Assessment Under Initial Rate Conditions of the Selectivity and Time Course of Cytochrome P450 Inactivation in Pooled Human Liver Microsomes and Hepatocytes: Optimization of Inhibitor Conditions Used for Reaction Phenotyping Studies

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Introduction

The identification of the metabolic pathways (reaction phenotyping) for new drug candidates is an essential component of drug development, as these studies increase the understanding of how a drug is cleared and the potential for the drug to be subject to drug-drug interactions with co-administrated medications. Typically *in vitro* reaction phenotyping is conducted in early drug development by measuring disappearance of parent drug, as metabolite standards are typically unavailable. In the industry, these assays have been conducted with various approaches, including the use of recombinant CYP enzymes, antibody inhibition of CYP enzymes and correlation analysis.

Another approach used to identify which cytochrome P450 (CYP) enzymes are responsible for the metabolism of a drug is the use of specific chemical inhibitors in test systems such as pooled human liver microsomes (HLM) or pooled cryopreserved human hepatocytes (CHH) (Ogilvie *et al.*, 2008; Khojasteh *et al.*, 2011; Nirogi *et al.*, 2014). These studies are typically more cost effective and offer logistical advantages over the other approaches. However, the design of these studies is critical because of factors such as metabolic depletion of the inhibitor, protein binding and insufficient enzyme inactivation. Additionally, the measurement of enzyme inactivation under initial rate substrate conditions is crucial to obtain an accurate assessment of the true levels of inhibition (Parkinson *et al.*, 2011).

In the present study, we examined the selectivity and duration of CYP inactivation, with a range of protein concentrations and incubation times, for a variety of commonly used CYP inhibitors all under initial rate probe substrate conditions in both HLM and CHH with the goal of optimizing assay conditions.

Materials & Methods

Chemicals

Furafylline, ketoconazole, mibefradil, paroxetine, phencyclidine and quinidine were purchased from Sigma-Aldrich (St. Louis, MO). Esomeprazole, gemfibrozil glucuronide and CYP3cide were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Tienilic acid was purchased from Cypex (Dundee, Scotland, UK). Troleandomycin was purchased from US Pharmacopeia (Rockville, MD). The sources of all other reagents have been described previously (Parkinson *et al.*, 2011).

Test system

Pooled human liver microsomes (HLM, n = 200, mixed gender) and pooled cryopreserved human hepatocytes (CHH, n = 100, mixed gender) were prepared from non-transplantable livers and characterized at XenoTech, LLC (Lenexa, KS) as described previously (Pearce *et al.*, 1996; Parkinson *et al.*, 2004).

***In vitro* chemical inhibition under initial rate conditions in HLM and CHH**

Briefly as described in **Figure 1**, NADPH-fortified pooled HLM (n = 200) at 0.1, 0.5 and 1 mg/mL or pooled CHH (n = 100) at 1 million cells/mL plated in 48-well plates, were pre-incubated for 30 min at 37°C with various CYP inhibitors (as shown in **Table 1**), namely furafylline (10 µM), phencyclidine (up to 30 µM), gemfibrozil glucuronide (100 µM), tienilic acid (20 µM), esomeprazole (10 µM), quinidine (5 µM), paroxetine (1 or 5 µM), ketoconazole (1 or 4 µM), CYP3cide (2.5 µM), mibefradil (1 µM) and troleandomycin (50 µM). Following the pre-incubation step, marker substrate (≈ K_m) incubations were performed for up to 5 min (10 min for CHH) at three time points (0, 30, 120 min; simulating the time course of an unknown drug) following the pre-incubation step to determine CYP1A2 (phenacetin), CYP2B6 (bupropion), CYP2C8 (amodiaquine), CYP2C9 (diclofenac), CYP2C19 (*S*-mephenytoin), CYP2D6 (dextromethorphan), CYP2E1 (chlorzoxazone) and CYP3A4/5 (midazolam) residual activities under initial rate conditions. Reactions were terminated with the addition of one volume of acetonitrile containing deuterated internal standard, followed by protein precipitation (10 min, 920 RCF). Metabolite formation was determined by LC-MS/MS analysis as described previously (Parkinson *et al.*, 2011).

Figure 1. Incubation process for the assessment of chemical inhibition at initial rate conditions

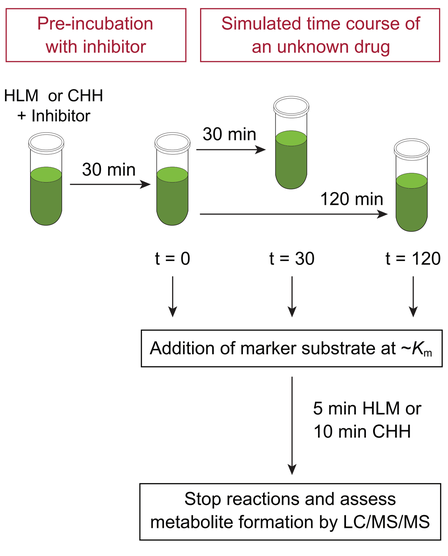


Table 1. Summary of inhibitor concentrations in each test system

Inhibitor	HLM	CHH
Furafylline	10 µM	
Phencyclidine	30 µM	10 µM
Gemfibrozil gluc	100 µM	
Tienilic acid	20 µM	
Esomeprazole	10 µM	
Paroxetine	5 µM	1 µM
Quinidine	5 µM	
Mibefradil	1 µM	
CYP3cide	2.5 µM	
Troleandomycin	50 µM	
Ketoconazole	1 µM	4 µM

Results

Results indicated that, in both HLM and CHH, inhibition of specific CYP enzymes can be accomplished over the duration of 120 minutes after pre-incubation with the inhibitors used. As summarized in **Figure 2**, in HLM at 0.1, 0.5 and 1 mg/mL over the time course of the incubation, furafylline selectively inactivated CYP1A2 activity by 63-80%; phencyclidine selectively inactivated CYP2B6 activity by 48-91%; gemfibrozil glucuronide selectively inactivated CYP2C8 activity by 96-98%; tienilic acid selectively inactivated CYP2C9 activity by 96-100%; paroxetine inactivated CYP2D6 activity by 55-90% with moderate inhibition of CYP2B6; quinidine selectively inactivated CYP2D6 by 55-90%; and ketoconazole, CYP3cide, mibefradil and troleandomycin inactivated CYP3A4/5 activity by 78-97%, 81-88%, 72-91% and 92-94% respectively. Esomeprazole was found to potently inhibit CYP2C19 activity by 85-100% with some moderate inhibition of CYP2E1 (53-66%) and CYP3A4/5 (31-68%).

Figure 3 summarizes the results from CHH (1 million cells/mL) over the incubation time course. Briefly, furafylline selectively inactivated CYP1A2 activity by 83-87%; phencyclidine inactivated CYP2B6 activity by 63-90% with moderate inhibition of CYP2D6 (37-56%); gemfibrozil glucuronide selectively inactivated CYP2C8 activity by 32-97%; tienilic acid selectively inactivated CYP2C9 activity by 100%; paroxetine inactivated CYP2D6 activity by 72-85% with moderate inhibition of CYP2B6; quinidine selectively inactivated CYP2D6 activity by 87-94%; and ketoconazole, CYP3cide, mibefradil, and troleandomycin inactivated CYP3A4/5 activity by 96-97%, 83-91%, 63-89% and 92-95% respectively. Esomeprazole was found to potently inhibit CYP2C19 activity by 89-95% with some moderate inhibition of CYP2E1 (68-69%), consistent with results from HLM.

Figure 2. Time course of inactivation of CYP enzymes in HLM at 0.1, 0.5 and 1 mg/mL by various chemical inhibitors under initial rate conditions

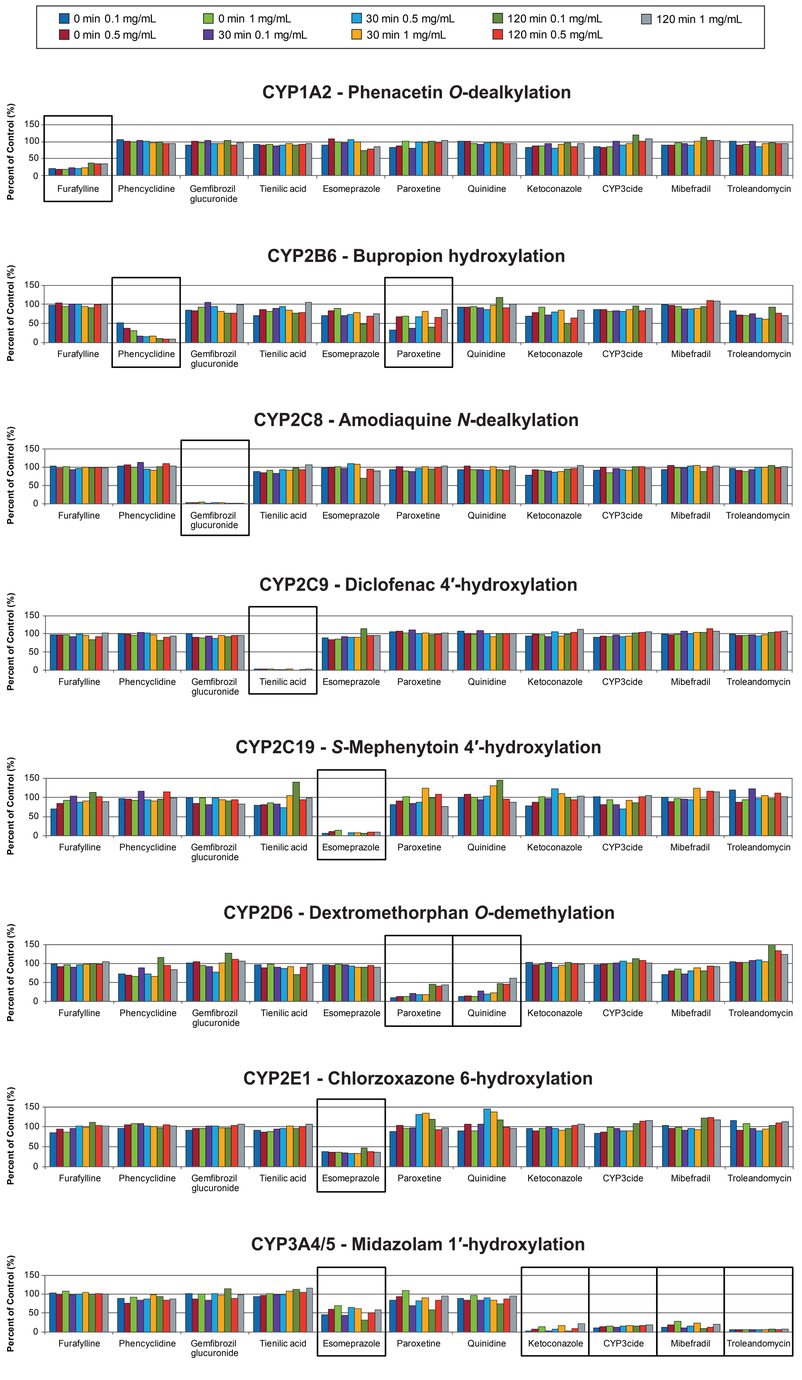
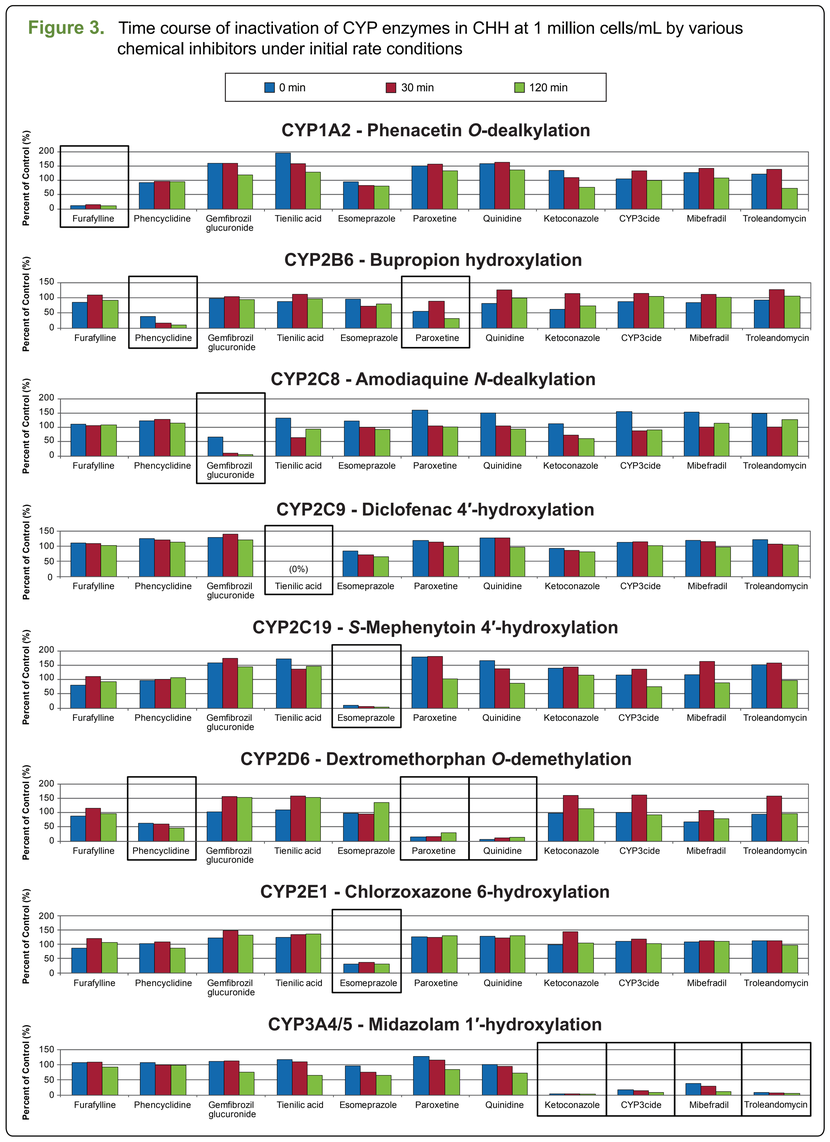


Figure 3. Time course of inactivation of CYP enzymes in CHH at 1 million cells/mL by various chemical inhibitors under initial rate conditions



Conclusions

- 11 chemical inhibitors were found to be selective for various CYP enzymes over 120 min after pre-incubation in both HLM at multiple protein concentrations and CHH.
- In summary, these findings demonstrate the suitability and optimized conditions for the use of common chemical inhibitors in CYP reaction phenotyping studies, with implications for the design of such studies.

References

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