Time-Course of Cytochrome P450 (CYP450) Induction in Cultured Human Hepatocytes: Evaluation of Activity and mRNA Expression Profiles for Six Inducible CYP450 Enzymes

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Introduction

Enzyme induction potential for new drug candidates is typically evaluated in vitro with cultured human hepatocytes with a demonstrated utility for the quantitative prediction of drug interactions involving cytochrome P450 (CYP450) enzymes, particularly CYP3A4. Recently, many researchers have begun to measure CYP450 mRNA expression in vitro for prediction of clinical outcomes for CYP3A4 with relative success. Furthermore, recent regulatory guidance from the FDA and EMA now recommends CYP450 mRNA expression as the primary in vitro endpoint as opposed to CYP450 activity.

The purpose of this study was to evaluate the time-course of CYP450 induction in cultured human hepatocytes to determine the adequate treatment period and establish CYP450 mRNA expression or enzymatic activity as reasonable endpoints for each of the six inducible hepatic CYP450 enzymes, namely CYP1A2, 2B6, 2C8, 2C9, 2C19 and 3A4.

Materials and Methods

Chemicals and Reagents:
DMSO, rifampin, phenobarbital, and omeprazole were obtained from Sigma-Aldrich (St. Louis, MO).

Hepatocyte cultures:
Cryopreserved human hepatocytes were isolated and prepared from non-transplantable donor livers at XenoTech, LLC. Three lots of cryopreserved hepatocytes (HC3-15, HC3-17 and HC4-1) were plated on collagen I pre-coated 48-well plates, with a Matrigel overlay, and cultured in supplemented Modified Chee's Medium (MCM+). Cultures were allowed to adapt to culture conditions for one day, after which they were treated once daily for up to three consecutive days (72 hrs) with a vehicle control (0.1% DMSO), five concentrations of rifampin (0.05 – 10 µM), and a single concentration of omeprazole (50 µM) or phenobarbital (750 µM) with media replacement every 24 hrs, as appropriate. CYP450 enzyme activities were measured in situ at 0, 24, 48 and 72 hrs with a two-step cocktail approach and CYP450 mRNA expression was evaluated at 0, 6, 12, 24, 30, 36, 48, 54, 60 and 72 hrs by RT-PCR.

In situ CYP450 enzyme activity assays:
CYP450 enzyme activities were evaluated with a two-step cocktail. Briefly, following each 24 hr treatment period (*i.e.* 24, 48 and 72 hrs) media containing controls or prototypical inducer was removed and the cells were washed with 1X PBS. Following removal of the wash media, 200 µL of fresh incubation medium was added containing cocktail #1 (See table below) and incubated for 45 minutes at 37°C. Following incubation with cocktail #1, medium (150 µL) was removed from each well into an equal volume of acetonitrile containing the appropriate internal standard. The cells were rinsed twice with 1X PBS between incubation periods and fresh incubation medium (200 µL) was added containing cocktail #2 (See table below) and incubated for 30 minutes at 37°C. Following incubation with cocktail #2, medium (150 µL) was removed from each well into an equal volume of acetonitrile containing the appropriate internal standard. Metabolite formation was analyzed by LC/MS/MS for the formation (pmol/inc/min) of specific metabolites (See table below).

Cocktail #	Enzyme	Marker Substrate ([µM])	Metabolite(s) Monitored
1	CYP1A2	phenacetin (100 µM)	acetaminophen
	CYP2C9	tolbutamide (150 µM)	4´-hydroxy- and carboxy-tolbutamide*
	CYP2C19	*S*-mephenytoin (400 µM)	4´-hydroxymephenytoin
2	CYP2B6	bupropion (500 µM)	hydroxybupropion
	CYP2C8	amodiaquine (20 µM)	*N*-desethylamodiaquine
	CYP3A4	midazolam (30 µM)	1´-hydroxymidazolam

*CYP2C9 activity was evaluated by combining the formation of sequential CYP2C9 metabolites, namely 4'-hydroxytolbutamide and carboxytolbutamide.

Analysis of mRNA expression:
Following substrate incubations, hepatocytes were lysed in Buffer RLT reagent with β-mercaptoethanol (1:100), and cell lysates were stored at −80 ± 10 °C. Lysates from each treatment group were pooled. Total RNA was purified using the RNeasy Mini Kit (Qiagen Inc.). RNA quality and concentration were determined by measuring absorbance at 260 and 280 nm on a NanoDrop 8000 Spectrophotometer (software version 2.2, Thermo Scientific). Single-stranded cDNA was prepared from RNA (10 ng RNA/reaction) with the RT Master Mix (Applied Biosystems) using the AB 7900HT Fast Real Time PCR System thermocycling program (Applied Biosystems). The cDNA samples were stored at −20 ± 5 °C prior to analysis by qRT-PCR. Quantitative PCR was performed with TaqMan® Gene Expression Assays (10 ng cDNA/reaction) for CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4 and TaqMan® Fast Advanced Master Mix (Applied Biosystems, Carlsbad, CA). The relative expression of each CYP was normalized to the GAPDH endogenous control with the ΔΔCt method. For CYP2C19, two Taqman Gene expression assays (assay # Hs01013970_mH and Hs00426380_m1) were used to verify mRNA expression. EC_{50} and E_{max} values calculated with the Sigmoid, 3-parameter fit model with Sigma Plot version 12 (Systat Software, Inc., San Jose, CA).

Results

The time course of EC_{50} and E_{max} values for CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 mRNA expression are described in **Table 1**. In general, EC_{50} values (measure of potency) for CYP3A4 mRNA induction by rifampin were consistent over time as measured from 6 to 72 hrs of treatment in all three cultures of hepatocytes (**Figure 1A**). However, time-dependent effects on E_{max} values were observed (typically increases) in CYP3A4 mRNA induction by rifampin (**Figure 1B**). In general, similar observations for rifampin EC_{50} and E_{max} values calculated from CYP3A4 enzyme activities (**Table 1**). In cases where rifampin EC_{50} and E_{max} values could be calculated for non-CYP3A4 enzymes, trends similar to the CYP3A4 results were observed.

Table 2 lists the time course of induction for CYP450 activity and mRNA expression in three cultures of cryopreserved human hepatocytes treated with the prototypical inducer, rifampin (10 µM). Furthermore, **Figure 2** illustrates the time course (24, 48 and 72 hrs) and dose-response for rifampin (0.05-10 µM) induced CYP3A4 activity and mRNA expression in the same three hepatocytes cultures, namely HC3-15, HC4-1 and HC3-17.

As expected, treatment of all three lots of hepatocytes with rifampin caused a time-dependent increase in enzyme activities of CYP2B6, all CYP2C enzymes, and CYP3A4 inasmuch as the highest fold-induction values compared to vehicle controls were typically observed at 72 hrs (**Table 2** and **Figure 3**). Similar results were observed for CYP1A2 activity following treatment with omeprazole (**Table 2**).

For CYP450 mRNA expression, the highest fold-induction values compared to vehicle controls were oftentimes reached by 48 hrs following initiation of treatment by prototypical inducers and, in some cases, as early as 24hrs. These results suggest that, when mRNA is a primary endpoint, a 48 hr or shorter treatment period may be suitable. In most cases, the maximal fold-induction (E_{max}) values observed for mRNA expression were equal to, or greater than, the fold-induction values determined by CYP450 activity measurement (**Table 2** and **Figure 3**). The exception was the observed results for CYP2C19 inasmuch as the fold induction observed for enzyme activity was greater than that for mRNA expression.

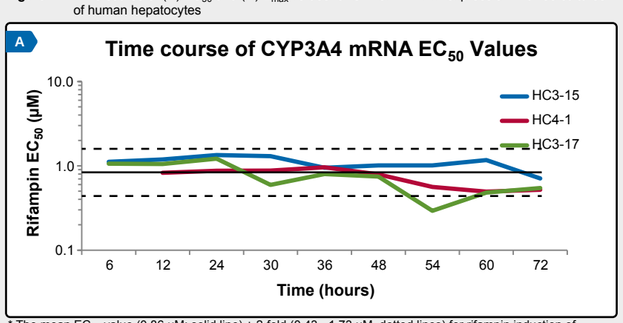
Tables and Figures

Table 1. Time course of EC_{50} and E_{max} values for rifampin-induced CYP3A4, CYP2B6, CYP2C8, CYP2C9 and CYP2C19 mRNA and activity in three cultures of human hepatocytes

		HC3-15				HC4-1				HC3-17			
		mRNA		Activity		mRNA		Activity		mRNA		Activity	
Enzyme	Time (hr)	EC_{50} (µM)	E_{max} (Fold)	EC_{50} (µM)	E_{max} (Fold)	EC_{50} (µM)	E_{max} (Fold)	EC_{50} (µM)	E_{max} (Fold)	EC_{50} (µM)	E_{max} (Fold)	EC_{50} (µM)	E_{max} (Fold)
CYP3A4	24	1.34 ± 0.17	33.2 ± 1.6	NC		0.87 ± 0.17	17.8 ± 1.6	NC		1.22 ± 0.16	88.0 ± 5.5	0.12 ± 0.10	2.1 ± 0.1
	48	1.01 ± 0.13	134 ± 11	0.85 ± 0.12	5.6 ± 0.3	0.79 ± 0.28	42.5 ± 6.8	0.12 ± 0.06	2.4 ± 0.1	0.75 ± 0.14	46.7 ± 6.6	0.65 ± 0.09	11.8 ± 0.8
	72	0.71 ± 0.14	110 ± 13	0.71 ± 0.07	20.7 ± 1.1	0.52 ± 0.07	18.7 ± 1.2	0.24 ± 0.03	3.8 ± 0.1	0.54 ± 0.18	16.8 ± 2	0.33 ± 0.08	15.4 ± 1.3
CYP2B6	24	1.59 ± 0.56	5.8 ± 0.4	0.52 ± 0.00	3.0 ± 0.0	0.65 ± 0.07	3.1 ± 0.1	NC		1.09 ± 0.07	15.9 ± 0.5	0.62 ± 0.11	2.6 ± 0.1
	48	0.63 ± 0.07	14.1 ± 0.8	0.94 ± 0.08	16.7 ± 0.8	0.85 ± 0.05	14.3 ± 0.5	0.61 ± 0.03	5.5 ± 0.1	0.88 ± 0.18	25.3 ± 2.8	0.92 ± 0.07	23.9 ± 1.1
	72	0.82 ± 0.82	19.2 ± 0.6	0.65 ± 0.05	19.8 ± 0.8	0.70 ± 0.10	13.1 ± 1.0	0.74 ± 0.09	11.6 ± 0.7	0.54 ± 0.09	16.1 ± 1.3	0.82 ± 0.13	26.7 ± 2.4
CYP2C8	24	0.21 ± 0.21	3.8 ± 0.5	NC		NC		NC		0.38 ± 0.04	6.2 ± 0.2	NC	
	48	0.09 ± 0.09	2.9 ± 0.1	0.30 ± 0.12	4.2 ± 0.4	0.10 ± 0.02	3.2 ± 0.1	NC		0.18 ± 0.04	3.7 ± 0.1	0.37 ± 0.06	7.2 ± 0.4
	72	0.12 ± 0.12	3.6 ± 0.3	0.10 ± 0.04	3.2 ± 0.2	0.45 ± 0.11	5.4 ± 0.5	0.23 ± 0.06	2.9 ± 0.1	0.09 ± 0.03	2.7 ± 0.1	0.23 ± 0.02	5.2 ± 0.1
CYP2C9	24	0.82 ± 0.11	6.7 ± 0.4	NC		NC		NC		0.59 ± 0.10	3.2 ± 0.2	NC	
	48	0.26 ± 0.03	4.3 ± 0.2	NC		*0.79 ± 0.39	*2.8 ± 0.3	NC		0.19 ± 0.07	2.4 ± 0.2	0.30 ± 0.05	3.0 ± 0.1
	72	0.41 ± 0.25	4.4 ± 0.6	0.26 ± 0.06	2.8 ± 0.1	0.31 ± 0.33	3 ± 0.5	0.27 ± 0.03	2.9 ± 0.1	0.15 ± 0.03	2.2 ± 0.03	0.30 ± 0.08	2.9 ± 0.1
CYP2C19	24	NC		NC		NC		NC		0.04 ± 0.01	2.1 ± 0.03	0.64 ± 0.06	3.3 ± 0.1
	48	NC		NC		NC		0.09 ± 0.14	2.4 ± 0.1	NC		0.48 ± 0.06	4.2 ± 0.2
	72	NC		0.37 ± 0.00	3.1 ± 0.0	NC		0.58 ± 0.03	5.2 ± 0.1	NC		0.41 ± 0.08	5.1 ± 0.3

EC_{50} and E_{max} values calculated with the Sigmoid, 3-parameter fit model unless otherwise indicated.
NC: Not calculated E_{max} < 2-fold
Values presented as ± standard error calculated in Sigma Plot.
*Values calculated with a simple, non-linear fit EC_{50} model with GraFit (version 4.0).

Figure 1. Time course of (**A**) EC_{50} and (**B**) E_{max} values for CYP3A4 mRNA expression in three cultures of human hepatocytes



* The mean EC_{50} value (0.86 µM; solid line) ± 2-fold (0.43 - 1.73 µM, dotted lines) for rifampin induction of CYP3A4 mRNA expression at all time points observed across three cultures of hepatocytes

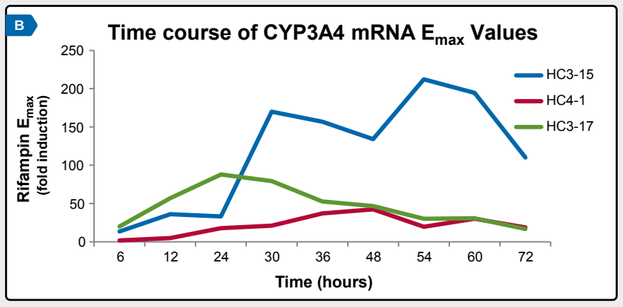


Table 2. Time course of CYP3A4 mRNA expression and enzyme activity in cultured human hepatocytes treated with rifampin (10 µM). Fold induction compared to vehicle controls

Enzyme	Hepatocyte Culture	Enzyme Activity			mRNA Expression		
		24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
CYP3A4	HC3-15	1.7	5.6	20.9	33.2	134	112
	HC4-1	1.3	2.4	3.8	17.9	43.0	19.2
	HC3-17	2.1	11.9	16.8	88.1	46.3	17.1
CYP2B6	HC3-15	3.0	16.8	19.9	5.8	14.3	19.3
	HC4-1	1.5	5.5	11.7	3.1	14.3	13.2
	HC3-17	2.6	23.9	26.9	16.0	25.5	16.8
CYP2C8	HC3-15	1.0	4.4	3.0	4.2	2.8	3.6
	HC4-1	1.0	1.5	3.0	1.4	3.0	5.4
	HC3-17	1.4	7.5	5.3	6.2	4.0	2.5
CYP2C9	HC3-15	1.1	1.9	2.8	6.8	4.5	4.4
	HC4-1	1.2	1.9	3.0	2.0	2.7	3.0
	HC3-17	1.6	3.0	2.9	3.2	2.4	2.2
CYP2C19	HC3-15	1.4	1.7	3.1	0.9	0.4	0.8
	HC4-1	1.1	2.4	5.2	1.6	2.0	1.1
	HC3-17	3.3	4.3	5.2	2.1	1.1	1.3
CYP1A2**	HC3-15	2.2	17.5	95.9	43.9	87.0	164
	HC4-1	0.3	3.1	34.9	13.6	61.7	89.0
	HC3-17	3.4	54.3	99.4	733	227	75.8

** Fold induction by Omeprazole (50 µM)

Figure 2. Time course of CYP3A4 mRNA expression and enzyme activity in cyropreserved human hepatocytes following treatment by multiple concentrations rifampin (0.05-10 µM) for 24, 48 or 72 hours

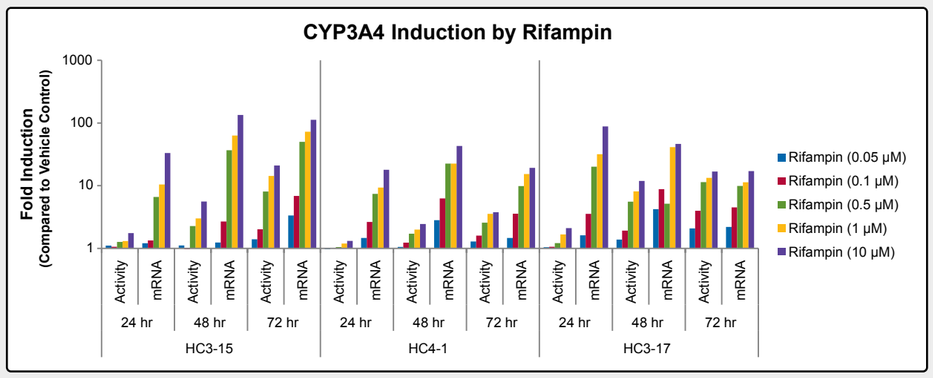
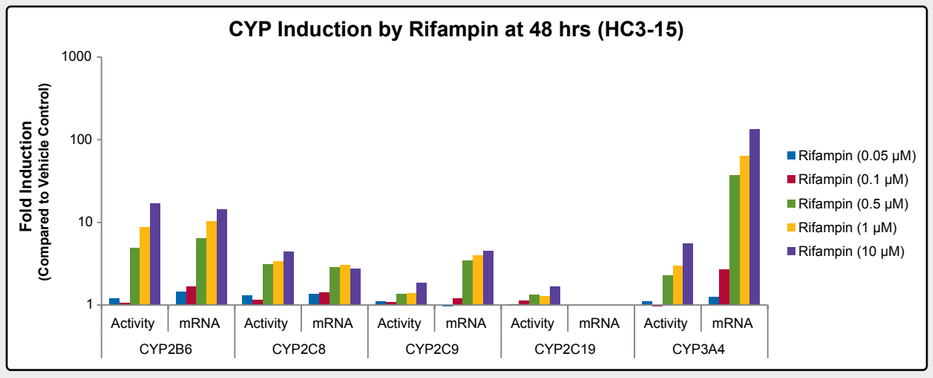


Figure 3. Induction of CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 mRNA expression and enzyme activity in a single lot of hepatocytes (HC3-15) following treatment by multiple concentrations rifampin (0.05-10 µM) for 48 hours



Conclusions

- These results suggest that when mRNA is a primary endpoint a 48 hr treatment period, or shorter, may be suitable.
- In general, E_{max} values for CYP3A4 mRNA induction by rifampin varied (~6 fold) between the three human hepatocyte cultures tested in this study; however, EC_{50} values for CYP3A4 mRNA induction by rifampin were less variable (~3-fold) between cultures.
- In most cases, the maximal fold-induction (E_{max}) values observed based on mRNA expression were equal to, or greater than, the fold-induction values determined by enzymatic activity measurement. However, in the case of CYP2C19, treatment of all three lots of hepatocytes with rifampin up to 72 hrs caused a greater than two-fold induction in enzyme activity compared to vehicle controls but did not cause more than two-fold induction of CYP2C19 mRNA compared to vehicle controls. There is no mechanistic explanation for these data at this time.
- The results presented here provide significant insight into the selection of both appropriate treatment duration and endpoint for in vitro CYP450 induction experiments.