Transcript Regulation of 42 ADME Genes by Prototypical Inducers in Human Hepatocytes

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ABSTRACT

Due to recent FDA draft guidance, a fundamental change from measuring enzyme activities to transcripts will have profound implications for studying induction drug-drug interactions (DDI). Discrete enzymatic assays for individual CYP enzymes are replaced by 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 the QuantiGene Plex Assay to simultaneously measure 42 ADME transcripts spanning Phase I (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, FMO1, FMO3, FMO5, AOX1, XDH, MAOA, MAOB) and Phase II (UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, 2B15, SULT1A1, 2A1, 2B1) enzymes, transporters (ABCA1, B1, B11, C1, C2, G2, SLC10A1, 22A1, 2A1, 3A1, O1B1, O1B3, O2B1) and eight control genes (ACTB, GAPDH, GUSB, HRPT1, PGK1, POLR2A, PPIB, TBP). We screened 10 donors of human cryoplateable hepatocytes at single concentration of omeprazole, phenobarbital and rifampicin after 48-hour exposure in 96-well format to observe individual variations in induction and suppression potentials. Inducers did modulate certain control gene expression levels. Selective use of control genes was necessary to normalize the data. For omeprazole, induction greater than 2-fold was observed for CYP1A1, 1A2, 2B6, 3A4, UGT1A1 and 1A4. Phenobarbital induced CYP2A6, 2B6, 2C8, 2C9, 3A4, 3A5, UGT1A1 and 1A4. Rifampicin induced CYP1A1, 2A6, 2B6, 2C8, 2C9, 3A4, UGT1A1 and 1A4. Suppression greater than 30% was observed in several genes.

For two donors, concentration response curves were generated and EC50 values were calculated where applicable. Omeprazole EC50 value for CYP1A2 was 2 µM, phenobarbital for CYP2B6 was 1.3 mM., and rifampicin for CYP3A4 was 260 nM , and CYP2C9 170 nM. Dose dependent suppression was measured for several genes. The data shows the power of generating 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 life style influences is one type of DDIs where 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 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 has 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 of *in situ* metabolism has been replaced with transcript measurements which was born out of articles touting its sensitivity and high correlation to clinical outcomes[5, 6, 12] . This new focus has altered the previous screening method for 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 to measure transcript levels by probing with primers with capture extender and label extender 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 use bDNA technology to probe for 42 ADME genes and 8 control genes to screen across 10 individual donors for induction with three prototypical inducers, omeprazole, phenobarital and rifampicin. Individual responses, as well as general trends, were assessed. For two donors, 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 BioreclamationIVT. Procedure for the thawing and plating of cryoplateable hepatocytes in *InVitro*Gro™ CP medium, and the culturing and dosing in *InVitro*Gro HI followed instructions for use as prescribed by BioreclamationIVT. 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 phenobarital, or 25 µM rifampicin as positive controls (PC) in *InVitro*Gro HI medium for 10 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 the QuantiGene Plex assay using the Sample Processing Kit for cultured cells (QS0100) as directed in 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. Sample plates were shipped on dry ice to the Panomics Division of Affymetrix for analysis.

QuantiGene 2.0 Plex Assay:
The QuantiGene 2.0 Plex assay from Affymetrix was used to quantitate 50 genes simultaneously for use on the Luminex platform. The 50-plex specific oligo probe sets for capture of the targeted RNA genes containing unique zip code and the label extension oligos of the targeted RNA genes were incubated overnight at 54 °C to unique fluorescent beads with the corresponding zip codes (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.

Data Analysis
The Median Fluorescent Intensity Units (MFI) for the samples were normalized by geometric average of GUSB, HPRT1, POLR2A, PPIB and TBP gene expression. ACTB and GAPDH signal was saturated and could not be used to normalize the data The adjusted MFI's were used to determine fold induction by dividing 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.

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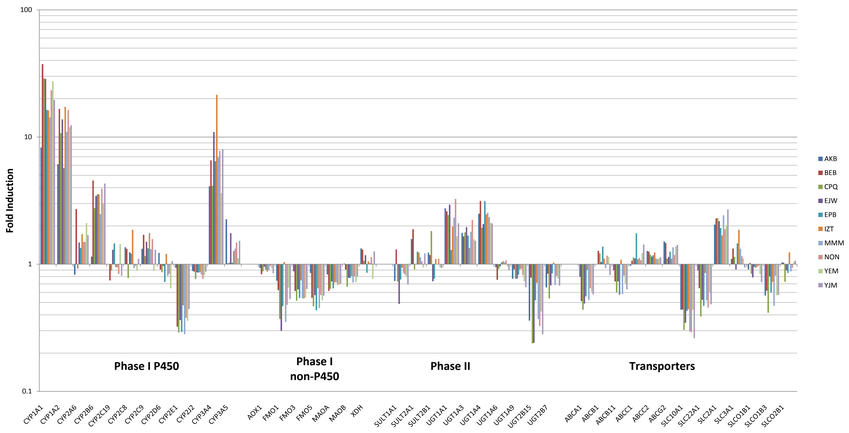
Omeprazole

Single Concentration Induction: Omeprazole 50 µM induced mRNA levels of CYP1A2, the surrogate marker for AHR-mediated induction, in all lots tested. The range was 5.7 to 17.2 fold and average was 12.2 fold. CYP1A1 too was induced with a range of 16.1 to 29.0. Omeprazole induced CYP2B6 greater than 2-fold in 9 of the 10 donors with a range of 1.1 to 4.6 fold. CYP3A4 was induced greater than 2-fold in all 10 donors with a range of 3.6 to 21.5 fold. Phase II enzymes UGT1A1 and UGT1A4 where induced greater than 2-fold in the majority of the lots .

Phase I non-P450s and transporters were not induced in any lot greater than 2-fold. Many of which experienced suppressed expression. Further investigation is necessary to confirm and explain this observation.

Discussion and Conclusion: The induction of CYP1A2 was induced by omeprazole as expected as well as the induction of CYP1A1.[3] Several publications have note similar 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. UGT1A1 has been reported to be induced via AhR receptor.[18] UGT1A4 induction appears to be novel with no literature references linking omeprazole to its induction.



Graph 2. Fold induction of 42ADME genes grouped by Phase I P450, Phase I non-P450, Phase II or Transporters after 48-hr exposure of 50 µM omeprazole.

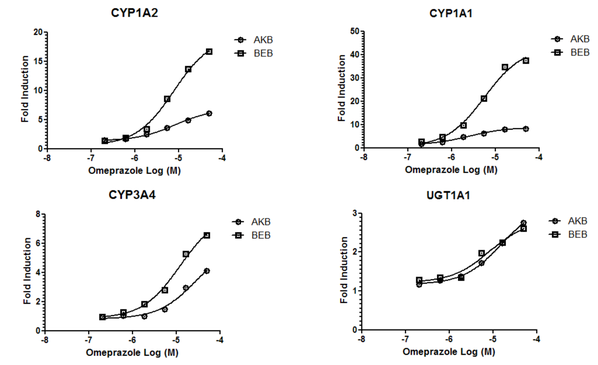
Concentration Response Curves: Omeprazole was dosed between 50 and 0.21 µM. Four genes , CYP1A1, CYP1A2, CYP3A4 and UGT1A1 and shown below (Graph 3A-D). EC_{50} values where calculated fro two lots, AKB and BEB. EC_{50} values for AKB and BEB are: CYP1A2 8.1 and 8.0 µM, CYP1A1 2.4 and 5.7 µM, CYP3A4 28 and 12 µM and UGT1A1 15 and 8.3 µM.

Minor responses of greater than 2-fold at highest concentration in a dose-dependent manner to CRC were observed in CYP2A6 and SLC2A1 (data not shown).

Several genes exhibited a concentration dependent reduction greater than 30%: FMO1, FMO3, FMO5, CYP2E1, SLC22A1, SLCO1B3, and SLC10A1 (data not shown).

All other genes remain relatively constant across the CRC.

Discussion and Conclusion: As previously noted, CYP1A2 CYP1A1, CYP3A4 and UGT1A1 responses were as expected. Responses in CYP2A6, UGT1A4 and SLC2A1, have not been cited in literature and appear to be concentration dependent. Therefore, these may represent novel responses warranting further study.



Graph 3 A-D. Induction concentration response curves of CYP1A2, CYP1A1, CYP3A4 and UGT1A1 with exposure to omeprazole in lots AKB and BEB.

Phenobarbital

Single Concentration Induction: Phenobarbital 1 mM induced mRNA levels of CYP2B6, the surrogate marker for CAR-mediated induction, in all lots tested except lot AKB. The range was 1.3 to 9.7 fold and average was 6.7 fold. Phenobarbital induced CYP3A4 greater than 2-fold in all donors with the range of 8.9 to 84 fold and an average of 32 fold. CYP2C9 was induced greater than 2-fold in 9 donors with a maximum of 3.6 fold. CYP2A6 was induced in all lots with a range fold of 2.1 to 10.9. UGT1A1 was induced in 9 donors with a maximum induction of 3.7 fold, as well as UGT 1A4 in 10 of the donors with the maximum induction of 5.

Phase I non-P450s and transporters were not induced in any lot greater than 2-fold. Many of which experienced suppressed expression. Further investigation is necessary to confirm and explain this observation.

Discussion and Conclusion: The induction of CYP2B6 was induced by phenobarital as expected[3] as well as the induction of CYP2A6, CYP3A4 and CYP2C9.[10, 15, 19] 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 phenobarital as well as 3-fold in humanized mouse model.[17]

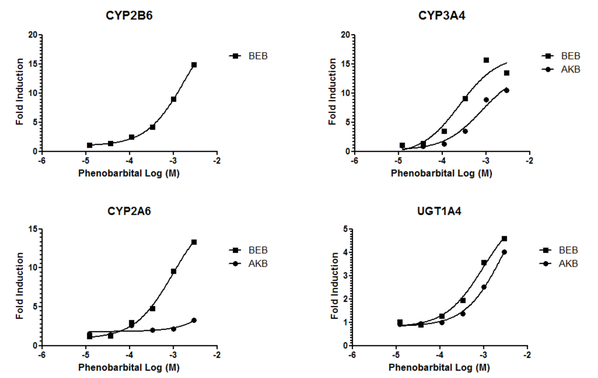
Graph 4. Fold induction of 42ADME genes grouped by Phase I P450, Phase I non-P450, Phase II or Transporters after 48-hr exposure of 1 mM phenobarbital.

Concentration Response Curves: Phenobarbital was dosed between 3 and 0.01 mM. Eight genes, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP3A4, CYP3A5, SULT2A1, UGT1A1 and 1A4, exhibited induction greater than 2-fold within the CRC. The CRC and EC_{50} values for four of the key genes, CYP2B6, CYP2A6, CYP3A4 and UGT1A1, are shown below (Graph 5A-D) . The EC_{50} values for the four enzymes shown for lots AKB and BEB are: CYP2B6 (not calculated) and 1.8 mM, CYP3A4 0.8 and 0.2 mM, CYP2A6 1 and 0.9 mM, and UGT1A4 2.8 and 1 mM.

Four genes exhibited a concentration dependent reduction greater than 30%: CYP2E1, ABCA1, SLCO1B3 and SLC10A1 (data not shown).

All other genes remain relatively constant across the CRC.

Discussion and Conclusion: As previously noted, CYP2B6, 2C8, 2C9 and 3A4[3] responses were expected due to there involvement in the CAR and PXR pathways, as well as UGT1A1 and CYP2A6. [19, 23] CYP3A5 has been shown to be induced in lung tissue as well as liver and intestines.[25, 26] SULT2A1 induction by phenobarbital has been observed human hepatocyte cultures. [27]



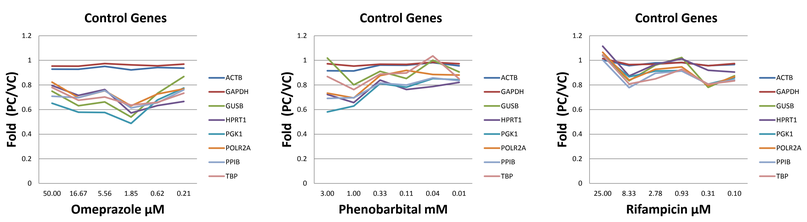
Graph 5 A-D. Concentration response curves for CYP2B6, CYP3A4, CYP2A6 and UGT1A4 exposed to phenobarital in lots AKB and BEB.

Control Genes

Phase I P450 Enzymes	Phase I Non-P450 Enzymes	Phase II Enzymes	Transporters	Control Genes
CYP1A1	AOX1	SULT1A1	ABCA1	ACTB
CYP1A2	FMO1	SULT2A1	ABCB1	GAPDH
CYP2A6	FM03	SULT2B1	ABCB11	GUSB
CYP2B6	FM05	UGT1A1	ABCC1	HRPT1
CYP2C8	MAOA	UGT1A3	ABCC2	PGK1
CYP2C9	MAOB	UGT1A4	ABCG2	POLR2A
CYP2C19	XDH	UGT1A6	SLC2A1	PPIB
CYP2D6		UGT1A9	SLC3A1	TBP
CYP2E1		UGT2B7	SLCO1B1	
CYP2J2		UGT2B15	SLC01B3	
CYP3A4			SLCO2B1	
CYP3A5			SLC10A1	
			SLC22A1	

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

Discussion and Conclusion: Eight control gene were monitored in the 50-gene set. The MFI values were corrected for background but were not normalized to other control genes. The graphs (1A-C) are the relative change in expression compared to the vehicle control for lot AKB. Lot BEB had similar pattern though MFI were approximately twice the values of AKB, except for ACTB and GAPDH. ACTB and GAPDH MFI values were saturated and deemed unreliable as a control gene due to their over expression. GUSB, HPRT1, PGK1, POLR2A and PPIB were used to normalize probe genes.



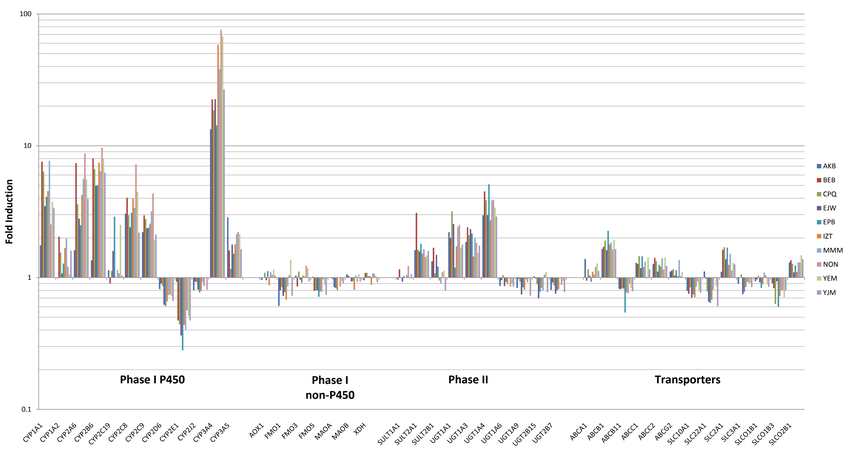
Graphs 1A-C. Relative fold change of control genes in the presence of inducers.

Rifampicin

Single Concentration Induction: Rifampicin 25 µM induced mRNA levels of CYP3A4, the surrogate marker for PXR-mediated induction, in all lots tested. The range was 13 to 75 fold and the average was 35.8 fold. Rifampicin induced CYP2B6 greater than 2-fold in 9 donors with the maximum induction of 9.7 fold. CYP2C8 was induced in all donors with a maximum of 7.2 fold, CYP2C9 in 9 donors with a maximum of 4.3 fold, CYP2A6 in 9 donors with a maximum of 8.7 fold, and CYP3A5 in 3 donors with a maximum of 2.9 fold. UGT1A4 induced greater than 2-fold in all donors with a maximum induction of 5.1 fold, UGT1A1 in 6 donors with maximum induction of 3.2, and UGT1A3 in 5 donors with maximum induction of 2.4.

Phase I non-P450s and transporters were not induced in any lot greater than 2-fold. Some of which experienced suppressed expression. Further investigation is necessary to confirm and explain this observation.

Discussion and Conclusion: The induction of CYP3A4 with rifampicin was as expected[3] as well as the induction of CYP2A6, CYP2B6, CYP2C8, CYP2C9.[13, 15, 20, 21] Phase II enzymes UGT1A1, UGT1A3 and UGT1A4 induction by rifampicin has been cited in the literature.[22, 28, 29]



Graph 6. Fold induction of 42ADME genes grouped by Phase I P450, Phase I non-P450, Phase II or Transporters after 48-hr exposure of 25 µM rifampicin.

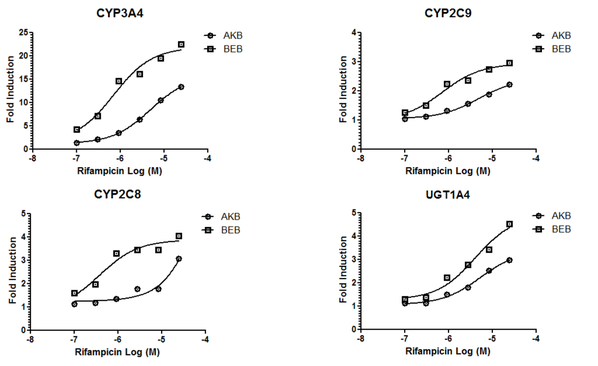
Concentration Response Curves: Rifampicin was dosed between 25 and 0.1 µM. Nine genes, CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9 CYP3A4, UGT1A1, UGT1A3, UGT1A4 and SULT2A1 exhibited induction greater than 2-fold within the CRC (Graph 2A-C). EC_{50} values for lots AKB and BEB of the four genes in Graph6A-D are CYP3A4 4.9 and 0.7 µM, CYP2C8 (not calculated) and 38 µM, CYP2C9 4.7 and 0.7 µM, and UGT1A4 5.2 and 4.1 µM.

CYP2E1 was the only gene that exhibited a suppression greater than 30 % in a dose dependent manner (data not shown).

All other genes remain relatively constant across the CRC.

Discussion and Conclusion: CYP3A4, 2B6, 2C8 and 2C9 responses were as expected.[3] The EC_{50} for CYP3A4 was close to values of 0.57 to 2.6 µM reported by Fahmi[4] , 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.

Phase II enzyme induction of UGT1A1, UGT1A3, UGT1A4 and SULT2A1 have been reported as noted in previous discussion



Graph 6 A-D. Concentration response curves of CYP3A4, CYP2C9, CYP2C8 and UGT1A4 with rifampicin in lots AKB and BEB.