Temporal changes in CYP3A4 mRNA and activity following treatment of cultured human hepatocytes with interleukin-6 (IL-6): Implications for study design and endpoint selection

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INTRODUCTION

In recent years the focus of pharmaceutical drug development (once dominated by small molecule (NCE) therapies) has shifted and is now shared with a significant number of new therapies emerging from biological (New Biological Entities or NBE's) development. Since the approval of the first biological treatment in the United States (recombinant insulin, 1982), more than 250 biologics have reached the market, representing roughly one-quarter of all new drugs approved by U.S. and European Union authorities (Trusheim *et al*, 2010). Biologics include a broad range of therapies including (but not limited too) vaccines, cell or gene therapies, therapeutic protein hormones, cytokines and tissue growth factors, and monoclonal antibodies.

The impact, both *in vitro* and *in vivo* of biologics, either those that act directly (cytokines or interleukins, as in inflammation or disease states) or those that act indirectly (cytokine modulation), on drug metabolizing enzymes (DMEs) has been well documented and this presents a potential new area of concern surrounding safety testing and the disposition of concomitantly administered drugs (Morgan, 2001; Renton KW, 2004; Mammood I and Green MD, 2007; Morgan *et al*, 2008; Kacevska M et al, 2008). Currently, draft guidance released by the Food and Drug Administration (FDA 2006), European Medical Agencies (EMA 2010) and other regulatory bodies, primarily focus on the drug-drug interaction potential for two or more concomitantly administered small molecule drugs, but provides little or no recommendations on the methods of evaluating possible interactions between small molecule drugs and therapeutic proteins (TPs), or biologics. However, the FDA suggests that biologics should be investigated, either *in vitro*, *in vivo*, or both; for the potential to cause biologic-drug interactions (BDI) (Huang SM *et al*, 2010).

Like their small molecule counterparts, therapeutic proteins, such as cytokines, monoclonal antibodies and soluble receptors, can increase systemic exposure to concomitantly administered small drug molecules. Biologics primarily act by suppressing hepatic cytochrome P450 levels through activation of transcription factors like NFκB either directly (as occurs with IL-1β, IL-6, IFNγ and TNFα) or indirectly, by stimulating the release of these pro-inflammatory cytokines from Kupffer (liver) or peripheral blood mononuclear cells (PBMCs) (Zhou *et al*, 2006; Gu *et al*, 2006; Mammood I and Green MD, 2007).

In order to assess the robustness of plated human hepatocytes to a pro-inflammatory cytokine, namely IL-6, and to provide guidelines for both endpoint selection and duration of test article exposure; we examined the time course of changes in CYP3A4 activity (and mRNA levels) in primary cultures of human hepatocytes treated for either 24 hours or once daily for three consecutive days with IL-6.

METHODS

Chemicals and reagents:
The sources of the reagents used in this study have been described previously (Madan et al., 2003). Rifampin and interleukin 6 (IL-6) were purchased from Sigma Aldrich and EMD Chemicals, respectively.

Hepatocyte Cell Culture:
Primary human hepatocytes were isolated and plated on collagen-coated dishes at XenoTech, essentially as described by Paris et al. (2009). The hepatocytes were allowed to adapt to culture conditions for two to three days, after which they were treated once daily for one or three consecutive days with dimethyl sulfoxide (DMSO, 0.1% v/v, vehicle control), or one known CYP3A4/5 prototypical inducer, namely, rifampin (10 µM, prepared in DMSO) or one pro-inflammatory cytokine, interleukin-6 (IL-6, 10 ng/mL prepared in acetic acid). Treated hepatocytes were either harvested for the preparation of microsomes (for subsequent analysis of CYP3A4/5 enzymatic activity) or lysed with TRIzol® reagent (Invitrogen) to isolate RNA (for analysis of CYP3A4 mRNA expression by qRT-PCR) following 24, 48 and 72 hours.

Microsomal CYP Enzyme Activities:
Following the treatment period, microsomes from human hepatocytes were assayed for CYP3A4/5 activity by testosterone 6β-hydroxylation. Microsomes isolated from the cultured hepatocytes (40 ug/mL) were incubated with testosterone (250 µM) for 10 minutes at 37°C. Reactions were stopped with acetonitrile containing internal standard and metabolite formation was determined by LC/MS/MS.

CYP Enzyme mRNA Analysis:
Total RNA was phase extracted with Trizol® reagent followed by purification with the RNeasy Mini Kit (Qiagen) according the manufacturer's protocol. Purified RNA was reverse transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the Applied Biosystems 7300 Real-Time PCR System (AB7300). Semi-quantitative PCR was performed with the AB7300 or AB7900 with the Applied Biosystems Universal Master Mix and TaqMan® Gene Expression Assays. CYP3A4 mRNA levels were normalized with GAPDH as the endogenous control. CYP mRNA levels were analyzed for fold induction caused from treatment with rifampin and IL-6 compared to the control (0.1% DMSO).

RESULTS

Table 1 and 2 show that treatment of three individual cultured human hepatocyte preparations with a single or daily treatment (up to three days) of the prototypical CYP3A4 inducer, rifampin, caused anticipated induction of CYP3A4/5 enzyme activity and CYP3A4 mRNA expression.

Additionally, **Table 1** and **Figure 1 and 3** show that treatment of three individual cultures of human hepatocytes with a single (24 hr) treatment of IL-6 caused a decrease in CYP3A4/5 activity and CYP3A4 mRNA expression. Following a single treatment with IL-6, the decrease in CYP3A4/5 activity was most pronounced (to 63.3% of control) after 48 hours and the activity began to increase by 72 hours (to 83.0% of control). The decrease in CYP3A4 mRNA expression was most pronounced immediately following IL-6 exposure at 24 hours (to 18.5% of control) and began to recover/increase after 48 and 72 hours (34.3 and 188%, respectively).

These results demonstrate the temporal relationship between CYP3A4/5 activity and CYP3A4 mRNA expression following treatment with a pro-inflammatory cytokine (IL-6). As expected, the decrease in CYP3A4 mRNA expression occurred prior to the subsequent decrease in enzyme activity.

Table 2 and **Figure 2 and 4** illustrate that treatment of three individual cultures of human hepatocytes with IL-6 for three consecutive days caused a decrease in CYP3A4 activity and mRNA expression; however, the decreases were more pronounced than those following a single treatment with IL-6. The decrease in CYP3A4/5 activity following a 3-day treatment with IL-6 was progressive by day and was most pronounced at 72 hours (to 31.4% of control). In a similar manner, the decrease in CYP3A4 mRNA expression was progressive by day and the decrease was most dramatic at 72 hours (7.27% of control).

Table 1

The effect of a single treatment of cultured human hepatocytes with IL-6 or Rifampin on CYP3A4 expression (activity and mRNA) at 24, 48 or 72 hours post treatment

Single Treatment Regimen							
			Activity			mRNA	
Treatment duration and regime	Treatment	Donor #	Fold Increase over Control Sample	% of Control	Mean % of Control	Fold over Control Sample (RQ)	% of Control	Mean % of Control
24 Hours Single Treatment	10 µM Rifampin	Donor 1	56.6	NA	NA	328	NA	NA
		Donor 2	3.59	NA		6.12	NA	
		Donor 3	2.38	NA		65.2	NA	
	10 ng/mL IL-6	Donor 1	0.638	63.8	86.2	0.291	29.1	18.5
		Donor 2	0.519	51.9		0.111	11.1	
		Donor 3	1.43	143		0.153	15.3	
48 Hours Single Treatment	10 µM Rifampin	Donor 1	71.7	NA	NA	88.5	NA	NA
		Donor 2	6.22	NA		16.6	NA	
		Donor 3	11.1	NA		260	NA	
	10 ng/mL IL-6	Donor 1	0.488	48.8	63.3	0.314	31.4	34.3
		Donor 2	0.280	28.0		0.485	48.5	
		Donor 3	1.13	113		0.230	23.0	
72 Hours Single Treatment	10 µM Rifampin	Donor 1	37.0	NA	NA	11.0	NA	NA
		Donor 2	9.04	NA		9.96	NA	
		Donor 3	17.8	NA		50.1	NA	
	10 ng/mL IL-6	Donor 1	0.717	71.7	83.0	1.07	107	188
		Donor 2	0.748	74.8		2.60	260	
		Donor 3	1.02	102		1.97	197	

Table 1

The effect of daily treatment (up to three days) of cultured human hepatocytes with IL-6 or Rifampin on CYP3A4 expression (activity and mRNA) at 24, 48 or 72 hours post initial treatment

Daily Treatment Regimen							
			Activity			mRNA	
Treatment duration and regime	Treatment	Donor #	Fold Increase over Control Sample	% of Control	Mean % of Control	Fold over Control Sample (RQ)	% of Control	Mean % of Control
24 Hours Daily Treatment	10 µM Rifampin	Donor 1	56.6	NA	NA	328	NA	NA
		Donor 2	3.59	NA		6.12	NA	
		Donor 3	2.38	NA		65.2	NA	
	10 ng/mL IL-6	Donor 1	0.638	63.8	86.2	0.291	29.1	18.5
		Donor 2	0.519	51.9		0.111	11.1	
		Donor 3	1.43	143		0.153	15.3	
48 Hours Daily Treatment	10 µM Rifampin	Donor 1	41.6	NA	NA	24.5	NA	NA
		Donor 2	5.48	NA		10.7	NA	
		Donor 3	12.1	NA		208	NA	
	10 ng/mL IL-6	Donor 1	0.285	28.5	48.2	0.411	41.1	15.3
		Donor 2	0.136	13.6		0.0130	1.30	
		Donor 3	1.02	102		0.0350	3.50	
72 Hours Daily Treatment	10 µM Rifampin	Donor 1	17.0	NA	NA	7.57	NA	NA
		Donor 2	5.27	NA		10.9	NA	
		Donor 3	21.4	NA		86.4	NA	
	10 ng/mL IL-6	Donor 1	0.169	16.9	31.4	0.0520	5.20	7.27
		Donor 2	0.0755	7.55		0.105	10.5	
		Donor 3	0.697	69.7		0.0610	6.10	

Figure 1

The effect of treating individual cultured human hepatocytes with IL-6 (single exposure) on CYP3A4 activity and mRNA expression at 24, 48 and 72 hours post treatment

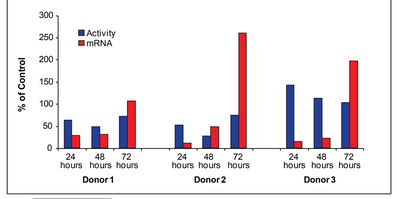


Figure 2

The effect of treating individual cultured human hepatocytes with IL-6 (daily, up to three days) on CYP3A4 activity and mRNA expression at 24, 48 and 72 hour treatment duration

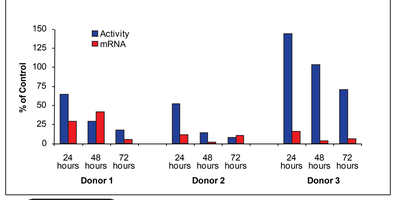


Figure 3

The mean effect of treating individual cultured human hepatocytes with IL-6 (single exposure) on CYP3A4 activity and mRNA expression at 24, 48 and 72 hours post treatment

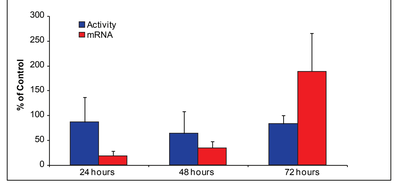
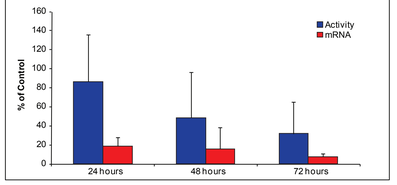


Figure 4

The effect of treating individual cultured human hepatocytes with IL-6 (daily, up to three days) on CYP3A4 activity and mRNA expression at 24, 48 and 72 hour treatment duration



CONCLUSIONS

1. Treatment of human hepatocytes with a single or multiple applications of IL-6 suppressed CYP3A4 mRNA levels and, to a lesser extent, CYP3A4/5 activity in all three hepatocyte preparations.
2. Following a single application of IL-6, suppression of CYP3A4 mRNA was maximal after 24 hours whereas suppression of CYP3A4/5 activity was maximal after 48 hours. Likewise, three days after a single application of IL-6, recovery of CYP3A4 mRNA levels was greater than the recovery of CYP3A4/5 activity.
3. Compared with a single application, multiple applications of IL-6 (once a day for 3 days) caused greater suppression of both CYP3A4/5 activity and CYP3A4 mRNA expression with no recovery over the time-course of the study (72 hrs following first exposure).
4. These data suggest that for a single application of pro-inflammatory cytokine, mRNA may be most suitable when examined after 24 hours whereas CYP activity may be best evaluated 48 hours post-treatment.
5. Consequently, the selection of a treatment regimen (single vs. multiple daily treatments) is an important factor in the selection of both time points and endpoints (mRNA or activity) when evaluating therapeutic proteins for the potential to modulate CYP3A4 expression.

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