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-administered 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; Norigi 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.

### 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%; phenycyclidine 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 87-94%; troleandomycin inactivated CYP2E1 activity by 96-97%, 83-91%, 63-89% and 92-95% respectively. Esomeprazole was found to potent inhibit CYP2C19 activity by 85-100% with some moderate inhibition of CYP1A2 (53-66%) and CYP3A4/5 (31-48%).

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%; phenycyclidine inactivated CYP2B6 activity by 63-90% with moderate inhibition of CYP2C8 (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, CYP3A4/5, troleandomycin and tienilic acid inactivated CYP3A4/5 activity by 96-97%, 83-91%, 63-89% and 92-95% respectively. Esomeprazole was found to potent inhibit CYP2C19 activity by 89-95% with some moderate inhibition of CYP2E1 (68-69%), consistent with results from HLM.

### Materials & Methods

#### Chemicals

Furafylline, ketoconazole, mibefradil, paroxetine, phenycyclidine 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 Cyrex (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), phenycyclidine (up to 30 μM), gemfibrozil glucuronide (100 μM), tienilic acid (20 μM), esomeprazole (10 μM), quinidine (5 μM), paroxetine (1 or 5 μM), ketocanazole (1 or 4 μM), CYP3cide (2.5 μM), mibefradil (1 μM) and troleandomycin (50 μM). Following the pre-incubation step, marker substrate (~Kc) 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-mephentoin), CYP2D6 (dextromethorphan), CYP2E1 (chlorozoxazone) 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, 500 RCF). Metabolite formation was determined by LC-MS/MS analysis as described previously (Parkinson et al., 2011).

### 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