Anti-CD28 Monoclonal Antibody–Stimulated Cytokines Released from Blood Suppress CYP1A2, CYP2B6, and CYP3A4 in Human Hepatocytes In Vitro

Maciej Czerwiński, Faraz Kazmi, Andrew Parkinson, and David B. Buckley

XenoTech, LLC, Lenexa, Kansas (M.C., F.K., D.B.B.); and XPD Consulting, Shawnee, Kansas (A.P.)

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ABSTRACT

Like most infections and certain inflammatory diseases, some therapeutic proteins cause a cytokine-mediated suppression of hepatic drug-metabolizing enzymes, which may lead to pharmacokinetic interactions with small-molecule drugs. We propose a new in vitro method to evaluate the whole blood-mediated effects of therapeutic proteins on drug-metabolizing enzymes in human hepatocytes cocultured with Kupffer cells. The traditional method involves treating hepatocyte co-cultures with the therapeutic protein, which detects hepatocyte- and macrophage-mediated suppression of cytochrome P450 (P450). The new method involves treating whole human blood with a therapeutic protein to stimulate the release of cytokines from peripheral blood mononuclear cells (PBMCs), after which plasma is prepared and added to the hepatocyte co-culture to evaluate P450 enzyme expression. In this study, human blood was treated for 24 hours at 37°C with bacterial lipopolysaccharide (LPS) or ANC28.1, an antibody against human T-cell receptor CD28. Cytokines were measured in plasma by sandwich immunoassay with electrochemiluminescence detection. Treatment of human hepatocyte cocultures with LPS or with plasma from LPS-treated blood markedly reduced the expression of CYP1A2, CYP2B6, and CYP3A4. However, treatment of hepatocyte cocultures with ANC28.1 did not suppress P450 expression, but treatment with plasma from ANC28.1-treated blood suppressed CYP1A2, CYP2B6, and CYP3A4 activity and mRNA levels. The results demonstrated that applying plasma from human blood treated with a therapeutic protein to hepatocytes cocultured with Kupffer cells is a suitable method to identify those therapeutic proteins that suppress P450 expression by an indirect mechanism—namely, the release of cytokines from PBMCs.

Introduction

Small-molecule drugs and therapeutic proteins are frequently coadministered to patients with cancer or immune-mediated inflammatory disease such as rheumatoid arthritis. Although the absorption, distribution, metabolism, and excretion properties of small-molecule drugs and therapeutic proteins (also known as biologic drugs) are distinct, the two classes of drugs can alter each other’s disposition, and therefore have the potential for drug-drug interactions (DDIs) (Evers et al., 2013). Changes in the clearance of small-molecule drugs caused by certain therapeutic proteins can be a consequence of the suppression of drug-metabolizing enzymes (DMEs) or transporters (Huang et al., 2010; Lee et al., 2010; Zhou and Mascelli, 2011). The decrease in victim drug clearance, and consequently increase in drug exposure, can lead to toxicity, loss of efficacy, or exaggerated pharmacological effects, as shown with cyclosporine, tacrolimus, and simvastatin (Vasquez and Pollak, 1997; Strehlau et al., 2000; Sifontis et al., 2002; Schmitt et al., 2011). Suppression of DME and transporters by certain biologic drugs involves a release of cytokines from the peripheral blood mononuclear cells (PBMCs), followed by activation of nuclear factor κ-light-chain-enhancer of activated B cells and downregulation of the aryl hydrocarbon receptor, constitutive androstane receptor, and pregnane X receptor (Ke et al., 2001; Morgan et al., 2002; Riddick et al., 2004; Morgan, 2009). Additionally, Kupffer cells can release cytokines such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) which modulate suppression of CYP3A4 in hepatocytes in vitro (Hoebe et al., 2001; Sunman et al., 2004). Therapeutic proteins that are cytokines or cytokine modulators can suppress DME by acting directly on hepatocytes (Ghose et al., 2011) and/or Kupffer cells, as has been shown for interferon-α-2B (IFNα-2B) with theophylline and methadone clearance (Iafolla, 2002), whereas other therapeutic proteins...
such as OKT3 antibody, cause systemic reaction by stimulating cytokine release from PBMCs (Abramowicz et al., 1989; Chatenoud et al., 1990; Herbelin et al., 1999; Wolf et al., 2012). The effects of therapeutic proteins that directly suppress drug metabolism can be examined in a traditional hepatocyte culture (Kraynov et al., 2011). In contrast, for those therapeutic proteins that stimulate a cytokine response in blood, a traditional hepatocyte culture treated with the drug alone may be an inadequate test system to assess DDI potential (Dallas et al., 2012; Evers et al., 2013).

The in vitro effects of numerous proinflammatory cytokines on cytochrome P450 (P450) enzymes have been demonstrated (Aitken and Morgan, 2007; Dickmann et al., 2011; Dallas et al., 2013). However, such studies usually examine the effects of only a single or a limited combination of cytokines (Dickmann et al., 2012b). It has been noted that many cytokines cross-regulate their own expression, and that cytokines can suppress or induce P450 expression in vitro (Marie et al., 1996; Liptrott et al., 2009; Barker et al., 2011; Dickmann and Morgan, 2007; Dickmann et al., 2011; Dallas et al., 2013). Despite the understanding that cytokines act as a network of signaling molecules, the effects of a full complement of cytokine et al., 2012a). Despite the understanding that cytokines act as a network of signaling molecules, the effects of a full complement of cytokine response to a therapeutic protein on DME have not been studied in vitro.

The 2012 Food and Drug Administration Draft Guidance for Industry on drug interaction specified that studies for the prediction of therapeutic protein–small-molecule drug DDI may be performed in vitro (http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm292362.pdf). In the present study, we propose a novel method that comprises stimulation of whole human blood with a therapeutic protein, followed by separation and subsequent incubation of the plasma with human hepatocytes cocultured with Kupffer cells to assess the potential of the therapeutic protein to alter the disposition of small-molecule drugs. All cultures of hepatocytes used in this study were in fact cocultures of hepatocytes and Kupffer cells isolated from the same donors in routine perfusion and plating procedures. The test system accounts for cumulative effects of all cytokines stimulated by therapeutic protein in blood on P450 expression. We selected lipopolysaccharide (LPS) and ANC28.1, a monoclonal antibody against T-cell receptor CD28, to demonstrate proof of concept for the new method (Fig. 1). Our choice of ANC28.1 was based on another anti-CD28 antibody, TeGenero humanized monoclonal antibody #1412 (TGN1412), which caused cytokine storm and severe toxicity in a first-in-man study and dramatically highlighted the need to improve preclinical safety assessment of therapeutic proteins (Suntharalingam et al., 2006). We stimulated release of cytokines in whole human blood with LPS and ANC28.1, quantified the cytokines in plasma, and examined their effects on P450 mRNA levels and enzyme activities in human hepatocytes cocultured with Kupffer cells. This study demonstrates the feasibility of an in vitro DDI evaluation of therapeutic proteins that stimulate cytokine release in blood.

Materials and Methods

Chemicals and Reagents. ANC28.1, a mouse monoclonal antibody against human T-cell receptor CD28 (anti-CD28), and MOPC 31C, a control mouse monoclonal antibody (of unknown antigen specificity) with the same subtype as ANC28.1, namely IgG1-κ, were purchased from Ancell Corp. (Bayport, MN) and diluted with purified saline. Escherichia coli LPS was purchased from Sigma-Aldrich (St. Louis, MO). Endotoxin Removal Gel was purchased from Pierce Biotechnology (Rockford, IL). Blood was donated by healthy volunteers who gave informed consent to participate in the study. IL-6 and TRIZol were purchased from EMD Biosciences (La Jolla, CA) and Invitrogen (Grand Island, NY), respectively. The sources of the other reagents used in this study have been described elsewhere (Paris et al., 2009).

Cytokine Release Assay. Normal sterile saline was purified with Endotoxin Removal Gel as described by Pierce Biotechnology. A stock solution of LPS (5 μg/ml) was prepared in purified saline. Blood was drawn into sterile glass 10-ml vacutainers containing sodium heparin (Becton-Dickinson, Franklin Lakes, NJ), transferred into sterile 50-ml polypropylene tubes (BD Biosciences, San Diego, CA), and aliquoted into sterile polypropylene microtubes (Sarstedt, Newton, NC). Saline (2%, v/v), LPS (50 ng/ml), or the antibodies (2 μg/ml) were added to blood in an aqueous solution, gently mixed, and incubated at 37°C for 24 hours. Plasma was separated from whole blood by centrifugation at 600g for 10 minutes, aliquoted, and stored at −80°C. IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and granulocyte-macrophage colony-stimulating factor were measured in the plasma by sandwich immunoassay with electrochemiluminescence detection with a Human Proinflammatory 9-Plex Ultra-Sensitive Kit (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer’s instructions. The measurements were conducted by Viracor-IBT Laboratories (Lee’s Summit, MO) with a SECTOR Imager 2400 (Meso Scale Discovery). For all cytokines measured with the 9-Plex Kit, the lower and upper limits of quantitation were 0.6 and 2500 pg/ml, respectively.

Hepatocyte-Kupffer Cell Coculture and Treatments. Nontransplantable livers were obtained from nine donors. Donor information is provided in Supplemental Table 1. Hepatocytes and Kupffer cells were isolated by a two-step collagenase perfusion method and plated on collagen-coated 24-well plates, as described previously (Madan et al., 2003). This isolation and plating procedure routinely resulted in a coculture of hepatocytes and liver macrophages (Lambert Li et al., 2013). Kupffer cells were stained with anti-CD68 antibody followed by Alexa Fluor 488–conjugated secondary antibody and fluorescent microscopy (Supplemental Fig. 1), and comprised ~4.7% of the plated cells, although in vivo macrophages may be present at a higher proportion (4–8%) (Racanelli and McAteer, 2011). Fig. 1. Experimental approach to an in vitro evaluation of whole blood-mediated effects and direct and Kupffer cell–mediated effects of therapeutic proteins on the expression of P450 enzymes in human hepatocyte–Kupffer cell cocultures. To examine the immune system–mediated effects of therapeutic proteins on gene expression or enzyme activity, whole human blood containing PBMCs was incubated with the therapeutic protein ex vivo, after which plasma was prepared and added to cultured human hepatocytes at a final concentration of 10–50% (v/v). To examine direct and Kupffer cell–mediated effects, the therapeutic protein was added directly to the coculture of hepatocytes in the absence of PBMCs. After 72-hour incubation, changes in P450 expression were determined based on measurements of mRNA levels or enzyme activities (measured in situ with P450-selective probes).
Rehermann et al., 2006). Cells were treated according to published protocols (Robertson et al., 2000; Paris et al., 2009). In brief, the confluent cocultures were achieved with approximately 4 × 10^6 cells per well, overlaid with Matrigel 2–3 hours after seeding, and were adapted to culture conditions for 2–3 days with daily changes of medium. Following the adaptation period, cells were treated once daily for 3 consecutive days with modified Chee’s medium (MCM+, negative control), IL-6 (10 ng/ml, positive control), LPS (25 ng/ml), ANC28.1 antibody (2 μg/ml), or plasma from whole blood treated with biologics or their respective controls (saline and MOPC 31C antibody). For hepatocyte treatments, plasma samples from blood treated with LPS, saline, or the antibodies were pooled within each treatment group. These pooled plasma samples are referred to as LPS plasma, saline plasma, ANC28.1 plasma, or MOPC 31C plasma. Aliquots of pooled plasma samples were thawed on the day of treatment. The saline plasma, LPS plasma, and plasma samples from antibody-treated blood were added to cultured cells at 10, 20, or 50%, v/v. Treatment solutions of LPS and the antibodies were prepared fresh daily. The cells maintained variability and culture confluency throughout the duration of the treatments. For studies of the temporal effects of the LPS plasma on CYP1A2, CYP2B6, and CYP3A4 mRNA, hepatocytes were treated for up to 72 hours with daily changes of medium or medium containing 10% plasma and examined for P450 expression at 12-hour intervals. Following the adaptation period, cells were treated for up to 72 hours with daily changes of medium or medium containing 10% plasma and examined for P450 expression at 12-hour intervals. Following each treatment, the cell culture medium was removed and the cells were washed with 1× phosphate-buffered saline. After removal of the phosphate-buffered saline wash, 200 μl of fresh incubation medium without plasma was added containing cocktail of phenacetin (100 μM), bupropion (50 μM), and midazolam (30 μM). After 30-minute incubation with these probe substrates, 150 μl of the medium was mixed with an equal volume of acetonitrile containing an appropriate internal standard, as described in Paris et al. (2009). Metabolite formation was analyzed by liquid chromatography–tandem mass spectrometry for the formation of acetaminophen (CYP1A2), hydroxybupropion (CYP2B6), and 1′-hydroxymidazolam (CYP3A4). Details of the analytical methods are given in Supplemental Table 2. Additional cells were lysed with TRIzol for isolation of mRNA (see next section). The time course of CYP1A2, CYP2B6, and CYP3A4 mRNA suppression by LPS plasma was studied in hepatocytes from three donors.

mRNA Analysis. Total RNA was phase extracted with TRIzol followed by purification with an RNeasy Mini Kit (Qiagen, Valencia, CA). Purified RNA was reverse transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and the Applied Biosystems 7300 Real-Time PCR System (AB7300). Quantitative polymerase chain reaction was performed with the AB7300 with the Applied Biosystems Universal Master Mix and TaqMan Gene Expression assays. P450 mRNA levels were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase mRNA, which were assumed to remain unaffected by any of the in vitro treatments.

Data Processing and Statistical Analysis. All data were processed and graphed with Microsoft Excel software (Office 2007; Microsoft, Redmond, WA). The effects of MOPC 31C and ANC28.1 antibodies on the level of cytokines in the blood were examined by Student’s one-tailed t test (Microsoft Excel).

Results

Effects of Biologies on Cytokine Release in Human Whole-Blood Cultures. The effects of LPS, ANC28.1 antibody, and their respective controls (saline and MOPC 31C antibody) on the release of IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and granulocyte macrophage colony-stimulating factor from PBMCs in whole blood are compared in Table 1. The concentration of ANC28.1 was based on a 0.1-mg/kg dose of TGN1412 given to human volunteers (Stebbings et al., 2007). In general, the levels of cytokines in plasma from blood treated with saline for 24 hours were consistent with endogenous levels reported by the assay’s manufacturer (http://www.mesoscale.com/CatalogSystemWeb/WebRoot/literature/applications/pdf/HUMAN_Cytokine_2009.pdf). Plasma from whole blood treated with MOPC 31C contained less IL-10 compared with the saline plasma. Treatment of whole blood with LPS caused the expected release of all cytokines examined. Treatment of whole blood with ANC28.1 also stimulated cytokine release but not to the same extent as LPS, with the notable exception of IL-2, the release of which was stimulated by ANC28.1 to a greater extent than by LPS (749 and 107 pg/ml, respectively). Treatment of whole blood with ANC28.1 caused a much greater release of most cytokines than MOPC 31C, the control for nonspecific effects of murine IgG1-κ.

Effects of Plasma from Saline- or LPS-Treated Blood on P450 mRNA Levels in Human Hepatocytes In Vitro. The time course of changes in the expression of CYP1A2, CYP2B6, and CYP3A4 mRNA in hepatocytes incubated with saline plasma or LPS plasma (10%, v/v) is shown in Fig. 2. Data from hepatocytes incubated with saline plasma-treated blood were normalized to MCM+-treated cultures at corresponding times. The data from hepatocytes incubated with plasma from LPS-treated blood were normalized to cultures incubated with plasma from saline-treated blood at corresponding times. These data were generated to distinguish the effects of normal plasma from those of plasma exposed to biologics. Data shown in Fig. 2A illustrate that treatment of hepatocytes with saline plasma increased CYP1A2 mRNA by about 50% at 48 hours, although it returned to 63% of the MCM+ control at 72 hours. Treatment of hepatocytes with LPS plasma suppressed CYP1A2 mRNA to 4–15% of the saline plasma control at ~36 hours. Data presented in Fig. 2B demonstrate that, on average,
saline plasma elevated levels of CYP2B6 mRNA during the 3-day treatment period by as much as 3-fold. There was considerable interindividual variation in the effects of saline plasma on CYP2B6 expression, as evident from the large standard deviations in Fig. 2B. CYP2B6 mRNA was suppressed by the LPS plasma, with high variability among donor cultures, to 40–80% of the control level. Data in Fig. 2C exemplify that treatment of hepatocytes with saline plasma increased CYP3A4 mRNA about 2.2-fold at 60 hours, and it remained elevated by about 50% of the MCM+ control at 72 hours. A considerable interindividual variation in the effects of saline plasma on CYP3A4 expression was observed. Treatment of hepatocytes with the LPS plasma suppressed CYP3A4 mRNA to less than 0.5% of the control at 36–48 hours. In subsequent experiments, we treated hepatocytes with human plasma for 72 hours with daily changes of medium, which conforms to the typical protocol for in vitro studies of P450 induction.

Effects of IL-6, LPS, or ANC28.1 on P450 Enzymes in Human Hepatocytes In Vitro. The direct and Kupffer cell–mediated effects of IL-6, a positive control for enzyme suppression, and LPS or ANC28.1 on P450 mRNA expression and enzyme activity in human hepatocytes after 72 hours are shown in Table 2. Because different sets of cell cultures were used in the LPS and ANC28.1 experiments, they are presented separately. Hepatocytes treated with cell culture medium (MCM+) with

Fig. 2. Time course of changes in CYP1A2 (A), CYP2B6 (B), and CYP3A4 (C) mRNA levels in human hepatocytes incubated in vitro with medium containing 10% plasma from whole human blood treated with saline or LPS. Whole human blood from eight healthy donors was incubated for 24 hours at 37°C with LPS (50 ng/ml) or saline (2%, v/v), after which plasma was prepared by centrifugation, as described in Materials and Methods. Human hepatocytes (H1127, H1130, and H1134) were incubated with cell culture medium containing MCM+, 10% saline plasma, or 10% LPS plasma for 12–72 hours, after which mRNA levels were determined by quantitative polymerase chain reaction (normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA levels). The levels of P450 mRNA in cultures treated with saline plasma were normalized to those in the MCM+ cultures. The levels of P450 mRNA in cultures treated with LPS plasma were normalized to those in the saline plasma–treated cultures. Values are the mean and standard deviation of measurements in the three cultures of human hepatocytes.
no plasma served as control. The concentration of marker substrate metabolites formed in the control cultures was used to normalize results from the cultures treated with IL-6, LPS, or ANC28.1. IL-6 caused suppression of CYP1A2 and CYP2B6 mRNA to 28 and 24% of control, respectively. The greatest effect of IL-6 was suppression of CYP3A4 mRNA to less than 1% of control. Treatment of human hepatocytes with IL-6 decreased CYP1A2, CYP26, and CYP3A4 enzyme activity to 30, 24, and 17% of control, respectively. In human hepatocytes, suppression of P450 mRNA levels and enzyme activity by LPS was comparable to that of the IL-6 treatment. LPS suppressed CYP1A2, CYP2B6, and CYP3A4 mRNA to 62, 44, and 29% of control, respectively. As expected, LPS reduced enzyme activity of CYP1A2, CYP2B6, and CYP3A4 to 28 and 24% of control, respectively. In contrast to IL-6 and LPS, the ANC28.1 antibody had little or no effect on P450 mRNA levels or enzyme activity.

Effects of Plasma from Saline- or MOPC 31C–Treated Blood on P450 Enzymes in Human Hepatocytes In Vitro. The effects of plasma (0–50%, v/v) from saline- or MOPC 31C–treated blood (saline plasma or MOPC 31C plasma) on P450 mRNA expression and enzyme activity in human hepatocytes after 72 hours are shown in Fig. 3 and Supplemental Table 3. Cells treated with medium (MCM+) with no plasma served as control. Saline plasma and MOPC 31C plasma both demonstrated a concentration-dependent trend toward suppression of CYP1A2 mRNA levels (to about 60% of control at 50% plasma) (Fig. 3A). Saline plasma caused an even greater suppression of CYP3A4 mRNA levels (to about 2% of control at 50% plasma) (Fig. 3B). MOPC 31C plasma also demonstrated a trend toward suppression of CYP3A4 mRNA levels, but to a lesser extent than saline plasma (to about 33% of control at 50% plasma) (Fig. 3B). Large culture-to-culture variation in plasma-mediated suppression was observed, as evident from the large standard deviations associated with data for all three P450 mRNA transcripts. In contrast to the effects on CYP1A2 and CYP3A4, saline plasma and MOPC 31C plasma both induced CYP2B6 mRNA levels about 2-fold (Fig. 3C). Treatment of human hepatocytes with saline plasma or MOPC 31C plasma had little or no effect on CYP1A2 activity with the exception of the highest concentration of saline plasma (50% v/v in media), which reduced enzyme activity of CYP1A2 to 65% of control (Fig. 3D). Treatment of human hepatocytes with saline plasma or MOPC 31C plasma decreased CYP3A4 enzyme activity to 52 and 76% of control, respectively (Fig. 3E). Saline plasma and MOPC 31C plasma both induced CYP2B6 activity about 3-fold (Fig. 3F). Large standard deviations were associated with data for all three P450 activities. The mechanism underlying the difference between the two control plasma samples was not investigated, although the levels of many of the cytokines in saline plasma were 2- to 4-fold higher than those in MOPC 31C plasma (Table 1).

Effects of Plasma from LPS-Treated Blood Treated on P450 Enzymes in Human Hepatocytes. Plasma from LPS-treated blood was applied to hepatocytes (three donors) to assess the whole-blood-mediated effects of endotoxin on P450 enzymes. As a positive control for P450 suppression, hepatocytes were treated with IL-6 at 10 ng/ml, which was comparable to the concentration of IL-6 in LPS plasma.

**TABLE 2**

Effects of IL-6, LPS, and ANC28.1 on P450 mRNA expression and enzyme activities in human hepatocytes in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CYP1A2</th>
<th>CYP2B6</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P450 mRNA (RQF)</td>
<td>P450 mRNA (RQF)</td>
<td>P450 mRNA (RQF)</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>IL-6 (10 ng/ml)</td>
<td>LPS (25 ng/ml)</td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.00</td>
<td>0.83 ± 0.791</td>
<td>0.617 ± 0.150</td>
</tr>
<tr>
<td>P450 activity (%) of control</td>
<td>100% (value = 43.9 ± 12.8 ng/ml)</td>
<td>100% (value = 45.3 ± 21.6 ng/ml)</td>
<td>NA</td>
</tr>
<tr>
<td>CYP2B6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>IL-6 (10 ng/ml)</td>
<td>LPS (25 ng/ml)</td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.00</td>
<td>0.240 ± 0.166</td>
<td>0.439 ± 0.0877</td>
</tr>
<tr>
<td>P450 activity (%) of control</td>
<td>100% (value = 77.3 ± 30.3 ng/ml)</td>
<td>100% (value = 55.2 ± 18.4 ng/ml)</td>
<td>NA</td>
</tr>
<tr>
<td>CYP3A4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>IL-6 (10 ng/ml)</td>
<td>LPS (25 ng/ml)</td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.00</td>
<td>0.00433 ± 0.000404</td>
<td>0.0230 ± 0.0283</td>
</tr>
<tr>
<td>P450 activity (%) of control</td>
<td>100% (value = 477 ± 132 ng/ml)</td>
<td>100% (value = 260 ± 26.6 ng/ml)</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable; RQF, relative quantification factor.

a RQF is a measure of a change in mRNA expression in a test sample relative to that in a control sample (e.g., medium).

b Values are the mean ± standard deviation of triplicate determinations of RQF.

c Values are the mean ± standard deviation of triplicate determinations of phenacetin O-dealkylation (CYP1A2), bupropion hydroxylation (CYP2B6), or midazolam 1'-hydroxylation (CYP3A) expressed as percent of control (medium alone).
Cultures treated with medium only or 10, 20, or 50% (v/v) saline plasma served as controls. The data are presented in Fig. 4 and Supplemental Table 3. Treatment of human hepatocytes with the LPS plasma revealed the cytokine-mediated effect of endotoxin. The LPS plasma suppressed CYP1A2 mRNA to 2% and CYP1A2 activity to 29% of control (observed with 50% v/v plasma; Fig. 4A), suppressed CYP2B6 mRNA to 12% and CYP2B6 activity to 11% of control (observed with 50% v/v plasma), and suppressed CYP3A4 mRNA to 1% and CYP3A4 activity to 31% of control (observed with 10% of plasma in medium). Increasing the concentration of LPS plasma from 10 to 50% did not increase the extent to which CYP1A2 activity was suppressed (Supplemental Table 3) and actually decreased (not increased) the extent to which CYP3A4 activity was reduced (Fig. 4; Supplemental Table 3).

Fig. 3. Effects of incubating human hepatocytes with various concentrations of plasma prepared from whole human blood treated with saline or control antibody (MOPC 31C) on CYP1A2, CYP2B6, and CYP3A4 mRNA levels and enzyme activity. Primary human hepatocyte–Kupffer cell cocultures from three donors were incubated for 72 hours with medium containing zero, 10, 20, or 50% plasma prepared from whole human blood treated with saline or MOPC 31C. The levels of CYP1A2 mRNA (A), CYP3A4 mRNA (B), and CYP2B6 mRNA (C) were measured by quantitative polymerase chain reaction and normalized first to the levels of glyceraldehyde 3-phosphate dehydrogenase mRNA and then to the levels of CYP1A2, CYP2B6, and CYP3A4 mRNA in hepatocytes incubated with cell culture medium alone (i.e., without plasma). The enzymatic activity of CYP1A2 (D), CYP3A (E), and CYP2B6 (F) was determined in situ with 30-minute incubations with phenacetin, midazolam, or bupropion (i.e., selective probes of CYP1A2, CYP3A, and CYP2B6), respectively, as described in Materials and Methods. Values are the mean and standard deviation of measurements in the three cultures of human hepatocytes. Numerical values are given in Supplemental Table 3.
of LPS (to 46% of control) (Table 2) was similar to that caused by 50% LPS plasma (29% of control) (Fig. 4; Supplemental Table 3; Table 2). The reduction in CYP3A4 enzyme activity caused by direct application of LPS (to 29% of control) was also similar to that caused by 10% LPS plasma (31% of control) and greater than that caused by 50% LPS plasma (54% of control) (Fig. 4; Supplemental Table 3; Table 2).

Effects of Plasma from ANC28.1-Treated Blood on P450 Enzymes in Human Hepatocytes. Plasma from ANC28.1-treated blood (ANC28.1 plasma) was applied to hepatocytes (three donors) to assess the whole-blood-mediated effects of ANC28.1 on P450 enzyme expression. Cultures treated with IL-6 served as positive control for P450 suppression (Table 2). Cultures treated with MCM+ or MOPC

Fig. 4. Effects of incubating human hepatocytes with LPS or various concentrations of plasma prepared from whole human blood treated with LPS on CYP1A2, CYP2B6, and CYP3A4 mRNA levels and enzyme activity. Primary human hepatocyte–Kupffer cell cocultures from three donors were incubated for 72 hours with medium alone, medium containing IL-6 (10 ng/ml), LPS (25 ng/ml), or 10, 20, or 50% plasma prepared from whole human blood treated with LPS. The levels of CYP1A2 mRNA (A), CYP3A4 mRNA (B), and CYP2B6 mRNA (C) in cultures treated with IL-6 or LPS were measured by quantitative polymerase chain reaction and normalized first to the levels of glyceraldehyde 3-phosphate dehydrogenase mRNA and then to the levels of CYP1A2, CYP3A4, and CYP2B6 mRNA in hepatocytes incubated with cell culture medium alone (i.e., without plasma). The mRNA levels in cultures treated with 10, 20, or 50% of LPS plasma were further normalized to cultures treated with corresponding concentrations of saline plasma. The enzymatic activity of CYP1A2 (D), CYP3A (E), and CYP2B6 (F) was determined in situ with 30-minute incubation with phenacetin, midazolam, or bupropion (i.e., selective probes of CYP1A2, CYP3A, and CYP2B6), respectively, as described in Materials and Methods. The enzyme activity levels in cultures treated with IL-6 or LPS were normalized to cultures treated with medium alone. The enzyme activity levels in cultures treated with 10, 20, or 50% of LPS plasma were normalized to cultures treated with corresponding concentrations of saline plasma. Values are the mean and standard deviation of measurements in the three cultures. Numerical values are given in Table 2 and Supplemental Table 3.
31C plasma served as controls. The data are presented in Fig. 5 and Supplemental Table 3. The effects of the ANC28.1 plasma on P450 expression were normalized to MOPC 31C plasma. In contrast to the effects of LPS, the direct addition of ANC28.1 to hepatocytes had little or no effect on CYP1A2, CYP2B6, and CYP3A4 mRNA levels or enzyme activity, although in some cases standard deviations associated with the data were approaching 40 or 50% of the mean (Fig. 5; Table 2). This observation is in agreement with expression of CD28 being restricted to T cells (Su et al., 2004). In contrast, treatment of human hepatocytes with plasma from ANC28.1-treated blood revealed the suppressive effect of cytokines released from ANC28.1-stimulated PBMCs. Compared with the control (MOPC 31C) plasma, ANC28.1 plasma suppressed CYP1A2 mRNA and enzyme activity to 31 and 51% of control, respectively; suppressed CYP2B6 mRNA and enzyme activity to 42 and 32% of control, respectively; and suppressed CYP3A4 mRNA and enzyme activity to 11 and 40% of control, respectively (Fig. 5; Supplemental Table 3). In most cases, a trend toward concentration-dependent suppression was observed. Error bars associated with the data in Fig. 5 indicated that the suppression of P450 was associated with significant interindividual variability.

Discussion

The suppression of P450 enzymes and other DMEs by certain therapeutic proteins and the rise in the coadministration of therapeutic proteins with small-molecule drugs create the potential for clinical DDIs. We investigated a new in vitro method for evaluating therapeutic proteins as perpetrators of DDIs with small-molecule drugs (Fig. 1). The method consists of adding the therapeutic protein to human blood ex vivo to permit the release of cytokines from PBMCs followed by the preparation of plasma and an assessment of its effects on DME in human hepatocytes cocultured with Kupffer cells. Suppression of DME by certain therapeutic proteins commonly involves the release of cytokines from PBMCs (Morgan et al., 2002, 2008). Current procedures to assess therapeutic proteins as perpetrators of DDIs rely primarily on clinical studies because the predictive value of applying cytokines individually or in a limited combination to hepatocytes in vitro is not well established (Girish et al., 2011; Evers et al., 2013). However, the current in vitro approach may be inadequate because a cytokine response comprises multiple inductive and suppressive molecules (Marie et al., 1996; Liptratt et al., 2009; Barker et al., 2011; Dickmann et al., 2012b). The complexity of the regulation of DME is exemplified by studies of interferon-α2B. This cytokine downregulated the mRNA encoding CYP1A2, CYP2B6, CYP2E1, UDP-glucuronosyltransferase UGT2B7, sulfotransferase SULT1A1, and the organic anion transporter OAT2, but it upregulated the mRNA encoding CYP3A4 and multidrug resistance–associated protein 2 (MRP2) in cocultures (parenchymal and nonparenchymal cells) of human hepatocytes in vitro (Chen et al., 2011). Similarly, IL-β and IL-6, when applied individually, suppressed P450 enzymes, but when coadministered with TNFα these two interleukins induced several P450 enzymes in mouse hepatocytes (Dickmann et al., 2012a). In light of these observations, we propose that the full complement of therapeutic protein–stimulated cytokines should be applied to human hepatocytes to assess the potential for DDI.

When added to whole human blood ex vivo, LPS and ANC28.1 both increased the plasma levels of IFN-γ, IL-1β, IL-2, IL-6, and TNF-α in agreement with published reports with monoclonal antibody TGN1412, which is also directed against CD28 (Shedlofsky et al., 1994; Suntharalingam et al., 2006; Wolf et al., 2012). Signaling by LPS is mediated by binding to the CD14/TLR4/MD2 receptor complex that is expressed by hepatocytes, Kupffer cells, and stellate cells in the liver, as well as extrahepatically in monocytes, intestine, heart, and multiple other organs (Shimazu et al., 1999; Paik et al., 2003; Scott and Billiar, 2008). In a study in six healthy subjects, LPS stimulated release of circulating TNF and IL-6 followed by a reduction in clearance of the P450 substrate antipyrine, the extent of which correlated with peak levels of plasma cytokines in six healthy subjects (Shedlofsky et al., 1994). Wolf and coauthors (2012) added ANC28.1 to whole human blood in aqueous solution for 24 hours and observed increases in IFN-γ, IL-2, IL-6, IL-8, IL-10, and TNF-α that matched our results. In a first-in-man study, the anti-CD28 antibody TGN1412 caused marked increases (up to 5000-fold) in INF-γ, IL-1β, IL-2, IL-6, IL-8, and TNF-α in six humans (Suntharalingam et al., 2006). In our study, ANC28.1, added to human blood ex vivo, increased the plasma levels of the same proinflammatory cytokines, although to a lesser extent than TGN1412 in vivo. Addition of ANC28.1 or TGN1412 to isolated human T cells stimulated the release of high levels of the proinflammatory cytokines INF-γ, TNF-α, IL-2, IL-5, and the anti-inflammatory cytokines IL-4 and, to a lesser extent, IL-10 (Waibler et al., 2008). In our study, the 93-fold increase in IL-2 in whole blood ex vivo was one of the qualitative differences in the cytokine response to ANC28.1 compared with LPS (Table 1). The temporal effects of 10% LPS plasma or saline plasma on CYP1A2 and CYP2B6 mRNAs were comparable in hepatocytes from six livers at 72 hours (Fig. 2; Supplemental Table 3). In contrast, 10% saline plasma reduced CYP3A4 mRNA at 72 hours in one set of hepatocyte cultures but increased it in the other set (Fig. 2; Supplemental Table 3). This discrepancy can be attributed to interindividual variability in the response of cocultured hepatocytes to cytokines. The suppression of CYP1A2 and CYP3A4 mRNA reached a plateau after 48–72 hours of exposure to LPS plasma. In contrast to its effects on CYP1A2 and CYP3A4, the effects of LPS plasma on CYP2B6 were without a clear time dependency.

The effects of the cytokines released from the whole blood treated with LPS on P450 mRNA levels varied between the enzymes, indicating gene-specific mechanisms of suppression and cautioning against extrapolating individual results to multiple DMEs. Aitken and Morgan (2007) observed downregulation of multiple human P450 mRNAs and proteins by LPS, as well as recombinant IL-6, following 24-hour treatment of hepatocytes. These investigators observed variable and suppressor-specific effects on CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, and CYP3A4 mRNA levels. Suppression of CYP3A4 mRNA in response to treating freshly plated hepatocytes with LPS, which was comparable to our results obtained with LPS plasma, was observed in one of two cultures studied by Aitken and Morgan, although the cultures were not analyzed for the presence of Kupffer cells. Suppression of CYP2B6 in response to treatment of hepatocytes with LPS (10 μg/ml) was not observed (Aitken and Morgan, 2007). In general, results of the two studies indicated the importance of gene- and hepatocyte culture–specific factors for P450 enzyme suppression in vitro.

In our method, the therapeutic protein–stimulated cytokines are applied to the hepatocytes in plasma; consequently, we normalized the effects of LPS plasma and ANC28.1 plasma to saline plasma and MOPC 31C plasma, respectively. This design isolates the effects of the therapeutic protein–stimulated cytokines from the effects of other components of human plasma. In our study, proteins present in saline plasma (50% plasma in medium) significantly suppressed CYP1A2 and had a pronounced suppressive effect on CYP3A4 mRNA. In contrast to these suppressive effects, saline plasma actually induced CYP2B6 mRNA levels (Fig. 3; Supplemental Table 3). These effects were, in general, dependent on the concentration of plasma in the cell culture medium (most pronounced in cultures exposed to 20 or 50% of plasma in the medium) and were paralleled by changes in P450 enzyme activities. The effects of saline plasma were comparable to those of naïve plasma (data not shown). Inclusion of isotype-specific IgG control for evaluation of ANC28.1.
allowed us to isolate the effects of CD28-mediated stimulation of T cells from nonspecific effects of the immunoglobulin.

The effects of plasma during an extended culture of human hepatocytes on DMEs have not been reported in the literature, but the effects of human serum have been described by Takeba et al. (2011). Compared with medium alone, human serum (10% in Williams’ E medium) reduced the levels of immunoreactive CYP3A enzymes in fresh-plated human hepatocytes cultured for up to 7 days. The authors did not investigate the identity of the serum component(s) responsible for this effect. In contrast to suppression of CYP3A4 mRNA and activity, serum did not suppress

![Fig. 5. Effects of incubating human hepatocytes with ANC28.1 or various concentrations of plasma prepared from whole human blood treated with ANC28.1 on CYP1A2, CYP2B6, and CYP3A4 mRNA levels and enzymatic activity. Primary cultures of human hepatocytes from three donors were incubated for 72 hours with medium alone, medium containing IL-6 (10 ng/ml), ANC28.1 (2 μg/ml), or 10, 20, or 50% plasma prepared from whole human blood treated with ANC28.1. The levels of CYP1A2 mRNA (A), CYP3A4 mRNA (B), and CYP2B6 mRNA (C) in cultures treated with IL-6 or ANC28.1 were measured by quantitative polymerase chain reaction and normalized first to the levels of glyceraldehyde 3-phosphate dehydrogenase mRNA and then to the normalized levels of CYP1A2, CYP3A4, and CYP2B6 mRNA in hepatocytes incubated with cell culture medium alone (i.e., without plasma). The mRNA levels in cultures treated with 10, 20, or 50% of ANC28.1 plasma were normalized to cultures treated with corresponding concentrations of MOPC 31C plasma. Values are the mean and standard deviation of measurements in the three cultures. Numerical values are given in Table 2 and Supplemental Table 3.](https://www.mdpi.com/2073-4409/3/9/1532/s1)
the levels of immunoreactive albumin, HNF-4α, CYP2C enzymes, or CYP2D6 (Takeba et al., 2011). In our study, although the two control plasma samples had similar effects on CYP1A2, their effects on CYP3A4 mRNA differed (Fig. 3). The saline plasma had a greater suppressive effect on CYP3A4 mRNA than MOPC 31C plasma. The saline plasma contained more IL-10 (and other cytokines) than MOPC 31C plasma. It remains to be explored whether IL-10 contributed to suppression of CYP3A4 mRNA. The inductive effect of both control plasma on CYP2B6 is a novel observation. For all three genes, the effects of the control plasma on P450 mRNA levels were more pronounced than their effects on P450 activity. This finding might reflect differences between mRNA and protein degradation rates. In general, changes in P450 mRNA levels and enzyme activities were in good agreement.

When added directly to hepatocyte cultures, LPS directly suppressed CYP1A2, CYP2B6, and CYP3A4 mRNA levels and enzyme activity of CYP1A2 and CYP3A4 (Fig. 4; Table 2). The suppressive effects were greater on CYP3A4 than CYP1A2, as previously observed in freshly plated hepatocytes (Aitken and Morgan, 2007). A comparison of the effects of LPS and the LPS plasma indicated that, in general, the cytokines augmented the effects of the endotoxin (Table 1). In contrast to LPS, ANC28.1 antibody had little or no direct effect on CYP1A2, CYP2B6, and CYP3A4 mRNA levels or enzyme activity (Fig. 4; Table 2). This negative effect contrasts with the suppression of CYP1A2 and CYP3A4 observed with ANC28.1 plasma. We attribute this effect to the cytokines released from ANC28.1-stimulated whole blood. In general, the ANC28.1 plasma contained fewer cytokines than LPS plasma (with the notable exception of IL-2), which correlated with less mRNA suppression (Fig. 4; Table 1).

In the present study, we established proof of concept for the new in vitro method to identify therapeutic proteins that can suppress hepatic DMEs by stimulating the release of cytokines in whole blood. We demonstrated that, when added to whole blood ex vivo, LPS and ANC28.1 antibody caused the release of multiple cytokines capable of suppressing the mRNA and the enzymatic activity of hepatic CYP1A2, CYP2B6, and CYP3A4 in vitro. Furthermore, we showed that the DDI potential of certain therapeutic proteins, such as ANC28.1, can be identified in vitro provided that cytokine release from whole blood is taken into account. The direct application of ANC28.1 to hepatocytes, which caused no suppression of CYP1A2, CYP2B6, or CYP3A4, would be interpreted as a lack of potential to affect DMEs in vivo—a false-negative assessment. At the current stage of our investigation, we believe that a method comprising ex vivo treatment of human blood followed by an evaluation of the effects of cytokine-containing plasma on DME expression in human hepatocyte cultures can expand the utility of in vitro methods to identify those therapeutic proteins with the potential to alter the disposition of small-molecule drugs.

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Authorship Contributions

Participated in research design: Czerwiński, Kazmi, Parkinson, Buckley.
Conducted experiments: Czerwiński.
Performed data analysis: Czerwiński, Parkinson, Buckley.
Wrote or contributed to the writing of the manuscript: Czerwiński, Kazmi, Parkinson, Buckley.

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Address correspondence to: Dr. Maciej Czerwiński, XenoTech, LLC, 16825 West 116th Street, Lenexa, KS 66219. E-mail: mczerwinski@xenotechllc.com