ApplicationNote

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Transcript regulation of 18 ADME genes by prototypical inducers in human hepatocytes

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Abstract

Due to recent FDA draft guidance, the importance of measuring mRNA to assess induction potential is paramount. The fundamental change from measuring enzyme activities to transcripts will have profound implications for workflow in studying induction drug-drug interactions (DDI). As with previous guidance, representative genes associated with the major induction pathways may be used to measure the induction potential of test articles. CYP1A2 is the marker for aryl hydrocarbon receptor (AhR) activation, CYP2B6 for constitutive androstane receptor (CAR), and CYP3A4 for pregnane X receptor (PXR) with potential of using CYP2C9 as a secondary marker. However, discrete enzymatic assays for individual CYP enzymes are replaced by a multiplexed assay to measure several genes from a single induction sample. This increase in data from a simplified system allows for basic screening with target markers as well as broader transcript surveys.

In this study, we used QuantiGene® Plex Assay to simultaneously measure 18 ADME transcripts (CYP1A2, 2B6, 2C9, 2D6, 3A4, 3A5; ABCB1, C2, C3, C4; SLC01B1, 01B3, 22A1; UGT1A1, 1A4, 1A9, 2B7, and SULT1E1) and two control genes (GAPDH and HRPT). We screened 11 donors of human cryoplateable hepatocytes at single concentrations of omeprazole (AhR), phenobarbital (CAR), and rifampicin (PXR) after 48-hour exposure in a 96-well format to observe individual variations in induction and suppression potentials. For omeprazole, induction greater than 2-fold was observed for CYP1A2, 2B6, 3A4, UGT1A1, and 1A4. Phenobarbital induced CYP2B6, 2C9, 3A4, 3A5, ABCB1, UGT1A1, and 1A4. Rifampicin induced CYP2B6, 2C9, 3A4, UGT1A1, and 1A4. Suppression greater than 30% was observed for SLC22A1 and UGT2B7 with omeprazole, for SULT1E1 for phenobarbital, and for SLC22A1 and UGT2B7 with rifampicin. No responses (<2 and >0.7) were observed for ABCC2, C3, C4, and SCC01B1 from all inducers. Mixed responses were seen in other genes.

For one donor, concentration response curves were generated for the prototypical inducers. EC_{50} values were calculated where applicable. Omeprazole EC_{50} value for CYP1A2 was 2 µM. Phenobarbital EC_{50} for CYP2B6 was 1.3 mM. Rifampicin EC_{50} for CYP3A4 was 260 nM and CYP2C9 170 nM. Dose dependent suppression was measured for several genes, such as SLC01B3 and UGT2B7 for omeprazole, SLC01B3 and SULT1E1 for phenobarbital, and ABCC3 and UGT1A9 with rifampicin.

The data shows the power of QuantiGene Plex Assay to generate profiles of transcript levels from a single concentration or a concentration response curve of an inducer in a multiplex format. Primary markers such as CYP1A2, 2B6, 2C9, and 3A4 can be used to fulfill regulatory requirements with a potential of adding secondary markers such as phase II enzymes or transporters to probe gene regulation from test articles.

Introduction

Drug-drug interactions (DDIs) are of particular concern for regulatory agencies and the pharmaceutical industry for drug safety. Induction of drug metabolizing enzymes by pharmaceuticals, nutraceuticals, and lifestyle influences is one type of DDI in which the influence of a perpetrator molecule increases the enzyme capacity that can metabolize a victim molecule, rendering it ineffective as a therapy. To evaluate this potential, screening assays have been developed, such as the use of hepatocytes or liver cell lines to measure *in situ* metabolism between control and exposed wells. As well, reporter gene assays such as PXR-linked luciferase construct assay have been employed.[1,2]
The FDA and other regulatory agencies have provided guidance documentation to summarize procedures and expectations in these matters. The use of human primary hepatocytes is the gold standard for evaluating induction potential of a test article as compared to prototypical inducers. Three key markers for the three major nuclear receptor pathways are measured to profile the induction potential: CYP1A2 for AhR, CYP2B6 for CAR, and CYP3A4 for PXR. However, in the most recent FDA guidance,[3] a major shift was documented. The reliance on *in situ* metabolism has been replaced with transcript measurements that were born out of articles touting their sensitivity and high correlation to clinical outcomes.[5,6,12] This new focus has altered the previous screening method for the pharmaceutical industry and for vendors who supply the primary hepatocytes.

The sensitivity of measuring distinct transcripts offers another benefit over *in situ* activities. A wide array of genes can be profiled to get a broader picture of the effects of a potential inducer beyond the three harbingers of the AhR, CAR, and PXR pathways. Several arrays have been published characterizing *in vitro* and *in vivo* responses.[7,8,9] In this approach, a broad assessment of gene regulation can be made from a single lysate and a single condition. Cell culture arrays can provide time-dependent and concentration-dependent analysis for the up- and down-regulation of affected genes. Branched DNA (bDNA) is one method that measures transcript levels by probing with capture extenders and label extenders in order to amplify the signal. It has been used successfully to probe for CYP3A4 induction across varying parameters.[9,10] The technology can be multiplexed to measure several transcripts from a single well in a single read.

Herein, we used bDNA technology to probe for 18 ADME genes and 2 control genes to screen across 11 individual donors for induction with three prototypical inducers: omeprazole, phenobarbital, and rifampicin. Individual responses, as well as general trends, were assessed. For one donor, a concentration response curve was used to determine EC_{50} values for those genes induced. The key biomarkers, CYP1A2, 2B6, and 3A4, were used to fulfill FDA regulatory guidance, as well as adding off-target markers to view trends in gene regulation associated with phase I and II metabolism, and transporter expression.

Materials and methods

Hepatocyte cultures – Human cryoplateable hepatocytes lots were obtained from Celsis In Vitro Technologies. Procedure for the thawing and plating of cryoplateable hepatocytes in InVitroGro™ CP medium, and the culturing and dosing in InVitroGro HI followed instructions for use as prescribed by Celsis In Vitro Technologies. The 96-well plates seeded with 50,000 viable hepatocytes per well were cultured for two days prior to induction. Several wells were left unseeded for controls in the QuantiGene® assay. For single concentration induction, wells were dosed with 0.1 mL of 50 µM omeprazole, 1 mM phenobarbital, or 25 µM rifampicin as positive controls (PC) in InVitroGro HI medium for 11 of the lots. Vehicle controls (VC) were 1% acetonitrile for omeprazole and rifampicin or media-only for phenobarbital. Concentration response curves (CRC) were performed on one lot (CDP) with omeprazole between 150 and 0.21 µM, phenobarbital between 9 and 0.01 mM, and rifampicin 75 and 0.01 µM. The inducers were exposed to the cells for 48 hours. Each condition was performed in duplicate.

mRNA preparation – At the end of the 48-hour induction, the cells were processed for QuantiGene Plex Assay using Sample Process Kit for Cultured Cells (QS0100) as directed in the package insert. Briefly, a lysis solution was prepared by mixing 130 µL of Proteinase K, 13 mL of Lysis Mixture, and 26 mL of InVitroGro KHB. Media was removed and 150 µL of lysis solution (37°C) was added to each well. The plate was incubated at 60°C for 30 minutes. After incubation, the contents of the well were pipetted up and down 10X to ensure thorough lysis of the cells. The plates were sealed and stored at <-70°C.

QuantiGene® 2.0 Plex Assay – QuantiGene Plex Assay from Affymetrix was used to quantitate 20 genes simultaneously on the Luminex® platform. Target-specific capture extenders and label extenders were incubated overnight at 54°C with the cell lysates and unique fluorescent beads. The beads are coated with capture probes specific to the capture extenders, thus cooperatively hybridizing each target gene to a unique bead. (Table 1). After overnight incubation, the bDNA signal amplification portion was initiated by first washing the beads followed by a 1-hour incubation with the pre-amplifier DNA mix at 50°C. The beads were next washed followed by a 1-hour incubation with the amplifier DNA solution mix at 50°C. Biotinylated DNA label probe was added to the wells and incubated at 50°C for 1 hour. Finally, the beads were washed followed by incubation at room temperature for 30 minutes with streptavidin phycoeurythin (SAPE). The beads were washed and read on the Luminex® instrument.

Phase I enzymes	Phase II enzymes	Transporters	Control genes
CYP1A2	UGT1A1	ABCB1	GAPDH
CYP2B6	UGT1A4	ABCC2	HPRT
CYP2C9	UGT1A9	ABCC3	
CYP2D6	UGT2B7	ABCC4	
CYP3A4	SULT1E1	SLCO1B1	
CYP3A5		SLCO1B3	
		SLC22A1	

Table 1: List of probe genes and their associated function.

Data analysis

The median fluorescence intensity units (MFI) for the samples were normalized to HPRT1 gene expression. GAPDH signal was saturated and could not be used to normalize the data. The adjusted MFIs were used to determine fold induction by dividing the average of treated wells by the average of the vehicle control wells for the associated inducers (PC/VC). Bar graphs were created in Excel for single concentrations. Concentration response curves were analyzed using Prism 5.0 for non-linear fit to determine EC_{50} value where applicable.

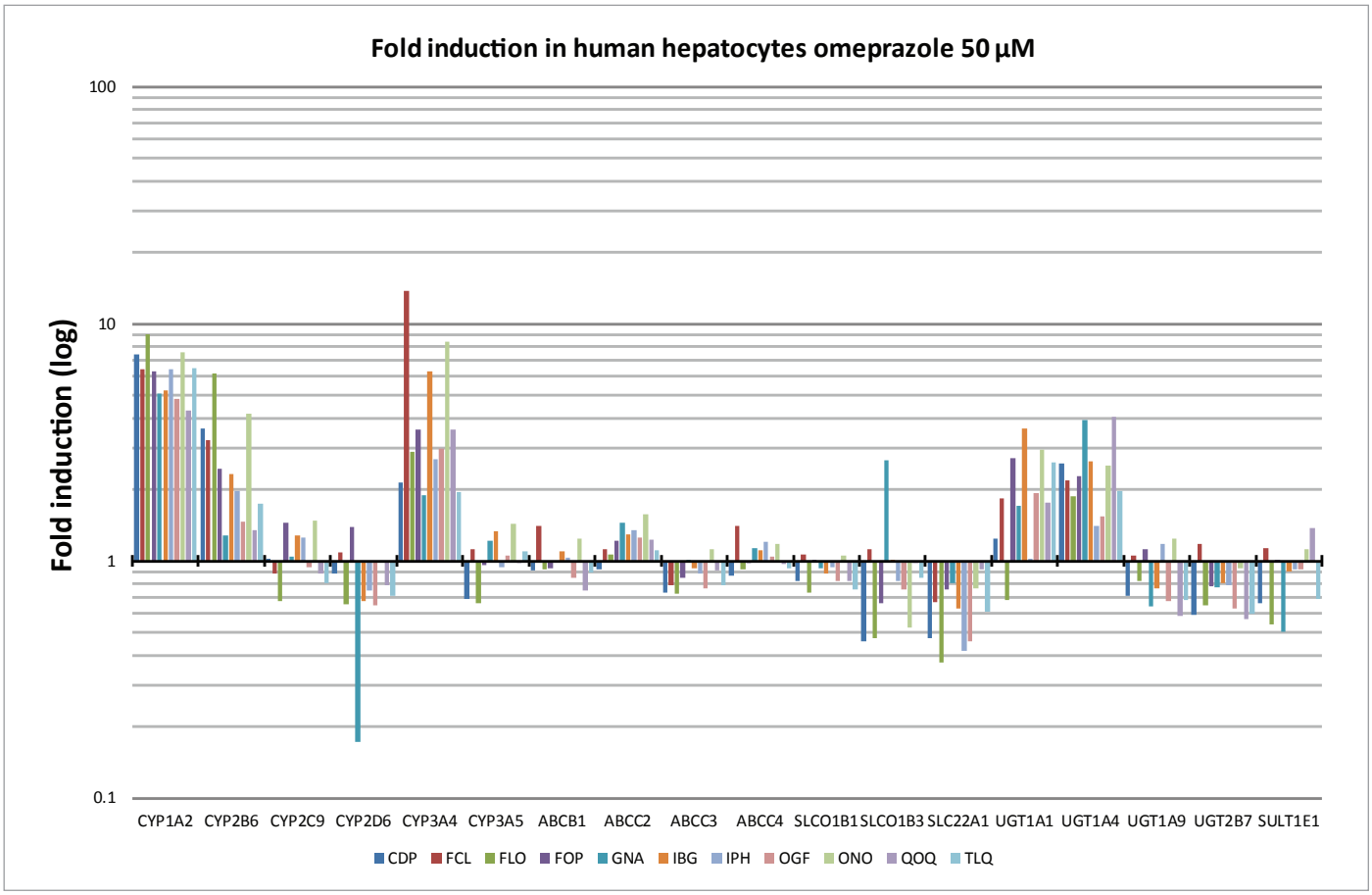
Results for single concentration induction – omeprazole

Omeprazole 50 µM induced mRNA levels of CYP1A2, the surrogate marker for AHR-mediated induction, in all lots tested. The range was 4.3- to 9-fold, and average was 6.3-fold. Omeprazole induced CYP2B6 greater than 2-fold in 6 of the 11 donors with a range of 1.3- to 6.2-fold. CYP3A4 was induced greater than 2-fold in 9 donors with a range of 1.9- to 13.7-fold. UGT1A4 induced greater than 2-fold in 7 of the 11 donors, ranging from 1.4- to 4-fold.

Significant reduction in mRNA of SLC22A1 was observed in 10 donors with a fold of less than 0.8. The minimum fold was 0.4 with an average of 0.6. UGT2B7 was reduced in 8 donors with a minimum of 0.6-fold. CYP2D6 showed an induction of less than 0.8 in 7 of the donors with the minimum fold of 0.2.

Discussion and conclusion for single concentration induction – omeprazole

The induction of CYP1A2 was induced by omeprazole as expected[3] as well as the induction of CYP3A4.[11,12] Induction of CYP2B6 confirmed results found by some researchers[8,13] while contradicting a previous literature report,[14] albeit the folds were modest and not all donors responded. UGT1A4 induction appears to be novel with no literature references linking omeprazole to its induction. Suppression of SLC22A1, UGT2B7, and CYP2D6 is unreported in the literature as well. Further study is needed to better elucidate these new responses.



Graph 1: Fold induction of 18 ADME genes after 48-hr exposure of 50 µM omeprazole.

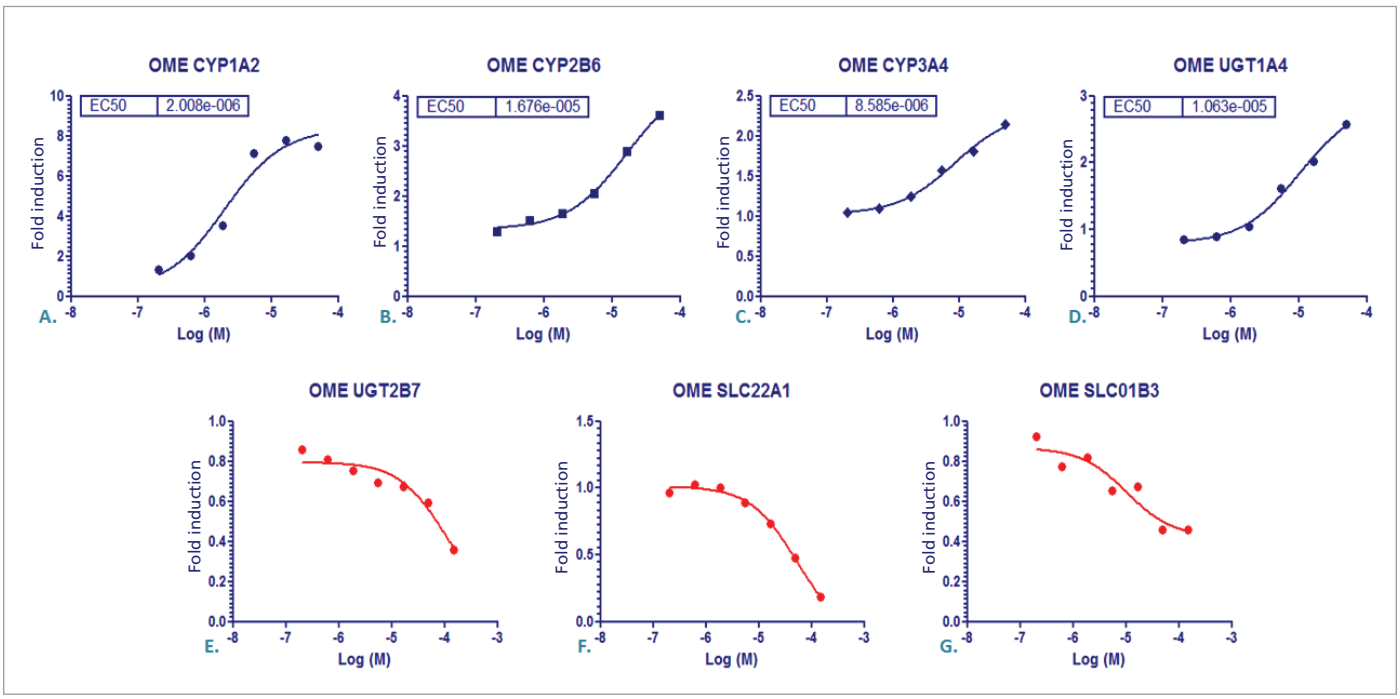
Results for concentration response curves – omeprazole

Omeprazole was dosed between 150 and 0.21 µM. The 150 µM induction result was removed from the EC_{50} calculations due to lower induction observed across all genes, possibly from toxicity but not confirmed. The highest concentration for the CRC was 50 µM for all calculations. Four genes, CYP1A2, 2B6, 3A4, and UGT1A4, exhibited induction greater than 2-fold within the CRC (Graph 2A–D).

Several genes exhibited a concentration-dependent reduction greater than 20% (Graph 2E–G). UGT2B7 had a modest reduction from 0.86- to 0.6-fold from low to high concentrations. SLC22A1 had a fold of 0.86 at 5.5 µM and continued to fall to 0.47 at 50 µM. SLC01B3 had reductions from 0.92 to 0.46 across the CRC. CYP2D6 was not significantly reduced in lot CDP (0.86-fold at 50 µM), and therefore, no concentration-dependent response was observed. All other genes remain relatively constant across the CRC except at the 150 µM concentration.

Discussion and conclusion for concentration response curves – omeprazole

As previously noted, CYP1A2 and CYP3A4 responses were as expected. Responses in CYP2B6, UGT1A4, UGT2B7, SLC22A1, and SLC01B3 have not been cited in literature and appear to be concentration dependent. Therefore, these may represent novel responses warranting further study.



Graph 2A–G: Induction concentration response curves and EC_{50} value determination of (A.) CYP1A2, (B.) CYP2B6, (C.) CYP3A4, (D.) UGT1A4, and reduction of mRNA for (E.) UGT2B7, (F.) SLC22A1, and (G.) SLC01B3 with omeprazole in Lot CDP.

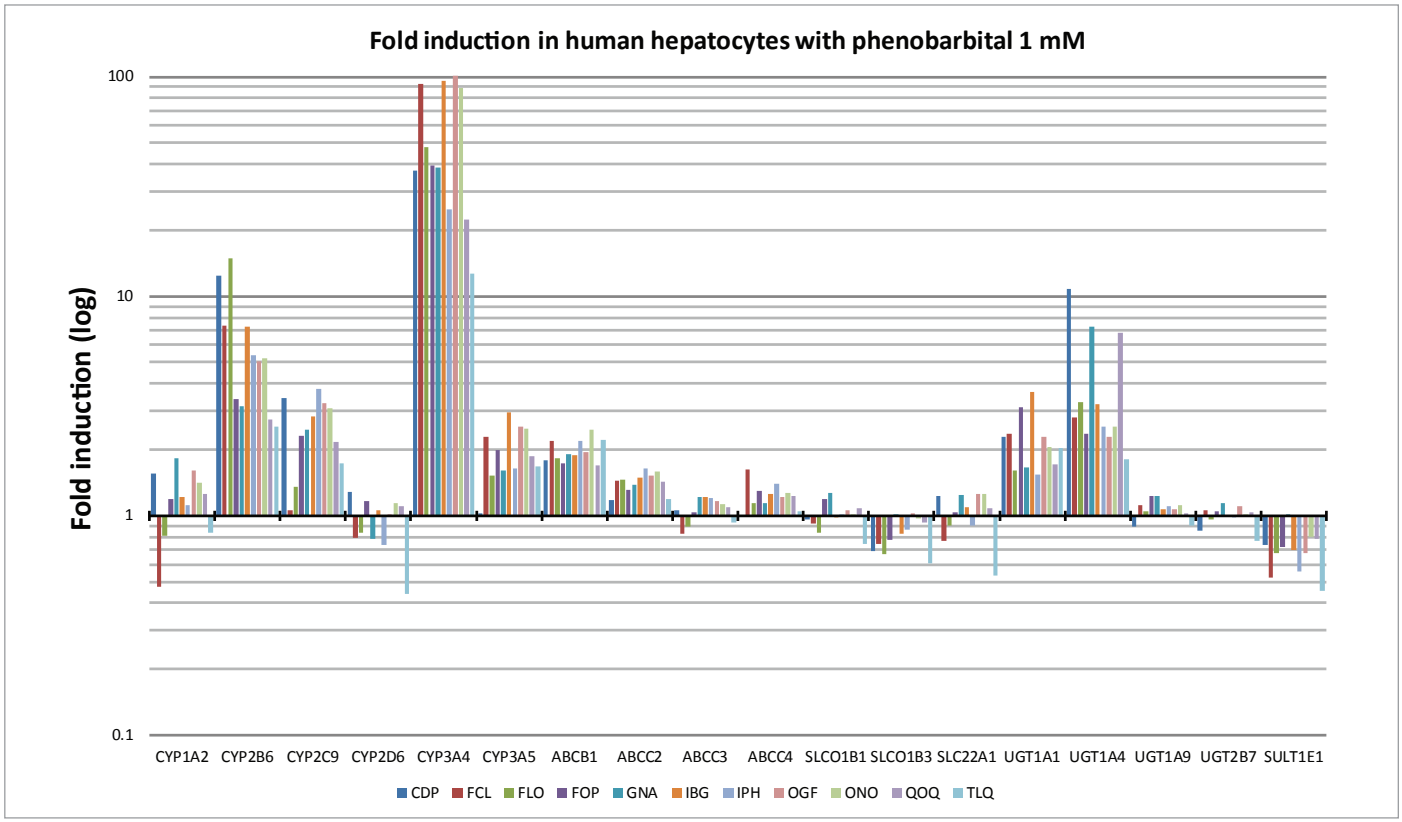
Results for single concentration induction – phenobarbital

Phenobarbital 1 mM induced mRNA levels of CYP2B6, the surrogate marker for CAR-mediated induction, in all lots tested. The range was 2.53- to 14.9-fold, and average was 6.3-fold. Phenobarbital induced CYP3A4 greater than 2-fold in all donors with the maximum induction of 104-fold. CYP2C9 was induced greater than 2-fold in 8 donors with a maximum of 3.5-fold. UGT1A1 was induced in 7 donors with a maximum induction of 3.7-fold as well as UGT 1A4 in 10 of the donors with the maximum induction of 10.8. Significant reduction in mRNA was observed with SULT1E1 with 10 donors with a fold of less than 0.8. The minimum fold was 0.45 with an average of 0.7.

Discussion and conclusion for single concentration induction – phenobarbital

As expected, phenobarbital induced CYP2B6[3] as well as CYP3A4[10] and CYP2C9.[15] UGT1A1 has been cited in literature as being inducible by phenobarbital.[16] UGT1A4 induction has been noted in clinical findings of 2-fold increase in metabolism of lamotrigine with co-administration of phenobarbital as well as 3-fold in humanized mouse model.[17]

SULT1E1 reduction was unexpected due to previous citations implicating PB induction via CAR in mouse model.[18] One explanation may be PXR activation repression of SULT1E1 observed in HuH7 cells[19] and may have a link via phenobarbital-PXR interaction. Further investigation is required to confirm this finding.



Graph 3: Fold induction of 18 ADME genes after 48-hr exposure of 1 mM phenobarbital.

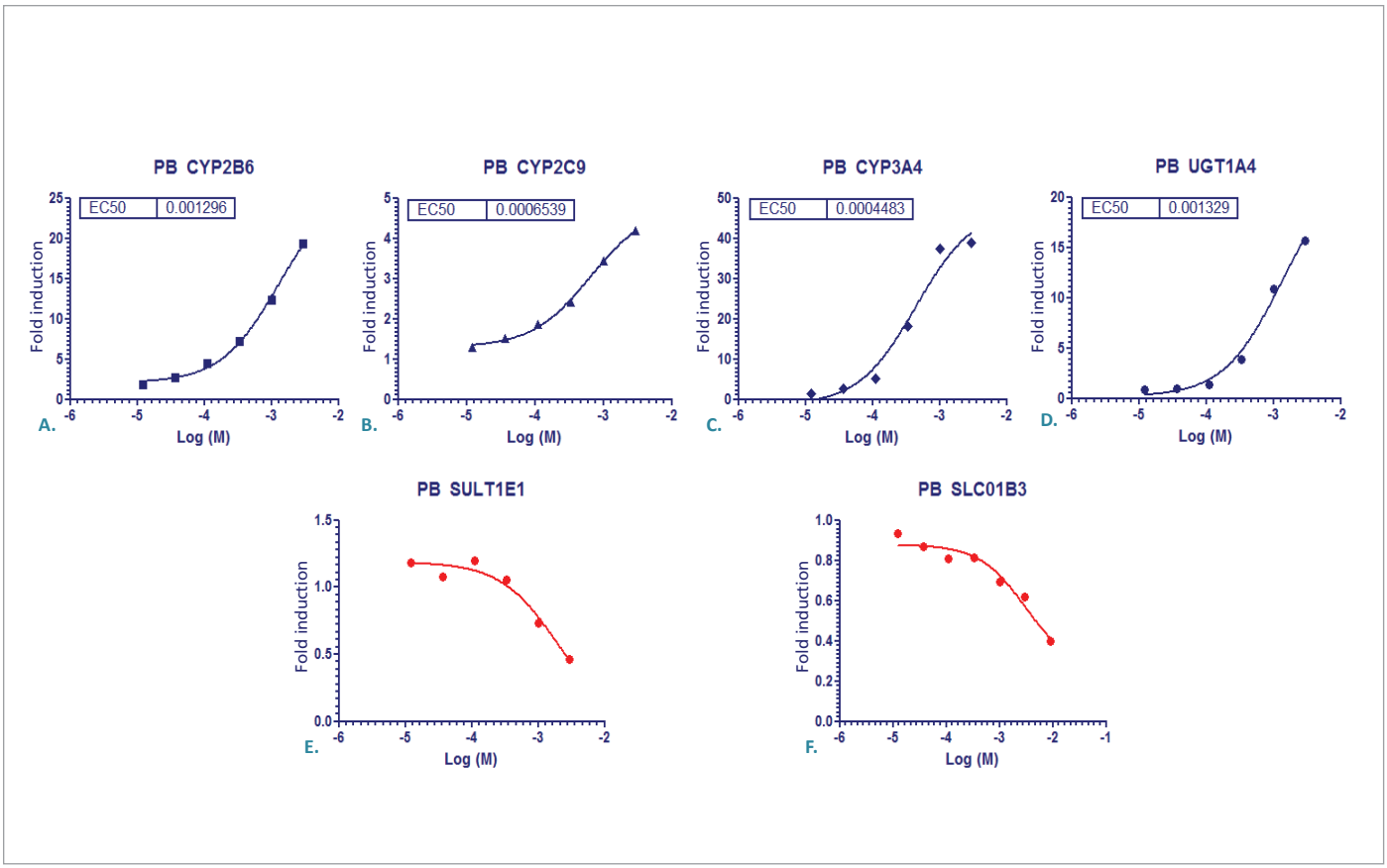
Results for concentration response curves – phenobarbital

Phenobarbital was dosed between 9 and 0.01 mM. The 9 mM induction result was removed from the EC_{50} calculations due to lower induction observed across all genes, possibly from toxicity but not confirmed. The highest concentration for the CRC was 3 mM for all calculations. Six genes, CYP1A2, 2B6, 2C9, 3A4, UGT1A1, and 1A4, exhibited induction greater than 2-fold within the CRC. The CRC and EC_{50} values for three of the key genes, CYP2B6, 2C9, and 3A4, are shown on page 6 (Graph 4A–C). UGT1A4 had significant induction with an Emax of 15.7 at 3 mM (Graph 3D). UGT1A1 showed minor induction with 2.3- and 2.8-fold at 1 and 3 mM, respectively. CYP1A2 had a minor induction at 3 mM with a fold increase of 2.5.

Two genes exhibited a concentration-dependent reduction greater than 20%. SULT1E1 showed a reduction in expression to 0.46-fold at 3 mM (Graph 4D). SLC01B3 was reduced to 0.62-fold at 3 mM (Graph 4E). All other genes remain relatively constant across the CRC except at the 9 mM concentration.

Discussion and conclusion for concentration response curves – phenobarbital

As previously noted, CYP2B6, 2C9, and 3A4 responses were expected due to their involvement in the CAR and PXR pathways, as well as UGT1A1. The reduction of SULT1E1 was unexpected as previously discussed and is in need of further investigation to confirm and explain the results. SLC01B3 did show a concentration-dependent reduction in Lot CDP; however, a reduction at 1 mM was only observed in 5 of the 11 donors. This response will need to be retested to confirm a common mechanism and consistency between donors.



Graph 4A–E: Concentration response curves and EC_{50} value determination of (A.) CYP2B6, (B.) CYP2C9, (C.) CYP3A4, and (D.) UGT1A4 and reduction of mRNA for (E.) SULT1E1 and (F.) SLC01B3 with phenobarital in Lot CDP.

Results for single concentration induction – rifampicin

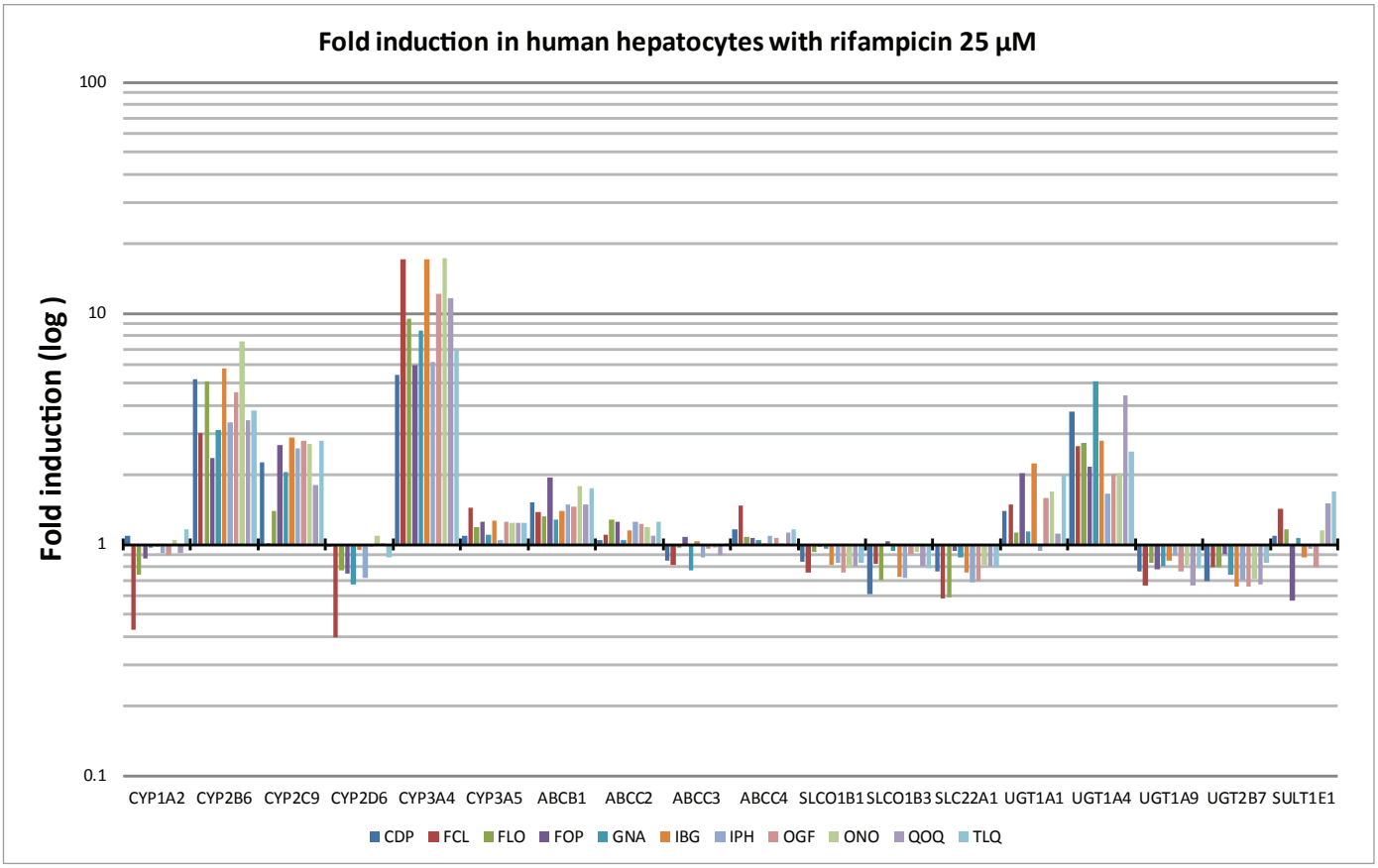
Rifampicin 25 µM induced mRNA levels of CYP3A4, the surrogate marker for PXR-mediated induction, in all lots tested. The range was 5.4- to 17.3-fold, and the average was 10.7-fold. Rifampicin induced CYP2B6 in all donors with the maximum induction of 7.6-fold. CYP2C9 was induced greater than 2-fold in 8 donors with a maximum of 2.9-fold. UGT1A4 induced greater than 2-fold in 10 of the 11 donors with a maximum induction of 5.1-fold.

A reduction in mRNA fold of less than 0.8 was observed with UGT2B7 (7 donors), UGT1A9 (6 donors), SLC22A1 (6 donors), and SLC01B3 (5 donors). The minimum fold was 0.59 for SLC22A1.

Discussion and conclusion for single concentration induction – rifampicin

The induction of CYP3A4 with rifampicin was as expected[3] as well as the induction of CYP2C9.[15,20] CYP2B6 induction has been previously reported.[15,21] UGT1A4 induction by rifampicin has been cited in the literature.[22]

The inductive effects of rifampicin on UGT2B7 and UGT1A9 have not been well described in literature. UGT1A9 had less than 2-fold induction,[13] but no suppression has been reported. UGT2B7 has not been reported to be up- or down-regulated by rifampicin. Likewise, SLC22A1 showed a slight decrease (0.95-fold) in hepatocytes exposed to rifampicin.[13] More studies will need to be conducted to determine the significance of these findings.



Graph 5: Fold induction of 18 ADME genes after 48-hr exposure of 25 µM rifampicin.

Results for concentration response curves – rifampicin

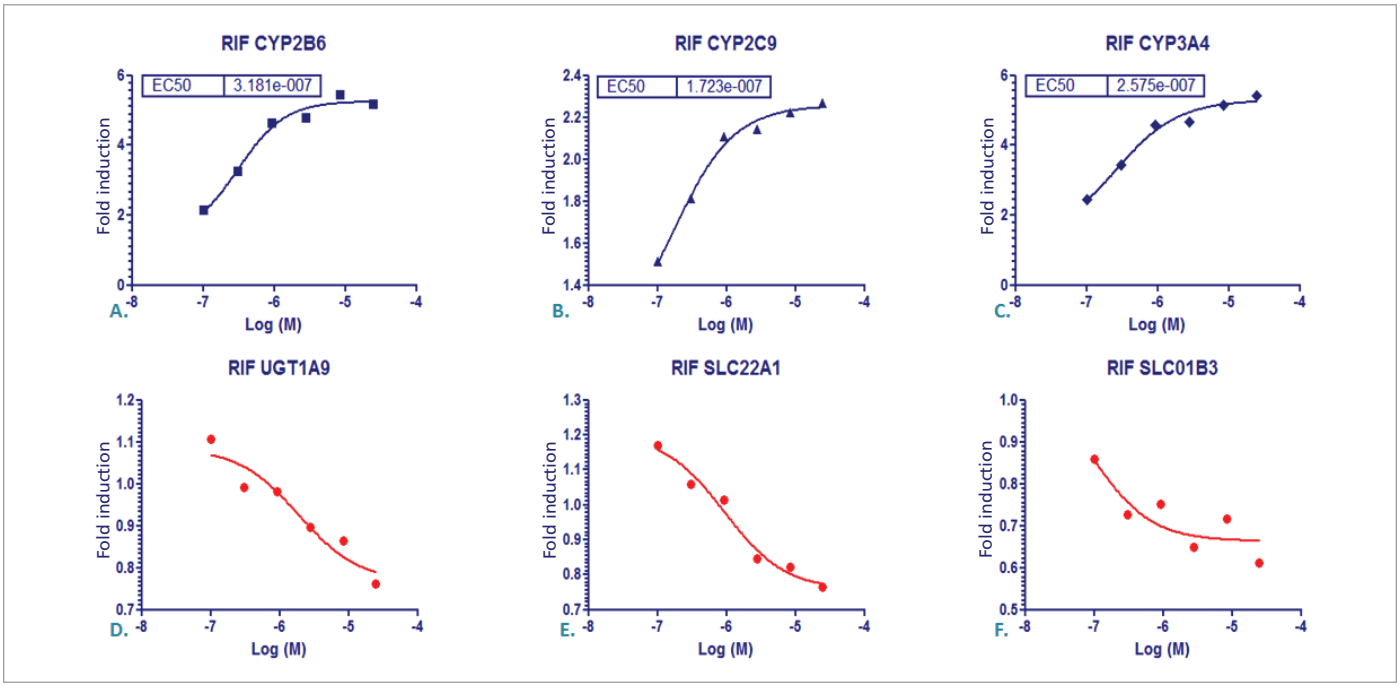
Rifampicin was dosed between 75 and 0.1 µM. The 75 µM induction result was removed from the EC_{50} calculations due to lower induction observed across all genes, possibly from toxicity but not confirmed. The highest concentration for the CRC was 25 µM for all calculations. Four genes, CYP2B6, 2C9, 3A4, and UGT1A4, exhibited induction greater than 2-fold within the CRC (Graph 6A–C). EC_{50} values were determined to be 0.32, 0.17, 0.26, and 0.16 µM, respectively.

Several genes exhibited a concentration-dependent reduction greater than 20%. UGT1A9 had a modest reduction to 0.76-fold at 25 µM (Graph 6D). SLC22A1 had a fold of 0.77 (Graph 6E). SLC01B3 had reductions from 0.86- to 0.61 across the CRC (Graph 6F). All other genes remain relatively constant across the CRC except at the 75 µM concentration.

Discussion and conclusion for concentration response curves – rifampicin

CYP3A4, 2B6, and 2C9 responses were as expected with EC_{50} of 0.26, 0.32, and 0.17 µM, respectively. The EC_{50} for CYP3A4 was close to values of 0.57 to 2.6 µM reported by Fahmi4 and within the range of 0.1 to 0.6 µM reported by McGinnity[5] and the value 0.847 ± 0.749 µM reported by Kato.[12] Further lots will need to be tested to see the variation between donors and repeated experiments between assays.

Though suppression of UGT1A9, SLC22A1, and SLC01B3 has not been described in literature nor are the reductions significantly reduced, they appear to be concentration-dependent. This suggests a biological event and not an assay artifact and warrants further investigation.



Graph 6A–F: Concentration response curves and EC_{50} value determination of (A.) CYP2B6, (B.) CYP2C9, (C.) CYP3A4 and reduction of mRNA for (D.) UGT1A9, (E.) SLC22A1, and (F.) SLC01B3 with rifampicin in Lot CDP.

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