EVIDENCE FOR CYP2C9:CYP3A4 ENZYME ACTIVITY INTERACTIONS IN HUMAN HEPATOCYTES THROUGH MODULATION OF ENZYME LEVELS

Diane B. Ramsden[1], Timothy S. Tracy[2], Tom S. Chan[1]

[1]Boehringer Ingelheim Pharmaceuticals, 175 Briar Ridge Rd., Ridgefield CT, 06877-0368.
[2]College of Pharmacy, University of Kentucky, 789 S. Limestone, Lexington KY, 40536-0596

Boehringer Ingelheim

ABSTRACT

Protein-protein interactions have been well documented for recombinant CYP450s[1-5]. This interaction has not been as clearly demonstrated in a more physiological in vitro system such as human liver microsomes or cultured hepatocytes. In this study we evaluated this interaction in human hepatocytes. Firstly, an analysis of vendor-supplied CYP2C9 and CYP3A4 activity levels from cryopreserved human hepatocytes was performed. This analysis indicated a direct correlation between CYP2C9 and CYP3A4 activities. This observation is inconsistent with the finding in recombinant systems where increased CYP3A4 levels led to decreased CYP2C9 activity[5]. Therefore, an attempt to dissociate the co-regulation of CYP2C9 and CYP3A4 constitutive protein expression, using siRNA, was undertaken. By utilizing a long-term, micropatterned, human hepatocyte and mouse fibroblast co-culture (HepatoPac®)[6], we were able to determine the modulation of CYP2C9 activity in response to selective knockdown and recovery of CYP3A4. CYP3A4 specific gene silencing resulted in a 70% decrease in CYP3A4 activity, as determined by monitoring midazolam hydroxylation, which was commensurate with a 67% increase in CYP2C9 activity, as determined by monitoring diclofenac hydroxylation. Conversely, once the siRNA was removed, both CYP3A4 and CYP2C9 activities returned to pre-knockdown levels. CYP2C9 and CYP3A4 activities were also evaluated following induction by rifampicin, with and without concomitant siRNA-mediated knockdown of CYP3A4 using SMARTpool® Accell® siRNA®. Knockdown of CYP3A4 levels, in the presence of rifampicin resulted in a 89% increase in CYP2C9 activity above the induced activity from vector control treated wells [3.5-fold (-siRNA) to 6.6-fold (+siRNA)]. While the absolute CYP3A4 activity of siRNA treated wells was lower than vector control treated wells there was no measurable increase in the magnitude of induction for CYP3A4 activity when compared to their respective non-induced control levels [4.7-fold (-siRNA) to 5.1-fold (+siRNA)]. These results demonstrate that modulation of CYP3A4 expression can lead to appreciable changes in CYP2C9 activity in cultured human hepatocytes. The clinical consequence of these findings is unclear. Contributions to this work, provided by T.S.T, were supported by a grant from the National Institutes of Health (GM 086891).

INTRODUCTION

CYP450 protein-protein interactions have been characterized in recombinantly expressed CYP450 systems for the inhibition of CYP2C9 activity in the presence of CYP3A4 [5]. The clinical implications of such an interaction are unclear, partly due to lack of data demonstrating this effect in a more physiologically relevant model. Moreover, the overpowering effect of the co-regulation of both CYP3A4 and CYP2C9 by the pregnane-X-receptor may obscure the inhibiting effect of CYP3A4 on CYP2C9. This is consistent with the well-known observation that PXR activation in human hepatocytes can increase CYP3A4 activity more than CYP2C9 activity[7].

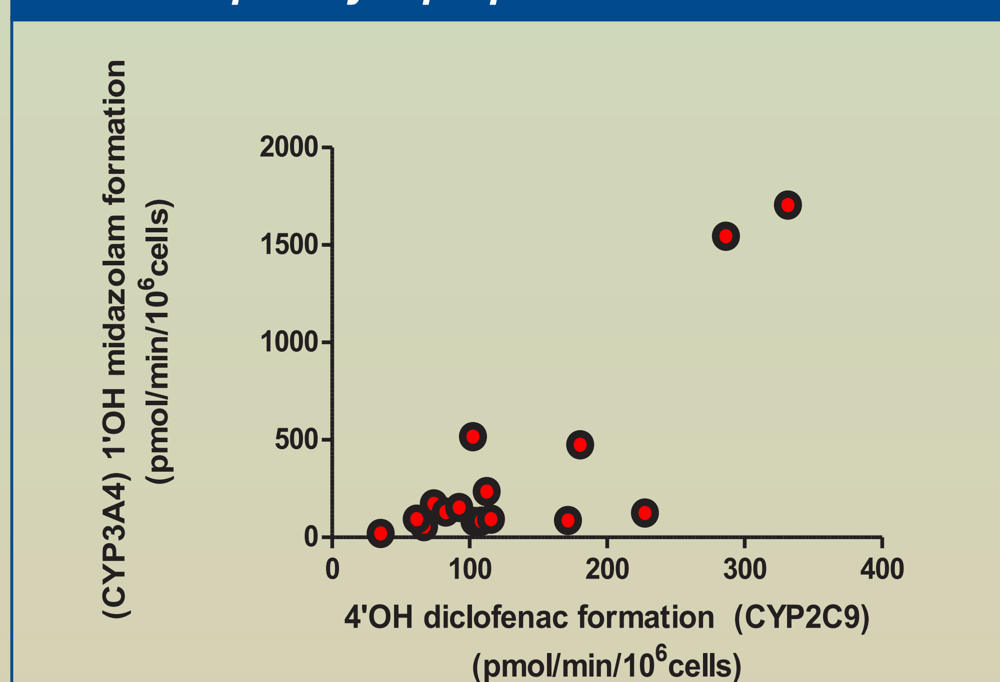
HepatoPac is a newly developed primary hepatocyte model for use in drug development studies. It has longevity in viability that is superior to suspended hepatocytes and maintains high enzyme activity for several weeks after seeding[6]. Using HepatoPac, we have provided strong evidence of the existence of CYP2C9:CYP3A4 protein-protein interactions in human hepatocytes by monitoring CYP2C9 activity while modulating the expression of CYP3A4 using siRNA and PXR induction (rifampicin).

METHODS

HepatoPac® co-cultures were prepared at Hepregen Corporation (Medford, MA) and shipped to Boehringer Ingelheim Pharmaceuticals in Ridgefield, CT. Dharmacon® SMARTpool® Accell® siRNA (Thermo Scientific, Lafayette, CO) was used at a concentration of 1 µM as described in the product kit insert. Initial studies were performed to optimize the conditions for maximal knockdown (data not shown). After 72 hours of siRNA treatment cells were washed and enzyme activity was assessed under linear time point conditions. In all cases the probe substrate concentration was considered to be at V_{max} concentrations with the exception of Midazolam (15 µM) due to an apparent concentration-dependent decrease in activity at V_{max} concentrations observed during kinetic experiments (data not shown).

RESULTS

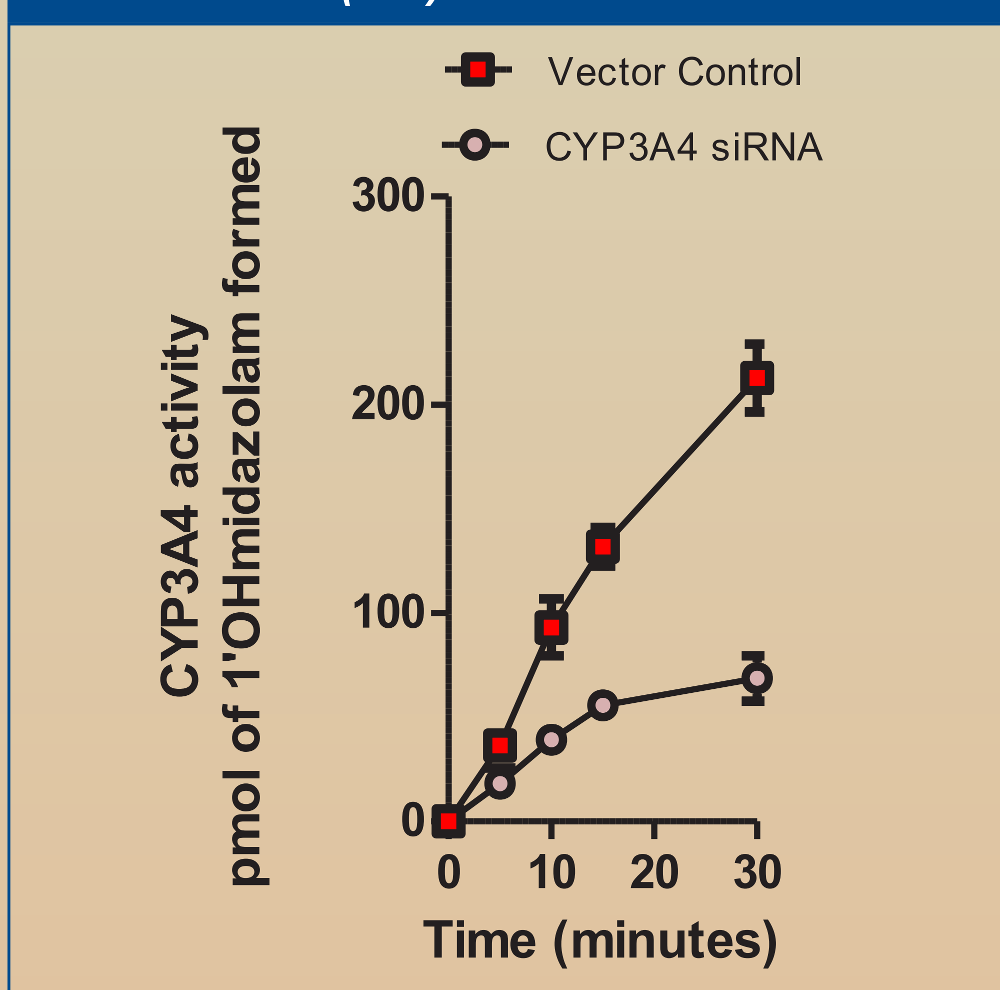
Figure 1: Correlation analysis for CYP2C9 and CYP3A4 activities in individual cryopreserved human hepatocyte preparations[8]



CYP2C9:CYP3A4 interaction could not be observed in suspended hepatocytes from 16 donors

- *Pearson correlational analysis indicated a significant positive correlation (r2=0.69, P<0.0001) between CYP2C9 and CYP3A4 activity*
- *This is an expected result, since both enzymes are co-regulated by PXR.*

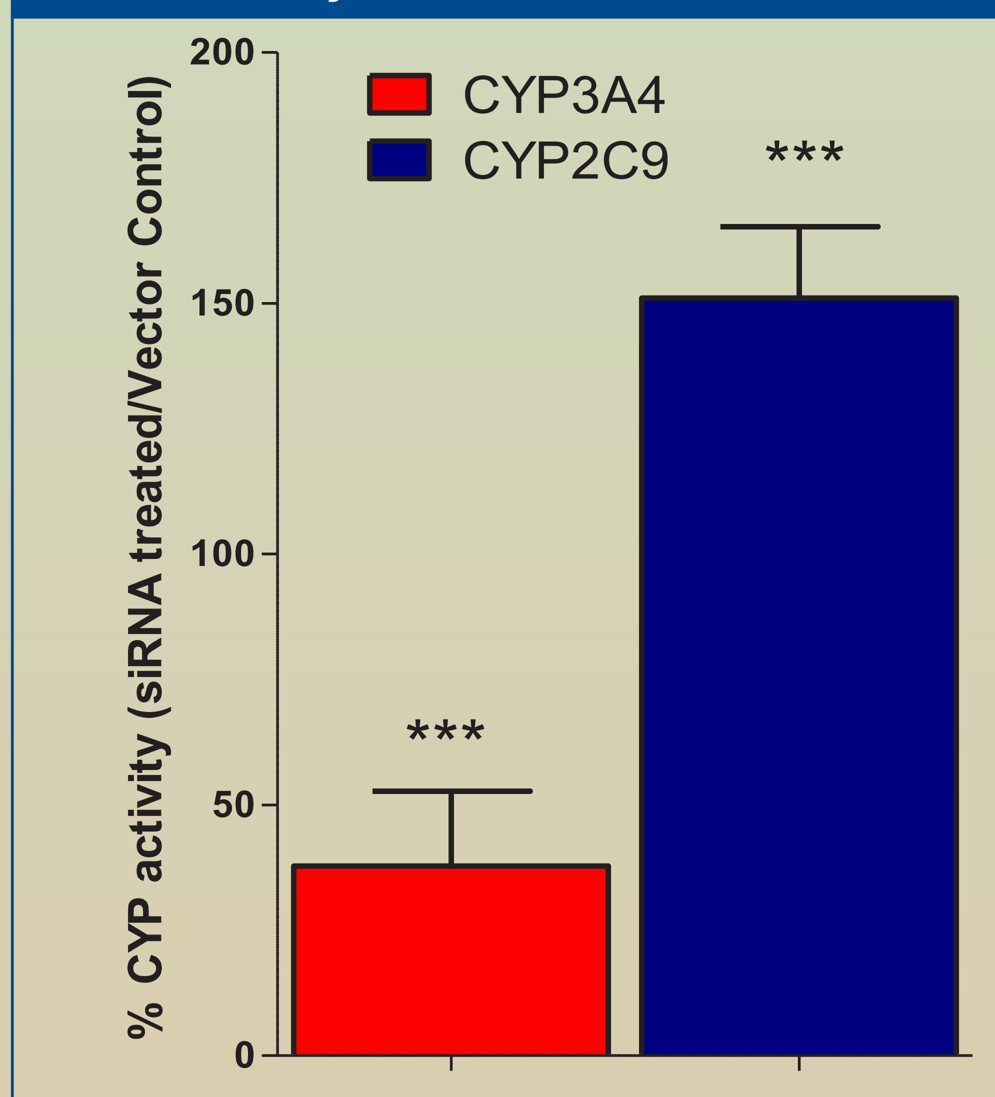
Figure 2: Demonstrating functional knockdown of CYP3A4 activity from 72 hour treatment with CYP3A4 siRNA (n=3)



CYP3A4 siRNA is effective for knocking down CYP3A4 activity in human HepatoPac cultures.

- *Treatment with siRNA leads to an 80% reduction in CYP3A4-mediated hydroxylation of midazolam.*

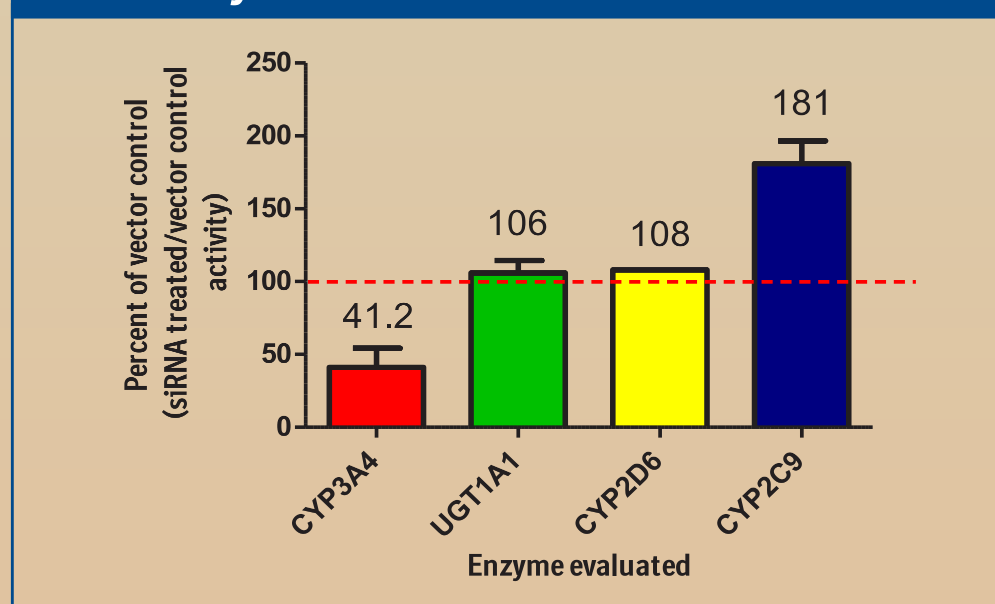
Figure 3: Increase in CYP2C9 activity was associated with siRNA-mediated knockdown of CYP3A4 activity



After specific knockdown of CYP3A4, the activity of CYP2C9 increased up to 150% of the vector control

- *CYP3A4 activity was determined by measuring the hydroxylation of midazolam, CYP2C9 activity was determined by measuring the hydroxylation of diclofenac. Data represents the average change in activity levels from 4 preparations of two different HepatoPac donors.*

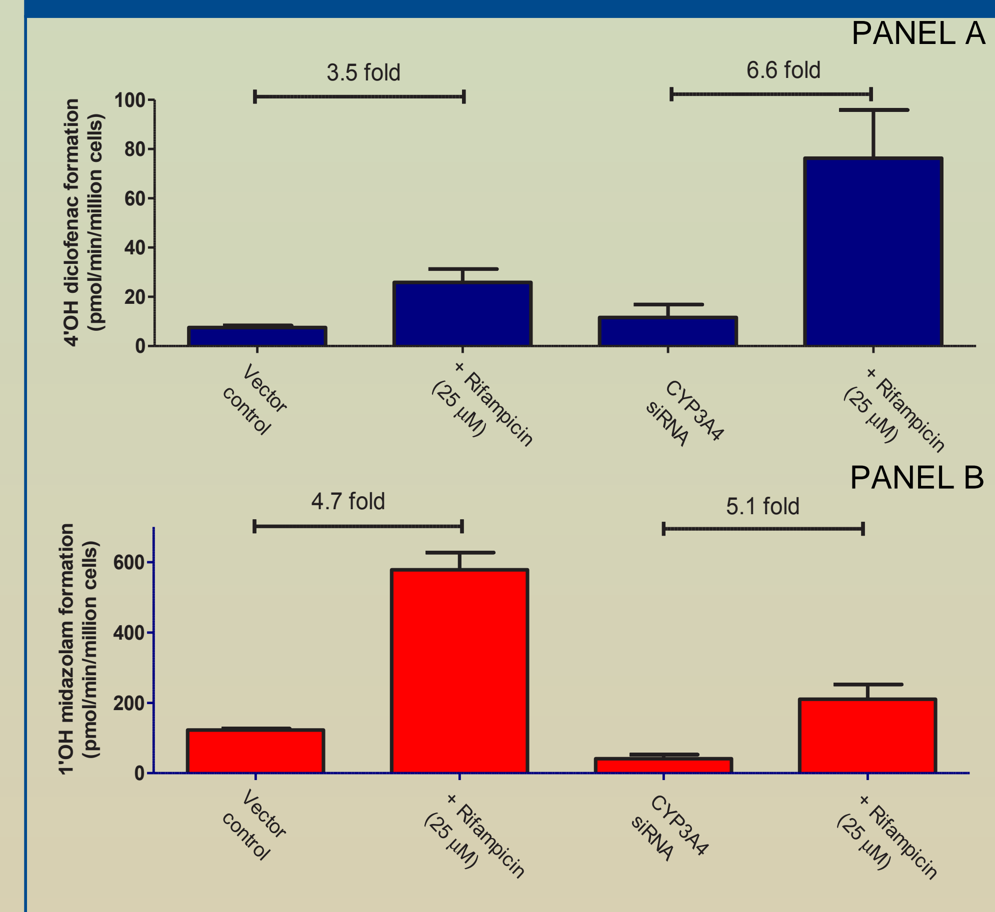
Figure 4: CYP3A4 targeted siRNA did not affect the activity of UGT1A1 and CYP2D6



Enhancement of enzyme activity associated with CYP3A4 knockdown is unique to CYP2C9

- *There was no effect of CYP3A4 siRNA treatment on UGT1A1 (estradiol 3-glucuronidation) or CYP2D6 (dextromethorphan o-demethylation) enzyme activities. (n=3)*

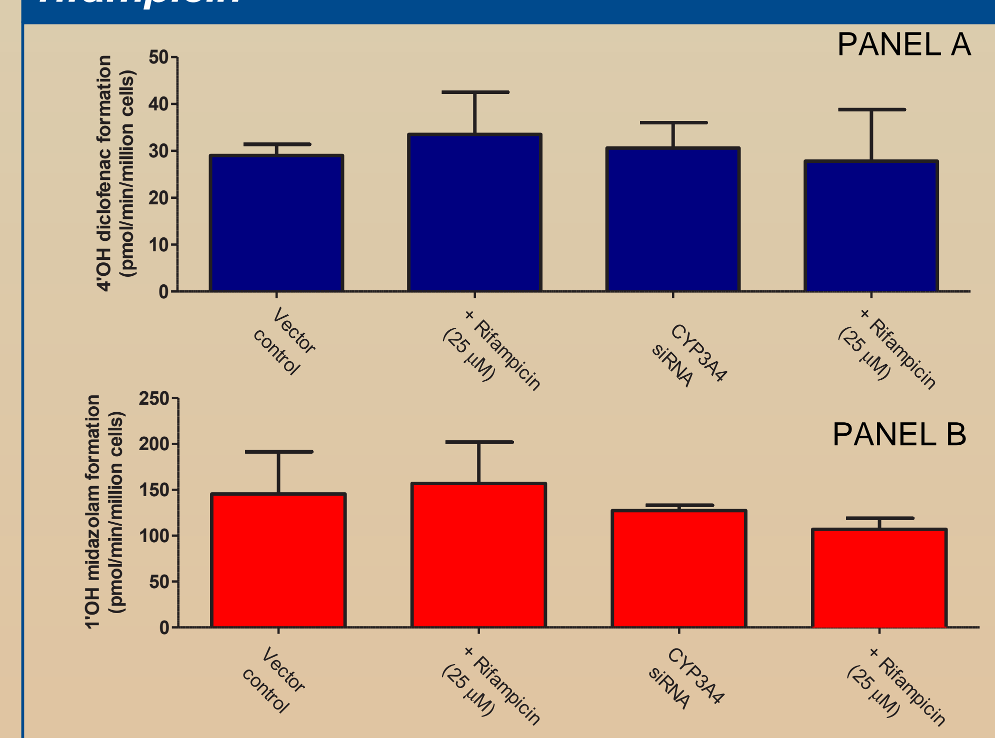
Figure 5: Effects of CYP3A4 knockdown on CYP2C9 and CYP3A4 induction



CYP3A4 knock down augments CYP2C9 induction by rifampicin

- *Rifampicin increases CYP2C9 activity 3.5 fold with the vector control, but 6.6 fold with CYP3A4 knockdown (Panel A)*
- *Rifampicin increases CYP3A4 activity by approximately 5 fold in the presence or absence of CYP3A4 knockdown (Panel B)*

Figure 6: Return of CYP3A4 and CYP2C9 activity to control levels following removal of siRNA and rifampicin



Both knockdown of CYP3A4 activity and accompanying enhancement of CYP2C9 activity are reversible

- *CYP2C9 activity returns to the level of the vector control after removal of the CYP3A4 siRNA and or rifampicin (Panel A)*
- *CYP3A4 activity returns to the level of the vector control after removal of the CYP3A4 siRNA and or rifampicin (Panel B)*

DISCUSSION AND CONCLUSIONS

We have provided compelling evidence that CYP3A4 decreases CYP2C9 through a protein-protein interaction in human hepatocytes. This novel finding was supported by:

1. Knockdown of CYP3A4 activity which increases CYP2C9 activity but not UGT1A1 or CYP2D6 activity.
2. A return of CYP2C9 activity to baseline levels after recovery of CYP3A4 activity following its knockdown.
3. Knockdown of CYP3A4 activity enhanced rifampicin-mediated induction of CYP2C9, but did not affect the magnitude of CYP3A4 induction.
4. A return of normal CYP2C9 activity or inducibility following the recovery of siRNA-suppression of CYP3A4 activity.

This study also suggests that the failure to observe a negative correlation between CYP3A4 and CYP2C9 activity in suspended hepatocytes is due to the co-regulation of CYP3A4 and CYP2C9 by PXR. In most cases PXR-mediated co-induction of CYP2C9 and CYP3A4 leads to a net increase in CYP2C9 activity. However, it is possible that selectively changing CYP3A4 activity (by some other mechanism) could unexpectedly lead to a change in the clearance of CYP2C9-metabolized drugs.

Additional studies are on-going and will include analysis of mRNA levels and protein levels to further support the findings from enzyme activity assessments.

REFERENCE LIST

1. Backes WL, Batie CJ, Cawley GF. Interactions among P450 enzymes when combined in reconstituted systems: formation of a 2B4-1A2 complex with a high affinity for NADPH-cytochrome P450 reductase. *Biochemistry* 1998;37(37):12852-12859.
2. Hazai E, Bikadi Z, Simonyi M, Kupfer D. Association of cytochrome P450 enzymes is a determining factor in their catalytic activity. *J Comput Aided Mol Des* 2005;19(4):271-285.
3. Kelley RW, Reed JR, Backes WL. Effects of ionic strength on the functional interactions between CYP2B4 and CYP1A2. *Biochemistry* 2005;44(7):2632-2641.
4. Subramanian M, Low M, Locuson CW, Tracy TS. CYP2D6-CYP2C9 protein-protein interactions and isoform-selective effects on substrate binding and catalysis. *Drug Metab Dispos* 2009;37(8):1682-1689.
5. Subramanian M, Tam H, Zheng H, Tracy TS. CYP2C9-CYP3A4 protein-protein interactions: role of the hydrophobic N terminus. *Drug Metab Dispos* 2010;38(6):1003-1009.
6. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. Nat *Biotechnol* 2008;26(1):120-126.
7. Sahi J, Shord SS, Lindley C, Ferguson S, LeCluyse EL. Regulation of cytochrome P450 2C9 expression in primary cultures of human hepatocytes. *J Biochem Mol Toxicol* 2009;23(1):43-58.
8. Ramsden D, Wallace G, Smith C, Chan T. A distribution analysis of cytochrome P450 activities in cryopreserved human hepatocytes from over 400 individuals. *Drug Metab Rev* 2009;41(s3):110-111.