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

Lydia M. M. Vermeer, Caleb D. Isringhausen, Brian W. Ogilvie, and David B. Buckley

Sekisui XenoTech, LLC Kansas City, Kansas

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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 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 European Medicines Agency recommended suspension of ketoconazole use in DDI studies in 2013. The FDA specifically recommended use of clarithromycin or itraconazole as alternative strong CYP3A4/5 inhibitors 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) toward 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findings were translated into clinical context with the basic static model approaches outlined by the FDA in its 2012 draft guidance on DDI. 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 toward 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.

Ketoconazole is an orally available, synthetic, broad-spectrum antifungal of the imidazole class that was initially approved by the 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 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 European Medicines Agency 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, 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 nonspecific 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 clinical alternatives.

1L.M.M.V. and C.D.I. contributed equally to this work.

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ABBREVIATIONS: Cmax, maximum plasma concentration; DDI, drug-drug interaction; DMEM, Dulbecco’s modified Eagle’s medium; FDA, Food and Drug Administration; HBSS, Hanks’ balanced salt solution; Ks, substrate concentration that gives a reaction rate equal to half of Vmax; NMQ, N-methylquinidine; UW DIB, University of Washington Metabolism and Transport Drug Interaction Database.
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 interlaboratory differences in reported IC₅₀ values that are dependent on test system, assay conditions, and probe substrates, among other factors. For instance, the reported range of IC₅₀ values for ketoconazole varies 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, IC₅₀ values for ritonavir vary by 85-fold and 117-fold for the inhibition of P-gp and OATP1B1, respectively (Keogh and Kunta, 2006; Herédí-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, hydroxyitraconazole, 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), MRPs (ABCC2, MRP2, MRPC3, and BSEP (ABCB1)).

Materials and Methods

Chemicals and Reagents. Methanol, estradiol-17β-glucuronide, estrone-3-sulfate, p-aminohippurate, verapamil, Ko143, cyclosporin, benzobromarone, cimetidine, rifampen, quinidine, ritonavir, clarithromycin, sulfate, p-desalkyl itraconazole were purchased from Sigma-Aldrich (St. Louis, MO), [3H]-estradiol-17β-glucuronide, [3H]o-aminohippuric acid, and [3H]-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-methylpyridine (NMP) was purchased from Solvo Biotechnologies (Boston, MA). [14C]-tetraethylammonium bromide was purchased from American Radiolabeled Chemicals (St. Louis, MO), and [3H]-metformin was purchased from Moravek Biochemicals (Brea, CA). The 10× Hank’s 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), SLC22A1 (OCT1), and SLC22A2 (OCT2) or empty vector (HEK-293 control) were purchased from American Type Culture Collection (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. Membrane vesicles expressing ABCB1 (BSEP), ABCB2 (MRP2), and ABCB3 (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 7 × 10^4 cells/mL in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum (10%), L-glutamine (10%), and penicillin-streptomycin (0.89%) at 37°C, 95% relative humidity, and 5% CO₂. After 24 hours, OATP1B1 and OATP1B3 cells were supplemented with 0.220 mg/mL (2 mM) sodium butyrate in cell culture medium to increase transporter expression in vitro. OAT1, OAT3, OCT1, and OCT2 media were replaced with supplemented DMEM. MATE1 and MATE2-K cells were thawed from cryopreservation and directly seeded onto 96-well plates at a density of 1 × 10^5 cells/mL in DMEM supplemented with glucose, MEM nonessential 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; Kimura, 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 itraconazole, clarithromycin, ritonavir, or the solvent control (dimethylsulfoxide) was added for 15 minutes. Inhibitor solutions were removed and replaced with HBSS containing the inhibitors (or solvent control, dimethylsulfoxide) and probe substrate for the designated time. Substrate solutions were removed and cells were washed once with 0.2% BSA in PBS and twice with 1× PBS. After the final wash, cells were lysed with 0.1 N 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, [3H]-estradiol-17β-glucuronide and rifampin (50 nM and 10 μM, respectively); OAT1, [3H]-p-aminohippurate and probenecid (1 μM and 100 μM, respectively); OAT3, [3H]-estrone-3-sulfate and probenecid (50 nM and 100 μM, respectively); OCT1, [14C]-tetraethylammonium bromide and quinidine (5 μM and 300 μM, respectively); OCT2, [14C]-metformin and quinidine (10 μM and 300 μM, respectively); and MATE1 and MATE2-K, [3H]-metformin and cimetidine (10 μM and 1000 μM, respectively). For all assays, following the 15-minute preincubation with inhibitor, the inhibitor solution was aspirated and fresh inhibitor plus the substrate was added and incubated for 2 minutes, with the exception of OCT1 and OCT3, which were incubated for 1 minute and 15 minutes, respectively. For each inhibitor, three assays were performed in triplicate. IC₅₀ values were determined from the average percent inhibition values from each experiment ± S.E. 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, MRPs, and BSEP was 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-itraconazole, keto-itraconazole, N-desalkyl-itraconazole, clarithromycin, or ritonavir were added to the plate and incubated for 15 minutes. 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 hour, after which scintillation cocktail was added to the filter plate wells and incubated for 1 hour 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, NMQ, and verapamil (0.5 μM and 60 μM, respectively); BCRP, [3H]-estrone-3-sulfate and Ko143 (1 μM and 1 μM, respectively); MRPs and MRPs, [3H]-estradiol-17β-glucuronide and benzobromarone (50 nM and 100 μM, respectively); and BSEP, [3H]-taurocholic acid and cyclosporine (0.4 μM and 20 μM, respectively). Following the 15-minute preincubation time with the inhibitor, the probe substrate was added for 3 minutes (P-gp), 1 minute (BCRP), or 5 minutes (MRPs, MRPs, and BSEP). For each inhibitor, two assays were performed in triplicate. IC₅₀ values were determined from the average percent inhibition values from each experiment ± S.E. 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 (NMQ) for data collection and integration, which were then processed with Microsoft Excel 2010 (Microsoft, Redmond, WA). Calibration standards were employed to calculate concentration based on analyst/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 × 2.1 mm, Milford, MA) column (at 40°C) and Luna C8 guard column (4.0 × 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 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 that were incubated with radioactive compounds was carried out with a MicroBeta² liquid scintillation counter (Perkin Elmer), according to manufacturer’s instructions.

Data Analysis. All data processing and statistical analyses were conducted with Microsoft Excel 2010. As described above, IC₅₀ values were determined.
from the average percent inhibition values from each experiment ± S.E. of the measurement. Nonlinear fitting and determination of IC50 values were conducted with GraFit 7.0.2 (Erithacus Software, Horley, Surrey, UK). Calculation of R values, [I]1 (maximum plasma concentration [Cmax], t)/IC50, unbound Cmax/IC50, and [I]2/IC50 values was performed as described in the 2012 FDA guidance document (FDA, 2012). Equations are available in (Supplemental Equation 1).

**Results**

**IC50 Determinations.** A summary of the inhibitory effects of ketoconazole, clarithromycin, ritonavir, and itraconazole (and its CYP3A4-inhibitory metabolites) toward OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2, MATE1, and MATE2-K in over-expressing HEK-293 cell lines is shown in Table 1, with individual bar graphs shown in (Supplemental Figs. 1–8). Similarly, a summary of the inhibitory effects of these same compounds toward P-gp, BCRP, MRP2, MRP3, and BSEP in transporter-expressing membrane vesicles is also shown in Table 1, with individual bar graphs shown in (Supplemental Figs. 9–11). Positive control inhibitors were used in each experiment in both test systems, and the results are located in (Supplemental Fig. 12). At the substrate concentrations used in these assays (Supplemental Tables 1 and 2), which are low relative to their Km values (i.e., ~0.05–10% of the Km), the IC50 values are expected to be similar to the extrapolated Ki 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 IC50 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 IC50 values into clinical context, the basic model approaches outlined by the FDA in their 2012 guidance (FDA, 2012) as described in detail (Supplemental Equation 1). These equations were applied as follows: 1) hepatic uptake and efflux transporters: [I]1/IC50 for P-gp, BCRP, BSEP, MRP2, MRP3, OATP1B1, OATP1B3, and OCT1 (Fig. 1A); 2) calculation of R values for hepatic uptake transporters (i.e., OATP1B1 and 1B3) (Fig. 1B); 3) renal and/or blood-brain barrier uptake and efflux transporters: unbound Cmax/IC50 for OAT1, OAT3, OCT1, OCT2, MATE1, MATE2-K, P-gp, and BCRP (Fig. 1C); 4) intestinal efflux transporters: [I]2/IC50 (Fig. 1D).

The clinical Cmax values that were used in the calculations are shown in Table 2 (derived from studies and values shown in Supplemental Table 3), with calculated R values, etc., shown in Table 3 and Fig. 1.

For hepatic P-gp, BCRP, BSEP, OATP1B1, OATP1B3, and OCT1, the majority of compounds examined for which IC50 values were determined have [I]1/IC50 values >0.1 (Fig. 1; Table 3). Particularly high [I]1/IC50 values (i.e., >100-fold the 0.1 cutoff) 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/IC50 values for itraconazole and its metabolites was >100-fold the cutoff 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 to 3.5 (Fig. 1; Table 3). 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 Cmax/IC50 values >0.1 for all but OAT1 (Fig. 1; Table 3). For the basic in vitro to in vivo extrapolation for inhibition of the intestinal efflux transporter P-gp, [I]2/IC50 values were approximately 30 and 50 times the cutoff 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 cutoff value. For the basic in vitro to in vivo extrapolation for inhibition of P-gp and BCRP at the blood-brain barrier, [I]1,unbound/IC50 values were greater than the 0.1 cutoff value only for ritonavir and itraconazole toward P-gp (Fig. 1; Table 3).

**Discussion**

As previously reviewed by Ke and colleagues (2014), of the approximately 20 strong CYP3A4/5 inhibitors (i.e., midazolam AUC ratio ≥5), the majority are unsuitable as clinical alternatives to ketoconazole because they 1) are not approved drugs in the US; 2) are known to be nonspecific 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 in this work 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).

### TABLE 1

<table>
<thead>
<tr>
<th>Transporter</th>
<th>IC50 (μM)²</th>
<th>Ketoconazole</th>
<th>Itraconazole</th>
<th>Hydroxy-itraconazole</th>
<th>Keto-itraconazole</th>
<th>N-Desalkyl-itraconazole</th>
<th>Clarithromycin</th>
<th>Ritonavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1</td>
<td>1.8 ± 0.2</td>
<td>&gt;10</td>
<td>0.23 ± 0.03</td>
<td>0.29 ± 0.04</td>
<td>&gt;0.2</td>
<td>5.3 ± 1.3</td>
<td>0.68 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>OATP1B3</td>
<td>3.9 ± 0.6</td>
<td>&gt;10</td>
<td>0.10 ± 0.01</td>
<td>0.088 ± 0.035</td>
<td>&gt;0.2</td>
<td>14 ± 2</td>
<td>2.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>OAT1</td>
<td>5.7 ± 0.5</td>
<td>&gt;10</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;0.2</td>
<td>&gt;50</td>
<td>17 ± 3</td>
<td></td>
</tr>
<tr>
<td>OAT3</td>
<td>0.86 ± 0.68</td>
<td>&gt;10</td>
<td>2.0 ± 0.3</td>
<td>&gt;3</td>
<td>&gt;0.2</td>
<td>&gt;50</td>
<td>&gt;30</td>
<td></td>
</tr>
<tr>
<td>OCT1</td>
<td>0.13 ± 0.03</td>
<td>0.74 ± 0.24</td>
<td>0.01 ± 0.00</td>
<td>0.04 ± 0.01</td>
<td>&gt;0.2</td>
<td>&gt;50</td>
<td>4.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>OCT2</td>
<td>0.89 ± 0.35</td>
<td>&gt;10</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;0.2</td>
<td>&gt;50</td>
<td>&gt;30</td>
<td></td>
</tr>
<tr>
<td>MATE1</td>
<td>0.37 ± 0.03</td>
<td>&gt;10</td>
<td>0.84 ± 0.21</td>
<td>1.1 ± 0.2</td>
<td>&gt;0.2</td>
<td>&gt;50</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>MATE2-K</td>
<td>&gt;2</td>
<td>&gt;10</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;0.2</td>
<td>&gt;50</td>
<td>15 ± 2</td>
<td></td>
</tr>
<tr>
<td>P-gp</td>
<td>5.6 ± 0.4</td>
<td>0.048 ± 0.04</td>
<td>0.49 ± 0.14</td>
<td>0.12 ± 0.12</td>
<td>0.26 ± 0.05</td>
<td>8.9 ± 0.5</td>
<td>0.24 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>BCRP</td>
<td>12 ± 9</td>
<td>1.9 ± 0.3</td>
<td>0.44 ± 0.03</td>
<td>0.10 ± 0.01</td>
<td>&gt;0.2</td>
<td>&gt;50</td>
<td>6.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>MRP2</td>
<td>&gt;20</td>
<td>&gt;10</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;0.2</td>
<td>&gt;50</td>
<td>&gt;30</td>
<td></td>
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<tr>
<td>MRP3</td>
<td>&gt;20</td>
<td>&gt;10</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;0.2</td>
<td>&gt;50</td>
<td>&gt;30</td>
<td></td>
</tr>
<tr>
<td>BSEP</td>
<td>2.4 ± 0.6</td>
<td>1.8 ± 0.0</td>
<td>1.2 ± 0.8</td>
<td>0.11 ± 0.02</td>
<td>&gt;0.2</td>
<td>59 ± 8</td>
<td>6.1 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

²Values are displayed to three significant figures, ± S.E. of the measurement.
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. 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 UW DIDB was developed by the University of Washington’s Department of Pharmaceutics, School of Pharmacy, with input from preclinical and clinical pharmaceutical scientists and is a large curated collection of in vitro and in vivo human drug-drug interaction data. The UW DIDB integrates experimental conditions and results of DDI studies from peer-reviewed journal articles, FDA Prescribing Information, and New Drug Application Reviews. The goals of these data analyses were threefold, as follows: 1) to define the range of observed plasma $C_{\text{max}}$ values for ketoconazole, clarithromycin, ritonavir, and itraconazole (and its CYP3A4-inhibitory

| Perpetrator Molecular Weight (g/mol) Dose and Regimen $C_{\text{max}}$ (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 (Templeton et al., 2008) |
| Itraconazole (oral solution) 705.64 100 mg qd 7 days 1.15 (Templeton et al., 2008) |
| Hydroxyitraconazole 721.63 0.608 0.005 (Templeton et al., 2008) |
| Keto-itraconazole 719.62 0.023 0.053 (Templeton et al., 2008) |
| N-Desalkylitraconazole 649.53 0.022 0.0012 (Templeton et al., 2008) |
Ketoconazole and the sum of these values for itraconazole and its metabolites show that the pharmacokinetics of OCT1 substrates are altered by OCT1 inhibitors (Cho et al., 2014; Müller et al., 2015), the high [I]/1/IC50 examined exhibited IC50 values where the [I]/1/IC50 values...
For the basic model of inhibition for the intestinal efflux transporter P-gp, $[I]_{50,IC_{50}}$ values were approximately 30 and 50 times the cutoff 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 cutoff 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., 2013; 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 $[I]_{50,IC_{50}}$ values toward BCRP that were approximately 25-, 8-, and 30-fold the cutoff value of 10. However, a survey of the UW DDB 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 currently there is no standardized or agreed upon methodology within the field for accurately determining the unbound concentrations of inhibitor in the system. As such, the experimental design for these assays used a consistent design, including a preincubation with the inhibitor (as described in Materials and Methods), and is similar to recent publications (Taub et al., 2011; Brouwer et al., 2013; Izumi et al., 2013, 2015; Shtara et al., 2013; Zamek-Gliszczynski et al., 2013).

The data presented in this work 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 toward the 13 drug transporters evaluated. Although 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.
Supplemental Materials

“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”

Lydia M.M. Vermeer, Caleb D. Isringhausen, Brian W. Ogilvie, and David B. Buckley
Figure 1 - OATP1B1

Ketoconazole

Hydroxy Itraconazole

Keto Itraconazole

Clarithromycin

Ritonavir
Figure 2 - OATP1B3

- **Ketoconazole**
  - OATP1B3 vs. Control
  - Uptake (pmol/mg) vs. Ketoconazole concentration [μM]

- **Hydroxy Itraconazole**
  - OATP1B3 vs. Control
  - Uptake (pmol/mg) vs. Hydroxy Itraconazole concentration [μM]

- **Keto Itraconazole**
  - OATP1B1 vs. Control
  - Uptake (pmol/mg) vs. Keto Itraconazole concentration [μM]

- **Clarithromycin**
  - OATP1B3 vs. Control
  - Uptake (pmol/mg) vs. Clarithromycin concentration [μM]

- **Ritonavir**
  - OATP1B3 vs. Control
  - Uptake (pmol/mg) vs. Ritonavir concentration [μM]
Figure 3 - OAT1

Ketoconazole

Ritonavir

DMD #67744
Figure 4 - OAT3

[Graph showing the uptake of Ketoconazole and Hydroxy Itraconazole with OAT3 and Control conditions]
Figure 5 - OCT1

- **Ketoconazole**
  - OCT1 vs Control
  - Dose response

- **Itraconazole**
  - OCT1 vs Control
  - Dose response

- **Hydroxy Itraconazole**
  - OCT1 vs Control
  - Dose response

- **Keto Itraconazole**
  - OCT1 vs Control
  - Dose response

- **Ritonavir**
  - OCT1 vs Control
  - Dose response
Figure 6 - OCT2

![Graph showing the uptake of Ketoconazole at different concentrations.](image-url)
Figure 7 - MATE1

- **Ketoconazole**
  - MATE1
  - Control

- **Hydroxy Itraconazole**
  - MATE1
  - Control

- **Keto Itraconazole**
  - MATE1
  - Control

- **Ritonavir**
  - MATE1
  - Control
Figure 8 - MATE2-K

![Bar graph showing the uptake of Ritonavir in MATE2-K and Controludo

Y-axis: Uptake (pmol/mg)

X-axis: Ritonavir [μM]

Legend:
- □ MATE2-K
- □ Control
Figure 9 - P-gp

**MDR1 Inhibition**

- **Ketoconazole [μM]**
  - +ATP
  - +AMP

- **Itraconazole [μM]**
  - +ATP
  - +AMP

- **Hydroxy Itraconazole [μM]**
  - +ATP
  - +AMP

- **Keto Itraconazole [μM]**
  - +ATP
  - +AMP

- **N-Desalkyl Itraconazole [μM]**
  - +ATP
  - +AMP

- **Ritonavir [μM]**
  - +ATP
  - +AMP

**DMD #67744**
Figure 10 - BCRP
Figure 12 - Positive control inhibitors

OATP1B1

OATP1B3

OAT1

OAT3

MATE1

MATE2-K

DMD #67744
Figure 12 cont. - Positive control inhibitors

**OCT1**
- Positive Control Inhibitors
  - OCT1
  - Control
  - Solvent Control, 300 μM Quinidine

**OCT2**
- Positive Control Inhibitors
  - OCT2
  - Control
  - Solvent Control, 300 μM Quinidine

**P-gp**
- Positive Control Inhibitor
  - +ATP
  - +AMP
  - Solvent Control, 100 μM Verapamil

**BCRP**
- Positive Control Inhibitor
  - +ATP
  - +AMP
  - Solvent Control, 1 μM Ko143

**MRP2**
- Positive Control Inhibitor
  - +ATP
  - +AMP
  - 2-[3H]E217pG Uptake (pmol/mg protein)
  - Solvent Control, 100 μM Benz bromarone

**MRP3**
- Positive Control Inhibitor
  - +ATP
  - +AMP
  - 2-[3H]E217pG Uptake (pmol/mg protein)
  - Solvent Control, 100 μM Benz bromarone
Figure 12 cont. - Positive control inhibitors

BSEP

<table>
<thead>
<tr>
<th>Positive Control Inhibitor</th>
<th>+ATP</th>
<th>+AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent Control</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>20 µM Cyclosporin</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

[^]TCA Uptake (pmol/mg protein)
## Supplemental Table 1: Concentration Ranges

<table>
<thead>
<tr>
<th>CYP3A4 inhibitor</th>
<th>OATP1B1</th>
<th>OATP1B3</th>
<th>OAT1</th>
<th>OAT3</th>
<th>OCT1</th>
<th>OCT2</th>
<th>MATE1</th>
<th>MATE2-K</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ketoconazole</strong></td>
<td>0.1, 0.3, 1, 3, 10, 20 µM</td>
<td>0.1, 0.3, 1, 3, 10, 20 µM</td>
<td>0.1, 0.3, 1, 3, 10, 20 µM</td>
<td>0.1, 0.3, 1, 3, 10, 20 µM</td>
<td>0.1, 0.3, 1, 3, 10, 20 µM</td>
<td>0.1, 0.3, 1, 3, 10, 20 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 2 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 2 µM</td>
</tr>
<tr>
<td><strong>Itraconazole</strong></td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
</tr>
<tr>
<td><strong>Hydroxyitraconazole</strong></td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.001, 0.003, 0.1, 0.3, 1, 3 µM</td>
<td>0.001, 0.003, 0.1, 0.3, 1, 3 µM</td>
<td>0.001, 0.003, 0.1, 0.3, 1, 3 µM</td>
<td>0.001, 0.003, 0.1, 0.3, 1, 3 µM</td>
</tr>
<tr>
<td><strong>Keto-itraconazole</strong></td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
</tr>
<tr>
<td><strong>N-desalkyl itraconazole</strong></td>
<td>0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM</td>
<td>0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM</td>
<td>0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM</td>
<td>0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM</td>
<td>0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM</td>
<td>0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM</td>
<td>0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM</td>
<td>0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM</td>
</tr>
<tr>
<td><strong>Clarithromycin</strong></td>
<td>0.3, 1, 3, 10, 30, 50 µM</td>
<td>0.3, 1, 3, 10, 30, 50 µM</td>
<td>0.3, 1, 3, 10, 30, 50 µM</td>
<td>0.3, 1, 3, 10, 30, 50 µM</td>
<td>0.3, 1, 3, 10, 30, 50 µM</td>
<td>0.3, 1, 3, 10, 30, 50 µM</td>
<td>0.3, 1, 3, 10, 30, 50 µM</td>
<td>0.3, 1, 3, 10, 30, 50 µM</td>
</tr>
<tr>
<td><strong>Ritonavir</strong></td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
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</table>
### Supplemental Table 2: Concentration Ranges

<table>
<thead>
<tr>
<th>CYP3A4 inhibitor</th>
<th>Transporter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-gp</td>
</tr>
<tr>
<td><strong>Ketoconazole</strong></td>
<td>0.1, 0.3, 1, 3, 10, 20 µM</td>
</tr>
<tr>
<td><strong>Itraconazole</strong></td>
<td>0.03, 0.1, 0.3, 1, 3, 10 µM</td>
</tr>
<tr>
<td><strong>Hydroxyitraconazole</strong></td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
</tr>
<tr>
<td><strong>Keto-itraconazole</strong></td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
</tr>
<tr>
<td>N-desalkyl itraconzole</td>
<td>0.001, 0.003, 0.01, 0.03, 0.1, 0.2 µM</td>
</tr>
<tr>
<td><strong>Clarithromycin</strong></td>
<td>0.3, 1, 3, 10, 30, 50 µM</td>
</tr>
<tr>
<td><strong>Ritonavir</strong></td>
<td>0.01, 0.03, 0.1, 0.3, 1, 3 µM</td>
</tr>
</tbody>
</table>
Supplemental Table 3: CYP3A inhibitors in clinical DDI studies

<table>
<thead>
<tr>
<th>Perpetrator</th>
<th>Dose and regimen</th>
<th>$C_{\text{max}}$ (µM)</th>
<th>$C_{\text{max,ss}}$ (µM)</th>
<th>$[I]_{\text{gut}}$ (µM)</th>
<th>Midazolam dose and regimen</th>
<th>Max Midazolam AUCR</th>
<th>Review PMID</th>
<th>Primary PMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole (high-dose)</td>
<td>400 mg qd 4 days</td>
<td>2.82</td>
<td>NR</td>
<td>3010</td>
<td>7.5 mg PO, day 4 +1h</td>
<td>16.7</td>
<td>23584886</td>
<td>8181191</td>
</tr>
<tr>
<td></td>
<td>2.8 – 13.7</td>
<td>2.8 – 13.7</td>
<td>NR</td>
<td></td>
<td></td>
<td></td>
<td>UW DIDB</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>200 mg bid 1.5 to 10 days</td>
<td>7.84 – 19.8</td>
<td>2.37 – 17.7</td>
<td>1505</td>
<td>Various</td>
<td>6.5-13.6-fold</td>
<td>UW DIDB</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>200 mg qd 3 days</td>
<td>3.57</td>
<td>NR</td>
<td></td>
<td>NA</td>
<td>NA (Ke reports 9.2-fold)</td>
<td>UW DIDB</td>
<td>15116057</td>
</tr>
<tr>
<td></td>
<td>200 mg qd 4 to 14 days</td>
<td>5.23-11.84</td>
<td>1.88 – 3.38</td>
<td></td>
<td>Various</td>
<td>5.2-8.7-fold</td>
<td>UW DIDB</td>
<td>NA</td>
</tr>
<tr>
<td>Clarithromycin (high-dose)</td>
<td>500 mg bid 7 days</td>
<td>3.03 – 3.12</td>
<td>2.27 (4 days)</td>
<td>2674</td>
<td>8 mg PO, day 0 and 7</td>
<td>8.4</td>
<td>24747234</td>
<td>16432272</td>
</tr>
<tr>
<td>Clarithromycin (low-dose)</td>
<td>250 mg bid 2.5-4.5 days</td>
<td>1.5-2.35</td>
<td>1.18</td>
<td>1337</td>
<td>15 mg PO</td>
<td>3.6-fold</td>
<td>NA</td>
<td>8880291</td>
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<tr>
<td>Ritonavir (low-dose)</td>
<td>100 mg bid 6 – 150 days</td>
<td>1.03-3.50</td>
<td>NA (0.08 at 100 mg QD, 17 days)</td>
<td>555</td>
<td>Various</td>
<td>23.8-26-fold</td>
<td>UW DIDB</td>
<td>e.g.: 20002087</td>
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<tr>
<td>Itraconazole</td>
<td>200 mg qd 3-42 days</td>
<td>0.28-4.34</td>
<td>0.22-1.92</td>
<td>1134</td>
<td>7.5 mg PO day 4</td>
<td>10.8 (with 200 mg qd 4 days)</td>
<td>UW DIDB</td>
<td>8181191</td>
</tr>
<tr>
<td>Itraconazole (oral solution)</td>
<td>200 mg bid 142 days</td>
<td>2.32</td>
<td>NA</td>
<td>1134</td>
<td>NA</td>
<td>NR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxyitraconazole (oral solution)</td>
<td>200 mg bid 142 days</td>
<td>3.28</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NR</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Itraconazole (oral solution)</td>
<td>100 mg qd 7 days</td>
<td>1.15</td>
<td>NA</td>
<td>567</td>
<td>NR</td>
<td>NR</td>
<td></td>
<td>NA</td>
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<tr>
<td>Hydroxyitraconazole</td>
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<td>0.608</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Keto-itraconazole</td>
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<td>0.023</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>N-desalkyl-itraconazole</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td>NA</td>
</tr>
</tbody>
</table>
Supplemental Equations 1

1. \( R value = 1 + (f_u \times \frac{I_{in,\text{max}}}{IC_{50}}) \)
   Where:
   - \( f_u \): fraction unbound of the inhibitor
   - \( I_{in,\text{max}} \): estimated maximum inhibitor concentration at the inlet to the liver
     - \( I_{in,\text{max}} = C_{\text{max}} + (k_a \times \text{Dose} \times \frac{F_a F_g}{Qh}) \)
     - \( k_a \): absorption rate constant of the inhibitor (assumed 0.1)
     - \( F_a F_g \): fraction of the dose of inhibitor absorbed (assumed 1)
     - \( C_{\text{max}} \): maximum systemic plasma concentration of inhibitor

OATP1B1/ OATP1B3

2. \( \frac{[I]_1}{IC_{50}} \)
   Where:
   \([I]_1\): mean steady state total (free and unbound) \( C_{\text{max}} \) following administration of highest proposed clinical dose of inhibitor

P-gp, BCRP, BSEP, MRP2, MRP3, OATP1B1, OATP1B3, OCT1

3. \( \frac{[I]_2}{IC_{50}} \)
   Where:
   \([I]_2\): Dose of the inhibitor (in mol)/250 mL

P-gp, BCRP, MRP2

4. \( \frac{\text{Unbound } C_{\text{max}}}{IC_{50}} \)

OAT1, OAT3, OCT1, OCT2, MATE1, MATE2-K

5. \( \frac{I_{1,\text{unbound}}}{IC_{50}} \)

P-gp, BCRP