Poster # P207

Evaluation of Metabolite Synthesis for Sixteen Commercial Drugs Using the Hypha Discovery System

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ABSTRACT

Identification and quantification of metabolite(s) at the earliest stage of drug discovery and development is crucial in assessing its contribution to pharmacological, toxicological and drug-drug interaction potential. There are many approaches, including chemical, hepatic subcellular systems, expressed CYP systems, bacterial and fungal strains etc. used to synthesize metabolites, but there hasn't been any published report where these systems are compared head to head for the ability to produce metabolites. In recent years, Hypha Discovery using a variety of microbial and fungal strains and inactivated yeast-based CYP powders (hrCYP) has provided an ideal platform to evaluate production of various metabolites using these three different systems simultaneously. In the present study, metabolism and production of various metabolites of sixteen test compounds were evaluated using this approach. Results obtained from screening of these test compounds demonstrated that the microbial biotransformation and the hrCYP system used are capable of providing metabolites of all but one of the substrates evaluated; yields varied from full conversions to those weakly detectable by LCMS. Production of weakly detectable metabolites to provide usable quantities (> 5mg) may require further optimization or greater production volume or number of batches. One of other major advantages of this approach is that many of the secondary, tertiary as well as glucuronide/sulfate conjugate metabolites of many of these test articles were produced in significant amounts in addition to primary metabolite produced by various other more common systems.

BACKGROUND

Characterization of the metabolites of a drug candidate is critical for drug discovery and development process. The major purposes of these characterizations are to (1) ensure that human metabolites are adequately tested in toxicology species, (2) determine whether the major metabolite has pharmacological activity, and 3) determine drug-drug interaction potential [http://www.fda.gov/cder/guidance]. The main objective of this study was to compare and contrast the ability of human liver microsomes, human hepatocytes, and Hypha's bacterial and fungal strains and inactivated yeast-based CYP powders to generate various human metabolites of sixteen test compounds and find a method of choice that can used for a large scale production of a drug metabolites.

METHODS

Microsomal incubations were carried out by adding liver microsomes and compound to 100 mM sodium phosphate buffer (pH 7.4) to give a final concentration of 1 mg/mL and 50 µM, respectively. The incubations were initiated with the addition of NADPH (1 mM final concentration). After 30 minutes of incubation at 37 °C, the incubations were quenched with an equal volume of water-acetonitrile (50:50, v/v). Samples were vortexed, centrifuged at 4,000 rpm for 10 minutes, and 10 µL of the supernatant were used for analysis. Hepatocyte incubations were carried out by adding thawed cryopreserved hepatocytes to hepatocyte maintenance media. Dilute stock solution of compound was added to media to give a final incubated concentration of 2 µM. After 4 hours of incubation at 37 °C in a humidified CO_2 incubator, they were quenched with an equal volume of acetonitrile. Samples were centrifuged at 4,000 rpm for 10 minutes and 10 µL of the supernatant were used for LC-MS analysis.

For screening bacterial and fungal strains, bacterial strains were initially recovered from cryo-storage directly into shake-flasks (50ml medium, M3G) of appropriate growth medium. Fungi were cultured in a similar manner, except via an intermediate agar-culture into the specific liquid medium (C95). Flask cultures were grown at 27°C, 200rpm. Substrates were added to strains in MicroBioreactors after 48hr pre-growth periods. MicroBioreactor fermentations proceeded at 27°C, 300rpm and were sampled at 48 & 120hrs. Samples were extracted with an equal volume of acetonitrile, centrifuged to clarify and dried by Genevac at 40°C, and lyophilized for future analysis. In addition to the microbial panel, four hrCYPs were also assessed for metabolite production. These are an inactivated yeast-based product. Yeast-hrCYP powders were suspended in phosphate buffer containing magnesium chloride to give 50 mg/ml in 100 µL reaction volume and then substrates, with formulation where needed, at 100mg/L final concentration. Substrates and Yeast-hrCYP were incubated in the presence of a NADPH regenerating system (G6P, NADP+ and G6PDH) at 27°C, 300rpm for 4hrs, followed by extraction with an equal volume of methylcyanide and processed as described above for future analysis. For analysis, the screening samples were re-suspended in 100 µL of water-acetonitrile and further diluted 10-fold before analysis LC/MS or LC/MS/MS on either a Waters Xevo or the Thermo Orbitrap Velos Pro with an Accela LC. Parent compound retention time was optimized by using a linear gradient with either 0.2 % formic acid in water or 10 mM ammonium bicarbonate in water for the aqueous side and either acetonitrile or methanol for the organic side. The data from the LC/MS analysis was processed by Metabolynx XS version 4.1 SCN 884. Metabolites of interest were further confirmed by LC/MS/MS on either the Thermo Orbitrap Velos Pro or the Waters Xevo Q-ToF.

Instrumentation					
Water System:	Thermo System:				
Masslynx 4.1 SCN 884	Xcalibur 2.2 SP1 48				
Xevo G2-S Q-ToF	OribTrap Velos Pro				
Acquity I Class Binary Solvent Manager	Accela 1250 Pump				
Acquity FTN Sample Manager	Accela Open AS				

HPLC Conditions					
Mobile Phase A:	0.2 % Formic Acid (FA) or 10 mM Ammonium Bicarbonate(AB)				
Mobile Phase B:	Acetonitrile (M1, M2, M3) or Methanol (M4, M5, M6)				
Gradient:	M1, M4: 0/5, 0.4/5, 3.0/ 50, 3.5/90, 4.0/90, 4.25/90, 4.5/5, 6/stop				
	M2, M5: 0/5, 0.4/5, 3.0/ 75, 3.5/90, 4.0/90, 4.25/90, 4.5/5, 6/stop				
	M3, M6: 0/10, 0.4/10, 3.5/90, 4.0/90, 4.25/90, 4.5/10, 6/stop				
Flow Rate(mL/min):	0.5	Injection Volume(µL):	5	Divert:	0.8 min
Analytical Column:	Waters Acquity MS BEH C18, 1.7 µM, 2.1 x 100 mm				

RESULTS

Table 1. A comparison of major metabolites produced following incubations of selected sixteen compounds with Hypha Microbes, Fungi and/or hrCYP, human liver microsomes and human cryo-preserved hepatocytes.

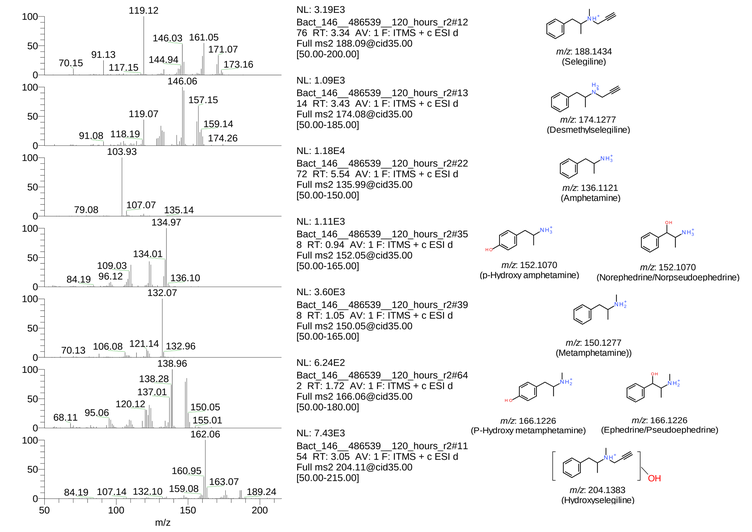
#	Substrate	Possible Metabolites	Observed *m/z*	Type of Metabolism	Hypha	HLM	Hepatocytes
1	Phenacetin	APAP	152.0706	Dealkylation	yes	yes	yes
		Hydroxy	196.0968	Hydroxylation	yes	yes	yes
		Hydroxy Glucuronide	372.1289	Oxidation + Glucuronidation			
2	Bupropion	Hydroxy	256.1099	Hydroxylation	yes	yes	yes
		Reduced	242.1306	Reduction	yes	yes	yes
		Hydroxy + Reduced	258.1255	Reduction + Hydroxylation	yes	yes	yes
3	Amodiaquine	Desethyl	328.1211	Dealkylation	yes	yes	yes
		Didesethyl	300.0898	Didealkylation			
		Hydroxy Desethyl	344.116	Dealkylation + hydroxylation			
		Aldehyde	299.0582	Dealkylation oxidation	Yes		
4	Diclofenac	Hydroxy	312.0189	Hydroxylation	yes		
		Hydroxy	312.0189	Hydroxylation		yes	
		Acyl glucuronide	472.056	Glucuronidation	yes		
5	S-Mephenytoin	Hydroxy	235.1077	Hydroxylation	yes		yes
		Desmethyl	205.0972	Dealkylation			
		Hydroxy + Glucuronide	411.1398	Oxidation + Glucuronidation	yes		
6	Aripiprazole	Reduced	446.1396	Reduction		yes	yes
		Hydroxy	464.1502	Hydroxylation	yes		yes
7	Rosuvastatin	Lactone	464.1650	Esterification	yes		yes
		Desmethyl	468.1599	Dealkylation	yes		yes
		Glucuronide	658.2076	Glucuronidation	yes		
8	Esomeprazole	Hydroxy/Sulfone	362.1169	Hydroxylation	yes	yes	
		Hydroxy/Sulfone	362.1169	Hydroxylation	yes	yes	
		Desmethyl	332.1063	Dealkylation	yes	yes	
		Desmethyl + Hydroxy	348.1012	Dealkylation + Hydroxylation	yes		
9	Sitagliptin	Sulfate	488.0822	Sulfation			
		Carbomyl Glucuronide	628.1473	Glucuronidation + CO2			
		Hydroxy	424.1203	Hydroxylation	yes		
		Cyclic	406.1097	Cyclization	yes		
10	Haloperidol	Reduced	378.1631	Reduction	yes	yes	yes
		Piperidine	212.0837	Dealkylation	yes		
		O-/N-Glucuronide	552.1795	Glucuronidation	yes		
11	Amitriptyline	Nortriptyline	264.1747	Dealkylation	yes	yes	yes
		Hydroxy Nortriptyline/ (Demethylation + hydroxy)	280.1696	Dealkylation + Hydroxylation	yes		
		Hydroxy	294.1852	Hydroxylation	yes	yes	yes
12	Propranolol	Hydroxy	276.1954	Hydroxylation	yes	yes	yes
		Ndesisopropylpropranolol	218.1176	Dealkylation	yes		
		Glucuronide	436.1966	Glucuronidation	yes		yes
13	Tamoxifen	N-Desmethyl	358.2271	Dealkylation	yes	yes	yes
		4-Hydroxy	388.2271	Hydroxylation	yes	yes	yes
		α-Hydroxy	388.2271	Hydroxylation	yes		
14	Carvedilol	Hydroxy	422.1914	Hydroxylation	yes	yes	
		Hydroxy	422.1941	Hydroxylation	yes	yes	
		Desmethyl	392.1809	Dealkylation	yes	yes	
		Glucuronide	582.2286	Glucuronidation	yes		yes
15	Selegiline	Amphetamine	136.1121	Didealkylation	yes		
		Hydroxyamphetamine	152.1070	Didealkylation + Hydroxylation	yes		
		Metamphetamine	150.1277	Dealkylation	yes		
		Hydroxymetamphetamine	166.1226	Dealkylation + Hydroxylation	yes		
16	Troglitazone*	Quinone	440.1537	Oxidation	yes		
		Hydroxy	456.1486	Hydroxylation	yes	yes	
		Sulfate	520.1105	Sulfation			yes
		Glucuronide	616.1858	Glucuronidation	yes		

*: Troglitazone was analyzed in negative ion mode

Table 2. Phase II metabolites produced following incubations of selected sixteen compounds with Hypha Microbes, Fungi and/or hrCYP (without any addition of UDPGA/PAPS during the incubations).

#	Substrate	Observed metabolite based up on m/z and MS-MS
1	Phenacetin	Oxidation + Glucuronidation
2	Amodiaquine	Glucuronidation
3	Diclofenac	Glucuronidation
		Hydroxylation + Glucuronidation
		Hydroxylation + Sulfation
4	Aripiprazole	Hydroxylation + Glucuronidation
5	Rosuvastatin	Glucuronidation
6	Esomeprazole	Glucuronidation
		Hydroxylation + Glucuronidation
		Dealkylation + Glucuronidation
		Dealkylation + Hydroxylation + Glucuronidation
7	Sitagliptin	Sulfation
8	Haloperidol	Reduction + dehydration + Glucuronidation
		Dehydration + Glucuronidation
		Glucuronidation
		Hydroxylation + Glucuronidation
9	Amitriptyline	Glucosidation
		Glucuronidation
		Hydroxylation + Glucuronidation
10	Propranolol	Glucuronidation
11	Tamoxifen	Glucuronidation
		Hydroxylation + Glucuronidation
		Hydroxylation + Glucuronidation
12	Carvedilol	Glucuronidation
		Dealkylation + Glucuronidation
		Dealkylation + Hydroxylation + Glucuronidation
		Hydroxylation + Glucuronidation
13	Troglitazone	Sulfation

Figure 1. Possible metabolites of selegiline generated by Hypha's system as determined by accurate mass and MS/MS.



CONCLUSIONS

- A comparison of results following incubation of 16 compounds with three systems showed that Hypha's microbial/fungal/hrCYP system was successful in generating approximately 83% of the major human metabolites, while human liver microsomes and human cryo-preserved hepatocytes were only able to generate approximately 40% each of the major human metabolites (Table 1).
- Hypha system was also able to generate many of the phase II metabolites (specifically glucuronides) without any addition of cofactor such as UDPGA/PAPS (Table 2).
- Hypha system was consistently better in generating in secondary and tertiary metabolites compared to human liver microsomes and human cryo-preserved hepatocytes (see Figure 1 as an example).

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References:

- Ho-Sang Shin, Metabolism of Selegiline in human: Identification, Excretion, and Stereochemistry of Urine Metabolites, Drug Metab Dispos. 1997 Jun;25(6):657--662.

All test compounds used in this study were either obtained from Lilly Research Center or purchased from Sigma-Aldrich.

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