The In Vitro Evaluation of Ketoconazole and its Alternative Clinical CYP3A4/5 Inhibitors (Ritonavir, Clarithromycin and Itraconazole) as Inhibitors of Non-CYP Enzymes

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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. 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).

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. Because of these observations, the FDA and EMA recommended the complete suspension of marketing authorization on the same day (FDA 2013; EMA 2013; Vermeer et al., 2016 and references within). 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. In addition to itraconazole and clarithromycin, ritonavir has also been suggested by some authors as a possible alternative to ketoconazole (Greenblatt and Harmatz, 2015).

Although the effects of these clinically used CYP3A4/5 inhibitors on other CYPs are largely established, the effects on other non-CYP drug metabolizing enzyme activities could confound the results of clinical DDI studies for some CYP3A4/5 substrates. The purpose of this study was to evaluate the inhibitory effects of ketoconazole, clarithromycin, ritonavir and itraconazole (and its CYP3A4-inhibitory metabolites, hydroxy-, keto- and *N*-desalkyl itraconazole) towards several clinically-relevant non-CYP drug metabolizing enzymes.

Materials & Methods

Chemicals and Reagents. Ritonavir, clarithromycin, itraconazole, and ketoconazole were purchased from Sigma-Aldrich (St. Louis, MO). [hydroxy-itraconazole, keto-itraconazole, and *N*-desalkyl itraconazole were purchased from Toronto Research Chemicals (Ontario, Canada). Pooled human liver microsomes (HLM; n = 200), pooled human liver S9 fractions (n=200) and pooled human liver cytosol (n = 200) were prepared by XenoTech (Kansas City, KS). rUGT enzymes (Corning® Supersomes™) were purchased from Corning Life Sciences (Corning, NY). Substrates, buffer components, cofactors and metabolite standards (listed in **Tables 1 and 2**) were all purchased from commercial sources.

Enzyme inhibition experiments. All experiments were conducted under initial rate conditions (previously determined; internal data) such that the concentration of marker substrate in reaction was at, or below, its experimentally determined K_m value and the rate of metabolite formation was linear for the duration of the marker substrate incubations. Incubation conditions are described in Tables 1 and 2. In summary, a two-concentration inhibition screen was first conducted with each of the seven inhibitors under the conditions described. In cases where inhibition ≥50% was observed, subsequent experiments were conducted to determine the inhibition profile inasmuch as a full IC_{50} determination was conducted with ≥7 concentrations of inhibitor based on findings from the preliminary experiments. All marker substrate reactions were stopped with organic solvent (≥50% v:v organic) containing internal standard, protein precipitated (10 min at 920 RCF) and the supernatant was analyzed for metabolite formation by LC-MS/MS.

Data analysis. All data processing (IC_{50} values) were determined from the average percent (%) inhibition values from each experiment (± the standard error of the measurement) based on the nominal concentration of inhibitor added to the incubation. Non-linear fitting and determination of IC_{50} values were conducted with GraFit 7.0.2 (Erithacus Software Ltd., Horley, Surrey, UK). Calculation of $[I]_1$ ($C_{max,t}$)/IC_{50} and Unbound C_{max}/IC_{50} values were calculated as described in the 2012 FDA guidance document (FDA, 2012).

Table 1. Summary of incubation conditions for the inhibition screen and IC_{50} determinations with human UGT enzymes.

Enzyme	Substrate	[Substrate] µM	Test System (mg/mL protein)	Incubation time (min)	Cofactor	[Alamethicin] (15 min)	Metabolite
UGT1A1	Estradiol	12	HLM (0.1)	5	10 mM UDPGA	25 µg/mg	Estradiol 3-O-glucuronide
UGT1A3	CDCA	25	HLM (0.1)	10	10 mM UDPGA	25 µg/mg	CDCA 24-O-glucuronide
UGT1A4	Trifluoperazine	10	HLM (0.1)	5	10 mM UDPGA	25 µg/mg	Trifluoperane N-glucuronide
UGT1A6	Naphthol	2	HLM (0.005)	5	10 mM UDPGA	25 µg/mg	Napthyl-glucuronide
UGT1A7	4MU	14	rUGT (0.1)	5	10 mM UDPGA	25 µg/mg	4MU-glucuronide
UGT1A8	4MU	730	rUGT (0.1)	5	10 mM UDPGA	25 µg/mg	4MU-glucuronide
UGT1A9	Propofol	5	HLM (0.1)	5	10 mM UDPGA	25 µg/mg	Propofol glucuronide
UGT1A10	4MU	31	rUGT (0.1)	30	10 mM UDPGA	25 µg/mg	4MU-glucuronide
UGT2B4	4MU	1200	rUGT (0.25)	15	10 mM UDPGA	25 µg/mg	4MU-glucuronide
UGT2B7	Morphine	400	HLM (0.1)	5	10 mM UDPGA	25 µg/mg	Morphine 3-O-glucuronide
UGT2B10	Levomedetomidine	3	HLM (0.1)	5	10 mM UDPGA	25 µg/mg	LMTD N-glucuronide
UGT2B15	Oxazepam	20	HLM (0.1)	5	10 mM UDPGA	25 µg/mg	S-Oxazepam glucuronide
UGT2B17	Testosterone	2.5	HLM (0.1)	5	10 mM UDPGA	25 µg/mg	Testosterone 17-O-glucuronide

Table 2. Summary of incubation conditions for the inhibition screen and IC_{50} determinations with various drug metabolizing enzymes.

Enzyme	Substrate	[Substrate] (µM)	Test system (mg/mL protein)	Incubation time (min)	Cofactor	Metabolite
AO	Phthalazine	2	Cytosol (0.1)	10	NA	Phthalazinone
AO	Zaleplon	80	Cytosol (0.25)	30	NA	5-Oxozaleplon
XO	Xanthine	60	Cytosol (0.5)	60	NA	Uric acid
FMO	Benzydamine	50	HLM (0.1)	5	NADPH-RS	Benzydamine N-oxide
CES1	Clopidogrel	15	Cytosol (0.1)	10	NA	Clopidogrel acid
CES2	Methylprednisolone 21-hemisuccinate	150	Cytosol (0.1)	10	NA	Methylprednisolone
NAT1	4-Aminobenzoic acid	15	Cytosol (0.5)	10	0.1 mM Acetyl-CoA	N-acetyl-p-aminobenzoic acid
NAT2	Sulfamethazine	65	Cytosol (0.2)	10	0.1 mM Acetyl-CoA	N-acetyl-sulfamethazine
MAO-A	5-Hydroxytryptamine	50	Mitochondria (0.1)	5	NA	5-Hydroxytryptophol
MAO-B	4-DMBA	30	Mitochondria (0.1)	5	NA	4-dimethylaminobenzaldehyde
SULTs	7-Hydroxycoumarin	2.5	L+I HS9 (0.1)	5	0.2 mM PAPS	7-Hydroxycoumarin sulfate
GSTs	1-Chloro-2,4-dinitrobenzene	100	Cytosol (0.02)	3	1 mM GSH	1-(Glutathione-S-yl), 2-4-dinitrobenzene
COMT	Dopamine	110	Cytosol (0.25)	10	0.25 mM SAM	3-Methoxytyramine

Results

Table 3. Summary of inhibition results (IC_{50} values) towards UGTs by clinically-relevant CYP3A4/5 inhibitors.

IC_{50} (µM)

Enzyme	Ketoconazole	Itraconazole	Hydroxy-itraconazole	Keto-itraconazole	N-Desalkyl-itraconazole	Clarithromycin	Ritonavir
UGT1A1	6.92 ± 0.44	0.940 ± 0.147	5.07 ± 0.65	5.73 ± 0.99	25.0 ± 2.8	>100	2.22 ± 0.08
UGT1A3	10.5 ± 1.6	>100	>300	>300	32.7 ± 15.1	>100	1.19 ± 0.19
UGT1A4	7.16 ± 1.22	0.854 ± 0.331	2.30 ± 0.46	0.881 ± 0.329	1.00 ± 0.26	>100	1.05 ± 0.08
UGT1A6	45.7 ± 5.3	97.4 ± 18.1	>300	>300	>25	>100	>100
UGT1A7	7.86 ± 0.50	>100	40.4 ± 11.5	>300	>25	>100	43.8 ± 4.3
UGT1A8	>100	>100	>300	>300	>25	43.5 ± 2.5	9.66 ± 2.67
UGT1A9	3.25 ± 0.63	>100	2.22 ± 0.26	3.73 ± 1.03	11.3 ± 2.0	>100	37.4 ± 4.5
UGT1A10	20.4 ± 3.5	>100	63.0 ± 26.7	>300	>25	>100	22.6 ± 1.7
UGT2B4	11.8 ± 1.6	>100	>200	>200	>25	>100	>100
UGT2B7	12.1 ± 1.3	>100	>300	>300	>25	>100	32.3 ± 5.6
UGT2B10	43.8 ± 10.4	>100	>300	>300	>25	>100	>100
UGT2B15	5.22 ± 1.01	>100	21.6 ± 8.6	>300	14.5 ± 3.5	>100	21.1 ± 1.3
UGT2B17	2.65 ± 0.13	>100	8.27 ± 0.97	>300	23.2 ± 5.45	>100	7.27 ± 0.74

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Table 4. Summary of inhibition results (IC_{50} values) towards various drug metabolizing enzymes by clinically-relevant CYP3A4/5 inhibitors.

IC_{50} (µM)

Enzyme	Ketoconazole	Itraconazole	Hydroxy-itraconazole	Keto-itraconazole	N-Desalkyl-itraconazole	Clarithromycin	Ritonavir
AO (P^1,0 min)	3.00 ± 0.26	>100	NI	NI	NI	63.7 ± 6.1	28.7 ± 4.4
AO (P^1, 30 min)	3.87 ± 0.28	>100	NI	NI	NI	61.7 ± 6.4	37.5 ± 6.1
AO (Z^2, 0 min)	3.81 ± 0.24	>100	NI	NI	NI	>100	43.3 ± 6.8
AO (Z^2, 30 min)	5.39 ± 0.34	>100	NI	NI	NI	>100	42.6 ± 5.3
XO	NI	NI	NI	NI	NI	NI	NI
FMO	NI	NI	NI	NI	NI	NI	NI
CES1	NI	NI	NI	NI	NI	NI	NI
CES2	>100	NI	NI	NI	NI	NI	>100
NAT1	NI	NI	NI	NI	NI	NI	NI
NAT2	>100	NI	NI	NI	>25	NI	NI
MAO-A	8.33 ± 1.30	NI	NI	NI	NI	NI	NI
MAO-B	5.95 ± 0.79	50.8 ± 9.0	>60	>60	6.28 ± 0.57	58.8 ± 9.0	NI
SULTs	NI	NI	NI	NI	NI	NI	NI
GSTs	>100	NI	NI	NI	NI	NI	NI
COMT	NI	NI	NI	NI	NI	NI	NI

Table 5. 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	3.5	(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)		
Hydroxy-itraconazole	721.63		0.608		0.005	
Keto-itraconazole	719.62		0.023		0.053	
N-Desalkyl-itraconazole	649.53		0.022		0.0012	

Table 6. DDI predictions based on a basic, static model approach calculated with total inhibitor C_{max} and in vitro IC_{50} values.

$[I]$ ($C_{max,\ t}$) / IC_{50}

Enzyme	Ketoconazole	Itraconazole	Hydroxy-itraconazole	Keto-itraconazole	N-Desalkyl-itraconazole	Clarithromycin	Ritonavir
UGT1A1	0.41	4.6	0.12	0.004	0.00088	NC	1.6
UGT1A3	0.27	NC	NC	NC	0.00067	NC	2.9
UGT1A4	0.39	5.1	0.26	0.0261	0.022	NC	3.3
UGT1A6	0.062	0.045	NC	NC	NC	NC	NC
UGT1A7	0.36	NC	0.015	NC	NC	NC	0.08
UGT1A8	NC	NC	NC	NC	NC	0.072	0.36
UGT1A9	0.87	NC	0.27	0.0062	0.0019	NC	0.09
UGT1A10	0.14	NC	0.01	NC	NC	NC	0.15
UGT2B4	0.24	NC	NC	NC	NC	NC	NC
UGT2B7	0.23	NC	NC	NC	NC	NC	0.11
UGT2B10	0.064	NC	NC	NC	NC	NC	NC
UGT2B15	0.54	NC	0.028	NC	0.0015	NC	0.17
UGT2B17	1.1	NC	0.074	NC	0.00095	NC	0.48
AO[1]	0.94	NC	NC	NC	NC	0.049	0.12
MAO-A	0.34	NC	NC	NC	NC	NC	NC
MAO-B	0.47	0.085	NC	NC	0.0035	0.053	NC

Yellow: $[I]/IC_{50} \geq 0.1$ and ≤ 1.0
Red: $[I]/IC_{50} \geq 1.0$
NC: Not calculated. No inhibition observed or the IC_{50} value was greater than the highest concentration tested.
C_{max} and fraction unbound values listed in Table 5. [1]Aldehyde oxidase substrate was phthalazine without preincubation.

Table 7. DDI predictions based on a basic, static model approach calculated with unbound inhibitor C_{max} and in vitro IC_{50} values.

$[I]$ ($C_{max,u}$) / IC_{50}

Enzyme	Ketoconazole	Itraconazole	Hydroxy-itraconazole	Keto-itraconazole	N-Desalkyl-itraconazole	Clarithromycin	Ritonavir
UGT1A1	0.013	0.17	0.0006	0.00021	0.0000011	NC	0.032
UGT1A3	0.0086	NC	NC	NC	0.00000081	NC	0.059
UGT1A4	0.013	0.18	0.0013	0.0014	0.000026	NC	0.067
UGT1A6	0.002	0.0016	NC	NC	NC	NC	NC
UGT1A7	0.011	NC	0.000075	NC	NC	NC	0.0016
UGT1A8	NC	NC	NC	NC	NC	0.02	0.0072
UGT1A9	0.028	NC	0.0014	0.00033	0.0000023	NC	0.0019
UGT1A10	0.0044	NC	0.000048	NC	NC	NC	0.0031
UGT2B4	0.0076	NC	NC	NC	NC	NC	NC
UGT2B7	0.0075	NC	NC	NC	NC	NC	0.0022
UGT2B10	0.0021	NC	NC	NC	NC	NC	NC
UGT2B15	0.017	NC	0.00014	NC	0.0000018	NC	0.0033
UGT2B17	0.034	NC	0.00037	NC	0.0000011	NC	0.0096
AO[1]	0.03	NC	NC	NC	NC	0.014	0.0024
MAO-A	0.011	NC	NC	NC	NC	NC	NC
MAO-B	0.015	0.0031	NC	NC	0.0000042	0.015	NC

Yellow: $[I]/IC_{50} \geq 0.1$ and ≤ 1.0
Red: $[I]/IC_{50} \geq 1.0$
NC: Not calculated. No inhibition observed or the IC_{50} value was greater than the highest concentration tested.
C_{max} and fraction unbound values listed in Table 5. [1]Aldehyde oxidase substrate was phthalazine without preincubation.

Conclusions

- Several UGT enzymes were inhibited by ketoconazole, itraconazole and ritonavir with one or more $[I]_{tot}/IC_{50}$ values > 1.0. When evaluated based on f_u from plasma protein binding, only itraconzole resulted in $[I]_u/IC_{50}$ values >0.1.
- Aldehyde oxidase activity was inhibited by ketoconazole and ritonavir with resultant $[I]_{tot}/IC_{50}$ values > 0.1; however, these values were <0.1 when $C_{max,u}$ was incorporated.
- MAO-A and MAO-B activities were inhibited by ketoconazole with resultant $[I]_{tot}/IC_{50}$ values > 0.1; however, these values were <0.1 when $C_{max,u}$ was incorporated.
- The results presented here suggest modest potential for clinical CYP3A4/5 inhibitors to inhibit non-CYP enzymes in vivo, most notably several UGT enzymes.

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