Automated Multiplexed Cell-Based Cytochrome P450 Assays

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Overview

Cytochrome P450 enzymes are key players in the metabolism of drugs within the body. Therefore, it is essential to understand how these enzymes can be affected by xenobiotics with regards to inhibition, induction, and toxicity to avoid potential drug-drug interactions. Using a multiplexed format, where multiple pieces of data are obtained from a single well, can facilitate this process and provide more reliable results. Here we examine data from two separate projects combining assays to examine CYP activity, as well as toxicity, in a cell-based format. The first demonstrates the ability to monitor induction and inhibition of the CYP3A4 enzyme, as well as cytotoxicity from a single assay well, using DPX-2 cells. The second demonstrates the ability to monitor CYP1A2 and 3A4 induction, combined with cytotoxicity measurements, from a single well using cryopreserved hepatocytes. Each assay utilized simple automation that can easily fit into existing biosafety cabinets. Data shown confirms how the combination of assay, cells, and instrumentation provide a simplified, robust method to obtain this valuable information.

Introduction

Drug-drug interactions (DDI) are of serious concern to the pharmaceutical industry and associated regulatory agencies. One parameter of DDI is induction, the upregulation of enzyme levels that increase metabolism, reducing a drug's therapeutic efficacy. Aryl hydrocarbon receptor (AHR), constitutively active receptor (CAR) and pregnane X receptor (PXR) are nuclear receptors responsible for transcription of the majority of enzymes and transporters associated with drug metabolism and excretion. However, all the enzymes and transporters do not need to be measured. To quantify this risk, the FDA guidance states that *in vitro* CYP1A and CYP3A activities may be used to monitor induction potential of all drug metabolizing CYPs. Though there are cell lines and reporter gene assays that can be used, the gold standard is the use of hepatocytes to assess the induction potential of a drug by measuring the enzymatic activity of CYP1A and CYP3A with specific substrates and comparing the activity to the basal rate of the enzyme.

Many drugs, as previously stated, upregulate the expression of the CYP3A4 gene via PXR. In contrast, a large set of drugs have also shown inhibitory effects on the CYP3A4 enzyme. The upregulation of the CYP3A4 gene or inhibition of the enzyme can result in adverse drug-drug interactions. Less understood and more difficult to assess is how certain drugs can induce the expression of the CYP3A4 gene and also inhibit the activity of the enzyme. The DPX2 cell line is a useful tool to assess the effects of a xenobiotic on gene induction and enzyme inhibition. DPX2 cells contain human PXR and a luciferase-linked CYP3A4 promoter reporter construct. Upon activation, PXR binds to the native promoter and the luciferase-linked promoter. Increased CYP3A4 enzyme expressed from the native gene, as well as luciferase expressed from the reporter construct is then expressed.

Typically this information has been gathered from separate wells of cells, and by combining the data from multiple experiments. This process is costly and time-consuming. And it may lead to misinterpretation when combining the data due to the variability within each assay. A multiplexed format provides a method to obtain multiple readouts from a single microplate well, which may attenuate these concerns.

Here we demonstrate the ability to monitor induction and inhibition data, combined with cytotoxicity measurements, from a single well. The assay procedure was automated using simple, yet robust robotic instrumentation, including cell manipulations, compound titration and transfer, and reagent dispensing. Results show how the ability to measure three critical responses from a single sample streamlines work flow, derives maximum value from costly reagents, and provides a means for reaching more biologically relevant conclusions.

BioTek Instrumentation

BioTek Liquid Handling

A. The EL406™ offers fast, accurate media removal and plate washing capabilities through its Dual-Action™ Manifold. It also offers reagent dispensing capabilities through the use of its peristaltic or syringe pumps, with volumes ranging from 1-3000 µL/well. The instrument was used for cell dispensing, media exchange and removal, as well as dispensing reagents to the cell plates.

B. The Precision™ XS combines a single-channel sample processing head, an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate compounds across a 96-well PP plate, transfer compounds to 96- and 384-well cell plates and media from the cell plates to luminometer plates, as well as dispense Luciferin Detection Reagent to all luminometer plates.

BioTek Detection

A. The Synergy™ H4 combines a filter-based and monochromator-based detection system in the same unit. The filter-based system was used to read the luminescent signal from the P450-Glo™ and CellTiter-Glo® assays, and the fluorescent signal from the Resorufin Ethyl Ether assay using a 530/20 nm excitation filter, 590/35 nm emission filter, and 550 nm cutoff dichroic mirror.

B. The Synergy™ Mx incorporates a quadruple monochromator system which selects wavelengths with a repeatability of plus or minus 0.2 nm, along with a dedicated optical system, separate from the fluorescence optics, for high-performance luminescence detection. The instrument was used to detect the luminescent P450-Glo™ and ONE-Glo™ signals, as well as the fluorescent AFC signal (Ex. 400/20 nm; Em. 505/20 nm).

CYP1A/-3A Triplex Assay

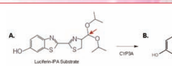

Figure 1 – ***A.*** Luciferin-IPA substrate, 3 µM, is added to hepatocytes following the 48 hour compound incubation period. The cell-permeable substrate enters the cells. Activation of the PXR receptor causes an increase in CYP3A enzyme expression. CYP3A oxidizes the luminogenic substrate to create luciferin. Luciferin is also cell-permeable, and is able to move out of the cells, into the media. ***B.*** Upon addition of P450-Glo™ Luciferin Detection Reagent, the luciferin combines with luciferase and ATP to create light that is proportional to CYP3A activity.

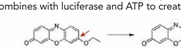

Figure 2 – Resorufin ethyl ether (7-ER), 2 µM, is added to hepatocytes following the 48 hour compound incubation period. The cell-permeable substrate enters the cells. Activation of the AHR receptor causes an increase in CYP1A enzyme expression. CYP1A cleaves the substrate to create the fluorescent substrate resorufin. Upon excitation at 570 nm, a fluorescent signal is then given off at 585 nm, which is proportional to CYP1A activity.

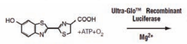

Figure 3 – The CellTiter-Glo® cell viability reagent is added to hepatocytes following the resorufin fluorescent plate read. ATP from viable cells combines with luciferase and luciferin from the detection reagent to create a luminescent signal that is proportional to the number of viable cells in the well.

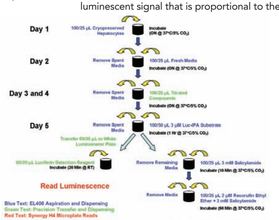

Figure 4 – 96- and 384-Well CYP1A/3A Triplex Assay Automated Workflow.

Cells

Hepatocytes are the gold standard for induction studies because hepatocytes, the primary cell of the liver, have the full complement of enzymes, nuclear factors and co-factors at physiological levels that offer the best correlation to *in vivo* activity compared to cell lines and reporter gene systems. Plateable cryopreserved hepatocytes from Celsis In Vitro Technologies, were used with this project, due to the convenience, reproducibility and flexibility they offer, which is amendable to screening protocols.

CYP1A/-3A Triplex Assay Validation

Automated Assay Validation

The ability of the EL406 to accurately dispense hepatocytes to 96- and 384-well plates was verified by analyzing the %CV of the CellTiter-Glo® values across all wells to contain cells in the assay (96-well: Columns 1-11; 384-well: Columns 1-22). 50,000 cells/well, and 10,000 cells/well were dispensed in 96- and 384-well formats, respectively. %CV values were 4.37% for 96-well dispensing, and 5.68% for 384-well dispensing.

Z'-Factor assays were performed to validate the CYP1A and -3A assays. Omeprazole was used as the control inducer for the CYP1A assay, while Rifampicin was used as the control inducer for the CYP3A assay. Forty-eight replicates of 10 µM or 0 µM compound were used as the positive and negative control, respectively. Z'-Factor values equaled 0.73 and 0.77 for the CYP1A and -3A assays, respectively.

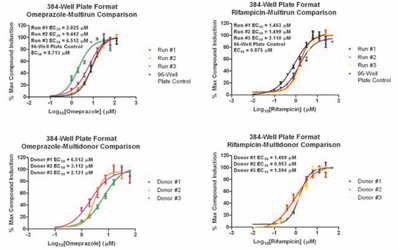

Figure 5 – Multirun/Multidonor EC50 Validation Data.

EC_{50} values for the control compounds Omeprazole and Rifampicin were computed to further validate the assay. Three runs from a single hepatocyte donor were performed to ensure data consistency. The assay was also performed in 96-well format as an additional control for the 384-well data. The EC_{50} values for each run fell within the average +/- ½ log concentration range of 2-21 µM for Omeprazole and 0.5-5.0 µM for Rifampicin.

The assay was also run using hepatocytes from other cell lots to analyze data consistency across multiple donors. The results for each test were once again within the concentration range determined from the original data set.

Fold Induction Values

	Omeprazole @ 62.5 µM		β-Napthoflavone @ 31.2 µM		3-Methylcholanthrene @ 1.6 µM		Lansoprazole @ 3.6 µM	
	CYP1A	CYP3A	CYP1A	CYP3A	CYP1A	CYP3A	CYP1A	CYP3A
Run #1	28	0.5	25	0.4	31	0.5	9	1.9
Run #2	43	0.9	24	1.7	31	2.1	11	1.5
Run #3	23	1.1	19	0.3	26	0.4	9	1.4
Average	31	0.8	22	0.8	29	1.0	10	1.6
Donor #1	43	0.9	24	1.7	31	2.1	11	1.5
Donor #2	14	0.5	24	0.9	31	0.5	8	2.5
Donor #3	14	0.9	22	-3.5	26	2.7	8	2.5
Average	24	0.8	23	-0.3	26	1.9	9	2.2
% Well Control	14	1	16	0.7	14	1.4	6	1.4
FDA Guidelines								
Fold Induction Range*	16-24	No Guidelines Given	4-23	No Guidelines Given	6-26	No Guidelines Given	10	No Guidelines Given
Concentration Range (µM)	25-100		33-50		1-2		10	

	Phenylbutazone @ 12.5 µM		Rifampicin @ 5.5 µM		SR12813 @ 0.63 µM		Dexamethasone @ 62.5 µM	
	CYP1A	CYP3A	CYP1A	CYP3A	CYP1A	CYP3A	CYP1A	CYP3A
Run #1	1.9	1.4	0.8	33	0.6	18	1.4	8.1
Run #2	1.8	1.9	1	26	0.9	18	2.1	7.9
Run #3	1.4	2.6	1.6	29	0.7	27	2.2	9.9
Average	0	20	1.1	26	0.7	20	1.9	8.0
Donor #1	1.4	26	1.6	29	0.7	27	2.2	9.9
Donor #2	1.1	14	0.7	22	1	14	1.6	4.5
Donor #3	1.1	16	1.2	20	1.1	14	2.8	6
Average	0	19	1.2	23	0.9	18	2.2	6.8
% Well Control	6		11		2.1			4.5
FDA Guidelines								
Fold Induction Range*	No Guidelines Given		No Guidelines Given	4-31	No Guidelines Given		No Guidelines Given	2.94-9
Concentration Range (µM)				10-50				33-260

*Guidance for Industry - Drug Interaction Studies - Study Design, Data Analysis, and Implications for Dosing and Labeling. (Draft September 2006). Department of Health and Human Services, Food and Drug Administration.

Table 1 – CYP1A and -3A fold induction values for control compounds. Values generated using the following formula: ((Bkg Subtr. Treated Well/Cell#)/(Avg(Bkg Subst. Basal Well))/Cell#)). FDA guidelines for fold induction and concentration range listed where applicable.

Eight compounds were tested with the triplex assay in dose response mode, including the known CYP1A inducers Omeprazole, β-Napthoflavone, 3-Methylcholanthrene, and Lansoprazole, and the known CYP3A inducers Phenylbutazone, Rifampicin, SR 12813, and Dexamethasone. CYP1A and -3A enzyme activity, and cell viability were computed for each compound. Resorufin and P450-Glo™ fold induction over basal activity was used to determine the effect of the compound on enzyme activity. Results were once again compared across multiple runs of the same hepatocyte donor, as well as across three separate hepatocyte donors.

CYP1A/-3A Triplex Assay Validation

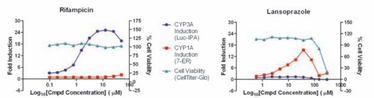

Figure 6 – CYP1A/-3A/CellViability Representative Data.

Fold induction and cell viability graphs were generated to visualize the effect that each compound had on each assay across the entire concentration range tested. The graph for Rifampicin represents a typical graph generated from each data set. The Lansoprazole graph demonstrates how the cell viability measurement can be used to help explain the changes in fold induction that are seen across each compound titration. Lansoprazole induces CYP1A at lower concentrations of the compound, but then this effect decreases dramatically above 50 µM. By including the viability measurement, it becomes obvious that the decrease is due to cytotoxicity from the compound at higher concentrations.

CYP3A4 Ind./Inh. Triplex Assay

Assay Chemistries

Luciferin-IPA substrate and P450-Glo™ Luciferin Detection Reagent were used as previously described.

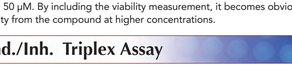

Figure 7 – A luciferin derivative, and ATP, from the ONE-Glo™ reagent combine with luciferase enzyme created within the DPX2 cells. The luminescent signal that is generated is proportional to PXR activation, and CYP3A gene expression within the DPX2 cells.

Figure 8– CellTiter-Fluor™ reagent is added to DPX2 cells to detect cell viability and normalize other assay results to cell number. A fluorogenic, cell-permeable, peptide substrate is added to the cells. Live cells cleave the substrate to create fluorescent AFC, which is also cell-permeable. Upon excitation at 400 nm, a fluorescent signal given off at 505 nm, which is proportional to cell viability.

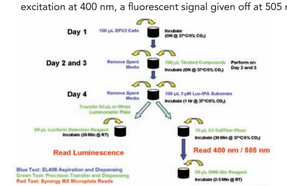

Figure 9 – CYP3A4 Induction/ Inhibition Triplex Assay Automated Workflow.

Cells

DPX2 cells, from Puracyp, Inc., are based on the HepG2 cell line. They contain a vector which constitutively expresses a PXR cDNA, as well as a stably transfected pGL3 plasmid with a CYP3A4 promoter upstream of the luciferase gene. When PXR is activated, PXR joins with RXR to create a heterodimer. This then binds to the 3A4 promoter on the pGL3 plasmid, causing transcription and translation of the luciferase gene and enzyme, respectively. The heterodimer also binds to endogenous CYP3A response elements, causing increased transcription of CYP3A genes, and eventually increased CYP3A enzyme activity.

Figure 10 –DPX2 Cell Diagram

Compound Induction/Inhibition Analysis

A set of compounds was tested in dose response mode. CYP3A4 gene induction, enzyme activity, and cell viability was computed for each compound. One-Glo™ and P450-Glo™ fold induction over basal activity was used to determine the effect of the compound on CYP3A4 expression and enzyme activity. The latter was also used to analyze any inhibitory effects that the compound might have on enzyme activity.

Compound % Rifampicin Transactivation Values

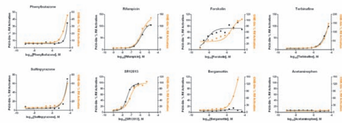

Figure 11 – % Rifampicin Transactivation values at the gene expression level were created for compounds to compare fold induction values to the control compound, Rifampicin, using the following formula: ((Fold induction (Compound Well)*100)/(Fold Induction 5.5 µM Rifampicin)). The 5.5 µM concentration was chosen because of its ability to show maximum fold induction of the compound, with no visible cytotoxicity.

Compound	Triplex ONE-Glo Assay Values: Max%TA	Concentration at Max%TA (µM)	EC_{50} (µM)	Literature Steady-Gls Values: Max%TA	Concentration at Max%TA (µM)	EC_{50} (µM)
Phenylbutazone	41	50	37	54	50	39.7
Fenbufen	101	50	3.72	61	50	1.49
Hyperforin	160	0.6	0.37	120	0.4	0.04
Rifampicin	127	16.7	1.26	114	16.7	1.19
Sulfinpyrazone	69	50	Not Determined	54	50	Not Determined
SR12813	98	5.5	0.09	114	1.9	0.22
Terbinafine	156	50	Not Determined	83	16.7	Not Determined
Bergamottin	141	5.5	22.6	109	16.7	3.23
Acetaminophen	No Transactivation Seen			No Transactivation Seen		

= > 40% Transactivation compared to positive control (5.5 µM Rifampicin-Triplex Assay/10 µM Rifampicin Literature Assay)
= < 40% Transactivation compared to positive control (5.5 µM Rifampicin-Triplex Assay/10 µM Rifampicin Literature Assay)

*Sinz M, Kim S, Zhu Z, Chen T, Anthony M, Dickinson K, Rodrigues A. *Evaluation of 170 Xenobiotics as Transactivators of Human Pregnane X Receptor (hPXR) and Correlation to Known CYP3A4 Drug Interactions*. Current Drug Metabolism 2006; 7: 375-388.

Table 2 – % Rif. Transactivation values for triplex assay compound values previously generated using a singleplex format. FDA guidelines for classification of CYP3A4 inducers (≥ 40% change compared to positive control = enzyme inducer) used to classify compounds as inducers or non-inducers.

Compound Fold Induction/Cell Viability Values

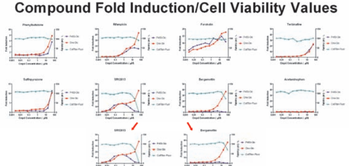

Figure 12 – CYP3A4 Gene Expression/Enzyme Activity/Cell Viability Data.

Fold induction and cell viability graphs were once again generated to visualize the effect that the compounds had on each assay across the entire concentration range tested. Two compounds, SR12813 and Bergamottin, initially show induction at the gene expression level, and inhibition at the enzyme activity level. By examining the cell viability values, however, it becomes apparent that the loss of CYP3A4 enzyme activity with SR12813 is due to cytotoxicity at high compound concentration levels, and not due to inhibition. In contrast, Bergamottin does not show cytotoxic effects until the 50 µM concentration level. Therefore, the lack of enzyme activity can be attributed to a true inhibitory effect.

Conclusions

- The instrumentation used to perform the triplex assay provides an easy to use, dependable solution for dispensing cells, titrating compounds, delivering reagents, and reading the signal from assay plates.
- The triplex assays, incorporating Promega's P450-Glo™, ONE-Glo™, CellTiter-Fluor™, and CellTiter-Glo® reagents, as well as Resorufin Ethyl Ether, provides a straightforward way to assess the effects a compound may have on CYP1A and -3A activity.
- Cryoplateable hepatocytes offer a reproducible and convenient alternative to freshly isolated hepatocytes and are amenable to HTS formats.
- DPX2 cells provide an easy, dependable way to assess a compounds effects on CYP3A4 gene expression and enzyme activity.
- The combination of instrumentation, assay chemistry, and cells create ideal solutions to help fully understand the stimulatory, inhibitory, and cytotoxic effects of a drug.