Comparison of Inhibition of CYP1A2, 2C9 and 3A4 using Human Liver Microsomes and Hepatocytes

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ABSTRACT

Determination of inhibition of drug metabolizing enzymes is a key component to assessing the risk of drug-drug interactions (DDI). Inhibition may lead to greater bioavailability and lower clearance of concomitant drugs which will alter efficacy and duration that may lead to toxicities and dysfunction. Due to this importance, drug candidates are screened for potential inhibition during drug discovery and development. Typically, this is performed using human liver microsomes (HLM), a simple system of membrane bound enzymes such as cytochrome P450s (CYPs) which are responsible for the majority of phase I drug metabolism in humans. However, growing evidence indicates CYP inhibition may be influenced by factors that are absent from microsomes. Passive membrane impermeability, transporters and cytosolic enzymes may affect the intracellular concentration of drug and thereby alter its effective inhibition potential. The drug, too, may be sequestered in intracellular compartments such as lysosomes which will decrease the availability of the drug to elicit inhibition of an enzyme. To better predict in vivo inhibition potential, whole cell incubations with hepatocytes are recommended to incorporate such influences that are lacking in microsomal preparations.

Here, we designed an automated high-throughput screen (HTS) system utilizing both HLM and human hepatocytes to be performed in 384-well format with luminescent probes for CYP1A2, 2C9 and 3A4. Z'-factor for both HLM and hepatocytes utilizing the three substrates were all greater than 0.7, indicating an excellent assay system. Twelve inhibitors (four specific inhibitors per CYP) were profiled against the three CYPs in an 11-point concentration response curve. IC_{50} values were determined and compared between HLM and hepatocyte incubations. Alpha-naphthoflavone provided approximately 9-fold higher IC50 in hepatocytes versus HLM, while less than 3-fold difference for fluvoxamine, furafylline and propranolol. The inhibitors for CYP2C9 (diclofenac, fluconazole, fluoxetine and sulfaphenazole) provided similar IC_{50} between the two systems. For CYP3A4, ketoconazole IC_{50} in HLM was 4-fold lower than in hepatocytes. Ritonivir and verapamil IC_{50} were higher in HLM than hepatocytes by 8- and 11-fold, respectively. Troleandomycin provided similar IC_{50} between the two systems.

The design of parallel experiments using HLM and hepatocytes allows for comprehensive profiling of inhibition potentials. Differences between the two systems may be elucidated to determine mechanism of action which may aid in designing a more efficient and informative *in vivo* study.

Introduction

Drug-drug interactions (DDIs) are of particular concern for regulatory agencies and the pharmaceutical industry for drug safety. Inhibition is one form of DDI that involves a drug interacting with an enzyme of transporter that alters the clearance of a second drug. Most common concern of DDI occurs when a perpetrator drug inhibits metabolizing enzymes slowing the clearance of a prey drug which may increase potential toxicity, extend therapeutic effects of the prey drug and increase accumulation of the prey drug. Traditionally, microsomes have been used as the gold standard for determining inhibition potential. Microsomes are a subcellular fraction that contains membrane bound proteins of the endoplasmic reticulum such as P450 enzymes and associated proteins, UDP-glucuronosyl transferases (UGTs) and flavin-containing monooxygenases (FMO). It is a simple system that allows free access of the drug to the enzymes allowing for direct measurement of potential inhibitor-substrate interactions. However, microsomes are missing components found *in vivo* that may alter inhibition potential such as an intact cell membrane, transporters and intracellular compartments. The use of hepatocytes in determining inhibition potential has increased extolling their usefulness to study time dependent inhibition, *in vitro-in vivo* correlation and comparison between microsomal and hepatocytic derived data.[1, 2, 3] Further, a study from Brown denoted direct interplay of transporters and altered K_i which highlights the benefits of using hepatocytes for inhibition studies.[4] To date, no publication has compared microsomes and hepatocytes in a high through-put screen (HTS) protocol.

Herein, we describe a system to compare human liver microsomes (HLM) and hepatocytes to investigate inhibition of CYP1A2, CYP2C9 and CYP3A4 using luminescent probe substrates in 384-well format. IC_{50} values were derived from 11-point curve of 12 known inhibitors (four specific per target P450 enzyme) in parallel with HLM and human pooled hepatocytes without the need for bioanalytical equipment and expertise. This simple and robust system demonstrates an automated solution to run luminescent CYP450 inhibition assays using primary hepatocytes in a profiling format for CYP1A2, CYP2C9 and CYP3A4. Validation and pharmacology data prove how the combination of cells, assay, and instrumentation provide rapid, dependable information on the inhibition of select CYP450-based drug metabolism in a cell-based format as compared to traditional microsomal assays.

Materials and Methods

Inhibitor Solution Preparation. Twelve inhibitors were dissolved in DMSO at stock concentration 100X of desired concentrations (Table 1). Intermediate dilutions were prepared in KHB to provide a 2X solution to the final desired concentration. Dilution series was made using Precision™ Pipeting System (BioTek).

HLM Preparation: HLM of a 50-donor pool provided by BioreclamationIVT were thawed and stored on ice. Dilution was made into KHB to provide 4X stock of 0.08 µg/µL of total protein.

Hepatocyte Preparation: LiverPool™ Pooled Human Hepatocytes (BioreclamationIVT) were prepared according to vendor's instruction. Quickly, cells were thawed, diluted into 50 mL of InVitroGRO™ HT medium, and centrifuged for 5 minutes at 50X gravity. The supernatant was removed and cell pellet was resuspended in KHB. Cell count was performed by Trypan blue exclusion and suspension diluted to 10^6 viable cells/mL.

Automated Assay Validation: See Figure 1 for workflow chart. Multiflo™ FX Microplate Dispenser (BioTek) was used to transfer solution to 384-well plate. Hepatocytes and HLMs were dispensed into 384-well microtiter plate with specific inhibitors α-naphthoflavone (CYP1A2), sulfaphenazole (CYP2C9) or ketoconazole (CYP3A4) at 10 µM or vehicle control. NADPH was added to HLM wells. The microtiter plates were warmed to 37°C prior to addition of P450-Glo ™ (Promega) substrate for CYP1A2 (Luciferin-1A2), CYP2C9 (Luciferin-H) or CYP3A4 (Luciferin-IPA). The plates were incubated for 60 minutes and Luciferin Detection Reagent (Promega) were added to each well. The plate was incubated at ambient temperature for 15 minutes and read on Synergy H4 plate reader (BioTek). Data was used to derive Z'-factor value and other statistical information.

Inhibitor Profiling Assay: Work flow was similar to assay validation (Figure 1) however concentration response curves were employed (Table 1). Hepatocytes and HLMs were dispensed into 384-well microtiter plate with CRC of inhibitors or vehicle control. NADPH was added to HLM wells. The microtiter plates were warmed to 37°C prior to addition of P450-Glo ™ (Promega) substrate for CYP1A2, CYP2C9 or CYP3A4. The plates were incubated for 60 minutes and Luciferin Detection Reagent (Promega) were added to each well. The plate was incubated at ambient temperature for 15 minutes and read on Synergy H4 plate reader (BioTek). Assay was performed in triplicate on three separate days and each condition was performed in quadruplicate within each data point. Data was used to derive IC_{50} values from non-linear regression using Graphpad Prism 5.

Inhibitors	Target CYP	Concentration Range [M]
α-Naphthoflavone	CYP1A2	$1.9 \times 10^{-9} – 5 \times 10^{-4}$
Fluvoxamine	CYP1A2	$2.9 \times 10^{-10} – 7.5 \times 10^{-4}$
Flurafylline	CYP1A2	$5.7 \times 10^{-10} – 1.5 \times 10^{-4}$
Propanol	CYP1A2	$1.9 \times 10^{-9} – 5 \times 10^{-4}$
Diclofenac	CYP2C9	$1.9 \times 10^{-9} – 5 \times 10^{-4}$
Fluconazole	CYP2C9	$1.9 \times 10^{-9} – 5 \times 10^{-4}$
Fluoxetine	CYP2C9	$1.9 \times 10^{-9} – 5 \times 10^{-4}$
Sulfaphenazole	CYP2C9	$2.4 \times 10^{-10} – 5 \times 10^{-4}$
Ketoconazole	CYP3A4	$9.5 \times 10^{-11} – 2.5 \times 10^{-4}$
Ritonivir	CYP3A4	$9.5 \times 10^{-11} – 2.5 \times 10^{-4}$
Troleandomycin	CYP3A4	$1.1 \times 10^{-9} – 3 \times 10^{-4}$
Verapamil	CYP3A4	$1.9 \times 10^{-9} – 5 \times 10^{-4}$

Table 1. List of inhibitors, their reported specificity and concentration range of the CRC.

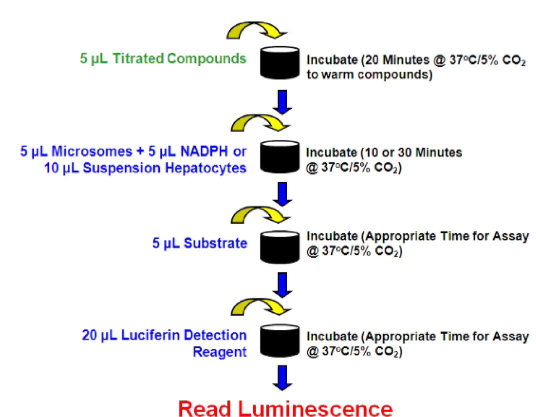

Figure 1. Workflow chart with associated instrumentation.

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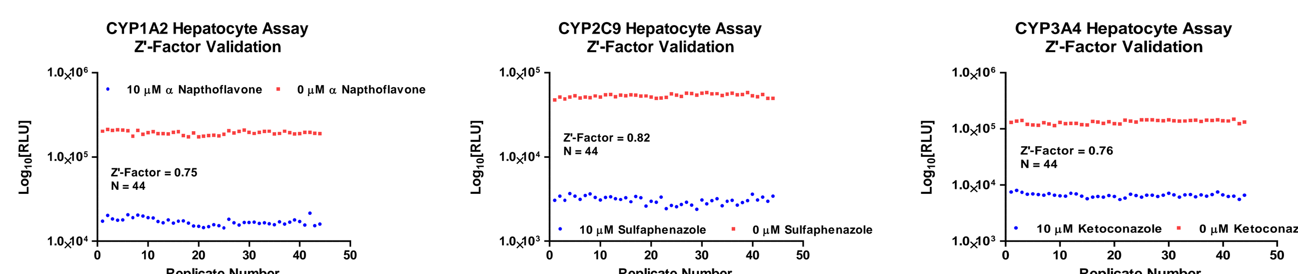
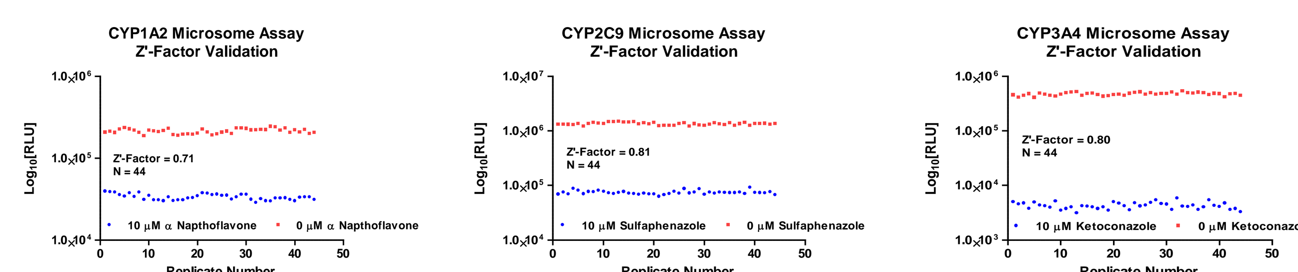
Automated Assay Validation

Results and Discussion: The system was tested to ensure that reagents and liquid handling equipment would provide a reproducible assay and that sufficient signal would be available in order to determine IC_{50} values. Single concentrations of known inhibitors for CYP1A2, CYP2C9 and CYP3A4 were used at sufficiently high concentrations to inhibit the majority of enzyme activity. Vehicle control samples measured total available activity as measured by specific luminescent substrates for the three CYPs.[5, 6] Graphs 2 and 3 are individual well RLU values for inhibited and uninhibited activities as measured in 384-well microtiter plate from pooled human hepatocytes and HLM, respectively. The results were used to derive a Z'-factor value which were > 0.5 for all assays indicating an excellent assay system.



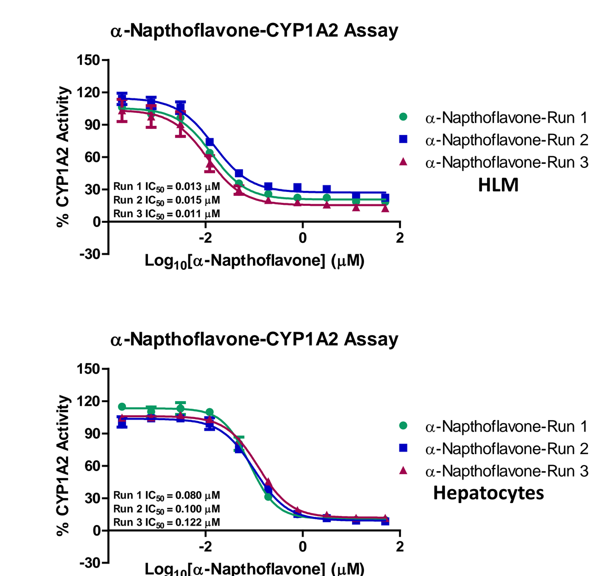
Graph 2 A-C. Graphical representation of raw RLU values from 44 wells of uninhibited and inhibited activities for CYP1A2, CYP2C9 and CYP3A4, and associated Z'-Factor for pooled human hepatocytes.



Graph 3 A-C. Graphical representation of raw RLU values from 44 wells of uninhibited and inhibited activities for CYP1A2, CYP2C9 and CYP3A4, and associated Z'-Factor for HLM.

CYP1A2

Inter-day Variation: IC_{50} values were calculated for prototypical inhibitors from three different days using pooled human hepatocytes and HLM. Graphs 2A-B depict the CRC and associated IC_{50} values for α-naphthoflavone from individual runs. Variation was minimal, ranging from 0.011 – 0.015 µM for HLM and 0.08 – 0.122 µM for pooled human hepatocytes. Similar consistency was observed from fluvoxamine, furafylline and propanol (graphs not shown). HLM provided less variation in IC_{50} values as compared to pooled human hepatocytes (Table 2). Since Z'-factor was similar (0.75:0.71 , hepatocytes:HLM), preparation variation such as cell counting may explain inter-day variation. However, the robustness of the assay may be observed with IC_{50} values that were statistically distinct. The hepatocyte-to-HLM phenomenon may be substrate specific due to the fact that the same trend was not observed for CYP2C9 nor CYP3A4 assay.



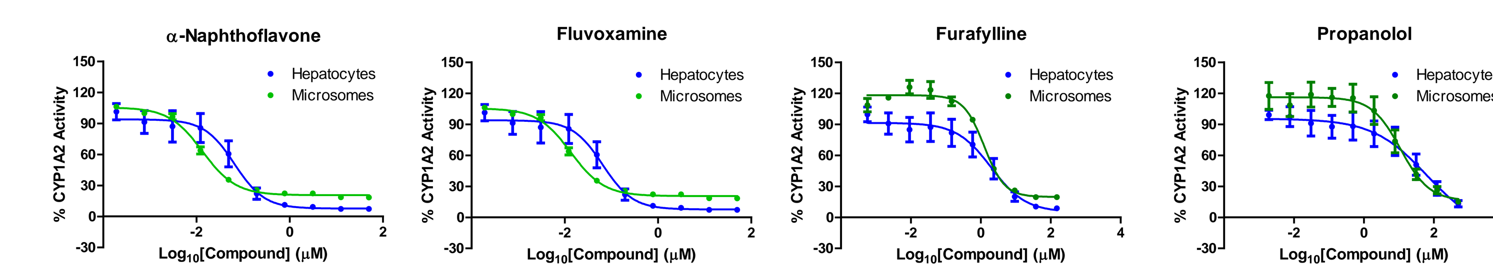
Graph 4 A-B. Comparison of inter-day results inhibited CYP1A2 activity from HLM and pooled human hepatocytes.

HLM and Hepatocyte Comparison: IC_{50} values were compared between pooled human hepatocytes and HLM. Graphs 5A-D depict the CRC and associated IC_{50} values for α-naphthoflavone, fluvoxamine, furafylline and propanolol. The CRC ranged across six log orders. As such, plateaus were achieved at highest and lowest percent of activities for most of the inhibitors allowing good curve fits and IC_{50} determinations. Propanolol did not achieve a low end plateau, however values were derived that were statistically acceptable. The IC_{50} values compared favorably to literature values for HLM (Table 2). Since inhibitor values are dependent upon probe substrate, this indicates that the probe substrate Luciferin-1A2 interacts similarly to CYP1A2 as drug probe substrate phenacetin, allowing for translation between traditional drug probes and Luciferin-1A2 and confidence in relevant data generated by Luciferin-1A2.

Furafylline and propanolol showed no major differences in IC_{50} values between HLM and hepatocytes with fold difference less than 2. Furafylline is a known mechanistic inhibitor and may confound any secondary variables that could influence inhibitor availability at the enzyme site such as uptake transporters. Attempts for time-dependent inhibition with a 30 minute pre-incubation were investigated but were flawed due to inability to wash out the inhibitor or dilute its presence, which were limitations of HTS screening in 384 well format.

α-Naphthoflavone was more potent in HLM than hepatocytes and had the greatest fold difference at 7.4. Though previously used by other researchers with hepatocytes as inhibitor of CYP1A, differences between hepatocytes and HLM have not been published. Further investigation is warranted to explain this difference.

Fluvoxamine inhibition has previously been described in rat HLM and hepatocytes, however as an inhibitor of CYP2C9, not CYP1A.[3] As a lipophilic base, fluvoxamine has been shown to be partitioned in the liver, possibly through lysosomal uptake which would account for a less inhibition in hepatocytes than HLM. Though K_i values were not altered between HLM and hepatocytes in Brown article (< 2-fold), a difference of 3.7 fold of IC_{50} was observed here. K_i value may need to be generated for CYP1A2 to fully characterize the difference.



Graph 5 A-D. IC_{50} graphs from concentration response curves of know CYP1A2 inhibitors as measured using HLM and human pooled hepatocytes.

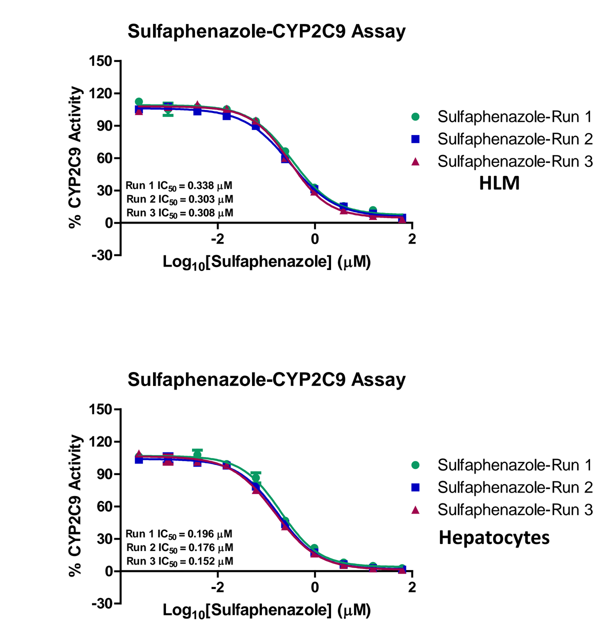
IC_{50} Values (µM)

Inhibitor	HLM Literature Values	Hepatocytes	Microsomes	Fold Difference
	CYP1A2			
α-Napthoflavone	0.01 [7]	0.096 ± 0.029	0.013 ± 0.002	7.4
Fluvoxamine	0.24 – 0.48 [8]	0.42 ± 0.23	0.114 ± 0.032	3.7
Furafylline	0.6 – 0.73 [7]	2.44 ± 0.44	1.20 ± 0.097	2.0
Propanolol	8.9 – 77.5 [9]	48.25 ± 12.62	16.52 ± 5.11	2.9

Table 2. IC_{50} values of know CYP1A2 inhibitors as measured using HLM and human pooled hepatocytes and the fold difference between the two values.

CYP2C9

Inter-day Variation: IC_{50} values were calculated for prototypical inhibitors from three different days using pooled human hepatocytes and HLM. Graphs 6A-B depict the CRC and associated IC_{50} values for sulfaphenazole from individual runs. Variation was minimal, ranging from 0.303 – 0.338 µM for HLM and 0.152 – 0.196 µM for pooled human hepatocytes. Similar consistency was observed from diclofenac, fluconazole and fluoxetine (graphs not shown). Hepatocytes provided less variation in IC_{50} values as compared to HLM (Table 3) in contract to CYP1A2 data, however the range was minimal (5% - 14% for hepatocytes and 6% - 21% for HLM). Likewise, the Z'-factor was similar (0.82:0.81 , hepatocytes:HLM) concurring with the robustness of this assay.

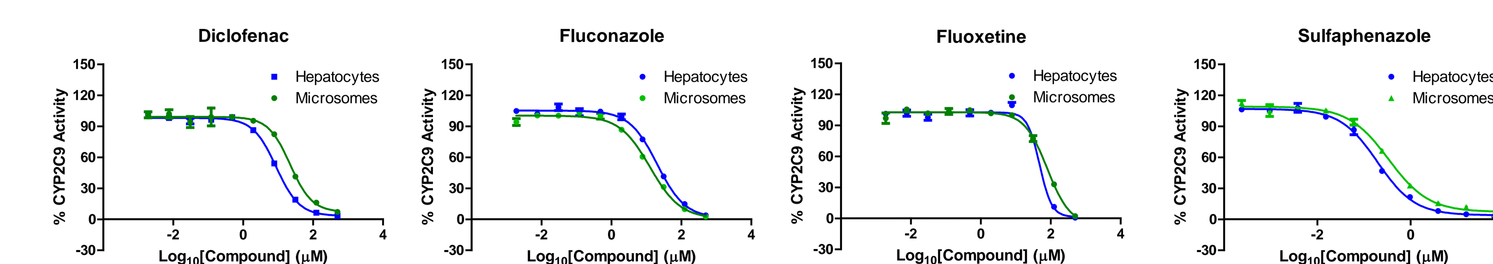


Graph 6 A-B. Comparison of inter-day results inhibited CYP2C9 activity from HLM and pooled human hepatocytes.

HLM and Hepatocyte Comparison: IC_{50} values were compared between pooled human hepatocytes and HLM. Graphs 7A-D depict the CRC and associated IC_{50} values for diclofenac, fluconazole, fluoxetine and sulfaphenazole. The CRC ranged across six log orders. Plateaus were achieved at lowest percent of activities for all the inhibitors, however, diclofenac, fluconazole and fluoxetine reached near 0% activity only at the highest concentration. Non-linear regression of the data proved good curve fits and IC_{50} determinations within reasonable limits. Sulfaphenazole achieved sigmoidal curve with IC_{50} value near the center of the CRC.

No significant difference was observed between HLM and hepatocytes IC_{50} values (< 2.5 fold) indicating no cellular interplay for the four inhibitors. Brown, too, found know significant difference in comparison of fluoxetine K_i values.[3] Diclofenac provided a difference of 2.4 which may be significant if the Brown's threshold of 2 is utilized. One potential reason is that diclofenac is a substrate for OATP1B3 which may increase intracellular concentration as compared to HLM concentrations.[10]

The IC_{50} values compared favorably to literature values for HLM (Table 3). As with Luciferin-1A2, Luciferin-H appears to interact with CYP2C9 similarly to traditional drug probes, such as diclofenac, flurbiprofen and tolbutamide. This observation has been documented by other researchers.[11]



Graph 7 A-D. IC_{50} graphs from concentration response curves of know CYP2C9 inhibitors as measured using HLM and human pooled hepatocytes.

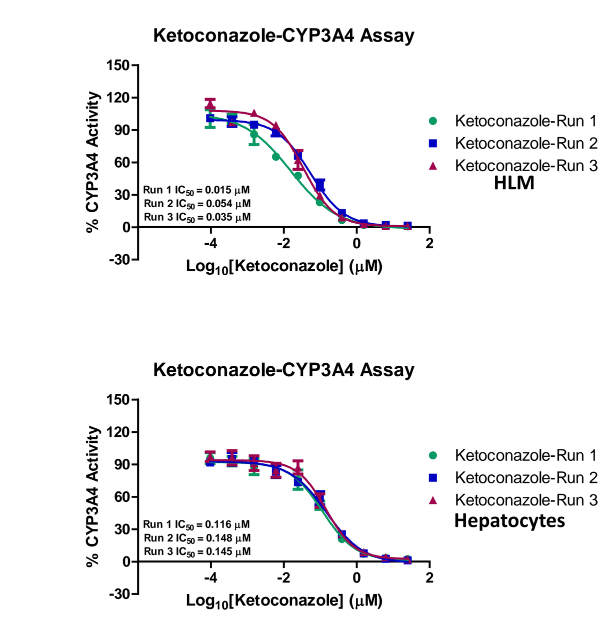
IC50 Values (µM)

Inhibitor	HLM Literature Values	Hepatocytes	Microsomes	Fold Difference
	CYP2C9			
Diclofenac	3.4 - 52 [7]	7.85 ± 1.11	18.62 ± 4.04	2.4
Fluconazole	7 [7]	18.94 ± 1.53	10.96 ± 1.74	1.7
Fluoxetine	18 - 41 [7]	45.49 ± 2.47	73.80 ± 9.42	1.6
Sulfaphenazole	0.3 [7]	0.17 ± 0.022	0.32 ± 0.019	1.9

Table 3. IC_{50} values of know CYP2C9 inhibitors as measured using HLM and human pooled hepatocytes and the fold difference between the two values.

CYP3A4

Inter-day Variation: IC_{50} values were calculated for prototypical inhibitors from three different days using pooled human hepatocytes and HLM. Graphs 8A-B depict the CRC and associated IC_{50} values for ketoconazole from individual runs. Some variation was observed with HLM ranging from 0.015 – 0.054 µM, a 3.6-fold difference. Hepatocytes provided consistent data between runs with an IC_{50} range of 0.116 – 0.148 µM for pooled human hepatocytes. The variation observed with ketoconazole in HLM may be isolated since ritonavir, troleandomycin and verapamil variation was minimal (graphs not shown). Standard error reflected the variation with microsomal ketoconazole error of 54% and hepatocytic troleandomycin error of 34%. The Z'-factor was similar (0.76:0.80 , hepatocytes:HLM) concurring with the robustness of this assay.

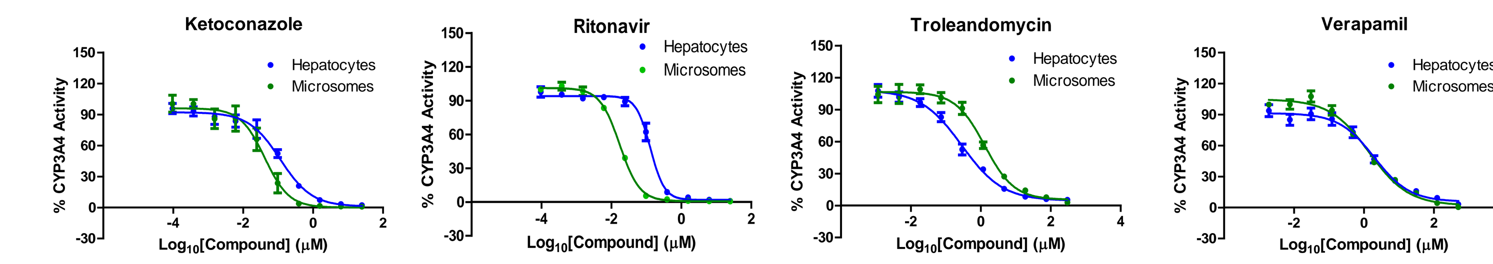


Graph 8 A-B. Comparison of inter-day results inhibited CYP3A4 activity from HLM and pooled human hepatocytes.

HLM and Hepatocyte Comparison: IC_{50} values were compared between pooled human hepatocytes and HLM. Graphs 9A-D depict the CRC and associated IC_{50} values for ketoconazole, ritonavir, troleandomycin and verapamil. The CRC ranged across six log orders. Plateaus were achieved at highest and lowest percent of activities for the inhibitors allowing good curve fits and IC_{50} determinations. Non-linear regression of the data proved good curve fits and IC_{50} determinations within reasonable limits.

Ketoconazole, ritonavir and verapamil had fold differences greater than 3 (Table 4). Ketoconazole and ritonavir IC_{50} values were lower in hepatocytes than HLM. This trend was observed in rat hepatocytes for ketoconazole by Brown and was attributed to intracellular binding since uptake was significant but K_i was higher than microsomal determination.[3] No published data on inhibition difference between hepatocytes and microsomes have been found. Uptake and intracellular binding would have to be studied to better elucidate this observation. Ritonavir is an mechanistic inhibitor, as well, which may influence IC_{50} values. In contrast, inhibition with verapamil in hepatocytes was greater then in HLM which concurred with previous reports.[12]

The IC_{50} values compared favorably to literature values for HLM (Table 4). As with Luciferin-1A2 and Luciferin-H, Luciferin-IPA appears to interact with CYP3A4 similarly to traditional drug probes, such as testosterone and midazolam.[5, 13, 14]



Graph 9 A-D. IC_{50} graphs from concentration response curves of know CYP3A4 inhibitors as measured using HLM and human pooled hepatocytes.

IC50 Values (µM)

Inhibitor	HLM Literature Values	Hepatocytes	Microsomes	Fold Difference
	CYP3A4			
Ketoconazole	0.0037 – 0.18 [7]	0.14 ± 0.028	0.035 ± 0.019	4.0
Ritonavir	0.41 – 0.9 [7]	0.13 ± 0.02	0.016 ± 0.001	8.1
Troleandomycin	0.5 [1]	0.61 ± 0.21	1.26 ± 0.26	2.1
Verapamil	10 – 24 [7]	1.60 ± 0.36	17.45 ± 4.11	10.9

Table 4. IC_{50} values of know CYP3A4 inhibitors as measured using HLM and human pooled hepatocytes and the fold difference between the two values.