Investigations into Definitive In Vitro DDI Assays to Satisfy FDA Guidelines

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Introduction

- Drug-drug interactions (DDI) may often confound clinical predictions of drug exposure extrapolated from in vitro drug metabolism data. The FDA recommendations outline strategies for evaluating perpetrators and victims of cytochrome P450 (CYP) and drug transporter DDI. Clear definitive results for drug candidates reduce the risk of negative clinical outcomes.
- We have developed definitive assays for evaluating drug candidates as CYP substrates, inhibitors, and inducers, as well as transporter substrates and inhibitors to satisfy the FDA guidelines.
- Here, we describe a drug candidate case study, conducted in two stages for a comprehensive examination of DDI liabilities: Stage I to identify CYP or transporter interactions and Stage II to confirm observations or generate DDI magnitude parameters

Methods

- CYP Reaction Phenotyping

Stage I: Recombinant Enzymes: Recombinant CYP (rCYP) Supersomes (Corning) were incubated with test compound and NADPH cofactor in a 45 minute time course at 37°C. Crashed sample supernatants were analyzed by LC-MS/MS. The first order elimination rate constant, k, was calculated from the disappearance of parent. Intrinsic clearance was calculated by scaling the amount of rCYP to the abundance in human liver microsomes (HLM). The fraction contribution of each CYP was calculated as a percentage of the sum of all intrinsic clearances.

Stage II: Chemical Inhibition in Human Liver Microsomes (HLM): The K_m value was determined using the substrate depletion method by calculating k from incubations with a range of substrate concentrations in HLM (BioIVT) and cofactor in a 45 minute time course at 37°C. k_{dep} was calculated from the k value as the substrate concentration approaches 0, and K_m was determined as the substrate concentration, S, at half the k_{dep} value. The test compound was incubated below the K_m value with CYP specific and selective inhibitor concentrations.

- CYP Inhibition in Human Liver Microsomes (HLM)

Stage I: IC_{50}: A range of test compound concentrations were incubated with CYP specific substrates with NADPH cofactor in HLM (BioIVT) at 37°C for 10 minutes. Crashed sample supernatants were analyzed by LC-MS/MS. Remaining enzyme activity was calculated from the formation of substrate metabolite at each test compound concentration against the vehicle control. The IC_{50} was determined as the test compound concentration at half the maximum activity.

Stage II: K_I: A range of test compound concentrations were incubated and analyzed as above with a range of CYP specific substrate concentrations. K_I values were calculated from the formation of substrate metabolite, test compound concentrations, and inhibitor concentrations by fitting to inhibition models.

- CYP Time Dependent Inhibition in HLM

Stage I: IC_{50} shift: A range of test compound concentrations were pre-incubated for 30 minutes with and without NADPH cofactor in HLM (BioIVT) at 37°C. At the end of the pre-incubation, the samples were spiked with CYP specific substrates for an additional incubation for 10 minutes. Crashed sample supernatants were analyzed by LC-MS/MS. Remaining enzyme activity was calculated from the formation of substrate metabolite at each test compound concentration against the vehicle control. The IC50 was determined as the compound concentration at half the maximum activity. The IC_{50} shift was calculated from the ratio of the IC50 value without and with cofactor pre-incubation.

Stage II: K_I/k_{inact}: A range of test compound concentrations were pre-incubated as above. At various time points during the pre-incubation, the samples were spiked into CYP specific substrates for an additional incubation and analyzed as above. The pseudo first order inactivation rate constant, k_{obs}, was calculated for each test compound concentration from the decrease in remaining activity with pre-incubation time. k_{inact} was calculated from the maximum inactivation rate plotted from k_{obs} against the test compound concentration, and the K_I was determined as the concentration of test compound at half the maximum inactivation.

- CYP Induction in Human Hepatocytes

Stage I: Fold Induction: Cryopreserved human hepatocytes (BioIVT) were seeded overnight on collagen coated plates at 37°C and overlaid with extracellular matrix. The cells were treated everyday with fresh compound dosing solution for 72 hours. At the end of the treatment, mRNA was isolated according to the Qiagen Rneasy kit instructions. cDNA was generated by reverse transcription and CYP1A2, 2B6, and 3A4 transcripts were measured by qPCR using specific Taqman (Thermofisher) primers. Fold induction of mRNA was calculated from the abundance of the targeted transcript of test compound treated samples over the vehicle control treated samples.

Stage II: EC_{50}/E_{max}: Plated cryopreserved human hepatocytes were treated and mRNA isolated as above with a range of test compound concentrations. E_{max} was calculated from the maximum fold induction of mRNA plotted from the fold induction against the test compound concentration. and the EC_{50} was determined as the concentration of test compound at half the maximum induction.

Methods *(cont.)*

- P-gp and BCRP Transport in Caco-2 Cells

Stage I: Substrate or Inhibitor: Caco-2 cells were seed onto permeable multi-well plate inserts and cultured for 21 days. Cells were pretreated with inhibitors for 30 minutes. For the substrate test, cells were then treated on the basolateral or the apical sides with test compound and known P-gp and BCRP inhibitors and incubated at 37°C for 60 minutes. For the inhibitor tests, cells were treated on the basolateral or the apical sides with test compound and known P-gp and BCRP substrates and incubated at 37°C for 60 minutes. Crashed apical and basolateral sample supernatants were analyzed by LC-MS/MS. Apparent permeability was calculated from amount of compound in the apical to basolateral direction (A > B) and the basolateral to apical direction (B > A). Efflux ratio (ER) was determined as the permeability B > A over A > B.

Stage II: IC_{50}: A range of test compound concentrations were incubated with P-gp and BCRP specific substrates in Caco2 cells and analyzed as above. The IC_{50} was determined as the test compound concentration at half the ER.

Results

CYP Reaction Phenotyping

Stage I
Recombinant Enzymes

Test Compound	CYP Isoform	Intrinsic Clearance in Supersomes (Clint, rcyp) (µL/min/pmol rCYP)	Calculated Intrinsic Clearance (CYPint, HLM) (µL/min/mg HLM protein)	Fraction Percent of Each CYP to the Overall Metabolism in HLM (Fm,cyp) (%)
XXXXX	1A2	0.000	0.00	0.00
	2B6	0.000	0.00	0.00
	2C8	0.023	0.54	4.63
	2C9	0.045	3.27	28.01
	2C19	0.000	0.00	0.00
	2D6	0.047	0.38	3.22
	3A4	0.067	7.49	64.14

Significant contribution (>25%) by CYP2C9 and 3A4

Stage II
Chemical Inhibition in HLM

Km Vmax Determination (Substrate Depletion)

Test Compound	K_m (µM)	V_{max} (µM/min)
XXXXX	40.81	0.30

Test Compound	Isoform	Inhibitor	Human Liver Microsomes Intrinsic Clearance (CLint) (mL/min/g liver)	Percent Remaining Activity
XXXXX	CYP2C9	NA	0.301	100%
		Sulfaphenazole	0.273	91%
Diclofenac		NA	3.860	100%
		Sulfaphenazole	0.999	26%
XXXXX	CYP3A4	NA	0.301	100%
		Azamulin	0.081	27%
Testosterone		NA	2.814	100%
		Azamulin	0.322	11%

Consistent with recombinant enzymes

CYP Inhibition in HLM

Stage I
IC_{50}

Test Compound	Isoform (Substrate), IC_{50} (µM)							
	CYP1A2 (Phenacetin)	CYP2B6 (Bupropion)	CYP2C8 (Paclitaxel)	CYP2C9 (Diclofenac)	CYP2C19 (S-Mephenytoin)	CYP2D6 (Dextromethorphan)	CYP3A4 (Midazolam)	CYP3A4 (Testosterone)
XXXXX	>33	>33	1.5990	12.3240	2.1217	>33	>33	14.3163
Positive Control	0.1465	0.0837	0.8446	0.2546	0.4458	0.0352	0.0196	0.0085

Inhibition of CYP2C8, 2C9, 2C19, and 3A4

Stage II
K_I

Test Compound	Isoform	Inhibition Model							
		Competitive		Non-Competitive		Un-Competitive		Mixed	
		K_I (µM)	R^2	K_I (µM)	R^2	K_I (µM)	R^2	K_I (µM)	R^2
XXXXX	CYP2C8	0.67	0.99	4.70	0.98	3.30	0.95	1.27	0.99
Quercetin		0.27	0.96	2.13	0.96	1.53	0.93	0.55	0.97
XXXXX	CYP2C9	7.10	0.95	49.70	0.92	36.30	0.90	5.46	0.95
Sulfaphenazole		0.09	0.99	0.54	0.96	0.34	0.93	0.09	0.99
XXXXX	CYP2C19	1.45	0.99	11.49	0.97	8.86	0.95	2.29	0.99
Ticlopidine		0.06	0.92	0.59	0.96	0.45	0.94	0.28	0.97
XXXXX	CYP3A4	17.49	0.99	52.37	0.99	25.79	0.98	25.22	0.99
Ketoconazole		0.011	0.99	0.036	0.98	0.019	0.97	0.011	0.99

Inhibition magnitude consistent with IC_{50} experiments

CYP Time Dependent Inhibition in HLM

Stage I
IC_{50} Shift

Test Compound	Pre-inc	Isoform (Substrate)															
		CYP1A2 (Phenacetin)		CYP2B6 (Bupropion)		CYP2C8 (Paclitaxel)		CYP2C9 (Diclofenac)		CYP2C19 (S-Mephenytoin)		CYP2D6 (Dextromethorphan)		CYP3A4 (Midazolam)		CYP3A4 (Testosterone)	
		IC_{50} (µM)	IC_{50} shift	IC_{50} (µM)	IC_{50} shift	IC_{50} (µM)	IC_{50} shift	IC_{50} (µM)	IC_{50} shift	IC_{50} (µM)	IC_{50} shift	IC_{50} (µM)	IC_{50} shift	IC_{50} (µM)	IC_{50} shift	IC_{50} (µM)	IC_{50} shift
XXXXX	+	>50	ND	>50	ND	2.15	0.975	19.5	0.995	3.17	1.35	>50	ND	>50	ND	12.4	3.43
	-	>50		>50		2.09		19.4		4.28		>50		>50		42.4	
Positive Control	+	0.874	5.29	0.071	20.8	3.72	4.06	0.104	5.84	0.555	2.45	0.125	5.01	1.48	7.00	1.50	13.8
	-	4.63		1.487		15.1		0.610		1.36		0.626		10.4		20.7	

Time dependent inhibition of CYP3A4

Stage II
K_I / k_{inact}

Test Compound	CYP3A4 (Testosterone)	
	K_I (µM)	K_{inact} (min^{-1})
XXXXX	21.7	0.0180
Troleandomycin	1.0	0.0948

Magnitude consistent with IC_{50} shift

Results *(cont.)*

CYP Induction in Hepatocytes

Stage I
Fold Induction

Test Compound	Donor ID	Isoform	mRNA Fold Induction		
			1 µM	10 µM	100 µM
XXXXX	JCG	CYP1A2	1.56	7.18	5.06
	AIH		4.40	11.94	6.32
	WWV		1.64	3.59	3.20
	JCG	CYP2B6	1.56	8.22	21.60
	AIH		2.96	8.54	3.92
	WWV		2.65	10.21	16.95
	JCG	CYP3A4	4.33	26.93	47.60
	AIH		2.06	10.60	7.49
	WWV		4.31	15.31	18.92
Omeprazole	JCG	CYP1A2	46.75		
	AIH		142.55		
	WWV		31.12		
Phenobarbital	JCG	CYP2B6	96.03		
	AIH		19.64		
	WWV		44.40		
Rifampicin	JCG	CYP3A4	14.25		
	AIH		36.91		
	WWV		12.11		

Significant fold induction (>4) of all three isoforms

Stage II
EC_{50} / E_{max}

Test Compound	Donor ID	Isoform	EC_{50} (µM)	E_{max} (Fold Induction)
XXXXX	JCG	CYP1A2	0.74	6.28
	AIH		0.58	3.40
	WWV		1.47	5.75
	JCG	CYP2B6	13.5	31.5
	AIH		0.33	3.71
	WWV		2.39	15.1
	JCG	CYP3A4	5.45	32.1
	AIH		1.87	13.2
	WWV		1.63	26.2

Magnitude consistent with Stage I

P-gp and BCRP Transport in Caco-2

Stage I
Substrate or Inhibitor

Transporter	Control Substrate	Control Inhibitor
P-gp	Digoxin	Zosuquidar
BCRP	Estrone-3-sulfate	Ko143

Compound Substrate (+Inhibitor)	P_{app} Efflux Ratio (ER)
XXXXX	2.63
XXXXX (+zosuquidar)	1.83
XXXXX (+Ko143)	2.06
Digoxin	292.67
Digoxin (+XXXXX)	2.79
Digoxin (+zosuquidar)	1.18
Estrone-3-sulfate	59.72
Estrone-3-sulfate (+XXXXX)	1.14
Estrone-3-sulfate (+Ko143)	3.03

Little effect compound as substrate

Large effect compound as perpetrator

Stage II
IC_{50}

Test Compound (Substrate)	IC_{50} (µM)
XXXXX (Digoxin)	0.0586
Zosuquidar (Digoxin)	0.0008
XXXXX (Estrone-3-Sulfate)	0.1894
Ko143 (Estrone-3-Sulfate)	0.0116

Conclusions

- The FDA guidelines describe the assays and parameters necessary for the determination of CYP and transporter DDI potential. The assays presented here tested a drug candidate as CYP substrate, inhibitor, and inducer as well as drug transporter substrate and inhibitor in two stages.
- The stage I assay results identified multiple CYP and transporter isoform liabilities.
- The stage II assays results confirmed the contributions of CYP2C9 and 3A4 to the test compound metabolism, generated K_i values for inhibition of CYP2C8, 2C9, 2C19, and 3A4, the K_I and k_{inact} for TDI of CYP3A4, EC_{50} and E_{max} values for induction of CYP1A2, 2B6, and 3A4, and IC_{50} values for inhibition of P-gp and BCRP
- The definitive design provides a comprehensive assessment of DDI.

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