Ketoconazole is a known CYP3A4/5 inhibitor and was previously used in this role for clinical drug-drug interaction (DDI) studies. The FDA and EMA recently recommended the suspension of ketoconazole in clinical DDI studies and suggested that clarithromycin or itraconazole be used. It is well-established that these alternatives are moderate or strong inhibitors of CYP3A4/5, but the effect on drug transporters is limited. Aim: 1) Determine the inhibitory effects of ketoconazole, clarithromycin, ritonavir, and verapamil towards 13 clinically relevant drug transporters. 2) To aid in the selection of clinical CYP3A4/5 inhibitors when drug transporters contribute to a drug candidate’s pharmacokinetics. Methods: HEK293 cell lines or membrane vesicles expressing the transporters were used. A range of concentrations for each inhibitor was used and cells or vesicles were preincubated for 15 min in the presence of the inhibitor and then the probe substrate was added. Samples were analyzed using LSC or LC-MS/MS. Results: Similar to ketoconazole, results indicate that ritonavir, clarithromycin, and itraconazole have differing transporter inhibition profiles. The data demonstrate that no single alternative to ketoconazole indicates a clean inhibition profile towards all 13 drug transporters tested. The results show the majority of the inhibitors tested may interact with one or more transporters in clinical studies (i.e. some values were calculated to be greater than the cutoff values specified by the FDA and EMA). Conclusion: The results provide guidance for the selection of clinical CYP3A4/5 inhibitors when transporters are involved in a drug candidate’s pharmacokinetics.

**Background & Purpose**

Ketoconazole is a only available, synthetic, broad-spectrum, antifungal agent. Approved in 1982 by the FDA for use in fungal infections, it is a known substrate and strong inhibitor of cytochrome P450 (CYP) 3A4 and 3A5. Previously, a high dose of ketoconazole was considered the gold standard for use in clinical drug-drug interaction (DDI) as a strong CYP3A4/5 inhibitor. By 2013, ketoconazole use in clinical studies had been banned due in part to evidence demonstrating the potential for liver injury following long dosing periods. Typically, patients would exhibit asymptomatic, reversible liver function test abnormalities. As early as 1984, Van Tyle demonstrated evidence of DDI in approximately 0.1% to 1% of patients, with results indicating that there was no association with the dose, but with the duration of dosing. In later estimates, studies showed that ~134 per 100,000 persons, 4.9 cases per 10,000 patients, and 3.6 to 4.2% demonstrated liver abnormalities. After ketoconazole was banned in clinical study use, the FDA recommended clarithromycin or itraconazole as an alternative, indicating that the alternative may be used. Ritonavir was suggested by some as an alternative CYP3A4/5 inhibitor. Following an extensive study by Ke et al, where inhibitors were systematically evaluated, only ritonavir, clarithromycin, and verapamil were considered acceptable. Exclusion criteria (included, the drug not being approved in the U.S., known non-specific inhibition of CYP safety issues, exclusive use with ritonavir, or only moderate information of CYP3A4/5). There is minimal drug transporter data in the literature, which includes ketoconazole and hydroxy-itraconazole. In a unique study, the goal of this study was to allow for a more informed choice of a strong CYP3A4/5 inhibitor for clinical DDI studies involving a drug candidate known to be a substrate of one or more of the transporters and to help reduce confounding DDI results.

**Materials & Methods**

Chemicals and Reagents: Methanol, estradiol-17β-glucuronide, estrone-3-sulfate, p-aminophenol, verapamil, Ko143, cyclosporin, benzamidone, cimetidine, rifampin, propranolol, quinidine, ritonavir, cyclosporin, clarithromycin, itraconazole, and ketoconazole were purchased from Sigma-Aldrich (St. Louis, MO). [17β estrone-3-sulfate, [17β estradiol-17β-glucuronide, [17β aminogluconic acid, and [17β-tauronic acid were purchased from Perkin Elmer (Oak Brook, IL), hydroxy-itraconazole, tetrahydro-itaconazole, and 3-desalkyl itraconazole were purchased from Toronto Research Chemicals (Ontario, Canada). N-methylpiperazine was purchased from Novo BioTechnologies (Boston, MA). [17β-tetrahydroammonium bromide was purchased from American Radiolabeled Chemicals (St. Louis, MO). and [17β metformin was purchased from Moravek Biochemicals (Brea, CA). 10x Hanks balanced salt solution (HBSS) was purchased from Invitrogen (Waltham, MA). HEK-293 inhibition assays, inhibition of OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2, MATE1, and MATE2-K in transfected and control HEK-293 cells were performed as previously described with some modifications. Briefly, cells were washed once with HBSS (pH 7.4) and then HBSS containing the inhibitor or solvent control was added for 15 min. Inhibitor solutions were removed and replaced with HBSS containing the inhibitors (or solvent control, DMEM) and probe substrate for the designated time. Substrate solutions were removed and washed once with 0.2% BSA in PBS and twice with 1x PBS. After the final wash, cells were lysed with 0.1% NaOH for liquid scintillation analysis. Vessel inhibition assays. Inhibition of probe substrate 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 ketocaphezone, itraconazole, hydroxy-itraconazole, keto-itraconazole, N-desalkyl-itraconazole, chlorothiazidine, ritonavir were added to the plate and incubated for 10 min. Substrate solutions containing either MgATP or MgMgP and probe substrate were added to the plate for the designated time. The incubation was ended by the addition of 10x cold wash mix. The sample solution was transferred to a filter plate and washed five times with wash mix plates. 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.

**Results**

Table 1. List of inhibitors tested in HEK-293 and membrane vesicles for inhibition profiles of 13 clinically relevant drug transporters

<table>
<thead>
<tr>
<th>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>
<th>Clathrin</th>
<th>Ritonavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>3 x 10^{-6}</td>
<td>&gt;10</td>
<td>0.01 x 0.01</td>
<td>&gt;10</td>
<td>0.66 ± 0.05</td>
<td>&gt;10</td>
<td>&lt;0.2</td>
<td>&gt;50</td>
<td>&gt;0.2</td>
<td>5.3 ± 1.3</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>0.37 ± 0.03</td>
<td>&gt;10</td>
<td>2.94 ± 0.14</td>
<td>&gt;10</td>
<td>&lt;0.2</td>
<td>&gt;50</td>
<td>&gt;0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>&lt;0.2</td>
<td>&gt;50</td>
<td>&gt;30</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

Figure 2. R-value determination for DDI prediction of OATP-mediated hepatic uptake.

Using an FDA cutoff value of 1.25, only ketoconazole and clarithromycin are predicted to affect OATP1B1 and OATP1B3. Ritonavir and itraconazole (and metabolites) demonstrated R-values of 1.25.

**Conclusions**

• None of the alternatives to ketoconazole provided a clean inhibition profile towards all 13 drug transporters evaluated.

• Each alternative to ketoconazole for potential use in a clinical DDI study has a unique transporter inhibition profile (MPR2 and MRP3 were not inhibited by any alternative inhibitors).

• Ritonavir and itraconazole may be the best alternative for CYP3A4/5 substrates which are transported by OATP1B1/1B3.

• CYP3A4 substrates transported by OAT1 may not be affected by any of the tested alternative inhibitors.

• Clarithromycin may be best choice for substrates of renal transporters or substrates of P-gp or BCRP (P-gp may still be affected).

• Overall: The best choice of a strong clinical CYP3A4/5 inhibitor will depend on the unique transporter substrate profile of the drug candidate.

**References**

2. Greenblatt and Greenblatt (2014) JCP 94 1321
4. Ke et al. (2014) CPT 85 473

**Figure 3.** DDI predictions for hepatic uptake and efflux transport using FDA baseline value (IC50) for each transporter: [Ritonavir] (µM)