Evaluation of Strategies for the Assessment of Drug-Drug Interactions involving Cytochrome P450 Enzymes

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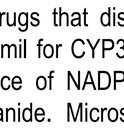
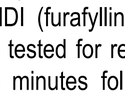
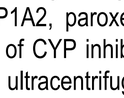
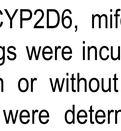
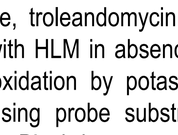
1 BACKGROUND

Drug-drug interactions (DDIs) can occur when one drug alters the metabolism of a second drug. Since it is nowadays common for patients to receive at least two or more drugs at the same time the likelihood of the occurrence of DDIs has increased significantly. Drug metabolism mediated by Cytochrome P450 enzymes (CYPs) is responsible for the majority of the metabolism of known drugs in humans and inhibition of CYP enzymes is a well-recognized cause of DDIs. As a result, the European Medicines Agency (EMEA) and the U.S. Food and Drug Administration (FDA) have introduced guidelines for *in vitro* studies to assess the ability of drug candidates to cause inhibition of CYP enzymes.

CYP inhibition is caused by two general mechanisms: direct inhibition by the drug itself or metabolism-dependent inhibition (MDI) by its metabolite(s). MDI is divided into three subcategories: reversible inhibition (a metabolite is a more potent inhibitor than the parent drug), irreversible inhibition (a reactive metabolite binds covalently to the heme or apoprotein to inactivate the enzyme), and quasi-irreversible inhibition (a reactive metabolite forms a stable metabolic intermediate complex (MIC) through a coordinate bond with the ferrous heme iron). The latter two categories are both a result of mechanism-based inactivation (MBI). In the current study, the use of various human liver microsomes (HLM)-based methods to determine occurrence of CYP-mediated MDI and possible follow-up studies was evaluated.

Reversibility assay	MIC formation	CO-difference spectra	GSH adduct formation	Covalent binding
Distinguish which type of MDI is involved	Measure if a Metabolic Intermediate complex (MIC) is formed in time	Measure if CO binding is reduced when inhibitor is incubated in presence of NADPH	Monitor formation of GSH adducts when inhibitor is incubated in presence of GSH	Measure covalent binding of radiolabeled inhibitor in absence and presence of GSH
				
UPLC-PDA-MS/MS	Double beam UV/vis spectrophotometer	Double beam UV/vis spectrophotometer	LTQ Orbitrap	Liquid Scintillation Counter

2 IC_{50}-SHIFT ASSAYS

Reversible inhibition (RI)
Time dependent inhibition (TDI)
Metabolism dependent inhibition (MDI)

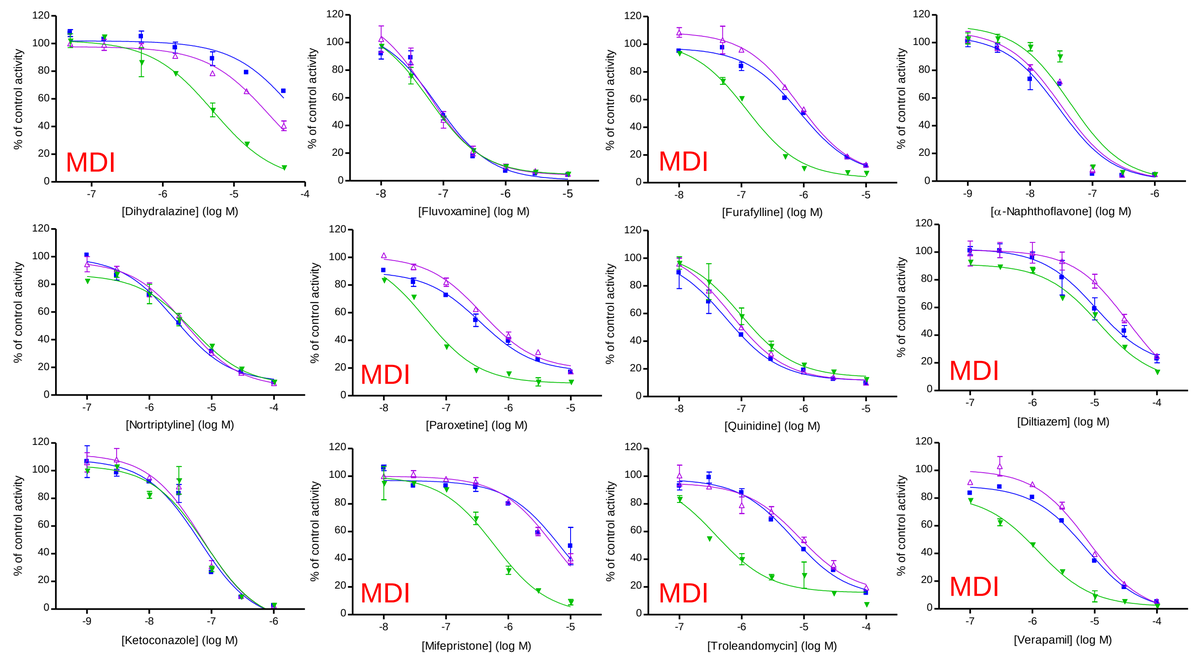


Figure 1 Evaluation of the inhibition of CYP activities by selected drugs using the non-dilution approach
Effects of dihydralazine, fluvoxamine, furafylline and α-naphthoflavone on CYP1A2 activity, nortriptyline, paroxetine and quinidine on CYP2D6 activity and diltiazem, ketoconazole, mifepristone, troleandomycin and verapamil on CYP3A4 activity.

3 FOLLOW-UP STUDY RESULTS

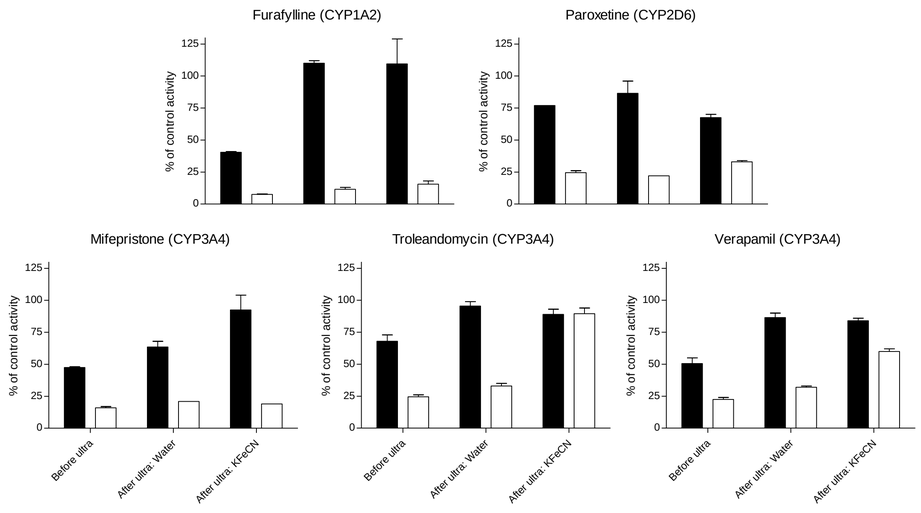


Figure 2 Results of the ferricyanide-based reversibility assay
Five drugs that displayed MDI (furafylline for CYP1A2, paroxetine for CYP2D6, mifepristone, troleandomycin and verapamil for CYP3A4) were tested for reversibility of CYP inhibition. Drugs were incubated with HLM in absence or presence of NADPH for 30 minutes followed by ultracentrifugation with or without CYP oxidation by potassium ferricyanide. Microsomes were re-isolated and residual CYP activities were determined using probe substrates (CYP1A2: phenacetin, CYP2D6: bufuralol, CYP3A4: midazolam) at their respective K_m values. Black bars represent values from HLM incubated with inhibitor in absence of NADPH (TDI). White bars represent the values from HLM incubated with inhibitor in presence of NADPH (MDI). Activity is expressed as the percentage of activity remaining as compared with a solvent control sample. Data show the mean ± % CV of duplicate incubations.

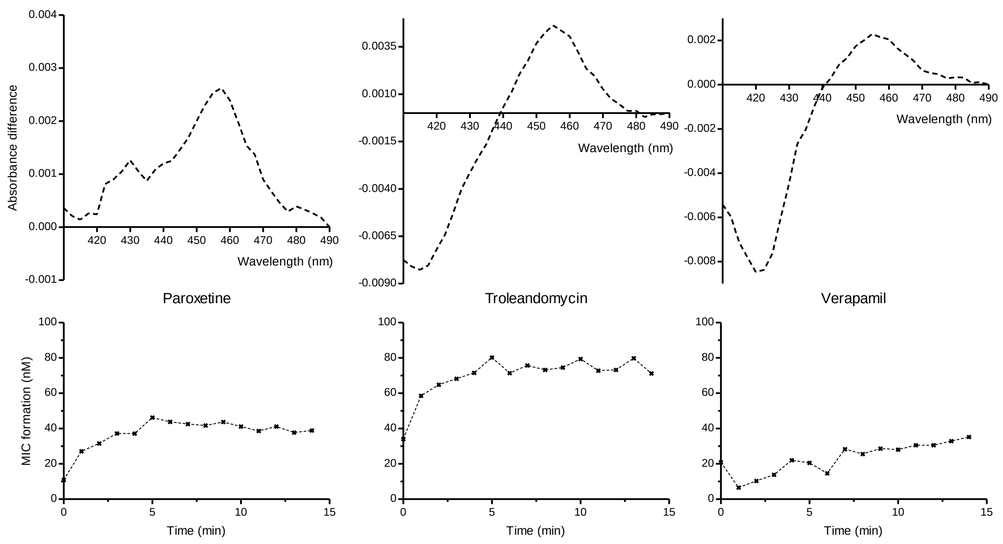


Figure 3 Metabolic intermediate complex formation by various drugs using recombinant human CYPs
MIC formation by paroxetine (2.5 µM) using human recombinant CYP2D6 (100 nM) and troleandomycin (25 µM) and verapamil (50 µM) using human recombinant CYP3A4 (100 nM). Incubations were performed in the presence of NRS (0.5 mM NADPH, 10 mM G6P and 10 U/mL G6P-DH). Absorbance difference spectra were obtained each minute for 15 minutes and the spectra obtained after 15 minutes of incubation are displayed in the upper graphs. The lower graphs display the time course monitoring of the MIC formation which was calculated using the observed increase in absorbance at 455 nm and the corresponding absorbance at 490 nm.

Figure 4 Reduced CO-difference spectra
Apparent human liver microsomal CYP content following 30 minutes of incubations with furafylline (5 µM), paroxetine (2.5 µM), mifepristone (25 µM), troleandomycin (25 µM) and verapamil (50 µM) in presence (MDI) or absence (TDI) of NADPH. Reduced CO difference spectra were determined by first reducing the enzyme with sodium dithionite. The reduced mixtures were divided between two cuvettes and CO was gently bubbled through the sample cuvette for 30 seconds. The spectrum of the reduced CO complex was recorded between 400 and 500 nm..

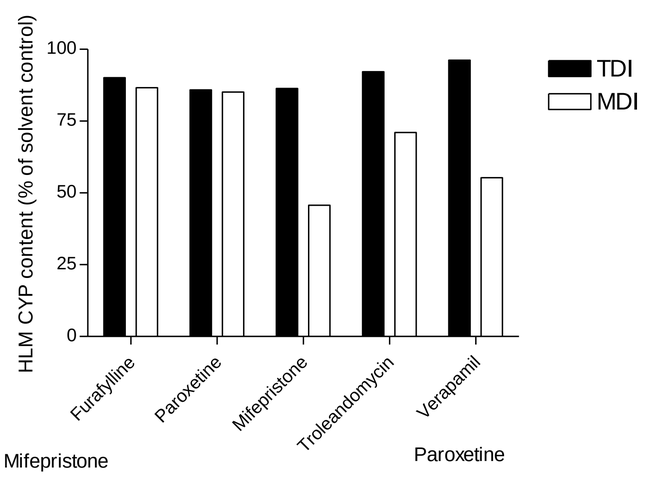
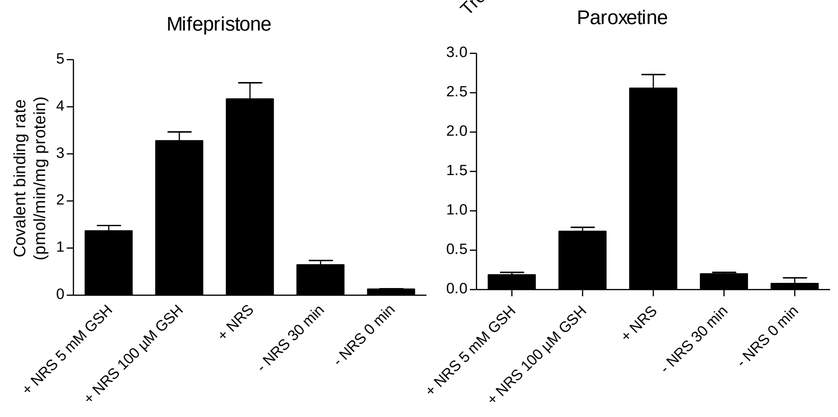


Figure 5 Covalent binding rate to HLM
In vitro covalent binding of [N-methyl ^{3}H]-mifepristone and [phenyl-6-^{3}H]-paroxetine to HLM (0.5 mg/mL) was evaluated. Incubations were performed at a 20 µM concentration for (a) 30 minutes in presence of NRS and 5 mM GSH; (b) 30 minutes in presence of NRS and 100 µM GSH; (c) 30 minutes in presence of NRS; (d) 30 minutes in absence of NRS or (e) 0 minutes in absence of NRS.



4 CONCLUSIONS

	Experimental Results					Literature Reports	
Drug	Reversibility	MIC formation	CO-spectra	GSH adducts	Covalent binding	Reactive intermediate	Site of action
Furafylline	Irreversible	×	No effect	-	-	Epoxide	Apoprotein
Mifepristone	Irreversible	×	Reduced	√	√	Epoxide	Apoprotein (near the heme)
Paroxetine	Irreversible	√	No effect	√	√	Carbene *Ortho*-quinone	MIC Covalent binding
Troleandomycin	Quasi-irreversible	√	Reduced	×	-	Nitroso	MIC
Verapamil	Quasi-irreversible	√	Reduced	×	-	Nitroso	MIC

– = Not performed.

In conclusion, several experimental strategies to study CYP inhibition and yield a greater understanding of the underlying mechanisms involved have been presented and evaluated. HLM-mediated direct, time- and metabolism-dependent inhibition of CYP1A2, CYP2D6 and CYP3A4 was studied for twelve drugs.

It was demonstrated that the combined use of follow-up experiments is a very useful tool to determine the exact inhibition mechanism responsible for CYP inactivation. This poster gives a representative overview of the current methodologies that can be used to study metabolism-dependent inhibition (MDI) in more detail. The here presented strategy can be used as a tool during risk evaluation of CYP-mediated DDIs.