APPLICATION NOTE

QuantiGene Plex Assays

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

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Abstract

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 drug–drug interactions (DDI) involving induction. 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 the potential to use *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 testing with target markers as well as broader transcript surveys.

In this study, we used Invitrogen™ QuantiGene™ Plex Assays to simultaneously measure 18 ADME transcripts (*CYP1A2*, *2B6*, *2C9*, *2D6*, *3A4*, *3A5*; *ABCB1*, *C2*, *C3*, *C4*; *SLC01B1*, *01B3*, *22A1*; *UGT1A1*, *1A4*, *1A9*, *2B7*; *SULT1E1*) and two controls (*GAPDH* and *HRPT*). We tested human cryoplateable hepatocytes from 11 samples at single concentrations of omeprazole (AhR ligand), phenobarbital (CAR ligand), and rifampicin (PXR ligand) 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*, *CYP2B6*, *CYP3A4*, *UGT1A1*, and *UGT1A4*. Phenobarbital induced *CYP2B6*, *CYP2C9*, *CYP3A4*, *CYP3A5*, *ABCB1*, *UGT1A1*, and *UGT1A4*. Rifampicin induced *CYP2B6*, *CYP2C9*, *CYP3A4*, *UGT1A1*, and *UGT1A4*. Suppression greater than 30% was observed for *SLC22A1* and *UGT2B7* with omeprazole, for *SULT1E1* with phenobarbital, and for *SLC22A1* and *UGT2B7* with rifampicin. No responses (<2 and >0.7) were observed for *ABCC2*, *ABCC3*, *ABCC4*, 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 was 2 µM for *CYP1A2*. Phenobarbital EC_{50} value was 1.3 mM for *CYP2B6*. Rifampicin EC_{50} value was 260 nM for *CYP3A4* and 170 nM for *CYP2C9*. Dose-dependent suppression was measured for several genes, such as *SLC01B3* and *UGT2B7* for omeprazole, *SLC01B3* and *SULT1E1* for phenobarbital, and *ABCC3* and *UGT1A9* for rifampicin.

The data show the power of the 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*, *CYP2B6*, *CYP2C9*, and *CYP3A4* can be used to fulfill regulatory requirements with the potential to add secondary markers such as phase II enzymes or transporters to probe gene regulation from test articles.

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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, testing assays have been developed, such as the use of hepatocytes or liver cell lines to measure *in situ* metabolism between control and exposed wells. Additionally, reporter gene assays such as PXR-linked luciferase construct assays 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 [4–7]. This new focus has altered the previous testing 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 [8–10]. 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 [10,11]. 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 absorption, distribution, metabolism, and excretion (ADME) genes and 2 control genes to test across 11 individual samples for induction with three prototypical inducers: omeprazole, phenobarbital, and rifampicin. Individual responses, as well as general trends, were assessed. For one sample, a concentration response curve was used to determine EC_{50} values for those genes induced. The key biomarkers, *CYP1A2*, *CYP2B6*, and *CYP3A4*, were used to fulfill FDA regulatory guidance, and other markers were added to view trends in gene regulation associated with phase I and II metabolism, and transporter expression.

Materials and methods

Hepatocyte cultures

Human cryoplateable hepatocyte lots were obtained from Celsis In Vitro Technologies. Procedures for the thawing and plating of cryoplateable hepatocytes in *InVitro*GRO™ CP Medium, and the culturing and dosing of cells in *InVitro*GRO™ HI Medium, were performed following the instructions for use from Celsis In Vitro Technologies. The 96-well plates seeded with 50,000 viable hepatocytes per well were cultured for 2 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 *InVitro*GRO 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 between 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 Assays using the Sample Process Kit for Cultured Cells (Cat. No. 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 *InVitro*GRO™ KHB medium. Next, the medium 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 10 times to ensure thorough lysis of the cells. The plates were sealed and stored below –70°C.

QuantiGene Plex Assay

The QuantiGene Plex Assay 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 preamplifier 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 phycoerythrin (SAPE). The beads were washed and read on the Luminex® instrument.

Table 1. Target genes and their associated functions.

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	

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 Microsoft™ Excel™ software for single concentrations. Concentration response curves were analyzed using Prism 5.0 software for nonlinear fit to determine EC_{50} value where applicable.

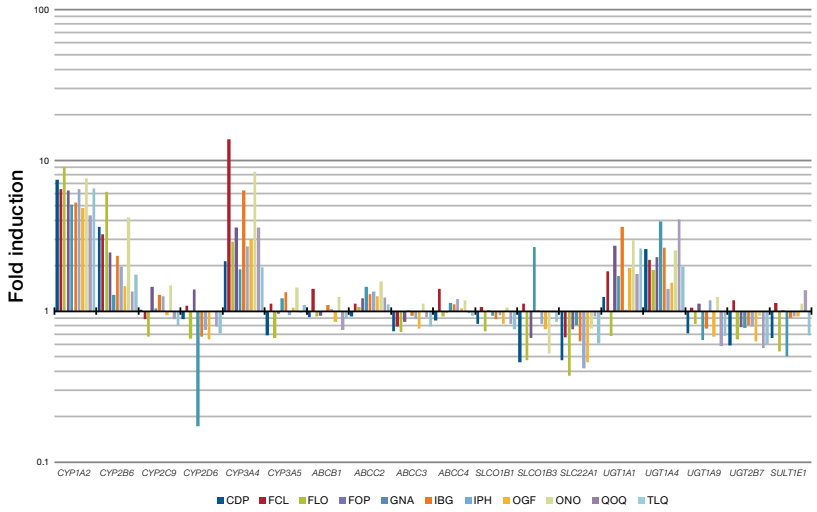

Figure 1. Fold induction of 18 ADME genes after 48 hr exposure of cells to 50 µM omeprazole.

Results and discussion

Single-concentration induction: omeprazole

Omeprazole at 50 µM induced mRNA levels of *CYP1A2*, the surrogate marker for AHR-mediated induction, in all lots tested (Figure 1). 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 samples with a range of 1.3- to 6.2-fold. *CYP3A4* was induced greater than 2-fold in 9 samples with a range of 1.9- to 13.7-fold. *UGT1A4* was induced greater than 2-fold in 7 of the 11 samples, ranging from 1.4- to 4-fold.

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

Omeprazole induced *CYP1A2* as expected [3] as well as *CYP3A4* [7,12]. Induction of *CYP2B6* confirmed results found by some researchers [9,13] while contradicting a previous literature report [14], albeit the changes were modest and not all samples 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.

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. Lower induction was possibly due to toxicity, but this was not confirmed. The highest concentration for the CRC was 50 µM for all calculations. Four genes, *CYP1A2*, *CYP2B6*, *CYP3A4*, and UGT1A4, exhibited induction greater than 2-fold within the CRC (Figure 2A–D).

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

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

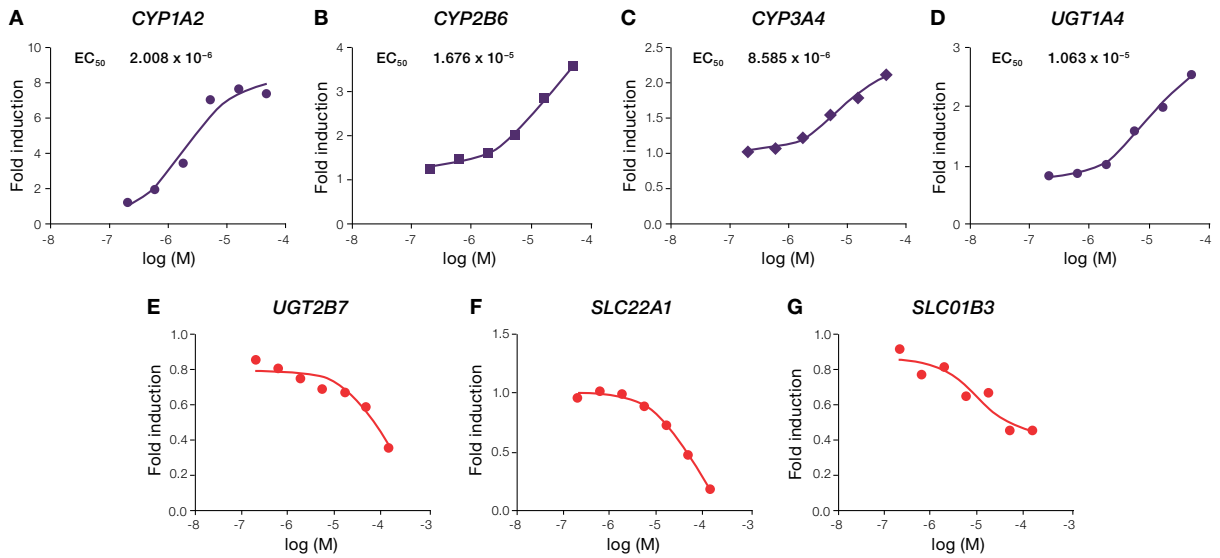

Figure 2. Omeprazole concentration response curves in lot CDP. Concentration response curves and EC_{50} values are shown for **(A)** *CYP1A2*, **(B)** *CYP2B6*, **(C)** *CYP3A4*, and **(D)** *UGT1A4*. Reduction of mRNA is shown for **(E)** *UGT2B7*, **(F)** *SLC22A1*, and **(G)** *SLC01B3*.

Single-concentration induction: phenobarbital

Phenobarbital at 1 mM induced mRNA levels of *CYP2B6*, the surrogate marker for CAR-mediated induction, in all lots tested (Figure 3). The range was 2.53- to 14.9-fold, and average was 6.3-fold. Phenobarbital induced *CYP3A4* greater than 2-fold in all samples with a maximum induction of 104-fold. *CYP2C9* was induced greater than 2-fold in 8 samples with a maximum of 3.5-fold. *UGT1A1* was induced in 7 samples with a maximum induction of 3.7-fold, and *UGT1A4* was induced in 10 of the samples with a maximum induction of 10.8-fold. Significant reduction in mRNA was observed with *SULT1E1* with 10 samples with a change of less than 0.8-fold. The minimum change was 0.45-fold with an average of 0.7-fold.

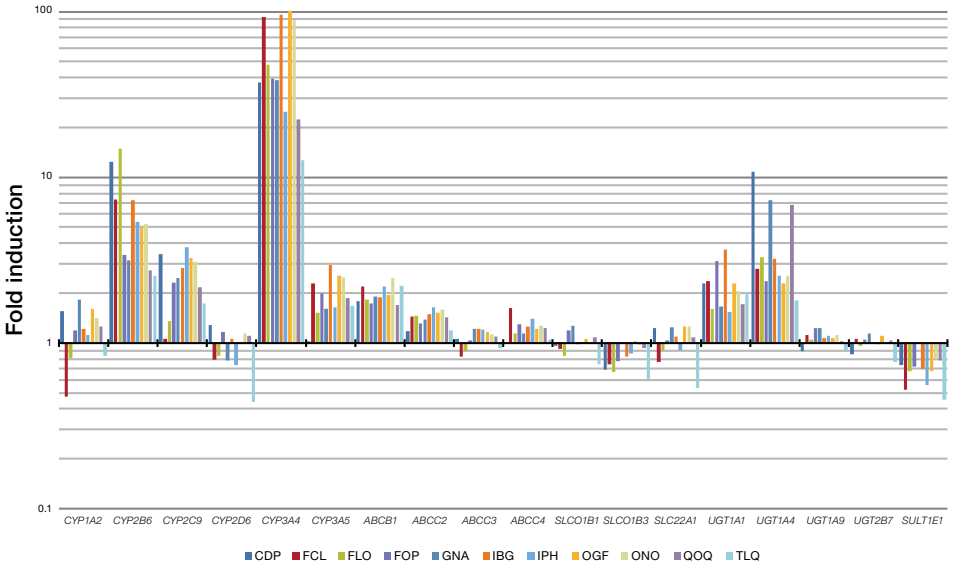

Figure 3. Fold induction of 18 ADME genes after 48 hr exposure of cells to 1 mM phenobarbital.

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

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

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. Lower induction was possibly due to toxicity, but this was not confirmed. The highest concentration for the CRC was 3 mM for all calculations. Six genes, *CYP1A2*, *CYP2B6*, *CYP2C9*, *CYP3A4*, *UGT1A1*, and *UGT1A4*, exhibited induction greater than 2-fold within the CRC. The CRC and EC_{50} values for three of the key genes, *CYP2B6*, *CYP2C9*, and *CYP3A4*, are shown in Figure 4A–C. *UGT1A4* had significant induction with an E_{max} of 15.7 at 3 mM (Figure 4D). *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 (Figure 4E). *SLC01B3* was reduced to 0.62-fold at 3 mM (Figure 4E). All other genes remained relatively constant across the CRC except at the 9 mM concentration.

As previously noted, *CYP2B6*, *CYP2C9*, *CYP3A4*, and *UGT1A1* responses were expected due to their involvement in the CAR and PXR pathways. 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 samples. This response will need to be retested to confirm a common mechanism and consistency between samples.

Single-concentration induction: rifampicin

Rifampicin at 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 samples with a maximum induction of 7.6-fold. *CYP2C9* was induced greater than 2-fold in 8 samples with a maximum of 2.9-fold. *UGT1A4* was induced greater than 2-fold in 10 of the 11 samples with a maximum induction of 5.1-fold (Figure 5).

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

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 the literature. *UGT1A9* had less than 2-fold induction [13], but no suppression has been reported. *UGT2B7* has not

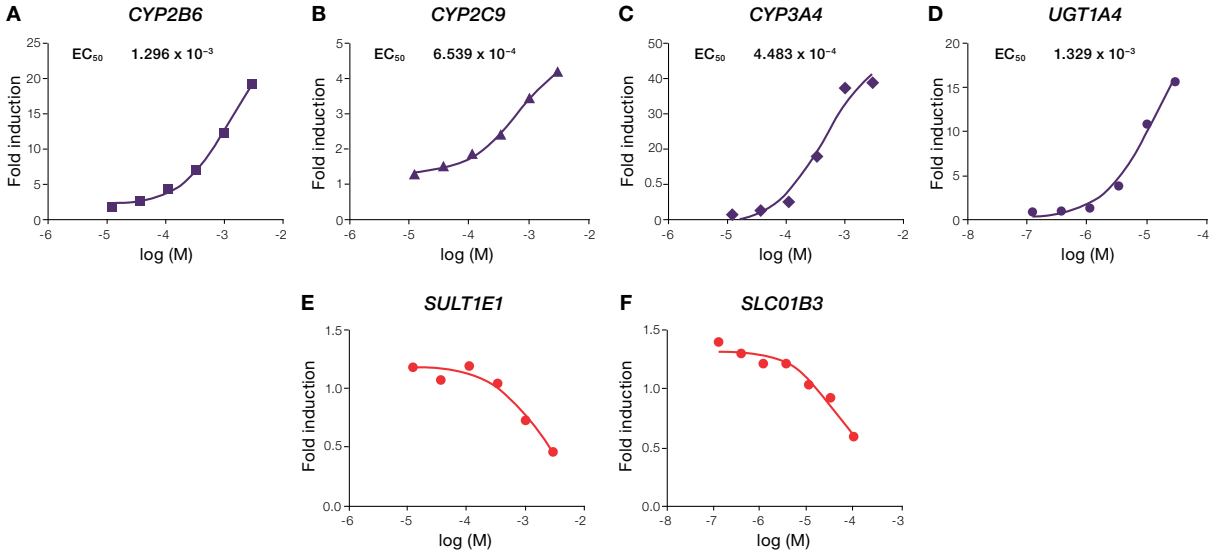

Figure 4. Phenobarbital concentration response curves in lot CDP. Concentration response curves and EC_{50} values are shown for **(A)** *CYP2B6*, **(B)** *CYP2C9*, **(C)** *CYP3A4*, and **(D)** *UGT1A4*. Reduction of mRNA is shown for **(E)** *SULT1E1* and **(F)** *SLC01B3*.

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.

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. Lower induction was possibly due to toxicity, but this was not confirmed. The highest concentration for the CRC was 25 µM for all calculations. Four genes, *CYP2B6*, *CYP2C9*, *CYP3A4*, and *UGT1A4*, exhibited induction greater than 2-fold within the CRC (Figure 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 (Figure 6D). *SLC22A1* had a reduction of 0.77-fold (Figure 6E). *SLC01B3* had reductions from 0.86- to 0.61-fold across the CRC (Figure 6F). All other genes remained relatively constant across the CRC except at the 75 µM concentration.

CYP3A4, *CYP2B6*, and *CYP2C9* responses were as expected, with EC_{50} values 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 Fahmi [4], 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 [7]. Further lots will need to be tested to see the variation between samples.

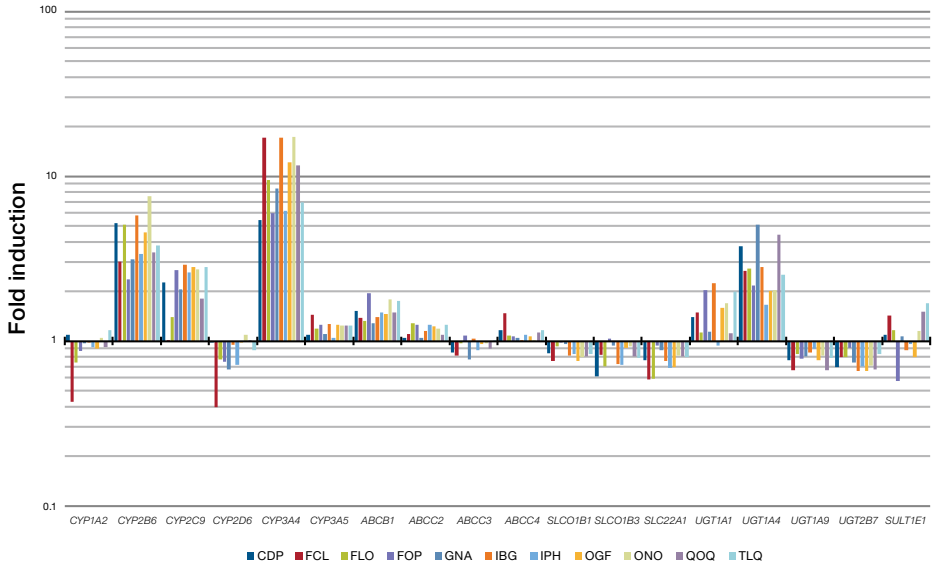

Figure 5. Fold induction of 18 ADME genes after 48 hr exposure of cells to 25 µM rifampicin.

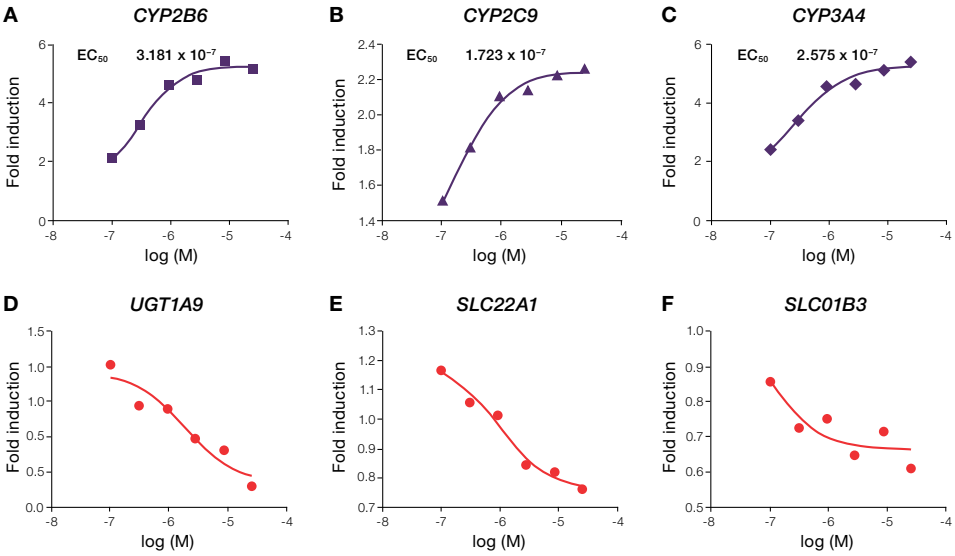

Figure 6. Rifampicin concentration response curves in lot CDP. Concentration response curves and EC_{50} values are shown for **(A)** *CYP2B6*, **(B)** *CYP2C9*, and **(C)** *CYP3A4*. Reduction of mRNA is shown for **(D)** *UGT1A9*, **(E)** *SLC22A1*, and **(F)** *SLC01B3*.

Though suppression of *UGT1A9*, *SLC22A1*, and *SLC01B3* has not been described in the literature, the reduction observed was concentration-dependent. This suggests a biological event and not an assay artifact, warranting further investigation.

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