cross-inhibition by these clinical CYP3A4/5 inhibitors may confound the results of clinical studies by altering pharmacokinetics through mechanisms other than CYP3A4/5 inhibition.

In conclusion, the current study provides a comprehensive *in vitro* evaluation of the inhibitory effects of ketoconazole and its potential replacement with strong clinical CYP3A4/5 inhibitors against 13 drug transporters that are key functional mediators of drug absorption,

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distribution and excretion. The results presented here may aid in the selection of clinical CYP3A4/5 inhibitors, especially when drug transporters are also potential mediators of a drug's pharmacokinetics.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Vermeer, Isringhausen, Ogilvie and Buckley.

Conducted experiments: Vermeer and Isringhausen.

Contributed new reagents or analytic tools: NA

Performed data analysis: Buckley, Ogilvie, Vermeer and Isringhausen.

Wrote or contributed to the writing of the manuscript: Vermeer, Isringhausen, Ogilvie and Buckley.

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FOOTNOTES

Authors Lydia M.M. Vermeer and Caleb D. Isringhausen shared equally in contribution to this manuscript.

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FIGURE LEGENDS

Figure. 1: **Basic and static model DDI predictions based on in vitro IC_{50} values.** In vitro IC_{50} values were incorporated into the basic and static models for transporter inhibition proposed in the FDA's 2012 DDI guidance. The potential for clinically-significant interactions was evaluated for (A) hepatic uptake and efflux transporters with $[I]_1$ ($C_{max,t}$)/IC_{50}, (B) hepatic OATP1B1 and OATP1B3 with the R-value, (C), renal and/or blood-brain barrier uptake and efflux transporters with unbound C_{max}/IC_{50}, and (D) and intestinal efflux transporters with $[I]_2/IC_{50}$.

Table 1: Summary of inhibition results (IC_{50} values) towards various transporters by clinically-relevant CYP3A4/5 inhibitors.

Transporter	IC_{50} (μM)[a]						
	Ketoconazole	Itraconazole	Hydroxy-itraconazole	Keto-itraconazole	*N*-Desalkyl-itraconazole	Clarithromycin	Ritonavir
OATP1B1	1.8 ± 0.2	>10	0.23 ± 0.03	0.29 ± 0.04	>0.2	5.3 ± 1.3	0.68 ± 0.17
OATP1B3	3.9 ± 0.6	>10	0.10 ± 0.01	0.088 ± 0.035	>0.2	14 ± 2	2.3 ± 0.4
OAT1	5.7 ± 0.5	>10	>3	>3	>0.2	>50	17 ± 3
OAT3	0.86 ± 0.68	>10	2.0 ± 0.3	>3	>0.2	>50	>30
OCT1	0.13 ± 0.03	0.74 ± 0.24	0.01 ± 0.00	0.04 ± 0.01	>0.2	>50	4.1 ± 0.6
OCT2	0.89 ± 0.35	>10	>3	>3	>0.2	>50	>30
MATE1	0.37 ± 0.03	>10	0.84 ± 0.21	1.1 ± 0.2	>0.2	>50	1.2 ± 0.2
MATE2-K	>2	>10	>3	>3	>0.2	>50	15 ± 2
P-gp	5.6 ± 0.4	0.048 ± 0.04	0.49 ± 0.14	0.12 ± 0.12	0.26 ± 0.05	8.9 ± 0.5	0.24 ± 0.02
BCRP	12 ± 9	1.9 ± 0.3	0.44 ± 0.03	0.10 ± 0.01	>0.2	>50	6.6 ± 0.5
MRP2	>20	>10	>3	>3	>0.2	>50	>30
MRP3	>20	>10	>3	>3	>0.2	>50	>30
BSEP	2.4 ± 0.6	1.8 ± 0.0	1.2 ± 0.8	0.11 ± 0.02	>0.2	59 ± 8	6.1 ± 0.9

[a] Values are displayed to three significant figures, ± standard error of the measurement

Table 2: Clinical parameters for CYP3A4/5 inhibitors used in clinical DDI studies: Dose C_{max} and fraction unbound.

Perpetrator	**Molecular weight** (g/mol)	**Dose and regimen**	**C_{max}** (μM)	**Reference**	**Fraction Unbound** (f_u)	**Reference**
Ketoconazole (high-dose)	531.431	400 mg qd 4 days	2.82	(Olkkola et al., 1994)	0.032	(Kuroha et al., 2002)
Clarithromycin (high-dose)	747.953	500 mg bid 7 days	3.12	(Calabresi et al., 2004)	0.28	(Davey, 1991)
Ritonavir (low-dose)	720.946	100 mg bid 15 days	3.50	(Reddy et al., 2007)	0.02	(Lee et al., 2004)
Itraconazole (capsules)	705.64	100 mg bid 4 days	4.34	(Jaakkola et al., 2005)	0.036	
Itraconazole (oral solution)	705.64		1.15			
Hydroxy-itraconazole	721.63	100 mg qd 7 days	0.608	(Templeton et al., 2008)	0.005	(Templeton et al., 2008)
Keto-itraconazole	719.62		0.023		0.053	
N-Desalkyl-itraconazole	649.53		0.022		0.0012	

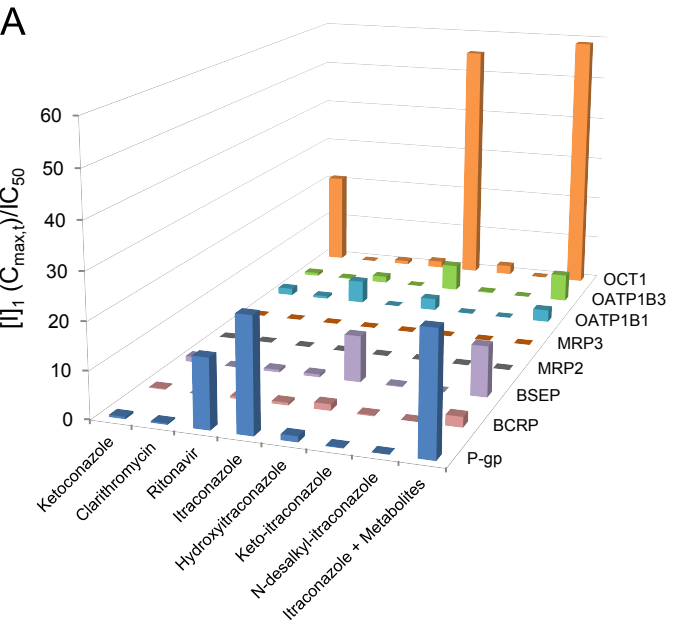
Table 3: DDI predictions based on in vitro IC_{50} values and the FDA's recommended basic and static models.

Transporter	Clinical CYP3A4/5 Inhibitors							
	Ketoconazole	**Clarithromycin**	**Ritonavir**	**Itraconazole**	**Hydroxy-itraconazole**	**Keto-itraconazole**	**N-Desalkyl-itraconazole**	**Itraconazole + Metabolites**
	$[I]_1$ ($C_{max,t}$)/IC_{50} *(≥ 0.1 indicates potential for clinically relevant interaction)*							
P-gp	0.51	0.35	15	24	1.3	0.19	0.0022	25
BCRP	0.23	NC	0.53	0.60	1.4	0.24	NC	2.2
BSEP	1.2	0.053	0.57	0.65	10	0.20	NC	11
MRP2	NC	NC	NC	NC	NC	NC	NC	0.00
MRP3	NC	NC	NC	NC	NC	NC	NC	0.00
OATP1B1	1.6	0.59	5.1	NC	2.7	0.08	NC	2.8
OATP1B3	0.72	0.22	1.5	NC	6.0	0.26	NC	6.2
OCT1	21	NC	0.85	1.6	55	2.1	NC	59
	R-values *(≥ 1.25 indicates potential for clinically relevant interaction)*							
OATP1B1	1.95	3.5	1.2	NC	1.0	1.0	NC	1.03
OATP1B3	1.44	2.0	1.1	NC	1.0	1.0	NC	1.04
	Unbound C_{max}/IC_{50} *(> 0.1 indicates potential for clinically relevant interaction)*							
OAT1	0.016	NC	0.0041	NC	NC	NC	NC	NC
OAT3	0.11	NC	NC	NC	0.0022	NC	NC	0.0022
OCT1	0.67	NC	0.017	0.056	0.28	0.11	NC	0.44
OCT2	0.10	NC	NC	NC	NC	NC	NC	NC
MATE1	0.25	NC	0.060	NC	0.73	0.022	NC	0.75
MATE2-K	NC	NC	0.0046	NC	NC	NC	NC	NC
P-gp	0.016	0.10	0.29	0.87	0.011	0.010	0.00003	0.88
BCRP	0.007	NC	0.010	0.022	0.011	0.013	NC	0.04

$[I]_2/IC_{50}$ *(≥ 10 indicates potential for clinically relevant interaction)*								
P-gp	541	301	2332	11860	NA	NA	NA	NA
BCRP	246	NC	84	294	NA	NA	NA	NA
MRP2	NC	NC	NC	NC	NA	NA	NA	NA

NC: Not calculated. No inhibition observed or the IC_{50} value was greater than the highest concentration tested.
NA: Not applicable. Exposure of intestinal efflux transporters to itraconazole metabolites assumed to be negligible upon oral dosing of itraconazole.
R-value = $1 + (fu * I_{inlet,max}/IC_{50})$; (FDA, 2012; and references within).
$[I]_2$ = dose/250 mL
C_{max} and fraction unbound values listed in Table 2.
Summation of itraconazole and its metabolites adapted from approached used by (Templeton et al., 2010).

Figure 1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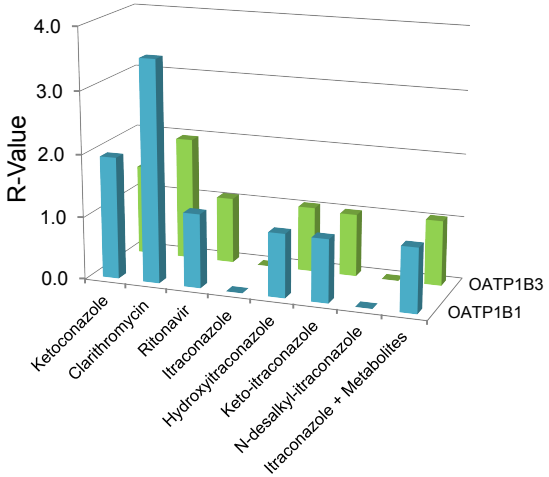
Itraconazole + Metabolites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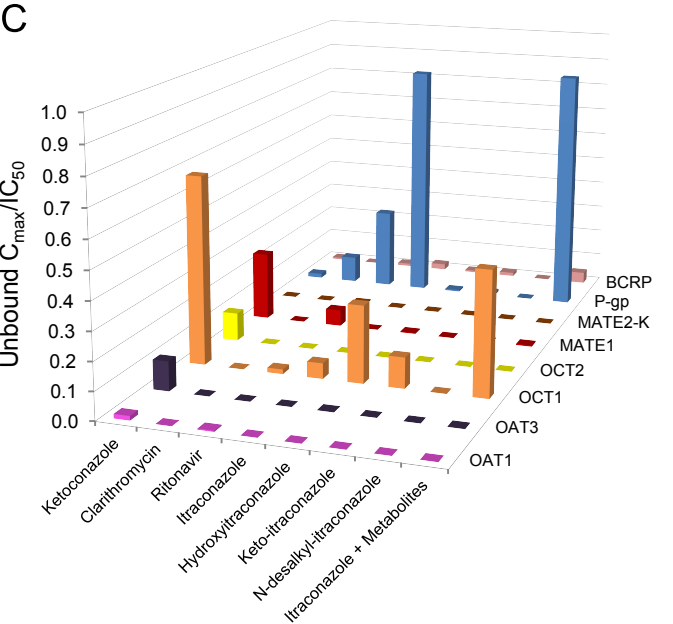
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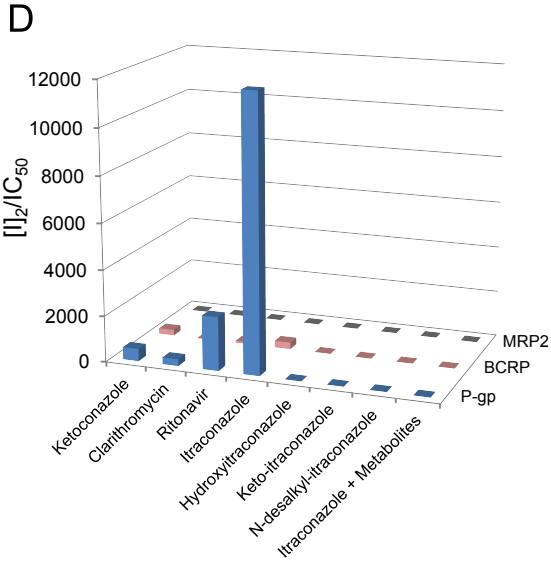
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