DMD Fast Forward. Published on May 9, 2011 as doi:10.1124/dmd.111.038679

Effects of Interleukin 6 (IL-6) and an anti-IL-6 Monoclonal Antibody on Drug Metabolizing Enzymes in Human Hepatocyte Culture

Leslie J. Dickmann, Sonal K. Patel, Dan A. Rock, Larry C. Wienkers and J. Greg Slatter

Pharmacokinetics and Drug Metabolism

Amgen, Inc., 1201 Amgen Court West, Seattle, WA 98119

DMD Fast Forward. Published on May 9, 2011 as DOI: 10.1124/dmd.111.038679
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Running Title: Interleukin-6 Effects on Human Hepatocytes

Corresponding Author: Leslie J. Dickmann

Address: 1201 Amgen Court West, Seattle, WA 98119

Tel. (206) 265-8897

Fax: (206) 217-0492

Email: ldickman@amgen.com

Number of text pages: 33

Number of tables: 6

Number of supplemental tables: 1

Number of figures: 5

Number of supplemental figures: 2

Number of references: 40

Number of words in Abstract: 245

Number of words in Introduction: 740

Number of words in Discussion: 1,499

Non-standard abbreviations: CRP, C-reactive protein; CYP, cytochrome P450; INFγ, interferon γ; IL-6, interleukin 6; KinExA, kinetic exclusion assay; LPS, bacterial lipopolysaccharide; mAb, monoclonal antibody; SAA, serum amyloid A; Kd, dissociation

constant; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α,

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Abstract

The cytokine-mediated suppression of hepatic drug metabolizing enzymes by inflammatory disease and the relief of this suppression by successful disease treatment have recently become an issue in the development of drug interaction labels for new biologic products. This study examined the effects of the inflammatory cytokine interleukin 6 (IL-6) on drug metabolizing enzymes in human hepatocyte culture, and the abrogation of these effects by a monoclonal antibody (mAb) directed against IL-6. Treatment with IL-6 in human hepatocytes (N=9 donors) revealed pan-suppression of mRNA of 10 major cytochrome P450 isoenzymes, but with EC_{50} values that differed by isoenzyme. Some EC_{50} values were above the range of clinically-relevant serum concentrations of IL-6. Marker activities for CYP1A2 and CYP3A4 enzyme were similarly suppressed by IL-6 in both freshly isolated and cryopreserved hepatocytes. IL-6 suppressed induction of CYP1A2 enzyme activity by omeprazole and CYP3A4 enzyme activity by rifampicin but only at supra-physiological concentrations of IL-6. Glycosylated and non-glycosylated IL-6 did not significantly differ in their ability to suppress CYP1A2 and CYP3A4 enzyme activity. A monoclonal antibody directed against IL-6 abolished or partially blocked IL-6-mediated suppression of CYP1A2 and CYP3A4 enzyme activity, respectively. These data indicate that experimentation with IL-6 and anti-IL-6 monoclonal antibodies in human hepatocyte primary culture can quantitatively measure cytochrome P450 suppression and de-suppression and determine EC_{50}s for IL-6 against individual cytochrome P450 isoenzymes. However, the complex biology of inflammatory disease may not allow for quantitative *in vitro–in vivo* extrapolation of these simple *in vitro* data.

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Introduction

The cytokine-mediated suppression of hepatic drug metabolizing enzymes by inflammatory disease and the relief of suppression ("de-suppression") by successful disease treatment have recently become an issue in the development of drug interaction labels for new biological products (Huang et al., 2010). In a bellwether example, treatment of rheumatoid arthritis patients with the anti-IL6R monoclonal antibody, tocilizumab (Actemra™, Roche/Genentech), resulted in a 1.8-fold (57% of control) decrease in the plasma AUC of the CYP3A4 substrate simvastatin (Genentech, 2010). Along with some related *in vitro* characterization of IL-6 effects in human hepatocytes, this resulted in a drug interaction label that recommends caution for Tocilizumab use with narrow therapeutic index cytochrome P450 substrates (Genentech, 2010). This is a drug-disease interaction, where the perceived risk of successful disease treatment is therapeutic failure due to an increase in the clearance of cytochrome P450 substrates. Subsequently, regulatory authorities have requested *in vitro* and/or *in vivo* study data for other cytokine-modulating biologics such as Golimumab (CDER, 2009). Unfortunately, there is only a limited understanding of the potential utility of the preclinical tools that might be used to predict the occurrence and magnitude of clinical de-suppression of cytochromes P450 enzymes by anti-cytokine biologic agents.

Interleukin-6 (IL-6) is an important mediator in the acute phase response (APR) (Morrone et al., 1988; Andus et al., 1989) and is relevant to the treatment of chronic diseases such as rheumatoid arthritis, as shown by the approval of Tocilizumab (Rose-John et al., 2007). IL-6 is secreted by Kupffer cells in the liver and acts through its receptor, IL-6R (CD126), on hepatocytes to elicit a variety of effects including mediating components of the APR, hepatic regeneration and anti-apoptotic and anti-necrosis effects (Taub, 2004). The IL-6R can bind IL-6 as a soluble or as a membrane bound protein (Rose-John et al., 2007) and the IL-6/IL-6R

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complex must bind two monomers of gp130 membrane protein to elicit signal transduction (Waetzig et al.; Rose-John et al., 2007).

Hepatic cytochrome P450 enzymes are known to be down-regulated during infection and inflammatory stress and this occurs across the spectrum of cytochrome P450 isoenzymes (Shedlofsky et al., 1994; Morgan et al., 1998; Cheng et al., 2003). Cytochrome P450 down-regulation is attributed to cytokine release, since various cytokines mimic the effects of infection when injected *in vivo* or added to hepatocytes in primary culture (Renton and Nicholson, 2000; Morgan, 2001; Aitken and Morgan, 2007). The mechanism of cytochrome P450 pan-suppression is apparently transcriptional, related to the APR, and may involve Nf-κB, although a unifying mechanism for pan-suppression is not fully elucidated. Ghose et al. (Ghose et al., 2004) have proposed that the broad down-regulation of cytochrome P450 isoenzymes may be due to inflammation-induced alteration of the subcellular location of RXRα, which is an obligate partner in the heterodimeric nuclear receptors LXR, FXR, RAR, CAR, PXR and PPARα. These nuclear receptors regulate enzymes controlling the metabolism of lipids, bile acids, steroids and xenobiotics.

Human hepatocytes have been used to investigate the effects of cytokines involved in the acute phase response on cytochrome P450 regulation (Sunman et al., 2004; Aitken and Morgan, 2007). IL-6, LPS, TNFα, INFγ and TGFβ all caused a marked decrease in cytochrome P450 mRNA and activity levels for CYP3A4, CYP2C isoforms, and CYP2B6, albeit with different magnitudes of suppression. Studies conducted to date were done at supra-physiological concentrations of IL-6 (> 2,000 pg/mL). In healthy individuals, serum IL-6 concentrations range from 1.3-10.3 pg/mL and are increased to 2.6-123 pg/mL in some patient populations presenting with inflammation based diseases (Roytblat et al., 2000; Hidaka et al., 2001; Yilmaz et al., 2001; Arican et al., 2005; Mastroianni et al., 2005; Ataseven et al., 2006; Wang et al., 2008).

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To discern the strengths and limitations of *in vitro* experimentation on cytokine-mediated suppression of cytochrome P450s, this study assessed the cytochrome P450 expression concentration-response to IL-6 in primary human hepatocytes at physiologically relevant concentrations, the influence of interindividual variability across donors, the effects of cryopreservation, concommitant induction, reagent glycosylation and the abrogation of IL-6 mediated suppression by a monoclonal antibody against IL-6.

This study shows that inclusion of IL-6 and an anti-IL-6 monoclonal antibody in human hepatocyte primary culture allows quantitative measurement of cytochrome P450 suppression and de-suppression. However, these data represent only a small component of the complicated biology of the acute phase response and chronic inflammation in the intact liver. This limits the value of the model for quantitative prediction of clinical drug-disease interactions related to cytochrome P450 suppression and de-suppression.

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Materials and Methods

Reagents and Chemicals. William's E Medium, Dubelcco's Modified Eagle's Medium (DMEM), dexamethasone, phenacetin, acetaminophen, 6β-hydroxytestosterone, omeprazole, rifampicin, and HEK 293 expressed IL-6 were purchased from Sigma-Aldrich (St. Louis, MO). Testosterone was purchased from Steraloids (Newport, RI). Human hepatocytes, Cryopreserved Recovery Medium (CHRM), and media supplements were purchased from CellzDirect (Durham, NC). Matrigel was purchased from BD Biosciences (San Jose, CA) and Kreb-Henseleit buffer (KHB) from Celsis/In Vitro Technologies (Chicago, IL). Human *E. coli* expressed IL-6 and human IL-6 neutralizing antibody, (clone 1936), were purchased from R&D Systems (Minneapolis, MN). Nuclease free water, RNA later, MagMax 96 RNA isolation kit, High Capacity cDNA Transcription Kit, TaqMan primer and probe sets and all TaqMan reagents and consumables were purchased from Applied Biosystems (Foster City, CA). IL-6R protein and mouse monoclonal and goat polyclonal antibodies to human IL-6 used in KinExA experiments were purchased from Abcam (Cambridge, MA). Cy5 labeled goat anti-mouse IgG was purchased from Jackson Immunoresearch (West Grove, PA), and NHS-activated sepharose from GE Healthcare (San Francisco, CA).

Cell culture. Suspension hepatoyctes from the vendor were spun at 100 x *g* for 10 minutes and resuspended in plating media (William's E plus plating supplements: 5% fetal bovine serum, 100 nM dexamethasone, 100 U/mL penicillin and streptomycin, 4µg/mL insulin, 2mM GlutaMAX™, 15mM HEPES, pH 7.4). Cryopreserved hepatocytes were thawed at 37ºC, spun at 100 x *g* in CHRM and resuspended in plating media. Viability and density were measured by trypan blue exclusion and 52,000 cells/well were plated onto 96-well collagen I coated plates. Hepatocytes were allowed to attach for 4-6 hours, plating media was removed and replaced with maintenance media (DMEM plus maintenance supplements: 100 U/mL penicillin and streptomycin, 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL

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bovine serum albumin, 5.35 µg/mL linoleic acid, 2mM GlutaMAX™, 15mM HEPES, pH 7.4) containing 0.25 mg/mL Matrigel. Cells were treated the following day. Media plus appropriate concentrations of cytokine and/or chemical drug was replaced daily. Unless otherwise noted, *E. coli* expressed IL-6 was used for all studies.

Analytical Conditions. The LC-MS/MS system was comprised of an Applied Biosystems 4000 Q-Trap equipped with an electrospray ionization source (Applied Biosystems, Foster City, CA) coupled to an HPLC system consisting of two LC-20AD pumps with an in-line CBM-20A controller and DGU-20A_5 solvent degasser (Shimadzu, Columbia, MD) and a LEAP CTC HTS PAL autosampler equipped with a dual-solvent self-washing system (CTC Analytics, Carrboro, NC). The injection volume was 20 µL for each analyte. LC separation was achieved using a Gemini C18 2.0 x 30 mm 5 µm column (Phenomenex, Torrance, CA). Gradient elution (flow rate = 500 µL/min) was carried out using a mobile phase system consisting of (A) 5 mM ammonium formate with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid with the following gradient 0-0.5 min, 5% (B); 0.5-1.75 min, 100% (B); 1.75-2 min, 5% (B). Source and gas parameters are as follows: Curtain gas: 10, collision gas: medium, ionspray voltage: 4500 V, temperature: 450°C.

Analysis of Acetaminophen Formation. Cells were washed with 100µL of KHB for 5 minutes. After removal, 100µL of KHB containing 100µM phenacetin was added to the cells and incubated on a rocking platform for 15 minutes in a 37°C incubator with 5% CO_2. After the incubation period, 50µL was removed and added to 50µL acetonitrile containing 100nM tolbutamide (internal standard). Acetaminophen was measured by using multiple reaction monitoring with the following conditions: Q1: 151.9, Q3: 110, DP: 56, CE: 25, CXP: 20. Mode: Positive for acetaminophen and Q1: 271.2, Q3: 91.1, DP: 71, CE: 43, CXP: 14, Mode: Positive for tolbutamide.

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Analysis of 6β-Hydroxytestosterone Formation. Cells were washed with 100µL of KHB for 5 minutes. After removal, 100µL of KHB containing 150µM testosterone was added to the cells and incubated on a rocking platform for 15 minutes in a 37°C incubator with 5% CO_2. After the incubation period, 50µL was removed and added to acetonitrile containing 100nM tolbutamide (internal standard). 6β-hydroxytestosterone was measured by using multiple reaction monitoring with the following conditions: Q1: 305, Q3: 269, DP: 55, CE: 25, CXP: 13. Mode: Positive.

mRNA analysis. Immediately following activity assays, remaining KHB was removed, 100µL of RNA later was applied and the cells were frozen at -30°C until mRNA isolation (no longer than one week). Total RNA was isolated using the MagMax96 RNA isolation kit according to manufacturer's protocol and RNA quantity and quality were assessed using the Agilent RNA 6000 Nano Chip kit and/or NanoDrop. All RNA samples were then normalized to 5 ng/µL with nuclease free water. cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit with a final volume of 40µL and 132 ng total RNA according to manufacturer's protocol. After synthesis, the cDNA reactions were diluted to 160µL total volume with nuclease free water. TaqMan reactions were run on a 7900HT Real-time PCR system in a 384-well optical reaction plate. Each reaction contained 10µL 2x Gene Expression Master Mix, 5µL nuclease free water, 1µL 20x Primer and Probe mix, and 4µL of cDNA. All reactions were run in duplicate using default cycling parameters. A commercially available panel of endogenous control genes from Applied Biosystems was run initially and showed that both 18s and beta-2 microglobulin levels were unaltered at all IL-6 concentrations.

Binding Constant Determination. Kd values for *E. coli* expressed and HEK293 expressed IL-6 binding to IL-6R were determined using Kinetic Exclusion Assay (KinExA). Briefly, a goat polyclonal to IL-6 (25 µg/1mL beads) was conjugated to NHS activated sepharose for 4 hours at room temperature and then blocked with BSA for an additional 2 hours at room temperature. A

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mouse monoclonal antibody directed towards IL-6 (2 µg/mL) along with a Cy5 labeled goat anti-mouse IgG (1 µg/mL) were used for detection. A constant IL-6 concentration (0.2, 1, or 5µM) was incubated with increasing concentrations of soluble IL-6R protein (12 concentrations, 3pM-529nM) at room temperature for 24 hours in PBS buffer containing 0.5 mg/mL BSA. Samples were run in triplicate using the standard Kd method outlined in the KinExA manual and analyzed using the KinExA n-Curve Analysis software.

Data analysis. Mass spectrometry data were analyzed using Analyst (Applied Biosystems, Foster City, CA) and Excel (Microsoft, Redmond, WA). mRNA data were analyzed using SDS 2.3 RQ Manager software from Applied Biosystems with beta-2 microglobulin as the endogenous control and untreated cells (depending on treatment) as the calibrator. Dose response curves, EC_{50}, E_{max}, and E_{min} values were obtained using GraphPad Prism software (GraphPad Software, La Jolla, CA) using the log(inhibitor) vs dose, variable slope model ($Y = E_{min} + (E_{max}-E_{min})/(1 + 10^{((LogEC_{50}-X)*Hill\ slope))}$) or the bell-shaped dose response model ($Y = E_{max\_plateau} + ((E_{max\_plateau} – E_{max})/(1 + 10^{((LogEC501-X)*Hill\ slope\ 1))})) + ((E_{min} – E_{max\_plateau})/(1 + 10^{((X – LogEC502)*Hill\ slope\ 2))})$). Kd values for IL-6 binding to its receptor were calculated using KinExA n-Curve Analysis software (Sapidyne Instruments, Boise, ID).

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Results

Effects of IL-6 Treatment on the Expression of Acute Phase Response Proteins. Primary hepatocytes from Donor Hu1153 were exposed to IL-6 for 72 hours. Over the concentration range 5 pg/mL-50 ng/mL IL-6, microscopic examination and an ATP assay showed that IL-6 was not toxic to hepatocytes from this donor (data not shown). IL-6 caused a marked increase in the acute phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA) (Table and Figure 1). The EC_{50} for CRP induction by IL-6 was 73.4 pg/mL with a maximum fold increase of 1021 over untreated cells (Table 1). SAA induction had a maximum fold induction of 19.9 and an EC_{50} of 22.9 pg/mL (Table 1).

Time Dependent Effects of IL-6 on CYP1A2 and CYP3A4 Activity. Primary hepatocytes from Donor Hu1153 were exposed to IL-6 for 48, 72, or 96 hours, at which point acetaminophen (CYP1A2 activity) and 6β-hydroxytestosterone (CYP3A4 activity) formation were assessed. At all three time points, both acetaminophen and 6β-hydroxytestosterone formation decreased with increasing concentration of IL-6 (Figure 2). After 48 hours, maximal suppression of acetaminophen formation was observed with an E_{min} of 10.7% of untreated cells and an EC_{50} value of 409 pg/mL (Table 2). Maximal suppression of 6β-hydroxytestosterone formation occurred at 96 hours with an E_{min} of 21.5% and an EC_{50} of 17.1 pg/mL, although the 72 and 96 hour time points were similar. The 72 hour treatment duration was chosen to standardize conditions for further investigation.

Effect of IL-6 on mRNA Expression of Cytochrome P450 Isoforms and IL-6R. mRNA levels were assessed in Donor Hu1153 hepatocytes exposed to IL-6 for 72 hours. IL-6 caused a global down-regulation of all cytochrome P450 isoforms examined. The EC_{50} and E_{min} data indicated that the potency and extent of suppression varied among the cytochrome P450

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isoforms (Table 3). CYP1A1 was the least sensitive to suppression by IL-6, with an EC_{50} of 1,620 pg/mL while CYP3A4 was the most sensitive with an EC_{50} of 3.2 pg/mL. While many P450 isoforms, such as CYP2B6, CYP2C8, CYP3A4, and CYP3A5, were down-regulated to below 5% of untreated cells, other isoforms such as CYP1A1, CYP1A2, CYP2C19, and CYP2D6 had a maximum suppression (E_{min}) of between 15-40% of untreated cells. The EC_{50} for down-regulation of IL-6R mRNA was similar at 107 pg/mL (data not shown), showing that cells respond in a dynamic way to sustained stimulation by IL-6.

Donor Variability in the Effects of IL-6 on CYP1A2 and CYP3A4 Activity. To understand variability in the effect of IL-6 suppression on marker assays for two representative cytochrome P450 isoforms, three cryopreserved donors (Hu1001, Hu8064, and Hu4151) and two additional fresh donors (Hu1146 and Hu1150) were assessed. Donor characteristics can be found in the supplemental data (Supplemental Table 1). As shown in Table 4 and the supplemental data (Supplemental Figure 1), all donors had decreasing formation of both acetaminophen and 6β-hydroxytestosterone formation with increasing IL-6 concentrations. The EC_{50} range for acetaminophen formation was 142-4070 pg/mL and 4.2-176 pg/mL for 6β-hydroxytestosterone formation. Maximal suppression for CYP1A2 activity ranged from 9.3-52.9% of untreated cells and for CYP3A4 activity the range was 10.7-41.7% of untreated cells.

The Effects of IL-6 on the Induction of CYP1A2 and CYP3A4. To examine IL-6-mediated suppression of CYP1A2 and CYP3A4 activity at different expression levels of cytochrome P450, hepatocytes from donor Hu4151 were treated for 72 h with IL-6 in the presence or absence of the CYP1A2 inducer omeprazole (50 μM) or CYP3A4 inducer rifampicin (10μM). Figure 3 shows that in the absence of IL-6, 8- and 4-fold induction occurred in omeprazole and rifampicin-treated cells, respectively. IL-6 overcame the effects of both inducers, but only at concentrations greater than 500 pg/mL. The EC_{50} of suppression of both CYP1A2 and CYP3A4 activity was shifted in the presence of inducer from 730 to 1120 pg/mL (1.5-fold) for CYP1A2 and from 82.8

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to 1574 pg/mL (19-fold) for CYP3A4 (Table 4). The dynamic range of suppression by IL-6 was also affected in induced cells, with E_{min} increasing almost 15-fold for CYP1A2 activity and 2-fold for CYP3A4 activity. IL-6-mediated suppression of the induced cytochrome P450 isoforms occurred at IL-6 concentrations that were well above those normally encountered in serum from patients with inflammatory disease (2.6-123 pg/mL, (Hidaka et al., 2001; Yilmaz et al., 2001; Arican et al., 2005; Ataseven et al., 2006; Wang et al., 2008). Based on these *in vitro* data, suppression of cytochrome P450 activity by IL-6 does not override cytochrome P450 induction at physiological concentrations of IL-6.

The Effect of IL-6 Glycosylation Status on Cytochrome P450 Suppression. Because most commercial IL-6 is expressed in an *E. coli* system that lacks the capability to glycosylate proteins, IL-6 expressed in a human cell line, HEK293, was used to compare the extent of cytochrome P450 suppression between non-glycosylated and glycosylated versions of IL-6. IL-6 expressed in the HEK293 cell line had an observed molecular weight between 26-30 kDa and had variable glycosylation (manufacturer's data). Glycosylated and non-glycosylated IL-6 were examined using a kinetic exclusion assay (KinExA) to measure the dissociation constant (Kd) from the soluble IL-6 receptor. The measured Kd for the non glycosylated *E. coli*-expressed IL-6 was 5.1 nM (2.5-9.7 nM; 95% confidence interval) and was 3.2 nM (1.9-5.1 nM; 95% confidence interval) for HEK293 expressed IL-6. Figure 4 and Table 5 data from Donor Hu1190 show that the glycosylation state of IL-6 did not markedly change the EC_{50} or E_{min} data for suppression of CYP1A2 or CYP3A4 activity. Cytochrome P450 mRNA data for several cytochrome P450 isoforms gave similar results (Table 5). Of these isoforms, no statistically significant change in EC_{50} values was observed between glycosylated and non-glycosylated IL-6. There was also no statistically significant difference in the maximum extent of suppression (E_{min}, data not shown).

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De-suppression of Cytochrome P450 *In Vitro*: The Effect of an IL-6 Neutralizing Antibody on IL-6 Mediated Suppression of CYP1A2 and CYP3A4. To assess whether cytochrome P450 suppression by IL-6 can be blocked by a monoclonal antibody against non-glycosylated IL-6, 100 ng/mL of IL-6 neutralizing monoclonal antibody was added to hepatocytes in the presence of increasing concentrations of IL-6. To better understand the ability of this particular antibody to neutralize IL-6 activity and cause cytochrome P450 de-suppression, a dissociation constant was measured between non-glycosylated IL-6 and the monoclonal antibody. The Kd value was 1.7 nM, similar to the Kd value of IL-6 binding to IL-6R.

In two donors, the antibody blocked IL-6 suppression of CYP1A2 activity completely such that an EC_{50} value could not be calculated for hepatocytes treated with both IL-6 and the anti-IL-6 antibody (Figure 5, Table 6). In donor Hu1242, CYP1A2 activity data in the absence of antibody was best fit to a bell-shaped dose response model with CYP1A2 activity initially increasing between 10 and 1000 pg/mL IL-6 and then decreasing to an E_{min} of 20%. In the presence of antibody, this effect was right-shifted with an increase in CYP1A2 activity of 171% of untreated cells at 1000 pg/mL and a subsequent decrease to 128% at 10 ng/mL.

Treatment with the IL-6 antibody did not completely abolish CYP3A4 suppression by IL-6, but did shift the EC_{50} curve 19-fold from 92.5 to 1738 pg/mL in Donor Hu1242 and 13-fold from 63.6 to 849 pg/mL in Donor Hu8110 (Figure 5, Table 6). The maximum level of suppression was not markedly altered by the IL-6 antibody at the highest IL-6 concentrations.

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Discussion

Model Characterization. Human hepatocyte culture is the *in vitro* system of choice to study the regulation of cytochrome P450 enzymes and transporters by various pro-inflammatory mediators (Aitken and Morgan, 2007; Lee et al., 2009; Vee et al., 2009). This model has also been used as a model to investigate cell signaling by NF-κB, p38, JNK, LIF/LAF, C/EBP, and other proteins involved in cytokine signaling (Henklova et al., 2008; Vrzal et al., 2008; Wanninger et al., 2009). IL-6 dependent up-regulation of the acute phase response markers, C-reactive protein and serum amyloid A, is dependent on signaling through the JAK/STAT3 pathway with involvement of NF-κB subunits p50 and p65 (Zhang et al., 1996; Jensen and Whitehead, 1998), and there is also a complex synergistic interaction between STAT3 and C/EBP that activates transcription of C-reactive protein. Therefore, the ability of IL-6 to cause a dose dependent increase in both C-reactive protein and serum amyloid A in our study is indicative of an acute phase response and is consistent with previous reports indicating that our human hepatocyte culture conditions were suitable for studying IL-6 signaling.

Under the standardized conditions established to monitor the effects of IL-6 on hepatocytes, dexamethasone was not added to the culture medium, as it complicates the hepatocyte model. Dexamethasone acts as a potent anti-inflammatory agent by controlling levels of leukotrienes and prostaglandins and enhancing cytochrome P450 levels (Pascussi et al., 2000a; Pascussi et al., 2000b; Brunton, 2011). It also up-regulates the levels of IL-6 receptor on certain cell types (Snyers et al., 1990; Hoffmann et al., 1994) and enhances the effects of cytokines (Steel and Whitehead, 1994; Thorn and Whitehead, 2002). In a pilot experiment, the addition of 100 nM dexamethasone increased IL-6R mRNA by 3.5-fold and CYP3A4 mRNA by 5-fold (Supplemental Figure 2). These increases are within the natural variation seen in donor populations. In this study, the absence of dexamethasone in the cell culture medium did not substantially alter basal cell stress levels. This was shown by similar ATP and GSH production

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at IL-6 concentrations up to 500 ng/mL. The mRNA levels of ER stress markers, CHOP and BIP, were slightly higher in IL-6 naïve, dexamethasone-treated cells (Supplemental Figure 2).

Effects of IL-6 on Cytochromes P450. This study expands upon previous studies exploring IL-6 regulation of cytochrome P450 enzymes in human hepatocyte culture (Yang et al.; Aitken and Morgan, 2007; Lee et al., 2009). In part because of regulatory interest, dissecting out the components of the APR is of industrial interest, since companies working on an anti-cytokine drug want to know whether their cytokine alone elicits this effect on cytochrome P450s. Thus our approach to reconstituting the APR in this model is cytokine-centric and differs from more complicated disease-specific/multi-cytokine (i.e. LPS) approaches.

We focused on several key aspects that have not been previously reported: A) the use of physiologically relevant IL-6 concentrations; B) biochemical characterization of commercial protein reagents and; C) the ability of an anti-IL-6 monoclonal antibody to block the effects of IL-6 on cytochrome P450s. Our data were comparable to previously published results in that cytochrome P450s were generally down-regulated by treatment with IL-6, although direct comparison among studies are problematic due to different commercial sources of cytokines and cytokine concentrations.

CYP3A4 was chosen as a marker for four reasons: 1) It is the enzyme responsible for metabolizing the majority of marketed drugs, 2) mRNA data indicated it was the most sensitive isoform to IL-6 challenge; 3) it is the responsible enzyme in the 1.8-fold tocilizumab:simvastatin clinical drug interaction and; 4) it is a PXR-regulated CYP isoform. CYP1A2 was chosen as a secondary marker because of its regulation, through the AhR receptor and not the PXR or CAR receptor (Montellano, 2005). Although individual EC_{50} values among the cryopreserved donors had greater variability than fresh donors, the average EC_{50} values between cryopreserved and

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fresh hepatocytes were not significantly different, suggesting they are both appropriate for investigating cytochrome P450 regulation by IL-6.

To address the role of basal expression of CYP3A4 in IL-6 mediated CYP3A4 expression, we examined suppression of CYP3A4 activity by IL-6 in hepatocytes induced by rifampicin. The EC_{50} for suppression of CYP3A4 activity by IL-6 was shifted by rifampicin from 82.8 to 1574 pg/mL (19 fold, Table 4). Based on normal ranges for IL-6 concentrations in serum, suppression of cytochrome P450 activity by IL-6 only overrode cytochrome P450 induction at supra-physiological concentrations of IL-6.

Treatment of human hepatocytes with IL-6 generally caused pan-suppression of mRNA for all cytochrome P450 isoforms with EC_{50} and E_{min} values varying amongst cytochrome P450 isoforms and for the same isoform between donors. IL-6 itself was not measured in the media and this precludes adjustment for possible substrate depletion. However, the EC_{50} data did define differences in responsiveness of individual cytochrome P450s at identical IL-6 concentrations and enabled rank ordering of cytochrome P450 sensitivity to IL-6. PXR/CAR regulated isoforms CYP3A4 and CYP2B6 were the most sensitive to down-regulation by IL-6, whereas AhR-regulated isoforms CYP1A1 and CYP1A2 were the least sensitive. Given the EC_{50} values of suppression for the individual cytochrome P450 isoforms and the physiological serum concentrations of IL-6, we speculate that CYP1A2 levels will not be altered significantly by anti-cytokine treatments for inflammatory disease.

Because IL-6 is differentially glycosylated *in vivo*, we investigated the effect of IL-6 glycosylation on cytochrome P450 suppression in human hepatocytes. EC_{50} values for CYP1A2 and CYP3A4 activity showed that glycosylation status of IL-6 did not markedly alter the potency of cytochrome P450 suppression. These data are in agreement with the Kd values for IL-6 binding to its soluble receptor. The measured Kd values of glycosylated and non-

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glycosylated forms were within 2-fold of one another. These data indicate that once bound both the glycosylated and non-glycosylated forms of IL-6 dissociate from the IL-6 receptor with equal potency.

An anti-IL-6 antibody was able to block IL-6 suppression of CYP1A2 activity in two donors such that an EC_{50} value could not be calculated. At supratherapeutic concentrations (500-10,000 pg/mL), IL-6 anomalously increased CYP1A2 activity relative to initial conditions. The mechanism behind this observation is currently not understood. Treatment with the anti-IL-6 antibody did not completely abolish CYP3A4 suppression by IL-6, but did shift the EC_{50} curve 19-fold and 13-fold in two different donors, respectively. The dissociation constant for the antibody used in this study was 1.7 nM, which is similar to the measured Kd values between IL-6 and its receptor (3.2-5.1 nM). However, the Kd value for the IL-6/IL-6R/gp130 complex, the active complex for IL-6 signaling, has been measured at 50 pM (Hibi et al., 1990). This could explain why CYP3A4 suppression was only partially blocked by the antibody used in this study and highlights the importance of biochemical characterization of large molecule reagents for proper interpretation of results.

Limitations to the Hepatocyte Culture Model. Cytokines are known to work in consort in an autocrine/paracrine fashion. Several complicating factors in studying cytochrome P450 suppression by cytokines in hepatocyte culture are the lack of other cytokine responsive cell types normally found in the liver, such as Kupffer cells, the absence of other cytokines that may work in consort with IL-6, a limited time frame to examine adaptive responses, and multiple hepatic functions of cytokines that signal through STAT3 (Taub 2004, Ki et al., 2010). Since Kupffer cells secrete IL-6 and constitutively express IL-6R, one could envisage that hepatocyte co-culture with Kupffer cells would give a different EC_{50} for cytochrome P450 suppression by IL-6. However, in a study by Sunman et al., the addition of Kupffer cells to hepatocyte culture did

not affect CYP3A4 suppression by IL-6 or IL-1, suggesting that co-culture may not be necessary for investigating cytochrome P450 suppression by IL-1 or IL-6 alone.

We demonstrated that IL-6 down-regulates its own receptor in human hepatoycte culture suggesting a potential adaptive response mechanism. Cultured hepatocytes are viable for a relatively short time period and therefore may not be the best model for discerning adaptive responses to cytokine stimulation. Advanced tissue engineering technologies such as three-dimensional liver culture or the HepatoPac™ platform might better define the adaptive response of hepatocytes to IL-6 and other cytokines.

This study shows that inclusion of IL-6 and an anti-IL-6 monoclonal antibody in human hepatocyte primary culture allows for quantitative measurement of cytochrome P450 suppression and de-suppression. However, the experiments also illustrate that these data cannot fully address the complicated biology of the acute phase response and/or chronic inflammation in human liver. Factors that complicate the predictive value of the experiments are the biology of the individual cytokine, relevant hepatic concentrations of the cytokine, the binding affinities of relevant monoclonal antibodies, the absence of other interacting cytokines and cell types in primary hepatocyte culture and the nuances of patient and disease-specific aspects of inflammatory disease.

We conclude that experimentation with a single cytokine in human hepatocyte primary culture will be useful for hypothesis generation. However, the complicated biology of inflammatory disease treatment precludes quantitative *in vitro* to *in vivo* prediction of the clinical effects of anti-cytokine treatment on the pharmacokinetics of small molecule concomitant medications.

Acknowledgements

The authors would like to thank Josh Pearson for KinExA expertise.

Authorship Contributions

Participated in research design: Dickmann, L.J., Slatter, J.G, Wienkers, L.C.

Conducted experiments: Dickmann, L.J., Patel, S.K., Rock, D.A.

Performed data analysis: Dickmann, L.J. Patel, S.K., Rock, D.A.

Wrote or contributed to the writing of the manuscript: Dickmann, L.J., Slatter, J.G., Wienkers, L.C.

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Figure 1. Induction of C-reactive protein and serum amyloid A levels in human hepatocytes treated with IL-6. Hepatocytes from Donor Hu1153 were treated with IL-6 concentrations ranging from 5 pg/mL to 50 ng/mL for 72 hours with daily media changes and IL-6 replenishment. mRNA was isolated, transcribed into cDNA, and analyzed using real-time PCR as described in the Materials and Methods section. Data were fit to a variable slope dose response model, and error bars represent the standard deviation of three individual wells. (A) C-reactive protein (B) serum amyloid A.

Figure 2. Time dependent suppression of CYP1A2 and CYP3A4 activity in human hepatocytes treated with IL-6. Hepatocytes from Donor Hu1153 were treated with IL-6 concentrations ranging from 5 pg/mL to 50 ng/mL for 48, 72, and 96 hours with daily media changes and IL-6 replenishment. At the allotted time point, cells were incubated with either 100 µM phenacetin (A) or 150 µM testosterone (B) for 15 minutes. Metabolites were analyzed by LC-MS/MS as described in the Materials and Methods section. Data were normalized to untreated cells and fit to a variable slope dose response model, and error bars represent the standard deviation of three individual wells.

Figure 3. The effects of inducers on CYP1A2 and CYP3A4 suppression by IL-6. Hepatocytes from Donor Hu4151 were incubated with increasing amounts of IL-6 with or without 50 µM omeprazole (A) or 10 µM rifampicin (B) with daily media changes and IL-6 and inducer replenishment. After 72 hours of treatment, cells were incubated with either 100 µM phenacetin (A) or 150 µM testosterone (B) for 15 minutes. Metabolites were analyzed by LC-MS/MS as described in the Materials and Methods section. Data were normalized to untreated cells and fit to a variable slope dose response model, and error bars represent the standard deviation of three individual wells. The dashed vertical lines represent approximate physiological serum ranges (10-125 pg/mL) in healthy individuals and those presenting with chronic inflammatory disease. See text for references.

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Figure 4. Comparison of glycosylated versus non-glycosylated IL-6 on the ability to down-regulate CYP1A2 and CYP3A4 activity. Hepatocytes from Donor Hu1190 were treated with either *E. coli* expressed IL-6 (non-glycosylated) or HEK293 (glycosylated) expressed IL-6 for 72 hours with daily media changes and IL-6 replenishment. Cells were incubated with either 100 µM phenacetin (A) or 150 µM testosterone (B) for 15 minutes. Metabolites were analyzed by LC-MS/MS as described in the Materials and Methods section. Data were normalized to untreated cells and fit to a variable slope dose response model, and error bars represent the standard deviation of three individual wells.

Figure 5. The effects of an anti-IL-6 monoclonal antibody on IL-6 mediated suppression of CYP1A2 and CYP3A4. Hepatocytes from Donor Hu8110 (A, B) and Donor Hu1242 (C, D) were treated with either IL-6 (0.5 - 10,000 pg/mL; $2.5x10^{-5}$ – 0.49 nM) alone or in combination with 100 ng/mL (0.67 nM) of anti-IL-6 antibody (measured Kd = 1.7 nM) for 72 hours with daily media changes and replenishment of IL-6 and antibody. Cells were incubated with either 100 µM phenacetin (A, C) or 150 µM testosterone (B, D) for 15 minutes. Metabolites were analyzed by LC-MS/MS as described in the Materials and Methods section. Data were normalized to untreated cells and fit to a variable slope dose response model, and error bars represent the standard deviation of three individual wells. The dashed vertical lines represent approximate physiological serum ranges of IL-6 (10-125 pg/mL) in healthy individuals and those presenting with chronic inflammatory disease. See text for references.

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Table 1. Induction of acute phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA), in human hepatocytes treated with IL-6. Donor Hu1153 was exposed to IL-6 for 72 hours.

	EC_{50} (pg/mL)	E_{max} (Fold increase)	E_{min} (Fold increase)	Hill slope	R^2
CRP	73.4	1021	7.14	1.59	0.98
SAA	22.9	19.9	1.06	1.02	0.91

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Table 2. Time dependent suppression of acetaminophen and 6β-hydroxytestosterone formation in IL-6 treated hepatocytes. Donor Hu1153 was used in this study. Data were normalized to untreated values.

	Acetaminophen Formation				
Timepoint (hrs)	EC_{50} (pg/mL)	E_{max} (% untreated cells)	E_{min} (% untreated cells)	Hill slope	R^2
48	409	90.3	10.7	-1.5	0.98
72	443	99.1	12.5	-1.3	0.99
96	1,260	94.1	23.3	-1.7	0.96
	6β-Hydroxytestosterone Formation				
Timepoint (hrs)	EC_{50} (pg/mL)	E_{max} (% untreated cells)	E_{min} (% untreated cells)	Hill slope	R^2
48	56.3	84.1	41.6	-1.3	0.90
72	20.6	96.5	28.6	-0.9	0.94
96	17.1	97.5	21.5	-0.8	0.98

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Table 3. mRNA levels measured in hepatocytes treated with IL-6. Donor Hu1153 was treated with IL-6 for 72 hours. Data were normalized to untreated values.

CYP Isoform	EC_{50} (pg/mL)	E_{max} (% untreated cells)	E_{min} (% untreated cells)	Hill slope	R^2
1A1	1,620	93.1	37.2	-1.05	0.91
1A2	271	101	15.7	-1.03	0.98
2B6	70.0	95.4	3.11	-0.95	0.98
2C8	153	84.1	3.86	-0.82	0.97
2C9	121	95.6	5.25	-0.88	0.98
2C19	71.3	101	21.4	-0.90	0.96
2D6	151	103	30.2	-0.75	0.96
3A4	3.23	157	0	-0.77	0.99
3A5	51.0	85.8	3.43	-0.93	0.98
4A11	160	105	2.47	-1.16	0.96

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Table 4. Donor variability and inducibility of acetaminophen and 6β-hydroxytestosterone formation in IL-6 treated fresh and cryopreserved hepatocytes. All donors were treated for 72 hours with IL-6, while donor Hu4151 was treated with and without a fixed amount of inducer at each concentration of IL-6. Data were normalized to untreated values. Donors Hu1001, 4151, and 8064 are cryopreserved hepatocytes and Hu1146 and 1150 are fresh hepatocytes.

Acetaminophen Formation					
Donor	EC_{50} (pg/mL)	E_{max} (% untreated cells)	E_{min} (% untreated cells)	Hill slope	R^2
Hu1001	142	91.4	23.0	NA[a]	0.91
Hu8064	4070	89.8	52.9	-5.97	0.79
Hu1146	262	99.3	6.22	-0.69	0.97
Hu1150	1050	78.1	23.7	-6.16	0.97
Hu4151	730	89.5	9.33	-1.58	0.98
Hu4151 + 50μM Omeprazole	1120	850	136	-1.42	0.97
6β-Hydroxytestosterone Formation					
Donor	EC_{50} (pg/mL)	E_{max} (% untreated cells)	E_{min} (% untreated cells)	Hill slope	R^2
Hu1001	4.23	111	41.7	-0.64	0.88
Hu8064	176	98.3	21.1	-0.94	0.96
Hu1146	59.7	89.6	10.7	-0.88	0.97
Hu1150	43.1	86.6	29.7	-1.16	0.93
Hu4151	82.8	81.2	17.1	-1.95	0.99
Hu4151 + 10μM Rifampicin	1574	374	38.3	-1.23	0.98

[a]NA, Not applicable, data fit to fixed slope dose response model.

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Table 5. Comparison of CYP1A2 and CYP3A4 activity and mRNA levels in hepatocytes treated with glycosylated (HEK293 expressed) or non-glycosylated (*E. coli* expressed) IL-6. Donor Hu1190 was treated with IL-6 for 72 hours. Data were normalized to untreated values. *P* values were calculated using a two-tailed, unpaired t-test.

EC_{50} value (pg/mL)			
CYP Isoform	***E. coli* expressed IL-6**	**HEK293 expressed IL-6**	***P* value**
Activity			
1A2	575	693	0.76
3A4	78.1	24.3	0.72
mRNA			
1A1	ND[a]	ND	ND
1A2	616	565	0.77
2B6	1.57	21.1	0.32
2C8	271	269	0.18
2C9	303	257	0.19
2C19	77.1	62.4	0.48
2D6	ND	ND	ND
3A4	102	88.6	0.27
3A5	193	270	0.55
4A11	112	95.7	0.61

[a]Not determined due to low observed decrease in mRNA levels.

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Table 6. The effects of an IL-6 neutralizing monoclonal antibody (mAb) on the suppression of CYP1A2 and CYP3A4 activity in human hepatocytes treated with IL-6.

Hepatocytes were treated for 72 hours with IL-6 in the presence or absence of 100 ng/mL of IL-6 neutralizing antibody (clone 1936, R&D Systems). The measured Kd value of the monoclonal antibody was 1.7 nM.

Acetaminophen formation					
Donor	EC_{50} (pg/mL)	E_{max} (% untreated cells)	E_{min} (% untreated cells)	Hill slope	R^2
Hu1242	1350	118	23.8	NA[a]	0.80
Hu1242 + mAb	ND[b]	ND	ND	ND	ND
Hu8110	879	105	29.0	-2.79	0.84
Hu8110 + mAb	ND[b]	ND	ND	ND	ND
6β-Hydroxytestosterone formation					
Donor	EC_{50} (pg/mL)	E_{max} (% untreated cells)	E_{min} (% untreated cells)	Hill slope	R^2
Hu1242	92.5	92.1	30.3	-2.07	0.96
Hu1242 + mAb	1738	98.1	10.8	-1.04	0.96
Hu8110	63.6	83.9	21.6	-1.10	0.88
Hu8110 + mAb	849	93.5	23.0	-1.08	0.92

[a]Not applicable. Hu1242 was fit to a bell-shaped dose response model as described in the Materials and Methods section.

[b]Not determined. Data could not be fit to any dose response model.

Figure 1

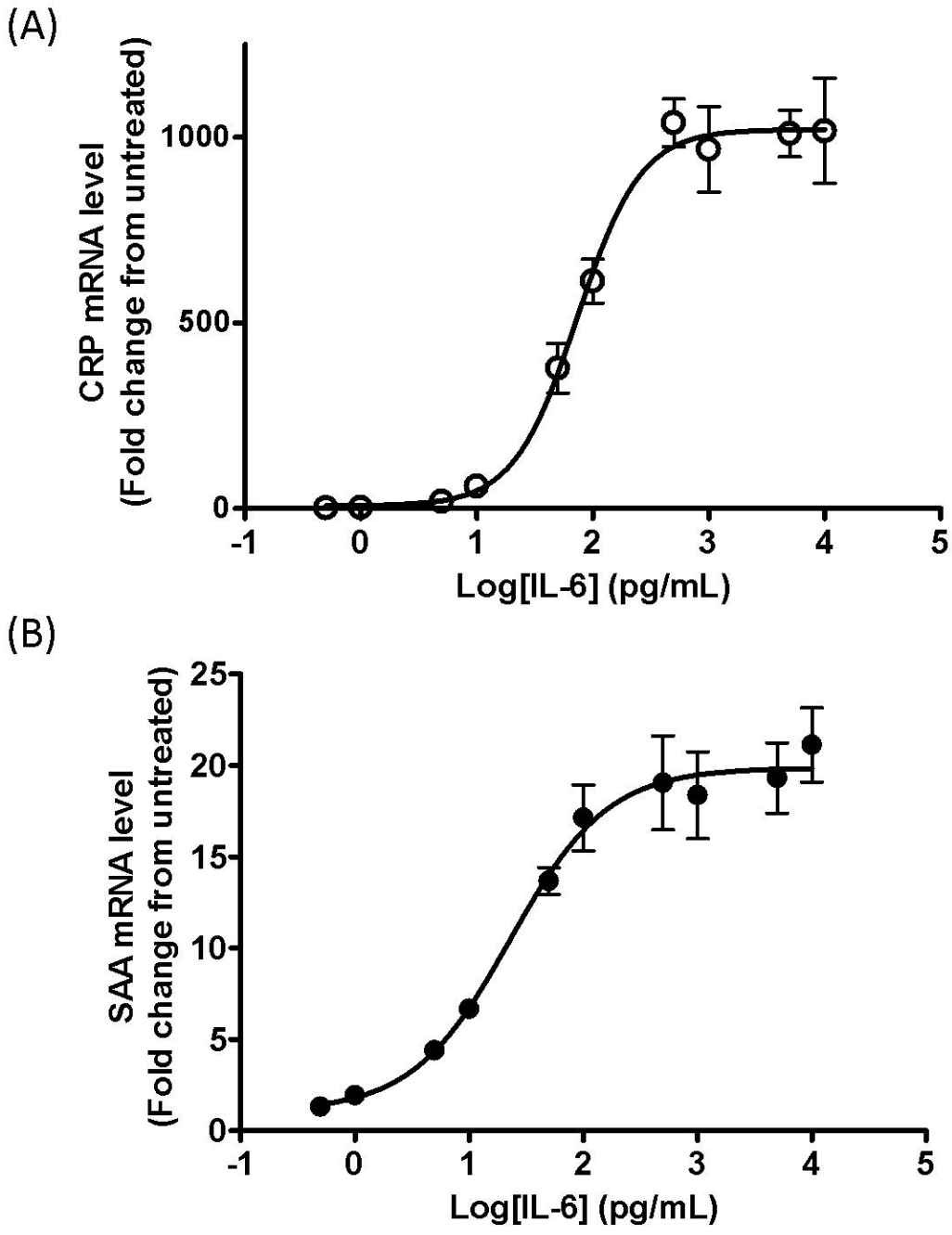

Figure 2

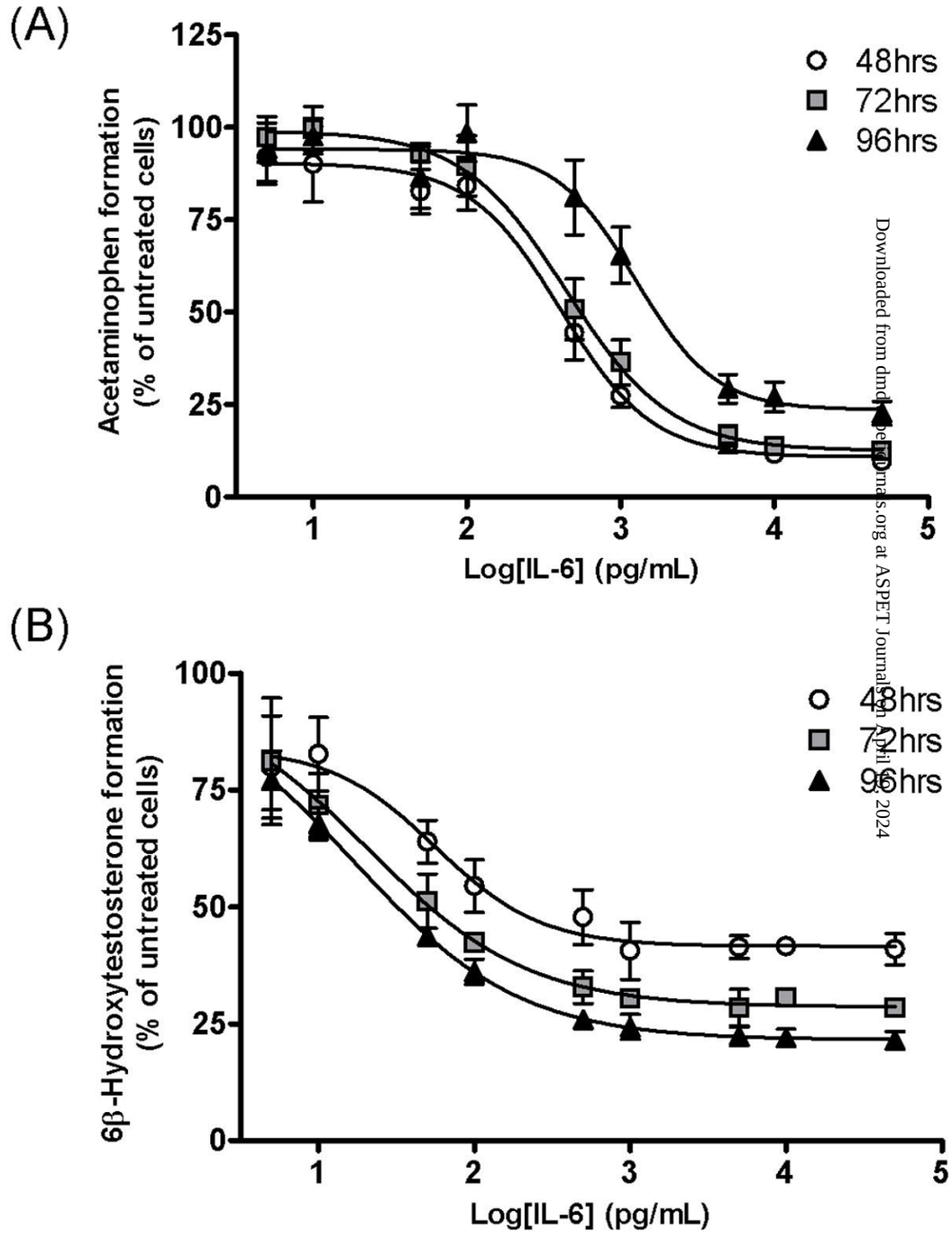

Figure 3

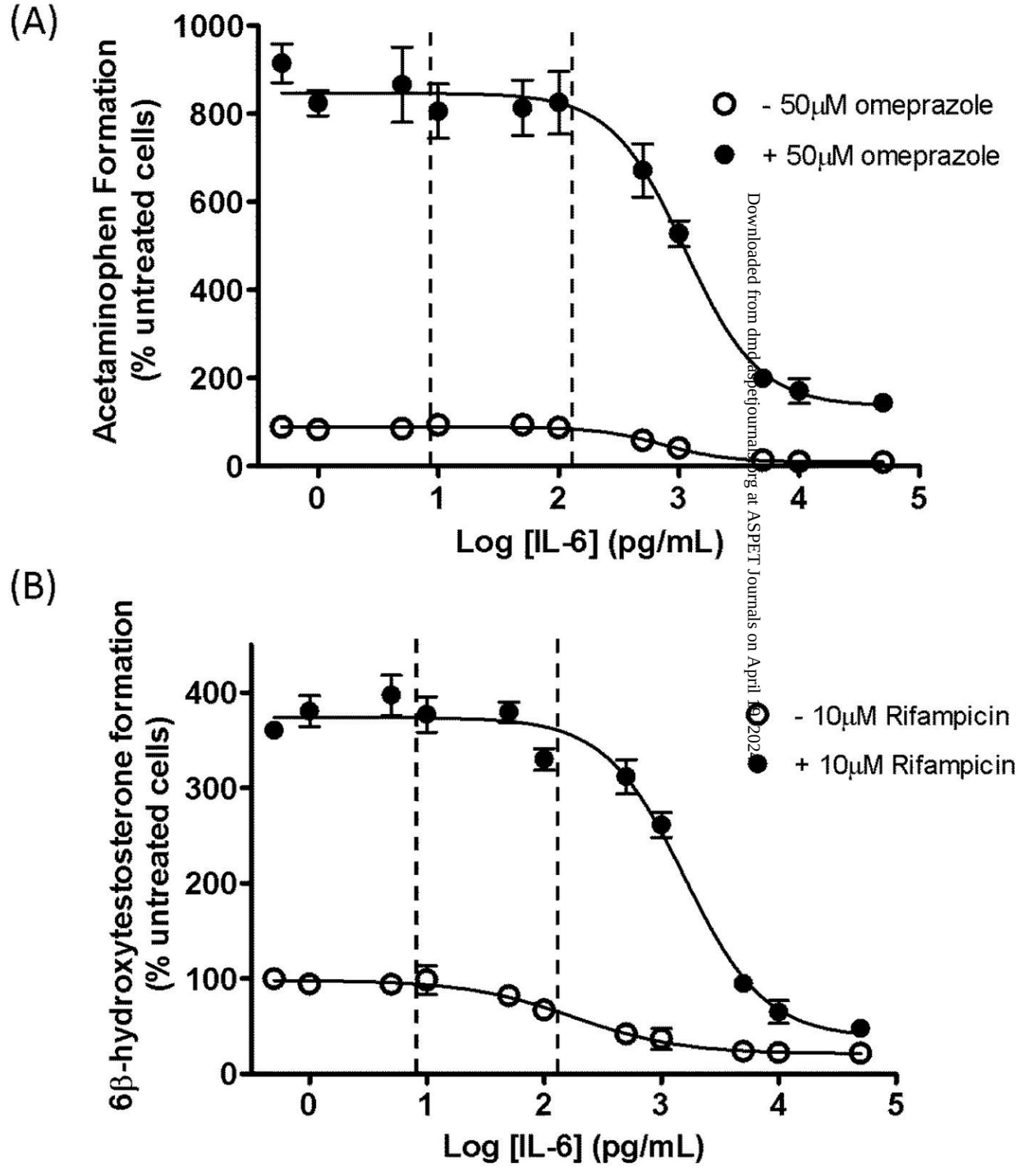

Figure 4

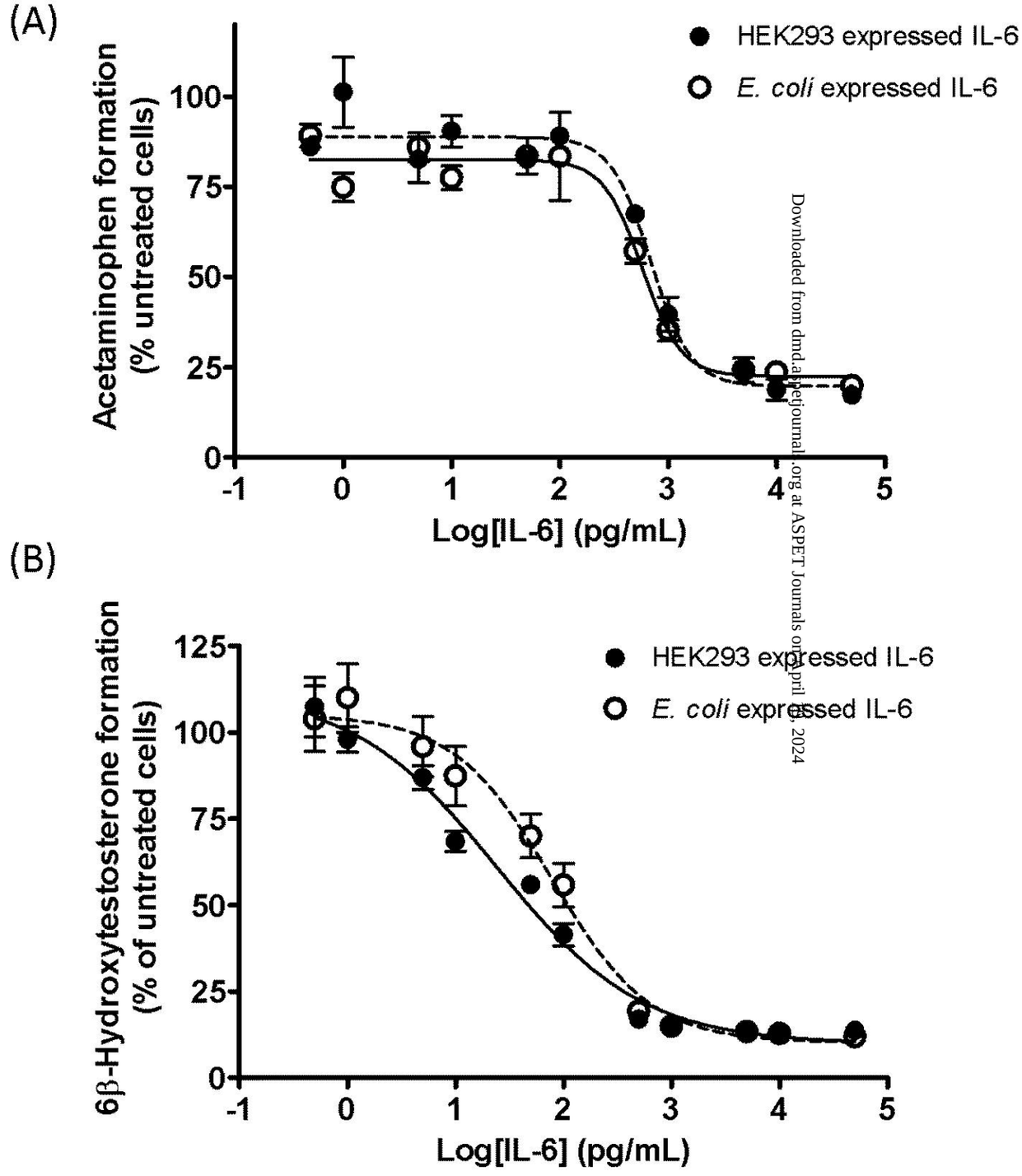

Figure 5